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Forward and reverse genetic approaches for characterisation of tillering genes in cereals

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Dedication

To my Sun and Moon ... To my Jupiter and Venus

To my Seas and Mountains ... To my Sky and Land

To my Family

To my wife **Asmaa**

To my daughter **Sarah**

To my son **Youssef**

To my father **Sayed**

To my brothers **Wessam** and **Wael**

To martyrs of freedom in **Tahrir** square, **Rabaa**, **Nahda**,

and all squares of struggle in my land ... **Egypt**

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Summery

Recessive mutations in *Cul4* locus was described as causing low tillering phenotype in barley. The locus was previously mapped to 0.22 cM genetic interval and Candidate Gene (CG) was proposed. In this study, we mapped the locus into 0.09 cM interval and proved the correspondence between the CG and *cul4* phenotype by showing the recombination between CG and adjacent annotated genes. Also, we isolated three independent mutant alleles for *Cul4* in the available mutant stocks, and we showed their correlation to *cul4* phenotype. Detailed phenotyping of *cul4* mutant lines revealed various effects on plant vegetative, reproductive, and developmental parameters. The *Cul4* gene was identified as encoding BTB/ANK domain protein highly related to Arabidopsis *BOP1* and *BOP2* genes. To assess the role of *Cul4/BOP*-like genes in other plant species, protein sequences similar to *Cul4* were used to carry out phylogenetic analysis. Cul4/BOP-like proteins appear to be organised in two main clades corresponding to monocot and dicot divergence. Two sub-clades exist within monocot clade, representing relatively ancient duplication after monocot and dicot separation; Cul4 orthologs and paralogs. Recent duplications appear within Cul4 monocot paralogs, as well as for Arabidopsis BOP1 and BOP2 proteins. Publicly available transcriptomics data for *Cul4* rice ortholog support involvement in axillary meristem development, leaf morphogenesis, and floral organ development. Rice mutant lines carrying T-DNA inserts in *Cul4*-like genes were identified and initially characterised. Screening of mutagenized TILLMore barley collection identified 12 lines with significant defect in tillering. Further histological characterisation of 3 lines displaying the most severe low tillering phenotype, showed inhibition in tiller outgrowth rather than axillary bud formation. Initial steps in constructing mapping populations for selected mutant lines were performed by crossing them to different wild-type parents.

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CHAPTER I

INTRODUCTION

1.   **Introduction**

At the global level, the most important cereal crops are maize (*Zea mays*), rice (*Oryza sativa*), wheat (*Triticum aestivum*) and barley (*Hordeum vulgare* spp. *vulgare*), with a total of 2.4 billion tons produced annually at a value of more than 446 billion Int. $ (FAOSTAT 2012). All four of these crops are members of the Poaceae family. Much research to increase productivity, particularly of rice and wheat, is performed both by publicly funded plant breeding programs and by seed companies (Shiferaw et al., 2013). Meanwhile, the demand for cereal grains may double by 2050 (Tilman et al., 2011), which will require an average yield increase of 2.4% per year for the major cereals (Ray et al., 2012). To achieve this, multidisciplinary approaches are needed with genetic improvement playing a major role.

Tillering is a key component of yield for major cereals such as wheat, rice, barley, and rye (*Secale cereale*) (Sakamoto and Matsuoka, 2004; Sreenivasulu and Schnurbusch, 2012). In grasses, tillers are side branches (i.e. culms) that grow at the ground level from nodes of non-elongated internodes, determining the overall shoot architecture of the plant and affecting important agronomical features like competition with weeds, herbicide treatment, spacing, and the ease of harvesting (Donald, 1968; Seavers and Wright, 1999). Although sharing some key steps in their development, tillers differ from lateral branches in eudicots in that they can produce adventitious roots and grow independently from the main plant shoot.

While the molecular mechanisms controlling branching in dicots have been widely investigated (Wang and Li, 2008; Domagalska and Leyser, 2011), they are not thought to be completely conserved with tiller development in monocots, particularly in the cross-talk among hormonal pathways and other genetic networks (Kebrom et al., 2013). For instance, *TEOSINTE BRANCHED1* (*TB1*)-like genes in dicots and monocots are known to work downstream of strigolactones (SL). But while the mRNA level of *TB1*-like genes in *Arabidopsis* and pea was down-regulated by SL, experimental evidence showed no transcriptional regulation for *FC1/OsTB1* by SL in rice (Aguilar-

Martínez et al., 2007; Minakuchi et al., 2010; Kebrom et al., 2013). Another example is offered by the rice gene *MONOCULM1* (*MOC1*) and its orthologs in *Arabidopsis* and tomato. Although they are all required for axillary meristem (AXM) initiation during the vegetative phase, *MOC1* plays additional functions in bud outgrowth and control of plant height (Li et al., 2003). There are also examples of the genes identified in eudicots that have not been identified to date in monocots such as the reduced-branching mutations in the *REGULATOR OF AXILLARY MERISTEMS1, 2, 3* and *BLIND* genes of *Arabidopsis* and tomato (Schmitz et al., 2002; Keller et al., 2006; Müller et al., 2006). These examples illustrate the importance of identifying and characterizing the genes involved in the control of tillering in cereals.

Tiller development is regulated by a complex network of genetic, hormonal and environmental factors, making tillering a highly plastic trait that allows wild cereals to adapt to different environmental conditions. It is also a major target for manipulation of plant architecture in breeding programs (Kebrom et al., 2013). Tillers can develop inflorescences and contribute to grain yield, but tillers that grow late in the season will not produce any grain and will lower the overall harvest index (Sakamoto and Matsuoka, 2004; Mäkelä and Muurinen, 2011), indicating that a balance between number and vigour of tillers is required. A model plant with reduced height, low number of unproductive tillers, higher number of grains, and erect leaves has been proposed as the ideotype for cereal breeding (Donald, 1968; Khush, 2001). Indeed, reduced tillering accompanied increased productivity in the domestication of maize (Doebley et al., 2006). Cereal varieties grown before the Green Revolution were responding to nitrogen fertilizer by profuse tillering and stem elongation, thus increasing biomass. Ideotype breeding led to shorter plants with more productive tillers and improved response to nitrogen, and increased harvest index to 0.5 (Khush, 2003). In rice, this ideotype is associated with certain alleles of the *wealthy farmer's panicle* (*wfp*)/*ideal plant architecture1* (*ipa1*) locus, which confer low tillering coupled with desired inflorescence features resulting in higher yield (Jiao et al., 2010; Miura et al., 2010). Reduced tillering has also been associated with improved kernel weight under terminal water deficit environments in wheat near-isogenic lines carrying the *tiller*

inhibition (*tin*) gene (Atsmon and Jacobs, 1977; Kebrom and Richards, 2013; Mitchell et al., 2013).

In addition to being important crops, barley and rice are genetic model systems for the Poaceae family (Izawa and Shimamoto, 1996). Rice has a small genome (ca. 430 Mb) with a high degree of chromosomal synteny with other major cereal crops (Bolot et al., 2009). Barley, with its diploid genome, represents another convenient model, particularly for Triticeae crops which share high genomic co-linearity, providing a basis for genetic and genomic analyses in polyploid wheat (Bennetzen and Freeling, 1997; Hayes et al., 2003; Kumlehn and Hensel, 2009).

The conservation of synteny and integration of genomic tools among small grain cereals, particularly rice, barley and wheat, allows the transfer of knowledge among these major crops.

2.   Tiller development in rice and barley

Shoot apical meristem (SAM) and axillary meristems (AXMs) (Wang and Li, 2008). Tiller development in grasses comprises three main stages: (1) establishment of an AXM marked by formation of stem cell population in the leaf axil (2) production of leaf primordia from the AXM to form an axillary bud, and (3) outgrowth of the axillary bud to form a tiller (**Fig. 1)** (Schmitz and Theres, 2005). Classically, shoot development is considered to occur in repeated units called phytomers, each consisting of an internode (stem segment), a node, a leaf and an axillary bud (Weatherwax, 1923; Sharman, 1942). Elaborations on this basic model have been proposed for barley to account for floral organs and paired structures present at branching points (e.g. prophyll and palea) (Bossinger et al., 1992; Forster et al., 2007).

In barley and rice, the first AXMs are formed during embryogenesis. The mature barley embryo generally contains two axillary buds one each in the axils of the coleoptile and of the first leaf primordium (Kirby and Appleyard, 1987), while the mature rice embryo generally contains one axillary bud in the axil of the coleoptile (Saha, 1957). Upon germination, both plants continue to produce more leaves and AXMs in an ordered and coordinated progression, as described in detail in rice (Itoh et al., 2005; Oikawa and Kyozuka, 2009). Leaves derive from leaf founder cells at the flanks of the SAM, which undergo a developmental transition from an indeterminate to a determinate cell fate as they are recruited into incipient leaf primordia. The time interval between the formation of two successive leaf primordia is called a plastochron and leaf developmental stages are identified by their plastochron number (Pi) with the youngest visible leaf primordium indicated as P1, the next youngest as P2 and so on (reviewed in Itoh et al., 2005). The AXM develops in the leaf axil and stages of AXM formation are indicated with Pi of the subtending leaves. In rice, the first visible indication of AXM development is a slight protrusion on the stem surface towards the P2 leaf primordium (Oikawa and Kyozuka, 2009). Cell proliferation continues through the P3 and P4 stages when cells undergo a critical transition acquiring meristematic

fate. The process is completed by the P5 stage when the new AXM has initiated its own leaf primordia originating an axillary bud (Oikawa and Kyozuka, 2009).

Depending on endogenous and environmental signals, an axillary bud may remain dormant or grow into a tiller. Each tiller is a new axis of growth, organized like the main culm in phytomer units. Each tiller harbours new axillary buds that may in turn develop new tillers in a reiterative pattern. Tillers, therefore, develop in acropetal succession with primary tillers arising from axillary buds of the main culm, secondary tillers growing out of leaf axils of primary tillers, and so on (Kirby and Appleyard, 1987). After the transition of the main culm SAM from a vegetative to a reproductive state, young tillers undergo senescence, possibly because nutrients are routed away from developing tillers to the elongating internodes (Mohapatra et al., 2011). The early developing primary tillers benefit from higher sink/source ratio, sink capacity, leaf area, spikelet number, and filled grain percentage (Choi and Kwon, 1985).

AXM establishment and formation of the axillary bud are mostly under genetic control, while bud outgrowth is regulated by a complex network of genetic, hormonal, and environmental factors (Kebrom et al., 2013), making it highly responsive to environmental conditions, such as shading and nutrient availability (Agusti and Greb, 2013). Being sessile, plant fitness in varied environmental conditions depends on their ability to translate external signals into developmental responses. Several distinct signals are involved in the regulation of shoot branching, with key roles played by auxin, cytokinins (CK), and strigolactones (SL) (Kebrom et al., 2013).

Upon transition to the reproductive phase, the SAM of each tiller is transformed into an inflorescence meristem and differentiates into an inflorescence, called a panicle in rice and spike in barley, organized around a main axis called a rachis (Fig. 1). In rice, each inflorescence meristem produces several AXMs that develop into rachis branches, and the AXMs generated subsequently may develop into the next order of rachis branches or lateral spikelets, resulting in a branched structure (**Fig. 1**) (Itoh et al., 2005; Oikawa and Kyozuka, 2009). In barley, AXMs arising from the rachis differentiate into spikelet triplet meristems, that in turn will develop into three spikelet meristems (Bossinger et al., 1992), one central and two lateral. In wild barley and two-rowed

cultivars only the central spikelet is fertile, while the lateral spikelets are sterile and remain underdeveloped. In six-rowed barley cultivars and mutants all three spikelets mature to produce grains (Kirby and Appleyard, 1987; Komatsuda et al., 2007).

Fig. 1 Tiller formation in barley and shoot architecture of barley and rice. (A) Longitudinal section of a barley shoot apex showing a shoot apical meristem (SAM), an axillary meristem (AXM, the first stage in tiller formation) and axillary buds (AXB, the second stage of tiller formation). (B) Barley plant showing outgrowth of primary (1°T) and secondary tillers (2°T), the third stage of tiller formation. (C) Barley shoot architecture showing the main culm (MC) and tillers (T), (D) Rice shoot architecture showing the main culm (MC) and tillers (T), (E) Barley two-rowed spike showing the flag leaf (FL), rachis (R), spikelet (S) and awn (AW), (F) Rice panicle showing the flag leaf (FL), the rachis (R), primary branch (PB), secondary branch (SB), and spikelet (S).

3.   Genetic and hormonal control of tillering in rice

Thanks to its importance as a crop and also as a model system for other cereals, the genetic basis of shoot branching in rice has received growing attention and over the last decade a number of genes involved in tiller development have been identified (**Table.1**). Some of these genes specifically affect tiller development, while others affect other agronomically-important traits such as inflorescence (panicle) architecture and plant height. Here we review the isolated and characterized genes involved in rice tillering and related hormonal pathways including auxin, cytokinins (CK), gibberellins (GA) and strigolactones (SL).

3.1 Genes involved in AXM formation

MOC1 was the first tillering gene to be identified in rice (Li et al., 2003). Plants carrying loss-of-function mutations in *MOC1* have only one main culm as a result of failure to establish AXMs, affecting both tiller and panicle branches (Li et al., 2003). *MOC1* encodes a GRAS (named after the first three members: G̲IBBERELLIC-ACID INSENSITIVE, R̲EPRESSOR of G̲A̲I and S̲CARECROW) transcription factor homologous to tomato LATERAL SUPPRESSOR (LS) (Schumacher et al., 1999), and Arabidopsis LATERAL SUPPRESSOR (LAS) (Greb et al., 2003). However, in *ls* and *las* mutants, AXM defects are mainly observed in the vegetative phase, while *moc1* shows suppression of all types of AXM, indicating that AXM control mechanisms differ at least in part between monocots and eudicots. *MOC1* expression marks the initiation of the AXM and is maintained in the developing bud, consistent with a role in axillary bud initiation and outgrowth (Li et al., 2003). Recently, a protein mediating the degradation of MOC1 was identified from analysis of the allelic mutants *tillering and dwarf1* (*tad1*; Xu et al., 2012) and *tillering enhancer* (*te*; (Lin et al., 2012). These mutants are characterized by high tillering and reduced plant height, similar to plants overexpressing *MOC1* (Li et al., 2003). The *TAD1/TE* gene encodes a Cdh1-type co-activator orthologous to the dicot

CCS52A (Vinardell et al., 2003), which interacts with the anaphase-promoting complex (APC/C), a multi-subunit E3 ligase. TAD1/TE forms a complex with APC/C-OsAPC10, targeting MOC1 for degradation via the ubiquitin-26S proteasome pathway, thus repressing AXM initiation (Xu et al., 2012). *MOC1* Interacting Protein 1 (*MIP1*) may be another player in this circuit (Sun et al., 2010). When overexpressed, the *MIP1* gene causes enhanced tillering and semi-dwarf stature similar to *MOC1* overexpression (Li et al., 2003). *MIP1* is a member of the *Bre1* family, that includes *At_HUB1*, which encodes an E3 ligase involved in H2B (Histone 2B; a subunit of the nucleosome) mono-ubiquitination in *Arabidopsis* (Fleury et al., 2007; Liu et al., 2007).

Soon after the first visible appearance of AXM formation, the *LAX PANICLE1 (LAX1)* gene plays a role in maintenance of AXM development. Plants carrying loss-of-function mutations in *LAX1* are characterized by reduced numbers of rachis branches and spikelets on the panicle, and reduced number of tillers (Komatsu et al., 2001; Oikawa and Kyozuka, 2009). *LAX1* encodes a putative basic-Helix-Loop-Helix (bHLH) transcription factor required for formation of AXMs in both vegetative and reproductive phases (Komatsu et al., 2001; Oikawa and Kyozuka, 2009). *LAX1* acts in a non-cell autonomous manner to maintain cell proliferation during AXM formation. Oikawa and Kyozuka, 2009 proposed a two-step regulation of spatial and temporal *LAX1* expression and activity. Spatially, *LAX1* mRNA is specifically expressed in the boundary region at the adaxial side of the developing AXM, and later, LAX1 protein is trafficked toward the AXM. Temporally, *LAX1* mRNA is expressed in leaf axils from P4 to later stages, while LAX1 protein movement is restricted to the P4 stage, accompanying the acquisition of meristematic fate. Plants carrying mutations in the *LAX2* gene show similar phenotypes to *lax1* mutants, with reduced branching in the vegetative and reproductive phases. *LAX2* encodes a novel nuclear protein with a plant-specific conserved domain and was shown to physically interact with LAX1 (Tabuchi et al., 2011). Double mutant analyses suggest that *MOC1*, *LAX1*, and *LAX2* function in partially independent but overlapping pathways to regulate AXM establishment and maintenance (Tabuchi et al., 2011).

3.2 Auxin.

The main shoot apex suppresses the outgrowth of axillary buds via auxin that is produced in young expanding leaves and actively transported basipetally through the shoot, acting indirectly on axillary buds (Agusti and Greb, 2013). The movement of auxin from developing leaves to stem (auxin sink) is termed Polar Auxin Transport (PAT) and depends on auxin efflux carriers of the ATP-Binding Cassette B (ABCB) and the PIN-FORMED (PIN) protein families (Zazímalová et al., 2010). PINs are integral membrane proteins with a topology similar to transporter proteins (Zazímalová et al., 2010). Auxin is transported to the organ initiation sites through the outermost epidermal layer of the shoot apex and is directed via the developing primordia into the basipetal stream of the main shoot. PIN1 proteins are localized in xylem parenchyma cells and maintain the auxin supplement to the basipetal stream of the main shoot, playing a role in PAT (Petrásek and Friml, 2009). In agreement with the role of PAT in inhibition of bud outgrowth, rice plants underexpressing *OsPIN1b* (previously known as *REH1*) showed increased number of tillers (Xu et al., 2005; Chen et al., 2012). Conversely, increased tillering and reduced stature were caused by overexpression of *OsPIN2*, suggesting that *OsPIN1b* and *OsPIN2* play distinct roles in the control of shoot architecture (Chen et al., 2012).

Additional insight into the role of auxin comes from analysis of the *ABERRANT SPIKELET AND PANICLE1 (ASP1* or rice *RAMOSA1 ENHANCER LOCUS2*, *OsREL2)* gene that encodes a transcriptional co-repressor proposed to act in auxin signalling (Yoshida et al., 2012). A variety of developmental alterations were described for *asp1* mutant plants, including de-repression of axillary bud outgrowth and disturbed phyllotaxy in the vegetative phase, disorganized panicle branching and spikelet morphology, indicating a general role for *ASP1* in controlling meristem fate (Yoshida et al., 2012).

A member of the MIR393 miRNA family known to regulate expression of auxin receptors also affects tillering ability in rice. Plants over-expressing *OsmiR393* showed reduced expression of the auxin receptors *OsTIR1* and *OsAFB2*, which sequentially repress the auxin transporter *OsAUX1* (Xia et al., 2012). This ultimately downregulates

OsTB1, a tillering repressor (see below), explaining the increased outgrowth of tillers in *OsmiR393* overexpressing plants.

Another auxin-dependent pathway regulating rice tillering involves *TLD1*, an indole-3-acetic acid (IAA)-amido synthetase that converts active auxin (IAA) to its inactive form via conjugation with amino acids (Zhang et al., 2009). The associated reduction of IAA concentration affects developmental traits and also influences plant resistance to biotic and abiotic stress via the abscisic acid (ABA), and salicylic acid (SA) pathways. Gain-of-function *tld1-D* mutants show pleiotropic phenotypes including increased number of tillers, reduced plant height, shorter panicle and reduced number of spikelets (Zhang et al., 2009).

3.3 Cytokinins

Cytokinins (CKs) are key regulators of many plant developmental processes including: cell division, activation of axillary buds, inhibition of root growth, and delay of senescence (Mok, 1994). CKs are mainly synthesized in the root and transported upwards along the xylem (Wang and Li, 2006). CK biosynthesis and signalling are affected by nutrient availability and environmental stresses, such as drought and high salinity (Krouk et al., 2011; Ha et al., 2012). The *supershoot* (*sps*) mutants in *Arabidopsis* show massive shoot overproliferation and 3- to 9-fold increase in levels of Z-type CKs that indicates a role of SPS in suppression of AXM initiation and growth through the localized attenuation of CK levels at sites of bud initiation (Tantikanjana et al., 2001).

The fundamentals of CK biosynthesis were originally studied in *Arabidopsis* (Sakakibara, 2004) where the central rate-limiting step is prenylation of adenosine $5'$ phosphates at the N^6-terminus with dimethylallyldiphosphate (DMAPP), catalysed by adenosine phosphate ISOPENTENYL TRANSFERASE (IPT). Auxin can down-regulate CK levels by inhibiting the expression of *IPT* genes (Ferguson and Beveridge, 2009). Other studies showed that the *Arabidopsis* KNOTTED-like homeobox (KNOX) protein SHOOTMERISTEMLESS (STM) induces expression of *AtIPT7* (Jasinski et al., 2005; Yanai et al., 2005) and down-regulates GA biosynthesis genes, creating low-GA and

high-CK status in the meristem, which may be essential for the maintenance of meristematic activity (Jasinski et al., 2005). Eight *OsIPT* genes have been identified and studied in the rice genome (Sakamoto et al., 2006). Transgenic rice plants over-expressing *OsIPTs* have increased axillary bud activity and reduced root formation, which are typical of CK overexpression. Also, *OsIPT3* and *OsIPT2* transformants showed elevated content in 12 different CK species, highlighting the overall stimulation of *de novo* CK biosynthesis (Sakamoto et al., 2006).

3.4 Gibberellins

Gibberellins (GAs) interact with auxin in the regulation of stem elongation, with apically-derived auxin regulating GA synthesis (O'Neill and Ross, 2002). In rice, GAs participate in controlling many plant developmental processes, positively regulating germination, stem and root elongation, and flower development, while negatively regulating *OSH1* (a rice *KNOX* gene) and *OsTB1* (see below) (Lo et al., 2008). The main catabolic pathway for GAs is the 2-β-hydroxylation reaction catalysed by C19- and C20-GA 2-oxidases (GA2oxs), which inactivate endogenous bioactive GAs, affecting GA-dependent developmental processes (Sakamoto et al., 2004). In total, 10 putative *GA2ox* genes were identified in the rice genome and rice lines overexpressing some C20 GA2oxs exhibit semi-dwarfing, early and increased tillering, and adventitious root growth (Lo et al., 2008).

The *ERF protein associated with tillering and panicle branching* (*OsEATB*) gene belongs to the <u>APETALA2</u> (AP2)/<u>E</u>thylene-<u>R</u>esponsive <u>E</u>lement Binding Factor (ERF) family of transcription factors. Transformed plants over-expressing *OsEATB* showed enhanced tillering, reduced height, enhanced panicle branching, and overall higher grain yield (Qi et al., 2011). These phenotypes were accompanied by decreased levels of different GAs, confirming the negative correlation between GA and tillering.

3.5 Strigolactones

Strigolactones (SLs) have been recently identified as phytohormones which inhibit side branching in plants (Gomez-Roldan et al., 2008; Umehara et al., 2008). SLs are synthesised in roots and move acropetally (Wang and Li, 2006). They interact with auxins in a dual-loop pathway to control axillary bud outgrowth, but the nature of this regulatory loop is unclear (Kebrom et al., 2013). Two hypotheses have emerged, mostly from studies in dicots: 1) strigolactones act downstream of auxin (Foo et al., 2005; Bainbridge et al., 2005; Brewer et al., 2009; Hayward et al., 2009); 2) strigolactones negatively regulate auxin transport in the main stem, and inhibit the establishment of axillary bud PAT into the stem (Bennett et al., 2006; Crawford et al., 2010; Domagalska and Leyser, 2011). In rice, mutations in SL biosynthesis gene *Dwarf27* (*D27)* result in increased PAT (Lin et al., 2009) and exogenous auxin application can upregulate expression of *Dwarf10* (*D10)* and *Dwarf17* (*D17)* (Zou et al., 2006; Arite et al., 2007), indicating a complex interplay between auxin and SL.

Genes acting in the SL pathway have been identified from analysis of rice *dwarf* mutants (*d*) (or *htd* for *high tillering dwarf*), and Arabidopsis *more axillary growth* (*max*) mutants, characterized by reduced plant height and increased branching (Brewer et al., 2013). Five *dwarf* rice mutants were initially identified and characterised to have reduced levels of SLs or impaired in SL response (Ishikawa et al., 2005): *dwarf3* (*d3*), *dwarf10* (*d10*), *dwarf14* (*d14*) (also reported as *d88* or *htd2*), *dwarf17or dwarf1* (*d17 or d1*) and *dwarf27* (*d27*) (Ishikawa et al., 2005; Zou et al., 2006; Arite et al., 2007; Lin et al., 2009; Hamiaux et al., 2012). Studies on *D* genes and their homologues in other plants (e.g. Arabidopsis *MAX2, MAX4, MAX3*, and *MAX1*) helped to clarify their roles in SL biosynthesis and signal transduction (Challis et al., 2013). *D17/D1* (*MAX3* in Arabidopsis) and *D10* (*MAX4* in Arabidopsis) encode carotenoid cleavage deoxygenase (CCD) family proteins CCD7 and CCD8, respectively (Zou et al., 2006; Arite et al., 2007), These enzymes are capable of sequentially cleaving the carotenoid 9-cis-b-carotene to produce a novel compound, carlactone, a putative strigolactone intermediate (Alder et al., 2012) . *D27* also acts in SL biosynthesis in rice (reverse genetics identified the *D27* orthologue in Arabidopsis, called *AtD27*; (Waters et al., 2012b; a), and encodes an iron-

containing beta-carotene isomerase protein that converts all-trans-beta-carotene into 9-cis-beta-carotene, which is then cleaved by *D17* (*CCD7*) into a 9-cis-configured aldehyde (Alder et al., 2012). Although 5 rice homologs of Arabidopsis *MAX1* cytochrome P450-type gene were identified by sequence similarity and phylogenetic analysis, but *max1* mutant phenotype has not been identified in rice (Challis et al., 2013).

Rice mutants *d14* and *d3* show insensitivity to SL application, indicating the corresponding genes act in SL signalling rather than biosynthesis (Ishikawa et al., 2005; Arite et al., 2009). *D14* encodes a hydrolase/esterase that was proposed to participate in the perception of SL (Ishikawa et al., 2005; Beveridge and Kyozuka, 2010), and *D3* encodes an F-box protein that mediates signalling of SL and karrikins (Ishikawa et al., 2005; Hamiaux et al., 2012). Recent findings have revealed the mechanism by which D14 - as a catabolic enzyme and SL receptor - is involved in integrating SLs signal perception into signal transduction and cross-talk with the GA signalling pathway (Nakamura et al., 2013). In a novel finding, Jiang et al., (2013) have identified a new mutant of the rice dwarf family, *D53*, which participates in SL signalling as a promoter of tillering, and they have proposed a model for SL signalling that is centred around a D14–D3–D53 signalling axis. In this model the D53 protein was found to serve as target for degradation by SL through D14 and D3, that subsequently activate ubiquitin proteasome system to degrade D53 (**Fig. 2**; Smith, 2013). In turn, degradation of D53 would release the repression of downstream target genes and suppress tillering (Jiang et al., 2013; Zhou et al., 2013).

More details regarding the SL pathway can be found in some excellent reviews (Beveridge and Kyozuka, 2010; Domagalska and Leyser, 2011; Brewer et al., 2013).

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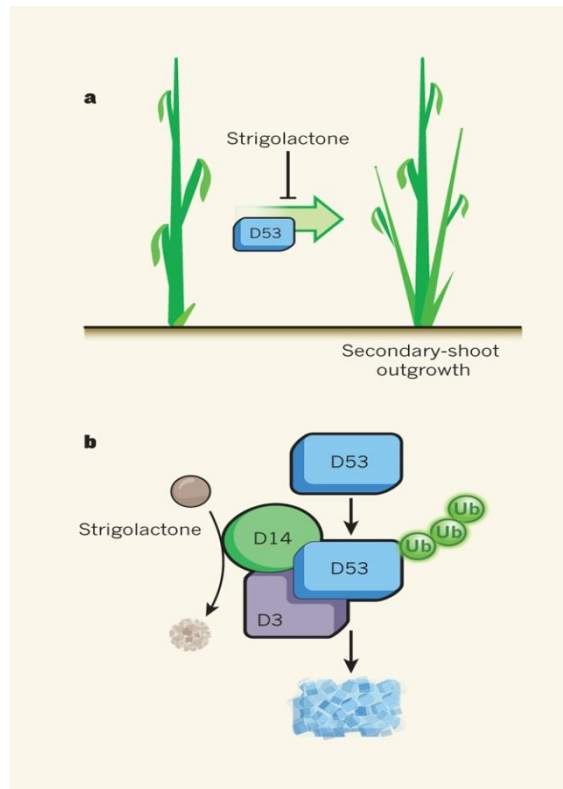
Fig. 2 Strigolactones hormones inhibit the axillary buds outgrowth, and act as responsive signal to nutrient deficiency. This pathway is modulated through the destruction of D53 protein that naturally promotes side branch out-growth. D14 serves as receptor that binds and hydrolyses SLs, leading to D53 and D3 to be recruited to the complex. This interaction leads to breaking down D53, and induce downstream effect that suppress side branching (Fig from Smith, 2013).

3.6 Brassinosteroids

Along with GAs, brassinosteroids (BRs) are viewed as major players in the control of plant height. BRs are phytohormones with a structure similar to animal steroid hormones, and mutants defective in BR biosynthesis and signalling generally exhibit a dwarf phenotype and other abnormalities in leaves and branches (Clouse and Sasse, 1998; Bishop and Koncz, 2002; Gendron et al., 2012). In contrast to the general negative correlation between plant height and tiller number (Hong et al., 2003; Booker et al., 2004), rice mutants impaired in the BR pathway such as *dwarf and low tillering* (*dlt*) show reductions in both tillering and stature. The *DWARF AND LOW-TILLERING* (*DLT* or *OsGRAS32*) gene encodes a GRAS transcription factor involved in feedback inhibition of BR biosynthesis (Tong et al., 2009, 2012). BRs can down-regulate the expression of *DLT*, via the OsBRI1 receptor-like protein, which regulates both DLT and

OsBZR1, a transcription factor that in turn regulates BR-responsive genes (Tong et al., 2009, 2012).

OsBZR1 is also controlled by *GSK3/SHAGGY-like kinase* (*GSK2*), which is the ortholog of *Arabidopsis GSK3/SHAGGY-like kinase BRASSINOSTEROID-INSENSITIVE2 (BIN2)*. Rice *GSK3/SHAGGY-like kinase* (GSK2) over-expression results in dwarf plants with dark green leaves, compact structure and fewer tillers (Tong et al., 2012).

3.7 Strigolactone pathway regulation and integration of different signals.

Recent studies are providing insight into the regulation of the SL pathway and its cross-talk with other hormonal pathways.

The rice mutant *fine culm1 (fc1)* exhibits thin culm and excessive tillering due to a loss-of-function mutation of the *OsTB1* gene, which encodes a TEOSINTE BRANCHED1/CYCLOIDEA/PCF (TCP) transcription factor homologous to *TEOSINTE BRANCHED1* (*TB1*) in maize, and *Arabidopsis BRANCHED 1/TEOSINTE BRANCHED 1-LIKE 1* (*BRC1/TBL1*) (Takeda et al., 2003; Choi et al., 2012). Thus, *FC1/OsTB1* functions as a negative regulator of lateral branching in rice, similar to maize *TB1* (Minakuchi et al., 2010). Based on insensitivity of the *fc1* mutant to exogenous application of SL and epistatic interaction with *d17*, Minakuchi et al., (2010) proposed that *FC1/OsTB1* acts downstream of SL. Negative regulation of *FC1/OsTB1* expression by CK and GA (Lo et al., 2008) further led to the hypothesis that *FC1/OsTB1* may act as an integrator of multiple pathways controlling tillering (Minakuchi et al., 2010). More details about the interplay between *OsTB1* and SL have recently emerged. OsMADS57, a MADS-box domain protein was shown to interact with OsTB1 and repress *D14*, a gene involved in SL signalling and possibly encoding a SL receptor (Guo et al., 2013). *OsmiR444a*, a member of the MIR444 miRNA family, post-transcriptionally regulates *OsMADS57* transcript accumulation (Guo et al., 2013). In contrast to the function of *FC1/OsTB1*, genetic and molecular analyses of *OsMADS57* indicate that it functions as a repressor of SL signalling and a positive regulator of tillering. Thus, interaction between the two transcription factors may act to fine-tune *D14* expression

and consequently tiller outgrowth (Guo et al., 2013). Recently, *OsTB1* was shown to be directly regulated by *OsSPL14*, a member of the *SQUAMOSA Promoter Binding Protein* gene family (Lu et al., 2013) previously associated with the semidominant *WEALTHY FARMERS PANICLE/IDEAL PLANT ARCHITECTURE1* quantitative trait locus (QTL) (Jiao et al., 2010; Miura et al., 2010). Increased *OsSPL14* expression results in reduced tillering, prolonged plastochron duration, sturdier stems, increased number of primary branches in the panicle and, ultimately, higher grain yield, corresponding to the ideotype for rice breeding (Jiao et al., 2010; Miura et al., 2010). In the vegetative phase, *OsSPL14* is specifically expressed in leaf primordia, indicating that *OsSPL14* may affect tillering through regulation of plastochron duration, an activity shared with the highly related *Arabidopsis* genes *SPL9* and *SPL15* (Luo et al., 2012). A genome-wide screen for OsSPL14 binding sites recovered various developmental genes including *OsPIN1b*, suggesting that OsSPL14 may also be involved in regulation of PAT (Lu et al., 2013). Negative control of *OsSPL14* expression involves cleavage by *OsmiR156,* whose over-expression transformants show increased number of tillers, and reduced number of spikelets and grains per panicle (Luo et al., 2012). *ABERRANT PANICLE ORGANIZATION1 (APO1*) and *APO2* also act upstream of *OsSPL14* (Luo et al., 2012). *APO1* encodes the ortholog of an *Arabidopsis* F-box protein UNUSUAL FLORAL ORGANS (UFO) while *APO2* is homologous to the *Arabidopsis LEAFY* (*LFY*) gene, and the two proteins were shown to interact *in vitro* (Ikeda et al., 2007; Ikeda-Kawakatsu et al., 2012). Rice *apo2* mutants have shorter plastochron duration, increased number of tillers, and small panicles with reduced number of primary branches (Ikeda-Kawakatsu et al., 2012). Together, analyses of these genes indicate that regulation of plastochron duration also affects the number of tillers formed by a plant as a result of the number of leaf/axils formed on the shoot (Wang and Li, 2011).

3.8 Other genes

More genetic factors that participate in the control of tillering have been recently characterized (**Table.1**). Future work will help to better understand if and how they interact with the aforementioned pathways. Among them, *FRIZZY PANICLE* (*FZP*) was initially identified as a repressor of AXM formation in the panicle, necessary to establish floral meristem identity in rice spikelets (Komatsu et al., 2003). The *FZP* gene encodes an ethylene-responsive element-binding factor (ERF) transcription factor highly related to *Branched Silkless1* (*BD1*) that was shown to play a similar role in maize inflorescence development (Chuck et al., 2002). Noteworthy is a loss-of-function allele of *FZP* that shows defects in tillering, suggesting that *FZP* plays additional roles in controlling vegetative AXM development (Kato and Horibata, 2011).

OsTEF1 (*transcription elongation factor- like 1*) was identified from loss-of-function mutant which showed reduced tillering ability, retarded growth of seminal roots, and less tolerance to salt (Paul et al., 2012). More than 100 genes were differentially expressed compared to wild-type and may mediate control of tillering by *OsTEF1*, including a gene coding for a cytochrome P450 protein. One cytochrome P450 gene in Arabidopsis, MAX1, is known to be involved in the SL biosynthesis pathway, but further work is needed to establish whether *OsTEF1* may be a regulator of SL biosynthesis in rice (Paul et al., 2012).

Ascorbic acid (Asc) is an important co-factor for a number of enzymes that affect plant growth and development. Rice RNAi transformants down-regulated for expression of mitochondrial flavoenzyme *l-galactono-1,4-lactonedehydrogenase* (*GLDH*) show reduced tillering ability and seed set, premature senescence, reduced plant growth rate, and reduced plant height. GLDH catalyses the last step of Ascorbic acid (Abc) biosynthesis by converting l-GalL into Asc. Low Asc concentrations are known to activate ABA- and JA-dependent signalling pathways (Liu et al., 2013).

reduced culm number1 (*rcn1*) rice mutants show low number of tillers, reduced panicle branching, and plant height. *RCN1* encodes rice OsABCG5, an ATP-binding cassette protein belonging to subfamily G (ABCG subfamily), also known as the white–

brown complex (WBC) subfamily. The *Arabidopsis* ortholog is *TERMINAL FLOWER 1* (*TFL1*), a gene assumed to be involved in transportation of lipid. While for Arabidopsis TFL1 an effect on leaf wax was identified, the same function was not recognised in rice (Yasuno et al. 2009).

In summary, since the first gene involved in tillering in rice was identified ten years ago (Li et al., 2003), many genes involved in tillering have been rapidly identified in this species (**Table.1**) revealing a complex genetic and hormonal network regulating tiller development. The growing number of genes associated with the regulation of tillering in rice provides a molecular framework for this process, while opening new questions about the interplay of the different factors involved.

Table.1. List of genes/mutants involved in rice tillering with their phenotypes, homologs, molecular function and pathway

Gene/mutant name-Protein/gene family	Phenotype[†, ‡, §, ¶, #]	Homologs	Molecular function/Pathway	References
AXM establishment & maintenance				
*Monoculm1 (MOC*1)- GRAS family	Reduced tillers [†]. Increased tillers and reduced plant height [‡]	Tomato *Lateral suppressor* (*Ls*), Arabidopsis *Lateral Suppressor* (*LAS*)	Transcription factor (TF)/ Up-regulates *OsTB1* and *OSH1*, works with LAX1 and LAX2 in AXM establishment /degraded by TAD1	Li et al. 2003, Xu et al. 2012, Lin et al. 2012
Tillering and Dwarf 1 (TAD1)/Tiller Enhancer (TE)- Cdh1-type co-activators	Increased tillers and reduced plant height [†]	Arabidopsis *Cell Cycle Switch52A (CCS52A)*	Co-activator of Anaphase-Promoting Complex/Cyclosome (APC/C), a multi-subunit E3 ligase /Forms a complex with OsAPC10, activates APC/C to target MOC1 for degradation by the ubiquitin–26S proteasome pathway	Xu et al. 2012, Lin et al. 2012
MOC1 Interacting Proteins (MIP1)- Brefeldin A-sensitivity protein 1 (*OsBre1A* and *OsBre1B*)	Increased tillers and reduced plant height[‡]	Arabidopsis *At_HUB1*, Maize *Zm_Bre1*	Bre1 protein family is E3 ubiquitin-protein ligase /At_HUB1 involved in chromatin modification and gene regulation /Interacts with MOC1, and regulates downstream *OSH1* and *OsTB1*	Sun et al. 2010
Lax Panicle1 (LAX1)- containing Basic Helix-Loop-Helix (bHLH) domain	Reduced tillers and panicle branches[†]	Arabidopsis *AtBA1*/*LAX1* Maize *Barren Stalk1* (*BA1*)	TF/Interacts with MOC1 and LAX2 in AXM establishment/maintenance	Komatsu et al. 2003, Oikawa and Kyozuka 2009
Lax Panicle2 (LAX2)- Novel plant nuclear protein	Reduced tillers and panicle branches[†]	Arabidopsis *DRIP1* and *DRIP2* (although lack the amino acid stretches)	Potentially works as co-activator of LAX1/Interacts with MOC1 and LAX1 in AXM establishment/maintenance	Tabuchi et al. 2011
Auxin				
Pin-Formed1 (OsPIN1)-PIN1 family	Increased tillers[§]	Arabidopsis *AtPIN1*	Auxin efflux carrier/Transports Auxin	Xu et al. 2005
Pin-Formed2 (OsPIN2)-PIN2 family	Increased tillers and tiller angle, reduced plant height[‡]	Arabidopsis *AtPIN2*	Auxin efflux carrier/Transports Auxin, down-regulates *OsLazy1* (negative regulator of polar auxin transport)	Chen et al. 2012
Increased number of Tillers/Enlarged Leaf angles/Dwarfism TLD1/ Gretchen Hagen3 OsGH3.13- GH3	Increased tillers, leaf angle and drought tolerance, reduced panicle length, plant height and spikelets no	Arabidopsis *WES1*/*GH3.5*	Multifunctional acetyl-amino synthetase/ Conjugates IAA to amino acids, Regulates negatively free IAA, Arabidopsis homologs respond to ABA and salicylic acid pathways	Zhang et al. 2009

Aberrant Spikelet and Panicle1(ASP1)/Ramos a1 Enhancer Locus2 (OsREL2)- Transcriptional co-repressor	Increased primary tillers and reduced secondary tillers/disrupted phyllotaxy of tillers arrangement[†]	Arabidopsis *Topless* (*TPL*), Maize *Ramosa Enhancer Locus2* (*REL2*)	Transcriptional co-repressor/De-repression of axillary bud growth /Involved in Auxin signalling	Kwon et al. 2012, Yoshida et al. 2012
OsmiR393- MIR393 non-coding miRNA	Increased tillers, early flowering, salt and drought susceptibility [‡]	Arabidopsis *miR393*	Represses *OsTIR1* and *OsAFB2*, that further represses *OsAUX1*, auxin transport to axillary buds and down-regulates *OsTB1*	Xia et al. 2012
Cytokinins (CK)				
IsopentenylTransferase (OsIPT1,2,3,4,7)- Adenosine 5' phosphate isopentenyl transferase	Overproliferation of axillary shoots[‡]	Arabidopsis *Isopentenyl Transferase* genes	Catalyses prenylation of adenosine 5' phosphates at the N6-terminus with dimethyl allyldiphosphate (DMAPP)/Involved in CK biosynthesis	Sakamoto et al. 2006
Gibberellin (GA)				
Gibberellin 2-Oxidases5/6 (*OsGA2ox5/6*) – Oxidases	Increased tillers and adventitious root growth, semi-dwarfing [‡]	Arabidopsis *Gibberellin 2-Oxidases* (*AtGA2oxs*) Maize *Gibberellin 2-Oxidases* (Zm*GA2oxs*)	Catalyse 2-β-hydroxylation reaction/Inactivate bioactive GA	Lo et al. 2008
Strigolactones (SL)				
Dwarf1 (D1/D17)/ High Tillering Dwarf1 (HTD1)- Carotenoid Cleavage Dioxygenase (CCD7)	Increased tillers[†]	Arabidopsis *More Axillary Growth3* (*MAX3*)	Cleaves β-carotene into 13-apo-β-carotenone/ Involves in SL biosynthesis/Up-regulated by Auxin	Ishikawa et al. 2005, Kebrom et al. 2013, Zou et al. 2006
Dwarf10 (D10/CCD8b)- Carotenoid Cleavage Dioxygenase (CCD8) family	Increased tillers[†]	Arabidopsis *More Axillary Growth4* (*MAX4*)	Cleaves β-carotene into 13-apo-β-carotenone /Involves in SL biosynthesis/ Up-regulates by Auxin	Ishikawa et al. 2005, Kebrom et al. 2013, Arite et al. 2007
Dwarf27(D27)- Iron-containing protein with isomerase activity	Increased tillers[†]	Arabidopsis *Dwarf27 (AtD27)*	Produces 9-cis-β-carotine from all-trans-β-carotine in SL biosynthesis pathway/Involved in SL biosynthesis	Ishikawa et al. 2005, Lin et al., 2009; Kebrom et al. 2013
Dwarf14 (D14)- hydrolase/esterase	Increased tillers[†]	Arabidopsis *Dwarf14 (AtD14)*	Regulated by OsMADS57/ Involved in SL signalling, potentially as SL receptor	Ishikawa et al. 2005, Kebrom et al. 2013, Guo et al. 2013
Dwarf3(D3)- F-box leucin-rich repeat protein	Increased tillers[†]	Arabidopsis *MAX2*	Mediates signalling of SL and karrikins/Involved in SL biosynthesis	Ishikawa et al. 2005, Kebrom et al. 2013

Dwarf53 (*D53*) - substrate for SCF[D3] ubiquitination complex	Increased tillering[¶]	Arabidopsis *SMXL6*, *SMXL7* and *SMXL8*	Negative regulator for SL /substrate for *D14* hydrolysis	(Jiang et al., 2013; Zhou et al., 2013)
SL pathway regulation and integration of different signals				
SQUAMOSA Promoter Binding Protein-Like 14 (OsSPL14)/ rice Ideal Plant Architecture1 (Os IPA11)/ Wealthy Farmer's Panicle (WFP) QTL – SPL family	Reduced tillering, increased lodging resistance and higher yield [#]	Arabidopsis *SQUAMOSA Promoter Binding Protein-Like* (*AtSPLs*) and maize *ZmSPLs*	TF/ regulates OsTB1	Miura et al. 2010, Chen et al. 2010, Lu et al. 2013
Teosinte Branched1 (OsTB1)/Fine culm1 (FC1)- class II proteins of TCP family	Excessive tillers and thin culm[†]	Maize *Teosinte Branc*hed (TB), Arabidopsis *Branched 1/Teosinte Branched 1-Like 1 (BRC1/TBL1)*	TF/Regulates SL signal transduction by interaction with OsMADS57 and reduces its inhibition for *Dwarf14 (D14)*/ Target for repression by CK and GA/Up-regulated by Auxin	Minakuchi et al. 2010, Guo et al. 2013, Takeda et al. 2003
OsMADS57- MADS-box domain protein	Increased tillers[‡], Reduced tillers[§]		Involved in SL signalling by inhibiting D17/ Inhibits directly D14 and its inhibition activity reduced by interaction with OsTB1	Guo et al. 2013
OsMIR444a- MIR444 non-coding miRNA	Reduced tillers[‡]		Enhances the SL biosynthesis by repressing *OsMADS57*	Guo et al. 2013
Increased Tillering, Reduced Height, and Infertile Spikelets (THIS) - Class III lipase family	Increased tillers, shorter plants, and less seed set[†]	Arabidopsis *PRLIP8*	May be involved in Auxin and SL signalling	Liu et al., 2013
Nodulation Signaling Pathway1 (NSP1) and Nodulation Signaling Pathway2 (NSP2)	Reduced tillers[†]	Legume NSP1 and NSP2	TFs/down-regulates D27	Liu et al., 2011
OsMIR156- non-coding miRNA	Increased tillers, reduced spikelets and grains no[‡]	Arabidopsis *MIR156*	microRNA cleavage of *OsSPL14* mRNA	Luo et al. 2012
Brassinosteroid				
Dwarf and Low-Tillering (DLT)/ OsGRAS32- GRAS	Reduced tillers and plant height[†]	Arabidopsis *AtGRAS8*	TF/ Involved in inhibition of Brassinosteroid (BR) biosynthesis through interaction with OsBZR1 that perceives BR signal by OsBRI1	Tong et al. 2009 and 2012
Glycogen synthase kinase2 (GSK2)- GSK family	Reduced tillers, dark green and dwarf plants [‡]	Arabidopsis *GSK3/SHAGGY-like kinase Brassinosteroid-Insensitive2 (BIN2)*	Regulates DLT gene by phosphorylation	Tong et al. 2012

Other factors				
Transcription Elongation Factor-like 1 (OsTEF1)- Elf1 superfamily	Reduced tillers, seminal roots growth and salt tolerance[†]	Arabidopsis *Transcription elongation factor-like 1*	Involved in transcription elongation by RNA polymerase II/ Induces cytochrome P450, potentially a member of SL pathway in Arabidopsis	Paul et al. 2012
ATP-Binding Cassette protein-G5 (OsABCG5)/reduced culm number1 (rcn*1*) - ATP-Binding Cassette protein subfamily G (ABCG)/ White–Brown Complex (WBC) subfamily	Reduced tillers, reduced panicle branches[†]	Arabidopsis *Terminal Flower 1 (TFL1)*	Half-transporter/ Arabidopsis *TFL1* potentially involved in transportation of lipid	Yasuno et al. 2009
Frizzy Panicle (FZP)- Ethylene-Responsive Element-binding factor (ERF) TFs	Reduced tillers and plant height, increased rachis-branches[†]	Maize *Branched Silkless1 BD1* gene	Involved in transition from axillary meristem identity to spikelet meristem	Xing and Zhang 2010, Kato et al. 2012
Aberrant Panicle Organization 2 (APO2)/ OsRFL	Increased tillers and leaf number, reduced panicle branching[†]	Arabidopsis *LEAFY (LFY)* gene	TF/Interacts with APO1	Ikeda-Kawakatsu et al. 2012
Aberrant Panicle Organization 1 (APO1)-F-box protein	Increased tillers and leaf number, reduced panicle branches[†]	Arabidopsis *Unusual Floral Organs (UFO)*	Interacts with APO2/ represses class-C genes	Ikeda et al. 2007 Ikeda et al. 2005
l-Galactono-1,4-Lactone dehydrogenase (GLDH)- Mitochondrial enzyme with FAD domain	Reduced tillers and plant height, premature senescence[§]	Arabidopsis *GLDH*	Catalyse the last step of Ascorbic acid (Abc) biosynthesis by converting l-GalL into Asc , potentially involved in ABA and JA pathway	Liu et al. 2013
ERF Protein Associated With Tillering and Panicle Branching (OsEATB)/AP2/ERF gene - APETALA2(AP2)/ERF	Increased tillers and panicle branches, reduced plant height[‡]	Arabidopsis *AtERFs*	TF/Involves in the cross talk between ethylene and GA/ Down-regulates ethylene-induced enhancement of GA synthase	Qi et al. 2011

† Loss of function mutation, ‡ Over-expression transformation, § Down-expression, ¶ Gain-of-function, # QTL

4.   Genetic control of tillering in barley

Several barley mutants exhibiting tillering abnormalities have been identified and characterized providing some initial insight into the genetic and hormonal regulation of tillering in the Triticeae (Dabbert et al., 2010) (**Table.2** and **Fig. 3**). These can be classified into four classes, according to their AXM activity. The first class includes mutants that fail to develop axillary buds, and consequently, develop no tillers. The mutant *uniculm2* (*cul2*) is the best characterized example of this class with a phenotype similar to that of *moc1* mutant in rice (Babb and Muehlbauer, 2003). The second class of mutants exhibit lower number of tillers due to compromised axillary bud outgrowth including *low number of tillers1* (*lnt1;* Dabbert et al., 2010), *absent lower laterals1* (*als1;* Dabbert et al., 2010) and *uniculme4* (*cul4*) (Babb and Muehlbauer, 2003). The third group comprises mutants with modestly reduced tillering, such as the *intermedium-b* (*int-b*) and *semi-brachytic* (*uzu*) mutants (Babb and Muehlbauer, 2003). The fourth class of mutants exhibits enhanced tillering, suggesting a defect in controlling tiller bud outgrowth. The mutants *granum-a* (*gra-a*), *grassy tillers* (*grassy*), *intermedium-c* (*int-c*), *many noded dwarf1* (*mnd1*), and *many noded dwarf6* (*mnd6*) represent the high tillering class (Babb and Muehlbauer, 2003; Druka et al., 2011). Additional loci altering inflorescence architecture may also affect tillering, although such defects have not been explored in detail in barley. One example is the barley *INT-C* gene, the ortholog of maize and rice *TB1* genes which acts in controlling spike row-type and also has an effect on seedling tiller number (Ramsay et al., 2011).

Uniculm2 (*cul2*) mutants possess one main culm without any tiller outgrowth (Babb and Muehlbauer, 2003). The *cul2* mutation appears to be epistatic to low tillering mutations (*lnt1*, *als1*, *cul4*, *int-b* and *uzu*) and high tillering mutations (*mnd1*, *mnd6*, and *gra*). Barley *cul2* mutants also show disorganization in the distal end of the developing inflorescence, and deviation from wild-type timing of reproductive developmental steps (Babb and Muehlbauer, 2003). Overall similarities between *cul2* and the rice *moc1* mutant include failure to develop axillary buds, reduced plant height, reduced

inflorescence branching, and the epistatic effects to mutations in other loci. However, in contrast to *moc1* where AXMs are not initiated, AXMs are present in *cul2* leaf axils but do not progress to axillary buds, indicating that *Cul2* acts at the stage of bud development. The *cul2* locus was positioned on chromosome 6H near the centromeric region, but no candidate genes have been identified (Okagaki et al., 2013). Transcriptome analysis of *cul2* mutants and corresponding wild-type plants using the Affymetrix Barley1 GeneChip (Close et al., 2004) indicated that *CUL2* is necessary for coordinating signalling pathways and stress response and integrating them into AXM development (Okagaki et al., 2013).

The *als1*, *lnt1*, and *cul4* mutants typically develop only 1 to 3 tillers compared to their wild-type background (Babb and Muehlbauer, 2003; Dabbert et al., 2009). These loci, mapped at different positions on chromosome 3H, are epistatic to the high tillering mutants, based on the low tillering phenotype of the double mutants (Dabbert et al., 2009, 2010; Muehlbauer, unpublished data). Transcriptomics studies showed that stress-related genes are up-regulated in the *als1*, but not in the *lnt1* mutant. Gene expression, mapping, and sequence analyses supported the *JuBel2* gene as a candidate for the *Lnt1* locus (Dabbert et al., 2010). The JuBEL2 protein is a member of BELL-family of homeodomain transcription factors (Müller et al., 2001), sharing high similarity with qSH1, a rice QTL for seed shattering on chromosome 1 (Konishi et al., 2006) and the transcription factor *Arabidopsis BELLRINGER (BLR)* in *Arabidopsis* (Smith and Hake, 2003; Byrne, 2003; Roeder et al., 2003). The mutant *qSH1* allele in cultivated rice is a regulatory mutation that prevents *qSH1* expression in the pedicle at the base of the rice seed, resulting in resistance to shattering (Konishi et al., 2006). No effect of this mutation was detected on expression of *qSH1* in other tissues and no phenotypic effect on tillering was reported. However, a *qSH1* loss-of-function allele has not been isolated in rice, and the *qSH1* locus may control other traits in addition to shattering (Konishi et al., 2006). In *Arabidopsis*, *blr* mutants show reduced plant height, additional axillary branches and leaves, and disrupted phyllotaxy in vegetative and inflorescence units (Smith and Hake, 2003; Byrne, 2003; Roeder et al., 2003). A two-hybrid protein-protein interaction experiment showed that barley JuBEL2 can bind to class I KNOX proteins including *Hooded/BKN3*, which is a barley homolog of *Arabidopsis STM* (Müller et al.,

2001). Similar results were obtained in *Arabidopsis* where BLR interacts with the STM protein, assisting in its transportation to the nucleus (Cole et al., 2006; Rutjens et al., 2009). *STM* is required to maintain the meristematic identity of cells in both the SAM and AXM (Long and Barton, 2000). Plants mutated in *BLR* and two other related *BELL-like* genes, *POUND-FOOLISH* and *ARABIDOPSIS THALIANA HOMEOBOX GENE 1,* lack a SAM, indicating that BELL-like proteins are essential for normal *STM* function (Rutjens et al., 2009). Together, these results suggest that interactions between *JuBel2* and class I *KNOX* genes may promote AXM developmentand tiller development in barley.

Recessive mutations in the *Int-b* (located on chromosome 5H) and *Uzu* (located on chromosome 3H) genes also reduce tiller number, but to a lesser extent compared to the mutants of the previous class (Babb and Muehlbauer, 2003). The *Uzu* gene encodes a putative BR receptor HvBRI1 (Chono et al., 2003) and its use as a dwarfing gene has become widespread in Asian breeding programs (Hoskins and PoehlmaN, 1971; Tsuchiya, 1976; Zhang, 1994, 2000; Saisho, D, Tanno, K, Chono, M, Honda, I, Kitano, H, Takeda, 2004). A similar correlation between tiller number and plant height has been associated with altered BR responses in rice *dwarf and low tillering (dlt)* mutants (Tong et al., 2009, 2012), indicating BR plays a role in tillering and plant height in both barley and rice. Rice *d61* is a mutant of the *BRASSINOSTERD-INSENSITIVE1* (*OsBRI1*) gene, the ortholog of barley *HvBRI1.*Reduced plant height in *uzu* and *d61* mutants indicate that these BR-receptor genes play similar roles in regulation of stem elongation in barley and rice (Chono et al., 2003). However, no effect on tillering was reported for *OsBRI1*-defective mutants and severe alleles cause complete loss of fertility and malformed dark green leaves (Nakamura et al., 2006), pointing to diverse developmental roles of *BRI* genes in the two species.

In barley, a different link between hormones, plant stature and tillering is supported by *semidwarf1 (sdw1)/denso* (mapped on chromosome 3H), an agronomically important dwarfing gene with pleiotropic effects on tillering (Jia et al., 2011). Although *sdw1* and *denso* are known to be allelic, the two alleles exhibit different phenotypic effects. A barley GA 20-oxidase gene (*Hv20ox2*) has been proposed as a candidate for

sdw1/denso (Jia et al., 2009). Compared to wild-type, *Hv20ox2* expression is reduced four and 60-fold in the *denso* and *sdw1* mutants, respectively. These data indicate that low expression decreased plant height while increasing tillering (Jia et al., 2009) and are consistent with a negative correlation between GA and tillering observed in rice (see above). The rice genomic region collinear to the *denso/sdw1* locus hosts the rice *sd1/Os20ox2* gene, the likely ortholog of *Hv20ox2* (Jia et al., 2009). While *denso/sdw1* and *sd1* have similar phenotypic effects on plant height (Sasaki et al., 2002; Spielmeyer et al., 2002), we could not find information about the involvement of the rice gene in tillering.

Semi-dwarf phenotypes and excessive development of tillers also characterize plants carrying recessive mutations in the *Gra-a* (3H), *Mnd1* (7HL) and *Mnd6* (5HL) genes (Druka et al., 2011). In histological studies, *gra-a* mutants exhibit increased numbers of AXMs and axillary buds, with occasional appearance of two shoot apical meristems (Babb and Muehlbauer, 2003), while the *mnd6* mutant is characterized by the development of side branches from aerial nodes (Babb and Muehlbauer, 2003). Although the genes that correspond to *gra-a* and *mnd* mutations have not been identified, their phenotypes resemble those of rice mutants defective in SL biosynthesis and signal transduction pathways (Ishikawa et al., 2005; Zou et al., 2006; Arite et al., 2007). Characterization of these mutants may thus offer a foundation for the study of the SL pathway in barley.

Abnormal formation of lateral shoots from aerial nodes was also observed upon Virus Induced Gene Silencing of the *P23k* gene, involved in synthesis of (1,3;1,4)-β-D-glucan (Oikawa et al., 2009), suggesting a link between cell wall polysaccharide synthesis and branch development.

Beside these classical mutants, screening of novel mutagenized populations (see below) may uncover new loci and alleles conditioning tiller development. Screening of TILLMore - a population obtained from sodium azide mutagenesis of the reference genotype Morex (IBSC, 2012; Talamè *et al.*, 2008, http://www.dista.unibo.it/TILLMore/) - identified a set of lines exhibiting decreased tillering that our group is currently characterizing.

In addition to mutants, QTLs involved in barley tillering have also been identified. Three QTLs for tiller number were mapped on chromosomes 1H bin 6-8, 2H bin 3 and 6H bin 10-11, each explaining 10.3-15.7% of the phenotypic variation in a cross between cultivated and wild barley (Gyenis et al., 2007) and also four QTLs for tiller number were detected on 1H, 2H, 3H and 4H in a cross of a Syrian barley line, "Arta", with a wild barley (*Hordeum vulgare* ssp. *spontaneum)* (Baum et al., 2003).
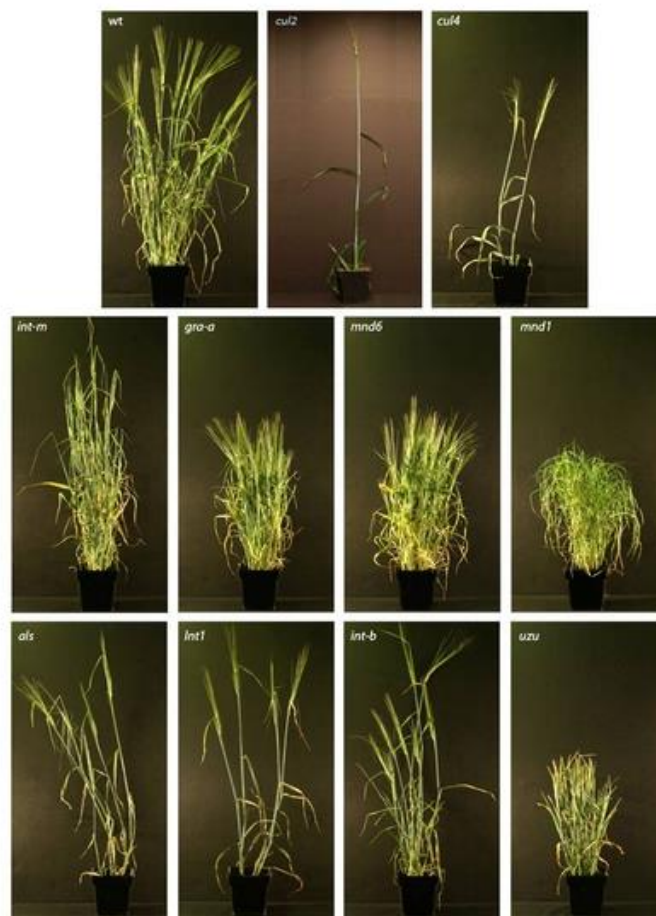
Fig. 3. Phenotypes of barley tillering mutants. The first row contains the wild-type cv. Bowman (wt), and the low-tillering mutants *uniculm2* (*cul2*) and *uniculme4* (*cul4*). The second row represents the high-tillering mutants *inter-medium spike-m* (*int-m*), *granum-a* (*gra-a*), *many noded dwarf6* (*mnd6*), *many noded dwarf1* (*mnd1*). The last row contains the low-tillering mutants *absent lower laterals* (*als*), *low number of tillers1* (*lnt1*), *intermedium spike-b* (*int-b*) and *semi rachitic* (*uzu*). (Photo: courtesy of M. Munoz-Amatriain, Department of Agronomy and Plant Genetics, University of Minnesota (USA))

Table.2. List of barley tillering mutants. For each mutant, the chromosomal position and relevant references are indicated, along with the corresponding (candidate) gene and rice ortholog when known. ND, not determined; INF, inflorescence.

Barley mutant	Map position	Mutant phenotype	Gene (rice ortholog)	References
uniculm2 (*cul2*)	6HL	No tiller, irregular INF	ND	Babb and Muehlbauer, 2003; Okagaki et al., 2013
low number of tillers1 (*lnt1*)	3HL	Few tillers, irregular INF	*JuBel2* (*qSH1*)	Dabbert et al., 2010
absent lower laterals1 (*als1*)	3HL	Few tillers, irregular INF	ND	Dabbert et al., 2009
uniculme4 (*cul4*)	3HL	Few tillers	ND	Babb and Muehlbauer, 2003; Rossini et al., 2006
intermedium spike-b (*int-b*)	5HL	Reduced tillering, irregular INF	ND	Babb and Muehlbauer, 2003; Okagaki and Muehlbauer, unpublished data
semi-brachytic (*uzu*)	3HL	Reduced tillering and plant height, shorten spike	*HvBRI1* (*OsBRI1*)	Babb and Muehlbauer, 2003; Chono et al., 2003; Nakamura et al., 2006
granum-a (*gra-a*)	3HL	High tillering, dwarf, shorten spike	ND	Babb and Muehlbauer, 2003
intermedium -c (*int-c*)	4HS	High tillering, Reduced lateral spikelet development	*HvTB1* (*OsTB1*)	Ramsay et al., 2011
many noded dwarf1.a (*mnd1.a*)	7HL	High tillering, dwarf, irregular INF	ND	Babb and Muehlbauer, 2003; Nice and Muehlbauer, unpublished data
many noded dwarf6 (*mnd6*)/*densinodosum6* (*den6*)	5HL	High tillering, short spike	ND	Babb and Muehlbauer, 2003; Druka et al., 2011; Nice and Muehlbauer, unpublished data
semidwarf1 (*sdw1*)/*denso*	3H	High tillering, reduced plant height	SD1	Jia et al., 2011
intermedium spike-m (*int-m*)	ND	High tillering, irregular INF	ND	Babb and Muehlbauer, 2003

5.   **Concluding remarks**

In this chapter, we aimed to review recent advances in the molecular and hormonal control of tillering in barley and rice, as being two important crops and model plants. We also tried to show the similarities and differences between the two systems, in order to allow for explore the likelihood of transferring knowledge between the two experimental systems, and the opportunities offered by studying the rice genetic and genomic system.

6.   **Scope of the thesis**

Tillering is an important trait for cereal crops and directly affects their productivity. It also represents a model trait to study developmental plasticity in the Poaceae. For these reasons, tillering in cereals has received increasing attention recently While significant progress was achieved in rice (Chapter 1), the complexity and size of the barley genome have delayed the scientific progression of studying genetic components controlling tillering in barley. However, recent genomic advancements have provided novel tools, established numerous databases with billions of entries, creating new opportunities in genetics and genomics. Worth to mention, comparative genomics and synteny studies (Bolot et al., 2009; Jaiswal, 2011) provide researchers with powerful tools to transfer information between related genetic systems.

The overall goal of this PhD project is to contribute to our understanding of the genetic mechanisms controlling tillering in cereal crops like barley and rice, identifying genetic factors involved in regulation of tiller development, and producing a starting point and basic materials for further dissection of tillering in barley and rice.

Among the described tillering mutants in barley, *cul4* was identified as producing very few tillers with pleitropic effects on other morphological traits (Franckowiak and Lundqvist, 2012). Initial efforts mapped the *Cul4* locus on the proximal end of the long arm on chromosome 3 (Pozzi et al., 2003), and defined flanking AFLP markers within 5.5 cM interval. In another study an introgression line of the *cul4* mutation into Bowman background (*cul4*-Bowman) was obtained (Druka et al., 2011). Before the start of this PhD project, our group was able to map the locus between 2 SNP markers 0.22 cM apart, and to identify Candidate Gene (CG) that co-segregated with locus *Cul4* in a fine mapping population (Tavakol et al. unpublished Data,).

Based on this starting point, this PhD project deployed a combination of forward, reverse and comparative genetic approaches to address the following specific objectives:

1) Validation of the aforementioned *Cul4* candidate gene and characterization of its function by analysis of different *cul4* mutant alleles (Chapter 2);

2) Identification and initial characterization of *Cul4* homologues in rice (Chapter 3);

3) Characterization of a novel set of tillering defective lines identified from the TILLMore mutagenized collection (Talamè et al., 2008) as a basis to identify new tillering genes in barley (Chapter 4).

CHAPTER II

VALIDATION OF CANDIDATE GENE FOR LOCUS *CUL4*

1. **Introduction**

As sessile organisms challenged by different environmental conditions, plants have to develop the ability to fit within changing natural habitats. Side branching is an important trait that shapes plant architecture and affects multiple aspects of plant development and productivity. For small grain cereals, tillering– a particular type of side branching – is a key component in plant yield and developmental plasticity under different growing conditions. While, tillering in the Poaceae has received increasing attention, progress in understanding the mechanisms that control this trait came mainly from studies on rice (See chapter 1). Although genetic analyses in rice are facilitated by genomic size and available resources, integrating genetic information from other important crops, like barley and wheat, will provide a broader view of the how cereals incorporate multiple endogenous and environmental signals to control tiller development. A number of barley tillering mutants have been identified and their characterisation has provided insight into the genetic mechanism of vegetative axillary development and tillering in this important crop plant (See chapter 1). However, despite recent progress in genetic and genomic technologies, most genes underlying tillering in the Triticeae tribe still await identification.

Tiller development can be divided into three main stages: i) establishment of the axillary meristem (AXM) in the leaf axil; ii) development of the axillary bud; iii) and outgrowth of the bud to form a tiller (Schmitz and Theres, 2005). The first two stages are mainly controlled by genetic factors, while tiller bud outgrowth is under control of complex network of genetic, hormonal and environmental factors (Kebrom et al., 2013). Development of the AXM follows a precise progression in parallel to the development of the subtending leaf (Oikawa and Kyozuka, 2009), although little is known about the mechanisms coordinating these processes in grasses. A typical grass leaf is organised in three distinct regions along the proximal-distal axis: 1) the proximal sheath is off-set from the 2) distal blade, which are separated by 3) a hinge-like structure comprising

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two wedge-shaped auricles, whereas a fringe-like epidermal outgrowth called ligule occurs on the adaxial leaf surface at the base of the auricle (Sylvester et al., 1990).

In leaf development, primordia are first established at specific sites on the periphery of the meristem. Then local patterning occurs along the three main axes: abaxial-adaxial, proximal-distal, and central-lateral (**Fig.4**). Asymmetrical development along organ axes is a central element of organ patterning. The development of asymmetry is divided into discrete phases. First, an axis is specified by long-range signals or unequal partitioning of a determinant within a cell before division. Second, specification of different identities occurs in domains along the axis. This usually results from activation of different transcription factors that may interact to re-establish the distinctions between domains. Third, short-range interactions can then elaborate the pattern, a process that may be coupled with growth (Hudson, 2000). For leaf particular case, during the vegetative development, primordia are first established at specific sites on the periphery of the meristem. Then local patterning occurs along the three main axes; abaxial-adaxial, proximal-distal, and central-lateral. From that patterning, the cells acquire divergent developmental identities. Finally, cell division and expansion guide organ growth, promoting leaf to obtain its size and shape (Byrne et al., 2000).
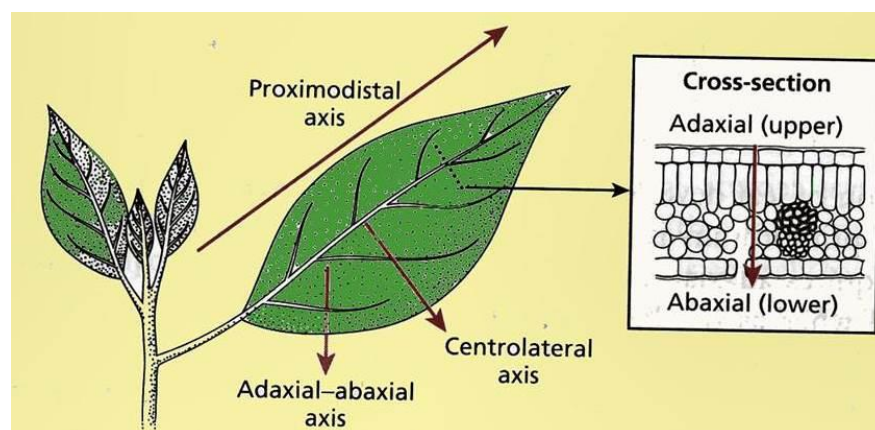
Fig.4. Illustration showing the 3 main developmental axes of dicot leaf: abaxial-adaxial; proximal-distal; and central-lateral axes.

Barley plants carrying recessive mutations in the barley *Uniculme4* (*Cul4*) gene show reduced tillering and characteristic defects in proximal-distal leaf patterning (Franckowiak and Lundqvist, 2012). Objective of the work described in this chapter was the validation and functional characterization of a Candidate Gene (CG) for the barley *uniculme4 (cul4)* mutant.

In a previous project, low-resolution mapping based on AFLP (Amplified Fragment Length Polymorphism) markers positioned the *Cul4* locus at the distal end of chromosome 3HL (Pozzi et al., 2003). Before the start of this PhD project, our group had developed a segregating population comprising 9,949 plants from a cross between a *cul4.5* Nearly Isogenic Line (NIL, Bowman background) and the wild-type parent Morex, delimiting the position of *Cul4* between two SNP markers (Close et al., 2009) 0.22 cM apart. In addition, synteny analysis between the barley genetic region and the collinear Brachypodium, rice and sorghum genomic regions (Mayer et al., 2011) was used to identify one co-segregating Candidate Gene (CG), encoding a protein with BTB/POZ domain (for Broad complex, Tramtrack, Bric à brac, Pox virus and Zinc finger) (Bardwell and Treisman, 1994) and ankyrin repeat (ANK), sharing high similarity with Arabidopsis *BLADE-ON-PETIOLE1* (*BOP1*) and *BOP2* (Ha et al., 2003), pea *COCHLEATA* (*COCH*), and *Medicago truncatula NODULE ROOT* (*NOOT*) (Couzigou et al., 2012). BTB domain proteins are evolutionarily conserved from Drosophila to mammals and are involved in transcriptional regulation (Bardwell and Treisman, 1994; Albagli et al., 1995; Ahmad et al., 1998). Although the BTB/POZ domain is known as DNA-binding motif, but in Drosophila, the BTB domain was proposed not to be directly involved in DNA binding, but rather modulate DNA binding through protein-protein interaction with other BTB domain or other family of proteins (Zollman et al., 1994). Some BTB-domain containing proteins are also known to modulate chromatin condensation (Bardwell and Treisman, 1994). In Drosophila BTB proteins affect pattern formation along the proximal-distal axis of the leg and antenna, and are also required during the morphogenesis of the ovary (Zollman et al., 1994). In Arabidopsis, BOP1 and BOP2 proteins which carry BTB domain were proved to be

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involved in leaf formation along proximal-distal axis by repressing the expression of knotted-like homeobox (*KNOX*) genes (Ha et al., 2003, 2007). In addition to the BTB domain, Arabidopsis BOP1 and BOP2 proteins contain ankyrin domain, making them similar to NPR proteins (<u>N</u>ONEXPRESSOR OF <u>P</u>ATHOGENICITY-RELATED GENES) that are required for Systemic Acquired Resistance (SAR) to many pathogens (Ha et al., 2004; Hepworth et al., 2005; Canet et al., 2012).

Arabidopsis *bop1 bop2* double mutant shows defects in leaf patterning and produces ectopic, lobed blades along the adaxial side of petioles of the cotyledon and rosette leaves, indicating that the corresponding genes are required for correct leaf patterning along the proximal-distal axis (Ha et al., 2003). The Arabidopsis BTB-ankyrin BOP proteins were proved to be involved in leaf formation along proximal-distal axis through the repression of the expression of three class I knotted-like homeobox genes; *BREVIPEDICELLUS* (*BP*), *KNAT2* (for *KNOTTED-like from Arabidopsis thaliana 2*), and *KNAT6* (for *KNOTTED-like from Arabidopsis thaliana 6*) (Ha et al., 2007).

BOP1 and *BOP2* genes showed regulatory effect on cell proliferation and differentiation by up-regulating ASYMMETRIC LEAVES2 (AS2) (Jun et al., 2010). *AS2* encodes a Lateral Organ Boundary (LOB) protein that interacts with AS1 to form a chromatin-remodelling complex required to repress expression of class I *KNOX* genes (Jun et al., 2010). The AS1-AS2 complex acts in silencing of three class I *KNOX* genes in developing leaf primordia: *BREVIPEDICELLUS (BP)*, *KNOTTED-like from Arabidopsis thaliana2* (*KNAT2*), and *KNAT6* (Jun et al., 2010). Suppression of *KNOX* genes in the leaf primordium is required for proper organ differentiation and formation (Ha et al., 2010). Conversely, another class I *KNOX* gene, *SHOOT MERISTEM LESS (STM)*, is known to negatively regulate *AS1, AS2, BOP1*, and *BOP2* in the SAM to maintain meristem cells in a proliferative state (**Fig. 5**; Jun et al., 2010; Ha et al., 2010). Additional players in the *BOP1* and *BOP2* network are two members of the *YABBY* (*YAB*) family of transcription factors, *FILAMENTOUS FLOWER (FIL)* and *YAB3*, which are down-regulated by *BOP1* and *BOP2* (Ha et al., 2010). *FIL* and *YAB3* are known for their role in establishment of organ abaxial identity and leaf lamina expansion (Ha et al., 2010).
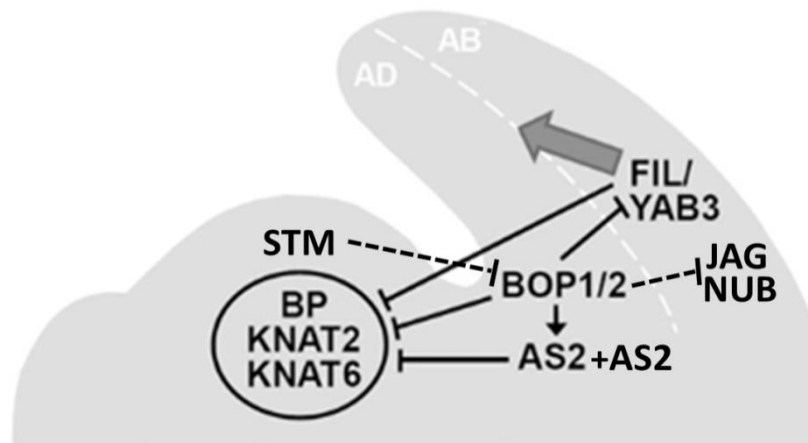
Fig. 5. Model for molecular function of Arabidopsis *BOP1* and *BOP2* genes. BOP1 and BOP2 directly up-regulate AS2, that interact with AS1 to down-regulate class I KNOX genes (*BP*, *KNAT2*, and *KNAT6*), that are also direct target for suppression by BOP1 and BOP2. Members of *YAB* family, *FIL* and *YAB3*, showed to be down-regulated by BOP1 and BOP2,in addition to directly or indirectly repression of *JAG* and *NUB* expression in the proximal domain to pattern the proximal-distal axis (Jun et al., 2010; Ha et al., 2010).

Although BOP1 and BOP2 share significant similarity with wider class of NPR proteins that are involved in salicylic acid (SA)-dependent plant systematic acquired resistance, but phylogenetically, BOP1 and BOP2 are clearly separated and form monophyletic well-supported group. Recently, an additional role of *BOP1* and *BOP2* in plant defence system against pathogens was revealed (Canet et al., 2012). When inoculated with *Pseudomonas* spp., Arabidopsis plants carrying mutant alleles *bop1 bop2* did not show any deviation from wild-type susceptibility (Hepworth et al., 2005), in addition to normal perception of Salicylic Acid (SA) (Canet et al., 2010). Canet et al., (2012) showed that *BOP1* and *BOP2* take part in plant resistance-inducing ability of methyl Jasmonate MeJA (called RIM). Beside affecting leaf development, pea (*Pisum sativum*) and *Medicago truncatula BOP* genes *COCHLEATA (COCH)* and *NODULE ROOT (NOOT)*, play a role in nodule meristem identity and maintenance, which is essential process in the establishment of nitrogen-fixing nodules (Couzigou et al., 2012). Together, available data from dicot species indicate that *BOP* genes are involved in diverse processes and some functional diversification has occurred in this gene family.

2.   **Scope of the chapter**

In order to map the locus at higher resolution, and validate the CG affiliation to *cul4* phenotype, our group planned to use the physical map data provided from partners in FP7 EU project TriticeaeGenome (Schulte et al., 2011), to develop new tight markers, and to design for amplification and sequencing the CG in different mutant stock available for us. The CG was identified as a BTB/ANK gene; homolog to Arabidopsis *BOP* genes, and our attempts in this chapter was to describe - for the first time - the functional characterization of a monocot *BOP*-like gene.

Proving the co-segragation of CG with *Cul4* locus, in addition to showing the recombination between locus *Cul4* and the putative genes in proximity of the CG, would provide mapping-based validation of the CG. Moreover, identifying polymorphism in CG sequence among different mutant and wild-type alleles that corresponds to variation in phenotype will provide additional validation approach for the responsibility of CG for *cul4* phenotype. To carry out those validation plans, we set the following objectives: (1) developing new tight markers based on the physical map and contig sequence provided by partners. Markers would allow mapping the gene at higher resolution, and proving co-segregation of *cul4* with the CG, in addition to estimation of physical-map distance ration; (2) amplifying the CG sequences from different genetic stocks (mutants and wild-type) through genomic PCR, and sequence the reaction product to identify polymorphism, and possible mutations explaining the deviation in phenotype; (3) detailed quantitative and qualitative phenotyping for *cul4* mutant lines in comparison to their respective wild-type backgrounds, to identify the different traits affected by mutation in locus *Cul4*.

3. Materials and Methods

3.1 Phenotyping analysis

Plant materials and growth conditions

For phenotyping, different *cul4* mutants and their respective wild-type backgrounds were planted in greenhouse in Tavazzano (Lodi, Italy) over two growing seasons; first of February 2011 till end of June 2011, and end of January 2012 till end of June 2012, in randomised complete block experiment.

Year 2011

The genotypes investigated during year 2011 are listed in (**Table. 3a,b).**

Table. 3a. Mutant genotypes investigated in the phenotypic analysis year 2011

Mutant name	Background	Mutagen	Collection	Accession number	Author
uniculm4.5 (***cul4.5/cul5***)	Bonus	x-ray	Nordic Genetic Resource Center	NGB115063	Udda Lundqvist
*unicum4.5-*Bowman	5 backcrosses of *cul4.5* into Bowman background to obtain near isogenic line				
uniculm4.15 (***cul4.15/cul15***)	Foma	N-ethyl-N-nitrosourethane	Nordic Genetic Resource Center	NGB115064	Udda Lundqvist
uniculm4.16 (***cul4.16/cul16***)	Bonus	Neutrons	Nordic Genetic Resource Center	NGB115065	Udda Lundqvist

Table. 3b. Wild-type genotypes investigated in the phenotypic analysis year 2011

Cultivar	Row type	Growth habit
Bonus	2-rows	spring
Bowman	2-rows	spring

Around 30 seeds were sown for each genotype in 2011 experiment. A single seed was sown in each plastic pot (13.5 × 13.5 × 15 cm, ca. 2 L) filled with normal field soil from Tavazzano, Lodi (Italy). The environmental conditions were the natural conditions

of temperature and photoperiod in Lodi (Italy) between first of February and end of June 2011 in the first experiment, and end of January 2012 to the end of June 2012 in the second experiment. Irrigation was carried out 2-3 times a week to keep soil wet at 60% field holding capacity. The fertilization programme was performed to keep favourable nutritional conditions for seeds germination and growth, and was applied as following: post-emergence (3-4 weeks after sowing), Urea solution (200 g/100 L); during tillering stage (5-6 weeks after emergence), Ammonium Nitrate (50 g/100 L), Calcium Nitrate (50 g /100 L), Magnesium Sulphate (50 g/100 L) Potassium Phosphate (50 g/100 L), Fe and microelements (10 g/100 L); during ear emergence (16-20 weeks after emergence), the same fertilizers and doses as during tillering application. During the ear emergence (16-20 weeks after emergence), plants were sprayed with Folicur® Bayer (active ingredient: Tebuconazole 4.3 %) fungicide at rate of 150 ml/ha (plant spacing was 30 plant per m^2).

Year 2012

The genotypes investigated during year 2012 are listed in (**Table. 4a,b**). Around 20 seeds were sown for each genotype in 2011/2012 experiment. The same experimental design, soil mixture, programme of irrigation, fertilization, and pesticide treatment were used as described in Year 2011 experiment settings.

Table. 4a. Mutant genotypes investigated in the phenotypic analysis year 2012

Mutant genotype	Background	Mutagen	Collection	Accession number	Author
uniculm4.3 (*cul4.3/cul3*)	Bonus	Ethylene oxide	Nordic Genetic Resource Center	NGB115062	Udda Lundqvist
uniculm4.5 (*cul4.5/cul5*)	Bonus	x-ray	Nordic Genetic Resource Center	NGB115063	Udda Lundqvist
uniculm4.15 (*cul4.15/cul15*)	Foma	N-ethyl-N-nitrosourethane	Nordic Genetic Resource Center	NGB115064	Udda Lundqvist
uniculm4.16 (*cul4.16/cul16*)	Bonus	Neutrons	Nordic Genetic Resource Center	NGB115065	Udda Lundqvist
uniculm4.24 (*cul4.24/cul24*)	Flare	Neutrons	Nordic Genetic Resource Center	NGB119360	Udda Lundqvist

Table.4b. Wild-type genotypes investigated in the phenotypic analysis year 2012

Cultivar	Row type	Growth habit
Bonus	2-rows	spring
Flare	2-rows	spring

Quantitative and qualitative measurements

Year 2011

At the end of the flowering stage phase (end-May, 5 months after sowing) in 2011, 25 parameters were measured (see **Table. 5 and Fig. 6**), and developmental phases were recorded on 3 dates.

Year 2012

At the end of the flowering stage phase (end-May, 5 months after sowing) in 2012, 22 parameters were measured (see **Table. 6 and Fig. 6**), and developmental phases were recorded on 8 dates.

Table. 5. Parameters measured in year 2011

no	Parameter	Description
Vegetative parameters		
1	height	The distance from the ground level to spike collar of the main culm (the highest culm)
2	no nodes	Considering ground nodes of the main culm as one node, and counting upward till spike collar (not counted as node)
3	1st lowest internode	Counting the ground nodes in the main culm as the 1st lower node, and the upward internodes as the 1st lowest internode and moving upward counting the next as 2nd lowest, then 3rd lowest.
4	2nd lowest internode	
5	3rd lowest internode	
6	1st highest internode	Counting the internode between spike collar and the downward node (1st highest node) in the main culm as the 1st highest internode, then the successive as the 2st highest internode and moving downward counting the next as 3nd highest internode.
7	2nd highest internode	
8	3rd highest internode	
9	leaf blade 2nd upper length	Counting the flag leaf as the 1st upper leaf in the main culm of the plant, then moving downward counting 2nd upper leaf, then 3rd upper, … The length of the leaf blade was measured from tip of leaf blade till ligule, Leaf blade width was measured in the middle one-third of the leaf
10	leaf blade 2nd upper width	
11	leaf blade 4th upper width	
12	leaf blade 4th upper length	
13	leaf blade 6th upper length	
14	leaf blade 6th upper width	
15	max tiller	The tiller with the greatest culm height
16	min tiller	The tiller with the lowest culm height
17	no tiller	Total number of side branches
18	leaf with auricle-like ectopic growth	Number of leaves showing ectopic auricle-like growth along the leaf sheath in the main culm
Spike parameters		
19	Spike length	The length between spike collar and spike tip in the main culm
20	Spike with awns length	The length between spike collar to the awns tip in the main culm
21	no spikelets	Total number of spikelets with floral organs (not counting infertile lateral spikelets in 2-rows cultivars
22	spike weight	The weight of the whole spike (include grains, infertile spikelets, awns, and rachis)
23	fertility	The number of grains was subtracted from the total number of spikelets, and the result was calculated as percentage to the total number of spikelets
24	No grains	Number of grains was counted in the main culm spike
25	Total grain weight	All grains were detached from the spike and were weighed

no	Parameter	Description
Calculated parameters		
26	One grain weight	Total grain weight/no of grains

Table. 6. Parameters measured in year 2012

no	Parameter	Description
Vegetative parameters		
1	height	The distance from the ground level to spike collar of the main culm (the highest culm)
2	no nodes	Considering ground nodes of the main culm as one node, and counting upward till spike collar (not counted as node)
3	1st lowest internode	Counting the ground nodes in the main culm as the 1st lower node, and the upward internodes as the 1st lowest internode and moving upward counting the next as 2nd lowest, then 3rd lowest.
4	2nd lowest internode	
5	3rd lowest internode	
6	1st highest internode	Counting the internode between spike collar and the downward node (1st highest node) in the main culm as the 1st highest internode, then the successive as the 2st highest internode and moving downward counting the next as 3nd highest internode.
7	2nd highest internode	
8	3rd highest internode	
9	1st upper (flag) leaf blade lenght	Counting the flag leaf as the 1st upper leaf in the main culm of the plant, then moving downward counting 2nd upper leaf, then 3rd upper, … The length of the leaf blade was measured from tip of leaf blade till ligule, Leaf blade width was measured in the middle one-third of the leaf on proximodistal axis
10	1st upper (flag) leaf blade width	
11	2nd upper leaf length	
12	2nd upper leaf width	
13	3rd upper leaf length	
14	3rd upper leaf width	
15	4th upper leaf length	
16	4th upper leaf width	
17	max tiller	The tiller with the greatest culm height
18	min tiller	The tiller with the lowest culm height
19	no tiller	Total number of side branches
20	leaf with auricle-like ectopic growth	Number of leaves showing ectopic auricle-like growth along the leaf sheath in the main culm
Spike parameters		
21	Spike length	The length between spike collar and spike tip in the main culm
22	Spike with awns length	The length between spike collar to the awns tip in the main culm

Fig. 6. Schematic diagram representing the main parts of the barley plant, and highlight the morphological position of parameters measured in the phenotypic analysis experiment.

The measurements were analysed statistically using XL Toolbox add-in Excel sheet. Test for outliers were made to exclude all data point with extreme values by Grubbs' test (on-line calculator; http://www.graphpad.com/quickcalcs/Grubbs1.cfm), ANOVA was run, and post-hoc Bonferroni-Holm test carried out. Normality of data was tested graphically to ensure the eligibility of data to run ANOVA test.

3.2 Candidate gene validation: high-resolution mapping

Previous work (before the start of this PhD project) using markers developed based on synteny with Brachypodium chromosome 2 and rice chromosome 1 genomic regions, resulted in mapping of the *Cul4* locus to an interval of 0.22 cM and identification of a Candidate Gene (CG) co-segregating with the locus (Bradi2g608710), and two flanking genes (Bradi2g60705 and Bradi2g60720). The mapping population consisted of 4,949 F3 individuals from a cross between *cul4.5*-Bowman and Morex, which yielded the most polymorphic markers. Fine mapping was based on analysis of 55 plants carrying recombination events around the *cul4* locus. To further define the position of the *cul4* locus, Bacterial Artificial Chromosome (BAC) contig (FPcontig_460) of the barley physical map (Schulte et al., 2011) was identified by sequence homologous search of the candidate gene (Bradi2g60710) and one of the flanking genes in Brachypodium (Bradi2g60705) sequences on the barley genomic sequence information (IBSC, 2012). A BAC clone (HVVMRXALLeA0131P08) (ca. 170 kb) was fully sequenced using a 454/Roche GS FLX platform (courtesy of IPK, Gatersleben, Germany) and sequence reads were assembled into contigs after trimming adaptors and vector sequences, and excluding short reads that may cause ambiguity, following the methodology described by Steuernagel et al. (2009). Contigs sequences were annotated to identify additional genes using Triannot pipline gene prediction programme (Leroy et al., 2012; http://wheat-urgi.versailles.inra.fr/Tools/Triannot-Pipeline) in the *Cul4* region. The new markers were developed by designing specific genomic PCR primers on the contigs sequences that will amplify the target region in the two parents Morex and *cul4.5*-Bowman. The PCR amplicons were Sanger sequenced at the Genomic Platform (Parco Tecnologico Padano), and sequences from the parents were compared to identify polymorphism (Single Nucleotide Polymorphism 'SNP'). Polymorphic markers were then mapped on 55 recombinants (F4 heterozygous individual carry recombination event between 2825-1609 and 3_1500 markers that were developed in previous work before this PhD project was initiated). Primers that were used to amplify the polymorphic markers are stated in **Table. 7**.

Table. 7. Primers used to identify SNP markers on the sequence of the two parents (*cul4.5-*Bowman and Morex) and genotype the mapping population (55 individuals showing recombination around the CG location (*cul4.5*-Bowman x Morex)

Marker name	Brachypodium ortholog/ primer	Forward primer	Reverse primer
ET_umil0110	Bradi2g60720	TTTCATGGCTGTGCTTTCAG	GGCAGCCAGTAATTTCGTGT
ET_umil0106	Bradi2g60705	AACCCTGGCGATTACTTGTG	GTACCGTACGTCGGTCTCGT
ET_umil0108	Bradi2g60710	AGCATGAACCTGAGCTTGGA	TGAATGTAGAGCCTAACGAACA
EP_umil0107	ISBP1	TTTCCTTTCTTGCCAGCCTA	ACATCACGGGCATCACCTAA
EP_umil0109	ISBP2	TTTATTCCGTTTGGACTCCG	AGGAGCCCAAGAAAATCGTT

3.3 Candidate gene validation: allelic comparison

The candidate gene was re-sequenced in the three available allelic mutant stocks *cul4.5* (KF151193), *cul4.16* (KF151195) and *cul4.24* (KF151196) and the respective backgrounds Bonus (KF151192) and Flare (KF151194) using primers described in **Table. 8**, and the amino acids substitution impact on the protein function was evaluated using http://sift.bii.a-star.edu.sg/www/SIFT_seq_submit2.html

Table. 8. Primers used to amplify the CG in different genetic stocks for allelic comparison

Primer name	Forward primer	Reverse primer
Exon 1	ACGGCTTCTTCCACTCCTCT	GGTTGGTTATGTTGGGATCG
Exon2	CGGTCTCTCCATGCCATATT	GAGATCGGTCGACGAGAATG
Intron	GTGCTCCAGTTCCTGTACA	GAGGACGTGATGAAGGTGCT
5'upstream-1	TTTGAGGTTGCAATGGCTCT	ATCAAAAGAGATCGGGCGAT
5'ustream-2	CAGTCAAAGCATGGCACACT	GGTTGGTTATGTTGGGATCG
5'upstream-3	CAGTGAAGTCACGGCAAGAA	GGTTGGTTATGTTGGGATCG

DNA extraction

Leaf tissue samples were lyophilised under vacuum at -50°C (Christ ALPHA 1-2 LD plus) for a minimum of 3 days, then stored at -20°C in vacuum bags. For DNA extraction, 20 mg of lyophilized tissue were initially ground with a QIAGEN Tissuelyser Retsch®MM300 Mixer Mill with metal tungsten carbide beads in QIAGEN Collection Microtubes (1.2 ml) capped with Collection Microtube Caps in TissueLyser

Adapter Sets, shaking at 30 rps for 3 min. The tissue powder was then supplemented with 600µl MATAB solution (20 mM EDTA pH 8.0, 100 mM Tris-HCl pH 8.0, NaCl 1.5 M, 0.5% (W/V) Na_2SO_3, 2% (W/V) hexadecyltrimethylammonium bromide (MATAB), Polyethylene glycol (PEG) solution 1% (W/V) pre-heated at 65°C. The suspension was incubated at 65°C for 15 min, then 10µl of RNase RNasiA (20mg/ml Invitrogen) were added to each sample, and the sample was incubated for 30 min at 65°C. The sample was then centrifuged at 4500 xg for 10 min. The supernatant was taken by pipetting to new tube, and precipitation of tissue debris was discarded. Mixture of chloroform/isoamyl (24/1) is added by 450 µl rate for each sample. The sample was mixed by inverting gently and then centrifuged at 6,200 xg for 10 min. The supernatant was pipetted into new tube (1.5 ml), and 150µl of isopropanol (pre-chilled to -20°C) were added to each sample. The sample was incubated at -20°C to allow precipitation of nucleic acids for 30 min, and then centrifuged to collect them at 4,500 xg for 10 min at 4°C. The isopropanol phase is discarded, while paying attention not to disturb the pellet of the nucleic acids. After addition of 800µl of ethanol 70%, each sample was mixed well and centrifuged again at 4,500 xg. This washing step was repeated twice. The pellet of nucleic acids was left to dry in a Vacufuge® vacuum concentrator (Eppendorf) at 35°C and 35 xg for 20 min., and finally resuspended in 40-50 µl of sterilised water. DNA quantity and quality was measured on NanoDrop 1000 Spectrophotometer (Thermo Scientific).

Genomic PCR

The purified DNA extracted from plant seedlings were adjusted to the concentration of 50ng/µl, and were used as a template to amplify the CG in different genetic stocks. Also the same Genomic PCR protocol was used to amplify genomic regions in parents (Morex and *cul4.5*-Bowamn) to develop new markers, and later to map them on the mapping population. The PCR reaction mixture was combined as presented in **Table. 9**

Table. 9. Genomic PCR cocktail

Reagent	Vol. per reaction (total vol 25μl)	Final concentration
ReadyMix™ Taq PCR Reaction Mix 2x (Sigma)	12.5μl	1x (1.5 units *Taq* DNA polymerase, 10 mM Tris-HCl, 50 mM KCl, 1.5mM MgCl$_2$, 0.001% gelatin, 0.2 mM dNTP, stabilizers
Primer Forward	1μl	0.4μM
Primer Reverse	1μl	0.4μM
DNA sample (50ng/μl)	1.5μl	3ng/μl
Molecular Grade water	9μl	

The thermal cycler machine (BIORAD PCR thermal cycler™) was programmed for the following: 94°C for 4 min, then 35 cycles of 94°C for 30 seconds, then annealing temperature between 52°C and 57°C (depend on melting temperature of the primers; the PCR annealing temperatures were 3-4 degrees less than the lowest melting for the primer pair) for 30 seconds, then 72°C for 1.5 minutes, then final extension phase at 72°C for 10 min. The PCR products were analysed on agarose gel 1.5% in TAE buffer 1x run at 80 V. The agarose gel was supplemented with 4μl of ethidium bromide solution (3%) to stain the PCR product that was visualised under UV light.

RNA extraction

Around 200 mg of freshly collected barley seedlings (2nd leaf not completely unfolded) were ground in pre-chilled mortars. The tissue powder was collected in tube, and 1ml of TRIzol (Invitrogen) reagent was added to each sample and mixed by inverting the tube gently. The mixture was incubated at room temperature for 5 min, then 200 μl of chloroform were added to each sample, slowly on the tube wall, the tube was inverted repeatedly to mix, and left at room temperature for 10 min. Samples were centrifuged at 15,000 xg for 10 min at 4°C. The supernatant (the aqueous phase) was transferred by pipetting into a clean tube, and 500 μl of pre-chilled isopropanol (-20°C) were added to each sample. Samples were left at 4°C for 10 min and centrifuged at 15,000 xg for 10 min at 4°C to precipitate nucleic acids. The supernatant was discarded

by pipetting; minding not to disturb the nucleic acid pellet, then the pellet was washed with 1 ml of 70% ethanol and centrifuged at 15,000 xg to re-precipitate RNA. The washing step was repeated twice, then the RNA pellet was left to dry at room temperature for 10-20 min. RNA was re-suspended by adding 40-50 µl of ultrapure water. RNA quality and quantity were evaluated by NanoDrop 1000 Spectrophotometer (Thermo Scientific), as well as checking RNA integrity on 1.5% agarose gel to ensure the typical migration pattern of two RNA bands (28s and 18s rRNA).

cDNA synthesis

The starting material for cDNA synthesis was 1 µg of RNA in a 3µl volume. The first step was to degrade any genomic DNA by adding 2µl of gDNA wipeout (Qiagen) plus 9µl of ultrapure water to each sample (to total volume of 15 µl), and then samples were incubated at 42°C for 5 min. Quanti tech RT kit (Qiagen) was used for the reverse transcriptase reaction. The reaction mixture was as following: 1µl of reverse transcriptase enzyme, 4µl of the buffer solution (Mg^{2+} and dNTPs), 1µl of primer mixture (containing oligo dT and random primers), and 14µl of the processed sample. The mixture was incubated at 42°C for 30 min, and then the reaction was blocked by incubating at 95°C for 3 min.

Sanger sequencing

The DNA sample to be sequenced was treated initially with ExoSap (New England Biolabs) by mixing 5µl of sample to 2µl of the reagent, and incubated at 37°C for 15 min followed by 80°C for 15 min. The BigDye® Terminator Cycle Sequencing Kit (Applied Biosystems) was used for sequencing. The reaction mixture was as constituted as presented in **Table. 10**

Reactions were incubated in 96 well plates, in a BIORAD PCR thermal cycler™ under the following thermal conditions: 25 cycles of [96°C for 10 sec, 50°C for 10 sec, 60°C for 4 min]. The reaction mixture then purified by ethanol precipitation as follows:

for each reaction, 2.5µl of EDTA and 30µl of absolute ethanol were added, then the whole 96-well plate was centrifuged for 45 min at 625 xg. The supernatant was discarded by inverting the plate, then 30µl of pre-chilled ethanol (-20°C) were added to each well, and centrifugation was run again at 625 xg for 15 min. The supernatant was discarded, and the plate was incubated at room temperature in the dark to allow ethanol residues to evaporate, while protecting the reaction dye from being degraded by light. At the end, 10 µl of formamide was added to each well, and reactions were submitted to the Genomic Platform at Parco Tecnologico Padano, Lodi, for capillary electrophoresis on an AB3730 capillary instrument DNA analyser (Applied Biosystems).

Table. 10. Sanger sequencing reaction mixture

Regent	Initial conc.	Volume added	Final conc.
BIG DYE v. 3.1 (Ready Reaction Mix)	10 ×	1	1 ×
Sequence buffer	5×	2	1x
Primer	10 µM	0.8	800 nM
ultra-pure molecular grade water	-	3.2	-
purified DNA		3	
Total reaction mix		10	

4.   Results

4.1 Detailed phenotyping of *cul4* mutants

In order to evaluate the phenotypic effects of mutations in the *Cul4* gene and compare severity of different alleles in relation to their molecular lesions (see section 3.3), a total of 9 different genotypes were investigated; 3 wild-type background varieties, and 6 different *cul4* mutant lines, over two growing seasons in 2011 and 2012.

Effects of mutations at the Cul4 locus on plant vegetative phenotypes

Plants carrying mutations at the *Cul4* locus generally have stiff thick stem, dark green curling leaves that rolled on their main vein axis (**Fig. 7** and **Fig. 9**). Plants homozygous for the *cul4.3*, *cul4.5* (both in the Bonus and Bowman background), *cul4.15*, and *cul4.16* alleles showed ectopic outgrowths of auricle-like tissue on the sheath margin of some leaves (**Fig. 8**). Such ectopic outgrowths appear especially more pronounced in the flag leaf, and the 2-3 leaves below the flag leaf. All *cul4* mutant lines were reported to have a liguleless phenotype, in contrast to wild-type backgrounds that develop normal ligules (**Fig. 10**) (Tavakol et al., unpublished data). Although all *cul4* mutant alleles show liguleless phenotype, the boundary region between the sheath and blade remains intact with auricle tissue grow at the normal location (Tavakol et al., unpublished data) (**Fig. 10**). Strong bending at nodes was occasionally observed in some mutant lines, particularly *cul4.5*, *cul4.15*, and *cul4.16*, and sometimes the bending affected also the internode showing more than 40º degrees bending angle (**Fig. 7**).

Compared to their wild-type backgrounds, *cul4* mutant lines showed significant reduction in number of tillers in both years 2011 and 2012, and often those fewer tillers were distorted and retarded in development (**Fig. 7** and **Fig. 9**). Mutant lines *cul4.3*, *cul4.5*, *cul4.5*-Bowman and *cul4.15* -carrying deletion mutations (see section 4.3 in this chapter) - showed the most severe reduction in number of tillers (**Fig. 11**). As for point

mutations (see below), *cul4.16* and *cul4.24* showed outgrowth of significantly less number of tillers, and compared to their respective wild-type background Bonus and Flare, respectively.

Plant height is another important agronomic trait affected by mutations at the *Cul4* locus (**Fig. 12**). Mutant line *cul4.24* showed significant increase in plant height reaching (118±15 cm), which is double the height of the Flare wild-type background (61±3.5cm). A significant difference in plant height was also detected between the *cul4.5*-Bowman line and wild-type Bowman. Deletion and point mutation lines in Bonus and Foma e.g. *cul4.3*, *cul4.5*, *cul4.15*, *cul4.16*, showed slight increase in plant average height, although not consistently significant over the two years of experiments, and obviously is affected by the genetic background and other unknown factors. Also, in terms of variability in number of nodes, mutant lines did not show significant differences compared to wild-type varieties (**Fig. 13**). Each internode was measured and compared to other mutant and wild-type lines in order-based way, e.g. comparing apical stem internodes among different genotypes, and comparing basal stem internodes among different genotypes, considering the order rather than the internode number. In mutant lines that showed significant increase in plant height, it was shown than most of the difference in height came from elongation in the top (apical) stem internodes, particularly the internode below the spike: in the *cul4.24* mutant length of this internode reached 2.5x fold the length of the respective internode in the wild-type background (16.5 cm). Similar differences were seen for the 2nd and 3rd upper internode. The same trend was seen in *cul4.5*-Bowman and its corresponding wild-type background Bowman (**Fig. 14, Fig. 15**, and **Fig. 16**).

Leaves showed variable phenotypes depending on the type of allele and the genetic background. In 2011, the leaf measurements were for 2nd, 4th, and 6th upper leaves, while the upper 4 leaves were measured in 2012. Significant differences in flag leaf length and width were observed in deletion mutation *cul4.3*, *cul4.5*, and *cul4.15* compared to the Bonus and Foma backgrounds, while relatively less increase was seen in *cul4.16* compared to the wild-type background in 2012 experiment. In contrary, flag

leaf dimensions did not show any change in *cul4.24* compared to Flare (**Fig. 17**). The leaf directly below the flag leaf, the 2nd upper leaf, showed variability in terms of length and width in mutant lines compared to wild-type background, with non-consistently significant increase in leaf length and width over the two years experiments in case of *cul4.5* and *cul4.16* (**Fig. 18**), while *cul4.5*-Bowman and *cul4.24* did not show any significant difference in leaf length. The 3nd upper leaf – that was measured only in 2012 - showed increase in leaf blade length and width in *cul4.3*, *cul4.5*, *cul4.15*, and *cul4.16* compared to wild-type background, while no significant difference was seen in *cul4.24* in terms of leaf blade length in 2012 data (**Fig. 19**). In older leaf, the 4rd upper leaf, no significant differences were noticed in terms of leaf blade length between the mutant lines and the wild-type cultivars, although all mutant alleles of *cul4* showed significant increase in 4th upper leaf width (**Fig. 20**).

Fig. 7. *cul4.5* mutant plant compared to wild-type Bonus. a: whole plant *cul4.5*, b: the ground nodes showing no outgrowth of tillers, c: *cul4.5* spike with distorted, twisted and lax appearance, d: whole plant wild-type Bonus, e: the ground nodes showing multiple growth of tiller, f: spike of wild-type Bonus with normal architecture.
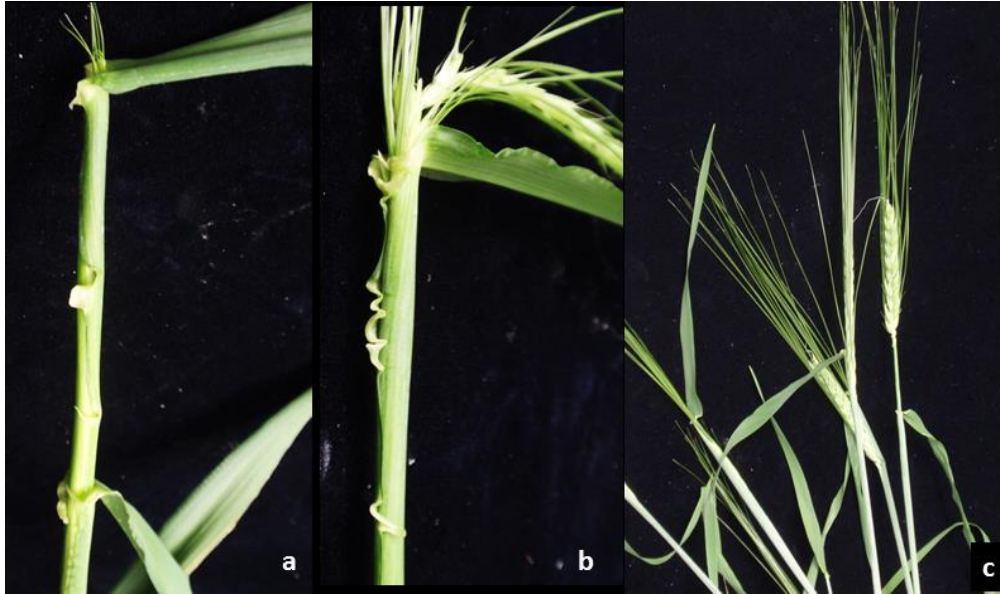
Fig. 8. Ectopic auricle-like growth along the leaf sheath of *cul4* mutants. a: auricle-like growth in *cul4.5*, b:*cul4.15*, c: wild-type Bonus
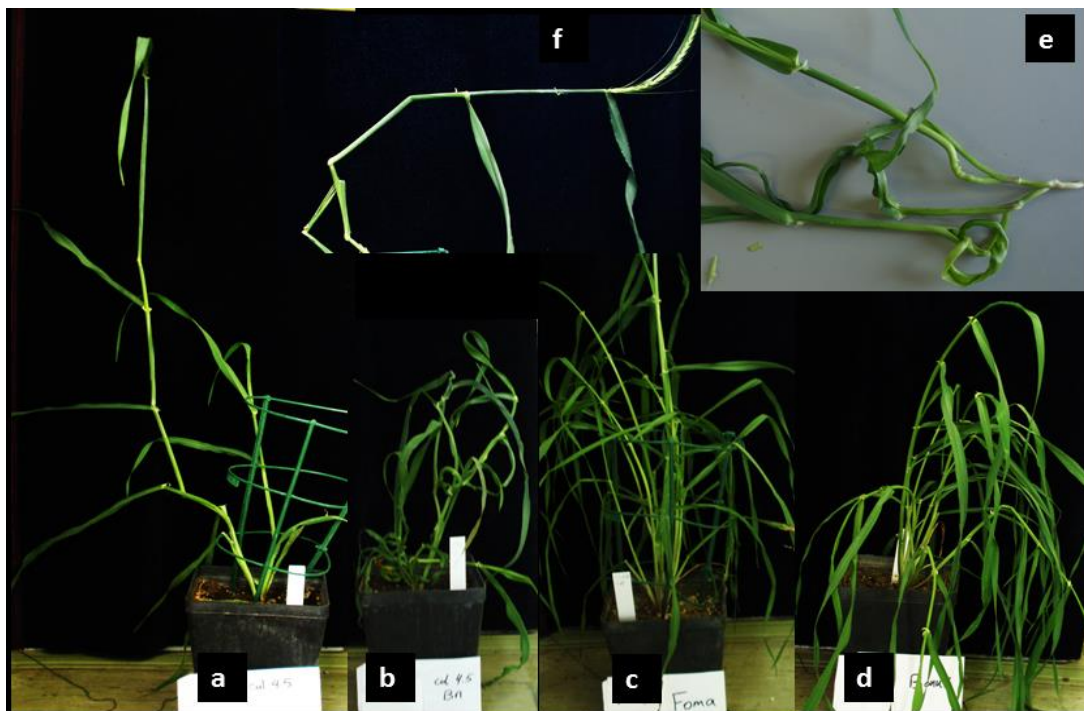
Fig. 9. Low number of tillers and bend internodes. a: *cul4.5*-Bowman, b: *cul4.5*-Bonus, c: Foma: d: Bonus, e: cul*4.15*
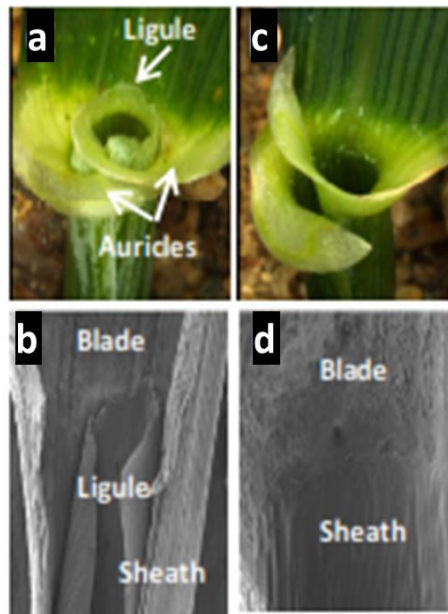
Fig. 10. Liguleless phenotype of *cul4* mutant. a: Wild-type Bowman development of boundary region between leaf sheath and leaf blade, where the ligule position and development appear normal, b: scanning electron microscopy image for the boundary region in wild-type Bowman, c: *cul4.5*-Bowman mutant line showing liguleless phenotype at the boundary region, d: scanning electron microscopy image for the boundary region in *cul4.5*-Bowman mutant line (Photo: courtesy to Elahe Tavakol; Tavakol et al. unpublished data).

Fig. 11. Effect of mutations in *cul4* locus on the number of tillers in two experiments, 2011 and 2012.

Fig. 12. Effect of mutations in *cul4* locus on plant height in two experiments, 2011 and 2012.

Fig. 13. Effect of mutations in *cul4* locus on plant number of nodes in two experiments, 2011 and 2012.

Fig. 14. Effect of mutations in *cul4* locus on the length of the most upper internode in two experiments, 2011 and 2012.
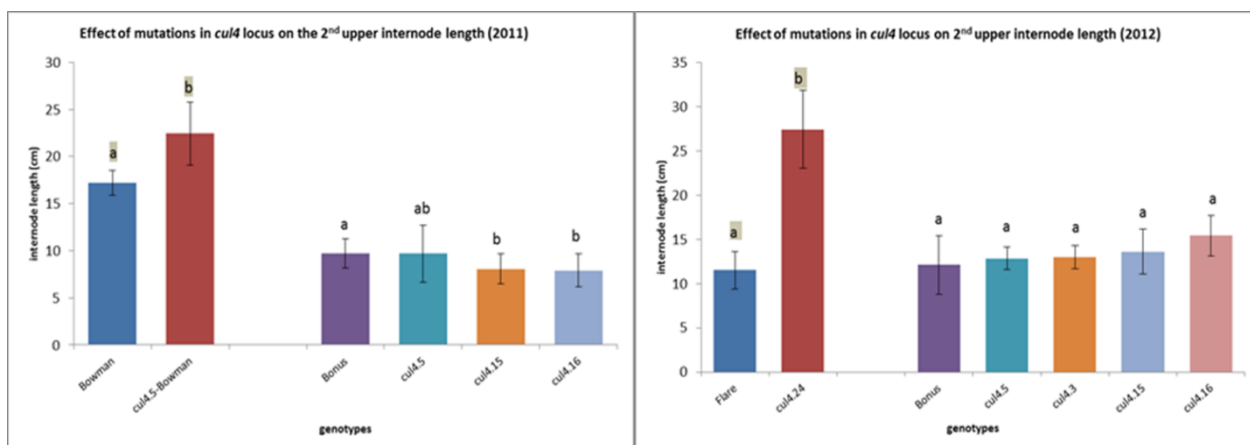
Fig. 15. Effect of mutations in locus *cul4* on the length of the 2nd upper internode in two experiments, 2011 and 2013.
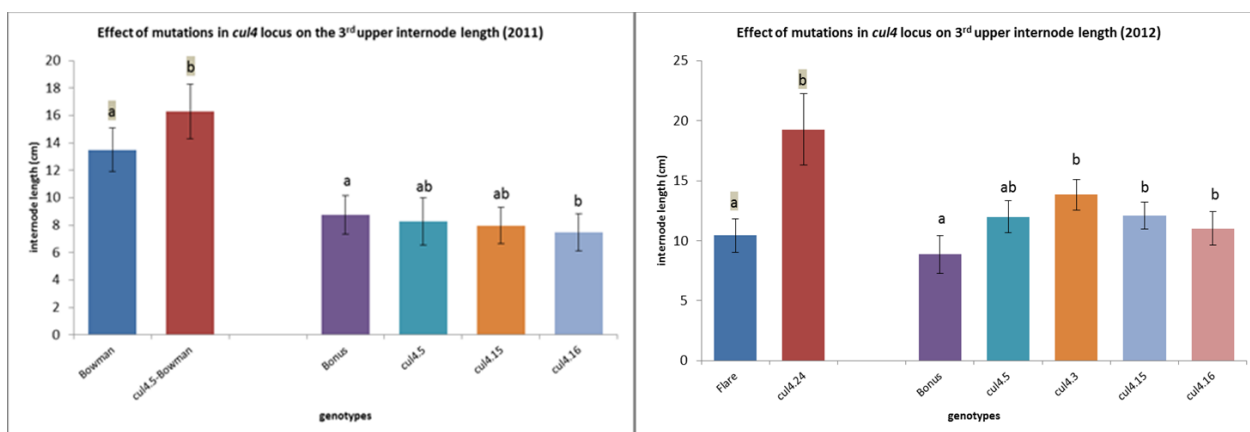
Fig. 16. Effect of mutations in *cul4* locus on the length of 3rd upper internode in two experiments, 2011 and 2012.
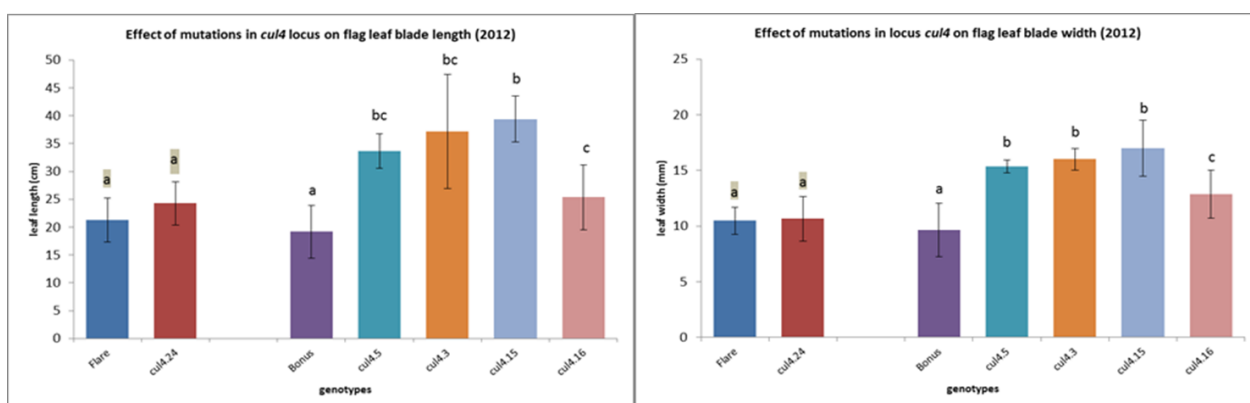
Fig. 17. Effect of mutations in *cul4* locus on flag leaf blade dimensions in 2012 experiment.
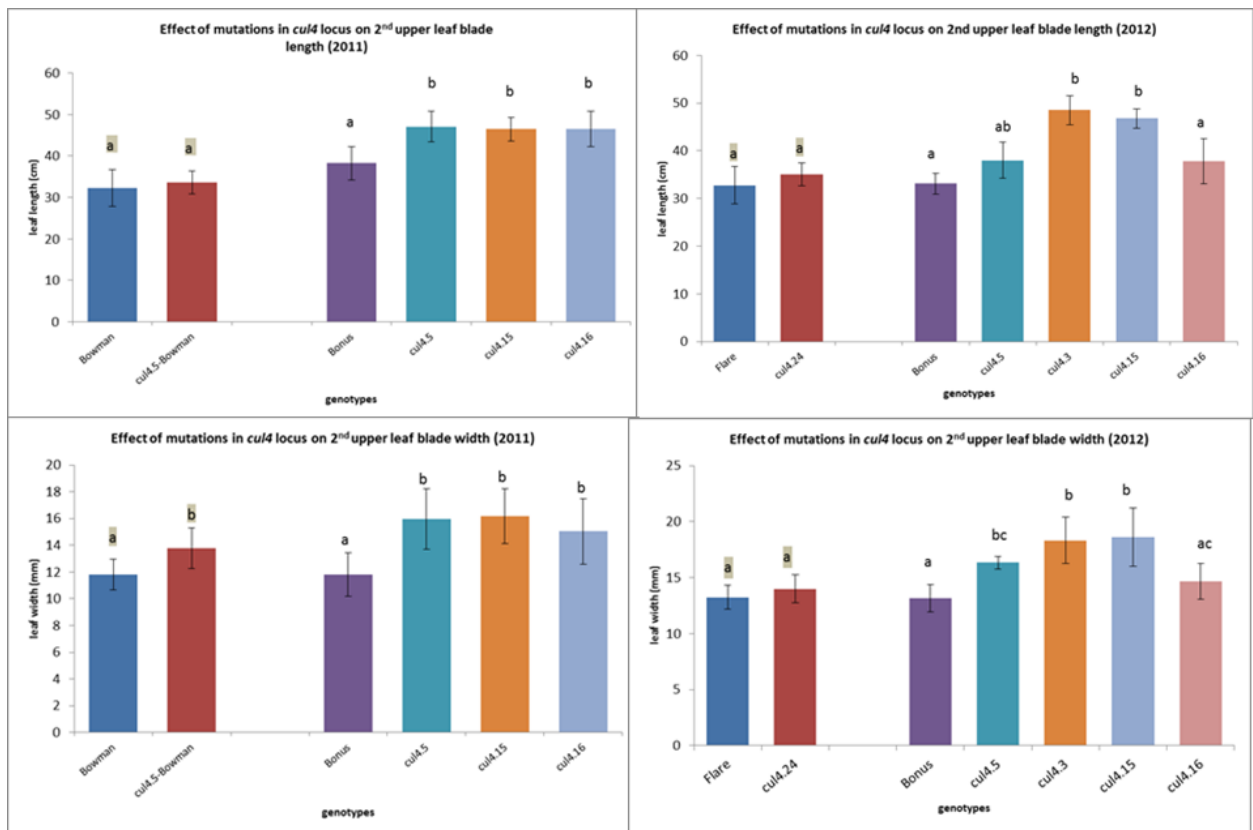
Fig. 18. The effect of mutations in *cul4* locus on 2nd upper leaf blade dimensions in two experiments 2011 and 2012.
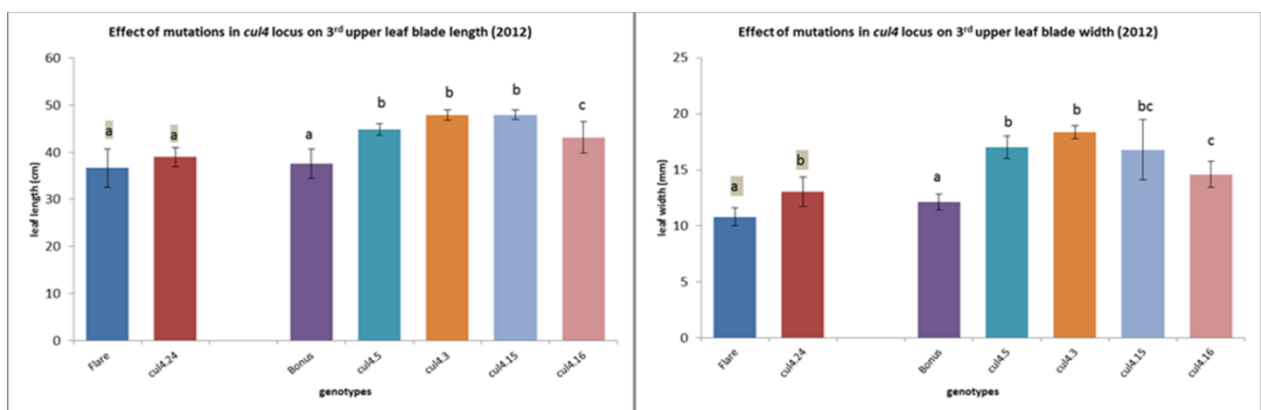
Fig. 19. Effect of mutations in *cul4* locus on 3rd upper leaf blade dimensions in 2012 experiment.
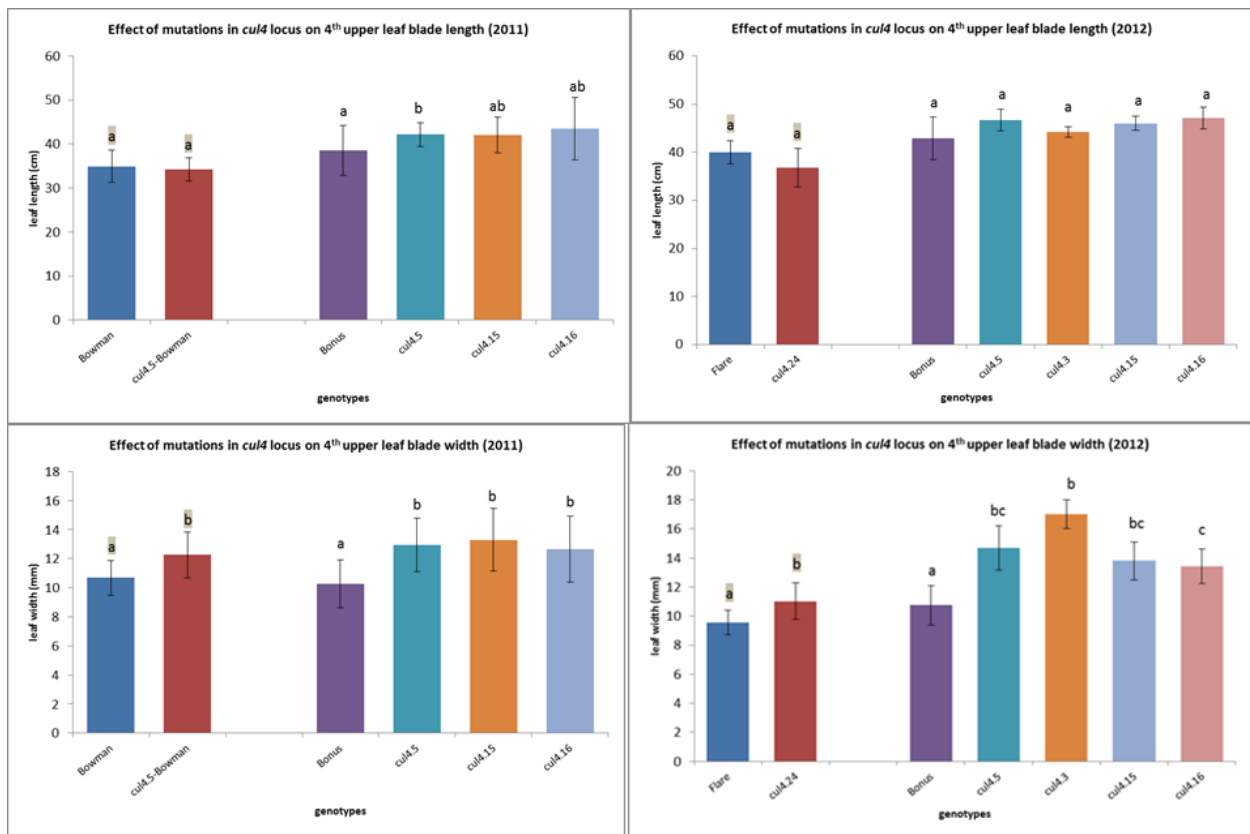
Fig. 20. Effect of mutations in *cul4* locus on 4th upper leaf dimensions in two experiments, 2011 and 2012.

Effects of mutations in Cul4 locus on overall plant development

Mutations at the *Cul4* locus affect plant development during the transition from vegetative to reproductive phases, particularly during booting, ear emergence, and flowering phases. Zadoks' cereal development decimal scale was used to express the development stage of the plants (**Fig. 22**, Zadoks et al., 1974), and data were collected on 3 dates in 2011, and on 8 dates in 2012. Mutant lines *cul4.3, cul4.5*-Bonus, *cul4.15,* and *cul4.16*, start booting (flag leaf sheath extending GS41) 11-8 days before their respective wild-type backgrounds, ear emergence (GS51-GS59), flowering (GS61-69), and grain watery ripe (GS71) stage are also anticipated compared to the respective background wild-types (**Fig. 21**). Similar trends were evident also for *cul4.24* compared to Flare,

from booting through flowering, watery ripening, early and late dough and up to complete ripening stage (**Fig. 21**).
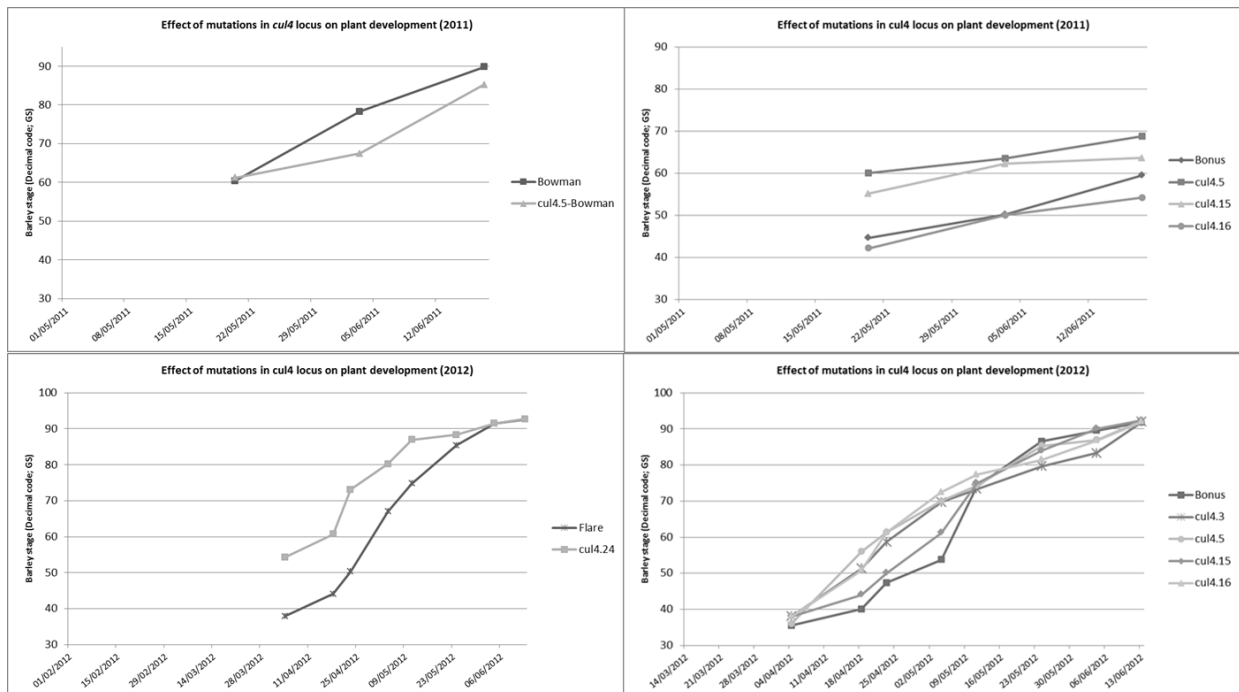
Fig. 21. Effect of mutations in *cul4* locus on plant development in two experiments, 2011 and 2012.

Zadoks Growth Stage	GS 00 - 09	GS10 - 19	GS20 - 29	GS30 - 39	GS40 - 49
Development phase	Germination	Seedling growth	Tillering	Stem elongation	Booting

Zadoks Growth Stage	GS 50 - 59	GS60 - 69	GS70 - 79	GS80 - 89	GS90 - 99
Development phase	Ear emergence	Flowering	Milk Development (grain fill period)	Dough Development (grain fill period)	Ripening
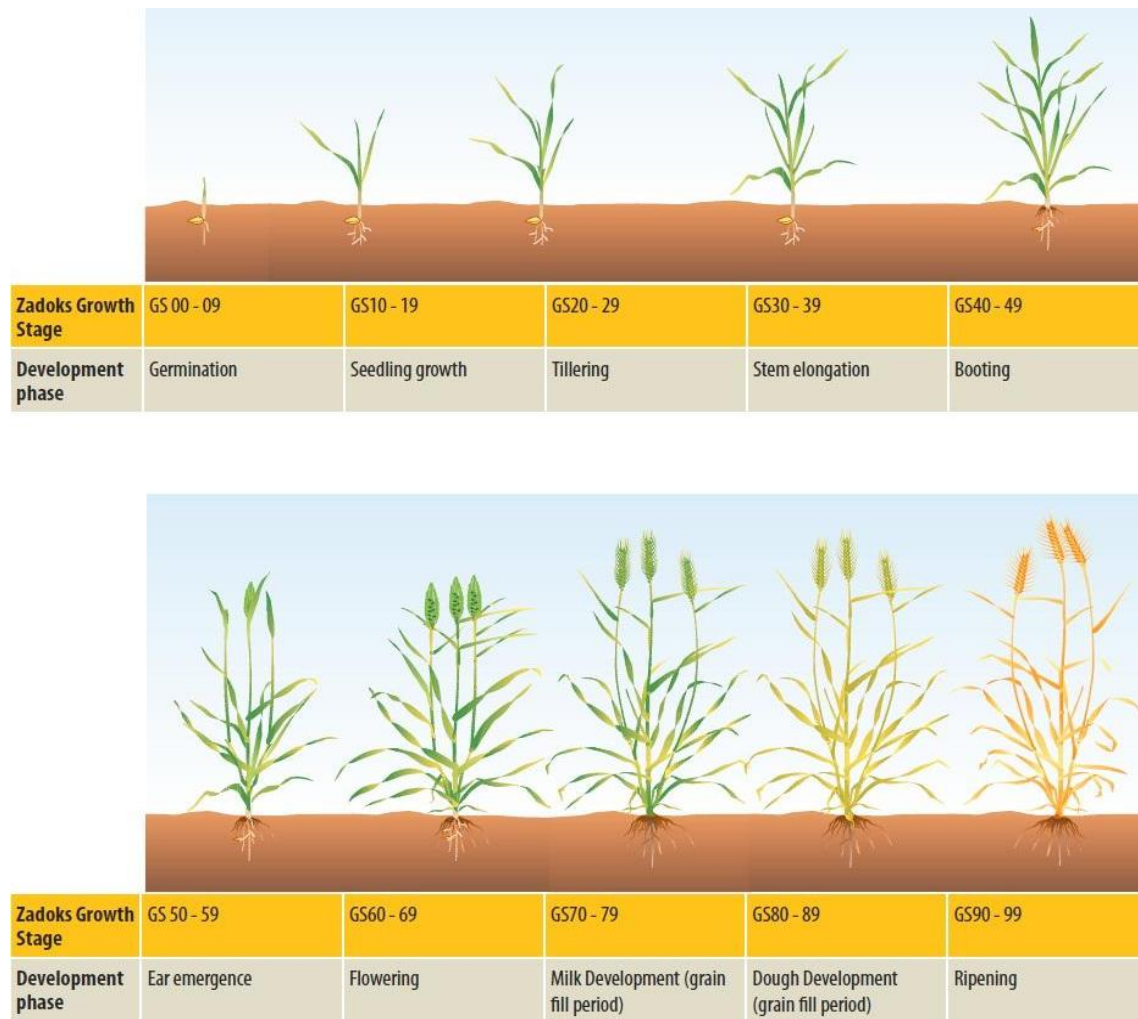
Fig. 22. Zadoks' cereal development decimal scale (Zadoks et al., 1974).

Effects of mutations in Cul4 locus on inflorescence phenotype:

The mature spikes of *cul4* mutants generally are longer with a lax appearance and longer rachis internodes which appear sometimes distorted and twisted (**Fig. 23**). The spikes of severe mutations like in *cul4.5*-Bonus and *cul4.15* often had difficulties in emergence and remained enclosed in the flag leaf sheath, until fully mature and dry. The awns of *cul4.5*-Bonus and *cul4.15* sometimes showed twisted and disorganised appearance (**Fig. 23)**. The spike length varies among *cul4* mutants reflecting the severity of mutations. Deletion mutations in Bonus and Foma backgrounds result in longer spikes e.g. *cul4.5* and *cul4.15*, and less severity in point mutation lines, e.g. *cul4.16*. The

cul4.24 mutant (substitution mutation) in Flare background showed average spike length significantly different from other deletion mutations. The *cul4.5*-Bowman line showed significant increase in spike length compared to the respective wild-type background, and also different from the same mutation in the Bonus backgrounds, indicating that genetic background influences the effect of this *cul4* allele on spike length (**Fig. 24**). The effect of *cul4* mutations on "spike with awn" length was less consistent: longer "spike with awns" were seen in mutants *cul4.5*, *cul4.15*, and *cul4.16* compared to their corresponding backgrounds Bonus and Foma statistically significant but more modest increase was observed for *cul4.5*-Bowman vs. the Bowman background, while for *cul4.24* no significant difference with the Flare background was observed (**Fig. 25**).
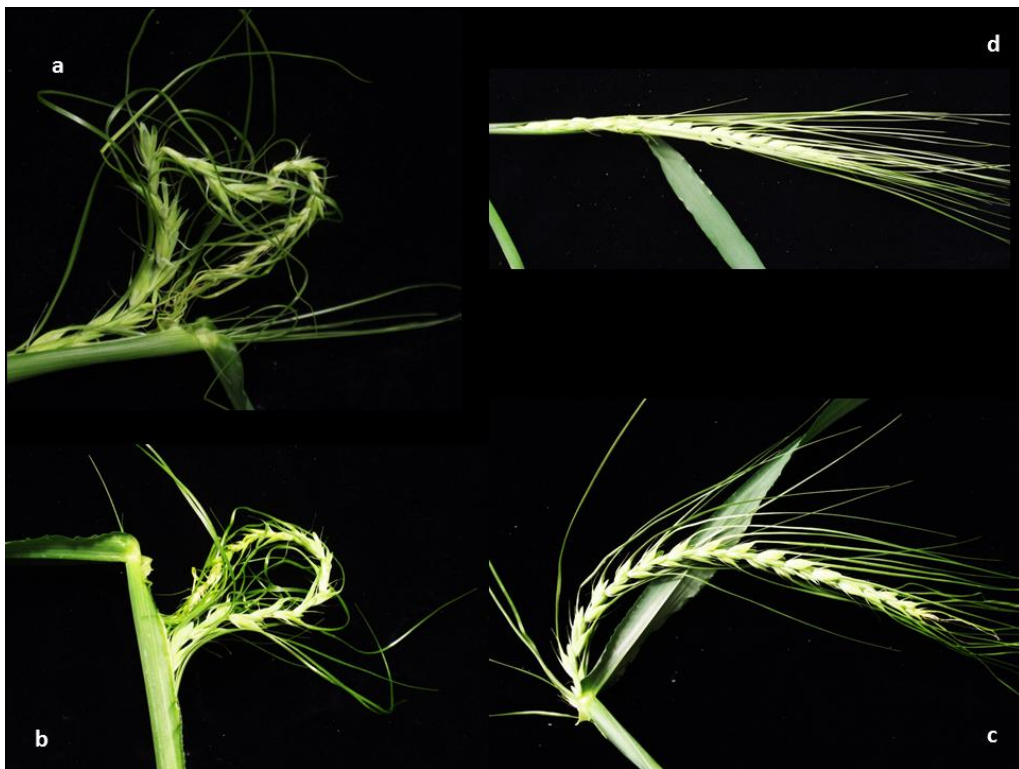
Fig. 23. Defects of spike architecture in *cul4* mutant lines. a: spike of *cul4.5* showing twisted appearance and malformation, b: spike of *cul4.15* showing defect in architecture and failure of growing out the flag leaf sheath with disorganised awns growth, c: spike of *cul4.5*-Bowman illustrating the increase in total spike length, rachis internode elongation, and loose organisation of spikelets, d: spike of wild-type Bonus showing the normal development.
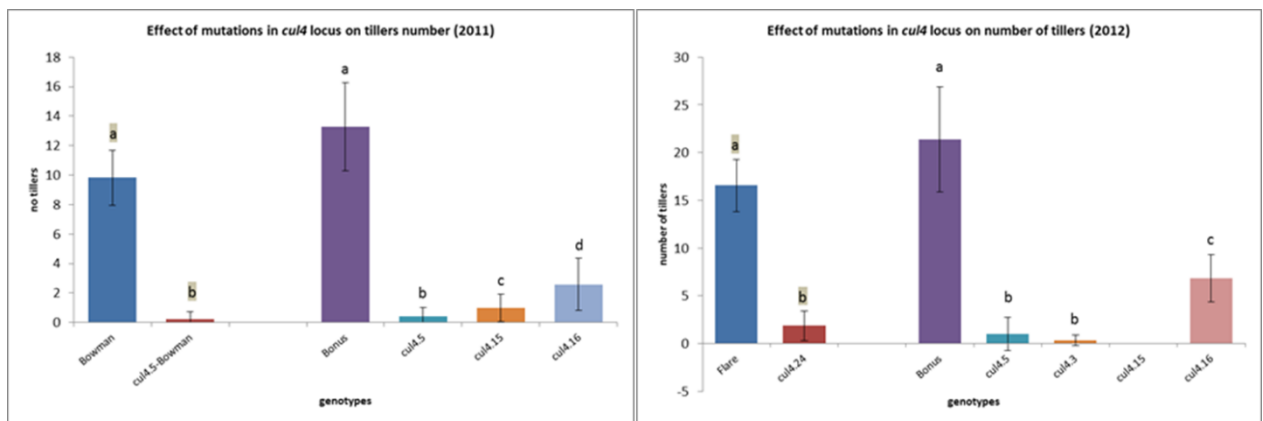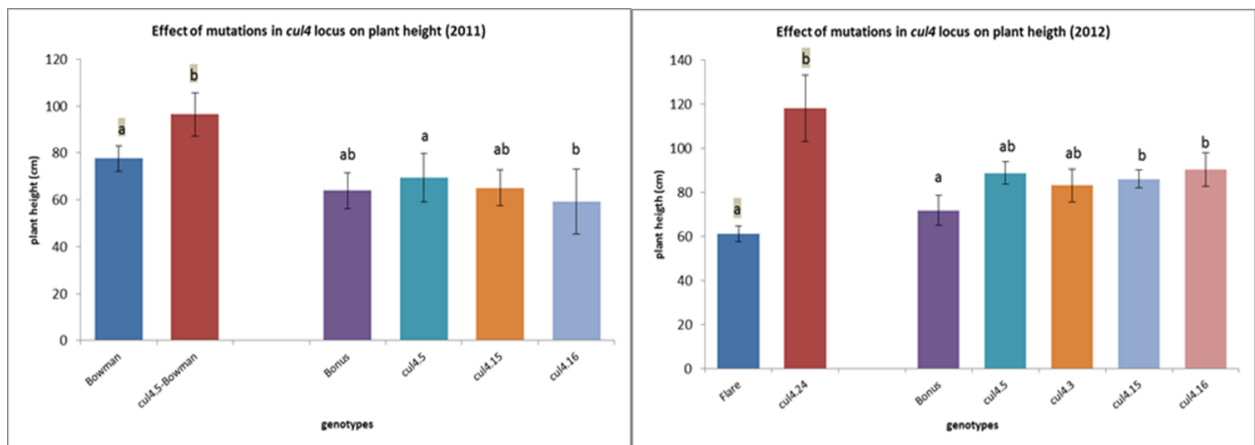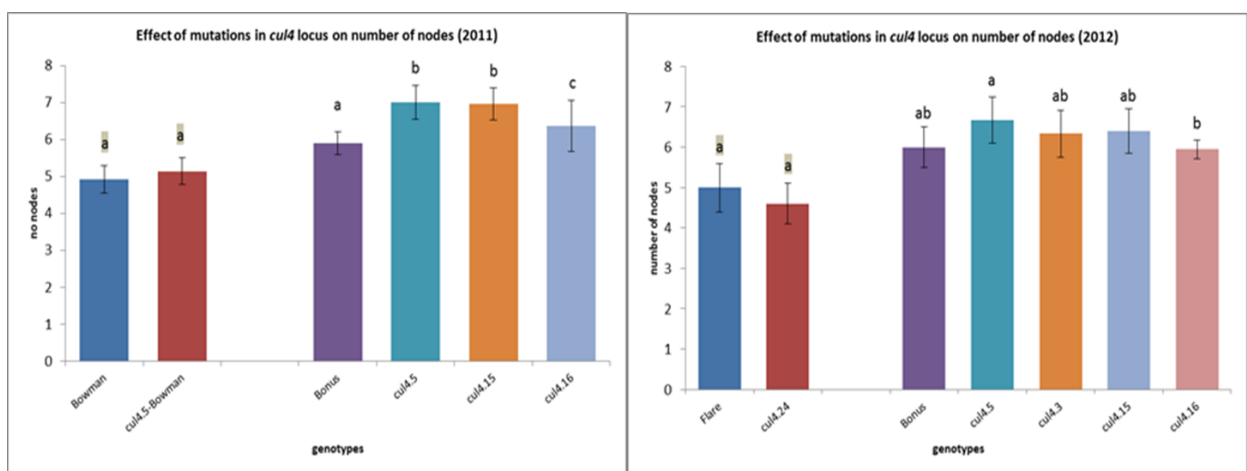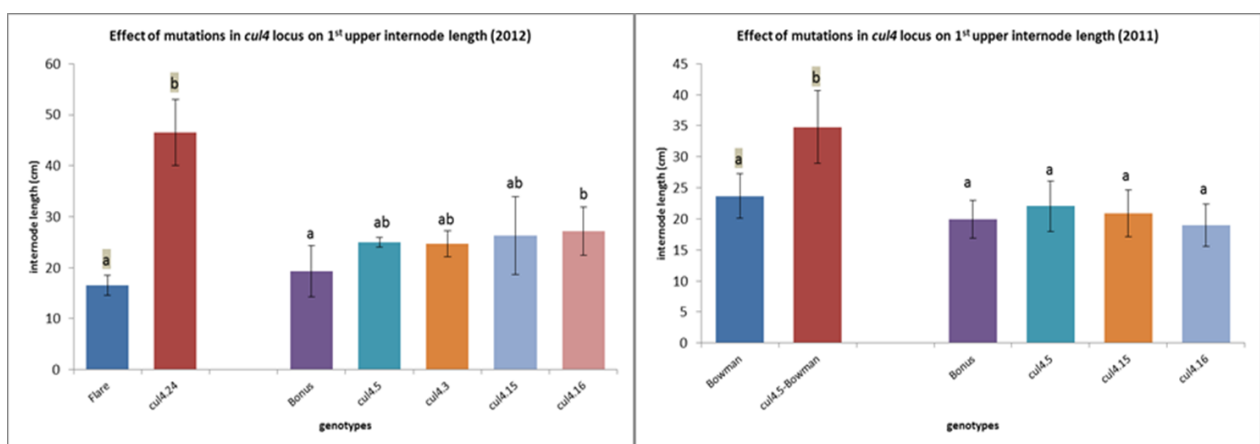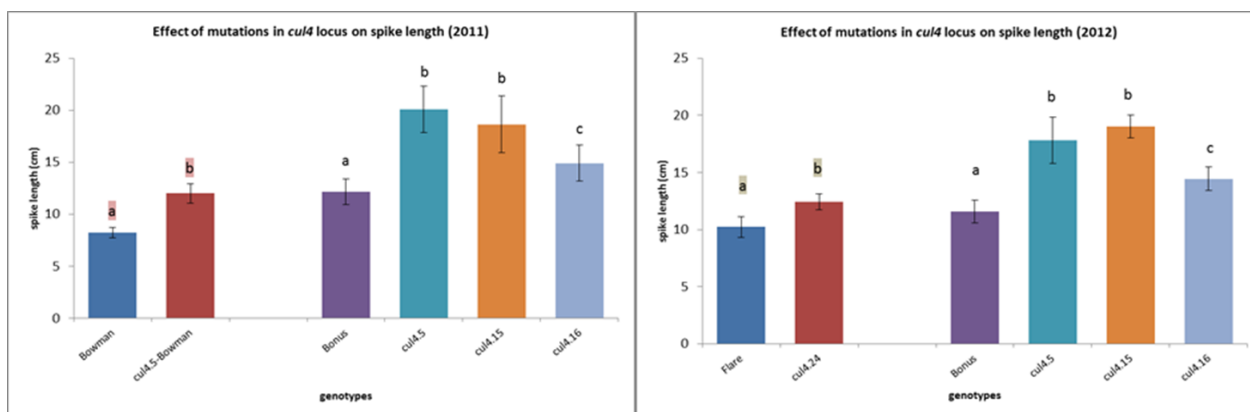
Fig. 24. Effect of mutations in *cul4* locus on spike length in two experiments, 2011 and 2012
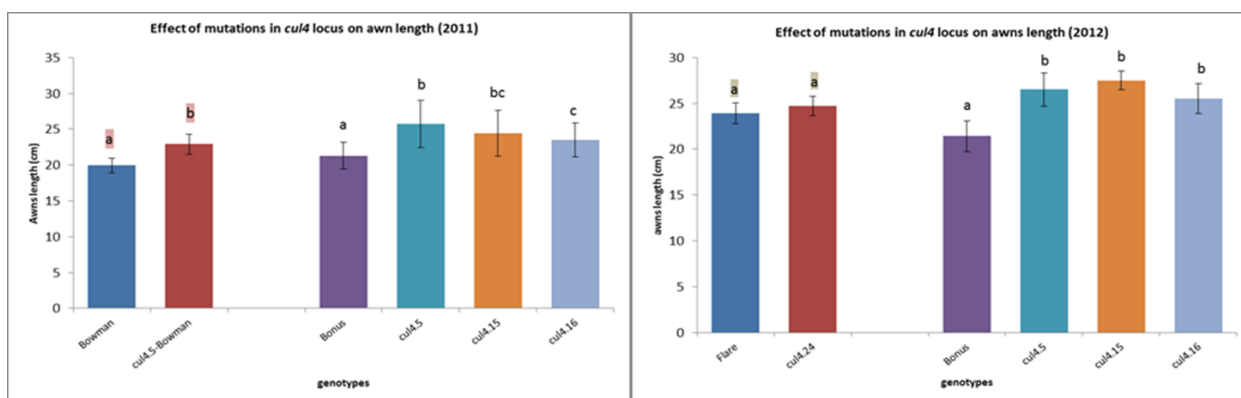
Fig. 25. Effect of mutations in *cul4* locus on "spike with awns" length in two experiments, 2011 and 2012.

Some of the effects of the mutations in locus *cul4* on the reproductive organs were only investigated in one year experiment, e.g. infertility ratio, number of spikelets, number of grains, weight of spike, and total weight of grains. Severe mutations in locus *cul4*, e.g. *cul4.5* and *cul4.15*, reduced the fertility in almost 99% of spikelets compared to 40% in wild-type Bonus and Foma. The low fertility in wild-type (40%) was due to drought stress challenged the plants during the flowering stage. While *cul4.5*-Bowman and *cul4*.16 did not show significant difference in fertility compared to Bowman and

Bonus respectively (**Fig. 26)**. High infertility in mutants *cul4.5* and *cul4.15* was also reflected in the reduction of the overall spike weight - ranging 0.4-0.45 g compared to spike weight of 0.6-0.8 g in wild-type Bonus and Foma (**Fig. 27**). Interestingly, for mutant plants with no significant difference in fertility compared to wild-type, the overall weight of spikes was significantly higher than wild-type, e.g. *cul4.16* showed spike weight of almost 1 g compared to 0.6-0.8 g in the wild-type background, and 1.5 g for *cul4.5*-Bowman compared to 1 g in the wild-type Bowman background (**Fig. 27**). To gain more insight into these phenotypic effects, average grain weight was calculated by dividing the total grain weight by the number of grains. All investigated mutant lines showed significant increases in grain weight, ranging between 0.03-0.05 g in *cul4.5* and *cul4*.16 compared to 0.025-0.035 g per grain in wild-type Bonus and Foma. Mutant *cul4.5*-Bowman also showed increase in grain weight, compared to Bowman (**Fig. 28**). Severe deletion mutants *cul4.5* and *cul4.15* also showed significant increase in number of spikelets ranging 32-34 spikelet per spike, compared to 26-28 spikelets in wild-type Bonus and Foma. However deletion mutation *cul4.5*-Bowman showed no difference in spikelet number compared to wild-type Bowman, suggesting the effect was influenced by the genetic background. No difference was found also in the case in less severe substitution mutation *cul4.16* (**Fig. 29**).
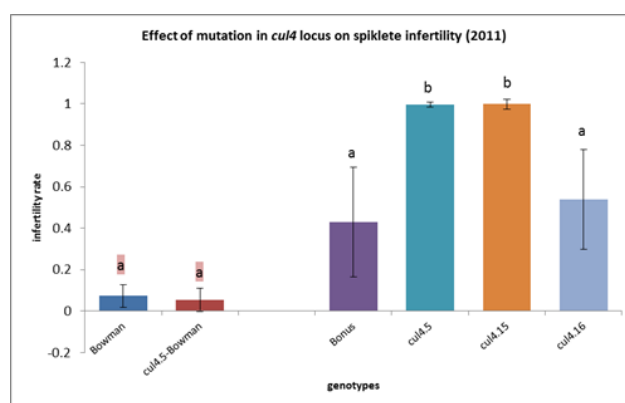
Fig. 26. Effect of mutations in *cul4* locus on reproductive organs fertility in 2011 experiment.
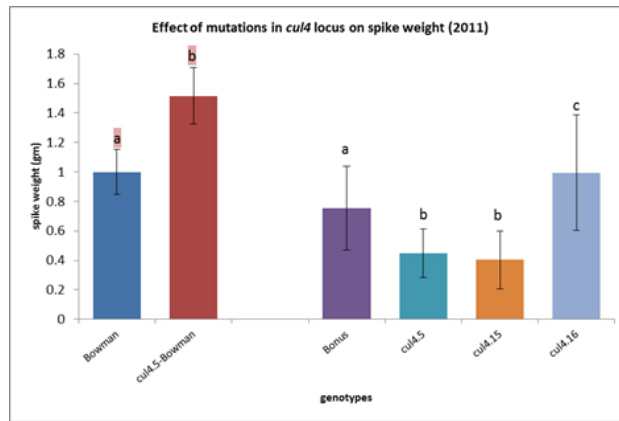
Fig. 27. Effect of mutations in *cul4* locus on spike weight in 2011 experiment.
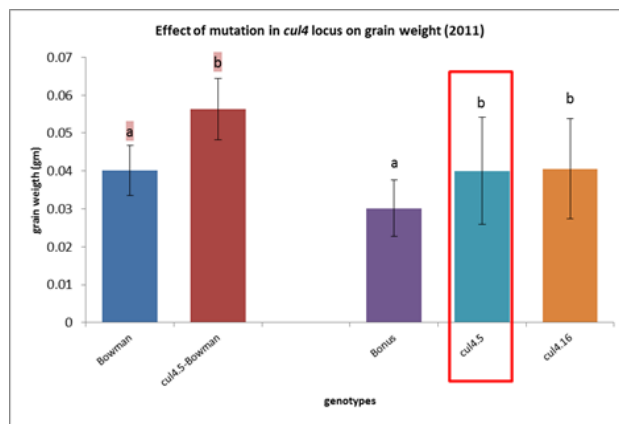
Fig. 28. Effect of mutations in *cul4* locus on one grain weight (calculated from dividing total grain weight/number of grains. The data from *cul4.5* was highlighted because it is based only on 3 grain weight, which weakened the confidence in the analysis.
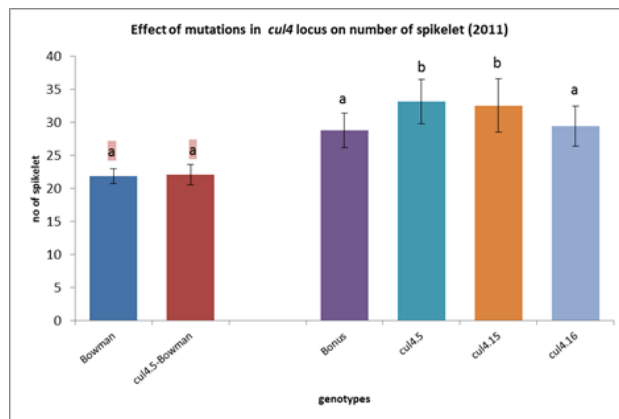
Fig. 29. Effect of mutations in *cul4* locus on number of spikelets per one spike of the main culm, in 2011 experiment.

4.2 Fine mapping of *Cul4*

Previous work carried out by our group allowed mapping of the *cul4* locus within a 0.22 cM interval, flanked by two genes that were identified on Brachypodium genome (Bradi2g60705 and Bradi2g60720), and co-segregating with the barley ortholog of BTB-ankyrin candidate gene Bradi2g608710 (see **Fig. 30** and **Fig. 31**).

To further refine the position of the *cul4* locus, identify and evaluate recombination/cosegregation of *cul4* with additional genes in the target genomic interval, sequence information from the barley physical map (collaboration with Dr. Nils Stein, IPK, Gatersleben, Germany) was queried with the co-segregating and flanking genes leading to the identification of a Bacterial Artificial Chromosome (BAC) clone (HVVMRXALLeA0131P08) matching the candidate gene and barley ortholog of the proximal flanking Brachypodium gene Bradi2g60705. Data from 454 sequencing of the BAC clone were obtained from IPK, and sequence reads were assembled into contigs with 22x coverage. The sequence was used to develop new markers by designing specific primers for genomic PCR amplification of the target region in the two parents; Morex and *cul4.5*-Bowamn. The amplicons were Sanger sequenced and compared to identify SNP markers that were then mapped on the mapping population of 55 recombinants. Successful amplification and sequencing of the target region allowed identification of tightly linked two polymorphic markers 'SNP'. Mapping the newly developed 2 markers on the fine-mapping population yielding better mapping resolution with new flanking markers 0.02 cM distal and 0.07 cM proximal to *cul4* proving recombination events between the co-segregating candidate gene and the other flanking annotated genes (**Fig. 31**). The two predicted genes annotated from this BAC (encoding a pentatricopeptide repeat (PPR-like)-containing protein and a hypothetical protein, respectively) showed recombination with the *cul4* locus, reinforcing the proposed correspondence between the BTB-ankyrin candidate gene and the *cul4* mutant locus. The fine-mapping allowed the estimation of physical-genetic distance in the region to be 1 cM ~ 1.1 Mb (**Fig. 31**).

67
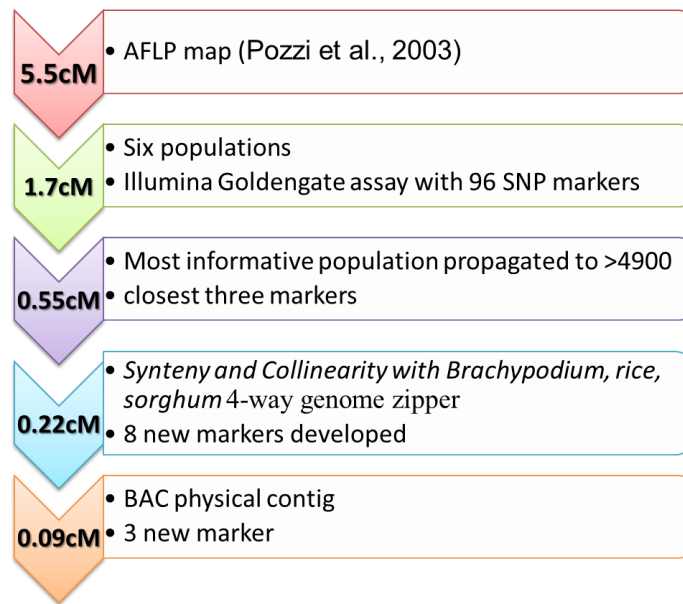
Fig. 30. The step-wise progress in mapping locus *Cul4*. The locus was initially mapped to 3HL telomerase end using AFLP map. Then, by genotyping 6 different populations with 96 SNP markers identified in the 42.4 cM interval spanning locus *Cul4*, the locus was mapped to 1.7 cM interval. The most informative population (*cul4.5*-Bowman x Morex) showing the highest number of polymorphic markers around the target locus was propagated to F3 (4949 individuals). The mapping population was genotyped by the closest 3 markers, which allowed to map the locus to 0.55 cM interval. Using synteny information with other cereal plants available on Genome Zipper, 8 more markers were developed and permitted the mapping resolution to arrive to 0.22 cM (Tavakol et al. unpublished data). In this work, the attempt to map *Cul4* locus at higher resolution was achieved through identifying the BAC clone spanning the CG for locus *Cul4*, and the sequence data was available to develop more tight 3 markers, and finally accomplishing fine mapping resolution of 0.09 cM.
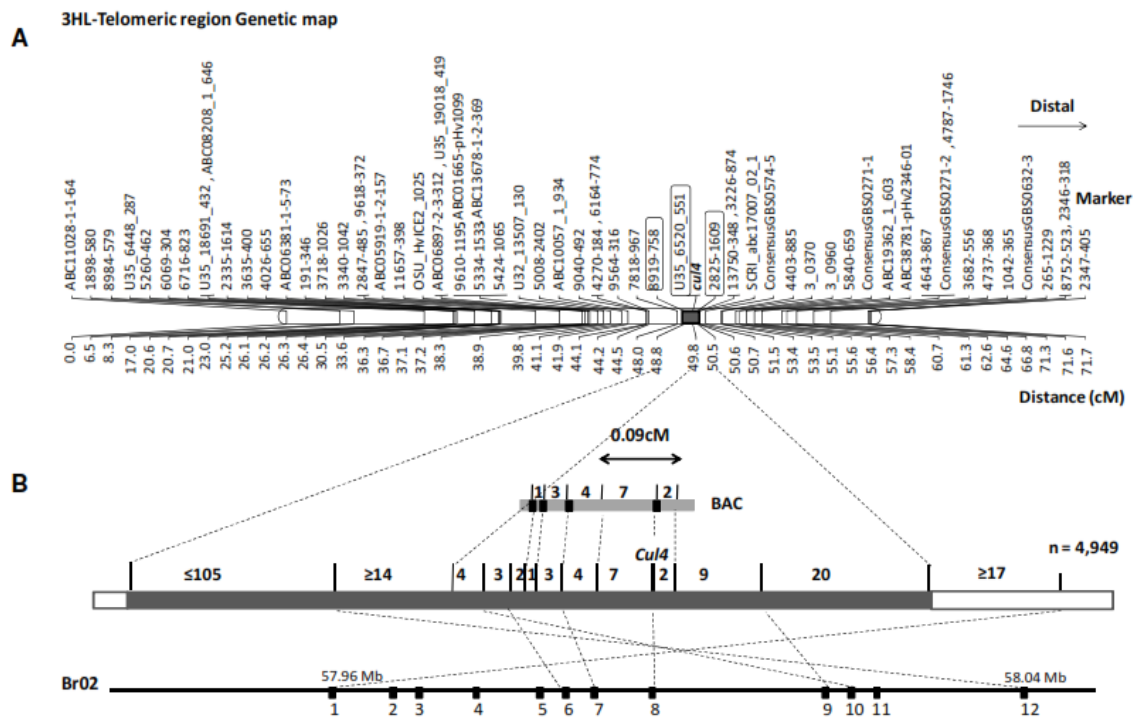
Fig. 31. Map-based cloning of locus *Cul4*. A: During previous stages (before the start of this PhD project), genotyping a mapping population (Morex x *cul4.5*-Bowman) of 4949 F3 individuals allowed defining locus *cul4* within 0.55 cM interval (markers 8919-758, U35_6520_551, and 2825-1609). The recombination events are indicated as numbers on the barley map. B: From co-linearity with Brachypodium chromosome 2, rice chromosome 1, and sorghum chromosome 3; seven flanking genes and one co-segregating gene were identified on Brachypodium chromosome 2 (the black line in the bottom Br02; flanking genes are presented as black square blocks and anchored to the barley genome by dashed lines; the closest are 7 = Bradi2g60705 and 9 = Bradi2g60720, and the co-segregating gene 8 = Bradi2g60710). The barley ortholog of flanking gene in Brachypodium, and the barley ortholog of the co-segregating gene (candidate gene) were identified on BAC clone (grey rectangle upward to the *cul4* locus region), that was sequenced, and primers were designed to anneal and amplify the target sequence by genomic PCR from the parents Morex and *cul4.5*-Bowman. Comparing the sequences, 3 new markers were developed and mapped on the selected 55 recombinants, defining the *cul4* locus on 0.09 cM interval.

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4.3 Allelic comparison

Comparing genomic and cDNA sequences of the *Cul4* candidate gene, we could identify two exons and one intron in the gene (consistent with published full length cDNA sequences AK360734.1 and AK355716.1). The gene spans 2,632 bp, from the start codon to the stop codon (**Fig. 32** and **Fig. 33**). The coding sequence is 1,542 bp in length, and is predicted to encode a peptide of 513 amino acids with estimated molecular weight of 54 kDa. The first exon is 546 bp long encoding a sequence of 182 amino acids that carry a conserved BTB/POZ domain of the length 114 amino acids. The intron is 1090 bp long, and the second exon is 996 bp coding for 331 amino acids, containing conserved sequence for 4 ankyrin repeats (ANK, 74 amino acids long).

Re-sequencing of candidate gene in different mutant and wild-type stocks revealed three independent mutations. Mutant alleles *cul4.5*, *cul4.3* and *cul4.15* turned out to carry an identical deletion of 3141 bp spanning most of exon 1 and 5′upstream region (**Fig. 33**). Sequences of the BTB-ankyrin Candidate Gene in mutant alleles *cul4.16* and *cul4.24* identified non-synonymous substitution mutations, compared to wild-type allele in Bonus and Flare (**Fig. 33**).

In the *cul4.16* allele, a non-synonymous substitution was identified in amino acid position 354, changing a leucine residue to glutamine in the ankyrin repeat region. The *cul4.24* allele carried two non-synonymous substitutions; leucine 420 to glutamine, in addition to another substitution mutation at position 441 from methionine to threonine. Running the SIFT program to predict for any deleterious effect for those mutation, the two amino acid substitutions L354Q and L420Q in *cul4.16* and *cul4.24*, respectively, were predicted to have a deleterious impact on the biological function of the protein at probability level P≤0.05.
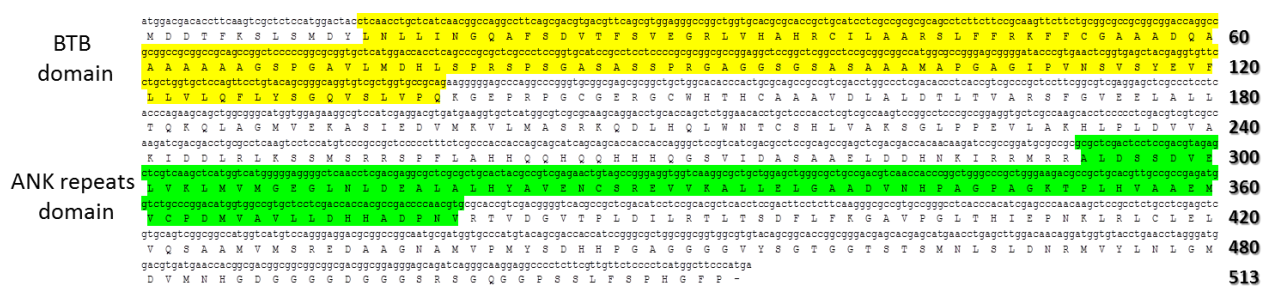
Fig. 32. The molecular characterisation of *Cul4* gene, showing the BTB domain (yellow highlighted) and the ANK repeats domain (green highlighted)
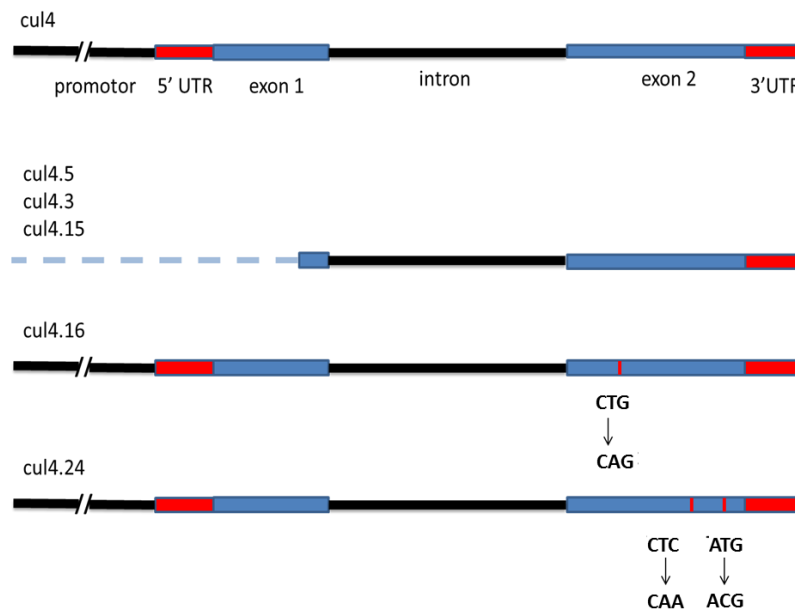
Fig. 33. The structure of *Cul4* gene, and the mutations identified in the independent mutant stocks (*cul4.5*, *cul4.3*, *cul4.15*, *cul4.16*, and *cul4.24*).

In summary, recovery of three distinct mutations in independent *cul4* alleles indicates that this BTB-ankyrin gene is responsible for the *cul4* phenotype. In addition, we observed that severity of the *cul4* mutation was consistent with the severity of the tillering and other phenotypes. Consistent with their similar phenotypic defects, alleles *cul4.3*, *cul4.5* and *cul4.15* (previously considered independent and distinct) appeared to carry an identical deletion spanning the first intron and upstream region of the gene.

5.   **Discussion**

Tillering is one of the most important traits that affect yield in Poaceae crops. Tillering contributes to whole plant architecture, and is controlled by different genetic, hormonal, and environmental factors, making it a highly plastic trait. The ability of plant monocot species to fit within particular environmental circumstances is greatly influenced by this trait, making it an important target for studies and breeding programmes. Many barley mutant lines have been identified to carry defect in loci involved in tillering, but until recently, none of them were isolated and characterised.

To characterize the functions of the *Cul4* gene in barley development, six different mutant lines were initially characterised; *cul4.3, cul4.5, cul4.5*-Bowman NIL, *cul4.15, cul4.16*, and *cul4.24*. Mutations in *Cul4* showed pleiotropic effects on multiple traits, often influenced by the genetic background. Mutations at the *cul4* locus caused reduction in number of tillers, ectopic growth of auricle-like tissue along the leaf sheath, liguleless leaves, longer spikes due to elongated rachis internodes giving the spike a lax appearance, dark green wider and longer leaves, acceleration in floral development, and reduced fertility. The negative relationship between plant tillering ability and plant height was previously observed from many other barley mutant lines, e.g. *uniculm2* (*cul2*) (no tillers and increased height), and *multi noded dwarf* (*mnd1*) (enhanced tillering and reduced height) (Babb and Muehlbauer, 2003; Druka et al., 2011). Similar results were obtained for rice mutants, e.g., *monoculm1* (*moc1*) (no tillers and increased plant height), and *dwarf* (*d*) mutants (enhanced tillering and reduced height) (Li et al., 2003; Arite et al., 2007) . A complex network of hormonal pathways were proven to be involved in tillering outgrowth in rice (chapter 1). Different component of hormonal synthesis and signalling pathways, for example auxin and gibberellin, are well known for their role in tillering as well as other traits like plant height in rice (Domagalska and Leyser, 2011; Tanaka et al., 2013). It will be interesting to explore the possible interactions of *Cul4* with these pathways. All *cul4* mutant alleles exhibited a liguleless

phenotype, although the boundary between the sheath and blade remained intact with auricles observed at the proper location. In addition, ectopic auricle tissue often developed on the margins of leaf sheaths in some *cul4* alleles, altering the proximal-distal development of the mutant leaf. These phenotypes demonstrate that *Cul4* is required for ligule outgrowth and coordinating the proximal-distal patterning of the barley leaf. In this respect, *Cul4* has similar functions to previously characterized *BOP* genes that are required for correct morphogenesis of the proximal region of the leaf in Arabidopsis, pea and Medicago (Ha et al., 2003; Couzigou et al., 2012).

Another important plant trait that is tightly correlated with tillering is inflorescence architecture, particularly branching. Many mutants in barley and rice show overall decreased branching in vegetative and reproductive stages, ex., *lax1*, *lax2* and *Reduced culm number1 (rcn1)* in rice, and *cul2* and *low number of tillers1 (lnt1)* show reduced branching vegetatively and reproductively (Komatsu et al., 2001; Nakagawa et al., 2002; Babb and Muehlbauer, 2003; Dabbert et al., 2010), while for other mutants, ex., *frizzy panicle (fzp)and Ideal Plant Architecture (IPA1)* QTL in rice, and *intermedium-b* (*int-b*) and *semi-brachytic* (*uzu*) in barley, they show reduced tillering and enhanced inflorescence fertility and branching (Komatsu et al., 2001; Chono et al., 2003; Miura et al., 2010; Dabbert et al., 2010). Here, we found that *cul4* mutations impact spike length, fertility and grain weight indicating that the BTB-ankyrin gene is required for normal inflorescence development.

Starting from the map position of *Cul4* in an genetic interval of 0.22 cM, work carried out during this PhD project contributed to attain a final mapping resolution of 0.09 cM, delimiting the target locus within a single BAC clone. This was considerably facilitated by the physical map (Schulte et al., 2011) that was built to guide barley genome sequencing in the framework on Triticeae Genome and other international projects (IBSC, 2012).

The map-based barley genome sequencing project yielded invaluable resources for researchers and breeders (IBSC, 2012) and will further simplify positional identification of important agronomic genes in the future, although this will also

depends on the ratio between physical and genetic distances in different parts of the genome. In our case, the physical distance to genetic distance ratio was estimated to be 1 cM ~ 1.1 Mbp

Identification of distinct mutations in the Candidate Gene (CG) in the three independent *cul4* mutant lines proves that the proposed CG is responsible for the *cul4* phenotype. In addition, we observed, as was shown in phenotyping characterisation of number of tillers, spike length and other phenotypes, that severity of *cul4* mutations was consistent with the severity of *cul4* phenotypes.

In summary, this work contributed to show that *Cul4* is required for correct development of tillers, leaves and other plant organs in barley. Together with other results produced in our group, conclusive evidence was obtained showing that *Cul4* encodes a BTB-ankyrin protein highly related to Arabidopsis *BOP1* and *BOP2*: 1) physical mapping and recombination with adjacent genes identified within the BAC clone spanning the *Cul4* locus; 3) identification of three independent mutant alleles confirming that *Cul4* mutations account for recessive *cul4* phenotypes of different severity. This is the first indication of the involvement of a BTB-ankyrin-type gene in shoot branching in plants. Noteworthy, the role of *Cul4* in proper proximal-distal leaf patterning is functionally similar to the role of Arabidopsis *BOP* genes in the proximal domain of the leaf, indicating that this aspect of leaf development is conserved between monocots and dicots.

CHAPTER III

CHARACTERIZATION OF RICE *BOP*-LIKE GENES

1. Introduction

1.1 Rice as important crop and model plant

Cereal crops represent more than 60% of total worldwide agricultural production (Harlan, 1995), and rice is one of the most important cereal crops with total world production of 718 million tonnes (FAOSTAT 2012). Rice provides over 50% of the caloric diet for one third of the human population. (Khush, 1997)

Rice is also considered as a model species for cereal genetics and genomics because of its relatively small genome (Izawa and Shimamoto, 1996). Along with efficient tools for plant transformation (Kim et al., 2003; Yang et al., 2004; Chen et al., 2008), plentiful genetic and genomic resources have been developed for rice including physical and genetic maps (Chen et al., 2002; Yu et al., 2004; Fekih et al., 2013), large-scale collections of expressed sequence tags (ESTs) and full length cDNAs (Yamamoto and Sasaki, 1997; Wu et al., 2002) and a high quality sequence of the genome (International Rice Genome Sequencing Project, 2005; Kawahara et al., 2013).

1.2 Rice genetics and genomics

The small genome and predicted high gene density of rice make it an attractive target for cereal gene discovery efforts and genome sequence analysis. The international efforts for rice genome sequencing resulted in valuable draft genomic information (Chen et al., 2002; Goff et al., 2002; Yu et al., 2002), concluded by the complete high-quality rice genome sequence (International Rice Genome Sequencing Project, 2005), and the annotation of the genome sequence is an active growing field of study (Sakai et al., 2013). In addition, more than 1.2 million rice ESTs from rice are deposited in GenBank (http://www.ncbi.nhm.nih.gov/dbEST/), providing verified and valuable information about gene structure and gene density in the genome (Yamamoto and

Sasaki, 1997; Wu et al., 2002; Yu et al., 2004). These ESTs have also been used for gene identification, developing molecular markers, and designing probes in microarrays (Yang et al., 2013). All these resources allowed for large scale projects of gene and QTL identification (Han and Huang, 2013). Despite this progress, the functions of large numbers of genes remain unknown. Inducing loss-of-function mutations and studying the resulting phenotypes is an efficient way to obtain information on the function of a gene.

1.3 Reverse genetics approach

Two main approaches are used in genetic analysis, forward and reverse genetics. Forward genetics begins with a mutant phenotype and tries to move forward into the genomic locus to ask the question "which gene is responsible for the altered phenotype and what is the nature of the mutation at the DNA level?". Conversely, reverse genetics starts from the gene sequence and attempts to link it to a biological function by generating a mutation or altering the expression of the gene and examining the resulting phenotypic changes. Forward genetics has been a classical strategy for more than a century employing classical and novel mutagenized populations. In this PhD thesis, applications of the forward genetics approach are presented in Chapters 2 and 4, while work reported in this Chapter illustrates the reverse genetics approach. Recently, advances in genomics methodology, including the Next Generation Sequencing (NGS) technology, have resulted in massive expansion in genomics and sequences information, which created new opportunities for novel strategies in both reverse genetics (An et al., 2005; Mieulet et al., 2013) and forward genetics (Schneeberger and Weigel, 2011; del Viso et al., 2012; Hartwig et al., 2012).

The first application of reverse genetics in plants was based on the use of the antisense method to down-regulate a ribulose bisphosphate carboxylase small subunit gene expression in tobacco plants (Rodermel et al., 1988). Currently, various methods are applied in plant reverse genetics including antisense and RNA interference (RNAi)

technologies (Chuang and Meyerowitz, 2000) that degrade gene transcripts and lead to decreased levels of available mRNA for translation. Another well-established method is based on random insertional mutagenesis, for example using T-DNA or transposons (Feldmann, 1991; Jeon et al., 2000). Classical insertional mutagenesis is often used for producing knockout mutants, or null mutations, providing a basis for the direct study of gene function. Modified methods of insertional mutagenesis have been developed for promoter/gene trapping and activation tagging (Jeong et al., 2002; Wu et al., 2003; Yang et al., 2004; Chen et al., 2008). In a promoter/gene trapping system, insertional mutations are generated randomly across the genome using a vector (e.g. T-DNA) carrying a gene trapping cassette: this consists of a promoterless reporter gene; *β-glucuronidase* (*GUS)* or *Green Fluorescent Protein* (*GFP*), and a selectable marker –usually antibiotic/herbicide resistance – used for selecting successfully transformed lines. The promoterless reporter gene flanked by an upstream 3′ splice site (splice acceptor and donor) and a downstream transcriptional termination sequence (polyadenylation sequence). When the gene trapping cassette inserts into either exon or intron of an expressed gene, the reporter gene is transcribed by the endogenous promoter of that gene in the form of a chimeric fusion transcript between the gene exons upstream of the insertion site with the reporter gene. The host gene transcript is terminated prematurely at the inserted terminator sequence, therefore the chimeric transcript encodes a truncated and non-functional version of the endogenous protein. Accordingly, gene traps simultaneously inactivate and report the expression of the trapped gene, and also provide a sequence tag for the rapid identification of the disrupted gene (Springer, 2000). Activation tagging is a powerful gain-of-function approach to study the functions of genes, especially those with high sequence similarity to other members in the gene family, that cause genetic function redundancy, and usually problematic to pursue by loss-of-function mutant analyses. Activation tagging involves randomly insertion of a T-DNA construct containing four copies of an enhancer element – e.g. multimerized transcriptional enhancers from the *Cauliflower Mosaic Virus* (*CaMV*) gene promoter - into a plant genome to activate transcription of flanking genes, followed by selection for the desired phenotype (Weigel et al., 2000; Gou and Li, 2012).

1.4 Insertional mutagenesis in rice

In rice, insertional mutagenesis has involved the use of either transposable elements (Piffanelli et al., 2007) or T-DNA (An et al., 2005). The DNA insert will disrupt the normal expression of the target gene, as well as serving as a tag for identifying the location of the insertion. The effectiveness of this system comes from the simplicity of detecting the insertion within the target gene among large populations of mutagenized plants, just by PCR using oligonucleotide primers from the insertional element and the gene of interest (Jeon et al., 2000). The number of T-DNA-tagged lines that would be required for saturating the rice genome can be estimated using the formula suggested by Krysan et al. (1999). Three main factors affect the number: (1) the mean size of rice genes, which is estimated to be 3.0 kb (Jeon et al., 2000); (2) the mean number of T-DNA inserts in one line, which is estimated to be 1.4; (3) and the haploid genome size of rice which is estimated to be 420 Mb with estimated number of 32,000-50,000 genes (Goff et al., 2002). Depending on these factors, and if the desired probability of locating a T-DNA within a gene is 99%, 660,000 insertions or 471,000 tagging lines are required (Jeon et al., 2000). As can be deduced from the figures, and given that the probability of insertion within a genetic region is lower than 99%, one can appreciate the difficulty of creating a mutagenized population to cover all the generic regions. The mutagenized rice collection generated by Jeon et al., 2000 approaching 18,358 fertile lines, is estimated to provide a 20% probability of finding a T-DNA insertion within a given gene of size 3 kb.

1.5 T-DNA mutagenized populations in rice

Efforts in developing novel tools for rice reverse genetics have led to important advances, particularly in building insertional mutagenized populations utilizing T-DNA constructs (Jeon et al., 2000; Sallaud et al., 2003), including promoter traps, enhancer traps, and activation tags (Jeong et al., 2002; Wu et al., 2003; Yang et al., 2004; Ayliffe and Pryor, 2007; Chen et al., 2008). Studies of transgene structure, distribution

and characterization of rice T-DNA transformed lines (Kim et al., 2003; Chen et al., 2003) improved transformation procedures (Sallaud et al., 2003), development of bidirectional gene trap vectors (Ryu et al., 2004), identification the flanking sequences and establishment of databases for insertions location (An et al., 2003; Sha et al., 2004; Ryu et al., 2004; Sallaud et al., 2004; Jeong et al., 2006) permit the ambitious call to identify the function of all rice genes by 2020 through the international project RICE2020 (Zhang et al., 2008).

In this context, the Plant Functional Genomics Laboratory at Kyung Hee University, Korea, has become a leader in the production of T-DNA insertional and activation tagging lines, using different vectors for transformation of *japonica* rice [*O. sativa* cv. Dongjin (DJ) or Hwayoung (HY)]. The vectors used to transform rice plants include pGA2707 (GUS trapping vector), pGA2717 (GUS and GFP trapping vector), and activation tagging vectors pGA2715 and pGA2772 (Jeon et al., 2000; Jeong et al., 2002). Vector pGA2707 (GUS trapping vector) contains the hygromycin phosphotransferase gene (*hph*) - conferring hygromycin resistance - expressed by the rice *α-tubulin* (*OsTubA1-1*) promoter, and a promoterless reporter gene *β-glucuronidase* (*GUS*) with an *OsTubA1* intron 2 (I2) and multiple splicing donors and acceptors in each of the three reading frames immediately next to the T-DNA right border, allowing *GUS* expression when the insertion occurs in either an exon or intron (Jeong et al., 2002) (see **Fig. 34**). Vector pGA2717 contains the same selection marker (*hph*), and the GUS reporter gene with I2 and multiple splicing donors and acceptors, in addition to *Green Fluorescence Protein* (*GFP*) reporter gene with *OsTubA1* intron 3 (I3) next to the left border. Therefore, inserting T-DNA into a gene, in any orientation, can result in the activation of either *GUS* or *GFP* (Ryu et al., 2004), resulting in the formation of a chimeric transcript with the mutagenized gene (see **Fig. 35**). Vector pGA2715 and pGA2772 carry the same major elements of vector pGA2707, in addition to tetramerized transcriptional enhancers from the *Cauliflower Mosaic Virus 35S* (*CaMV 35S*) gene promoter next to the left border (Jeong et al., 2002, 2006) (see **Fig. 36** and **Fig. 37**). The enhancer elements function both upstream and downstream of the insertional mutagenized gene, and in either

orientation, and at a considerable distance from the coding regions (1.5kb to 4.3kb). The CaMV 35S enhancer can cause enhanced expression of the endogenous gene without affecting the expression pattern (Jeong et al., 2006).
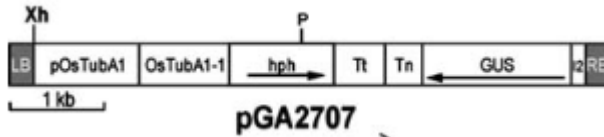
Fig. 34. Map of the T-DNA region of tagging vector pGA2707. RB and LB in the gray bar represent the right and left borders of T-DNA, respectively. I2, *OsTubA1*, rice *α-tubulin* intron 2 carrying three putative splicing acceptor and donor sites; GUS, *β-glucuronidase* gene; Tn, *nos* terminator; Tt, *OsTubA1* terminator; *hph*, *hygromycin phosphotransferase* gene; OsTubA1-1, the first intron of *OsTubA1*; pOsTubA1, rice *OsTubA1-1* gene promoter (Ryu et al., 2004).
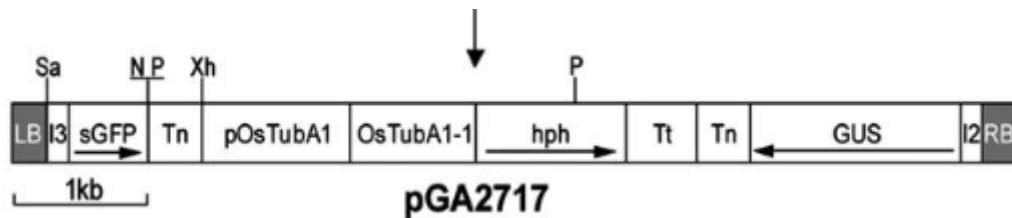
Fig. 35. Map of the T-DNA region of tagging vector pGA2717. RB and LB in the gray bar represent the right and left borders of T-DNA, respectively. I2, rice *α-tubulin OsTubA1* intron 2 carrying three putative splicing acceptor and donor sites; GUS, *β-glucuronidase* gene; Tn, *nos* terminator; Tt, *OsTubA1* terminator; *hph*, *hygromycin phosphotransferase* gene; OsTubA1-1, the first intron of *OsTubA1*; pOsTubA1, rice *OsTubA1-1* gene promoter; sGFP, modified form of *Green Fluorescence Protein* gene; I3, *OsTubA1* intron 3 carrying three putative splicing acceptor and donor sites (Ryu et al., 2004).
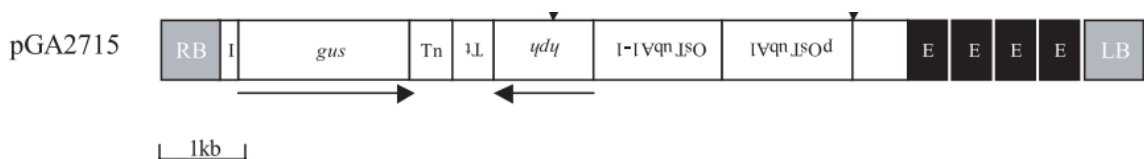
Fig. 36. Map of the T-DNA region of tagging vector pGA2715. RB and LB in the gray bar represent the right and left borders of T-DNA, respectively. I, rice *α-tubulin OsTubA1*intron 2 carrying three putative splicing acceptor and donor sites; GUS, *β-glucuronidase* gene; Tn, *nos* terminator; Tt, *OsTubA1* terminator; *hph*, *hygromycin phosphotransferase* gene; OsTubA1-1, the first intron of *OsTubA1*; pOsTubA1, rice *OsTubA1-1* gene promoter; E, transcriptional enhancers from the *Cauliflower Mosaic Virus 35S* (*CaMV 35S*) gene promoter (Jeong et al., 2002).
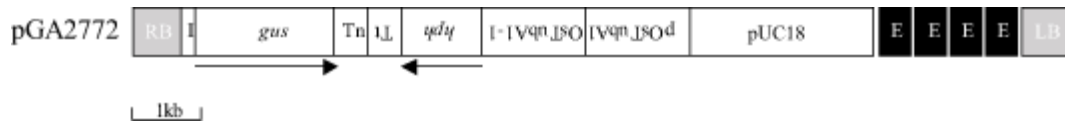
Fig. 37. Map of the T-DNA region of tagging vector pGA2715. RB and LB in the gray bar represent the right and left borders of T-DNA, respectively. I, rice *α-tubulin OsTubA1* intron 2 carrying three putative splicing acceptor and donor sites; GUS, *β-glucuronidase* gene; Tn, *nos* terminator; Tt, *OsTubA1* terminator; *hph*, *hygromycin phosphotransferase* gene; OsTubA1-1, the first intron of *OsTubA1*; pOsTubA1, rice *OsTubA1-1* gene promoter; E, transcriptional enhancers from the *Cauliflower Mosaic Virus 35S* (*CaMV 35S*) gene promoter; pUC18, fragment of pUC18 vector used to retrieve flanking sequences by inverse PCR (Jeong et al., 2006).

To facilitate the identification of lines carrying insertions in genes of interest, the Korean group has determined the locations of 27,621 T-DNA inserts by amplifying the flanking sequences by inverse-PCR or Thermal Asymmetric Interlaced (TAIL) PCR (see **Fig. 38** and **Fig. 39**) and Sanger sequencing of the amplification products; these sequences (called Flanking Sequence Tags "FST") are collected into a database (An et al., 2003; Jeong et al., 2006) that can be queried at http://signal.salk.edu/cgi-bin/RiceGE, using sequence similarity searching tools.
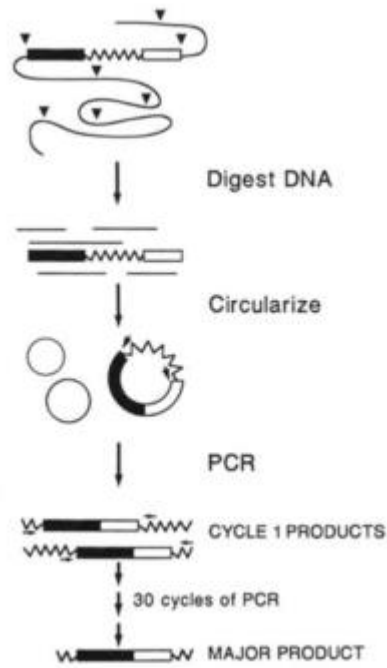
Fig. 38. Schematic diagram of the inverse-PCR procedure. The core region is depicted as a jagged line. Filled and open boxes represent upstream and downstream flanking regions, respectively. DNA is digested with a restriction enzyme (restriction sites denoted by triangles), circularized under conditions that favour the formation of monomeric circles, and enzymatically amplified using PCR. Oligonucleotide primers (constructed to anneal to the core region) and the direction of DNA synthesis are shown by arrows (Ochman et al., 1988).
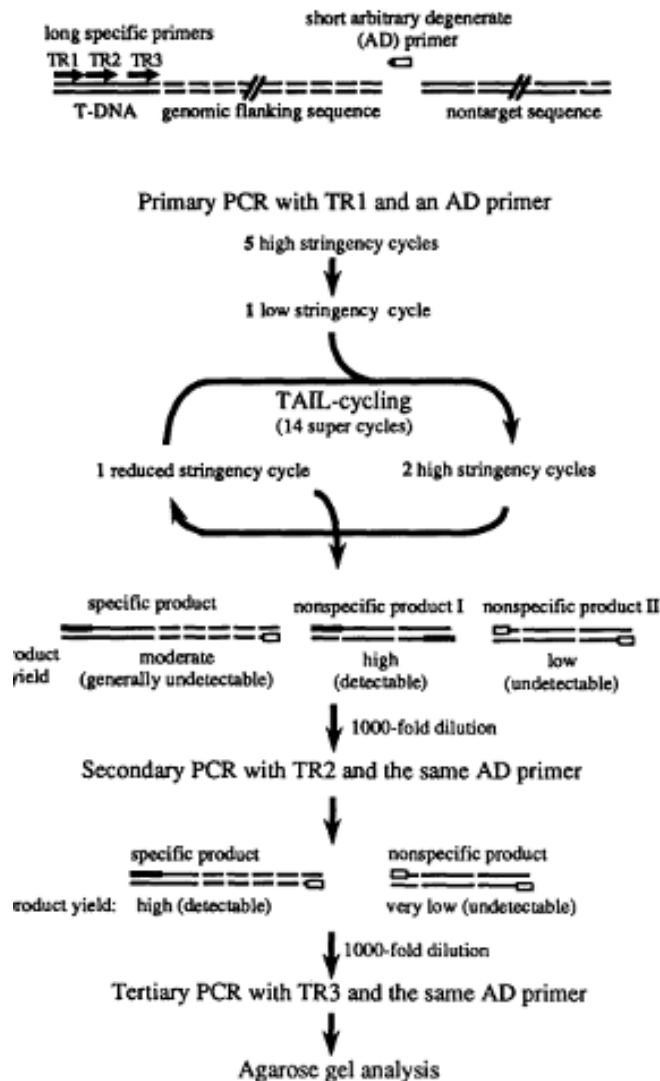
Fig. 39. TAIL-PCR procedure for amplification of flanking sequence of T-DNA insert. Three PCR are carried out to amplify target sequence using nested T-DNA-specific primers (bold segments) on one side and a short arbitrary degenerated primer (AD9) (small open rectangles) on the other. One or more sites within the flanking sequence are adapted for annealing to the AD primer through a low-stringency cycle. Even after creation of sites adapted for the AD primer, however, high temperature annealing still favours the specific primer, resulting in a linear amplification of target molecules. To achieve adequate thermal asymmetry, the specific and AD primers are designed to have tm of 57°-62°C and 44°-46°C, respectively. By interspersing reduced-stringency cycles to allow AD priming, double-strand molecules can be formed, and the preferential linear amplification of target molecules becomes logarithmic. In the secondary and tertiary reactions, non-specific product fails to be re-amplified and thus is not shown (Liu et al., 1995).

1.6 Central role of rice in comparative genetics of cereals

The two major groups of flowering plants, monocots and dicots, diverged 200 million years ago (Wolfe et al., 1989), and the cereals have been evolving independently from a common ancestral species for 50 to 70 million years (Kellogg, 1998). Many studies that compared the physical and the genetic maps of the grass genomes have revealed strong synteny, i.e. conservation of gene order and orientation along chromosomes (Chandler and Wessler, 2001; Freeling, 2001; Yu et al., 2004; Bolot et al., 2009; Galvão et al., 2012; Feuillet et al., 2012). Despite gene similarity and genome synteny, cereal genome sizes and organization vary greatly, e.g. the genomes of sorghum, maize, barley, and wheat are estimated at 1,000, 3,000, 5,000, and 16,000 Mb, respectively, while rice has a much smaller genome, estimated at 420 Mb (Goff et al., 2002). The relative ease of manipulating rice genetic and genomic tools, in addition to the significant synteny between rice genomic regions and other cereals make rice an important "hub" for many studies on cereal crops (Devos, 2005; Bolot et al., 2009). Twenty years ago, the genetic information generated on one certain cereal species was limitedly applied to improve genetic studies in other cereal species, until pioneer research achievements in 1993/1994 demonstrated high synteny between rice, maize and wheat genomic regions (Ahn and Tanksley, 1993; Kurata et al., 1994). Recently, the rice genome has become extensively used as a source of information for positional cloning and for studying gene family organization in other crop plants, while divergence from the micro-colinearity is helping to study the mechanisms of grass genome evolution (Raghuvanshi et al., 2010).

2.   **Scope of the chapter**

Most of the knowledge available on genetic and hormonal control of tillering in Poaceae come from studies on rice and maize (Kebrom et al., 2013). Rice represents a particular model plant for small grains crops because of its simple and small genome, in addition to availability of various genetic and genomic resources (Izawa and Shimamoto, 1996). Extending the studies of tillering genetic elements to comprise additional homolog components in different plant systems will provide bases to understand the conserved and flexible elements in tillering pathways, which is useful for basic sciences and also applied breeding. Studying barley *Cul4* homolog genes in rice will provide additional data and allow insight assessment of conserved pathway elements among cereal plants. *BOP* genes encode a group of proteins that function as transcription factors with diverse biological roles, e.g. leaf morphogenesis (Ha et al., 2003), and root nodule development (Couzigou et al., 2012) in dicots, leaf patterning, tillering and other developmental traits in barley (Chapter 2). While *BOP* genes have been extensively studied in Arabidopsis (Ha et al., 2003, 2004, 2007, 2010; Norberg et al., 2005; Hepworth et al., 2005; Jun et al., 2010; Xu et al., 2010; Saleh et al., 2011; Canet et al., 2012) no *BOP*-like gene was functionally characterized in rice. Analysis of rice *BOP* genes is expected to provide valuable information on the evolution and diversification of this gene family, allowing comparison with information obtained in barley and dicots, and providing a basis for further work on rice, barley and other cereal crops.

To extend our vision, and gain comprehensive view of *Cul4* functions in other cereal crops, we planned to follow reverse genetic approach to identify mutant lines in homolog genes in rice, and to investigate possible effects on similar or different functions. Rice T-DNA insertional mutational libraries provide researchers with excellent starting point for reverse genetics (Jeon et al., 2000; Sallaud et al., 2003; Wu et al., 2003; Ryu et al., 2004; An et al., 2005; Piffanelli et al., 2007; Larmande et al., 2008; Chen et al., 2008) , given that searching for mutant lines made easy by simple sequence

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homologous search on databases that contain the flanking sequences to the insert, which allow identify the location/gene where insertion exist (Hirochika et al., 2004; Sha et al., 2004; Jeong et al., 2006; Zhang et al., 2006). Our work was organised into the following steps: (1) identifying *Cul4* ortholog genes in different plant species and study the phylogenetic relationships among them; (2) performing data mining in publicly available data for *Cul4* homolog genes in rice including information on gene annotation, transcriptomics or phenotyping data; (3) identifying the insertion lines carrying mutation in the *Cul4* homolog genes in rice; (4) order plant material and grow them for propagation and phenotyping; (5) screen the mutant lines with simple genomic PCR experiments to exclude wild-type homozygotes alleles for *Cul4*-like gene.

3. **Materials and Methods**

3.1 Phylogenetic analysis of *BOP* genes

A phylogenetic tree was built from aligned protein sequences retrieved from publicly available genome sequences databases. The *Cul4* peptide sequence (513 aminoacids, Chapter 2) was used as a query in BLASTP 2.2.22+ (Altschul et al., 1990) searches versus Phytozome v9.1 http://www.phytozome.net/ (Goodstein et al., 2012) for the following species: *Arabidopsis thaliana*, *Brachypodium distachyon, Glycine max*, *Mimulus guttatus*, *Oryza sativa*, *Sorghum bicolor*, *Vitis vinifera*, *Zea mays*. For *Hordeum vulgare*, blastp was used on IPK barley blast server, on HC_genes_AA_Seq database http://webblast.ipk-gatersleben.de/barley/viroblast.php (IBSC, 2012). And for *Pisum sativum*, the sequence JN180860 was retrieved from the Genbank http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&PAGE_TYPE=BlastSearch &LINK_LOC=blasthome (Benson et al., 2013). For genes with alternative splicing forms, the form best supported by EST sequences was considered. EST sequences are searchable through Genebank searching tool (Boguski et al., 1993). ScanProsite tool http://prosite.expasy.org/prosite.html (de Castro et al., 2006) was used to scan each polypeptide against Prosite database (Sigrist et al., 2013) to ensure the presence of BTB and Ankyrin domains.

The evolutionary history was inferred using the Neighbour-Joining method (Saitou and Nei, 1987). The optimal tree with sum of branch length = 0.94055521 was achieved. A bootstrap test with 1000 replicates was run (Efron et al., 1996). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling, 1965) and are in the units of the number of amino acid substitutions per site. The analysis involved 21 amino acid sequences from 10 species. All ambiguous positions were

removed manually for each sequence. There were a total of 440 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011).

3.2 Transcription data

The Genevestigator database https://www.genevestigator.com/gv/ allows to search by gene name on expression analysis experiments submitted to the database. In order to explore the expression patterns of rice *BOP* genes and evaluate their compatibility with a possible role in shoot development as is the case for *Cul4*, publicly available rice transcriptomics data produced by Affymetrix GeneChip®Rice Genome Array 51K, were searched through the Genevestigator database (Hruz et al., 2008). The rice genome array (Affymetrix) contains probe sets designed from approximately 51,279 transcripts; 48,564 *japonica* and 1,260 *indica* sequences (http://www.affymetrix.com/estore/browse/products.jsp?productId=131497#1_1). Sequence information for this array was derived from the National Center for Biotechnology Information (NCBI) UniGene Build number 52 (http://www.ncbi.nlm.nih.gov/UniGene), GenBank mRNAs, and 59,712 gene predictions from TIGR's osa1 version 2.0.

3.3 Identification of rice lines carrying insertions in *Cul4*-like *BOP* genes

The Cul4 protein sequence (513 aa) was used as query in a tBLASTn search (Altschul et al., 1997) on http://signal.salk.edu/cgi-bin/RiceGE (An et al., 2003; Jeong et al., 2006) .

The most similar Flanking Sequences Tags (FSTs) deriving from this search were used to identify the corresponding genes in the rice genome, and to identify the mutagenized lines carrying the insertion. Seeds of selected lines were ordered from the T-DNA insertion mutagenized rice collection curated by the group of Dr. Gynheung An, Kyung Hee University, Crop Biotech Institute, Korea (Jeon et al., 2000; Jeong et al.,

2002). These insertional T-DNA lines were generated in two *O. sativa* spp. *japonica* Korean cultivars, Dongjin and Hwayoung (mid- to late-flowering photoperiod sensitive cultivars, flower 100-110 days after germination under field conditions, 85-90 days under long-day conditions, 55-65 days under short-day conditions). Seeds of these cultivars were ordered along with the insertional mutant lines, and grown for comparison and as control group.

Information regarding the type of T-DNA constructs and the position of the insertions were recovered from the FST database: schematic representations of the inserts, their locations, and orientation are presented in **Fig. 43**, **Fig. 44**, and **Fig. 45** in the Results section of this chapter.

3.4 Validation of T-DNA insertion in line 1B-17402

Plant materials

Seeds for insertional mutagenized rice lines and their corresponding wild-type backgrounds were surface sterilised by soaking in bleach solution (1:1) for 3 min, then rinsing with sterilised distilled water for 5 times, each for 3 min. The seeds were left to dry under biosafety cabinet and 6 seeds for each genotype were sown initially on Mourashige and Skoog (MS30) media (Murashige and Skoog, 1962).

The basic medium contains macro-elements (N, P, K, Ca, and Mg), micro-elements (B, Co, Cu, S, Fe, Mn, I, Mo, Zn, and Na), and some vitamins and organic materials (Inositol, Niacin, Pyridoxine, Thiamine, IAA, Kinetin, Glycine, and Edamine). A commercialised form (Murashige & Skoog Medium -Micro and Macro elements including Vitamins-, Duchefa Biochemie) was used at recommended rate of 4.4 g per litre, supplemented with 3% sucrose and 1% agar to obtain semi-solid medium. The medium was sterilized by autoclaving (121°C for 20min), and poured into plastic boxes dedicated for in vitro culture (ECO2box white filter, Micropoli).

For germination, seeds were incubated at room temperature (24-28°C) under dark conditions for 10 days. Rice seedlings were then transplanted into peat-moss media in seedlings plastic tray (well vol.~20ml), at room conditions (natural photoperiod of 15 hours day/ 9 hrs night and temperature 24-28°C) for 2 days. The seedlings were then transplanted into growing media (peat-moss: volcanic stones 3:1), filled into plastic pots (vol. ~1L), and moved to a growth chamber under controlled conditions (24-28°C, humidity 80%, 16hr day) at University of Milan. Iron solution (1% chelate) was supplied to each pot – after transplantation – at rate of 10 ml per pot, and pots were then covered with plastic cover for 6 days. Rice plants were irrigated day after day to keep favourable conditions for rice as a semi-aquatic plant, and a fertilizer solution (ALGOFLASH ®; NPK + B, Cu, Fe, Mn, Mo, Zn; 0.4% solution in water) was added every two weeks at rate of 5-8 ml to each pot. Plants were kept in the same growth chamber conditions (long-day photoperiod) through tillering and stem elongation phase, to encourage the maximum production of tillers, and accordingly panicles. When plants reached the 6th/7th leaf stage (60 days after emergence), they were transferred to the greenhouse under short day conditions to induce flowering (26-32°C, 12hrs day, RH: 70-85%) at University of Milan, Botanic Garden.

Plant samples and DNA extraction

Plant leaf samples were collected 60 days after emergence of seedlings. Leaf tissue samples were frozen on dry ice then kept at -20°C till DNA extraction was carried out.

For DNA extraction from fresh plant material, 200-250 mg of fresh leaf tissue were homogenized with a mortar and pestle using liquid nitrogen. The tissues were ground to a fine powder then collected in 2 ml eppendorf tubes. Each ground sample yielded between 150-200 mg of fine powder. Each sample was supplemented with 600 µl of pre-heated (65°C) MATAB (20 mM EDTA pH 8.0, 100 mM Tris-HCl pH 8.0, NaCl 1.5 M, 0.5% (W/V) Na_2SO_3, 2% (W/V) hexadecyltrimethylammonium bromide (MATAB), Polyethylene glycol (PEG) solution 1% (W/V)) and mixed well by vortexing

for 3 min, then incubated at 65oC for 15 min. RNase (20 mg/ml) was added at rate of 10µl to each sample, and then incubated at 65oC. Samples were then centrifuged at 4,500 xg for 10 min. The supernatant was taken by pipetting into new tube, and supplemented with 450 µl chloroform/isoamyl alcohol (24/1). The sample was mixed by inverting gently then centrifuged at 6,200 xg for 10 min. The supernatant was taken by pipetting into a new tube, and supplemented with 150µl of isopropanol (pre-chilled to -20oC) and 100µl of a high-salt precipitation solution (0.8M sodium citrate and 1.2M NaCl). The sample was incubated at -20oC to allow precipitation of nucleic acids, and then centrifuged to collect them at 6,200 xg for 10 min at 4oC. The isopropanol was discarded, and the pellet was rinsed with 800µl of 70% ethanol and re-collected by centrifugation at 4,500 xg for 10 min. This step of washing the pellet was repeated twice. The pellet of nucleic acids was left to dry in a Vacufuge® vacuum concentrator (Eppendorf) at 35oC and 35 xg for 20 min., and finally resuspended in 40-50 µl of sterilised water. DNA quantity and quality was measured on NanoDrop 1000 Spectrophotometer (Thermo Scientific).

Primer design for screening for T-DNA insertion

The public available reference sequence of rice *japonica* cv. Nipponbare was used to design the primers to work either on the wild-type allele of the gene or on the insert and the gene flanking sequences. Schematic diagrams were constructed based on available data of (FST) to aid in designing the primers and hypothesised the expected size of the amplicons for either the presence or the absence of the inserts (**Fig. 43**, **Fig. 44**, and **Fig. 45**). Specific primers for the wild-type form of the gene (no insert) anneal to the target gene sequences across the point of insertion. Therefore, if the insert present, the distance between the forward and reverse primer is too long to be amplified under the PCR conditions used. To detect the presence of the insert, a primer designed on the insert sequence was used in combination with a primer designed on the adjacent gene sequence. In multiplex PCR, three pattern could be observed: a single fragment from the wild-type allele (homozygous wild-type), a single fragment from the insert

(homozygous for the insertion), or both fragments in case of heterozygosity of the insertion mutation.

The Primer-BLAST tool (Ye et al., 2012) was used for designing the primers and testing any non-specific amplification. The Primer-Blast tool on NCBI website http://www.ncbi.nlm.nih.gov/tools/primer-blast uses PRIMERS3 (Koressaar and Remm, 2007; Untergasser et al., 2012) for designing the primers, and uses BLAST and global alignment algorithm against Genbank DNA sequence database of *O. sativa* to test for non-specific amplification. Primers are listed in **Table. 11**.

Table. 11. Primers used to screen the mutant lines for the presence of the T-DNA insert

Primer	Melting temp. (^{o}C)	Sequence	Mutant line
pGA2717_LB-F	60.25	GGCGGACTGGGTGCTCAGGTA	
Os01g72020_int_1118-R	57.99	ATATTCCCCCACCTCCATGCTCG	1B-17402
Os01g72020_3UTR_89_F	55.74	GCCCAAAAGGATCATTCTCATCTCC	
Os12g04410_int_536-R	57.04	ACGTCGTGTTCCTGAATTTTTCGC	
pGA2772_RB-F	57.35	GCCACGTAAGTCCGCATCTTCA	2D-10109
Os12g04410_5'UTR_833-F	58.50	GCCCATTCACTTCGTTCCTCGC	
Os11g04600_int_823-F	58.08	GCGAGCTGAGCTGCACGATT	
pGA2707_22_RB-R	57.52	TGGTACCTCGGATCCGTGTTTGA	1D-04839
Os11g04600_int_1356-F	51.08	CAGAACTGAAGAAAGTACTACTACTAC	
Os11g04600_3'UTR_281_R	55.74	TGCTCATCTCCTGGTGGATCG	
pGA2715_1195_RB-F	58.23	GCCATGTTCATCTGCCCAGTCG	
Os11g04600_int_1143-R	58.36	AGCAAGCATGGGGATGGATGTGA	3A-50737
Os11g04600_5'UTR_21_F	55.99	CCGGCGCTTTTATCTGGTGG	
Os11g04600_int_1356-R	51.08	GTAGTAGTAGTACTTTCTTCAGTTCTG	

Genomic PCR

The DNA samples extracted from rice mutant lines were adjusted to the concentration of 50 ng/μl and used in genomic PCR. The reaction mixture is described in **Table. 12**.

Table. 12. Reaction mixture used in PCR to screen the rice mutant lines.

Reagent/stock	Vol per reaction (total vol 25μl)	Final concentration
ReadyMix™ Taq PCR Reaction Mix 2X (Sigma)	12.5μl	1X (1.5 units *Taq* DNA polymerase, 10 mM Tris-HCl, 50 mM KCl, 1.5mM $MgCl_2$, 0.001% gelatin, 0.2 mM dNTP, stabilizers.
Primer Forward (10μM)	1μl	0.4μM
Primer Reverse (10μM)	1μl	0.4μM
DNA sample (50ng/μl)	1.5μl	3ng/μl
Molecular Grade water	9μl	

PCR was carried out in a (BIORAD PCR thermal cycler™) following the following thermal protocol:

Initial denaturation at 95°C for 4min
Denaturation 95°C for 30sec
Annealing (2-3°C less than the lower primer Tm) for 30sec
Elongation 72°C for 1 min
Terminal elongation 72°C for 4min

35 cycles
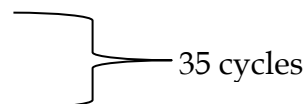
The PCR products were separated on 1.5% Agarose gel in TAE buffer at 70 volt for 1hr.

DNA extraction from gel

PCR products were gel-purified using the QIAquick Gel Extraction Kit following the manufacturer's protocol (Qiagen).

The DNA band was excised with sharp and sterilized scalpel and put into a 2 ml eppendorf tube. The weight of the gel bands was measured to be between 150-300mg. Accordingly, 3 volumes of buffer QG were added and the sample was incubate at 50°C

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for around 10 min (until the gel slice has completely dissolved), periodically mixing by vortexing. One volume of isopropanol (150-300 µl) was added to each tube, then the solution was loaded onto QIAquick column inside a 2 ml collection tube, and centrifuged for 1 min at 11,000 xg. The flow-through was discarded, and 0.5 ml QG buffer was added to the spin column. To wash out any residues of the gel, 0.75 ml of PE Buffer was loaded into the column and centrifuged for 1 min at 11,000 xg. The flow-through was discarded, and the centrifugation step was repeated to ensure dryness of the column. After adding 30 µl of molecular grade water, the QIAquick column was centrifuged for 1 min at 11,000 xg. The DNA is collected at the bottom of the tube in the elution solution. The purified PCR products were cloned and sequenced to investigate the flanking sequence and exact location and orientation of insertion.

Cloning - Ligation

Gel-purified PCR products were ligated into the pGEM®-T Easy Vector Systems (Promega), following the manufacturer's protocol. The ligation cocktail was composed as indicated in **Table. 13**.

Table. 13. The ligation mixture components

Reagent	Volume added	Final conc.
2X Rapid Ligation Buffer, T4 DNA Ligase (Promega)	5µl	1x
pGEM®-T Easy Vector (50ng)	1µl	5ng/µl
T4 DNA Ligase (3 Weiss units/µl)	1µl	0.33U/µl
PCR product	1.5µl	
Mol.grad. water	1.5µl	
Total vol.	10µl	

Ligation reaction mixture was incubated at 4°C for 3-4 hours, then at room temperature for another 1-2 hours.

Cloning – Preparation of chemically competent E. coli cells

Bacterial suspension (5 µl) of *E. coli* (TOP10 strain) with concentration $\sim 10^8$–10^7 Colony Forming Units (CFUs) was used to inoculate 3 ml Luria Broth (LB) broth, and

incubated at 37°C overnight to allow bacterial growth. Luria Broth (LB) broth was prepared by adding 20 g from readymade LB substrate mixture (Lennox) (Sigma-Aldrich) in 1 L of distilled water. One litre of LB liquid medium contains: 10 g/L Tryptone, 5 g/L Yeast Extract, and 5 g/L NaCl. The 3 ml of bacterial culture – that was incubated overnight - were used to inoculate 15 ml of LB liquid medium. The bacterial cells were incubated at 37°C for 3-5 hours under agitation at 150 rpm, and then the bacterial suspension turbidity was measured with a spectrophotometer (at wavelength λ= 600 nm, the optical density was 0.45). The bacterial cells were harvested by centrifugation at 990 xg for 15 min at 4°C, supernatant was discarded and bacterial pellet was suspended in 16.7 ml of sterilised RF1 solution (100 mM Potassium Chloride, 50 mM Manganese Chloride, 30 mM Potassium Acetate, 10 mM Calcium Chloride, and 15% glycerol; pH 5.8); all further steps of preparing *E. coli* competent cells were performed on ice. The bacterial suspension was mixed well with RF1, and incubated on ice for 30 min with repeated mixing by pipetting. The bacterial cells were harvested again at 990 xg for 15 min at 4°C, the supernatant was discarded, and the bacterial pellet was re-suspended in 4 ml of sterilised RF2 solution (10 mM 3-(N-morpholino) propanesulfonic acid "MOPS", 75 mM Calcium Chloride, mM Potassium Chloride, 15% glycerol; pH 6.8). The bacterial suspension was aliquoted in 50 μl and snap-frozen in liquid nitrogen, and then stored at -80°C.

Cloning - Transformation of competent E. coli cells

For transformation, 10μl of ligation mixture (see preparation of ligation mixture above) was mixed with competent cells (50μl), then incubated on ice for 30 min, and mixed by inverting every 5 min. Next, the mixture was incubated at 42°C for 1.5 min, and put back on ice for 2 min. Following addition of 1 ml LB broth, the tube was incubated at 37°C for 1.5 hour under agitation at 150 rpm. After the incubation, 500μl of the bacterial culture in LB broth were spread on LB Agar plates for screening. The LB Agar substrate for screening contains: 80 mg/l IPTG, 100 mg/l Ampicillin, and 100 mg/l X-Gal, and 20 g/l Agar. Plates were left to dry under the hood then incubated up-side-down at 37°C overnight.

The transformed bacterial cells carrying the insert appeared as white colonies. These colonies were picked and inoculated into LB broth supplemented with ampicillin 100 mg/l and incubated at 37°C under agitation at 150 rpm overnight. The transformed bacterial colonies, in parallel with inoculation into the LB broth, were checked with colony-PCR using primers targeting the insert before proceeding to miniprep extraction protocol (see PCR protocol above) to ensure the presence of the target insert.

Miniprep protocol

The bacterial cultures that were positive for the presence of the insert in the transformation (see above) were collected by centrifugation at 990 xg for 10 min and plasmid DNA was extracted using the Wizard®Plus SV Minipreps DNA purification System (Promega).

After resuspension of bacterial pellets in 250 μl of Cell Re-Suspension Solution provided by the commercial supplier (Promega), 250μl of Cell Lysis Solution were added to each tube, and the tubes were mixed well by inverting 5 times and incubated at room tempreture for 5 min. Then 10 μl of Alkaline Protease Solution were added to each tube, and the tubes were mixed by inverting 5 times and incubated for 5 minutes at room temperature. Then 350 μl of the Neutralization Solution were added and the tubes were mixed by inverting 5 times. Bacterial lysates were centrifuged for 10 minutes at 11,000 xg, and the clear lysates were transferred to spin columns, centrifuged for 1 minute at 11,000 xg. The flow-through was removed, and the spin columns were washed twice, first with 750 μl and then with 250 μl of Column Wash Solution and finally centrifuged for 2 minutes at 11,000 xg. Spin columns were transferred to a new tubes and DNA was eluted by adding 50 μl of molecular grade water and centrifuging for 1 minute at 11,000 xg.

Sanger Sequencing

The DNA samples that resulted from miniprep extraction procedure were sequenced using the Sanger method to investigate the exact location of T-DNA insert, and the integration pattern of T-DNA insert. The BigDye® Terminator Cycle

Sequencing Kit (Applied Biosystems) was used for sequencing. The reaction mixture was constituted as in **Table. 14**.

Table. 14. Sanger sequencing reaction mixture

Reagent	Initial conc.	Volume added	Final conc.
BIG DYE v. 3.1 (Ready Reaction Mix)	10 ×	1	1 ×
Sequence buffer	5×	2	1x
Primer	10 μM	0.8	800 nM
Ultra-pure molecular grade water	-	5.2	-
purified DNA vector with insert		1	
Total reaction mix		10	

Reactions were incubated in 96 well plates, in a BIORAD PCR thermal cycler™ under the following thermal conditions: 25 cycles of [96ºC for 10 sec, 50ºC for 10 sec, 60ºC for 4 min]. The reaction mixture then purified by ethanol precipitation as follows: for each reaction, 2.5μl of EDTA and 30μl of absolute ethanol were added, then the whole 96-well plate was centrifuged for 45 min at 625 xg. The supernatant was discarded by inverting the plate, then 30 μl of pre-chilled ethanol (-20ºC) were added to each well, and centrifugation was run again at 625 xg for 15 min. The supernatant was discarded, and the plate was incubated at room temperature in the dark to allow ethanol residues to evaporate, while protecting the reaction dye from being degraded by light. At the end, 10 μl of formamide were added to each well, and reactions were submitted to the Genomics Platform at Parco Tecnologico Padano, Lodi, for capillary electrophoresis on an AB3730 capillary instrument DNA analyser (Applied Biosystems).

Chromatogram *.ab1 files were aligned by ClustalW (Larkin et al., 2007) in MEGA 5.1 programme (Tamura et al., 2011). For comparison with the reference, the Os01g72020 sequence was retrieved from Rice Genome Annotation Project database (O. *sativa* sp *japonica* cv. Nipponbare) http://rice.plantbiology.msu.edu/cgi-bin/gbrowse/rice/ (Kawahara et al., 2013) and the insert left border obtained from the

vector sequence available on http://signal.salk.edu/rice/PosTech_Vector_T-DNA_Sequence.htm#2717 .

3.5 Phenotyping measurements

Phenotypic measurements were taken when plants reached the end of the flowering stage at the beginning of the milk grain filling stage (110 days after emergence). The parameters measured were: plant height (from ground level to panicle base); stem thickness (the internode below the flag leaf was measured); leaf blade length (the leaf below the flag leaf was measured); leaf blade width (the leaf below the flag leaf was measured); and number of tillers. Plants were also evaluated for any defects in leaf patterning, such as disruptions of the boundary between leaf blade and leaf sheath, ligule or auricles.

4. Results

4.1 The Phylogenetic analysis of *BOP* genes

In order to study the evolutionary relationships of *Cul4* and other *BOP* genes, a phylogenetic analysis was conducted considering functionally characterized genes from *Arabidopsis*, pea *COCH* and *Medicago truncatula NOOT,* along with sequences from monocots and dicots genomes. The phylogeny was initially targeting all BTB/ANK proteins in 12 different species; *Sorghum bicolor*, *Brachypodium distachyon*, *Oryza sativa*, *Zea mays, Hordeum vulgaris, Musa, Arabidopsis thaliana, Glycine max, Mimulus guttatus, Vitis vinifera, Solanum lycopersicum* and *Pisum sativum*. The resulting phylogenetic tree contained 64 protein sequences, that were organised in major dichotomy between BTB/ANK proteins, and BTB/ANK/NPR protein (data not shown; see Annexes). Based on this initial analysis, we decided to focus further analysis on the division of BTB/ANK that contain the characterised genes for our interest; *BOP*1, *BOP*2, *COCH*, and *Cul4*, in a subset of 10 plant species. The phylogeny of BOP proteins (**Fig. 40**) showed two distinct clades corresponding to monocot and dicot sequences, indicating divergent evolution of this gene family after the split between the monocot and dicot lineages 200 million years ago (Wolfe et al., 1989). Monocot *BOP* genes appear to be grouped in two monophyletic groups that may represent an early duplication event after the separation from dicots. Besides *Cul4*, a close paralog was identified in the barley genome (MLOC_61451.6). In agreement with the close evolutionary relationship with barley, a similar situation is found in Brachypodium with Bradi2g60710 and Bradi4g43150 representing putative orthologs of *Cul4* and MLOC_61451.6, respectively. Orthology between *Cul4* and Bradi2g60710 is also supported by chromosomal synteny as described in Chapter 2. In rice, maize, and sorghum, the situation appears more complex due to the presence of additional genes, possibly deriving from recent duplication events, as is the case for Arabidopsis *BOP1* and *BOP2* (Shi et al., 2012). In particular for rice, gene Os01g72020 has the most similar sequence to *Cul4* and is

located in a syntenic block in rice chromosome 1 with barley chromosome 3. While rice paralogs Os12g04410 and Os11g04600 cluster with the barley *Cul4* paralog MLOC_61451 in one group separated from the *Cul4* group by a well-supported node. The high sequence similarity between rice Os12g04410 and Os11g04600 and their position within recently duplicated chromosomal blocks between chromosomes 11 and 12 (Bolot et al., 2009) suggest possible functional redundancy as in *BOP1* and *BOP2* genes. The BOP proteins phylogeny reflects also the general evolutionary relationships between species, placing –for example- barley *BOP* genes closer to Brachypodium sequences, with both barley and Brachypodium closer to rice than to maize and sorghum.
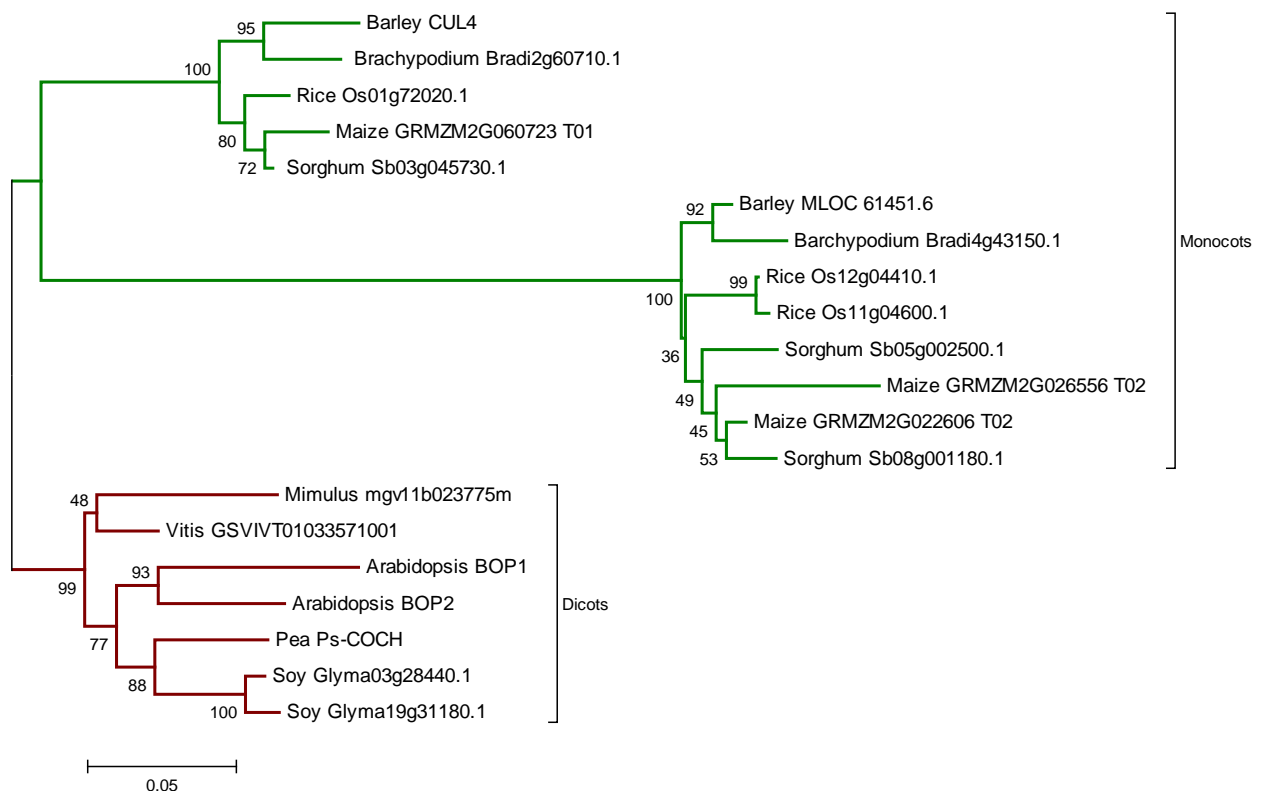
Fig. 40. Evolutionary relationships among BOP-like (NBCL clade of BTB/ANK) proteins. Phytozome v9.1 database was used to retrieve BOP-like protein sequences http://www.phytozome.net/; HC_genes_AA_Seq database http://webblast.ipk-gatersleben.de/barley/viroblast.php was used to retrieve barley sequences; Genbank http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome. Barley (*Hordeum vulgare*) CUL4 = AK360734, Pea (*Pisum sativum*) Ps-

COCH = JN180860, *Arabidopsis thaliana* BOP1 = AT3G57130, *Arabidopsis thaliana* BOP2 = AT2G41370.

4.2 Expression profiles of rice *BOP* gene Os01g72020

For LOC_Os01g72020, the rice ortholog of barley *Cul4*, two probes were found: Os.49276.1.S1_at and OsAffx.24010.1.S1_x_at (Walia et al., 2005). The gene appears to have two maximum expression peaks: the first during early seedling emergence (before first leaf completely unfolded stage), and the second during the flowering (**Fig. 41).** According to the anatomical classification, the gene shows highest expression in stigma, followed by the collar of the flag leaf, leaf sheath, and stem crown (**Fig. 42**). For the other two rice *BOP*-like genes, Os11g04600 and Os12g04410, no probes were found in the Genevestigator database.
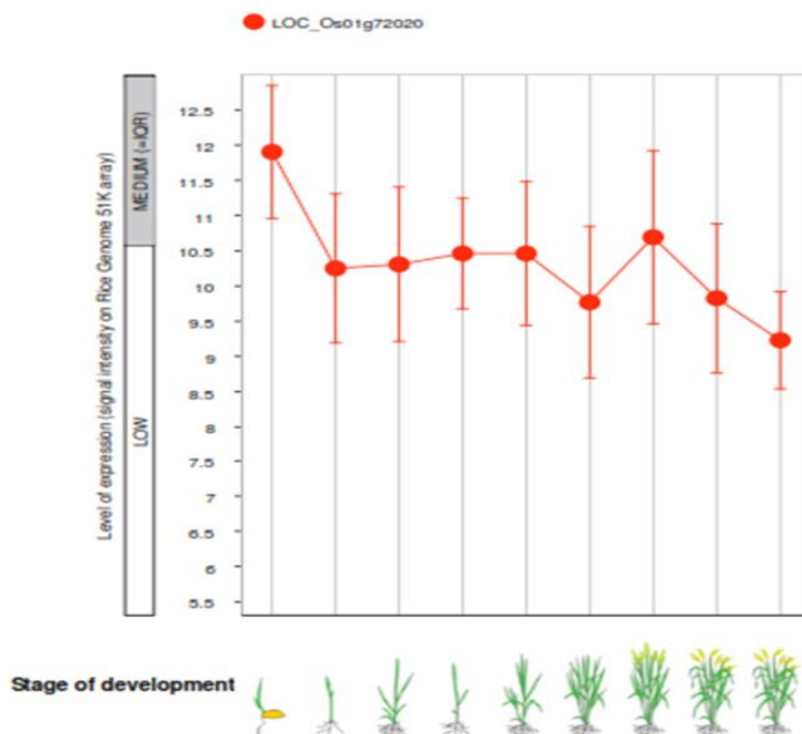
Fig. 41. The expression profile for LOC_Os01g72020 gene during different plant developmental stages.
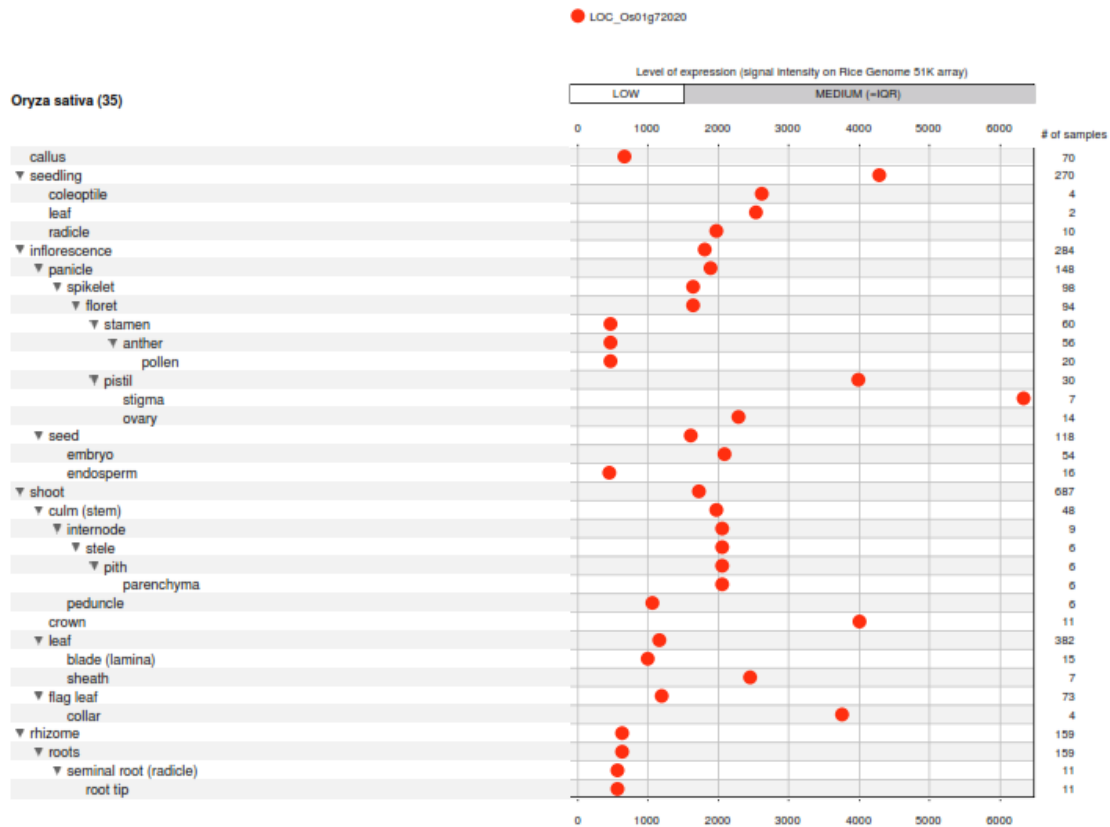
Fig. 42. The expression profile for LOC_Os01g72020 gene according to the anatomical classes.

4.3 Identification of rice line carrying insertions in *Cul4*-like *BOP* genes

In order to explore the biological functions of rice *BOP* genes and compare them with the role of *Cul4* in barley, we undertook a reverse genetics strategy taking advantage of available collections of rice insertional mutants. Similarity searches were conducted on the flanking sequence tags (FSTs) database http://signal.salk.edu/cgi-bin/RiceGE and lines carrying insertions in rice *BOP* genes were identified (**Table. 15**). Analysis of FST information allowed to predict the insertion structure and to draw a schematic representation for each line (**Fig. 43**, **Fig. 44**, and **Fig. 45**).

Table. 15. The results of homologous search using Cul4 protein sequence as query against rice database.

Chrom	gene	score	e-values	Max similar	Gene function	Insertion lines	Type of cassette	location
Chr1	Os01g72020	463	e-129	75%	BTB/ Ankyrin	1B-17402	GUS and GFP trapping	exon
Chr11	Os11g04600	343	3e-93	66%	BTB/ Ankyrin	2D-10109	activation tagging and GUS trapping	exon
						3A-52261		promoter
						3A-50737		intron
						1D-04839	GUS trapping	intron
Chr12	Os12g04410	343	3e-93	66%	BTB/ Ankyrin	2D-10109	Activation tagging	exon

For line 1B-17402, the FST database showed that the T-DNA left border is adjacent to exon 2 of gene Os01g71010 and the downstream sequence, indicating that the insert is in inverted orientation; thus the transcription from the gene promoter through the gene trap construct is expected to result in expression of the *GFP* reporter gene, forming chimeric fusion with exon 2 of gene Os01g71010.

For insertion line 2D-10109, FST information points to a possible insertion in exon 1 in gene Os12g04410 or Os11g04600. As indicated by phylogenetic analysis (Chapter III paragraph 4.1: The Phylogenetic analysis of BOP genes), these two genes are very highly related making it difficult to discriminate between these two possibilities. In either case, the orientation of the insertion would be inverted, and the GUS marker gene would not be expressed.

In the case of line 1D-04839, FST information indicates the T-DNA right border flanks an intron sequence in gene Os11g04600, with inverted orientation of the insertion. In this case the GUS reporter gene is in the transcription direction, therefore it could be expressed.

In insertion line 3A-50737 the insert right border is adjacent to the intron sequence of gene Os11g04600, and the insertion appears to be in forward orientation.

In summary, database searches resulted in the identification of 6 rice lines putatively carrying insertions in *Cul4*-like *BOP* genes.
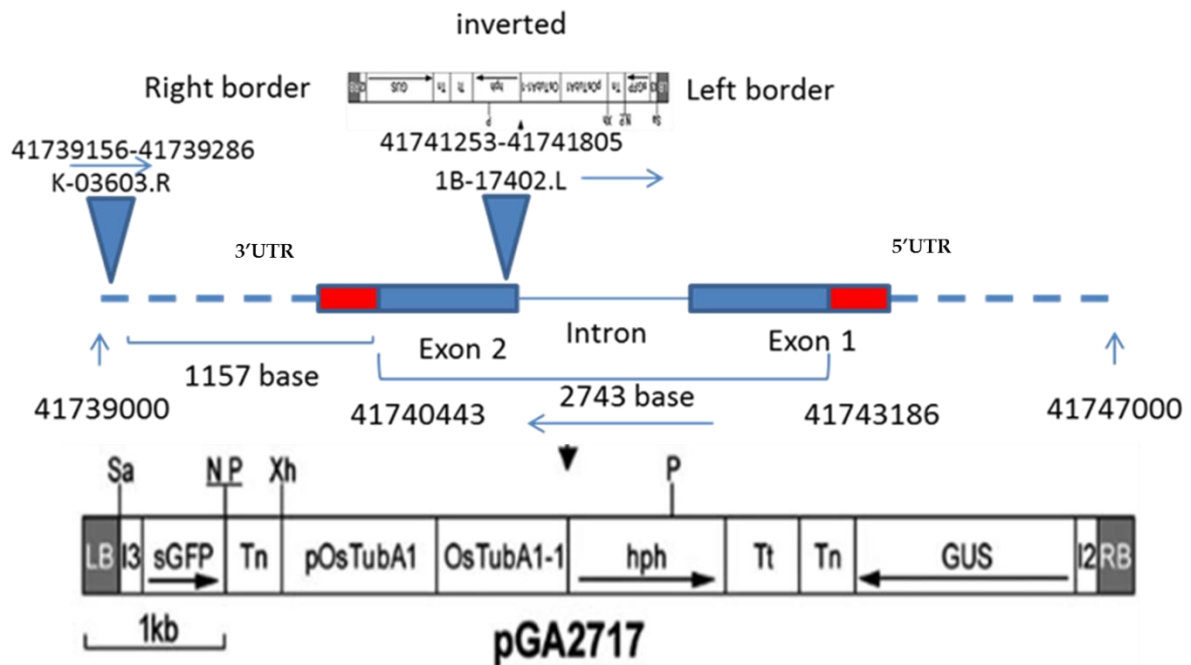
Fig. 43. Schematic diagram for GUS and GFP trapping cassette in insertion line 1B-17402 in gene Os01g72020. The blue rectangular blocks represent gene transcribed sequences, while red rectangular blocks are untranslated sequence (UTR). The numbers on diagram represent the physical positions and distances on rice genome. The inverted blue triangle represents the location of insertion, and the name of the mutant line is reported above the location of insertion with the orientation of insertion (forward or inverted). The blue arrow beside the mutant line name represents the sequence information available on FST database. The transformation cassette is illustrated below the gene diagram.
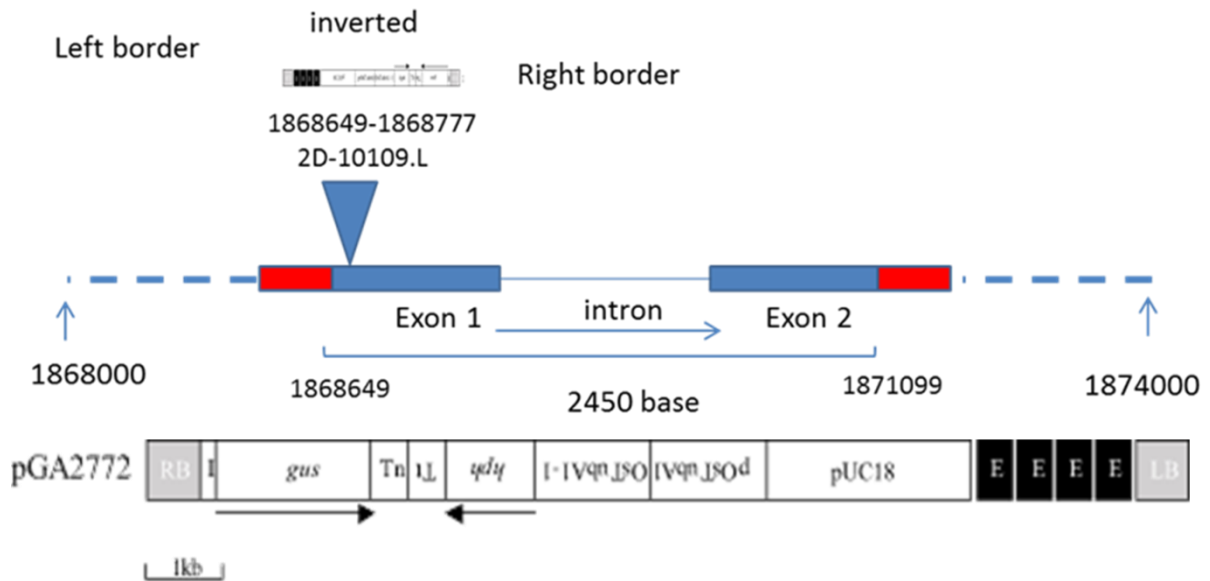
Fig. 44. Schematic diagram for activation tagging and GUS trapping cassette in insertion line 2D-10109 in gene Os12g04410. The blue rectangular blocks represent gene transcribed sequences, while red rectangular blocks are untranslated sequences (UTR). The numbers on diagram represent the physical positions and distances on rice genome. The inverted blue triangle represents the location of insertion, and the name of the mutant line is reported above the location of insertion with the orientation of insertion (forward or inverted). The blue arrow beside the mutant line name represents the sequence information available on FST database. The transformation cassette is illustrated below the gene diagram.
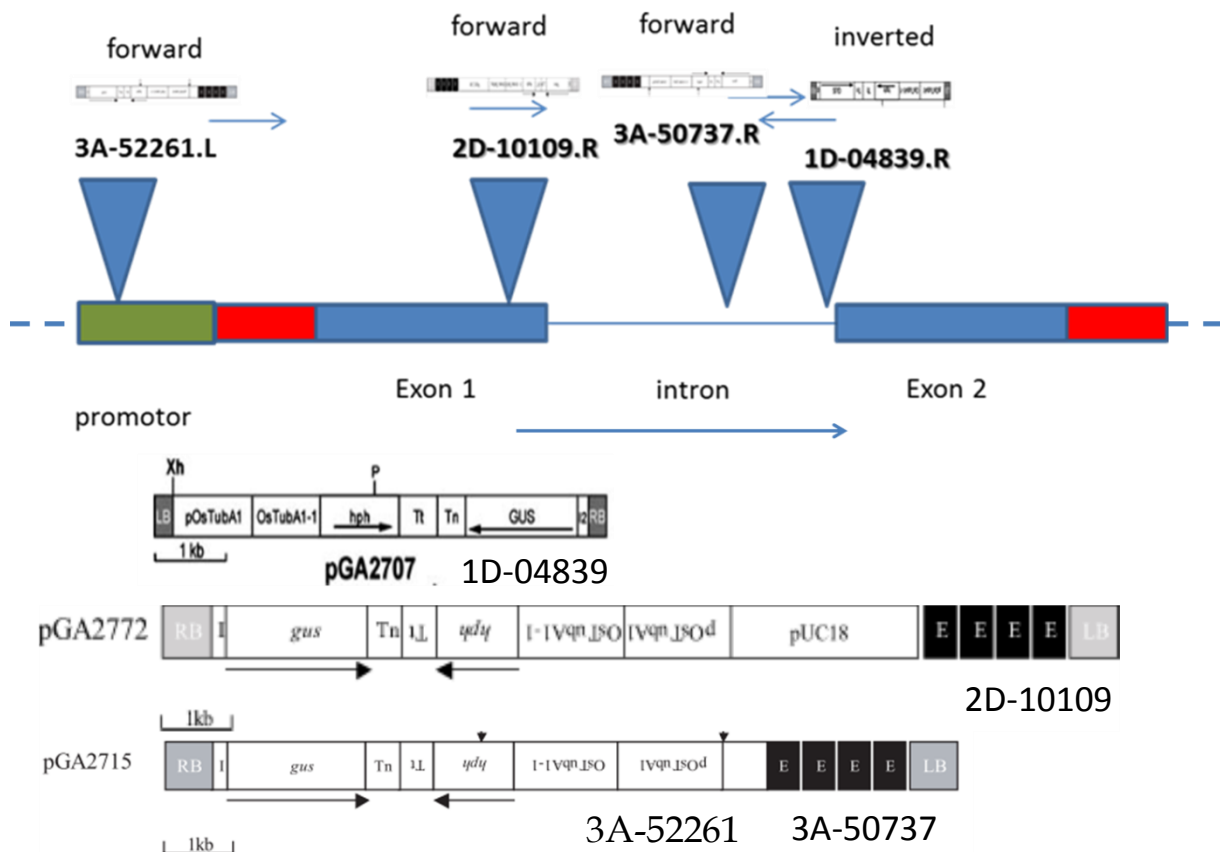
Fig. 45. Schematic diagram for GUS trapping cassette in line 1D-04839, and activation tagging and GUS trapping cassettes in lines 2D-10109, 3A-50737, and 3A-52261, in gene Os11g04600. The blue rectangular blocks represent gene transcribed sequences, while red rectangular blocks are untranslated sequences (UTR). The green rectangular block represents promoter region. The inverted blue triangles represent the locations of insertion, and the name of the mutant lines are reported above the location of insertion with the orientation of insertion (forward or inverted). The arrow beside the mutant line name represents the sequence information available on FST database. The transformation cassettes are illustrated below the gene diagram.

4.4 Validation of T-DNA insertion in line 1B-17402

Phylogenetic reconstruction supports orthology between Os01g71010 and *Cul4*, while indicating that the paralogous genes Os11g04600 and Os12g04410 may have arisen from a recent duplication, a situation which is often associated with functional redundancy. The sequence information on FST database concerning line 2D-10109 does not allow to discriminate the location of the insert in either of the paralog genes Os11g04600 and Os12g04410. Experimental attempts to resolve this by genomic PCR gave non-reproducible results (data not shown). For these reasons, we decided to proceed to the molecular characterization of mutant line 1B-17402.

Four plants of the line 1B-17402 putatively carrying a T-DNA insertion in gene Os01g71010 were screened with primers pGA2717_LB-F, Os01g72020_int_1118-R, and Os01g72020_3UTR_89_F. Genomic PCR results are shown in **Fig. 46**. Plants carrying the insert yielded a band with primers pGA2717_LB-F and Os01g72020_int_1118-R. In case of primers Os01g72020_3UTR_89_F and Os01g72020_int_1118-R, a band indicates presence of the wild-type sequence. Multiplex PCR results indicate this insertion line is heterozygous for the presence of the T-DNA in Os01g72020. The wild-type amplicon from primers Os01g72020_3UTR_89_F and Os01g72020_int_1118-R was compatible with the expected size of 1209 bp. In contrast, the ca. 1100 bp amplicon obtained with primers pGA2717_LB-F and Os01g72020_int_1118-R was smaller than the expected size [1369 bp = 894 bp from the T-DNA insert (including 156 bp from intron 3 of β-tubulin, and 692 bp from the GFP gene) + 452 from the exon 2 sequence of gene Os01g71010]. In order to better characterize the insertion, the 1100 bp fragments obtained from 3 plants of line 1B-17402 were extracted from the gel and cloned into the pGEM-Teasy vector (Promega). Purified plasmid DNAs were subjected to restriction enzyme digestion and sequencing. The restriction patterns along with sequence analyses showed the existence of a 320 bp deletion spanning the left border of the insert and the flanking sequence of the gene.
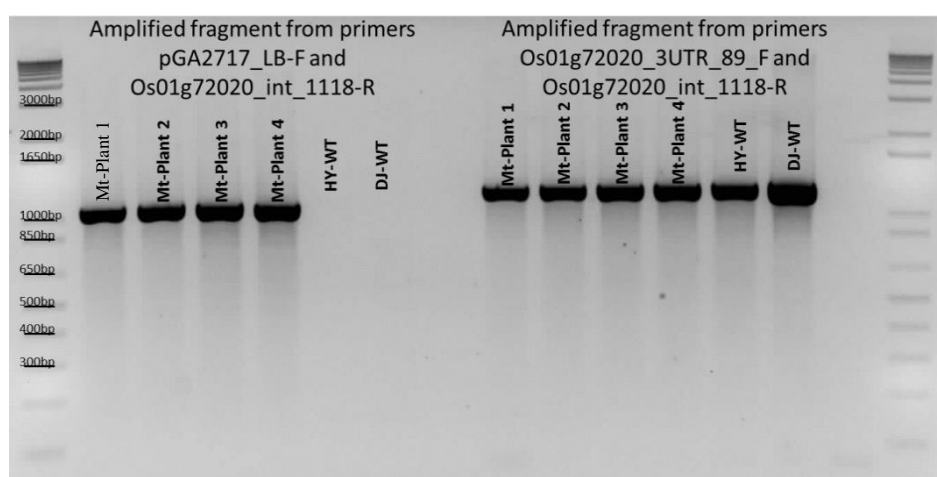
Fig. 46. Electrophoresis of genomic PCR products amplified from line 1B-17402, expected to carry T-DNA insert in gene Os01g72020.

4.5 Phenotypic analysis

Comparison of 1B-17402 plants with their respective background showed that this line did not have any of the defects in leaf patterning or the boundary between leaf blade and leaf sheath seen in barley *cul4* mutants. Leaf polarity and development appeared normal in all plants. Quantitative measurements showed no significant difference between lines 1B-17402 and 3A-50737 and their wild-type background cv. Dongjin, while line 2D-10109 showed shorter plants, shorter leaves, normal stem thickness and normal number of tillers compared to Dongjin, (**Fig. 47** and **Fig. 48**). The activation-tagging vector used to transform plant line 2D-10109 carries *CaMV 35S* enhancer elements that could activate gene transcription up to 4 kb downstream or upstream. Investigating up to 5 kb upstream and downstream the point of insertion in the MSU Rice Genome Annotation database (Kawahara et al., 2013), using Genome Browser tool, no genes have been annotated in that range. On the other hand, line 1D-04839 that carries insertional mutation in rice gene Os11g04600, showed shorter plant stature, thicker stems, and more tillering ability compared to the respective wild-type background Hwayoung.
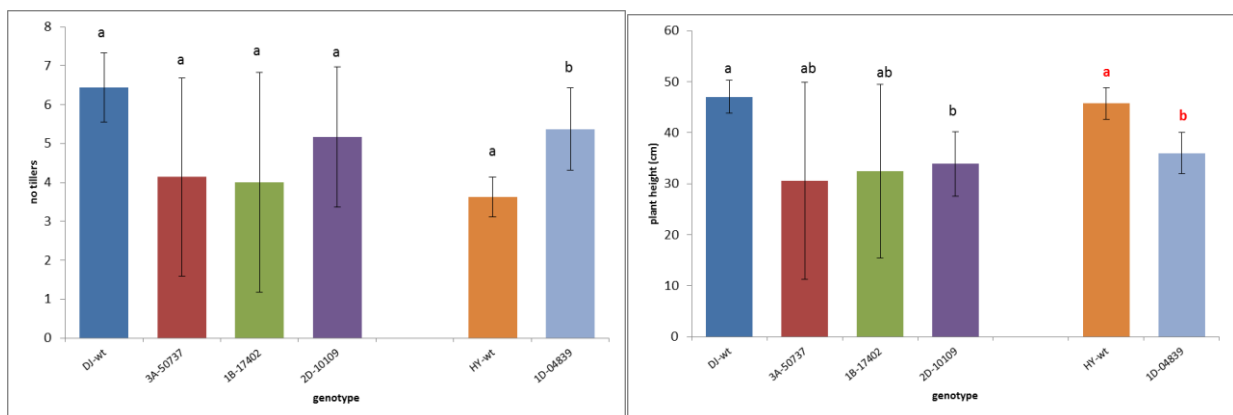
Fig. 47. The number of tillers (left graph) and plant height (right graph) measurements recorded for T-DNA insertional mutant lines in growth chamber experiment, and compared to wild-type background cultivars DJ and HY. The significance group are based on ANOVA and post-hoc Bonferroni-Holm analysis at $p \leq 0.05$.
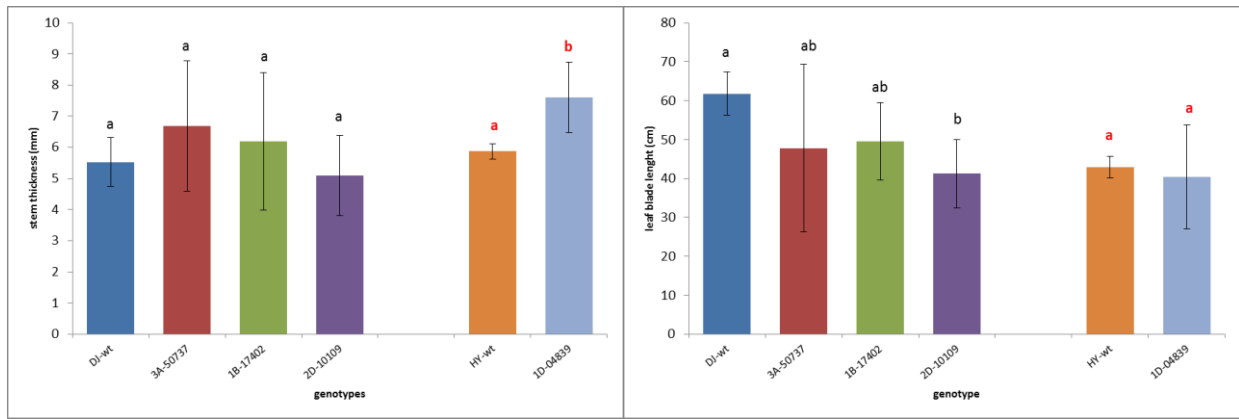
Fig. 48. The stem thickness (left graph) and leaf blade length (right graph) measurements recorded for T-DNA insertional mutant lines in growth chamber experiment, and compared to wild-type background cultivars DJ and HY. The significance group are based on ANOVA and post-hoc Bonferroni-Holm analysis at p≤0.05.

5.   **Discussion**

The Phylogenetic analysis of BTB/ANK genes supports the hypothesis that an ancient duplication resulted in the divergence of the two main clades, NPR and BOP (Shi et al., 2012). The phylogenetic reconstruction presented in this chapter showed that all dicot *BOP* genes cluster in one group, while monocot *BOP* genes assembled into two main clades that appear to have separated relatively early. The barley *Cul4* gene clade includes also a single *BOP* gene from Brachypodium, rice, maize and sorghum supporting orthology among these genes. The other monocot cluster includes, in some cases, pairs of close paralogs, e.g. rice (Os11g04600 and Os12g04410), maize (GRMZM2G022606 and GRMZM2G026556), and sorghum (Sb05g002500 and Sb08g001180). In the case of rice, genes Os11g04600 and Os12g04410 are located within duplicated chromosomal blocks in rice chromosomes 11 and 12 (Bolot et al., 2009). A similar situation applies to *Zea mays* genes GRMZM2G022606 and GRMZM2G026556 located on duplicated segments of chromosomes 2 and 10 (Wei et al., 2007), and sorghum Sb05g002500 and Sb08g001180 on chromosomes 5 and 8 (Paterson et al., 2009).

Mostly, dicot plant species tested carry one *BOP* gene, but for Arabidopsis and soybean, the genome carries two copies that appear to have diverged recently (Shi et al., 2012). In agreement with a recent duplication event, Arabidopsis *BOP1* and *BOP2* are highly related and play redundant functions (Norberg et al., 2005). The pea *BOP* gene *COCH* acts in root architecture, particularly nodule identity during symbiotic relationship with nitrogen-fixing bacteria (Couzigou et al., 2012).

Together, the phylogenetic relatedness of barley *Cul4*, and rice Os01g72020, in addition to the synteny relationship of their chromosomal positions, may suggest molecular function similarity for rice Os01g72020 and *Cul4* as transcriptional repressor involved in axillary meristem development and lateral organ fate (Tavakol et al. unpublished Data; Ha et al., 2003, 2007; Jun et al., 2010; Xu et al., 2010; Couzigou et al., 2012). Possible functional redundancy of the rice genes Os11g04600 and Os12g04410

may be also supported by paralogous sequence similarity and recent chromosomal block duplication on rice chromosomes 11 and 12, as seen for the recent duplication and function redundancy in Arabidopsis *BOP1* and *BOP2* (Norberg et al., 2005; Hepworth et al., 2005).

In order to gain further insight into the possible involvement of rice *BOP* genes in shoot developmental processes, we searched publicly available transcriptomic data generated from Affymetrix Rice Genome 51K microarray comparing two *indica* rice genotypes, FL47 and IR29, under salinity stress conditions (Walia et al., 2005). Out of the three rice *BOP*-like genes, data only existed for Os01g72020 – the ortholog of *Cul4*. Transcripts accumulation was variable among the different developmental stages, with highest expression during the early germination stage, and later during the flowering stage. Plants as sessile organisms produce embryos that have to cope with diverse environmental conditions, and therefore, and in contrast to animal embryos, they establish only the basic body plan and the main axes, while further development is left to be responsive to the post-embryonic inconstant environment (Leyser, 2009). Accordingly, two transitional stages in plant development represent signposts in terms of genetic regulation; the transitions from embryonic to germinative growth and from vegetative to reproductive growth (Finkelstein et al., 2002). Many hormonal and genetic factors are involved in those two transition stages including: ABA, gibberellic acid (GA), cytokinins, or auxin, with interactions between ABA and ethylene signalling, brassinosteroid, light, and sugars (Holdsworth et al., 2001, 2008; Finkelstein et al., 2002). Many of these genetic and hormonal factors are known to contribute to the plasticity of lateral organs development, particularly side branching and leaf formation. Expression of the Os01g72020 gene during germination and flowering, are in agreement with its putative involvement in early development of vegetative organs, and also the establishment and early development of reproductive organs as verified by the second peak in expression during flowering. A role in flowering is also played by Arabidopsis *BOP* genes (Ha et al., 2003, 2007; Hepworth et al., 2005; Xu et al., 2010). *BOP* genes are known to act in floral meristem identity maintenance, and regulation of flowering-time

genes (e.g., AGL24, SOC1, and SVP). Functional similarly can be assumed between rice Os01g72020 and Arabidopsis *BOP* genes depending on temporal transcription pattern, in addition to sequence similarity.

Further analysis of Os01g72020 expression showed that the organ with highest transcripts accumulation is the stigma in the floret, followed by other organs such as the collar of flag leaf, leaf sheath, and stem crown. The anatomical expression pattern of rice Os01g7202 can be attributed to three main anatomical classes: reproductive organs (stigma), leaf organs (collar of flag leaf and leaf sheath), and meristem tissues (stem crown at early stages). The transcriptomic data support the role of Os01g72020 in floral meristem fate and development, leaf morphogenesis, and meristem maintenance and development as in Arabidopsis *BOP* genes (Ha et al., 2003, 2004, 2007; Hepworth et al., 2005; Xu et al., 2010).

Similar expression patterns were seen in barley *Cul4* expression analysis by qRT-PCR (Tavakol et al. unpublished data). *Cul4* transcripts accumulation was detected in the crown of young seedlings, where axillary buds and leaf primordia are developing. Also, *Cul4* higher expression was identified in leaf organs during later stages (4th leaf stage), the expression was intensified in the ligular region of fully expanded leaves. The expression experiments showed comparable results between *BOP* genes, *Cul4*, and Os01g72020, which indicate possible function similarities between these genes. However, microarray "Affymetrix" data for rice Os01g72020 only provide an initial indication and need to be validated by further analysis, particularly with qRT-PCR, RNAseq or *in situ* expression analyses.

Molecular characterization of 1B-17402 insertion showed the T-DNA construct is located in exon 2 and that a deletion exists spanning ~320 bp of the left border (LB) of the insert, and the flanking part of the gene exon 2. This is not an uncommon event as Kim et al. (2003) reported that the LB junction point was not conserved in all tested lines. Such events were also reported in Arabidopsis and tobacco T-DNA lines (Tinland, 1996) where up to 1500 bp could be deleted at the left border integration site.

Phenotypic characterization of insertion line 1B-17402 did not uncover any alterations in leaf patterning and tillering, as seen in *cul4* barley mutants (chapter 2). However, this is expected as phenotyped plants were heterozygote for the T-DNA insertion. Screening of the segregating progenies of these plants is necessary to identify homozygous plants and evaluate their phenotypes. *BOP* genes form a diverse family in terms of biological functions, e.g. *Cul4* affects side branching in barley (Tavakol et al. unpublished Data,), while in pea and *Medicago truncatula*, they affect root architecture, particularly nodule formation (Couzigou et al., 2012), which was not identified in either *Cul4* in barley, or *BOP1* and *BOP2* in Arabidopsis. Also, *BOP1* and *BOP2* have been proposed to act in some plant resistance mechanisms that are mediated by methyl jasmonate (Canet et al., 2012). Such a function has not been proven/studied for *Cul4*, *COCH*, or *NOOT* On the other hand, *BOP*-like genes form a conserved clade, structurally and functionally, and share some common functions in leaf morphogenesis (Tavakol et al. unpublished Data, Ha et al., 2003, 2004, 2007; Hepworth et al., 2005; Xu et al., 2010; Couzigou et al., 2012). Future work will allow to explore and compare more comprehensively *BOP* gene functions in different species to gain a better understanding of the evolution of this gene family.

Insertion lines identified in this work offer a basis for functional characterization of rice *BOP* genes. Although, some insertion lines showed interesting phenotype, the current lack of molecular data on these lines does not allow classifying the type of mutation. Of particular interest, line 1D-04839, that putatively carries insertional mutation in rice gene Os11g04600, showed shorter plant stature with excessive tillering. The produced seeds for that line represent a good starting material for molecular screening and further characterisation.

In addition, the progeny of the heterozygote 1B-17402 can be screened to identify homozygote mutants in the rice *Cul4* ortholog Os01g72020, and identify their phenotype. Line 1B-17402 carries bi-directionally gene trap system that is assumed to express *GFP*. Although, initial efforts were carried out to detect *GFP* expression and the localisation of the chimeric protein (data not shown; see Annexes), no conclusive results

could be presented, given the time limitation imposed by the long growth cycle of rice, and difficulties in growing the target cultivars (long life cycle and photo-period sensitivity) under growth chamber conditions, a problem that has been reported from different laboratories (Kim et al., 2013). The primary work carried out in this PhD project is ideally to be followed by further analysis of the mutant lines and exploiting the numerous and useful genetic and genomic resources that exist for rice.

CHAPTER IV

IDENTIFICATION AND CHARACTERISATION OF TILLERING MUTANTS IN NEW MUTAGENIZED COLLECTION (TILLMORE)

1.   Introduction

1.1 Classical mutant collections in barley

Mutagenesis has been a main driving force in basic and applied genetic research for over 80 years (Jiang and Ramachandran, 2010) and has resulted in thousands of improved crop varieties cultivated worldwide.

Since the pioneering work of Stadler on ionizing radiation mutagenesis in barley (Stadler, 1928), plant genetic research has extensively used mutagenesis to identify mutants to study the fundamental processes of plant physiology and development. Mutagenized collections were produced for a number of plant species (Perry et al., 2003; Wu et al., 2005; Martin et al., 2009; Saito et al., 2011; Meinke, 2013). Barley mutant collections grew to contain thousands of accessions; some with detailed phenotypic descriptions (Franckowiak and Lundqvist, 2012), but most are not yet characterised. The Scandinavian mutation research program established by the Swedish geneticists H. Nilsson-Ehle and A. Gustafsson provided a collection of more than 10,000 different characterized mutants stored in the NordGen genebank (http:// www.nordgen.org/) (Lundqvist, 2009). Among these, a few barley mutants altered in tillering have been identified and characterized (reviewed in chapter 1). Classically, barley tillering mutants are categorised based on the type of deviation from normal development i.e. for producing less tillers or more tillers (Schmitz and Theres, 2005; Bennett and Leyser, 2006; Dabbert et al., 2010). The mutation could disturb either axillary meristem establishment and bud development, or the tiller bud outgrowth. Axillary meristem establishment and bud development are mainly controlled by intrinsic genetic factors. Axillary bud outgrowth to form a tiller is largely controlled by genetic and hormonal factors resulting in a highly plastic and responsive trait (Kebrom et al., 2013).

The genetic and hormonal elements controlling branching among dicots and monocots have shown many similarities and relatedness, but also diverted and

particularized pathways exist (see chapter 1), which impose scientific quests to identify the evolutionary conserved components of branching in monocots and dicot, and also to understand the particular plasticity of developmental programme for each species under its unique environmental habitat. Given the importance of Poaceae family as economic crops, and the direct effect of tillering on their yield, comprehensive account for identifying the main conserved and flexible elements of tillering mechanisms in cereals is a crucial need for both applied and basic science research (Sakamoto and Matsuoka, 2004; Sreenivasulu and Schnurbusch, 2012). Although, rice as a model plant for Poaceae has received increasing importance for studying tillering (Izawa and Shimamoto, 1996; Wang and Li, 2011), but extensive comparison of genes molecular and functional characteristics identified among other cereals is essential. In this context, barley represents a valuable model system to study tillering, due to both, importance as cereal crop and model plant for Triticeae tribe, and also for the cumulative knowledge of genetic and genomic data (Sreenivasulu and Schnurbusch, 2012; Feuillet et al., 2012). Although several conserved genomic and developmental features are common between rice and barley (Mayer et al., 2011), fewer barley genes/loci were identified to be involved in tillering, in comparison to the more number of genes/loci identified and characterised rice. Therefore, many genetic components governing tillering in barley presumably exist and await for identification and characterisation. Screening of barley mutagenized populations, particularly newly developed populations, is expected to result in isolating new mutant lines, and consequently new genes/loci that control tillering in barley. In a classical forward genetics approach (Peters et al., 2003), new mutants are isolated by screening phenotype in mutagenized population, followed by detailed description of the mutant phenotype, identifying the mode of inheritance (e.g. dominant or recessive), ensuring novelty of the locus by performing complementary test against known loci, constructing mapping population by crossing to wild-type parents and propagation of mapping materials, and ultimately, mapping the locus and identifying the molecular components underling the phenotype.

Barley mutants were originally induced or discovered in different backgrounds; which may retard the comparisons between mutant lines, and in order to "standardise" the approach repeated backcross-based transfer of the mutant locus into the common recurrent genetic background was performed. That approach resulted in Near Isogenic Lines (NILs) that carry a relatively small genetic interval from the donor mutant parent which contains the mutated locus, inserted in the genomic background of the recurrent parent (Druka et al., 2011). Extensive backcrossing program in barley was initiated in the mid-1980s to introgress mutated loci from the worldwide collection of barley mutants into a common genetic background; cv. Bowman (Franckowiak et al., 1985). Druka and co-workers developed more than 800 NILs in barley cv. Bowman background (Druka et al., 2011), and the NIL collection includes 17 mutant independent lines indicated as defect in bud development represent 10 different loci.

As has been stated above, the starting point for forward genetics is a screen for a desired phenotype within a mutagenized population, which can be the result of natural or induced mutagenesis. Where possible, some approaches used for mutagenesis can facilitate the subsequent identification of the mutation position, as in the case of T-DNA or transposon mutagenesis in various plant species (Yoder et al., 1988; Ishida et al., 1996; Cheng et al., 1997; Jeon et al., 2000; Alonso et al., 2003; Tadege et al., 2005). Despite some success (Ramachandran and Sundaresan, 2001; May and Martienssen, 2003), such approaches have proven impractical in the case of barley due to difficulties in plant transformation and the lack of efficient transposon systems (Harwood, 2012; Wang et al., 2012). Thus most barley mutants have been identified from chemical or radiation-mutagenesis. In order to isolate the underlying genes, map-based cloning (Muller et al., 1995; Chono et al., 2003; Komatsuda et al., 2007; Tonooka et al., 2009; Taketa et al., 2011; Ramsay et al., 2011; Houston et al., 2012; Yuo et al., 2012) and comparative-genomics/candidate gene approaches (Rossini et al., 2006) are then used. Chemical and radiation mutagenesis can be applied to basically any plant species easily and the spectrum of induced mutants is broader than with tagging approaches (Peters et al., 2003).

1.2 A new take on mutagenesis: TILLING

Besides being exploited in forward genetics, chemical mutagenesis is also a powerful tool for reverse genetic analyses: new high throughput screening methods for the discovery of sequence variants have been applied to identify mutations in genes of interest from mutagenized populations - an approach called TILLING (Targeting Induced Local Lesions IN Genomes (McCallum et al., 2000) – which has been applied in animals and plants, e.g. zebrafish, Arabidopsis, rice, wheat and barley (Rawat et al., 2012) where next generation sequencing allows fast and efficient recovery of desired mutations (Tsai et al., 2011). To this end, different mutagens have been used e.g. Ethyl Methane Sulfonate (EMS) induces 99% C-to-T changes resulting in GC-AT substitution; treatment with N-Nitroso-N-methylurea (MNU) usually resulted in 90% GC-AT transition changes, and when combined with sodium azid resulted in 20% AT-GC changes; Gamma irradiation produces 30% small indels of only a few base pairs, which is effecting for generating knockouts of single genes; fast neutrons produces mainly big deletions ranging in size from hundreds to thousands of base pairs (Wang et al., 2012).

In a classical TILLING protocol (see **Fig. 49**), after chemical mutagenesis, plants are self-fertilized, and DNA samples are collected from M2 or M3 individual plants, pooled and subjected to PCR using primers that hybridize to a region of interest. To detect mutations, PCR reaction products are subjected to digestion by CEL I, then separated by gel-based denaturing electrophoresis and sequenced (Mejlhede et al., 2006; Wang et al., 2012).
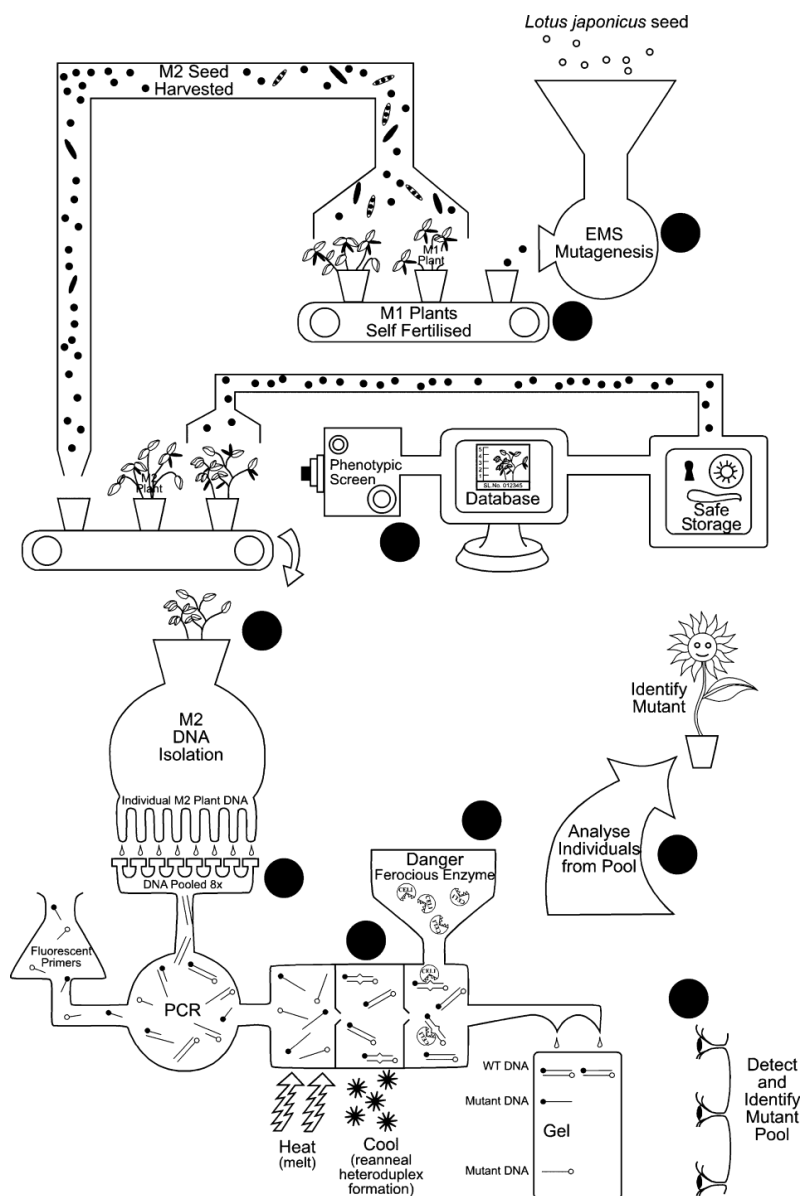
Fig. 49. A summary of the TILLING process embracing forward genetic screening. The pure seed line (a) is treated with EMS at an appropriate concentration (determined by testing small batches of seed with a range of concentrations), then (b) seeds are sown, grown (M1 plant generation) and harvested as individuals for M2 seed. This seed is then re-sown as families (12 seed usually) and the plants screened for segregating phenotypes. A single fertile plant from each family (c) is used to collect leaves for DNA extraction and (d) the seed harvested and stored appropriately. Siblings can be screened for a variety of phenotypes by eye or by high-throughput (bio)chemical screens, e.g. (Takos et al., 2010; Vriet et al., 2010). Seeds from sibling plants bearing useful phenotypes are also stored. Individual DNAs are pooled (e) in microtitre plates and pools used for PCR amplification using a mixture of labelled and unlabelled primers. Products are heated, cooled and (f) the annealed products cut (g) with CEL1 enzyme. The resultant products are purified and run on gels (h) or capillaries to detect the mismatched products. Individuals are then selected from the pools (i) by re-sequencing and their seed sown to identify mutant plants (Wang et al., 2012; reproduced in that article with the permission of Jillian Perry).

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In barley, a few TILLING collections have been produced. For example, a TILLING population was generated using ethylmethanesulphonate (EMS) as chemical mutagenesis in variety 'Optic', producing 9,216 M2 lines, 1152 eight-plant DNA pools and estimated mutation rate of 1 mutation per 1 Mb. For phenotypic data, 12–16 M3 progeny from each of the M2 plants were evaluated visually for phenotypes, and the data made available on (http://bioinf.scri.sari.ac.uk/distilling/distilling.html). A visible phenotype abnormality was recorded for over 20% of the M3 families (Caldwell et al., 2004). Another mutagenized population in the Danish variety 'Lux' induced by sodium azide producing 9,575 lines of M3 plants with estimated mutation rate 1 mutation per 2.5 Mb. The M3 were scored for 4 phenotypes: no growth, chlorophyll defects, dwarfs and necrotic spots on leaves. The phenotype scoring revealed rate of 3.5% abnormal plants (Lababidi et al., 2009). Mutagenized population using EMS was also build in cv. 'Barke' producing 10,279 M2 lines. In this population, mutation rate was estimated to be one mutation per 0.5 Mb, and 20% of the plants of M2 displayed visible phenotype (Gottwald et al., 2009). Additional population was developed in cv. 'Sebastian' using MNU in combined with sodium azide as mutagens to produce 10,000 lines of M2 plants with estimated high frequency of 1 mutation per 0.235 Mb (Kurowska et al., 2011). A good review for the recently developed TILLING populations in barley could be accessed from Kurowska et al., 2011.

The University of Bologna, Department of Agro-Environmental Sciences and Technology (DiSTA) (Italy) produced another TILLING population by treating seeds of cv. 'Morex' with sodium azide (NaN) causes single nucleotide G/C-to-A/T transitions producing 4,906 M2 lines of at high frequency of 1 mutation per 0.374 kb (Talamè et al., 2008). DNA extraction resulted in 4,906 DNA samples, and the morphological alterations are scored regularly and organized in the main following categories: habitus, tillering, plant colour, glossy/waxy, plant height, plant morphology, leaf appearance and heading data. The M3 population show high frequency of morphological defects; spike morphology mutants show 28% of the total number of morphological variants. This population is especially attractive as it is based on 'Morex', a Manchurian six-row

barley cultivar chosen for genome sequencing by the IBSC (IBSC, 2012). Using TILLING approach to screen the TILLMore population, 29 mutations in five starch-related barley genes were identified (Bovina et al., 2011b), and 4 barley genes involved in root development were analysed revealing average of total 20 mutations (6 alleles per gene). Alternatively, forward genetics approaches could be applied to screen TILLMore mutagenized population to identify mutants and follow map-based cloning to isolate genes. The TILLMore population was used to identify desired root morphology for drought tolerance; screening ca. 1000 M4 families allowed to identify ca. 70 lines with altered root phenotypes, and more detailed phenotyping at the histological level is in progress (Bovina et al., 2011a).

2.   **The scope of the chapter**

In order to identify new mutants potentially representing additional genes controlling shoot branching in barley, we searched available phenotypic information for the TILLMore collection (http://www.dista.unibo.it/TILLMore/) for low tillering phenotypes. Lines of interest were obtained from the University of Bologna (courtesy of Dr Silvio Salvi and Dr. Valentine Talamè) phenotyped and propagated by single-seed descent to purify the mutation. In addition to scoring the number of tillers, histological characterisation was carried out for some mutant lines to identify the stage at which the mutation affects tiller development. The initial steps of construction mapping population were performed by crossing the mutant lines to wild-type parents in two growing seasons.

3.   **Materials and Methods**

3.1 Phenotyping

The TILLMore collection was developed at University of Bologna (Talamè et al., 2008) where lines were screened visually and a list of mutant families that showed defects in tillering was published via http://www.dista.unibo.it/TILLMore/. Thirty three mutant lines (listed in **Table. 16**) were ordered and propagated in 2011 in the growth chamber at Parco Tecnologico Padano (Lodi, Italy). The conditions of the growth room were for temperature 22ºC/18ºC, day/night, and for photo-period 18/6 hrs, and relative humidity RH: 60%. The seeds were sown in planting medium contains 4:4:1 sand : peat-moss : clay, one seeds per each pot (13.5 × 13.5 × 15 cm, ca. 2 L), and were irrigated 3 times per week. After 5 months of sowing the seeds, the plants were screening for number of tillers, leaf blade-sheath boundary, plant stature, leaf appearance, and any obvious abnormality (e.g., retarded development). At the end of plant development cycle – 6 months after sowing – the spikes were collected and threshed.

Table. 16. Mutant lines from TILLMore collection that were ordered and grown for propagation and initial phenotyping. For the phenotyping in 2012 and 2013, the actually analysed number of individuals is indicated between brackets in the last two columns.

Mutant code (UNIBO)	Genotype code (UNIMI)	Phenotype UNIBO (from the online database)	Phenotype UNIMI (growth chamber 2011)	no of seeds harvested 2011	Sown in 2012 for characterisation (analysed)	Sown in 2013 for characterisation (analysed)
45	GM1126	Poor tillering, very rich surface wax coating, very early heading	no tillers, normal height	100	15 (14)	-
217	GM1127	Albino seedling, poor tillering, necrotic leaf spots.	no tillers	77	15 (10)	58 (8)
516	GM1128	Single stem (Uniculm)	no tillers	171	20 (13)	-
663	GM1129	Poor tillering	1-3 tilers	106	20 (14)	-
734	GM1130	Erect growth habit, poor tillering, Short leaf, Late heading	Erect growth, few distorted tillers, short plants, late maturity	26	15 (11)	-
948	GM1131	Single stem (Uniculm)	no tillers, shorter than normal	57	20 (14)	27 (7)
1176	GM1132	Semi-dwarf, Poor tillering, Late heading	no tillers, normal height	80	20 (15)	-
1712	GM1133	Single stem (Uniculm)	no tillers, leaf necrosis	133	20 (12)	42 (2+15)
1720	GM1134	Semi-dwarf, Poor tillering, Late heading, Long basal rachis internode	0-3 distorted tillers, plant are shorter than normal, leaf necrosis	56	15 (12)	48 (2)
1798	GM1135	Dwarf, Poor tillering	no tillers, shorter than normal	58	20 (10)	10 (4)
2039	GM1136	Single stem (Uniculm)	NO GERMINATED PLANTS	0	-	-
2083	GM1137	Single stem (Uniculm)	NO GERMINATED PLANTS	0	-	-
2406	GM1138	Albino seedling, Single stem (Uniculm)	weak vegetative tillers, abnormal branching from above ground nodes, plant are very short and late maturity	0	-	-
2793	GM1139	Poor tillering, Long basal rachis internode	no tillers	74	20 (14)	15 (2)
2866	GM1140	Poor tillering, Long basal rachis internode	NO GERMINATED PLANTS	0	2 (original stock; not considered in analysis)	-
3125	GM1141	Single stem	no tiller	33	20 (14)	20 (10)

		(Uniculm), Collapsing leaf blade				
3226	GM1142	Poor tillering	0-1 tillers	190	15 (15)	-
3253	GM1143	Semi-dwarf,Single stem (Uniculm), Narrow leaf	no tillers, narrow leaves, short plants	5	3 (3)	10 (0)
3565	GM1144	Semi-dwarf, Single stem (Uniculm)	NO GERMINATED PLANTS	0	-	-
3617	GM1145	Albino seedling, Poor tillering	NO GERMINATED PLANTS	0	-	-
3621	GM1146	Multinoded, Branched tillers, Irregular spike	no tillers	50	15 (13)	-
3833	GM1147	Poor tillering	no tillers	153	20 (15)	-
4054	GM1148	Albino seedling, Poor tillering	0-1 tillers	58	20 (10)	-
4174	GM1149	Single stem (Uniculm)	NO GERMINATED PLANTS	0	-	-
4188	GM1150	Albino seedling, Poor tillering	very weak plant, slow growing, no tillers	4	-	-
4216	GM1151	Dwarf, Poor tillering	one tiller with spike well developed	74	15 (11)	-
4267	GM1152	Single stem (Uniculm)	no tillers	55	20 (15)	-
4308	GM1153	Short culm, Poor tillering, Necrotic leaf spots	0-1 tillers, short tiller if present	82	15 (11)	-
4401	GM1154	Single stem (Uniculm)	NO GERMINATED PLANTS	0	-	-
5109	GM1155	Single stem (Uniculm)	no tillers, shorter than normal, leaf necrosis	97	20 (14)	-
5166	GM1156	Semi-dwarf, Poor tillering, Short spike	NO GERMINATED PLANTS	0	-	-
5361	GM1157	Narrow culm and leaves, Poor tillering	NO GERMINATED PLANTS	0	-	-
5943	GM1158	Poor tillering, Necrotic leaf spots	no tillers, leaf necrosis	43	15 (14)	-
Morex			1-3 tillers	>100	20 (19)	50 (23)

Lines that had produced sufficient seeds were used for further phenotyping experiments. Around 15-20 seeds from each line were sown in mid-December 2011 in Milan University greenhouse in Tavazzano (Lodi, Italy) for a total of 23 mutant lines and the wild-type background Morex. A single seed was sown in each plastic pot (13.5

× 13.5 × 15 cm, ca. 2 L) filled with normal field soil from Tavazzano, Lodi, Italy. The environmental conditions were the natural conditions of temperature and photoperiod in Lodi, Italy, between December 2011 and June 2012. Irrigation was carried out 2-3 times a week to keep soil wet at 60% of the field holding capacity. The fertilization programme was performed to keep favourable nutritional conditions for seeds germination and growth, and was applied as following: post-emergence (3-4 weeks after sowing), urea solution (200 g/100 L); during tillering stage (5-6 weeks after emergence), Ammonium Nitrate (50 g/100 L), Calcium Nitrate (50 g/100 L), Magnesium Sulphate (50 g/100 L) Potassium Phosphate (50 g/100 L), Fe and microelements (10 g /100 L); during ear emergence (16-20 weeks after emergence), the same fertilizers and doses as during tillering application. During the ear emergence (16-20 weeks after emergence), plants were sprayed with Folicur® Bayer (active ingredient: Tebuconazole 4.3 %) fungicide at rate of 150 ml/ha (plant spacing was 30 plant per m²). The number of tillers per each plant was recorded at the end of May 2012 when flowering was completed.

The same experiment was repeated in 2012/2013, sowing seeds in mid-December and plants were harvested in June, but only 8 mutants lines with the background wild-type Morex were planted. The selected 8 lines were chosen based on the strength of the reduction in tillering, and the availability of seeds.

Statistical analyses were carried out using XL Toolbox add-in in Excel environment. Test for outliers was used to exclude data points with extreme values by Grubbs' test (on-line calculator; http://www.graphpad.com/quickcalcs/Grubbs1.cfm), ANOVA was run, and post-hoc Tukey test carried out. Normality of data was tested graphically to ensure the eligibility of data to run ANOVA test.

3.2 Histological analyses

Based on results of low tillering phenotyping in 2012 and 2013, 3 mutant lines were selected for further histological phenotyping. Seeds were sown in small pots (50

ml) in perlite medium, and put in tank filled with nutrient solution to cover plantlet roots (hydroponic system). Liquid fertilizers (ALGOFLASH ®; NPK + B, Cu, Fe, Mn, Mo, Zn) were added to distilled water at rate of 10 ml per 2L and filled the tank, and were changed once a week. Seedlings were grown in the growth chamber at Parco Tecnologico Padano. The conditions of the growth room were for temperature 22°C/18°C, day/night, photo-period 18/6 hrs, relative humidity RH: 60%. One and two weeks after germination, plant samples were collected for histological dissection. For 1-week old seedlings, the first leaf was completely unfolded (developmental stage; GRO:0007060), while in 2-weeks old seedlings the 2nd leaf completely unfolded, and the tip of 3nd leaf was starting to grow out (GRO:0007061). The roots and leaves were cut off, and a 2 cm sample corresponding to the crown was kept in the fixation solution. Fixation solution (100 ml) was composed of: Ethanol (final conc. 50%), Acetic acid (final conc. 5%), Formaldehyde (final conc. 3.7-3.8%). Crown samples were kept in the fixation solution at 4°C overnight, then dehydrated in alcohol series as follows: 2 steps in Ethanol 85%(30 min), two steps with Ethanol 90%, (30 min) then with Ethanol 95% (30 min), then with Ethanol absolute. The sample was further processed to expose the coleoptile bud by properly hand-cut with a blade. This step was performed to mark the position of coleoptile bud, and to allow the specimen to stay on the cut side into the embedding mould (**Fig. 50**)
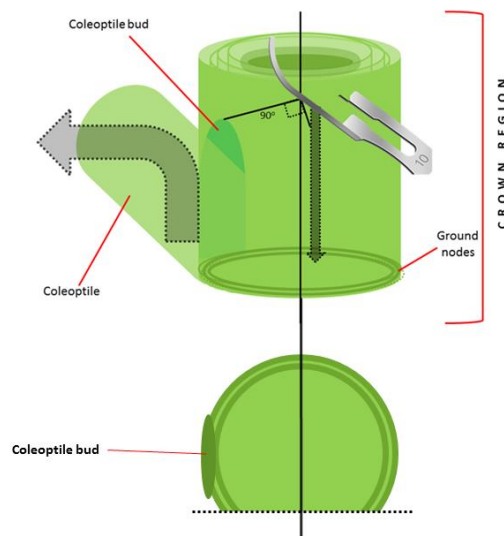
Fig. 50 Diagram illustrating marking the crown sample by hand-cut. The samples for histology were initially processed by peeling off the coleoptile leaf, and mark the position of the coleoptile bud by longitudinal hand-cut with blade.

Embedding was carried out using cold-polymerizing resin Technovit 7100 kit (Hetaeus Kulzer)) following the manufacturer protocol that consists of 3 steps: pre-infiltration, infiltration, and polymerisation. For pre-infiltration, samples were immersed in the mix of 50% Base Solution (Technovit 7100) and 50% ethanol (96%) for 2 hours. In the infiltration step, samples were immersed in preparation solution (1g hardener I in 100 ml base solution) overnight; For embedding and polymerisation, 1 ml of hardener II was added to 15 ml of preparation solution, then about 1 ml of this mixture was poured inside each well of the Teflon embedding mould (Histoform, Fisher scientific), and the plant specimens were positioned so that the coleoptile bud had a chosen orientation to facilitate later dissection. After some hours, the cured samples were mounted by Technovit 3040 (quickly hardening 2-component plastic) that tightly bond the specimen with the plastic holder Histobloc; and after about 10 min., the Histobloc and the fixed specimens were removed from the Histoform.

The embedded specimens were cut by Leica RM2245 microtome to 8 μl thick longitudinal serial sections through the apical meristem region. Sections were spread on water film on glass slides, and then inspected under a light microscope to ensure the

correct cut. The selected slides were incubated in oven at 50°C for 15 min, then stained by Toluidine blue (0.05%; Fisher scientific) for 5 min. After repeated washes with water to wash-off all excess of stain, slides were left to dry and coverslips were mounted over the stained tissue sections and examined using light microscopy. For each specimen serial sections were analysed and the number of axillary buds were recorded.

3.3 Crossing

In order to evaluate the inheritance of the phenotype and start constructing a mapping population, the lines that showed most significantly reduced tillering ability were used in crossing with wild-type parents. In 2012, twelve lines were crossed to four wild-type varieties, Harrington, Barke, Dicktoo, and Steptoe, performing 24 crosses, in which 58 spikes were used (see **Table. 17**). While in 2013, work focused on seven lines for the crossing with wild-type varieties Barke, Steptoe, Dicktoo, and Harrington, performing 17 crosses in which 50 spikes were used (see **Table. 18**). Those seven mutant lines showed the most significant and evident reduction in tillering in 2012 phenotyping. The progeny of 2012 crosses (F1) were sown in the greenhouse in Tavazzano in December 2012, phenotyped for tillering and number of rows in the end of May, and harvested in July 2013.

Table. 17. List for crosses in 2012 stating the parents: the mutant "code" and the wild-type cultivars, and indicating the male and female parents

Mutant genotype code	X	Wild-type
GM1126 ♀	X	Harrington ♂
GM1126 ♀	X	Barke ♂
GM1128 ♀	X	Harrington ♂
GM1130 ♂	X	Harrington ♀
GM1130 ♂	X	Barke ♀
GM1134 ♂	X	Harrington ♀
GM1135 ♂	X	Harrington ♀
GM1135 ♂	X	Barke ♀
GM1135 ♂	X	Dicktoo ♀
GM1141 ♀	X	Harrington ♂
GM1141 ♀	X	Diktoo ♂

GM1141 ♀	X	Barke ♂
GM1143 ♀	X	Harrington ♂
GM1147 ♀	X	Diktoo ♂
GM1148 ♀	X	Diktoo ♂
GM1151 ♀	X	Harrington ♂
GM1151 ♀	X	Barke ♂
GM1152 ♀	X	Diktoo ♂
GM1152 ♀	X	Barke ♂
GM1152 ♀	X	Harrington ♂
GM1152 ♀	X	Steptoe ♂
GM1153 ♀	X	Barke ♂
GM1153 ♀	X	Harrington ♂
GM1153 ♀	X	Steptoe ♂

Table. 18. List for crosses in 2013 stating the parents: the mutant "code" and the wild-type cultivars, and indicating the male and female parents

Mutant genotype code	X	Wild-type
GM1127 ♀	X	Barke ♂
GM1127 ♀	X	Steptoe ♂
GM1130 ♀	X	Barke ♂
GM1130 ♀	X	Steptoe ♂
GM1130 ♀	X	Harrington ♂
GM1131 ♀	X	Barke ♂
GM1131 ♀	X	Steptoe ♂
GM1131 ♀	X	Harrington ♂
GM1131 ♀	X	Dicktoo ♂
GM1133 ♀	X	Steptoe ♂
GM1134 ♀	X	Barke ♂
GM1134 ♀	X	Barke ♂
GM1134 ♀	X	Steptoe ♂
GM1135 ♀	X	Steptoe ♂
GM1135 ♀	X	Dicktoo ♂
GM1141 ♀	X	Barke ♂
GM1141 ♀	X	Steptoe ♂

4.   Results

4.1 Phenotyping

In end of January 2012, 23 mutant lines (TILLMore lines) were sown under greenhouse conditions. For one line (GM1140) only one seeds germinated, and therefore was excluded from the phenotyping analysis. Out of the remaining 22 lines investigated, 12 lines showed significant reduction in tillering (avg. 3.6±1) compared to Morex wild-type background (avg. 7±2.5) (**Fig. 51**). In particular, 6 lines showed the most pronounced reduction in tillering (avg. 2.7±1), with line GM1135 showed the most severe effect (avg. 1.2±1.6).
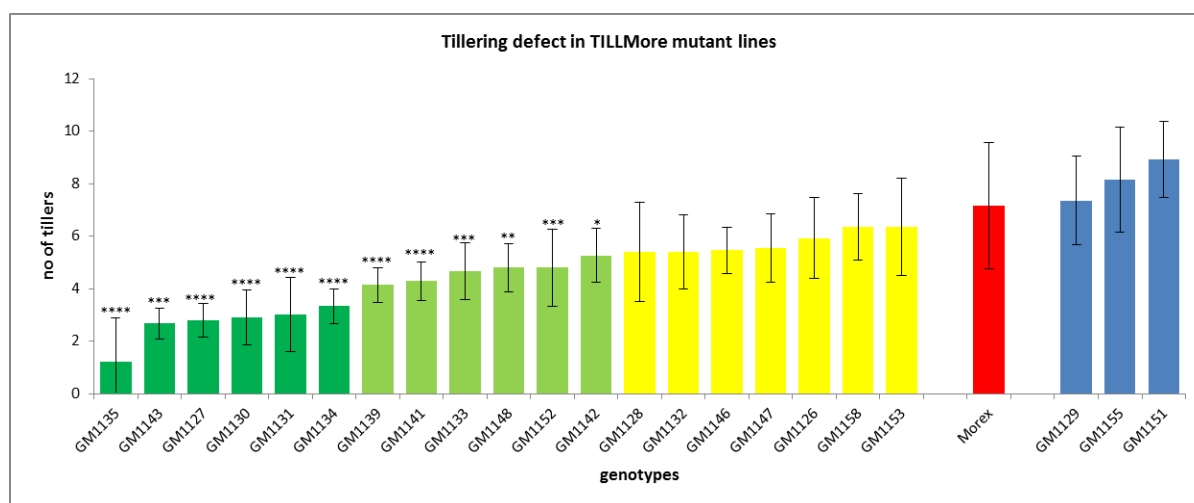
Fig. 51. Number of tillers scored for 22 mutant lines and Morex wild-type background in 2012. The significance indicated as: *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001. The error bars show ±standard deviation. The wild-type Morex (background) is highlighted in red.

In December 2012, 8 mutants lines with the background wild-type Morex were planted, and number of tillers were recorded. According to some uncontrolled problems in the greenhouse (rat attacks), most of the barley seedlings were lost, and in order to compromise for the losses, re-sowing some seeds was carried out in March 2013. Sowing date is known factor affecting number of tiller (late sowing date, less

tillering) (Hay, 1986), and accordingly, it was not possible to compare number of tillering for late-sown plants to the early-sown plants. Moreover, comparing to last year data, although TILLMore lines have comparable measurements for the number of tillers, the wild-type Morex tillered dramatically less than in the previous season (**Fig. 52**). Together, this second experiment helped to propagate the lines but phenotypic data are not considered as they are not reliable due to uncontrolled problems.
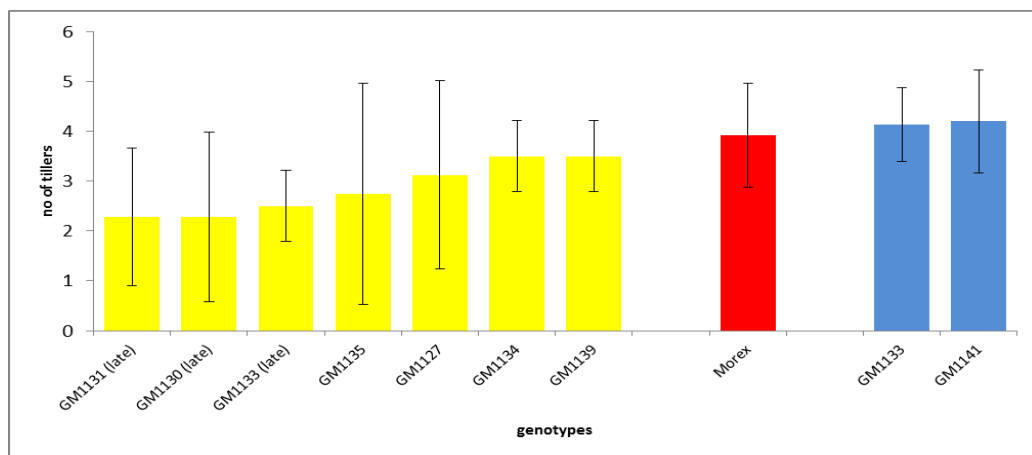
Fig. 52. Number of tillers scored for 8 mutants lines and Morex wild-type background in 2013. Only plants grew from seeds that were sown in December 2012 are presented in this figure. Plants that were sown later were analysed separately (indicated by "late").

4.2 Histological phenotyping

Three mutant lines showing clear reductions in tiller number compared to the wild-type parent Morex, and with sufficient production of seeds (GM1127, GM1130, and GM1131) were further investigated at the histological level to identify at which stage the axillary development is affected. Number of axillary buds were counted for each genotype using light microscopy (**Fig. 53**).
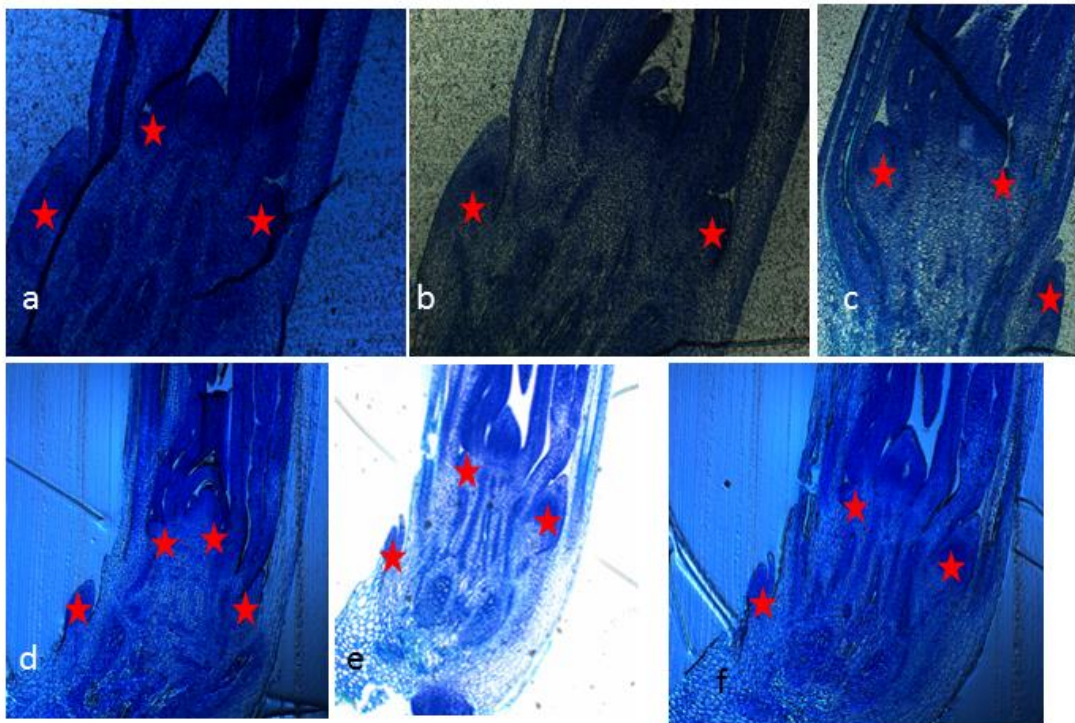
Fig. 53. Histological longitudinal sections (10μm thickness) through apical meristem in mutants and wild-type Morex, showing number of axillary buds. a: GM1127 mutant 1-week-old (3 tiller buds), b: GM1131 mutant 1-week-old (2 tiller buds) c: wild-type Morex 1-week-old (3 tiller buds) d: GM1127 mutant 2-weeks-old (4 tiller bud) e: wild-type Morex 2-weeks-old (3 tiller buds), f: GM1130 mutant 2-weeks-old (3 tiller buds). Red asterisks indicate the tiller buds.

As shown in **Table. 19**, no differences were noticed in number of axillary buds between mutant lines and their background wild-type Morex. Barley seedlings of Morex showed development of 2-3 axillary buds by the end of the first week after germination. The axillary buds grew in the axil of the coleoptile, first leaf, and second leaf. The same number and location of axillary buds were seen in the 3 mutant lines specimens. While in the 2nd week age, axillary meristem developed to form visually distinguishable bud in the 3rd leaf axil.

Table. 19. Number of axillary tillers in 3 mutant lines and the wild-type Morex in one and two weeks old plants.

STAGE	GENOTYPE			
	Morex	**GM1127**	**GM1130**	**GM1131**
1-week old	2-3	2-3	2-3	2-3
2-weeks old	3-4	3-4	3-4	3-4

4.3 Crossing

The TILLMore lines have been generated in the six-rows Morex background. In barley, the 2/6-rows trait is controlled by a single Mendelian locus with six-rows recessive (*vrs1*) vs. two-rows (*Vrs1*) (Komatsuda et al., 2007). We used two-rows wild-type genotypes (*Vrs1 Vrs1*) as pollen parents for crosses (Harrington and Barke), in order to discriminate between seeds deriving from true crosses (i.e. Morex x two-rows F1, *vrs1 Vrs1*, two-rows phenotype) vs. self-fertilization of TILLMore lines (*vrs1 vrs1*, six-rows phenotype). An additional advantage of using two-rows parents is that they represent a distinct genetic pool compared to six-row barleys, maximizing polymorphic marker diversity for future mapping. For example the cv. Barke has been crossed to Morex to produce a dense SNP map to anchor the barley gene space (Comadran et al., 2012). On the other hand, to circumvent possible epistatic effects of the 2/6-row locus on tiller number (Ramsay et al., 2011), the tillering mutants were also crossed with six-row parents Dicktoo and Steptoe, that was used for construction of the Steptoe x Morex SNP map (Muñoz-Amatriaín et al., 2011). A total of 58 spikes were crossed in 2012, but only 28 spikes produced seeds. The produced seeds were sown in the greenhouse in December 2012: scoring for row type indicated that only 4 spikes produced true F1 hybrids **Table. 20**. In order to identify true crosses derived from crosses of six-rows parents screening by molecular marker is necessary. In 2012/2013, 50 spikes were crossed, in which 22 set seeds. The resulted seeds will be planted in 2013/2014 to evaluate true F1s.

Table. 20. List of results of performed crosses in 2012, indicating no of produced seeds, resulted plants, and true F1 hybrids based on 2/6-rows marker. na= not applicable to use row-type as marker, because the two parents are six-rows.

Mutant genotype code	X	Wild-type	Produced seeds	No. of grown plants	No. of true F1 hybrids
GM1126	X	Harrington	11	6	1
GM1126	X	Barke	3	2	0
GM1128	X	Harrington	33	15	10
GM1130	X	Harrington	5	4	0
GM1130	X	Barke	13	3	0
GM1134	X	Harrington	4	0	-
GM1135	X	Harrington	0	-	-
GM1135	X	Barke	0	-	-
GM1135	X	Dicktoo	0	-	-
GM1141	X	Harrington	35	8	4
GM1141	X	Diktoo	6	1	na
GM1141	X	Barke	12	4	1
GM1143	X	Harrington	0	-	-
GM1147	X	Diktoo	8	1	na
GM1148	X	Diktoo	16	1	na
GM1151	X	Harrington	0	-	-
GM1151	X	Barke	0	-	-
GM1152	X	Diktoo	20	14	na
GM1152	X	Barke	11	1	0
GM1152	X	Harrington	21	13	0
GM1152	X	Steptoe	10	2	na
GM1153	X	Barke	3	2	0
GM1153	X	Harrington	2	2	0
GM1153	X	Steptoe	0	-	-

5.   **Discussion**

For many years mutant collections have propelled barley genetics leading to the identification of many loci controlling a wide range of phenotypic traits (Peters et al., 2003). However, classical mutant collections may have not represented all possible loci for certain traits. Comparison between rice and barley tillering loci (Chapter 1) suggests that so far only a subset of tillering loci have been identified in barley. The TILLMore collection hosted by Bologna University, Italy (Talamè et al., 2008) represents an attractive resource for forward genetics approaches in identification of novel barley tillering loci, as preliminary phenotypic information is available (http://www.dista.unibo.it/TILLMore/) and the Morex background was targeted for genome sequencing by the International Barley Sequencing Consortium (2012). In the present research, TILLMore families previously indicated as low tillering or monoculm, were re-evaluated allowing to confirm low tillering phenotypes for 12 out of 23 lines. The seeds were M3 or M4; but due to the growing plan used (bulked mutant family), the mutation may still segregate in the progenies. In order to stabilise the low tillering phenotype and purify sub-lines for further genetic analyses, we propagated materials by single-seed descent. During the two seasons 2011/2012 and 2012/2013, we could see comparable figures for the number of tillers, except for the wild-type Morex, which is more probably caused by uncontrolled environmental effects in the growing season 2012/2013.

The 3 mutant lines (GM1127, GM1130, and GM1131) with the most significant and evident defect in tiller development, and that also produced enough seeds were selected for further histological characterisation. Line GM1127 indicated as producing albino seedlings, poor tillering, and necrotic leaf spots in TILLMore database, and showed reduced tillering produce only 2.8±0.6 tillers in 2012, and 3±1.8 tillers in 2013. While the number of tillers were significantly less than the corresponding wild-type background 'Morex', which produced 7±2.5, the histological dissection of axillary buds

at 1-week and 2-weeks old seedlings showed no differences to the wild-type tiller number. Similar results were obtained for GM1130 with 2.9±1 tillers in 2012, and 2.2±1.7 tillers in 2012, and line GM1131 with 3±1.4 tillers in 2012, and 2.2±1.3 tillers in 2013, but both showed the same number of axillary buds like Morex at 1-week and 2-weeks old seedlings.

Low tillering or monoculm mutants identified in barley and rice were either lacking axillary meristems/buds, or the axillary buds were suppressed from growing out to form tiller (Bennett and Leyser, 2006). In rice, *moc1*, *lax1*, and *lax2* mutants showed defect in axillary meristem establishment and development, therefore, no axillary buds were formed (Li et al., 2003; Oikawa and Kyozuka, 2009; Tabuchi et al., 2011). While other mutants, particularly those have impaired biosynthesis or signalling in hormonal pathways, showed defect in axillary bud out-growth rather than axillary meristem establishment or development (Jain et al., 2006; Arite et al., 2007; Lo et al., 2008; Tong and Chu, 2009; Lin et al., 2009; Beveridge and Kyozuka, 2010; Qi et al., 2011; Chen et al., 2012). The function homologous of rice *moc1*, *lax1*, and *lax2* mutants have not been identified in barley yet (Babb and Muehlbauer, 2003; Dabbert et al., 2009, 2010). In barley, the characterised tillering mutants have defect either in lower number of axillary buds, e.g. *als* and *lnt* (Dabbert et al., 2009, 2010) , delayed developmental, e.g. *als* and *lnt* (Dabbert et al., 2009, 2010) or the outgrowth of axillary buds, e.g. *als*, *lnt*, *cul2*, and *cul4* (Tavakol et al. unpublished Data; Dabbert et al., 2009, 2010; Druka et al., 2011; Ramsay et al., 2011), as those steps are widely controlled by various complex genetic and hormonal networks (Kebrom et al., 2013). Our results showed that the 3 investigated lines have the same number of developed axillary buds as the wild-type, indicating the bud outgrowth defect in these mutants is in tiller outgrowth rather than axillary bud formation.

In order to select lines of interest for further analyses, in parallel to characterization of phenotypic effects, the inheritance of the phenotype needs to be verified by crossing with wild-type parents. To this end, we crossed total of 12 mutant lines to 4 wild-type varieties. The wild-type varieties Barke and Harrington were used

as two-rows parents and row-type was used as visual marker for successful crosses. In addition, six-rows parents Dicktoo and Steptoe were used because the loci controlling row type are also known to affect number of tillers (Ramsay et al., 2011). A total of 108 spikes were used during the crossing work in 2012 and 2013, but only 50 spikes were productive. In 2012 crossing work produced total of 213 seeds, but only 79 were successfully grown, 16 plants of them are proved to be true F1 hybrids, and 29 await to be confirmed by molecular genotyping and 44 proved to be failed cross. The seeds produced from crosses in 2012 and 2013 will be propagated and screened in the coming year.

Together this work produced useful materials and information for further characterization of tillering in the TILLMore mutant lines. Constructing adequate mapping populations is the most important and crucial step in forward genetics. The crosses obtained can be used in the future to genetically map the corresponding tillering loci in barley. However, allelism tests with known classical mutants are needed first to be performed in order to verify new tillering loci.

Conclusions

Since the first rice gene involved in tillering was isolated (Li et al., 2003), a number of genetic and hormonal factors were identified as regulators of tillering in grasses (Kebrom et al., 2013). The overall objective of this PhD project was to contribute to the understanding of the genetic bases of tillering in barley and rice, as model species for cereal crops. The recessive *cul4* locus had previously been described as causing a low tillering phenotype in barley (Franckowiak and Lundqvist, 2012)and mapped within a 0.22 cM genetic interval on chromosome 3HL, where a Candidate Gene (CG) was identified as cosegregating with the trait in a population of 4949 F3 plants (Tavakol et al. unpublished Data). In this PhD project, the correspondence between this CG and the *cul4* phenotype was validated by: 1) mapping of the *cul4* locus at higher resolution (0.09 cM) within a single BAC clone and showing recombination with adjacent genes ; 2) identification of three independent mutant alleles confirming that mutations in the *Cul4* gene account for recessive *cul4* phenotypes of varying severity The *Cul4* gene was thus shown to encode a BTB/ANK domain protein highly related to Arabidopsis BOP1 and BOP2 (Ha et al., 2003; Norberg et al., 2005), providing the first proof that *BOP*-like genes are involved in tillering in monocots. In addition, mutations of the *Cul4* gene resulted in perturbations of leaf morphology (absence of ligules, ectopic outgrowth of auricle tissue on proximal leaf sheath), longer spikes, high infertility, increase in grain weight and acceleration of floral development, indicating that *Cul4* controls multiple aspects of vegetative and reproductive development, as well as sharing with dicot homologs a role in leaf patterning.

To evaluate the role of *Cul4/BOP*-like genes in other cereals, *Cul4* homologs were identified in rice and a phylogenetic analysis was carried out using protein sequences from 5 monocot and 5 dicot plant species. BOP-like proteins organised in two main clades, corresponding to the partition between monocots and dicots. Within monocots, BOP-like proteins are grouped in two clades, possibly reflecting an ancient duplication.

The Cul4 sub-clade contains the rice ortholog Os01g72020, while rice paralogs Os12g04410 and Os11g04600 belong to the other monocot sub-clade and may have arisen from a recent duplication, as also in the case of Arabidopsis BOP1 and BOP2 (Norberg et al., 2005). Publicly available transcriptomics data from the Genevestigator database (Hruz et al., 2008) provided an initial indication that gene expression patterns of the rice *Cul4* ortholog Os01g72020 are compatible with a possible role in axillary meristem development, leaf morphogenesis, and floral organ development. *In silico* searches of rice FST information (Jeong et al., 2006), allowed the identification of mutagenized lines carrying T-DNA insertions within rice *BOP*-like genes. The lines were ordered and grown for propagation and phenotyping. Molecular screening of line 1B-17402 revealed that it is heterozygous for the insertional mutation, and that a deletion of ~320 bp occurred in the left border of the insert and the upstream sequence of the gene. Molecular data and materials resulting from this work form the bases for further studies on rice *BOP*-like genes.

In the attempt of identifying novel genes involved in tillering in barley, we took advantage of the barley mutagenized collection TILLMore, developed and hosted by University of Bologna (Talamè et al., 2008). This TILLING population is especially attractive for barley genetic studies it was developed in cv. "Morex" the reference sequenced cultivar. Phenotypic data on TILLMore mutant families were searched online and thirty three lines classified as exhibiting tillering defects were ordered to be propagated and phenotyped. Out of the 33 lines, 12 showed significant reduction in number of tillers, and were subjected to further phenotyping analyses. Among them, three lines showed the most evident and consistent low tillering phenotype and were thus selected for histological analyses. Histological sections through apical and axillary meristems demonstrated normal early development of axillary buds, which indicate that the mutant lines are impaired in tiller bud outgrowth rather than axillary bud development. Selected lines, based on obvious low tillering phenotype, were crossed to 4 wild-type parents, in order to study the inheritance of the tillering trait and build mapping populations for further forward genetics studies.

In summary, this PhD project contributed to improve our knowledge on genetic factors controlling tillering in barley, and offered starting information and material for further analysis of this important trait in rice and barley.

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Annexes

Evolutionary relationships of BTB/ANK/NPR1 proteins

The tree was built from aligned protein sequences retrieved from publicly available genome sequences databases. The cul4 peptide sequence (513 aa) was used as enquiry in program BLASTP 2.2.22+. searching tool on Phytozome v9.1 for the following species: *Arabidopsis thaliana*, *Brachypodium distachyon*, *Glycine max*, *Mimulus guttatus*, *Oryza sativa*, *Sorghum bicolor*, *Vitis vinifera*, *Zea mays, solanum lycopersicum, Musa spp.*. For *Hordeum vulgare*, blastp was used on IPK barley blast server, on HC_genes_AA_Seq database. All alternative splicing forms of genes are pesented. ScanProsite tool was used to scan each polypeptide against Prosite database to ensure the presence of BTB, NRP1, MATH and Ankyrin domains. BOP proteins are indicated with green arrows.

The evolutionary history was inferred using the Neighbour-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to each branch. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. The analysis involved 71 amino acid sequences from 11 species. All ambiguous positions were removed for each sequence. There were a total of 375 positions in the final dataset. Evolutionary analyses were conducted in MEGA5. All splicing formed are marked with U-turn arrows. Splicing forms can carry different protein domains as indicated in the tree. The BTB proteins are organised in two main clades, BTB/ANK (13 proteins) and BTB/NPR1 (16 proteins) in one clade, and BTB/MATH (5 proteins) in another clade. BTB/ANK and BTB/NPR1 diverted later forming two speared well supported groups. Proteins represented in BTB/MATH clade appear as non-comprehensive list, as they are presented only in Sorghum. For detailed discussion on BTB/ANK clade see Chapter 2.
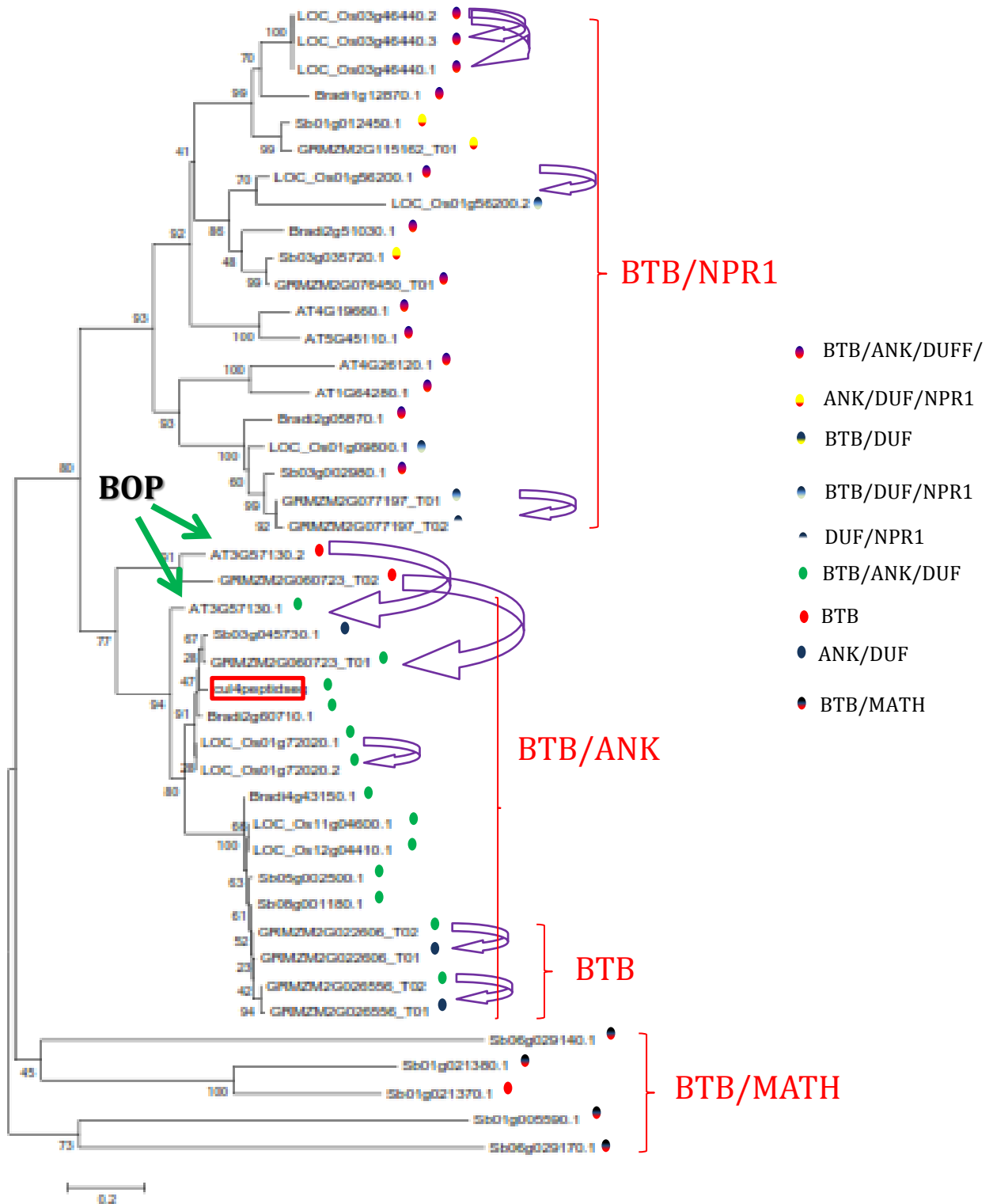
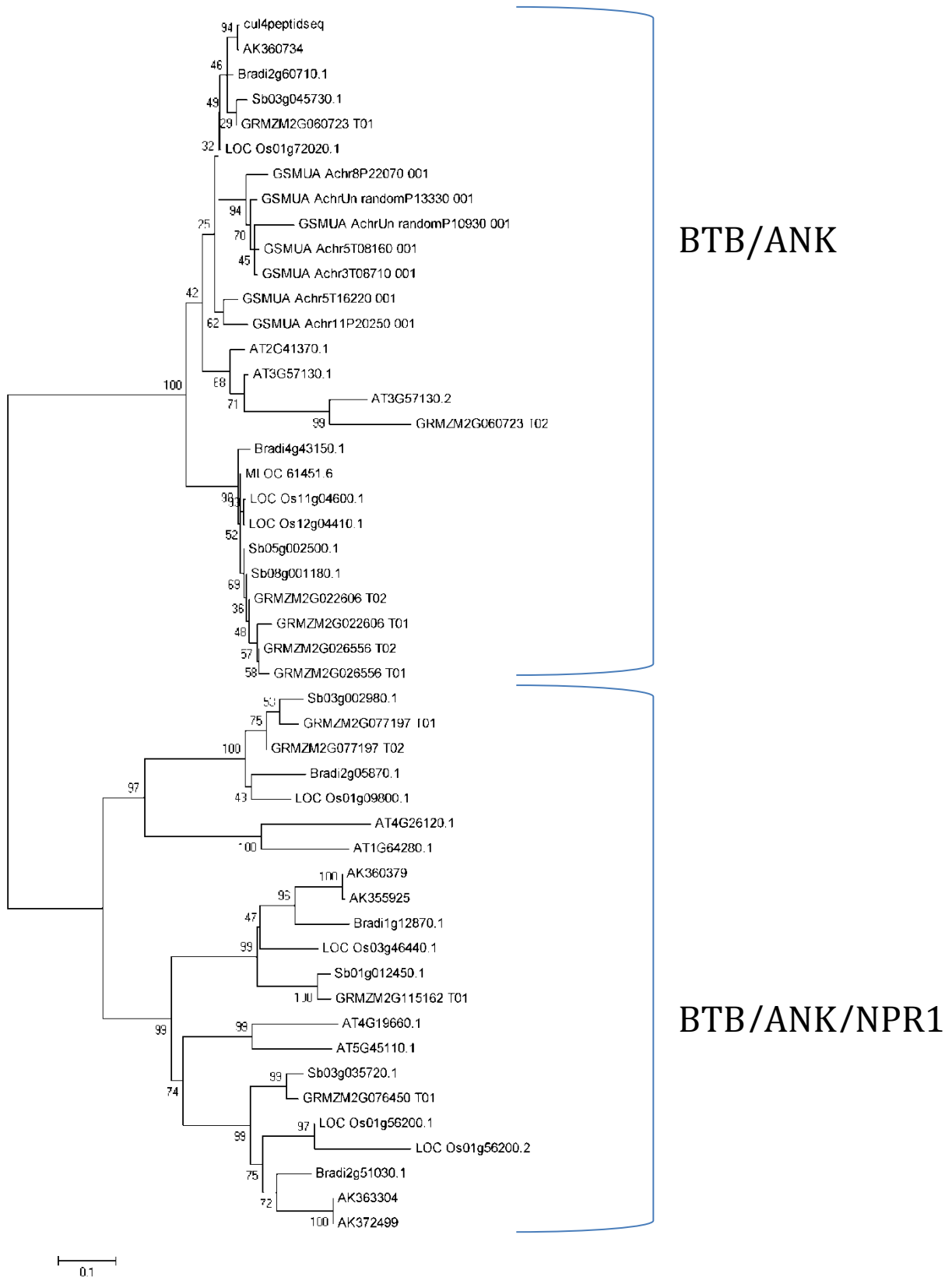Fig. Annex 1. Phylogenetic relationships of BTB proteins from 11 plant species.

Fig. Annex 2. Phylogenetic relationships of BTB/ANK proteins from 11 plant species.
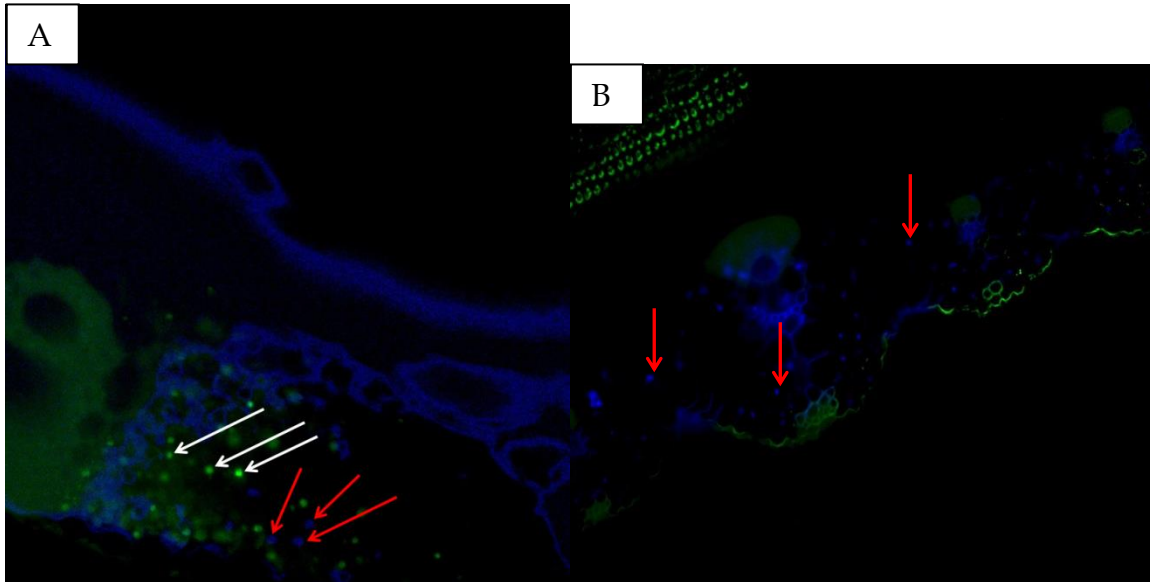
GFP expression analysis

Fig. Annex 3. Epi-flourcence microscopy images for A: transverse hand section of leaf blade of *GFP* transformed rice plant. B: transverse hand section of leaf blade wild-typ rice plant. The plant sections were subjected to DAPI staining. The cell nuclei show blue emission of DAPI (red arrows), and presumed GFP expression appear in green emission (white arrows).

Microscopy image for T-DNA transformed rice plant leaf blade in comparison to wild-type DJ rice plant leaf. The T-DNA cassette contains GFP in frame with endogenous gene Os01g72020 expression. GFP assumed to form chimeric protein with truncated host protein (exon 2). The plant specimen was hand-cut, stained with DAPI (staining cell nuclei with blue), and examined under epi- fluorescence microscope. A: transformed plant line 1B-17402, the white arrows indicates possible GFP expression, while red arrows represent cell nuclei. B: Wild-type cv. DJ rice plant with cell nuclei in blue, and non-specific emission of green fluoresce from auto-fluoresce components of cell.