# Expression Analyses of Barley *Knox* Homeobox Genes and Characterization of Putative Upstream Regulators of *BKn3* (*Barley Knox3*), the *Hooded* Gene

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**Yamei WANG**

aus Changchun, China

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Berichterstatter: PD. Dr. Richard Thompson
Prof. Dr. Wolfgang Werr

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*To my home country – China,*

*my parents*

*and my husband – Awen*

# Abbreviations

| | |
|---|---|
| **APS** | Ammonium persulphate |
| **bp** | base pair |
| **BSA** | Bovine Serum Albumin |
| **CaMV** | Cauliflower mosaic virus |
| **cDNA** | Complementary deoxyibonucleic acid |
| **DAP** | day after pollination |
| **DEPC** | diethlypyrocarbonate |
| **DIG** | digoxingenin |
| **DNA** | deoxyribonucleic acid |
| **DNase** | deoxyribonuclease |
| **DTT** | dithiothreitol |
| **EDTA** | ethylene diamine tetraacetic acid |
| **EtBr** | ethidium bromide |
| **GFP** | green fluorescent protein |
| **GST** | glutathione-*S*-transferase |
| **GUS** | β-glucuronidase |
| **HEPES** | 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid |
| **IPTG** | isoproyethio-β-o-galactopyranoside |
| **kb** | kilo base |
| **MES** | 2-(*N*-Morpholino)ethane·sulfonic acid |
| **MOPS** | 3-(*N*-morpholino)-propanesulfonic acid |
| **MS** | Murashige and Skoog |
| **mRNA** | messenger ribonucleic acid |
| **4-MUG** | 4-methylumbelliferyl-β-D-glucuronide |
| **NAA** | α-naphethalene acetic acid |
| **NBT/BCIP** | 4-Nitobluetetrazoliumchloride/5-Bromo-4-chloro-3-indolyl-phosphate |
| **PCR** | polymerase chain reaction |
| **PEG** | polyethylene glycol |
| **pfu** | plaque forming units |
| **RNA** | ribonucleic acid |
| **RNase** | ribonuclease |
| **RNasin** | RNase inhibitor |
| **RT-PCR** | reverse transcrition-polymerase chain reaction |
| **TEMED** | tetramethylethylenediamine |
| **SDS** | sodium dodecyl sulfate |
| **SDS-PAGE** | sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| **ssDNA** | single-stranded deoxyribonucleic acid |

# CONTENTS

# INTRODUCTION

# *CHAPTER 1*

## 1.1 The discovery of the homeobox and homeobox genes in animals

'Homeotic' transformations are developmental anomalies in which one part of the body develops in the likeness of another. Many cases of homeosis were described by William Bateson in his book (Bateson, 1894). Within the large collection of such homeotic variations, there are a few striking examples: in the sawfly *Cimbex*, the antenna is transformed into a foot; the *Zygaena* moth shows the transformation of a hind leg into a hind wing; even more striking is the transformation of an eye into an antenna-like structure in the crab *Palinurus*. *Bithorax* (*bx*), the first homeotic mutant in *Drosophila* arose spontaneously in the laboratory and shows a partial duplication of the thorax. Several homeotic mutations causing the transformation of an antenna to a leg, named *Antennapedia*, were found in nature or induced by neutron or X-ray irradiation (Gehring, 1998).

During chromosome walking across the *Antennapedia* locus, it was found that *Antennapedia* cDNA also hybridized to the neighboring *fushi tarazu* (*ftz*) and *Ultrabithorax* (*Ubx*) genes. The homology sequence causing the cross-hybridization was the homeobox a 180bp DNA fragment with 75-77% sequence identity between these genes (McGinnis *et al*., 1984a; Scott and Weiner, 1984). The three homeoboxes encode a similar protein sequence, which is a 60 amino acid protein segment, designated the homeodomain. Soon after this discovery other homeobox-containing genes were cloned from frogs, mice, humans, sea urchins and nematodes using homeobox DNA cross-hybridization (see review Gehring, 1998).

Nuclear magnetic resonance (NMR) analysis and X-ray crystal structures of several homeodomains have provided direct evidence for a helix-turn-helix structure (Qian *et al*., 1989; Billeter *et al*., 1990; Otting *et al*., 1990). There are three well-defined α–helices in the Antennapedia homeodomain: helix 1 followed by a loop connecting it to helix 2, helix 2 is connected with helix 3. The three helical regions are folded into a tight globular structure, forming a well-defined hydrophobic core. Helix 3 was named 'recognition helix' because it has been shown to be required for recognition of specific target DNA sequences by fitting into the major groove of DNA (Gehring *et al*., 1994a, 1994b; Scott *et al*., 1989). However, the DNA-binding action and the functional specificity of homeodomain proteins also reside to a large extent in the N-terminal arm of the homeodomain (reviewed by Gehring, 1998). The yeast MATα2 homeodomain, which shares only 28 percent amino acid sequence identity to that of Antennapedia, has a very similar three-dimensional structure (see review Gehring, 1998). From these studies it can be concluded that although the primary homeodomain sequence can be quite divergent among different genes, the secondary structures are remarkably similar.

Homeobox genes play a vital role in the control of a vast diversity of cellular and developmental processes in animals. In *Drosophila*, the clustered homeotic genes of the

*Antennapedia* and *bithorax* complexes determine segment identity by promoting the morphogenesis of appropriate anatomical structures within particular segmental or parasegmental domains of the body (Lawrence and Morata, 1994). The vertebrate *HOX* genes also instruct cells to undergo appropriate development decisions. *HOX* genes are expressed in nearly every cell type, but have been most extensively studied for their roles in the developing central nervous system and axial skeleton (Manak and Scott, 1994). Novel and divergent homeobox genes are continually being identified. They may diverge in function and control the development of the forebrain, brachial arches, sensory organs, teeth, and limbs and may be involved in oncogenesis (Duboule 1994).

## 1.2 Plant homeobox genes

By analogy to the functional roles of animal homeobox genes, plant homeobox genes are thought to play important roles in plant morphogenesis. Many homeodomain proteins have been cloned from various plants in efforts to address the biological functions of homeobox genes in plant development.

The most important criterion for designating a novel gene to an homeodomain gene superclass, class or family is the structure of the homeodomain itself, due to its important functional implications, mainly in the protein-DNA interaction. In many instances, however, domains outside the homeodomain are conserved and are also used in the designation of the individual homeobox gene. In plants, homeodomain proteins are rather diverged and presently fall into six different families according to amino acid sequence similarities within the homeodomain or conserved protein motifs outside the homeodomain, they are (1) Knox, (2) Bel, (3) HD-Zip, (4) PHD-finger, (5) Glabra 2, and (6) PALE families (Fig. 1-1).

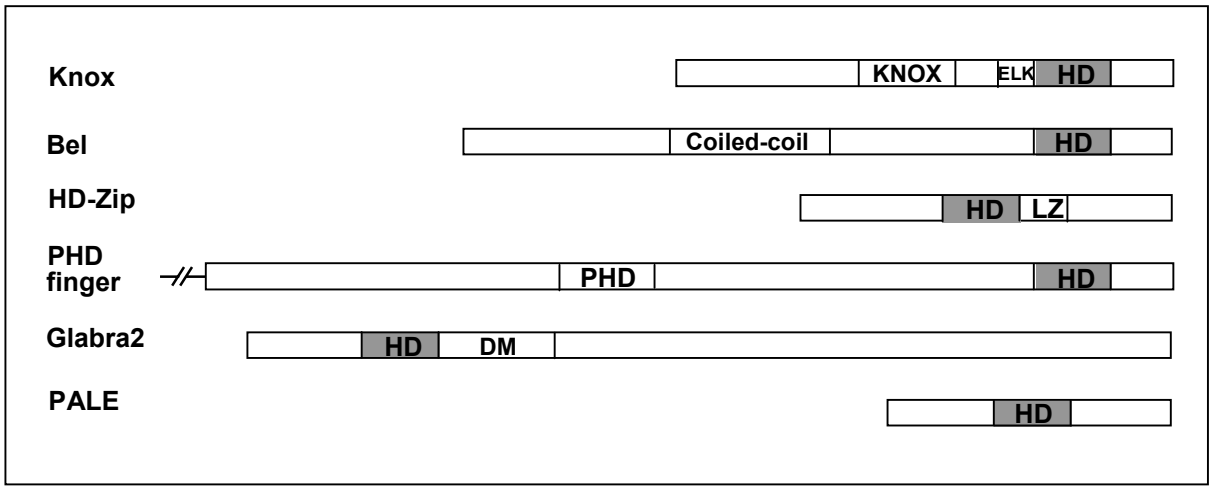



**Fig. 1-1 Scheme of representative members of each family of plant homeodomains.** Conserved motifs are shown: HD, homeodomain; KNOX, Knox domain; ELK, ELK domain; Coiled-coil, coiled-coil domain; LZ, leucine zipper; PHD, PHD-finger; DM, dimerization motif. (adapted from Chan *et al*., 1998 with some modifications)

### 1.2.1 Knox family

Knox (*Knotted-1* like homeobox) proteins all have the homeodomain located near the C-terminal end (Fig. 1-1). The homeodomain of all members of this family has three extra amino acids between helix 1 and helix 2. They belong to the TALE (three amino acid loop extension) superclass (Bertolino *et al*., 1995), which consists of two classes in plants (KNOX and BEL), two classes in fungi (the mating type genes and the CUP genes) and four classes in animals (PBC, MEIS, TGIF and IRO) (Bürglin, 1997).

Just upstream of the homeodomain, there is a second motif which is highly conserved in all members of the Knox family. This motif, termed ELK after the first three amino acids, has been postulated to play a role in protein-protein interactions or serve as a nuclear localization signal. The similarity between the KNOX domain and the MEIS domain from a TALE class in animals suggests that the KNOX and the MEIS domains are both derived from the same common ancestral domain, the MEINOX domain. A smaller, less conserved element, the GSE box, is present between the KNOX domain and the ELK domain (Bürglin, 1997). Sequence comparisons of various *Knox* genes have shown extensive conservation further upstream, the KNOX domain of about 100 amino acids, which has been shown to be important for protein-protein interactions (Müller, 1999; Müller *et al*., 2001).

The *Knox* family defines a large gene family and has been divided in class I and class II genes based on sequence similarities and expression patterns (Kerstetter *et al*., 1994). Mutants of *Knox* genes in maize, barley, rice and *Arabidopsis* suggest that class I *Knox* genes play important roles in plant development (more details in section 1.3).

### 1.2.2 Bel family

The *Bel* family consists of two genes isolated from *Arabidopsis* (Reiser *et al*., 1995; Quadvlieg *et al*., 1995) and two genes from barley (Müller, 1999; Müller *et al*., 2001) so far. BELL1 has a homeodomain located within the C-terminal third of the protein (total length of 611 amino acids) and is involved in ovule development (Reiser *et al*., 1995). It is similar to ATH1 (*Arabidopsis thaliana* homeobox 1), which is 473 amino acids long, and lacks most of the C-terminal region found in BELL1 (Quadvlieg *et al*., 1995). Two barley cDNAs encoding two BELL1 like proteins JUBEL1 and JUBEL2, were isolated in an attempt to identify interaction partners of barley *Hooded* gene product BKN3 by yeast two-hybrid screening (Müller, 1999; Müller *et al*., 2001). They belong to the TALE superclass of homeodomain proteins, but form a distinct class separated from the KNOX proteins. Outside the homeodomain, similarities between the BEL proteins include a region folding into a coiled-coil structure, considered to be a putative protein-protein interaction surface (Reiser *et al*., 1995). The coiled-coil domains of JUBEL1 and JUBEL2 are involved in protein-protein interactions, but the coiled-coil domain alone is not sufficient for the interaction (Müller,

1999; Müller *et al*., 2001).

### 1.2.3 HD-Zip family

HD-Zip (Homeodomain leucine-zipper) proteins are distinguished by the presence of a leucine zipper adjacent to the homeodomain (Fig. 1-1). This feature is found only in plant homeodomain proteins (Sessa *et al*., 1998). The leucine zipper motif was shown to be involved in the formation of protein dimers (Sessa *et al*., 1993). In general, an acidic domain is found N-terminal to the homeodomain, which could be the transcriptional activation domain. HD-Zip proteins constitute a large family of homeodomain-containing proteins and have been further subdivided into three or four classes, depending on whether the Glabra2 family is considered as HD-Zip class IV or not (see review Chan *et al*., 1998).

Expression analyses showed that HD-Zip genes are expressed in different plant organs and different developmental stages (see review Chan *et al*., 1998). They may have functions in several processes, including photomorphogenesis (Carabelli *et al*., 1996), vascular development (Tornero *et al*., 1996; Scarpella *et al*., 2000), and defense gene regulation (Frank *et al*., 1998).

### 1.2.4 PHD-finger family

PHD-finger (plant homeodomain-finger) proteins are defined by the presence of a $Cys_4$-His-$Cys_3$ zinc finger (PHD-finger motif) N-terminal to the homeodomain. ZMHOX1 in maize (Bellmann and Werr, 1992) and HAT3.1 in *Arabidopsis* (Schindler *et al*., 1993) are two members of this family. The ZMHOX1a homeodomain protein binds to the *Shrunken* feedback-control element, further supporting the notion that plant homeodomain proteins function as DNA-binding regulators (Bellmann and Werr, 1992).

Overexpression of *Zmhox1a* and *Zmhox1b* in tobacco causes reduction in plant size, the formation of adventitious shoots and homeotic floral transformations (Uberlacker *et al*., 1996). These observations suggest that *Zmhox1* genes may be involved in regulating important aspects of plant development throughout the life cycle of the maize plant. The interaction between the *Zmhox1a* gene product and the 14-3-3 protein as revealed by the yeast two-hybrid system suggested that PHD-finger proteins might be potential targets for 14-3-3 singalling. This protein-protein interaction may be of general importance and incorporate homeodomain transcription factors into plant signalling pathways (Halbach *et al*., 2000).

### 1.2.5 Glabra2 family

The Glabra2 family contains *Glabra2*, *ATML1* (*Arabidopsis thaliana* meristem layer 1) in *Arabidopsis* (Rerie *et al*., 1994; Lu *et al*., 1996) and *O39* in orchid (Nadeau *et al*., 1996).

Proteins in this family contain a truncated leucine zipper-like segment adjacent to the homeodomain. This motif is present at the same position as that found in HD-Zip I and II proteins, interrupted by a loop and followed by another leucine zipper-like segment. Domain exchange experiments indicate that the two coiled coils connected by the loop in GLABRA2 can replace the leucine zipper motif of one HD-Zip gene and promote dimerization and consequent DNA binding (DiCristina *et al*., 1996).

Mutant analysis shows that *Glabra2* is necessary for normal trichome formation in *Arabidopsis* (Rerie *et al*., 1994). The expression of *ATML1* gene restricted to the protoderm suggests that it may be involved in the definition of morphogenetic boundaries of positional information necessary for controlling cell specification and pattern formation (Lu *et al*., 1996). *O39* is specifically expressed in placental epidermis and protoderm (Nadeau *et al*., 1996). These studies suggested that genes of the *Glabra2* family may play a role in development of the epidermis and related structures.

### 1.2.6 PALE family

The *Pale* (penta loop extension) family is a newly defined family containing to date only two genes isolated from *Populus tremula* × *tremuloides* (Hertzberg and Olsson, 1998). The PALE proteins are about 200 amino acids long and have a homeodomain located at the center of the proteins. The homeodomain is 65 amino acids long, due to an extra 5 amino acids inserted between helices 1 and 2. There is no other distinctive feature known outside the homeodomain. These two *Pale* genes may be involved in the regulation of secondary xylem development or vascular cell development.

## 1.3 *Knox* genes in plants

As mentioned before, the plant *Knox* genes fall into two classes based on sequence similarities and expression patterns (Kerstetter *et al*., 1994). The homeodomains of *Knox* class I and class II proteins show a high level of identity, suggesting that they interact with similar DNA sequences. When class I and II proteins are compared, identical amino acids are found mainly in helix 3 (the recognition helix), the loop, the turn, and the last four amino acids of the N-terminal arm. This may indicate that, although the main contacts with the DNA backbone may be similar for members of the two classes, subtle changes in structure may exist which may result in difference in DNA binding and/or interaction with other protein partners (Chan *et al*., 1998).

Class I genes have been isolated from maize (Vollbrecht *et al*., 1991; Kerstetter *et al*., 1994; Schneeberger *et al*., 1995), barley (Müller *et al*., 1995), rice (Matsuoka *et al*., 1993), soybean (Ma *et al*., 1994), *Arabidopsis* (Lincoln *et al*., 1994; Chuck *et al*., 1996; Long *et al*., 1996), tobacco (Müller, 1997; Tamaoki *et al*., 1997) and tomato (Hareven *et al*., 1996; Janssen *et*

*al*., 1998). They are mainly expressed in apical tissues and the available evidence suggests that they may act to regulate basic morphogenetic programs in the apex. For class II genes, there is much less evidence on their role. In general, class II genes have a more diverse pattern of gene expression than class I genes.

### 1.3.1 Mutations of class I *Knox* genes

Many class I *Knox* genes were isolated through the study of mutants. Mutant analyses have provided very useful clues on *Knox* gene functions in plant development.

#### 1.3.1.1 Gain-of-function mutants

***Dominant mutants in maize***

The maize *Knox* genes *Knotted1*, *Rough sheath1*, *Liguleless3* and *Gnarley1* were first defined by a series of dominant mutations exhibiting similar, yet distinguishable phenotypes in the leaf (Freeling, 1992). These mutations all show perturbations at the blade-sheath boundary as shown in Fig.1-2.

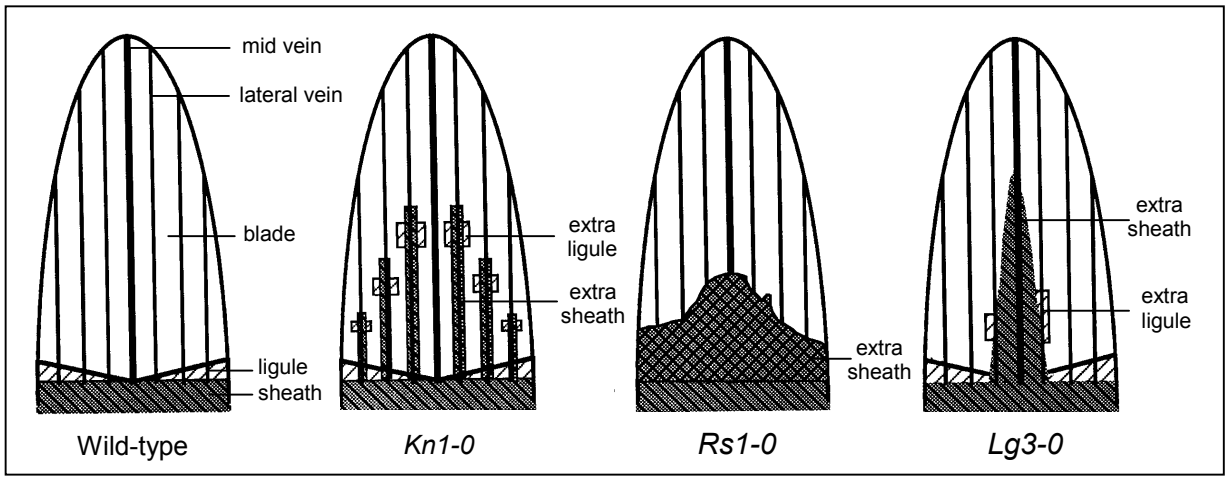



**Fig. 1-2 Schematic representation of adult, vegetative leaves of wild-type and *Kn1-0*, *Rs1-0* and *Lg3-0* mutants.** All mutant leaves show a proliferation of sheath-like tissue that would normally have been blade. (adapted from Freeling, 1992)

*Knotted-1* (*Kn1*) mutations are gain-of-function (neomorphic), dominant mutations that have been interpreted as retarding maturation of cells specifically around lateral veins such that these cells act immature when signalled to differentiated (Freeling and Hake, 1985). This immaturity is characterized by excessive leaf growth (knots) and sheath extending around lateral veins, thereby replacing normal blade identity with sheath identity, and leading to ectopic ligule formation at the new blade-sheath boundaries. *Kn1* mutants are known to function in the provascular tissue of the leaf, and to induce surrounding tissues to differentiate inappropriately. *Kn1* has been cloned by transposon tagging (Hake *et al*., 1989) and was the first plant gene shown to contain a homeobox (Vollbrecht *et al*., 1991).

The *Rough sheath1* (*Rs1*) mutant is phenotypically similar to *Kn1*, but the phenotype is confined to the ligular area of the leaf (Fig. 1-3) and causes a proliferation of sheath-like tissue at the base of the blade and throughout the ligular region (Becraft and Freeling, 1994).

The *Liguleless3* (*Lg3*) mutant causes a blade-to-sheath transformation at the midrib region of the maize leaf (Fig. 1-3). The LG3 protein is highly homologous to maize KN1 and RS1 (Muehlbauer *et al*., 1999).

Most of *Kn1*, *Rs1* and *Lg3* mutant alleles were caused by transposon insertion in introns or in the 5´ UTR of the corresponding genes. These *Knox* genes are normally expressed in the shoot apical cells. The dominant phenotypes are caused by their ectopic expression in the leaf (Smith *et al*., 1992; Schneeberger *et al*., 1995; Muehlbauer *et al*., 1999).

*Gnarley1* (*Gn1*) is a dominant mutation in the *Knox4* homeobox gene. Mutants show reduction in internode and sheath length caused by an overall reduction in cell length in these tissues (Foster *et al*., 1999). The cause of the mutant phenotype is not clear. Two mutant alleles have unique polymorphisms 5´ of the coding regions that distinguish them from their respective progenitors. The insertion of a *Mu* element 21bp 5´ of the start of *Knox4* transcription causes the reversion of the dominant phenotype.

***The barley Hooded mutant***

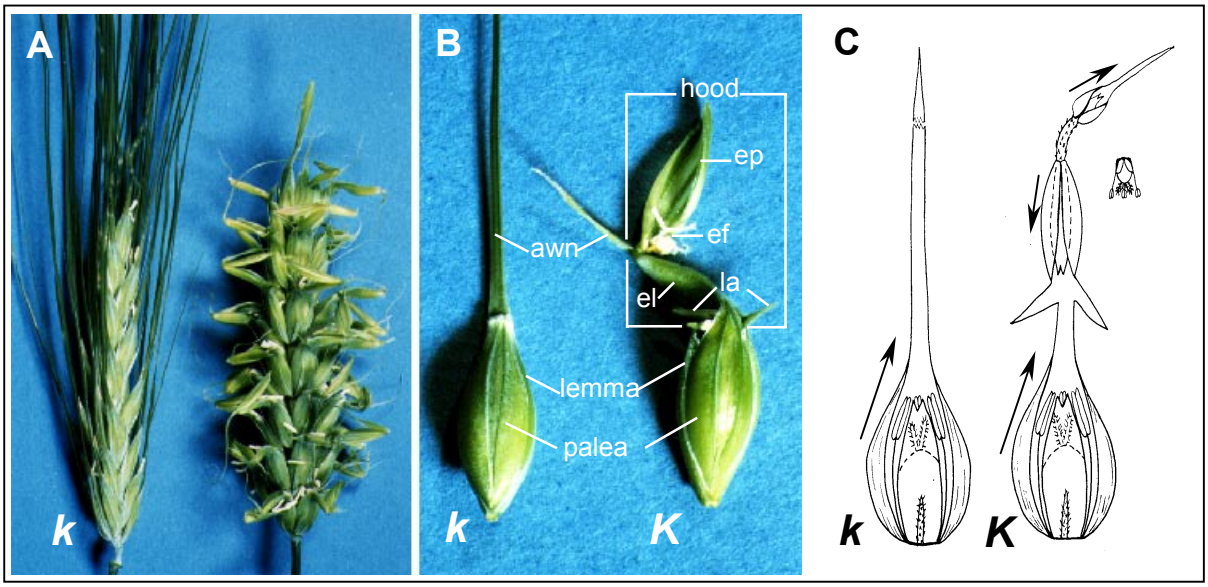



**Fig. 1-3 Phenotypes of *awned* (*k*) and *Hooded* (*K*) barley.** (A) Inflorescences of *awned* (*k*) and *Hooded* (*K*) barley. (B) Single florets with the awn shown for *k* and with the hood shown for *K*. ef, extra floret; ep, extra palea; el, extra lemma; la, lateral appendages (photographs kindly provided by Dr. Judith Müller). (C) The hood is inversely oriented with respect to the direction of lemma growth. Arrows indicate the orientation of the lemma growth. (adapted from Stebbins and Yagil, 1966)

In the dominant gain-of-function barley mutant *Hooded*, periclinal cell divisions in the subepidermal layer of the awn primordium give rise to a meristematic cushion, which

differentiates the hood consisting of an extra flower with the organs seen in Fig. 1-3. The hood is inversely oriented with respect to the direction of lemma growth (Fig. 1-3C). The German translation of hooded, Kapuze, was used for the symbol of this mutation (*K*). The homeotic mutation of awned (*k*) barley to the *Hooded* (*K*) phenotype occurred only once naturally (Müller *et al*., 1995). The *Hooded* mutation results from the ectopic expression of *HvKnox3* (later named *BKn3* (*Barley Knox* 3)) in the lemma, which is caused by a 305bp duplication in intron IV of this class I *Knox* gene (Müller *et al*., 1995).

***Dominant mutants in tomato***

Two dominant mutants in tomato, *Curl (Cu)* and *Mouse-ear (Me)*, show ramification of the compound leaf, suppression of apical dominance, and retardation of growth (Parnis *et al*., 1997). These mutations are associated with two aberrant modes of the transcription of *TKn2*, a class I *Knox* gene. Overexpression of the two in-frame wild-type transcripts of *TKn2* is associated with the *Cu* mutation, whereas misexpression of an abundant and oversized fusion mRNA is associated with the *Me* mutation (Parnis *et al*., 1997).

The phenotype of these dominant mutants suggests a role for class I *Knox* genes in cell fate acquisition and in the switch between indeterminate and determinate cell fates. But it has been realized that dominant mutant phenotypes do not necessarily allow a prediction of the function of the corresponding wild-type gene and may not be sufficient to define gene function (Smith and Hake, 1994). This is because dominant, gain-of-function mutations typically result from overexpression or misexpression of a gene or from a change in the gene sequence that results in production of a novel protein with different functional properties from its wild-type counterpart. Recessive, loss-of-function mutations would permit a more direct analysis of gene function.

### 1.3.1.2 Loss-of-function mutants

The *Arabidopsis SHOOTMERISTEMLESS* (*STM*) gene encodes a KN1-like homeodomain protein (Long *et al*., 1996). Loss-of-function mutations at the *STM* locus result in plants that can form cotyledons, but are unable to form subsequent leaves, clearly indicating that *STM* is necessary to initiate or maintain the shoot meristem (Barton and Poethig, 1993; Clark *et al*., 1996; Endrizzi *et al*., 1996).

Loss-of-function mutations at the *Kn1* locus only exhibit subtle phenotypes including fewer branches and spikelets on the tassel, absent or small ears with few spikelets, extra carpels in female florets, abnormally proliferated ovule tissue, and extra leaves in the axils of vegetative leaves. This may indicate that the loss of *Kn1* uncovers a redundant factor in the maize genome (Kerstetter *et al*., 1997). The normal role of *Kn1* is still unclear and may be broader than that revealed by loss-of-function mutants, if expression of other *Knox* genes can partially compensate for its loss.

Three recessive *lg3* mutant alleles, each of which carries a *Mu* element in an exon, have not shown an obvious effect on the phenotype, indicating that the phenotype of the recessive *lg3* mutation may be subtle. Alternatively, it is well possible that a *lg3* homologue exists and provides genetically redundant functions (Muehlbauer *et al*., 1999).

Loss-of-function mutations in the rice class I *Knox* gene *OSH15* were identified from a library of retrotransposon-tagged lines of rice. Internodes of the mutants are short and squat, with a corresponding change in cell shape (Sato *et al*., 1999). These mutants demonstrated a role of *OSH15* in the development of rice internodes. But no abnormality related to shoot apical meristem formation or maintenance was observed during early embryogenesis in the *OSH15* loss-of-function mutants. This does not lead to the conclusion that *OSH15* is not related to shoot apical meristem formation and/or maintenance at this stage, because the redundant gene(s) may compensate for this process in the early embryogenesis (Sato *et al*., 1999).

### 1.3.2 Expression patterns of *Knox* genes

While the general rules on the role of class I *Knox* genes begins to emerge, defining the exact function of each of the numerous genes that compose this family may be a more difficult task. The problems of gene redundancy and of mutual interactions between different members may obscure the results. Therefore, the definition of precise expression patterns may be helpful. As an example, the expression of *Kn1* and *Rs1* in different groups of cells within the meristem is most likely indicative of different functions, mainly in the generation of boundaries within the meristem, defining groups of cells that will acquire determinate fates (Jackson *et al*., 1994) (Fig. 1-4).

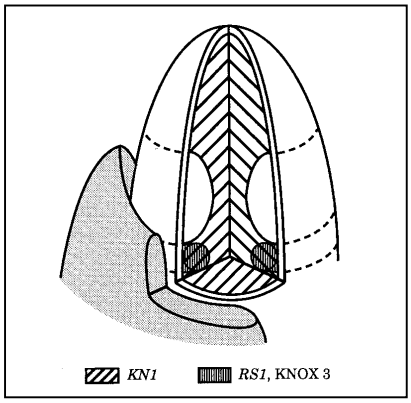



**Fig. 1-4 Schematic representation of gene expression of maize *Knox* genes in the shoot meristem.** *Kn1* expression throughout the meristem and excluded from $P_0$ leaf and the L1 layer. *Rs1* and *Knox3* gene expression in the meristem is restricted to positions between the lateral organs. (adapted from Jackson *et al*., 1994)

In general, class I genes are expressed in meristematic tissues. In contrast to class I genes, class II genes are expressed in various locations throughout the plant. The expression patterns of class I and class II *Knox* genes from different plant species are summarized in Table 1-1.

**Table 1-1 Expression patterns of *Knox* genes**

| Plant | Gene | Expression pattern | Reference |
|---|---|---|---|
| **Monocots** | **Class I** | | |
| maize | *Kn1* | shoot meristems (absent at the young leave primordia), immature stems, floral shoots[(i) (im)] | Smith *et al*., 1992 |
| | | shoot meristems (absent at the leave primordia), developing vascular, inflorescence meristems[(i)] | Jackson *et al*., 1994; |
| | | embryonic shoot meristems, embryonic roots[(i) (im)] | Smith *et al*.,1995 |
| | *rs1* | shoot meristems (the base of the disc of leaf insertion), inflorescence meristems[(i)] | Jackson *et al*., 1994; |
| | | roots, mesocotyl, immature inflorescences, shoots[(n)] shoot meristems (the base of the leaf insertions)[(i)] | Schneeberger *et al*., 1995 |
| | *lg3* | vegetative meristems, shoots, roots, immature ears and tessels, mature tessels and embryos[(r)] | Muehlbauer *et al*., 1999 |
| | *gn1/Knox 4* | shoot meristems, inflorescences[(n)] shoot apical meristems (absent from young leaf primordia)[(im)] | Kerstetter *et al*., 1994; Forster *et al*., 1999 |
| | *Knox3* | shoot meristems, inflorescences[(n)] | Kerstetter *et al*., 1994 |
| | | shoot meristems (the base of the disc of leaf insertion), inflorescence meristems[(i)] | Jackson *et al*., 1994; |
| | *Knox8* | shoot meristems, inflorescences[(n)] | Kerstetter *et al*., 1994 |
| rice | *OSH1* | globular embryos, embryonic meristems, epiblasts, radicles[(i)] | Sato *et al*., 1996 |
| | *OSH15* | vegetative shoots, inflorescences, stems, rachis[(n)] the boundaries between the embryonic organs[(i)] | Sato *et al*., 1998 |
| | *Oskn2* | shoot meristems of 5 DAP embryos, epiblasts, the boundary of the scutellum and the coleoptile[(i)] | Dorien *et al*., 1999 |
| | *Oskn3* | shoot meristems of 4-6 DAP embryos, the boundaries of different embryonic organs[(i)] | Dorien *et al*., 1999 |
| barley | *HvKnox3/ BKn3* | the lemma, the palea and vascular tissues of wild type and *hooded* inflorscences[(i)] | Müller *et al*., 1995 |
| | *BKn1* | inflorescences, stems, shoot apices, seedling roots[(r)] [(n)] embryos, embryonic apices, radicules, vegetative apices, leaf primorida, stems, inflorescences, floral organs[(i)] | this thesis |
| wheat | *WKnox1* | shoots containing meristems, young spikes[(r)] | Takumi *et al*., 2000 |
| orchid | *DOH1* | stems, vegetative apices, transitional buds and floral buds[(n)] apical region of vegetative shoot meristem, provascular stands of leaf primordia, inflorescence meristems and floral primodia[(i)] | Yu *et al*., 2000 |
| **Dicots** | **Class I** | | |
| *Arabidopsis* | *KNAT1* | flowers, inflorescence stems, seedlings[(n)] peripheral zone of shoot meristems, inner layers of the cortex of inflorescence stems[(i)] | Lincoln *et al*., 1994 |
| | *KNAT2* | flowers, inflorescence stems, seedlings, roots[(n)] | Lincoln *et al*., 1994 |
| | *STM* | four types of shoot apical meristems (vegetative, axillary, inflorescence and floral), (absent in leaf primordia), stems (around the vascular strands)[(i)] | Long *et al*., 1996 |

**Table 1-1** *Continued*

| Plant | Gene | Expression pattern | Reference |
|---|---|---|---|
| soybean | *SBH1* | early-stage somatic embryos, weakly in stems[(n)] | Ma *et al*., 1994 |
| tobacco | *NTH15* | flower buds, flowers and stems[(n)], the corpus of shoot meristems, stem internodes[(i)] | Tamaoki *et al*., 1997 |
| tomato | *TKn1* | apices, stems, immature flowers, floral organs[(n)], shoot apical meristems, provascular strands, floral meristems and mature flowers[(i)] | Hareven *et al*., 1996 |
| | *TKn2/ LeT6* | stems, apices, floral buds[(n)], apical meristems and leaf primordia[(i)] | Parnis *et al*., 1997 |
| | | vegetative buds, ovaries, young fruits, seed and pericarp[(n)], specific location of developing ovules and immature fruits[(i)] | Janssen *et al*., 1998 |
| apple | *KNAP1/ KNAP2* | stems[(n)] | Watillon *el at*., 1997. |
| *Antirrhinum* | *AmSTM* | shoot apical meristem[(n)] | Waites *et al*., 1998 |
| **Gymnosperms** | **Class I** | | |
| *Picea albies* | *HBK1* | the central zone of the vegetative shoot meristems (excluded from developing needle primordia)[(i)] | Sandas-Larsson *et al*., 1998 |
| **Unicellular alga** | | | |
| *Acetabularia acetabulum* | *AaKnox1* | all phases of development, highest level during early reproductive phase[(n)] | Serikawa & Mandoli, 1999 |
| **Monocots** | **Class II** | | |
| maize | *KNOX1* | strong in roots and weak in all other tissues examined (embryos, young leaves, inflorescences, shoot meristems)[(n)] | Kerstetter *et al*., 1994 |
| | *KNOX2* | strong in all tissues examined (embryos, young leaves, shoot meristems, inflorescences, roots)[(n)] | Kerstetter *et al*., 1994 |
| | *KNOX6/7* | strong in all tissues examined (embryos, young leaves, shoot meristems, inflorescences, roots)[(n)] | Kerstetter *et al*., 1994 |
| rice | *OSH44/ OSH45* | all tissues tested (shoot meristems, leaves, roots, stems, flowers, rachis) [(n) (r)] | Tamaoki *et al*., 1995 |
| | *OSH42* | leaves, stems, rachis [(n)] | Tamaoki *et al*., 1995 |
| barley | *BKn7* | all tissues examined (roots, shoots, stems , leaves, and floral organs)[(n) (r)] | this thesis |
| **Dicots** | **Class II** | | |
| *Arabidopsis* | *KNAT3* | all tissues examined, strongest in young siliques, inflorescences and roots[(n)] | Serikawa *et al*., 1996 |
| | *KNAT4* | all tissues examined, strongest in leaves and young siliques[(n)] | Serikawa *et al*., 1996 |
| | *KNAT5* | all tissues examined, strongest in roots[(n)] | Serikawa *et al*., 1996 |
| tomato | *LeT12* | all tissues examined[(n)] specific location of developing ovules and immature fruits[(i)] | Janssen *et al*., 1998 |
| apple | *KNAP3* | wide range of both vegetative and reproductive organs[(n)] | Watillon *et al*., 1997 |

(n), (r), (i) and (im) indicate that the results obtained from Northern blot analysis, RT-PCR analysis, *in situ* hybridization and immunolocalization, respectively.

### 1.3.3 Overexpression of class I *Knox* genes in transgenic plants

The class I *Knox* genes often cause severe morphological abnormalities when overexpressed in tobacco, *Arabidopsis* and tomato.

Overexpression in tobacco of several *Knox* genes, including *Kn1*, *OSH1*, *BKn3* driven by the constitutive cauliflower mosaic virus (CaMV) 35S promoter results in leaf phenotypes such as rumpling, reduced lamina, and formation of ectopic shoots on the leaves (Sinha *et al*., 1993; Kano-Murakami *et al*., 1993; Müller *et al*., 1995). Tobacco plants with severe phenotypes lack apical dominance and are dwarfed in overall height and leaf size. Small shoots originated from the surface of these diminutive leaves.

*KNAT1*, a class I *Knox* gene from *Arabidopsis*, induces modifications in leaf shape, producing lobed leaves with ectopic meristems in the margins in close vicinity to the veins when overexpressed in *Arabidopsis* (Chuck *et al*., 1996). Constitutive expression of *Kn1* in *Arabidopsis* results in plants with similar phenotypes (Lincoln *et al*., 1994). However, constitutive expression of *STM* or *OSH1* in *Arabidopsis* results in a phenotype that is different from *Kn1* or *KNAT1* overexpression (Matsuoka *et al*., 1993). In particular, ectopic *STM* expression results in severely stunted plants with a highly disorganized shoot meristem. The shoot meristem has many bulges that resemble leaf primordia but do not grow into mature leaves (Williams, 1998). Therefore, ectopic expression of closely related *Knox* genes can affect plant growth in different ways.

Transgenic tomato plants overexpressing the *Kn1* gene displayed altered morphogenesis; mature leaves are subdivided to the fourth, fifth, or sixth order, forming supercompound leaves. The appearance of supercompund leaves is always associated with growth retardation and the loss of apical dominance, resulting in dwarfed, bushy plants. Unlike the leaves, morphology of the inflorescences, flowers and floral organs of tomato are not visibly affected by the overexpression of the *Kn1* gene (Hareven *et al*., 1996). Overexpression of a homologous *Knox* gene *LeT6* in tomato produces more phenotypic variabilities. The transgenic tomato plants display not only multiple orders of compounding in the leaf, but also numerous shoots, inflorescences, and floral meristems on leaves and the conversion of rachis-petiole junctions into “axillary” positions where floral buds can arise (Janssen *et al*., 1998).

Overexpression of *Kn1* and *Kn1*-like genes in monocots has caused different phenotypes from those observed when the same or similar constructs were expressed in dicots. Barley plants overexpressing the maize *Kn1* gene under the control of the ubiquitin promoter showed no abnormal leaf phenotype, although ectopic meristems were formed on the adaxial surface near the lemma/awn transition zone (Williams-Carrier *et al*., 1997).

Rice plants transformed with five rice class I *Knox* genes (*OSH1*, *OSH6*, *OSH15*, *OSH43* and *OSH71*) under the control of either the CaMV 35S or the rice *Act1* gene promoter were found to have severely malformed leaves with ectopic knots on their adaxial side (Sentoku *et al*., 2000). Knot formation and ligule displacement occurring in transgenic rice plants were similar to those seen in spontaneous dominant *Kn1* mutants.

These studies suggest that class I *Knox* gene products may regulate the expression of genes related to morphogensis acting at an early stage of tissue or organ differentiation. However, the molecular mechanism(s) by which class I *Knox* genes regulate plant morphogenesis remains to be determined.

A variety of specific morphological abnormalities of transgenic plants overexpressing *Knox* genes is reminiscent of those induced by hormone changes. Tobacco plants that overexpress the *NTH15* gene, a tobacco class I *Knox* gene, show a drastic decrease of $GA_1$ and an increase of cytokinin (Tamaoki *et al*., 1997). Decreased $GA_1$ content caused by the overexpression of the rice *Knox* gene *OSH1* in transgenic tobacco is accompanied by suppression of $GA_{20}$-oxidase gene expression (Kusaba *et al*., 1998a, b). Lettuce plants overexpressing *KNAT1* from *Arabidopsis* under the control of the pea plastocyanin promoter have accumulated a high content of cytokinin in leaves (Frugis *et al*., 1999). These studies suggest an interplay between homeobox genes and hormones in establishing developmental patterns in plants. However, it is difficult to ascertain whether altered hormone levels are the result of direct or indirect action of homeodomain proteins, or whether overexpressing plants are altered in hormone metabolism or sensitivity.

In cytokinin-overproducing *Arabidopsis* plants, the two class I *Knox* genes *STM* and *KNAT1* are up-regulated (Rupp *et al*., 1999). This result led the authors to hypothesize that cytokinins act upstream of *KNAT1* and *STM*. This result together with those from overexpression of *Knox* genes in transgenic plants make the relationship of *Knox* genes and phytohormone more intriguing. Thus, the challenge now remains to determine the interrelationship between developmental pathways that regulate *Knox* gene expression and those that coordinate the action of plant growth regulators.

### 1.3.4 The regulation of class I *Knox* gene expression

Attempts to define the roles for *Knox* genes based on ectopic expression have uncovered several potential levels of gene regulation. What makes certain tissues competent to respond to *Knox* genes? Why do ectopic meristems form along the midvein for the lamina in tobacco, in the sinuses of lobed leaves in *Arabidopsis* and on the awn of barley flowers? Why does *Kn1* make knots in maize but not in barley leaves? It is likely that *Knox* gene expression requires the cooperation of, or is inhibited by, other factors that are expressed in a spatially or

temporally restricted pattern during development (reviewed by Reiser *et al*., 2000).

Despite the fact that the transgenes are presumably under the control of constitutive promoters, the transgene is not detected uniformly throughout the plant. While it is possible that these differences are a consequence of transgene silencing in specific domains, the most probable explanation is post-transcriptional regulation of *Knox* accumulation (Williams-Carrier *et al*., 1997).

The fact that *Knox* gene expression is excluded from lateral organ primordia led to the assumption that its expression is negatively regulated in the meristem prior to organ initiation (Jackson *et al*., 1994). The analysis of the recessive mutation *rough sheath2* in maize provided some evidence for this assumption. The recessive *rough sheath2* mutant has a phenotype similar to that of dominant *Knox* gene mutations (Schneeberger *et al*., 1998). Ectopic expression of *Rs1*, *Knotted1*, and *Liguleless3* can be detected as early as $P_1$ in *rs2* mutant leaf primordia. Therefore, *rs2* acts to negatively regulate *Knox* genes in immature leaves (Schneeberger *et al*., 1998). The ability to negatively regulate *Knox* genes in the $P_0$ and later stages of development in *rs2* mutants may be due to the presence and function of a duplication factor for *rs2*. Other loci may be required, such as *narrow sheath* (*ns*) and *leafbladeless* (*lbl*), both of which are thought to participate in founder cell recruitment (Scanlon *et al*., 1996; Timmermans *et al.*, 1998). Double mutants between *ns* and *rs2* were additive, indicating that these genes act in separate pathways to restrict *Knox* gene expression in the leaf primordia (Schneeberger *et al*., 1998). *rs2* was cloned and the predicted protein encodes a myb-like transcription factor similar to the *Phantastica* (*Phan*) gene product from *Antirrhinum* (Timmermans *et al.*, 1998; Waites *et al*., 1998). Lateral organs in *phan* mutants are abaxialized and have radial symmetry (Waites and Hudson, 1995). The *Phan* expression domain complements that of an *Antirrhinum Kn1*-like gene, consistent with a role in delimiting the parameters of *Knox* gene expression (Waites *et al*., 1998). These data suggest that a common mechanism to regulate *Knox* gene expression exists in *Antirrhinum* and maize.

The possibility of an interplay between *Knox* and *Phan* and phytohormones, which influence plant development and morphogenesis, is intriguing. A strict correlation between *Knox* expression and cytokinins levels has been observed, either suggesting that cytokinins may regulate *Knox* expression or act as a secondary signal regulated by *Knox* gene products (Frugis *et al*., 1999; Rupp *et al*., 1999). Furthermore, ectopic *Knox* expression in maize was correlated with an aberrant polar auxin transport (Tsiantis *et al*., 1999a). Therefore, the spatial expression of *Knox* genes in shoot meristems may be coupled to the alteration of hormone levels.

## 1.4 Towards an understanding of barley *Knox* genes

### 1.4.1 Sequence comparison of barley *Knox* genes with other members of the *Knox* gene family

Previously, seven *Knox* genes were isolated from barley by using the maize *Kn1* homeobox sequence as a hybridization probe (Müller, 1993; Müller, 1997). They were named *BKn1* (*Barley Knox*1) to *BKn7*. Based on sequence similarity to other members of *Knox* gene family they can be divided into class I and class II groups. *BKn1*, *2*, *3*, *4*, *5* belong to class I; *BKn6* and *7* belong to class II. The sequences of the ELK and homeodomains of seven barley *Knox* genes were compared with those of other members of *Knox* family. The resulting dendrogram is shown in Fig. 1-5.

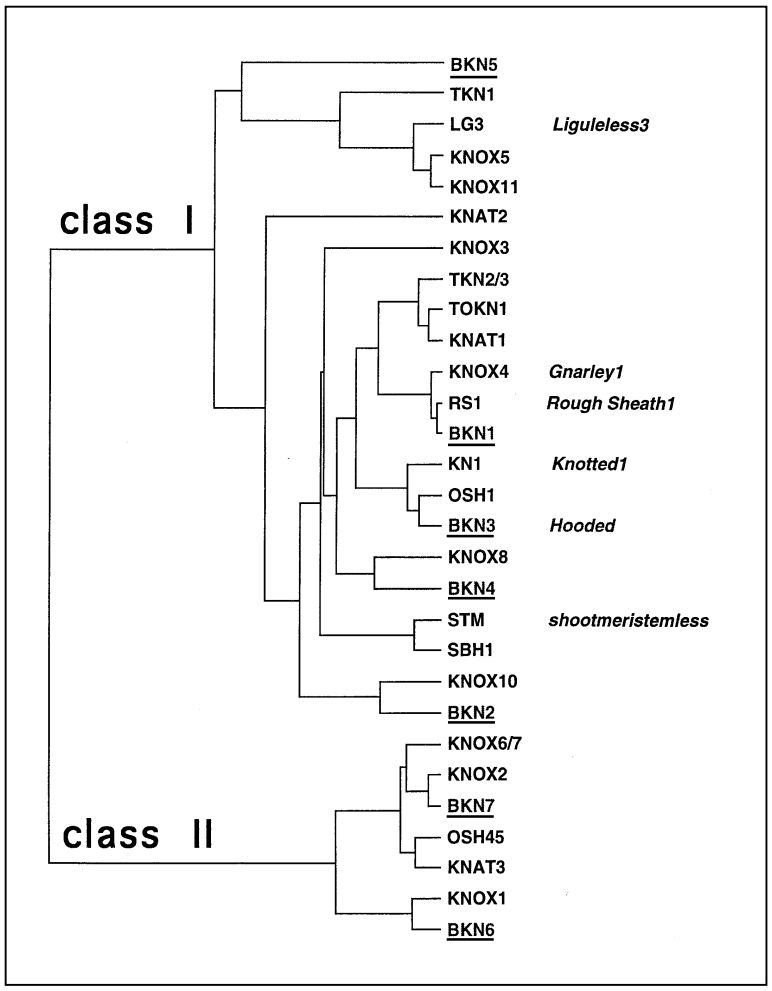



**Fig. 1-5 *Knox* genes and developmental mutants.** A dendrogram comparison of the ELK and homeodomains of barley Knox proteins with other plant Knox proteins. Protein sequences are from barley (BKn1, 2, … 7, underlined), maize (KN1, RS1, LG3, KNOX1, 2, … 11), rice (OSH1, OSH45), *Arabidopsis* (STM, KNAT1, KNAT2, KNAT3), tobacco (TKN1, TKN2/3), tomato (TOKN1) and soybean (SBH1). The identified developmental mutants are indicated at the right. (kindly provided by Dr. Kai Müller)

Among the seven isolated barley *Knox* genes, two class I genes, *BKn1* and *BKn3*, and one class II gene *BKn7* were selected for further analysis. In Fig. 1-6, the ELK domain and homeodomain sequences of *BKn1*, *BKn3* and *BKn7* are aligned with those of other members of *Knox* gene family.

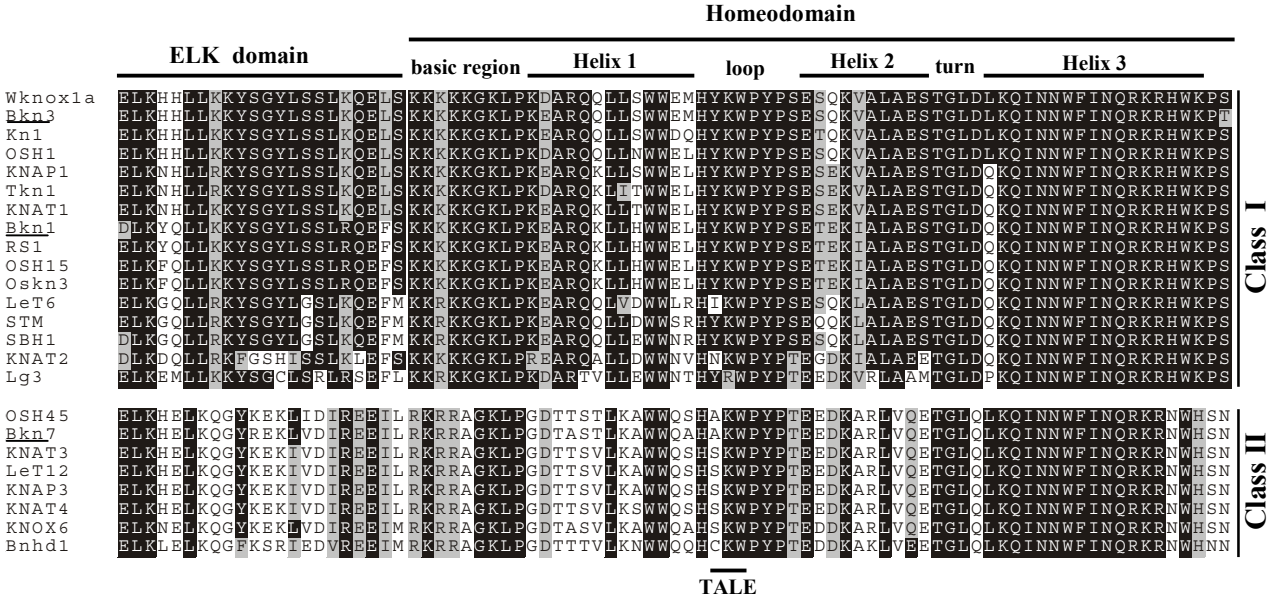



**Fig. 1-6 The ELK domain and homeodomain sequences of BKn1, BKn3 and BKn7 aligned with those of other members of the Knox family.** The position of ELK domain and the helix-loop-helix-turn-helix structure of homeodomain are indicated above the sequences. The proteins are subdivided into class I and class II according to the sequence similarity. Three extra amino acids between helix1 and helix2 are indicated underneath the sequences with TALE (three amino acid loop extension).

## 1.4.2 Strategy for studying barley *Knox* genes

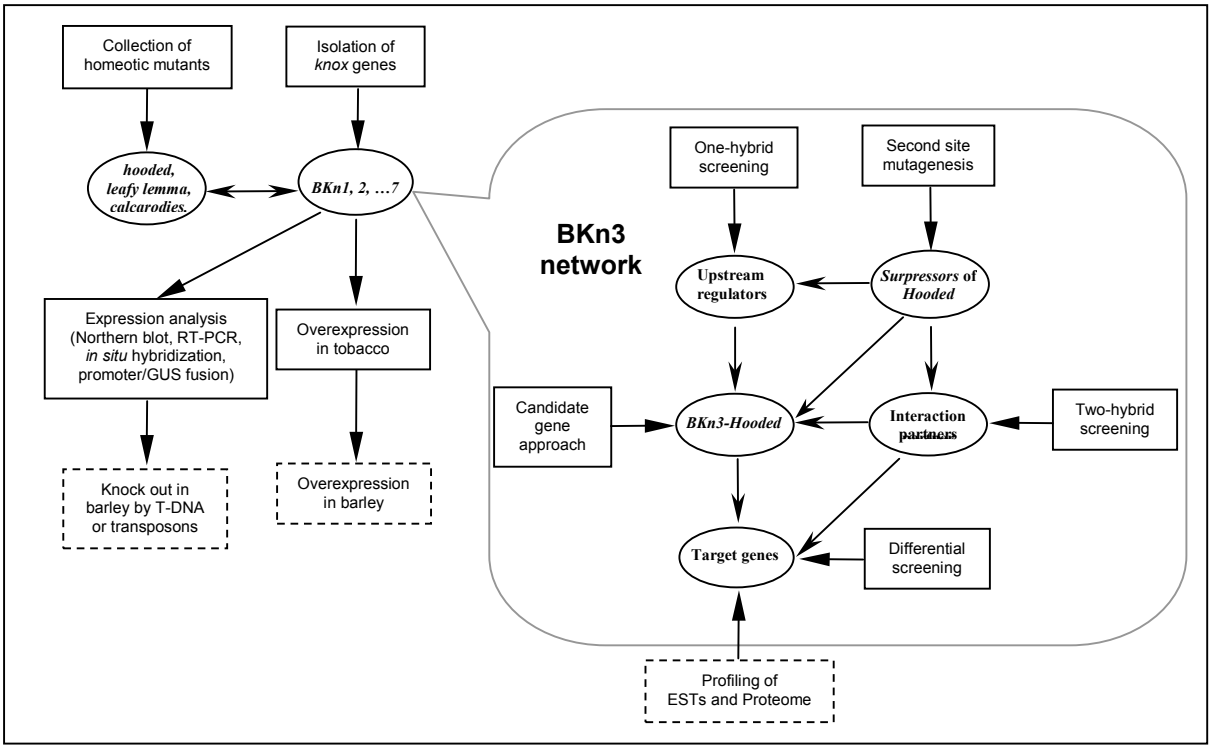



**Fig. 1-7 The strategy for studying barley *Knox* genes.** Approaches which have been applied in the laboratory are listed in squares with black lines, those listed in squares with dashed lines are approaches which are going to be performed and will be very informative as well. Genes and mutants are listed in ellipses. See details in the following sections.

Fig. 1-7 depicts the strategy for studying barley *Knox* genes isolated. At the Department of Prof. Dr. Salamini, a large number of barley mutants affecting flower and leaf development were collected. These developmental mutants and isolated barley *Knox* genes are the starting point for the project. Approaches being attempted to understand the functions of barley *Knox* genes are described in the following sections.

### 1.4.2.1 Expression analysis

In terms of understanding the functions of genes, knowing when, where and to what extent a gene is expressed is important to understand the activity and biological roles of its encoded protein. The expression pattern of an individual *Knox* gene might consequently give a clue to its function. Several different techniques have been used for the expression analysis of barley *Knox* genes.

Northern blot and reverse transcription-polymerase chain reaction (RT-PCR) are conventional methods to measure mRNA abundance in different tissues. Northern blot analysis revealed *BKn3* expression was 2.5 fold higher in *Hooded* barley than in wild-type barley (Müller *et al*., 1995). The expression patterns of *BKn1* and *BKn7* was studied in this thesis.

The analysis of promoter-GUS fusions is one of the most widely used techniques for identifying sequences that control the temporal and spatial regulation of cloned genes. Indeed, this approach allows researchers to resolve characteristic and striking patterns of tissue-specific and/or developmentally regulated expression that are consistent with the known activity of the promoter *in vivo*. However, one has to deal with this technique carefully, because there are increasing numbers of experiments demonstrating that promoter-GUS fusion can be prone to artifactual expression that does not accurately reflect the *in vivo* regulation of the gene of interest (Taylor, 1997). The promoters of *BKn1*, *BKn3* and *BKn7* genes fused to GUS reporter gene have been analyzed in transgenic tobacco plants (Kai Müller, unpublished data).

*In situ* hybridization, which measures the abundance of mRNA, has definite advantages over promoter-GUS fusions to study gene expression (Taylor, 1997). Gene-specific probes make it possible to study the expression of individual genes. However, it also has some drawbacks. It is difficult to compare different cell types, especially if they differ in cytoplasmic content or the relative volume taken up by the vacuole. Thus the intensity of the signal reflects the abundance of cytoplasm as well as the abundance of the mRNA under study. Furthermore, abundance of mRNA does not always translate into abundance of protein, because of post-transcriptional regulation of gene expression (Barrieu *et al*., 1998). *BKn3* gene expression in the barley inflorescence was previously analyzed by *in situ* hybridization (Müller *et al*., 1995). *BKn1* and *BKn7* gene expression in different barley tissues were studied by *in situ* hybridization in this thesis.

### 1.4.2.2 Functional analysis in transgenic plants

Since mutants are not available for most of the *Knox* genes isolated up to now, the effects of either overexpressing (using strong promoter) or knocking out (by antisense constructs, T-DNA or transposon insertion) the corresponding genes can be assayed in transgenic plants to elucidate the biological function of *Knox* gene products.

Because at present barley can not be transformed efficiently, the coding region of barley *Knox* genes was introduced into heterologous systems such as tobacco or *Arabidopsis* under the control of the CaMV 35S promoter. Overexpression of *BKn1*, *BKn3* and *BKn7* genes was analyzed in transgenic tobacco plants. Tobacco plants overexpressing *BKn1* and *BKn3* (two class I *Knox* genes) displayed a severely dwarfed phenotype and ectopic shoots and flowers on the leaves (Müller *et al*., 1995). Overexpression of *BKn7*, one of class II *Knox* gene did not cause any detectable phenotypic changes (Müller, unpublished data).

Since dicots respond to *Knox* genes in a different way from monocots, overexpression of barley *Knox* genes in the homologous system would give more information on their functions in barley development. Overcoming the difficulties of barley transformation will greatly facilitate the functional studies of barley *Knox* genes in the future.

### 1.4.2.3 Candidate gene approach, gene-mutant association

The candidate gene approach correlates a phenotype with its underlying biochemical or physiological basis by demonstrating that candidate genes are tightly linked to the genetic locus of interest. It is a powerful and robust method compared to the genome wide mapping strategy, since the selection of candidate gene markers is based on known relationships between biochemistry, physiology and the agronomic character under study.

In this approach, a newly cloned gene is mapped in order to determine where it is equivalent to a locus that has been previously mapped by genetic experiments. The candidate genes are chosen on the prior belief, based on assumptions concerning the biological or physiological system involved, that they can be associated with the trait of interest. This approach was successfully used to associate the *Hooded* mutation to the class I *Knox* gene *BKn3* in barley (Müller *et al*., 1995). The fact that both *Knotted-1* and *Hooded* mutations are dominant and link to an alcohol dehydrogenase (*Adh*) gene suggested that two mutants are homologous and further supported the consideration of a homeobox gene as the genetic locus for *Hooded* (*K*) alleles.

However, the candidate gene approach is sometimes limited by the understanding of the physiology and biochemistry of the trait of interest and by the requirement of prior identification of genes that potentially involved in the trait expression. The confirmation of the causal relationship between a specific trait and candidate genes has proven to be difficult.

The candidate gene approach for the barley *Knox* genes may prove successful, once all of the available homeotic barley mutants have been mapped to the barley linkage map.

#### 1.4.2.4 Yeast two-hybrid screening

Since protein-protein interactions are critical to most biological processes the identification of protein-protein interactions is very helpful in understanding biological phenomena. The yeast two-hybrid system has rapidly become an attractive method for both the identification of potential interactions and their characterization because it allows the genetic selection of genes encoding potential interacting proteins without the need for protein purification (reviewed by Vidal and Legrain, 1999). It allows a very high number of potential coding sequences to be assayed in a convenient microorganism *in vivo*.

In yeast two-hybrid system, the DNA-binding domain (DB) and the activation domain (AD) of yeast Gal4p or LexA can fuse to any protein from any organism. In the configuration of two-hybrid system, DB-X/AD-Y, DB-X hybrid protein is often referred to as the 'bait' and AD-Y hybrid could be AD-Y libraries, genes encoding proteins that potentially interact with DB-X. Potential interactions between proteins can be identified on the basis of the transcriptional activation of a 'gene required for growth' or a 'gene required for the production of an enzyme' which confers a selective advantage.

This technique has been successfully used to identify the interaction partners of *BKn3* gene product in the group. Two BELL1-like barley proteins were isolated during the yeast two-hybrid screening. Another two barley *Knox* gene products, BKN1 and BKN7 also showed interactions with BKN3 in the yeast two-hybrid system (Müller, 1999; Müller *et al*., 2001).

#### 1.4.2.5 Yeast one-hybrid screening

Although protein-protein interactions form the basis of many biological processes, other macromolecular interactions such as DNA-protein and RNA-protein interactions are also critical. The one-hybrid system used to detect DNA-protein interactions is an extension, by simplification, of the two-hybrid concept. The original two-hybrid DB-X/AD-Y configuration is modified. The DB-X hybrid is eliminated and the DNA Gal4p or LexA-binding sites are replaced by a specific DNA sequence identified as an important binding site in the relevant biological system. The AD fusion libraries can be used for identifying the DNA-binding proteins corresponding to this site (reviewed by Vidal and Legrain, 1999).

In the laboratory, the yeast one-hybrid system has been used for identifying the putative upstream regulators of *BKn3*, the *Hooded* gene. The dominant barley *Hooded* mutant is caused by the 305bp duplication in the fourth intron of *BKn3* (Müller *et al*., 1995). Most of *Kn1* alleles characterized to date are associated with insertions of transposable elements into the large third intron that is homologous to *BKn3* intron IV (Greene *et al*., 1994). When the

305bp intron sequence was fused to the CaMV 35S minimal promoter or *BKn3* gene promoter, it was able to activate GUS expression in the vegetative apex, vascular strands, lateral shoot branching points and the inflorescence of transgenic tobacco plants (Kai Müller, unpublished data). It seems that this region carries *cis*-acting regulatory elements that are important for the formation of epiphyllous flowers on the lemma of the *Hooded* barley. Based on this assumption, one and three copies of 305bp fragment were used as 'baits' in the yeast one-hybrid system to screen for putative DNA-binding proteins which could regulate the expression of *BKn3* gene through the binding to the 305bp intron sequence. By this approach four barley cDNAs were obtained, they are *BEIL* (Barley Ethylene Insensitive Like), *BAPL* (Barley Aptala2 Like), *BBR* (Barley Brain) and *BGRF* (Barley Growth Regulating Factor), named after their homologous genes in other organisms (Müller, unpublished data, see details in section 3.2.2). Further characterization and analysis of these barley proteins were performed in this thesis.

#### 1.4.2.6 Second site mutagenesis

Second site mutagenesis is a powerful genetic approach to identify the genes involved in a specific developmental pathway and create loss-of-function mutants. It has been successfully used for isolating enhancers and suppressors of activated *Notch* in *Drosophila* (Esther *et al*., 1996) and loss-of-function alleles of *Knotted-1* (Kerstetter *et al*., 1997).

At the department of Prof. Dr. Salamini, a second site mutagenesis screen was performed to isolate recessive suppressors of the *Hooded* phenotype. The *Hooded* phenotype is ideal for a second-site modifier screen, since it is dominant and reflects the transmission of intracellular signals in the absence of an extracellular stimulus. Several extragenic mutations (mutant alleles of genes interacting with *BKn3* or regulating the expression of *BKn3*, required for hood formation on the barley lemma) and 1 intragenic mutation (loss-of-function alleles of *BKn3*) have been identified to date (Müller *et al*., 2000). The identified barley proteins that interact with the *BKn3* gene product (by the yeast two-hybrid screening) and the 305bp fragment in *BKn3* intron IV (by the yeast one-hybrid screening) could represent potential candidates for these suppressors (Müller *et al*., 2000). The association of these barley proteins with the suppressors will be carried out by a candidate gene approach.

#### 1.4.2.7 Other methods

*Knox* gene products control plant morphogenesis through regulation expression of certain target gene(s). However, little is known about the target genes of plant homeodomain containing proteins. Differential screening of wild-type and *Hooded* barley inflorescence cDNA library was performed to identify the target gene(s) of BKN3, *Hooded* gene product (Müller, 1997).

The recent growth of genome and ESTs databases, DNA microarray and proteomics approaches has now made it possible to explore the physiological significance of transcriptional regulators by identifying the complement of genes that they regulated. Analysis of gene expression profiles will aid our understanding of barley *Knox* genes in the future.

## 1.5 Objectives of this thesis

Following the strategy being carried out in the laboratory, this thesis attempted to learn more about barley *Knox* genes through two approaches.

### 1.5.1 Expression analysis of barley *Knox* genes

This thesis set out to analyze the expression patterns of *BKn1*, a class I *Knox* gene, and *BKn7*, a class II *Knox* gene, in different barley tissues using Northern blot, RT-PCR and *in situ* hybridization. This detailed analysis was expected to provide a solid basis of information concerning the possible biological functions of these genes.

### 1.5.2 Identification of putative upstream regulators of *BKn3*

Previous experiments have demonstrated that the 305bp sequence in *BKn3* intron IV has tissue specific enhancer activity and may contain *cis*-acting elements essential for *BKn3* gene expression (Kai Müller, unpublished data). When one and three copies of 305bp fragment were used as 'baits' in the yeast one-hybrid screening, four barley cDNA clones named *BEIL*, *BAPL*, *BBR* and *BGRF* were isolated (Kai Müller, unpublished data).

In this thesis, to get more insight how proteins encoded by these cDNA clones interact with the 305bp intron sequence to regulate the *BKn3* gene expression, the detailed molecular characterization of these barley cDNA clones was performed on the following aspects:

— isolation and sequencing of genomic DNA sequences of *BEIL*, *BAPL*, *BBR* and *BGRF*;

— mapping of the transcription initiation site of *BBR*, whose cDNA sequence obtained from one-hybrid screening was not full-length;

— expression analysis of *BEIL*, *BAPL*, *BBR* and *BGRF* in different wild-type barley tissues and *Hooded* barley inflorescences using Northern blot and RT-PCR;

— determination of *in vitro* DNA-binding properties of BEIL, BAPL, BBR and BGRF by EMSA (electrophoretic mobility shift assay) and identification of the DNA-binding site of BBR; (collaborated with Luca Santi)

— genome mapping of *BEIL*, *BAPL*, *BBR* and *BGRF* by SSCP (single-strand conformation polymorphism);

— further characterization of BBR by testing its nuclear localization and transactivation activity in tobacco protoplasts.

# MATERIALS AND METHODS

## *CHAPTER 2*

## 2.1 Chemicals, enzymes and oligonucleotides

Enzymes were obtained from Boehringer-Mannheim currently renamed as Roche (Mannheim), Biolab (England), Life Technologies (Freiburg) or MBI Fermentas (St. Leon-Rot) and used with the 10× buffer supplied, unless otherwise stated.

Laboratory reagents were obtained from following companies: Life Technologies, Pharmacia (Freiburg), Sigma (Deisenhofen), Merck (Darmstadt), Roth (Karlsruhe), Serva (Heidelberg), Biomol (Hamburg), Fluka (Neu-Ulm), Promega (Madison), Duchefa (Haarlem) and Bio-Rad (Munich). Nylon membranes were obtained from either Amersham (Braunschweig) or Macherey-Nagel (Düren). Radioisotopes [$\alpha^{32}$P]-dCTP (10$\mu$Ci/$\mu$l), [$\gamma^{32}$P]-ATP (10$\mu$Ci/$\mu$l) and [$\alpha^{33}$P]-dCTP (10$\mu$Ci/$\mu$l) were purchased from Amersham Buchler (Braunschweig).

Oligonucleotides were synthesized by MWG-Biotech (Munich), Life Technologies and Metabion (Martinsried).

## 2.2 Plant materials

*Hordeum vulgare* L. The wild-type barley variety Atlas (accession number of MPI collection is G1002) and mutant *Hooded-Atlas* (accession number of MPI collection is G1005) were grown at 18°C, 14 hours light and 16°C, 10 hours dark in the greenhouse.

The 100 doubled haploid barley lines (DH lines) used for mapping were originated from a cross between the varieties Proctor and Nudinka (Heun *et al*., 1991). Seeds were provided, together with the parental lines, by M. Heun in 1991 and were maintained at the Max-Planck-Institute in Cologne.

*Nicotiana tabacum* L. (cv. Petit Havana line) SR1 tobacco plants were grown in MS medium under sterile condition with a photoperiod of 15 hours light at 26°C and 9 hours dark at 24°C.

## 2.3 Bacterial strains

***Escherichia coli***

| | |
|---|---|
| DH10B | $F^-$, *mcr*AΔ(*mrr-hsd*RMS-*mcr*BC )Φ80*lacZ*ΔM15, Δ*lac*X74, *deo*R, *rec*A1, *end*A1, *ara*D139, Δ(*ara*, *leu*)7607, *gal*U, *gal*K, $\lambda^-$*rps*1, *nup*G |
| K803 | $F^-$, e14$^-$ (McrA$^-$ ), *lac*Y1, or, Δ(*lac*)6, *sup*E44, *gal*K2, *gal*T22, *rfb*D1, *met*B1, *mcr*V1, *hsd*S3, ($r_k^-$, $m_k^+$). |
| BL21 | $F^-$, *omp*T, *hsd* S ($r_B^-$ $m_B^-$), *gal*, *dcm*. |

***Agrobacterium tumefaciens***

| | |
|---|---|
| LBA4404 | $Sm^r$, ($Rif^r$). |

## 2.4 Cloning vectors

| | |
|---|---|
| pBluescript KS (+) | (Stratagene) |
| pBluescript SK (+) | (Stratagene) |
| pGEX-5X-1 | (Pharmacia Biotech) |
| pRT100 | (Töpfer *et al*., 1987) |
| pRT104 | (Töpfer *et al*., 1987) |
| pRT104GUS | (Töpfer *et al*., 1988) |
| pBI 101.2 | (Clontech) |
| pCATgfp | (Dr. Guido Jach's lab at the Max-Planck-Institute in Cologne) |

## 2.5 Southern Blot

### 2.5.1 Plant genomic DNA extraction

Genomic DNA was extracted from barley plants by established protocols (Junghans and Metzlaff, 1990). In brief, 1-3g plant materials were ground under liquid nitrogen to fine powder. 10ml extraction buffer with RNase (A+T, 10mg/ml) was added to the powder and the mixture was shaken gently for 30min at 37°C. The lysate was extracted with phenol/chloroform and centrifuged for 30min at 5,000rpm, 4°C. After centrifugation the supernatant was added to 1 volume of isopropanol and centrifuged at 20,000g for 15min. Precipitated DNA was washed with 70% ethanol, dried at room temperature and dissolved in 10mM Tris-HCl (pH 8.5) buffer. Extracted DNA was quantified by comparison of band-intensity on ethidium bromide stained agarose gels with a DNA molecular weight standard.

***Extraction buffer***

50mM Tris-HCl (pH 9.0)
10mM EDTA
2% (w/v) SDS
100mM NaCl
200$\mu g$/ml proteinase K

### 2.5.2 DNA digestion, separation and transfer to membranes

Purified DNA was digested at 37°C overnight with *EcoR*I, *EcoR*V, *Bam*HI, *Hind*III, *Xba*I and *Xho*I and separated in 0.7% agarose gels. After electrophoresis the agarose gels were soaked for 15min in 0.2M HCl, 30 min in denaturing solution (1.5M NaCl, 0.5M NaOH) and 30min in neutralizing solution (1M Tris-HCl, 1.5M NaCl, pH7.5) prior to DNA transfer onto nylon membranes using 20×SSC by vacuum blotting (Appligene-Oncor Vacuum Blotter). The transferred DNA was immobilized by UV cross-linking (Stratagene UV crosslinker, 12mJ).

### 2.5.3 Radioactive labelling of probes

***PCR labelling***

Radiolabelled probes were prepared by PCR from *BKn1* and *BKn7* cDNA clones with gene-

specific primers for non-conserved regions. For amplication of *BKn1* cDNA representing the 260bp region downstream the homeobox, primers 5'-GACATGGAATTCGTCATGATGGA AGGATTC-3' and 5'-CCCACCGAATTCACATGACAATTGACAAC-3' were used. For *BKn7*, primers 5'-GACCGCGAATTCGTCGGACAAGAGCAAG-3' and 5'-GACGCCGAA TTCTCCCAACACGCAGCAC-3' were used to amplify the 300bp region downstream the homeobox.

***PCR reaction (10μl)***

1μl template DNA (about 10ng)
1μl 10× PCR buffer
1μl dNTPs (20μM dATP, dGTP, dTTP)
0.5μl 20mM $MgCl_2$
1μl primer mix (5pmol each primer)
0.5μl *Taq*-polymerase (2U/μl)
5μl [$\alpha^{32}$P]dCTP (10μCi/μl)

***PCR conditions***

94°C denature 4min
55°C annealing 2min
*35 cycles of*
72°C extension 30sec
92°C denaturation 1min
55°C annealing 1min
72°C extension 5min
10°C hold

***Random primer labelling***

*BEIL*, *BAPL*, *BBR* and *BGRF* cDNA fragments purified from agarose gel by NucleoSpin column (Macherey-Nagel) were used in the labelling reaction.

***Random primer labelling reaction***

50-100ng template DNA
in 39μl volume, *heat to 95°C for 5min*
*add* 5μl 10× oligo mix
5μl [$\alpha^{32}$P]dCTP (10μCi/μl)
1μl Klenow enzyme (2U/μl)
*incubate at 37°C for 1 hour*

***10× Oligo mix***

0.2mM dATP, dGTP, dTTP
4.0mg/ml $(dN)_6$
2.0M HEPES, pH 6.6
0.4M Tris-HCl, pH 8.0
$7.4\times10^{-5}$M $MgCl_2$
0.7% (v/v) β-Mercaptoethanol

## 2.5.4 Hybridization

Pre-hybridization and hybridization were carried out in hybridization solution in glass tubes or plastic boxes at 65°C. Pre-hybridization was performed for at least 2 hours. After adding the denatured probe, the hybridization was performed overnight under the same condition. After hybridization the membranes were washed with decreasing concentrations of SSC in 0.1% SDS with a final wash of 0.5 or 0.1×SSC, 0.1% SDS at 65°C and exposed to Kodak X-ray films (X-Omat AR) at -70°C using intensifying screens.

***Hybridization solution***

5× Denhardt's solution
5× SSC
0.1% (w/v) SDS
100μg/ml Herring sperm DNA
*or*
7% SDS
0.25M $Na_2HPO_4$ (pH 7.2)
40μg/ml Herring sperm DNA

## 2.6 Northern Blot

### 2.6.1 Plant total RNA extraction

Total RNA from various barley tissues was extracted using Total RNA Isolation Reagent (Biomol) or RNeasy plant minikit (Qiagen) following the supplier's instructions. The concentration of extracted RNA was estimated by checking rRNA band intensity on ethidium bromide stained agarose gels or alternatively, by the absorbance at 260nm.

### 2.6.2 Plant polyA$^+$ mRNA extraction

1-3g barley tissues were ground under liquid nitrogen to fine powder. 10ml lysis buffer was added to the powder and the mix was shaken at 37°C for 30min. The lysate was extracted with equal volume of phenol/chloroform. After centrifugation at 4,000rpm for 5min the aqueous phase was taken off and mixed with 1/10 volume of 4M NaCl and 10-30mg Oligo(dT)Cellulose (Biolab) and shaken at room temperature for 30-60min.

The cellulose was collected by centrifugation at 4,000rpm for 1min and washed three times with 50ml of washing buffer I and subsequently three times with washing buffer II. PolyA$^+$ mRNA was eluted with 10ml elution buffer (0.01M Tris-HCl, pH 7.5) at 55°C. 1/20 volume of 4M NaCl or $NH_4OAc$ and 2.5 volume of ethanol were added to the eluate. After precipitation overnight at -20°C the polyA$^+$ mRNA was spun down at 10,000rpm for 20min. The pellet was washed two times with 70% ethanol containing 0.2M NaOAc, dried and dissolved in $H_2O$. The concentration of polyA$^+$ mRNA was measured by the absorbance at 260nm.

| | |
|---|---|
| ***Lysis buffer*** | 0.1M NaCl<br>0.05M Tris-HCl (pH 9.0)<br>0.01M EDTA<br>2% (w/v) SDS<br>2mg/10ml proteinase K |
| ***Washing buffer I*** | 0.4M NaCl<br>0.01M Tris-HCl (pH 7.5)<br>0.2% (w/v) SDS |
| ***Washing buffer II*** | 0.1M NaCl<br>0.02M Tris-HCl (pH 7.5)<br>0.01% (w/v) SDS |

### 2.6.3 RNA separation and transfer to membranes

20$\mu$g total RNA or 2$\mu$g polyA$^+$ mRNA were mixed with 3 volumes of RNA loading buffer

and denatured at 65°C for 5min before loaded and separated on a 1% denaturing agarose gel.

***RNA loading buffer***

66% formamide (deionized)
0.5× MOPS
8% formaldehyde
0.1% bromophenol blue
with (for total RNA) or
without (for polyA$^+$ mRNA) 0.1mg/ml EtBr

After electrophoresis the agarose gel was soaked for 1 hour with gentle shaking in 4 volumes of water with two time changes to remove the formaldehyde. The transfer of the RNA to a nylon membrane was performed as described above (2.5.2).

***5×MOPS***

41.7g MOPS
4.1g NaOAc
10ml 0.5M EDTA
*add* $H_2O$ to 1L, *adjust pH to 7.0*
*filter sterilize*

***RNA running buffer***

3% formaldehyde
1× MOPS

***RNA denaturing gel***

1% agarose
6.6% formaldehyde
1× MOPS

### 2.6.4 Radioactive labelling of probes

The methods used for labelling are the same as those for Southern bloting. Probes used in Northern blot for investigation of expression of *BKn1*, *BKn7*, *BEIL*, *BAPL*, *BBR* and *BGRF* are identical to those used in Southern blot.

### 2.6.5 Hybridization

Pre-hybridization and hybridization were carried out in hybridizing solution in glass tubes or plastic boxes at 42°C.

Pre-hybridization was performed for at least 2 hours. After adding the denatured probe, the hybridization was performed overnight under the same condition.

***Hybridization solution***

50% formamide
5× Denhardt's solution
5× SSC
0.1% (w/v) SDS
100 $\mu$g/ml Herring sperm DNA

After hybridization, washing of membranes and detection of radioactive signals were performed as described above (2.5.4).

## 2.7 RT-PCR

### 2.7.1 Reverse transcription (RT)

10$\mu$g of total RNA from different barley tissues was digested with 10U of DNase I at 37°C for 1 hour. DNase I activity was removed by phenol/chloroform extraction. 3$\mu$g of total RNA was transcribed with 200U of Superscript reverse transcriptase (Life Technologies). Oligo $(dT)_{15}$ was used as downstream primer.

***Reverse transcription reaction***

3$\mu$g total RNA
1$\mu$g oligo $(dT)_{15}$
in 11.5$\mu$l volume, *heat to 70°C for 10min, quick-chill on ice*
*add* 1$\mu$l dNTP (10mM each)
4$\mu$l 5× reverse transcriptase buffer
2$\mu$l 0.1M DTT
0.5$\mu$l RNasin
*incubate for 3min at 42°C*
*add* 1$\mu$l Superscript reverse transcriptase (200U/$\mu$l)
*incubate for 50min at 42°C*
*heat to 95°C for 5min, quick-chill on ice*
*add 10$\mu$l DEPC treated $H_2O$*

### 2.7.2 cDNA PCR amplification

***PCR reaction***

1$\mu$l RT reaction product
2.5$\mu$l 10× PCR buffer
0.5$\mu$l dNTPs (10mM each)
0.75$\mu$l 50mM $MgCl_2$
0.5$\mu$l *Taq* Polymerase (2U/$\mu$l)
2$\mu$l primers (5pmol each)
17.75$\mu$l distilled $H_2O$

The product of the reverse transcription reaction was amplified by PCR using gene specific primers. For amplification of 3' region of *BKn1* cDNA, primers 5'-GAAGGTGTTGGCTCTTCT-3' and 5'-CATTGCAACCA GTGCTGA-3' were used and PCR product is a 527bp DNA fragment. For *BKn7*, primers 5'-TGAGAGATCCTT GGTCGA-3' and 5'-ATCCACGACACACGTTAC-3' were used to amplify a 644bp fragment at the 3' region of the gene. For BBR cDNA amplification, primers 5'-TCTGCATGCTGCACAACTTCT-3' and 5'-GGTCATAACAGACATTGAAG-3' were used and the resulting PCR product is a 478bp DNA fragment. For BGRF, primers 5'-CTGCACATGGACACTGCTG-3' and 5'-GGCCACGAGTCCCTGTCCT-3' were used to amplify a 492bp fragment at the 3' end of the gene.

Amplification of GAPDH (glyceraldehyde-3-phosphate dehydrogenase) cDNA by using GAPDH primers specific for the barley GAPDH gene (Chojecki, 1986) was performed as an internal control to ensure that equal amounts of cDNA were added to each PCR.

Two different PCR conditions were used depending on two different detection methods. The

PCR products amplified using conventional PCR condition described above (2.5.3) were analysed by agarose gel electrophoresis and DNA fragments were visualized by EtBr staining. The PCR condition and detection method were performed for amplification of *BKn1* and *BKn7* cDNAs. Another PCR condition was used with reduced number of PCR cycles to 20 cycles if the PCR products would be subsequently detected by radioactive labelled cDNA probes. After PCR amplification the PCR products were run on a 0.7% agarose gel, blotted to membranes and hybridized to $^{32}$P-labelled cDNA probes and visualized by autoradiography. The amplification and detection of *BBR* and *BGRF* cDNAs were carried out in this way.

## 2.8 *In situ* hybridization

### 2.8.1 Fixation and section of tissues

Various barley tissues at different developing stages were fixed in 4% FAE (4% formaldehyde, 10% acetic acid, 50% ethanol) for 16 hours at 4°C, dehydrated in an increasing ethanol series, infiltrated with xylene and embedded in Histowax (Leica) by standard methods. Microtome sections (10$\mu$m thick) were mounted on (3-aminopropyl)-trimethoxysilane-coated slides.

### 2.8.2 Preparation of Digoxigenin(DIG)-labelled RNA probes

Digoxigenin(DIG)-labelled RNA probes were obtained by *in vitro* transcription of linearized plasmid DNA containing the cDNA fragments subcloned into pBluescriptKS(+) vector. The *BKn1* probe spanned 740bp 3' to the ATG; two *BKn7* gene-specific probes included 107bp upstream and 383bp downstream of the ATG and 265bp of the 3'-untranslated region, respectively.

***In vitro transcription reaction***

| |
|---|
| 3$\mu$l linearized plasmid DNA (500ng) |
| 2$\mu$l 10× transcription buffer |
| 2$\mu$l DIG-UTP mix (Boehringer) |
| 1$\mu$l RNasin (20U/$\mu$l) |
| 1$\mu$l T3 or T7 RNA polymerase (10U/$\mu$l) |
| 11$\mu$l DEPC-treated $H_2O$ |

After the reaction was incubated at 37°C for 2 hours, 1$\mu$l of DNase I (1U/$\mu$l, RNase free) was added to the reaction and incubated at 37°C for another 30min. 2$\mu$l 0.5M DEPC-treated EDTA were used to stop the reaction. After a single phenol/chloroform extraction, the *in vitro* transcribed RNA was precipitated overnight at -20°C by using 2 volume of ethanol in the presence of 1.33M LiCl and 30ng/$\mu$l yeast tRNA. Precipitated RNA was washed with 70% ethanol, dried and dissolved in 50$\mu$l DEPC-treated water. The concentration of RNA was measured on an agarose gel by EtBr staining.

When we used sense RNA probe as a negative control, we got weak signals due to the contamination of antisense transcripts, demonstrated by Northern blot hybridization with single-stranded DNA probes. So an unrelated gene, Lamda DNA 250bp *Pst*I fragment was subcloned into pBluescript KS+ and used as the template of RNA probes. For an additional

negative control, the sections were digested with RNase prior to hybridization. *Histon2a* gene with ubiquitous expression and one barley MADS-box gene with a known expression pattern (Schmitz *et al*., 2000) specifically in the inflorescence were used as positive controls.

### 2.8.3 Hybridization

Prior to hybridization the wax was removed by xylene and the sections were re-hydrated in a decreasing ethanol series and treated with 10$\mu$g/ml of proteinase K for 5 to 10 min at 37°C in a solution of 2×SSC, 0.1% SDS. Proteinase K digestion was stopped by addition of 0.1M glycine in 2×SSC and the sections were fixed for 5min in 4% formaldehyde containing 0.1M phosphate buffer (pH 7.0), washed in 2×SSC and dehydrated in ethanol.

Hybridization was performed for 16 hours at 50°C in hybridization solution with 0.5-1ng/$\mu$l probe concentration.

***Hybridization solution***
*(100$\mu$l for one slide)*

50$\mu$l formamide (deionized)
2.5$\mu$l yeast tRNA (20mg/ml)
1$\mu$l poly(A) (10mg/ml)
6$\mu$l 5M NaCl
4$\mu$l 50× TE (pH 7.0)
2$\mu$l 50× Denhardt's solution
20$\mu$l 50% (w/v) dextran sulphate
50-100ng DIG-labelled RNA probe
*add DEPC-treated $H_2O$ to 100$\mu$l*

Post-hybridization washes were performed in a decreasing series of SSC from 3× to 0.5× at 45°C or 50°C. Excess single-stranded DIG-labelled RNA was removed by digestion with 20$\mu$g/ml of RNase A in 0.5M NaCl, 1×TE (pH 8.0) at 37°C for 30min.

### 2.8.4 Detection of hybridization signals

Detection of hybridized DIG-labelled RNA probes was done according to the instructions of Boehringer Mannheim. In brief, the sections were rinsed for 5min in buffer I (100mM Tris-HCl, 150mM NaCl, pH 7.5) and blocked for 1 hour in 0.5% (w/v) blocking reagent prior to incubation with anti-DIG-alkaline phosphatase-conjugate antibody (1:2,000 in 0.5% blocking reagent) for 1 hour. The sections were subsequently washed for 20 min in buffer II (100mM Tris-HCl, 150mM NaCl, 0.3% (v/v) Triton X-100, pH 7.5) four times, for 5min in buffer I and for 5 min in buffer III (100mM Tris-HCl, 100mM NaCl, pH 9.5). The sections were incubated in NBT/BCIP solution for 3 hours to 36 hours in the dark. The enzyme reaction was stopped by incubating in water for 5min. The hybridization signals were visualized under a Nikon light microscope.

| | |
|---|---|
| ***NBT/BCIP solution*** | 100mM Tris-HCl (pH 9.5)<br>100mM NaCl<br>150$\mu$g/ml NBT<br>75$\mu$g/ml BCIP |

## 2.9 Genomic library screening and DNA sequencing

### 2.9.1 Screening of Barley gDNA library on λEMBL3

A λEMBL3 genomic library prepared from barley *Calc15* inflorescences was screened essentially according to Sambrook *et al*. (1989). *E. coli* strain K803 was used for λEMBL3 phage infection and propagation.

#### 2.9.1.1 λ phage plating and transfer

A single colony of *E. coli* K803 was incubated overnight in 5ml LB containing 0.2% maltose. 2ml of overnight culture was added to 18ml of LB containing 0.2% maltose and 10mM $MgSO_4$ and incubated at 37°C for 3 hours. Bacteria were collected by centrifugation at 4,000rpm for 5min. The cell pellet was resuspended in 10mM $MgSO_4$ to $OD_{600}$ 0.5.

For phage plating, 600$\mu$l of *E. coli* suspension were mixed with 100$\mu$l SM containing $2.5\times10^5$pfu of phage suspension. The mixture was left for 30min at 37°C, added to 35ml of LB top agarose kept at 45°C, and plated on one 245×245cm$^2$ petri-dish containing LB agar. Eight plates prepared for one screening were incubated overnight at 37°C and stored at 4°C before membrane transfer.

About $2\times10^6$ plaques were transferred onto nylon membranes followed by soaking for 5min in denaturing solution, 5min in neutralizing solution and 5min in 2×SSC. The membranes were dried at room temperature and the DNA was UV cross-linked to the membranes. The screening probes were [$\alpha^{32}$P]-dCTP labelled full-length cDNA of *BEIL*, *BAPL*, *BBR* and *BGRF*. Hybridization and washing were performed as described above (2.5.4).

#### 2.9.1.2 Selection and rescreening of λ clones

Plaques strongly hybridizing to the probes were selected, placed in Eppendorf tubes containing 1ml of SM solution plus 3 drops of chloroform and the λ phages were left to elute overnight at 4°C.

For λ plating, three different volumes (0.1$\mu$l, 1$\mu$l or 10$\mu$) of the phage suspension in a total volume of 100 $\mu$l of SM were added to 200$\mu$l of *E. coli* cells. The mixture was left for 30min at 37°C, added to 3ml of LB top agarose kept at 45°C, and plated on 85mm∅ petri-dishes containing LB agar. The plates were incubated overnight at 37°C and stored at 4°C before membrane transfer.

The $^{32}$P-labelled probes from 2.9.1.1 were reused in the re-screening. The re-screening was

performed till a single positive plaque could be picked easily. Afterwards another re-screening was used to confirm the single plaque picked was the correct one.

#### 2.9.1.3 Extraction of λEMBL3 phage DNA

50ml of NZCYM media for phage culture were inoculated with 100$\mu$l *E. coli* K803 suspension (described in 2.9.1.1) and 100$\mu$l of the isolated phage eluate. After overnight incubation at 37°C, 250$\mu$l of chloroform were added and cell debris was precipitated by centrifugation at 4,000rpm for 10min. 50$\mu$g DNase I and 50$\mu$g RNase were added to the supernatant and incubated for 30min at room temperature.

Phage particles were precipitated by incubating with 2.8g NaCl and 5g PEG6,000 for 2 hours on ice. After centrifugation at 4,000rpm for 30min at 4°C, the pellet was resuspended in 5ml TE buffer containing 1mg proteinase K, 0.05% SDS, 40mM EDTA, 100mM NaCl and incubated for 1 hour at 56°C, then 20min at 70°C. The isolated phage DNA was extracted twice with phenol/chloroform and precipitated with 0.2M NaCl and 0.7 volume of isopropanol. The resulting pellet was washed with 70% ethanol and resuspended in 200$\mu$l TE buffer (10mM Tris-HCl, 1mM EDTA, pH 7.6).

#### 2.9.1.4 Subcloning of λ clone inserts

Insert size was determined by digestion of 1$\mu$g purified phage DNA with *Sal*I. Restriction maps of genomic clones were created by digestion of phage DNA with different restriction enzymes. Digested DNA was checked on 0.7% agarose gel and subsequently blotted to membranes and hybridized with $^{32}$P-labelled probes (2.9.1.1.) The digested bands hybridizing with the probes were extracted from the agarose gel and cloned into pBluescript KS$^{+}$ vector. Plasmid DNA was prepared using the alkaline-lysis method (Sambrook *et al*., 1989) and used for DNA sequencing.

### 2.9.2 DNA sequencing

DNA sequences were determined by the dideoxynucleotide chain termination method using the ABI PRISM Dye Terminator cycle Sequencing Ready Reaction Kit (Applied Biosystems) on 377 DNA sequencer (Applied Biosystems).

Sequence analyses were performed using sequence analysis software package GCG (Wisconsin University, Version 9.1, UNIX, September 1997). For sequence comparison, the *Bestfit* or *Pileup* programs were used. Updated versions of the sequence databases GenBank and EMBL were searched for the sequences available in the databases using the *FASTA* or *BLAST* program.

## 2.10 Detection of single strand conformation polymorphism (SSCP)

### 2.10.1 PCR amplification of genomic DNA and digestion

A Nudinka × Proctor mapping population of 100 doubled-haploid (DH) lines was used for the mapping of *BEIL*, *BAPL*, *BBR* and *BGRF*. Genomic DNA fragments spanning introns were amplified with gene-specific primers from genomic DNA of Nudinka , Proctor and each of the 100 DH lines. PCR conditions were performed as described above (2.5.3).

***PCR reaction***

1$\mu$l genomic DNA (100ng)
2.5$\mu$l 10× PCR buffer
0.75$\mu$l 50mM $MgCl_2$
0.5$\mu$l dNTPs (10mM each)
0.5$\mu$l *Taq* polymerase(2U/$\mu$l)
2$\mu$l primers (5pmol each)
*add deionized $H_2O$ to 25$\mu$l*

4$\mu$l of PCR products were digested in 10$\mu$l volume by *Alu*I, *Dpn*I, *Hae*III, *Mse*I, *Rsa*I, *Taq*I or other restriction enzymes which were 4-bp cutter.

### 2.10.2 Detection of SSCP

SSCP polymorphism was detected by separating digested PCR products on non-denaturing acrylamide gels according to the protocol of the supplier (MDE gel solution, FMC BioProducts, Rockland, USA). After adding 10% APS and TEMED the gel mix was poured to a 20cm wide, 25cm high and 0.7mm thick gel mold consisting of two glass plates, one of which was treated with antistick silan (dichlorodimethylosilan) and the other with stick silan (γ-methacryloxypropyl-trimethoxysilan). The gel was polymerized for at least 2 hours at room temperature before loading samples.

4$\mu$l of digested PCR product mixed with 9$\mu$l sample buffer was denatured at 95°C for 3min and quick-chilled on ice. 5$\mu$l of this mixture were loaded on the gel. The electrophoresis was run in 0.6×TBE at 2W for 15-16 hours at room temperature. After electrophoresis the gel was fixed for 3min in fixation solution (10% ethanol, 0.5% acetic acid), stained for 5min in silver staining solution, rinsed shortly in distilled water and soaked for 10 to 20min in developing solution (3% NaOH, 0.1% formaldehyde) till the clear bands appeared. The gel was subsequently fixed for 5min and washed with water to remove the background. Fragment patterns were visualized using a gel printer (SONY, UP-890CE) and the pictures were saved in computer disks.

***SSCP gel mix***

1.5g glycerol
1.8ml 10× TBE
2.5ml MDE gel solution
19.2ml deionized $H_2O$
150$\mu$l 10% APS
18.8$\mu$l TEMED

***10× TBE***

890mM Tris-HCl
890mM boric acid
20mM EDTA, pH 8.0

***Sample buffer***

95% formamide
0.01M NaOH
0.05% xylen cyanol
0.05% bromphenol blue

### 2.10.3 Data analysis

Once the polymorphism between Nudinka and Proctor was found the 100 progeny were then analysed for the presence or absence of Nudink- or Proctor- specific alleles and segregation analysis was performed using the MAPMAKER/EXP 3.0b software (Lander *et al.*, 1987) to integrate the map positions of the gene analysed into a combined AFLP/RFLP/ISTR linkage map (Castiglioni *et al.*, 1998)

## 2.11 Primer extension

The primer extension experiment was performed according to Current Protocols in Molecular Biology (Ausubel *et al.*, 1994) with some modifications.

### 2.11.1 Primer labelling

To localize the transcription initiation site of the *BBR* gene, an oligonucleotide, 5'-CCGAGG TTTCCTTTCATCGTCTCGTAG-3', which is complementary to the sense strand sequence of the *BBR* cDNA from +42 to +69 relative to the translation start site (ATG) was radiolabelled at its 5' terminus with T4 polynucleotide kinase (PNK) and [$\gamma$-$^{32}$P]ATP.

***Primer labelling reaction***

2$\mu$l deionized $H_2O$
1$\mu$l 10× $T_4$ PNK buffer
1$\mu$l 0.1M DTT
1$\mu$l 1mM spermidine
1$\mu$l 10pmol/ $\mu$l primer
3$\mu$l [$\gamma$-$^{32}$P]ATP
1$\mu$l $T_4$ PNK (10U/$\mu$l)
*mix the reagents in the order indicated above, incubate 1 hour at 37°C*

### 2.11.2 Hybridization

1$\mu$l of labelled primer was hybridized at 65°C for 90min with 10$\mu$g total RNA which was isolated from barely inflorescences and then cooled down slowly to 50°C by switching off the water bath.

***Hybridization reaction***

12.5$\mu$l total RNA(10$\mu$g)
1.5$\mu$l 10× hybridization buffer
1$\mu$l labelled primer

***10× Hybridization buffer***

1.5M KCl
0.1M Tris-HCl, pH 8.3
10mM EDTA

### 2.11.3 Reverse transcription

After hybridization, complementary DNA was synthesized from the annealed primer by the addition of reverse transcriptase and dNTP. The reverse transcription reaction was performed at 42°C for 1 hour.

***Reverse transcription reaction***

0.9$\mu$l 1M Tris-HCl, pH 8.3
0.9$\mu$l 0.5M $MgCl_2$
2.5$\mu$l 0.1M DTT
6.75$\mu$l 1mg/ml actinomycin D
1.33$\mu$l 5mM dNTP
17.75$\mu$l $H_2O$
1$\mu$l Superscript reverse transcriptase (200U/$\mu$l)
15$\mu$l annealed primer from 2.11.2

After reverse transcription, the primer extension product was precipitated by ethanol and the resulting pellet was dissolved in 7$\mu$l annealing buffer and 5$\mu$l stop solution provided by T7 Sequencing Kit (from USB Corporation) and denatured at 95°C for 3min just before loading on the gel.

### 2.11.4 DNA sequencing

In order to provide size markers, part of the BBR gene was sequenced with the same primer used in the primer extension experiment. The sequencing was done by using T7 Sequencing Kit following the supplier's instructions.

#### 2.11.4.1 Annealing of primer to double-stranded DNA template

1.5-2$\mu$g of double-stranded DNA template was denatured by 2M NaOH at room temperature, precipitated by ethanol and dissolved in 11$\mu$l of $H_2O$. 1$\mu$l of primer (10pmol/$\mu$l) mixed with 2$\mu$l of annealing buffer was added to the template DNA and incubated for 5min at 65°C, 10min at 37°C and more than 5min at room temperature.

#### 2.11.4.2 Sequencing reactions

3$\mu$l of labelling mix-dCTP, 2$\mu$l of diluted T7 DNA polymerase and 1$\mu$l of [$\alpha^{33}$P]-dCTP were added to the annealed template and primer, incubated for 5min at room temperature and subsequently divided into 4 parts and transferred to four pre-warmed sequencing mixes (G, A, T, C) respectively. The reactions were incubated at 37°C for 5min and stopped by adding

5$\mu$l of stop solution. The reactions were heated at 80°C for 2min before loaded on a gel.

### 2.11.5 Gel electrophoresis

The primer extension product and sequencing reactions were loaded on 8% polyacrylamide-6M urea gel.

***Gel mix***

12.6g urea
8.75ml 5× TBE
9.33ml 30% acrylamide/bisacrylamide (29:1)
*add deionized $H_2O$ to 35ml*

The gel mix was filtered through 0.2$\mu$m filter and degassed under vacuum for 5min. After adding 240$\mu$l 10% APS and 30$\mu$l TEMED and gently mixing, the gel was poured to a 20cm wide, 40cm high and 0.3mm thick gel mold. The samples were loaded on the gel after the gel was polymerized and pre-run for 30min in 1×TBE at running condition of 24mA/50W/1800V. The gel was run at the same condition till the Bromphenol Blue in the sample ran out of the gel.

After electrophoresis the gel was transferred to 3MM Whatman paper, dried at 65°C for 1 hour under the vacuum and visualized by autoradiography.

## 2.12 Electrophoretic mobility shift assay (EMSA)

### 2.12.1 Preparation of purified GST-fusion proteins

Full-length cDNA fragments of *BEIL*, *BAPL*, *BBR* and *BGRF* genes were subcloned into the pGEX-5X-1 vector and the resulting constructs were transformed into *E. coli* strain BL21.

A single colony of transformed BL21 bacteria was inoculated in 5ml LB media overnight. 2ml of overnight cultures were diluted in 100ml LB media and cultured at 37°C for 2 hours. Overexpression of fusion proteins was induced by the addition of IPTG to 0.1mM final concentration and the cultures were incubated at 37°C for another 4 hours.

The bacteria were harvested by centrifugation at 5,000 rpm for 15min at 4°C and pellet was washed twice with 6ml of 1×STE and resuspended in 6ml of 1×STE containing 100$\mu$g/ml lysozyme and incubated on ice for 15min. After adding DTT to final concentration of 5mM and Sarkosyl to 1.5% (v/v) final concentration, the resuspension was sonicated for 1min at 50% duty cycles and 4.5 power level (Branson sonifier 250, Heinmann Ultraschall-und Labortechnik) and clarified by centrifugation at 10,000rpm for 10min. The supernatant was taken off and Triton X-100 was added to the supernatant to 2% final concentration and incubated with 1ml swollen glutathione-sepharose beads for 60min at 4°C with gentle shaking. The beads were collected by centrifugation at 500rpm for 2 min and washed 6 times with 1×PBS. The GST-fusion proteins were eluted by incubation 5 times 10min each in 1ml

of 10mM Tris-HCl (pH 8.0) containing 10mM reduced glutathione.

***10× STE buffer***

100mM Tris-HCl (pH 8.0)
1.5M NaCl
10mM EDTA

***10× PBS***

1.37mM NaCl
27mM KCl
43mM $Na_2HPO_4 \cdot 7H_2O$
14mM $KH_2PO_4$

***1× Binding buffer***

0.5mM EDTA
10mM Tris-HCl (pH 7.5)
1mM $MgCl_2$
5mM NaCl
4% glycerol

The proteins were dialysed in 2l of 1× binding buffer overnight at 4°C and concentrated to 1ml by using Centricon-50 (Amicon, Witten). The concentration of purified protein was measured by comparison of the BSA standard and protein bands intensity on coomassie blue stained SDS-PAGE gel.

### 2.12.3 Preparation of radioactive-labelled probes

***5' end filling reaction***

100ng DNA template
2$\mu$l 10× Klenow buffer
1$\mu$l Klenow enzyme (2U/$\mu$l)
1$\mu$l 5mM dATP/dGTP/dTTP
3$\mu$l [$\alpha^{32}$P]dCTP (1$\mu$Ci/$\mu$l)
*add deionized $H_2O$ to 20 $\mu$l*

Probes used for DNA mobility shift assay were derived from digested plasmid DNA fragments or annealed oligonucleotides and radioactively labelled by filling in 5' protruding ends. The enzymes which produce 5' protruding ends were used to digest plasmid DNA and the desirable DNA fragments were purified from agarose gels. Two oligonucleotides (11.5$\mu$g of each, 1$\mu$g/$\mu$l) were mixed in the presence of 1$\mu$l of TE and 1$\mu$l 75mM NaCl and heated at 85°C for 10min in a heating block. The annealing reaction was left in the heating block overnight after the heating block was switched off. The annealed oligonuceotides were diluted to 100ng/$\mu$l, stored at 4°C and used for the labelling reaction.

The reaction was incubated at 37°C for 30min. Probes longer than 100bp were purified by NucleoSpin columns (Macherey-Nagel) and labelled oligonucleotides were purified by Nucleotrap kit (Macherey-Nagel).

### 2.12.3 *In vitro* translation

The cDNA sequences of the *BEIL*, *BAPL*, *BBR* and *BGRF* genes subcloned in pBluescritpKS+ vector were transcribed *in vitro* as described in section 2.8.2. The 100ng

aliquot of RNA was *in vitro* translated in a 50$\mu$l volume for 1 hour at 25°C by incubation with 25$\mu$l of wheat germ extracts, 4$\mu$l of amino acid mix devoid of methionine, and 1$\mu$l of 100mM potassium-acetate in the presence of 2$\mu$l $^{35}$S-methionine. 10$\mu$l of *in vitro* translated products were denatured at 100°C for 3min in 2× loading buffer and analyzed on 12.5% SDS-PAGE gels. After electrophoresis, gels were soaked in a solution containing 10% acetic acid and 40% methanol for 2 hours, followed by 100% DMSO for 1 hour with 2 changes, then in Rotifluorescence for 3 hours and in water for 1 hour. Gels were vacuum dried and exposed to X-ray films.

### 2.12.4 Binding reactions

Binding reactions were performed in a 20$\mu$l volume containing 10,000cpm $^{32}$P-labelled probes, 15-30ng of purified proteins, 0.5mM EDTA, 10mM Tris-HCl (pH 7.5), 1mM $MgCl_2$, 50mM NaCl, 4% glycerol, 0.5mM DTT and 1$\mu$g poly(dI-dC) at room temperature for 20 min. Competition experiments were conducted by adding an excess of unlabelled probe.

### 2.12.5 Gel electrophoresis

A 4% native polyacrylamide gel in 0.2×TAE buffer was used to separate DNA-protein complexes. The gel was pre-run in 0.2×TAE at 100V for 30min at 4°C and the voltage was increased to 150V before loading the samples. The samples were loaded without turning off the gel and run for 3 to 10 hours depending on the sizes of the DNA probes used. After electrophoresis the gel was transferred to 3MM Whatman paper and dried at 65°C under the vacuum. The mobility shift was visualized by autoradiograghy.

***Gel mix***

12ml 1× TAE
7.95ml 30% acrylamide/bisacrylamide (29:1)
5.295g glycerol
*add $H_2O$ to 60ml*
405$\mu$l 10% APS
40.5$\mu$l TEMED

***1× TAE***

40mM Tris
1mM EDTA
1.162ml acetic acid
*add $H_2O$ to 1L and adjust pH to 8.0*

## 2.13 Tobacco protoplast transfection

### 2.13.1 Preparation of tobacco protoplasts

Tobacco SR1 leaves from sterile cultures were digested for 16 hours at 25°C in K3 medium containing 0.4% cellulase and 0.2% macerozyme. The protoplasts were separated by a

100μm-mesh screen and washed three times in K3 medium. They were collected in W5 medium by centrifugation at 500rpm for 5min and resuspended to a final concentration of $1\times10^6$ protoplasts $ml^{-1}$ in transfection medium.

***K3 medium***

4.5g MS basal medium
100mg inositol
250mg xylose
136.92 sucrose
*add $H_2O$ to 1L*
*adjust pH to 5.6*

***W5 medium***

154mM NaCl
125mM $CaCl_2$
5mM KCl
5mM glucose
*adjust pH to 5.6*

***Transfection medium***

450mM mannitol
15mM $MgCl_2$
0.1% (w/v) MES
*adjust pH to 5.6*

### 2.13.2 Construction of *BBR* promoter/GUS and *BBR*/GFP fusion plasmids

A series of 5' deletions of the *BBR* promoter was amplified via PCR. For a transcriptional fusion of the *BBR* promoter to the GUS reporter gene, a *Bam*HI site was created just before the translation initiation site (ATG) of the *BBR* gene and a *Hind*III site was introduced to the 5' end of the deletions through PCR amplification. The resulting PCR products were introduced into the *Hind*III/*Bam*HI sites of pBI 101.2 vector to produce promoter deletions/GUS constructs (these constructs were used directly for *Agrobacterium*-mediate tobacco transformation, see section 2.14.1). The promoter deletion/GUS cassette was isolated from the pBI 101.2 vector by *Hind*III/*Bam*HI digestion and subcloned into the *Hind*III/*Bam*HI site of the pBluescript KS+ vector.

5' end primers used for construction of 5' deletion of *BBR* promoter were the following: for 5Del1, 5'-CGACGAAGCTTCCTGTCGCATCTTT-3'; for 5Del2, 5'-ACTTAAAGCTTAACGCGACCTCGT-3'; for 5Del3, 5'-GTCTTAAGCTTAGGAACGGAGAGAG-3'; for 5Del4, 5'-ATCCAAAGCTTGGCATTTTGTGTCAAAG-3'; for 5Del5, 5'-CATTGAAGCTTGAGGTATGGAGGCTG-3' and for 5Del6, 5'-GAAATAAGCTTCGTGGATTGGCGTTT-3'. 3' end primer used for introducing *Bam*HI site was 5'-TCGGCGGATCCAACAAACGAATCGAAAT-3'. *Hind*III and *Bam*HI sites are underlined.

To construct the translational fusion of BBR to GFP, *Nco*I sites were introduced by PCR to both ends of the *BBR* coding sequence. The translation initiation site of different *BBR* coding

regions was embedded in the 5' *Nco*I site. The PCR products were subsequently digested with *Nco*I and inserted to the *Nco*I site of pCATgfp which is downstream of double 35S promoters. The sequences of the primers used for PCR amplification of different regions of *BBR* coding sequence are GFP1, 5'-GGCAACCATGGACGACGACGGCAGCTTGA-3'; GFP2, 5'-GCATGCCATGGACCTGATTGTTACAAACTTG-3'; GFP3, 5'-ACAGGAGCCCATGGTGCCTGATGAGGAAAA-3' and GFP4, 5'-TTCCTCATCCATGGCAGGAGGCTCCTGTGG-3'. *Nco*I sites are underlined.

### 2.13.3 Transfection of tobacco protoplasts

In 15ml tubes, 300$\mu$l of protoplasts were incubated with 10$\mu$g of plasmid DNA (if the constructs were promoter/GUS fusions, 5$\mu$g of the CaMV35S/LUC (luciferase) control plasmid DNA were also included) and 5$\mu$g of carrier DNA (herring sperm DNA) for 5min at room temperature. Then 700$\mu$l of 25% PEG1,500 were added and incubated for a further 25min. 5ml K3 media containing 1$\mu$g/ml NAA were added to the transfected protoplasts and incubated at 25°C for 20 hours in the dark.

***25% PEG1,500***

25% PEG1,500
0.1M $MgCl_2 \cdot 6H_2O$
0.45M mannitol
0.02 M HEPES
*adjust pH to 6.0 and filter sterilize*

### 2.13.4 Detection of *BBR*/GFP fusion protein

The protoplasts floating on the surface of the medium were dropped on a slide, covered with a coverslip and examined under fluorescence microscopy (Zeiss, Germany). For fluorescence studies, filter block I (blue light exciter BP 450-490nm, beamsplitter RKP 510nm, emitter LP 520nm) was used. For the elimination of chlorophyll autofluorescence in tobacco SR1 mesophyll protoplasts, the filter set II (exciter BP 470/20, beamsplitter 493nm, emitter BP 505-530nm) was used. Images were acquired through a 20× objective with a JVC KY-F70 CCD camera.

### 2.13.5 Measurement of GUS activity

For GUS activity measurement, the protoplasts were harvested by mixing with 8ml of W5 media and centrifuging at 4,000rpm for 5min. The resulting pellet was frozen in liquid $N_2$ and extracted with LUC extraction buffer. The extract was used for GUS, LUC and Bradford assays.

72$\mu$l of extract were mixed with 8$\mu$l 10× 4-MUG and assayed as described by Jefferson *et al.* (1987). Standardization was done by measuring the protein concentration according to

Bradford (1976) and by determination of the LUC activity according to Kleines *et al*. (1999).

| | |
|---|---|
| ***LUC extraction buffer*** | 0.1M $K_3PO_4$<br>1mM DTT, pH 7.5 |
| ***10× 4-MUG*** | 10mM 4-MUG<br>50mM $NaH_2PO_4$<br>10mM EDTA<br>10mM β-mercaptoethanol<br>0.1% Triton X-100<br>pH 7.0 |

## 2.14 *Agrobacterium* mediated tobacco transformation

### 2.14.1 *Agrobacterium* transformation

A series of 5' deletions of the *BBR* promoter were introduced into the *Hind*III/*Bam*HI site of the pBI 101.2 vector to produce promoter deletion/GUS constructs (see section 2.13.2). These constructs were then introduced into the *Agrobacterium tumefaciens* strain LBA4404 by electroporation (Bio-Rad, GenePulser).

A single colony of *Agrobacterium* containing the transformation construct was inoculated in 5ml of YEB medium with selective antibiotics at 28°C with shaking for 2 days. 2ml of the cultures were added to 40ml of fresh YEB medium with selective antibiotics and grown at 28°C overnight with shaking. The bacteria were harvested by centrifuging at 4,000rpm for 15min and the resulting pellet was resuspended in 5ml of 10mM $MgSO_4$. The resuspension was centrifuged at 4,000rpm for 10min and the resulting pellet was resuspended in 70ml of MS I medium and used for tobacco transformation.

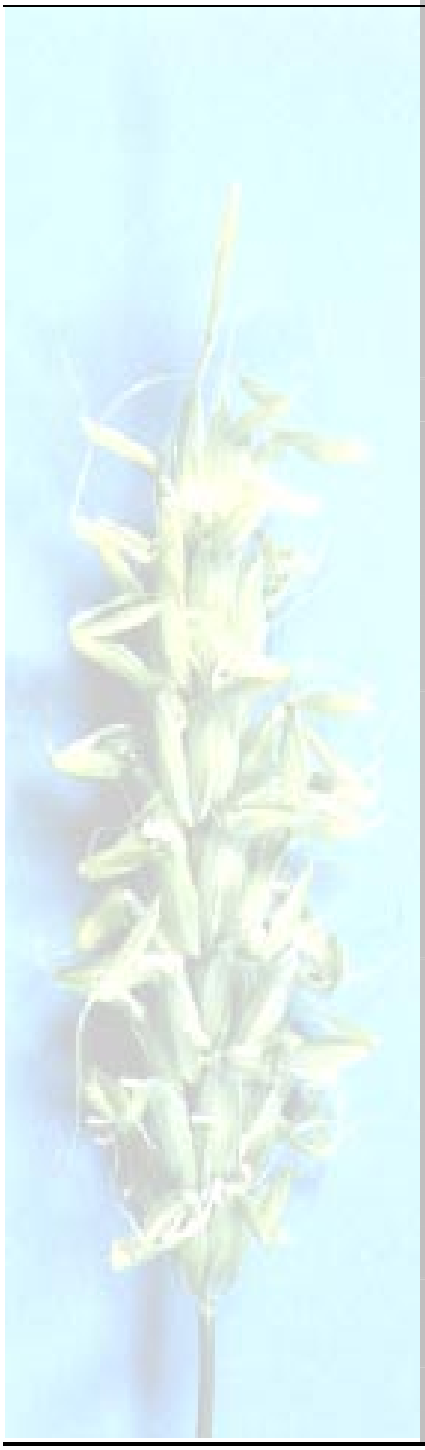
### 2.14.2 Tobacco transformation

Sterile SRI tobacco leaves were cut into 1-3$cm^2$ pieces, placed into the *Agrobacterium* suspension and incubated for 30min at 26°C. The leaf pieces were washed with 100ml of MS I medium two times and laid upside down on MS-Agar plates and incubated for 2 days at 26°C. The leaf pieces were transferred to fresh MS-Agar plates containing claforan (500mg/L), kinetin (0.2mg/L), auxin (1.0mg/L) and antibiotics to select for growth of transgenic cells. After 3 to 4 weeks calli formed at the periphery of the leaf pieces, and shoots subsequently appeared. Once the shoots were 0.5-1.0cm in size, they were removed from the calli and placed on MS medium with claforan, but lacking auxin and kinetin to form roots. When sufficient root formation had occurred, the plants were removed to pots and transferred to a greenhouse for further growth and genetic analysis.

* The methods which are absolutely routine in the laboratory and not described here in detail were performed as described in ***Molecular Cloning*** (Sambrook, J., Fritsch, E. F. and Maniatis, T., 1989).

# RESULTS

# *CHAPTER 3*



## 3.1 Expression of barley *Knox* genes during barley development

A total of 7 barley *Knox* genes had been cloned from barley inflorescence cDNA and genomic DNA libraries using a maize *Kn1* homeobox as a hybridization probe (Müller *et al.*, 1995; Müller, 1997). Based on the sequence similarity to other members of *Knox* gene family, they can be divided into class I and class II groups (Fig. 1-6). To gain insight of their functions in barley development, *BKn1*, one of class I genes, and *BKn7*, one of class II genes were selected for expression analysis.

To assess the copy number of *BKn1* and *BKn7* genes present in the barley genome, barley genomic DNA gel blots were prepared and hybridized at high stringency to respective cDNA probes without conserved ELK and homeodomains (see Materials and Methods). Fig. 3-1 shows the gene-specific probes hybridized to only one band, indicating that both *BKn1* and *BKn7* are single copy genes in the barley genome.

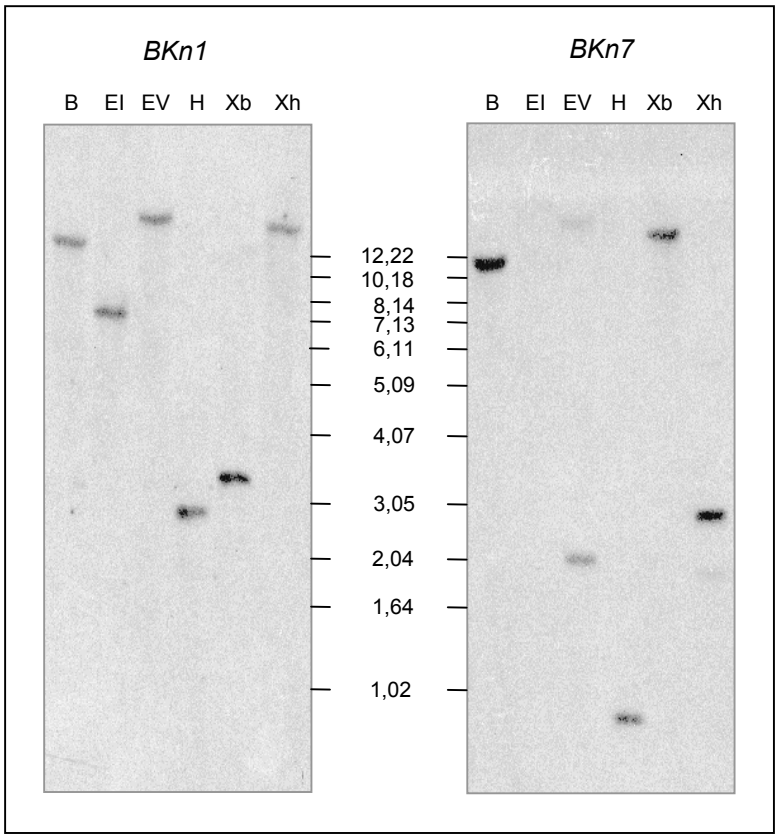



**Fig. 3-1 Genomic Southern blots of *BKn1* and *BKn7*.** Each lane contained 10$\mu$g of barley genomic DNA digested with the indicated restriction enzymes. B, *Bam*HI; EI, *Eco*RI; EV, *Eco*RV; H, *Hind*III; Xb, *Xba*I; Xh, *Xho*I. Genomic DNA was prepared from fully expanded barley seedling leaves (wild type *k-Atlas*). Gene-specific cDNA probes were prepared by PCR in the presence of [$\alpha^{32}$P]-dCTP. Hybridizations and washes were performed at high-stringency conditions. DNA size was marked in kilobases at the middle of the figure.

To investigate the expression of *BKn1* and *BKn7* in different tissues of wild-type barley plants, three different techniques were used. Northern blot analysis and RT-PCR were used to obtain direct estimations of *BKn1* and *BKn7* transcript levels present in total RNA samples extracted from different barley tissues. Furthermore, *in situ* hybridization was performed to determine the spatial pattern of *BKn1* and *BKn7* expression during different stages of barley development.

### 3.1.1 Northern blot analysis

To analyze the expression patterns of *BKn1* and *BKn7* in various organs of barley, Northern blot analyses were conducted using gene-specific cDNA probes (see Materials and Methods) and, as a control, membranes were reprobed with a ribosome DNA probe (pTA71) from wheat to monitor whether the same amount of RNA had been loaded. 20$\mu$g of total RNA extracted from 5-day old fully expanded seedling leaves, 5-day old seedling roots, 2-week old leaves, 2-week old roots, internodes, nodes, inflorescences at all stages and mature embryos, were loaded on the gels and transferred to membranes.

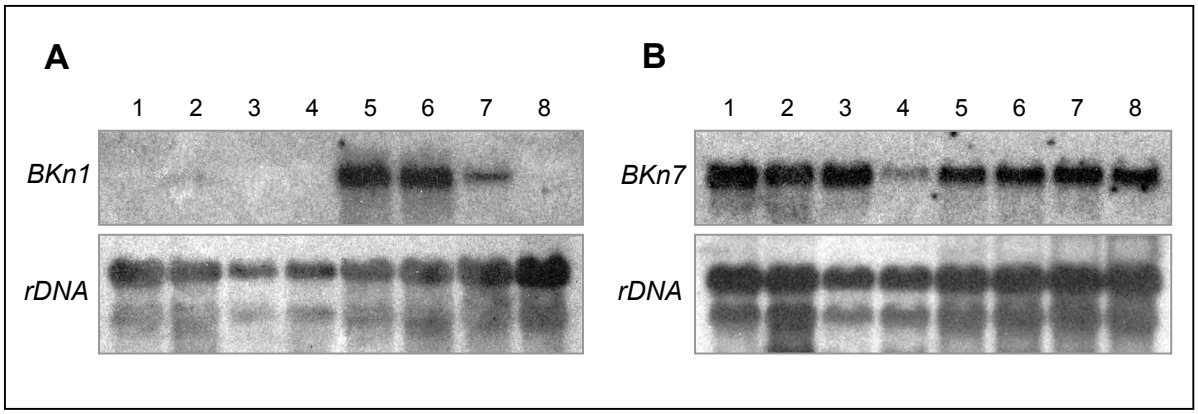



**Fig. 3-2 Northern blot analyses of *BKn1* and *BKn7*.** 20$\mu$g of total RNA were isolated from 5-day old fully expanded seedling leaves (lane 1), 5-day old seedling roots (lane 2), 2-week old leaves (lane 3), 2-week old roots (lane 4), internodes (lane 5), nodes (lane 6), inflorescences (lane 7) and mature embryos (lane 8). Hybridization was done with gene-specific probes of *BKn1* (A) and *BKn7* (B). Integrity of each RNA sample was determined by reprobing with an rDNA probe (pTA71) from wheat.

The hybridization pattern (Fig. 3-2A) showed the highest level of *BKn1* transcription in internodes and nodes and a relatively high level in inflorescences. A very week signal was seen in the lane where total RNA from 5-day old seedling roots was loaded. No expression of *BKn1* was detected in 5-day old fully expanded seedling leaves, 2-week old fully expanded leaves, 2-week old roots and mature embryos.

By contrast, the *BKn7* transcript appeared to be present at different levels in all tissues tested (Fig. 3-2B), as often observed for the *Knotted-1* like class II genes (Kerstetter *et al*., 1994, Serikawa *et al*., 1996). The lowest expression of *BKn7* was detected in 2-week old roots and higher expression was seen in all the other tissues examined in the experiment.

Northern blot data indicated that *BKn1* gene expression was mainly present in the stem and the inforescence and *BKn7* gene expression was present at different levels in all tissues tested.

### 3.1.2 RT-PCR analysis

To investigate *BKn1* and *BKn7* gene expression by RT-PCR, 3$\mu$g of total RNA from various barley tissues were digested with DNase I to avoid genomic DNA contamination and transcribed with reverse transcriptase. *BKn1* and *BKn7* gene-specific primers (see Material and Methods) were used for cDNA amplification. Amplification of *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) cDNA by using primers specific for the barley *GAPDH* gene was applied as an internal control for the quantification of the reaction.

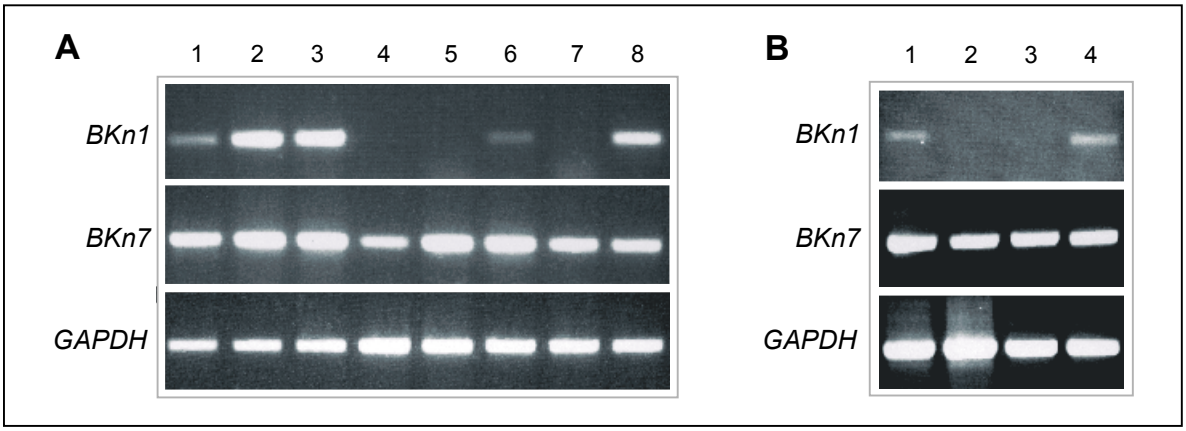



**Fig. 3-3 RT-PCR analyses of *BKn1* and *BKn7* expression in different barley tissues.** (A) cDNAs were synthesized from 3$\mu$g of total RNA isolated from mature embryos (lane 1), 4-week old shoot apices (lane 2), 6-week old shoot apices (lane 3), 2-week old roots (lane 4), 2-week old fully expanded leaves (lane 5), 5-day old seedling roots (lane 6), 5-day old fully expanded seedling leaves (lane 7) and inflorescences at all stages (lane 8) and amplified with *BKn1*, *BKn7* and *GAPDH* gene-specific primers. (B) cDNAs were synthesized from 3$\mu$g of total RNA isolated from palea (lane 1), lemma (lane 2), anther (lane 3) and stigma (lane 4) of fully developed florets and amplified with *BKn1*, *BKn7* and *GAPDH* gene-specific primers.

Fig. 3-3A indicates that, the highest level of *BKn1* expression was detected in shoot apices, relatively high level in inflorescences and the lowest level in mature embryos and seedling roots. *BKn1* expression in embryos detected by RT-PCR, compared to Northern blot, was probably due to the higher sensitivity of this technique. As observed by Northern blot analysis, the expression of *BKn7* could be detected in all tissues examined by RT-PCR.

In developed florets, RT-PCR revealed that *BKn1* gene expression was present in palea and stigma tissues at very low levels and *BKn7* was expressed in all floral organs at significant levels (Fig. 3-3B).

Taken together, the results from Northern blot and RT-PCR analyses indicated that *BKn1* expression is restricted to meristematic tissues, including the embryo, vegetative and reproductive apices and the stem, while *BKn7* is expressed throughout the life cycle of the barley plant.

### 3.1.3 *In situ* hybridization

To determine the spatial patterns of *BKn1* and *BKn7* gene expression during different stages of barley development, *in situ* hybridization was conducted with digoxigenin-labelled antisense RNAs as probes (see Material and Methods).

#### 3.1.3.1 *In situ* localization of *BKn1* mRNA in barley mature embryos

The formation of shoot and root meristems that ultimately give rise to all tissues of the plant body occurs for the first time during embryogenesis. In order to determine whether *BKn1* is expressed in the shoot meristem from its earliest inception, the expression of *BKn1* was examined by *in situ* hybridization in barley mature embryos.

The morphology of barley mature embryos is illustrated in Fig. 3-4. The coleoptile encloses the shoot apical meristem with its 4 to 5 leaf primodia. The root and its cap are enclosed in the coleorhiza. A procambial system interconnects the shoot and the root. The procambium is elaboratedly branched in the scutellum, the single cotyledon of the embryo and a shieldlike structure partly enclosing the embryo axis and the epicotyl.

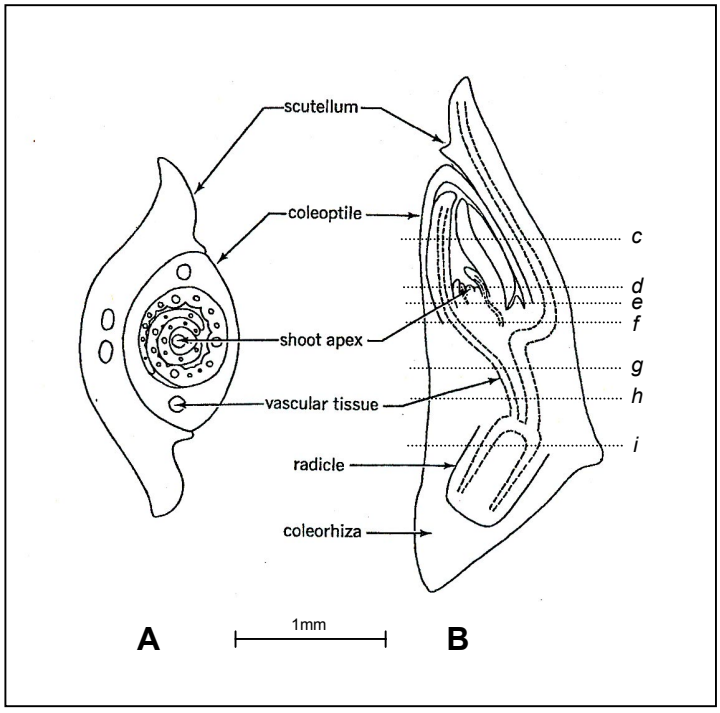



**Fig. 3-4 The schematic representation of a transverse (A) and a longitudinal (B) sections of a barley mature embryo.** (adapted from Merry, 1981) The procambium is outlined with dashed lines. Lines c, d, e, f, g, h and i indicate an approximate plane of the transverse sections shown in Fig. 3-5 C, D, E, F, G, H and I, respectively.

In a longitudinal section of a mature embryo shown in Fig. 3-5A, *BKn1* mRNA is abundant in the shoot meristem, leave primordia and the subjacent shoot axis. An enlarged view of the shoot portion (Fig. 3-5B) shows that the strong signals were detected in both the L1 layer (tunica) of the meristem and leaf primordia. It is noteworthy that *BKn1* expression in embryonic shoot meristem is not like those of class I *Knox* genes from maize and rice, for

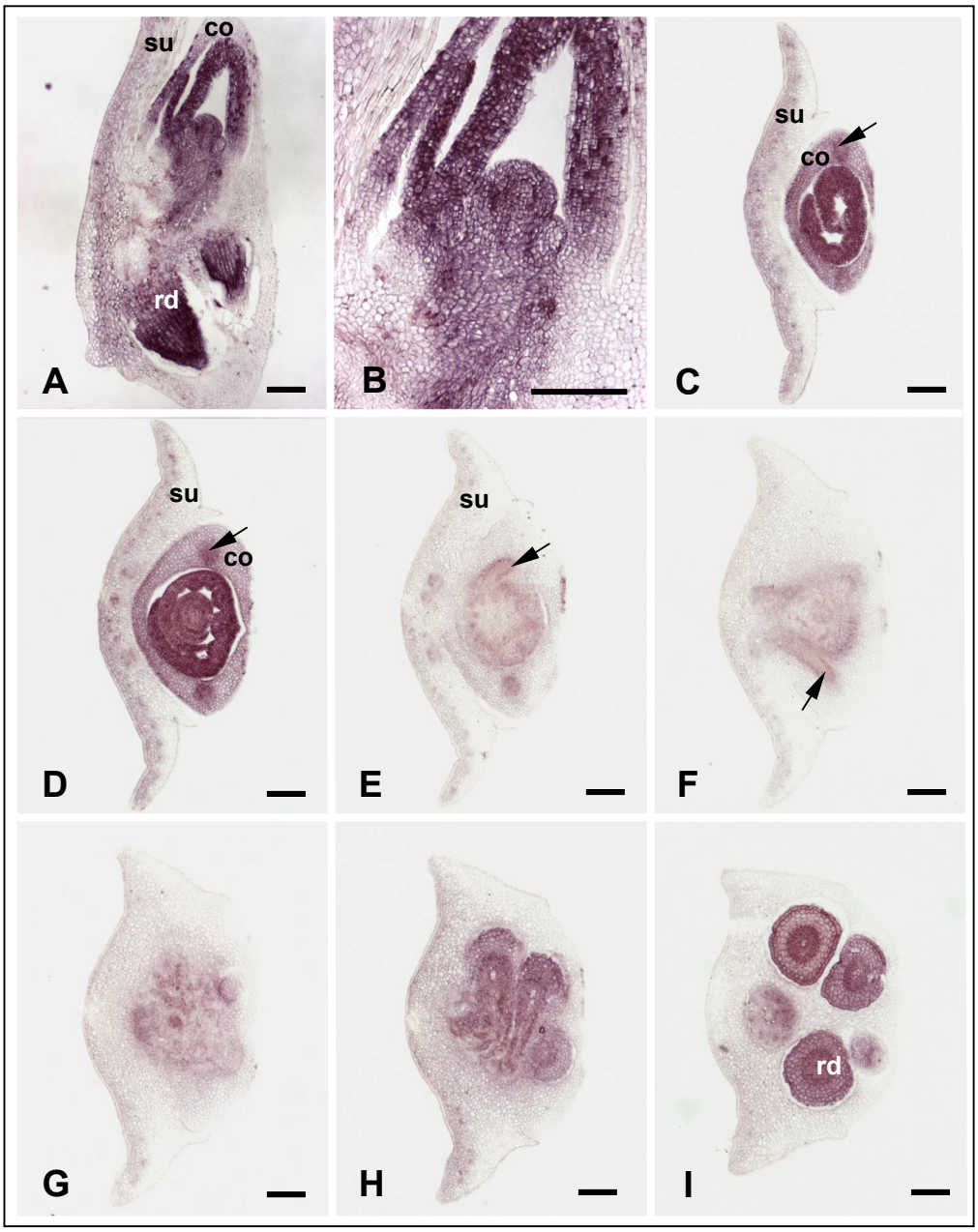



**Fig. 3-5 *In situ* localization of *BKn1* mRNA in barley mature embryos.** (A) A median longitudinal section of a mature embryo probed with a DIG-labelled *BKn1* antisense probe. Hybridization was visualized as the dark purple coloured product of the alkaline phosphatase reaction. (B) Higher magnification of the shoot portion of the embryo in (A). (C-I) A series of transverse sections of a barley mature embryo, sectioned sequentially from the apical end (c) to the basal end (i) in Fig. 3-4B, respectively, probed with a DIG-labelled *BKn1* antisense probe. su, scutellum; co, coleoptile; rd, radicle. Arrows indicate the procambial strand in the coleoptile. Bars, 200$\mu$m.

instance, *Kn1* (Simth *et al*., 1995), *OSH1* (Sato *et al*., 1996) and *OSH15* (Sato *et al*., 1998), as it is not suppressed in L1 layer and young leaf primodia. In the primary and adventitious roots of the embryo, *BKn1* expression was also detected by the dark purple coloured signals (Fig. 3-5A).

To constitute a three-dimensional image of *BKn1* expression in the embryo, a series of transverse sections of a mature embryo was examined. Lines c, d, e, f, g, h and i illustrated in Fig. 3-4B indicate the approximate planes of the transverse sections shown in Fig. 3-4 C, D, E, F, G, H and I, respectively. In Fig. 3-4 C, *BKn1* expression was observed in young leaf primordia and procambial strands in the coleoptile and the scutellum. In Fig. 3-4 D, signals could be seen in the shoot meristem, leave primordia and the procambium. Fig. 3-4 E, F, G and H showed that *BKn1* expression was detected throughout the procambial system interconnecting the shoot and the roots. At the anterior face of the embryo, signals were present in the primary and adventitious roots (Fig. 3-4 H).

*BKn1* expression, as revealed by *in situ* hybridization, was localized to the shoot, the roots and the procambial system in mature embryos. The expression of *BKn1* in the embryo was not specific for the shoot meristem as those of *Kn1* and *OSH1* (Smith *et al*.,1995; Sato *et al.*, 1996).

### 3.1.3.2 *In situ* localization of *BKn1* mRNA in barley vegetative shoot apices

Since *BKn1* is expressed in the embryonic shoot apex it was interesting to persue its expression pattern in the shoot apex after embryogenesis.

Fig. 3-6A presents an example for *BKn1* expression in a median longitudinal section through a shoot apex of a seedling at 5 days after germination. At this stage the meristem has initiated approximately five to six young leaf primodia. The pattern of *BKn1* expression in the shoot portion is very similar to that described previously for the mature embryo; its mRNA can be detected in the meristem (including the L1 layer), young leaf primordia, very young leaves and developing tissues in the stem. Higher magnification of the shoot apex revealed that unlike often observed in class I *Knox* genes, such as *Kn1* from maize (Jackson *et al*., 1994), *OSH1* from rice (Sentoku *et al*., 1999) and *STM* from *Arabidopsis* (Long *et al*., 1996), *BKn1* expression could be seen in all young leaf primordia (Fig. 3-6B). The signals were also observed in an adventitious root primordium at the base of the sixth leaf primodium (indicated by arrows in Fig. 3-6A). The longitudinal sections hybridized with an unrelated gene probe (lambda probe) did not show any signals higher than background (Fig. 3-6E).

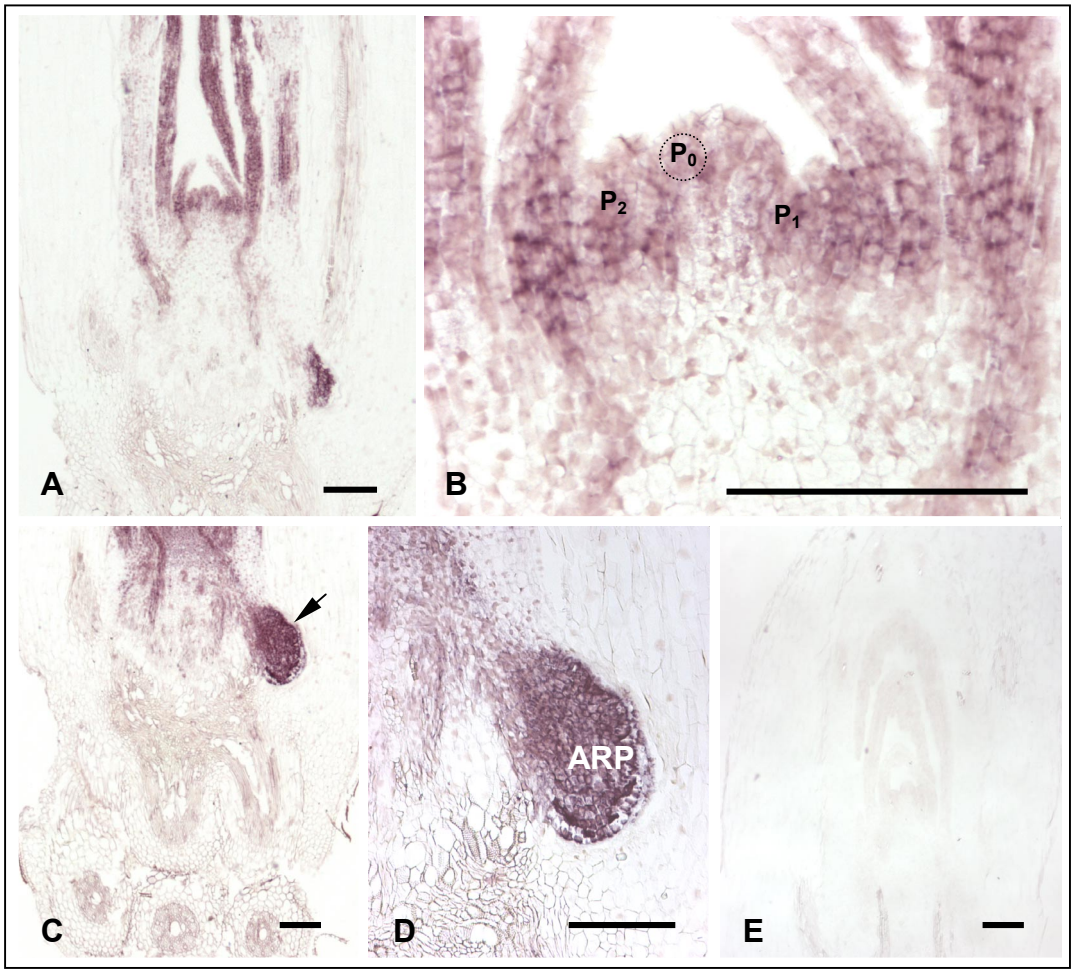



**Fig. 3-6 Expression of *BKn1* in barley vegetative shoot apices.** (A) A median section through a 5-day old seedling shoot apex, probed with a *BKn1* antisense RNA probe. (B) Closeup of the shoot apex in (A), surrounded by $P_0$-$P_2$ leaf primordia. (C) A longitudinal section through the base of the seventh leaf from the meristem, showing the developing adventitious root primordium (arrowed). (D) Higher magnification of the adventitious root primordium (ARP) in (C). (E) A longitudinal section of shoot apex hybridized with an unrelated probe (lambda probe) as a negative control. Bars, 200$\mu$m.

### 3.1.3.3 *In situ* localization of *BKn1* mRNA during barley floral development

Since both Northern blot and RT-PCR analyses revealed *BKn1* expression in barley inflorescences, *in situ* hybridization was also performed to examine *BKn1* mRNA localization in longitudinal and transverse sections of inflorescences representing different developmental stages.

Fig. 3-7 diagrammatically illustrates stages of barley inflorescence development. The vegetative apical meristem elongates to form inflorescence meristems after initiating a full complement of leaves (Fig. 3-7A-C). Each spikelet primordium will give rise to spikelets, two lateral and one median spikelets (Fig. 3-7D). The meristem of each spikelet primordium then initiates a pair of glumes (Fig. 3-7E), followed by lemma (Fig. 3-7F), stamen (Fig. 3-7G), palea, lodicule, carpel, rachilla and awn (Fig. 3-7H).

Inflorescences at the awn primordium stage display undifferentiated apical florets and young florets with differentiated organs at more basal regions (Fig. 3-7F). White anther stage inflorescences (Fig. 3-7I) allow expression analysis of further developed florets with further differentiated organs. Thus the investigation of barley ears at these two stages permits an expression analysis at flower developmental stages ranging from the floral meristem via florets with all organ primordia to florets with almost fully differentiated organs.

Fig. 3-8A shows a longitudinal section of a barley inflorescence at the awn primordium stage hybridized with a DIG-labelled *BKn1* antisense RNA probe. *BKn1* expression appeared in floret meristems and developing vascular tissues along the inflorescence stem. Transcripts were present uniformly in the floret meristem, including the tunica (Fig. 3-8B) and the region of developing glume primordia (Fig. 3-8C). At awn primordium stage, *BKn1* mRNA accumulation was observed in the floret meristem, all the floral organ primordia and vascular tissues (Fig. 3-8D). In a longitudinal section of an inflorescence at the white anther stage *BKn1* expression was localized in the developing floral organs and vascular tissues (Fig. 3-8E, F). In the transverse section of an inflorescence at the awn primordium stage, signals were detectable in all floral organ primordia and the developing vascular tissue of the inflorescence stem (Fig. 3-8G, H).

In summary, in barley inflorescences *BKn1* is expressed in floret meristem, including tunica, all floral organ primordia, developing floral organs and vascular tissuses. This result is in good agreement with the expression pattern of *BKn1* observed in embryonic shoot apices and seedling shoot apices (see Fig. 3-5 and Fig. 3-6).

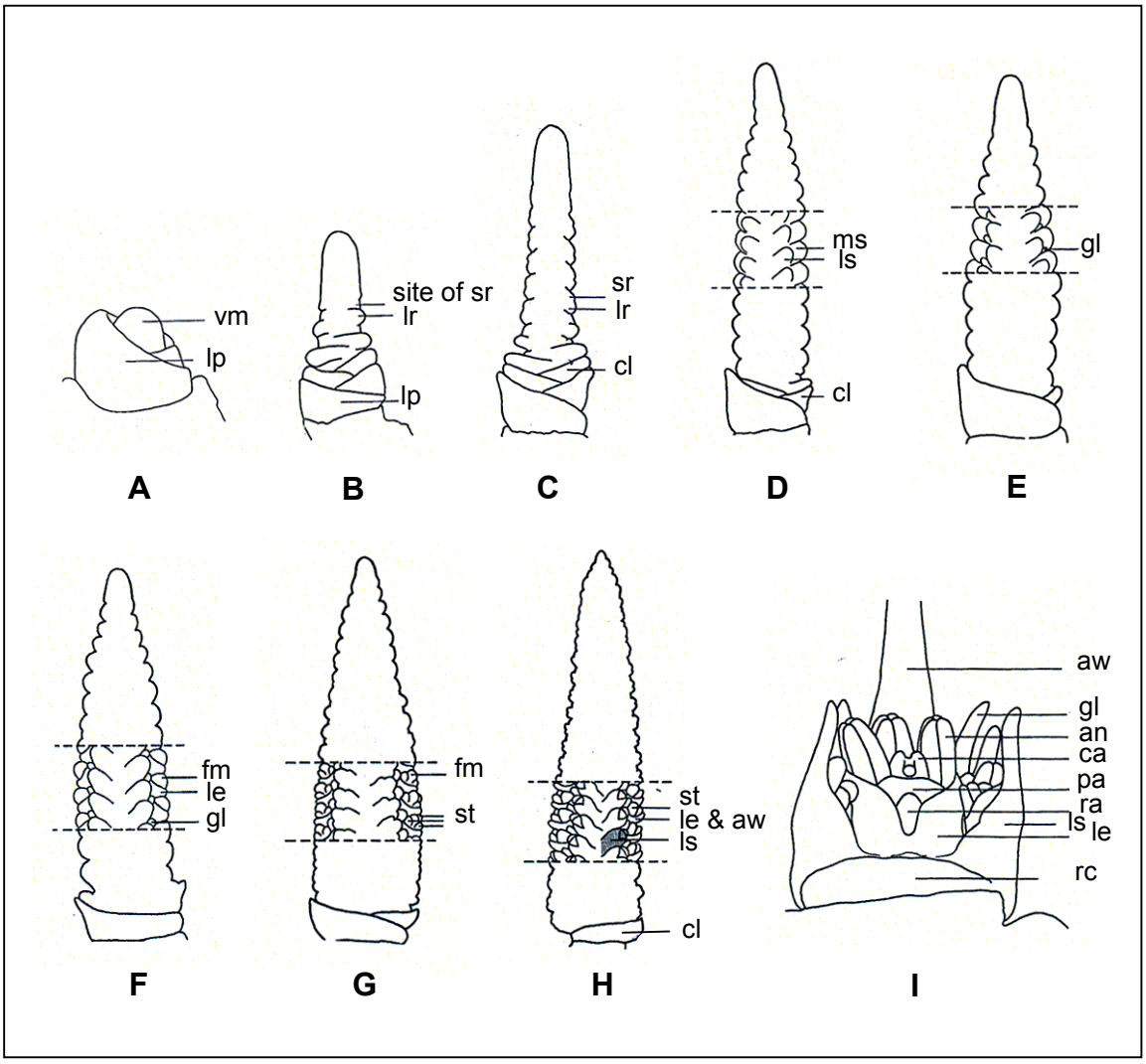



**Fig. 3-7 Diagrammatic representation of barley inflorescence development (adapted from Kirby and Appleyard, 1984).** (A) The vegetative meristem (vm) with leaf primordia (lp). (B) Late stage of vegative meristem. A spikelet ridge (sr) develops in the region immediately above each leaf ridge (lr). (C) Floral initiation at the double ridge stage. A spikelet primordium ridge (sr) appears above a leaf primordium ridge (lr).cl, collar. (D) Inflorescence meristem at the triple mound stage. A spikelet primordium differentiated into three distinct mounds. The central mound will form the median spikelet (ms) while the two mounds flanking it will become the lateral spikelets (ls). (E) Glume primordium stage. Spikelet meristems initiate a pair of bracts called glumes (gl). (F) Lemma primordium stage. Another bract, lemma (le), is initiated. fm, floret meristem. (G) Stamen primordium stage. Three stamen primordia are initiated. (H) Awn primordium stage. The ear has its full complement of spikelet primordia. aw, awn. (I) A spikelet at white anther stage cut through its rachis (rc). The awn and palea (pa) of the median floret surround the anthers and carpel. ra, rachilla.

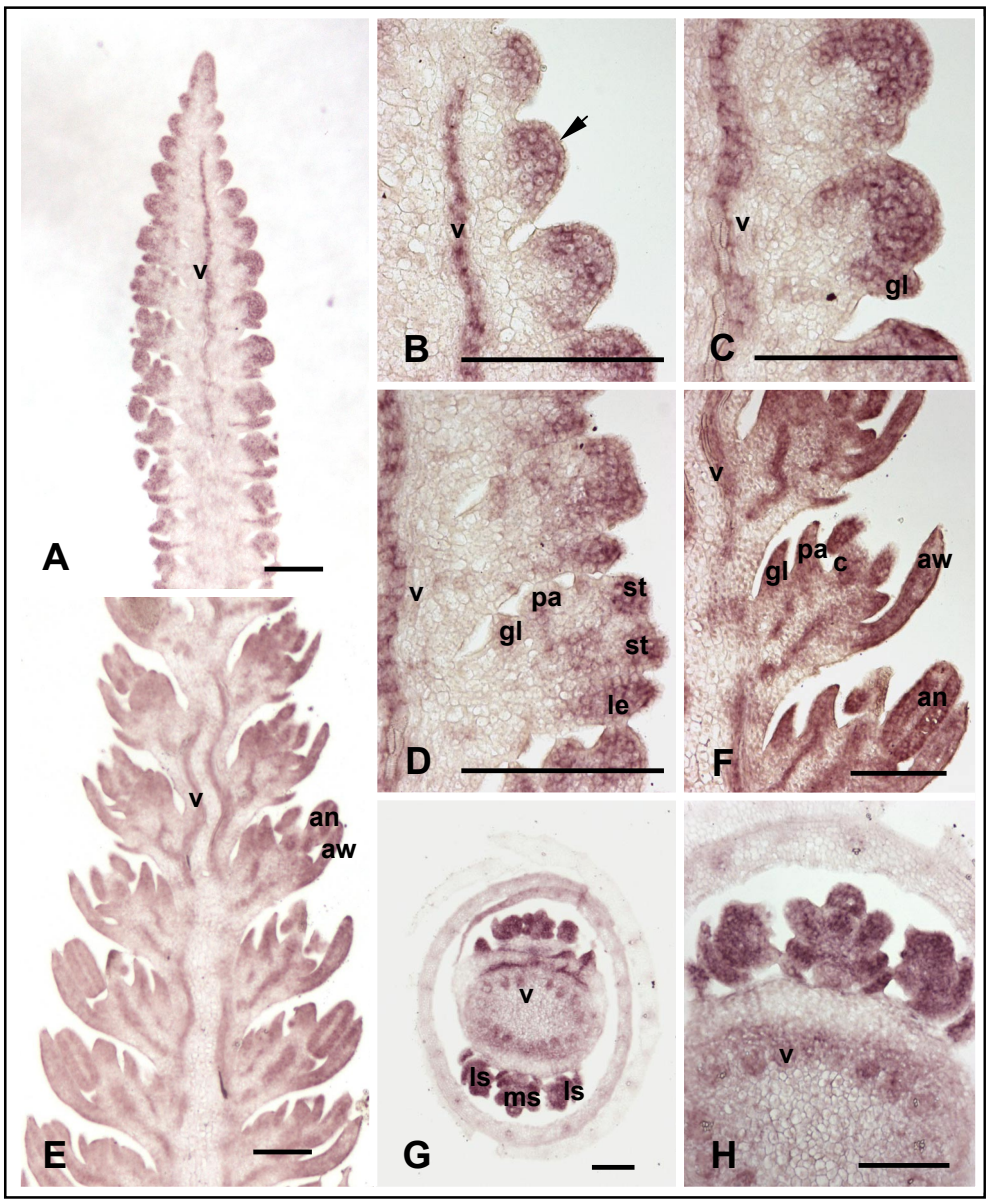



**Fig. 3-8 Spatial expression pattern of *BKn1* mRNA in barley inflorescences.** (A) A longitudinal section of a inflorescence at the awn primordium stage. The *BKn1* transcript was detected in floret meristems, floral organ primordia and the vascular system (va). (B) A longitudinal section through three spikelet meristems on the flank of the inflorescence, showing *in situ* localization of *BKn1* mRNA, note strong staining in the tunica (arrowed), corpus and the region where the glume primordiua are going to initiate on the flanks of the meristems. (C) A longitudinal section through spikelet meristems with glume primordia (gl). (D) A longitudinal section through florets showing the primordia of the lemma (le), the palea (pa) and the stamen (st). (E) A median longitudinal section through the axis of inflorescence at the white anther stage. an, anther; aw, awn. (F) A longitudinal section through spikelets at the white anther stage. ca, carpel. (G) A transverse section of an ear at the awn primordium stage. ms, median spikelet; ls, lateral spikelet. (H) Higher magnification of the transverse section in (G), showing the expression of *BKn1* in all the floral primordia and the vascular tissue. Bars, 200$\mu$m.

### 3.1.3.4 *In situ* localization of *BKn1* mRNA in barley stems

Northern blot analysis showed that *BKn1* was strongly expressed in the stem (Fig. 3-2A). Therefore, *in situ* hybridization was performed to precisely localize the expression of *BKn1* in barley stems.

Stem sections for *in situ* hybridization were obtained from barley plants at the eighth leaf mid-expansion stage. At this stage, the eighth leaf blade was at mid-expansion stage, whereas the ninth leaf, although already emerged, had not commenced expanding. Young stems just below the shoot apex were taken for analyzing the localization of *BKn1* mRNA.

In a longitudinal section of a barley stem shown in Fig. 3-9A, several nodes and internodes were visible, and leaves were inserted at the nodes. The outermost leaf sheath is of the eighth leaf (L8) and the inner layer is of the newly emerged ninth leaf (L9). L9 and L8 are inserted at the plates of node 9 (N9) and node 8 (N8), respectively. Internode 10 and 9 are positioned above and below the N9 respectively. *BKn1* transcripts were detected in procambial strands which connect internodes, nodes and young leaves. In internodes procambial strands are oriented in a parallel and a longitudinal manner, whereas in node 9 and 8, they are oriented transversely, forming a complicated network where they merge with the procambial strands from leaves 9 and 8. Weak expression of *BKn1* gene was also observed in rib meristem zones (RM) which locate in the central portion of internode 10 and 9. These rib meristem zones could be distinguished by short columns of cells in which the individual series were usually three or four cells in length (Fig. 3-9A'). In the rib meristem zone, repeated cell divisions in parallel series of cells and enlargement of the derivatives of these divisions largely account for internodal elongation.

Fig. 3-9 (B-H') shows a serial transverse stem sections selected at different levels below the shoot apex. In the section cut through internode 10 just above the insertion of leaf 9, the expression of *BKn1* appeared as a ring which might represent the rib meristem zone of internode 10 (Fig. 3-9B, B'). In internode 10, the vascular strands are not much differentiated. In the section cut though node 9 where the procambial strands from leaf 9 merge with those in the stem, the hybridization signals were observed in the transversely oriented procambial strands (Fig. 3-9C). Observations at a higher magnification revealed that *BKn1* transcripts were not detectable in the differentiated vascular bundles but in the cells flanking the vascular bundles (Fig. 3-9C'). In sections cut through internode 9, the hybridization signals were present in the cells flanking the vascular bundles of the stem (Fig. 3-9D, D', E, E'). In the section cut through internode 9 just above the insertion of leaf 8, more differentiated vascular bundles around the internode became visible (Fig. 3-9F). The expression of *BKn1* was restricted to the flanking cells of vascular bundles and part of the cortex parenchyma which might retain meristematic activity (Fig. 3-9F'). In two sections cut through node 8 and

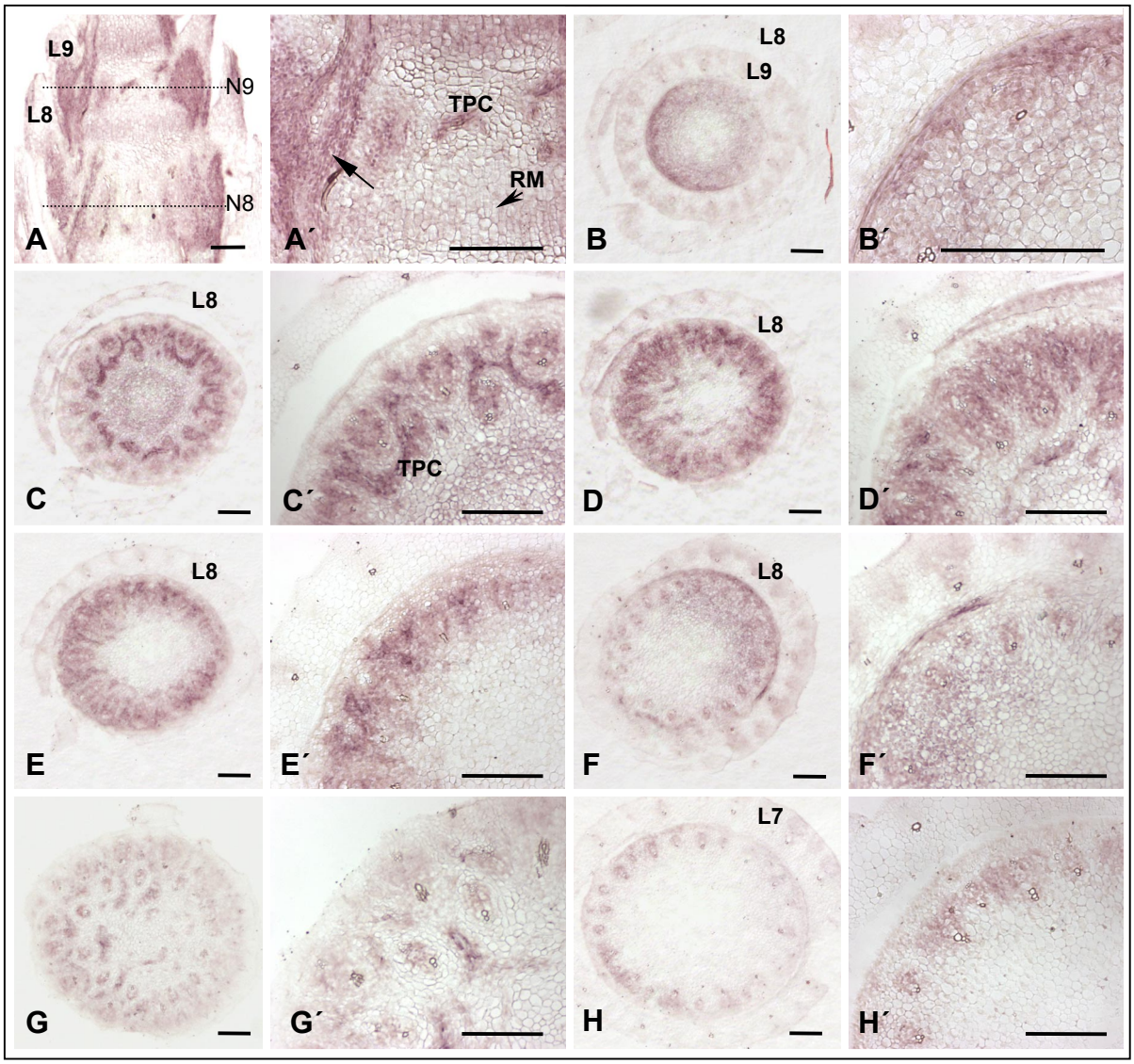



**Fig. 3-9 Localization of *BKn1* mRNA in barley stems.** Longitudinal and transverse sections of the young stem from barley plants at the eighth leaf mid-expansion stage were hybridized with a DIG-labelled antisense *BKn1* RNA probe. (A) A longitudinal section of the stem just below the shoot apex. The dotted lines indicate the plates of node 9 (N9) and node 8 (N8), respectively. (A´) Internode 10, node 9 and internode 9 of the stem section in (A) are shown at higher magnification. The arrow indicates a procambial strand. (B-H) Serial transverse stem sections selected at different levels below the shoot apex. Transverse sections cut through internode 10 just above the insertion of leaf 9 (B), node 9 (C), at level of upper portion of internode 9 (D), at level of lower portion of internode 9 (E), internode 9 just above the insertion of leaf 8 (F), node 8 (G) and internode 8 (H). (B´), (C´), (D´), (E´), (F´), (G´), and (H´) are higher magnification of sections shown in (B), (C), (D), (E), (F), (G), and (H), respectively. L7, the leaf sheath of the 7th leaf; L8, the leaf sheath of the 8th leaf; L9, the leaf sheath of the 9th leaf; TPC, transverse procambial strand; RM, rib meristem zone. Bars, 200$\mu$m.

internode 8, the expression of *BKn1* was weak and restricted to a few cells around the differentiated vascular bundles (Fig. 3-9G, G', H, H').

Thus, *BKn1* mRNA is located in the procambial strands of young barley stems, in the cells flanking the differentiated vascular bundles and in the rib meristem zone of internodes.

*In situ* localization of *BKn7* mRNA in barley inflorescences at the white anther stage was conducted using two *BKn7* gene-specific probes (see Material and Methods). It took four-fold longer incubation period to get signals. As observed for *BKn1* mRNA in the inflorescence, *BKn7* mRNA accumulation was detected in floral meristems, developing floral organs and vascular tissues of the inflorsecnce stem (data not shown). Localization of *BKn7* mRNA in barley mature embryos and vegatitive apices was further analyzed by Judith Müller and Rainer Franzen. The expression pattern of *BKn7* observed in these two tissues was similar to that of *BKn1* (Müller *et al*., 2001).

## 3.2 Characterization of cDNAs encoding DNA-binding proteins that interact with the 305bp intron sequence of *BKn3*

Four cDNA clones encoding DNA-binding proteins that interact with the 305bp intron sequence of *BKn3* gene were isolated in the yeast one-hybrid screening (Kai Müller, unpublished data). They are *BEIL* (Barley ethylene insensitive like), *BAPL* (Barley apetala2 like), *BBR* (Barley brain) and *BGRF* (Barley growth regulating factor), named after their homologues in other species (see details in section 3.2.2). To characterize these cDNA clones, their genomic and full-length cDNA sequences were determined, their genomic organization and their copy numbers in the barley genome were examined.

### 3.2.1 Isolation of genomic sequences

To obtain the sequence information necessary for full-length cDNA sequences, the isolation and sequencing of genomic clones of *BEIL*, *BAPL*, *BBR* and *BGRF* was required. The isolation of genomic sequences should also allow the cloning of their promoters, which are important for the *in vivo* characterization of the expression patterns of these genes. Furthermore, the sequence information can be very useful for mapping these genes by single nucleotide polymorphism (SNP) analysis.

To isolate the genomic clones of *BEIL*, *BBR*, *BAPL* and *BGRF*, a λEMBL3 genomic library prepared from barley mutant *Calc15* inflorescences was screened using the longest cDNA clones obtained from one-hybrid screening as probes. Among 2 million λ clones plated, 3 positive clones were obtained for *BEIL*, 5 clones for *BBR*, 3 clones for *BAPL* and 6 clones for *BGRF*.

In order to isloate the inserts of positive clones which might contain the genomic sequences of four genes, λ DNA of these clones was restricted with different enzymes, and the resulting fragments were analysed by Southern blot. Fig. 3-10 shows the analysis of two *BEIL* λ clones as an example. The probes used were identical to those prepared for the screening of the genomic DNA library. Fragments in the range of 2 - 6kb showing strong hybridizing signals were subcloned into pBluescript KS+ and fully sequenced in both directions.

Assembling of the sequence data from each subclone resulted in a sequence of 6870bp for *BEIL*, of 7834bp for *BAPL*, of 4957bp for *BBR* and of 5203bp for *BGRF*. All genomic sequences are shown in the appendix.

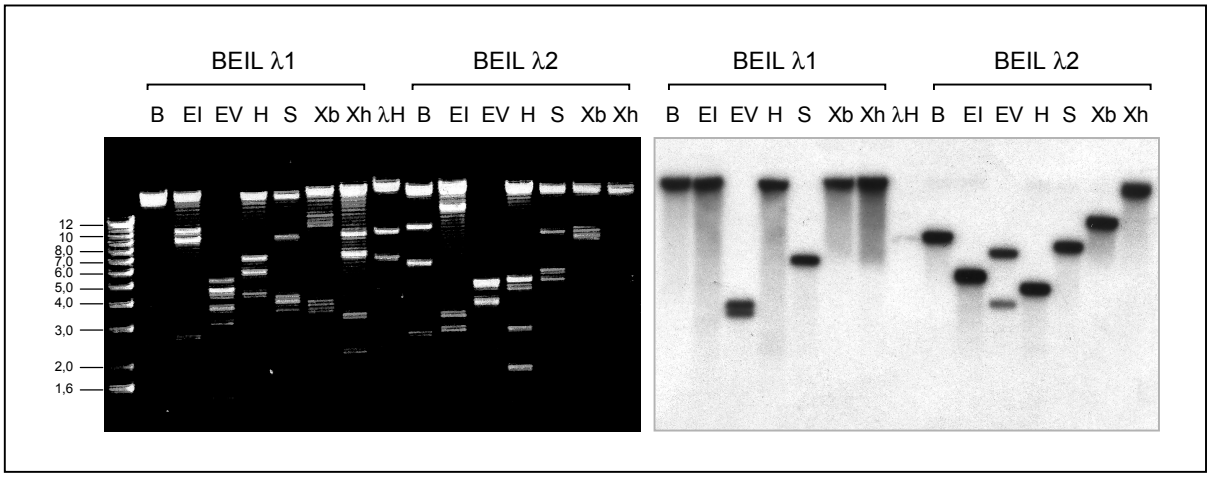



**Fig. 3-10 Analysis of the inserts of two *BEIL* λ clones.** (A) Restriction analysis of *BEIL* λ1 and λ2 with *Bam*HI (B), *Eco*RI (EI), *Eco*RV (EV), *Hind*III (H), *Sal*I (S), *Xba*I (XB) and *Xho*I (XH) restriction enzymes in 0.7% agarose gel stained with ethidium bromide. Lane λH is λ DNA digested by *Hind*III as a size marker. At the left side DNA size is marked in kilobases. (B) Southern blot using the longest *BEIL* cDNA fragment obtained from the yeast one-hybrid screening as a probe.

### 3.2.2 Isolation of full-length cDNA sequences

#### *BEIL*

The longest *BEIL* cDNA clone obtained from the yeast one-hybrid screening comprises 2218bp and the longest ORF corresponds to 505 amino acid residues with a predicted molecular mass of 55kDa (Kai Müller, unpublished data). This is likely to be the complete coding region, since several stop codons occur in frame 5' to the putative translation initiation codon ATG and the *BEIL* transcript detected by Northern blot in different barley tissues is about 2.2 kb in length (section 3.3.1 and Fig. 3-17A). In addition, a comparison of amino acid sequences with other EIN3/EIL (Ethylene insensitive 3/Ethylene insensitve 3 like) related proteins isolated from different plants (Fig. 3-11), such as EIN3, EIL1, 2, 3 from *Arabidopsis* (Chao *et al*., 1997) and TEIL from tobacco (see comment of Genbank AB015855), suggests that the longest cDNA clone is full-length. Clear consensus sequences for polyadenylation signals (AATAAA/T) are not present in 3' untranslated region. However, in many plant genes

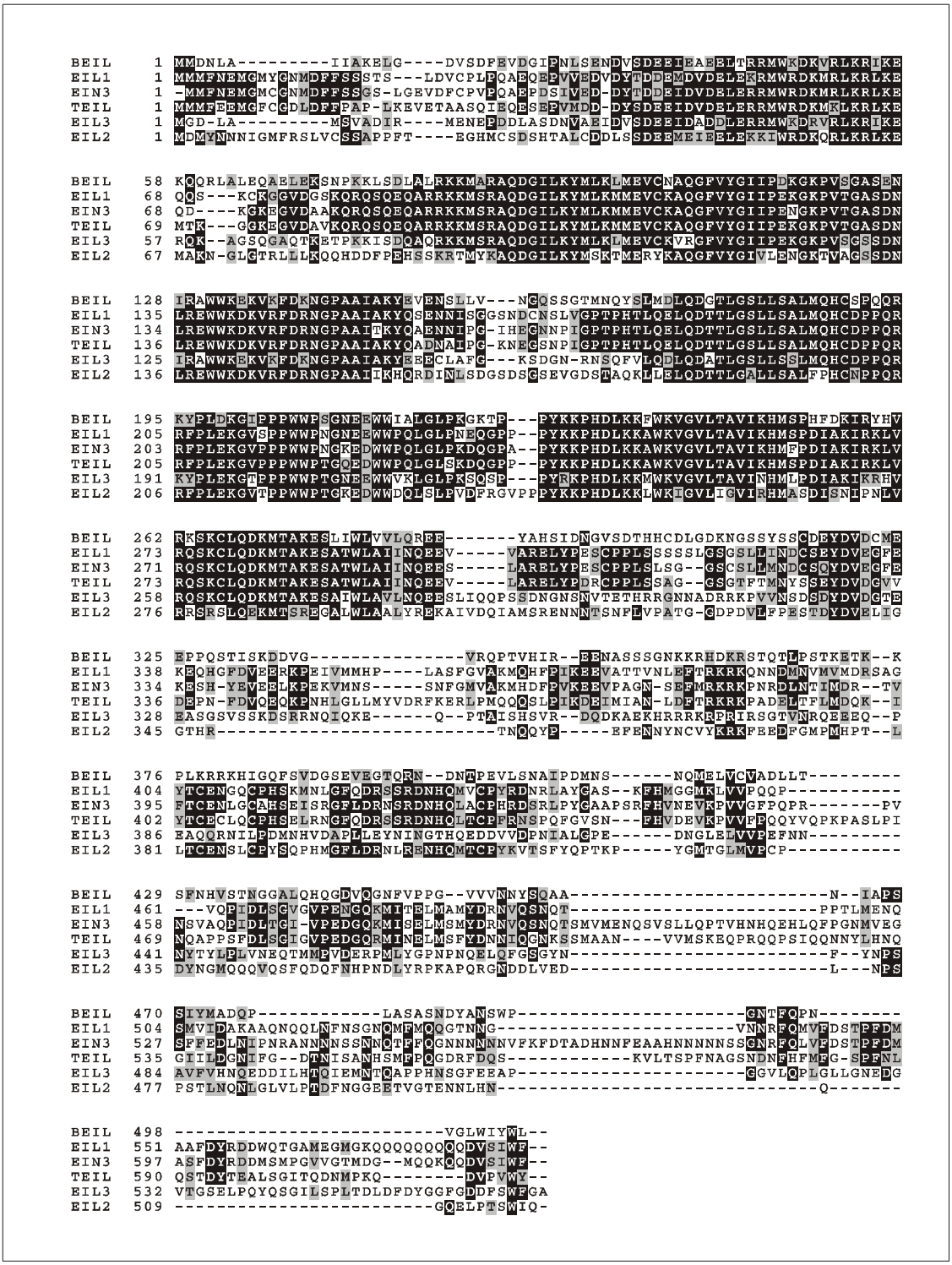


**Fig. 3-11 Amino acid sequence alignment of the BEIL protein compared with other members of the EIN3/EIL (Ethylene-insensitive 3/ethylene-insensitive 3 like) family.** The BEIL sequence is compared with EIL1 (Acession number, AF004213), EIL2 (AF004214), EIL3 (AF004215), EIN3 (AF004217), and TEIL (AB015855). Identical residues conserved among at least three sequences are displayed in reverse type and similar residues are in grey boxes. Gaps introduced to improve the alignment are indicated by dashes. The sequence alignment was done using the Multisequence alignment program. The boxes were drawn using BOXSHADE.

such signals appear diffuse, and it has not been possible to define a single, universal poly(A) signal (Rothnie, 1996).

An initial database search for related protein sequences revealed similarity between the BEIL and a number of other isolated EIN/EIL proteins (Fig. 3-11), including a small gene family from *Arabidopsis* containing four EIN3/EIL proteins (Chao *et al*., 1997) and TEIL protein from tobacco (see comment of Genbank AB015855). The similarity is most prominent within the 200 amino acids at the N-terminus of the sequences, where all EIN/EIL proteins are 82% or more identical (Fig. 3-11). This conservation might reflect the functional significance of this N-terminal sequences.

***BAPL***

The *BAPL* cDNA clone obtained from yeast one-hybrid screening comprises 1527 nucleotides and its largest ORF encodes a protein of 359 amino acid residues with a predicted molecular

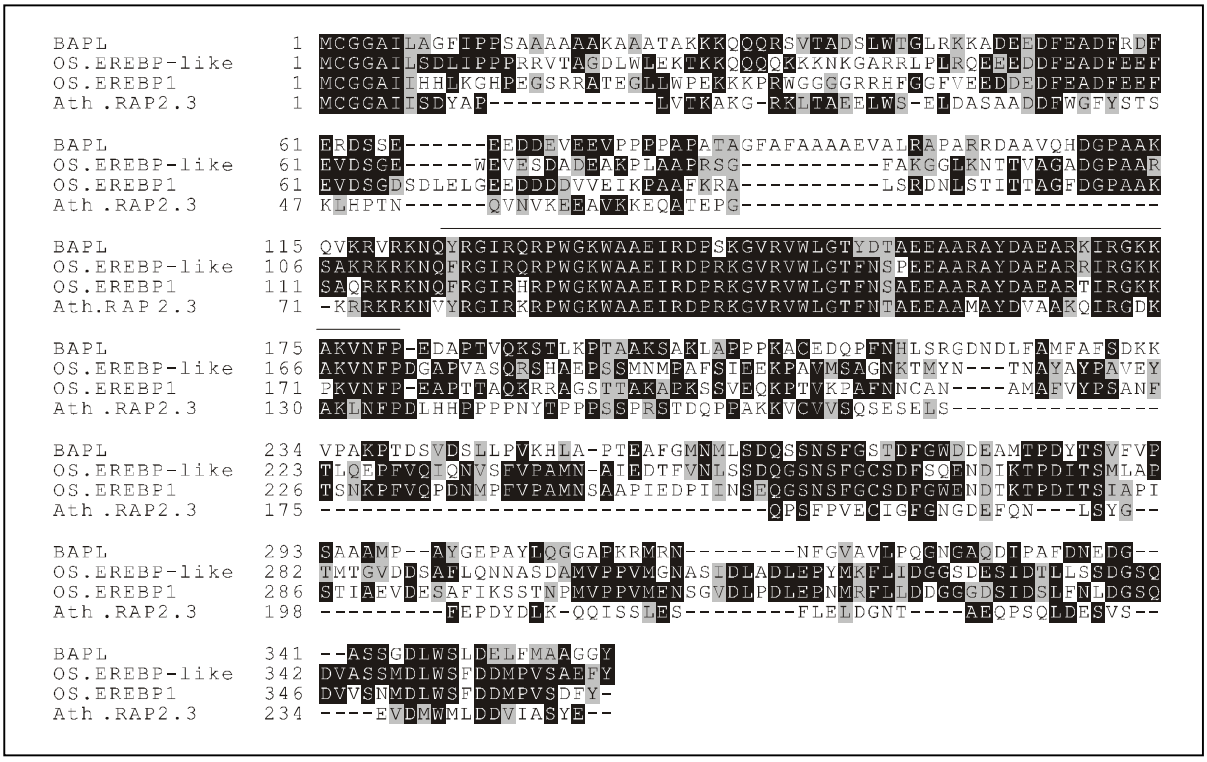



**Fig. 3-12 Amino acid sequence alignment of the BAPL protein with other members of EREBP/AP2 (ethylene response element binding protein/Apetala 2) family.** BAPL sequence is compared with EREBP-like (GenBank accession number AF193803) and EREBP1 (GenBank accession number AF190770) from rice and RAP2.3 (GenBank accession number AF003096) from *Arabidopsis*. The EREBP-like AP2 domain is marked by an overhead line.

weight of 40kDa (Kai Müller, unpublished data). The size of the *BAPL* transcript revealed by Northern blot analysis (Fig. 3-17A) and several stop codons present in frame 5' to the putative translation initiator ATG codon suggested that the cDNA clone is nearly full-length. In addition, the comparison of its amino acid sequence with those of other members of the

AP2/EREBP (Apetala2/Ethylene response element binding protein) family isolated from different plants (see Fig. 3-12) also confirmed that this cDNA clone contains the complete ORF.

Searches of the sequence databases with the predicted BAPL protein sequence identified that BAPL contains an EREBP-like AP2 domain which has been demonstrated to be sufficient for sequence-specific DNA-binding activity by *in vitro* studies (Ohme-Takagi and Shinshi, 1995). Within 57 amino acids of the EREBP-like AP2 domain, BAPL shares 82% or more similarity to two EREBP-like proteins from rice (see comments of AF193803 and AF190770) and RAP2.3 (related to AP2) from *Arabidopsis* (Okamuro *et al*., 1997) (Fig. 3-12).

### *BGRF*

The *BGRF* cDNA clone obtained from the one-hybrid screening is about 1000 nucleotides in length and does not contain an ATG start codon (Kai Müller, unpublished data). To get the

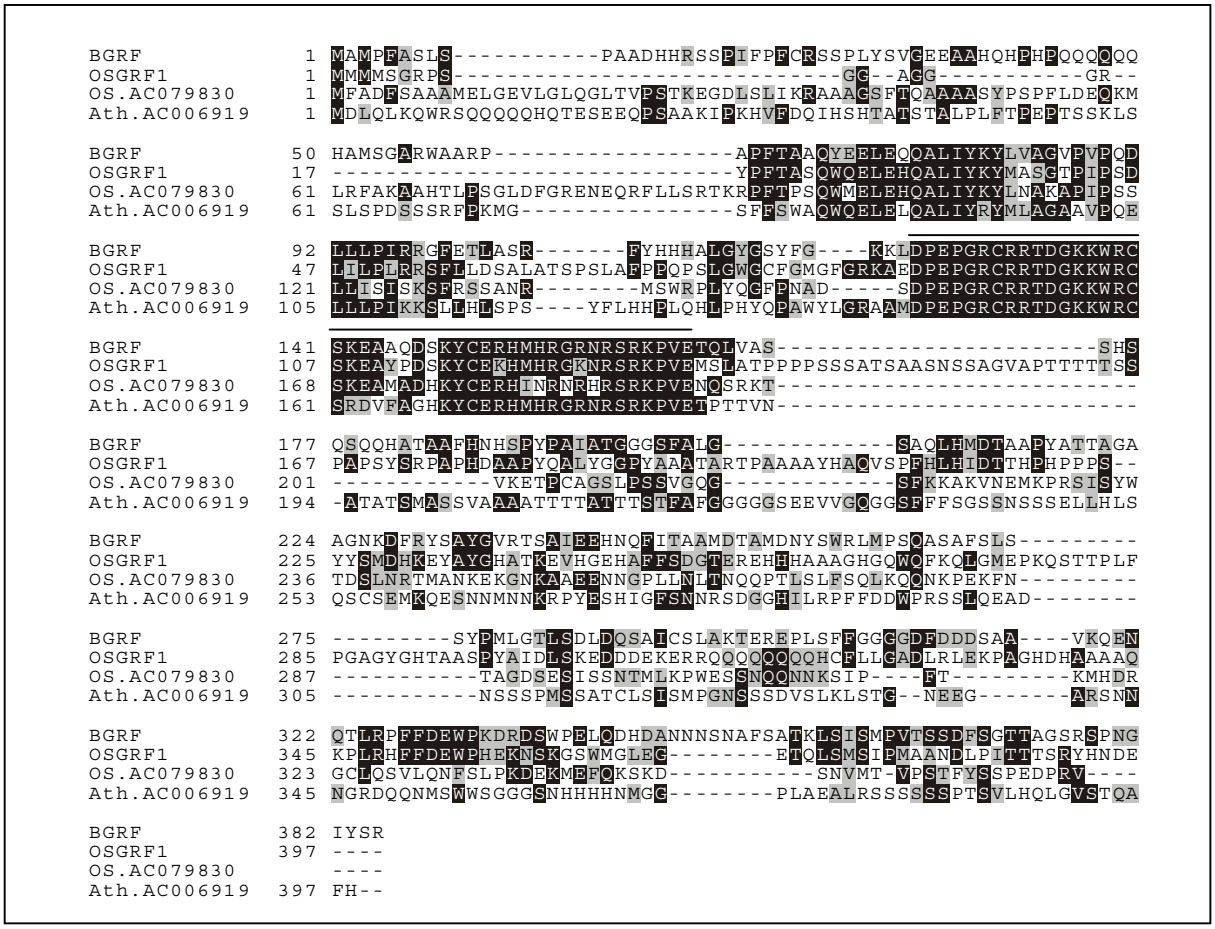



**Fig. 3-13 Amino acid sequence alignment of the BGRF protein with OSGRF1** (*Oryza sativa* growth regulating factor 1, GenBank accession number AF201895)**, a putative protein from rice** (GenBank accession number AC079830) **and a putative protein from *Arabidopsis*** (GenBank accession number AC006919)**.** The putative DNA-binding domain is marked by an overhead line.

full-length cDNA of *BGRF*, the cDNA fragment was used to screen an inflorescence cDNA library from the barley *Hooded* mutant (Luca Santi, unpublished data). The longest cDNA insert obtained from the screening comprises 1534bp and contains an ORF corresponding to 386 amino acid residues with a predicted molecular weight of 42kDa. Several stop codons present in frame 5' to the putative translation start codon ATG suggest that the cDNA clone is nearly full-length and the ORF is complete.

Database searches showed that the BGRF protein shares 58% sequence similarity to OSGRF1 (Growth Regulating Factor1, GenBank accession number AF201895) from rice (see Fig. 3-13). OSGRF1 is induced by GA and considered as a putative transcription factor (see comments in AF201895). In the center of the BGRF amino acid sequence, there is a stretch of 44 amino acids sharing 86-90% homology with OSGRF1 and other two putative proteins from rice (Genbank accession number AC079830) and *Arabidopsis* (GenBank accession number AC006919). This conserved region may represent the DNA-binding domain.

***BBR***

Comparison of the longest *BBR* cDNA clone obtained from the one-hybrid screening with the genomic sequence revealed that two additional translation start codons were present in frame upstream of the isolated cDNA sequence, suggesting that 5'end of this cDNA had not been isolated yet.

A primer extension analysis was carried out to identify the full-length cDNA sequence of the BBR gene and to localize its transcription initiation site. An oligonucleotide (see Materials and Methods) complementary to the sense strand of the BBR genomic DNA from +42 to +69 relative to the first considerate translation start site (ATG) in frame with the ORF was radiolabelled at its 5' terminus by T4 polynucleotide kinase (PNK) and [$\gamma$-$^{32}$P]ATP. The labelled primer was annealed to 10$\mu$g of total RNA isolated from young barley inflorescences (wild-type *k-Atlas*) and extended using reverse transcriptase. Electrophoresis revealed two major reverse transcription products (Fig. 3-14). The longer one was considered as the transcription initiation site of BBR, which co-migrated with a G residue in the sequencing ladder. It indicated that the transcription initiated mainly at a position which is located 18bp downstream from a putative CAAT box. The transcription initiation site was numbered +1 in the sequence indicated in Fig. 3-14. The full-length cDNA sequence obtained from primer extension experiment and the deduced amino acid sequence of the *BBR* gene is shown in Fig. 3-15A. Several acidic regions, which might represent transcription activation domains, and a short stretch of $(QH)_{14}$, were present in the predicted amino acid sequence of BBR (Fig. 3-15A).

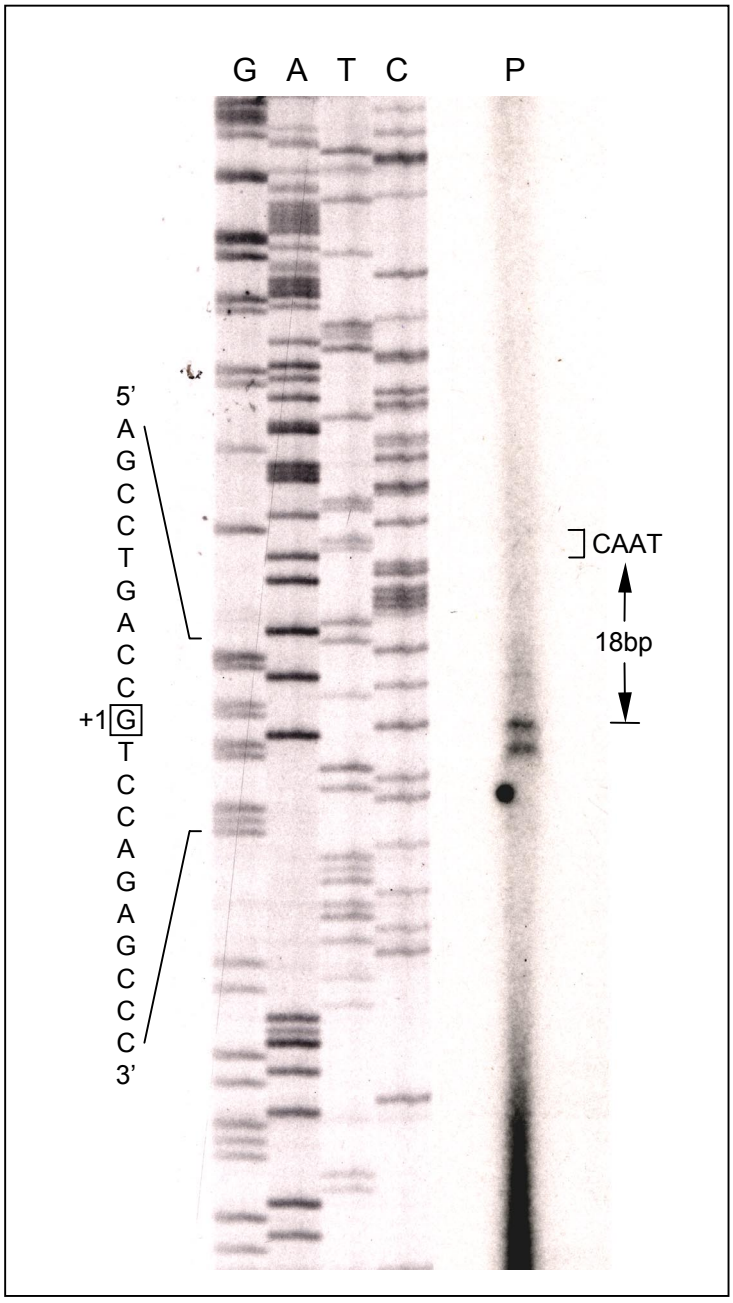



**Fig. 3-14 Primer extension analysis of the transcription initiation site of *BBR*.** A $^{32}$P-end labelled antisense primer was annealed to 10$\mu$g of total RNA isolated from barley young inflorescences (wild type *k-Atlas*) and extended using reverse transcriptase. The extended cDNA product was then analysed on a 8% sequencing gel (lane P) along with a *BBR* 5'-flanking genomic DNA sequencing ladder (G, A, T, C) generated by the same primer. The longer extension product was considered as the transcription initiation site of *BBR*. The number inside the vertical double-headed arrow on the right refers to the distance from the closest CAAT box to the primer extension product indicated by the horizontal line. The sense sequence around the product is shown on the left and the transcription initiation site is boxed and assigned +1.

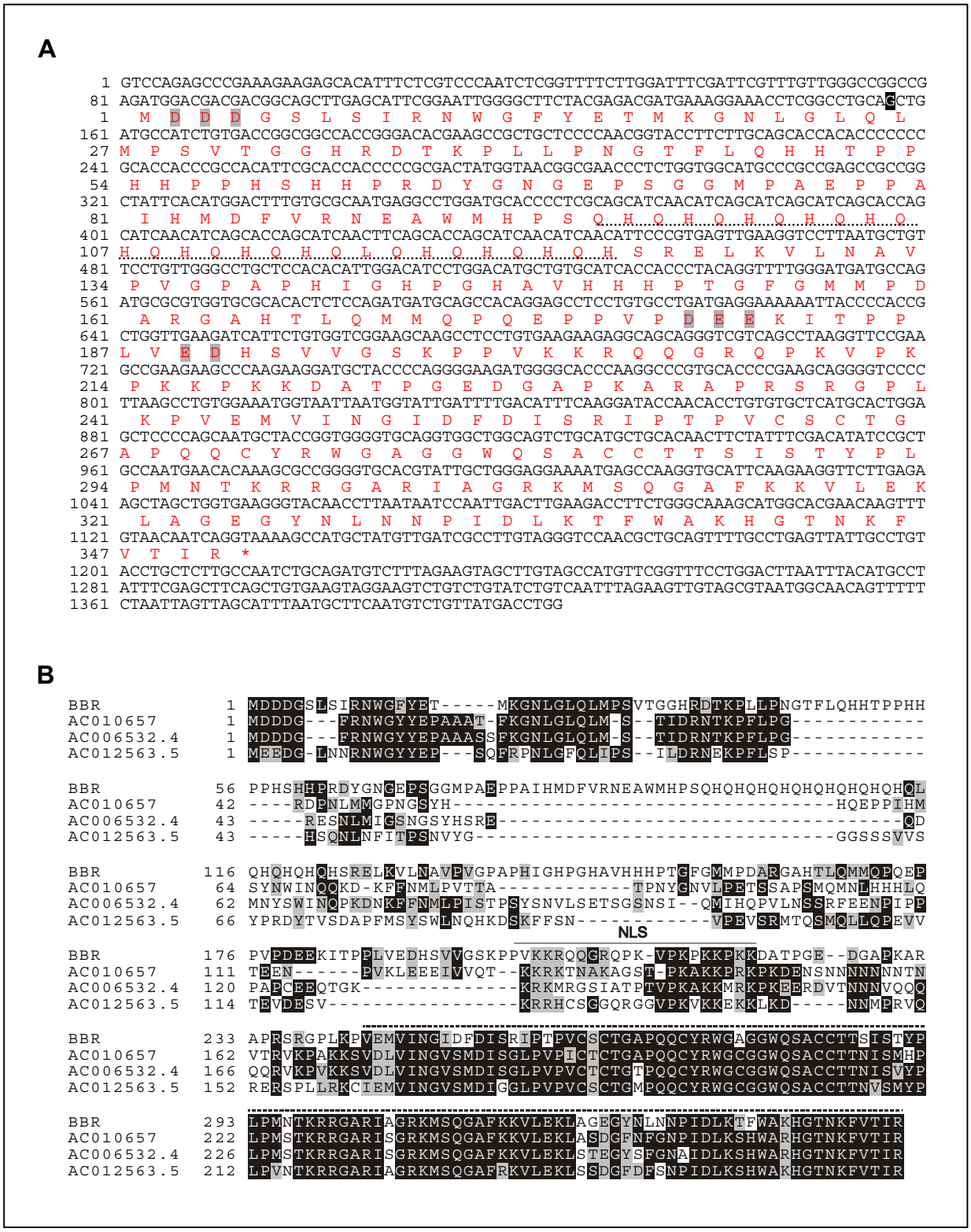



**Fig. 3-15 The cDNA and deduced amino acid sequences of the *BBR* gene and alignment of the BBR protein with three putative *Arabidopsis* proteins.** (A) cDNA and deduced amino acid sequences of the *BBR* gene. Acidic regions are shaded in grey. The first nucleotide of the longest one-hybrid clone is in reverse type. The Gln/His repeats are underlined with a dotted line. (B) Amino acid sequence alignment of the BBR protein with three putative *Arabidopsis* proteins with unknown function (Accession numbers: AC010657, AC006532.4, AC012563.5). The putative NLS is indicated by single line. The putative DNA-binding domain is marked by a dashed line.

Within the four BBR cDNA clones isolated from the yeast one-hybrid screening, two truncated cDNA clones encoding the C-terminal part of BBR protein retained the DNA-binding capability in the yeast one-hybrid system (Kai Müller, unpublished data). It indicated that the C-terminal part of BBR is responsible for DNA-binding and contains the DNA-binding domain. Computer-aided searches of the databases failed to reveal any significant sequence similarity between BBR and any known transcription factors or DNA-binding proteins, but only a stretch of 53 amino acids at the C-terminus sharing 40% similarity to the gene product of human *T-box brain1*, a DNA-binding nuclear protein (accession number, Q16650) (Bulfone *et al*., 1995), where BBR got its name 'Barley Brain'. BBR also does not contain a basic leucine zipper (bZIP) motif or a zinc finger motif, which are common to many plant DNA-binding proteins. The secondary structure of the DNA-binding domain, as predicted by computer analysis, suggested some possible α-helices, extended β-strands and random coils. However, no structural homology to helix-loop-helix or helix-turn-helix structure was found in the DNA-binding domains that have been reported in the database. However, the search revealed that BBR had high homology with three putative proteins in the *Arabidopsis* genome database (Fig. 3-15B). The C-terminal part of BBR, which is the putative DNA binding domain (243-350), shares 86% or higher sequence similarity to these putative proteins (Fig. 3-15B), which indicates that the DNA-binding domain of BBR is probably conserved in plants. All proteins also show high sequence homology in the N-terminal part and the putative nuclear localization sequence (NLS).

### 3.2.3 Genomic structure of *BEIL*, *BAPL*, *BBR* and *BGRF*

The genomic structure of the four genes established by comparing genomic sequences with the full-length cDNA sequences is summarized in Fig. 3-16A.

The *BEIL* gene spans 3084bp of 5' non-transcribed sequence (promoter), 3256bp of transcribed region consisting of three exons and two introns and 530bp of 3' non-transcribed sequence. The cDNA sequence is 99.5% identical to the corresponding genomic sequence. This is probably due to the different genetic stocks used in these two studies.

The *BAPL* gene has 3402bp of 5' non-transcribed sequence (promoter), 2227 bp of transcribed region consisting of four exons and three introns and 1205bp of 3' non-transcribed sequence. The cDNA sequence is identical to the corresponding genomic sequence.

The *BBR* gene has 1985bp of 5' non-transcribed sequence (promoter), 1404bp of transcribed region without introns and 1568bp of 3' non-transcribed sequence. The cDNA sequence is 100% identical to the corresponding genomic sequence.

Although the genomic sequence of *BBR* does not contain any introns we believe it is not a peseudogene. First of all the genomic sequence is 100% identical to the cDNA sequence

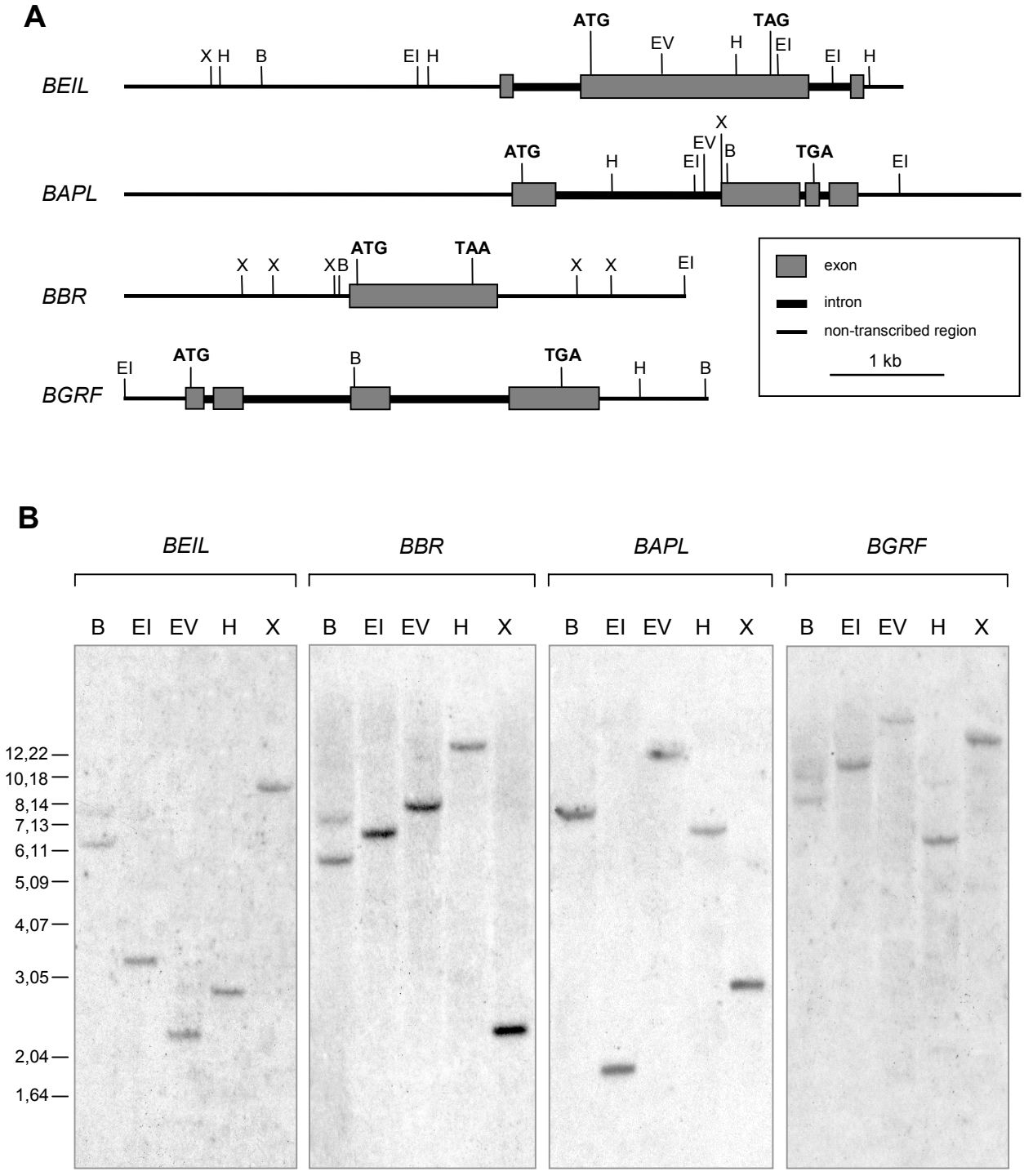



**Fig. 3-16 Genomic structures and genomic Southern blots of *BEIL*, *BAPL*, *BBR* and *BGRF*.** (A) Genomic structures. The big solid boxes indicate exons and the small black boxes denote introns, the thick black lines indicate the non-transcribed regions. (B) Genomic Southern blots. Each lane contained 10$\mu$g of barley genomic DNA digested with the indicated restriction enzymes. Genomic DNA was prepared from fully expanded barley seedling leaves (wild type *k-Atlas*). cDNA probes were prepared using the random priming DNA labelling method and [$\alpha^{32}$P]-dCTP. Hybridzations and washes were performed at high-stringency conditions. DNA size was marked in kilobases at the left-hand side of the figure. B, *Bam*HI; EI, *Eco*RI; EV, *Eco*RV; H, *Hind*III; X, *Xba*I.

obtained from one-hybrid screening. Secondly, the genomic Southern blot analysis showed that it is a single copy gene in the barley genome (Fig. 3-16B). Thirdly, PCR amplification of the BBR gene from other two barley wild-type varieties Proctor and Nudinka did not show any detectable introns. Finally, the genomic sequences of the three BBR homologous genes from *Arabidopsis* revealed that their ORFs are not interrupted by any intron either.

The genomic sequence isolated for the *BGRF* gene contains 524bp of 5' non-transcribed sequence (promoter), 3613bp of transcribed region with four exons and three introns and 1036bp of 3' non-transcribed sequence. The cDNA sequence is identical to the corresponding genomic sequence.

Table 3-1 shows the sequences around the exon/intron junctions in the *BEIL*, *BAPL*, and *BGRF* genes. The introns are relatively AT-rich compared to the exons, and all have the conserved splice site sequences at their 5' and 3' ends, following the 'GT…AG' rule of plant introns (Brown, 1986).

**Table 3-1 Sequences of exon/intron borders in the *BEIL*, *BAPL* and *BGRF* genes.**

| | ExonI | IntronI | ExonII | IntronII | ExonIII | IntronIII | ExonIV |
|---|---|---|---|---|---|---|---|
| BEIL | 74bp AG | gt… 616bp…ag | GT…1935bp…AC | gt…429bp…ag | C…150bp | — | — |
| BAPL | 117bp AC | gt…1448bp…ag | AT… 707bp…AC | gt…786bp…ag | GA…102bp…A | gt… 174bp…ag | GT…300b |
| BGRF | 164bp TC | gt… 125bp…ag | GG… 242bp…CC | gt…955bp…ag | TT…352bp…AC | gt…1031bp…ag | GT…773b |

Numbers indicate number of nucleotides between adjacent sequences.

Southern blot analyses were performed to determine the numbers of these genes in the barley genome. When blots were probed with the full-length cDNA fragments under high-stringency conditions, one strongly hybridized band was observed in most of the lanes (Fig. 3-16B). For *BEIL*, *BBR* and *BGRF*, in the lanes with *Bam*HI-digested genomic DNA, one weaker band was observed higher than the stronger band, probably caused by imcomplete digestion. The band patterns were in agreement with the restriction maps of the genomic DNA, suggesting that all bands were derived from a single genetic locus.

## 3.3 Expression of *BEIL*, *BAPL*, *BBR* and *BGRF* mRNAs

Since *BEIL*, *BAPL*, *BBR* and *BGRF* encode DNA-binding proteins which supposedly interact with the 305bp intron sequence of *BKn3*, one can expect that they should be expressed in barley inflorescences and probably show differential expression in the wild-type and the *Hooded* mutant. To analyze the transcription levels of the four genes in different wild-type

barley tissues and to compare their expression levels in wild-type and *Hooded* inflorescences, Northern blot and RT-PCR analyses were performed.

20$\mu$g of total RNA isolated from wild-type barley mature embryos, 5-day old fully expanded seedling leaves, 5-day old seedling roots, internodes, nodes, inflorescences and *Hooded* inflorescences were applied in Northern blot analysis using full-length *BEIL*, *BAPL*, *BBR* and *BGRF* cDNAs as probes, respectively. The pictures of ethidium bromide-stained RNA gels were documented for the control of RNA loading.

Fig. 3-17A shows the hybridization patterns of the *BEIL* and *BAPL* genes. The *BEIL* transcript is about 2.2 kb in length and was observed in all barley tissues examined at different levels. The highest level of *BEIL* gene expression was detected in both wild-type and *Hooded* inflorescences. In *Hooded* infloresences the expression level of the *BEIL* gene appeared relatively unchanged. The *BAPL* transcript is about 1.5 kb in length and was present in all barley tissues tested. A higher expression level of the *BAPL* gene was observed in the wild-type and *Hooded* inflorescences and wild-type stems. Differential expression of *BAPL* in wild-type and *Hooded* inflorescences was not significant.

Since *BBR* transcripts could not be detected by using 20$\mu$g of total RNA, 1-2$\mu$g of polyA$^+$ mRNA isolated from different barley tissues were used in the Northern hybridization with full-length *BBR* cDNA as a probe. The blot was reprobed with an *Anthirrhinum* actin cDNA fragment to compare the quantity of RNA in each lane. The *BBR* transcript is about 1.5kb in length and was observed in all barley tissues examined as shown in Fig. 3-17B. The expression level of the *BBR* gene in different barley tissues was normalized to the actin expression and expressed relative to the value obtained for the seedling leaves, which showed the lowest level. The diagram on the right in Fig. 3-17B shows the relative expression levels of *BBR* in different tissues. The highest expression level of *BBR* was detected in mature embryos and the lowest level in seedling leaves. It is noteworthy that the *BBR* expression in *Hooded* inflorescences is two-fold higher than that in wild-type inflorescences.

*BBR* gene expression in barley tissues was also examined by RT-PCR analysis. cDNA was synthesized from 3$\mu$g of total RNA isolated from different barley tissues and amplified with *BBR* gene-specific primers (see Material and Methods). The amplified PCR products were detected by Southern blot using the *BBR* cDNA fragment as a probe. Amplification of barley *GAPDH* gene using gene-specific primers was an internal control. The expression pattern of the *BBR* gene revealed by RT-PCR analysis was consistent with that obtained from Northern blot analysis (Fig. 3-17C) by showing that *BBR* mRNA was more abundant in *Hooded* inflorescences than in wild-type inflorescences.

When a radiolabelled *BGRF* cDNA probe was hybridized with blots prepared with total RNA or polyA$^+$ mRNA isolated from various barley tissues, it gave very high background and

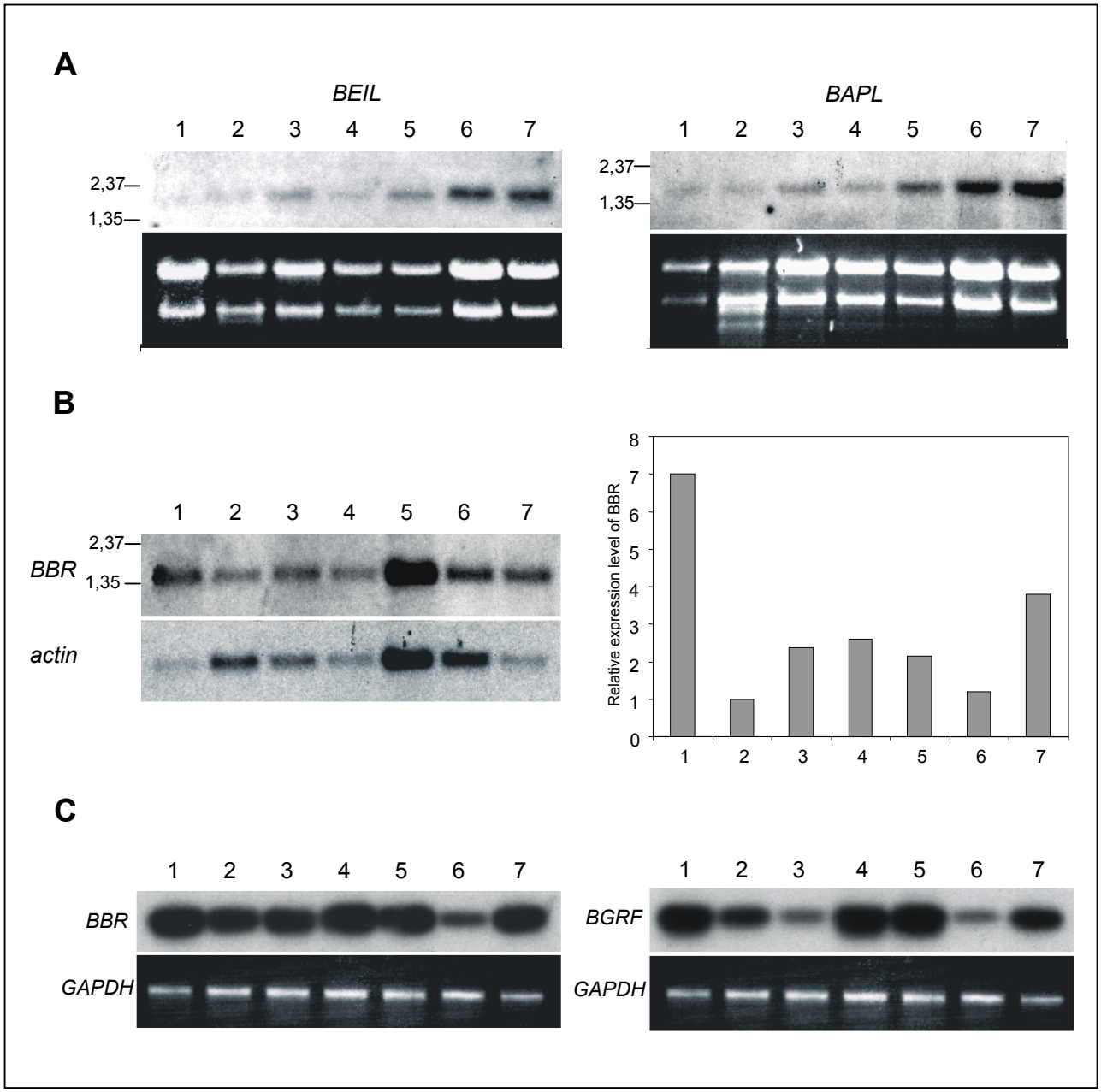



**Fig. 3-17 Expression of *BEIL*, *BAPL*, *BBR* and *BGRF* in different barley tissues.** In all panels lanes 1, 2, 3, 4, 5, 6 and 7 correspond to mature embryos, 5-day old fully expanded seedling leaves, 5-day old seedling roots, internodes, nodes, inflorescences from wild type barley and inflorescences from the *Hooded* mutant, respectively. (A) Northern blot analyses of the *BEIL* and *BAPL* genes. Northern blots were prepared from 20$\mu$g of total RNA. Blots were probed with a $^{32}$P labelled full-length *BEIL* cDNA probe and a *BAPL* cDNA probe. Ethidium bromide stained RNA gels are shown for the control of RNA loading. (B) Northern blot analysis of the *BBR* gene. 1-2$\mu$g of polyA$^+$ mRNA were loaded to each lane. The blot was hybridized with a full-length *BBR* cDNA probe. The amount of mRNA on the blot was determined by reprobing with an actin probe. The diagram on the right shows the relative expression level of *BBR* in different barley tissues calculated from comparison with actin expression by using the ImageQuant program. The relative amount of *BBR* mRNA in seedling leaves with lowest level was set at 1,0. (C) RT-PCR analyses of *BBR* and *BGRF* expression. cDNA was synthesized from 3$\mu$g of total RNA and amplified with *BBR* and *BGRF* gene-specific primers. The amplified PCR products were detected by Southern blot. Amplification with *GAPDH*-specific primers was used as an internal control.

several diffuse bands in all lanes. It might be due to several extremely GC rich regions throughout the cDNA probe cross-hybridizing with other RNA molecules. This cross-hybridization was also observed when the *BGRF* cDNA probe was used for genomic library screening. Two clones showing strong hybridization signals turned out to be false positives probably caused by the cross-hybridization within 80bp region with high GC content.

The expression of the *BGRF* gene was analyzed by RT-PCR. *BGRF* gene-specific primers spanning one intron (1020bp in length) (see Material and Methods) were used for amplification of the cDNAs, they were designed to help distinguish the amplification of genomic DNA. As shown in Fig. 3-17C the expression of *BGRF* was present in all barley tissues investigated and notably *Hooded* inflorescences rendered higher *BGRF* expression than wild-type inflorescences.

Together these results suggest that *BEIL*, *BAPL*, *BBR* and *BGRF* have broader expression patterns than *BKn3* and are expressed in all barley tissues examined, including embryos, leaves, roots, stems and inflorescences. *BEIL* and *BAPL* gene expression is not dramatically altered in *Hooded* inflorescences, however, *BBR* and *BGRF* are up-regulated in *Hooded* inflorescences.

## 3.4 Determination of DNA-binding properties of BEIL, BAPL, BBR and BGRF

*(This part of work was done with collaboration of Luca Santi. We both contributed equally.)*

To verify if BEIL, BAPL, BBR and BGRF proteins bind to the 305bp sequence of *BKn3* intron IV *in vitro*, purified GST-fusion proteins and *in vitro* translated products were used for *in vitro* binding studies.

### 3.4.1. Overexpression and purification of GST-fusion proteins in *E. coli*

To obtain GST fusion proteins for *in vitro* DNA-binding assays, the complete ORFs of the *BEIL*, *BAPL*, *BBR* and *BGRF* genes were subcloned into *Eco*RI/*Not*I sites of the expression vector pGEX-5X-1. The production of fusion proteins were induced by IPTG in the *E.coli* strain BL21 harbouring the recombinant plasmids. After induction for 4 hours there were high amounts of BBR and BGRF recombinant proteins produced by bacteria, but the induction of BEIL and BAPL GST-fusions was not efficent in *E.coli* as shown in Fig. 3-18.

The purification of the recombinant proteins was carried out by affinity chromatography on glutathione-Sepharose beads and the integrity and purity of the purified product was checked by SDS-PAGE (data not shown). Only low concentrated BBR and BGRF proteins were

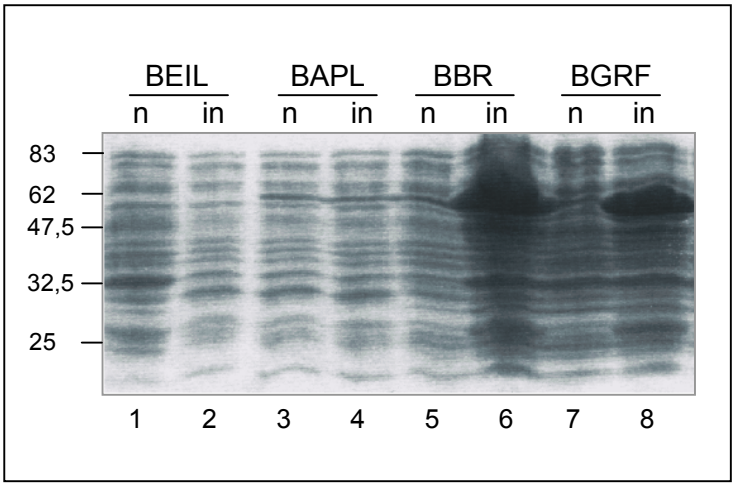



**Fig. 3-18 Expression of the GST fusion proteins from *E. coli*.** Crude bacterial extracts were checked by 12.5% SDS-PAGE. Lane1, 3, 5 and 7 show the extracts from BL21 harbouring pGEX-BEIL, BAPL, BBR and BGRF, respectively under noninduced conditions (n). Lane 2, 4, 6 and 8 show the extracts from BL21 harbouring pGEX-BEIL, BAPL, BBR and BGRF, respectively, under induced conditions (in). Molecular weight markers are indicated at the left side of the picture.

obtained after purification. Most of the proteins could not be eluted from the sepharose beads. For BEIL and BAPL, we could not see any proteins after elution. Considering the low amounts of protein needed for *in vitro* binding assays, purified recombinant proteins were tested in the *in vitro* binding assay although the purification of GST-fusion proteins was not fully successful.

### 3.4.2 *In vitro* translation of *BEIL*, *BAPL*, *BBR* and *BGRF*

Because the production of GST-fusion proteins was not really successful, *in vitro* translation was carried out as an alternative method. To get the *in vitro* translated products of the *BEIL*, *BAPL*, *BBR* and *BGRF* genes, the ORF of each gene was subcloned into the *Eco*RI/*Pst*I sites of pBluescript KS+. RNA templates for *in vitro* tanslation were obtained by *in vitro* transcription using T3 or T7 polymerase, and *in vitro* translation was done in the wheat germ extracts in the presence of $^{35}$S methionine. The *in vitro* translation products were analyzed on 12.5% SDS-PAGE gels (data not shown). Four major bands with the expected molecular weights of the four proteins were observed. Parallel *in vitro* translations were performed in the presence of unlabelled methionine. The extracts obtained were incubated with the 305bp fragment, and binding of four proteins was examined by electrophoretic mobility shift assay (EMSA).

### 3.4.3 *In vitro* binding studies of BEIL, BAPL, BBR and BGRF proteins

The DNA-binding properties of BEIL, BAPL, BBR and BGRF recombinant proteins and *in vitro* expressed proteins were examined by electrophoretic mobility shift assay with the 305bp intron sequence as a probe.

Incubation of purified BBR-GST fusion proteins with the labelled 305bp fragment gave two retarded bands in the gel, but purified protein extracts from cells expressing GST alone did not show these bands (Fig. 3-19). In addition, these two bands could be competed by an excess of

unlabelled 305bp fragment (Fig. 3-19). These results indicated that the BBR recombinant protein could bind to the 305bp sequence specifically. However, we could not detect shifted bands with BEIL, BAPL and BGRF recombinant proteins or any of the *in vitro* expressed proteins even if different binding conditions were performed in the binding reactions.

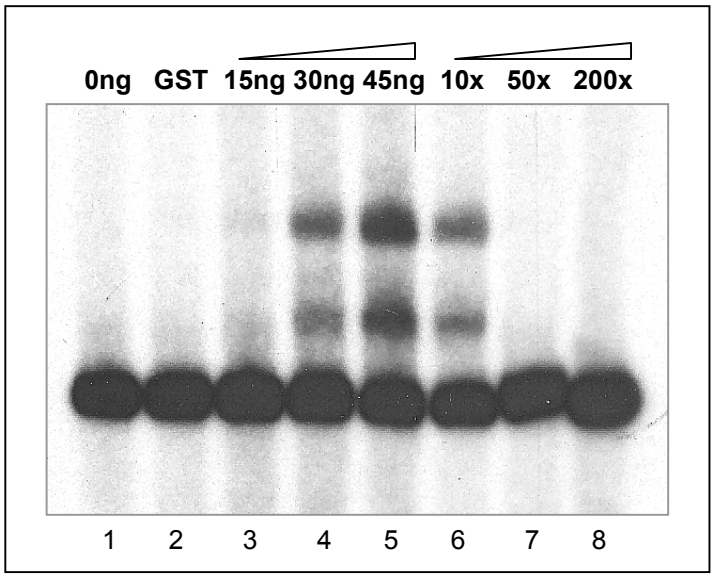



**Fig. 3-19 The BBR recombinant protein specifically binds to the 305bp intron sequence of *BKn3 in vitro*.** The gel retardation assay performed with the BBR-GST fusion protein and the 305bp intron fragment. 0.1ng of $^{32}$P labelled 305bp fragment (10,000c.p.m) was added to each lane. The sample in lane 1 corresponds to the free probe (control lane, no protein added). The sample in lane 2 contained 1μg of purified GST protein. Samples in lanes 3, 4, 5 contained 15ng, 30ng and 45ng of purified BBR-GST fusion protein, respectively. For the competition, 30ng of purified BBR-GST fusion protein and 0.1ng of labelled 305bp fragment were combined in the presence of 10-fold (lane 6), 50-fold (lane 7) and 200-fold (lane 8) excess unlabelled 305bp fragment.

In the future, different expression systems will be used to produce BEIL, BAPL and BGRF recombinant proteins, and truncated versions of *BEIL*, *BAPL* and *BGRF* cDNAs will be fused to the GST gene to eliminate the regions causing low expression of fusion proteins in bacteria. Proteins with better quality will be very helpful to prove their ability to bind to the 305bp intron sequence *in vitro*.

### 3.4.4 Identification of the BBR binding site in the 305bp intron sequence

Since recombinant BBR protein could bind to the 305bp intron sequence in the previous experiment, further experiments were performed to narrow down the binding site of BBR protein within the 305bp sequence (Fig. 3-20A).

First, three PCR fragments with at least 20bp overlapping to each other (Fig. 3-20B) were examined by EMSA under the conditions allowing strong binding of BBR to the 305bp fragment. Only the most 3' PCR fragment (from 180-305 nt) was sufficient to confer BBR binding and the protein-DNA complex formation could be completely competed by 200-fold excess unlabelled identical PCR fragment (data not shown).

Four double-stranded oligonucleotides with 10bp overlaps were designed based on the 305bp sequence from nucleotide 180 to 305 (Fig. 3-20B). When they were tested in EMSA, it turned

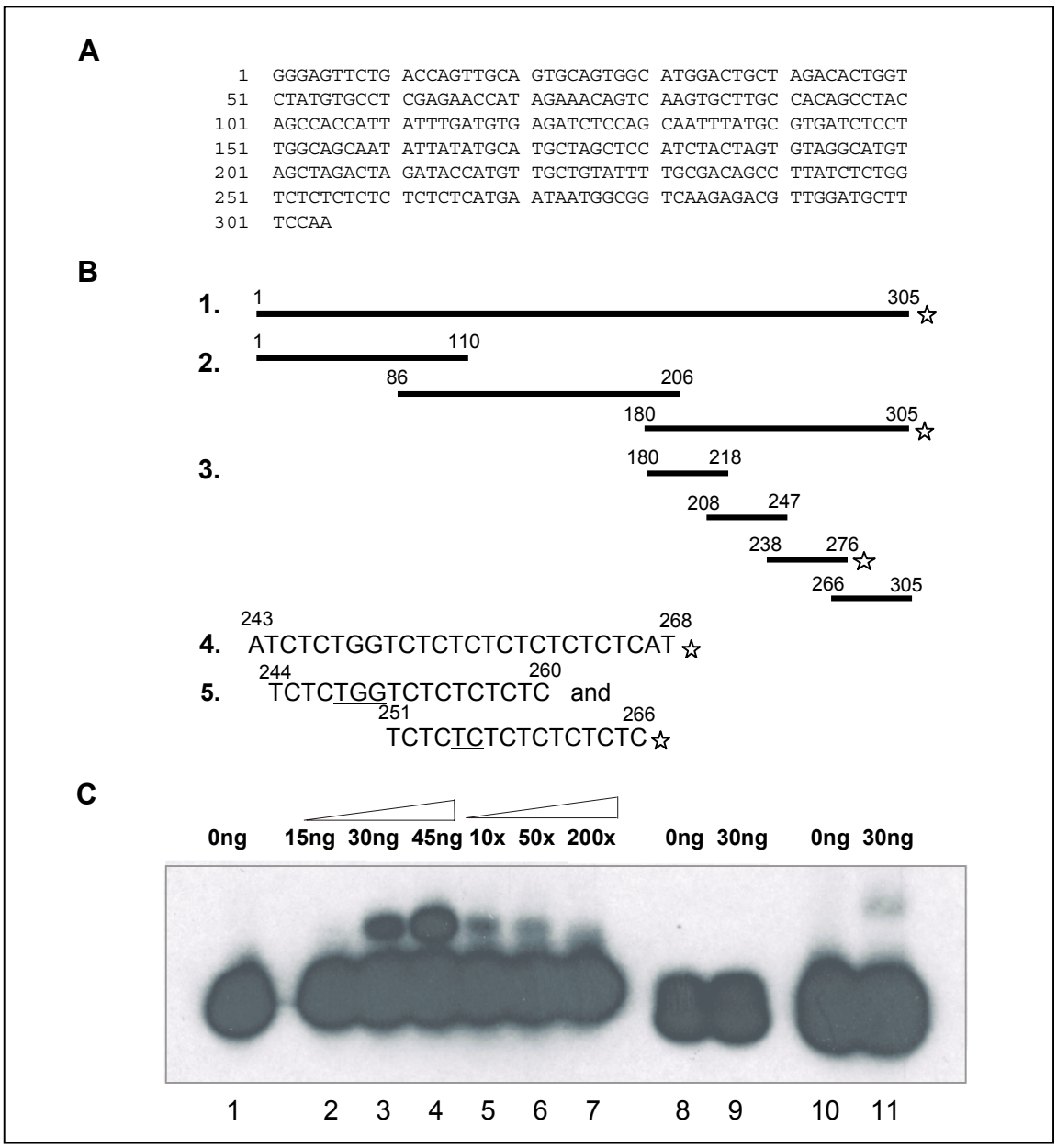



**Fig. 3-20 Determination of the DNA-binding sites of the BBR recombinant protein.** (A) The DNA sequence of the 305bp intron fragment. (B) DNA probes used for determining the DNA-binding site of the BBR protein in five individual experiments. Numbers indicate the positions of the probes in the 305bp intron sequence. Those marked with stars were bound by BBR protein. (C) EMSA of recombinant BBR protein with different DNA fragments in the 305bp intron sequence. (lane 1-7) 0.1ng of $^{32}$P labelled oligonucleotide `ATCTCTGGTCTCTCTCTCTCTCTCAT´ (243 to 268) (10,000 c.p.m) was added to each lane. The sample in lane 1 corresponds to the free DNA probe (control lane, no protein added). Samples in lane 2, 3 and 4 contained 15ng, 30ng and 45ng of purified BBR-GST fusion protein, respectively. For the competition, 30ng of purified BBR-GST fusion protein and 0.1 ng of oligonucletide were combined in the presence of 10-fold (lane 5), 50-fold (lane 6) and 200-fold (lane 7) excess unlabelled identical oligonucleotide. (lane 8, 9) 0.1ng of $^{32}$P labelled oligonucleotide `TCTCTGGTCTCTCTCTC´ (244 to 260) was added to each lane. The sample in lane 8 corresponds to the free probe. The sample in lane 9 contained 30ng of purified BBR-GST protein. (lane 10, 11) 0.1ng of $^{32}$P labelled oligonucleotide `TCTCTCTCTCTCTCTC´ (251 to 266) was added to each lane. The sample in lane 10 corresponds to the free probe. The sample in lane 11 contained 30ng of purified BBR-GST protein.

out that the BBR protein bound specifically to the oligonucleotides corresponding to nucleotide 238 to 276 in the 305bp sequence. The binding could be completely competed by a 200-fold excess of unlabelled identical oligonucleotides (data not shown).

A shorter oligonucleotide with the sequence 'ATCTCTGGTCTCTCTCTCTCTCTCAT' (Fig. 3-20C) was tested further in EMSA. It was still sufficient for BBR binding. When another two even shorter oligonucleotides 'TCTCTGGTCTCTCTCTC' and 'TCTCTCTCTCTCTCTC' (Fig. 3-20C) were examined under the same conditions, a weaker mobility shift was detected only when BBR recombinant protein was incubated with the latter oligonucleotides (Fig. 3-20C). It indicated that the binding affinity of BBR to this shorter oligonuclecotides decreased. The binding of BBR to 'TCTCTGGTCTCTCTCTC' could not be detected even after overexposure of the gel for 2-3 days, indicating that change from TC to TGG abolished BBR binding entirely.

These results demonstrated that the 'TCTCTCTCTCTCTCTC' was sufficient for BBR recognition. A search for similar DNA-binding motifs in the databases revealed that this DNA-binding site had not been reported for any DNA-binding proteins characterized so far. But it is reasonable to assume that a novel DNA-binding protein should have a novel DNA-binding site. Using *Blastn* to search for sequences showing an exact match of TCTCTCTCTCTCTCTC in the databases, we found that several genes contain this short sequence at their promoters or 5'UTR, including 5'UTR of *BKn3* (Müller *et al*., 1995) and *Superman* (Sakai *et al*., 1995). The promoter region of the *Carpel Factory* gene (Jacobsen *et al*., 1999) contains the sequence 5'-$(CT)_{22}$-3'. Near the beginning of transcription of the *LEAFY* gene, a sequence 5'-T$(CT)_6$ATC-3' is found very similar to the binding site of BBR protein (Weigel *et al*., 1992). In the sequence of the GA-regulated barley transcription factor *GAMyb* gene, there are two sequences 5'-$(CT)_{11}$-3' located in the promoter region (-118 to -97) and 5'UTR (Gomez-Cadenas *et al*., 2001). It will be very interesting to find out the role of this element in gene regulation.

## 3.5 Mapping of *BEIL*, *BAPL*, *BBR* and *BGRF* genes

Since it was previously assumed that some of *BEIL*, *BAPL*, *BBR* and *BGRF* could be candidate genes for the suppressors of *Hooded* (*suK*), mapping of these genes was carried out to possibly set up the association between genes and mutants.

SNPs, single nucleotide polymorphisms, have been shown to be the most common type of genetic variation in organisms, representing up to 80% of all possible DNA polymorphisms (Brookes, 1999). Among the methods for SNP detection, single-stranded conformation polymorphism (SSCP) analysis is the most commonly used gel based mutation detection method. SSCP relies on conformational intrastrand differences in different DNA sequences.

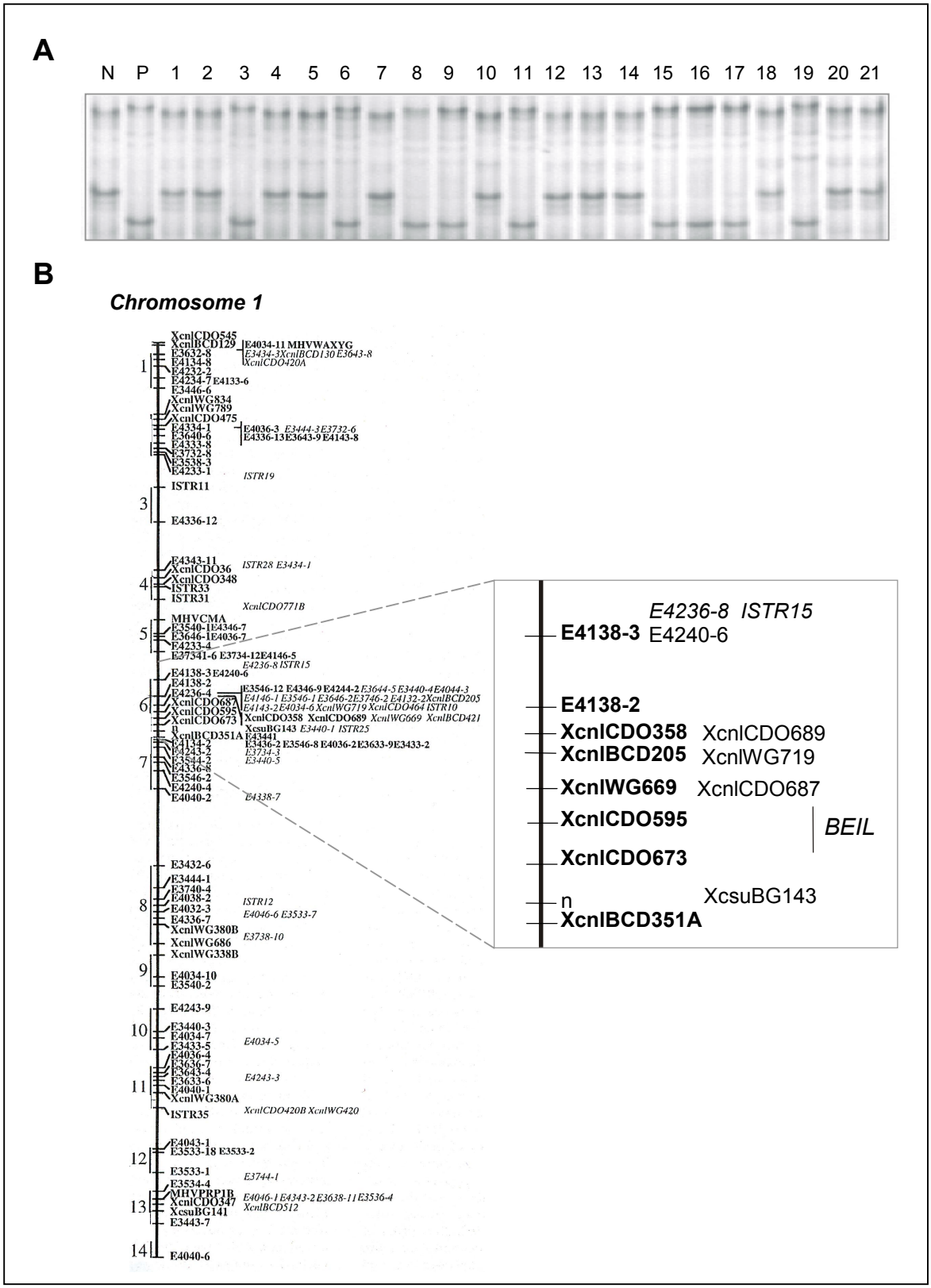



**Fig. 3-21 Analysis of single-nucleotide polymorphisms (SNP) by SSCP and mapping of the *BEIL* gene.** (A) The SSCP segregation pattern for *BEIL* in 21 out of 100 DH lines of the Nudinka × Proctor mapping population (1-21). The segregation pattern of the remaining 79 individuals is not shown. N and P are the digested PCR fragments of the *BEIL* gene for the parental lines Nudinka and Proctor, respectively. (B) Schematic presentation of the map position for *BEIL* on chromosome 1. The RFLP/AFLP/ISTR linkage map of barley chromosome 1 is shown at the left hand side of the figure.

Thus it can detect single nucleotide substitutions, insertions and deletions with high sensitivity (Sheffield *et al*., 1993). Therefore, the mapping of the four genes *BEIL*, *BAPL*, *BBR* and *BGRF* to the Nudika × Proctor genetic linkage map (Castiglioni *et al*., 1998) was undertaken through the detection of SNPs by SSCP analysis with the 100 available doubled-haploid (DH) lines of the mapping population.

According to the genomic sequence of *BEIL*, *BAPL*, *BBR* and *BGRF*, primers (sequences shown in the appendix) were designed along the genomic sequence and used to amplify Nudinka and Proctor genomic DNA in different combinations. Amplified PCR products were digested with *Alu*I, *Dpn*I, *Hae*III, *Mse*I, *Rsa*I, and *Taq*I and separated on non-denaturing acrylamide gels to detect single-stranded conformation polymorphisms between Nudinka and Proctor. Once polymorphisms were found between the two parental lines, the 100 progenies were then analyzed for the presence or absence of Nudinka- or Proctor- specific alleles.

Genomic DNA fragments spanning a 420bp intron from *BEIL*, amplified from Nudinka and Proctor by PCR using gene-specific primers (Bei27/Bei28, see sequences in the appendix), contained a single nucleotide polymorphism. The SNP observed for the two parental lines was based on single strand conformation polymorphism (SSCP) and segregated in the mapping population (Fig. 3-21A). This allowed the mapping of *BEIL* to the combined AFLP/RFLP/ISTR linkage map (Castiglioni *et al*., 1998). *BEIL* was mapped to chromosome 1 on linkage subgroup 6 in a region with high marker density (Fig. 3-21B).

SSCP analyses of *BAPL*, *BBR* and *BGRF* genomic PCR products did not reveal any polymorphisms in the two parental lines. Thus mapping of these genes will be carried out by a combined RFLP/AFLP approach that has been described previously (Castiglioni *et al*., 1998).

## 3.6 Further characterization of BBR

Since the BBR protein showed the capacity of binding to the 305bp intron sequence *in vitro*, further experiments were performed to find out its subcellular localization and to test its transactivation activity. The BBR promoter was analyzed in transient and stable expression systems.

### 3.6.1 Nuclear localization of BBR

The fact that the BBR gene product binds to the 305bp fragment *in vivo* and *in vitro* implies that BBR very likely functions as a transcription factor. Consistent with this prediction, a basic domain conforming to a bipartite nuclear localization motif (Chelsky *et al*., 1989) was identified in the region between residues 198 to 219 by database searching (Fig. 3-22). Due to the presence of an NLS in BBR it was assumed that BBR could be localized in the nucleus.

In order to define the region of BBR which is responsible for nuclear targeting, three different regions of BBR were fused to a green fluorescent protein (GFP) reporter gene (Fig. 3-22). To construct the translational fusion of BBR to GFP, *Nco*I sites were introduced by PCR to both ends of the BBR coding sequence. The translation initiation site of different BBR coding regions was embedded in the 5' *Nco*I site. The PCR products were subsequently excised with *Nco*I and integrated to the *Nco*I site of pCATgfp (kindly provided by Dr. Guido Jach), which is downstream of a double CaMV 35S promoter.

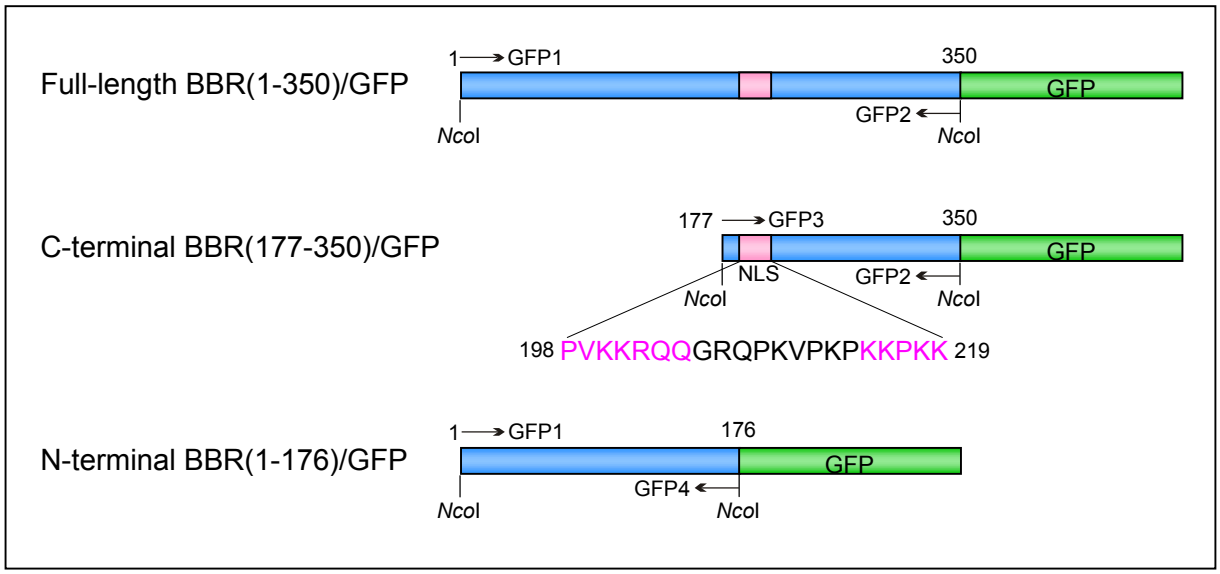



**Fig. 3-22 Schematic overview of BBR/GFP fusion constructs used in investigation of their localization in tobacco SR1 protoplasts.** Boxes in blue represent coding sequence of the *BBR* cDNA, boxes in pink represent the putative nuclear localization signal (NLS) within the coding region of BBR. The NLS sequence is indicated underneath C-terminal BBR/GFP construct. Boxes in green represent the GFP coding sequence. Boxes showing GFP are not drawn to scale. The positions of BBR amino acids are indicated above the constructs. Locations of primers used for amplifying different regions of the *BBR* coding sequence are shown by arrows. *Nco*I sites used for the construction of BBR fusions to GFP are indicated.

The gene constructs were introduced into tobacco SR1 protoplasts by PEG-mediated transfection, and the localization of the chimeric GFP proteins were observed by fluorescence microscopy. When 35S:GFP was expressed, GFP fluorescence was distributed throughout the cells (Fig. 3-23A). In contrast, the full-length BBR/GFP fusion protein was found exclusively in the nucleus (Fig. 3-23B). If the putative NLS located in BBR between amino acids 198-219 was functional in nuclear targeting, one would expect that the fusion protein consisting of GFP and the C-terminal region of BBR would be located in the nucleus, and this was indeed the case (Fig. 3-23C). For unclear reasons the fusion protein of GFP with the N-terminal region of BBR could not be detected in protoplasts. Cloning mistakes could be excluded because all the constructs were fully sequenced to confirm the correct translational fusion of BBR to GFP prior to the transfection.

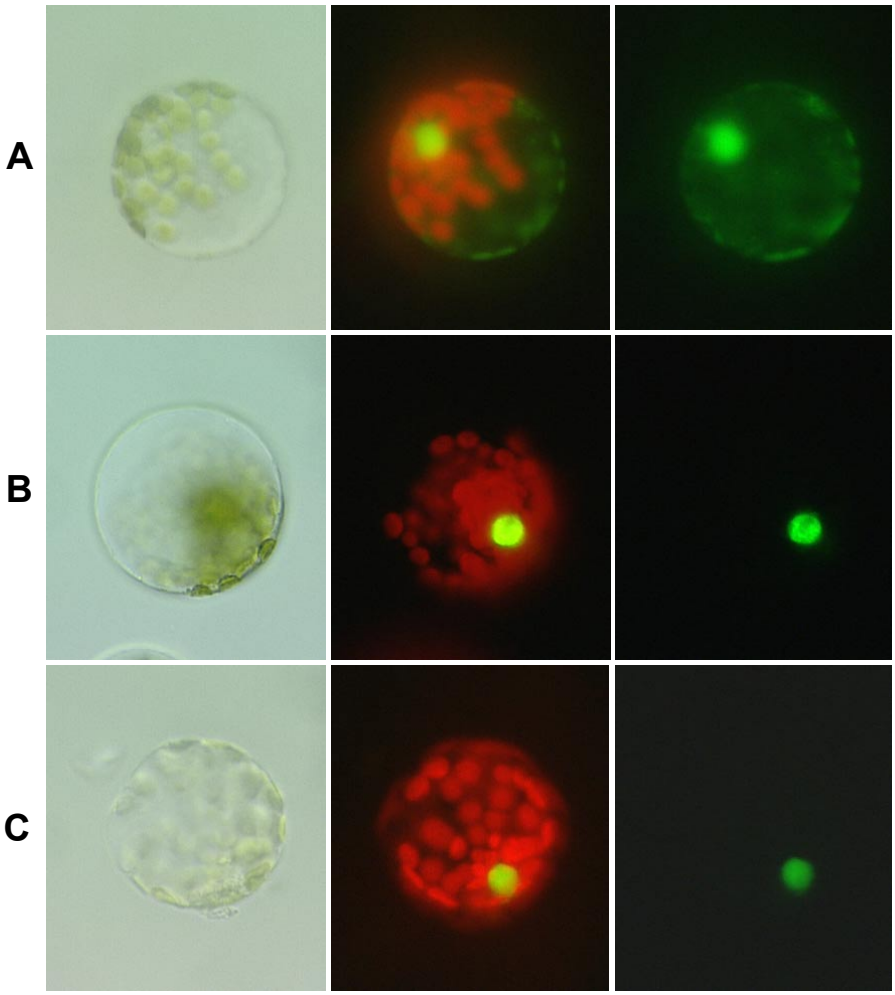



**Fig. 3-23 Localization of BBR/GFP fusion proteins in tobacco SR1 protoplasts.** Tobacco SR1 protoplasts were transiently transfected with BBR/GFP fusion constructs and were analyzed for the green fluorescence by fluorescence microscopy. pCATgfp vector (35S:GFP) (A) was used as a positive control to show GFP expression in the nucleus and the cytoplasm. Full-length BBR(1-350)/GFP (B) and C-terminal BBR(177-350)/GFP (C) fusion proteins are located in the nucleus. From left to right: identical protoplast viewed in bright field (left), with filter set I (blue light excitation, 450-490nm) (middle) and a GFP filter set which blocks the red autofluorescence from chlorophyll (right).

Taken together, these data indicated that the BBR protein is nuclear-localized and the C-terminal region of BBR is sufficient for nuclear targeting of BBR.

### 3.6.2 Transactivation activity of BBR

A typical plant transcription factor contains a transcription-regulation domain besides a DNA-binding domain and a nuclear localization signal. It was; therefore, expected that the BBR protein could also contain a transcription-regulation domain. Several acidic regions were found distributed throughtout the predicted amino acid sequence of BBR (Fig. 3-15A). The acidic domains are thought to function in transcriptional activation (Frankel and Kim, 1991).

To investigate the transactivation activity of BBR, co-transfection experiments and transient expression assays using tobacco SR1 protoplasts were performed. Since both the 305bp intron

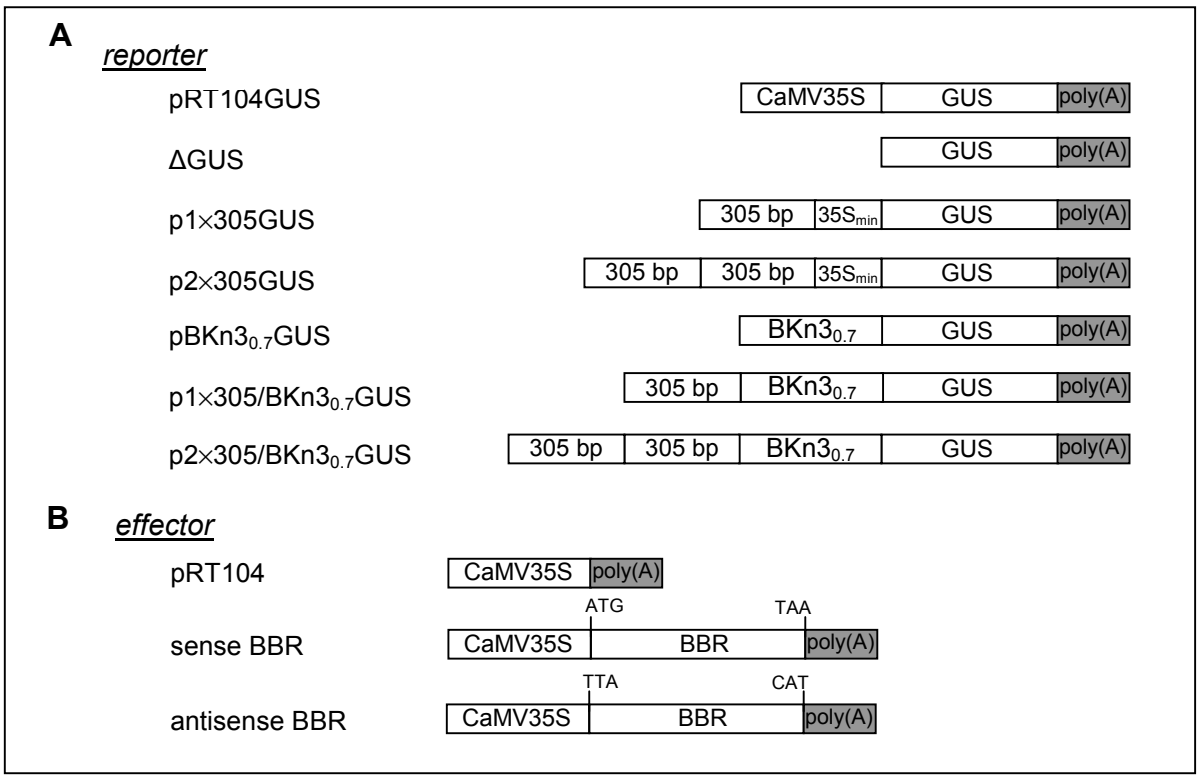



**Fig. 3-24 Schematic representation of reporter and effector constructs.** (A) The chimeric GUS reporter constructs used to analyze the interaction between BBR and the 305bp fragment in *BKn3* intron IV and *BKn3* 0.7-kb promoter fragment. pRT104GUS, a positive control; ΔGUS, the promoterless GUS gene as a negative control; p1×305 GUS, GUS placed under the control of one copy of the 305bp fragment fused to the CaMV35S minimum promoter; p2×305GUS, GUS placed under the control of two copies of the 305bp fragment fused to CaMV35S minimum protmoter; pBKn3$_{0.7}$ GUS, GUS placed under the control of the 0.7-kb *BKn3* promoter fragment; p1×305/ BKn3$_{0.7}$ GUS, GUS placed under the control of one copy of the 305bp fragment fused to the 0.7-kb *BKn3* promoter fragment; p2×305/BKn3$_{0.7}$ GUS, GUS placed under the control of two copies of the 305bp fragment fused to the 0.7-kb *BKn3* promoter fragment. (B) The effector constructs. pRT104, a negative control; sense *BBR*, the full-length *BBR* cDNA placed in sense orientation under the control of the CaMV35S promoter; antisense *BBR*, the full-length *BBR* cDNA placed in antisense orientation under the control of the CaMV35S promoter. All boxes are not drawn to scale.

sequence and 5'UTR of *BKn3* gene contain the putative BBR binding site, reporter constructs containing the GUS reporter gene under the control of one or two copies of the 305bp sequence fused to the CaMV 35S minimal promoter or a 0.7kb *BKn3* promoter fragment (0.7kb fragment 5'of *BKn3* translation initiation site ATG) (kindly provided by Dr. Kai Müller), were used in co-transfection of protoplasts. Fig. 3-24 shows the reporter and effector constructs (including controls) used in the experiments.

When reporter constructs containing either the 305bp sequence or the 0.7kb *BKn3* promoter fragment were co-transfected with the effector construct containing sense BBR into tobacco protoplasts, no significant increase of GUS activity could be detected although the positive control construct pRT104GUS gave high GUS activity in the experiments.

### 3.6.3 Promoter analysis of *BBR*

The 2 kb 5'-flanking non-transcribed region of the *BBR* gene was isolated by genomic library screening. A number of potential *cis*-regulatory elements were found in this region (Fig. 3-25). The proximal promoter region from nucleotide -300 to -18 contains three GC boxes (CCGCCC) which are common promoter components recognized by transcription factor Sp1 in mammals (Mikami *et al*., 1987), and four CAAT boxes which are the first common type of promoter elements to be described. In addition, this region contains one SV40 core enhancer (GTGGATTG) which is present in virus and plant gene promoters (Donald and Cashmore 1990); one Pyrimidine box HV (TTTTTTCC) which is found in the barley EPB-1 gene promoter and required for GA induction (Cercos *et al*., 1999); and one amylase box (TTTGTTA) which is a conserved sequence found in 5'-upstream regions of all GA-inducible α–amylase genes of rice, wheat and barley (Gomez-Cadenas *et al*., 2001). In a region further upstream, a sequence identical to a Box C (CTCCCAC), found in the light-inducible AS1 promoter in pea (Nagai *et al*., 1997); a G Box (CACGTG), an element found within the upstream regions of a number of environmentally inducible genes (Hong *et al*., 1995; de Vottern and Ferl, 1995); and a MYB core (CGGTTG), the binding site known of all animal MYB and at least two *Arabidopsis* MYB proteins (Solano *et al*., 1995) were found.

To identify the 5'-flanking regions necessary for BBR gene expression, a series of 5' deletion derivatives of the *BBR* promoter (Fig. 3-26) was transcriptionally fused to GUS reporter gene (see Material and Methods), and plasmids were introduced in tobacco SR1 protoplasts or tobacco plants by *Agrobacterium*-mediated transformation. For unknown reasons, we could not detect a significant increase of GUS activity in protoplasts transfected with all the 5' deletion promoter/GUS constructs. It could be due to that tobacco mesophyll cells lacked the cofactor(s) which could interact with the *BBR* promoter to initiate the transcription of GUS gene. Alternatively, more upstream or downstream sequences of analyzed *BBR* promoter may be required for gene expression.

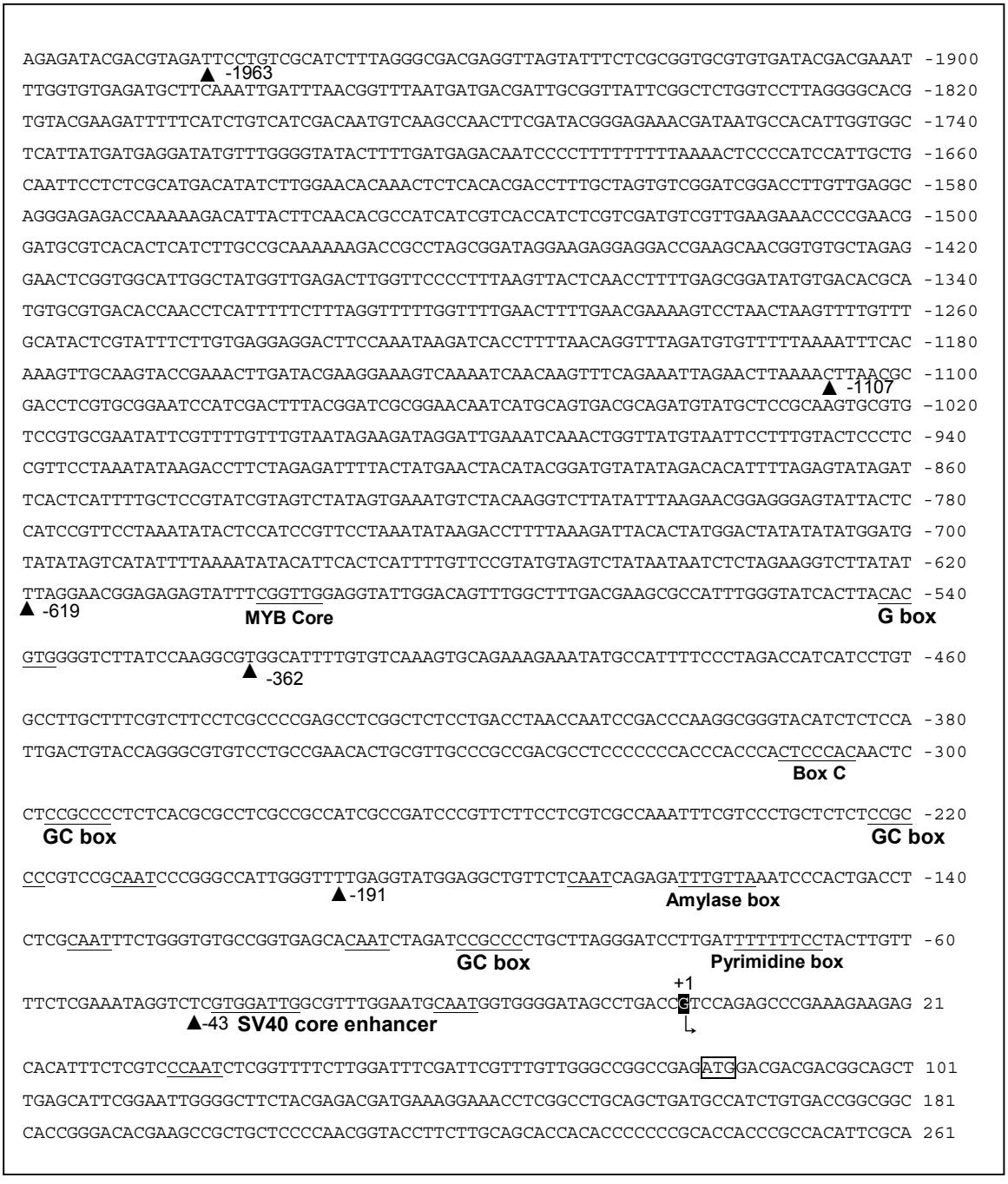



**Fig. 3-25 Nucleotide sequence of the *BBR* promoter.** Numbers indicate the distance relative to the transcription start site (+1) which is indicated by an arrow. Putative *cis* elements are indicated below the sequence and underlined. The end points of the 5' deletion constructs were indicated by arrowheads below the first nucleotides of the deletions. The translation initiation codon (ATG) is boxed.

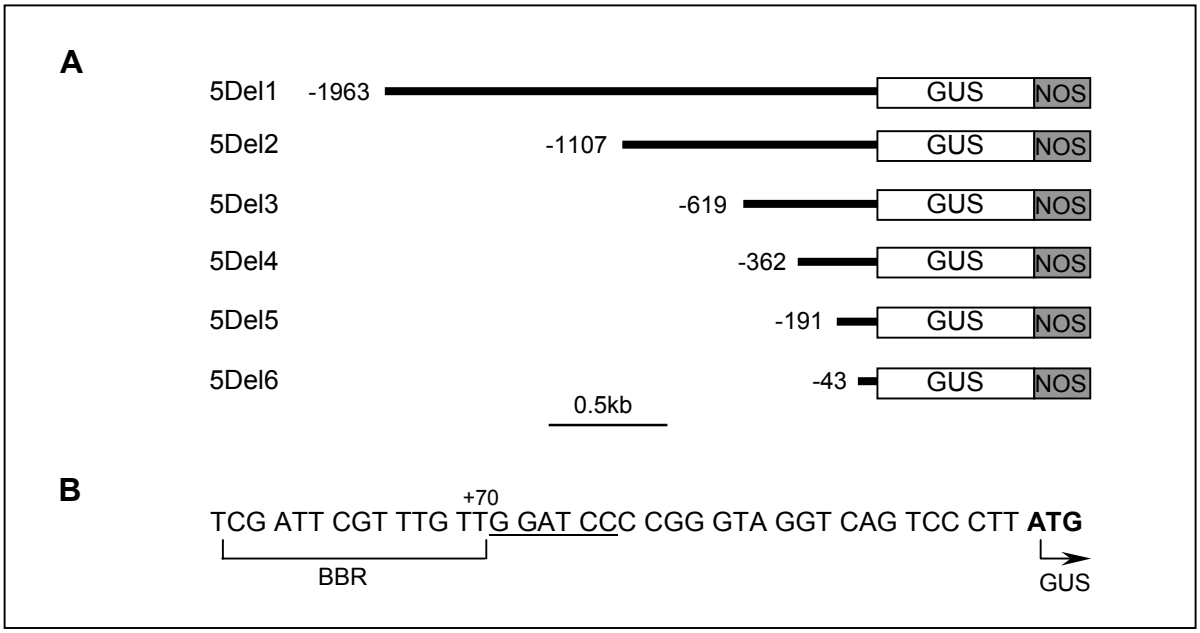



**Fig. 3-26 Schematic diagram of the 5' deletion chimeric constructs.** (A) The thick lines denote *BBR* promoter sequences. The numbers at left indicate deletion-end points relative to the transcription initiation site (+1) of the *BBR* gene. The open and grey boxes indicate the GUS gene and the NOS terminator, respectively. The boxes are not drawn to scale. (B) The junction sequences between the *BBR* gene and GUS. The *Bam*HI site used to join the two genes is underlined. The translation initiation site (ATG) of the GUS gene is indicated by boldface letters.

Testing these 5' deletion constructs in transformed tobacco plants is still going on. For each construct, forty calli which were able to grow on selective medium were selected and used for further culture and regeneration. Currently, shoots generating from the transgenic calli are transferred to new medium to form roots. The GUS activity and expression pattern will be analyzed in $T_0$ transgenic tobacco plants in the near future.

# DISCUSSION

## *CHAPTER 4*

## 4.1 The *BKn1* gene is expressed in the barley shoot apex and young leaf primordia

Like other class I *Knox* genes, *BKn1* mRNA is found in all apical tissues examined by Northern blot and RT-PCR, including embryos, shoot apices, seedling roots, stems and infloresecencs, but not in differentiated tissues, like mature leaves (see sections 3.1.1 and 3.1.2). However, detailed expression analysis by *in situ* hybridization revealed that unlike some closely related class I *Knox* genes, *BKn1* is expressed in the L1 layer of meristems, young leaf primordia, very young leaves and lateral organs of inflorescences (see section 3.1.3).

Within the meristem, the expression patterns of different class I *Knox* genes are distinct as illustrated schematically in Fig. 4-1 (reviewed by Reiser *et al*., 2000). Expression of some class I *Knox* genes, including *Kn1* from maize, *OSH1* from rice and *STM* from *Arabidopsis*, is detected throughout the meristem and excluded from lateral organs (Fig. 4-1A). mRNA of another group of class I *Knox* genes, including *RS1*, *Knox4* and *Knox3* from maize, *OSH15* from rice, tends to be excluded from the central regions of the meristem and instead accumulates in the periphery, between lateral organs (Fig. 4-1B). Suppression of these *Knox* gene expression in the future initiation sites of leaves on the flanks of apices invoked the inference that such a down-regulation is a prerequisite for leaf initiation (reviewd by Hake *et al*., 1995).

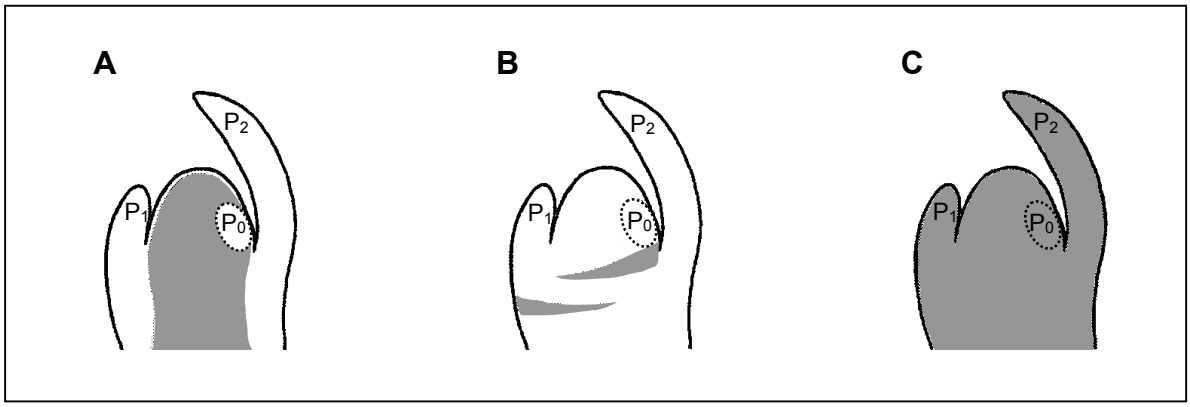



**Fig. 4-1 Schematic representation of the three major types of gene expression seen for *Knox* genes** (adapted from Reiser *et al*., 2000). The meristem outlined has a distichous phyllotaxy. (A) *Kn1*-like expression throughout the meristem and excluded from $P_0$ and L1 layer. (B) *RS1*-like gene expression in the meristem is restricted to positions between the lateral organs. (C) *Knox* gene pattern showing expression throughout the meristem including the L1 and $P_0$ is similar to *Knox* expression in tomato.

In contrast to those class I *Knox* genes desribed above, the expression pattern of two tomato class I *Knox* genes, *TKn1* and *Tkn2*/*LeT2*, were considered to be an exception. *TKn1* and *Tkn2*/*LeT2* mRNAs are not down-regulated in the $P_0$ and continue to be expressed in immature leaves (Chen *et al*., 1997) (Fig. 4-1C). The expression of these class I *Knox* genes in young

leaf primorida and immature leaves was explained as a contribution of *Knox* genes to the creation of compound leaves, which retain meristematic activity to organize leaflets.

The expression analysis of *BKn1* (this thesis) and *BKn3* (Müller *et al*., 2001) in barley shoot meristems revealed similar expression patterns as those of *TKn1* and *Tkn2*/*LeT2*, although barley has simple leaves. The sequence of *BKn1* is highly similar to those of *OSH15* and *RS1*, and *BKn3* was considered as the orthologous gene of *Kn1* and *OSH1*. Based on the sequence similarity, one would expect that *BKn1* and *BKn3* exhibit *RS1*- and *Kn1*-like expression patterns, respectively. This prediction is not supported by the detailed expression analysis presented in this thesis (see section 3.1.3). The expression patterns of *BKn1* and *BKn3* indicate different regulation mechanisms in barley as opposite to those in maize, rice and *Arabidopsis*. Alternatively, as it has been suggested that the $P_0$ downregulation of *Knox* genes in maize and *Arabidopsis* might be coincidental and inconsequential to leaf initiation (Chen *et al*., 1997), the downregulation of *Knox* genes in $P_0$ might not be the only prerequisite for leaf initiation and development in barley.

The inference that a downregulation of *Knox* gene expression in the leaf primordium is a prerequisite for leaf initiation is followed by that ubiquitous expression of *Knox* genes alters phyllotaxis (Hake *et al*., 1995). This prediction has not yet been confirmed. In plants bearing dominant overexpressing mutations such as *Kn1*, *Rough sheath1*, *Cu* or *Me*, or in transgenic tobacco, *Arabidopsis*, and tomato plants expressing *Knox* genes, phyllotaxis was never altered (Parnis *et al*., 1997).

When the expression of *Kn1*-like proteins is analyzed in 35S-*Kn1* transgenic tobacco shoot apices, there appears to be a downregulation of *Kn1*-like proteins in the $P_0$ region, but ectopic expression (presumably of the 35S-*Kn1* transgene) is still seen in the developing leaf primordia. Thus, even under synthetic overexpression conditions using strong constitutive promoters, downregulation of *Knox* gene expression is seen in the $P_0$ region of tobacco (Chen *et al*., 1997).

The *rs2* gene in maize is expressed in young leaf primordia and its gene product is considered as a negative regulator of several KNOX proteins. However, immunolocalization studies of even the most severe *rs2* mutant plants indicate that KNOX proteins are still down-regulated appropriately at $P_0$ (Schneeberger *et al*., 1998).

The morphogenic differences induced by ectopic expression of the two class I *Knox* genes, for example, *Knotted1* in leaves and *Hooded* in lemmas, might not be indicative of different functions, but of different regulation mechanisms in different species. We propose that the two genes are expressed under different spatial and temporal controls in two different species.

There is also evidence to suggest post-transcriptional regulation of *Knox* gene expression. In barley, the ubiquitin promoter drives constitutive gene expression. However, when *Kn1* was

expressed from the ubiquitin promoter, *Kn1* mRNA was detected only in the awn and ectopic flowers. The transgenic barley plants showed no abnormal leaf phenotype, although ectopic meristems formed on the adaxial surface near the lemma/awn transition zone (Williams-Carrier *et al*., 1997). Rice plants transformed with five rice class I *Knox* genes (*OSH1*, *OSH6*, *OSH15*, *OSH43* and *OSH71*) under the control of either the cauliflower mosaic virus 35S promoter or the rice *Act1* gene promoter exibitied severely malformed leaves with ectopic knots on their adaxial side (Sentoku *et al*., 2000). Knot formation and ligule displacement that occurred in transgenic rice plants, were similar to those seen in spontaneous dominant *Kn1* mutants. However, even in plants with severe phenotypes, meristem structures were never found in the lemma.

Thus mRNA levels alone may be the wrong parameter, and post-transcriptional, but otherwise species-specific regulation may operate in all plants to eliminate unwanted *Kn1* gene products. It is also possible that we are looking at a secondary phenomenon, that other unkown factors are of primary importance, and that correlation alone may sometimes be misleading (Parnis *et al*., 1997). One approach to clarify this issue, therefore, is to compare the effects of different *Knox* genes in the same species and under the control of the same regulatory sequences.

## 4.2 Possible developmental roles of *BKn1* and *BKn7*

### 4.2.1 Possible developmental roles of *BKn1*

In general, class I *Knox* genes show strong expression around the shoot apical meristem, and their ectopic expression in spontaneous mutants and in transgenic plants alters leaf and flower morphology (Freeling *et al*., 1992; Matsuoka *et al*., 1993, 1995; Lincoln *et al*., 1994; Müller *et al*., 1995; Schneeberger *et al*., 1995). Strong alleles of *STM* seedlings lack a visible shoot apical meristem and have cotyledons with fused petioles, and meristems are terminated prematurely in partial loss-of-function or weak alleles of *STM* (Barton and Poethig, 1993; Clark *et al*., 1996; Endrizzi *et al*., 1996). These data suggest a role for *STM* in meristem initiation or maintenance. However, all *Kn1* loss-of-function alleles only exhibit subtle phenotypes (Kerstetter *et al*., 1997). This may indicate that the loss of *kn1* uncovers a redundant factor in the maize genome. The normal role of *kn1* is still unclear and may be broader than that revealed by loss-of-function mutants, if expression of other *Knox* genes can partially compensate for its loss. Based on these observations, it has been proposed that the *Knox* genes are involved in determination of cell fate and patterning in the meristem and able to alter plant morphology profoundly when overexpressed (reviewed by Reiser *et al*., 2000).

*BKn1*, like other class I *Knox* genes, is expressed in shoot apical meristems and floral meristems (see section 3.1.3). Overexpression of *BKn1* in transgenic tobacco plants caused leaf phenotypes such as rumpling, reduced lamina and formation of ectopic shoots on the

leaves (Kai Müller, 1997). So it is likely that *BKn1* plays a similar role in barley development, although this remains to be determinated in the future.

Although most of the class I *Knox* genes are expressed in the developing vascular tissue of stems (see Table 1-1), the function of these genes in stems still remains to be elucidated. Four independently loss-of-functions of *OSH15* were isolated which are defective in internode elongation (Sato *et al*., 1999). Within the internodes of mutant plants, epidermal and hypodermal cellular morphology was defective; the cells were abnormally shaped and some cell types were absent. These data suggest that *OSH15* has a role in the devleopment of the rice internode. It is the first evidence that class I *Knox* genes control processes other than shoot apical meristem formation and/or maintenance in plant development. In our studies, *BKn1* expression is detected in barley stems at relatively high level and *BKn1* mRNA is localized in the rib meristem zones (see section 3.1.3). It is possible that *BKn1* gene expression in stems is responsible for regulating stem elongation like *OSH15*.

The role of the class I *Knox* genes in roots has not been well discussed, although studies demonstrated that some of class I *Knox* genes are indeed expressed in roots. *Rs1* in maize (Schneeberger *et al*., 1995), *Lg3* in maize (Muehlbauer *et al*., 1999), *KNAT2* in *Arabidopsis* (Lincoln *et al*., 1994) and three class I *Knox* genes in *Pharbitis* (Kobayashi *et al*., 2000) are expressed in roots. Expression of *Kn1* and *OSH1* in embryonic roots was detected by *in situ* hybridization (Smith *et al*.,1995; Sato *et al.*, 1996). The relatively strong expression of *BKn1* in embryonic roots and weak expression in seedling roots (see sections 3.1.1 and 3.1.2) support the possibility that *BKn1* might be involved in the development of roots as well as shoots. A detailed expression analysis of *BKn1* genes during postembryonic root development will give more information about its possible functions in roots.

### 4.2.2 *BKn7*, a class II *Knox* gene, may play a role in barley development

No mutant phenotypes have been associated with loss-of-function of any class II *Knox* gene. Redundant functions shared by members of this class may mask a loss-of-function phenotype. Alternatively, loss of function of some class II genes may have either a subtle effect or such a strong effect on development that they are lethal (reviewed by Reiser *et al*., 2000). Despite the lack of loss-of-function mutants, the variation of gene expression patterns for class II genes suggests diverse roles for members of this family. It may be possible to clarify the functions of these genes through more careful molecular approaches.

The expression analysis has revealed that *BKn7* has a more diverse pattern than class I *Knox* genes, like all the other class II *Knox* genes characterized to date. *BKn7* expression can be detected in all tissues examined, including embryos, shoot apices, leaves, roots, stems, inflorescences and mature floral organs, but with different levels (see sections 3.1.1 and 3.1.2).

The lowest level of *BKn7* expression was observed in roots, but in other tissues, *BKn7* is expressed at higher level. This expression pattern might reflect the functions of *BKn7* in barley development. But it is difficult to propose a single function that would require *BKn7* expression in so many different tissues at so many different times, especially the diverse patterns do not clearly indicate a common role for *BKn7*. It may be playing several different roles depending on where and when it is expressed in the developing plant. The possibility of diverse roles correlates with the observation that homeodomain-containing proteins often work as homo- or heterodimers with other transcription factors, and these interactions can result in very different specificities in terms of targets and their regulation (Goutte and Johnson, 1994; Wilson *et al*., 1993). It has been proved by the yeast two-hybrid system and *in vitro* binding assays that BKN7 can form homodimers as well as heterodimers with BKN1 and BKN3, two class I *Knox* genes (Müller, 1999; Müller *et al*., 2001). Further analysis of the network formed by these *Knox* genes and identification of their target genes will help us better understand the functions of class II *Knox* genes in barley development.

## 4.3 Putative upstream regulators of *BKn3*, discovered by the yeast one-hybrid screening

### 4.3.1 Putative enhancer element in *Bkn3* intronIV

Generally, *cis*-acting elements required for gene expression are found in regions upstream of the transcription initiation site (promoter); however, it is not unusual to find control elements located downstream of a gene as well (Dietrich *et al*., 1992; Larkin *et al*., 1993). Recently, an increasing number of studies showed that regulatory control elements can also reside within a gene. In *Drosophila*, human and mice, several genes have been identified that contain regulatory control elements within introns (Gremke *et al*., 1993; Hinz *et al*., 1992; Shamsher *et al*., 2000; Seul and Beyer, 2000). In plants, the presence of the first intron has been correlated with enhanced levels of gene expression for several different genes. For instance, the inclusion of the alcohol dehydrogenase 1-S (*Adh1-S*) intron 1 in the transcription unit of maize gene constructs was shown to increase gene expression in cultured maize cells (Luehrsen and Walbot, 1991); the first intron of the maize *Shrunken-1* gene can stimulate reporter gene expression in maize protoplasts up to 100-fold (Clancy *et al*., 1994). It has aslo been demonstrated that enhancer elements are present in the second large intron of *PLENA* (Bradley *et al*., 1993) and *AGAMOUS* (Sieburth and Meyerowitz, 1997; Deyholos and Sieburth, 2000), the C-function floral organ identity genes in *Antirrhinum* and *Arabidopsis*, respectively.

The homolog of *BKn3* from maize, *Knotted-1*, has a genomic organization that is nearly identical to that of *BKn3* except for an additional small intron present in the 5´UTR of *BKn3*

(Müller *et al*., 1995; Kai Müller, 1997) (Fig. 4-2).

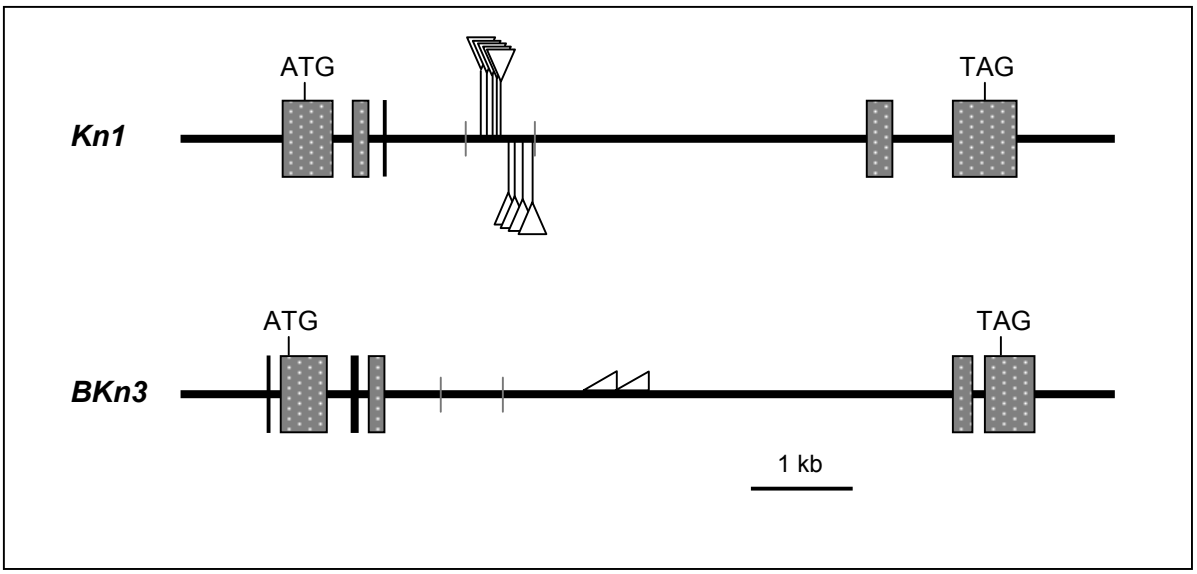



**Fig. 4-2 Genomic organization of the *Kn1* locus in maize displaying the positions of the *Mu* insertions** (*open triangles*) **and the *BKn3* locus in barley showing the position of the 305bp duplication** (*open triangles*) (adapted from Greene *et al*., 1994 and Müller *et al*., 1995). Exons are shown as shaded boxes. The regions between grey lines in *Kn1* and *BKn3* have 76.67% identity in their sequences.

The *Kn1-0* allele of dominant mutant *Knotted1* is a tandem duplication of *Kn1* in which a second coding region is associated with a novel 5´-upstream region (Veit *et al*., 1990; Mathern and Hake, 1997). Many of the other *Kn1* alleles characterized to date are associated with insertions of transposable elements into the large third intron (Greene *et al*., 1994) (Fig. 4-2). These transposon insertions point to at least two potential regulatory domains within the *Kn1* genomic regions that determine *Kn1* expression in leaves. Based upon the phenotype of certain dominant and revertant *Kn1* alleles, it was suspected that leaf silencing elements present at the 5´ end of the gene and in the large third intron. Mutation of either of these regulatory domains or of second-site loci that interact with these domains could alter the spatial or temporal pattern of *Kn1* gene expression (Greene *et al*., 1994; Mathern and Hake, 1997).

The dominant barley *Hooded* mutant is caused by a 305bp duplication in the fourth intron of *BKn3*. The appearance of *Hooded* is accompanied by a deletion of 33bp located 300bp 5´to the 305bp duplication in the fourth intron. The 5´ sequence of the fourth intron of *BKn3* shares 76.67% identity to the 5´sequence of the third intron of *Kn1* where transposons insert (Müller, 1997) (Fig. 4-2). The enhancer activity of the 305bp intron sequence has been examined in transgenic tobacco plants. In combination with a CaMV35S minimal promoter, the 305bp intron sequence induces tissue-specific expression of the GUS reporter gene in tobacco. Staining is observed in aerial vegetative and reproductive branching points and in the flower base (Kai Müller, unpblished data). It is very likely that this intron IV carries regulatory *cis*-

elements which are important for the formation of epiphyllous flowers on the lemma of the barley spikelets.

### 4.3.2 Putative upstream regulators of *BKn3*

In spite of increasing evidence for *cis*-acting elements within introns regulating the gene expression in many different systems, we are far from understanding its molecular basis. Identification of the DNA-binding proteins which bind to these *cis*-acting elements would be very helpful to get the answers. Using one copy or three copies of the 305bp intron sequence as a bait to screen *Hooded* barely inflorescence expression library in the yeast one-hybrid system led to the isolation of four interesting barley cDNA clones, which could be the putative upstream regulators of *BKn3* (Kai Müller, unpublished data).

Deduced amino acid sequence of *BEIL* share 60% sequence homology to the members of *Arabidopsis* EIN3/EIL family (Chao *et al*., 1997) (Fig. 3-11). *Arabidopsis EIN3* was cloned through the screening of *Arabidopsis* mutants which showed a much reduced response to ethylene treatment, indicating that wild-type *EIN3* gene product is an essential regulator in the ethylene signaling pathway. The other three *EIN3*-like genes were also isolated in the same screening. These four genes encode nuclear-localized proteins that share sequence similarity, structural features, and genetic function (Chao *et al*., 1997). The sequence similarity between *BEIL* and *EIN3/EIL* genes suggests that BEIL might be the homologous gene of *EIN3/EIL* and function in a similar way in barley.

*BAPL*, encodes a protein containing a putative DNA-binding domain found in several members of EREBP/AP2 family (Fig. 3-12). EREBPs mediating ethylene response gene activation in tobacco have been identified (Ohme-Takagi and Shinshi, 1995), and related genes have been observed in *Arabidopsis* (Ecker, 1995; Weigel, 1995). BAPL, a barley EREBP-like protein, might also involve in the ethylene response pathway.

*BGRF* gene encodes a protein showing 58% sequence similarity to OSGRF1 from rice (Fig. 3-13), which is induced by GA and considered as a putative transcription factor (see comments in AF201895).

Since BBR amino acid sequence does not show homology to any proteins characterized so far, it is difficult to predict its roles in barley development. In the proximal promoter of BBR (see Fig. 3-25) there are one Pyrimidine box (TTTTTTCC) (-75 – -68) and one amylase box (TTTGTTA) (-160 – -154), which are also found in the promoter of all GA-inducible α–amylase gene of rice, wheat and barley (Gomez-Cadenas *et al*., 2001). They might indicate that BBR gene expression is linked to the GA response although their importance for the BBR gene expression remains to be determined in the future.

Although we did not demonstrate that BEIL, BAPL and BGRF recombinant proteins and *in vitro* translated products can bind to the 305bp intron sequence in the *in vitro* DNA-binding assay yet, it does not mean that they can not bind to 305bp fragment *in vivo*. We believe that better quality of proteins will be helpful to improve the *in vitro* DNA-binding results in the future. The identification of *BKn3* as the target of these proteins in the plant cell nucleus would represent an interesting linkage between *Knox* genes and plant hormone respones, metabolism or transport pathways.

The genomic clones of all four putative upstream regulators of *BKn3* were isolated and sequenced. The genomic structures of the four genes were established by comparing genomic sequences with the full-length cDNA sequences. *BEIL*, *BAPL* and *BGRF* contain two, three and three introns, respectively. All introns have the conserved splice site sequences at their 5´ and 3´ ends, following the `GT...AG´ rule of plant introns (Table 3-1). The ORF of *BBR* is not interrupted by any introns. Genomic Southern blot analyses using full-length cDNAs as probes indicated that all of them are single copy genes in the barley genome (Fig. 3-16). Their promoters isolated in the genomic library screening can be used to further analyze their expression *in vivo* by promoter/reporter gene fusions.

Northern blot and RT-PCR analyses revealed that *BEIL*, *BAPL*, *BBR* and *BGRF* have broader expression patterns than *BKn3* and are expressed in all barley tissues examined, including embryos, leaves, roots, stems and inflorescences, at different levels (see Fig. 3-17). *BEIL* and *BAPL* gene expressions are not dramatically altered in *Hooded* mutant inflorescences, however, *BBR* and *BGRF* are up-regulated in *Hooded* mutant inflorescences. Currently it is difficult to explain why *BBR* and *BGRF* mRNAs are more abundant in *Hooded* inflorescences.

The mapping of putative upstream regulators of *BKn3* and recessive suppressors of the *Hooded* phenotype (*suK*) is being undertaken in an effort to associate genes to mutations. Presumably, *BEIL*, *BAPL*, *BBR* and *BGRF* could represent potential candidates for these suppressors obtained through second site mutagenesis (Müller et al., 2000). Up to now one of recessive suppressors has been mapped to linkage groups in proximity to AFLP markers (Pozzi, 1998). The SNP DNA marker type used in this study for gene mapping is based on polymorphisms produced by single point mutations and thus provides a higher potential to detect polymorphisms than the RFLP/AFLP approach (Castiglioni *et al*., 1998). The map positions for these four genes were determined by SSCP analysis with a mapping population derived from the barley lines Nudinka and Proctor. *BEIL* was mapped to chromosome 1 in an area of high marker density (see Fig. 3-21). The mapping of *BAPL*, *BBR* and *BGRF* and other suppressors is in progress. Once that all of the available recessive suppressors and putative upstream regulators of *BKn3* have been mapped to the barley linkage map, the potential cosegregation of identified genes and mutant phenotype can be investigated.

## 4.4. The *BBR* gene encodes a novel nuclear-localized DNA-binding protein

### 4.4.1 The BBR protein contains a novel DNA-binding domain

The BBR deduced amino acid sequence does not share any homology with known proteins in the existing databases, rendering predictions of a conserved biological function difficult to make. The results from the yeast one-hybrid screening and *in vitro* binding studies demonstrate that it is a novel DNA-binding protein. The C-terminal part of BBR, which is the putative DNA binding domain (243-350), shares 86% or higher sequence similarity to three putative *Arabidopsis* proteins in the *Arabidopsis* genome database (see section 3.2.2), which have not been analyzed yet. These three putative proteins also show high sequence homology with BBR protein at the N-terminal part and a nuclear localization sequence (NLS). So these putative *Arabidopsis* proteins might be also nuclear-localized due to the bipartite NLS near the putative DNA-binding domain. The middle part of all four proteins shows more diversity (Fig. 3-15B).

Three putative *Arabidopsis* proteins (Genebank accession numbers are AC010657, AC006532.2 and AC012563.5, respectively) share more homology to each other (Fig. 3-15B). AC010657, the most similar protein of BBR, seems to be the common ancestor of AC006532.2 and AC012563.5. AC006532.2, which is located on chromosome II, might be derived from AC010657, which is located on chromosome I, through a gene duplication event in different chromosomes happened more recently. AC012563.5, located on chromosome I, might be derived from AC01657 through a gene duplication event in the same chromosome. By analogy to the *BBR* gene, all three *Arabidopsis* genes have similar genomic structures without disruption by any intron.

It will be very interesting to know the functions of the three BBR homologues in *Arabidopsis* through a reverse genetics approach. But one has to keep in mind that the gene redundancy may complicate the loss-of-function studies.

It would be also very interesting to know the three-dimensional structure of this novel DNA-binding domain by crystalization study of the BBR protein.

### 4.4.2 BBR binds to the 305bp intron sequence *in vitro*

The BBR binding site in the 305bp intron sequence, established by *in vitro* binding studies (see details in section 3.4.4), is a pyrimidine-rich sequence 5′-$(TC)_8$-3′, which is also present in 5′UTR of *BKn3* in reverse orientation. A search for similar DNA-binding sites in published literature revealed that it has not been reported for any DNA-binding proteins characterized to date.

Our searches using Blastn for sequences showing an exact match of TCTCTCTCTCTCTCTC in the databases showed that several genes contain this short sequence in their promoters or 5′UTR. In the 5′UTR of the *Arabidopsis Superman* gene (Sakai *et al*., 1995), 5′-$(TC)_8$-3′ is present in the most densely methylated region of the gene. It has been supposed that a hairpin structure formed by this sequence and its neighbouring region is involved in targeting *Supermen* for hypermethylation (Jacobsen *et al*., 2000). Also the promoter region of the *Carpel Factory* gene (Jacobsen *et al*., 1999) contains 5′-$(CT)_{22}$-3′ (beginning at nucleotide 22869 in GenBank sequence AC007323). Near the beginning of transcription of the *LEAFY* gene, a sequence 5′-T$(CT)_6$ATC-3′ is found very similar to the binding site of the BBR protein (beginning at nucleotide 2742, GenBank sequence M91208). Methylation was not detected at either of the sequences for *Carpel Factory* and *LEAFY* (Jacobsen *et al*., 2000). It indicated that this pyrimidine-rich region is not necessarily related to the hypermethylation event. In the sequence of the barley transcription factor *GAMyb* gene, there are two sequences 5′-$(AG)_{11}$-3′ (5′-$(CT)_{11}$-3′ in the complementary strand) located at the promoter region (-118 to -97) and 5′UTR (Gomez-Cadenas *et al*., 2001). The *GAMyb* gene is a GA-regulated transcription factor acting as an activator of downstream GA-regulated genes encoding α-amylases and proteinases. The presence of the BBR binding site in the promoters and 5′UTRs of several genes involved in plant development, may indicate the importance of this *cis*-acting element in gene regulation, although further analysis is needed to confirm it.

*In vitro* binding studies also showed that BBR is able to bind to DNA without the involvment of another protein. It is not currently known whether BBR forms a homodimer or a heterodimer with another partner.

We think that this DNA-protein interaction is physiologically significant since the yeast one-hybrid system detects binding that occurs *in vivo*, and a natural intron sequence, not an artificial multimerized sequence, was employed in the screenings (Kai Müller, unpublished data). The fact that four different cDNA clones representing the *BBR* gene were obtained from the one-hybrid screening also indicates the interaction between BBR DNA-binding domain and its binding site in the 305bp sequence is not just an artifical event. The significance of this DNA-protein interaction will be elucidated by examining the transactivation activity of the BBR protein in the future.

### 4.4.3 The BBR protein is localized in the nucleus

A nuclear targeting assay showed that BBR is able to direct a GFP fusion protein into the nucleus, which is consistent with BBR's presumed function as a transcription factor.

The BBR portein contains a bipartite nuclear localization sequence (NLS) `PVKKRQQ GRQPKVPKP KKPKK′. It meets the criteria of a functional bipartite NLS, which comprises

two basic amino acids, a spacer region of any ten amino acids and a basic cluster in which three out of the next five amino acids must be basic (reviewed by Dingwall and Laskey, 1991). This NLS is located at the C-terminal part of the protein and near the conserved, putative DNA-binding domain at the C-terminal end. It has been suggested that the bipartite NLS may be the most prevalent NLS in all nuclear proteins (Varagona *et al*., 1992). Although we can not rule out that other functional NLSs may exist in other parts of the sequence, our data suggest that this bipartite NLS is sufficient for BBR nuclear targeting (Fig. 3-23).

In summary, it has been demonstrated that the BBR protein is localized in the nucleus and contains a novel DNA-binding domain, which binds to the 305bp intron sequence of *BKn3* in a sequence-specific manner. So probably it functions as a transcription factor regulating the expression of *BKn3* gene through binding to a *cis*-acting element within the 305bp intron sequence (see below).

The possible reasons that we could not detect the transactivation activity of the BBR protein in tobacco protoplasts in preliminary experiments could be the following: (1) BBR might not be a transcription activator but a repressor. The system used in the experiments is more suitable to detect transcription activators than repressors. (2) The tobacco mesophyll protoplast could lack the cofactors which are present in the transcription machinery of barley cells. (3) The reporter gene and the effector could be separated temporally and spatially.

To solve the problem, different constructs could be tested in both heterologous and homologous systems in future studies. For example, considering the first possible reason we could use reporter constructs which contain GUS reporter gene under the control of the 305bp intron sequence fused to strong expressed promoter; for the second possible reason, we could use barley endosperm suspension cells for transfection; for the third possible reason, we could integrate reporter gene and effector into one plasmid vector. The identification of BBR as an transcription activator or repressor will be very helpful to understand the possible regulation mechanism of *BKn3* by its intron IV in the future.

## 4.5 Models for *BKn3* regulation

We do not know the exact nature of the regulatory roles of the BBR protein. Although further studies are required to determine its specific roles, several models can be speculated for explaining the overexpression of *BKn3* caused by the 305bp duplication in intron IV at the transcriptional level (Fig. 4-3).

One is that, in the *Hooded* mutant, due to the duplication of the 305bp sequence, there would be two BBR binding sites within the duplicated region. The double amount of BBR protein would bind to intron IV. However, the amount of other *trans*-acting elements binding to intron

IV sequence outside the duplicated region would not be increased. If BBR protein and these *trans*-acting elements transactivate *BKn3* expression antagonistically, the expression of *BKn3* would be activated in the *Hooded* mutant due to the changed ratio between BBR protein and other *trans*-acting elements. It is not currently known whether the BBR protein is a transcriptional activator or repressor. But this will not prevent us to explain the regulation of *BKn3* expression using this model, since not only BBR is expected to bind to the 305bp duplication. Some other factors could also bind to the duplicated region and regulate *BKn3* gene expression together with the BBR protein.

Alternatively, *BKn3* gene expression might be regulated by *trans*-acting factors which bind to the intron IV sequence through chromatin remodeling (Fig. 4-3B). It has been demonstrated that in *Drosophila*, shaping the embryo depends critically on the precise temporal and spatial expression of homeotic genes, which is ensured by epigenetically relevant chromatin-remodeling mechanisms (reviewed by Müller and Leutz, 2001). For example, the restricted transcription of the homeotic genes located in the *Antennapedia* and *Bithorax* complexes in *Drosophila* embryogenesis is initially achieved by gap and pair-rule transcription factors that act only transiently early in development. Later on, the expression patterns of homeotic genes are maintained by Polycomb group (PcG) and trithroax group (trxG) proteins, which exert their functions by stabilizing distinct chromatin structures. PcG proteins are involved in gene repression and silencing; trxG proteins maintain gene activation and counteract repressive PcG functions (reviewed by Farkas *et al*., 2000). In plants similar mechanisms may also be present. It has been shown that the *CURLY LEAF* gene (*CLF*) of *Arabidopsis*, the first PcG gene identified in plants, is necessary for stable repression of the floral homeotic gene *AGAMOUS* (*AG*) in *Arabidopsis* leaves, inflorescence stems and flowers (Goodrich *et al*., 1997). Recently, more genes encoding PcG proteins have been isolated from *Arabidopsis* (Grossniklaus *et al*., 1998; Ohad *et al*., 1999).

An example for ordered recruitment of transcription factors, chromatin-modifying activities and the basal transcription machinery at a complex mammalian promoter has now been described (Agalioti *et al*., 2000). Binding of different transcription factors to the INFβ enhancer results in the recruitment of the GCN5 histone acetylase complex immediately followed by the recruitment of the CBP and the Pol II holoenzyme complex. Histone acetylation at the INFβ promoter stimulates chromatin remodeling mediated by the SWI/SNF complex which is recruited in a later step. Once chromatin remodeling has occurred, TFIID can enter the promoter and initiate transcription.

Based on the information regarding the chromatin mediated gene regulation, a hypothesis can be made for explaining the overexpression of *BKn3* gene caused by the 305bp duplication (Fig. 4-3B). In the wild-type, transcription factors with similar functions of PcG and trxG bind to intron IV and the promoter of *BKn3* and determine where and when *BKn3* gene is activated

through controling the chromatin structure. In the *Hooded* mutant, the double amount of BBR protein and other transcription factors binding to the 305bp duplication region, which have supposed functions of trxG, antagonize the repressive functions of PcG-like proteins binding to other parts of intron IV. This action results in the recruitment of the GCN5 histone acetylase complex followed by the establishment of active chromatin domains. Then the basal transcription machinery can access the promoter of *BKn3* to initiate its transcription.

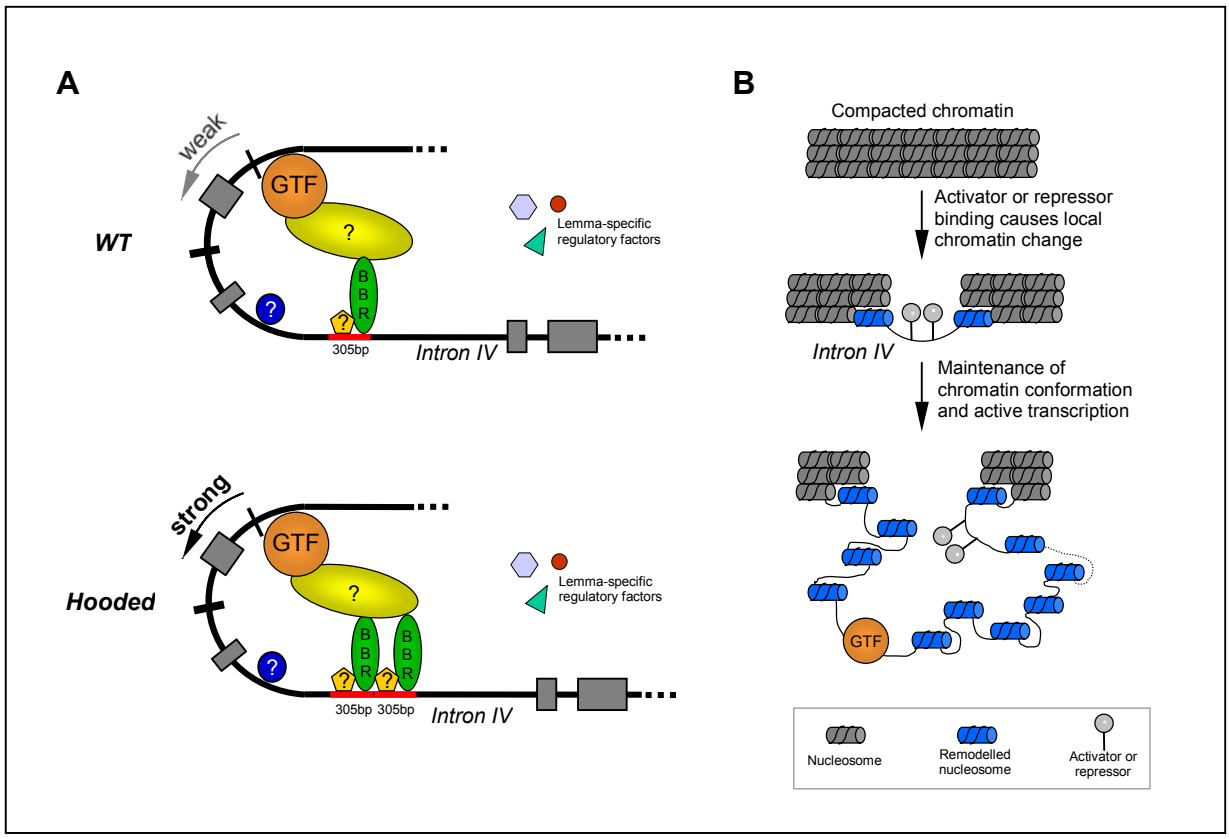



**Fig. 4-3 Models for *BKn3* regulation.** (A) The BBR protein and other *trans*-acting elements bind to intron IV of *BKn3*, regulating *BKn3* gene expression at the transcriptional level. Considering that *cis*-acting elements may exist 5′ upstream of the 305bp fragment in *BKn3* intron IV, additional factors are expected to be involved. There are also multiple binding sites for the factors within the *BKn3* promoter, thus implying potential combinatorial interactions between multiple *cis*- and *trans*-acting elements. In the lemma of wild-type inflorescences there is weak *BKn3* expression . However, in the lemma of *Hooded* mutant inflorescences there is strong *BKn3* expression caused by the double amount of BBR and other transcription factors binding to the 305bp duplication. It is supposed that some lemma-specific regulators are also involved in *BKn3* overexpression. (see more details in the text) (B) *BKn3* gene expression is regulated by BBR and other *trans*-acting elements through a chromatin-mediated gene regulation mechanism. (see more details in the text)

Based on the fact that the overexpression of *BKn3* causes the formation of extra florets on the lemma of the *Hooded* inflorescene, it is likely that *BKn3* gene expression requires the cooperation of, or is inhibited by, other factors that are expressed in a spatially or temporally restricted pattern during barley development.

Whatever the actual roles of BBR may be, it is suggested that the regulation of the *BKn3* gene is complex. It could be regulated at all possible levels, including transcriptional, post-transcriptional and post-translational level. It could also be regulated epigenetically. Models described above are pure hypotheses and could be much more simple than what they should be, or totally wrong. As is often the case, discoveries often yield new questions, and the study of plant *Knox* genes will likely lead in unexpected directions.

## 4.6 Future perspectives

Elucidating the function of a gene poses a major challenge. An ever increasing number of genes have currently been identified by numerous approaches including sequencing of complete genomes. Despite the relative efficiency of gene isolation, the methods available to dissect gene functions are still rather indirect, laborious and slow. A complete understanding of gene functions requires information on many levels: knowledge of transcriptional, post-transcriptional, translational and post-translational regulation, binding constants, structures, protein interactions and cellular networking.

As a first step to uncover the possible function of the barley *Knox* genes, we have performed detailed expression analyses of these genes. Several putative upstream regulators and interaction partners (Müller, 1999; Müller *et al*., 2001) of *BKn3* have been identified to form a small *BKn3* network. However, we are still far from understanding the functions of barley *Knox* genes and the formation of the hood on the lemma of barley inflorescences. Currently the isolation of further BKN3 interaction partners is being carried out by large-scale two-hybrid screening at the Department of Prof. Dr. Salamini. A combination of screening aproaches, such as DNA microarrays, proteomics and protein-interaction studies, will help us to build up a detailed *BKn3* network, which may provide us a comprehensive view of the action of *Knox* genes during barley development. In addition, new technologies and general knowledge of gene function and regulation generated by *Arabidopsis* functional genomics will certainly accelerate progress towards understanding barley *Knox* genes within the next decade.

# Summary

*Knox* (*Knotted-1* like homeobox) genes form a large gene family of plant homeobox genes. Previously, seven *Knox* genes were isolated from barley (*Hordeum vulgare* L.) by using the maize *Knotted-1* homeobox sequence as a hybridization probe (Müller, 1993; 1997).

As an initial step towards understanding their functions in barley development, this thesis has investigated the expression patterns of two barley *Knox* genes by Northern blot, RT-PCR and *in situ* hybridization. *BKn1*, a class I *Knox* gene, was shown to be expressed in meristematic tissues, including shoot apices, young leaf primordia, stems, floral meristems and developing floral organs. *BKn1* mRNA accumulation could also be detected in embryonic roots and seedling roots. In contrast, the class II *Knox* gene *BKn7* showed a broader expression pattern and its mRNA could be detected in all tissues tested, including embryos, leaves, roots, stems, inflorescences and developed floral organs. Based on the detailed analyses of their expressions in different barley tissues at different developmental stages, their roles in barley development are discussed.

*Hooded* is a dominant barley mutant, characterized by the appearance of an epiphyllous floret on the lemma of the flower and caused by a duplication of 305bp in intron IV of *BKn3*, a class I *Knox* gene (Müller *et al*., 1995). When one and three copies of this 305bp fragment were used as "baits" in the yeast one-hybrid screening, four different cDNAs encoding barley proteins binding to the 305bp sequence were isolated, designated *BEIL*, *BAPL*, *BBR* and *BGRF* (Kai Müller, unpublished data).

In this thesis, to get more insight how proteins encoded by these cDNAs interact with the 305bp intron sequence to regulate the *BKn3* gene expression, the detailed molecular characterization of these barley cDNAs was performed. The genomic clones of *BEIL*, *BAPL*, *BBR* and *BGRF* were isolated and sequenced, the transcription initiation site of *BBR* was mapped. Their expressions in different wild-type barley tissues and *Hooded* barley inflorescences were analyzed using Northern blot and RT-PCR. To verify if BEIL, BAPL, BBR and BGRF proteins bind to the 305bp sequence of *BKn3* intron IV *in vitro*, purified GST-fusion proteins and *in vitro* translated proteins were used for *in vitro* binding studies (collaborated with Luca Santi). We demonstrated that the BBR recombinant protein could specifically bind to the 305bp intron sequence *in vitro*. The DNA-binding site of the BBR protein was identified. Mapping of *BEIL*, *BAPL*, *BBR* and *BGRF* was carried out by single strand conformation polymorphism (SSCP) analysis. *BEIL* has been mapped to chromosome 1. The nuclear localization of BBR/GFP fusion protein and the transactivation activity of BBR was tested in tobacco SR1 protoplasts. The possible regulation mechanism of *BKn3* by the proteins binding to the intron IV at the transcriptional level is discussed.

# Zusammenfassung

*Knox* (*Knotted-1* like homeobox) Gene bilden eine große Familie innerhalb der Homöoboxgene der Pflanzen. Bisher wurden unter Verwendung der Homöobox von *Knotted-1* aus Mais als Hybridisierungssonde sieben *Knox* Gene aus Gerste (*Hordeum vulgare* L.) isoliert (Müller, 1993; 1997).

In dieser Arbeit wurden die Expressionsmuster von zweien der *Knox* Gene aus Gerste durch Northern Blot, RT-PCR und *in situ* Hybridisierung detailliert untersucht, um erste Hinweise auf mögliche Funktionen zu erhalten. Die Expression von *BKn1*, einem *Knox* Gen der Klasse I, konnte vor allem in meristematischen Geweben des Sproß-Apex, jungen Blattprimordien, Stengeln, Infloreszenz- und Blütenmeristemen und entstehenden Blütenorganen und in den Wurzeln von Embryonen und Keimlingen nachgewiesen werden. Im Vergleich dazu zeigte das Klasse II *Knox* Gen, *BKn7,* ein breiteres Expressionsmuster, das alle getesteten Gewebe wie Gerstenembryonen, Blätter, Wurzeln, Stengel, Infloreszenzen und sich entwickelnde Blütenorgane einschloß. Mögliche funktionale Konsequenzen dieser Expressionsmuster in verschiedenen Entwicklungsstadien der Pflanze werden diskutiert.

*Hooded* (zu deutsch: Kapuzengerste) ist eine dominante Gerstenmutante, die durch das Auftreten einer epiphyllen Blüte auf der Deckspelze der normalen Gerstenblüte charakterisiert ist. Der Phänotyp wird durch eine Duplikation von 305 Basenpaaren in Intron IV des Klasse I *Knox* Gens *BKn3* und eine damit verbundene Überexpression des Genproduktes verursacht (Müller *et al.*, 1995). Durch One-Hybrid Screening in Hefe mit einer bzw. drei Kopien dieses 305 bp Elementes als "Köder" konnten vier potentielle Regulatoren der *BKn3* Expresson isoliert werden: *BEIL, BAPL, BBR* und *BGRF* (Kai Müller, unveröffentlicht).

In der vorliegenden Arbeit wurden diese vier Gene molekular charakterisiert. Genomische Klone von *BEIL, BAPL, BBR* und *BGRF* wurden isoliert und sequenziert und der Transkriptionsstart von BBR wurde bestimmt. *BEIL* wurde durch SSCP (Single Strand Conformation Polymorphism) Analyse auf Chromosom 1 kartiert; für *BAPL, BBR* und *BGRF* konnten bisher keine zur Kartierung geeigneten Polymorphismen identifiziert werden. Die Expressionsmuster der vier Gene wurden durch Northern Blot und RT-PCR Analysen untersucht. Ferner konnte durch die Expression von BBR-GFP-Fusionen in Tabakprotoplasten gezeigt werden, daß das Protein im Zellkern vorliegt. In *E. coli* exprimierte GST-Fusionen und *in vitro* Translationssprodukte von BEIL, BAPL, BBR und BGRF wurden in Zusammenarbeit mit Luca Santi durch Bindungsstudien auf ihre Fähigkeit getestet, *in vitro* an die 305 bp Sequenz aus *BKn3* Intron IV zu binden. Im Falle der BBR-GST Fusion konnte eine spezifische Protein-DNA Interaktion bestätigt und die DNA-Bindestelle eingegrenzt werden. Anhand dieser Ergebnisse wird eine mögliche Rolle von *BEIL, BAPL, BBR* und *BGRF* in der Regulation der *BKn3* Expression diskutiert.

**Agalioti, T., Lomvardas, S., Parekh, B., Yie, J., Maniatis, T., Thanos, D.** (2000) Ordered recruitment of chromatin modifying and general transcription factors to the *IFN-beta* promoter. *Cell*. 103:667-678.

**Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., Struhl, K.** (1994) Current Protocols in Molecular Biology. Wiley, New York.

**Barrieu, F., Chaumont, F., Chrispeels, M.J.** (1998) High expression of the tonoplast aquaporin ZmTIP1 in epidermal and conducting tissues of maize. *Plant Physiol*. 117:1153-1163.

**Barton, K.M., Poethig, S.R.** (1993) Formation of the shoot apical meristem in *Arabidopsis thaliana*: an analysis of development in the wild type and in the *shoot meristemless* mutant. *Development* 119:823-831.

**Becraft, P.W., Freeling, M.** (1994) Genetic analysis of *Rough sheath1* developmental mutants of maize. *Genetics*. 136:295-311.

**Bellmann, R., Werr, W.** (1992) Zmhox1a, the product of a novel maize homeobox gene, interacts with the Shrunken 26bp feedback control element. *EMBO J*. 11:3367-3374.

**Bertolino, E., Reimund, B., Wildt-Perinic, D., Clerc, R.G.** (1995) A novel homeobox protein which recognizes a TGT core and functionally interferes with a retinoid-responsive motif. *J Biol Chem*. 270:31178-31188.

**Billeter, M., Qian, Y., Otting, G., Müller, M., Gehring, W.J., Wuthrich, K.** (1990) Determination of the three-dimensional structure of the *Antennapedia* homeodomain from *Drosophila* in solution by 1H nuclear magnetic resonance spectroscopy. *J Mol Biol*. 214:183-197.

**Bradford, M.M.** (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*. 72:248-254.

**Bradley, D., Carpenter, R., Sommer, H., Hartley, N., Coen, E.** (1993) Complementary floral homeotic phenotypes result from opposite orientations of a transposon at the plena locus of *Antirrhinum*. *Cell*. 72:85-95.

Brookes, A. J. **(1999) The essence of SNPs. *Gene*. 234: 177-186.**

**Brown, J.W.** (1986) A catalogue of splice junction and putative branch point sequences from plant introns. *Nucleic Acids Res*. 14:9549-9559.

**Bulfone, A., Smiga, S. M., Shimamura, K., Peterson, A., Puelles, L., Rubenstein, J. L.** (1995) T-brain-1: a homolog of Brachyury whose expression defines molecularly distinct domains within the cerebral cortex. *Neuron*. 15: 63-78.

**Burglin, T.R.** (1997) Analysis of *TALE* superclass homeobox genes (*MEIS*, *PBC*, *KNOX*, *Iroquois*, *TGIF*) reveals a novel domain conserved between plants and animals. *Nucleic Acids Res*. 25:4173-4180.

**Carabelli, M., Morelli. G., Whitelam, G., Ruberti, I.** (1996) Twilight-zone and canopy shade induction of the Athb-2 homeobox gene in green plants. *Proc. Natl. Acad. Sci.* 93: 3530-3535.

**Castiglioni, P., Pozzi, C., Heun, M., Terzi, V., Müller, K.J., Rohde, W., Salamini, F.** (1998) An AFLP-based procedure for the efficient mapping of mutations and DNA probes in barley. *Genetics*. 149:2039-2056.

**Cercos, M., Gomez-Cadenas, A., Ho, T.H.** (1999) Hormonal regulation of a cysteine proteinase gene, *EPB-1*, in barley aleurone layers: cis- and trans-acting elements involved in the co-ordinated gene expression regulated by gibberellins and abscisic acid. *Plant J*. 19:107-118.

**Chan, R.L, Gago, G.M., Palena, C.M., Gonzalez, D.H.** (1998) Homeoboxes in plant development. *Biochim Biophys Acta*. 1442:1-19.

**Chao, Q., Rothenberg, M., Solano, R., Roman, G., Terzaghi, W., Ecker, J.R.** (1997) Activation of the ethylene gas response pathway in *Arabidopsis* by the nuclear protein ETHYLENE-INSENSITIVE3 and related proteins. *Cell*. 89:1133-1144.

**Chelsky, D., Ralph, R., Jonak, G.** (1989) Sequence requirements for synthetic peptide-mediated translocation to the nucleus. *Mol Cell Biol*. 9:2487-2492.

**Chen, J.J., Janssen, B.J., Williams, A., Sinha, N.** (1997) A gene fusion at a homeobox locus: alterations in leaf shape and implications for morphological evolution. *Plant Cell*. 9:1289-1304.

**Chojecki, J.** (1986) Identification and characterisation of a cDNA clone for cytosolic glyceraldehyde-3-phosphate dehydrogenase in barley. *Carlsberg Res. Commun*. 51: 203-210.

**Chuck, G., Lincoln, C., Hake, S.** (1996) KNAT1 induces lobed leaves with ectopic meristems when overexpressed in *Arabidopsis*. *Plant Cell*. 8:1277-1289.

**Clancy, M., Vasil, V., Hannah, L.C., Vasil, I.K.** (1994) Maize *Shrunken-1* intron and exon regions increase gene expression in maize protoplasts. *Plant Sci*. 98:151-161.

**Clark, S.E., Jacobsen, S.E., Levin, J.Z., Meyerowitz, E.M.** (1996) The *CLAVATA* and *SHOOT MERISTEMLESS* loci competitively regulate meristem activity in *Arabidopsis*. *Development*. 122:1567-1575.

**de Vetten, N.C., Ferl, R.J.** (1995) Characterization of a maize G-box binding factor that is induced by hypoxia. *Plant J.* 7:589-601.

**Deyholos, M.K., Sieburth, L.E.** (2000) Separable whorl-specific expression and negative regulation by enhancer elements within the *AGAMOUS* second intron. *Plant Cell*. 12:1799-1810.

**Di Cristina, M., Sessa, G., Dolan, L., Linstead, P., Baima, S., Ruberti, I., Morelli, G.** (1996) The *Arabidopsis* Athb-10 (GLABRA2) is an HD-Zip protein required for regulation of root hair development. *Plant J*. 10:393-402.

**Dietrich, R.A., Radke, S.E., Harada, J.J.** (1992) Downstream DNA sequences are required to activate a gene expressed in the root cortex of embryos and seedlings. *Plant Cell*. 4:1371-1382.

**Dingwall, C., Laskey, R.A.** (1991) Nuclear targeting sequences--a consensus? *Trends Biochem Sci (TIBS)*. 16:478-481.

**Donald, R.G., Cashmore, A.R.** (1990) Mutation of either G box or I box sequences profoundly affects expression from the *Arabidopsis* rbcS-1A promoter. *EMBO J*. 9:1717-1726.

**Duboule, D.** (1994) "Guidebook to the Homeobox Genes". Oxford University Press, Oxford.

**Ecker, J.R.** (1995) The ethylene signal transduction pathway in plants. *Science*. 268:667-675.

**Endrizzi, K., Moussian, B., Haecker, A., Levin, J.Z., Laux, T.** (1996) The *SHOOT MERISTEMLESS* gene is required for maintenance of undifferentiated cells in *Arabidopsis* shoot and floral meristems and acts at a different regulatory level than the meristem genes *WUSCHEL* and *ZWILLE*. *Plant J*. 10:967-979.

**Farkas, G., Leibovitch, B.A., Elgin, S.C.** (2000) Chromatin organization and transcriptional control of gene expression in *Drosophila*. *Gene*. 253:117-136.

**Foster, T., Yamaguchi, J., Wong, B.C., Veit, B., Hake, S.** (1999) *Gnarley1* is a dominant mutation in the *knox4* homeobox gene affecting cell shape and identity. *Plant Cell*. 11:1239-1252.

**Frank, W., Phillips, J., Salamini, F., Bartels, D.** (1998) Two dehydration-inducible transcripts from the resurrection plant *Craterostigma plantagineum* encode interacting homeodomain-leucine zipper proteins. *Plant J*. 15:413-421.

**Frankel, A.D., Kim, P.S.** (1991) Modular structure of transcription factors: implications for gene regulation. *Cell*. 65:717-719.

**Freeling, M and Hake, S.** (1985) Developmental genetics of mutants that specify knotted leaves in maize. *Genetics.* 111: 617-634.

**Freeling, M.** (1992) A conceptual framework for maize leaf development. *Dev Biol*. 153:44-58.

**Frugis, G., Giannino, D., Mele, G., Nicolodi, C., Innocenti, A.M., Chiappetta, A., Bitonti, M.B., Dewitte, W., Van Onckelen, H., Mariotti, D.** (1999) Are homeobox *knotted-like* genes and cytokinins the leaf architects? *Plant Physiol*. 119:371-374.

**Gehring, W. J.** (1998) Master control genes in the development and evolution: the homeobox story. Yale University press, New Haven and London.

**Gehring, W.J.** (1987) Homeo boxes in the study of development. *Science*. 236:1245-1252.

**Gehring, W.J., Qian, Y.Q., Billeter, M., Furukubo-Tokunaga, K., Schier, A.F., Resendez-Perez, D., Affolter, M., Otting, G., Wuthrich, K.** (1994a) Homeodomain-DNA recognition. *Cell*. 78:211-223.

**Gehring, W.J., Affolter, M., Burglin, T.** (1994b) Homeodomain proteins. *Annu Rev Biochem*. 63:487-526.

**Gentzbittel, L., Mouzeyar, S., Badaoui, S., Mestries, E., Vear, F., Tourvieille De Labrouhe, D., Nicolas, P.** (1998) Cloning of molecular markers for disease resistence in sunflower, *Helianthus annuus* L. *Theor Appl Genet*. 96:519-525.

**Gomez-Cadenas, A., Zentella, R., Walker-Simmons, M.K., Ho, T.H.** (2001) Gibberellin/Abscisic acid antagonism in barley aleurone cells. Site of action of the protein kinase pkaba1 in relation to gibberellin signaling molecules. *Plant Cell*. 13:667-679.

**Goodrich, J., Puangsomlee, P., Martin, M., Long, D., Meyerowitz, E.M., Coupland, G.** (1997) A Polycomb-group gene regulates homeotic gene expression in *Arabidopsis*. *Nature*. 386:44-51.

**Goutte, C., Johnson, A.D.** (1994) Recognition of a DNA operator by a dimer composed of two different homeodomain proteins. *EMBO J*. 13:1434-1442.

**Greene, B., Walko, R., Hake, S.** (1994) Mutator insertions in an intron of the maize *knotted1* gene result in dominant suppressible mutations. *Genetics*. 138:1275-1285.

**Gremke, L., Lord, P.C., Sabacan, L., Lin, S.C., Wohlwill, A., Storti, R.V.** (1993) Coordinate regulation of *Drosophila tropomyosin* gene expression is controlled by multiple muscle-type-specific positive and negative enhancer elements. *Dev Biol* 159:513-527.

**Grossniklaus, U., Vielle-Calzada, J.P., Hoeppner, M.A., Gagliano, W.B.** (1998) Maternal control of embryogenesis by *MEDEA*, a polycomb group gene in *Arabidopsis*. *Science*. 280:446-450.

**Hake, S., Char, B., Chuck, G., Foster, T., Long, J., and Jackson, D.** (1995). *Homeobox* genes in the functioning of plant meristems. *Philos Trans R Soc Lond B Bil. Sci*. 350:45-51.

**Hake, S., Vollbrecht, E., Freeling, M.** (1989) Cloning *Knotted*, the dominant morphological mutant in maize using *Ds2* as a transposon tag. *EMBO J*. 8:15-22.

**Halbach, T., Scheer, N., Werr, W.** (2000) Transcriptional activation by the PHD finger is inhibited through an adjacent leucine zipper that binds 14-3-3 proteins. *Nucleic Acids Res*. 28:3542-3550.

**Hareven, D., Gutfinger, T., Parnis, A., Eshed, Y., Lifschitz, E.** (1996) The making of a compound leaf: genetic manipulation of leaf architecture in tomato. *Cell*. 84:735-744.

**Hertzberg, M., Olsson, O.** (1998) Molecular characterisation of a novel plant homeobox gene expressed in the maturing xylem zone of *Populus tremula* x *tremuloides*. *Plant J*. 16:285-295.

**Heun, M., Kennedy, A.E., Anderson, J.A., Lapitan, N.L., Sorrells, M.E., Tanksley, S.D.** (1991) Construction of a restriction fragment length polymorphism map for barley (*Hordeum vulgare*). *Genome*. 34:437-447.

**Hinz, U., Wolk, A., Renkawitz-Pohl, R.** (1992) Ultrabithorax is a regulator of beta 3 tubulin expression in the *Drosophila* visceral mesoderm. *Development*. 116:543-554.

**Hong, J.C., Cheong, Y.H., Nagao, R.T., Bahk, J.D., Key, J.L., Cho, M.J.** (1995) Isolation of two soybean G-box binding factors which interact with a G-box sequence of an auxin-responsive gene. *Plant J*. 8:199-211.

**Jacobsen, S. E., Running, M. P., Meyerowitz, E. M.** (1999) Disruption of an RNA helicase/RNAse III gene in

*Arabidopsis* causes unregulated cell division in floral meristems. *Development.* 126: 5231-43.

**Jacobsen, S. E., Sakai, H., Finnegan, E. J., Cao, X., Meyerowitz, E. M.** (2000) Ectopic hypermethylation of flower-specific genes in *Arabidopsis*. *Curr Biol*. 10: 179-86.

**Janssen, B.J., Williams, A., Chen, J.J., Mathern, J., Hake, S., Sinha, N.** (1998) Isolation and characterization of two *knotted-like* homeobox genes from tomato. *Plant Mol Biol*. 36:417-425.

**Jefferson, R.A., Kavanagh, T.A., Bevan, M.W.** (1987) GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J*. 6:3901-3907.

**Junghans, H., Metzlaff, M.** (1990) A simple and rapid method for the preparation of total plant DNA. *Biotechniques*. 8:176.

**Kadonaga, J.T., Tjian, R.** (1986) Affinity purification of sequence-specific DNA binding proteins. *Proc Natl Acad Sci USA*. 83:5889-5893.

**Kano-Murakami, Y., Yanai, T., Tagiri, A., Matsuoka, M.** (1993) A rice homeotic gene, *OSH1*, causes unusual phenotypes in transgenic tobacco. *FEBS Lett*. 334:365-368.

**Kaufman, P. B.** (1959) Development of the shoot of *Oryza Sativa* L. *Phytomorphology*. 9: 382-404.

**Kerstetter, R., Vollbrecht, E., Lowe, B., Veit, B., Yamaguchi, J., Hake, S.** (1994) Sequence analysis and expression patterns divide the maize *knotted1-like* homeobox genes into two classes. *Plant Cell*.6:1877-1887.

**Kerstetter, R.A., Laudencia-Chingcuanco, D., Smith, L.G., Hake, S.** (1997) Loss-of-function mutations in the maize homeobox gene, *knotted1*, are defective in shoot meristem maintenance. *Development*. 124:3045-3054.

**Kim, S.Y., Chung, H.J., Thomas, T.L.** (1997) Isolation of a novel class of bZIP transcription factors that interact with ABA-responsive and embryo-specification elements in the *Dc3* promoter using a modified yeast one-hybrid system. *Plant J*. 11:1237-1251.

**Kirby, E.J.M., Appleyard, M.** (1984) Cereal Development Guide, Arable Unit, National Agricultural Centre, Warwickshire, UK.

**Kleines, M., Elster, R.C., Rodrigo, M.J., Blervacq, A.S., Salamini, F., Bartels, D.** (1999) Isolation and expression analysis of two stress-responsive sucrose-synthase genes from the resurrection plant *Craterostigma plantagineum* (Hochst.). *Planta*. 209:13-24.

**Klinge, B., Uberlacker, B., Korfhage, C., Werr, W.** (1996) *ZmHox*: a novel class of maize homeobox genes. *Plant Mol Biol*. 30:439-453.

**Kobayashi, A., Kiyosawa, N., Suzuki, Y., Murofushi, N., Yamaguchi, I.** (2000) Pharbitis class-1 *knotted*-like homeobox gene, *PKn3*, shares similar characteristic to those of class-2 *knotted*-like genes. *Plant Cell Rep*. 19:911-920.

**Koltai, H., Bird, D.M.** (2000) Epistatic repression of *PHANTASTICA* and class 1 *KNOTTED* genes is uncoupled in tomato. *Plant J*. 22:455-459.

**Krumlauf, R.** (1994) Hox genes in vertebrate development. *Cell*. 78:191-201.

**Kusaba, S., Kano-Murakami, Y., Matsuoka, M., Tamaoki, M., Sakamoto, T., Yamaguchi, I., Fukumoto, M.** (1998a) Alteration of hormone levels in transgenic tobacco plants overexpressing the rice homeobox gene *OSH1*. *Plant Physiol*. 116:471-476.

**Kusaba, S., Fukumoto, M., Honda, C., Yamaguchi, I., Sakamoto, T., Kano-Murakami, Y.** (1998b) Decreased GA1 content caused by the overexpression of *OSH1* is accompanied by suppression of GA 20-oxidase gene expression. *Plant Physiol*. 117:1179-1184.

**Lander, E.S., Green, P., Abrahamson, J., Barlow, A., Daly, M.J., Lincoln, S.E., Newburg, L.** (1987) MAPMAKER: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. *Genomics*. 1:174-181.

**Larkin, J. C., Oppenheimer, D. G., Pollock, S., Marks, M. D.** (1993). *Arabidopsis GLABROUS1* gene requires downstream sequences for function. *Plant Cell.* 5:1739-1748.

**Lawrence, P.A., Morata, G.** (1994) Homeobox genes: their function in *Drosophila* segmentation and pattern formation. *Cell*. 78(2):181-189.

**Lefebvre, V., Kuntz, M., Camara, B., Palloix, A.** (1998) The capsanthin-capsorubin synthase gene: a candidate gene for the y locus controlling the red fruit colour in pepper. *Plant Mol Biol*. 36:785-789.

**Lincoln, C., Long, J., Yamaguchi, J., Serikawa, K., Hake, S.** (1994) A *knotted1-like* homeobox gene in *Arabidopsis* is expressed in the vegetative meristem and dramatically alters leaf morphology when overexpressed in transgenic plants. *Plant Cell*. 6:1859-1876.

**Liu, Q., Kasuga, M., Sakuma, Y., Abe, H., Miura, S., Yamaguchi-Shinozaki, K., Shinozaki, K.** (1998) Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought- and low-temperature-responsive gene expression, respectively, in *Arabidopsis*. *Plant Cell*. 10:1391-1406.

**Long, J.A., Moan, E.I., Medford, J.I., Barton, M.K.** (1996) A member of the KNOTTED class of homeodomain proteins encoded by the *STM* gene of *Arabidopsis*. *Nature*. 379:66-69.

**Lu, P., Porat, R., Nadeau, J.A., O'Neill, S.D.** (1996) Identification of a meristem *L1* layer-specific gene in *Arabidopsis* that is expressed during embryonic pattern formation and defines a new class of homeobox genes. *Plant Cell*. 8:2155-2168.

**Luehrsen, K.R., Walbot, V.** (1991) Intron enhancement of gene expression and the splicing efficiency of introns in maize cells. *Mol Gen Genet*. 225:81-93.

**Ma, H., McMullen, M.D., Finer, J.J.** (1994) Identification of a homeobox-containing gene with enhanced expression during soybean (*Glycine max* L.) somatic embryo development. *Plant Mol Biol*. 24:465-473.

**Manak, J.R., Scott, M.P.** (1994) A class act: conservation of homeodomain protein functions. *Dev Suppl*. 61-77.

**Mathern, J., Hake, S.** (1997) Mu element-generated gene conversions in maize attenuate the dominant *knotted* phenotype. *Genetics*. 147:305-314.

**Matsuoka, M., Ichikawa, H., Saito, A., Tada, Y., Fujimura, T., Kano-Murakami, Y.** (1993) Expression of a rice homeobox gene causes altered morphology of transgenic plants. *Plant Cell*. 5:1039-1048.

**McGinnis, W., Levine, M.S., Hafen, E., Kuroiwa, A., Gehring, W.J.** (1984a) A conserved DNA sequence in homoeotic genes of the *Drosophila Antennapedia* and *bithorax* complexes. *Nature*. 308:428-433.

**McGinnis, W., Garber, R.L, Wirz, J., Kuroiwa, A., Gehring, W.J.** (1984b) A homologous protein-coding sequence in *Drosophila* homeotic genes and its conservation in other metazoans. *Cell*. 37:403-408.

**Menke, F.L., Champion, A., Kijne, J.W., Memelink, J.** (1999) A novel jasmonate- and elicitor-responsive element in the periwinkle secondary metabolite biosynthetic gene *Str* interacts with a jasmonate- and elicitor-inducible AP2-domain transcription factor, ORCA2. *EMBO J*. 18:4455-4463.

**Merry, J.** (1981) Studies on the embryo of *Hordeum sativum*. I. The development of the embryo. *Bull Torrey Bot Club.* 68: 586-598.

**Mikami, K., Tabata, T., Kawata, T., Nakayama, T., Iwabuchi, M.** (1987) Nuclear protein(s) binding to the conserved DNA hexameric sequence postulated to regulate transcription of wheat histone genes. *FEBS Lett*. 223:273-278.

**Muehlbauer, G.J., Fowler, J.E., Girard, L., Tyers, R., Harper, L., Freeling, M.** (1999) Ectopic expression of the maize homeobox gene *liguleless3* alters cell fates in the leaf. *Plant Physiol*. 119:651-662.

**Müller, C., Leutz, A.** (2001) Chromatin remodeling in development and differentiation. *Curr Opin Genet Dev*. 11:167-174.

**Müller, J.** (1999) Protein-Protein Interaktionen zwischen Homöodomänenproteinen der Gerste: Identifizierung

und Charakterisierung interagierender Partner von BKN3, dem Genprodukt des Hooded-Lokus. Dissertation, Universität zu Köln.

**Müller, J., Müller, K., Pozzi, C., Santi, L., Wang, Y., Salamini, F., Rohde, W.** (2000) Networking around the barley *Hooded* locus: molecular analysis of potential partners for epiphyllous flower formation. *Barley Genetics VIII*. 114-116.

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

**Müller, K., Pozzi, C., Müller, J., Salamini, F., Rohde, W.** (2000) Molecular analysis of homeotic genes involved in barley development. *Eur. J. Physiology*. 439 (suppl): R14-R15.

**Müller, K.J.** (1993) Untersuchungen zu homöotischen Gerstengenen der knotted-Familie. Diplomarbeit, Philipps-Universität, Marburg.

**Müller, K.J.** (1997) Die Homöboxgene der Knox-Familie in Gerste (*Hordeum vulg*. L.): Molekulare Characterisierung, transgene Expression und Assoziationversuche mit Homöotischen Mutationen. Dissertation, Universität zu Köln.

**Müller, K.J., Romano, N., Gerstner, O., Garcia-Maroto, F., Pozzi, C., Salamini, F., Rohde, W.** (1995) The barley *Hooded* mutation caused by a duplication in a homeobox gene intron. *Nature*. 374:727-730.

**Nadeau, J.A., Zhang, X.S., Li, J., O'Neill, S.D.** (1996) Ovule development: identification of stage-specific and tissue-specific cDNAs. *Plant Cell*. 8:213-239.

**Ngai, N., Tsai, F.Y., Coruzzi, G.** (1997) Light-induced transcriptional repression of the pea *AS1* gene: identification of cis-elements and transfactors. *Plant J*. 12:1021-1034.

**Ohad, N., Yadegari, R., Margossian, L., Hannon, M., Michaeli, D., Harada, J.J., Goldberg, R.B., Fischer, R.L.** (1999) Mutations in *FIE*, a *WD* polycomb group gene, allow endosperm development without fertilization. *Plant Cell*. 11:407-416.

**Ohme-Takagi, M., Shinshi, H.** (1995) Ethylene-inducible DNA binding proteins that interact with an ethylene-responsive element. *Plant Cell*. 7:173-182.

**Okamuro, J.K., Caster, B., Villarroel, R., Montagu, M. V., Jofuku, K. D. (1997)** The AP2 domain of APETALA2 defines a larger new family of DNA binding proteins in *Arabidopsis*. *Proc. Natl. Acad. Sci. USA*. 94: 7076-7081.

**Otting, G., Qian, Y.Q., Billeter, M., Müller, M., Affolter, M., Gehring, W.J., Wuthrich, K.** (1990) Protein-DNA contacts in the structure of a homeodomain-DNA complex determined by nuclear magnetic resonance spectroscopy in solution. *EMBO J*. 9:3085-3092.

**Parnis, A., Cohen, O., Gutfinger, T., Hareven, D., Zamir, D., Lifschitz, E.** (1997) The dominant developmental mutants of tomato, *Mouse-ear* and *Curl*, are associated with distinct modes of abnormal transcriptional regulation of a *Knotted* gene. *Plant Cell*. 9:2143-2158.

**Poethig, R.S.** (1988) Heterochronic mutations affecting shoot development in maize. *Genetics*. 119:959-973.

**Postma-Haarsma, A.D., Verwoert, I.I., Stronk, O.P., Koster, J., Lamers, G.E., Hoge, J.H., Meijer, A.H.** (1999) Characterization of the *KNOX* class homeobox genes *Oskn2* and *Oskn3* identified in a collection of cDNA libraries covering the early stages of rice embryogenesis. *Plant Mol Biol*. 39:257-271.

**Pozzi, C.** (1998) Morphological, genetic and molecular approaches to map-based cloning of barely developmental genes. Dissertation, Universität zu Köln.

**Qian, Y.Q., Billeter, M., Otting, G., Müller, M., Gehring, W.J., Wuthrich, K.** (1989) The structure of the Antennapedia homeodomain determined by NMR spectroscopy in solution: comparison with prokaryotic repressors. *Cell*. 59:573-580.

**Quaedvlieg, N., Dockx, J., Rook, F., Weisbeek, P., Smeekens, S.** (1995) The homeobox gene *ATH1* of *Arabidopsis* is derepressed in the photomorphogenic mutants *cop1* and *det1*. *Plant Cell*. 7:117-129.

**Reiser, L., Modrusan, Z., Margossian, L., Samach, A., Ohad, N., Haughn, G.W., Fischer, R.L.** (1995) The *BELL1* gene encodes a homeodomain protein involved in pattern formation in the *Arabidopsis* ovule primordium. *Cell*. 83:735-742.

**Reiser, L., Sanchez-Baracaldo, P., Hake, S.** (2000) Knots in the family tree: evolutionary relationships and functions of *knox* homeobox genes. *Plant Mol Biol*. 42:151-166.

**Rerie, W.G., Feldmann, K.A., Marks, M.D.** (1994) The *GLABRA2* gene encodes a homeo domain protein required for normal trichome development in *Arabidopsis*. *Genes Dev*. 8:1388-1399.

**Rothnie, H.M.** (1996) Plant mRNA 3´-end formation. *Plant. Mol. Biol*. 32:43-61.

**Rupp, H.M., Frank, M., Werner, T., Strnad, M., Schmulling, T.** (1999) Increased steady state mRNA levels of the *STM* and *KNAT1* homeobox genes in cytokinin overproducing *Arabidopsis thaliana* indicate a role for cytokinins in the shoot apical meristem. *Plant J*. 18:557-563.

**Sakai, H., Medrano, L.J., Meyerowitz, E.M.** (1995) Role of *SUPERMAN* in maintaining *Arabidopsis* floral whorl boundaries. *Nature*. 378:199-203.

**Sambrook, J., Fritsch, E.F., Maniatis, T.** (1989) Molecular Cloning: A Laboratory Manual. 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

**Sato, Y., Hong, S.K., Tagiri, A., Kitano, H., Yamamoto, N., Nagato, Y., Matsuoka, M.** (1996) A rice homeobox gene, *OSH1*, is expressed before organ differentiation in a specific region during early embryogenesis. *Proc Natl Acad Sci U S A*. 93:8117-8122.

**Sato, Y., Sentoku, N., Miura, Y., Hirochika, H., Kitano, H., Matsuoka, M.** (1999) Loss-of-function mutations in the rice homeobox gene *OSH15* affect the architecture of internodes resulting in dwarf plants. *EMBO J*. 18:992-1002.

**Scanlon, M.J., Schneeberger, R.G., Freeling, M.** (1996) The maize mutant *narrow sheath* fails to establish leaf margin identity in a meristematic domain. *Development*. 122:1683-1691.

**Scarpella, E., Rueb, S., Boot, K.J., Hoge, J.H., Meijer, A.H.** (2000) A role for the rice homeobox gene *Oshox1* in provascular cell fate commitment. *Development*. 127:3655-3669.

**Schindler, U., Beckmann, H., Cashmore, A.R.** (1993) HAT3.1, a novel *Arabidopsis* homeodomain protein containing a conserved cysteine-rich region. *Plant J*. 4:137-150.

**Schneeberger, R., Tsiantis, M., Freeling, M., Langdale, J.A.** (1998) The *rough sheath2* gene negatively regulates homeobox gene expression during maize leaf development. *Development*. 125:2857-2865.

**Schneeberger, R.G., Becraft, P.W., Hake, S., Freeling, M.** (1995) Ectopic expression of the *knox* homeobox gene *rough sheath1* alters cell fate in the maize leaf. *Genes Dev*. 9:2292-2304.

**Scott, M.P., Tamkun, J.W., Hartzell, G.W.** (1989) The structure and function of the homeodomain. *Biochim Biophys Acta*. 989:25-48.

**Scott, M.P., Weiner, A.J.** (1984) Structural relationships among genes that control development: sequence homology between the *Antennapedia*, *Ultrabithorax*, and *fushi tarazu* loci of *Drosophila*. *Proc Natl Acad Sci U S A*. 81:4115-4119.

**Sentoku, N., Sato, Y., Kurata, N., Ito, Y., Kitano, H., Matsuoka, M.** (1999) Regional expression of the rice KN1-type homeobox gene family during embryo, shoot, and flower development. *Plant Cell*. 11: 1651-64.

**Sentoku, N., Sato, Y., Matsuoka, M.** (2000) Overexpression of rice *OSH* genes induces ectopic shoots on leaf sheaths of transgenic rice plants. *Dev Biol*. 220:358-364.

**Serikawa, K.A., Mandoli, D.F.** (1999) *Aaknox1*, a *kn1-like* homeobox gene in *Acetabularia acetabulum*, undergoes developmentally regulated subcellular localization. *Plant Mol Biol*. 41:785-793.

**Serikawa, K.A., Martinez-Laborda, A., Kim, H.S., Zambryski, P.C.** (1997) Localization of expression of *KNAT3*, a class 2 *knotted1-like* gene. *Plant J*. 11:853-861.

**Serikawa, K.A., Martinez-Laborda, A., Zambryski, P.** (1996) Three *knotted1-like* homeobox genes in *Arabidopsis*. *Plant Mol Biol*. 32:673-683.

**Serikawa, K.A., Zambryski, P.C.** (1997) Domain exchanges between KNAT3 and KNAT1 suggest specificity of the *kn1-like* homeodomains requires sequences outside of the third helix and N-terminal arm of the homeodomain. *Plant J*. 11:863-869.

**Sessa, G., Morelli, G., Ruberti, I.** (1993) The Athb-1 and -2 HD-Zip domains homodimerize forming complexes of different DNA binding specificities. *EMBO J*. 12:3507-3517.

**Sessa, G., Steindler, C., Morelli, G., Ruberti, I.** (1998) The *Arabidopsis Athb-8*, *-9* and *-14* genes are members of a small gene family coding for highly related HD-ZIP proteins. *Plant Mol Biol*. 38:609-622.

**Seul, K.H., Beyer, E.C.** (2000) Mouse *connexin37*: gene structure and promoter analysis. *Biochim Biophys Acta*. 1492:499-504.

**Shamsher, M.K., Chuzhanova, N.A., Friedman, B., Scopes, D.A., Alhaq, A., Millar, D.S., Cooper, D.N., Berg, L.P.** (2000) Identification of an intronic regulatory element in the human protein C (PROC) gene. *Hum Genet*. 107:458-465.

**Sheffield, V. C., Beck, J. S., Kwitek, A. E., Sandstrom, D. W., Stone, E. M.** (1993) The sensitivity of single-strand conformation polymorphism analysis for the dtection of single base substitutions. *Genomics*. 16: 325-332.

**Sieburth, L.E., Meyerowitz, E.M.** (1997) Molecular dissection of the AGAMOUS control region shows that cis elements for spatial regulation are located intragenically. *Plant Cell*. 9:355-365.

**Sinha, N.R., Williams, R.E., Hake, S.** (1993) Overexpression of the maize homeo box gene, *KNOTTED-1*, causes a switch from determinate to indeterminate cell fates. *Genes Dev*. 7:787-795.

**Smith, L., Jackson, D., Hake, S.** (1995) Expression of *knotted1* marks shoot meristem formation during maize embryogenesis. *Dev. Genet.* 16: 344-348.

**Smith, L.G., Greene, B., Veit, B., Hake, S.** (1992) A dominant mutation in the maize homeobox gene, *Knotted-1*, causes its ectopic expression in leaf cells with altered fates. *Development*. 116:21-30.

**Smith, L.G., Hake, S.** (1994) Molecular genetic approaches to leaf development: *Knotted* and beyond. *Can J Bot*. 72:617-625. .

**Solano, R., Nieto, C., Avila, J., Canas, L., Diaz, I., Paz-Ares, J.** (1995) Dual DNA binding specificity of a petal epidermis-specific MYB transcription factor (MYB.Ph3) from *Petunia hybrida*. *EMBO J*. 14:1773-1784.

**Stebbins, G.L., Yagil, E.** (1966) The morphogenetic effects of the *Hooded* gene in barley. I. The course of development in *Hooded* and *Awned* genotypes. Genetics. 54:727-741.

**Sundas-Larsson, A., Svenson, M., Liao, H., Engstrom, P.** (1998) A homeobox gene with potential developmental control function in the meristem of the conifer *Picea abies*. *Proc Natl Acad Sci U S A*. 95:15118-15122.

**Takumi, S., Kosugi, T., Murai, K., Mori, N., Nakamura, C.** (2000) Molecular cloning of three homoeologous cDNAs encoding orthologs of the maize KNOTTED1 homeobox protein from young spikes of hexaploid wheat. *Gene*. 249:171-181.

**Tamaoki, M., Kusaba, S., Kano-Murakami, Y., Matsuoka, M.** (1997) Ectopic expression of a tobacco homeobox gene, *NTH15*, dramatically alters leaf morphology and hormone levels in transgenic tobacco. *Plant Cell Physiol*. 38:917-927.

**Tamaoki, M., Tsugawa, H., Minami, E., Kayano, T., Yamamoto, N., Kano-Murakami, Y., Matsuoka, M.** (1995) Alternative RNA products from a rice homeobox gene. *Plant J*. 7:927-938.

**Timmermans, M.C., Schultes, N.P., Jankovsky, J.P., Nelson, T.** (1998) *Leafbladeless1* is required for dorsoventrality of lateral organs in maize. *Development*. 125:2813-2823.

**Töpfer, R., Matzeit, V., Gronenborn, B., Schell, J., Steinbiss, H.H.** (1987) A set of plant expression vectors for transcriptional and translational fusions. *Nucleic Acids Res*. 15:5890.

**Töpfer, R., Pröls, M., Schell, J., Steinbiß, H-H.** (1988) Transient gene expression in tobacco protoplasts: II. Comparison of the reporter gene systems for CAT, NPT II, and GUS. *Plant Cell Rep*. 7:225-228.

**Tornero, P., Conejero, V., Vera, P.** (1996) Phloem-specific expression of a plant homeobox gene during secondary phases of vascular development. *Plant J*. 9:639-648.

**Tsiantis, M., Brown, M.I., Skibinski, G., Langdale, J.A.** (1999a) Disruption of auxin transport is associated with aberrant leaf development in maize. *Plant Physiol*. 121:1163-1168.

**Tsiantis, M., Schneeberger, R., Golz, J.F., Freeling, M., Langdale, J.A.** (1999b) The maize *rough sheath2* gene and leaf development programs in monocot and dicot plants. *Science*. 284:154-156.

**Uberlacker, B., Klinge, B., Werr, W.** (1996) Ectopic expression of the maize homeobox genes *ZmHox1a* or *ZmHox1b* causes pleiotropic alterations in the vegetative and floral development of transgenic tobacco. *Plant Cell*. 8:349-362.

**Ulmasov, T., Hagen, G., Guilfoyle, T.J.** (1997) ARF1, a transcription factor that binds to auxin response elements. *Science*. 276:1865-1868.

**Varagona, M.J., Schmidt, R.J., Raikhel, N.V.** (1992) Nuclear localization signal(s) required for nuclear targeting of the maize regulatory protein Opaque-2. *Plant Cell*. 4:1213-1227.

**Veit, B., Vollbrecht, E., Mathern, J., Hake, S.** (1990) A tandem duplication causes the *Kn1-O* allele of *Knotted*, a dominant morphological mutant of maize. *Genetics*. 125:623-631.

**Verheyen, E.M., Purcell, K.J., Fortini, M.E., Artavanis-Tsakonas, S.** (1996) Analysis of dominant enhancers and suppressors of activated *Notch* in *Drosophila*. *Genetics*. 144:1127-1141.

**Vidal, M., Legrain, P.** (1999) Yeast forward and reverse 'n'-hybrid systems. *Nucleic Acids Res*. 27:919-929.

**Vollbrecht, E., Veit, B., Sinha, N., Hake, S.** (1991) The developmental gene *Knotted-1* is a member of a maize homeobox gene family. *Nature*. 350:241-243.

**Waites, R., Hudson, A.** (1995). *phantastica*: a gene required for dorsoventrality of leaves in *Antirrhinum majus*. *Development* 121:2143-2154.

**Waites, R., Selvadurai, H.R., Oliver, I.R., Hudson, A.** (1998) The *PHANTASTICA* gene encodes a MYB transcription factor involved in growth and dorsoventrality of lateral organs in *Antirrhinum*. *Cell*. 93:779-89.

**Watillon, B., Kettmann, R., Boxus, P., Burny, A.** (1997) *Knotted1-like* homeobox genes are expressed during apple tree (*Malus domestica* [L.] Borkh) growth and development. *Plant Mol Biol*. 33:757-763.

**Weigel, D.** (1995) The APETALA2 domain is related to a novel type of DNA binding domain. *Plant Cell*. 7:388-389.

**Weigel, D., Alvarez, J., Smyth, D. R., Yanofsky, M. F. Meyerowitz, E. M.** (1992) LEAFY controls floral meristem identity in *Arabidopsis*. *Cell*. 69: 843-859.

**Williams, R.W.** (1998) Plant homeobox genes: many functions stem from a common motif. *Bioessays*. 20:280-282.

**Williams-Carrier, R.E., Lie, Y.S., Hake, S., Lemaux, P.G.** (1997) Ectopic expression of the maize *kn1* gene phenocopies the *Hooded* mutant of barley. *Development*. 124:3737-3745.

**Wilson, D., Sheng, G., Lecuit, T., Dostatni, N., Desplan, C.** (1993) Cooperative dimerization of paired class homeo domains on DNA. *Genes Dev*. 7:2120-2134.

**Yu, H., Yang, S.H., Goh, C.J.** (2000) *DOH1*, a class 1 *knox* gene, is required for maintenance of the basic plant architecture and floral transition in orchid. *Plant Cell*. 12:2143-2160.

## 7.1 Genomic sequences of *BEIL*, *BAPL*, *BBR* and *BGRF*

### 7.1.1 *BEIL*

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### 7.1.2 *BAPL*

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### 7.1.3 *BBR*

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   1 AGAGATACGA CGTAGATTCC TGTCGCATCT TTAGGGCGAC GAGGTTAGTA TTTCTCGCGG TGCGTGTGAT ACGACGAAAT TTGGTGTGAG ATGCTTCAAA
 101 TTGATTTAAC GGTTTAATGA TGACGATTGC GGTTATTCGG CTCTGGTCCT TAGGGGCACG TGTACGAAGA TTTTTCATCT GTCATCGACA ATGTCAAGCC
 201 AACTTCGATA CGGGAGAAAC GATAATGCCA CATTGGTGGC TCATTATGAT GAGGATATGT TTGGGGTATA CTTTTGATGA GACAATCCCC TTTTTTTTTA
 301 AAACTCCCCA TCCATTGCTG CAATTCCTCT CGCATGACAT ATCTTGGAAC ACAAACTCTC ACACGACCTT TGCTAGTGTC GGATCGGACC TTGTTGAGGC
 401 AGGGAGAGAC CAAAAAGACA TTACTTCAAC ACGCCATCAT CGTCACCATC TCGTCGATGT CGTTGAAGAA ACCCCGAACG GATGCGTCAC ACTCATCTTG
 501 CCGCAAAAAA GACCGCCTAG CGGATAGGAA GAGGAGGACC GAAGCAACGG TGTGCTAGAG GAACTCGGTG GCATTGGCTA TGGTTGAGAC TTGGTTCCCC
 601 TTTAAGTTAC TCAACCTTTT GAGCGGATAT GTGACACGCA TGTGCGTGAC ACCAACCTCA TTTTTCTTTA GGTTTTTGGT TTTGAACTTT TGAACGAAAA
 701 GTCCTAACTA AGTTTTGTTT GCATACTCGT ATTTCTTGTG AGGAGGACTT CCAAATAAGA TCACCTTTTA ACAGGTTTAG ATGTGTTTTT AAAATTTCAC
 801 AAAGTTGCAA GTACCGAAAC TTGATACGAA GGAAAGTCAA AATCAACAAG TTTCAGAAAT TAGAACTTAA AACTTAACGC GACCTCGTGC GGAATCCATC
 901 GACTTTACGG ATCGCGGAAC AATCATGCAG TGACGCAGAT GTATGCTCCG CAAGTGCGTG TCCGTGCGAA TATTCGTTTT GTTTGTAATA GAAGATAGGA
1001 TTGAAATCAA ACTGGTTATG TAATTCCTTT GTACTCCCTC CGTTCCTAAA TATAAGACCT TCTAGAGATT TTACTATGAA CTACATACGG ATGTATATAG
1101 ACACATTTTA GAGTATAGAT TCACTCATTT TGCTCCGTAT CGTAGTCTAT AGTGAAATGT CTACAAGGTC TTATATTTAA GAACGGAGGG AGTATTACTC
1201 CATCCGTTCC TAAATATACT CCATCCGTTC CTAAATATAA GACCTTTTAA AGATTACACT ATGGACTATA TATATGGATG TATATAGTCA TATTTTAAAA
1301 TATACATTCA CTCATTTTGT TCCGTATGTA GTCTATAATA ATCTCTAGAA GGTCTTATAT TTAGGAACGG AGAGAGTATT TCGGTTGGAG GTATTGGACA
1401 GTTTGGCTTT GACGAAGCGC CATTTGGGTA TCACTTACAC GTGGGGTCTT ATCCAAGGCG TGGCATTTTG TGTCAAAGTG CAGAAAGAAA TATGCCATTT
1501 TCCCTAGACC ATCATCCTGT GCCTTGCTTT CGTCTTCCTC GCCCCGAGCC TCGGCTCTCC TGACCTAACC AATCCGACCC AAGGCGGGTA CATCTCTCCA
1601 TTGACTGTAC CAGGGCGTGT CCTGCCGAAC ACTGCGTTGC CCGCCGACGC CTCCCCCCCA CCCACCCACT CCCACAACTC CTCCGCCCCT CTCACGCGCC
1701 TCGCCGCCAT CGCCGATCCC GTTCTTCCTC GTCGCCAAAT TTCGTCCCTG CTCTCTCCGC CCCGTCCGCA ATCCCGGGCC ATTGGGTTTT GAGGTATGGA
1801 GGCTGTTCTC AATCAGAGAT TTGTTAAATC CCACTGACCT CTCGCAATTT CTGGGTGTGC CGGTGAGCAC AATCTAGATC CGCCCCTGCT TAGGGATCCT
1901 TGATTTTTTT CCTACTTGTT TTCTCGAAAT AGGTCTCGTG GATTGGCGTT TGGAATGCAA TGGTGGGGAT AGCCTGACCG TCCAGAGCCC GAAAGAAGAG
2001 CACATTTCTC GTCCCAATCT CGGTTTTCTT GGATTTCGAT TCGTTTGTTG GGCCGGCCGA GATGGACGAC GACGGCAGCT TGAGCATTCG GAATTGGGGC
2101 TTCTACGAGA CGATGAAAGG AAACCTCGGC CTGCAGCTGA TGCCATCTGT GACCGGCGGC CACCGGGACA CGAAGCCGCT GCTCCCCAAC GGTACCTTCT
2201 TGCAGCACCA CACCCCCCCG CACCACCCGC CACATTCGCA CCACCCCCGC GACTATGGTA ACGGCGAACC CTCTGGTGGC ATGCCCGCCG AGCCGCCGGC
2301 TATTCACATG GACTTTGTGC GCAATGAGGC CTGGATGCAC CCCTCGCAGC ATCAACATCA GCATCAGCAT CAGCACCAGC ATCAACATCA GCACCAGCAT
2401 CAACTTCAGC ACCAGCATCA ACATCAACAT TCCCGTGAGT TGAAGGTCCT TAATGCTGTT CCTGTTGGGC CTGCTCCACA CATTGGACAT CCTGGACATG
2501 CTGTGCATCA CCACCCTACA GGTTTTGGGA TGATGCCAGA TGCGCGTGGT GCGCACACTC TCCAGATGAT GCAGCCACAG GAGCCTCCTG TGCCTGATGA
2601 GGAAAAAATT ACCCCACCGC TGGTTGAAGA TCATTCTGTG GTCGGAAGCA AGCCTCCTGT GAAGAAGAGG CAGCAGGGTC GTCAGCCTAA GGTTCCGAAG
2701 CCGAAGAAGC CCAAGAAGGA TGCTACCCCA GGGGAAGATG GGGCACCCAA GGCCCGTGCA CCCCGAAGCA GGGGTCCCCT TAAGCCTGTG GAAATGGTAA
2801 TTAATGGTAT TGATTTTGAC ATTTCAAGGA TACCAACACC TGTGTGCTCA TGCACTGGAG CTCCCCAGCA ATGCTACCGG TGGGGTGCAG GTGGCTGGCA
2901 GTCTGCATGC TGCACAACTT CTATTTCGAC ATATCCGCTG CCAATGAACA CAAAGCGCCG GGGTGCACGT ATTGCTGGGA GGAAAATGAG CCAAGGTGCA
3001 TTCAAGAAGG TTCTTGAGAA GCTAGCTGGT GAAGGGTACA ACCTTAATAA TCCAATTGAC TTGAAGACCT TCTGGGCAAA GCATGGCACG AACAAGTTTG
3101 TAACAATCAG GTAAAAGCCA TGCTATGTTG ATCGCCTTGT AGGGTCCAAC GCTGCAGTTT TGCCTGAGTT ATTGCCTGTA CCTGCTCTTG CCAATCTGCA
3201 GATGTCTTTA GAAGTAGCTT GTAGCCATGT TCGGTTTCCT GGACTTAATT TACATGCCTA TTTCGAGCTT CAGCTGTGAA GTAGGAAGTC TGTCTGTATC
3301 TGTCAATTTA GAAGTTGTAG CGTAATGGCA ACAGTTTTTC TAATTAGTTA GCATTTAATG CTTCAATGTC TGTTATGACC TGGATGTTTA TGGCTTTATG
3401 TAGAGCTTTT CTCCATTGAA AGTTGAAAGA TAATCACCAC CTCTTCTAGG GCTAGTTGTA ATTTGGTATT TCCTGACATC TGTCAAGTTT AAATATGAAA
3501 TACCCACATG ACTCGTGCAG TTCTTATCTT TGACCTGCTA TTAAGTTGTG CTCTCATTGA TCGAAGCAAG TAAAATGTCT GATAATTTCT GGTTGTTCCG
3601 TTCTGGTGTT AATGTGCACA TAACCGAGTA TTTTTATTTG TGCCTCTACC TTAATACCTT GGACATAATA TAACCTTCTG TGTTGGACTT AACATATGTC
3701 GTGAAAAAAT TGTTCACCCA TAGTTTGCAT ATCTTAACTC AATATGTGTA GTAGTTGCCT TTTGAAATAG CATGAACTTT AAGTTGTGGT GTTTGATCGC
3801 CCCTTTTCCT AGAATCATGC AAGGGAGTAG TGAAAGGTTT GCTACCAGAG GTCAGTAAGA TCCTGCAAAT GTGTGGCCTT GGCCTCATTG TGAAAAAAAA
3901 ACTTCTATTC CTCTCATTGC ATAGGTGCCA TGTGCCCACA ATAAAGAAGC TGTCCATACC TTTTCTGTTC TGAAAGATGT GAGAGTCCCT ATCCTCTTGC
4001 CACCAGTCTT CCAGTTCACT ATCAAGAGAG GGTGTTAAGC ATTCAAACCA GTCCATCTCT AGATGAAGAA GTTCAAATTT TGCAAGTCTC TGACAATTCT
4101 GAGTTGTTGA AAACCTGATT ACATGACGTT CTGTGAACAT CTTACTAGCT ACTTACGCAA CTTCCAGAAG TACGAGGATA GGTCTTTGGT CATGGTTCTG
4201 TATTTGGAAA TCAAGGTTTA GTTGTGGTGT GTTCGCAAAT ATTTATCCAT ACTTACAAAC CGAGCAGGTT TGATTGCAAT GAGAAAAGGA CTTGCTCTGT
4301 AAACAAATAT AAGAGCGTTT AGATCACTAC TTTAGTGATC TAGACACTCT TATATTTCTT TGCGGAGTGA GTATATGGGT AGCAAAACTA AGTAATATGC
4401 ATATTGTTTG GTTTAGCCAA TTGAACAAAA GTCCATTTGC TTGCCTGCTC TGGTTTGTTT CTTCTTTTAG CTAACAGACA TAATGAGCTA CTACCAACCT
4501 TTTTGCATTT ACGTTGGTTG GCTTTCTGAA AGCACTTTTC AGTTTCTATA TTTGGGCGTT TCAATCATCT TAAGATCTAC CACCGTGCAG AAAGCGCTTT
4601 CTTATTTCTA TTTTTGGATG TATCAGTTAC TTTACGATAC TAGAAGCGCT CGGCTGCTTC CACTATTCGA TGTATTATTA GTTAGCTGGA AAGTACCGGT
4701 ATGCTTTTGG AAGTAACATC AAAATCTTGC GAAAGTTAGC AAGGTTTCTC TATCACAGTA CACATCGGCC GGATATGCCA TGGGTCTATT TGAAGAAACT
4801 GGCATGGCGG TGTAGGAGGT TGGGCCCCCA ACCAAATTAA GTAAAAACAA TGATAGACCA CTCTCTATCG CTAGCTGTAT ACAACTTCAC AGCTGCAGTC
4901 GAGATGAAAC CAGCGTCCTC CCATCGCGCC CTTGGTTTTG TTATTG
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### 7.1.4 *BGRF*

```
   1 GAATTCCCGT TACAACAACT TTACACACGA GGTCAGACCC CTACCGCCAT AGTTCCTAGT GGTAGGGTCT TGCATCCTAT CGTCTTATAC TTGGCGGTAC
 101 GGCCGTTACG CCACGTGAGC CCTTCGGCTG GCAGTTGACG GCCGCTGTTG TTACTCGACT GTCAGATACC TATAAACCTA TCGCCAACCT GTGTAACAAT
 201 GAGAAACGGT CAAATCCCGA AAAAATTTCG AAGCAGGATC GCATCCTGCT AAACTTTTGA CAAATGGTCA AAACACGAAA TTTTTGCCGC TCGTTGTGCC
 301 TCTGTAAGGT GGAAGCCTAC GGTGTCGGCC TCACCCCCAC ACGGTGCTGC CGCTGCTGCG CCTATCGCCA GCGCTTCACG CTATATATCC ACCCCGTCGT
 401 CGTCTGAGTC TCACCAGGCA GATCGAGCCC TGCGCAGCGA GGGGAAAGAG ACACACACAG CGCCACCAGG CAAGTAGTAG TAAAAGGCAA AAGCACGGCA
 501 CATTAAAAGA GAGGCCAGCC CAGCCCCGGA CCGGACCGGA GCCAAGCCGC AGCCGCAGCC GCAGCAGAGG AGAGAGAGAG GGAGGGAGAA GCATATATGG
 601 CGATGCCCTT TGCCTCCCTG TCAACGGCAG CCGACCACCA CCGCTCCTCC CCCATCTTCC CCTTCTGCCG CTCCTCCCCT CTCTACTCGT AAGCCGGCCG
 701 GCCGGCCGGC CAACCGCCTC ACTTCTTTCT TCGTATCTGC TTCCATCTTA GCTCGAGGGG TTCGCTAATG CGGTGACCGT CTCCGGCGCC TGTGTTCGTG
 801 TTCGTGTGTG CAGGGTAGGG GAGGAGGCGG CGCATCAGCA TCCTCATCCT CAGCAGCAGC AGCAGCACGC GATGAGCGGC GCGCGGTGGG CGGCGAGGCC
 901 GGCGCCCTTC ACGGCGGCGC AGTACGAGGA GCTGGAGCAG CAGGCGCTCA TCTACAAGTA CCTCGTCGCC GGCGTCCCCG TCCCGCAGGA TCTCCTCCTC
1001 CCCATCCGCC GCGGCTTCGA GACCCTCGCC TCGCGCTTCT ACCACCACCA CGCCCGTACG TACCCCATCC CTTCCTCCTC CTACCCCGGC CAGGAGTAGT
1101 ACTTGCTTTT TTGCATTCGC CATGCGATCT GCCCGGTTGT TTATTCGGAT CGAGCACTTG CTTTTGCATT CGCCATGCGA TTTGCCCGGC TTGTTTATTG
1201 GGATCGAGAG ATTCAGGTGT GCTCGACCCC CATCCCATGA TTCCCATCTC TTTGTTAATT GCTCCGGTCA TTTGTTAATC CCTCCCCGGA TTTGGCCGAG
1301 CAAAAGTCTC ATTATTCTAA TCCGAGCAAG CCTCGTGCCC CTGTTCAAAG ATTTGCTCCT ACCATCACCA CCTACCACCA TCCAGCAAGC ATCTCCTGCC
1401 TCGCCGGGTC TTTTAATTTA CTTGGGATTT CATTCTCATG TCATGTCATG TGCTATGATT TGATTAGATG GCGCTAGTCG AGTCTTGGGT TAGTTTCCAT
1501 TGGTCCTTCC GTGGCAAGGG GGTTATTCCT GTCTGGTTGT TGGGAGCCTC ACCCACGCAT TCACTCGCTC GCTCGCTGGT CATGTCCTGC CACGGCCGAT
1601 CTCACCGATC CATCCTGCAT CGCATCACAT GGACCCCCGA CGAAAAAGAT CGGCAATCAA CCACGCACAG CTCCTCCTTT CCCCGGAAAT TATTTCGCAT
1701 ACGTCCTTCC TTCCTTCGTT CCTTCCTTCT TGCGGGGTAA ATGATTGGTT TGGTGGGGTG GGCACACAGA TAGATCCAGG ACGAGGACGA CCGCCTTCGT
1801 CCGTCCCTCC GGCCGGCCGG CGTCATGATT GATTGCTACC TGCTACGGCC TTGGACTGGA CGCGTCTCCG TTCTTCCGAT CTCGCGTCTC CTCCTGAGTT
1901 GATTTCTTAG TCCCTCCGGA TGAGCACCGA CACATTCCAA GTGCGTACAG ATGTATGGGG TATTTATCAT GAAAAAGCAT TCTTGACGTG GGTGTTTTTC
2001 GTTGTTTGCA GTTGGGTACG GGTCCTACTT CGGGAAGAAG CTGGATCCGG AGCCGGGGCG GTGCCGGCGG ACGGACGGCA AGAAGTGGCG GTGCTCCAAG
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2101 GAGGCCGCTC AGGACTCCAA GTACTGCGAG CGCCACATGC ACCGCGGCCG CAACCGTTCA AGAAAGCCTG TGGAAACGCA GCTCGTCGCC AGCTCCCACT
2201 CCCAGTCCCA GCAGCACGCC ACCGCCGCCT TCCACAACCA CTCGCCGTAT CCGGCGATCG CCACTGGCGG TGGCTCCTTC GCCCTGGGGT CTGCTCAGCT
2301 GCACATGGAC ACTGCTGCGC CTTACGCGAC GACCGCCGGT GCTGCCGGAA ACAAAGATTT CAGGTGACCT CCATCTGCCT TCTCTGCATA TACTCTGCTG
2401 CCGTCGTGTT GATTAGTTTT TTTTTTTTAA CCTAGTCGTT GTTGTCAGGG GCGTTGAGGT GGCCGATAAC ATCCGATGTT CCCATTTTTG TGCTTGATTG
2501 CATCAGTAGA CAGTAGAGCA TGTGTTGATC AGACTAGTAC CAGTGCTCTT CTGACACTGT TAGTATGATC TGTGCTCGCT TAGGTAATTG CTTGCGTTGC
2601 AGTTTCTGGC CATGCTTCCT GGGGCCGGGT TTTAGACTGG GAAGATTGCG AAACAGAGAG GTGCTCTGCT CTGCTCTGCT CTGATGCAAG TTCAGAGCAG
2701 AGTGTTCTAA ACTAGATTCA CACGCATAGA ACTTTATCTG AGTTTTATGC TCAGAGCCTG CTCTCTGTTC CTGCTCCATC TTAGGCCTGG CATTTATTTT
2801 GCTCCTTTTT CCCTCTGCTG AACCCTGTTT CTTTCCTCTT GTTTGGGTCT GCACTTAGAA AAAATAACGC CTGTTCTGCT CCAGCAAAAC TTACCTGTTT
2901 CTGCCTTTCT CGTCGGGGAC GCCTCATCTC ATTCTACGCC TGAAACCATC TGAAAAGGCT AGCATGATTA TTGAAGCCCA TCACCTTTTG CAATCCATCC
3001 TCTAGCTTTT ATTTTTAGTT TCATCAGAAC TTTGTGATGA GTTACCAGCA ATGTCCCTTT GCCCAAGCTC TGTCATTGTA GTAGTAGGGT TACCTGCGGG
3101 GCAGTCAGCA CATGCATGAT GCTGCATTTC TGCCAATCAT GTTTGCTTGG ATGTCTGGCT TCCTATTTTT GTTTTGTTTT GTTTTTCTCC TGTCACGAGC
3201 TCCTGCTTTA AAATGTGCAG CAACAATTGC TAGCATGTCC CTTTTGTTTT GTCTACTACT CCTAGTAGAC AAGTTTAGAG GTAACGTGAA GCATACCGTA
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3801 ACCAGCTCCG ACTTCTCCGG CACCACCGCC GGCTCCCGCT CGCCCAACGG TATATACTCC CGGTGAACGG CGTCGGCCGG CCTGATCTAC TGCTGATTTG
3901 CCGTGGTCAC GACGGACGTC CTCCAAATCA TCACAGATGA GCGAACCGGC CCGACCCGAT CGAATGTGTC TGTGAGCCGA CTGCAGCTTG CTTGCTCATT
4001 TTGTATGGAT CGTCGTCCAG CAGGAACAAA ACACTACTCC TTTAATTTCC TTTCTTTAAT TTCACAACGT TTTTTCTGGG TTTTGCCGTG TATCGGCCGG
4101 AACTGTACTA CCAAGTTTTC TATAGCCTCG ATGGTCATGC ACGACATCGT TGACTGTTTC CCGCGCACTT ACTGTTGAAA TAATCTTCCA TTTTTGGCTA
4201 TCAATTTACC GTCAGTTCTC TGAATAAACC TCCGAACTAG TAGTAGTTGT AGATGCGATG GTCTTTACTT GATGTTTAAA TGTTCGGGCT TGATAAAACA
4301 CACTCGGCTT TTGCAACATT TTATGCCAAC TTTTGCAACA TTTTGTCCTA GAGATTGAAG TATTCGTCTT GTTGGCCCTC ATCGGTTGAG ACTTCGTTAA
4401 ATCTCAGTCG ATTGAGTTTT AGACGGACAT TTAAATGTTT AATCGAATGC TAGACGGACC CTTAAATATC CAATCGATCT TTGGATGGAT CACATGGACG
4501 GGTAGGGTTG TCAGCAGGGT CACGCGTGTT GCTTCATGGT GGAGGCAAGC CCCGATCCGG GGCAAAGCTT CATGCGCAAT CCTCCGACGA GCAAGCATCT
4601 TTTTTTCTTT CTTTTGATGG GCGGGTTATG GGAGCATTCG GGGCACAGAT GGGCATTCGT GCTTCCTCTT TTTCGACTTG CTTTGGCGTG TGAGACGCAA
4701 GGGGACACGG TTCGCTGCCC CTGGTCATGC AGGCTTACTT TAAGAGGAGG AGAAAGTACA ATGATGCACT GACGCTGGCT GGATCTGCCC GCGCAAAGCC
4801 TCCGTAGTTG GTGGAGGAAC AGGCTGTGGT AAGCCCTTGC TGACCGTGGG CCCGTGCCTG AACTTGCCGT GTTTGTGGCC TTGGCTTGGA AGCACACGAC
4901 GCGGCAGCAT CCGGTTCAGT TAACTCGGTC CGTGCGGGGT GGGCAAAACT GACAAATTTG ACCCAAAAAC CAAACTAATT CATAAACTGA ATTACGTTCG
5001 AAAAAATATT CATCCAACTG ACCCTTTTAT GTGATGCTTT TGTCTTAGGC GTCACGCCAC GTTGTGTGAC GTCTGAGACC TATTGTGGCG CCTAAGGCAT
5101 AGGCCCCATA TACACTGTGT GGCGTCTAAG ACAAAGGTGT TACACAGTGT AGTGTGACAG CTGACTGTGG GACGTCACAC AAAAGGATCC GTTGACCTGC
5201 AGG
```

## 7.2 Oligonucleotides used for single strand conformation polymorphism (SSCP) analyses of *BEIL*, *BAPL*, *BBR* and *BGRF*

### 7.2.1 *BEIL*

Bei23 5′-GATATTCTTCTTCGATTCATG-3′
Bei24 5′-CTGACATCATTCTCAGAGAGG-3′
Bei25 5′-CTACGGATAACCATGTGCCTG-3′
Bei26 5′-CATCATCATCCAGCAGTTCAG-3′
Bei27 5′-CTCTACTGTGGCCGAATTAG-3′
Bei28 5′-ACCTCATCGGTATTAATAGTCC-3′
Bei29 5′-TGGCCAGCAAAGAAAGATTGT-3′
Bei30 5′-TCCTTGATCCTCTTGAGCCT-3′
Beip1 5′-CTATCACATCAGGGATCTCAGG-3′
Beip2 5′-ATCATCATGCGCAACAACCAAT-3′
Beip3 5′-ATGACCTACCTTACCTGAGCAC-3′
Beip4 5′-TGCTGCATTCTAGTAAGTGCTG-3′
Beip5 5′-TTGAGCTTCAGGGTTGCATAT-3′

### 7.2.2 *BAPL*

BAPL5 5′-GGACATACGACACTGCTGAG-3′
BAPL6 5′-CGGAATGAACATGCTCTCTG-3′
BAPL7 5′-TCCAGAGCGAGTCTGCCGTCA-3′
BAPL8 5′-TATGCTGCTAGCAGTGGGAA-3′
BAPL9 5′-TCGGTTGCACACTGGATTT-3′
BAPL10 5′-TGCACAAGACATCCCTGCT-3′
BAPL11 5′-CAAGCACATTACATCAGGCA-3′
BAPL12 5′-TCCATGACAGCACATCCT-3′
BAPL13 5′-GTTCGGTTCAGTGCACTGA-3′

BAPP1 5′-TACATCAGAATTAGGTGGATGC-3′
BAPP2 5′-TCGACGTCAGCAGCAACAACAA-3′
BAPP3 5′-ACTGATGGTACAACAATCCCAA-3′
BAPP4 5′-AGATGCATGCATATCATACCAC-3′
BAPP5 5′-TGAGACCACGAGGGGCACATT-3′
BAPP6 5′-AAATCGTACGGTTATGGACG-3′
BAPP8 5′-AGACCTCGTGTGTACTACTA-3′
BAPP10 5′-TGTGATCCGTCCATCCATCT-3′
BAPP11 5′-TGGTGTATTGTCATCTTGAG-3′
BAPP12 5′-GAACGAACGACATGCCTACA-3′
BAPP18 5′-AGGGATGGAGTCACTGCTT-3′

### 7.2.3 *BBR*

BBR10 5′-GCACGAGGCTGATGCCATC-3′
BBR11 5′-CGGCGGGCATGCCACCAGAG-3′
BBR12 5′-GCCTGAGTTATTGCCTGTAC-3′
BBR13 5′-GGTCATAACAGACATTGAAG-3′
BBR14 5′-TGCTGCAAGAAGGTACCGT-3′
BBRP1 5′-GTTGCGTAAGTAGCTAGTAAG-3′
BBRP2 5′-AACAATCATGCAGTGACGCAG-3′
BBRP3 5′-AACATTCCGGTCTTACAGACA-3′
BBRP4 5′-TACATCTCTCCATTGACTGTA-3′
BR1 5′-TGCGTGTGATACGACGAA-3′
BR2 5′-AGGAACGGAGGGAGTACAA-3′
BR3 5′-AGAGAGCAGGGACGAAAT-3′
BR4 5′-TGCTGGTGCTGATGTTGA-3′
BR5 5′-TCACATGACTTTGTGCGCA-3′
BR6 5′-ACCAGTCTTCCAGTTCACT-3′
BR7 5′-AGGACGCTGGTTTCATCT-3′

### 7.2.4 *BGRF*

H1 5′-CATGCTGGGCACGCTGAGCG-3′
H2 5′-CGCTCAGCGTGCCCAGCATG-3′
H7 5′-CTGCAAGACCACCTCGACTCTA-3′
H8 5′-AGGCAAACCTCGTGCCGAAT-3′
H6-1 5′-CTGTCGCCGGCAGCCGACCA-3′
H6-2 5′-GGCCACGAGTCCCTGTCCT-3′
H6-3 5′-GACGTCCTCACTCCATAGGC-3′
H6-4 5′-GCGCATCAGCATCCTCATCC-3′
H6-5 5′-CACTCGTCGAAGAAGGGCCC-3′
H14 5′-GTTCGCTCATCTGTGATG-3′
H15 5′-AGACGATAGGATGCAAGAC-3′
HP1 5′-AACAGTGAGAGGTGAACGAT-3′
HP2 5′-AACTCATTTTGGGGTACTCA-3′
HP3 5′-TCGACCTCATTCACGGGA-3′
HP4 5′-AAGCTGGTGATGCTGAGCA-3′
HP5 5′-AGCCAGGTAACACAGTGTT-3′
HP6 5′-CACGGATCTATGACAATGC-3′
HP7 5′-TCCAGAACATCTCGCTGGT-3′

# Erklärung

Ich versichere, daß ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit – einschließlich Tabellen, Karten und Abbildungen -, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; daß diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; daß sie – abgesehen von unten angegebenen Teilpublikationen – noch nicht veröffentlicht worden ist sowie, daß ich eine solche Veröffentlichung vor Abschluß des Promotionsverfahrens nicht vornehmen werde.

Die Bestimmungen dieser Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Priv.-Doz. Dr. Richard Thompson betreut worden.

Köln, den 02.05.2001

WANG Yamei

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

# Lebenslauf

| | | |
|---|---|---|
| **Name** | WANG Yamei<br>Kolibriweg 14,<br>50829 Köln | |
| **Geburtsdatum und -ort** | 22.08.1970<br>Changchun, Jilin Provinz, V R. China | |
| **Nationalität** | Chinesisch | |
| **Eltern** | Vater<br>Mutter | WANG Xinyue<br>ZHAO Xiouhui |
| **Familienstand** | veheiratet | |
| **Schule** | 1976-1981<br>1981-1984<br>1984-1987 | Grundschule<br>Vorbereitungsschule<br>Höherschule |
| **Studium** | Sep.1987-<br>Jul.1991 | Studium der Biologie an der<br>Northeast Normal University,<br>Changchun, V R. China |
| | Sep.1991-<br>Jun.1994 | Magisterarbeit der Genetik an der<br>Northeast Normal University,<br>Changchun, V R. China |
| | Jul.1994-<br>Jun.1997 | Teaching assistant an der<br>Beijing Agricultural College,<br>Beijing, V R. China |
| **Promotion** | Jul.1997-<br>Dec.2000 | Ph.D. Genetik<br>Universität zu Köln/Max-Planck Institut,<br>Köln, Deutschland |

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