Identification and Characterization of the *BRX* Gene Family of *Arabidopsis thaliana*: Investigation of Functional Redundancy in the Root and Emerging Roles in Shoot Gravitropism.

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Abstract

The model plant *Arabidopsis thaliana* has been very successful thus far as a tool for understanding and studying the genetics of plant development. Analysis of its sequenced genome revealed the occurrence of duplicated chromosome blocks, resulting in duplicated genes. Duplicated genes, high in sequence and/or structure similarity, form gene families. One such family, of *BRX*-like genes, is presented in this thesis. All members contain a characteristic "BRX" domain that is required for BRX activity *in planta. BRX* (*BREVIS RADIX*), is a novel regulator of root growth in *Arabidopsis*. However, analyses of *Arabidopsis* single and double mutants with other gene family members, suggests that *BRX* is the only member with a dominating role in root development. Interestingly, *BRXL1,* although having BRX activity in the root, does not act redundantly with *BRX in vivo*, presumably because it is expressed at much lower levels than *BRX*. These two gene family members demonstrate the uncommon phenomenon of unequal genetic redundancy in plants. Another gene family member, *BRXL4,* although non- redundant with regards to BRX activity in the root, did display novel shoot-related phenotypes when over-expressed. In these lines the lateral shoots and hypocotyls showed increased Gravitropic Set-Point Angles resulting in the downward growth of the adult lateral shoots and a wide range of growth directions in the hypocotyls. Over-expression lines of *BRXL4* also displayed seedling agravitropism. Auxin-induced transcription as monitored by the DR5::GUS reporter, is altered in these lines compared to wild-type. Additionally, hypocotyl curvature, stem bending and amyloplast localization profiles in response to a change in gravity vector, are also altered in these over-expression lines and in the *brxl4* mutant compared to wild-type.

Résumé

Le modèle végétale *Arabidopsis* thaliana a été jusqu'à présent très utile comme outil pour comprendre et étudier la génétique du développement de la plante. L'analyse du génome séquencé d'*Arabidopsis* a révélé la présence de blocs de chromosomes redondants, aboutissant à la duplication de gènes. Ces gènes dupliqués, qui présentent une forte homologie de séquence et/ou de structure, forment des familles de gène. Une de ces familles, la famille de gènes *BRX*-like est présentée dans cette thèse. Tous les membres de cette famille contiennent un domaine caractéristique, le domaine "*BRX*" qui est indispensable pour l'activité *BRX* in planta. *BRX* (Brevis Radix), est un nouveau régulateur de la croissance des racines chez *Arabidopsis*. Cependant, les analyses de mutants simples et doubles sur d'autres membres de la famille de gènes suggèrent que *BRX* est la seule région ayant un rôle dominant dans le développement des racines. Il est intéressant de constater que in vivo et bien qu'ayant une activité *BRX* dans la racine, *BRXL1* n'agit pas de façon redondante avec *BRX*, vraisemblablement parce qu'il est exprimé à un niveau bien plus faible que BRX. Ces deux membres de la famille de gènes mettent en évidence le phénomène inhabituel de redondance génétique inégale chez les plantes. Un autre membre de la famille, *BRXL4*, bien que non redondant en ce qui concerne l'activité *BRX* dans la racine, a permis de mettre en évidence de nouveaux phénotypes au niveau de la pousse quand il est surexprimé. Dans ces lignées, les pousses latérales et les hypocotyles démontrent une croissance altérée en réponse à la force de gravité (Gravitropic Set-Point Angles, GSA's) aboutissant à la croissance vers le bas des pousses latérales et à la croissance des hypocotyles dans des directions variées. Les lignées qui sur-expriment *BRXL4* ont aussi démontré une croissance agravitropique

des plantules. De plus, et comparativement aux plantes sauvages, la transcription induite d'auxine contrôlée par le gène reporter GUS (DR5::GUS) est altérée dans ces lignées. Enfin, la courbure de l'hypocotyle, de la tige et les profils de localisation des amyloplastes en réponse à un changement du vecteur de la force de gravité sont aussi modifiés dans les lignées sur- exprimées et dans les lignées mutantes *BRXL4* et ce en comparaison au type sauvage.

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Contribution of Authors

Chapter 2: G.C.B was involved in the layout, literature mining and writing of the manuscript.

Chapter 3: C.S.H. conceived this study, wrote the manuscript and contributed data for Figure 1A-E and 3A. C.F.M. contributed data for Figures 1F-G, 2, 3B-G, 4A-F, 4I, 5, 7A-B and Table 1, and established the introgression and recombinant inbred lines. G.C.B. contributed data for Figures 4C, 4G, 6 and 7C, and analyzed the *brxl* single and double mutants.

Chapter 4: G.C.B. contributed results for Figs. 1, 3A-B, 3E-F, 4, 5, and 6. C.F.M. contributed results for Fig. 3C-D. C.S.H. gathered data for Figs. 1 and 2. C.S.H. and G.C.B conceived this study and wrote the manuscript.

Chapters 5 and 6: G.C.B performed all data mining, research and experiments

Chapter 1

INTRODUCTION AND THESIS RATIONALE

During the process of embryogenesis, the basic axes and tissues of animals and plants are established. In the mature plant embryo (seed), the fundamental components of the adult plant form, including an embryonic root (radicle); an embryonic stem (hypocotyls) and some leaf-like storage organs (cotyledons) are present. For plant survival, these organs are further elaborated during processes of post-embryonic growth, development and morphogenesis. During these processes, plants also develop more tissues and organs (secondary growth axes), forming lateral roots and lateral shoots (branches).

The cell wall is a defining structure in plants and is largely responsible for the sessile nature of these organisms. In addition to affecting growth and development, this physical immobility means that plants are forced to deal with and adapt to various environmental influences. As a result, environmental signals such as light, water availability and especially gravity, play important roles in plant developmental processes (Raghavan, 2000) and plants, in response, are highly plastic in nature. This phenomenon is commonly referred to as phenotypic plasticity and links the processes of plant physiological mechanisms, gene regulation and morphological plasticity (Schlichting, 2002). At the genome level, this plasticity is also observed and is thought to be related to genome and gene duplication events. These duplication events, although occurring in various forms all involve the rearrangement of genetic material. The repeated occurrence of these duplication events during the evolutionary history of the organism, can lead to the creation of large gene families. The commonality within these families may be structural, functional or both. The duplication process is thought to result in the relaxation of selection on one gene copy (Ohno, 1970). This, in essence, creates a situation where some gene family members may be capable of compensating for the functional loss of other genes, a

phenomenon known as genetic redundancy (Avery and Wasserman, 1992; Martienssen, 1999). This redundancy in itself makes the genome more robust and flexible. This increased flexibility or plasticity of the genome, may aid in buffering the cell against errors in metabolism thereby adding versatility to its metabolic regulatory strategy (Martienssen, 1999).

The model plant organism *Arabidopsis thaliana* has been extensively studied due to its relatively small and sequenced genome (128Mb) and its comparatively short life-cycle. Genome analysis has revealed that most of its genes belong to gene families or have closely related sequences in the genome (The *Arabidopsis* Genome Initiative, 2000). In line with this observation, duplication of chromosomal segments appears to have occurred several times during the evolutionary history of this species, making it an excellent model for the study of genetic redundancy arising from gene duplication (Vision *et al.,* 2000; Simillion *et al.,* 2002; Blanc *et al.,* 2003). Even with all the resources that the availability of the *Arabidopsis* genome sequence presents, including the identification of gene knock-outs or disruptions, assigning functions to genes is still the largest challenge to date. The major pitfall in this task is the fact that the majority of gene knock-outs do not result in phenotypes (Bouche and Bouchez, 2001), a problem that is largely due to the presence of these gene families. It has therefore been suggested that more research be devoted to the study of the evolutionary and functional divergence of gene families, ideally combining loss-of-function as well as gain-of-function approaches (Somerville and Dangl, 2000; Bouche and Bouchez, 2001; Hirschi, 2003). This is particularly important for the characterization of the roughly 45% of *Arabidopsis* genes that can neither be assigned a biological nor a biochemical function, because of a lack of homology with functionally defined genes or protein domains (Somerville and Dangl, 2000).

The focus of my thesis research has been twofold. First, I undertook an examination of the phenomenon of genetic redundancy in

plants. In this study I contribute to the exploration of plant gene families and their function by identifying and characterizing the *BRX* gene family, results of which are presented in two published papers (Chapters 3 and 4). The second area of my research stemmed out of my first analysis of the *BRX* gene family, and focused on *BRXL4*, which from phenotypic observations and analyses, appears to have a novel role in shoot development. For this work, I present unpublished data to support the proposed involvement of *BRXL4* in shoot gravitropism and the specification of the gravitropic set-point angle of lateral shoots (Chapter 5).

Chapter 2

LITERATURE REVIEW

Duplication, Functional Redundancy and Shoot Gravitropism

My thesis research focused on the analysis and characterization of the *BRX* gene family in *Arabidopsis thaliana*. Under the broad umbrella of the *BRX* gene family analysis, two distinct areas of research are discussed. Because of the separate nature of these topics, I will first present a literature review on gene duplication and functional redundancy, Chapter 2.1, and a second literature review on shoot development and gravitropism, Chapter 2.2

2.1 Genome and Gene Duplication

Genome duplication or genome doubling has been significant not only in the speciation process in plants, but also in the evolution of all vertebrates and many eukaryotes (Wendel, 2000 and references within). Genome doubling, as the term implies, involves the doubling of the host genome. This process involves obtaining an initial temporary allopolyploidy (different genomes) or autopolyploidy (same genome) state followed by a series of genome diploidization events. Diploidization involves the successive return from tetraploidy to the diploid state. It is not an instantaneous process and may in fact occur on a chromosome-by-chromosome basis as observed in maize, an allotetraploid. In this regard, many plant species considered to be diploid, are actually stabilized or chromosomally diploidized polyploids (Wendel, 2000). The diploidization process is a cyclical one, involving rounds of duplication and divergence. It may also involve other processes such as chromosomal re-patterning and loss of duplicated segments, with resulting evolutionary consequences such as changes in duplicate gene expression levels and functional divergence.

Ohno (1970) believed that one of the evolutionary consequences of genome doubling, i.e duplication, leads to the relaxation of selection on one gene copy and allows divergence between duplicated copies to occur, thereby facilitating the acquisition of new function (Wendel, 2000; Ohno 1970). Gene silencing is also highlighted by Ohno as another evolutionary consequence of genome doubling (Ohno, 1970). Here the influence of immediate gene silencing or epigenetic mechanisms is thought to play a pivotal role in the initial stabilization of the polyploidy state. Other silencing mechanisms via mutational and/or deletion events, are considered long-term evolutionary consequences of polyploidy. Polyploidy increases the number of duplicated sequences in the genome, thus providing the material for homology or non-homology dependent recombination, which

may result in chromosomal rearrangements (described below). Therefore, gene duplication events, in the form of single genes or larger duplication blocks, occur on an already altered genomic background. This background, which contains many homologous regions, is now biased towards future homology-based recombination and duplication events.

Gene duplication, as described above, mainly results from the rearrangement of genetic material and several different classes of chromosomal rearrangements can occur in the genome. These may take the form of deletions, duplications, inversions and translocations, just to name a few, and may result from transposition, illegitimate recombination, and chromosomal non-disjunction or genome fusion events. These rearrangements, balanced or unbalanced, may also occur via crossing-over events between repetitive DNA elements.

The occurrence of *balanced rearrangements* neither affects chromosome number nor do they result in any dosage effects. Instead they simply result in a change in gene order on the chromosome. These processes however, generate duplication and deletion products at the end of meiosis. For example, in a unidirectional translocation event, one segment of a chromosome is directly inserted into another and subsequent segregation of this insertional heterozygote with a normal copy can result in a duplication event. Another type of balanced rearrangement, an inversion event, involves the physical breakage, 180 degree rotation and reattachment of an internal chromosome segment.

Imbalanced rearrangements on the other hand do result in gene dosage changes in part of the affected chromosomes. These may include whole genome changes as previously discussed, but may also include processes that result in duplication or deletion of genes or chromosome segments. The most common forms of imbalanced rearrangements are the result of tandem repeats through slippage mechanisms during the recombination process (thought to be followed by periods of local chromosome rearrangement and extensive gene silencing in one of the

duplication blocks). Other forms of imbalanced rearrangements include gene conversion, gene transpositions e.g. horizontal transfer, and segmental duplication of chromosomes. Large-scale imbalances resulting from the loss or gain of whole chromosomes can also occur in the genome and results in aneuploidy. Chromosomal non-disjunction, which involves the failure of homologous chromosomes or chromatids to separate to opposite poles during the course of meiosis or mitosis, occurs spontaneously and is the most common cause of aneuploidy.

Overall, the repeated occurrence of these duplication events during the evolutionary history of the organism can lead to the creation of large gene families and, by extension, the occurrence of functional redundancy. This process and its implications on plant development are discussed in more detail in the following section.

"Unequal genetic redundancies in *Arabidopsis* - a neglected phenomenon?"

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Abstract

Genetic redundancy is a common phenomenon in *Arabidopsis* and thought to be responsible for the absence of phenotypes in the majority of single loss-of-function mutants. Here, we highlight an increasing number of examples where redundancy between homologous genes is limited or absent, despite functional equivalence of the respective proteins. In particular, we focus on cases of unequal redundancy, where the absence of a mutant phenotype in loss-of-function mutants of one gene contrasts with a strong phenotype in mutants of its homolog. In the double mutants, this phenotype is strongly enhanced. Possible explanations for such scenarios are discussed. We propose that the study of unequally redundant gene pairs offers a unique opportunity to understand global patterns of functional genome evolution.

2.1.0 Genetic redundancy in plant development

Genetic analysis of development in *Arabidopsis thaliana* has been tremendously successful in isolating mutants that are impaired in specific processes, and the analysis of the affected genes has produced much insight into the molecular basis of plant development. However, the lack of distinguishable mutant phenotypes in the majority of loss-of-function mutants identified by reverse genetics suggests that the mutagenesis approach is limited in scope. This absence of mutant phenotypes has been largely attributed to genetic redundancy (Bouche and Bouchez 2001; Somerville and Dangl 2000; Hirschi 2003), a notion that is supported by analyses of the *Arabidopsis* genome sequence, which demonstrate that over 80% of the 125Mb genome represent duplicated regions (Blanc *et al.* 2003; Bowers *et al.* 2003; Simillion *et al.* 2002; Vision *et al.* 2000). Interestingly, large-scale genome duplication events, such as polyploidization, are a common feature of plant genomes and are mainly responsible for the large number of duplicated individual loci (Bowers *et al.* 2003, Langham *et al.* 2004; Paterson *et al.* 2004; Maere *et al.* 2005).

2.1.1 The evolutionary fate of duplicated genes

Since genetic redundancy is usually observed between homologous genes (Pickett and Meeks-Wagner 1995), its occurrence follows gene duplication events in the evolutionary history of the organism. In principal, gene duplication creates two functionally identical copies, which should act fully redundantly immediately after the duplication event. Generally it is assumed that one of the redundant copies is initially free of all selective restraint (Ohno 1970). Since this could in principal be either copy, we refer to the copy that is under selection to retain all or most of the original functions as the ancestral gene, while we refer to the other copy as the duplicate gene. Because it is more likely that the duplicate gene acquires deleterious rather than advantageous mutations, classic

evolutionary theory predicts that most of the time, it is subsequently lost or becomes a pseudogene (Ohno 1970; Wagner 1998; Tautz 1992; Weintraub 1993). This process is called nonfunctionalization. Alternatively, the duplicate gene might acquire advantageous mutations that become subject to selection and lead to a new function, a process termed neofunctionalization. Finally, both genes can accumulate mutations that might lead to the sub-division of the functions of the ancestral gene. This process is called subfunctionalization. Both neo- and subfunctionalization presumably occur mainly through *cis*-acting mutations in regulatory sequences, rather than mutations in the coding sequence (Langham *et al.* 2004; Wang *et al.* 2004; Blanc and Wolfe 2004; Casneuf *et al.* 2006; Haberer *et al.* 2004), although one has to caution that this conclusion might simply reflect a bias in the field towards analyzing the divergence of gene expression rather than protein activity. Because expression pattern differences between duplicated genes can occur rapidly after the duplication event, probably driven by epigenetic changes, neo- and subfunctionalization might be more frequent than previously suspected (Adams *et al.* 2003; Wang *et al.* 2004; Casneuf *et al.* 2006).

2.1.2 Full and partial genetic redundancy

The level of genetic redundancy between two homologous genes correlates with the fate of the duplicate gene (Figure 2.1A). Immediately after duplication, the homologs are presumably fully redundant. Thus, loss-of-function of either copy will not by itself result in a mutant phenotype. Full redundancy is, however, considered to be genetically unstable, because of the accumulation of deleterious mutations that can result in rapid loss of the duplicate gene (Ohno 1970; Wagner 1998; Tautz 1992; Weintraub 1993). Nevertheless, full genetic redundancy in *Arabidopsis* has been documented on several occasions (e.g. Liljegren *et al.* 2000; Wang *et al.* 2005). Fully redundant genes are mostly discovered through reverse genetic approaches, based on sequence homology and

overlapping expression patterns (Liljegren *et al.* 2000) because the likelihood to hit two redundant genes simultaneously in a mutagenesis experiment is extremely slim. In fact, only one such case has been reported for *Arabidopsis* (Aida *et al.* 1997).

Examples of partial redundancy are more numerous (e.g. Aida *et al.* 2004; Bernhardt *et al.* 2003). This could either reflect the fact that loss-of-function mutants in partially redundant genes have a detectable phenotype, or that partial redundancy is evolutionarily more stable (Tautz, D. 1992; Weintraub, H. 1993). In this context, we would like to note that in this review, we use the term partial redundancy for the situation where both gene copies become partially compromised so that both copies are required to provide the overall function that was previously provided by the single ancestral gene (Figure 2.1A).

2.1.3 Unequal genetic redundancy

As discussed above, the common fate of duplicate genes is assumed to be nonfunctionalization. It is conceivable that non-functionalization is not always a sudden event, such as a missense mutation, but could involve transition stages in which the duplicate gene possesses only a fraction of the functions of its homolog. However, in such a situation, one would already expect to observe that loss-of-function of the ancestral gene would result in a mutant phenotype, while loss-of-function of the duplicate gene would have no effect. Depending on the residual function retained in the duplicate gene, different scenarios could be envisioned for the double mutant phenotype. These scenarios imply that the trait affected by the genes is sensitive to their activity in a continuous, quantitative manner, as observed in many developmental processes. If a certain threshold gene activity is required for the genes to have any influence on the trait at all, and if the residual activity of the duplicate gene is below this threshold, then the double mutant phenotype should resemble the single mutant of the ancestral gene (Figure 2.1B). If

however the residual activity of the duplicate gene is above this threshold, then the double mutant would display an enhanced, "true" null phenotype, and possibly a novel phenotype. The latter would apply in any case if the process in question is influenced by the gene activity in a continuous quantitative manner, without the need for a threshold (Figure 2.1C), as long as the duplicate gene has some residual activity. Thus, in these situations the duplicate gene, although principally dispensable, still contributes significantly to the overall activity of the gene pair, as revealed by double mutant analysis. Here, we would like to highlight this type of scenario, for which we use the term unequal redundancy.

The classic example for unequal redundancy in *Arabidopsis* is the genetic interaction between *APETALA1* (*AP1*) and *CAULIFLOWER* (*CAL*) (Bowman *et al.* 1993; Kempin *et al.* 1995). *ap1* mutants display a floral homeotic phenotype, while *cal* mutants resemble wild type. The double mutants however display a strongly enhanced *ap1* phenotype with novel aspects, the *cauliflower* phenotype (Kempin *et al.* 1995). indicating that *CAL* activity contributes to *AP1*-controlled processes. Recently, an increasing number of examples for unequal redundancy in *Arabidopsis* have been reported (Table 2.1), suggesting that this phenomenon is more common than previously suspected.

2.1.4 Cross-regulation in unequal redundancy: weak "double mutants"

Interestingly the *AP1–CAL* example is consistent with the idea that expression pattern changes contribute in large part to the diversification of duplicate genes (Langham *et al.* 2004; Adams *et al.* 2003; Wang *et al.* 2004; Blanc and Wolfe 2004; Casneuf *et al.* 2006; Haberer *et al.* 2004), because the non-dispensable gene, *AP1*, is expressed at much higher level than the dispensable one, *CAL* (Kempin *et al.* 1995). However, for other unequally redundant gene pairs, expression levels could be largely similar. Nevertheless, only loss-of-function of one of the genes results in a

mutant phenotype. Such a situation can occur if cross-regulation between the homologous genes exists (Figure 2.2).

For instance, mutants of *LONG HYPOCOTYL 5* (*HY5*) display strong photomorphogenesis and root system defects, whereas mutants of its close homolog *HY5 HOMOLOG* (*HYH*) resemble wild type. In *hy5 hyh* double mutants however, the *hy5* phenotypes are strongly enhanced, and even novel phenotypes appear (Holm *et al.* 2002; Richard Sibout, unpublished). The expression patterns and levels of the two genes are similar (Richard Sibout, unpublished), but interestingly, HYH activity is much higher in wild type than in *hy5* mutants, indicating that it partially depends on the presence of *HY5* (Holm *et al.* 2002). Moreover, constitutive expression of *HYH* in a *hy5* background rescues the mutant phenotypes. Therefore, *hy5* can be considered a sort of weak *hy5 hyh* double mutant.

Another recent example demonstrated crucial dosage dependence of the expression of the auxin response factor (ARF) *ARF19* on its unequally redundant homolog *NON-PHOTOTROPIC HYPOCOTYL 4* (*NPH4/ARF7*). While *nph4* mutants display defective tropic hypocotyl responses and lateral root formation (Okushima *et al.* 2005; Stowe-Evans *et al.* 1998), *arf19* mutants have no mutant phenotype (Okushima *et al.* 2005; Stowe-Evans *et al.* 1998). However, in the double mutant, *nph4* phenotypes are strongly enhanced and even novel phenotypes appear. Interestingly, *NPH4* is required for auxin-responsiveness of *ARF19* expression, suggesting that *ARF19* activity is sub-optimal in *nph4* (Li *et al.* 2006). This notion is confirmed by the striking observation that a genomic fragment of *ARF19* largely rescues an *nph4* single mutant. Thus, conceptually *nph4* can be considered a weak *nph4 arf19* double mutant.

The cross-regulation in the above examples offers an immediate explanation for the observed unequal redundancies. It also indicates that these situations might represent a transition stage between full

redundancy and nonfunctionalization. Nevertheless, the possibility of sub- or neofunctionalization of the duplicate genes still exists.

2.1.5 Maintenance of unequal redundancy by sub-or neo-functionalization

For instance it has been suggested that *CAL* is under selection (Purugganan and Suddith 1998). This finding seems in disagreement with the nature of the original *cal* allele, which was found rather serendipitously as a natural, accession-specific variant and has as little activity as unequivocal null alleles (Bowman *et al.* 1993; Kempin *et al.* 1995). These observations could be reconciled by the finding that *CAL* might be recruited for novel, adaptive but dispensable functions, supporting the idea that unequal redundancy could also reflect a transition state between full redundancy and sub- or neofunctionalization.

The idea that *CAL* is being recruited for novel functions is supported by its non-neutral intra-specific evolution and the existence of functionally distinct alleles (Bowman, J.L., *et al.* 1993; Purugganan and Suddith 1998), which differentially influence the branching pattern of inflorescences. In this case, neofunctionalization appears to result from changes in the protein sequence. However, expression pattern divergence predicted to accompany sub- or neofunctionalization in *Arabidopsis* (Wang *et al.* 2004; Blanc and Wolfe 2004; Casneuf *et al.* 2006; Duarte *et al.* 2006) can be observed in other cases of unequal redundancy. For instance, the brassinosteroid receptor BRASSINOSTEROID INSENSITIVE 1 (BRI1) and its homolog BRI1-LIKE 1 (BRL1) share overlapping but distinct expression patterns (Cano-Delgado *et al.* 2004). *BRI1* is generally expressed in a broader domain and at higher level than *BRL1*. Expression of *BRL1* from the *BRI1* promoter rescues a *bri1* null mutant, indicating that the protein products are in principal functionally equivalent, although BRL1 is less active than BRI1 (Cano-Delgado *et al.* 2004; Zhou *et al.* 2004). Unlike *bri1* mutants, *brl1* mutants do not display a mutant phenotype. However, the severe dwarfism of *bri1* mutants, but not

their vascular defect, is enhanced in *bri1 brl1* double mutants (Cano-Delgado *et al.* 2004). Interestingly, this unequal redundancy is background-specific (Cano-Delgado *et al.* 2004) in the Ws-2 background, but not in the Col background, *brl1* single mutants have a vascular phenotype. Therefore, in the Ws-2 background, *BRL1* is subfunctionalized, contributing non-dispensable activity in a tissue where *BRI1*'s expression level is sub-optimal, possibly because of background-specific genetic modifiers.

2.1.6 Adding complexity: unequal redundancies involving more than two genes

The *BRI1–BRL1* example also demonstrates that unequal redundancies can involve more than two genes, because another gene, *BRL3*, also encodes a functional brassinosteroid receptor that complements *bri1* when expressed under control of the *BRI1* promoter (Cano-Delgado *et al.* 2004). In the Col background, the vascular defects of *bri1* are not enhanced in *bri1 brl1* or *bri1 brl3* double mutants, but they are in *bri1 brl1 brl3* triple mutants. Similar situations of one non-dispensable gene versus two dispensable homologs might be more frequent than expected, since this has already been observed repeatedly (Cano-Delgado *et al.* 2004; Muller *et al.* 2006; Shpak *et al.* 2004). If differential unequal redundancies between the possible pairs of more than two homologs exist, this can stabilize rather complex genetic interactions. Such differential relations have been observed for *NPH4*, exemplifying sub- and possibly neofunctionalization in different developmental processes.

As mentioned above, *NPH4* and *ARF19* display unequal redundancy (Okushima *et al.* 2005; Li *et al.* 2006), but interestingly, *NPH4* also acts unequally redundantly with another ARF, *MONOPTEROS/ARF5* (*MP*) (Hardtke *et al.* 2004). However, this unequal redundancy is observed with respect to a different process, embryogenesis. *mp* null

mutants display a strong seedling lethal phenotype, i.e. absence of the hypocotyl and primary root, because of an embryonic patterning defect. However, because *MP* is required for vascular differentiation throughout the life cycle, adult *mp* plants, generated by adventitious rooting (Przemeck *et al.* 1996) display numerous shoot defects. By contrast, *nph4* mutants are viable and have no seedling patterning defects (Stowe-Evans *et al.* 1998) but display defective tropic responses and root system growth. In *mp nph4* double mutants however, the seedling defects of *mp* are strongly enhanced (Hardtke *et al.* 2004) indicating that *NPH4* activity supplements *MP* activity during embryogenesis.

The example of *NPH4–MP* is different when compared to the previous ones, because the unequal redundancy between them is restricted to particular processes. Thus, although *NPH4* is not essential for embryogenesis, it is subject to selection, since it is essential for other processes. For instance, *MP* and *NPH4* act partially redundantly in differential growth responses in the hypocotyl (Hardtke *et al.* 2004). Therefore, *NPH4* is an example of subfunctionalization. Moreover, unlike in the previous examples, the NPH4 and MP proteins are not fully exchangeable. While constitutive ectopic expression of *MP* can complement the *nph4* hypocotyl defects, constitutive ectopic expression of *NPH4* cannot complement the vascular defects of *mp* plants (Hardtke *et al.* 2004). Therefore, *NPH4* has become sub-functionalized both because of its differential expression pattern and differential protein activity (Hardtke *et al.* 2004). Finally, whether *MP* can replace *NPH4* in lateral root development has not been tested, thus the involvement of *NPH4* in this process could result from neofunctionalization.

2.1.7 Absence of redundancy between highly homologous genes

While unequal redundancies appear to be relatively common in *Arabidopsis*, surprising absence of redundancy has also been discovered in a number of cases, possibly representing the final stage of

nonfunctionalization. For instance, *BREVIS RADIX* (*BRX*) and its close homolog *BRX-LIKE 1* (*BRXL1*) encode highly similar proteins, whose loss-of-function alleles, however, have very different effects on plant development: while *brx* mutants display strongly reduced primary root growth, *brxl1* mutants display no apparent phenotype (Briggs *et al.* 2006; Mouchel *et al.* 2004). Therefore, *BRXL1* seems to be dispensable for wild type development. Nevertheless, constitutively expressed *BRXL1* can complement the *brx* mutant, suggesting that the genes do not act redundantly *in vivo* because of a difference in expression pattern or level. Indeed, *BRXL1* is expressed at much lower levels than *BRX*, and its activity might be below the critical threshold needed to compensate for the absence of *BRX* (Briggs *et al.* 2006). A similar scenario has been described for the proteasome subunits RPN1A and RPN1B: while *RPN1A* loss-of-function results in embryo lethality, *rpn1b* mutants are fully viable and resemble wild type (Brukhin *et al.* 2005). The double mutant resembles *rpn1a*, indicating that *RPN1B* has no role in processes that require *RPN1A*. Again, the non-dispensable gene, *RPN1A*, is expressed at much higher levels than *RPN1B*, and additionally, the *RPN1B* expression pattern is contained within the broader expression of *RPN1A* (Brukhin *et al.* 2005). Nevertheless, expression of *RPN1B* under control of the *RPN1A* promoter rescues an *rpn1a* mutant, demonstrating functional equivalency of the proteins.

2.1.8 Why are dispensable duplicate genes maintained?

In the above examples of absence of redundancy and unequal redundancy, the duplicate gene of a homologous pair appears to be clearly dispensable as determined by mutant analysis, with the exception of cases of subfunctionalization in non-overlapping processes. Just as in full redundancy, one might ask why such a fully functional but dispensable gene copy is maintained through generations? On the one hand, the described redundancies might be transient and get lost over evolutionary

time, representing a transition stage, where full redundancy has already been lost although the gene products are still functionally equivalent. On the other hand, the fact that these duplicate genes are so well conserved argues against their nonfunctionalization. Indeed, evidence for selection pressure on duplicate genes soon after a duplication event has been found (Moore and Purugganan 2003; Moore and Purugganan 2005) and favors this idea. Thus, the high level of sequence conservation in the discussed gene pairs might indicate that the non-essential duplicate gene is indeed subject to selection. From the double mutant analyses it is clear that the duplicate gene contributes to the overall activity of the gene pair, and it is conceivable that this contribution could be close to being essential. This might, however, not become apparent in a laboratory setting, and in this case the mutant analyses would be misleading. For instance, the *HY5-HYH* and *NPH4–ARF19* gene pairs are at least in part involved in responses to environmental stimuli. Thus, testing the fitness of the different genotypes in natural conditions might reveal a requirement for *HYH* or *ARF19* that escapes laboratory assays.

Further, it is also possible that the mutant analyses might not be complete enough, because phenotypic analyses often focus on strong phenotypes and/or do not cover the whole *Arabidopsis* life cycle. For instance, while the *HY5–HYH* pair is a prime example of unequal redundancy with respect to the seedling phenotypes, both genes have a partially redundant, but minor role in determining flowering time (Holm *et al.* 2002). In another example, the homologs *ERECTA-LIKE 1* (*ERL1*) and *ERL2* are dispensable copies of *ERECTA* (*ER*), which display unequal redundancy with *ER* in growth control (Shpak *et al.* 2004). However, *erl1* and *erl2* single mutants also display slight defects in stomata development, acting partial redundantly with *er* in this process (Shpak *et al.* 2005). Thus, selection pressure created by the essential, albeit minor involvement of *HYH* and *ERL1/2* in other processes could be sufficient to maintain their unequally redundant roles observed elsewhere in

development. In addition, the issue is complicated by the possibility that neither the ancestral nor the duplicate gene might be strictly non-dispensable. For instance, null alleles of *CAL* and *BRX* have been isolated in natural accessions (Bowman *et al.* 1993; Kempin *et al.* 1995; Mouchel *et al.* 2004), indicating that despite their respectively minor and major developmental roles, they might not be required for survival in the wild.

2.1.9 Conclusion

In summary, unequal genetic redundancies of homologous gene pairs appear to be a common phenomenon in *Arabidopsis* and likely represent a transition stage between full redundancy and non-, sub- or neofunctionalization. In the absence of molecular evolutionary analyses, the developmental and evolutionary significance of the presumably dispensable duplicate genes discussed in this article is hard to judge. This issue can only be addressed by combining careful phenotypic and gene expression analyses throughout the life cycle with ecological fitness assays and surveys of the natural allelic variation in the genes of interest over a wide sample of haplotypes. Such systems level studies of unequally redundant gene pairs should be an excellent opportunity to shed light on the global patterns of functional genome evolution.

essential gene	*non-essential gene(s)*	*affected processes*	*references*
ABI5	***ABF3***	**germination**	**47**
AP1	***CAL***	floral development	26, 27
BRI1	***BRL1***	**overall growth**	**34**
BRI1	***BRL1/3***	overall growth, vascular differentiation	34
CSN5A	***CSN5B***	**photomorphogenesis, overall growth**	**46**
ER	***ERL1***	silique, pedicel elongation	37
ER	***ERL2***	**internode elongation**	**37**
ER	***ERL1/2***	overall growth	37
HY5	***HYH***	**Photomorphogenesis, root development**	**28, R.S.**
NPH4	***ARF19***	hypocotyl tropisms, lateral root formation	29, 31
MP	***NPH4***	**embryogenesis**	**38**
RAX1	***RAX2***	axillary meristem formation	36
RAX1	***RAX3***	**axillary meristem formation**	**36**
RAX1	***RAX2/3***	axillary meristem formation	36
SPA1	***SPA2***	**photomorphogenesis**	**48**

Table 2.1 A, by no means complete, list of examples of unequally redundant gene pairs in *Arabidopsis*.

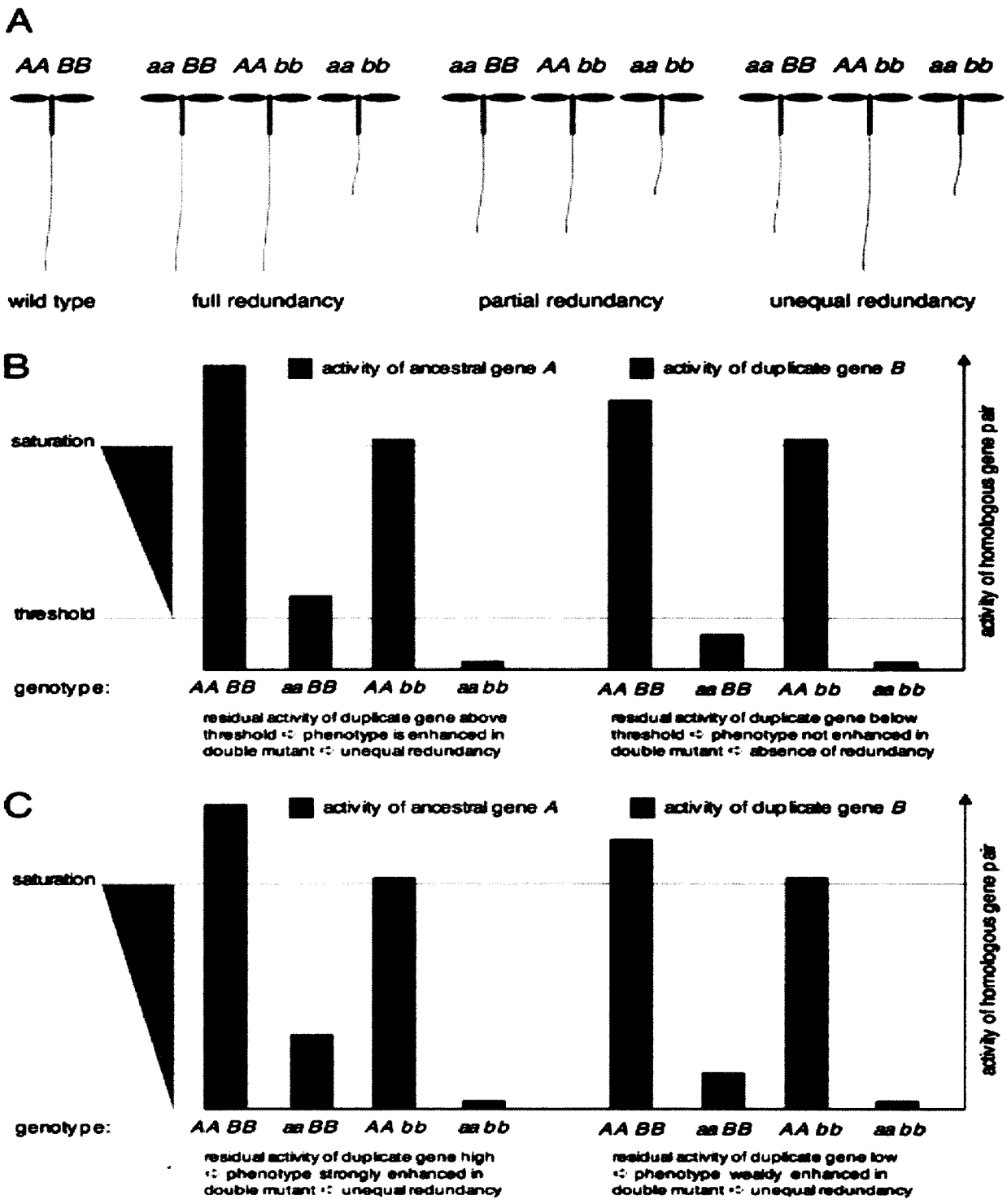

A
AA BB
aa BB
AA bb
aa bb
aa BB
AA bb
aa bb
aa BB
AA bb
aa bb
wild type
full redundancy
partial redundancy
unequal redundancy
B
activity of ancestral gene A
activity of duplicate gene B
saturation
threshold
genotype:
AA BB
aa BB
AA bb
aa bb
AA BB
aa BB
AA bb
aa bb
activity of homologous gene pair
residual activity of duplicate gene above threshold ⇨ phenotype is enhanced in double mutant ⇨ unequal redundancy
residual activity of duplicate gene below threshold ⇨ phenotype not enhanced in double mutant ⇨ absence of redundancy
C
activity of ancestral gene A
activity of duplicate gene B
saturation
genotype:
AA BB
aa BB
AA bb
aa bb
AA BB
aa BB
AA bb
aa bb
activity of homologous gene pair
residual activity of duplicate gene high ⇨ phenotype strongly enhanced in double mutant ⇨ unequal redundancy
residual activity of duplicate gene low ⇨ phenotype weakly enhanced in double mutant ⇨ unequal redundancy

Figure 2.1 (A) The different types of genetic redundancy between two homologous genes, as exemplified by the trait *primary root growth* in *Arabidopsis*. *A* represents the ancestral gene, *B* represents the duplicate gene (see text). In full redundancy, both single mutants display no mutant phenotype, but the double mutant does. In partial redundancy, both single mutants display a mutant phenotype, which is enhanced in the double mutant. In unequal redundancy, only the single mutant of the ancestral gene displays a mutant phenotype, while the single mutant of the duplicate gene resembles wild type. Nevertheless, the phenotype of the ancestral gene single mutant is enhanced in the double mutant. (B) Possible explanations for unequal genetic redundancy in traits whose expression correlates quantitatively with gene activity, but requires a threshold gene activity to be modulated. Unequal redundancy only occurs if the residual activity of the duplicate gene is above the threshold. (C) As in B, without requirement for a threshold gene activity. Unequal redundancy only occurs as long as the duplicate gene possesses residual activity. The level of enhancement of the double mutant phenotype as compared to the single mutant depends on the level of residual activity of the duplicate gene.

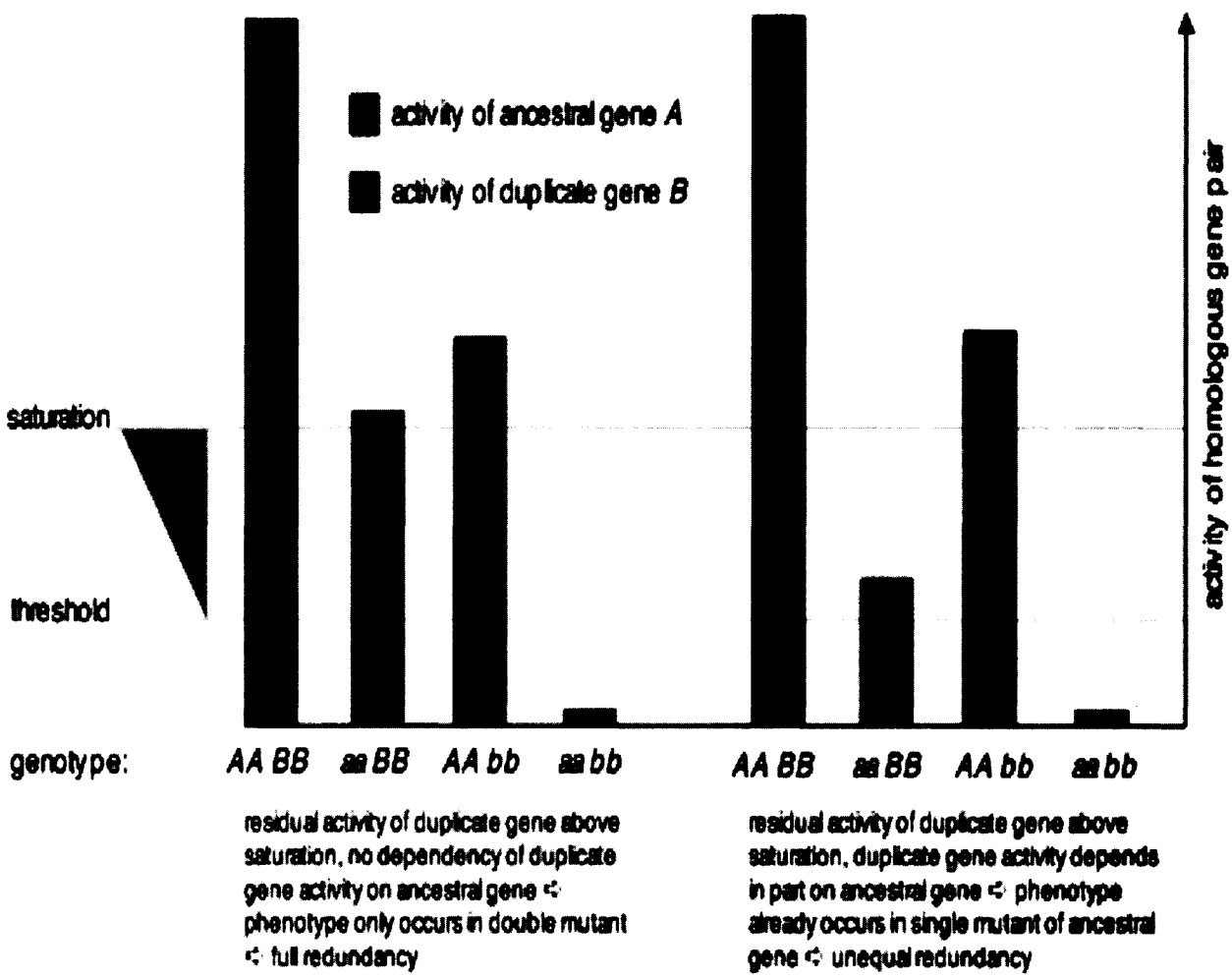

activity of ancestral gene A
activity of duplicate gene B
saturation
threshold
genotype:
AA BB
aa BB
AA bb
aa bb
AA BB
aa BB
AA bb
aa bb
activity of homologous gene pair
residual activity of duplicate gene above saturation, no dependency of duplicate gene activity on ancestral gene ⇨ phenotype only occurs in double mutant ⇨ full redundancy
residual activity of duplicate gene above saturation, duplicate gene activity depends in part on ancestral gene ⇨ phenotype already occurs in single mutant of ancestral gene ⇨ unequal redundancy

Figure 2.2 Cross-regulation between homologous genes as a possible explanation for unequal genetic redundancy in traits whose expression correlates quantitatively with gene activity, requires a threshold gene activity to be modulated, and can be saturated once an excess of gene activity is reached. The residual activity of the duplicate gene is above the saturation level. Unequal redundancy only occurs if the activity of the duplicate gene depends on the activity of the ancestral gene.

2.2 Plant Growth and Gravitropism

Plants are sessile organisms. Their lack of mobility requires that they adapt mechanisms to deal with the elements of nature and to respond appropriately in order to survive. Most plant responses are the result of various environmental stimuli. Stimuli are first perceived by the organism and changed or transduced into another form or signal, the transmission of which results in a response. Tropisms are directed growth responses to environmental stimuli. Plant development is regulated by many environmental stimuli including light, temperature, gravity, nutrients, water and oxygen. However, the impact of these individual factors on plant form vary considerably, with some having more severe effects than others (Leyser and Day, 2003). The overall form of higher plants is quite diverse and complex and can consist of a combination of single and multiple branching axes. Plant form, especially of aerial systems, encompasses spatial developmental requirements such as directional growth, mainly influenced by light and gravity. The directional guidelines of the primary versus the secondary (lateral) organs are substantially different, and result in the spatial separation of these organs. For plants, light and gravity provide directional growth guides (Sedbrook *et al.,* 1999). However, the gravity force is constantly exerted on each individual plant cell in an almost uniform manner. It is therefore the very persistent and unvarying nature of this vectorial force that allows gravity to have such a major effect on plant form (Leyser and Day, 2003). Gravitropism in plants is separated into three stages (1) Gravity perception, where the plant senses the gravity force (2) Signal production and transmission, where a physiological signal is generated and transmitted from the point of perception to a spatially distinct response region, and (3) Gravitropic response, where the transduced signal elicits a response, usually involving the asymmetric bending of the organ (Kato *et al.,* 2002; Leyser and Day, 2003).

As far back as the days of Darwin, observations and experiments examining the effect of the gravity vector on plants have been performed. Today, considerable progress has been made towards understanding the physiological and mechanical processes governing the gravitropic response. Defects in gravitropism have been observed in both the root and the shoot system of plants, however, to date, molecular and genetic analyses of gravitropism have been far more successful and well studied in the root system.

2.2.0 Root Structure and Root Gravitropism

Adult plants consist of an underground root system and an aerial shoot system. In higher plants, the root is highly symmetrical and includes a specified number of layers designed in a concentric manner. These layers represent specific tissue types and from the outermost region of the root are: epidermis, cortex, endodermis, pericycle and a central stele containing the vascular tissues (Figure 2.3A) (Boonsirichai *et al.,* 2002). The root apical meristem (RAM) is located at the most distal and central position of the root (Figure 2.3B). On the apical-basal axis, the root can be separated into functionally distinct zones. These are defined by the most basal proliferation or dividing zone (PZ), which contains the meristematic tissue (RAM); the elongation zone (EZ) which borders the PZ and consists of cells undergoing rapid decreases in cell division but rapid increases in cell elongation and finally the most apical Mature zone (MZ), in which cells cease to elongate, but begin differentiating.

The root cap is a renewable cellular structure that covers the extreme tip of the root. It is composed of central columella, lateral and tip cells (Figure 2.3B). In the most apical layers of the RAM, cell divisions result in the formation of cells that replenish the root cap. One of the most important functions of the root cap is the protection of the RAM, it also serves many other sensory functions and is essential to the gravitropic

response (Masson *et al.,* 2002; Blancaflor and Masson, 2003; Boonsirichai *et al.,* 2002).

2.2.1 The starch-statolith hypothesis of gravity perception

The plant root has been used as a genetic model for almost 50 years in the study of gravitropism. The specificity of the root cap to the gravitropic response was demonstrated in early experiments by Juniper (1966) where decapped roots were no longer responsive to the gravity vector, although proper plant growth was still maintained (Waisel *et al.,* 2006). In the root system, the columella cells of the central root cap function as specialized cells called statocytes. In these cells, plastids containing large starch granules known as amyloplasts function as gravity-sensing structures (statoliths) during the perception phase of the gravitropic response (Figure 2.3B). In these gravity perceiving cells, amyloplasts actively sediment in response to gravity and are believed to be essential to the root gravitropic response. The starch–statolith hypothesis describes this gravity perception phase of gravitropism and postulates that starch-filled amyloplasts perceive the gravitational force and sediment in the direction of gravity. Because amyloplasts always sediment in the direction of gravity, any change in the direction of the gravity vector is thus followed by a change in the direction of the sedimenting amyloplasts (Figure 2.4B). This change is believed to produce a signal that is then transmitted to the elongation zone where the gravitropic response occurs. Non-statocyte cells of the root cap are highly vacuolated, possess a centrally positioned amyloplast-encircled nucleus and contain endoplasmic reticulum (ER) throughout the cells. However, statocyte cells of the root cap are relatively unvacuolated with the ER pushed-back against the borders of the cell. Amyloplasts lie in the central and distal portions of the cell, while the nucleus is at the end of the cell closest to the root meristem (Figure 2.3B).

2.2.2 Mutant analysis and the elucidation of the gravitropic response

The starch-less class of mutants:

Molecular analysis of isolated *Arabidopsis* mutants has yielded considerable support for the contributions of starch to the gravity perception phase of gravitropism. Reduction of starch by certain treatments leads to a reduction in amyloplast sedimentation, and ultimately a reduced gravitropic response (Weise and Kiss, 1999). Further, starch deficient *Arabidopsis* mutants such as *phosphoglucomutase (pgm)* cannot synthesis starch and show an altered response to gravity in the root, hypocotyl and shoot (Morita and Tasaka 2004). Interestingly, the response of each starch-altered mutant correlates to the level of starch accumulation in each organ, which is linked to the amount of starch present in the gravity-sensing amyloplast (Kato *et al.,* 2002). Weise and Kiss reported sedimentation of plastids in endodermal cells of the wild type and reduced-starch mutants but not in the starchless mutant, which was still capable of responding to a changed gravity stimulus (Weise and Kiss, 1999). Somerville and co-workers also reported that a starch-less mutant was still capable of responding to gravity. It was thus initially thought if starchless plasmids are unable to sediment there must be another mechanism for gravity perception. Later experiments published by Yamamoto *et al.,* 2002, however, demonstrated that the starchless plastids of the mutant did settle in the statocytes, but at a much slower rate than amyloplasts of the wild-type plants (Yamamoto *et al.,* 2002). Taken together, these genetic analyses support the starch-statolith theory of gravity perception.

The agravitropic class of mutants:

In the root gravitropic response, the region of gravity perception is physically distinct from the region of gravity response. Therefore, the physical movement of the amyloplasts within the statocytes is converted

into a physiological signal via a still unknown mechanism. This signal must then be transduced to the region of the organ where the gravity response occurs (Sedbrook *et al.,* 1999). Screens for abnormal gravitropic responses have revealed mutants in the gravity signal transduction aspect of the gravitropic response. *altered response to gravity*, *arg* mutants are specifically altered in root and hypocotyl gravitropism responses, demonstrating a slower re-orientation to a gravity change. Additionally, these mutants are not affected in their phototropic responses and show a wild-type response to all hormone treatments including application of exogenous auxin. Further, *arg* mutants showed no defect in amyloplast accumulation in the root, indicating that *ARG* does not affect starch accumulation (Fukaki *et al.,* 1997; Sedbrook *et al.,* 1999, Guan *et al.,* 2003). In *Arabidopsis*, *ARG* belongs to a small gene family containing thus far, two other members *ARG-Like1* (*ARL1)* and *ARG-Like2* (*ARL2).* The *ARG* and *ARL* genes, encode DnaJ-like proteins. DnaJ proteins can complex with HSP70 and bind actin filaments in the cytoskeleton in a calcium dependent manner (Sedbrook *et al.,* 1999). As such, they contain a coil-coiled structure similar to those found in cytoskeleton interacting proteins. As a result, it has been proposed that these *ARG* and *ARG-like* genes may facilitate the transmission of gravity signals from the site of gravity perception, via interactions with the cytoskeleton (Sedbrook *et al.,* 1999). Although the *ARG* family members are expressed ubiquitously in the plant, altered phenotypes are only observed at the root at hypocotyl levels, indicating possibly that the signal relay mechanism involved in stem gravitropism may be significantly different to that of the root.

The auxin-related class of mutants:

A screen to identify roots insensitive to inhibitory amounts of an exogenously applied artificial auxin 2,4-D, led to the identification of the *AUX1* gene (Martindale and Maher, 1980). *Arabidopsis AUX1* encodes an amino acid permease-like protein. It is thought to function in auxin uptake

and examined *aux1* mutants show a variety of auxin insensitive phenotypes and a general reduction in the root gravitropic response (Bennet *et al.,* 1996; Leyser *et al.,* 1996; Marchant *et al.,* 1999). *aux1* mutants respond normally to the membrane-permeable auxin NAA, the addition of which restores the root graviresponse to *aux1* mutant (Bennett *et al.,* 1996; Marchant *et al.,* 1999). A similar screen performed by Estelle and Wilson (1990), revealed another class of mutants with altered auxin sensitivity that function in a separate auxin-responsive pathway than that of *AUX1* (Timpte *et al.,* 1994, 1995; Wilson *et al.,* 1990). These loci defined as *AXR1*, *AXR2*, *AXR3*, *AXR4*, and *AXR6*, show a range of developmental defects ranging from decreases in plant height, root gravitropism, hypocotyl elongation, and fertility to altered shoot phenotypes (Lincoln *et al.,* 1990; Leyser *et al.,* 1996; Timpte *et al.,* 1992). Taken altogether, these mutants support the involvement of auxin in the gravitropic response.

The involvement of the PIN family of efflux carriers in root gravitropism has also been studied in quite some detail and putative working models have been put forward (Mueller *et al.,* 1998; Chen *et al.,* 1998; Friml *et al.,* 2002). A screen to identify roots showing abnormal gravitropic responses (agr), with a corresponding normal sensitivity to auxin, revealed three mutants *agr1*, *agr2* and *agr3*, later shown to all be alleles of the same gene (Utsuno *et al.,* 1998). *agr1* has a root specific agravitropic phenotype and its functional protein was shown to encode a membrane-bound transporter. Further analyses have shown *agr1* to be allelic to *eir1*, *wav6* and *pin2*. *PIN2* encodes a member of the PIN family of auxin efflux carriers, and its involvement in the root gravitropic response has also been demonstrated (Chen *et al.,* 1998; Shin *et al.,* 2005). Furthermore, it has been shown that the polar localization of PIN proteins is required to direct auxin flow in plants (Wisniewska et al, 2006), suggesting that with respect to gravitropism, transduction of the auxin

signal via PIN protein localization may be required to effect the gravity response.

Auxin has thus been implicated as being the uncharacterized physiological signal transmitted from the region of graviperception to the graviresponse zone. Auxin is synthesized in the shoot tip and transported basipetally via the network of auxin transport factors that are tightly associated with the vascular system of the plant, including PIN proteins, which have been shown to act in gravitropism. The application of auxin transport inhibitors to wild-type roots also blocks the gravitropic response, supporting this proposed role.

2.2.3 The Cholodny-Went hypothesis and gravitropic bending

The bending response to a gravitational stimulus is the final and only visual step of a plant's response to a change in the gravity vector. The gravity response zone is spatially distinct from the point of gravity perception and the response is explained by the Cholodny-Went hypothesis, formulated in the 1930's. This hypothesis proposed that perception of a unilateral stimulus in the form of light or gravity, results in the lateral redistribution of auxin (Firn *et al.,* 2000).

The visual bending response occurs downstream of the auxin re-distribution process and involves structural changes at the cellular level resulting in bending. Auxin is distributed in a differential manner on opposing flanks of the responsive organ accumulating on the lower side with respect to gravity and diminishing from the upper portions (Cosgrove, 1997). In the root, cellular auxin accumulation reduces the rate of cell elongation, whereas cells with reduced auxin levels, undergo rapid elongation (Figure 2.4A).

Auxin supply and redistribution plays a central role in the gravitropic response because of the direct and rapid effect of auxin on cell expansion. Auxin controls cell expansion by regulating the activity of plasma membrane pumps resulting in cell wall acidification and increased

extensibility (Masson *et al.,* 2002 and references therein). The establishment of asymmetry in the wall pH during the bending response, was demonstrated in a clever experiment by Mulkey *et al.,* in 1982. By plating maize and sunflower seedlings onto growth media containing a pH indicator dye, the upper, rapidly growing portion of the organ corresponded to a high acidic pH in the media (Mulkey *et al.,* 1982). This pH differential, acting as a secondary messenger, is thought to activate one or more biochemical mechanisms which in turn translates into asymmetrical wall relaxation and extension in the affected cells (Cosgrove, 1997).

Another candidate functioning as a secondary messenger is the inorganic ion calcium (Ca^{2+}). There is a growing body of evidence to support the indirect involvement of Ca^{2+} and calmodulin in gravity signal transduction (Boonsirichai *et al.,* 2002 and references therein; Perrin *et al.,* 2005 and references therein; Waisel *et al.,* 2006). Statocytes of the root cap were noted to contain elevated levels of Ca^{2+} and upon gravistimulation, in oat and maize pulvini, levels of inositol 1,4,5-trisphosphate (IP_3), an activator of Ca^{2+} release, fluctuates on opposing sides of the responsive organ (Boonsirichai *et al.,* 2002; Perrin *et al.,* 2005).

2.2.4 Shoot Structure and Shoot Gravitropism

The plant shoot system consists of individual units called phytomers. Each phytomer contains an internode and a node with a leaf and lateral bud (Tsukaya, 2006 and references therein). Plants obtain an indeterminate growth state by repeated stacking of these phytomers (Figure 2.5a-b).

The embryonic shoot system in dicoteyledons is the epicotyl, which consists of the shoot apical meristem (SAM) centrally positioned between two cotyledons. The hypocotyl is just below the cotyledons and post-embryonically grows solely via cell elongation. Structurally, the hypocotyl

is almost identical to the adult inflorescence stem. Inflorescence stems are made up of several tissue types patterned in a circular, concentric manner. From the outside inwards, the tissues include one epidermal layer, three layers of cortex, one layer of endodermis and a central stele containing the vascular tissue (Masson et al, 2000). Vascular bundles in the dicoteyledonous stem form a continuous cylinder or a series of bundles containing phloem cells nearest to the epidermis and xylem cells towards the center of the stem (Leyser and Day, 2003). The hypocotyl contains two instead of three layers of tissue in the cortex. Initially, the young inflorescence stem of *Arabidopsis* undergoes considerable cell elongation throughout most of its length. Later in development, this decreases in the basal regions, where mature cells are typically lignified, and increases in the upper regions of the younger elongating stem (Masson et al, 2000).

Nodes on the stem are the sites at which leaves and axillary buds emerge. The most common leaf pattern or phyllotaxy (i.e. the regular arrangement of leaves) is one leaf per node. Almost simultaneous with the development of a leaf at a node, is the development of an axillary meristem at the base of the leaf (Figure 2.5c). Further development results in the transformation of axillary meristems into lateral buds. The bud is formed via the initiation of several leaves by the axillary meristem which upon completion arrests in development to become dormant. The future activity of the bud is strongly influenced by its proximity to the primary shoot tip. This phenomenon is known as apical dominance, the only reprieve from which is attained by increased distance from the apex or by removal of the shoot tip altogether. Reactivation of the dormant bud, results in its subsequent growth into a lateral shoot (Leyser and Day, 2003).

Unequal subdivisions or unequally developed subdivisions of the apex results in branching, and lateral axes arise from buds situated in the axils of leaves (Figure 2.5c). Although axillary meristems are positioned on

the developing stem, *Arabidopsis* internodes produced during juvenile and adult development fail to undergo elongation, resulting in a compact stem structure with a surrounding rosette of leaves. These rosette leaves gradually change in size and shape as they progress from juvenile to adult, and are attached to the stem by characteristic long petioles. The rapid transition from vegetative to reproductive phase in the plant is characterized by a dramatic increase in cell elongation in the stem, resulting in the primary inflorescence. Newly formed leaves also develop on the basal portions of the stem. These petiole-less, cauline leaves subtend the emerging axillary buds that later form secondary inflorescences (lateral shoots) (Leyser and Day, 2003) (Figure 2.5c).

In the stem, statocytes are formed from the endodermal layer, sometimes referred to as the starch sheath (Leyser and Day, 2003) (Figure 2.4B). While the endodermal layer is the site of gravity perception in the shoot, changes in rates of elongation are only clearly observed in the peripheral layers of the cortex and the epidermal layers. This suggests that there must be some radial movement of the signal from the inner to outermost tissue layers of the stem (Blancaflor and Masson, 2003; Leyser and Day, 2003).

In hypocotyls and shoots, the entire length of the stem contains the capacity to respond to a gravity stimulus as observed in experiments on stem segments (Fukaki *et al.,* 1996). Although responses vary depending on the origin of the segment (basal or apical), the results suggest that all stem segments contain the essential components necessary for the gravitropic response (Masson *et al.*, 2000). The bending response itself, however, is local, rapid and reversible, and has been recorded in less than five minutes in coleoptiles and 11 minutes for cucumber hypocotyls. In *Arabidopsis*, however, gravitropic response in the stem requires 90 minutes. This relatively short lag-time between perception and response is too rapid to reflect cell division and cell displacement, suggesting the

involvement of a more rapid mechanism, likely cell elongation (Cosgrove, 1997).

2.2.5 Mutant analysis and the shoot gravitropic response

The starch-less class of mutants

As previously discussed, starch deficient *Arabidopsis* mutants cannot synthesize starch and show an altered response to gravity in the root, hypocotyl and shoot (Morita and Tasaka 2004).

The shoot gravitropic class of mutants

A series of specific shoot gravitropism (*sgr*) mutants in *Arabidopsis* have been isolated. To date, seven independent loci have been identified, with most isolated from a screen for abnormal shoot inflorescence gravitropic response. Although shoot gravitropism is affected in these mutants, their root gravitropic response is unaltered (Kato *et al.,* 2002). Interestingly, the shoot phototropic response is unaffected in these mutants. The shoot phototropic response is very similar to the shoot gravitropic response, differing mainly in the nature of the stimulus (light instead of gravity) and the stimulus receptors. This result therefore suggests that mechanisms for polar auxin transport and auxin distribution, required in both responses, are functional and not greatly affected in these plants (Fukaki *et al.,* 1998). Two of these mutants, *sgr1* and *sgr7* are allelic to the GRAS transcription factors *scarecrow (scr)* and *short-root (shr),* respectively. *SCR* and *SHR* were previously identified through root mutants and determined to be regulators of radial patterning in the root, such that, in both mutants, the endodermal layer is absent (van den Berg *et al.,* 1995; Di Laurenzio *et al.,* 1996). Further studies have demonstrated the requirement for both *SCR* and *SHR* in the radial organization of the shoot as well, such that *scr* and *shr* mutants are shoot agravitropic. These two mutants generate overwhelming support for the necessity of the endodermal layer to shoot gravitropism (Fukaki *et al.,* 1998). Although the hypocotyl and stem share similar tissue structure and arrangement, these

organs are not identical, and, as such, their reactions to gravity are not always correlated. These observed differences in gravitropic responses may be derived from the different origins of the two organs. The hypocotyl differentiates from the central domain of the early embryo whereas the stem is a post-embryonic organ that differentiates from the SAM. Therefore, although these organs are structurally similar, they may be governed by different developmental programs. The *sgr* class of can be used to dissect these differences. *sgr1*, *sgr2*, *sgr4* and *sgr7* show agravitropism in both stems and hypocotyls; *sgr3*, *sgr5* and *sgr6,* however, are stem agravitropic but show a normal hypocotyl response to gravistimulation. Overall, these mutants support the existence of separate genetic mechanisms governing the gravitropic response in the different organs, and also confirm the fact that the endodermal layer is necessary for some phase of the gravity response in the shoot (Kato *et al.,* 2002).

In wild-type plants, the amyploplasts in the endodermis are almost completely enwrapped by a thin cytoplasmic layer and vacuolar membrane (Figure 2.6). Amyloplast movement can be observed, and occurs within these boundaries. These narrow areas of cytoplasm enclosed by the vacuolar membrane and continuous with the peripheral cytoplasm, are referred to as transvacuolar strands (Saito *et al.,* 2005). The large size and density of these specialized amyloplasts facilitates their easy movement through the cytoplasm after organ re-orientation. In *sgr* mutants, amyloplasts instead move within the transvacuolar strands both before and after gravistimulation by reorientation, and remain in the peripheral region of the endodermal cells (Morita *et al.,* 2002).

In addition to the endodermal layer and the requirement of starch-filled amyloplasts, other factors, such as vacuolar membrane dynamics, also play an important role in stem amyloplast movement and by extension, the graviperception phase of the gravitropic response. As discussed earlier, the endodermal cells of the shoot are mostly filled with large central vacuoles. It has been proposed that these vacuoles

contribute to gravity perception by affecting amyloplast sedimentation (Morita and Tasaka, 2004). Investigation of a series of *sgr* mutants, has recently confirmed this involvement of these vacuoles in gravity perception. These mutants include *sgr2*, *sgr3* and *sgr4*, all of which encode genes related to the biogenesis or integrity of the vacuoles (Kato *et al.,* 2002; Yano *et al.,* 2003). SGR2, a phopholipase A1-like protein localizes to the vacuolar membrane (Kato *et al.,* 2002); SGR3 and SGR4 are members of the SNARE family involved in intracellular protein trafficking and membrane fusion (Kato *et al.,* 2002; Yano *et al.,* 2003). SNAREs present on transport vesicles (v-SNARES), interact with those on target compartments (t-SNARES) to form stable complexes. SGR3 encodes a t-SNARE involved in vacuolar trafficking and SGR4 encodes a v-SNARE. These two SNAREs form a complex in the shoot endodermal cells (Kato et al., 2002, Yano *et al.,* 2003). Mutants for each of these genes demonstrate abnormal amyloplast sedimentation when compared to the wild-type (Saito *et al.,* 2005), suggesting involvement of vacuoles for proper amyloplast sedimentation and thus the gravitropic response.

The PIN3 mutant

As previously discussed, molecular examination of the root gravitropic response has progressed significantly, while work on clarifying the molecular nature of the shoot gravitropic response has been more elusive. Friml and co-workers addressed this issue (2002), and demonstrated the asymmetrical accumulation of auxin in the hypocotyls during differential growth. Here the auxin efflux regulator, *PIN3* plays a central role as it is expressed in the gravity sensing tissues in the root and shoot, accumulating predominantly at all lateral cell surfaces (Friml *et al.,* 2002). Anti-PIN3 immunogold labeling suggests a rapid cycling mechanism of the PIN3 protein between vesicles and the plasma membrane. This cycling mechanism may provide a relocation mechanism during gravistimulation, since, 2 minutes after gravistimulation in the root,

PIN3 distribution becomes laterally asymmetric (Friml *et al.,* 2002). Although *pin3* mutants displayed defects in differential growth as seen in a reduced gravitropic response, the nature of the gravitropic response in the adult stem was not reported (Friml *et al.,* 2002). Therefore it is possible that genetic redundancy within the PIN gene family may be acting in the regulation of stem gravitropism.

2.2.6 Hormone treatments influence trophistic responses in roots, shoots and hypocotyls

Auxin distribution and transport are key components to the gravitropic response. As a result, factors that directly or indirectly affect the distribution and/or transport of auxin, may also affect the overall gravitropic response of the plant. This is clearly observed with the addition of auxin transport inhibitors e.g. TIBA and NPA that notably reduce or prevent the bending response of hypocotyls and roots to a 90 degree re-orientation. Recent reports have shown that other hormones, namely the brassinosteroid e-BL, is also capable of affecting auxin distribution and transport (Bao *et al.*, 2004; Li *et al.*, 2005). In work performed by Li and workers, it was shown that the treatment of WT seedlings with BR results in the promotion of the gravitropic response. Further, they demonstrated that this increased trophistic response, correlated to an enhancement of the distribution of some PIN proteins, namely PIN1, PIN2 and PIN3 and an acceleration in the asymmetric accumulation of auxin, resulting in an enhanced gravitropic bending response (Li *et al.*, 2005). Their work also indicated that accumulation of the ROP GTPase, ROP2 is also affected by the presence of exogenous BR, such that ROP2 accumulation increased in the presence of the hormone.

ROP GTPases are known to function as molecular switches in varying signal transduction pathways (Molendijk *et al.,* 2004; Xu and Scheres, 2005). ROP2 accumulates in columella cells where it controls F-actin dynamics and directional cell expansion. Although the function of

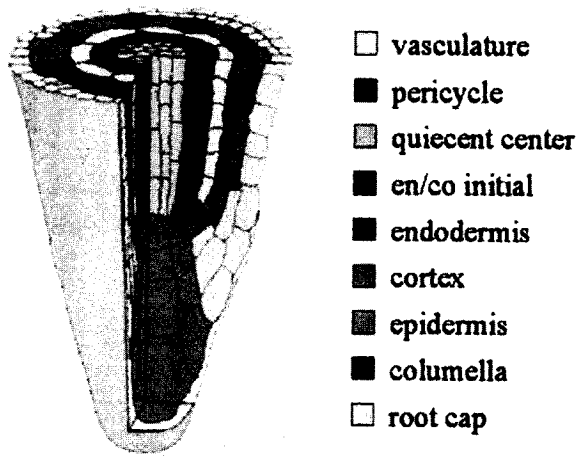
ROP2 has only been noted in the root thus far, over-expression of ROP2 using a 35S::ROP2 construct, results in increased gravitropic bending (over-bending) not only in the root but also in the hypocotyl and adult stem (Li et al., 2005 and references therein). Cytoskeletal dynamics and thus the influence of F-actin may aid in controlling the targeting of influx/efflux carriers to specific membrane domains and thus may affect the cycling and/or vesicle sorting of these proteins at membrane surfaces (Molendijk *et al.,* 2004). Therefore the effect of BR on auxin transport and distribution can be both direct and indirect. In a direct manner BR application may affect the Polar Auxin Transport (PAT) process, ultimately altering the gravitropic response. Alternatively, BR, acting through its regulation of ROP2, could indirectly alter auxin distribution and transport through the effect of ROP2 on the cycling and distribution of auxin transport carriers.The detailed mechanisms of these processes are yet to be determined.

2.2.7 Conclusion

For over half of a century, researchers have investigated the genetic and molecular nature of the root gravitropic response, with little attention placed on that of the shoot. Within the last 5-10 years, however, a significant amount of research focusing on shoot gravitropism, has lead to important observations indicating both similarities and possible differences between the gravitropic responses of the root and that of the shoot. Most notably, work by Friml and workers demonstrating the asymmetrical accumulation of auxin in shoot bending, mechanistically unifies root and shoot gravitropism and further implicates auxin as a key component of the gravitropic response in these two organs. With the new and emerging roles of the vacuoles, cell wall components and the involvement of calcium in the gravitropic response, we are beginning to uncover the molecular and genetic components of the gravitropic response in both the root and the shoot.

The gravisensing cells of the root and shoot are structurally very distinct in terms of the relative positioning and distribution of their organelles. In the root, amyloplast interact strongly with the endoplasmic reticulum (ER), which lines the cell periphery. The shoot however, contains a large central vacuole that occupies most of the cell volume. Amyloplast movement occurs within this vacuole as the amyloplasts are engulfed into the vacuolar lumen. These significant differences in subcellular ultrastucture between the root and shoot may result in the activation of different sensors that create the proposed physiological signal in response to amyloplast sedimentation. Therefore, one can argue that despite the common use of amyloplasts as statoliths, other components of the gravitropic responses in these two systems may differ considerably at the genetic level.

A


vasculature
pericycle
quiecent center
en/co initial
endodermis
cortex
epidermis
columella
root cap

B

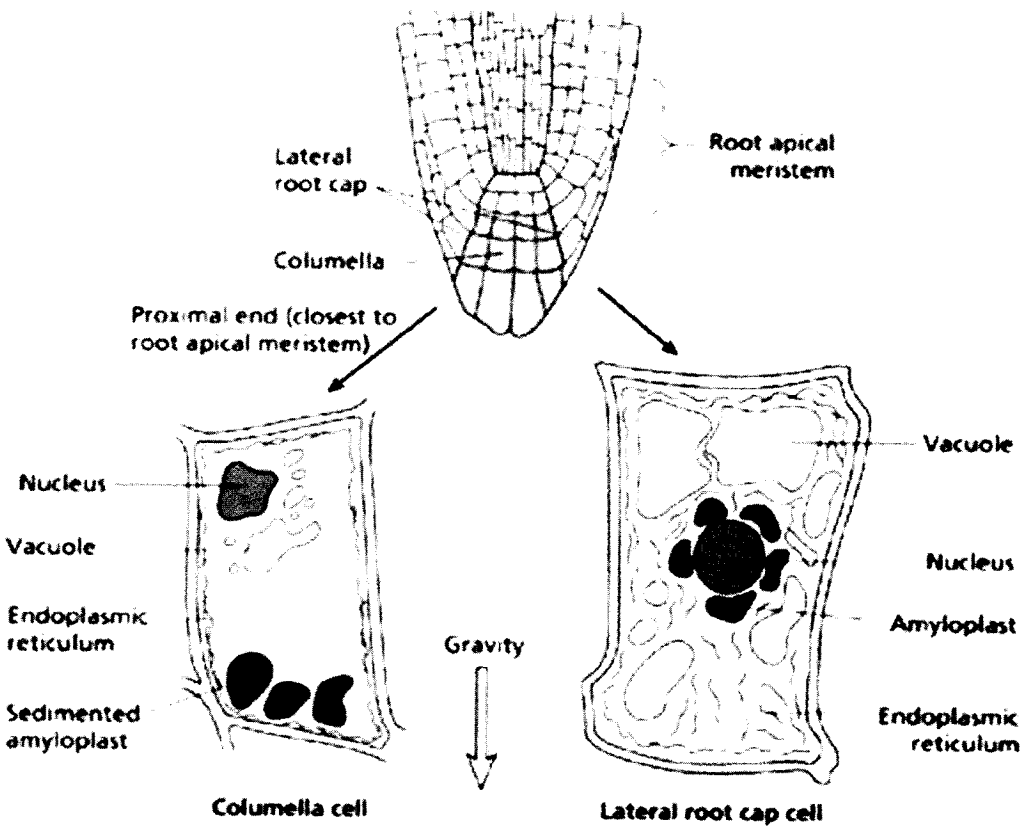
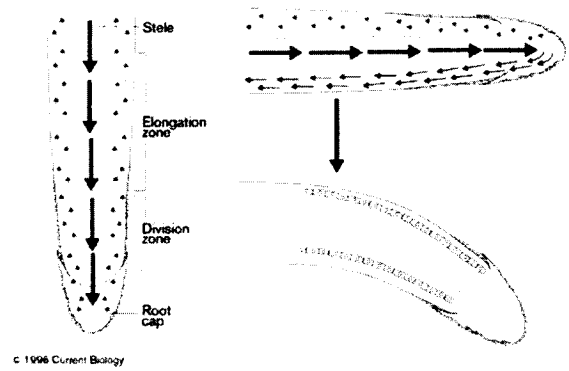
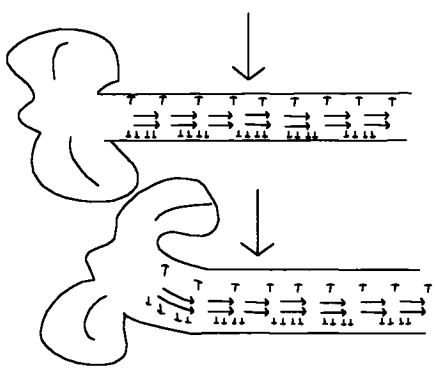

Lateral root cap
Root apical meristem
Columella
Proximal end (closest to root apical meristem)
Nucleus
Vacuole
Endoplasmic reticulum
Sedimented amyloplast
Gravity
Columella cell
Vacuole
Nucleus
Amyloplast
Endoplasmic reticulum
Lateral root cap cell

Figure 2.3 Root structure and Gravitropism. (A) Illustration of the cellular structure of the root tip in *Arabidopsis*. Cross and Longitudinal sections indicate the cell layers organized in a concentric manner. *(Adapted from Benfey and Scheres, 2000)* (B) Schematic of the root tip indicating the columella (gravity sensing) cells of the central root cap. The differences between the cellular structure of these cells and the cells of the lateral root cap are indicated. *(Leyser and Day, 2003)*

A


Stele
Elongation zone
Division zone
Root cap
c 1996 Current Biology



B

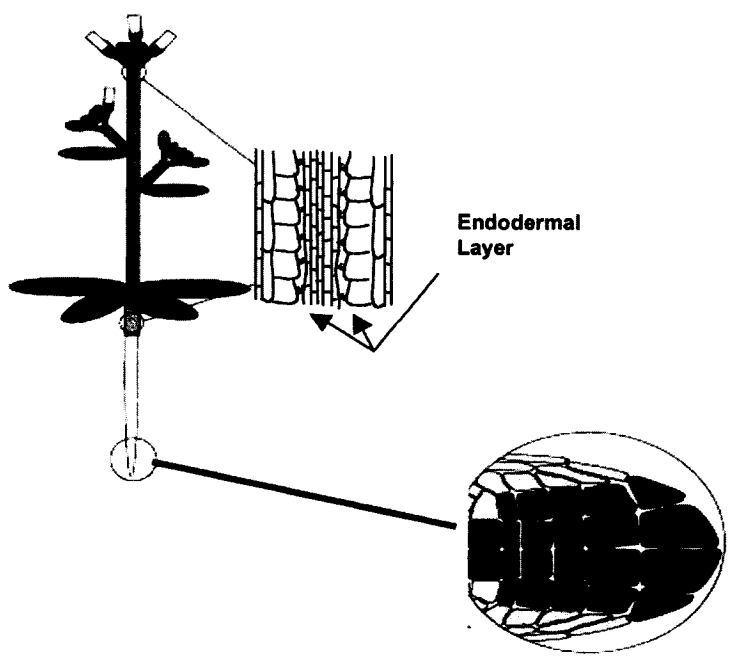
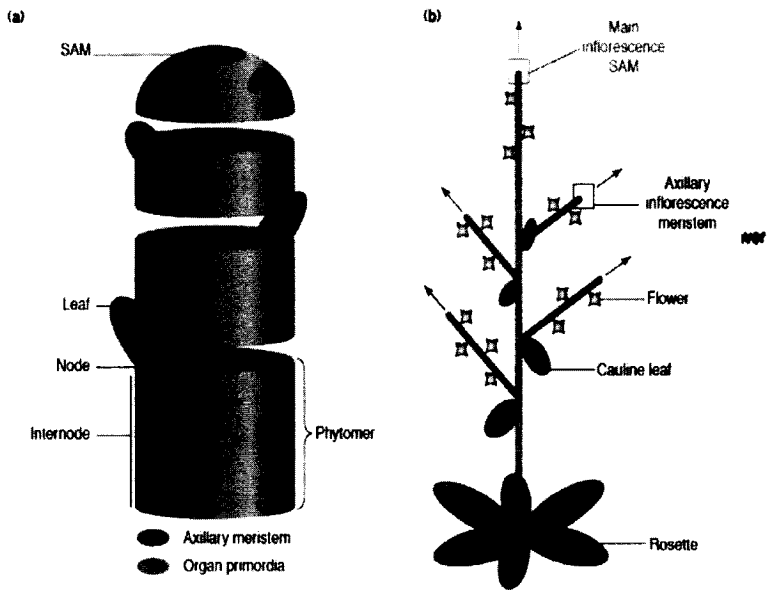

Endodermal Layer
Central Columella Cells (gravisensing)

Figure 2.4 Gravity Perception and Response in Roots and Shoots. (A) The bending response results from the differential distribution of auxin (red arrows in root and small black arrows in shoot). Roots have positive gravitropic bending in the direction of gravity (large black arrows) whereas shoots have negative gravitropic bending away from the gravity vector. In the root, the root tip perceives the gravity force however the bending response occurs in the elongation zone as indicated in the illustration. *(Adapted from Estelle, 1996)* (B) *Arabidopsis* plant indicating the gravity perception regions in the root and shoot (encircled). In stem the gravity sensing endodermal layer with sedimenting amyloplasts (black dots) are clearly indicated. Upon rotation, the amyloplasts now sediment in the direction of the new gravity vector as indicated by the columella cells of the root tip *(Adapted from Morita and Tasaka 2004).*


(a)
SAM
Leaf
Node
Internode
Phytomer
Axillary meristem
Organ primorda
(b)
Main inflorescence SAM
Axillary inflorescence meristem
Flower
Cauline leaf
Rosette

(c)

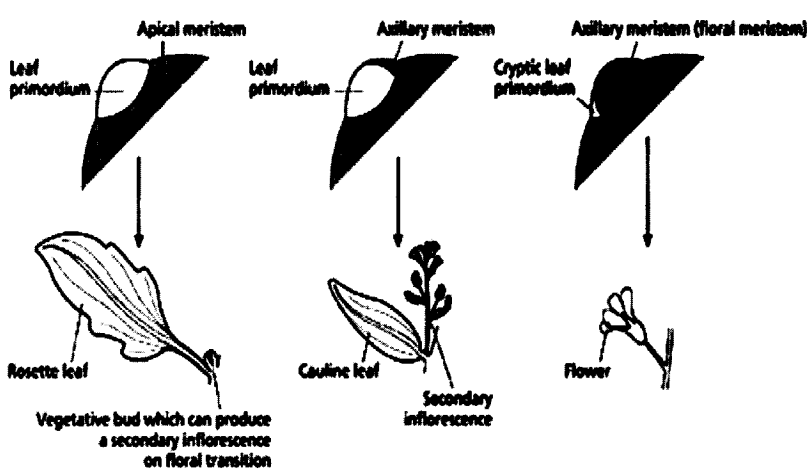

Apical meristem
Leaf primordium
Rosette leaf
Vegetative bud which can produce a secondary inflorescence on floral transition
Axillary meristem
Leaf primordium
Cauline leaf
Secondary inflorescence
Axillary meristem (floral meristem)
Cryptic leaf primordium
Flower

Figure 2.5 Phytomer Unit and Node Types in *Arabidopsis* (a) Illustration of a phytomer unit indicating the node, internode, leaf and positions of the primary and axillary meristems (b) the adult form of *Arabidopsis*, involves repetition of these phytomer units and outgrowth from the meristems *(http://www.plantsci.cam.ac.uk/Haseloff/teaching/CDB_2006/CDB_lect3/CDB_lect3.html)* (c) unequal subdivisions of the meristematic tissue results in the formation of different node types *(Leyser and Day, 2003)*

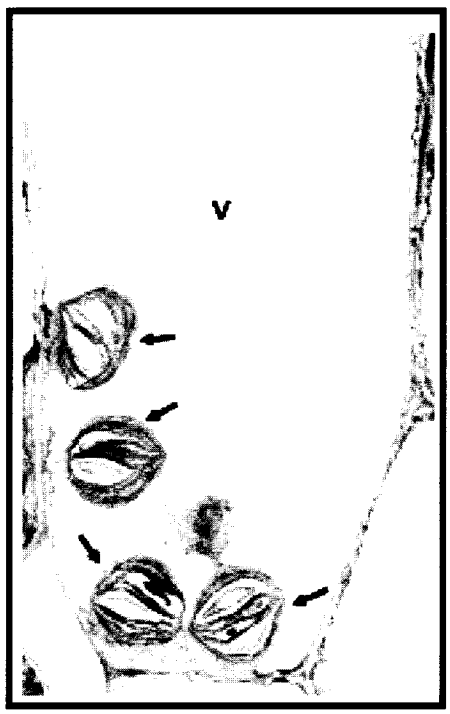


Figure 2.6 Amyloplast movement within endodermal cells of *Arabidopsis*. An electron micrograph of an endodermal cell showing the amyloplasts (lateral and basal) surrounded by a large vacuole (v) and encased in cytoplasm. (Saito *et al.*, 2005).

Chapter 3

Natural Genetic Variation in *Arabidopsis* Identifies *BREVIS RADIX*, a Novel Regulator of Cell Proliferation and Elongation in the Root

This chapter represents results published in a research article in the scientific journal *Genes and Development* in 2004. Here we demonstrate the use of natural genetic variation in *Arabidopsis thaliana* to reveal a novel regulator of root growth, *BRX*. We show further that *BRX* is a quantitative trait locus and is essential for proper cell elongation and proliferation in the root. Further I show that *BRX* is part of a small, highly conserved gene family, where *BRX* plays a dominating role in root development.

Natural genetic variation in *Arabidopsis* identifies *BREVIS RADIX*, a novel regulator of cell proliferation and elongation in the root

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Abstract

Mutant analysis has been tremendously successful in deciphering the genetics of plant development. However, less is known about the molecular basis of morphological variation within species, which is due to naturally occurring alleles. In this study, we succeeded in isolating a novel regulator of root growth by exploiting natural genetic variation in the model plant *Arabidopsis*. Quantitative trait locus analysis of a cross between isogenized accessions revealed that a single locus is responsible for approximately 80% of the variance of the observed difference in root length. This gene, named *BREVIS RADIX (BRX)*, controls the extent of cell proliferation and elongation in the growth zone of the root tip. We isolated *BRX* by positional cloning. *BRX* is a member of a small group of highly conserved genes, the *BRX* gene family, which is only found in multicellular plants. Analyses of *Arabidopsis* single and double mutants suggest that *BRX* is the only gene of this family with a role in root development. The BRX protein is nuclear localized and activates transcription in a heterologous yeast system, indicating that BRX family proteins represent a novel class of transcription factors. Thus we have identified a novel regulatory factor controlling quantitative aspects of root growth.

Introduction

The past years have witnessed tremendous advances in the genetic analysis of plant development, thanks to the rigorous application of mutagenesis approaches. However, much less is known about the molecular basis for the variation observed within species. This variation is due to natural genetic heterogeneity, which is the result of selection pressures that are created by environmental conditions. For sessile terrestrial plants, adaptation to local conditions is especially important and has been observed on a temporally and geographically very small scale (Linhart and Grant 1996). Such natural variation can be exploited to isolate novel genes or alleles involved in plant physiology and development, for instance by analysis of isogenized accessions of the model plant *Arabidopsis thaliana* (Alonso-Blanco and Koornneef 2000). This approach has been successful in isolating both novel genes (Johanson et al. 2000) and novel alleles of known genes (El-Din El-Assal et al. 2001; Maloof et al. 2001). A distinct advantage of exploiting natural genetic variation is its ability to detect alleles that have been subjected to selection in the wild. This approach, in essence, counter-selects against alleles that are detrimental to plant survival and can thus complement the more common mutagenesis approaches, which often target genes that are essential for the trait of interest. Here we have exploited natural variation in *Arabidopsis* to isolate a novel regulator of root growth.

The root system has a pivotal role for the survival of higher plants. Roots provide the plant with physical support as well as essential nutrients and water, which they take up from the soil. *Arabidopsis thaliana* is a dicotyledonous plant and has a typical allorhiz root system. Initially, growth is restricted to a primary root, which is formed during embryogenesis. Later in development the root system expands by forming lateral roots, which originate from the pericycle, an inner cell layer of the primary root. Eventually, adventitious roots might also be formed at the hypocotyl-root junction. At the cellular level, *Arabidopsis* roots have a simple

organization, consisting of concentric layers of epidermis, cortex and endodermis, surrounding the stele that contains the vascular tissues (Dolan et al. 1993). These tissue layers are formed through the action of a growth zone at the distal tip of the root, the apical root meristem. Within this meristem, signals emanating from a quiescent center of slowly dividing cells organize a region of stem cells, which give rise to the cell files of the tissue layers by stereotypic divisions in a reiterative fashion (van den Berg et al. 1997; Sabatini et al. 2003). The daughter cells continue to divide several times in the distal meristematic zone, before entering a zone of rapid cell elongation and differentiating to maturity.

Genetic analysis has provided evidence that plant hormone signaling pathways are fundamentally important for root development. An intact auxin signaling pathway, for example, is required for proper root growth (Davies 1995; Sabatini et al. 1999), a growth-promoting effect that is mediated via signaling through another plant hormone, gibberellic acid (Fu and Harberd 2003). In addition, root patterning requires correctly localized peaks of auxin concentration gradients (Sabatini et al. 1999) as well as the action of two transcription factors, SCARECROW (SCR) and SHORT ROOT (SHR). The latter are needed for the asymmetric division of initials that give rise to the cortex and endodermis cell layers, as well as for the differentiation of these tissues (Di Laurenzio et al. 1996; Helariutta et al. 2000; Nakajima et al. 2001). Interestingly, SCR and SHR also have a fundamental role in the maintenance of the quiescent center and thereby the stem cell population (Sabatini et al. 2003).

The ontogenesis of the root system is highly plastic and sensitive to changes in environmental conditions. In particular, the availability of rate-limiting nutrients for plant growth, such as phosphate and nitrate, results in profound changes in root system architecture. Root systems can react to localized supplies of these nutrients by adjusting their rate and direction of growth, as well as their extent of branching and their extent of root hair formation (Zhang and Forde 1998; Malamy and Ryan 2001; Linkohr et al.

2002; Lopez-Bucio et al. 2002). These localized growth responses are mediated by pathways that appear to be coordinated with phytohormone signaling, allowing for their coordination with the cell elongation and proliferation events that underlie all growth phenomena (Lopez-Bucio et al. 2003).

Although environmental inputs have an important influence on root system architecture, it is conceivable that root growth is limited by inherent genetic boundaries. Such boundaries are for instance set by the cellular mechanisms controlling cell elongation and proliferation (Beemster et al. 2003). For instance, cell proliferation is a particularly important factor in the determination of root growth rate, since transgenic interference with cell cycle progression has profound effects on growth rate and sometimes also on meristem organization of the root (Doerner et al. 1996; Cockcroft et al. 2000; De Veylder et al. 2001). Furthermore, cell production is an important component of root growth rate in natural accessions of *Arabidopsis* (Beemster et al. 2002). To a significant degree, the effect of plant hormones on root growth also appears to be mediated by modulation of cell cycle duration (Beemster and Baskin 2000; Stals and Inze 2001; Werner et al. 2003). At the organ level, the outputs of the cellular mechanisms that control the size of the root meristem, the rate of cell proliferation and the extent of cell elongation, are integrated to determine the overall rate of growth. However, whether or to what degree these mechanisms are acting independently from one another is not clear (Beemster et al. 2003).

The aim of this study was to isolate novel regulators of quantitative aspects of root growth that are responsible for the intra-specific variation of root system morphology in *Arabidopsis*. Therefore, we exploited natural genetic variation rather than mutagenesis of a particular wild type background. This strategy also avoids the isolation of alleles that affect basic properties of the root system, such as the formation of certain tissue layers or physiological responses to nutrient availability. We were

successful in isolating a novel gene, which regulates the extent of cell proliferation and elongation in the root. It represents a member of a novel, plant-specific gene family and encodes a novel type of nuclear protein, which appears to be involved in transcriptional regulation.

Results

Root growth parameters vary among isogenized Arabidopsis wild type lines

To determine natural genetic variation of root system morphology, we compared 44 arbitrarily chosen *Arabidopsis* accessions in tissue culture experiments. A sample of 20 seedlings of each line was grown under constant illumination on solid medium containing basic macro- and micronutrients and agar. Nine days after germination (dag) the length of the primary root, the number of lateral roots and the number of adventitious roots were recorded. An overall two- to three-fold variation in primary root length and lateral root number was observed between accessions. Adventitious roots were very rare in all accessions, however they were observed more frequently in Umkirch-1 (Uk-1). This accession also developed a significantly shorter primary root than average (Fig. 3.1A) and a generally more branched root system at later stages. Because of its clearly distinct root system phenotype, we chose to analyze this line in further detail.

The short primary root of Uk-1 seedlings is largely due to a single locus

To test whether the alleles conferring the root phenotype of Uk-1 are of a dominant or recessive nature, we crossed Uk-1 into Slavice-0 (Sav-0), an accession with an average root system as compared to other accessions in our assays. In the F2 generation of our cross we noticed that the short primary root phenotype of Uk-1 segregated as a recessive in a ratio close to 3:1. Root development is highly plastic, and although the

average primary root lengths of the Uk-1 and Sav-0 lines are clearly distinct (Fig. 3.1A), their ranges of root length in individuals overlap. By analysis of the F3 progeny however, it was possible to unequivocally determine the phenotype of the parental F2 plants, confirming the suspected 3:1 ratio. Thus, the short root phenotype of Uk-1 appears to be largely caused by a single locus, which we named *BREVIS RADIX (BRX)*, latin for "short root".

Starting from the F2 progeny of two different F1 plants, we also established a recombinant inbred line population of 206 lines by repeated selfing for six generations. The primary root length of these lines was measured and each line was genotyped for a set of simple sequence length polymorphism (SSLP) markers spread over the *Arabidopsis* genome (Tab. 1). The data were then subjected to quantitative trait locus (QTL) analysis. The results indicate that a major QTL for primary root length is located on the upper arm of chromosome I and identical with *BRX* (see below; Fig. 3.5).

The Uk-1 short root phenotype does not depend on shoot-derived signals

Morphological differences between accessions were not only observed in the root system, but also in the shoot system. Because it has been shown that communication between shoot and root tissues can significantly influence each other's growth rate and branching pattern (Turnbull et al. 2002; Sorefan et al. 2003), we wanted to determine whether the Uk-1 root phenotype is autonomous from shoot-derived signals. To this end, we introgressed the short primary root phenotype into an Sav-0 background, whose shoot morphology is very different from Uk-1. Sav-0 plants flower early, approximately after the 6^{th} true leaf (under constant illumination), and form multiple shoots. By contrast, in the same conditions Uk-1 plants flower late (approximately after the 24^{th} true leaf) and form a single shoot. From a sample of the F2 generation resulting from our Uk-1 X Sav-0 cross we selected the seedling with the shortest

primary root. This plant was then back-crossed into the parental Sav-0 line, a scheme that was in total repeated four times. From this introgression we derived plants whose genome consists of approximately 97% of Sav-0 DNA and only 3% of Uk-1 DNA. In the following we refer to individuals with a short root phenotype that have been derived from this introgression into an Sav-0 background as *brx*^{S}.

The roots of *brx*^{S} seedlings are as short as those of Uk-1 seedlings, both when grown in the light (Fig. 3.1B) or in darkness (data not shown). In the adult root system of *brx*^{S} plants the primary root is slightly longer and the root system is less branched than in Uk-1. This is true for root systems grown in tissue culture (Fig. 3.1C) as well as for soil-grown roots (Fig. 3.1D, E). In contrast to the root system, the shoot system morphology and flowering time of *brx*^{S} plants resembles the Sav-0 shoot system (Fig. 3.1D). Moreover, grafts between Sav-0 shoots and Uk-1 roots and vice versa, do not influence the respective root system morphologies (data not shown). Therefore, the short root phenotype conferred by the Uk-1 allele of the *BRX* locus is independent from shoot-derived signals.

Physiological responses of the root system are intact in brx^S *plants*

Because the influence of patterning genes, plant hormones and environmental stimuli on root growth are well documented, we checked whether *brx*^{S} plants are impaired in any of the corresponding pathways. Transverse sections of Uk-1 roots indicate that the cortex and endodermis cell layers are present (Fig. 3.1F), ruling out defects in the *SCR* or *SHR* genes. Also, *brx*^{S} seedlings respond to exogenous application of plant hormones, such as auxins, gibberellins or cytokinins, in roughly the same proportional range as the parental Sav-0 line (e.g. Fig. 3.1G). Notably, the application of plant hormones was in no instance able to rescue the short root phenotype (Fig. 3.1G), even when very low concentrations were applied (data not shown). Finally, we also tested the response of *brx*^{S}

seedlings to different nutrient conditions, since nutrient availability has been demonstrated to affect root system architecture (Lopez-Bucio et al. 2003). However, we did not observe any apparent defects in the numerous assays that we conducted, including examination of the responses to low or high nitrate or phosphate levels, or to different ratios of nitrogen to carbon source. Again, *brx*^{S} seedlings responded in proportional ranges similar to those of the parental Sav-0 line (data not shown). In summary, the short root phenotype of *brx*^{S} plants is not due to a major defect in basic hormone or physiological response pathways.

brx^{S} *seedlings have shorter and fewer root cells*

To characterize the *brx*^{S} phenotype in further detail, we analyzed the primary roots of *brx*^{S} seedlings at the cellular level. In principal, the *brx*^{S} short root phenotype could be due to one of two phenomena, either shorter cells or fewer cells. To distinguish between these two possibilities, we microscopically analyzed mature epidermal cell files (i.e. the root hair-bearing region distal to the meristem). Analysis of the size and number of epidermal cells revealed that *brx*^{S} roots are composed of shorter (Fig. 3.2A) as well as fewer (Fig. 3.2B) cells. These parameters remained relatively constant throughout the period of observation (3-8 dag). In Sav-0, the production rate of mature epidermal cells was 19-24 cells per day and their average length was 110-117 µm, while in *brx*^{S} 11-13 cells per day with a length of 76-87 µm were produced. Since the root growth rate in both genotypes remained roughly the same up to 21 dag, it is reasonable to assume that these parameters did not change throughout development. In line with the observations in epidermal cell files, confocal microscopy revealed that the more evenly sized cortical cells are also shorter in *brx*^{S} roots (Fig. 3.2C, D). In summary, both cell elongation and cell production rate are decreased in *brx*^{S} seedlings, contributing approximately one and two thirds respectively, to the overall difference in root length as compared to Sav-0 seedlings.

The BRX locus affects cell proliferation in the apical root meristem

To visualize the meristematic region of the root, we crossed a transgenic reporter of cell proliferation, a fusion protein between cyclin B1;1 (CYCB1;1) and beta-glucuronidase (GUS) expressed under control of the *CYCB1;1* promoter (de Almeida Engler et al. 1999), into the brx^S and Sav-0 lines. GUS staining of roots of these seedlings revealed that the root meristems of brx^S seedlings are smaller than Sav-0 meristems (Fig. 3.3A). When investigated by confocal microscopy, the organization of brx^S root meristems appears normal (Fig. 3.3B). However, compared to Sav-0 meristems, cells in the meristematic zone in brx^S appear to increase in size earlier and the number of cells undergoing division appears to be reduced (Fig. 3.3C). This phenotype (shown for 0.5% sucrose concentration in Fig. 3.3B, C) becomes more pronounced in growth-promoting conditions. In our physiological assays we noticed that the difference in root length between Sav-0 and brx^S seedlings increased when root growth rate was stimulated by increasing the amount of sucrose in the medium (Fig. 3.3D, Benfey et al. 1993). This correlates with a further size reduction of brx^S meristems at a higher growth rate (e.g., 2% sucrose; Fig. 3.3E). In these conditions, they are composed of fewer cells that are less organized and not as isodiametric (Fig. 3.3F).

To quantify our observations, we measured the size of the meristematic and elongation zones of Sav-0 and brx^S seedlings that were grown on 2% sucrose at 6 dag by analyzing cell files. We took the number of cortical cells, counted from the initial cell up to the first rapidly elongating cell, as an indicator of root meristem size (Casamitjana-Martinez et al. 2003). By this measure, brx^S root meristems consist of about 25% of the number of cells in Sav-0 meristems (Fig. 3.3G). We also took the number of cortical cells, counted from the first rapidly elongating cell up to the first cell of mature size, as an indicator of elongation zone size. By this measure, brx^S elongation zones consist of approximately 40% of the number of cells in Sav-0 elongation zones (Fig. 3.3G).

Therefore, the ratio between the number of cells in the meristematic zone versus the number of cells in the elongation zone is shifted close to 1.0 in *brx*^{S} from approximately 1.7 in Sav-0. Thus, the size of both the meristematic and elongation zones of the root tips of *brx*^{S} seedlings are decreased, but the meristematic zone is affected more severely.

Isolation of the BRX gene by positional cloning

In order to identify the *BRX* gene at the molecular level, we followed a positional cloning approach. To this end, genomic DNA was isolated from 860 individuals of the F2 population from the Uk-1 X Sav-0 cross and genotyped with molecular markers that showed polymorphism between the two accessions. The root phenotype of the F2 plants was unequivocally scored by analysis of the F3 progeny. Recombination mapping placed the *BRX* locus on the upper arm of chromosome I. Subsequently, novel markers were generated from PCR-amplified DNA fragments arbitrarily chosen from the *Arabidopsis* genome sequence. This strategy allowed us to locate the *BRX* gene in a zero recombination interval of ca. 45 kb, flanked by proximal and distal markers indicating three and one recombination events, respectively (Fig. 3.4A).

Crosses of Uk-1 with the *Arabidopsis* reference accession Columbia (Col) result in segregation of a recessive short root phenotype as well. Thus, we tested five of the ten candidate genes in the 45 kb interval by analyzing respective T-DNA insertion mutants in Col background that were available (Alonso et al. 2003). A short root phenotype was not observed in any of these mutants (Fig. 3.4B). We also analyzed eight of the ten *BRX* candidate genes by comparing the sequence of the Uk-1 alleles with the corresponding Col alleles. We found no Uk-1 alleles with obvious implications for gene functionality (Fig. 3.4B), with the exception of the gene represented by unicode At1g31880. This gene contains a base pair change in the fourth exon, which results in a pre-mature stop codon in the open reading frame and therefore a

truncated protein missing about two thirds of the C-terminus (Fig. 3.4C). This stop codon is not present in the respective alleles of other accessions with long primary roots (determined for accessions Sav-0, Wassilewskaja, Landsberg *erecta*, Freiburg-1, Eilenburg-0, Loch Ness-0, Chisdra-0, Goettingen-0 and Kindalville-0). Moreover, the stop codon is also missing from the sequence of the accessions Uk-2, Uk-3 and Uk-4, whose *BRX* alleles are nearly identical to the Col allele apart from very few silent polymorphisms and/or one conserved substitution. These three accessions have long primary roots and were collected in the immediate vicinity of Uk-1 (www.arabidopsis.org). Introduction of a transgenic construct expressing the open reading frame of At1g31880 under control of the 35S cauliflower mosaic virus gene promoter *(35S)* into brx^S seedlings largely rescues the short root phenotype (Fig. 3.4D) and restores the meristem size to Sav-0 dimensions (Fig. 3.4E). Finally, this is also true for a transgene expressing a *BRX* open reading frame in its native start codon context (i.e. including the untranslated exons and introns up to the ATG; Fig. 3.4C) under control of a 1.9 kb fragment of the *BRX* promoter (data not shown). Thus, the combined evidence demonstrates that At1g31880 and *BRX* are identical.

BRX is expressed in the root at very low levels

From the brx^S phenotype it can be expected that *BRX* is expressed in the root. To determine whether this is the case, we analyzed whole seedlings, shoots and roots by reverse transcription PCR (RT-PCR). In these experiments, *BRX* expression can be detected in all three samples (Fig. 3.4G). In order to visualize *BRX* expression at spatiotemporal resolution, we also constructed transgenic plants expressing the green fluorescent protein (GFP) or a fusion protein of BRX and GFP under control of the *BRX* promoter (constructs *BRX:GFP* and *BRX::BRX:GFP*, respectively). Importantly, the *BRX::BRX:GFP* transgene rescues the brx^S root phenotype, demonstrating expression and functionality of the

BRX:GFP fusion protein (Fig. 3.4H). However, in (confocal) fluorescence microscopy neither the BRX:GFP fusion protein nor native GFP could be detected. In line with these observations, Western analysis of the transgenic lines using an anti-GFP antibody yields a very faint signal, and only so if an excess amount of protein extract is loaded, while GFP produced in a *35S::GFP* line is readily detectable in very little extract (data not shown). Therefore, in summary our results indicate that *BRX* is expressed in the shoot and root, albeit at very low levels.

BRX explains most of the variance in primary root length between Uk-1 and Sav-0

We observed rescue of the short root phenotype of brx^S seedlings in several transgenic lines derived from independent primary transformants. However, we noticed that rescue was not complete in any of these lines (e.g. Fig. 3.4F, H). This finding is consistent with the idea that *BRX* represents the major QTL for primary root length predicted on chromosome I from regression analysis of our recombinant inbred line population. The creation of a BseGI restriction enzyme polymorphism by the base pair change in the Uk-1 allele of *BRX* allowed us to directly score the *BRX* genotype in the recombinant inbred lines and include this information in the regression analysis. The results indicate that the *BRX* locus explains ca. 80% of the observed variance in primary root length in the population (Fig. 3.5).

BRX is a member of a novel, plant-specific gene family

At the time of its identification, the *BRX* gene was not correctly annotated in public databases, with most of the open reading frame predicted to be fused with the neighboring gene and consequently considered a novel type of aquaporin (Johanson et al. 2001). However, the annotation of a related gene, which we named *BRX-like 1* (*BRXL1*; unicode At2g35600), enabled us to determine the correct intron-exon

structure of *BRX* by comparison, including two non-coding exons representing 5' untranslated regions (Fig. 3.4C). Based on the gene structure of *BRX* and *BRXL1*, we were able to identify and annotate three more genes of this type in the *Arabidopsis* genome, *BRXL2*, *BRXL3* and *BRXL4* (unicode or fusion of parts of unicodes At3g14000, At1g54180-At1g54190 and At5g20530-At5g20540, respectively). Subsequently, full length cDNA clones became available for four out of the five genes and confirmed the predicted gene models. The *BRX* family genes and the proteins they encode are highly conserved (Fig. 3.6A) and found in all higher plants for which data are available, but are absent from unicellular organisms or animals. Therefore, this gene family appears to be specific to multicellular plants.

To test whether other *BRX-like* genes act partially redundant with *BRX* in root growth, we obtained presumed null mutants for *BRXL1*, *BRXL2* and *BRXL3* from the SALK T-DNA insertion mutagenesis project (Alonso et al. 2003). Insertions in the *BRXL4* gene could not be confirmed. Interestingly, none of these mutants display a *brx* root phenotype. However, partial and asymmetric redundancy has been observed in other cases and might only become apparent in a *brx* mutant background. Thus, we created double mutants between the Uk-1 *brx* allele, twice introgressed into a Col background (we refer to these plants as brx^{C}), and the other *brxl* mutants. In our analysis, we focused on the brx^{C};*brxl1* double mutant, because of the high similarity of *BRXL1* to *BRX* both in gene structure (only these two *BRX-like* genes possess the untranslated exons) and amino acid sequence (Fig. 3.6A, B). In this double mutant, we did not observe any abnormalities in the root system that would indicate an enhancement of the brx^{C} phenotype (Fig. 3.6C, D). Similar results were obtained for the brx^{C};*brxl2* and brx^{C};*brxl3* double mutants (data not shown). Therefore, *BRX* likely is the only gene in this family with a role in root development.

The BRX protein is nuclear localized and can activate transcription in yeast

The BRX protein does not contain any previously characterized motifs that would indicate its biochemical function. However, sequence alignment of the BRX family proteins reveals that all five of them contain three highly conserved domains (Fig. 3.6A). One domain is located at the N-terminus, between amino acids 28 and 45 of BRX, whereas two more domains that are highly similar to each other are located between amino acids 169 and 182, and 320 and 334, respectively. Interestingly, in secondary structure predictions these three domains all contain regions that have a high probability of forming alpha-helical secondary structures (Fig. 3.7A).

Alpha helices are characteristic for transcription factor proteins and are often found in DNA binding and protein interaction domains (Luscombe et al. 2000). Transcription factors are nuclear proteins, and therefore we tested whether BRX accumulates in the nucleus. To this end, a fusion between GFP and BRX was transiently expressed in epidermal onion cells and its subcellular localization was monitored by fluorescence microscopy. In this assay, the BRX:GFP fusion protein is found primarily in the nucleus (Fig. 3.7B), unlike GFP by itself, indicating that BRX is actively transported into the nucleus.

We also tested whether the BRX protein can activate transcription in a heterologous yeast system. To this end, we cloned the *BRX* open reading frame into a yeast expression vector, in frame with the lexA DNA binding domain of *E. coli*. Expression of this fusion protein in the presence of a beta-galactosidase reporter gene controlled by lexA promoter binding sites results in strong reporter activity (Fig. 3.7C). This is not the case if a control fusion protein between the *Arabidopsis* transcription factor HY5, which lacks transactivation potential (Ang et al. 1998), and lexA is expressed instead. The transactivation potential is largely reduced in a truncated BRX protein comprising the 100 N-terminal amino acids. Thus,

the data indicate that BRX contains a transcription activation domain.

Discussion

Natural genetic variation in root system morphology of Arabidopsis

The goal of our study was to isolate novel regulators of root growth that are responsible for the intra-specific variation in root system morphology. Such genes should not be essential for root development *per se*, based on the assumption that alleles that are selected in the wild are not detrimental to basic plant development and that evolution preferentially acts on genes controlling non-essential aspects of growth. Because of the well-developed genetic resources and the ease of manipulation, we chose to analyze natural genetic variation in isogenized accessions of the model plant *Arabidopsis*.

Mutagenesis approaches in *Arabidopsis* have been tremendously successful in isolating genes involved in different aspects of root development, such as pattern formation, growth rate or cell shape (e.g., Benfey et al. 1993; Hauser et al. 1995). While the analysis of these genes has greatly enhanced our knowledge of root development, less is known about the factors that specifically control quantitative aspects of root biology, such as the rate of growth. Although it is clear that control of cell proliferation has an important role in root growth (Beemster et al. 2003), to our knowledge loss of function mutants that are specific to cell proliferation in the root meristem have not been isolated to date. Rather, experimental evidence for pathways controlling root growth has been gathered from transgenic gain of function approaches, which usually involve the ectopic and/or overexpression of candidate genes. By these means, for example, the control of cell cycle progression (Doerner et al. 1996; Cockcroft et al. 2000; De Veylder et al. 2001) and CLAVATA-type pathways (Casamitjana-Martinez et al. 2003; Hobe et al. 2003) have been implicated in the control of root growth and/or meristem size.

Notably, it has been observed that there is detectable variation in root growth between *Arabidopsis* accessions and that this is to a significant degree due to differences in mature cell size or the rate of cell proliferation (Beemster et al. 2002), supporting the notion that genetic analysis of natural variation can identify factors controlling these processes. Consistent with this previous report, we observed an average two- to three-fold variation in root growth parameters of *Arabidopsis* accessions. The reduction of growth in the Uk-1 line as compared to average was, however, remarkable. The occurrence of this phenotype in the wild might be related to the fact that the Uk-1 accession has been reportedly collected from a river embankment (http://www.arabidopsis.org). Thus, water availability might not be an as limiting growth factor in the natural environment of this line, and this might have permitted the evolution of a shorter root as compared to accessions that grow in more arid environments.

The characterization of QTLs by genetic mapping is a well-established procedure, however isolation of a gene corresponding to a QTL of interest is still an arduous task. Our success in isolating the *BRX* gene was greatly aided by two factors. First, the effect of the Uk-1 allele of *BRX* on root growth is a strong one and therefore easily detectable. Second, the unmatched genomic resources for *Arabidopsis* enable fine mapping within a reasonable time frame (Borevitz and Nordborg 2003). Nevertheless, it can be expected that increased availability of molecular markers and automatization of mapping procedures will soon enable the routine isolation of small effect QTLs in *Arabidopsis* (Borevitz et al. 2003; Schmid et al. 2003; Torjek et al. 2003).

Specificity of the brx phenotype

By introgression of the Uk-1 allele of *BRX* into the Sav-0 background we have demonstrated that the brx^S phenotype does not depend on shoot-derived signals. Moreover, in our phenotypic analysis we

could not detect any abnormalities in the shoot system of *brx*^{S} plants. Thus *BRX* activity is specifically needed in the root. Although many genes influencing root growth have been isolated by mutagenesis approaches, such specificity is still rare. Notably, the majority of root growth mutants isolated to date are involved in hormone signaling pathways. Generally, they also display conspicuous defects outside the root system. For instance, root growth is impaired in the *gai* and *rga* mutants, which disrupt gibberellic acid signaling (Fu and Harberd 2003). However, these genes also have a central role in the growth of stems. The issue is further complicated in mutants affecting the auxin signaling pathway, which include several gain-of-function mutants that might occasionally represent neomorphic phenotypes (Leyser 2002). An auxin signaling gene that appears to be required only in the root is *SHY2* (Tian and Reed 1999). Although *shy2* gain-of-function mutants have shoot and root phenotypes, corresponding loss-of-function mutants only display a root growth phenotype. In addition, *shy2* loss-of-function results in reduced root growth only in light-grown conditions, and this reduction can be rescued by exogenous application of auxin (Tian and Reed 1999). By contrast, the phenotype of *brx*^{S} seedlings is not conditional and cannot be rescued by plant hormone application. It also has to be stressed that under all growth conditions tested, *brx*^{S} roots always grow at a rate that is two- to three-fold lower than in roots of seedlings carrying the functional Sav-0 allele. Further, the reduction in meristem size in *brx*^{S} can be observed early in development, does not change as the roots become older and does not result in growth arrest. This differs significantly from other studies (Casamitjana-Martinez et al. 2003; Hobe et al. 2003), where the root meristem has normal size in early stages and becomes consumed over time, eventually resulting in the shutdown of growth. In summary, compared to other root growth mutants the phenotype of *brx*^{S} seedlings is unique in many aspects and *BRX* appears to be a very basic factor, required for an optimal rate of root growth in any condition.

The brx phenotype: cell proliferation versus cell elongation

The slow primary root growth of *brx*^{S} seedlings is due to a reduction in mature cell size as well as cell proliferation. The reduced cell proliferation quantitatively contributes more to the *brx*^{S} phenotype than the reduced cell size. It has to be noted however, that in our introgression we always selected the seedlings with the shortest primary root, thereby likely introducing all the genetic factors affecting root growth in Uk-1 into *brx*^{S} seedlings. Since transgenic expression of *BRX* in *brx*^{S} seedlings restores mature cortical cell size to wild type dimensions, but does not rescue total root length to a 100%, we must assume that indeed additional, smaller effect QTLs have been introgressed and would have to be complemented to fully restore the cell proliferation rate to Sav-0 levels.

The different contributions of cell proliferation and cell elongation to overall root growth have been difficult to dissect. To date, it is not clear whether these processes are controlled independently (Beemster and Baskin 1998; De Veylder et al. 2001). This issue is also complicated by the fact that cells still divide, although at a much lower frequency, in the elongation zone (Beemster et al. 2003). It is, however, conceivable that a reduction of cell proliferation in the meristematic region results in a decreased supply of cells to the elongation zone, thus decreasing its size. It also has been suggested that it is the time a cell spends as part of the elongation zone rather than elongation zone size *per se* that determines final mature cell length (Beemster and Baskin 1998; Beemster and Baskin 2000). Since decreased cell proliferation in the meristem would also result in slower displacement of cells from the elongation zone, the time they spend elongating consequently might not change dramatically, even if the elongation zone is physically smaller. This explanation accounts for the observation that interference with cell proliferation in the root meristem, resulting in reduced size of the meristematic region, always results in a reduction of elongation zone size, while mature cell size is usually not affected to the same degree (Beemster and Baskin 2000; De Veylder et

al. 2001; Casamitjana-Martinez et al. 2003; Werner et al. 2003).

Genetically, we cannot separate the roles of *BRX* in cell proliferation and elongation. However, several arguments support the notion that the reduced mature cell size might be a secondary consequence of reduced cell proliferation. In brx^S seedlings, the growth zone of the root is reduced in size. This phenotype is enhanced if cell proliferation is stimulated by increased sucrose concentration of the medium. Compared to Sav-0, the cell number in the meristematic zone is affected to a greater extent in brx^S seedlings than the cell number in the elongation zone. Interestingly, this phenotype shows significant similarity to root tips of seedlings in which cell proliferation has been slowed down, for instance, by cytokinin treatment (Beemster and Baskin 2000) or by overexpression of inhibitors of cell cycle progression (De Veylder et al. 2001). Finally, previous analyses suggest that the rate of root growth is primarily controlled at the step of cell proliferation (Beemster and Baskin 1998; Beemster et al. 2002; Beemster et al. 2003). Thus, the primary cause of the brx^S phenotype might be the reduction of cell proliferation in the root meristem.

Implications from the low expression level of BRX

Our expression analyses determined that *BRX* is expressed in the root as well as the shoot of young seedlings. Thus, *BRX* might also have a yet unknown function in the shoot, which could be masked by redundantly acting *BRX-like* genes in brx^S plants.

We could not detect GFP fluorescence in our reporter lines *in situ*. In this context it is important to note that we demonstrate that the *BRX:BRX::GFP* transgene can substitute for native *BRX*. The transgenic proteins, i.e. BRX:GFP or native GFP, are also barely detectable in Western blots, supporting our conclusion that the *BRX* expression level is very low. This result is corroborated independently by the very rare occurrence of *BRX* cDNAs in public databases (two hits at time of

publication) and *BRX* signatures in MPSS experiments (mpss.udel.edu/at/java.html). Finally, based on the low expression level of *BRX* and the transgenic rescue of brx^S seedlings with a *35S::BRX* construct it can be concluded that ectopic overexpression of *BRX* does not stimulate root growth beyond the rate observed in Sav-0, therefore indicating that BRX is one of several factors that determine the rate of root growth.

The BRX gene family of Arabidopsis: a novel class of transcription factors?

The BRX family proteins are remarkably well conserved in *Arabidopsis* (64-93% similarity at amino acid level), indicating that most of their structure is important for their function. However, with the possible exception of *BRXL4*, for which we could not confirm a T-DNA insertion mutant, only *BRX* appears to have a role in root growth, as demonstrated by the analysis of the single and double mutants with *brxl1*, *brxl2* and *brxl3*. This could indicate that, despite the similarity between these genes, there are functional differences in the activity of the encoded proteins, or that these genes act only partially redundantly due to differential expression patterns. Which of these possibilities is the case will be the subject of future investigations. Orthologous *BRX-like* genes can be found in all other multicellular plant species for which data are available, but not in unicellular organisms or animals. Notably, the corresponding proteins are very well conserved within and between species (C.S.H., K.F.X. Mayer and co-workers, unpublished results). Therefore, we propose that *BRX* is part of an important gene family with conserved functions in general plant development.

In the absence of previously defined functional domains it is difficult to assign a biochemical activity to the BRX protein. However, the high level of conservation of distinct domains between BRX family proteins indicates that these regions might be especially important for their activity.

These domains are predicted to form alpha-helical structures, which are often found in protein-protein interaction or DNA binding domains (Luscombe et al. 2000). Our findings that BRX can localize to the nucleus and can activate transcription in a heterologous system support the notion that BRX family proteins represent a novel class of transcription factors. Thus, although their exact biochemical activity remains elusive for now, BRX family proteins are novel nuclear localized regulatory factors of plant development.

Conclusion

The development of plant organs is intrinsically linked to the localized control of cell proliferation (Beemster et al. 2003). Although considerable progress has been made in the characterization of the components of the cell cycle machinery and their differential activity throughout development, several studies clearly suggest that higher level controls modulating cell proliferation in a tissue-specific manner must exist. Accelerating or slowing down the cell cycle results in enhanced or reduced overall growth, respectively (Doerner et al. 1996; Cockcroft et al. 2000; De Veylder et al. 2001). However, in both cases the relative shape and size of plant organs is largely maintained, indicating that additional factors regulate the relative levels of cell proliferation in a highly localized fashion. The *BRX* gene represents such a regulatory factor with respect to root growth.

Materials and Methods

Plant material and tissue culture

Seeds of *Arabidopsis* accessions and T-DNA insertion mutants were obtained from the *Arabidopsis* Biological Resources Center. Unless otherwise stated, seedlings were grown at 22°C under constant illumination on culture medium containing 0.5 x MS salts, 0.5 g/l MES and 0.9% agar (pH adjusted to 5.8 - 6.0 with 1M KOH), plus the indicated amount of sucrose and any hormone supplements (Sigma-Aldrich). The light intensity was approximately 140 µmole m^{-2} sec^{-1}. Grafting experiments were performed as described (Turnbull et al. 2002).

Root length measurements

To determine root lengths, seedlings were grown on vertically oriented plates, which were either scanned on a flat bed scanner or photographed with a digital camera to produce image files suitable for quantitative analysis using the NIH Image software (v 1.63).

GUS staining

To visualize GUS reporter activity, seedlings were incubated in 90% acetone for 1 hr at room temperature and then washed once in GUS staining buffer (100 mM phosphate buffer pH 7.0; 1mM K-ferricyanide; 1 mM K-ferrocyanide; 0.1% Triton X-100) for 15 min. The wash was replaced by GUS staining buffer that contained 1 mg/ml of X-Gluc and the samples were incubated in darkness at 37°C. The reaction was stopped by replacing the staining solution with 20% EtOH.

Molecular markers and mapping procedures

For mapping purposes, PCR-based molecular markers detecting polymorphisms between Uk-1 and Sav-0 genomic DNA were generated by sequence or restriction analysis of described markers or arbitrarily

chosen genomic fragments. SSLP markers were scored on 4% gels using high resolution agarose (Amresco).

For fine mapping of the *BRX* locus, genomic DNA was prepared from 860 F2 plants using the DNeasy™ Plant Genomic DNA Isolation Kit (Qiagen) and genotyped with molecular markers. The *BRX* genotype was deduced from phenotypic analysis of a sample of approximately 20 seedlings of the F3 progeny. Recombination mapping followed standard procedures.

To generate marker data for the QTL analysis, genomic DNA was isolated from plants of the S6 generation and genotyped. Phenotypic measurements were taken from a sample of 16 seedlings of the same generation.

Transgenic analysis

The coding regions of the *BRX* open reading frame were amplified from genomic DNA by PCR, using Pfu polymerase (Fermentas). These fragments were then connected by subsequent directed ligation reactions and re-amplifications to produce the full length open reading frame. The open reading frame was then cloned into the binary vector pTCSH1 (Hardtke et al. 2000) and verified by sequencing to serve as a basis for further manipulations, such as replacement of the promoter driving expression. The GFP version used in our constructs is mGFP5. The transgenic constructs were transformed into brx^S plants via the floral dip method and transgenic lines were selected by screening the seed progeny for glufosinate ammonium resistance (15 mg/l, "BASTA", Sigma-Aldrich) on medium containing 0.3% sucrose.

RT-PCR

Total RNA was prepared from Col seedlings with the RNeasy™ kit (Qiagen) according to the manufacturer's instructions and RT-PCR reactions were performed according to standard procedures using

Superscript II™ reverse transcriptase (Invitrogen). PCR reactions were performed with 5µl RT reaction as a template. Oligonucleotides for the detection of *BRX* and actin4 were chosen to amplify fragments spanning an intron-exon border, in order to permit detection of genomic contamination.

QTL analysis

For QTL analysis, a recombinant inbred line population was established starting from the F2 progeny of two F1 plants resulting from a Uk-1 x Sav-0 cross. From the F2 plants, 206 lines were established by selfing and single seed descent for six generations. The genotypes for SSLP markers were then determined for plants of the S6 generation and root length was measured by analysis of 16 seedlings of the same generation. Genotype data and average root lengths were entered into a matrix and free marker regression analysis for selfed recombinant inbred lines was performed using the MapManager QTX for Macintosh software, version 0.27 (mapmgr.roswellpark.org/mmQTX.html).

Transient transformation of onion epidermis cells

Constructs for transient expression were generated by replacing the GUS gene in vector pTCSH1 with the open reading frame of the green fluorescent protein (mGFP5). For expression of a GFP-BRX fusion protein, the *BRX* open reading frame was inserted in frame at the C-terminus of the GFP. Transient transformation of onion epidermis cells was performed using a PDS1000 helium particle gun (Bio-Rad). After 24 hr incubation in darkness the cells were examined by fluorescence microscopy.

Transactivation assay

To test the transactivation potential of BRX, the full length or part of the open reading frame was inserted into the vector pEG202 (Clontech),

resulting in an in frame fusion to the lexA DNA binding domain of *E. coli*. The HY5 control construct has been described (Hardtke et al. 2000). Plasmids were then introduced into the *S. cerevisiae* strain EGY48 (Clontech), together with the reporter construct pSH18-34 (Clontech), which carries the beta-galactosidase reporter gene under control of lexA binding sites. Transformants were grown in liquid culture overnight, diluted in the morning and incubated for six more hours before beta-galactosidase activity was measured by standard assay.

Bioinformatic analyses

The *BRX-like* genes were identified by homology searches of the *Arabidopsis* genome sequence with the BLAST search tools (www.ncbi.nlm.nih.gov/BLAST/). The intron-exon structure of *BRX-like* genes, sequence alignments, phylogenetic trees and secondary structure predictions of BRX-like proteins were generated by using the analysis tools provided by the European Institute for Bioinformatics (www.ebi.ac.uk/Tools/).

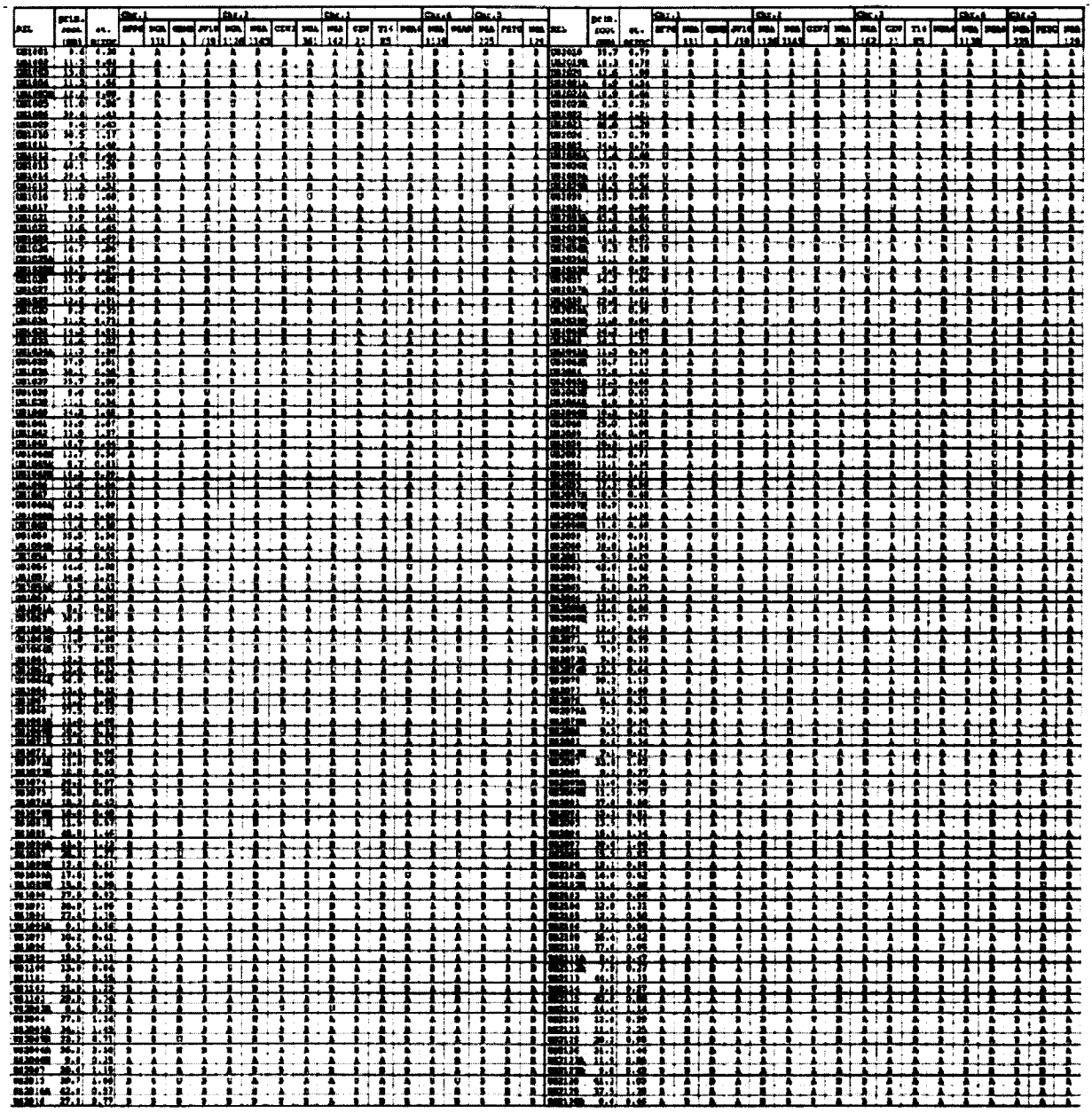


Table 3.1 Analysis of a recombinant inbred line (RIL) population derived from a cross between the Sav-0 and Uk-1 accessions. Columns indicate the line number, average primary root length determined from a sample of 16-20 seedlings in the S6 generation and the genotype at simple sequence length polymorphism markers distributed throughout the *Arabidopsis* genome. Genotypes: A = Uk-1 allele; B = Sav-0 allele; H = heterozygous; U = unknown.

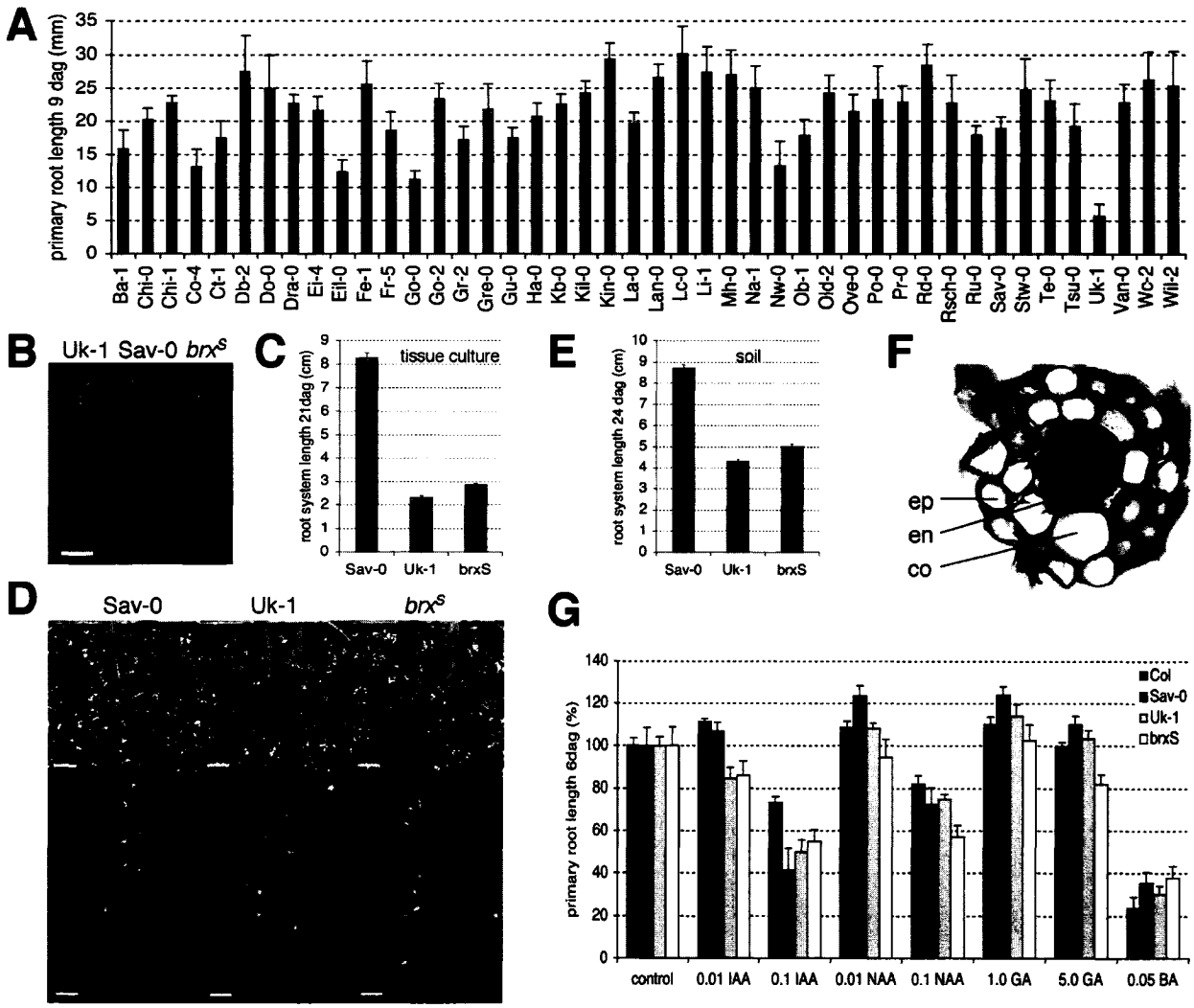

A
primary root length 9 dag (mm)
0
5
10
15
20
25
30
35
Ba-1
Chi-0
Chi-1
Co-4
Ct-1
Db-2
Do-0
Dra-0
Ei-4
Eil-0
Fe-1
Fr-5
Go-0
Go-2
Gr-2
Gre-0
Gu-0
Ha-0
Kb-0
Kil-0
Kin-0
La-0
Lan-0
Lc-0
Li-1
Mh-0
Na-1
Nw-0
Ob-1
Old-2
Ove-0
Po-0
Pr-0
Rd-0
Rsch-0
Ru-0
Sav-0
Stw-0
Te-0
Tsu-0
Uk-1
Van-0
Wc-2
Wil-2
B
Uk-1 Sav-0 brxS
C
tissue culture
root system length 21dag (cm)
Sav-0
Uk-1
brxS
E
soil
root system length 24 dag (cm)
Sav-0
Uk-1
brxS
F
ep
en
co
D
Sav-0
Uk-1
brxS
G
primary root length 6dag (%)
Col
Sav-0
Uk-1
brxS
control
0.01 IAA
0.1 IAA
0.01 NAA
0.1 NAA
1.0 GA
5.0 GA
0.05 BA

Figure 3.1 Natural variation in root system morphology among *Arabidopsis* accessions. *(A)* Primary root length of *Arabidopsis* seedlings at 9 dag, grown in 8 hr dark – 16 hr light cycle on 0.5 x MS medium. n >= 10. *(B)* Representative seedlings of the Uk-1 and Sav-0 accessions, and a seedling resulting from introgression of the Uk-1 short root phenotype into an Sav-0 background (*brx*^{S}), 9 dag grown in constant light on 0.5 x MS medium containing 0.3% sucrose. Size bar = 1 cm. *(C)* Primary root length of plants of the three genotypes grown in constant light on 0.5 x MS medium containing 1.0% sucrose, 21 dag. n = 6. *(D)* Top: Representative rosette phenotypes of the three genotypes at 24 dag, grown on soil under constant illumination. Bottom: Root system belonging to the shoots shown in top panel, dug out from the soil and cleaned. Size bar = 1 cm. *(E)* Approximate primary root length of plants of the three genotypes grown on soil under constant illumination, 24 dag. n >= 7. *(F)* Transverse cryosection through the mature part of a primary root of a 7 day old Uk-1 seedling. ep = epidermis; co = cortex; en = endodermis; *(G)* Relative response of Col, Sav-0, Uk-1 and *brx*^{S} seedlings to different exogenous plant hormone applications, 6 dag. Seedlings were grown in constant light on 0.5 x MS medium containing 2.0% sucrose plus indicated hormone supplement. IAA = indole acetic acid; NAA = naphtalene acetic acid; GA = gibberellic acid; BA = benzylaminopurine; Error bars are standard error.

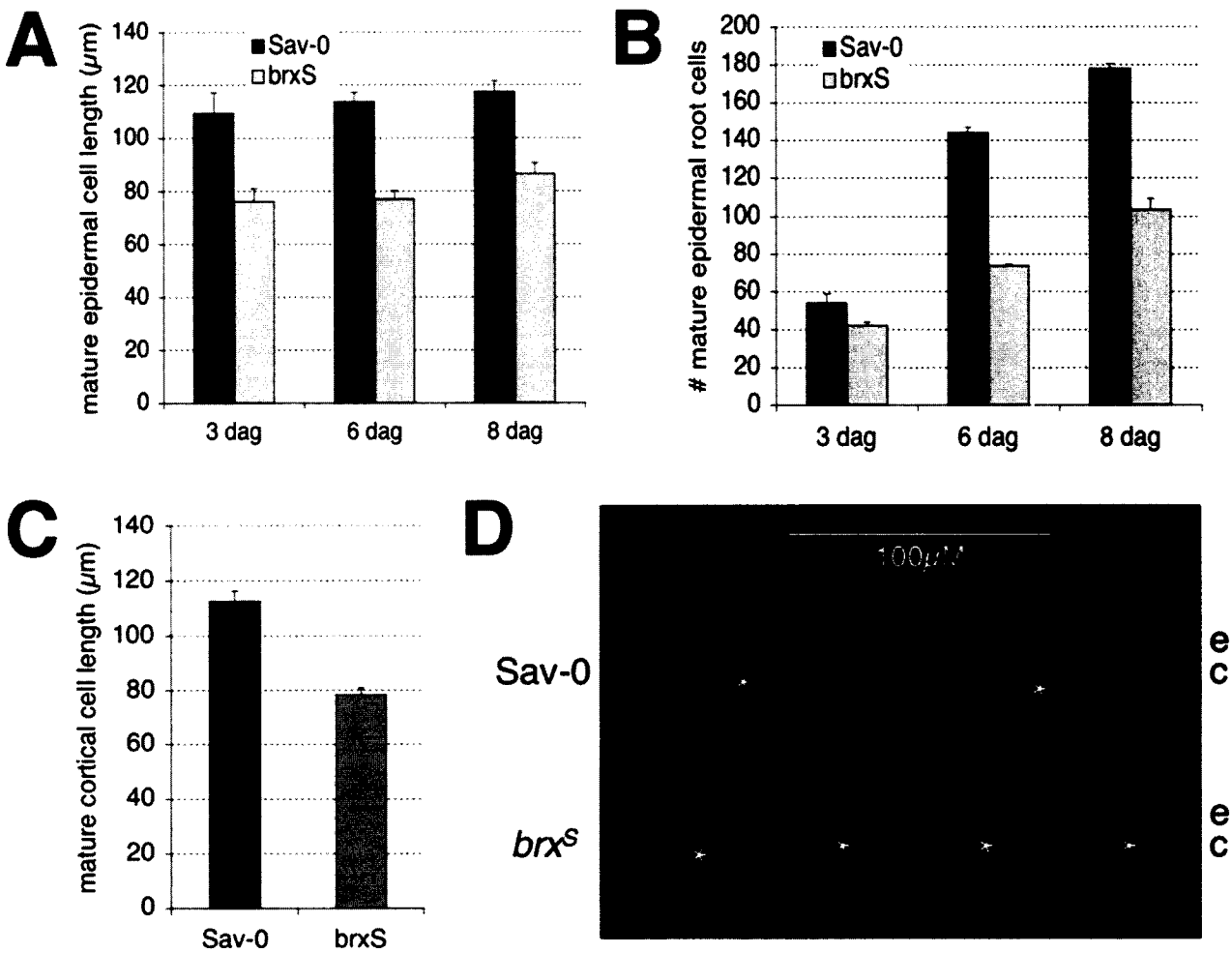

A
mature epidermal cell length (μm)
140
120
100
80
60
40
20
0
Sav-0
brxS
3 dag
6 dag
8 dag
B
mature epidermal root cells
200
180
160
140
120
100
80
60
40
20
0
Sav-0
brxS
3 dag
6 dag
8 dag
C
mature cortical cell length (μm)
140
120
100
80
60
40
20
0
Sav-0
brxS
D
100μM
Sav-0
brxS
e
c
e
c

Figure 3.2. Mature cell size and number in the primary roots of Sav-0 and *brx*^{S} seedlings. *(A)* Mature epidermal cell length at 3, 6 and 8 dag. For each genotype, 3 seedlings were measured per time point. The number of cells measured in each seedling was: >= 13 at 3 dag; >= 46 at 6 dag; >= 34 at 8 dag; *(B)* Number of mature epidermal cells in a cell file of the root at 3, 6 and 8 dag. For each genotype, 3 seedlings were counted per time point. *(C)* Mature cortical cell length at 6 dag. For each genotype, 3 seedlings were measured. The number of cells measured was >= 50. *(D)* Confocal microscopy images of the mature region of Sav-0 and *brx*^{S} roots. Asterisks mark cortical cells. e = epidermis; c = cortex; Error bars are standard error.

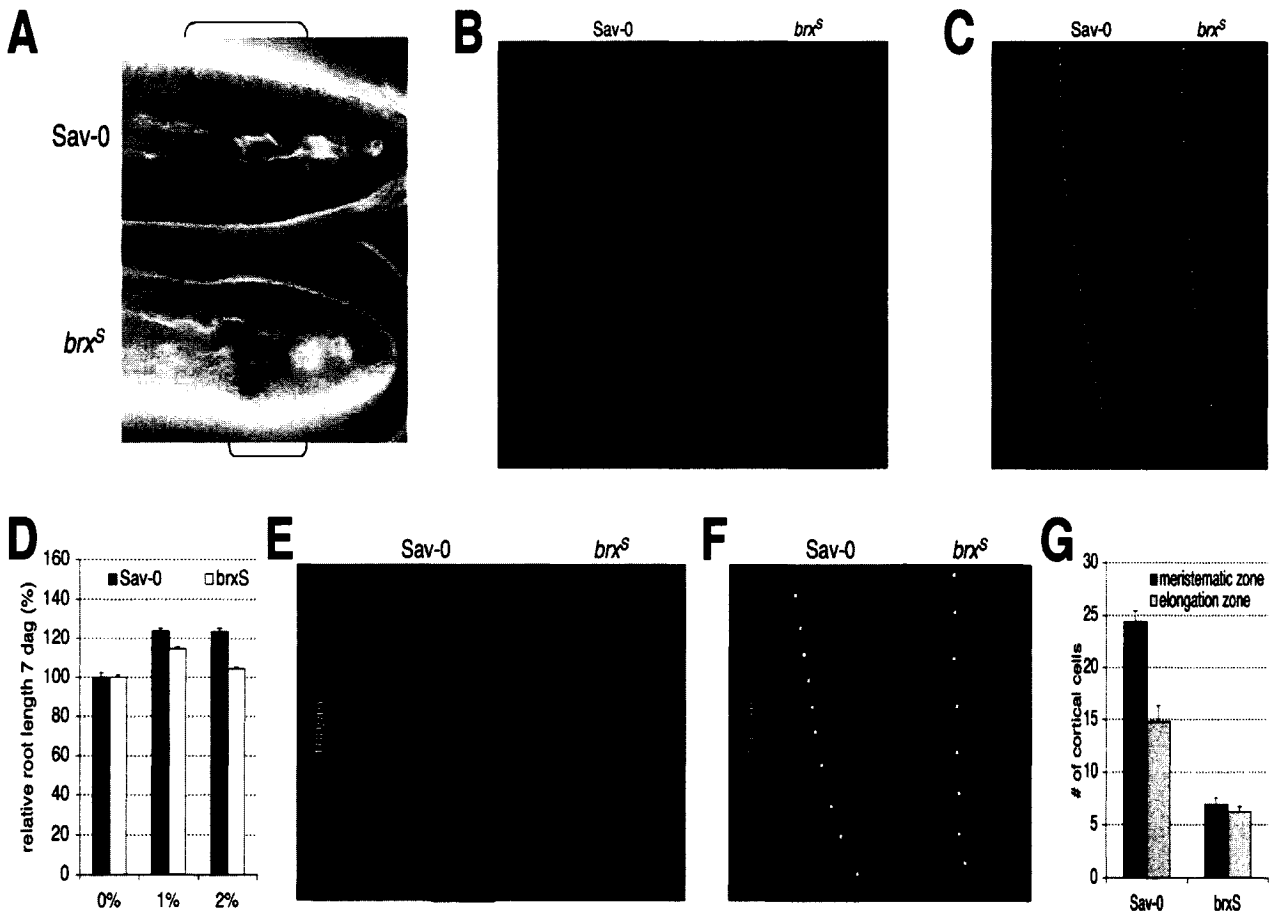
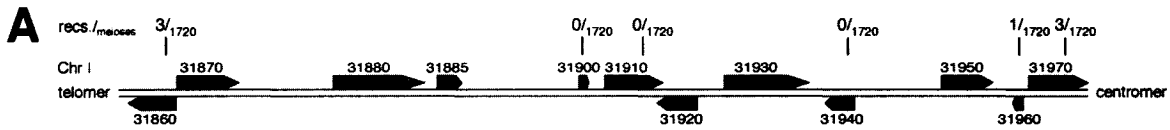

A
Sav-0
brxS
B
Sav-0
brxS
C
Sav-0
brxS
D
relative root length 7 dag (%)
Sav-0
brxS
0%
1%
2%
E
Sav-0
brxS
F
Sav-0
brxS
G
# of cortical cells
meristematic zone
elongation zone
Sav-0
brxS

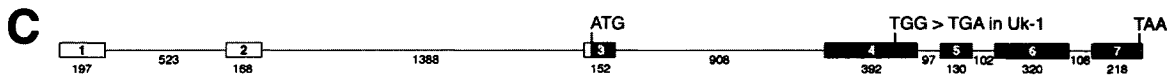
Figure 3.3 Root meristem morphology and size in the primary roots of Sav-0 and *brx*^{S} seedlings. *(A)* Activity of a *CYCB1;1::CYCB1;1:GUS* reporter gene in the meristems of *brx*^{S} and Sav-0 seedlings, detected by GUS staining. Brackets indicate the meristematic region as defined by the GUS signal. *(B)* Confocal images of root meristems grown on 0.5 x MS medium containing 0.5% sucrose. *(C)* Magnification of cortical cell files (marked by white dots), starting from the initial cell, shown in *B*. *(D)* Response of root growth of Sav-0 and *brx*^{S} seedlings to increasing amounts of sucrose (given in %) in the medium, scored 7 dag. n >=8. *(E)* Confocal images of root meristems grown on 0.5 x MS medium containing 2.0% sucrose. *(F)* Magnification of cortical cell files (marked by white dots), starting from the initial cell, shown in *E*. *(G)* Number of cells in cortical cell files of the root meristematic and elongation zones as defined in the text, grown on 0.5 x MS medium containing 2.0% sucrose and scored 6 dag. n >= 10. Error bars are standard error.

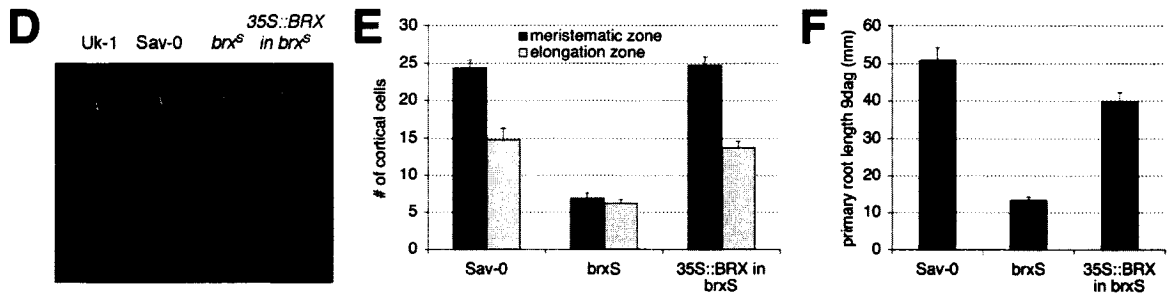


B

Sequence analysis Uk-1 vs. Col

	At1g31870	At1g31880	At1g31885	At1g31890	At1g31900	At1g31910	At1g31920	At1g31930	At1g31940	At1g31950	At1g31960
T-DNA insertion line (Col): short root phenotype?	n.a.	n.a.	n.a.	NO	n.a.	NO	NO	NO	n.a.	NO	n.a.
conserved substitutions?	1	1	10	n.d.	NO	11	2	38	NO	n.d.	start codon is missing; ORF contains frameshift due to 2bp deletion; both features are also present in Sav-0
non-conserved substitutions?	2	0	1	n.d.	NO	3	2	7	NO	n.d.	
insertions?	NO	NO	NO	n.d.	NO	NO	72bp in 3' UTR (also present in Sav-0)	NO	NO	n.d.	
deletions?	3bp (1 amino acid)	NO	NO	n.d.	NO	NO	5bp in 3' UTR (also present in Sav-0)	NO	NO	n.d.	
premature STOP?	NO	YES	NO	n.d.	NO	NO	NO	NO	NO	n.d.	
Transgenic rescue?	n.d.	YES (35S::cDNA)	n.d.	n.d.	n.d.	n.d.	NO	NO	n.d.	n.d.	n.d.





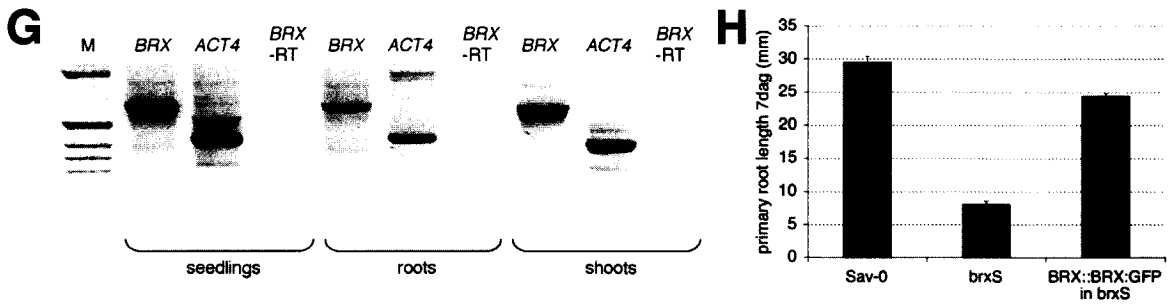


Figure 3.4. Positional cloning of the *BRX* gene. *(A)* Schematic representation of recombination mapping of the *BRX* locus to an approximately 45 kb interval on chromosome I of *Arabidopsis*. Solid bars indicate predicted genes, numbers indicate their unicode. *(B)* Summary of the genetic and sequence analysis of the genes in the region of interest. n.a. = not available; n.d. = not determined; *(C)* Schematic presentation of the intron-exon structure of the *BRX* gene. Boxes represent exons, lines represent introns, their sizes are given in nucleotides below. The shaded boxes indicate the open reading frame. The position of the mutation resulting in a premature stop codon in the Uk-1 accession is shown. *(D)* Representative Uk-1, Sav-0 and brx^S seedlings, and brx^S seedlings carrying a *35S::BRX* transgene, 9 dag grown in constant light on 0.5 x MS medium containing 0.3% sucrose. *(E)* Number of cells in cortical cell files of the root meristematic and elongation zones as defined in the text, grown on 0.5 x MS medium containing 2.0% sucrose and scored 6 dag. n >= 10. *(F)* Primary root length of seedlings grown in constant light on 0.5 x MS medium containing 1.0% sucrose, 9 dag. n >= 15. *(G)* RT-PCR of *BRX* and the control gene actin4 (*ACT4*) from RNA isolated from different sources. Control reactions for *BRX* in which the reverse transcriptase was lacking (*BRX*-RT) are shown as well. M = DNA size marker. *(H)* Primary root length of seedlings grown in constant light on 0.5 x MS medium containing 1.0% sucrose, 7 dag. n >= 15. Error bars are standard error.

A

chromosome	locus	likelihood ratio statistic	P value	% of variance explained	chromosome	locus	likelihood ratio statistic	P value	% of variance explained
I	JV18/19	47.8	0.00	21	III	nga162	0.0	0.97	0
I	ZFPG	69.0	0.00	28	III	T16K5	1.2	0.26	1
I	At1g31880	327.2	0.00	80	III	ciw21	2.3	0.12	1
I	AtGENEA	2.0	0.16	1	III	nga6	0.5	0.49	0
I	nga111	7.3	0.00	4	IV	nga8	0.5	0.47	0
II	nga1145	1.6	0.20	1	IV	nga1139	0.0	0.92	0
II	ciw3	0.6	0.42	0	V	nga225	1.4	0.22	1
II	nga1126	0.0	0.94	0	V	PHYC	0.0	0.90	0
II	nga361	0.3	0.57	0	V	nga129	2.3	0.13	1

B

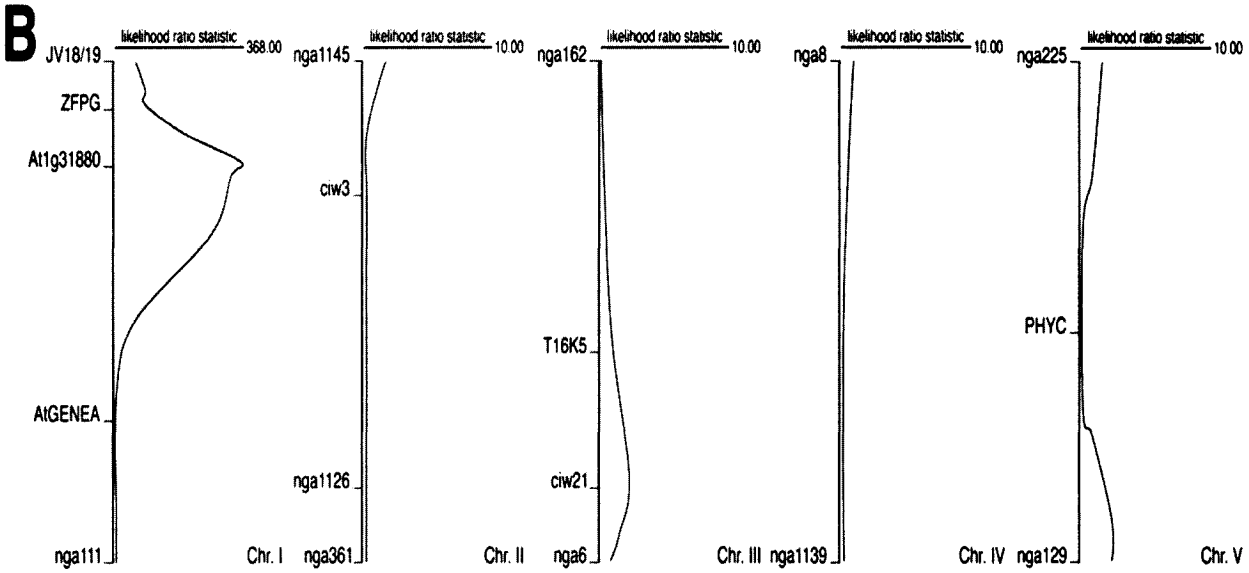


Figure 3.5 Quantitative trait locus analysis of the RIL population. *(A)* Results from the regression analysis of the data presented in Table 1 plus the genotypes at the *BRX* (At1g31880) locus, with respect to primary root length. *(B)* Graphical presentation of the data shown in *A)*. The different chromosomes and the relative position of the scored simple sequence length polymorphism markers are indicated, along with the likelihood statistics for the positions of QTLs.

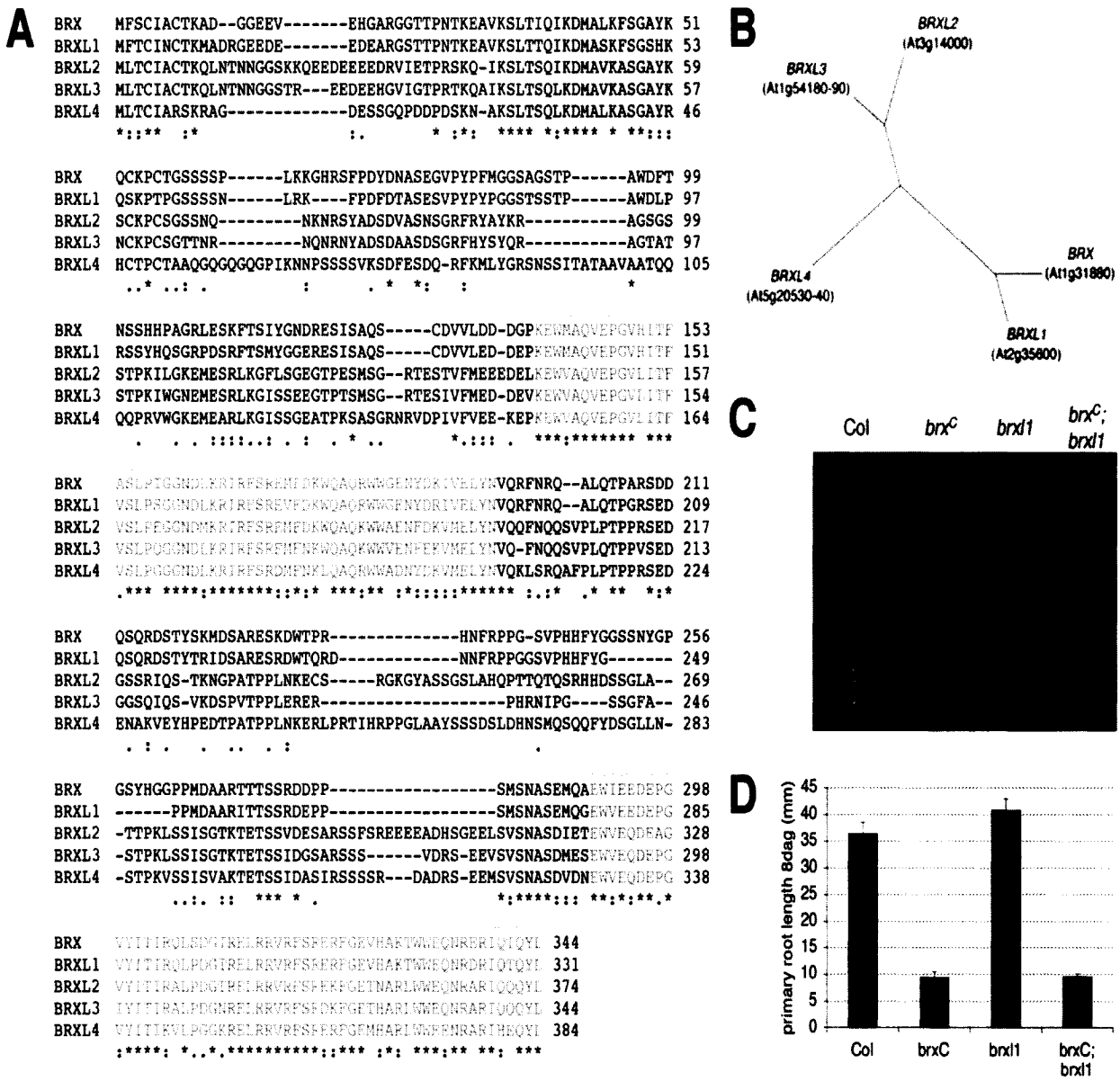
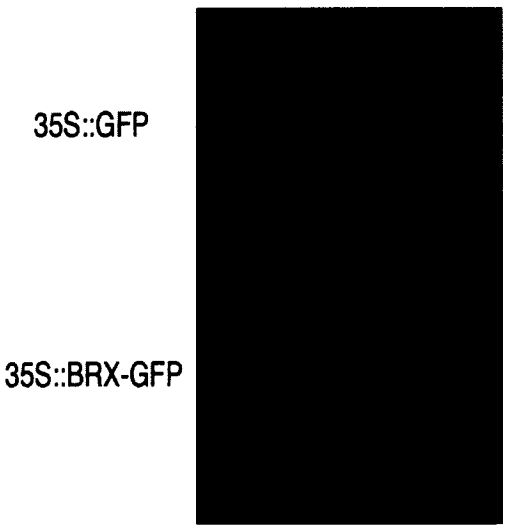

A
BRX MFSCIACTKAD--GGEEV-------EHGARGGTTPNTKEAVKSLTIQIKDMALKFSGAYK 51
BRXL1 MFTCINCTKMADRGEEDE-------EDEARGSTTPNTKEAVKSLTTQIKDMASKFSGSHK 53
BRXL2 MLTCIACTKQLNTNNGGSKKQEEDEEEEDRVIETPRSKQ-IKSLTSQIKDMAVKASGAYK 59
BRXL3 MLTCIACTKQLNTNNGGSTR---EEDEEHGVIGTPRTKQAIKSLTSQLKDMAVKASGAYK 57
BRXL4 MLTCIARSKRAG-------------DESSGQPDDPDSKN-AKSLTSQLKDMALKASGAYR 46
BRX QCKPCTGSSSSP------LKKGHRSFPDYDNASEGVPYPFMGGSAGSTP------AWDFT 99
BRXL1 QSKPTPGSSSSN------LRK----FPDFDTASESVPYPYPGGSTSSTP------AWDLP 97
BRXL2 SCKPCSGSSNQ---------NKNRSYADSDVASNSGRFRYAYKR----------AGSGS 99
BRXL3 NCKPCSGTTNR---------NQNRNYADSDAASDSGRFHYSYQR----------AGTAT 97
BRXL4 HCTPCTAAQGQGQGQGPIKNNPSSSSVKSDFESDQ-RFKMLYGRSNSSITATAAVAATQQ 105
BRX NSSHHPAGRLESKFTSIYGNDRESISAQS-----CDVVLDD-DGP 153
BRXL1 RSSYHQSGRPDSRFTSMYGGERESISAQS-----CDVVLED-DEP 151
BRXL2 STPKILGKEMESRLKGFLSGEGTPESMSG--RTESTVFMEEEDEL 157
BRXL3 STPKIWGNEMESRLKGISSEEGTPTSMSG--RTESIVFMED-DEV 154
BRXL4 QQPRVWGKEMEARLKGISSGEATPKSASGRNRVDPIVFVEE-KEP 164
BRX VQRFNRQ--ALQTPARSDD 211
BRXL1 VQRFNRQ--ALQTPGRSED 209
BRXL2 VQQFNQQSVPLPTPPRSED 217
BRXL3 VQ-FNQQSVPLQTPPVSED 213
BRXL4 VQKLSRQAFPLPTPPRSED 224
BRX QSQRDSTYSKMDSARESKDWTPR-------------HNFRPPG-SVPHHFYGGSSNYGP 256
BRXL1 QSQRDSTYTRIDSARESRDWTQRD-------------NNFRPPGGSVPHHFYG------- 249
BRXL2 GSSRIQS-TKNGPATPPLNKECS-----RGKGYASSGSLAHQPTTQTQSRHHDSSGLA-- 269
BRXL3 GGSQIQS-VKDSPVTPPLERER-------------------PHRNIPG----SSGFA-- 246
BRXL4 ENAKVEYHPEDTPATPPLNKERLPRTIHRPPGLAAYSSSDSLDHNSMQSQQFYDSGLLN- 283
BRX GSYHGGPPMDAARTTTSSRDDPP-----------------SMSNASEMQA 298
BRXL1 ------PPMDAARITTSSRDEPP-----------------SMSNASEMQG 285
BRXL2 -TTPKLSSISGTKTETSSVDESARSSFSREEEEADHSGEELSVSNASDIET 328
BRXL3 -STPKLSSISGTKTETSSIDGSARSSS------VDRS-EEVSVSNASDMES 298
BRXL4 -STPKVSSISVAKTETSSIDASIRSSSSR---DADRS-EEMSVSNASDVDN 338
BRX 344
BRXL1 331
BRXL2 374
BRXL3 344
BRXL4 384
B
BRXL2
(At3g14000)
BRXL3
(At1g54180-90)
BRX
(At1g31880)
BRXL4
(At5g20530-40)
BRXL1
(At2g35600)
C
Col
brxC
brxl1
brxC; brxl1
D
primary root length 8dag (mm)
45
40
35
30
25
20
15
10
5
0
Col
brxC
brxl1
brxC; brxl1

Figure 3.6 The *BRX* family of genes. *(A)* Sequence alignment of the predicted sequences of BRX family of proteins. Asterisks indicate identity, two dots indicate conserved substitutions, one dot indicates substitutions with similar basic characteristics. A highly conserved domain, occurring twice in each protein, is highlighted. *(B)* Unrooted phylogenetic tree based on the amino acid sequences shown in *A*. *(C)* Analysis of brx^{C};*brxl1* double mutants. Representative seedlings of the indicated genotypes, grown in constant light on 0.5 x MS medium containing 1.0% sucrose, are shown at 8 dag. *(D)* Primary root length of seedlings grown in constant light on 0.5 x MS medium containing 1.0% sucrose, scored 8 dag. n >= 9. Error bars are standard error.

A

```
         10        20        30        40        50        60        70        80
MFSCIACTKADGGEEVEHGARGGTTPNTKEAVKSLTIQIKDMALKFSGAYKQCKPCTGSSSSPLKKGHRSFPDYDNASEGVPYPFMGG
.........................HHHHHHHHHHHHHHHHHH.....................................
90        100       110       120       130       140       150       160       170
SAGSTPAWDFTNSSHHPAGRLESKFTSIYGNDRESISAQSCDVVLDDDGPKEWMAQVEPGVHITFASLPTGGNDLKRIRFSREMFDKW
.................................................EEEEEE....EEEEEEE.......EEEEE..HHHHHHH
   180       190       200       210       220       230       240       250       260
QAQRWWGENYDKIVELYNVQRFNRQALQTPARSDDQSQRDSTYSKMDSARESKDWTPRHNFRPPGSVPHHFYGGSSNYGPGSYHGGPP
HHHHHHHHHHHHH.....................................................................
    270       280       290       300       310       320       330       340
MDAARTTTSSRDDPPSMSNASEMQAEWIEEDEPGVYITIRQLSDGTRELRRVRFSRERFGEVHAKTWWEQNRERIQTQYL
.....................EEEEEE....EEEEEEE.....EEEEEEEE.HHHHHHHHHHHHHHHHHHHHHHHH..
```

B



C

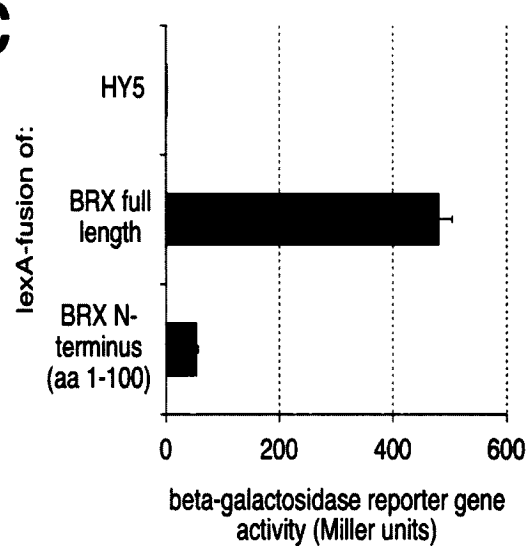


Figure 3.7 Analysis of the BRX protein. *(A)* Secondary structure prediction for the BRX protein. The domains highly conserved between BRX-like proteins are indicated in green. Regions with a high probability of forming alpha-helical structures are indicated by H, regions with high probability of forming extended beta-sheets by E. *(B)* Nuclear localization of BRX. Fluorescent microscopy of transiently transformed epidermal onion cells expressing a BRX-GFP fusion protein or GFP alone. *(C)* Reporter gene activity in yeast expressing the indicated lexA fusion proteins. n = 8. Error bars are standard error.

ADDENDUM TO CHAPTER 3

Attempts to demonstrate the DNA binding capacity of BRX were performed by CFM, via SELEX experiments. These however were unsuccessful and the additional highly unstable nature of the BRX protein (Mouchel et al., 2006) made further analyses difficult. Sequence examination of BRX and BRXL proteins did not reveal any previously characterized DNA or protein binding motifs. In addition to this, a known nuclear localization sequence could not be determined for these proteins. However, the observed localization of the 35S::BRX-GFP fusion protein to the nucleus of onion epidermal cells, indicated to us that the nuclear localization signal may simply be a novel, uncharacterized one.

PRELUDE TO CHAPTER 4

Bioinformatic searches and analyses revealed that *BRX* is a member of a small gene family in *Arabidopsis thaliana*. From our initial work, it was evident that *BRX* appeared to also be the only member with an assayable function in primary root development.

Preliminary support for this was demonstrated by the fact that single mutants of *BRXL 1, 2* and *3* all had normal length primary roots. Furthermore, double mutants of *brxl* genes with *brx* do not result in any enhancement of the short root phenotype of *brx* single mutant plants. The exception at this point is that of *BRXL4*, for which a reliable T-DNA insertion line was not verified.

Therefore, despite apparently high sequence similarity, significant divergence in function appears to have occurred to prevent the occurrence of complete or partial genetic redundancy amongst *brx* and *brxl* genes in *Arabidopsis*. This might be in part attributed to the absence or alterations of as yet undefined functional domains in the proteins. We have additionally identified common regions of the protein that are highly conserved in the gene family. These domains are predicted to form alpha-helical structures and could possibly mediate protein-protein interactions.

To further investigate the biochemical role and *in planta* activity of *BRX*, we decided to examine more closely the importance and significance of the highly conserved BRX repeats to BRX function.

Chapter 4

Characterization of the Plant-Specific *BREVIS RADIX* Gene Family Reveals Limited Genetic Redundancy Despite High Sequence Conservation

This chapter represents results published in a research article in the scientific journal *Plant Physiology* in 2006. Here I present a comprehensive analysis of the *BRX* gene family of *Arabidopsis* and show that despite the high levels of sequence conservation amongst *BRX* gene family members, functional redundancy between *BRX* and *BRXL* genes in the root is lacking. I also show that the highly conserved BRX repeat domain mediates interactions between gene family members, and is required for *BRX* function *in planta*.

Characterization of the plant-specific *BRX* gene family reveals limited genetic redundancy despite high sequence conservation.

Georgette C. Briggs, Céline F. Mouchel and Christian S. Hardtke*

Department of Plant Molecular Biology, University of Lausanne, Biophore Building, CH–1015 Lausanne, Switzerland, and Biology Department, McGill University, 1205 Dr. Penfield Avenue, Montréal, Québec H3A 1B1, CanadaThis work was funded by an NSERC Strategic Projects Grant and operating grant 3100AO-107631/1 from the Swiss National Science Foundation.

Abstract

To date, the function of most genes in the *Arabidopsis* genome is unknown. Here we present the first analysis of the novel, plant-specific *BRX* gene family. *BRX* has been identified as a modulator of root growth through a naturally occurring loss-of-function allele. The biochemical function of BRX is enigmatic, however several domains in BRX are conserved in the proteins encoded by the related *BRX-like (BRXL)* genes. The similarity between *Arabidopsis* BRXL proteins within these domains ranges from 84-93%. Nevertheless, analysis of *brx brx-like* multiple mutants indicates that functional redundancy of *BRXL*s is limited. This results mainly from differences in protein activity, as demonstrated by assaying the propensity of constitutively expressed *BRXL* cDNAs to rescue the *brx* phenotype. Among the genes tested, only *BRXL1* can replace *BRX* in this assay. Nevertheless, *BRXL1* does not act redundantly with *BRX in vivo*, presumably because it is expressed at much lower level than *BRX*. BRX and BRXL1 similarity is most pronounced in a characteristic tandem repeat domain, which we named "BRX domain". One copy of this domain is also present in the PRAF-like family proteins. The BRX domain mediates homo- and heterotypic interactions within and between the BRX and PRAF protein families in yeast, and therefore likely represents a novel protein-protein interaction domain. The importance of this domain for BRX activity *in planta* is underscored by our finding that expression of the C-terminal fragment of BRX, comprising the two BRX domains, is largely sufficient to rescue the *brx* phenotype.

Introduction

Genetic redundancy is a common phenomenon in both plants and animals (Pickett and Meeks-Wagner, 1995). Although occasionally observed between non-homologous genes (e.g. Weigel *et al.,* 1992; e.g. Shannon and Meeks-Wagner, 1993), it generally occurs between members of the same gene family. In *Arabidopsis thaliana*, chromosomal segments have been duplicated several times during evolution (Vision *et al.,* 2000; Simillion *et al.,* 2002; Blanc *et al.,* 2003), contributing to the creation and extension of gene families. Only a fraction of those gene families have been experimentally assigned functions (Somerville and Dangl, 2000), mainly by analysis of single loss-of-function mutants. However, this approach is limited, since the majority of gene knock-outs do not result in phenotypes (Bouche and Bouchez, 2001), presumably due to genetic redundancy. Thus, it has been suggested that more research be devoted to study the evolutionary and functional divergence of gene families (Somerville and Dangl, 2000; Bouche and Bouchez, 2001; Hirschi, 2003). This is particularly important for characterizing the ~45% of *Arabidopsis* genes that can neither be assigned biological nor biochemical function, because of a lack of homology to functionally defined genes or protein domains (Somerville and Dangl, 2000). With this study we contribute to the exploration of plant gene families by presenting the first comprehensive analysis of the novel, plant-specific *BRX* gene family.

We previously identified a regulator of root growth, the *BREVIS RADIX (BRX)* gene (Mouchel *et al.,* 2004), through a naturally occurring allele in the accession Uk-1. Initially, *BRX* was annotated incorrectly, because of its low expression and particular intron-exon structure. The same was true for *BRX* homologs, with exception of one gene, for which a full-length cDNA clone was available. Based on this clone and genomic homology searches, the correct intron-exon structure of *BRX* was determined and three more *BRX* homologs were identified. Together, the

five genes, *BRX* and *BRX-like* (*BRXL*) *1-4*, constitute the *BRX* gene family. The respective proteins are highly similar and contain four highly conserved domains of unknown function.

In this study, we present a broader characterization of the *BRX* gene family in plants. We determine the functional overlap between *Arabidopsis BRX* family genes, and we assign a putative function to the conserved repeat domain, which is a prominent feature of BRX family proteins.

Results

BRXL genes form a highly conserved, plant-specific gene family

We previously isolated *BRX* (Mouchel *et al.,* 2004), founding member of the novel family of plant-specific *BRX-like* genes. *Arabidopsis BRXL* genes are transcribed at low levels (Meyers *et al.,* 2004), however expressed sequence tags (ESTs) have by now been found for all five family members, with full length clones confirming our annotations of four genes. The open reading frames (ORFs) of all *BRX* family genes are composed of 5 exons, whose lengths are conserved between genes (Fig. 4.1A). The encoded proteins are 35-40kDa in size and show extensive conservation of domain structure and primary sequence. The overall similarity between *Arabidopsis* BRXL proteins is at least 50% and can be as high as 81%. Thus, BRXL proteins are remarkably well conserved.

BRXL genes are found in all higher plants for which data are available, but not in unicellular organisms and animals. Based on EST and genomic searches, we defined the full set of *BRXL* genes in the entirely sequenced plant genomes of poplar and rice (Fig. 4.1A). In both species, as in *Arabidopsis*, 5 *BRXL* genes can be found, which we named *PtBRXL1-5* and *OsBRXL1-5* in poplar and rice, respectively, ordered according to their similarity to *Arabidopsis BRX (AtBRX)*. ESTs exist for two poplar and four rice genes (Tab. 4.1). The intron-exon structure of

PtBRXL and *OsBRXL* genes is mostly similar to *AtBRXL* genes (Fig. 4.1A). An exception is *OsBRXL5*, which appears to be the result of transposition of a *BRXL* cDNA and has acquired numerous non-synonymous mutations in absolutely conserved amino acid (aa) residues (see below). Except OsBRXL5, all rice and poplar BRXL proteins are well conserved. Compared to AtBRX, overall aa similarity ranges from 55% to 76% in poplar and 39% to 56% in rice. In summary, *BRXL* genes are highly conserved both in di- and monocotyledons, indicating their possible orthologous origin.

Domain structure of BRXL proteins

Four regions of high conservation can be distinguished in BRX family proteins. So far however, no function has been described for these domains. The homology among BRX family proteins within and between species is especially conserved in these regions, with aa similarity to AtBRX ranging from 84 to 93% in *Arabidopsis*, 86 to 96% in poplar and 84 to 87% in rice (OsBRXL5 excluded). At the N-terminus, short stretches of ~10 and ~25 aas are conserved (Fig. 4.1B). In most BRX family proteins, they contain conserved cysteines, whose spacing is indicative of a potential zinc-binding motif. The middle region of BRX family proteins contains a highly conserved domain of ~55 aas, with 33 invariant positions (Fig. 4.1C). Following a variable spacer of ~100-150 aas, another highly conserved domain of ~55 aas is present, with 27 invariant positions (Fig. 4.1D). This domain is homologous to the middle domain (56% amino acid similarity). Therefore, the two domains constitute a novel type of tandem repeat, which is the main characteristic of BRX family proteins. Thus we named the repeat domain the "BRX domain". Aa similarity between any two BRX domains is at least 84% for the N-terminal domains (Fig. 4.1C), and 81% for the C-terminal ones (Fig. 4.1D). This indicates that selection pressure maintains the structural integrity of BRX domains, since they are

nearly invariant in proteins from distantly related species that can be clearly separated in a phylogenetic tree (Fig. 4.2).

Analysis of multiple mutants for Arabidopsis BRXL genes

The high level of conservation within the *BRX* gene family could mean that these genes act redundantly, as frequently observed in other gene families (e.g. Kempin *et al.,* 1995; Holm *et al.,* 2002; e.g. Hardtke *et al.,* 2004). Redundancy in a developmental context often requires overlapping domains of gene activity. Thus, we investigated whether *BRXL* genes are expressed in the root by quantitative real time PCR (qPCR). In these assays, expression of all *BRXLs* was detected in seedling roots (see below), suggesting that they might indeed be (partially) redundant with *BRX* in root growth. To clarify this issue, we analyzed different combinations of *brxl* mutants.

Many putative mutants are available for *BRX-like* genes, however, the same T-DNA insertions are often annotated for in several genes, because of their high sequence similarity. We analyzed the available SALK lines (Alonso *et al.,* 2003) by PCR and Southern blot analysis and confirmed T-DNA insertions in *BRXL1, 2* and *3* (Fig. 4.3A) (Mouchel *et al.,* 2004). No insertions were confirmed for *BRXL4*. SALK line 038885 carries a T-DNA insert in an intron of *BRXL1*. The T-DNA in line 032250 is located a few nucleotides 5' to the transcription start of *BRXL2*. Finally, line 017909 carries a T-DNA in an exon of *BRXL3*. Wild type transcript was not detectable for any of the mutant loci (G.C.B., data not shown), suggesting that the mutants represent partial or total loss-of-function alleles. *brxl1*, *brxl2* and *brxl3* did not show any conspicuous root system defect, suggesting that, unlike *BRX*, these genes have no role in primary root growth. However, potential synergistic action of *BRX* and *BRXL* genes in the root might only become evident in a *brx* background. Therefore, we generated multiple mutants using the T-DNA mutants and

the original *brx* allele from Uk-1, introgressed into a Col background (brx^C) (Mouchel *et al.*, 2004).

As reported previously (Mouchel *et al.*, 2004), double mutants between brx^C and *brxl1, 2* or *3* did not show an enhanced brx^C root phenotype. The same is true for brx^C *brxl1 brxl2* triple mutants and brx^C *brxl1 brxl2 brxl3* quadruple mutants (Fig. 4.3B). Rather, all multiple mutant lines had slightly longer roots than brx^C, likely because of insufficient introgression of the *brx* mutation into Col. Additional root system QTLs from the Uk-1 background retained in brx^C (Mouchel *et al.*, 2004) should have been largely lost in subsequent crosses for the creation of double and multiple mutants, leading to slightly longer roots. Irrespective of background influence, the data suggest that *brxl1*, *brxl2* and *brxl3* do not enhance the brx^C root phenotype.

Finally, because all *BRX* family genes are also expressed in all the shoot organs (data not shown), we also inspected the shoot morphology of the different mutants throughout their life cycle. However, we did not detect any apparent phenotypes.

BRXL1 has BRX activity

A lack of redundancy between *BRX* and *BRXL* genes could be due to non-overlapping expression patterns or differences in protein activity. To determine whether differential expression patterns could play a role, we expressed *BRX* and *BRXL1, 2* and *4* under control of the *35S* promoter, which confers strong expression in root tissues (Benfey *et al.*, 1990, 1990), in brx^S plants and analyzed their root growth. *35S:BRX* rescued the root length to ~78% of wild-type (Sav-0) (Fig. 3C), consistent with the QTL nature of the *BRX* locus in the brx^S background (Mouchel *et al.*, 2004). Strikingly, rescue of the same magnitude was obtained with *35S:BRXL1* (Fig. 3D). By contrast, over-expression of *BRXL2* (Fig. 4.3E) or *BRXL4* (Fig. 4.3F) was not able to rescue brx^S root length. Thus, *BRXL1*, but not *BRXL2* or *BRXL4*, can replace *BRX* when expressed

constitutively. Therefore, despite their high degree of similarity, BRX family proteins have functionally diversified.

The original brx allele is a loss-of-function

One explanation for the lack of redundancy between *BRX* family genes in root development could be a dosage-dependent dominant negative effect of the *brx* allele in a homozygous state, similar for instance to the recessive interfering alleles of *SLEEPY* (Strader *et al.,* 2004). Alternatively, the *brx* allele might simply be hypomorphic or null. If *brx* is null, this would facilitate discovery of possible redundancy. However, if it is hypomorphic, the residual activity could have masked the redundancy. Therefore, it is important to clarify the nature of the *brx* allele.

The *brx* allele carries a point mutation that creates an early stop codon at aa 141 (Mouchel *et al.,* 2004). Full length *BRX* mRNA can be detected in homozygous brx^{C} seedlings (Fig. 4.4A), indicating that truncated BRX comprising the N-terminal 140 amino acids could be produced from the mutant allele (Fig. 4.4B). To test whether this BRX^{N140} fragment has residual activity *in planta*, we raised its quantity in brx^{S} plants by expressing a respective cDNA fragment under control of the *35S* promoter. However, this did not significantly rescue root growth (Fig. 4.4C). Next, we tested whether BRX^{N140} could act in a dominant negative fashion by introducing the construct into the wild-type control Sav-0. No consistent, and quantitatively only small effects were observed (Fig. 4.4D), arguing against a dominant negative effect of the BRX^{N140} fragment. In summary, our results suggest that the *brx* allele is a loss-of-function and most likely null allele.

Expression levels of BRXL genes in the root

Lack of redundancy between *BRX* and *BRXL1 in planta* despite functional equivalence of the proteins could be due to non-overlapping expression patterns or differences in transcription level of the two genes.

In fact, both genes are expressed at very low levels (Meyers *et al.,* 2004; Mouchel *et al.,* 2004). Moreover, qPCR experiments show that *BRX* is expressed at a 9-10 times higher level than *BRXL1* in roots (Fig. 4.5A). Thus, *BRXL1* activity might be too low to compensate a loss of *BRX* activity *in vivo*. Notably, the qPCR experiments confirmed the very low expression levels of the two genes (Fig. 5B), even compared to the other *BRXLs*, which can be detected in the same samples at much higher levels (5-8 fold as compared to *BRX*). However, in general all *BRX* family genes are poorly expressed, as demonstrated by their relative expression as compared to a housekeeping gene, *eIF4* (Fig. 4.5C).

The BRX domain is a novel protein-protein interaction domain

Since our data indicate diverse functions of BRX family proteins, we sought to characterize them in more detail in order to define functionally relevant domains. Previously, secondary structure predictions identified alpha-helical regions, which are characteristic of DNA binding and protein-protein interaction domains, within the conserved domains (Mouchel *et al.,* 2004). Interestingly, many known protein-protein interaction domains mediate homodimerization (Fan *et al.,* 1997; Ulmasov *et al.,* 1999; Holm *et al.,* 2002). We tested whether the BRX domain mediates homodimerization using a yeast two hybrid assay. Full length BRX and C-terminally truncated versions display a high transactivation potential (Mouchel *et al.,* 2004) and could thus not be used as baits. However, a bait containing the BRX C-terminus, starting at aa 101, was stably expressed and did not result in significant background activation (Fig. 4.6A). We used this bait to test interactions with prey fusion proteins of BRX and BRXL1. Strikingly, highly significant interactions were observed for both BRX and BRXL1 (Fig. 4.6B), indicating that these proteins can homo- and heterodimerize. Further, a truncated prey protein containing only the first BRX domain of BRX interacted as well, suggesting that interaction is mediated by the BRX domain.

Interestingly, one copy of the BRX domain is also present in the PRAF protein family of *Arabidopsis*, although this has not been noticed so far (van Leeuwen *et al.,* 2004). Thus, we also tested interaction with a prey construct that contained the C-terminus (aas 857 to 1104) of PRAF1 (At1g76950) (Jensen *et al.,* 2001). This fragment includes the BRX domain but otherwise shows no homology to BRX. Again, highly significant interaction was observed (Fig. 4.6B), indicating that the BRX domain is necessary and sufficient for this interaction. Thus, our results suggest that the BRX domain is a novel protein-protein interaction domain, which likely mediates homo- and heterodimerization within and/or between the BRXL and PRAF-like protein families.

The BRX domains are largely sufficient for BRX activity

The early stop codon in the *brx* loss-of-function allele is located at aa 141, immediately in front of the first BRX domain, suggesting that the BRX domains are absolutely required for BRX function. To test whether this is indeed the case, we expressed transgenes of BRX fragments under control of the *35S* promoter in a brx^S background, and assayed their ability to normalize root growth. As a control, the BRX^{N140} transgene was assayed in parallel and again had no significant effect on brx^S root length. By contrast, expression of the BRX N-terminus including the first BRX domain (*35S:*BRX^{N205}) partially rescued brx^S root length, and this rescue was highly significant (p value < 0.001) (Fig. 6C). On average, root length was restored to ~49% (±1.1%) of the wild-type control, as compared to a value of ~34% for the brx^S background line. Strikingly, expression of the C-terminal two thirds of BRX including both BRX domains (*35S:*BRX^{C244}) was even more effective, restoring root length to ~63% (±4.2%) of the wild-type control. Notably, in a few lines root growth was restored to the level of full rescue (as observed with full length *BRX*, ~78% (±3.4%) of control, see above). Thus, our data suggest a minor role for the N-

terminus in BRX activity and support the notion that the presence of two BRX domains is of critical importance for BRX function *in planta*.

Discussion

Genetic redundancy in plant development

Rearrangements of genetic material during species evolution can result in gene duplication, which, if occurring repeatedly in a phylogeny, leads to the creation of gene families. Genome analyses indicate that this has been the case in *Arabidopsis thaliana* (Vision *et al.,* 2000; Simillion *et al.,* 2002; Blanc *et al.,* 2003). In fact, all *BRX* family genes, except *BRXL4*, are located on duplicated chromosome segments, suggesting that they are true paralogs. In principle, gene duplication creates functionally identical copies, which should be fully redundant. However, positive selection pressure on one of the copies can decrease, thus providing a template for the evolution of a gene with novel function. In fact, models of evolutionary selection argue against the maintenance of full genetic redundancy (Tautz, 1992; Weintraub, 1993). Indeed, partial redundancy is often observed among gene family members (e.g. Holm *et al.,* 2002; e.g. Hardtke *et al.,* 2004), indicating that duplicated genes can evolve new, mutually exclusive roles while preserving a shared set of functions. Interestingly, shared functions often become unequally distributed between duplicated genes, ultimately allowing one of them to predominantly perform new functions and eventually leading to diversification of protein activity (e.g. Hardtke *et al.,* 2004). This might have happened in the *BRX* gene family.

Genetic redundancy in Arabidopsis BRXL genes

In the context of redundancy, it was important to clarify the nature of the so far only available *brx* allele (Mouchel *et al.,* 2004). Our finding that this allele likely is a loss-of-function excludes the possibility of full

genetic redundancy between *BRX* and *BRXL* genes. Further, we did not observe root phenotypes in *brxl1*, *brxl2* and *brxl3* mutants. Since even quadruple *brx brxl1 brxl2 brxl3* mutants do not display an enhanced *brx* phenotype, these *BRXL* genes likely do not play a role in modulating root growth. This should also be true for *BRXL4*, since it cannot replace *BRX* when expressed constitutively (see below). Whether *BRX* family genes have a role in shoot development remains to be determined. Although the genes do not act redundantly in the root, this might be different in the shoot. However, no shoot phenotypes were observed in the quadruple mutant. Thus, a so far unavailable quintuple mutant of all five genes would be necessary to clarify this issue.

We also employed a gain-of-function approach to compare *BRXL* genes, by assaying their ability to rescue the *brx* phenotype. We show that *BRXL1*, but not *BRXL2* or *BRXL4*, when expressed under control of the *35S* promoter, can rescue as well as *BRX* itself. Thus, *BRXL1* in principle possesses *BRX* activity, suggesting that a lack of redundancy between the genes results from insufficient *BRXL1* activity in tissues where *BRX* is required. Therefore, *BRX* and *BRXL1* must have diverged due to the evolution of differential expression, rather than differential protein activity. In light of our qPCR results, a likely explanation for the lack of redundancy between *BRX* and *BRXL1 in planta* is the 8-9 fold lower level of *BRXL1* expression in roots. This level might not be able to saturate the threshold of required *BRX* activity. Alternatively, *BRX* and *BRXL1* expression patterns might be mutually exclusive. To address this issue, the *in situ* expression patterns of *BRX* and *BRXL1* will have to be determined, which is hampered by the very low expression level of these genes.

High degree of conservation of BRXL proteins

Our results demonstrate that BRX and BRXL1 have equivalent activity, which is lost in BRXL2 and BRXL4. This functional diversification contrasts with the high degree of structural and aa similarity between

BRXL proteins. Generally, one would expect novel protein functions to arise from mutations in functionally relevant domains. In the case of *BRXL* proteins, these are presumably the conserved domains found in *BRX* family proteins of all species investigated. Considerable positive selection acts on these domains, implying that they are essential for the function of *BRXL* proteins, and also that *BRX* family genes are important for plant development. Although the lack of apparent morphological phenotypes in investigated *brxl* mutants argues against the latter conclusion, one has to consider that plants have evolved mechanisms that are critical for survival in the wild, but not necessarily needed in a laboratory environment (e.g. Devlin *et al.,* 1998). Thus, important roles for *BRXL* genes might be revealed in future assays that monitor physiological and environmental responses. Whether functional diversity among BRX family proteins is due to aa substitutions in the conserved domains remains to be seen. Compared to *BRX* and *BRXL1*, the conserved domains of *BRXL2* and *BRXL4* contain only 8 non-similar substitutions, out of 143 aas covered by the domains. Thus, either these aas are critical for the specialization of domain function, or differential activity of *BRXL* proteins is due to features in their more variable, non-conserved regions.

Interactions mediated by the BRX domain

Our finding that the BRX domain likely is a novel protein-protein interaction domain is a first hint toward the biochemical function of *BRXL* proteins. The strength of interaction between BRX domains is in the range of the interactions among IAA and ARF proteins (Hardtke *et al.,* 2004), and could reflect rather transient interaction. Also, because of the high sequence similarity of BRX domains, it appears likely that in yeast interaction occurs between any two BRX domains, again similar to interactions among IAA and ARF proteins. Thus, whether *BRX* family proteins and PRAF family proteins interact *in vivo* remains to be determined. Nevertheless, the observation that the BRX domain also

occurs in PRAF family proteins is of particular interest. PRAF family proteins contain other conserved domains, such as the PH and FYVE domains, which are assumed to bind phosphoinositides and target proteins to the plasma membrane. *In vitro* phosphoinositide binding of PRAF proteins has been demonstrated (Jensen *et al.,* 2001), however, the *in vivo* relevance of this observation is not clear. In fact, most PH or FYVE domain containing proteins are not targeted to membranes and PRAF family proteins are thought to be nuclear localized (Drobak and Heras, 2002; Hayakawa *et al.,* 2004; Lemmon, 2004). PRAF-like proteins also contain RCC1 repeats, which are implicated in multiple cellular processes (Renault *et al.,* 1998). RCC1 repeats often provide guanine nucleotide exchange activity, which has also been demonstrated for PRAF1 (Jensen *et al.,* 2001). Clearly, the notion that BRX domain-mediated interaction between BRXL proteins and PRAF family proteins could be relevant *in planta* is an attractive working hypothesis. Analysis of single and multiple mutants in *PRAF-like* genes, combined with verification of BRXL-PRAF interaction *in vivo*, will enable us to determine whether this hypothesis is valid and might help us to elucidate the biochemical and cell biological activity of BRXL proteins.

Relevance of the BRX domains for BRX function

The BRX domains are the most conspicuous feature of *BRXL* proteins and therefore it is not surprising that they play a role in *BRX* function. Their importance is already evident from the fact that the Uk-1 *brx* allele could at best direct synthesis of a truncated protein lacking the BRX domains. Our finding that adding one BRX domain to the respective BRX^{N140} fragment results in partial rescue emphasizes however that both BRX domains are absolutely required for *BRX* activity. Nevertheless, it is surprising that transgenic expression of the BRX C-terminus including both BRX domains rescues the *brx* phenotype in many lines just as well as full length BRX. Therefore, the conserved N-terminal domains of *BRX*

family proteins might only have a minor functional role. The importance of the BRX domains notwithstanding, the specific activity of *BRX* and *BRXL1* as opposed to other *BRXL* proteins seems less likely to be localized in the BRX domains, since they are so highly conserved (see above). Rather, it might be the region between the BRX domains that differentiates those two proteins from the others. The entire C-terminus is quite conserved between *BRX* and *BRXL1*, but has diverged in the other BRXL proteins. Clearly, extensive domain swapping and site-directed mutagenesis experiments will be required to determine the functionally important residues within the *BRXL* proteins, shedding more light on the structural requirements of this novel, highly conserved protein domain of higher plants.

Materials and Methods

Bioinformatic analyses

ESTs and genomic sequences of *BRX-like* genes were identified using the tBLASTn search tool (www.ncbi.nlm.nih.gov/BLAST/) in the indices of The Institute for Genomic Research (www.tigr.org) and the poplar genome database (genome.jgi-psf.org/Poptr1/Poptr1.home.html) (versions as of February 22nd, 2005). *BRXL* genes of rice and poplar were further annotated and analyzed using splice site prediction programs and tools included in MacVector™ (Accelrys, v. 7.2.2).

Plant material and tissue culture

Seedlings were grown at 22°C under constant illumination on culture medium (0.5 x MS salts, 0.5 g/l MES, 0.9% agar, 1% sucrose, pH 5.8). Light intensity was ~90µE.

Nucleic acid isolation, RT-PCR and qPCR

Nucleic acids were prepared using RNeasy™ and DNeasy™ kits from Qiagen according to the manufacturer's instructions. RT-PCR reactions were performed according to standard procedures with Superscript II™ reverse transcriptase (Invitrogen). qPCR analysis was performed with a Stratagene MPx3000 instrument according to the Standard Curve Method (Rutledge and Cote, 2003) using SyberGreen mix (Stratagene) and the reference dye ROX. In all biological and technical replicates, two standard curves were done concurrently with unknown samples to minimize experimental and machine variance. Ct values obtained for each amplicon tested were plotted onto relevant standard curves to infer initial amounts (Rutledge and Cote, 2003).

Creation and analysis of transgenic plants

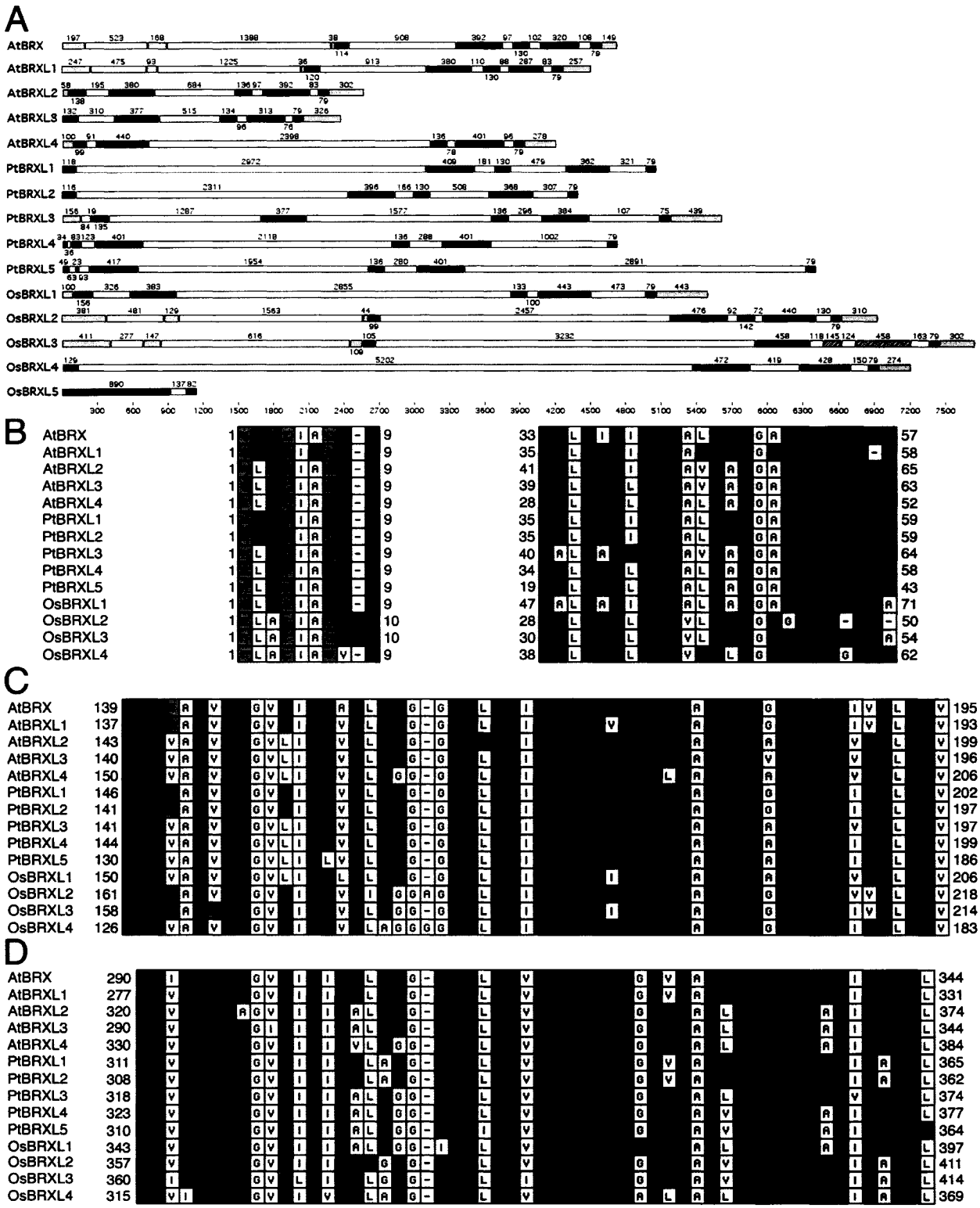
Plasmids were created by amplification of ORF fragments of *BRXL* genes from cDNA templates with Pfu polymerase (Fermentas), followed by cloning into binary vector pTCSH1 (Hardtke *et al.,* 2000). For truncated fragments, stop codons were introduced through the respective oligonucleotides. All constructs were verified by sequencing.

Binary constructs were transformed into brx^S plants via floral dip using Agrobacterium, and transgenic lines were selected by screening seed progeny for glufosinate ammonium resistance (15 mg/l) on medium containing 0.3% sucrose. Because rescue of the brx^S root phenotype by *35S:BRX* is not dosage-dependent (Mouchel *et al.,* 2004) and because of frequent problems with transgene silencing in subsequent generations, the T2 generation was chosen for analysis. Independent transgenic lines segregating single locus insertions were selected. A minimum of 20 seedlings per line was investigated. In rescue experiments, one quarter of the sample with root lengths in the range of the brx^S control was typically discounted to eliminate non-transgenic seedlings from the analyses.

Transgene (over)-expression was verified by RT-PCR, using 3'UTR-specific primers to differentiate transgenes from endogenous genes. To determine primary root length, seedlings were grown on vertical plates and scanned on a flat bed scanner to produce image files suitable for analysis in ImageJ software (v 1.3). Seedlings were analyzed 9 days after germination. To compare lines measured in separate experiments, control lines were grown for each experiment and relative root lengths were calculated with respect to wild type (Sav-0 or Col) as a 100% reference.

Yeast two hybrid interaction assays

BRX-derived baits were produced in vector pEG202. Prey plasmids were constructed by cloning cDNA fragments of *BRX*, *BRXL1* and *PRAF1* into vector pJG4-5. HY5 and COP1 control constructs have been described (Hardtke *et al.,* 2000). Plasmids were introduced into *S. cerevisiae* strain EGY48, together with reporter construct pSH18-34. Transformants were grown in liquid culture overnight on selective medium. Cultures were diluted in the morning and prey expression was induced in one sub-sample. Cultures were incubated for 6 more hours before lacZ activity was measured by standard liquid assay. A minimum of 8 independent transformants was assayed for each construct combination. Stable expression of all bait proteins was confirmed by Western blot analysis.


A
AtBRX
AtBRXL1
AtBRXL2
AtBRXL3
AtBRXL4
PtBRXL1
PtBRXL2
PtBRXL3
PtBRXL4
PtBRXL5
OsBRXL1
OsBRXL2
OsBRXL3
OsBRXL4
OsBRXL5
B
AtBRX
AtBRXL1
AtBRXL2
AtBRXL3
AtBRXL4
PtBRXL1
PtBRXL2
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PtBRXL5
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AtBRXL4
PtBRXL1
PtBRXL2
PtBRXL3
PtBRXL4
PtBRXL5
OsBRXL1
OsBRXL2
OsBRXL3
OsBRXL4

Figure 4.1 Structure of *BRX* family genes and proteins in *Arabidopsis*, poplar and rice. **(A)** Intron-exon structure of confirmed and predicted *BRXL* genes in *Arabidopsis thaliana (AtBRXL)*, *Populus trichocarpa (PtBRXL)* and *Oryza sativa (OsBRXL)*, drawn to scale. Numbers indicate the size of features in nucleotides. If available, 5' and 3' UTRs are included. Grey boxes: exon, untranslated; black boxes: exon, coding; open boxes: intron; Patterned boxes in *OsBRXL3* indicate predicted coding exons, which are however missing in the only available cDNA clone for this gene. **(B)** Aa alignment of conserved domains in the N-terminus of BRXL proteins. Numbers indicate aa position for the first and last residue shown. **(C)** As in (B), showing alignment of the 1st BRX repeat domain. **(D)** As in (B), showing alignment of the 2nd BRX repeat domain.

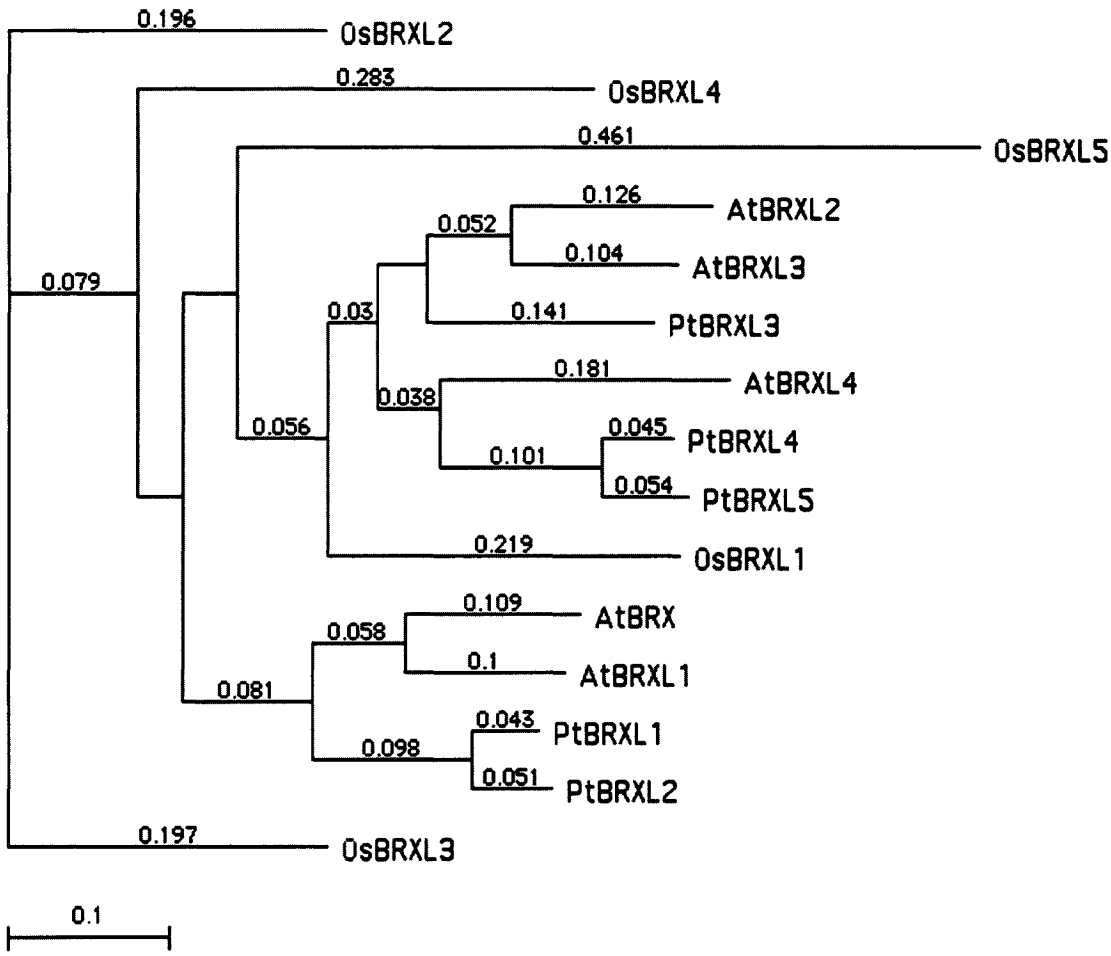


Figure 4.2 Phylogenetic tree of BRXL proteins. Unrooted phylogenetic tree diagram of BRXL proteins from *Arabidopsis*, poplar and rice (see Fig. 1), based on full length protein sequences.

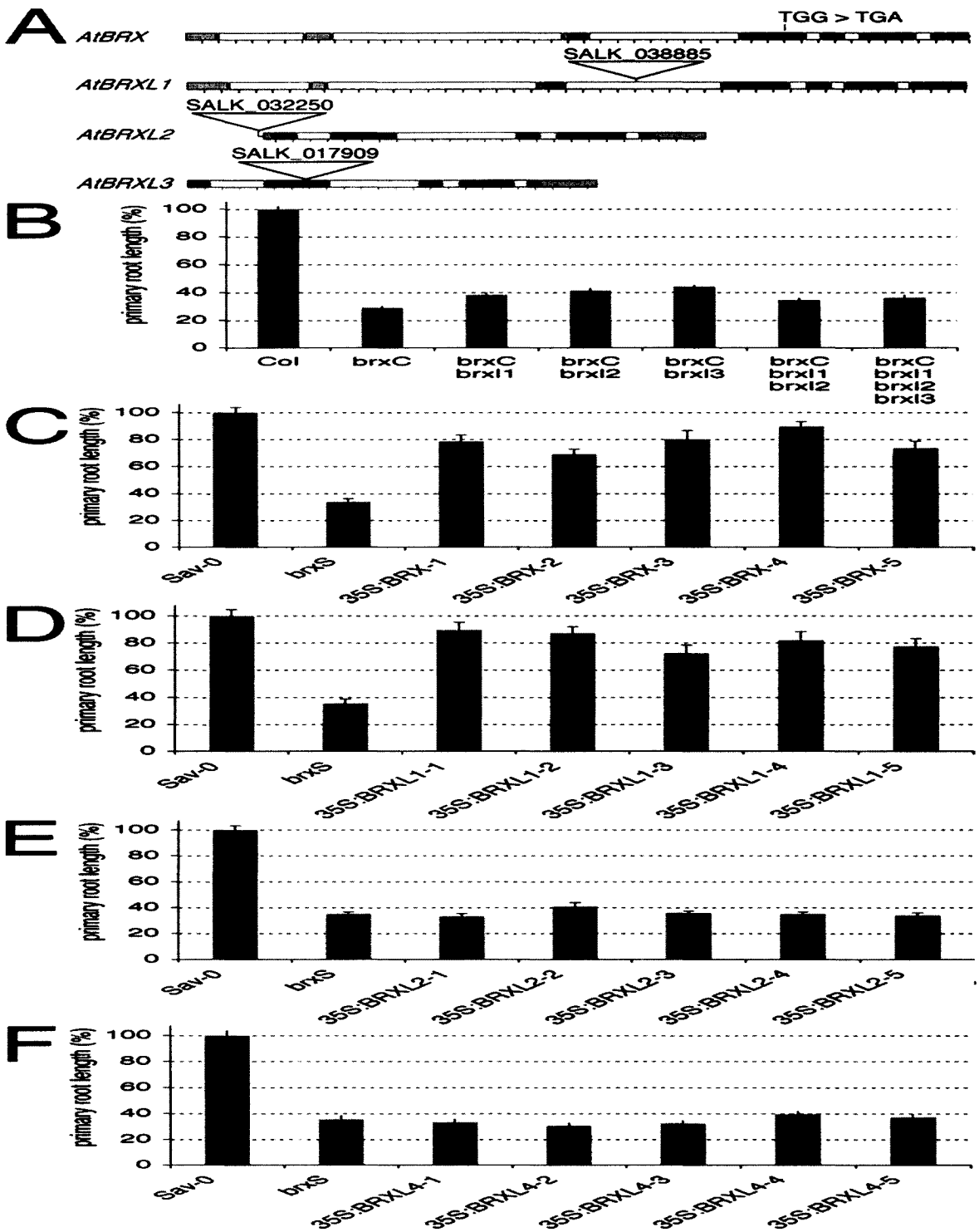

A
AtBRX
TGG > TGA
SALK_038885
AtBRXL1
SALK_032250
AtBRXL2
SALK_017909
AtBRXL3
B
primary root length (%)
100
80
60
40
20
0
Col
brxC
brxC brxl1
brxC brxl2
brxC brxl3
brxC brxl1 brxl2
brxC brxl1 brxl2 brxl3
C
primary root length (%)
Sav-0
brxS
35S:BRX-1
35S:BRX-2
35S:BRX-3
35S:BRX-4
35S:BRX-5
D
primary root length (%)
Sav-0
brxS
35S:BRXL1-1
35S:BRXL1-2
35S:BRXL1-3
35S:BRXL1-4
35S:BRXL1-5
E
primary root length (%)
Sav-0
brxS
35S:BRXL2-1
35S:BRXL2-2
35S:BRXL2-3
35S:BRXL2-4
35S:BRXL2-5
F
primary root length (%)
Sav-0
brxS
35S:BRXL4-1
35S:BRXL4-2
35S:BRXL4-3
35S:BRXL4-4
35S:BRXL4-5

Figure 4.3 Redundancy of *BRXL* genes of *Arabidopsis*. **(A)** Schematic representation of *Arabidopsis* mutants for different *BRXL* genes. The stock numbers of respective SALK T-DNA insertion lines are indicated. **(B-F)** Relative primary root length of 9 day old seedlings grown in tissue culture with respect to wild type control. **(B)** Different single and multiple *brxl* mutant lines. **(C-F)** Values for independent transgenic lines each expressing a **(C)** *35S:BRX*, **(D)** *35S:BRXL1*, **(E)** *35S:BRXL2* or **(F)** *35S:BRXL4* transgene in brx^{S} background. Error bars indicate standard error of the mean.

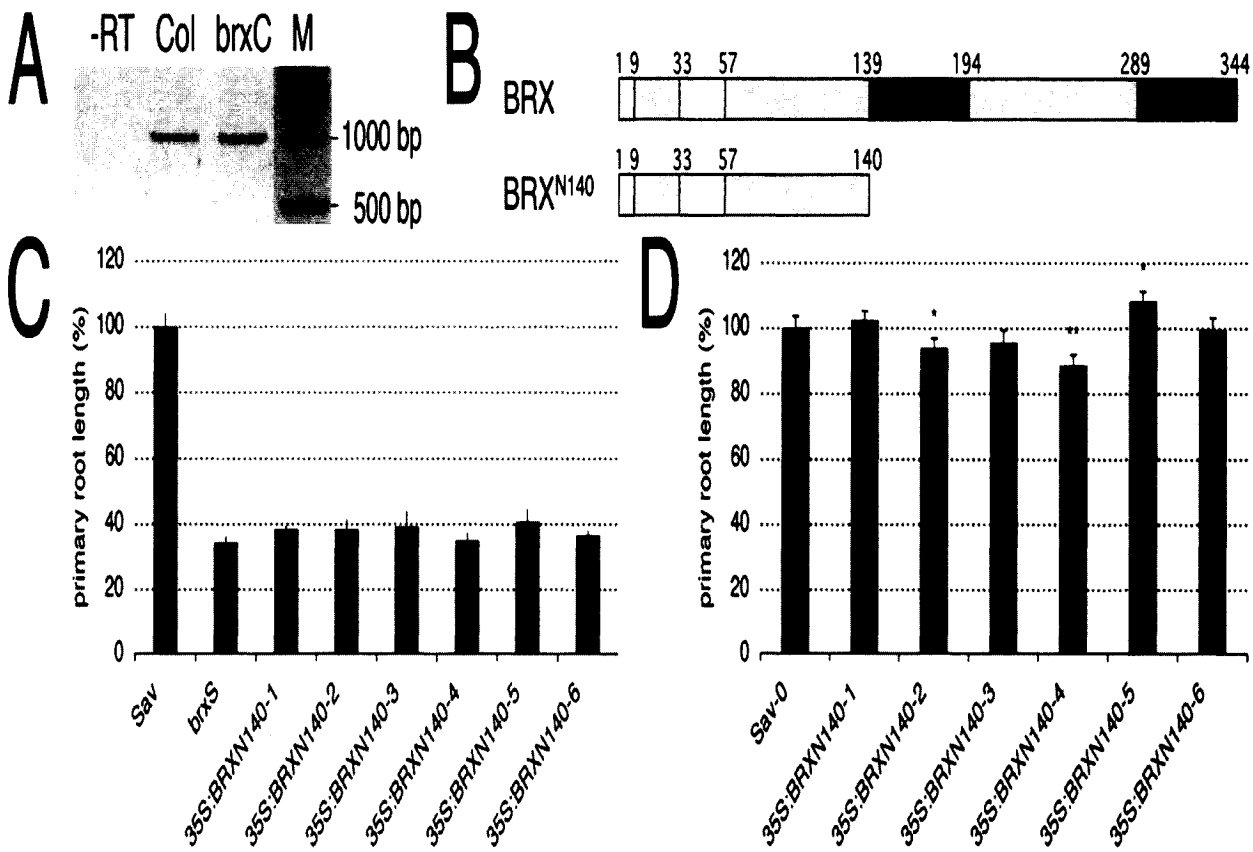

A
-RT
Col
brxC
M
1000 bp
500 bp
B
BRX
1 9 33 57 139 194 289 344
BRX^N140
1 9 33 57 140
C
primary root length (%)
120
100
80
60
40
20
0
Sav
brxS
35S:BRXN140-1
35S:BRXN140-2
35S:BRXN140-3
35S:BRXN140-4
35S:BRXN140-5
35S:BRXN140-6
D
primary root length (%)
120
100
80
60
40
20
0
Sav-0
35S:BRXN140-1
35S:BRXN140-2
35S:BRXN140-3
35S:BRXN140-4
35S:BRXN140-5
35S:BRXN140-6

Figure 4.4 The original *brx* allele is a loss-of-function allele. **(A)** RT-PCR of *BRX* mRNA, amplified from total RNA isolated from wild type (Col) or brx^{C} mutant seedlings. –RT: control reaction without reverse transcriptase. **(B)** Schematic presentation of BRX full length and potential truncated mutant protein. Light grey boxes indicate the position of conserved N-terminal domains in BRX (see Fig. 1B), dark grey boxes the position of the conserved BRX domains (see Fig. 1C, D). Respective aa positions are indicated. **(C-D)** Relative primary root length of 9 day old seedlings grown in tissue culture with respect to wild type control (Sav-0). **(C)** Values for independent transgenic lines expressing the $35S{:}BRX^{N140}$ transgene in brx^{S} background. **(D)** Values for independent transgenic lines expressing the $35S{:}BRX^{N140}$ transgene in Sav-0 background. Error bars indicate standard error of the mean. Asterisks indicate p values of Student's t-test, with one asterisk signifying $p<0.05$ and two asterisks signifying $p<0.01$.

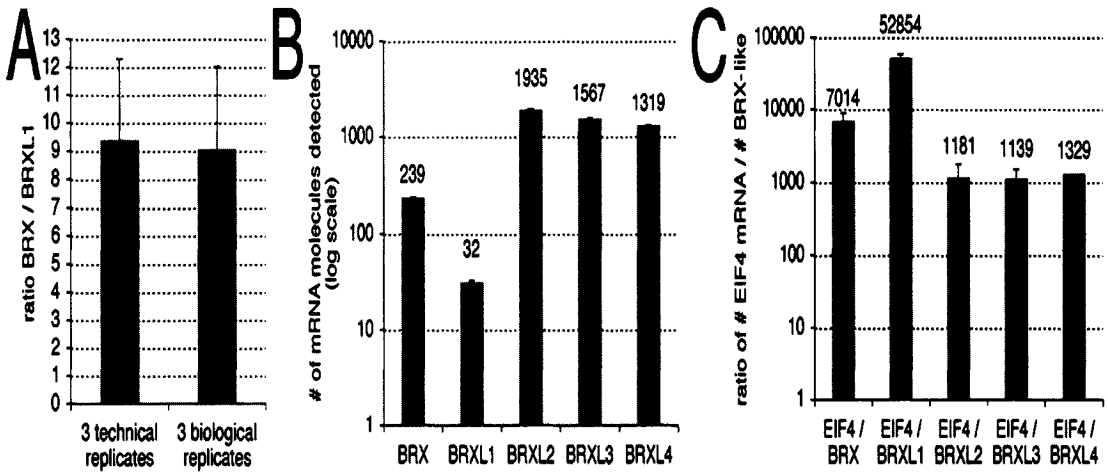


Figure 4.5 Quantification of expression levels of *BRXL* genes in the root. Quantification of *BRXL* expression levels by qPCR using total RNA isolated from roots of 9 day old seedlings. **(A)** Relative expression of *BRX* compared to *BRXL1* as determined in identical samples. **(B)** Absolute number of mRNA molecules detected in 1µg of total RNA. Average of 3 samples assayed for all 5 genes. **(C)** Relative expression of *BRXL* genes as compared to housekeeping gene *EIF4*, in the samples analyzed in (B). Error bars indicate standard error of the mean.

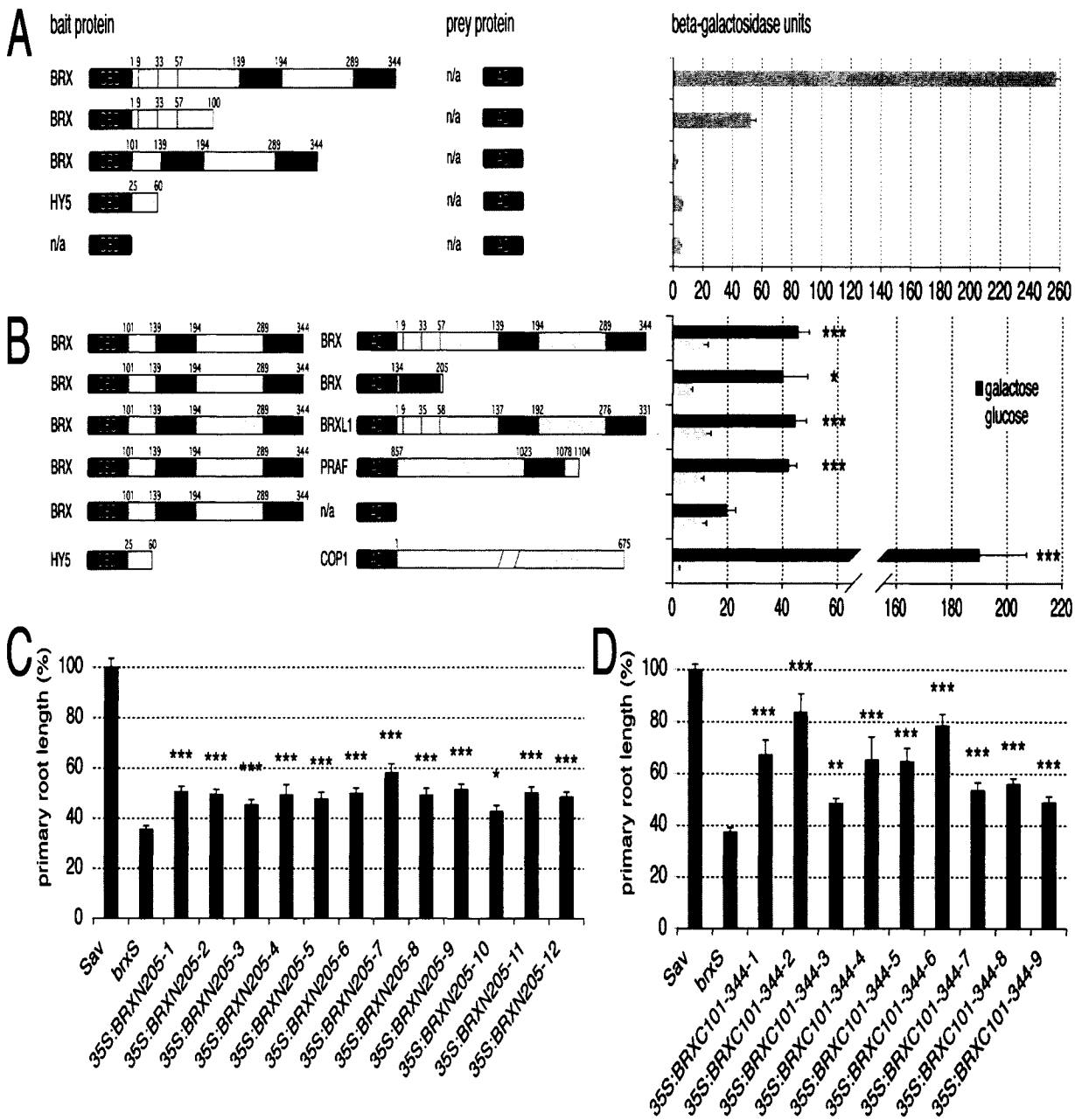

A
bait protein
prey protein
beta-galactosidase units
BRX
HY5
n/a
B
BRXL1
PRAF
COP1
galactose
glucose
C
primary root length (%)
Sav
brxS
35S:BRXN205-1
35S:BRXN205-2
35S:BRXN205-3
35S:BRXN205-4
35S:BRXN205-5
35S:BRXN205-6
35S:BRXN205-7
35S:BRXN205-8
35S:BRXN205-9
35S:BRXN205-10
35S:BRXN205-11
35S:BRXN205-12
D
primary root length (%)
35S:BRXC101-344-1
35S:BRXC101-344-2
35S:BRXC101-344-3
35S:BRXC101-344-4
35S:BRXC101-344-5
35S:BRXC101-344-6
35S:BRXC101-344-7
35S:BRXC101-344-8
35S:BRXC101-344-9

Figure 4.6 The BRX domain is a protein-protein interaction domain and functionally significant *in planta*. **(A)** Autoactivation of BRX-derived bait proteins fused to lexA DNA binding domain (DBD) in yeast. Aas of BRX are indicated. Light grey boxes indicate the position of conserved N-terminal domains in BRX (see Fig. 1B), dark grey boxes the position of the conserved BRX domains (see Fig. 1C, D). **(B)** Yeast two hybrid interaction assays of BRX-derived bait protein fused to lexA DBD and different prey proteins fused to an activation domain (AD). Light grey boxes indicate the position of conserved N-terminal domains in BRX, dark boxes the position of the conserved BRX domains in BRX, BRXL1 or PRAF. lacZ reporter gene activity measured in liquid assays is indicated in Miller units. **(C-D)** Relative primary root length of 9 day old seedlings grown in tissue culture with respect to wild type control (Sav-0). **(C)** Values for independent transgenic lines expressing the *35S:BRX*^{N205} transgene (see text) in *brx*^{S} background. **(D)** Values for independent transgenic lines expressing the *35S:BRX*^{C244} transgene in *brx*^{S} background. Error bars indicate standard error of the mean. Asterisks indicate p values of Student's t-test, with one asterisk signifying $p<0.05$, two asterisks signifying $p<0.01$ and three asterisks signifying $p<0.001$.

Gene name	Gene ID	ESTs exist?
AtBRX	At1g31880	yes
AtBRXL1	At2g35600	yes
AtBRXL2	At3g14000	yes
AtBRXL3	fuse At1g54180 + At1g54190	yes
AtBRXL4	At5g20540	yes
PtBRXL1	C-scaff-290001	yes
PtBRXL2	LG_III000279	no
PtBRXL3	LGIII1601	yes
PtBRXL4	LGVI000394	no
PtBRXL5	LGXVIII000005	no
OsBRXL1	Os08g36020	yes
OsBRXL2	Os02g47230	yes
OsBRXL3	Os04g51170	yes
OsBRXL4	Os03g63650	yes
OsBRXL5	Os12g09080	no

Table 4.1 *BRX-like* genes from different plants. Table of *BRX-like* genes identified in *Arabidopsis* (At), poplar (Pt) and rice (Os), with respective gene ID numbers and existence of expressed sequence tags indicated.

PRELUDE TO CHAPTER 5

At the end of the first examination of the roles of *BRXL* genes in root development, the lack of scorable phenotypes in the root or shoot in any of the *brx*^{S} multiple mutant lines implied that the issue of genetic redundancy could not be investigated any further. At the time of this analysis, a reliable insertion mutant line for *BRXL4* had not been obtained, thus assays involving this gene member were not previously discussed.

At the time, I only had the over-expression construct for *BRXL4*, which, when introduced into the *brx*^{S} background, did not result in the rescue of the short-root phenotype. However, upon further examination, the over-expression lines were found to have a novel shoot phenotype consisting of an alteration in the growth direction of lateral inflorescences. Recent isolation of a confirmed insertion mutant line for *BRXL4* then allowed further analysis of the developmental roles of the *BRXL* genes in *Arabidopsis*.

Chapter 5

Observations and Phenotypic Characterization of *BRXL4*

In this chapter I present unpublished observations and experiments performed in the examination of the *BRXL4* over-expression lines and the *brxl4* mutant of *Arabidopsis*. I show that *BRXL4* is involved in the shoot gravitropic response and propose its possible involvement in the regulation of the GSA of lateral shoots.

INTRODUCTION

The sessile nature of plants requires that they adapt to changes or cues in their environment for survival. Tropisms form an important class of responses to these environmental cues or stimuli. Any response that causes a change in the direction of growth of a plant organ is called a tropism. These stimuli may include, but are not limited to, temperature, light, water, mechanical forces and nutrient availability. Gravity is another environmental signal that also has a significant effect on plant form, since plants use the gravity vector as a directional guide to growth. Gravitropism involves a bending action of the stimulated organ and is confined to elongating regions of the plant body. Defects in shoot gravitropism were recognized as early as the 1930's with the observation of the "lazy" phenotype. The "lazy" phenotype was observed in several horticultural plants and many agronomically important crops such as maize (1931), pepper (1935) and barley (1938) (Firn and Digby and references therein, 2000). The shoots of these mutants display a very dramatic trailing or "drooping" habit with no other deleterious effect to the plant. It has been suggested that the phenotype of these mutants is a result of a defect in the Gravitropic Set-Point Angle (GSA) of the shoot (Firn and Digby, 2000; Digby and Firn, 1997).

It has been postulated that plant organs must possess a mechanism that allows them to attain a stable gravitropic position at any angle. In addition, the angle at which any part of an organ is maintained as a result of gravitropism is characteristic of the organ and possibly controlled by a combination of developmental and environmental factors (Digby and Firn, 1997). This angle is referred to as the Gravitropic Set-Point Angle (GSA), and attempts to provide a unifying model to explain the fact that not all organs grow vertically. Further, the GSA describes the equilibrium angle from the vertical at which an organ, within the plane of a gravity vector, shows no gravity-induced differential growth (Mullen and Hangarter, 2003). The model defines primary roots as having a GSA of 0

degrees, and primary shoots 180 degrees, i.e. the two extremes of the continuum. As a result, lateral roots have a GSA between 90-180 degrees and lateral shoots between 0-90 degrees. Lateral organs in *Arabidopsis* initially grow away from the main axis of the primary organ in a horizontal manner. This growth habit facilitates many other physiological processes such as light, water and nutrient acquisition and additionally increases mechanical support (Mullen and Hangarter, 2003). As the organs elongate, a yet unknown trigger causes a change in the mainly horizontal growth habit of the lateral organs and differential growth is initiated, resulting in the upward growth of lateral shoots and the downward growth of lateral roots. This action on its own possesses a significant challenge to the aforementioned theories governing gravitropic perception and response, especially since young horizontally oriented lateral roots of the beans *Phaseolus vulgaris* and *Ricinus communis,* contain sedimented amyloplasts in their root caps (Ransom and Moore, 1983). Yamamoto *et al.,* 2002 described this growth behavior as a gradual acquisition of gravitropic sensitivity. Work by Mullen and Hangarter, however, demonstrated that newly emerged lateral roots of *Arabidopsis* (approximately 0.5mm in length) are gravitropically competent (Mullen and Hangarter, 2003). These laterals not only actively maintain their orientation at a determined GSA, but are also capable of both positive and negative gravitropic responses with respect to the gravity vector.

In this report, I describe the *Arabidopsis brxl4* mutant and the novel phenotypes identified in transgenic lines containing the *35S::BRXL4* over-expression construct. *brxl4* plants showed an overall reduction in both the root gravitropic response and in the GSA of the hypocotyls and adult lateral shoots. By contrast, stems of *35S::BRXL4* lines showed an increased response to a 90 degree rotation (overbending) and the lateral shoots had increased GSA's, such that they grew vertically or downward. Because of the well established involvement of auxin in the gravitropic response, I also examined the activity of the DR5::GUS reporter in these

lines and observed significant differences in the levels and patterns of auxin induced transcription associated with this reporter. Interestingly, treatment of wild-type lines with the brassinosteroid e-BL an enhancer of the trophistic response (Li *et al.,* 2005), results in a comparable DR5::GUS reporter expression profile similar to that of untreated *35S::BRXL4* lines. These and other results are discussed in more detail.

RESULTS

Over-expression BRXL4 phenotypes

Using a gain-of-function approach to examine possible redundancy in the *BRX* gene family, we expressed all of the available *BRX*-like cDNAs under the control of the CaMV 35S promoter (Briggs *et al.,* 2006). As previously discussed, the over-expression of *BRXL4* in this case did not rescue the short-root phenotype of brx^S roots. The 35S::*BRXL4* construct was also introduced into several other accessions, namely Columbia and Slavice-0. In all of the transgenic lines, novel phenotypes were observed both at the seedling level and in the adult shoot system. Seedlings grown both under continuous white light (Figure 5.1B) and in the dark (Figure 5.1A) showed a significant degree of agravitropism when compared to wild-type seedlings grown under the same conditions. This gravitropism defect was visible in both the primary and lateral roots and also both in the hypocotyls (dark-grown) and seedling shoots. During the adult stage of growth, defects in the shoot system became even more severe. At the rosette stage, the rosettes of over-expression lines appeared smaller and more compact compared to wild-type plants (Figure 5.1D). This was determined to be the result of not only smaller leaves (i.e surface area), but also shorter petiole lengths of the rosette leaves (Figure 5.1E&F). This phenotype however was only observed in the Sav0 and brx^S backgrounds. In these same backgrounds, primary shoots initially emerged or bolted at an almost 45 degree angle to the vertical. (Figure 5.1H). Later in shoot

development when lateral shoots begin to emerge, growth occurs at an angle of 90 degrees or more. This results in a right angle growth orientation to the primary shoot or in most cases a downward growth orientation (>90 degrees) of the lateral shoots (Figure 5.1G). I also observed that the penetrance of some phenotypes was more significant in some accessions than others. These phenotype/accession differences are highlighted in Table 5.1

Identification of the brxl4 allele

BRXL4 had previously been identified in a bioinformatics search for *BRX*-like genes in *Arabidopsis thaliana* (Mouchel *et al.,* 2004). Similar to *BRX* and the other members of the *BRX* gene family, *BRXL4* is expressed in almost all tissue as shown via RT-PCR analysis (G.C.B. data not shown). Although expressed in the root, *BRXL4* did not show any *BRX* activity as assayed by its inability to rescue the short root phenotype of brx^{S} seedlings when over-expressed (Briggs *et al.,* 2006). One T-DNA insertion line, SALK_147349, had been identified with an insertion in the fourth exon of *BRXL4* (Figure 5.2 A). The presence of a T-DNA insert in *BRXL4* was confirmed by PCR. RT-PCR analysis of independent lines using primers for the full-length mRNA (Figure 5.2B) and primers immediately flanking the T-DNA insertion site (data not shown), did not result in the production of any detectable *BRXL4* mRNA transcripts in the mutant lines as compared to WT.

Gravity response of 35S-BRXL4 and brxl4 young seedlings and adult plants

In order to further characterize the agravitropic phenotype of the 35S::*BRXL4* lines, I began by comparing the hypocotyl gravity responses of the mutant *brxl4* and wild-type lines. First, the hypocotyl response of dark grown seedlings to a 90-degree rotation, measured as the deviation angle from vertical, was recorded after 16-20 hours. I observed that

hypocotyls of *brxl4* seedlings showed a reduced gravity response when compared to wild-type seedlings. However, the 35S::*BRXL4* lines showed a wider scope of responses to the gravity stimulus, a range that includes responses of both wild-type and mutant lines (Figure 5.3A). This wide-ranging response was similarly observed for hypocotyls in the brx^S and Sav0 backgrounds (data not shown).

When subjected to a gravirotation of 90 degrees, adult plants containing the 35S::*BRXL4* construct in the brx^S and Sav0 backgrounds also behaved differently compared with wild-type plants. After 3 hours in the dark, stems in both Sav0 and brx^S control plants rotated in the opposite direction of the new gravity force, moving towards a final 90-degree angle. Lines containing the over-expression construct in the Sav0 background, however, showed a prominent over-rotation of the stems in response to the same gravity change (Figure 5.3C). As seen in Figure 5.3C, the stem bending phenotype of these lines is also modified in that the angles are not directly parallel to the direction of gravity. Additionally, over-expression lines in the Sav0 background exhibited an almost permanent change in the stem GSA in response to the 3-hour gravitational change as these stems maintained their orientation up to 3 days even when re-oriented in the gravitational field (data not shown). Overall, lines containing the over-expression construct are capable of effecting a gravity response indicating that all the components of the gravity sensing and response machinery are functioning. However, the nature of the response is different in the over-expression lines, which responded to a greater extent than wild-type, resulting in some cases in a change in the GSA of the organ.

Reduced Apical Hook Formation in Dark Grown Hypocotyls

When grown in the dark, *Arabidopsis* seedlings develop a hook-like structure at the apical part of the hypocotyl. This apical hook is established by differential elongation rates of cells within the hypocotyl. It is thought

that the hook evolved as a mechanism to protect the delicate shoot meristem while the seedling navigates through the soil. Light exposure of the emerged seedling, triggers the irreversible opening of this structure (Raz and Ecker 1999).

In this experiment, seedlings were grown in the dark for 5 days and their apical hook angles recorded. As seen in Figure 5.3D, the angle of the apical hook in Columbia wild-type lines was 65 degrees. The *brxl4* lines, on average, showed a reduced apical hook angle of 30 degrees and the 35S::*BRXL4* lines in the Columbia background showed increased angles of 120 degrees,. The apical hook angle was measured as the angle formed between the hypocotyl neck and the most terminal mid-center point of the paired cotyledons. The apical hook is initially formed in the 35S::*BRXL4* lines, however when assayed after 5 days, the hook is not maintained in these lines. These results possibly indicate improper differential growth in this region, which is necessary to maintain the hook-like structure.

The Gravitropic Set Point Angle (GSA) of the hypocotyls increases in the BRXL4 over-expression lines but decreases in the brxl4 mutant

Experiments were performed to determine the GSA of dark-grown hypocotyls. In these analyses, seedlings were grown for 24hrs in the light (to synchronize germination) then placed in the dark for 5 days. Plates were scanned and the angles of the hypocotyls relative to a defined zero vertical were measured. On average, most of the wild-type seedlings had a GSA in the range of 16 degrees, while *brxl4* mutant lines had GSA's in the range of 4-8 degrees (Figure 5.4A). The over-expression lines however, had a completely random distribution as indicated in Figure 5.4B, where almost half of the seedlings maintained a GSA greater than 90 degrees.

The node angles of brxl4 plants are reduced compared to wild-type plants

The lateral branching phenotype of the *BRXL4* over-expression lines is very striking (Figure 5.5B), and can be viewed as an increase in the GSA of the lateral shoots. To examine if this is a specific effect of the activity of *BRXL4*, I then looked more closely at the lateral organ GSA of the *brxl4* mutant lines. Node angle measurements taken on the mutant lines were significantly smaller (40 degrees) than wild-type node angles (60 degrees) at the same developmental stage (Figure 5.5A). This is in contrast to the large increase in the GSA (110 degrees) of the lateral shoots in the over-expression lines (Figure 5.5A).

Auxin- induced transcription in brxl4 and 35S::BRXL4 transgenic lines

Auxin plays a pivotal role in the gravitropic response of plants. It has been shown that the differential distribution of auxin underlies the observed bending response induced by a change in the gravity vector. As such I decided to investigate the degree of auxin induced transcription in the *brxl4* and the 35S::*BRXL4* lines assayed by monitoring DR5::GUS reporter expression in both backgrounds. DR5 is a synthetic auxin-inducible promoter containing an auxin responsive element (TGTCTC). The TGTCTC element functions as Auxin Response Elements (AuxREs) when multimerized and properly spaced as palindromic repeats or direct repeats in either orientation (Ulmasov et al., 1997). DR5 is much more active than natural AuxREs, and is therefore more informative for studying auxin responsive gene expression. In the DR5::GUS reporter, the auxin inducible DR5 promoter is driving the expression of the beta-glucuronidase (GUS) enzyme.

In the assays performed, wild-type lines containing the DR5::GUS construct showed detectable auxin-induced transcription at the cotyledon tips (Figure 5.6A) of light and dark grown seedlings and at the root tips of both emerging seedlings and 5 DAG old seedlings (Figure 5.6B&C). Examination of the 35S::*BRXL4* lines in the *brx*^{S} and Sav0 backgrounds

under these same conditions, however, showed an increase in the domain of auxin-induced transcription in the cotyledons of both light and dark-grown seedlings and an almost saturated auxin-induced transcription profile in the hypocotyl neck region (Figure 5.4A&B). No staining was observed in the region of the hypocotyl neck in either the *brx*^{S} or the Sav0 lines. Further, the intensity of the DR5::GUS reporter was significantly higher in the root tips of both emerging and 5 DAG seedlings of the over-expression lines compared to WT, with additional intense staining in the vasculature in the over-expression lines.

This spread in the domain of auxin-induced transcription as noted in the over-expression lines, had been previously observed in the treatment of seedlings with the brassinosteroid (e-BL) hormone (Bao *et al.,* 2004; Li *et al.*, 2005), I therefore decided to investigate this in more detail. In these additional experiments, I found that the application of e-BL to the control lines *brx*^{S} and Sav0, resulted in DR5::GUS expression profiles similar to that observed in untreated 35S::*BRXL4* lines. Further, the addition of e-BL to the over-expression lines did not lead to any change in the DR5::GUS expression profile compared to untreated seedlings (Figure5.6A&D, **rightmost panels),** possibly implying an insensitivity to the presence of exogenously applied steroid.

DISCUSSION

The agravitropic phenotype in seedlings of the over-expression lines may be attributed to several observed factors. The roots of emerging seedlings in the over-expression lines showed intense DR5::GUS activity at this stage (compared to wild-type), and also showed asymmetric auxin accumulation initiated at the root tip and spreading to one side of the root (Figure 5.6C). Usually, the asymmetric accumulation of auxin in the root is synonymous with the auxin differential observed in the gravity response. Here, the root bends in the direction corresponding to auxin accumulation. This may be what is occurring in the emerging root and results in the

further bending of the primary root to horizontal and sometimes upward directions instead of the straight downward growth expected of the emerging primary root. In older seedlings, this continues and staining can be seen at the bending portions of agravitropic roots (data not shown). Additionally, amyloplast sedimentation in hypocotyls of the over-expression lines appeared unorganized and quite dispersed (data not shown). This observation can be associated with the range of degrees of bending in response to the 90 degree change in the gravity vector (Figure 5.3A). Conversely, the *brxl4* mutant lines appeared to have fewer, more dispersed amyloplasts in their hypocotyls compared to wild-type lines (data not shown). This may result in a reduction in the amount of transmissible signal produced via amyloplast sedimentation, which can further be translated into a reduced bending response, as observed with the hypocotyl response of these *brxl4* mutant lines.

In dark-grown *Arabidopsis* seedlings, the apical hook is formed 24 hours after germination and is maintained for about 5 days by a process of differential growth. The hook structure undergoes several stages including formation, maintenance and opening (Raz and Ecker, 1999). Apical hook development is largely regulated by the hormones auxin and ethylene, where auxin promotes hook opening and ethylene promotes hook curvature. The opening of the apical hook involves the process of differential growth. It has been demonstrated that trophic stimuli induce the lateral re-distribution of auxin, resulting in its unequal accumulation on opposing flanks of the responding organ and promoting auxin-mediated differential growth. In our apical hook assay, the *brxl4* mutant lines showed a reduction in the apical hook angle after 5 days compared to wild-type. Conversely, the over-expression lines showed an increased apical hook angle under the same conditions. This result was supported by the DR5::GUS expression profile analysis in these lines, where the apical hook and hypocotyl neck region of these plants where saturated by the reporter after 4hours. Total saturation may indicate a lack of

establishment or maintenance of proper asymmetric auxin, required for organ bending. However, this cannot be definitely stated, as the expression profile in dark- grown *brxl4* mutant lines was not assayed. At the same time, one could hypothesize, based on the expression profiles of the light grown seedlings, that there is possibly reduced reporter expression in these lines. This would help explain the persistence of a small apical hook angle in these lines maintained even after 5 days of growth.

The observations of larger node angles in the 35S::*BRXL4* lines and the converse decrease in the node angles in the *brxl4* mutant lines, allows us to hypothesize the possible involvement of the *BRXL4* gene product in the determination and/or maintenance of the GSA of the lateral shoots. Similar observations were also made with respect to the GSA of the hypocotyls, where the mutant *brxl4* lines showed a reduction in hypocotyl GSA compared to wild-type plants and the over-expression lines showed a converse increase in hypocotyl GSA. Recently, Zhi-Yong-Wang's group has implicated the hormone, brassinosteroid (BR), in developmental patterning and organ separation. In their unpublished work (Gendron *et al.,* 2006), they describe a dominant mutation in an activator of BR signaling that has a nodal phenotype where the primary stem bends or 'kinks' toward the lateral stem. Examination of these lines reveal changes in boundary specifications between axil cells and elongating cells. These lines also show organ fusion defects in other plant parts such as in the stamens. The involvement of BR in node angle specification poses an alternate hypothesis for the action of *BRXL4* as it could, possibly through the action of BR, be regulating organ boundary specification or possibly angle maintenance at the nodes. This is especially interesting since at the seedling stage, the DR5::GUS expression profile of the 35S::*BRXL4* over-expression lines is similar to e-BL treated wild-type seedlings, and further, the addition of e-BL to 35S::*BRXL4* plants, does not change the expression profile of the reporter construct compared to

untreated plants. These results may indicate that the BR status of these over-expression lines is endogenously altered.

BR has also been implicated in the alteration of auxin transport and/or distribution required for the gravitropic response and has been demonstrated to affect the accumulation of ROP2 and some PIN proteins (Bao et al., 2004; Li *et al.*, 2005). BR associated increases in PIN2 and PIN3 localization have been associated with increases in basipetal and lateral auxin transport resulting in an increased gravitropic response (Li *et al.*, 2005). Additionally, the over-expression of ROP2, a ROP GTPase, via a 35S::ROP2 construct, has been shown to result in the over-bending of adult stems of *Arabidopsis* in response to a change in the gravity vector (Li *et al.*, 2005). It might be possible that the over-bending phenotype observed in the 35S::*BRXL4* lines may be attributed to an endogenously altered BR status of these plants, which may be affecting auxin transport and/or distribution directly via PIN protein cycling dynamics or via ROP2 accumulation.

Notably, the response of the adult stems and hypocotyls to a 90 degree gravity change was different in the 35S::*BRXL4* lines. In the adult stem rotation assay, these lines clearly showed an over-rotation compared to wild-type plants, whereas the hypocotyls did not show any over-bending when assayed for a similar response. These results highlight the possible differences in the gravitropic response mechanisms of the hypocotyls verses the stem as seen with the mutant analysis.

In summarizing all of the results discussed, a possible common theme of differential growth, probably via cell expansion, appears to connect all observed phenotypes, this however has not been examined in detail. It is known that BR is involved in the process of cell expansion, and recent work supporting its involvement in the regulation of auxin distribution and/or transport, added to it newly proposed role in organ boundary formation, can place the question of maintenance of BR homeostasis in the shoot as a possible mechanistic function of BRXL4.

The overall implication of BR regulation as a putative role for BRXL4 in these processes may not be unrealistic, especially since the founding member of the gene family, BRX has been recently shown to be a regulator of BR biosynthesis in the root (Mouchel *et al.,* 2006).

MATERIALS AND METHODS

Plant Material and Tissue Culture

Seeds of *Arabidopsis* accessions and T-DNA insertion mutants were obtained from the *Arabidopsis* Biological Resources Center. Seedlings were grown at 22°C under constant illumination on culture medium (0.5 X Murashige and Skoog salts, 0.5 g/L MES, 0.9% agar, 1% Suc, pH 5.8). Light intensity was approximately 90 microEinsteins. For analysis of adult plant growth,10 DAG seedlings were transferred from plates to soil and grown under constant light for 4-8 weeks.

Creation and Analysis of Transgenic Plants

The 35S::BRXL4 lines were created as previously described (Briggs *et al.,* 2006). Binary constructs were transformed into Columbia, Sav0 and brxS plants via floral dip using Agrobacterium, and transgenic lines were selected by screening seed progeny for glufosinate ammonium resistance (15 mg/L) on medium containing 0.3% Sucrose. Because of frequent problems with transgene silencing in subsequent generations, the T2 generation was chosen for analysis. Independent transgenic lines segregating single-locus insertions were selected.

Verification of SALK Insertion Lines by PCR and RT-PCR

T-DNA insertion lines were verified by PCR as previously described (Briggs *et al.,* 2006). Total RNA was extracted from established homozygous lines. Nucleic acids were prepared using RNeasy™ and DNeasy™ kits from Qiagen according to the manufacturer's instructions.

RT-PCR reactions were performed according to standard procedures with Superscript II™ reverse transcriptase (Invitrogen). Primers for amplification of the full-length mRNA were BRXL4D 5' ATGCTGACGTGT ATAGCTCGTTCG 3' and BRXL4U 5' GGAGTGTCTTCTGGATGGTAC 3'. And primers for amplification of the truncated portion flanking the T-DNA insertion site were BRXL4EX4DQRT_1 5' TTATATCACCATTAAAGTTTT ACCA 3' and BRXL4U.

Gravitropism Assays

Seeds were surface sterilized, plated and imbibed for 3 nights at 4°C in the dark. To synchronize seedling germination, plates were placed vertically in the gravity vector and exposed to continuous light at 22°C for 24 hours. Plates were then wrapped in several layers of foil and placed again vertically in the gravity vector in a square tray at 22 degrees in a dark chamber. After 4 days, plates were removed from the chamber and scanned to record seedling positions. Under sterile conditions, the seedlings were re-oriented vertically upright on the plates for uniformity. The plates were then re-wrapped in foil and placed again in the same orientation in the chamber. After 24hrs, the plates were rotated 90 degrees clockwise and allowed to germinate for a further 24hrs. After this time, the plates were removed and scanned and the rotation relative to the gravity vector was measured using NIH Image Software.

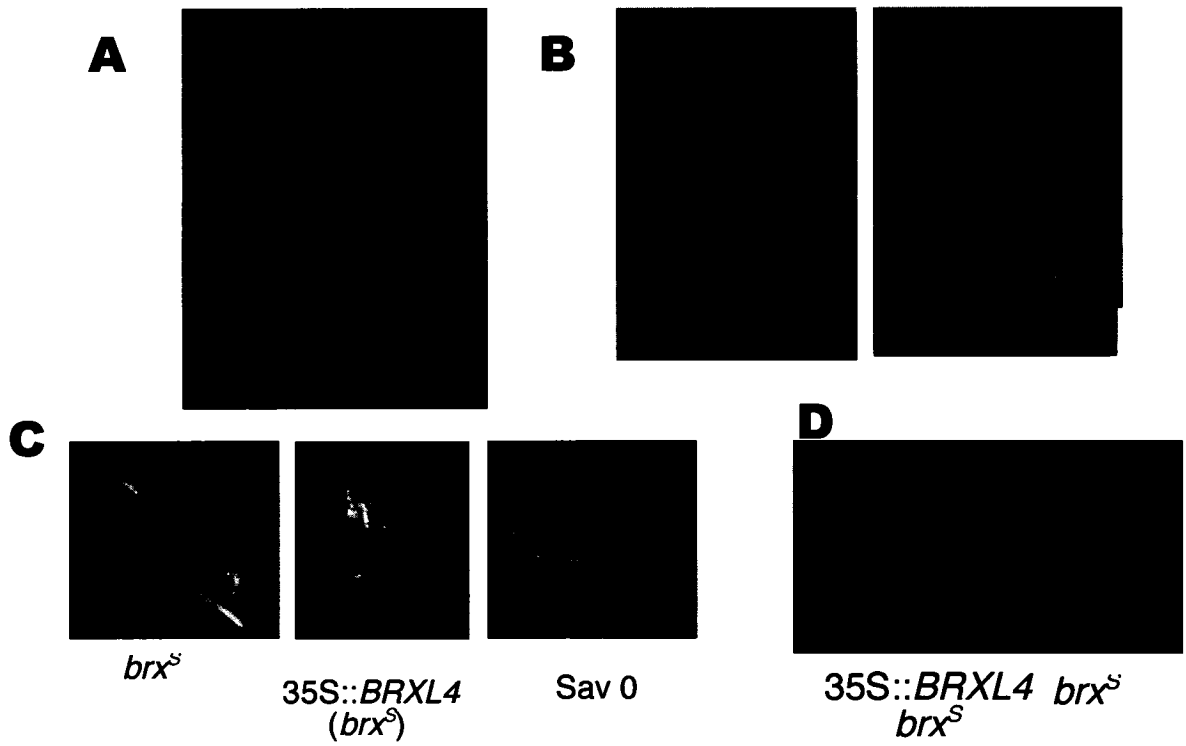
To measure the stem gravity response, adult plants were grown for 4 weeks on soil and then subjected to a gravitational rotation. In this experiment, the pots were placed on their sides, photographed, and placed in the dark for 3hrs. The plants were again photographed after this time to record the angles of stem rotation. Stem angles were measured using NIH image software.

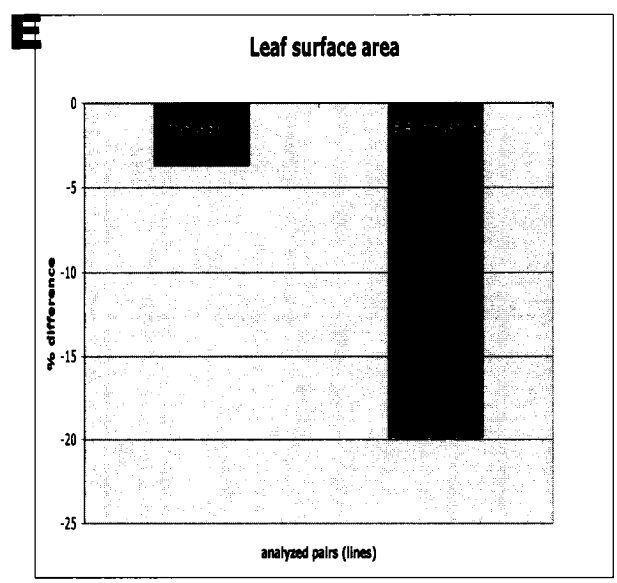
Gravitropic Set-Point Angle (GSA) Measurements

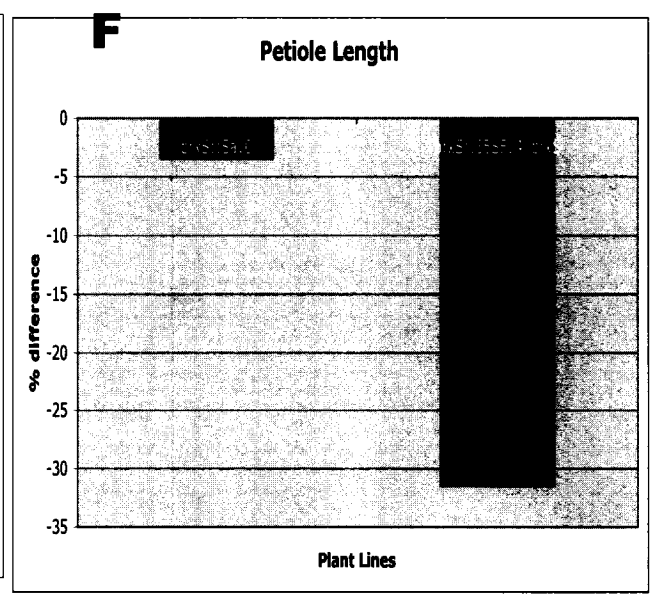
The GSA of seedlings was analyzed and measured using NIH Image Software. This assay measured the degree of randomness of emergence of the etiolated hypocotyls. As such, measurements of the GSA of the emerged hypocotyls relative to the vertical were recorded. For adult plants, the GSA was measured from photographic records of plant sections. Stem portions spanning approximately 5cm on both sides of the node were excised. Sections were freshly cut and placed in sterile water. Photos were taken with a digital camera at the same magnification. NIH Image software, was used to analyze images and measurements of the GSA of the lateral shoots (secondary inflorescence) were recorded.

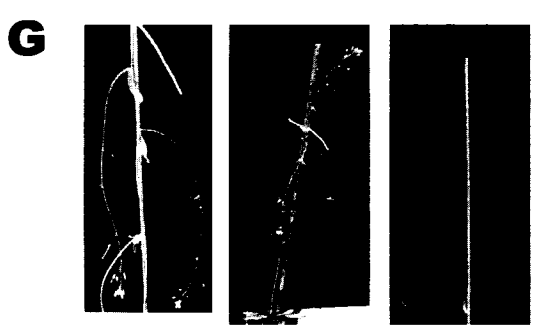
DR5::GUS Reporter Analysis

Crosses were performed between DR5::GUS Columbia plants (Tom Guilfoyle, Univ. of Missouri) and homozygous lines of the *brxl4* mutant and the 35S::*BRXL4* lines (brxS, Sav0). T2 seedlings were grown as specified under the different assay conditions. Where indicated, seedlings were treated with and 5mM e-BL. To assay for GUS activity, seedlings were placed in cold 70% acetone for 15-20 minutes, then washed twice in GUS washing buffer (100 mM Sodium Phosphate, pH 7, 10 mM EDTA, 0.1% (w/v) Tween 20, 1mM potassium ferricyanide, 1mM potassium ferrocyanide) for 20 minutes each. This solution was replaced with fresh GUS staining buffer, (GUS wash buffer; 1 mg/mL of 5-bromo-4-chloro-3-indolyl beta-D-Glucuronide) and vacuum infiltrated for 20 minutes. Samples were incubated at 37°C for 4 hours. The reaction was stopped by the addition of 70% ethanol. Samples were maintained at 4°C until ready for microscope work.


A
B
C
D
brx^S
35S::BRXL4
(brx^S)
Sav 0
35S::BRXL4 brx^S
brx^S


E
Leaf surface area
% difference
0
-5
-10
-15
-20
-25
analyzed pairs (lines)


F
Petiole Length
% difference
0
-5
-10
-15
-20
-25
-30
-35
Plant Lines


G

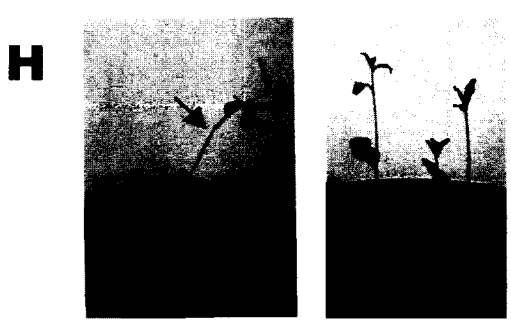

H

Figure 5.1 35S::*BRXL4* over-expression phenotypes in *Arabidopsis*. **(A)** Hypocotyls of wild-type Sav0 seedlings in the dark (above) and agravitropic seedlings of 35S::*BRXL4* (Sav0) **(B)** Light grown seedlings of Columbia (left panel) and agravitropic seedlings of 35S::*BRXL4* in Columbia (above) and brxS (below) **(C)** Leaves of control and over-expression lines showing differences in leaf surface area and pedicle length **(D)** Smaller and more compact rosettes observed in brx^S lines containing the over-expression construct compared to brx^S **(E)** Graph indicating the observed 20% reduction in leaf surface area in over-expression lines compared to wild type **(F)** Graph indicating the 30% reduction in petiole length observed in the over-expression lines compared to the wild type **(G)** Older plants containing the 35S construct in both the brx^S and Sav0 backgrounds showed an increased angle at the lateral nodes, resulting in a downward bending of the lateral shoots. **(H)** Young adult plants containing the 35S::*BRXL4* construct in the Sav0 background, have a characteristic bending phenotype (red arrow), compared to wild-type plants.

Observed Phenotypes	Slavice-0	*brxS*	Columbia
Seedling agravitropism	+	+	+
Hypocotyl GSA			
brxl4	N/A	N/A	+
35S::*BRXL4*	+	+	+
Rosette phenotype	+	+	-
Node angles (adult)			
brxl4	N/A	N/A	(+)
35S::*BRXL4*	+	+	(+)

Table 5.1 *BRXL4* phenotype differences in different accessions of *Arabidopsis*. The table highlights the observed differences in the penetrance of the *BRXL4* phenotypes in different genetic backgrounds. + indicates a strong positive response; (+) a weakly positive response; - indicates absence of a phenotype and N/A indicates not applicable.

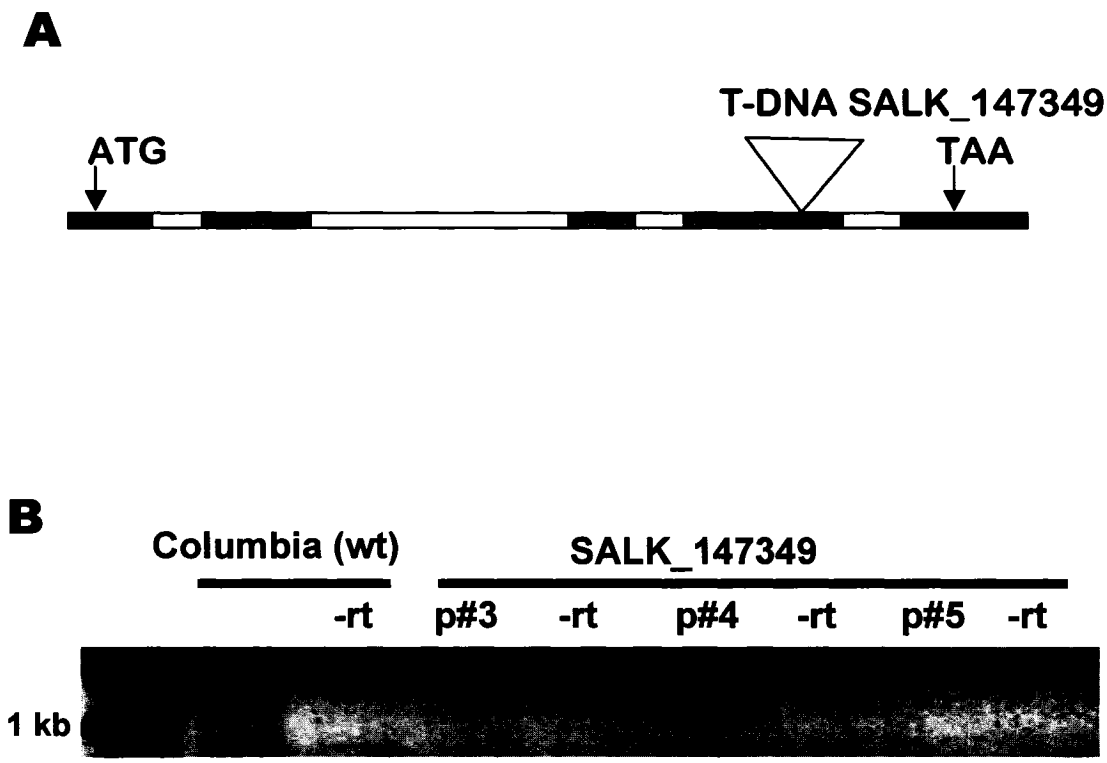
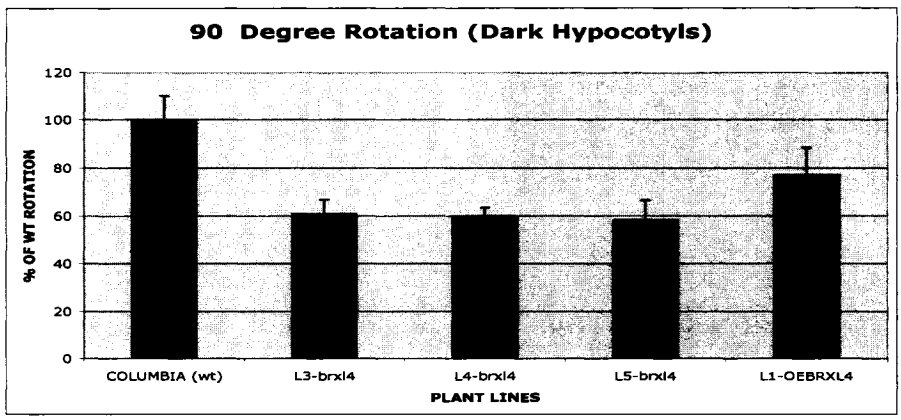
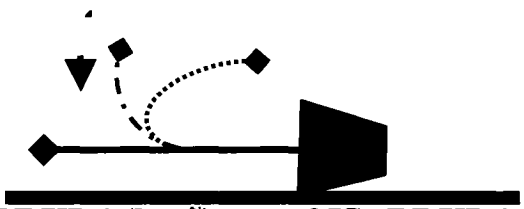

A
T-DNA SALK_147349
ATG
TAA
B
Columbia (wt)
SALK_147349
-rt
p#3
-rt
p#4
-rt
p#5
-rt
1 kb

Figure 5.2 Identification of the *brxl4* allele in *Arabidopsis*. **(A)** Gene structure of BRXL4 indicating positioning of the T-DNA insertion in Exon 4 **(B)** RT-PCR indicating the detection of *BRXL4* transcript in Columbia wild-type lines but not in the insertion lines.(–rt) RT negative control; p#3, p#4,p#5 represent independent T-DNA insertion homozygous lines

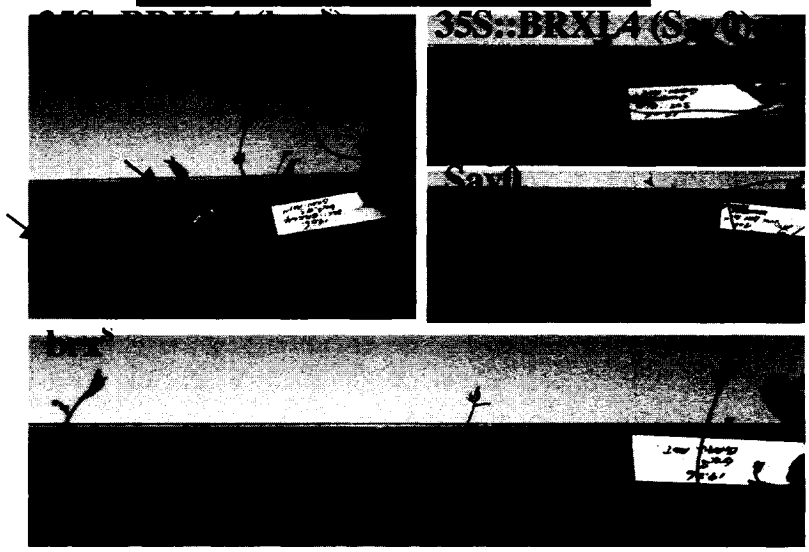
A


90 Degree Rotation (Dark Hypocotyls)
% OF WT ROTATION
120
100
80
60
40
20
0
COLUMBIA (wt)
L3-brxl4
L4-brxl4
L5-brxl4
L1-OEBRXL4
PLANT LINES

B



C


35S::BRXL4 (S

D

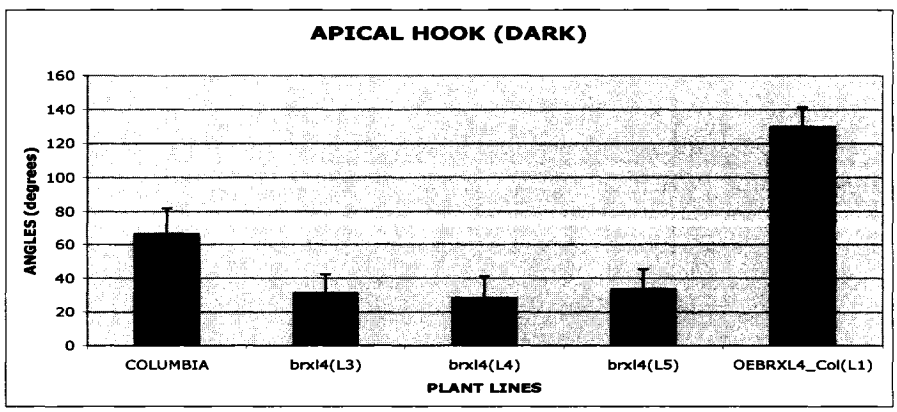
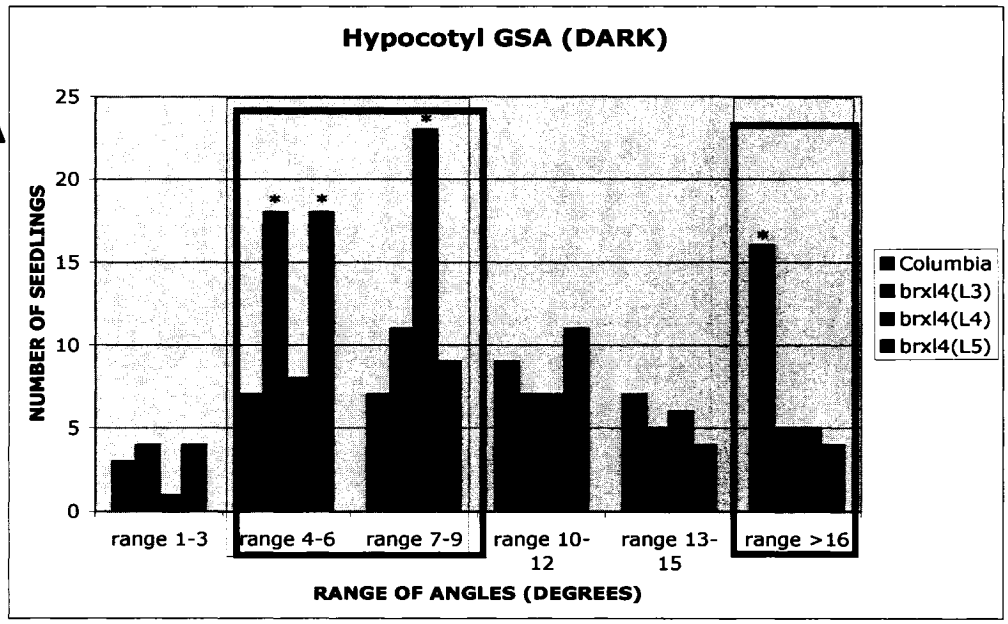

APICAL HOOK (DARK)
ANGLES (degrees)
160
140
120
100
80
60
40
20
0
COLUMBIA
brxl4(L3)
brxl4(L4)
brxl4(L5)
OEBRXL4_Col(L1)
PLANT LINES

Figure 5.3 Gravirotation of Columbia, *brxl4* and 35S::*BRXL4* young seedlings and adult plants. **(A)** 90 degree rotation of young seedlings in the dark. **(B)** Over-bending in the over-expression lines, G indicates the direction of the new gravity vector, red dashed line indicates the over-bending stems compared to the wild-type response (black dashed lines). **(C)** Photos indicating the over-bending phenotype of adult plants containing the over-expression construct in response to the gravity vector change. **(D)** Apical hook formation in the dark.

A


Hypocotyl GSA (DARK)
NUMBER OF SEEDLINGS
25
20
15
10
5
0
range 1-3
range 4-6
range 7-9
range 10-12
range 13-15
range >16
RANGE OF ANGLES (DEGREES)
Columbia
brxl4(L3)
brxl4(L4)
brxl4(L5)

B

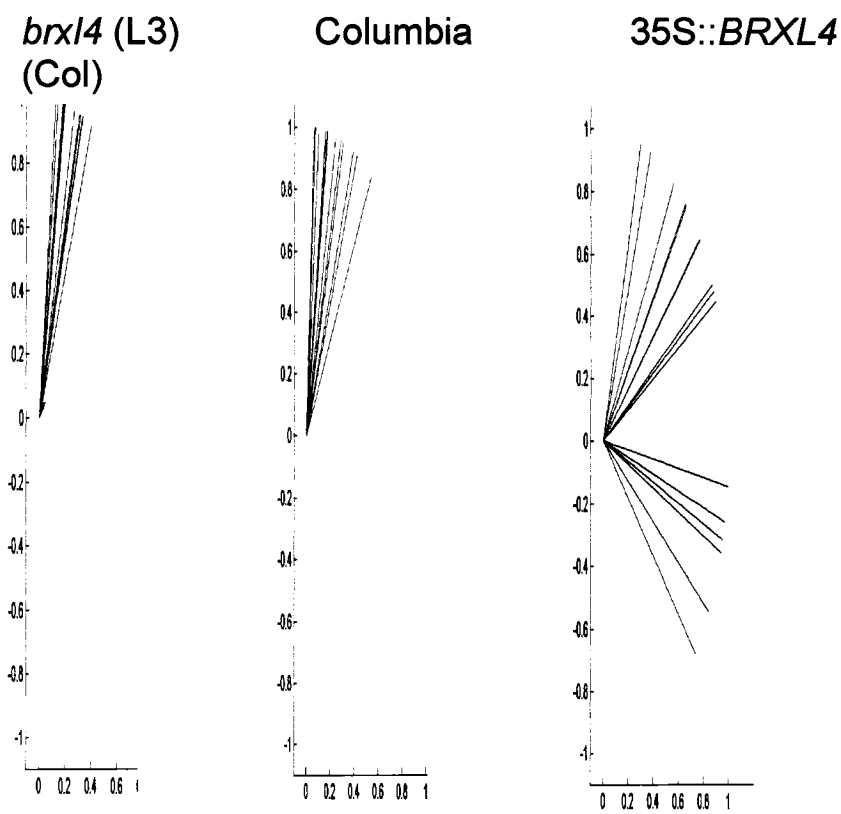
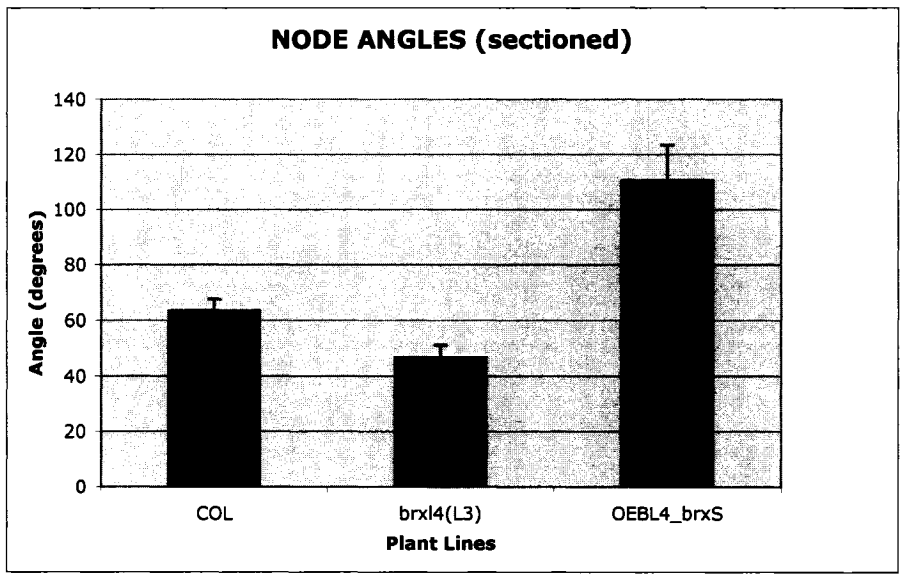
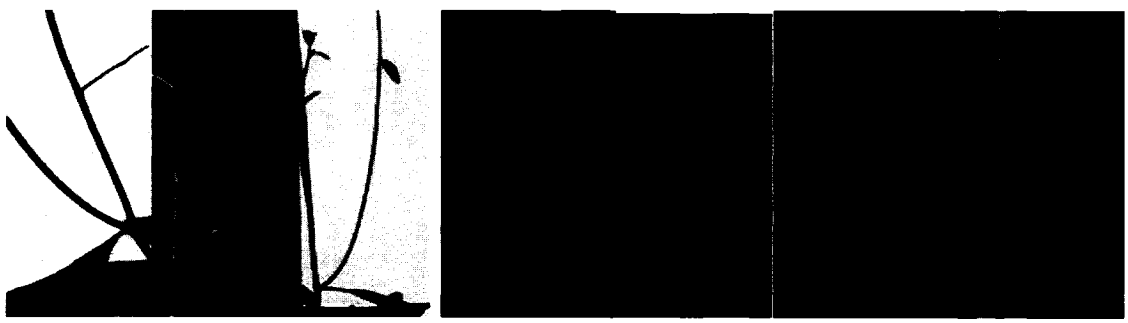

brxl4 (L3) (Col)
Columbia
35S::BRXL4

Figure 5.4 The Gravitropic Set-Point Angle (GSA) of the hypocotyl and lateral shoot. (A) Graphical representation of the hypocotyl GSA of wild-type and *brxl4* mutant lines in Columbia. The majority of wild-type seedlings have a GSA of about 16 degrees (indicated in red box), whereas the *brxl4* mutant lines show a reduction to 4-8 degrees (indicated in the blue box). (B) Diagrammatic representation of the hypocotyl GSA of the wild-type, mutant and over-expression lines in Columbia. Lines indicate the position from the vertical and in the case of the over-expression lines, the increased angles of hypocotyl growth are also visible.

A



B



brxl4 (Col) | 35S::*BRXL4* (Col) | Col | 35S::*BRXL4* (Sav0) | Sav0 | 35S::*BRXL4* (*brx*^{S}) | *brx*^{S}

Figure 5.5 Node angles / lateral shoot GSA of adult plants (A) The GSA of the lateral shoots of adult plants are shown in the graph, indicating a significant reduction in the nodal angle of the *brxl4* mutant lines (43 degrees) compared to the wild-type (60 degrees) and a corresponding increase in the nodal angles of the over-expression lines (110 degrees). (B) Photos of control *brxl4* mutant and over-expression lines showing differences in the nodal angles/lateral shoot GSA

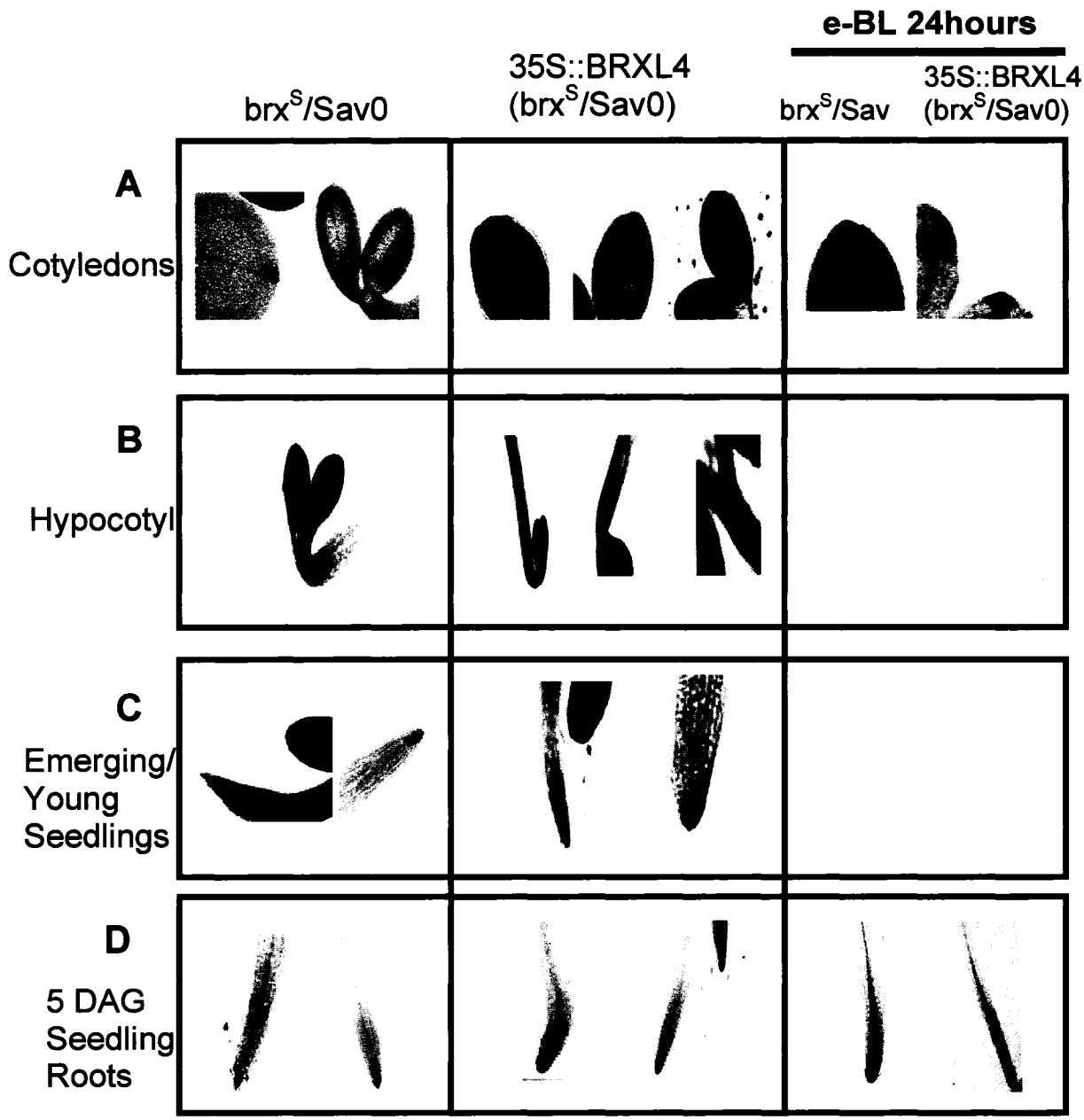

e-BL 24hours
brx^S/Sav0
35S::BRXL4 (brx^S/Sav0)
brx^S/Sav
35S::BRXL4 (brx^S/Sav0)
A
Cotyledons
B
Hypocotyl
C
Emerging/ Young Seedlings
D
5 DAG Seedling Roots

Figure 5.6 DR5::GUS expression patterns in 35S::*BRXL4* lines. DR5::GUS staining profile of untreated control lines and over-expression lines in (A) cotyledons (B) hypocotyls (C) emerging seedlings and (D) 5DAG seedling roots. As seen in the further right panels, 24hr treatment of the control lines and over-expression lines with e-BL results in a DR5::GUS expression profile, similar to that of untreated over-expression lines.

Chapter 6

Conclusion

Examination of the natural genetic variation among accessions of *Arabidopsis thaliana* allowed us to identify a novel factor regulating primary root growth. This gene called *BREVIS RADIX* (*BRX*) resulted in a short primary root when in its inactive form (*brx*). As part of my contribution to the initial analysis, I have demonstrated that *BRX* is part of a small gene family in *Arabidopsis* (Chapter 3). The discovery of the *BRX* gene family allowed me to examine not only the putative roles of the *BRX*-Like genes in development, but also to contribute to the understanding of genetic and functional redundancy in *Arabidopsis*. This study was initiated in Chapter 3, where I constructed double mutants with the *brx* allele in an attempt to identify possible synergistic interactions between gene pairs. This, however, was not observed and the *BRX* gene was determined to be the only member of the *BRX* gene family with an assayable function in the root. Further, construction of triple and quadruple mutants amongst *BRX*-Like genes did not reveal any novel insights into issue of functional redundancy in root development.

A more detailed examination and analysis into the *BRX* gene family of *Arabidopsis* revealed that they represented a small, plant-specific, highly conserved group of genes (Chapter 4). *BRX*-like genes were found not only in *Arabidopsis*, but also in other plant species, including rice and poplar and further, putative homologs were identified in almost all plant databases for which sequence data was available (Chapter 4). *BRX* gene family members contain a characteristic "BRX" domain. The BRX domain was shown to be necessary for *BRX* function *in planta* through transgenic analysis (Chapter 4). Yeast interaction assays performed using different portions of the *BRX* protein, both with and without the BRX domain, showed that the *BRX* is capable of mediating homologous and heterologous interactions between gene family members. I further show that these interactions are mediated through the conserved BRX domain (Chapter4). In Chapter 4, I also demonstrate that the originally isolated *brx* allele of Uk-1 is a true null allele.

BRX gene family members are highly similar both at the level of gene structure and sequence. We hypothesized that this similarity might translate into functional similarity and thus explored extensively the question of functional redundancy in this gene family (Chapters 3&4). This approach via single, double, triple and quadruple mutant analysis in combination with an over-expression approach, although informative, was not as revealing as we hypothesized. Of all four *BRX* homologs, only *BRXL1* was determined to have *BRX* activity and qRT-PCR analysis revealed that significantly lower expression levels of this gene *in planta* is possibly a major reason for the lack of observable phenotypes in single and double mutants with *brxl1* (Chapter4). Recently it has been shown that *OsBRXL1* and *OsBRXL2*, *Oryiza sativa* (rice) homologs of *BRX* and *BRXL1* respectively, are also capable of rescuing the short root phenotype of brx^S plants. These results demonstrate a possible functional conservation of *BRX* genes even in the monocotyledons (Julien Beuchat, personal communication). The lack of assayable phenotypes in the multiple mutant lines did not allow us to explore the question of functional redundancy amongst *BRX* genes any further. At the same time however, careful examination of the *BRXL4* over-expression lines, revealed some new and interesting phenotypes in *Arabidopsis* (Chapter 5).

When over-expressed, one of the gene members, *BRXL4*, although not rescuing the short-root phenotype of brx^S plants, showed an abnormal shoot phenotype. The later acquisition of a reliable T-DNA insertion line for this gene allowed us to correlate the over-expression or absence of this gene to the observed phenotypes. Lines containing the over-expression construct of *BRXL4* showed strong seedling agravitropism and abnormalities in the GSA of hypocotyls and lateral stems. Over-expression lines also showed more compact rosettes, corresponding to shorter petioles and smaller leaf surface area. Apical hook abnormalities and altered gravity responses were also observed in these lines. These phenotypes can all be linked to problems of cell elongation, cell

differentiation and alterations in hormone distribution and/or response. However, this is not conclusive and has not been measured or examined in detail at the cellular level.

Project Extension and Future Experiments

The *BRX* gene family work is currently being investigated in more detail and verification of a quintuple mutant is already ongoing. Further, experiments to determine the portions of the protein that have diverged between *BRX* and members without *BRX* activity, are already in progress. Additionally, work on identification and functional characterization of *BRX* and *BRX*-like genes in other plant models (e.g. tomato, rice, and medicago) are also in progress.

The observations and experiments performed on the *BRXL4* over-expression lines and involving the *brxl4* mutant were performed over a few months. As a result, this portion of my thesis research, although proving very interesting and promising from initial analyses, is still in its preliminary stages. Overall, molecular analysis is yet to be performed to confirm several discussed observations. Firstly, because the *brxl4* mutant phenotype in the adult plant is not very strong in the Columbia background, I have begun introgression of the *brxl4* mutant into the Sav0 background in an attempt to observe a stronger phenotype for the lateral shoots. This would then serve as a good assay system for the rescue construct required to confirm the involvement of *BRXL4* in the control of the GSA in the hypocotyl and shoot. Secondly, more stringent histology, sectioning and amyloplast profiles to examine the cell state, structure and any changes in localization of polarized proteins (e.g. PIN1::GFP), could aid in determining the nature of the change that occurs at the lateral shoot node including the possible involvement of cell expansion and/or elongation in these processes. Also, because of time constraints, proper analysis of the adult plant phenotype in terms of DR5::GUS expression especially at the nodes, was not performed. For this the *brxl4* adult plants

containing the DR5::GUS construct need to be genotyped for proper comparison to the over-expression and WT lines in the same accession background. Additionally, examination of amyloplast sedimentation at these nodes and in the lateral shoots should also prove informative. Further, the hormone ethylene is known to have an effect on apical hook formation and organ curvature, therefore a closer examination of the possible involvement of this hormone and its effects on the observed *BRXL4* phenotypes, should also be investigated using characterized ethylene mutants like *ein2, etr1* and *ctr1*.

Previous mutants have been isolated in *Arabidopsis* with putative defects in the GSA of different organs (lateral roots, lateral shoot, primary shoots etc.) (Mullen and Hangarter 2003; Digby and Firn, 2000). However, to date, solid characterization of these mutants in an effort to dissect the possible molecular and genetic components of the GSA pathway is lacking. With this analysis, I have initiated a more detailed approach to the analysis of the GSA especially in lateral shoots and hypocotyls of *Arabidopsis*. The continuation of this work would certainly add to the existing knowledge and aid in addressing the question of GSA determination in plants. Considering the role of *BRX*, the founding member of the *BRX* gene family, as a regulator of root growth via regulation of brassinosteroid biosynthesis in the root (Mouchel *et al.*, 2006), it is possible to hypothesis that *BRXL4* being the only member of the gene family thus far with an obvious shoot phenotype, may also be acting as a putative regulator of brassinosteroids and thus GSA determination in the shoot.

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