Calcium- and BTB domain protein-modulated PINOID protein kinase directs polar auxin transport

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Chapter 4

BT scaffold proteins: a crucial function in plant development

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Abstract

BTB domain scaffold proteins are present in many organisms. In *Arabidopsis thaliana* they form a family of eighty proteins that have been implied in a variety of signaling pathways. We have identified the BTB and TAZ domain protein1 (BT1) as interactor and regulator of the PINOID (PID) kinase. BT1 has a typical domain structure that is only observed in land plants. Here we present a detailed functional analysis of the Arabidopsis *BT* family, which comprises five members, named BT1 to BT5, of which at least four are encoded by auxin responsive genes. BT1 is a short-lived protein, which characteristic as target for degradation by the 26S proteasome is linked to the BTB domain. Expression pattern, genomic structure and sequence alignment analyses indicate that BT1 and BT2 are close relatives that localize both to the nucleus and the cytosol, whereas BT4, BT5 and probably also BT3 are cytosolic. Detailed molecular and phenotypic analysis of plants segregating for loss-of-function mutations in the different *BT* genes shows that there is considerable functional redundancy among the *BT* members, and that BT function is crucial for early steps in both male and female gametophyte development. *BT2* is the dominant gene in this process, in which it is functionally replaced by *BT3* and also partially by *BT1* in *bt2* loss-of-function mutants, through reciprocal transcription regulation. This expression compensation is an important mechanism in the functional redundancy among *BT* genes. Based on our findings and recent data of other research groups we speculate that BT proteins, as regulators of PID activity, are likely part of a feed-back loop that affects auxin distribution.

Introduction

Effector proteins in basic cellular processes, such as protein kinases in signal transduction, transcription factors in gene expression and F-box proteins in protein degradation, act generally as part of a protein complex that is held together by one of more scaffold- or linker proteins. Although the importance of scaffold proteins has for a long time been undervalued, the finding that loss-of-function leads to lethality, for example for CULLIN1 and CULLIN3 (CUL3) in targeted proteolysis (Hellmann et al., 2003, Gingerich et al., 2005), Tic proteins in plastid biogenesis (Inaba et al., 2005) and the extensin RSH in cytokinesis (Hall and Cannon, 2002), has stressed their importance and revived the interest in them.

Scaffold proteins are characterized by their protein-protein interaction domains, which are conserved and form the basis of their classification. One of the largest families of scaffold proteins is formed by proteins that contain a so-called BTB domain. This domain mediates protein-protein interactions, and has first been identified in *Drosophila melanogaster* proteins that are part of Bric-à-brac, Tramtrack and Broad (BTB) protein

complexes of transcriptional regulators. It is also referred to as POZ domain because of its occurrence in many POx virus Zinc finger proteins (Albagli et al., 1995). Currently BTB proteins have been identified in many other eukaryotes including yeasts, *C. elegans*, mammals and plants (Bardwell and Treisman, 1994, Stogios et al., 2005).

The genomes of the model plants *Arabidopsis thaliana* and rice encode, respectively, 80 and 149 BTB proteins classified in ten subfamilies (Gingerich et al., 2005, Gingerich et al., 2007), of which only a few have been studied in detail. Most but not all Arabidopsis BTB proteins combine the BTB domain with at least one other protein-protein interaction domain that assigns a specific cellular function to these proteins. For example, the ankyrin and armadillo domains that are found in BTB proteins are involved in transcriptional regulation (Cao et al., 1997, Ha et al., 2004, Hepworth et al., 2005, Norberg et al., 2005), whereas the MATH and TPR domains in BTB/POZ-MATH, ETHYLENE OVERPRODUCER1 (ETO1) and ETO1-like proteins EOL1 and 2 form E3 ubiquitin protein ligase complexes with CUL3 that label proteins for degradation by the 26S proteasome (Wang et al., 2004, Dieterle et al., 2005, Gingerich et al., 2005, Weber et al., 2005). Furthermore, NON-PHOTOTROPIC HYPOCOTYL3 (NPH3) and Transcriptional Adaptor Zinc finger (TAZ) domains were found in scaffold proteins that organize protein complexes (Motchoulski and Liscum, 1999, Kemel Zago, 2006). These additional domains are not exclusive for BTB proteins, but are also found in other proteins. For example, the TAZ domain of the BTB and TAZ domain (BT) proteins (Du and Poovaiah, 2004) is also present in histone acetyltransferases of the p300/CREB binding protein (CBP) family. The CBP-type histone acetyltransferases 1, 2, 4, 5 and 12 are multidomain proteins with up to two TAZ and two ZZ zinger finger domains and a CBP-type histone acetyltransferase domain (Pandey et al., 2002). The TAZ and ZZ zinc finger domains are known to mediate protein-protein interaction with transcriptional factors (Ponting et al., 1996). Plant BTB proteins take part in a variety of cellular processes, such as phototropic responses for NPH3 (Motchoulski and Liscum, 1999), leaf and flower morphogenesis for BLADE-ON-PETIOLE1 and 2 (Ha et al., 2004, Hepworth et al., 2005, Norberg et al., 2005), and in the abscissic acid and ethylene responses for ARIA and for ETO1, EOL1 and EOL2, respectively (Wang et al., 2004, Kim et al., 2004).

The BTB domain protein BT1 (At5g63160) consists of an N-terminal BTB domain, a TAZ domain and a C-terminal Calmodulin Binding domain (CaMBD), a combination that is plant specific. We identified BT1 as an interacting partner of the protein serine/threonine kinase PINOID (PID), and we therefore initially named the protein PINOID BINDING PROTEIN2 (PBP2) (Benjamins, 2004). Besides BT1/PBP2, four other proteins with the same protein structure have been identified, namely BT2/PBP2H1 (At3g48360), BT3/PBP2H2 (At1g05690), BT4/PBP2H4 (At5g67480) and BT5/PBP2H3 (At4g37610) (Du and Poovaiah, 2004). All five BT proteins are interacting with the potato Calmodulin6 (CaM6) in a calcium-dependent manner through the CaMBD (Du and

Poovaiah, 2004), BT1, 2 and 4 were found to bind to the bromodomain proteins BET10 (Bromodomain and extraterminal domain protein), also called GTE11 (Global Transcriptional Factor group E), and BET9/GTE9 (Florence and Faller, 2001, Pandey et al., 2002, Du and Poovaiah, 2004). Besides PID, BT1 was found to interact with several transcriptional factors and cytoskeleton binding proteins (Kemel Zago, 2006). Furthermore BT2 seems to be part of a feed-back loop that enhances auxin responses, such as root growth inhibition or telomerase activation in vegetative tissues by exogenous auxin (Ren et al., 2007).

Here we present a detailed functional analysis of the BT family in Arabidopsis. We show that the expression of specific *BT* genes is upregulated in loss-of-function mutants of other *BT* family members. The subcellular localization of the BT proteins in Arabidopsis protoplasts is either nuclear, nuclear and cytoplasmic or only cytoplasmic, and correlates well with the presence of nuclear localization and export signals in the different BT proteins sequences. In addition, by generating plants containing multiple loss-of-function mutations in the different *BT* genes, we show that this protein family is essential for plant development. Both male and female gametophyte development relies on BT proteins, because strict segregation distortion and gametophyte development defects occurred in plants containing multiple mutations. *BT2* appeared to be the penultimate regulator of gametophyte and potentially plant development, as the absence of *BT2* could not be achieved in the quintuple loss-of-function mutant, nor in the *bt2 bt3* double mutant. Our results demonstrate that BT proteins play an essential role during megagametogenesis, and possibly throughout plant development.

Results

The BT protein family is land-plant specific

In *Arabidopsis thaliana*, the BT family comprises five members. Orthologues of the BT proteins are found in rice (Gingerich et al., 2007), Medicago, red clover, Solanaceae (SOL genomic network, http://www.sgn.cornell.edu) and Physcomitrella, but not in algae, yeast, fungi or animals, indicating that the domain structure of the BT family is restricted to land plants (Figure 1A).

A comparison of the amino acid sequences of BT1 homologues from *Arabidopsis* distinguishes three groups (Figure 1A) (Du and Poovaiah, 2004). The first group comprises BT1 and BT2, the second one BT3 and the last one BT4 and BT5. The sequence identity between the groups in *Arabidopsis* is between 38% and 49%. Interestingly, BT1 and BT2, as well as BT4 and BT5, are part of segmental genome duplications between chromosomes 3 and 5, and chromosomes 4 and 5, respectively (http://www.tigr.org/tdb/e2k1/ath1/duplication_listing.html), something that is reflected in

the exon/intron gene structure (Figure 1B), and suggests that these genes are likely to be functionally redundant. BT1 and BT2 possess, respectively, three and one nuclear localization signals (NLS: aa57-60, aa193-203 and aa342-345, PSORT prediction) (la Cour et al., 2004) and one and two leucine-rich Nuclear Export Signals (NES, NetNES prediction), whereas the three other BT proteins do not contain clear subcellular targeting signals.

Different studies indicate that BT proteins may act as scaffold proteins in various signaling pathways and may be functionally redundant (Du and Poovaiah, 2004, Kemel Zago, 2006, Ren et al., 2007). In order to further investigate the possible function of these proteins in Arabidopsis, lines with T-DNA- or transposon insertions in the corresponding genes were obtained from available collections and loss-of-function of the disrupted gene was verified. Phenotypic analysis indicated that all insertion mutants, shown to be complete loss-of-function, were indistinguishable from their parental wild type ecotype (Figures 1B-G and data not shown). These results corroborate the conclusion drawn from the BT family analysis that there is strong redundancy among BT family members.

Subcellular localization of BT proteins reflects the predicted nuclear im- and export signals

Up to now the subcellular localization of BT proteins have not been analyzed in detail. We therefore made a C-terminal fusion of BT1 to 5 with YFP, and transfected protoplasts. A C-terminal YFP fusion to BT1 transfected in Arabidopsis protoplasts showed that BT1:YFP is cytoplasmic in 38 % of the protoplasts (n = 21, Figure 2A), whereas it is both cytosolic and nuclear in 62 % of the protoplasts (Figure 2B). The predominant BT1 nuclear localization is in line with previous localization studies using tobacco BY-2 cells (Du and Poovaiah, 2004). When the C-terminal YFP fusions for BT2 was expressed in Arabidopsis protoplasts, 90 % (n = 31) of the cells showed cytoplasmic localization (Figure 2C), and 10 % of the cells showed both nuclear and the cytosolic localization (Figure 2D). The less predominant nuclear localization of BT2 compared to BT1 reflects the ratio of NLSs and NESs, which is 3:1 in the case of BT1, and 2:2 in the case of BT2 (Figure 1B), and confirms the functionality of the NLS and NES in BT1 and BT2 (Figure 1B). BT4:YFP and BT5:YFP were only found in the cytoplasm (n = 40 and 33, respectively, Figures 2E and 2F), corresponding to the fact that no NLS is found in these proteins. Western blot analysis showed the integrity of the protoplast expressed BT:YFP fusion proteins (Figure 2H). Unfortunately, we were not able to clone the *BT3* cDNA to complete this analysis, but based on the absence of predicted NLS (Figure 1) we expect this protein to be cytosolic.

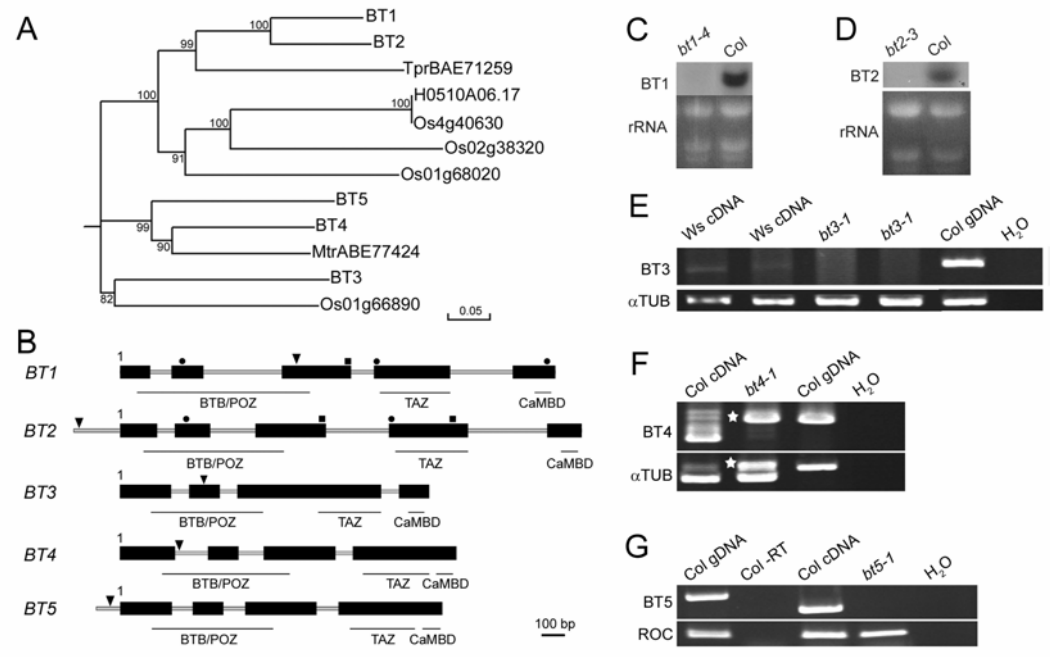

Figure 1. The Arabidopsis BT family comprises 5 members that classify into three groups.

(A) Phylogenic tree showing the relationship between BT1, its four *Arabidopsis* homologous and its orthologues from Rice (Japonica nipponbare and Indica cultivars), *Medicago truncatula* and *Trifolium pratense*. The tree is based on an alignment of the protein sequences using ClustalW and is constructed using the Neighbor-Joining method with a Bootstrap test of 10 000 iterations.

(B) The structure of the five *BT* genes is depicted. The black boxes represent the exons. The parts encoding the N-terminal BTB domain, the TAZ domain and the C-terminal CaMBD are underlined. The positions of predicted Nuclear Localization Signals (NLSs, PSORT prediction) and of the leucine-rich Nuclear Export Signals (NESs, NetNES prediction) are indicated by a circle and a square, respectively. For BT1, three NLSs and one NES are found at positions aa57-60, aa193-203, aa342-345 and aa181-183, respectively. For BT2, two NLSs and two NESs are found at position aa65-81, aa203-212 and aa191-197, aa294-302, respectively The position of the T-DNA or transposon insertion is indicated by a black arrowhead for each gene: *bt1-4* (GT2847), *bt2-3* (SALK_084471), *bt3-1* (Flag_396E01), *bt4-1* (SALK_045370) and *bt5-1* (GABI-Kat 771C08) at position +748 bp, -173 bp, +357 bp, +430 bp and -42 bp relative to the ATG.

(C-G) Northern blots (C, D) and RT-PCR (E-G) analyses on 8-day old seedlings showing the complete loss-of-function of the mutant alleles used in this study for BT1 (C), BT2 (D), BT3 (E), BT4 (F), BT5 (G). Positive controls are the Col and Ws cDNA and genomic DNA (gDNA). Negative controls are a reverse transcription reaction in which the enzyme was omitted (Col -RT) and water (H_2O). rRNA, αTubulin and ROC expression were used as loading controls. Note that for the *bt4-1* sample, some genomic DNA contamination was amplified (stars) but not the cDNA.

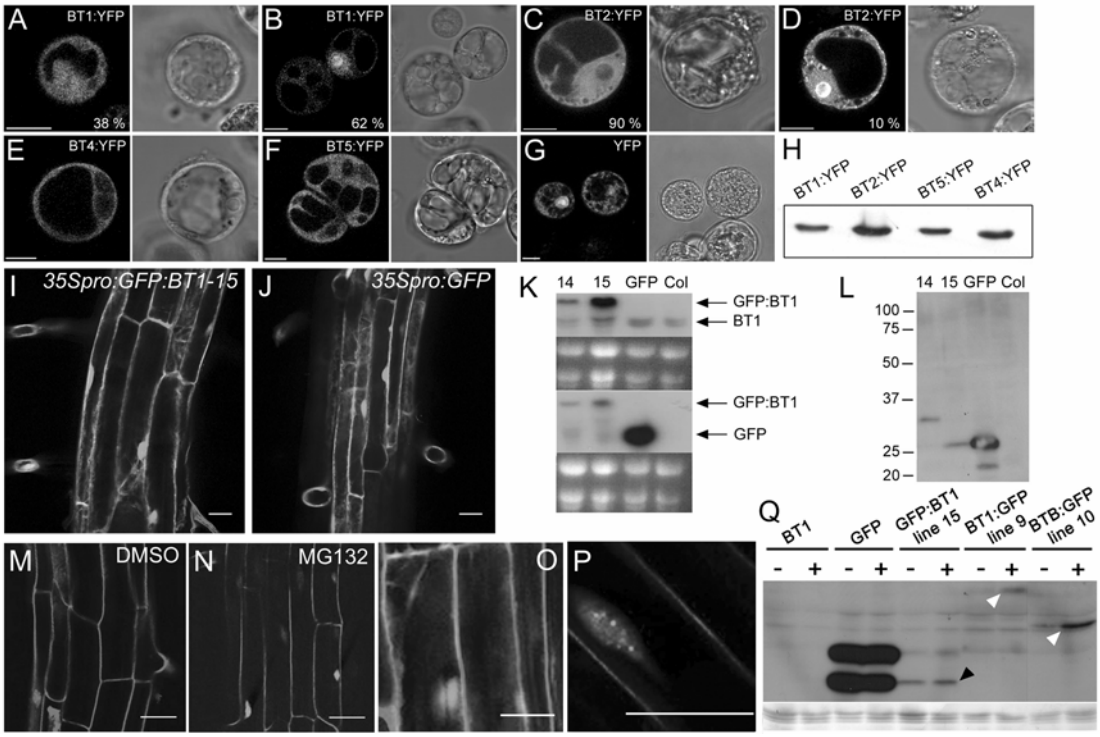

Figure 2. The subcellular localization of Arabidopsis BT proteins.

(A-G) A confocal fluorescent image (left) and the corresponding transmitted light image (right) of Arabidopsis protoplasts expressing C-terminal YFP:HA fusions of BT1 (A, B), BT2 (C, D), BT4 (E) and BT5 (F), or YFP:HA alone (G). For BT1:YFP and BT2:YFP, the percentage of cells showing cytoplasmic (A, C) or both cytoplasmic and nuclear localization (B, D) is indicated. The scale bars represent 10 µm.

(H) Western blot analysis of extracts of cells shown in (A-F) with an anti-HA confirms the expression of full-length BT:YFP fusion proteins.

(I, J) Confocal images of root epidermal cells of the lines *35Spro:GFP:BT1-15* (I) and *35Spro:GFP* (J) show identical cytoplasmic and nuclear localized GFP signal.

(K) Northern blot analysis with the *BT1* (top) or the *GFP* probe (bottom) shows that a full-length *GFP:BT1* mRNA is produced in *35Spro:GFP:BT1* lines 14 and 15.

(L) Western blot probed with anti-GFP antibodies showing that the strong fluorescent signal in lines *35Spro:GFP:BT1-14* and *-15* is caused by respectively a partial GFP:BT1 fusion (30 kDa) or unfused GFP (27 kDa; GFP:BT1 is 69 kDa).

(M-P) BT1:GFP is detected in line *35Spro:BT1:GFP-9* after 4 h of MG132 treatment (N-P), but not in the DMSO treated control (M). MG132 treatment results in a GFP signal in both the cytoplasm (N, O) and the nucleus (N, P).

(Q) Western blot analysis using anti-GFP antibodies confirming the instability of the BT1:GFP and BTB:GFP fusions. Samples were treated 4 h with MG132 (+) or DMSO (-). Plants overexpressing BT1 or GFP were used as negative and positive controls, respectively. Note that the stability of the GFP protein in line *GFP:BT1-15* is not enhanced by MG132 treatment (black arrow head), whereas the full length C-terminal fusions become more abundant after MG132 treatment (white arrow heads).

Scale bars are 10 µm in (A-G) and 20 µm in (I, J, M-P).

BT1 is a short-lived protein in Arabidopsis

Next, we generated stable transformants using both *35Spro:BT1:GFP* and *35Spro:GFP:BT1* constructs. For each construct at least twenty-five independent T2 lines were generated and studied. All lines showed wild-type phenotypes, and none of the *35Spro:BT1:GFP* lines and only few of the *35Spro:GFP:BT1* lines showed a clear cytosolic fluorescent signal in the root (Figure 2I), resembling that of soluble GFP (Figure 2J). Further analysis demonstrated that no full-length fusion protein could be detected in these GFP positive lines, even though a full-length *GFP:BT1* mRNA was produced (Figures 2K and L). The above result together with the lack of fluorescent signal in all the lines carrying the C-terminal fusion (Figure 2M) suggested to us that BT1 and the BT1:GFP fusions were unstable. To test this, seedlings expressing BT1:GFP were treated with the 26S proteasome inhibitor MG132. After 4 hours of treatment, a fluorescent signal was observed both in the nucleus and the cytoplasm of root cells of different lines (Figures 2N-P). Under higher magnification, the nuclear localization was not uniform but consisted of bright dots (Figure 2P) possibly corresponding to MG132-stabilized BT1:GFP present in nuclear bodies of the ubiquitin-proteasome pathway (Tao et al., 2005). Western blot analysis confirmed the presence of the full-length fusion protein in the MG132 treated samples (Figure 2Q). Although the fusion protein in untreated samples could be detected by Western blot, the amount was not sufficient to be observed by confocal microscopy. Different treatments with auxin or auxin transport inhibitors did not influence the stability, nor the subcellular localization of the BT1:GFP fusion protein in the MG132-treated samples (data not shown). Fluorescent microscopy on other tissues than the root (leaves, inflorescences) did not identify tissue-specific stabilization of the BT1:GFP fusion. In conclusion, our *in planta* results indicate that BT1 is a short-lived target for the 26S proteasome pathway, and that this predominantly nuclear protein is also present in the cytoplasm.

Also plants overexpressing a C-terminal fusion of the BTB domain of BT1 with GFP (*35Spro:BTB:GFP*) were indistinguishable of wild type plants. The BTB:GFP fusion protein, like BT1:GFP, was not detectable by confocal microscopy or Western blotting in untreated plants, but could be stabilized by MG132 treatment (Figure 2Q). The subcellular localization of stabilized BTB:GFP is identical to that observed for BT1:GFP (Figures 2M-P), showing predominant nuclear (the NLSs aa57-60 and aa192-203 are included in the fusion) but also cytoplasmic localization (data not shown). These data corroborate our observations that BT1 is an inherently unstable protein, and indicate that its proteasome-mediated degradation is linked to the N-terminal BTB domain-containing part of the protein.

"Expression compensation" among BT family members possibly mediated by auxin

In addition to the subcellular localization of the BT proteins, we investigated the expression of Arabidopsis *BT* family members by Northern blot analysis (Figure 3A). The four *BT* genes analyzed are most abundantly expressed in rosette leaves. *BT1 and BT5* are also strongly expressed in stems, and at low levels in the other tissues tested. *BT2* is highly expressed in seedlings, whereas *BT4* is strongly expresses in flowers and siliques.

Interestingly, *bt1-4* loss-of-function caused a clear increase of expression of *BT2* and *BT5* in seedlings, roots, stems and flowers, whereas *BT4* expression was enhanced in stems. Likewise, *bt2* loss-of-function enhanced the expression of *BT1* and *BT5* in roots, whereas *BT5* expression in stems was significantly reduced (Figure 3A). The observed *bt* loss-of-function induced changes in gene expression of *BT* family members indicates that the genes are under reciprocal transcription regulation, where the expression of one gene has a direct compensatory effect for the loss of another *BT* gene expression. This could be through a direct effect of the BT proteins on the transcription of the other BT family members, or through indirect feed-back control on *BT* expression.

Recently, evidence was presented that BT2 potentiates auxin responses (Ren et al., 2007), and this opens the possibility that the feed back control on *BT* expression is mediated by auxin. We therefore tested whether the *BT* genes are auxin responsive. *BT1*, *BT2* and *BT5* showed a rapid and transient induction of expression, at different strength levels, with a peak at 30 minutes after auxin (IAA) treatment. *BT4* expression was also enhanced at this time point, but the RNA levels increased until 4 hours, and enhanced levels persisted at least until 24 hours after auxin treatment (Figure 3B). These results indicate a clear link between auxin signaling and *BT* gene expression, and suggest that the reciprocal transcription regulation within the *BT* gene family is at least in part mediated by feed back control on auxin signaling.

BT proteins are essential for gametophyte development

Since the single loss-of-function mutants did not show a phenotype, mutant combinations were generated to bypass the possible functional redundancy of the BT proteins and show their relevance for plant development. Unfortunately, the different combinations of double and triple mutants produced were indistinguishable from wild type (data not shown). To definitively establish or distinguish whether BT proteins are essential for plant development, the generation of a quintuple mutant was initiated by crossing a triple, *bt1 bt2 bt4*, and a double mutant, *bt3 bt5*. In the progeny of this cross, only two combinations of homozygous quadruple mutants out of the five possible could ever be obtained: *bt1 bt2 bt4 bt5* and *bt1 bt3 bt4 bt5*. This implied that quadruple mutants where *bt2* and *bt3* alleles were both homozygous could not be obtained. Furthermore in an attempt to identify the quintuple loss-of-function mutant, only *bt1 bt2/+ bt3/+ bt4 bt5* and *bt1 bt2/+ bt3 bt4 bt5*

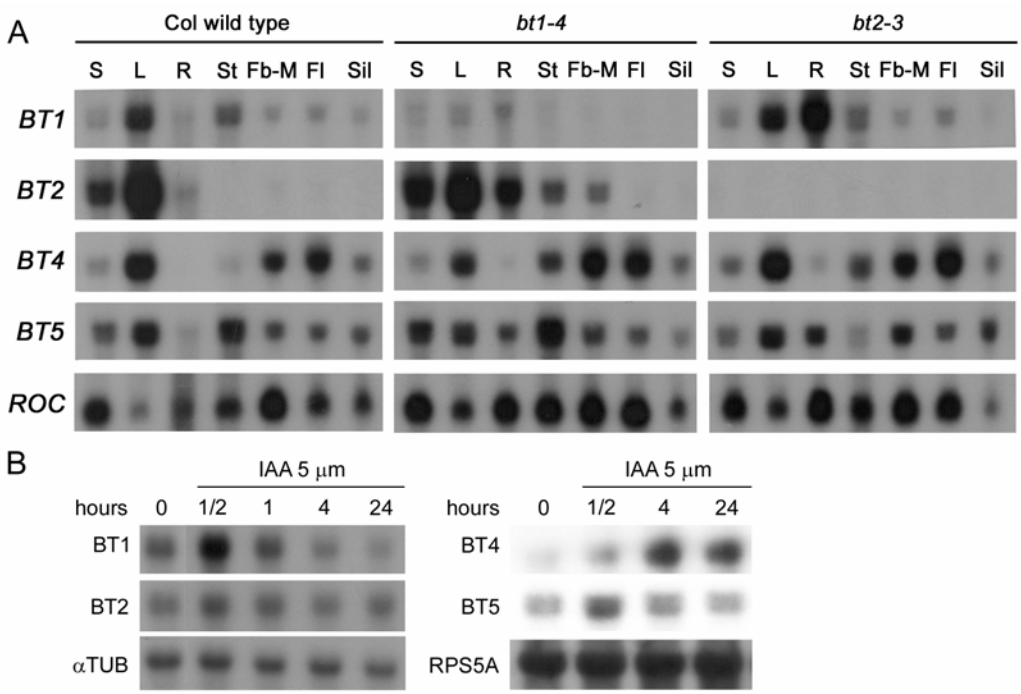

Figure 3. *BT* gene expression shows reciprocal transcriptional regulation

(A) Northern blot analysis of the expression pattern of four of the *BT* genes (*BT1*, *BT2*, *BT4*, *BT5*) in 8-day old seedlings (S), or rosette leaves (L) and roots (R) of 3-week old seedlings or stems (St), flower buds and inflorescence meristems (Fb-M), flowers (Fl) and siliques (Sil) from 6-week old plants of Col wild-type (right), *bt1-4* (middle) and *bt2-3* (left). The *ROC* expression was used as control. The blots probed with *BT1* and *BT2* were exposed overnight, with *ROC* for 48 hours and with *BT4* and *BT5* for 72 hours.

(B) Northern blot analysis of the BT gene expression (*BT1, BT2, BT4, BT5*) after auxin (5 µm IAA) treatment for the indicated time. The *αTubulin* and *RPS5A* expression were used as control. The blot probed with *BT1* was exposed for 48 hours, with *BT2* for 6 hours, with *BT4*, *BT5*, *αTubulin* and *RPS5A* for 16 hours.

were obtained. As *BT2* and *BT3* are located on chromosomes 3 and 1, respectively, physical linkage can not explain the absence of seedlings carrying both insertion alleles. The self-pollinated population of *bt1 bt2/+ bt3/+ bt4 bt5* was screened in order to analyze the observed segregation distortion. In over 200 seedlings that were screened from this genotype (Table 1), neither quintuple homozygous mutants, nor *bt1 bt2 bt3/+ bt4 bt5* seedlings were found. As the expected segregation ratio for these genotypes is 1/16 and 2/16, respectively, their absence from the test population is highly significant. Furthermore, quadruple mutants homozygous wild type for either *BT2* or *BT3* were obtained in a higher proportion than expected (25 % and 15 % against 6.3 %, respectively, Table 1). Allele frequencies for the other possible genotypes were in range with the expected ratios.

Table 1. Segregation ratio of the progeny of the quintuple *bt1 bt2/+ bt3/+ bt4 bt5* mutant.

bt1 bt2/+ bt3/+ bt4 bt5 progeny	observed proportion	expected proportion
bt1 bt2 bt3 bt4 bt5	**0 %**	**6.3 %**
bt1 bt2 bt3/+ bt4 bt5	**0 %**	**13 %**
bt1 bt2 BT3 bt4 bt5	**15 %**	**6.3 %**
bt1 bt2/+ bt3 bt4 bt5	**8 %**	**13 %**
bt1 bt2/+ bt3/+ bt4 bt5	32 %	25 %
bt1 bt2/+ BT3 bt4 bt5	10 %	13 %
bt1 BT2 bt3 bt4 bt5	**25 %**	**6.3 %**
bt1 BT2 bt3/+ bt4 bt5	**6 %**	**13 %**
bt1 BT2 BT3 bt4 bt5	5 %	6.3 %

Based on this analysis we hypothesized that it is impossible to produce gametes containing both *bt2* and *bt3* loss-of-function mutations. To confirm this hypothesis, reciprocal backcrosses between *bt1 bt2/+ bt3/+ bt4 bt5* and Col wild type were analyzed. Genotyping of the F1 progeny of the two reciprocal backcrosses showed a segregation ratio of 1:2:1 for the gamete genotypes *BT2 bt3* : *BT2 BT3* : *bt2 BT3* ($n = 96$, Chi-square < 9.348 for $p = 0.01$ in both cases), whereas the *bt2 bt3* genotype was never found. The results suggest that the mutations do not lead to embryo arrest, since plants heterozygous for both mutations are fully viable, and indicate that the single *bt2* and *bt3* mutations hamper gametophyte development, thus leading to segregation distortion, and that megagametogenesis and microsporogenesis, respectively female and male gametophyte development, arrest when both loss-of-function mutations are present. Similar segregation ratios have been observed for other mutations affecting both male and female gametophyte development (Yadegari and Drews, 2004).

To confirm the results of the segregation and backcrosses analyses, embryo and ovule development was studied in *bt1 bt2/+ bt3/+ bt4 bt5* plants. Cleared siliques of *bt1 bt2/+ bt3/+ bt4 bt5* showed empty spaces (Figures 4A and 4C), whereas *bt1 bt3 bt4 bt5* siliques contained full seed set (Figures 4A and 4B). Closer inspection showed that in *bt1 bt2/+ bt3/+ bt4 bt5* siliques, about 70 % of the ovules were not fertilized and desiccated, and that the seed set in these plants was reduced to 30 % (Figure 4C and Table 2). The embryos and endosperm in the seeds that succeeded to develop were normal (data not shown). All these data are consistent with two mutations affecting female gametophyte development (Christensen et al., 1998). As a result of the reduced seed set, the silique length was significantly reduced in *bt1 bt2/+ bt3/+ bt4 bt5* plants (8.7 mm +/- 1.6 mm, $n = 7$), compared to Col wild type (15 mm +/- 1.3 mm, $n = 5$, Student's t-test, $p < 0.01$) or *bt1 bt3 bt4 bt5* plants (14.6 mm +/- 1.7 mm, $n = 5$, Student's t-test, $p < 0.01$) (Table 3).

Table 2. Seed set in the different mutant combinations.

	seeds per silique(%)[1]	s. d.	n
Col	96.5	3.8	2
bt2	95.8	4.1	4
bt1/+ bt2	92	9.2	1
bt1 bt2/+	**50.1**	10.5	5
bt1 bt2	98.3	2.8	2
bt1 bt5	98.8	1.4	1
bt1 bt2 BT3 bt4 bt5	93.6	5.7	11
bt1 BT2 bt3 bt4 bt5	96.6	5.2	7
bt1 bt2/+ bt3/+ bt5	**31.4**	10.1	3
bt1 bt2/+ bt3/+ bt4 bt5	**30**	6	10

[1] mean of the developed seeds versus total seed per silique for four siliques per plant

Table 3. *bt* mutant has a reduced silique length.

	silique length (mm)*	s. d.	n
Col	15	1.3	5
bt1 BT2 bt3 bt4 bt5	14.6	1.7	5
bt1 bt2/+ bt3/+ bt4 bt5	8.7[*] [†]	1.6	7

[*] Significantly different from Col wild type (Student's t-test, $p < 0.01$)

[†] Significantly different from *bt1 BT2 bt3 bt4 bt5* (Student's t-test, $p < 0.01$)

BT function essential during early stages of female gametophyte development

To analyze during which stages of megagametogenesis BT function is essential, we searched mature ovules at terminal development stage (FG7) (Christensen et al., 1997) for aberrant morphology of the female gametophyte. Unfertilized ovules were dissected out of emasculated carpels and observed by confocal microscopy after propidium iodide staining. Wild type gametophytes are highly polarized structure made of seven cells (Figures 4D and 4E). At the micropyle pole (bottom on Figures 4D-J), two synergid cells are surrounding the egg cell. The nuclei of the synergids are facing the micropyle whereas the nucleus of the egg cell is located toward the chalazal pole (top left on Figures 4D-J). In the middle of the gametophyte, a bi-nucleate cell with a huge vacuole is forming the central cell composed of two fused nuclei, called polar nuclei, facing the egg cell. Three remaining cells, the antipodal cells, are located at the chalazal pole and will degenerate during the final stages of megagametogenesis (Figures 4D and 4E) (Christensen et al., 1997, Drews and Yadegari, 2002).

In the quintuple *bt1 bt2/+ bt3/+ bt4 bt5* gynoecium, besides wild type gametophytes, three different mutant phenotypes were observed. First, gametophytes with unfused polar nuclei

were found (9 out of 42 [counts include the wild type phenotype] counted on two siliques, Figures 4F and 4G). Unfused polar nuclei can also be found in immature gametophytes (stage FG5) and in mutants of the category 4 such as *magatama1* and *3*, *gametophytic factor2* (*gfa2*), and *gfa3* (Christensen et al., 1998, Shimizu and Okada, 2000, Drews and Yadegari, 2002) or seed shortly after fertilization (stage F2) (Faure et al., 2002). Therefore either these ovules are delayed in growth, or their polar nuclei failed to fuse. In some other gametophytes, the synergid cells were collapsed (7 out of 42, Figure 4H). Collapse of the synergid cells does not occur if pollination is prevented, but it is usually observed at the time of, or shortly before, the fertilization in wild type seeds due to the pollen tube penetration and discharge of the sperm cells (Faure et al., 2002). Viable synergid cells are required for the pollen guidance and attraction to the gametophyte for a proper fertilization (Higashiyama et al., 2001). The segregation distortion observed in *bt1 bt2/+ bt3/+ bt4 bt5* plants may in part be explained by a reduction of the pollen tube attraction due to the collapsed synergid cells in some of the gametophytes. Finally the last and more striking phenotype was a total absence of gametophyte (19 out of 42 [7 out of 42 were n/a], Figures 4I and 4J) and the presence of some degenerated structures instead of the synergid cells, suggesting that the defect occurred very early during the megagametogenesis. Based on the segregation ratios of this last phenotype, it is likely that the quintuple *bt* loss-of-function mutant belongs to the category 1 of gametophytic mutations, such as *gfa4, gfa5*, *female gametophyte2* and *3* (Christensen et al., 1998, Drews and Yadegari, 2002) , where the mutants are affected at the earliest step of the gametophyte development and do not progress after the one-nuclei stage (FG1) (Christensen et al., 1997). The presence of the BT proteins seems to be essential during early stages of female gametophyte development. The male gametophyte development was not analyzed in detail but according to the results obtained in the backcrosses analysis, it is likely that BT function is also essential during early stages of male gametophyte development.

BT2 ***acts redundantly with*** **BT3** ***and*** **BT1** ***during female gametophyte development***

Because it appeared that the gametophytic phenotype in the quintuple *bt* mutant was caused by the absence of *BT2* and *BT3* expression, as the combination of *bt2* and *bt3* loss-of-function could not be obtained, we analyzed more in detail whether the female gametophyte development was also impaired in the different mutant combinations we had generated. Reduced seed set and shorter siliques were observed in *bt1 bt2/+, bt1 bt2/+ bt3 bt4 bt5 and bt1 bt2/+ bt3/+ bt5* plants (Table 2), indicating that the phenotype is more strongly linked to *bt2* loss-of-function. Interestingly, however, the siliques were full in the quadruple *bt1*

Figure 4. (continued)
small arrow), or is completely absent except for a white fluorescent structure at the micropyle pole (I,J). MC: micropyle pole, CHZ: chalazal pole, PN: polar nuclei, EC: egg cell, Sy: synergid cell, UPN: unfused polar nuclei, CSy: collapsed synergids. Scale bars represent 20 µm.

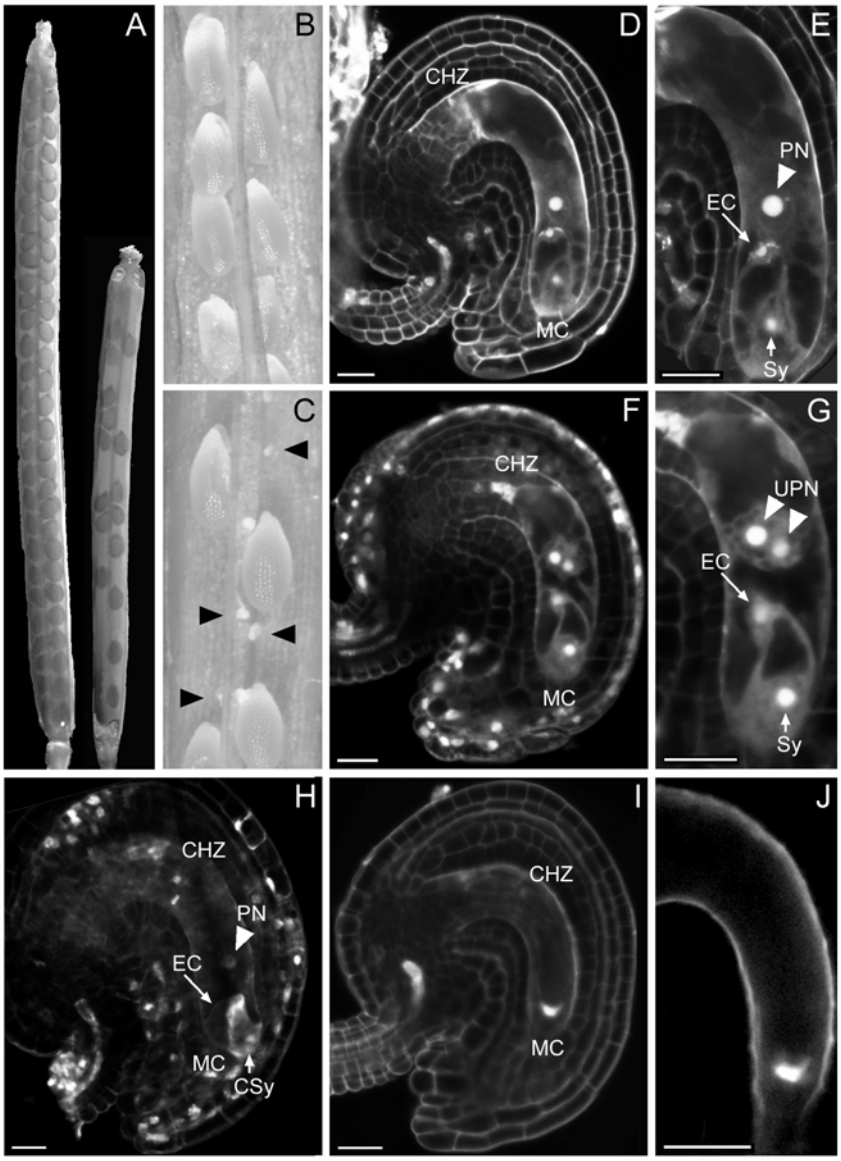

Figure 4. *bt* loss-of-function is gametophytic lethal.

(A) Cleared siliques of a *bt1 bt2*/+ *bt3*/+ *bt4 bt5* plant (right) show many random empty positions, whereas a silique of a *bt1 bt3 bt4 bt5* plant (left) shows full seed set. Note that the *bt1 bt2*/+ *bt3*/+ *bt4 bt5* silique is significantly shorter than the one with full seed set.

(B-C) Details of opened siliques of a *bt1 bt3 bt4 bt5* plant (B) and a *bt1 bt2*/+ *bt3*/+ *bt4 bt5* plant (C). The empty spaces in the quintuple mutant are from unfertilized ovules, as indicated by the dried and white structures (black arrowheads).

(D-J) Confocal sections of unfertilized female gametophytes at mature stage (FG7) stained with propidium iodide. (D, E) Mature wild type female gametophytes are characterized by fused polar nuclei (arrowhead), the egg cell (large arrow) and synergid cells (small arrow). (F-J) *bt1 bt2*/+ *bt3*/+ *bt4 bt5* ovules in which the female gametophyte shows unfused polar nuclei (double arrowheads) in the central cell (F,G), collapsed synergid cells (H,

bt2 bt4 bt5, suggesting in this case that *BT3* is the redundant copy of *BT2*.

To test whether *BT2* and *BT3* are the crucial and redundant *BT* genes during female gametophyte development, the progeny of a cross between *bt2* and *bt3* were analyzed. Remarkably, only crosses with *bt3* as pollen donor and *bt2* as ovule acceptor did lead to germinating seeds. The short silique and reduced seed set phenotypes were observed in 14 plants out of 20 of the F2 progeny of *bt2/+ bt3/+* F1 plants (not shown). This corroborates our hypothesis that the absence of these two genes is sufficient to induce defective female gametophyte development, and also indicates that the three other *bt* genes are not able to fully complement the double mutant. The 14 plants showing the phenotype were double heterozygous for *bt2* and *bt3*. The 6 remaining wild type looking F2 plants were homozygous for one of the *bt* mutations and wild type for the other *BT* gene, confirming that the two mutations can only coexist in one plant when heterozygous.

Interestingly, when we compared seed set in *bt2*, *bt1 bt2* or *bt1 bt2/+* plants, the first two mutants showed full siliques, whereas the seed set was reduced to 50 % in *bt1 bt2/+* plants (Table 2). These results indicate that expression compensation by *BT1* (Figure 3) is needed to functionally replace BT2 in the *bt2/+* background. Apparently, the *bt2/+* heterozygous situation is not sufficient to trigger the full expression compensation by *BT3* that is clearly observed in the *bt1 bt2* double and *bt1 bt2 bt4 bt5* quadruple mutant plants. As suggested by the possibility to get the double homozygous *bt1 bt2* mutant and by the segregation analysis of the quintuple mutant *bt1 bt2/+ bt3/+ bt4 bt5*, the penetrance of the gametophytic phenotype is partial in double, triple and quadruple mutants because of functional redundancy, until the complete gene family is knocked out. Then the phenotype is fully penetrant and gametophytic lethal.

Discussion

BTB domain proteins form a large family of scaffold proteins that are found in a wide range of organisms. Here we studied the function of a specific subfamily, the BT proteins, in Arabidopsis. Based on their structure BT proteins are land plant-specific BTB domain proteins. Previous work indicated that these proteins are multi-functional scaffolds that interact with a variety of other proteins such as a protein kinase, transcriptional factors and cytoskeleton motor proteins (Du and Poovaiah, 2004, Kemel Zago, 2006). BT2 was found to be involved in the induction of the telomerase activity ((Du and Poovaiah, 2004, Kemel Zago, 2006, Ren et al., 2007). In addition, we have shown that PBP2/BT1 interacts with and regulates the activity of PID, a protein kinase that directs the transport of the plant hormone auxin (Chapter 5, this thesis, Friml et al., 2004). Here we present a detailed functional analysis of the Arabidopsis *BT* gene family, and demonstrate that there is considerable functional redundancy among the family members. Detailed analysis of

Arabidopsis plants segregating loss-of-function mutations of the five *BT* genes indicated that the BT proteins are essential for gametophyte development, and probably also for other stages of plant development.

Redundancy of the BT proteins

Since the loss-of-function allele *bt1-4* is wild type looking, functional redundancy among the BT protein family was hypothesized and demonstrated using expression pattern analysis and a genetic analysis with T-DNA/transposon insertion lines. First, each of the *BT* genes is expressed in a large panel of tissues where their respective expression pattern overlaps rather than being tissue-specific. Secondly, these expressions are cross-regulated within the gene family, since in the loss-of-function mutants *bt1-4* and *bt2-3*, the absence of *BT1* and *BT2* expression, respectively, is compensated by the other functional family members, which may explain the absence of phenotype in these mutants. Similar redundancy and cross-regulation of gene expression have previously been observed for the *PIN* auxin efflux carrier gene family (Vieten et al., 2007). For the *PIN* genes, expression compensation is achieved via their auxin responsiveness, since *pin* loss-of-function leads to alterations in auxin distribution. The *BT* genes were also found to be auxin responsive, and given that BT2 was found to alter auxin responses, and that BT1 to regulate the activity of PID (Chapter 5, this thesis), a protein kinase regulating auxin transport, it is likely that the reciprocal transcriptional regulation of the *BT* genes is auxin-controlled. Finally, to get insight in the function of the BT proteins, single insertion lines which disrupt gene expression of each of the five genes were isolated. None of them as well as combinations of double, triple or quadruple mutants with *bt1-4*, *bt3-1*, *bt4-1* and *bt5-1* resulted in mutant phenotypes. Combinations with *bt2-3* showed an alteration in the gametophyte development which was fully penetrant only when the *bt3-1* mutation was present. A total absence of the BT proteins in the plant is lethal since it results in an absence of female gametophyte formation. These data together indicate that the BT protein family is able to compensate for the lost of one of the family members and is functionally redundant.

***Impaired gametophytic development in* bt1 bt2/+ bt3/+ bt4 bt5**

Our attempt to knock-out BT function using Arabidopsis plants segregating for multiple *bt* loss-of-function mutations demonstrated that the BT2 protein is essential for female and male gametophyte development, and that in the mutant background either *BT1* or *BT3* can compensate for *bt2* loss-of-function. *bt2* loss-of-function could co-exist with the *bt3-1* allele in plant only in the heterozygous state, but their co-occurrence in the haploid megaspore resulted in an aberrant gametophyte development (Drews and Yadegari, 2002). Ovules are developed from a diploid megaspore that undergoes meiosis giving rise to four haploid megaspores from which three die. The remaining one (stage FG1) follows three round of mitosis and becomes an eight-nucleate cell (stage FG5), which, after nuclear

migration and fusion, and cellularization, turns into a seven-cell structure (stage FG7) (Christensen et al., 1997). Segregation of *BT2* and *BT3* in *bt1 bt2/+ bt3/+ bt4 bt5* gave rise to four different kinds of female gametophytes: (1) wild type looking, presumably derived from *bt1 BT2 BT3 bt4 bt5* megaspores; (2) with unfused polar nuclei and (3) with collapsed synergid cells, presumably derived from *bt1 BT2 bt3 bt4 bt5* or *bt1 bt2 BT3 bt4 bt5* megaspores*;* and (4) with an empty embryo sac, most likely derived from *bt1 bt2 bt3 bt4 bt5* megaspores. According to the segregation analysis, it seems likely that the three first phenotypes are able to develop normally to give fertile ovules, whereas the last one collapsed. Additional backcrosses analysis indicated that it is likely that the pollen microspore with *bt1 bt2 bt3 bt4 bt5* haplotype is not able to develop either. Together these data suggest that the BT proteins are essential during female and male gametophyte development, probably as early as the first meiosis.

BT proteins and the telomerase activity in gametes

Recently, the *BT2* gene has been shown to be directly and specifically induced by TELOMERASE ACTIVATOR1 (TAC1) transcriptional factor, indicating that BT2 is required for the activation of the telomerase activity in Arabidopsis mature leaves (Ren et al., 2007). Telomerases are enzymes that synthesize and maintain telomeric DNA at the end of the chromosomes, mainly during the transition between the vegetative to reproductive phase, meaning in the gametophytes (Riha and Shippen, 2003). Telomerase activity is usually absent in leaves but can be induced by exogenous auxin as well as by TAC1 overexpression (Ren et al., 2004). And BT2 is sufficient and necessary to stimulate TAC1-dependent telomerase activity in leaves by inducing the expression of *Arabidopsis thaliana TELOMERASE REVERSE TRANSCRIPTASE* (*ATTERT*) (Ren et al., 2007). One hypothesis could be that the gametophyte phenotype observed in the quintuple *bt* mutant is caused by the absence of telomerase activity. However, Arabidopsis *attert* mutants can survive up to the tenth generation without telomerase activity, during which they accumulate phenotypes from leaf morphology aberration at the sixth generation to vegetative termination at the tenth generation (Riha et al., 2001). In contrast, *bt1 bt2/+ bt3/+ bt4 bt5* plants are initially wild type looking, but their defected gametophyte development eventually causes a reduction in seed set and silique length. Quintuple homozygous plants could not be produced. Hence the gametophyte lethality observed in the quintuple homozygous *bt* mutant was probably not the direct result of the absence of activation of *ATTERT* expression by BT2, but rather the consequence of a more general function of these proteins as scaffolds in a variety of signaling pathways.

BT proteins are multifunctional scaffold proteins

Functional analyses of some BTB proteins in yeast and *C. elegans* indicated that these proteins are involved in targeting proteins for degradation as part of CULLIN3 (CUL3)

containing E3 Ubiquitin ligases (Geyer et al., 2003, Furukawa et al., 2003, Pintard et al., 2004, Moon et al., 2004). In such E3 ligases, the BTB protein acts as a scaffold protein that interacts with CUL3 through the BTB domain, and selects the target proteins for ubiquitination through its affinity for the second protein-protein interaction domain (Krek, 2003, Moon et al., 2004). In Arabidopsis, members of several BTB proteins subfamilies, such as NPH3 and the BTB-MATH protein family, were found to interact with CUL3, but for the BT clade the reports are contradictory (Wang et al., 2004, Dieterle et al., 2005, Figueroa et al., 2005, Gingerich et al., 2005, Weber et al., 2005). Moreover, in a yeast two-hybrid screen with BT1, we have not identified CUL3 as BT1 interactors (Kemel Zago, 2006). Interestingly, the experiments presented here indicate that the BT1 protein itself is a target for degradation by the 26S proteasome pathway, as the proteasome inhibitor MG132 is able to stabilize the protein. The BT1 instability and degradation by the 26S proteasome could be part of a feed back regulation of BT1 function. Considering the variety of interacting proteins identified for the BT proteins, the presence of two protein-protein interaction domains in the structure and the drastic effect of the loss of the BT function in Arabidopsis, it is likely that BT proteins are multifunctional scaffolds that act in, or maybe even interconnect, multiple cellular pathways. The finding that BT proteins interacts with PID through their BTB domain (Chapter 5), that BT2 has been found to potentiate auxin responses (Ren et al., 2007), and that the reciprocal transcriptional control between *BT* genes is possibly mediated by auxin (this chapter), make it tempting to hypothesize that their key role is to modulate auxin distribution by regulating the activity of the PID protein kinase. Further research on this hypothesis is presented in Chapter 5 of this thesis.

Material and methods

Arabidopsis lines, plant growth, transformation and protoplast transfections

The loss-of-function *bt2-3* (SALK_084471) and *bt4-1* alleles (SALK_045370) were obtained from NASC (Alonso et al., 2003). The loss-of-function *bt1-4* allele (Ds transposon line GT2847) was obtained from the Cold Spring Harbor Laboratory (Sundaresan et al., 1995). The loss-of-function *bt3-1* allele (Flag 396E01) was obtained from INRA (Versailles, France) (Samson et al., 2002). The loss-of-function *bt5-1* allele *(*GABI-Kat 771C08) was provided by MPI for Plant Breeding Research (Cologne, Germany) (Rosso et al., 2003). For detection of the insertion, we used gene-specific primers 5'TCTCTTCCGCCAGGTAAAAA3' and 5'CGCAAGACTCGTTGGAAAAG3' for *pid-14*, 5'TTCTCCGAGGTTCGTCTTTC3' and 5'GGACACGGCAAGATTCAGAT3' for *bt1-4,* 5'TCATGATCTCCACGGACCAAA3' and 5'GGACGGACATTGCGACAAGA3' for *bt2-3,* 5'TGAGGTTGCATCAGATTAGGG3' and 5'TCATCACTTTCCATCCCTCTG3' for *bt3-1*; 5'CACAACACATCTCATTTCTCCGC3'

and 5'TACATTAAGCTCGTAAGCGACAGA3' for *bt5-1*, 5'GGCTAAAGAATCGACAATAT3' and 5'TACGGTGAGATATGAGGCTA3' for *bt4-1* and the insertion-specific primers LBaI, Ds3-2, LB4 and GABI-LB for respectively the SALK, the Ds transposon, the FLAG and the GABI-Kat lines (Sundaresan et al., 1995, Samson et al., 2002, Rosso et al., 2003, Alonso et al., 2003). The flanking region of each insertion was sequenced to confirm the insertion position and Northern blot or RT-PCR analysis was performed to determine if the insertion resulted in a complete loss-of-function allele.

Arabidopsis seeds were surfaced-sterilized by incubation for 15 min in 50 % commercial bleach solution and rinsed four times with sterile water. Seeds were vernalized for 2 to 4 days before germination at 21°C with a 16-hour photoperiod and 3000 lux on solid MA medium (Masson and Paszkowski, 1992) supplemented with antibiotics when required. Two- to three-week old plants were transferred to soil and grown at 21°C with a 16-hour photoperiod of 10000 lux and at 70 % relative humidity.

Protoplasts were obtained from *Arabidopsis thaliana* Col cell suspension cultures that were propagated as described (Schirawski et al., 2000). Protoplast isolation and PEG-mediated transfections were performed as initially described (Axelos et al., 1992) and adapted by Schirawski and coworkers (Schirawski et al., 2000). Transfections were performed with 10 µg of plasmid DNA, after which the cells were incubated for at least 16 h prior observation using confocal laser scanning microscopy.

Molecular cloning and constructs

Molecular cloning was performed following standard procedures (Sambrook et al., 1989). Bacteria were grown on LC medium containing 100 µg/ml carbenicillin (Cb) or 50 µg/ml Km for *E. coli* strains DH5α or Rosetta (Novagen) containing typical high copy cloning plasmids or the binary vector pCambia1300, respectively, or 20 µg/ml rifampicin and 50 µg/ml Km for Agrobacterium strains containing binary vectors. The *BT1* cDNA was amplified, using the Expand High Fidelity kit (Roche) from a cDNA batch made from Arabidopsis seedlings, using primers 5'CC-*Sal*I-GCTATAAACCGCCACTCA3' and 5'CCGGAACAAGTTAATGTGA-*Pst*I-AA3'. The amplified cDNA was cloned in the *Sal*I and *Pst*I sites in pBluescript-SK+ giving rise to pSDM6014. The *BT2* cDNA was amplified from pUNI10183 (Yamada et al., 2003) with the primers 5'G-*Eco*RI-ATGGAAGCTGTTCTTGTCGC3' and 5'CG-*Bam*HI-TTAAACCCCTTGTGCTTGTT3' and cloned (*Eco*RI-*Bam*HI) into pUC28, giving rise to pSDM6069. The *BT4* cDNA was cloned (*Stu*I-*Nco*I) from pUNI13579 (Yamada et al., 2003) into pUC28 giving rise to pSDM6092. For the sub-cellular localization analysis in protoplast cells, *35Spro:BT1:YFP*, *35Spro:BT2:YFP*, *35Spro:BT4:YFP and 35Spro:BT5:YFP* were constructed using the Gateway Technology (Invitrogen). Genes of interest were PCR amplified from pSDM6014, pSDM6069, pSDM6092 and BX827434 (Castelli et al., 2004), respectively with primers

containing *att*B recombination sites (underlined): 5'GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGGCTATAACCGCCACT3' and 5'GGGGACCACTTTGTACAAGAAAGCTGGGTACATTAACTTGTTCCGGAT3' for BT1; 5'GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGGAAGCTGTTCTTGTC3' and 5'GGGGACCACTTTGTACAAGAAAGCTGGGTAAACCCCTTGTGCTTGTTC3' for BT2; 5'GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGGTGACAGGTTGTGTT3' and 5'GGGGACCACTTTGTACAAGAAAGCTGGGTAACAGTTTGTCACCGGTAA3' for BT4 and 5'GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGGAGAACATGGACGAT3' and 5'GGGGACCACTTTGTACAAGAAAGCTGGGTATAAAGTAACATCAATTGC3' for BT5. BP reactions were performed in pDONOR207 according to manufacturer's instructions (Invitrogen). Recombinant plasmids were isolated and sequenced. LR reactions were performed in pART7 plasmids containing a Gateway cloning cassette in frame with a YFP:HA tag coding region (C. Galván-Ampudia, unpublished data). For the *35Spro:BT1* construct (pSDM6086), the *BT1* cDNA was cloned as a *SalI-SpeI* fragment from pSDM6014 into the pCambia1300int-35Snos binary vector. To create *35Spro:GFP:BT1* (pSDM6025), the *BT1* coding sequence was cloned as a *Xho*I-*Sma*I fragment from pSDM6014 in pTH2 (Chiu et al., 1996). For *35Spro:BT1:GFP* (pSDM6063), the complete cDNA of *BT1*, including the start codon and excluding the stop codon, was amplified from pSDM6014 using the M13 forward primer and BT1-R-minusTGA-*Sal*I (5'TAGC-*Sal*I-CATTAACTTGTTCCG3'). The amplified fragment was cloned as *Sal*I fragment in pTH2 (pSDM6062). For *35Spro:BTB:GFP* (pSDM6066), the BTB domain containing part of BT1 was cloned as a *Nco*I fragment from pGEX-BT1 into pTH2 (pSDM6098). The N- and C-terminal *GFP* fusions were cloned as *Eco*RI-*Hin*dIII fragments into the binary vector pCambia1300.

Northern blot analysis

Auxin treatments were done on 8-day old Col wild type seedlings with 5 µm IAA for the indicated time. Total RNA was purified using the RNeasy Plant Mini kit (Qiagen). Subsequent RNA blot analysis was performed as described (Memelink et al., 1994) using 10 µg of total RNA per sample. The following modifications were made: pre-hybridizations and hybridizations were conducted at 65^{o}C with 10 % Dextran sulfate, 1 % SDS, 1 M NaCl, 50 µg/ml of single stranded Herring sperm DNA as hybridization mix. The hybridized blots were washed for 20 min at 65^{o}C in 2x SSPE 0.5 % SDS, and for 20 min at 42^{o}C in respectively 0.2x SSPE 0.5 % SDS, 0.1x SSPE 0.5 % SDS and 0.1x SSPE. Blots were exposed to X-ray film FUJI Super RX. Probes were PCR amplified and column purified (Qiagen) using primers 5'CATCCCAAACATTACAAAGGGC3',

5'TTCTCCGAGGTTCGTCTTTC3' for *BT1* from pSDM6006, *5'CTCACCATGGGTTTCCACAAGATCATA3', 5'GGCAACATCCTGGGGCACAA3' for GFP from pSDM6025,* 5'TGTTCCTCACCACGCTCTTC3', 5'TGCTCAAAACATTCCTCACC3' for *BT5, ,* 5'GACGCCGAATCGGTAAACT3', 5'TTTGTAATCGCGAAACAACGGA3' for *BT2*, 5'GGCTAAAGAATCGACAATAT3', 5'TACGGTGAGATATGAGGCTA3' for *BT4,* 5'CTCTCATTCGCGCGACGCAAACG3', 5'GGGTTCAAGTCAGACAAGAGGTGG3' for *RPS5A* and 5'CGGGAAGGATCGTGATGGA3', 5'CCAACCTTCTCGATGGCCT3' for *AtROC* from Col wild type genomic DNA. Probes were radioactively labeled with α-^{32}P-ATP (Amerscham) using the Prime-a-gene kit (Promega).

RT-PCRs were performed as described (Weijers et al., 2001) using 10 µg of total RNA from 8-day old seedlings for the RT reaction. The PCR reactions were performed with one tenth of the RT volume with the same gene specific primers used for the probe amplification in the Northern blot analysis and with 5'ATGTCTAGTAGTACCAAGAACATTCCAAAAC3', 5'TATCAAACCAGAAGAACGTGACGAG3' for *BT3* and 5'CGGAATTCATGAGAGAGATCCTTCATATC3', 5'CCCTCGAGTTAAGTCTCGTACTCCTCTTC3' for *αTubulin*. A RT reaction from Col seedlings RNA in which the reverse-transcriptase was omitted served as a negative control.

Western blot analysis

To check YFP-tagged proteins integrity, total protein was extracted from transfected protoplasts. Half volume of the transfected cells were pelleted at full-speed at 4^{o}C, resuspended in 30 µl of 1x Laemmeli sample buffer and boiled for 5 min. Proteins (half volume) was separated on a SDS-PAGE gel (10 %) with PageRuler Prestained Protein Ladder (Fermentas) as a size marker. To analyze the GFP-tagged BT1 in Arabidopsis, total protein was extracted from 7-day old seedlings as previously described (Kurata et al., 2005). The protein concentration was determined by Bradford assay and per sample 40 µg of protein was separated on a SDS-PAGE gel (12 %) with PageRuler Prestained Protein Ladder (Fermentas) as a size marker. A parallel gel was run and stained with Coomassie to correct for loading differences. Gels for Western blot analyses were transferred to nitrocellulose membranes (Immobilon-P, Millipore) which were incubated with rat HRP-conjugated anti-HA (3F10) antibody (1/2000, Roche) or rabbit anti-GFP primary antibody (1/5000, Molecular Probes) and an anti-rabbit horse radish peroxidase (HRP)-conjugated secondary antibody (1/5000, Promega). Detection followed the protocol of the Phototope-HRP Western Blot Detection Kit (New England Biolabs).

Microscopy and phenotypic analysis

The 26S-proteasome inhibitor MG132 (Sigma) was used at 50 µM during 4 h. Propidium iodide (0.1 µg/ml in distilled water) was used to stain the cell walls of living cells and the nuclei of the female gametophyte. The female gametophyte phenotypes and GFP fusion lines were observed using 40x dry and oil objectives on a ZEISS Axioplan microscope equipped with a confocal laser scanning unit (MRC1024ES, BIO-RAD, Hercules, CA). The GFP fluorescence was monitored with a 522-532 nm band pass emission filter (488 nm excitation). Propidium iodide was visualized using the 585 nm long pass emission filter (568 nm excitation). All images were recorded using a 3CCD Sony DKC5000 digital camera. For the subcellular localization of the BT proteins in protoplast cells, a Leica DM IRBE confocal laser scanning microscope was used with a 63x water objective. The fluorescence was visualized with an Argon laser for excitation at 514 nm and with a 522-532 nm (YFP) emission filter. Images were processed by ImageJ (http://rsb.info.nih.gov/ij/) and assembled in Adobe Photoshop 7.0.

Silique seed set was determined in cleared siliques after treatment with a derivative of Hoyer's solution (Boisnard-Lorig et al., 2001) using a Leica MZ12 stereomicroscope equipped with a 3CCD Sony DKC-5000 digital camera. Quantification of seed set was performed by slitting opened siliques for at least 4 siliques of 5 plants per genotype and counting the number of seeds and ovules. Silique length was measured for 10 mature siliques per plant.

Accession Numbers

The *Arabidopsis* Genome Initiative locus identifiers for the genes mentioned in this article are as follows: *BT1/PBP2* (At5g63160), *BT2/PBP2H1* (At3g48360), *BT3/PBP2H2* (At1g05690), *BT4/PBP2H4* (At5g67480), *BT5/PBP2H3* (At4g37610), *ROC* (At4g38740), *αTubulin* (At5g44340), *RPS5A* (At3g11940), *NPH3* (At5g64330), *RPT2* (At2g30520), *CUL3a* and *b* (At1g26830 and At1g69670), *BET10/GTE11* (At3g01770), *BET9/GTE* (At5g14270) and *TAC1* (At3g09290). The TIGR *Rice* Genome initiation locus identifiers for the japonica nipponbare cultivar *BT* family genes used the Figure 1B are as follows: *Os01g66890*, *Os01g68020*, *Os02g38320* and *Os04g40630*. The GeneBank locus names of the *BT* homologues are H0510A06.17 for the rice indica cultivar *BT* gene, AC146856.8 (protein ABE77424) for the *Medicago truncatula BT* gene and AB236807 (protein BAE71259) for the *Trifolium pratense BT* gene.

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