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BABY BOOM target genes provide diverse entry points into cell proliferation and cell growth pathways

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Abstract Ectopic expression of the *Brassica napus* BABY BOOM (BBM) AP2/ERF transcription factor is sufficient to induce spontaneous cell proliferation leading primarily to somatic embryogenesis, but also to organogenesis and callus formation. We used DNA microarray analysis in combination with a post-translationally regulated BBM:GR protein and cycloheximide to identify target genes that are directly activated by BBM expression in Arabidopsis seedlings. We show that BBM activated the expression of a largely uncharacterized set of genes encoding proteins with potential roles in transcription, cellular signaling, cell wall biosynthesis and targeted protein turnover. A number of the target genes have been shown to be expressed in meristems or to be involved in cell wall modifications associated with dividing/growing cells. One of the BBM target genes encodes an ADF/cofilin protein, ACTIN DEPOLYMERIZING FACTOR9 (ADF9). The consequences of BBM:GR activation on the actin cytoskeleton were followed using the GFP:FIMBRIN ACTIN BINDING DOMAIN2 (GFP:FABD) actin marker. Dexamethasone-mediated BBM:GR activation induced dramatic changes in actin organization resulting in the formation of dense actin networks with high turnover rates, a phenotype that is consistent with cells that are rapidly undergoing cytoplasmic reorganization. Together the data suggest that the BBM transcription factor activates a complex network of developmental pathways associated with cell proliferation and growth.

Keywords AP2/ERF transcription factor · BABY BOOM · Arabidopsis · Somatic embryo · Actin

Abbreviations

GR	Glucocorticoid receptor
CHX	Cycloheximide
DEX	Dexamethasone
EFS	Embryo forming structure
2,4-D	2,4-Dichlorophenoxyacetic acid
qRT-PCR	Quantitative real-time RT-PCR

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Introduction

The ability to regenerate whole plants via tissue culture is a well-described and much applied phenomenon. The most commonly used methods for plant regeneration from somatic cells involve culturing explants on medium containing growth regulators to induce callus formation, followed by organogenesis or embryogenesis from the dedifferentiated callus (de Klerk et al. 1997). In some systems, growth regulators are applied, but the callus phase is bypassed in favor of direct regeneration. Dissecting the phenomenon of in vitro regeneration at the molecular-genetic level has been difficult due to the length of the regeneration process and the many developmental events that take place as explants dedifferentiate and then redifferentiate new tissues and organs. Research in Arabidopsis has greatly facilitated our knowledge on multiple aspects of the regeneration process, including how competent cells are formed and induced to regenerate (Che et al. 2006, 2007). Mutant screens and functional studies have been particularly helpful in identifying proteins that control developmental pathways leading to both direct and indirect regeneration, either by promoting spontaneous (hormone-free) regeneration or by stimulating regeneration under sub-optimal conditions (Chuck et al. 1996; Gallois et al. 2002; Banno et al. 2001; Zuo et al. 2002; Marsch-Martinez et al. 2006). Not surprisingly many of these genes normally function in hormone-dependent pathways to control cell proliferation, the establishment of meristem identity and/or organ identity.

Among the proteins controlling regeneration are those that induce somatic embryogenesis from competent somatic cells. The BABY BOOM (BBM) AP2/ERF domain protein is a seed and root-meristem expressed transcription factor that was identified as marker for embryo development in *Brassica napus* microspore-derived embryo cultures (Boutilier et al. 2002), as a gene showing preferential expression in the basal region of the Arabidopsis embryo (Casson et al. 2005), and as an auxin-inducible root expressed gene in *Medicago truncatula* (Imin et al. 2006). Ectopic expression of *BBM* in Arabidopsis primarily induces spontaneous somatic embryo formation from seedlings, although ectopic shoots and callus also develop at a lower frequency (Boutilier et al. 2002). In tobacco heterologous BBM expression induces spontaneous shoot and callus formation, while a cytokinin pulse is required for somatic embryo formation (Srinivasan et al. 2007).

Ectopic expression of the seed-specific CCAAT-box binding factor LEAFY COTYLEDON1 (LEC1) and the B3 domain transcription factor, LEC2 also promote somatic embryo formation on seedlings (Lotan et al. 1998; Stone et al. 2001). Loss-of-function *lec1 and lec2* mutants are impaired in embryo identity and maturation, indicating their broad role as regulators of embryo development (reviewed in Harada 2001). These *lec* mutants are also strongly impaired in their ability to form somatic embryos in response to 2,4-dichlorophenoxyacetic acid (2,4-D; Gaj et al. 2005). Other such proteins play a broader role in controlling cell identity and differentiation. The VP1/ABI3-LIKE (VAL) proteins are a class of B3 domain proteins with a possible chromatin-related repressor function. Double *val1 val2* or *val1 val3* mutants and single *val1* mutants treated with GA biosynthesis inhibitors develop embryonic characteristics on seedlings, a phenotype that correlates with derepression of *LEC1* and other genes encoding embryo-expressed B3 domain factors (Suzuki et al. 2007). The WUSCHEL (WUS) homeodomain protein specifies stem cell fate in the shoot and floral meristem (Laux et al. 1996; Mayer et al. 1998), but also promotes somatic embryo development in seedlings when ectopically expressed (Zuo et al. 2002; Gallois et al. 2004). WUS function is not directly linked to embryo identity, but rather to the maintenance of an undifferentiated cell state that responds to different stimuli to change the developmental fate of tissues (Gallois et al. 2002, 2004). Loss-of-function mutants of the PICKLE (PKL) CHD3 chromatin-remodeling factor convert seedling roots into embryogenic tissues (Ogas et al. 1999). PKL activity is not restricted to seedlings, as *pkl* mutants also exhibit GA-deficient shoot phenotypes (Ogas et al. 1997; Henderson et al. 2004), as well as ectopic activation of primordium and meristem identity genes in the carpel (Eshed et al. 1999).

It is becoming clear that the developmental pathways in which some of these embryo promoting/repressing genes function are interconnected (Ogas et al. 1999; Zuo et al. 2002; Rider et al. 2003; Harding et al. 2003). Dissecting the complexity of these networks is a major challenge that requires the detailed analysis of each individual pathway. Here we provide insight into BBM-mediated control of plant regeneration through the identification of candidate direct target genes in Arabidopsis. We show that ectopic expression of a BBM:GR fusion protein activates expression of a diverse collection of largely uncharacterized genes, many of which are associated with cell proliferation and growth pathways.

Materials and methods

Transformation and plant material

The ligand-binding domain of the rat glucorticoid receptor (GR; Lloyd et al. 1994) was cloned in frame at the 3′ end of the *B. napus BBM1* coding region (AF317904) in the *35S::BBM1* binary vector (Boutilier et al. 2002) to create

35S::BBM:GR. The *35S::BBM:GR* chimeric gene was then cloned together with the bar marker gene in the polylinker between the T-DNA borders of pGSC1700 (Cornelissen and Vandewiele 1989) to create *pTLH250*. Arabidopsis ecotype C24 was used as the background for all experiments. Plant transformations were carried out using *Agrobacterium tumefaciens* C58C1 strain MP90, essentially as described in Clough and Bent (1998).

For dexamethasone and cycloheximide treatments, dexamethasone (DEX) was dissolved in either 70% (v/v) ethanol or 0.1% DMSO and cycloheximide (CHX) in 70% (v/v) ethanol. The percentage of embryo forming seedlings in DEX-induced *35S::BBM:GR* lines is reduced by up to 80% when using bleach vapor sterilized seeds, therefore all seeds were sterilized in liquid bleach. *35S::BBM:GR* seeds for the time course induction were sterilized, imbibed for 4 days at 4°C and then transferred to 8 cm diameter glass jars containing 75 ml 0.5× MS-10 medium. The jars were placed on a rotating platform (30 rpm) under standard growth conditions. DEX was added to the medium at defined days after the start of culture. The number of embryo-forming seedlings was counted 14 days after the addition of DEX.

In vitro grown material was cultured in a controlled environment room at 23°C on a 16 h/8 h day/night cycle at 3,300 lux and 55% relative humidity. For the microarray experiments, sterile wild-type and *35S::BBM:GR* seeds were germinated on a 150 micron nylon mesh placed on top of 0.5× MS-10 agar in 9 cm Petri dishes. Four days later, the nylon mesh with the germinated seedlings was transferred to a 9 cm Petri dish containing 5 ml water supplemented with DEX and CHX or the corresponding amount of 70% ethanol. Seedlings were incubated with gentle rotation for 8 h. To ensure the DEX responsiveness of the *35S::BBM:GR* transgenic lines, the same seed batch was germinated directly on solid 0.5× MS-10 medium containing 10 μM DEX and then scored for somatic embryo formation.

The Arabidopsis *pickle* mutant (*pkl1-1*) was obtained from the Arabidopsis Biological Resource Center (stock number CS3840).

Microarray experiments

Two independent *35S::BBM:GR* lines were used (biological replicates) and seedlings from each of the two lines were plated in duplicate (technical replicates) and each replicate treated with DEX + CHX. Technical replicates were not performed for the two untreated *35S::BBM:GR* control lines. DEX + CHX-treated wild-type seedlings were harvested from multiple dishes and then pooled for analysis, while untreated (control) wild-type seedlings were harvested from two replicated dishes and analyzed independently. Total RNA was isolated using the RNeasy Plant Mini kit (Qiagen).

For target labeling, 1 μg of seedling total RNA was linearly amplified in the presence of 5-(3-aminoallyl)-UTP (Sigma-Aldrich) using the MessageAmp™ aRNA Kit (Ambion). Cy3 and Cy5 mono-reactive Dyes (Amersham Biosciences) were coupled to the amplified RNA (aRNA) in 0.1 M sodium carbonate buffer, pH 9.3 for 30 min at room temperature. Labeled aRNA was fragmented prior to hybridization using RNA Fragmentation Reagents (Ambion). RNA amplification, labeling and fragmentation efficiencies were monitored by agarose gel electrophoresis and by measurement of the Cy5 and Cy3 fluorescence emissions at 635 and 532 nm, respectively, using a Molecular Imager FX Pro Plus scanner (BioRad). Labeled target samples were hybridized to a 26,000 element Arabidopsis oligonucleotide microarrays (Qiagen-Operon Arabidopsis Genome Oligo Set Version 1.0), provided by the University of Arizona. Immobilization of the oligonucleotide array elements was performed as described at http://ag.arizona.edu/microarray/protocol1.doc. Slides were pre-hybridized at 50°C in 120 μl SlideHyb™ Glass Array Hybridization Buffer (1 (Ambion) using the HybArray 12™ hybridization station (Perkin Elmer Life and Analytical Sciences). The pre-hybridization mixture was replaced after 2 h by 120 μl pre-warmed SlideHyb™ Glass Array Hybridization Buffer (1 (Ambion) containing heat-denatured labeled target (2.5 μg Cy3-labeled aRNA and 1.25 μg Cy5-labeled aRNA) and incubated overnight at 50°C. The slides were then washed at room temperature down to 0.1× SSC in the hybridization station and scanned separately for the two fluorescent dyes with a GenePix® 4000B scanner (Axon Instruments). The integrated optical density of each probe was measured using the AIS software (Imaging Research). Normalization was performed using the median of the background corrected ratios for all spots excluding negative controls and blanks. Differential expression in each of the six DEX + CHX-treated 35S::BBM:GR versus DEX + CHX-treated wild-type seedling microarray hybridizations, expressed as the 2Log value of the normalized signal ratios, was tested for statistical significance using a simple *t*-test per clone ($P = 0.05$), an Analysis of Variance (Anova) and Significance Analysis of Microarrays (Tusher et al. 2001). The microarray data is deposited in NCBI's Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) and is accessible through GEO Series accession number GSE3399.

Real-time quantitative RT-PCR

Total RNA was DNaseI-treated for 30 min at 37°C using RQ1 RNase-free DNase (Promega) in the presence of RNaseOUT™ Recombinant Ribonuclease Inhibitor (Invitrogen).

After phenol-chloroform extraction and ethanol precipitation, 1 μg of DNase-treated RNA was reverse-transcribed with either an oligod(T) primer or random hexamers using TaqMan® Reverse Transcription Reagents (Applied Biosystems) according to the manufacturer's instructions. The cDNA was used as a 160× diluted stock for PCR amplification. Gene-specific primer pairs were designed to span an intron and to generate circa 150 bp products (Primer3: http://biotools.umassmed.edu/bioapps/primer3_www.cgi). The primer sequences are listed in Supplementary Table 1. The annealing specificity of the primers was verified using a BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/), by PCR amplification of Arabidopsis genomic DNA and by sequencing of the PCR products. DNA amplification efficiencies were assessed for each primer pair using a cDNA dilution series under Real-Time PCR conditions (MyiQ™ Single-Color Real-time PCR Detection System with the iQ SYBR Green Supermix; Bio-Rad). PCR reactions were carried out in a reaction volume of 20 μl using the following cycling parameters: 95°C for 3 min, 40 cycles consisting of 95°C for 15 s and 60°C for 1 min. PCR amplification was followed by a DNA melting curve analysis (Ririe et al. 1997) to verify primer specificity.

A ΔΔCt calculation (Livak and Schmittgen 2001) was used for validation of the Arabidopsis microarray data. cDNA was synthesized using an oligo(dT) primer and the expression level of each target gene in seedlings from the four replicated *35S::BBM:GR* samples was determined using the *ACTIN2* and *ACTIN8* genes as the reference (An et al. 1996) and the DEX + CHX-treated wild type seedling sample as the calibrator.

To determine the developmental expression profiles of the candidate BBM target genes, cDNA was synthesized using random hexamers and the expression level of each target gene was normalized using 18S rRNA as the reference (ΔCt calculation). The relative expression level for each gene in the series of different tissues was calculated by subtracting the lowest ΔCt value from each ΔCt value in the series, converting the obtained values from a 2log scale to the original scale and then normalizing these values to the largest value (100%) in the series.

Relative *ADF9* expression in DEX-treated *35S::BBM:GR* was determined using a $2^{-\Delta\Delta Ct}$ calculation (C value or fold change) in which DEX-treated wild-type seedlings were used as the calibrator and 18S rRNA as the reference.

The standard deviations were calculated according to the Applied Biosystems manual "Guide to Performing Relative Quantitation of Gene Expression Using Real-Time Quantitative PCR."

Microscopy and data analysis

Confocal analysis of actin organization and dynamics was performed using an inverted microscope (Axiovert 200M, Zeiss) connected to a Zeiss LSM510 META confocal scanning system. A Zeiss 63× α-Plan Fluar oil objective (NA 1.45) was used for all imaging experiments. For confocal imaging, we used a 30 mW Ar-laser (488 nm) set to 3–5% with dichroic mirror DM488/543 and band pass emission filter EM BP505-530. A pinhole of 1 Airy Unit (~1 μm) was used. Maximal projections were made of collected Z-stacks using the Zeiss LSM Image Browser software (version 4.0.0.157). Images were pseudo-colored and overlays were produced in Image J version 1.38a (National Institutes of Health, USA; http://rsb.info.nih.gov/ij).

Results

De novo induction of somatic embryogenesis by BBM

A glucocorticoid-regulated *B. napus* BBM protein (BBM:GR) was used in combination with microarray analysis to identify candidate genes that are directly activated by BBM. The ability of the *35S::BBM:GR* construct to induce somatic embryogenesis in Arabidopsis seedlings was verified by phenotypic observation of *35S::BBM:GR* seeds that were germinated and grown in the presence of 10 μM dexamethasone (DEX). As was shown for *35S::BBM* seedlings (Boutilier et al. 2002), we observed somatic embryo formation on the cotyledons, first leaves and shoot meristems of DEX-treated *35S::BBM:GR* seedlings (Fig. 1a). This phenotype was observed in 5 of the 15 tested *35S::BBM:GR* T1 lines. *35S::BBM:GR* seedlings that were not treated with DEX (Fig. 1b) and DEX-treated wild-type seedlings (not shown) did not show any obvious morphological abnormalities. The penetrance of the somatic embryo phenotypes in two of the five embryogenic *35S::BBM:GR* T1 lines was extremely high with respect to the percentage of embryo forming seedlings (EFS) and ranged from 80 to 100% for both lines depending on the seed batch. The activation of the BBM:GR fusion protein was phenotypically visible as early as 4 days after seed plating; DEX-treated *35S::BBM:GR* seedlings at this stage were smaller and more compact than wild-type or non DEX-treated seedlings, and retained embryonic features, such as a thickened non-elongated hypocotyl, fleshy cotyledons and lack of root outgrowth, as previously described (Boutilier et al. 2002). In the two highly penetrant lines, the seedling root consisted entirely of small dense green cells that resemble those seen in *pkl* roots (Fig. 1a; Ogas et al. 1997). These pickle-like roots were rarely observed in *35S::BBM* transgenic lines. Non-embryogenic DEX-treated *35S::BBM:GR* seedlings exhibit the same pleiotropic phenotypes as previously described for *35S::BBM* lines (Boutilier et al. 2002), including cell proliferation at the root-hypocotyl transition zone leading to callus

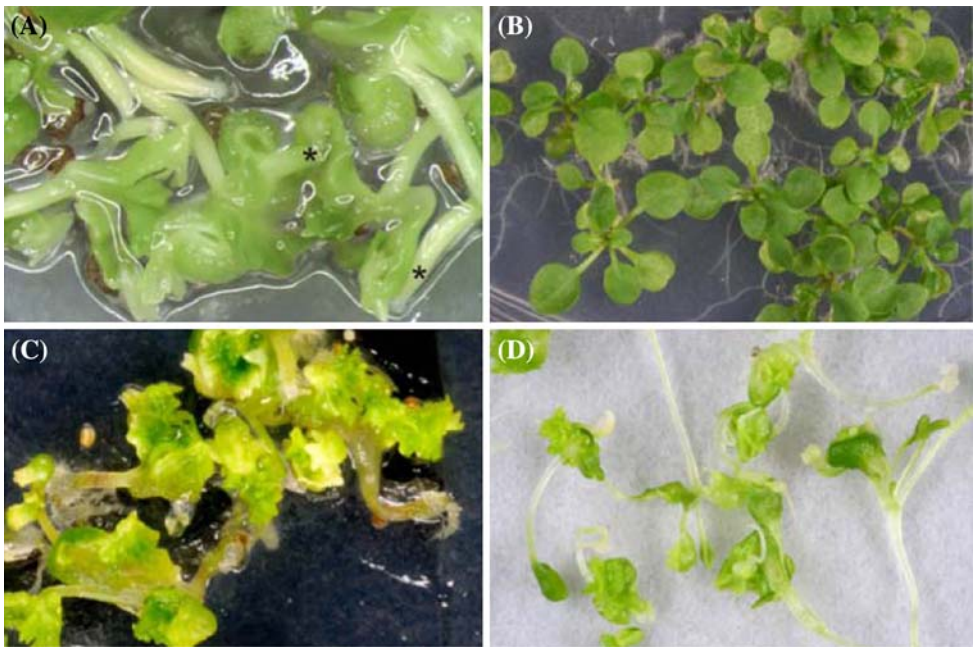

Fig. 1 *35S::BBM:GR* DEX-dependent somatic embryo formation in *Arabidopsis*. (**a**) Embryogenic seedlings of a DEX-induced *35S::BBM:GR* line. *pickle*-like roots are prevalent (*). (**b**) Wild-type looking *35S::BBM:GR* seedlings develop in the absence of DEX treatment. (**c**) Embryogenic seedlings of a *35S::BBM* line. (**d**) Embryogenic response of *35S::BBM:GR* seedlings grown for 4 days in liquid culture on minimal media, followed by culture in liquid media containing DEX. All seedlings were photographed 14 days after the start of culture

formation, lobed cotyledons and occasional ectopic shoots (data not shown).

The developmental competence of Arabidopsis seedlings to respond to the BBM signal and form somatic embryos was examined using a time-course experiment in which 10 μM DEX was added on successive days to *35S::BBM:GR* seeds or seedlings growing in liquid culture. As shown in Table 1, mature seeds and developing seedlings continued to form somatic embryos in response to a de novo BBM signal up to at least 6 days after the start of the culture period. DEX application up to 2 days after the start of the culture led to the production of ball-like clusters of somatic embryos and no or minimal leaf outgrowth, while seedlings exposed to DEX from the fourth day of culture onward showed leaf outgrowth and developed small clusters of somatic embryos or single somatic embryos on the cotyledon/first leaf edge (Fig. 1d).

Table 1 Embryogenic competence of *Arabidopsis* seedlings

DEX addition (day)	% EFS
0	100 ($n = 222$)
2	99 ($n = 167$)
4	79 ($n = 211$)
6	13 ($n = 158$)
8	0 ($n = 108$)

Ten micromolar dexamethasone (DEX) was added to *35S::BBM:GR* seeds growing in liquid medium on the indicated day after the start of the culture period

The number of seedlings counted (n) for each treatment is indicated

EFS, Embryo-forming seedlings

BBM activates a diverse set of target genes

We used the approach described by Lloyd et al. (1994) to identify candidates for direct downstream BBM targets. In this system, dexamethasone (DEX) and cycloheximide (CHX) are applied together to respectively, induce nuclear localization of the BBM:GR protein and prevent translation of the primary target mRNAs. Direct downstream targets were identified by comparing gene expression profiles between DEX + CHX-treated *35S::BBM:GR* seedlings and DEX + CHX-treated wild-type seedlings. Non-treated wild-type and *35S::BBM:GR* seedlings were used as controls to determine respectively, whether the DEX + CHX treatment affected expression of any of the candidate target genes and whether any of the candidate target genes was activated by the BBM:GR protein in the absence of DEX. The experimental set-up of the microarray hybridizations is shown in Fig. 2. We treated four-day-old wild-type and *35S::BBM:GR* seedlings for 8 h with 10 μM DEX and 10 μM CHX and compared their mRNA populations using Arabidopsis long oligo microarrays. The two independent lines used in this analysis showed 100% EFS when germinated and grown continuously on media with DEX. Four-day-old seedlings were chosen as the experimental material because they are highly responsive to BBM:GR activation and provide sufficient RNA for the microarray experiments.

Significance Analysis of Microarrays (Tusher et al. 2001) identified 2449 probes (with an estimate of 4.8 false positives) showing significant differential expression in all six DEX + CHX-treated *35S::BBM:GR* seedling versus DEX + CHX-treated wild-type seedling microarray

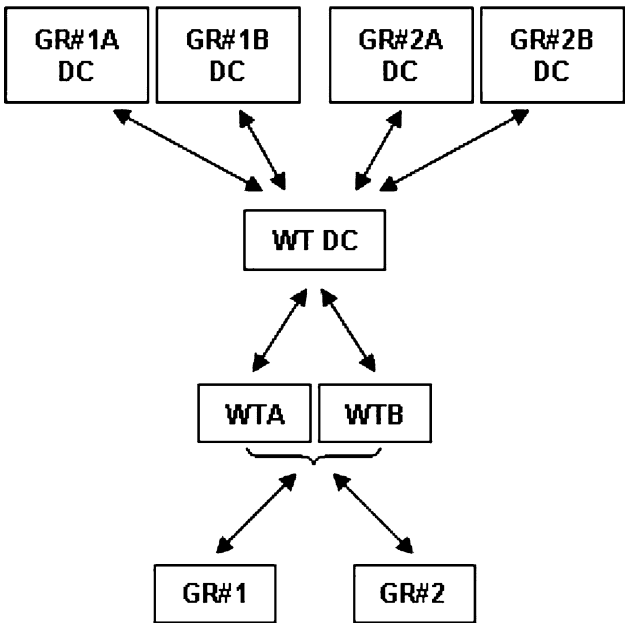

Fig. 2 Microarray hybridization set-up. A dual-colored microarray hybridization scheme was used in which two amplified target samples (boxed samples joined by double-headed arrows) were hybridized together on one array. Two independent single insertion *35S::BBM:GR* lines were used (GR1 and GR2). Lines grown in duplicate and for which RNA was isolated, amplified and hybridized independently are denoted 'A' and 'B.' Samples treated with dexamethasone (D) and cycloheximide (C) are indicated by 'DC.' Reciprocal hybridizations (dye-swaps) were performed for one technical replicate of each of the two *35S:BBM-GR* DC-treated lines. The remaining hybridizations were carried out in one direction only

analyses. Approximately half of the differentially expressed probes were up-regulated in DEX + CHX-treated *35S::BBM:GR* seedlings as compared to DEX + CHX-treated wild-type seedlings. In consideration of the large number of significantly up-regulated probes, we also performed a t-test and an Anova. Limiting the number of candidate BBM targets to those probes that were significantly up-regulated in all three statistical analyses and which also showed a four-fold upregulation (2Log ratio $\geq$ 2) reduced the number of up-regulated probes to 139 (Supplementary Table 2). The large number of significant differentially expressed genes is a consequence of the low variance between replicates (data not shown). The number of candidate probes presented here was arbitrarily reduced by selecting the 43 most highly expressed probes (Table 2). With the exception of BBM (see below), none of these 43 candidate target genes was significantly expressed in non-treated *35S::BBM:GR* lines (GEO Series accession number GSE3399).

Quantitative real-time RT-PCR (qRT-PCR) was used to validate the BBM-activated expression of 22 of the selected target genes (Table 2). Expression of two of these genes, a SNF7 protein (At5g44560) and a CCR4-NOT transcription complex protein (At5g10960), was up-regulated in DEX + CHX-treated *35S::BBM:GR* seedlings as compared to DEX + CHX-treated wild-type seedlings, but not above the arbitrary four-fold cut-off ($\Delta\Delta$Ct values $\geq$2). The remaining 20 genes were up-regulated by at least four fold in both transgenic lines as compared to the wild-type. The relative increase in gene expression measured for these 20 probes by qRT-PCR was comparable to that observed in the microarray hybridizations.

The Arabidopsis *BBM* gene (*AtBBM*, At5g17430) was identified as one of the probes that is significantly up-regulated upon BBM:GR activation. The *BBM* probe on the microarray does not discriminate between mRNAs from the *35S::BBM:GR* transgene and the endogenous Arabidopsis *BBM* gene, therefore we performed qRT-PCR with *AtBBM* specific primers on the same samples used for the microarray experiments. *AtBBM* expression does not differ significantly between untreated or DEX + CHX-treated wild-type and *35S::BBM:GR* samples (data not shown), but increases approximately 60 fold in the DEX + CHX-treated *35S::BBM:GR* seedlings compared to DEX + CHX-treated wild-type seedlings (Table 2). The data suggest that the increase in *BBM* expression observed after DEX + CHX treatment of the *35S::BBM:GR* lines results from a positive feedback loop involving transcriptional activation of the Arabidopsis *BBM* gene by the *B. napus* BBM:GR protein.

To facilitate classification of the BBM target genes, gene annotations were retrieved from The Arabidopsis Information Resource (TAIR; http://www.arabidopsis.org) and subsequently refined based on the identified InterPro domains (http://www.ebi.ac.uk/interpro/). The majority of BBM targets fall into one of four major function/process categories: transcription, signaling, protein–protein interactions, and cell wall/cell membrane localized proteins (Table 2). Probes corresponding to proteins involved in transcription and signal transduction processes account for about one quarter of the BBM target genes indicating that BBM occupies a hierarchically high position in the developmental pathways in which it functions. Six of the predicted BBM primary targets encode enzymes involved in cell wall modification (pectate lyase, xyloglucan endotransglycosylase, *O*-methyltransferase, laccase-like multicopper oxidase, polygalacturonase, endo-β-1,4-glucanase), suggesting that BBM also acts directly at the level of basal cellular processes.

The probes assigned to protein–protein interaction category are particularly interesting in that a number of them may play a role in ubiquitination. Proteins containing BTB-POZ (Bric a brac, Tramtrack, Broad complex/Pox virus and Zinc finger) domains, RING (Really Interesting New Gene) and U-box domains have been shown to function as E3 ligases or as subunits of multisubunit E3 ligase

Table 2 Candidate BABY BOOM target genes

Rank	AGI No.	Annotation	Microarray (2Log ratio)	*t*-test (*P*-value)	qRT-PCR (ΔΔCt)
Transcription					
1	At5g17430	BABY BOOM (BBM)	6.08 ± 0.09	6.42E-06	5.98 ± 1.00
4	At5g39820	NAC domain protein (ANAC094)	4.89 ± 0.04	1.10E-06	6.02 ± 0.48
6	At1g16070	Tubby family protein (TLP8)	4.78 ± 0.07	8.46E-06	8.40 ± 0.09
19	At1g65300	MADS-box protein (PHERES2)	3.46 ± 0.51	7.72E-04	n.d.
22	At5g46640	AT-hook protein (AHL8)	3.28 ± 0.11	8.07E-05	3.52 ± 0.23
23	At3g60580	Zinc finger protein C2H2-type	3.27 ± 0.08	3.68E-05	3.76 ± 0.14
37	At1g51140	Basic Helix-Loop-Helix protein (BHLH122)	2.95 ± 0.08	3.89E-05	3.43 ± 0.25
39	At5g10960	CCR4-NOT transcription complex protein	2.92 ± 0.22	9.77E-04	0.59 ± 0.30
Signaling					
9	At5g45780	Leucine-rich receptor-like kinase, LRRII group	4.39 ± 0.14	6.53E-05	5.14 ± 0.26
10	At2g34020	Calcium-binding EF hand protein	4.14 ± 0.06	6.72E-06	5.66 ± 0.21
13	At4g11320	Cysteine proteinase	3.69 ± 1.01	5.87E-03	n.d.
33	At1g61610	S-locus lectin protein kinase	3.07 ± 0.19	5.10E-04	4.18 ± 0.80
41	At5g59100	Subtilisin-like serine protease, S8 family	2.91 ± 0.16	3.92E-04	6.25 ± 0.93
Protein–protein interactions					
2	At5g48130	BTB-POZ domain protein, NPH3 family (NRL27)	5.21 ± 0.25	2.37E-04	5.54 ± 1.72
8	At3g54780	RING H2 domain protein	4.58 ± 0.15	7.82E-05	5.57 ± 0.54
16	At5g48510	BTB-POZ domain protein, speckle-type	3.64 ± 0.23	1.17E-04	n.d.
18	At4g38140	RING H2 domain protein	3.56 ± 0.29	1.22E-04	n.d.
25	At3g19380	U-box/armadillo domain protein	3.22 ± 0.66	1.50E-03	n.d.
26	At4g35070	RING/U box domain protein	3.20 ± 0.36	5.41E-04	n.d.
40	At3g15680	Zinc finger protein, RanBP2-type	2.91 ± 0.46	2.15E-04	n.d.
Cell wall/cell membrane-localized					
11	At5g47440	PH domain-containing protein (PH16)	4.06 ± 0.19	2.18E-04	4.63 ± 0.36
12	At5g03260	Laccase-like multicopper oxidase	3.90 ± 0.43	2.80E-04	n.d.
15	At4g03210	Xyloglucan endotransglycosylase (XTH9)	3.64 ± 0.56	1.03E-03	3.78 ± 0.85
20	At5g48900	Pectate lyase (PLL21)	3.36 ± 0.58	9.69E-04	n.d.
28	At5g01870	Lipid-transfer protein (LTP6-like)	3.13 ± 0.42	1.78E-04	n.d.
31	At1g76790	*O*-Methyltransferase	3.09 ± 0.23	8.27E-05	n.d.
32	At4g02290	Endo-β-1,4-glucanase	3.09 ± 0.29	3.41E-05	n.d.
34	At4g27520	ENOD-like GPI-anchored arabinogalactan protein (AGP)/phytocyanin	3.04 ± 0.23	2.45E-04	3.36 ± 0.69
36	At5g48140	Polygalacturonase	3.0 ± 0.65	1.89E-03	n.d.
Other					
3	At3g26200	Cytochrome P450 (CYP71B22)	4.94 ± 0.22	1.95E-04	5.99 ± 0.27
5	At2g03830	Unknown protein	4.79 ± 0.42	2.08E-04	n.d.
7	At4g34970	Actin-depolymerizing factor (ADF9)	4.78 ± 0.09	1.56E-05	9.45 ± 0.24
14	At5g11890	Harpin-induced1 family protein (NHL28)	3.67 ± 0.30	9.55E-05	n.d.
17	At4g14690	EARLY LIGHT INDUCED PROTEIN2 (ELIP2)	3.59 ± 0.75	8.59E-04	n.d.
21	At5g62490	AtHVA22b	3.29 ± 0.28	1.18E-04	n.d.
24	At1g64590	Short-chain dehydrogenase/reductase (SDR)	3.23 ± 0.09	5.41E-05	1.85 ± 0.58
27	At5g02550	Expressed protein	3.15 ± 0.11	1.05E-04	4.93 ± 0.19
29	At4g02360	Expressed protein, DUF538	3.11 ± 0.13	1.73E-04	3.92 ± 0.64
30	At5g44560	SNF7 protein	3.10 ± 0.13	1.69E-04	0.75 ± 0.43
35	At3g18800	Expressed protein	3.01 ± 0.10	7.50E-05	3.61 ± 0.80
38	At2g41800	Unknown protein, DUF642	2.92 ± 0.39	6.10E-04	n.d.

Table 2 continued

Rank	AGI No.	Annotation	Microarray (2Log ratio)	*t*-test (*P*-value)	qRT-PCR (ΔΔCt)
42	At3g02960	Copper chaperone (ATX1)	2.90 ± 0.46	1.15E-03	n.d.
43	At3g60150	Hypothetical protein, DUF598/498	2.89 ± 0.14	2.63E-04	3.39 ± 0.41

Four-day-old *Arabidopsis* seedlings from two *35S::BBM:GR* lines (GR) treated in duplicate for 8 h with dexamethasone and cycloheximide (DC) were compared with a pool of wild-type seedlings (WT) treated in the same way. The 2Log ratios represent the average of the normalized 2Log ratios obtained for the replicated microarray hybridizations as described in Fig. 2

Relative expression levels, as determined by quantitative RT-PCR on the same RNA samples, are represented as the average of the ΔΔCt values (ΔCt WT DC − ΔCt *35S::BBM:GR* DC). n.d., not determined

complexes. E3 ligases control the last step in the ubiquitination process, transfer of ubiquitin from a ubiquitin conjugating enzyme (E2) to a lysine in the target protein, and also control the target specificity for ubiquitin transfer. The BTB-POZ protein encoded by At5g48510 interacts with CUL3A, a scaffold protein in a multiprotein E3 ligase complex that is involved in protein degradation via the 26S proteasome pathway and that together with CUL3B is essential for embryo development in Arabidopsis (Figueroa et al. 2005).

BBM candidate genes are predominantly expressed in somatic embryos

The relative expression profiles of 19 validated candidate genes were determined by qRT-PCR in wild-type tissues and in embryogenic *pickle* seedlings and somatic embryos derived from *35S::BBM* seedlings. As shown in the heat map in Fig. 3, 12 of the 19 target genes show the highest relative expression level in *35S::BBM*-induced somatic embryos (Group I), while the remaining seven genes are preferentially expressed in other tissues (Group II). The 12 genes that are preferentially expressed in BBM-induced somatic embryos can be subdivided into two groups, one of which comprises genes expressed at lower levels in a relatively broad range of tissues (Group IA), and a second group whose expression is largely restricted to BBM-induced somatic embryos (Group IB) and other embryo-containing samples, such as seeds. A number of target genes are expressed at relatively higher level in the embryogenic *pkl* mutant compared to wild-type seedlings, suggesting that their expression is specifically enhanced in embryogenic tissue. Microarray analysis using Arabidopsis long oligo arrays and RNA targets from excised zygote-to-

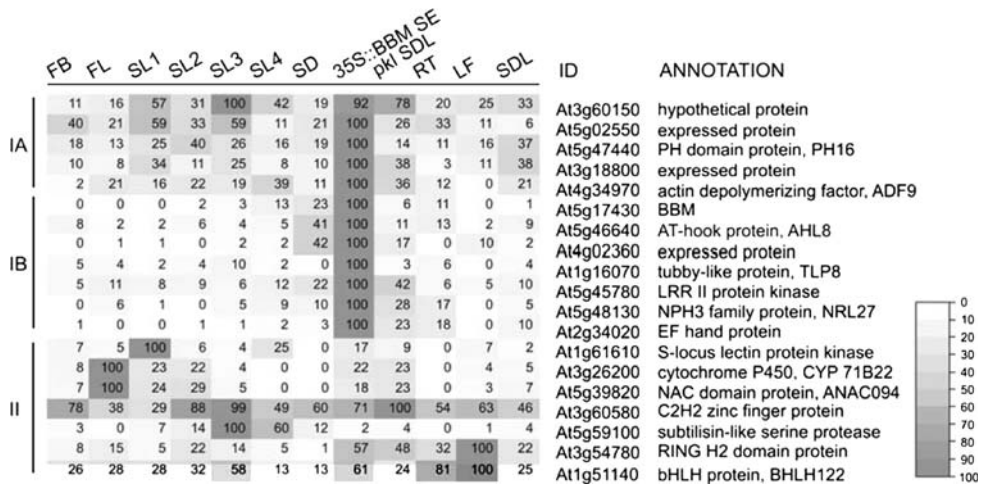

Fig. 3 Heat map showing the relative gene expression levels of BBM target genes in wild-type and mutant tissues. The expression profiles for each gene were normalized to the highest expression level for that gene in the series of tissues examined as described in Materials and methods. The values shown are relative i.e., they do not reflect the absolute expression levels of these genes in the analyzed tissues. The heat map was created in GeneMaths version 2.01 (Applied Maths). The gray-scale bar is shown on the right. Gene nomenclature is as in Table 2.The groups IA, IB and II are described in the text. FB, flower buds (pooled stages before anthesis); FL, flowers at anthesis; SL, siliques containing embryos up to the early globular stage (SL1), late globular to heart stage (SL2), torpedo stage (SL3), and bent cotyledon stage (SL4), SD, seeds containing mature cotyledon-stage embryos; 35S::BBM SE, somatic embryo clusters derived from *35S::BBM* seedlings; pkl SDL, 14 day old pickle seedlings; RT, roots from 14 day seedlings; LF, rosette leaves from non-bolted plants; SDL, 14-day-old seedlings

mature stage *Brassica napus* embryos and globular-to-mature stage Arabidopsis embryos indicates that 88% of the (corresponding) BBM target genes examined by qRT-PCR are expressed from the zygote stage onward in *Brassica napus* and 95% from the globular stage onward in Arabidopsis (Raju Datla, personal communication).

BBM dynamizes the actin cytoskeleton

One of the genes that is preferentially expressed in DEX-induced *35S::BBM:GR* seedlings is *ACTIN DEPOLYMERIZING FACTOR9 (ADF9)*, which encodes an ADF/cofilin protein. ADF/cofilin binds both to G- and F-actin and enhances actin dynamics by severing actin filaments and increasing their depolymerization from the pointed end (Carlier et al. 1997; Gungabissoon et al. 1998). Since the activity of ADF/cofilin on the actin cytoskeleton has been studied in detail, and since ADF9 provides a potential link from BBM signaling to actin dynamization, we chose to further characterize the up-regulation of *ADF9* in *35S::BBM:GR* seedlings. Firstly, the time course of *ADF9* induction was determined by qRT-PCR in four-day-old DEX-treated *35S::BBM:GR* seedlings as compared to DEX-treated wild-type seedlings (Fig. 4). *ADF* expression is rapidly induced after BBM activation and transcript levels are maintained over the next 2 days, where after they begin to drop. A second DEX treatment 72 h after the initial DEX application did not lead to an increase in *ADF9* transcript levels (data not shown). The inability of BBM to reactivate *ADF9* expression in response to a second DEX application may be due to a number of factors, including reduced competence of the tissue to respond to the BBM signal, or to the inability of the 35S promoter to direct sufficient BBM expression at this developmental stage.

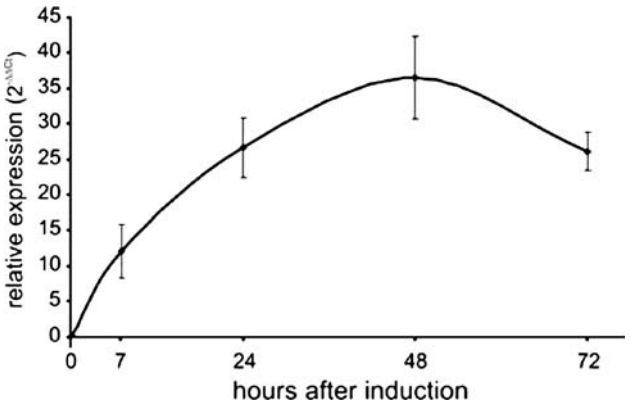

Fig. 4 *ADF9* expression is rapidly activated by BBM. Quantitative real-time RT-PCR analysis of BBM:GR-induced *ADF9* expression. *35S::BBM:GR* and wild-type seedlings were grown in liquid culture and then transferred at day 4 of culture to the same medium containing 20 μm DEX. Shown is the fold change in *ADF9* gene expression in *35S::BBM:GR* seedlings relative to wild-type seedlings

Next, to follow the consequences of BBM activation on the actin cytoskeleton, we crossed *35S::BBM:GR* plants with plants expressing a 35S promoter-driven actin marker, GFP:FIMBRIN ACTIN BINDING DOMAIN 2 (GFP:FABD) (Ketelaar et al. 2004). We selected F1 and F2 offspring that expressed both the *35S::BBM:GR* and *35S::GFP:FABD* constructs. The first true leaves of these plants (6–7 days after germination) and of control plants containing single constructs were incubated in water containing 20 μM DEX or 0.1% DMSO as a control and then examined 1–2 h later using a confocal microscope. We visualized actin organization and dynamics in leaf pavement cells by collecting subsequent Z-stacks. When two subsequent Z-stacks are projected, pseudo-colored and superimposed, the changes in actin organization over time can be observed. In control samples comprising DMSO-treated *35S::BBM:GR/35S::GFP:FABD* seedlings (Fig. 5a) and DEX-treated *35S::GFP:FABD* seedlings (Fig. 5b), we observed thick bundles of actin filaments that traversed the leaf pavement cells. After 1.5 min, most of

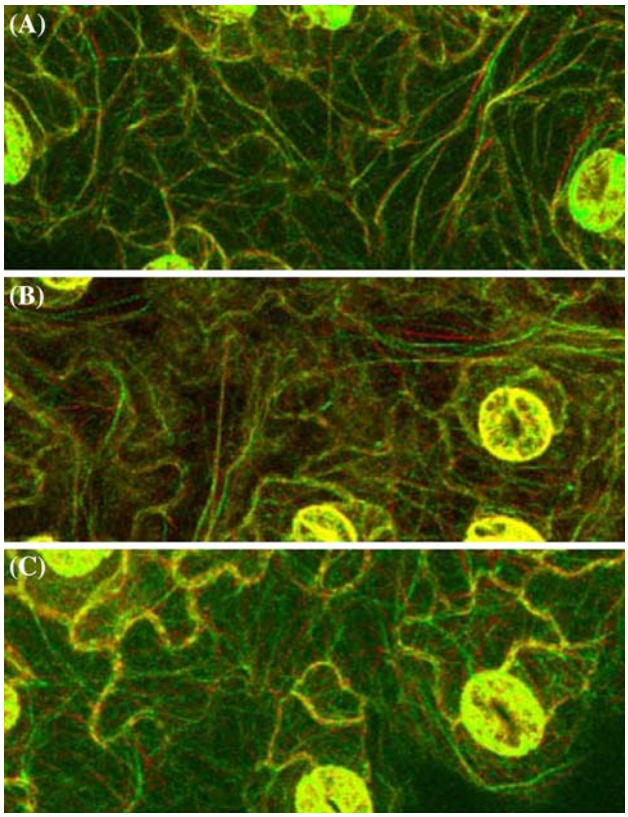

Fig. 5 BBM reorganizes the actin cytoskeleton. The effect of transcriptional activation by BBM on the actin cytoskeleton was visualized using a GFP:FABD actin marker and confocal microscopy. The images are projections of a *z*-series taken over a 1.5 min interval. (**a**) *35S::BBM:GR/35S::GFP FABD* seedlings treated with 0.1% DMSO. (**b**) *35S::GFP FABD* seedlings treated with 20 μM DEX. (**c**) *35S::BBM:GR/35S::GFP FABD* seedlings treated with 20 μM DEX

the actin bundles were still intact, although some of the bundles had dislocated slightly, and a few bundles had disappeared. New actin bundles only appeared occasionally. In contrast to control plants, actin organization and dynamics changed dramatically in the DEX-activated *35S::BBM:GR* line. Upon DEX treatment, most of the thick actin bundles disappeared and were replaced by a dense network mostly consisting of finer actin bundles (Fig. 5c). These fine actin bundles reorganized rapidly, with most of the fine actin bundle network being completely reorganized within 1.5 min. In addition, the cytoplasmic staining of GFP:FABD increased, indicating that either the actin filaments were too dynamic for the GFP:FABD to bind, or that the total amount of filamentous actin had decreased (Fig. 5c). This data suggests that BBM activation is associated with rapid and effective dynamization of the actin cytoskeleton.

Discussion

A conditionally active form of the BBM protein, BBM:GR, was used to identify candidate BBM target genes that could play a role in BBM-mediated regeneration. We have shown that the BBM:GR protein is functional by its ability to induce somatic embryo formation after DEX application. Using this inducible system allowed us to identify the developmental window in which mature seeds and seedlings are competent to respond to the BBM protein. Somatic embryos formed in *35S::BBM:GR* lines when DEX was applied as long as 6 days after the start of the liquid cultures, however the number of seedlings that formed somatic embryos, as well as the number of somatic embryos formed per seedling decreased as the seedlings aged. Arabidopsis seedlings maintain the ability to form embryos well after the completion of seed development and maturation indicating that somatic embryo formation is not simply a prolongation of the embryonic program, as was previously suggested for other somatic embryo-inducing proteins and mutants (de Vries 1998), but rather reflects an inherent capacity of seedlings to respond de novo to the BBM protein and reinitiate embryo development. The developmental window in which somatic embryos are induced by the BBM:GR protein in Arabidopsis may reflect the developmental competence of the seedling to respond to a signal that keeps cells in a relatively undifferentiated or juvenile state. Older tissues continue to respond to BBM, as evidenced by altered leaf and floral morphology but rarely produce somatic embryos (Boutilier et al. 2002). This suggests that a specific developmental state, defined by factors such as the cellular growth regulator status, chromatin structure or presence/absence of interacting proteins, is required for BBM-mediated reversion to cell proliferation and somatic embryogenesis. However, we have observed that the activity of the 35S promoter used in this study decreases after the seedling stage until flowering (data not shown), raising the possibility that in addition to the developmental competence of the tissue, a threshold level of BBM protein may be required to induce somatic embryogenesis.

The identities of the BBM target genes do not provide insight into the specific pathways activated by this transcription factor, as to our knowledge none of the genes, with the exception of *XTH9*, have been functionally characterized, and most of the BBM targets belong to large multigene families, the members of which are involved in diverse processes. None of the immediate BBM target genes correspond to any genes known to induce (*LEC1*, Lotan et al. 1998; *LEC2*, Stone et al. 2001; *WUS*, Zuo et al. 2002) or enhance (*SERK1*, Hecht et al. 2001; *AGL15*, Harding et al. 2003) somatic embryogenesis in Arabidopsis. There appear to be many routes through which seedlings can be reprogrammed to form embryos, thus it is likely that these different pathways eventually merge to direct the same developmental outcome. Target genes activated by LEC2 have been described (Braybrook et al. 2006). LEC2 expression activates genes involved in auxin response and seed maturation, and arguments have been made to link these two pathways to the initiation of somatic embryo development (Stone et al. 2008). We did not identify any of the LEC2 target genes in our experiments, suggesting that BBM and LEC activate different sets of primary target genes, and perhaps different initial signaling pathways. WUS target genes have been described (Leibfried et al. 2005), and two of the BBM targets, a short-chain dehydrogenase/reductase gene (SDR; At1g64590) and a gene encoding an expressed protein (At5g02550) are among the 148 candidate WUS-upregulated genes (Leibfried et al. 2005). One of the lower ranked BBM target genes not presented here, encoding a glycine-rich protein (Supplementary Table 2: At2g05520), was shown to be up-regulated in *pkl* embryogenic roots (Rider et al. 2003). A causal relationship between these target genes and BBM-mediated cell proliferation has not been established.

A number of the BBM target genes have been identified in other microarray-based expression studies. Notably, at least six of the BBM target genes have been identified in screens for meristem-expressed genes. Expression of *BBM*, *TUBBY-LIKE PROTEIN 8* (*TLP8*), an LRR kinase gene (At5g45780), *XTH9* (At4g03210) and a gene coding for a PH domain-containing expressed protein (At5g47440) is enriched in the quiescent center (QC), a group of four to seven cells that give rise to the root initial/meristem cells (Nawy et al. 2005). *TLP8* was also recently identified as a direct target of LEAFY (LFY), a transcription factor that controls the switch from vegetative-to reproductive meristem development (William et al. 2004). The activation of

root meristem-expressed genes by BBM is intriguing. BBM, like the related AP2/*ERF PLETHORA1 (PLT1)* and *PLT2* genes is expressed in the root meristem (Aida et al. 2004), but unlike PLT1/PLT2 overexpression, BBM overexpression does not appear to induce ectopic root or root meristem formation in Arabidopsis. This suggests that many of the root meristem-expressed BBM target genes may play a more general role in maintaining cells in an undifferentiated state.

BBM:GR also activates a number of genes with roles in the development of the primary and secondary cell wall. Primary cell walls are produced by dividing and growing cells and provide a flexible structural support for cell division and elongation. They are composed of a rigid network of cellulose fibrils, hemicelluloses (e.g., xyloglucans) and pectin, and also contain structural proteins. Secondary cell walls are extremely rigid due to the presence of lignin and are characteristic of cells that have attained their final shape and size, for example vascular cells. The BBM:GR protein activates expression of proteins that are structural components of the cell wall (AGP) or that are involved in the synthesis or modification of cell wall polysaccharides including cellulose (endo-β-1,4-glucanase), hemicellulose (xyloglucan endotransglycosylase), pectin (pectate lyase, polygalacturonase) and lignin (laccase-like multicopper oxidase, *O*-methyltransferase). Loosening of the primary cell wall could be necessary to support the prolific cell division and growth that occur in BBM overexpression lines. A role for BBM in activating genes involved in secondary cell wall modification seems contradictory, given that secondary cell walls form in cells that have stopped growing. However, in Arabidopsis, BBM is also expressed in the vascular stem cells (procambium) of the embryo and seedling root (Galinha et al. 2007), possibly reflecting a role for BBM in vascular tissue development and differentiation.

One way in which BBM may reprogram cell division and growth is through the actin cytoskeleton. After BBM:GR activation in Arabidopsis leaf cells, the actin organization changes from mostly thick, stabile bundles that remain intact during the imaging to a dense network of fine actin bundles that appears to be rapidly turning over, judged by the disappearance of actin filaments in one location and the de novo appearance of actin filaments in other locations. Dynamization of the actin cytoskeleton underlies many cellular processes including cell division, cell elongation, polar tip growth and intracellular trafficking (Hussey et al. 2006). ADF, together with a battery of other actin binding proteins, is thought to play a central role in these different processes by modulating the rate of actin depolymerization. A role for ADF in actin filament turnover is well supported by studies on single cell systems, including plant cells (van der Honing et al. 2007), however there is little direct evidence linking ADF proteins to complex cellular events during the plant lifecycle. The actin cytoskeleton is the backbone of cytoplasmic organization. Cytoplasmic strands and accumulations cannot exist in the absence of a functional actin cytoskeleton (van der Honing et al. 2007). Dynamic reorganization of the actin cytoskeleton is characteristic for cells that are undergoing rapid cytoplasmic reorganization, for example during localized expansion in tip growing pollen tube and root hair cells (Hussey et al. 2006). It is likely that the actin dynamization that we observe after BBM:GR nuclear localization, correlates with the trans-differentiation to meristematic cells that are re-entering the cell cycle. The actin cytoskeleton is highly dynamic in meristematic cells, whereas the dynamicity of actin reorganization is reduced in fully grown cells (Voigt et al. 2005).

Recently Ruzicka et al. (2007) characterized the expression patterns of the 11 Arabidopsis *ADF* gene family members using qRT-PCR and GUS reporter gene fusions. They show that *ADF9* is expressed at low levels in a broad range of tissues during plant development, and within these tissues shows the strongest expression in rapidly dividing and/or growing cells, such as those of the root and shoot meristem, leaf margins, emerging leaves and root elongation zone. Interestingly, *ADF9* is the most abundantly expressed *ADF* gene in callus, showing a ten-fold higher expression level as compared to other tissues. This data combined with the role of ADF proteins in filament turnover suggests a correlation between actin dynamization and cell proliferation. The observation that *ADF9* is up-regulated in BBM overexpression lines is intriguing, however a direct link between BBM-mediated cell proliferation and *ADF9* upregulation remains to be proven.

Conclusion

Use of a DEX induced BBM:GR protein together with the protein synthesis inhibitor, cycloheximide, allowed us to identify candidate genes that may be directly activated by the BBM:GR protein. A number of new interesting target genes have been identified, however additional studies need to be performed to resolve a number of issues, including whether these candidates are indeed direct BBM targets and whether the set of genes identified here also corresponds to the physiological targets of the native BBM protein. Finally, given the dual role of BBM in both early embryo and root meristem development, it will be important to determine the developmental context in which each of the individual target genes function.

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