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ORIGINAL ARTICLE

Heterologous expression of the *BABY BOOM* AP2/ERF transcription factor enhances the regeneration capacity of tobacco (*Nicotiana tabacum* L.)

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Abstract Gain-of-function studies have shown that ectopic expression of the *BABY BOOM* (*BBM*) AP2/ERF domain transcription factor is sufficient to induce spontaneous somatic embryogenesis in *Arabidopsis* (*Arabidopsis thaliana* (L.) Heynh) and *Brassica napus* (*B. napus* L.) seedlings. Here we examined the effect of ectopic *BBM* expression on the development and regenerative capacity of tobacco (*Nicotiana tabacum* L.) through heterologous expression of *Arabidopsis* and *B. napus BBM* genes. *35S::BBM* tobacco lines exhibited a number of the phenotypes previously observed in *35S::BBM Arabidopsis* and *B. napus* transgenics, including callus formation, leaf rumpling, and sterility, but they did not undergo spontaneous somatic embryogenesis. *35S::BBM* plants with severe ectopic expression phenotypes could not be assessed for enhanced regeneration at the seedling stage due to complete male and female sterility of the primary transformants, therefore fertile *BBM* ectopic expression lines with strong misexpression phenotypes were generated by expressing a steroid-inducible, post-translationally controlled BBM fusion protein (BBM:GR) under the control of a 35S promoter. These lines exhibited spontaneous shoot and root formation, while somatic embryogenesis could be induced from in-vitro germinated seedling hypocotyls cultured on media supplemented with cytokinin. Together these results suggest that ectopic *BBM* expression in transgenic tobacco also activates cell proliferation pathways, but differences exist between *Arabidopsis*/*B. napus* and *N. tabacum* with respect to their competence to respond to the BBM signalling molecule.

Keywords AP2/ERF transcription factor · *Arabidopsis* · BABY BOOM · *Brassica* · Competence · *Nicotiana* · Organogenesis · Somatic embryogenesis

Abbreviations

DEX	Dexamethasone
BAP N^6	Benzylaminopurine
NAA	Napthaleneacetic acid
TDZ	Thidiazuron (*N*-phenyl-*N′*-1,2,3,-thiadiazol-5-ylurea)
TEM	Transmission electron microscopy
SEM	Scanning electron microscopy

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Introduction

Plant regeneration is an important tool in modern plant breeding and crop biotechnology. Plant regeneration through somatic embryogenesis or organogenesis generally depends on the addition of one or more plant growth regulators to the culture medium. Manipulation of the ratio or concentration of these growth regulators is often the key factor leading to successful regeneration protocols (Thorpe 2000). However, there is a large difference in the response of individual plant tissues and genetic backgrounds to in vitro regeneration conditions, with the result that the empirical approach often fails and several important crops remain recalcitrant for in vitro regeneration (Srinivasan et al. 2005).

The genetic basis for the differences in regeneration capacity is still poorly understood, however, a number of genes have been identified that positively influence the regenerative competence of plant cells for somatic embryogenesis and/or adventitious shoot formation. (Kakimoto 1996; Lotan et al. 1998; Hewelt et al. 2000; Banno et al. 2001; Stone et al. 2001; Boutilier et al. 2002; Zuo et al. 2002). Most of these genes have been identified in *Arabidopsis* [*Arabidopsis thaliana* (Heyhn) L.] and many of them encode transcription factors or proteins involved in signal transduction. Over-expression of these genes effectively substitutes for plant hormones such as cytokinins or auxins, or enhances the regeneration responsiveness of plant tissues to these hormones.

Several of these genes belong to the AP2/ERF multigene family of transcription factors (reviewed in Riechmann and Meyerowitz 1998). The *Arabidopsis* AP2/ERF family comprises a large class of 144 transcription factors with diverse roles throughout the plant life cycle. The members of this class of regulatory proteins all contain the conserved AP2/ERF domain, a 60–70 amino acid region that is thought to mediate DNA binding and protein–protein interactions (Jofuku et al. 1994; Okamura et al. 1997). The AP2/ERF family has been organized into five phylogenetically distinct subfamilies that differ in the number of AP2/ERF domains, as well as the amino acid similarity between these domains (Sakuma et al. 2002). Genes belonging to two of these subfamilies have been shown to enhance in vitro regeneration (Banno et al. 2001; Boutilier et al. 2002), while others play a role in related processes controlling meristem cell fate and organ development (Jofuku et al. 1994; Elliot et al. 1996; Chuck et al. 1998; van der Graaff et al. 2000). One of these genes, *ENHANCER OF SHOOT REGENERATION1* (*ESR1*), was identified in an over-expression screen for *Arabidopsis* genes that promote adventitious shoot formation from callus both in the absence and presence of cytokinins (Banno et al. 2001). Over-expression of another AP2/ERF transcription factor gene *BABY BOOM* (*BBM*) also bypasses the requirement for plant growth regulators to induce regeneration. Ectopic expression of a *B. napus BBM* gene (*BnBBM*) in *B. napus* and *Arabidopsis* under control of the CaMV 35S promoter primarily induces spontaneous somatic embryogenesis. Ectopic *BBM* expression in *B. napus* and *Arabidopsis* also stimulates occasional ectopic shoot production, and in *Arabidopsis*, ectopic *BBM* expression stimulates regeneration via organogenesis from leaf explants (Boutilier et al. 2002).

Here we report on the effect of heterologous expression of *Arabidopsis* and *B. napus BBM* genes on tobacco (*Nicotiana tabacum*) development and in vitro regeneration responses. We show that heterologous *BBM* expression induces many of the developmental alterations observed in *Arabidopsis* and *B. napus*, including enhanced competence of tissues to undergo organogenesis and somatic embryogenesis.

Materials and methods

Plant vectors and transformation

The entire *Arabidopsis BBM* (*AtBBM*) cDNA coding region was cloned by RT-PCR based on the published sequence (GenBank accession AF317907). The amplified gene fragment was verified by DNA sequencing and cloned between the CaMV 35S promoter and the *nos* 3′ terminator in pBIN19. The *35S::AtBBM* construct was subsequently electroporated into *Agrobacterium tumefaciens* GV3101 for plant transformation. The isolation of the *B. napus BnBBM1* cDNA and the construction of the *35S::BnBBM* binary vector have been described previously (Boutilier et al. 2002). The *35S::BBM:GR* construct was made by creating a translational fusion between the *BnBBM1* cDNA (GenBank accession AF317904) and the ligand-binding domain of the rat glucocorticoid receptor (GR). The BBM:GR fusion protein is sequestered in the cytoplasm thereby preventing the fusion protein from entering the nucleus and activating gene expression. Addition of the synthetic glucocorticoid steroid DEX promotes dissociation of this complex and enables translocation of the BBM:GR fusion protein to the nucleus (Schena et al. 1991). The *35S::BnBBM* and *35S::BBM:GR* constructs were electroporated into *A. tumefaciens* C58C1 carrying the helper plasmid pMP90.

Leaf discs excised from in vitro germinated seedlings of *N. tabacum* cvs Wisconsin 38 and Petit Havana SR1 (seeds produced by our laboratory) were transformed as described by Hörsch et al. (1985). Transgenic shoots were selected on 100–200 mg/l kanamycin, rooted in vitro and the transgenic plants acclimated in the greenhouse.

Plant growth and culture conditions

Transgenic and wild-type seeds were surface sterilized and germinated on agar-gelled MS (Murashige and Skoog 1962) basal medium plus 2% sucrose (MS-20). DEX was prepared as a 10 mM stock in 70% ethanol and added to MS-20 medium to a final concentration of 10 μM. DEX-containing media were refreshed every 2–4 weeks. For the somatic embryo induction experiments, seeds of homozygous *35S::BBM:GR* and wild-type plants were sown on solidified MS-20 medium supplemented with 0.25–2 mg/l zeatin or 1 mg/l BAP with or without 10 μM dexamethasone. All cultures were incubated in the light (3,000–5,000 lux, 16 h/8 h day/night cycle) at 25°C. Experiments involving DEX treatments were carried out as described above, but under dim light conditions (300–500 lux)

Histological analyses and microscopy

Leaves of *35S::BBM* transgenics were examined using light microscopy, TEM and SEM. Samples of fully expanded leaves of severe *35S::AtBBM* lines and wild-type untransformed plants were fixed for 1 h in 4% formaldehyde and 1% gluteraldehyde in 0.1 M phosphate buffer, and post-fixed in 1% aqueous osmium tetroxide. After dehydration through an ethanol series samples were embedded in Epon–Araldite resin for TEM and light microscopy. One-micron thick sections were cut and stained with 2% toluidine blue-borax stain and observed under the light microscope. Sixty nanometre thin sections were cut for TEM, stained with saturated aqueous uranyl acetate and Reynolds lead citrate and observed in a TEM (JEOL 100 CX II) operated at 60 KV. For SEM, dehydrated samples were critical point dried with CO_2, mounted on a stub with adhesive tabs, gold coated with 30 nm gold particles and observed in a SEM operated at 20 KV (Philips 515).

Real-time RT-PCR

Quantitative real-time RT-PCR was used to determine the relative expression levels of the *AtBBM* transgene in *35S::AtBBM* over-expression lines. Fully expanded leaves were collected, immediately frozen in liquid nitrogen and stored at −80°C. Total cellular RNA was extracted using a Purescript RNA isolation kit (Gentra System Inc., Minneapolis, MN, USA). Contaminating DNA was removed from these samples using a DNA-Free kit (Ambion). RT-PCRs were performed using Taqman® One-Step RT-PCR Master Mix Reagents following the manufacturer's instructions and were monitored by the ABI7900HT Sequence Detection System (Applied Biosystems, Hayward, CA, USA). Primers and probes were designed using Primer Express software (Applied Biosystems). FAM/MGB-NFQ Taqman® probes were synthesized by Applied Biosystems. Relative *AtBBM* gene expression levels were determined using the comparative $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen2001). The expression level of the *AtBBM* gene in different transgenic lines was normalized using the *N. tabacum RIBOSOMAL L25* gene (GenBank accession L18908) as the reference (Volkov et al. 2003) and the normalized *AtBBM* expression value from the transgenic line with the lowest *AtBBM* expression level as the calibrator.

The primer and probe sequences used for real-time PCR are as follows: *AtBBM* forward primer, 5′-GAG AGCCCGAGTCTACCTATTGG-3′; *AtBBM* reverse primer, 5′-GAACCGGATTGTTAACGTCCTT-3′; *AtBBM* probe, 5′-AGTTCTGCGAAACGTC-3′; *RIB OSOMAL L25* forward primer, 5′-GGCCTGATGG GACGAAGA-3′; *RIBOSOMAL L25* reverse primer, 5′-CAACGTCCAAAGCATCATAGTCA-3′; *RIBOS OMAL L25* probe, 5′–5′-AGCATATGTGAGGTTG AC-3′.

Results

Eight transgenic tobacco lines expressing the *35S::AtBBM* construct (cv Wisconsin 38) and 20 transgenic tobacco lines expressing the *35S::BnBBM* construct (cv Petit Havana SR1) were regenerated, and are collectively referred to as *35S::BBM* lines. The primary transgenics were divided into moderate and severe classes based on their combined vegetative and reproductive phenotypes. Moderate *35S::BBM* lines were normal in stature, showed mild vegetative phenotypes and reduced fertility. Severe *35S::BBM* lines displayed extreme vegetative and floral phenotypes. They are dwarf, show reduced apical dominance and were both male and female sterile. Flowers displayed long sepals, light pink to white petals, short filaments and delayed dehiscence of anther lobes. The vegetative phenotypes are described in more detail below, while

the reproductive phenotypes are presented as supplemental data (Suppl. Fig. 1). Both the moderate and the severe phenotypes were observed in the primary transformants. In fertile transgenic lines, the ectopic *BBM* expression phenotypes co-segregated with the transgene in subsequent generations. Real-time RT-PCR analyses of the eight *35S::AtBBM* transgenic lines, comprising seven moderate lines and one severe line (line #7), showed that *AtBBM* gene expression was highest in the severe line (Fig. 1).

BBM alters the vegetative growth of tobacco

Perturbed leaf development was a highly penetrant trait in both the Petit Havana SR1 and Wisconsin 38 tobacco cultivars transformed with the *35S::BBM* construct. Wild-type plants of both tobacco genotypes produced lanceolate shaped leaves (Fig. 2a), while the *35S::BBM* plants had broad oblong leaves that are rumpled and with a wavy margin (Fig. 2b). Moderate *35S::BBM* phenotypes differed from severe phenotypes in the degree of leaf rumpling. The rumpled appearance of *35S::BBM* leaves resulted from increased interveinal depression of the leaf blade, and was particularly evident in the lines with a severe phenotype (Fig. 2c). Root development was also affected in all *35S::BBM* transgenics. *35S::BBM* plants produced only fibrous roots while the control plants had several long thick roots in addition to fibrous roots (Fig. 2d). *35S::BBM* plants with moderate phenotypes were approximately the same height as wild-type plants (Fig. 2e) whereas severe *35S::BBM* phenotypes showed reduced apical dominance and short internodes (Fig. 2f). Moderate *35S::BBM* phenotypes were not significantly different from those of wild-type

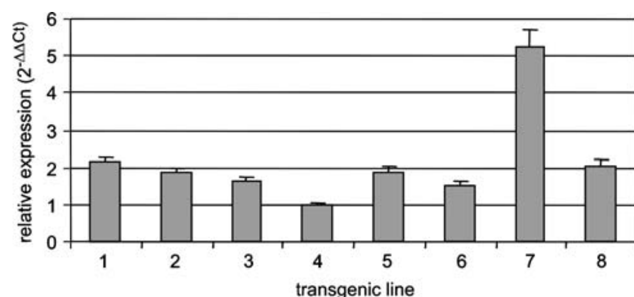


Fig. 1 Relative expression levels of the *AtBBM* transgene in tobacco. The relative expression level of the *AtBBM* transgene was measured in eight independent *35S::AtBBM* lines using quantitative real-time RT-PCR. Relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method in which the *AtBBM* gene expression level was normalized to that of *RIBOSOMAL L25* RNA and then calibrated using the *35S::BBM* transgenic line with the lowest *AtBBM* expression (line #4)

plants with respect to days to flowering, while severe *35S::BBM* lines flowered slightly later than wild-type plants.

To gain insight into the nature of alteration of leaf development in *35S::BBM* plants, we examined the anatomy and ultrastructure of the control and *35S::AtBBM* tobacco plants using light microscopy, SEM and TEM. SEM observations of *35S::AtBBM* leaves showed that both the overall number and size of the trichomes were reduced as compared to wild-type leaves (Fig. 3a, b). The trichomes in the depressed area of the leaf were smaller and more sparsely distributed than in the non-depressed areas of the leaf. Light microscopic examination of cross sections of fully expanded leaves showed that there were eight cell layers in wild-type leaves as compared to six cell layers in *35S::AtBBM* leaves (Fig. 3c, d). The cells in *35S::AtBBM* leaves were more loosely packed and had more air spaces than wild-type leaves, and they did not develop the characteristic adaxial layer of palisade parenchyma cells. The number of chloroplasts per cell was also slightly reduced in *35S::AtBBM* leaves (4.9 ± 2.1 per cell, $n = 25$ cells) as compared to wild-type leaves (5.9 ± 2.1 per cell, $n = 25$ cells). Both light microscopic thin sections and TEM observations of *35S::AtBBM* leaves showed the presence of large number of starch granules (Fig. 3d, f) whereas the wild-type leaf cells contained very few or no starch granules (Fig. 3c, e).

The *35S::BBM* tobacco plants were generated from leaf explants, therefore *35S::BBM* seedling phenotypes could only be examined in the offspring of fertile *35S::BBM* lines. Seed germination was delayed in *35S::BBM* lines as compared to wild-type plants, and the *35S::BBM* seedlings were smaller than wild-type seedlings at the same developmental stage. Wild-type tobacco seedlings had round cotyledons, whereas cotyledons of moderate *35S::BBM* seedlings were longer and narrower than wild-type seedlings (Fig. 4a, b). The first true leaves of *35S::BBM* seedlings showed the characteristic rumpling that was also seen in older *35S::BBM* leaves. The cotyledons of *35S::BBM* seedlings often did not unfurl initially, being constrained at their tip or around their middle by a mass of proliferating tissue that remained associated with the seed coat. This callus-like tissue may have been derived from the aleurone layer of the tobacco endosperm. In wild-type seeds, the aleurone layer of the persistent endosperm degenerated shortly after germination, but in *35S::BBM* seeds this layer remained viable for a few weeks (Fig. 4c) and then eventually degenerated. This callus-like tissue is highly regenerative (see below).

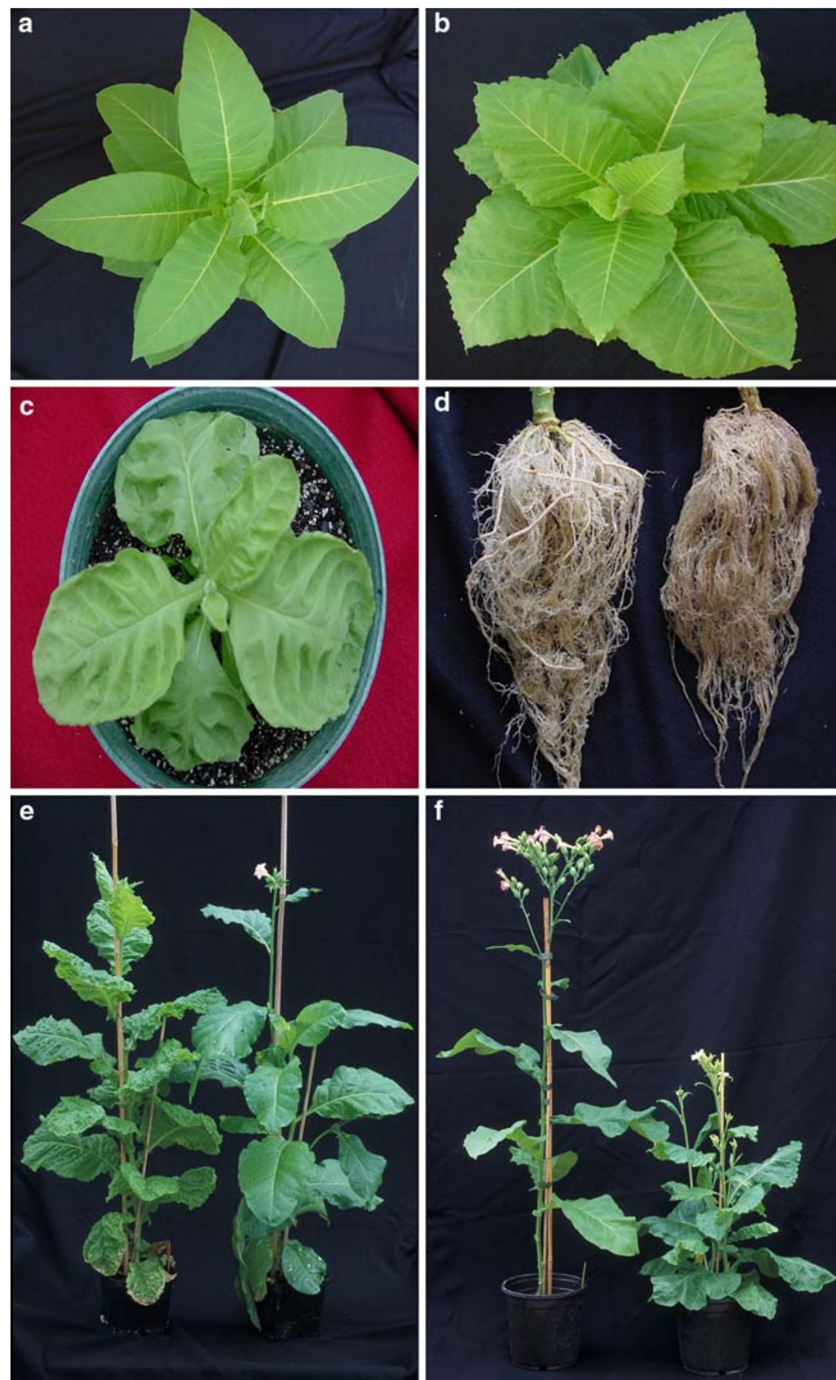


Fig. 2 Morphological alterations induced by expression of the *35S::BBM* transgene in tobacco. **a** Wild-type plant. **b** *35S::AtBBM* plant showing mild leaf rumpling and a wavy margin. **c** Leaves of a severe *35S::AtBBM* plant showing deep interveinal depressions. **d** Root systems of wild-type (*left*) and *35S::AtBBM* (*right*) plants. **e**, **f** Comparison of wild-type plants at flowering with moderate (**e**) and severe (**f**) *35S::BnBBM* transgenics. The wild-type plants are shown on the right in **e** and on the left in **f**. The plant cultivars are Wisconsin 38 in **a**, **b**, **c**, **d** and Petit Havana SR1 in **e** and **f**

BBM enhances the regenerative capacity of tobacco

Neither somatic embryo formation nor adventitious shoot formation were observed on young leaves of the primary moderate and severe tobacco *35S::BBM* lines, nor on seedlings in subsequent generations of fertile moderate lines. No difference in the frequency of shoot regeneration was observed between *35S::BnBBM* and wild-type tobacco explants placed on shoot induction medium containing 1 mg/l BAP (data not shown); however, indications were obtained that *35S::BnBBM* plants do have a higher regenerative capacity than wild-type plants. Firstly, moderate *35S::BnBBM* leaf explants placed on basal medium without growth regulators remained green and enlarged, while wild-type leaf explants gradually became chlorotic and necrotic (Fig. 4d). Secondly, seedlings derived from microspore-derived embryo cultures of (sterile) severe *35S::BnBBM* lines produced ectopic shoots from the seedling leaf surface (data not shown).

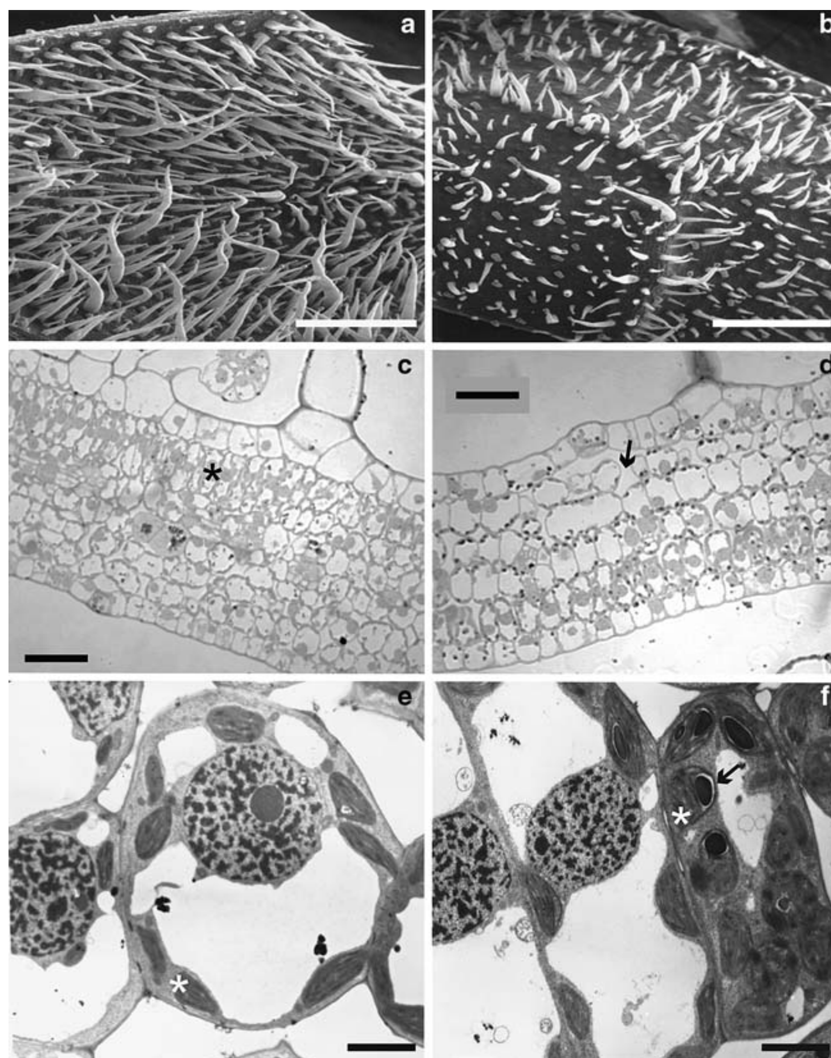
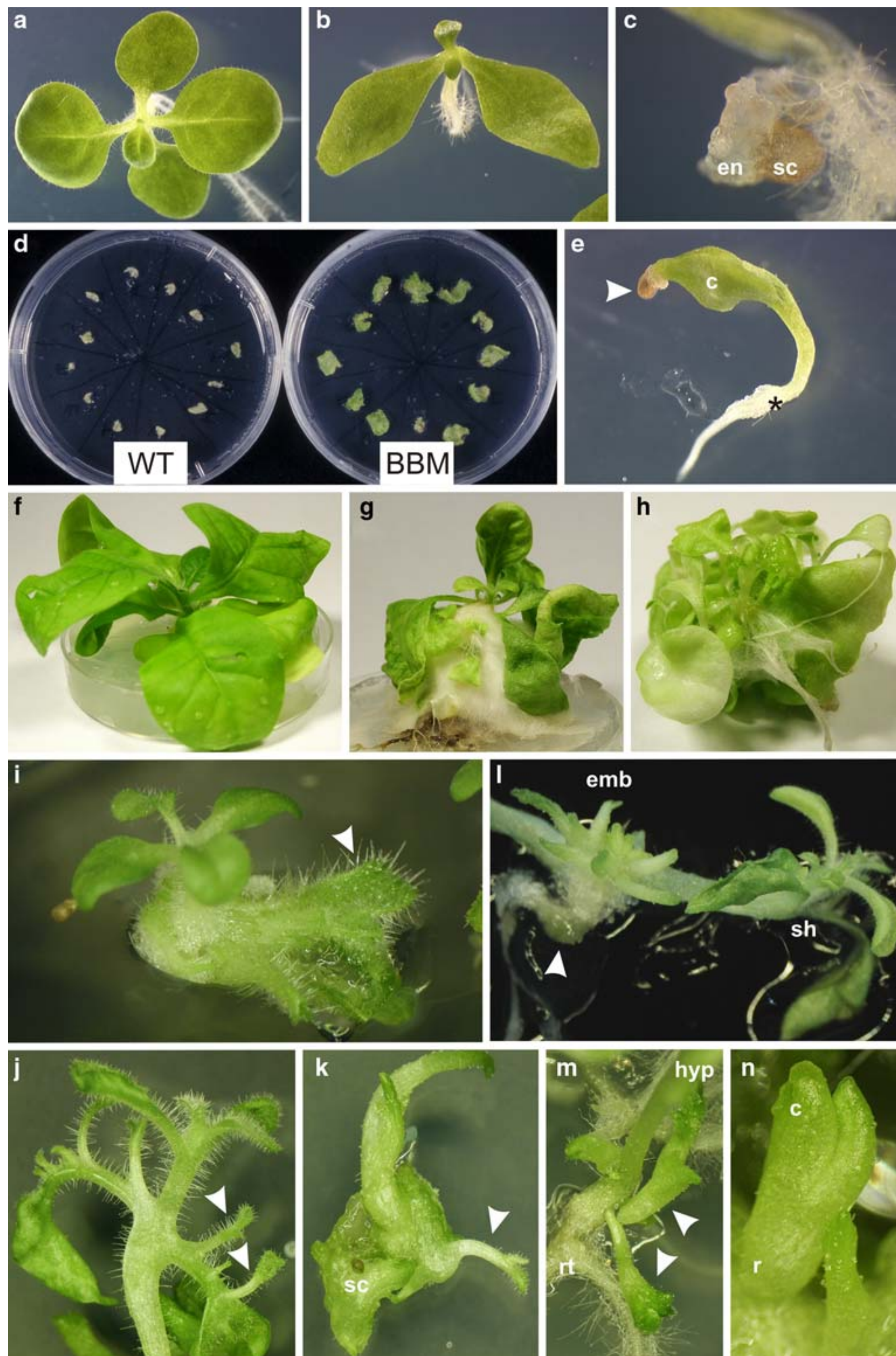


Fig. 3 Ultrastructural analyses of leaf development in *35S::BBM* tobacco plants. Images in **a**, **c**, and **e** are wild-type plants. Images in **b**, **d**, and **f** are *35S::BBM* plants. **a**, **b** SEM of wild-type and *35S::BBM* leaf surfaces. The wild-type leaf has longer and more densely packed trichomes than the transgenic. **c**, **d** Cross sections of leaves. The transgenic leaf has fewer and more loosely packed cell layers, more air spaces (*arrow*) and starch grains (visible as *dark dots*) than the wild-type leaf. The palisade parenchyma cells (*asterisk*) that are present in wild-type leaves are not clearly visible in the leaf sections from *35S::BBM* plants **e**, **f** TEM of leaves from wild-type and *35S::BBM* plants. The transgenic leaf has fewer chloroplasts (*asterisk*) and accumulates more starch granules (*arrow*) than the wild-type leaf. The *scale bars* are 1 mm (**a**, **b**), 20 μm (**c**, **d**) and 4 μm (**e**, **f**). All SEM, TEM and light microscopic sections of *35S::BBM* plants were from the depressed interveinal portion of the leaf

We were interested to assess the regenerative capacity of seedlings from *BBM* ectopic expression lines with severe phenotypes, however, as mentioned above, *35S::BBM* tobacco lines with severe phenotypes were sterile. We therefore used a conditionally active form of the BBM protein to obtain transgenic seedlings from

Fig. 4 **a**–**n** Seedling phenotypes and regenerative capacity of *BBM* misexpression lines. **a** Wild-type and **b**, **c** *35S::BBM* seedlings, 14 days after sowing. The cotyledon leaves of the wild-type seedling are rounder than those of the transgenic. The endosperm (*en*) of *35S::BBM* seeds (**c**) continues to divide after germination and forms callus-like cells. *sc* Seedcoat. **d** Leaf explants of wild-type (*WT*) and moderate *35S::BBM* (*BBM*) plants cultured for 14 days on MS-20 medium without growth regulators. **e** A 14-day-old DEX-treated *35S::BBM*:GR seedling showing similar phenotypes as *35S::BBM* seedlings, including elongated cotyledons (*c*), and post-germination endosperm proliferation (*arrow*). Callus formation (*asterisk*) at the transition between the hypocotyl and root is frequently observed. **f** Wild-type and **g**, **h** severe *35S::BBM*:GR plants grown on 10 μM DEX. Wild-type plants grown on DEX for 28 days show no phenotypic alterations (*f*), while severe *35S::BBM:GR* lines produce ectopic roots and shoots (**g**, **h**). A leaf (h) was removed from the *35S::BBM:GR* DEX-induced plant for easier observation of the ectopic shoots. **i** Adventitious shoots (*arrow*) develop from callus formed at the shoot-root transition zone of non DEX-activated *35S::BBM:GR* ▶ seedlings growing on medium containing 1 mg/zeatin. **j** Adventitious shoots (*arrows*) are formed on the cotyledons and leaves of *35S::BBM:GR* seedlings grown on medium containing zeatin and DEX. **k** The endosperm-derived callus of DEX-treated *35S::BBM:GR* germinated seeds proliferates in the presence of zeatin and forms adventitious shoots (*arrow*). The shed seed coat (*sc*) is indicated. l Somatic embryos (*emb*) are formed at the shoot-root transition zone of *35S::BBM:GR* seedlings grown on medium containing zeatin and DEX. The shoot region (*sh*) and hypocotyl (*hyp*) of the maternal seedling are indicated. Roots and callus (*arrow*) develops at the radicle end of the somatic embryos. **m** Somatic embryos (*arrows*) developing at the transition zone of *35S::BBM:GR* seedlings grown on medium containing cytokinin and DEX. The seedling hypocotyl (*hyp*) and root regions (*rt*) are indicated. Unlike adventitious shoots (see **k**), the somatic embryos are easily detached from the underlying tissue. **n** Bipolar *35S::BBM:GR*-derived somatic embryo. The cotyledons (*c*) and radicle end (*r*) are indicated



tobacco lines showing severe *BBM* ectopic expression phenotypes. Tobacco transformants were generated that expressed a fusion protein between the BnBBM protein and the ligand binding domain of the rat glucocorticoid receptor (GR) under control of the 35S promoter (*35S::BBM:GR*).

Transgenic plants containing the *35S::BBM:GR* gene were obtained that showed no-, moderate- and severe *BBM* ectopic expression phenotypes after DEX induction. The most striking phenotypes observed in DEX-treated *35S::BBM:GR* seedlings grown in vitro were the high frequencies of spontaneous callus and

ectopic root and shoot formation (Fig. 4e–h). Ectopic shoots emerged from the leaf axils, petioles and leaf blade of the plant, while ectopic roots developed from the callus tissue formed at the hypocotyl-root transition zone, from the main stem, and from the stems of ectopic shoots.

35S::BBM:GR seedlings with severe *BBM* ectopic expression phenotypes were highly regenerative via organogenesis, however, spontaneous somatic embryo formation was never observed in these lines. This is in contrast to *Arabidopsis*, where the *35S::BBM:GR* construct induces a high penetrance of somatic embryo formation (data not shown). However, a series of experiments revealed that addition of 1–2 mg/l of zeatin or 1 mg/l BAP to the culture medium was sufficient to induce somatic embryogenesis in DEX-activated *35S::BBM:GR* tobacco seedlings. Our observations on wild-type and *35S::BBM:GR* seedlings treated with 1 mg/l zeatin are described below.

Germination of both wild-type and *35S::BBM:GR* seeds was slightly delayed on zeatin-containing media in comparison to the non-cytokinin treated controls. After 4 weeks both the wild-type and *35S::BBM:GR* seedlings on medium supplemented with zeatin developed a short single main root and produced callus tissue and adventitious shoots at the transition zone between the root and hypocotyl (Fig. 4i). Addition of DEX to the zeatin-containing medium induced changes in the development of *35S::BBM:GR* seedlings, while the response of the wild-type seedlings was unchanged. The root system of zeatin-plus DEX-treated *35S::BBM:GR* seedlings expanded, and adventitious shoots developed along the vascular tissue of the cotyledons (Fig. 4j) and from the endosperm-derived callus (Fig. 4k), even when the endosperm was not in contact with the medium. Callus and shoot formation at the hypocotyl-root transition zone of DEX-plus zeatin-treated *35S::BBM:GR* seedlings was drastically reduced and was replaced by somatic embryo formation (Fig. 4l). Zeatin concentrations below 1 mg/l were not sufficient for a reliable somatic embryo induction. Somatic embryos first become visible 21–24 days after sowing on zeatin-containing medium and become very obvious by 28 days. The somatic embryos that formed at the transition zone between the root and hypocotyl of the DEX-induced *35S::BBM:GR* seedlings developed as either single embryos or clusters, and arose directly from the seedling transition zone rather than indirectly via callus (Fig. 4m). Unlike adventitious shoots, *35S::BBM:GR*-induced somatic embryos are generally bipolar, comprising two cotyledons, and an axis with a distinct basal/radicle end. The somatic embryos are easily detached from the maternal tissue, indicating that they are not attached to the underlying seedling vasculature (Fig. 4m, n). Transfer of the somatic embryos to hormone- and DEX-free medium induced root formation and further vegetative development. A number of developmental abnormalities in cotyledon formation, including altered number of cotyledons and fused cotyledons, were routinely observed in the DEX- and zeatin-induced *35S::BBM:GR* somatic embryos (Fig. 4l, m).

Discussion

The BBM AP2/ERF transcription factor induces embryogenic cell development in *Arabidopsis* and *B. napus*. *BBM* is expressed during the early stages of in vitro and seed embryogenesis, and in the seedling root meristem (Boutilier et al. 2002; Casson et al. 2005; Nawy et al. 2005). Ectopic *BBM* expression in *Arabidopsis* is sufficient to induce spontaneous somatic embryogenesis and shoot development from seedlings and explants, and its expression in older tissues such as leaves and flowers induces pleiotropic morphological alterations. These observations suggest a developmental context dependent role for *BBM* in promoting cell proliferation processes. Here we examined the effect of expressing *Arabidopsis* and *B. napus BBM* genes on the development and regeneration capacity of a heterologous species, *N. tabacum* (tobacco). Constitutive *BBM* expression in tobacco recapitulates many of the developmental alterations observed in *Arabidopsis* and *B. napus 35S::BBM* transgenics, including de-regulated cell growth and differential growth of leaf and floral organs, but also induces new phenotypes, including adventitious root production from vegetative tissues. Spontaneous BBM-induced somatic embryogenesis was notably absent in the *35S::BBM* transgenics, but could be induced on seedling hypocotyls using concentrations of zeatin or BAP that were unable to induce somatic embryogenesis in wild-type plants. The results support the idea that the effect of *BBM* expression on cell proliferation processes is tissue/cell-dependent, and also illustrate that fundamental differences exist in the ability of different species to respond to the same signalling molecule.

There are number of possible explanations as to why different phenotypes are observed in tobacco and *Arabidopsis*/*B. napus* after expression of the crucifer *BBM* genes. One possibility is that the crucifer and tobacco *BBM* orthologues may differ enough in their sequence, such that heterologous *BBM* gene expression in tobacco activates a different set of targets genes than would normally be activated by the endogenous

tobacco *BBM* genes. A second possibility is that inherent differences exist in the developmental competence of tobacco cells and tissues to undergo organogenesis and somatic embryogenesis. Tobacco is competent for BBM-mediated organogenesis, but might lack the specific molecular or physiological environment required for spontaneous BBM-mediated somatic embryogenesis.

The phenotypes that arise from constitutive expression of *BBM* in tobacco are pleiotropic and therefore difficult to ascribe to alterations in a single specific signalling pathway or developmental process. However, a number of the phenotypes observed in transgenic tobacco lines that constitutively express *BBM* have been described for tobacco plants with altered hormone levels. Classical tissue culture experiments have demonstrated that exogenous application of a low ratio of auxin to cytokinin promotes shoot regeneration from tobacco callus, while a high auxin to cytokinin ratio stimulates root formation (Skoog and Miller 1957). These observations are supported and extended by numerous studies in which heterologous expression of bacterial cytokinin-(isopentenyltransferase; *ipt*) and auxin biosynthesis enzymes (tryptophan monooxygenease, *iaaM;* indoleacetamide hydrolase; *iaaH*) has been used to alter endogenous levels of these hormones (Smigocki and Owens 1988; Sitbon et al. 1992; Hewelt et al. 1994; Eklöf et al. 2000). *BBM* over-expression transgenics show elements of both cytokinin and auxin over-production phenotypes including ectopic shoot production and leaf wrinkling (cytokinin) and ectopic root production (auxin). The simultaneous production of ectopic roots and shoots in a single plant has to our knowledge only been reported in the offspring of crosses between cytokinin- and auxin-overproducing transgenic lines (Eklöf et al. 2000). Endogenous cytokinin and auxin levels in these plants were similar to wild-type hormone levels, suggesting that the observed phenotypes are due to local physiological responses to auxin and cytokinin levels/ratios at the cellular rather than at the organ level.

A number of the pleiotropic BBM-induced phenotypes we observed in ectopic expression lines have also been described for tobacco plants that ectopically express Class 1 *KNOX* (knotted-like) homeobox genes. *KNOX* homeobox genes are expressed in the shoot meristem where they are required for maintenance of the meristem (reviewed in Hake et al. 2004). Ectopic expression of *KNOX* genes induces species-specific alterations in leaf shape and morphology, as well as ectopic meristem/shoot production. Many of these phenotypes are similar to transgenics expressing bacterial *ipt* genes, and indeed ectopic *KNOX* expression alters not only cytokinin, but also gibberellin and auxin metabolism (Tamaoki et al. 1997; Hewelt et al. 2000; Frugis et al. 2001; Sakamoto et al. 2001). In tobacco, mild *KNOX* misexpression phenotypes that are similar to *BBM* misexpression phenotypes include adventitious shoot formation, rumpled leaves with a disorganized or absent palisade parenchyma layer and flowers that are pale to white in colour and that have stamens that are shorter than those from wild-type plants (Kano-Murakami et al. 1993; Sinha et al. 1993; Tamaoki et al. 1997; Sato et al. 1998; Postma-Haarsma et al. 1999).

Adventitious root (stem cell) formation, in addition to being stimulated by auxin, is also induced by ectopic expression of the BBM-related *Arabidopsis* AP2/ERF transcription factors, PLETHORA1 (PLT1) and PLT2 (Aida et al. 2004). *PLT1/PLT2* expression does not appear to alter auxin levels, but rather is upregulated in response to auxin accumulation (Aida et al. 2004). *PLT1* and *BBM* are similar in their sequence and gene expression patterns, raising the possibility that the adventitious root production observed in *35S::BBM:GR* lines arises from activation of *PLT1* target genes.

Plant growth regulators are required for the induction of somatic embryogenesis from non-embryo tissue and explants in the majority of plants (Gaj 2004). Only a few studies examined the tissue culture conditions that induce somatic embryogenesis in tobacco. TDZ, a substituted phenylurea that replaces the need for cytokinin and possibly auxin in cell cultures, as well as a combination of cytokinin (BAP) and auxin (NAA), are efficient inducers of somatic embryos from tobacco leaf discs (Stolarz et al. 1991; Gill and Saxena 1993). Here we show that ectopic BBM expression in combination with cytokinin is sufficient to induce somatic embryogenesis in tobacco seedlings, suggesting that BBM acts by bypassing the need for one or more of these plant growth regulators. Spontaneous somatic embryogenesis has been observed in a number of *Arabidopsis* mutants, including the gain-of-function *LEAFY COTYLEDON1* (*LEC1*) *and LEC2* mutants (Lotan et al. 1998; Stone et al. 2001) and the *pickle* (*pkl*) loss-of-function mutant (Ogas et al. 1997). Both LEC proteins and PKL appear to function in the context of hormone signalling pathways to activate spontaneous somatic embryogenesis. PKL acts together with gibberellin to repress embryo identity genes during germination (Ogas et al. 1997). Auxin-induced somatic embryogenesis in *Arabidopsis* requires *LEC1* and *LEC2* expression (Gaj et al. 2005). Normal auxin distribution (as measured by a *DR5::GUS* reporter construct) was observed in *lec2-1* plants treated with somatic embryogenesis induction medium containing 2,4-D, leading

Gaj et al. (2005) to suggest that *LEC2* functions downstream of auxin in this hormone-dependent somatic embryogenesis system. However, Braybrook et al. (2006) showed that ectopic expression of a *35S::LEC2:GR* transgene activates *IAA30* gene expression, indicating a potential link between auxin signaling and *LEC2*-induced somatic embryogenesis. Whether *BBM* also alters hormone levels or hormone signalling pathways to induce somatic embryogenesis and organogenesis remains to be determined.

In conclusion, we showed that *BBM* over-expression in *N. tabacum* induces both similar and unique cell proliferation and differentiation responses as compared to *Arabidopsis* and *B. napus*. In this respect, the BBM signalling pathway can serve as a model for understanding the physiological and molecular factors that determine the competence of different cell types and germplasm for in vitro regeneration.

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