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BRIEF COMMUNICATION

Expression of the *BBM* gene during somatic embryogenesis of *Arabidopsis thaliana*

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Abstract

The relationship between somatic embryogenesis (SE) and the expression of the *BABY BOOM (BBM)* gene was studied in cultured immature zygotic embryos (IZEs) using a transgenic line of *Arabidopsis thaliana* containing a *BBMPro::GUS* construct. Results showed spatio-temporal differences in *BBM* expression in explants during culture. *BBM* promoter activity was observed in freshly isolated IZEs except distal parts of cotyledons. At the beginning of culture, considerable increase of GUS staining intensity was observed in all parts of explants, which maintained at high level over next few days and coincide with cell divisions. Gradual decrease of GUS distribution in explants was observed at about the 5th day of culture. *BBM* promoter activity became largely restricted to dividing cells, then to developing somatic embryos, shoot-like structures and callus. In parts of explants not involved in morphogenesis *BBM* promoter activity was absent or hardly seen. Thus the *in vitro* expression of *BBM* coincides with cell proliferation and morphogenesis.

Additional key words: GUS construct, immature zygotic embryos.

Somatic embryogenesis (SE) is a model system to investigate different aspects of embryogenesis and mechanisms controlling this process (for a review see Zimmermann 1993, Feher *et al*. 2003, Karami *et al*. 2009). Plant development and cell differentiation, including somatic embryogenesis, are regulated directly or indirectly by changes in gene expression (Goldberg *et al*. 1989). Attempts to understand mechanisms underlying the induction of somatic embryogenesis have resulted in identification of several genes, the overexpression of which have been shown to enhance effectively the regenerative competence of plant cells for somatic embryogenesis (Lotan *et al*. 1998, Hecht *et al*. 2001, Stone *et al*. 2001, Boutilier *et al*. 2002, Zuo *et al*. 2002).

BABY BOOM (*BBM*) gene, among others like *LEAFY COTYLEDON (LEC1* and *LEC2*) and *SOMATIC EMBRYOGENESIS RECEPTOR KINASE (SERK),* is postulated to be involved in the acquisition of embryogenic competence by somatic cells (Schmidt *et al*. 1997, Lotan *et al*. 1998, Somleva *et al*. 2000, Boutilier *et al*. 2002, Gaj *et al*. 2005). BBM is a transcription factor of AP2/ERF family expressed in seed and root meristem (Nole-Wilson *et al*. 2005). It was originally isolated as a marker for embryogenic cells in tissue culture (Boutilier *et al*. 2002), which activates a complex network of developmental pathways associated with cell proliferation and growth (Passarinho *et al*. 2008). *BBM* expression was observed in microspore-derived embryos of *Brassica napus* (Boutilier *et al*. 2002) and basal region of *Arabidopsis thaliana* zygotic embryos (Casson *et al.* 2005). It was also identified in root meristems and lateral roots of *Arabidopsis* seedlings (Casson *et al*. 2005, Galinha *et al*. 2007) and as an auxin-inducible gene in *Medicago truncatula* roots (Imin *et al*. 2007). The *BBM* gene was expressed early during microspore embryogenesis of *Brassica napus*, and it was postulated that the *BBM* gene was involved in the conversion from vegetative to embryogenic state of development (Boutilier *et al*. 2002). Ectopic expression of *BBM* in

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Abbreviations: 2,4-D - 2,4-dichlorophenoxyacetic acid; DSE - direct somatic embryogenesis; GUS - β-glucuronidase; ISE - indirect somatic embryogenesis; IZE - immature zygotic embryo; SAM - shoot apical meristem; SE - somatic embryogenesis.

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Arabidopsis thaliana and *Brassica napus* induced spontaneous somatic embryos and cotyledon-like structures formation from the vegetative tissues of young seedlings (Boutilier *et al*. 2002). Similar effect was induced by constitutive expression of *GmBBM* in *Arabidopsis* (El Ouakfaoui *et al*. 2010). In tobacco, heterologous *BBM* expression led to spontaneous formation of shoots and callus indicating its role in promoting cell proliferation and morphogenesis (Srinivasan *et al*. 2007). Mutant phenotypes of *bbm* and *plt2* have shown that *AtBBM* and *PLT2* function as redundant partners in early *Arabidopsis* embryogenesis (Galinha *et al*. 2007). Deletion and domain swap analyses revealed that bbm-1, highly conserved motif specific to *BBM-like* genes in different species, was important for somatic embryogenesis (SE) and acted cooperatively with at least one other motif (El Ouakfaoui *et al*. 2010). Phenotype generated by deletion of the bbm-1 motif was characterized by the complete loss of SE on cotyledons as well as the accompanying pleiotropic effects on seedlings, emerged at later stages of plant development (El Ouakfaoui *et al*. 2010). What is more, *BBM* gene expression in seeds is under strict spatial and developmental regulation (Boutiler *et al*. 2002, Nole-Wilson *et al*. 2005, Imin *et al*. 2007, El Ouakfaoui *et al*. 2010). Results presented in this paper concern histological analysis of spatio-temporal expression of the *BBM* gene within the explant and somatic embryos of *Arabidopsis thaliana* transgenic line carrying the *BBM::GUS* construct. Studies were also performed to answer the question if there is any correlation between cells which express *BBM* gene activity and cells engaged in somatic embryogenesis.

Arabidopsis thaliana L. Columbia ecotype transgenic line carrying the *BBM::GUS* construct (for more information see Casson *et al*. 2005; line At5g17430) were used as an explant source for *in vitro* culture. Plants were grown in soil mixed with *Vermiculite* (1:1) at 20 - 23 °C, 16-h photoperiod and irradiance of 100 μmol m^{-2} s^{-1} (white fluorescent tubes). In order to establish embryogenic cultures, siliques containing immature zygotic embryos (IZEs) at the late cotyledonary stage were cultured following the standard protocol to induce SE (Gaj 2001). The IZEs were cultured in Petri dishes with modified solid induction medium (Gamborg *et al*. 1968) supplemented with 5 μM 2,4-dichlorophenoxy-acetic acid (2,4-D; *Sigma*, St. Louis, MO, USA) and pH adjusted to 5.8. The culture was kept in a growth chamber at 22 °C and a 16-h photoperiod for 3 weeks. The IZEs in a late cotyledonary stage of development (day 0 of the culture) and explants at every day of 21-d culture on induction medium were taken for histological analysis and assayed for *GUS* expression. A histochemical assay for β-glucuronidase (GUS) enzyme activity was performed according to method of Jefferson *et al*. (1987). Analysis of GUS distribution in IZEs, explants and somatic embryos was carried out under a stereomicroscope (*Nicon SMZ 1500*, Tokyo, Japan) equipped with a digital camera (*Nikon DS-U2*). Explants were fixed in ethanol-acetic acid (9:1, v/v) overnight at 4 °C and embedded in Steedman's wax (Vitha *et al*. 1997). The samples were cut into a series of sections with a *Zeiss Hydrax M40* (Jena, Germany) microtome. Sections 8 - 10 μm thick were attached to microscopic slides with Haupt's adhesive and mounted under coverslips in 50 % glycerol. Sections were examined under an *Olympus BX42* (Tokyo, Japan) microscope equipped with an *Olympus XC50* photo camera. At least 400 explants were analyzed.

To validate use of the *BBM* gene as a molecular marker for somatic embryogenesis, we analyzed expression of the *BBM::GUS* construct in IZEs and explants from day 1 to 21 of culture and in somatic embryos at different stages of development. Blue colouring indicated the GUS signal.

Freshly isolated IZEs displayed weak GUS staining in the mid part of cotyledons, hypocotyl and root (Fig. 1*A*). GUS expression did not occur in the distal part of cotyledons (Fig. 1*A*). Histological analysis of GUS distribution confirmed explant surface observations. GUS staining was detected in all explant cells, except cells in the distal parts of cotyledons (Fig. 1*B*). Strong GUS staining was visible in shoot and root apical meristems (Fig. 1*C*,*D*). In roots, especially intensive GUS staining characterized cells of the quiescent centre (Fig. 1*D*).

After 1 d of culture, analysis of explant surface revealed stronger GUS staining in whole explants compared to IZEs (Fig. 1*E*). Histological analysis of these explants showed strong GUS staining in protodermal cells including the distal part of cotyledons (Fig. 1*F*). A fairly high expression level was observed in shoot apical meristem (SAM; Fig. 1*G*) and in roots, especially in root cap cells (Fig. 1*H*). During next two days of culture GUS expression remained at the same, relatively high level in whole explant tissues. The strongest GUS signal was still in protodermis, SAM and fairly high in provascular tissue.

Differences in *BBM* promoter activity between different parts of explants coincided with swellings forming in proximal parts of cotyledons. Surface analysis showed that intense GUS staining was displayed in developing SAM and swellings (Fig. 1*I*). A slight decrease in *BBM* promoter activity in distal parts of cotyledons, hypocotyl and in roots was observed (Fig. 1*I*). Histological analysis of explants at this stage of culture, showed groups of protodermal and subproto-dermal cells undergoing periclinal an anticlinal cell divisions. Dividing cells displayed strong GUS staining in comparison to surrounding cells (Fig. 1*J*).

On the following days of culture, GUS expression became more restricted and occurred in parts of explants engaged in developmental processes and cell division. Very often intensive GUS staining was observed only on adaxial side of cotyledons in middle and proximal parts, which were areas considered as being embryogenic in this culture system (Fig. 1*K*). No or very weak expression was observed in non-embryogenic parts of explants.

From about day 10 of culture, the first somatic

embryos and the shoot-like structures developed. *BBM* promoter activity became weaker in root, hypocotyl, and parts of the cotyledons not involved in morphogenesis. GUS staining was observed in somatic embryos and shoot-like structures (Fig. 1*L*,*M*). The strongest GUS staining was still visible in external cell layers of shoot-like structures and somatic embryos. Especially intense GUS staining was observed in explant-derived callus tissues developing in proximal parts of cotyledons (Fig. 1*M*). Cells of explants lying below developing structures (organ-like, embryos) and callus also displayed GUS staining. Likewise, in a small number of explants, which did not form somatic embryos and organ-like structures, GUS staining was very weak or absent (data not shown).

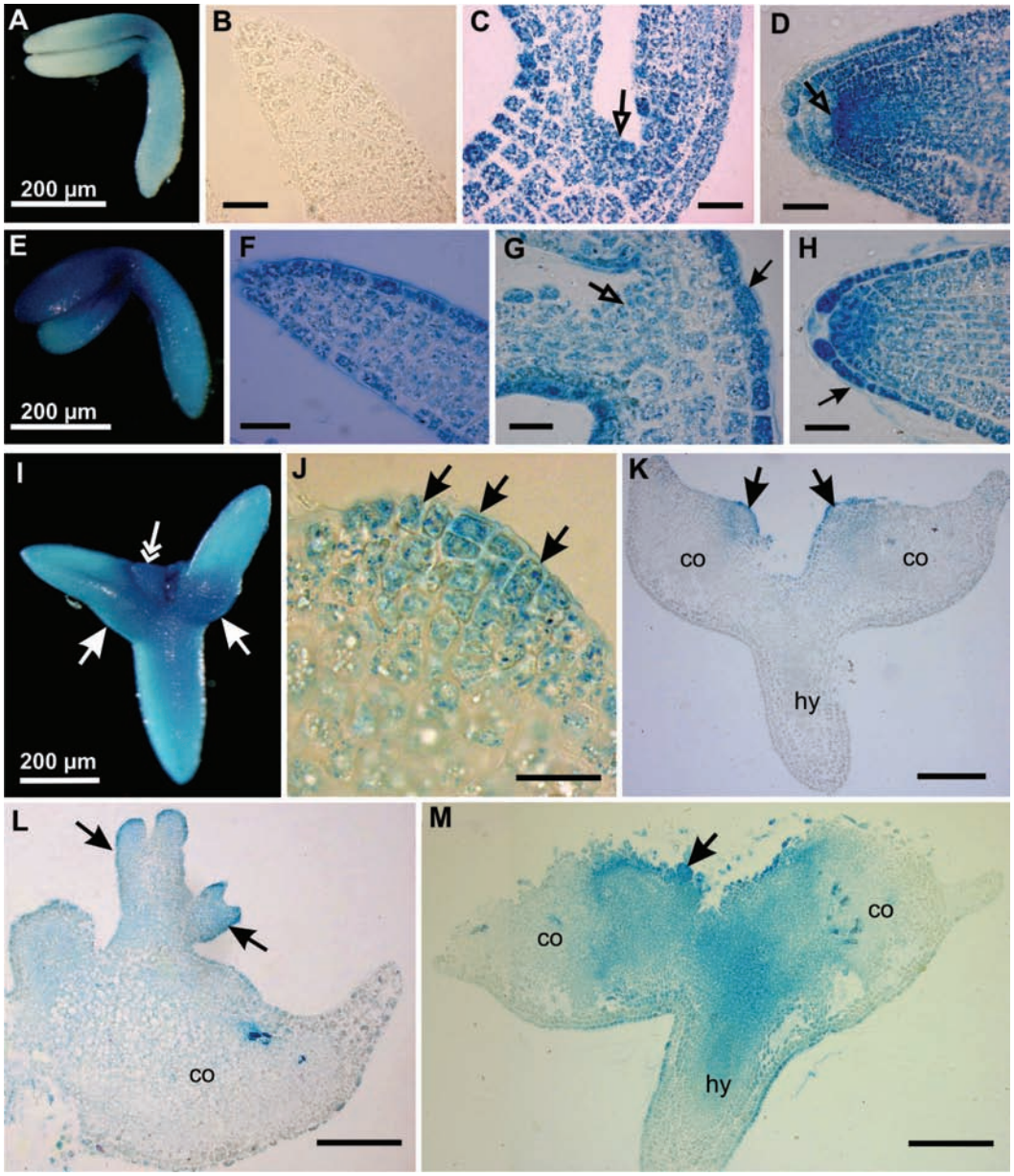

Fig. 1. GUS distribution in IZEs and explants. *A* - surface view of GUS distribution in whole IZE and histological analysis of particular parts of IZE, *B* - distal part of cotyledon, *C* - SAM (*empty arrow*), *D* - root with marked quiescent centre (*empty arrow*), *bars* = 20 µm, *E* - GUS distribution in the explant observed under stereomicroscope, *F* - histological analysis of the explant after 1 d of culture: distal part of cotyledon, *G* - SAM (*empty arrow*), protodermis (*arrow*), *H* - root with root cap (*bars* = 20 µm), *I* - surface view of GUS distribution in explant at 6th day of culture; strong GUS expression in swellings (*arrows*) and shoot-like structure (*double arrow*), *J* - dividing protodermal cells in the area of cotyledon node (*arrows, bar* = 20 µm), *K* - GUS staining in cells of adaxial side in proximal parts of cotyledons (co) and hypocotyl (hy), *bar* = 100 µm, *L* - shoot-like structures developing on proximal part of cotyledon (co) after 1 week of culture (*arrow*), *bar* = 100 µm, *M* - strong GUS staining in callus in proximal parts of cotyledons (co) and in hypocotyl (hy) at 10th day of culture, visible developing indirect somatic embryo (*arrow*), *bar* = 100 µm.

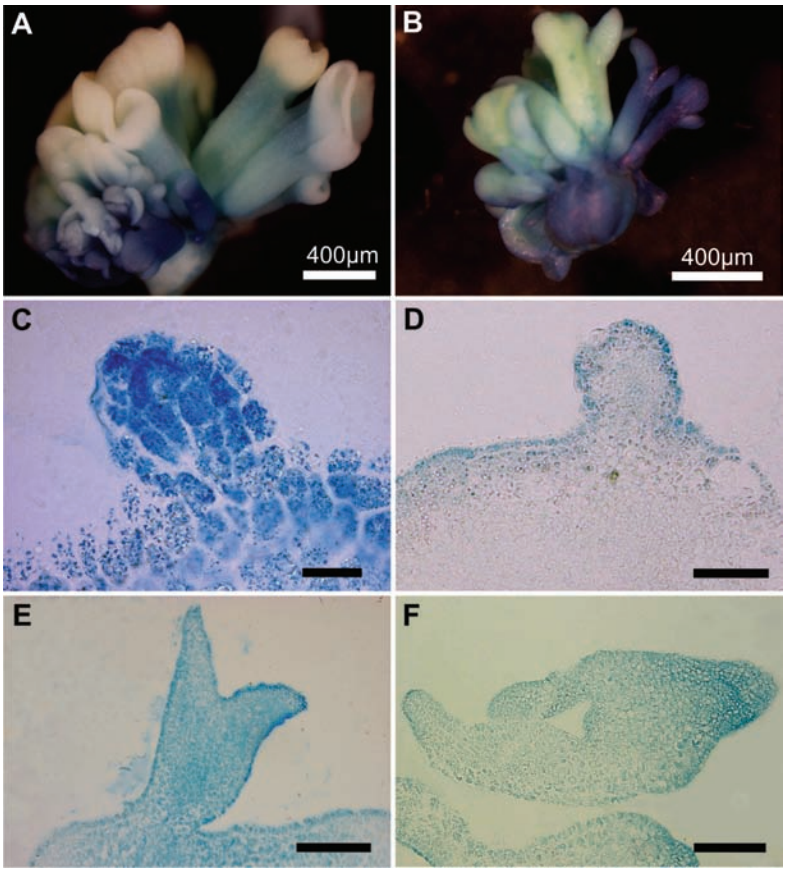

Fig. 2. Changes of *BBM::GUS* construct expression in somatic embryos. *A* - GUS distribution in somatic embryos at different stages of development – surface view; *B* - differences in GUS distribution among somatic embryos at cotyledonary stage; *C* - GUS staining in somatic embryos at early globular stage, *bar* = 20 µm, *D* - at late globular stage**,** *E* - at torpedo stage, *F* - at cotyledonary stage, *bars D*, *E*, *F* = 100 µm.

The GUS signal was investigated in somatic embryos at all developmental stages (Fig. 2*A*). Somatic embryos at an equivalent stage of development as IZEs displayed similar GUS distribution in root pole, hypocotyl and in proximal parts of cotyledons and no GUS staining in distal parts of cotyledons (Fig. 2*A*). Some of embryos at cotyledonary stage displayed very weak or no GUS expression, even when neighbouring, younger embryos exhibited strong GUS signal (Fig. 2*B*). During embryo development strongest GUS staining was observed at the early globular stage. At this stage reporter gene expression was homogenous in all cells of the somatic embryo and was higher than in explant tissue (Fig. 2*C*). At later stages of development, changes in GUS distribution within embryos were observed. Somatic embryos at the late globular stage displayed strong GUS staining only in protodermal cells and weaker in ground tissue, but still *BBM* promoter activity was higher in somatic embryos than in explant tissue (Fig. 2*D*). In embryos at early torpedo stage GUS signal was also strongest in the protodermis and weaker in ground tissue (Fig. 2*E*). Embryos at the cotyledonary stage displayed relatively stronger GUS staining in both root pole and SAM cells (Fig. 2*F*).

The aim of our study was to describe the distribution of *BBM* promoter activity in *Arabidopsis thaliana* explants during SE. This analysis showed spatio-temporal changes of this gene expression. Observed distribution of *BBM* gene expression indicated that its activity increased at the beginning of culture, which was probably related to auxin present in medium. Such conclusion confirm previous results showing that the AP2-domain containing *PLETHORA* and *BABY BOOM* were strongly induced by auxin (Imin *et al*. 2007). During later stages of the culture, high expression of this gene was associated with explant cells undergoing division including callus. Such results are in accordance with earlier studies which postulated the role of *BBM* gene in promoting cell proliferation and specifying an undifferentiated fate (Boutilier *et al*. 2002, Nole-Wilson *et al*. 2005, Srinivasan *et al*. 2007, Passarinho *et al*. 2008).

Analysis of *BBM* gene expression in somatic embryos of *Arabidopsis* showed expression of the *BBM* gene from the earliest stages of embryogenesis, which is in agreement with previous studies (Boutilier *et al*. 2002), and confirms the hypothesis that *BBM* is an important gene in embryo development (Boutilier *et al*. 2002, El Ouakfaoui *et al*. 2010). *BBM* expression pattern presented by Galinha *et al*. (2007) showed *BBM* promoter activity in provascular tissue and in the lens-shaped quiescent

centre progenitor cell at the heart stage of the zygotic embryo. A similar expression pattern was shown using a *BBM::GUS* fusion construct (Casson *et al*. 2005). Those studies are in contrast to results presented by Boutilier *et al*. (2002), which showed *BBM* gene expression in whole embryos at different stages of development. Results presented in this paper showed that in IZEs and in somatic embryos at an equivalent stage GUS expression pattern was similar to that shown by Casson *et al*. (2005). Our results showed that whole young somatic embryos displayed GUS expression what was in accordance to Boutilier *et al*. (2002).

It was suggested that *BBM* gene may play distinct roles at different stages of development and induce different pathways of development depending on the genetic and cellular environment (El Ouakfaoui *et al*. 2010). It may be the reason why BBM promoter activity was observed in embryos but also in other structures like shoots and callus.

We also tested the hypothesis that there was a correlation between explant cells expressed *BBM* gene and cells engaged in somatic embryogenesis. Our results showed some similarities between *BBM* and *LEC2* gene expression. Both genes are characteristic for periclinally dividing protodemal and subprotodermal cells in area of cotyledon node, so the part of explants which was postulated to be embryogenic (Kurczyńska *et al*. 2007). It was already suggested that *BBM* and *LEC* genes can have overlapping functions (Boutilier *et al*. 2002).

Such results may suggest that the *BBM* gene is involved in changes taking place in cells engaged in somatic embryogenesis because these protodermal cells which undergo ectopic occurrence of periclinal divisions are postulated to be cells generating the somatic embryos in *Arabidopsis* (Kurczyńska *et al*. 2007). In natural conditions epidermal cells divide anticlinally (Considine and Knox 1981). A distinct characteristic of protodermal/ epidermal cells is the direction of divisions (anticlinal) which is postulated to be a mechanism of self-maintenance of the surface layer of the plant embryo or body. In explant tissue, cells competent to change their fate are recognized as cells which respond to external signals to enter a specific, new pathway of development (Meins and Binns 1979, Lo *et al*. 1997, Namasivayam, 2007). It was suggested that the occurrence of cell divisions is needed for dedifferentiation of cells, which was shown for regenerative processes leading to bud formation (Chlyah et al. 1974, Lo *et al*. 1997). It was shown that periclinal divisions of epidermal/protodermal cells in the culture lead to the formation of somatic embryos (Uzelac *et al*. 2007, Chiappetta *et al*. 2009), and shoots (Lo e*t al.* 1997). Such divisions represented the dedifferentiation phase during which competent cells are formed (Lo *et al*. 1997). Higher expression of *BBM* gene in protodermal cells, which undergo periclinal divisions may suggest that the *BBM* gene is a marker of embryogenically competent cells in the protodermis/ epidermis.

To conclude, our results show that prior to the expression in somatic embryos, organ-like structures and callus, *BBM::GUS* was expressed in dividing single cells and cell clusters. This indicates that *BBM* marks cells re-entering the cell cycle which is in agreement with previous research (Boutilier *et al*. 2002, Nole-Wilson *et al*. 2005, Passarinho *et al*. 2008).

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