Applied and Fundamental Aspects of BABY BOOM-mediated Regeneration

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Chapter 1

General Introduction

Iris Heidmann

From botanical observations to totipotency

At the end of the 18th century the German philosopher Johann Wolfgang van Goethe (1749-1832) wrote in the introduction of his book "The Metamorphosis of Plants": *"Wenn wir nun bemerken, daß es auf diese Weise der Pflanze möglich ist, einen Schritt rückwärts zu tun und die Ordnung des Wachstums umzukehren, so werden wir auf den regelmäßigen Weg der Natur desto aufmerksamer gemacht, und wir lernen die Gesetze der Umwandlung kennen, nach welchen sie einen Teil durch den anderen hervorbringt und die verschiedenen Gestalten durch Modifikation eines einzigen Organs darstellt"* (Goethe 1790). ("Hence we may observe that the plant is capable of taking this sort of backward step, reversing the order of growth. This makes us all the more aware of nature's regular course; we will familiarize ourselves with the laws of metamorphosis by which nature produces one part through another, creating a great variety of forms through the modification of a single organ" (Miller 2009)). Goethe stated more specifically that everything on a plant resembles a leaf or can be described as modified leaves. He based his thoughts on Linné's "Philosophia Botanica" (Linnaeus 1751) realising that plants, unlike animals, have the ability to change their body plan easily by forming an organ from another one. This and many other botanical observations by researchers and philosophers at that time inspired the 'cell theories' of Schleiden for plants (Schleiden 1838) and Schwann for plants and animals (Schwann 1839), in which cells within an organism can act autonomously and form a new individual. Experimental evidence for this forerunner to the 'totipotency theory' was provided by researchers like Vöchting, Sachs, and Haberlandt, who are regarded as the founders of plant tissue culture (Gautheret 1983). Meristems, which they called "*Vegetationspunkte*" were regarded as the consequence of the "continuity of the embryonic substance" ("*Continuität der embryonalen Substanz*"), which was thought to comprise remnants of the fertilised egg cell within the developing zygotic embryo. The structural changes occurring during zygotic embryo development, already described in great detail at the end of the 19th century by von Hanstein (von Hanstein 1870), were regarded as "form giving exercises to prepare for the real tasks" ("Gestaltungs-Vorübungen zur Vorbereitung der eigentlichen Aufgaben").

The totipotency theory has its origin in the cell theory, which states that all tissues develop from individual cells, that the first step towards a new cell is a nucleus, and that each organism carries an inherent power in which the arrangement of molecules is controlled in relation to the purpose of the tissue (Schwann and Schleiden 1847). In animals, the zygote, which is formed after the fusion of the egg cell with the sperm cell, is considered to be the only naturally totipotent cell, that is, a cell that can develop or differentiate into any other cell type. Pluripotent and multipotent cells, like stem cells or blastomeres (four to eight celled embryo), are more restricted as they can only differentiate into a few cell types, although some blastomeres can revert to totipotency (Ishiuchi and Torres-Padilla

2013). Unipotent cells are fully differentiated cells that can develop into a single, different cell type (transdifferentiation) if the conditions are right. Mammals have a limited number of stem cells and an additional cellular memory of the previous differentiation process that prevents a complete reversion to totipotency *in vivo* (Kim et al. 2010). The way in which the different levels of potency are acquired in mammals *in vivo* and *in vitro* is summarized in the Waddington model (Waddington 1957). The model describes how a totipotent cell, represented by a marble, gradually loses its totipotency by rolling through a descending epigenetic landscape of deep valleys by which it becomes a pluripotent, multipotent, and finally a differentiated cell. In later versions of the model, a cell has to "overcome a threshold" (cross one of the deep valleys) to transdifferentiate from one unipotent cell type to another unipotent cell type (Eguizabal et al. 2013). The reverse process, dedifferentiation of the unipotent cell, takes place in an uphill direction, and the cell has to be reprogrammed to regain a higher level of potency (Sánchez Alvarado and Yamanaka 2014), for example, towards pluripotency. This process is considered more complex and is also not included in the original Waddington model (Eguizabal et al. 2013).

Pluripotency in plants

Waddington's model is less frequently applied to plant development, perhaps because it is not well known (Slack 2002) or because plants exhibit a higher adaptive capacity or plasticity with respect to cell fate than the animal systems on which the model was based. Plant stem cells are produced by organising centres and are found in the shoot, root, flower and vascular meristems. Stem cells divide to renew their own identity and at the same time drive the continuous development of new tissues and organs. Plant meristems are maintained by a context-dependent network of plant hormones, transcription factors, and other signalling proteins (Verdeil et al. 2007; Heidstra and Sabatini 2014).

Although stem cells drive plant growth, their function can be taken over by other meristematic cells. For example, root tips can regenerate after wounding in the absence of stem cells, but not in the absence of a meristem (Sena et al. 2009), indicating that these remaining meristematic cells can reorganize to adopt new fates. Likewise, a new root organising centre can be formed from adjacent stem cells after it is ablated (Van Den Berg et al. 1997; Sabatini et al. 1999; Xu et al. 2006). Transdifferentiation from one cell fate directly into another cell fate also occurs naturally in plants, for example, when epidermal cells transdifferentiate into root hairs or trichomes (Tominaga-Wada et al. 2011).

The best illustration of the enormous plasticity of plants can be seen during *in vitro* culture systems where various explants can be induced to form completely different cell types, tissues and

organs (Thorpe 2012). Organogenesis, the process by which organs are formed from a group of tissues, forms the basis for many plant transformation and propagation protocols (Motte et al. 2014). Organogenesis can occur directly or indirectly via a 'callus' phase. The term callus is generally used to describe proliferating, unorganized tissue, that can be induced e.g. after wounding and/or by the addition of plant growth regulators from any part of the plant (Ikeuchi et al. 2013). Different callus types can be induced depending on the explant and growth regulator regime, including the highly prolific and often loose callus of suspension cultures (Moscatiello et al. 2013), as well as organogenic and embryogenic callus (Ikeuchi et al. 2013).

Gene expression and mutant analysis suggest that organogenic callus resembles a collection of lateral root meristems (Sugimoto et al. 2010; Atta et al. 2009). Lateral root meristems are derived from xylem-pole pericycle (stem) cells during normal plant development, but can also be formed in tissue culture from pericycle (-like) cells in a variety of explants, usually in response to a high auxin to cytokinin ratio (Atta et al. 2009; Che et al. 2007). When callus is left on the same auxin-rich medium it forms roots, while shoot formation usually requires application of a new hormone regime in the form of cytokinin or a high cytokinin to auxin ratio. The switch to cytokinin-rich medium is thought to repress lateral root differentiation in favour of shoot meristem development (Atta et al. 2009).

Stem cell-free transdifferentiation has also been reported in tissue culture, where *Xinnia* protoplasts directly transdifferentiate into trachery elements in response to auxin and cytokinin (Kohlenbach and Schoepke 1981).

Totipotency in plants

Pluripotency is the ability of one cell type to form another cell type, tissue or organ, while totipotency is the ability of a single cell to develop via embryogenesis into an entire organism. Totipotency during plant sexual reproduction is restricted to the zygote, which is formed in the ovule through fusion of an egg cell and a sperm nucleus. However, plant cells other than the zygote may also show natural or induced totipotency. During gametophytic embryogenesis, embryos form from the haploid gametes or accessory cells within the ovule (gynogenesis) or pollen grain (androgenesis). Gynogenesis and androgenesis are most commonly induced in tissue culture, but also occur *in planta* in apomictic plants (Dunwell 2010; Hand and Koltunow 2014), and after wide hybridisation or interspecific crosses with specific genotypes (Pichot et al. 2008; Murovec and Bohanec 2012; Lermontova and Schubert 2013). Somatic embryogenesis is another form of asexual reproduction in which embryos develop from diploid vegetative cells, rather than from haploid gametophytic cells. As such, somatic embryos are clones of the mother plant. Like gametophytic embryogenesis, somatic embryogenesis is most commonly induced *in vitro*, although somatic embryos may also form

naturally from the ovule in apomictic plants (Koltunow et al. 2013) or even from the zygotic embryo, so-called twin embryos (Vernon and Meinke 1994). Gametophytic or somatic embryos can be distinguished from adventitious shoots and roots by the absence of leaf trichomes, the lack of a vascular connection to the explant, and the accumulation of species-specific seed storage products, such as proteins, carbohydrates and lipids. Unlike regenerated roots or shoots, somatic embryos are bipolar, containing both a shoot and a root meristem.

The changes that a differentiated cell undergoes during somatic embryo induction resemble those of Waddington's reverse model, where a differentiated cell is triggered to switch back to an embryogenic state (Fehér 2008). The steps that take place as a differentiated plant cell develops into a totipotent cell are difficult to follow for a number of reasons. One problem is that somatic embryogenesis is usually induced from cells that are part of a highly complex tissue or organ that can comprise a mixture of different cell types. Imaging or cell tracking can also be difficult when the cell in question is embedded within an explant. Isolated plant cells such as protoplasts would be the perfect system to study the acquisition of totipotency, providing the efficiency of this system can be improved (Luo and Koop 1997).

One unanswered question in plant biology is whether totipotent cells are stem cells. Canonical stem cell niches are only formed after a number of divisions in zygotic embryos (Heidstra and Sabatini 2014), thus the cells that develop before and outside of these niches are not considered to be stem cells. A classical stem cell niche has not been identified for totipotent embryogenic cells in culture (Verdeil et al. 2007). Rather, these niches are established after the removal of auxin from culture when the embryo differentiates (Su et al. 2009). However, Verdeil *et al*. argue for the existence of a totipotent stem cell in tissue culture that differs from classical embryonic and post-embryonic stem cells with respect to cellular organisation, physiology and molecular aspects (Verdeil et al. 2007).

In general, there are two ways to induce somatic embryos, either directly from the explant or indirectly from the explant via a callus phase (George et al. 2008), but both forms can co-exist in the same explant (Yang and Zhang 2010). The first histological change observed during direct somatic embryogenesis is the formation of cell clusters in the epidermal or sub-epidermal layers of the explant, which are surrounded by a thick cell wall. The embryogenic cells within these clusters are generally rounder and smaller and have a larger nucleus and denser cytoplasm than their surrounding cells (Williams and Maheswaran 1986). The formation of condensed, cytoplasmic-rich cell clusters separated from the surrounding tissue is characteristic in some species for areas competent for somatic embryo formation or areas where somatic embryos have formed, but not yet

differentiated (Namasivayam et al. 2006; Bassuner et al. 2007; Solís-Ramos et al. 2010; Rocha et al. 2012; Kurczyńska et al. 2007).

During indirect somatic embryogenesis, so-called pro-embryogenic masses (PEMs), clusters of undifferentiated embryogenic cells, develop from the embryogenic callus and give rise to somatic embryos (Kocak et al. 2014; Ma et al. 2003; Solís-Ramos et al. 2010). Although universal characteristics of embryogenic and non-embryogenic callus have not been defined, embryogenic callus is often characterized by thick cell walls (Lee et al. 2013), can have a nodule-like structure and can contain higher levels of specific compounds including sugars, pectin, auxin or abscisic acid (Endress et al. 2009; Shang et al. 2009; Jiménez and Bangerth 2001). Non-embryogenic callus lacks these characteristics, although it might still be able to undergo organogenesis (Bibi et al. 2011). In practice it can be quite difficult to differentiate between direct and indirect somatic embryogenesis, and it is not clear if somatic embryos in the direct system directly switch to their new cell fate or whether they first pass through other developmental states before embryo fate is established.

Role of auxin and stress in somatic embryogenesis

The most common method by far to induce somatic embryogenesis is by exposing explants to synthetic derivatives of the plant growth regulator auxin, such as 1-naphthalene acetic acid (NAA) or auxinic herbicides such as 2,4-dichlorophenoxy acetic acid (2,4-D), 3,6-dichloro-2-methoxybenzoic acid (dicamba), or 4-amino-3,5,6-trichloro-2-pyridinecarboxylic acid (picloram) (Jiménez 2005). These auxins are either used alone or in combination with other plant growth regulators to induce somatic embryos directly from the explant or indirectly via embryogenic callus (Jiménez 2005).

Auxinic herbicides are used at high concentrations to kill dicot weeds in fields of monocot crops, but at lower concentrations in tissue culture they can induce callus, adventitious root formation or somatic embryogenesis. The success of auxinic herbicides for somatic embryo culture lies in their mode of action. Auxinic herbicides are thought to be more stable and therefore to elicit a stronger effect than natural auxins applied at the same concentration. Natural auxins are inactivated either by oxidation or by sugar or amino acid conjugation (Normanly 2010), while 2,4-D and dicamba are not inactivated (Kelley and Riechers 2007). A few studies have shown that genotypes recalcitrant to somatic embryo formation have a lower uptake of auxinic herbicides, alterations in auxin metabolism and biosynthesis or a different auxin binding affinity (Ceccarelli et al. 2002; Bai et al. 2013; Lee et al. 2014).

Application of herbicide-level auxin doses induces stress-related processes like ethylene synthesis and ABA accumulation as early as a few hours after application (Grossmann 2000). It is not clear how lower doses of the same herbicides induce somatic embryogenesis, but a stress response has also

been proposed to play a role (Fehér 2014). This conclusion is based in part on the observation that various abiotic stress treatments, including temperature or osmotic shocks and changes in pH are also able to induce somatic embryogenesis (Ikeda-Iwai et al. 2003; Cabrera-Ponce et al. 2014; Teixeira da Silva and Malabadi 2012), and in part on the observation that tissue culture concentrations of synthetic auxins also induce stress and defence-related responses (Galland et al. 2001; Hoenemann et al. 2012; Stasolla 2010; Maillot et al. 2009; Gomez-Garay et al. 2013; Raghavan et al. 2006), including genes involved in ABA biosynthesis and signaling and ethylene signaling (Raghavan et al. 2006). Down-regulating the natural antioxidative response in the presence of 2,4-D enhances auxin-induced somatic embryogenesis in *Arabidopsis thaliana* (Arabidopsis (Becker et al. 2014)), suggesting that the inability to neutralize stressful reactive oxygen species (ROS) might partly underlie this switch in developmental pathways. The similarities between stress- and 2,4-D responses suggest that stress plays a role during reprogramming, but whether it is directly involved or a secondary consequence needs to be investigated in more detail.

Arabidopsis as a model system

Arabidopsis provides an excellent model system for studying somatic embryogenesis because of the wealth of genetic resources, including mutant and reporter-lines and ecotypes (Koornneef et al. 2004), the ease of performing functional genomics (Clough and Bent 1998), and the availability of different somatic embryogenesis systems. With respect to the latter, a large number of explants can be used for somatic embryo culture, including immature zygotic embryos (Wu et al. 1992; Gaj 2011), mature zygotic embryos (Kobayashi et al. 2010), meristems (Ikeda-Iwai et al. 2003), and even protoplasts (Luo and Koop 1997). The efficiency of somatic embryogenesis from zygotic embryos depends strongly on the developmental stage of the embryo. The optimal developmental stage for direct somatic embryogenesis is between the heart- (Luo and Koop 1997) and bent cotyledons stage (Gaj 2001), although this can vary with respect to the ecotype, the culture conditions and the 2,4-D concentration (Gaj 2004). Yields of close to 100% responding explants have been described (Nowak et al. 2012; Gaj 2001). The regulatory factors that promote zygotic embryo identity are down-regulated during seed germination (Jia et al. 2014; Braybrook and Harada 2008), which might be the cause of the lower efficiency of somatic embryo induction from older tissues such as mature, desiccated embryos (Kobayashi et al. 2010), leaves, or floral explants (Gaj 2004), where these factors are no longer expressed.

Regulation of totipotency at the chromatin level

Gene expression is regulated at both the DNA and chromatin level. Gene expression is usually activated by transcription factors when the associated chromatin is less condensed and transcriptional start sites or specific binding sites are free of nucleosomes. These nucleosomes, the structural unit of chromatin, consist of DNA wound around histone proteins. Proteins that modify DNA and histones alter the ability of other proteins, e.g. transcription factors, to bind DNA and can therefore block or promote transcription (Engelhorn et al. 2014). Mutants of a large number of Arabidopsis DNA or histone modifying proteins spontaneously form somatic embryos on seedlings. These genes encode ATP-dependent CHD chromatin remodeling factors like PICKLE (PKL) (Henderson et al. 2004; Ogas et al. 1999), POLYCOMB REPRESSIVE COMPLEX1 (PRC1) proteins like ATBMI1A/B (Bratzel et al. 2010), PRC2 complex proteins like CURLY LEAF (CLF), SWINGER (SW), EMBRYONIC FLOWER2 (EMF2), FERTILISATION-INDEPENDENT ENDOSPERM (FIE) and VERNALISATION2 (VRN2) (Bouyer et al. 2011; Chanvivattana et al. 2004), as well as the HISTONE DEACETYLASEs HDA6 and HDA19 (Tanaka et al. 2008) and the B3 domain VP/ABI3-LIKE (VAL) proteins HIGH-LEVEL EXPRESSION OF SUGAR INDUCIBLE GENE2 (HSI2)/VAL1 and HSL1/VAL2 (Suzuki et al. 2007). All of these proteins function to repress embryo identity during the transition to seedling growth, indicating that pathways essential for maintaining totipotency are shut down during this phase transition.

Role of transcription factors in plant cell totipotency

While chromatin modifying proteins play a role in repressing totipotency, a number of proteins have been identified that play a role in promoting totipotency (Harada et al. 2010), some of which are known targets of these repressive chromatin modifiers. The proteins that promote totipotency can be grouped into two classes: proteins that enhance somatic embryogenesis in the presence of growth regulators and proteins that spontaneously induce somatic embryogenesis. Here I focus on the role of the transcription factors LEAFY COTYLEDON (LEC) and BABY BOOM (BBM) (Lotan et al. 1998; Stone et al. 2001; Boutilier et al. 2002), both of which induce spontaneous somatic embryogenesis.

The *LAFL* network

Ectopic expression of the transcription factors *LEC1* or *LEC2* induces spontaneous somatic embryo formation (Lotan et al. 1998; Stone et al. 2001). *LEC1* encodes a HEME-ACTIVATED PROTEIN3 (HAP3)/CCAAT box binding factor, and *LEC2* encodes a B3-domain transcription factor. Both LEC1 and LEC2 are part of the LAFL transcription factor network (for LEC1/LEC1-LIKE (LIL), ABSCISIC INSENSITVE3 (ABI3), FUSCA (FUS3), and LEC2). LAFL proteins are essential for the normal progression

of zygotic embryo development and maturation (Braybrook and Harada 2008). *LEC1* and *LEC2* are first expressed during early zygotic embryogenesis, and also play a role in seed maturation later in development, where they stimulate seed storage protein and fatty acid biosynthesis (Junker et al. 2012; Stone et al. 2008; Mu et al. 2008; Baud et al. 2007). *FUS3* and *ABI3* expression increases during zygotic embryo development and peaks at the maturation *(FUS3)* and dormancy *(ABI3)* phases, when *LEC1* and *LEC2* expression has already declined (Yamamoto et al. 2014).

The loss of *LEC1, LEC2* or *FUS3* causes defects in embryo development, including desiccation intolerance and premature formation of leaf features like trichomes and vascular tissue in cotyledons (Meinke et al. 1994). Ectopic expression of *LEC1* and *LEC2* in seedlings induces somatic embryogenesis and promotes seed storage product accumulation (Lotan et al. 1998; Stone et al. 2001; Guo et al. 2013), while *FUS3* overexpression promotes cotyledon identity (Gazzarrini et al. 2004), and ectopic expression of *ABI3* enhances abiotic stress tolerance and induces seed storage production (Shiota and Kamada 2000; Tamminen et al. 2001). These data suggest that the *LEC* and *FUS* genes promote both embryo identity and maturation, while *ABI3* plays a more restricted role in seed maturation.

LAFL genes are required for auxin-induced somatic embryogenesis. *lec1, lec2, fus3,* and *abi3* mutants are completely recalcitrant for somatic embryogenesis when used as double (*lec1*;*lec2*, *lec1*;*fus3*, *lec2*;*fus3*) and triple (*fus3*;*lec1*;*lec2*) mutant combinations in an Arabidopsis 2,4-D-induced direct somatic embryogenesis system (Gaj et al. 2005; Gaj et al. 2006). The *lec1*, *lec2* and *fus3* single mutants are severely limited in their ability to form somatic embryos both in terms of the number of responding explants and the number of embryos formed per explant, and mainly produce watery callus and root hairs. In addition, while wild type zygotic embryos at different developmental stages are competent to form somatic embryos in response to 2,4-D, the *lec1* and *lec2* mutants are only responsive at heart and torpedo stages, while the *fus3* mutant is only responsive at the bent cotyledon stage. Adventitious shoot formation was not affected in these mutants (Gaj et al. 2006; Gaj et al. 2005).

It has been implied that stress-induced processes characteristic for the maturation phase of seed development might be induced by *LEC2* expression (Harada et al. 2010) and that this underlies the ability of LEC proteins to induce somatic embryogenesis (Stone et al. 2008). On the other hand, auxin biosynthesis and signalling genes are quickly upregulated in response to the LEC2 protein (Stone et al. 2008). Overexpression of *LEC2* in the presence of auxin can compensate for too little or the wrong type of auxin in somatic embryo culture (Wójcikowska et al. 2013), while high *LEC2* expression in combination with normal concentrations of 2,4-D is detrimental (Wójcikowska et al. 2013; Ledwon and Gaj 2009).

These results demonstrate that the LAFL network, which has an important role in early zygotic embryo development and maturation, also has an important role during somatic embryo formation, but it is not known whether it is their embryo identity, auxin signalling and/or maturation functions that are critical factors for somatic embryo induction.

AINTEGUMENTA-LIKE gene family

Overexpression of the BABY BOOM (BBM) AINTEGUMENTA-LIKE (AIL) AP2/ERF transcription factor also promotes spontaneous somatic embryogenesis (Fig. 1; (Boutilier et al. 2002)), and has also been used in different species in combination with exogenous growth regulators to enhance regeneration through organogenesis or somatic embryogenesis (Deng et al. 2009; Ananiev et al. 2009; Lutz et al. 2011; Boutilier et al. 2002; Heidmann et al. 2011; Srinivasan et al. 2007). The *AIL*-group comprises eight genes of which *AINTEGUMENTA* (*ANT*) and *AIL1* are phylogenetically distinct from the cluster of *PLETHORA1* (*PLT1*), *PLT2*, *AIL6/PLT3*, *CHOTTO1/AIL5/EMBRYOMAKER/PLT5*, *AIL7/PLT7,* and *BBM* (Kim et al. 2006). AIL proteins regulate many pathways, including organ size and positioning, specification of the stem cell niche, meristem maintenance, zygotic embryo development and growth (Horstman et al. 2014).

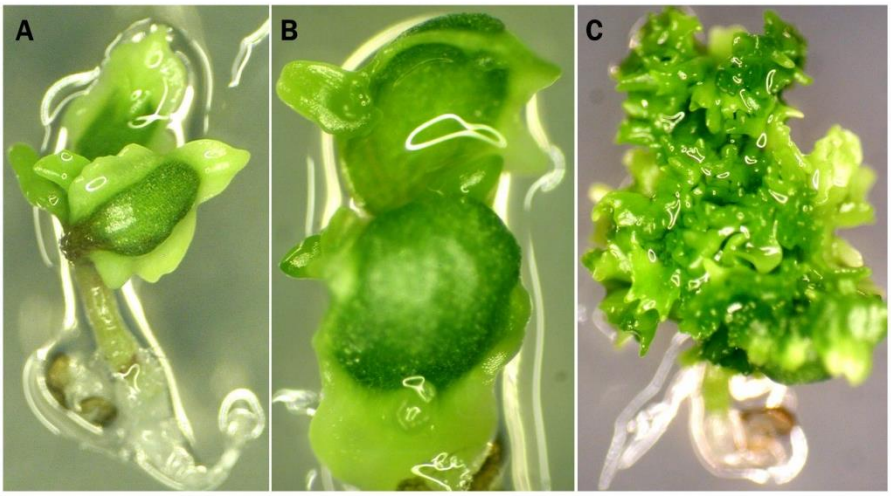

Figure 1: *BBM* induces rapid and prolific formation of embryogenic tissue and somatic embryos in Arabidopsis seedlings
A: A four-day-old *35S::BBM* seedling with embryogenic tissue at the margin of the cotyledon;
B: Formation of the first somatic embryos from the cotyledon margin in a seven-day-old *35S::BBM* seedling;
C: Primary and secondary somatic embryo formation at the cotyledons of a ten-day-old *35S::BBM* seedling.

BBM is expressed throughout the embryo from the two-celled stage to the globular stage, at which point it becomes predominantly expressed in the basal region of the embryo. BBM expression remains restricted to the root meristem after germination (Casson et al. 2005; Galinha et al. 2007). Single *bbm* loss-of-function mutants do not show abnormal phenotypes, while homozygous

bbm;plt2 mutants are arrested early in embryo development and seedlings segregating for different *plt* and *bbm* combinations show reduced root meristem growth or lack a root (Galinha et al., 2007).

Although BBM and PLT2 share functions during embryo and root development, they have different overexpression phenotypes. *BBM* overexpression induces somatic embryo or adventitious shoot formation (Boutilier et al. 2002; Srinivasan et al. 2007), while *PLT2* overexpression induces ectopic root formation (Aida et al. 2004; Galinha et al. 2007). Overexpression of *PLT5/AIL5/EMK/CHO1* also induces somatic embryogenesis, but the double mutant *bbm;cho1/ail5/emk/plt5* does not have a mutant phenotype, indicating that either BBM and EMK act independently or share functions with other AIL proteins (Tsuwamoto et al. 2010)..

Currently, not much is known about how AIL proteins promote regeneration. AIL proteins regulate and are regulated by auxin-dependent pathways and promote stem cell niche and meristem development during normal plant development (Horstman et al. 2014). PLT3, PLT5 and PLT7 regulate de novo shoot regeneration in a two-step process involving the induction of shoot progenitor cells followed by shoot initiation from these progenitor cells (Kareem et al. 2015). It is not known how these natural and induced pathways relate to BBM- and AIL5/CHO1/EMK/PLT5-mediated somatic embryogenesis.

Microarray analysis of BBM direct target genes in five-day-old seedlings showed that BBM activates its own expression, as well as expression of a broad range of genes with roles in cytoskeleton formation, cell proliferation, transcription, signalling, protein interactions and in cell wall/membrane formation (Passarinho et al. 2008), but no direct links between these genes and somatic embryo induction could be identified. Recently, BBM was shown to interact with L1-expressed HOMEODOMAIN GLABROUS (HDG) transcription factors, which function antagonistically to BBM, with BBM promoting cell proliferation and HDGs promoting differentiation (Horstman et al. 2015). Overexpression of *HDG1* antagonizes BBM-mediated somatic embryogenesis (Horstman et al. 2015), but the specific role of this interaction during the acquisition of totipotency is not known.

Somatic embryos are induced by exposure to auxin, and this process shows some similarity to the way in which BBM induces somatic embryogenesis. One AIL family member, CHO1/EMK/AIL5/PLT5 directly activates the expression of auxin biosynthesis genes, while other AIL proteins act upstream and downstream of auxin (Horstman et al. 2014; Pinon et al. 2013; Aida et al. 2004). LEC1 and LEC2 also activate auxin biosynthesis and signalling (Stone et al. 2008; Junker et al. 2012). Combined, these data suggest a link between BBM, auxin and somatic embryo induction.

Outline of the thesis

Somatic embryogenesis is not only a fascinating process to study at the fundamental level, but also has many applications in plant breeding, where knowledge on embryo formation can be applied to many regeneration, propagation and production processes. This thesis highlights applications of BBM-mediated somatic embryogenesis for two commercially important crops, tobacco and sweet pepper, and attempts to explain some of the mechanisms underlying BBM-mediated changes in cell fate in the model plant Arabidopsis.

Chapter 1 gives a short historical summary of how botanical observations supported the development of the totipotency theory, briefly outlines the different totipotency pathways in plants, and then provides a more detailed description of somatic embryo initiation and development in tissue culture, especially in relation to auxin and transcription factors.

Chapter 2 demonstrates that overexpression of a *Brassica napus BBM* gene in tobacco *(Nicotiana tabacum L.)* induces somatic embryogenesis and other phenotypes that were first identified after overexpression of the same gene in Arabidopsis. However, in tobacco, somatic embryogenesis was only achieved after the addition of cytokinin, and embryos formed at the transition zone between the hypocotyl and root, rather than at the cotyledons and meristems as with Arabidopsis seedlings. This study also highlights the utility of using a post-translationally inducible BBM-GR fusion protein to restrict BBM overexpression phenotypes to specific developmental time points.

In **Chapter 3** we show how *BBM* overexpression can be used to improve transformation in a recalcitrant species. We demonstrate that controlled overexpression of *BBM* closes the gap between sweet pepper (*Capsicum annuum*) tissues that are competent for *Agrobacterium tumefaciens* infection and tissues that are able to regenerate. By restricting BBM activity to the transformation and early regeneration phase we were able to regenerate numerous independent transgenic pepper plants whose offspring also showed highly efficient and prolific somatic embryo formation. As with tobacco, application of a cytokinin was required to induce somatic embryo formation.

Chapter 4 describes the BBM-mediated sweet pepper transformation protocol in more detail.

Chapter 5 explores the influence of developmental context and protein dose on AIL-mediated somatic embryogenesis. First we show that overexpression of all members of the BBM clade of AIL proteins induces somatic embryogenesis. Next we demonstrate, using *BBM* and its homologue *PLT2*,

that the developmental outcome of *AIL* overexpression depends on the protein dose and timing of activation of these transcription factors. A low dose of BBM or PLT2 protein promotes organogenesis, while a high dose promotes somatic embryogenesis. We also demonstrate that expression of *BBM* at different time points in seedling development induces two different somatic embryogenesis pathways, namely direct and indirect. Finally, using protein-DNA binding studies, gene expression and genetic analysis, we show that the LAFL proteins are important components of BBM-mediated somatic embryogenesis.

The study in **Chapter 6** investigates the role of auxin biosynthesis and transport in BBM-mediated somatic embryogenesis. We identified auxin biosynthesis and transport genes that are directly bound and transcriptionally regulated by BBM. We show that BBM induces an enhanced auxin response and that both auxin biosynthesis and transport play roles in BBM-mediated somatic embryogenesis.

Chapter 6 summarizes and discusses the major findings of this thesis, places them in context with each other and recent publications, and outlines options for future research and applications.

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Chapter 2

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, posttranslational 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 N6 Benzylaminopurine

NAA Napthaleneacetic acid

TDZ Thidiazuron (N-phenyl-N-1,2,3,-thiadiazol5-ylurea)

TEM Transmission electron microscopy

SEM Scanning electron microscopy

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; Banno et al. 2001; Stone et al. 2001; Boutilier et al. 2002; Zuo et al. 2002; Hewelt et al. 1994). 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 (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; Okamuro 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; Elliott 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). Overexpression 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* L.) 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 amplifed 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 (Gen-Bank 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 (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 basal medium (Murashige and Skoog 1962) plus 2% sucrose (MS20). Dexamethasone (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 DEX. 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 wildtype untransformed plants were fixed for 1 h in 4% (w/v) formaldehyde and 1% (w/v) gluteraldehyde in 0.1 M phosphate buffer, and post-fixed in 1% (w/v) 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 CO2, 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 RTPCR 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/MGBNFQ Taqman® probes were synthesized by Applied Biosystems. Relative AtBBM gene expression levels were determined using the comparative $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). 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; RIBOSOMAL L25 forward primer, 5-GGCCTGATGG GACGAAGA-3; RIBOSOMAL L25 reverse primer, 5-CAACGTCCAAAGCATCATAGTCA-3; RIBOSOMAL 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).

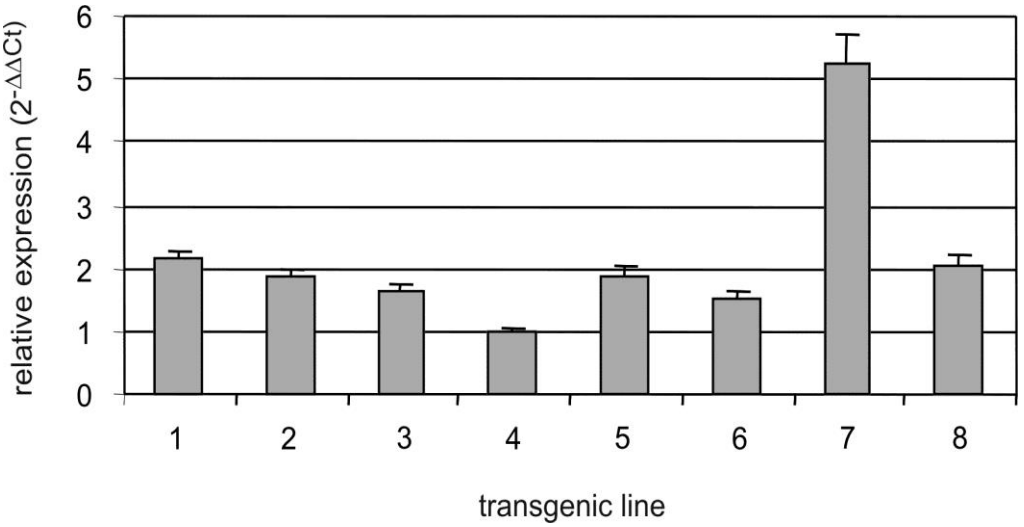

Figure 1: Relative expression levels of the *AtBBM* transgene in tobacco.
The relative expression level of the *AtBBM* transgene was measured in eight independent *35S::AtBBM* lines using quantitative real-time RT-PCR. Relative expression levels were calculated using the $2^{-\Delta\Delta ct}$ method 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).

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.

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.

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

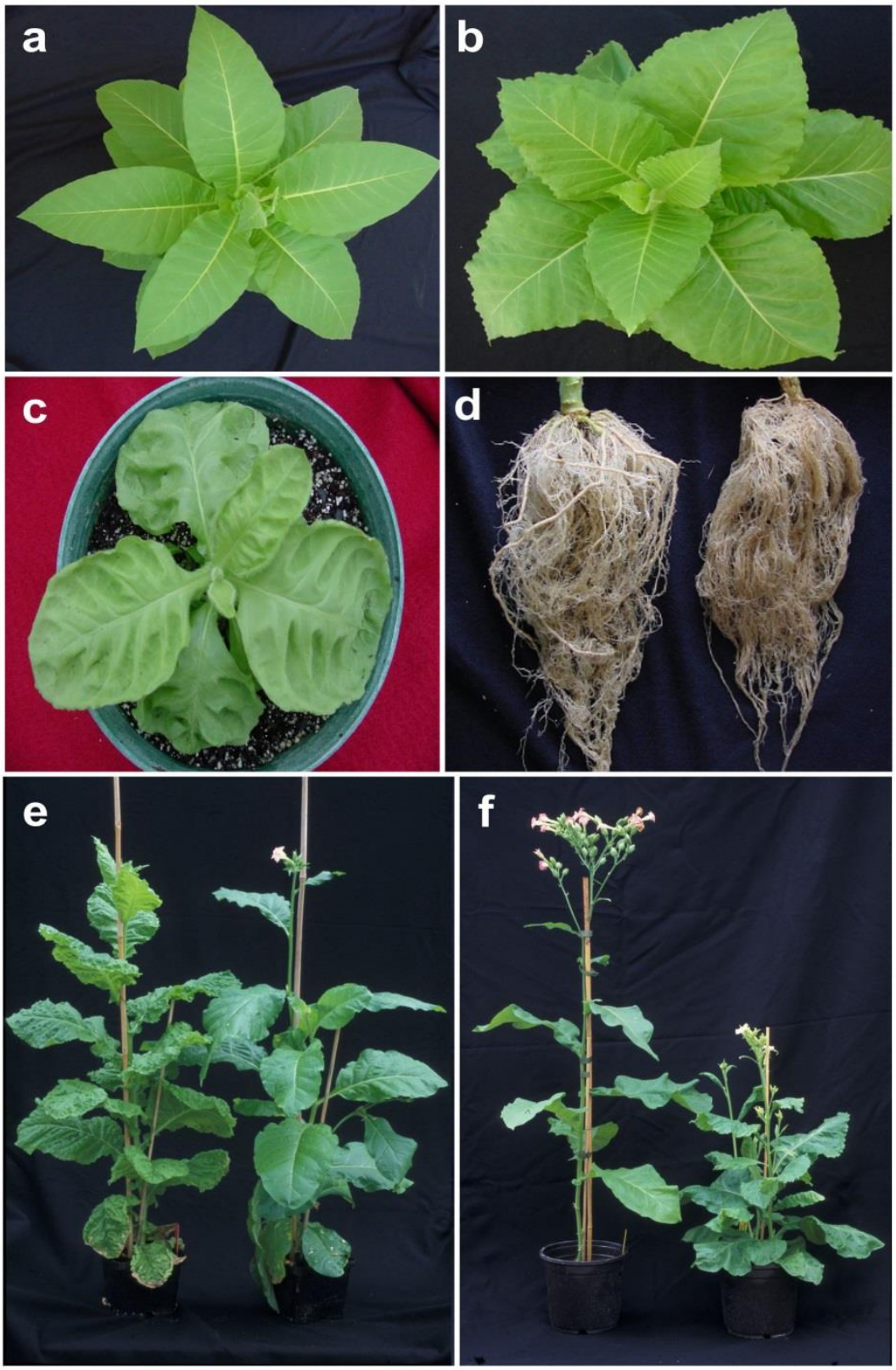

Figure 2: Morphological alterations induced by expression of the *35S::BBM* transgene in tobacco.
a, wild-type plant. **b**, *35S::AtBBM* plant showing mild leaf rumpling and a wavy margin. **c**, leaves of a severe *35S::AtBBM* plant showing deep interveinal depressions. **d**, root systems of wild-type (left) and *35S::At-BBM* (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**.

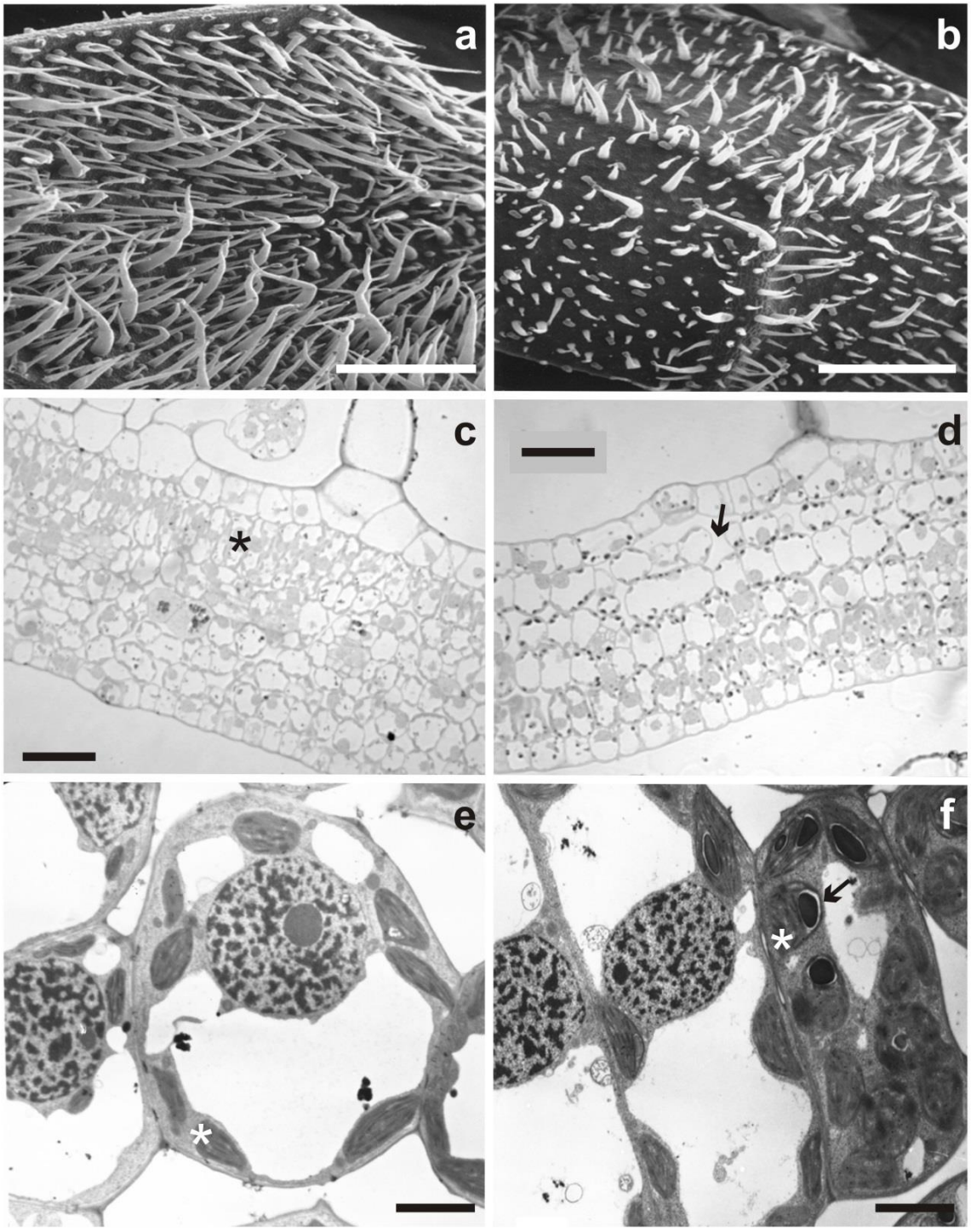

Figure 3: Ultrastructural analyses of leaf development in *35S::BBM* tobacco plants.
Images in **a**, **c**, and **e** are wild type plants. Images in **b, d,** and **f** are *35S::BBM* plants. **a, b** SEM of wild-type and *35S::BBM* leaf surfaces. The wild-type leaf has longer and more densely packed 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 m (**c, d**) and 4 m (**e, f**). All SEM, TEM and light microscopic sections of *35S::BBM* plants were from the depressed interveinal portion of the leaf.

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).

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).

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 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

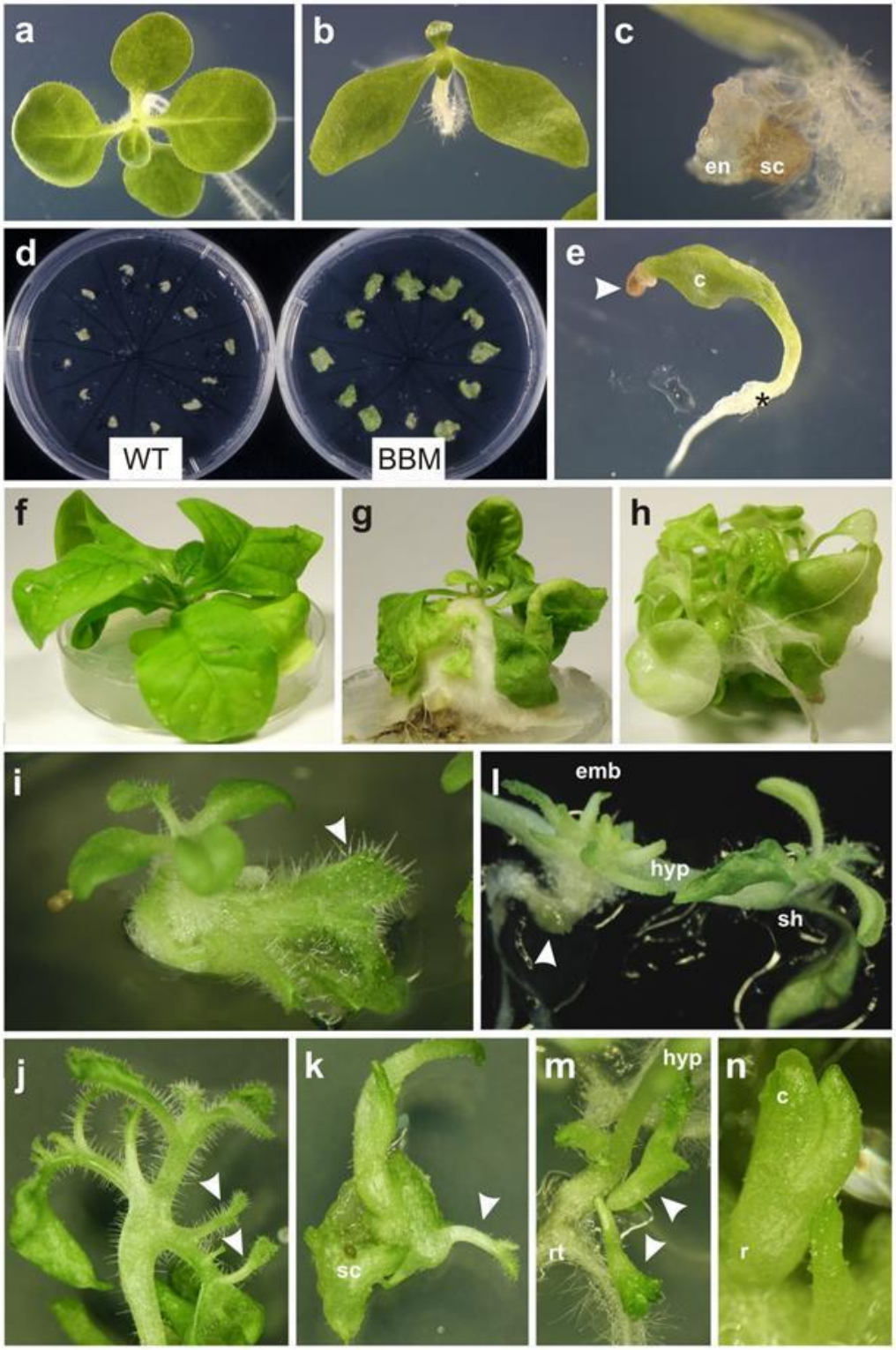

Figure 4: Seedling phenotypes and regenerative capacity of BBM misexpression lines.
a, Wild-type; **b** and **c** *35S::BBM* seedlings, 14 days after sowing. The cotyledon and leaves of the wild-type seedling are rounder than those of the transgenic. The endosperm (en) of *35S::BBM* seeds (**c**) continues to divide after germination and forms callus-like cells. sc seed coat; **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 *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 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.

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

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 (Hewelt et al. 1994; Eklof et al. 2000; Sitbon et al. 1992; Smigocki and Owens 1988). *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 (Eklof 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. (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 (Hewelt et al. 1994; Frugis et al. 2001; Sakamoto et al. 2001; Tamaoki et al. 1997). 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; Postma-Haarsma et al. 1999; Sato et al. 1998; Tamaoki et al. 1997; Sinha et al. 1993).

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 (Gill and Saxena 1992; Stolarz et al. 1991). 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. (Gaj et al. 2005) to suggest that LEC2 functions downstream of auxin in this hormone-dependent somatic embryogenesis system. However, Braybrook et al. (Braybrook et al. 2006) showed that ectopic expression of a *35S::LEC2:GR* transgene activates *IAA30* gene expression, indicating a potential link between auxin signalling 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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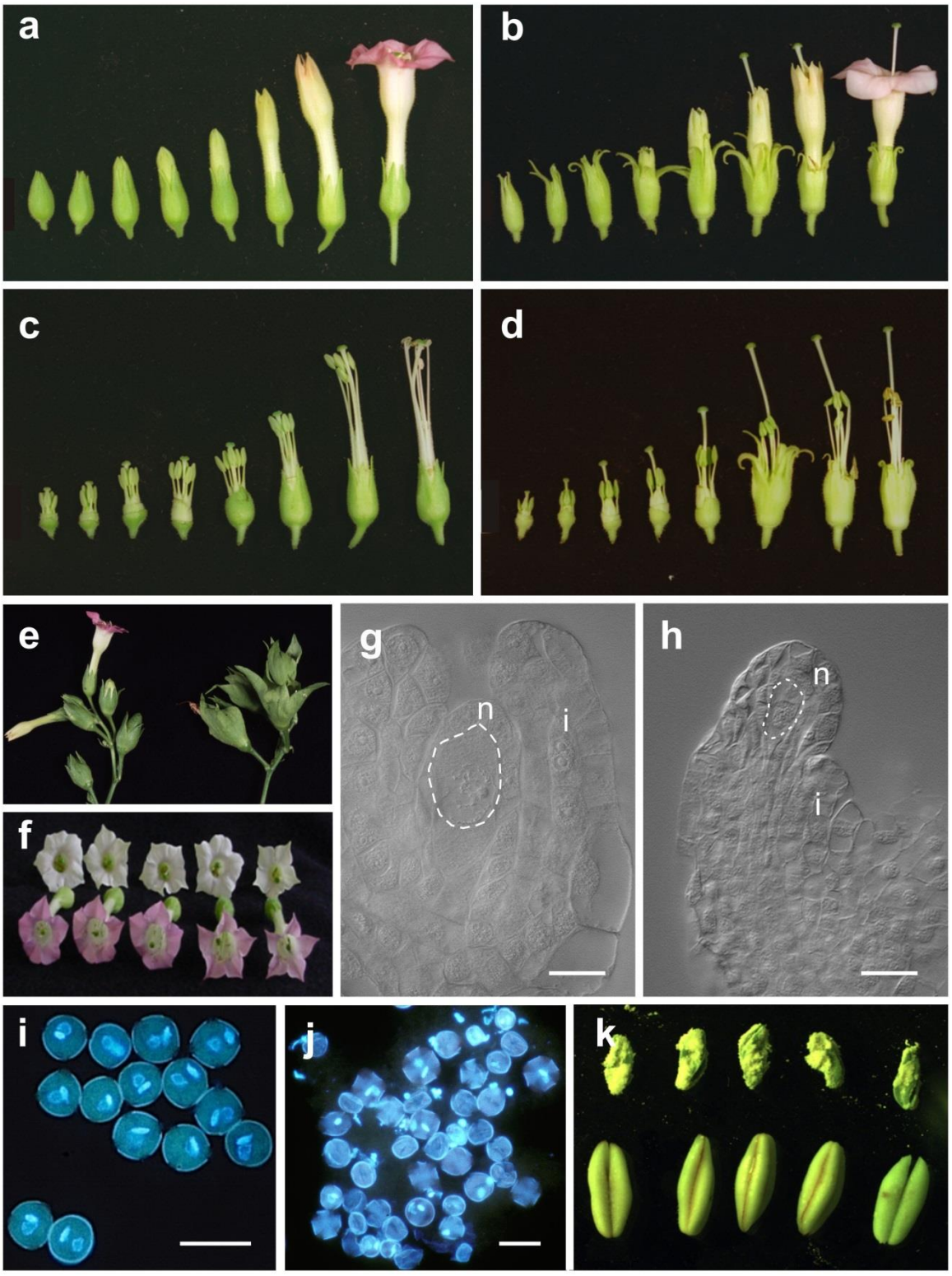

Supplementary Figure 1: Floral phenotypes of *35S::BBM* tobacco transgenics. **a**, Wild-type flower buds at progressive stages of development; **b**, Flower buds of a moderate *35S::BBM* lines; **c**, Wild-type and **d** *35S::BBM* flower buds, from which the corolla, and in some cases the calyx, was removed; **e**, Wild-type (left) and severe *35S::BBM* (right) flower buds showing the enlarged calyx in the transgenic lines; **f**, Wild-type (bottom) and *35S::BBM* (top) flowers. The transgenic petals are white; **g**, Ovules of a wild-type; **h**, sterile *35S::BBM* plant showing the megaspore mother cell (dashed line), the integument (*i*) and the nucellus (*n*). The integument of

the transgenic ovules is underdeveloped; **i**, binucleate pollen from a wild-type plant and **j** a severe *35S::BBM* plants. The wild-type pollen has a large diffuse staining vegetative nucleus and a smaller brightly staining generative nucleus. The transgenic pollen grains have precociously burst and released their content; **k**, anthers of wild-type (top) and *35S::BBM* plants (bottom). The wild-type anthers have dehisced to release mature pollen grains, whereas the transgenic anthers remained closed. The scale bars are 50µm.

Chapter 3

Efficient Sweet Pepper Transformation Mediated by the BABY BOOM Transcription Factor

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ABSTRACT

Pepper (*Capsicum* L.) is a nutritionally and economically important crop that is cultivated throughout the world as a vegetable, condiment and food additive. Genetic transformation using *Agrobacterium tumefaciens* (agrobacterium) is a powerful biotechnology tool that could be used in pepper to develop community-based functional genomics resources and to introduce important agronomic traits; however, pepper is considered to be highly recalcitrant for agrobacterium-mediated transformation, and current transformation protocols are either inefficient, cumbersome or highly genotype dependent. The main bottleneck in pepper transformation is the inability to generate cells that are competent for both regeneration and transformation. Here we report that ectopic expression of the *Brassica napus* BABY BOOM AP2/ERF transcription factor overcomes this bottleneck and can be used to efficiently regenerate transgenic plants from otherwise recalcitrant sweet pepper (*C. annuum*) varieties. Transient activation of BABY BOOM in the progeny plants induced prolific cell regeneration and was used to produce a large number of somatic embryos that could be converted readily to seedlings. The data highlight the utility of combining biotechnology and classical plant tissue culture approaches to develop an efficient transformation and regeneration system for a highly recalcitrant vegetable crop.

Keywords

Sweet pepper (*Capsicum annuum*), transformation, *Agrobacterium*, BABY BOOM, somatic embryogenesis, regeneration

Abbreviations

TDZ	Thidiazuron
BBM	BABY BOOM
CCM	Co-cultivation medium
EM	Elongation medium
GR	Rat glucocorticoid receptor ligand binding domain
GUS	β-glucuronidase
MS	Murashige and Skoog medium
PRM	Pre-rooting medium
RM	Rooting medium
SE	Somatic embryo
SLS	Shoot-like structures

INTRODUCTION

The genus *Solanaceae* comprises some of the most economically important vegetable species, including potato (*Solanum tuberosum*), tomato (*Solanum lycopersicon*), eggplant (*Solanum melongena*), and pepper (*Capsicum spp*). More than forty species belong to the genus *Capsicum*. Five pepper species, *C. annuum, C. frutescens, C. baccatum, C. chinense,* and *C. pubescens*, are valuable crops plants that are cultivated and consumed throughout the world, with *C. annuum* being the most widely cultivated species. Pepper is second only to tomato in terms of vegetable production in developed countries and its breeding and production, as with other major crops, is constantly challenged by numerous pests, diseases and abiotic stresses (Djian-Caporalino et al. 2007). Trait development in the genus *Capsicum* is hampered by interspecific crossing barriers, as well as by the general lack of an efficient regeneration system, which is a prerequisite for the introduction of new traits by genetic transformation. The major bottlenecks in *Capsicum* regeneration are the general low frequency of shoot formation and the development of malformed shoot buds and shoots (variously referred to in the literature as rosette shoots, leafy shoots or blind leaves) that fail to elongate, most likely due to the absence of a shoot apical meristem (Dabauza and Pena 2003; Mihálka et al. 2003; Liu et al. 1990; Wolf et al. 2001; Engler et al. 1993; Kothari et al. 2010). In general, the chili (hot) pepper types are much more responsive for *in vitro* regeneration than the sweet pepper types (Zapata-Castillo et al. 2007; Solís-Ramos et al. 2010; Ochoa-Alejo and Ramirez-Malagon 2001; Dabauza and Pena 2001; Engler et al. 1993; Khan et al. 2006; Lopez-Puc et al. 2006), although a strong genotype dependency has been observed in both.

Agrobacetrium tumefaciens (agrobacterium)-mediated transformation of *C. annuum* has been described in the literature, however in most reports only a few transgenic lines were obtained and/or the transformation efficiency and heritability of the transgene were not reported (Dabauza and Pena 2003; Manoharan et al. 1998; Mihálka et al. 2003; Shivegowda et al. 2002; Zhu et al. 1996). Reproducible agrobacterium-mediated transformation is currently limited to a few responsive chili pepper genotypes (Ko et al. 2007; Lee et al. 2004) and one sweet-mini pepper genotype (Engler et al. 1993; Harpster et al. 2002). The difficulties associated with pepper transformation have been attributed to its low regeneration capacity, and the poor overlap between the tissues that are competent for agrobacterium infection and those that are competent for regeneration (Wolf et al. 2001). This is a general phenomenon that has been observed in plants that are recalcitrant for transformation (Potrykus 1991). A system that supports transformation and regeneration of the same tissues could therefore provide the basis for an efficient pepper transformation protocol.

A number of genes encoding transcription factors, cell cycle proteins, and components of hormone biosynthesis and signalling pathways have been shown to enhance plant regeneration

responses when mutated or ectopically expressed (Banno et al. 2001; Zuo et al. 2002; Lotan et al. 1998; Catterou et al. 2002; Riou-Khamlichi et al. 1999). One of these genes, *BABY BOOM* (*BBM*), encodes an AP2/ERF transcription factor that induces regeneration under culture conditions that normally do not support regeneration in wild-type plants. Ectopic expression of *Brassica napus BBM* (*BnBBM*) genes in *B. napus* and the related crucifer Arabidopsis (*Arabidopsis thaliana*) induces spontaneous somatic embryogenesis and organogenesis from seedlings grown on hormone-free basal medium (Boutilier et al. 2002). In tobacco, ectopic *BBM* expression is sufficient to induce adventitious shoot and root regeneration on basal medium, however exogenous cytokinin is required for somatic embryo (SE) formation (Srinivasan et al. 2007). Ectopic *BBM* expression has also been used to generate transgenic Chinese white poplar (*Populus tomentosa* Carr.) plants (Deng et al. 2009). Poplar callus transformed with a *B. rapa BBM* gene developed SEs that could be converted into plantlets, while untransformed callus failed to regenerate. The system was combined with heat shock-inducible FRT/FLP-mediated excision of the transgene to produce marker-free lines.

Transformation strategies based on standard tissue culture approaches have not led to efficient pepper transformation protocols. We therefore examined whether the positive influence of *BBM* expression on regeneration that is observed in other plant species could be transferred to pepper. Here we describe the efficient regeneration of large numbers of fertile transgenic plants of two *C. annuum* sweet pepper varieties by combining a classical tissue culture approach with transient activation of a BnBBM protein.

MATERIALS AND METHODS

Explant pre-culture

Surface sterilized seeds of the F1 hybrids Fiesta, Ferrari and Spirit (Enza Zaden, The Netherlands) were sown on full strength MS medium (Murashige and Skoog 1962) with 2 % (w/v) sucrose, (pH 5.8, MS20) solidified with 0.8 % (w/v) Microagar. Ten-day old petiole-free cotyledons were cut twice, transverse to the mid-rib, to generate three explants, which were then pre-cultured on solid (0.7 % Microagar) co-cultivation medium (CCM) supplemented with 40 mg/l acetosyringone (Acros Organics) for one-to-two days under dim light conditions (1500 lux) at 23 ˚C. CCM is a modified R medium (Sibi et al. 1979) supplemented with 1.6 % (w/v) glucose, 2 mg/l zeatin riboside and 0.1 mg/l indole-3-acetic acid to promote shoot regeneration.

Agrobacterium and vectors

Agrobacterium strain GV3101 carrying the pMP90 Ti plasmid was used in all experiments. Agrobacterium containing the *35S::BnBBM:GR* (Srinivasan et al. 2007) and *35S::GUS* binary vectors

were grown with the appropriate antibiotics in 100 ml YEB medium at 28 ˚C. Prior to transformation the agrobacterium suspension was diluted to OD_{660} 0.3-0.4 with liquid CCM supplemented with freshly prepared 40 mg/l acetosyringone.

Transformation and regeneration

The diluted agrobacterium culture was added to the pre-cultured cotyledon explants and incubated at room temperature for 30-60 minutes. Explants were blotted dry and further co-cultured on CCM supplemented with 40 mg/l acetosyringone for two-to-three days under dim light conditions (1500 lux) at 23 ˚C before transfer to selection medium consisting of CCM supplemented with 1 mg/l thidiazuron (TDZ; Murthy et al. 1998), 100 mg/l kanamycin sulfate and 500 mg/l cefotaxime. Explants were transferred to full light conditions (3000 lux) on a 16 h/8 h day/night cycle at 23 ˚C for two months. The medium was refreshed after four weeks. Explants with emerging shoots or shoot-like structures (SLS) were transferred for four weeks to elongation medium (EM) consisting of MS macro- and microsalts (Murashige and Skoog 1962), B5 vitamins (Gamborg et al. 1968), 1.6 % (w/v) glucose, 1 mg/l inositol, 20 mg/l adenine sulfate, 200 mg/l casein hydrolysate, 10 mg/l gibberellic acid 3, 4 mg/l benzylaminopurine and 30 µM silverthiosulfate. Elongated shoots were transferred to pre-rooting medium (PRM), comprising MS20 medium supplemented with 30 mg/l glutathione, 60 ml/l kanamycin sulfate, and 300 mg/l cefotaxime. The shoots were transferred after one month to rooting medium (RM;(Rugini 1984)) supplemented with 2 % (w/v) sucrose, 50 mg/l kanamycin. Rooted shoots were transferred into the greenhouse for seed set. All media used in experiments involving the *35S::BnBBM:GR* construct were supplemented with 10 µM dexamethasone (DEX; Sigma) up to the point where shoots were transferred to EM, after which DEX-free media was used. Except where noted, all tissue culture chemicals were supplied by Duchefa Biochemicals (Haarlem, The Netherlands).

β-Glucuronidase (GUS) staining

Histochemical GUS staining (Jefferson 1987) of *35S::GUS* explants was performed after three weeks on selection medium.

Evaluation of stable transgenic lines of *35S::BnBBM:GR*

Surface sterilized seeds were sown on MS20 medium supplemented with either 10 µM DEX, 1 mg/l TDZ, or 10 µM DEX plus 1 mg/l TDZ. The response of the seedlings was evaluated 24 days after sowing. Root, hypocotyl, and cotyledon explants from 10-day old seedlings and leaf explants from four-week old plantlets were obtained from donor material grown on MS20 without any

supplements. Explants were placed on MS20 or MS20 supplemented 10 µM DEX, either alone or in combination with 1 mg/l TDZ, benzylaminopurine or zeatin riboside. The response of the explants was evaluated after two weeks. Conversion of SEs into plantlets was assessed by placing embryos onto RM. Conversion into plantlets was evaluated four weeks after transfer to RM.

RESULTS

BABY BOOM-mediated regeneration

We examined the utility of a *BnBBM* gene as a tool to enhance regeneration during agrobacterium-mediated transformation of *C. annuum* sweet, blocky pepper types. Ectopic BBM expression induces pleiotropic phenotypes such as adventitious growth and sterility that are likely to interfere with the regeneration process and subsequent growth of transgenic plants (Boutilier et al. 2002). To avoid generating plants with detrimental phenotypes, we expressed a fusion between the BnBBM protein and the ligand binding domain of the rat glucocorticoid receptor (BBM:GR), which sequesters the BBM transcription factor in the cytoplasm in the absence of glucocorticoid steroid e.g. DEX (Passarinho et al. 2008; Srinivasan et al. 2007). Explants were co-cultivated with agrobacterium carrying either the 3*5S::BnBBM:GR* construct or a control *35S::GUS* construct carrying the scorable GUS marker. Both constructs confer kanamycin resistance via the *nptII* selection marker. Explants were co-cultivated with agrobacterium on CCM, and then transferred to CCM medium containing kanamycin until shoots appeared, at which point the shoots were transferred to EM. Elongated shoots were then transferred to PRM and subsequently to RM when rooting did not occur already on PRM. Approximately 5,600 explants from two cultivars, were used in the transformation experiments with the *35S::GUS* plasmid, and approximately 6,400 explants from three cultivars in the transformation experiments with the *35S::BnBBM:GR* plasmid (Table 1).

Cotyledon explants that were co-cultivated with agrobacterium containing the *35S::GUS* construct behaved as previously described for poorly regenerating or non-transformable genotypes (Liu et al. 1990; Lee et al. 2004). The explants increased in size about two-fold during the first three weeks on selection medium. Small calli became visible at the cut edge of the explants during the following two to-four weeks, accompanied by a few dense rosette-forming SLS (Fig. 1a, b).

SLS transferred to EM failed to elongate and therefore did not root when transferred to rooting medium (RM). *In vitro* grafting of the SLS onto a wild-type rootstock did not promote further shoot development or elongation, suggesting that the SLS lacked a functional apical meristem. In a separate experiment, histochemical staining of three-week old explants (n = 225) that formed both SLS and callus showed that 6 % of the explants exhibited GUS activity and that the GUS activity was restricted to the developing callus (Figure 1c). This observation supports the hypothesis of Wolf et al.

(2001) that under these conditions, pepper cells that are susceptible for agrobacterium transfection lack regeneration capacity and *vice versa*.

Table 1 Regeneration response and transformation efficiency of sweet pepper varieties

Construct/Cultivar	No. explants	Explants with SLS (% of total explants used)	Explants with elongated shoots	No. transgenic shoots[a]	Transformation efficiency[b]
35S::GUS					
Fiesta	5150	64 (1.2)	0		
Spirit	475	0 (0)	0		
Total	5625	64	0	0	0
35S::BnBBM:GR					
Fiesta	4448	798 (17.9)	26	78	0.6
Ferrari	805	29 (3.6)	9	20	1.1
Spirit	1179	67 (5.7)	0		0
Total	6432	894	35	98	

[a]Individual explants produce multiple shoots
[b]Transformation efficiency = (no. explants with transgenic shoots/ total no. explants) x 100 %

In contrast to the control experiments with the *35S::GUS* construct, co-cultivation of sweet pepper cotyledon explants with agrobacterium carrying the *35S::BnBBM:GR* construct allowed us to generate numerous transgenic shoots that stably transmitted the transgene to the next generation. The transformation experiments with the *35S::BnBBM:GR* plasmid were carried out as described above, except that 10 µM DEX was included in the selection medium to localize the BBM:GR protein to the nucleus. Explants co-cultivated with the *35S::BnBBM:GR* agrobacterium behaved essentially the same as in the 3*5S::GUS* experiments during the first seven weeks of culture, except that they produced more SLS in each experiment compared to the *35S::GUS* control (Table 1). Unlike the control experiments with *35S::GUS*, the SLS that formed after co-cultivation with the *35S::BBM:GR* agrobacterium elongated and proliferated within four weeks after transfer to EM (Fig. 1d). SEs developed occasionally on the leaves of elongated shoots and/or SLS that remained in contact with the medium (Fig. 1e), and in turn produced elongated shoots. Plantlet formation from elongated shoots was enhanced after transfer to PRM (Fig. 1f). Shoots that did not root on PRM formed roots

within two weeks after transfer to RM. In total, 98 rooted shoots regenerated from 35 independent explants were transferred to the greenhouse. PCR analysis of the first 39 plantlets showed that all carried the *BBM* transgene (examples shown in Supplementary Fig. 1). Plating of the T1 seed on kanamycin-containing medium showed that the progeny of the 39 PCR-confirmed plants and of the remaining 59 plants were all transgenic (Supplementary Table 1). An overview of the workflow for our sweet pepper transformation protocol is shown in Table 2.

Table 2: Workflow for *35S::BnBBM:GR* –mediated sweet pepper transformation

Step	Days (n)	Medium
Growth of donor material	10	MS20
Explant pre-culture	1-2	CCM + ZR + IAA
Co-cultivation of explants	3-4	CCM + ZR + IAA+ACS
Shoot regeneration on selective medium	2 x 30	CCM + TDZ + DEX
Shoot elongation	30	EM
Pre-rooting of elongated shoots	30	PRM
Rooting of elongated shoots	14	RM
Total	150	

CCM, co-cultivation medium; MS20, (Murashige and Skoog 1962) with 2 % (w/v) sucrose; ZR, zeatin riboside; IAA, indole-3-acetic acid; ACS, acetosyringone; TDZ, thidiazuron; DEX, dexamethasone; EM, elongation medium; PRM, pre-rooting medium; RM, rooting medium.

Regeneration and transformation efficiency y

The regeneration response of the three tested varieties was greatly enhanced in the experiments with the *35S::BnBBM:GR* plasmid (Table 1), although genotypic differences were observed among the varieties with respect to the different steps in the regeneration protocol. In all cases, co-cultivation with the *35S::BnBBM:GR* plasmid was able to relieve one or more bottlenecks in the regeneration/transformation procedure for each of the varieties tested. Transgenic plantlets were generated for two of the three varieties, whereas no transgenics were obtained from any variety in the control experiments. Numerous elongated shoots could be produced from a single SLS, however as multiple shoots may arise from a single transformation event, only a single elongated shoot per SLS was used to calculate the transformation efficiency (Table 1). Based on this criterion we obtained average transformation efficiencies of 0.6 and 1.1 % for the two varieties, although the transformation efficiency can be much higher in individual experiments (up to 3.8 %, data not

shown). Different segregation patterns were often observed in the progeny of the multiple shoots derived from a given SLS (Supplementary Table 1), suggesting that multiple independent transgenic plants can be regenerated from a single SLS. Based on this observation, we expect the actual transformation efficiency to be even higher than calculated above.

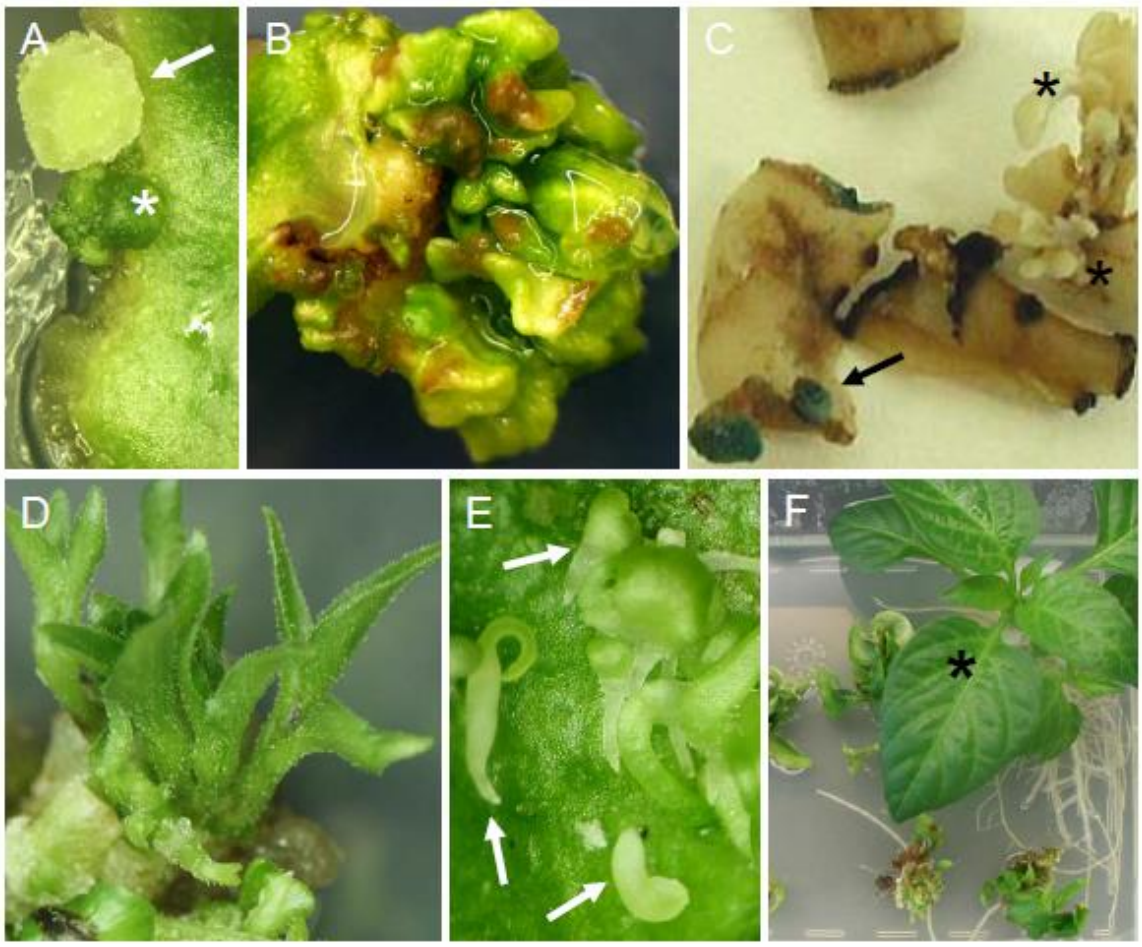


Figure 1: Regeneration response of sweet pepper explants. Response of sweet pepper 'Fiesta' explants to co-cultivation with agrobacterium carrying either the *35S:GUS* construct (a-c) or the *35S::BnBBM:GR* construct (d-f).
(a) callus formation (arrow) and shoot like structures (SLS; asterisk) after 3 weeks of culture;
(b) leaf-like structures developing at the wounded edge of a cotyledon after 4 weeks of culture;
(c) explant with callus (arrow) and SLS (asterisk) histochemically stained for GUS activity;
(d) SLS, four weeks after treatment;
(e) somatic embryo formation (arrows) on a newly emerged leaf, after six weeks of culture;
(f) elongating SLS (asterisk) after four weeks on elongation medium.

Stable *35S::BnBBM:GR* transformants are highly regenerative

Two single locus, homozygous *35S::BnBBM:GR* lines were selected for further phenotypic analysis. Seeds were sown on either MS20, MS20 supplemented with either DEX or TDZ or MS20 supplemented with both DEX and TDZ. Seedlings of the two *35S::BnBBM:GR* lines were

indistinguishable from the wild-type when grown on MS20 (Fig. 2a). *35S::BnBBM:GR* seedlings grown on DEX-containing medium were severely delayed in their initial growth as compared to wild-type plants growing on the same medium. Seedlings from one of the two lines germinated, but failed to develop further. Seedlings of both lines showed a thickened root, a pronounced apical hook (Fig. 2b) and were agravitropic. The cotyledons that remained in contact with the medium eventually swelled and formed irregular protruberances lacking a defined structure. Wild-type and *35S::BnBBM:GR* seedlings plated on TDZ-containing medium developed as on the control MS20 medium (Fig. 2c). Wild-type and *35S::BnBBM:GR* seedlings growing on medium supplemented with both DEX and TDZ showed a combination of the phenotypes observed in the presence of the individual compounds (Fig. 2d). In addition, cotyledons of *35S::BnBBM:GR* seedlings that remained in contact with the medium eventually developed into a callus mass and produced a few SEs (data not shown).

The ability of *35S::BnBBM:GR* explants to form SEs prompted us to assess the regenerative capacity of different organs. Segments of roots and hypocotyls and feather-cut cotyledons from 10-day old seedlings, and feather-cut leaves from four-week old homozygous *35S::BnBBM:GR* plants were placed onto MS20 or MS20 supplemented with TDZ, DEX or both TDZ and DEX. Root and hypocotyls segments formed callus but did not regenerate under any of the conditions tested (data not shown). Cotyledons and leaves cultured on MS20 formed white callus at the wounded edges of the explant (Fig. 2e, i). TDZ stimulated white callus production in cotyledons and leaves, and also induced SLS formation at the wounded edges of cotyledons (Fig. 2g, k). Culturing cotyledons and leaves on medium with DEX (Fig. 2f, j) or DEX and TDZ (Fig. 2h, l) induced prolific SE formation.

SE induction was greatly diminished when uncut leaves and cotyledons were used as explants (data not shown). Quantitative differences in SE production were not observed when TDZ was replaced by the cytokinins benzylaminopurine and zeatin riboside (data not shown). SE induction was mainly observed on the surface of the explant adjacent to the cut edge, and SEs appeared to develop directly from the underlying tissue, rather than indirectly through an intermediate callus phase. SEs formed on DEX-containing medium generally developed to the globular stage (Fig. 2f, j), while SEs growing on medium containing TDZ (or other cytokinins) and DEX progressed further to the heart-shaped stage and beyond, in which the cotyledons are visible (Fig. 2h, l). SEs from *35S::BBM:GR* explants induced on TDZ (or other cytokinins) and DEX have a clear bipolar structure (Fig. 2m), and are more similar to wild-type zygotic embryos (Fig. 2n) than to standard SEs derived from immature wild-type zygotic embryos (Fig. 2o). *35S::BBM:GR* SEs could be converted into plantlets by plating them on RM. While individual cytokinins did not affect SE production quantitatively, they did influence the ability of SEs to convert into plantlets. The highest conversion rate (50%) was obtained in embryos that were induced in the presence of benzylaminopurine (Supplementary Table 2).

Figure 2: Regeneration response of stable *35S::BBM:GR* sweet pepper lines.
(a-d) 24-day-old seedlings germinated on medium with the indicated supplements;
(e-h) feather-cut cotyledons of 10-day-old seedlings incubated for 14 days on medium with the indicated supplements. Shoot-like structures are indicated by an arrow;
(i-l) feather-cut leaves of 4-week-old plants incubated for 14 days on medium with the indicated supplements;
(m) *35S::BBM:GR* somatic embryos;
(n) immature wild-type zygotic embryo;
(o) somatic embryo formation on wild-type zygotic embryos.
MS20, Murashige and Skoog medium with 2 % (w/v) sucrose; TDZ, thidiazuron; DEX, dexamethasone

DISCUSSION

Pepper is a major crop that is grown world-wide and whose production is threatened by various biotic and abiotic stresses. Resistances can often be found in wild relatives, however there are often strong breeding incompatibilities between *Capsicum* species that are not easy to circumvent (Onus and Pickersgill 2004; Jae et al. 2006). Traits from incompatible wild relatives could be introduced into cultivated peppers via genetic transformation, however peppers, especially the sweet genotypes, are highly recalcitrant for transformation.

Here we describe a reliable and efficient transformation protocol for sweet pepper genotypes that takes advantage of the enhanced regeneration response conferred by the BBM AP2/ERF transcription factor. The protocol is straightforward in that additional measures such as grafting SLS onto a rootstock (Mihálka et al. 2003), a long phase of shoot elongation ("normalization") (Engler et al. 1993) or the addition of auxin to enhance rooting (Khan et al. 2006; Engler et al. 1993) were not required. Direct comparison of our sweet pepper transformation efficiencies with published protocols is difficult as in practice there are no routine, reliable and reproducible protocols that are applicable to more than one genotype. The comparison is further complicated by the lack of information on the transformation efficiency (Harpster et al. 2002; Zhu et al. 1996) or the heritability of the transgene (Engler et al. 1993; Dabauza and Pena 2003; Manoharan et al. 1998; Mihálka et al. 2003; Shivegowda et al. 2002). Our transformation efficiencies of 0.6 and 1 % obtained with two recalcitrant sweet pepper genotypes are higher on average than the 0.03 to 0.6 % reported for the most responsive '*C. annuum.* Chili' pepper genotypes (Lee et al. 2004; Manoharan et al. 1998; Ko et al. 2007).

This and previous studies (Wolf et al. 2001) suggest that the few regenerating structures that are obtained in standard pepper transformation protocols are not susceptible for agrobacterium-mediated transformation. The mechanism by which the BBM protein closes the gap in competencies for transformation and regeneration in pepper is not clear. BBM might exert a positive effect on the transformation efficiency by creating a cellular environment that is both susceptible to transformation and regeneration, or by increasing the total number of regenerating cells (SLS), thereby increasing the probability that both processes coincide in one cell. *35S::BBM:GR* explants not only produce a higher number of SLS than control explants, they also elongate to produce shoots at a higher frequency. Again, the underlying mechanism is not clear. Both *35S::GUS* and *35S::BBM:GR* explants initially produce morphologically similar SLS that grow in dense rosettes with no clear boundaries between them, suggesting a non-functional or missing SAM. Somewhere during the regeneration of BBM-SLS, a functional SAM and vascular bundles are formed that allow the

shoots to elongate and develop into a normal plant. The ability of BBM to induce direct regeneration i.e. without an intervening callus phase, may promote improved shoot polarity and differentiation.

Efficient *in vitro* regeneration systems based on somatic embryogenesis can facilitate the classical breeding process by providing large amounts of clonal material for propagation, as well as explants for transformation. SE production from immature zygotic embryos on solid medium has been reported in *C. annuum* (chili and sweet pepper types) and C. *chinense*, although the induction frequencies are low (maximum 8 SE/explant) (Harini and Lakshmi Sita 1993; Binzel et al. 1996; Steinitz et al. 2003), and the SEs can exhibit a high frequency of morphological defects that affects their conversion into seedlings (Steinitz et al. 2003). Solís-Ramos et al.. used inducible expression of the arabidopsis WUSCHEL (WUS) homeobox transcription factor (Zuo et al.., 2002) to enhance SE formation in *C. chinense* L. (Solís-Ramos et al. 2009). A small number of globular structures could be induced to form on primary stem explants transformed with the inducible WUS construct, however the embryos failed to develop further and eventually died. In contrast, stable *35S::BBM:GR* transgenics exhibited an extremely high regeneration capacity, producing hundreds to thousands of well-formed embryos per explant that could be converted at a high frequency into seedlings.

A number of strategies can be used to implement a BBM-based transformation technology. For example, a second gene of interest can be co-transformed along with the *35S::BBM:GR* construct or stable, highly regenerative *35S::BBM:GR* transformants could be used as explants for the introduction of a second gene of interest. In both examples, the positive effect of the BBM protein on the regeneration process can serve as a selectable marker during (co-)transformation of a second gene of interest, which can itself be selected in the classical way (e.g. using antibiotic- or herbicide resistance) or via PCR. For some purposes it may be desirable to avoid stable integration of the *35S::BBM:GR* transgene. This could be circumvented by transient expression techniques (Vergunst et al. 2000) or by segregation of the *35S::BBM:GR* transgene in progeny lines.

The sweet pepper transformation system described here opens up possibilities for the introduction of new disease and abiotic stress resistances, as well as for important reproductive and architecture traits. In addition to these practical applications, this transformation system provides opportunities for building up fundamental research tools that can be used to understand gene function in *Capsicum* spp.

SUPPLEMENTARY MATERIALS

Supplementary Methods

Progeny analysis

Only the 34 progeny of SLS that produced >50 seeds were analysed. A minimum of 50 T1 seeds from each line were surface sterilized and sown onto MS20 and 100 mg/l kanamycin sulfate. Segregation of the transgene(s) was evaluated four weeks after sowing. A χ^2 test was used to identify lines with a single locus insertion.

Molecular analysis

DNA was isolated from leaves of T0 plants using a CTAB protocol (Rogers, SO, Bendich, AJ (1985) Extraction of DNA from milligram amounts of fresh, herbarium and mummified plant tissues. Plant Mol. Biol. 5: 69-76).

Transgene confirmation

Specific primers were designed to amplify the *35S::BBM* and *nos::nptI*I fragments, and the pepper *CAPSANTHIN/CAPSORUBIN SYNTHASE* gene (*CCS*; GU122936), which was used as a control (Supplementary Table 3). The *p35S::BBM* (365 bp) and the *nos::nptII* (464 bp) genes were each amplified in the same reaction as the *CCS* gene (105 bp).

TAIL PCR

TAIL-PCR (Liu, Y-G, Chen, Y (2007) High-efficiency thermal asymmetric interlaced PCR for amplification of unknown flanking sequences. BioTechniques 43:649-656) was used to determine if transgenic shoots derived from a single SLS represent the same or independent transformation events. TAIL PCR was performed on the progeny of the multiple transgenic lines obtained from 31 of the 34 SLS (Supplementary Table 1) using published primers for the T-DNA right border (RB) and *35S::BBM:GR* specific primers. (Supplementary Table 3). The TAIL-PCR was performed using the RB1and LAD1-1 or LAD1-3 primers for the pre-amplification and the RB2 and AC primers for the nested PCR.

The single fragment obtained from the progeny of SLS10 and one of the two fragments obtained from the progeny of SLS12 (SLS12b) were cloned into pCR4- (Invitrogen), verified by PCR with the RB3 and AC primers and sequenced. SLS10 and SLS12 primers were designed based on the genomic sequence at the T-DNA integration site and used with RB2 and RB3, respectively, to amplify genomic DNA from all of the SLS10 and SLS12 progeny. The *CCS* gene and DNA of a non-transgenic pepper plant were used as controls.

Supplementary Tables

Supplementary Table 1 Segregation analysis of germinating seed from *35S::BnBBM:GR* transgenics

Individual SLS	No. progeny lines analysed	Segregation pattern	
		one locus	other
1	3	3	0
2	2	1	1
3	6	3	3
4	4	4	0
5	4	0	4
6	1	1	0
7	1	1	0
8	4	4	0
9	1	1	0
10	5	5	0
11	1	1	0
12	7	3	4
13	3	1	2
14	1	1	0
15	1	0	1
16	1	1	0
17	1	1	0
18	1	1	0
19	1	0	1
20[a]	2	2	0
21[a]	1	1	0
22	1	0	1
23	1	0	1
24	1	1	0
25[a]	1	0	1
26[a]	4	1	3
27[a]	1	0	1
28[a]	1	1	0
29[a]	1	1	0
30[a]	1	1	0
31[a]	1	1	0
32	1	1	0
33	1	1	0
34	1	1	0
total	67	44	23

[a]'Ferrari', remaining lines are 'Fiesta'

Supplementary Table 2 Conversion of *35S::BBM:GR* somatic embryos into plantlets

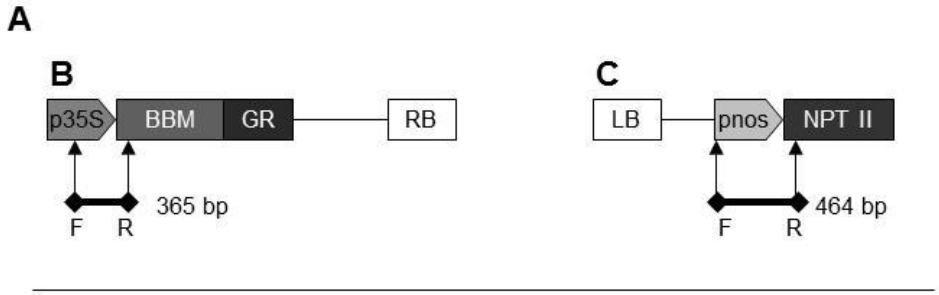
SE induction medium	No. Embryos	Conversion efficiency[a] (%)
CCM + DEX	100	3
CCM + DEX + TDZ	100	4
CCM + DEX + BAP	100	50
CCM + DEX + ZR	100	26

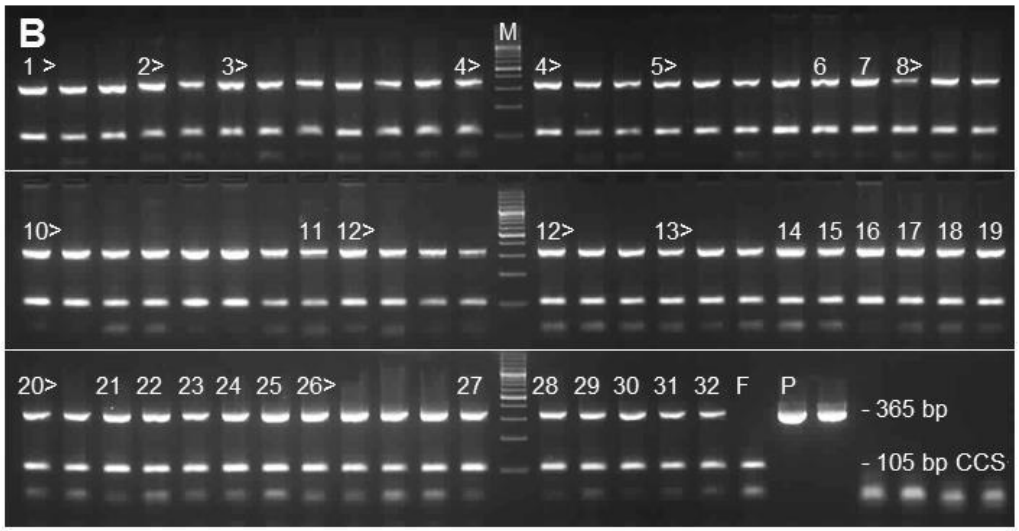
[a]Conversion efficiency = (no. converted plantlets/no. embryos) x 100 %
CCM, co-cultivation medium; DEX, dexamethasone; TDZ, thiadiazuron; BAP, benzylaminopurine: ZR, zeatin riboside

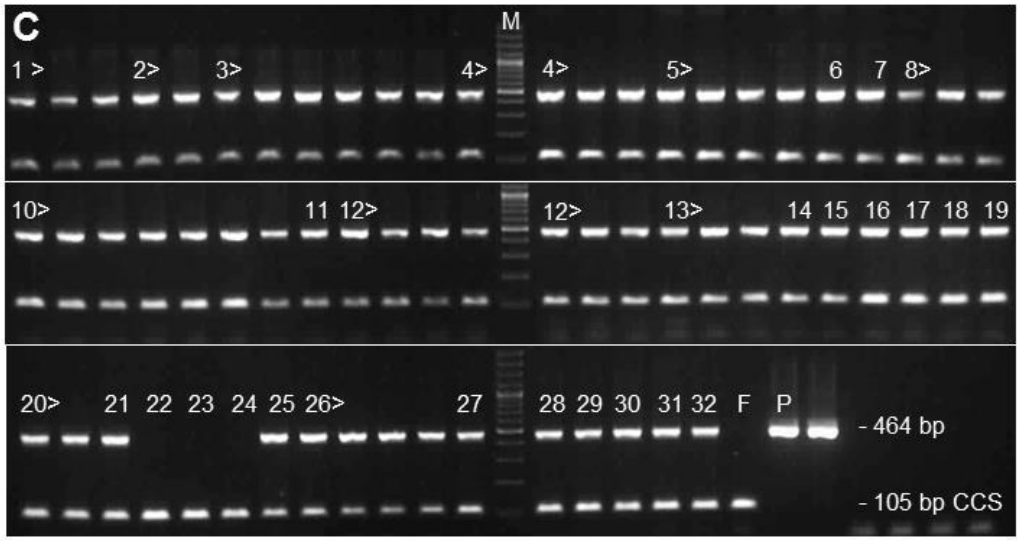
Supplementary Table 3: Overview of primers

	Primer
Transgenic confirmation	
35S::BBM:GR	Forward: caatcccactatccttcgcaagaccc Reverse: cccaatctcgggagtgactattgttg
nos::NPTII	Forward: aagggcgaaaaaccgtctat Reverse: tgtctgttgtgcccagtcat
CCS	Forward: gtctgtcaaagaacttgctg Reverse: agtttaaccaaggggacagt
TAIL PCR	
RB1	gctggcgtaatagcgaagag
RB2	tccctttagggttccgattt
RB3	ggttcacaaactatcagtg
LAD 1-1	acgatggactccagagcggccgc(g/c/a)n(g/c/a)nnnggaa
LAD 1-3	acgatggactccagagcggccgc(g/c/a)n(g/c/a)n(g/c/a)n nnccaa
AC	acgatggactccagag
Cloned T-DNA insertions	
SLS 10	ttgacctttgccaatggaat
SLS 12	tatgtcggggcatatgatga

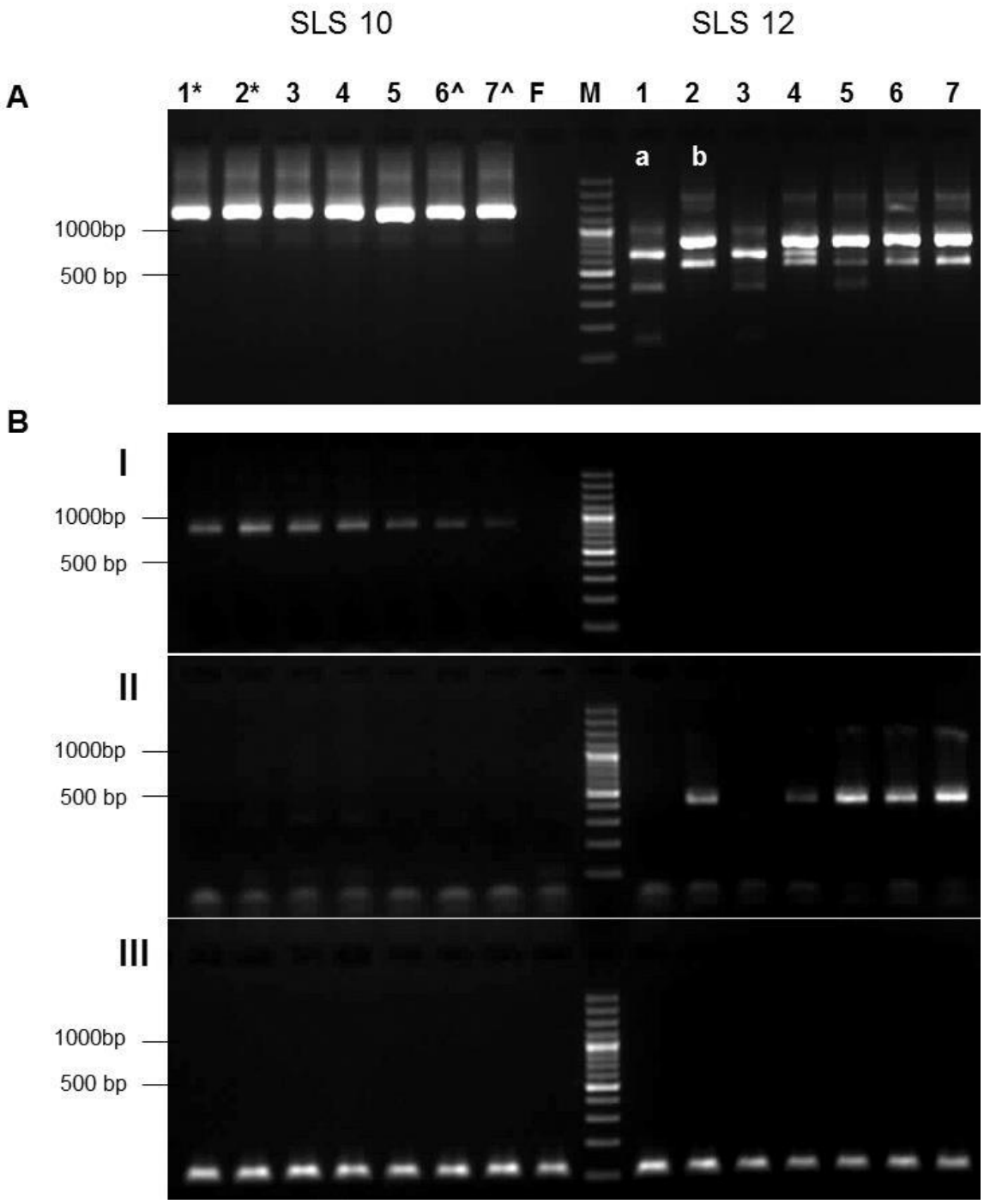
Supplementary Figures







Supplementary Figure 1 PCR analysis of *35S::BBM:GR* T1 progenies.
A. Schematic overview of the primers used to amplify fragments from the *35S::BBM:GR* (365 bp) and *nos::nptII* (464 bp) constructs
B-C. Agarose gels showing PCR amplification *35S/BBM* (**B**) and *nos/nptII* (**C**) primers. Each PCR was performed together with the construct-specific and control *CCS* primers. Numbers above the lanes correspond to the SLS as shown in Supplemental Table 1, with each lane corresponding to the individual lines selected from a given SLS. N.B. not all SLS/progeny lines were included in the analysis. M = molecular size marker; F, non-transgenic Fiesta; P, *35S::BBM:GR* plasmid. Lines marked with (*) and (^) represent technical replicates within a line.



Supplementary Figure 2 Characterization of *35S::BBM:GR* T-DNA insertion sites

A. TAIL-PCR of T1 progeny from shoots collected from SLS10 and SLS12. The same sized fragment was isolated from the SLS10 lines, while two different-sized fragments (a and b) were amplified from the SLS12 lines.
B. Reamplification of the cloned SLS10 and SLS12b TAIL-PCR DNA fragments in all SLS10 and SLS12 progeny lines
I, PCR amplification with SLS10 and RB2 primers;
II, PCR amplification with SLS12b and RB3 primers. The primers do not amplify the DNA in lines carrying the SLS12a T-DNA insertion
III, PCR amplification with CCS primers
F, non-transgenic Fiesta; M, molecular size marker. Lines marked with (*) and (^) represent technical replicates.

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Chapter 4

Pepper, Sweet (*Capsicum annuum*)

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SUMMARY

Capsicum (pepper) species are economically important crops that are recalcitrant to genetic transformation by Agrobacterium (*Agrobacterium tumefaciens*). A number of protocols for pepper transformation have been described but are not routinely applicable. The main bottleneck in pepper transformation is the low frequency of cells that are both susceptible for Agrobacterium infection and have the ability to regenerate. Here, we describe a protocol for the efficient regeneration of transgenic sweet pepper (*C. annuum*) through inducible activation of the BABY BOOM (BBM) AP2/ERF transcription factor. Using his approach, we can routinely achieve a transformation efficiency of at least 0.6 %. The main improvements in this protocol are the reproducibility in transforming different genotypes and the ability to produce fertile shoots. An added advantage of this protocol is that BBM activity can be induced subsequently in stable transgenic lines, providing a novel regeneration system for clonal propagation through somatic embryogenesis.

Keywords: *Agrobacterium tumefaciens*, BABY BOOM, regeneration, somatic embryogenesis, sweet pepper (*Capsicum annuum*), transformation,

1. INTRODUCTION

The ability of *Agrobacterium tumefaciens* (Agrobacterium) to transfer part of its DNA to the plant nuclear genome is widely exploited for plant transformation; however many plant species are recalcitrant to transformation by Agrobacterium, mainly due to the poor regeneration capacity of the transformed cells. Pepper (*Capsicum annuum, C. frutescens, C. baccatum, C. chinense,* and *C. pubescens*) is an economically important crop. The genus *Capsicum* shows a number of crossing barriers that hamper the introduction of biotic and abiotic resistances, as well as important developmental traits (Djian-Caporalino et al. 2007). The introduction of these traits by genetic transformation is limited by the widespread recalcitrance of this genus for Agrobacterium-based transformation. A number of transformation protocols have been described for hot and sweet pepper species or cultivars, but these are either inefficient, highly genotype dependent, or are associated with non-inheritance of the transgene or fertility problems (Dabauza and Pena 2003; Manoharan et al. 1998; Shivegowda et al. 2002).

Deregulation of hormone biosynthesis and signaling pathways, cell cycle proteins, or transcription factors have been shown to enhance the regeneration response of model plants like *Arabidopsis thaliana* (Banno et al. 2001; Qiao et al. 2012; Lotan et al. 1998; Stone et al. 2001; Zuo et al. 2002***)***. Members of the AINTEGUMENTA-LIKE (AIL) group of AP2/ERF transcription factors play important role in shoot and root meristem maintenance (Galinha et al. 2007) and when ectopically expressed can induce spontaneous organogenesis or embryogenesis (Aida et al. 2004; Boutilier et al. 2002; Tsuwamoto et al. 2010). Inducible, ectopic expression of BABY BOOM (BBM), one member of the AIL-group of proteins, induces somatic embryogenesis and regeneration in *Arabidopsis thaliana*, *Brassica napus*, *Nicotiana tabacum*, and sweet pepper (*C. annuum*) (Heidmann et al. 2011; Boutilier et al. 2002; Srinivasan et al. 2007).

Here we describe a protocol for sweet pepper (*C. annuum*) transformation that makes use of constitutive expression of an inducible BBM protein ***(***Passarinho et al. 2008). In this approach the BBM protein is fused in-frame to the ligand binding domain of the rat glucocorticoid receptor (GR; (Schena et al. 1991)) and expressed under control of the *35S* promoter (*35S::BBM:GR*). BBM is a nuclear protein, but fusion of BBM to GR sequesters BBM in the cytoplasm. Addition of the glucocorticoid steroid dexamethasone (DEX) causes release of the BBM:GR protein, allowing translocation to the nucleus and transcriptional activation. In this way the BBM protein can be activated specifically during the transformation and/or regeneration process, thereby avoiding the pleiotropic phenotypes associated with BBM constitutive overexpression (Srinivasan et al. 2007). Agrobacterium strain GV3101 carrying the *35S::BBM:GR* construct in the binary vector pBIN+ (Engelen et al. 1995), which harbors the kanamycin selection gene, was used to transform cotyledon explants of the sweet pepper F1 hybrids Fiesta and Ferrari, as well as non-commercially available

parental lines. DEX-induced nuclear translocation of BBM was applied from the start of culture to the appearance of shoots (ca. 80 days). The transformation efficiency ranges from 0.6 - 4% (defined as: (the number of explants with transgenic shoots/total number of explants) x 100. The entire process- from sowing of the donor material to the harvest of transgenic seeds- takes approximately 9 months. Using this approach, we have generated more than one hundred fertile transgenic plants from different genotypes that are indistinguishable from wild type plants and that also transmit the transgene to the next generation (Heidmann et al. 2011).

2. MATERIALS

All chemicals used in this protocol are of analytical grade. All media are prepared with purified deionized water (18 MΩ) and are autoclaved at 114 °C for 20 minutes (*see* **Note1**). The media can be stored at room temperature (20-22 °C) in the dark for three weeks and re-heated once in a microwave. Plant hormones or antibiotics (tissue culture-approved grade) are added to the medium after autoclaving/re-heating, when the medium has reached ca. 55 °C. Medium containing hormones or antibiotics should not be reheated.

The use of genetically modified organisms (GMO) is regulated by national and local government rules.

2.1. General equipment:

1. Water purification system to produce deionized water (18 MΩ, Millipore);
2. pH meter and solutions (1N HCl, 1N KOH, 1N NaOH) to adjust the pH;
3. Autoclave (temperature range 114-120°C) allowing for a pressure of 1.2 bar;
4. Incubator at 28°C without light (Sanyo);
5. Rotary shaker allowing 28°C and 200 rpm (Thermo Fisher);
6. Microwave;
7. Laminar flow cabinet (Hereaus/Thermo Fisher);
8. Bunsen burner;
9. Photospectrometer plus cuvettes;
10. Labware, including autoclavable glassware (bottles, Erlenmeyer flasks), long-handled forceps, razor blades and holder, sterile filters (0.25 µm pore size, Millipore) plus syringes, sterile filter paper, sterile screw-cap disposable tubes (50 and 15ml, Greiner), sterile tissue culture vessels like Steri Vent containers (Duchefa), Petri dishes (9 cm in diameter, Greiner), sterile disposable pipettes of various volumes (Greiner), cling film or Parafilm, micropipette and tips for small-volume dispensing (Gilson), Eppendorf tubes (Greiner);
11. Solvents (ethanol, methanol and dimethylsulfoxide (DMSO));

12. Growth chamber with light (at least 42 µmol/m^2/s)) at 23°C, on a 16/8 h day/night cycle, preferentially with air ventilation within the shelves to prevent condensation;

2.2. Agrobacterium strain and plasmid

1. Agrobacterium strain GV3101 containing the pMP90 T1 plasmid (Koncz and Schell 1986) (see **Note 2**).

2. Binary vector p35S::BBM:GR (Fig. 1, (Engelen et al. 1995)). The vector contains a duplicated-enhancer CaMV 35S promoter, a translational enhancer from the 5' untranslated leader sequence from Alfalfa Mosaic Virus subgenomic RNA4 (AMV), and the *Brassica napus BBM1* coding region ligated in frame to the ligand binding domain of the rat glucocorticoid receptor, all in the $pBIN^+$ background (Srinivasan et al. 2007; Datla et al. 1993; Schena et al. 1991) (*see* **Note 3**)**.**

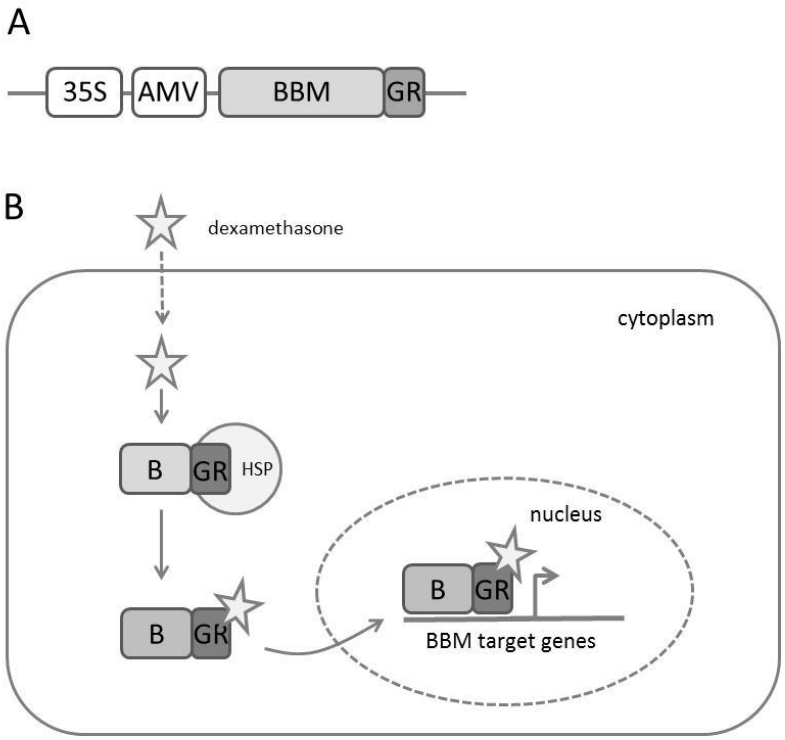

Figure 1: Inducible BABY BOOM activation. (A) The BABY BOOM (BBM) binary vector comprises a doubled-enhanced CaMV 35S promoter, an AMV translational enhancer, and a translational fusion between the protein-coding regions of the *Brassica napus* BBM transcription factor and the ligand-binding domain (LBD) of the rat glucocorticoid steroid. (B) The BBM:GR fusion protein is sequestered in the cytoplasm by a heat-shock protein (HSP)-containing complex in the absence of exogenous glucocorticoid steroid. Addition of the glucocorticoid steroid dexamethasone (DEX) to the culture medium induces translocation of the BBM:GR fusion protein form the cytoplasm to the nucleus, where it regulates expression of its target genes.

2.3. Plant Material

Seeds of the F1 hybrids Fiesta and Ferrari or their parental lines (supplied by Enza Zaden, The Netherlands) were used for the transformation procedure.

2.4. Stock solutions

2.4.1. Stock solutions for Agrobacterium media

1. $MgSO_4$ (1 M): dissolve 24.65 g of $MgSO_4 \bullet 7H_2O$ in 100 ml of deionized water. The solution is autoclaved at 120ºC, 1.2 bar, for 20 minutes. Keep at room temperature until use.

2. Rifampicin (20 mg/ml): dissolve 1 g in 50 ml of methanol. Keep stock solution in the dark at -20°C until required.

3. Gentamycin (25 mg/ml): dissolve 250 mg in 10 ml of deionized water. Filter-sterilize stock solution (0.25 µm pore size) and keep at -20°C until use.

4. Kanamycin (100 mg/ml): dissolve 1g in 10 ml of deionized water. Filter-sterilize stock solution (0.25 µm pore size) and keep at -20°C until use.

2.4.2. Stock solutions for plant culture media:

1. Zeatine riboside (ZR, 2 mg/ml) : dissolve 20 mg of ZRin a few drops of 1 M KOH and then fill-up to 10 ml with absolute ethanol. Keepstock solution at -20°C until use.

2. Indole-3-acetic acid (IAA, 1 mg/ml): dissolve 10 mg of IAA in 10 ml of absolute ethanol. Keep stock solution at -20°C until use**.** IAA stock solutions older than six months should not be used.

3. Acetosyrringone (ACS, 40 mg/ml): dissolve 40 mg of ACS (Acros) in 1 ml of DMSO. Prepare a fresh ACS stock solution before use.

4. Thidiazuron (TDZ, 1mg/ml): dissolve 10 mg of TDZ in 10 ml of DMSO. Keep stock solution at -20°C until use.

5. Myo-inositol (1 mg/ml): dissolve 10 mg of myo-inositol in 10 ml of deionized water. Filter-sterilize stock solution and keep at room temperature in the dark until use.

6. Cefotaxime (100 mg/ml): dissolve 1 g of cefotaxime in 10 ml of deionized water. Filter-sterilize stock solution and keep at -20°C until use.

7 Gibberellic acid 3 (GA3, 10 mg/ml): dissolve 100 mg of GA3 in 10 ml of absolute ethanol. Keep stock solution at -20°C until use.

8. Benzylaminopurine (BAP, 1 mg/ml): dissolve 40 mg BAP in a few drops of 1 M KOH and fill up to 10 ml with absolute ethanol. Keep stock solution at -20°C until use.

9. Silver thiosulfate (STS, 20mM): mix two 100 mM stock solutions of each sodium thiosulfate and silver nitrate in a 4:1 ratio. Sodium thiosulfate (100 mM): dissolve 158 mg of sodium thiosulfate in 10 ml of deionized water. Silver nitrate (100 mM): dissolve 170 mg of silver nitrate in 10 ml deionized water. To make the STS stock solution, transfer 8 ml of the 100 mM sodium thiosulfate stock into a new tube and add 2 ml of 100 mM silver nitrate drop by drop while stirring. Filter-sterilize stock solution prior use. Silver nitrate is quickly photo-oxidized leaving dark brown strains on most surfaces, with the exception of glass and plastics. Protective gloves and clothes are recommended

during its preparation. The STS stock solution can be kept for 30 days in the dark at 4°C if necessary, but it is recommended to prepare it directly before use.

10. Dexamethasone (DEX, 10 mM): dissolve 39.2 mg of DEX in 7 ml of absolute ethanol and fill up to 10ml with sterile deionized water. Keep stock solution at -20°C until use.

2.5. Culture media

2.5.1. Agrobacterium

1. Yeast/Beef extract medium (YEB) (1 liter):dissolve 1g yeast extract (Difco), 5 g beef extract (Sigma), and 5 g sucrose in 900 ml of deionized water, adjust to pH 7.2 with 1 M HCl or 1 M NaOH and filld-up to 1000 ml with deionized water. This medium should be prepared in both a liquid and an agar-solidified form.

2. Divide liquid YEB medium into 100 or 200 ml aliquots (in autoclavable bottles) and add 10 g/lagar (Difco) for agar plates.

3. Autoclave medium at 114ºC, 1.2 bar for 20 minutes and store in the dark at room temperature until use. Add antibiotics and magnesium sulfate from prepared stock solutions, as needed, after autoclaving and right prior culture (*see* **Note 3**).

2.5.2. Sweet pepper plants

Plant tissue culture media can be made by combining the individual chemicals (*see* **Table 1**) or from ready-made mixes provided by commercial suppliers. Ready-made mixes are stored at 4°C in the dark and prepared according to the manufacturer's instructions. When using ready-made media the appropriate amount of medium and sucrose or glucose is dissolved in deionized water in 95 % of the final volume. The pH is adjusted to 5.8 with 1 M KOH or 1 M HCl. The medium is filled-up to the final volume with deionized water before being transferred to autoclavable bottles that contain 8 g/l microagar. The medium is then autoclaved for 20 minutes at 114°C. The medium should be cooled to ca. 55°C before adding supplements from prepared stock solutions. Media for germination, elongation and rooting are poured into high Steri Vent containers. All other media are poured into 9 cm diameter Petri dishes.

1. Germination medium (GM): Full strength MS medium (Murashige and Skoog 1962), supplemented with 2% (w/v) sucrose, solidified with 0.8 % (w/v) micro agar.

2. Co-cultivation medium (CCM): full strength R medium (Sibi et al. 1979) supplemented with 1.6 % (w/v) glucose, 2 mg/l zeatin riboside (ZR), 0.1 mg/l indole-3-acetic acid (IAA) and 40 mg/l acetosyringone (ACS). This medium should be prepared in both a liquid and an agar-solidified form.

3. Selection and regeneration medium (SRM): full strength R medium (Sibi et al. 1979***)*** supplemented with 1.6 % (w/v) glucose, 1 mg/l thidiazuron (TDZ) (Murthy et al. 1998), 100 mg/l kanamycin sulfate, 500 mg/l cefotaxime and 10 µM DEX.

4. Elongation medium (EM): full strength MS macro- and micro salts (Murashige and Skoog 1962), B5 vitamins (Gamborg et al. 1968), 1.6 % (w/v) glucose, 1 mg/l inositol, 20 mg/l adenine sulfate, 200 mg/l casein hydrolysate, 10 mg/l gibberellic acid 3 (GA3), 4 mg/l benzylaminopurine (BAP), 30 µM silver thiosulfate (STS) and 10 µM DEX.

5. Pre-rooting (PRM): full strength MS20 medium supplemented with 30 mg/l glutathione, 60 mg/l kanamycin sulfate and 300 mg/l cefotaxime.

6. Rooting medium (RM): full strength Rugini salts and vitamins (Rugini 1984) supplemented with 2 % (w/v) sucrose and 50 mg/l kanamycin.

3. METHODS

3.1. Growth of Agrobacteria

1. Maintain Agrobacterium strain plus binary vector on 9 cm Petri dishes with YEB agar medium containing the appropriate antibiotics, and 250 mM $MgSO_4$ (*see* **Note 3**). Start maintenance culture from a single colony deriving from a fresh Agrobacterium transformation, a previous maintenance plate, or a glycerol stock (*see* **Note 4**). Keep Petri dishes containing Agrobacteria at 28°C in the dark for 1-2 days until bacterial growth is clearly visible. The bacterial plates can be stored at 4°C for two weeks.

Table1: Basal medium salt and vitamin composition (mg/l)

Compound	MS	R medium	MS B5	Rugini
NH_4NO_3	1650	1238	1650	412
$(NH_4)_2SO_4$		34		
KNO_3	1900	2150	1900	1100
KH_2PO_4	170	142	170	340
$NaH_2PO_4 \bullet H_2O$		38		
$CaCl_2 \bullet 2H_2O$	440	313	440	440
$CaNO_3 \bullet 4H_2O$		50		600
KCl		7		500
$MgSO_4 \bullet 7H_2O$	370	444	370	1500
$ZnSO_4 \bullet 7H_2O$	8.6	3.225	8.6	14.3
$MnSO_4 \bullet H_2O$	22.3	20.13	22.3	22.3

NaFeEDTA	37.5	37.5	37.5	36.7
H_3BO_3	6.2	3.15	6.2	12.4
KI	0.83	0.33	0.83	0.83
$Na_2MoO_4 \bullet 2H_2O$	0.25	0.138	0.25	0.25
$CoCl_2 \bullet 6H_2O$	0.025	0.016	0.025	0.025
$CuSO_4 \bullet 5H_2O$	0.025	0.011	0.025	0.25
thiamine HCl	0.1	0.6	10	0.5
pyridoxine HCl	0.5	5.5	1	0.5
nicotinic acid	0.5	0.7	1	5
myo-inositol	100	50	100	100
glycine	2	1.4		2
biotin		0.05		0.05
folic acid				0.5
Ca-D-panthothenate		0.5		

2. Start liquid culture from the maintenance plate by transferring a couple of colonies (collected with the tip of a sterile 1ml pipette) of Agrobacterium into 100 ml liquid YEB medium (in a sterile 250 ml Erlenmeyer flask) supplemented with 100 mg/l kanamycin for maintenance of the *35S::BBM:GR* plasmid. Grow Agrobacterium overnight at 28°C in the dark on a rotary shaker set at 200 rpm.

3. Prior to transformation, transfer culture into sterile 50 ml tubes and centrifuge at 4000 rpm for 20 minutes. Discard supernatant and resuspend the bacterial pellet completely by repetitive vortexing in liquid CCM. Adjust the OD_{600} to 0.3-0.4 using a photospectrometer.

3.2. Growth of donor plants

3.2.1. Seed sterilization

1. Place 100-200 pepper seeds in a 50 ml screw cap tube.

2. Add twenty ml of 90% ethanol and shake the tube briefly for one minute to surface sterilize the seeds.

3. Remove ethanol is removed and replace by 30 ml of household bleach (1% active NaOCl plus detergent).

4. Shake the tube briefly 2-3 times during the 20 minute incubation at room temperature. From this point onwards all procedures should be performed under aseptic conditions i.e. in a laminar flow cabinet with sterile tools.

5. Remove the bleach completely with a sterile pipette and replace by 50 ml of sterile water. Shake the closed tube a couple of times and allow the seeds to sink to the bottom of the tube.

6. Replace the sterile water three times in the same way (*see* **Note 5**).

3.2.2. Sowing and culture

1. Place twenty to twenty-five seeds at 1-2 cm distance from each other using sterile forceps into a Steri Vent container containing about 80 ml of GM.

2. Let seeds germinate at 25°C in the dark for 3-4 days and then transfer containers to the growth chamber (see 2.1.) for another 6-7 days or until the cotyledons have expanded, but before the first pair of leaves has emerged (*see* **Note 6**).

3.3. Transformation procedure

An overview of the workflow and time schedule is given in Table 2.

3.3.1. Explant pre-culture

1. Remove the cotyledons from the petioles (seedlings from step 2 in Subheading 3.2.2.) with a razor blade and forceps and transfer them with the abaxial (lower side, away from the meristem) side down onto CCM medium.

Table2: Workflow and estimated time schedule for sweet pepper transformation

Step	**Day**
Sterilisation and sowing of seeds for donor material	0
Transfer of donor material into the light	3
Start of pre-culture of explants (CCM) and start liquid overnight culture of Agrobacterium	10
Transformation and start of co-cultivation (CCM)	11
Transfer of explants to selection medium	13
Sub-culture on fresh selection medium	43
Transfer of explants to elongation medium (EM)	73
Transfer to pre-rooting medium (PRM)	106
Transfer to rooting medium (RM)	136
Transfer to greenhouse	150-172

2. Cut each cotyledon twice, transverse to the mid-rib, to generate three explants (Fig. 2A) (*see* **Note 6**). About twenty cotyledons can be collected, cut and placed onto medium in one 9 cm Petri dish (60 explants).

3. Seal the Petri dishes with cling film or Parafilm and place under dim light at 23°C for 24 hours (*see* **Note 7**).

3.3.2. Transformation and regeneration

1. Pour the Agrobacterium culture (from step 3 in Subheading 3.1.3) onto the pre-cultured cotyledon explants until they are just submerged.

2. Incubate the explants and Agrobacterium in the closed Petri dish within the laminar flow at room temperature for 30-60 minutes without shaking.

3. Briefly dry the explants on sterile filter paper and transfer them with the abaxial side down onto new CCM. The explants can be placed close to each other, but should not touch. Seal the Petri dishes with cling film or Parafilm.

4. Co-cultivate the explants with the Agrobacterium for two days under dim light conditions (21 µmol/m^2/s) at 23˚C (*see* **Note 8**).

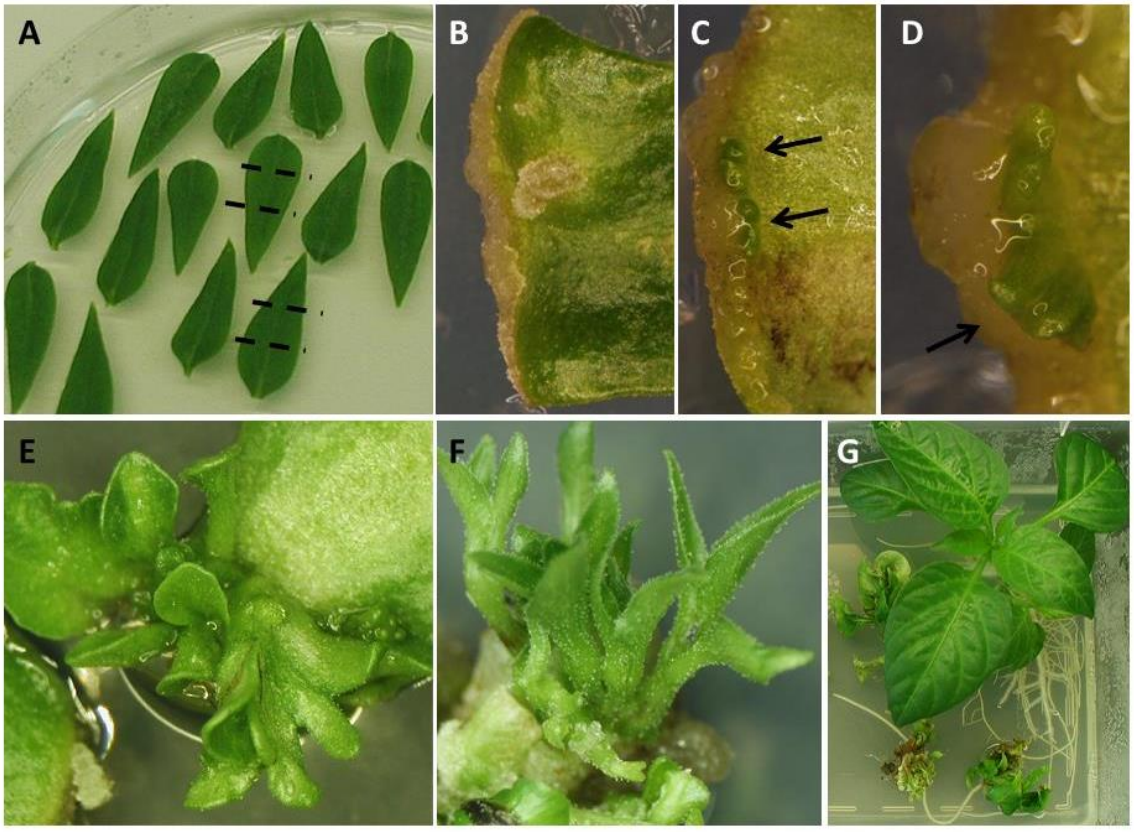

Figure 2: Transgenic sweet pepper regeneration. **A**, cotyledons showing positions of cuts used to generate donor explants (dotted lines); **B**, adaxial side of two week-old explant showing callus formation; **C** and **D**,

abaxial sides of two week old explants with young SLS (arrows); **E**, four week old explants with SLS; **F**, elongated shoots derived from SLS; **G**, rooted transgenic shoot on selection medium.

5. After the co-cultivation period transfer the explants (abaxial side down) to selection and regeneration medium (SRM). Place twelve to sixteen explants per Petri dish onto SRM for 4 weeks and sub-culture on the same, freshly prepared medium for another four weeks at 23˚C (42 µmol/m^2/s). During this period the explants will form callus at the wound sites (Fig. 2B) and primordia-like protuberances on the abaxial side (Fig. 2C and D).
6. Transfer explants with shoots or shoot-like structures (SLS; Fig. 2E) to EM for four weeks (*see* **Note 9**).
7. Transfer elongated shoots (Fig. 2F) to PRM for four weeks to allow further elongation before transfer to RM (*see* **Note 10**).

3.3.3. Seed production from transgenic sweet pepper plants

Transfer rooted shoots of about 5 cm height (Fig. 2G) to either soil or wet rock wool-blocks and kept in a plastic tent for about one week before starting the acclimatization to the greenhouse conditions. Keep acclimatized plants at a constant temperature of 23°C until flowering. Under optimal conditions 50-200 seeds can be harvested from a single sweet pepper fruit of the blocky type e.g. Fiesta or Ferrari (*see* **Note 11**). .

3.3.4. Analysis of transgenic plants

Confirm the presence of the transgene by a PCR reaction on DNA that has been extracted (Rogers and Bendich 1985) either from the original shoot or from its offspring (*see* **Note 10**). A Southern blot (Southern 1975) or a TAIL-PCR (Liu and Chen 2007) can be performed to distinguish between independent transgenic events and clones of the same event (*see* **Note 12**). The number of unlinked integration sites can be determined by sowing sterilized T1 seeds onto GM supplemented with 100 mg/l kanamycin sulphate (*see* **Note 13**) and scoring the ratio of kanamycin susceptible to kanamycin resistant plants. Analyze the functionality of the BBM protein in pepper by placing young leaves taken from T1 plants on germination medium supplemented with 10 µM DEX and 1 mg/l BAP (*see* **Note 14**).

4. NOTES

1. We autoclave all media at 114ºC to prevent caramelization of the sugars. The pH of plant tissue culture media is always adjusted with KOH rather than NaOH to prevent the formation of NaCl. We autoclave media in 500 ml autoclavable bottles (Schott), as these fit in most microwaves and are

easier to pour than larger bottles. The medium is approximately 55 °C when the bottles can be held with bare hands.

2. The Agrobacterium strain GV3101 with the pMP90 Ti plasmid is suitable for transformation of most plant species. Other Agrobacterium strains such as GV2260 or LBA could work as well. If a second cassette is built into the BBM construct then it is advisable to control the functionality of the BBM protein in Arabidopsis prior to starting the more laborious pepper transformation.

3. The *35S::BBM:GR* plasmid is prone to rearrangements during transfer to Agrobacterium by electroporation. The integrity of the *35S::BBM:GR* plasmid should be confirmed at the DNA level by PCR or preferably, at the functional level by transformation to Arabidopsis

The antibiotics used depend on the Agrobacterium strain, the Ti plasmid and the binary vector. In our case we used 100 mg/l rifampicin (GV3101), 25 mg/l gentamycin (pMP90), and 100 mg/l kanamycin (*35S::BBM:GR*).

4. It is advisable to prepare a glycerol stock of Agrobacterium strain for long-term storage at -80 °C. The glycerol stock is prepared by mixing a fresh liquid culture of Agrobacterium ($O.D_{600}$ = 1) in a 1:1 ratio with 100% glycerol (previously autoclaved at120ºC, 1.2 bar for 20 min) in sterile Eppendorf tubes.

5. Some brands of bleach do not contain detergents. In this case, a few drops of Tween 20 (ca. 0.03%) should be added to the bleach.

6. One seedling will give six explants but the tip of cotyledon is not used as it usually regenerates poorly. A minimum of 200 explants will be needed to obtain one transgenic plant. It is advisable to check seed batches beforehand for slow growing bacterial contaminations, as they can interfere later with the transformation and regeneration process. Do not grow the donor plants on medium with antibiotics that act on gram-negative bacteria, as this may interfere with the Agrobacterium infection.

7. For the preparation of the explants we prefer razor blades that provide a clean cut e.g. double-sided razor blades. Torn edges resulting from blunt and/or old blades will cause the explants to disintegrate quickly, resulting in poor regeneration. Additionally, Agrobacteria that reside in these tears can escape selection pressure and overgrow the explants. Razor blades are dipped into 100% ethanol and quickly passed through the flame of a Bunsen burner to burn off the ethanol. The razor blade should be replaced by a new one when its color changes from shiny to dark.

8. Explants should be handled with care, i.e. they should not be damaged by pinching or squeezing them with forceps. We prefer to use bent forceps that allow the explant to be scooped up from below. To create dim light conditions, Petri dishes are covered with one layer of 60 x 60cm filter paper. The co-cultivation of explants with agrobacteria can vary between two and four days depending on the plants species and genotype, culture conditions and Agrobacterium strain. Initially,

the experimental set-can be optimized by performing the transformation with an intron-containing GUS-reporter construct and staining the explants after two or three days of co-cultivation to assay for stable integration of the transgene (Jefferson et al. 1987). Under our conditions a three-day co-cultivation period is optimal for transformation.

9. The explants will increase four to five times in size after transfer to SRM The wounded edges will swell slightly, turn yellowish-brownish and produce a small amount of callus. After subculture, some explants will produce a number of leafy structures in a rosette that resemble shoots, but without a clear meristem. We call these structures SLS. Any transformation protocol can produce escapes (non-transgenic shoots), which can also be observed in sweet pepper. However, only shoots with the BBM construct will be able to form a stem on EM, which will then allow rooting. Non-transgenic escape shoots will remain as a rosette or SLS.

10. It takes about six to nine months from the start of the protocol to this point. If shoots are well elongated they can be directly placed onto rooting medium. Multiple shoots can be regenerated from a single SLS and in most cases they will be clones. However, two or more transformation events can occur in close proximity, making it difficult to distinguish between independent transformation events. Therefore, prior to molecular analysis each shoot transferred to rooting medium should be considered as an independent transformation event.

11. Tissue culture boxes have a high humidity, which limits the formation of a wax layer on the leaves. The plants need to be adapted slowly to the lower humidity conditions of growth chambers and greenhouses. An easy way to gradually lower the humidity is to punch holes in the plastic tent and increase the hole size daily once the plants start to form new leaves.

Plants on rock wool require added nutrients, which should be applied regularly in liquid form. Depending on the genotype, plants will start flowering at around a height of 50 cm. For optimal flowering and seed production sweet pepper should be kept at 23˚C during the day and at 18-20˚C during the night. The plants will usually self-pollinate, but seed production can be enhanced by shaking the whole plant twice per week. The number of branches should be reduced to two for good seed production. Additional light should be provided (16 hour day length) by 1000W SON-T lamps (70-140W/m^2) to achieve a light intensity of at least 108 µmol/m^2/s.

12. The same primer combinations and protocol that were used for checking the Agrobacterium strain can be used to confirmation the presence of the transgene.

13. Kanamycin resistant seedlings can be distinguished from kanamycin sensitive plants at the first leaf stage. Sensitive seedlings remain at the cotyledon stage i.e. do not develop true leaves, while the resistant seedlings develop further. It is advisable to sow at least 100 seeds per offspring for a proper segregation analysis.

14. Leaves of transgenic BBM plants are preferentially cut with a razor blade into feathers. Somatic embryos will form along the wounded edges after about 10 days of culture on MS medium supplemented with 3% sucrose, 0.8% microagar, 1 mg/l BAP and 10 µM DEX.

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Chapter 5

BABY BOOM and PLETHORA2 induce somatic embryogenesis in a dose- and context-dependent manner via the LAFL pathway

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ABSTRACT

Somatic embryogenesis (SE) is an example of cellular totipotency, where embryos develop from vegetative cells rather than from gamete fusion. The *AINTEGUMENTA-LIKE* (*AIL*) transcription factor family comprises eight genes, which redundantly regulate meristem identity and growth. Ectopic expression of the *AIL* genes *BABY BOOM* (*BBM*) or *PLETHORA5/AIL5,* is sufficient to induce SE in *Arabidopsis thaliana* seedlings, but the roles of the other AIL genes in this process, as well as the signalling pathways underlying AIL-mediated SE, are not known. Here, we show that overexpression of all *AIL* genes, except for the phylogenetically-distinct *AIL1* and *AINTEGUMENTA*, induces SE, suggesting extensive overlap in AIL function. Using *BBM* and *PLT2* as representatives of AIL function, we show that AIL-mediated SE is dose-dependent, where a relatively high dose induces SE and a relatively low dose induces shoot (BBM) or root (PLT2) organogenesis. AIL-induced SE is also context-dependent, as early expression of BBM or PLT2 induces SE directly from seedling tissues, whereas late expression induces SE indirectly from callus. Analysis of BBM regulatory pathways shows that BBM binds to and regulates genes with roles in maintaining embryo identity and/or somatic embryo induction including the *LAFL* genes, *LEC1*, *LEC2*, *FUS3* and *ABI3*, as well as *AGL15*. Mutant analysis identified these genes as positive regulators of BBM-mediated SE and their chromatin mediated repressors *PKL* and *VAL1* as negative regulators. Our results demonstrate that AIL proteins regulate overlapping pathways in a context- and dose-dependent manner to modulate plant development and place BBM and PLT2 upstream of other known inducers of SE.

INTRODUCTION

AINTEGUMENTA-LIKE (*AIL*) genes form a small clade of eight members within the AP2 group of APETALA2/ethylene-responsive element–binding factor (AP2/ERF) transcription factors (Kim et al. 2006), and comprise *AINTEGUMENTA* (*ANT*), *AIL1*, *PLETHORA1* (*PLT1*), *PLT2*, *PLT3/AIL6*, *CHOTTO1* (*CHO1*)*/EMBRYOMAKER* (*EMK*)*/PLT5/AIL5* (hereafter named *PLT5/AIL5*), *PLT7* and *BABY BOOM* (*BBM*). *AIL* genes are expressed in dividing tissues, including root, shoot and floral meristems (Nole-Wilson et al. 2005), where they act in a redundant manner to maintain a meristematic state (reviewed in Horstman et al. 2014). Single knock-out mutants of *AIL* genes show only minor defects, but double or triple mutants have stronger phenotypes related to reduced cell proliferation or altered cell identity. For example, the *ant* single mutant has smaller floral organs with partial loss of identity, a phenotype that is enhanced in the *ant;plt3/ail6* double mutant (Sharma et al. 2013; Klucher et al. 1996; Krizek 2009). Combinations of *plt1*, *plt2*, *plt3/ail6* and *bbm* mutants are embryo lethal (*plt2;bbm*), rootless (*plt1;plt2;plt3/ail6*) or have a short root (*plt1;plt2*) (Aida et al. 2004; Galinha et al. 2007), and the *ant;plt3/ail6;plt7* triple mutant is impaired in shoot meristem maintenance (Mudunkothge and Krizek 2012).

In line with their loss-of-function phenotypes, overexpression of AIL transcription factors induces cell overproliferation phenotypes. Ectopic overexpression of *PLT5/AIL5* promotes somatic embryo and ectopic organ formation on seedlings (Boutilier et al. 2002; Tsuwamoto et al. 2010), while overexpression of *PLT1* and *PLT2* leads to ectopic development of hypocotyls, roots and quiescent centre cells (Aida et al. 2004). Besides promoting enhanced pluripotency and totipotency, *AIL* overexpression can also lead to an enlarged root meristem (*PLT2*) (Galinha et al. 2007) and to increased floral organ size due to increased cell number, as shown for *ANT*, *PLT5/AIL5* and *PLT3/AIL6* overexpression (Krizek 1999; Krizek and Eaddy 2012; Nole-Wilson et al. 2005). In contrast, sepals of seedlings expressing higher levels of *PLT3/AIL6* are small and undifferentiated, suggesting that high PLT3/AIL6 levels inhibit cell differentiation (Krizek and Eaddy 2012).

Genetic analysis shows both specific and overlapping roles for *AIL* genes, and that AIL proteins can partially or fully complement phenotypes of other *ail* mutants (Galinha et al. 2007), but it has been difficult to assign specific AIL functions based on the overexpression studies. *AIL* genes that show redundancy in loss-of-function studies, such as *BBM* and *PLT2*, do not show the same overexpression phenotypes (Aida et al. 2004; Boutilier et al. 2002), while overexpression of the same gene e.g. *PLT5/AIL5*, can result in different overexpression phenotypes (Nole-Wilson et al. 2005; Tsuwamoto et al. 2010; Yano et al. 2009). Whether these different phenotypes are due to differences in the expression level of the transgene or due to the screening approach is not clear. AIL target genes have only been identified for BBM (Passarinho et al. 2008), thus it is not known whether AIL proteins have the same or partially overlapping target genes.

Here, we focus on the role of *AIL* genes in somatic embryo induction. Besides AIL proteins, a number of other transcription factors have been identified that can induce or enhance somatic embryogenesis (SE) when ectopically expressed (Fehér 2014). These include two *LEAFY COTYLEDON 1 (LEC1)/LEC1-LIKE; ABSCISIC ACID (ABA)-INSENSITIVE3 (ABI3); FUSCA3 (FUS3); LEC2* (*LAFL*) seed maturation genes (Jia et al. 2013), LEC1 and LEC2 (Lotan et al. 1998; Stone et al. 2001), and the MADS-domain transcription factor AGAMOUS-LIKE15 (AGL15) (Harding et al. 2003; Zheng et al. 2009). The developmental programs regulated by AGL15 and LEC2 have been well characterized and their pathways are interconnected, as LEC2 and AGL15 positively regulate each other's function (Braybrook et al. 2006; Zheng et al. 2009). Similar embryogenic phenotypes are observed in loss-of-function mutants of epigenetic regulators, including the CHD3 protein PICKLE (PKL) (Ogas et al. 1999), the B3-domain proteins VP1/ABI3-LIKE1 (VAL1) and VAL2 (Suzuki et al. 2007), and the Polycomb Group proteins CURLY LEAF (CLF), SWINGER (SWN), EMBRYONIC FLOWER2 (EMF2), VERNALIZATION2 (VRN2), and FERTILIZATION INDEPENDENT ENDOSPERM (FIE) (Bouyer et al. 2011; Chanvivattana et al. 2004), which function to repress *LAFL* gene expression during the transition to post-embryonic growth.

Here, we show that the BBM clade of AIL proteins are potent inducers of SE that this function is dose- and context-dependent. In addition, we show that that AIL-induced SE is mediated in part by direct activation of *LAFL* genes and indirect activation of other components of the LAFL network.

MATERIALS AND METHODS

Plant material and growth conditions

The *lec2-1* (CS3868), *lec1-2* (CS3867), *fus3-3* (CS8014), *agl15-3* (CS16479), *fie* (SALK_042962), and *pkl-1* (CS3840) mutants were obtained from Nottingham Arabidopsis Stock Centre. The *val1-2* (*hsi2-5*), *val1-2;val2-1, abi3-8*, *abi3-9*, *abi3-10* and *abi5-7* mutants have been previously described (Nambara et al. 2002; Sharma et al. 2013; Suzuki et al. 2007). The *LEC1::LEC1-GFP* (Li et al. 2014) marker and the *35S::BBM* and *35S::BBM-GR* constructs were described previously (Boutilier et al. 2002; Passarinho et al. 2008). The *35S::BBM-GR* construct was introduced into the mutant lines by floral dip transformation (Clough and Bent 1998).

Seeds were sterilized with liquid bleach (1 minute in 70% ethanol, followed by 20 minutes in commercial bleach (4%) containing 0.03% Tween-20, and then washed 4-5 times with sterile MilliQ water) before plating on solid medium (½MS-10: half-strength Murashige and Skoog salts and vitamins, pH 5.8, with 0.8% agar and 1% sucrose). Embryo rescue of the *lec1-2* mutant was performed by culturing ovules from sterilized siliques on solid ½MS-10 medium. For some experiments, sterilized seeds were dispensed in 190 ml containers (Greiner) with 30 ml liquid ½MS-10 medium. DEX and CHX (both Sigma) were added to the medium as described in the text. Solid and

liquid (rotary shaker, 60 rpm/min) cultures were kept at 21 ˚C and 25 ˚C, respectively (16 hour light/8 hour dark regime). Plants were grown for seed collection at 21 ˚C (16h light/8h dark regime) on rockwool plugs (Grodan) supplemented with 1 g/L Hyponex fertilizer.

Vector construction and transformation

The *ANT*, *PLT3/AIL6*, *PLT7* and *PLT1* protein coding regions were amplified from Arabidopsis Col-0 genomic DNA and the *PLT2* protein coding region from cDNA, using the primers listed in Supplemental Table 1. The DNA fragments were cloned into the Gateway (GW) binary vector pGD625, which contains a double-enhanced cauliflower mosaic virus *35S* promoter and an AMV translational enhancer (Immink et al. 2002). *BBM-GFP* was amplified from the *BBM::BBM-GFP* plasmid (Horstman et al 2015). The GW-compatible destination vector pARC146 (Danisman et al. 2012) was used for inducible ectopic activity of *PLT2* and *BBM-GFP*. This vector contains a double-enhanced cauliflower mosaic virus *35S* promoter and an AMV translational enhancer, as well as the coding region of the ligand binding domain of the rat glucocorticoid receptor (GR) downstream of the GW cassette.

Leaf imaging and quantification of stomatal development

The first leaf pairs of nine day-old untreated or 0.1 µM DEX-treated *35S::BBM-GR* seedlings were placed overnight in 70% ethanol at 4 ˚C, then transferred to 85% ethanol for 6 hours, and subsequently to 3% bleach overnight or until imaging. Leaves were mounted in HCG solution (80 g chloral hydrate, 10 ml glycerol, 30 ml water) prior to imaging with a Nikon Optiphot microscope.
The stomatal, meristemoid and stomatal lineage indices (SI, MI and SLI) were calculated as previously described (Peterson et al. 2013): SI = (number of stomata/(total number of stomata + non-stomatal epidermal cells)) x 100. For the SI, only mature stomata with a pore were counted. MI = (number of meristemoids/(total number of stomata + non-stomatal epidermal cells)) x 100. SLI = (number of stomata and stomata precursors/(total number of stomata + non-stomatal epidermal cells)) x 100.

Tissue sectioning

35S::BBM-GR and *35S::PLT2-GR* seedlings were fixed overnight in 3:1 ethanol (100%):acetic acid and dehydrated stepwise from 70 to 100% ethanol. The samples were then infiltrated in Technovit 7100 (including hardener 1) in three steps (Hereaus Kulzer, Germany), followed by Technovit 7100 plus hardeners 1 and 2 (Hereaus Kulzer, Germany). Four micron-thick sections were prepared using a rotary microtome (Zeiss HM340E) and Technovit blades (Adamas, The Netherlands). Sections were stained with 0.05% Toluidine Blue (Merck, Germany) for three minutes, and then rinsed well with water and air-dried. The sections were mounted in Euparal (Roth, Germany) and images were taken

using an IX70 microscope (Olympus) with a DP70 camera and CellSens software (Olympus). Seven to ten seedlings per line per treatment were observed.

Confocal laser scanning microscopy

LEC1::LEC1-GFP seedlings were fixed for one week at 4 °C in 1x microtubule stabilizing buffer (MTSB: 50mM PIPES, 5mM MgS04, 5 mM EGTA, pH7.4) containing 4% paraformaldehyde. Fixed seedlings were washed three times with 0.2x MTSB and mounted in the same buffer containing 1% glycerol prior to imaging. Roots were counterstained with 10 µg/mL propidium iodide (PI). Confocal laser scanning microscopy was performed with a Leica SPE DM5500 upright microscope using the LAS AF 1.8.2 software. GFP was excited with a 488-nm solid-state laser and its emission was detected at a band width of 500–530 nm. PI (roots) and red autofluorescence (cotyledons) were used as a background signals (excited with a 532 nm laser and detected at 600-800 nm).

ChIP-seq

ChIP-seq experiments and data analysis were carried out as described in Horstman et al (Horstman et al 2015). Somatic embryo material generated from either 2,4-dichlorophenoxyacetic acid (2,4-D)-induced cultures or from a *BBM* overexpression line were used for ChIP. Somatic embryos from a *BBM::NLS-GFP* line, or embryogenic *35S::BBM* seedlings served as negative controls for the *BBM::BBM-YFP* and *35S::BBM-GFP* ChIPs, respectively. ChIP-seq results were visualized using Integrated Genome Browser (IGB) 8.1.11 (Nicol et al. 2009). The ChIP-seq data is available via NCBI (GEO accession: GSE52400).

Expression analysis of BBM/PLT2 target genes

One- and five-day-old Col-0, *35S::BBM-GR* and *35S::PLT2-GR* seedlings (3 biological replicates of each) were treated for 3 hours with 10 µM DEX plus 10 µM CHX. RNA was extracted using the NucleoSpin RNA kit (Machery-Nagel) kit in combination with Plant RNA Isolation Aid (Ambion), treated with DNA-*free* (Ambion) and then used for cDNA synthesis with M-MLV Reverse Transcriptase (Life Technologies). Quantitative real-time RT-PCR (qPCR) analysis of BBM/PLT2 target genes was performed using the BioMark HD System (Fluidigm) as previously described (Horstman 2015). The data were normalized against the *SAND* gene (Czechowski et al. 2005) and relative gene expression was calculated according to Livak and Schmittigen (Livak and Schmittgen 2001) by comparison with DEX + CHX-treated wild-type Col-0. The DNA primers are shown in Supplemental Table 1.

RESULTS

All BBM and PLT proteins induce SE

BBM and *PLT2* have redundant roles in embryogenesis and root meristem maintenance (Galinha et al. 2007), but show different overexpression phenotypes (Boutilier et al. 2002; El Ouakfaoui et al. 2010; Galinha et al. 2007). This observation, together with reported differences in the overexpression phenotypes described for the same *AIL* gene (*PLT5/AIL5*) (Nole-Wilson et al. 2005; Tsuwamoto et al. 2010; Yano et al. 2009), prompted us to investigate the overexpression phenotypes of the AIL family members using the same overexpression vector and under the same growth conditions. We generated Arabidopsis *35S::AIL* overexpression lines for the six *AIL* genes that have not been reported to induce SE when overexpressed, namely *ANT*, *AIL1*, *PLT1*, *PLT2*, *PLT3/AIL6* and *PLT7*, and found that overexpression of all these genes except the phylogenetically-distinct *ANT1* and *AIL1* (Kim et al. 2006) induced somatic embryogenesis in 7-26% of the primary transformants (Supplemental Fig. 1, Supplemental Table 2). These numbers are in line with the percentage of embryogenic seedlings obtained after transformation with *35S::BBM* (Supplemental Table 2). We observed the large flower phenotype that has been reported previously for *35S::ANT* (Krizek 1999; Mizukami and Fischer 2000), demonstrating that the protein is expressed, but did not observe the previously reported conversion of the shoot apical meristem (SAM) into root identity in *PLT1* or *PLT2* overexpression lines (Aida et al. 2004; Galinha et al. 2007), neither in the primary transformants nor in subsequent generations. No mutant phenotypes were observed upon *AIL1* overexpression. These results show that all AIL proteins, except for ANT and AIL1, have the capacity to induce SE, and suggest that all BBM-clade proteins are functionally interchangeable with respect to somatic embryo induction.

BBM and PLT2 have dose-dependent overexpression phenotypes

PLT2 functions in a dose-dependent manner in the root, with different levels of PLT2 protein instructing different cellular outputs (Galinha et al. 2007). We employed fusions between two representative AIL proteins, BBM and PLT2, and the glucocorticoid receptor ligand-binding domain (GR, *35S::AIL-GR*) to investigate the dose-dependency of AIL overexpression phenotypes. The amount of nuclearly localized BBM-GFP-GR protein could be controlled by the DEX concentration. In the absence of DEX, GFP was localized to the cytoplasm, but became increasingly nuclear-localized with higher DEX concentrations, such that cytoplasmic GFP could no longer be detected in the presence of 1 µM DEX (Supplemental Fig. 2). These experiments demonstrated that the proportion of a nuclear-localized GR fusion protein, and by extension AIL-GR protein, can be controlled by exposing plant tissue to different amounts of DEX.

We used the same DEX concentration range to regulate AIL-GR activity in *35S::AIL-GR* seedlings (Fig. 1). Control seedlings (wild-type seedlings + DEX) did not show aberrant phenotypes when grown on DEX, whereas *35S::BBM-GR* and *35S::PLT2-GR* seedlings showed dose-dependent mutant phenotypes. The DEX concentration required to induce a specific phenotype was dependent on the strength of the transgenic line.

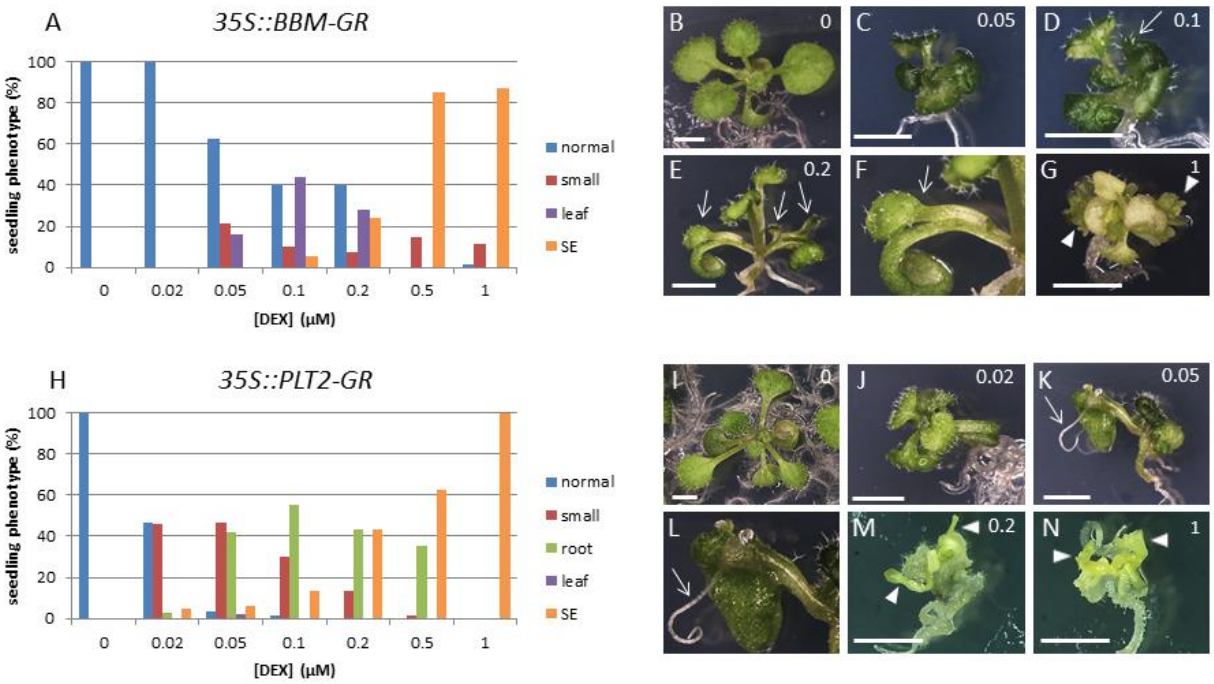


Figure 1: BBM and PLT2 have dose-dependent overexpression phenotypes
35S::BBM-GR and *35S::PLT2-GR* seedlings were grown for two weeks on medium containing different DEX concentrations. Frequency of *35S::BBM-GR* (**A**) and *35S::PLT2-GR* (**H**) phenotypes (*n*=62 to 196 seedlings). No additional phenotypes were observed in treatments above 1 µM DEX. Leaf, ectopic leaves; root, ectopic root; SE, somatic embryogenesis.
(**B-G**) Representative phenotypes of *35S::BBM-GR* seedlings grown on the DEX concentration (µM) indicated in each picture. (**B**) A normal looking seedling grown without DEX. (**C**) A small seedling showing epinastic growth of leaves and cotyledons. (**D**) A small, epinastic seedling with a trichome-bearing ectopic leaf (arrow) on the cotyledon petiole. (**E**) A seedling with ectopic leaves on the petioles of both cotyledons (arrows). (**F**) A magnified view of the ectopic leaf (arrow) in (**E**). (**G**) A seedling with somatic embryos on the cotyledon margins (arrowheads).
(**I-N**) Representative phenotypes of *35S::PLT2-GR* seedlings grown on the DEX concentration (µM) indicated in each picture. (**I**) A wild-type seedling grown in the absence of DEX. (**J**) A small seedling showing epinastic growth of leaves and cotyledons. (**K**) A small epinastic seedling with ectopic root formation on the cotyledon (arrow). (**L**) A magnified view of the ectopic root (arrow) shown in (**K**). (**M, N**) Seedlings with somatic embryos on the cotyledons (arrowheads). Scale bars represent 2.5 mm.

The dose-dependent phenotypes of strong AIL-GR lines (i.e. lines that show highly penetrant SE at a high DEX dose) are shown in Fig. 1. At the lowest effective DEX concentrations *35S::BBM-GR* seedlings were stunted, with epinastic leaves (Fig. 1A). Analysis of the first leaf pair and stomatal development suggested that a low BBM/PLT2 dose inhibits cell differentiation (Supplemental Fig. 3). At intermediate DEX concentrations the seedlings were still small, but now formed leaf-like structures from their cotyledon petioles, which ranged from trichome-bearing protrusions (Fig. 1D)

to ectopic leaves (Fig. 1E, F). At the highest effective DEX concentration, *35S::BBM-GR* seedlings also developed somatic embryos on their cotyledons (Fig. 1A, G) (Passarinho et al. 2008). *35S::PLT2-GR* seedlings also showed stunted growth and somatic embryo formation at the lowest and highest effective DEX concentrations tested, respectively, but ectopic roots were more prevalent than shoots at intermediate DEX concentrations (Fig. 1H, K, L). Phenotypically weaker *35S::BBM-GR* and *35S::PLT2-GR* transgenic lines showed a similar dose-dependent response, but the penetrance and severity of the phenotypes was lower (Supplemental Fig. 4). For example, although the number of SE-forming seedlings was high in these weaker lines, they only produced a few somatic embryos at the tip of the cotyledon.

Our data suggest that BBM and PLT2 overexpression phenotypes are dose-dependent, with similar phenotypes at relatively low (stunted) and high doses (embryogenesis) and divergent phenotypes at an intermediate dose (shoot or root organogenesis).

BBM and PLT2 promote context-specific embryogenesis

Previously, we showed that there is an optimal developmental window for BBM-mediated SE; a significant drop in the number of seedlings that form somatic embryos is observed when DEX is added four days after seed germination (Passarinho et al. 2008). We examined this developmental competence in more detail by activating BBM-GR and PLT2-GR at different time points before and after germination. Germination is defined as the emergence of the radicle through the surrounding structures (Bewley 1997) and is a two-step process in Arabidopsis, comprising testa rupture (d1) followed by radicle protrusion through the endosperm (endosperm rupture, d2).

When *35S::BBM-GR* seeds were placed directly in DEX-containing medium prior to or at endosperm rupture (d0-d2), 100% of the seedlings formed somatic embryos directly on their cotyledons after circa one week (Fig. 2A; Fig. 3A). In contrast, post-germination DEX treatment (d3-d4) induced callus formation on the adaxial side of the cotyledons, from which visible somatic embryos developed approximately 14 days after BBM activation (ca. 40%; Fig. 2A; Fig. 3A). *35S::PLT2-GR* seedlings treated with DEX at the same time points, showed similar phenotypes (Fig. 2B; Fig. 3B) with two exceptions. Firstly, when *35S::PLT2-GR* seedlings were DEX-treated at endosperm formed a whitish protrusion at the SAM that contained leaf-like tissue on its distal end (Fig. 2B; Fig. 3B), which developed somatic embryos 12 days after PLT2 activation (Fig. 2B; Fig. 3B).

Secondly, post-germination (d3-d4) DEX treatment of *35S::PLT2-GR* plants induced callus and somatic embryo formation on both the petioles and the cotyledons (Fig. 2B; Fig. 3B). These results suggest that the response to BBM and PLT2 ectopic expression depends on the developmental context in which the proteins are expressed.

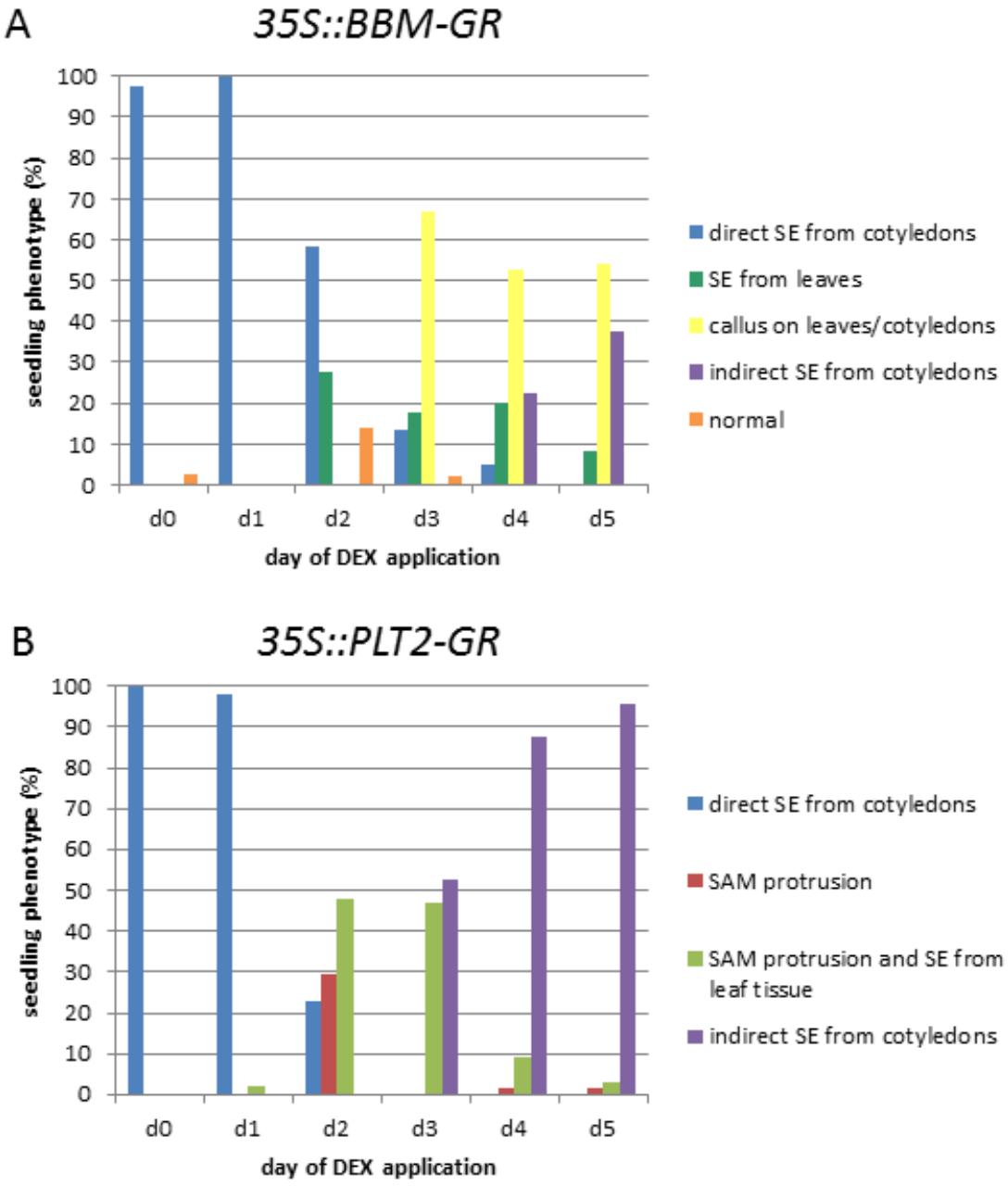


Figure 2: BBM and PLT2 induce stage-specific phenotypes
The effect of applying of a relatively high BBM (**A**) or PLT2 dose (**B**) at different time points after sowing (d0-d5). The seedling phenotypes were scored two weeks after the DEX application. For each time point, between 31 and 70 seedlings were analysed. The quantification is shown for single *35S::BBM-GR* and *35S::PLT2-GR* lines, but similar results were obtained with other independent lines (Supplemental Fig. 4). SE, somatic embryogenesis, SAM, shoot apical meristem.
rupture (d2), they did not form somatic embryos directly from the cotyledon as for BBM, but rather

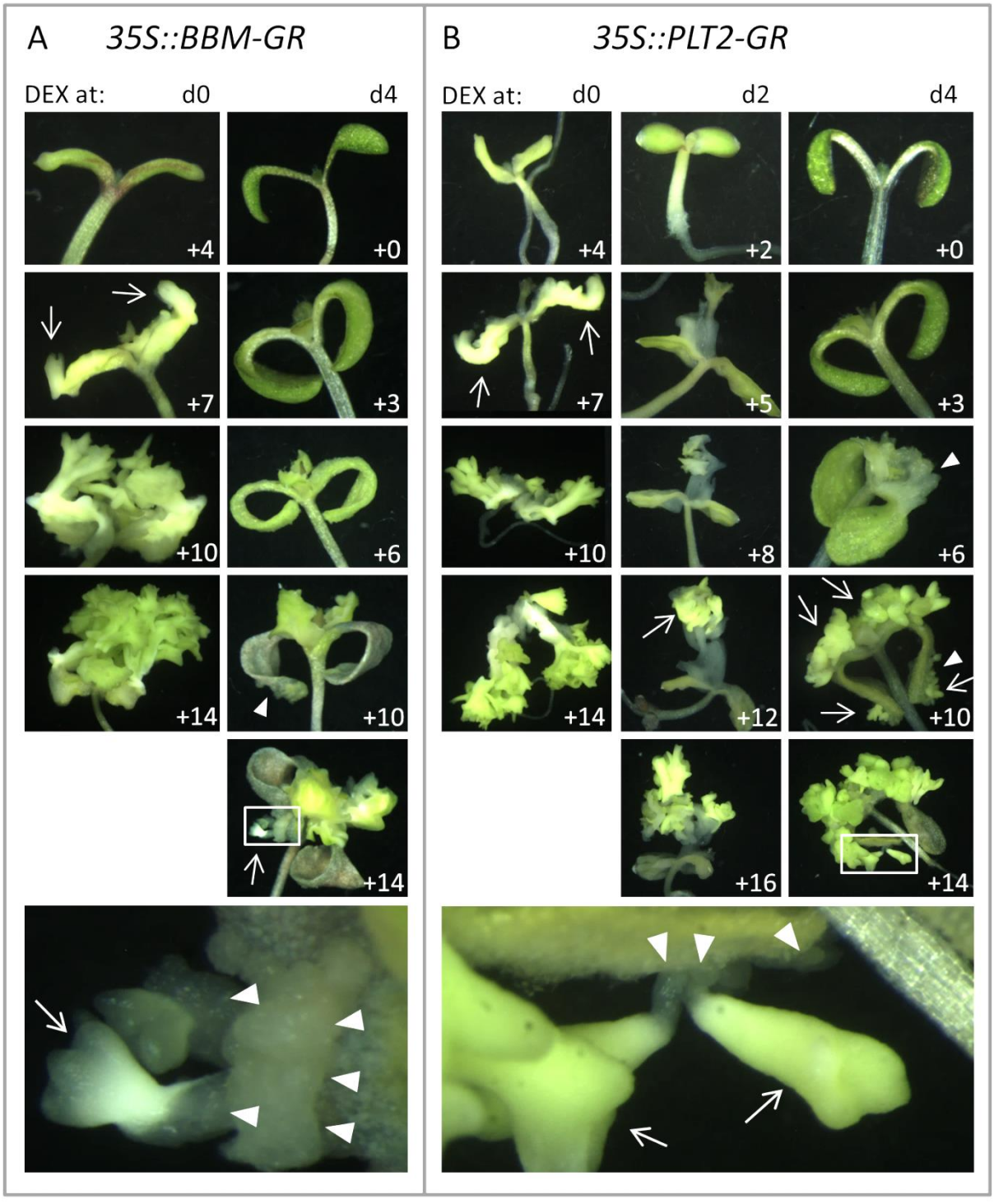


Figure 3: BBM and PLT2 promote context-specific embryogenesis
35S::BBM-GR (**A**) and *35S::PLT2-GR* (**B**) lines were treated with 10 µM DEX at different time points (d0,d2, and d4), and the development of the seedlings was followed in time. The culture time after DEX application is indicated on the bottom right of each picture. The images at different individuals are from different individuals. The arrowheads and arrows indicate callus and somatic embryos/embryogenic tissue, respectively. The lower-most images in (**A**) and (**B**) are magnifications of the boxed regions in the respective '+14' images, and show the indirect development of somatic embryos from callus.

The timing and origin of somatic embryo formation in *35S::BBM-GR* and *35S::PLT2-GR* seedlings was examined in more detail using tissue sections. *35S::BBM-GR* seedlings that were DEX-induced at d0 showed anti- and periclinal cell division in the sub-epidermal layers on the adaxial side of the cotyledon, resulting in the formation of small cells at the position where elongated palisade cells are found in wild-type seedlings (Fig. 4A).

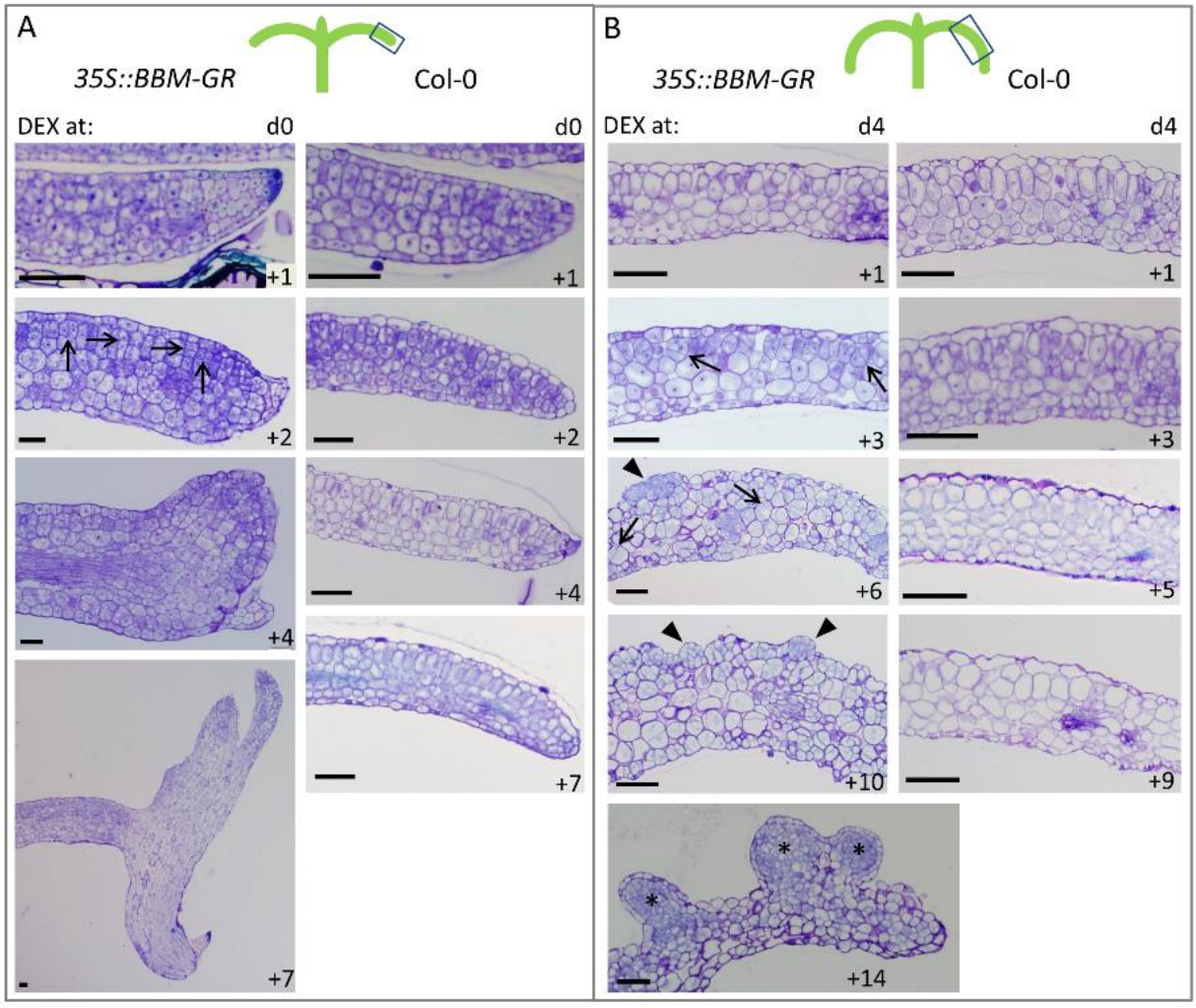


Figure 4: BBM induces direct and indirect SE
Sections of *35S::BBM-GR* and wild-type Col-0 seedlings that were DEX-induced at d0 (**A**) or d4 (**B**), and cultured for the additional period indicated at the bottom right of each image (+1 to +14 days). The schematic illustrations depict the cotyledon regions (blue boxes) that were sectioned in the images below. BBM-GR activation at both d0 and d4 induces anticlinal, periclinal and oblique cell divisions, indicated by the horizontal, vertical and oblique arrows, respectively, on the adaxial side of the cotyledon. BBM-GR activation at d0 (**A**) induces cell divisions (+1, +2), and thickening of the cotyledon tip (+4), followed by the direct development of a somatic embryo from this area (+7). By contrast, BBM-GR activation at d4 (**B**) induces oblique and less compact cell divisions (+1, +3) and the formation of more compact cell masses (arrowheads) from which globular somatic embryos with a distinct epidermis (asterisks) develop. Scale bars, 100 µm.

After about four days of BBM-GR activation, a bump formed on the tip of the cotyledon that developed into a bipolar somatic embryo a few days later (Fig. 4A). Later, somatic embryos also developed on more proximal parts of the cotyledon and secondary embryos formed on the primary somatic embryo on the cotyledon tip (Fig. 3A +10 and +14). PLT2-GR activation at d0 induced a similar developmental change (Fig. 3B). BBM-GR activation at d4 predominantly induced oblique

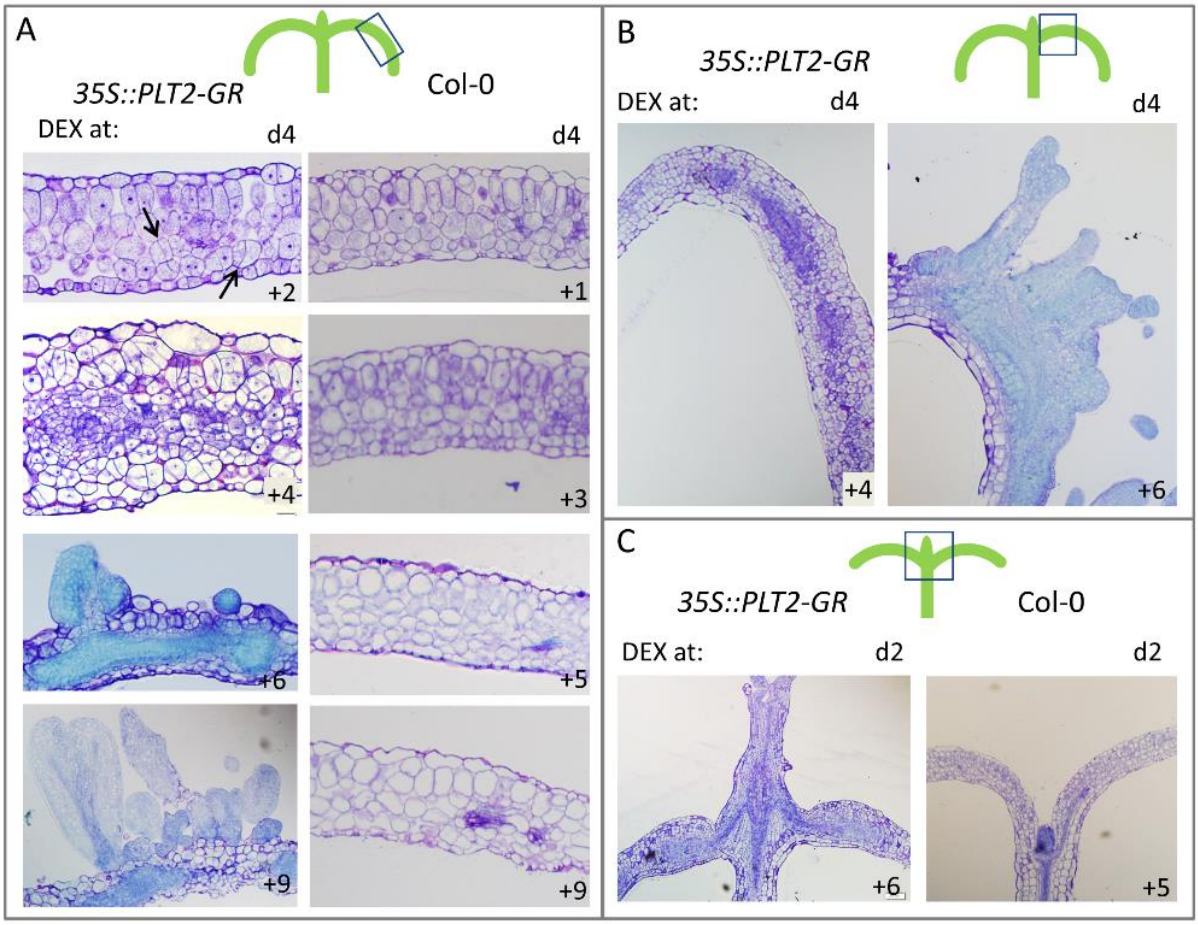


Figure 5: Post-germination activation of PLT2
Sections of *35S::PLT2-GR* and wild-type Col-0 seedlings grown in medium supplemented with 10 µM DEX at d4 (**A, B**) or d2 (**C**), and cultured for the additional period indicated on the bottom right of each image (+2 to +9 days). The schematic illustrations depict the cotyledon regions (blue boxes) that were sectioned in the images below.
(**A, B**) PLT2-GR activation at d4 induces cell divisions (+2, arrows) in/around the cotyledon vasculature (+4) in both the distal (**A**) and proximal (**B**) parts of the cotyledon. Extensive callus production is observed after 6 days, from which somatic embryos arise later (+9).
(**C**) PLT2-GR activation at d2 induces growth of the region below the SAM and swelling of the cotyledons.

cell divisions in the subepidermal cell layers on the adaxial side of the cotyledon and did not induce cell division at the cotyledon tips (Fig. 4B). Moreover, in contrast to early BBM induction, larger, irregularly-shaped, vacuolate cells were formed proximal to the tip, resulting in a rough cotyledon surface (Fig. 4B). Small clusters of small, cytoplasm-rich cells were observed on the cotyledon surface

around seven days after BBM activation (Fig. 4B). Ten days after BBM-GR activation, we observed larger globular-shaped structures enclosed by a smooth epidermis, which were set off from the underlying tissue by a thicker cell wall. These structures are reminiscent of globular-stage somatic embryos (Fig. 4B). We observed the same phenotype after post-germination PLT2-GR activation, although somatic embryos developed faster (Fig. 5A). Notably, BBM-GR and especially PLT2-GR activation induced proliferation of the cotyledon vasculature (Fig. 5B). Somatic embryos always formed above this tissue, but we did not observe a direct connection between the proliferating vascular tissue and the somatic embryos.

We conclude that AIL-mediated SE is induced in two ways depending on the developmental stage of the explant: directly from cotyledons in a narrow window surrounding germination, and indirectly via a callus phase after germination. The data imply that the developmental competence for SE relies on context-specific co-factors.

BBM activates embryogenesis regulators

To understand the regulatory networks underlying AIL-mediated SE, we identified genes that were directly bound by BBM during somatic embryo development. Chromatin immunoprecipitation coupled to next-generation sequencing (ChIP-seq) (Horstman et al 2015, Horstman 2015) showed that BBM bound to the promoter regions of transcription factor genes that have roles in promoting zygotic and/or somatic embryo development, including the *LAFL* seed maturation genes, *LEC1*, *LEC2*, *ABI3* and *FUS3* (but not *LEC1-LIKE*), and the MADS box transcription factor *AGL15* (Fig. 6A).

We examined whether BBM binding regulates the expression of these genes during direct and indirect SE by inducing one day-old (early, direct) and five day-old (late, indirect) *35S::BBM-GR* and *35S::PLT2-GR* seedlings with DEX in the presence of the translational inhibitor cycloheximide (CHX) (Gorte et al. 2011) and examining target gene expression using quantitative RT-PCR (qPCR). Early activation of BBM/PLT2-GR was characterized by upregulation of *LEC1*, *LEC2*, *FUS3* and *ABI3* gene expression (Fig. 6B). In contrast, expression of *LEC1*, *LEC2*, *FUS3* and *ABI3* was not detected in five day-old induced Col-0 seedlings, nor was it detected in DEX-induced *35S::BBM-GR* and *35S::PLT2-GR* seedlings (Fig. 6B). *AGL15* expression was not much affected by BBM/PLT2-GR activation at either of the two time points (Fig. 6B). It might be that *LEC* genes are in an epigenetically silent state in five day-old seedlings and only become accessible after re-differentiation of the cells into callus.

Next, we used the *LEC1::LEC1-GFP* reporter (Li et al. 2014) to chart the dynamics of *LEC1* expression during BBM-induced SE. When DEX is added before germination (d1) *35S::BBM-GR* seedlings form somatic embryos directly on the cotyledon tip. Under these conditions, LEC1-GFP was

observed one day after BBM-GR activation, in small patches of cells on the abaxial side of the cotyledon (Fig. 7C, d1+1).

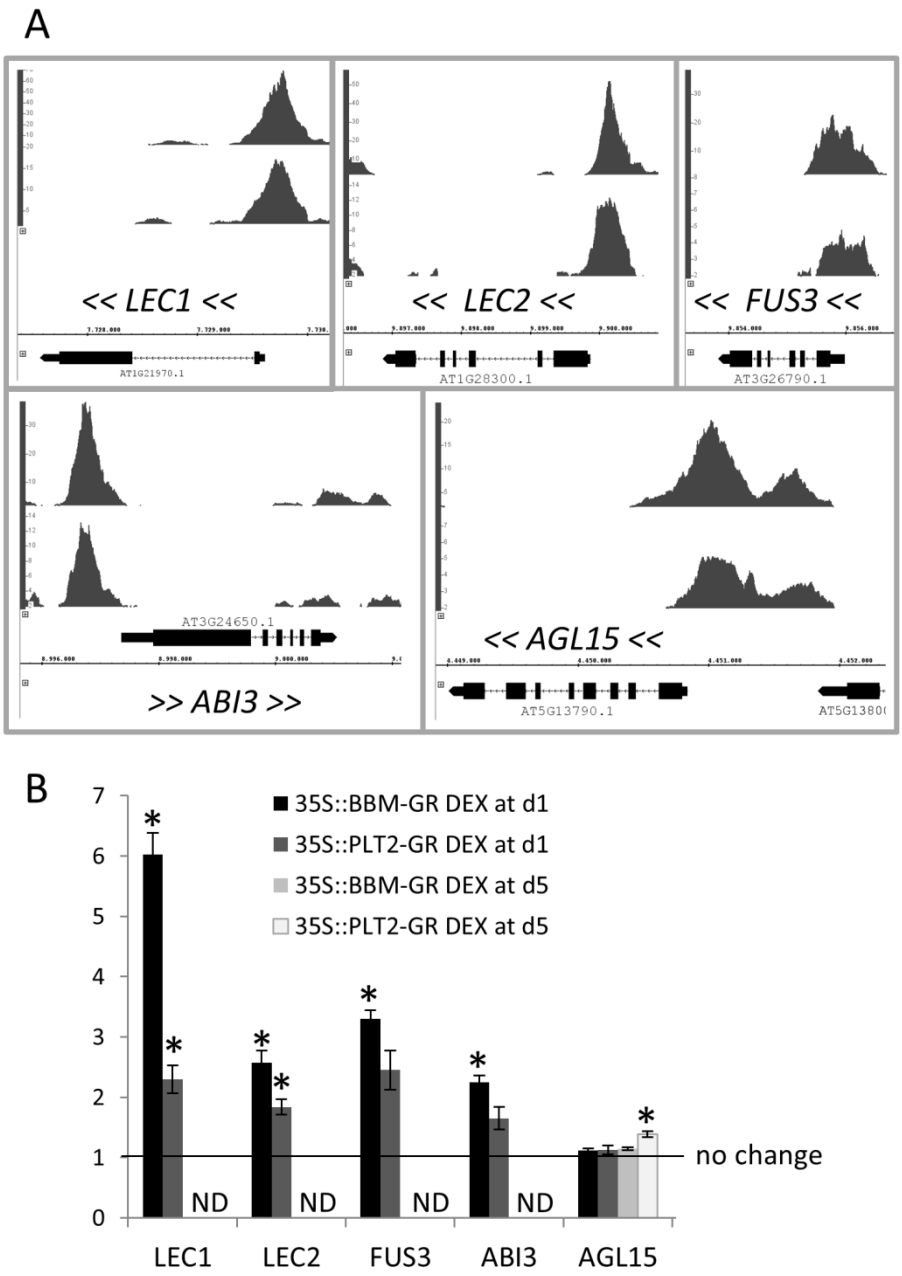


Figure 6: BBM binds and activates embryo-specific genes

(**A**) ChIP-seq BBM binding profiles for embryo-expressed genes in somatic embryo tissue. The binding profiles from the *35S::BBM-GFP* (upper profile) and *BBM::BBM-YFP* (lower profile) ChIP-seq experiments are shown. The x-axis shows the nucleotide position of DNA binding in the selected genes (TAIR 10 annotation), the y-axis shows the ChIP-seq score, and the brackets indicate the direction of gene transcription. Peaks with scores above 1.76 (for *35S::BBM-GFP*) and 3.96 (for *pBBM::BBM-YFP*) are considered statistically significant (FDR<0.05).

(**B**) The relative expression of embryo-specific genes was determined by quantitative real-time RT-PCR for DEX+CHX treated *35S::BBM-GR* and *35S::PLT2-GR* seedlings at d1 and d5 using DEX+CHX treated Col-0 as the calibrator and the *SAND* gene (Czechowski et al., 2005) as the reference. Error bars indicate standard errors of the three biological replicates. Statistically significant differences (*) between *35S::BBM-GR*/*35S::PLT2-GR* and Col-0 were determined using a Student's *t*-test (p<0.01). ND, not detected.

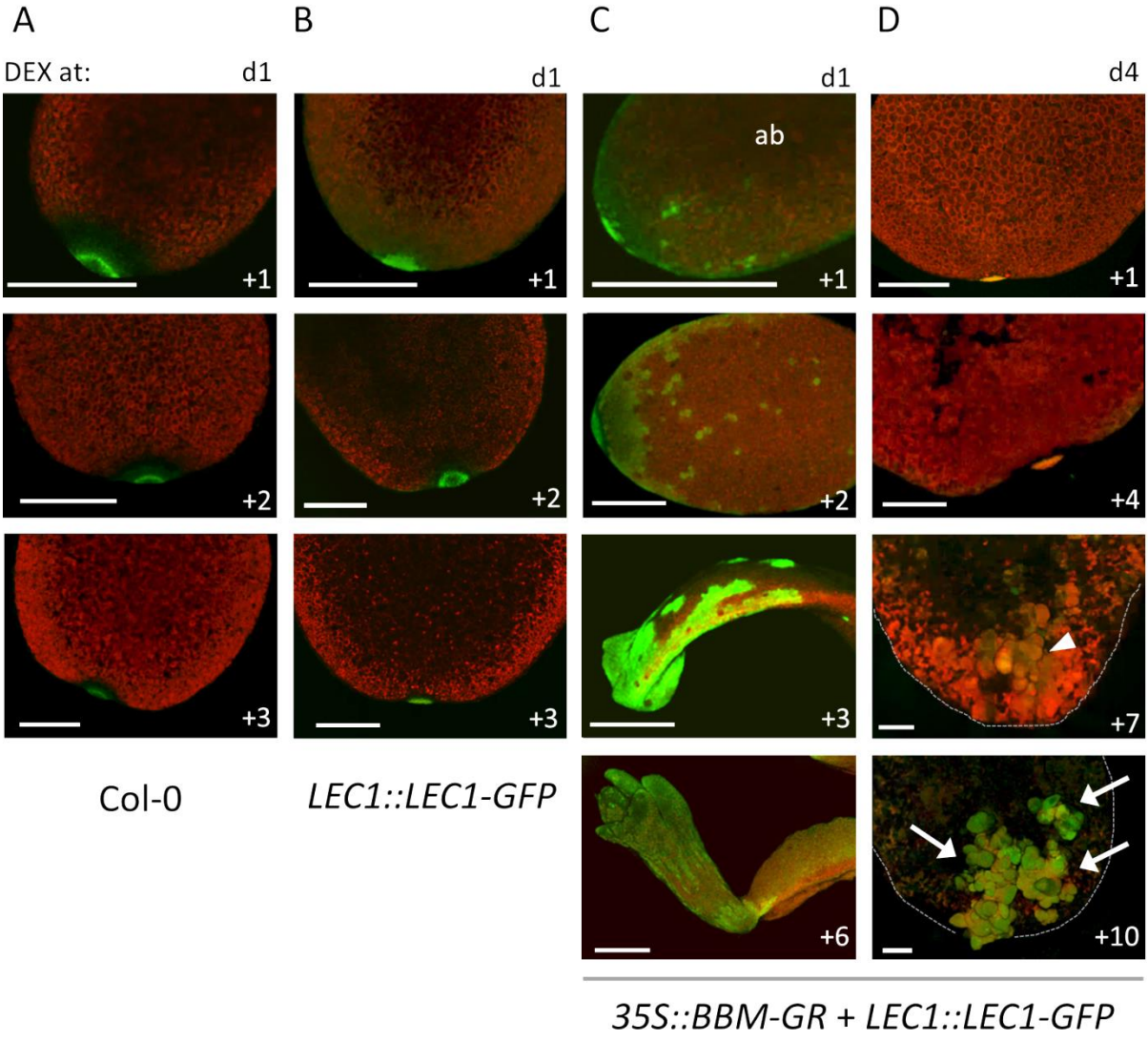


Figure. 7: BBM-GR activates *LEC1* expression in a developmentally specific manner
Wild-type Col-0 (**A**), *LEC1::LEC1-GFP* (**B**) and *LEC1::LEC1-GFP* + *35S::BBM-GR* (**C, D**) seedlings were treated with 10 µM DEX at d1 or d4 and the GFP signal was observed from one to 10 days later (indicated on the bottom right of each picture). The images show the adaxial sides of cotyledons, unless indicated otherwise (ab, abaxial side). The green signal in Col-0 (**A**) and *LEC1::LEC1-GFP* (**B**) cotyledon tips is autofluorescence. Seedlings that were treated with DEX before germination show the first patches of ectopic *LEC1* expression one day after BBM activation (**C**). Seedlings that were treated with DEX after seed germination (**D**) show *LEC1* expression around 10 days after BBM-GR activation (d4+10), when embryogenic clusters are visible (arrows). The arrowhead in (d4+7) indicates the callus that is formed on the distal end of the cotyledon blade. The outline of the cotyledon margins in (D) is shown with dashed lines. Red autofluorescence was used to delineate the tissue. Scale bars, 250 µm.

LEC1 expression expanded to the cotyledon tip and in patches of cells on the adaxial cotyledon blade (Fig. 7C, d1+2), and then became stronger in the cotyledon and extended to the first leaves at the time when the cotyledon tip began to swell (Fig. 7C, d1+3). Later, *LEC1* expression was observed in the outer layer of the somatic embryos, but not in the underlying seedling cotyledon (Fig. 7C, d1+6).

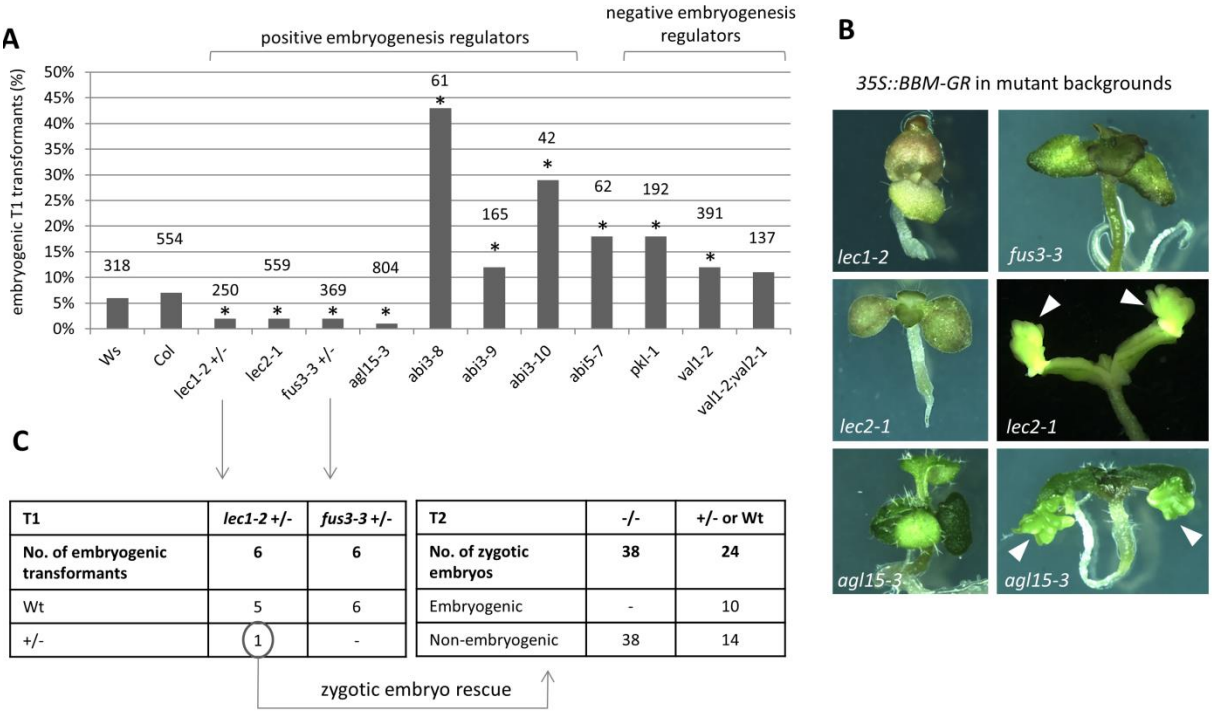
When DEX is added after germination (d4), *35S::BBM-GR* seedlings form callus on the cotyledon blade from which somatic embryos develop. LEC1-GFP could only be detected 10 days after DEX-induction (Fig. 7D, 4+7, 4+10), where it was localized to the large globular-like embryo structures (Fig. 5B). These results reinforce our qPCR-based expression analysis in which we observed rapid *LEC* expression when BBM was activated before germination, but no *LEC* expression when BBM is activated after germination. The observation that LEC1-GFP is initially absent from the callus that forms after post-germination BBM-GR activation, suggests that somatic embryo identity is established much later in this indirect pathway.

LAFL genes and _AGL15_ are important for BBM-mediated direct SE

We investigated the genetic relationship between BBM and its direct gene targets. Both *LEC1* and *LEC2* overexpression induces spontaneous SE in seedlings, while the LEC2 target *AGL15* enhances the embryogenic potential in 2,4-D induced SE tissue culture when overexpressed (Harding et al. 2003; Lotan et al. 1998; Stone et al. 2001; Zheng et al. 2009). The other two LAFL proteins FUS3 and ABI3 do not induce SE when overexpressed, but FUS3 overexpression confers cotyledon identity to leaves (Gazzarrini et al. 2004), and ABI3 overexpression increases the expression of seed storage protein genes in leaves in response to ABA (Parcy and Giraudat 1997; Parcy et al. 1994). Since BBM overexpression lines cannot be outcrossed without loss of the BBM phenotype, we introduced the *35S::BBM-GR* construct into the *lec1-2*$^{+/-}$, *lec2-1*, *fus3-3*$^{+/-}$, *agl15-3* and *abi3* (three alleles) mutant backgrounds via transformation. These mutants, except *agl15-3*, display defects during the later stages of embryogenesis with regard to storage protein accumulation, the acquisition of desiccation tolerance and dormancy (Meinke et al. 1994; Nambara et al. 2002). The *lec1-2* and *fus3-3* seeds are desiccation intolerant (Meinke et al. 1994), therefore heterozygous mutants were used for transformation.

In wild-type Arabidopsis, 6-7% of the primary (T1) *35S::BBM-GR* transformants was embryogenic when grown on DEX (Fig. 8A). Transformation of the *lec1-2*$^{+/-}$, *lec2-1, fus3-3*$^{+/-}$ and *agl15-3* mutants, resulted in a reduced percentage of *35S::BBM-GR* seedlings that formed embryogenic tissue (Fig. 8A). *35S::BBM-GR* also severely inhibited growth and caused swelling of the cotyledons in the *lec1-2*, *fus3-3* and *lec2-1* backgrounds (15-20%; Fig. 8B), a phenotype which was not observed in DEX-activated *35S::BBM-GR* lines. Growth inhibition was also in the *agl15-3* mutants, but not cotyledon swelling (Fig. 8B), a phenotype that was also observed in the wild-type background and that resembles *35S::BBM-GR* seedlings treated with low DEX concentrations (Fig. 1C). Of the few embryogenic seedlings that were found in the *lec1-2*$^{+/-}$ and *fus3-3*$^{+/-}$ segregating populations none contained the *fus3-3* mutant allele, and only one contained the *lec1-2* mutant allele in the heterozygous state (Fig. 8C). Immature embryos from this *lec1-2*$^{+/-}$*/35S::BBM-GR* plant were rescued

to bypass the *lec1-2* desiccation intolerance. The embryos were separated phenotypically into *lec1-2* homozygous mutant and *lec1-2* heterozygous mutant/wild-type classes and placed on DEX-containing selective medium.



T1	*lec1-2* +/-	*fus3-3* +/-
No. of embryogenic transformants	**6**	**6**
Wt	5	6
+/-	1	-

T2	-/-	+/- or Wt
No. of zygotic embryos	**38**	**24**
Embryogenic	-	10
Non-embryogenic	38	14

Figure 8: The efficiency of BBM-induced SE in embryogenesis mutants
(**A**) Percentage of primary embryogenic transformants obtained after transformation of the *35S::BBM-GR* construct to wt (Ws/Col) or the indicated mutants. Statistically significant differences (*) between the mutant and the corresponding wt line were determined using a Pearson's chi-squared test (p<0.05). The total number of transformants per line is indicated above each bar.
(**B**) Phenotypes of embryogenesis mutants that contain the *35S::BBM-GR* construct. In the *lec1-2* and *fus3-3* mutants, BBM-GR activation leads to severe growth inhibition, the *lec1-2* mutant was obtained via embryo rescue (**C**). Severe growth inhibition was also observed in the *lec2-1* mutant (left), but also embryogenic seedlings could be obtained (right). In the *agl15-3* mutant, BBM-GR activation leads to milder growth inhibition (left) and SE (right). Arrowheads indicate the somatic embryos formed on the cotyledon tips.
(**C**) Phenotype and genotype of the (progeny of) the embryogenic transformants obtained in the *lec1-2*$^{+/-}$ and *fus3-3*$^{+/-}$ segregating populations. The numbers of rescued embryos do not reflect the *lec1-2* phenotype segregation ratio. Phosphinothricin-resistance was used to select the *35S::BBM-GR* transgene.

Somatic embryos formed in wild-type/heterozygous *lec1-2* seedlings, but not in the homozygous *lec1-2* mutant seedlings (Fig. 8B, C). Instead, growth was severely inhibited in the *lec1-2*/*35S::BBM-GR* mutants (Fig. 8C). We could also obtain a few homozygous *fus3-3/35S::BBM-GR* lines, showing that the *fus3-3* seed maturation phenotype is not fully penetrant. However, no SE was observed in these lines (Fig. 8B). These results suggest that *LEC1*, *LEC2*, *FUS3* are positive regulators of BBM-mediated SE, and that LEC1 and FUS3 are absolutely required for this process. Surprisingly, we found that *AGL15* also is a positive regulator of BBM-induced SE even though it is not transcriptionally regulated by BBM overexpression at the two time points examined; *AGL15* might be regulated by

BBM at a later time point or function downstream of the *LAFL* genes (Braybrook et al., 2006) in BBM-induced SE.

In contrast to the results obtained with the *fus3*, *lec* and *agl15* mutants, transformation of the *35S::BBM-GR* construct to three different *abi3* mutants led to an enhanced SE response (Fig. 8A). Notably, *abi3* is the only *LAFL* mutant that is insensitive to ABA and overexpression of *ABI3* does not lead to somatic embryogenesis (Parcy and Giraudat 1997; Parcy et al. 1994). Of the three examined *abi3* alleles, *abi3-9* had the mildest effect on BBM-induced SE (Fig. 8A). Interestingly, the *abi3-9* mutant was also found to be sensitive to ABA in the presence of glucose, in contrast to *abi3-8* and *abi3-10*, which were ABA-insensitive under these conditions (Nambara et al. 2002). In order to separate the effects of ABA-insensitivity and other embryo defects of *abi3* mutants on the BBM phenotype, we tested another ABA-insensitive mutant, *abi5-7*, which does not have any other reported embryo defects (Nambara et al. 2002). We also observed an enhanced BBM phenotype in the *abi5-7* mutant compared to wild-type (Fig. 8A). These data suggest that BBM-mediated SE is suppressed by ABA signalling and that the enhanced BBM response in the *abi3* mutants is due to ABA-insensitivity, rather than to other defects in the *abi3* mutants.

Finally, we tested whether transcriptional repressors of the *LAFL* genes, PKL and VAL proteins, have an effect on the BBM phenotype. We observed that *pkl-1* and *val1-2* (*hsi2-5*) mutants enhanced the efficiency of BBM-mediated SE, as measured by a higher percentage of embryogenic primary transformants (Fig. 8A). In the *val1-2;val2-1* double mutant, no significant change in SE-induction could be observed, which may be due to the lower number of transformants obtained in this mutant.

Together, the data show that members of the *LAFL* network, as well their upstream and downstream regulators are important components of the BBM signalling pathway during somatic embryo induction (Fig. 9).

DISCUSSION

AIL transcription factors play key roles throughout plant development, where they regulate processes such as meristem identity and maintenance, cell proliferation, organ size and organ development (Horstman et al. 2014). Functional redundancy among AILs has been demonstrated using loss-of-function mutants, but these shared functions have been difficult to reconcile with the range of phenotypes observed in AIL overexpression studies. Using the same overexpression and growth conditions, we have shown that AIL proteins have overlapping functions that are expressed in a dose-dependent manner. Our data suggest that the variety of overexpression phenotypes observed in different studies can be explained in part by differences in transgene expression levels.

Dose-dependent AIL function

We have shown that relatively high expression of the six BBM clade of AIL proteins induced somatic embryogenesis. By contrast, overexpression of the remaining two AIL proteins, ANT and AIL1, was not sufficient to induce SE. The *ANT* and *AIL1* genes comprise the basalANT lineage within the *AIL* family, while the remaining proteins belong to the euANT lineage (Kim et al. 2006). The expression pattern of *ANT* also differs from that of other *AIL* genes; *ANT* is expressed at the meristem periphery in the shoot apical and flower meristems, while the other *AIL*/*PLT* genes are expressed throughout these meristems (Mudunkothge and Krizek 2012; Prasad et al. 2011). This suggests that the two groups of AIL proteins regulate distinct processes.

We showed that a high BBM/PLT2 dose induces SE, a lower dose induces organogenesis and the lowest dose inhibits differentiation. Although we did not examine the dose-dependency of the other AILs, it is likely that they have similar dose-response phenotypes. It was suggested that PLT2 and, by extension, other AIL proteins act as morphogens, regulating root meristem size and maintenance in a dose-dependent manner through a protein concentration gradient, with a high AIL dose instructing stem cell fate, an intermediate AIL dose leading to cell division, and a low AIL dose causing differentiation (Galinha et al. 2007). Our results on seedling cotyledons and leaves also support a dose-dependent AIL output in these tissues, but suggest that a low AIL dose prevents differentiation rather than promoting differentiation. In analogy, a low AIL dose in the root might not actively instruct cellular differentiation, rather, it might simply be ineffective, thereby allowing cellular differentiation. We showed that a high AIL dose induces SE in cotyledons, but it is not known whether this proceeds through a stem cell pathway as instructed by a high AIL dose in the root. Likewise, it is not known whether higher AIL concentrations than are found in the stem cell niche are required for organogenesis and embryogenesis under normal growth conditions/*in planta*. Measurement of cellular AIL protein levels would help to relate the endogenous protein expression levels to those in overexpression lines.

It is currently unclear how different AIL concentrations instruct separate cellular outputs. The AIL dose-dependent phenotypes could result from different expression levels of the same target genes and/or from dose-dependent activation of specific target genes. A transcription factor gradient can regulate different sets of target genes through differences in binding site number and affinity (Rogers and Schier 2011). In this model, target genes with many or high-affinity binding sites are activated by low levels of the transcription factor, whereas genes with few or low-affinity binding sites are only activated at high transcription factor levels. For example, the transcription factor Bicoid regulates anterior-posterior axis patterning in *Drosophila* embryos through a protein gradient, and Bicoid target genes with high-affinity binding sites were expressed at lower Bicoid levels in contrast to

targets with low-affinity binding sites (Driever et al. 1989). Genome-wide AIL-DNA binding studies using different AIL dosages could reveal whether such high- and low-affinity AIL binding sites exist.

We observed some differences in the dose- and context- dependent overexpression phenotypes of BBM and PLT2. For example, intermediate doses of BBM and PLT2 mainly (though not exclusively) induce ectopic shoot and root formation, respectively. It is not clear how AIL specificity is determined. The *in vitro* DNA binding sites of ANT and PLT5/AIL5 appear to be very similar (Nole-Wilson and Krizek 2000; Yano et al. 2009), but they need to be better defined for each AIL protein. We have previously shown that multiple AILs interact with HDGs, however the individual AIL-HDG interactions differed (Horstman et al 2015). Defining the overlapping and unique target genes for each AIL transcription factor and the protein complexes in which they function may shed light on how specificity is achieved.

AILs trigger two distinct SE pathways

We observed that the developmental context in which AILs are expressed also affects the SE process. BBM and PLT2 can induce SE in two ways: either directly and quickly or indirectly and slowly. Direct SE was observed when BBM and PLT2 are activated before or during germination, and indirect SE when activated after germination. During direct SE, cells in the L1/L2 layers of the cotyledon divide, and somatic embryos develop from the cotyledon tips. The indirect SE pathway seems to take a different route: the upper layers become rough and irregular, the underlying tissue proliferates and somatic embryos are formed on the cotyledon blade. Previously, it was shown that organogenesis from aerial tissues starts from pericycle-like cells around the vasculature and proceeds via a lateral root pathway (Atta et al. 2009; Che et al. 2007; Sugimoto et al. 2010). Embryogenic callus can also be derived from pericycle-like cells (Sticklen 1991; Yang et al. 2010). Indirect BBM/PLT2-induced somatic embryogenesis does not appear to originate from vascular-derived callus, but rather from the ground tissue. However, future research should focus on whether this embryogenic callus originates from a similar lateral root pathway or a completely different developmental program.

In Arabidopsis, late zygotic embryo stages and dry seeds are the only stages that have been reported to undergo direct SE. All other tissues form callus and then somatic embryos, regardless of the inducing factor (2,4-D/transcription factor; discussed in Horstman 2015). Our results reinforce the existence of such a developmental window of competence for direct SE, and the idea that tissues outside this window require more extensive reprogramming, callus formation, before the embryo program can be initiated.

BBM-mediated SE requires LEC and FUS3 gene expression

Besides AILs, the LEC1/LEC2 and AGL15 transcription factors can also induce or enhance SE respectively, when overexpressed (Harding et al. 2003; Lotan et al. 1998; Stone et al. 2001; Zheng et al. 2009). *LEC* genes are important regulators of seed maturation and it was previously suggested that LEC2 overexpression might promote SE through dehydration stress resulting from the ectopic activation of seed maturation processes in vegetative tissues (Stone, 2008). Overexpression of *FUS3* does not induce SE, but does induce cotyledon identity in leaves: they have a rounder shape, lack trichomes and accumulate seed storage proteins (Gazzarrini et al. 2004). Loss of function of the LAFL repressors PKL and VAL also induces spontaneous SE (Ogas et al. 1999; Suzuki et al. 2007). We showed that BBM acts upstream of these embryogenesis regulators and that their expression is important for SE (Fig. 9). BBM overexpression in *lec1*, *lec2*, *fus3* and *agl15* mutants either eliminated or reduced SE. The importance of *FUS3* for BBM-mediated SE was unexpected as *FUS3* overexpression does not enhance or induce SE, however, the *fus3* mutant is also impaired in 2,4-D induced SE (Gaj et al. 2005). The reduction of BBM-mediated SE in the mutants could be explained in two ways: (1) the developmental defects in the mutants change the physiological state of the seed in such a way that it is no longer responsive for BBM-mediated SE, or (2) that BBM-induced SE relies on suitable transcriptional activation of these target genes, which is hampered in the mutants. Several lines of evidence support the latter scenario. First, we observed a reduced responsiveness to BBM in segregating *lec1* and *fus3* populations, which contain wild-type and heterozygous plants. However, the few embryogenic transformants in these populations were mainly wild-types, suggesting that the *lec1* and *fus3* mutations already affect BBM-induced SE in the heterozygote state. Heterozygous *lec1* and *fus3* mutants do not show reported growth defects, suggesting that reduced *LEC1* or *FUS3* expression in the heterozygous mutants, rather than a change in the physiological state of the tissue, reduces the response to BBM overexpression. Secondly, the *abi3* mutant shows similar maturation defects as the other *LAFL* mutants, yet we observed no negative effect of *abi3* mutations on the BBM overexpression phenotype. Therefore, we hypothesize that the lack of elevated expression of the *LAFL* genes reduces BBM-induced SE in the mutants. This hypothesis is further strengthened by our observations that mutations in LAFL repressors (PKL/VAL; Fig. 9) enhance BBM-mediated SE, probably by facilitating elevated *LAFL* gene expression. The enhanced BBM response in the *abi3* and *abi5* mutants is intriguing. Exogenous ABA application is reported to either inhibit or promote somatic embryo induction, depending on the experimental system (Rai et al. 2011). In Arabidopsis, the ABA-insensitive *abi3* and *abi5* mutants have a negative effect on 2,4-D induced direct SE from immature zygotic embryos, but so do ABA hypersensitive mutants (Gaj et al. 2006), making it difficult to assign a single role to ABA in this system.

It was previously shown that transcriptional feedback loops exist within the LAFL network between known regulators of SE (Fig. 9) (Jia et al. 2013; To et al. 2006). Recently, it was also shown that the *Phaseolus vulgaris* ABI3-like factor (Pv-ALF), which binds to the promoter of Arabidopsis *PLT5/AIL5 in vitro*, and that PLT5/AIL5 is required for activation of seed storage genes by Pv-ALF (Sundaram et al. 2013). In addition, FUS3 binds to the first exon/intron of *BBM in vivo*, although direct transcriptional regulation by FUS3 was not investigated (Wang and Perry 2013). Here, we uncovered another regulatory layer in which BBM stimulates the expression of *LAFL* genes during the induction of SE.

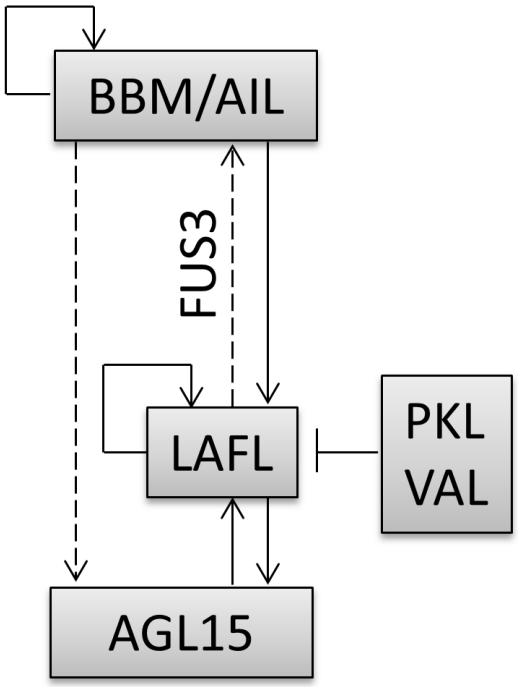


Figure 9: SE gene regulatory networks
Schematic representation of the genetic interactions between genes involved in SE. The solid lines indicate DNA binding plus transcriptional activation or repression, while the dashed lines indicate DNA binding in the absence of transcriptional regulation.

ACKNOWLEDGEMENTS

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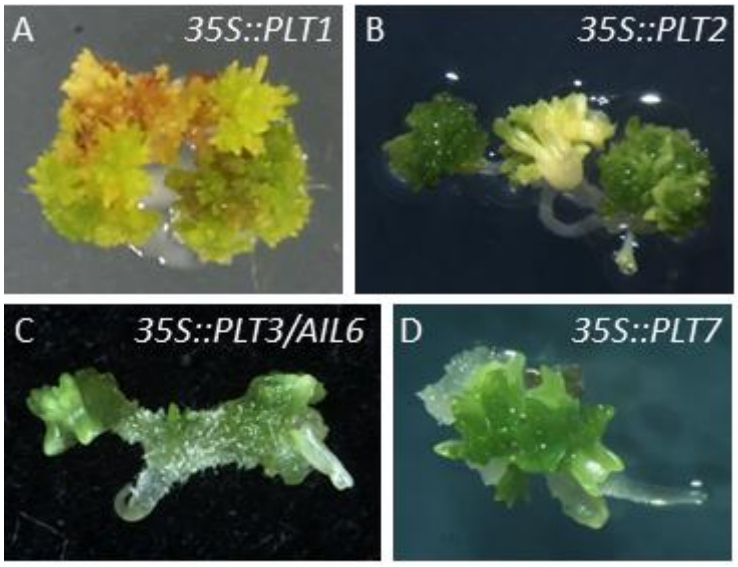
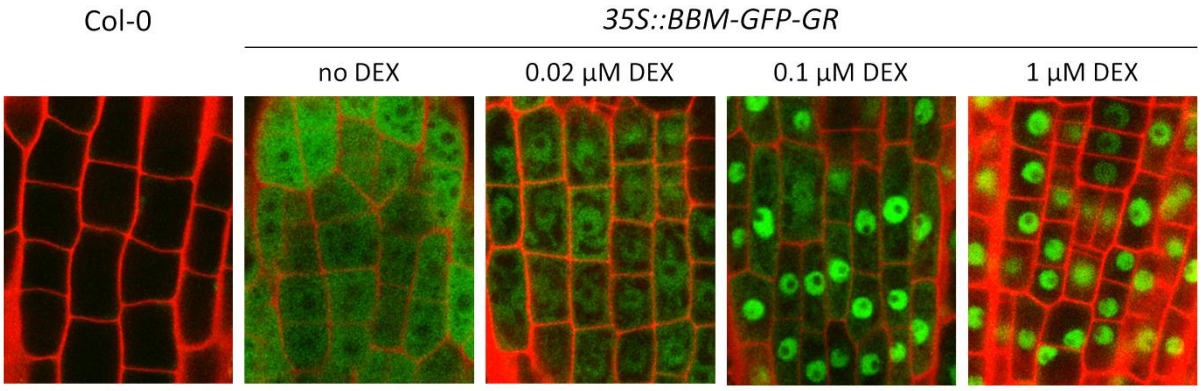
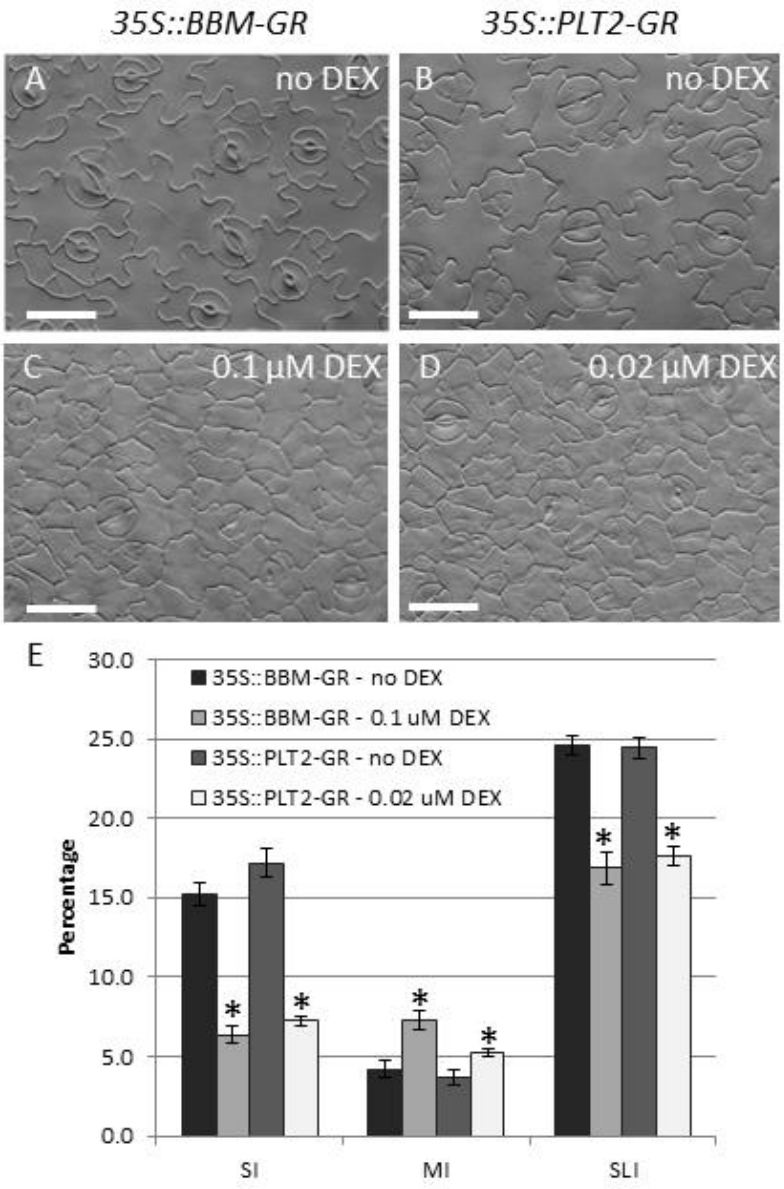
Supplemental material



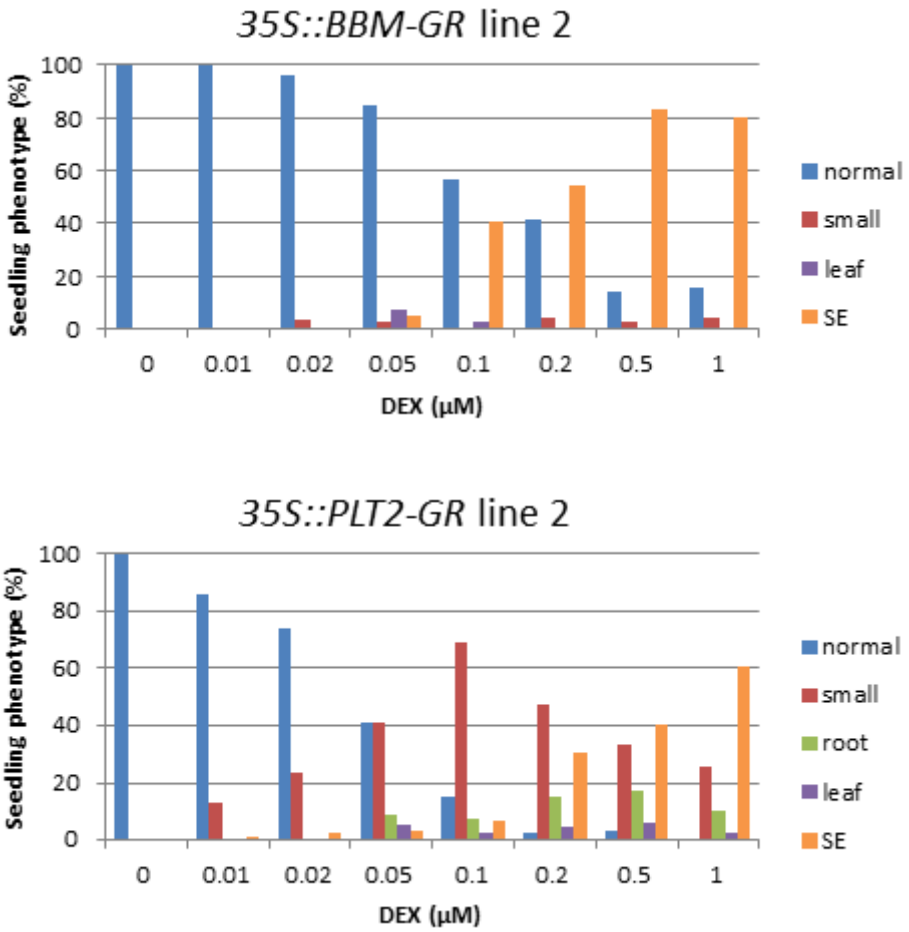
Supplemental Figure 1: Overexpression of AIL/PLT proteins induces somatic embryogenesis
Somatic embryo phenotypes of Arabidopsis primary transformants: *35S::PLT1* (**A**); *35S::PLT2* (**B**); *35S::PLT3/AIL6* (**C**); and *35S::PLT7* (**D**). Seedlings were grown on selection medium for 12 days (**C**), 3 weeks (**D**), 4 weeks (**B**) or 7 weeks (**A**).



Supplemental Figure. 2: BBM-GFP-GR nuclear localization increases with increasing dexamethasone concentration
The effect of dexamethasone (DEX) on BBM localization in roots of *35S::BBM-GFP-GR* seedlings grown for seven days in medium containing the indicated DEX concentration. Non-DEX treated (Col-0) roots are shown as a GFP-negative control. Green, GFP. Red, propidium iodide.



Supplemental Figure. 3: A low BBM/PLT2 dose inhibits differentiation of leaf epidermal cells
The abaxial sides of cleared first leaves of nine day-old *35S::BBM-GR* (**A, C**) and *35S::PLT2-GR* (**B, D**) seedlings grown on medium without DEX (**A, B**) or with 0.1 or 0.02 µM DEX (**C, D**). A relatively low BBM or PLT2 dose leads to the development of smaller and less-lobed leaf pavement cells compared to the control. Scale bars, 25 µm.
(**E**) Stomatal differentiation in DEX-treated *35S::BBM-GR* and *35S::PLT2-GR* seedlings is reduced compared to untreated seedlings. Fewer mature stomata were found in leaves of DEX-treated 35S::BBM-GR/PLT2-GR seedlings, as reflected by a lower stomatal index (SI), while the number of stomatal meristemoids was increased (meristemoid index, MI). The stomatal lineage index (SLI), reflecting the total number of stomata and stomatal precursors, was lower in DEX-treated 35S::BBM-GR/PLT2-GR leaves than in the control. For each index, eight images were analysed with total cell numbers between 125 and 350 per image. Error bars indicate standard errors. *, statistically significant difference compared to the control (p<0.05 in Student's t-test).



Supplemental Figure 4: BBM/PLT2 dose-dependent overexpression phenotypes in independent transgenic lines

Effect of DEX dose on a the development of additional, independent *35S::BBM-GR* and *35S::PLT2-GR* lines. The experimental conditions were the same as for the lines shown in Fig. 1. No additional phenotypes were observed in treatments above 1 µM DEX. *n*=200 to 350 seedlings. Leaf, ectopic leaves; root, ectopic root; SE, somatic embryogenesis.

Supplemental Table 1. Primers used in this study

Cloning		
ANT	FW	GGGGACAAGTTTGTACAAAAAAGCAGGCTCAATGAAGTCTTTTTGTGATAATG ATGA
	RV	GGGGACCACTTTGTACAAGAAAGCTGGGTATCAAGAATCAGCCCAAGCAG
AIL1	FW	GGGGACAAGTTTGTACAAAAAAGCAGGCTCAATGAAGAAATGGTTGGGATTT T
	RV	GGGGACCACTTTGTACAAGAAAGCTGGGTATTAGTGGCCGGCGC
PLT3/AIL6	FW	GGGGACAAGTTTGTACAAAAAAGCAGGCTCAATGATGGCTCCGATGACG
	RV	GGGGACCACTTTGTACAAGAAAGCTGGGTATTAGTAAGACTGATTAGGCCAG AGG
PLT7	FW	GGGGACAAGTTTGTACAAAAAAGCAGGCTCAATGGCTCCTCCAATGACG
	RV	GGGGACCACTTTGTACAAGAAAGCTGGGTATTAGTAAGACTGGTTAGGCCAC AA
PLT1	FW	GGGGACAAGTTTGTACAAAAAAGCAGGCTCAATGAATTCTAACAACTGGCTT GG
	RV	GGGGACCACTTTGTACAAGAAAGCTGGGTATTACTCATTCCACATAGTGAAAA CAC
PLT2	FW	GGGGACAAGTTTGTACAAAAAAGCAGGCTCAATGAATTCTAACAACTGGCTC G
	RV+stop	GGGGACCACTTTGTACAAGAAAGCTGGGTATTATTCATTCCACATCGTGAAAA C
	RV-stop	GGGGACCACTTTGTACAAGAAAGCTGGGTATTCATTCCACATCGTGAAAAC
BBM-GFP	FW	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGAACTCGATGAATAACTGG TT
	RV-stop	GGGGACCACTTTGTACAAGAAAGCTGGGTACTTGTACAGCTCGTCCATGC
Gene expression analysis		
SAND	FW	AACTCTATGCAGCATTTGATCCACT
	RV	TGATTGCATATCTTTATCGCCATC
LEC1	FW	ACAAGAACAATGGTATCGTGGTCC
	RV	GAGATTTTGGCGTGAGACGGTAA
LEC2	FW	ATCGCTCGCACTTCACAACAG
	RV	AACAAGGATTACCAACCAGAGAACC
FUS3	FW	TCTTCTTCCTTTAACCTTCTCTCTTTCC
	RV	ACCGTCCAAATCTTCCATTCTTATAGG
ABI3	FW	GGCAGGGATGGAAACCAGAAAAGA
	RV	GGCAAAACGATCCTTCCGAGGTTA
AGL15	FW	GAACGATTGCTGACTAACCAACTTG
	RV	GCAAAGTTGTGTCTGAATCGGTGTT

Supplemental Table 2. The efficiency of AIL-induced SE

Construct	No. of primary transformants	No. of transformants with SE	% SE
35S::ANT	89	0	-
35S::AIL1	228	0	-
35S::AIL6	171	45	26%
35S::AIL7	57	10	18%
35S::PLT1	136	9	7%
35S::PLT2	96	10	10%
35S::BBM	81	19	22%

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Chapter 6

Auxin biosynthesis and transport are required for BABY BOOM-mediated somatic embryogenesis

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ABSTRACT

Exogenous application of the plant growth regulator auxin is often used to induce somatic embryogenesis in plant tissue culture. Alternatively, somatic embryogenesis can be induced by overexpression of the BABY BOOM (BBM) AINTEGUMENTA-LIKE AP2/ERF domain transcription factor. Here we provide a link between the effect of exogenous auxin in Arabidopsis somatic embryo culture and BBM-induced somatic embryogenesis by showing that BBM binds to and regulates the expression of *YUCCA* (*YUC*) and *TRYPTOPHAN AMINOTRANSFERASE ARABIDOPSIS1* (*TAA1*) auxin biosynthesis genes. The changes in auxin biosynthesis were accompanied by an enhanced *DR5* auxin response along the margin of the cotyledon, followed by a loss of *DR5* expression just prior to and at the site of somatic embryo formation. Chemical inhibition of YUC and TAA1 activity abolished BBM-mediated somatic embryo formation, indicating a role for auxin biosynthesis in this process. BBM also bound and activated expression of the auxin efflux transport genes *PIN1, PIN4*, *MDR1/ABCB19/PGP19* and *NPY4*. Blocking polar auxin transport with NPA also inhibited visible somatic embryo formation, but only at a relatively high concentration. Our data show that auxin biosynthesis and polar auxin transport are essential steps in BBM-mediated somatic embryogenesis.

INTRODUCTION

Somatic embryogenesis is the process whereby embryos are formed from somatic cells, rather than from the fusion of an egg and a sperm cell. Somatic embryo formation occurs naturally in apomictic species via adventitious embryony (Mendes-Rodrigues et al. 2005), but is most commonly induced *in vitro* (Fehér 2006) to regenerate and/or clonally propagate plants (Litz and Gray 1995; Chugh et al. 2009). Somatic embryogenesis is usually obtained by exposing cells or tissues to abiotic stress (Fehér 2014) or exogenous growth regulators, in particular the synthetic auxin 2,4-dichlorophenoxy acetic acid (2,4-D; (Nolan and Rose 2010; Gaj 2004). In plant tissue culture, application of 2,4-D alone or in combination with other growth regulators activates cell division and induces callus formation, adventitious root formation or somatic embryogenesis, depending on the type of explant and the culture regime (Jiménez and Thomas 2006). The mechanism by which 2,4-D induces somatic embryogenesis is not known, but it has been suggested that 2,4-D induces a stress response and changes in chromatin modifications (Fehér 2014).

In the model plant Arabidopsis (*Arabidopsis thaliana*), somatic embryos are most efficiently induced after exposure to 2,4-D and emerge either directly from the explant (Luo and Koop 1997; Gaj 2001; Kobayashi et al. 2010) or indirectly from embryogenic callus (Ikeda-Iwai et al. 2003). In the direct system, fully differentiated embryos with root and shoot meristems and cotyledons form in the presence of 2,4-D, while in the indirect system removal of 2,4-D from the culture medium is usually required to promote differentiation (patterning) of pro-embryogenic masses (PEMs), which are multicellular embryos lacking radial and apical-basal patterning (Von Recklinghausen et al. 2000; She et al. 2013; Mordhorst et al. 1998; Gaj 2011; Su et al. 2009).

Auxin is synthesised in plants by a number of different pathways (Normanly et al. 2010; Zhao 2014). The major auxin In Arabidopsis is indole-3-acetic acid (IAA), which is mainly synthesized via the tryptophan (TRP)-dependent pathway (Woodward and Bartel 2005). Enzymatic activity of TRYPTOPHAN AMINOTRANSFERASE ARABIDOPSIS1 (TAA1) and TAA1 RELATED PROTEINS (TAR) convert TRP into the intermediate product indole-3-puryvic acid (IPyA), from which indole-3-acetic acid (IAA) is synthesized via YUCCA flavin-dependent mono-oxygenases (Stepanova et al. 2011). Arabidopsis has eleven YUCCA monooxygenases (YUC1-YUC11) (Cheng et al. 2006), which are differentially expressed throughout plant development (Robert et al. 2013; Cheng et al. 2007; Hentrich et al. 2013; Wang et al. 2011; Cheng et al. 2006). Arabidopsis YUCs function in a redundant manner, with the result that their functions only become evident in higher order mutant combinations (Cheng et al. 2007, 2006; Wang et al. 2011; Robert et al. 2013).

Auxin biosynthesis genes are expressed as early as the 16-cell embryo stage during zygotic embryo development in both the embryo proper and suspensor. TAA1 is expressed in the apical-most cells of the embryo proper and *YUC3, YUC4* and *YUC9* in the basal suspensor (Robert et al.

2013; Stepanova et al. 2008). At the globular stage apical *TAA1* expression (Stepanova et al. 2008) overlaps with the broader apical expression of *YUC1, YUC4, YUC10* and *YUC11* (Cheng et al. 2007). Later *YUC4* and *TAA1* are also expressed at the basal pole (Stepanova et al. 2008; Robert et al. 2013). Loss of auxin biosynthesis gene expression at the apical pole is associated with basal embryo defects and vice versa (Robert et al. 2013; Cheng et al. 2007; Stepanova et al. 2008; Wabnik et al. 2013). Additional mutant analysis and mathematical modelling suggest that local auxin biosynthesis at one embryo pole is required to establish a new auxin response maximum and promote patterning at the opposite pole (Robert et al. 2013; Wabnik et al. 2013).

Cells or tissues undergoing 2,4-D-induced somatic embryo formation often show elevated levels of endogenous auxin, mainly IAA (Charriere et al. 1999; Pasternak et al. 2002; Michalczuk et al. 1992). In the Arabidopsis direct somatic embryogenesis system, exposure of immature zygotic embryos to 2,4-D induces expression of *TAA1, YUC1, YUC4* and *YUC10* (Wójcikowska et al. 2013). Single *YUC* mutants have no obvious phenotype under normal growth conditions, but in 2,4-D-induced somatic embryo cultures *yuc2* and *yuc4* mutants have fewer responding explants and produce fewer somatic embryos per explant (Wójcikowska et al. 2013). By contrast, in the indirect somatic embryogenesis system *YUC* gene expression *(YUC1, YUC2, YUC4,* and *YUC6)* is detected late in the development of embryogenic callus and then increases significantly after transfer of the callus to 2,4-D-free medium (Bai et al. 2013). The quadruple *yuc1;yuc2;yuc4;yuc6* mutant shows a normal progression of somatic embryogenesis, while the *yuc1;yuc4;yuc10;yuc11* mutant produces only a few malformed somatic embryos (Bai et al. 2013). Endogenous auxin biosynthesis therefore plays a significant role in somatic embryo induction even in the presence of exogenous auxin.

Differential auxin transport throughout the plant creates auxin gradients, characterized by auxin maxima and minima, which guide meristem and organ formation (Wang et al. 2014; Cucinotta et al. 2014). Auxin transport is regulated in part by the PIN family of efflux transporters (Adamowski and Friml 2015). While the importance of auxin transport during zygotic embryo formation (Vieten et al. 2005; Weijers et al. 2005; Steinmann et al. 1999) and plant development (Adamowski and Friml 2015) has been well documented, the role of auxin transport during somatic embryo formation is not fully understood. In Arabidopsis 2,4-D-treated indirect somatic embryo cultures, PIN1-GFP is initially expressed in an apolar fashion in cells on the surface of the embryogenic calli (Su et al. 2009), but becomes polarized a few days after 2,4-D removal. Inhibition of auxin efflux transport by 1-naphthylphthalamic acid (NPA) disrupts PIN1 localisation at the apical region of embryogenic calli and inhibits somatic embryo formation, although it was not shown whether somatic embryo induction or only embryo differentiation was inhibited (Su et al. 2009). In Norway spruce (*Picea abies*), callus and PEMs are induced in the presence of both auxin and cytokinin and differentiate into somatic embryos after the depletion of both plant growth regulators from the medium (Larsson et al.

2008). Application of NPA to callus and PEMs does not affect somatic embryo induction, but rather increases IAA content and twin-embryo formation. NPA treatment at later stages e.g. during embryo differentiation or maturation induces patterning defects in a concentration-dependent manner (Larsson et al. 2008; Hakman et al. 2009), as with NPA-treated zygotic embryos (Hadfi et al. 1998; Liu et al. 1993).

Overexpression of specific transcription factors can also induce somatic embryo formation on seedlings in the absence of exogenous hormones, for example, the LEAFY COTYLEDON 1 (LEC1) HAP3/CCAAT binding protein, and the LEC2 B3-domain protein (Gaj et al. 2005; Lotan et al. 1998; Stone et al. 2001). LEC1 and LEC2 have dual roles in maintaining embryo identity and promoting maturation during zygotic embryo development (Meinke et al. 1994). How LEC1 and LEC2 overexpression induces somatic embryos is not known, but a role has been shown for LEC1 and LEC2 in auxin biosynthesis, through the activation of *YUC* gene expression (Stone et al. 2008; Junker et al. 2012) and auxin signalling, through the activation of *INDOLE-3-ACETIC ACID INDUCIBLE (IAA)* gene expression (Stone et al. 2008; Braybrook et al. 2006). During direct Arabidopsis somatic embryogenesis, overexpression of *LEC2* can compensate for a suboptimal dosage of 2,4-D or for ineffective auxins, such as indole-3-acetic acid (IAA) or 1-Naphthaleneacetic acid (NAA) (Wójcikowska et al. 2013). Conversely, ectopic expression of *LEC2* in the presence of an optimal concentration of 2,4-D negatively affects somatic embryo formation, as it delays and reduces embryo induction and induces callus and shoot-like structures (Ledwon and Gaj 2009). The *lec1* and *lec2* loss-of-function mutants show a severe reduction of the number of responding explants in the presence of 2,4-D, as well as a shift from direct to indirect somatic embryogenesis (Gaj et al. 2005). These results imply that a specific auxin balance is required to induce somatic embryogenesis.

Another transcription factor that induces somatic embryogenesis in the absence of exogenous hormones is BABY BOOM (BBM), a member of the AINTEGUMENTA-LIKE (AIL) clade of APETALA2/ETHYLENE RESPONSE FACTOR (AP2/ERF) transcription factors (Boutilier et al. 2002; Passarinho et al. 2008). BBM and PLETHORA2 (PLT2) are redundantly required to maintain embryo identity in Arabidopsis, as embryo development arrests after the few-celled stage in the *bbm;plt2* double mutant. BBM also has a redundant role with other PLT proteins in root meristem maintenance. These phenotypes are in line with the general role of AILs in maintenance of meristem identity and control of organ development (Horstman et al. 2014). Genetic analysis has shown that *AIL* genes function both upstream and downstream of auxin signalling (Horstman et al. 2014). To date, only AIL5/PLT5/EMBRYOMAKER (EMK)/CHOTTO1 (CHO1) is known to directly regulate auxin-biosynthesis by binding to and transcriptionally regulating *YUC4* (Pinon et al 2013).

We have shown previously that BBM directly binds to and activates expression of the *LAFL* embryo identity and maturation genes, which include *LEC1* and *LEC1-LIKE (L1L)* and three B3-domain

transcription factor genes *ABSCISIC ACID 3* (*ABI3*), *FUSCA3* (*FUS3*) and *LEC2* (Jia et al. 2014), Chapter 5)). While *LEC1, LEC2,* and *FUS3* are required for BBM-mediated somatic embryogenesis, ABI3 negatively regulate this process (Chapter 5). Here we show that in addition to directly regulating *LAFL* gene expression, BBM also binds to and activates the expression of auxin biosynthesis and transport-related genes. Using pharmacological inhibition of auxin biosynthesis and efflux pathways in combination with an auxin response marker and phenotypical observations, we show that both auxin biosynthesis and transport are required for BBM-mediated somatic embryogenesis.

MATERIALS AND METHODS

Plant material and growth conditions

The *DR5::GFP (Ruzicka et al. 2007)*, *DR5::GUS* (Benkova et al. 2003), *PIN1::GUS, PIN1::PIN1-GFP* (Benkova et al. 2003), *PIN4:GU*S (Vieten et al. 2005) and *BBM::GUS* reporter lines, the *QC46 GUS* promoter trap line (Sabatini et al. 1999) and the *35S::BBM-GR* overexpression line (Passarinho et al. 2008) were previously described. All reporter lines were crossed with a homozygous *35S::BBM-GR* line. The progeny were selected over four generations until homozygous lines with at least 90% somatic embryo formation and 100% reporter gene expression were obtained.

Seeds for *in vitro* culture were surface sterilized with liquid bleach. Sterilized seeds were dispensed in 190 ml containers (Greiner) with 30 ml liquid ½MS medium (half-strength Murashige and Skoog salts and vitamins (Murashige and Skoog 1962), pH 5.8) with 1% sucrose and germinated at 23˚C on a rotary shaker (80 rpm/min) under a 16 hour light/8 hour dark regime.

Dexamethasone (DEX) and cycloheximide (CHX) (Sigma, dissolved in 70% ethanol), kynurenine (kyn) and yucasin (yuc) (Sigma, dissolved in DMSO), and 1-naphthylphthalamic acid (NPA) (Duchefa, dissolved in 0.1N KOH were added to the medium according to the experimental design.

Molecular analyses

Chromatin immunoprecipitation of BBM-bound DNA complexes was performed using a GFP antibody on 2,4-D-induced (*BBM::BBM-YFP*) and *35S::BBM-GFP*-induced somatic embryo tissue, followed by high throughput sequencing (ChIP-seq, (Horstman 2015)).

The effect of BBM overexpression on BBM target gene expression was examined by quantitative RT-PCR (qPCR) after treating one-day-old Col-0 and *35S::BBM-GR* seedlings for three hours with 10 µM DEX and 10 µM CHX. RNA was isolated using the RNA Nuclear Spin kit (Machery & Nagel) with the addition of 25 µl RNA Aid (Ambion/Life Technologies) and 175 µl RA1 buffer, followed by an additional DNAse treatment (Ambion/Life Technologies). cDNA was synthesised using M-MLVRT (Invitrogen/Life Technologies) following the manufacturer's instructions. DNA primers were designed using the QuantPrime software (Arvidsson et al. 2008). qPCR was performed using the BioMark HD

System (Fluidigm), as described in Chapter 5. The data were normalized against the *SAND* gene (Czechowski et al. 2002) and relative gene expression was calculated according to Livak and Schmittigen (Livak and Schmittgen 2001) by comparing DEX+CHX-treated *35S::BBM-GR* samples with DEX+CHX-treated Col-0 samples. Three biological replicates and eight technical replicates were performed for each treatment.

IAA measurements

Wild-type seeds and seeds from two independent *35S::BBM-GR* lines (two replicates per line) were grown for 24 hours in liquid ½MS medium with 1% sucrose and then grown for an additional three days in the same medium in the presence or absence of 10 µM DEX. IAA extraction and measurements were performed as described in Ruyter-Spira et al. (Ruyter-Spira et al. 2011) using ca. 100-250 mg fresh weight per sample.

Microscopy

Whole seedlings were stained for GUS activity for six to eight hours using standard buffer conditions (Jefferson 1987) and 2.5 mM iron. Seedlings were mounted and cleared in Hoyers' solution (Meinke 1994) after chlorophyll removal with 70% ethanol. Starch granules were visualised by staining roots for five minutes in Lugol's solution (Sigma) and mounted in Hoyers' solution after a brief wash in culture medium.

Light images of whole seedlings were taken with a DP70 camera (Olympus) mounted on a SZX16 binocular. GUS- and Lugol-stained samples were imaged with the same camera, but mounted on an Olympus IX70 microscope. All images were processed with CellSens software (Olympus).

Confocal laser scanning microscopy for GFP analysis was performed as described in Chapter 4. Hand sections were performed with a single-edged razor blade by cutting though propidium iodine (PI)- or FM4-64- (Life Technologies) counterstained seedlings.

RESULTS

BBM binds and transcriptionally regulates auxin biosynthesis and transport genes

We identified BBM DNA binding sites in somatic embryo tissue by ChIP-seq. Two complementary constructs were used for the analysis, a native *BBM* promoter driving the expression of a *BBM-YFP* fusion (*BBM::BBM-YFP*) in 2,4-D-induced somatic embryo cultures, and a *BBM-GFP* fusion under the control of a *35S* promoter (*35S::BBM-GFP*), which also induces somatic embryo formation. BBM-bound DNA was immunoprecipitated from somatic embryos derived from both *BBM-YFP* lines using a GFP antibody (Horstman et al. 2015) (Chapter 5). This allowed us to identify an overlapping set of

BBM target genes in somatic embryos. Here we focus on BBM-bound genes with roles in auxin biosynthesis and auxin transport (Fig. 1).

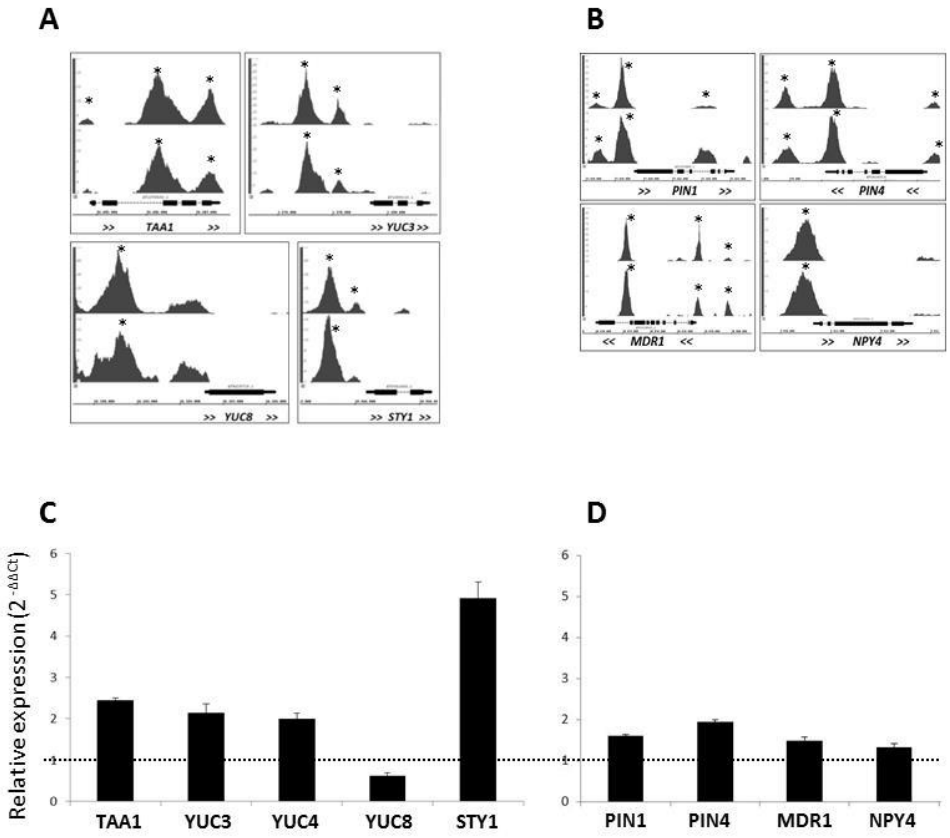


Figure 1: BBM binds and regulates the expression of auxin-related genes
A, B, ChIP-seq BBM binding profiles for auxin-related genes in somatic embryo tissue. The binding profiles from the *35S::BBM-GFP* (upper profile) and *BBM::BBM-YFP* (lower profile) ChIP-seq experiments are shown. The x-axis shows the nucleotide position of DNA binding in the selected genes (TAIR 10 annotation), the y-axis shows the ChIP-seq score, and the arrowheads indicate the direction of gene transcription. Peaks with scores above 1.76 (for *35S::BBM-GFP*) and 3.96 (for *pBBM::BBM-YFP*) were considered statistically significant (* = FDR <0.05).
C, D, The relative expression of auxin-related genes was determined by qPCR for dexamethasone and cycloheximide (DEX+CHX)-treated *35S::BBM-GR* seedlings using DEX+CHX treated Col-0 as the calibrator and the *SAND* gene (Czechowski et al., 2005) as the reference. Error bars indicate standard errors of the three biological replicates of the same genetic background. The dotted line indicates no difference in gene expression between *35S::BBM-GR* and Col-0 seedlings. All genes showed significantly different expression levels (Student's *t*-test p<0.05 (*)) in DEX+CHX-treated *35S::BBM-GR* plants compared to the DEX+CHX-treated Col-0 control.
A, C show data for auxin biosynthesis genes
B, D show data for auxin transport-related genes.

BBM bound to *TRYPTOPHAN AMINOTRANSFERASE ARABIDOPSIS 1 (TAA1)* and two *YUCCA (YUC)* flavin monooxygenase genes, *YUC3* and *YUC8*, both of which are involved in the TRP-dependent pathway for IAA biosynthesis ((Mashiguchi et al. 2011; Stepanova et al. 2008), Supplemental Fig. 1). BBM also bound to the *STYLISH1* (*STY1*) gene, which encodes a RING-like zinc finger transcription factor. STY1 activates auxin biosynthesis via *YUC4* (Eklund et al. 2010) and down-regulates gibberellic acid biosynthesis (Fridborg et al. 1999). Significant BBM binding peaks were identified in the

promoter regions of *YUC3, YUC8* and *STY1*, including positions close to the transcriptional start site, and also in the second intron and last exon/3' UTR of *TAA1*. Significant ChIP-seq peaks were also observed for the *PIN1, PIN4, MDR1,* and *NPY4* genes, which are all involved in auxin transport. Although the value of the broad peaks in YUC8 and PIN1 are above the threshold for significance they are not recognized as such by the CSAR script software (Muiño et al. 2011) probably due to their shape. PIN1 and PIN4 are auxin efflux carriers with overlapping functions throughout plant development, including early zygotic embryo development (Vieten et al. 2005). *MULTIDRUG RESISTANT1 (MDR1)/ABCB19/PGP19* encodes an ABCB auxin efflux carrier that mediates gravitropic response (Titapiwatanakun et al. 2009) and auxin efflux into expanding cotyledons (Lewis et al. 2009). The interaction between MDR1 with PIN1 is thought to stabilize PIN1 in micro-domains at the plasma membrane, thereby enhancing the efflux transport activity in the basal direction (Titapiwatanakun et al. 2009). *NPY4* (*MEL4, MACCHI-BOU/ENHANCER OF PINOID/NAKED PINS IN YUC MUTANTS-LIKE)* and its homologue *NPH3*, act together with PINs and AGC-kinases to control auxin transport in response to light (Wan et al. 2012; Galván-Ampudia and Offringa 2007). *PIN1, PIN4, MDR1,* and *NPY4* had significant ChIP-seq peaks in their promoter regions, while *PIN4* had an additional peak at the 3' untranslated region, and *MDR1* in the last intron (Fig. 1 A and B).

Our BBM-binding profiles were obtained from developing somatic embryos, therefore we used qPCR to determine whether BBM alters the expression of these auxin biosynthesis and transport genes during the initial phase of somatic embryo induction in seedlings. We treated imbibed *35S::BBM-GR* seeds for three hours with dexamethasone (DEX) in the presence of the translational inhibitor cycloheximide (CHX). DEX application up to one day after germination induces direct, highly penetrant, and prolific somatic embryogenesis from the cotyledon margin and shoot apical meristem (Chapter 4). Under these conditions BBM induced *TAA1, YUC3*, and *STY1* gene expression and down-regulated *YUC8* expression (Fig. 1C). *YUC4* expression was also significantly up-regulated although the BBM binding peak in this gene was just under the significance cut-off (Fig. 1C). In addition to the auxin biosynthesis genes, BBM induced significantly enhanced expression of the *PIN1, PIN4*, *MDR1,* and *NPH4* genes that are involved in auxin transport, although with the exception of *PIN4* the changes in gene expression were minimal (Fig. 1D).

Overexpression of BBM induces an auxin response

We used the synthetic auxin reporter *DR5* to follow the timing and spatial localization of auxin response during BBM-mediated somatic embryogenesis. DEX was applied to *35S::BBM-GR* seeds directly after imbibition (day 0, D0). We observed previously that early BBM activation (D0-D1) results in fast and direct somatic embryo formation at tip of the cotyledon after six to seven days of culture, and in the following days additional somatic embryos also form at the margin and the SAM,

and secondary somatic embryos develop from the primary somatic embryos at the cotyledon tip (Chapter 5).

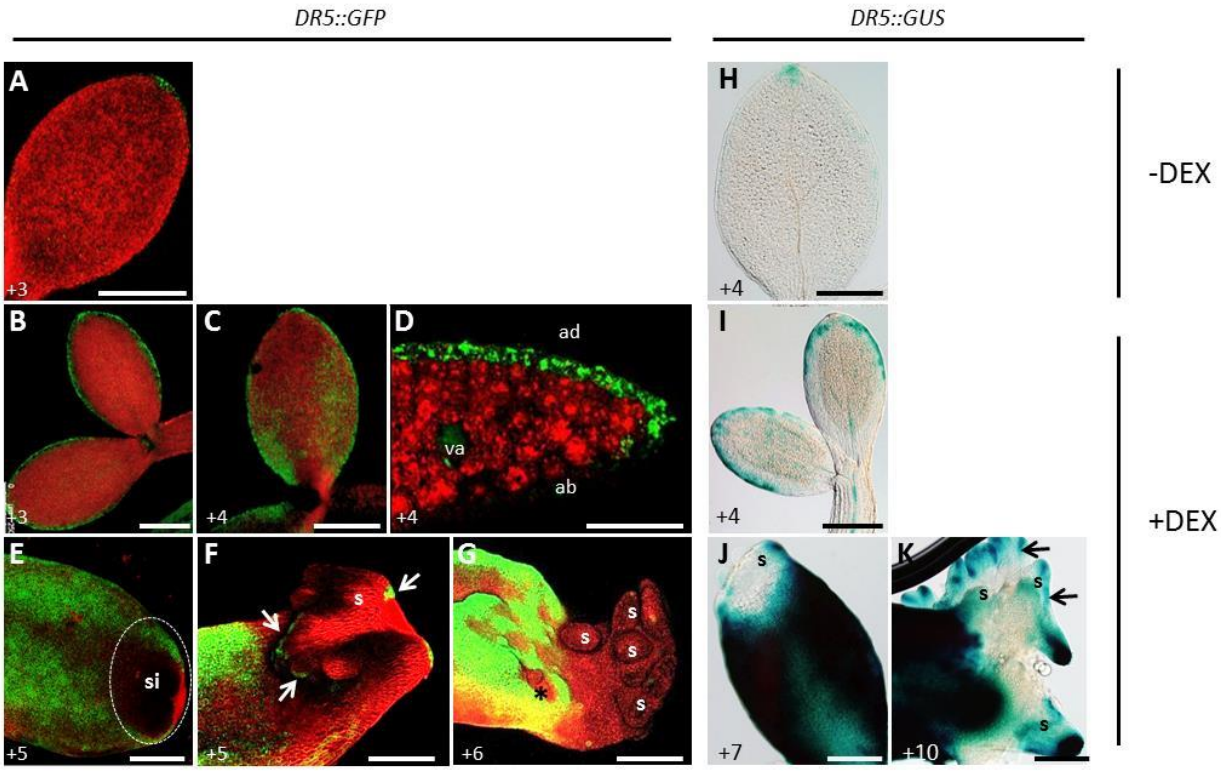


Figure 2: BBM overexpression enhances *DR5* auxin response
Seeds were imbibed and then grown with (+DEX) or without (-DEX) dexamethasone for the number of days indicated in each image.
A-G, confocal images of *DR5::GFP* expression (green). Samples were counter stained with FM4-64 (red);
H-K, *DR5::GUS* expression in cleared cotyledons;
A, *DR5::GFP* expression in cotyledons in the absence of DEX*;*
B-G, *DR5::GFP* expression in cotyledons of DEX-treated seedlings;
A-C, adaxial side of cotyledons;
D, transverse section through a cotyledon; ad, adaxial; ab, abaxial; va, vascular tissue
E-G, *DR5::GFP* expression at the adaxial side of cotyledon during somatic embryo initiation (si) and formation (s). White arrows, GFP in cotyledon tips and root poles of a somatic embryo; Asterisk, growth protrusion; Circle, region with a *DR5* minimum and early somatic embryo formation;
H, *DR5::GUS* expression in a cotyledon in the absence of DEX;
I-K, *DR5::GUS* expression in cotyledons of DEX-treated seedlings. Black arrows indicate somatic embryos.
Note: Single somatic embryos develop faster (**F**) than embryos formed in a cluster (**G**).
Scale bars, 250 µm.

Non-DEX-treated *35S::BBM-GR* seedlings showed *DR5::GFP* or *GUS* expression at the tip of the cotyledon (Fig. 2A, H). Enhanced *DR5* expression was observed after 24 hours of DEX treatment along the adaxial surface of the cotyledon margin (Fig. 2B), where it remained for the first three to four days after DEX treatment (Fig. 2I). Thereafter, *DR5* expression spread quickly to cover most of the adaxial cotyledon surface, except for the centre of the cotyledon (Fig. 2C). Hand-sections through the cotyledon showed that *DR5::GFP* expression was localized to the adaxial epidermis and vascular

bundles (Fig. 2D). After five to six days of DEX treatment *DR5* expression disappeared at the cotyledon tip, the region where the first somatic embryos usually appear (Fig. 2E-G, J). *DR5* was also expressed in broad growth protrusions that formed next to the central vein, but somatic embryos never formed from these structures. Small protrusions lacking *DR5* expression were observed within these broad areas of *DR5* expression (Fig. 2G). The flanks of the cotyledon did not show *DR5* expression, but it was not clear if this area was part of the abaxial surface, which has a much lower *DR5* expression, that became visible due to the strong swelling of the cotyledon (Fig. 2G). *DR5* expression was not observed in globular-shaped somatic embryos (Fig. 2G), but could be seen in the cotyledon and root poles of differentiated embryos (Fig. 2F). These data suggest that BBM induces a broad auxin response along the margin and at the surface of cotyledon, which is followed by a local auxin minimum prior to somatic embryo formation.

We determined whether increased expression of auxin biosynthesis genes contributed to the enhanced *DR5* response by measuring IAA levels in four-day-old wild-type seedlings and four-day-old seedlings from two independent *35S::BBM:GR* lines treated with or without DEX. Approximately 50% and 100% of the seedlings formed somatic embryos in lines 1 and 2, respectively. Seedlings of both *35S::BBM-GR* lines treated with DEX showed increased IAA levels compared to the control wild-type and *35S::BBM-GR* seedlings, but only line 2 had a significantly higher IAA level than the controls (Fig. 3). The natural IAA catabolite oxindole-3-acetic acid (oxIAA) was also increased in response to BBM overexpression (Fig. 3). Oxidation of IAA to the oxIAA, which has no or little auxin activity, plays an important role in maintaining auxin homeostasis (Pěnčík et al. 2013).

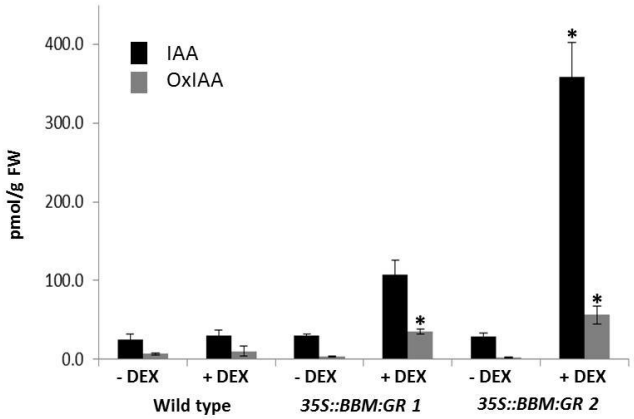


Figure 3: BBM overexpression induces auxin biosynthesis
IAA and IAAox concentrations in seedlings of wild type (Col-0) and two *35S::BBM-GR* lines grown in the absence (-DEX) or presence (+DEX) of dexamethasone. Asterisk, *35S::BBM-GR* samples that showed significantly different IAA or oxIAA concentrations compared to the non-DEX-treated *35S::BBM-GR* control. Student's *t*-test, *p*-value <0.05).

Our data suggest that the broad *DR5* auxin response along the margin and at the surface of cotyledon observed in *35S::BBM-GR* seedlings is induced in part by enhanced auxin biosynthesis gene expression.

Overexpression of BBM partially rescues auxin biosynthesis defects in roots

Our ChIP-seq data suggested that BBM binds to and regulates expression of the *TAA1* and *YUC* auxin biosynthesis genes. We examined the role of auxin biosynthesis in BBM-mediated somatic embryogenesis using chemical inhibitors of TAA1/TAR and YUC enzyme activity. The TRP analogue kynurenine (kyn) inhibits the activity of TAA1 and TAR (He et al. 2011), while the putative methimazole analogue yucasin (yuc) inhibits the enzymatic activity of flavin-containing monooxygenases (Supplemental Fig. 1) (Nishimura et al. 2014).

To obtain initial insight into the effect of auxin biosynthesis inhibitors on BBM function, we examined the effect of kyn and yuc on root development. Previously, it was shown that kyn-treatment destroys the root meristem of wild type Arabidopsis seedlings, and that this phenotype can be rescued by applying IAA (He et al. 2011). yuc-treatment rescues the auxin overproduction phenotypes induced by *YUC1* overexpression, disrupts root development by reducing the length of the root meristem, and inhibits columella cell differentiation (Nishimura et al. 2014). *35S::BBM-GR* seedlings were grown for four or seven days with or without the inhibitors in the absence or presence of DEX. Root meristem development was evaluated by monitoring *DR5* expression, expression of the *QC46 GUS* quiescent cell (QC) marker (Sabatini et al. 1999), and starch accumulation in differentiated columella cells. Roots of *35S::BBM-GR* seedlings grown in the absence of both DEX and auxin biosynthesis inhibitors developed normally and showed *QC46* expression in the QC (Sabatini et al., 1999; Fig. 4A), starch accumulation in the four layers of differentiated columella cells (Fig. 4B), and *DR5* expression in the QC and columella cells (Fig. 4C). DEX treatment of *35S::BBM-GR* seedlings did not affect root meristem structure during the four day period (Fig. 4C). Neither the *QC46* expression pattern nor starch accumulation was altered compared to the non-DEX-treated control, while the number of *DR5*-expressing columella cells was more variable than in in the control seedlings (Fig. 4A-C). A seven day treatment with 25 µM kyn or a four-day treatment with 100 µM kyn completely destroyed the root meristem (Fig. 4A-C). *DR5* and *QC46* expression and starch accumulation were reduced or eliminated, accordingly (Fig. 4A–C). By contrast, roots of *35S::BBM-GR* seedlings treated with both DEX and kyn did not degenerate, and showed a variable recovery in *DR5/QC46* expression and starch accumulation (Fig. 4A-C). Similar results were obtained after yuc treatment, where BBM overexpression rescued the yuc-mediated reduction in *QC46* expression and columella starch staining. *DR5* expression was not altered in response to BBM overexpression (Fig. 4C). Thus BBM overexpression is able to partially rescue the effects of auxin biosynthesis inhibitors on root meristem marker expression.

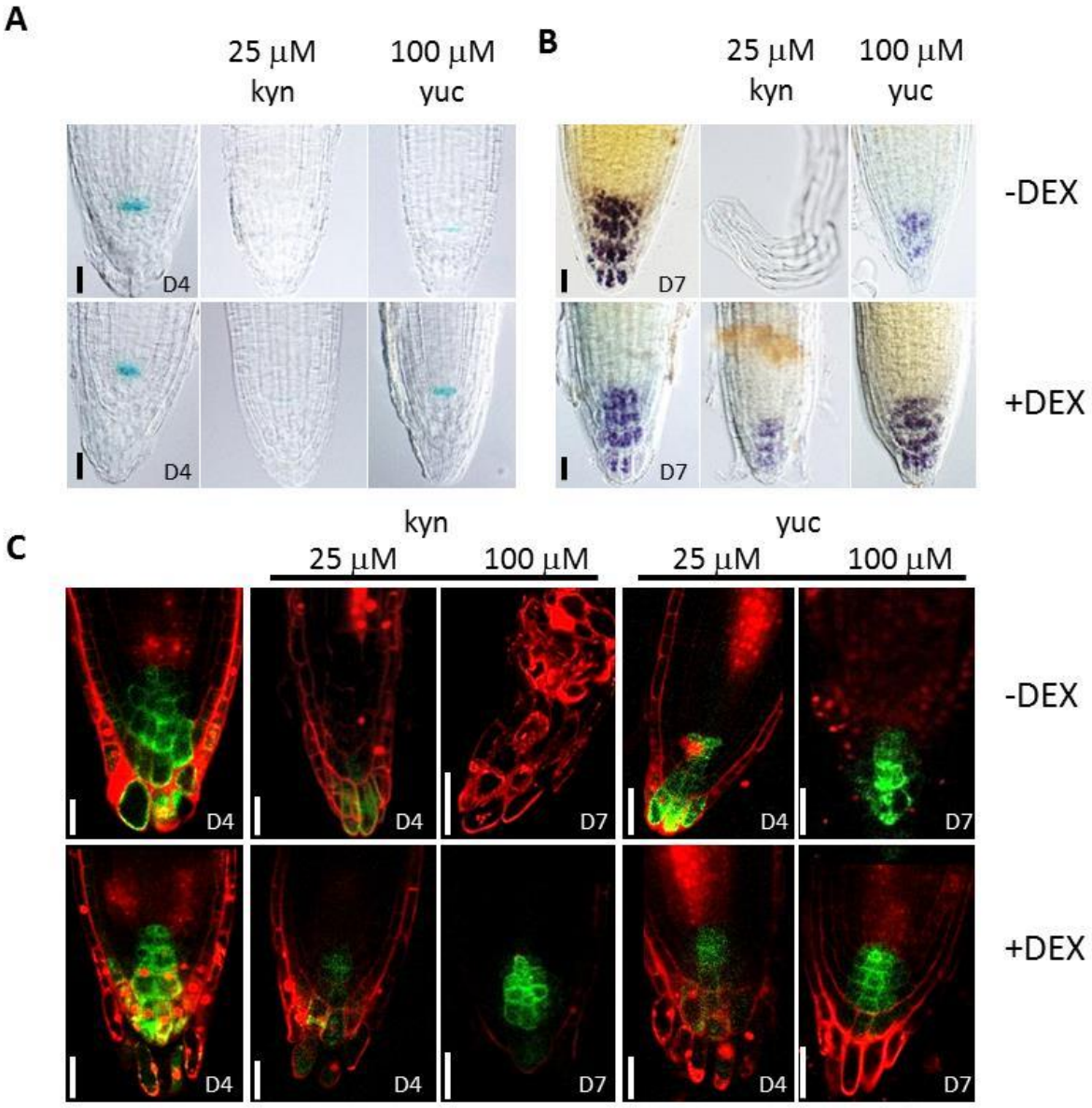


Figure 4: Effect of auxin biosynthesis inhibitors on BBM overexpression in roots
35S::BBM-GR seeds were grown in the absence (-DEX) or presence (+DEX) of dexamethasone, with or without the auxin biosynthesis inhibitors kynurenine (kyn) or yucasin (yuc) for the indicated amount of time. Root development was assessed by QC46-driven GUS expression (**A**), Lugol staining of differentiated columella cells (**B**) and *DR5::GFP* expression (**C**).
A, expression of *QC46* in four day old seedlings in the presence of kyn or yuc;
B, Lugol staining of root tips of seven day old seedlings in the presence of kyn or yuc;
C, *DR5::GFP* expression in root tips of four-day-old seedlings (D4) after treatment with kyn or yuc, and seven-day-old seedlings (D7) after treatment with kyn or yuc.
DR5::GFP expression (green) was observed after counter staining with propidium iodide (red). Scale bars: **A** and **B**, 100 µm; **C** , 250 µm.

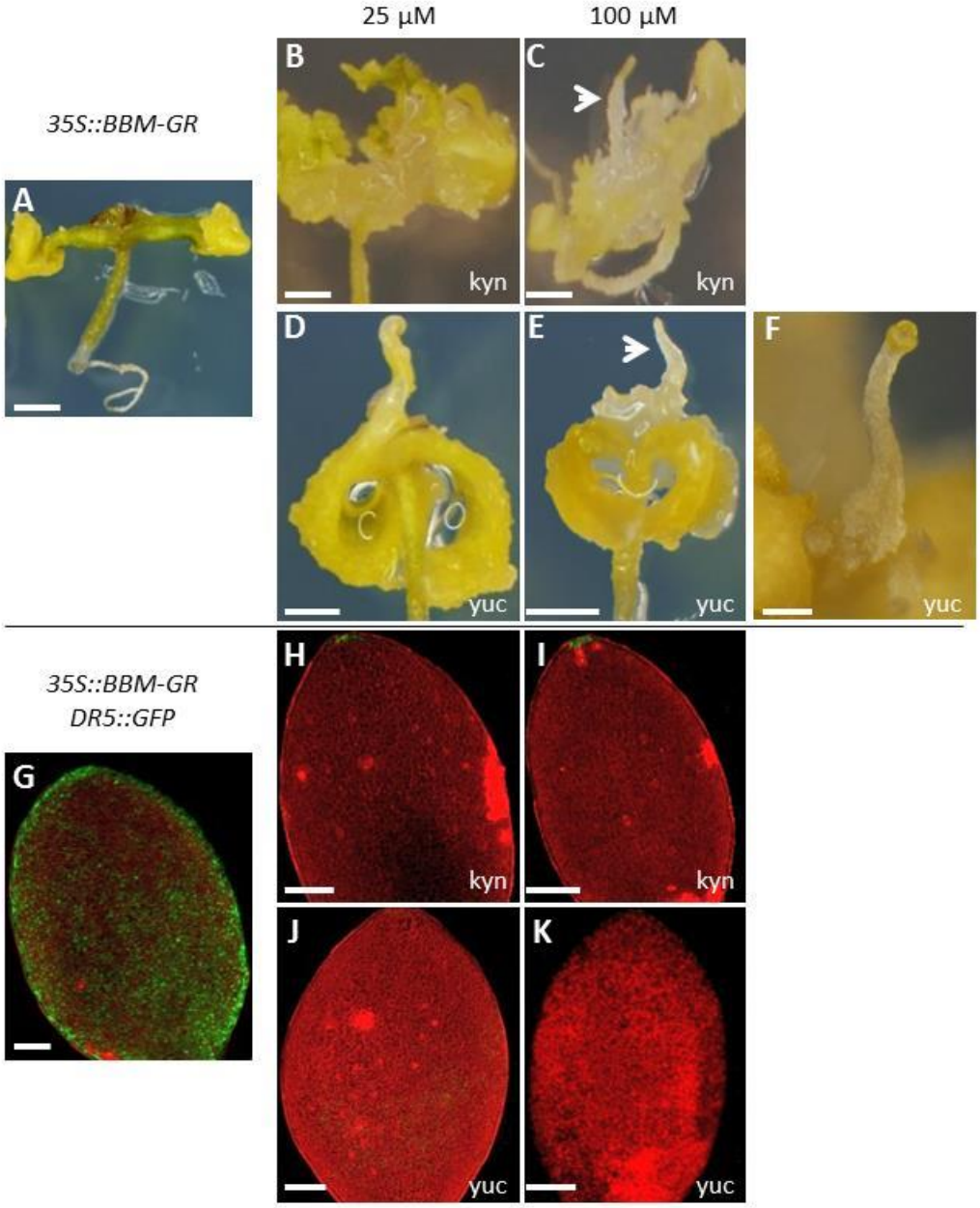


Figure 5: Auxin biosynthesis is required for BBM-mediated somatic embryogenesis
35S::BBM-GR seeds were grown in the presence of dexamethasone with or without the auxin biosynthesis inhibitors kynurenine (kyn) or yucasin (yuc).
A-E, 14-day-old seedlings; Arrowheads, pin-like structures
F, pin-like structure after removal of DEX and auxin biosynthesis inhibitors from the medium;
G-K, *DR5::GFP* expression (green) in four-day-old cotyledons; Seedlings were counter-stained with propidium iodide (red);
A, G, DEX-treated controls (without auxin biosynthesis inhibitor);
Scale bars: **A-E**, 250 µm; **F-K**, 100 µm.

Auxin biosynthesis is required for somatic embryo development

Next we examined the effect of chemically inhibiting TAA1 and YUC activity on BBM-mediated somatic embryogenesis. *35S::BBM-GR* seedlings that were treated with DEX after imbibition (D0) developed somatic embryos at the tip of the cotyledon around seven days after the start of the experiment (Fig. 5A). Treatment with DEX in combination with kyn blocked visible somatic embryo formation at the tip of the cotyledon at all concentrations tested. Instead, a white, translucent, watery callus developed from the central part of the cotyledon and petiole (Fig. 5B, C). yuc treatment also blocked visible somatic embryo formation and induced callus formation, but to a lesser extent than in the kyn treatments (Fig. 5D, E). In addition, the shoot apical meristem (SAM) of the inhibitor-treated seedlings formed a pin-like structure (Fig. 5C-E) The pin-like structures formed shoots at their tips when both DEX and the inhibitors were removed from the culture (Fig. 5F), which is in line with the requirement for local auxin accumulation for organ primordia initiation (Reinhardt et al. 2000; Cheng et al. 2006). By contrast, the cotyledon callus continued to proliferate after DEX and inhibitor removal, but did not form somatic embryos or other organs. The enhanced *DR5* expression in cotyledons activated by BBM in four-day-old seedlings (Fig. 5G) was drastically reduced after kyn and yuc treatment (Fig. 5H-K), suggesting that yuc and kyn treatment reduced auxin levels in the cotyledon. Together these results suggest that both TAA1/TAR and YUC protein activity is required for BBM-mediated somatic embryogenesis.

BBM enhances the expression of auxin efflux transporters

We showed that BBM binds to auxin transport genes in somatic embryo tissue, although it was not clear whether auxin transport gene expression was activated early in somatic embryo induction (Fig. 1D). We therefore examined the role of auxin transport in BBM-mediated somatic embryogenesis in more detail. We focussed initially on the role of two auxin efflux carriers, PIN1 and PIN4, in BBM-mediated somatic embryogenesis because of the genetic relation between *AILs* and *PINs* (Blilou et al. 2005; Galinha et al. 2007), and the role of PIN1 in zygotic- and *in vitro*- embryo development (Su et al. 2009; Soriano et al. 2014; Weijers et al. 2005). We used GUS and/or GFP-based reporters to mark *PIN1* (*PIN1::GUS* and *PIN1::PIN1-GFP*) and *PIN4* expression (*PIN4::GUS*).

PIN1 and *PIN4* expression during root development has been well described. *PIN1* is predominantly expressed in the vascular stele, while *PIN4* is mainly expressed around the QC with some expression in the stele (Friml et al. 2002; Vieten et al. 2005). DEX-treated *35S::BBM-GR* seedlings showed increased *PIN1* and *PIN4* expression in roots, with *PIN4* expression being activated earlier than *PIN1* expression (Fig. 6). After DEX treatment *PIN1* expression was restricted to its wild-type expression domain, while the *PIN4* expression domain was broader. Thus, BBM overexpression activated *PIN1* and *PIN4* expression in roots and altered the *PIN4* spatial expression pattern.

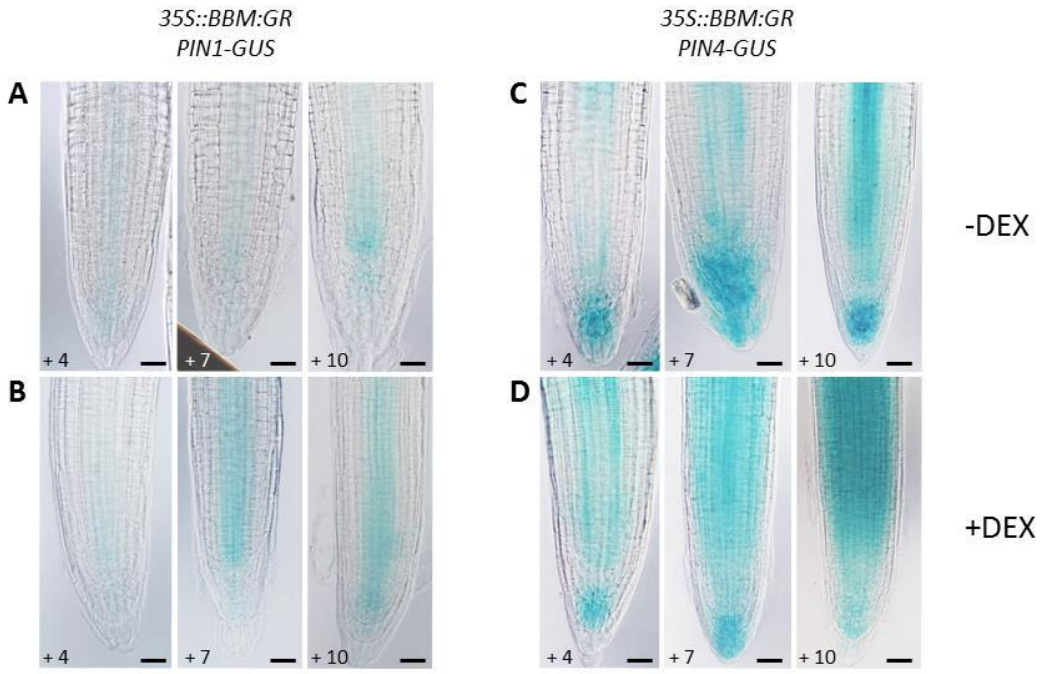


Figure 6: BBM overexpression activates *PIN* gene expression in roots
35S::BBM-GR seeds were grown in the absence (-DEX) or presence (+DEX) of dexamethasone for the amount of time indicated in the images.
A, B, *PIN1::GUS* expression in *35S::BBM-GR* roots;
C, D, *PIN4::GUS* expression in *35S::BBM-GR* roots.
Scale bars, 100 µm.

PIN1 and *PIN4* expression in aerial parts of the plant has also been described. While *PIN1* expression can be easily detected in vascular bundles, the SAM and young leaf primordia, *PIN4* expression is undetectable (Vieten et al. 2005; Guenot et al. 2012). In cotyledons of non-DEX-treated *35S::BBM-GR* seedlings, *PIN1*-driven *GUS* expression was as previously described (Fig. 7A). *PIN1::GUS* expression was first enhanced in cotyledons four days after DEX-treatment, where it had spread throughout the cotyledon and increased in the vasculature (Fig. 7B-D). Somatic embryos were initiated at the tip of the cotyledon after seven days of DEX treatment (Fig. 7C). At this time point it became difficult to distinguish between GUS staining in the somatic embryo and the underlying cotyledon explant (Fig. 7C, D). Unlike *PIN1::GUS* expression, *PIN1::PIN1-GFP* expression could only be detected in the vascular bundles and emerging somatic embryos after DEX treatment (Fig. 7E-H). The discrepancy in *PIN1*-driven GUS and GFP accumulation might be due to differences in sensitivity of the two markers or to diffusion of GUS reaction intermediates. *PIN4::GUS* expression was barely detectable in cotyledons of non-DEX-treated *35S::BBM-GR* seedlings (Fig. 7K), as previously reported. After DEX treatment, *PIN4* was expressed in cotyledons in a similar pattern as *PIN1,* but was initially detected at a later time point and was not as strongly expressed as *PIN1* (Fig. 7L-N).

We demonstrated that the expression of both *PIN1* and *PIN4* is enhanced during BBM-mediated somatic embryogenesis, and that *PIN* expression is induced much later than *DR5* expression.

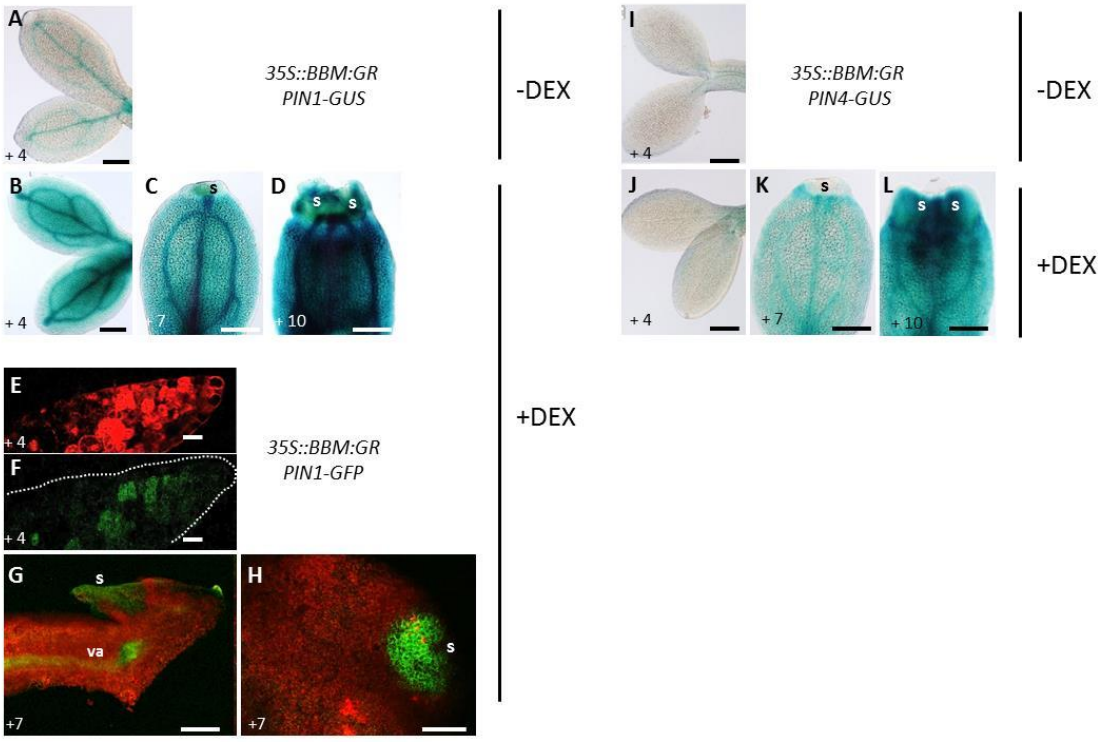


Figure 7: BBM overexpression activates *PIN* gene expression in cotyledons
35S::BBM-GR seeds were grown in the absence (-DEX) or presence (+DEX) of dexamethasone. The day of analysis relative to the start of experiment is indicated in the images.
A-D, Differential interference contrast (DIC) images of *PIN1::GUS* expression in cotyledons of *35S::BBM-GR* seedlings in the absence of DEX (**A**) and presence of DEX (**B-D**);
E-H, Confocal images of *PIN1::GFP* expression in cotyledon tips of DEX-treated *35S::BBM-GR* seedlings; **E and F,** longitudinal hand sections through the tip of the cotyledon. **E** overlay red and green channel; **F**, green channel only. The dotted line indicates the tissue boundary; **G,** cotyledon tip showing PIN1-GFP expression in the vascular tissue (va) and in a somatic embryo (s); **H**, adaxial side of a cotyledon tip showing PIN1-GFP at the apical part of a somatic embryo.
Samples were counterstained (red) with propidium iodide;
I-L, Differential interference contrast (DIC) images of *PIN4::GUS* expression in cotyledons of *35S::BBM-GR* seedlings in the absence (**I**) and presence of DEX (**J-L**). Scale bars, 100 µm.

BBM overexpression seedlings are partially resistant to NPA

We investigated the effect of chemically inhibiting auxin transport on BBM-mediated somatic embryogenesis by growing *35S::BBM-GR* seedlings in relatively low (5 µM) and high (50 µM) concentrations of NPA. Wild-type Arabidopsis seedlings treated with NPA show a stunted phenotype comprising a shorter root and a swollen root tip with enhanced *DR5* expression (Ruzicka et al. 2007; Lewis et al. 2009). We observed the same root phenotypes and increase in *DR5* expression when *35S::BBM-GR* seedlings were treated with NPA in the absence of DEX (Fig. 8A, B) however, these phenotypes were partially rescued after DEX treatment (Fig. 8C-D). NPA did not induce swelling in

DEX-treated *35S::BBM-GR* roots and NPA did not enhance *DR5* expression or stunt growth to the same extent in DEX-treated *35S::BBM-GR* roots as non-DEX-treated roots. NPA resistance could be the result of both direct and indirect effects. For example, NPA treatment might be insufficient to overcome BBM-induced *PIN1*, *PIN4, MDR1* or *NPY4* expression, while indirect (downstream) effects such as reduced auxin perception, biosynthesis, signalling, or stabilisation of the PIN proteins might also confer NPA resistance (Robert et al. 2015; Yamada et al. 2009; Geisler et al. 2003).

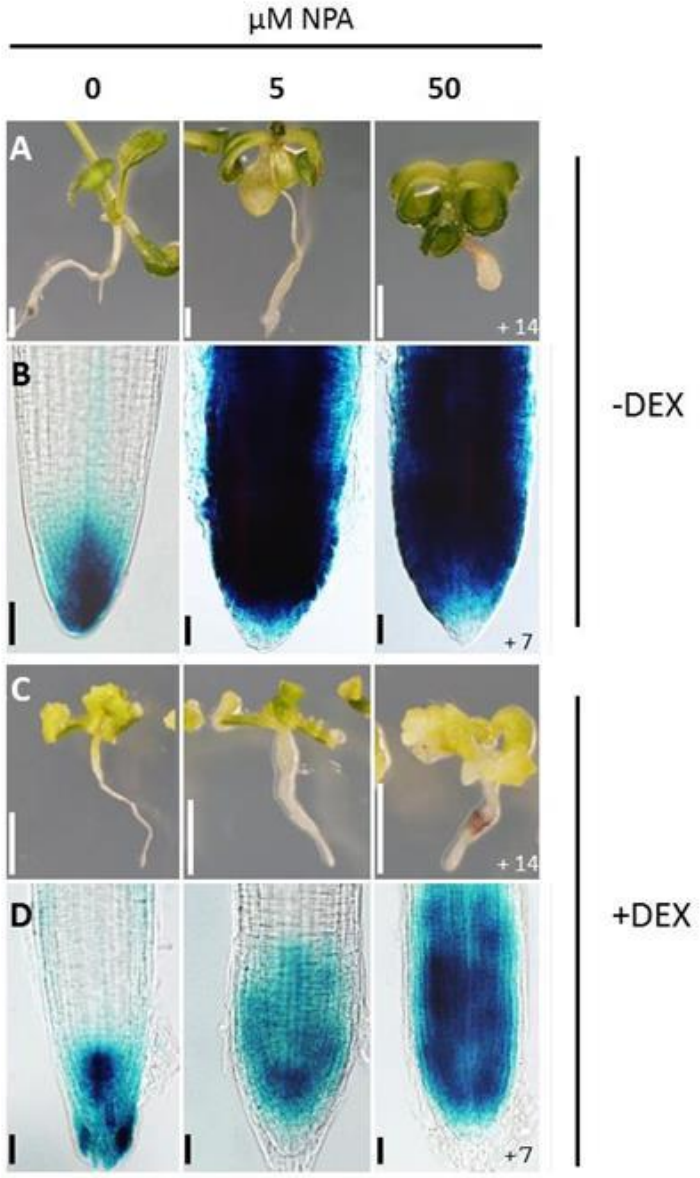


Figure 8: BBM overexpression rescues NPA-mediated root defects
35S::BBM-GR seeds were grown in the absence (-DEX) or presence (+DEX) of dexamethasone with or without the polar auxin transport inhibitor NPA. The NPA concentration is indicated at the top of each column.
A, C, effect of NPA on 14-day-old seedlings;
B, D, Effect of NPA on *DR5::GUS* expression in seven-day-old roots;
Scale bars: **A** and **C**, 2 mm; **B** and **D**, 100 µm.

Auxin transport is required for embryo outgrowth rather than embryo initiation

We investigated the effect of NPA on BBM-mediated somatic embryogenesis by culturing seeds after imbibition in medium containing DEX with and without NPA. The first somatic embryos were observed after seven days of culture in DEX-treated *35S::BBM-GR* seedlings in the absence of NPA (Fig. 9C). Treatment with DEX and 5 µM NPA delayed the appearance of embryos and also reduced their size (Fig. 9A), while callus formed at the surface of the cotyledon after treatment with DEX and 50 µM NPA, (Fig. 9A). Treatment with DEX and NPA initially induced a shift in *DR5::GUS* expression from the cotyledon margin to the blade (Fig. 9B), and at a later time point caused an overall reduction in *DR5::GUS* expression (Fig. 9C, D). These experiments suggest that NPA can block the formation of BBM-mediated somatic embryos.

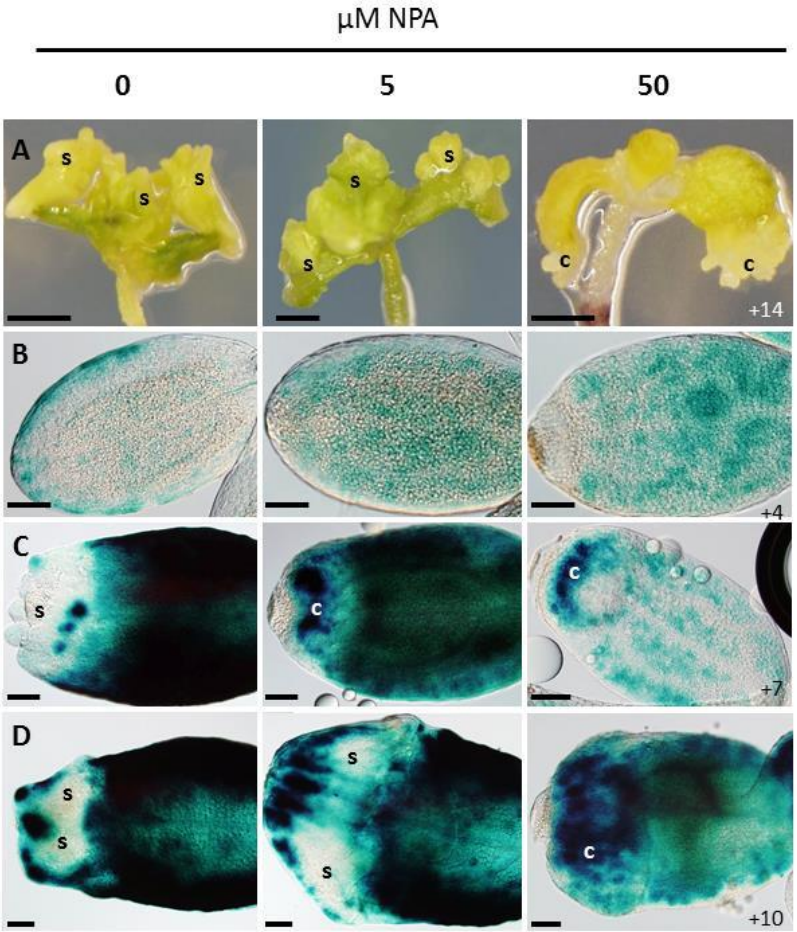


Figure 9: Polar auxin transport is required for somatic embryogenesis
Effect of NPA on BBM-mediated somatic embryogenesis and *DR5* auxin response. *35S::BBM-GR* seeds were grown in the presence of dexamethasone with or without the polar auxin transport inhibitor NPA. The NPA concentration is indicated at the top of each column and the age of the seedling in the images.
A, Aerial region of 14-day-old seedlings;
B, C, *DR5::GUS* expression in cotyledons after 4, 7, and 10 days of culture;
c, callus; s, somatic embryo. Scale bars: **A** and **C**, 1mm; **B** and **D**, 100 µm.

DISCUSSION

The plant hormone auxin plays an important role in many aspects of plant development (Zhao 2010), including somatic embryogenesis, where treatment with an exogenous auxin is often needed to initiate the process. The AINTEGUMENTA-LIKE (AIL) transcription factor BABY BOOM (BBM) induces somatic embryogenesis in Arabidopsis in the absence of exogenously applied auxin (Boutilier et al. 2002; Heidmann et al. 2011; Srinivasan et al. 2007), while auxin treatment is normally a prerequisite for somatic embryogenesis in wild type Arabidopsis explants. A genetic relationship between AILs and auxin, as well as direct transcriptional regulation of *YUC* expression has been shown for AIL-family members (Horstman et al. 2014), but not in the context of somatic embryogenesis. Here we have shown that BBM binds to and regulates the expression of auxin biosynthesis and auxin transport genes, and that both processes play roles in BBM-mediated somatic embryogenesis.

We demonstrated that BBM activates the expression of auxin biosynthesis genes at two steps of the tryptophan-dependent auxin biosynthesis pathway (Supplemental Fig. 1), through *TAA1* and through *YUC3*. Our expression data suggests that BBM also directly regulates *YUC4* expression in germinating seeds. BBM might also indirectly regulate *YUC4* through its direct targets *STY1* (Eklund et al. 2010), *LEC1* and *LEC2* (Chapter 5) (Junker et al. 2012; Stone et al. 2008). *YUC4* expression can be activated by STY1, LEC2, and BBM and is therefore be a good candidate for further analysis, although additional proof for BBM binding to *YUC4* is required. Additional support for auxin biosynthesis genes as direct BBM targets came from our observation that IAA levels were increased in response to BBM overexpression, with a phenotypically stronger overexpression line showing higher IAA levels than a weaker line. Both lines had also higher levels of the degradation product OxIAA indicating that IAA was actively reduced.

We also demonstrated the importance of auxin biosynthesis in BBM signalling by showing that auxin biosynthesis inhibitors block BBM-mediated somatic embryo formation: a callus-like tissue was formed rather than morphologically-discernible embryos. Zygotic embryo development in *taa1/tar* and quadruple *yuc* mutants is terminated at the transition from globular to heart stage (*taa1/tar*) and heart- to torpedo stage (*yuc*) (Robert et al. 2013; Stepanova et al. 2008). A complete description of all *YUC* gene expression patterns and functions during zygotic embryogenesis is currently not available, thus it is not known if non-characterized *YUC* genes also function earlier in establishment of the embryo proper. Analysis of embryo reporters is required to determine at which stage somatic embryo development is blocked in yuc/kyn-treated BBM overexpression seedlings. Recently it was shown that TRP-independent IAA biosynthesis is essential for early zygotic embryo development, while TRP-dependent IAA biosynthesis is important for late embryo development (Wang et al. 2015). The role of the TRP-independent pathway in BBM-mediated somatic embryogenesis or other forms

of *in vitro* somatic embryogenesis requires further investigation, however, genetic components of the TRP-independent pathway have not been identified as direct BBM targets.

Somatic embryogenesis was inhibited at the highest yuc/kyn concentrations tested, which are higher than those that affect root development in wild type and BBM overexpression plants (He et al. 2011; Stepanova et al. 2008), but similar to the yuc concentration range (20-100 µM) that complemented the *YUC1* overexpression phenotype (Nishimura et al. 2014). The overall IAA level in our BBM lines was much lower than that in *TAA1*, *YUC1* or *YUC6* overexpression lines (Nishimura et al. 2014; Mashiguchi et al. 2011), yet only a high inhibitor concentration could suppress BBM-mediated somatic embryo formation. This suggests that BBM induces high local IAA levels in cotyledons or that embryogenesis is less sensitive to inhibitor treatment. Additional support for a role of auxin biosynthesis in BBM-mediated somatic embryogenesis should be obtained through local auxin biosynthesis measurements, analysis of TAA1/YUC auxin biosynthesis reporters and mutants, and complementation of the yuc/kyn phenotypes with exogenous IAA.

Polar auxin transport has a role in embryo patterning and initiation in both zygotic embryos and cultured embryos. In zygotic embryos, inhibition of auxin transport negatively affects pattern formation and outgrowth of the embryo (Hadfi et al. 1998; Benkova et al. 2003; Blilou et al. 2005). *pin* mutants *(pin1;pin3;pin4;pin7)* fail to develop past the globular stage (Vieten et al. 2005). In microspore embryo culture, polar auxin transport is required for embryo proper formation when embryos develop from a suspensor-like structure (Soriano et al. 2014), in analogy with the effect of a *pin7* mutation on embryo proper formation in zygotic embryos (Friml et al. 2003). NPA treatment does not affect the initiation of suspensor-less microspore-derived embryos, but rather affects root meristem formation and radial patterning later in development (Soriano et al. 2014; Blilou et al. 2005). In somatic embryo cultures of Norway spruce, NPA treatment also negatively affects the development of cotyledons and the shoot apical meristem (Palovaara and Hakman 2009; Larsson et al. 2008). Polar auxin transport is however required for somatic embryo initiation from embryogenic callus in Arabidopsis (Su et al. 2009), although, a detailed analysis of embryo marker lines would be required to determine to what extent the embryo pathway is activated when auxin transport is inhibited.

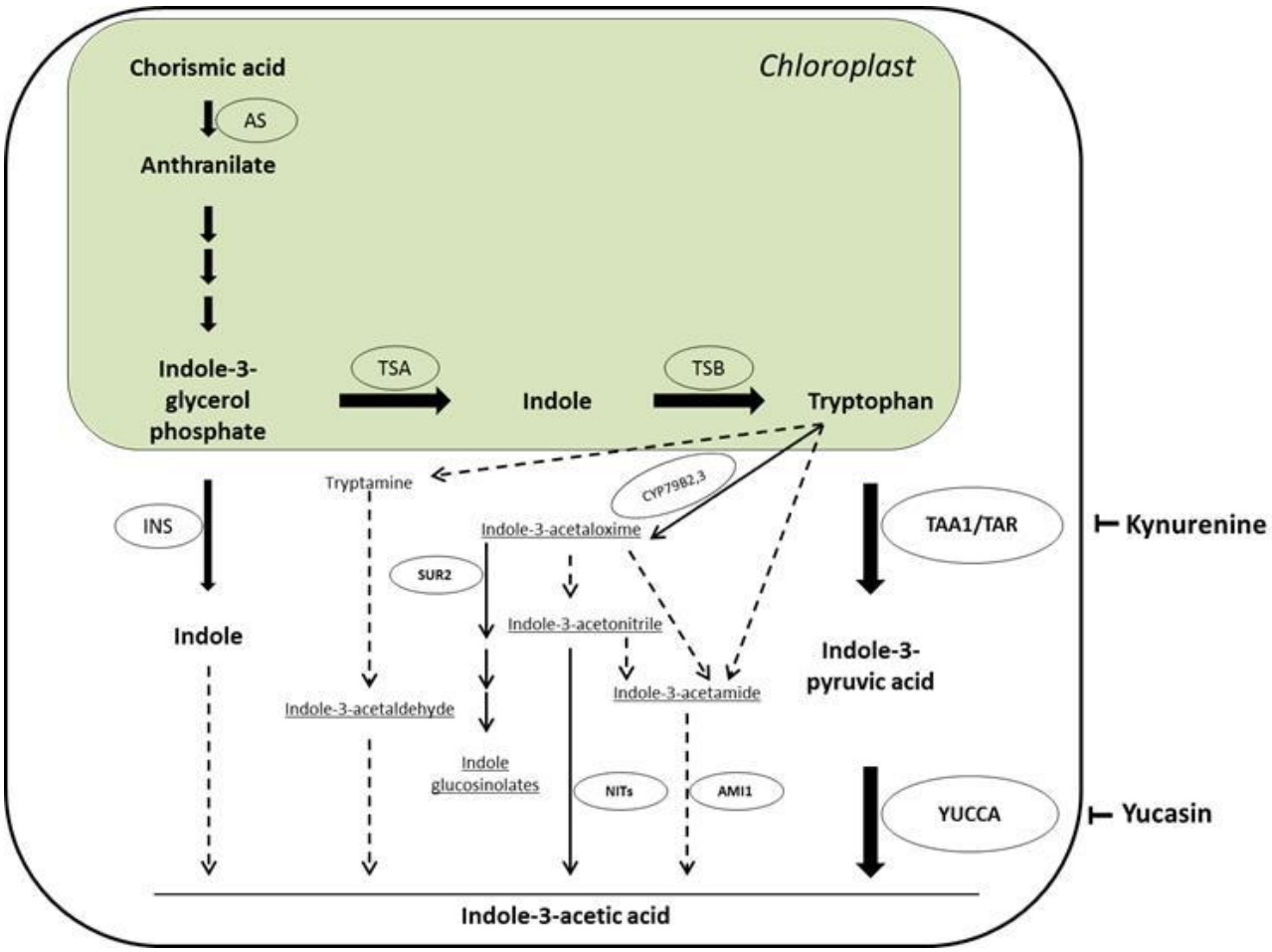
Our ChIP-seq data suggest that BBM also binds to the auxin transport genes *PIN1*, *PIN4*, *MDR1* and *NPY4*. qPCR analysis revealed weak, albeit direct transcriptional activation of *PIN1*, *PIN4, MDR1* and *NPY4* by BBM, while the *PIN1* and *PIN4* reporters did not show major changes in expression within the first few days of DEX treatment. Thus, although *PIN1* and *PIN4* are bound by BBM they might not be transcriptionally regulated by BBM during this early time point. *PIN1* and *PIN4* expression increased at later stages of BBM-mediated somatic embryogenesis, but it is not known if this is a direct or indirect effect of *BBM* overexpression. Notably, a *DR5* minimum was observed at

the tip of the cotyledon concomitant with the appearance of the first somatic embryos. If auxin efflux away from the site of somatic embryo formation underlies the observed *DR5* minimum and drives somatic embryo initiation, then treating BBM overexpression seedlings with the polar auxin transport inhibitor NPA should block somatic embryo formation. Visible somatic embryos were not formed after treatment of a DEX-treated *35S::BBM-GR* line with 50 μM NPA, and unlike shoot development, removal of NPA and DEX did not allow outgrowth of pre-existing structures. The possibility remains that embryo development was not initiated in the presence of high concentrations of NPA or that few-celled embryos were formed, but were unable to grow further in the absence of DEX. Additional experiments to assess the effect of NPA removal alone, as well as analysis of embryo reporters and the subcellular localisation of PIN proteins, are required to determine whether the *DR5* minimum that coincides with somatic embryo initiation is accompanied by relocalisation of PIN proteins and whether this minimum is actually required somatic embryo initiation.

In conclusion, we showed that auxin biosynthesis and signalling genes are directly regulated by BBM and that chemically disturbing auxin biosynthesis or transport has a negative effect on BBM-mediated somatic embryogenesis.

Supplemental Table 1: DNA primers

Gene	Primer orientation	Sequence (5' to 3')
Reference gene		
SAND	Forward	AACTCTATGCAGCATTTGATCCACT
	Reverse	TGATTGCATATCTTTATCGCCATC
	Auxin biosynthesis	
TAA1	Forward	TTCGTGGTCAATCTGGATCATGG
	Reverse	ACCACGTATCGTCACCGTACAC
YUCCA 3	Forward	ATGGTCGTTCGTAGCGCTGTTC
	Reverse	GCGAGCCAAACGGGCATATACTTC
YUCCA 4	Forward	CGTTCTTGATGTCGGTGCCATTTCTTTAATCC
	Reverse	GGCGTCATAGGCTGTTCCCGAAAGTC
YUCCA 6	Forward	AAACTCCGGTTCTCGACGTTGG
	Reverse	CCCGAACACACCTTAATGTCTCC
YUCCA 8	Forward	TGCGGTTGGGTTTACGAGGAAAG
	Reverse	GCGATCTTAACCGCGTCCATTG
STY1	Forward	TCGCATACCTTCTCATTCAGGGCT
	Reverse	CACCTAACACCGCCGATGAACT
Auxin transport genes		
PIN1	Forward	GGCATGGCTATGTTCAGTCTTGGG
	Reverse	ACGGCAGGTCCAACGACAAATC
PIN4	Forward	ACAACGTGGCAACGGAACAATC
	Reverse	GCCGATATCATCACCACCACTC
MDR1	Forward	TAAAGGCTACGACACACAGGTTG
	Reverse	TTGCAATTCTCTGCTTCTGTCCAC
NPY4	Forward	TGCATAAGTTCCCATTGCTGTCG
	Reverse	AGCAACAGAACCACCAGGAATCTC



Supplemental Figure 1: Auxin biosynthesis pathways and inhibitors
Indole-3-acetic acid (IAA) is synthesized via multiple steps and intermediates (Normanly et al. 2010). Chorismic acid is metabolised by anthranilate synthase (AS) to anthranilate, which is further converted via intermediates to indole-3-glycerol-phsophate (IGP). In the tryptophan (TRP)-independent pathway IGP is converted to indole by the indole synthase (INS) and further to IAA by an unknown mechanism. In the TRP-dependent pathway the tryptophan synthase α (TSA) converts IGP to indole from which TRP is synthesized via the tryptophan synthase β (TSB). The synthesis route from TRP to IAA via TRYPTOPHAN AMINO TRANSFERASE ARABIDOPSIS (TAA1)/TAA1-RELATED (TAR) and YUCCA is considered as the main pathway in Arabidopsis (Mashiguchi et al. 2011), although alternative pathways via various indole-3-variants and enzymatic activity of CYP79B2,3, SUPERROOT2 (SUR2), INDOLE-3-ACETAMIDE HYDROLASE1 (AMI1), and NITRILASE (NIT) co-exist (Zhao 2014). TAA1/TAR is inhibited by kynurenine (He et al. 2011) and YUCCA by yucasin (Nishimura et al. 2014).

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Chapter 7

General Discussion

Iris Heidmann

Plant regeneration systems are not only of tremendous economic interest to the plant breeding industry, nurseries, and production labs, but also play an important role in conservation programs and are fascinating systems to study pluri- and totipotency. Improvements in plant regeneration have been achieved mainly through empirical approaches rather than through knowledge of the underlying mechanisms. Somatic embryogenesis is an efficient form of plant regeneration as both root and shoot meristems are present simultaneously thereby eliminating the need to induce these organs in a step-wise fashion to obtain a complete plantlet. Somatic embryos can be induced by applying plant growth regulators, especially auxins, or by the overexpression of embryo-expressed transcription factors like LEAFY COTYLEDON1 (LEC1), LEC2 (Lotan et al. 1998; Stone et al. 2001) and BABY BOOM (BBM) (Boutilier et al. 2002; Deng et al. 2009). This thesis shows that BBM is an effective inducer of somatic embryogenesis across plant species, as overexpression of a *Brassica napus BBM* gene, induces somatic embryogenesis in tobacco, sweet pepper, and Arabidopsis (Chapter 2, (Srinivasan et al. 2007); Chapter 3, (Heidmann et al. 2011); Chapter 5). BBM-mediated somatic embryogenesis shows developmental similarities to auxin- and LEC-induced somatic embryogenesis, but no molecular mechanism has been identified that connects these three pathways. This thesis provides evidence that these pathways are intertwined by showing that BBM binds to and transcriptionally regulates *LEC1* and *LEC2* and two other members of the LAFL network (Jia et al. 2013), *ABSCISIC ACID INSENSITIVE3* (*ABI3)* and *FUSCA3 (FUS3)* (Chapter 5), as well as auxin biosynthesis and transport genes (Chapter 6). Genetic and chemical inhibition studies suggest roles for these genes in somatic embryo induction. Finally, this thesis also describes the influence of both tissue context and AIL protein dose on BBM-mediated somatic embryogenesis (Chapter 5).

Applied aspects of somatic embryogenesis

Classically, *in vitro* plant regeneration via somatic embryogenesis is induced by the application of plant growth regulators (PGRs), especially auxinic herbicides, or stress (Motte et al. 2014; Fehér 2014), but can also be induced by the ectopic expression of transcription factors such as BBM. Exposure of explants to PGRs such as auxins and cytokinins promotes cell division followed by callus, root-, shoot-, or even somatic embryo formation, depending on the type and/or ratio of different PGRs. Unlike shoots, somatic embryos are not connected to the underlying vascular system of the donor tissue and have both a shoot and root pole. However, as with adventitious organogenesis, somatic embryogenesis can be induced directly from the explant or indirectly via an intervening callus phase. Both direct and indirect somatic embryogenesis can occur on one explant (Yang and Zhang 2010).

Clonal propagation via somatic embryogenesis is of commercial interest because of the potential for high throughput automatisation (Ibaraki and Kurata 2001), the ability to store embryos (Bonga

2015), and the ability to efficiently convert somatic embryos into seedlings. As with micropropagation via organogenesis, somatic embryogenesis is used to clonally propagate plant species with a long life cycle, genetic material that cannot be propagated via seeds, including male sterile plants, and products of interspecific crosses or endangered species (Dunwell 2010). Somatic embryogenesis is also used in some species as a way to circumvent classical shoot regeneration for plant transformation (Obembe et al. 2011). Finally, somatic embryogenesis is used as a model system to study totipotency (Verdeil et al. 2007).

Although somatic embryogenesis can be useful in plant breeding and propagation programs (Pasqual et al. 2014), it often difficult to implement efficiently as there is a strong species- and genotype-dependence for tissue culture response. In addition, long term culture of explants is often required, which can lead to somaclonal variation and generation of off-types (Hitomi et al. 1998; Borchert et al. 2007), the loss of embryogenic competence of cell cultures and a reduced ability to convert the somatic embryos into plantlets (Christianson and Warnick 1983; Hazubska-Przybył et al. 2014; Bonga 2015).

BBM-mediated somatic embryogenesis in crop plants

Ectopic expression of BBM induces somatic embryo formation in *B. napus*, Arabidopsis (Boutilier et al. 2002), Chinese white poplar (*Populus tomentosa* Carr.) (Deng et al. 2009) and cacao (*Theobroma cacao*) (Florez et al. 2015), and has also been used to regenerate plastid-transformed plants (Lutz et al. 2011). In Chapters 2 and 3 we show that heterologous expression of *BBM* can be used to induce somatic embryogenesis in the crop species tobacco (*Nicotiana tabacum*) and pepper (*Capsicum annuum*).

BBM overexpression in tobacco and sweet pepper

Nicotiana species are not only used as crop plants by the tobacco industry, but are also used for pharmaceutical production (da Cunha et al. 2014; Ikramt et al. 2015) due to the ease with which they can be transformed and grown (Ganapathi et al. 2004). Spontaneous BBM-induced somatic embryogenesis was not observed in *BBM* overexpression lines, but could be induced after treatment with cytokinin, like zeatin or BAP (Chapter 2) (Srinivasan et al. 2007). Application of cytokinin to BBM overexpression seedlings (*35S::BBM-GR*) induces somatic embryogenesis at the root-hypocotyl transition zone, while treatment of wild type tobacco seedlings with cytokinin induces shoot formation at the same position.

The observation that BBM overexpression enhances regeneration and supports somatic embryo formation in tobacco (Chapter 2) (Srinivasan et al. 2007) lead to the idea that BBM overexpression could be used to improve regeneration in the related solanaceous species, sweet pepper (*Capsicum*

annuum). Sweet pepper is an important vegetable crop (Djian-Caporalino et al. 2006) that is notoriously recalcitrant to transformation (Wolf et al. 2001). In our experiments, activation of BBM during the selection and regeneration phases enhanced shoot formation with occasional somatic embryo formation on primary shoots or shoot-like structures. Prolific somatic embryogenesis was only observed in the subsequent generation. As with tobacco, exogenous cytokinin was required to induce somatic embryo formation in sweet pepper; only callus formed in the absence of exogenous cytokinin. Unlike tobacco and Arabidopsis, strong somatic embryogenesis was observed on leaf explants.

BBM response in solanaceous species versus Arabidopsis

Somatic embryos can be induced from tobacco callus or explants in response to cytokinin and IAA (Haccius and Lakshmanan 1965; Stolarz et al. 1991) or thidiazuron (Gill and Saxena 1992), a plant growth regulator with a cytokinin- and auxin-like mode of action (Murthy et al. 1998).

Somatic embryogenesis in *Capsicum* is restricted to a few species that require either 2,4 D alone or additional cytokinin for the induction, however, morphological defects as the lack of a shoot apical meristem, needed for the conversion into seedlings, are observed (Santana-Buzzy et al. 2012).

These observations suggest that there are species-specific requirements for somatic embryo induction and differentiation.

At the time we performed the tobacco and sweet pepper experiments we did not know that BBM directly activates auxin biosynthesis genes, resulting in increased endogenous IAA levels (Chapter 5 and 6). In retrospect, some of the phenotypes observed in Arabidopsis and tobacco seedlings, including stunted growth, epinastic cotyledons, and enhanced root formation might be explained by auxin overproduction. It is therefore conceivable that BBM overexpression in tobacco and sweet pepper alleviates the need for exogenous auxin to induce somatic embryogenesis, but not the requirement for exogenous cytokinin. Why both increased auxin and cytokinin are required to induce somatic embryogenesis in these solanaceous plants and not in Arabidopsis is not known. Perhaps local levels and/or ratios of endogenous hormones differ in explants from different species or families.

In contrast to Arabidopsis, somatic embryos were rarely observed in primary tobacco and pepper transformants. At the time we performed the pepper experiments, the dose-response effect of BBM (Chapter 5) was not known. The lack of prolific somatic embryogenesis in primary pepper and tobacco transformants might be explained by a low BBM dose. Higher levels of BBM expression, sufficient to induce somatic embryogenesis, might only be achieved in the subsequent generation, perhaps in part due to the number of transformed cells or the type of explant that is used.

Somatic embryos can also be induced in Arabidopsis by overexpression of the LEC1 and LEC2 transcription factors (Lotan et al. 1998; Stone et al. 2001). Guo et al (Guo et al. 2013) reported that overexpression of DEX activated LEC-GR fusions in tobacco stimulates the formation of small, embryo-like seedlings (LEC1-GR) or callus formation from the shoot meristem followed by somatic embryo formation (LEC2-GR). However, the LEC2-induced 'somatic embryos' shown by Guo et al. appear more shoot-like than embryo-like. *LEC2* overexpression did however induce expression of embryo maturation genes. This data suggests that, as with BBM overexpression, *LEC1* or *LEC2* overexpression in tobacco induces embryo identity, but is not sufficient to induce a complete conversion of vegetative cells to differentiated embryos. It would be interesting to determine whether exogenous cytokinin promotes LEC-mediated somatic embryogenesis in tobacco.

The different reactions of tobacco/pepper and Arabidopsis seedlings to BBM or LEC overexpression might be explained by differences in the structure of the seed and/or the speed of germination between these two species. While Arabidopsis zygotic embryos are surrounded by a single-layer of endosperm and germinate quickly, solanaceous embryos are embedded in a thick, persistent endosperm and germinate slower. The thickness of the endosperm might act as a barrier, delaying and reducing DEX-uptake by the zygotic embryo with the consequence that less BBM protein is transported to the nucleus and a subsequent lower activation of BBM-target genes during the optimal window for somatic embryo induction i.e. that the seedlings are too mature to be responsive for BBM-mediated somatic embryogenesis. In Arabidopsis *lec1, lec2 and fus3* zygotic embryos show leaf characteristics (Meinke 1992) and these zygotic embryos do not form 2,4-D-induced somatic embryos (Meinke 1992; Gaj et al. 2005). By analogy, tobacco cotyledons might be more leaf-like and less 'embryo-like' than Arabidopsis cotyledons, making them less competent for BBM-mediated somatic embryogenesis.

Introducing embryo, shoot/callus, auxin and cytokinin markers into tobacco and pepper BBM overexpression lines, analysis of tissue specific hormone profiles, and inhibition of auxin biosynthesis (Chapter 6) would help determine how BBM overexpression in combination with cytokinin induces seedlings to produce somatic embryos, where wild-type seedlings would normally develop shoots or callus.

Somatic embryo quality

For practical purposes, somatic embryo cultures should generate genetically identical clones of the donor plant and, like seeds, should be able to produce large numbers of embryos that can be stored and easily converted to seedlings. However, a number of problems occur that reduce either the quantity or the quality of the embryos produced in tissue culture.

Somaclonal variation refers to induced genetic variation among cultured plants, which results in the production of genetic off-types i.e. plants that do not breed true to type. Somaclonal variation is encountered in many plant tissue systems and is not specific to somatic embryogenesis. Somaclonal variation is thought to be caused by high concentrations of plant growth regulators and/or an extended tissue culture period (Us-Camas et al. 2014; Miguel and Marum 2011; Paszkowski 2015; Rodriguez-Enriquez et al. 2011). Somaclonal variation has been reported after auxin-induced regeneration (Miguel and Marum 2011), but not after transcription-factor induced regeneration, although admittedly no studies are available for the latter.

In *Capsicum annuum* (sweet and pungent types), the efficiency of auxin-induced somatic embryogenesis is quite low, and embryos often exhibit morphological defects, including embryo fusion and lack of a functional shoot apical meristem (Steinitz et al. 2003), both of which reduce the number of converted seedlings. By contrast, BBM-induced somatic embryos are more zygotic embryo-like without the mentioned morphological defects (Chapter 3, Heidmann et al., 2011). The reduced time required to produce somatic embryos combined with the avoidance or lower concentrations of exogenous auxin (2,4-D) might be responsible for the improvements in embryo and plant quality.

Despite the missing knowledge on how BBM induces regeneration in species other than Arabidopsis, BBM can still be used as a regeneration tool in recalcitrant species. However, as in classical tissue culture, BBM-based regeneration protocols need to be adapted for each species or even genotype with respect to the choice of tissue explant, the application of additional growth regulators, and the timing of BBM overexpression.

To date, all published BBM or AIL-mediated regeneration or somatic embryogenesis events are based on stable transgene integration (Boutilier et al. 2002; Heidmann et al. 2011; Srinivasan et al. 2007; Deng et al. 2009; Lutz et al. 2011; Kareem et al. 2015). As such, these plants including their offspring are considered genetically modified organisms (GMOs) and their use is restricted by GMO regulations. Plants produced by transient expression of BBM from a DNA construct are also considered as GMOs in Europe. The challenge for the future is to develop transient, non-DNA-based systems for BBM expression and/or activation of its endogenous targets to promote regeneration. Protein transfer via peptides coated nanoparticles as used in cancer therapy (Eudes et al. 2014; Estanqueiro et al. 2015), or Agrobacterium VirB protein translocation (Vergunst et al. 2000) would not fall under the current European GMO regulations might allow deregulation of the regenerated plantlets.

AILs and regeneration response

The function and role of the eight known Arabidopsis AIL family members has been reviewed most recently (Horstman et al. 2014). The AIL members *PLT3*, *PT5* and *PLT7* are required for shoot regeneration from growth-regulator induced callus from where they mediate organogenesis in a two-step process (Kareem et al. 2015). These PLTs first induce expression of the root stem cell regulators *PLT1* and *PLT2*, which in turn induce the formation of the lateral root primordia that comprise the shoot progenitor cells in 'rooty' organogenic callus (Atta et al. 2009; Sugimoto et al. 2010; Che et al. 2007). This first step is followed by activation of *CUP-SHAPED COTYEDON2 (CUC2)* gene expression, which is required for shoot formation from the progenitor cells in the callus (Kareem et al. 2015).

We and others have shown that overexpression of all *AIL* genes except *ANT* and *AIL1* induces somatic embryogenesis (Tsuwamoto et al. 2010), Chapter 5). Nonetheless, somatic embryogenesis is not observed during AIL-mediated *in vitro* organogenesis (Kareem et al. 2015), even in stable transgenic lines (Kareem et al. 2015). This suggests the hormone regime that favours adventitious organogenesis is not optimal for somatic embryogenesis. This might be similar to the situation in which overexpression of *LEC* genes in the presence of 2,4-D has a negative effect on somatic embryo formation (Ledwon and Gaj 2009).

The tissue response to AIL activation also depends on the level of AIL expression (Chapter 5). Post-translational regulation of BBM and PLT2 overexpression suggests that a relatively high AIL protein dose promotes somatic embryogenesis, while a relatively moderate dose favours organogenesis, and a relatively low dose promotes dedifferentiation. Part of this dose response could be regulated by the interaction between BBM and members of the epidermis-expressed HOMEODOMAIN GLABROUS (HDG) protein family. BBM interacts with HDG1, HDG11, and HDG12, in an antagonistic manner, with BBM promoting cell proliferation and HDG proteins promoting cell differentiation. Overexpression of HDG1 suppresses BBM-mediated somatic embryogenesis in a dose-dependent manner (Horstman et al. 2015). A high AIL protein dose might physically outcompete HDG proteins on common or HDG-specific gene target genes, thereby promoting cell division and dedifferentiation.

With respect to sweet pepper and tobacco, mature, non-embryogenic explants such as those used in transformation should have a balance of HDG and AIL proteins that promotes differentiation (Chapter 3). The higher level of differentiation might therefore require relatively high levels of BBM protein to shift growth back to a less differentiated, totipotent state.

Consequences of early and late BBM activation

We found that somatic embryo formation was direct and most efficient when AILs were activated early, before seed germination. AIL activation after germination induced callus formation that was eventually followed by indirect somatic embryogenesis (Chapter 5). Early AIL activation prior to seed

germination initially induced the palisade layer of the cotyledon to divide in an anticlinal instead of periclinal orientation, creating new cells layers below the epidermis and eventually leading to somatic embryo formation at the tip of the cotyledon. During late (post-germination) BBM activation the first divisions occurred mainly in vascular bundles and in the palisade layer in close proximity to the shoot apical meristem, which is typical for auxin-induced somatic embryogenesis (Raghavan 2004). Late BBM activation induces mainly oblique cell divisions in the palisade layer, which appeared to divide single cells into small clusters. These small clusters might be the source of the callus tissue observed after post-germination BBM induction. Somatic embryos formed from the uppermost layers of this callus in close proximity- but not connected to veins (Chapter 5). Division of vascular pericycle cells leading to ectopic root primordia formation is associated with hormone-induced rooty callus formation that precedes shoot organogenesis (Sugimoto et al. 2010; Atta et al. 2009). We did not observe a physical connection between vascular tissue and somatic embryos after late BBM activation (Chapter 5), but we cannot exclude that a root-like organogenesis pathway in the underlying tissue might have a cell non-autonomous effect on somatic embryo formation.

It is unclear why the first cell divisions after early BBM activation occur predominantly in the specific cell layers or regions of the seedling. Assuming the *35S* promoter is active in all cells, an interacting protein like HDG or a physiological factor (e.g. plant growth regulator balance) might restrict BBM activity or function to specific tissues or layers. Alternatively, the cotyledon palisade cells might react differently due to differences in their connectedness. For example, the palisade cells are in closer contact before germination than they are after germination. This close contact of individual cells could promote cell-to-cell communication, leading to direct embryogenesis. The palisade cells are more loosely connected after germination. Close contact and communication between palisade cells might only be re-established during late BBM activation after an initial period of cell proliferation and the establishment of a physical boundary around cell clusters.

Role *LAFL* gene expression in tissue competence

The differential tissue competence may be due in part to interplay between auxin levels and LAFL gene expression. LAFL proteins have major roles in zygotic embryo development/identity and maturation (Jia et al. 2013), and are required for 2,4-D induced somatic embryogenesis (Gaj et al. 2005). LAFL proteins function in an overlapping network and their gene expression is down-regulated at germination (Jia et al. 2013). *AGL15* is also a direct BBM target, although no evidence was found for transcriptional activation by BBM at the time points examined (Chapter 5). Both FUS3 and LEC2 activate *AGAMOUS-LIKE 15* (*AGL15*) expression (Wang and Perry 2013; Braybrook et al. 2006), which enhances the competence for somatic embryogenesis.

Early BBM activation (before germination) increases auxin (IAA) biosynthesis (Chapter 5). In parallel, BBM induces the expression of *LEC1, LEC2,* and *FUS3* (Chapter 5), which would maintain the embryo identity of the tissue and, support direct somatic embryo formation together with auxin-mediated cell division (Fig. 1).

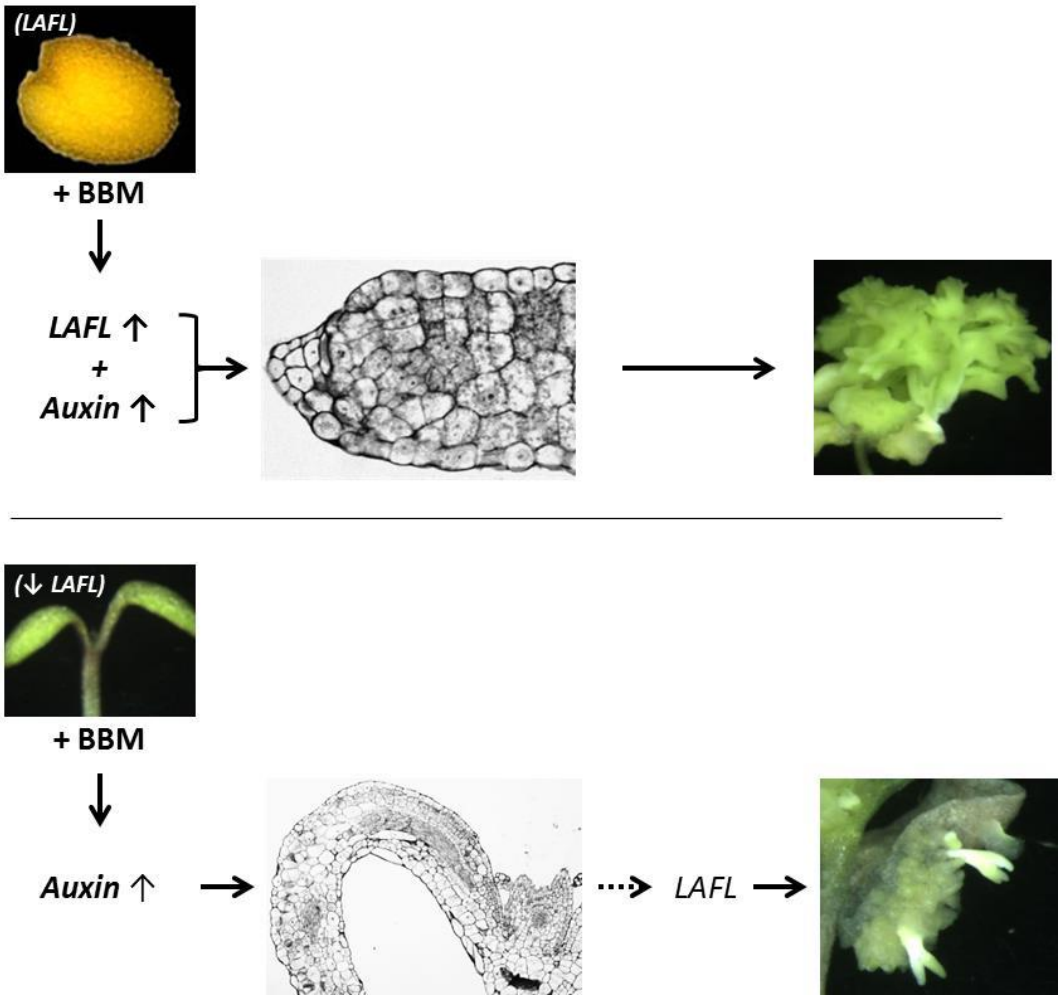

Figure 1: Consequences of early and late BBM activation.
During early BBM activation both *LAFL* and auxin biosynthesis gene expression is simultaneously activated, which leads to enhanced embryogenic cell divisions in sub-epidermal layers of the cotyledon. Late BBM activation activates auxin biosynthesis gene expression, which leads first to callus formation from epidermal and/or subepidermal layers followed by LAFL gene expression and somatic embryogenesis.

Late BBM activation (after germination) does not induce *LAFL* gene expression (Chapter 5) and *LEC1::LEC1:GFP* expression is only detected after approximately seven days, when embryogenic cells emerge from the callus (Chapter 5). In wild type plants, *LAFL* gene expression decreases at germination (Jia et al. 2013). Mutants of certain chromatin remodelling proteins (*pickle*, *bmi1a;bmi1b, ring1a;ring1b vrn2;emf2*, *clf;swn* and *fie*) show elevated LAFL gene expression and form somatic embryos in seedlings, suggesting that chromatin level modifications repress embryo identity and embryo gene expression programs during the transition from seed to seedling

development (Jia et al. 2014). With respect to BBM-mediated somatic embryogenesis, *LAFL* genes might be transcriptionally accessible in a narrow developmental window before germination, allowing early, direct BBM-mediated somatic embryogenesis, but transcriptionally inaccessible after germination. Late BBM-mediated somatic embryo induction would therefore rely first on the activation of auxin biosynthesis to stimulate callus formation, followed by modification of LAFL genes and associated chromatin and- reactivation of *LAFL* gene expression (Fig. 1).

BBM and auxin

Auxin is the most commonly used plant growth regulator that is used for somatic embryo induction (Nolan and Rose 2010; Gaj 2004). We have shown that BBM binds to and regulates the expression of auxin biosynthesis (*TAA1, YUCCA*) and transport genes (*PIN1, PIN4, MULTRIDRUGRESISTANT1(MDR1)/ABCB19, NPY4*), as well as the RING-like transcription factor gene *STY1*, which also activates auxin biosynthesis (via YUC4 and YUC8), while down-regulating GA response (Fridborg et al. 2001; Eklund et al. 2010; Sohlberg et al. 2006). Like the *LAFL* genes, these genes are also differently transcribed upon early and late BBM activation (Chapter 5 and 6). We observed higher IAA levels in BBM-overexpression seedlings, an enhanced auxin response shortly after BBM activation, and an auxin response minimum prior to somatic embryo formation. We also found that inhibiting TAA1 by kynurenine (He et al. 2011) and YUCCA by yucasin (Nishimura et al. 2014) completely abolished visible somatic embryo formation (Chapter 6).

BBM-overexpression seedlings treated with auxin biosynthesis inhibitors formed a pin-like structure from the shoot apical meristem, a phenotype that was not observed in wild type seedlings treated with auxin biosynthesis inhibitors (He et al. 2011; Nishimura et al. 2014). Pin-like structures are observed in inflorescences of Arabidopsis mutants with reduced or altered auxin transport such as *pin1* and *pinoid (pid)* (Gälweiler et al. 1998; Benjamins et al. 2001), either alone or combined with *naked pins in yucca (npy)* mutants or *yuc* mutants (*npy1;yuc1;yuc4* (Cheng et al. 2007). The BBM overexpression phenotype observed in the presence of auxin biosynthesis inhibitors suggests that BBM interferes with establishment of the auxin maximum necessary for organ boundary formation and outgrowth. This phenotype might be explained by a pharmacological (yucasin/kynurenine) reduction of auxin biosynthesis in combination with BBM-enhanced auxin transport away from the SAM. Treating BBM-overexpression seedlings with both auxin biosynthesis inhibitors and the auxin transport inhibitor 1-N-Naphthylphthalamic acid (NPA), might rescue this phenotype by preventing any residual auxin from being transported away from the SAM.

Somatic embryos are not observed after treatment with either auxin biosynthesis or transport inhibitors. Auxin biosynthesis and transport processes might be individually important for somatic embryo growth, but can also be required together for establishment of a local auxin maximum

followed by an auxin minimum. Detailed analysis of the spatial and temporal expression of auxin biosynthesis and transport genes and mutant analysis together with biochemical inhibition studies will be needed to explain the role of the different auxin biosynthesis pathways and dynamics during somatic embryo formation.

BBM network

This thesis demonstrates that BBM/AIL genes can induce somatic embryogenesis in both crop and model species, but that the efficiency and type of embryogenesis is influenced by the donor tissue, and species-specific factors. We have identified a number of genetic and biochemical factors that play a role in BBM-mediated somatic embryogenesis, which when combined with published data can be used to construct a BBM network. This network comprises direct activation of *LAFL* and auxin biosynthesis and transport genes, and an indirect regulation of other genetic and biochemical components (Fig. 2).

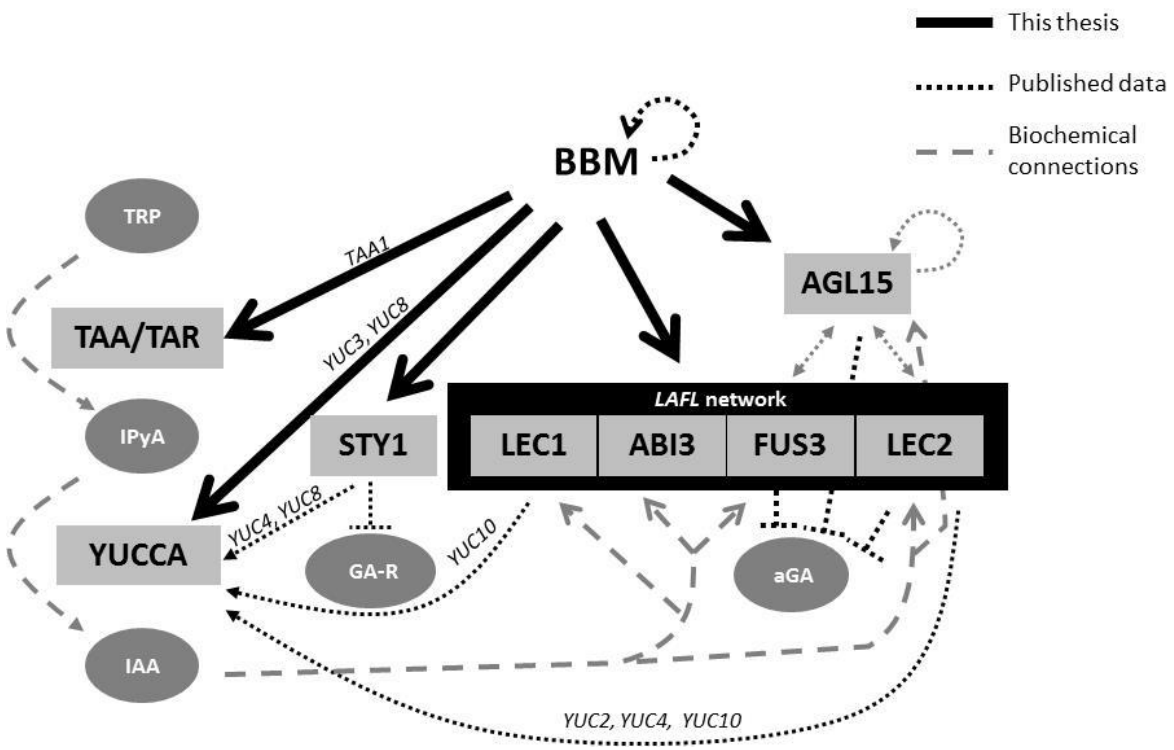

Figure 2: BBM network during somatic embryo induction.
The genetic and biochemical network is based on published data and data from this thesis. BBM activates auxin biosynthesis genes at two steps of the TRP-dependent auxin biosynthesis pathway. The BBM target *STY1* activates the *YUC4 and 8* auxin biosynthesis genes and inhibits gibberellic acid response (GA-R) (Eklund et al. 2010; Fridborg et al. 2001). The LAFL genes *LEC2*, *FUS3* and *ABI3*, are direct BBM targets, and function in a network with each other and with another BBM target, *AGL15* (Jia et al. 2014). *LEC2*, *FUS3*, *ABI3* and *AGL15* expression is regulated by auxin (Gaj et al. 2005; Gazzarrini et al. 2004; Liu et al. 2013), and LEC1 and LEC2 activate auxin biosynthesis gene expression and signalling (Junker et al. 2012; Stone et al. 2008). Bioactive gibberellic acid (aGA) promotes the transition to germination and promotes differentiation (Yamaguchi et al. 2014). Active GA can be

reduced 1.) by AGL15, which directly activates *GA2ox6* expression (GA metabolism) (Wang et al. 2004) while directly repressing the transcription of *GA3ox2* (GA biosynthesis) (Zheng et al. 2009) and recruiting histone acetylase complexes (Hill et al. 2008) or 2.) by the transcriptional down-regulation of *GA3ox2* by *FUS3* and *LEC2* (Curaba et al. 2004).

Our data allow us further to postulate that the simultaneous expression of *LAFLs* and auxin biosynthesis genes underlies BBM-mediated direct somatic embryogenesis, while the initial lack or insufficient expression of *LAFL* genes combined with auxin biosynthesis underlies BBM-mediated indirect somatic embryogenesis (Fig. 1). In line with this, endogenous ABA and GA levels and/or signalling could also be important components of BBM-mediated somatic embryogenesis, as these hormones regulate respectively embryo maturation and seed germination.

Future research on the interaction of BBM with candidate targets that were not discussed in this thesis and the involvement of interacting proteins will increase the knowledge on BBM's activation network.

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Summary

Somatic embryogenesis is an efficient method to regenerate and propagate plants from somatic tissue. Somatic embryo formation is usually initiated under a plant growth regulator regime, which can have a negative impact on the quality of the product and the amount of labour involved, and quite often shows species- and genotype-specific recalcitrance. Alternatively, high quality somatic embryos can be induced rapidly by the overexpression of transcription factors like BABY BOOM (BBM) in the absence or with a reduced amount of plant growth regulators. The scope of this thesis was to investigate the utility of BBM-mediated somatic embryogenesis in the crop plants tobacco and sweet pepper, and to explore the underlying mechanism in the model plant Arabidopsis.

Chapter 1 provides an introduction to the field of somatic embryogenesis. It summarises how observations in nature supported development of the totipotency theory and describes the different potency forms in plants, with reference to the Waddington model. The role of auxin and stress during somatic embryogenesis and the role of embryo identity and maturation genes during zygotic and somatic embryo development are discussed. This chapter also describes how totipotency is acquired in the model plant Arabidopsis, as well as the functions of the *AINTEGUMENTA-LIKE (AIL)* gene family, to which BBM belongs, during development and *in vitro* regeneration.

We first studied the potential of using a *Brassica napus BBM* gene (*BnBBM1*) to induce somatic embryo formation in a heterologous species, the model and crop plant *Nicotiana tabacum* (tobacco) **(Chapter 2**). Somatic embryogenesis was induced in tobacco seedlings by overexpressing a post-translationally-regulated BBM-GR protein (*35S::BBM-GR*). Unlike in Arabidopsis and *Brassica napus*, tobacco somatic embryo formation required exogenous cytokinin and embryos formed at the transition zone between the root and hypocotyl rather than on cotyledons or leaves. The use of the BBM-GR fusion protein prevented pleiotropic phenotypes such as stunted growth and sterility that were previously observed in constitutive *35S::BBM* overexpression lines. Our results demonstrated not only the benefits of an inducible system, but also the functionality of BnBBM in a non-cruciferous species.

Subsequently, we showed in **Chapter 3** that that ectopic overexpression of *BnBBM1* in the vegetable crop sweet pepper, a species that is notoriously recalcitrant to transformation, improved the frequency of transgenic plant regeneration. Our data supports the idea that the bottleneck in sweet pepper transformation is the lack of regenerative competence in tissues susceptible for *Agrobacterium tumefaciens* infection (Wolf et al 2001). The use of the post-translationally inducible BBM-GR fusion protein closed this gap in competence and allowed the regeneration of numerous independent transgenic pepper plants that were fully fertile and transmitted the transgene to the next generations. As with tobacco, additional cytokinin was required for BBM-mediated somatic embryogenesis. While classically induced somatic embryos of sweet pepper showed organ fusions or other deformations that hampered their conversion to seedlings, BBM-induced somatic embryos had no morphological alterations. This BBM-based pepper transformation protocol (**Chapter 4**) and somatic embryogenesis system can now be used to study the function of individual genes and processes in sweet pepper.

To understand the underlying mechanism of BBM-induced somatic embryogenesis we continued with studies in Arabidopsis. In **Chapter 5** we show that overexpression of all AIL-family members, except for ANT and AIL1, induces somatic embryo formation. We demonstrated that changing the BBM or PLETHORA 2 (PLT2) protein dose and developmental stage at which they are overexpressed induces different developmental outcomes. While a low AIL dose prevented differentiation, a medium dose promoted shoot regeneration, and a high dose induced somatic embryogenesis.

BBM/PLT2 activation prior to germination induced prolific and direct somatic embryogenesis, while post-germination BBM/PLT2 activation promoted callus formation followed by less prolific indirect somatic embryo formation. Chromatin immunoprecipitation followed by high throughput sequencing (ChIP-seq) showed that the LAFL genes *LEC1, LEC2, ABI3* and *FUS3* are direct BBM targets. We hypothesized that the different reaction to BBM in developing seedlings (direct or indirect somatic embryogenesis) can be explained by the differential regulation of these target genes by BBM. Mutant analysis demonstrated that the *LEC1* and *FUS3* LAFL genes are required for BBM-mediated direct somatic embryogenesis, whereas *LEC2* enhances the efficiency of this process.

Besides the regulation of the LAFL genes, BBM binds to and transcriptionally regulates auxin biosynthesis and transport genes **(Chapter 6**). *BBM*-overexpressing seedlings have an enhanced *DR5* auxin response and elevated auxin levels. The auxin efflux transport genes *PIN1* and *PIN4* were induced upon BBM overexpression, although more slowly than *DR5*. Biochemical inhibition of auxin biosynthesis and auxin transport prevents the formation of visible somatic embryos, suggesting that these two processes are important steps in BBM-mediated somatic embryogenesis.

The study described in this thesis demonstrate the potential of somatic embryo induction by BBM in crop species, while the study in Arabidopsis reveals that BBM transcriptionally controls a set of embryo-expressed genes that are important for embryo identity, differentiation and maturation. Furthermore, we demonstrated that BBM controls auxin biosynthesis and transport pathways, and that these pathways and these pathways are important components of BBM-mediated somatic embryogenesis.

Samenvatting

Somatische embryogenese is een efficiënte manier om planten vanuit somatisch weefsel te regenereren en te vermeerderen. In het algemeen worden somatische embryo's door plantengroeiregulatoren geïnduceerd, maar deze kunnen van negatieve invloed zijn op de kwaliteit van het product, en niet alle soorten of genotypen reageren even goed of zijn juist ongevoelig. Bovendien vergt deze methode veel handwerk. Een alternatieve wijze om snel somatische embryo's van goede kwaliteit te induceren is door overexpressie van transcriptiefactoren, zoals BABY BOOM (BBM), waarbij geen of nauwelijks extra groeiregulatoren nodig zijn voor de inductie. Het doel van dit promotieonderzoek is de toepasbaarheid van BBM in commerciële gewassen, zoals tabak en paprika, te onderzoeken en tegelijkertijd in het modelgewas Arabidopsis te begrijpen hoe BBM somatische embryogenese induceert.

Hoofdstuk 1 introduceert het onderzoeksgebied van de somatische embryogenese, geeft een kort overzicht hoe observaties van de natuur de basis legden voor de totipotency theorie en beschrijft de verschillende vormen van totipotency met verwijzing naar het Waddington model. De rol van auxine en stress gedurende inductie en ontwikkeling van een somatische embryo, alsook de rol van embryonale identiteitsgenen tijdens de zygotische- en somatische embryo-ontwikkeling worden besproken. Dit hoofdstuk beschrijft ook hoe de modelplant Arabidopsis de staat van totipotency bereikt en de functie van de *AINTEGUMENTA-LIKE (AIL)* genfamilie, tot welke *BBM* behoord, tijdens de plantontwikkeling en *in vitro* regeneratie.

Allereerst is de potentie voor somatische embryogenese van het *BBM*-gen uit koolzaad (*BnBBM1, Brassica napus*) in een heterologe soort zoals tabak (*Nicotiana tabacum)* onderzocht(Hoofstuk2). Overexpressie van een post-translationeel gereguleerd BBM-GR eiwit (*35S::BBM-GR*) induceerde somatische embryogenese in tabak zaailingen, maar anders dan in Arabidopsis en koolzaad, had tabak extra cytokinine nodig om somatische embryo's te vormen. Bovendien werden de embryo's op de overgangszone van wortel naar hypocotyl gevormd in plaats van op de kiemlobben of bladeren. Het gebruik van het BBM-GR fusie eiwit voorkwam ongewenste (pleiotrope) effecten zoals gereduceerde groei en steriliteit, die voorheen werden waargenomen in lijnen met constitutieve overexpressie van *35S::BBM*.

Vervolgens wordt in **Hoofdstuk 3** aangetoond dat overexpressie van *BnBBM1* de regeneratie van transgene paprika planten verbetert. Paprika is een groentegewas dat normalerwijze niet of heel moeizaam te transformeren is. Onze data ondersteunen de stelling dat de weefsels die door *Agrobacterium tumefaciens* geïnfecteerd kunnen worden van zichzelf het vermogen missen om te regenereren (Wolf et al 2001). Het gebruik van het BBM-GR fusie heft dit onvermogen op en maakte de regeneratie van talrijke, onafhankelijk transgene paprika planten mogelijk. Net als bij tabak was ook bij paprika extra cytokinine nodig om de somatische embryogenese door BBM te induceren. Terwijl het klassieke protocol voor somatische embryo inductie in paprika tot orgaanfusie en andere deformaties leidt welke de uitgroei van de embryo's belemmert, was dit bij BBM-geïnduceerde somatische embryo's niet het geval. Het op BBM gebaseerde transformatieprotocol voor paprika (**Hoofdstuk 4**) en het bijbehorende inductiesysteem voor somatische embryogenesis kan nu worden gebruikt om andere genfuncties in paprika te bestuderen.

Om de mechanismen waardoor BBM somatische embryogenese induceert te verklaren, zijn onze studies in Arabidopsis voortgezet. **Hoofdstuk 5** laat zien dat de overexpressie van alle AIL-familie leden, behalve ANT en AIL1, somatische embryogenese induceert. Er wordt aangetoond dat zowel eiwitdosis van BBM of PLETHORA2 (PLT2) als ook het ontwikkelingsstadium, waarin de overexpressie plaatsvindt elk een ander effect geven op het eindresultaat. Terwijl een lage dosis AIL differentiatie voorkomt en een middelhoge dosis AIL scheutregeneratie bevordert, induceert een hoge dosis AIL somatische embryogenese. BBM/PLT2 activatie voor de kieming induceert een sterke en directe somatische embryogenese, terwijl BBM/PLT2 activatie na de kieming callusvorming bevordert en dit tot een minder frequente en indirecte somatische embryo formatie leidt. Immunoprecipitatie van chromatine en daarop volgend grootschalige DNA sequentiebepaling (ChIP-seq) toonde aan dat het BBM-eiwit direct aan de *LAFL*-genen *LEC1, LEC2, ABI3 en FUS3* bindt. Dit leidt tot de hypothese dat het verschil in reactie van ontwikkelende zaailingen op de BBM-activatie (directe of indirecte somatische embryogenese) verklaard kan worden door een verschil in regulatie van deze LAFL-genen. Een

analyse van mutanten voor *lec1* en *fus* liet zien dat deze *LAFL*-genen heel belangrijk zijn voor direct somatische embryogenese geïnduceerd door BBM, terwijl LEC2 de efficiëntie van dit proces verhoogt.
Naast de regulatie van de *LAFL*-genen, bindt BBM ook aan auxinebiosynthese- en transportgenen en reguleert de expressie van deze genen (**hoofdstuk 6**). Zaailingen met *BBM*-overexpressie geven een verhoogde *DR5* auxine respons en hebben een verhoogde concentratie aan auxine. De expressie van de auxine-efflux-transportgenen *PIN1* en *PIN4* werd eveneens door BBM geïnduceerd, maar langzamer dan die van het *DR5*-gen. Zowel de remming van de auxinebiosynthese alsook van auxinetransport voorkomt de zichtbare vorming van somatische embryo's, wat eveneens suggereert dat deze beide processen belangrijk zijn tijdens de BBM-geïnduceerde somatische embryogenese.
Dit promotieonderzoek laat zien dat BBM de potentie heeft om somatische embryogenese in verschillende gewassen te induceren. BBM reguleert een aantal genen, die belangrijk zijn voor de identiteitsbepaling en verdere ontwikkeling van een zygotische embryo. Tenslotte laat dit proefschrift zien dat BBM naast embryo-identiteitsgenen ook auxine-gerelateerde genen bindt en reguleert, en dat beide processen een belangrijk rol spelen tijdens de BBM-geïnduceerde somatische embryogenese.

Acknowledgements

Fifteen lab journals, almost 400 experiments and thousands of analysed plantlets fallen during the BBM-battle later, it is finally done: The booklet has been completed!
This would have been impossible without the understanding and support of a number of people to whom I am deeply grateful.

Joep, about eight years ago I approached you with the idea to dive deeper into the mechanism of somatic embryogenesis, by which I had become fascinated after some experiments with material provided by Kim. You fully understood my motivation, agreed that Arabidopsis and tobacco are not really crops for a vegetable breeding company and therefore no problem to publish. I always loved (and still do) our brainstorm sessions ("*voeten op tafel overleg*"), however, I don't know how you manage to read so many and such a broad range of papers while at the same time being a R&D director. Maybe this is due to you having time to read while you travel or that we just simply share the same fascination. Therefore, a big THANK YOU to you, Joep, and also to the Enza Zaden organisation for giving me the opportunity to follow my passion.

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Kim, BABY BOOMer of the first hour, I still remember your enthusiastic reaction when I sent you the images of the first BBM-somatic embryos in tobacco. You understood my curiosity to discover the underlying factors and my wish to become a PhD student in order to do so. As my supervisor you taught me to look more precisely at my data than I was used to, to think more in a transcription-factor-like manner, and to keep me focussed. Thanks for all your help to put my, sometimes chaotic, thoughts onto paper and pep-talks in between. What I most liked was when we sat together and brainstormed about new projects, of which we hopefully will have a number in the future.

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Curriculum vitae

Iris Heidmann was born on July 3rd, 1963 in Bremen, Germany. She, was trained as a technician at the college of the Ministry of Agriculture of Schleswig-Holstein at the Institute for Horticultural Plant Breeding at Ahrensburg, Germany, after which she worked as a technician at the University of Konstanz (Department of Plant Biochemistry and Physiology) and at the Max-Planck Institute for Plant Breeding Research, Cologne (Department of Molecular Plant Breeding and Max Delbrück Laboratory) before taking a research position at Enza Zaden, Enkhuizen, The Netherlands, in 1999. In 2007 she started her PhD (part-time) in the group of Kim Boutilier and Gerco Angenent at Wageningen University and Research Centre, on applied and fundamental aspects of BBM-mediated regeneration, in parallel to her position as senior researcher at Enza Zaden, where she is still working.

Selected publications

I. Heidmann and K. Boutilier (2015) Pepper, Sweet (Capsicum annuum). In: Agrobacterium protocols Vol. 1233:321-334. Ed. K Wang. Humana Press

I. Heidmann, B. De Lange, J. Lambalk, G. Angenent, K. Boutilier (2011) Efficient sweet pepper transformation mediated by the BABY BOOM transcription factor. Plant Cell Rep 30:1107-1115

C. Srinivasan, Z. Liu, **I. Heidmann**, E. Supena, H. Fukuoka, R. Joosen, J. Lambalk, G. Angenent, R. Scorza, J. Custers, K. Boutilier (2007) Heterologous expression of the BABY BOOM AP2/ERF transcription factor enhances the regeneration capacity of tobacco (*Nicotiana tabacum* L.). *Planta* 225:341-351

I. Heidmann, N Efremova, H Saedler, Z Schwarz-Sommer (1998) A protocol for transformation and regeneration of *Antirrhinum majus*. *Plant J* 13:723-728

M. ten Lohuis, H. Galliano, **I. Heidmann**, P. Meyer (1995) Treatment with propionic and butyric acid enhances expression variegation and promoter methylation in plant transgenes. *Biological Chemistry Hoppe-Seyler* 376:311-320

P. Meyer, **I. Heidmann** (1994) Epigenetic variants of a transgenic petunia line show hypermethylation in transgene DNA: An indication for specific recognition of foreign DNA in transgenic plants. *Mol Gen Genet* 243:390-399

P. Meyer, **I. Heidmann**, I. Niedenhof (1993) Differences in DNA-methylation are associated with a paramutation phenomenon in transgenic petunia. *Plant J* 4:89-100

P. Meyer, **I. Heidmann**, I. Niedenhof (1992) The use of African cassava mosaic virus as a vector system for plants. *Gene* 110:213-217

F. Linn, **I. Heidmann**, H. Saedler, P. Meyer (1990) Epigenetic changes in the expression of the maize A1 gene in *Petunia hybrida*: Role of numbers of integrated gene copies and state of methylation. *Mol Gen Genet* 222:329-336

P. Meyer, S. Kartzke, I. Niedenhof, **I. Heidmann**, K. Bussmann, H. Saedler (1988) A genomic DNA segment from *Petunia hybrida* leads to increased transformation frequencies and simple integration patterns. *Proc Natl Acad Sciences* 85:8568-8572

P. Meyer, **I. Heidmann**, G. Forkmann, H. Saedler (1987) A new petunia flower colour generated by transformation of a mutant with a maize gene. *Nature* 330:677-678

Education statement

Education Statement of the Graduate School

Experimental Plant Sciences

Issued to: **Iris A. Heidmann**
Date: **27 October 2015**
Group: **Molecular Biology, and Bioscience - Plant Developmental Systems**
University: **Wageningen University & Research Centre**

1) Start-up phase	*date*
► **First presentation of your project**	
BBM's network	Feb 01, 2007
► **Writing or rewriting a project proposal**	
TKI-HDAC (2013, granted)	2013
► **Writing a review or book chapter**	
Methods in Molecular Biology (Springer), Kan Wang (ed.), Agrobacterium protocols, Chapter 26, Pepper, Sweet (Capsicum annuum), vol. 1223, pp 321-336	2015
► **MSc courses**	
► **Laboratory use of isotopes**	
Subtotal Start-up Phase	*10.5 credits**

2) Scientific Exposure	*date*
► **EPS PhD student days**	
PhD student days 2015, Get2Gether, Soest, NL	Jan 29-30, 2015
► **EPS theme symposia**	
EPS Theme 1 Symposium 'Developmental Biology of Plants', Leiden, NL	Jan 20, 2011
EPS Theme 1 Symposium 'Developmental Biology of Plants', Wageningen, NL	Jan 19, 2012
EPS Theme 1 Symposium 'Developmental Biology of Plants', Leiden, NL	Jan 17, 2013
EPS Theme 1 Symposium 'Developmental Biology of Plants', Wageningen, NL	Jan 24, 2014
► **NWO Lunteren days and other National Platforms**	
NWO-ALW meeting 'Experimental Plant Sciences, Lunteren, NL	Apr 04-05, 2011
NWO-ALW meeting 'Experimental Plant Sciences, Lunteren, NL	Apr 02-03, 2012
NWO-ALW meeting 'Experimental Plant Sciences, Lunteren, NL	Apr 22-23, 2013
NWO-ALW meeting 'Experimental Plant Sciences, Lunteren, NL	Apr 14-15, 2014
► **Seminars (series), workshops and symposia**	
Japanese-German JPSP and DFG funded workshop, Frontiers of Plant Chromosome Research "Centromeres and Artificial Chromosomes (Gatersleben, Germany)	Oct 31-Nov 03, 2011
Invited seminar John Harada, Keygene, Wageningen	Sep 24, 2012
EPS Frontiers in Plant Development, Wageningen	Nov 13, 2012
Workshop on molecular precision breeding, Gatersleben, Germany	Sep 12-13, 2013
Invited seminars Thomas Laux and Kaoru Sugimoto, Keygene, Wageningen	Sep 19, 2013
Workshop on somatic embryogenesis and molecular breeding, Einbeck, Germany	May 11-13, 2015
► **Seminar plus**	
► **International symposia and congresses**	
Keystone symposia "Plant hormone and signalling" (Keystone, USA)	Feb 10-15, 2008
8th International Symposium on the plant Hormone Ethylene (Ithaca, USA)	Jun 21-25, 2009
Auxins and Cytokinins in plant development (Prague, Cech Republic)	Jul 10-15, 2009
International conference "Molecular aspects of plant development" (Vienna, Austria)	Feb 23-26, 2010
International conference "Plant transformation technologies (Vienna, Austria)	Feb 19-22, 2011
Keystone symposia "Nuclear Events in Plant Gene Silencing and Signaling (Taos, USA)	Mar 06-11, 2012
Auxin Sail (Leiden, TheNetherlands)	Jun 08-09, 2013
23rd ICSPR (International Conference on Sexual Plant Reproduction), Porto, Portugal	Jul 13-18, 2014
► **Presentations**	
Keystone symposia "Plant hormone and signalling" (Poster, Why do BBM cells go Bananas ?)	Feb 10-15, 2008
International conference "Plant transformation technologies"(Poster, BBM-mediated Sweet pepepr transformation)	Feb 19-22, 2011
NVPZ (national) "BBM-mediated Pepper transformation" (Talk)	Jun 28, 2012
Symposium Plant Developmental Biology, Wageningen (Talk)	Oct 14, 2013
23rd ICSPR (International Conference on Sexual Plant Reproduction), Porto, Portugal (2 Posters, Key-elements in BBM-mediated SE; Impedance flow cytometry: A novel method for pollen viability determination)	Jul 13-18, 2014
► **IAB interview**	
Meeting with a member of the International Advisory Board of EPS	Jan 05, 2015
► **Excursions**	
Subtotal Scientific Exposure	*24.3 credits**

3) In-Depth Studies	*date*
► **EPS courses or other PhD courses**	
EPS-Transcription factors	May 09-11, 2011
Microscopy and Spectroscopy in Food and Plant Science	May 06-09, 2014
Basic Statistics	Dec 10,11, 16-18, 2013
► **Journal club**	
Journal club of the PRI cluster every two weeks	2011-2014
► **Individual research training**	
Subtotal In-Depth Studies	*4.6 credits**

4) Personal development	*date*
► **Skill training courses**	
Writing A Scientific Article (VU)	May 2008
Scientific integrity, Wagenngen Language center	Jun 05, 2013
Presentation skills, Wageningen Language center	Sep 17, Oct 01 & 08, 2013
Voice and presentation skills by Mariska Wessel, Voice matters	Oct 01.& 15, 2013
► **Organisation of PhD students day, course or conference**	
► **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*2.7 credits**

TOTAL NUMBER OF CREDIT POINTS*	**42.1**
Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 ECTS credits	
** A credit represents a normative study load of 28 hours of study.*	

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