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ORIGINAL ARTICLE

Steroid-inducible BABY BOOM system for development of fertile *Arabidopsis thaliana* plants after prolonged tissue culture

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Abstract
Key message **We describe a steroid-inducible BABY BOOM system that improves plant regeneration in *Arabidopsis* leaf cultures and yields fertile plants.**
Abstract Regeneration of *Arabidopsis thaliana* plants for extended periods of time in tissue culture may result in sterile plants. We report here a novel approach for *A. thaliana* regeneration using a regulated system to induce embryogenic cultures from leaf tissue. The system is based on BABY BOOM (BBM), a transcription factor that turns on genes involved in embryogenesis. We transformed the nucleus of *A. thaliana* plants with BBM:GR, a gene in which the BBM coding region is fused with the glucocorticoid receptor (GR) steroid-binding domain. In the absence of the synthetic steroid dexamethasone (DEX), the BBM:GR fusion protein is localized in the cytoplasm. Only when DEX is included in the culture medium does the BBM transcription factor enter the nucleus and turn on genes involved in embryogenesis. BBM:GR plant lines show prolific shoot regeneration from leaf pieces on media containing DEX. Removal of DEX from the culture media allowed for flowering and seed formation. Therefore, use of BBM:GR leaf tissue for regeneration of plants for extended periods of time in tissue culture will facilitate the recovery of fertile plants.

Keywords *Arabidopsis thaliana* · BABY BOOM · Plant regeneration · Tissue culture

Abbreviations

A. thaliana	*Arabidopsis thaliana*
ARM	*A. thaliana* tissue culture media
BBM	BABY BOOM
BA	Benzyladenine
DEX	Dexamethasone
GR	Glucocorticoid receptor
IAA	Indoleacetic acid
IPA	Isopentenyladenine
L*er*	*Landsberg erecta*
ptDNA	Plastid genome

Introduction

In *Arabidopsis thaliana* regeneration of plants for several months in tissue culture from leaf tissue resulted in sterile plants (Sikdar et al. 1998). Possible reasons for the sterility of the plants are: polyploidy or aneuploidy caused by 2,4D treatment necessary to trigger uniform leaf cell division; somaclonal variation caused by the prolonged tissue culture regeneration protocol; and natural polyploidy of mature *A. thaliana* leaf cells that were the source of the regenerating plants (Galbraith et al. 1991; Melaragno et al. 1993; Zoschke et al. 2007). Therefore, we were looking for a tissue culture system that enables induction of uniform

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cell division, sustained capacity for plant regeneration and maintenance of the diploid state. Meristematic cells in a shoot apex or cells of a developing embryo meet these criteria. We decided to test whether or not embryogenic cultures obtained by ectopic expression of transcription factors are suitable for regeneration of fertile plants after prolonged tissue culture selection.

BABY BOOM (BBM) is a member of the AP2/ERF family of transcription factors (Nole-Wilson et al. 2005; Riechmann et al. 2000) and it activates developmental pathways associated with cell proliferation and growth (Passarinho et al. 2008). Constitutive expression of BBM was reported to sustain spontaneous production of somatic embryos but did not yield fertile plants (Boutilier et al. 2002). Direct fusion of plant transcription factors with a steroid nuclear receptor has been successfully employed to regulate expression, for example, of the maize transcriptional regulator R (Lloyd et al. 1994), APETALA1 (Wagner et al. 1999), SHOOT MERISTEMLESS (Gallois et al. 2002) and KNOTTED1 (Hay et al. 2003). Although the fusion proteins are constitutively expressed, transcription of the downstream target genes is dependent on the supply of steroid hormones in the culture medium. In the absence of the steroid hormone, the receptor associates with cellular regulatory proteins, including Hsp90, and becomes anchored in the cytosol as a monomer. Association of the steroid with the hormone-binding domain leads to the release of HSP90 from the receptor. The receptor subsequently dimerizes, translocates into the nucleus and binds to the target DNA to activate transcription (Fig. 1a) (Zuo and Chua 2000). Post-translational control of BBM import into the nucleus by fusion to a glucocorticoid receptor (GR) resulted in improved regeneration and yielded fertile plants in chocolate tree, pepper and tobacco (Florez et al. 2015; Heidmann et al. 2011; Srinivasan et al. 2007). Therefore, we decided to regulate BBM function by fusing it with the GR steroid-binding domain (BBM fusion).

Here, we report that *A. thaliana* plants carrying the BBM fusion protein were indistinguishable from non-transformed plants in the absence of the inducer. However, when the synthetic steroid dexamethasone (DEX) was included in the culture medium, prolific shoot regeneration on leaf segments and formation of somatic embryos on seedlings was obtained. Removal of DEX from the media allowed for regeneration of fertile plants after extended periods of tissue culture. We believe that the use of this improved tissue culture system will allow for improved regeneration in ecotypes that are recalcitrant to regeneration in tissue culture. This protocol will also enable experiments in *A. thaliana* that require lengthy cultivation in tissue culture, such as antibiotic selection and may aid in the development of a reproducible plastid transformation protocol for *A. thaliana*.

Materials and methods

Construction of BBM fusion plant lines

Two steroid-inducible BBM constructs were created. Plasmid pKO214 contains the BBM coding region translationally fused with the ligand-binding domain of the rat glucocorticoid receptor (GR) and plasmid pKO216 contains the *BBM* coding region translationally fused with the coding region of *gfp*, the *Aequorea victoria* green fluorescent protein, and the GR (Fig. 1b). The fusion proteins are contained in a *Bam*HI-*Xba*I fragment. The BBM coding

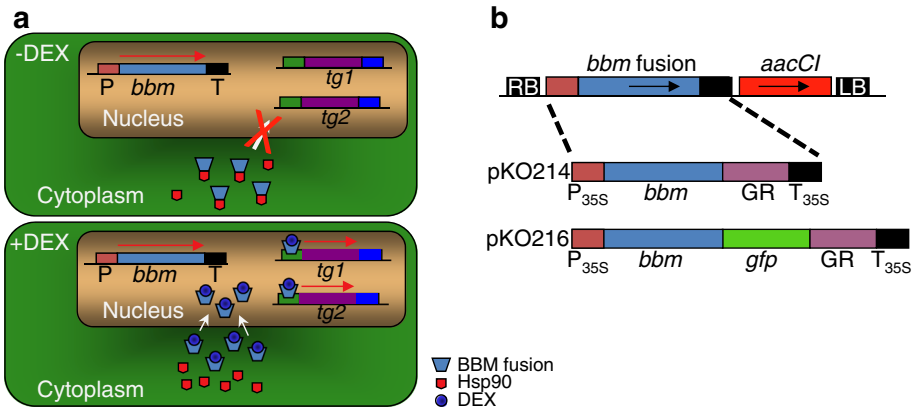

Fig. 1 Steroid-inducible BBM system. **a** The BBM fusion protein remains in the cytoplasm in the absence of dexamethasone (−DEX). After introduction of dexamethasone (+DEX), the BBM fusion protein enters the nucleus and activates target genes (tg). **b** A map of the *Agrobacterium* binary vector pKO214/216 T-DNA region. Shown are the BBM:GR (in plasmid pKO214) and BBM:GFP:GR (in plasmid pKO216) coding region expressed in a 35S promoter–terminator (P_{35S}/T_{35S}) cassette, the *aacC1* gentamycin-resistant gene expressed in the P_2/T_{nos} cassette, and the T-DNA left (LB) and right (RB) border regions

region was PCR amplified from cDNA (Genbank Accession Number AF317907). The *gfp* gene, derived from plasmid PSMGFP, is a soluble modified version of GFP (GenBank Accession No. U70495) and was obtained from the Arabidopsis Biological Resource Center, Columbus, OH, under Stock Number CD3-326 (Davis and Vierstra 1998). The ligand-binding domain of the rat glucocorticoid receptor (508–795) was derived from plasmid pBI-ΔGR constructed by Alan Lloyd (Lloyd et al. 1994). Plasmids pKO214 and pKO216 are pPZP200 binary plasmid derivatives (Hajdukiewicz et al. 1994) in which the plant gentamycin resistance marker is expressed in a 2′ promoter and *nos* terminator cassette. The BBM:GR (pKO214) and BBM:GFP:GR (pKO216) coding regions were cloned as a *Bam*HI–*Xba*I fragments in the Cauliflower Mosaic Virus 35S promoter–terminator cassette derived from plasmid pFF19G (Timmermans et al. 1990). Plasmids pKO214 and pKO216 were independently transformed into either *A. thaliana* ecotype RLD or *Landsberg erecta* (L*er*) by the floral dip protocol (Clough and Bent 1998). Gentamycin-resistant seedlings were transferred to pots where they developed into normal plants, flowered and produced seed. Lines were designated by the ecotype, the plasmid name and a serial number, for example RLD-216-22. Both BBM:GR and BBM:GFP:GR lines will be referred to as BBM fusion plants for the remainder of the text.

Tissue culture media

Tissue culture protocols were adopted from the literature (Czakó et al. 1993; Márton and Browse 1991). We describe here a protocol for plant regeneration from leaves; a variant protocol that regenerates from roots is described elsewhere (Lutz et al. 2011). *A. thaliana* tissue culture media (ARM) are derivatives of the Murashige and Skoog MS medium (Murashige and Skoog 1962). ARM medium: MS salts, 3 % sucrose, 0.8 % agar (A7921; Sigma, St. Louis, MO, USA), 200 mg l^{-1} myo-inositol, 0.1 mg l^{-1} biotin (1 ml l^{-1} of 0.1 mg m l^{-1} stock), 1 ml vitamin solution (10 mg vitamin B1, 1 mg vitamin B6, 1 mg nicotinic acid, 1 mg glycine per ml), pH 5.8. ARM-B medium: ARM medium containing 3 mg indoleacetic acid (IAA), 0.6 mg benzyladenine (BA) and 0.3 mg isopentenyladenine (IPA) per liter. The stocks of plant hormones were filter sterilized, and added to media cooled to 45 °C after autoclaving. Dexamethasone (D4902) purchased from Sigma, St. Louis, MO, USA, was used at 5 μM final concentration in the culture medium. The 10 mM stock solution was prepared by dissolving 4 mg DEX in 1 ml DMSO and filter sterilized using DMSO Safe Acrodisc Syringe Filter (Pall Corporation, Ann Arbor, MI, USA).

Flow cytometry

Plant nuclei were isolated as described (Dolezel et al. 2007) from 10 mg of leaf tissue. The DNA was stained by adding 100 μg/ml propidium iodide; RNaseA (100 μg/ml) was added with the propidium iodide to degrade any RNA present, since propidium iodide can also bind to dsRNA. A FACsCalibur (B.D.) flow cytometer with a doublet-discrimination module was used and nuclei were collected for 5 min. Samples were analyzed with the CellQuest analysis software (BD Biosciences).

Results

Construction of *A. thaliana* plants with an inducible BABY BOOM gene

Plasmid pKO214 contains a translational fusion of the BABY BOOM transcription factor with the glucocorticoid receptor (GR) steroid-binding domain (BBM:GR) and plasmid pKO216 has a BBM:GFP:GR fusion (Fig. 1b) to allow visualization of the movement of BBM from the cytoplasm to the nucleus after introduction of DEX. Plasmids pKO214 and pKO216 were independently transformed into either *A. thaliana* ecotype RLD or L*er* by the floral dip protocol (Clough and Bent 1998). Three-week-old gentamycin-resistant seedlings were transferred to pots where they developed into normal plants, flowered and produced seed. Twenty-one pKO214 (BBM:GR) and sixteen pKO216 (BBM:GFP:GR) independent nuclear transformants were identified by gentamycin resistance. PCR amplification of the BBM gene confirmed gentamycin resistance results. Lines were designated by the ecotype, the plasmid name and a serial number, for example RLD-216-22. Four lines had few (1–2) or no gentamycin-resistant seedlings in the T3 generation. These lines were not analyzed for the ability to induce plant regeneration. Seed from three lines (RLD-pKO216-21, L*er*-pKO216-42 and L*er*-pKO216-81) were germinated on ARM media containing DEX. Response to DEX was inconsistent in these lines and no further characterization was performed. Leaf assays were performed on seven lines (L*er*-pKO214-8, L*er*-pKO214-11, RLD-pKO214-35, L*er*-pKO214-38, RLD-pKO216-1, L*er*-pKO216-2 and RLD-pKO216-22; Table S1). Three lines (RLD-pKO214-35, L*er*-pKO214-38 and RLD-pKO216-22) were identified as consistently responding to DEX by plant regeneration (see below), segregating for gentamycin resistance in a Mendelian fashion in T1 progeny indicating that they are single locus insertion lines, and the lack of segregation for gentamycin resistance upon selfing in T3 and T4 generations of seed

progeny. No further characterization of the remaining BBM lines was performed.

The BBM fusion protein translocates from the cytoplasm to the nucleus after addition of DEX to the culture media

When DEX is present, it associates with the GR hormone-binding domain and triggers translocation of the BBM fusion protein into the nucleus where it can activate transcription from target DNA (Fig. 1a). To confirm movement of the BBM fusion protein from the cytoplasm to the nucleus after induction by DEX, localization of GFP fluorescence in RLD-pKO216-22 plants (containing the BBM:GFP:GR protein) was performed. Nuclei were isolated from leaves grown in the presence or absence of DEX and stained with propidium iodide. Figure 2 shows GFP accumulation in nuclei from leaves grown in the presence of DEX, but no accumulation when grown in the absence of DEX. Nuclei isolated from *A. thaliana* wild-type (RLD-wt) leaf tissue have no detectable GFP fluorescence. Localization of GFP to the nucleus only in the presence of DEX confirms movement of the BBM protein to the nucleus where it can activate genes involved in embryogenesis.

BBM potentiates shoot regeneration from leaves

Previous experiments required short-term cultivation of explants on a medium containing 2,4-D, a synthetic auxin, to induce cell division and efficient shoot regeneration from both leaves (Feldmann and Marks 1986) and roots (Márton and Browse 1991; Valvekens et al. 1988). Since BBM is known to induce somatic embryogenesis in the absence of plant hormones (Boutilier et al. 2002), we cultured leaf sections with and without the inducer, 5 μM DEX, on ARM-B medium (ARM I medium lacking 2,4-D; Márton and Browse 1991) to test if BBM expression can replace the short-term 2,4-D treatment. In 1 month most leaf sections of wild-type L*er* (L*er*-wt) turned brown and died, whether or not the inducer was included in the culture medium (Fig. 3a). In dramatic contrast, L*er*-pKO214-38 leaf sections produced prolific shoots and some callus in the presence of DEX. Activation by DEX is somewhat leaky, since some shoot regeneration is also seen in the absence of the inducer (No DEX, L*er*-214-38; Fig. 3a). The BBM response in the RLD background is similar, but reflects an overall better potential for shoot regeneration from leaf sections (RLD 214-35 and RLD-216-22; Fig. 3a). Thus, significantly enhanced, uniform shoot regeneration could be obtained in both ecotypes in the presence of the inducer indicating that BBM expression could replace induction by 2,4-D.

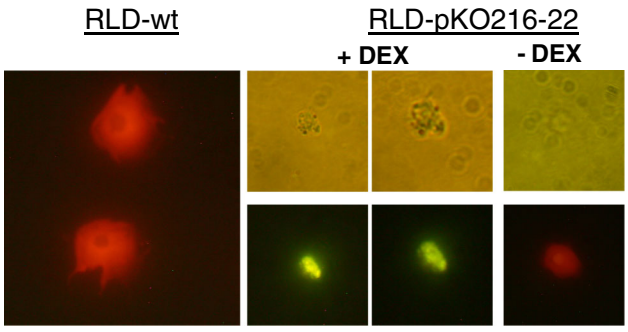

Fig. 2 GFP fluorescence is detected in the nucleus after treatment with the steroid DEX. Nuclei isolated from *A. thaliana* wild-type (RLD-wt) leaf tissue have no detectable GFP fluorescence. Nuclei isolated from RLD-pKO216-22 (BBM:GFP:GR) leaf tissue exhibit GFP fluorescence in the presence of DEX (+DEX). Whereas, nuclei isolated from RLD-pKO216-22 leaf tissue grown in the absence of DEX (−DEX) do not show GFP fluorescence. RLD-wt and RLD-pKO216-22 − DEX nuclei appear red from staining with 10 μg/ml propidium iodide (PI). RLD-pKO216-22 + DEX nuclei were also stained with 10 μg/ml PI, but the GFP fluorescence masks the *red color* (color figure online)

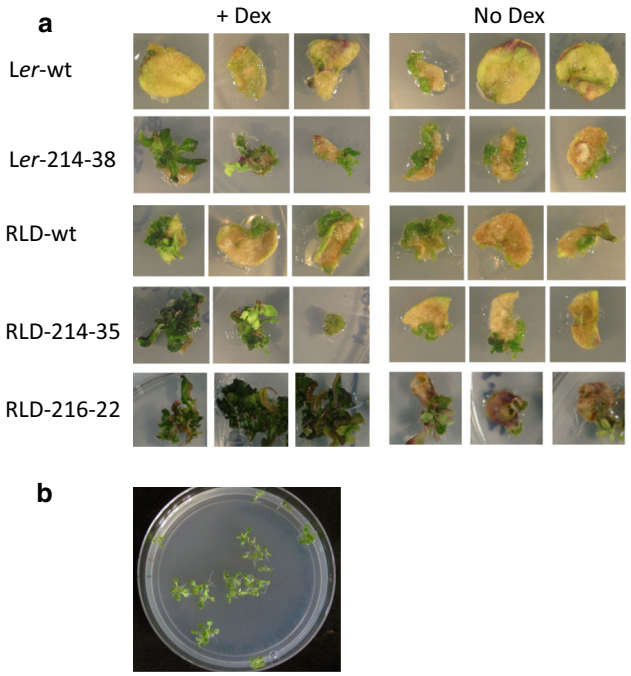

Fig. 3 Homozygous BBM plant lines respond to DEX and regenerated plants are fertile. **a** Dexamethasone-inducible plant regeneration. Wild-type (L*er*-wt and RLD-wt) leaf pieces placed on ARM-B medium containing 5 μM DEX show limited plant regeneration, whereas BBM fusion lines (L*er*-214-38, RLD-214-35 and RLD-216-22) induces prolific shoot regeneration. **b** RLD-216-22-38-6E seedlings formed from regenerated leaf pieces germinate on ARM media

Seed set on regenerated plants are viable and diploid

The *A. thaliana* tissue culture protocol needed improvement in two areas. First the frequency of shoot regeneration from leaves had to be increased and secondly the regenerated shoots need to be fertile. Expression of the BBM fusion protein has resulted in increased shoot regeneration (see above) when leaf tissue is grown in the presence of DEX. To determine if the regenerated plants can develop viable seed, shoots were regenerated from RLD-216-22 leaf pieces by cultivation on ARM-B medium containing DEX. Shoots that formed were cut into small pieces and regenerated multiple rounds on the same media for a minimum of 3 months. Throughout the experiment, tissue was transferred every 2 weeks to fresh media. Seventeen shoots were placed into Magenta boxes containing ARM media lacking DEX to allow for seed formation. Seeds were collected from 13 plants 3 months after transfer to the Magenta box. No seed formed from four of the plants (RLD-216-22-176-3-3D-1, RLD-216-22-176-3-3D-2, RLD-216-22-176-3-9D-4 and RLD-216-22-176-3-9D-5; Table 1). Seed from the 13 plants were sterilized and placed on ARM media containing gentamycin to confirm the presence of the BBM. Resistant seedlings germinating on gentamycin are green, whereas sensitive seedlings are pigment deficient. Seed germinated from all 13 lines indicating that the seeds were viable. All seeds that germinated were gentamycin resistant, confirming that the lines are non-segregating and homozygous. Eleven lines contained some seed that did not germinate and three plant lines (RLD-216-22-176-3-9D-6, RLD-216-22-626-18-2 and RLD-216-22-626-19-5) only produced one viable seed (Table 1). Shown in Fig. 3b are seedlings derived from the RLD-216-22-38-6E line.

In *A. thaliana* it has been shown that as plants develop, ploidy levels in the leaf tissue change. Tetraploid and octoploid nuclei were found in leaf samples as young as 2 days old and up to 128 genome copies were seen in older leaf samples (Zoschke et al. 2007). Since plants regenerated from BBM leaf tissue are fertile, we decided to test if seedlings developed from regenerated plants maintain the diploid state. Flow cytometry was used to analyze the DNA content of single nuclei stained with propidium iodide, a fluorescent dye that binds to DNA stoichiometrically. The diploid state would be represented as the first peak and would indicate the cell is in the G_1 stage of the cell cycle. Each progressive peak would correspond to a doubling of the DNA content of the nuclei (Dolezel et al. 2007). Flow cytometry has been used by plant scientists to determine nuclear DNA content (Arumuganathan and Earle 1991), ploidy level (DeLaat et al. 1987) and to study the cell cycle (Galbraith et al. 1983). To test whether or not the seedlings obtained from the regenerated plants were diploid, flow cytometry was performed on two-week-old seedlings grown on ARM media. Nuclei were isolated from leaves of 2–3 seedlings from 12 of the 13 regenerated lines that produced viable seed. Flow cytometry was not performed on line RLD-216-22-176-3-9D-6. Flow cytometry shows a diploid peak in all samples tested indicating that the regenerated BBM plants are able to form viable seed that develop into diploid plants. Figure 4 shows the peak profile for RLD-wt seedlings and seedlings from two regenerated plant lines (RLD-216-22-38-6E-4-2 and RLD-216-22-626-19-4). Peaks corresponding to increased ploidy levels were

Table 1 Seed progeny from RLD-216-22 regenerated plant lines

Plant line	No. of seeds germinated	No. of seeds not germinated
RLD-216-22-38-6E-3-T3	3	1
RLD-216-22-38-6E-3-T1	13	36
RLD-216-22-38-6E-3-T6	37	38
RLD-216-22-38-6E-4-6	22	22
RLD-216-22-38-6E-4-2	34	5
RLD-216-22-176-3-9D-7	4	0
RLD-216-22-176-3-9D-6	1	20
RLD-216-22-176-3-9D-5	No seed	No seed
RLD-216-22-176-3-9D-4	No seed	No seed
RLD-216-22-176-3-5B-3	5	0
RLD-216-22-176-3-3D-2	No seed	No seed
RLD-216-22-176-3-3D-1	No seed	No seed
RLD-216-22-626-19-5	1	8
RLD-216-22-626-19-4	9	34
RLD-216-22-626-19-3	6	11
RLD-216-22-626-19-2	22	10
RLD-216-22-626-18-2	1	3

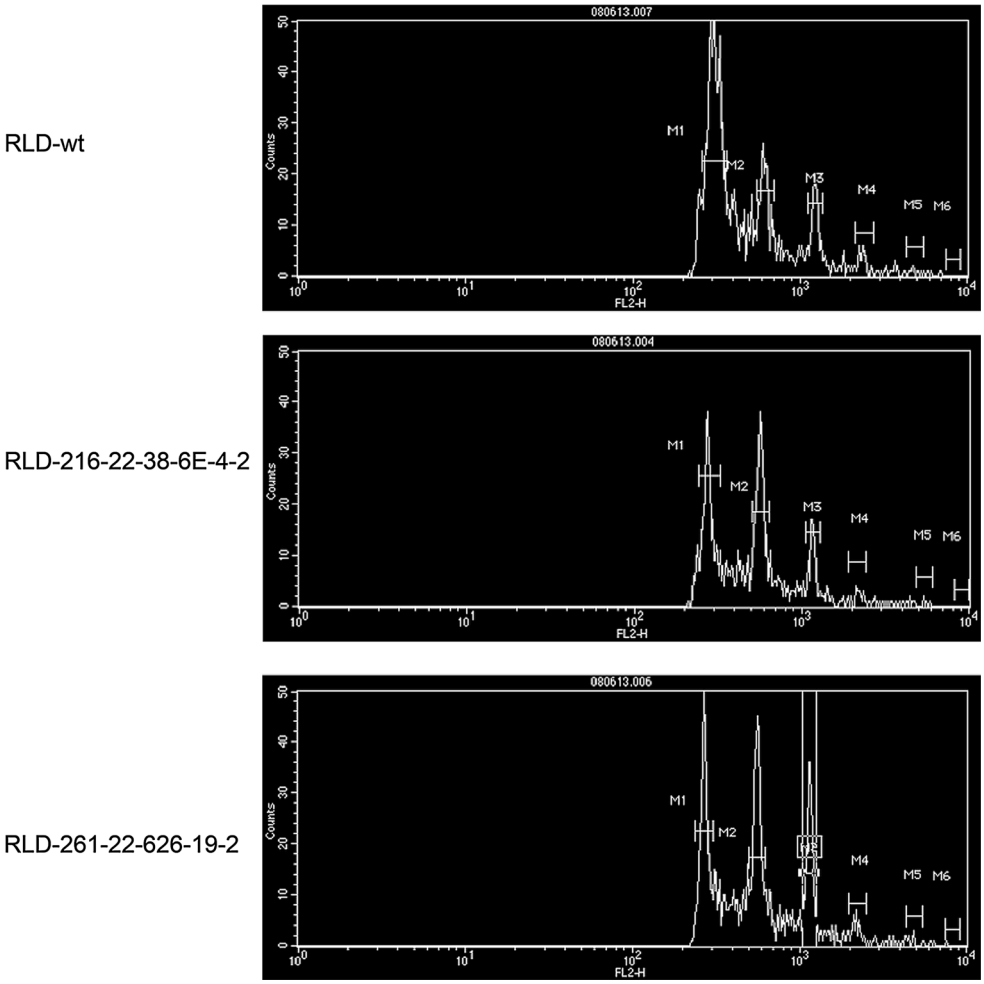

Fig. 4 Seedlings developed from regenerated plants maintain the diploid state. Nuclei were isolated from leaf tissue of 2-3 seedlings from wild-type (RLD-wt) and regenerated lines (RLD-216-22-38-6E-4-2 and RLD-216-22-626-19-2). In all results, the first peak corresponds to diploid cells (2n), and each corresponding peak is due to a doubling of the ploidy (4n, 8n, 8n, 16n, etc.)

also seen in the seed progeny of regenerated plants, as well as in wild-type RLD seedlings, as expected.

Discussion

We report here improved plant regeneration in *A. thaliana* based on a post-translationally controlled BBM fusion protein that results in fertile, diploid plants. Plants expressing the *BBM* fusion gene from a constitutive promoter are normal in the absence of DEX, the GR ligand, but exhibit enhanced shoot regeneration on medium containing DEX. Several other genes have been used in inducible systems to improve plant regeneration in *A. thaliana*. The WUSCHEL gene, when expressed in a transcriptionally regulated system, promoted the vegetative-to-embryonic transition and yielded fertile plants after removal of the inducer (Zuo et al. 2002). LEC1 and LEC2 are two additional seed-expressed transcription factor genes which, when expressed constitutively, promoted spontaneous embryo formation on vegetative tissues (Lotan et al. 1998; Stone et al. 2001; Wojcikowska et al. 2013). Interestingly, regulated over-expression of LEC1 cited in ref. Zuo et al. (2002) and induction of a LEC2:GR fusion (Santos Mendoza et al. 2005) did not result in formation of embryo-like structures. Regulated induction of BBM also induced embryogenesis and improved plant regeneration in tobacco (Srinivasan et al. 2007) and sweet pepper (Heidmann et al. 2011).

The efficiency of shoot regeneration in *A. thaliana* leaf culture is ecotype dependent (Luo and Koop 1997; Schmidt and Willmitzer 1988). Thus, the BBM system can be used to boost shoot organogenesis in ecotypes which are recalcitrant to plant regeneration from cultured cells. Capacity for plant regeneration is also dependent on explant type: roots regenerate plants faster than leaves (Márton and Browse 1991; Valvekens et al. 1988). The efficient flower dip protocol eliminated the need for a reliable tissue culture system for nuclear gene transformation (Clough and Bent 1998). Still, there is a need for a reliable tissue culture system for manipulation of organellar traits (Lutz et al. 2011; Day and Goldschmidt-Claremont 2012; Maliga 2012).

Utility of fertile plastid mutants is shown by selection of a plastid-encoded spectinomycin-resistant mutant (At-RLD-Spc1) in leaf culture to demonstrate pollen transmission of plastids in *A. thaliana* (Azhagiri and Maliga 2007). A similar spectinomycin-resistant *A. thaliana* mutant was isolated in root culture in the *Landsberg erecta* background (Lutz et al. 2011). The steroid-inducible BBM plant regeneration system results in fertile plants after regeneration from leaf pieces for several months and thus would be a suitable system for use in protocols that require extended cultivation in tissue culture. Plastid transformation protocols, which require cultivation in vitro for up to 12 weeks (Maliga and Bock 2011; Bock 2015), would benefit most from this new system.

Author contribution statement KL and PM designed research. KL constructed plasmids, transformed plants and characterized transformants. KL and CM performed flow cytometry. KL and SK performed plant regeneration experiments. KL and PM wrote the paper.

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Conflict of interest The authors declare that they have no conflict of interest.

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