

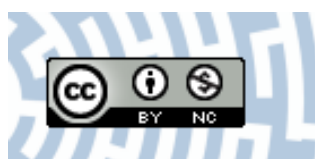


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Title: LEAFY COTYLEDON1, FUSCA3 expression and auxin treatment in relation to somatic embryogenesis induction in Arabidopsis

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LEAFY COTYLEDON1*, *FUSCA3* expression and auxin treatment in relation to somatic embryogenesis induction in *Arabidopsis

Agnieszka Ledwoń · Malgorzata D. Gaj

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Abstract The expression pattern of the *LEC1* and *FUS3* genes during somatic embryogenesis in *Arabidopsis* explants (immature zygotic embryos) induced in vitro was analysed, using Real-time quantitative PCR (qRT-PCR). The analysis revealed differential expression of *LEC1* but not *FUS3* within a 30 day time course of somatic embryo development, and a significant auxin-dependent upregulation of *LEC1* was found over the time course. In contrast to embryogenic culture, the level of *LEC1* and *FUS3* expression was noticeably lower in non-embryogenic callus of Col-0 and hormonal mutants (*cbp20* and *axr4-1*) with low SE-efficiency. In addition, the expression profile of *LEC1* and *FUS3* was followed in the embryogenic culture derived from 35S::LEC2-GR explants. A significant increase of *LEC1* but not *FUS3* activity was observed under LEC2 overexpression induced in auxin-treated explants. The work provides further experimental evidence on LEC gene involvement in the embryogenic response in *Arabidopsis* somatic cells, and also implicates *LEC1* function in more advanced stages of SE culture in relation to somatic embryo differentiation and development.

Keywords Auxin · Embryogenic transition · *FUSCA3* · Hormonal mutants · *LEAFY COTYLEDON1*, *LEC2* overexpression

Abbreviations

2,4-D 2,4-dichlorophenoxyacetic acid
E Induction medium
IZE Immature zygotic embryo

SE Somatic embryogenesis
ZE Zygotic embryogenesis

Introduction

Somatic embryogenesis (SE) manifests a unique ability of plant cells to reprogram the normal pattern of somatic development into an embryogenic one, leading to the formation of structures resembling zygotic embryos. The SE process therefore provides an attractive system for studies on totipotency mechanisms and the developmental switch of somatic cells towards embryogenesis. Moreover, in plant biotechnology the phenomenon of SE is widely applied for micropropagation and genetic transformation.

The availability of a rich resource of genomic data, plus a number of versatile molecular tools, coupled with a simple in vitro culture system, predisposes *Arabidopsis* to molecular studies on SE (Gaj 2004). In addition, the implementation of genomic expression methods in the recent decade has greatly speed up the identification of numerous genes with potential involvement in the determination of SE. With the use of transcriptome analysis, candidate SE-specific genes were identified in a number of plant species, including gymnosperms (Stasolla et al. 2004; Aquea and Arce-Johnson 2008), *Glycine max* (Thibaud-Nissen et al. 2003), *Triticum aestivum* (Singla et al. 2007), cotton (Zeng et al. 2006), maize (Che et al. 2006), potato (Sharma et al. 2008), oil palm (Low et al. 2008) and *Cyclamen persicum* (Hoenemann et al. 2010). The available data confirmed a substantial complexity of gene networks involved in the onset and progress of SE (Zeng et al. 2007), in which genes with regulatory functions, such as transcription factors (TFs), are predominantly represented

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and predicted to have an essential role (Singla et al. 2007). However, the great majority of these genes needs further analysis to annotate their function in embryogenic development taking place in plant somatic cells. Only a small set of TF-specific genes have been experimentally proven to function in this way, including *BABY BOOM* (*BBM*, Boutilier et al. 2002), *WUSCHEL* (*WUS*, Zuo et al. 2002), *AGAMOUS-LIKE15* (*AGL15*, Harding et al. 2003), and *LEAFY COTYLEDON2* (*LEC2*) (Stone et al. 2001; Ledwoń and Gaj 2009).

Aside from *LEC2*, two other *LEC* genes, *LEC1* and *FUS3*, master regulators of the morphogenic and maturation phase of ZE (Harada 2001), are believed to be involved in SE. The *LEC* genes are postulated to link the maturation phase of ZE and initiation of SE via the establishment of the proper environment for cellular differentiation (Braybrook and Harada 2008). In the context of SE process, *LEC2* is the best recognized regulator in terms of possible mechanisms involved in the reprogramming of somatic cells towards embryogenesis (Stone et al. 2001; Braybrook et al. 2006; Stone et al. 2008; Ledwoń and Gaj 2009).

LEC1 and *FUS3* on the other hand are less well known in their role in SE. *LEC1* and *FUS3* proteins are built up from distinctly different structural domains. *LEC1* contains a homolog of yeast HAP3, or the mammalian NF-YB/CBF-A trimeric CCAAT-binding factor subunit, CBF, which consists of three domains (Lotan et al. 1998). During carrot embryo development C-*LEC1* was indicated to be a component of the CCAAT box-binding complex together with C-HAP2B and C-HAP5A or C-HAP5B (Yazawa and Kamada 2007). *LEC1*, and a closely related *LEC1-LIKE* (*L1L*), constitute a class of *LEC1-LIKE* (*L1L*) proteins (Kwong et al. 2003). In a similar way to *LEC1*, *L1L* is expressed during seed development, but suppression of *L1L* expression resulted in a phenotype different to the *lec1* mutant suggesting that *LEC1* and *L1L* play quite different roles in embryogenesis (Kwong et al. 2003). In ZE *LEC1* expression is referred to as embryo maturation (Meinke et al. 1994; West et al. 1994; Parcy et al. 1997), although activity of the gene has also been reported at earlier stages of zygotic embryo development (Wang et al. 2007). Knowledge on the molecular action of *LEC1* and its target genes is limited. *LEC1* was implicated in seed storage protein accumulation via control of *FUS3* and *ABI3* expression (Kagaya et al. 2005a), and the regulation of *LEC2* by *LEC1*, in a rather indirect manner, was suggested by Kagaya et al. 2005a and Santos-Mendoza et al. 2005. Recently, it was shown that *LEC1*, together with *L1L*, can activate CRUCIFERIN C (*CRC*), a seed storage protein, and SUCROSE SYNTHASE 2 (*SUS2*) via functional coupling with the ABA-response element (*ABRE*) binding factor (Yamamoto et al. 2009).

FUS3, together with *ABI3* and *LEC2* belongs to a AFL B3 subfamily of the plant-specific B3 domain of TFs, and its involvement in the transition between embryogenesis and vegetative phase of development, as was strongly proposed by Suzuki and McCarty (2008). These authors revealed the extensive role of the AFL B3 genes in regulating the principle hormone signaling pathways in seeds, including ABA, GA and auxin. Accordingly, *FUS3* negatively regulates GA accumulation through repression of the GA-biosynthesis genes, *GA3ox1* and *GA3ox2* (Gazzarrini et al. 2004, Curaba et al. 2004), and up-regulates ABA accumulation (Gazzarrini et al. 2004). In a similar way to other *LEC* genes, *FUS3* controls the synthesis of storage proteins and lipids during the maturation phase of ZE (Kagaya et al. 2005b), and the gene was found to be activated by *LEC1* (Kagaya et al. 2005a) and *LEC2* (Stone et al. 2008).

In plant development *LEC1* and *FUS3* expression is limited to zygotic embryos, and declines during germination (Lotan et al. 1998; Reidt et al. 2001). The tight and multidirectional interactions between *LEC1*, *FUS3* and *LEC2* genes during different stages of ZE has been documented to include a superior function of *LEC1* and *LEC2* over *FUS3* (Wang et al. 2007) and mutual regulation of *LEC1* and *LEC2* (Santos-Mendoza et al. 2005; Kagaya et al. 2005a). It has not yet been elucidated whether similar interactions between *LEC* genes exist in somatic cells induced to undergo embryogenesis.

In the present work, to gain more insight into the possible functions of *LEC* genes in SE, the expression profiles of *LEC1* and *FUS3* at various stages of in vitro induced embryogenic culture were defined, in relation to exogenous auxin treatment and tissue capacity for SE.

Materials and methods

Plant material

The Columbia (Col-0) genotype of *Arabidopsis thaliana* (L.) Heynh., and two hormonal mutants (*axr4-1* and *cbp20*) of low capacity to produce somatic embryos under in vitro conditions (Gaj et al. 2006) were studied. The *axr4-1* form belongs to a class of auxin resistant mutants (Hobbie and Estelle 1995), while *cbp20* is hypersensitive for abscisic acid and is drought tolerant (Papp et al. 2004). Seeds of the *axr4-1* mutant and Col-0 parental genotype were supplied by NASC (The Nottingham Arabidopsis Stock Centre), and the *cbp20* mutant was kindly provided by Z. Szwedkowska-Kulińska, University of Adam Mickiewicz—Poland. The *cbp20* mutant carries a knock-out mutation (Papp et al. 2004) and the *axr4-1* possesses a T-DNA insertion at the beginning of the *AXR4* gene

(Dharmasiri et al. 2006). Additionally, the Col-0 transgenic line (12/1/8) harboring a single copy of the 35S::LEC2-GR construct, and expressing a high and stable level of *LEC2* transcript (Ledwoń and Gaj 2009), was also used in the experiments.

Somatic embryogenesis induced in vitro

Immature zygotic embryos (IZEs) with fully developed green and bent cotyledons were cultured in vitro following a standard protocol which enables efficient and direct somatic embryo development in the control Col-0 genotype (Gaj 2001). Ten IZEs were cultured in a Petri dish (35 mm) on Phytigel-solidified (3.5 g l^{-1}) induction medium (E) containing basal B5 micro and macro-elements (Gamborg et al. 1968) and 20 g l^{-1} sucrose. E media with auxin, $5 \text{ }\mu\text{M}$ 2,4-D, Sigma (E5) and without auxin (E0) were used.

Plant growth and in vitro culture conditions

Plants used as a source of explants for in vitro culture were grown in mixture of the soil and vermiculite (1:1), at 22°C under a 16 h photoperiod of $100 \text{ }\mu\text{M m}^{-2}\text{s}^{-1}$ white fluorescent light; and embryogenic cultures were kept at 23°C under a 16 h photoperiod of $40 \text{ }\mu\text{M m}^{-2}\text{s}^{-1}$ white fluorescent light.

Induction of LEC2 protein activity in transgenic plants

To induce activity of the LEC2 protein a synthetic glucocorticoid, dexamethasone (water soluble DEX, Sigma), was added to the media at a concentration of $30 \text{ }\mu\text{M}$, as described by Ledwoń and Gaj (2009).

RNA isolation and qRT-PCR analysis

An RNAqueous Kit (AMBION) was used to isolate RNA. The concentration and purity of RNA was evaluated with a ND-1000 spectrophotometer (NanoDrop). To control for DNA contamination RNAs were treated with RQ1 RNase-free DNase I (Promega), following the manufacturer's instructions. First-strand c-DNA was produced in a $40 \text{ }\mu\text{l}$ reaction volume using the RevertAid First Strand cDNA Synthesis Kit (Fermentas). The product of reverse transcription was diluted with water in 1:1 ratio, and $1 \text{ }\mu\text{l}$ of this solution was used for quantitative RT-PCR (qRT-PCR). qRT-PCR was done in a $10 \text{ }\mu\text{l}$ reaction volume with the use of LightCycler FastStart DNA Master SYBR Green I (Roche) and appropriate primers.

The following primers were used for qRT-PCR reactions of the analysed genes:

LEC1 (R): 5'-CTGGACCACGATACCATTGTT-3'
(F): 5'-GTGGAGCTCCCTTCTCTCACT-3'
FUS3 (R) TGAAGGTCCAAACGTGAAAAC
(F):GTCAGCTCTCTCCGACGTATG
At4g27090 (R) 5'-CCTCGATCAAAGCCTTCTTCT-3';
(F) 5'-F:GTCGTTATCGTCGACGTTGTT-3'

The LightCycler 2.0 (Roche) real-time detection system was used with the following reaction conditions: denaturation one repeat of 10 min at 95°C , followed by 45 repeats of 10 s at 95°C , 8 s at 55°C , 12 s at 72°C and 5 s at 80°C . Denaturation for melt curve analysis was conducted at 95°C followed by 15 s at 65 and 95°C (0.1°C/s for fluorescence measurement).

Primary data analysis was performed with LightCycler Software 4.0 (Roche). Relative RNA levels were calculated and normalized to an internal control, the *At4g27090* gene encoding 60S ribosomal protein (Thellin et al. 1999). In all analyzed tissue samples the control gene exhibited a constant expression pattern with $C_p = 18 \pm 1$.

The expression values normalized to the internal control were standardized differently as indicated in the figure legends.

Total RNA was isolated from freshly isolated (0 day) explants induced on E5 and E0 media. Tissue sampling included 1, 3, 5, 10, 15 and 30 day for E5-induced cultures, and 3, 5 and 10 day for E0-growing explants. Depending on culture age, 250 (0 day)–2 (30 day) tissue samples were used per treatment. The plant tissues for the qRT-PCR analysis were produced in 3–4 independent biological replicates, and 2 technical replicates of each repetition were carried out.

Statistical analysis

ANOVA rang Kruskal–Wallis and U Mann–Whitney's statistical tests were applied to calculate significant differences (at $P = 0.05$) between samples.

Results

Col-0 embryogenic culture

The analysis of *LEC1* and *FUS3* expression profile took place during the time course of embryogenic cultures induced in vitro on auxin and auxin-free medium. Under in vitro conditions inductive for SE in Arabidopsis, i.e. in cultures of Col-0 IZEs on E5 auxin medium, the explant tissues undergo successive morphological changes characteristic of early, transient and advanced stages of embryogenesis (Fig. 1). The Real-Time PCR analysis

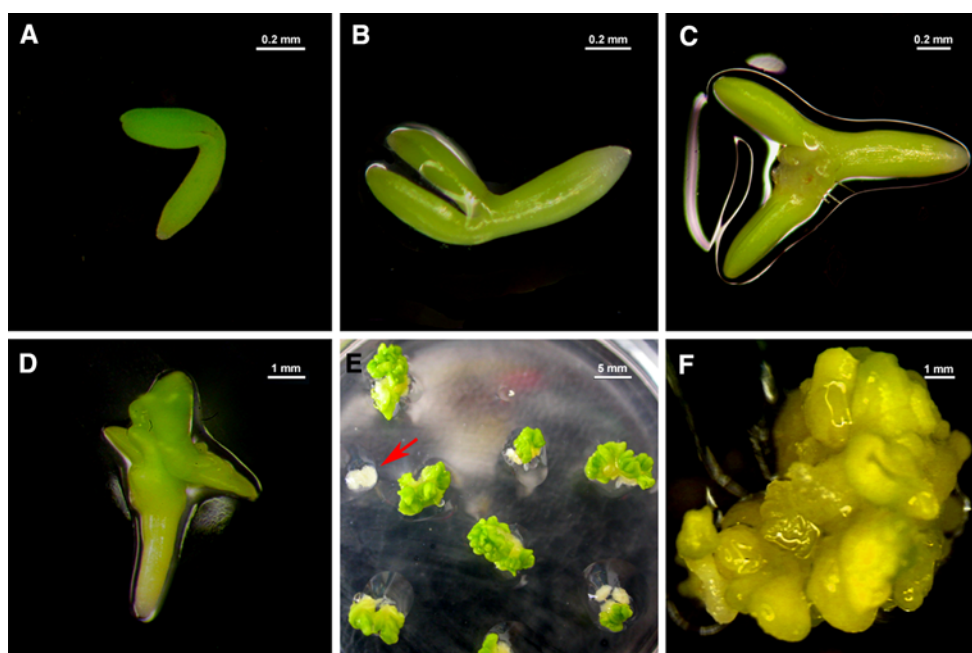


Fig. 1 Developmental stages of Col-0 IZE explants cultured on E5 medium (**a–f**) and analysed with Real-Time qPCR. **a** freshly isolated IZE explant of late cotyledonary stage (0 day); **b** straightening of cotyledons (3 day); **c** swelling and expanded cotyledons (5 day);

d embryo-like protuberances on the surface of cotyledons (10 day); **e** explants covered with fully developed somatic embryos and callus (an *arrow*) formed sporadically from 10% of explants (15 day); **f** aging of culture and callus development (30 day)

focused on selected stages of SE, and included: initiation phase (3 and 5 day-old cultures), manifested by distinct changes of cotyledon morphology (straightening, enlargement and swelling); emergence of the first embryos (10 day); development of numerous embryos (15 day) and aging of the cultures manifested by callus overgrowth due to the re-differentiation of somatic embryos. Beside embryogenic cultures induced on auxin medium, the IZEs induced on auxin-free medium (E0) were analysed up to day 10, when instead of the embryogenic response, a regular development of seedlings is observed.

The expression level of the *LEC1* gene in the tissues sampled between 0 and 30 day of Col-0 culture on E5 revealed a progressive expression pattern. A significant but temporary increase of expression was observed at the 1st day of culture, followed by a drastic decrease at 3 day and a subsequent successive increase of activity up to 15 day (Fig. 2a). In contrast to a period of embryogenic induction and intensive embryo development (3–15 day), related to stimulation of *LEC1* activity, a significant fall of the gene activity was detected in aging 30 day-old cultures.

A distinctly different pattern of *LEC1* expression was observed on auxin-free medium where, except for the 3rd day, the explants cultured on E0 medium presented up to sixfold lower (10 day) expression compared with auxin-induced cultures (Fig. 2b). The observed differences in the

pattern and level of expression found in E0 and E5 induced cultures demonstrates the SE-specific and auxin-mediated stimulation of *LEC1* in embryogenic culture.

In order to further verify the suggestion that up-regulation of the *LEC1* gene observed on auxin medium is specific to embryogenic tissue, the level of transcripts was also analysed in non-embryogenic Col-0 callus produced sporadically on E5 medium by some explants unable to undertake embryogenic transition. qRT-PCR analysis confirmed up to a tenfold lower expression level of *LEC1* in 15 day callus compared with embryogenic cultures of the same age (Fig. 2c).

As for *LEC1*, the activity of *FUS3* was detected at all stages of SE, but unlike *LEC1* the expression level of *FUS3* was not differentiated during SE and remained at the level observed in freshly isolated explants (Fig. 3a). Moreover, it was found that the expression profiles of *FUS3* on E5 and E0 media were similar, implying that the gene activity is not influenced by exogenous auxin (Fig. 3b). Regardless of auxin presence in the medium, *FUS3* transcripts displayed a steady level at the monitored stages of SE, suggesting that the gene activity is not specific to any particular stage, and that a higher expression level accompanies embryogenic cultures. Accordingly, *FUS3* expression was increased in cultures producing somatic embryos above the level observed in non-embryogenic callus of Col-0 (Fig. 3c).

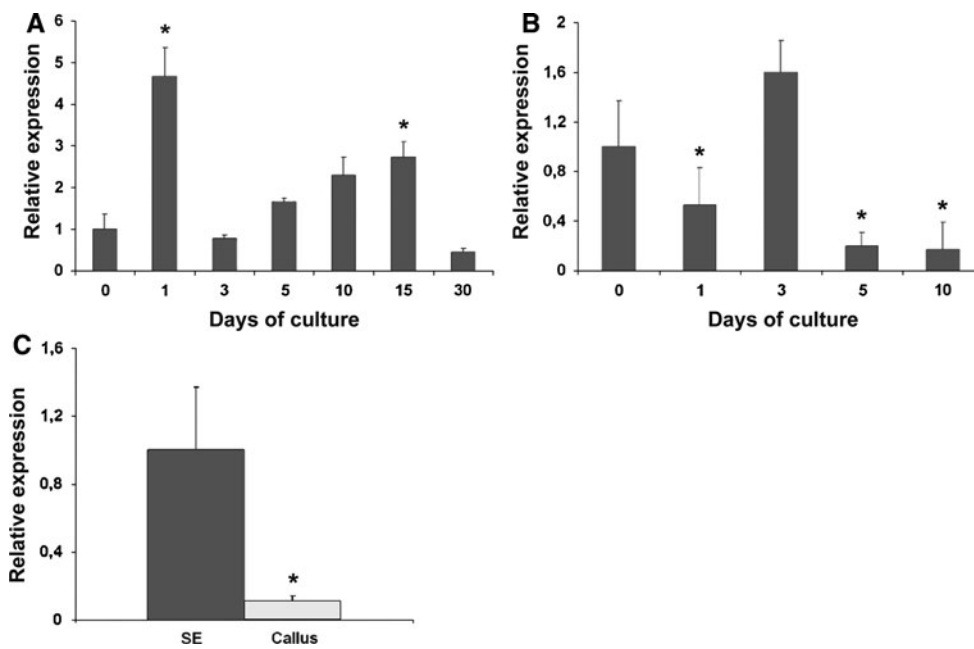


Fig. 2 Expression patterns of the *LEC1* gene in IZE-derived cultures. **a** embryogenic and **(b)** non-embryogenic cultures of Col-0 explants on auxin, E5 **(a)** and auxin-free, E0 **(b)** medium, respectively. **c** embryogenic (SE) and non-embryogenic (callus) tissue of Col-0 explants on E5 medium at 15 day. The activity of the gene was standardized to: E5

culture at 0 day **(a)**, E5 culture **(b)**, E5 embryogenic culture at 15 day **(c)**. *Expression level significantly differs from those observed in: culture at 0 day **(a)**; culture on E5 medium **(b)**, embryogenic culture at 15 day **(c)**; $P < 0.05$. RNA levels were normalized to that of *At4g27090*. Bars show standard deviation

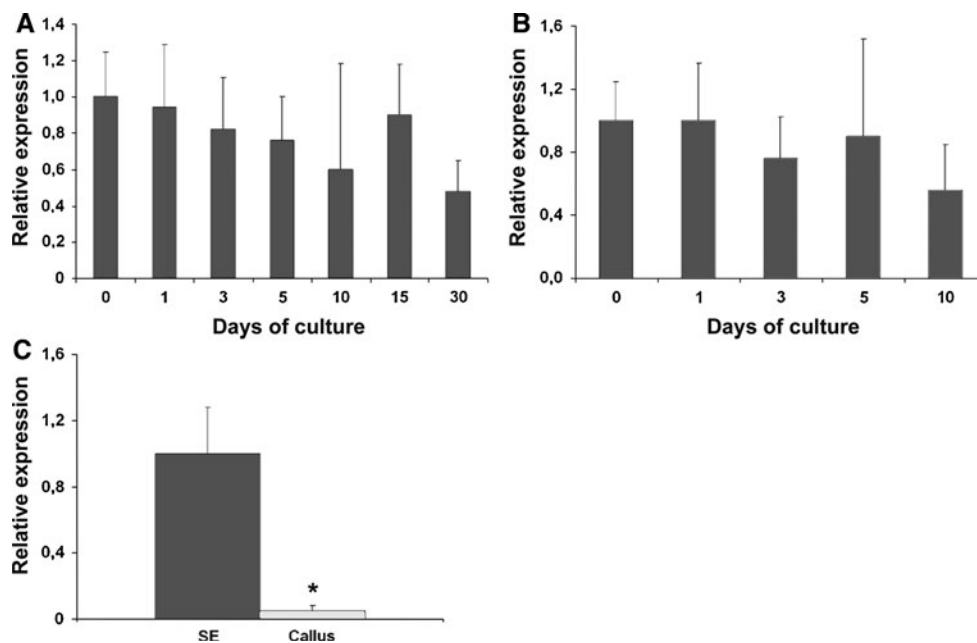


Fig. 3 Expression patterns of the *FUS3* gene in IZE-derived cultures. **(a)** embryogenic and **(b)** non-embryogenic cultures of Col-0 explants on auxin, E5 **(a)** and auxin-free, E0 **(b)** medium, respectively. **c** embryogenic (SE) and non-embryogenic (callus) tissue of Col-0 explants on E5 medium at 15 day. The activity of the gene was

standardized to: E5 culture at 0 day **(a)**, E5 culture **(b)**, E5 embryogenic culture at 15 day **(c)**. *Expression level significantly differs from those observed in: culture at 0 day **(a)**; culture on E5 medium **(b)**, embryogenic culture **(c)**; $P < 0.05$. RNA levels were normalized to that of *At4g27090*. Bars show standard deviation

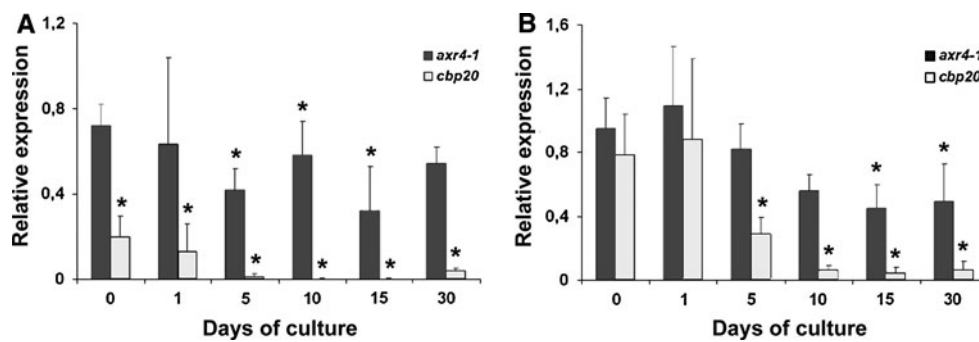


Fig. 4 Expression level of *LEC1* (a) and *FUS3* (b) genes in cultures of SE-impaired mutants (*cbp20* and *axr4-1*) induced on E5 medium. The activity of the gene was standardized to Col-0 culture on E5. *Expression level differs significantly from observed in Col-0

embryogenic culture ($P < 0.05$). RNA levels were standardized to embryogenic Col-0 culture and normalized to that of *At4g27090*. Bars show the standard deviation

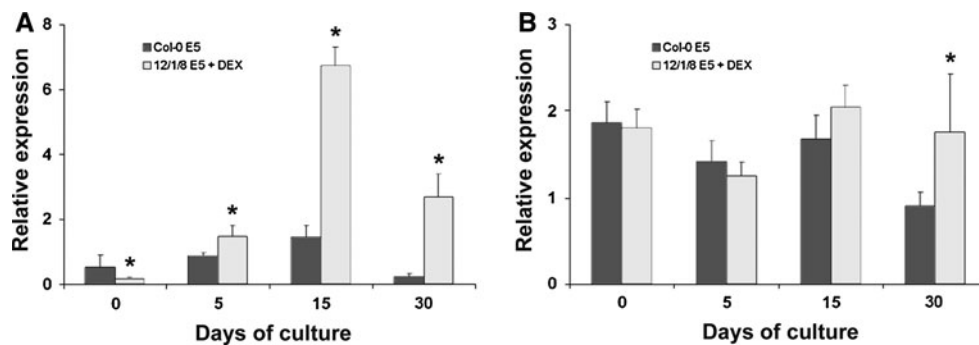


Fig. 5 Expression pattern of *LEC1* (a) and *FUS3* (b) genes in embryogenic cultures induced on E5 medium in explants overexpressing the *LEC2* gene (35S::*LEC2-GR*, Col-0). The activity of the gene was standardized to Col-0 culture on E5. *Expression level

differs significantly from observed in Col-0 embryogenic culture ($P < 0.05$). RNA levels were normalized to that of *At4g27090*. Bars show standard deviation

Mutants impaired in SE capacity

In addition to the Col-0 highly embryogenic genotype, *LEC1* and *FUS3* expression was also determined in cultures of *cbp20* and *axr4-1* hormonal mutants (Fig. 4). The mutants were selected for their strongly impaired capacity for SE, which results in a decreased frequency of explants producing somatic embryos and abundant callus formation (Gaj et al. 2006). Both mutant cultures displayed patterns and levels of *LEC1* (Fig. 4a) and *FUS3* (Fig. 4b) expression, manifested by a significant reduction of their activity, different to Col-0. In *cbp20* there was an especially strong inhibition of gene activity, with highly reduced SE-efficiency and productivity (Gaj et al. 2006). This mutant also displayed a drastic decrease in *LEC1* transcript level in all monitored time points of the culture, including freshly isolated explants, while *FUS3* transcripts were significantly down-regulated from day 5 onwards.

Embryogenic culture overexpressing *LEC2*

The activity of the *LEC1* and *FUS3* genes was also monitored during a 0–30 day period of embryogenic culture

derived from IZEs of the 35S::*LEC2-GR* transgenic line. The transgene encodes a fusion *LEC2-GR* protein which enters a nucleus only upon DEX treatment (Sablowski and Meyerowitz 1998). We proved a constitutive and highly increased *LEC2* expression level (up to 3000-fold) in transgenic plants and in vitro cultured explants regardless of DEX treatment. Simultaneously, DEX treatment induced developmental abnormalities in transgenic seedling and somatic embryo development *in planta* and in vitro (Ledwoń and Gaj 2009). Moreover, functionality of the construct was also indicated by observation that DEX treatment induced expression of *LEC1* and *FUS3* in 7- and 28-day old transgenic plants (data not presented).

Analysis of *LEC1* and *FUS3* transcripts indicated that their general expression pattern in transgenic culture followed that observed in the Col-0 control. Accordingly, a significant increase of *LEC1* (Fig. 5a), and a constant *FUS3* (Fig. 5b) expression level between 5 and 15 day of culture, were detected. However, under *LEC2* overexpression a significant up-regulation (2–11-folds) of the *LEC1* transcription level was recorded in cultures induced to initiate embryos. Contrary to *LEC1*, expression level of *FUS3* was not elevated in transgenic culture within a period

corresponding to SE induction and embryo development. However, a significant up-regulation of *FUS3* over the level detected in non-transgenic culture was observed in aging 30 day tissue.

Discussion

In agreement with an early postulate on the genetic similarity between somatic and zygotic embryogenesis (Zimmerman 1993), there is growing evidence confirming a close relationship between these apparently different embryogenic pathways triggered in two different cell types. Among the key genes controlling embryo development in zygotic and somatic plant cells, the *LEC* genes of Arabidopsis were postulated to play a crucial role (Braybrook and Harada 2008). The mechanisms involved in *LEC*-related control of ZE have been extensively investigated and recognized (Harada 2001; Braybrook and Harada 2008; Suzuki and McCarty 2008), while the function of *LEC* in SE was already revealed. Recent experimental data strongly confirmed the link between the *LEC2* gene and the induction of SE (Stone et al. 2008; Ledwoń and Gaj 2009), but much less is known about two other closely related *LEC* genes, *LEC1* and *FUS3*, in terms of molecular mechanisms driven by them in somatic cells.

The suggestion on the involvement of *LEC1* and *FUS3* in SE are based on the following observations: (1) mutants of these genes lacking activity could not bring about effective SE induction (Gaj et al. 2005) and (2), *LEC1* overexpression resulted in spontaneous somatic embryo formation *in planta* (Lotan et al. 1998). Moreover, the expression of *LEC1*, and the closely related to *LEC1-LIKE* (*LIL*) ortholog, during *in vitro* induced SE, has been reported in numerous plants, including maize (Zhang et al. 2002), carrot (Yazawa et al. 2004), *Helianthus annuus* (Fambrini et al. 2006), *Medicago sativa* (Domoki et al. 2006), *Theobroma cacao* (Alemanno et al. 2008) and *Vitis vinifera* (Schellenbaum et al. 2008; Maillot et al. 2009). *LEC1* expression was also found to be associated with ectopic formation of somatic embryos induced by overexpression of *LEC2* (Stone et al. 2001) and *MYB118* (Wang et al. 2009).

The present work has provided further evidence of a close relation between the embryogenic potential of tissues and the activity of *LEC1* and *FUS3*. Real-time qRT-PCR analysis proved a high level of activity of *LEC1* and *FUS3* in Arabidopsis embryogenic culture, and established that their transcript level is significantly increased in highly embryogenic cultures compared with cultures impaired in SE.

The activity of *LEC1* and *FUS3* was found in all monitored stages of embryogenic cultures, but the distinctly

different expression pattern displayed by the genes suggests their likely disparate functions in SE. *LEC1* showed a differential expression pattern over the time course of SE, while *FUS3* activity was constant at all monitored stages. The expression profile of *LEC1* observed in Arabidopsis embryogenic culture suggests the involvement of the gene in the process of differentiation and development, rather than induction of somatic embryos; although a drastic increase of *LEC1* activity was observed in the first day of culture. However, the transient character of this up-regulation indicates that general stress, not specific to SE, but imposed by *in vitro* conditions, is responsible for this stimulation, as was also observed in Arabidopsis for *LEC2* (Ledwoń and Gaj 2009). Embryogenic culture stress-related stimulation of *LEC1*, and some other SE-associated genes, at the first day of embryogenic culture has also been reported in alfalfa (Domoki et al. 2006). The SE-related expression of *LEC1* revealed in the present work indicated a progressive and auxin-dependent increase in gene transcript level, which peaked at day 15 of embryogenic culture i.e. at a stage relevant to efficient development of somatic embryos. Consistent with Arabidopsis, in carrot (Yazawa et al. 2004) and alfalfa (Domoki et al. 2006) embryogenic cultures, *LEC1* expression reached maximum in advanced cultures efficiently producing somatic embryos in callus tissue. Considering these observations *LEC1* is likely to be involved in somatic embryo differentiation and development. However, the possible function of *LEC1* in initiation of somatic embryos cannot be excluded, due to the spontaneous formation of somatic embryos *in planta* under its overexpression (Lotan et al. 1998). Accordingly, in a recent network of interacting LEC factors *LEC1* was positioned very early during seed development, and upstream of the B3 network (Suzuki and McCarty 2008). A possible indirect link between *LEC1* and the induction of embryogenesis can be expected in the light of a recent postulate that in ZE processes *LEC1* may be activated by the presumptive carboxypeptidase *ALTERED MERISTEM PROGRAM 1* (*AMP1*) involved in a general differentiation-promoting activity (Suzuki et al. 2008). Interestingly, the mutation in *AMP1* results in higher capacity for SE manifested by embryo production in seedling culture (Mordhorst et al. 1998), and the recent finding that *AMP1* interacts with the MP/ARF5 auxin response factor (Vidaurre et al. 2007) provides a new perspective on a possible links between auxin, *LEC1* and embryogenesis.

In terms of the predicted *LEC1* and auxin relationship, our work documents that expression of *LEC1* during SE is auxin-stimulated. Similarly, in alfalfa embryogenic culture, up-regulation of *MsLEC1* gene upon auxin treatment has also been demonstrated (Domoki et al. 2006). Furthermore, it was recently indicated that in sunflower, overexpression of a closely related *LEAFY COTYLEDON1-LIKE* (*Ha-LIL*)

marked the putative founder cells of ectopic embryos, and co-localized with IAA accumulation (Chiappetta et al. 2009). These observations are consistent with auxin promoted activity of *LEC1* postulated in studies on the *turnip*, a gain-of-function mutation of *LEC1*, leading to altered embryogenic response (Casson and Lindsey 2006). Thus it seems conceivable that *LEC1* acts in concert with auxin to potentiate embryogenic pathways *in planta* and *in vitro*, and further analysis is needed to reveal other interacting factors, among which AMP1 may play an important role (Suzuki et al. 2008).

Besides auxin-related factors, ABA-controlled proteins are also expected to interplay with *LEC1* during embryogenesis; and in this respect an interaction between *LEC1/L1L* and a seed-specific ABA-response element (ABRE)-binding factor, *bZIP67*, was recently reported in Arabidopsis seed development (Yamamoto et al. 2009). Interestingly, *bZIP67* was also found to be expressed in embryogenic culture induced *in vitro* in Arabidopsis (M. Kurczyk, M. Gliwicka, MDG, unpublished data).

In addition to *LEC1* activity, the present analysis also indicates expression of *FUS3* in Arabidopsis embryogenic cultures, as previously reported (Ikeda-Iwai et al. 2002; Gaj et al. 2005). However, it was found that contrary to *LEC1* and *LEC2* (Ledwoń and Gaj 2009) the level of *FUS3* expression is constant, and not related with exogenous auxin treatment. Although the activity of the *FUS3* gene does not seem to be related with any particular SE stage, its activity proved to be specific to embryogenic cultures. According to this, in Arabidopsis explants induced *in vitro* on cytokinin medium for adventitious shoot regeneration, a progressive decline of *FUS3* expression was observed (M. Wajand, AL and MDG, unpublished), and the loss-of-function *fus3* mutant was found to be highly effective in shoot regeneration (Gaj et al. 2005).

During ZE, in contrast to SE, a differential expression pattern of *FUS3* was described. Transcripts were not detected at early stages of zygote development, and the gene expression was indicated from the second week after fertilization up to the dry seed stage (Kroj et al. 2003; Baumbusch et al. 2004; Wang et al. 2007). Expression of *FUS3* at the very early stages of SE, found here, can reflect the gene activity observed in explant tissue, the immature zygotic embryos at the late developmental stage used to induce embryogenesis, and seems not to be related with the gene involvement in SE inductive mechanisms. Moreover, considering that in ZE *FUS3* activity is proposed to be regulated by *LEC1* (Kagaya et al. 2005a), its transcription during SE can result from its auxin-stimulated expression found in embryogenic culture. Interestingly, the present analysis indicates that auxin treatment was shown not to influence *FUS3* expression level in cultured explants. In

contrast to SE, in zygotic embryos *FUS3* activity monitored with *GUS* was reported to be up-regulated by exogenous auxin (Gazzarrini et al. 2004). Thus, the possible link between *FUS3* and auxin in zygotic and somatic cells remain unclear, and further studies are needed to elucidate the mechanism.

The activity of *FUS3* in embryogenic culture was found to be different to that observed *in planta*, as well as in terms of its interactions with *LEC2*. It has been shown that as a consequence of overexpression of *LEC2*, two other *LEC* genes, *LEC1* and *FUS3* were induced *in planta* in vegetative organs (Santos-Mendoza et al. 2005; Stone et al. 2008). Recently, it has also been postulated that this *LEC2*-driven expression is mediated by auxin, as elevated production of this hormone can be expected under *LEC2* overexpression (Stone et al. 2008). In the present work, however, it was observed that in cultures of 35S::*LEC2*-GR explants the increased activity of *LEC1*, but not *FUS3*, was induced over a time course related to induction, differentiation and development of somatic embryos. Given up-regulation of *FUS3* by *LEC2*, as indicated in ZE (Kroj et al. 2003; To et al. 2006), and included in a recently proposed network of interactions between *LEC* (Suzuki and McCarty 2008; Braybrook and Harada 2008), a constant *FUS3* expression level, not influenced by *LEC2* expression during SE, suggests that complex and cell type-dependent relationships involving *LEC* genes can be expected.

Taken all together, the present results on expression patterns of *LEC1* and *FUS3* in embryogenic culture provide further evidences that these genes, together with *LEC2* (Ledwoń and Gaj 2009), determine embryogenic potential in Arabidopsis tissue. Discussing the possible mechanisms used by *LEC1* and *FUS3* in determination/enhancing of embryogenic capacity in somatic cells, the well recognized links between *LEC* genes and hormones such as auxin, GA and ABA (reviewed by Braybrook and Harada 2008), the key factors for SE induction (Jimenez 2005), are crucial. In support for this hypothesis, a significantly altered expression profile of *LEC2* (Ledwoń and Gaj 2009) as well as *LEC1* and *FUS3* (the present work) was found in the *cbp20* and *axr4-1* hormonal mutants with impaired capacity for SE (Gaj et al. 2006). The *cbp20* mutation disturbs abscisic acid signaling (Papp et al. 2004), possibly by influencing microRNAs involved in the regulation of developmental processes (Kim et al. 2008), while *axr4-1* interferes with auxin influx in Arabidopsis cells (Dharmasiri et al. 2006). Thus, both mutations are expected to alter the hormonal environment of somatic cells, and to negatively interfere with their embryogenic capacity. How a significantly decreased *LEC* expression level observed in the mutant explants and cultures is related with the *cbp20* and *axr4-1* mutations it remains to be revealed.

Although the presented data strongly confirmed the key role of *LEC* factors in somatic embryogenesis in *Arabidopsis*, species-specific requirements in genetic mechanisms involved in embryogenic transition of somatic cells should also be considered. In this respect, in contrast to the recent data on the diverse effects caused by overexpression of *LEC* genes documented in *Arabidopsis*, ectopic expression of *LEC1* homolog gene in white spruce did not result in somatic embryo formation (Klimaszewska et al. 2010). Similarly, overexpression of *LEC2* and *BBM* genes reported to induce SE in *Arabidopsis* plants (Stone et al. 2001; Boutilier et al. 2002) did not promote embryo development in tobacco (Rashid et al. 2007; Srinivisan et al. 2007).

Conclusions

Current advances in cellular and molecular mechanisms of SE in higher plants indicate the complexity of the factors involved in embryogenic transition, and among them the regulatory genes encoding transcription factors are of special interest (Yang and Zhang 2010). Within TFs, crucial for enabling embryogenic transition of somatic cells, the *LEC* gene master regulators of ZE, were indicated. Accordingly, in *Arabidopsis*, only the cells of immature zygotic embryo with high activity of *LEC* genes are able to produce somatic embryos under in vitro culture. Differential and auxin-dependent expression of *LEC1* (the present work) and *LEC2* (Ledwoń and Gaj 2009) displayed during SE demonstrates their vital role in induction (*LEC2*), differentiation and development of somatic embryos (*LEC1*). The activity of *FUS3* was also indicated to be associated with SE, although the mechanisms which link the gene with capacity of somatic cells for embryogenesis are much less clear, and seem not to be directly related to auxin.

Given the key role of *LEC* genes in defining embryogenic potential of somatic cells, a challenging perspective for controlling plant totipotency is associated with mechanisms of chromatin-based repression system, which restricts *LEC* function to seed development (Zhang and Ogas 2009). To release embryogenic potential in any tissue of interest, the control of the key players essential for embryogenic transition must include TFs operating during SE and, on the other hand, the factors repressing their activity. Manipulation of the repressors of *LEC* may provide a new approach for triggering embryogenic development in somatic cells (Suzuki et al. 2007; Tanaka et al. 2008).

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