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POLSKA AKADEMIA NAUK

ACTA BIOLOGICA CRACOVIENSIA Series Botanica 53/2: 59–67, 2011
DOI: 10.2478/v10182-011-0028-x

IMMATURE ZYGOTIC EMBRYO CULTURES OF *ARABIDOPSIS*. A MODEL SYSTEM FOR MOLECULAR STUDIES ON MORPHOGENIC PATHWAYS INDUCED IN VITRO

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Received July 25, 2011; revision accepted November 9, 2011

To understand the molecular mechanism controlling in vitro plant morphogenesis, a culture system enabling induction of alternative morphogenic pathways (somatic embryogenesis, SE; shoot organogenesis, ORG) in a well defined population of somatic cells is needed. *Arabidopsis* is the most useful model plant for genomic studies, but a system in which SE or ORG can be induced alternatively in the same type of explant has not been proposed. Immature zygotic embryos (IZEs) of *Arabidopsis* provide the only explants with embryogenic potential, and have been recommended for studying mechanisms of SE induced in vitro. This study was aimed at defining culture conditions promoting induction of alternative morphogenic pathways: shoot ORG in IZE explants. The established protocol involves pretreatment of IZE explants with liquid auxin-rich callus induction (CIM) medium, followed by subculture on solid cytokinin-rich shoot induction medium (SIM). The method enables efficient shoot induction in Columbia (Col-0) and Wassilewskija (Ws), genotypes commonly used in molecular studies. During 3 weeks of culture up to 90% of Col-0 and 70% of Ws explants regenerated shoots via an indirect morphogenic pathway. We analyzed the qRT-PCR expression patterns of the *LEC* (*LEC1*, *LEC2* and *FUS3*) genes, the key regulators of *Arabidopsis* embryogenesis, in the IZE explants induced to promote shoot ORG. The sharp decline of *LEC* expression on SIM medium confirmed that culture of *Arabidopsis* IZE explants enables experimental manipulation of the morphogenic response of somatic cells. A scheme illustrating various in vitro morphogenic responses of IZEs in relation to hormonal treatment is presented.

Key words: *Arabidopsis*, immature zygotic embryos, in vitro morphogenesis, shoot regeneration.

INTRODUCTION

In vitro plant regeneration systems are widely used in plant biotechnology as valuable methods for micropropagation of commercial species, production of transgenic forms, and also in basic research on different aspects of plant morphogenesis. With the advent of genomic and molecular tools, in vitro regeneration methods have substantially contributed to studies of the molecular aspects of plant morphogenesis (Che et al., 2002; Che et al., 2006; Su et al., 2009). Two alternative morphogenic pathways can be induced in vitro: somatic embryogenesis (SE), resulting in the formation of bipolar structures with defined shoot and root meristems, and organogenesis (ORG), resulting in adventitious shoot or root regeneration. Both pathways can proceed directly or indirectly when dedifferentiation of explant cells and callus formation is initially observed (Hicks, 1994).

Since the first demonstration by Skoog and Miller (1957) that a specific hormonal treatment is required to induce a certain type of morphogenic response the procedure has become widely accepted. However, the molecular mechanisms that are induced following hormone treatment of cultured cells to determine ORG or SE processes remain obscure. To understand the totipotency/pluripotency of somatic plant cells, and to control in vitro regeneration, the genetic factors need to be identified, especially those engaged in a specific morphogenic response induced in vitro. Recent studies

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Abbreviations: IZE – immature zygotic embryo; ORG – organogenesis; SE – somatic embryogenesis; CIM – callus induction medium; SIM – shoot induction medium; LC – late cotyledonary; *LEC* – *LEAFY COTYLEDON*; 2,4-D – 2,4-dichlorophenoxyacetic acid; BAP – benzyl adenine; NAA – 1-naphthaleneacetic acid

PL ISSN 0001-5296

of genes involved in plant regeneration suggest the existence of independent mechanisms triggering SE and ORG in cells cultured in vitro. For example, high efficiency of ORG was found to be associated with the failure of SE in some *Arabidopsis* mutants (Gaj et al., 2005). Differential expression of genes during SE and ORG found in rice cultures (Su et al., 2007) points to specific reorganization of the transcriptome in relation to a particular morphogenic pathway induced in vitro.

The use of model plants is recommended in research aimed at identifying the genetic mechanisms that determine the organogenic or embryogenic response of cultured tissue. Among dicots, *Arabidopsis* has been widely adopted as a standard reference plant in all of biology (Koornneef and Meinke, 2010), including molecular studies on plant development and morphogenic processes induced in vitro (Ezhova, 2003; Kuromori et al., 2009; Luo and Lam, 2009; Irish, 2010).

Different types of *Arabidopsis* explants have been shown to produce shoots in vitro via ORG in response to specific hormonal treatments, including fragments of leaves (Lloyd et al., 1986; Feldman and Marks, 1986; Sheikholeslam and Weeks, 1987; Schmidt and Willmitzer, 1988), stems (An et al., 1986), roots (Feldman and Marks, 1986; Valvekens et al., 1988; Akama et al., 1992), hypocotyls (Akama et al., 1992) and protoplasts (Damm and Willmitzer, 1988). In contrast to easy induction of shoot ORG in a variety of somatic tissues, the alternative process of plant morphogenesis, via SE, is restricted in *Arabidopsis* to only one explant type – immature zygotic embryos (IZEs). IZEs in the late cotyledonary (LC) stage of development, corresponding to the maturation phase of zygotic embryogenesis, were found to be most suited for rapid, efficient and reproducible production of somatic embryos (Gaj, 2001). IZEs in early developmental stages (globular to torpedo) and mature zygotic embryos infrequently produce somatic embryos, and SE proceeds via callus development (Wu et al., 1992; Luo and Koop, 1997; Gaj, 2001; Gaj et al., 2005). Culture of LC IZEs on solid medium supplemented with auxin as the sole plant regulator results mainly in direct SE (DSE) (Kurczynska et al., 2007). Alternatively, indirect SE (ISE) can be induced in LC IZE explants, where prolonged culture on liquid media with increased auxin level led to abundant formation of embryogenic callus (Pillon et al., 1996; Ikeda-Iwai et al., 2002; Su et al., 2009).

Different *Arabidopsis* explants have been used in molecular studies on morphogenic processes induced in vitro, that is, SE and ORG. Molecular events related to shoot ORG induced in vitro have been analyzed in cultures of root explants (Barton and Poethig, 1993; Ozawa et al., 1998; Cary et al., 2001; Cary et al., 2002; Zuo et al., 2002; Gordon et al., 2007; Zhao et al., 2008; Bao et al., 2009). To reveal the molecular mechanisms involved in the embryogenic transition and somatic embryo formation, culture of IZE explants of *Arabidopsis* has been recommended (Gaj, 2004) and successfully applied (Gaj et al., 2005; Su et al., 2009). To identify the genetic factors responding to specific morphogenesis-related culture conditions it would be helpful to manipulate the culture systems to enable alternative morphogenic pathways to be induced in the same explant. In *Arabidopsis*, the plant most amenable to molecular analysis, a system in which SE or ORG are induced alternatively in the same tissue has not yet been explored.

Such a system for induction of SE or ORG was established in sunflower: in cultures of IZE explants, somatic embryos or shoots could be induced in vitro on culture media with differing sucrose concentrations (Jeannin et al., 1995). However, the application of this unique in vitro system in genetic studies is limited in sunflower due to the poor availability of genomic data.

To take advantage of the availability of genomic studies in *Arabidopsis*, we propose the use of IZE culture to reveal the molecular mechanisms controlling in vitro plant morphogenesis. IZE explants present high embryogenic potential (Gaj, 2011), and the culture conditions promoting induction of shoot ORG, the alternative morphogenic pathway, are already well defined in IZE culture (Elhiti and Stasolla, 2011). To assess the proposed in vitro system's usefulness for identifying the genetic mechanisms specific to the morphogenic pathway, we analyzed the expression patterns of *LEC* genes, key regulators of *Arabidopsis* embryogenesis, in IZE culture induced towards shoot ORG.

Here we present the results and discuss the potential of *Arabidopsis* IZE-derived cultures for research on the molecular mechanisms controlling morphogenic processes induced in vitro in plant cultures.

MATERIALS AND METHODS

PLANT MATERIAL AND EXPLANTS FOR IN VITRO CULTURE

Arabidopsis thaliana (L.) Heynh. plants of the Columbia (Col-0) and Wassilewskija (Ws) genotypes were used. The IZE explants with fully developed green and bent cotyledons at late cotyledonary (LC) stage (Gaj, 2001) were isolated from siliques of 8–10-week-old plants. The siliques were surface-sterilized for 20 min in 20% solution of sodium hypochlorite with three drops of Tween 20 per 100 ml, and rinsed three times in sterile water. Sterile siliques were opened with very fine needles, and IZEs were excised under a dissecting microscope.

TABLE 1. Primers used for qRT-PCR reactions of the analyzed genes

Gene	Reverse primer	Forward primer
LEC1	(R)5'- CTGGACCACGATACCATTGTT-3'	(F)5'-GTGGAGCTCCCTTCTCTCACT-3'
LEC2	(R)5'-CAGTGGTGAGGTCCATGAGAT-3'	(F)5'-AGGGAAAGGAACCACTACGAA-3'
FUS3	(R)5'-TGAAGGTCCAAACGTGAAAAC-3'	(F)5'-GTCAGCTCTCTCCGACGTATG-3'
At4g27090	(R)5'-CCTCGATCAAAGCCTTCTTCT-3'	(F)5'-GTCGTTATCGTCGACGTTGTT-3'

IN VITRO ORGANOGENESIS AND CULTURE MEDIA

The IZE explants were incubated for 7 days in liquid callus induction medium (CIM), and the cotyledons were then cut off and transferred to solid shoot induction medium (SIM-C). The CIM medium contained the microsalts, macrosalts and vitamins of B5 medium (Gamborg et al., 1968), 0.5 g l^{-1} MES, 20 g L^{-1} glucose, 2.2 μM 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.2 μM kinetin. The SIM-C medium included MS (Murashige and Skoog, 1962), the microsalts, macrosalts and vitamins of B5 medium (Gamborg et al. 1968), 20 g l^{-1} glucose, 0.5 μM naphthalene acetic acid (NAA) and 4.4 μM benzyl adenine (BAP) (Patton and Meinke, 1988). To induce rooting and conversion to plants, regenerated shoots were transferred to hormone-free ½ MS basal medium with sucrose (10 g l^{-1}) and agar (8 g l^{-1}). The pH of all media was adjusted to 5.8.

The culture system's capacity for shoot ORG induction was evaluated in 3-week-old cultures on SIM-C medium, in terms of the frequency of explants producing shoots and the average number of shoots formed per explant. We also calculated the percentage of shoots developing roots. Rooted plantlets were transferred to soil in pots for further development and seed harvest.

Thirty explants were analyzed in each of 3 replicates for each genotype analyzed.

PLANT GROWTH AND IN VITRO CULTURE CONDITIONS

The plants used as the source of explants and the regenerants were grown in soil in pots at 20–22°C under a 16 h photoperiod (100 μmol m^{-2} s^{-1} white fluorescent light). Plant material cultured in sterile conditions was kept at 23°C under a 16 h photoperiod (40 μmol m^{-2} s^{-1} white fluorescent light).

RNA ISOLATION AND qRT-PCR ANALYSIS

We used an RNAqueous Kit (AMBION) to isolate RNA. Its concentration and purity were checked with an ND-1000 spectrophotometer (NanoDrop). To control for DNA contamination the RNA was treated with RQ1 RNase-free DNase I (Promega) according to the manufacturer's instructions. First-strand c-DNA was produced in a 40 μl reaction volume using the RevertAid First Strand cDNA Synthesis Kit (Fermentas). The product of reverse transcription was diluted 1:1 with water, and 1 μl of this solution was used for real-time quantitative RT-PCR (qRT-PCR). qRT-PCR was carried out in a 10 μl reaction volume with the use of LightCycler Fast-Start DNA Master SYBR Green I (Roche) and appropriate primers. The primers were designed for qRT-PCR reactions of the analyzed genes (Tab. 1).

The LightCycler 2.0 (Roche) real-time detection system was used with the following reaction conditions: one denaturation cycle of 10 min at 95°C, followed by 45 cycles 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°C and 95°C (0.1°C/s for fluorescence measurement).

Primary data analysis was performed with LightCycler 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 the analyzed tissue samples the control gene exhibited a constant expression pattern with Cp = 18±1.

Total RNA was isolated from freshly isolated (day 0) and induced in vitro Col-0 explants. Tissue sampling was done on days 1, 3 and 5 for CIM-induced cultures and on days 10 and 15 for SIM-C regenerating explants. Depending on culture age, between 250 (day 0) and 4 (day 15) explants were used for RNA isolation. The plant tissues for qRT-PCR analysis were produced in 3 biological repetitions, and two technical replicates of each repetition were carried out.

RESULTS

SHOOT ORGANOGENESIS INDUCTION IN IZE EXPLANTS

To induce shoot organogenesis in cultures of *Arabidopsis* IZE explants we applied two media of different hormonal composition. IZEs at late cotyledonary stage (Fig. 1a) incubated for 7 days in liquid auxin-rich CIM medium became straightened, thickened and distinctly enlarged, with callus tissue formed on the cotyledons (Fig. 1b). Before subculture to SIM-C medium, with superior cytokinin over auxin concentration, the cotyledons

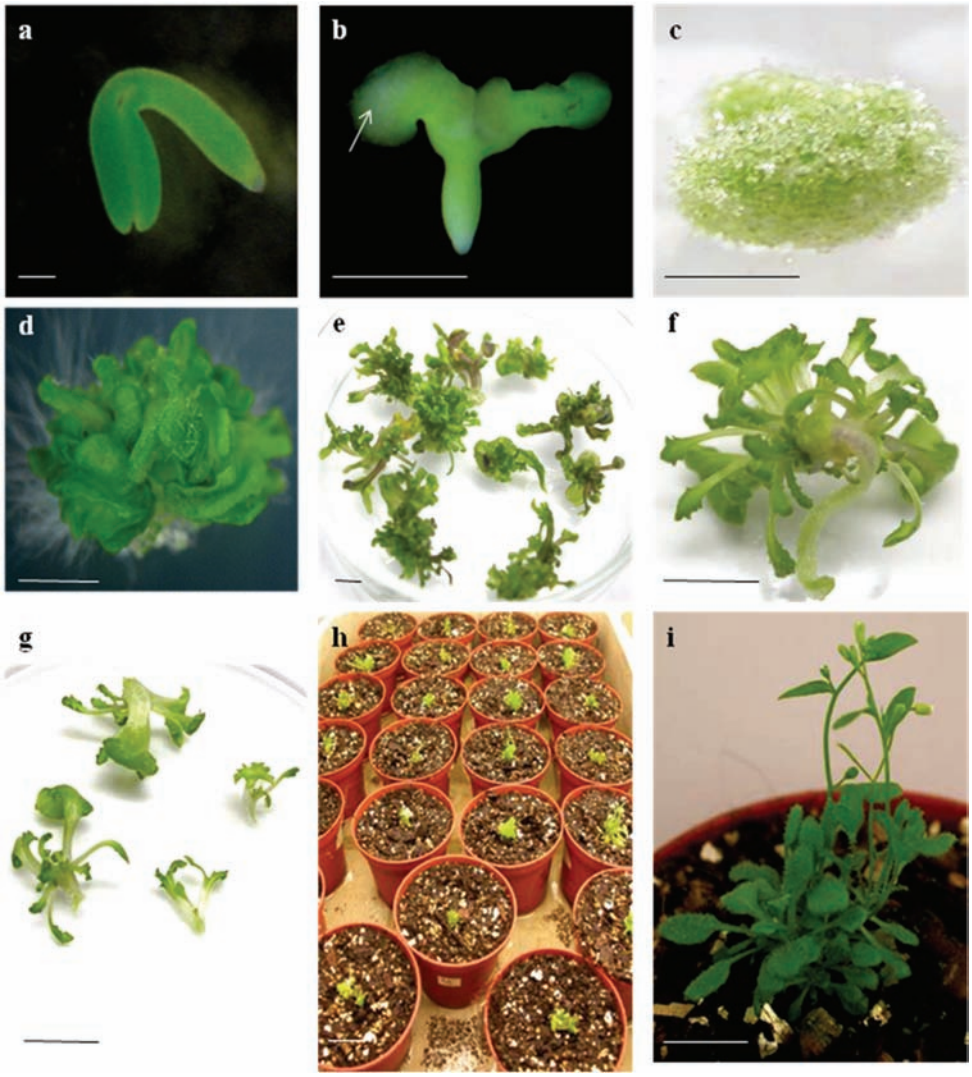

Fig. 1. Regeneration of *Arabidopsis* plants via shoot organogenesis in cultures of IZE-derived cotyledons. (**a**) IZE explants, (**b**) Enlargement of IZE explant and callus formation on cotyledons (arrow) after 7 days in CIM medium, (**c**) Abundant callus formation during 10-day culture on SIM-C medium, (**d**) First shoot primordial regenerated at day 15 on SIM-C, (**e**) Shoot organogenesis in cultures of IZE cotyledons of Col 0, (**f**) Numerous shoots regenerated by one cotyledon explant at day 30 on SIM-C medium, (**g**) Individual shoots rooted on MS medium, (**h**) Regenerants transferred to pots, (**i**) Flowering regenerant. Bars = 0.1 mm in (a), 1 mm in (b–d), 0.5 cm in (e–g), 1 cm in (h,i)

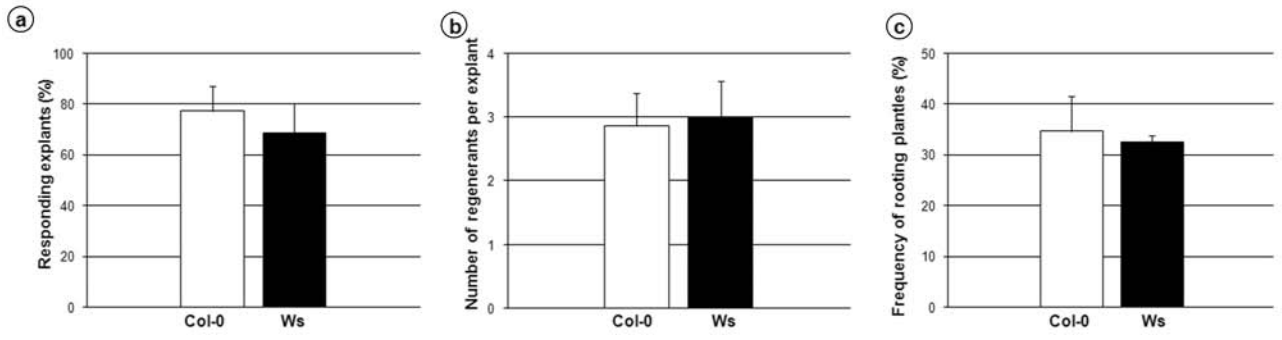

Fig. 2. Shoot organogenesis capacity in IZE explant cultures of Col-0 and Ws genotypes. (**a**) Frequency (%) of shoot-regenerating explants, (**b**) Average number of regenerated shoots per explants, (**c**) Frequency (%) of rooted regenerants.

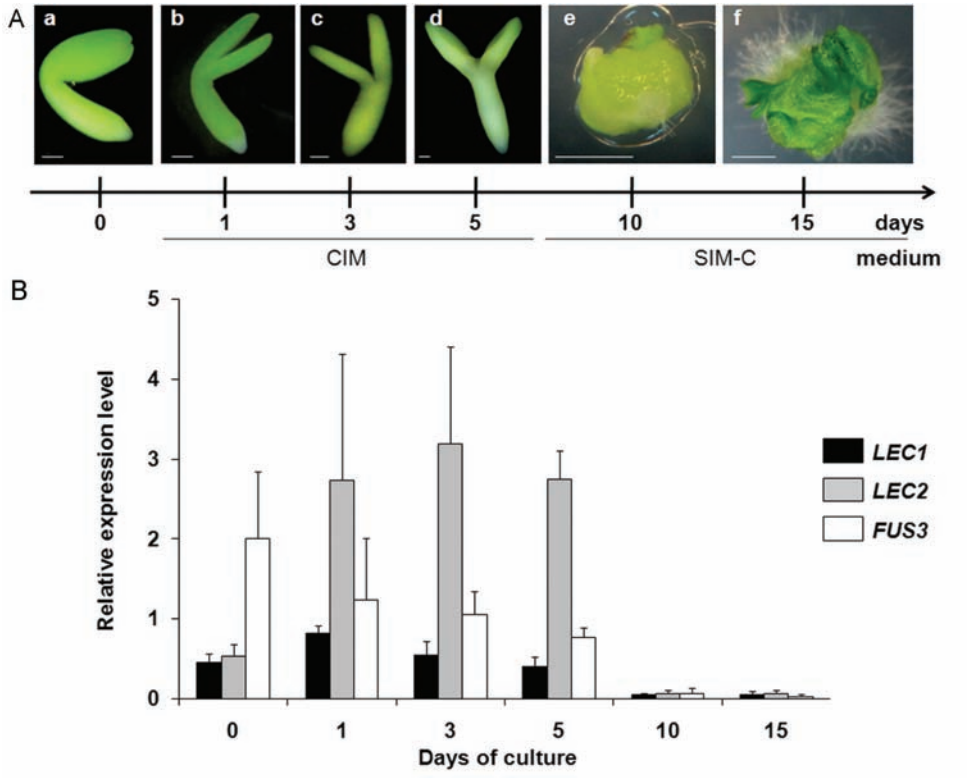

Fig. 3. Analysis of *LEC* gene expression pattern in culture of IZE Col-0 explants induced to undergo shoot organogenesis (ORG). A – Time points for tissue sampling and RNA isolation and the respective developmental stages of Col-0 IZE explants cultured on CIM (b–d) and SIM-C (e,f) media. (**a**) Freshly isolated IZE explant (0d), (**b**) Explants after one day of culture, (**c**) Straightening of cotyledons (day 3), (**d**) Swelling and enlargement of cotyledons (day 5), (**e**) Callus developing on an isolated cotyledon (day 10), (**f**) Shoot primordia emerging (day 15). Bars = 0.1 mm in (a–d), 1.0 mm in (e,f). B – Expression pattern of *LEC* genes during ORG evaluated by qRT-PCR. Freshly isolated (day 0), CIM (days 1, 3, 5) and SIM-C (days 10, 15) induced explants were analyzed. RNA levels were normalized to that of *At4g27090*.

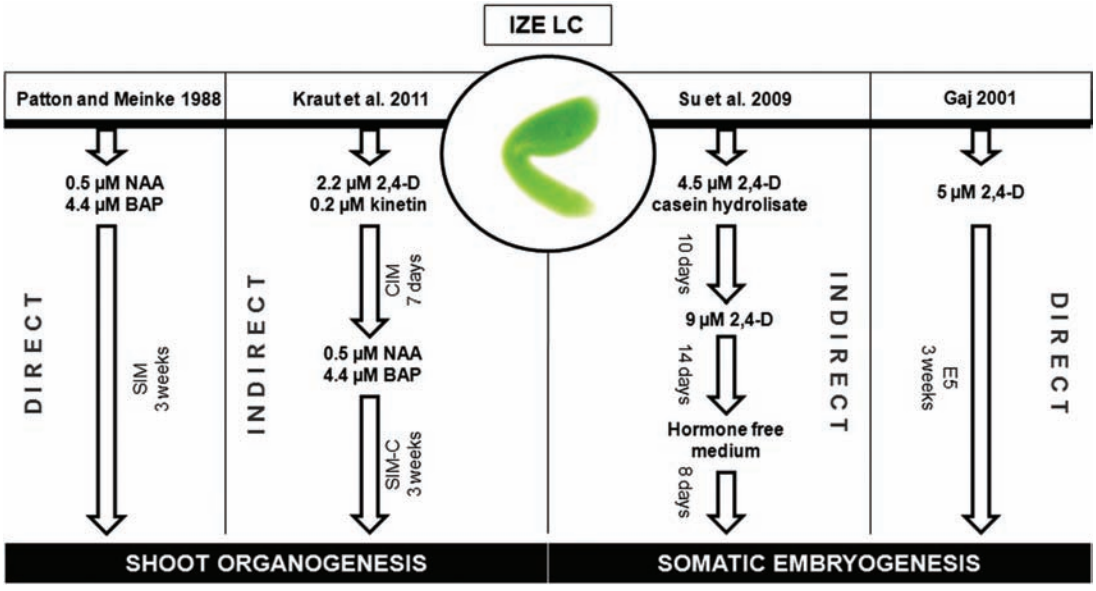

Fig. 4. *Arabidopsis* IZE explant culture system recommended for studies of the molecular mechanisms controlling alternative morphogenic pathways induced in vitro.

were cut off from the initial explants. Culture on cytokinin-rich medium resulted in the formation of green callus covering the cotyledon explants (Fig. 1c), and the first regenerated shoot primordia were seen on day 10 of SIM-C culture (Fig. 1d). Two weeks later the explants were covered with numerous shoots (Fig. 1e). The regenerated shoots were separated and transferred to hormone-free medium for rooting (Fig. 1f). The plantlets were then transferred to soil in pots and grown to maturity (Fig. 1g,h).

The shoot regeneration procedure was applied in the most commonly used ecotypes: Col-0 and Ws. The efficiency of shoot ORG induction was especially high in Col-0 cultures, in which up to 90% of the cotyledons regenerated shoots (Fig. 2a). On average, three shoots per cotyledon were regenerated (Fig. 2b) in cultures of both genotypes. On hormone-free medium, less than half of the regenerated shoots produced roots (Fig. 2c).

EXPRESSION PATTERN OF *LEC* GENES IN CULTURES UNDERGOING SHOOT ORGANOGENESIS

We used the qRT-PCR reaction to assess the expression level of *LEC* genes in Col-0 tissues sampled at different stages of the shoot ORG process (from days 0 to 15 of culture). The time points selected for this analysis allowed us to monitor gene activity from initiation of culture (day 0) through to callus induction (days 1, 3, 5) and shoot induction and development (days 10, 15) (Fig. 3a).

The level of *LEC* gene expression observed during the time course of the shoot ORG process was related to the transcriptional activity of the reference gene. The results of qRT-PCR analysis indicated activity of all of the genes in freshly isolated IZE explants (0d). Under the in vitro conditions applied to promote ORG, the *LEC* genes differed in their levels of expression, and the expression levels changed through time (Fig. 3b). Treatment of the explants with CIM medium resulted in a gradual decrease of *LEC1* and *FUS3* expression, while *LEC2* activity significantly increased on the first day of culture and remained relatively high during culture on CIM medium. The expression levels of all *LEC* genes abruptly changed upon transfer of the explants to SIM-C medium, falling sharply from the first days of subculture on this cytokinin-rich medium.

DISCUSSION

The early events induced in explant tissue which trigger a specific morphogenic response are the most fascinating and obscure phenomena of plant regeneration in vitro. *Arabidopsis*, as a model in plant genomics, and an in vitro system able to induce different morphogenic pathways in a defined population of somatic cells, provides the means to investigate these phenomena. In this work we showed that culture of *Arabidopsis* IZE explants fulfills the requirements of a culture system suitable for studying plant development in vitro. Here we demonstrated that *Arabidopsis* IZEs at the strictly defined late cotyledonary developmental stage can be induced to engage an alternative morphogenic pathway, shoot ORG, in addition to SE (Gaj, 2001). IZEs of other plant species have also been used successfully as explants for adventitious shoot induction (Elhiti and Stasolla, 2011).

As in SE (Kurczynska et al., 2007), in shoot ORG induction the cotyledons of IZEs are involved; the hypocotyl and root parts of the explant are not responsive. *Arabidopsis* IZE cotyledons in the maturation phase of development provide highly responsive totipotent cells for in vitro culture, and the type of morphogenic response induced depends on the hormonal treatment applied. In the protocol given here to induce efficient shoot ORG, the proposed sequence of two media involves short treatment with auxin-rich liquid medium and subsequent culture on cytokinin-rich shoot regeneration medium. The use of an auxin-rich medium promotes rapid de-differentiation of explant tissue, manifested in callus formation; thus the shoots developing on SIM-C medium result from indirect morphogenesis. Moreover, pretreatment of IZE explants in CIM medium facilitates isolation of cotyledons from IZEs, as it substantially enlarges the explants. Isolation of cotyledons from the IZE primary explant prior to transfer to SIM-C medium is recommended to enhance the regeneration capacity of the cultures (data not presented). In contrast to the indirect shoot ORG induced with the CIM-SIM protocol, direct shoot development was reported in cotyledons isolated from *Arabidopsis* IZEs and cultured on a single cytokinin-rich medium (Patton and Meinke, 1988).

The duration of explant treatment on auxin-rich medium has been found to influence the efficiency of shoot ORG in *Arabidopsis* explants (Schmidt and Willmitzer, 1988). In the present protocol, 7 days of pre-incubation of IZE cotyledons in the presence of 2.2 μM 2,4-D promoted the shoot ORG response. A similar auxin concentration and pre-incubation time (8 days) in CIM medium has been recommended to induce adventitious shoots in culture of cotyledons isolated from 10-day-old seedlings (Akama et al., 1992). Our results, however, indicate that cotyledons isolated from IZEs have much higher regeneration potential, as measured by the frequency of responding explants (80%), than seedling-derived cotyledons (50%) (Akama et al., 1992).

The proposed IZE-based system of shoot ORG was more effective for Col-0 than for Ws explants.

The high efficiency of shoot ORG observed in Col-0 IZE culture should be viewed in the light of other work showing that it is also superior to Ws in its capacity for SE (Gaj, 2001). These two highly efficient protocols for induction of shoot ORG (this paper) and SE (Gaj, 2001) in IZE culture make the Col-0 genotype very useful material for studies of plant morphogenesis induced in vitro.

The efficiency of the proposed IZE-based protocol is comparable to the most efficient shoot regeneration systems established in *Arabidopsis* with the use of various seedling- or leaf-derived explants (Schmidt and Willmitzer, 1988; Akama et al., 1992). More importantly, the efficiency of shoot induction obtained in the proposed IZE-based protocol is similar to that reported in seedling root culture (Feldman and Marks, 1986), which thus far has been the one most frequently employed in genetic studies on regeneration induced in vitro (Yasutani et al., 1994; Banno et al., 2001; Zhao et al., 2002; Daimon et al., 2003). In both of these systems, IZE and root-derived, a sequence of two media with opposite auxin-to-cytokinin ratios results in de-differentiation of primary explant tissue and subsequent shoot induction during 3-week culture. In contrast to root culture, which fails to undergo SE induction, the IZE-derived culture displayed high embryogenic potential (Gaj, 2011). This unique feature in *Arabidopsis* makes IZE culture a more attractive tool for genetic studies of plant morphogenesis.

Shoot induction in this protocol was relatively high but the frequency of rooting among regenerants was rather low; the majority of adventitious shoots failed to develop roots after subculture on hormone-free medium. Weak rooting response has also been observed in *Arabidopsis* shoots induced directly from immature cotyledons in spite of additional auxin treatment (Patton and Meinke, 1988). SE-derived *Arabidopsis* plants, on the other hand, displayed high rooting ability (Gaj, 2001), but somatic embryos recently were found to have a high frequency of defective root meristem development (Nowak et al., 2011). The difference in rooting capacity between SE-derived and ORG-derived plants seems attributable to the different hormonal treatments applied in these alternative culture systems.

To assess the utility of the proposed IZE-based system for molecular studies on shoot ORG we analyzed the expression pattern of *LEC* genes, master regulators of ZE (Harada, 2003), in shoot-induced tissue. In plant development the expression of these genes is related to zygotic embrygenesis, and declines during seed germination (Lotan et al., 1998; Reidt et al., 2001). *LEC* genes have also been found to be active during SE induced in IZE-derived culture, and *LEC2* expression was stimulated in embryogenic culture (Gaj et al., 2005; Ledwoń and Gaj, 2009). In agreement with the embryogenesis-specific pattern of *LEC* gene expression, we observed a drastic reduction of *LEC* expression in IZE cultures subjected to shoot ORG induction on cytokinin-rich SIM-C medium. We also noted that in auxin-rich CIM medium, which is believed to promote de-differentiation of tissue and callus formation (Feldman and Marks, 1986), *LEC* genes were active. Expression of *LEC2* (Ledwoń and Gaj, 2009) together with *LEC1* and *FUS3* (Ledwoń and Gaj, 2011) was observed in callus tissue developing on auxin-rich medium. As in sunflower (Fambrini et al., 2006; Chiappetta et al., 2009), *LEC1* expression was not observed in cultures induced to regenerate shootlike structures, but the gene was highly active in embryogenic cells.

One way to investigate the molecular mechanisms underlying in vitro plant morphogenesis is to compare the transcriptomes specific to the fates of organogenic and embryogenic development. Using genome-wide expression analysis, a set of genes discriminating the processes of in vitro induced shoot, root and somatic embryo formation was identified in the model monocot rice (Su et al., 2007). No similar global analysis of transcriptomes in *Arabidopsis* cultures undergoing alternative morphogenic responses has yet been reported. We recommend *Arabidopsis* IZE explants for such studies, as they can enable experimental manipulation of a population of differentiated somatic cells into either shoot ORG or SE (via direct or indirect pathways), depending on the hormonal treatment employed (Fig. 4).

ACKNOWLEDGMENTS

Author BW was supported by a scholarship within the framework of the UPGOW project of the European Social Fund, implemented at the University of Silesia, Katowice, Poland.

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