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ORIGINAL PAPER

Expression of seed storage product genes (*CRA1* and *OLEO4*) in embryogenic cultures of somatic tissues of Arabidopsis

**Marta Gliwicka · Katarzyna Nowak ·
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Abstract In Arabidopsis, immature zygotic embryos (IZEs) at the late cotyledon stage provide the only explants that can be used to induce in vitro somatic embryogenesis (SE) with high efficiency. The most conspicuous characteristic of SE-competent IZEs is the accumulation of seed storage reserves (SSR), as proteins and lipids. In order to elucidate an assumed role of these compounds in the mechanisms involved in tissue capacity for SE, the genes encoding the main seed storage protein, cruciferin (*CRUCIFERIN1, CRA1*) and the lipid body-related protein oleosin (*OLEOSIN4, OLEO4*), were studied. Significantly higher transcriptional activity of both genes, *CRA1* and *OLEO4*, in embryogenic than in non-embryogenic cultures, were indicated. However, their activity under in vitro culture were found not to be induced by auxin treatment or *LEC2* expression, and were unspecific for SE induction. In addition, the results on mutants severely impaired in SE response indicated that high activity of *SSR* genes in explant tissue is not sufficient for SE induction. On the other hand, the *cra1* and *oleo4* insertional mutants were found to be defective in their capacity for SE. In addition, it was found that the mutants displayed lower tolerance to high salinity and osmotic stress. Altogether, the results suggest an indirect influence of *SSR* genes on the embryogenic capacity of cultured tissues possibly via improvement of cell response to stress imposed in vitro.

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Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
2iP	6-(α,α-Dimethylallylamino)-purine
BAP	Benzyl adenine
CIM	Callus induction medium
IAA	Indole-3-acetic acid
IZE	Immature zygotic embryo
MZE	Mature zygotic embryo
NAA	Naphthalene acetic acid
ORG	Organogenesis
PSV	Protein storage vacuoles
SE	Somatic embryogenesis
SIM	Shoot induction medium
SSP	Seed storage protein
SSR	Seed storage reserves
TAG	Triacylglycerols
ZE	Zygotic embryogenesis

Introduction

Since not all somatic cells have capacity for totipotency, and therefore in order to regenerate plants in vitro, it is challenging to identify the internal and external factors which determine developmental plasticity of vegetative cells, and especially their embryogenic transition (Costa and Shaw 2006). Among the various factors that influence the induction of somatic embryogenesis (SE), such as plant growth regulators and other media components, and

physical culture conditions, or the donor genotype, the choice of explant type is considered to be crucial for the establishment of embryogenic culture, including in Arabidopsis (Gaj 2004). The embryogenic response is usually limited to specific tissues, strictly defined in age and developmental stage, and thus a selection of the proper explant type is a prerequisite for successful SE induction.

In many plants zygotic embryos provide explants with high SE potential, although their developmental stage allowing for embryogenic transition varies considerably between species (Elhiti and Stasolla 2011). Mature zygotic embryos (MZEs) are efficient in SE induction in some species (Prakash and Gurumurthi 2010; Chen et al. 2010; You et al. 2011; Pinto et al. 2011), mainly in cereals (Sharma et al. 2005); but immature zygotic embryos (IZEs) have been most frequently used to start embryogenic culture in plants of various taxonomic groups (Gaj 2004). Importantly, a strictly defined developmental stage of IZE determines high capacity for SE, and in most plants the competency of IZE for SE decreases with their age (Ogita et al. 1999; Wendt dos Santos et al. 2002; Rai et al. 2010; Kong et al. 2011). In woody species especially, a declining competence for SE is correlated with IZE age, along with the accumulation of seed storage reserves (SSR) (Keinonen-Mettala et al. 1996; Kärkönen 2000; Corredoira et al. 2002).

In contrast to these species, and much less frequently, the IZEs advanced in maturation with accumulated nutrient reserves display higher embryogenic capacity, as described in *Brassica napus* (Turgut et al. 1998) and *Panax ginseang* (Choi et al. 1998). Similarly, in *Feijoa sellowiana* efficient SE induction can be achieved only in the culture of cotyledons, which have already accumulated storage proteins and lipids (Canhoto and Cruz 1996). In Arabidopsis, a model species in plant genomics, the highest competency for SE induction is provided by IZEs at the late cotyledon stage (Gaj 2001). This stage of seed development, spanning between 12 and 16th DAP (day after pollination), corresponds to the mid maturation phase of ZE, and correlates with high synthesis and accumulation of proteins in cotyledons (Ruuska et al. 2002; Baud et al. 2008). The culture of IZEs at this stage results in rapid and efficient development of somatic embryos, mostly via the direct pathway induced in cotyledons (Kurczynska et al. 2007). On the contrary, IZEs of Arabidopsis at early stages of development (globular to torpedo) display drastically lower competency for SE, and abundant callus formation precedes somatic embryo development (Gaj 2001). The most distinct difference between poorly embryogenic IZEs, at the globular-torpedo stage, and highly embryogenic IZEs of late cotyledon stage, is the synthesis and accumulation of storage materials in the cotyledons (Mansfield and Briarty 1992).

There are also other observations suggesting a positive correlation between the accumulation of seed storage proteins (SSPs) in tissues and the competence for SE in Arabidopsis. Accordingly, beside IZEs, the only postembryonic vegetative tissues in Arabidopsis able to develop somatic embryos were found in some genotypes which ectopically accumulate the SSRs, due to mutation or induced over-expression of some specific embryogenesis-related regulatory genes. The *pickle* mutant shows spontaneous production of somatic embryos in seedling roots which display ectopic expression of *SSPs* genes (Ogas et al. 1997). The spontaneous or enhanced somatic embryo formation on seedling aerial parts, accompanied by SSR ectopic expression, was found in transgenic lines over-expressing transcription factors involved in SE induction, including: *LEAFY COTYLEDON2* (Stone et al. 2001), *BABY BOOM* (Boutilier et al. 2002), *AGL15* (Harding et al. 2003), as well as *MYB118* and *MYB115* (Wang et al. 2009). Conversely, the *abi3*, and *lec* mutants, with severely reduced SSPs accumulation in seeds (Fujiwara et al. 2002), were found to be significantly defective in SE response (Gaj et al. 2005, 2006).

All these observations lead to the hypothesis that the seed maturation process, mainly the SSP accumulation, provides a suitable or even indispensable environment for the initiation of SE in Arabidopsis (Stone et al. 2008). However, the nature and the mechanisms involved in the relation between embryogenic competency of somatic tissue and SSRs, remain to be revealed.

In Arabidopsis, as in other crucifers, synthesis of large amounts of storage reserves, including proteins and lipids, is related with seed development. These compounds are accumulated in the protein storage vacuoles (PSVs) and oil bodies (oleosomes), respectively, and each of these structures can constitute 30–40% of MZE dry weight (North et al. 2010). In a post embryonic growth the SSPs and triacylglycerols (TAGs) stored in oleosomes are used as a reservoir of nutrients, and a source of energy during germination and plantlet development, before the autotrophic mode of feeding is switched on.

The main proteins stored in PSVs of Arabidopsis and other crucifer seeds are 12S globulins (cruciferins). Globulins constitute a large fraction of dicotyledon, monocotyledon and gymnosperm SSPs, and belong to a large protein superfamily called cupins, which include a diverse array of prokaryotic as well as eukaryotic proteins that play a variety of different roles (Dunwell et al. 2000; Wan et al. 2007). Many of the cupin functions are dependent on enzymatic activity, but SSPs have lost this feature (Dunwell et al. 2000). The Arabidopsis Col-0 genome contains four cruciferin loci: *At1g03880* (*CRB*), *At1g03890* (*CRU2*), *At4g28520* (*CRC*) and *At5g44120* (*CRA1*), with the latter two contributing most to the cruciferin seed content (Wan et al. 2007; Baud

et al. 2008). Oleosomes are spherical structures of 0.2–2.0 μm in diameter, and they consist of a TAG core surrounded by phospholipid monolayer, which in turn is encapsulated by unique proteins called oleosins. Up to 50% of the oleosome surface is built up of proteins, mostly the plant specific oleosins, but also caleosins and steroleosin (Frandsen et al. 2001; Lin et al. 2002; North et al. 2010). Oleosins are known to prevent the coalescence of oil bodies, and thus help to maintain the small size of the oleosomes which stabilizes oil bodies and supports efficient oil metabolism during germination (Frandsen et al. 2001). Moreover, the proteins embedded in the lipid monolayer of oil bodies, including oleosins, play critical roles during degradation of storage lipids after germination (Baud and Lepiniec 2010). In the Arabidopsis genome 16 genes encode oleosins specific to seeds, microspores and tapetum (Kim et al. 2002). Among the seed-specific oleosins there are four proteins: OLEO1 (At4g25140), OLEO2 (At5g40420), OLEO3 (At5g51210) and OLEO4 (At3g27660). The most abundant is OLEO1, which constitutes 65% of Arabidopsis oleosome-associated proteins, and two others (OLEO2 and OLEO4) which contribute up to 30% of the oil-bodies proteome (Siloto et al. 2006).

The goal of the presented study was to gain insights into the relation between expression of genes encoding SSRs and tissue embryogenic potential. Two genes encoding *CRUCIFERIN1* (At5g44120), and *OLEOSIN4* (At3g27660) were selected for the analysis, due to their high expression level in embryogenic culture indicated in cDNA RDA (representational difference) analysis (MG and MDG, data not published). The *SSR* gene expression patterns in vitro in relation to auxin treatment were analyzed, and genotypes of different embryogenic potential were evaluated. Moreover, the *cra1* and *oleo4* mutants were evaluated in respect to their in vitro culture capacity and stress response. The involvement of SSR genes in the improvement of embryogenic potential of explant tissue is discussed in the context of stress responses of in vitro cultured cells.

Materials and methods

Plant material and growth conditions

Several genotypes of *Arabidopsis thaliana* (L.) Heynh. with different capacities for SE were used. The wild type Col-0 ecotype that shows a high capacity in standard in vitro culture conditions (Gaj 2001) and the mutants impaired in SE were analyzed, including *axr4-1*, *cbp20* (Gaj et al. 2006), *lec2-1* (Gaj et al. 2005) and *tan1-2* (Baster et al. 2009). Additionally, *cra1-1* (SALK004699), *cra1-2* (SALK002668) and *oleo4* (SALK011533) insertional mutants were used. The mutants

carry T-DNA insertion in exons which almost always disturbs protein expression (Wang 2008). The *cra1* and *oleo4* mutants were evaluated in terms of their capacity for in vitro induced morphogenesis and stress responses.

The parental Col-0 ecotype, *axr4-1*, *cra1-1*, *cra1-2* and *oleo4* mutants were purchased from NASC (The Nottingham Arabidopsis Stock Center). The *tan1-2* and *lec2* mutants were kindly provided by J. J. Harada (California University, Davies, USA), while the *cbp20* mutant is part of the Max-Planck Institut für Züchtungsforschung Köln collection, and was kindly provided by Z. Szweykowska-Kulinska, Institute of Molecular Biology and Biotechnology, AMU, Poznań.

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.

Plants used as the source of explants for SE culture and DNA extraction were grown in Jiffy-7 peat pots of 42 mm diameter (Jiffy), under controlled conditions: 22°C, long day photoperiod (16h/8h L/D) and light intensity of 100 μmol m^{-2} s^{-1} in a 'walk-in' type growth chamber.

Explants and growth conditions for in vitro culture

IZEs and roots of seedlings were used as explants for in vitro culture. The IZE explants were isolated from the siliques of 8–12 week old plants. The siliques were surface-sterilized for 20 min in a 20% solution of sodium hypochloride with three drops of Tween 20 per 100 ml, and rinsed three times in sterile water. Sterile siliques were opened with fine needles, and IZEs were excised under a dissecting microscope.

To produce root explants, seeds were surface-sterilized in a 20% solution of commercial bleach for 20 min and rinsed three times in sterile water. The seeds were germinated on ½ MS medium with half concentration of Murashige and Skoog (1962) basal macro- and microelements, 10 g L^{-1} sucrose and 8 g L^{-1} agar. The roots were excised from 14 day old seedlings.

In vitro cultures were maintained in the controlled conditions of a growth chamber: 22°C, long day photoperiod, light intensity 50 μmol m^{-2} s^{-1}. All experiments were carried out in three replicates of at least 30 explants per each (10 explants/Petri dish).

Somatic embryogenesis

SE was induced in cultures of IZEs under in vitro conditions, according to the standard protocol for Arabidopsis (Gaj 2001). The IZEs isolated from the siliques were placed on SE induction E5 solid medium with B5 salts and

vitamins (Gamborg et al. 1968), and supplemented with 5 μM 2,4-D (2,4-dichlorophenoxyacetic acid), 20 g L^{-1} sucrose and 3,5 g L^{-1} Phytagel (Sigma). In addition to auxin medium, explant cultures were also established on E0 hormone-free medium.

The capacity for SE was evaluated by two parameters: SE efficiency (the percent of explants forming somatic embryos) and productivity (the average number of somatic embryos produced by a responding explant), calculated at 21st day of culture.

Shoot organogenesis

The capacity for in vitro shoot organogenesis (ORG) was evaluated in cultures of two explant types: IZEs and seedling roots. The explants were placed for 7 days in a standard callus induction medium, CIM, with 2.2 μM 2,4-D and 0.2 μM kinetin (Feldman and Marks 1986). Subsequently, the shoot induction media (SIM) of different hormonal composition were used, according to the explant type. SIM-R medium with 5 μM 2iP (6-(α,α-dim-ethylallylamino)-purine) and 0.8 μM IAA—indole-3-acetic acid (Feldman and Marks 1986), or SIM-C medium supplemented with 0.5 μM naphthalene acetic acid (NAA) and 4.4 μM benzyl adenine (BAP) were used to induce ORG in cultures of root and IZE explants, respectively. The capacity for ORG was evaluated in three-week old cultures on SIM. ORG efficiency was calculated as the percent of explants regenerating shoots. ORG productivity referred to the average number of shoots formed on the responding IZE or developed by 1 cm of root explant.

Induction of LEC2 protein activity in transgenic plants

To induce activity of the LEC2 protein a synthetic gluco-corticoid, dexamethasone (water soluble DEX, Sigma), was added to the media at a concentration of 30 μM, as described by Ledwoń and Gaj (2009).

Analysis of stress responses in seed germination assay

Seeds were sterilized with chlorine gas in a dessicator jar and plated onto ¼ MS (Murashige and Skoog 1962) salts with 10 g L^{-1} sucrose and 8 g L^{-1} agar. The percentage of seed germination was scored on media with different concentrations of NaCl (0; 50; 150 and 200 mM) and mannitol (0; 100; 200 and 300 mM). Plates were chilled at 4°C in the dark for 3 days and moved to 22°C with a 16h–light/8h-dark cycle with light intensity of 100 μmol m^{-2} s^{-1}. Germination was defined by emergence of the radicle through the seed coat and the evaluation was carried at 4th day after stratification. In each experimental combination 80–120 seeds in three replications were evaluated.

RNA isolation and RT-qPCR analysis

Total RNA for RT-qPCR analysis was isolated with the RNAqueous kit (Ambion) at the selected time points of SE and ORG cultures (0, 1, 3, 5, 10 and 15 day), and during IZE-development on E0 medium (0, 1, 3, 5, 10 day). The concentrations and purity of RNA were evaluated with a ND-1000 spectrophotometer (NanoDrop). RNAs were treated with RQ1 RNase-free DNase I (Promega), following the manufacturer's instructions. cDNA synthesis was carried out with the RevertAid^{TM} First Strand cDNA Synthesis Kit (Fermentas). Standard cDNA samples with sevenfold serial dilution were used for PCR efficiency calculations. Amplification curves were generated for all used primers pair. The relative expression level of the genes was determined with the LightCycler 2.0 system (Roche), and gene specific primers. The *At4g27090* gene encoding ribosomal 60S L14 protein was used as the standard control (Thellin et al. 1999; Gaj et al. 2005; Yamagishi et al. 2005; Braybrook et al. 2006; Baster et al. 2009; Ledwoń and Gaj 2009), and the following primers were used for RT-qPCR reactions of the analysed genes: *OLEO4* (R)-5′-GCCAAGATTCCCGTCACAGC-3′; *OLEO4* (F)-5′-CGCG GCCCCTCCACTAAC-3′; *CRA1* (R)-5′-TGGTTCCTGTCC GTCGTTGT-3′; *CRA1* (F)-5′-TCGTGGCTAAGGGAC-GAGGT-3′; *At4g27090* (R)-5′-CCTCGATCAAAGCCTTCT TCT-3′; *At4g27090* (F)-5′-GTCGTTATCGTCGACGTTG TT-3′.

RT-qPCR reactions contained the fluorescent dye SYBR Green (LightCycler FastStart DNA Master SYBR Green I—Roche), and the results were analyzed with the Light-Cycler 4 Software (Roche). In all analyzed tissue samples the control gene exhibited a constant expression pattern with Cp = 18 ± 1. All experiments were conducted with at least 3 biological replicates, and the Real-time qPCR reactions were repeated twice.

Identification of insertional mutants

The seeds of *cra1-1*, *cra1-2* and *oleo4* insertional mutants were purchased as part of the T$_3$ segregating lines (Alonso et al. 2003). To identify homozygous mutants DNA was isolated from individual T$_3$ plants using a modified micro-CTAB method (Doyle and Doyle 1987). PCR reactions containing two genes and one insert specific primer (http://signal.salk.edu/tdnaprimers.html) were then conducted. The size of amplified products indicated the homo- or hemizygous status of the plants, in terms of the presence of the insert within the gene of interest.

Statistical analysis

The Kruskal–Wallis ANOVA test was employed for the analysis, followed by U Mann–Whitney test. The average

value for three biological replicates with the standard deviation is presented.

Results

Expression of *CRA1* and *OLEO4*

Callus versus embryogenic culture

To answer the question whether the transcription of *SSR* genes is correlated with embryogenic tissue the explants at day 15 of culture on E5 medium, efficiently forming somatic embryos, and those occasionally producing only non-embryogenic callus (Fig. 1), were compared in terms of *CRA1* and *OLEO4* transcription level. It was found that the expression level of *CRA1* and *OLEO4* was significantly higher (up to 17 fold) in Col-0 culture efficiently forming somatic embryos than in callus developing from explants which failed in embryogenic induction (Fig. 2).

Alternative morphogenic pathways induced in vitro

IZEs of Col-0 treated with auxin, 5 μM 2,4-D in E5 medium, undergo efficient SE induction, while the use of auxin and cytokinin in CIM-SIM-C medium combination triggers effective shoot regeneration. In contrast, hormone free medium (E0) enables development of IZEs into seedlings. Real-time qPCR analysis indicated that *CRA1* and *OLEO4* displayed a similar expression pattern in IZEs cultured under alternative hormonal treatment (Fig. 3). At the first day (1 day) of culture, regardless of medium applied, the transcription level of the both genes dropped significantly. Then, a gradual increase of *CRA1* and *OLEO4* expression was noticed during seedling development on hormone-free medium. In contrast to E0 medium, explants induced in presence of auxin to induce SE or auxin combined with cytokinin to induce ORG displayed
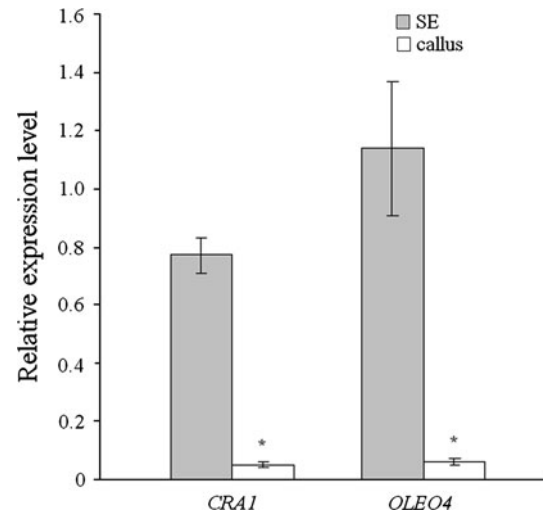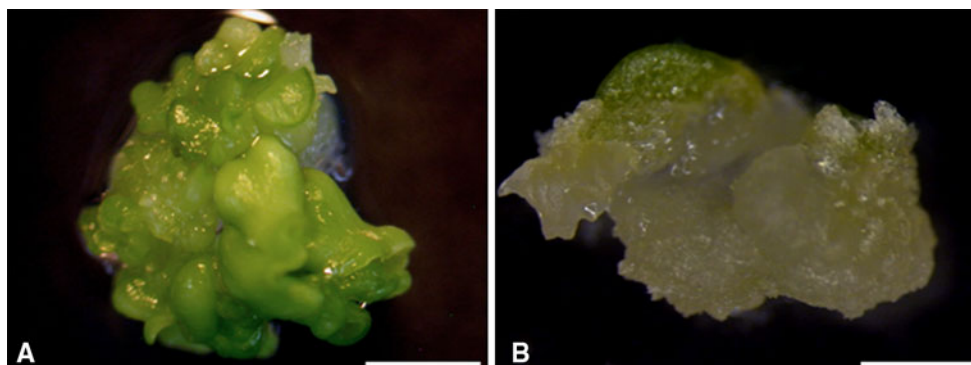

Fig. 2 *CRA1* and *OLEO4* expression level in 15 days old Col-0 cultures of different embryogenic capacity: highly embryogenic culture (SE) and non-embryogenic callus. *Values significantly different from those observed in SE culture ($P < 0.05$)

relatively constant and significantly lower than in freshly isolated explants, transcription level. The results indicated that *CRA1* and *OLEO4* expression pattern is not specific for embryogenic culture, and instead of auxin-stimulation assumed for SE-specific genes down-regulation of transcription was observed.

LEC2 overexpression

The expression level of *CRA1* and *OLEO4* was also investigated in E5 cultures derived from 35S::LEC2-GR; Col-0 explants to reveal the relation between *LEC2* expression and activity of *SSR* genes under in vitro conditions. The results indicated that the transcription level of *CRA1* and *OLEO4* dropped in SE culture, irrespective of *LEC2* expression induced by inclusion of DEX into E5 medium (Fig. 4).

Fig. 1 IZE-derived on E5 medium Col-0 cultures at 15 days of different embryogenic capacity **a** highly embryogenic culture producing numerous somatic embryos; **b** non-embryogenic callus. *Scale bars* = 0.4 cm
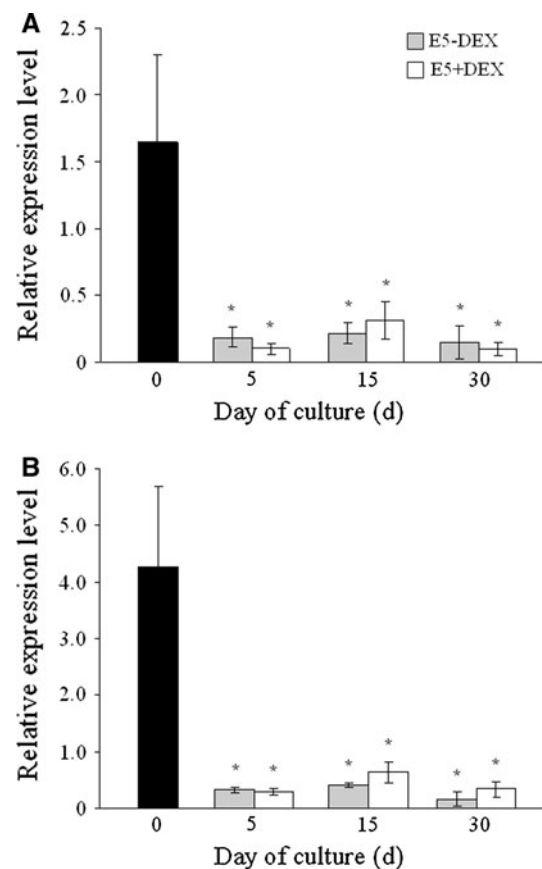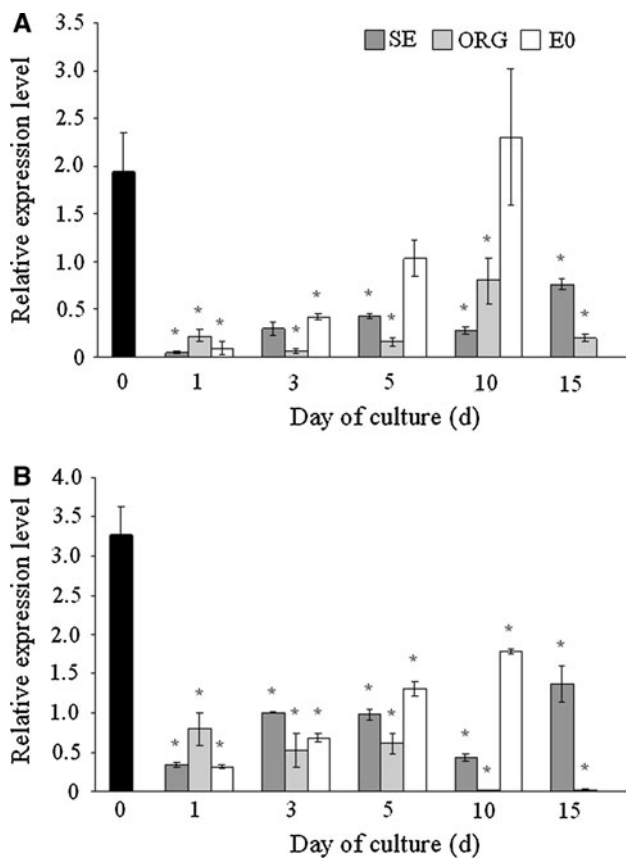
Fig. 3 Expression level of *CRA1* (**a**) and *OLEO4* (**b**) genes during IZE-derived cultures of the Col-0 induced towards somatic embryo formation (SE), shoot regeneration (ORG) or seedling development (E0). *Values significantly different from those observed at 0 day ($P < 0.05$)

Fig. 4 Expression level of *CRA1* (**a**) and *OLEO4* (**b**) genes in IZE cultures of 35S::LEC2-GR, Col-0 induced on E5 medium in respect to DEX treatment. *Values significantly different from those observed at 0 day ($P < 0.05$)

Mutants impaired in SE

We found it interesting to compare the transcription level of the *CRA1* and *OLEO4* genes in IZE cultures of highly embryogenic Col-0 genotype and severely impaired in SE mutants, a*xr4-1*, *cbp20*, *lec2* and *tan1-2*. It was observed that transcript level of both genes in freshly isolated explants (0 day) of the SE-impaired mutants was not reduced in comparison to highly embryogenic Col-0 (control) explants, but was found even significantly higher for *OLEO4* in IZEs of *cbp20* and *tan1-2* mutants (Table 1). During E5 culture, the expression pattern of *CRA1* and *OLEO4* in mutant-derived cultures was similar to that displayed by Col-0. Accordingly, significant down-regulation of their activity was observed during the culture, however the decrease in *CRA1* and *OLEO4* transcription was more drastic in SE-impaired mutant cultures than in highly embryogenic control culture. Especially, expression of *CRA1* and *OLEO4* was dramatically inhibited in culture of *tan1-2* mutant.

In vitro morphogenic capacity of *cra1* and *oleo4* mutants

In order to further investigate the relationship between genes encoding SSR, and embryogenic response of somatic tissue, the morphogenic potential of lines with mutations in *OLEO4* and *CRA1* genes were assessed. Two insertional mutant lines for the *CRA1* gene (*cra1-1*, *cra1-2*), and one line (*oleo4*) for *OLEO4*, were studied under in vitro culture. The phenotypes of the mutants *in planta* were carefully inspected and no distinct morphological changes were noticed. Morphology of zygotic embryos, seedlings and plants including growth rate, flowering time and fertility were found similar to Col-0 control plants. In contrast, both mutations were found to significantly impair embryogenic response of the IZE explants, and a significantly reduced frequency (over 50%) of SE-responding explants was noticed in the mutant cultures (Fig. 5a). Additional to the deleterious effect on SE efficiency, mutations in *CRA1* gene did not significantly reduce embryo number (Fig. 5b).
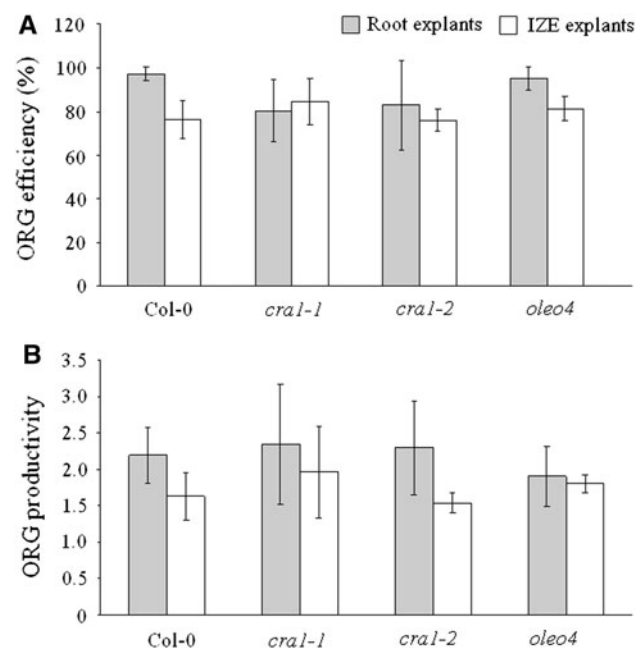
Table 1 Expression level of *OLEO4* and *CRA1* genes during in vitro culture on E5 medium of SE-impaired mutants (*axr4-1, cbp20, lec2, tan1-2*), compared with the Col-0 genotype.
* Values significantly different from those observed in Col-0 culture at the same stage of culture ($P < 0.05$)

	0 day	1 day	5 days	10 days	15 days
OLEO4					
Col-0	2.07 ± 0.86	0.32 ± 0.03	0.92 ± 0.1	0.47 ± 0.07	0.87 ± 0.29
axr4-1	2.05 ± 0.75	0.24 ± 0.1	0.53 ± 0.05*	0.23 ± 0.04*	0.34 ± 0.07*
cbp20	3.53 ± 1.97*	0.22 ± 0.01*	0.22 ± 0.1*	0.04 ± 0.02*	0.05 ± 0.01*
lec2	2.47 ± 0.0	0.8 ± 0.32*	0.34 ± 0.06*	0.06 ± 0.02*	0.09 ± 0.02*
tan	5.87 ± 2.33*	0.35 ± 0.1	0.09 ± 0.03*	0.0012 ± 0.0001*	0.002 ± 0.003*
CRA1					
Col-0	1.48 ± 0.85	0.04 ± 0.02	0.39 ± 0.08	0.25 ± 0.06	0.56 ± 0.36
axr4-1	0.59 ± 0.42	0.04 ± 0.04	0.35 ± 0.07	0.23 ± 0.1	0.26 ± 0.08
cbp20	3.87 ± 2.03	0.05 ± 0.03	0.14 ± 0.03*	0.03 ± 0.02*	0.03 ± 0.01*
lec2	1.86 ± 0.0	0.43 ± 0.36*	0.27 ± 0.03	0.04 ± 0.02*	0.06 ± 0.03*
tan	2.94 ± 1.1	0.29 ± 0.07*	0.09 ± 0.07*	0.00003 ± 0.0002*	0.00004 ± 0.000039*
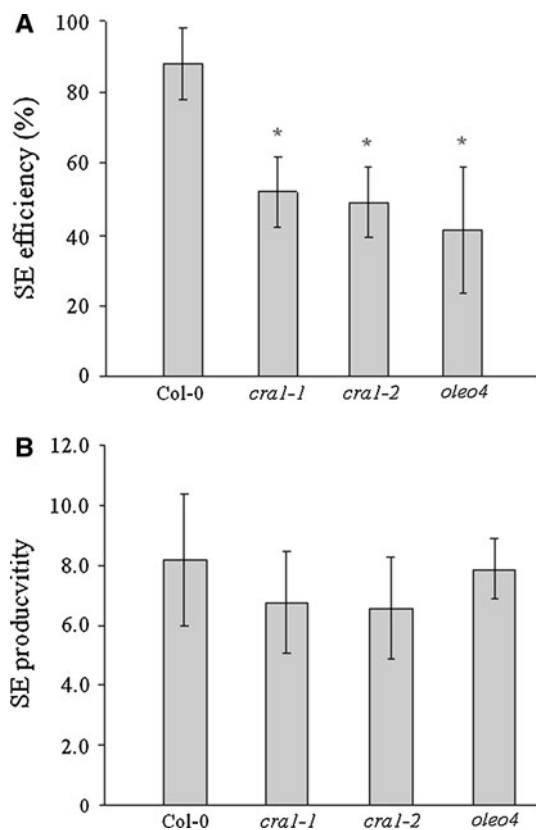
Fig. 5 Capacity for SE in cultures of *cra1* (*cra1-1* and *cra1-2*) and *oleo4* mutants and their parental Col-0 genotype. **a** SE efficiency; **b** SE productivity. *Values significantly different from those observed for Col-0 ($P < 0.05$)

Fig. 6 High efficiency (**a**) and productivity (**b**) for shoot ORG in 21 days root and IZE-derived cultures of Col-0 genotype and *cra1-1; cra1-2* and *oleo4* mutants

To verify whether the reduction in morphogenetic potential of *cra1* and *oleo4* mutants is specific for SE induction, the capacity of the insertional mutants for in vitro ORG was also evaluated. Two explant types which require different hormone treatment to induce shoot regeneration were used—cotyledons of IZEs and seedling roots. In contrast to SE inductive conditions, the mutant explants subjected to shoot induction displayed an efficiency similar to Col-0 culture capacity for shoot regeneration, in terms of efficiency (Fig. 6a) and productivity (Fig. 6b) of organogenesis. This observation suggests that mutations in *OLEO4* and *CRA1* genes specifically impair tissue capacity for SE.

cra1 and oleo4 mutant responses to stress treatment

To reveal mechanism of SE-impaired response observed in culture of the *cra1* and *oleo4* mutants we evaluated sensitivity of these mutants to stress factors. To this end, the frequency of germination in the mutant and control seeds
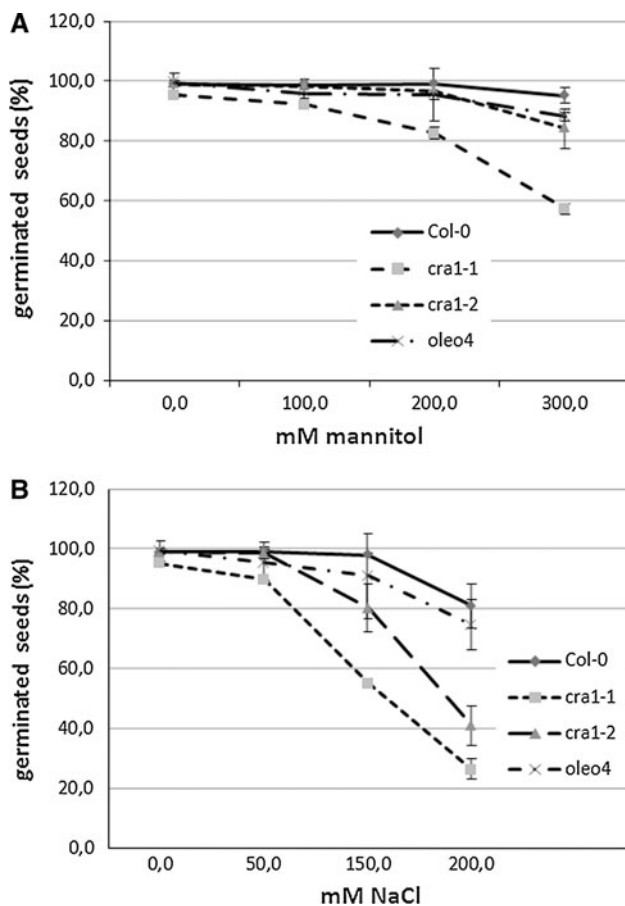
Fig. 7 Seed germination frequency of *cra1-1*, *cra1-2* and *oleo4* mutants under stress induced with mannitol (**a**) and NaCl (**b**)

were evaluated on media supplemented with different concentrations of NaCl and mannitol (Fig. 7). It was noticed that both *cra1* mutants displayed decreased tolerance to NaCl. On average, 50–75% less of *cra1-1* and *cra1-2* than Col-0 seeds germinated on medium with 200 mM NaCl. Beside NaCl, *cra1-1* seeds were also found more sensitive to mannitol, and its inclusion into the medium at a concentration of 300 mM of inhibited germination of over 40% of mutant seeds. The inhibitory effect of NaCl and mannitol on seed germination of other mutants tested were also noticed, however it was less pronounced.

Discussion

In Arabidopsis, it was proposed that the cellular environment conferring cell competence to switching on the embryogenic program of development in somatic cells is similar to that of the maturation phase zygotic embryos (Stone et al. 2008; Braybrook and Harada 2008). As the most distinct phenotype of ZE maturation phase is the

accumulation of storage materials, the question remains as to whether these compounds are related with the determination of embryogenic potential in Arabidopsis.

Hence, in this study the genes encoding representatives of the major storage compounds of Arabidopsis seeds, cruciferin and oleosin were studied, in terms of their transcription profiles in in vitro cultured tissue. It was indicated that although *SSR* genes display significantly higher activity in cultures efficiently producing somatic embryos than in callus, their expression was found to be not stimulated by auxin treatment, the key factor in SE induction. Moreover, the expression pattern of *CRA1* and *OLEO4* was revealed to be nonspecific to embryogenic culture. These observation rendered the hypothesis of direct involvement of *SSR* genes in SE induction rather unlikely. Thus, it can be assumed that transcription of *SSRs* in cultured somatic tissue may result from the activity of their regulatory genes, including those of essential function in SE induction. Accordingly, *LEC2*, a master regulator of ZE (Harada 2001), which directly controls 12S storage protein genes, including *CRA1* gene (Braybrook et al. 2006; Stone et al. 2008), was shown to determine SE induction in IZE culture (Gaj et al. 2005; Ledwoń and Gaj 2009). Several observations show that during ZE expression the pattern of *SSR* did not directly follow *LEC2* expression. In seed development the highest expression of *LEC2* was detected from the early to the mid stages of ZE (Stone et al. 2001; Santos-Mendoza et al. 2008; Stone et al. 2008), and preceded the highest accumulation of *SSR* transcripts observed in mature seeds (Ruuska et al. 2002). Our observations on SE indicated that, in parallel to ZE, up-regulation of *LEC2* activity is characteristic for the early phases of embryogenic culture (Ledwoń and Gaj 2009), while increased *SSR* expression was detected in advanced embryogenic cultures producing cotyledon-stage somatic embryos (present results).

Moreover, it was found that an elevated *LEC2* expression induced in 35S::LEC2 transgenic explants did not stimulate *CRA1* expression in embryogenic culture. Similarly, up-regulation of *CRA1* and *OLEO* was not observed in seeds of transgenic lines, in contrast to stimulation of these genes in postembryonic organs overexpressing *LEC2* (Stone et al. 2008). These observations suggest that some similarities exist in relations between *SSR* and *LEC2* in terms of their expression pattern during ZE and SE.

In addition, the analysis of SE-defective mutants revealed that high activity of *CRA1* and *OLEO4* found in some of them is not sufficient to define SE-promoting environment in explant tissue.

Collectively, the results on expression patterns of *SSR* genes analyzed in relation to various tissue culture conditions and genotypes did not implicate a direct or specific role of these genes in SE induction, but rather illustrate some similarities to their activity during ZE.

However, the observations that *cra1* and *oleo4* mutations significantly impaired SE induction in IZE-derived cultures suggest that *SSR* genes may contribute to embryogenic induction. Possibly, the activity of *CRA1* and *OLEO4* genes can improve the SE-conducive cellular environment, and support the perception of an embryogenic signal by explant tissue resulting in the onset of embryogenic development. A possible role of *SSR* in SE induction should be considered in the context of a stress-related concept of SE induction. An immense body of recent data strongly supports the idea that mechanisms governing cell reprogramming and transcriptome alteration necessary for somatic cells to acquire an embryogenic state imitate stress-related responses (Fehér et al. 2003; Zavattieri et al. 2010; Karami and Saidi 2010; Grafi et al. 2011). In support of the postulated positive relation between *SSR* responsiveness to stress and tissue competence for SE, storage product genes were indicated among those induced in response to several abiotic and biotic stresses, to which various plants were subjected (Gong et al. 2001; Rossel et al. 2002; Shewry 2003; Vooková et al. 2006; Zhang et al. 2009; Sobhanian et al. 2010; Sharma et al. 2010), including Arabidopsis (Berger et al. 2002; Baud et al. 2008). Relevantly to the genes analysed in the present work, water stress induced the transcription of cruciferin genes in rapeseed (Moons et al. 1997), and oleosins were documented to be involved in plant response to abiotic stress exerted by freezing (Shimada et al. 2008).

Thus, it can be speculated that impaired stress-related mechanisms are responsible for decreased embryogenic responses presented by the mutants defective in cruciferin and oleosin expression. In support for this assumption, we observed reduced tolerance to high salinity and osmotic pressure in *cra1* mutants. Interestingly, these stress factors were reported to enhance SE-capacity of Arabidopsis seedling tissue treated with auxin (Ikeda-Iwai et al. 2003). Further support to the possible link between *SSR* genes, stress tolerance and capacity for SE is provided by observation that in conifers, germin-like proteins recognized as stress-related proteins, were suggested to play a central role in somatic embryo formation via the regulation of cell wall remodeling (Mathieu et al. 2003).

Postulation on the positive influence of *SSR* genes on enhanced embryogenic capacity of cultured tissue, possibly via improvement of cell response to stress imposed in vitro, is also in agreement with the recent view on seed maturation phenomenon. It is postulated that the biological processes found in ZE, and leading to mature seeds, may not contribute to the function of the seed *per se* (Angelovici et al. 2010). In this perspective, it is speculated that the maturing seeds, which accumulate vast number of various proteins and transcripts, acquire higher developmental plasticity. Some recent observations suggest that apart from providing energy

to the post-germinative seedling (Penfield et al. 2006), SSR may have unexpectedly new, not yet defined functions during post-embryonic development of plants. Accordingly, the transcription of caleosin, a storage protein believed to be seed specific, was observed transiently in root tips of Arabidopsis (Næsted et al. 2000), while oleosin transcripts were detected at a relatively high level in young root meristems of *Brassica napus* (Murphy et al. 2001).

Based on our results, indicating that the defective embryogenic responses of *cra1* and *oleo4* mutants is coupled with their impaired tolerance to stress factors, it is conceivable that high activity of *SSR* genes in IZEs of Arabidopsis indirectly increases embryogenic response of somatic tissue, through providing a better endogenous environment to respond under stress conditions. Mechanisms which relate high activity of *SSR* and improved stress responsiveness of somatic tissue remain to be elucidated.

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