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RESEARCH NOTE

LEAFY COTYLEDON2-mediated control of the endogenous hormone content: implications for the induction of somatic embryogenesis in Arabidopsis

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Abstract The overexpression of *LEAFY COTYLEDON2* (*LEC2*) in Arabidopsis, results in the induction of somatic embryogenesis (SE) in an auxin-free environment and the stimulation of auxin biosynthesis was postulated as being involved in this response. To gain further insight into the hormone-related functions of *LEC2* in SE, the effect of *LEC2* overexpression on the hormone content in Arabidopsis plants and in vitro cultured explants was analysed. In addition to indole-3-acetic acid (IAA) and cytokinins (CKs), which are hormones that play a key role in plant development, the stress-related hormones, abscisic acid (ABA) and salicylic acid (SA), which are involved in the stress response that is related to SE-induction, were analysed. Together with the observations that *LEC2* activity can compensate for the auxin treatment required for SE induction (Ledwoń and Gaj in Plant Tissue Cell Org Cult 28:1677–1688, 2009) and *LEC2* may control auxin biosynthesis pathway during SE induction (Wójcikowska et al. in Planta 238:425–440, 2013), a significant increase in the IAA content in response to *LEC2* overexpression found in the present study supply further evidence that LEC2-controlled auxin biosynthesis may be involved in the mechanism that triggers embryogenic development in somatic cells. Moreover, LEC2-controlled SE induction was shown to be associated with a decrease in the total content of CKs and an accumulation of some specific CK types, including isopentenyl-adenin and *cis*-zeatin. Additionally, an increase in SA and a decrease in ABA content were also found to be related to *LEC2* activity in embryogenically induced tissue. The obtained results provide further proof of the close link between *LEC2* and the establishment of the hormonal environment that is required for the promotion of SE.

Keywords Auxin · Cytokinins · ABA · SA · *LEC2* overexpression · In vitro culture

Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
ABA	Abscisic acid
CKs	Cytokinins
cZ	*cis*-zeatin
DEX	Dexamethasone
DZR	Dihydrozeatin-ribosid
FW	Fresh weight
IAA	Indolilic-3-acetic acid
iP	Isopentenyl-adenin
IZE	Immature zygotic embryo
LEC2	*LEAFY COTYLEDON2*
SA	Salicylic acid
SE	Somatic embryogenesis
tZ7G	*trans*-zeatin-7-glucoside
tZ9G	*trans*-zeatin-9-glucoside
tZROG	*trans*-zeatin-riboside-*O*-glucosid
ZR	*trans*-zeatin-ribosid

The *LEC2* gene, which is a master regulator of zygotic embryogenesis in Arabidopsis, has been suggested as being in control of embryogenic development in somatic cells and the SE-promoting effect of *LEC2* overexpression in plants and explants cultured under auxin-free conditions was reported (Stone et al. 2001; Ledwoń and Gaj 2009). Hence, the overexpression of *LEC2* was postulated as

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compensating for the auxin treatment and accordingly, *LEC2*-stimulated expression of the *YUC* (*YUCCA*) genes, which encode the key enzymes in tryptophan dependent pathway of auxin biosynthesis, was recently indicated in SE (Wójcikowska et al. 2013).

To gain further insight into the auxin-related function of *LEC2* in SE, we evaluated the effect of *LEC2* overexpression on the IAA content in: (1) plants that are able to produce somatic embryos *in planta* and (2) immature zygotic embryo (IZE) explants that were cultured in vitro on auxin medium. Considering the fact that the extensive interactions between auxin and other hormones are associated with plant development and that a variety of hormone-related genes were reported to be LEC2-controlled (Braybrook et al. 2006), we found it to be reasonable to also analyse the CKs, which also play an essential role in plant development and the stress hormones (ABA and SA), which are believed to affect in vitro induced SE.

Transgenic plants of *Arabidopsis thaliana* (L.) Heynh., which carry a 35S::LEC2-GR transgene, were used in the study (Ledwoń and Gaj 2009). The plants display a high and stable level of the *LEC2* transcripts after treatment with 30 μM of water soluble dexamethasone (DEX) (Sablowski and Meyerowitz 1998).

The plants that were used as the source of the IZE explants were grown in Jiffy-7 pots at 22 °C under a 16 h photoperiod of 100 μM m^{-2} s^{-1} white, fluorescent light. The IZEs were used as explants for the in vitro culture. In order to induce SE, the standard protocol for Arabidopsis was used (Gaj 2001). Accordingly, ten IZEs were cultured in a Petri dish on an agar-solidified (8 g L^{-1}) induction medium (E5) containing basal B5 macro and micro-elements (Gamborg et al. 1968), 20 g L^{-1} sucrose and 5 μM 2,4-dichlorophenoxyacetic acid (2,4-D) (Sigma). The IZE explants that were cultured for 5 days on an E5 medium were used for HLPC analysis.

To produce plants for induction of SE *in planta*, seeds of the 35S::LEC2-GR transgenic line were sterilised in a 20 % solution of commercial bleach for 20 min and then rinsed three times in sterile water. Seeds were then germinated on a 1/2 MS medium (Murashige and Skoog 1962) containing 10 g L^{-1} sucrose and 8 g L^{-1} agar. Four-week-old plants that produced somatic embryos after DEX-treatment were used for the HLPC analysis.

Plants and IZE-cultures were grown at 23 °C under a 16 h photoperiod of 40 μM m^{-2} s^{-1} white, fluorescent light.

HPLC analysis was used to evaluate the content of different hormones including: indole-3-acetic acid (IAA), abscisic acid (ABA), salicylic acid (SA) as well as ten forms of cytokinins: *trans*-zeatin-7-glucoside (tZ7G), *trans*-zeatin-9-glucoside (tZ9G), *cis*-zeatin (cZ), *trans*-zeatin-riboside-*O*-glucosid (tZROG), dihydrozeatin-ribosid (DZR), *trans*-zeatin-ribosid (ZR) and isopentenyl-adenin (iP), *trans*-zeatin (tZ), dihydrozeatin (DHZ) and *trans*-zeatin-*O*-glucosid (tZOG). Hormones were extracted from tissue that had been frozen in liquid nitrogen and 100 mg of tissue was used per sample. The plant tissues for HPLC analysis were produced in two biological replicates, and two technical replicates of each repetition were carried out. HPLC analysis followed the method described by Großkinsky et al. (2014) and was performed in the Department of Plant Physiology, Institute of Plant Sciences, University of Graz, Austria.

Statistical analyses were performed using the student's *t* test embedded in microsoft excel. Only a return of $P < 0.05$ was designated as being statistically significant.

The HLPC analysis indicated a significant modulation in the hormone content and profile in tissue that overexpressed *LEC2*. A dramatic increase in the IAA content in the plants and IZE-culture in response to *LEC2* overexpression was observed. The level of IAA, which was undetected in the control plants, increased significantly in the DEX-treated plants that produced somatic embryos (Fig. 1a). A significant, more than a three-fold increase in the IAA content was also observed in the transgenic IZE-culture that were induced on the auxin medium and treated with DEX. This result supports our earlier postulation that the reduced SE efficiency and prominent callus formation that was observed in the transgenic IZEs cultured on auxin-medium results from over-optimal for SE induction production of IAA (Ledwoń and Gaj 2009). A substantially higher IAA level was accumulated in the plants (508.7 ng g^{-1} FW) than in the IZE-culture (29.8 ng g^{-1} FW), which possibly results from the tissue-specific genetic components and regulatory pathways that are involved in the LEC2-controlled auxin biosynthesis (Zhao 2010). In addition, exogenous 2,4-D, which was used in the IZE culture to induce SE, may strongly affect the endogenous IAA accumulation (Pasternak et al. 2002). Thus, there may be some differences in the auxin-related mechanisms that are involved in SE induction *in planta* (plants) and in vitro (auxin-cultured IZEs).

Out of the ten CK-types that were analysed, three (tZ, DH and ZOG) were not detected in any of the tissue samples. The analysis showed that in contrast to auxin, *LEC2* overexpression negatively affected the level of CKs in both the plants and the IZE-culture (Fig. 1b). An *LEC2*-induced reduction in the total CK content was especially dramatic in plants (2.5-fold) and was coupled with a considerably high IAA accumulation. *LEC2* overexpression was also found to significantly limit the diversity of the CK-types that accumulated. Three of the CKs (tZ9G, tZROG, ZR) were not detected in the DEX-treated tissue while the level of two others (tZ7G, DZR) was found to be significantly reduced in response to *LEC2* overexpression. In contrast, the content of iP and cZ was found to be

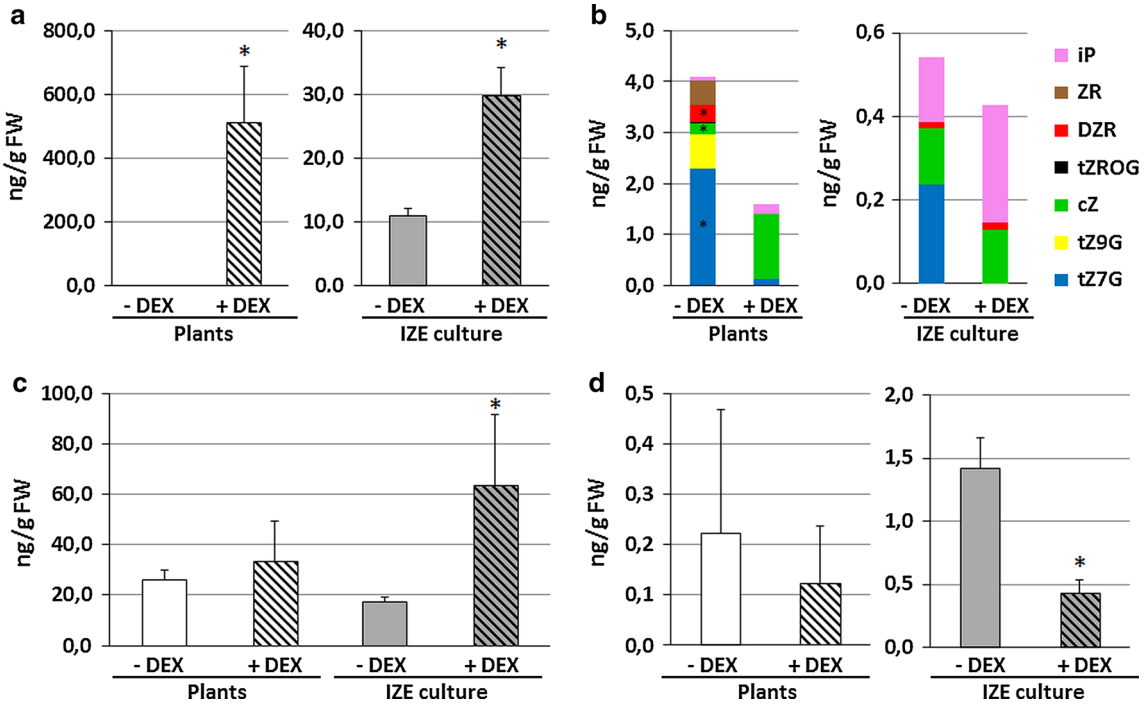

Fig. 1 Level of hormones in plants and IZE-culture of 35S::LEC2-GR transgenic line in response to DEX-induced (+DEX) overexpression of *LEC2*, including: IAA (**a**); cytokinins: *trans*-zeatin-7-glucoside (tZ7G), *trans*-zeatin-9-glucoside (tZ9G), *cis*-zeatin (cZ), *trans*-zeatin-riboside-*O*-glucosid (tZROG), dihydrozeatin-ribosid (DZR), *trans*-zeatin-ribosid (ZR) and isopentenyl-adenin (iP) (**b**); SA (**c**) and ABA (**d**). Values represent the mean ± SD from two independent sets of samples. *Asterisks* indicate significant differences between untreated and DEX-treated cultures ($P < 0.05$, student's *t* test). FW, fresh weight

increased in both the plants and in the IZE-culture that overexpressed *LEC2*. These CKs belong to the isoprenoid CKs, which is the most abundant class of CKs and iP is expected to have a higher biological activity than cZ (Sakakibara 2006). Collectively, the analyses of CKs indicated that although LEC2-directed SE induction is associated with a decrease in total content of CKs specific types of CKs such as iP and cZ may be positively related to the embryogenic response and their biological function in the mechanism of SE-induction remains to be revealed.

The extensive cross-talk between CKs and auxin in plant development (Moubayidin et al. 2009) might account for the significant inhibition of the CK accumulation that was observed in tissue in which the overexpression of *LEC2* enhanced auxin. A modulation in the level of CKs in response to the auxin treatment was demonstrated in different plants, including Arabidopsis (Nordström et al. 2004). In conclusion, a negative relation between CKs content and embryogenic potential of explant tissue seems to exist in Arabidopsis and an *LEC2*-mediated reduction in the content of CKs may have a beneficial effect on the induction of SE. Similarly, a low level of CKs was reported to be related to a high embryogenic response in some other plants (Rajasekaran et al. 1987; Wenck et al. 1988).

The present analysis indicated that *LEC2* overexpression stimulates a high, threefold increase in the SA concentration in the IZE culture (Fig. 1c). In contrast, the increase in SA content was not significant in DEX-treated plants implying that SA accumulation in response to *LEC2* overexpression seems to be specific to in vitro conditions. This observation suggests that similarly to other plants it can also be assumed that there is a positive relation between SA and embryogenic response in Arabidopsis (Quiroz-Figueroa et al. 2001; Hao et al. 2006). Among the SA-induced mechanisms that promote SE, the inhibition of ethylene production (Roustan et al. 1990) and an increase in the hydrogen peroxide level (Luo et al. 2001), which have positive effect on SE (Kairong et al. 2002), may be considered. The LEC2-stimulation of SA content that was observed may result from an accumulation of *AtMES9* and *AtMES12* transcripts of methyl salicylate esterases (MeSA), involved in a SA biosynthesis pathway, that was observed in response to *LEC2* overexpression (Braybrook et al. 2006).

The accumulation of ABA in the highly embryogenic cotyledonary stage of the IZEs implied a positive relation between the ABA level and the embryogenic potential of Arabidopsis tissue (Braybrook and Harada 2008). The HPLC results showed a significant reduction in the ABA content in response to *LEC2* overexpression and a sharp (45–70 %) decrease in the ABA level was observed in both the DEX-treated plants and the IZE-culture (Fig. 1d).

Similarly, a low level of ABA during the initial phases of SE induction was described in other plants (Pescador et al. 2012). In support of the involvement of *LEC2* in the reduction of the ABA level during the induction of SE, the genes encoding ABA 8′-hydroxylases (*CYP707A1*, *CYP707A2*, *CYP707A3*) responsible for ABA catabolism were reported to be up-regulated in response to *LEC2* overexpression (Braybrook et al. 2006). The presence of the RY motif, which is characteristic of LEC2-controlled genes in a promoter region of the *CYP* genes further supports the assumption of the LEC2-mediated negative control of ABA activity during SE induction.

One possible scenario of SE induction in Arabidopsis is that the stimulation of *LEC2* activity, which can result from the overexpression of the gene (Stone et al. 2001) or 2,4-D treatment (Ledwoń and Gaj 2009), exerts an embryogenic response via the up-regulation of the auxin biosynthesis *YUC* genes (Wójcikowska et al. 2013). In response to IAA accumulation, substantial changes in the content and profiles of other hormones, including CKs, SA and ABA (present results), are induced and finally, the hormone-controlled SE-effectors are triggered. Further experiments are needed to identify the SE-specific targets of the hormone signalling pathways that are involved in the embryogenic transition of somatic cells.

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