

The content of endogenous hormones and sugars in the process of early somatic embryogenesis in the tree fern *Cyathea delgadii* Sternb.

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Abstract Somatic embryogenesis (SE) of *Cyathea delgadii* presents a model system for investigating the mechanisms associated with the acquisition of embryogenic competence by single epidermal cells of stipe explants cultured on plant growth regulator-free medium. The present work reveals relationship between endogenous hormone and sugar content in the process of early SE in *C. delgadii*. By comparing two types of initial explants, i.e. incapable (non-etiolated) and capable (etiolated) of SE, it was established that in etiolated explants, the glucose, fructose, sucrose, and abscisic acid (ABA) contents diminished, but indole-3-acetic acid (IAA) and cytokinins (CKs; i.e. *cis/trans* zeatin, *cis/trans*-zeatin riboside, kinetin, kinetin riboside, isopentenyladenosine) contents increased. The ratios between phytohormones revealed that a high concentration of ABA is the main factor inhibiting SE induction. Because

of explant excision, a dramatic reduction in concentration of all phytohormones studied was observed, but hormonal balance and sugar content remained almost unchanged. During the 14-day-long culture, the ABA/CKs and ABA/IAA ratios remained constant, whereas the greatest differences were detected for the IAA/CKs and Z-type/iPA cytokinin ratios. Excluding day 6 of culture, cytokinins were found to be the predominant phytohormones over IAA. An almost 12-fold increase in soluble sucrose concentration at day 6 of culture might be the switch to the SE expression phase. Frequent cell divisions leading to somatic embryo formation are clearly associated with increase in *trans*-zeatin riboside content.

Keywords Etiolation · Explant excision · High-performance liquid chromatography · PGR-free medium · Phytohormones

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Abbreviations

ABA	Abscisic acid
<i>c/t-Z</i>	<i>Cis/trans</i> zeatin
<i>c/t-ZR</i>	<i>Cis/trans</i> -zeatin riboside
CKs	Total concentration of cytokinins
FW	Fresh weight
HPLC	High-performance liquid chromatography
IAA	Indole-3-acetic acid
iPA	Isopentenyladenosine
Kin	Kinetin
KinR	Kinetin riboside
PGR	Plant growth regulator
SE	Somatic embryogenesis

Introduction

Somatic embryogenesis (SE) is an important pathway for the regeneration of plants, as well as an attractive system for the study of the morphology, physiology, and genetic mechanisms of embryo induction and development (Elhiti et al. 2013; Fehér 2015). Although this process was first described many years ago, our present knowledge about the acquisition of embryogenic competence by somatic cells remains unknown, and it is unexplored in ferns.

Understanding of the physiological mechanism that underlies the induction of SE is essential for the regulation of this process. Phytohormones are employed as signalling molecules for the induction of SE by mediating the signal transduction cascade leading to the reprogramming of gene expression patterns (Elhiti et al. 2013). Some hormones, such as abscisic acid (ABA) and cytokinins have been shown to help regulate the sugar metabolism and transport. On the other hand, sugars regulate the synthesis, conjugation and transport of phytohormones (for example ABA), and also the expression of components of phytohormone-response pathways (Gibson 2004). A direct link between sugar signalling and hormone biosynthesis has been described by Eveland and Jackson (2011). At present, it is not surprising that the availability of sugars in the induction medium helps regulate developmental processes in ferns (Mikuła et al. 2015a). However, the changes in endogenous sugar contents during SE induction have not yet been studied.

The relationships between endogenous hormone contents and SE have been the subject of many studies. However, most of them are limited to the comparisons of the non-embryogenic and embryogenic callus (Jiménez and Bangerth 2000; Pintos et al. 2002; Jiménez et al. 2005), the non-embryogenic explants and those with somatic embryos developed on the explant surface (Pérez-Jiménez et al. 2013), or the genotypes/explants with different embryogenic capability (Wenck et al. 1988; Centeno et al. 1997; Limanton-Grevet et al. 2000; Jiménez and Bangerth 2001a). Only a few reports on the progressive hormonal changes that occur during SE are available, but they differ in the type of explant, method (plant growth regulators or other stress factors) and manner of somatic embryo induction (i.e. direct or indirect), and culture conditions (photoperiodic or darkness) employed (Grieb et al. 1997; Charrière et al. 1999; Jiménez and Bangerth 2001b; Yang et al. 2012; Ayil-Gutiérrez et al. 2013). In these studies, the dynamic changes in the content of indole-3-acetic acid (IAA) (Ayil-Gutiérrez et al. 2013), or IAA and ABA and cytokinins (Grieb et al. 1997; Charrière et al. 1999), and also in gibberellin concentrations (Jiménez and Bangerth 2001a) during the induction of SE were described for *Coffea canephora* Pierre ex Froehner, *Daucus carota*

L., *Helianthus annuus* L., and *Zea mays* L., respectively. Because the ratio of endogenous hormones controls a mode of morphogenetic response, the ratio of IAA or ABA to cytokinins (Žur et al. 2015), or the ratio of ABA to IAA (Centeno et al. 1997), or of various types of cytokinins (e.g. zeatin (Z)-type and isopentenyl (iP)-type; Grieb et al. 1997; Pintos et al. 2002) tend to be good indicators of the embryogenic competence of explants.

In plant material, endogenous hormone contents may be affected by many factors. For example, exogenously applied plant growth regulators (PGRs) can interact with phytohormones and change their concentrations (Ayil-Gutiérrez et al. 2013). Another factor causing hormonal balance disorders is explant excision (Thornburg and Li 1991; Yang et al. 2012). It was showed that wounding is the first event that provides signals for triggering the entire regenerative process (Iwase et al. 2011; Chen et al. 2016). Although excision is a commonly used procedure for the introduction of explants into *in vitro* culture, little attention has been paid to this treatment so far. Phytohormone concentrations and their signalling pathways can also be regulated by changes in the photoperiodic conditions (Symons and Reid 2003; Pacholczak et al. 2005). As a result of the light deficit, plants show a dramatic elongation of the stem (Suzuki and Kerbauy 2006). The plant response is often linked to reduced leaf development, enhanced apical dominance, and a re-allocation of resources from storage-favouring shoot elongation to reproductive growth (Suzuki and Kerbauy 2006; Mikuła et al. 2015b). Dark treatment of donor plantlets was reported to favour SE in *Helianthus annuus* L. (Fiore et al. 1997), *Dendranthema grandiflora* Tzvelev. (May and Trigiano 1991) and *Cyclamen persicum* Mill. (Takamura and Tanaka 1996). Moreover, Pacholczak et al. (2005) discovered a correlation between light intensity and total soluble sugars and endogenous ABA and IAA contents.

By culturing stipe explants of the tree fern *Cyathea delgadii* Sternb. on PGR-free half-strength Murashige and Skoog's (1/2MS) medium, in darkness, we established a unique model system for the study of SE (Mikuła et al. 2015b). In this system, somatic embryos regenerate directly from the single epidermal cells of stipe explants. However, the capacity of *C. delgadii* explants to undergo SE depends on the prevailing photoperiodic conditions during the development of plantlets (Mikuła et al. 2015a). Explants excised from sporophytes that developed under 16/8 photoperiod conditions are incapable of SE. In contrast, in etiolated explants, the first cell divisions typically starts at 10th day of culture, and during the next 4–6 days, many somatic embryos at linear developmental stage are observed (Mikuła et al. 2015b). The present study was undertaken to find out if the etiolation, which is a key factor in the capacity of stipe explants to form somatic embryos, has an effect

on the content and balance of hormones and sugars. The study was also designed to determine possible relationships between the concentration and balance of above-mentioned compounds in etiolated explants during their 14-day-long culture on PGR-free medium.

Materials and methods

Plant material and culture initiation

Embryogenic culture of *C. delgadii* was established following the procedure described earlier by Mikuła et al. (2015b). The somatic embryo-derived sporophytes were cultured on PGR-free medium containing half-strength Murashige and Skoog's (Murashige and Skoog 1962) macro- and micro-nutrients and full complement of vitamins (1/2MS) and 2% (w/v) sucrose, solidified with 0.7% plant agar (Duchefa Biochemie); pH 5.8. The cultures were maintained under the photoperiodic conditions (16/8 light–dark regime; $50 \mu\text{M m}^{-2} \text{s}^{-1}$; defined as non-etiolated) or in constant darkness (defined as etiolated) for 5 months.

In experiments described here, sporophytes that had developed 4 or 5 leaves were used as a source of explants. The stipe explants measuring 2.5 mm in length were excised from the first frond of non-etiolated and etiolated sporophytes (Mikuła et al. 2015a) and used for high-performance liquid chromatography (HPLC) analysis. Moreover, etiolated explants were cultured on 1/2MS induction medium supplemented with 1% (w/v) sucrose, in constant darkness for 14 days. The plant material was collected for HPLC analysis every 2 days.

Samples preparation for HPLC analysis

Excised explants were immediately frozen in liquid nitrogen. The plant samples (about 80 explants per sample; 35 mg of FW) were lyophilised and homogenised in an ice-cold mixture of methanol, water, and formic acid (15/4/1) according to Dobrev and Kamínek (2002). An internal isotopic standard mixture consisting of deuterated IAA and ABA, and kinetin labelled with nitrogen ^{15}N was added to each sample during homogenisation. The prepared extract was fractionated using solid phase extraction (SPE) columns Oasis MCX (Waters), and three fractions were collected: “acidic” eluted with methanol for IAA and ABA analyses according to HPLC method of Štefančíč et al. (2007), “basic” eluted with methanolic solution of ammonia for cytokinins analyses according to Žur et al. (2015), and “flow through” for carbohydrates analyses according to Hura et al. (2016).

Quantification of IAA and ABA

After IAA and ABA elution from SPE column by pure methanol, each sample was evaporated to dryness and reconstituted in 50 μl methanol (Žur et al. 2015). Finally, samples were analysed using a Supelco Ascentis RP-Amide column (7.5 cm \times 2.1 mm, 2.7 μm). Mobile phases consisted of 0.1% formic acid solution in water (solvent A) and acetonitrile:methanol (1:1) mixture (solvent B). Analyses were performed using gradient elution at a flow rate of 1.5 ml min^{-1} with gradient starting from 20% B at 0 min, rising to 80% B at 3 min, and back to 20% at 3.5 min, total analysis time was 4 min. The HPLC apparatus was Agilent Technologies 1290 Infinity equipped with Agilent Technologies 6460 Triple Quad LC/MS with Jet Stream. Capillary voltage was set to 4000 V, two the most abundant product ions were monitored using MRM (Multiple Reaction Monitoring) mode for each analysed compound. The most abundant product ion was used for quantification (quantifier), whereas the other ion was used to confirm the identity of the phytohormones (qualifier). The MRMs were set as follows in Supplementary Table 1. Agilent Technologies Mass Hunter 5 software was used for apparatus control, data collection, and processing.

Quantification of cytokinins

Cytokinins, such as t-Z, c-Z, t-ZR, c-ZR, Kin, KinR and iPA, were separated from the samples. Their sum was defined as the total content of cytokinins and indicated by the abbreviation CKs. For their structural similarity, cytokinins were categorised into Z-type (i.e. t-Z + c-Z + t-ZR + c-ZR).

Fractions of cytokinins were flushed out from the SPE column after collecting IAA and ABA. Impurities were first washed out with 0.35 M ammonia in water and then cytokinins were eluted with 0.35 M ammonia in 60% methanol (Žur et al. 2015). The collected fraction was evaporated to dryness, reconstituted in 50 μl methanol, and analysed using the same chromatographic system and HPLC column as described above. The solvent system consisted of water with 0.001% acetic acid (solvent A) and acetonitrile with 0.001% acetic acid (solvent B) at a flow rate of 1.5 ml min^{-1} , gradient profile was 2.5% B to 1 min, 10% B at 3 min, 25% B at 6 min, 75% B at 8 min and 2.5% B at 8.5 min. Capillary voltage was set to 4000 V, two the most abundant positive secondary ions were monitored as follows in Supplementary Table 2.

Quantification of soluble sugars

The “flow through” fraction collected from the SPE column was lyophilised and reconstituted according to the

procedure described by Janeczko et al. (2010). Samples were analysed on Agilent Technologies 1200 HPLC equipped with an amperometric detector ESA Coulochem II Analytical Cell 5040 with gold electrode. The Hamilton RCX-10 250×4.1 mm (Hamilton, Reno, NV, USA) HPLC column was used. The mobile phase comprised 100 mM sodium hydroxide solution in water, and the flow rate was set to 1.5 ml min⁻¹. The detector was set at an analytical potential of 200 mV, an oxidising potential of 800 m, and a reducing potential of -900 mV (with reference to a palladium electrode). Agilent Technologies ChemStation B04 software was used for apparatus control, data collection, and processing.

Statistical analysis

Data for endogenous hormone and sugar contents are presented as mean ± SD for three replicates (35 mg FW, i.e. about 80 explants, was considered as one replicate). Statistical analysis for etiolated and non-etiolated explants was performed using the Student's *t* test embedded in microsoft excel. Only a return of *p* < 0.05 was designated as being statistically significant. As the number of data for analysis conducted during the 14 days of culture was limited it was not possible to execute normality tests, thus a non-parametric Analysis of Variance (Kruskal Wallis test) was applied to calculate the significant differences between the

comparisons (corrected *p* ≤ 0.05). The parameters that contributed significantly to the final score were determined by Multiple comparisons of mean ranks. All statistical analyses were performed using STATISTICA version 6.0 (Stat Soft Inc., USA, 2001) package.

Results

The effect of etiolation on endogenous hormone and soluble sugar contents in initial explants

The concentration of phytohormones and soluble sugars in stipe explants derived from sporophytes developed under photoperiodic conditions (non-etiolated) and in those developed in darkness (etiolated) was significantly different (Fig. 1). Etiolated explants (capable of SE) were characterised by almost 12-fold lower concentration of ABA, 2-fold greater IAA content and 9.4-fold greater concentration of total cytokinins in comparison with non-etiolated explants (incapable of SE; Fig. 1a, b). Moreover, the content of some individual cytokinins such as KinR and *c*-ZR was more than 450 times greater in etiolated explants than in non-etiolated ones. The concentrations of hexoses and sucrose were more than 2- and 39-fold lower in etiolated explants than in the plant material kept under photoperiodic conditions, respectively (Fig. 1b).

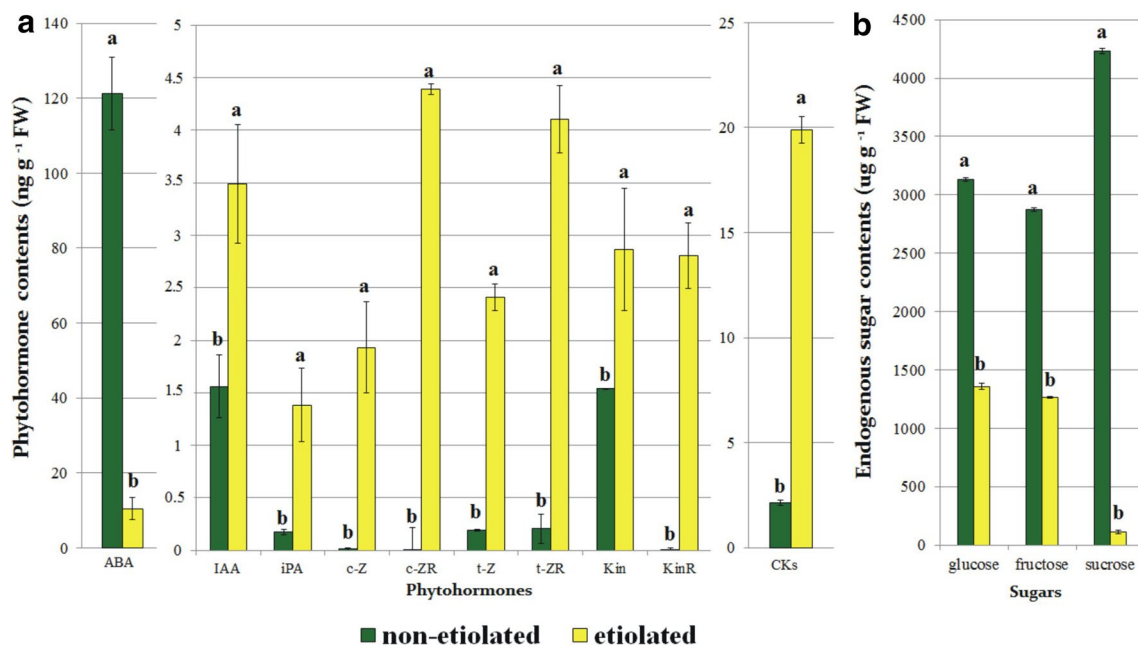


Fig. 1 Contents of **a** endogenous hormones and **b** soluble sugars in non-etiolated (incapable of somatic embryogenesis) and etiolated (capable of somatic embryogenesis) explants of *C. delgadoi*. Data are the means (±SD) of three replicates. The Student's *t* test was used independently for each type of phytohormones and sugars. Mean values marked with the same letter do not differ significantly at the 0.05

confidence level. ABA abscisic acid, CKs the sum of *c*-Z, *c*-ZR, iPA, Kin, KinR, *t*-Z and *t*-ZR concentrations, *c*-Z cis zeatin, *c*-ZR cis zeatin riboside, IAA indole-3-acetic acid, iPA isopentenyladenosine, Kin kinetin, KinR kinetin riboside, *t*-Z trans zeatin, *t*-ZR trans zeatin riboside

In order to assess the hormonal balance in both types of plant material, the interaction between endogenous hormones was expressed by their ratios (Table 1). In non-etiolated explants, the ratios of IAA and ABA to individual cytokinins (also to CKs), as well as ABA to IAA, were much greater than those in etiolated ones. This was especially evident in the case of KinR, c-ZR and c-Z. Etiolation of donor plantlets resulted in equalisation of all ratios mentioned above (excluding IAA/Kin). Only in case of the ratio of Z-type cytokinin to iPA, its value was lower in non-etiolated explants (2.4) than in etiolated explants (9.3) (Table 1).

Changes in endogenous hormone and soluble sugar contents during 14-day-long culture

To characterise the physiological changes that occur during early SE in etiolated explant of *C. delgadii*, the content of endogenous hormones (Fig. 2a–e) and sugars (Fig. 2f) was

Table 1 Ratios of different phytohormones in non-etiolated and etiolated initial explants of *C. delgadii*

Phytohormone ratios	Non-etiolated explants	Etiolated explants	Non-etiolated/etiolated explants
IAA/CKs	0.73 ± 0.1a	0.18 ± 0.1b	4.0
IAA/t-Z	8.1 ± 2.1a	1.5 ± 0.7b	5.4
IAA/c-Z	145.1 ± 13.9a	1.8 ± 1.1b	80.6
IAA/t-ZR	7.6 ± 2.8a	0.9 ± 0.5b	8.4
IAA/c-ZR	159.6 ± 6.9a	0.8 ± 0.5b	199.5
IAA/Kin	1.0 ± 0.1a	1.2 ± 0.2b	0.8
IAA/KinR	435.3 ± 12.4a	1.2 ± 0.5b	362.8
IAA/iPA	9.0 ± 1.9a	2.5 ± 0.5b	3.6
ABA/CKs	56.8 ± 3.2a	0.53 ± 0.2b	107.2
ABA/t-Z	626.3 ± 44.1a	4.3 ± 1.9b	145.7
ABA/c-Z	11,275.9 ± 63.1a	5.4 ± 1.6b	2088.1
ABA/t-ZR	591.8 ± 16.6a	2.6 ± 0.7b	227.6
ABA/c-ZR	12,400.5 ± 24.1a	2.4 ± 0.6b	5166.9
ABA/Kin	78.9 ± 9.7a	3.7 ± 2.0b	21.3
ABA/KinR	33,829.6 ± 80.6a	3.7 ± 1.8b	9143.1
ABA/iPA	695.8 ± 24.2a	7.6 ± 3.0b	91.6
ABA/IAA	77.7 ± 12.0a	2.7 ± 0.4b	28.8
Z-type/iP-type	2.4 ± 0.4a	9.3 ± 4.9b	0.3

Data are the means (±SD) of three replicates. The Student's *t* test was used independently for each type of ratio determined. Mean values marked with the same letter do not differ significantly at the 0.05 confidence level

Values marked with the same letter differ insignificantly at the 0.05 confidence level according to Student's *t* test

ABA abscisic acid, CKs the sum of c-Z, c-ZR, Kin, KinR, t-Z, t-ZR, and iPA concentrations, c-Z cis zeatin, c-ZR cis zeatin riboside, IAA indole-3-acetic acid, iPA isopentenyladenosine, Kin kinetin, KinR kinetin riboside, t-Z trans zeatin, t-ZR trans zeatin riboside, Z-type the sum of c-Z, c-ZR, t-Z, t-ZR concentrations

determined during 14 days of culture. The content of all phytohormones studied diminished rapidly after excision of explant (i.e. at day 2 of culture). Between days 4 and 14, their concentrations remained relatively stable, excluding IAA, whose concentration decreased at day 10 (Fig. 2b), and Kin, whose concentration increased at day 12 (Fig. 2e), and t-ZR, whose amount increased starting on day 8 of culture (Fig. 2d).

The hormonal balance during induction of SE is presented in Table 2. Results showed that the ratios of IAA to the individual cytokinins (as well as to CKs) increased reaching its maximum at day 6 of culture. The ratio of Z-type cytokinin to iPA was significantly lower between days 2 and 8 of culture. After that, its value increased gradually. The values of the ABA/CKs (as well as to individual cytokinins) and ABA/IAA ratios remained almost unchanged during 14-day-long culture of stipe explants (Table 2).

The explant excision did not influence significantly the content of endogenous sugars (Fig. 2f). Intensive sucrose accumulation (with a mean 12-fold increase) was observed at day 6 of culture. Over the next 2 days, the level diminished by 37%, and remained relatively stable up to day 14. An increase in the hexose concentrations was observed at day 4. Then, content of both the glucose and fructose gradually diminished.

Discussion

The morphogenetic response of plant material *in vitro* is greatly influenced by the ontogenetic age of the tissue or organ which is used as the source of the initial explant (Gaj 2004). Along with changes in the degree of tissue differentiation, the physiological state of cells is modified. The basis of these changes is an altered pattern of endogenous hormone synthesis, conjugation and/or inactivation, which can be modulated by the use of many stress-related factors (Fehér 2015) as well as by etiolation and explant excision.

Etiolation resulting in altered concentration and balance of endogenous hormones

Light affects the biosynthesis and signalling of endogenous hormones (Symons and Reid 2003). In cryptogamic plants, light is a crucial factor in controlling the regeneration pathways, including SE (Mikuła et al. 2015a). Therefore, we carried out quantitative analysis of ABA, IAA and cytokinin contents in etiolated (capable of SE) and non-etiolated (incapable of SE) stipe explants of *C. delgadii*, and found extreme differences in both the hormone content and hormonal balance. Our studies showed significantly lower concentration of ABA in etiolated plant material.

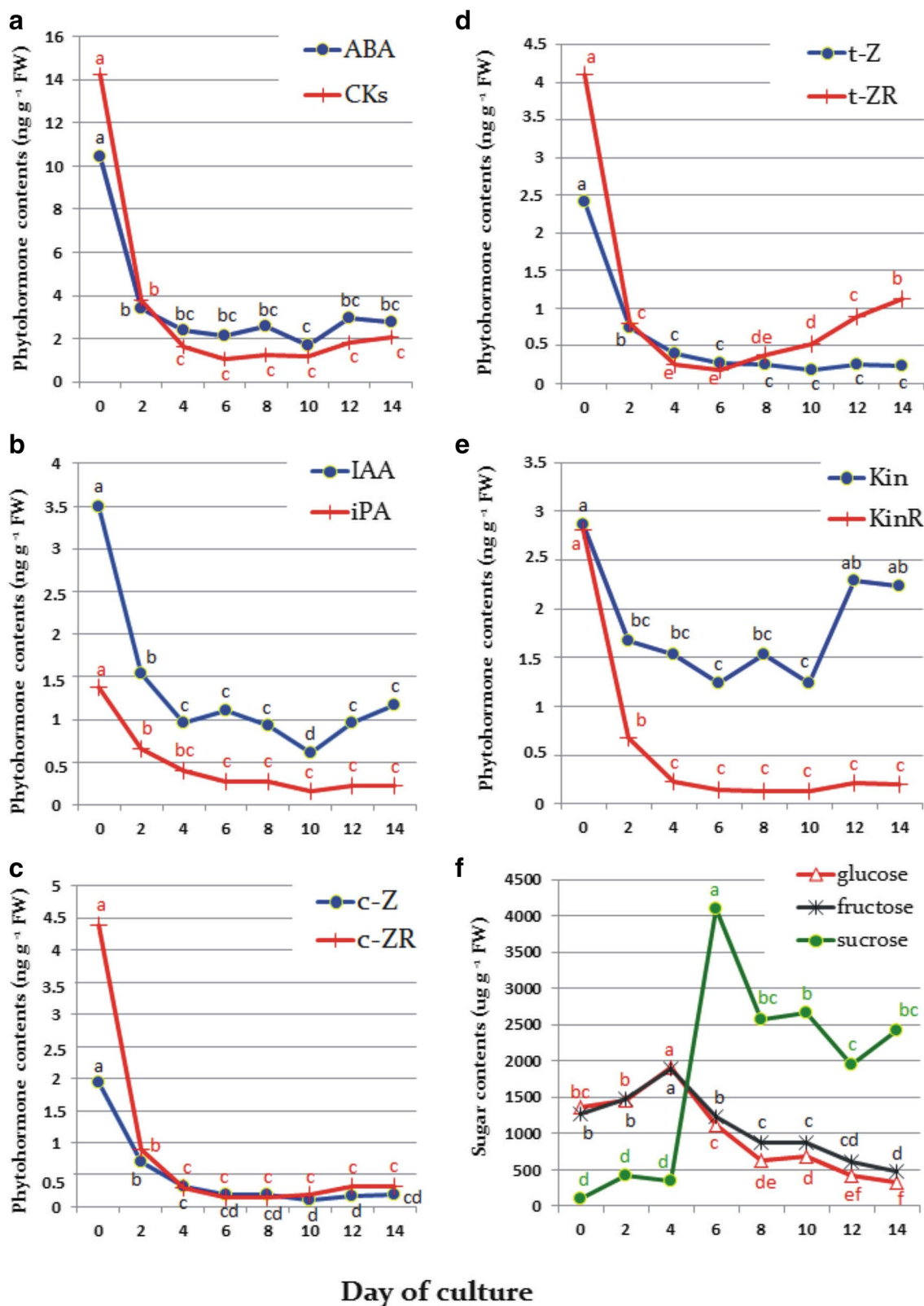


Fig. 2 Concentrations of phytohormones and soluble sugars during early somatic embryogenesis (14 days of culture in darkness). Data are the means of three replicates. Mean values marked with the *same letter* do not differ significantly at the 0.05 confidence level according to Kruskal–Wallis ANOVA and Median test. *ABA* abscisic acid, *CKs*

the sum of *c-Z*, *c-ZR*, *iPA*, *Kin*, *KinR*, *t-Z* and *t-ZR* concentrations, *c-Z* cis zeatin, *c-ZR* cis zeatin riboside, *IAA* indole-3-acetic acid, *iPA* isopentenyladenosine, *Kin* kinetin, *KinR* kinetin riboside, *t-Z* trans zeatin, *t-ZR* trans zeatin riboside

Table 2 Ratios of different phytohormones in etiolated explants during 14-day-long culture of *C. delgadii*

Phytohormone ratios	Day of culture								
	0	2	4	6	8	10	12	14	
IAA/CKs	0.5 ± 0.06c	0.7 ± 0.01b	0.8 ± 0.02b	1.4 ± 0.16a	1.0 ± 0.16b	0.8 ± 0.12b	0.6 ± 0.04c	0.8 ± 0.01c	
IAA/t-Z	1.4 ± 0.50c	2.0 ± 0.09c	2.4 ± 0.22bc	4.2 ± 1.73ab	3.9 ± 0.43b	3.5 ± 1.31b	3.6 ± 0.44b	4.9 ± 0.74a	
IAA/c-Z	1.8 ± 0.79d	2.1 ± 0.13d	3.1 ± 0.37c	6.17 ± 2.5a	5.5 ± 2.0b	5.7 ± 1.5ab	5.6 ± 1.5ab	6.1 ± 0.5a	
IAA/t-ZR	0.8 ± 0.37c	2.0 ± 0.17bc	3.8 ± 0.75b	5.9 ± 1.74a	2.4 ± 0.28b	1.2 ± 0.21c	1.1 ± 0.48c	1.0 ± 0.1c	
IAA/c-ZR	0.8 ± 0.37e	1.7 ± 0.1d	3.1 ± 0.38c	7.3 ± 2.22a	6.7 ± 1.13b	3.6 ± 1.81c	3.0 ± 0.4c	3.8 ± 0.49c	
IAA/iPA	2.4 ± 0.39c	2.3 ± 0.17c	2.4 ± 0.05c	4.2 ± 1.66ab	3.5 ± 0.54b	3.9 ± 1.11b	4.2 ± 0.51ab	5.4 ± 0.69a	
IAA/Kin	1.2 ± 0.17a	0.9 ± 0.14ab	0.6 ± 0.02b	0.9 ± 0.30ab	0.6 ± 0.15ab	0.6 ± 0.39ab	0.4 ± 0.03ab	0.5 ± 0.02ab	
IAA/KinR	1.2 ± 0.38d	2.3 ± 0.19c	4.1 ± 0.60b	8.3 ± 3.91a	7.8 ± 2.40ab	5.5 ± 3.35b	4.5 ± 0.09b	6.1 ± 1.17ab	
ABA/CKs	0.5 ± 0.17b	0.6 ± 0.23ab	0.7 ± 0.22ab	0.9 ± 0.32a	0.9 ± 0.39a	0.7 ± 0.13ab	0.7 ± 0.38ab	0.6 ± 0.17ab	
ABA/t-Z	4.4 ± 1.32c	4.7 ± 0.96c	6.0 ± 0.86bc	7.7 ± 0.97b	10.7 ± 2.3a	9.4 ± 1.67ab	11.0 ± 0.71a	11.3 ± 0.15a	
ABA/c-Z	5.4 ± 1.12e	5.0 ± 0.93e	7.7 ± 1.3d	11.2 ± 1.39c	15.9 ± 7.3bc	15.4 ± 1.14b	17.1 ± 3.66a	14.1 ± 0.77b	
ABA/t-ZR	2.6 ± 0.52c	4.7 ± 1.6bc	9.5 ± 2.35a	11.1 ± 2.59a	6.7 ± 0.14b	3.2 ± 0.04c	3.4 ± 1.32c	2.5 ± 0.58c	
ABA/c-ZR	2.4 ± 0.42e	4.1 ± 1.27d	7.8 ± 1.36c	13.8 ± 3.13b	19.01 ± 5.1a	9.53 ± 2.961c	9.4 ± 0.68c	8.8 ± 0.08c	
ABA/iPA	8.4 ± 4.3c	5.4 ± 0.93d	5.9 ± 0.43d	7.7 ± 1.04c	9.8 ± 2.49b	10.6 ± 0.9ab	12.9 ± 0.81a	12.5 ± 0.13a	
ABA/Kin	4.1 ± 2.17a	2.1 ± 0.18b	1.5 ± 0.12b	1.7 ± 0.34b	1.8 ± 0.60b	1.6 ± 0.66b	1.3 ± 0.03b	1.2 ± 0.11b	
ABA/KinR	3.9 ± 1.31e	5.5 ± 1.84d	10.2 ± 2.01c	14.7 ± 0.84b	22.3 ± 9.07a	14.2 ± 6.03b	14.2 ± 0.58b	14.0 ± 0.77b	
ABA/IAA	2.7 ± 0.4a	2.2 ± 0.8ab	2.5 ± 0.2a	1.9 ± 0.5ab	2.8 ± 0.4a	2.8 ± 0.8a	3.0 ± 0.3a	2.3 ± 0.5ab	
Z-type/iPA	9.9 ± 3.1a	4.8 ± 0.4c	3.2 ± 0.3c	2.9 ± 0.1c	3.6 ± 0.3c	6.3 ± 0.0b	7.5 ± 1.6a	8.6 ± 1.2a	

Data are the means (\pm SD) of three replicates. Mean values marked with the same letter do not differ significantly at the 0.05 confidence level according to Kruskal–Wallis ANOVA and Median test

Values marked with the same letter differ insignificantly at the 0.05 confidence level according to Kruskal–Wallis ANOVA and Median test

ABA abscisic acid, CKs the sum of c-Z, c-ZR, Kin, KinR, t-Z, t-ZR, and iPA concentrations, c-Z cis zeatin, c-ZR cis zeatin riboside, IAA indole-3-acetic acid, iPA isopentenyladenosine, Kin kinetin, KinR kinetin riboside, t-Z trans zeatin, t-ZR trans zeatin riboside, Z-type the sum of c-Z, c-ZR, t-Z, t-ZR concentrations

The results are in agreement with previously published data (Volmaro et al. 1998; Pacholczak et al. 2005). ABA is biosynthesised especially in chloroplasts and appears to be a cleavage product in the biosynthetic pathway of carotenoids (Seo and Koshiba 2002). In view of this, the reduction in ABA concentration in response to darkness seems to be related to changes in the structure and/or function of chloroplasts. Researchers also agree that the exposure of plants to darkness increases the concentration of endogenous IAA, even twice (Liu et al. 1996; Volmaro et al. 1998; Suzuki and Kerbauy 2006), and it is in agreement with the results obtained for *C. delgadii*. The negative effect of the light on IAA concentration is presumably because light induces IAA-peroxidase and IAA-oxidase activity, the key enzymes involved in the breaking down of IAA (Liu et al. 1996). Dark treatment also greatly stimulates cytokinin accumulation. In shoots of dark-grown *Catsetum fimbriatum*, the total cytokinin concentration was almost threefold greater than in those growing under photoperiodic conditions (Suzuki and Kerbauy 2006). In stipe explants of *C. delgadii*, this difference reached more than 9 in favour of the dark. Similarly, the concentrations of Z, ZR, iP, and iPA were significantly higher after the exposure to darkness than to continuous light (Macháčková et al. 1996). Our data

confirmed this to be true also for other cytokinins, i.e. Kin and its riboside. Although the occurrence of Kin has been considered to be an artefact, its presence had already been found in plant material such as coconut chunks (Barciszewski et al. 1996) and water extracts (Ge et al. 2005), root nodules of Australian pine (Raman and Elumalai 1996) as well as triticale anthers (Žur et al. 2015). The importance of Kin in controlling different physiological processes in plants may be associated with its antioxidant and ABA-antagonistic properties (Barciszewski et al. 1996; Žur et al. 2015).

The key question of our research is how the content/ratio of phytohormones, established as a result of etiolation of the donor plantlets, promotes SE induction? The hormonal state of somatic cells determines the morphogenetic response of explants by genes classified as hormone-related transcription factors (Gliwicka et al. 2013). Some authors postulate that elevated level of ABA reduces/inhibits the explant ability to SE (Ivanova et al. 1994; Jiménez and Bangerth 2001a). According to this, our results showed the many times higher ABA/CKs and ABA/IAA ratios in non-embryogenic explants than in embryogenic explants. On the level of molecular studies, the negative relationship between the ABA content and the *LEC2* (*LEAFY*

COTYLEDON2) activity during SE induction was recently revealed by Wójcikowska and Gaj (2015). On the other hand, *LEC2* activation induces *YUC2* and *YUC4* (*YUCCA* 2 and 4) genes involved in auxin biosynthesis (Stone et al. 2008). The connection between auxin and genes that are specific for early SE, such as *AGL15* (*AGAMOUS-Like15*) and *FUS3* (*FUSCA3*) has also been documented (Stone et al. 2008; Zheng et al. 2013). It is commonly known that an appropriate level of auxin is required for SE induction (Ayil-Gutiérrez et al. 2013; Gliwicka et al. 2013). Thus, the competence of *C. delgadii* explants to undergo SE may relate to low ABA level together with high content of IAA and cytokinins achieved by etiolation.

Explant excision resulting in altered endogenous hormone concentration

Our earlier research revealed that the tissue damage caused by aging and dying of the upper part of etiolated fronds can spontaneously induce direct or indirect SE in *C. delgadii* on PGR-free medium (Mikuła et al. 2015b). In plant tissue culture, wounding is one of the most important factors affecting the developmental programme of somatic cells (Iwase et al. 2011). Sometimes, this stress treatment may be enough to initiate SE (Smith and Krikorian 1989). Following a wound, the level of IAA in tobacco leaves declined 2- to threefold (Thornburg and Li 1991). Moreover, enhanced endogenous ABA, jasmonic acid, and ethylene production were detected (León et al. 2001). Wounding also enhanced the endogenous cytokinin activity (Crane and Ross 1986; Iwase et al. 2011). However, the hormonal response to excision seems to be completely different than to wounding. An excision releases explants from the influence of apical meristems that are the sources of hormone synthesis. Therefore, in excised stipes of *C. delgadii* about threefold reduction in the concentration of IAA, ABA and CKs was observed. The similar decline in IAA content was also noticed in excised cotton hypocotyl explants (Yang et al. 2012). Furthermore, it is worth mentioning that although a dramatic decline in the hormone concentration occurred, the balance between IAA/CKs, ABA/CKs and ABA/IAA ratios remained almost unchanged, and significantly greater concentration of CKs than IAA was still maintained in *C. delgadii* explants.

Summary of physiological events during early SE of *C. delgadii*

Analysis of the induction phase of SE from the viewpoint of physiological changes reveals three sub-phases (I–III) that can be observed in *C. delgadii* explants, and these are triggered by etiolation, explant excision, and a massive increase in endogenous sucrose concentration (Fig. 3).

Our results provide evidence that by etiolation of donor plantlets it is possible to achieve the hormonal balance which requires only a slight further modification to switch the developmental path of somatic cells. The etiolation reduces a high concentration of ABA, the main factor inhibiting somatic embryo production (Charrière et al. 1999; Jiménez and Bangerth 2001a), and increases the content of IAA and cytokinins. As a result of explant excision a dramatic decline in the concentration of studied hormones occurs, but the hormonal balance and sugar content remain almost unchanged. Among those phytohormones, the rapid decline in IAA concentration seems to be directly related to the sugar influx into the explant cells. It was shown that the low level of IAA leads to an increase in apoplastic pH and the activation of invertases (Mohr and Schopfer 1995). Seeing that, almost 12-fold increase in sucrose concentration due to the influx of glucose and fructose into the explant cells of *C. delgadii*, may represent an osmotic signal that is required for the expression of embryogenic competence (Kikuchi et al. 2006). We suggest that the short-term osmotic signal is a final switch to the massive genetic reprogramming towards embryogenesis. The studies also showed that the predominant class of hormones found in the stipe explants during early SE are cytokinins. In all likelihood, they favour this type of morphogenetic response, however, initiation of third sub-phase of induction phase seems to be supervised by auxin, as apparent from the highest IAA/CKs ratio on day 6 of culture.

In *C. delgadii*, the hormonal balance established at 6th day of culture (Fig. 3) promotes transition from the induction to the expression phase four days later. During next four days of culture many somatic embryos at the linear stage of development were present. This phase requires a considerable amount of metabolic energy and carbon skeletons (Gomes et al. 2014; Cangahuala-Inocente et al. 2009). Our results confirmed it by observation of intensive utilisation of soluble sugars. Third part of acquisition phase and an early stage of SE expression were also correlated with a greater concentration of cytokinins Z-type compared with iPA. This result resembles previous data indicating that the ratio of these two types of cytokinins is a good index of both the explant and callus embryogenic capacity (Centeno et al. 1997; Pintos et al. 2002). Among Z-type cytokinins, t-ZR appears to be particularly associated with frequent cell divisions leading to the production of linear stage somatic embryos in *C. delgadii*.

Conclusion

1. The capacity of stipe explants to form somatic embryos in *C. delgadii* is determined by the endogenous level and balance of hormones that are modulated by the

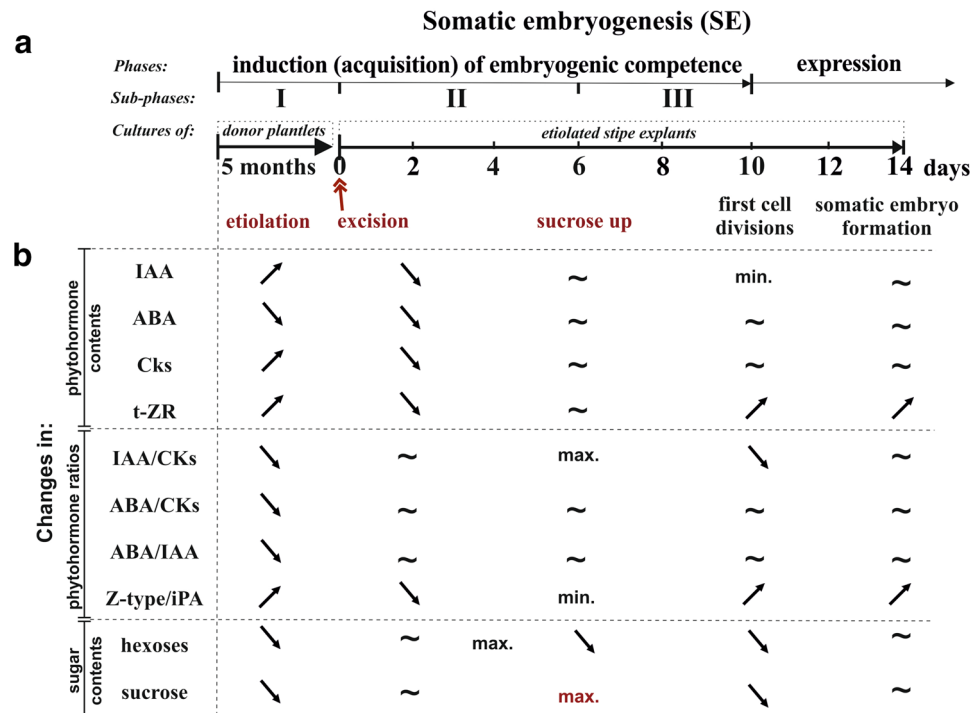


Fig. 3 Schematic summary of physiological events happened during early somatic embryogenesis in *C. delgadii*. **a** Time-line of embryogenic pathway development illustrating the two different phases, i.e. the induction (acquisition) of embryogenic competence and the expression of SE. The acquisition phase is divided into three sub-phases (I–III). There are also three different stimuli: etiolation, excision (exogenously applied), and a massive increase in endogenous sucrose concentration (sucrose up). They switch these sub-phases on

and off. **b** Changes in endogenous hormone and sugar contents, and in hormone ratios, showing the influence of the stimuli. *ABA* abscisic acid, *CKs* the sum of *c-Z*, *c-ZR*, *iPA*, *Kin*, *KinR*, *t-Z* and *t-ZR* concentrations, *c-Z* cis zeatin, *c-ZR* cis zeatin riboside, *IAA* indole-3-acetic acid, *iPA* isopentenyladenosine, *Kin* kinetin, *KinR* kinetin riboside, *t-Z* trans zeatin, *t-ZR* trans zeatin riboside, *Z-type* the sum of *t-Z*, *c-Z*, *t-ZR*, *c-ZR* concentrations, *min./max.* minimum/maximum content, $\uparrow/\downarrow/\sim$ increasing/decreasing/constant trend

conditions of light under which the donor plantlets have developed.

2. Drastic reduction in hormone contents caused by excision is sufficient to trigger embryogenic competence in epidermal cells of *C. delgadii* stipe explants.
3. Rapid increase in sucrose content can be considered as a switch to the SE expression phase.
4. *Trans*-zeatin riboside is clearly associated with SE expression phase of *C. delgadii* in which the frequent cell divisions happen and the somatic embryos at linear stage appear.

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Author Contributions MG performed the experiments and prepared the samples for HPLC analysis. AK performed HPLC analyses of carbohydrates, PW carried out HPLC separation of phytohormones. AK and PW performed all chromatographic data analysis.

MG and AM designed the experiments and wrote the manuscript. AM conceived the study. All authors read and approved the final manuscript.

Compliance with ethical standards

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

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