Original Paper Open Access

Comparison of dynamic changes of endogenous hormones between calli derived from mature and immature embryos of maize

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Abstract

Mature and immature embryos of maize inbred lines 87-1 and 137 were used as explants to induce callus on improved N6 medium. The contents of endogenous hormones abscisic acid (ABA), indoleacetic acid (IAA), gibberellic acid (GA3) and cytokinins (ZR) of immature, mature embryos and their corresponding calli were detected by method of enzyme-linked immunosorbant assay (ELISA). At the beginning of culture, IAA and GA3 levels decreased rapidly and reached their lowest levels at day 7, indicating that large amounts of IAA and GA3 are needed for germination. Levels of IAA and GA3 were highest at the beginning of embryonic callus formation from immature embryos, suggesting high levels of IAA and GA3 were beneficial to induction of embryonic callus from immature embryos (CIME). The IAA, GA3 and ABA contents and ration of IAA to ABA (IAA/ABA), GA3 to ABA (GA3/ABA) in callus of mature embryos (CME) were higher than those of CIME after the 14th day from culture initiation and the changes of ratios IAA/ABA and GA3/ABA increased rapidly in CME while they remained low in CIME during the whole experimental period. This inferred that high levels of IAA, GA3 or ABA and large increases in IAA/ABA and GA3/ABA might hinder the induction and maintenance of embryonic calli from mature embryos.

Keywords: callus induction, endogenous hormone, immature embryos, maize, mature embryos

Abbreviations: ABA: abscisic acid, CIME: callus of immature embryos, CME: callus of mature embryos, GA3: gibberellin acid, IAA: indoleacetic acid, ZR: cytokinin, 2,4-D: 2,4-Dichlorophenoxyacetic acid

Introduction

Maize (*Zea Mays* L.) is one of the most important crops around the world because of its importance in direct and indirect production of food for human consumption. Because of limited land resources, expanding population, plant diseases and insect pests stresses, traditional breeding methods have not consistently met the demand for maize, considering both quality and quantity. Consequently, improvement through biotechnological approaches has received more emphasis, and genetically transformed maize varieties have been obtained by various approaches, such as particle bombardment (Klein et al, 1989) and Agrobacterium-mediated (Schlapp and Hohn, 1992; Valdez-Ortiz et al, 2007). However, success or failure of maize genetic transformation largely depends on the ability of transformed tissues to proliferate and subsequently to regenerate into whole plants. Considerable work has been done on tissue culture and plant regeneration in maize, primarily using immature tissues (Green and Philips, 1975; Ting et al, 1981; Conger et al, 1987; Pareddy and Petolino, 1990; Ray and Gosh, 1990; Zhong et al,1992; O'Connor-Sánchez et al, 2002; Zhang et al, 2002; Valdez-Ortiz et al, 2007).

Immature embryo-derived callus is more efficient for plant regeneration than callus from other explants. Immature embryo production is time-dependent and difficult to obtain all seasons of the year. Conversely, mature embryos from dry seeds are available any time throughout the year. As explants, mature embryos have been used successfully to induce callus and regenerate plants (Green et al, 1974; Wang, 1987; Huang and Wei, 2004). However, only certain genotypes could be regenerated from mature embryos and with a generally low frequency of regeneration. Consequently, it is necessary to improve the culture conditions by adjusting elements of culture medium to develop better procedures for maize plant regeneration from mature embryos. The type, amounts and relative proportions of endogenous hormones are key factors to callus induction and differentiation for rice, and the concentration of 2,4-D influences both viability of callus and levels of endogenous hormones (Liu et al, 2007). Only a few attempts have been performed to estimate hormone contents in

 RECEIVED 24/01/2011

maize embryos. Fu et al (2006) estimated levels of abscisic acid (ABA), indoleacetic acid (IAA), gibberellins (GA3, GA4) and cytokinins (DHZR, IPA, ZR) of immature embryos and embryonic and non-embryonic calli during culture using enzyme-linked immunosorbent assay (ELISA). However, little is known about hormone levels in isolated maize mature embryos, and little work has been done comparing endogenous hormones of immature and mature embryos and their corresponding calli in maize.

The objectives of this study were: (i) to estimate contents and to analyze the differences in dynamic range of endogenous hormones abscisic acid (ABA), indoleacetic acid (IAA), gibberellic acid (GA3) and cytokinin (ZR) of immature, mature embryos and their corresponding calli during culture process. and (ii) to provide a theoretical instruct for improvement of embryonic callus induction from mature embryos by adding particular exogenous plant growth regulator and thus coordinating levels of endogenous hormones according to endogenous hormones levels of immature embryos which have a high frequency of embryonic induction.

Materials and Methods

Plant material and culture initiation

Female ears of maize inbred lines 87-1 and 137 were picked on day 14 after artificial pollination. Ears were sterilized with 70% ethanol (v/v) after bract removal. Immature embryos were isolated by excision and cultured immediately on callus induction medium. Callus induction medium was prepared by using N6 basal salts (Chu et al,1975) and B5 vitamins (Gamborg et al, 1968), supplemented with 2.0 mg/l glycine, 690 mg/l proline, 1 g/l casein hydrolysate, 3% (w/v) maltose, and (0-13.6) μM 2,4-D. All medium components were mixed together, adjusted to pH 5.8, and solidified with 0.7% (w/v) agar prior to autoclaving at 121°C for 20 min. Sixteen isolated immature embryos were placed embryonic axis-side down in full contact with 25-30 ml of primary callus induction medium in a Petri dish (92 x 16 mm) and cultured in the dark at 25±1°C for callus induction. Treatments consisted of the basal medium plus 0, 2.5, 4.5, 6.8, 9.0, 11.3, and 13.6 μM 2,4-D, respectively. Each treatment was replicated three times. Calli were evaluated 4 weeks after culture initiation and the results were the means ± SE of three replicates.

Mature embryos were isolated from dry seeds after surface sterilization as previously described (Huang and Wei, 2004) and soaking in sterile water at 4°C for 2 days. To estimate levels of endogenous hormones, approximately one hundred of isolated mature embryos, with a similar number of immature embryos were cultured on callus induction medium in the dark at 25±1°C with the embryo axis in contact with the media. Callus induction medium was as above described except that the concentration of 2,4-D was 11.3 μM.

Hormone extraction, purification and quantification

Cultured calli that were incubated in the dark at 25±1°C were sampled at the 0th, 7th, 14th, 21th and 28th day after culture initiation and immediately frozen in liquid nitrogen after determination of fresh weight. They were then stored at -20°C until analysis. The extraction, purification and determination of endogenous hormone levels of IAA, GA3, ZR and ABA by an indirect ELISA technique were performed as previously described (He et al, 2005; Yang et al, 2006; Teng et al, 2006) with some modifications. Briefly, about 0.5 g fresh weight of callus selected at random was extracted and homogenized in 4 ml 80% methanol (containing 1μM butylated hydroxytoluene) and stored at 4°C for 4 h. After centrifugation at 20,000 g for 15 min, sediments were resuspended in 1 ml 80% methanol at 4°C for 1 h. The combined extracts were passed through C18-Sep-Pak cartridges (Waters, Milford, USA) for purification. Afterwards, samples were dried under a stream of N2. The residues were then dissolved in 0.01 M phosphate buffer solution (pH 7.5) and levels of IAA, GA3, ZR and ABA were determined. ELISA was performed on a 96-well microtitration plate. Each well on the plate was coated with synthetic IAA, GA3, ZR or ABA ovalbumin conjugates in $NaHCO_3$ buffer (50 mM pH 9.6) and stored overnight at 37°C. Ovalbumin solution (10 mg/ml) was added to each well for the purpose of blocking nonspecific binding. After incubation for 30 min at 37°C, IAA, GA3, ZR and ABA standards (0-2,000 ng/mldilution range), samples and antibodies were added and incubated for a further 45 min at 37°C. The antibodies against IAA, GA3, ZR and ABA were produced at the Phytohormones Research Institute (China Agricultural University; see He, 1993). Then horseradish peroxidase-labelled goat antirabbit immunoglobulin was added to each well and incubated for 1 h at 37°C. Finally, orthophenylenediamino (OPD) substrate solution was added to each well of the plates, and the enzyme reaction was carried out in the dark at 37°C. The reaction progress was stopped by adding of 50 μl 3M H_2SO_4 per well when the 2,000 ng/ml standard had a pale colour, and the 0 ng/ml standard had a deep colour in the wells. Calculations of the enzyme-immunoassay data were performed as described by Weiler et al (1981). The results were the means ±SE of three replicates.

In this study the percentage recovery of each hormone was calculated by adding known amounts of standard hormone to a split extract. Percentage recoveries were all above 90%, and all sample extract dilution curves paralleled the standard curves, showing the absence of nonspecific inhibitors in the extracts.

Statistical analysis

The significance of differences among means of treatments was tested by ANOVA (Univariate) and Duncan's multiple range test ($P < 0.05$). All these sta-

tistical analyses were conducted on SPSS 11.5 software (USA).

Results

Effect of 2,4-D on callus initiation from immature embryos

The highest frequency of both primary and embryogenic callus formation was observed from immature embryos cultured on medium F (Table 1). Therefore, callus induction medium used in the estimation of endogenous hormones consisted of N6 basal salts (Chu et al, 1975), B5 vitamins (Gamborg et al, 1968), 2.0 mg/l glycine, 690 mg/l proline, 1 g/l casein hydrolysate, 3% (w/v) maltose, and 11.3 μM 2,4-D.

Differences between calli from immature and mature embryos in growth traits

After 3 days, the cultured immature and mature embryos swelled, enlarged in size with the elongation of the radicle and plumule and, after 5-7 days, loose, soft and yellowish primary callus formation along with germination was observed. Shoots and roots were cut out completely, and the callus-responding explants were plated again in the same Petri dish for callus proliferation. During the 14th - 28th day from culture initiation, callus from immature embryos (CIME) enlarged in volume and was compact, friable, regularly shaped, light-yellow or creamy in color, all of which indicated embryogenic calli formation. During the 14th - 28th day from culture initiation, callus of mature embryos (CME) also enlarged in volume but remained soft, watery, bruised, brown in color, which indicated non-embryogenic calli.

Hormone levels in embryos and their corresponding calli of maize inbred line 87-1

In cultured calli from both immature and mature embryos of inbred line 87-1, trends of IAA levels over days of development were similar. In these calli, IAA levels decreased rapidly and reached their nadir at the 7th day; then they increased sharply to day 14 (Figure 1A). After day 14, IAA levels in CME increased gradually while those of CIME did not show a similar gradual increase (Figure 1A). During the entire callus induction process, CME contained more IAA than CIME except for days 0-7 from culture initiation.

Trends of GA3 levels over days of development were similar to those of IAA in both immature and mature embryos during days 0-14 from culture initiation (Figure 1B). During days 14-28, GA3 levels in CME gradually decreased while those of immature embryos decreased rapidly from the 14th day to the 21th day and then increased gradually from the 21th day to the 28th day. GA3 levels were higher in mature embryos and their corresponding calli than those of immature embryos during the entire experimental period except for day 7 (Figure 1B).

Levels of ABA were greater in mature embryos compared to immature embryos (Figure 1C). Over the days of development, the ABA levels in CME decreased rapidly and reached their nadir on day 7. Subsequently, they exhibited a relatively constant trend that resembled those found in immature embryos and their corresponding calli. Levels of ABA in calli from both immature and mature embryos exhibited a similar trend after day 7, but ABA levels in CME were higher than those of immature embryos during the entire experimental period except for day 28 (Figure 1C).

Levels of ZR in CME were relatively constant during days 0-21, then decreased rapidly. Levels of ZR in CIME fluctuated without any apparent trend (Figure 1D).

Although levels of IAA, ABA, GA3 and ZR in maize inbred line 137 were different from those in inbred

Table 1 - Frequency of primary and embryogenic callus induction from immature embryos of different maize inbred lines 87-1 and 137[a].

Medium	Concentration of 2,4-D (μM)	Immature embryos forming			
		87-1		137	
		Primary callus (%)[b,d]	Embryogenic callus (%)[c,d]	Primary callus (%)[c,d]	Embryogenic callus (%)[c,d]
A	0	0[f]	0[f]	0[f]	0[g]
B	2.5	47.53±0.56[e]	21.32±0.83[e]	41.63±0.30[e]	20.15±0.62[f]
C	4.5	80.60±1.55[d]	45.73±0.73[d]	75.21±1.32[d]	43.36±1.03[e]
D	6.8	88.39±0.49[bc]	47.28±0.86[d]	82.87±1.62[c]	50.69±0.99[d]
E	9.0	90.76±0.64[b]	66.83±0.73[b]	86.40±1.13[b]	65.77±0.42[b]
F	11.3	96.86±0.87[a]	82.28±1.86[a]	90.08±0.65[a]	79.43±0.72[a]
G	13.6	86.72±0.68[c]	59.45±0.70[c]	75.18±1.34[d]	57.83±0.32[c]

[a] Each treatment was replicated three times and the results were the means ± SE.
[b] Frequency of primary callus induction was evaluated by counting the immature embryos that formed callus out of the total number of immature embryos cultured ×100.
[c] Frequency of embryogenic callus formation was assessed by the percentage of immature embryos forming embryogenic callus and the total immature embryos explanted.
[d] Values followed by the same letters are not significantly different at P=0.05 according to the ANOVA test.

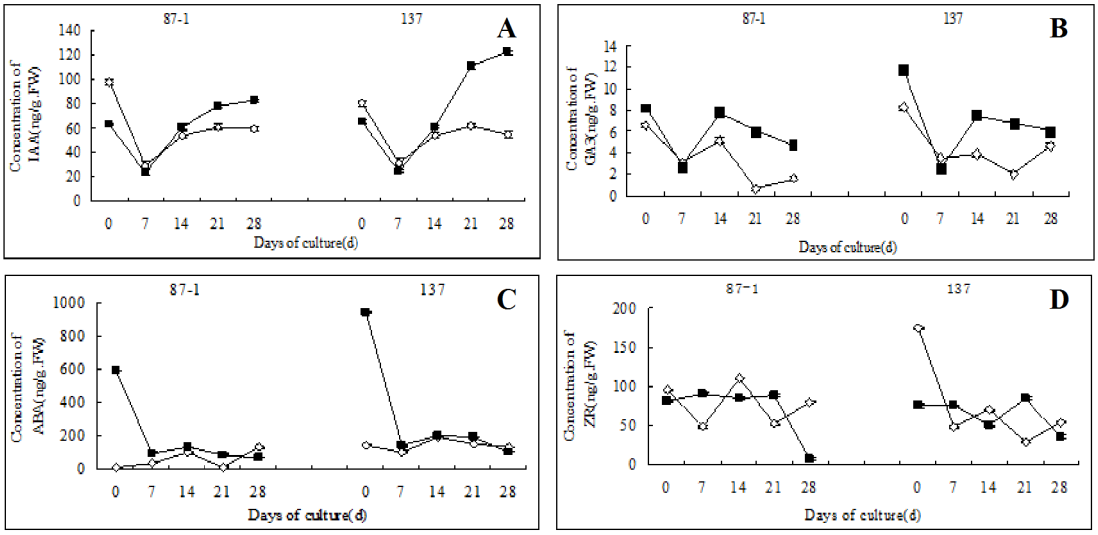

Figure 1 - A,B,C,D respectively denoted the contents of endogenous hormone IAA, GA3, ABA, ZR in calli of immature embryos (open squares) and mature embryos (closed squares) at different stages of callus induction culture. Error bars indicate ±S E of the mean, n=3

line 87-1, similar trends in changes over time of four endogenous hormones were observed in both inbred lines (Figure 1A-D).

Changes of ratios IAA to ZR (IAA/ZR), IAA to GA3 (IAA/GA3), IAA to ABA (IAA/ABA), GA3 to ABA (GA3/ABA) during callus induction period from embryos of maize inbred line 87-1

Physiological events during plant growth and development are regulated by not only levels of a particular hormone but also by relative levels among the different hormones. Study of ratios between different hormones may provide us some useful information about their roles in callus induction. The dynamic changes of the ratio IAA/ZR levels in CME resembled those of immature embryos during days 0-21 from culture initiation (Figure 2E) and both CME and CIME exhibited a relatively constant trend in this ratio. During days 21-28 the ratio in CME increased rapidly and reached their peak value at the 28th day, which was higher than those in CIME (Figure 2E).

The ratio of IAA/GA3 levels is shown in Figure 2F. Changes of IAA/GA3 in CME over time were different from those of immature embryos. In CIME IAA/GA3 decreased gradually and reached their nadir at day 7 and remained at that level day 14; subsequently, the ratio increased to highest levels at day 21 and then decreased sharply afterward. In contrast, the ratio of IAA/GA3 in CME showed little evidence of a trend during the whole experimental period (Figure 2F).

Trends in the ratio IAA/ABA in calli of both mature and immature embryos resembled those of GA3/ABA. Levels of ratios IAA/ABA and GA3/ABA in CME gradually increased during days 0-14 and slightly decreased afterward, then they began to increased rapidly and reached highest ratios on day 28. Levels of ratios IAA/ ABA and GA3/ABA in CIME were low and relatively constant during the whole experimental period (Figure 2G, H).

Although levels of ratios IAA/ZR, IAA/GA3, IAA/ABA and GA3/ABA in inbred line 137 were different from those in inbred line 87-1, similar trends in time of the four ratios were observed in both inbred lines (Figure 2E-H).

Discussion

Endogenous hormones are involved in control and regulation of gene expression, thus influencing metabolic pathways and, ultimately, capacity for formation, maintenance and expression of embryogenic callus (Xi, 1978). Results from this study demonstrated that CIME could grow normally and therefore could continue to induce and maintain embryogenic callus. Conversely, CME appeared watery and brown during the latter period of callus induction, and callus formation from mature embryos was difficult. The results of endogenous hormone analysis indicated that the levels of ABA and GAs in CIME peaked and IAA rapidly increased to high levels at the beginning (about the 14th day from culture initiation) of embryonic calli formation from immature embryos. It was concluded that higher levels of IAA, ABA and GA3 benefitted induction of embryonic calli from immature embryos. In contrast to results of this study, Fu et al (2006) reported that low levels of GA3 facilitated induction of embryonic calli from immature embryos. This difference might be caused by different genotypes of explants or by different experimental set up. However, it is notable that levels of endogenous hormones in CIME were moderate compared to CME which did not readily form viable calli. The higher levels of IAA, GA3 and ABA did not apparently benefit the CME in contrast to conclusions of Fu et al (2006).

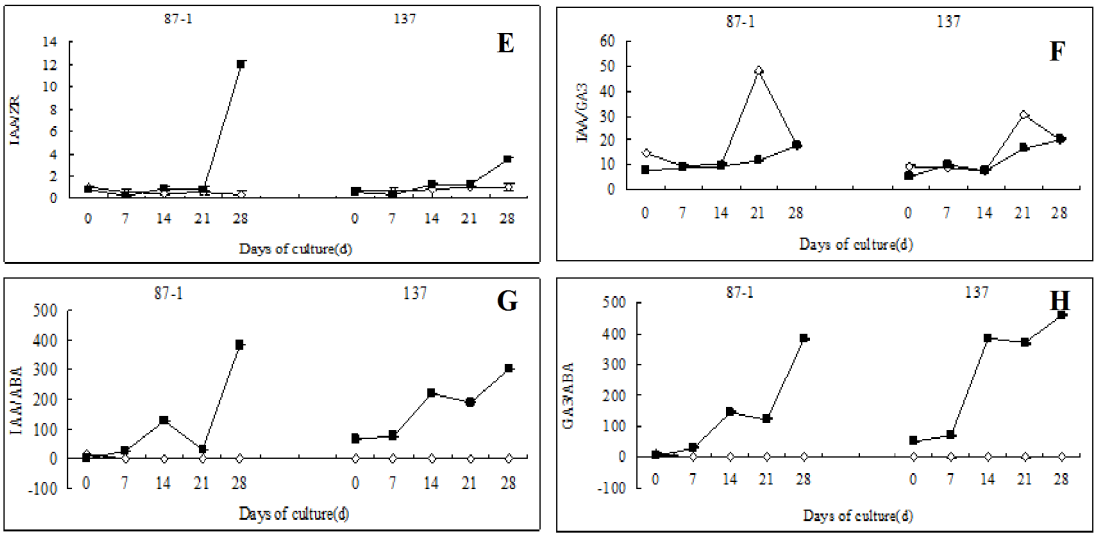

Figure 2 - E,F,G,H respectively denoted the ratio of endogenous hormones IAA to ZR, IAA to GA3, IAA to ABA, and GA3 to ABA in calli of immature embryos (open squares) and mature embryos (closed squares) at different stages of callus induction culture. Error bars indicate ±S E of the mean, n=3.

During days 14-28, levels of endogenous hormones IAA, GA3 and ABA in CIME were lower than those in mature embryos (Figure 1A-C), which indicated that moderate levels of IAA, GA3 and ABA facilitate the formation and maintenance of embryonic calli. Higher levels of IAA, GA3, ABA were evident in CME, which might be one of main physiological reasons of failure to induce embryonic calli from mature embryos.

Current philosophy in plant physiology has focused on hormonal interaction to explain plant growth regulation (e.g. Wu et al, 2007). In this study, ratios of IAA/ABA and GA3/ABA in CIME exhibited a low, relatively constant trend compared to the ratios in CME during the whole experimental period (Figure 2G, H). In CME, these ratios significantly increased, which indicated that, relative to ABA, the levels of IAA and GA3 increased rapidly and the balance among those three endogenous hormones in CME was abnormal in comparison with that in CIME. This might be another physiological reason of failure to induce embryonic calli from mature embryos. The content of endogenous hormone ZR and the ratio of IAA/ZR in CIME changed erratically during the whole experimental period. It was concluded that the endogenous ZR does not play key roles in induction and maintenance of embryonic calli.

Many scientists reported that by application of exogenous hormones, the balance among different endogenous hormones of explants was improved, thus promoting the viability of explants (Li and Liu, 1994; Zhang et al, 2000; Fu et al, 2003). By evaluating differences in endogenous hormone levels among explants with different viabilities, the viability of explants might be enhanced by adding different exogenous hormones. It has been reported that the culture of explants in medium containing 2,4-dichlorophenoxyacetic acid (2,4-D), the classic induction treatment for many species, increases the endogenous auxin levels in the responsive explants (Michalczuk et al, 1992; Pasternak et al, 2002), being this synthesis one of the crucial signals determining embryogenic fate of cultured cells (Thomas et al, 2002). In this report, the endogenous hormone levels in mature embryos and their corresponding calli were different from those of immature embryos, which suggests the frequency of embryogenic calli formation from mature embryos might be raised by adding suitable levels and ratios of different exogenous hormones to embryogenic calli induction medium. Further work is under going to prove the reliability of this hypothesis.

Acknowledgements

This work was supported by Article 2004AA207140 of National High Technological and Developmental Plan (863). We appreciate the reviews, editing, and helpful suggestions of Dr. M. A. Brown, USDA-ARS, Grazinglands Research Laboratory, El Reno, OK, USA.

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