

Abscisic Acid and High Osmoticum Regulation of Development and Storage Reserve Accumulation in Sugarcane Somatic Embryos*

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Abstract : Both abscisic acid and high osmoticum influence (somatic) embryogenesis in plants. However, the specific effects of these bioregulators on general aspects of embryogenesis and storage reserve accumulation, in particular, remain quite unclear. Our results showed that abscisic acid and sorbitol could, depending upon their concentrations, act in ways that are either beneficial or detrimental to the frequency of somatic embryogenesis in sugarcane (*Saccharum officinarum* L.) callus cultures. Complementary studies of sectioned scutellar tissues, using transmission electron microscopy were conducted to examine the *in situ* localization of storage reserves in cells of somatic embryos treated with : (i) 10^{-5} M ABA ; (ii) 9% sorbitol ; (iii) 10^{-5} M ABA and 9% sorbitol ; and (iv) untreated embryos (control) for 14 days. They indicated that these substances elicited storage reserve accumulation differently. Whilst ABA-treated embryos allocated significantly more of their cell volumes to protein bodies with no visible amyloplasts, sorbitol-treated embryos allocated more cell volumes to prominent starch grains and numerous lipid bodies. It is concluded that the specific control mechanism(s) of influence due to these compounds is probably different, as reflected in the varying patterns of amyloplasts (or starch grains), protein- and lipid bodies visualized in the scutellar cells.

Key words : Absciscic acid, High osmoticum, Protein body, *Saccharum officinarum*, Somatic embryogenesis, Ultrastructure.

アブシジン酸および高浸透圧物質によるサトウキビ体細胞胚の発達と貯蔵物質の蓄積の制御 : Ebiamadon Andi BRISIBE・三宅 博・谷口 武・前田英三 (名古屋大学農学部)

要 旨 : アブシジン酸 (ABA) および高浸透圧物質は、ともに植物の体細胞胚発生に影響を与える。しかし胚発生、特に胚発生時の貯蔵物質蓄積に対するこれらの物質の作用の相違については明らかでない。そこでサトウキビ (*Saccharum officinarum* L.) の培養カルスからの胚発生に対するABAとソルビトールの作用を比較した。胚発生促進において、ABAもソルビトールも最適濃度範囲があった。処理開始14日目の体細胞胚の胚盤を電子顕微鏡で観察したところ、 10^{-5} M ABA処理では細胞のかんりの体積がプロテインボディで占められ、アミロプラストは観察されなかったのに対し、9% ソルビトール処理では多数のデンプン粒と脂質粒が観察され、プロテインボディは認められなかった。従って胚発生促進におけるABAとソルビトールの作用機構は異なっていると結論された。

キーワード : アブシジン酸、高浸透圧物質、サトウキビ、体細胞胚発生、微細構造。

Recently there has been a great deal of interest in somatic embryogenesis, understandably because somatic embryos, like their zygotic counterparts provide an excellent experimental process for investigation of cell and plant differentiation¹³⁾. However, unlike zygotic embryos they develop from somatic cells¹⁴⁾ instead of zygotes, and therefore, potentially represent a significant step which could

enable the production of novel varieties of many economic crops. In sugarcane, and many other vegetatively propagated species somatic embryogenesis would be useful for breeding purposes, especially with regard to cell fusion, transfer of genetic information, herbicide tolerance etc. Knowledge of somatic embryodifferentiation is also fundamental in order to enhance high frequency of plantlet regeneration or the utilization of somatic embryos as inocula for synthetic seed production⁸⁾. However, to achieve their full potential for the production of synthetic seeds, somatic embryos must continue to maintain their development in the embryogenic state ; without precociously germinating, and be replete

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with large quantities of the common storage reserves (that is, carbohydrates, proteins and lipids) to reflect those found in orthodox seeds.

Absciscic acid and high osmoticum are two clear candidates which have commonly been implicated in the regulation of many aspects of somatic embryogenesis, including embryo morphogenesis and storage reserve accumulation^{1,7,15,16,17}). Although our current understanding of the roles of ABA and high osmoticum has increased considerably within the last decade (reviewed by Black²) and Kermode⁹), certain aspects of their activities are still poorly understood. This especially applies to our overall understanding of the effects of exogenously applied ABA and high osmoticum on storage reserve accumulation, and naturally has raised some fundamental questions. How are the regulatory mechanisms of these bioregulators modulated during storage reserve accumulation?⁹) Do these compounds influence storage reserve accumulation in an identical or distinctly different manner? While most of our current knowledge about the regulatory role(s) of ABA and high osmoticum has come from biochemical and molecular assays, ultrastructural studies have been profoundly limited in both number and scope. It is possible that some of these biochemical analyses do not allow proper distinction between the effects of either of these compounds, hence the disparate (and sometimes confusing) aspects on their regulatory roles in the literature. Transmission electron microscopy offers several advantages to complement current knowledge using biochemical assays.

Ultrastructurally, it enables *in situ* visualization of storage reserves as they are deposited at the single-cell level because of the distinct morphological appearances of the cellular compartments in which they are stored. In our continuing investigation into somatic embryo differentiation in sugarcane (*Saccharum officinarum* L.), the present study therefore provides data concerning the influence of ABA and sorbitol in the regulation of their overall development and, especially, *in situ* localization of storage reserves in scutellar cells.

Material and Methods

1. Plant material and tissue culture

Primary explants used in this study were derived from young leaf segments of *in vitro*-grown sugarcane (cv. NCo 310) plantlets which were regenerated from a callus stock that has been maintained in this laboratory for over three years. Explants were identified and prepared as described by Brisibe et al⁴).

The callus induction medium (CIM) consisted of Murashige and Skoog's¹¹) basal salts at double strength containing 30 μ M dicamba (3, 6-dichloro-O-anisic acid), myo-inositol (250 mg/l), casein hydrolysate (1 g/l), thiamine-HCl (5 mg/l), nicotinic acid (5 mg/l), pyridoxine-HCl (0.5 mg/l) and 4% (w/v) sucrose. All explants were cultured for 21 days and at the end of this period, three morphologically distinct: (i) soft and non-embryogenic, (ii) mucilaginous, and (iii) pearl-white and compact embryogenic callus types had developed.

Embryogenic calli were transferred to an embryo formation and development medium (EFDM) consisting of double strength MS basal salts that was supplemented with dicamba (10 μ M), proline (10 mM), casein hydrolysate (1 g/l), maltose (3%) and sucrose (3%) for 14 days. Somatic embryos were later initiated according to procedures described in preliminary studies^{3,4}). Briefly, callus pieces showing characteristic presence of somatic embryo(id)s were subsequently transferred onto an embryo maturation medium (EMM) containing 3% sucrose and 0.2% (w/v) Gelrite, either with filter-sterilized ABA or sorbitol or both, at the concentrations stated in the text and were cultured for 14 days.

All cultures were maintained at 25°C in darkness, and the development of somatic embryos was followed and regularly examined under a stereomicroscope over a period of 4 weeks. Developing and fully developed somatic embryos were isolated with as little callus tissues as possible, immediately dipped in sterile distilled water for 2 to 5 minutes, and thereafter fixed for microscopical procedures as described below.

2. Electron microscopy

For observations of the different stages of morphological differentiation by scanning electron microscopy, samples were prepared and examined using the method previously described⁴).

Table 1 Influence of varying concentrations of ABA and sorbitol on frequency of somatic embryogenesis in callus maintenance cultures of sugarcane.

Treatment	Frequency of somatic embryogenesis ^a		
	No. of callus pieces cultured	No. showing embryo development	Percentage
Control	10	0*	0
ABA (M)			
10 ⁻⁷	10	0*	0
10 ⁻⁶	30	14*	47
10 ⁻⁵	30	26	87
10 ⁻⁴	20	2	10
10 ⁻³	10	0	0
Sorbitol (%)			
3	10	0*	0
6	20	11*	55
9	20	15	75
12	20	9	45
15	10	0	0

^a Frequency of somatic embryogenesis was assessed after 21 days of culture on medium containing double strength MS basal salts and 30 μ M dicamba. Values given are the means of three replicates.

* Most of the callus pieces had developed leafy shoots/plantlets by the end of the culture period, hence values showing presence of somatic embryos appear considerably low.

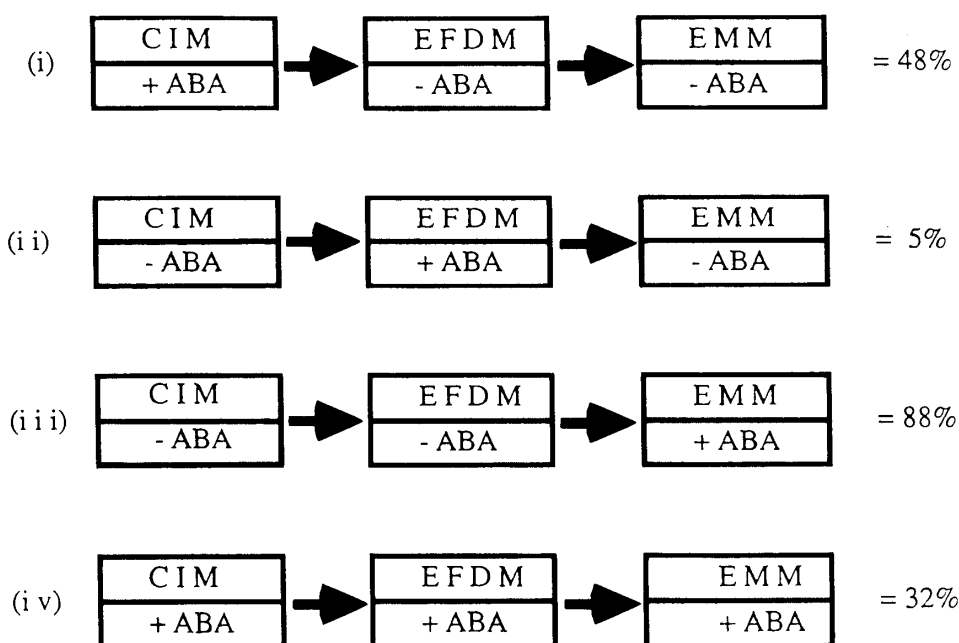


Fig. 1. Schematic representation of ABA (10^{-5} M) treatments necessary to optimize frequency of somatic embryogenesis (%) in callus cultures of sugarcane. Each developmental phase was exposed to ABA as outlined under Materials and Methods. CIM : callus induction medium, EFDM : embryo formation and development medium, EMM : embryo maturation medium.

Somatic embryos for transmission electron microscopy were fixed at 4°C for 4 to 6 h in 4% glutaraldehyde buffered at pH 7.0 with 0.1 M sodium cacodylate buffer. They were thoroughly washed in several changes of the buffer and postfixed for 2 h in 1% aqueous osmium tetroxide. The samples were dehydrated in a graded ethanol series and finally in propylene oxide, and embedded in an epoxy resin. Ultrathin sections of scutellar tissues for transmission electron microscopy were cut with diamond knives on a Reichert-Nissei Ultracut N ultramicrotome at a thickness of 80–90 nm, collected on copper grids, and post-stained with uranyl acetate followed by lead citrate. Tissues were examined and photographed using a Hitachi H-600 transmission electron microscope at 100 kV.

Results

1. High concentrations of ABA and sorbitol inhibit somatic embryogenesis

In the first experiment, embryogenic callus cultures were incubated for 21 days in a range of ABA (between 10^{-7} and 10^{-3} M) and sorbitol (from 3 to 15% ; w/v) concentrations. This was with the intent of determining the concentrations that were : (i) supportive, and (ii) totally suppressive of somatic embryogenesis. The results, shown in Table 1, indicate that there was a dose-dependent relationship between regulator concentration and the frequency of somatic embryo development. Absciscic acid at 10^{-5} M and sorbitol at 6–12% generally supported embryogenesis. Higher levels of ABA (10^{-4} M or greater) and sorbitol (about 12%) in the medium suppressed embryogenic growth. At these concentrations, most of the callus pieces either simply became hard and brownish or completely developed necrosis. Lower concentrations of both substances also supported somatic em-

bryogenesis but most of the embryos germinated precociously.

The responsiveness of somatic embryo(id)s at different stages of morphological differentiation to applied ABA or sorbitol was also investigated (as illustrated in Fig. 1) during proembryonic and globular embryo formation on CIM, embryo formation and development on EFDM and embryo maturation phases on EMM (see Figs. 2 to 5). Embryogenic cultures at each of these stages were exposed to 10^{-5} M ABA or 9% sorbitol in darkness. The results showed that, overall, the highest frequency of somatic embryogenesis was observed when 10^{-5} M ABA was added to the embryo maturation medium (EMM) rather than to the embryogenic callus initiation (CIM) or embryo formation and development (EFDM) media (Fig. 1). Inclusion of 9% sorbitol at the same periods of development gave similar responses, though the frequency (values) of fully developed somatic embryos observed with this treatment were lower than those observed with 10^{-5} M ABA (data not shown). Preliminary experiments revealed that embryo(id) response to ABA and osmoticum is developmental stage-dependent ; a reflection of differential sensitivity of developing somatic embryo(id)s to exogenously applied ABA and sorbitol.

2. Influence of ABA and sorbitol on storage reserve localization in somatic embryos

Although considerable progress in understanding the regulatory roles of ABA and osmotic potential on storage reserve synthesis and accumulation during *in vitro* embryogenesis in dicots has been achieved^{1,7)}, less is known about their exact regulatory influences during somatic embryogenesis in monocots. Therefore, with the aim of comparing *in situ* localization of storage reserves in

Explanation of Figures

Figs. 2 to 5. Scanning electron micrographs showing sugarcane somatic embryogenesis at the different phases of differentiation that were treated with 10^{-5} M ABA and/or 9% sorbitol.

Fig. 2. Proembryonic and globular embryo formation stage on CIM ($\times 600$).

Fig. 3. Embryoid formation phase on EFDM ($\times 300$).

Fig. 4. Embryo development phase on EFDM ($\times 600$).

Fig. 5. Embryo maturation phase on EMM ($\times 420$).

Figs. 6 to 16. Transmission electron micrographs of sugarcane somatic embryos derived from cultures on EMM with 10^{-5} M ABA and/or 9% sorbitol.

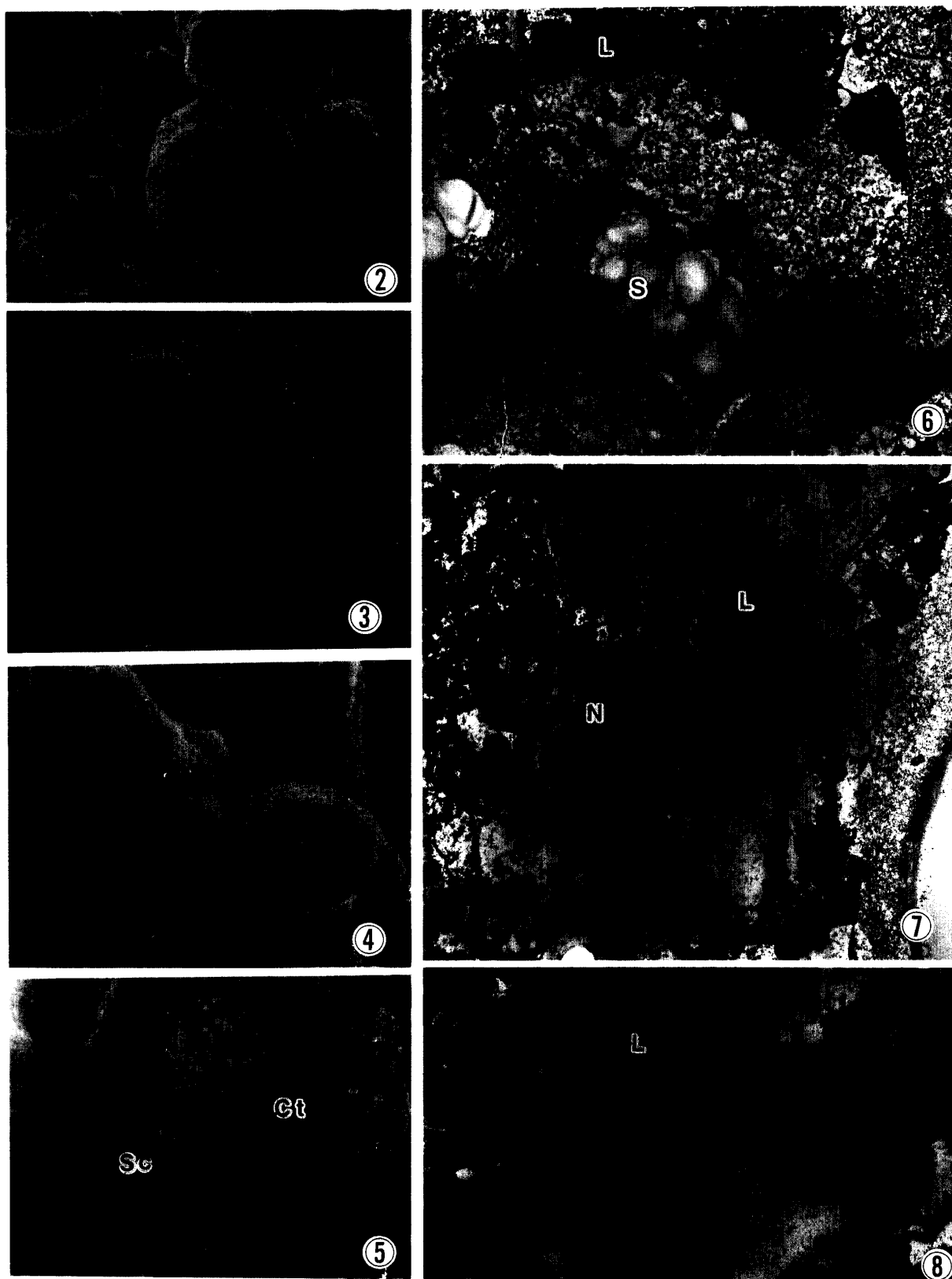
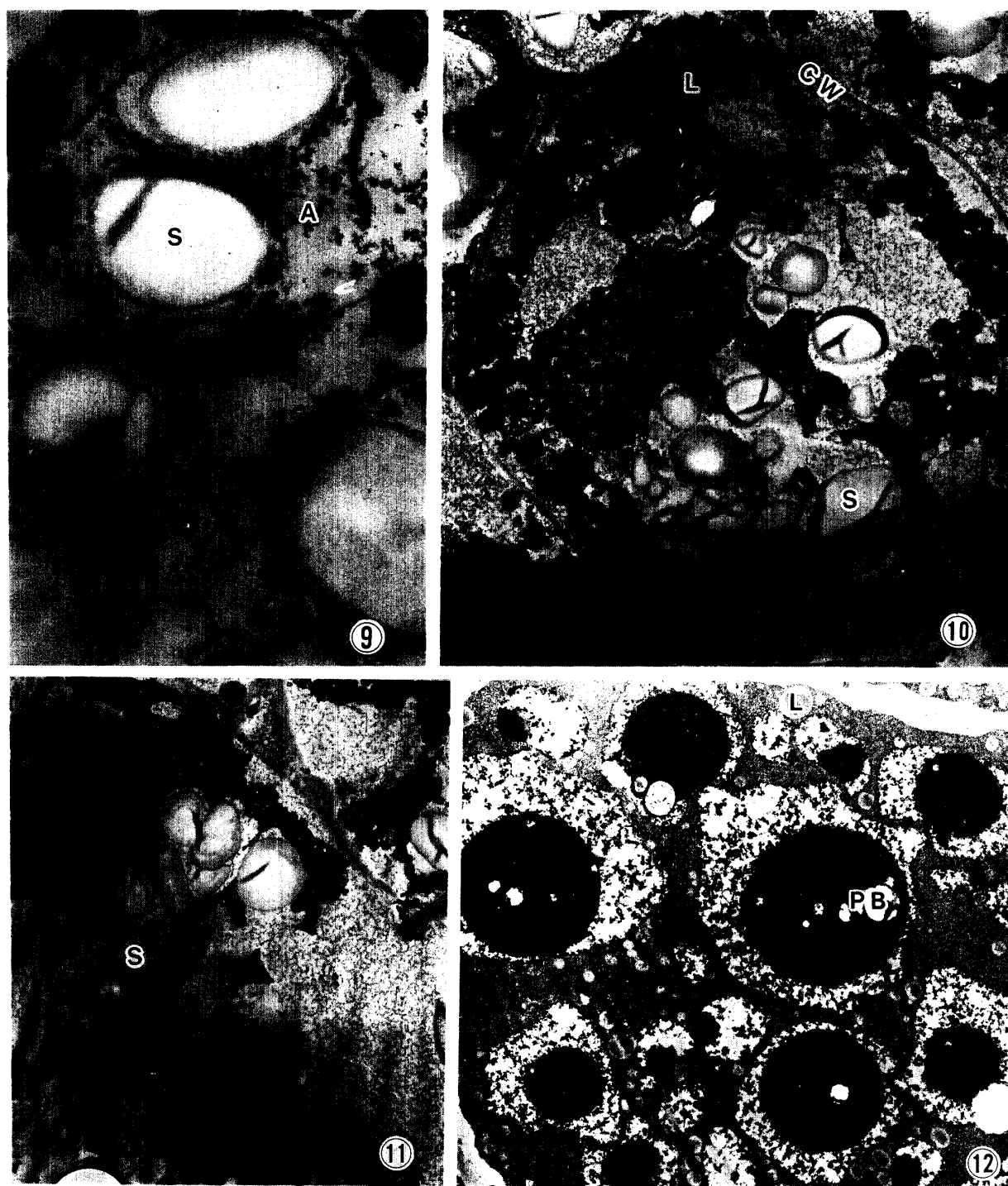


Fig. 6. Scutellar cells in an untreated (control) somatic embryo. The cells appear electron dense. Starch (S) and lipid (L) are also visible ($\times 3000$).

Fig. 7. Scutellar cell in an untreated (control) sugarcane somatic embryo showing tightly packed lipid bodies (L) ($\times 3000$).

Fig. 8. Higher magnification of lipid bodies observed in Fig. 7 ($\times 4500$).



- Fig. 9. Scutellar cell in an untreated (control) sugarcane somatic embryo showing amyloplasts (A) with one or two conspicuous starch grains (S) ($\times 9000$).
- Fig. 10. Scutellar cell in a sugarcane somatic embryo treated with sorbitol. Note the cytoplasmic density of the cell and the conspicuous localization of starch (S) and lipid bodies (L). Also note the conspicuous absence of protein bodies in the cell ($\times 4500$).
- Fig. 11. Scutellar cells in a sugarcane somatic embryo treated with sorbitol showing a high frequency of starch grains (S). Note that protein bodies are also absent in these cells ($\times 2300$).
- Fig. 12. Scutellar cell in a sugarcane somatic embryo treated with ABA. This cell contains compactly arranged protein bodies (PB) which occupy almost its entire cytoplasmic matrix. Some lipid bodies (L), quite small in size and only faintly stained, are also observed. Note that the cell does not contain any starch grains ($\times 3800$).

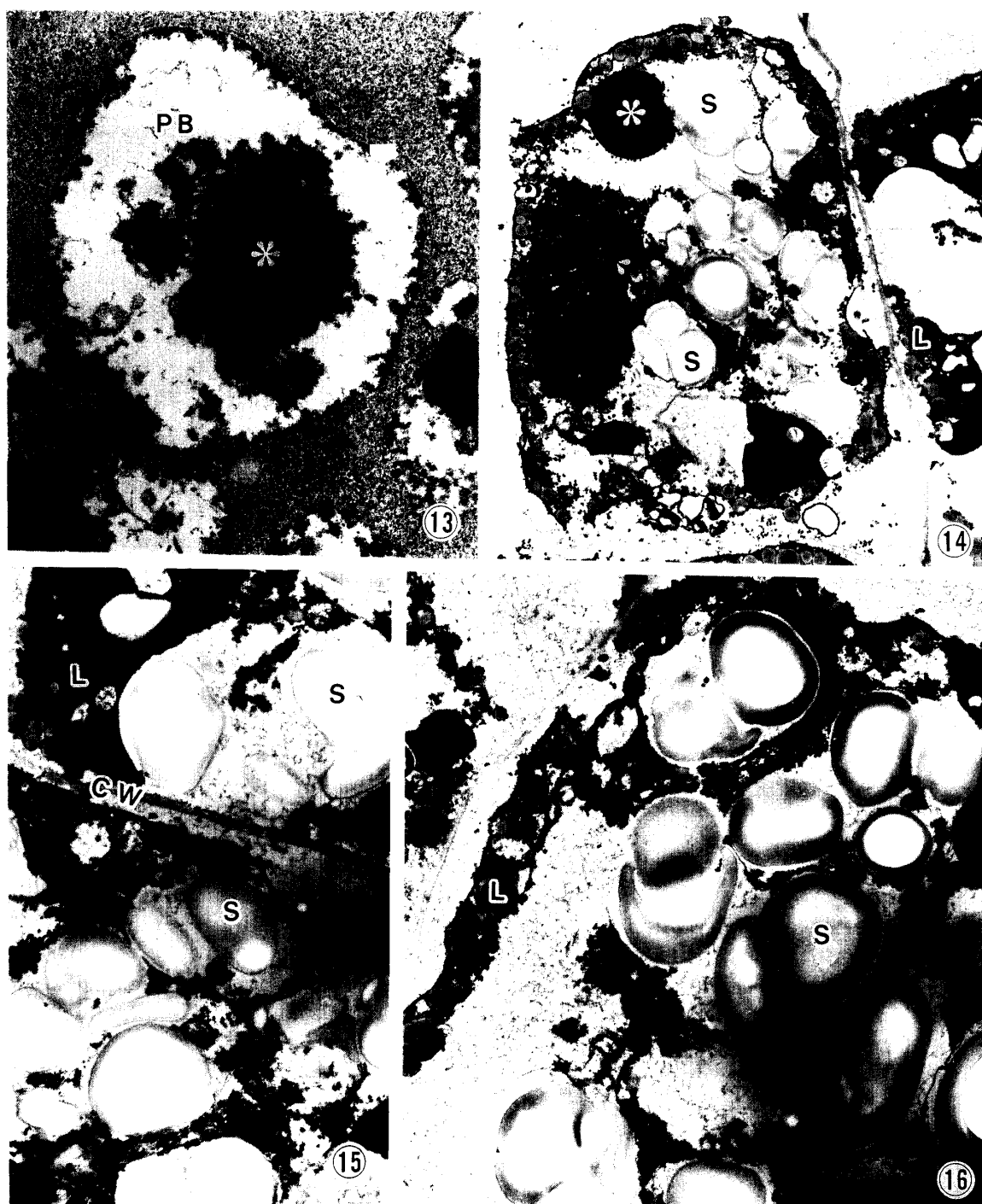


Fig. 13. Magnification of a protein body (PB). Note that the contents are densely stained. Also note the conspicuous presence of both globular (*) and flocculent precipitates ($\times 12800$).

Fig. 14. Scutellar cells in a sugarcane somatic embryo treated with both 10^{-5} M ABA and 9% sorbitol in combination showing the localization of starch grains (S) and lipid bodies (L), mainly along the cell periphery. Also note the presence of a globular precipitate (*) in the cell ($\times 2400$).

Figs. 15 & 16. Storage deposits localized in scutellar cells treated with ABA and sorbitol in combination. Note the rather distinct nature of starch (S) and lipid bodies (L) ($\times 6400$).

Abbreviations used in Figures

A : amyloplast, Ct : coleoptile, CW : cell wall, L : lipid body, N : nucleus, PB : protein body, S : starch grain, Sc : scutellum.

response to the addition of ABA (10^{-5} M) and sorbitol (9%), scutellar tissues of fully developed and mature somatic embryos treated with these substances for 14 days were examined by transmission electron microscopy (Fig. 14). A comparison of the qualitative localization of storage reserves (that is, starch, proteins, and lipids) in cellular compartments of somatic embryos of the control treatment, as well as those treated with these substances, is illustrated in Figs. 6 to 16.

In control embryos on regulator-free medium, scutellar cells (Figs. 6 to 9) were electron dense with lipid bodies that were either scattered (Fig. 6) or tightly aggregated (Figs. 7 and 8) and amyloplasts which contained few starch grains (Fig. 9). The corresponding cells of embryos treated with sorbitol are shown in Figs. 10 and 11. In contrast to those of control embryos, these cells allocated significantly large proportions of their cytoplasmic matrices to visible starch grains and lipid bodies which were mainly localized along the cell periphery (Fig. 10). It is significant that though protein bodies were not visualized in these cells, they were present in scutellar cells of ABA-treated embryos.

Figure 12 shows the profile of protein bodies visualized in scutellar cells of ABA-treated embryos. It also shows that unlike sorbitol-treated embryos, ABA-treated embryos characteristically lacked amyloplasts and had considerably fewer and smaller lipid bodies which were faintly stained. Figure 13 is a high magnification illustration of a typical protein body seen in Fig. 12. It is composed of globular (asterisk) and flocculent precipitates; evidence of storage protein deposition in these membrane bound cellular compartments.

It is important to recognize that scutellar cells of embryos treated with ABA and sorbitol in combination (Figs. 14 to 16) did completely reflect the pattern of visualization observed with their individual applications. Cells contained prominent starch grains and peripherally localized lipid bodies (just as seen with sorbitol treatment). Globular precipitates of protein bodies can also be visualized (see Fig. 14).

Discussion

Within the last one or two decades, several reports have already demonstrated that ab-

scisic acid and high osmoticum regulate various aspects of *in vivo* and *in vitro* embryogenesis in many plant species^{1,5,6,7,17}. These authors provided strong evidence to show that ABA and/or high osmoticum promote a cascade of reactions that are necessary for normal embryogenesis to occur. Like many of these studies, the current investigation demonstrates that the inclusion of ABA and/or sorbitol in sugarcane embryogenic cultures elicits several developmental responses which are reflected in the dynamics of somatic embryo differentiation. The results reported in this study particularly indicate that, within the ranges evaluated, 10^{-5} M ABA and 6–12% sorbitol promote an optimum response in the parameters tested. These concentrations are well within the ranges reported previously to enhance *in vitro* embryo development in rape^{5,6}.

That increasing the concentration of ABA and sorbitol adversely affects the frequency of somatic embryogenesis may be a common expectation, but it is clearly shown by the results of this study that these effects are most severe during the early stages of embryo(id) differentiation. This quantitative evaluation, comparing the sensitivity of somatic embryo(id)s at different growth stages to 10^{-5} M ABA and 9% sorbitol as a measure of their effects on embryo differentiation, assumes that somatic embryo(id)s respond differently to ABA and sorbitol, depending on the morphological maturity of the embryos. Such a comparison is justified in light of the finding that, depending on the stage of development, elevated levels of ABA decrease the rate of endosperm cell division in cultured maize kernels¹². High levels of ABA have also been correlated with the inhibition of the initial steps during embryogenesis and growth in wheat¹⁰. Undoubtedly, our data in Fig. 1 could thus be interpreted on the basis that ABA (and sorbitol) may actually co-ordinate various aspects of somatic embryo development by acting either in an inhibitory (if applied early) or stimulatory (when applied late during differentiation) capacity. Such differing effects of ABA (and sorbitol) may, in part, be explained by the fact that their application creates an osmotic stress component which perhaps drastically reduces cell division and cell number due to reduction in turgor-driven growth processes during the

initial stages of embryo differentiation.

The influence of ABA and sorbitol on *in situ* storage reserve localization, as seen with transmission electron microscopy, presents several interesting features with respect to the type of storage products identified. Whilst lipid bodies were localized in embryos treated separately with ABA and sorbitol, it is apparent that not all the cells contained either protein bodies or starch grains. A closer look at the pattern of storage reserve localization in ABA treated embryos indicates that one very striking feature is the significant visualization of protein bodies in scutellar cells (see Fig. 12). This is not surprising since it fits with previous observations that ABA participates in regulating the synthesis of several kinds of proteins, which are either laid down as reserves or are thought to enable the maturing seed (or embryo during unorthodox embryogenesis²) to withstand the rigours of desiccation². The absence of starch grains (amyloplasts) in these cells is characteristic and may be an implicit indication that ABA either does not stimulate the accumulation of starch in sugarcane somatic embryos or it enhances the accumulation of protein bodies at the expense of starch grains. These two explanations need not be mutually exclusive. On the other hand, the prominence of distinctive starch grains and numerous lipid bodies in sorbitol treated embryos, which lack protein bodies (at least, during the duration of this study) may be an indication that sorbitol does not enhance protein synthesis.

Of greater interest and significance of the differences in metabolic response(s) between the effects of ABA and sorbitol is the observation that starch grains, lipids and protein bodies are localized when these substances are applied in combination. Collectively, the ultrastructural data presented in this report illustrating the altered patterns of cell development is a confirmation of the point earlier proposed by Black²) that "ABA and highly negative osmotic potentials might not necessarily act by the same mechanism". On account of this, it is tempting to speculate that sugarcane somatic embryos perceive physiological signals from ABA and sorbitol differently during embryogenesis. This implies, therefore, that for the embryos to be replete with the highest levels of storage reserves in

order to ensure best postgerminative growth and seedling vigor, it would be necessary to treat them with a combination of ABA and osmoticum.

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