Calcium/Calmodulin Regulated Cell Wall Regeneration in *Zea mays* Mesophyll Protoplasts

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The composition of newly synthesized cell walls starting from enzymatically isolated *Zea mays* mesophyll protoplasts was studied. The contents of pectin, cellulose and hemicellulose in addition to the wall-associated proteins were followed in the presence of increasing concentrations of calcium ions with or without the calcium channel blockers like lanthanum, lithium ions, verapamil, nifedipine and the calmodulin antagonist trifluperazine. Pectin accumulation was inhibited by all antagonists except lanthanum. Cellulose formation, however, was increased by organic antagonists trifluperazine, verapamil and maximally by nifedipine while it was not affected by the inorganic ions lanthanum or lithium. Hemicellulose accumulated with nifedipine present but significantly decreased by all other antagonists. Added Ca^{2+} (5–10 mM) reversed most of the blockers-induced inhibition on pectin and hemicellulose. In the case of cellulose, however, calcium concentration which reversed the inhibitory action of these antagonists was dependent on the antagonist. Starch exhibited little alterations indicating its minor role in deposition of wall components. Cell wall-associated proteins were negatively affected by lanthanum and verapamil and positively by lithium and nifedipine. Generally, this fraction was found in a negative correlation with pectin levels. Chlorophyll contents were lowered after growth for 48 h; this might be due to repeated cell division. These results are discussed in relation to the mechanism of antagonists and effects of calcium.

Introduction

Calcium ions are involved in the stabilization of cell wall structures, acidic growth, ion exchange properties and control of the activities of wall enzymes. All these properties originate from the tight binding of calcium ions to cell wall pectins (Demarty *et al.*, 1984). Cell wall metabolism is an important process in plant growth not only because cell walls compose a large proportion of the cell biomass but also because the role of cell walls in determining cell growth (Zhong and Läuchli, 1988). Pectin loss was observed in *Nitella* walls when the absorbed divalent cations were exchanged for monovalents (Gillet and Liners, 1996). However, high concentrations of Ca^{2+} almost totally inhibited cellulose deposition (Eklund and Eliasson, 1990). Also, the formation of 1,3 β-glucans, which can serve as a transient cell wall material during various steps of plant growth and development, is strictly dependent on $[Ca^{2+}]$ (Fink *et al.*, 1987).

On the other side, calcium channel blockers can modify the endogenous Ca^{2+} level/distribution in different compartments (Sharma *et al.*, 1992) although they vary in their modes of action. In addition, calmodulin/Ca^{2+} is required during the differentiation process posssibly to allow secondary wall deposition (Roberts and Haigler, 1992).

Since most of the cell wall studies which have been published up to now have been performed using entire plants or cells, the present paper was designed to focus on wall regeneration starting from protoplasts. The role of Ca^{2+} and CaM (calmodulin) on regenerated wall components of *Zea mays* mesophyll protoplasts was followed by applying various Ca/CaM antagonists, having various mechanisms to control Ca^{2+} level/distribution, with or without successively increasing concentrations of calcium.

Materials and Methods

Zea mays L. was grown for 35 days under field conditions in the garden of the Department. Pro-

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toplasts were isolated and cultured essentially according to Hahne *et al.* (1983). 5 g of de-ribbed leaves were sectioned and incubated at 30 °C for 3 h in 20 ml of a solution containing 0.2% pectinase (macerozyme), 2% cellulase (R-10, Yakult Honsha Tokyo) and 0.4 M mannitol at pH 5.8. Intact protoplasts were then freed from debris by collecting them from the surface of 0.6 M sucrose solution using a Pasteur pipette after sedimentation of the cell deberis and the broken ones. After washing twice in 5 ml 0.4 M mannitol they were resuspended in 10 ml of a culture medium described by Nagata and Takebe (1970) at a density of 5x 10^3 cell/ml at 30 °C in diffuse light (*ca.* 7.0 μE). The cultures were treated with 0.1, 1, 5 or 10 mM Ca^{2+} (Cl_2) with or without one of the following Ca/CaM antagonists:

– 1.0 mM La or Li; as lanthanum or lithium chloride.

– 5x 10^{-4} mM verapamil, nifedipine, or trifluperazine hydrochloride.

– Control cultures contained none of the above treatments.

After 48 h, the protoplasts were collected by centrifugation (*ca.* 250 x *g* for 5 min). Cell wall fractionation was conducted essentially according to Dever *et al.*, (1968) and Galbraith and Shields (1981). Aliquots of protoplast cultures were extracted twice in 80% ethanol to remove soluble metabolites. The precipitate was then extracted in 2 ml 0.5 N NaOH for starch, 0.5% ammonium oxalate-oxalic acid (90 °C for 24 h) for pectins, 17.5% NaOH for hemicellulose and in 72% H_2SO_4 (with 15 min autoclaving) for cellulose extraction. After that, no precipitate was found. According to Dever *et al.* (1968) such a precipitate was ascribed to the lignin fraction. In the present study, a period of 48 h may not be sufficient for lignin deposition by the protoplasts. Cell wall-associated proteins were extracted with 3 M LiCl in citrate phosphate buffer, pH 5.5 according to Acebes and Zarra (1992) and were quantified with the Folin phenol reagent (Lowry *et al.*, 1951). Contents of starch and wall polysaccharides were determined by the anthrone sulfuric acid reagent using glucose as a standard. Chlorophyll was measured according to Mackinney (1941) using the absorbance of 90% acetone extracts. The data of three replicates were then subjected to one way analysis of variance for the effect of antagonists (without supplemental calcium) and a second time for the effect of increased concentrations of Ca^{2+} on each antagonist.

Results

The regenerated wall materials of *Zea mays* mesophyll protoplasts were followed in the presence of the calcium antagonists lanthanum, lithium, verapamil, nifedipine or the calmodulin antagonist trifluperazine with and without various concentrations of Ca^{2+}. Pectin, cellulose and hemicellulose contents were variably affected by Ca^{2+} as well as by the antagonists applied (Table Ia, b and c). Pectin deposition was inhibited by all of the applied Ca/CaM antagonists other than lanthanum. The calmodulin antagonist trifluperazine (TFP) was more potent in decreasing pectins than the Ca^{2+} antagonists. Increasing the concentration of external Ca^{2+} (up to 5 mM) did not affect pectin accumulation but the concentration of 10 mM was inhibitory. However, this concentration stimulated pectin accumulation when combined with the various Ca/CaM antagonists; lanthanum, nifedipine, lithium, and even with trifluperazine (Table Ia). From the data, it can be concluded that this high concentration (10 mM) is necessary only in the presence of the antagonists to overcome their inhibitory action. In the case of verapamil, however, the stimulatory concentration was lower (5 mM Ca^{2+}).

Unlike pectin, cellulose accumulation was significantly stimulated by the addition of trifluperazine, verapamil and maximally by nifedipine while lanthanum and lithium were strictly ineffective in comparison with that of the control (Table Ib). With calcium being added to lanthanum, lithium or also to trifluperazine, cellulose accumulation was enhanced but the enhancing concentration was high (10 mM with lithium) and low (0.1 mM with lanthanum). However, with nifedipine or verapamil, the additional Ca^{2+} lowered cellulose contents.

Hemicellulose accumulation was significantly stimulated only by nifedipine. It was, however, maximally inhibited by verapamil and to a smaller extent by lanthanum, trifluperazine and lithium. Generally, the concentration of 5 mM Ca^{2+} maximized hemicellulose contents in all cultures of *Zea mays* protoplasts (Table Ic). In the case of lantha-

Table I. Cell wall polysaccharides (mg/culture containing $2.5x10^4$cells) of *Zea mays* protoplasts as influenced by various Ca/CaM antagonists: 1 mM La or Li and 0.5 mM verapamil (V), nifedipine (N) or trifluperazine (TFP).

a: Pectin

mM Ca^{2+}	0	La	Li	V	N	TFP
0.0	0.57±0.02Ab	0.65±0.01Ba	0.43±0.03 Bc	0.49±0.02Bc	0.46±0.02Cc	0.35±0.02Cd
0.1	0.57±0.01A	0.63±0.01B	0.38±0.01C	0.51±0.01B	0.58±0.03A	0.43±0.02A
1.0	0.52±0.01A	0.62±0.01B	0.34±0.01D	0.45±0.02B	0.59±0.01A	0.43±0.02A
5.0	0.57±0.01A	0.61±0.01C	0.44±0.02B	0.95±0.03A	0.57±0.01B	0.42±0.01B
10.0	0.38±0.01B	0.89±0.01A	0.63±0.02A	0.40±0.01C	0.65±0.03A	0.50±0.02A

b: Cellulose

mM Ca^{2+}	0	La	Li	V	N	TFP
0.0	0.66±0.03Cc	0.64±0.03Cc	0.64±0.03Cc	0.78±0.01Ab	1.01±0.01Aa	0.79±0.01Bb
0.1	0.63±0.02C	1.01±0.01A	0.67±0.01C	0.68±0.03B	0.79±0.03B	0.57±0.01C
1.0	1.01±0.02A	0.52±0.01D	0.60±0.01D	0.60±0.01C	0.74±0.01B	0.75±0.03B
5.0	0.89±0.02B	0.89±0.02B	0.78±0.01B	0.57±0.01D	0.62±0.02C	0.78±0.02B
10.0	0.65±0.03 C	0.55±0.02D	1.00±0.01A	0.64±0.02B	0.56±0.00C	0.95±0.02A

c: Hemicellulose

mM Ca^{2+}	0	La	Li	V	N	TFP
0.0	0.59±0.02Ab	0.46±0.02Bc	0.54±0.02Bb	0.35±0.01Dd	0.68±0.02Ca	0.43±0.01Cc
0.1	0.47±0.01B	0.38±0.01C	0.47±0.01C	0.42±0.01B	0.68±0.02C	0.52±0.01B
1.0	0.48±0.01B	0.83±0.03A	0.56±0.01A	0.44±0.01B	0.80±0.01B	0.49±0.01B
5.0	0.63±0.02A	0.79±0.02A	0.60±0.01A	1.04±0.02A	1.22±0.02A	0.63±0.02A
10.0	0.56±0.02A	0.53±0.03B	0.57±0.01A	0.38±0.01C	0.64±0.02C	0.46±0.02B

The data were subjected to one way analysis of variance and the mean values of three replicates±s.e. are presented. Means which are not significantly different (p = 0.05) are followed by the same letter (small letters- effect of antagonists without $CaCl_2$, capital letters – effect of $CaCl_2$ with each single antagonist).

num, the concentration with the strongest enhancing effect was of 1 mM Ca^{2+}.

Starch as a reserve polysaccharide might play an important role also for monosaccharides pool. Therefore, its content was also followed and found to be the least fraction to be altered by the applied treatments (Table IIa). Trifluperazine, verapamil or lanthanum did not impose significant changes on starch content. It was only significantly decreased by lithium and nifedipine. Consequently, Ca^{2+} did not cause any significant alterations in control, La^{3+} or TFP treated cultures. Only its highest concentration decreased starch contents in the presence of lithium or verapamil.

Proteins associated with the cell walls of *Zea mays* protoplasts were severely lowered by lanthanum in comparison with that of the control (Table IIb). All other antagonists, maximally lithium, increased the levels of this protein fraction. 10 mM Ca^{2+} doubled the wall proteins compared with that of the control. However, in combination with the antagonists, Ca^{2+} effect and concentration on wall proteins varied from one antagonist to the other. With nifedipine calcium had no significant effect while with trifluperazine or lithium and supplemental calcium the amount of cell wall proteins was decreased. They were, however, maximally increased when 5 mM Ca^{2+} were combined with verapamil or lanthanum.

Zea mays protoplast cultures started growing with a chlorophyll (a and b) content of 13.8±0.3 µg which corresponds to $5x10^3$ cells/ml. All protoplast cultures exhibited lowered contents of chlorophyll after growth for 48 h despite the alterations recorded in response to the applied treatments. The antagonists lanthanum and lithium caused chlorophylls to decrease in comparison with that of the control (Table IIc) while verapamil, nifedipine as well as the calmodulin antagonist trifluperazine inreased chlorophyll contents. The added Ca^{2+} caused a dramatic decrease in chlorophyll contents in nifedipine treated cultures while in combination with other antagonists chlorophyll was more preserved. However, when not

Table II. Starch, cell wall associated proteins and chlorophyll (a and b) of *Zea mays* protoplasts ($2.5x10^4$ cells) as influenced by various Ca/CaM antagonists:1 mM La or Li and 0.5 mM verapamil (V), nifedipine (N) or trifluperazine (TFP).

a: Starch (mg/culture)

mM Ca^{2+}	0	La	Li	V	N	TFP
0.0	1.53±0.05Aa	1.55±0.07Aa	1.23±0.08Dc	1.67±0.06Ab	1.35±0.02Ab	1.60±0.11Aa
0.1	1.44±0.06A	1.64±0.08A	1.99±0.01A	1.47±0.09B	0.83±0.09B	1.48±0.11A
1.0	1.55±0.07A	1.77±0.08A	1.67±0.06B	1.94±0.05A	1.21±0.05A	1.21±0.01A
5.0	1.75±0.06A	1.61±0.04A	1.85±0.02A	1.45±0.09B	1.10±0.04A	1.50±0.05A
10.0	1.45±0.09A	1.62±0.06A	1.43±0.07C	0.85±0.07C	1.40±0.01A	1.40±0.00A

b: Cell wall associated proteins (mg/culture)

mM Ca^{2+}	0	La	Li	V	N	TFP
0.0	0.31±0.01Bd	0.15±0.01De	1.24±0.04Aa	0.25±0.02Dc	0.63±0.01Ab	0.50±0.01Ac
0.1	0.26±0.01B	0.23±0.01C	0.91±0.01B	0.39±0.01C	0.66±0.03A	0.53±0.02A
1.0	0.24±0.02C	0.22±0.01C	0.55±0.01C	0.51±0.01B	0.65±0.01A	0.14±0.02B
5.0	0.33±0.02B	0.49±0.01A	0.63±0.02C	0.67±0.02A	0.68±0.01A	0.15±0.01B
10.0	0.61±0.02A	0.33±0.02B	0.61±0.02C	0.23±0.03D	0.65±0.02A	0.14±0.01B

c: Chlorophyll (μg/culture)

mM Ca^{2+}	0	La	Li	V	N	TFP
0.0	7.5±0.03Bb	4.4±0.1Bc	4.4±0.3Bc	9.1±0.3Aa	9.1±0.1Aa	8.9±0.4Ba
0.1	7.5±0.1B	4.4±0.1B	4.4±0.2B	7.3±0.2C	5.6±0.2B	12.1±0.4A
1.0	7.0±0.1B	6.2±0.03A	4.9±0.1B	8.5±0.1B	3.7±0.1C	10.0±0.2B
5.0	8.3±0.3A	6.2±0.2A	7.2±0.2A	8.9±0.3A	3.3±0.1C	8.0±0.1C
10.0	7.5±0.2B	4.6±0.1B	6.7±0.3A	9.9±0.1A	2.8±0.05D	8.0±0.4C

The data were subjected to one way analysis of variance and the mean values of three replicates±s.e. are presented. Means which are not significantly different ($p = 0.05$) are followed by the same letter (small letters-effect of antagonists without $CaCl_2$, capital letters-effect of $CaCl_2$ with each single antagonist).

combined with the antagonists, added Ca^{2+} did not exert marked changes on chlorophyll (a and b).

Discussion

In this work, various Ca/CaM antagonists were studied for their effect on the composition of the newly synthesized wall materials. The applied antagonists have different modes of action and they refer to various classes of Ca^{2+} channels. All of them, however, were reported to control the level/distribution of Ca^{2+}. Inhibited pectin accumulation by the Ca/calmodulin antagonists and its increase upon addition of Ca^{2+} may indicate that this process is controlled by apoplastic calcium, which is in contact with the outer surface of the membranes and its associating enzymes. Comparatively, *Nitella* cell walls exhibited a major loss of pectin when the adsorbed bivalent ions are exchanged for monovalents (Gillet and Liners, 1996). Cellulose levels, however, were not decreased by any of the antagonists. On the contrary, they even increased in comparison to the control *i.e.* lower calcium, induced by the action of the antagonists, favoured cellulose accumulation. Eklund and Eliasson (1990) reported that high concentrations of Ca^{2+} totally inhibited cellulose deposition. Choi and Ljungdahl (1996) found that intact cellulosomes hydrolyze cellulose in the presence of calcium and this activity was inhibited by EDTA (ethylenediaminetetraacetic acid). Organic antagonists seem to have a mechanism different from that of the inorganic ones. Lanthanum and lithium, as inorganic ions, may exchange with calcium of the cellulosomes leading to loss of their ability to hydrolyze cellulose in a similar action to that of ethylenediaminetetraacetic acid. The hemicellulose fraction exhibited its highest levels in the presence of 5 mM Ca^{2+}; whatever the antagonist applied. However, the mechanism by which calcium interfers with hemicellulose is generally lacking in the literature. Starch exhibited little alterations in response to the treatments which indicates that starch as a sugars pool or, alternatively a sink, plays a minor

role in determining the deposition of wall components.

The cell wall associated proteins were enriched by all antagonists, except lanthanum. Valero and Labrador (1993) found that similarly extracted proteins are able to release sugars from cell wall materials. In accordance with that, wherever cell wall associated proteins were high, pectin levels were lowered (Fig. 1a). This negative correlation indicates that this protein may determine the level of pectin deposition; as this fraction contains polygalacturonase (Acebes and Zarra 1995). This relationship, however, was disturbed by calcium, particularly in the presence of organic antagonists (Fig. 1b). According to the 'steric hinderance' hypothesis, the inhibitory effects of calcium on the hydrolysis of polygalacturonic acids works *via* the formation of calcium bridges cross-linking galacturonan polysaccharides with one another, thus decreasing their enzyme hydrolysis and/or the mobility of the enzyme and its access to the substrate (Januneau *et al.*, 1994). Variation in Ca^{2+} requirement seems to be dependent on the mode of action of each antagonist. In addition, different classes of Ca^{2+} channels appear to be involved for Ca^{2+} effect (Reiss and Beale, 1995).

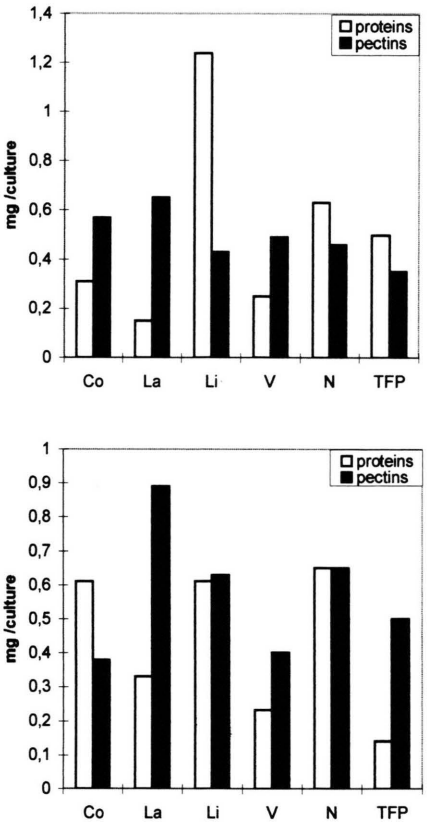

Fig. 1. Relationship between cell wall associated proteins and pectin levels in regenerated cell walls of *Zea mays* protoplast cultures (each culture contained $2.5x10^4$ cells) as affected by Ca/CaM antagonists without supplemental calcium (a) and Ca/CaM antagonists combined with 10 mM calcium (b).

All protoplast cultures exhibited lowered contents of chlorophyll after growth for 48 h. Earlier, Nagata and Takebe (1970) recorded that the cultured protoplasts had lost much of their green color and became yellowish due to repeated cell divisions. Apart from nifedipine, Ca^{2+} variously abolished most of the effects of blockers on chlorophyll contents. It was found that Ca^{2+} inhibits some of the chlorophyllase activity (Abdel-Basset *et al.*, 1995) and thus Ca^{2+} supplemented cells preserved more chlorophylls.

Finally it can be concluded that calcium antagonists imposed significant alterations on newly synthesized wall components: both pectins and hemicellulose were decreased while cellulose was increased. Secondly, added Ca^{2+} not only participates in pectin polymerization but it also affected the level, and possibly the activity, of the cell wall associated proteins which contain enzymes capable of hydrolyzing cell wall polysaccharides. Since the applied antagonists vary in their modes of action, concentrations of Ca^{2+} to overcome their effects are also dependently vary.

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