Cell Research (1998), **8**, 41-50

# The effect of external $Ca^{2+}$ and $Ca^{2+}$-channel modulators on red-light-induced swelling of protoplasts of *Phaseolus radiatus* L.

LONG CHENG[1], XIAO JING WANG, RUI CHI PAN
*Department of Biology, South China Normal University, Guangzhou 510631, China*

## ABSTRACT

Red-light-induced swelling of the protoplasts isolated from hypocotyl of etiolated mung bean (*Phaseolus radiatus* L.) was observed only when $Ca^{2+}$ ions were present in the medium. The optimal $CaCl_2$ concentration was 250 $\mu M$. Swelling response declined when $Ca^{2+}$ was supplied into the medium after red light irradiation. The $Ca^{2+}$-chelator EGTA eliminated the red-light-induced swelling and $^{45}Ca^{2+}$ accumulation in the protoplasts. In contrast, $A_{23187}$, a $Ca^{2+}$-ionophore, could mimic the effect of red light in darkness. These results indicate that $Ca^{2+}$ may play a role in light signal transduction. In addition, swelling response was prevented by TFP and CPZ (both are CaM antagonists), implying the involvement of CaM in red-light-induced and $Ca^{2+}$ -dependent protoplast swelling.

**Key words:** *Calcium ions, red light, protoplast swelling, $^{45}Ca^{2+}$ accumulation, Phaseolus radiatus*

## INTRODUCTION

Most of the processes in plant growth and development depend on the presence of calcium ions. During the past few decades, it has been demonstrated that cal-

1. Present address and to whom correspondence should be sent: Department of Biology, East China Normal University, Shanghai 200062, China. Fax: 021-6257 6217, E-mail: biol1@ ecnu.edu.cn

**Abbreviations:** R, red light; D, darkness; CaM, calmodulin; CPZ, chlorpromazine; TFP, trifluoperizine; MES, 2-(N-morpholine) ethanesulphonic acid; EGTA,ethylene glycol-bis( $\beta$ -aminoethyl ether) N,N,N',N'-tetracetic acid

cium, as a 'secondary messenger', participates the transduction of environmental signals[1-5]. The hypothesis that a phytochrome-induced increase in intracellular $Ca^{2+}$ concentration is necessary for the promotion of some phytochrome responses was supported by two best characterized phytochrome responses: light chloroplast rotation in the alga, Mougetia[6] and spore germination in the fern, *Onoclea*[5]. In monoco plants such as Avena sativa, Triticum aestivum, Zea may and Vallisneria, etiolated leaf protoplasts swelled in response to red light[7-9]. Red light increased the rate of $^{45}Ca^{2+}$ uptake by corn leaf protoplasts, and the response was reversed by far-red light[7]. Recent evidence has shown that this physiological response was preceded by a transient increase in cytosolic free calcium[10] and *illumination* of the protoplast increased the open probability of ion channel[11]. Little evidence has been obtained so far in dico plants, except that chloroplasts and protoplasts from spinach leaves exhibited a light-induced $Ca^{2+}$ influx using the metallochromic indicator arsenazo III[12, 13]. In our previous reports[14, 15], we have shown that phytochrome induced swelling of protoplasts of mung bean hypocotyls. The study reported here was designed to elucidate the role of calcium ions in the red-light-induced swelling of protoplasts of *Phaseolus radiatus* L.

## MATERIALS AND METHODS

### *Plant material*

Seeds of mung bean (Phaseolus radiatus L.) were surface sterilized with 70% ethanol for 1 min and 0.1% $HgCl_2$ for 5 min, then washed with sterile water (3 changes). Sterilized seeds were grown in darkness at 25± 1℃ . Etiolated seedlings were harvested after 64 h.

### *Protoplast isolation*

Hypocotyl segments (5 mm in length) were excised from 3 mm below the hook, then cut transversely into 0.5 mm slices, and plasmolysed (1 h) in a solution of 13% (w/v) mannitol containing $KH_2PO_4$ 27.2, $KNO_3$ 101, $CaCl_2 \cdot 2H_2O$ 1480, $MgSO_4 \cdot 7H_2O$ 240, KI 0.16, $CuSO4 \cdot 5H_2O$ 0.025 mg/L, (pH 5.6). Plasmolysed tissue from 50 hypocotyls were incubated on a rotary shaker (50 ~ 60 r/min, 25 ± 1 ℃) for 5 ~ 6 h in an enzyme solution containing 2% (w/v) cellulase, 1% (w/v) hemicellulase, 0.5% pectinase, 5 m*M* MES, 9% (w/v) mannitol and the other salt components as used for plasmolysis (pH 5.2). The enzyme-protoplasts mixture was filtered through a nylon sieve (64 $\mu M$ pore size), and centrifuged (100 × g, 10 min). The pellet was suspended in a 21% (w/v) sucrose solution (other salt components were same as plasmolysis solution) and centrifuged (100 × g, 5min). Floating protoplasts were washed twice with a solution of 9% mannitol containing MES 5 m*M* , with or without $CaCl_2$ 250 $\mu M$, pH 5.6. The final pellet was suspended to the desired concentration ($10^5$ to 5 × $10^5$ cells per ml) in a suspension medium containing 9% (w/v) mannitol, MES 5 m*M*. Protoplast quantification was performed using haemocytemeter and all protoplast suspension used showed > 95% viability as assessed by fluorescein diacetate (FDA) staining[16]. All manipulations were performed under a dim green light (<0.2 $Wm^{-2}$).

In all experiments, performed in the present work, protoplasts were maintained in darkness or irradiated with 3 min red light (10.5 $Wm^{-2}$), then incubated for 30 min in darkness at 20±2℃ before volume measurement.

### *Light sources*

The red light source was obtained as described by Long et al[14] with some modification. Briefly, monochromatic light was selected from six 40-W red fluorescent lamps in a custom-built projector using a red interference filter ( $\lambda$ =660 nm, 25 nm half bandwidth) (Roscolene No. 823 Kliegle Brothers, USA). The fluence rate was 10.5 $Wm^{-2}$ and was measured with Kettering Radiant Power Meter.

### *Protoplast volume measurement*

The size of protoplasts was determined by photographing 100 $\mu$l protoplast suspension that was revolved at random by a disposable pipette from the treated population after an incubation period of 30 min in darkness at 20 ±2℃, and placed on a haemocytometer. At least 100 intact protoplasts were measured for each datum of the protoplast volume of each experiment.

### *$^{45}Ca^{2+}$ transport assay*

Accumulation of $^{45}Ca^{2+}$ in protoplasts was determined as described in the previous report[15]. 194 $\mu$l of protoplast suspension was incubated with 6 $\mu$l of $^{45}Ca^{2+}$ (74 MBq/ml), the reaction was stopped by adding 5 ml of cold washing solution containing 9% (w/v) mannitol, 5 m*M* MES and with or without 250 $\mu M$ $CaCl_2$. The protoplasts were collected immediately on glass fiber filters and washed with 10 ml of fresh cold washing solution and counted in a G-M counter.

### *Protein content*

The content of protein was determined by Folin-phenol assay.

### *Chemicals*

Hemicellulase H-2125, pectinase, EGTA, verapamil, $A_{23187}$, CPZ, TFP and Folin-B were purchased from Sigma Chemical Co., St. Louis, MO, USA. Cellulase Onzuka R-10 was purchased from Kinki Yakult Manuf. Co. Ltd. Japan. MES was purchased from Serva Feinbiochemica Heideberg, New York. $^{45}CaCl_2$ was purchased from Institute of Atomic Energy, Chinese Academy of Sciences, Beijing. All other chemicals were analytical grade.

The data presented represent mean ± S.E. of three independent experiments. Statistical analysis was based on a one-way analysis of variance and significant differences at the 5% level were calculated.

## RESULTS

### *Effect of $Ca^{2+}$ concentration on red-light-induced protoplast swelling and $^{45}Ca^{2+}$ accumulation in protoplasts*

We have demonstrated previously that red-light-induced protoplast swelling occurred only when $Ca^{2+}$ ions were present, other divalent ($Ba^{2+}$, $Mg^{2+}$ and $Zn^{2+}$) and monovalent ($K^+$) cations had no visible effects on protoplast swelling[14]. To investigate the effect of different external $CaCl_2$ concentration on swelling and $^{45}Ca^{2+}$ accumulation, protoplasts were suspended in the media containing 0 (plus EGTA), 100, 250, 500 and 1000 $\mu M$ of $CaCl_2$, separately, and were irradiated with red light. Protoplast volume were determined after 30 min of incubation in darkness. As shown in Tab 1, both the volume and the accumulation of $^{45}Ca^{2+}$ of the protoplasts increased when $CaCl_2$ was present in the medium. The most effective concentration was 250 $\mu M$. the protoplast volume was 30.83% larger than that incubated in calcium-free control (+EGTA), while $^{45}Ca^{2+}$ accumulation was 61.09% higher than

dark control in the same medium. Therefore this concentration was used in all subsequent experiments.

**Tab 1.** The effect of different $CaCl_2$ concentration on red-light-induced protoplast swelling and $^{45}Ca^{2+}$ accumulation in protoplasts. Standard errors are shown for all data.

| Medium | Light regime | $^{45}Ca^{2+}$ accumulation (cpm $\mu g^{-1}$ protein) | Volume ($\times 10^3$ $\mu M^3$) |
|---|---|---|---|
| $CaCl_2$ (1 *M*) | D | 165.78 ± 5.98 | 20.19 ± 0.47 |
| EGTA (1000 m*M*) | R | ND | 19.72 ± 0.59 |
| $CaCl_2$ (100 m*M*) | R | 241.39 ± 10.14 | 22.41 ± 0.62 |
| $CaCl_2$ (250 m*M*) | R | 271.37 ± 11.43 | 25.80 ± 0.68 |
| $CaCl_2$ (500 m*M*) | R | 263.34 ± 10.50 | 25.47 ± 0.73 |
| $CaCl_2$ (1000 m*M*) | R | 258.66 ± 9.57 | 24.95 ± 0.66 |

ND = not determined, D = dark, R = red light irradiation.

## *$Ca^{2+}$ -dependence of protoplast swelling induced by red light*

To investigate further the relationship between $Ca^{2+}$ effect and the light signal, $CaCl_2$ (250 *M*) was added into calcium-free medium before or after red light treatment. It was shown that when the calcium was added 1 min after red light irradiation, swelling response decreased about 60% comparing to that added before light treatment. No significant swelling response occurred when $CaCl_2$ was supplied 5, 10, 20 min after red light irradiation (Fig 1).

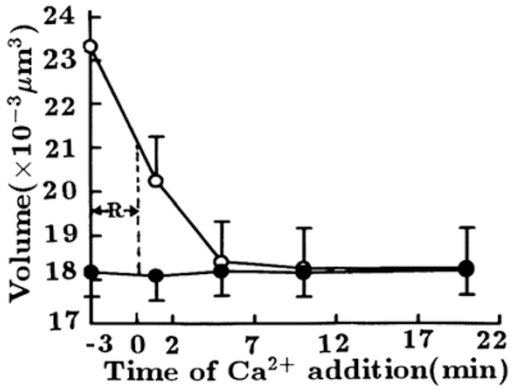


**Fig 1.** The effect of $Ca^{2+}$ addition before or after red light irradiation on red-light-induced protoplast swelling. D (•); R (o). The final concentration of $Ca^{2+}$ was 250 $\mu M$. Values and error bars were the mean and standard error of three replicate samples.

## *The specificity of $Ca^{2+}$ on red-light-induced protoplast swelling*

EGTA enter the cell membrane with difficulty, but it can chelate $Ca^{2+}$ in the medium and specifically reduce the efficiency of external $Ca^{2+}$[17]. When 0.25, 0.5 or 1 m*M* EGTA were added into $Ca^{2+}$-containing medium, the red-light-induced protoplast swelling was inhibited. The stimulatory effect of red light was totally nullified at the concentration of 0.5 m*M* EGTA (Fig 2). In our experiments, EGTA at concentrations higher than 1.5 mM led to protoplast rupture.

## *Effect of verapamil on swelling and $^{45}Ca^{2+}$ uptake of protoplasts*

Verapamil as a $Ca^{2+}$-channel blocker can prevent selectively the influx of $Ca^{2+}$ across the plasma membrane in some plant systems[8]. When 1, 5 and 10 $\mu M$ verapamil were added, respectively, into the $Ca^{2+}$-containing medium, $^{45}Ca^{2+}$ uptake was reduced and protoplast swelling was inhibited even though the protoplasts was irradiated with red light. At the concentration of 10 $\mu M$ verapamil, 84.26% $^{45}Ca^{2+}$ uptake and 81.57% swelling response was inhibited (Fig 3).

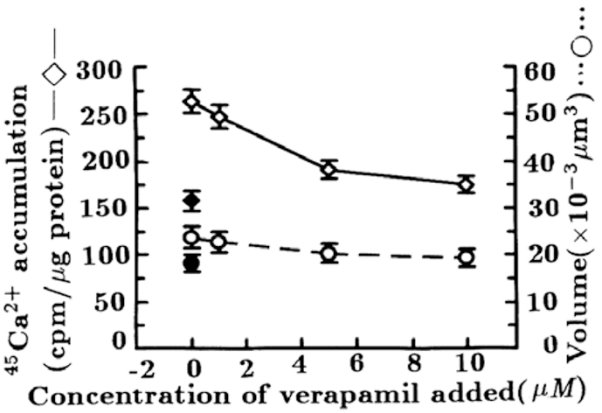


**Fig 2.** The effect of EGTA on red-light- induced protoplast swelling. D (●); R (o).

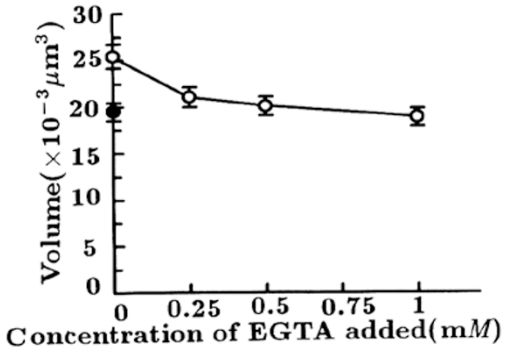


**Fig 3.** The effect of verapamil on red-light-induced protoplast swelling and $^{45}Ca^{2+}$ accumulation in protoplasts. D ( ●, ◆); R (○, ◇).

## *Effect of $LaCl_3$ on swelling and $^{45}Ca^{2+}$ uptake of protoplasts*

$La^{3+}$, an inorganic $Ca^{2+}$-antagonist, can not enter plant cell, but it inhibits the movement of $Ca^{2+}$ through the plasma membrane[18]. Treatment of the protoplasts with 10, 50 or 100 $\mu M$ $LaCl_3$ did indeed inhibit $^{45}Ca^{2+}$ uptake and swelling response of protoplasts irradiated with red light in the $Ca^{2+}$-containing medium. At the concentration of 100 $\mu M$ of $LaCl_3$, 89.35% of $^{45}Ca^{2+}$ uptake and protoplast swelling was totally inhibited (Fig 4).

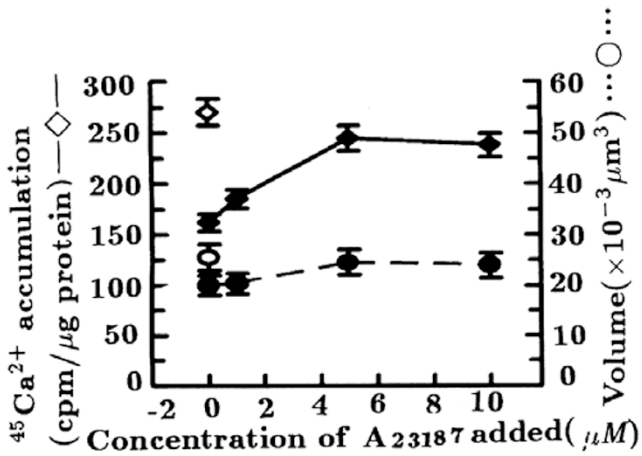


**Fig4.** The effect of $LaCl_3$ on red-light-induced protoplast swelling and $^{45}Ca^{2+}$ accumulation in protoplasts. D ( ●, ◆); R (○, ◇).

## *Effect of $A_{23187}$ on swelling and $^{45}Ca^{2+}$ uptake of protoplasts*

$A_{23187}$, a compound transporting $Ca^{2+}$ across membrane down the concentration gradient[19], was a kind of carboxylic antibiotic $Ca^{2+}$ ionophore. In darkness, when 1, 5 or 10 $\mu M$ of $A_{23187}$ were supplied into the $Ca^{2+}$-containing medium, $^{45}Ca^{2+}$ uptake was improved, and protoplasts swelled. The volume and $^{45}Ca^{2+}$ uptake in protoplasts were 82.91% and 90.62% ,respectively, as those of red light treatment when 5 $\mu M$ of $A_{23187}$ was added to medium (Fig 5).

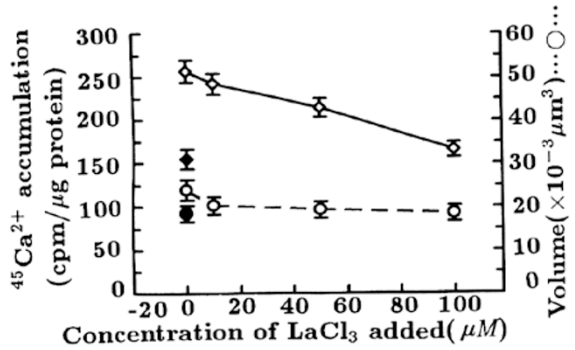


**Fig 5**. Effect of $A_{23187}$ on swelling and $^{45}Ca^{2+}$ uptake of protoplasts. D ( ●, ◆); R (○, ◇).

*The involvement of CaM in protoplast swelling*

It has been shown that $Ca^{2+}$ may act as secondary messenger, and combine with CaM in cytoplasm. The $Ca^{2+}$-activated CaM then activates enzymes which regulates the physiological processes of plants[3]. To identify whether or not CaM involves in physiological modulation, CaM antagonists are generally used to test their inhibitory action. The previous results showed that treatment of protoplasts with 1, 5 or 10 $\mu M$ of trifluoperizine (TFP) or chlorpromazine (CPZ), antagonists of the $Ca^{2+}$-CaM complex, did indeed abolish the swelling of protoplasts irradiated with red light, and it was strongly blocked at the dose of 5 $\mu M$ (Tab 2). Protoplasts rupture was observed at the concentration higher than 20 $\mu M$.

**Tab 2.** The effect of TFP and CPZ on red-light-induced protoplast swelling. The concentration of $CaCl_2$ was 250 $\mu M$

| Medium | Light regime | Volume($\times 10^3 \mu m^3$) | △Volume($\times 10^3 \mu m^3$) |
|---|---|---|---|
| $CaCl_2$ | D | 18.63±0.55 | 0 |
| $CaCl_2$ | R | 24.03±0.63 | 5.4 |
| $CaCl_2$ + TFP (1 $\mu M$) | R | 19.54±0.38 | 0.95 |
| $CaCl_2$ + TFP (5 $\mu M$) | R | 19.55±0.57 | 0.92 |
| $CaCl_2$ + TFP (10 $\mu M$) | R | 18.54±0.43 | -0.09 |
| $CaCl_2$ + CPZ (1 $\mu M$) | R | 19.25±0.36 | 0.62 |
| $CaCl_2$ + CPZ (5 $\mu M$) | R | 18.65±0.39 | 0.02 |
| $CaCl_2$ + CPZ (10 $\mu M$) | R | 18.12±0.41 | -0.51 |

## DISCUSSION

It has been demonstrated in plants that the environmental signals outside-cell are transduced to relative processes inside-cell via the change of $Ca^{2+}$ concentration in cytosol. However, it is very significant but rather difficult to measure static or activated $Ca^{2+}$ concentration in cytosol. So the participation of this well defined cellular signalling molecule in light signal transduction has been tested inside plant cells by using agonists or antagonists to examine their effects on light-mediated responses[20]. $Ca^{2+}$ influx into or efflux from plasma membrane or organ has been reviewed[3]. Hepler and Wayne[5] showed that the changes of $Ca^{2+}$ transport system meant the change of the concentration of intracellular $Ca^{2+}$ when the spores of *Onoclea* were stimulated by red light. Recently, by means of laser scanning confocal microscopy, Fluo-3 was used to examine red-light-induced cytosolic free $Ca^{2+}$ changes[10]. In our experiments, red-light-induced $Ca^{2+}$ influx into protoplasts has been measured using $^{45}Ca^{2+}$. When $CaCl_2$ was at the concentration of 250-1000 $\mu M$, swelling response and the $^{45}Ca^{2+}$ uptake were positively correlated to concentration within certain limit, and the optimum concentration of external $CaCl_2$ on red-light-induced swelling response of protoplasts was found to be 250 $\mu M$ (Tab 1). The results demonstrated that $Ca^{2+}$ was absolutely required for red-light-induced swelling

of protoplasts. Moreover, full response was only observed when $Ca^{2+}$ was added to the medium before red light treatment (Fig 1). Reduced response occurred when $Ca^{2+}$ was added 1 min after light treatment and the swelling was totally prevented when $Ca^{2+}$ was added to the calcium-free medium 5 min after red-light-irradiation, implying a pre-request of $Ca^{2+}$ in red-light-induced swelling and this request is time dependent. Shacklock et al.[10] reported that a transient rise in $[Ca^{2+}]_i$ lasting no more than 1 min was sufficient to elicit the prolonged physiological response of a change in cell volume.

If $Ca^{2+}$ was chelated by EGTA, no protoplast swelling after red light-irradiation occurred (Fig 2). When the agents such as verapamil and $LaCl_3$ were used to block the $Ca^{2+}$-channel, the red-light-induced $^{45}Ca^{2+}$ uptake and protoplast swelling were inhibited significantly. There was a good dose-relationship between percent of inhibition and concentrations of channel blockers (Fig 3 and 4). In darkness, $A_{23187}$ could partially mimic red-light-induced swelling and promoting $^{45}Ca^{2+}$ influx in the presence of $Ca^{2+}$ (Fig 5). All these facts indicates that protoplast swelling is a consequence of a rise in cytoplasmic $Ca^{2+}$ concentration, and its inhibition by various inhibitors imply opening of $Ca^{2+}$ channel stimulated by red light. Therefore our results support the hypothesis that red light induces an entrance of $Ca^{2+}$ into protoplasts. Takagi and Nagai[8] found that $La^{3+}$ inhibit $Ca^{2+}$ influx through $Ca^{2+}$ channel in the cell membrane of protoplasts from Vallisneria mesophyll cell. Akerman et al[21] observed that $A_{23187}$ enhanced net $Ca^{2+}$ accumulation by protoplasts. Mehta et a1[22] suggested a role of ion channels and pumps in phytochrome-controlled $Ca^{2+}$ fluxes and that modulation of $[Ca^{2+}]_i$ is likely to be achieved through changes in the activity of $Ca^{2+}$ channels. These data suggested that dico and monoco plants share a common biochemical mechanism for at least part of protoplast swelling. Broad similarities between swelling of protoplasts induced by red light in mung bean and more well-studied monoco plants were apparent. However, there also existed considerable differences in detail, such as initial time, level and maintaining period of $Ca^{2+}$ influx and protoplast swelling.

The participation of well-characterized cellular signalling molecules in light signal transduction has been tested as inside plant cells by using agonists or antagonists and by examining their effects on light-mediated responses[20]. $Ca^{2+}$ influx or efflux in plasma membranes or organs were studied[3]. The ability of TFP and CPZ to inhibit red-light-induced protoplast swelling (Tab 2) indicates that CaM might be involved in the transduction of light signal. When CaM is activated by $Ca^{2+}$, it may then activate other target enzymes and physiological processes which result in the swelling of protoplasts. These drugs at higher concentrations may lead to disruption of protoplasts. In this case, their action is due to a non-specific disruption of membrane function rather than a specific CaM antagonism. These actions were highly similar to those found in monocotyledons. Similar phenomena have been noted by others[23].

In summary, this study provides a strong demonstration of calcium acting as a

signaling molecule for phytochrome action in dico plants.

*ACKNOWLEDGEMENT*

The project was supported by grant from National Science Foundation of China (No. 39170038).

## REFERENCES

[1] Bush DS. Calcium regulation in plant cells and its role in signaling. Annu Rev Plant Physiol Plant Mol Biol 1995; **46:**95-122.
[2] Gilroy S, Trawavas T. A decade of plant signals. Bio Essays 1994; **16:**677-82.
[3] Sun DY, Gao YL. Intracelluar signal $Ca^{2+}$. In Signal system in cell. Ed: Liang SW, Science Press: Beijing **1993:**140-203
[4] Hao LL, Yu SW. *Messenger* and function in plant cell. In: Plant physiology molecular biology. Ed: Yu SW, Science Press: Beijing **1992:**123-40.
[5] Hepler PK, Wayne RO. Calcium and plant development. Annu Rev Plant Physiol 1985; **36:**397-439.
[6] Serlin BS, Roux SJ. Modulation of chloroplast movement in the green alga Mougeotia by the $Ca^{2+}$ ionphore $A_{23187}$ and by calmodulin antagonists. Proc Nat Acad Sci USA 1984; **81:**6368-72
[7] Das R, Sopory SK. Evidence of regulation of calcium uptake by phytochrome in maize protoplasts. Biochem Biophy Res Comm 1985; **128:**1455-60.
[8] Takagi S, Yamamoto KT, Furuya M, Nagai R. Cooperative regulation of cytoplasmic streaming and $Ca^{2+}$ fluxes by Pfr and photosynthesis in *Vallisneria mesophyll* cells. Plant Physiol 1990; **94:**1702-8.
[9] Tretyn A, Kendrick RE, Bossen ME. The effect of a calcium-channel antagonist, nifedipine and agonist, Bay K-8644, on the phytochrome-controlled swelling of etiolated wheat protoplasts. Physiol Plant 1990; **78:**230-5.
[10] Shacklock PS, Read ND, Trewavas AJ. Cytosolic free calcium mediates red-light-induced photomorphorgensis. Nature 1992; **358:**753-5.
[11] Deng XW. Fresh view of light signal transduction plants. Cell 1994;**76:**423-6.
[12] Heimann K, Kreimer G, Melkonian M, Latzko E. Light-induced $Ca^{2+}$ influx into spinach protoplasts. Zeitschrift Naturforschung 1987; **42C:**283-7.
[13] Kreimer G, Melkonian M, Latzko E. An electrogenic uniport mediates light-dependent $Ca^{2+}$ influx into intact spinach choroplast. FEBS Lett 1985; **180:**253-8.
[14] Long C, Wang XJ, Pan RC. Regulation of phytochrome on swelling of protoplasts isolated from hypocotyl of etiolated mung bean seedlings. Acta Bot Sinica 1994; **36:**765-72.
[15] Long C, Wang XJ, Pan RC. The role of calcium ions red-light-induced swelling of protoplasts of mung bean. Chinese Sci Bull 1995; **40:**248-51.
[16] Larkin PJ. Purification and viability determinations of plant protoplasts. Planta 1976; **128:**213-6.
[17] Gilroy S, Hughes WA, Trewavas AJ. The measurement of intracellular calcium levels in protoplasts from higher plant cell. FEBS Lett 1986; **199:**217-21.
[18] Thomson WW, Platt KA, Campbell N. The use of lanthanum to delineate the apoplectic continuum in plants. Cytobios 1973; **8:**57-62.
[19] Reed PW, Lardy HA. $A_{23187}$: a divalent cation ionphore. J Bio Chem 1972; **247:**6970-7.
[20] Elzenga JT, Van Volkenburgh E. Characterization of a light-controlled anion channel in the plasma membrane of messophyll cells of pea. Plant Physiol 1997; **113:**1419-26.
[21] Akerman KEO, Proudlove MO, Moore AL. Evidences for a $Ca^{2+}$ gradient across the plasma membrane of wheat protoplast. Biochem and Biophy Res Comm 1983; **113:**171-7.

[22] Mehta M, Malik MK, Khurana JP, Maheshwari SC. Phytochrome modulation of calcium fluxes in wheat (Triticum aestivum L.) protoplasts. Plant Growth Reg 1993; 12:293-302.
[23] Gilroy S, Hughes WA, Trewavas AJ. Calmodulin antagonists increase free cytosolic calcium levels in plant protoplasts *in vivo*. FEBS Lett 1987; 212:133-7.

*Received Aug-27-1997. Revised Jan-22-1998. Accepted Feb-18-1998.*