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Inositol(1,4,5)trisphosphate production in plant cells: an early response to salinity and hyperosmotic stress

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Abstract Salinity and hyperosmotic stress are environmental factors that severely affect the growth and development of plants. Adaptation to these stresses is known to be a complex multistep process, but a rise in cytoplasmic Ca^{2+} and increased polyphosphoinositide turnover have now been identified as being amongst the early events leading to the development of tolerance. To determine whether a causal link exists between these two events we have investigated the effects of several salts and osmotic agents on levels of inositol(1,4,5)trisphosphate (Ins(1,4,5)P_3) in plant cells. Our data show that salts as well as osmotic agents induce a rapid and up to 15-fold increase in cellular Ins(1,4,5)P_3 levels. The increase in Ins(1,4,5)P_3 occurs in a dose-dependent manner and levels remain elevated for at least 10 min. These data indicate that increased Ins(1,4,5)P_3 production is a common response to salt and hyperosmotic stresses in plants and that it may play an important role in the processes leading to stress tolerance.

Key words: Inositol(1,4,5)trisphosphate; Calcium; Phospholipase C; Salt stress; Osmotic stress; Higher plant

1. Introduction

Salt and drought stresses are widespread challenges met by plants in natural ecosystems as well as in agriculture [1,2]. Development of tolerance to such stress situations by higher plants involves a complex set of cellular interactions which ultimately result in altered expression patterns of numerous genes involved in the adaptive responses. Relatively little is known about the early events that trigger the induction of adaptive processes but rapid changes in cytosolic free Ca^{2+} concentrations have been shown to be one of the early responses to both salinity and drought stress in *Arabidopsis thaliana* [3]. In addition it has recently been demonstrated that salt and hyperosmotic stress result in increased turnover of polyphosphoinositides in *Chlamydomonas* and a number of higher plants [4,5]. These findings suggest that a link might exist between the activation of the plant phosphoinositide system [6] and the elevation of cytosolic Ca^{2+}. Although it has been known for some time that induction of genes encoding enzymes of the plant phosphoinositide cycle can be triggered by hyperosmotic stress [7,8] little detailed information is currently available about the immediate effects of salt stress and hyperosmotic stress on phosphoinositide-metabolising enzymes and inositol(1,4,5)trisphosphate (Ins(1,4,5)P_3) generation. We now describe the effect of salt and osmotic stress on Ins(1,4,5)P_3 production in suspension-cultured carrot (*Daucus carota* L.) cells. Our data show that stresses induced by both salt and hyperosmotic shock lead to a rapid and sustained elevation of cellular levels of Ins(1,4,5)P_3 and that the increases in Ins(1,4,5)P_3 occur in a dose-dependent manner. A direct effect of salt upon phospholipase C activity in vitro was not observed which supports the idea that a more complex set of mechanisms is involved in the sensing of salt and osmotic stresses. These findings also suggest that in plants the activation of the phosphoinositide system, Ins(1,4,5)P_3 production, and changes in cytosolic Ca^{2+} may act in concert to transduce the signals caused by changes in the extracellular ionic or osmotic environment.

2. Materials and methods

2.1. Cell culture and the induction of salt and osmotic stress

Carrot (*Daucus carota* L) var. 'Oxford' suspension cultures were maintained as described previously [9]. Cell suspensions were used for experiments 4–5 days after subculturing. Cells were pelleted by gentle centrifugation and adjusted to a concentration of 300–500 mg/ml of culture medium and incubated at room temperature with gentle agitation. After temperature equilibration for 5 min, experiments were initiated by the sampling of three 100 μl control samples at 0.5 min intervals. Immediately after the sampling of the third control sample the concentration of either salt or osmoticum was increased in the incubation medium by addition of a smaller volume of temperature-equilibrated growth medium containing increased salt/osmoticum. The final concentration of salts or osmoticum was 370 mM unless otherwise stated. The salt/osmoticum-containing medium was added slowly (5–10 s) and with appropriate mixing to avoid localised extremes. Further aliquots of 100 μl were removed after incubations for the appropriate time and analysed for Ins(1,4,5)P_3 content as described below. The slight dilution caused by the addition of growth medium has been accounted for in the calculation of data.

2.2. Extraction and assay of Ins(1,4,5)P_3

All samples were immediately quenched with 100 μl ice-cold $HClO_4$ (10% w/v). After 15 min on ice, protein and cellular debris was sedimented by centrifugation (15 000 × *g*) for 7 min and 100 μl of the supernatant removed and neutralised by 1.5 M KOH/60 mM HEPES. After the removal of precipitated $KClO_4$ by centrifugation the Ins(1,4,5)P_3 concentration was determined in the supernatant by the receptor binding assay described by Palmer and Wakelam [10]. Commercially available preparations of Ins(1,4,5)P_3 binding protein from bovine adrenal glands (Amersham, UK, TRK 1000) were used for Ins(1,4,5)P_3 determinations and standard curves were constructed using [2-^{3}H]Ins(1,4,5)P_3 (Amersham, UK) as tracer. We have found that high salt concentrations (notably $MgCl_2$) carried through the Ins(1,4,5)P_3 extraction procedure in some cases can affect the properties of the binding protein. Therefore we carried out a number of control experiments to investigate the effect of adding cell-free growth

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Abbreviations: Ins(1,4,5)P_3, inositol(1,4,5)trisphosphate; PtdIns-(4,5)P_2, phosphatidylinositol(4,5)bisphosphate; PI-PLC, phosphoinositide-specific phospholipase C

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medium containing either salt solutions or mannitol/sucrose solutions to the assays. None of the salts or other osmotic agents used in this study were found to significantly affect the binding characteristics of the adrenal protein at the concentrations employed.

2.3. Isolation of plasma membranes

Preparation of plasma membrane vesicles from bean leaves was carried out exactly as described by Drøbak et al. [11].

2.4. Assay of plasma membrane-associated phosphatidylinositol(4,5)bisphosphate (PtdIns(4,5)P_2)-specific phospholipase C activity

PtdIns(4,5)P_2 phospholipase C activity was assayed essentially as described by Drøbak et al. [11]. In brief, after temperature equilibration, plasma membranes were incubated (25°C) in assay buffer: 50 mM Tris–malate (pH 6.0), 10 μM $CaCl_2$ in the presence of a sonicated solution of PtdIns(4,5)P_2 spiked with 0.86 kBq [^{3}H]PtdIns(4,5)P_2 (specific activity 325.6 GBq/mmol). After appropriate incubation times subsamples were collected and the reaction stopped by 1 ml chloroform/methanol (2:1, v/v) and tubes were placed on ice for 5 min. 0.25 ml 0.6 N HCl was then added to facilitate separation of phases. Tubes were vortexed vigorously and centrifuged at 15 000×*g* for 2 min. 400 μl of the top phase was removed from each tube and radioactivity in the water-soluble components was determined by liquid scintillation spectrometry (Wallac 1410, Turku, Finland) after addition of scintillation fluid (Hionic-Fluor, Hewlett-Packard, Beds., UK).

2.5. Light microscopy of NaCl-stressed carrot cells

All microscopical examinations were carried out using a Zeiss Axiovert 25 microscope and a ×40 objective. Four-day-old cells were pelleted gently in a Denley swing-out centrifuge and some of the growth medium removed. Cells were reconstituted in growth medium adjusted to final concentrations of 0, 200, 400 and 1000 mM NaCl. Cells were incubated for 20 min with agitation at room temperature. Evans blue (0.0125 % w/v, final concentration) was added and the cell suspensions were examined after a further 5 min. Photographs were taken using Kodak EDT/160T colour reversal film. Montages were prepared using PhotoShop 5.0 software.

2.6. Miscellaneous methods

Protein concentrations were determined using the Bio-Rad (Bio-Rad, UK) dye binding assay using bovine serum albumin as standard.

3. Results and discussion

3.1. Salt and osmotic shock induce rapid Ins(1,4,5)P_3 increases in carrot cells

The effect of increased NaCl concentration upon Ins(1,4,5)P_3 production in carrot cells is shown in Fig. 1A. Prior to the elevation of the salt level in the culture medium the cellular levels of Ins(1,4,5)P_3 were approximately 0.20 pmol/mg fresh weight, a figure in good agreement with data from earlier investigations [9]. However, the increase of NaCl to a final concentration of 370 mM leads to a rapid and substantial increase in cellular Ins(1,4,5)P_3 with the highest levels occurring approximately 5–10 min after salt addition. That the magnitude of the salt-induced Ins(1,4,5)P_3 increase is dose-dependent is illustrated by the data in Fig. 1B. An EC_{50} of approximately 150 mM can be derived. To investigate whether the observed Ins(1,4,5)P_3 increases were specifically induced by NaCl stress we tested the effect of an equimolar concentration of mannitol. As the results in Fig. 2A show the addition of mannitol to the growth medium (final concentration 370 mM) also led to an increase in cellular Ins(1,4,5)P_3 levels although only to levels around half those induced by NaCl. Although the concentrations of mannitol and NaCl were equimolar, mannitol only has half the osmotic strength. This suggests that the osmotic effect overall is a more important determinant for Ins(1,4,5)P_3 generation than the absolute solute concentration. The timing of mannitol-induced Ins(1,4,5)P_3 elevation was similar to that observed for NaCl-induced increases with maximum levels attained 3–10 min after induction of stress. A mannitol EC_{50} of approximately 150 mM was also derived although increases in the mannitol concentration to around 100 mM only had a negligible effect on Ins(1,4,5)P_3 levels (Fig. 2B).

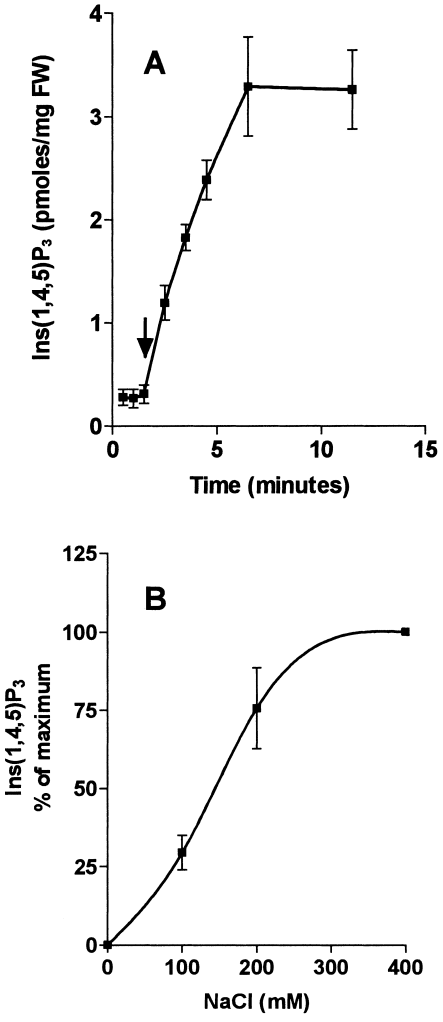

Fig. 1. A: Effect of 340 mM NaCl on the production of Ins(1,4,5)P_3 in carrot suspension cells. The increase of NaCl in the growth medium was effectuated immediately after sampling of the third control sample and is indicated by the vertical arrow. Data are the mean of four independent experiments and the vertical bars indicate S.D. B: Dose–response curve for NaCl-induced Ins(1,4,5)P_3 production. Ins(1,4,5)P_3 levels measured 3 min after induction of salt stress (400 mM NaCl) have been set at 100%. Data represent the mean from two independent experiments and the vertical bars indicate range.

Fig. 3 shows a comparison of Ins(1,4,5)P_3 levels induced by various monovalent salts and the two osmotic agents, mannitol and sucrose (final concentration 370 mM). LiCl was found to be as effective as NaCl in inducing Ins(1,4,5)P_3 elevations. This is a little surprising as Li^+ is known to inhibit plant inositol monophosphatase [12] and affect salt-induced Ca^{2+} transients in *Arabidopsis* [3]. However, we find it unlikely that the short-term exposure to LiCl in our experiments could dramatically affect cellular levels of inositol and subsequently

the size of the phosphoinositide pool. It should also be borne in mind that the growth medium used in our experiments is supplemented with D-*myo*-inositol. The effect of KCl on Ins-(1,4,5)P_3 production was consistently observed to be about half of that induced by NaCl and LiCl although both the concentration and osmotic strength were identical. We have currently no conclusive explanation for this phenomenon but speculate that the elevated levels of K^+ may somehow interfere with the mechanisms leading to Ins(1,4,5)P_3 production. It is interesting to note that an outward-rectifying K^+ channel (SKOR), which is thought to play an important role in plant adaptation to drought, is inwardly permeable to Ca^{2+} [13]. If a significant synergy exists between trans-plasma membrane Ca^{2+} fluxes and Ins(1,4,5)P_3 production perturbations of transmembrane K^+/Ca^{2+} gradients may help to explain the unexpected results observed during the KCl-induced salt stress. Mannitol and sucrose had equal effects on Ins(1,4,5)P_3 production as would be expected of two osmotica present at isoosmotic strength.

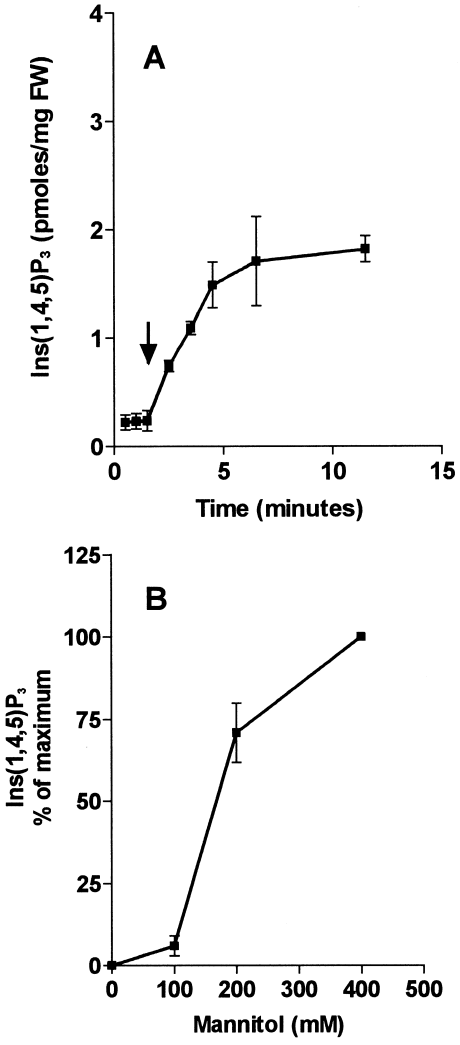

Fig. 2. A: Effect of 340 mM mannitol on the production of Ins-(1,4,5)P_3 in carrot suspension cells. The increase of mannitol in the growth medium was effectuated immediately after sampling of the third control sample and is indicated by the vertical arrow. Data are the mean of four independent experiments and the vertical bars indicate S.D. B: Dose–response curve for mannitol-induced Ins-(1,4,5)P_3 production. Ins(1,4,5)P_3 levels measured 3 min after induction of osmotic stress (400 mM mannitol) have been set at 100%. Data represent the mean from two independent experiments and the vertical bars indicate range.

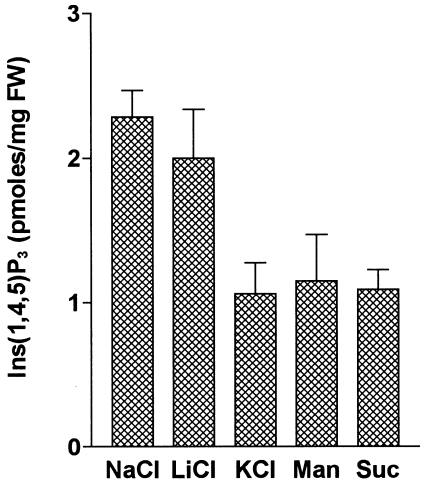

Fig. 3. Effect of various salts and osmotica (370 mM final concentration) on Ins(1,4,5)P_3 production in suspension-cultured carrot cells. Man: mannitol; Suc; sucrose. Data represent the mean of three independent determinations and vertical bars indicate S.D.

To investigate whether the carrot cells were being irreversibly damaged by the stress treatment we carried out a number of experiments where cell cultures were subjected to salt/osmotic stress (370 mM NaCl or 370 mM mannitol) for 20 min. Following a brief centrifugation step and resuspension in fresh growth medium with normal levels of salts and osmotica the cells were allowed to resume normal growth. Cell cultures were inspected after 24 and 48 h growth under standard conditions and in no case did we observe any significant effects on viability or growth rate of the stress exposure (data not shown). These conclusions are supported by the light microscopy data shown in Fig. 4. The image in panel A shows cells in normal growth medium and panel B shows cells subjected to 200 mM NaCl for 25 min. No discernible differences in morphology are evident. The cells shown in Fig. 4C had been subjected to 400 mM NaCl and early stages of plasmolysis are evident in a number of cells in which the protoplasts can be seen to be noticeably reduced in size. The cells shown in Fig. 4D (treated with 1000 mM NaCl) have nearly all undergone plasmolysis. It is worth noting that even at the highest salt concentration (panel D) the cells still appear to be able to exclude the dye, Evans blue, indicating that a high degree of cell integrity in maintained. As the levels of mannitol and sucrose used in these studies are known not to damage plant cells (see e.g. [9]) we did not carry out microscopic examinations of cells exposed to these osmotic agents. Knight et al. [3] have shown that mannitol concentrations of up to 660 mM do not adversely affect normal cell function.

3.2. *Effect of NaCl on the in vitro activity of phospholipase C in isolated plasma membranes*

The simplest explanation of the observed data would be that NaCl and other osmotically active agents have a direct effect on the enzyme responsible for the synthesis of Ins-(1,4,5)P_3, i.e. PtdIns(4,5)P_2-PLC (PI-PLC). In previous experiments we have not observed any significant effect of elevation of osmotica on the in vitro activity of plant PI-PLC

and the results illustrated in Fig. 5 show that 200 mM NaCl has a negligible effect on plant PI-PLC activity in vitro whereas 400 mM NaCl leads to an approximate 30–50% reduction in enzyme activity. These data indicate that the PI-PLC activation and subsequent Ins(1,4,5)P_3 production triggered by salt and osmotic challenges are unlikely to result from simple ionic and osmotic effects on PI-PLC activity. It is far more likely that one or more, yet unidentified, salt/osmotic sensors act as primary perceivers of the stress signals and mediate the downstream PI-PLC activation.

3.3. A role for Ins(1,4,5)P_3 and Ca^{2+} in the adaptive response to salt and hyperosmotic stress

The precise links between Ins(1,4,5)P_3 production, Ca^{2+} transients and initiation of downstream adaptive responses remain largely obscure but the recent identification of a genetic locus in *Arabidopsis* necessary for salt tolerance may help towards further insight in this area. A mutation in the salt tolerance locus, *sos3*, results in hypersensitivity to both NaCl and LiCl but increased Ca^{2+} levels can abrogate the hypersensitivity response and suppress the mutant phenotype [14]. The *sos3* gene has been found to encode a protein related to the yeast calcineurin B subunit and a neuronal Ca^{2+} sensor [15]. In yeast cells, Ca^{2+}-activated calcineurin interacts with the transcription factor TCN1/CRZ1 and triggers the transcription of the *ENA1/PMR2A* gene which encodes a plasma membrane Na^+-ATPase responsible for the extrusion of Na^+. That a similar set of events may also occur in higher plants now seems plausible due to the recent discovery that activated yeast calcineurin also facilitates salt stress adaptation in tobacco [16]. It should also be mentioned that several Ca^{2+}-dependent protein kinases are likely to be involved in the elicitation of adaptive processes through their activation of a stress-responsive promoter [17]. It is still not clear whether

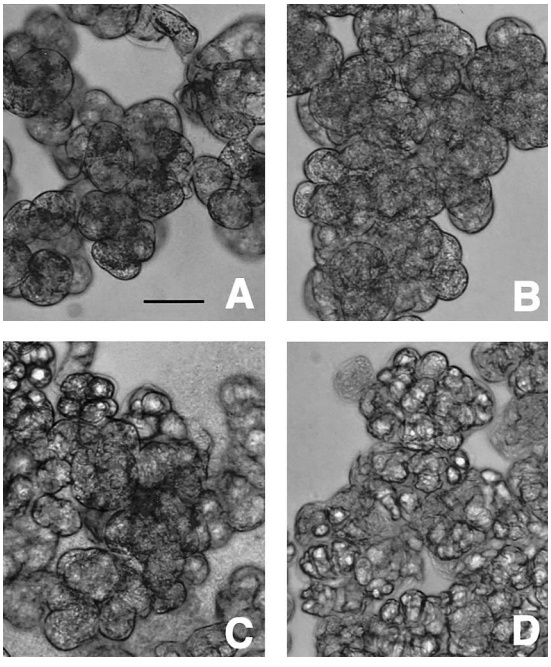

Fig. 4. Light micrographs of suspension-cultured carrot cells subjected to various concentrations of NaCl for 25 min. A: No added NaCl. B: 200 mM NaCl. C: 400 mM NaCl. D: 1000 mM NaCl. The vital dye, Evans blue, was in all cases included in the incubation medium. The horizontal bar in A has a length of 40 μm.

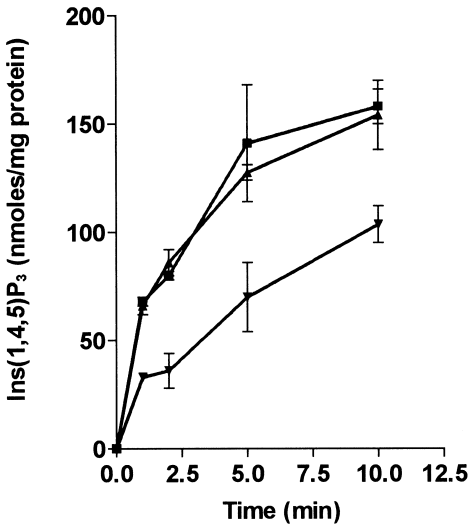

Fig. 5. Effect of NaCl on the activity of plasma membrane-associated PI-PLC activity. Data are the mean from three independent experiments and the vertical bars indicate S.D. ■: control; ▲: 200 mM NaCl; ▼: 400 mM NaCl.

stress-induced cytoplasmic Ca^{2+} increases are due to influx of extracellular Ca^{2+} or mobilisation of Ca^{2+} from intracellular stores. However, the results of Knight et al. [3], using a number of approaches and pharmacological tools, suggest that both processes are likely to be involved.

A point to notice is the timing of the Ins(1,4,5)P_3 increases. Although an increase is evident already shortly after stress induction the cellular Ins(1,4,5)P_3 levels were consistently observed to peak approximately 3–10 min later. In their study of salt and osmotic effects on Ca^{2+} increases in *Arabidopsis*, Knight et al. [3] showed that the peak levels of cytosolic Ca^{2+} occurred as early as 5–10 s after the induction of stress. This suggests that the rise in cytoplasmic Ca^{2+} precedes the onset of Ins(1,4,5)P_3 production rather than being a consequence thereof. However, the data obtained by Knight et al. [3] also showed that although the peak Ca^{2+} levels were reached very quickly the cytoplasmic Ca^{2+} levels remained elevated for periods exceeding 60 s. Lynch et al. [18] equally showed that the elevation of cytoplasmic Ca^{2+} in NaCl-stressed maize protoplasts can persist for up to 10 min. As the presently known PtdIns(4,5)P_2-specific phospholipase C enzymes in plants have all been found to be strictly Ca^{2+}-dependent enzymes (thus resembling members of the mammalian PLC-δ family) a scenario can be envisaged where an early Ca^{2+} spike (trigger Ca^{2+}) can lead to the activation of PI-PLC and Ins(1,4,5)P_3 synthesis. Ins(1,4,5)P_3 produced in this manner has the potential to release Ca^{2+} from additional/other spatially distinct Ca^{2+} pools – a scenario similar to that described by Franklin-Tong et al. [19].

It is known that plants have the ability to discriminate between osmotic and salt stresses [20] but the underlying mechanisms have yet to be identified. It is now generally recognised that the creation of distinct spatio-temporal Ca^{2+} profiles within cellular microdomains is utilised by mammalian cells to distinguish between both the nature and intensity of a wide range of cellular stimuli [21] and, as has recently been pointed out by several researchers, a similar situation is likely to exist in plant cells [22,23]. It is thus an attractive hypothesis that the ability to distinguish between salt and

hyperosmotic stress somehow is encoded by the Ins(1,4,5)P_3 production shown in the present study and the Ca^{2+} transduction pathways previously identified [3,18]. However, considerable more work is obviously needed to test this idea.

3.4. Conclusion

In summary, our data support the idea that the first stages of the perception of salt and osmotic stress signals in plants involve a yet unidentified salt/osmotic stress sensor(s) and that this perception process is followed by the transduction of the primary signals by Ca^{2+}- and Ins(1,4,5)P_3-mediated pathways. Our findings also agree with previous data showing that inhibitors of components of the phosphoinositide signalling pathway severely affect stress-induced Ca^{2+} transients [3].

As several of the key components of the early plant response to salt and osmotic stress have now been identified, the time seems ripe to start more detailed studies of how their individual functions and interactions are orchestrated and how they trigger the onset of stress-adaptive processes.

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