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REVIEW ARTICLE

Inositol trisphosphate receptor in higher plants: is it real?

Ondřej Krinke[1,3], Zuzana Novotná[1], Olga Valentová[1] and Jan Martinec[2,*]

[1] *Department of Biochemistry and Microbiology, Institute of Chemical Technology Prague, Technická 3, 166 28 Prague 6, Czech Republic*

[2] *Institute of Experimental Botany, The Academy of Sciences of the Czech Republic, Rozvojová 135, 165 02 Prague 6, Czech Republic*

[3] *Université Pierre et Marie Curie-Paris 6 and Centre National de la Recherche Scientifique, Formation de Recherche en Evolution 2846, Laboratoire de Physiologie Cellulaire et Moléculaire des Plantes, Ivry-sur-Seine, F-94200 France*

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Abstract

The receptor for D-*myo*-inositol 1,4,5-trisphosphate ($InsP_3$-R) has been well documented in animal cells. It constitutes an important component of the intracellular calcium signalling system. Today the corresponding genes in many species have been sequenced and the antibodies against some of the $InsP_3$-Rs are available. In contrast, very little is known about its plant counterpart. Only a few published works have dealt directly with this topic. This review summarizes the available relevant data and determines some properties of putative plant receptor(s) including the *in silico* search for its gene in plant genomes, *in vivo* evidence, its electrophysiology, the parameters of $InsP_3$-induced calcium release and $InsP_3$ binding, immunological cross-reactivity, and subcellular localization. Future progress in this area seems to be inevitable as, despite the efforts, its gene in plants has not been identified yet.

Key words: Ca^{2+} signalling, higher plants, inositol trisphosphate receptor, ligand-gated Ca^{2+} channels.

Introduction

The activation of phospholipase C leads to the hydrolysis of phosphatidylinositol 4,5-bisphosphate (Fig. 1) and the production of D-*myo*-inositol 1,4,5-trisphosphate ($InsP_3$). $InsP_3$ as a second messenger in the cytoplasm acts on its specific receptor/Ca^{2+}-permeable ion channel on endomembranes. Opening of the Ca^{2+}-permeable channel temporarily increases the cytosolic concentration of Ca^{2+} ($[Ca^{2+}]_{cyt}$) near the channel mouth. The specific spatiotemporal pattern of the calcium signal is then the key to the physiological response of the cell. In the case of the inositol trisphosphate receptor ($InsP_3$-R), this elevation is more like a trigger for opening other ion channels than the principal response to $InsP_3$. The $InsP_3$-induced Ca^{2+} release (IICR) is well documented in animal systems; it has also been reported in fungi (Cornelius *et al*., 1989; Belde *et al*., 1993) and in a number of plant species during the last two decades.

The properties of $InsP_3$-R in plants are reviewed and discussed with respect to its animal counterpart. This further supports the idea that the phosphoinositide signalling system in plants and animals is quite similar. No matter how much evidence for the phosphoinositide signalling pathway in plants has been gathered, the final step has not been realized as the gene corresponding to plant $InsP_3$-R has not yet been identified.

In silico search for the $InsP_3$-R gene in plants

The first step in characterizing the $InsP_3$-R should be looking for its gene. From the time when the first plant genome sequencing project was completed, it has become clear that the search for the plant $InsP_3$-R gene will not be an easy task. To date (September 2006), no plant gene has been annotated as $InsP_3$-R. Lin *et al*. (2004), in an article dealing with the mapping of the inositide signalling

* To whom correspondence should be addressed. E-mail: martinec@ueb.cas.cz

Abbreviations: ABA, abscisic acid; ER, endoplasmic reticulum; IICR, $InsP_3$-induced calcium release; $InsP_3$, D-*myo*-inositol 1,4,5-trisphosphate; $InsP_3$-R, $InsP_3$ receptor; RyR, ryanodine receptor.

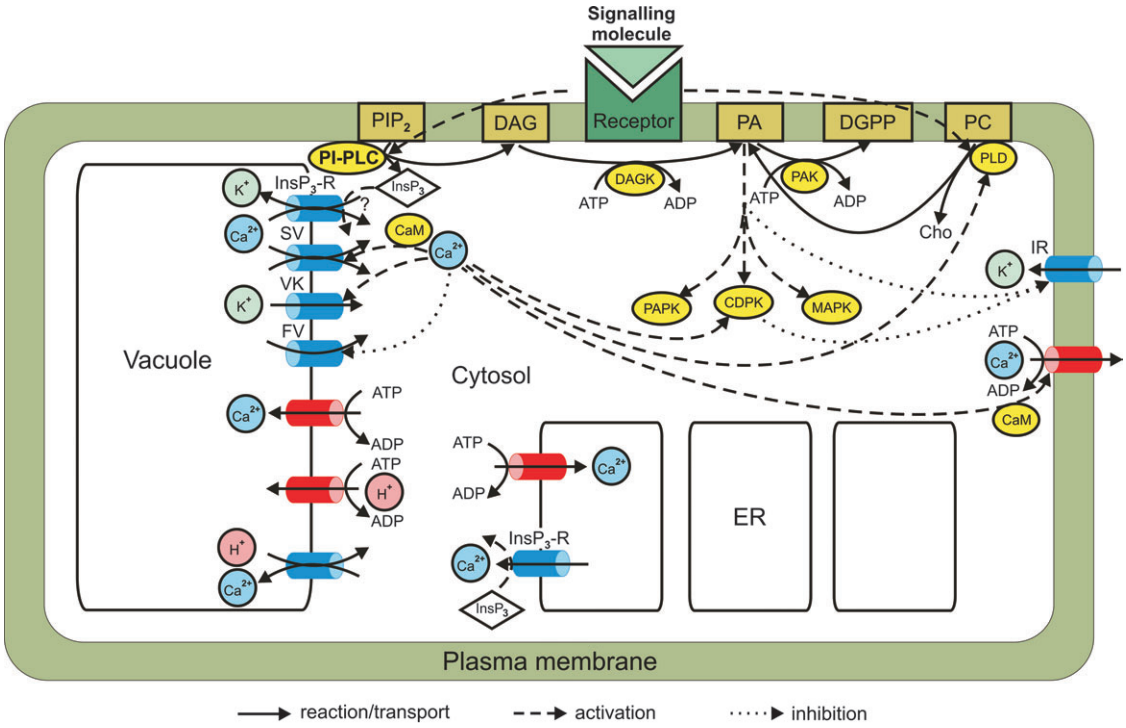

Fig. 1. Proposed physiological role of the $InsP_3$-R in the cell of higher plants. Upon binding of a signalling molecule (e.g. elicitor) to its receptor on the plasma membrane, PI-PLC is activated and it hydrolyses the membrane phospholipid PIP_2 to DAG and $InsP_3$. $InsP_3$ opens the Ca^{2+} channel on the tonoplast and/or on the ER, thus increasing the $[Ca^{2+}]_{cyt}$. In *Acer pseudoplatanus*, this Ca^{2+} transport seems to be coupled with the antiport of K^+ (see the text). The increased Ca^{2+} concentration together with the pH shift and with the membrane depolarization activates, via CaM, the SV channel which is permeable for K^+ and Ca^{2+}. Increased cytosolic Ca^{2+} activates the CDPK, PLD, and the VK channel, and it inhibits the FV K^+ channel. The inward rectifying K^+ channel on the plasma membrane is also indirectly inhibited by the action of CDPK. The resting levels of Ca^{2+} are subsequently restored by the Ca^{2+}-ATPases on the tonoplast, ER, and plasma membrane, and by the coupling of H^+-ATPase with the H^+/Ca^{2+} antiporter on the vacuole. The Ca^{2+}-ATPase on the plasma membrane is activated by the Ca^{2+}–CaM complex. The signalling network becomes even more complex when considering the fate of DAG, which might be rapidly transformed to PA, an important signalling molecule acting on ion channels and on several protein kinases. CaM, calmodulin; CDPK, Ca^{2+}-dependent protein kinase; Cho, choline; DAG, diacylglycerol; DGK, DAG kinase; DGPP, diacylglycerolpyrophosphate; FV, fast activating vacuolar cation channel; $InsP_3$, inositol 1,4,5-trisphosphate; $InsP_3$-R, receptor for $InsP_3$; IR, inward rectifying; MAPK, mitogen-activated protein kinase; PA, phosphatidic acid; PAK, PA kinase; PAPK, PA-dependent protein kinase; PC, phosphatidylcholine; PIP_2, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; PLD, phospholipase D; SV, slow vacuolar channel; VK, vacuolar K^+-selective channel.

pathways in plants by the DNA microarray technique, published two AGI annotations supposed to be the $InsP_3$-R genes in *Arabidopsis*, but the first one (At3g10380) is now annotated as a probable component Sec8 of the exocyst complex and the second one (At5g27230) is a membrane electron transport protein according to its domain composition. A simple NCBI Blast search (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) for the $InsP_3$-R protein sequence found no plant protein bearing a significant homology. A more sensitive method for detection of remote protein homologies is based on the profile hidden Markov model algorithm (Eddy, 1998). The profile hidden Markov model turns a multiple sequence alignment into a position-specific scoring system suitable for searching databases for remotely homologous sequences. Profile hidden Markov model analyses complement standard pairwise comparison methods for large-scale sequence analysis. After aligning the input sequences, the algorithm generates a set of consensual motifs typical for the protein family. These sequence motifs are then compared with the library of already annotated protein domains. It is a way to run 'Blast' using only important traits of the studied protein family. Generating a homology profile for the three isoforms of the rat $InsP_3$-R using a profile hidden Markov model algorithm and searching all the protein databases (http://motif.genome.jp/MOTIF2.html) with this profile did not give any significant hits for any plant proteins.

The mammalian $InsP_3$-R is a surprisingly large molecule (~2700 amino acids) and has a well-defined domain structure, shown in Fig. 2. The so-called RIH [ryanodine receptor (RyR) and $InsP_3$-R homology] domain is very specific for $InsP_3$-R and for its close relatives, the RyRs. Based on the presence of this domain, no homologous protein in plants has been found in InterPro (http://www.ebi.ac.uk/interpro/) or the Conserved Domain Database (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). The presence of an ion transport domain is more general, and several proteins possessing a similar domain can be

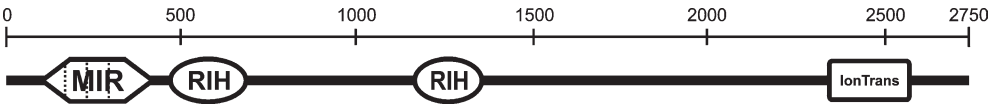

Fig. 2. Domain structure of the rat type 1 $InsP_3$-R based on the domain structure proposed by Pfam (St Louis, MO, USA) (http://pfam.wustl.edu/hmmsearch.shtml). The RIH domain is responsible for $InsP_3$ binding. $InsP_3$, inositol 1,4,5-trisphosphate; $InsP_3$-R, receptor for $InsP_3$; IonTrans, ion transport domain; this protein family contains sodium, potassium, and calcium ion channels and typically they are constituted by six transmembrane helices in which the last two helices flank a loop which determines the ion selectivity of the channels. MIR, domain found in ryanodine receptors; RIH, ryanodine receptor and $InsP_3$-R homology domain.

found in *Arabidopsis* and rice genomes, but those represent mostly the genes previously annotated as K^+ channels with a quite different domain architecture from what would be expected for a true $InsP_3$-R homologue. The lack of domain homology with any plant protein implies that IICR in plants is on a different molecular basis from that in animals. Despite the failure of the sequence database mining, it is very likely now that IICR exists in plants. The *in vivo* evidence for this mechanism is quite convincing.

In vivo evidence for $InsP_3$-induced Ca^{2+} release in plants

Before considering the *in vitro* properties of the putative $InsP_3$-R, it should be clear that $InsP_3$ has some physiological relevance *in vivo*. The biochemistry of inositol phosphates in plants differs somewhat from that described in animal systems (van Leeuwen *et al*., 2004) and has been reviewed from the metabolic (Drøbak, 1992; Loewus and Murthy, 2000) and the signalling point of view (Coté and Crain, 1993; Stevenson *et al*., 2000). Metabolism of $InsP_3$ in plants was also studied (Coté *et al*., 1987; Morse *et al*., 1987; Drøbak *et al*., 1991; Brearley and Hanke, 1996*a*, *b*; DePass *et al*., 2001). Changes in the intracellular $InsP_3$ level have been reported in many species of higher plants in response to various stimuli such as light, cold, gravistimulation, oxidative stress, hyperosmotic stress, plant hormones, G-protein activation, and pathogenic elicitors (for a summary, see Table 1). The best documented system seems to be the abscisic acid (ABA)-stimulated stomatal closure which was thoroughly studied in this context (Staxen *et al*., 1999; Hunt *et al*., 2003). Release of caged $InsP_3$ was shown to cause Ca^{2+} influx in growing *Agapanthus* pollen tubes (Monteiro *et al*., 2005). A good review on how the sustained increase of $InsP_3$ and Ca^{2+} leads to gravitropic growth response can be found in Stevenson *et al*. (2000). Plants often show elevated $InsP_3$ levels upon stimulation. This suggests $InsP_3$ to be a positive regulator of many signalling pathways. Nevertheless it has been shown in *in vivo* studies that the phosphoinositide turnover in higher plants is quite rapid (van der Luit *et al*., 2000) and some steady-state level of $InsP_3$ is thus present even in non-stimulated cells. This $InsP_3$ level may serve as a constitutive repressor or enhancer of gene expression, and its decrease would also lead to altered gene expression, as was shown in mutants with a constitutively decreased $InsP_3$ level (Perera *et al*., 2002, 2006; Burnette *et al*., 2003).

Changes in $InsP_3$ levels have often been linked with changes in activity of phosphoinositide-specific phospholipase C (PI-PLC). The role of this enzyme in plants has been reviewed several times (Munnik *et al*., 1998; Mueller-Roeber and Pical, 2002). An exhaustive study of the tissue and stress expression pattern of all nine *Arabidopsis* PI-PLC isoforms with suggestions as to how they may intervene in various stress responses was recently published by Hunt *et al*. (2004). Its mode of catalysis, domain structure, and possible cellular functions were reviewed by Wang (2001) and later in a more general context of phospholipid signalling (Wang, 2004). The fact that the $InsP_3$ concentration *in vivo* varies upon stimulation points to its significance in plant cell signalling.

It was also necessary to demonstrate that elevation of the cytoplasmic concentration of $InsP_3$ triggers some physiological response in living cells. This was done mainly by the photolysis of the caged $InsP_3$ or by microinjection of $InsP_3$. Release of $InsP_3$ from its caged form in stomatal guard cells of *Commelina communis* led to an increase of $[Ca^{2+}]_{cyt}$ and to the closure of stomata (Gilroy *et al*., 1990, 1991). A similar experiment was done with guard cells of *Vicia faba* (Blatt *et al*., 1990). They showed that the application of exogenous $InsP_3$ reversibly inactivates the plasma membrane-located inward rectifying K^+ channel in guard cells by releasing calcium into the cytoplasm. Microinjections of free $InsP_3$ in algal cells resulted in a change in the plasma membrane conductance (Förster, 1990; Thiel *et al*., 1990). Microinjection of $InsP_3$ was used to prove the cell–cell communication through oscillations of $[Ca^{2+}]_{cyt}$ (Tucker and Boss, 1996).

Additional data about the importance of $InsP_3$ have been gathered thanks to the characterization of mutants with constitutively increased levels of $InsP_3$ (Xiong *et al*., 2001; Carland and Nelson, 2004) or transgenic plants with constitutively decreased $InsP_3$ levels due to overexpression of $InsP_3$ 5-phosphatase (Perera *et al*., 2002, 2006; Burnette *et al*., 2003). One of the mutants with a constitutively increased $InsP_3$ level is *fry1*, a loss-of-function mutant in a bifunctional enzyme which exhibits both 3′(2′), 5′-bisphosphate nucleotidase and inositol polyphosphate 1-phosphatase activities (Xiong *et al*., 2001). The other

Table 1. *Involvement of $InsP_3$ in plant physiological processes*

Plant	Tissue	Stimulus[a]	Reference
(A) Level of $InsP_3$ increased			
Arabidopsis thaliana	Whole plants	Hyperosmotic and salt stress	DeWald *et al*., 2001
Arabidopsis thaliana	Whole plants	Heat shock	Liu *et al*., 2006
Arabidopsis thaliana	Cell suspension culture	Hyperosmotic and salt stress	Takahashi *et al*., 2001
Arabidopsis thaliana	Cell suspension culture	Cold shock	Ruelland *et al*., 2002
Avena sativa	Shoot pulvini	Gravistimulus	Perera *et al*., 2001
Beta vulgaris	Tap root	Hyperosmotic stress	Srivastava, 1989
Brassica napus L. var. *oleifera*	Leaves	Cold shock	Smoleńska-Sym and Kacperska, 1994
Brassica napus L. var. *oleifera*	Non-acclimated leaves	Cold shock, ABA, dehydration	Smoleńska-Sym and Kacperska, 1996
Brassica oleracea	Etiolated seedlings	Light	Acharya *et al*., 1991
Brassica oleracea	Hypocotyls from seedlings	NAA, BAP	Durejamunjal *et al*., 1992
Citrus limon	Seedlings	Fungal infection by *Alternaria alternata*	Ortega and Perez, 2001; Ortega *et al*., 2005
Daucus carota L.	Cell suspension culture	Melittin, mastoparan	Drøbak and Watkins, 1994
Daucus carota L.	Cell suspension culture	Mastoparan	Cho *et al*., 1995
Daucus carota L.	Cell suspension culture	Hyperosmotic and salt stress	Drøbak and Watkins, 2000
Dianthus caryophyllus L.	Petals	Dehydration	Drory *et al*., 1992
Digitaria sanguinalis	Mesophyll protoplasts	Light and cytoplasmic pH increase in C_4 plants	Coursol *et al*., 2000*a*, *b*
Glycine max	Cell suspension culture	Mastoparan, polygalacturonic acid elicitor	Legendre *et al*., 1993
Malus domestica	Apple buds	Decapitation of shoots	Wang and Faust, 1995
Medicago sativa	Cell suspension culture	Glycoprotein elicitor from *Verticillium alboatrum*	Walton *et al*., 1993
Nicotiana tabacum	Cell suspension culture	Elicitor from *Phytophthora nicotiniae*	Kamada and Muto, 1994
Nicotiana tabacum	Cell suspension culture	Mastoparan	Perera *et al*., 2002
Nicotiana tabacum	Cell suspension culture	G-protein-coupled receptor or G-protein α subunit overexpression	Apone *et al*., 2003
Oryza sativa	Leaf	Elicitor from *Pyricularia oryzae*	Kanoh *et al*., 1993
Oryza sativa	Aleurone layer	Gibberellin	Kashem *et al*., 2000
Papaver rhoeas	Pollen tube	Mastoparan	Franklin-Tong *et al*., 1996
Pisum sativum L.	Epicotyls	Elicitor from *Mycosphaerella pinodes*	Toyoda *et al*., 1993
Rubia tinctorum L.	Cell suspension culture	Chitosan elicitation	Vasconsuelo *et al*., 2005
Samanea saman	Leaf pulvini	Light	Morse *et al*., 1987
Samanea saman	Leaf pulvini protoplasts	Light-induced K^+ channel closure	Kim *et al*., 1996
Triticum aestivum	Root tips	H_2O_2	Jones and Kochian, 1995
Vicia faba	Guard cell protoplasts	ABA	Lee *et al*., 1996
Zea mays L.	Coleoptiles	Fungal phytotoxin fusicoccin	Aducci and Marra, 1990
Zea mays L.	Etiolated leaves	Amine oxidase inhibitor 5-hydroxytryptamine	Chandok and Sopory, 1994
Zea mays L.	Internodal pulvini	Gravistimulus	Perera *et al*., 1999
(B) Level of $InsP_3$ decreased			
Brassica oleracea	Hypocotyls from seedlings	Spermidine	Durejamunjal *et al*., 1992
Glycine max	Cell suspension culture	Infection by *Pseudomonas syringae* pv. *glycinea*	Shigaki and Bhattacharyya, 2000
Pisum sativum L.	Epicotyls	Suppressor from *Mycosphaerella pinodes*	Toyoda *et al*., 1993
Triticum aestivum	Root tips	Al^{3+}	Jones and Kochian, 1995

[a] ABA, abscisic acid; BAP, benzylaminopurine; NAA, naphthylacetic acid.

mutant is *cvp2* which is affected in inositol polyphosphate 5-phosphatase activity. These two inositol polyphosphate phosphatase activities probably act on different levels of $InsP_3$ signal attenuation, as proposed by Xiong *et al*. (2002), where the inositol polyphosphate 5-phosphatase acts directly on $InsP_3$ and FRY1 hydrolyses the resulting $Ins(1,4)P_2$. Slowing down the $InsP_3$ breakdown on each level causes stress hypersensitivity, especially hypersensitivity to ABA, in these mutants. Consistently, the inositol polyphosphate 5-phosphatase gain-of-function mutant showed insensitivity to ABA (Burnette *et al*., 2003). Both of these observations indicate a role for $InsP_3$ in ABA signalling. Besides ABA signalling, a role in cold and hyperosmotic stress was proposed for FRY1, and premature vascular termination was observed in *cvp2*. The other phenotypes reported for these mutants may be caused by the other enzymatic activity of FRY1 in the case of *fry1*, by very specific tissue localization of CVP2 in the case of *cvp2*, and by their intervention on different levels of $InsP_3$ catabolism. Based on these data, it is clear that $InsP_3$ elicits a physiological response in plants probably by mobilizing Ca^{2+} from either intracellular or extracellular stores. However, a role for other inositol polyphosphates in Ca^{2+} mobilization cannot be excluded.

Potential role of $InsP_6$ in intracellular calcium signalling

In a very few cases, $InsP_6$ (known as phytate) was shown to be involved in plant cell signalling as an ion channel agonist. Lemtiri-Chlieh *et al*. (2000) showed that $InsP_6$ is active in inhibiting the inward rectifying K^+ ion channel in a Ca^{2+}-dependent manner when delivered through patch electrode to guard cells in submicromolar concentrations. This inhibitory effect is very specific to *myo*-$InsP_6$ which is 100-fold more potent than $InsP_3$. The effect was documented in the response of guard cells to ABA in at least two distinct species of higher plants (*Solanum tuberosum* and *V. faba*). Lemtiri-Chlieh *et al*. (2003) later confirmed these findings by the release of caged $InsP_6$, and found that it also releases Ca^{2+} from internal stores and not from extracellular space through the plasma membrane.

It is clear that this Ca^{2+}-releasing pathway is unique to plants, as no data for $InsP_6$-induced Ca^{2+} release in animal species have been published so far. Although the temptation exists to interpret $InsP_3$ findings as hidden $InsP_6$ action, considering the *in vitro* studies of IICR it is more likely that these two pathways exist in parallel and this dichotomy may have some physiological relevance only in specific cell types such as the guard cells.

In vitro measurements of electrophysiological properties of the $InsP_3$-induced calcium release

Once the relevance of the *in vivo* $InsP_3$ action has been shown, the next step is to prove the existence of such an ion channel *in vitro*. The conductometric measurement of ion channels (patch-clamp technique) is the most reliable one, but it can be performed only with some limitations of the biological material, especially the need for large membrane patches, which can be achieved only with plasma membrane of protoplasts or with large plant vacuoles. That might be the reason why these experiments have been successfully accomplished with only one type of membrane, the tonoplast from *Beta vulgaris* storage root. Alexandre *et al*. (1990) found that the channel has a conductance of 30 pS (after activation by 1 μM $InsP_3$) at –80 mV (referenced to the vacuolar lumen) and it can be opened only by depolarization (i.e. it is voltage dependent). The Ca^{2+} transport was oriented and required outside-out patches with a higher Ca^{2+} concentration inside the membrane vesicles. The density of Ca^{2+} channels was estimated to be ~1200 per vacuole of an average diameter 45±5 μm. Alexandre and Lassalles (1990) showed that by the membrane depolarization the $InsP_3$-released Ca^{2+} closes the non-specific ion channels on the tonoplast. They also speculated that the depolarization might be sometimes more physiologically important than the increase of the $[Ca^{2+}]_{cyt}$ itself. Alexandre and Lassalles (1992) discovered a higher level of the single channel conductance of 50 pS.

Allen and Sanders (1994) found three levels of the single channel conductance of 11, 51, and 182 pS at –80 mV. This discrepancy was not satisfactorily explained. The whole vacuole ion permeability ratio was estimated as 200:1 (P_{Ca}:P_K). The permeability ratio of a single channel was estimated to be in the range from 100:1 to 800:1 (P_{Ca}:P_K). The channel opening was shown to be independent of the $[Ca^{2+}]_{cyt}$ and reversible, although the ligand dissociation was very slow. The Ca^{2+} current of the whole vacuole patch-clamp was also shown to be cytosol directed and appreciably enhanced after hyperosmotic pretreatment of the tissue. This might originate in *de novo* synthesis or in functional phosphorylation of the $InsP_3$-R, or simply in more efficient extraction of the large $InsP_3$-responsive vacuoles from the shrunken cytoplasm. The need for hyperosmotic pretreatment could explain the negative findings published for the vacuoles of the *B. vulgaris* cell suspension culture (Gelli and Blumwald, 1993). In contrast to the finding of Allen and Sanders (1994) on *B. vulgaris*, the IICR in *Acer pseudoplatanus* was found to be stimulated by a decrease of the osmotic pressure. This stimulation was explained by the expansion of the vacuolar surface which made the $InsP_3$-R molecules more accessible and/or changed the conformation of the receptor to the active form. No Ca^{2+} channel opening upon 2–8 μM $InsP_3$ stimulation at –120 mV occurred in the patch-clamp experiments with the tonoplast from tobacco cells (*Nicotiana tabacum* L. cv. BY-2) carried out by Ping *et al*. (1992).

The positive findings for the $InsP_3$-R were later reviewed in the context of the cyclic adenosine diphosphate-ribose (cADPR)-induced Ca^{2+} release. The release was shown to be additive in the whole vacuole patch-clamp of *B. vulgaris*. The additivity was confirmed by the radiometric measurements; both ligands could release 15% of the releasable Ca^{2+} (Allen *et al*., 1995). Both of the mechanisms are assumed to trigger the calcium-induced calcium release (CICR) in plants. The CICR itself is then performed by the slow vacuolar (SV) channels during the depolarization of the membrane caused by the opening of either the $InsP_3$-R itself (Sanders and Johannes, 1990) or of the tonoplast Ca^{2+}-activated K^+ channels. The $InsP_3$- and cADPR-induced Ca^{2+} currents were not spontaneously deactivated, which means that the closure of the channels is induced by the voltage change, and later the metabolic degradation of the ligand occurs, which prevents further Ca^{2+} channel reactivation (Allen *et al*., 1995).

Muir *et al*. (1997) failed to measure the IICR from the whole vacuole of guard cells and from the whole vacuole of cells from the inflorescence of *Brassica oleracea* L. using the patch-clamp technique under the same conditions used for *B. vulgaris*. The cause of the failure with the guard cells could be the small diameter of the vacuole. The increase in current under the given conditions would

be far below the resolution of the technique. The failure in the case of vacuoles from the inflorescence of *B. oleracae* could originate in the different subcellular localization of $InsP_3$-R in this plant tissue, as documented elsewhere (Muir and Sanders, 1997).

In vitro estimated parameters of the $InsP_3$-induced Ca^{2+} release

More detailed insight into the IICR can be obtained by looking at the effect on Ca^{2+} transport after application of some Ca^{2+} channel blockers or $InsP_3$ isomers. The *in vitro* Ca^{2+} transport can be measured by the previously discussed patch-clamp approach or by monitoring Ca^{2+} transport using radioactive $^{45}Ca^{2+}$ or using Ca^{2+}-specific fluorescent dyes.

In animal cells, the IICR was shown to be a quantal process which means that the submaximal dose of the ligand does not fully empty the Ca^{2+} store even after a longer period of time. This phenomenon can be explained by the voltage-dependent channel closing described above, by the relatively slow dissociation of $InsP_3$ from $InsP_3$-R, or by the [$InsP_3$]-driven ratio of the $InsP_3$-R_{bound}/$InsP_3$-R_{free} molecules. The result of these processes is simple Michaelis–Menten saturation kinetics of the IICR. An apparent saturation constant (IC_{50}) that defines the concentration of $InsP_3$ which gives half-maximal Ca^{2+} release is one of the basic features of IICR. The second one is the percentage of mobilizable Ca^{2+}. These characteristics for various plant materials are summarized in Table 2.

Johannes *et al*. (1992*a*) found that the $InsP_3$-regulated Ca^{2+} channels represent only a minor portion of the Ca^{2+} channels residing on the tonoplast of *B. vulgaris*, the majority of those Ca^{2+} channels being voltage gated and $InsP_3$ independent. Alexandre *et al*. (1990) showed that the $InsP_3$ binding in *B. vulgaris* exhibits no cooperativity, unlike the binding to its animal analogue. The values of the IC_{50} differ greatly, ranging from 0.2 to 15 μM (Table 2); the lower values are in accord with those reported for animal $InsP_3$-R, but the high values for *Zea mays* L. coleoptiles, *Daucus carota* L. cell suspension culture, and *Cucurbita pepo* L. hypocotyls were neither reproduced later nor satisfactorily explained. However, Drøbak and Ferguson (1985) warned that their $InsP_3$ preparation was probably contaminated by a considerable amount of Ins(2,4,5)P_3. The amount of released Ca^{2+} was usually in the range of a few nanomoles per mg of protein. The percentage of the $InsP_3$-releasable Ca^{2+} normally ranges from 10% to 20% for the microsomal fraction and from 20% to 40% for the tonoplast-enriched microsomal fraction. A relatively small part of the mobilizable Ca^{2+} can be released by $InsP_3$. The density of $InsP_3$-binding sites in the microsomal fraction mirrors to some extent the density of $InsP_3$-R. This value, called B_{max}, is generally expressed in pmoles of specifically bound $InsP_3$ per mg of proteins in the studied sample. In animal cerebellum, this value ranges from 10 to 70 depending on the species and on the age of the animals (Simonyi *et al*., 1998; Vanlingen *et al*., 1999; Coquil *et al*., 2004). The relatively small portion of $InsP_3$-releasable Ca^{2+} can be explained using the example of *B. vulgaris* (the corresponding value of B_{max} can be found in Table 4). Its B_{max} is at least 10 times lower than that of microsomes from animal brain. The relatively small number of $InsP_3$-R molecules per vacuole leads, after the reconstitution of the tonoplast vesicles, to the fact

Table 2. *The IC_{50} values ($InsP_3$) for the IICR*

Plant	Tissue	Membrane type	Technique	IICR/mobilizable Ca^{2+} (%)	IC_{50} (μM)	Reference
Acer pseudoplatanus	Cell suspension culture	Vacuole	Quin2	ND[a]	0.2	Ranjeva *et al*., 1988
Avena sativa L.	Root	Tonoplast-enriched MF[a]	$^{45}Ca^{2+}$	35	0.6	Schumaker and Sze, 1987
Beta vulgaris	Storage root	Tonoplast-enriched MF	$^{45}Ca^{2+}$	20.8±0.8	0.54±0.11	Brosnan and Sanders, 1990
Beta vulgaris	Storage root	Vacuole	Patch-clamp	ND	0.22	Alexandre *et al*., 1990
Beta vulgaris	Storage root	Tonoplast-enriched MF	$^{45}Ca^{2+}$	10±1	ND	Johannes *et al*., 1992*a*
Beta vulgaris	Storage root	Tonoplast-enriched MF	$^{45}Ca^{2+}$	15	ND	Allen *et al*., 1995
Brassica oleracea L. var. *botrytis*	Inflorescence	MF	$^{45}Ca^{2+}$	19.0±1.3	0.59±0.14	Muir and Sanders, 1997
Brassica oleracea L.	Inflorescence	MF	Fluo-3	2.5±0.5	ND	Krinke *et al*., 2003
Chenopodium album L.	Cell suspension culture	Vacuole	Indo-1	10	ND	Lommel and Felle, 1997
Cucurbita pepo L.	Hypocotyl	MF	$^{45}Ca^{2+}$	30	15±3	Drøbak and Ferguson, 1985
Daucus carota L.	Cell suspension culture	MF	$^{45}Ca^{2+}$	10±2	12±2	Zbell *et al*., 1989
Daucus carota L.	Cell suspension culture	MF; tonoplast-enriched MF	$^{45}Ca^{2+}$	10; 40	ND; 1	Canut *et al*., 1993
Zea mays L.	Coleoptile	MF	$^{45}Ca^{2+}$	50	8	Reddy and Poovaiah, 1987

[a] ND, not determined; MF, microsomal fraction.

that only one vesicle from about eight contains at least one $InsP_3$-R molecule. This happens because the diameter of the reconstituted vesicle is ~1% of that of the intact vacuole (Brosnan, 1990).

The animal $InsP_3$-R is regulated by calmodulin (CaM), either free or bound to Ca^{2+}, which inhibits $InsP_3$ binding to the receptor; no direct regulation by Ca^{2+} was observed (Bultynck *et al*., 2003). The dependence of IICR on $[Ca^{2+}]_{free}$ in plants was also examined in a few studies. In experiments with *C. pepo* L., the $[Ca^{2+}]_{free}$ during the Ca^{2+} release was only 10 nM, indicating no regulation by calcium ions in this case. Allen and Sanders (1994), using the patch-clamp technique, found that the IICR in *B. vulgaris* is not affected whether the $[Ca^{2+}]_{free}$ is 100 nM or 1 mM. However, the IICR in *Z. mays* was maximal at 100 nM $[Ca^{2+}]_{free}$. Even this finding is questionable because the dependence on $[Ca^{2+}]_{free}$ was not bell-shaped as for the animal $InsP_3$-R (Finch *et al*., 1991), and it was not measured for concentrations lower than 50 nM. This may mean that, in contrast to the animal $InsP_3$-R, the plant $InsP_3$-R might not be regulated by the $[Ca^{2+}]_{free}$.

A wide range of different inhibitors was used as a tool to study the IICR. The effect of some well-known inhibitors of Ca^{2+} release is reviewed in Table 3. Nifedipine, the mammalian L-type Ca^{2+} channel blocker, ryanodine, the sarcoplasmic reticulum Ca^{2+} channel blocker and the antagonist of RyR, and ruthenium red, the blocker of Ca^{2+} uptake by mitochondria and another antagonist of RyR, were all ineffective in inhibition of IICR in plants. Surprisingly caffeine, an activator of RyR, acted as an $InsP_3$-R inhibitor. Thus it is not a good inhibitor either because of its dual specificity. EDC [1-ethyl-3-(3-dimethylaminopropyl)carbodiimide], a carboxyl amino acid modifier, was shown to abolish the IICR, thus confirming the need for free aspartate or glutamate residues for the proper function of the receptor (Samanta *et al*., 1993). Trifluoperazine, the CaM antagonist, was not a good inhibitor because of the existence of the Ca^{2+}-ATPase in the tonoplast which is also sensitive to it (Pfeiffer and Hager, 1993; Lommel and Felle, 1997). To achieve inhibition comparable with animal $InsP_3$-R, a relatively high concentration of TMB-8 [8-(*N,N*-diethylamino)octyl 3,4,5-trimethylbenzoate], an inhibitor of some endomembrane Ca^{2+} channels and protein kinase C, is required. This points to a lower specificity for plant Ca^{2+} channels than reported for those of animals. Verapamil, a Ca^{2+} channel blocker, was effective in *B. vulgaris*, but totally ineffective in *A. pseudoplatanus*. The reason could be the insufficient concentration used or the different nature of the receptor in *A. pseudoplatanus*. In *A. pseudoplatanus*, the dependence of the IICR on the presence of K^+ was proved, as well as coupling of Ca^{2+} influx with K^+ efflux (Canut *et al*., 1989). The coupling with K^+ efflux was not reported for other plant species, which further supports the idea of the different nature of the IICR in *A. pseudoplatanus* compared with that of other higher plants. Taken together, none of the inhibitors mentioned above seems to be specific enough for plant $InsP_3$-R, and their use in plants will probably not give meaningful results.

Low molecular weight heparin is a potent inhibitor of the IICR in plants and also competes more strongly than other inositol phosphates. The IC_{50} values are in close quantitative agreement with those reported for rat cerebellum (Worley *et al*., 1987; Challiss *et al*., 1991) although the affinity of $InsP_3$-R from animal peripheral tissues for heparin is somewhat weaker (Guillemette *et al*., 1989; Tones *et al*., 1989). Heparin of higher molecular weight is a markedly less potent inhibitor of IICR, both in animals (Chopra *et al*., 1989) and in plants (Sanders *et al*., 1990; Johannes *et al*., 1992*b*). Some difficulties were observed in the case of heparin and the tonoplast of *B. vulgaris* because its competitive inhibitory effect on the Ca^{2+} release observed with $^{45}Ca^{2+}$ (Brosnan and Sanders, 1990; Johannes *et al*., 1992*b*) was not confirmed by the patch-clamp techniques (Alexandre and Lassalles, 1992) as the inhibitory effect there was rather non-specific. In that work, it might have been a problem of inappropriate molecular weight of the heparin preparation used; unfortunately this information was missing. Nevertheless, the use of low molecular weight heparin may be a good tool for the study of plant IICR.

The IICR in *Vigna radiata* was also enabled by $Ins(2,4,5)P_3$ (EC_{50}=0.8 μM) but the maximal release thus gained was about three times lower than for $InsP_3$, which is consistent with the findings of Alexandre *et al*. (1990) and with the data obtained for the animal $InsP_3$-R. The IICR was stimulated when $InsP_3$ was complexed with phytase (Samanta *et al*., 1993). Dasgupta *et al*. (1996) found that this enhancement comes from the presence of the allosteric site on phytase with high affinity for $InsP_3$. This allosteric site changes the conformation of the phytase upon binding of $InsP_3$, thus enabling its effective interaction with the $InsP_3$-R.

In vitro estimated parameters of $InsP_3$ binding

The receptor part of the $InsP_3$-R can also be characterized by the affinity of the ligand ($InsP_3$) for the $InsP_3$-binding site. However, unless the gene for the receptor is known, it cannot be excluded that the studied binding site does not belong to the $InsP_3$-R molecule. Just the expression of the receptor part of the $InsP_3$-R and a clear demonstration that its binding properties are the same as those of the natural samples can prove that it is in fact the receptor part of the $InsP_3$-R that is studied under the given conditions.

The experiments are usually carried out as the $[^3H]InsP_3$ competitive displacement assay. It is supposed that, under certain conditions, only the specifically bound $[^3H]InsP_3$ can be displaced by the excess of the other ligand and the non-specifically bound $[^3H]InsP_3$ cannot as the

Table 3. *The effect of various Ca^{2+} channel blockers on the IICR*

Plant	Tissue	Membrane type	Technique	IICR/ mobilizable Ca^{2+} (%)	Ca^{2+} channel blocker	Concentration used	Inhibition by (%)	IC_{50} (μM)	Reference
Acer pseudoplatanus	Cell suspension culture	Vacuole	Quin2	ND[a]	TMB-8	250 μM	80	ND	Ranjeva *et al*., 1988
Acer pseudoplatanus	Cell suspension culture	MF[a]	$^{45}Ca^{2+}$	13	Bepridil	50 μM	No inh.[a]	–	Canut *et al*., 1989
					Verapamil	NI[a]	No inh.	–	Canut *et al*., 1989
Avena sativa L.	Root	Tonoplast-enriched MF	$^{45}Ca^{2+}$	35	TMB-8	250 μM	85	50	Schumaker and Sze, 1987
Beta vulgaris	Storage root	Vacuole	Patch-clamp	ND	Verapamil	20 μM	66±5	ND	Alexandre *et al*., 1990
					TMB-8	100 μM	44±3	ND	Alexandre *et al*., 1990
Beta vulgaris	Storage root	Tonoplas-enriched MF	$^{45}Ca^{2+}$	20.8±0.8	TMB-8	200 μM	Full inh.	ND	Brosnan and Sanders, 1990
					Nifedipine	NI	No inh.	–	Brosnan and Sanders, 1990
					Ryanodine	NI	No inh.	–	Brosnan and Sanders, 1990
					Heparin (mol. wt 5000)	1 μM	87	0.086±0.02	Brosnan and Sanders, 1990
Beta vulgaris	Storage root	Vacuole	Patch-clamp	ND	Heparin (mol. wt NI)	1 mg ml^{-1}	60	ND	Alexandre and Lassalles, 1992
Beta vulgaris	Storage root	Tonoplast-enriched MF	$^{45}Ca^{2+}$	10±1	Ruthenium red	20 μM	No inh.	–	Johannes *et al*., 1992*a*
					Ryanodine	10 μM	No inh.	–	Johannes *et al*., 1992*a*
					Zn^{2+}	1 mM	No inh.	–	Johannes *et al*., 1992*a*
					Gd^{3+}	100 μM	34±8	ND	Johannes *et al*., 1992*a*
					TMB-8	200 μM	85±7	ND	Johannes *et al*., 1992*a*
					Heparin (mol. wt NI)	1 μM	96±2	ND	Johannes *et al*., 1992*a*
Beta vulgaris	Storage root	Tonoplast-enriched MF	$^{45}Ca^{2+}$	ND	Ruthenium red	NI	No inh.	–	Johannes *et al*., 1992*b*
					Heparin (mol. wt 6000–20 000)	ND	ND	1.4	Johannes *et al*., 1992*b*
Brassica oleracea L.	Inflorescence	MF	$^{45}Ca^{2+}$	ND	Ruthenium red	30 μM	No inh.	–	Muir *et al*., 1997
					Ryanodine	100 μM	No inh.	–	Muir *et al*., 1997
					Caffeine	5 mM	70	ND	Muir *et al*., 1997
					TMB-8	200 μM	90	ND	Muir *et al*., 1997
					Heparin (mol. wt NI)	10 μM	70	ND	Muir *et al*., 1997
Brassica oleracea L.	Inflorescence	MF	fluo-3	2.5±0.5	Heparin (mol. wt 3000)	3 nM	Full inh.	ND	Krinke *et al*., 2003
Chenopodium album L.	Cell suspension culture	Tonoplast-enriched MF	Ca^{2+}-selective mini-electrode	10	Heparin (mol. wt NI)	25 μg ml^{-1}	Full inh.	ND	Lommel and Felle, 1997
Daucus carota L.	Cell suspension culture	Protoplast	$^{45}Ca^{2+}$	17	Trifluoperazine	15 μM	Full inh.	ND	Rincon and Boss, 1987
Vigna radiata	Hypocotyl	MF	Quin2	80	EDC[a]	NI	NI	ND	Samanta *et al*., 1993
					Heparin (mol. wt NI)	NI	NI	ND	Samanta *et al*., 1993

[a] ND, not determined; NI, not indicated; inh., inhibition; MF, microsomal fraction; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide.

non-specific binding is not saturable. The data for various plant materials are summarized in Table 4 together with the pH values used and with the densities of the binding sites per mg of protein (B_{max}) where available.

These types of experiments are difficult to interpret because they are usually performed under non-physiological conditions (alkaline pH, high [Ca^{2+}], absence of ATP, solubilized $InsP_3$-R molecules) and their results cannot be interpreted in terms of the function of $InsP_3$-R; they only give us limited information about the receptor part of the ion channel. However, this approach is very suitable for the evaluation of the purification process as it does not require maintaining the $InsP_3$-R in its native conformation.

The IC_{50} values for $InsP_3$ can be compared with the EC_{50} only in the case of *B. vulgaris*. The IC_{50} is lower perhaps because of no ATP in the $InsP_3$ binding assay. It was previously shown that ATP, at the concentration used for the Ca^{2+} transport assay, has a considerable affinity for the $InsP_3$-binding site in this plant (IC_{50}=980 μM; Johannes *et al*., 1992*b*). The influence of ATP is further confirmed by the lower EC_{50} value obtained by the patch-clamp technique where ATP is also not present (Alexandre *et al*., 1990).

The affinity of plant $InsP_3$-R for ATP observed in some studies is a property reflecting that of its rat counterpart where the IC_{50} for ATP ranges from 0.35 to 0.79 mM (Nunn and Taylor, 1990; Challiss *et al*., 1991). As ATP is present in millimolar concentrations in living cells, this affinity may also have some physiological consequences *in vivo*. This concentration of ATP anticipates the $InsP_3$ binding to its receptor, and this might account for the higher than expected concentrations of $InsP_3$ in animal cells (Nunn and Taylor, 1990). However, the higher $InsP_3$ concentration in plants compared with animals may just be a simple consequence of the lower affinity of plant $InsP_3$-R for $InsP_3$ combined with the need for a comparable physiological impact of phosphoinositide signalling in plant cells. Another explanation could be that the true binding ligand is $InsP_6$ and that the $InsP_3$ serves only as a precursor for its synthesis, thus being more abundant in the plant cell

Table 4. *Binding parameters for various agonists of $InsP_3$-R*

Plant	Tissue	Membrane type	Technique	pH	Agonist	IC_{50}	B_{max} (pmol mg^{-1})	Reference
Beta vulgaris	Storage root	Tonoplast-enriched MF[a]	Solubilization and PEG[a] precipitation	8.0	$InsP_3$	56±6 nM	0.009±0.001	Johannes *et al*., 1992*b*
				8.0	ATP	980 μM	NI[a]	Johannes *et al*., 1992*b*
				8.0	Heparin (mol. wt 5000)	320 nM	NI	Johannes *et al*., 1992*b*
Beta vulgaris	Storage root	MF	Solubilization and PEG precipitation	8.0	$InsP_3$	121±10 nM	0.84	Brosnan and Sanders, 1993
				8.0	Heparin (mol. wt 5000)	301±72 nM	NI	Brosnan and Sanders, 1993
Brassiea oleracea L.	Inflorescence	MF	Solubilization and PEG precipitation	8.0	$InsP_3$	61±10 nM	0.86	Muir *et al*., 1997
Chenopodium rubrum	Leaf	MF	Filtration	9.0	$InsP_3$	142±17 nM	47	Scanlon *et al*., 1995
				9.0	L-$InsP_3$	691±114 nM	NI	Scanlon *et al*., 1995
				9.0	Ins(1,4)P_2	24.8±6.9 μM	NI	Scanlon *et al*., 1996
				9.0	ATP	241±25 μM	NI	Scanlon *et al*., 1996
				9.0	Heparin (mol. wt NI)	534±142 nM	NI	Scanlon *et al*., 1996
Vigna radiata	Hypocotyl	MF	Centrifugation	9.0	$InsP_3$	125 nM	NI	Samanta *et al*., 1993
				9.0	Ins(2,4,5)P_3	400 nM	NI	Samanta *et al*., 1993
Vigna radiata	Hypocotyl	MF/purified $InsP_3$-R	Centrifugation for MF/PEG precipitation for solubilized $InsP_3$-R	8.5	$InsP_3$	125 nM	1.1	Biswas *et al*., 1995
				8.5	Heparin (mol. wt 5000)	260 nM	NI	Biswas *et al*., 1995
Vigna radiata	Hypocotyl	Purified $InsP_3$-R	Fluorescence quenching	8.0	$InsP_3$	90±10 nM	NI	Dasgupta *et al*., 1996
				8.0	Ins(2,4,5)P_3	110±5 nM	NI	Dasgupta *et al*., 1996
Vigna radiata	Hypocotyl	Purified $InsP_3$-R	Fluorescence quenching	8.0	$InsP_3$	82±20 nM	NI	Dasgupta *et al*., 1997
					Ins(2,4,5)P_3	115±30 nM	NI	Dasgupta *et al*., 1997

[a] NI, not indicated; MF, microsomal fraction; PEG, polyethylene glycol.

and less efficient in receptor binding. Further studies where $InsP_3$ and $InsP_6$ would be studied in parallel could substantially contribute to resolving this dilemma. If both these ligands have a physiological role in plant cells, their mutual interaction and interpretation of the two different signals by the plant cell are definitely points of future interest. Plant $InsP_3$ and more generally phosphoinositide signalling is one of the current topics under discussion, and more and more researchers tend to abandon the idea of simple transformation of the animal model of phosphoinositide signalling to plants (van Leeuwen *et al*., 2004).

The specific binding is usually calculated as the difference between the total and non-specifically bound [^{3}H]$InsP_3$. The non-specific binding usually accounted for 35–50% for the tonoplast-enriched microsomal fraction from *B. vulgaris* (Johannes *et al*., 1992*b*) and 10–50% for the microsomal fraction from *Chenopodium rubrum* (Scanlon *et al*., 1996) which is higher than that reported for the animal membrane preparations where it represents 5–30% (Wilcox *et al*., 1994; Vanlingen *et al*., 1999). By comparing the specific [^{3}H]$InsP_3$ binding to the microsomal fraction from the inflorescence of *B. oleracea* L., from the coleoptiles of *Z. mays* L., and from the storage root of *B. vulgaris*, Muir *et al*. (1997) found the inflorescence of *B. oleracea* L. to be the best material for further studies because it has the highest specific $InsP_3$ binding under the given conditions. The polyethylene glycol (PEG) precipitation technique was criticized by Scanlon *et al*. (1996) due to the possible underestimation of B_{max} values, and the rapid filtration method was suggested instead. However, the degree of this possible underestimation was not specified.

[Ca^{2+}] was either not controlled in the above experiments or it was set to high non-physiological values (e.g. 10 mM in the case of *C. rubrum*; Scanlon *et al*., 1995, 1996). It was also found that EGTA acts as the antagonist of $InsP_3$ binding either by decreasing the $[Ca^{2+}]_{free}$ or by direct interaction with the $InsP_3$-binding site (Richardson and Taylor, 1993; Scanlon *et al*., 1996).

The sulphydryl reagents 1 mM *N*-ethylmaleimide and 50 μM *p*-chloromercuribenzoic acid (PCMB) were shown to reduce the $InsP_3$ binding in *B. vulgaris* (Brosnan and Sanders, 1993). This was further confirmed by the finding that 1 mM pCMBS (*p*-chloromercuribenzoylsulphonate), another sulphydryl reagent, abolishes the $InsP_3$ binding in *V. radiata* (Biswas *et al*., 1995). Scanlon *et al*. (1996) found that it is critical to add at least 5 mM DTT (dithiothreitol) to ensure the reproducibility of the results with the microsomal fraction of *C. rubrum*, suggesting some role for the cysteine residues in the binding site of $InsP_3$-R. In contrast, 400 μM TMB-8, an inhibitor of the IICR, did not alter the $InsP_3$ binding in *B. vulgaris* (Brosnan and Sanders, 1993) which means that it does not act as a competitive inhibitor of $InsP_3$ but rather acts directly on the Ca^{2+} channel part of the plant $InsP_3$-R.

Samanta *et al*. (1993) described that phytase increased the affinity of inositol trisphosphates for the $InsP_3$-R (it decreased their IC_{50}). This finding was further confirmed by Dasgupta *et al*. (1996) who found that the phytase from *V. radiata* cotyledons has an allosteric site for $InsP_3$/$Ins(2,4,5)P_3$ and that the phytase with $InsP_3$ bound to its allosteric site has a higher affinity for the $InsP_3$-R than the $InsP_3$ itself (the IC_{50} value for both $InsP_3$ and $Ins(2,4,5)P_3$ was 75 ± 10 nM using the [^{3}H]$InsP_3$ competitive displacement assay). However, the physiological relevance of this finding remains unclear.

Employing the $InsP_3$ binding assay, Biswas *et al*. (1995) found that the $K_d(InsP_3)=1.5$ nM in *V. radiata*. This significant difference from the IC_{50} value might be explained by the fact that the [^{3}H]$InsP_3$ concentration used in this experiment (25 nM) was relatively high (the IC_{50} value approximately matches the K_d only when the [^{3}H]$InsP_3$ concentration is lower than $0.1\times K_d$). The pellet after Triton X-100 solubilization was shown to inhibit the $InsP_3$-specific binding by 30%, suggesting the modulation by some non-identified factor (perhaps present in the membrane microdomains; Peskan *et al*., 2000). Biswas *et al*. (1995) were also the first to report the purification of the $InsP_3$-R to apparent homogeneity using affinity chromatography with heparin-modified agarose. Analysis of the purified $InsP_3$-R revealed that the receptor is a homotetramer of 110 kDa subunits. The native molecular mass of the animal $InsP_3$-R is 310 kDa, with the equivalent band of 260 kDa on SDS–PAGE. *In vivo* the animal receptor also functions as a homotetramer (Ferris and Snyder, 1992; Pozzan *et al*., 1994; Taylor and Traynor, 1995). The reported molecular mass of that plant $InsP_3$-R is rather in the range of an $InsP_3$-R from the olfactory cilia of the fish *Ictalurus punctatus* (Kalinoski *et al*., 1992).

The interaction of $InsP_3$ with the $InsP_3$-R in *V. radiata* was further studied by Dasgupta *et al*. (1997). He found that $Ins(1,3,4)P_3$ and $Ins(1,5,6)P_3$ do not significantly bind to the $InsP_3$-binding site and that both $InsP_3$ and $Ins(2,4,5)P_3$ bind to this site, but interestingly each of them causes a different conformational change of the $InsP_3$-R, leading to a different functional response (only $InsP_3$ affects the membrane-spanning helical domain in the $InsP_3$-R). The binding stoichiometry for $InsP_3$ was ascertained to be one molecule of $InsP_3$ per one subunit of $InsP_3$-R.

The animal $InsP_3$-R has supramicromolar affinity for $InsP_2$, $InsP_4$, and $InsP_6$ (Worley *et al*., 1987; Challiss *et al*., 1991). However, this affinity has not been confirmed for the commercially available $InsP_2$ (Maeda *et al*., 1990), pointing to the necessity for highly purified batches of inositol phosphates for this type of study. This is consistent with the finding that $InsP_2$ failed to bind to the $InsP_3$-R from *B. vulgaris* even at millimolar concentrations (Brosnan and Sanders, 1993), thus emphasizing its high specificity for $InsP_3$.

Immunological cross-reactivity between the animal and putative plant $InsP_3$-R

The immunological cross-reactivity of the putative plant $InsP_3$-R might point to some structural similarities with its animal counterpart and thus might serve as a valuable tool for purification of the corresponding plant homologue. Two studies examined this possibility. Muir and Sanders (1997) studied the cross-reactivity in the inflorescence of *B. oleracea* L. Using two different antibodies (T210, the polyclonal antibody raised against the C-terminal part of animal $InsP_3$-R1; and NT, the polyclonal antibody raised against the N-terminal part of animal $InsP_3$-R1), they have identified a 200 kDa protein in the microsomal fraction but numerous other cross-reactive smaller fragments were detected with both antibodies. The 200 kDa protein band on SDS–PAGE can be correlated with the size of the animal $InsP_3$-R subunit of 260 kDa. However, the effort to perform a subcellular localization using these antibodies failed and only putative proteolytic fragments were detected in the tested membrane fractions.

Cramer *et al*. (1998) tested two different plant materials, leaves from *V. faba* L. and *Z. mays* L. Polyclonal antibody raised against the C-terminus of the animal $InsP_3$-R (Calbiochem) interacted with a 260 kDa protein in the microsomal fraction and tonoplast of *V. faba* L., but not in the plasma membrane. A 252 kDa protein was also found in the microsomal fraction of *Z. mays* L. However, smaller cross-reactive fragments were also detected in both plant species.

Immunological cross-reactivity experiments have also been done in our laboratory (Feltlová, unpublished data) with *C. rubrum* as the plant material, but their outcome was not interpretable. Unfortunately, in no case were these cross-reactive proteins shown to co-purify with the IICR activity. This more or less general failure of efforts to immunolocalize the plant $InsP_3$-R by antibodies raised against the animal receptor is most probably a good example of non-specific binding of antibodies raised against animal proteins. Antibodies raised against animal $InsP_3$-R will certainly not be useful for purification of its plant homologue.

Subcellular localization of the putative plant $InsP_3$-R

The subcellular localization of $InsP_3$-R was studied in a considerable number of publications, and various separation techniques were applied to reach this goal (the results are summarized in Table 5). It is clear that the $InsP_3$-R is localized on the tonoplast in the majority of the plants; its presence on this membrane was proved by various techniques. It is not a very surprising finding as the vacuole is the largest intracellular Ca^{2+} store and, moreover, its volume represents >90% of the cytoplasm.

Table 5. *Subcellular localization of the putative plant $InsP_3$-R*

Plant	Tissue	Separation	Technique	Localization	Reference
Acer pseudoplatanus	Cell suspension culture	Ficoll/dextran gradient	Quin2	Tonoplast	Ranjeva *et al*., 1988
Avena sativa L.	Root	Dextran gradient	$^{45}Ca^{2+}$	Tonoplast	Schumaker and Sze, 1987
Beta vulgaris	Storage root	Extraction	Patch-clamp	Tonoplast	Alexandre *et al*., 1990; Alexandre and Lassalles, 1992; Allen and Sanders, 1994; Allen *et al*., 1995
Beta vulgaris	Storage root	None[a]	$^{45}Ca^{2+}$	Tonoplast	Brosnan and Sanders, 1990
Beta vulgaris	Storage root	Sucrose gradient	[^{3}H]$InsP_3$	Tonoplast	Johannes *et al*., 1992*b*; Brosnan and Sanders, 1993
Beta vulgaris	Storage root	Sucrose gradient	$^{45}Ca^{2+}$	Tonoplast	Allen *et al*., 1995
Brassica oleracea L.	Inflorescence	Sucrose gradient	$^{45}Ca^{2+}$/antibody	Smooth ER/ tonoplast Rough ER/PM	Muir and Sanders, 1997
Chenopodium album L.	Cell suspension culture	Ficoll gradient	Indo-1	Tonoplast	Lommel and Felle, 1997
		Sucrose gradient	Ca^{2+}-selective mini-electrode	Tonoplast	Lommel and Felle, 1997
Chenopodium rubrum	Leaf	Sucrose gradient/ free-flow electrophoresis	[^{3}H]$InsP_3$	ER[b]	Martinec *et al*., 2000
Daucus carota L.	Cell suspension culture	Free-flow electrophoresis	$^{45}Ca^{2+}$	Tonoplast	Canut *et al*., 1993
Vicia faba L.	Leaf	Sucrose gradient	Antibody	Tonoplast	Cramer *et al*., 1998
		Two-phase partitioning	Antibody	PM[b]	Cramer *et al*., 1998
Vigna radiata	Hypocotyl	None[a]	$^{45}Ca^{2+}$	Tonoplast	Biswas *et al*., 1995

[a] The origin of the $InsP_3$-sensitive Ca^{2+} stores was identified only by pharmacological approach.
[b] ER, endoplasmic reticulum; PM, plasma membrane.

In addition to this, evidence is emerging that there are other $InsP_3$-sensitive Ca^{2+} stores in the plant cell. Some studies have pointed to the plasma membrane (Muir and Sanders 1997; Cramer *et al*., 1998), but these results may be artefacts to some extent because the plasma membrane is in close appositional contact with the membrane system of the cortical endoplasmic reticulum (ER) and this contact is further stabilized by the structures of the cytoskeleton (Hepler *et al*., 1990). This artefact can be removed by using the microfilament-disruptive drug cytochalasin B (Lièvremont *et al*., 1994). By preparing the ER-enriched fraction from the microsomes of *C. rubrum*, Martinec *et al*. (2000) showed that the specific [^{3}H]$InsP_3$ binding is co-localized with this fraction. It seems clear that in many plants there is another $InsP_3$-sensitive Ca^{2+} store besides the central vacuole. Only the ER and plasma membrane bear a sufficient Ca^{2+} gradient (Bush, 1995); the role of the ER seems to be proved, but the possible involvement of the plasma membrane remains to be elucidated in the future.

The $InsP_3$-R is probably localized in the membrane microdomains (Muir and Sanders, 1997) or its function is at least modified by some factor present in the microdomains (Biswas *et al*., 1995). In summary, although the IICR seems to be a well established Ca^{2+}-releasing mechanism in plants, its subcellular localization has not been satisfactorily unravelled so far.

Conclusions and future perspectives

In vivo evidence for IICR is strong and it should encourage researchers to persevere in their efforts in identifying the plant protein(s) and corresponding gene(s) responsible for it. The overwhelming majority of the experiments presented in this review were carried out with $InsP_3$; nevertheless, a physiological role also undoubtedly exists for $InsP_6$, at least in guard cells. In the light of this finding, some *in vivo* experiments (Gilroy *et al*., 1990, 1991) should be re-evaluated with consideration given to the potential conversion of $InsP_3$ into $InsP_6$. One can imagine engineering mutants compromised in $InsP_6$ synthesis and their crossing with *cvp2* to show that the *cvp2* phenotype in these hybrids remains conserved and thus it is really a result of an elevated $InsP_3$ level and not a result of a consecutively increased $InsP_6$ level.

The electrophysiological approach has been used only by two laboratories and it gave some positive results only with *B. vulgaris* vacuoles. Even though this approach is the most reliable one, such limitations prevent some general conclusions about plant cell signalling to be made. The lack of published positive experimental data may point to a number of negative unpublished results which makes the whole issue even hazier. The parameters of IICR obtained by *in vitro* Ca^{2+} transport assay are quite reliable too as they measure the function of the putative $InsP_3$-R, unlike the $InsP_3$ binding assay which may mix up the $InsP_3$-R with other $InsP_3$-binding proteins (e.g. $InsP_3$-metabolizing enzymes).

Immunological data are quite confusing, reporting fragments of various molecular masses which makes one doubt about any relevant structural similarities between the animal and plant $InsP_3$-R. This heterogeneity is further supported by the failure of the *in silico* approach. Subcellular localization studies point mainly to the vacuole as the prevalent $InsP_3$-sensitive Ca^{2+} store, but even at this point there is some reliable evidence for other localizations such as the ER. The observed discrepancies may originate in the diversity of the studied plant materials or in their different developmental stages.

All presented studies are based on those performed earlier on the animal $InsP_3$-R. It is clear that the animal receptor has higher affinity for $InsP_3$ (between 1 and 100 nM) and it is present in microsomes at at least 10 times higher relative abundance (Simonyi *et al*., 1998; Vanlingen *et al*., 1999; Coquil *et al*., 2004) than the plant receptor, with the exception of *C. rubrum* (Scanlon *et al*., 1996). Also the $InsP_3$-releasable Ca^{2+} pool is much larger in animal cells where it represents up to 90% of the Ca^{2+} mobilizable from the ER (Prentki *et al*., 1985). This finding points to the existence of another mechanism for Ca^{2+} mobilization in plants and implies IICR as its potential trigger.

Several strategies in identifying the responsible gene(s) can be proposed. Provided that the signalling molecule is really $InsP_3$, one can imagine a screening of a ethylmethylsulphonate-mutated population of *Arabidopsis* plants with stable expression of aequorin. The first level of such screening should reveal mutants with lower Ca^{2+} influx upon ABA treatment. Among these mutants, a second screening should be carried out to select only those which have the same basal level and the same degree of $InsP_3$ induction in the first seconds upon ABA treatment as the original non-mutated aequorin transformants. This second round of selection should exclude mutations in $InsP_3$-metabolizing enzymes. The point mutations in these selected mutants can then be mapped to genes and these genes can be studied in detail, for example by complementation in yeast. This approach would certainly be very costly and time-consuming but has a good chance to succeed and finally identify the $InsP_3$-R gene(s). It is quite probable that the responsible protein is not a canonical receptor/ion channel molecule as in animals and it would not be surprising if the IICR would be assured by a heteromeric subunit complex. Some exciting strategies using heterologous expression systems have been demonstrated recently such as the one for identification of a novel plant cell surface calcium-sensing receptor (Han *et al*., 2003). These strategies would require a system where the whole $InsP_3$-mobilizing machinery would work and where the IICR would be

absent or minor. Even if such a system existed, this approach would fail if the $InsP_3$-R was a subunit complex, which is quite likely. Purification of the whole receptor/ion channel monitored by its function is not a good idea as this would be experimentally extremely costly with an uncertain outcome. One would need to reconstitute the membrane vesicles with the purified fraction in each step and still the whole complex could require some cytosolic factor which can be easily lost during the purification process. Another strategy would be to look for high-affinity binding sites for $InsP_3$ in the plant microsomal fraction and then try to purify these sites. This would certainly reveal some ordinary $InsP_3$-metabolizing enzymes associated with membranes, but one of these sites could be the receptor subunit or directly a part of the putative $InsP_3$-R. Then, by means of the classical proteomics approach, the gene could be identified. If this turns out to be only one subunit of the whole complex, it would be necessary to look for its binding partners by pull-down experiments or yeast two-hybrid screens. Even though investigators have been very close to reaching this goal (Biswas *et al*., 1995), this final step has not been done yet. As soon as the putative gene(s) is found by any of the approaches, *Arabidopsis* T-DNA knock-out or RNA interference (RNAi) mutants should confirm that deletion of the corresponding protein(s) diminishes or even abolishes the *in vivo* observed IICR. Plant $InsP_3$-R is a missing piece in the plant phosphoinositide signalling puzzle and its identification awaits those willing to accept the challenge.

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References

Acharya MK, Durejamunjal I, Guhamukherjee S. 1991. Light-induced rapid changes in inositolphospholipids and phosphatidylcholine in *Brassica* seedlings. *Phytochemistry* **30,** 2895–2897.

Aducci P, Marra M. 1990. IP_3 levels and their modulation FY fusicoccin measured by a novel [^{3}H]IP_3 binding assay. *Biochemical and Biophysical Research Communications* **168,** 1041–1046.

Allen GJ, Muir SR, Sanders D. 1995. Release of Ca^{2+} from individual plant vacuoles by both $InsP_3$ and cyclic ADP-ribose. *Science* **268,** 735–737.

Allen GJ, Sanders D. 1994. Osmotic stress enhances the competence of *Beta vulgaris* vacuoles to respond to inositol 1,4,5-trisphosphate. *The Plant Journal* **6,** 687–695.

Alexandre J, Lassalles JP. 1990. Effect of D-*myo*-inositol 1,4,5-trisphosphate on the electrical properties of the red beet vacuole membrane. *Plant Physiology* **93,** 837–840.

Alexandre J, Lassalles JP. 1992. Intracellular Ca^{2+} release by $InsP_3$ in plants and effect of buffers on Ca^{2+} diffusion. *Philosophical Transactions of the Royal Society B: Biological Sciences* **338,** 53–61.

Alexandre J, Lassalles JP, Kado RT. 1990. Opening of Ca^{2+} channels in isolated red beet root vacuole membrane by inositol 1,4,5-trisphosphate. *Nature* **343,** 567–570.

Apone F, Alyeshmerni N, Wiens K, Chalmers D, Chrispeels MJ, Colucci G. 2003. The G-protein-coupled receptor GCR1 regulates DNA synthesis through activation of phosphatidylinositol-specific phospholipase C. *Plant Physiology* **133,** 571–579.

Belde PJM, Vossen JH, Borst-Pauwels GWFH, Theuvenet APR. 1993. Inositol 1,4,5-trisphosphate releases Ca^{2+} from vacuolar membrane vesicles of *Saccharomyces cerevisiae*. *FEBS Letters* **323,** 113–118.

Biswas S, Dalal B, Sen M, Biswas BB. 1995. Receptor for *myo*-inositol trisphosphate from the microsomal fraction of *Vigna radiata*. *The Biochemical Journal* **306,** 631–636.

Blatt MR, Thiel G, Trentham DR. 1990. Reversible inactivation of K^+ channels of *Vicia* stomatal guard cells following the photolysis of caged inositol 1,4,5-trisphosphate. *Nature* **346,** 766–769.

Brearley CA, Hanke DE. 1996*a*. Metabolic evidence for the order of addition of individual phosphate esters to the *myo*-inositol moiety of inositol hexakisphosphate in the duckweed *Spirodela polyrhiza* L. *The Biochemical Journal* **314,** 227–233.

Brearley CA, Hanke DE. 1996*b*. Inositol phosphates in barley (*Hordeum vulgare* L.) aleurone tissue are stereochemically similar to the products of breakdown of $InsP_6$ *in vitro* by wheat-bran phytase. *The Biochemical Journal* **318,** 279–286.

Brosnan JM. 1990. Opening plant calcium channels. *Nature* **344,** 593–593.

Brosnan JM, Sanders D. 1990. Inositol trisphosphate-mediated Ca^{2+} release in beet microsomes is inhibited by heparin. *FEBS Letters* **260,** 70–72.

Brosnan JM, Sanders D. 1993. Identification and characterization of high-affinity binding sites for inositol trisphosphate in red beet. *The Plant Cell* **5,** 931–940.

Bultynck G, Sienaert I, Parys JB, Callewaert G, De Smedt H, Boens N, Dehaen W, Missiaen L. 2003. Pharmacology of inositol trisphosphate receptors. *European Journal of Physiology* **445,** 629–642.

Burnette RN, Gunesekera BM, Gillaspy GE. 2003. An *Arabidopsis* inositol 5-phosphatase gain-of-function alters abscisic acid signaling. *Plant Physiology* **132,** 1011–1019.

Bush DS. 1995. Calcium regulation in plant cells and its role in signalling. *Annual Review of Plant Physiology and Plant Molecular Biology* **46,** 95–122.

Canut H, Carrasco A, Graziana A, Boudet AM, Ranjeva R. 1989. Inositol trisphosphate-stimulated calcium release from *Acer* microsomal fractions involves the uptake of potassium. *FEBS Letters* **253,** 173–177.

Canut H, Carrasco A, Rossignol M, Ranjeva R. 1993. Is vacuole the richest store of IP_3-mobilizable calcium in plant cells? *Plant Science* **90,** 135–143.

Carland FM, Nelson T. 2004. Cotyledon Vascular Pattern2-mediated inositol (1,4,5) triphosphate signal transduction is essential for closed venation patterns of *Arabidopsis* foliar organs. *The Plant Cell* **16,** 1263–1275.

Challiss RA, Willcocks AL, Mulloy B, Potter BV, Nahorski SR. 1991. Characterization of inositol 1,4,5-trisphosphate and inositol 1,3,4,5-tetrakisphosphate-binding sites in rat cerebellum. *The Biochemical Journal* **274,** 861–867.

Chandok MR, Sopory SK. 1994. 5-Hydroxytryptamine affects turnover of polyphosphoinositides in maize and stimulates nitrate reductase in the absence of light. *FEBS Letters* **356,** 39–42.

Cho MH, Tan Z, Erneux C, Shears SB, Boss WF. 1995. The effects of mastoparan on the carrot cell plasma membrane

polyphosphoinositide phospholipase C. *Plant Physiology* **107,** 845–856.

Chopra LC, Twort CH, Ward JP, Cameron IR. 1989. Effects of heparin on inositol 1,4,5-trisphosphate and guanosine 5′-*O*-(3-thiotriphosphate) induced calcium release in cultured smooth muscle cells from rabbit trachea. *Biochemical and Biophysical Research Communications* **163,** 262–268.

Coquil JF, Blazquez S, Soave S, Mauger JP. 2004. Regulation of the cerebellar inositol 1,4,5-trisphosphate receptor by univalent cations. *The Biochemical Journal* **381,** 423–428.

Cornelius G, Gebauer G, Techel D. 1989. Inositol trisphosphate induces calcium release from *Neurospora crassa* vacuoles. *Biochemical and Biophysical Research Communications* **162,** 852–856.

Coté GG, Crain RC. 1993. Biochemistry of phosphoinositides. *Annual Review of Plant Physiology and Plant Molecular Biology* **44,** 333–356.

Coté GG, Morse MJ, Crain RC, Satter RL. 1987. Isolation of soluble metabolites of the phosphatidylinositol cycle from *Samanea saman*. *Plant Cell Reports* **6,** 352–355.

Coursol S, Giglioli-Guivarc'h N, Vidal J, Pierre JN. 2000*a*. An increase in phosphoinositide-specific phospholipase C activity precedes induction of C-4 phosphoenolpyruvate carboxylase phosphorylation in illuminated and NH_4Cl-treated protoplasts from *Digitaria sanguinalis*. *The Plant Journal* **23,** 497–506.

Coursol S, Pierre JN, Vidal J. 2000*b*. Role of the phosphoinositide pathway in the light-dependent C-4 phosphoenolpyruvate carboxylase phosphorylation cascade in *Digitaria sanguinalis* protoplasts. *Biochemical Society Transactions* **28,** 821–823.

Cramer H, Meinhard M, Schulz M, Schanbl H. 1998. Immunological detection of an inositol 1,4,5-trisphosphate receptor in microsomes from *Vicia faba* (L.) and *Zea mays* (L.) and broadbean vacuoles. *International Journal of Plant Sciences* **159,** 750–752.

Dasgupta S, Dasgupta D, Chatterjee A, Biswas S, Biswas BB. 1997. Conformational changes in plant $Ins(1,4,5)P_3$ receptor on interaction with different *myo*-inositol trisphosphates and its effect on Ca^{2+} release from microsomal fraction and liposomes. *The Biochemical Journal* **321,** 355–360.

Dasgupta S, Dasgupta D, Sen M, Biswas S, Biswas BB. 1996. Interaction of myoinositol trisphosphate–phytase complex with the receptor for intracellular Ca^{2+} mobilization in plants. *Biochemistry* **35,** 4994–5001.

DePass AL, Crain RC, Hepler PK. 2001. Inositol 1,4,5 trisphosphate is inactivated by a 5-phosphatase in stamen hair cells of *Tradescantia*. *Planta* **213,** 518–524.

DeWald DB, Torabinejad J, Jones CA, Shope JC, Cangelosi AR, Thompson JE, Prestwich GD, Hama H. 2001. Rapid accumulation of phosphatidylinositol 4,5-bisphosphate and inositol 1,4,5-trisphosphate correlates with calcium mobilization in salt-stressed *Arabidopsis*. *Plant Physiology* **126,** 759–769.

Drøbak BK. 1992. The plant phosphoinositide system. *The Biochemical Journal* **288,** 697–712.

Drøbak BK, Ferguson IB. 1985. Release of Ca^{2+} from plant hypocotyl microsomes by inositol 1,4,5-trisphosphate. *Biochemical and Biophysical Research Communications* **130,** 1241–1246.

Drøbak BK, Watkins PAC. 1994. Inositol(1,4,5)trisphosphate production in plant cells—stimulation by the venom peptides, mellitin and mastoparan. *Biochemical and Biophysical Research Communications* **205,** 739–745.

Drøbak BK, Watkins PAC. 2000. Inositol(1,4,5)trisphosphate production in plant cells: an early response to salinity and hyperosmotic stress. *FEBS Letters* **481,** 240–244.

Drøbak BK, Watkins PAC, Chattaway JA, Roberts K, Dawson AP. 1991. Metabolism of inositol(1,4,5)trisphosphate by a soluble enzyme fraction from pea (*Pisum sativum*) roots. *Plant Physiology* **95,** 412–419.

Drory A, Borochov A, Mayak S. 1992. Transient water-stress and phospholipid turnover in carnation flowers. *Journal of Plant Physiology* **140,** 116–120.

Durejamunjal I, Acharya MK, Guhamukherjee S. 1992. Effect of hormones and spermidine on the turnover of inositol-phospholipids in *Brassica* seedlings. *Phytochemistry* **31,** 1161–1163.

Eddy SR. 1998. Profile hidden Markov models. *Bioinformatics* **14,** 755–763.

Ferris CD, Snyder SH. 1992. Inositol 1,4,5-trisphosphate-activated calcium channels. *Annual Review of Physiology* **54,** 469–488.

Finch EA, Turner TJ, Goldin SM. 1991. Calcium as a coagonist of inositol 1,4,5-trisphosphate-induced calcium release. *Science* **252,** 443–446.

Förster B. 1990. Injected inositol 1,4,5-trisphosphate activates Ca^{2+}-sensitive K^+ channels in the plasmalemma of *Eremosphaera viridis*. *FEBS Letters* **269,** 197–201.

Franklin-Tong VE, Drøbak BK, Allan AC, Watkins PAC, Trewavas AJ. 1996. Growth of pollen tubes of *Papaver rhoeas* is regulated by a slow-moving calcium wave propagated by inositol 1,4,5-trisphosphate. *The Plant Cell* **8,** 1305–1321.

Gelli A, Blumwald E. 1993. Calcium retrieval from vacuolar pools. *Plant Physiology* **102,** 1139–1146.

Gilroy S, Fricker MD, Read ND, Trewavas AJ. 1991. Role of calcium in signal transduction of *Commelina* guard cells. *The Plant Cell* **3,** 333–334.

Gilroy S, Read ND, Trewavas AJ. 1990. Elevation of cytoplasmic calcium by caged calcium or caged inositol trisphosphate initiates stomatal closure. *Nature* **346,** 769–771.

Guillemette G, Lamontagne S, Boulay G, Mouillac B. 1989. Differential effects of heparin on inositol 1,4,5-trisphosphate binding, metabolism and calcium release activity in the bovine adrenal cortex. *Molecular Pharmacology* **35,** 339–344.

Han S, Tang R, Anderson LK, Woerner TE, Pei ZM. 2003. A cell surface receptor mediates extracellular Ca^{2+} sensing in guard cells. *Nature* **425,** 196–200.

Hepler PK, Palevitz BA, Lancelle SA, McCauley MM, Lichtscheidl I. 1990. Cortical endoplasmic reticulum in plants. *Journal of Cell Science* **96,** 355–373.

Hunt L, Mills LN, Pical C, Leckie CP, Aitken FL, Kopka J, Mueller-Roeber B, McAinsh MR, Hetherington AM, Gray JE. 2003. Phospholipase C is required for the control of stomatal aperture by ABA. *The Plant Journal* **34,** 47–55.

Hunt L, Otterhag L, Lee JC, *et al.* 2004. Gene-specific expression and calcium activation of *Arabidopsis thaliana* phospholipase C isoforms. *New Phytologist* **162,** 643–654.

Johannes E, Brosnan JM, Sanders D. 1992*a*. Parallel pathways for intracellular Ca^{2+} release from the vacuole of higher plants. *The Plant Journal* **2,** 97–102.

Johannes E, Brosnan JM, Sanders D. 1992*b*. Calcium channels in the vacuolar membrane of plants: multiple pathways for intracellular calcium mobilization. *Philosophical Transactions of the Royal Society B: Biological Sciences* **338,** 105–112.

Jones DL, Kochian LV. 1995. Aluminum inhibition of the inositol 1,4,5-trisphosphate signal transduction pathway in wheat roots—a role in aluminum toxicity. *The Plant Cell* **7,** 1913–1922.

Kalinoski DL, Aldinger SB, Boyle AG, Huque T, Marecek JF, Prestwich GD, Restrepo D. 1992. Characterization of a novel inositol 1,4,5-trisphosphate receptor in isolated olfactory cilia. *The Biochemical Journal* **281,** 449–456.

Kamada Y, Muto S. 1994. Stimulation by fungal elicitor of inositol phospholipid turnover in tobacco suspension-culture cells. *Plant and Cell Physiology* **35,** 397–404.

Kanoh H, Haga M, Iwata M, Seikizawa Y. 1993. Causal analysis of reaction cascades in the induced defense mechanisms of rice plants. Transmembrane signalling operated at rice blade cells stimulated by blast fungus elicitor. Operation of the phospholipase C system. *Journal of Pesticide Science* **18,** 299–308.

Kashem MA, Itoh K, Iwabuchi S, Hori H, Mitsui T. 2000. Possible involvement of phosphoinositide–Ca^{2+} signalling in the regulation of alpha-amylase expression and germination of rice seed (*Oryza sativa* L.). *Plant and Cell Physiology* **41,** 399–407.

Kim HY, Cote GG, Crain RC. 1996. Inositol 1,4,5-trisphosphate may mediate closure of K^+ channels by light and darkness in *Samanea saman* motor cells. *Planta* **198,** 279–287.

Krinke O, Novotná Z, Valentová O, Martinec J. 2003. *In vitro* fluorimetric measurement of ligand gated calcium channels in plants (in Czech). *Biologické listy* **3,** 195–199.

Lee YS, Choi YB, Suh S, Lee J, Assmann SM, Joe CO, Kelleher JF, Crain RC. 1996. Abscisic acid-induced phosphoinositide turnover in guard cell protoplasts of *Vicia faba*. *Plant Physiology* **110,** 987–996.

Legendre L, Yueh YG, Crain R, Haddock N, Heinstein PF, Low PS. 1993. Phospholipase C activation during elicitation of the oxidative burst in cultured plant cells. *Journal of Biological Chemistry* **268,** 24559–24563.

Lemtiri-Chlieh F, MacRobbie EAC, Brearley CA. 2000. Inositol hexakisphosphate is a physiological signal regulating the K^+-inward rectifying conductance in guard cells. *Proceedings of the National Academy of Sciences, USA* **97,** 8687–8692.

Lemtiri-Chlieh F, MacRobbie EAC, Webb AAR, Manison NF, Brownlee C, Skepper JN, Chen J, Prestwich GD, Brearley CA. 2003. Inositol hexakisphosphate mobilizes an endomembrane store of calcium in guard cells. *Proceedings of the National Academy of Sciences, USA* **100,** 10091–10095.

Lièvremont J-P, Hill A-M, Hilly M, Mauger J-P. 1994. The inositol trisphosphate receptor is localised on specialised subregions of the endoplasmic reticulum in rat liver. *The Biochemical Journal* **300,** 419–427.

Lin WH, Ye R, Ma H, Xu ZH, Xue HW. 2004. DNA chip-based expression profile analysis indicates involvement of the phosphatidylinositol signalling pathway in multiple plant responses to hormone and abiotic treatments. *Cell Research* **14,** 34–45.

Liu HT, Gao F, Cui SJ, Han JL, Sun DY, Zhou RG. 2006. Primary evidence for involvement of IP_3 in heat-shock signal transduction in *Arabidopsis*. *Cell Research* **16,** 394–400.

Loewus FA, Murthy PPN. 2000. *Myo*-inositol metabolism in plants. *Plant Science* **150,** 1–19.

Lommel C, Felle HH. 1997. Transport of Ca^{2+} across the tonoplast of intact vacuoles from *Chenopodium album* L. suspension cells: ATP-dependent import and inositol-1,4,5-trisphosphate-induced release. *Planta* **201,** 477–486.

Maeda N, Niinobe M, Mikoshiba K. 1990. A cerebellar Purkinje cell marker P_{400} protein is an inositol 1,4,5-trisphosphate ($InsP_3$) receptor protein. Purification and characterization of $InsP_3$ receptor complex. *EMBO Journal* **9,** 61–67.

Martinec J, Feltl T, Scanlon CH, Lumsden PJ, Macháčková I. 2000. Subcellular localization of a high affinity binding site for D-*myo*-inositol 1,4,5-trisphosphate from *Chenopodium rubrum*. *Plant Physiology* **124,** 475–483.

Monteiro D, Liu Q, Lisboa S, Scherer GE, Quader H, Malhó R. 2005. Phosphoinositides and phosphatidic acid regulate pollen tube growth and reorientation through modulation of $[Ca^{2+}]_c$ and membrane secretion. *Journal of Experimental Botany* **56,** 1665–1674.

Morse MJ, Crain RC, Satter RL. 1987. Light-stimulated inositol-phospholipid turnover in *Samanea saman* leaf pulvini. *Proceedings of the National Academy of Sciences, USA* **84,** 7075–7078.

Mueller-Roeber B, Pical C. 2002. Inositol phospholipid metabolism in *Arabidopsis*. Characterized and putative isoforms of inositol phospholipid kinase and phosphoinositide-specific phospholipase C. *Plant Physiology* **130,** 22–46.

Muir SR, Bewell MA, Sanders D, Allen GJ. 1997. Ligand-gated Ca^{2+} channels and Ca^{2+} signalling in higher plants. *Journal of Experimental Botany* **48,** 589–597.

Muir SR, Sanders D. 1997. Inositol 1,4,5-trisphosphate-sensitive Ca^{2+} release across nonvacuolar membranes in cauliflower. *Plant Physiology* **114,** 1511–1521.

Munnik T, Irvine RF, Musgrave A. 1998. Phospholipid signalling in plants. *Biochimica et Biophysica Acta* **1389,** 222–272.

Nunn DL, Taylor CW. 1990. Liver inositol 1,4,5-trisphosphate-binding sites are the Ca^{2+}-mobilizing receptors. *The Biochemical Journal* **270,** 227–232.

Ortega X, Perez LM. 2001. Participation of the phosphoinositide metabolism in the hypersensitive response of *Citrus limon* against *Alternaria alternata*. *Biological Research* **34,** 43–50.

Ortega X, Velasquez JC, Perez LM. 2005. IP_3 production in the hypersensitive response of lemon seedlings against *Alternaria alternata* involves active protein tyrosine kinases but not a G-protein. *Biological Research* **38,** 89–99.

Perera IK, Heilmann I, Chang SC, Boss WF, Kaufman PB. 2001. A role for inositol 1,4,5-trisphosphate in gravitropic signalling and the retention of cold-perceived gravistimulation of oat shoot pulvini. *Plant Physiology* **125,** 1499–1507.

Perera IY, Heilmann I, Boss WF. 1999. Transient and sustained increases in inositol 1,4,5-trisphosphate precede the differential growth response in gravistimulated maize pulvini. *Proceedings of the National Academy of Sciences, USA* **96,** 5838–5843.

Perera IY, Hung CY, Brady S, Muday GK, Boss WF. 2006. A universal role for inositol 1,4,5-trisphosphate-mediated signaling in plant gravitropism. *Plant Physiology* **140,** 746–760.

Perera IY, Love J, Heilmann I, Thompson WF, Boss WF. 2002. Up-regulation of phosphoinositide metabolism in tobacco cells constitutively expressing the human type I inositol polyphosphate 5-phosphatase. *Plant Physiology* **129,** 1795–1806.

Peskan T, Westermann M, Oelmuller R. 2000. Identification of low-density Triton X-100-insoluble plasma membrane microdomains in higher plants. *European Journal of Biochemistry* **267,** 6989–6995.

Pfeiffer W, Hager A. 1993. A Ca^{2+}-ATPase and a Mg^{2+}/H^+-antiporter are present on tonoplast membranes from roots of *Zea mays* L. *Planta* **191,** 377–385.

Ping Z, Yabe I, Muto S. 1992. Identification of K^+, Cl^-, and Ca^{2+} channels in the vacuolar membrane of tobacco cell suspension cultures. *Protoplasma* **171,** 7–18.

Pozzan T, Rizzuto R, Volpe P, Meldolesi J. 1994. Molecular and cellular physiology of intracellular calcium stores. *Physiological Reviews* **74,** 595–636.

Prentki M, Corkey BE, Matschinsky FM. 1985. Inositol 1,4,5-trisphosphate and the endoplasmic reticulum Ca^{2+} cycle of a rat insulinoma cell line. *Journal of Biological Chemistry* **260,** 9185–9190.

Ranjeva R, Carrasco A, Boudet AM. 1988. Inositol trisphosphate stimulates the release of calcium from intact vacuoles isolated from *Acer* cells. *FEBS Letters* **230,** 137–141.

Reddy ASN, Poovaiah BW. 1987. Inositol 1,4,5-trisphosphate induced calcium release from corn coleoptile microsomes. *Journal of Biochemistry* **101,** 569–573.

Richardson A, Taylor CW. 1993. Effects of Ca^{2+} chelators on purified inositol 1,4,5-trisphosphate ($InsP_3$) receptors and $InsP_3$ stimulated Ca^{2+} mobilization. *Journal of Biological Chemistry* **268,** 11582–11587.

Rincon M, Boss WF. 1987. *Myo*-inositol trisphosphate mobilizes calcium from fusogenic carrot (*Daucus carota* L.) protoplasts. *Plant Physiology* **83,** 395–398.

Ruelland E, Cantrel C, Gawer M, Kader JC, Zachowski A. 2002. Activation of phospholipases C and D is an early response to a cold exposure in *Arabidopsis* suspension cells. *Plant Physiology* **130,** 999–1007.

Samanta S, Dalal B, Biswas S, Biswas BB. 1993. Myoinositol tris-phosphate–phytase complex as an elicitor in calcium mobilization in plants. *Biochemical and Biophysical Research Communications* **191,** 427–434.

Sanders D, Johannes E. 1990. Opening plant calcium channels. *Nature* **344,** 593–594.

Sanders D, Miller AJ, Blackford S, Brosnan JM, Johannes E. 1990. Cytosolic free calcium homeostasis in plants. *Current Topics in Plant Biochemistry and Physiology* **9,** 20–37.

Scanlon CH, Martinec J, Macháčková I, Rolph CE, Lumsden PJ. 1996. Identification and preliminary characterization of a Ca^{2+}-dependent high-affinity binding site for inositol-1,4,5-trisphosphate from *Chenopodium rubrum*. *Plant Physiology* **110,** 867–874.

Scanlon CH, Martinec J, Rolph CE, Lumsden PJ. 1995. Characterisation of inositol 1,4,5-trisphosphate receptors from *Chenopodium rubrum*. *Biochemical Society Transactions* **23,** S574–S574.

Schumaker KS, Sze H. 1987. Inositol 1,4,5-trisphosphate releases Ca^{2+} from vacuolar membrane vesicles of oat roots. *Journal of Biological Chemistry* **262,** 3944–3946.

Shigaki T, Bhattacharyya MK. 2000. Decreased inositol 1,4,5-trisphosphate content in pathogen-challenged soybean cells. *Molecular Plant–Microbe Interactions* **13,** 563–567.

Simonyi A, Xia J, Igbavboa U, Wood WG, Sun GY. 1998. Age differences in the expression of metabotropic glutamate receptor 1 and inositol 1,4,5-trisphosphate receptor in mouse cerebellum. *Neuroscience Letters* **244,** 29–32.

Smoleńska-Sym G, Kacperska A. 1994. Phosphatidylinositol metabolism in low temperature-affected winter oilseed rape leaves. *Physiologia Plantarum* **91,** 1–8.

Smoleńska-Sym G, Kacperska A. 1996. Inositol 1,4,5-trisphosphate formation in leaves of winter oilseed rape plants in response to freezing, tissue water potential and abscisic acid. *Physiologia Plantarum* **96,** 692–698.

Srivastava A, Pines M, Jacoby B. 1989. Enhanced potassium uptake and phosphatidylinositol 4,5-bisphosphate turnover by hypertonic mannitol shock. *Physiologia Plantarum* **77,** 320–325.

Staxen I, Pical C, Montgomery LT, Gray JE, Hetherington AM, McAinsh MR. 1999. Abscisic acid induces oscillations in guard-cell cytosolic free calcium that involve phosphoinositide-specific phospholipase C. *Proceedings of the National Academy of Sciences, USA* **96,** 1779–1784.

Stevenson JM, Perera IY, Heilmann I, Persson S, Boss WF. 2000. Inositol signaling and plant growth. *Trends in Plant Science* **5,** 252–258.

Takahashi S, Katagiri T, Hirayama T, Yamaguchi-Shinozaki K, Shinozaki K. 2001. Hyperosmotic stress induces a rapid and transient increase in inositol 1,4,5-trisphosphate independent of abscisic acid in *Arabidopsis* cell culture. *Plant and Cell Physiology* **42,** 214–222.

Taylor CW, Traynor D. 1995. Calcium and inositol trisphosphate receptors. *Journal of Membrane Biology* **145,** 109–118.

Thiel G, MacRobbie EAC, Hanke DE. 1990. Raising the intracellular level of inositol 1,4,5-trisphosphate changes plasma membrane ion transport in characean algae. *EMBO Journal* **9,** 1737–1741.

Tones MA, Bootman MD, Higgins BH, Lane DA, Pay GF, Lindahl U. 1989. The effect of heparin on the inositol 1,4,5-trisphosphate receptor in rat liver microsomes: dependence on sulphate content and chain length. *FEBS Letters* **252,** 105–108.

Toyoda K, Shiraishi T, Yamada T, Ichinose Y, Oku H. 1993. Rapid changes in polyphosphoinositide metabolism in pea in response to fungal signals. *Plant and Cell Physiology* **34,** 729–735.

Tucker EB, Boss WF. 1996. Mastoparan-induced intracellular Ca^{2+} fluxes may regulate cell-to-cell communication in plants. *Plant Physiology* **111,** 459–467.

van der Luit AH, Piatti T, van Doorn A, Musgrave A, Felix G, Boller T, Munnik T. 2000. Elicitation of suspension-cultured tomato cells triggers the formation of phosphatidic acid and diacylglycerol pyrophosphate. *Plant Physiology* **123,** 1507–1516.

van Leeuwen W, Ökrész L, Bögre L, Munnik T. 2004. Learning the lipid language of plant signalling. *Trends in Plant Science* **9,** 378–384.

Vanlingen S, Sipma H, Missiaen L, De Smedt H, De Smet P, Casteels R, Parys JB. 1999. Modulation of type 1, 2 and 3 inositol 1,4,5-trisphosphate receptors by cyclic ADP-ribose and thimerosal. *Cell Calcium* **25,** 107–114.

Vasconsuelo A, Morelli S, Picotto G, Giulietti AM, Boland R. 2005. Intracellular calcium mobilization: a key step for chitosan-induced anthraquinone production in *Rubia tinctorum* L. *Plant Science* **169,** 712–720.

Walton TJ, Cooke CJ, Newton RP, Smith CJ. 1993. Evidence that generation of inositol 1,4,5-trisphosphate and hydrolysis of phosphatidylinositol 4,5-bisphosphate are rapid responses following addition of fungal elicitor which induces phytoalexin synthesis in lucerne (*Medicago sativa*) suspension culture cells. *Cellular Signalling* **5,** 345–356.

Wang X. 2001. Plant phospholipases. *Annual Review of Plant Physiology and Plant Molecular Biology* **52,** 211–231.

Wang X. 2004. Lipid signaling. *Current Opinion in Plant Biology* **7,** 329–336.

Wang SY, Faust M. 1995. Changes of inositol phosphates during decapitation-induced lateral bud break of *Malus domestica*. *Physiologia Plantarum* **93,** 471–475.

Wilcox RA, Challiss RA, Traynor JR, Fauq AH, Ognayanov VI, Kozikowski AP, Nahorski SR. 1994. Molecular recognition at the *myo*-inositol 1,4,5-trisphosphate receptor. 3-Position substituted *myo*-inositol 1,4,5-trisphosphate analogues reveal the binding and Ca^{2+} release requirements for high affinity interaction with the *myo*-inositol 1,4,5-trisphosphate receptor. *Journal of Biological Chemistry* **269,** 26815–26821.

Worley PF, Baraban JM, Supattapone S, Wilson VS, Snyder SH. 1987. Characterization of inositol trisphosphate receptor binding in brain. *Journal of Biological Chemistry* **262,** 12132–12136.

Xiong LM, Lee BH, Ishitani M, Lee H, Zhang CQ, Zhu JK. 2001. FIERY1 encoding an inositol polyphosphate 1-phosphatase is a negative regulator of abscisic acid and stress signalling in *Arabidopsis*. *Genes and Development* **15,** 1971–1984.

Xiong L, Schumaker KS, Zhu JK. 2002. Cell signaling during cold, drought, and salt stress. *The Plant Cell* **14,** S165–S183.

Zbell BA, Walter-Back C, Bucher H. 1989. Evidence of an auxin-mediated phosphoinositide turnover and an inositol(1,4,5)trisphosphate effect on isolated membranes of *Daucus carota* L. *Journal of Cellular Biochemistry* **40,** 331–340.