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Fusarium elicitor-dependent calcium influx and associated ros generation in tomato is independent of cell death

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Abstract *Fusarium oxysporum* f. sp. *lycopersici* elicitor (EFOL-2) treatment induces cytosolic influx of calcium in *Fusarium*-resistant tomato suspension culture. The calcium signature was found to be biphasic, which is characteristic of recognition of oligosaccharides in the elicitor preparation. Further, several lines of evidence such as, (i) attainment of saturation level of the $[Ca^{2+}]_{cyt}$ at a definite extra-cellular calcium concentration (ii) prominent reduction in EFOL-2-induced influx in $[Ca^{2+}]_{cyt}$ on treatment with the calcium channel blockers verapamil and diltiazem and (iii) establishment of a refractory stage of $[Ca^{2+}]_{cyt}$ level upon repeated stimulation by EFOL-2, is indicative of receptor-mediated activation of the calcium channel for cytosolic elevation. In addition, inhibition of EFOL-2-induced $[Ca^{2+}]_{cyt}$ increase by protein kinase inhibitor staurosporine and wortmannin indicate phosphorylation is a regulatory event of calcium influx. Additionally, monitoring of cell death on EFOL-2 treatment indicated that the degree of ROS generation is not capable of inducing cell death. Inhibition of ROS generation on two separate occasions such as, calcium-free media and on treatment with inhibitors causing calcium channel occlusion revealed ROS generation as a successive event of calcium influx.

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Abbreviations

BAPTA	1,2-bis- (2- aminophenoxy) ethane N,N,N`,N`-tetraacetic acid
BAP	Benzyl amino purine
$[Ca^{2+}]_{cyt}$	Cytosolic-free calcium
CDPK	Calcium-dependent protein kinase
H_2DCFDA	dihydro dichloro fluorescein diacetate
DCFDA	2`,7`-Dihydrodichlorofluorescein diacetate
EFOL-2	Elicitor of *Fusarium oxysporum* f. sp. *lycopersici* Race 2
ETI	Effector-Triggered Immunity
EGTA	ethylene glycol-bis-(2-aminoethylether)-N, N, N`, N`-tetraacetic acid
FDA	Fluorescein di acetate
FACS	Fluoresence-Assisted Cell Sorter
hpt	Hours Post-Treatment
HR	Hypersensitive Response
IP_3	inositol 1,4,5-triphosphate
MFI	Mean Fluorescence Intensity
NAA	Napthelene Acetic Acid
PAMPs	Pathogen-Associated Molecular Patterns
PTI	PAMP-Triggered Immunity
Ptdins-3 kinase	phosphatidylinositol kinase
PI	phosphatidylinositol

PIP	phosphatidylinositol 4 monophosphate
PIP_2	phosphatidylinositol 4,5 bisphosphate
ROS	Reactive Oxygen Species
TFP	Trifluoroperazine

Introduction

Plants on interaction with pathogens initiate a plethora of signals as a component of the innate defence system. The first line of response is triggered by a ligand-specific recognition of the pathogen associated molecular patterns (PAMPs) by the receptors on the cell surface in a process of PAMP triggered immunity (PTI) (Chisholm et al. 2006; Mucyn et al. 2006). Concurrently, resistance gene or R gene-mediated recognition of pathogen-derived effectors by the plant, form the second line of response, leading to gene-for-gene resistance (Jia et al. 2000; Dey et al. 2006) or more precisely effector-triggered immunity (ETI) (Chisholm et al. 2006). Conserved microbial features initiating PTI include bacterial lipopolysaccharide, flagellin as well as fungal cell wall components, commonly referred to as general elicitors of plant defence, which however are not found in host cells (Nurnberger et al. 2006; Ali et al. 2007). Primary perception of these 'non-self' factor(s) trigger kinase activation, production of reactive oxygen species (ROS), induction of pathogen responsive genes, and deposition of callose (Nurnberger et al. 2006), which collectively lead to plant defence. However, most of these responses often encounter a variation in cytosolic calcium concentration, which remains the most critical step in the induction of plant innate immunity (Ali et al. 2007; Ma and Berkowitz 2007).

PAMP-induced increase in cytosolic calcium concentration has been detected in tobacco (Chandra and Low 1997; Lecourieux et al. 2002), tomato (Gelli et al. 1997), *Arabidopsis* (Tegg et al. 2005), rice (Kurusu et al. 2005) and parsley (Blume et al. 2000), though the amplitude and duration of the transient increase is variable in different plants. The change in levels of cytosolic calcium, manifested as specific 'signatures', help in conveying signals received across the cell surface to the intracellular milieu (Kurusu et al. 2005) which in turn are decoded by internal calcium sensors, thereby initiating a response (Karita et al. 2004). In addition, calcium has been observed to play a pivotal role as a secondary messenger in signalling events coordinating myriad physiological processes in plants including mechanical and low temperature signals, oxidative stress, hypoosmotic shock, light, hormones and *Nod* factors (Sanders et al. 1999). Physiological studies have revealed that the influx of calcium leads to changes in membrane potential at the initial stage of the defence response (Blume et al. 2000; Kadota et al. 2004) and is often accompanied by the generation of reactive oxygen species (ROS) (Link et al. 2002) mediated hypersensitive response (HR) and associated cell death (Levine et al. 1994).

The generation of ROS and cognate redox signalling play a central role in the integration of an array of defence regulatory pathways which include oxidative cross-linking, lignification of the host cell wall, transcription of defence-related genes, secondary metabolite biosynthesis, and direct pathogen cytotoxity (Grant and Loake 2000). Several lines of evidence suggest the role of ROS as an intracellular diffusible signal for the activation of downstream responses upon wounding and biotic stress (Orozco-Cardenas et al. 2001). In a cultured cell system, the oxidative burst is promoted by isolated elicitors, like harpin (Chandra et al. 1996) oligouronides (Legendre et al. 1993), elicitins (Virad et al. 1994), purified fungal peptides (Vera-Estrella et al. 1992), and other molecules from the extracts of pathogens (Schwacke and Hager 1992).

Pathogen-induced calcium influx has been reported to occur both before (Schwacke and Hager 1992; Davies et al. 2006) and after (Kawano and Muto 2000; Rentel and Knight 2004) ROS generation. The *Trichoderma viride* xylanase-induced ROS generation and cell death in rice cell suspension was severely compromised in the presence of the calcium chelator ethylene glycol-bis- (2-aminoethylether)-N, N, N`, N`-tetraacetic acid (EGTA) and 1,2-bis- (2- aminophenoxy) ethane N,N, N`,N`-tetraacetic acid (BAPTA) (Kurusu et al. 2005). Similarly, calcium channel blockers and chelators in tobacco (Chandra and Low 1997), *Arabidopsis* (Grant et al. 2000) and parsley (Blume et al. 2000) prevented the expression of elicitor-induced oxidative burst. ROS generation in cultured spruce cells induced by elicitors from mycorrhizal fungi was suppressed in calcium-depleted media. Besides, insertional mutagenesis of the rice Ca^{2+} permeable channel (OsTPC1) revealed suppression of elicitor-mediated cell death, due to ROS,

and activation of MAP kinase (Kurusu et al. 2005) suggesting the necessity of intracellular-free calcium for the generation of ROS (Schwacke and Hager 1992; Kurusu et al. 2005). Calcium has also been reported to be required for MAP kinase activation upon *Fusarium*-derived elicitor treatment in tomato cell suspension culture (Link et al. 2002), although information on the mode and nature of variation of intracellular-free calcium as well as dependence of calcium on generation of ROS is not available.

The present study thus aims at different aspects of the involvement of intra-cellular calcium store(s), the role of calcium-bound proteins and cognate phosphorylation / dephosphorylation, on transient increase in intracellular calcium concentration and its relation to the one of the most important defence components such as ROS generation and associated HR mediated cell death upon *Fusarium*-derived elicitor treatment in *Fusarium*-resistant tomato (*Lycopersicon esculentum* cv. C295) cell suspension culture. Findings reveal the unequivocal requirement of calcium as a regulatory component of the signalling mechanism.

Materials and methods

Proliferation of callus and growth of cell suspension culture

Callus of the tomato var. C295 was induced from cotyledon and hypocotyl explants in Murashige and Skoog's medium supplemented with NAA (5 mg l^{-1}) and BAP (2 mg l^{-1}). After three cycles of subculture for a period of 21 days in each culture, photoautotrophic cell suspension culture was established in half concentration of MS medium supplemented with NAA (0.5 mg l^{-1}) and BAP (0.2 mg l^{-1}), set at pH 5.5. Cells of the exponential growth phase were considered for experimental sampling. To ensure uniform experimental conditions, the cultures were mixed one day prior to treatment. An initial cell concentration of 10^5 cells ml^{-1} was maintained for all experiments.

Preparation of elicitor from *Fusarium oxysporum* f.sp. *lycopersici*

Fusarium oxysporum f.sp. *lycopersici*, Fol1007, Race 2 was obtained from the Swammerdam Institute of Life Sciences, The Netherlands. The fungus was cultured in the medium in accordance with Sinha et al. (2002). After 4 days of shaking at 29°C, the culture was autoclaved to kill the fungus, washed several times with double distilled water and lyophilised. The elicitor (EFOL-2) stored at −20°C was used at a concentration of 150 mg l^{-1} of tomato cell suspension culture, according to Sinha et al. (2002).

Measurement of cytosolic-free calcium

The cytosolic-free calcium was measured using FURA 2 fluorescence according to Grynkiewicz et al. (1985). Cell suspension was loaded with 50 μM Fura 2 AM (Sigma) and incubated at 37°C in dark for 1 h in ½ MS media, pH 5.5. The cells were hen washed with the media twice to remove non-hydrolysed Fura 2 AM and transferred to a 3 ml capacity quartz cuvette of a spectrofluorometer (Hitachi model 3010). Fluorescence was measured at 37°C with excitation at 380 nm and emission at 510 nm. To convert fluorescence values into cytosolic calcium ion concentration, calibration was performed at the end of each experiment. Influx of calcium on treatment of Fura-2 loaded cells with 5 μM calcium ionophore A23187 (Sigma) was considered as F_{max}. Subsequently, Fura-2 fluorescence of the intracellular calcium was quenched by addition of 300 μM Mn^{2+} and considered as F_{min}. The concentration of cytosolic-free calcium was then obtained from the values of F, F_{max} and F_{min} by using the following formula:

$$\left[Ca^{2+}\right]_{cyt} = K_d(F - F_{min})/(F_{max} - F),$$

where, K_d was the dissociation constant of calcium with Fura 2 (224 nM) (Grynkiewicz et al. 1985). Each experiment was repeated with at least three biological replicates with five technical replicates under similar conditions. Fluorescence intensities were expressed as an average of sample numbers (*n*). The comparative change in fluorescence intensity with respect to the control (EFOL-2 treated), was estimated by calculating the total peak area using Microcal Origin version 6.0. Inhibitors, obtained from Sigma; these were added 10 min prior to EFOL-2 treatment.

Measurement of intracellular reactive oxygen species (ROS)

Aliquots (5 ml) of tomato cell suspension were dispensed into 25 ml conical flasks for individual

treatment. Cells were treated with EFOL-2 in such a way, so that all the samples could be harvested at one time-point after the treatment at various time-points according to the experimental requirement, to ensure uniform experimental conditions. For measurement of ROS, the cells were stained with H_2DCFDA (2`,7` - Dihydrodichlorofluorescein diacetate), which has been used as a highly sensitive fluorescent indicator for the formation of peroxides such as H_2O_2 and lipid hydroperoxides (Ezaki et al. 2000). The cells were incubated in the dark at 37°C for 1 h in the presence of 50 μM H_2DCFDA (Sigma) in 0.1 M phosphate-buffered saline (PBS, pH 7.0). Intracellular ROS generation was monitored in a cell population of 10,000 cells, against control in a flow cytometer (FACS Calibur; Becton Dickinson, San Jose CA). Excitation of DCF was monitored at 488 nm and emission was detected at 530±15 nm as FL1 fluoresecensce. For a comparative estimate of the degree of ROS generation, the Mean Fluorescence Intensity (MFI) at the FL1 region was calculated as M1 beyond the logarithmic scale of 10^1, to minimise the interference of the cellular auto fluorescence at the FL1 range.

Determination of cell viability

Cells were stained with fluorescein diacetate (FDA) (Sigma) at a final concentration of 50 mg l^{-1} in 0.1 M phosphate-buffered saline (PBS, pH 7.0). Intracellular lipases cause the degradation of FDA to fluorescein leading to fluorescence of intact viable cells. The cells were incubated for 15 min and subsequently washed with the same buffer and observed under a fluorescence microscope. For quantification, cell viability was monitored in a population of 10,000 cells, against control by flow cytometry using similar configurations as stated previously. Cellular viability was monitored at 0, 6, 12 and 24 h post-treatment (hpt) and expressed as the percentage of cells showing maximum Fl-1 fluorescence, beyond the logarithmic scale of 10^1 in a scatter plot.

All flow cytometry results were obtained in triplicate using high flow rates. List mode data were converted from Hewlett Packard to Apple (Macintosh) computer format using the programme FACS-Convert (Becton Dickinson, San Jose, CA). Data were then analysed using the CellQuest software (version 3.3; Becton Dickinson). All data were obtained in triplicate and the Newman Keuls *post-hoc* test was done to check the level of significance.

Results

Fusarium elicitor triggers a biphasic increase in cytosolic calcium concentration $[Ca^{2+}]_{cyt}$ in tomato cells

Prominent 'calcium signatures' consisting of two distinct peaks were observed within 30–300 s of EFOL-2-treated cells with 10 mM extra cellular calcium, as evident from the spectro-fluorimetric data. The first rise within 50 s of treatment, persisted for 50–65 s, resulting in $(Ca^{2+})_{cyt}$ peak values of 85.63± 0.66 n moles. The second increase in cytosolic calcium occurred within 150–300 s of elicitor treatment, which persisted for 135–150 s, attaining a peak value of 77.9±1.50 n moles, which ultimately attained baseline levels within 400 s (Fig. 1a). The biphasic calcium signature was highly reproducible (n=20), though no variation was observed upon further EFOL-2 treatment within the next 30 min (data not shown) indicating attainment of a refractory stage for the said period.

EFOL-2-driven mobilisation of extracellular and intracellular pools of calcium

To check whether variation in extra cellular calcium levels may influence difference in EFOL-2-induced intracellular calcium fluxes, different calcium concentrations starting from 0.5–25 mM calcium chloride in HEPES- mannitol buffer pH 5.6 was used in separate experimental sets. A distinct variation in amplitude of the peaks was observed in different extracellular concentrations, which attained maximum peak area at 10 mM concentration. The variation is represented in Fig. 1b as total peak area vs. difference in cytosolic-free calcium. Further, no change in intracellular-free calcium was evident in calcium-free media and both peaks originating due to EFOL-2 treatment were suppressed. To further determine the mode of calcium influx, cells were treated with the calcium channel blockers (verapamil, diltiazem and nifedipine) 10 min prior to EFOL-2 treatment, keeping EFOL-2-treated cells as the control. Results revealed that intracellular-free calcium fluxes were severely compromised in the

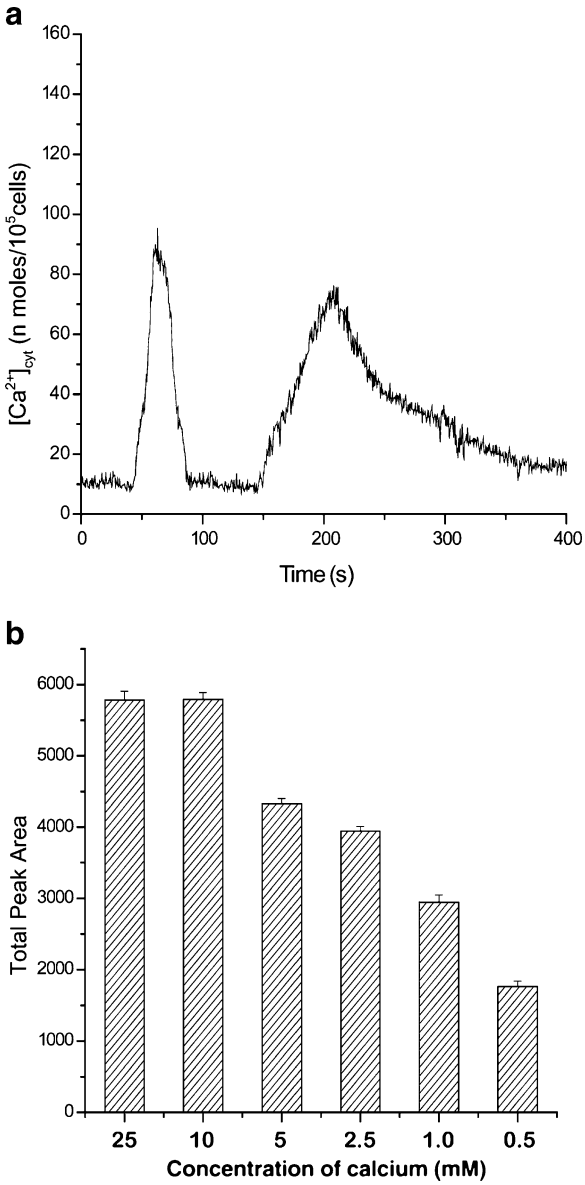

Fig. 1 EFOL-2-induced increase in levels of intracellular free calcium in suspension-cultured *L. esculentum* var. C295. (a) Variation in EFOL-2-induced cytosolic calcium fluxes and (b) histogram showing total peak area due to different concentrations (mM) of extracellular calcium added in calcium-free media. The error bars indicate SE (n=15)

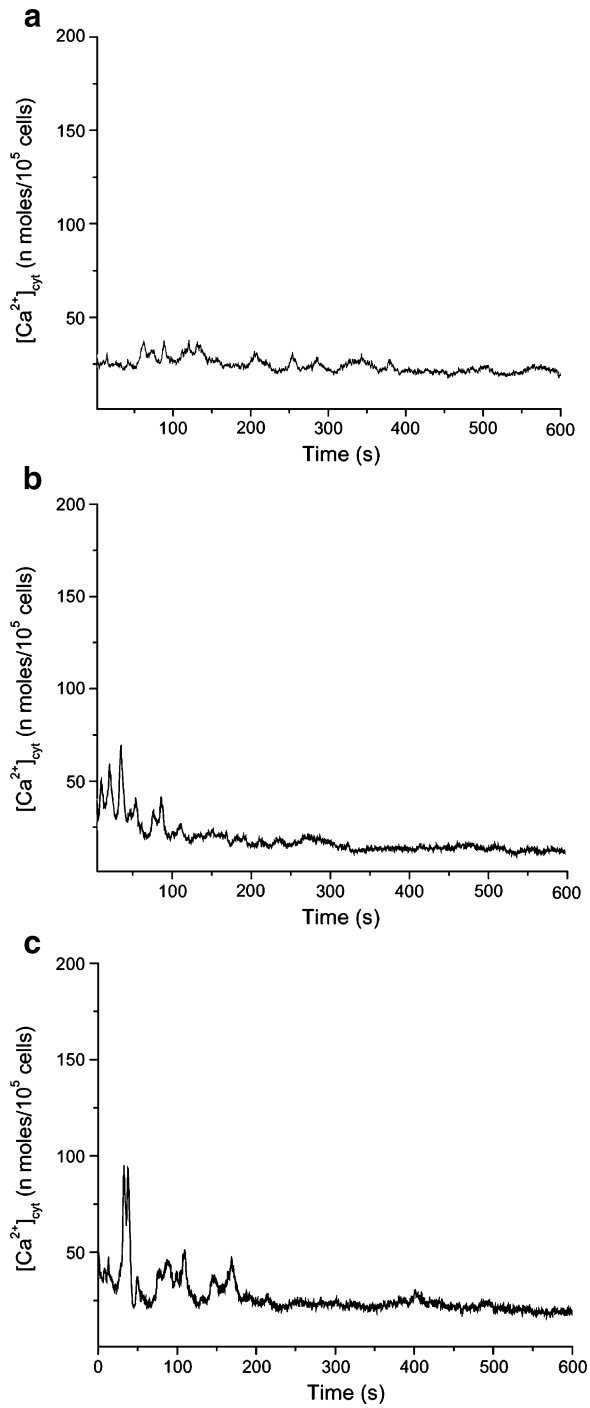

Fig. 2 Variation in EFOL-2-induced cytosolic calcium fluxes in *L. esculentum* var. C295 due to treatment with various calcium channel blockers: (a) verapamil, (b) diltiazem, and (c) nifedipine

presence of verapamil (Fig. 2a) and diltiazem (Fig. 2b) (79.11% and 73.37% decrease over elicitor-treated cells) at 100 μM concentration respectively. While minor changes in baseline levels were observed upon treatment with verapamil, multiple small fluxes within the first 50 s were observed with diltiazem,

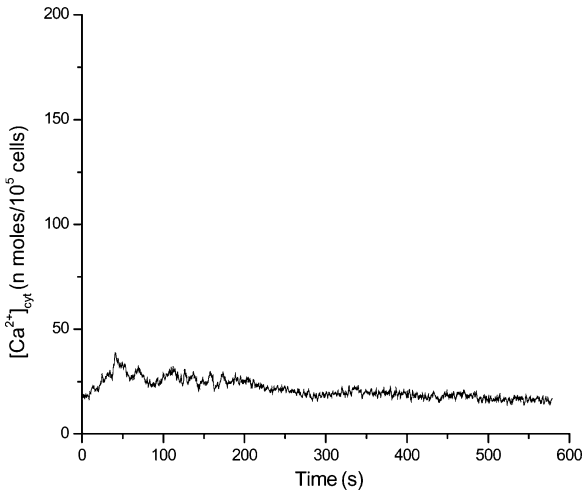

Fig. 3 Variation in EFOL-2-induced calcium fluxes in the presence of trifluoroperazine

although no variation in calcium fluxes was observed for either of the inhibitors used in the absence of EFOL-2 within the same time frame. In spite of the primary variation, no secondary peak was observed in either case. In case of nifedipine, although variation in the form of a primary flux within the first 50 s, along with consequent minor peaks within the next 200 s was observed (Fig. 2c)(59.54% decrease over control), it seems that the efficiency of this channel blocker is not so pronounced compared to the effects of verapamil and diltiazem. The confirmation of calcium-dependent activation was further observed upon treatment with Trifluoroperazine (TFP), an inhibitor of the calcium-binding domain containing protein including calmodulin, where calcium fluxes were severely compromised (Fig. 3).

To investigate the release of calcium from internal stores in elicitor-induced increase in $[Ca^{2+}]_{cyt}$, the cells were treated with neomycin, an inhibitor of phospholipase C involved in the inositol 1,4,5-triphosphate (IP_3)-mediated intracellular Ca^{2+} release (Franklin-Tong et al. 1996). Addition of 50 μM neomycin, 10 min prior to elicitor treatment revealed diminishing secondary calcium release (Fig. 4). The primary peak was, however, not affected, thereby suggesting that the first $[Ca^{2+}]_{cyt}$ increase which peaked within 50 s of elicitor treatment, resulted from extracellular calcium influx, while the second peak at 150–300 s possibly resulted due to release of calcium from internal stores.

Role of protein phosphorylation/dephosphorylation in $[Ca^{2+}]_{cyt}$ increase

The kinase inhibitor staurosporine was used to monitor phosphorylation-dependent intracellular calcium influx in tomato cells upon EFOL-2 treatment. Staurosporine treatment (50 μM) indicated 86.06% inhibition in total peak area of cytoplasmic-free calcium over the control (Fig. 5a). Wortmannin was used to further investigate the role of phosphatidylinositol kinase (Ptdins-3 kinase), which functions in phosphorylation of the membrane-bound phosphatidylinositol (PI), phosphatidylinositol 4 monophosphate (PIP) and phosphatidylinositol 4,5 bisphosphate (PIP_2), products of which may in turn act as secondary messengers (Mueller-Roeber and Pical 2002). Addition of 50 μM wortmannin revealed 79.7 % suppression of calcium fluxes upon elicitor treatment (Fig. 5b). For better representation, the effect of all the inhibitors used on EFOL-2-induced calcium fluxes is summarised in Fig. 6.

EFOL-2-mediated ROS generation in relation to intracellular calcium fluxes and cell death

Prominent ROS generation in elicitor-induced cell suspension culture was measured by the DCF fluorescence at the FL-1 range (Fig. 7). Significant amount (revealed from one-way ANOVA and the Newman Keuls *post-hoc* test) of ROS generation ($P<0.001$)

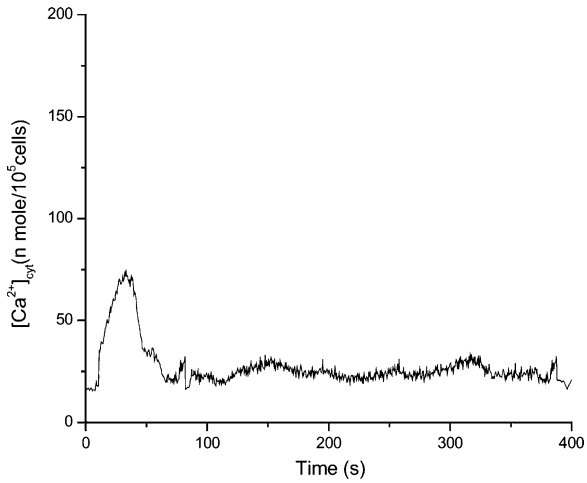

Fig. 4 Effect of neomycin on $[Ca^{2+}]_{cyt}$ changes in EFOL-2-treated cells

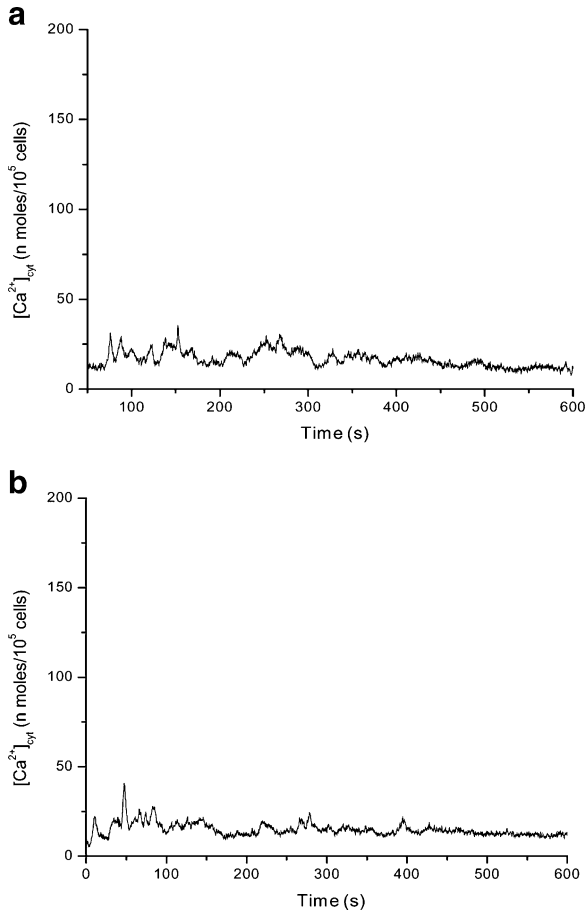

Fig. 5 Variation in EFOL-2-induced calcium fluxes upon treatment with protein kinase inhibitors (a) staurosporine and (b) wortmannin

was observed with 203.71% in Mean Fluorescence Intensity (MFI) over the control, within 1 h of elicitor treatment. Gradual decrease in the degree of ROS generation to control levels was attained within 12 h of elicitor treatment (Figs. 7, and 8a).

To determine the influence of extra cellular calcium in the development of elicitor-induced intracellular ROS generation, cells were suspended in HEPES- mannitol buffer, pH 5.6 (without calcium). ROS generation was subsequently monitored in cell suspensions culture, up to 3 h after elicitor treatment. Results indicated a significant decrease ($P<0.001$) in EFOL-2-induced ROS generation in calcium-deprived media (Fig. 8b) with 39.57% of ROS generation over the control, in comparison to 203.71% in calcium-supplemented media (Fig. 8a), 1 h after EFOL-2 treatment.

Various calcium channel inhibitors were used to further establish the relationship between the influx of calcium in the cytosol through plasma membrane-bound calcium channels with the induction of intracellular ROS generation. The cell suspension was treated with the pharmacological inhibitors 10 min prior to elicitor treatment (Fig. 8). Significant decrease in ROS generation was observed upon treatment with the calcium channel blockers verapamil ($P<0.01$) and diltiazem ($P<0.001$) but there was no significant decrease in ROS generation in the presence of nifedipine within a period of 1 h. Furthermore, a significant decrease in ROS generation ($P<0.01$) was observed in the presence of Trifluoroperazine (TFP), an inhibitor of calcium-binding proteins, like calmodulin and CDPKs (Fig. 8).

Although EFOL-2 treatment induces transient ROS generation, no apparent cell death was observed upon FDA treatment (as observed by fluorescence microscopy) or quantitative measurement (by flow cytometry) in tomato cell suspension culture, showing about 83.71% cell viability 24 hpt, in comparison to 84.48% at 0 hpt (Fig. 9). These results reveal that neither calcium fluxes, nor ROS generation have been part of a cytotoxic effect or induce programmed cell death.

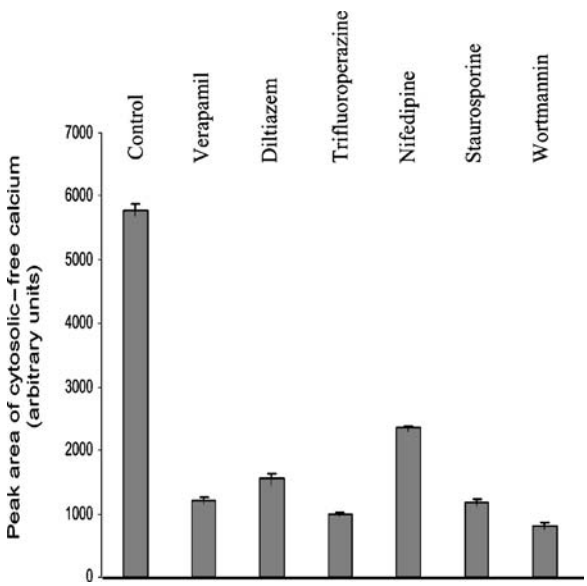

Fig. 6 Bar diagram representing the peak area of cytosolic-free calcium upon EFOL-2 treatment in suspension-cultured cells of *L. esculentum* var. C295 in the presence of inhibitors as indicated. The error bars indicate SE ($n=15$)

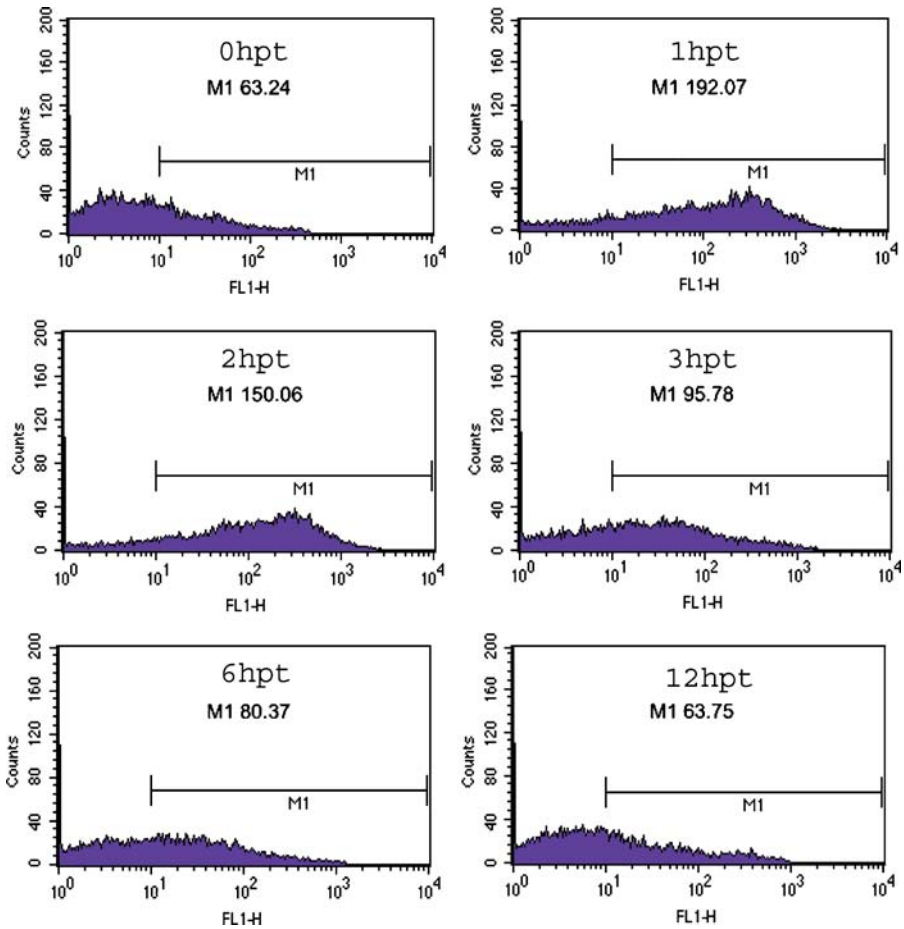

Fig. 7 Flow cytometric estimation of relative FL1 fluorescence of DCF detected upon EFOL-2-induced variation in intracellular ROS generation in cell suspension culture of C295 at the various time-points (hours post treatment, hpt) as indicated

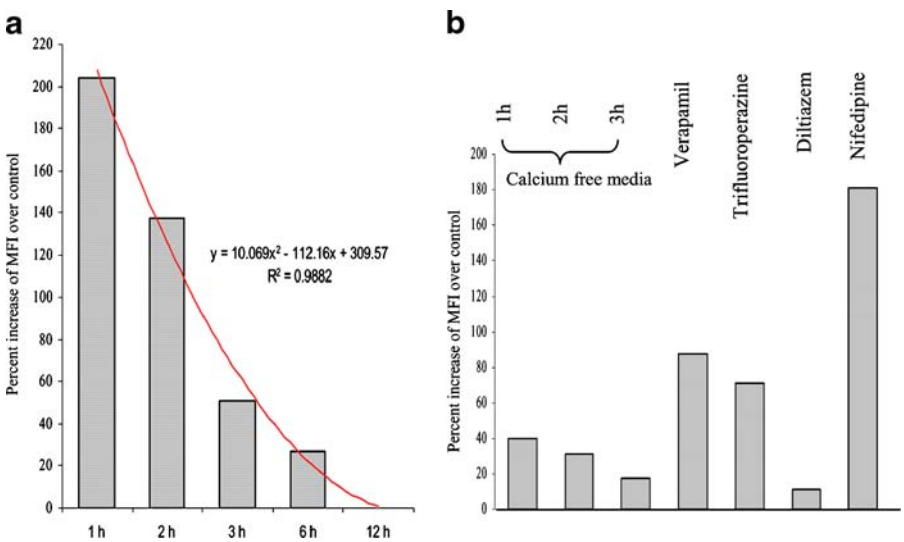

Fig. 8 EFOL-2-induced time-dependent ROS generation in cell suspension-cultured *L. esculentum* var. C295, represented as percent increase; (a) Mean Fluorescence Intensity (MFI) over control (untreated) and (b) ROS generation in calcium-free media and in the presence of various calcium channel blockers at 1 hpt

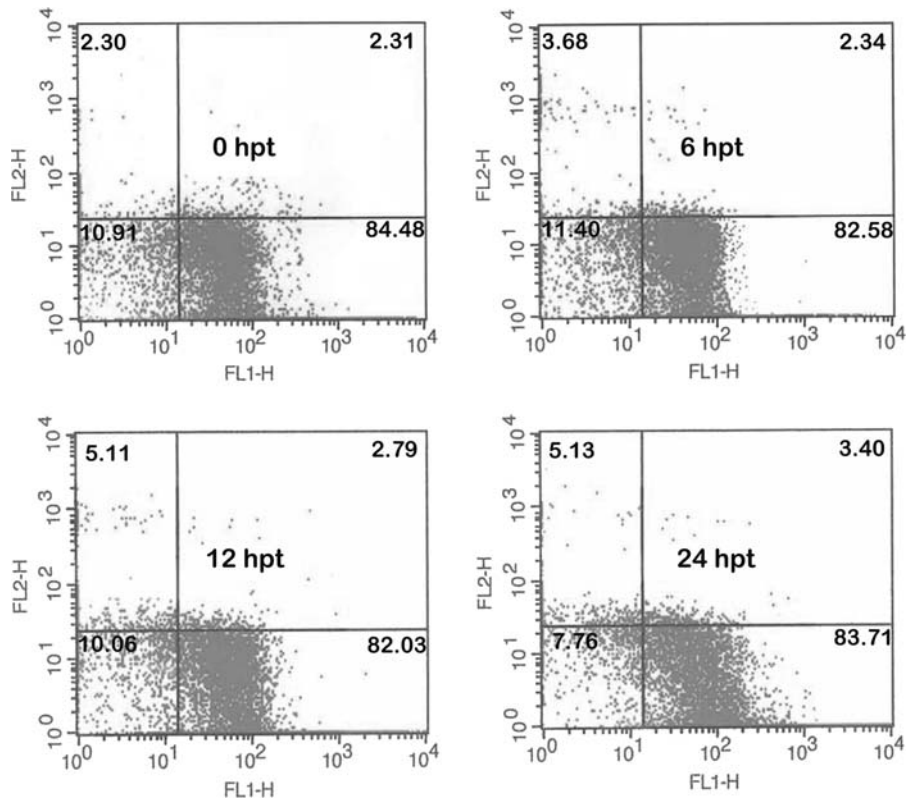

Fig. 9 Scatter plot of EFOL-2-treated cells upon FDA staining by flow cytometry at different time-points (hpt) as indicated. Live cells were detected as FL1 fluorescence beyond 10^1. Numbers in quadrants indicate percentage of cells in each. The figure is a representative assay of a sample of three

Discussion

Fusarium wilt is a devastating pathological syndrome causing huge yield loss in a wide range of crops worldwide. Considering the enormity of the problem, the time-dependent response to pathogen recognition was studied to understand the primary messengers upon Fusarium elicitation in tomato. Elevation in levels of cytosolic calcium on perception of pathogen-derived factors forms a fundamental entity as a secondary messenger coupling the extracellular stimuli to intracellular global responses in plant systems (Ma and Berkowitz 2007). Analogous to animals, the cytosolic elevation of calcium in plants on interaction with a pathogen has been ascribed to influx across the plasma membrane and intracellular release from the tonoplast and / or endoplasmic reticulum (Blume et al. 2000). While calcium influx has been observed upon both PTI and ETI-mediated defences, evidence suggests that PAMP alone is capable of activating inward plasma membrane calcium currents in plant cells (Ali et al. 2007). Thus, in the present study, variation in levels of cytosolic calcium in tomato cells on treatment with Fusarium elicitor, EFOL-2 was monitored using the flourogenic dye Fura 2 AM, since Fura 2 AM binds to calcium in a stoichiometry of 1:1 (Bush and Jones 1990) and has been used widely for monitoring the levels of intracellular calcium in oilseed rape, tomato, barley, corn root and *Santalum album* (Bush and Jones 1990; Anil and Rao 2000).

The transient biphasic calcium signature upon elicitation of tomato cell culture by EFOL-2 is reported here for the first time and focuses on the possibility of recognition of the oligosaccharide-derived factors in the elicitor preparation. This is contrary to an initial peak followed by a second 'peak' which was sustained and did not attain baseline levels within a certain time frame, usually attributed to proteinaceous elicitors (Lecourieux et al. 2002, 2005). Considering the mode of EFOL-2 preparation, the fungal cell wall-derived non-metabolisable sugar derivatives may be the major signalling component and has already been reported to transduce specific defence-related stimuli in tomato (Sinha et al. 2002). Particular calcium signatures have been identified to act as an encrypted signal, unique to the nature of

gene activation and subsequent cellular response in *Nicotiana* and *Arabidopsis (*Lecourieux et al. 2002; Rentel and Knight 2004) on interaction with either elicitor or pathogen. In the present study, transient cytosolic calcium elevation, manifested as a distinct signature, characterised by magnitude, frequency and duration instead of repeated oscillations, has been studied which does specify a distinct signalling machinery precisely to *Fusarium* elicitation in tomato.

Several lines of evidence suggest that the increase in $[Ca^{2+}]_{cyt}$ is a successive event of a specific recognition. Attainment of saturation level of the $[Ca^{2+}]_{cyt}$ at 10 mM extra-cellular concentration of calcium as well as prominent reduction in EFOL-2-induced influx in $[Ca^{2+}]_{cyt}$ on treatment with the calcium channel blockers verapamil and diltiazem suggest the possibility of EFOL-2 recognition-activated calcium channel activity for cytosolic elevation. Diverse calcium channels have been found in plant cell membranes, which actively function as a principal component of the cell signalling machinery (White and Broadley 2003). Establishment of a refractory stage of $[Ca^{2+}]_{cyt}$ level upon repeated stimulation by EFOL-2 is further indicative of receptor-mediated activation of the calcium channel, a phenomenon reported earlier using fungal chitooligosaccharides in tomato cells (Boller 1995). In this regard, the dependence of EFOL-2-mediated calcium influx on extracellular calcium concentration has been substantiated by no variation in EFOL-2-stimulated calcium fluxes in calcium-free media, and sustained increment upon increase in extracellular calcium concentration. Another important observation was the inhibition of EFOL-2-induced $[Ca^{2+}]_{cyt}$ increase by the broad spectrum protein kinase inhibitor staurosporine (Rashotte et al. 2001), which establishes that maintenance of the active phosphorylated state of proteins through reversible phosphorylation and dephosphorylation is perhaps a key process in the activation of calcium channels. Phosphorylation-dependent intracellular calcium influx has already been reported in elicitin-dependent signal transduction in tobacco (Lebrun-Garcia et al. 1998) but activation of the same kind of channels by EFOL-2 in tomato is not yet shown. Prominent inhibition of EFOL-2-driven cytosolic calcium fluxes in the presence of wortmannin reflects the possibility of Ptdins-3 kinase-mediated phosphorylation of the membrane-bound PI, PIP and PIP_2, products which may act as secondary messengers (Mueller-Roeber and Pical 2002) leading to activation of the calcium channels during this process. However, specific identification of the type of kinase responsible for the process needs further experimentation.

In addition, TFP-mediated attenuation of calcium influx proves the role of the calcium binding domain containing proteins in EFOL-2-mediated activation of calcium channels. This is consistent with findings of calcium, calmodulin-binding sites at the C terminal domain of human voltage-gated calcium channels (Le Blanc et al. 2004; Jurkat-Rott and Lehmann-Horn 2004). The neomycin-dependent suppression of the second peak at 3 min probably indicates release of free calcium from intracellular stores in a phospholipase C-mediated pathway. This phenomenon was also observed in tobacco (Lecourieux et al. 2002) and soybean (Chandra et al. 1996; Legendre et al. 1993) suspension culture on elicitation. Furthermore, suppression of the both the peaks on treatment with the calcium channel blockers indicates the primary requirement of calcium influx from an extracellular source through calcium channels for the intracellular calcium release.

The concomitant activation of ROS with increase in $[Ca^{2+}]_{cyt}$ is functionally associated with other defence-related responses in plants (Kurusu et al. 2005). Reduction in levels of ROS in calcium-free media in addition to compromised ROS generation upon treatment with the calcium channel blockers verapamil and diltiazem and the calcium-binding protein inhibitor TFP reveal the upstream requirement of calcium for signalling. Moreover, no significant effect on ROS generation upon treatment with comparatively less sensitive calcium channel blocker nifedipine, further confirms the quantitative requirement of free cytosolic calcium for the induction of ROS. These results are consistent with reports of receptor-mediated ROS generation upon recognition of chitooligosaccharides in rice (Kaku et al. 2006).

Interestingly, knockdown mutants of the chitin affinity binding protein-encoding gene in rice and a chitin receptor like kinase in *Arabidopsis* (Miya et al. 2007) resulted in the suppression of the elicitor-induced oxidative burst (Kaku et al. 2006) as well as the gene responses (Miya et al. 2007), showing that the oligosaccharide receptor plays a key role in the perception and signal transduction of the chitin oligosaccharide elicitor in rice and *Arabidopsis* cells. Moreover, functional characterisation of the *Arabidopsis* NADPH oxidase homologue revealed calcium-

dependent conformational change of the EF-hands to be distinctly responsible for ROS production (Ogasawara et al. 2008). Our observations within the whole cell population substantiate that EFOL-2 treatment does not cause cell death in resistant tomato cell suspension culture in contrast to earlier findings of *Cladosporium fulvum* specific elicitor treatment in tomato (Vera-Estrella et al. 1992) and AVR9 elicitor treatment in tobacco cell suspension culture (Piedras et al. 1998). Considering the fact that no cell death was observed either upon *Fusarium* infection in intact plants during an incompatible interaction (data not shown), we propose that the resistance response is independent of programmed cell death, while the calcium-dependent transient ROS generation is possibly part of the signalling machinery.

In conclusion, our data indicate for the very first time that *Fusarium*-derived elicitor recognition in tomato causes a distinct biphasic calcium signature indicative of a precise defence signalling mechanism. This recognition involves phosphorylation-dependent activation of calcium channels, resulting in calcium influx and the subsequent mobilisation of calcium from intracellular pools. While ROS generation, an established entity of the plant resistance response, is also induced upon EFOL-2 treatment, the phenomenon is unique as it does not stimulate programmed cell death in tomato. The data suggest that the Ca^{2+} fluxes associated with PAMP perception and the associated ROS generation exhibit the properties of a true secondary messenger which precedes initiation of the biological response but is not required to maintain it.

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References

Ali, R., Ma, W., Lemitri-Chlieh, F., Tsaltas, D., Leng, Q., von Bodman, S., et al. (2007). Death don't have no mercy and neither does calcium: *Arabidopsis* cyclic nucleotide gated channel and innate immunity. *Plant Cell, 19*, 1081–1095.

Anil, V. S., & Rao, K. S. (2000). Calcium-mediated signaling during sandalwood somatic embryogenesis. Role for exogenous calcium as second messenger. *Plant Physiology, 123*, 1301–1312.

Blume, B., Nürnberger, T., Nass, N., & Scheel, D. (2000). Receptor mediated increase in cytosolic free calcium required for activation of pathogen defense in parsley. *Plant Cell, 12*, 1425–1440.

Boller, T. (1995). Chemoperception of microbial signals in plant cells. *Annual Review of Plant Physiology and Plant Molecular Biology, 46*, 189–214.

Bush, D. S., & Jones, R. L. (1990). Measuring intracellular Ca^{2+} levels in plant cells using the fluorescent probes, Indo-1 and Fura-2. *Plant Physiology, 93*, 841–845.

Chandra, C., Heinstein, P. F., & Low, P. S. (1996). Activation of Phospholipase A by plant defense elicitors. *Plant Physiology, 110*, 979–986.

Chandra, S., & Low, P. S. (1997). Measurement of Ca^{2+} fluxes during elicitation of the oxidative burst in aequorin transformed tobacco cells. *Journal of Biological Chemistry, 272*, 28274–28280.

Chisholm, S. T., Coaker, G., Day, B., & Staskawicz, B. J. (2006). Host microbe interactions: Shaping the evolution of the plant immune response. *Cell, 124*, 803–814.

Davies, D. R., Bindschedler, L. V., Strickland, T. S., & Bolwell, G. P. (2006). Production of reactive oxygen species in *Arabidopsis thaliana* cell suspension cultures in response to an elicitor from *Fusarium oxysporum*: implications for basal resistance. *Journal of Experimental Botany, 57*(8), 1817–1827.

Dey, S., Ghose, K., Gangopadhyay, G., & Basu, D. (2006). Assessment of genomic diversity of wild and cultivated tomato through Resistance Gene Analogue Polymorphism and *I2* homologues. *Euphytica, 154*, 219–230.

Ezaki, B., Gardner, R. C., Ezake, Y., & Matsumoto, H. (2000). Expression of aluminum-induced genes in transgenic *Arabidopsis* plants can ameliorate aluminum stress and/or oxidative stress. *Plant Physiology, 122*(3), 657–666.

Franklin-Tong, V. E., Drobak, B. K., Allan, A. C., Watkins, P., & Trewavas, A. J. (1996) Growth of pollen tubes of papaver rhoeas is regulated by a slow-moving calcium wave propagated by inositol 1,4,5-trisphosphate. *Plant Cell, 8*, 1305–1321.

Gelli, A., Higgins, V. J., & Blumwald, E. (1997). Activation of plasma membrane Ca^{2+}-permeable channels by race-specific fungal elicitors. *Plant Physiology, 113*, 269–279.

Grant, M., Brown, I., Adams, S., Knight, M., Ainslie, A., & Mansfield, J. (2000). The *RPM1* plant disease resistance gene facilitates a rapid and sustained increase in cytosolic calcium that is necessary for the oxidative burst and hypersensitive cell death. *Plant Journal, 23*, 441–450.

Grant, J. G., & Loake, G. J. (2000). Role of reactive oxygen intermediates and cognate redox signaling in disease resistance. *Plant Physiology, 124*, 21–29.

Grynkiewicz, G., Poenie, M., & Tsein, R. Y. (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *Journal of Biological Chemistry, 260*(6), 3440–3450.

Jia, Y., McAdams, S. A., Bryan, G. T., Hershey, H. P., & Valent, B. (2000). Direct interaction of resistance gene and avirulence gene products confers rice blast resistance. *EMBO Journal, 19*, 4004–4014.

Jurkat-Rott, K., and Lehmann-Horn, F. (2004) The impact of splice isoforms on voltage-gated calcium channel α_1 subunits. *Journal of Physiology*, *554*, 609–619.

Kadota, Y., Goh, T., Tomatsu, H., Tamauchi, R., Higashi, K., Muto, S., et al. (2004). Cryptogein-induced initial events in tobacco BY-2 cells; pharmacological characterization of molecular relationship among cytosolic Ca^{2+} transients, anion efflux and production of reactive oxygen species. *Plant Cell Physiology, 45*, 160–170.

Kaku, H., Nishizawa, Y., Ishii-Minami, N., Akimoto-Tomiyama, C., Dohmae, N., Takio, K., et al. (2006). Plant cells recognize chitin fragments for defense signaling through a plasma membrane receptor. *Proceedings of the National Academy of Science USA, 103*(29), 11086–11091.

Karita, E., Yamakawa, H., Mitsuhara, I., Kuchitsu, K., & Ohashi, Y. (2004). Three types of tobacco calmodulins characteristically activate plant NAD kinase at different Ca^{2+} concentrations and pHs. *Plant Cell Physiology, 45*, 1371–1379.

Kawano, T., & Muto, S. (2000). Mechanism of peroxidase actions for salicylic acid- induced generation of active oxygen species and an increase in cytosolic calcium in tobacco cell suspension culture. *Journal of Experimental Botany, 51*, 685–693.

Kurusu, T., Yagala, T., Miyao, A., Hirochika, H., & Kuchitsu, K. (2005). Identification of a putative voltage gated Ca^{2+}channel as a key regulator of elicitor- induced hypersensitive cell death and mitogen-activated protein kinase activation in rice. *Plant Journal, 42*, 798–809.

Le Blanc, C., Mironneau, C., Barbot, C., Henaff, M., Bondeva, T., Wetzker, R., et al. (2004). Regulation of vascular L type Ca2+ channels by phosphatidyl inositol 3, 4, 5-triphosphate. *Circulation Research, 95*, 300.

Lebrun-Garcia, A., Ouaked, F., Chiltz, A., & Pugin, A. (1998). Activation of MAPK homologues by elicitors in tobacco cells. *Plant Journal, 15*, 773–781.

Lecourieux, D., Lamotte, O., Bourque, S., Wendehenne, D., Mazars, C., Ranjeva, R., et al. (2005). Proteinaceous and oligosaccharide elicitors induce different calcium signatures in the nucleus of tobacco cells. *Cell Calcium, 38*, 527–538.

Lecourieux, D., Mazars, C., Pauly, N., Ranjeva, R., & Pugin, A. (2002). Analysis and effects of cytosolic free calcium increases in response to elicitors in *Nicotiana plumbaginifolia* cells. *Plant Cell, 14*, 2627–2641.

Legendre, L., Rueter, S., Heinstein, P. F., & Low, P. S. (1993). Phospholipase C activation during elicitation of the oxidative burst in cultured plant cells. *Plant Physiology, 102*, 233–240.

Levine, A., Tenhaken, A., Dixon, R., & Lamb, C. (1994). H_2O_2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. *Cell, 79*, 583–593.

Link, V. L., Markus, G. H., Sinha, A. K., Ehness, R., Strnad, M., & Roitsch, T. (2002). Biochemical evidence for the activation of distinct subsets of mitogen- activated protein kinases by voltage and defense-related stimuli. *Plant Physiology, 128*, 271–281.

Ma, W., & Berkowitz, G. A. (2007). The grateful dead: calcium and cell death in plant innate immunity. *Cellular Microbiology, 9*(11), 2571–2585.

Miya, A., Albert, A., Shinya, T., Desaki, Y., Ichimura, K., Shirasu, K., et al. (2007). CERK1, a LysM receptor kinase, is essential for chitin elicitor signaling in *Arabidopsis*. *Proceedings of the National Academy of Science (USA), 104*(49), 19613–19618.

Mucyn, T. S., Clemente, A., Andriotis, V. M. E., Balmuth, A. L., Oldroyd, G. E. D., Staskawicz, B. J., et al. (2006). The tomato NBARC-LRR protein Prf interacts with the Pto kinase in vivo to regulate specific plant immunity. *Plant Cell, 18*, 2792–2806.

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.

Nurnberger, T., Brunner, F., Kemmerling, B., & Piater, L. (2006). Innate immunity in plants and animals: striking similarities and obvious differences. *Immunolgical Reviews, 198*(1), 249–266.

Ogasawara, Y., Kaya, H., Hiraoka, G., Yumoto, F., Kimura, S., Kadota, Y., et al. (2008). Synergistic activation of the *Arabidopsis* NADPH oxidase AtrbohD by Ca^{2+} and phosphorylation. *Journal of Biological Chemistry, 283*, 8885–8892.

Orozco-Cardenas, M. L., Vasquez, J. N., & Ryan, C. A. (2001). Hydrogen peroxide acts as a second messenger for the induction of defense genes in tomato plants in response to wounding, systemin, and methyl jasmonate. *Plant Cell, 13*, 179–191.

Piedras, P., Hammond-Kosack, K. E., Harrison, K., & Jones, J. D. G. (1998). Rapid *Cf-9* and *Avr-9* dependent production of active oxygen species in tobacco suspension cultures. *Molecular Plant Microbe Interactions, 11*, 1155–1166.

Rashotte, A. M., DeLong, A., & Muday, G. K. (2001). Genetic and chemical reductions in protein phosphatase activity alter auxin transport, gravity response, and lateral root growth. *Plant Cell, 13*, 1683–1697.

Rentel, M. C., & Knight, M. R. (2004). Oxidative stress–induced calcium signaling in *Arabidopsis*. *Plant Physiology, 135*, 1471–1479.

Sanders, D., Brownlee, C., & Harper, J. F. (1999). Communicating with calcium. *Plant Cell, 11*, 691–706.

Schwacke, R., & Hager, A. (1992). Fungal elicitors induce a transient release of active oxygen species from cultured spruce cells that is dependent on Ca^{2+} and protein kinase activity. *Planta, 187*(1), 136–141.

Sinha, A. K., Hofmann, M. G., Romer, U., Kockenberger, W., Elling, L., & Roitsch, T. (2002). Metabolizable and non-metabolizable sugars activate different signal transduction pathways in tomato. *Plant Physiology, 128*, 1480–1489.

Tegg, R. S., Melian, L., Wilson, C. R., & Shabala, S. (2005). Plant cell growth and flux responses to the streptomycete phytotoxin thaxtomin A: Calcium and hydrogen flux patterns revealed by the non-invasive MIFE technique. *Plant Cell Physiology, 46*(4), 638–648.

Vera-Estrella, R., Blumwald, E., & Higgins, V. J. (1992). Effect of specific elicitors of *Cladosporium fulvum* on tomato suspension cells: Evidence for the involvement of active oxygen species. *Plant Physiology, 99*, 1208–1215.

Virad, M. P., Martin, F., Pugin, A., Ricci, P., & Blein, J. P. (1994). Protein phosphorylation is induced in tobacco cells by the elicitor cryptogein. *Plant Physiology, 104*, 1245–1249.

White, P. J., & Broadley, M. R. (2003). Calcium in plants. *Annals of Botany, 92*, 487–511.