Running Head : SA-induced $[Ca^{2+}]_c$ elevation in *Arabidopsis*

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Title : An investigation into the role of cytosolic free Ca^{2+} in Salicylic acid mediation of disease resistance in *Arabidopsis*

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ABSTRACT

Salicylic acid (SA) accumulation upon pathogen attack is a fundamental requirement for the activation of numerous plant defence mechanisms. Cytosolic free Ca^{2+} ($[Ca^{2+}]_c$) is a ubiquitous signalling molecule involved in a host of cellular processes. Using transgenic *Arabidopsis thaliana* seedlings expressing the Ca^{2+}-sensitive photoprotein aequorin, we previously reported a rapid and transient increase in $[Ca^{2+}]_c$ upon application of exogenous SA. We now investigated the characteristics of the SA-induced $[Ca^{2+}]_c$ increase and report that the majority of the response is derived from internal stores. It appears likely that SA triggers Ca^{2+}-induced Ca^{2+}-release. Preliminary evidence suggests a role for the SA-induced $[Ca^{2+}]_c$ increase in the regulation of *NPR1* expression since modulation of the SA-induced $[Ca^{2+}]_c$ response with the extracellular Ca^{2+} chelator BAPTA causes a reduction in *NPR1* mRNA levels. We have isolated two putative mutants that are defective in their ability to produce a SA-induced $[Ca^{2+}]_c$ increase. Characterisation of these mutants is underway and will prove invaluable in identifying the components or events that cause the SA-induced $[Ca^{2+}]_c$ transient, thereby aiding in the understanding of the role of $[Ca^{2+}]_c$ in SA-mediated signal transduction.

INTRODUCTION

Owing to the diversity of environmental stimuli imposed upon plants, the flexibility and plasticity of plant metabolism is essential for their survival (Somssich and Hahlbrock 1998). It is this phenomenon that allows plants to undergo transcriptional reprogramming when challenged with pathogens and mount an effective defence response (Somssich and Hahlbrock 1998; Maleck *et al.* 2000). The complexity of defence responses includes the potentiation of host cell death, containment of the pathogen and the synthesis of numerous proteins essential for the establishment of defence responses (Dempsey *et al.* 1999). The endogenous accumulation of salicylic acid (SA) following pathogen attack has been shown to be essential for the establishment of these defence responses and limitation of plant disease (Yalpani *et al.* 1991; Gaffney *et al.* 1993; Delaney *et al.* 1994; Dempsey *et al.* 1999; Nawrath and Metraux 1999), the most intriguing of which being the establishment of systemic acquired resistance (SAR). SAR confers the plant with long-lasting and broad spectrum immunity to subsequent pathogen attack of bacterial, viral or fungal origin (Ross 1961; Cao *et al.* 1994; Dempsey *et al.* 1999).

Characteristic to the establishment of SAR is an increase in the expression of a family of low molecular weight proteins called pathogenesis-related (PR) proteins, of which some members are effective antimicrobial agents against certain bacteria and fungi (Dempsey *et al.* 1999). PR proteins are expressed in both infected and uninfected (systemic) plant tissue and consequently utilized as markers for the induction of SAR (Ward *et al.* 1991). Furthermore, it was demonstrated that application of exogenous SA induced both SAR

and the expression of *PR* genes in the absence of an invading pathogen (Uknes *et al.* 1992). Extensive research is focussing on elucidating the downstream components that mediate SA-induced signal transduction. Aiding in this investigation is the isolation and characterisation of mutants that are either defective in their defence responses to pathogen attack (Cao *et al.* 1994; Zhou *et al.* 1998; Nawrath and Metraux 1999) or show enhanced disease resistance (Rate and Greenberg 2001).

The *Arabidopsis NPR1* gene, which is predicted to encode a protein containing ankyrin-like repeats (Cao *et al.* 1997; Ryals *et al.* 1997), has been shown to be essential in the activation of systemic, inducible plant defence responses induced by SA and is required for *PR1* expression (Cao *et al.* 1994). Additionally the binding of *NPR1* to the basic leucine zipper transcription factor TGA2 was illustrated to be critical for the activation of defence genes (Despres *et al.* 2000).

A novel superfamily of plant-specific transcription factors called WRKY proteins, with up to 100 representatives, have recently been identified and shown to play a role in regulating a wide variety of physiological responses (Eulgem *et al.* 2000). In a recent study some members of the WRKY gene family were shown to be induced by SA and to act upstream of *NPR1* positively regulating its transcription. Others were demonstrated to be induced by SA but to function independently of *NPR1*, for example *AtWRKY7* (Yu *et al.* 2001). Furthermore, it was illustrated that there was a significant over-representation of W-box motifs (consensus sequence to which WRKY proteins bind), and their clustering, in the PR1 regulon (groups of genes with common regulatory patterns) gene

promoters, which suggests that WRKY factors are crucial in the co-regulation of these genes (Maleck *et al.* 2000).

Cytosolic free Ca^{2+} ($[Ca^{2+}]_c$), is a well established second messenger in both plant and animal systems implicated in a wide variety of cellular processes (Bush 1995; Clapham 1995; Pandey *et al.* 2000). Different environmental stimuli such as cold, touch and fungal elicitors (Knight *et al.* 1991), abscissic acid (ABA) (McAinsh *et al.* 1990) and hypo-osmotic shock (Cessna *et al.* 1998) each produce distinct transient increases in $[Ca^{2+}]_c$ otherwise known as specific Ca^{2+} signatures (McAinsh and Hetherington 1998). The ability of the different Ca^{2+} signatures to elicit a specific downstream response is thought to reside in the amplitude, temporal and spatial change in $[Ca^{2+}]_c$ (McAinsh and Hetherington 1998; Pandey *et al.* 2000). Recently it was demonstrated that the pattern of $[Ca^{2+}]_c$ oscillations induced by ABA in *Commelina communis* guard cells was concentration dependent and correlated with the final stomatal aperture (Staxen *et al.* 1999). Furthermore, studies utilizing a 'calcium clamp' to systematically vary the $[Ca^{2+}]_c$ oscillation parameters in *Arabidopsis* guard cells illustrated that there is a defined range of frequency, transient number, duration and amplitude of $[Ca^{2+}]_c$ oscillations that determines the extent of steady-state stomatal closure (Allen *et al.* 2001).

It has been suggested that Ca^{2+} functions as a second messenger of SA with the observation that extracellular Ca^{2+} was required for the induction of SA-induced chitinase accumulation (at the translational level) in both tobacco leaves (Raz and Fluhr 1992) and carrot suspension culture (Schneider-Mullar *et al.* 1994). The early steps in SA-mediated signal transduction have recently been elucidated by providing evidence that exogenous

SA induces a rapid and transient generation of superoxide anion (O_2^-) followed by a transient increase in $[Ca^{2+}]_c$ in tobacco cell culture (Kawano *et al.* 1998). Using transgenic *Arabidopsis* seedlings constitutively expressing apoaequorin, we previously reported a SA-induced $[Ca^{2+}]_c$ increase. Preliminary evidence suggested a role for both Ca^{2+} influx across the plasma membrane and Ca^{2+} release from internal stores in the mediation of the SA-induced $[Ca^{2+}]_c$ increase (Petersen 2000).

In this study we aim to further characterise the SA-induced $[Ca^{2+}]_c$ increase and report that the majority of the response is derived from internal stores. Additionally, it appears likely Ca^{2+}-induced Ca^{2+}-release (CICR) is triggered by SA. We have investigated the effect of modulating the SA-induced $[Ca^{2+}]_c$ response on the expression pattern of SA-induced genes. We have also isolated two putative mutants that are defective in their ability to produce a SA-induced $[Ca^{2+}]_c$ increase and characterisation of these mutants will prove invaluable in deciphering the role of $[Ca^{2+}]_c$ in SA-mediated signal transduction and how this transient is produced.

RESULTS

SA-induced $[Ca^{2+}]_c$ increase occurs in both roots and leaves

Using transgenic *Arabidopsis* seedlings expressing the Ca^{2+}-sensitive photoprotein aequorin in the cytosol, we previously reported that the application of exogenous SA (to a final concentration of 1 mM) induces a specific Ca^{2+} signature. The $[Ca^{2+}]_c$ immediately rises from basal levels reaching a maximum at approximately 2 min followed by a gradual decline within 8 to 10 min (Petersen 2000). We now investigated whether this Ca^{2+} signature differs in different parts of the plant. Following overnight reconstitution in 5 μM coelenterazine, the roots and leaves of 7 day old transgenic seedlings were separated and individually treated with SA to a final concentration of 1 mM. The SA-induced $[Ca^{2+}]_c$ response of the roots is significantly larger than that of the leaves, reaching maximal levels of 1.2 μM $[Ca^{2+}]_c$ by 200 sec and rapidly declines compared to the leaves where the $[Ca^{2+}]_c$ gradually increases only reaching levels of 0.35 μM by 200 sec and maintained at that level for duration of the experiment (Figure 1). The response in intact whole seedlings is less pronounced than that of the roots but greater than that of leaves. This data suggests that the SA-induced $[Ca^{2+}]_c$ increase occurs in one tissue type with the largest contribution occurring from response in the roots.

The majority of the SA-induced $[Ca^{2+}]_c$ response is derived from an intracellular Ca^{2+} store

Preliminary evidence suggested that the SA-induced $[Ca^{2+}]_c$ increase is derived from both extra- and intracellular Ca^{2+} pools (Petersen 2000). To investigate this further, here we varied the $[Ca^{2+}]$ in the plant growth media in an attempt to reduce the apoplastic $[Ca^{2+}]$ and determine the effect on the SA-induced $[Ca^{2+}]_c$ response. There was no significant difference observed in the SA-induced $[Ca^{2+}]_c$ response even with a 20-fold reduction of $[Ca^{2+}]$ in the growth medium (Figure 2A). Similarly, increasing the $[Ca^{2+}]$ in the plant growth medium above the normal 2 mM, produced no significant difference in the SA-induced $[Ca^{2+}]_c$ response (data not shown). In order to illustrate that the apoplastic $[Ca^{2+}]$ was affected, transgenic seedlings grown on varying $[Ca^{2+}]$ were treated with mannitol, which imposes drought stress, to a final concentration of 0.6 M. The $[Ca^{2+}]_c$ increase in response to mannitol is largely due to Ca^{2+} influx across the plasma membrane (Knight *et al.* 1997). A significant reduction in the magnitude of the $[Ca^{2+}]_c$ increase in response to mannitol was observed with a 20-fold reduction in the $[Ca^{2+}]$ of the growth media (Figure 2B). These results suggest that, unlike the response to mannitol, intracellular Ca^{2+} stores are the principle players in the SA-induced $[Ca^{2+}]_c$ increase.

SA may cause Ca^{2+}-induced Ca^{2+}-release (CICR)

The external Ca^{2+} chelator BAPTA was shown to completely inhibit the SA-induced $[Ca^{2+}]_c$ increase both in tobacco cell culture (Kawano *et al.* 1998) and intact whole transgenic *Arabidopsis* seedlings constitutively expressing cytosolic aequorin (Petersen 2000). SA treatment of transgenic *Arabidopsis* seedlings (C24 ecotype), which report

increases in $[Ca^{2+}]_c$ around the microdomain adjacent to the tonoplast (Knight *et al.* 1996), produced a large increase in $[Ca^{2+}]_c$ reaching magnitudes similar to transgenic plants expressing cytosolic aequorin (Figure 3A). This data supports the observation the intracellular Ca^{2+} stores contributes a large part to the SA-induced $[Ca^{2+}]_c$ increase.

However, treatment of C24 seedlings with 5 mM BAPTA prior to application of 1 mM SA completely abolishes the SA-induced $[Ca^{2+}]_c$ increase (Figure 3A). Since BAPTA is a known extracellular Ca^{2+} chelator we had to test that it was not leaching intracellular Ca^{2+}. Treatment of cytosolic aequorin expressing seedlings with 5 mM BAPTA for 5 min followed by 1 mM SA yielded no response and immediate treatment of cold stress to these plants resulted in a cold-induced $[Ca^{2+}]_c$ increase (Figure 3B). The cold-induced response has been shown to be largely due to release from an intracellular Ca^{2+} pool (Knight *et al.* 1996). This demonstrates that the absence of the SA-induced $[Ca^{2+}]_c$ increase after treatment with BAPTA is due to the chelation of extracellular Ca^{2+} and not leaching by internal stores. Therefore we suggest that SA firstly induces Ca^{2+} influx across the plasma membrane and this increase in $[Ca^{2+}]_c$ causes a release of Ca^{2+} from the vacuole.

CICR is the phenomenon whereby Ca^{2+} itself mediates the release of Ca^{2+} from internal stores. In animal cells, both inositol triphosphate (IP_3) and ryanodine-mediated Ca^{2+} channels located on intracellular membranes has the capacity to be activated by Ca^{2+}, which underlies their involvement in CICR (Rudd and Franklin-Tong 1999; Sanders *et al.* 1999). A model for the involvement of IP_3 Ca^{2+} channels in the formation of Ca^{2+} waves, dependent on the concentration of both IP_3 and $[Ca^{2+}]_c$, in plants was recently proposed

(Trewavas 1999). Preliminary evidence indicates a role for IP_3-activated Ca^{2+} channels in the SA-induced $[Ca^{2+}]_c$ elevation. Pre-treatment of cytosolic aequorin expressing seedlings with 0.3 mM neomycin, which blocks metabolism of phosphoinositides reducing IP_3 synthesis (Knight *et al.* 1996), causes a slight reduction in the magnitude of the SA-induced $[Ca^{2+}]_c$ response (Figure 3C). This result strengthens the hypothesis for a putative mechanism of CICR in operating the SA-induced $[Ca^{2+}]_c$ increase.

The effect of the SA-induced $[Ca^{2+}]_c$ elevation on gene expression

SA is known to induce the expression of some pathogenesis related (PR) proteins, which are commonly used as markers for the establishment of systemic acquired resistance (Dempsey *et al.* 1999). We reported that the SA-induced $[Ca^{2+}]_c$ increase was dependent on the concentration of SA (Petersen 2000) and consequently investigated the expression pattern of PR1 at different [SA] using Western analysis. As expected the expression of PR1 increases with increasing concentrations of SA in 2 week old *Arabidopsis* seedlings (Figure 4A). However, seedlings preincubated in 5 mM BAPTA followed by treatment with 1 mM SA did not show a reduction in the amount of PR1 protein present compared to seedlings treated only with 1 mM SA. This suggests that PR1 expression in response to SA is independent of the $[Ca^{2+}]_c$ increase.

We therefore decided to investigate the expression profile of genes that are induced much earlier in response to SA. NPR1 has been shown to be essential for mediating SA-induced disease resistance in *Arabidopsis thaliana* and functions upstream of PR proteins (Cao *et al.* 1994; Zhang *et al.* 1999). The RNA blots for northern analysis in Figure 4B and 4C were transferred and hybridised together under the exact same conditions. Therefore

despite the unequal RNA loading, we observed that SA induces *NPR1* by 2 hours with transcript levels being maintained until 12 hours (Figure 4B). In addition, there is a distinct reduction in *NPR1* expression in samples treated with 5 mM BAPTA prior to application of exogenous SA specifically at the 12 hour time point (Figure 4C). We have noted that the endogenous *NPR1* levels in tissue treated with 5 mM BAPTA for 12 hours is high (compared to the loading) and may be due to possible contamination that was not visible at the time of treatment (Figure 4C, lane 3). These results suggest a link between the SA-induced $[Ca^{2+}]_c$ increase and the regulation of *NPR1*, however further investigation is required to ensure these preliminary findings are reproducible and to reconcile the effect of long-term incubation in BAPTA.

Cloning and expression analysis of *AtWRKY7*

The WRKY proteins are a superfamily of transcription factors that are unique to plants and involved in the regulation of a variety of physiological programmes (Eulgem *et al.* 2000). Numerous WRKY proteins have been shown to be induced by SA in *Arabidopsis thaliana*, within as little as 2 hours (Yu *et al.* 2001). Some of these (including *AtWRKY7*) show induction independently of *NPR1*, suggesting they may be expressed upstream of *NPR1* in a SA signalling pathway (Eulgem *et al.* 2000; Yu *et al.* 2001). We were therefore interested in investigating whether *AtWRKY7*, one of these early SA-induced genes, was responding to the SA-induced $[Ca^{2+}]_c$ increase.

We designed primers (see materials and methods) to amplify a cDNA fragment of *AtWRKY7* of approximately 969 bp in length, spanning the coding region of *AtWRKY7* closest to the 3'-end of the gene. After PCR of cDNA synthesised from 3 week old 1 mM

SA treated leaves, which gave a single PCR product (Figure 5), the resulting DNA fragment was cloned into pGEM®-T easy vector. Sequence analysis verified that the correct fragment had been cloned (data not shown).

We determined the expression pattern of *AtWRKY7* in 2 week old seedlings treated with 1 mM SA (Figure 6). Whereas published data revealed that *AtWRKY7* is induced within 2 hours and decreases to basal levels by 8 hours in 4 week old *Arabidopsis* plants sprayed with 2 mM SA (Yu *et al.* 2001), we observed that *AtWRKY7* reached maximal expression at 12 hours in response to 1 mM SA under our experimental conditions. Furthermore, the endogenous *AtWRKY7* expression is induced in the H_2O samples at 2 and 12 hours, although to a lesser extent than that of the SA treated samples at the same time points (compare loadings of Figure 6: lanes 2 and 3 to lanes 8 and 10 respectively). Therefore *AtWRKY7* may be responding to other additional stress signals under our experimental conditions. In order to reconcile this data and determine whether *AtWKRY7* responds to the SA-induced $[Ca^{2+}]_c$ increase further investigation into its induction is required.

Isolation and characterisation of mutants defective in ability to produce the SA-induced $[Ca^{2+}]_c$ elevation

Transgenic *Arabidopsis* seedlings (RLD ecotype) constitutively expressing cytoplasmic aequorin were EMS mutagenised and screened for their lack of ability to produce an increase in $[Ca^{2+}]_c$ in response to 2 mM SA. Following SA treatment, seedlings were chilled and those that showed a reduced cold response were discarded to ensure mutants were chosen in which the reduction in the SA-induced $[Ca^{2+}]_c$ increase was not due to failure to reconstitute functional aequorin. Mutant M_2 plants were selected and the M_3

generation plants were screened with 1 mM SA using luminometry to confirm the phenotype. Two putative mutants were selected for further study and there was no obvious morphological difference between putative mutants and wild type *Arabidopsis* seedlings.

Both mutants, 7-102 and 1-126, exhibited reduced magnitudes of 0.5 ± 0.03 μM $[Ca^{2+}]_c$ compared to 0.7 ± 0.05 μM $[Ca^{2+}]_c$ observed in the parental transgenic line upon treatment of 1 mM SA. Additionally, peak maxima were established slightly quicker in both mutant lines, around 120 sec for the mutants compared to 180 sec observed in the control (Figure 7A).

In order to ascertain whether the observed reduction in the SA-induced $[Ca^{2+}]_c$ increase in the mutants is due to a general inability to establish $[Ca^{2+}]_c$ increases or specific to SA, we investigated the effect of other stresses known to cause an increase in $[Ca^{2+}]_c$ in these mutants. In response to cold 7-102 and 1-126 displayed similar kinetics to wild type (Figure 7B and 7C). Similarly, imposing drought stress, by treatment with 1 M mannitol, results in both 7-102 and 1-126 reaching magnitudes not significantly different from that of the parental line (Figure 7D).

In response to salt stress both putative mutants exhibit altered kinetics compared to those of the control (Figure 7E). Here the mutants show a biphasic Ca^{2+} signature whereas RLD produced the characteristic single $[Ca^{2+}]_c$ transient in response to salt (Knight *et al.* 1997). This data suggests that the mutations in both mutants have an effect on the salt-induced $[Ca^{2+}]_c$ increase and that salt and SA may operate via a similar $[Ca^{2+}]_c$-based signalling mechanism. The mutants appear to have an increased sensitivity to oxidative stress since

the magnitude of the $[Ca^{2+}]_c$ increase in both mutants is significantly higher than that of the wild type RLD but the kinetics of the responses are similar (Figure 7F). Although the Ca^{2+} signatures produced by both mutants in response to the stresses tested in this report were not always identical to those of the parent line, it is evident that 7-102 and 1-126 are not defective in mounting an increase in $[Ca^{2+}]_c$ in response to these stresses and none of the responses are lower than wild type when altered. Thus further characterisation of these mutants could provide significant clues in SA-mediated signal transduction and the issue of specificity of the SA-induced $[Ca^{2+}]_c$ response.

RLD 7-102 shows reduced *NPR1* expression in response to SA

In this study we reported that treatment of *Arabidopsis* seedlings with the extracellular Ca^{2+} chelator BAPTA prior to SA treatment caused a reduction in NPR1 gene expression. Therefore we investigated the effect of the mutation in 7-102 on *NPR1* expression in response to SA. Since BAPTA completely inhibited the SA-induced $[Ca^{2+}]_c$ increase but only partially inhibited NPR1 gene expression, we did not expect a large reduction in *NPR1* expression in the mutant.

NPR1 expression was maximal at 6 hours following SA application and decreased slightly at 12 and 24 hours in the RLD ecotype (Figure 6A). The expression pattern in 7-102 is similar to that of wild type also reaching maximal levels at 6 hours but there is a reduction in the level of *NPR1* induction in 7-102, with a significant reduction observed at the earlier time points (15 min to 2 hours) as well as at 12 and 24 hours in the mutant compared to the wild type samples (Figure 6B). The level of *NPR1* in the 24 hour H_2O sample of 7-102 is unexpectedly high (Figure 6B, lane 4), however, for each sample an

individual plate of *Arabidopsis* seedlings is utilized and it is possible that plate could have been contaminated leading to *NPR1* induction. This experiment would need to be repeated in order to confirm the observed reduction in *NPR1* expression in 7-102 compared to wild type.

These results together with the expression pattern in the BAPTA treated samples holds promise for linking the SA-induced $[Ca^{2+}]_c$ to an end response but further investigation is required to confirm these preliminary findings.

DISCUSSION

The versatility of Ca^{2+} enables it to function in a host of physiological responses either through chemical stabilization of biological molecules or the ability of $[Ca^{2+}]_c$ to act as a second messenger (Taiz and Zeiger 1998; Pandey *et al.* 2000). There are extensive reports which demonstrate an increase in $[Ca^{2+}]_c$ in response to various environmental stimuli (Knight *et al.* 1991; Cessna *et al.* 1998; Xu and Heath 1998) and current research is focussing on identifying the sensing components that convert the $[Ca^{2+}]_c$ change into a specific downstream response.

Recently it has been demonstrated that specific kinetics of $[Ca^{2+}]_c$ oscillations are required for initiating stomatal closure in response to extracellular Ca^{2+} and hydrogen peroxide (Allen *et al.* 2000; Allen *et al.* 2001). However, nothing is known about how specific oscillations are sensed or transduced. Furthermore, recent evidence illustrated quantitative differences in changes in the $[Ca^{2+}]_c$ between the functionally diverse cell

types of the *Arabidopsis* root in their response to cold, osmotic and salt stress (Kiegle *et al.* 2000), which suggests that there are specific roles for certain cell types in the sensing and/or response to the same environmental cue. Thus evidence is ever accumulating to support the hypothesis that specificity of Ca^{2+} based signal transduction resides in the physiological condition of the cell, source of Ca^{2+} mobilization and the spatial and temporal patterns of $[Ca^{2+}]_c$ change (McAinsh and Hetherington 1998; Pandey *et al.* 2000). Our research is focused on determining whether the SA-induced $[Ca^{2+}]_c$ increase is a key regulator in mediating SA-dependent defence responses in plants and how specificity of the $[Ca^{2+}]_c$ increase is achieved.

We investigated whether the SA-induced $[Ca^{2+}]_c$ increase differed in the various parts of the plant and report that the response is greater in the root compared to the leaves (Figure 1). This suggests that the root either is the primary effector of the SA-induced $[Ca^{2+}]_c$ increase or responds faster than the leaves. It is possible that the lesser response of the SA-induced $[Ca^{2+}]_c$ increase in the leaves could be due to the accessibility of SA to these organs owing to the greater complexity of cells within the leaves compared to the roots. In support for the argument of accessibility, the kinetics of the SA-induced $[Ca^{2+}]_c$ increase observed in tobacco cell culture (Kawano *et al.* 1998) is reminiscent of that seen in the roots. Furthermore, *in vivo* studies in tobacco showed that upon infection with necrotizing pathogen SA accumulates in the both the inoculated and upper uninoculated leaves but little or no increase in SA is observed in the roots (Shulaev *et al.* 1995). Similarly, following pathogen infection SA has been shown to accumulate in cucumber (Meuwly and Metraux 1993) and *Arabidopsis* leaves (Nawrath and Metraux 1999). These

data support the possibility that the actual response of cells to SA is an immediate increase in $[Ca^{2+}]_c$ and is dependent on the accessibility of SA to the individual cells.

Previously we reported a role for both extracellular and intracellular Ca^{2+} stores for the SA-induced $[Ca^{2+}]_c$ response (Petersen 2000). We have provided evidence that the intracellular Ca^{2+} stores are primarily responsible for the observed SA-induced $[Ca^{2+}]_c$ increase since varying the apoplastic Ca^{2+} had no significant effect on this response (Figure 2) and transgenic C24 seedlings expressing aequorin targeted to the microdomain of the tonoplast yielded a large increase in $[Ca^{2+}]_c$ of similar magnitude as the cytoplasmic aequorin expressing line (Figure 3A). However, the involvement of extracellular Ca^{2+} cannot be excluded and subsequent experiments suggest the mechanism of CICR, which is established in animal systems (Sanders *et al.* 1999) and has recently been briefly reviewed in plants (Rudd and Franklin-Tong 1999), in operating the SA-induced $[Ca^{2+}]_c$ response.

The extracellular Ca^{2+} chelator BAPTA was shown here and previously to completely abolish the SA-induced $[Ca^{2+}]_c$ increase in both the cytosolic (Petersen 2000) and microdomain expressing aequorin lines (Figure 3A). However, BAPTA failed to inhibit the $[Ca^{2+}]_c$ increase in response to cold (Figure 3B), which was reported to be largely due to Ca^{2+} release from the vacuole (Knight *et al.* 1996), illustrating that BAPTA is not leaching intracellular Ca^{2+}. Therefore, if the observed Ca^{2+} influx and Ca^{2+} release in response to SA were separate responses, BAPTA would be unable to inhibit the SA-induced $[Ca^{2+}]_c$ increase in the C24 seedlings. The fact that BAPTA can inhibit this response in C24 seedlings indicates that the two stores are not acting independently in the

SA-induced $[Ca^{2+}]_c$ response. Furthermore, preliminary evidence implicates a role for IP_3-activated Ca^{2+} channels in the SA-induced $[Ca^{2+}]_c$ response (Figure 3C). These channels have been shown to be regulated by both IP_3 and local Ca^{2+} concentrations and are involved in CICR in animal systems (Marchant and Taylor 1997; Sanders *et al.* 1999; Trewavas 1999). We therefore propose that the mechanism for the SA-induced $[Ca^{2+}]_c$ increase is CICR where SA firstly induces Ca^{2+} influx across the plasma membrane causing an increase in $[Ca^{2+}]_c$ which, in conjunction with IP_3 synthesis, activates IP_3-activated Ca^{2+} channels on the tonoplast resulting in Ca^{2+} release from the vacuole.

SA mediates a host of responses to confer disease resistance in plants including the establishment of systemic acquired resistance (SAR) characterised by the up regulation of PR proteins (Dempsey *et al.* 1999). Western analysis revealed that PR1 expression increases with increasing concentration of SA in *Arabidopsis* seedlings (Figure 4A), which correlated to the ability of the various [SA] to induce changes in $[Ca^{2+}]_c$ (Petersen 2000). However, pre-treatment with 5 mM BAPTA did not cause a reduction in the level of PR1 present. This implies either that PR1 expression is independent of the SA-induced $[Ca^{2+}]_c$ elevation or other compensatory pathways are activating PR1 expression in the absence of Ca^{2+}, suggesting that changes in $[Ca^{2+}]_c$ in response to SA might be one of two or more parallel SA signalling pathways. This is not without precedent, for example, the expression of *PR* genes and disease resistance were shown to be induced by SA in the dominant *ssi1* mutation independently of *NPR1* (Shah *et al.* 1999).

A clearer picture in linking changes in $[Ca^{2+}]_c$ to SA-mediated signal transduction may be provided by investigating the effects of the SA-induced $[Ca^{2+}]_c$ increase on genes that are induced more directly and more rapidly by SA. We chose *NPR1* as a candidate gene since *NPR1* operates upstream of *PR1* expression (Cao *et al.* 1994). Our data suggests that the SA-induced $[Ca^{2+}]_c$ increase affects *NPR1* expression since modulation of the $[Ca^{2+}]_c$ response with BAPTA causes a reduction in *NPR1* especially at later time intervals (compare figures 4B and 4C). However, these experiments must be repeated to confirm these preliminary findings which suggest a role for the SA-induced $[Ca^{2+}]_c$ response in the regulation of *NPR1* expression.

Additionally, we were interested in investigating the effect of the SA-induced $[Ca^{2+}]_c$ response on the plant specific transcription factor WRKY7, because it is early responsive to SA, reaching maximal levels by 2 hours following SA treatment (Yu *et al.* 2001). More importantly its regulation is independent of *NPR1* (Yu *et al.* 2001) suggesting *AtWRKY7* functions upstream of *NPR1* or in an independent SA signalling pathway. We have successfully cloned a 969 bp cDNA fragment of *AtWRKY7* and shown that *AtWRKY7* is induced by SA in 2 week old *Arabidopsis* seedlings but at a later time interval (12 hours as opposed to 2 hours) compared to a previous report (Yu *et al.* 2001). Future experiments will entail modulating the SA-induced $[Ca^{2+}]_c$ response to determine if this response is able to regulate *AtWRKY7* expression. These data together with *NPR1* expression studies may provide evidence that the SA-induced $[Ca^{2+}]_c$ response is able to regulate the expression of SA-inducible genes suggesting a role for $[Ca^{2+}]_c$ in the establishment of defence responses when plants are challenged with pathogen.

Another important approach we adopted in order to elucidate the early events of SA-mediated signal transduction, as well as the issue of specificity of the SA-induced $[Ca^{2+}]_c$ response, is the isolation of putative mutants which lack the $[Ca^{2+}]_c$ elevation in response to SA. These mutants should prove to be useful tools to identify the components or events leading to the $[Ca^{2+}]_c$ change in response to SA. We have isolated two putative mutants, 7-102 and 1-126, that display reduced magnitude of the SA-induced $[Ca^{2+}]_c$ response. No mutants were isolated in which the response is completely abolished possibly since both SA and $[Ca^{2+}]_c$ are ubiquitous in signalling cascades (Dempsey *et al.* 1999; Pandey *et al.* 2000) and such a mutation could prove lethal. Furthermore, we have shown that the observed reduction in the SA-induced $[Ca^{2+}]_c$ elevation is not due to a general deficiency in Ca^{2+} signalling since other stresses, known to induce increases in $[Ca^{2+}]_c$, produced similar increases in $[Ca^{2+}]_c$ in the mutants and wild type. However, the Ca^{2+} signature of the mutants in response to salt stress displayed altered kinetics to that of wild type plants. Similarly, in response to oxidative stress the mutants produced increases in $[Ca^{2+}]_c$ that were significantly larger than that produced in wild type. These observations cause us to speculate that Ca^{2+}-based signalling involves a complex network of components and various environmental cues, for example SA, salt and H_2O_2, might utilize the same components but in a different manner in order to elicit a specific change in $[Ca^{2+}]_c$.

We have also observed a partial reduction in *NPR1* expression in 7-102, which was consistent with the expression studies utilizing BAPTA that completely inhibits the SA-induced $[Ca^{2+}]_c$ increase. These preliminary findings strengthens the hypothesis that *NPR1* is regulated by the SA-induced $[Ca^{2+}]_c$ response and further characterisation of

these mutants may provide insightful clues into the role of $[Ca^{2+}]_c$ in SA-mediated signal transduction.

In this study we have further characterised the SA-induced $[Ca^{2+}]_c$ transient, showing that the response differs in the different parts of plant and that majority of the response derives from intracellular Ca^{2+} stores. We also provide evidence for a mechanism of CICR in this response, with the involvement of IP_3-activated Ca^{2+} channels requiring verification. The initial *NPR1* expression studies reported here suggests a role for the SA-induced $[Ca^{2+}]_c$ increase in the regulation of this SA-inducible gene. Microarray analysis of the mutants versus wild type would reveal whether the SA-induced $[Ca^{2+}]_c$ increase acts as a global regulator of SA-mediated gene expression and assist in the elucidation of a direct link between the SA-induced $[Ca^{2+}]_c$ elevation and an end response/s. Furthermore, future characterisation of the mutants will help in the identification of components or events that leads to the SA-induced $[Ca^{2+}]_c$ increase thereby aiding in the understanding of the role of $[Ca^{2+}]_c$ in SA-mediated signal transduction and establishment of disease resistance.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Unless otherwise stated, experiments were performed using seedlings of transgenic *Arabidopsis* Columbia ecotype constitutively expressing recombinant apoaequorin, a Ca^{2+}-sensitive photoprotein, in their cytosol as described by Knight *et al.* (1996). Where stated, transgenic Arabidopsis of the RLD ecotype expressing cytoplasmic apoaequorin and C24 ecotype expressing apoaequorin-pyrophosphatase fusion protein that targets aequorin to the cytoplasmic face of the vacuolar membrane (both a kind gift from Dr. H. Knight, Department of Plant Sciences, University of Oxford, Oxford, UK) were used. All plants were grown on nutrient media (Haugh and Sommerville, 1986) under fluorescent light (80-100 μmol photon/sec/m^2) in a controlled environment chamber at 21°C. Where the calcium concentration was varied, the [$Ca(NO_3)_2.4H_2O$] was altered. Columbia ecotype seedlings were grown on soil for 3 weeks under fluorescent light (80-100 μmol photon/sec/m^2) in a controlled environment chamber at 21°C.

Chemicals

Chemically synthesized coelenterazine (Prolume) was stored in 20 nmole aliquots at –20°C. Coelentrazine was dissolved in methanol and dilute in sterile H_2O to obtain the desired concentration. Salicylic acid, 4-hydroxybenzoic acid (4-HBA), 1,2-bis(2-aminophenoxy)-ethane-*N,N,N',N'*-tetraacetic acid (BAPTA), neomycin and sodium salicylate were purchased from Sigma. SA and 4-HBA were dissolved in ethanol and

diluted to desired concentrations with sterile H_2O to a final ethanol concentration of 0.5 % (v/v).

In vivo Reconstitution of Aequorin and $[Ca^{2+}]_c$ measurements using luminometry

Reconstitution of aequorin was performed essentially as described by Knight *et al.* (1991) with individual seedlings incubated in microtitre plates in a final concentration of 5 μM coelenterazine in the dark overnight at 20°C. Transgenic *Arabidopsis* C24 ecotype seedlings were individually incubated in 10 μM coelenterazine under the same conditions. Following reconstitution, seedlings were placed individually in a plastic cuvette in 0.5 ml H_2O for approximately 10 min or in 0.5 ml Ca^{2+} channel inhibitors and Ca^{2+} chelators for an hour (unless otherwise stated) prior to addition of SA. SA, mannitol, NaCl, H_2O_2 and ice cold H_2O were delivered through a light tight port to the final concentrations specified in individual experiments. For cold stress, seedlings were individually placed in a plastic cuvette and subjected to 1 ml ice cold H_2O. To determine SA-induced $[Ca^{2+}]_c$ increase in roots and leaves, the roots and leaves were separated following reconstitution and individually placed in plastic cuvettes for 1 hour prior to SA application. Bioluminescence counts were recorded over 5 sec intervals (1 sec intervals for treatments other than SA), recorded as average light units per second, with a digital luminometer (*Luminoskan TL Plus Luminometer*, Labsystems, Finland). At the end of each experiment the remaining aequorin was discharged by addition of 1 ml 2 M $CaCl_2$ in 20 % ethanol (Knight *et al.* 1997). Calibration of $[Ca^{2+}]_c$ was performed as described by Knight *et al.* (1996) using the calibration equation: $pCa = 0.332588(-logk) + 5.5593$,

where k is a rate constant equal to luminescence counts per second divided by the total remaining counts.

Protein Extraction and Western Blot Analysis

Seedlings were grown on nutrient medium for 2 weeks post germination and 250 mg plant tissue was place in microtitre plates for the following treatments. Seedlings were incubated in various SA concentrations for 30 min and subsequently placed in H_2O or incubated in 5 mM BAPTA for 5 min prior to addition of SA to a final concentration of 1 mM and subsequently placed into 5 mM BAPTA. For the control treatments seedlings were incubated in 4-HBA or sterile H_2O for 30 min followed by subsequent placement in H_2O, incubated in 5 mM BAPTA for duration of experiment or immediately harvested without any treatment in liquid N_2. Tissue was harvested in liquid N_2 after 48 hours and homogenized in a 1 X TBS protein extraction buffer (2 mM PMSF, 2 mM benzamide, 10 mM ε amino caproic acid and 1 mM EDTA). Protein concentrations were determined using the Bradford assay (Bradford 1976). 23 μg of protein was electrophoresed in a 12 % polyacrylamide gel. Proteins were transferred overnight onto nitrocellulose membranes and probed with anti-PR1 antiserum at a dilution of 1:62.5 for 4 hours at 37°C. Chemiluminescent detection was performed as previously described (Durrant and Fowler 1994).

RNA Extraction and Northern Blot Analysis

Seedlings (250 mg) were treated 2 weeks post germination with a final concentration of 1 mM SA for 30 min following subsequent placement in H_2O. For samples pre-treated

with 5 mM BAPTA, following SA application samples were subsequently placed into 5 mM BAPTA for duration of experiment. Tissue was harvested in liquid N_2 at indicated time points. Total RNA was isolated utilizing Trizol (Life Technologies) as per manufacturer's instructions. 10 μg RNA of each sample was electrophoresed in formaldehyde-agarose gels and blotted onto Hybond N^+ membranes by capillary action using standard procedures (Peters *et al.* 1996). Membranes were probed with α-[^{32}P]dCTP-labelled cDNA fragments (Megaprime DNA labelling kit, Amersham) of the *NPR1* gene from the L35 clone set (Mendel Biotechnology) or the *AtWRKY7* cDNA.

Isolation of mutants defective in SA-induced $[Ca^{2+}]_c$ response

Transgenic *Arabidopsis* seedlings (RLD ecotype) constitutively expressing cytoplasmic apoaequorin were mutagenised with ethyl methanesulfonate (EMS). The initial screen entailed identifying individual seedlings that lacked the ability to produce an increase in $[Ca^{2+}]_c$ in response to 2 mM SA. Seedlings were subsequently chilled and those showing a reduced end response were discarded to ensure mutants were chosen in which the reduction in SA-induced $[Ca^{2+}]_c$ increase was not due to failure to reconstitute functional aequorin. Mutant M_2 plants were chosen and M_3 generation plants were screened with 1 mM SA to confirm the phenotype. K. Denby and N. Adams carried out the primary screen and secondary screen. For characterization of putative mutants, all subsequent luminometry and northern analysis were performed as described for that of wild type (Columbia ecotype) treatments.

Cloning of *AtWRKY7* cDNA

Three week old *Arabidopsis* (Columbia ecotype) leaves were excised and treated with 1 mM SA for 30 min followed by incubation for 2 hours in H_2O. Tissue was harvested in liquid N_2 and total RNA was isolated utilizing Trizol (Life Technologies) as per manufacturer's instructions. cDNA was synthesized from total RNA utilizing the oligo(dT) protocol of the Gibco BRL SuperScript™ kit (Life Technologies) as per manufacturer's instructions. To isolate the AtWRKY7 gene probe used for RNA gel blot analysis, we designed two primers (5'-CGGAGGAGGAGGTGATGGTT-3' and 5'-TGGTCTCCTTCGTACGTCACG-3') for PCR amplification. *AtWRKY7* cDNA was amplified under the following conditions: 90°C for 3 min followed by 35 cycles of 94°C for 2 min, 63°C for 1 min, 72°C for 1.5 min, ending with 10 min at 72°C. A single product of the expected size (approximately 969 bp) was obtained and following agarose gel purification (Qiagen gel extraction kit) the fragment was cloned into pGEM®-T easy vector (Promega). DNA sequence analysis was carried out by the Sequencing Unit in the Department of Molecular and Cell Biology at UCT.

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Figure 1: SA-induced $[Ca^{2+}]_c$ elevation in the roots, leaves and intact whole seedlings of transgenic *Arabidopsis thaliana* expressing the photoprotein aequorin

Seven day old *Arabidopsis* seedlings (Columbia ecotype) were reconstituted overnight in 5 μM coelenterazine and either left intact (whole) or the roots and leaves were separated and individually treated 1 hour after separation with SA to a final concentration of 1 mM. Bioluminescence measurements from several experiments (n=5 each treatment) were converted to $[Ca^{2+}]_c$ (Knight *et al.* 1996) . The vertical line represents the standard error of the mean at peak maxima.

Figure 2: Effect of varying apoplastic $[Ca^{2+}]$ on the SA-induced $[Ca^{2+}]_c$ increase

Transgenic *Arabidopsis* seedlings were grown on plant growth media ranging in $Ca(NO_3)_2.4H_2O$ concentration from 2 mM to 0.1 mM. Seven days post germination seedlings were reconstituted in 5 μM coelenterazine and treated with (A) 1 mM SA or (B) 0.6 M mannitol (n=10 for each trace).

Figure 3: SA-induced $[Ca^{2+}]_c$ increase possibly caused by Ca^{2+}-induced Ca^{2+}-release

Transgenic *Arabidopsis* expressing aequorin in the cytosol (cyt) and seedlings expressing aequorin targeted to the microdomain adjacent to the tonoplast (MD) seven days post germination were reconstituted in 5 and 10 μM coelenterazine respectively. Seedlings were preincubated in Ca^{2+} channel inhibitors and chelators followed by application of 1 mM SA.

A: 5 mM BAPTA for 5 min, followed by 1 mM SA at time 0 (n=11).

B: 5 mM BAPTA for 5min, 1 mM SA at time 0 and 500 µl ice cold H_2O at time 275 sec (n=5).

C: 0.3 mM Neomycin for 1 hour, followed by 1 mM SA at time 0 (n=5).

Figure 4: The effect of SA-induced $[Ca^{2+}]_c$ increase on PR1 and NPR1 gene expression

A: Protein blot showing PR1 protein levels. 14 day old *Arabidopsis* (Columbia ecotype) seedlings were treated with different concentrations of SA, ranging from 0.1 to 1 mM, for 30 min followed by subsequent placement in H_2O. Seedlings pre-treated with 5 mM BAPTA for 5 min were subsequently placed in 5 mM BAPTA, following 1 mM SA treatment. Lanes: Time 0 hr (1); H_2O (2); 1 mM 4-HBA (3); 0.1, 0.25, 0.5, 0.75 and 1 mM SA (4-8); 1 mM Sodium salicylate (9); 5 mM BAPTA (10) and 5 mM BAPTA + 1 mM SA (11).

B: RNA blot probed with NPR1 cDNA. 14 day old *Arabidopsis* (Columbia ecotype) seedlings were treated with 1 mM SA or H_2O for 30 min followed by subsequent placement in H_2O. Lanes: Time 0 hr (1); 15 min, 2 hr and 12 hr H_2O (2-4); 15 min, 30 min, 1, 2, 6 and 12 hr 1 mM SA (5-10).

C: RNA blot probed with NPR1 cDNA. 14 day old *Arabidopsis* (Columbia ecotype) seedlings were treated with 5 mM BAPTA prior to treatment with 1 mM SA for 30 min followed by subsequent placement in 5 mM BAPTA. Control samples were incubated in 5 mM BAPTA for the time points indicated. Lanes: 15 min, 2

and 12 hr 5 mM BAPTA (1-3); 15 min, 30 min, 1, 2, 6 and 12 hr 5 mM BAPTA + 1 mM SA (4-9).

Figure 5: PCR amplification of *AtWRKY7* cDNA yields single DNA fragment

Primers were designed to amplify a 969 bp DNA fragment of *AtWRKY7* from cDNA synthesized from 3 week old SA treated leaves. PCR reactions were electrophoresed on a 1 % agarose gel. Lanes: (1) λ DNA digested with Eco R1 and Hind III (2-3) PCR reactions with 1.5 and 2 mM $MgCl_2$ respectively (4) H_2O control PCR reaction.

Figure 6: SA induces *AtWRKY7* in transgenic Arabidopsis seedlings

RNA blot probed with AtWRKY7 cDNA. 14 day old *Arabidopsis* (Columbia ecotype) seedlings were treated with 1 mM SA or H_2O (control samples) for 30 min followed by subsequent placement in H_2O. Lanes: Time 0 hr (1); 15 min, 2 and 12 hr H_2O (2-4); 15 min, 30 min, 1, 2, 6 and 12 hr 1 mM SA (5-10).

Figure 7: Characterisation of the $[Ca^{2+}]_c$ increase in response to various stresses in the putative mutants *7-102* and *1-126*

Transgenic seedlings (RLD ecotype) expressing cytoplasmic aequorin and the mutant lines 7-102 and 1-126 seven days post germination were incubated overnight in 5 μM coelenterazine and treated with the following solutions:

A: 1 mM SA (n=10 each trace)

B: Cold stress: 1 ml ice cold H_2O (n=5 each)

C: Cold stress: 1ml ice cold H_2O (n=5 each)

D: Drought: 1 M Mannitol (n=5 each)

E: Salt stress: 0.5 M NaCl (n=5 each)

F: Oxidative stress: 10 mM H_2O_2 (n=5 each)

Figure 8: The effect of reduced SA-induced $[Ca^{2+}]_c$ elevation on *NPR1* gene expression

RNA blot probed with NPR1 cDNA. 14 day old *Arabidopsis* seedlings, RLD (A) and 7-102 (B), were treated with 1 mM SA for 30 min and subsequently placed into H_2O and harvested at the indicated time points. Lanes for both A and B: Time 0 hr (1); 2, 12 and 24 hr H_2O (2-4); 15 min, 30 min, 1, 2, 6, 12 and 24 hr 1 mM SA (5-11).

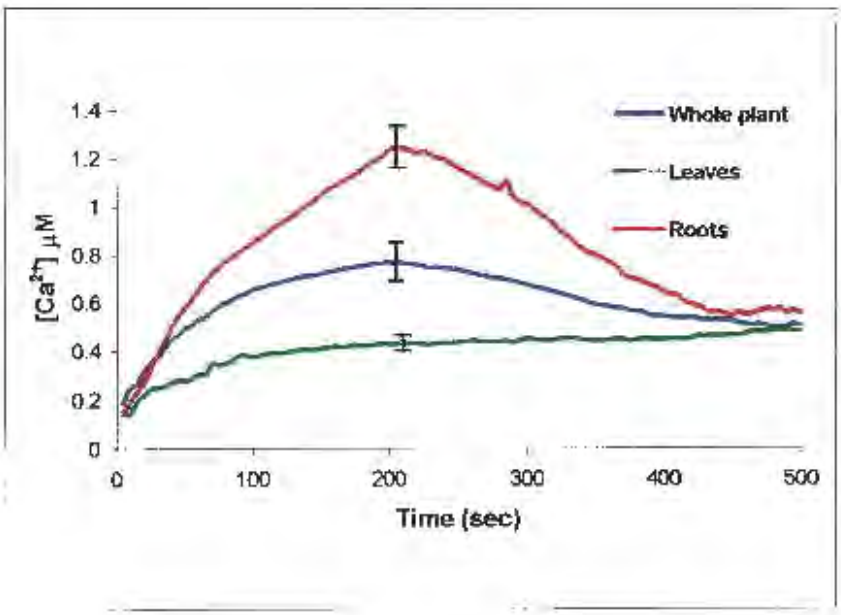
1.4
1.2
1
0.8
0.6
0.4
0.2
0
[Ca2+] µM
0
100
200
300
400
500
Time (sec)
Whole plant
Leaves
Roots

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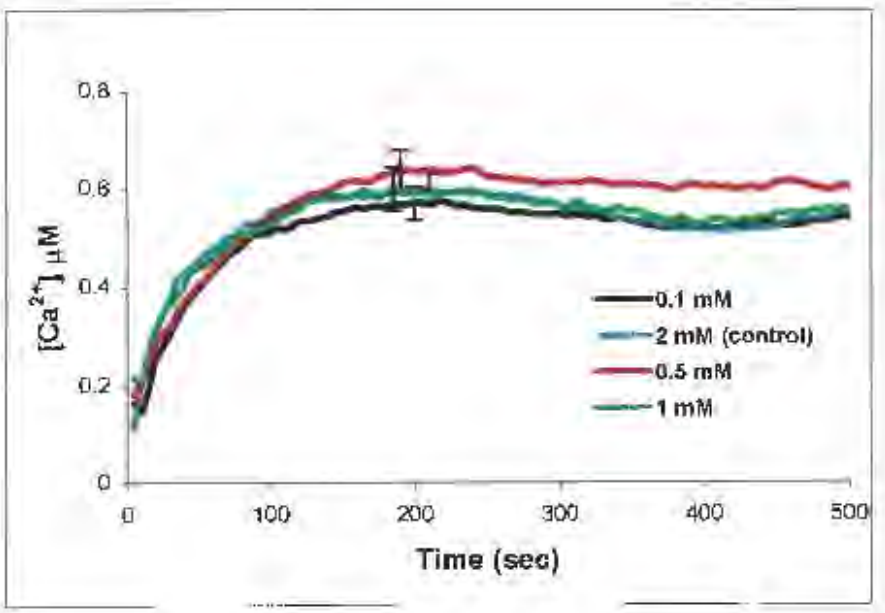
Figure 1

A


0.8
0.6
0.4
0.2
0
[Ca2+] µM
0.1 mM
2 mM (control)
0.5 mM
1 mM
0
100
200
300
400
500
Time (sec)

B

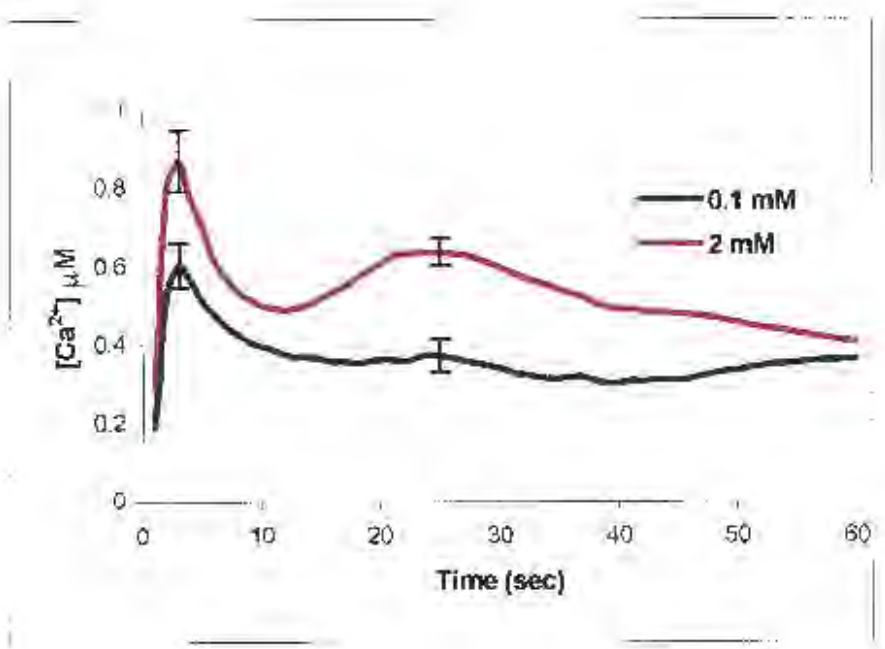

0.8
0.6
0.4
0.2
0
[Ca2+] µM
0.1 mM
2 mM
0
10
20
30
40
50
60
Time (sec)

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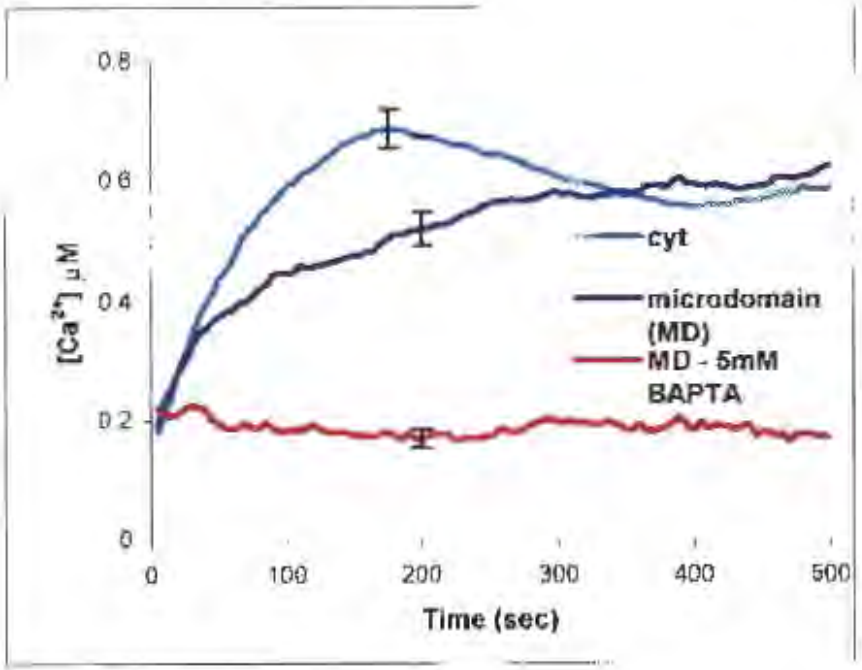
Figure 2A and 2B

A


0.8
0.6
0.4
0.2
0
[Ca2+] µM
0
100
200
300
400
500
Time (sec)
cyt
microdomain (MD)
MD - 5mM BAPTA

B

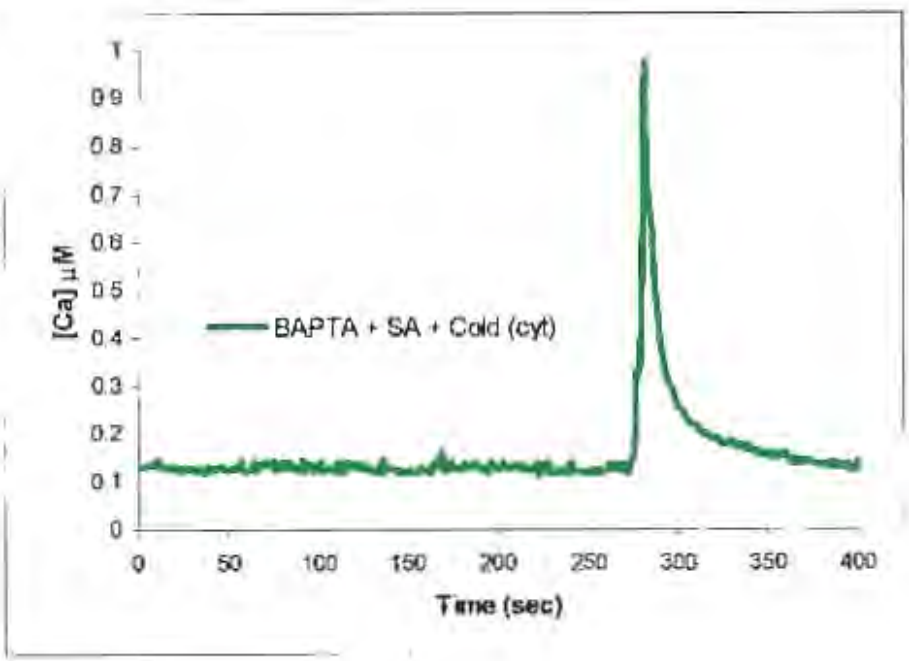

1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
[Ca] µM
0
50
100
150
200
250
300
350
400
Time (sec)
BAPTA + SA + Cold (cyt)

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Figure 3A and 3B

C

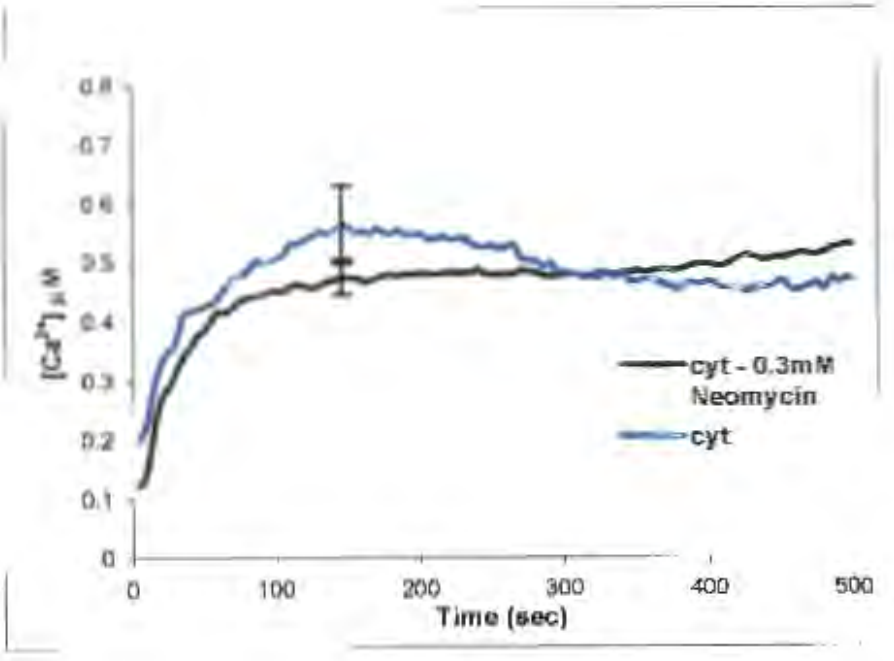
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
[Ca2+]i µM
0
100
200
300
400
500
Time (sec)
cyt - 0.3mM Neomycin
cyt

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Figure 3C

A


1 2 3 4 5 6 7 8 9 10 11
PR 1

B


1 2 3 4 5 6 7 8 9 10
NPR1
rRNA

C


1 2 3 4 5 6 7 8 9
NPR 1
rRNA

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Figure 4A, 4B and 4C

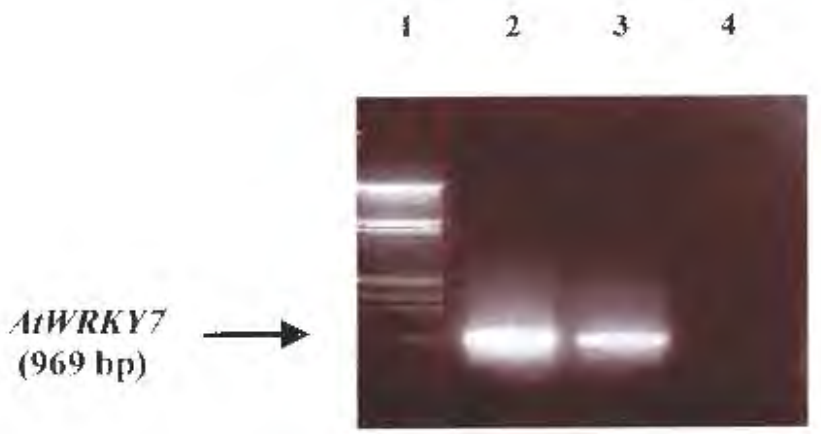
1
2
3
4
AtWRKY7
(969 bp)

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Figure 5

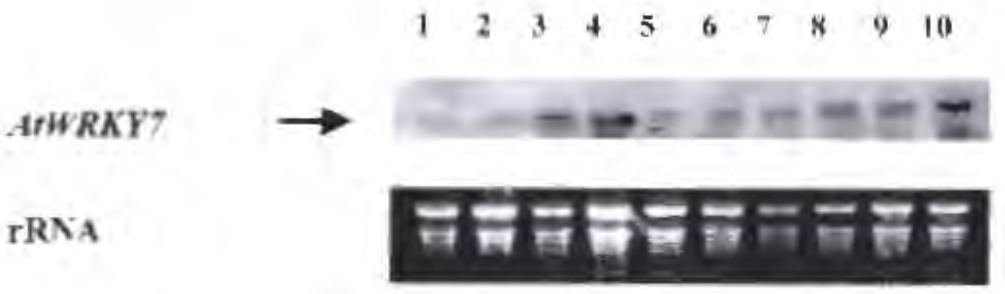
1 2 3 4 5 6 7 8 9 10
AtWRKY7
rRNA

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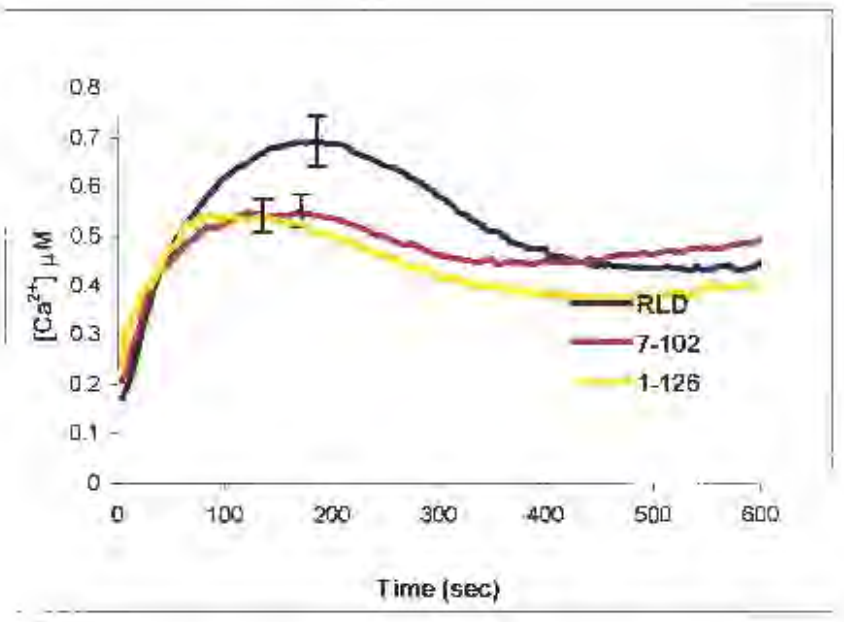
Figure 6

A


0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
[Ca2+] µM
0
100
200
300
400
500
600
Time (sec)
RLD
7-102
1-126

B

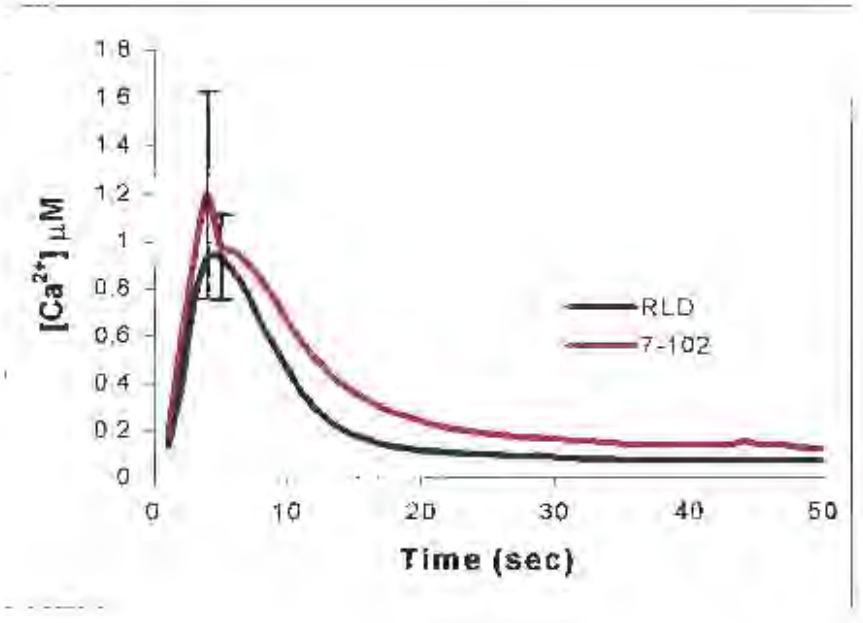

1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
[Ca2+] µM
0
10
20
30
40
50
Time (sec)
RLD
7-102

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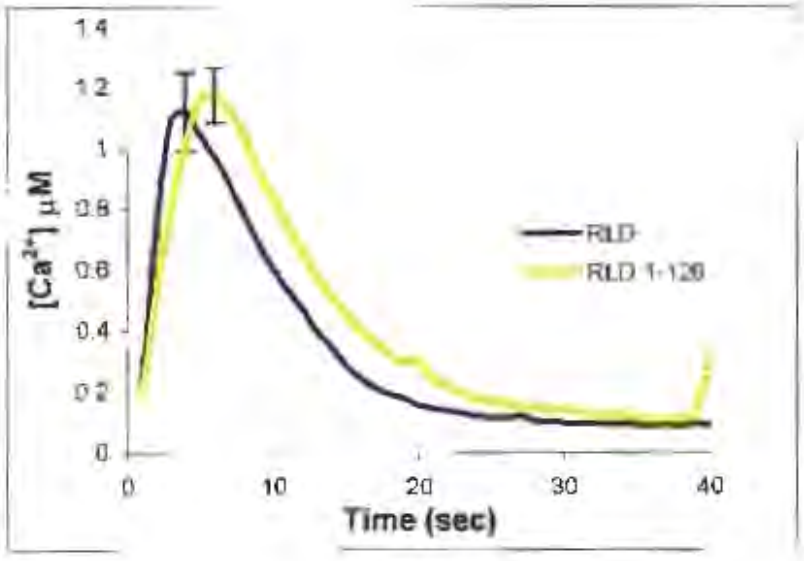
Figure 7A and 7B

C


1.4
1.2
1
0.8
0.6
0.4
0.2
0
[Ca2+] µM
0
10
20
30
40
Time (sec)
RLD
RLD 1-126

D

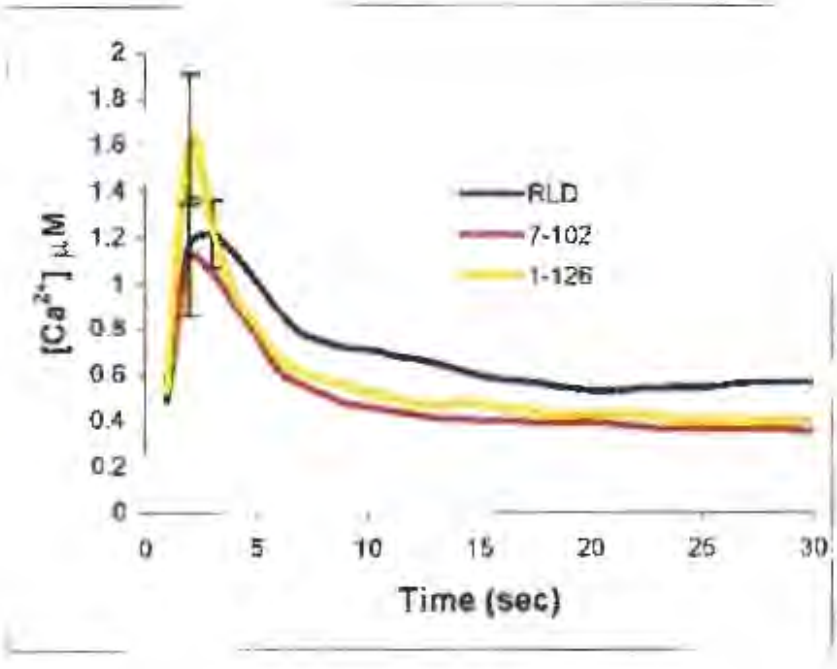

2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
[Ca2+] µM
0
5
10
15
20
25
30
Time (sec)
RLD
7-102
1-126

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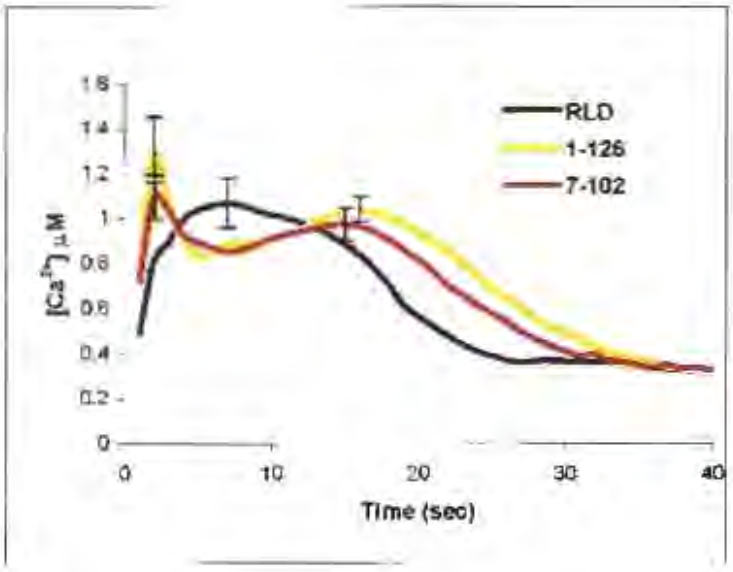
Figure 7C and 7D

E



F

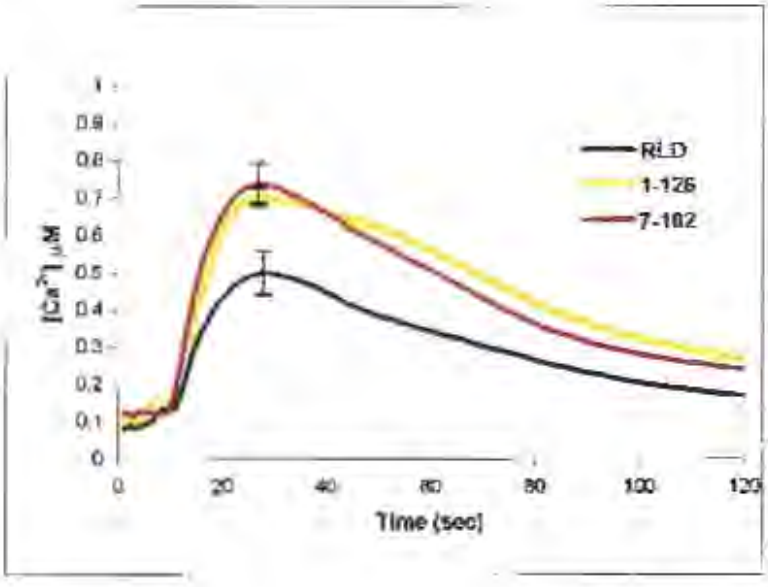


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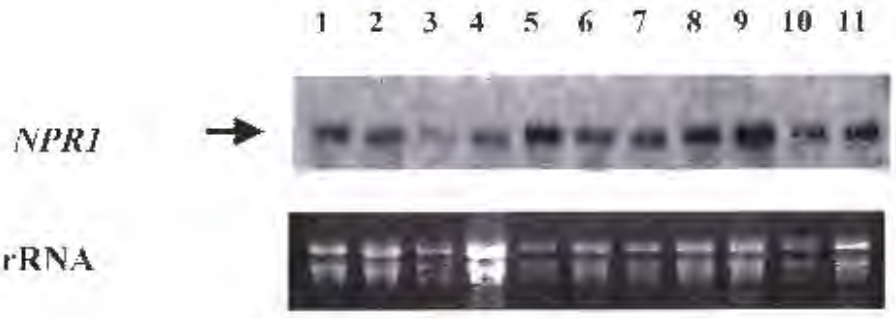
Figure 7E and 7F

A



B

Petersen, L.N. and Henby, K.J.

Figure 8A and 8B