Chapter 7

Chitosan-induced expression of nitrate reductase and NAD(P)H oxidase genes in leaves of Arabidopsis wild types and mutants
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Chitosan initiates signalling cascades, which include generation of reactive oxygen species (ROS) and reactive nitrogen species such as H$_2$O$_2$ and nitric oxide (NO) (Lee et al. 1999; Srivastava et al. 2009; Manjunatha et al. 2009), besides altered gene expression (Povero et al. 2011). The generation of ROS and NO is impaired in mutants such as AtrbohD/F (NADPH oxidase) and nia1 and nia2 (Desikan et al. 2002; Bright et al. 2006; Modolo et al. 2006) respectively. The plant membrane-bound respiratory burst oxidase homologue (Rboh, NADPH oxidase), which shows homology to mammalian gp91phox, has been identified as an important source of ROS. Rboh is involved in plant defence against pathogen and various abiotic stresses (Torres et al. 2006; Miller et al. 2008) and in hormonal (Kwak et al. 2003; Bright et al. 2006) and developmental responses (Torres and Dangl 2005). In Arabidopsis, NR was encoded by two genes NIA1 and NIA2. NIA2 accounts for 90% of the total NR activity, while NIA1 is responsible for the remaining 10% of NR activity (Yu et al. 1998).

A number of Arabidopsis genes are induced upon chitin elicitation (Ramonell et al. 2005; Miya et al. 2007; Wan et al. 2008; Povero et al. 2011) and some genes are also induced following elicitation by chitosan/chitooligosaccharides (Povero et al. 2011; Malerba et al. 2012). These include defense-related genes (such as, phenylalanine ammonia-lyase, chitinase, peroxidase and some camelexin biosynthetic genes) as well as other genes with functions not yet identified. ABA-induced biphasic expression of RBOH A–D in Zea mays (Lin et al. 2009), while the highest induction of RBOHD expression was by ABA in Arabidopsis guard cells (Kwak et al. 2003). In ABA-deficient mutants,
ABA-induced stomatal closure and expression of some of these genes are impaired leading to a wilty phenotype (Hoth et al. 2002). Since, chitosan is similar in action to that of ABA, it is possible that it may also affect the genes related to NO and H$_2$O$_2$ generation.

This chapter describes our efforts to determine the effect of chitosan on the expression of genes, involved in NO and H$_2$O$_2$ generation, namely *NIA1*, *NIA2*, *RBOHD* and *RBOHF*.

**Results**

The effects of chitosan on the expression of the genes required for H$_2$O$_2$ and NO generation, namely *RBOH* and *NIA1*, was determined, as also the reciprocal functional requirement of each of these genes on the chitosan-induced expression of each of the others. It was not possible to carry out these experiments with guard cells due to the technical limitation arising with the preparation of sufficiently large numbers of isolated guard cells. Thus, the effects of chitosan were determined on whole leaves.

The overall picture of *NIA1*, *NIA2*, *RBOHD* and *RBOHF* gene transcript accumulation are shown as gel images in Figures 7.1 and quantitative assessment of transcripts are presented in the next five Figures of 7.2 to 7.5.

*The levels of NIA1 transcripts in the mutants nia2 and rbohd/f were induced quickly by chitosan*

Accumulation of *NIA1* transcripts was elicited rapidly following chitosan treatment with a 5-fold increase occurring in leaves of both wild types Col-0 and Ler and approximately
10-fold increase in the \textit{nia2} and \textit{rbohD/F} mutants after 30 minutes (Fig. 7.1 A and Fig. 7.2 A). This accumulation peaked in 1 h and thereafter declined. Relative \textit{NIA1} transcript accumulation was highest in leaves of the \textit{rbohD/F} mutant, and was also higher in those of the \textit{nia2} mutant when compared to the corresponding wild type (Fig. 7.2 B). In all the plants treated, with increasing time, the level of the \textit{NIA1} transcript returned to levels prior to the treatment of the leaves with chitosan. The actual time taken for this to occur and the rate at which this happened depended on the plant genotype being investigated, being slow in the \textit{nia2} and \textit{rbohD/F} mutants, compared to the wild type.

\textit{The chitosan-induced increase in the levels of NIA2 transcripts was delayed in the mutants nia1 and rbohdf/}

The \textit{NIA2} transcript accumulation profile in response to chitosan followed a similar pattern to that of \textit{NIA1}, but with different overall kinetics (Fig. 7.1 B and 7.3 A, B). Chitosan-induced increases in \textit{NIA2} transcript accumulation occurred at slower rate and peaked later in 6 h. Again, the peak of \textit{NIA2} transcripts was higher in the leaves of the two mutants, \textit{nia1} and \textit{rbohD/F}, than in those of the wild type Col-0. Similarly, after reaching a peak level of accumulation, the level of \textit{NIA2} transcripts declined. However, in contrast with the rapid post-peak decline exhibited by \textit{NIA1} transcripts, those encoded by \textit{NIA2} declined at a slower rate such that in the leaves of the \textit{nia1} and \textit{rbohD/F} mutants they were still significantly elevated above the pre-treatment levels at the 48 h sampling time.
The levels of RBOHD transcripts in the mutants nia1 and nia2 when exposed to chitosan

The accumulation of transcripts encoded by the gene RBOHD was also followed in the leaves of wild type Col-0 and nia1 and nia2 mutant plants following their treatment with chitosan (Fig. 7.1 C and 7.4 A). The accumulation of RBOHD transcripts in the leaves of the mutants nia1 and nia2 started to increase by 1 h and reached its maximum in 2 h, remained higher till 6 h and then started to decline by 12 h and 24 h. However, the transcript accumulation in the leaves of nia2 mutants were lower than those of Col-0 and nia1. Relative RBOHD expression was highest in the wild types Col-0 and the mutants nia1 but in the nia2 was lower when compared to its wild type (Fig. 7.4 B).

The chitosan-stimulated levels of RBOHF transcripts follow a similar pattern with that of RBOHD in the mutants nia1 and nia2

The accumulation of transcripts encoded by the gene RBOHF followed a similar pattern to those of RBOHD when induced by chitosan in the wild type Col-0 and the mutants nia1 and nia2 (Fig. 7.1 D and 7.5 A). A3-fold increase in RBOHF in the leaves of nia1 mutants increased by 0.5 h and reached its maximum by 1 h and remained higher till 6 h and then declined in 24 h. While the RBOHF expression in the nia2 mutants increased their expression by 0.5 h and reached their maximum by 1 h and remained higher till 2 h and then declined by 6 h till 24 h. Transcript accumulation of RBOHF in both nia1 and nia2 mutants levels are almost similar but two fold higher than those of wild type Col-0.
The relative gene expression of *RBOHF* in the wild types Col-0 and the mutants *nia1* and *nia2* was somewhat similar to the fold change increase (Fig. 7.5 B).
Figure 7.1. Accumulation of *NIA1, NIA2, RBOHD* and *RBOHF* gene transcript in chitosan treated *Arabidopsis* whole leaves, as observed with RT-PCR (A) *NIA1* transcript levels in the leaves of wild types Col-0 and Ler and the mutants *nia1* and *rbohD/F*. (B) *NIA2* transcript levels in the leaves of wild type Col-0 and the mutants *nia1* and *rbohD/F*. (C & D) *RBOHD* and *RBOHF* transcript levels in the leaves of the wild type Col-0 and the mutants *nia1* and *nia2*. Total RNA was extracted from treated and control leaves and used to perform RT-PCR analysis. Treatment was 20 µg mL⁻¹ chitosan or solvent control.
Figure 7.2. Transient increase in NIA1 gene expression on exposure to chitosan in wild type and mutants of Arabidopsis. 

A. Effects of chitosan on NIA1 gene expression (expressed as fold change) in leaves of Col-0 (●) and Ler (○) wild types and nia2 (▼) and rbohdf (▽) mutants. Fold changes in expression of individual transcripts due to the treatments were calculated at each time point by dividing the normalised accumulation level of the transcript in the RNA samples derived from the treated plants by that from the untreated controls.

B. Effects of chitosan on NIA1 relative gene expression (expressed as %) in leaves of Col-0 (black bars) and Ler (white striped bars) wild types and nia2 (criss cross bars) and rbohdf (bricked bars) mutants. Total RNA was extracted and used to perform quantitative RT-PCR for NIA1. Treatment was 20 µg mL⁻¹ chitosan or solvent control. Each experiment included three replicates for each time point.
Figure 7.3. Transient increase in NIA2 gene expression on exposure to chitosan in wild type and mutants of Arabidopsis. A. Effects of chitosan on NIA2 gene expression (expressed as fold change) in leaves of Col-0 (●) and nia1 (○) and rbohdf (▼) mutants. B. Effects of chitosan on NIA2 relative gene expression (expressed as %) in leaves of Col-0 wild type (black bars) and nia1 (blocked bars) and rbohdf (bricked bars) mutants. Further details are given in Fig. 7.2 and Methods.
Figure 7.4. Transient increase in RBOHD gene expression on exposure to chitosan in wild type and mutants of Arabidopsis. A. Effects of chitosan on RBOHD gene expression (expressed as fold change) in leaves of Col-0 (●) and nia1 (○) and nia2 (▼) mutants. B. Effects of chitosan on RBOHD relative gene expression (expressed as %) in leaves of Col-0 wild type (black bars) and nia1 (blocked bars) and nia2 (crossed bars) mutants. Further details are given in Fig. 7.2 and Methods.
Figure 7.5. Transient increase in RBOHF gene expression on exposure to chitosan in wild type and mutants of Arabidopsis. A. Effects of chitosan on RBOHF gene expression (expressed as fold change) in leaves of Col-0 (●) and nia1 (○) and nia2 (▼) mutants Arabidopsis leaves. B. Effects of chitosan on RBOHF gene expression (expressed as %) in leaves of Col-0 wild type (black bars) and nia1 (blocked bars) and nia2 (crossed bars) mutants. Further details are given in Fig. 7.2 and Methods.
Discussion

Chitosan-induced gene expression in Arabidopsis leaves may not be strictly dependent on H$_2$O$_2$ production

The significant increase in the levels of *NIA1* and *NIA2* transcripts by chitosan, in *rbohD/F* mutant (Fig. 7.2 and Fig. 7.3) indicates that the H$_2$O$_2$ generation associated with these isoforms of NADPH oxidase was not required for the downstream chitosan signaling that resulted in altered gene expression. However, whether or not this precluded an absolute requirement for H$_2$O$_2$ in such chitosan signaling is unclear, since multiple other isoforms of NADPH oxidase exist in *Arabidopsis* (Capper & Dolan 2006) and may have compensated for the lack of the D and F isoforms here. What is clear though, is that in the guard cells at least, chitosan failed to induce detectable levels of H$_2$O$_2$ in the *rbohD/F* mutant as determined by H$_2$DCFDA fluorescent imaging (Fig. 6.4 L). Thus, it may be possible to conclude that there is no requirement for H$_2$O$_2$ in order for chitosan to induce the expression of *NIA1* in *Arabidopsis* leaves.

The lack of *NIA1* is compensated by enhancement of *NIA2* during chitosan-induced gene expression in Arabidopsis leaves

The increase in the levels of either *NIA2* and *RBOH*, or *NIA1* and *RBOH* transcripts when induced by chitosan, in either the *nia1* or *nia2* mutants (Fig. 7.3, 7.4 and 7.5) respectively, indicates that neither *NIA1* nor *NIA2* expression and thus, the synthesis of NO was required. However, it is possible that other sources of NO exist.

It may be argued that *NIA1* is not the only source of NO in plants and plants may also possess a mammalian type nitric oxide synthase (NOS) which can convert arginine
to citrulline and NO (Corpas et al. 2009). However, the evidence for the existence of such a plant NOS has been on the basis of a non-specific inhibitor, L-nitro-arginine methyl ester (L-NAME). There is neither biochemical nor genetic evidence to support its existence (Neill et al. 2008). Whichever may be the case, chitosan did not induce NO accumulation in the guard cells of either the nia1 or rbohD/F mutants (Fig. 6.2 D and L).

We therefore feel that the presence of NO may not by itself required for the expression of NIA1 and RBOHD/F after exposure to chitosan. It is possible that chitosan treatment initially leads to an alteration in gene expression initially via an alternative signaling pathway that does not functionally depend on the presence of NO.

**NIA1 and NIA2 genes are differentially expressed in Arabidopsis whole leaves**

The transcripts of NIA1 and NIA2 were all higher in chitosan-treated leaves of the rbohD/F mutant than in those of similarly treated wild types (Fig. 7.1), indicating a possible role for NIA1 in NO generation. The genetic evidence to date suggests that only NIA1 is required for NO synthesis, at least in guard cells (Bright et al. 2006). However, here the increase in the accumulation of NIA2 transcripts in leaves following chitosan treatment lagged behind that of those encoded by NIA1 (Fig. 7.2). This suggest a differential expression of NIA1 and NIA2 genes when induced by chitosan in Arabidopsis leaves. Other reports have similarly demonstrated differential expression patterns for NIA1 and NIA2 in response to NO−3, light, circadian rhythm and cytokinin (Cheng et al. 1991; Yu, Sukumaran & Marton 1998).

The elevated chitosan-induced accumulation of NIA2 transcripts in the leaves of the nia1 mutant (Fig. 7.3 A, B) compared to those of wild types suggests that expression
of NIA2 may compensate for a lack of NIA1. Further experiments are necessary to validate our suggestions. It would be interesting to determine the efficacy of NO mediated acclimation responses to stress in the nia2 mutant. The present data suggests that the signaling molecules NO and H$_2$O$_2$ might not be required during chitosan-induced gene expression in leaves. The focus of future work may determine which signaling pathways are involved during this process.

**Conclusions**

1. Chitosan-induced gene transcript accumulation in *Arabidopsis* leaves is not dependent on RBOH (and possibly H$_2$O$_2$).
2. The increased levels of NIA2 transcripts in the nia1 mutants suggest that NIA2 expression may compensate for a lack of NIA1 and that NO production may not be essential for NIA1 during chitosan-induced gene transcript accumulation in *Arabidopsis* leaves.
3. The expression of NIA1 and NIA2 genes are differentially modulated when exposed to chitosan in *Arabidopsis* leaves.
4. The ability of chitosan to induce stomatal closure and to activate expression of defense-related genes emphasizes its potential use for modulating the stomatal movement and defense responses.

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