CHAPTER SIX

NPR1-DEPENDENT *PR-1* TRANSCRIPTION REQUIRES A FUNCTIONAL GLUTATHIONE BIOSYNTHETIC PATHWAY

6.1 Abstract

The glutathione-ascorbate cycle is a major pathway for reactive oxygen species (ROS) scavenging especially during abiotic and/or biotic stress conditions when syntheses of ROS escalate. This interaction further influences the activation of downstream defense signaling processes for *PR* gene activation. Most *PR* gene induction processes are dependent on the activation of cytosolic NPR1 which in itself is redox dependent. Using *Arabidopsis* mutants deficient in cytosolic reduced glutathione (GSH), the interplay between GSH and ascorbic acid (As)A in *NPR1*-dependent *PR-1* gene induction was studied. Following pathogen infection, a decreased GSH content in mutants led to an increased oxidized glutathione (GSSG) content lowering the GSH/GSSG redox pool. The GSH deficit in these plants was compensated by an increased dehydroascorbate (DHA). However, this compensatory effect had no significant effect on *NPR1* gene transcription. Interestingly, the presence of cytosolic glutathione in wild-type plants coupled with a higher GSH/GSSG ratio significantly affected the amounts of *NPR1* transcripts measured after infection. *PR-1* transcription was further substantially higher in wild-type plants than in the mutants enabling the wild-type plants to efficiently limit the spread of pathogens after infection. This study shows that *NPR1* transcription does require a functional cytosolic GSH biosynthetic pathway for an effective *PR-1* transcription and biotic stress response

6.2 Introduction

Glutathione, ascorbate and tocopherols are low molecular weight antioxidants involved in plant growth and developmental processes, as well as during plant-pathogen interactions (Foyer and Noctor, 2005; Choi *et al*., 2007). These antioxidants interact with reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), whose concentrations are known to increase when plants are exposed to various forms of stressors (Foyer and Noctor, 2005; Gills and Tuteja, 2010; Liu *et al*., 2010). H_2O_2 generated in this way during pathogen-induced oxidative burst has been associated with the hypersensitive response (HR) and systemic acquired resistance (SAR) playing a pivotal role in conferring tolerance (Mellersh *et al*., 2002; Hahlbrock *et al*, 2003; Foyer and Noctor, 2005; Davies *et al*., 2006; Liu *et al*., 2010). The major point of detoxification of H_2O_2 is the ascorbate-glutathione (AsA-GSH) cycle which occurs predominately in the mitochondria, chloroplasts, peroxisomes and the cytoplasm (Noctor and Foyer, 1998; Asada, 2000; Foyer and Noctor 2005; Kotchoni and Gachomo, 2006). During this processes, the enzyme ascorbate peroxidase (APX) uses electrons from AsA to degrade the H_2O_2 produced either from superoxide dismutase (SOD), or ROS into water and monodehydroascorbate (MDHA). The MDHA, however is relatively unstable and quickly dissociates to dehydroascorbate (DHA) in a reaction catalyzed by monodehydroascorbate reductase (MDHAR). The DHA is in a next step reduced by DHAR into AsA using electrons donated by GSH. GSH is reduced during this process to oxidized glutathione (GSSG) and in a reaction catalyzed by glutathione reductase (GR), together with electrons from NADPH; GSH is again regenerated (Noctor and Foyer, 1998; Asada, 2000). Thus, the ability of the AsA-GSH cycle to directly interact with ROS and to control the AsA and

GSH redox state of the plant has rendered the cycle a key determinant of other downstream defense response (Kiddle *et al*., 2003).

Recently, a new family of *Arabidopsis* thiol transporter has been isolated by Maughan *et al*. (2010). This transporter, which has a high homology to the *Plasmodium falciparum (Pf*) chloroquine-resistance transporter *(Pf*CRT) and designated CRT-Like Transporter1 (CLT1), has been implicated in the translocation of GSH and *gamma*-glutamylcysteine (γ EC) from the chloroplast to the cytosol. Analysis of these genotypes revealed that, while the glutathione content of the roots of the triple mutant (*clt1clt2clt3*) are significantly reduced compared to the wild-type, shoot glutathione contents are similar in both genotypes. However, it appears that most, if not all, of the GSH in the leaves of the triple *clt1clt2clt3* mutant is localized in the chloroplast and hence the cytosol is deficient in GSH. This triple mutant is therefore an ideal tool, with which to study the role of cytosolic glutathione and other redox determinants like AsA in the *NPR1*-dependent defense response pathway.

Studies aimed at delineating the role of GSH and more specifically of cytosolic GSH on *NPR1*-dependent *PR-1* activation are limited. This part of the study was therefore aimed at elucidating the effect of a deficit in cytosolic GSH on the *NPR1*-dependent *PR-1* transcription using *clt1clt2clt3 Arabidopsis* mutants and to investigate the role of cytosolic GSH in conferring resistance to plants against *Pseudomonas syringae* and *Botrytis cinerea* pathogens.

6.3 Materials and methods

6.3.1 Growth of *Arabidopsis* plants

Except indicated otherwise, *Arabidopsis* wild-type and *clt1clt2clt3* triple mutant Columbia seeds, were surface sterilized for 30 min in a 1.5% (v/v) sodium hypochlorite solution. Seeds were thoroughly rinsed (four times) in distilled water and re-suspended in 0.1% agar (w/v). The surface sterilized seeds were plated in 90-mm Petri dishes (Plastpro Scientific, South Africa) containing Murashige and Skoog (MS) agar medium and vernalized at 4^{o}C for 3-days in the dark. Petri dishes containing vernalized seeds were later transferred to a 10 h day/14 h dark regime for 10 d before sowing individual seedlings in moist 44-mm Jiffy-7 pots (Jiffy International AS, Norway). The Jiffy pots (30 each) where placed in 35 x 25 x 9 cm trays without holes to facilitate sub-irrigation. Trays were covered with cling wrap to ensure for maximum humidity. These were then transferred to controlled environment chambers.

Each chamber was provided a photosynthetic photon flux of 200 µmol. m^{-2} s^{-1}, a photoperiod of 10 h day/14 h dark regime and a relative humidity of 65 ± 5%. The cling wrap was removed from the trays after 2-weeks when the plants had developed at least two true leaves. Plants were then maintained under the above mentioned growth conditions for a further 2-weeks. Throughout the experimental period, the Jiffy-7 pots were kept moist by sub-irrigating them twice a wk. All experiments were performed on 4-week-old soil grown plants.

6.3.2 Bacteria growth, plant treatment and sampling

Except stated otherwise, all reagents used for these experiments were purchased from Sigma Aldrich, USA.

6.3.2.1 *Growth of pathogens used for infection*

Arabidopsis plants were infected with either a virulent strain of *Pseudomonas syringae* pv. *maculicola* DG3 (*Pma* DG3; Guttman and Greenberg, 2001), *P. syringae* pv. *tomato* - luxCDABE (*Pst*-luxCDABE; JIC, England), the avirulent *P. syringae* pv. *tomato* avrRPM1 (*Pst*avrRPM1; JIC, England), or *Botrytis cinerea* B06.10 (kindly provided by Dr Henk-Jan Schoonbeek, University of Fribourg).

All *P. syringae* strains were initially cultured from glycerol stocks in 90-mm Petri dishes (Plastpro Scientific, South Africa) containing King B's medium [20 gL^{-1} proteose peptone (w/v), 1.5 gL^{-1} Di-potassium hydrogen phospohate (w/v), 1.5 gL^{-1} magnesium sulphate (w/v), 1.5% glycerol (v/v) and 1.2% bacterio agar (w/v)]. Petri dishes were incubated at 28 ^{o}C for 48 h.

Pma DG3 was selected on medium supplemented with 50 mgL^{-1} streptomycin and kanamycin, while the virulent *Pst*-luxCDABE strain and the avirulent *Pst*avrRPM1 strain was selected on King B's medium supplemented with 50 mgL^{-1} kanamycin only.

Twenty-four hours post infection, bacteria strains were re-suspended in a 10 mM $MgCl_2$ solution. This inoculum (100 µL), was spread on Petri dishes containing King B's medium supplemented with the appropriate antibiotic and incubated at 28°C.

B. cinerea B05.10 (Büttner *et al*. 1994) was cultured at 20°C on Malt extract agar (30 gL^{-1}) and yeast extract (2 gL^{-1}) medium (MEYA; Oxoid, UK) as described by Schoonbeck *et al*. (2003) and in section 5.3 of this thesis.

6.3.2.2 *Plant infection*

Plants for antioxidant measurements and qRT-PCR were sprayed generously with an inoculum of *Pma*DG3 (OD600nm =0.05) re-suspended in a buffer consisting of 10 mM $MgCl_2$ and supplemented with 0.02% Silwett L-77 [(v/v); Lehle seeds, USA]. Another set of plants representing the controls were sprayed in the same way with the re-suspension buffer only. Entire wild-type and *clt1clt2clt3* mutant rosettes (three per time point) were harvested at 0, 24 and 48 h post treatment. Upon harvesting, samples were immediately weight, frozen in liquid nitrogen and stored at -70°C until required. The antioxidant experiments were repeated seven times, while the qRT-PCR experiment was repeated twice with independent plants grown and infected as described above.

For the bioluminescence assay to determine bacteria growth *in planta* and qRT-PCR, a single leaf from each plant was initially syringe infiltrated with either 10 mM $MgCL_2$ followed 48 h by a secondary infiltration of three additional leaves per plant with the virulent *Pst*-DC3000-Lux

($5x10^5$ cfu.ml^{-1} in 10mM $MgCl_2$) bacteria. This represented the non-SAR inducing treatment (MV treatment). In the SAR-inducing treatment (AV), *Pst avrRPM1* ($5x10^6$ cfu.ml^{-1} in 10mM$MgCl_2$) was used for primarily infiltration of a single leaf per plant and 48 h following primary infiltration, three additional leaves were infiltrated with the virulent *Pst*-DC3000-Lux strain. Growth of *Pst*-DC3000-Lux was determined *in planta* 48 h after the secondary challenge using 8-mm leaf discs excised from *Pst*-DC3000-Lux-infiltrated leaves as described by Fan *et al*. (2008). A total of 12 plants were used to for each time point an bacteria growth was represented as the number of photon counts per second (CPS). The severity of damage caused by the pathogen was also observed visually and digital pictures of selected representative infected leaves were taken. Samples for RNA extraction were harvested 48 h after the primary and secondary infection, frozen in liquid nitrogen and stored at -70C until required. The experiment was repeated twice with independent samples.

For infection with *B. cinerea* B06.10, an innoculum (resuspended in ¼ strength MEYA medium) with a final concentration of 2.5 x 10^5 spores/mL was prepared. For each plant to be infected six leaves were used and 5 µL droplets of the inoculums were pipetted onto one side of each leaf. Trays containing plants were covered with a plastic dome to achieve close to 100% humidity. The severity of damage caused by the fungus to the plant was assessed 3 dpi. Plants for the control experiment were treated in the same way with 5 µL droplets of the re-suspension solution. Data was collected from a total of eight plants to represent a single time point and disease severity caused by the pathogen was also observed visually and pictures of selected infected leaves were digitally taken. The experiment was repeated twice with independent plants grown and infected as described above.

6.3.3 Chlorophyll, glutathione and ascorbate measurements

The methods for these assays were adapted from Queval and Noctor, (2007) with slight modifications. Except where stated otherwise, each 1 mg of plant material was homogenized in 1 mL of extraction buffer. Centrifugation steps were carried out at 4^{o}C and 12.000 revolutions per minute (rpm). Sample was assayed in triplicates in 96-welled UV-transparent micro titer plates (Corning, USA) and absorbencies were read at 25^{o}C using a SPECTRAmax plus 384 plate reader (Molecular device ltd, Ireland). Each experiment was repeated seven times with different samples that were treated and sampled in the same manner as described above in section 6.3.2.2.

Each *Arabidopsis* rosette was ground to a fine powder with a pestle in a mortar containing liquid nitrogen. The metabolite in the sample was extracted in a total volume of 2 mL using a 1-M hypochloric acid ($HClO_4$) solution and using two aliquots of the acid as follows: to each sample, 1 mL of 1 M $HClO_4$ was added, mixed properly and transferred to a 2-mL Eppendorf tube. The mortar and pestle was rinsed with the remaining 1 mL of the 1 M $HClO_4$ and this was added to the first extract in the same tube. Samples were thoroughly mixed before centrifugation at 12. 000 rpm for 10 min. The clear supernatant was retained for ascorbate and glutathione assays while the pellet was used to assay chlorophyll measurements.

6.3.3.1 *Chlorophyll measurement*

Each pellet obtained from the above centrifugation step was re-suspended in a total volume of 10 mL of an 80% acetone solution in two steps: in the first step, the pellet was re-suspended in 1 mL

of 80% acetone and mixed by vortexing. This was transferred to a 14-mL Falcon tube containing 8 mL of 80% acetone. The Eppendorf tube was rinsed with an additional 1 mL of 80% acetone and this was added to the components in the Falcon tube. The chlorophyll extract was thoroughly mixed and all samples were wrapped in foil paper and incubated in the dark at 4^{o}C overnight.

The following day, the chlorophyll extract were again mixed and a 1-mL aliquot of plant extract was transferred to a 1.5 mL Eppendorf tube. This was centrifuged at 12.000 rpm for 10 min. Samples in which the pellet still appeared green were re-suspended in an additional 1 mL of 80% acetone, mixed and centrifuged under the same conditions as the first. Both supernatants were combined and the volume noted. Once a clear pellet was obtained, 2-mL aliquots of the supernatant were used to assay chlorophyll (phaeophytin) using a quartz cuvette. Absorbance was read at 655 nm and 666 nm and these were corrected against blank readings obtained from 2 mL of an 80% acetone solution.The concentration of phaeophytin in each sample was calculated using the formula:

Phaeophytin (mg.L^{-1}) = (26.03 x A_{655}) + (6.75 x A_{666}).

6.3.3.2 *Measurement of total, reduced* and *oxidized glutathione*

Reagents for GSH assays consisted of 50 mM nicotinamide adenine di-nucleotide phosphate reduced tetra-sodium salt (NADPH; EC: 220-163-3) and 6 mM 5'5'-dithiobis-2-nitobenzoic acid (DTNB; EC: 200-714-4). These reagents were prepared fresh in a 0.12 M NaH_2PO_4 (pH 7.5) buffer which had been supplemented with 6 mM EDTA. Thereafter, aliquots were made from the NADPH solution and stored at -20^{o}C, while the DTNB solution was stored in the dark at 4^{o}C.

The enzyme for this reaction glutathione reductase (GR) was prepared fresh daily by spinning 25 µL of the GR stock and re-suspending the pellet in 567 µL of reaction buffer to obtain a final concentration of 20 UmL^{-1}.

Glutathione (reduced and oxidized glutathione) standards were prepared in a buffer consisting of 0.5 mL of a 1 M $HClO_4$ solution containing 0.1 mL of 0.12 M NaH_2PO_4 (pH 7.6). The pH of this buffer was adjusted to 6.0 with sufficient amounts of 2.5 M K_2CO_3. The pH was verified using pH strips. Excess $KClO_4$ resulting from the reaction was removed from the solution by centrifugation for 10 min and the supernatant was decanted into a clean 50-mL Falcon tube.

Standards for total glutathione were prepared from a 1 M GSH (EC:200-725-4) solution which was diluted to 200 µM to obtain a stock amount of 2 nmol in a volume of 10 µL. Serial dilutions of 2.0, 1.0, 0.5, 0.25 and 0.125 nmol were prepared from the stock using the standard buffer.

Each reaction mixture consisted of 0.16 mL of 0.12 M NaH_2PO_4 (pH 7.5), 6 mM EDTA, 0.01 mL of 6 mM DTNB, 0.01 mL of 50 mM NADPH and 0.01 mL of either standards or neutralized plant sample. Reference samples (blanks) contained the above-mentioned solutions but lacked the standard and plant supernatant. The samples were mixed in the plate reader and the absorbance at 412 nm read for zero. The reaction was initiated by adding 10 µL of GR into each well and the increase in absorbance at 412 nm was monitored for 5 min after automatic mixing.

For GSSG measurements, an aliquot of the neutralized plant supernatant was first treated with 2-vinylpyridine. This was achieved by adding aliquots of 0.2 mL neutralized plant extract to a tube

containing 5 µL of 2-vinylpyridine. The reaction mixture was incubated at 25°C for 30 min. After incubation, samples were centrifuged for 15 min and 0.15 mL of the supernatant was decanted into a fresh tube. The centrifugation step was repeated using this second supernatant and 0.13 mL of the final supernatant decanted into a fresh tube.

For GSSG standards, 0.1 M GSSG (EC: 248-170-7) was diluted to a final concentration of 4 µM to obtain a stock solution of 80 pmol in every 20 µL. The stock solution (0.2 mL) was treated with 2-vinylpyridine in the same way as the plant extract. Standards for the reaction were prepared to final amounts of 80, 40, 20, 10, 5 and 2.5 pmol from the treated stock solution. All samples were assayed as for total glutathione.

The amount of GSH for both the wild-type and *clt1clt2clt3* mutant *Arabidopsis* plants was calculated by subtracting the GSSG content obtained for each time point from those of the total GSH content.

6.3.3.3 *Measurement of reduced, total ascorbate* and *dehydroascorbate content*

AsA contents were measured using the same acid extracts as GSH. Each supernatant (0.5 mL) obtained after centrifugation of the 1 M $HClO_4$ crude extract was decanted into a fresh tube containing 0.1 mL of a 0.12 M NaH_2PO_4 (pH 7.6) solution. The pH of the supernatant was adjusted to 6.0 by adding in a drop-wise manner an aliquot (60 µL) of 2.5 M K_2CO_3. The pH was determined using pH strips. The supernatants were thoroughly mixed after each addition to ensure a uniform and a rapid change in the pH of the extracts. The mixtures were centrifuged

again and the insoluble pellets were discarded. The final supernatants, which were used for the ascorbate and glutathione determinations, were at a pH of 6.0.

A stock solution of ascorbate oxidase (AO; EC: 232-852-6) was prepared by dissolving eight mg of the enzyme powder in a 0.2 M NaH_2PO_4 (pH 6.6) solution so as to obtain a final concentration of 40 UmL^{-1} AO. This stock solution was stored in aliquots of 0.2 mL at -20^{o}C until required. Each reaction mixture for the AsA measurement consisted of 40 µL of the neutralized plant extract (pH 6.0) and 0.16 mL of 0.12 M NaH_2PO_4 (pH 6.6) which, had been supplemented with 1 mM EDTA. The reaction was properly mixed and their absorbencies measured at $A_{266.}$ The oxidation of the AsA was initiated by adding 0.2 U AO. The reactions were again mixed and the decrease in absorbance at A_{265} was monitored until the reaction was complete.

For measurement of total ascorbate (DHA plus AsA), DHA was first reduced to AsA. This was accomplished using 1-4-dithiothreitol (DTT; EC: 222.4687) as follows: aliquots of 0.1 mL of the neutralized plant extract were added to tubes containing 0.14 mL of 0.12 M NaH_2PO_4 (pH 7.5) and 0.01 mL of 0.5 M DTT solution. The mixtures were incubated for 30 min at 25^{o}C and the samples were then assayed as described above for reduced ascorbate.

The amount of ascorbate in both wt and *clt1clt2clt3* mutant *Arabidopsis* rosettes was calculated using the extinction coefficient: A_{265} = 14000 mol/L where, mol/L = mol per litre. The amount of DHA for both the wt and *clt1clt2clt3* mutant *Arabidopsis* plants were calculated by subtracting the reduced ascorbate content obtained for each time point from those of the total ascorbate content.

6.3.4 Quantitative real time-polymerase chain reaction

Quantitative real time polymerase chain reaction (qRT-PCR) was conducted to measure the relative transcript amounts of the *Arabidopsis NPR1* (*AtNPR1*; AT1g64280) and *AtPR-1* gene (NM_127026.2) in the leaves of both wt and *clt1clt2clt3* mutant *Arabidopsis* plants treated with *P. syringae* as specified above. Primers for these reactions were designed using the Netprimer3 program (Premier Biosoft, Palo Alto, CA, USA) and following specifications described in chapter 4.3. *Arabidopsis* actin (AT3g18780) was used as the housekeeping gene. Following verification of resultant primers as described in section 4.3, the following primer combinations were used for the reaction: qNPR1_forward -TCTATCAGAGGCACTTATTGG and qNPR1_reverse – TGCCTTATGTACATTCGAGAC; qPR-1_forward – CGGAGCTACGCAGAACAACT and qPR-1_reverse – CTCGCTAACCCACATGTTCA; qActin_forward – AGTGGTCGTACAACCGGTATTGT and qActin_reverse – GATGGCATGGAGGAAGAGAGAAAC.

QRT-PCR was carried out using cDNA synthesized from total RNA of leaf material from *Pst* avrRPPM1, *Pst* avrRPPM1/*Pst*-luxCDABE and treated plants harvested at 0 and 48 h post-treatment. By use of the manufacturer's software program, the relative concentrations of the samples were determined. All samples were normalized using their endogenous actin values and the relative transcript amounts for each time point was expressed relative to the controls which served as the calibrator. The Applied Biosystems User Bulletin No. 2, (2000) and steps described in Livak and Schmittgen (2001) were used for data analysis.

6.3.5 Statistical analysis

The statistical differences from data obtained between the two genotypes and between the different time points in each experiment were calculated using the Statistical Analysis Software (SAS$^{(R)}$, USA). This was done using ANNOVA and Tukey studentized range (HSD) test and means with similar letters were considered as not being statistically different from one another. Percentage differences between two independent samples were calculated using the Student's T-Test. In both procedures, the cut-of value was set at $P>0.05$ for samples which were not significantly different from each other.

6.4 Results

6.4.1 Chlorophyll content

The chlorophyll content in the two *Arabidopsis* genotypes was measured as phaeophytin after acid extraction of plant extract. SAS$^{(R)}$ analysis showed no significant (P>0.05) differences in chlorophyll turnover in both genotypes during the time points used for this study (Table 6.1). All antioxidants measured in the following study were therefore expressed in terms of their chlorophyll content.

Table 6.1 Chlorophyll content in *Pseudomonas*-treated wild-type and *clt1clt2clt3* mutant *Arabidopsis* plants.

Chlorophyll content (mgg^{-1}FW)			
Genotype	**Post *Pseudomonas* infection (h)**		
	0	**24**	**48**
Wild type	**1.2 ± 0.11^{a}**	**1.19 ± 0.07^{a}**	**1.10 ± 0.15^{a}**
***clt1clt2clt3* mutant**	**1.2 ± 0.09^{a}**	**1.21 ± 0.17^{a}**	**1.21 ± 0.13^{a}**

Values represent the means ± standard error of the mean (SEM) of seven independent experiments. Means with similar letters are not significantly different (P>0.05).

6.4.2 Glutathione content

GSSG content was measured after treatment of plant extracts with 2-vinyl pyridine to complex the GSH present. At the start of the experiment (0 hpi), the GSSG content was not significantly ($P>0.05$) different between the wild-type and the *clt1clt2clt3* mutant *Arabidopsis* genotypes (Table 6.2). In the mutant, a 84% significant ($P<0.05$) increase in GSSG content was found 24 hpi when compared to the GSSG amount at the 0 h time point. This increment was also 74% significantly ($P<0.05$) higher than the GSSG content in the wild-type plants 24 h post *Pseudomonas* treatment. At the 48 h time point, the GSSG content in the mutant had again increased in comparison to the 24 h GSSG content, however this was not significantly ($P>0.05$) different from that of the wild-type *Pseudomonas* treated plants analyzed 48 h after infection.

Table 6.2 GSSG content in *Pseudomonas*-treated wild-type and *clt1clt2clt3* mutant *Arabidopsis* plants.

GSSG content ($nmolmg^{-1}$Chlorophyll)			
Genotype	**Post *Pseudomonas* infection (h)**		
	0	**24**	**48**
Wild type	**21.63 ± 2.14^{c}**	**24.20 ± 1.20^{c}**	**46.43 ± 7.16^{ab}**
***clt1clt2clt3* mutant**	**23.27 ± 1.12^{c}**	**42.32 ± 6.06^{ab}**	**57.29 ± 6.79^{a}**

Values represent the means ± standard error of the mean (SEM) of seven independent experiments. Means with similar letters are not significantly different (P>0.05).

Similar to the GSSG content, the GSH content between the two *Arabidopsis* genotypes was not significantly ($P>0.05$) different at the start of the experiment (Fig. 6.1). After 24 hpi, the GSH content in the mutant was significantly ($P<0.05$) reduced by 28% in comparison to the GSH content at the beginning of the experiment (Fig 6.1). This decrease was also significantly lower (56%) than the GSH content measured in the infected wild-type plants 24 hpi. At 48 hpi, the GSH content in both genotypes decreased without being significantly ($P>0.05$) different from each other.

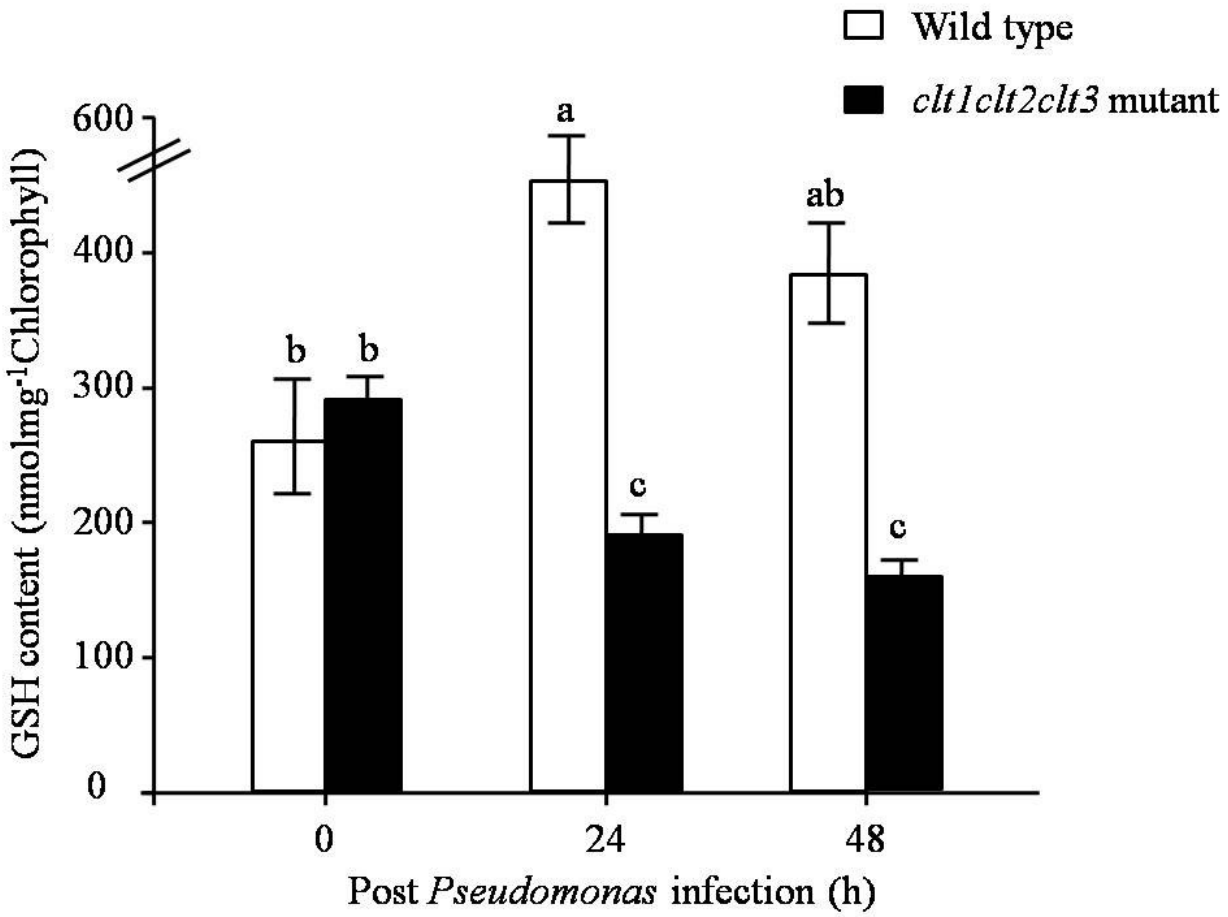

Figure 6.1 Reduced glutathione content in *Pseudomonas*-infected wild-type and *clt1clt2clt3* mutant *Arabidopsis* plants. Metabolites were extracted in 1 N of a $HClO_4$ solution using *Arabidopsis* rosettes harvested at 0, 24 and 48 h post *Pma*DG3 infection. Total glutathione was measured directly from the neutralized supernatant while reduced glutathione was calculated by subtracting the GSSG content (measured after treatment of the neutralized plant supernatant with 2-vinyl pyridine) from that of the total glutathione content. All samples were assayed in triplicates by reading the absorbance at 412 nm before and after initiating the reaction with GR. The mean values ± standard error of the mean (SEM) for a total of seven repeats were plotted on the graph. Means with the same letters are not significantly different (P>0.05).

During the time points investigated, the GSH/GSSG ratio in *Pseudomonas*-treated mutant plants significantly (P<0.05) decreased by 77% at 24 hpi compared to the ratio at the start of the experiment. This was followed by a continuous significant (P<0.05) decrease in the GSH/GSSG ratio of the mutant of a further 75% at 48 hpi when compared to the GSH/GSSG ratio at 24 hpi. At both time points (24 and 48 hpi), the GSH/GSSG ratio in the mutant was always significantly (P<0.05) lower than that of the *Pseudomonas*-treated wild-type plants (Table 6.3). In the wild-type plants, the GSH/GSSG ratio initially increased significantly (P<0.05) by 46% 24 hpi in comparison to the GSH/GSSG ratio at the start of the experiment. This was followed by a 57% significant (P<0.05) decrease in this ratio 48 hpi.

Table 6.3 GSH/GSSG ratio in *Pseudomonas*-treated wild-type and *clt1clt2clt3* mutant *Arabidopsis* plants.

	GSH/GSSG		
Genotype	**Post *Pseudomonas* infection (h)**		
	0	**24**	**48**
Wild type	**14.70 ± 2.35^{b}**	**21.59 ± 2.07^{a}**	**08.66 ± 1.17^{c}**
***clt1clt2clt3* mutant**	**14.65 ± 1.79^{b}**	**4.89 ± 1.55^{d}**	**2.52 ± 0.09^{de}**

Values represent the means ± standard error of the mean (SEM) of seven independent experiments. Means with similar letters are not significantly different (P>0.05).

6.4.3 Ascorbate content

Similar to the GSH content, the absence of a functional GSH transporter in the mutant plants did not affect the total ascorbate and reduced ascorbate content at the start of the experiment (Fig. 6.2). Twenty-four h after treatment with *Pseudomonas*, both genotypes had significantly ($P<0.05$) increased amounts of total ascorbate (61%) in comparison to the content at the 0 h time point (Fig 6.2A). Thereafter, the total ascorbate content decrease in both genotypes at 48 hpi with a more dramatic and significant ($P<0.05$) decrease in the mutant plants which, was also 37% significantly ($P<0.05$) less than that of the wild-type plants.

Following pathogen infection, the content of AsA also increased significantly ($P<0.05$) by 33% in the wild-type plants when compared to the mutant plants (Fig. 6.2B) whose AsA content remained constant between the 0 and 24 hpi. At 48 hpi, the AsA content in the mutant decreased significantly ($P<0.05$) by 76% which was significantly ($P<0.05$) lower when compared to the content in wild-type plants.

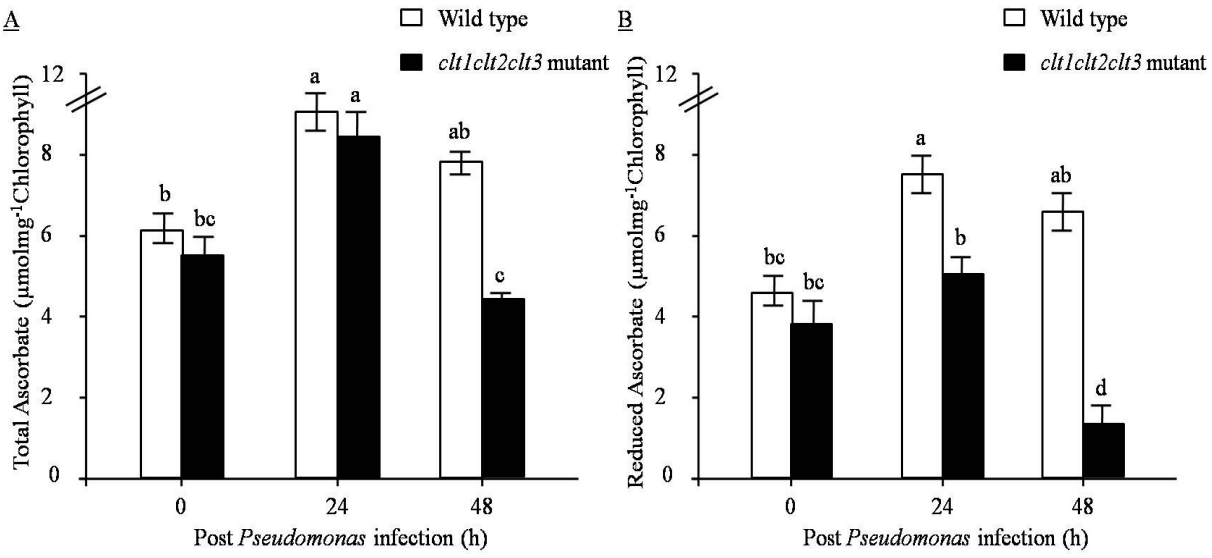

Figure 6.2 Total and reduced ascorbate content in *Pseudomonas*-treated wild-type and *clt1clt2clt3* mutant *Arabidopsis* plants. Leaf material from three plants were pooled to represent a single time point and harvested from the two genotypes at 0, 24 and 48 h after infection with *Pma*DG3. Metabolite extraction was done in a 1 M $HClO_4$ solution and, the acid supernatant was neutralized to a pH of 6.0 with 2.5 M K_2CO_3. Total ascorbate (A) measurement was performed using samples that had been treated with DTT. Reduced ascorbate (B) content was measured directly using untreated neutralized acid supernatants. All samples were assayed by reading the absorbance at 265 nm before and after initiating the reaction with ascorbate oxidase. The mean values ± SEM for a total of seven repeats were plotted on the graphs. Means with the same letters on individual graphs are not significantly different (P>0.05).

The DHA content was measured by subtracting the AsA content at each time point from that of the total ascorbate measured. While no significant (P>0.05) difference was obtained in the DHA content between both genotypes at 0 hpi, a significant (P<0.05) 65% increase in DHA was obtained at 24 hpi for the mutant when compared to the DHA content in the wild-type (Table 6.4). The DHA content in the mutant again increased significantly (P<0.05) by 179% at 48 hpi when compared to the DHA content in the wild-type at this time point.

Table 6.4 DHA content in *Pseudomonas*-treated wild-type and *clt1clt2clt3* mutant *Arabidopsis* plants.

DHA content ($\mu molmg^{-1}$Chlorophyll)			
Genotype	**Post *Pseudomonas* infection (h)**		
	0	**24**	**48**
Wild type	**1.67 ±0.15^{c}**	**2.53 ± 0.11^{b}**	**1.18 ± 0.07^{c}**
***clt1clt2clt3* mutant**	**1.79 ± 0.79^{c}**	**4.19 ± 0.42^{a}**	**3.52 ± 0.87^{ab}**

Values represent the means ± standard error of the mean (SEM) of seven independent experiments. Means with similar letters are not significantly different (P>0.05).

6.4.4 *NPR1* and *PR-1* transcription

Treatment of *Arabidopsis* wild-type plants and *clt1clt2clt3* mutant plants with only $MgCl_2$ (control) resulted in no significant ($P>0.05$) changes in *NPR1* transcript amounts 48 h after treatment. In the MV- and AV-treated plants, there was a slight but significant ($P<0.05$) 1.5-fold and 1.3-fold increase in *NPR1* transcript amounts for the wild-type plants compared to the $MgCl_2$ (control) plants. In the mutant plants no significant ($P<0.05$) differences in *NPR1* transcription was measured in comparison to the control plants (Fig. 6.3A). *PR-1* transcript amounts were significantly ($P<0.05$) higher in both the wild-type and mutant *Arabidopsis* genotypes after pathogen infection in comparison to the $MgCl_2$ treated samples (Fig. 6.3B). Primary infection of the plants with *Pst*-luxCDABE led to a 41-fold significant ($P<0.05$) increase of *PR-1* transcripts in the wild-type plants. This was significantly ($P<0.05$) higher than the only 5-fold increase in *PR-1* transcript amounts obtained for the mutant when compared to their $MgCl_2$ treated samples. The SA-inducing treatment (AV-treatment) resulted 48 h after the secondary infection to a significant ($P<0.05$) 77-fold increase in wild-type *PR-1* transcripts compared to a 3-fold increase in transcripts measured in the mutant.

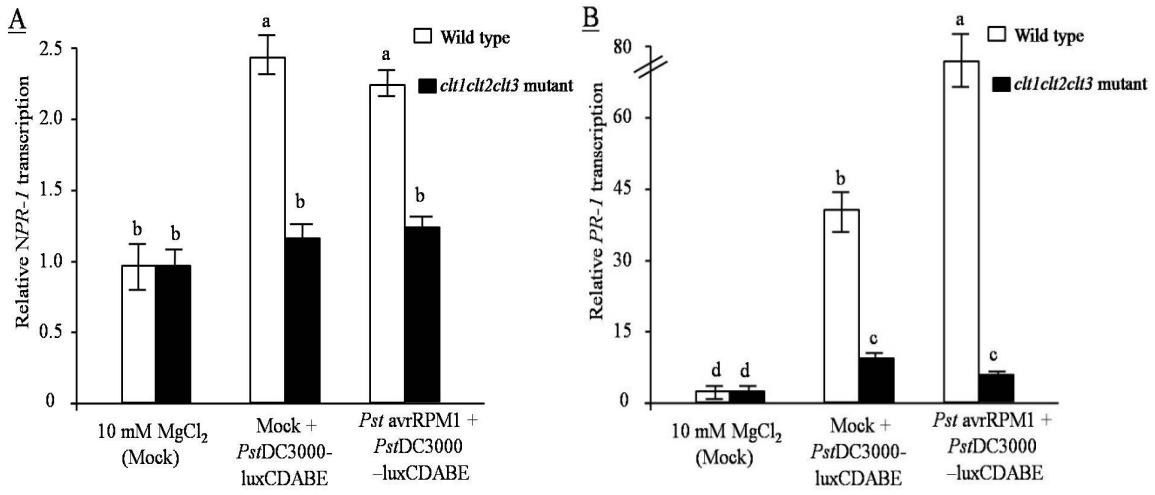

Figure 6.3 Relative *NPR1* and *PR-1* transcript amounts in *Pseudomonas*-treated wild-type and *clt1clt2clt3* mutant *Arabidopsis* plants. Plants of the two genotypes were treated with either $MgCl_2$ (control), *Pst*DC3000-luxCDABE or *Pstavr*RPM1/*Pst*DC3000-luxCDABE. cDNA from leaf material harvested 48 h post treatment with 10 mM $MgCl_2$ or 48 h following either a primary or the secondary challenge with *Pst*DC3000-luxCDABE were used for qRT-PCR. The transcript amounts for the *Arabidopsis* (At)-*NPR1* (A) and *At-PR-1* (B) were calculated relative to the endogenous levels of actin (reference gene) and expressed relative to the transcript amounts obtained from their respective control plant samples. The experiment was repeated twice using different sets of plant material treated and harvested as described above. The mean values ± SEM were plotted on the graph. Means with the same letters (a, b, c, d) on the same graph are not significantly different (P>0.05).

6.4.5 Disease progression

Pst-luxCDABE bacteria growth was measured 48 h post secondary infection in the MV- and AV-treated (Fig 6.4). In MV-treated (non-SAR induced) plants, a significant ($P<0.05$) 1.5-fold reduction in *Pst*-luxCDABE bacteria number was found for the wild-type plants when compared to the mutant plants 48 h post secondary infection. Similarly, in AV-treated (SAR induced) plants, growth of *Pst*-luxCDABE was significantly ($P<0.05$) suppressed (3.2-fold) in the wild-type plants in comparison to the mutant plants.

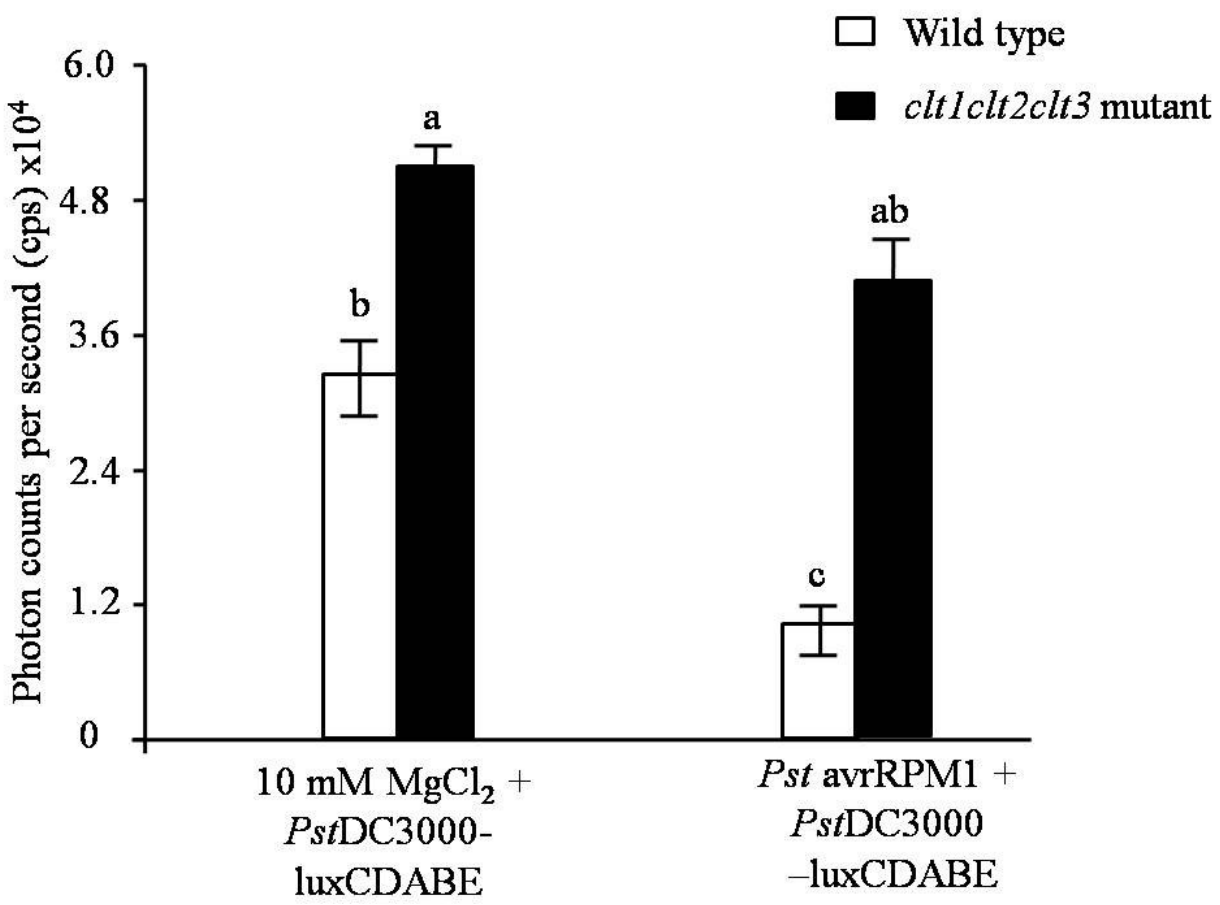

Figure 6.4 Bacteria growth measurement in wild-type and *clt1clt2clt3* mutant *Arabidopsis* plants after treatment with *Pseudomonas syringae*. Plants of the two genotypes were pre-treated (one leaf per plant) with either $MgCl_2$ (Control) or *Pstavr*RPM1. This was followed 48 h later by a secondary challenge (three non-inoculated leaves per plant) using the virulent *Pst*DC3000-luxCDABE strain. Leaf disks were punched from the *Pst*DC3000-luxCDABE infected leaves using a 4-mm cork borer 48 h after the secondary challenge and the luminescence detected using a luminomenter. For each experiment, 36 leaf disks per genotype were used and the experiment was repeated three-times. Values plotted represent the mean ± SEM. Means with the same letters are not significantly different (P>0.05).

The difference in bacterial growth between both genotypes was also visually apparent from the degree of damage caused on the infected leaves. The mutant leaves were more severely damaged compared to the wild-type leaves after pathogen infection (Fig. 6.5). Furthermore, wild-type plants infected with only *Pst*avrRPM1 had a significantly ($P<0.05$) reduced lesion length compared to the mutant plants even on the fourth day after infection. At this time, almost 70% of the mutant leaf had been damaged by the avirulent pathogen while the lesion on the wild-type leaf was still very much localized around the point of infection.

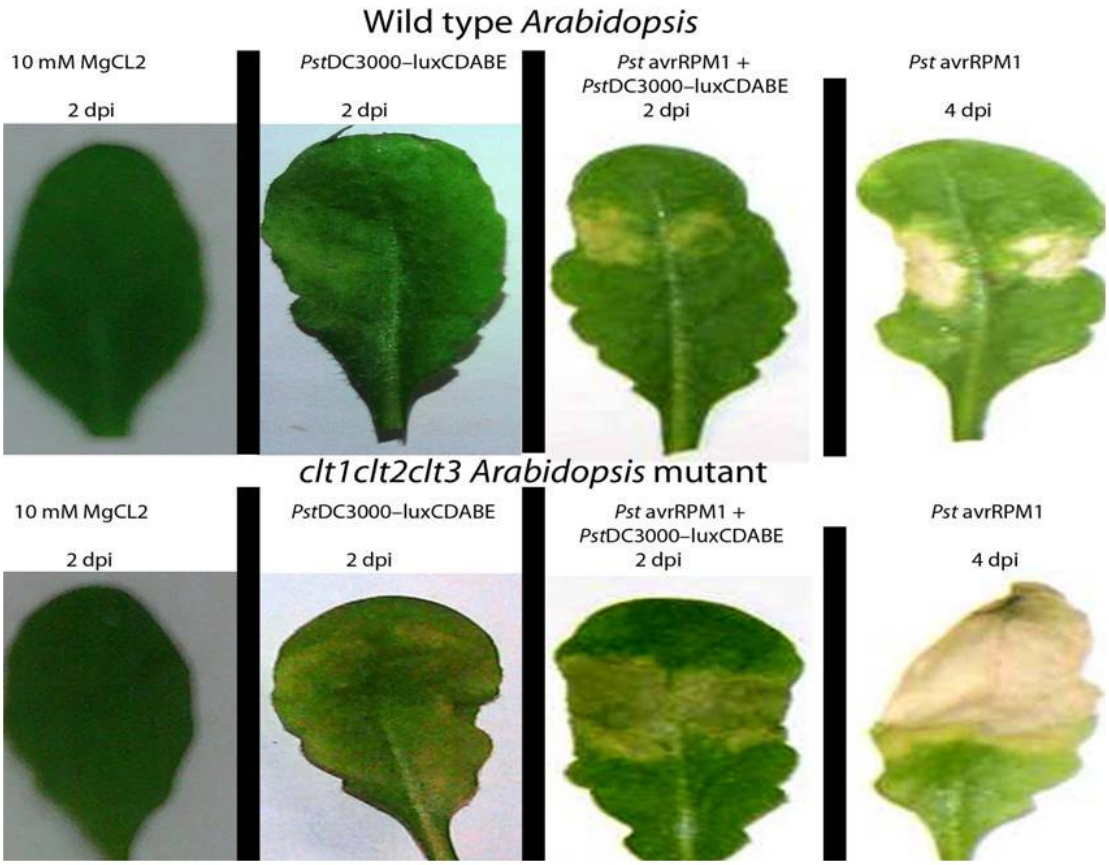

Figure 6.5 Disease symptoms in wild-type and *clt1clt2clt3* mutant *Arabidopsis* plants after treatment with *Pseudomonas syringae*. Plants of the two genotypes were pre-treated (one leaf per plant) with either 10 mM of $MgCl_2$ (control) or *Pstavr*RPM1. This was followed 48 h later by a secondary challenge (three non-inoculated leaves per plant) using the virulent *Pst*DC3000-luxCDABE strain. Disease progression was visually observed in infected plants at 2 d and 4 d post challenge. Pictures depict symptoms from selected leaves treated with 10 mM of $MgCl_2$ only, *Pst*DC3000-luxCDABE, *Pstavr*RPM1 + *Pst*DC3000-luxCDABE, or *Pstavr*RPM1. The experiment was repeated twice with similar results obtained for each repeat.

Disease development in the experimental plants following infection with the necrotrophic fungi *B. cinerea* was determined by measuring the lesion length formed on infected leaves 3 dpi. In wild-type infected plants, the lesion lengths formed were significantly 2.3-fold lower than those that developed on infected leaves of the mutant plants (Fig. 6.6A). These disease symptoms were also visually apparent as a greater portion of the mutant leaves became colonized by the fungi with some of their leaves becoming completely bleached compared to the spotted lesions present on the leaves of the wild-type plants (6.6B).

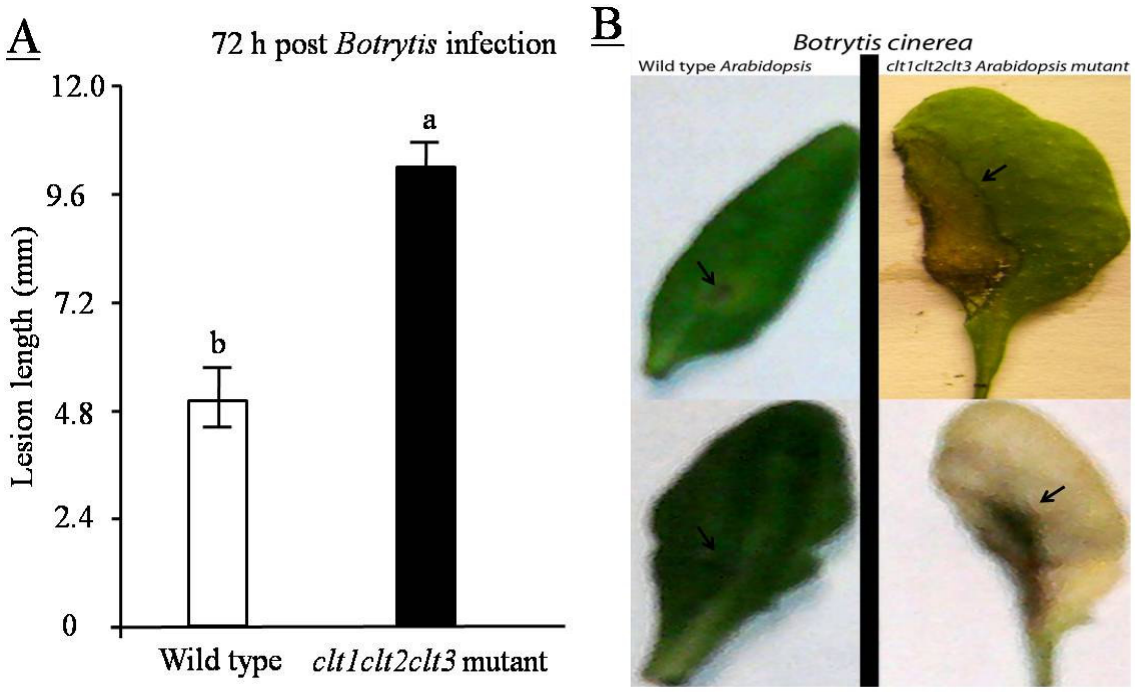

Figure 6.6 Lesion length and disease symptoms in wild-type and *clt1clt2clt3* mutant *Arabidopsis* plants after treatment with *B. cinerea*. Plants of the two genotypes (six leaves per plant) were treated with 5 µL of a 2.5 x 10^5 spores/mL inoculums of *B. cinerea* B06.10. The severity of damage caused by the fungus to the plant was assessed 3 dpi by measuring the lesion length (A) and visually with pictures of selected leaves taken using a digital camera (B). Data was collected from a total of eight plants to represent a single time point and the experiment was repeated twice. Values plotted represent the mean ± SEM. Means with the same letters are not significantly different (P>0.05).

6.5 Discussion

In this study, *NPR1* transcription was not significantly affected after pathogen infection in *Arabidopsis* mutant plants that were deficient in cytosolic GSH (*clt1clt2clt3*) and unable to synthesize GSH in the cytosol which affects the cellular redox state. Furthermore, the GSH amount of wild-type plants increased but decreased in *Arabidopsis* mutant plants. Such GSH increase in wild-type *Arabidopsis* plants following exposure to a pathogen, but also ozone, is well-documented in literature (Mou *et al*., 2003; Yoshida *et al*., 2009). However as a new result, this study provides evidence that the absence of the γ EC/GSH transporter in *clt1clt2clt3* mutant *Arabidopsis* plants prevents the plant's natural ability to synthesize foliar GSH to amounts comparable to the amount found in wild-type plants following pathogen infection. This reduced GSH amount in mutant plants was further directly related to an increase in the amount of oxidized glutathione (GSSG). This resulted in a greatly reduced GSH/GSSG ratio in this mutant genotype. The GSH/GSSG ratio also plays a key role in determining the redox state of cells (Pavet *et al*., 2005; Solomon *et al*., 2010) and this directly affects NPR1 oligomerization (Mou *et al*., 2003) and, as found in this current study, *NPR1* transcription was also affected.

Further, the DHA amount, but not the AsA amount, increased when the GSH amount was reduced in these mutant plants after pathogen infection. Although evidence exists for compensatory antioxidant effects with *Arabidopsis* plants deficient in either GSH (*cad2* mutant) or AsA (*vtc* mutant) having a higher AsA (GSH-deficient) or a higher GSH (AsA-deficient) amount when exposed to stresses like high light intensity (Kanwischer *et al*., 2005; Colville and Smirnoff, 2008), GSH deficiency was not compensated in the *clt1clt2clt3* mutant *Arabidopsis*

plants by an increased AsA amount. In general, in the AsA-GSH cycle for H_20_2 scavenging, AsA is directly oxidized to DHA and, regenerated in a reaction necessitating GSH (Asada, 2000). The lower AsA content measured in the *clt1clt2clt3* mutants could have been due to the usage of AsA for scavenging of H_20_2 generated by pathogen infection. In addition, a lower GSH content found in mutant plants following infection might also have limited the supply of electrons required for an adequate recycling of AsA. Unlike AsA, the DHA content in mutant plants increased during pathogen treatment causing a continuous oxidation of AsA with only a slow reduction of DHA in the AsA-GSH cycle (Asada, 2000).

In contrast to mutant plants, in wild-type *Arabidopsis* plants, which are able to synthesize GSH in both the chloroplast and the cytosol, a small but significant increase in *NPR1* transcription was measured 48 hpi not found in mutant plants. This provides evidence for a relationship between presence of cytosolic GSH and transcription of cytosol-located NPR1 and that both a functional GSH biosynthetic pathway and NPR1 transcription very likely are required for efficient protection against a pathogen such as *P. syringae*. Recent findings have already indicated such a relationship and, tobacco plants over-expressing a tomato *gamma*-glutamylcysteine synthetase gene (γ ECS) had a 2-fold higher *NPR1* transcription and synthesized more GSH, which resulted in an increased SA amount when compared to wild-type non-transformed plants (Ghanta *et al.*, 2011). Also, tobacco plants over-expressing a tomato or maize *gamma*-glutamylcysteine synthetase gene (γ ECS), or treated with a GSH solution, transcribed more *PR-1* in comparison to non-transformed or untreated plants (Gomez *et al.*, 2004). Furthermore, when Spoel *et al.* (2009) used complimented *Arabidopsis npr1-1* plants expressing the *NPR1* gene and further treated these plants with SA, an effective recruitment of NPR1 to the nucleus and a continuous

degradation and turnover occurred which promoted *PR-1* transcription. In the present study such processes have not been investigated and future research might therefore include investigating if mutant plants are also affected in proteasome-mediated degradation of already used-up nuclei localized NPR1. This might affect NPR1 turnover in the nucleus which promotes the supply of newly transcribed NPR1 transcription factors to their target promoter regions.

Downstream *PR-1* transcription was further significantly reduced in cytosolic GSH-deficient *Arabidopsis* mutant plants. These plants exhibited also a higher pathogen sensitivity following infection when compared to wild-type *Arabidopsis* plants. This confirms previous results obtained by Maughan *et al*. (2010) that *clt1clt2clt3* mutant plants have a reduced amount of *PR-1* transcripts and are more susceptible to pathogens in comparison to wild-type plants. Furthermore, findings in this study also confirm results found with *cad2 Arabidopsis* mutants that a reduced GSH content increases susceptibility to a pathogen when compared to wild-type plants (Ball *et al*., 2004). Upon pathogen infection, the GSH amount of wild-type plants increased in the present study but decreased when mutant plants were infected. When the equilibrium between GSH and GSSG production and recycling is shifted towards accumulation of oxidized glutathione (GSSG) as found for the mutant plants used in this study, the cell's environment will be more oxidized. This favors NPR1 proteins to exist predominately as an oligomer which is, in comparison to a monomer NPR1, unable to move to the nucleus to activate transcription factors such as TGA2 and TGA3 (Kinkema *et al*., 2000; Zhou *et al*., 2000; Mou *et al*., 2003). The ultimate redox dependency of transcription factors to which NPR1 monomers would bind are affected under oxidized conditions with the likely consequence of decreased

NPR1-dependent *PR-1* transcription and higher pathogen susceptibility also found in this study (Mou *et al*., 2003; Després *et al*., 2003; Pavet *et al*., 2005).

In mutant plants a reduced, but not completely blocked, *PR-1* transcription was observed 48 hpi whereas *PR-1* transcription was greatly increased at this time point in wild-type plants. This indicates that, in spite of the low GSH and also low AsA content, as well as the lack of *NPR1* transcription, *PR-1* transcription still occurred in mutant plants following infection with a pathogen. Such reduced *PR-1* transcription has also been previously reported by Colville and Smirnoff (2008) using AsA-deficient *vtc2-1* mutants with a reduced AsA content in comparison to wild-type plants. Following inhibition of GSH biosynthesis in these *vtc2-1* mutant plants by treatment with the inhibitor D,L-buthionine-[S,R]-sulphoximine (BSO), *PR-1* transcription in these mutants was still not completely blocked. Also Ball *et al*. (2004) found by using *rax1-1* and *cad2 Arabidopsis* mutants with reduced GSH amounts, that *PR-1* transcription was not completely blocked. Since *clt1clt2clt3* mutant plants also produced some *PR-1* transcript, this indicates that *PR-1* transcription might also occur independently of *NPR1* transcription. However, reduced *PR-1* transcription in mutant plants was overall not sufficient to render mutant plants more resistant to *B. cinerea* and *P. syringae* pathogens in comparison to wild-type plants in which pathogen progression were successfully prevented.

Overall, data from this study have confirmed that a functional GSH biosynthetic pathway is required to effectively mediate an *NPR1*-dependent *PR-1* biotic defense responses network in *Arabidopsis* plants and that lack of GSH causes increased susceptibility of plants against a pathogen.

CHAPTER SEVEN

GENERAL DISCUSSION AND FURTHER PERSPECTIVE

The study aimed to functionally characterize *NPR1*-like genes isolated from banana through comparative sequence analysis and complementation studies, evaluation of their expression in response to pathogen infection and their role in the pathogen response cascade through assessment of expression of downstream genes in the SA/JA responsive pathways. Also the role of known antioxidants in *NPR1* expression was explored. Overall, the study has provided novel information on the functional role of two banana *NPR1*-like homologues which highlights their involvement in mediating defense response. Further, first evidence has been provided on a possible direct relationship between cytosolic glutathione and *NPR1* transcription important for biotic stress tolerance.

New knowledge contributed was that the two *MNPR1* genes were transcribed in response to a hemi-biotrophic pathogen *Xanthomonas campestris* pv. *musacearum.* This addressed the set objective to study transcription of the two banana genes in *Xanthomonas*-infected banana. Data from this aspect of the study showed that the MeJA-inducible *MNPR1A* gene and the SA/MeJA-inducible *MNPR1B* gene were sequentially transcribed following treatment of banana plants with the hemi-biotroph *X. campestris* (Endah *et al*., 2010). This new finding is also supported by previous reports where investigations with heterologous plant systems revealed that hemi-biotrophs elicit both a SA- and JA/ET-dependent pathway (Oliver and Ipcho, 2004; Glazebrook, 2005; Spoel *et al*., 2007) and where associated increases in JA and SA in response to pathogen attack have been measured (Delannoy *et al*., 2005).

A further contribution was that *Arabidopsis npr1-2* mutants were successfully complemented via genetic transformation with both banana *NPR1* coding sequences under the control of the 35S

CaMV promoter. These transformed plants were used for the functional characterization of the two coding sequences with particular emphasis on their involvement in controlling distinct defense response pathways (SA or JA/ET-mediated defenses). Data obtained from the study revealed that both banana *NPR1*-like homologues complemented the *NPR1* function in the mutants, restoring the pathogen resistance phenotype of the *npr1 Arabidopsis* mutants to a degree comparable to the wild-type *Arabidopsis* phenotype as reported for other *NPR1*-like homologues (Kinkema *et al*., 2000; Shi *et al*., 2010).

However, both *MNPR1* coding sequences under the control of the CaMV 35S promoter did not show a differential response to the biotrophic pathogen *Hyaloperonospera arabidopsidis* and the necrotrophic pathogen *Botrytis cinerea* although both coding sequences were transcribed in the presence of these pathogens. This observation suggested that differential expression of the genes, if it exists in banana, is possibly regulated by transcriptional elements that interact with the promoter region, or with the promoter and coding sequence. Analysis of regulatory *cis* elements within the two *MNPR1* coding (MNPR1A and B) regions unveiled the existence of multiple JA, SA, ET and ABA regulatory *cis*-elements within their structure. A comparison of the MNPR1 amino acid coding sequences with those of other NPR1-like amino acid coding sequences provided additional information on the level of amino acid sequence similarity shared by these MNPR1 proteins especially in relation to conserved regions which have been well characterized in *Arabidopsis*. In the future, a follow-up mutational study using dissimilar amino acid sequence(s) from these conserved regions might provide additional information on their contribution towards defense. Further, since plant-pathogen responses are influenced by the cellular environment as well as other genes that are specific to a plant species, it is also possible

that the plant-pathogen system (*Arabidopsis* instead of banana) might have influenced the expression of these two coding sequences in *Arabidopsis*. Further studies to be carried out with banana might provide more elaborate insight into a possible differential function of the two *MNPR1* coding sequences. Such studies could entail transient expression using the agro-infiltration technique for the delivery of these *MNPR1* cDNAs or the mutated versions of these cDNA into banana plants. Transient expression systems are indeed becoming a method of choice for a more rapid and effective characterization of genes and this has been successfully applied in the characterization of grape vine *NPR1*-like genes (Le Hananff *et al*., 2009; Leicke and Stewart, 2011). Future investigations might also involve creating *MNPR1*-GUS or *MNPR1*-GFP translational fusions under the control of a 35S promoter sequence for easy visualization and sub-cellular localization of *MNPR1*. Furthermore, since the 35S promoter is also inducible by SA (Qin *et al*., 1994), this might also have contributed in this present study to trigger the transcription of the MeJA-inducible *MNPR1A* gene following infection with the biotrophic pathogen *H. arabidopsidis* which induces a SA mediated response. A transient expression study in banana using the banana *NPR1*-like genes, or the mutated versions of these genes under the control of their native promoter, might therefore provide a more robust system to study *MNPR1* expression. In such a transient expression system, all the interacting banana *cis* elements that are required to influence, or be influenced by these *MNPR1* genes would also be available allowing a complete interaction of the native elements with native transcription factors.

Finally, this study has also shown that functional GSH biosynthesis in the cytosol is required for *NPR1*-dependent *PR-1* transcription in *Arabidopsis*. This addressed the objective of shedding light on the role of GSH in *NPR1* transcription and *PR1* expression. GSH and especially the

GSH/GSSG ratio, is known to be a key indicator of the cellular redox state. This redox state controls NPR1 oligomer/monomer activity (Mou *et al*., 2003; Pavet *et al*., 2005; Solomon *et al*., 2010), which is critical for the expression of *PR* genes further downstream. *Arabidopsis* mutants deficient in cytosolic glutathione served as an ideal tool for characterization of this system. NPR1 oligomers are located in the cytosol during ambient conditions and are activated and translocated to the nucleus for the induction of downstream defense responses only during stress conditions (Kinkema *et al*., Mou *et al*., 2003; 2000; Speol *et al*., 2009). New evidence from this study shows that a dysfunctional GSH biosynthetic pathway in the cytosol, as demonstrated by the cytosolic deficient GSH mutant (*clt1clt2clt3*) *Arabidopsis* plants, compromises the plant's ability to adequately transcribe both *NPR1* and downstream *PR-1* genes. Both are important components of SAR for efficient limitation of pathogen colonization. Results obtained with wild-type *Arabidopsis* plants further confirmed previous reports of GSH-dependent *NPR1* transcription for limitation of pathogen growth. This highlights the importance of a functional GSH biosynthetic pathway for the efficient activation of *PR-1* (Gomez *et al.*, 2004; Maughan *et al*., 2010) and possibly its direct role in the process.

Overall, this study has shown that the two *NPR1*-like coding sequences from banana (*MNPR1A* and *MNPR1B*), could compliment the mutant phenotype of the *npr1-2 Arabidopsis* mutants restoring resistance to levels similar to those of wild-type *Arabidopsis* plants. However, there was no differential response patterns to the classes of pathogens investigated thereby implying that the coding regions of the *MNPR1A* and *MNPR1B* genes might not be sufficient or necessary in controlling the differential response of these genes to distinct classes of pathogens. Also, the

plant-pathogen system might also play an important role in the further evaluation of the genes to various pathogens.