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# **Investigating the Role of Cyclic Nucleotide Gated Channels in Plant-Pathogen Interactions**

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## **Abstract**

### **Investigating the Role of Cyclic Nucleotide Gated Channels in Plant-Pathogen Interactions**

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The presence of twenty putative cyclic nucleotide gated channels in *Arabidopsis thaliana* suggests that cyclic nucleotides may play an important function in various plant processes. This research used various molecular and biochemical techniques to identify transcripts affected by cyclic nucleotide signalling in leaf tissue and characterise members of the cyclic nucleotide gated channel family for their ion transport capabilities and role in the defence signalling pathway initiated by the necrotrophic pathogen, *Botrytis cinerea*.

A transcriptomics approach was used to identify downstream targets of cyclic nucleotide signalling in leaf tissue and a number of transcripts regulated by cyclic nucleotides can be linked to processes that also require functional cyclic nucleotide gated channels. Bioluminescent and histochemical reporter gene assays were used to identify calcium transport capabilities and tissue specific expression of cyclic nucleotide gated channels respectively. Heterologous expression of *Cyclic Nucleotide Gated Channel 19* in yeast, harbouring the calcium reporting Aequorin protein, resulted in increased cytosolic calcium levels in response to salt stress and transgenic plants expressing the  $\beta$ -glucuronidase reporter gene driven by the *Cyclic Nucleotide Gated Channel 19* promoter displayed staining in roots throughout development. Transient expression of *Cyclic Nucleotide Gated Channel 19* fused to the yellow fluorescent protein showed plasma membrane targeting in onion epidermal cells and co-localised with a known plasma membrane marker. A reverse genetics approach using T-DNA insertion lines to identify cyclic nucleotide gated channels needed for full resistance to *Botrytis cinerea*, revealed four members with altered defence phenotypes. Semi-quantitative analysis of defence gene expression, quantification of secondary metabolite accumulation using high-pressure liquid chromatography and histochemical staining to detect spontaneous lesion formation was used to further characterize the *cyclic nucleotide gated channel 19* mutant lines and establish the role of *Cyclic Nucleotide Gated Channel 19* in defence signalling.

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God gave you a gift of 86,400 seconds today. Have you used one to say, "Thank You"?  
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### **List of Abbreviations**

|                                    |                                                                                            |
|------------------------------------|--------------------------------------------------------------------------------------------|
| [Ca <sup>2+</sup> ] <sub>cyt</sub> | Cytosolic calcium concentration                                                            |
| 8-Br-cGMP                          | 8-Bromoguanosine-3', 5'-cyclomonophosphate                                                 |
| A                                  | Adenine                                                                                    |
| <i>A. tumefaciens</i>              | <i>Agrobacterium tumefaciens</i>                                                           |
| ABA                                | Abscissic acid                                                                             |
| <i>Acd</i>                         | <i>Accelerated cell death</i>                                                              |
| Aeq                                | Aequorin                                                                                   |
| AGI                                | <i>Arabidopsis</i> genome initiative                                                       |
| <i>Akt1-1</i>                      | <i>Arabidopsis</i> potassium transporter 1-1                                               |
| aRNA                               | Amplified ribonucleic acid                                                                 |
| Avr                                | Avirulent                                                                                  |
| <i>B. cinerea</i>                  | <i>Botrytis cinerea</i>                                                                    |
| BASTA                              | Glufosinate ammonium                                                                       |
| bp                                 | Base pair                                                                                  |
| BSA                                | Bovine serum albumin                                                                       |
| Ca <sup>2+</sup>                   | Calcium or calcium ion                                                                     |
| CaCl <sub>2</sub>                  | Calcium chloride                                                                           |
| CaM                                | Calmodulin                                                                                 |
| CaMBD                              | Calmodulin binding domain                                                                  |
| cAMP                               | Cyclic adenosine monophosphate                                                             |
| CaMV                               | Cauliflower mosaic virus                                                                   |
| CAPS                               | Cleaved amplified polymorphic sequence                                                     |
| CATMA                              | Complete <i>Arabidopsis</i> transcript microarray                                          |
| cDNA                               | Complementary deoxyribonucleic acid                                                        |
| CFU                                | Colony forming units                                                                       |
| cGMP                               | Cyclic guanosine monophosphate                                                             |
| CN                                 | Cyclic nucleotide                                                                          |
| CNBD                               | Cyclic nucleotide binding domain                                                           |
| CNGC                               | Cyclic nucleotide gated channel                                                            |
| Col-0                              | Columbia-0                                                                                 |
| <i>Cpr22</i>                       | <i>Constitutive PR-1 expression 22</i>                                                     |
| C-terminus                         | Carboxy-terminus                                                                           |
| CTR1                               | Constitutive triple response 1                                                             |
| DAG                                | Diacyl glycerol                                                                            |
| dCTP                               | Deoxycytidine triphosphate                                                                 |
| <i>Dnd1</i>                        | <i>Defence no death 1</i>                                                                  |
| DPI                                | Days post infection                                                                        |
| EDS1                               | Enhanced disease susceptibility                                                            |
| Ef-Tu                              | Elongation factor Tu                                                                       |
| ER                                 | Endoplasmic reticulum                                                                      |
| Eth                                | Ethylene                                                                                   |
| EV                                 | Empty vector                                                                               |
| FM4-64                             | N-(3-triethylammoniumpropyl)-4-(6-(4-(diethylamino)phenyl)hexatrienyl)pyridinium dibromide |
| FW                                 | Fresh weight                                                                               |
| G                                  | Guanidine                                                                                  |
| GABI-Kat                           | Genomanalyse im biologischen – Kölner <i>Arabidopsis</i> TDNA                              |
| GFP                                | Green fluorescent protein                                                                  |
| GO                                 | Gene ontology                                                                              |

|                               |                                                                               |
|-------------------------------|-------------------------------------------------------------------------------|
| GUS                           | β-glucuronidase                                                               |
| <i>H. arabidopsidis</i>       | <i>Hyaloperonospora arabidopsidis</i>                                         |
| H <sub>2</sub> O <sub>2</sub> | Hydrogen peroxide                                                             |
| HEK                           | Human embryonic kidney                                                        |
| <i>Hlm-1</i>                  | <i>Hypersensitive-like mimic-1</i>                                            |
| HPI                           | Hours post infection                                                          |
| HPLC                          | High pressure liquid chromatography                                           |
| HR                            | Hypersensitive response                                                       |
| IP <sub>3</sub>               | Inositol-1,4,5-triphosphate                                                   |
| JA                            | Jasmonic acid                                                                 |
| K <sup>+</sup>                | Potassium ion                                                                 |
| KB                            | Kilobase                                                                      |
| KCl                           | Potassium chloride                                                            |
| LRR                           | Leucine rich repeat                                                           |
| <i>Lsd</i>                    | <i>Lesion simulating disease</i>                                              |
| LZ                            | Leucine zipper                                                                |
| MAANOVA                       | Microarray analysis of variance                                               |
| MeJA                          | Methyl jasmonate                                                              |
| <i>Δmid1Δcch1</i>             | <i>Mating pheromone induced death1 calcium channel protein 1</i> yeast mutant |
| min                           | Minute(s)                                                                     |
| mRNA                          | Messenger ribonucleic acid                                                    |
| MS                            | Murashige and Skoog                                                           |
| Na <sup>+</sup>               | Sodium ion                                                                    |
| NaCl                          | Sodium chloride                                                               |
| NASC                          | Nottingham <i>Arabidopsis</i> stock centre                                    |
| NBS                           | Nucleotide binding sequence                                                   |
| NO                            | Nitric oxide                                                                  |
| NOS                           | Nitric oxide synthase                                                         |
| <i>Npr1</i>                   | <i>Non-expressor of PR genes 1</i>                                            |
| NR                            | Nitrate reductase                                                             |
| N-terminus                    | Amino-terminus                                                                |
| OE                            | Over-expressor                                                                |
| <i>P. syringae</i>            | <i>Pseudomonas syringae</i>                                                   |
| PA                            | Phosphatidic acid                                                             |
| <i>Pad3</i>                   | <i>Phytoalexin deficient 3</i>                                                |
| PAMP                          | Pathogen associated molecular pattern                                         |
| Pb <sup>+</sup>               | Lead ion                                                                      |
| PCD                           | Programmed cell death                                                         |
| <i>Pdf1.2</i>                 | <i>Plant defensin 1.2</i>                                                     |
| pEG-101                       | pEarleyGate-101                                                               |
| <i>Pers. Comm.</i>            | Personal communication                                                        |
| PLC                           | Phospholipase C                                                               |
| PLD                           | Phospholipase D                                                               |
| PM                            | Plasma membrane                                                               |
| <i>PR-1</i>                   | <i>Pathogenesis related-1</i>                                                 |
| QTL                           | Quantitative trait loci                                                       |
| R                             | Resistance                                                                    |
| ROS                           | Reactive oxygen species                                                       |
| <i>S. cerevisiae</i>          | <i>Saccharomyces cerevisiae</i>                                               |

|            |                                                                   |
|------------|-------------------------------------------------------------------|
| SA         | Salicylic acid                                                    |
| SAIL       | Syngenta <i>Arabidopsis</i> insertion library                     |
| SAR        | Systemic acquired resistance                                      |
| SD         | Synthetic defined                                                 |
| SDS        | Sodium dodecyl sulphate                                           |
| SIGNAL     | SALK Institute genomic analysis laboratory                        |
| sqRT-PCR   | Semi-quantitative reverse transcriptase polymerase chain reaction |
| SSC        | Sodium citrate, sodium chloride                                   |
| TAE        | Tris-acetate ethylenediaminetetraacetic acid                      |
| TAIR       | The <i>Arabidopsis</i> information resource                       |
| T-DNA      | Transfer deoxyribonucleic acid                                    |
| TIR        | TOLL and interleukin-1                                            |
| tRNA       | Transfer ribonucleic acid                                         |
| Ubq        | Ubiquitin                                                         |
| UTR        | Untranslated region                                               |
| v/v        | Volume per volume                                                 |
| Vir        | Virulent                                                          |
| w/v        | Weight per volume                                                 |
| Ws-4, Ws-2 | Wassilewskija-4 or -2                                             |
| X-GlcA     | 5-bromo-4-chloro-3-indolyl glucuronide                            |
| YFP        | Yellow fluorescent protein                                        |

## Chapter 1

### Second Messengers of Plant Defence Signalling

All living cells have a network of signalling events to decode an external or internal stimulus into a physiological response. A crucial component of signalling cascades involve the production or release of second messengers, which are characterised as being readily accessible or rapidly mobilised, at the correct spatio-temporal level needed to amplify and relay the appropriate message both within the cell and to neighbouring cells by binding to and activating downstream targets. The majority of information concerning second messenger systems has been elucidated from animal studies since the first second messenger was discovered in liver tissue over fifty years ago (Rall *et al.* 1957, Sutherland and Rall 1957). Unfortunately, some of the techniques and methods used to study second messengers in animals were not suitable for plant research and this resulted in questionable data being produced and published. Information from animal studies demonstrated that second messengers have to be readily available and rapidly metabolised to perform their transient but essential function and the lack of identification of plant homologues to perform these duties fuelled the controversy regarding the existence of certain second messenger systems in plants. With the advance of scientific equipment and the continual discovery of plant proteins involved in second messenger regulation, the uncertainty surrounding some plant second messengers, i.e. cyclic nucleotides (CNs), is slowly dispersing.

There are a few second messenger systems in plants of which the calcium ( $\text{Ca}^{2+}$ ) second messenger system is best characterised. The hydrophilic  $\text{Ca}^{2+}$  ion is one of the most extensively studied second messenger molecules found in higher plants and is maintained at its lowest concentration in the cytosol so that it can be readily accessed down its concentration gradient from the apoplast or internal cellular compartments via  $\text{Ca}^{2+}$  channels (Bush 1995). Countless signalling events utilise  $\text{Ca}^{2+}$  as a second messenger and each stimulus causes a unique spatio-temporal  $\text{Ca}^{2+}$  pattern to bring about the desired physiological outcome. The use of various  $\text{Ca}^{2+}$  reporting technologies allows the visualisation of the  $\text{Ca}^{2+}$  signal and these

studies have revealed the huge variety in  $\text{Ca}^{2+}$  responses to numerous stimuli (Gilroy *et al.* 1993, Lecourieux *et al.* 2006).

The dynamics and timing (when does it begin and for how long?) of  $\text{Ca}^{2+}$  influx in relation to  $\text{Ca}^{2+}$  sensor molecules ('responder' or 'relay') present in the cell will dictate the outcome of the signalling cascade (Sanders *et al.* 2002). Enzymes that are directly activated by  $\text{Ca}^{2+}$  binding are termed 'responders' whereas  $\text{Ca}^{2+}$  sensor 'relay' molecules, e.g. calmodulin (CaM), bind  $\text{Ca}^{2+}$  so that they can go on to activate effector proteins, such as enzymes or transcription factors. The presence of a large family of CaM proteins allows further fine-tuning of signalling cascades as each CaM molecule is present in varying concentrations and locations within the cell and each isoform may have specific downstream targets (Ling *et al.* 1991, Snedden and Fromm 2001). In *Arabidopsis thaliana*, a large family of cyclic nucleotide gated ion channels (CNGCs) are good candidates for downstream targets of CaM signalling as they have CaM binding domains, are expressed in various plant tissues and have been shown to be involved in various signalling cascades such as defence signalling (Kaplan *et al.* 2007). Plant CNGCs have a CaM binding domain which overlaps with a binding domain for the notorious second messenger, CNs, and this review will focus on the CN second messenger system, its putative downstream targets, CNGCs, and their roles in defence.

### 1.1 Cyclic Nucleotides

The CN second messenger system is well characterised in bacterial, fungal and animal cells but only recently, has the scientific community accepted that CNs are playing a critical signalling role in higher plants (Newton and Smith 2004). CN signalling in higher plants was believed to be a myth due to various shortfalls in methodology and misconstrued data from earlier studies which used assays designed for animals tissue to detect CNs in plants (Spiteri *et al.* 1989). Levels of plant CNs were found to be low, near detection limits of the animal based assays, and there was some ambiguity in the purified molecule using these methods. However, a series of techniques have been developed to detect the levels of CNs found in plants more reliably and also irrevocably established the molecule as a 3'5' CN.

As mentioned before, second messengers have to be rapidly synthesised and degraded to perform their transient function. Enzymes which synthesise (adenylyl cyclase or guanylyl cyclase) and breakdown (CN phosphodiesterase) CNs in animal tissue are well characterised with many methods in place to measure the activity of these enzymes and their products. In animal tissue a stimulus, such as light, results in the activation of adenylyl or guanylyl cyclase which converts adenosine triphosphate (ATP) or guanosine triphosphate (GTP) into the second messengers: 3', 5' – cyclic adenosine monophosphate (cAMP) or 3' 5' – cyclic guanosine monophosphate (cGMP) respectively. These molecules are rapidly produced to bind to and activate their target proteins but they are also quickly hydrolysed to their respective monophosphates (AMP or GMP) by the action of CN phosphodiesterases. The search for homologous proteins in plant genomes to synthesise and breakdown cAMP and cGMP was not very successful and is probably due to the fact that plant CN signalling has diverged considerably from other organisms and therefore homologous sequences cannot be found.

One study used a motif search containing a conserved amino acid sequence of the catalytic site of known guanylyl cyclases from a variety of organisms to perform a blast search of the *Arabidopsis* genome, and identified the first plant protein with guanylyl cyclase activity (Ludidi and Gehring 2003). The first adenylyl cyclase enzyme, ZmPsiP, was discovered in *Zea mays* and adenylyl cyclase activity has been measured in various plant species including *Nicotiana tabacum* and *Vicia faba* (Li *et al.* 1994, Moutinho *et al.* 2001, Witters *et al.* 2004, Witters *et al.* 2005). Proteins displaying CN phosphodiesterase activity has also been identified in a range of plant species and provides more evidence that plants have the ability to synthesis and breakdown CN, which is a critical component of a second messenger system (Vandepeute *et al.* 1973, Ashton and Polya 1975, Abel *et al.* 2000).

Besides the synthesis and breakdown of CNs, downstream targets of CNs displaying direct binding need to be identified to confirm that it is a *bona fide* second messenger system in plants. The lack of clarity regarding CN 'responder' proteins that are activated by direct binding of CNs, such as protein kinase A (PKA) found in animals and fungi, still casts some doubt on how CNs are regulating downstream targets *in planta* as no homologous PKA and PKG sequences can be found in plants (Bridges

*et al.* 2005). Most plant proteins with putative CN binding domains (CNBDs) are ion transport channels and a recent review speculates that plant CN signalling could be converted into a  $\text{Ca}^{2+}$  signal by the action of CNGCs, which are non-selective cation channels harbouring overlapping CN and CaM regulatory domains on their carboxy-terminus (C-terminus) (Martinez-Atienza *et al.* 2007). Heterologous expression studies have shown that plant CNGCs can be activated in the presence of CNs and most of the CNGCs characterised thus far display the ability to transport  $\text{Ca}^{2+}$  which makes them the good candidates for this role (Leng *et al.* 2002, Mercier *et al.* 2004, Ali *et al.* 2006). The abundance of  $\text{Ca}^{2+}$  sensor 'responder' proteins, which are encoded by thirty-four putative  $\text{Ca}^{2+}$  dependent protein kinase (CDPK) genes found in *Arabidopsis*, and lack of CN sensor 'responder' proteins in plants lends support for the CN to  $\text{Ca}^{2+}$  signal conversion hypothesis via the action of CNGCs.

## 1.2 Cyclic Nucleotide Gated Channels

CNGCs in higher plants were discovered just over a decade ago and putative CNGC genes can be found in many plant genomes including barley, tobacco and rice (Schuurink *et al.* 1998, Sunkar *et al.* 2000, Talke *et al.* 2003). The model plant, *Arabidopsis*, has twenty putative CNGC genes and heterologous expression of these genes in *Xenopus* oocyte or HEK cells for electrophysiological analysis indicates that many of them are voltage-independent, non-selective cation channels allowing the passage of  $\text{Ca}^{2+}$ , potassium ( $\text{K}^+$ ) and sodium ( $\text{Na}^+$ ) ions similarly to their animal homologues (Leng *et al.* 1999, Leng *et al.* 2002, Balague *et al.* 2003). Plant CNGCs are also similar to their animal homologues having six transmembrane domains with a non-selective ion pore between the fifth and sixth transmembrane domain. The only physical difference between plant and animal CNGCs is the position of the CN and CaM regulatory domains. Plant CNGCs are thought to be under tighter regulation than their animal homologues as the CNBD and CaM binding domain (CaMBD) overlap at the C-terminus, whereas animal CNGCs have a regulatory domain on either end of the protein (Zagotta and Siegelbaum 1996, Leng *et al.* 1999).

Evidence to support the hypothesis that plant and animal CNGCs are under similar regulatory control, i.e. opened by CNs and closed by CaM, has come from electrophysiological studies, where a current is observed by a single plant CNGC only after CN treatment, and indirectly from yeast complementation assays. The

immediate activation of these channels in electrophysiological assays also implies that the CN molecule is binding directly to the channel (Leng *et al.* 2002). Baxter *et al.* (2008) used computational modelling to indicate a binding pocket for CNs and an *in vitro* cAMP binding assay shows that a fused CNGC11/12 protein can bind cAMP. A previous study from the same group showed that addition of cAMP, but not cGMP, enhanced the growth of a yeast potassium mutant expressing the *Arabidopsis* CNGC11 or CNGC12 proteins, which suggests that only cAMP activates these channels to allow the influx of potassium and provides evidence that each CN may have specific CNGC targets and physiological roles *in planta* (Yoshioka *et al.* 2006). However, unlike animal CNGCs, a study by Maathuis and Sanders (2001), suggests a contradictory mode of action for CN regulation in plants as CN treatment of voltage-independent channels (VICs) found in *Arabidopsis* root protoplasts, which could include CNGCs, inhibited a Na<sup>+</sup> current. This study also indicated that addition of CN to a high salinity medium provided increased tolerance to salinity stress. The only protein, other than the CNGCs, that contains a CNBD and has been shown to play an important role in salt tolerance is Salt Overly Sensitive 1 (SOS1), a Na<sup>+</sup>/H<sup>+</sup> antiporter (Shi *et al.* 2000). Therefore, the inhibition of a Na<sup>+</sup> current in response to cGMP treatment could be due to the action of the SOS1 protein.

Many studies used heterologous expression of plant CNGCs in wildtype and mutant *Saccharomyces cerevisiae* strains to determine the ion transport capability of the CNGC being characterised via complementation analysis. Analysis of the data from these studies indicates that it was not always necessary to activate the plant channel by supplying extra CN molecules and this could be due to the presence of native yeast CN molecules binding to the plant CNGC and causing channel opening. In some experiments, where partial complementation of a yeast mutant is observed, the addition of CN allows enhanced ion transport ability causing full complementation of the yeast mutant by CNGC activity. This further supports the hypothesis that CNGCs are activated by CNs. As mentioned before, there are many CaM isoforms and each of them may have specific downstream target proteins. Studies have shown that there is differing affinity for CaM isoforms between the CNGCs, which could contribute to the tight regulation of such a big channel family. Protein-protein interactions using yeast-2-hybrid assays show that *Arabidopsis* CNGC1 and CNGC2 display different affinities to three CaM isoforms found in yeast and *Arabidopsis*

(Köhler and Neuhaus 2000, Ali *et al.* 2006). CNGC1 has high affinity for the yeast CaM isoforms and therefore was unable to transport ions when expressed in yeast, unless its CaMBD was removed. This further supports the theory that plant CNGCs are regulated similarly to their animal homologues.

Thus far, no study has categorically shown that plant CNGCs form heterotetrameric channels, like their animal homologues, but the heterotetrameric nature of plant CNGCs has been inferred from computational protein modelling and it is one of the theories used to explain why the expression of a single CNGC in *Xenopus* oocyte or HEK cells for electrophysiological analysis is temperamental (Leng *et al.* 2002). Determining which CNGC pairs form heterotetrameric channels could be insinuated from analysis of CNGCs which are co-expressed during development or in response to external stimuli. A number of studies used various techniques to determine the tissue specific expression of *Arabidopsis* CNGCs and their sub-cellular localisation (Gobert *et al.* 2006, Christopher *et al.* 2007, Frietsch *et al.* 2007). Data available from a vast number of microarray projects can also be mined for information to identify potential CNGC pairs which may be functioning as heterotetramers in response to certain stimuli.

Analysis of CNGC1 protein levels in root and shoot tissue of Wassilewskija-4 (Ws-4) wildtype plants indicated that CNGC1 is mainly expressed in roots and absent from shoot tissue (Ma *et al.* 2006). However, publicly available expression data show that CNGC1 mRNA levels appear to be similar in root and shoot tissue. These differences in shoot mRNA and protein levels could be due to post-translational regulation or natural variation in ecotype gene expression as the publicly available microarray experiments used Columbia-0 (Col-0) plants. Differences in ecotype gene regulation is also observed for CNGC4 expression as pathogen attack causes induction of CNGC4 expression in Col-0 but not Ws-4 plants (Balague *et al.* 2003). Other than CNGC1, CNGC3, CNGC5 and CNGC10 are all expressed in roots but these channels could also have different functions in various root tissue types such as root hairs or the root tip (Gobert *et al.* 2006, Christopher *et al.* 2007). This is apparent when looking at expression of CNGC3 which was displayed specific expressing in the root hairs but was absent from the root tip of a transgenic CNGC3::*GUS* line (Gobert *et al.* 2006). CNGC5 protein levels are similar in root and leaf tissue whereas

the CNGC10 protein was found to be more abundant in roots than leaves which may exclude them from being a potential heterotetrameric pair in roots (Christopher *et al.* 2007). The uptake of  $K^+$  via the roots is crucial for plant development and both CNGC3 and CNGC10 display the ability to transport  $K^+$  when expressed in yeast mutants that lack  $K^+$  uptake channels (Li *et al.* 2005, Gobert *et al.* 2006). Therefore these channels are perfect candidates to function as a heterotetrameric pair *in planta*, but studies of single knockout mutants of either CNGC3 or CNGC10 show that the loss of either protein causes a  $K^+$  related phenotype indicating their non-redundant roles (Li *et al.* 2005, Gobert *et al.* 2006).

Analysis of CNGC2p::GUS plants indicate that the light and developmentally regulated CNGC2 gene is expressed in all tissues except roots throughout development which suggests that it will not form a heterotetrameric pair with CNGCs expressed in root tissue (Köhler *et al.* 2001). The CNGC2 gene transcript levels were induced in tissue that is undergoing programmed cell death or maturing as increased expression of CNGC2 is apparent in leaf tissue once the plant has started flowering, in floral tissue once they start withering and in the dehiscence zone of mature siliques (Köhler *et al.* 2001). No other study has reported any CNGC with a similar pattern of expression to CNGC2 but analysis of information from the public microarray database identified a few other CNGCs, e.g. CNGC13, which displayed significant up-regulation in older leaves.

CNGC18 expression is only found in pollen grains and it has been shown to play a role in pollen tube growth (Chang *et al.* 2007, Frietsch *et al.* 2007). This is the only *Arabidopsis* pollen-specific CNGC to be characterised in the literature to date but transcriptomics data from various microarray experiments does show that a few other CNGCs are exclusively expressed in pollen. Thus far, the tissue localisation of the CNGCs characterised shows a positive correlation with proposed function *in planta* but no studies have conclusively shown that plant CNGCs are functioning in heterotetrameric pairs.

Sub-cellular localisation of CNGCs is also important in understanding its exact function *in planta*. Many studies used heterologous expression of *Arabidopsis* CNGC proteins fused to green or yellow fluorescent protein (GFP or YFP) in yeast, tobacco

and onion cells to identify which membranes these proteins are targeted to. Analysis of C-terminal tagged CNGC1:GFP, CNGC2:GFP, CNGC11:GFP and CNGC12:GFP proteins expressed in yeast shows that these CNGC proteins are expressed at the plasma membrane of yeast cells (Mercier *et al.* 2004, Ali *et al.* 2006, Urquhart *et al.* 2007). Transient expression of CNGC3:GFP in onion cells displayed plasma membrane localisation and the amino-terminus (N-terminus) tagged YFP:CNGC18 protein was targeted to the plasma membrane of a tobacco pollen tube (Gobert *et al.* 2006, Frietsch *et al.* 2007). Frietsch *et al.* (2007) also looked at the sub-cellular localisation of three other CNGCs, CNGC7; CNGC8 and CNGC16, that are hypothesised to be specifically expressed in pollen and found that all three were targeted to endomembrane structures but not the plasma membrane of tobacco pollen tubes (Frietsch *et al.* 2007). This indicates that even though tissue specific expression may suggest that these four pollen-specific CNGCs could be potential heterotetrameric pairs, their differences in sub-cellular localisation of these CNGCs immediately rejects that theory.

One drawback of using GFP tagged proteins under the control of a 35S promoter is the potential for mis-targeting. A study of CNGC5 and CNGC10 sub-cellular localisation used primary anti-bodies specific to the respective CNGC and visualised with the appropriate fluorescent secondary antibody (Christopher *et al.* 2007). Both CNGC5 and CNGC10 were localised to the plasma membrane of *Arabidopsis* leaf protoplasts. This study also looked at CNGC10 localisation in root cells using immuno-localisation and found that CNGC10 was targeted to the plasma membrane, the endoplasmic reticulum (ER) and Golgi vesicles (Christopher *et al.* 2007). This indicates that CNGC10 may be differentially targeted in root and leaf tissue in accordance with its function.

Most of the CNGCs that have been characterised display the ability to transport  $K^+$  and  $Ca^{2+}$  and thus far only CNGC2 displayed a bias against  $Na^+$  transport (Leng *et al.* 2002, Mercier *et al.* 2004, Li *et al.* 2005, Yoshioka *et al.* 2006, Frietsch *et al.* 2007, Urquhart *et al.* 2007). In accordance with the suggested role of plant CNGCs converting a CN signal into a  $Ca^{2+}$  signal, it could be hypothesised that plant CNGCs are opened by CN binding allowing  $Ca^{2+}$  to enter the cell, thereby decoding the CN encrypted message to a  $Ca^{2+}$  message. The  $Ca^{2+}$  ions could directly activate  $Ca^{2+}$

dependent target proteins or bind CaM molecules which can activate downstream proteins and/or act as a feedback loop to inhibit CNGC ion flow.

Several studies have characterised nearly half of the *Arabidopsis* CNGCs and the members of this protein family are involved in defence signalling, development, nutrient uptake, pollen tube growth and ion homeostasis. Characterisation of plants which mis-express a single CNGC protein results in an altered phenotype and therefore the myth of functional redundancy can be discarded for at least these members of this large protein family. In the final section of this review, I will give a brief description of the plant defence signalling cascade and discuss how second messenger systems act in concert to regulate this signalling pathway.

### **1.3 Plant Defence Signalling**

Biochemical and molecular characterisation of numerous plant mutants displaying altered defence responses has helped piece together the elaborate defence signalling cascade and this section will give a concise summary of the key players known to date. Plants employ various mechanisms to defend themselves against pathogen attack; basal resistance, Resistance (*R*) gene-mediated defence and systemically induced defence responses. The efficiency of the defence response relies on the plant's ability to identify or recognise the invading pathogen before it causes disease by rapidly initiating the appropriate defence signalling pathway. Plants recognise the invading pathogen by its pathogen associated molecular pattern (PAMP) molecules or effector proteins that they secrete (Flor 1971, Ingle *et al.* 2006). Plant pathogens are divided into two broad groups, biotrophic (including hemi-biotrophic) and necrotrophic, based on their mode of infection and this correlates to the defence signalling pathway induced and sustained by plant hormones (Glazebrook 2005). Biotrophic pathogens, such as *Pseudomonas syringae* and *Hyaloperonospora arabidopsidis*, are associated with the salicylic acid (SA) defence signalling pathway and can be further classified as virulent (able to cause disease) or avirulent (unable to cause disease) with respect to a specific host. The jasmonic acid (JA) / ethylene (Eth) signalling pathway is induced for basal defence against virulent necrotrophic pathogens, such as *Botrytis cinerea* and *Alternaria brassicicola*.

Immediately after pathogen recognition has taken place, various second messenger molecules are mobilised in the form of ion flux across cellular membranes, production of nitric oxide (NO) and a phosphatidic acid (PA) induced oxidative burst (Zimmermann *et al.* 1999, Sang *et al.* 2001, Arasimowicz and Floryszak-Wieczorek 2007). The potency of these early signalling events is thought to confer specificity to the response as slight differences in the accumulation of reactive oxygen species (ROS), dynamics of ion flux and level of NO production will specify which downstream defence responses are induced. Accumulation of NO and ROS, such as hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and superoxide anion (O<sup>-</sup>), and the alteration in membrane potential by Ca<sup>2+</sup> and proton (H<sup>+</sup>) influx and K<sup>+</sup> and chloride (Cl<sup>-</sup>) efflux induces a range of downstream effects, which could include localised cell death and the production of anti-microbial proteins and secondary metabolites. The second messenger, PA, is thought to induce the oxidative burst whereas NO and Ca<sup>2+</sup> are well known second messengers. Many studies have tried to tweeze apart these early signalling events to identify the order in which they occur but thus far, it remains unclear.

Biotrophic pathogens utilise the plants' cellular machinery to proliferate and an incompatible reaction occurs if the host plant harbours an R protein corresponding to the pathogen's effector (or avirulence, Avr) protein. This recognition event, also known as the gene-for-gene interaction, is critical in establishing an effective defence response to prevent both local and systemic infection. Therefore plants have to co-evolve with the invading pathogen by adapting their R proteins in response to the pathogen's strategy of modifying its Avr proteins to evade detection. There are numerous plant genes encoding R proteins but the known Avr proteins outnumber them suggesting that R proteins recognise more than one Avr protein (Keen 1990). A majority of the characterised R proteins have a leucine rich repeat (LRR) domain on the C-terminus and the N-terminus harbours a nucleotide binding sequence (NBS) with some exhibiting a TOLL and interleukin-1 (TIR) or leucine zipper (LZ) domain. R proteins have been shown to be located in the cytosol or in the plasma membrane where they can quickly identify the effector protein or its activity as it is released into the cell by the pathogen. A direct interaction between R and Avr proteins has been demonstrated (Scofield *et al.* 1996, Tang *et al.* 1996) but a study by Mackey *et al.* (2002) also provides evidence for the 'Guard model' which demonstrates that an R

protein recognises the conformational change of another plant protein that binds the Avr protein.

The proposed recognition event of PAMPs is thought to occur via an interaction with the extracellular portion of plant membrane-bound receptor kinases. The cytosolic kinase domain of the receptor could directly activate cytosolic proteins to induce the basal defence response. Defence signalling pathways that are initiated by gene-for-gene interactions are much more effective in preventing disease symptoms than those initiated by PAMP recognition. Pathogens can employ various deceptive tactics to delay the appropriate defence signalling cascade being activated if the plant does not accurately recognise the PAMP of the invading pathogen. Once the recognition event has occurred between plant and pathogen proteins, plants have to induce both local and systemic defence mechanisms to prevent primary and secondary infections occurring by production of ROS and NO and allowing ion flux.

As stated earlier, biotrophic pathogens require living host tissue to proliferate and if the plant is able to recognise that it is being invaded by a biotrophic pathogen, through gene-for-gene interactions, it can limit the spread of this pathogen by inducing localised cell death at the site of infection known as the hypersensitive response (HR). The HR is the first visual characteristic of an incompatible reaction and many mutants, such as the accelerated cell death (*acd*) and lesion simulating disease (*lsd*) mutants, display constitutive activation of defence and spontaneous lesion formation in the absence of pathogen attack (Greenberg and Ausubel 1993). This provides a positive correlation between HR formation and pathogen defence. However, the *defence no death mutants* (*dnd*), show uncoupling of HR and resistance as these mutants display heightened resistance to avirulent biotrophic pathogens in the absence of an HR (Yu *et al.* 1998, Yu *et al.* 2000). This indicates that the HR may not be critical for systemic acquired resistance (SAR) but is a localised defence strategy to contain the pathogen at the site of infection.

The importance of SA accumulation in SAR was explicitly shown when transgenic *Arabidopsis* or tobacco plants, expressing the bacterial salicylate hydroxylase gene, *nahG*, displayed increased susceptibility to biotrophic pathogen attack (Delaney *et al.* 1994). The use of *nahG* transgenic plants as a tool to understand SA-dependent

defence signalling was ground-breaking but comparison of phenotypes due to *nahG* expression or the loss of an endogenous SA biosynthetic plant gene indicated that the bacterial gene may be causing additional side-effects due to the SA breakdown product, catechol (Gaffney *et al.* 1993, van Wees and Glazebrook 2003). Accumulation of SA is vital for the induction of defence gene expression in both the infected leaf and systemic tissue. Plants that display constitutively high levels of SA are normally more resistant to both virulent and avirulent biotrophic pathogens, indicating that both basal and R gene-mediated defence mechanisms are under the control of SA signalling.

Increasing SA levels cause the activation of transcription factors, which induce expression of the *pathogenesis related 1 (PR-1)* gene whose protein product has displayed anti-microbial activity *in vitro* (Van Loon and Van Strien 1999). The non-expressor of PR genes 1 (NPR1) protein has protein-protein binding capabilities and is translocated to the nucleus upon SA accumulation to activate transcription factors, such as TGA2 or WRKY70, needed for activation of *PR* genes (Kinkema *et al.* 2000, Fan and Dong 2002, Zhang *et al.* 2006). Many studies have isolated *NPR1* alleles, such as *non-inducible immunity (NIM1)*, but very few new transcriptional regulators acting downstream of SA accumulation have been identified (Ryals *et al.* 1997). Plants that have non-functional *NPR1* proteins do not accumulate *PR-1* when treated with SA or infected with *P. syringae* but are able to express *PR-1* when infected with *H. arabidopsidis* (Cao *et al.* 1994). This indicates that there is an NPR1-independent SA defence signalling pathway specific to alternate stimuli. There are many other PR genes, besides *PR-1*, induced during SA dependent defence signalling but *PR-1* is used as the hallmark to determine or indicate an active SA defence signalling cascade as expression of the *PR-1* gene correlates with an effective defence outcome against most biotrophic pathogens (Van Loon and Van Strien 1999). Expression of *PR-1* in un-infected distal parts of a plant causing broad-spectrum resistance to secondary infection is a trademark of SAR.

Plant recognition of necrotrophic pathogens does not occur via gene-for-gene interaction and is thought to occur via PAMP detection, inducing basal defence responses. *B. cinerea* secrete toxins and enzymes to gain entry to the plant cell and it is hypothesised that this pathogen also tricks the plant into inducing an HR so that

it can utilise the dead tissue to proliferate. This hypothesis is supported by characterisation of some mutant plants displaying increased resistance to *B. cinerea* infection, as they are unable to induce HR formation (Govrin and Levine 2000). Induction of the opposing defence signalling pathways is a deceptive tactic deployed by both necrotrophic and virulent biotrophic pathogens. *B. cinerea* induces the SA pathway causing HR formation for its benefit and similarly, virulent *P. syringae* strains inject effector proteins that induce the JA defence signalling pathway to delay the activation of appropriate SA defence mechanisms (Nomura *et al.* 2005, Govrin *et al.* 2006). Induction of an HR occurs in parallel to an oxidative burst, NO production and ion flux, which result in the acquisition of second messengers. However, unlike R gene-mediated SA defence mechanisms, activation of these defence responses are more beneficial to the necrotrophic pathogen.

Similarly to basal defence mechanisms against virulent biotrophic pathogens, the production of second messengers cause induction of the necessary local defence responses but is unable to prevent disease symptoms occurring or induce SAR in distal parts of the plant. Plants lacking functional proteins for JA and Eth biosynthesis or signalling are more susceptible to *B. cinerea* attack indicating that accumulation and functional hormone signalling pathways are required for full *B. cinerea* defence. For example, the *jasmonic acid resistant 1* (*jar1*) and *ethylene insensitive 2* (*ein2*) mutants are more susceptible to *B. cinerea* infection and display reduced expression of the plant defensin 1.2 (PDF1.2) protein, indicating that functional JA and Eth signalling pathways are needed for PDF1.2 expression (Ferrari *et al.* 2003, Kishimoto *et al.* 2006). Induction of this anti-fungal peptide is characteristic of the JA/Eth defence signalling pathway but it cannot act alone to provide full defence. Plants also induce expression of other *PR* genes such as *PDF1.2* and accumulate antimicrobial metabolites to contain *B. cinerea* infection (Manners *et al.* 1998, Denby *et al.* 2004).

The major secondary metabolite produced by *Arabidopsis* in response to pathogen attack is the phytoalexin, camalexin, which displays antimicrobial activity *in vitro* (Darvill and Albersheim 1984, Tsuji *et al.* 1992, Rogers *et al.* 1996, Zook and Hammerschmidt 1997). The *Arabidopsis* mutant, *phytoalexin deficient 3* (*pad3*), is unable to produce camalexin as its cytochrome P450 (CYP71B15) enzyme, needed to catalyse the final reaction for camalexin production, is not functional (Glazebrook

*et al.* 1996). Camalexin is induced in response to both biotrophic and necrotrophic pathogens but it is only effective against necrotrophic pathogens as the *pad3* plants are more susceptible to certain strains of *B. cinerea* whereas they display wildtype resistance towards all *P. syringae* pathogens (Thomma *et al.* 1999, Denby *et al.* 2004). A study by Denby *et al.* (2004) shows a positive correlation between accumulation of camalexin and resistance to *B. cinerea* susceptibility in various *Arabidopsis* ecotypes, however, camalexin is not the main resistance strategy to deter infection. The defence signalling cascade leading to *B. cinerea* resistance is hypothesised to induce a suite of genes whose protein products work as a team to impart resistance. Analysis of quantitative trait loci (QTL) underlying *B. cinerea* defence has uncovered many genes that are yet to be functionally characterised but have also identified members of the BOS family, known to play important roles in *B. cinerea* defence (Denby *et al.* 2004, Veronese *et al.* 2004).

There has been some evidence regarding crosstalk between the SA and JA/Eth defence signalling pathways causing synergistic, antagonistic and additive defence responses (Kunkel and Brooks 2002). Effective defence responses require orchestrated and complex signalling events to produce a number of anti-microbial mechanisms that work together to stop initial and future disease from occurring. Plants are not able to escape their attackers and the continual activation of defence responses could be too energy consuming to take a 'prevention is better than cure' approach. Therefore, plants have intricate signal cascades to rapidly induce the appropriate defence response when necessary. The action of second messengers is vital to induce and amplify the signalling cascade needed for the correct defence response. The complexity of plant defence signalling cascades require the interplay of second messenger systems and a short summary is provided to give a general overview of how these second messenger systems could work together to provide an effective defence response.

#### **1.4 Hydrophobic Second Messengers of Plant Defence**

Lipids have shown versatility in the roles they play in various plant processes. They are best characterised for their importance as structural components of membranes but can also function as enzyme co-factors and has recently been identified as possible second messenger and defence molecules in various signalling cascades.

The plant cuticle is proposed to be a physical barrier to pathogen invasion and is made up of lipids and waxes. However, an *Arabidopsis* mutant displaying decreased cutin biosynthesis, which causes improper cuticle development, does not show increased susceptibility to opportunistic necrotrophic pathogens. Characterisation of the *long chain Acyl CoA synthetase 2 (lacs2)* *Arabidopsis* mutant led to the identification of lipid molecules with anti-fungal properties which can diffuse across the *lacs2* mutants more permeable cuticle and cause increased resistance to *B. cinerea* (Bessire *et al.* 2007).

In addition to lipid molecules acting as physical barriers or anti-microbial peptides they also form integral components of signalling cascades. The extensively studied plant hormone, JA, is synthesised from linolenic acid via the octadecanoid pathway and is an important lipid-derived molecule needed for basal defence signalling, as mentioned before. Expression of the *phospholipase A<sub>2</sub> (PLA<sub>2</sub>)* gene, whose protein products could play a role in JA biosynthesis, displays induction by a range of elicitors in various plant species and many studies have shown a link between phospholipid metabolism and plant defence pathways (Laxalt and Munnik 2002, Yang *et al.* 2007).

Mutations in plant genes that encode lipase - like proteins, phytoalexin deficient 4 (PAD4) and enhanced disease susceptibility 1 (EDS1), cause defence related phenotypes in *Arabidopsis*. Studies show that these proteins play a role in basal and R gene-mediated defence signalling pathways mediated by SA, but the lipase activity of these proteins has not yet been demonstrated experimentally. Loss of PAD4 or EDS1 function results in increased susceptibility to a number of biotrophic pathogens, which harbour effector proteins recognised by the TIR-NBS-LRR R proteins. Basal resistance to some virulent biotrophs and necrotrophic pathogens, such as *A. brassicicola* are also affected in these mutants. The *eds1* and *pad4* mutant plants are not able to accumulate SA and *pad4* plants induce lower levels of camalexin when attacked by a pathogen. These lipase-like genes demonstrate the antagonistic relationship between SA and JA/Eth signalling as mutant plants were able to induce higher levels of *PDF1.2* expression in response to *B. cinerea* infection than wildtype, due to lack of SA accumulation (Xing and Chen 2006).

A phospholipid-derived second messenger molecule, PA, is thought to play an important role in defence, acting upstream of the oxidative burst and defence hormone accumulation (Sang *et al.* 2001). PA is produced via two independent enzymatic pathways involving phospholipase C (PLC) or phospholipase D (PLD). Plants, unlike animals, do not utilise one of the products, diacylglycerol (DAG), from the PLC reaction but rapidly convert DAG to PA via the action of DAG kinase (DAGK). PA produced via PLD enzymatic activity can be biochemically distinguished from DAG derived PA, as PLD allows a direct conversion of phosphatidylcholine into PA. Therefore the presence of two PA producing pathways suggests that this second messenger can be utilised for different signalling cascades depending on which phospholipase is activated. Various studies on a range of plant species show that pathogen elicitors, such as xylanase, rapidly induce expression of PLD or accumulation of PA and one pharmaceutical study using a chemical inhibitor of PLC did not allow a pathogen induced oxidative burst. PA second messenger systems are therefore thought to act upstream of the oxidative burst as there is additional evidence for PA activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzyme and an oxidative burst can be triggered in *Arabidopsis* leaves following PA treatment (Sang *et al.* 2001).

Downstream targets of PA were identified by passing tomato and *Arabidopsis* suspension cell extracts through a PA-affinity matrix and analysing the bound soluble protein using mass spectrometry (MS). Cell extracts from elicitor treated suspension cells were also analysed to identify further, defence-related, PA protein targets. Many proteins displayed the ability to bind PA but only seven PA-binding proteins could be clearly identified and annotated using MS and bioinformatic analysis (Testerink *et al.* 2004). Heat shock protein 90 (HSP90) and two 14-3-3 isoforms, which have been linked to R gene-mediated SA defence signalling pathway, were three of the PA-binding proteins identified (Hubert *et al.* 2003). Members of the 14-3-3 family could be regulating other second messenger systems as they have been shown to interact with nitrate reductase (NR) needed for NO biosynthesis and  $\text{Ca}^{2+}$  binding proteins (Moorhead *et al.* 1996). The second product of the PLC enzymatic breakdown of phosphatidylinositol 4,5-bisphosphate is inositol-1,4,5-trisphosphate ( $\text{IP}_3$ ), which is well known for causing  $\text{Ca}^{2+}$  influx into the cytosol, provides more evidence of crosstalk between phospholipid and  $\text{Ca}^{2+}$  second messenger systems. A later study

by Testerink *et al.* (2007) identified the negative regulator of Eth biosynthesis, constitutive triple response 1 (CTR1), as having high affinity for PA. CTR1 is a homologue of the PA binding mammalian Raf-1 protein but they appear to have different PA binding sites. The kinase activity of CTR1 is inhibited upon PA binding and it could be speculated that PA induced by pathogen recognition binds to CTR1 to inactivate it, allowing the Eth defence signalling pathway to be activated.

Thus far, identification of PA binding proteins has established the link between PA and the SA and Eth defence signalling pathways. Many of the downstream PA targets still need to be properly identified and characterised to give a better understanding of how it functions in propagating the defence signal and its involvement in regulating other second messenger systems.

### **1.5 Gaseous Second Messengers of Plant Defence**

The NO second messenger system is well defined in mammalian cells but the tradition of using mammalian based protocols to detect and quantify NO in plants has resulted in similar problems faced by scientists investigating plant CNs. NO has been recognised as an essential signalling molecule mediating plant defence cascades, a function which seems to be evolutionary conserved as NO is also involved in the mammalian immune response (Bogdan 2001, Mur *et al.* 2006). In particular, treatment of *Arabidopsis* or tobacco plants with avirulent strains of *P. syringae* results in a rapid increase in NO accumulation and pre-treatment of *Arabidopsis* leaves with inhibitors of mammalian NO synthetic pathways, suppressed HR formation (Mur *et al.* 2006). Furthermore, accumulation of NO is thought to be mainly involved in the R gene-mediated SA signalling pathway as the *hrp<sup>-</sup>* *P. syringae* mutant is unable to induce NO in *Arabidopsis* and *PR-1* expression, a SAR hallmark, can be induced by NO treatment. Therefore, NO accumulation occurs rapidly after pathogen attack and precedes the oxidative burst and formation of HR.

Two enzymatic pathways exist to produce NO in mammalian cells but thus far only one of these pathways can be clearly identified in plants. The NR pathway reduces nitrate to nitrite and characterisation of the *nia1 nia2* double mutant, which has reduced NR activity due to loss of two NR isoforms, displays an altered HR response with reduced NO accumulation at the site of infection (Modolo *et al.* 2006). A

previous study by these authors showed that the second enzymatic NO synthesis pathway is active in the *nia1 nia2* mutants, however the search for plant homologues of the mammalian NO synthase (NOS) gene has been unsuccessful and very recently the *Arabidopsis AtNOS* gene, which displayed homology to a snail NOS, was found to be a GTPase and renamed *nitric oxide associated protein1 (AtNOA1)* (Moreau *et al.* 2008). There is some evidence of a NOS-mediated NO biosynthetic pathway as mammalian NOS inhibitors suppress NO production and L-citrulline, a by product of NOS activity, is produced in a number of plant species but a plant NOS enzyme remains elusive. The defence signalling pathway is therefore thought to be reliant on NO produced via NR and non-enzymatic NO synthesis pathways.

The increase in NO levels is needed for the activation of a variety of defence mechanisms, but the exact molecular process of these reactions remains unclear. NO may act as an anti-oxidant by interacting with ROS and many studies has shown that a proper balance of ROS and NO is needed to execute the correct defence signal. NO treatment of tomato leaves caused increased susceptibility to *B. cinerea* infection and this coincided with a lower level of hydrogen peroxide accumulation (Malolepsza and Rozalska 2005). In mammalian cells, S-nitrosylation and NO binding of haem groups can cause activation or inhibition of NO target proteins (Takemura *et al.* 1999). In relation to plant defence systems, the mobile SAR signal has yet to be found but it has been suggested that a S-nitrosylated protein is responsible for inducing defence in systemic tissue (Tada *et al.* 2008). NO has been shown to bind to the haem group of mammalian P450 enzymes and therefore could play a similar role in plants, by inactivating members of the P450 family, such as camalexin. NO inhibition of camalexin activity would tie in with the observation that NO treatment of tomato leaves resulted in increased susceptibility to *B. cinerea* infection, making NO a negative regulator of *B. cinerea* defence.

In mammalian cells, soluble guanylyl cyclases are known to be activated by NO binding and although direct activation of a cyclase by NO has not been shown in plants, NO treatment of plants does cause accumulation of the second messenger, cGMP (Durner *et al.* 1998). As stated before, the CNGC family could be the main target for cGMP and studies have shown that two CNGC mutants display phenotypes that could be attributed to lack of NO production (Ma *et al.* 2006, Ali *et al.* 2007). The absence of HR formation in *cngc2* plants is proposed to be due to lack of NO

generation. Elicitor treated *cngc2* plants displayed lower levels of NO accumulation than wildtype plants and the NO donor, sodium nitroprusside (SNP), could partially restore HR formation in infected *cngc2* plants (Ali *et al.* 2007). Pharmaceutical methods were used to block  $\text{Ca}^{2+}$  signalling prior to elicitor treatment of wildtype plants and showed that NO accumulation is dependent on  $\text{Ca}^{2+}$  flux.

Rapid and transient induction of second messenger molecules is critical for initiating defence signalling cascades but they are also needed intermittently throughout the entire process until all the defence mechanisms have been put in place. Accurate co-ordination of the delicately tangled web of second messenger systems is vital to ensure that the complex defence signalling pathway is executed properly, and interestingly, both hydrophobic and gaseous second messenger systems display a link to  $\text{Ca}^{2+}$  signalling.

### 1.6 Hydrophilic Second Messengers of Plant Defence

Plant-pathogen interactions have been shown to cause  $\text{Ca}^{2+}$  oscillations, sustained  $\text{Ca}^{2+}$  elevation or a slow  $\text{Ca}^{2+}$  spike in accordance with the type of invading pathogen (Lecourieux *et al.* 2006). Therefore it is hypothesised that slight differences in the rate of  $\text{Ca}^{2+}$  and ion flux are interpreted by the plant cell to produce the correct downstream effector molecules specific to the invading pathogen.

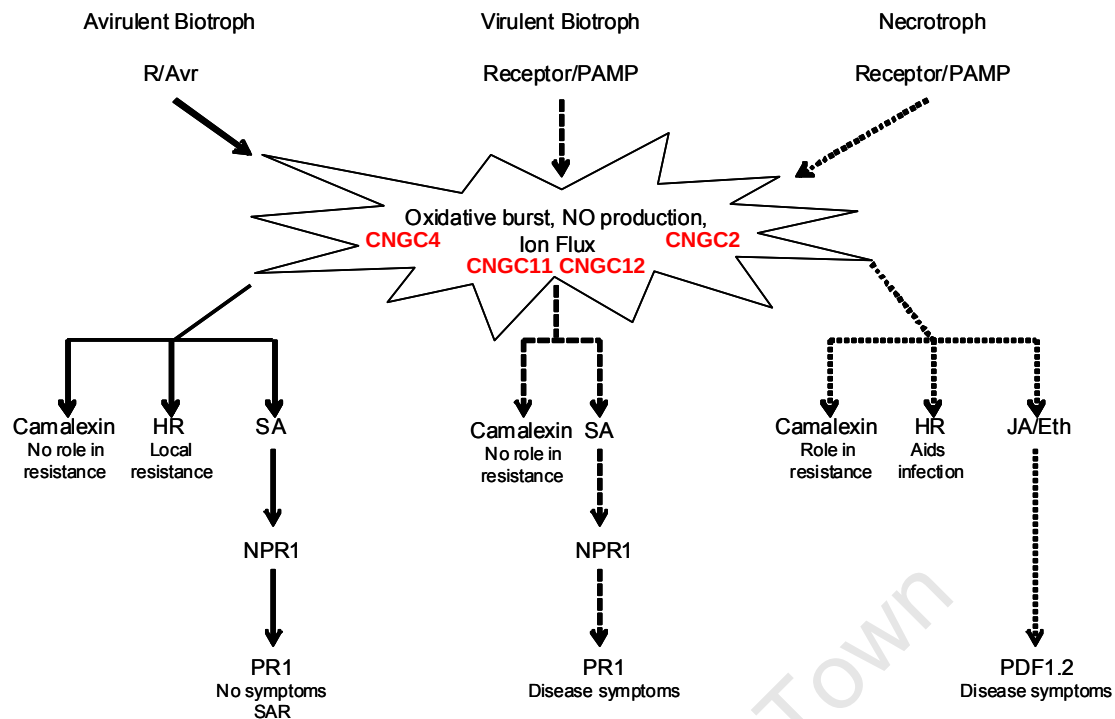
CNs are also hydrophilic second messenger molecules and it has been proposed that CN signalling is converted to  $\text{Ca}^{2+}$  *in planta*, by the action of CNGCs. Single mutations in *CNGC2*, 4, 11 and 12 cause defence-related phenotypes indicating that some CNGCs have non-redundant roles in defence signalling. Each of these studies examined CNGCs role in the SA signalling defence pathway and only one study looked at the role of CNGC2 in the necrotrophic pathogen pathway. Loss of CNGC2 or CNGC4 activity causes constitutive defence gene expression and results in increased resistance to both virulent and avirulent biotrophic pathogens. The increased resistance phenotype of *cngc2* and *cngc4* plants against virulent strains of *P. syringae* is dependent on a functional PAD4 protein as *pad4 cngc2* and *pad4 cngc4* double mutants were more susceptible to infection, however most of the other defence-related phenotypes displayed by *cngc2* and *cngc4* were not dependent on PAD4 function (Jirage *et al.* 1999). For example, conditional lesion development, and

increased SA accumulation were not affected in the *pad4 cngc* double mutants but higher *PR-1* expression and camalexin accumulation due to the *cngc* mutation was slightly repressed by the *pad4* mutation. The decreased resistance phenotype of the double mutants indicates that *cngc2* and *cngc4* are negative regulators of an alternate SA signalling pathway, uncoupled from *PR-1* expression. Both CNGC2 and CNGC4 have shown the ability to transport  $K^+$  but only CNGC2 has been characterised for  $Ca^{2+}$  transport.

A fused CNGC11/CNGC12 protein results in increased defence to *P. syringae* attack which is dependent on EDS1 and PAD4 function but once again, the high SA levels brought on by the *cngc11/12* mutation is independent of these lipase-like proteins. Both CNGC11 and CNGC12 proteins have the ability to transport  $Ca^+$  and  $K^+$  but a study has suggested that the defence phenotypes are due to improper  $K^+$  flux (Baxter *et al.* 2008). Single knockout mutants, *cngc11* and *cngc12*, also display defence-related phenotypes in response to biotrophic pathogen attack but further studies have to be undertaken to establish the cause of these phenotypes. The loss or mis-expression of a single CNGC gene causes widespread disruption affecting both SA and JA/Eth defence signalling and supporting the idea that these channels are responsible for directing the incoming stimulus into the appropriate signalling cascade. These studies indicate that CNGCs probably function very early in defence signalling cascades, and this is in keeping with their suggested role as second messenger converters.

Thus far, both phospholipid and CN messenger systems display a connection to  $Ca^{2+}$  signalling by potentially causing inhibition or activation of CNGCs respectively. The  $IP_3$  induced  $Ca^{2+}$  influx could activate the CNGC inhibiting molecule, CaM and further evidence suggesting antagonism or crosstalk between  $Ca^{2+}$  and PA second messenger systems is found in mammalian cells, where PA has been shown to activate a CN phosphodiesterase which would deplete CN levels needed for CNGC activation (Grange *et al.* 2000, Baillie *et al.* 2002). A recent study of the extensively characterised *cngc2* mutant provides yet another link between  $Ca^{2+}$  to another second messenger system involving NO (Ali *et al.* 2007).

Ion channels play an important role in early signalling events as ion flux, especially  $\text{Ca}^{2+}$ , is critical for a proper defence signalling cascade to be set up. A study using Parsley cell cultures indicated that ion flux occurs as soon as four minutes after addition of an elicitor (Jabs *et al.* 1997a). This flux is critical for subsequent defence process as the use of various ion channel inhibitors or blockers did not allow defence gene expression or phytoalexin accumulation. The CNGC family of *Arabidopsis* has twenty of the fifty-seven known cation channels in this genome and they are therefore possible candidates to orchestrate some of the ion flux. CNGCs also seem to be playing a critical role in converting signals from phospholipid and CN second messenger systems into a customary  $\text{Ca}^{2+}$  signal, observed in almost every plant signalling pathway, and may also act as the mediator of feedback loops. Almost thirty studies concerning *Arabidopsis* CNGCs have been published since their discovery over a decade ago and only one investigated the role of CNGCs in necrotrophic pathogen attack. The main aim of this study was to identify CNGCs needed for full defence against the necrotrophic pathogen, *B. cinerea*, followed by defence and functional characterisation of a *cngc* T-DNA insertion line displaying an altered defence phenotype.



A simplified model displaying the trademark events of signal transduction cascades controlling the defence response to biotrophic (*P. syringae*) or necrotrophic (*B. cinerea*) pathogen attack in *Arabidopsis*. CNGCs are hypothesised the function early in the defence signalling pathway by contributing to various events, such as ion flux. A few studies have implicated CNGC2, CNGC4, CNGC11 and CNGC12 in the biotrophic defence signalling pathway.

## Chapter 2

### Materials and Methods

#### 2.1 Plant Growth Conditions

*Arabidopsis thaliana* seeds were stratified for 2 days at 4°C prior to germination and grown under long day conditions, 16 hours light ( $150 \mu\text{mol m}^{-2}\text{sec}^{-1}$ ) and 8 hours dark, at 23°C and 70% relative humidity. Seeds were sown on Levington, M2 pot and bedding compost (Scotts, UK) or on plant growth media in accordance with the nature of the experiment being conducted.

##### 2.1.1 Growth on soil

Plants used for pathogen assays, cyclic guanosine monophosphate (cGMP) treatment, *Agrobacterium tumefaciens* (*A. tumefaciens*) transformation, cross-fertilization, genotypic analysis, glufosinate ammonium (BASTA) selection, growth development (visual and reporter gene expression analysis), and general plant propagation for seed collection were grown on Intercept treated M2 compost. Stratified seeds were sown on the compost and germinated in 100% humidity for 7 days by covering the plant trays with cling film or a propagator lid.

BASTA selection: Ten day old seedlings were watered 3 times with a solution containing 150 mg/L BASTA (Harvest, Bayer Crop Science) 2 days apart.

##### 2.1.2 Growth on media in sterile conditions

Seeds were surface sterilised by agitation in 70% (v/v) ethanol, followed by washing in 10% (v/v) bleach, 0.02% (v/v) Triton X-100 and rinsed 5 times with sterile water before stratification in 0.1% (w/v) agar. Seeds were sown on 0.8% (w/v) agar solidified half strength Murashige and Skoog (MS, Sigma, UK) media containing Gamborg's vitamins (Murashige and Skoog 1962, Gamborg *et al.* 1968) for the purpose of transformant selection, high ionic stress growth assays and plant aequorin (Aeq) assays.

Transformant selection:

Seeds were germinated on half strength MS agar containing one of the following antibiotics: kanamycin (50  $\mu\text{g/ml}$ ), hygromycin (15  $\mu\text{g/ml}$ ) or BASTA

(10 µg/ml). Ten to 14 day old transformants were transplanted onto soil and maintained in 100% humidity for 7 days by covering plant trays with cling film or a propagator lid.

#### Ionic stress assays:

Thirty seeds per line were germinated and grown for 2 weeks on half strength MS media supplemented with varying concentrations of sodium chloride (NaCl, 60 – 140 mM) or potassium chloride (KCl, 80 – 120 mM). Fourteen day old seedlings were weighed to determine fresh weight (FW) and percentage weight was calculated by dividing the FW of treated seedlings by the FW of untreated seedlings.

#### Akt1-1 low potassium media

For growth analysis of *akt1-1* and *akt1-1* lines over-expressing *CNGC3* (*35S:CNGC3:akt1-1*), 25 seeds per line were single seeded on media supplemented with 10 µM KCl, as described by Hirsch *et al.* (1998). Fresh weight of 9 day old seedlings was measured as an indication of growth.

## **2.2 PCR Selection of Homozygous T-DNA Insertion Mutants**

Segregating T<sub>3</sub> T-DNA mutagenised seed generated by the SALK, SAIL and GABI-Kat initiatives was obtained from NASC (Sessions *et al.* 2002, Alonso *et al.* 2003, Rosso *et al.* 2003). DNA was extracted from leaf tissue of the segregating T<sub>3</sub> lines and their self-fertilised progeny using the superquick DNA extraction protocol (Edwards *et al.* 1991) for genotypic analysis to identify homozygous insertion mutants. The T-DNA primer design tool on the SIGnAL website (<http://signal.salk.edu/T-DNAprimers.html>) was used to design gene-specific primers (Refer to Table A) and the T-DNA-specific primer sequences can also be found at this web address. Products were amplified with Taq Polymerase (Invitrogen, UK) using the following thermal cycler conditions: initial denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 1 min; annealing at 55°C for 1 min; elongation for 1 min at 72°C and a final elongation step at 72°C for 10 min. Products were run on a 1% (w/v) agarose gel in 40 mM Tris-Acetate, 1 mM EDTA (1X TAE) buffer and visualised on a UV light box.

| Name   | AGI code       | Polymorphism | Primer sequence                                                           |
|--------|----------------|--------------|---------------------------------------------------------------------------|
| CNGC3  | At2g46430      | SALK_056832  | 5' ATCAGAACCTTTAAGCGGCCC 3'<br>5' CGATCAACGGACAAGAACCGT 3'                |
| CNGC5  | At5g57940      | SAIL_215_F04 | 5' TCACAGCCCAGGTCTTTTGA 3'<br>5' TGTCCACTGAAATTGGAGGCG 3'                 |
| CNGC6  | At2g23980      | SALK_042207  | 5' TCCCCACCACAAACAGAAGAA 3'<br>5' TCGCAGTATCCGTTGATCCTCTC 3'              |
| CNGC8  | At1g19780      | GABI_101C03  | 5' TCTTTGTCTTTCCTCCTCCACGA 3'<br>5' TTTGGATGTAACGTGCATGATGTG 3'           |
| CNGC10 | At1g01340      | SALK_071112  | 5' TGGTTGTTTGCTTTGATTTCTCA 3'<br>5' TCAGACGCCTCCATAGCAAGC 3'              |
| CNGC12 | At2g46450      | SALK_092622  | 5' ATTGCTTTTGGTGGGGTCTCC 3'<br>5' CAGGCAAGAGAATGAAACCTTCAA 3'             |
| CNGC13 | At4g01010      | SALK_082668  | 5' TCAGGGTTGATAAACGGGCAC 3'<br>5' CAGAGATTGGATTTCAGAGGGAACA 3'            |
| CNGC15 | At2g28260      | SALK_017995  | 5' TTCTTGAGATGATACTGGATTCCG 3'<br>5' CCAGAAGAAAAGGGAAGAAAGCG 3'           |
| CNGC17 | At4g30360      | SALK_041923  | 5' GAATCTGATGACATTTTCCTGTT 3'<br>5' TCAGCTCGAAGAGCGAATGCT 3'              |
| CNGC19 | At3g17690      | SALK_027306  | 5' CACAATGGACAAAGCTCTTGGA 3'<br>5' GCAAATCGCTTAGCCCAACCT 3'               |
| CNGC19 | At3g17690      | SAIL_757_E05 | 5' AAAATGGCTCACACTAGGACTTTCCTTC 3'<br>5' TCAAATTTAACGGTTGGAATTGGAGTGAG 3' |
| CNGC20 | At3g17700      | SALK_074919  | 5' TCAAGCTCTTGGTAAAAGGTAAACA 3'<br>5' TGGCATCCTTATCCTCGTCCC 3'            |
| GABI   | T-DNA Specific |              | 5' GTGGATTGATGTCAATATCTCC 3'                                              |
| SAIL   | T-DNA Specific |              | 5' TAACAATTTACACAGGAAACAGCTATGAC 3'                                       |
| SALK   | T-DNA Specific |              | 5' TTTTTCGCCCTTTGACGTTGTTGGAGT 3'                                         |

**Table A. List of gene-specific primers used for genotyping T-DNA insertion mutants.**  
The T-DNA insertion line used for each gene is indicated in the polymorphism column. The top and bottom sequence of each primer pair is the forward and reverse primer respectively.

The homozygous deletion mutant, *dnd1* (*cngc2*), was previously isolated and characterised by Yu *et al.* (1998) and seed was obtained from NASC. T-DNA mutant seed of *hlm1* (*cngc4*) and *cngc1* were generated by Bechtold *et al.* (1998) and characterised by Balague *et al.* (2003) and the Maathuis group (*Pers. comm.*) respectively.

### **2.3 Gene Expression Analysis**

Total mRNA was extracted from uninfected leaf tissue, infected leaf tissue or seedlings to analyse expression of *CNGC19*, defence genes (*PR-1* and *PDF1.2*) or

*CNGC3* respectively using semi-quantitative RT-PCR (sqRT-PCR). The RNeasy plant mini kit (Quiagen, UK) was used to extract total mRNA from plant material and a modified version of the Invitrogen protocol, which included an RNase H (NEB, UK) and RNase A (MBI, UK) treatment was used to produce cDNA using Superscript II Reverse transcriptase (Invitrogen, UK). Taq polymerase was used to amplify the desired product from cDNA using 27 cycles of amplification at the appropriate annealing temperature for each primer pair, listed in Table B. To establish that each sample had similar starting concentrations of cDNA, amplification of *Ef-Tu* and *Ubiquitin* mRNA was used for cDNA quantity control.

| Name               | AGI code  | Primer sequence                                                             |
|--------------------|-----------|-----------------------------------------------------------------------------|
| <i>CNGC3</i>       | At2g46430 | 5' ATCAGAACCTTTAAGCGGCCC 3'<br>5' CGATCAACGGACAAGAACCGT 3'                  |
| <i>CNGC19</i>      | At3g17690 | 5' AAAATGGCTCACACTAGGACTTTCAC TTC 3'<br>5' TCAAATTTAACGGTTGGAATTGGAGTGAG 3' |
| <i>PDF1.2</i>      | At5g44420 | 5' TCATGGCTAAGTTTGCTTCC 3'<br>5' AATACACACGATTIAGCACC 3'                    |
| <i>PR-1</i>        | At2g14610 | 5' GATTCTCGTAATCTCAGCTC 3'<br>5' CTGCAGACTCATACACTCTGG 3'                   |
| <i>Ubiquitin10</i> | At4g05320 | 5' CACACTCCACTTGGTCTTGCGT 3'<br>5' TGGTCTTTCCGGTGAGAGTCTTCA 3'              |
| <i>Ef-Tu</i>       | At5g60390 | 5' ATTACCGGAGCTGCACAGATG 3'<br>5' CTCTTTAGTCTGAGGCATAGGACCAT 3'             |

**Table B. List of gene-specific primers used for sqRT-PCR analysis**

## **2.4 Calcium Measurements**

All luminescence readings were measured on a Sirius single tube luminometer (Berthold Detection systems, Germany) in a dark room.

### **2.4.1 Transient expression of *CNGCs* in *Saccharomyces cerevisiae***

For heterologous expression of *CNGC* genes in yeast, the protocol described by Dohmen *et al.* (1991)) was used to prepare and transform competent yeast cells with an empty pYES2 vector (pYES2:EV) or pYES2 vectors harboring full length *CNGC* cDNA (pYES2:*CNGC*) under the control of a galactose inducible promoter (Dohmen *et al.* 1991). The pYES2 vector constructs were a kind gift from Dr A Gobert and Dr E Peiter provided the mutant yeast strain,  $\Delta mid1\Delta cch1$  pEVP11:Aeq (*MATa,112*, *his4*, *trp1*, *ura3-52*, *rme*) and its parental wildtype, JK9-3DA pEVP11:Aeq (*MATa,112*,

*his4*, *trp1*, *ura3-52*, *rme*) for transformation (Knight *et al.* 1991, Batiza *et al.* 1996, Fischer *et al.* 1997). The transformed yeast strains were maintained on synthetic defined (SD) medium lacking histidine and tryptophan and addition of geneticin (200 µg/ml) to the medium was needed for selection against wild type contaminants. Liquid cultures were grown in SD media containing galactose till early log phase and incubated with 5 µM coelentrastine (LUX biotechnology, UK) for 1 hour in the dark while shaking. A 100 µl culture aliquot was treated with an equal volume of 2 M NaCl (1 M NaCl effective) 20 seconds after luminescence measurements were started. Luminescence data was integrated every 0.2 seconds over a 5 min period. The experiment was stopped by adding 2 M CaCl<sub>2</sub> 20% (v/v) ethanol, which discharges the remaining coelentrastine. Luminescence data was converted to cytosolic Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]<sub>cyt</sub>) using a formula described by Knight *et al.* (1996).

#### 2.4.2 T-DNA insertion mutants expressing the apoaequorin reporter gene

*Arabidopsis* Col-0, *cngc10* and *cngc13* homozygous T-DNA insertion lines were transformed with *A. tumefaciens* carrying the pGIF:Aeq construct (Kaplan *et al.* 2006), which contains BASTA and hygromycin selectable markers, using the floral dip method (Clough and Bent 1998). BASTA selection was used to isolate subsequent self-fertilised generations expressing the apoaequorin gene. Plants were grown under sterile conditions on half strength MS agar and 10 day old plants were incubated in 5 µM coelentrastine in the dark over night. Seedlings were placed in a luminometer tube containing 100 µl of water and allowed to rest for at least 30 min to avoid a touch induced Ca<sup>2+</sup> response. Seedlings were placed in a luminometer and 20 seconds after readings began, a stimulus (1 mM hydrogen peroxide, H<sub>2</sub>O<sub>2</sub>, or 1 mM salicylic acid, SA) was carefully injected into the tube. Luminescence data was integrated every 0.2 seconds over a 5 min period. The experiment was stopped by adding 2 M CaCl<sub>2</sub> 20% (v/v) ethanol, which quenches the remaining coelentrastine. Luminescence data was converted to [Ca<sup>2+</sup>]<sub>cyt</sub> using a formula described by Knight *et al.* (1996).

#### 2.5 Sub-Cellular Localisation

Gateway<sup>TM</sup> cloning technology was used to construct the CNGC19:yellow fluorescent protein (YFP) fusion protein by following the protocols set out by the manufacturer

(Invitrogen, UK). Full length *CNGC19* cDNA was amplified with Platinum *Pfx* DNA polymerase (Invitrogen, UK) to incorporate *attB* sites flanking the *CNGC19* sequence (primer sequences can be found in Table C) and through 2 homologous recombination events, *CNGC19* was cloned into the pEarleyGate-101 (pEG-101, (Earley *et al.* 2006)) destination vector, downstream of a 35S cauliflower mosaic virus (CaMV) promoter and in frame with the C-terminal located YFP sequence. Tungsten particles were coated with pEG-101:*CNGC19* plasmid DNA and biolistic particle bombardment was used to transform onion cells with the construct. *CNGC19*:YFP expression was visualized 16 hours post transformation using an Olympus Fluoview IX 70 confocal microscope. As the plasma membrane dye, FM4-64, is endocytosed relatively quickly, the location of an onion cell displaying fluorescence from *CNGC19*:YFP expression was marked on the microscope slide to indicate its location before FM4-64 treatment of onion cells. Dr Volkan Cevik used biolistic transformation of onion cells to transiently co-express pEG-101:*CNGC19* and CD3-1008, a known plasma membrane protein fused to mCHERRY (Nelson *et al.* 2007), and viewed their co-localisation on the Olympus Fluoview IX 70 confocal microscope.

| Name          | Primer sequence ( <i>attB</i> sites are in bold)                               |
|---------------|--------------------------------------------------------------------------------|
| <i>CNGC19</i> | 5' <b>GGGGACAAGTTTGTACAAAAAAGCAGGCT</b> <i>AAATGGCTCACACTAGGACTTTCACTTC</i> 3' |
|               | 5' <b>GGGGACCACTTTGTACAAGAAAGCTGGGT</b> <i>GACGGTTGGAATTGGAGTGAGC</i> 3'       |

**Table C. Primer sequences used to introduce *CNGC19* cDNA into the pDONR<sup>TM</sup> 207 donor vector using Gateway<sup>TM</sup> technology.** Two adenine (A, in italics and underlined) base pairs were included in the forward primer and the guanidine (G, in italics and underlined) was included in the reverse primer to keep the gene-specific sequence in frame and remove the *CNGC19* stop codon respectively.

## **2.6 Tissue Specific *CNGC* Expression**

Seed of T<sub>2</sub> transgenic Col-0 plants harboring the  $\beta$ -glucuronidase (GUS) reporter gene under the control of *CNGC13* or *CNGC19* promoter sequences were provided by Dr A Gobert. The same methodology described by Gobert *et al.* (2006) was used to clone approximately 1 kb sequences upstream of the *CNGC13* and *CNGC19* translation start codon into the pBI101.3 vector (Jefferson 1987) and the same procedures were used to transform Col-0 and select the transgenic seed. A protocol adapted from Jefferson (1987) was followed to localize GUS expression by staining

and destaining in 5-bromo-4-chloro-3-indolyl glucuronide (X-GlcA, Sigma) and 70% (v/v) ethanol respectively. Detached leaves of 4 week old *CNGC13::GUS* and *CNGC19::GUS* plants were infected with *Botrytis cinerea* and stained for expression 2 and 3 days post infection (dpi). Tissue from *CNGC19::GUS* plants aged 1 – 6 weeks old was assayed for GUS expression to identify tissue specific CNGC19 expression throughout development. An Olympus digital camera attached to an Olympus SZ stereomicroscope was used to take pictures of the histochemically stained plants.

## **2.7 Pathogen Assays**

*B. cinerea* isolates were subcultured on sterile apricot halves (Tesco, UK) and spores from 11 - 14 day old cultures were suspended in half strength grape juice (Tesco, UK) at a final concentration of  $5 \times 10^4$  spores/ml. Fifteen or more detached leaves from each line was inoculated on the upper leaf surface with a 6  $\mu$ l droplet of the spore suspension and kept at 100% humidity in long day conditions. Lesion perimeter was measured by using Image J software (<http://rsbweb.nih.gov/ij/index.html>) to analyse digital photographs of leaves 2 - 5 dpi.

*Pseudomonas syringae* assays were performed as described in de Torres-Zabala *et al.* (2007).

## **2.8 Trypan Blue Staining**

Trypan blue staining of *B. cinerea* infected leaves, 0 hours post infection (hpi) and 48 hpi, was performed as described by Cao *et al.* (1998). Images were taken with an Olympus digital camera connected to an Olympus SZ stereomicroscope.

## **2.9 Secondary Metabolite Analysis**

Four leaf discs, from the border of the *B. cinerea* lesion, were harvested per infected leaf and camalexin was extracted and quantified according to conditions set out by Denby *et al.* (2004).

## **2.10 Plant Complementation and Site-Directed Mutagenesis**

### **2.10.1 Over-expression of CNGC3 in an *akt1-1* background**

Dr A Gobert cloned the full length *CNGC3* cDNA sequence into the pBART27 binary vector (Gleave 1992) and used this construct to transform *akt1-1* (Hirsch *et al.* 1998) plants via *A. tumefaciens* transformation using the floral dip method (Clough and Bent 1998). The T<sub>2</sub> 35S:*CNGC3:akt1-1* seed was kindly provided by Dr Gobert and BASTA selection of subsequent self-fertilised generations was used to identify a homozygous line which displayed constitutively high levels of *CNGC3* through sqRT-PCR.

#### 2.10.1 *Cngc19* complementation and site-directed mutagenesis

Complementation: Full length *CNGC19* cDNA was cloned into the pBART27 binary vector (Gleave 1992) and transformed into *A. tumefaciens* by Dr A Gobert. *Cngc19-1* plants were transformed using the floral dip method (Clough and Bent 1998) and successive transgenic progeny was selected with BASTA treatment.

Site-Directed Mutagenesis: Primers were designed to introduce base pair changes that truncated or mutated the putative regulatory domains of *CNGC19*, namely the cyclic nucleotide and calmodulin binding domains. These primers also incorporated *attB* sites needed for Gateway™ technology cloning and the sequences are listed in Table D. Platinum Pfx DNA polymerase (Invitrogen, UK) was used to amplify the site-directed mutagenised *CNGC19* cDNA sequences which were inserted into the pEG-100 destination vector via homologous recombination. The construct was transformed into *A. tumefaciens* for transformation into *cngc19-1* plants using the floral dip method (Clough and Bent 1998). Transgenic *cngc19:CaM* plants were selected with BASTA treatment.

| Name          | Primer sequence ( <i>attB</i> sites are in bold)                      |
|---------------|-----------------------------------------------------------------------|
| CNGC19 F      | 5' <b>GGGGACAAGTTTGTACAAAAAAGCAGGCT</b> CGAGATTAATGAAGGATCGGA 3'      |
| CNGC19-Both R | 5' <b>GGGGACCACTTTGTACAAGAAAGCTGGGT</b> CCTTATCTAAGTCCTCTGTTACAGCC 3' |
| CNGC19 F      | 5' <b>GGGGACAAGTTTGTACAAAAAAGCAGGCT</b> CGAGATTAATGAAGGATCGGA 3'      |
| CNGC19-CaM R  | 5' <b>GGGGACCACTTTGTACAAGAAAGCTGGGT</b> GCCTTCTTAAGAATCATGAAAACAAG 3' |
| CNGC19 F      | 5' <b>GGGGACAAGTTTGTACAAAAAAGCAGGCT</b> CGAGATTAATGAAGGATCGGA 3'      |
| CNGC19-CN R   | 5' TC <del>GAA</del> AGCTCTC <del>TATCTCAC</del> ATCTC 3'             |
| CNGC19-CN F   | 5' GAGA <del>TGTGAGAT</del> AGAGAGC <del>TTT</del> C <del>GA</del> 3' |
| CNGC19 R      | 5' <b>GGGGACCACTTTGTACAAGAAAGCTGGGT</b> AGAATGGGCCGAAATTATGAG 3'      |

**Table D. Primer sequences used to create truncated or mutated *CNGC19* cDNA sequences and allow insertion into the Gateway™ pDONR™ 207 donor vector.** The mutated base pairs are underlined and italicised. To truncate the *CNGC19* cDNA sequence,

a stop codon has been introduced using the CNGC19-Both R primer (removes both CNBD and CaMBD) or the CNGC19-CaM R primer (removes the CaMBD). To mutate the CNBD, 3 PCR reactions had to be performed. The CNGC19 F / CNGC19-CNR and CNGC19-CN F / CNGC19 R primer combinations were used to amplify CNGC19 cDNA and incorporate the mutated base pairs into both DNA strands. The products from both PCR reactions were used as template for CNGC19 F and CNGC19 R primers.

### **2.11 Gene Expression Profiling of cGMP Treated Leaves**

Leaf 7 of soil grown Col-0 plants was identified and tagged when plants were 21 days old. A week later, when plants were 4 weeks old, leaf 7 was pressure infiltrated with 10  $\mu$ M 8-Bromoguanosine-3', 5'-cyclomonophosphate (8-Br-cGMP, Sigma, UK) or sterile water until the leaf appeared water-soaked. Eight leaves per treatment were harvested in liquid nitrogen, 4 and 10 hours post treatment. Total mRNA was extracted from 4 replicates of each treatment per time point using a TRIzol (Invitrogen, UK) based protocol. Total mRNA concentration was determined using a Nanodrop (Labtech, UK) and 1  $\mu$ g of mRNA was amplified using the Message Amp<sup>TM</sup> II aRNA amplification kit (Ambion, UK). The RNA 6000 Nano assay (Agilent technologies, Germany) protocol was followed to check the integrity of the aRNA by running samples on the Agilent 2100 bioanalyzer (Agilent technologies, Germany). Five micrograms of aRNA was directly labelled with Cy3<sup>TM</sup>-dCTP and Cy5<sup>TM</sup>-dCTP (GE Healthcare, UK) using Superscript II reverse transcriptase as per manufactures guidelines but the dNTP mix contained 10 mM each of dATP, dGTP, dTTP and 2 mM dCTP (Invitrogen, UK). Labelled products were purified using a QIAquick PCR purification kit (Qiagen, UK). The concentration of Cy-labelled cDNA products was determined on the Nanodrop and the 30 pmol of cDNA of the appropriate samples were combined and freeze dried. CATMA version IV microarray slides (Sclep *et al.* 2007) were incubated in pre-hybridisation buffer (5X SSC, 0.1% (v/v) SDS, 10 mg/ml BSA) for 1 hour at 42°C and washed 5 times in deionised water before air-drying after a quick isopropanol treatment. The freeze dried Cy-labelled samples were resuspended in 50  $\mu$ l hybridisation buffer (5X SSC, 0.1% (v/v) SDS, 25% (v/v) formamide, 0.5 mg/ml yeast tRNA) and applied to the microarray slides. The slides were incubated at 42°C overnight in a humid chamber. To remove unbound labelled samples, microarray slides were washed in pre-warmed wash 1 solution (2X SSC, 0.1% (v/v) SDS) for 5 min at 42°C followed by room temperature washes in wash 2

solution (0.1X SSC, 0.1% (v/v) SDS) and wash 3 solution (0.1X SSC) for 10 and 1 min respectively. The third wash was repeated 4 times before slides were air-dried after isopropanol treatment. Microarray slides were scanned using the Affymetrix 428<sup>TM</sup> (Affymetrix, USA) array scanner and the images were analysed with ImaGene 7 software (BioDiscovery, USA).

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## Chapter 3

### Ion Transport and Expression Analysis of Cyclic Nucleotide Gated Channels

#### 3.1 Introduction

Regimented ion transport and homeostasis is vital for many plant processes and a large family of cyclic nucleotide gated channels (CNGCs) are hypothesised to be key players in this process in the model plant, *Arabidopsis thaliana*. Analysis of *Arabidopsis* CNGC expression patterns indicates that these ion channels could be involved in a variety of functions throughout plant development ranging from ion uptake in the roots during germination to ion signalling in pollen tubes during fertilization. Ten years after the first plant CNGC was identified, nearly half of the twenty *Arabidopsis* CNGC members have been tested for ion transport capabilities but their exact function and mode of regulation *in planta* is still unclear. Many different techniques, such as complementation of ion transport yeast mutants, electrophysiological studies and phenotypic analysis of plants mis-expressing a single CNGC protein have been used to investigate which ions they transport and how they are regulated (Leng *et al.* 2002, Chan *et al.* 2003, Gobert *et al.* 2006). This study utilises some of these techniques to determine or further investigate the ion transport capabilities of uncharacterised or previously characterised CNGCs respectively and looks at cyclic nucleotide (CN) regulated transcripts in leaf tissue in an attempt to indirectly link CN to CNGC function *in planta*, by using a transcriptomics approach.

Animal CNGCs have been more extensively studied and are therefore used as a benchmark for plant CNGC studies. Unlike animal CNGCs, which are known to transport monovalent and divalent cations non-selectively in a CN-dependent manner, some *Arabidopsis* CNGCs appear to preferentially transport potassium ( $K^+$ ) over sodium ( $Na^+$ ) ions and the exogenous application of CN is not always necessary for ion transport to occur when they are expressed in a heterologous system (Zagotta and Siegelbaum 1996, Leng *et al.* 2002). Animal CNGCs function as heterotetramers but thus far, this has not been proven for plant CNGCs (Zhong *et al.* 2002). Some studies which used electrophysiological techniques to assess ion transport of *Arabidopsis* CNGCs have suggested that they do form heterotetrameric channels

because it is difficult and sometimes impossible to get reliable data when a single CNGC gene is expressed (Leng *et al.* 2002). Overall, expression of *Arabidopsis* CNGC genes in human embryonic kidney (HEK) and *Xenopus* oocyte cells for electrophysiological analysis was considerably lower than the animal CNGCs and *Arabidopsis* ion channels used as positive controls to show that foreign transcripts can be successfully expressed in the heterologous system being used (Leng *et al.* 2002). Despite these difficulties, heterologous expression of *Arabidopsis* CNGCs in yeast, HEK and *Xenopus* oocyte cells has provided the most information regarding their ion transport capabilities.

Complementation studies using yeast mutants which lack specific ion transport channels have highlighted which ions the CNGCs may be capable of transporting *in planta*. However, there are some contrasting results regarding the activation and inactivation of these channels by CNs and calmodulin (CaM) when expressed heterologously. Some studies show that exogenous application of CN is necessary for ion transport to occur whereas other studies show ion transport in the absence of CN treatment (Leng *et al.* 1999, Urquhart *et al.* 2007). One study showed that the *Arabidopsis* CNGC proteins have different affinity for CaM isoforms and suggested that this could play a crucial role in the regulation of such a big channel family (Ali *et al.* 2006). The *Arabidopsis* CNGC1 protein, but not CNGC2, displayed high affinity to a specific yeast CaM isoform and it was suggested that the binding of CaM to the full length CNGC1 protein was responsible for its inability to complement an ion transport yeast mutant (Ali *et al.* 2006). To demonstrate that a plant CNGC could be regulated similarly to their animal homologues, a truncated form of the CNGC1 protein, which lacked the CaM binding domain, was used to successfully complement a potassium transport yeast mutant (Ali *et al.* 2006).

One of the most important ions found in plant cells is calcium ( $\text{Ca}^{2+}$ ) as it is a ubiquitous second messenger in many signalling pathways and altering the  $\text{Ca}^{2+}$  homeostasis causes many developmental and stress-related defects. To determine whether plant channels can transport  $\text{Ca}^{2+}$ , a *Saccharomyces cerevisiae* mutant that lacks high affinity  $\text{Ca}^{2+}$  uptake channels can be used for complementation analysis. MID1 and CCH1 are plasma membrane-bound channels that facilitate the transport of  $\text{Ca}^{2+}$  into the yeast cell in response to various external stimuli (Fischer *et al.* 1997).

The  $\Delta mid1\Delta cch1$  yeast mutant, which lacks these channels, is unable to grow in the presence of the  $\alpha$ -factor mating pheromone, as an influx of  $Ca^{2+}$  is needed for the signalling cascade induced by this pheromone. Channels which enable  $Ca^{2+}$  influx enable growth. Of the four CNGCs (*CNGC1*; *CNGC3*; *CNGC11*; and *CNGC12*) that were tested for the ability to transport  $Ca^{2+}$  by complementation analysis of  $\Delta mid1\Delta cch1$  using the  $\alpha$ -factor mating pheromone assay, two were able to do so (Ali *et al.* 2006, Gobert *et al.* 2006, Urquhart *et al.* 2007).  $\Delta Mid1\Delta cch1$  strains expressing *CNGC1* or *CNGC3* were not able to grow in the presence of  $\alpha$ -factor mating pheromone, which suggests that these CNGCs are not suitable  $Ca^{2+}$  channel substitutes in yeast and that they are not capable of transporting  $Ca^{2+}$  *in planta* (Ali *et al.* 2006, Gobert *et al.* 2006). However, as mentioned previously, the CNGC1 protein does display high affinity to yeast CaM isoforms which could inactivate it. Therefore, the investigators expressed a truncated CNGC1 protein that lacked the CaM binding domain and were able to show complementation of the  $\Delta mid1\Delta cch1$  yeast strain demonstrating the ability of CNGC1 to transport  $Ca^{2+}$  (Ali *et al.* 2006). Hence, yeast complementation assays are useful to functionally characterise single CNGCs but mis-targeting of the protein or inactivation by CaM in yeast could provide misleading results.

A more reliable way of determining which ions the individual CNGCs are transporting would be to characterise CNGC knockout mutants for altered germination or growth in response to ion stress. Functional redundancy within such a large channel family was suggested to negatively influence the chance of finding a phenotype but so far, a few studies have reported phenotypes resulting from the disruption of a single CNGC gene. Plants that lack *CNGC2* expression (*dnd1*) display an increased sensitivity to high  $Ca^{2+}$  concentrations whereas *cngc1* plants were more tolerant to high lead ( $Pb^{2+}$ ) levels (Sunkar *et al.* 2000, Chan *et al.* 2003). As the CNGCs are a large family of channels, prior knowledge of a specific CNGC's expression pattern could also assist in deciding which phenotype to look for in a plant that does not express the CNGC of interest.

Analysis of information from publicly available microarray data shows that CNGCs are expressed at varying levels, in different tissues and at different stages of

development suggesting that each member may have a specific task to fulfil. Maser *et al.* (2001) used amino acid sequence similarity to phylogenetically group the CNGCs into four main subgroups and members from the same subgroup could be performing similar functions *in planta*. CNGCs with similar expression patterns or function were identified and this chapter presents functional characterisation of the selected CNGCs using both yeast and plant systems. CNGCs constitute the largest family of proteins with CN binding domains but no study has unequivocally shown direct regulation of these channels by CN binding *in planta*. A study by Maathuis (2006a) used a transcriptomics approach to identify genes regulated by CNs in roots but as some CNGCs are specifically expressed in leaf tissue, this study used a similar approach to identify transcripts regulated by CN in mature leaves.

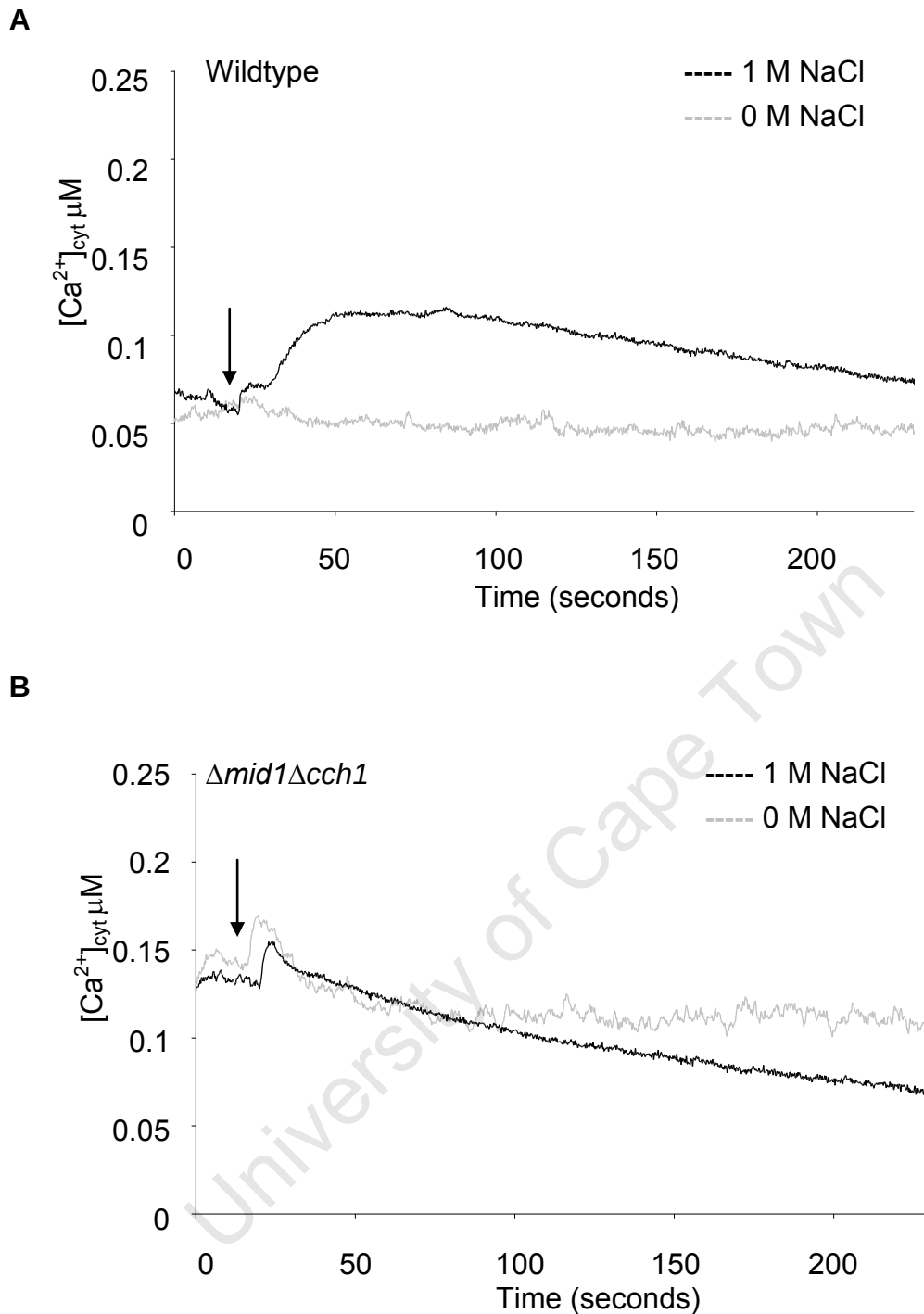
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## 3.2 Results

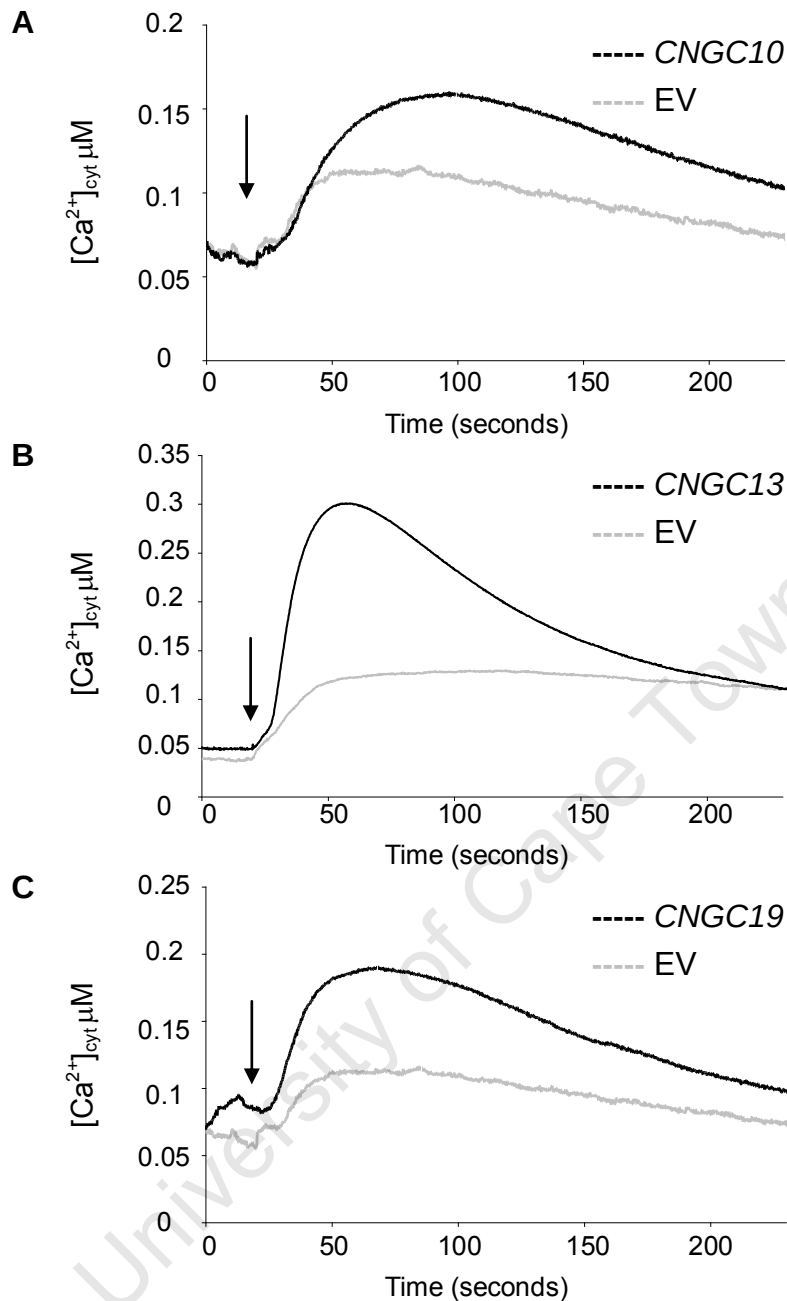
### 3.2.1 Ion transport ability of CNGC10, CNGC13 and CNGC19 in yeast

Uptake of ions via the roots is crucial for plant development. Three CNGCs that were identified from the public microarray database, Genevestigator (<https://www.genevestigator.ethz.ch>), as displaying expression in root tissue but have not been investigated for the ability to transport  $\text{Ca}^{2+}$  are *CNGC10*, *CNGC13* and *CNGC19*. *CNGC10* and *CNGC13* are the only two members of subgroup I that have not been investigated for the ability to transport  $\text{Ca}^{2+}$  and as both are expressed in root tissue they could be potential candidates for  $\text{Ca}^{2+}$  uptake during development. *CNGC19*, from subgroup IVa, is expressed in the roots throughout development and is hypothesised to be important for ion uptake as data from a salt stress microarray experiment indicates that *CNGC19* expression is significantly up-regulated in response to salinity stress (Maathuis 2006b).

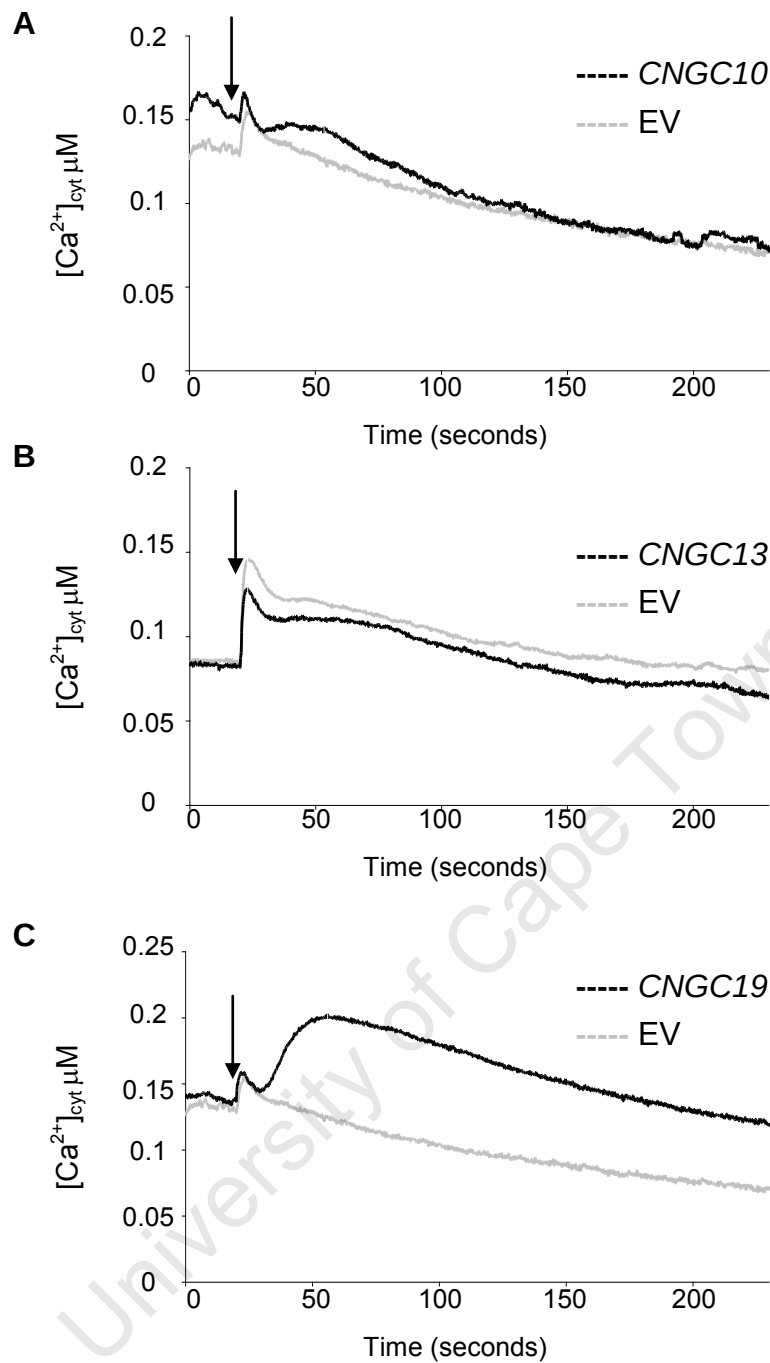
To determine whether these three CNGCs could transport  $\text{Ca}^{2+}$ , each *CNGC* was heterologously expressed in both wildtype and  $\Delta mid1\Delta cch1$  yeast strains for complementation analysis. A study by Matsumoto *et al.* (2002) showed that hypertonic stress treatment of a  $\Delta mid1\Delta cch1$  yeast strain harbouring the  $\text{Ca}^{2+}$  reporter construct apoaequorin, did not induce an increase in cytosolic  $\text{Ca}^{2+}$  levels ( $[\text{Ca}^{2+}]_{\text{cyt}}$ ) as displayed by the wildtype strain. As expected, in this study, the wildtype yeast strain displays a sustained increase in  $[\text{Ca}^{2+}]_{\text{cyt}}$  when treated with 1 M NaCl (Figure 1A) whereas the  $\Delta mid1\Delta cch1$  yeast strain displays only a very slight touch response which is seen when both salt and control treatments are administered (Figure 1B). Full length *Arabidopsis* *CNGC* cDNA was sub-cloned into the pYES2 galactose inducible yeast vector and expressed in wildtype and  $\Delta mid1\Delta cch1$  yeast lines (Fischer *et al.* 1997). As a negative control, an empty vector (EV) was introduced into the wildtype and mutant strains. Figures 2 and 3 display graphs of  $[\text{Ca}^{2+}]_{\text{cyt}}$  in both wildtype (Figure 2) and  $\Delta mid1\Delta cch1$  (Figure 3) strains that harboured either the EV or the specified *CNGC* construct. For simplicity, the negative control treatment, synthetic defined (SD) medium without NaCl, is not represented on the graphs. The peak  $[\text{Ca}^{2+}]_{\text{cyt}}$  after 1 M NaCl treatment of the wildtype strain expressing any of the *CNGC* genes was at least double the  $[\text{Ca}^{2+}]_{\text{cyt}}$  of the wildtype strain expressing an EV (Figure 2). This suggests that these three *CNGCs* have the ability to transport  $\text{Ca}^{2+}$ . A further experiment using the  $\Delta mid1\Delta cch1$  mutant yeast strain



**Figure 1.  $\Delta Mid1\Delta cch1$  does not display increased  $[Ca^{2+}]_{cyt}$  in response to salt stress.** Wildtype and  $\Delta mid1\Delta cch1$  yeast cells expressing the  $Ca^{2+}$  reporter protein, Aequorin, were treated with 1 M (black curve) or 0 M (grey curve) NaCl 20 seconds (black arrow) after luminescence measurements began. **(A)** Wildtype cells show a moderate increase in  $[Ca^{2+}]_{cyt}$  in response to 1 M NaCl. **(B)**  $\Delta Mid1\Delta cch1$  cells display similar  $[Ca^{2+}]_{cyt}$  levels in response to 1 M or 0 M NaCl. This experiment was repeated with similar results.



**Figure 2. Plant CNGCs affect the  $[Ca^{2+}]_{cyt}$  of wildtype yeast cells in response to salt stress.** Wildtype yeast expressing a plant CNGC (black curve: CNGC10 (A), CNGC13 (B), CNGC19 (C)) displayed higher levels of  $Ca^{2+}$  influx when treated with 1 M NaCl compared to yeast expressing an empty vector (EV, grey curve). The black arrow indicates the time of treatment. Experiments were repeated with similar results.



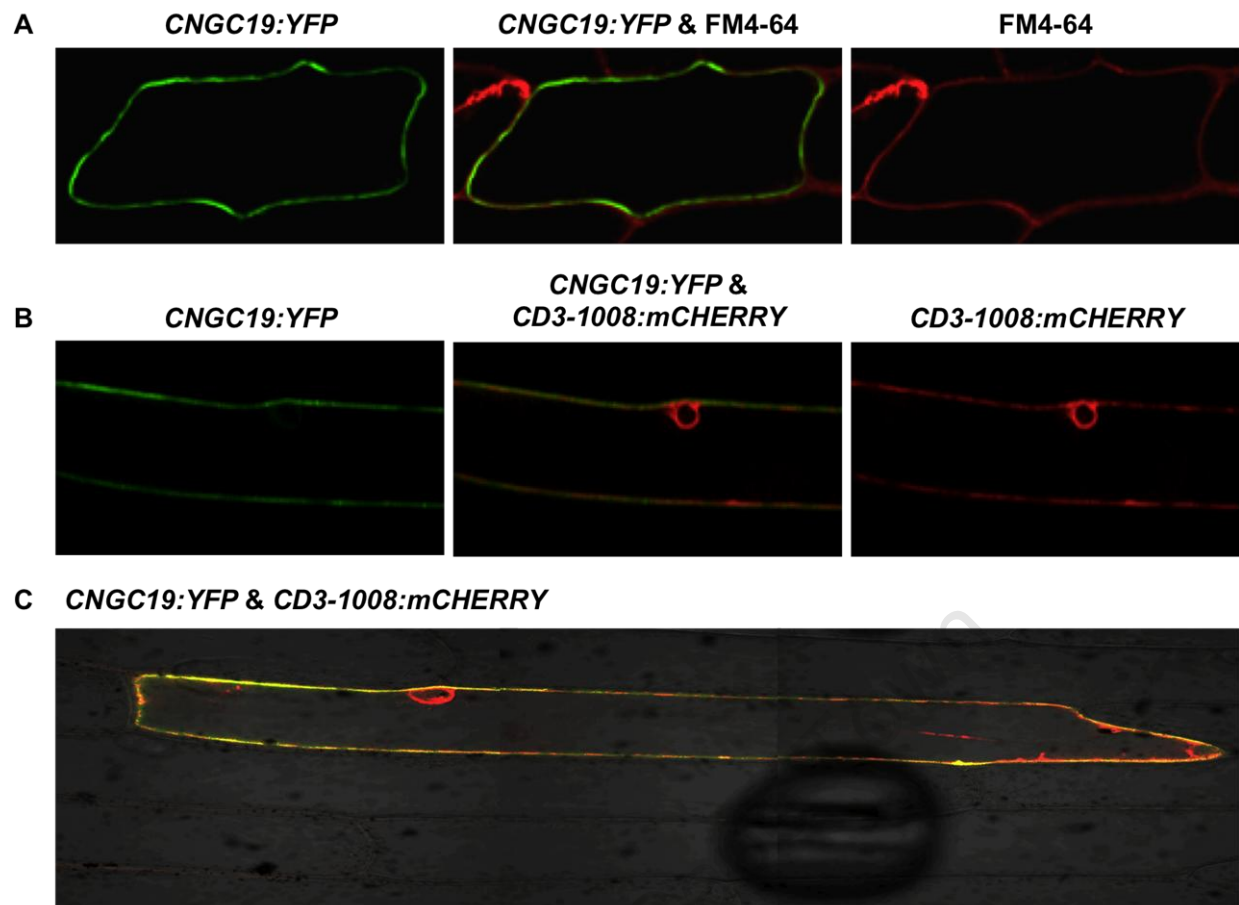
**Figure 3. *CNGC19* restores the  $[Ca^{2+}]_{cyt}$  influx of salt stressed  $\Delta mid1 \Delta cch1$  cells.**  $\Delta Mid1 \Delta cch1$  cells expressing plant CNGCs (black curve: *CNGC10* (A), *CNGC13* (B), *CNGC19* (C) or the empty vector (EV, grey curve) were treated with 1 M NaCl (arrow indicates time of treatment). *CNGC10* (A) and *CNGC13* (B) did not facilitate increased  $[Ca^{2+}]_{cyt}$  of salt stressed  $\Delta mid1 \Delta cch1$  cells whereas increased  $[Ca^{2+}]_{cyt}$  was seen in  $\Delta mid1 \Delta cch1$  cells expressing *CNGC19* (C). Experiments were repeated with similar results.

provides more insight into the transport ability and possible locations of these CNGCs.  $\Delta Mid1\Delta cch1$  expressing *CNGC19* reached a peak  $[Ca^{2+}]_{cyt}$  of 0.2  $\mu M$  which is approximately a 0.05  $\mu M$  increase from resting state (Figure 3C) whereas *CNGC10* (Figure 3A) and *CNGC13* (Figure 3B) failed to allow  $Ca^{2+}$  influx in response to NaCl treatment.

All three CNGCs tested display the ability to transport  $Ca^{2+}$  as increased  $[Ca^{2+}]_{cyt}$  is seen in wildtype yeast expressing these CNGCs but only *CNGC19* allows increased  $Ca^{2+}$  transport for  $\Delta mid1\Delta cch1$ . As *MID1* and *CCH1* are plasma membrane proteins, it could be hypothesised that only proteins which are specifically targeted to the plasma membrane can rescue this mutant (Iida *et al.* 1994, Locke *et al.* 2000). *CNGC10* has been shown to localise to the plasma membrane, ER and Golgi of plant cells (Christopher *et al.* 2007), however, it may be preferentially targeted to the ER and Golgi in yeast which allows it to transport  $Ca^{2+}$  from internal stores but not to rescue the  $\Delta mid1\Delta cch1$  phenotype which requires the influx of  $Ca^{2+}$  from external stores. The sub-cellular locations of *CNGC13* and *CNGC19* are unknown but it could be hypothesised that *CNGC19* has the ability to allow  $Ca^{2+}$  uptake in this mutant line because it is a plasma membrane protein whereas *CNGC13* is not.

### 3.2.2 Localisation of CNGC19

To determine the sub-cellular localisation of *CNGC19*, the *CNGC19* cDNA sequence was cloned into the pEarleyGate-101 (pEG-101) vector that harbours a CaMV 35S promoter and C-terminal yellow fluorescent protein (YFP) coding region (Earley *et al.* 2006). This construct was transiently expressed in onion epidermal cells by biolistic transformation and therefore fluorescence can only be seen in cells that have been successfully transformed. The C-terminally tagged *CNGC19:YFP* fusion protein was targeted to the plasma membrane as seen by fluorescence on the perimeter of the onion cell which co-localised with a plasma membrane specific dye, FM4-64 (Figure 4A). Further confirmation of the plasma membrane localisation of *CNGC19*, was found in a double-labelling experiment where onion cells were co-transformed with *CNGC19:YFP* and the plasma membrane marker construct, *CD3-1008* (Figure 4B). The *CD3-1008* construct contains the coding region of a plasma membrane aquaporin, *AtPIP2A*, fused to the N-terminus of the mCherry fluorescent protein and



**Figure 4. Transient expression of *CNGC19:YFP* in onion epidermal cells indicates that *CNGC19* is targeted to the plasma membrane.** The C-terminal YFP tagged *CNGC19* fusion protein accumulates on the margins of the onion cell and co-localises with the plasma membrane specific dye, FM4-64 (**A**), and the plasma membrane marker CD3-1008 (**B**, **C**). Nuclear localisation of the CD3-1008 marker could be an artefact of over-expressing the protein. The double fluorescence imaging of *CNGC19:YFP* and CD3-1008 was kindly done by Dr V. Cevik.

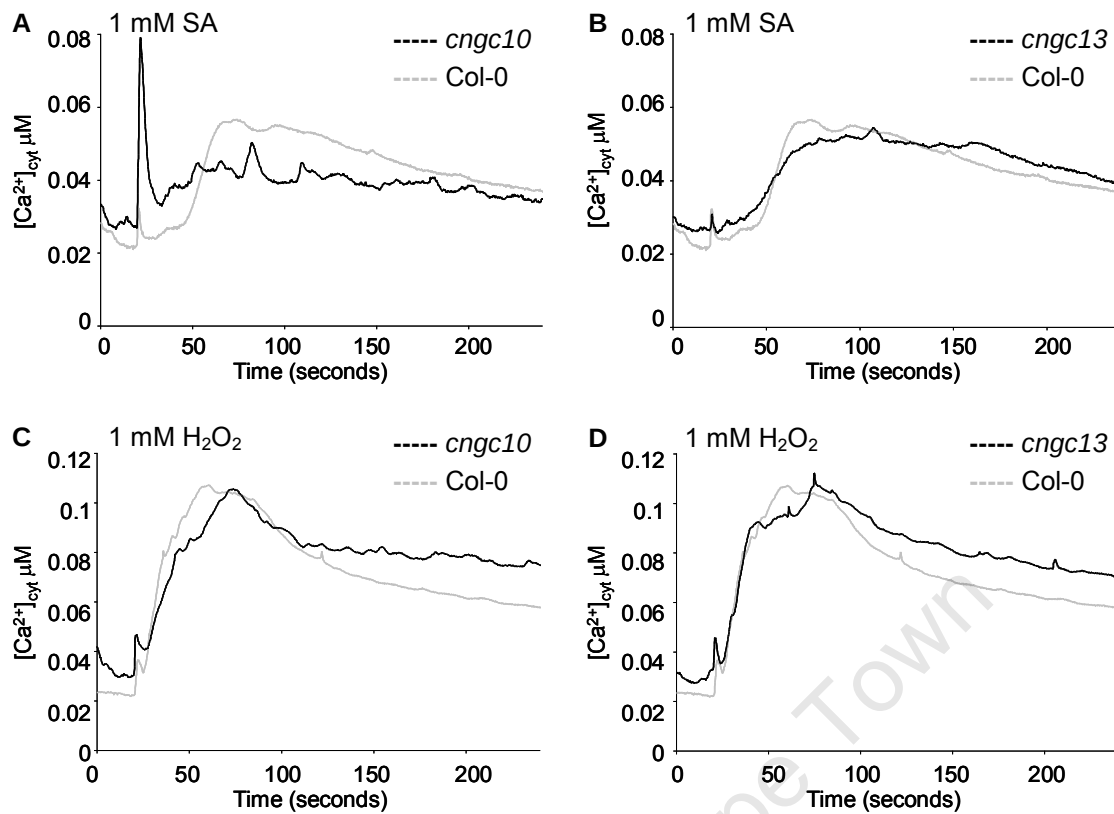
this protein fusion is driven by a 35S promoter (Nelson *et al.* 2007). *CNGC19:YFP* and *CD3-1008* displayed co-localisation at the plasma membrane which supports the hypothesis that CNGC19 rescues the  $\Delta mid1\Delta cch1$  yeast mutant because it is specifically targeted to the plasma membrane. Nuclear membrane localisation of the CD3-1008 construct could be due to mis-targeting related to over-expression of the protein. Further studies must be done to determine the sub-cellular localisation of CNGC13 and to confirm where these plant CNGCs are targeted in yeast cells.

### 3.2.3 Ion transport ability of CNGC10 and CNGC13 *in planta*

Previous studies of the  $Ca^{2+}$  transport ability of plant CNGCs in yeast have shown that three out of the four subgroup I members investigated were capable of transporting  $Ca^{2+}$  (Ali *et al.* 2006, Gobert *et al.* 2006, Urquhart *et al.* 2007). This study has shown that the remaining two members of this subgroup, *CNGC10* and *CNGC13*, also have the ability to transport  $Ca^{2+}$  in wildtype yeast. To investigate their  $Ca^{2+}$  transport ability *in planta*, homozygous T-DNA insertion lines of each CNGC was transformed with a *35S:Aeq* construct to test for altered  $Ca^{2+}$  signalling in response to hydrogen peroxide ( $H_2O_2$ ) and salicylic acid (SA) treatment. The stimulants used in this experiment are known to be produced by plants during defence signalling and are known to cause a  $Ca^{2+}$  response (Price *et al.* 1994, Lin *et al.* 2005, Lecourieux *et al.* 2006). As other members of subgroup I, *CNGC11* and *CNGC12*, are known to play a role in defence, we checked whether the  $Ca^{2+}$  signal would be altered by the absence of either *CNGC10* or *CNGC13* expression. Analysis of  $[Ca^{2+}]_{cyt}$  levels in ten day old *cngc10:Aeq* and *cngc13:Aeq* seedlings treated with 1 mM  $H_2O_2$  or 1 mM SA treatment indicates that the absence of these gene products do not affect  $H_2O_2$  or SA induced  $Ca^{2+}$  signalling (Figure 5). There was a bit of variation in the  $[Ca^{2+}]_{cyt}$  displayed by the three *cngc10:Aeq* line in response to SA and therefore the average  $[Ca^{2+}]_{cyt}$  level displayed is not significantly different to Col-0.

### 3.2.4 CNGC3 was unable to complement a potassium transport mutant of *Arabidopsis*

*CNGC10* was the first CNGC to display an ability to transport  $K^+$  *in planta* by rescuing the low  $K^+$  phenotype of *akt1-1* (Li *et al.* 2005). The AKT1 channel specifically transports  $K^+$  across membranes in the root tissue and a loss of this channel results in plants that cannot grow on media containing low levels of  $K^+$  (Hirsch *et al.* 1998).



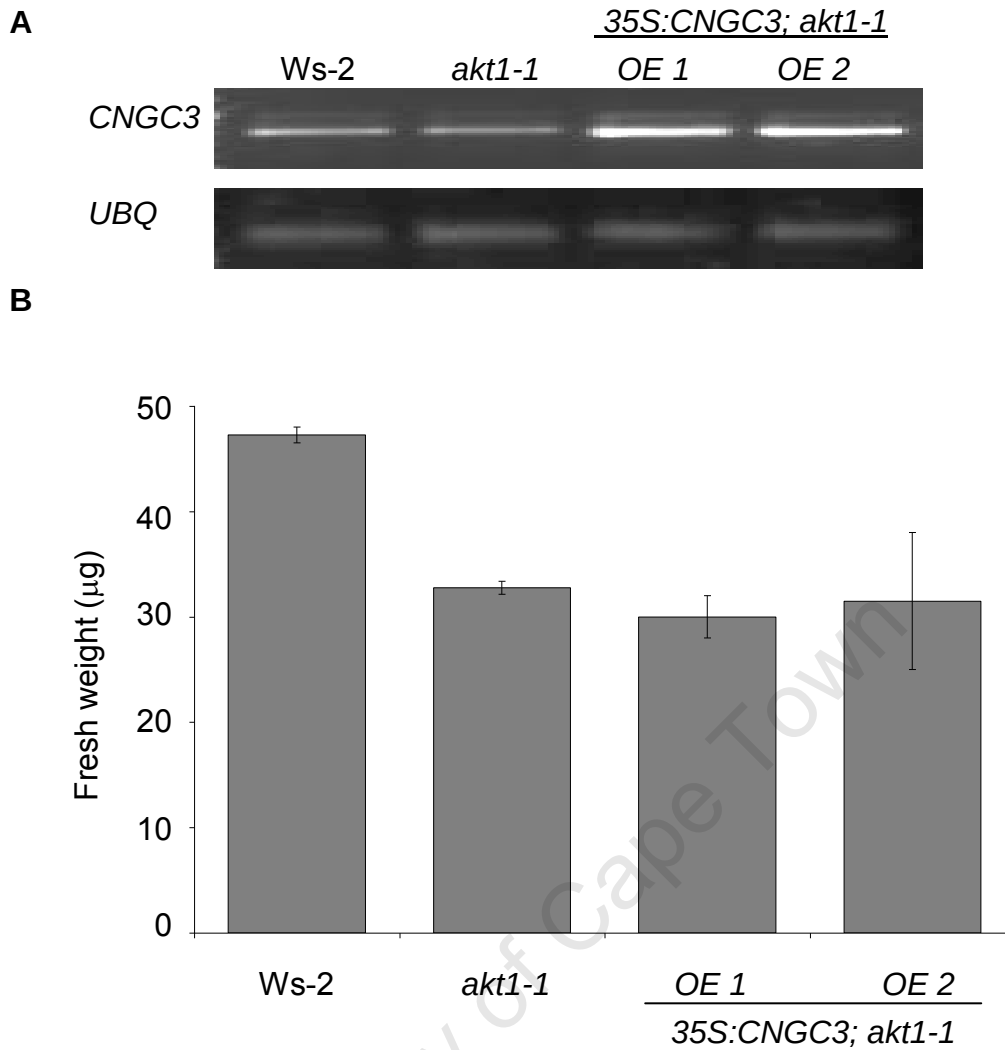
**Figure 5. *Cngc10* and *cngc13* T-DNA insertion mutants do not display altered defence related  $[Ca^{2+}]_{cyt}$  signalling.** Ten day old T-DNA insertion mutants & Col-0 seedlings expressing the  $Ca^{2+}$  reporter protein, Aequorin, were treated with 1 mM salicylic acid (SA, **A** & **B**) or hydrogen peroxide ( $H_2O_2$ , **C** & **D**) and monitored for aberrant  $[Ca^{2+}]_{cyt}$  due to the *cngc10* (**A** & **C**) or *cngc13* (**B** & **D**) mutation. Graphs display the average  $[Ca^{2+}]_{cyt}$  obtained from 3 seedlings in one experiment and neither T-DNA insertion mutant displayed significantly different  $[Ca^{2+}]_{cyt}$  compared to Col-0.

A previous study by Gobert *et al.* (2006) indicated that *CNGC3* could be involved in  $K^+$  transport as a *cngc3* T-DNA insertion mutant displayed increased tolerance on media with high  $K^+$  levels and expressing *CNGC3* cDNA in a  $K^+$  transport yeast mutant allowed the mutant to grow on media containing low  $K^+$  levels. To determine whether *CNGC3* could transport  $K^+$  *in planta*, *CNGC3* was over-expressed in an *akt1-1* background and the 35S:*CNGC3*; *akt1-1* plants were analysed for growth on media containing low  $K^+$ . Over-expressing lines of *CNGC3* were selected with semi-quantitative RT-PCR using *CNGC3* and *Ubiquitin* specific primers to monitor *CNGC3* expression relative to cDNA levels in each plant (Figure 6A). Over-expression of *CNGC3* did not compensate for the lack of  $K^+$  uptake by *akt1-1* as two over-expressing lines displayed similar fresh weight values to *akt1-1* when grown on low  $K^+$  (Figure 6B).

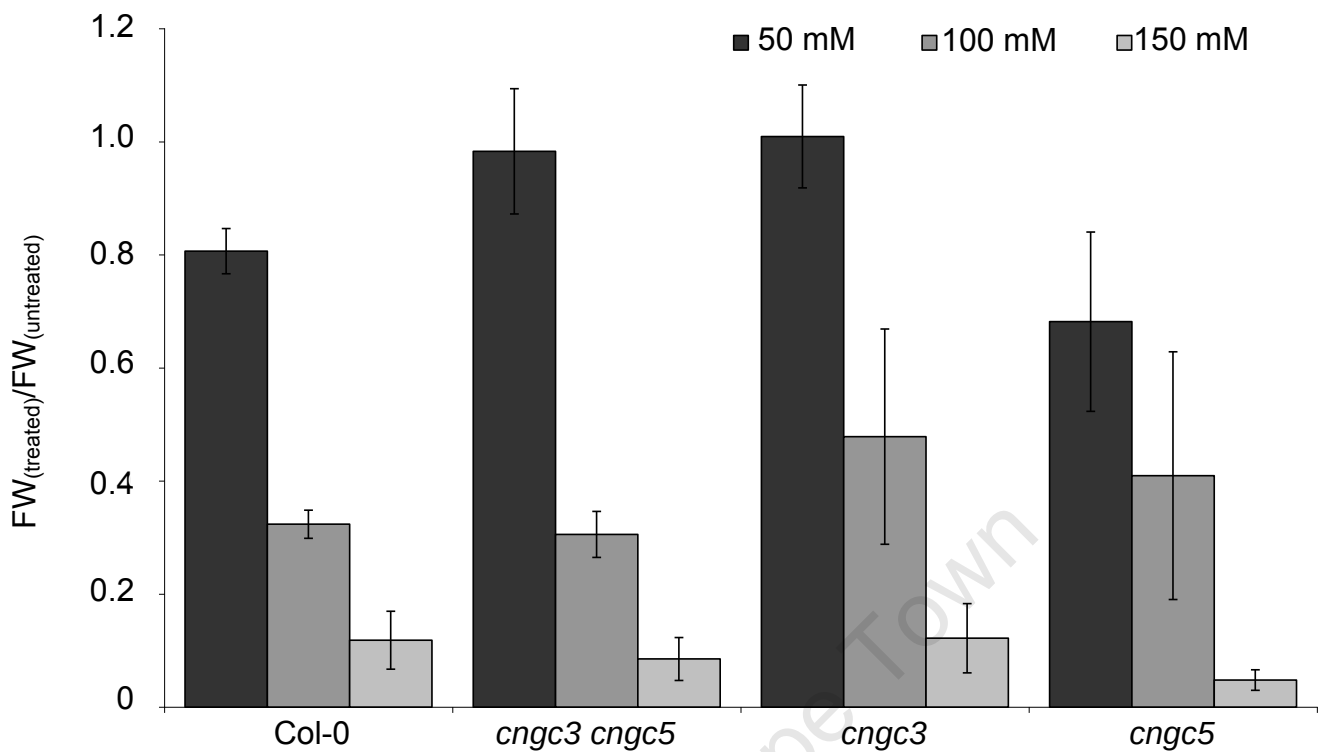
### 3.2.5 Salinity tolerance of *cngc* double T-DNA insertion mutants

Plant growth and development relies on effective nutrient uptake via roots to maintain correct ion homeostasis within plant cells. Uptake of ions such as  $K^+$  and  $Ca^{2+}$  is crucial for plant growth but excess ion uptake, especially of  $Na^+$  ions, can be detrimental. Altered germination and growth phenotypes of single *cngc* T-DNA insertion mutants subjected to atypical nutrient environments are not very conspicuous and therefore several double mutants were created to eliminate expression of two *CNGCs* which are normally expressed in root tissue. PCR based screening with gene and T-DNA-specific primers were used to identify or confirm double insertion mutants of *cngc3 cngc5*, *cngc5 cngc10* and *cngc8 cngc10*.

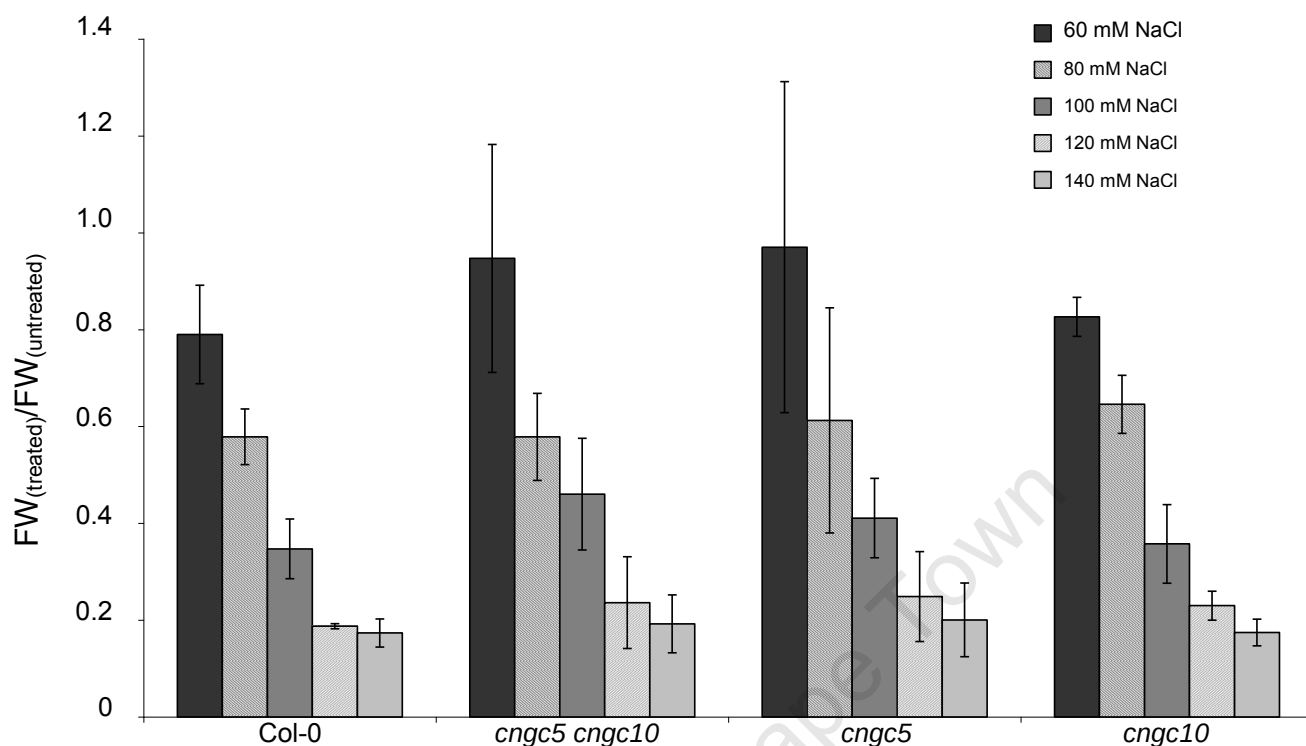
Plant growth assays on media containing increasing levels of NaCl were used to identify *CNGC* pairs that could be essential for salinity tolerance. Fresh weight measurements of two week old plants grown on media containing increasing NaCl concentrations were recorded and used to calculate the ratio of growth on media containing NaCl relative to growth on normal media containing no added NaCl. None of the double mutants displayed a significant difference in increased salinity tolerance when compared to both wildtype and the single insertion mutants (Figures 7 - 9). The *cngc3* single mutant displayed the increase in tolerance to NaCl as previously reported by Gobert *et al.* (2006) (Figure 7). Due to badly infected seed stocks of *cngc8*, only one set of data could be obtained (Figure 9).



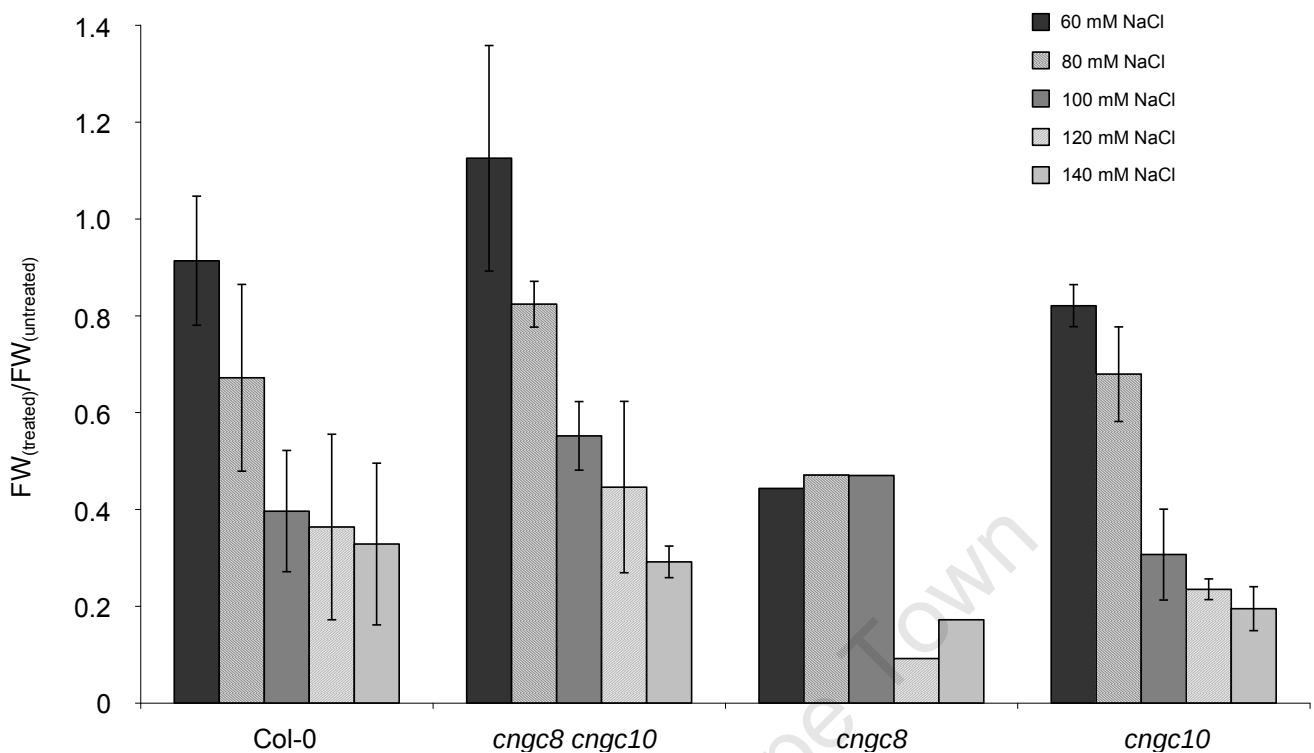
**Figure 6. Over-expression of CNGC3 does not complement the *akt1-1* low potassium phenotype.** *Akt1-1* lines over-expressing the *CNGC3* gene were selected with semi-quantitative RT-PCR using *CNGC3* and *ubiquitin (UBQ)* specific primers for gene expression and cDNA quantity controls respectively (A). Two over-expressing lines (*OE 1* and *OE 2*) were identified as displaying higher levels of *CNGC3* expression than *akt1-1* and its parental ecotype, *Ws-2* (A). Fresh weight measurements of seven day old seedlings indicated that *akt1-1* and the two over-expressing *35S:CNGC3; akt1-1* lines did not grow as well as *Ws-2* on media containing 10 µM KCl.



**Figure 7. *Cngc3 cngc5* double mutants do not display altered growth on media containing high levels of sodium chloride.** Seedlings of Col-0, single and double T-DNA insertion mutants were grown on media containing various concentrations of sodium chloride (NaCl) for 14 days and then weighed to determine fresh weight (FW). The bar graph represents the average ratio of fresh weight of plants grown on media containing NaCl ( $FW_{\text{treated}}$ ) relative to growth on media without NaCl ( $FW_{\text{untreated}}$ ). This experiment was repeated three times.



**Figure 8. Growth of *cngc5 cngc10* is similar to wildtype on media containing increasing sodium chloride levels.** The graph displays the average ratio of the fresh weight (FW) of seedlings grown on sodium chloride (NaCl) containing media ( $FW_{\text{treated}}$ ) relative to growth of seedlings on media without additional NaCl ( $FW_{\text{untreated}}$ ). The experiment was repeated three times.

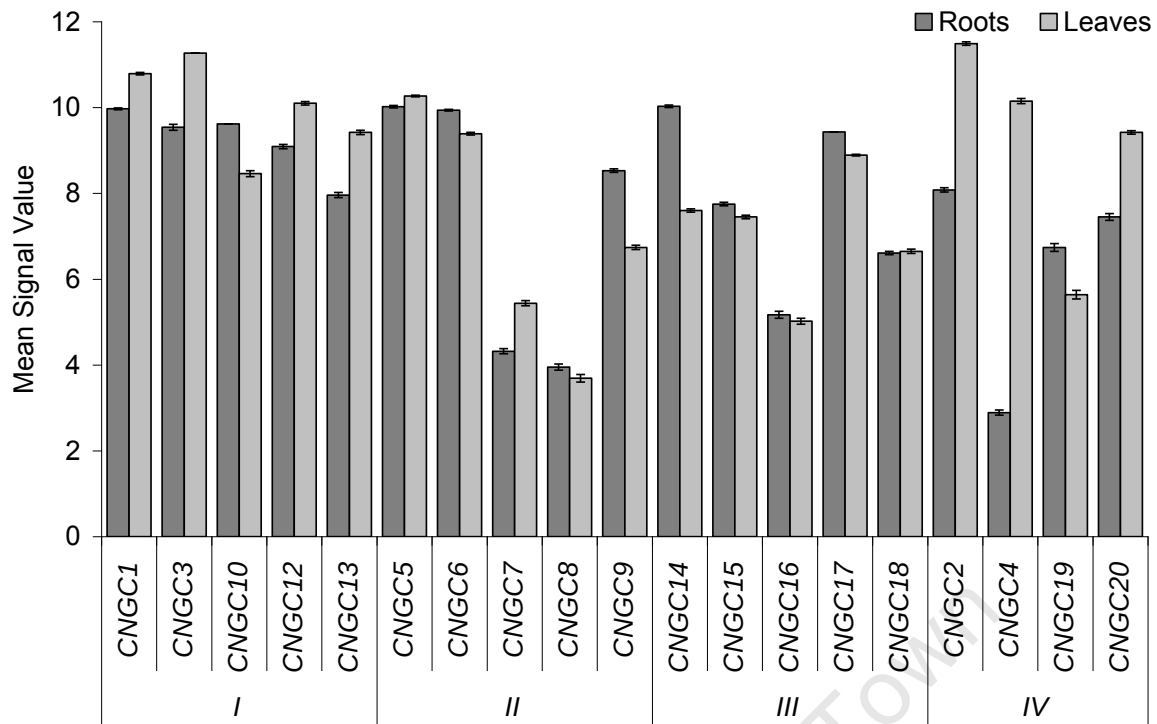


**Figure 9. *Cngc8* and *cngc10* single and double mutants did not display significantly different salinity stress phenotypes when compared to wildtype.** These graphs represent the average ratio of the fresh weight (FW) from plants grown on media containing various concentrations of sodium chloride (NaCl,  $FW_{\text{treated}}$ ) relative to growth on media containing no sodium chloride ( $FW_{\text{untreated}}$ ). The bar graphs for all lines, except *cngc8*, represent an average of three experiments and include standard error bars. The bar graph for *cngc8* represents results from one experiment.

### 3.2.6 Cyclic nucleotide regulated gene expression in leaves

The role of CNs in plants has been a controversial topic for many years (Newton and Smith 2004). The presence of a large ion channel family, which contains a CN binding domain, suggests that these second messengers are playing a role in a variety of signalling cascades in *Arabidopsis*. A study by Maathuis (2006a) investigated the effect of cyclic guanosine monophosphate (cGMP) treatment on the transcriptome of root tissue and found that many plant processes are affected. This study showed that six *CNGC* members (*CNGC6*, *CNGC12*, *CNGC15*, *CNGC16*, *CNGC17* and *CNGC20*) displayed over two-fold up regulation and one member, *CNGC2*, displayed two-fold down regulation in response to cGMP treatment. Thus far, this is the only study *in planta*, which shows CN regulation of *CNGC* expression in root tissue. *CNGCs* are, however, expressed at different levels during development and display unique patterns of expression and tissue specificity, indicating that they could be regulated slightly differently in other tissues. Figure 10 displays the mean signal value, obtained from Genevestigator microarray data, of all *CNGC* members' expression in root and adult leaf tissue. Both *CNGC2* and *CNGC4* display significantly higher expression in adult leaf tissue in comparison to roots (Figure 10) and loss of either *CNGC2* or *CNGC4* results in a *dnd* (*defence no death*) mutant, which displays constitutive induction of defence genes but is unable to induce the hypersensitive response when infected by avirulent biotrophic pathogens (Yu *et al.* 1998, Balague *et al.* 2003). This indicates that apart from *CNGCs* important role of ion uptake in the roots, *CNGCs* could also be involved in programmed cell death pathways, which take place in other plant organs, such as leaves. To complement data obtained through analysis of the CN regulated root transcriptome, transcripts affected by CN treatment of leaves was also determined.

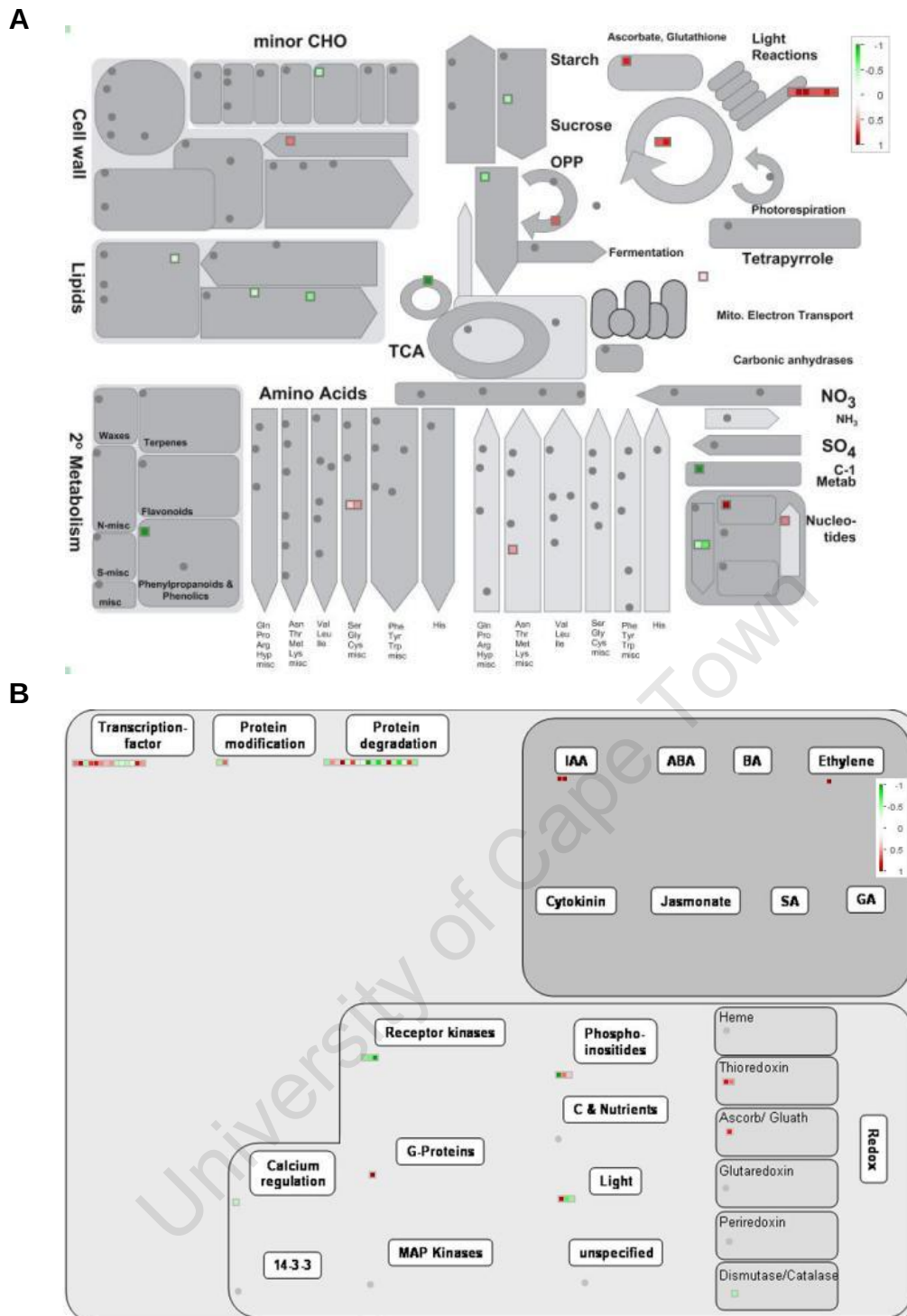
The abaxial surface of leaf seven from four week old plants was pressure infiltrated with either water or membrane permeable cGMP and harvested four and ten hours post treatment for microarray analysis using full genome CATMA arrays (Sclep *et al.* 2007). The same leaf from each plant was used to reduce variability between replicates. The MAANOVA statistical package (Wu *et al.* 2002) was used to normalise data and obtain an expression value per gene and time point from the 32 arrays, which included four biological replicates per treatment and time point, and four technical replicates with dye swaps.



**Figure 10. Expression of CNGCs in root and leaf tissue.** The mean signal value of CNGCs in root tissue and adult leaf tissue was obtained using microarray data, from at least 399 arrays, found on Genevestigator. CNGCs are clustered in their respective subgroups but one member of subgroup I, CNGC11, is not represented due to lack of microarray data. Standard error bars are depicted in the bar graph.

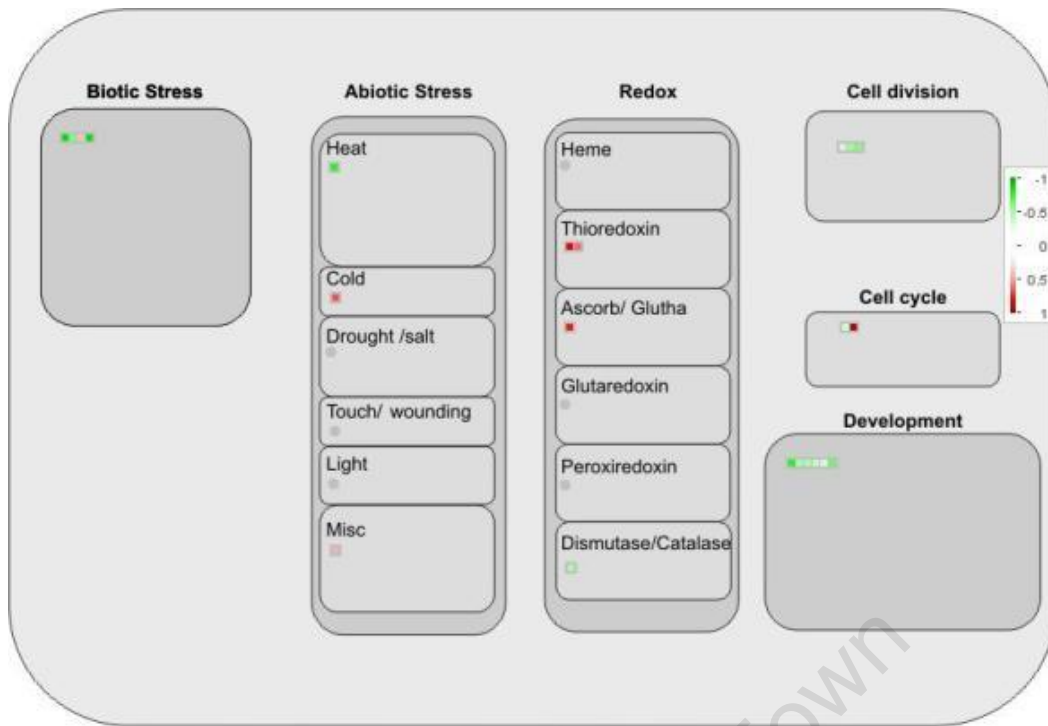
Two hundred and forty six genes displayed a significant difference, of at least 1.5 fold, in transcript level at the four hour time point whereas no transcripts were significantly affected at the ten hour time point (See Appendix A). The absence of transcripts affected by cGMP ten hours after treatment could indicate that cGMP signalling does not produce long lasting transcriptional effects in leaf tissue. The list of up- (129) or down- (117) regulated genes from the four hour time point were subjected to FATIGO analysis to find Gene Ontology (GO) annotations that were over- and under-represented in comparison to all other *Arabidopsis* genes (Al-Shahrour *et al.* 2004). Up-regulated transcripts associated with photosynthetic biological processes (GO:0015979) displayed over-representation as 9.84% of the 69 annotated up-regulated transcripts was linked with this term compared to 0.093% of genes from the rest of the *Arabidopsis* genome at a 95% confidence level. The over-representation of up-regulated genes involved in photosynthesis can be visualised when the significantly differentially expressed transcripts are displayed on the MAPMAN 'Overview of Metabolism' (Figure 11) (Thimm *et al.* 2004, Usadel *et al.* 2005). The MAPMAN program was also used to produce a visual representation of cGMP transcripts involved in an 'Overview of Regulation' and 'Response to Stress' (Figure 11 and 12). There were no significant under-represented GO biological process annotations for either up or down-regulated cGMP transcripts and none of the down-regulated transcripts displayed over-representation of any annotations. Over-representation of the GO molecular function annotation "ion transport" was identified for down-regulated genes. Down-regulation of cation, ammonium and sulphate transporters could be related to an increase in ion uptake through CN activated channels.

A comparison of data from the root and leaf microarray studies was performed and transcript levels of 27 genes were found to be significantly affected in both studies (Table 1). However, eight of the transcripts displayed contrasting regulation by cGMP in root and leaf tissue. None of the genes found to be significantly affected by cGMP treatment in both root and leaf tissue were part of the leaf specific, over-represented photosynthetic class. Unlike the transcriptome of roots, cGMP treatment of leaves did not affect expression of any *CNGC* genes nor genes involved in CN metabolism.

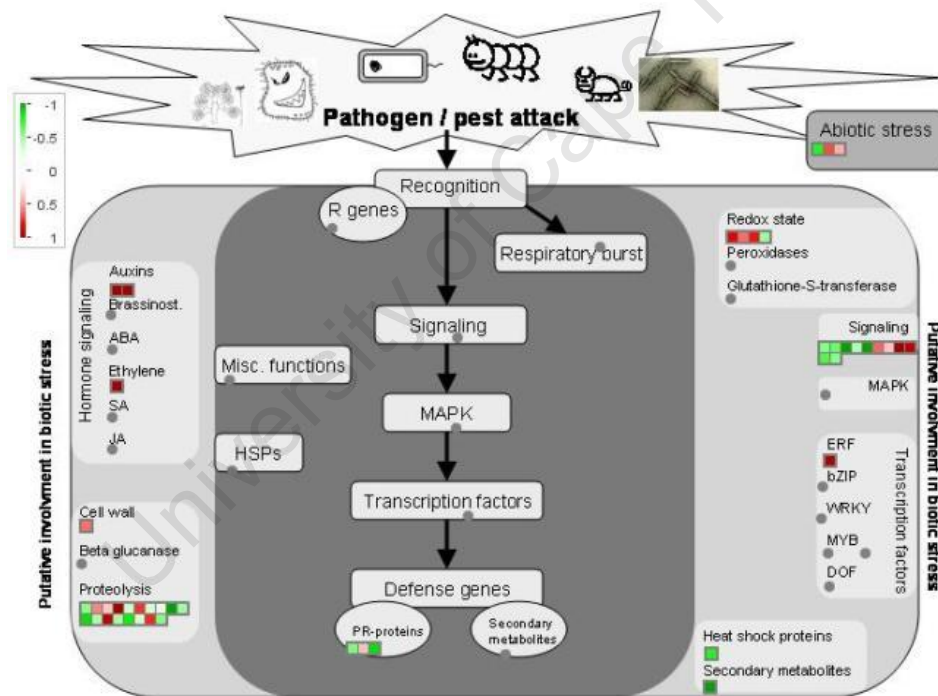


**Figure 11. A visual representation of processes that cGMP regulated transcripts are involved in.** MAPMAN analysis of the 246 significantly regulated cGMP genes in metabolism (**A**) and regulation (**B**) overview maps. Each square represents a gene involved in the particular process and they are colour-coded red or green to represent whether the gene is up- or down- regulated respectively.

A



B



**Figure 12. cGMP regulated transcripts are involved in stress responses. Red and green squares represent the up- and down- regulated cGMP transcripts involved in an overview of cell response (A) and biotic stress (B).**

| AGI       | Description                                                       | Root         | Leaf  |
|-----------|-------------------------------------------------------------------|--------------|-------|
| At3g04630 | <i>WDL1 (WVD2-LIKE 1)</i>                                         | up (2 hrs)   | up    |
| At2g31870 | <i>TEJ</i> , Poly (ADP-ribose) Glycohydrolase                     | up (5 hrs)   | down* |
| At1g05910 | <i>CDC48-related (Cell division cycle protein 48-related)</i>     | down (2 hrs) | down  |
| At1g70770 | Unknown protein                                                   | down (2 hrs) | down  |
| At5g01980 | Zinc finger (C3HC4-type RING finger) family protein               | down (2 hrs) | down  |
| At5g51280 | DEAD-box protein                                                  | up (5 hrs)   | down* |
| At1g49660 | Unknown protein; similar to cell death associated protein-related | down (2 hrs) | up*   |
| At3g09920 | <i>PIP5K9 (Phosphatidylinositol monophosphate 5 kinase)</i>       | up (2 hrs)   | down* |
| At2g31020 | Oxysterol-binding family protein                                  | up (5 hrs)   | down* |
| At1g43560 | <i>ATY2 (Arabidopsis thioredoxin y2)</i>                          | up (2 hrs)   | up    |
| At4g15530 | <i>PPDK (PYRUVATE ORTHOPHOSPHATE DIKINASE)</i>                    | up (2 hrs)   | down* |
| At1g22015 | <i>DD46</i> ; transferase                                         | down (2 hrs) | down  |
| At1g09640 | <i>Elongation factor 1B-gamma</i>                                 | down (both)  | down  |
| At1g07510 | <i>FTSH10 (FtsH protease 10)</i>                                  | down (2 hrs) | down  |
| At1g78000 | <i>SULTR1;2 (SULFATE TRANSPORTER 1;2)</i>                         | down (2 hrs) | down  |
| At1g63360 | Disease resistance protein (CC-NBS-LRR class)                     | down (2 hrs) | down  |
| At1g28340 | Leucine-rich repeat family protein                                | down (2 hrs) | down  |
| At3g47570 | Leucine-rich repeat transmembrane protein kinase                  | up (2 hrs)   | down* |
| At3g61830 | <i>ARF18 (AUXIN RESPONSE FACTOR 18)</i>                           | down (2 hrs) | down  |
| At4g13510 | <i>AMT1;1 (AMMONIUM TRANSPORT 1)</i>                              | down (2 hrs) | down  |
| At4g15780 | Synaptobrevin-related family protein                              | up (5 hrs)   | up    |
| At5g15980 | Pentatricopeptide (PPR) repeat-containing protein                 | up (2 hrs)   | down* |
| At3g09570 | Unknown protein                                                   | down (2 hrs) | down  |
| At3g08880 | Unknown protein                                                   | up (both)    | up    |
| At2g26340 | Unknown protein                                                   | up (2 hrs)   | up    |
| At4g24330 | Unknown protein                                                   | down (2 hrs) | down  |
| At5g48790 | Unknown protein                                                   | up (2 hrs)   | up    |

**Table 1. Transcripts displaying altered regulation by cGMP treatment in both root and leaf tissue.** The AGI code and gene description of twenty-seven cGMP regulated transcripts common to root and leaf tissue are listed with an indication of whether the gene was up- (up) or down-regulated (down) in response to cGMP treatment. As two time points were used for the root tissue analysis, the time point when a change in transcript level was observed is stated in brackets (hrs = hours; both = regulated at both time points). Altered regulation in leaf tissue was analysed at four hours post cGMP treatment and statistically significant ( $P < 0.05$ ) changes in expression are shown. Transcripts marked with asterisks (\*) show contrasting regulation in root and leaf tissue.

### 3.3 Discussion

A few members of the *Arabidopsis* CNGC family have been characterised to gain a better understanding of their function, expression and regulation *in planta*. There are many difficulties involved in studying a large family of integral membrane proteins ranging from functional redundancy *in planta* to unpredictable expression of the ion channel in a heterologous system. It is important to use a variety of methods to obtain as much information as possible which can be pieced together to give a clearer picture of a channel's function. The results presented in this chapter were obtained from reverse genetics approaches to identify CNGCs involved in salinity stress and those capable of transporting  $\text{Ca}^{2+}$  in yeast and plant systems. The response of plants to CNs was also explored using a transcriptomics approach.

*Arabidopsis* CNGCs lack the 'GYG'  $\text{K}^+$  selective signature in the pore region and therefore are thought to be non-selective cation channels (Hua *et al.* 2003). Most of the CNGCs characterised thus far display the ability to transport cations in a non-selective manner but CNGC2, which contains a unique selective filter, 'AND', in its predicted pore region, transports  $\text{K}^+$  preferably to  $\text{Na}^+$  (Hua *et al.* 2003). Studies show that the asparagine (N) and aspartic acid (D) residues confer the  $\text{K}^+$  selectivity of CNGC2 proteins (Hua *et al.* 2003). Clustal alignment of the putative pore regions of other CNGCs indicates that the predicted pore region of CNGC19 has a fairly unique selective filter, 'AGN', whereas CNGC10 and CNGC13 have the 'GQN', selectivity filter that is common to half of the CNGCs. These filters should allow the non-selective passage of  $\text{Ca}^{2+}$ ,  $\text{Na}^+$  and  $\text{K}^+$  ion through the pore region of CNGCs. To investigate  $\text{Ca}^{2+}$  transport capabilities of CNGC10, CNGC13 and CNGC19, which are expressed in root tissue, complementation analysis of the  $\Delta\text{mid1}\Delta\text{cch1}$  yeast mutant, which lacks plasma membrane-bound high affinity  $\text{Ca}^{2+}$  channels and displayed a higher basal level of cytosolic calcium, was performed. CNGC19 cDNA was used to successfully complement both wildtype and  $\Delta\text{mid1}\Delta\text{cch1}$  yeast lines whereas expression of CNGC10 and CNGC13 cDNA only increased  $[\text{Ca}^{2+}]_{\text{cyt}}$  in the wildtype line exposed to salinity stress. It is apparent that all three CNGCs are capable of transporting  $\text{Ca}^{2+}$  but they could be transporting it from different  $\text{Ca}^{2+}$  stores. Sub-cellular localisation of CNGC13 has not yet been investigated but CNGC10 localises to a few organelles *in planta* and therefore could be preferentially targeted to the ER or Golgi in yeast to release  $\text{Ca}^{2+}$  from internal stores (Christopher *et al.* 2007). A

previous study showed that CNGC10 can transport  $K^+$  (Li *et al.* 2005) and therefore it could also be hypothesised that CNGC10 and CNGC13 activate the endogenous putatively voltage-gated CCH1 channel by altering the membrane potential through  $K^+$  permeation, thereby allowing more  $Ca^{2+}$  influx in the wildtype yeast cells.

Sub-cellular localisation of the YFP tagged CNGC19 protein indicates that it is targeted to the plasma membrane of onion cells as CNGC19:YFP co-localised with a plasma membrane specific dye and the *CD3-1008* plasma membrane marker protein. Plasma membrane targeting of *CNGC19:YFP* in onion cells and *CNGC19*'s ability to complement a yeast mutant lacking two plasma membrane proteins provides strong evidence that CNGC19 is a plasma membrane bound protein *in planta*. Some studies indicate that CN treatment is needed for activation or to enhance the activity of plant CNGCs expressed in yeast and one study has shown that deletion of the calmodulin binding domain was required to prevent channel inhibition by the binding of a yeast calmodulin isoform (Mercier *et al.* 2004). No CN treatment or deletion of the CNGC19 calmodulin binding domain was needed to allow *CNGC19* mediation of  $Ca^{2+}$  fluxes in this specific assay. It could be hypothesised that the CNGC19 calmodulin binding domain does not have high affinity for any of the yeast calmodulin isoforms and the nature of this particular assay allows yeast cells to supply sufficient amounts of CNs which could activate CNGC19, if required.

Heterologous expression of a plant CNGC in yeast is a good tool to test which ions it is capable of transporting but the information obtained cannot be used in isolation to predict a role for that particular CNGC *in planta*. Over-expressing the protein could cause membrane mis-targeting and erroneous regulation of the channel could be caused by native yeast CN and CaM molecules. Electrophysiological assays or analysis of these channels *in planta* (complementation analysis or a reverse genetics approach) would impart better evidence of its ion transport capabilities, provided there is no functional redundancy between some members. Complementation of the *Arabidopsis*  $K^+$  mutant, *akt1-1*, by over-expression of *CNGC10* is the most convincing evidence that CNGC10 transports  $K^+$  *in planta* (Li *et al.* 2005). A previous study by Gobert *et al.* (2006) indicated that *CNGC3* may play a role in  $K^+$  uptake and therefore a similar approach was taken to over-express *CNGC3* in the *akt1-1* background. Both over-expressing lines displayed an *akt1-1* phenotype, which

suggests that *CNGC3* is not a key  $K^+$  channel in root tissue. The lack of complementation could be due to the fact that *CNGC3* protein levels may not correlate to the increased *CNGC3* transcript displayed in these lines. Therefore, *CNGC3* protein levels would need to be determined to confirm if the lack of complementation is due to lack of *CNGC3* protein expression. Instead of over-expressing a specific *CNGC*, T-DNA insertion lines disrupting the expression of one or more *CNGCs* could also be investigated for difference in ion transport or uptake. Previous studies show that only a few *cngcs* display robust phenotypes when exposed to various ionic stresses, such as the increased sensitivity of *cngc2* to high  $Ca^{2+}$  concentrations (Chan *et al.* 2003). One hypothesis for the lack of phenotypes from single *cngc* mutants is that plant *CNGCs* function as heterotetramers, like their animal homologues (Zhong *et al.* 2002) and may display functional redundancy. To try and overcome this problem, double T-DNA insertion mutants of *CNGCs* expressed in root but from different subgroups were created and exposed to increasing levels of  $Na^+$ . None of the double mutants displayed consistently significant growth differences to wildtype or the single mutant lines. This could be due to redundancy in more than two genes or the *CNGC* pair that was chosen is not involved in the same process. Analysis of microarray data from salinity stressed tissue could be used to identify which *CNGC* genes are co-expressed to potentially give a better indication of which *CNGCs* to use in double or triple knockout experiments.

The use of *cngc* T-DNA insertion lines expressing the  $Ca^{2+}$  reporting protein, *Aeq*, allows one to analyse whether the lack of that particular *CNGC* affects  $[Ca^{2+}]_{cyt}$  in response to various treatments. Chemicals associated with defence signalling were chosen to treat *cngc10:Aeq* and *cngc13:Aeq* as they are closely related to *CNGC11* and *CNGC12* which are known to be involved in defence. Both *Aeq* lines did not display altered  $[Ca^{2+}]_{cyt}$  when treated with 1 mM  $H_2O_2$  or 1 mM SA. This could be due to functional redundancy or that *CNGC10* and *CNGC13* are not involved in these signalling pathways. Further analysis using different concentrations of these chemicals could be performed before excluding these channels as a link between  $Ca^{2+}$  and defence signalling. It would be interesting to test both single and double T-DNA insertion *cngc:Aeq* lines with various treatments, such as osmotic stress,

pathogen infection and ionic stress, using a luminometer or low-light imaging camera to follow  $[Ca^{2+}]_{cyt}$  *in planta*.

There are various difficulties associated with studying ion channels as over-expression could cause mis-targeting and functional redundancy prevents any phenotypic analysis of single knockout mutants. Electrophysiological techniques are great for obtaining functional characterisation of single ion channels but previous studies have indicated that it was difficult to obtain good expression of *Arabidopsis* CNGCs (Leng *et al.* 2002, Balague *et al.* 2003). Thus far, electrophysiological studies have shown that CNGC1, CNGC2, CNGC4 and CNGC10 allow CN regulated  $K^+$  transport (Leng *et al.* 2002, Balague *et al.* 2003, Christopher *et al.* 2007) but heterologous expression of CNGCs for complementation analysis of various ion transport yeast mutants have been more widely used. These assays have provided most of the information regarding ion transport capabilities of CNGCs but there are some disadvantages regarding localisation and regulation of CNGCs in yeast. In this study, the combination of yeast complementation and a  $Ca^{2+}$  reporter assay provided information which could be used to propose both the function and localisation of the CNGCs tested. Transient expression of *CNGC19:YFP* in onion epidermal cells supported the data obtained from the yeast complementation analysis and shows that CNGC19 is yet another CNGC which localises to the plasma membrane. Most of the previous studies used transient expression of GFP tagged CNGCs in yeast, onion or tobacco cells to determine sub-cellular localisation and eight of the eleven CNGCs tested were targeted to the plasma membrane (Ali *et al.* 2006, Gobert *et al.* 2006, Chang *et al.* 2007, Christopher *et al.* 2007, Urquhart *et al.* 2007). CNGCs which do not display plasma membrane localisation are: CNGC7, CNGC8 and CNGC16, as they are targeted to endomembrane structures of pollen tubes (Chang *et al.* 2007). All plant processes require a well-orchestrated signalling cascade to transmit a stimulus into a response by means of an ion flux. CNGCs are good candidates to co-ordinate these signals as they are expressed in various tissues and membranes to allow ion transport of a specific amplitude. The regulation of these channels could also play a vital role in managing signals from  $Ca^{2+}$  and CN second messenger cascades.

A recent study used a microarray approach to identify downstream targets of CN signalling in root tissue (Maathuis 2006a). Analysis of the *Arabidopsis* root transcriptome, two and five hours post cGMP treatment indicated that many different biological processes, identified using the GO search tool on the TAIR website, could be affected by increased levels of this secondary signalling molecule. However, the significance of this data is difficult to interpret as analysis of over- and under-represented terms in these differentially expressed genes compared to the rest of the genome was not performed. As *CNGCs* and other genes, which contain putative CN binding domains, are also expressed in aerial organs of the plant, a similar approach was taken to identify transcripts in leaf tissue affected by CN signalling. The decision to harvest tissue at four and ten hours post treatment was based on the fact that a previous microarray experiment found no significantly regulated genes, one hour post treatment (*Pers. comm.* Dr K Denby) and the Maathuis paper (2006a) identified genes that were significantly up-regulated at both the two and five hour time points. Therefore an intermediary time point of four hours and a later time point of ten hours were chosen for analysis. Analysis of the microarray data using the MAANOVA statistical package identified no transcripts being significantly regulated ten hours post treatment and less than 1% of the total genes on the array as being significantly affected four hours after cGMP treatment. Analysis of the number of transcripts regulated by cGMP in both root and leaf tissue at the various time points indicates that cGMP signalling has a transient effect on the plant transcriptome as observed by the decreasing amount of significantly regulated transcripts over time. The highest number of transcripts affected by cGMP treatment was seen in root tissue harvested two hours post treatment and this was more than double the amount identified in the five hour root tissue samples. Similarly, of the two time points tested in cGMP treated leaf tissue, only the earlier samples displayed significantly regulated transcripts. A comparison of data from the four hour time point and the combined two and/or five hour time points from the Maathuis paper, identified twenty-seven transcripts common to both data sets with eight displaying contrasting regulatory profiles. These transcripts are therefore very strong candidates for regulation by cGMP with some displaying tissue specific regulation.

MAPMAN presentation of cGMP regulated transcripts involved in the cell's response to various stresses produced a good visual representation of the variety of CN

regulated transcripts involved in plant defence, albeit the fact that a significant GO term associated with defence was not identified through FATIGO analysis. cGMP regulated transcripts whose protein products could be playing a role in defence include anti-oxidants such as *catalase 3* (CAT3) and *glutathione peroxidase* (ATGPX10), two classes of disease resistance proteins (TIR-NBS-LRR and CC-NBS-LRR), reversion to ethylene 1 (RTE1) and members of the cytochrome P450 and leucine-rich repeat families (Resnick *et al.* 2006, Benschop *et al.* 2007, Tan *et al.* 2007, Chang *et al.* 2009). The defensin gene, *PDF1.2*, and senescence associated gene, *SAG101*, were down-regulated by cGMP treatment. Down-regulation of defence and program cell death related genes by cGMP treatment is mirrored by increased expression of defence genes and cell death pathways due to the loss of *CNGC* expression, suggesting a linkage between cGMP and these channels. The *cngc4* mutant displays constitutive *PDF1.2* expression and spontaneous senescent-like lesions in the absence of pathogen infection (Balague *et al.* 2003, Jurkowski *et al.* 2004). The majority of the published literature provides information of *CNGCs* involvement in defence against biotrophic pathogens and therefore, the next chapter explores the role of *CNGC19* in plant defence.

## Chapter 4

### Cyclic Nucleotide Gated Channels Required for Full Defence Against *B. cinerea*

#### 4.1 Introduction

Plants have both preformed and induced defence responses which are utilised in accordance to the life-style of the invading pathogen. Pathogens will either exploit or destroy the plant's cellular machinery to obtain nutrients for its proliferation within the host. Therefore, the host plant needs to recognise the type of pathogen by the specific proteins or elicitors that the pathogen produces at the site of infection, and deploy the appropriate counter-attack strategy. The induced defence signalling pathway has been scrutinized for many years (Metraux 2001, Durrant and Dong 2004) but the intricate signalling cascade is still being pieced together. What is known thus far is that an effective defence response is dependent on the ability of the plant to recognise the invading pathogen so that it can initiate the co-ordinated and overlapping action of the relevant defence genes and their protein products (Mishina and Zeier 2007). An activated defence signalling pathway results in accumulation of pathogenesis-related proteins and secondary metabolites, which correlate with the type of invading pathogen as they are tactically important to limit disease caused by that pathogen (Glazebrook 2005). The secondary metabolite, camalexin, is a good example of a defence strategy specifically required for full defence against *Botrytis cinerea* as it plays no role in *Pseudomonas syringae* defence (Ferrari *et al.* 2003). Conversely, a programme cell death (PCD) pathway, known as the hypersensitive response (HR), effectively limits the spread of avirulent strains of *P. syringae* but these dead cells could actually facilitate *B. cinerea* infection (Govrin and Levine 2000).

Numerous studies have identified genes encoding proteins that are necessary for the correct functioning of a defence signalling pathway. These include transcription factors, kinases, lipases and some of unknown function (Glazebrook 2001). Ion channels have also been shown to play a role in defence and include some members of the cyclic nucleotide gated channel (CNGC) family (Clough *et al.* 2000, Balague *et al.* 2003, Yoshioka *et al.* 2006). Studies show that cyclic nucleotide (CN) signalling (Bindschedler *et al.* 2001), calmodulin (CaM) signalling (Chiasson *et al.* 2005) and

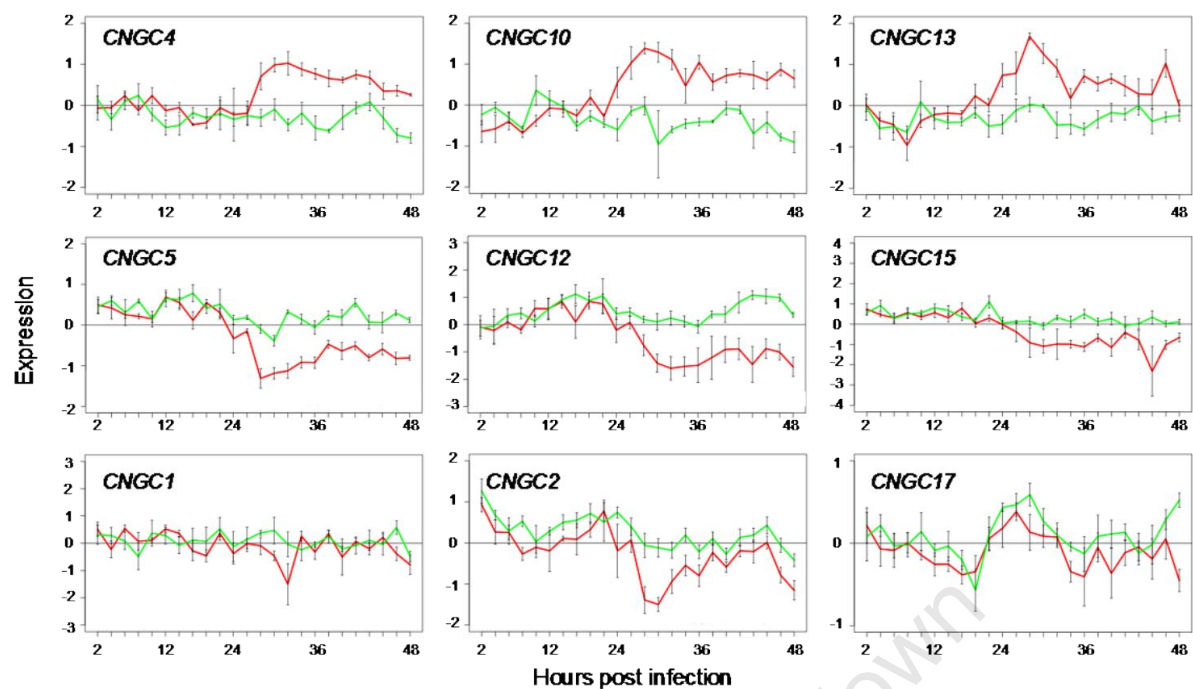
ion flux (Jabs *et al.* 1997b) are early responses required for effective defence. The CNGCs would be able to assimilate all three of these signalling events required for full defence against pathogen attack as they have both CN and CaM binding (CNB, CaMB) sites which may regulate the flow of ions through the pore region. The role of ion transporters is suggested to be critical for the early activation of the defence response to pathogen attack, as many studies have shown that altered regulation of either calcium ( $\text{Ca}^{2+}$ ) or potassium ( $\text{K}^+$ ) transport in a variety of plant species results in altered susceptibility (Lecourieux *et al.* 2006, Baxter *et al.* 2008).

Four CNGCs (CNGC2, CNGC4, CNGC11 and CNGC12) have been shown to play a role in defence through mutant characterisation (Clough *et al.* 2000, Balague *et al.* 2003, Yoshioka *et al.* 2006). The loss of a single CNGC results in a defence-related phenotype, which demonstrates that these members of the CNGC family have a specific non-redundant role in defence. This is also backed up by the unique spatio-temporal expression patterns of the individual CNGC genes and corroborates the hypothesis that each member plays a specific role in various signalling cascades for plant development and adaptation to abiotic and biotic stresses. Analysis of publicly available microarray data and publications indicate that the subgroup divisions also appear to correlate with expression profiles and function. Subgroup IVb (CNGC2 and CNGC4) is the most divergent group of CNGCs and mutating either member of subgroup IVb causes both developmental and defence-related phenotypes. *Cngc2* and *cngc4* plants exhibit stunted growth and increased resistance to a variety of pathogens (Yu *et al.* 1998, Clough *et al.* 2000, Balague *et al.* 2003). Both mutants display a *defence-no-death* (*dnd*) phenotype whereby they maintain resistance to avirulent biotrophic pathogen attack but fail to induce a HR. This study identifies other CNGCs that may be involved in the plant defence signalling pathway activated in response to *B. cinerea* infection.

## 4.2 Results

### 4.2.1 Expression profile of CNGCs during *B. cinerea* infection

Analysis of data in public microarray databases, such as Genevestigator (<https://www.genevestigator.ethz.ch/gv/index.jsp>) and Bio-Array Resource (BAR, <http://bar.utoronto.ca/>), together with microarray data obtained from a *B. cinerea* time-course performed in our group (Denby, manuscript in preparation) identified CNGCs which are up-regulated in response to *B. cinerea* infection. These were considered potential players in the defence pathway. The *B. cinerea* microarray data found on Genevestigator and BAR displays the log-ratio of expression from infected versus uninfected tissue of Col-0 leaves harvested forty-eight hours post infection thereby only giving a snapshot of a very rapid signalling pathway. The CNGCs displaying significant ( $P < 0.05$ ) differences in expression at forty-eight hours post infection are: *CNGC4*, *CNGC5*, *CNGC7*, *CNGC10*, *CNGC13*, *CNGC15*, and *CNGC16*. However, analysis of gene expression at one time point may result in some genes being overlooked as CNGCs are hypothesised to be involved in the early signalling events of the defence pathway. The time-course microarray data shows the normalised expression of every gene at two hour intervals from zero to forty-eight hours post infection and therefore one can identify genes which are significantly regulated before or after that time point. The time-course microarray data indicates that *CNGC4*, *CNGC10* and *CNGC13* are up-regulated whereas *CNGC5*, *CNGC12* and *CNGC15* are down-regulated from approximately twenty-four hours after *B. cinerea* infection and remain at those levels till forty-eight hours post infection (Figure 13). The time-course data also shows transient differences in expression of *CNGC1* and *CNGC2*. *CNGC2* transcript levels start dropping at about twenty-four hours post-infection and returns to wildtype levels approximately ten hours later whereas *CNGC1* displays a sharp decrease in transcript levels thirty hours post infection (Figure 13). These CNGCs could be playing an important role in *B. cinerea* defence but are not identified in the snapshot microarray experiments due to the time point that was used. Some CNGCs, such as *CNGC17*, displayed no difference in expression throughout the time-course which suggests that only certain CNGCs may be required for *B. cinerea* defence (Figure 13).

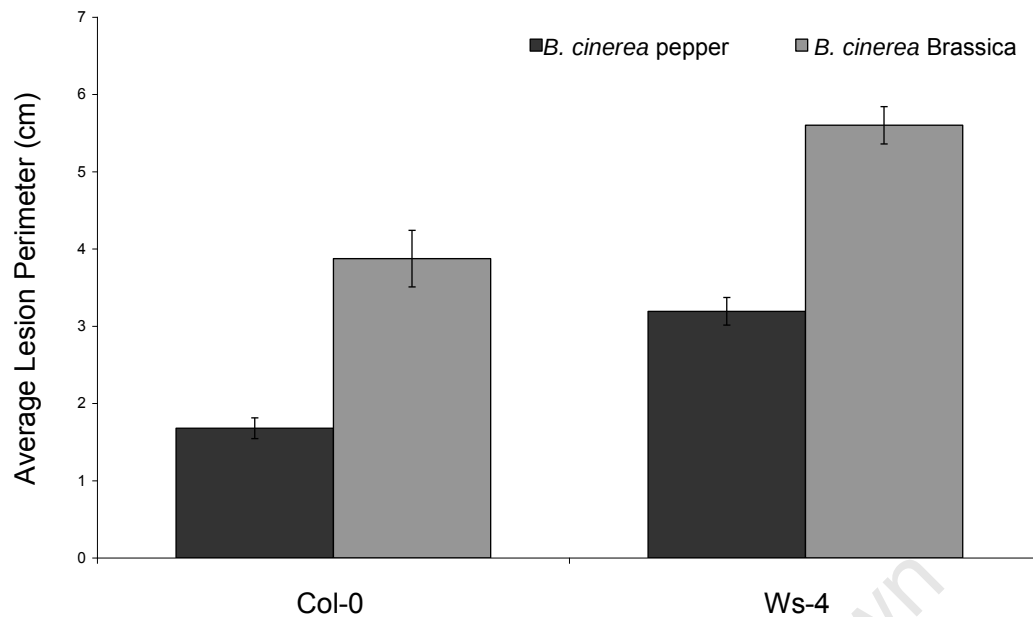


**Figure 13. Time-course analysis of *CNGC* transcripts in response to *B. cinerea* attack.** Each graph depicts the average expression with standard error bars of the specified *CNGC* from 4 biological repeats of infected (red) or control (green) samples which were harvested at 2 hour intervals from 2 to 48 hours post infection. *CNGCs* that are up- and down-regulated in response to *B. cinerea* infection are displayed on the top and middle rows respectively. The graphs on the bottom row depict *CNGCs* that display a transient difference (*CNGC1* and *CNGC2*) or no difference (*CNGC17*) between infected and control samples.

Two of the significantly differentially expressed genes identified from the microarray data, *CNGC4* and *CNGC12*, have been shown to play a role in defence signalling against biotrophic pathogens and therefore could be playing a key role in setting up a general defence signalling cascade in response to pathogen attack (Balague *et al.* 2003, Yoshioka *et al.* 2006). Thus far CNGCs from subgroup I (*CNGC11* and *CNGC12*) and IV (*CNGC2* and *CNGC4*) have been shown to be involved in biotrophic defence pathway but as CNGCs from each of the four subgroups, i.e. Subgroups I: *CNGC12* and *CNGC13*, II: *CNGC5*, III: *CNGC15*, IV: *CNGC4*, are represented in the six genes identified from the *B. cinerea* time-course microarray data, a reverse genetics approach was used to analyse *B. cinerea* susceptibility of at least two members from each subgroup.

#### **4.2.2 *B. cinerea* susceptibility phenotypes of *cngc* mutants**

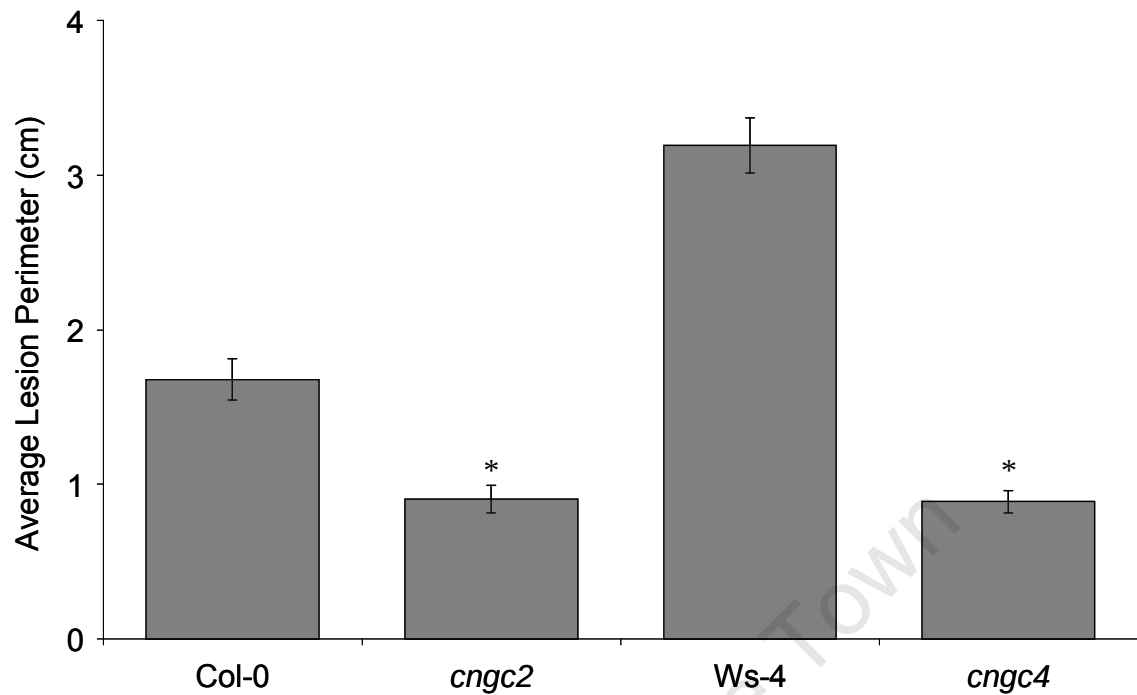
Both members of subgroup IVb, *CNGC2* and *CNGC4*, are known to be involved in the *P. syringae* defence signalling pathway but only *CNGC2* has been shown to play a role in defence against *B. cinerea* as it displayed increased resistance to the INRA isolate of *B. cinerea* (Clough *et al.* 2000, Govrin and Levine 2000, Balague *et al.* 2003). Therefore, the *dnd1* (*cngc2*) mutant was used as a control in the initial symptom based screen for susceptibility to the pepper isolate of *B. cinerea*. The *hlm1* (*cngc4*) mutant has already been shown to be involved in defence against *P. syringae* and as this mutant lacks expression of a gene which is from the same subgroup as *CNGC2*, it was tested for *B. cinerea* susceptibility. *Dnd1* (*cncg2*) and *hlm1* (*cngc4*) lines are from Col-0 and Ws-4 ecotypes respectively and studies have shown that the degree of susceptibility to *B. cinerea* infection by an *Arabidopsis* plant is dependent on both the *B. cinerea* isolate and the ecotype of the *Arabidopsis* host (Denby *et al.* 2004). This is apparent when comparing the average lesion size, four days post infection of the two parental ecotypes used in this study. The Ws-4 ecotype was more susceptible than Col-0 to infection by both pepper and Brassica isolates of *B. cinerea* and the Brassica isolate was more virulent than the pepper isolate on both ecotypes (Figure 14). The pepper isolate was used for the time-course microarray experiment and therefore it was also used for subsequent assays.



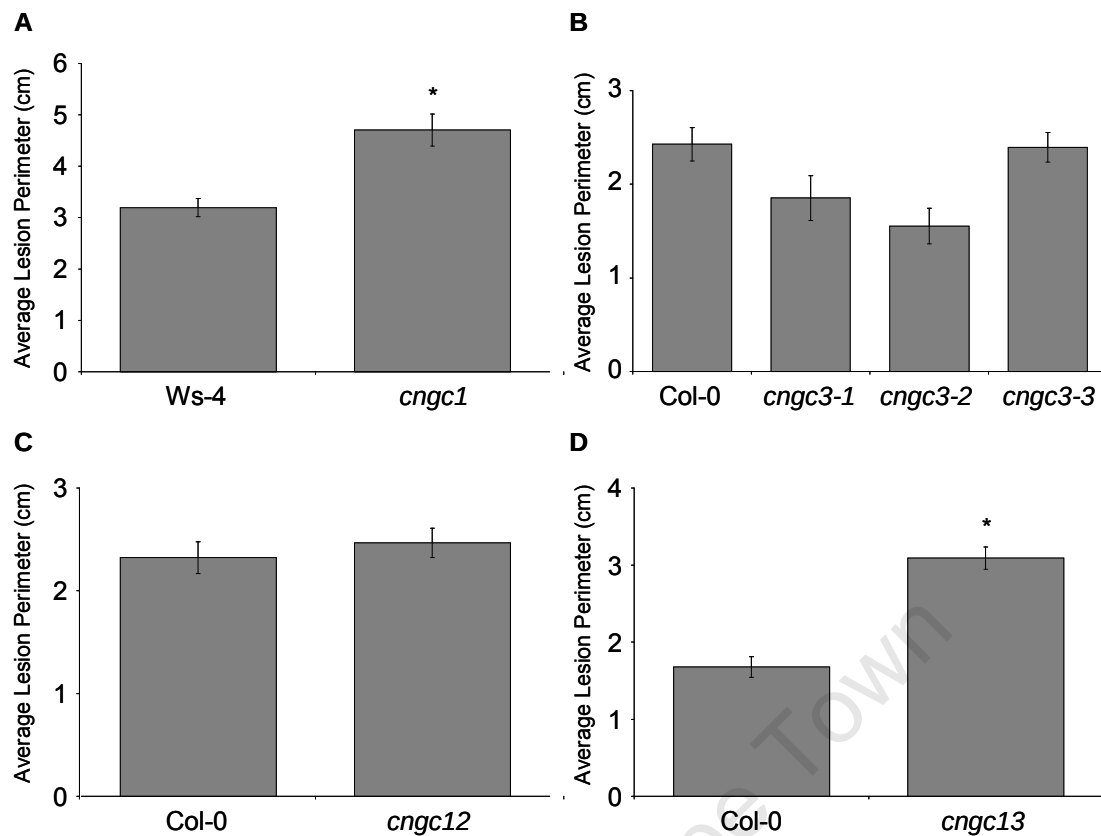
**Figure 14. *Arabidopsis* – *B. cinerea* interactions are affected by the genetic variation of both host and pathogen.** The average lesion size formed 4 days post infection on 4 week old Col-0 leaves was smaller than that formed on Ws-4 when infected with either isolate of *B. cinerea*. The Brassica isolate (grey bars) of *B. cinerea* caused larger lesions to appear on both *Arabidopsis* ecotypes. This graph displays the average perimeter of the lesion with standard error bars ( $n \geq 15$ ). This experiment was repeated with similar results.

Both *cngc2* and *cngc4* mutant lines were less susceptible to *B. cinerea* infection than their respective wildtype parents as seen by the smaller lesions formed on *dnd1* and *hlm1* leaves (Figure 15). The phenotypes displayed by *dnd1* and *hlm1* lines were very consistent even though there was slight variability of the lesion size formed on both mutant and wildtype leaves between independent experiments. This is the first report, to my knowledge, of *CNGC4*'s involvement in *B. cinerea* defence and provides further evidence of the role of *CNGC2* and *CNGC4* in defence against more than one specific pathogen.

Homozygous T-DNA insertion mutants of *CNGCs* from the other subgroups were selected by PCR screening with gene- and T-DNA-specific primers and used in *B. cinerea* susceptibility assays. Four members of subgroup I have either been previously shown to be involved in the biotrophic defence pathway, i.e. *CNGC11* and *CNGC12* (Yoshioka *et al.* 2006), or display significant transcriptional up-regulation in response to *B. cinerea* infection, i.e. *CNGC10* and *CNGC13* (Denby, unpublished data) but none have been tested for their involvement in *B. cinerea* defence. The remaining two members of subgroup I, *CNGC1* and *CNGC3*, displayed transient or no significant transcriptional regulation to *B. cinerea* infection respectively but this does not exclude them from playing a role in the defence signalling pathway. Analysis of *B. cinerea* susceptibility of available homozygous T-DNA insertion *cngc* mutants from subgroup I, *cngc1*, *cngc3*, *cngc12* and *cngc13*, indicated that at least two members are needed for full *B. cinerea* defence (Figure 16). The *cngc1* insertion mutant is in a Ws-4 background whereas all the other T-DNA insertion mutants are from a Col-0 background. The average lesion size of *cngc1* was significantly larger than Ws-4, indicating an increased susceptibility to the pepper isolate of *B. cinerea* (Figure 16A). Three independent *cngc3* T-DNA insertion lines were tested for altered *B. cinerea* susceptibility and none displayed a repeatable significant difference in lesion size when compared to Col-0 (Figure 16B). Analysis of lesion size four days post infection indicates that *cngc12* susceptibility was comparable to Col-0 (Figure 16C) whereas *cngc13* was more susceptible than Col-0 (Figure 16D). Hence, this study identified two more members of subgroup I involved in defence signalling and provides evidence to suggest that *CNGC12* could be specifically involved in the



**Figure 15. Members of subgroup IVb are involved in *B. cinerea* defence.** The average lesion size formed 4 days post infection of the *cngc* mutant lines and their respective wildtype parents are depicted in this graph. The *B. cinerea* pepper isolate was used to infect at least 15 leaves from each line. Lines identified with an asterisk are significantly different than its respective wildtype (T test,  $P < 0.01$ ). This experiment was repeated with similar results.

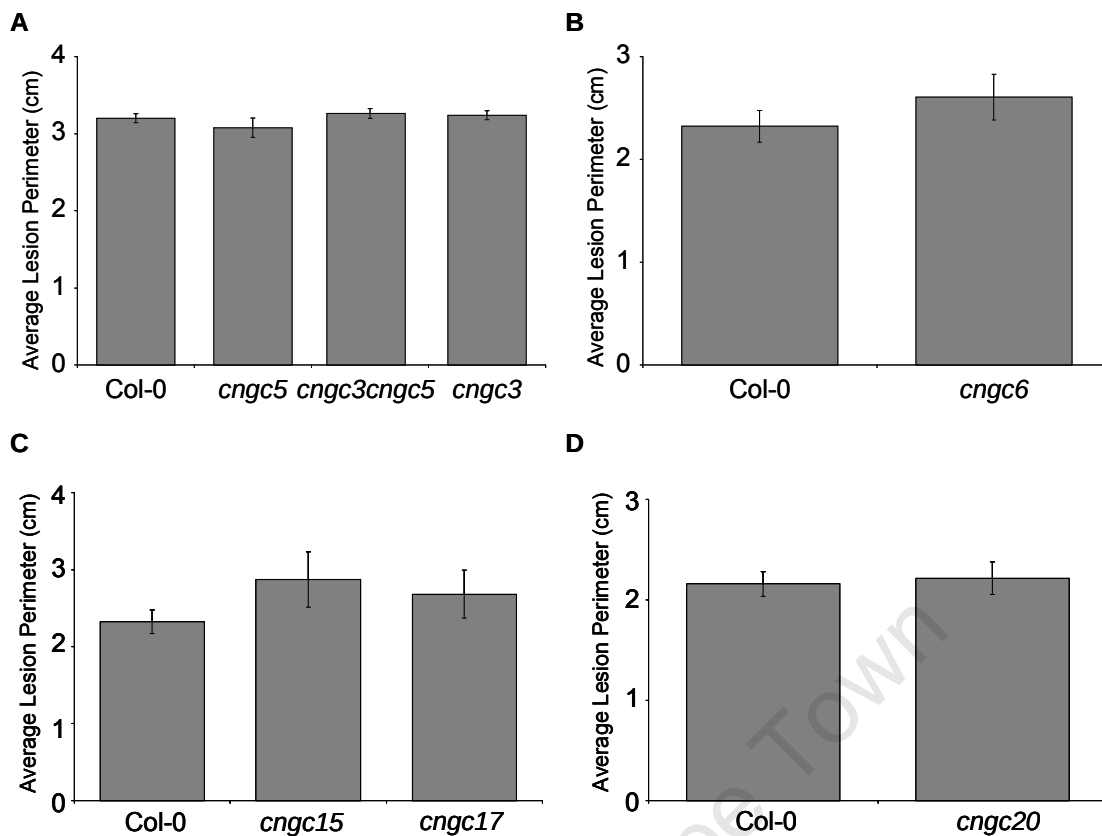


**Figure 16. *CNGC1* and *CNGC13* from subgroup I are involved in *B. cinerea* defence.** These bar graphs display the average lesion size 4 days post infection of T-DNA insertion mutants from subgroup I and their respective wildtype lines. The pepper isolate of *B. cinerea* caused larger lesions on *cngc1* leaves than Ws-4 (**A**). Three independent *cngc3* insertion mutants displayed similar lesion sizes to Col-0 (**B**). The slight decrease in susceptibility displayed by the *cngc3-2* line was not repeatable. *Cngc12* insertion mutants displayed lesions comparable to Col-0 (**C**) whereas the average lesion size formed on *cngc13* leaves was larger than Col-0 (**D**). Each graph displays the average lesion size with standard error bars of at least 15 leaves. *Cngc* lines that repeatedly displayed significantly different (T test,  $P < 0.05$ ) lesion sizes to wildtype are marked with an asterisk.

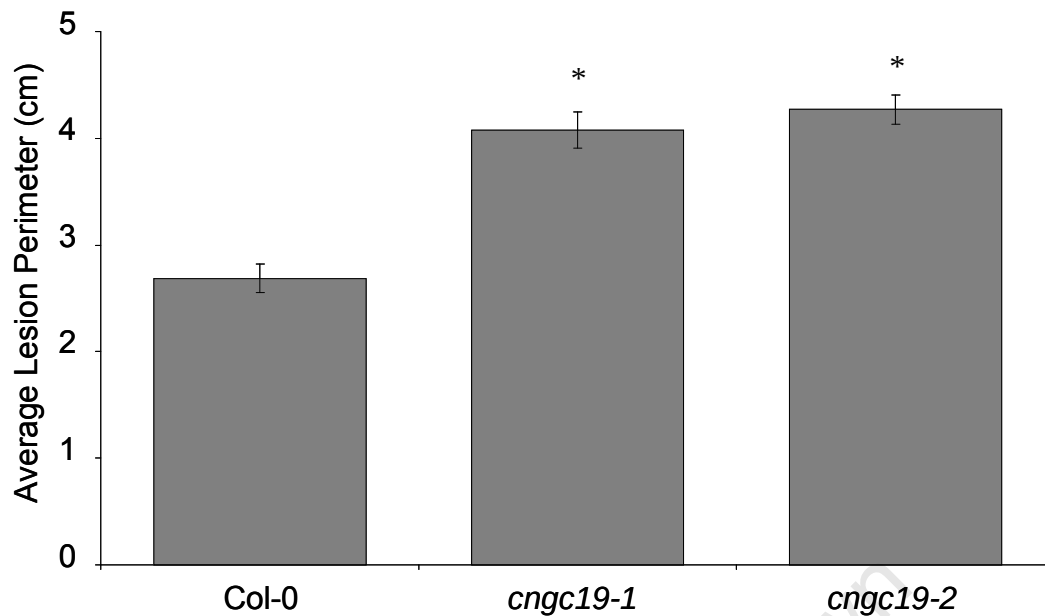
biotrophic defence pathway.

The time-course microarray data indicated that the expression of *CNGC5* and *CNGC15*, from subgroups II and III respectively, was down-regulated in response to *B. cinerea* attack. However, transcriptional regulation does not always correlate with proposed defence response *in planta*, as displayed by *CNGC1* and *CNGC2*, and therefore homozygous T-DNA insertion lines of two members from subgroups II (*CNGC5*, *CNGC6*) and III (*CNGC15*, *CNGC17*) were tested for altered *B. cinerea* susceptibility. All of the single *cngc* mutants were as susceptible to *B. cinerea* as Col-0 (Figure 17). As it was available, the double knockout mutant, *cngc3 cngc5*, where expression of members of subgroup I and II have been lost, was also tested. It also displayed lesion sizes comparable to both single mutants and Col-0 (Figure 17A). Loss of *cngc6*, another member of subgroup II, displayed wildtype lesion sizes (Figure 17B). Similarly, loss of *CNGC15* and *CNGC17* expression, from subgroup III, had no effect on symptom development compared to Col-0 (Figure 17C). The lack of *B. cinerea* phenotypes identified from the members in these subgroups suggests that they do not play a role in defence signalling, despite their down-regulation during defence. Plants are immobile and have to co-ordinate a number of signalling pathways to respond appropriately to multiple stresses they are constantly faced with (Fujita *et al.* 2006). The down-regulation of *CNGC5* and *CNGC15* could be due to defence signalling being prioritised over another signalling pathway and therefore analysis of plants constitutively over-expressing *CNGC5* and *CNGC15* may identify a role for these channels and/or explain their down-regulation during *B. cinerea* infection.

As stated before, at least two members from each subgroup were tested for *B. cinerea* susceptibility and therefore members of the last subgroup, *CNGC19* and *CNGC20*, which did not show any difference in transcriptional regulation in response to *B. cinerea* attack, were also tested. The *cngc20* mutant line showed wildtype symptoms in response to *B. cinerea* infection (Figure 17D). However, loss of *CNGC19* expression resulted in increased susceptibility to *B. cinerea* infection, as two independent T-DNA insertion lines were significantly more susceptible than Col-0 (Figure 18). The T-DNA insertion lines had lesions that were approximately 40%



**Figure 17. Insertion mutants of *CNGCs* from subgroup II (*CNGC5* & *CNGC6*), III (*CNGC15* & *CNGC17*) and IVa (*CNGC20*) do not display altered *B. cinerea* phenotypes.** The average lesion size 4 days post infection (dpi) of the *cngc5* single insertion mutant is not significantly different to Col-0 and the *cngc3 cngc5* double mutant displays a similar lesion size to the single *cngc* mutants (**A**). The *cngc3-1* line was used to create the double mutant (**A**). *Cngc6* insertion mutants displayed similar symptoms to Col-0 (**B**). *Cngc15* and *cngc17* T-DNA insertion mutants display similar lesion sizes to Col-0, 4 dpi (**C**). The average lesion size formed on *cngc20* leaves was similar to Col-0 (**D**). At least 15 leaves from each line was infected with the pepper isolate of *B. cinerea* and the average lesion size with standard error bars are displayed in these graphs.



**Figure 18. Two independent T-DNA insertion lines of *cngc19* display increased susceptibility to *B. cinerea*.** Analysis of lesion size 4 days post infection with the pepper isolate of *B. cinerea* shows that the average lesion size formed on Col-0 was significantly smaller than on 2 independent *cngc19* insertion lines (T test,  $P < 0.05$ ). At least 15 leaves of each line were infected and the average lesion sizes with standard error bars are depicted in the graph. This experiment was repeated with similar results.

larger than those formed on wildtype leaves. As both independent *cngc19* insertion lines display increased susceptibility to *B. cinerea* infection, it strongly suggests that the phenotype is a result of a single insertion that disrupts the *CNGC19* gene.

Susceptibility assays of *cngc* T-DNA insertion lines performed in this study identified four CNGCs, i.e. CNGC1, CNGC4, CNGC13 and CNGC19, which play a role in *B. cinerea* defence. These CNGCs are part of subgroup I (CNGC1 and CNGC13), subgroup IVa (CNGC19) and subgroup IVb (CNGC4). Other members of subgroup I and IVb are known to play a role in defence signalling which suggests that members of the same subgroup are performing similar functions *in planta* (Clough *et al.* 2000, Yoshioka *et al.* 2006). The second messenger,  $\text{Ca}^{2+}$ , plays a crucial role in defence and a previous study of CNGC2 identified a link between its  $\text{Ca}^{2+}$  transport capabilities and its role in defence (Ali *et al.* 2007). Two CNGCs, CNGC13 and CNGC19, were identified in this study as playing a role in *B. cinerea* defence and also displayed the ability to transport  $\text{Ca}^{2+}$ . Similarly to CNGC2, CNGC19 displayed localisation to the plasma membrane when transiently expressed in onion cells. *CNGC19* was chosen for further characterisation as this is the first study, to my knowledge, which identifies a member from subgroup IVa needed for defence.

#### 4.2.3 Characterisation of *CNGC19*

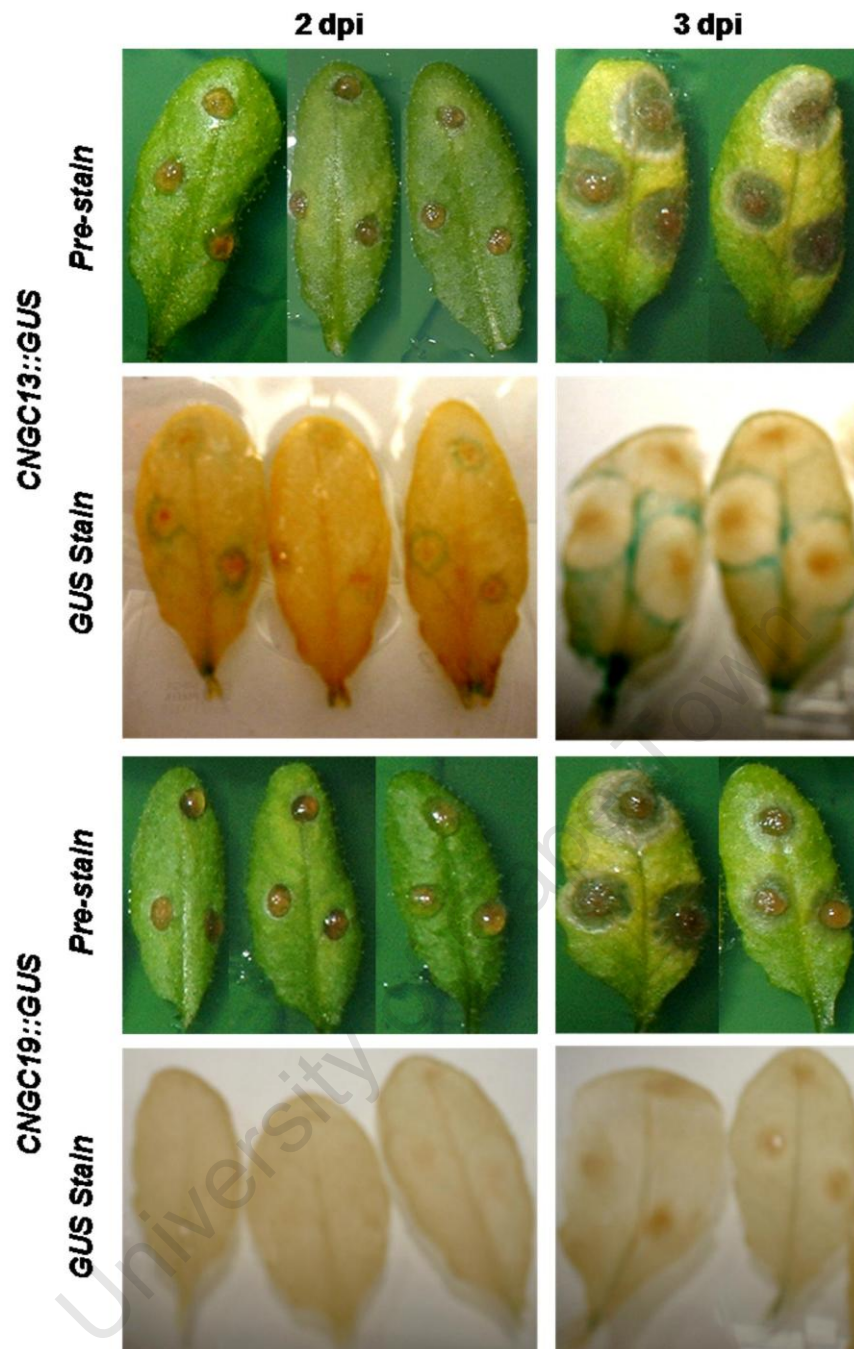
Microarray analysis indicates that *CNGC19* is not transcriptionally regulated by *B. cinerea* infection but up-regulated by salt stress which suggests a putative role for *CNGC19* in salinity stress. However, this study shows that *cngc19-2* is more susceptible to *B. cinerea* whereas a study by Maathuis (2006b) indicated that *cngc19-2* plants do not display altered germination or growth on media containing high salt concentrations. Therefore, CNGC19 is a good example where expression profiling does not give an accurate indication of the physiological function *in planta*.

Bioinformatic analysis of the putative promoter region of *CNGC19* identified cis-acting elements involved in methyl jasmonate (MeJA), abscissic acid (ABA) and salicylic acid (SA) response pathways which further suggests that it is regulated by various stress responses (<http://bionformatics.psb.ugent.be/webtools/plantcare/html>). Excised leaves of heterozygous four week old transgenic plants expressing the  $\beta$ -glucuronidase (*GUS*) reporter gene under the control of the *CNGC13* or *CNGC19*

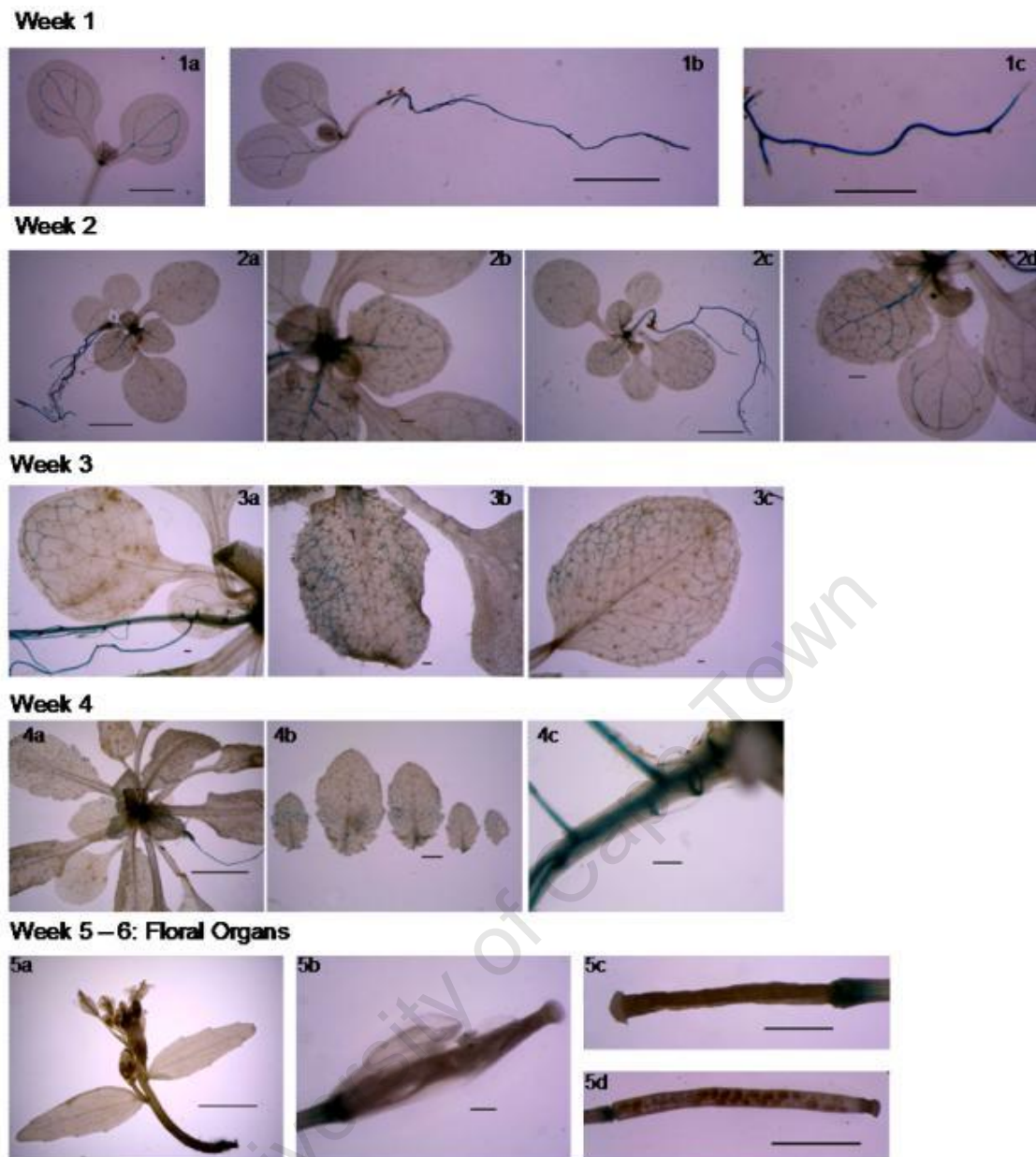
promoter (a 1 kb sequence upstream of the translational start codon) were used to verify expression of these *CNGCs* in response to infection by the *B. cinerea* pepper isolate. Leaves were drop inoculated with *B. cinerea* spores and harvested two and three days post infection for GUS staining. The *CNGC13::GUS* leaves displayed staining on the border of the forming lesion at two and three days post infection (Figure 19). *CNGC19* expression was not up-regulated in response to *B. cinerea* infection as no staining was observed on the infected leaves (Figure 19). This confirmed the data obtained from the microarray time-course data which show that *CNGC19* is not transcriptionally regulated in response to *B. cinerea* infection. It should be noted that the complete lack of GUS staining in infected *CNGC19::GUS* leaves appears to be due to the low level of expression of *CNGC19* in mature leaves, and not a defective GUS construct, as staining can be observed in other developmental tissues (Figure 20).

The *CNGC19::GUS* line was used to follow the tissue specific expression of *CNGC19* at various stages of development and results were very similar to the data on the public database, Genevestigator (Figure 20). GUS expression driven from the *CNGC19* promoter was visible in the roots throughout development, specifically in the vascular tissue. Expression of *CNGC19* in the vascular tissue of cotyledons and younger leaves is at similar levels to those found in roots. However, only a few of the older leaves of three and four week old plants display slight staining in the vascular tissue of indicating that *CNGC19* expression was either absent or below detectable limits of the GUS assay. Staining was observed at the base of siliques and as tiny spots on the anthers.

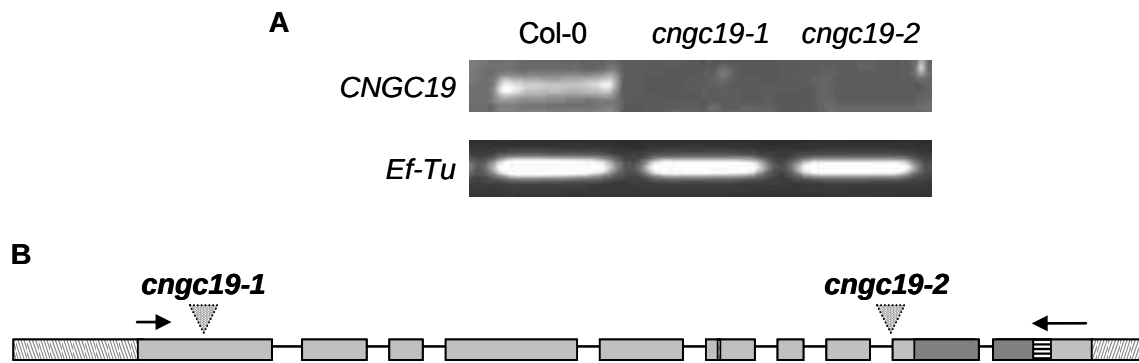
To determine whether *CNGC19* is expressed in mature leaves, albeit at a low level, and whether the *cngc19* T-DNA insertion lines display complete loss of *CNGC19* expression, sqRT-PCR analysis of four-week old Col-0, *cngc19-1* and *cngc19-2* leaf tissue was performed (Figure 21A). The SIGnAL website (<http://signal.salk.edu/cgi-bin/T-DNAexpress>) indicates that the SALK T-DNA insertion (*cngc19-1*)



**Figure 19.** The *CNGC13::GUS* and *CNGC19::GUS* transgenic lines confirm expression data obtained from microarray analysis of *B. cinerea* infected plants. Four week old *CNGC13::GUS* and *CNGC19::GUS* plants were infected with the pepper isolate of *B. cinerea* and stained for GUS activity 2 and 3 days post infection (dpi). Staining is only observed on the border of lesions formed on the *C13::GUS* leaves at 2 dpi and 3 dpi.



**Figure 20. Developmental expression of *CNGC19* was analysed using the *CNGC19::GUS* transgenic line. *CNGC19* is expressed in root tissue throughout development (1b, 1c, 2a, 2c, 3a, 4a, 4c), in the vasculature of younger leaves and cotyledons (1a, 1b, 2b, 2c, 3b, 3c, 4b). Expression of *CNGC19* was also observed at the base of a forming silique (5c, 5d) and in tiny spots in the middle of the anther (5b). The scale bar represents 1 mm.**



**Figure 21. Diagrammatical representation of the *At3G17690* (*CNGC19*) genomic sequence indicating the position of the T-DNA insertions, which cause loss of expression in the respective *cngc19* lines.** Semi-quantitative RT-PCR analysis of leaf material from 4 week old *cngc19* insertion lines and Col-0 for *cngc19* expression using *CNGC19* specific primers (**A**). The *Ef-Tu* house-keeping gene was used as a control. **B**) displays the 3487 bp *CNGC19* gene: comprised of 10 exons (light grey boxes), separated by 9 introns (lines) with the 5' UTR (left diagonal shading) and 3' UTR (right diagonal shading) on the left and right borders respectively. The positions of the 2 T-DNA insertions (dotted triangles) are indicated in the 1<sup>st</sup> and 9<sup>th</sup> exons. The predicted position of the 3 amino acids responsible for the selectivity filter in the pore region (black shading), cyclic nucleotide binding domain (dark grey shading) and calmodulin binding domain (striped shading) are indicated on exons 6, 9 and 10 respectively. Arrows indicates the position of primers used for qRT-PCR analysis.

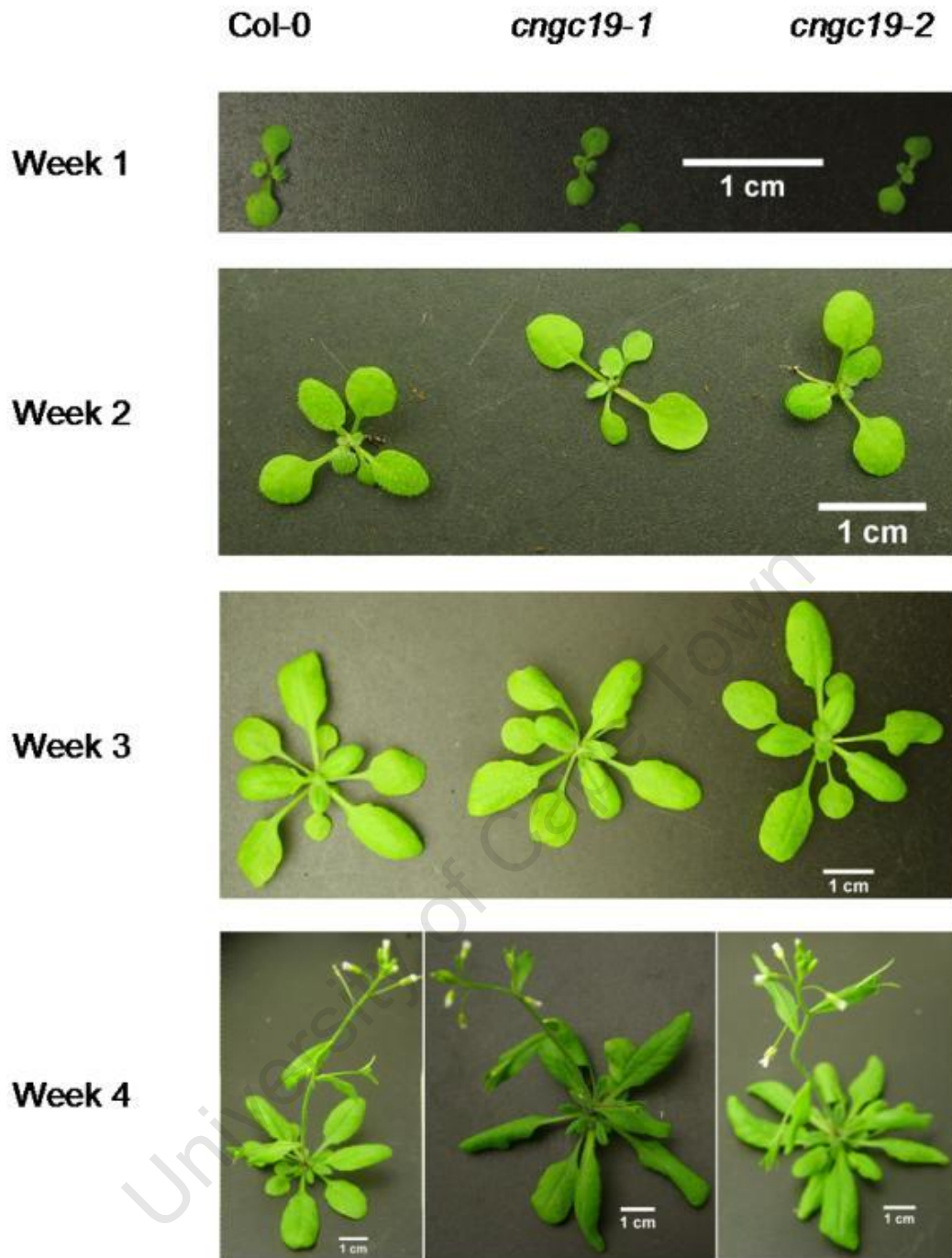
is located in the first exon and the SAIL T-DNA insertion (*cngc19-2*) disrupts the ninth exon (Figure 21B). These results show that *CNGC19* is expressed in mature leaves of wildtype plants, albeit at low levels, and that the T-DNA insertions cause complete knockout mutations of *CNGC19*.

Studies have shown that the loss of a single *CNGC* gene, such as *CNGC2* or *CNGC4*, caused severe developmental defects, which are exacerbated by certain environmental conditions, and lowered fertility (Yu *et al.* 1998, Balague *et al.* 2003, Jurkowski *et al.* 2004). The loss of *CNGC19* expression did not affect gross plant morphology or development as both *cngc19-1* and *cngc19-2* were indistinguishable from the parental wildtype line, Col-0 (Figure 22).

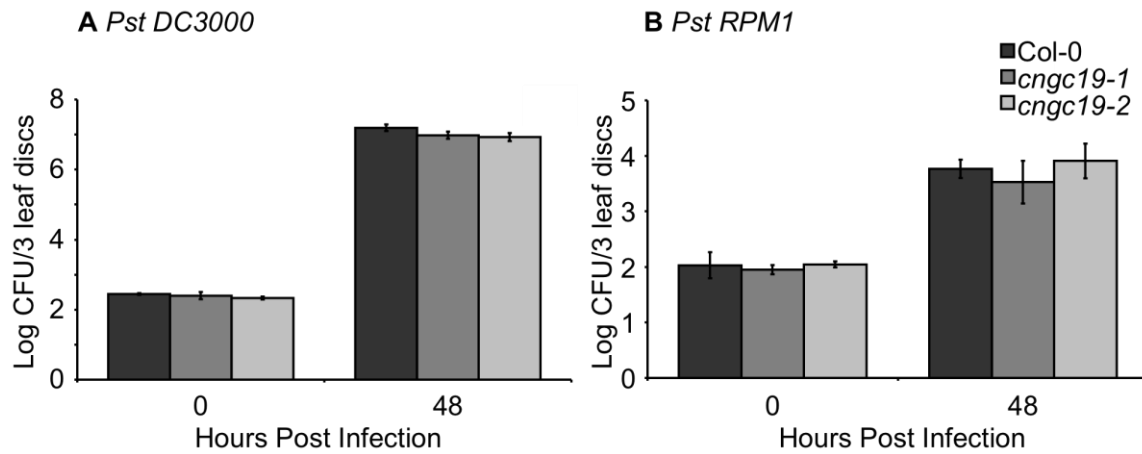
#### **4.2.4 *CNGC19*'s role in the defence signalling pathway**

Analysis of microarray data from Genevestigator indicates that *CNGC19* expression is up-regulated in response to infection with both virulent and avirulent *P. syringae* strains. To determine whether the loss of *CNGC19* expression affected susceptibility to biotrophic pathogens, four-week old *cngc19-1*, *cngc19-2* and Col-0 plants were challenged with the virulent (DC3000) and avirulent (*avrRPM1*) *P. syringae* pv tomato (*Pst*) strains. Analysis of *cngc19* lines and Col-0 infected with *Pst* DC3000 and *Pst avrRPM1* strains, showed that as expected the virulent strain displayed a higher bacterial titre compared to the avirulent strain in both wildtype and mutant lines. However, there was no difference in bacterial titres when comparing *cngc19* knockout lines to Col-0 (Figure 23). These results indicate that *CNGC19* expression is required for an effective defence signalling pathway against *B. cinerea* but not *P. syringae* infection.

To determine how the loss of *CNGC19* expression is affecting known components of the necrotrophic defence signalling pathway, a comparison of various defence hallmarks displayed by Col-0 and *cngc19* was undertaken. Previous studies show that altered PCD pathways, defence gene expression and accumulation of secondary metabolites can be affected by the loss of a single *CNGC* (Govrin and Levine 2000). A study by Govrin *et al.* (2000) showed that the lack of cell death induction rendered the *cngc2* mutant more resistant to *B. cinerea* infection. To determine if the increased



**Figure 22. *Cngc19* lines do not display gross morphological defects during development.** Col-0 and *cngc19* mutant lines were observed weekly. The plants were grown in long day conditions and a representative of each line is shown at weeks 1 to 4 after sowing.



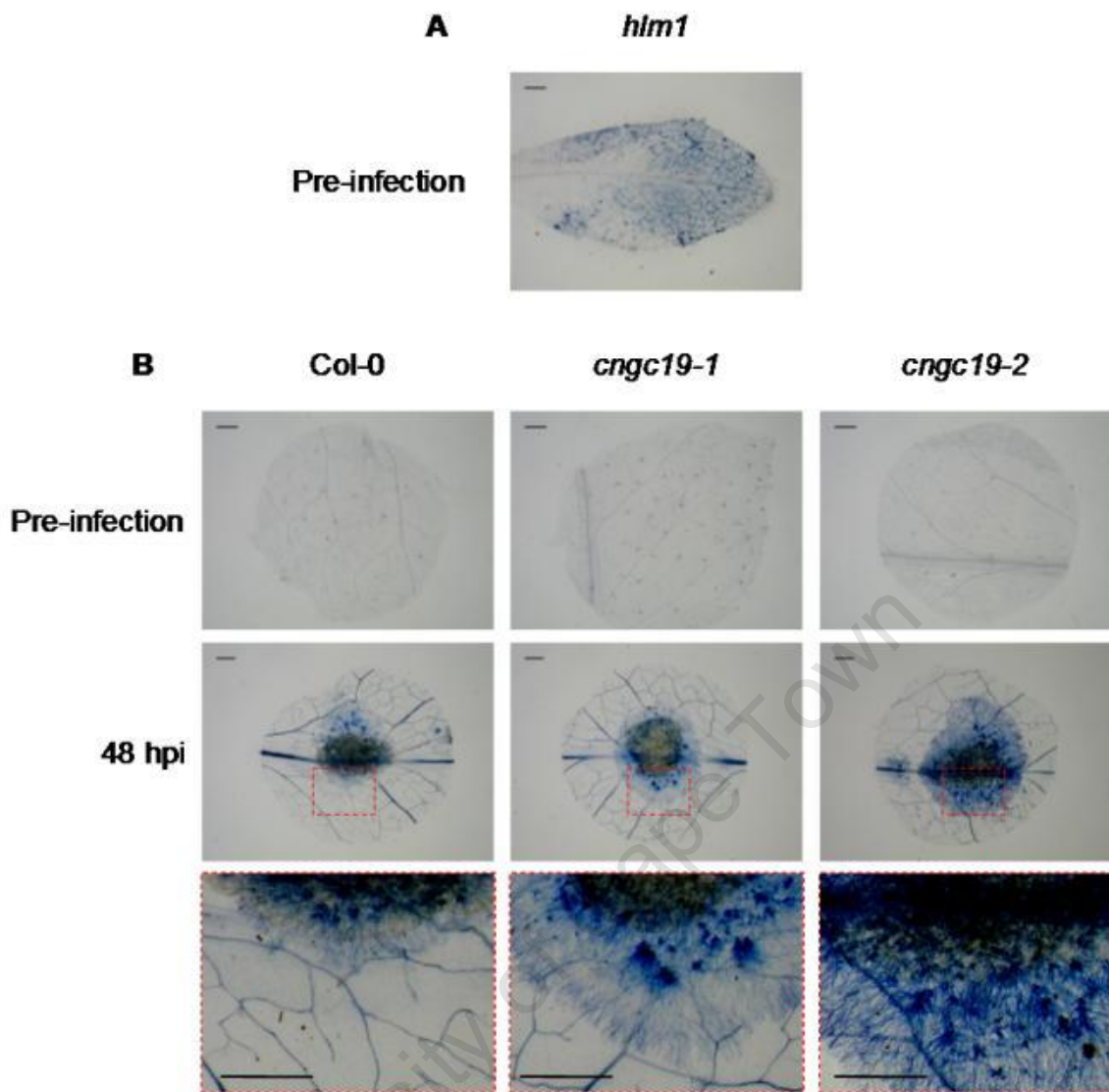
**Figure 23. Resistance to both virulent and avirulent strains of *P. syringae* is unaltered in the *cngc19* lines.** Growth of virulent *P. syringae* pv tomato (A) and the avirulent strain expressing the *avrRPM1* gene (B) in 4 week old Col-0, *cngc19-1* and *cngc19-2* plants. At the times indicated, 3 leaf discs were harvested from each of 3 independent plants, which had been pressure infiltrated with a  $1 \times 10^6$  cfu/ml bacterial suspension. The bar chart displays the average colony forming units (CFU) in 3 leaf discs from 3 independent plants of each line with standard error bars.

susceptibility of *cngc19* could be contributed by the presence of micro-lesions on the leaf surface, four week old leaves of Col-0 and *cngc19* were treated with trypan blue pre- and post-infection to stain for the presence of dead cells (Figure 24). The *hlm1* (*cngc4*) line was used as a positive control as it displays spontaneous lesions in the absence of pathogen infection (Balague *et al.* 2003). Both Col-0 and *cngc19* mutant plants did not display micro-lesions on the leaf surface prior to infection. The increased cell death and stained fungal hyphae observed on *cngc19* leaves correlates with the larger lesion size (Figure 24). This indicates that CNGC19's increase in susceptibility is not due to the presence of spontaneous lesion development on its leaf surface.

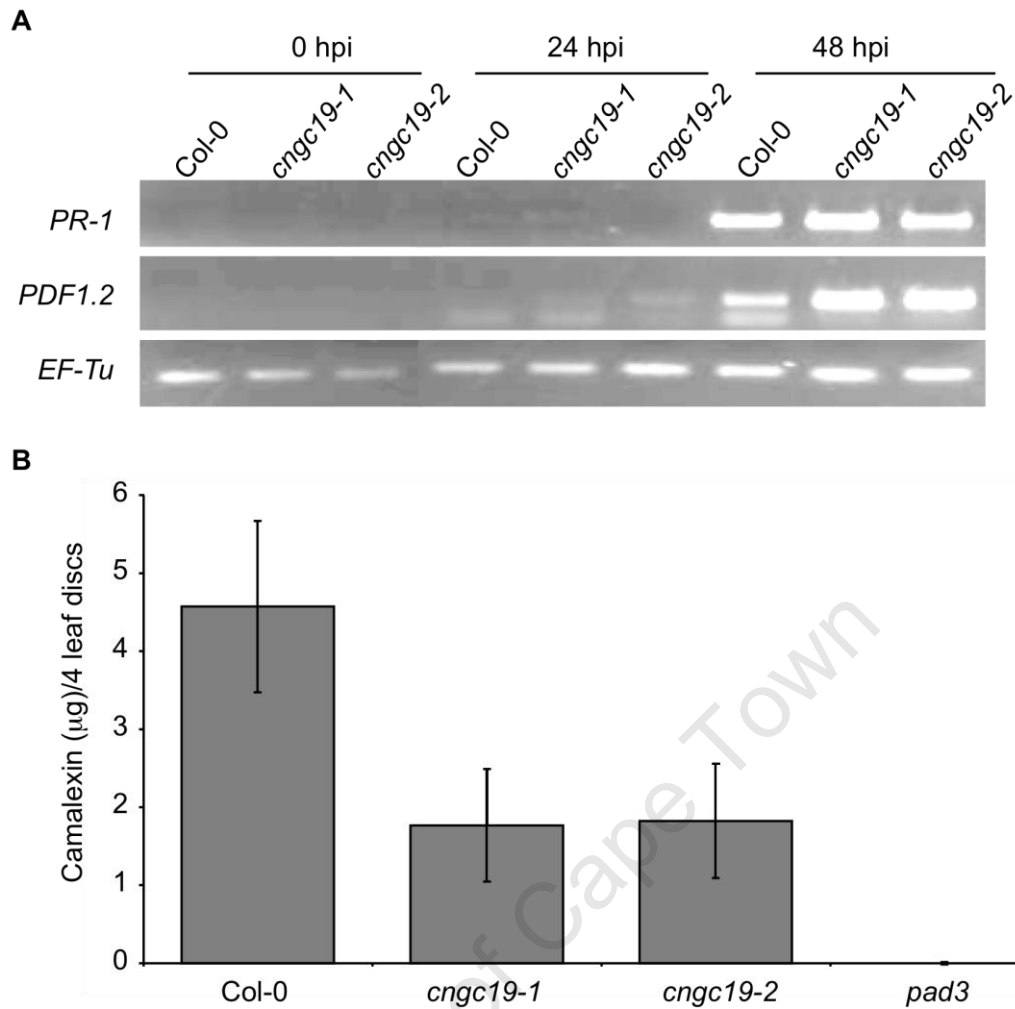
To determine whether the SA and/or JA/Eth defence signalling cascades are disrupted by a particular mutation, expression analysis of pathogenesis related (PR) gene, *PR-1* and a defensin gene, *PDF1.2*, can be used to indicate whether the respective hormone induced defence signalling cascades are functioning properly. The expression of *PR-1* was not significantly different between the *cngc19* lines and Col-0 but *PDF1.2* expression was slightly higher in the more susceptible *cngc19* lines, following *B. cinerea* infection, as expected. This indicates that the two key defence signalling pathways are not affected by the loss of CNGC19 expression.

As another test of induced defence responses, levels of the secondary metabolite, camalexin, was measured as it has been shown to be important for full *B. cinerea* defence (Denby *et al.* 2004). It accumulates at the border of the forming lesion and the loss of camalexin accumulation causes increased susceptibility to *B. cinerea* as shown by the *pad3* mutants, which are unable to produce camalexin (Thomma *et al.* 1999). Both *cngc19* lines displayed lower levels of camalexin than Col-0 forty-eight hours after *B. cinerea* infection (Figure 25B). Reduced levels of camalexin in these mutants may be responsible for the increased susceptibility.

Camalexin accumulates in response to biotrophic and necrotrophic pathogens but is only effective in minimising necrotrophic infection. A study by Denby *et al.* (2004) showed that wildtype ecotypes which are more resistant to *B. cinerea* infection produce more camalexin at the border of the forming lesion, suggesting a positive



**Figure 24.** Trypan blue staining indicates that *cngc19* plants do not develop spontaneous micro-lesions, as seen in *hlm1*, and *cngc19* lines support greater fungal growth than Col-0. The *hlm1* (*cngc4*) lesion mimic mutant produces spontaneous lesions in the absence of pathogen infection as revealed with trypan blue staining (**A**). *Cngc19* lines and Col-0 do not display micro-lesions prior to pathogen infection (**B, top row**). Trypan blue treatment of leaves 48 hours post infection (hpi) stained the dead plant cells and fungal hyphae growth (**B, middle row**). A close up of the infected area (red box) on the *cngc19* and Col-0 leaves illustrates the increased fungal growth on *cngc19* lines compared to Col-0 (**B, bottom row**). This experiment was repeated 3 times with similar results.

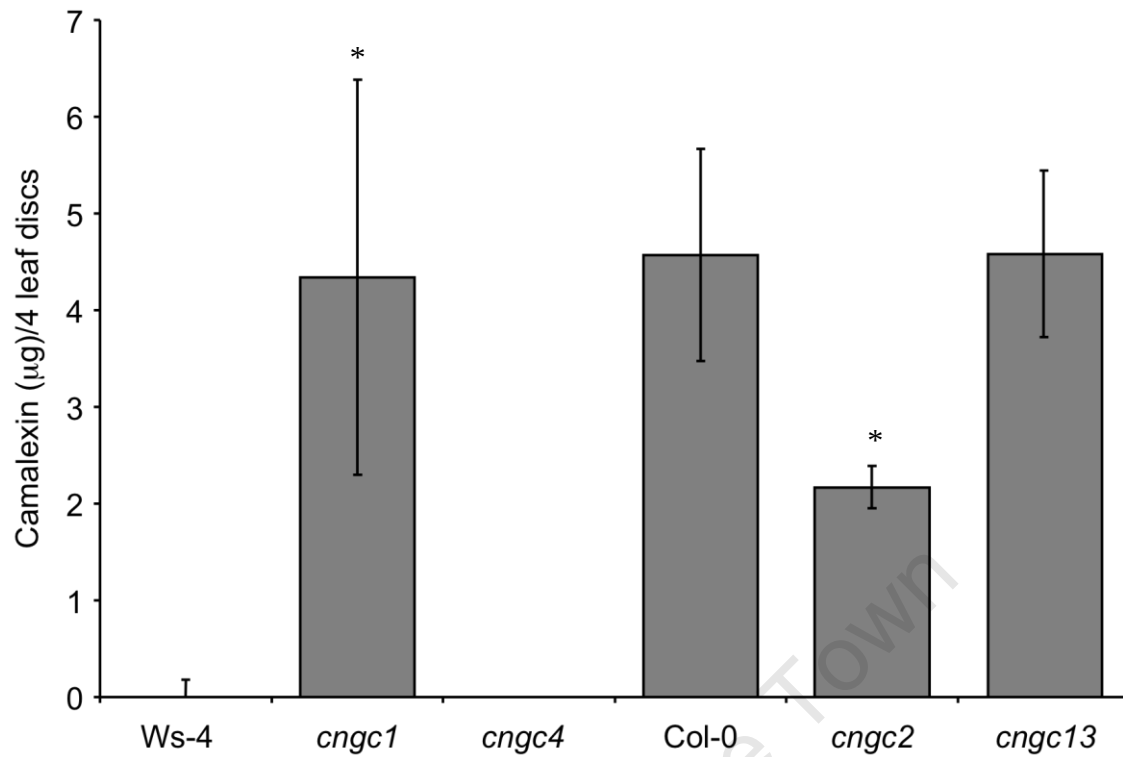


**Figure 25. *B. cinerea* infected *cngc19* plants display similar induction of defence gene expression but lower levels of camalexin accumulation than Col-0.** Defence gene expression in Col-0 and *cngc19* lines was analysed by performing semi-quantitative RT-PCR on infected leaf tissue at 0, 24 and 48 hours post infection (hpi) and *Ef-Tu* was used as a cDNA control **(A)**. The average camalexin levels in 3 *B. cinerea* infected leaves (4 discs per leaf) 48 hpi **(B)**. Camalexin levels in both *cngc19* lines and *pad3* were significantly lower than Col-0 (T test,  $P < 0.05$ ). Experiments were repeated with similar results.

correlation between *B. cinerea* susceptibility and low camalexin levels. A previous study has shown that *cngc* defence-related mutants, *cngc2* and *cngc4*, displayed wildtype camalexin levels in response to a virulent strain of *P. syringae* (Jirage *et al.* 2001) but to my knowledge, this is the first study to measure camalexin levels in *B. cinerea* infected *cngc* mutants. To determine whether other *cngcs* displaying a *B. cinerea* phenotype could be playing a role in camalexin induced defence responses, camalexin accumulation at the border of the forming lesions was analysed forty-eight hours post infection. The amount of fungal growth should not affect camalexin levels as four leaf discs of equal size were harvested from the border of each lesion but due to the dwarfed phenotype of *cngc2* and *cngc4*, the entire leaf had to be harvested.

Camalexin levels in *cngc2* plants were significantly lower than Col-0 whereas camalexin was barely detectable in *cngc4* and its wildtype line, Ws-4. (Figure 26). This indicates that other factors are involved in *B. cinerea* defence because the increased resistant phenotype of these two *cngcs* is not dependent on higher camalexin levels. The increased resistance phenotype of *cngc2* and *cngc4* could be due to lack of *B. cinerea* penetration or constitutive expression of the *PDF1.2* defence gene. Similarly, CNGC13 may also be involved in regulating a different step in the *B. cinerea* defence signalling pathway as the loss of *cngc13* expression does not affect camalexin accumulation in response to *B. cinerea* even though *cngc13* plants are more susceptible (Figure 26). The more susceptible Ws-4 ecotype displayed lower levels of camalexin at the border of its lesion than Col-0 which fits with the data obtained by Denby *et al.* (2004). Surprisingly, the loss of *cngc1* from the Ws-4 background caused high levels of camalexin to accumulate even though *cngc1* displayed increased susceptibility to infection (Figure 26).

A site-directed mutagenesis approach was used to determine whether either or both of the regulatory domains (CN and/or CaM) are critical for CNGC19 function by complementation analysis of *cngc19*. As both regulatory domains are found at the C-terminus, PCR methodology was used to alter a single base pair which introduced stop codons upstream of both regulatory domains to delete CNB and CaMB sites (*C19-Both*) or upstream of the CaMB domain, leaving the CNB domain intact (*C19-CaMBD*). To eliminate the CNB domain, specific base pairs had to be mutated (*C19-CNBD*). Two glycine residues thought to be important for the structural integrity of the

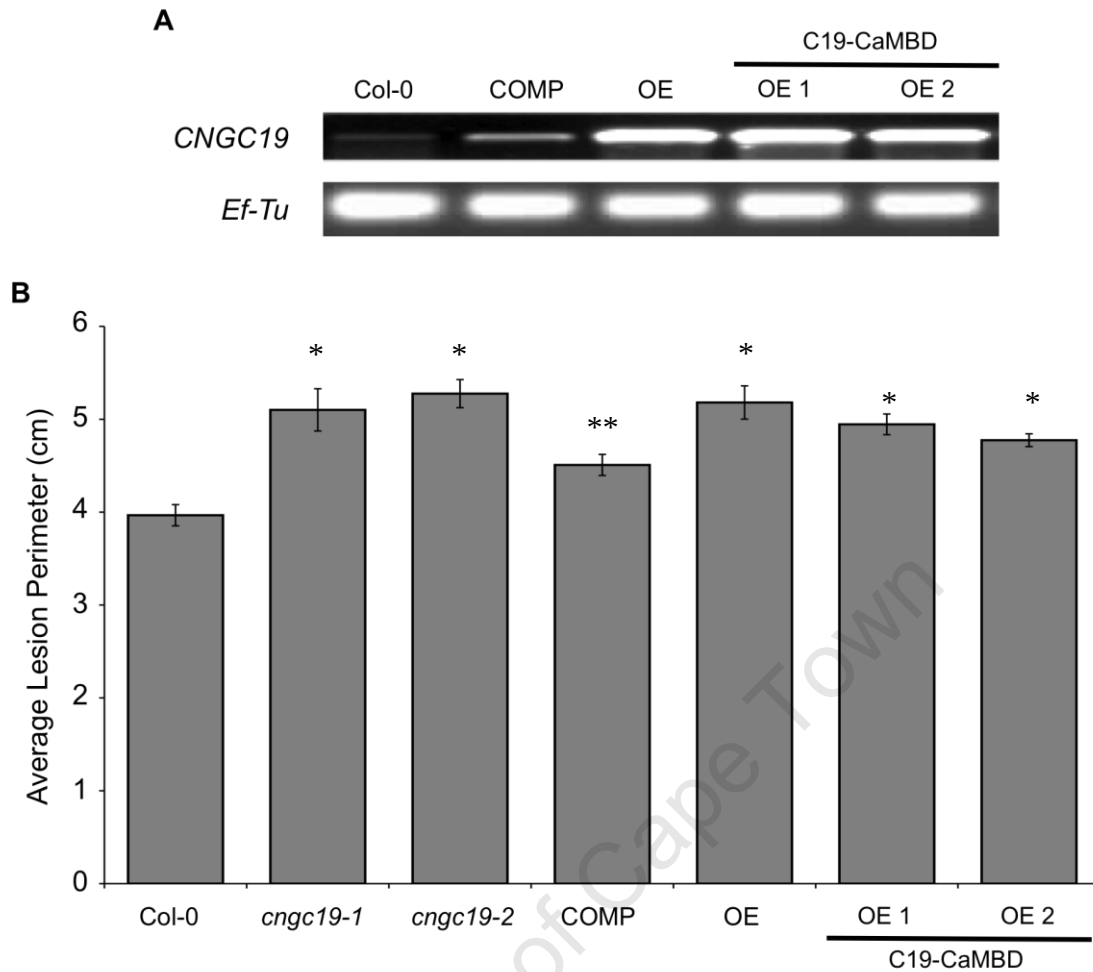


**Figure 26. Camalexin accumulation in *cngcs* displaying altered *B. cinerea* phenotypes.** The average camalexin levels found in 3 leaves 48 hours post infection. *Cngc1* and *cngc4* are in the Ws-4 background whereas *cngc2* and *cngc13* are from Col-0. The asterisk denotes a significant different to the respective wildtype line (T test,  $P < 0.05$ ). This experiment has repeated with similar results.

CNB pocket (Bridges *et al.* 2005) were mutated to cysteine and arginine to impair binding activity. The full length wildtype and mutated *CNG19* cDNA sequences were introduced into plant vectors which harboured 35S promoters to drive expression of the *CNGC19* cDNA sequences and contained the glufosinate ammonium (BASTA) selectable marker. As the *cngc19-1* and *cngc19-2* lines had kanamycin and BASTA resistance respectively, the *cngc19-1* line was transformed with the four constructs for ease of selection. As a 35S promoter was driving these constructs, semi-quantitative RT-PCR was used to screen for lines displaying levels of *CNGC19* similar to wildtype (denoted by: COMP) or over-expressing *CNGC19* (denoted by: OE) (Figure 27A). Unfortunately, due to time constraints, only three lines (COMP, OE and *C19-CaMBD*) were available for *B. cinerea* susceptibility assays and subsequent hypothesis were drawn on the assumption that the relative gene expression measured was indicative of the protein produced.

Analysis of lesion development four days post infection of Col-0, *cngc19*, and COMP lines indicated that the COMP line partially complemented the increased susceptibility phenotype of *cngc19* (Figure 27B). The COMP line does appear to express slightly higher levels of *CNGC19* than wildtype, but analysis of protein levels is required to determine whether the partial complementation is due to increased *CNGC19* levels. If so, it could be hypothesised that any mis-expression of *CNGC19* causes a disruption to the *B. cinerea* defence pathway. In keeping with the presumption that levels of mRNA determined directly equate to protein products, the increased susceptibility phenotype displayed by the OE line supports the hypothesis that increased *CNGC19* expression results in the same susceptibility phenotype as the knockout lines (Figure 27B).

Two independent *C19-CaMBD* lines displayed increased susceptibility to *B. cinerea* (Figure 27B) but as over-expression of *CNGC19* causes the same phenotype, it is not possible to distinguish whether the increased susceptibility is due to the lack of *CaMBD* or over-expression of *C19-CaMBD*.



**Figure 27. Altered expression of *CNGC19* disrupts *B. cinerea* defence.** The *cngc19-1* line was transformed independently with 2 constructs harbouring full length (complemented - COMP, over expresser - OE) or truncated (lack of CaM binding domain – C19-CaMBD OE 1; OE 2) *CNGC19* cDNA driven by a 35S promoter for complementation analysis. Semi-quantitative RT-PCR with *CNGC19* specific and *Ef-Tu* primers was used to identify complemented and over-expressing lines used in the pathogen assay **(A)**. These lines were tested for *B. cinerea* susceptibility and show that altering the *CNGC19* expression levels lowers the defence against *B. cinerea* **(B)**. A single asterisk denotes a significant difference in lesion perimeter relative to Col-0 (T test,  $P < 0.05$ ) and the double asterisk denotes a significant difference in lesion perimeter relative to Col-0 and *cngc19* lines (T test,  $P < 0.05$ )

### 4.3 Discussion

There has been a greater interest in the *Arabidopsis thaliana*-*Botrytis cinerea* interaction recently and therefore more information is publicly available to extract and analyse. This data can be used as a starting point to find candidates potentially involved in *B. cinerea* defence but it may overlook ones that are not transcriptionally regulated. In accordance with the hypothesised role of CNGCs in second messenger signalling, these channels would have to be constitutively present for rapid and early responses to stimuli. Therefore transcriptional regulation of a specific CNGC may not always correlate with its function *in planta* as its basal level of expression might be sufficient for transmitting an accurate signal.

This study identified three CNGCs (*CNGC1*, *CNGC2* and *CNGC19*) that were needed for full defence against *B. cinerea* attack but displayed either transient or no transcriptional regulation by *B. cinerea* infection. Some CNGCs are, however, regulated and there seems to be some specificity in which channels are regulated in response to certain stimuli. *CNGC13* and *CNGC12* displayed contrasting transcriptional regulation in response to *B. cinerea* infection, displaying up and down-regulation respectively. The contrasting transcriptional regulation of *CNGC13* and *CNGC12* in response to *B. cinerea* infection is suggestive of their respective roles in the antagonistic SA and JA defence signalling pathways. *CNGC12* has been shown to play a positive regulatory role in the SA induced defence signalling pathway (Yoshioka *et al.* 2006) and therefore the *B. cinerea* induced JA defence signalling pathway may cause *CNGC12* to be down-regulated to prevent any antagonism between the two defence signalling cascades.

The CNGC subgroups were formed based on amino acid similarity and therefore CNGCs in one subgroup could be functionally similar but thus far, few have displayed functional redundancy. This is apparent in subgroup I where four members display a role in defence signalling. A previous study identified *CNGC11* and *CNGC12*'s involvement in biotrophic defence (Yoshioka *et al.* 2006) and this study identified *CNGC1* and *CNGC13*'s involvement in *B. cinerea* defence. All four genes are hypothesised to be positive regulators of the respective defence signalling cascades as loss of expression causes increased susceptibility to attack. *CNGC1* has also been shown to play a role in nutrient uptake via the roots (Ma *et al.* 2006) and

similarly, CNGC10 and CNGC13 have also been shown to be involved in nutrient uptake and response to salinity stress (Li *et al.* 2005, Gobert *et al.* 2006, Guo *et al.* 2008). Therefore, subgroup I could be designated as the 'biotic and abiotic stress' group.

Thus far, phenotypes associated with members from subgroup II and III have only been described for CNGC18. A few of the CNGCs from these two subgroups are exclusively expressed in reproductive tissue and therefore the lack of studies showing a phenotype with insertion mutants of these two subgroups could be due to embryo lethal phenotypes or high functional redundancy in mutant lines. However, members of subgroup II (CNGC7 and CNGC8) and subgroup III (CNGC16) were shown to localise to distinct endomembrane structures of pollen tubes which suggests that they may have non-redundant roles based on their localisation (Chang *et al.* 2007). CNGC18 displayed plasma membrane localisation and has been shown to be required for pollen tube growth (Chang *et al.* 2007).

Both members of subgroup IVb, CNGC2 and CNGC4, have been shown to be involved in the SA-defence signalling pathway (Clough *et al.* 2000, Balague *et al.* 2003) and this study shows that both members are also involved in *B. cinerea* defence. This is the only subgroup where both members have similar physiological functions *in planta*. CNGC2 and CNGC4 are not found in tandem in the genome. CNGC members of subgroup IVa, CNGC19 and CNGC20, are the closest neighbours of CNGC2 and CNGC4 in terms of protein similarity. CNGC19 and CNGC20 are located in tandem on chromosome III and share 78% similarity in their coding sequence but unexpectedly do not have similar physiological functions. Only one, CNGC19, displays a positive regulatory role in *B. cinerea* defence signalling.

The precise action of CNGCs from different subgroups could be used to co-ordinate the intricate defence signalling pathway and bring about the desired pathogen-specific response. The fact that some CNGCs needed for defence are not transcriptionally regulated suggests that their presence is required for very early responses to initiate the defence signalling cascade whereas those that are transcriptionally regulated, such as CNGC13, could be required for downstream effects. Preliminary characterisation of one defence response in *cngcs* displaying

altered *B. cinerea* defence suggests that different channels have different effects and this could be brought about by a channel's specific localisation.

The phytoalexin, camalexin, has been shown to play an important role in resistance to *B. cinerea* and a study performed by Denby *et al.* (2004) showed a correlation between small lesions and high camalexin levels after infection with the pepper isolate of *B. cinerea*. Analysis of camalexin accumulation in *B. cinerea* infected *cngc*s suggests that only one CNGC is involved in camalexin accumulation as no correlation between camalexin levels and lesion size was found in the other *cngc* mutants. Unlike *cncg19*, erroneous camalexin production could not be attributed to the *B. cinerea* phenotypes displayed by the other *cngc*s.

The increased susceptibility of *cngc1* and *cngc13* plants is not due to a lack of camalexin accumulation as they display increased or wildtype levels of camalexin respectively. The loss of *CNGC1* and *CNGC13* expression may be affecting a different part of the *B. cinerea* defence signalling pathway and therefore further defence characterisation of these mutants would be useful to understand its role in *B. cinerea* defence and whether they are involved in defence to other pathogens. In the case of *CNGC13*, expression is induced at the border of the forming lesion as seen in infected *CNGC13::GUS* leaves and therefore *CNGC13* may be important for signalling events to maintain induced defence mechanisms at the border of the forming lesions to prevent further growth of *B. cinerea in planta*.

The *cngc2* and *cngc4* lines displayed lower camalexin levels yet were more resistant to *B. cinerea* infection. As these lines do produce dwarfed leaves, the entire leaf was harvested to keep the amount of tissue harvested constant between all lines. The degree of infection together with the increased leaf tissue harvested could have an effect on the camalexin measurement. Studies have shown that uninfected *cngc2* and *cngc4* plants constitutively express certain defence mechanisms but this does not include camalexin (Clough *et al.* 2000, Jirage *et al.* 2001, Balague *et al.* 2003). These plants are, however, able to accumulate camalexin in response to virulent *P. syringae* (Jirage *et al.* 2001) which indicates that the lack of camalexin accumulation after *B. cinerea* is not due to them being unable to produce camalexin *per se*. The

constitutive activation of other defence mechanisms could be responsible for the increased defence response to *B. cinerea*.

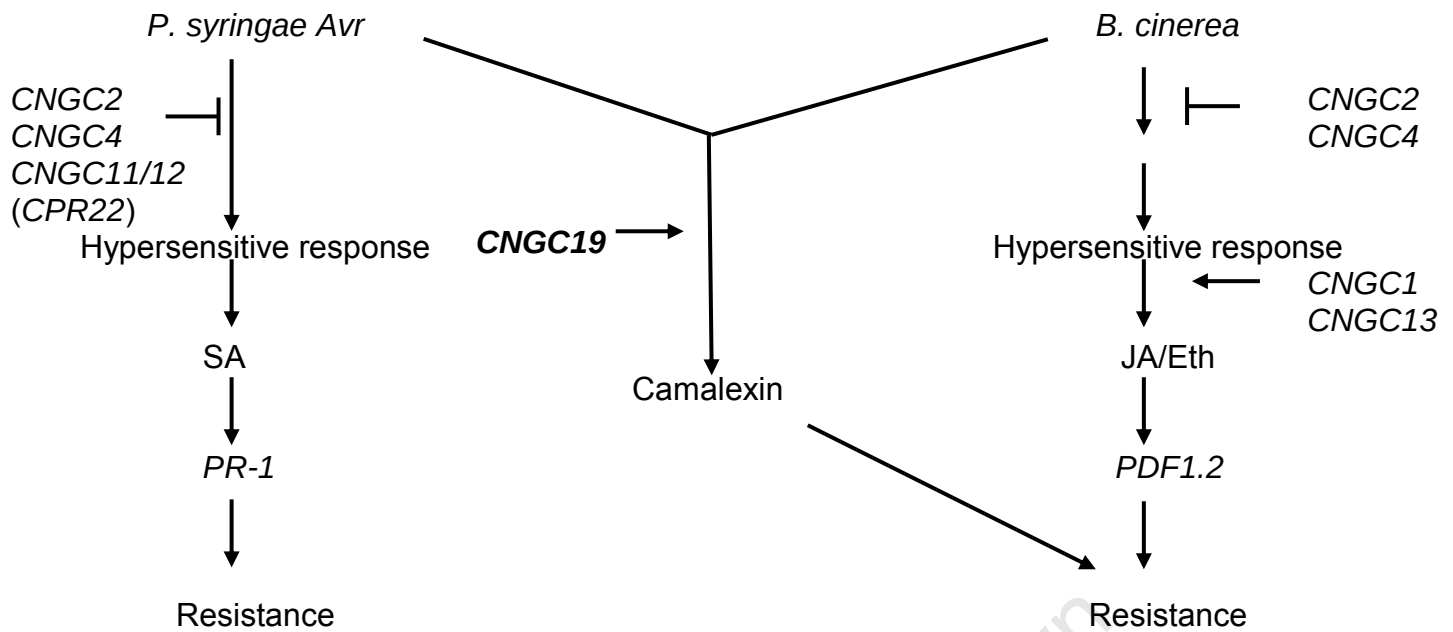
The *cngc19* plants appear to have functional JA/Eth induced defence signalling in response to *B. cinerea* infection but the lack of camalexin accumulation at the border of the forming lesion could be contributing to its increased susceptibility phenotype. Camalexin is not needed for full defence against *P. syringae* and therefore if the main function of *CNGC19* is to induce camalexin accumulation then a loss of *cngc19* expression will not affect *P. syringae* defence. This is consistent with the observation that *in planta* growth of virulent and avirulent strains of *P. syringae* was not significantly different between wildtype and *cngc19* mutant lines. The function of *CNGC19* may be to transduce the signal needed for camalexin accumulation by inducing expression of genes needed for camalexin biosynthesis, such as *CYP71B15* (*PAD3*).

$\text{Ca}^{2+}$  influx is critical for setting up an effective defence response and it has been suggested that the spatial and temporal patterns of this ion flux will determine which pathway is activated (Lecourieux *et al.* 2006). In the case of *CNGC19* the absolute level of protein expression, as inferred by its transcript abundance in the knockout and over-expressing lines, seems to be important for *B. cinerea* defence. As both the loss and gain of a plasma membrane targeted channel displaying the ability to transport  $\text{Ca}^{2+}$  causes a defence phenotype, it could be hypothesised that *CNGC19* allows the influx of a certain  $\text{Ca}^{2+}$  threshold needed for the downstream activation of camalexin accumulation. However, protein levels in the overexpressing lines must be determined to confirm whether *CNGC19* does accumulate to high levels in these lines.

The members of this large ion channel family are spread throughout the plant, at various stages of development and are expressed at different levels, which could suggest a way of fine-tuning signalling cascades of two very important second messenger systems. This study has identified four CNGCs, *CNGC1*, *CNGC4*, *CNGC13* and *CNGC19*, required for full defence against *B. cinerea* and preliminary defence characterisation indicates that they each have a unique role to play. Unlike *CNGC2*, which plays a role in both *B. cinerea* and *P. syringae* defence (Clough *et al.*

2000), CNGC19 was shown to be specifically involved in *B. cinerea* defence. In contrast to CNGC19's function, this study showed that CNGC12, which is needed for defence against biotrophic pathogens (Yoshioka *et al.* 2006), was not needed for *B. cinerea* defence. This study provides more evidence that CNGCs are not functionally redundant and have specific tasks to fulfil in response to certain pathogens. To summarise, a model which indicates 'when' and 'how' CNGCs function in defence signalling pathways is depicted in Figure 28.

This model does suggest that CNGC19 has a unique role in *B. cinerea* defence but further defence characterisation of *cngc19* is needed to understand how and why this increased susceptibility phenotype is caused. A construct containing *CNGC19* under its native promoter, must be used for complementation analysis of the *B. cinerea* susceptibility and camalexin accumulation phenotypes in the *cngc19* lines. Similarly, to gain some understanding of channel regulation during defence signalling, the site-directed mutagenised *CNGC19* gene must be expressed under its native promoter for complementation analysis of the *cngc19* defence phenotype. The site-directed mutagenised CNGC19 protein could also be heterologously expressed in various ion transport yeast mutants to determine the channel's regulatory properties. Finally, as *cngc19* has been shown to transport  $\text{Ca}^{2+}$ , a *cngc19:Aeq* transgenic line could be used to determine whether the  $\text{Ca}^{2+}$  signature brought about by *B. cinerea* infection has been disrupted in these mutants which would provide a direct link between  $\text{Ca}^{2+}$  and CNGC signalling during the defence response. The *cngc19:Aeq* line could also be used for complementation, over-expression and site-directed mutagenised analysis which would provide further details of CNGC19's role in co-ordinating the  $\text{Ca}^{2+}$  and CN second messenger signalling pathways.



**Figure 28. A model describing putative roles of CNGCs in defence signalling pathways.** *CNGC2*, *CNGC4* and the fused *CNGC11/12* (*CPR22*) genes are placed upstream of the hypersensitive response (HR) as mutations in these genes cause spontaneous lesion development and constitutive expression of defence genes, *PR-1* and *PDF1.2*. *CNGC1* and *CNGC13* are placed below the HR as loss of these genes do not affect cell death pathways but do cause increase susceptibility to *B. cinerea* infection. Loss of *CNGC19* expression does not cause cell death phenotypes or constitutive defence gene expression but does result in lower camalexin accumulation which could be the reason for the increased susceptibility of *cngc19* to *B. cinerea* infection.

## Chapter 5

### Concluding Remarks

The presence of approximately thirty cation channel proteins with cyclic nucleotide (CN) binding domains in *Arabidopsis*, suggests that CN signalling plays a vital role in maintaining and regulating ion homeostasis needed for a variety of plant processes and signalling cascades. Two-thirds of these channels belong to the cyclic nucleotide gated channel (CNGC) family and numerous studies have shown that detrimental plant phenotypes are associated with the loss of a single CNGC (Clough *et al.* 2000, Balague *et al.* 2003, Chang *et al.* 2007). Whether the function of CNGCs is CN dependent *in planta* is yet to be confirmed as no study has shown the direct interaction between a CN and CNGC protein. However, studies have shown direct binding between plant CNGCs and various calmodulin isoforms which does suggest that plant CNGCs are regulated similarly to their animal homologues (Köhler and Neuhaus 2000, Ali *et al.* 2006).

Gene expression profiling of leaf tissue treated with membrane soluble cyclic guanosine monophosphate (cGMP) identified transcripts that could be associated with various biological functions involving CNGCs and provides some evidence to link CNGCs and CN signalling. As CNs are thought to be second messenger molecules, its specific spatio-temporal pattern could be important for activating the correct signalling cascade and may be dependent on channels immediately available to transmit its message. Therefore, the increased CN levels could be interpreted slightly differently in root and leaf tissue depending on which CN responder molecules are present. In keeping with its role as a second messenger, cGMP has a transient effect on gene regulation as the number of transcripts affected by cGMP treatment decreases within a few hours of treatment. This was evident in the case of CNGCs where five were significantly regulated two hours post infection but only one displayed a difference three hours later in root tissue (Maathuis 2006a). No CNGCs were significantly regulated in leaf tissue four hours post treatment and the identification of no transcripts displaying a significant difference in regulation ten hours post treatment re-iterates the short-lived action of CNs on transcriptional regulation.

CNGCs are hypothesised to act very early in signalling cascades and therefore these channels should be specifically expressed when and where they are needed to perform their function and transcriptional regulation would not be efficient for this purpose. Besides the link between CNGCs and CNs, CNGCs are also implicated in another important second messenger pathway controlled by calcium ( $\text{Ca}^{2+}$ ). Stringent regulation of cation flux across cellular membranes is critical to obtain the desired effect initiated by internal or external stimuli and the importance of a precise ion flux can be clearly illustrated when characterising a plant that has lost or gained the function of a CNGC which has the ability to transport  $\text{Ca}^{2+}$ . CNGC19 displayed the ability to complement the  $\Delta\text{mid1}\Delta\text{cch1}$  yeast mutant, which lacks plasma membrane high affinity  $\text{Ca}^{2+}$  channels and this study also shows that altered CNGC19 expression, inferred by its transcript levels, causes increased susceptibility to *B. cinerea* attack. The actual level of expression, mode of regulation and ion transport ability of CNGC19 *in planta*, still needs to be investigated to determine its role *B. cinerea* defence. Defence characterisation indicates that the increased susceptibility phenotype of *cngc19* could be due to a lack of camalexin accumulation but further investigation is needed to link mis-regulated CNGC19 expression to altered camalexin levels after *B. cinerea* infection. To determine whether the regulatory domains of the CNGC affect its role in *B. cinerea* defence, complementation analysis of knockout mutants with a site-directed mutagenised CNGC19 under the control of the native promoter can be used for *B. cinerea* susceptibility assays and camalexin measurements.

This study also illustrates the non-redundant function of a few CNGCs in plant-pathogen interactions needed to orchestrate the complex defence signalling cascade. Some CNGCs are responsible for defence against specific pathogens and seem to be acting at different stages of the signalling pathway. This could be brought about by the specific localisation of CNGCs and which ions they transport to create a distinct ion flux or signature. Studies of defence-related phenotypes displayed by the *cpr22* mutant has shown that could be a result of improper potassium ( $\text{K}^+$ ) signalling caused by a fused CNGC11/CNGC12 protein (Baxter *et al.* 2008) whereas imprecise  $\text{Ca}^{2+}$  signalling is the cause of defence-related phenotypes displayed by the *cngc2* null mutant (Ali *et al.* 2007). The  $\text{Ca}^{2+}$  reporting protein, Aequorin (Aeq), is a good tool to identify whether  $\text{Ca}^{2+}$  signalling has been altered due to the loss of one or

more CNGC function. Luminometry or low-light imaging of *cngc:Aeq* plants treated with various stimuli could provide vital information linking CNGCs and  $\text{Ca}^{2+}$  to a specific signalling pathway. As CNGC19 has been shown to allow  $\text{Ca}^{2+}$  transport, comparison of low-light images of *B. cinerea* infected Col-0:*Aeq* and *cngc19:Aeq* plants over time could illustrate whether CNGC19 is responsible for critical points of  $\text{Ca}^{2+}$  signalling in defence. This data can also be supported with analysis of *cngc19:Aeq* lines which either over-express CNGC19 or express wildtype levels of site-directed mutagenised CNGC19 which would directly link CNGC19 expression or regulation to  $\text{Ca}^{2+}$  defence signalling respectively.

This study identified four CNGCs required for full defence against *B. cinerea* in addition to CNGC2 which had been identified in a previous study (Govrin and Levine 2000). Analysis of one *B. cinerea* defence hallmark indicates that these channels may have unique roles to play and epistasis tests between some *cngc* mutant lines may provide some vital information about the order in which these channels are functioning during defence.

Over the past ten years approximately thirty studies have been published which detail the characterisation of half the members of the *Arabidopsis* CNGC family. These non-selective cation channels bring together three important signalling molecules (ion flux,  $\text{Ca}^{2+}$  and CNs) essential for various plant processes and gradually more studies are likely to discover a unique role for each member of CNGC family and precisely how they act in specific signalling responses.

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## Appendix

**Appendix A: A list of 246 genes displaying at least 1.5 fold differences in expression between cGMP treated and control leaves four hour post treatment.** The genes are listed in descending order based on the  $\log_2$  ratio between treated and control samples.

| AGI       | Tair.7 description                                              | Log <sub>2</sub> ratio |
|-----------|-----------------------------------------------------------------|------------------------|
| At5g52900 | Unknown protein                                                 | 2.730                  |
| At1g07940 | Calmodulin binding / translation elongation factor              | 2.584                  |
| At3g50160 | Unknown protein                                                 | 2.005                  |
| At3g04630 | WDL1 (WVD2-LIKE 1)                                              | 1.865                  |
| At5g44710 | Unknown protein                                                 | 1.862                  |
| At2g23170 | GH3.3; indole-3-acetic acid amido synthetase                    | 1.825                  |
| At4g38860 | Auxin-responsive protein                                        | 1.674                  |
| At5g36260 | Aspartyl protease family protein                                | 1.543                  |
| At3g24620 | ATROPGEF8/ROPGEF8 (KINASE PARTNER PROTEIN-LIKE)                 | 1.322                  |
| At2g19590 | ACO1 (ACC OXIDASE 1); 1-aminocyclopropane-1-carboxylate oxidase | 1.226                  |
| At5g49320 | Unknown protein                                                 | 1.124                  |
| At3g30720 | Unknown protein                                                 | 1.093                  |
| At5g14320 | 30S ribosomal protein S13, chloroplast (CS13)                   | 1.062                  |
| At2g33450 | 50S ribosomal protein L28, chloroplast (CL28)                   | 1.055                  |
| At5g11160 | ADENINE PHOSPHORIBOSYLTRANSFERASE 5 (APT5)                      | 1.054                  |
| At3g14560 | Unknown protein                                                 | 1.034                  |
| At5g24920 | Unknown protein; similar to GLUTAMINE DUMPER 1 (GDU1)           | 1.014                  |
| At5g48630 | Cyclin family protein                                           | 0.992                  |
| At3g57600 | AP2 domain-containing transcription factor                      | 0.973                  |
| At3g16140 | Photosystem I subunit H-1 (PSAH-1)                              | 0.947                  |
| At1g67900 | Phototropic-responsive NPH3 family protein;                     | 0.946                  |
| At2g32560 | F-box family protein                                            | 0.943                  |
| At4g21445 | Unknown protein                                                 | 0.932                  |
| At2g24020 | Unknown protein                                                 | 0.918                  |
| At2g46820 | THYLAKOID MEMBRANE PHOSPHOPROTEIN OF 14 KDA (TMP14)             | 0.910                  |
| At1g18060 | Unknown protein                                                 | 0.895                  |
| At5g57500 | Transferase, transferring glycosyl groups                       | 0.863                  |
| At4g04840 | Methionine sulfoxide reductase domain-containing protein        | 0.862                  |
| At5g14410 | Unknown protein                                                 | 0.861                  |
| At1g43560 | Arabidopsis thioredoxin y2 (ATY2)                               | 0.858                  |
| At3g56910 | PLASTID-SPECIFIC 50S RIBOSOMAL PROTEIN 5 (PSRP5)                | 0.840                  |
| At4g09650 | ATP synthase delta chain, chloroplast                           | 0.839                  |
| At5g59520 | ZINC TRANSPORTER 2 PRECURSOR (ZIP2)                             | 0.837                  |
| At3g06740 | Zinc finger (GATA type) family protein                          | 0.826                  |

| AGI       | Tair.7 description                                                                                             | Log <sub>2</sub> ratio |
|-----------|----------------------------------------------------------------------------------------------------------------|------------------------|
| At1g73060 | Unknown protein                                                                                                | 0.811                  |
| At5g61410 | EMBRYO DEFECTIVE 2728 (RPE)                                                                                    | 0.811                  |
| At2g17972 | Unknown protein                                                                                                | 0.809                  |
| At5g13510 | Ribosomal protein L10 family protein                                                                           | 0.788                  |
| At2g25080 | GLUTATHIONE PEROXIDASE 1 (ATGPX1)                                                                              | 0.786                  |
| At1g32550 | Ferredoxin family protein                                                                                      | 0.776                  |
| At3g01950 | Unknown protein                                                                                                | 0.771                  |
| At5g48790 | Unknown protein                                                                                                | 0.765                  |
| At1g13510 | Unknown protein                                                                                                | 0.762                  |
| At5g22660 | F-box family protein                                                                                           | 0.749                  |
| At5g19940 | Plastid-lipid associated protein PAP-related / fibrillin-related                                               | 0.741                  |
| At2g41310 | RESPONSE REGULATOR 3 (ATRR3)                                                                                   | 0.732                  |
| At5g44990 | Unknown protein                                                                                                | 0.730                  |
| At3g05780 | Lon protease, putative                                                                                         | 0.727                  |
| At3g17120 | Unknown protein                                                                                                | 0.715                  |
| At3g52740 | Unknown protein                                                                                                | 0.712                  |
| At3g17670 | binding; similar to stress-inducible protein                                                                   | 0.710                  |
| At2g28740 | Histone H4 (HIS4)                                                                                              | 0.708                  |
| At1g70760 | Inorganic carbon transport protein-related                                                                     | 0.701                  |
| At2g15800 | Unknown protein                                                                                                | 0.692                  |
| At1g50900 | Unknown protein                                                                                                | 0.684                  |
| At3g22250 | UDP-glucuronosyl/UDP-glucosyl transferase family protein                                                       | 0.681                  |
| At3g53990 | Universal stress protein (USP) family protein                                                                  | 0.680                  |
| At4g03280 | Cytochrome B6-F complex iron-sulfur subunit                                                                    | 0.679                  |
| At4g33625 | Unknown protein                                                                                                | 0.675                  |
| At3g04790 | Ribose 5-phosphate isomerase-related                                                                           | 0.673                  |
| At4g04640 | ATP synthase gamma chain 1 (ATPC1)                                                                             | 0.671                  |
| At4g01150 | Unknown protein; similar to threonine endopeptidase                                                            | 0.668                  |
| At5g03140 | Lectin protein kinase family protein;                                                                          | 0.657                  |
| At2g37370 | Unknown protein                                                                                                | 0.654                  |
| At5g02120 | ONE HELIX PROTEIN (OHP)                                                                                        | 0.650                  |
| At5g59760 | Unknown protein                                                                                                | 0.639                  |
| At3g61970 | NGATHA2; transcription factor                                                                                  | 0.627                  |
| At1g49660 | Unknown protein; similar to cell death associated protein-related                                              | 0.626                  |
| At3g10620 | Diadenosine 5',5'''-P1,P4-tetraphosphate hydrolase, putative                                                   | 0.626                  |
| At3g60370 | Immunophilin / FKBP-type peptidyl-prolyl cis-trans isomerase family protein                                    | 0.625                  |
| At5g08410 | Ferredoxin/thioredoxin reductase subunit A (variable subunit) 2 (FTRA2)                                        | 0.618                  |
| At5g57345 | Unknown protein                                                                                                | 0.614                  |
| At2g28760 | UXS6; similar to UXS5 (UDP-Xyl synthase 5)                                                                     | 0.607                  |
| At2g39681 | Expressed gene                                                                                                 | 0.607                  |
| At4g29610 | Cytidine deaminase                                                                                             | 0.594                  |
| At4g22890 | Unknown protein                                                                                                | 0.587                  |
| At3g53470 | Unknown protein                                                                                                | 0.585                  |
| At4g17600 | Transcription factor; Encodes light-harvesting-like protein (LIL3:1)                                           | 0.570                  |
| At5g44650 | Unknown protein                                                                                                | 0.570                  |
| At1g33820 | Unknown protein                                                                                                | 0.565                  |
| At5g42750 | Unknown protein; Encodes a PM associated phosphoprotein that interacts directly with the kinase domain of BRI1 | 0.563                  |
| At5g64240 | Latex-abundant family protein (AMC3) / caspase family protein;                                                 | 0.563                  |

| AGI       | Tair.7 description                                                                                   | Log <sub>2</sub> ratio |
|-----------|------------------------------------------------------------------------------------------------------|------------------------|
| At5g65840 | Unknown protein                                                                                      | 0.559                  |
| At5g28785 | Hypothetical protein                                                                                 | 0.555                  |
| At1g04290 | Thioesterase family protein                                                                          | 0.551                  |
| At4g39710 | Immunophilin, putative / FKBP-type peptidyl-prolyl cis-trans isomerase, putative                     | 0.546                  |
| At3g60210 | Chloroplast chaperonin 10, putative;                                                                 | 0.540                  |
| At4g14880 | O-ACETYL SERINE (THIOL) LYASE (OAS-TL) ISOFORM A1 (OASA1)                                            | 0.538                  |
| At3g20800 | Rcd1-like cell differentiation protein, putative                                                     | 0.538                  |
| At5g16140 | Peptidyl-tRNA hydrolase family protein                                                               | 0.534                  |
| At5g25890 | IAA-ALANINE RESISTANT 2 (IAA28); transcription factor                                                | 0.532                  |
| At4g13650 | Pentatricopeptide (PPR) repeat-containing protein                                                    | 0.526                  |
| At5g42030 | ABL INTERACTOR-LIKE PROTEIN 4 (ABIL4)                                                                | 0.512                  |
| At1g50575 | Lysine decarboxylase family protein                                                                  | 0.512                  |
| At3g59490 | Unknown protein                                                                                      | 0.508                  |
| At5g18850 | Unknown protein                                                                                      | 0.498                  |
| At2g48070 | Unknown protein                                                                                      | 0.492                  |
| At2g13850 | Copia-like retrotransposon family                                                                    | 0.491                  |
| At3g17210 | Stable protein 1-related; Identical to Putative Pop3 protein                                         | 0.476                  |
| At1g32070 | NUCLEAR SHUTTLE INTERACTING (ATNSI)                                                                  | 0.475                  |
| At2g21990 | Unknown protein                                                                                      | 0.470                  |
| At5g12240 | Unknown protein                                                                                      | 0.464                  |
| At2g26340 | Unknown protein                                                                                      | 0.463                  |
| At4g18596 | Pollen Ole e 1 allergen and extensin family protein                                                  | 0.459                  |
| At3g18215 | Unknown protein                                                                                      | 0.452                  |
| At2g16930 | Ribosomal protein L27 family protein                                                                 | 0.450                  |
| At1g68585 | Metal ion binding                                                                                    | 0.442                  |
| At3g08880 | Unknown protein                                                                                      | 0.441                  |
| At4g28640 | Indoleacetic acid-induced protein 11 (IAA11) Auxin induced gene                                      | 0.437                  |
| At3g63490 | Ribosomal protein L1 family protein                                                                  | 0.425                  |
| At3g23400 | Plastid-lipid associated protein PAP / fibrillin family protein                                      | 0.421                  |
| At3g48185 | Unknown protein                                                                                      | 0.416                  |
| At2g26070 | REVERSION-TO-ETHYLENE SENSITIVITY1 (RTE1)                                                            | 0.410                  |
| At4g28025 | Unknown protein                                                                                      | 0.405                  |
| At1g67080 | Unknown protein; Involved in the photoprotection of PSII                                             | 0.402                  |
| At2g18876 | Unknown protein                                                                                      | 0.402                  |
| At1g58170 | Disease resistance-responsive protein-related / dirigent protein-related                             | 0.395                  |
| At5g63000 | Unknown protein                                                                                      | 0.392                  |
| At5g06340 | Diadenosine 5',5'''-P1,P4-tetraphosphate hydrolase, putative                                         | 0.389                  |
| At4g12890 | Gamma interferon responsive lysosomal thiol reductase family protein / GILT family protein           | 0.386                  |
| At4g00230 | XYLEM SERINE PEPTIDASE 1 (XSP1)                                                                      | 0.385                  |
| At5g24850 | CRYPTOCHROME 3 (CRY3); DNA binding / DNA photolyase/ FMN binding                                     | 0.361                  |
| At1g18490 | Unknown protein                                                                                      | 0.344                  |
| At3g08790 | VHS domain-containing protein / GAT domain-containing protein                                        | 0.339                  |
| At4g00560 | Methionine adenosyltransferase regulatory beta subunit-related                                       | 0.328                  |
| At1g62305 | Unknown protein                                                                                      | 0.320                  |
| At5g66510 | GAMMA CARBONIC ANHYDRASE 3(GAMMA CA3)                                                                | 0.314                  |
| At4g15780 | Synaptobrevin-related family protein, similar to Vesicle-associated membrane protein 722 (AtVAMP722) | 0.304                  |
| At2g18770 | Signal recognition particle binding                                                                  | 0.245                  |
| At3g12970 | Unknown protein                                                                                      | -0.168                 |

| AGI       | Tair.7 description                                                                                | Log <sub>2</sub> ratio |
|-----------|---------------------------------------------------------------------------------------------------|------------------------|
| At3g12100 | Cation efflux family protein / metal tolerance protein                                            | -0.247                 |
| At2g03820 | Nonsense-mediated mRNA decay NMD3 family protein                                                  | -0.256                 |
| At1g50140 | AAA-type ATPase family protein                                                                    | -0.267                 |
| At3g17450 | hAT dimerisation domain-containing protein                                                        | -0.273                 |
| At5g10610 | Cytochrome P450 family protein                                                                    | -0.297                 |
| At3g57590 | F-box family protein                                                                              | -0.299                 |
| At2g36330 | Unknown protein; similar to integral membrane protein                                             | -0.306                 |
| At1g71900 | Unknown protein; similar to permease-related                                                      | -0.311                 |
| At5g43920 | Transducin family protein / WD-40 repeat family protein                                           | -0.315                 |
| At1g05380 | DNA binding; similar to PHD finger transcription factor                                           | -0.321                 |
| At5g05520 | Outer membrane OMP85 family protein                                                               | -0.322                 |
| At3g04650 | Oxidoreductase; similar to amine oxidase-related                                                  | -0.325                 |
| At1g07510 | FtsH protease 10 (FTSH10); encodes an FtsH protease that is localized to the mitochondrion        | -0.327                 |
| At1g80550 | Pentatricopeptide (PPR) repeat-containing protein                                                 | -0.328                 |
| At3g13990 | Unknown protein                                                                                   | -0.336                 |
| At3g09570 | Unknown protein                                                                                   | -0.344                 |
| At1g05910 | Cell division cycle protein 48-related / CDC48-related; similar to AAA-type ATPase family protein | -0.355                 |
| At2g45000 | Structural constituent of nuclear pore                                                            | -0.361                 |
| At2g43970 | La domain-containing protein; similar to RNA-binding protein,                                     | -0.361                 |
| At5g56100 | Glycine-rich protein / oleosin                                                                    | -0.362                 |
| At3g53270 | Unknown protein                                                                                   | -0.362                 |
| At3g50810 | Integral membrane protein                                                                         | -0.364                 |
| At5g41770 | Crooked neck protein, putative / cell cycle protein                                               | -0.370                 |
| At3g44370 | Binding; similar to OXA1 family protein                                                           | -0.389                 |
| At3g05940 | Unknown protein                                                                                   | -0.394                 |
| At3g13672 | Seven in absentia (SINA) family protein                                                           | -0.394                 |
| At3g51460 | Phosphoinositide phosphatase family protein                                                       | -0.396                 |
| At3g24070 | Zinc knuckle (CCHC-type) family protein                                                           | -0.396                 |
| At1g53200 | Binding                                                                                           | -0.398                 |
| At1g09640 | Elongation factor 1B-gamma                                                                        | -0.408                 |
| At4g31410 | Unknown protein                                                                                   | -0.413                 |
| At4g38130 | HISTONE DEACETYLASE 19 (HD19)                                                                     | -0.423                 |
| At1g09830 | Phosphoribosylamine-glycine ligase (PUR2)                                                         | -0.423                 |
| At1g34220 | Unknown protein                                                                                   | -0.426                 |
| At5g15980 | Pentatricopeptide (PPR) repeat-containing protein                                                 | -0.428                 |
| At1g28340 | Leucine-rich repeat family protein                                                                | -0.432                 |
| At4g02150 | ATIMPALPHA3/MOS6; protein transporter                                                             | -0.435                 |
| At3g13320 | CATION EXCHANGER 2 (CAX2)                                                                         | -0.438                 |
| At1g08030 | Unknown protein                                                                                   | -0.440                 |
| At4g28600 | NO POLLEN GERMINATION RELATED 2 (NPGR2)                                                           | -0.444                 |
| At5g51280 | DEAD-box protein                                                                                  | -0.444                 |
| At5g01290 | mRNA guanylyltransferase                                                                          | -0.445                 |
| At5g19900 | PRLI-interacting factor                                                                           | -0.447                 |
| At5g01980 | Zinc finger (C3HC4-type RING finger) family protein                                               | -0.451                 |
| At2g19520 | FVE (FVE); Controls flowering                                                                     | -0.456                 |
| At5g22350 | Unknown protein                                                                                   | -0.456                 |
| At4g14180 | Unknown protein                                                                                   | -0.456                 |
| At1g75200 | Flavodoxin family protein / radical SAM domain-containing protein                                 | -0.458                 |

| AGI       | Tair.7 description                                                      | Log <sub>2</sub> ratio |
|-----------|-------------------------------------------------------------------------|------------------------|
| At5g61380 | TIMING OF CAB1 1 (TOC1)                                                 | -0.461                 |
| At1g55120 | BETA-FRUCTOFURANOSIDASE 5 (ATFRUCT5)                                    | -0.467                 |
| At3g12360 | Ankyrin repeat family protein                                           | -0.472                 |
| At3g19540 | Unknown protein                                                         | -0.473                 |
| At3g60630 | Scarecrow transcription factor family protein                           | -0.474                 |
| At5g25230 | Elongation factor Tu family protein                                     | -0.479                 |
| At4g24330 | Unknown protein                                                         | -0.484                 |
| At3g61830 | AUXIN RESPONSE FACTOR 18 (ARF18)                                        | -0.485                 |
| At2g27210 | Kelch repeat-containing serine/threonine phosphoesterase family protein | -0.486                 |
| At2g35390 | Ribose phosphate diphosphokinase                                        | -0.491                 |
| At2g14120 | Dynamin-like protein 2b (ADL2b)                                         | -0.492                 |
| At1g20620 | CATALASE 3 (CAT3)                                                       | -0.494                 |
| At3g19740 | ATPase; similar to AAA-type ATPase family protein                       | -0.496                 |
| At3g51895 | SULFATE TRANSPORTER 1 (SULTR3;1)                                        | -0.496                 |
| At4g22270 | Unknown protein                                                         | -0.508                 |
| At2g17510 | Ribonuclease II family protein                                          | -0.509                 |
| At5g44320 | Eukaryotic translation initiation factor 3 subunit 7                    | -0.518                 |
| At3g25800 | PDF1 (65 KDA REGULATORY SUBUNIT OF PROTEIN PHOSPHATASE 2A)              | -0.524                 |
| At1g49540 | Nucleotide binding; similar to WD-40 repeat family protein              | -0.534                 |
| At2g31870 | TEJ (SANSKRIT FOR 'BRIGHT')                                             | -0.543                 |
| At5g47800 | Phototropic-responsive NPH3 family protein                              | -0.549                 |
| At3g47570 | Leucine-rich repeat transmembrane protein kinase                        | -0.556                 |
| At1g13440 | GAPC-2                                                                  | -0.563                 |
| At3g09840 | CELL DIVISION CYCLE 48 (CDC48)                                          | -0.564                 |
| At4g02570 | CULLIN 1 (ATCUL1)                                                       | -0.565                 |
| At1g16010 | Magnesium transporter CorA-like family protein (MRS2-1)                 | -0.569                 |
| At5g08620 | DEAD box RNA helicase (RH25)                                            | -0.578                 |
| At4g15020 | Unknown protein; similar to DNA binding / protein dimerization          | -0.582                 |
| At1g63360 | Disease resistance protein (CC-NBS-LRR class)                           | -0.589                 |
| At1g22015 | DD46; transferase, transferring hexosyl groups                          | -0.590                 |
| At3g06860 | MULTIFUNCTIONAL PROTEIN (MFP2)                                          | -0.594                 |
| At5g13030 | Unknown protein                                                         | -0.595                 |
| At1g70770 | Unknown protein                                                         | -0.599                 |
| At5g35560 | DENN (AEX-3) domain-containing protein                                  | -0.601                 |
| At1g07920 | Elongation factor 1-alpha                                               | -0.602                 |
| At3g13690 | Protein kinase family protein                                           | -0.618                 |
| At5g14930 | SENESCENCE-ASSOCIATED GENE 101 (SAG101)                                 | -0.622                 |
| At2g31480 | Unknown protein                                                         | -0.662                 |
| At2g37690 | Phosphoribosylaminoimidazole carboxylase                                | -0.667                 |
| At3g18020 | Pentatricopeptide (PPR) repeat-containing protein                       | -0.682                 |
| At5g18960 | FAR1-RELATED SEQUENCE 12 (FRS12)                                        | -0.688                 |
| At5g11030 | ABERRANT LATERAL ROOT FORMATION 4 (ALF4)                                | -0.711                 |
| At3g17520 | Late embryogenesis abundant domain-containing protein                   | -0.751                 |
| At3g23590 | Unknown protein; similar to structural constituent of ribosome          | -0.754                 |
| At4g30200 | Unknown protein; similar to VIN3 (VERNALIZATION INSENSITIVE 3)          | -0.758                 |
| At5g12380 | Annexin, putative; similar to ANN7 (ANN7, ANNEXIN ARABIDOPSIS 7)        | -0.763                 |
| At5g47880 | EUKARYOTIC RELEASE FACTOR 1-1 (ERF1-1)                                  | -0.768                 |
| At1g02650 | DNAJ heat shock N-terminal domain-containing protein                    | -0.771                 |
| At1g10510 | EMBRYO DEFECTIVE 2004 (EMB2004)                                         | -0.780                 |

| AGI       | Tair.7 description                                           | Log <sub>2</sub> ratio |
|-----------|--------------------------------------------------------------|------------------------|
| At2g23150 | NRAMP metal ion transporter 3 (NRAMP3)                       | -0.782                 |
| At1g24340 | EMBRYO DEFECTIVE 2421 (EMB2421)                              | -0.798                 |
| At3g16210 | F-box family protein                                         | -0.816                 |
| At1g76390 | Armadillo/beta-catenin repeat family protein                 | -0.862                 |
| At5g36930 | Disease resistance protein (TIR-NBS-LRR class)               | -0.864                 |
| At3g18830 | ATPLT5POLYOL TRANSPORTER 5 (ATPLT5)                          | -0.886                 |
| At5g26340 | SUGAR TRANSPORT PROTEIN 13 (MSS1)                            | -0.925                 |
| At4g28570 | Alcohol oxidase-related                                      | -0.949                 |
| At4g13510 | AMMONIUM TRANSPORT 1 (AMT1;1)                                | -0.953                 |
| At3g47960 | Proton-dependent oligopeptide transport (POT) family protein | -1.038                 |
| At2g45500 | ATP binding; similar to ATPase                               | -1.040                 |
| At1g51805 | Leucine-rich repeat protein kinase, putative                 | -1.057                 |
| At2g31020 | Oxysterol-binding family protein                             | -1.058                 |
| At4g36220 | FERULATE-5-HYDROXYLASE 1 (FAH1)                              | -1.067                 |
| At4g15530 | PYRUVATE ORTHOPHOSPHATE DIKINASE (PPDK)                      | -1.071                 |
| At4g02510 | Translocon outer membrane complex 159 (TOC159)               | -1.100                 |
| At1g78000 | SULFATE TRANSPORTER 1;2 (SULTR1;2)                           | -1.131                 |
| At4g31120 | Skb1 methyltransferase family protein                        | -1.214                 |
| At3g09920 | PHOSPHATIDYL INOSITOL MONOPHOSPHATE 5 KINASE 9 (PIP5K9)      | -1.355                 |