Investigation of structure-function and signal transduction of plant cyclic nucleotide-gated ion channels

by

Kimberley Chin

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

Department of Cell and Systems Biology

University of Toronto

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2013

Abstract

Cyclic nucleotide-gated channels (CNGCs) are non-selective cation channels that were first identified in vertebrate photosensory and olfactory neurons. Although the physiological roles and biophysical properties of animal CNGCs have been well studied, much less is known about these channels in plants. The Arabidopsis genome encodes twenty putative CNGC subunits that are postulated to form channel complexes that mediate various physiological processes involving abiotic and biotic stress responses, ion homeostasis and development.

The identification of Arabidopsis autoimmune CNGC mutants, such as defense no death class (dnd1 and dnd2), and the constitutive expressor of pathogenesis related genes 22 (cpr22) implicate AtCNGC2, 4, 11 and 12 in plant immunity. Here, I present a comprehensive study of the molecular mechanisms involved in CNGC-mediated signaling pathways with emphasis on pathogen defense. Previously, a forward genetics approach aimed to identify suppressor mutants of the rare gain-of-function autoimmune mutant, cpr22, identified key residues that are important for CNGC subunit interactions and channel function.
First, I present a structure-function analysis of one of these suppressor mutants (S58) that revealed a key residue in the cyclic nucleotide binding domain involved in the stable regulation of CNGCs. Second, I present a new suppressor screen using AtCNGC2 T-DNA knockout mutants that specifically aimed to identify novel downstream components of CNGC-mediated pathogen defense signaling. In this screen, I successfully isolated and characterized the novel Arabidopsis mutant, repressor of defense no death 1 (rdd1), and expanded this study to demonstrate its involvement in AtCNGC2 and AtCNGC4-mediated signal transduction. Additionally, I demonstrated for the first time, the physical interaction of AtCNGC2 and AtCNGC4 subunits in planta.

The findings presented in this thesis broaden our current knowledge of CNGCs in plants, and provide a new foundation for future elucidation of the structure-function relationships and signal transduction mediated by these channels.
Acknowledgements

I would first like to extend my deepest gratitude to Dr. Keiko Yoshioka for providing me with the opportunity to be part of this incredible research project. It is her boundless enthusiasm for science, consistent mentorship and constant support that not only provided an exciting lab environment to work in, but also allowed for the many successes I have achieved during my doctoral candidacy.

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I further extend my gratitude to the members of my supervisory committee, Dr. Darrell Desveaux and Dr. Malcolm Campbell, for their invaluable insight and input into my research project(s), and for guiding me towards the successes I have achieved during my doctoral candidacy.

Lastly, I am immensely grateful to my beloved family and friends who have provided me with the endless encouragement and occasional kick to keep me focused throughout my academic career. To mum and dad, I acknowledge that it is hard at times to understand what it is that I do, and I am forever indebted to you for your continued patience and support in all of my endeavours. It is your sacrifices that inspire me to pursue everything that I desire without reservations. Words cannot describe how appreciative I am of all that you have done for me. Thank you.
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<tr>
<td>$\chi^2$</td>
<td>Chi-squared</td>
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<td>2D</td>
<td>2 Dimensional</td>
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<td>3D</td>
<td>3 Dimensional</td>
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<td>Ac-YVAD-CHO</td>
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<td>AOTF</td>
<td>Acousto-Optic Tunable Filter</td>
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<tr>
<td>A</td>
<td>Adenine</td>
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<td>AND</td>
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<td>AET</td>
<td>Alanine-Glutamic acid-Threonine</td>
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<tr>
<td>nYFP</td>
<td>Amino portion of yellow fluorescent protein</td>
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<tr>
<td>NH$_4^+$</td>
<td>Ammonium ion</td>
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<tr>
<td>AtCNGC</td>
<td>Arabidopsis thaliana cyclic nucleotide-gated channel</td>
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<tr>
<td>R</td>
<td>Arginine</td>
</tr>
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<td>D</td>
<td>Aspartic acid</td>
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<td>B$_1$</td>
<td>Backcross (first generation)</td>
</tr>
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<td>B$_2$</td>
<td>Backcross (second generation)</td>
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<td>BiFC</td>
<td>Bimolecular fluorescence complementation</td>
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<td>Cd$^{2+}$</td>
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<td>CaM</td>
<td>Calmodulin</td>
</tr>
<tr>
<td>CaMBD</td>
<td>Calmodulin binding domain</td>
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<tr>
<td>CML</td>
<td>Calmodulin-like</td>
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<td>cYFP</td>
<td>Carboxyl portion of yellow fluorescent protein</td>
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<td>C-terminus/terminal</td>
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<td>CLZ</td>
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<td>CaMV35S</td>
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<td>Cm</td>
<td>Centimeters</td>
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<td>Cs$^+$</td>
<td>Cesium ion</td>
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<td>CAPS</td>
<td>Cleaved amplified polymorphic sequences</td>
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<td>Cfu</td>
<td>Colony forming units</td>
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<td>Col-wt</td>
<td>Columbia wildtype</td>
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<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
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<td>cpr22</td>
<td>constitutive expresser of pathogenesis related genes 22</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine-3',5' triphosphate</td>
</tr>
<tr>
<td>cGMP</td>
<td>Cyclic guanosine-3',5' triphosphate</td>
</tr>
<tr>
<td>CNBD</td>
<td>Cyclic nucleotide binding domain</td>
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</table>
CNGA  Cyclic nucleotide-gated channel alpha
CNGB  Cyclic nucleotide-gated channel beta
C    Cysteine
C    Cytosine
°C  Degrees Celsius
DAMP Damage associated molecular pattern
    or
    Danger associated molecular pattern
dCAPS Derived cleaved amplified polymorphic sequences
dnd1 defense no death 1
dnd2 defense no death 2
DIC Differential Interference Contrast (Microscopy)
EF1α Elongation Factor 1 α
EV    Empty vector
E. coli Escherichia coli
EtOH  Ethanol
Eag   Ether-a-go-go
ET    Ethylene
EDTA  Ethylenediaminetetraacetic acid
EMS   Ethyl methanesulfonate
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<td>Epac1</td>
<td>Exchange proteins activated by cyclic AMP 1</td>
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<td>Filial third generation</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
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<tr>
<td>FMO</td>
<td>Flavin-containing monooxygenase</td>
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<tr>
<td>FLC</td>
<td>Flowering Locus C</td>
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<td>Gd&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Gadolinium ion</td>
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<td>GC-MS</td>
<td>Gas chromatography-mass spectrometry</td>
</tr>
<tr>
<td>Q</td>
<td>Glutamine</td>
</tr>
<tr>
<td>E</td>
<td>Glutamic acid</td>
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<td>GET</td>
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<td>hlm1</td>
<td>HR-like lesion mimic 1</td>
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<td>HEK</td>
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<td>HCl</td>
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<td>SpIH</td>
<td>(Sea urchin) isolated CNGC</td>
</tr>
<tr>
<td>SSLP</td>
<td>Simple sequence length polymorphism</td>
</tr>
<tr>
<td>SDM</td>
<td>Site directed mutagenesis</td>
</tr>
<tr>
<td>SPE</td>
<td>Solid phase extraction</td>
</tr>
<tr>
<td>NaOAC</td>
<td>Sodium acetate</td>
</tr>
<tr>
<td>Na⁺</td>
<td>Sodium ion</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>SNP</td>
<td>Sodium nitroprusside</td>
</tr>
<tr>
<td>SE</td>
<td>Standard error</td>
</tr>
<tr>
<td>TEA</td>
<td>Tetraethylammonium chloride</td>
</tr>
<tr>
<td>T</td>
<td>Thymine</td>
</tr>
<tr>
<td>TEV</td>
<td>Tobacco etch virus</td>
</tr>
<tr>
<td>T-DNA</td>
<td>Transfer DNA</td>
</tr>
<tr>
<td>S1-S6</td>
<td>Transmembrane segment 1 to 6</td>
</tr>
<tr>
<td>Y</td>
<td>Tyrosine</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
</tr>
<tr>
<td>v/v</td>
<td>Volume to volume</td>
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<tr>
<td>Ws</td>
<td>Wassilewskija</td>
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<tr>
<td>Wt</td>
<td>Wildtype</td>
</tr>
<tr>
<td>Symbol</td>
<td>Description</td>
</tr>
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<td>-------------------</td>
</tr>
<tr>
<td>YFP</td>
<td>Yellow fluorescent protein</td>
</tr>
<tr>
<td>Zn$^{2+}$</td>
<td>Zinc ion</td>
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</table>
Chapter 1

Introduction

Modified from:

Biological roles of cyclic-nucleotide-gated ion channels in plants: What we know and don’t know about this 20 member ion channel family


Botany 87: 668-677
1.1. Abstract

Cyclic-nucleotide-gated ion channels (CNGCs) are nonselective cation channels that were first identified in vertebrate retinal photoreceptors and olfactory sensory neurons. The *Arabidopsis thaliana* genome encodes twenty putative CNGC subunits that are postulated to form heterotetrameric channels at the plasma membrane. Current research has so far revealed their ability to transport cations that play a role in mediating various biotic and abiotic stresses, and developmental processes. This chapter briefly introduces the history of CNGCs in animal research, and discusses in large extent, the current knowledge of CNGCs in plants, focusing on functional aspects, with references to heterologous expression studies and reverse genetics analyses. In addition, structural aspects of these channels will also be covered.
1.2. Ion flux changes during pathogen defense in plants

As sessile organisms, plants lack the mobility and adaptive immune system of vertebrates to evade pathogen attack. As a consequence, plants have evolved a sophisticated signaling system that deploys two main branches of immune responses to defend against pathogen infection (Chisholm et al, 2006; Jones and Dangl, 2006). The first line of defense is a basal immune response that is induced upon recognition of highly conserved pathogen-associated molecular patterns (PAMPs) by pattern-recognition receptors (PRRs) at the cell surface (Zipfel and Felix, 2005; Jones and Dangl, 2006). In response, many phytopathogens have developed the ability to secrete effector proteins that work to enhance virulence and suppress host defense machinery (Bent and Mackey, 2007). To counteract this virulence strategy, plants in turn evolved the ability to induce a second line of defense that results in a more robust response mediated by resistance (R) genes that either interact with these effectors or recognize effector-mediated modifications of plant proteins (Bent and Mackey, 2007).

One of the earliest responses to pathogen recognition is ion flux changes across the plasma membrane (Nurnberger et al., 1994; Chandra et al., 1997; Gelli et al., 1997; Scheel, 1998; Blume et al., 2000). For instance, rapid transient increases in cytosolic Ca\(^{2+}\) levels ([Ca\(^{2+}\)]\(_{\text{Cyt}}\)) was observed in aequorin-transformed tobacco cells treated with crude elicitors from yeast and the phytopathogenic fungus, *Gliocladium deliquescens* (Knight et al., 1991). Another example of ion flux change is the rapid influxes of Ca\(^{2+}\) and H\(^{+}\), and effluxes of K\(^{+}\) and Cl\(^{-}\) reported in parsley cells after treatment with the *Phytophthora* elicitor, Pep-13 (Nurnberger et al., 1994; Hahlbrock et al., 1995; Blume et
al., 2000). Pharmacological analyses have implicate these ions, and in particular Ca\(^{2+}\), to be indispensable for the induction of downstream defense responses including the production of reactive oxygen species and activation of defense-related genes (Hahlbrock et al., 1995; Chandra et al., 1997).

Ca\(^{2+}\)-permeable channels in the plasma membrane have been suggested to provide a major pathway by which Ca\(^{2+}\) influx is mediated during plant immune responses (Gelli et al., 1997; Gelli and Blumwald, 1997; Zimmermann et al., 1997; Ali et al., 2007). Based on animal research, two major classes of Ca\(^{2+}\) channels have been postulated to exist in plants – voltage-dependent and ligand-gated Ca\(^{2+}\)-permeable channels (Hedrich et al., 2012). Although genome analyses revealed the lack of plant genes that are homologous to those encoding animal voltage-dependent Ca\(^{2+}\) channels, voltage-dependent Ca\(^{2+}\) currents occurring in different plant cell types have been reported (Kiegle et al., 2000; Véry and Davies, 2000; Stoelzle et al., 2003; Wu et al., 2007; Chen et al., 2012). With respect to plant immunity, electrophysiological analyses by Gelli et al. (1997) and Gelli and Blumwald (1997) have reported Ca\(^{2+}\) influxes at hyperpolarized membrane potentials at the plasma membrane of tomato suspension cells in response to fungal elicitors.

The second class of Ca\(^{2+}\)-permeable channels comprise of two families that display high homology to the animal cyclic nucleotide-gated (CNG) and ionotropic glutamate receptor (iGLR) channels (Maser et al., 2001; Forde and Lea et al., 2007; Ward et al., 2009). Genetic evidence from mutants of Arabidopsis GLRs implicate these channel proteins in mediating various physiological responses including pathogen
defense, and are hypothesized to be directly gated by amino acids, primarily glutamate and glycine (Chiu et al., 2002; Kang and Turano, 2003; Kang et al., 2004; Meyerhoff et al., 2005; Kwaaitaal et al., 2011; Li et al., 2013). The CNG channels (CNGCs) of Arabidopsis on the other hand, comprise a twenty member family that is postulated to be directly gated by cyclic nucleotides. Many CNGCs have been characterized to be involved in a variety of physiological responses, and have so far been established to be involved in plant immunity (Clough et al., 2000; Maser et al., 2001; Jurkowski et al., 2004; Yoshioka et al., 2006; Chin et al., 2009). Similar to GLRs, CNGCs are proposed to form channel complexes at the plasma membrane that mediate cation influxes into the cell (Dietrich et al., 2010). The following sections in this chapter introduces early research that identified CNGCs as prime candidates for Ca^{2+} signaling in plants, and discusses the current knowledge of the biophysical properties and physiological roles with respect to immune signaling, mediated by this ion channel family in plants.

1.3. Cyclic Nucleotide Gated Ion Channels (CNGCs)

1.3.1. CNGCs in animals

Cyclic nucleotide-gated ion channels (CNGCs) are nonselective cation channels that are gated by the direct binding of cyclic nucleotide monophosphates (cNMPs), and are predominantly found in vertebrate visual and olfactory signal transduction cascades (Craven and Zagotta 2006; Zagotta and Siegelbaum 1996; Yao and Baylor, 1989). Mutations in the genes encoding CNGC proteins have been shown to cause visual and olfactory neuronal disorders in animals such as retinitis pigmentosa, achromatopsia and loss of odour perception.
(Peng et al., 2003; Munger et al., 2001; Wissinger et al., 2001; Kohl et al., 1998; Brunet et al., 1996)

Fesenko et al. (1985) identified the first CNGC which activated a current directly stimulated by cyclic guanosine-3',5' monophosphate (cGMP) in the outer membrane of retinal rods. Similar channels were subsequently found in cone photoreceptors and olfactory sensory neurons that are activated by cyclic adenosine-3',5' monophosphate (cAMP) and cGMP (Yao and Baylor, 1989; Haynes et al., 1985). The gene encoding a bovine rod photoreceptor CNGC was first cloned by Kaupp et al. (1989), and the subsequent cloning of related genes from olfactory neurons (Goulding et al., 1992; Ludwig et al., 1990; Dhallan et al., 1990) demonstrated that these channels are structurally homologous to the voltage-dependent K⁺ family of channels although being directly gated by cNMPs and not by voltage. There are six CNGC genes in mammalian genomes that encode four “A” subunits and two “B” subunits (Pifferi et al., 2006; Hoffman et al., 2005; Bradley et al., 2001). The typical mammalian CNGC comprises of four CNGC subunits in varying stoichiometries that form around a central pore (Liu et al., 1996; Varnum and Zagotta, 1996; Gordon and Zagotta, 1995). Native rod and cone photoreceptors are composed of two types of subunits: CNGA1 and CNGB1 with a stoichiometry of 1:3 (CNGA1: CNGB1a splice variant) in rod, and CNGA3 and CNGB3, with a stoichiometry of 2:2 (CNGA3:CNGB3) in cone photoreceptors (Peng et al., 2004; Weitz et al., 2002; Zhong et al., 2002; Gertsner et al., 2000; Chen et al., 1993). Native olfactory sensory neurons on the other hand, are composed of three types of subunits: CNGA2, CNGA4 and CNGB1, with a stoichiometry of 2:1:1 (CNGA2:CNGA4:CNGB1b splice variant) (Zheng et al., 2004; Bradley et al., 1994; Liman et al., 1994; Dhallan et al., 1990; Ludwig et al., 1990). Similar to the family of voltage-dependent K⁺ channels, the topology of each CNGC subunit consists of
cytosolic N- and C-terminal domains that flank a six-transmembrane domain (S1-S6) with a pore-loop region situated between S5 and S6 (Pifferi et al., 2006; Matulef and Zagotta 2003; Henn et al., 1995; Molday et al., 1991; Kaupp et al., 1989). Later studies of native CNGCs identified additional modulations of channel activity by Ca\(^{2+}\)-calmodulin (CaM) and Ca\(^{2+}\)-free CaM (apocalmodulin) (Bradley et al., 2004; Kleene, 1999; Balasubramaniam et al., 1996; Gordon et al, 1995). This led to the subsequent identification of CaM binding domains (CaMBDs) within the N- and C-terminal regions of the varying CNGC subunits (Pifferi et al., 2006; Zheng et al., 2003; Grunwald et al., 1999; Chen et al., 1994).

Although extensive research has been conducted to elucidate the physiological and biophysical properties of CNGCs in animals, much less has been done in plants. The current knowledge and future perspectives of plant CNGC research will be discussed in the following sections of this chapter.

1.3.2. CNGCs in plants

Ion influx is central to signal transduction, and one of the potential pathways for the uptake of these ions is via cyclic-nucleotide-gated ion channels (CNGCs) (Talke et al., 2003). As mentioned above, the topology of CNGCs in animals composes of six transmembrane domains and a pore region between the fifth and sixth transmembrane domains (Figure 1-1A; Zagotta and Siegelbaum 1996; Henn et al., 1995; Molday et al., 1991; Kaupp et al., 1989). The fourth transmembrane domain has similarities to the Shaker-type voltage sensor (Köhler et al., 1999; Rehmann et al., 2007). The N-terminal domain extends into the cytosol and is believed to bind CaM while the C-terminal domain binds cNMPs. Plant CNGC has a similar basic structure but it has been
suggested to have differences in CaM binding domains. C-terminal CaMBDs were first proposed to occur in overlapping regions with the CNBD (Hua et al., 2003b; Kohler and Neuhaus, 2000). More recently, a novel IQ-like CaMBD was identified in a region after the CNBD in AtCNGC20 (Fischer et al, 2013), and N-terminal CaMBDs were predicted to occur in several other AtCNGCs as well (Abdel-Hamid Ph.D. thesis, 2013; DeFalco and Yoshioka, unpublished data). The role of animal CNGCs in the signal transduction of light and odorant perception is well documented and they are believed to localize to the plasma membrane in heterotetramers (Kaupp and Seifert 2002; Talke et al., 2003).

While six CNGC subunits have so far been identified in mammalian genomes, the Arabidopsis genome encodes 20 putative CNGC subunits (Mäser et al., 2001) that are thought to assemble in the same heterotetrameric form as animal subunits (Hua et al., 2003b; Talke et al., 2003). The current model of CNGC signaling is summarized in Figure 1-1. It is based on research on animal CNGCs and involves the opening of the channel by either cAMP or cGMP, which leads to an influx of cations such as K\(^+\) and Ca\(^{2+}\). Binding of Ca\(^{2+}\) activates CaM, allowing it to bind to the CaM-binding domain of the CNGC. This would close the channel, acting as a negative feedback mechanism.

Alignment of the 20 predicted amino-acid sequences categorized the members into four groups (I–IV), of which group IV is broken into two subgroups (IVA and IVB) (Mäser et al., 2001). Studies on some Arabidopsis CNGCs have so far revealed their ability to transport cations that play a role in mediating various environmental stresses, plant defense responses and development (Clough et al., 2000; Balagué et al., 2003; Chan et al., 2003; Gobert et al., 2006; Ma et al., 2006; Yoshioka et al., 2006; Borsics et al., 2007; Urquhart et al., 2011; Gao et al., 2012; Finka et al., 2012, Tunc-Ozdemir et al.,
2013a, 2013b, summarized in Table 1-1). The details of their biological functions are discussed in the following sections.

1.3.3. Ion selectivity

Ion channel function has typically been assessed by electrophysiological methods using *Xenopus laevis* oocytes or human embryonic kidney (HEK) cells. Some plant CNGCs have been functionally characterized by analyzing channel currents in voltage clamp studies. In 1999, Leng et al. demonstrated that the expression of AtCNGC2 in *X. laevis* oocytes resulted in cNMP-dependent K$^+$ currents in the presence of either lipophilic cAMP or cGMP. They further confirmed AtCNGC2 to be an inward-rectified cNMP-gated ion channel. Using guard cell plasma membranes, Ali et al. (2007) demonstrated (by patch clamp analysis) cAMP-activated currents in membranes from wild type plant but not *dnd1* mutants, which lack AtCNGC2 function. A report using *X. laevis* oocytes suggested that AtCNGC2 may also be selective for other monovalent cations (Li$^+$, Cs$^+$, Rb$^+$) to an extent nearly as great as K$^+$ (Leng et al., 2002).

Interestingly, expression of AtCNGC2 in both *X. laevis* oocytes and HEK cells showed a strong discrimination against Na$^+$ (Leng et al., 2002). In contrast, AtCNGC1 was found to be an inward rectified ion channel equally permeable to both K$^+$ and Na$^+$ (Hua et al., 2003a). It should be noted that the amino acid triplet motif GYG of the K$^+$ selectivity filters in K$^+$ selective channels has been demonstrated to be a strong basis for discrimination against Na$^+$ conductivity (Leng et al., 2002). Animal CNGCs, on the other hand, lack this motif. Instead, they possess a GET motif and have been shown to be nonselective for divalent and monovalent cations, and do not discriminate between K$^+$,
Figure 1-1. A) Structure of plant cyclic-nucleotide-gated ion channels (CNGC). CNGCs are composed of six transmembrane domains (S) and a pore region (P) between the fifth and sixth domains. The cytosolic C-terminus has a C-linker followed by the cyclic nucleotide binding domain (CNBD) with an overlapping CaM binding domain (CaMBD).

B) Model of CNGC-mediated signaling in plants. Upon perception of a stimulus, adenylate or guanylate cyclase produce cAMP or cGMP, respectively, which bind to the CNBD. Binding causes a conformational change leading to opening of the channel. This causes an influx of monovalent and divalent cations in the cell. This elevation in cation concentration activates downstream signaling components that result in a physiological response. Ca$^{2+}$-activated CaM binding leads to closing of the CNGC as a negative feedback mechanism.
Na⁺, and Ca²⁺ (Kaplan et al., 2007). Plant CNGCs however, do not contain the GYG or GET motifs (conserved in animals) (Hua et al., 2003a). Through computational modeling, Hua et al. (2003a) showed that AtCNGC1 and AtCNGC2 have a GQN and AND triplet, respectively. A GQN or similar selectivity filter motif is present in 16 out of 20 Arabidopsis CNGCs (Kaplan et al., 2007), while the AND motif was unique to only AtCNGC2. They further showed that by changing the AND filter of AtCNGC2 to AET, which is more closely related to animal CNGCs, Na⁺ conductance is recovered in both X. laevis oocytes and HEK cells (Hua et al., 2003a). It was thus suggested that AtCNGC2’s AND motif facilitates K⁺ conductance and Na⁺ exclusion in a fashion similar to the GYG triplet of K⁺ Shaker-like channels (Hua et al., 2003a). In the same year, Balagué et al. (2003) demonstrated that X. laevis oocytes expressing AtCNGC4 showed both K⁺ and Na⁺ outward rectified currents that are activated by both cAMP and cGMP, with the latter being more efficient. Additionally, these currents are blocked by Cs⁺ ions. Like AtCNGC2, the selectivity filter of AtCNGC4 is believed to differ from the rest of the family members and is exclusive to AtCNGC4. The expression of AtCNGC10 in HEK cells was also shown to conduct inward K⁺ currents that are activated by lipophilic cGMP (Christopher et al., 2007). More recently, Gao et al. (2012) demonstrated that AtCNGC6 activates a heat-induced Ca²⁺ flux using whole cell patch clamp techniques in Arabidopsis root protoplasts.

So far only a few plant CNGCs have been characterized by electrophysiological methods. This has been partially attributed to the instability of plant CNGCs in these cells as reported by Leng et al. (1999) and Balagué et al. (2003). Additionally, it requires specialized skills and costly infrastructure. Thus, in recent years the functional
properties of plant CNGCs have been characterized through the use of heterologous systems involving cation-uptake deficient yeast (*Saccharomyces cerevisiae*) and *Escherichia coli* mutants.

One commonly used yeast mutant is the CY162 strain, which lacks two major K\(^+\) transporters, TRK and TRK2, and cannot survive in low-K\(^+\) media without a complementing channel (Ko and Gaber, 1991). In 1998, Schuurink et al. (1998) demonstrated that the barley CNGC, HvCBT1, cannot complement the mutant on low-K\(^+\) media. Hydropathy analysis suggested that HvCBT1 possesses six transmembrane domains similar to those of K\(^+\) Shaker-type channels and animal CNGCs, but with a GQN instead of the conserved GYG consensus sequence of K\(^+\) selective channels in the pore region. By changing the pore to the GYG consensus sequence, yeast growth is further attenuated (Schuurink et al., 1998). In a similar assay, the expression of AtCNGC10 is able to rescue yeast growth (Li et al., 2005), suggesting that AtCNGC10 can form K\(^+\) permeable channels in yeast. Leng et al. (1999) demonstrated that the Arabidopsis AtCNGC2 partially complements the CY162 yeast mutant in the presence of membrane-permeable (dibutyryl)-cAMP or cGMP when grown on solid and liquid media with low K\(^+\). AtCNGC3 also partially complements a similar yeast strain deficient in the TRK1, TRK2, and TOK1 K\(^+\) transporters (Gobert et al., 2006). In a slightly modified analysis, Mercier et al. (2004) later demonstrated the ability of AtCNGC1, AtCNGC2, and AtCNGC4 to complement the hypersensitivity of the trk1/trk2-deficient WD3 yeast mutant to the cationic amino glycoside, hygromycin B, in low K\(^+\). The expression of AtCNGC1, AtCNGC2, and AtCNGC4 was shown to further enhance yeast growth in the presence of dibutyryl-cAMP. Using a similar protocol, Yoshioka et al.
reported the complementation of the WD3 yeast mutant with AtCNGC11, AtCNGC12, and the chimeric AtCNGC11/12. However, contrary to the finding of Leng et al. (1999) on AtCNGC2, the expression of AtCNGC11, AtCNGC12, and AtCNGC11/12 exhibits enhanced yeast growth in the presence of only lipophilic cAMP, suggesting that these channels are activated solely by cAMP and not cGMP. More recently, the same analysis incorporated into a structure-function study of AtCNGC11/12 demonstrated the physical binding of cAMP to AtCNGC12 and AtCNGC11/12, further supporting this notion (Baxter et al., 2008).

Plant CNGCs have also been functionally characterized with the use of yeast Ca\(^{2+}\)- and Na\(^{+}\)-channel mutants. Ali et al. (2005) utilized a Ca\(^{2+}\) uptake-deficient mutant lacking the Ca\(^{2+}\) transporters MID1 and CCH1 (ELY151). These transporters are involved in the response to α mating factor and the mutant strain exhibits growth arrest in the presence of this factor. Ali et al. (2005) demonstrated the ability of AtCNGC1 to complement the yeast mutant strain. The same assay was employed by Urquhart et al. (2007) to show that AtCNGC11, AtCNGC12, and AtCNGC11/12 also can conduct Ca\(^{2+}\) transport in yeast. Gobert et al. (2006) assessed Na\(^{+}\) uptake of AtCNGC3 by using the salt-sensitive G-19 yeast mutant strain that carries deletions in the ENA1–4 Na\(^{+}\)-extruding pumps. Mutant yeast expressing AtCNGC3 are hypersensitive to high salt concentrations and accumulate significantly higher amounts of Na\(^{+}\) compared with the empty vector control, indicating that AtCNGC3 can act as Na\(^{+}\) channels in yeast (Gobert et al., 2006).
The K⁺-uptake-deficient *E. coli* strain LB650 with mutations in the TRK H and TRK G genes has also been used to assess the K⁺ channeling of some CNGCs. Ali et al. (2005) demonstrated that AtCNGC1 can also rescue bacterial growth in this system. In the same year, Li et al. (2005) reported similar results when AtCNGC10 is expressed in LB650. A summary of the ion selectivity of each CNGC is listed in Table 1-1. However, as Kaplan et al. (2007) also pointed out, it is not completely clear how well these heterologous expression systems reflect their *in vivo* function. Therefore, careful interpretation of these results and further assessment are required.

**1.3.4. The role of CNGCs in cation homeostasis**

Arazi et al. (1999) first reported that the overexpression of a tobacco plasma membrane CNGC designated NtCBP4 conferred tolerance to Ni²⁺ and hypersensitivity to Pb²⁺. A later study demonstrated that transgenic tobacco plants expressing a truncated version of NtCBP4 has improved tolerance to Pb²⁺ and attenuated Pb²⁺ accumulation compared to those expressing the full-length gene (Sunkar et al., 2000). Furthermore, T-DNA insertion mutants of AtCNGC1, which encodes the homologous protein in Arabidopsis, exhibit Pb²⁺ tolerance, as well as significantly lower levels of Pb²⁺ accumulation (Sunkar et al., 2000). Unlike its tobacco counterpart, this tolerant phenotype is not observed with Ni²⁺, suggesting that AtCNGC1 elicits a response that is specific to Pb²⁺. Taken together, these results indicate that NtCPB4 and AtCNGC1 are likely involved in heavy metal uptake across the plasma membrane. More recently, Ma et al. (2006) demonstrated that the null mutation of AtCNGC1 reduces Ca²⁺ uptake in similar magnitude to the reduction in Pb²⁺ uptake reported by Sunkar et al. (2000). This
suggests that AtCNGC1 is similarly permeable to both Ca\(^{2+}\) and Pb\(^{2+}\). Since AtCNGC1 seems to be expressed primarily in Arabidopsis roots (Ma et al., 2006), it may be involved in ion uptake from the soil.

Two mutant alleles of AtCNGC2 have been tested for sensitivity to pH changes, Mg\(^{2+}\), K\(^{+}\), Na\(^{+}\) and Ca\(^{2+}\). Both null mutants are exclusively hypersensitive to Ca\(^{2+}\) (Chan et al., 2003). When compared to their wild type counterparts, the mutant plants have smaller rosette sizes, root mass and overall stature (Chan et al., 2003). Furthermore, it was shown that the Ca\(^{2+}\) hypersensitivity of CNGC2 knock out mutant (cngc2) mutants is not caused by the accumulation of Ca\(^{2+}\), but rather by a defective signaling pathway in the high Ca\(^{2+}\) environment. Genome wide transcriptional analysis of cngc2 and wild type plants under normal and elevated Ca\(^{2+}\) conditions revealed that the transcriptional profile of cngc2 plants under normal conditions is similar to that of wild type plants under high Ca\(^{2+}\) condition (Chan et al., 2008). These results indicate that the role of AtCNGC2 may not be Ca\(^{2+}\) uptake, but rather signal transduction and homeostasis. Interestingly, hierarchical clustering revealed similarities in expression patterns between cngc2 plants under high Ca\(^{2+}\) and various biotic and abiotic stresses, suggesting the involvement of Ca\(^{2+}\)/CNGCs in the signal transduction during these stress responses (Chan et al., 2008).
Table 1-1: Ion selectivity and physiological roles identified in plant CNGCs

<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene ID</th>
<th>Ion Selectivity</th>
<th>Suggested physiological role</th>
<th>Reference</th>
</tr>
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<tr>
<td>AtCNGC1</td>
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<td>K⁺, Na⁺, Ca²⁺, Pb²⁺</td>
<td>Cation uptake from soil Heavy metal uptake</td>
<td>Hua et al., 2003a; Mercier et al., 2004; Sunkar et al., 2000; Ali et al., 2005</td>
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<td>AtCNGC2</td>
<td>AT5G15410</td>
<td>K⁺, Ca²⁺, Li²⁺, Cs⁺, Rb⁺</td>
<td>Developmental cell death and senescence Growth and development Cell elongation and pollen tube guidance Pathogen resistance Heatshock response and thermotolerance</td>
<td>Leng et al., 1999; Mercier et al., 2004; Ma et al., 2010, 2011 Kohler et al., 2001; Chan et al., 2003; Chaiwongsar et al., 2009 Ali et al., 2007; Clough et al., 2000; Finka et al., 2012</td>
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<td>AtCNGC3</td>
<td>AT2G46430</td>
<td>K⁺, Na⁺</td>
<td>Distribute and translocate ions from xylem Translocate Na⁺ within embryo</td>
<td>Gobert et al., 2006</td>
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<td>AT5G54250</td>
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<td>Pathogen resistance</td>
<td>Genger et al., 2008; Mercier et al., 2004; Balague et al., 2003; Jurkowski et al., 2004</td>
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<td>Unknown</td>
<td>Male fertility and pollen tube growth</td>
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<td>Gene ID</td>
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<td>AtCNGC10</td>
<td>AT1G01340</td>
<td>K⁺, Na⁺, Ca²⁺, Mg²⁺</td>
<td>Light modulated development to shoots</td>
<td>Li et al., 2005; Borsics et al., 2007; 2008, 2010</td>
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<td>Pathogen resistance Gravitropism Negative regulation of senescence</td>
<td>Yoshioka et al., 2006; Urquhart et al., 2007, 2011</td>
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<td>Pathogen resistance Gravitropism Negative regulation of senescence</td>
<td>Yoshioka et al., 2006; Urquhart et al., 2007, 2011</td>
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<td>Polarized pollen tube growth</td>
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<td>Pb²⁺</td>
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<td>Arazi et al., 1999</td>
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<tr>
<td>HvCBT1</td>
<td>AJ002610</td>
<td>Unknown</td>
<td>Ion transport in aleurone</td>
<td>Schuurink et al., 1998</td>
</tr>
<tr>
<td>NEC1</td>
<td>AY972627</td>
<td>Unknown</td>
<td>Pathogen resistance</td>
<td>Rostoks et al., 2006</td>
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<td>Physcomitrella patens</td>
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<tr>
<td>PpCNGCb</td>
<td>Pp1s90_245F4.1</td>
<td>Ca²⁺</td>
<td>Heat shock response and Thermotolerance</td>
<td>Finka et al., 2012</td>
</tr>
</tbody>
</table>
AtCNGC3 has been recently found to be also involved in ion homeostasis in * planta*. Gobert et al. (2006) reported that *cngc3* null mutants exhibit impaired germination in response to high external levels of Na⁺. When compared with wild type, the three allelic mutant lines have significantly less germination in the presence of high external Na⁺, but not K⁺ or NH₄⁺ (Gobert et al., 2006). Furthermore, isomolar concentrations of sorbitol have a similar effect on mutant and wild type plants, suggesting that the impaired germination is caused by specific sensitivity to Na⁺ (Gobert et al., 2006). In addition, *cngc3* mutant plants grow better than wild type when exposed to intermediate concentrations of NaCl, whereas no discernible differences are observed with exposure to Zn²⁺, Pb²⁺, Cd²⁺, Ca²⁺, or sorbitol. Measurements of ion levels within tissue revealed that *cngc3* and wild type seedlings have similar Na⁺ concentrations but significantly lower K⁺ concentrations in *cngc3*, indicating that the mutant plants are capable of preventing K⁺ uptake (Gobert et al., 2006). This suggested that AtCNGC3 does not play a prominent role in salinity adaptation. GUS reporter gene analysis revealed AtCNGC3 expression to be prevalent in the embryo, leaf and root tissue throughout development and it has been suggested that AtCNGC3 plays a role in the distribution and translocation of ions from the xylem. Furthermore, germination results indicated that AtCNGC3 does not participate in ion uptake during germination, but rather is involved in transporting toxic levels of Na⁺ from sensitive to tolerant tissues within the embryo (Gobert et al., 2006). In addition, it was also suggested that AtCNGC3 is part of a Na⁺ uptake pathway during seedling development where a null mutation results in less accumulation of Na⁺ (Gobert et al., 2006).
Urquhart et al. (2011) demonstrated that AtCNGC11 and AtCNGC12 are involved in the endogenous movement of Ca$^{2+}$. It was previously reported that AtCNGC11 and AtCNGC12 can conduct Ca$^{2+}$ and K$^+$ in heterologous systems, but root growth experiments revealed that single knockout mutants for AtCNGC11 or AtCNGC12 are similarly sensitive as wild type to elevated levels of Ca$^{2+}$ and K$^+$. In contrast, the double knockout mutant of AtCNGC11 and AtCNGC12 (S90) displayed enhanced sensitivity to only Ca$^{2+}$, but not K$^+$. These results demonstrated redundancy in the biological function of both genes, and that they are involved in Ca$^{2+}$ signaling, not K$^+$ in planta (Urquhart et al., 2011). Furthermore, the endogenous levels of Ca$^{2+}$ were not altered in the mutants indicating that AtCNGC11 and AtCNGC12 are likely not involved in general Ca$^{2+}$ uptake and homeostasis, but rather its signaling and/or translocation (Urquhart et al., 2011). Similarly, AtCNGC19 and AtCNGC20 were shown by knockout mutant and GUS reporter gene analyses to potentially redistribute Na$^+$ within the plant during salinity stress, rather than maintaining Na$^+$ homeostasis (Kugler et al., 2009).

1.3.5. The role of CNGCs in development

The ion sensitivities and expression profile of different CNGCs reported above suggest that these ion channels play a prominent role in regulating various developmental pathways. GUS reporter gene analysis revealed the expression of AtCNGC2 to be prominent in cotyledons of 14-day-old seedlings, and in leaves of flowering plants (Köhler et al., 2001). Köhler et al also reported that AtCNGC2 expression is induced during the early stages of plant senescence, as well as in the dehiscence zone of mature siliques. Therefore, these results suggested that AtCNGC2 is a positive regulator of the developmental cell death program that is expressed
throughout the life cycle of Arabidopsis plants, starting from early seedling development up until the early stages of senescence (Köhler et al., 2001). More recently however, Ma et al. (2010, 2011) reported that AtCNGC2 conducts a Ca\(^{2+}\) signal that acts to negatively regulate the senescence signaling pathway through nitric oxide (NO). The AtCNGC2 loss-of-function mutant, dnd1, displays early senescence phenotypes that can also be observed in wild type plants treated with Ca\(^{2+}\) channel blockers (Ma et al., 2010). Additionally, the application of the nitric oxide (NO) donor, sodium nitroprusside (SNP), rescued the senescence-associated phenotypes in dnd1 resulting in reduced H\(_2\)O\(_2\) levels and lipid peroxidation in leaves, and reduced expression of senescence marker genes (Ma et al, 2010). cngc2 mutants have also been reported to be hypersensitive to Ca\(^{2+}\) that result in severe reductions in plant size, cell elongation and fertility, further suggesting that AtCNGC2 is involved in a variety of developmental processes (Chan et al, 2003). Most recently, AtCNGC2 and AtCNGC4 were discovered to be involved in thermal sensing (Finka et al., 2012) as well as floral transition where null mutants displayed late flowering phenotypes caused by alterations in expression of the central floral repressor, Flowering Locus C (FLC) (Chapter 3).

Borsics et al. (2007) demonstrated that AtCNGC10 antisense lines exhibit significant changes in growth and metabolism. Compared with wild type plants, the antisense lines have a 20% reduction in leaf surface area, laminar thickness, and palisade columnar cell length (Borsics et al., 2007). Furthermore, the leaves of the antisense plants accumulate almost double the amount of starch than wild type, and flowering occurs approximately 10 days earlier. The root lengths in the antisense seedlings are also significantly shorter than wild type, and exhibit impaired gravitropic responses (Borsics et al., 2007). Taken together, it was suggested that AtCNGC10 is
involved in a multitude of developmental pathways involving light modulation because of the similarities in phenotype to weak photoreceptor mutants (Weston et al., 2000; Chun et al., 2001; Borsics et al., 2007). The inability to generate homozygous T-DNA insertion mutant lines and a low frequency of antisense plant recovery further support the notion that AtCNGC10 is an essential gene with very low redundancy.

Apart from Ca$^{2+}$ translocation, Urquhart et al. (2011) demonstrated that AtCNGC11 and AtCNGC12 double mutants display a loss of gravitropic responsiveness and enhanced (dark-induced) senescence. Therefore, AtCNGC11 and AtCNGC12 are postulated to synergistically conduct a Ca$^{2+}$ signal that controls gravitropic response and negatively regulates the senescence signaling pathway (Urquhart et al., 2011).

Recently, Frietsch et al. (2007) investigated the role of AtCNGC18 in pollen tube development. GUS reporter gene analysis revealed that AtCNGC18 is primarily expressed in pollen grains, and not in the leaves or roots of young seedlings (Frietsch et al., 2007). Analysis of the pollen tubes from two allelic cngc18 mutants showed that they develop into short and thin tubes that grow non-directionally for a short distance before premature termination, resulting in male sterility (Frietsch et al., 2007). Heterologous expression of AtCNGC18 in E. coli results in an accumulation of Ca$^{2+}$, suggesting that AtCNGC18 transduces a signal that regulates pollen-tube growth. Interestingly, Moutinho et al. (2001) previously showed that cAMP acts as a signal for pollen tube growth and reorientation. In a study of transporter genes that are expressed during pollen development, AtCNGC7, 8, 16 and 18 were identified to be primarily expressed in pollen (Bock et al., 2006), indicating the importance of CNGCs in pollen development. To this end, AtCNGC7 and 8 were recently demonstrated to be important for male
fertility, where cngc7/8 double mutant pollen grains displayed a high frequency of bursting upon germination (Tunc-Ozdemir et al., 2013). Therefore, it is speculated that subunits of AtCNGC7, 8 and 18 interact in a multimeric complex that generates a signal involved in regulating pollen tube growth. Thus, it is evident that CNGCs are involved in a variety of important developmental pathways in plants, and it is very likely that further specific roles in plant development will be elucidated.

1.3.6. The role of CNGCs in pathogen defense

A number of CNGCs have been implicated in mediating pathogen defense responses in plants. Yoshioka et al. (2001, 2006) identified the cpr22 mutant (constitutive expresser of PR genes 22) that is generated by a 3kb deletion that fuses the 5’ portion of AtCNGC11 and the 3’ portion of AtCNGC12 to form the chimeric AtCNGC11/12 gene (Figure 1-2). This mutation confers a stunted morphology with curly leaves, hypersensitive response (HR)-like spontaneous lesion formation in leaves, salicylic acid (SA) accumulation, and enhanced disease resistance to oomycete and bacterial pathogens (Yoshioka et al., 2001). Subsequent work suggested that AtCNGC11 and AtCNGC12 are positive regulators of pathogen defense signaling (Yoshioka et al., 2006, Moeder et al., 2011). AtCNGC11 and AtCNGC12 loss of function mutants exhibit partial breakdown of resistance to avirulent oomycete and bacterial pathogens compared with wild type plants (Yoshioka et al., 2006; Moeder et al., 2011).

The cpr22 phenotype is not caused by the absence of either channel, AtCNGC11 or 12, but rather by the expression of the chimeric AtCNGC11/12 (Yoshioka et al., 2006). Furthermore, the requirement of functionally active AtCNGC11/12 for the cpr22 phenotype was also confirmed (Baxter et al., 2008). AtCNGC11/12, like its wild type
proteins, AtCNGC11 and AtCNGC12, conducts both Ca\(^{2+}\) and K\(^+\). In yeast complementation assays, no significant difference in ion conductance efficiency was found between AtCNGC11/12 and its wild type proteins. However, the Ca\(^{2+}\) channel blockers, Gd\(^{3+}\) and La\(^{3+}\), but not the K\(^+\) channel blocker tetraethylammonium chloride (TEA) suppress AtCNGC11/12-induced HR-like cell death formation (Urquhart et al., 2007), indicating that Ca\(^{2+}\) influx is required for HR-like cell death formation and is at least in part mediated by CNGCs. Involvement of Ca\(^{2+}\) in cpr22 mediated phenotype was also supported by chemical screening that can suppress cpr22 penoytypes (Abdel-Hamid, 2011). Urquhart et al. (2007) also showed that the cell death induced by AtCNGC11/12 is not due to unspecific perturbation of cellular physiology. Molecular and microscopic analyses revealed that AtCNGC11/12 activates programmed cell death (PCD) in plants.

Other defense-related CNGC mutants are dnd1 and dnd2/hlm1. They also have been shown to exhibit conditional spontaneous cell death formation and broad-spectrum disease resistance (Clough et al., 2000; Balagué et al., 2003; Jurkowski et al., 2004). The barley nec1 mutation, which induces necrotic lesions, was also mapped to the homolog of DND2/HLM1 in barley (Rostoks et al., 2006). Both dnd1 and dnd2/hlm1 have mutations that abolish channel function of AtCNGC2 and AtCNGC4, respectively (null mutations). They exhibit constitutive Pathogenesis-Related (PR) gene expression,
Figure 1-2. *cpr22* is caused by the fusion of *AtCNGC11* and *AtCNGC12*. The *cpr22* mutation is the result of a 3kb deletion that caused the in-frame fusion of the 5' end of *AtCNGC11* and the 3' end of *AtCNGC12* forming the chimeric *AtCNGC11/12* gene.
elevated levels of SA, and enhanced disease resistance to virulent pathogens similar to cpr22 (Clough et al., 2000; Balagué et al., 2003; Jurkowski et al., 2004). Furthermore, the dnd mutants maintain their gene-for-gene resistance to some avirulent pathogens even though HR formation after infection is attenuated (Yu et al., 1998; Clough et al., 2000; Balagué et al., 2003). Interestingly, as described above activation of resistance responses such as SA accumulation and PR gene expression seen in cpr22, are induced by expression of AtCNGC11/12 and by the lack of AtCNGC2 or AtCNGC4 in dnd1 and dnd2/hlm1, respectively. This suggests that a precise cation signal/homeostasis is required to activate PCD.

More recently, electrophysiological analysis by Ali et al. (2007) demonstrated that AtCNGC2 transduces a Ca$^{2+}$ current that leads to an increase in NO production. As mentioned above, the dnd1 mutant does not display HR after infection with some avirulent pathogens. However, exogenous NO treatment can restore HR formation indicating that NO formation is downstream of Ca$^{2+}$ influx (Ali et al., 2007). It is known that NO synthase (NOS) in animals is a Ca$^{2+}$/CaM dependent enzyme. Recently, Ma et al. (2008) provided some evidence that CaM-like 24 (CML24) is required for Ca$^{2+}$ -dependent activation of plant NOS as well. As described in the previous section, AtCNGC11/12 induces HR-like PCD in a Ca$^{2+}$-dependent manner (Urquhart et al., 2007). These data suggest a tight connection of CNGCs and Ca$^{2+}$ with pathogen resistance signal transduction. A number of studies have reported the importance of Ca$^{2+}$ influx in pathogen resistance responses (Kwaaitaal et al., 2011; Qi et al., 2010; Ma et al., 2008; Urquhart et al., 2007; Ali et al., 2007; Kang et al., 2006). However, concrete components/channels that regulate Ca$^{2+}$ fluxes after pathogen infection have yet to be
clearly characterized. Thus, the study of CNGCs in pathogen resistance may be a novel avenue for studying Ca\(^{2+}\) signal transduction in plant immune responses.

1.3.7. Structural analysis of molecular composition of CNGCs

Although structure–function analyses of plant CNGCs are still in its infancy, CNGCs in animals have been relatively well studied and documented. The structure of the cytoplasmic C-terminal region including the CNBD and the C-linker domains of a hyperpolarization-activated cyclic-nucleotide-gated channel 2 (HCN2) was solved by X-ray crystallography (Zagotta et al., 2003). The HCN channels belong to a superfamily of voltage-gated channels that play an essential role in relaying electrical signals involved in cardiac and neuronal pacemaker activities in animals (Zagotta et al., 2003). In addition, these channels are similar to those of the CNGC, eag- (ether a go-go), and KAT1-related Shaker-type K\(^{+}\) channels (Zagotta et al., 2003). The C-terminal region possesses a tetrameric configuration with the CNBD composed of four \(\alpha\)-helices and a \(\beta\)-roll in which cyclic nucleotides bind and interact with the C-linker. Furthermore, the CNBD of HCN2 has a similar fold to the CNBD of the \textit{E. coli} catabolite gene activator protein (CAP) and the regulatory unit of the cAMP dependent protein kinase A. In addition, HCN2 is specifically regulated by cAMP even though cGMP interactions can occur. It was postulated that cAMP binding causes a conformational change in the CNBD that is then coupled with a conformational change in the C-linker, leading to the opening of the channel (Zagotta et al., 2003). It has been shown that CNGCs likely function as heterotetramers in animals (Zheng and Zagotta 2004; Craven and Zagotta 2006), but whether this also is true in plants is still unknown. Furthermore, knowledge of the inter- and intra-subunit interactions in plants is also absent. In animals, the cytosolic
N-terminus of the olfactory CNGA2 channel subunit interacts either with its own or with its partner’s C-terminus and this interaction is necessary to promote channel opening through stabilizing the conformations of the C-linker and the CNBD (Varnum and Zagotta 1997; Kaupp and Seifert 2002; Trudeau and Zagotta 2003). In addition, similar interactions have been reported to occur between the cytoplasmic N- and C-terminal regions of the animal photoreceptor rod subunits CNGA1 and CNGB1 (Varnum and Zagotta 1997; Kaupp and Seifert 2002; Trudeau and Zagotta 2003).

In light of the large number of CNGCs in plants, the possible combinations that can form functional hetero/homomeric channels are immense. Thus, the identification of all the interacting subunit partners remains extremely challenging. So far, no crystal structure of a plant CNGC has been reported. Hua et al. (2003b) modeled the CNBD belonging to AtCNGC2. Using the previously solved structure of the cAMP dependent protein kinase A, R1α, it was shown that cAMP resides in the β barrel in the syn-conformation in AtCNGC2, which is different from the anti-conformation seen in animals (Hua et al., 2003b; Zagotta et al., 2003). Another notable difference from animal CNGCs is the bifunctionality of the CNBD. Through the identification of structural similarities between the truncated CNBD of R1α (domain B) and the CNBD of AtCNGC2, Hua et al, (2003b) suggested that plant CNGCs may have permitted the evolution of a CaM binding domain in this region while still retaining full cyclic nucleotide binding ability. This feature so far seems to be unique to plant CNGCs, as shown by previous work from Köhler and colleagues (Köhler et al., 1999; Köhler and Neuhaus 2000). For example, Köhler and Neuhaus (2000) mapped the CaM-binding domain of AtCNGC1 to the last αC-helix of the CNBD, and demonstrated efficient binding to AtCaM2 and AtCaM4. Similar binding was observed with AtCNGC2, although with different affinities.
from AtCNGC1 to both CaMs (Köhler and Neuhaus 2000). More recently, the CaM binding site in AtCNGC20 was mapped to an IQ motif located after the CNBD, and was shown to interact with all AtCaM isoforms except CaM-like (CML) 8 and 9 (Fischer et al., 2013). Lastly, two CaM binding domains were recently identified in AtCNGC12, each located within the N- and C-terminal cytosolic regions, respectively (Abdel-Hamid, 2013). In the same study, one CaM binding domain was identified in the N-terminal cytosolic region of AtCNGC11.

In 2005, Bridges et al. generated an in silico model from the alignment of the putative CNBDs of several ion channels (including AtCNGCs), and one class of cyclic nucleotide-gated thioesterases (AtCNTEs) across the Arabidopsis thaliana and Oryza sativa (rice) genomes. They were able to show that AtCNTE1 possessed the highest similarity to known crystal structures of Rlα, HCN2, CAP (Catabolite Activator Protein) and Epac1 (Exchange Protein Activated by cAMP 1) models very well against all CNBDs with a high level of conservation of all secondary structures (Bridges et al., 2005). Furthermore, phylogenetic analyses of the plant CNBDs suggested that they can be classed into three subfamilies (CNTEs, CNGCs, and Shaker-type channels) that completely mirror the phylogeny of full-length proteins. This suggested that the CNBDs arose prior to isoform duplication and their commonality to both Arabidopsis and rice further indicated that plant specific nucleotide responses likely developed prior to the monocot–dicot divergence (Bridges et al, 2005).

Detailed mutation studies of functional domains in CNGCs have also elucidated the structural and regulatory properties of these channels. Baxter et al (2008) identified a functionally essential amino acid for CNGCs by utilizing an intragenic suppressor
mutant of \textit{cpr22} (AtCNGC11/12). Computational modeling and \textit{in vitro} cAMP-binding assays indicated that E519 in AtCNGC11/12 (and also the equivalent residue in AtCNGC12) is a key residue for the structural stability and functionality of AtCNGCs through the interaction of the C-linker and the CNBD (Figure 2-1; Baxter et al., 2008). Interestingly, E519 aligns well to Y573 in human CNGA3 where a mutation in this residue causes achromatopsia (Wissinger et al., 2001). Furthermore, the Y573 residue is conserved among various organisms, suggesting that this amino acid position plays a functionally important role for CNGCs in general. Most recently, Abdel-Hamid et al. (2013) identified two residues of AtCNGC11/2 from the same suppressor screen that were demonstrated to be important for inter- and intra-subunit interactions. A fourth intragenic mutant was also identified that demonstrated the functional importance of the αC-helix in the stable regulation of CNGCs in plants (Chin et al., 2010). The details of that study will be discussed in Chapter 2.

1.4. Concluding remarks

As we discussed in this chapter, although only a handful of plant CNGCs have been investigated so far, it seems apparent that these channels play important roles for plant survival and adaptation by mediating multiple stress responses and developmental pathways. The molecular mechanisms and downstream signaling components of \textit{Ca}^{2+}-mediated signal transduction in animals are well studied. However, although numerous studies have shown the importance of \textit{Ca}^{2+} in many signaling pathways in plants, much remains unknown. It is suggested that CNGCs and glutamate receptors are prime candidates to facilitate the \textit{Ca}^{2+} influxes required for many cellular processes (White et al., 2002). Therefore, the study of CNGCs is required to understand \textit{Ca}^{2+} signal
transduction in plants. The following chapters present our efforts to broaden the current knowledge of plant CNGCs.
1.5. Thesis overview

The main objectives of my doctoral thesis were to elucidate the molecular mechanisms by which plant CNGCs are regulated, and to identify novel components in AtCNGC-mediated signaling pathways with an emphasis on pathogen defense. As mentioned above, the Arabidopsis CNGCs comprise of a large 20 member family in which many of them have been identified to be involved in a variety of physiological processes. Of these, 4 members (AtCNGC11 and 12 belonging to Group I, and AtCNGC2 and 4 belonging to Group IV) have so far been shown to be involved in pathogen defense signaling. To understand the regulatory mechanism of plant CNGCs in pathogen defense, I focused on the predicted αC helix of the CNBD. As these channel proteins are postulated to be directly gated by cNMPs, the CNBD is thought to be essential for channel function and regulation. Moreover, the predicted αC helix has been reported to contain an overlapping CaMBD that is hypothesized to affect cNMP binding, which differs from what has been observed in their animal counterparts. However, much remains unknown about the structure-function relationship of CNBDs in plant CNGCs. Therefore, I selected the intragenic suppressor mutant S58 from a cpr22 suppressor screen that has mutation in Arginine (R) 557 located in the putative CNBD and CaMBD of AtCNGC11/12 (AtCNGC12) (Chapter 2). As R557 is located within the putative regulatory domains of AtCNGC11/12 (AtCNGC12), I hypothesized that the R557C mutation conferred by S58, causes an alteration in AtCNG channel regulation rather than function. I present a structure-function analysis that identified R557 as an important residue for CaM binding, and hypothesized that the R557C mutation causes an alteration in CaM binding in AtCNGC11/12 which is attributable to the suppressor phenotypes observed in S58.
For the second research objective, I hypothesized that suppressor mutants of CNGC autoimmune mutants can be utilized. However, the aforementioned *cpr22* suppressor screen did not provide any intergenic mutants so far. Thus, a second suppressor screen using *cngc2* was performed, and an intergenic suppressor for *cngc2* mutants was identified. As mentioned, *cngc2* knockout mutants exhibit autoimmune phenotypes similar to those observed in *cpr22*. In this thesis, I present the identification of the novel suppressor mutant *repressor of defense no death 1 (rdd1)*, and extended this study to investigate the role of *AtCNGC4* in the same signaling pathway (Chapter 3). Furthermore, from double mutant analyses, I hypothesized that *AtCNGC2* and *AtCNGC4* act in the same signaling pathway involving *RDD1* to mediate plant immunity. Conventional map based cloning and whole genome sequencing identified 4 potential causative mutations in *At5G24680*, *At5G25590*, *At5G25620*, and *At5G26050*. Preliminary studies indicate that *At5G25620* encoding a flavin-containing monooxygenase-like protein may contain the causative mutation in *rdd1* (Chapter 4), which implicates the possibility of altered auxin physiology during *AtCNGC2* and *AtCNGC4*-mediated immune signaling.
Chapter 2

Importance of the αC-helix in the cyclic nucleotide binding domain of CNGCs in Arabidopsis

Modified from:

Importance of the αC-helix in the cyclic nucleotide binding domain for the stable channel regulation and function of cyclic nucleotide gated ion channels in Arabidopsis.

Calmodulin binding to Arabidopsis cyclic nucleotide gated ion channels.
H. Abdel-Hamid, K. Chin, D. Shahinas, W. Moeder and K. Yoshioka
(2010) Plant Signal Behav. 5: 1147-1149

Author contributions: K. Chin performed most of the experiments and H. Abdel-Hamid provided data for Figure. 2-10 and 2-11
2.1. Abstract

The involvement of cyclic nucleotide gated ion channels (CNGCs) in the signal transduction of animal light and odorant perception is well documented. Although plant CNGCs have recently been revealed to mediate multiple stress responses and developmental pathways, studies that aim to elucidate their structural and regulatory properties are still very much in their infancy. The structure–function relationship of plant CNGCs was investigated here by using the chimeric Arabidopsis AtCNGC11/12 gene that induces multiple defense responses in the Arabidopsis mutant constitutive expresser of PR genes 22 (cpr22) for the identification of functionally essential residues. A genetic screen for mutants that suppress cpr22-conferred phenotypes identified over 20 novel mutant alleles in AtCNGC11/12. One of these mutants, suppressor mutant 58 (S58) possesses a single amino acid substitution, arginine 557 to cysteine, in the αC-helix of the cyclic nucleotide-binding domain (CNBD). S58 lost all cpr22 related phenotypes, such as spontaneous cell death formation under ambient temperature conditions. However, these phenotypes were recovered at 16 ºC suggesting that the stability of channel function is affected by temperature. In silico modeling and site-directed mutagenesis analyses suggest that arginine 557 in the αC-helix of the CNBD is important for channel regulation through CaM binding. Furthermore, another suppressor mutant, S136 that lacks the entire αC-helix due to a premature stop codon, lost channel function completely. The data presented in this chapter indicate that the αC-helix is functionally important in plant CNGCs.
2.2. Introduction

For plant survival, cationic nutrients play essential roles in a wide variety of physiological aspects during growth and development. They also play important roles for signal transduction. For example, Ca\(^{2+}\) and K\(^{+}\) fluxes have been reported to be important for abiotic stress as well as biotic stress responses. Uptake and distribution of cationic nutrients mainly relies on membrane-localized cation transporter proteins. Based on genomic sequence data, Arabidopsis contains over 150 cation transport proteins (Mäser et al., 2001). Among them cyclic nucleotide-gated ion channels (CNGCs) form a large gene family consisting of 20 members that have been implicated in defense responses, development, and ion homeostasis in plants (Talke et al., 2003; Kaplan et al., 2007; Chin et al., 2009).

CNGCs were first discovered in retinal photoreceptors and olfactory sensory neurons and so far, six CNGC channel genes have been found in mammalian genomes (Zagotta and Siegelbaum, 1996; Zufall et al., 1994). The structure of CNGCs is similar to that of the voltage-gated outward rectifying K\(^{+}\)-selective ion channel (Shaker) proteins, including a cytoplasmic N-terminus, six membrane spanning regions (S1–S6), a pore domain located between S5 and S6, and a cytoplasmic C-terminus (Zagotta and Siegelbaum, 1996). However, CNGCs are opened by the direct binding of cyclic nucleotides (CN), such as cAMP and cGMP (Fesenko et al., 1985). The CN-binding domain (CNBD) of CNGCs is located at the cytoplasmic C-terminus and exhibits significant sequence similarity to that of protein kinase A, protein kinase G, and the catabolite activator protein of E. coli (Bridges et al., 2005). The cytoplasmic C-terminus contains a CNBD that is connected to the pore domain by a C-linker region. Important
functional features of CNGCs were extensively studied in animal systems and it has been suggested that the subunit composition of the respective channel complex is an important determinant for functional features such as ligand sensitivity, selectivity, and gating (Kaupp and Seifert, 2002).

The first plant CNGC, HvCBT1 (*Hordeum vulgare* calmodulin CaM-binding transporter), was identified as a CaM-binding protein in barley (Schuurink et al., 1998). Subsequently, several CNGCs were identified from Arabidopsis and *Nicotiana tabacum* (Arazi et al. 1999; Köhler et al., 1999). A precise analysis of the CaM binding site has been reported using the tobacco CNGC, NtCBP4 as well as Arabidopsis AtCNGC1 and AtCNGC2, and it has been suggested to be located at the αC-helix of the CNBD in these plant CNGCs (Arazi et al., 2000; Köhler and Neuhaus, 2000). However, only a handful of studies on the structure–function analysis of plant CNGCs have so far been published (Hua et al., 2003; Bridges et al., 2005; Kaplan et al., 2007; Baxter et al., 2008).

The Arabidopsis mutant *constitutive expressor of PR genes 22* (*cpr22*), which contains a novel chimeric CNGC, AtCNGC11/12, shows environmentally sensitive defense responses, such as heightened SA accumulation and a hypersensitive response (HR)-like programmed cell death (Yoshioka et al., 2001; Moeder and Yoshioka, 2009). It has been reported that the expression of AtCNGC11/12 and its channel activity is attributable to the *cpr22* phenotype (Yoshioka et al., 2006; Baxter et al. 2008). Interestingly, all SA-dependent phenotypes are suppressed under high humidity conditions and enhanced by low humidity (Yoshioka et al. 2001). This type of environmental sensitivity has been reported for various pathogen resistance mutants as
well as on defense responses in wild type plants (Moeder and Yoshioka, 2009). The importance of the interaction between the C-linker domain and the CNBD domain for basic channel function was reported previously by Baxter et al. (2008). In addition, it was shown that intragenic mutants of cpr22 are useful tools to study the structure–function relationship of CNGCs (Baxter et al., 2008). Here, the suppressor mutant 58 (S58) was isolated, and used in a structure–function study to show the importance of the αC-helix of the CNBD for stable channel function in planta.

2.3. Materials and Methods

2.3.1. Plant growth conditions and oomycete strains

Arabidopsis thaliana plants were grown on Pro-Mix soil (Premier Horticulture Inc., Red Hill, PA, USA) in a growth chamber under ambient humidity as described by Silva et al. (1999). Nicotiana benthamiana plants were grown on the same soil in a greenhouse under a 14:10 hour light:dark regimen at 25 °C (day) and 20 °C (night).

H. arabidopsidis isolate Emwa1 was propagated on Arabidopsis seedlings (ecotype Ws) and placed in a growth chamber in high humidity under a 16:8 hour light:dark regimen at 16°C. Conidiospores were harvested for infection assays 10 days after propagation.

2.3.2. Suppressor screening and identification of the S58 mutant

Approximately 10, 000 cpr22 homozygous seeds (M₀) were mutagenized with 0.3% (v/v) EMS solution (Sigma-Aldrich, http://www.sigmaaldrich.com/) for 8 hours at room temperature, followed by rinsing more than 15 times in water. The M₀ seeds were grown under high humidity (RH >90%) for M₁ plants. The M₂ seeds were collected and then
screened for suppressor plants under normal humidity. Suppressor 58 (S58) was identified based on wild type morphology. It was backcrossed twice with a homozygous cpr22 plant for genetic analysis.

2.3.3. Trypan blue staining

Leaf samples were taken from 3 – 4 week old plants grown on soil, and vacuum-infiltrated with trypan blue stain. Leaves were then boiled for 2 minutes and incubated overnight at room temperature. Leaves were then de-stained with chloral hydrate and mounted on 80% glycerol for light microscopy.

2.3.4. RNA extraction, RT-PCR, and Northern hybridization

Small-scale RNA extraction was carried out using the TRizol reagent (Invitrogen, Carlsbad, MO, CA), according to the manufacturer's instructions. Reverse transcriptase (RT)-PCR was performed using cDNA generated by SuperScript™ II Reverse Transcriptase (Invitrogen, Carlsbad, MO, CA) according to the manufacturer's instructions. For the detection of CNGC gene expression in N. benthamiana, CNGC and actin gene expression in yeast, and β-tubulin gene expression in Arabidopsis, the same sets of primers described by Baxter et al. (2008) were used (Appendix 1). Primers for the detection of PR-1 by RT-PCR are listed in Appendix 1. Northern hybridization of PR-1 gene was performed as previously described (Yoshioka et al., 2006). RT PCR was carried out for 25 cycles.
2.3.5. Pathogen infection

Cotyledons of 7 day old seedlings were inoculated with a fresh suspension of *H. arabidopsidis* isolate Emwa1 conidiospores in water (10^6 spores ml\(^{-1}\)). Seedlings were then grown for 7 days in 16°C, and susceptibility was evaluated based on the level of sporangiophore and hyphal growth visualized by trypan blue staining.

2.3.6. Plasmid construction

The yeast expression vector plasmid pYES2-empty vector (Invitrogen), pYES2-AtCNGC12, pYES2-AtCNGC11/12, and pYES2-AtCNGC11/12:E519K (S73) were constructed as previously described (Yoshioka et al., 2006; Baxter et al., 2008). For pYES2-AtCNGC11/12:R557C (S58), total RNA was extracted from S58 and cDNA was generated as described above. cDNA of AtCNGC11/12:R557C was then amplified by RT-PCR, and then cloned into pGEM-T Easy (Promega, Madison, WI). In the case of pYES2-AtCNGC11/12:Q543X (S136), AtCNGC11/12 cDNA was used as template for PCR. Both cDNA clones were then subcloned into pYES2 at the BamHI and NotI restriction enzyme sites. AtCNGC:R557C (S58) cDNA minus stop was generated by RT-PCR and subsequently cloned into pGEM-T easy (Promega, Madison, WI). This clone was then subcloned into the XbaI site in pMBP3 (Yoshioka et al., 2006; Urquhart et al., 2007). For site-directed mutagenesis, AtCNGC:R557C (S58) cDNA was excised using XbaI and subsequently subcloned into pBluescript (Stratagene, La Jolla, CA). The point mutation R557I (C1627T) was introduced into AtCNGC11/12 cDNA in pBluescript (Baxter et al. 2008) using the QuickChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. The AtCNGC11/12:R557I cDNA was then subcloned into pMBP3 for transient assays in *N. benthamiana*. All constructed plasmids
were sequenced for fidelity. All primers used for plasmid construction are listed in Appendix 1.

2.3.7. Agrobacterium-mediated transient expression

All pMBP3 plasmids were transformed into Agrobacterium tumefaciens strain, GV2260 by heat shock. Individual colonies were grown for 20 hours at 30°C in Luria Broth (LB) medium, and then subcultured into Medium 1 (LB, 10mM MES, and 20uM acetylsyringone) for 20 hours. Cultures were then washed in infiltration medium (10mM MgCl₂, 10mM MES and 150uM acetylsyringone), and resuspended to OD₆₀₀ = 0.5. Cultures were incubated overnight at room temperature and syringe-infiltrated into 6 week old N. benthamiana leaves. All cultures were co-infiltrated with HC-Pro of tobacco etch virus (TEV) to suppress post-transcriptional gene silencing. Cell death development was assessed between 30 - 48 hours. The expression of these genes was confirmed by RT-PCR (see RNA extraction and RT-PCR), and protein stability was confirmed by confocal microscopy (see GFP visualization by confocal microscopy).

2.3.8. Functional complementation in yeast

The K⁺-uptake deficient yeast mutant strain CY162 (MAT α, ura3-52, trk1,2) was transformed with the empty plasmid pYES2, pYES2-AtCNGC12, pYES2-AtCNGC11/12, pYES2-AtCNGC11/12:R557C, pYES2-AtCNGC12:R557I and pYES2-AtCNGC11/12:Q543X following the lithium acetate transformation protocol (Ausubel et al., 1987). Yeast transformants were grown in synthetic minimal media supplemented with 0.1 mM KCl (Leng et al., 1999) and hygromycin (5 mg l⁻¹) (Mercier et al., 2004) to
suppress background growth. Growth rates were monitored by determining the OD$_{600}$ of the growing yeast cultures at 20 and 40 hours.

A wild type strain of *S. cerevisiae* W303-1A (Wallis et al., 1989) and the Ca$^{2+}$ channel mutant (*cch1::TRP1* null mutant) strain K927 (Locke et al., 2000) were provided by Dr H. Iida (Tokyo Gakugei University). K927 was transformed with pYES2 empty vector, pYES2-AtCNGC12, pYES2-AtCNGC11/12, pYES2-AtCNGC11/12:R557C, pYES2-AtCNGC12:R557I, and pYES2-AtCNGC11/12:Q543X following the lithium acetate transformation protocol (Ausubel et al., 1987). To test for complementation of the *cch1* mutation, yeast transformants were grown to logarithmic phase in synthetic minimal media and were diluted to $10^6$ cells ml$^{-1}$ and exposed to 20 μM α-mating factor in modified synthetic minimal media containing 100 μM CaCl$_2$ (Muller et al., 2001). 100 μl aliquots of cells were harvested by centrifugation and resuspended in 10 mg ml$^{-1}$ trypan blue solution at 4, 8, and 12 hours. Yeast viability was measured by assessing the ratio of stained to unstained cells under brightfield microscopy. A minimum of 200 cells were counted for each transformant.

Ca$^{2+}$ uptake was measured by the method described by Kurusu et al., (2004) with slight modifications. K927 transformants were grown to logarithmic phase in synthetic minimal media and diluted to $10^7$ cells ml$^{-1}$. Cultures were then pre-incubated for 120 minutes at 30 °C in 10 mM MES-TRIS buffer (pH 6.0) containing 100 mM glucose. 45-CaCl$_2$ was added to a final concentration of 72 kBq ml$^{-1}$. 100 μl aliquots were harvested at 10, 20, and 30 minutes by centrifugation, and washed five times with washing solution (20 mM MgCl$_2$, 0.1 mM LaCl$_3$). Radioactivity in yeast cells was
measured using a liquid scintillation counter. All experiments have been conducted with three biological repeats, each with three technical repeats.

2.3.9. Green fluorescence protein (GFP) visualization by confocal microscopy

Agrobacterium-mediated transient expression in *N. benthamiana* was performed as described above, at 16 °C and 22 °C. Plants were shifted to 16 °C for 12 h after infiltration, and protein stability in either condition was confirmed by GFP expression at 30 hours. Small sections of the infiltrated area were excised and used for confocal microscopy. Confocal fluorescence images were acquired using a Leica TCS SP5 confocal system with AOBS® (HCS PL APO CS 40× immersion oil objective; NA, 1.25) with the AOTF for the argon laser (488 nm) set at 35% and the detection window at 500–600 nm (Leica Microsystems Inc., Wetzlar, Germany).

2.3.10. Computational modeling and sequence alignment

The tertiary structure modeling of AtCNGC11/12 was conducted as described previously (Baxter et al., 2008) using the crystallized structure of the cytoplasmic C-terminus of invertebrate CNGC, SpIH (Flynn et al., 2007, PDB no. 2PTM). The protein fold recognition server (Phyre; Kelley and Sternberg, 2009) was used to model the protein coordinates with estimated precision of 100%. All the images were generated using PyMOL (DeLano, 2002).

The sequence alignments of the CNBD amino acid sequences of all 20 Arabidopsis CNGCs were aligned using ClustalW (Thompson et al., 1994). The accession numbers of all protein sequences used for the alignment are indicated in Appendix 2.
2.3.11. Nuclear Magnetic Resonance (NMR) spectroscopy

Peptides corresponding to the C-terminus of AtCNGC11/12 (same as AtCNGC12) and AtCNGC11/12:S58 were synthesized (purification grade <90%). NMR data was collected at 20°C on a 800-MHz Bruker ADVANCE II spectrometer equipped with z-axis pulsed field gradient units, and room temperature shielded triple resonance probes using 100µl NMR tubes. NMR samples were prepared in a buffered solution (10mM TRIS:HCl pH 7, 10mM CaCl₂, 150mM NaCl, 10% D₂O). All samples were prepared as a 40µl reaction with a peptide concentration of 0.2mM.

2.4. Results

2.4.1. The intragenic suppressor S58 loses cpr22 -related phenotypes under ambient conditions

The suppressor screen of cpr22 was reported previously (Baxter et al., 2008). Through this screen, 29 novel mutant alleles in AtCNGC11/12 have been discovered so far (Figure 2-1, Abdel-Hamid, 2013). One of these mutants, suppressor S58 (S58) was found to be morphologically identical to the wild type (wt) plants (Figure 2-2A). The homozygosity of the cpr22 allele in S58 was confirmed by PCR-based marker analysis (data not shown). In contrast to homozygous cpr22, S58 is neither lethal, nor shows HR-like spontaneous lesion formation and constitutive PR-1 gene expression (Figure 2-2A, B). Pathogen resistance was evaluated using the oomycete pathogen, Hyaloperonospora arabidopsis, isolate Emwa1, which is virulent for the ecotype Wassilewskija (Ws, the background ecotype of cpr22). cpr22 showed enhanced
Figure 2-1. Location of the novel 29 mutations identified in AtCNGC11/12. Mutations are listed with respect to the proposed topological model of AtCNGC11/12 including the six transmembrane domains (S1–S6), the ion pore (P), the C-linker and the cyclic nucleotide-binding domain (CNBD). The R557C mutation conferring S58 resides in the CNBD (red box).
Figure 2-2. Characterization of the suppressor mutant S58. A) Morphological phenotypes and spontaneous HR cell death formation of wild type (Ws-wt), cpr22, and suppressor 58 (S58). A cpr22 homozygous plant is shown in the white square. Approximately 4-week-old plants were used. B) Northern blot analysis for PR-1 gene expression in Ws-wt, cpr22, and S58. The samples were taken from approximately 4-week-old plants. Ethidium bromide staining of ribosomal RNA (rRNA; lower panel) served as a loading control. C) Growth of *H. arabidopsis*, isolate Emwa1 in Ws-wt,
cpr22, and S58. Plants were infected by spraying a conidiospore suspension of $10^6$ ml$^{-1}$ on 7-d-old plants. Trypan blue analysis 8 d after infection was done to visualize pathogen growth.

### Table 2-1. Interaction phenotype with *H. arabidopsidis* isolate Emwa1$^a$

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Total No. of Plants</th>
<th>No. of Resistant Plants</th>
<th>No. of Susceptible Plants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ws-wt</td>
<td>33</td>
<td>3</td>
<td>30</td>
</tr>
<tr>
<td>Col-wt</td>
<td>22</td>
<td>22</td>
<td>0</td>
</tr>
<tr>
<td>cpr22/CPR22</td>
<td>26</td>
<td>26</td>
<td>0</td>
</tr>
<tr>
<td>S58</td>
<td>32</td>
<td>0</td>
<td>32</td>
</tr>
</tbody>
</table>

$^a$ Based on formation of sporangiophores; Resistance, no formation; Susceptible, formation.

### Table 2-2. Segregation analysis of the cpr22 phenotype

<table>
<thead>
<tr>
<th>Plant line$^a$</th>
<th>Total No.</th>
<th>Morphological phenotype</th>
<th>Hypothesis$^b$</th>
<th>$\chi^2$</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Wt</td>
<td>cpr22</td>
<td>Lethal</td>
<td></td>
</tr>
<tr>
<td>cpr22/CPR22</td>
<td>92</td>
<td>25</td>
<td>43</td>
<td>23</td>
<td>1:2:1</td>
</tr>
<tr>
<td>S58 x cpr22/cpr22 (B$_1$)$^d$</td>
<td>5</td>
<td>0</td>
<td>5</td>
<td>0</td>
<td>0:1:0</td>
</tr>
<tr>
<td>B$_2$$^e$</td>
<td>92</td>
<td>19</td>
<td>47</td>
<td>24</td>
<td>1:2:1</td>
</tr>
</tbody>
</table>

$^a$ S58 is the pollen accepting plant

$^b$ Both cpr22 and S58 are semi-dominant

$^c$ two degrees of freedom

$^d$ Backcross first generation of S58 and cpr22 homozygous plants

$^e$ Backcross second generation of S58 and cpr22 homozygous plants
resistance to Emwa1 (Yoshioka et al., 2006). As predicted, S58 lost cpr22-mediated enhanced resistance to this pathogen (Figure 2-2C; Table 2-1). Taken together; it is concluded that S58 lost all tested cpr22-related phenotypes under ambient temperature condition (22 °C). Sequence analysis of the cpr22 gene (AtCNGC11/12) revealed one nucleotide substitution, C to T in the cyclic nucleotide binding domain (CNBD) that caused an amino acid substitution, Arginine 557 to Cysteine (R557C) in S58 (Figure 2-1). The genetic nature of S58 was evaluated by backcrossing with cpr22 homozygous plants. As shown in Table 2-2, all B₁ (backcross, 1st generation) plants showed cpr22 heterozygous-like phenotypes, and the following self-pollinated B₂ generation showed a segregation of 1 (wildtype like):2 (cpr22 heterozygous like):1 (lethal). This indicated the semi-dominant nature of this mutation supporting the idea that the aforementioned intragenic mutation R557C is the cause of the phenotype suppression. RT-PCR in homozygous cpr22 and S58 mutants confirmed that there is no significant difference in expression of AtCNGC11/12 and AtCNGC11/12:R557C.

2.4.2. The S58 mutation (R557C) does not affect channel function in yeast

Growth enhancement of mutant yeast has been previously demonstrated upon expression of various plant CNGCs (Köhler et al., 1999; Leng et al., 1999; Ali et al., 2006). More recently, it was suggested that AtCNGC11, 12, and AtCNGC11/12 can function as K⁺ channels as well as Ca²⁺ channels when expressed in the same systems (Yoshioka et al., 2006; Urquhart et al., 2007). Therefore, it is possible that the loss of cpr22-related phenotypes in S58 can be attributed to either a loss in basic ion channel function, or a loss in the constitutive active character of AtCNGC11/12 while still maintaining basic channel function. To address this point, a heterologous expression
system using K\(^+\) and Ca\(^{2+}\) uptake-deficient yeast mutants was used to evaluate the channel function of AtCNGC11/12:R557C. The \textit{trk1, trk2} K\(^+\) uptake-deficient yeast mutant, CY162 (Leng et al., 1999), transformed with AtCNGC11/12 or AtCNGC11/12:R557C was tested in low external K\(^+\) and in the presence of the cationic antibiotic hygromycin. As shown in Figure 2-3A, \textit{AtCNGC11/12} and \textit{AtCNGC12} were able to complement the \textit{trk1, trk2} phenotype compared to the empty vector control. Interestingly, the mutants carrying \textit{AtCNGC11/12:R557C} were also able to complement the \textit{trk1, trk2} phenotype with the same efficiency as \textit{AtCNGC11/12}, whereas another CNBD mutant, S73 (E519K), which affects ion channel function (Baxter et al., 2008) did not, suggesting that the R557C mutation does not affect its basic ion channel function. To explore if this is also the case for Ca\(^{2+}\) channel function, a similar complementation analysis was conducted using the Ca\(^{2+}\)-uptake yeast mutant strain K927 (\textit{cchl::TRP1}; Locke et al., 2000). \textit{CCH1} has been implicated in Ca\(^{2+}\) influx in response to mating pheromones (Fischer et al., 1997). Previously, it has been shown that Ca\(^{2+}\) is important for cell death induction by the expression of \textit{AtCNGC11/12} (Urquhart et al., 2007). Therefore, it is possible that only Ca\(^{2+}\) channel function is affected by the S58 mutation. However as shown in Figure 2-3B by trypan blue staining, \textit{AtCNGC11/12, AtCNGC12,} and \textit{AtCNGC11/12:R557C} were able to rescue this yeast phenotype to comparable levels, indicating that \textit{AtCNGC11/12:R557C} is functional as a Ca\(^{2+}\) ion channel. These data were quantitatively confirmed in Figure 2-3C. In addition, \(^{45}\text{Ca}^{2+}\) uptake analysis was conducted using the same yeast mutant strain. Although the \(^{45}\text{Ca}^{2+}\) uptake did not reach the same level as wild-type yeast, K927 mutant yeast with \textit{AtCNGC11/12} or \textit{AtCNGC11/12:R557C} had a higher uptake rate of \(^{45}\text{Ca}^{2+}\) than K927 carrying the empty vector (Figure 2-4). The same result was observed in four independent experiments. No
significant difference between *AtCNGC11/12* and *AtCNGC11/12:R557C* was observed, suggesting that the R557 (S58) mutation does not impair the function of *AtCNGC11/12*. This result is consistent with the mating pheromone analyses using the same yeast mutant strain (Figure 2-3B). Taken together, it was concluded that the mutation R557C does not affect basic ion channel function but rather affects the regulation of *AtCNGC11/12*. 
**Figure 2-3.** Yeast complementation analyses. A) AtCNGC11/12, AtCNGC12, and S58 (AtCNGC11/12:R557C) complemented the K\(^+\)-uptake deficient mutant CY162, whereas S73 and the empty vector did not. Data are the average of three biological repeats ±SE. Student's \( t \) test shows significant differences between the empty vector/S73 and AtCNGC11/12, AtCNGC12, or S58 at 20 h and 40 h \((P<0.05)\). Experiments have been performed more than three times with similar results. B) AtCNGC11/12, AtCNGC12, and S58 (AtCNGC11/12:R557C) complemented the Ca\(^{2+}\)-uptake deficient mutant K927, whereas S73 and empty vector did not. Data are the average of three biological repeats ±SE. Student's \( t \) test shows significant differences between the empty vector and AtCNGC11/12, AtCNGC12, or S58 at 4, 8, and 12 h \((P<0.05)\). The experiment has been repeated more than three times with comparable results. C) Yeast viability analysis by Trypan blue staining. AtCNGC11/12, AtCNGC12, and S58 (AtCNGC11/12:R557C) rescued the cell death phenotype of the Ca\(^{2+}\)-uptake deficient mutant K927, whereas S73 and empty vector did not.
Figure 2-4. Radioactive Ca\(^{2+}\) uptake in yeast. AtCNGC11/12, AtCNGC12, and S58 (AtCNGC11/12:R557C) complemented the Ca\(^{2+}\)-uptake deficient mutant K927, whereas S73 and empty vector did not. Data are the average of three biological repeats ±SE. Student’s t test shows significant differences between the empty vector and AtCNGC11/12 or S58 at 10, 30, and 30 minutes (P < 0.05). The experiment has been repeated more than three times with comparable results.
2.4.3. AtCNGC11/12:R557C recovers the activity to induce cell death under low temperature

As mentioned above, cpr22-related phenotypes show temperature sensitivity. Relatively high temperatures (>28 °C) can suppress its phenotypes and on the other hand, relatively lower temperatures (<16 °C) enhanced them (Mosher et al., 2010). Since the R557C mutation seems to suppress the constitutive active character of AtCNGC11/12 but not basic ion channel function, the question was asked if lower temperature can restore this constitutive active character (channel activity). Strikingly, a temperature shift from 22 °C to 16 °C induced cpr22-like morphology, such as curly leaves 4 days after shift in S58. Chlorosis on leaves, which indicates cell death development, could also be observed (Figure 2-5A). Trypan blue staining as well as electrolyte leakage analyses further confirmed cell death development in S58 (Figure 2-5A, B). PR-1 gene expression also recovered after the shift from 22 °C to 16 °C (Figure 2-5C), further supporting the temperature sensitivity of this mutant.

To characterize the temperature sensitivity of S58 (AtCNGC11/12:R557C) further, Agrobacterium-mediated transient expression analysis was conducted in N. benthamiana. This is an established system to analyze HR cell death development and has been used to show that AtCNGC11/12 induces cell death in a synchronized manner (Urquhart et al., 2007). As shown in Figure 2-6A, cell death was induced by the transient expression of AtCNGC11/12 but not AtCNGC11/12:R557C at 22 °C (ambient temperature) indicating the inactivity of AtCNGC11/12:R557C in planta under this condition. On the other hand, AtCNGC11/12:R557C recovered cell death induction in plants that were shifted from 22 °C to 16 °C. This was also confirmed quantitatively
**Figure 2-5.** Temperature sensitivity of *cpr22*-related phenotypes in *cpr22*, S58, S73, and Ws-wt plants after a shift from 22 °C to 16 °C. A) S58 displayed *cpr22*-morphology after temperature shift. *cpr22* showed enhancement of HR cell death and S58 induced HR cell death and *cpr22*-related phenotypes after the temperature shift. No cell death induction was observed in another intragenic suppressor, S73 and Ws-wt under both conditions. Photographs were taken 7 days after the shift. B) Quantitative analysis of cell death by electrolyte leakage in *cpr22*, S58, S73, and Ws-wt. Samples were taken 7 days after the shift. C) RT-PCR analysis of *PR-1* gene expression in *cpr22*, S58, S73, and Ws-wt. Temperature shift induced *PR-1* gene expression in S58, whereas no significant change was observed in S73 and Ws-wt. *β-tubulin* served as a loading control. Samples were taken 7 days after the shift. Primers used for PR-1 gene analysis are listed in Appendix 1.
by electrolyte leakage analysis (Figure 2-6B). Transcript and protein levels were not significantly altered by the temperature shift as depicted by RT-PCR (Figure 2-5C) and GFP fluorescence (Figure 2-6D). Note that while we have observed enhanced cell death in cpr22 when it was shifted from 22 °C to 16 °C (Figure 2-5A), there is no significant difference between 22 °C and 16 °C when AtCNGC11/12 was transiently expressed in N. benthamiana. This is probably due to the sampling timing. The samples for Figure 2-5B were taken at 80 h after Agrobacterium infiltration. At this time point, cell death development induced by transient expression of AtCNGC11/12 is almost complete and also, it is very strong and uniform compared to the mutant itself due to the constitutive CaMV35S promoter.
**Figure 2-6.** Temperature sensitivity of cell death induction by transient expression of AtCNGC11/12, empty vector, S58 (AtCNGC11/12:R557C), and S73 (AtCNGC11/12:E519K) in *Nicotiana benthamiana*. A) Induction of cell death in *N. benthamiana* 24, 48, and 80 hours after Agrobacterium infiltration, either shifted from 22 °C to 16 °C at 12 hours after Agrobacterium infiltration (lower panels) or not shifted (upper panels). Cell death induction was observed in the leaf area expressing S58, but not empty vector (EV) or S73 after the temperature shift. Cell death induced by AtCNGC11/12 was enhanced by temperature shift. Red circles indicate HR development. B) Quantitative analysis of cell death in *N. benthamiana* by electrolyte leakage of leaf discs. S58 expression induced cell death after the temperature shift, but not empty vector (EV) or S73. Samples were taken 80 hours after Agrobacterium infiltration. C) RT-PCR analysis of leaf discs from *N. benthamiana* leaves expressing AtCNGC11/12, S58 or empty vector (EV). The temperature shift did not significantly affect gene expression of AtCNGC11/12 or AtCNGC11/12:R557C in *N. benthamiana* leaf discs. *actin* served as a loading control. Samples were taken 24 hours after Agrobacterium- infiltration (12 hours after the shift). D) The expression of AtCNGC11/12:GFP and AtCNGC11/12:R557C:GFP was not altered by the temperature shift. The samples were taken 30 hours after Agrobacterium infiltration. The fluorescence of the GFP-fusion proteins was monitored by confocal microscopy.
2.4.4. 3D computational modeling of AtCNGC:R557C

In order to predict the role of R557 in channel structure, a computational analysis of the three-dimensional structure of the cytoplasmic C-terminal region was conducted. Previously, the crystal structure of the cytoplasmic C-terminal region of a hyperpolarization-activated cyclic nucleotide-modulated channel, HCN2, has been used as a template (Zagotta et al., 2003, PDB ID: 1Q50, Baxter et al., 2008). The recently published crystal structure of another HCN, SpIH was used here (crystallized with cAMP; Flynn et al., 2007; PDB ID: 2PTM) that possesses higher structural similarity than HCN2 to AtCNGC12 (H Abdel-Hamid and D Shahinas, unpublished data). As shown in Figure 2-7A, R557 is located in the middle of the αC-helix in the CNBD. The importance of this αC-helix for cNMP binding has been reported in animal CNGCs (Rehmann et al., 2007). According to the crystal structure of the CNBD of HCN2 in the presence of cAMP, cAMP binds in the anti-configuration between the β-roll and the αC-helix of the CNBD. In our model, the side chain of R557 faces away from the interior of the cNMP binding pocket formed by the β-roll of the CNBD and therefore, likely does not bind to cNMPs directly (Figure 2-7A). Hydrophobic interactions between the αC-helix and the base of cNMPs were also postulated to stabilize cNMP binding (Rehmann et al., 2007), further suggesting that the hydrophilic R557 does not directly interact with cNMPs. A sequence comparison revealed that R557 is conserved in 17 out of 20 Arabidopsis CNGCs (Figure 2-7B). Only three CNGCs, AtCNGC1, 3, and 11 have a lysine (K) or glutamine (Q) that mediate ionic interactions similarly to arginine (R) in this position. All of them are hydrophilic (hydropathy index: −4.5). Cysteine is slightly polar due to its −SH group but does not mediate any ionic interactions (hydropathy index: +2.5). To address whether a more hydrophobic residue with less polarity than cysteine
such as isoleucine (hydropathy index: +4.5) can completely disrupt channel function, AtCNGC11/12:R557I was created by site-directed mutagenesis, and it was transiently expressed in *N. benthamiana* to assess cell death induction. As shown in Figure 2-8A, cell death is not induced by AtCNGC11/12:R557I at 22 °C, but is recovered at 16 °C similar to AtCNGC11/12:R557C. The expression of all constructs was confirmed by RT-PCR (Figure 2-8B). Therefore, even by altering R557 to isoleucine, complete suppression of channel function at 16 °C could not be obtained.
Figure 2-7. The location of R557 and Q543 in tertiary structure and amino acid sequence alignment. A) Ribbon diagram of the cytoplasmic C-terminal region of AtCNGC11/12 (AtCNGC12) (left panel) and close-up of the indicated area of the left panel (right panel). R557 is located in the αC-helix and Q543 is located in the αB-helix of the CNBD. cAMP is indicated by pink colour. B) Alignment of the area of R557 with 20 Arabidopsis CNGCs and tobacco NtCBP4. The NCBI accession numbers are listed in Appendix 2. The black box indicates the position of R557. The red box indicates the CaM binding domain and bold characters highlight the critical four amino acids for the CaM binding suggested by Arazi et al. (2000). The location of Q543 (S136) is indicated by a black dot.
Figure 2-8. AtCNGC11/12:R557I expression induces cell death similarly to S58 in *Nicotiana benthamiana* at lower temperature. A) Quantitative analysis of cell death in *N. benthamiana* was assessed by electrolyte leakage of leaf discs. AtCNGC11/12:R557I expression induced cell death to the same degree as AtCNGC11/12:R557C (S58) after the temperature shift from 22 °C to 16 °C but not at 22 °C. B) RT-PCR analysis of leaf discs from *N. benthamiana* leaves expressing AtCNGC11/12, S58 (AtCNGC11/12:R557C), AtCNGC11/12:R557I or empty vector (EV). The temperature shift did not significantly affect gene expression of AtCNGC11/12, AtCNGC11/12:R557C or AtCNGC11/12:R557I in *N. benthamiana* leaf discs. *Actin* (Act) served as a loading control.
2.4.5. The αC and αB helices in the CNBD are crucial for channel function

The αC-helix in the CNBD was suggested to play an important role for animal CNGC function/channel gating (Rehmann et al., 2007). However, so far there is almost no report about the importance of the αC-helix in plant CNGCs except one report showing that the CaM binding domain is located in the αC-helix (Arazi et al., 2000). To study the role of the αC-helix in channel function further, another intragenic suppressor mutant, S136, was used that has a premature stop codon at Q543 (C to T point mutation) in the CNBD. Our structural model revealed Q543 to be located at the middle of the αB helix, indicating that S136 does not have the entire αC-helix and only a partial αB helix (Figure 2-7A). S136 did not display cpr22 phenotypes under 22 °C or 16 °C (Figure 2-9A). It also lost constitutive expression of PR-1 and enhanced pathogen resistance (Figure 2-9B and C). Furthermore, heterologous expression in the previously mentioned trk1, trk2 K⁺ and cchl Ca²⁺ yeast mutants revealed that AtCNGC11/12:Q543X does not have channel function (Figure 2-9D; data not shown). Taken together, these data suggest that the αC-helix and possibly the αB-helix of the CNBD is functionally essential for CNGC function in plants.
Figure 2-9. Characterization of the premature stop codon mutant, S136. A) Morphological and cell death phenotypes of Ws-wt, cpr22 and S136 with and without temperature shift. S136, unlike S58 does not induce cell death after a shift from 22 °C to 16 °C. Samples were taken 7 d after the shift. B) RT-PCR analysis for PR-1 gene expression in Ws-wt, cpr22, and S136. The samples were taken from approximately 4-week-old plants. β-tubulin (β-tub) served as a loading control. C) Growth of H. arabidopsidis, isolate Emwa1 in Ws-wt, cpr22, and S136. Plants were infected by spraying a conidiospore suspension of $10^6$ ml$^{-1}$ on 7 day-old plants. Trypan blue analysis 8 days after infection was done to visualize pathogen growth. D) Yeast complementation analysis using the Ca$^{2+}$-uptake deficient mutant K927. Only AtCNGC11/12 but not S136 (AtCNGC11/12: Q543X) rescued the K927 phenotype. Data are the average of three biological repeats ±SE. Student’s $t$ test shows a significant difference between AtCNGC11/12 and both empty vector and S136 at 12 hours ($P <0.05$). The experiment has been repeated more than three times with similar results.
2.4.6. R557C results in the partial loss of CaM binding

In animal systems, it has been reported that cNMPs bind within the pocket formed by the αC-helix and the β-barrel composed of the eight β sheets in the CNBD (Weber and Steitz, 1987; Rehmann et al., 2007). The αC-helix was suggested to function as the lid of this pocket that stabilizes cNMP binding by forming hydrophobic interactions with the bound cNMP (Rehmann et al., 2007). However, because R557 is hydrophilic, we hypothesized that R557 might not participate in cNMP binding. Interestingly, a 19–20 amino acid sequence within this helix was suggested to be the CaM binding domain in AtCNGC1 and AtCNGC2 by Köhler and Neuhaus (2000) using yeast two hybrid analysis. Additionally, Arazi et al. (2000) biochemically demonstrated that a 23 amino acid sequence overlapping this 19–20 amino acid region is the CaM binding domain in the tobacco CNGC, NtCBP4. They also reported that four additional amino acids (W R T/S W) which are located just outside of the 19–20 amino acid sequence are crucial for efficient binding. As shown in the alignment in Figure 2-7B, R557 is located in this crucial sequence (indicated by bold characters). Considering that R557C mutation does not affect basic channel function, it can be hypothesized that R557C mutation may instead alter channel regulation by disrupting CaM binding. Therefore, to investigate this hypothesis, in silico models of CaM binding in the CNBD of AtCNGC12 (identical to AtCNGC11/12) and AtCNGC12:R557C (S58) were generated. We first modeled Arabidopsis CaM1 (AtCaM1) based on the crystal structure of a potato CaM, PCM6 (PDB# 1RFJ) (Yun et al., 2004). There are seven different CaM genes in Arabidopsis that encode two sets of identical isoforms (CaM1 and CaM4; CaM2, CaM3 and CaM5), and two additional distinct isoforms (CaM6 and CaM7) (McCormack and Bramm, 2003; DeFalco et al, 2009), all of which share high similarity with PCM6. Using
the AtCaM1 model, we modeled possible interactions between AtCaM1 and the αC-helix of the CNBD in AtCNGC12 and AtCNGC12:R557C. As shown in Figure 2-10A, AtCaM1 forms a hydrophobic pocket that binds to the αC-helix of the CNBD in AtCNGC12. In this model, R557 creates salt bridges with both D79 and E83 of AtCaM1 (indicated by a box) that appear to play an important role in binding. These salt bridges have been reported to be crucial for CaM binding with several target proteins (Ye et al, 2008). Interestingly, these salt bridges are no longer seen in AtCNGC12:R557C (Figure 2-10B), indicating that the R557C mutation may cause a change in affinity in CaM binding. To test this hypothesis experimentally, the cytosolic C-terminal region (S357 to K578) of AtCNGC12 and AtCNGC12:R557C were expressed in E. coli, and analyzed for CaM binding using NMR spectroscopy. NMR spectroscopy is a well established method for analyzing protein-protein and protein-ligand interactions (Takeuchi and Wagner, 2006), especially with CaM where its NMR spectra have been well resolved for over two decades (Mal and Ikura, 2006). The black rat CaM (PDB 3CLN) with greater than 95% identity to AtCaM’s was used in this analysis. As shown in Figure 2-11A, a significant chemical shift in the residues of CaM was observed upon addition of the C-terminal region of AtCNGC12 indicating that this region of AtCNGC12 is able to bind CaM. Interestingly, a significant loss in chemical shift was observed upon addition of the C-terminal region of AtCNGC12:R557C (Figure 2-11B), indicating an impairment of CaM binding. Therefore, data from the NMR analysis indicates that the predicted C-terminal domain in the αC-helix of AtCNGC12 can in fact bind CaM, and that the R557C mutation impairs this binding.
Figure 2-10. Computational structural modeling of CaM binding with AtCNGC12 and AtCNGC12:R557C. Modeling of the tertiary structure of AtCaM1, and the \( \alpha \)C-helix of AtCN GC12 and AtCN GC12:R557 was conducted using the crystallized structures of the potato CaM, PCM 6 (PDB# 1RFJ)22 and the cytoplasmic C-terminus of the invertebrate CN GC, SpI1H (Flynn et al. 2007, PDB# 2PTM),27 respectively, as templates. The protein fold recognition server Phyre 28 was used to model these proteins. The binding modeling was performed using an algorithm for molecular docking.
(PatchDock) 29. All the images were generated using PyMOL 30. CaM is colored in cyan and the αC-helix is shown in magenta. A) Left: overall binding model between AtCaM1 and AtCNGC12, Right: close up of the boxed area of the left part in AtCNGC12. B) The same area in AtCNGC12:R557C. M73, M52 and M37 of AtCaM1 create a hydrophobic pocket together with F562 and I564 of AtCNGC12, which is a typical binding configuration between CaM and target proteins. R557 creates salt bridges with both D79 and E83 (A; red box). These salt bridges are no longer seen between AtCaM1 and AtCN GC12:R557C (B; red box).
Figure 2-11. 2D NMR spectra of Ca$^{2+}$ activated CaM with the C-terminal region of AtCNGC12 and AtCNGC12:R557C (S58). A) NMR spectra of $^{15}$N-labelled Ca$^{2+}$/CaM (black) and in complex with unlabelled C-terminal region of AtCNGC12 (red). A significant chemical shift is observed for most of the CaM residues upon addition of AtCNGC12 C-terminus. B) NMR spectra of $^{15}$N-labelled Ca$^{2+}$/CaM (black) and in complex with unlabelled C-terminal region of AtCNGC12:R557C (red). Almost no shift is observed in the residues of CaM upon addition of AtCNGC12:R557C (S58) C-terminus.
2.5. Discussion

In this study, it was demonstrated that R557 in the αC-helix of the CNBD in AtCNGC11/12 (same as 12) plays an important role in stable channel regulation likely through CaM binding. Substitution of R557C in AtCNGC11/12 impaired almost all cpr22-related phenotypes suggesting a disruption of channel function by this mutation. However, the cpr22-related phenotypes as well as cell death induced by transient expression of AtCNGC11/12:R557C could be rescued under slightly lower temperatures (16 °C), suggesting an alteration in channel regulation rather than loss of channel function. Yeast complementation analysis demonstrated that AtCNGC11/12:R557C maintains its basic channel function for both K+ and Ca2+ conductance supporting this notion. R557 is highly conserved in the Arabidopsis CNGC family and all residues in this position share similar polarity and hydrophobicity. It has been examined if a change to an even more hydrophobic and less polar residue (R557I) would have a stronger effect that completely disrupts basic channel function. However, this channel remained to be conditional just like AtCNGC11/12:R557C, further strengthening the notion that R557 is important for the regulation of channel activity rather than basic channel function.

In animal systems, it has been reported that cNMPs bind within the pocket formed by the αC-helix and the β-barrel composed of the eight β sheets in the CNBD (Weber and Steitz, 1987; Rehmann et al., 2007). The αC-helix was suggested to function as the lid of this pocket that stabilizes the cNMP binding by forming hydrophobic interactions with the bound cNMP (Rehmann et al., 2007). However, because R557 is hydrophilic, it does not seem to participate directly in cNMP binding. Our computational modeling also supported this notion. Regarding the role of the αC-
helix in plant CNGCs, a 19–20 amino acid sequence of this region was suggested to be the CaM binding domain in AtCNGC1 and AtCNGC2 by Köhler and Neuhaus (2000) using yeast two hybrid analysis. As mentioned above, Arazi et al. (2000) biochemically demonstrated that a 23 amino acid sequence overlapping with this 19–20 amino acids is the CaM binding domain in the tobacco CNGC, NtCBP4. Furthermore, they reported that the four additional amino acids (W R T/S W) which are located just outside of the 19–20 amino acid sequence are crucial for efficient binding. As shown in the alignment in Figure 2-7B, R557 is located in this crucial sequence (indicated by bold characters). Considering the fact that AtCNGC11/12:R557C maintains basic channel function, we hypothesized that R557C alters channel regulation by disrupting CaM binding. This hypothesis was further supported by computational modeling, and experimentally tested using NMR spectroscopy. Significant chemical shifts for most of the residues of CaM were observed upon addition of the cytosolic C-terminal region of AtCNGC12 while they were not observed with AtCNGC12:R557C. Thus, the NMR analysis supports the notion that the R557C mutation alters channel regulation by disrupting CaM binding. It should be noted that preliminary NMR results showed the recovery of CaM binding in AtCNGC11/12:R557C under reducing conditions. Thus, it can be further speculated that disulphide bonds formed between the cysteine residues of the αC-helices in neighbouring subunits impairs CaM binding. Further analysis is required for precise molecular mechanisms to confer conditional phenotypes by R557C mutation.

The experimental results involving of suppressor S136 was unexpected, since the deletion of the part of the CNBD that includes the CaM binding site has been reported to enhance the AtCNGC1 channel function in yeast (Ali et al., 2006). Ali et al. (2006) demonstrated that two deletion constructs of AtCNGC1 showed enhanced K+...
channel function in a yeast complementation analysis. They hypothesized that the binding of the negative regulator CaM does not occur in these deletion constructs thereby promoting channel activity. Based on our computational modeling, the deletion of S136 is positioned between these two constructs (data not shown). However, we did not see any channel function using both K\(^+\) and Ca\(^{2+}\) yeast mutants in the case of S136, suggesting the importance of the αC-helix and possibly the αB-helix for its basic channel function. This discrepancy could be due either to differences in regulatory properties of different AtCNGC subunits or to unknown structural changes caused by the different deletion positions used in both studies. As of now, the answer remains unknown, and further analyses will be required to understand these differences.

In summary, it has been demonstrated that R557 in the αC-helix of the CNBD of CNGCs plays an important role for channel regulation likely through CaM binding. Our data here demonstrates the importance of the αC-helix in CaM binding in a plant CNGC for the first time.
2.6. Acknowledgements

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Chapter 3

AtCNGC2 and AtCNGC4 work in the same signaling pathway to regulate pathogen defense and floral transition

Modified from:

AtCNGC2 and AtCNGC4 work in the same signaling pathway to regulate pathogen defense and floral transition

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(Under revision by Plant Physiology)

Author contributions: K. Chin performed all experiments listed in Chapter 3 and wrote the manuscript with input and direction from W. Moeder and K. Yoshioka.
3.1. Abstract

*Arabidopsis thaliana* cyclic nucleotide-gated ion channels (CNGCs) form a large family consisting of 20 members and have been implicated in Ca$^{2+}$ signaling related to various physiological processes such as pathogen defense, development and thermotolerance. The null mutant of *AtCNGC2*, “*defense, no death*” (*dnd1*) exhibits autoimmune phenotypes, while it is impaired in mounting the hypersensitive response (HR) which is a hallmark of effector-triggered (a.k.a. *R*-gene mediated) resistance. It has been suggested that *AtCNGC2* is involved in defense responses and likely other aspects of physiology through its role as a Ca$^{2+}$-conducting channel. However, the downstream signaling components and its relation with *AtCNGC4*, which is the closest paralog of *AtCNGC2*, remain elusive. Despite the fact that *cngc4* mutants display almost identical phenotypes to those seen in *cngc2* mutants, not much is known about their relationship. Here, we report the identification and characterization of the Arabidopsis mutant, *repressor of defense no death1* (*rdd1*), obtained from a suppressor screen of a T-DNA insertion knockout mutant of *AtCNGC2* in order to identify downstream components of *dnd1*-mediated signal transduction. *rdd1* suppressed the majority of *dnd1*-mediated phenotypes except Ca$^{2+}$ hypersensitivity. In addition, *rdd1* also suppressed the *dnd1*-mediated late flowering phenotype that was discovered in this study. Our genetic analysis conducted to elucidate the relationship between *AtCNGC2* and *AtCNGC4* indicates that *RDD1* is also involved in *AtCNGC4*-mediated signal transduction. Lastly, bimolecular fluorescence complementation (BiFC) analysis suggests that *AtCNGC2* and *AtCNGC4* are likely part of the same channel complex.
3.2. Introduction

The Arabidopsis "defense, no death" mutant, dnd1, is a rare autoimmune mutant that was identified by its reduced ability to produce a hypersensitive response (HR) (Yu et al., 1998; Clough et al., 2000). The HR is a characteristic feature of effector-triggered (a.k.a. R-gene mediated) resistance by which plants undergoing an attack by an avirulent pathogen induce a type of programmed cell death around the sites of pathogen entry (Hammond-Kosack and Jones, 1996; Greenberg and Yao, 2004). Despite the impairment of HR, the dnd1 mutant displays typical autoimmune phenotypes, such as constitutive expression of Pathogenesis-Related (PR) genes, elevated levels of salicylic acid (SA) - an important signaling molecule for pathogen resistance, and conditional HR-like spontaneous lesions without pathogen infection. Consequently, dnd1 plants show enhanced broad spectrum resistance against several taxonomically unrelated pathogens (Yu et al., 1998; Clough et al., 2000; Genger et al., 2008). The mutation in dnd1 has been revealed to be a premature stop codon in the Arabidopsis cyclic nucleotide-gated ion channel, AtCNGC2, and thus is a loss of function mutant of this gene (Clough et al., 2000).

CNGCs are non-selective cation channels that were first identified in vertebrate photoreceptors and olfactory sensory neurons (Zagotta and Siegelbaum, 1996; Craven and Zagotta, 2006). In animals, the biological roles of CNGCs and their regulation have been well studied, and CNGC subunits have been shown to form tetrameric channels at the plasma membrane that are directly regulated by cyclic nucleotides and the Ca$^{2+}$ sensor protein, calmodulin (Kaupp and Siefert, 2002). On the other hand, plant CNGCs
have only been investigated much more recently, and the mechanisms by which they are regulated are just starting to be revealed (Chin et al., 2009; Moeder et al., 2011).

Based on the Arabidopsis genome sequence, there are 20 members in the Arabidopsis CNGC family that are classified into four groups (group I-IV), where group IV is further divided into subgroup IVA and IVB (Mäser et al, 2001). \textit{AtCNGC2} belongs to group IVB, together with only \textit{AtCNGC4}, and are distinctly different from the rest of the CNGC family members. Interestingly, loss of function mutants of \textit{AtCNGC4}, \textit{dnd2/hlm1}, show remarkably similar autoimmune phenotypes to \textit{dnd1}, including an alteration of HR after avirulent pathogen infection (Balagué et al., 2003; Jurkowski et al., 2004). Thus, this observation raises the question of whether these two closely related CNGCs share the same biological function by a similar molecular mechanism.

Experiments in heterologous expression systems suggested that \textit{AtCNGC2} can form ion channels that conduct both Ca\textsuperscript{2+} and K\textsuperscript{+} (Leng et al., 1999; 2002), although it has been mostly implicated in Ca\textsuperscript{2+} signaling (Urquhart et al., 2007; Ali et al., 2007; Finka et al., 2012). Furthermore, \textit{dnd1} shows hypersensitivity to elevated Ca\textsuperscript{2+}, exhibiting severe growth suppression in media containing 20mM CaCl\textsubscript{2} (Chan et al., 2003). Genome wide transcriptome analysis revealed that \textit{dnd1} displays a similar transcriptional profile to that of wild type plants grown in high- Ca\textsuperscript{2+} media (Chan et al., 2008). It is known that Ca\textsuperscript{2+} influx and a rise in cytoplasmic Ca\textsuperscript{2+} play crucial roles in the signal transduction of defense responses, and that pathogen recognition events can trigger a Ca\textsuperscript{2+} signal generated by plasma membrane-localized Ca\textsuperscript{2+}-conducting channels (McAinsh and Schroeder, 2009, Spalding and Harper , 2011). Using electrophysiology, \textit{AtCNGC2} was suggested to form such a channel that generates an
inward Ca\(^{2+}\) current upon recognition of the bacterial elicitor, lipopolysaccharide (LPS) leading to nitric oxide (NO) generation (Ali et al., 2007). Thus, this could be also the case with AtCNGC4.

In addition to \textit{AtCNGC2} and 4, \textit{AtCNGC11} and 12, members of group I, are also postulated to be involved in plant immune responses. This was revealed in the study of the rare gain-of-function mutant, \textit{constitutive expresser of PR genes22 (cpr22)} that resulted from the fusion of \textit{AtCNGC11} and \textit{AtCNGC12} (Yoshioka et al., 2006). Similar to \textit{dnd1} and \textit{hlm1/dnd2}, \textit{cpr22} displays autoimmune phenotypes with increased SA accumulation and constitutive \textit{PR} gene expression. Additionally, \textit{cpr22} mutants exhibit Ca\(^{2+}\)-dependent spontaneous cell death (Urquhart et al., 2007). However, unlike \textit{dnd1} and \textit{hlm1/dnd2}, they are able to induce an HR in response to avirulent pathogens (Yoshioka et al., 2006). Furthermore, \textit{atcngc11} and 12 knockout mutants showed a partial breakdown of resistance against avirulent pathogens indicating a striking difference in the molecular mechanisms that govern defense signaling mediated by \textit{AtCNGC11} and 12 from that of \textit{AtCNGC2} and 4 (Yoshioka et al., 2006; Moeder et al., 2011).

In order to better understand plant CNGC signal transduction, various epistatic analyses have been conducted with \textit{dnd1}, \textit{dnd2/hlm1} and \textit{cpr22}. These experiments revealed that mutations affecting the accumulation and/or perception of SA abolish enhanced resistance to the bacterial pathogen, \textit{Pseudomonas syringae}, and the oomycete pathogen, \textit{Hyaloperonospora arabidopsidis} observed in \textit{dnd1}, \textit{hlm1/dnd2}, and \textit{cpr22} (Clough et al., 2000; Jurkowski et al., 2004; Yoshioka et al., 2001; 2006; Genger et al., 2008). Therefore, despite the differences in HR responses, the enhanced
resistance phenotype in these mutants was established to be SA-dependent. Additionally, \textit{PAD4 (Phytoalexin Deficient4), EDS1(Enhanced Disease Susceptibility1),} and \textit{NDR1 (Non Race-Specific Disease Resistance1)} are important components of the resistance regulated by the aforementioned CNGCs (Jirage et al., 2001; Yoshioka et al., 2001; 2006; Genger et al., 2008). Alterations in jasmonic acid (JA) and ethylene (ET) signaling pathways, which mediate wounding, herbivory and the resistance response to necrotrophic pathogen attack, have also been observed in these mutants (Yoshioka et al., 2001; Jurkowski et al., 2004; Genger et al., 2008). Along with \textit{PR} genes, \textit{cpr22} constitutively expresses the JA inducible antifungal defensin gene \textit{PDF1.2}, which is suppressed when crossed to mutants of JA/ET signaling, thereby indicating that the mutation does not only activate SA-dependent signaling pathways, but JA/ET-dependent ones as well (Yoshioka et al., 2001). In contrast, \textit{PDF1.2} is not expressed in \textit{dnd1}, but is highly induced when SA-associated pathways are impaired (Jirage et al., 2001; Genger et al., 2008). However, the mechanism by which this redirection of both signaling pathways occurs remains unknown. Thus, there is increasing evidence that implicates CNGCs in regulating pathogen defense responses through separate but partially overlapping pathways. Many of the signaling components in these pathways however, still remain to be elucidated despite significant progress.

Here, we report the identification and characterization of the Arabidopsis mutant, \textit{repressor of defense no death1 (rdd1)}, obtained from a suppressor screen of a T-DNA knockout mutant of \textit{AtCNGC2} in order to identify downstream components of \textit{dnd1}-mediated signal transduction. \textit{rdd1} suppressed the majority of \textit{dnd1}-mediated phenotypes except \textit{Ca}^{2+} hypersensitivity. In addition, \textit{rdd1} suppressed the \textit{dnd1}-mediated floral transition phenotype that was discovered in this study. Our genetic
analysis conducted to elucidate the relationship between \textit{dnd1}-mediated and \textit{hlm1/dnd2}-mediated resistance signaling pathways indicates that \textit{RDD1} is also involved in \textit{AtCNGC4}-mediated signal transduction. The data presented here provides evidence of the overlapping nature of the two signaling pathways and possible hetero-tetramerization of \textit{AtCNGC2} and \textit{AtCNGC4}. Finally, BiFC analysis in \textit{Nicotiana benthamiana} suggests that \textit{AtCNGC2} and \textit{AtCNGC4} subunits form homo- and hetero-tetrameric channels \textit{in planta}.

3.3. Materials and Methods

3.3.1. Plant materials and growth conditions

The null mutant for \textit{AtCNGC2}, \textit{dnd1} was originally identified by Yu et al. (1998) and then was called \textit{dnd1-1} in Genger et al. (2008) and \textit{cngc2-1} in Chan et al. (2003). We used the original name of \textit{dnd1} in this chapter. Another allele, \textit{cngc2-2}, was also reported (Chan et al., 2003), thus we named our T-DNA insertion line (Salk Salk_066908) \textit{cngc2-3}. The allelic null mutants for \textit{AtCNGC4} have been reported in Balagué et al. (2003) as \textit{hlm1-1}, \textit{-2}, \textit{-3} and \textit{dnd2-1} in Jurkowski et al. (2004). We used \textit{dnd2-1} from Jurkowski et al. (2004) in this chapter, and used the name \textit{dnd2} to avoid confusion. The T-DNA insertion knockout line for \textit{AtCNGC4} that was used in this study (Salk Salk_081369) thus was named \textit{cngc4-5}. The seeds were planted on Sunshine Mix #1 (Sun Gro Horticulture Canada Ltd) and stratified at 4°C for 4 days. Plants were either grown in a growth chamber with a 9 hour photoperiod (9 hour light/ 15 hour dark) and a day/night temperature regime of 22°C/18°C, or a 16 hour (16 hour light/ 8 hour dark) photoperiod at 22°C.
3.3.2. Suppressor screening and identification of *rdd1*

Approximately 10,000 *cngc2* T-DNA (Salk_066908) mutant seeds were mutagenized with 0.3% (v/v) EMS solution (Sigma-Aldrich) for 8 hours, followed by rinsing more than 15 times in water. These *M*₀ seeds were grown under ambient humidity for *M*₁ plants. The *M*₂ seeds were collected and then screened for mutants suppressing the dwarf phenotype conferred by the *cngc2* mutation. *rdd1-1D cngc2-3* was identified based on its intermediate morphology between Col-wt and *cngc2-3* plants. It was backcrossed twice with a homozygous *cngc2-3* plant for genetic analysis.

3.3.3. Pathogen infection assays

Bacterial infection was conducted as previously reported (Yoshioka et al., 2006) using 5 to 6 week old plants. Infection with *Hyaloperonospora arabidopsis*, isolate Noco2, was performed as described previously with 8 x 10⁵ spores/ml (Yoshioka et al., 2006).

3.3.4. Ca²⁺ sensitivity assay

Wild type and mutant plants were grown on 0.5x Murishage and Skoog (MS) salt, 2.5mM MES, 1% (v/w) sucrose and 0.8% agar, pH 5.7. Calcium sensitivity was tested on MS media supplemented with 20mM CaCl₂. All plants were grown under continuous light.

3.3.5. RNA extraction and quantitative real time-PCR (qRT-PCR)

Small-scale RNA extraction was carried out using the TRIzol reagent (Invitrogen, Carlsbad, MO, CA) according to the manufacturer’s instructions. Quantitative real time-PCR (qRT-PCR) was performed as previously described (Mosher et al., 2010) using
**Elongation Factor-1α (EF-1α)** gene expression for normalization. Primer sequences are listed in Appendix 1.

### 3.3.6. Extraction and analysis of SA

Approximately 100mg of frozen leaf tissue of each genotype was homogenized in solution containing acetone:10mM citric acid (70:30) and 200ng of [²H₅] SA as the internal standard. Homogenates were then centrifuged and the supernatant was evaporated overnight at room temperature in a fume hood. Free SA was then separated from conjugated SA (SAG) through organic extraction with 2 volumes of ether. The organic phase containing free SA was then passed through solid phase extraction (SPE) columns (Supelclean LC-NH₂, Sigma-Aldridge) pre-equilibrated with ether. Samples were washed with chloroform:propanol (2:1), and then eluted with ether:formic acid (98:2) and dried under a nitrogen stream. The dried extract was resuspended in dichloromethane:methanol (80:20), and incubated, first with trimethylsilyl-diazomethane and then 2M acetic acid (with hexane) for 30 and 20 minutes respectively. The methylated samples were analyzed by GC-MS (Agilent Technologies). SAG was extracted from the same samples but with the addition of HCl and incubation at 90°C for 1 hour prior to solid phase extraction. Each genotype was extracted and measured in triplicates.

### 3.3.7. Bimolecular fluorescence complementation (BiFC) and Agrobacterium infiltration

For BiFC analysis, full length *AtCNGC2* and *AtCNGC4* cDNAs were subcloned into the Gateway entry vector, pDONR221 (Invitrogen, http://www.invitrogen.com).
Subsequently, all clones were subcloned into the destination vectors for plant expression, pK2GWYn9 (nYFP), pL2GWYc9 (cYFP) or pEARLEY101 (full length YFP; Earley et al., 2006) using LR Clonase (Invitrogen, http://www.invitrogen.com) according to the manufacturer’s instructions. All constructed plasmids were sequenced for fidelity. Constructs were then transformed into *Agrobacterium tumefaciens* strain, GV2260. Individual colonies were grown for 20 hours at 30°C in Luria Broth (LB) medium, and then subcultured into Medium 1 (LB, 10mM MES, and 20μM acetosyringone) for 20 h. Cultures were then washed in infiltration medium (10mM MgCl₂, 10mM MES and 150μM acetosyringone), and resuspended to OD₆₀₀ = 0.5. Cultures were incubated overnight at room temperature and syringe-infiltrated into 6 week old *N. benthamiana* leaves. N- and C-terminal YFP fusions were co-infiltrated with HC-Pro of tobacco etch virus (TEV). Leaf discs of infiltrated areas were excised 48 hours after inoculation and used for confocal microscopy.

### 3.3.8. Microscopy

HR-associated auto-fluorescence was monitored using the FITC/GFP filter on the Leica DMI3000 inverted microscope. Confocal fluorescence images were acquired using a Leica TCS SP5 confocal system with AOBS® (HCX PL APO CS 40x immersion oil objective; NA, 1.25) with the AOTF for the argon laser set at 20% and the detection window 525-600nm for YFP (Leica Microsystems Inc., Wetzlar, Germany). Auto-fluorescence of chloroplasts was detected at 650-700nm.
3.4. Results

3.4.1. Identification of *rdd1*

A T-DNA insertion homozygous line (Salk_066908) for *AtCNGC2* (At5g15410) was obtained from TAIR ([www.arabidopsis.org](http://www.arabidopsis.org)) (Figure 3-1A). We named this line *cngc2-3*. Homozygosity of the insertion was confirmed by PCR analysis and the knockout status (*i.e.* expression) was confirmed by RT-PCR (Figure 3-1B). As we expected, the morphological phenotype of this line is identical to the null mutant, *dnd1* (Figure 3-1C, upper panel). Approximately 10,000 *cngc2-3* seeds were mutagenized with ethyl methane sulfonate (EMS), and screened for plants suppressing the morphological phenotype of *cngc2* in the *M*₂ generation. *repressor of defense no death1* (*rdd1*) in the *cngc2-3* background, designated *rdd1 cngc2-3*, was identified based on its intermediate morphology between Col-wild type (Col-wt) and *cngc2* (Figure 3-1C, upper panel).

The *cngc2-3* background of *rdd1 cngc2-3* was confirmed by PCR analysis (Figure 3-1D). *dnd1* has been reported to exhibit conditional spontaneous lesion formation (Clough et al., 2000). Under our growth conditions, we observed this lesion formation in *dnd1* as well as *cngc2-3*, while this phenotype was suppressed in *rdd1 cngc2-3* (Figure 3-1C, lower panel). To determine the genetic nature, *rdd1 cngc2-3* plants were backcrossed to *cngc2-3* plants. As seen in Table 3-1, all B₁ (backcrossed, first generation) plants displayed *rdd1 cngc2-3* morphology, suggesting that *rdd1* is a dominant mutation. The subsequent self-pollinated B₂ generation (backcrossed, selfed) segregated with a 3:1 (*rdd1 cngc2-3:cngc2-3*) phenotypic ratio, indicating that the morphological phenotype of *rdd1 cngc2-3* segregates as a single locus that is dominant.
to the wild type $RDD1$ allele (Table 3-1). Thus, we renamed this mutant as $rdd1-1D$
cngc2-3.
A. T-DNA knockout mutant (cnge2-3) (Salk_066908)

B. AtCNGC2

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</tbody>
</table>

C. Col-wt  rdd1-1D  cnge2-3  dnd1

D. cnge2 T-DNA  CNGC2
Figure 3-1. *rdd1* suppresses *cngc2* (*dnd1*)-conferred morphological phenotype. A) The T-DNA insertion position in *cngc2-3* (Salk_066908) and the primer position used for RT-PCR. B) RT-PCR analysis for *AtCNGC2* in *cngc2-3*. “3’” indicates the result using forward primer “3’” and the reverse primer in (as indicated in A). “Full” indicates the result using forward primer “full” and reverse primer in (as indicated in A). β-*tubulin* was used as the loading control. Although *cngc2-3* shows an amplicon with the 3’ primers, there is no amplicon produced with the full primers, and shows identical phenotypes to the null mutant, *dnd1* (as shown in C), thus indicating that it is functionally null mutant. C) (Upper panel) Morphological phenotype of 5 week old Col-wild type (Col-wt), *rdd1-1D cngc2-3*, *cngc2-3* and *dnd1* plants. *cngc2-3* mutants are stunted in growth and exhibit conditional lesion formation similar to *dnd1*. *rdd1-1D cngc2-3* plants display intermediate rosette morphology between wt and *cngc2-3*. Bar = 1 cm. (Lower panel) Trypan blue staining reveals the reduction of cell death in *rdd1-1D cngc2-3* compared to *cngc2-3*. Bar = 50 µm. Approximately 5 week old leaves were used. Red arrows indicate cell death. D) Genotyping of *cngc2-3* (Salk_066908) for *AtCNGC2* (At5G15410). T-DNA-insertion status was confirmed by PCR using the CNGC2 LP3 and RP2 (CNGC2), and RP2 and LBb1 primers (T-DNA) listed in Appendix 1. http://signal.salk.edu/cgi-bin/tdnaexpress.
Table 3-1. Segregation analysis of the rdd1-1D cngc2-3 phenotype

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<th>Plant line</th>
<th>Total No.</th>
<th>Morphological phenotype</th>
<th>Hypothesis</th>
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<td>92</td>
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<td>3.04</td>
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<tr>
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<td>(B$_1$)$^b$</td>
<td>13</td>
<td>13</td>
<td>0</td>
<td>1:0</td>
</tr>
<tr>
<td>(B$_2$)$^c$</td>
<td>105</td>
<td>77</td>
<td>28</td>
<td>3:1</td>
<td>0.16</td>
</tr>
</tbody>
</table>

a One degree of freedom
b Backcross first generation of rdd1 and cngc2-3 plants
c Backcross second generation of selfed B$_1$ plants

3.4.2. rdd1-1D suppresses the HR-deficient phenotype in cngc2 (dnd1) mutants

The partial suppression of the cngc2-conferred dwarfism in rdd1-1D cngc2-3 prompted us to assess other dnd1-related phenotypes. As mentioned above, dnd1 plants display reduced or absent HR upon infection with avirulent pathogens. To examine the effect of the rdd1-1D mutation on HR formation, rdd1-1D cngc2-3 leaves were inoculated with a high titer of avirulent Pseudomonas syringae pv. glycinea expressing avrRpt2 ($Psg$ avrRpt2$^+$), and assessed for HR induction. At 24 hours post inoculation, Col-wt leaves displayed HR as visible leaf collapse, which was absent in rdd1-1D cngc2-3, cngc2-3 and dnd1 leaves (Figure 3-2A). Plant cells that undergo HR display significant increases in autofluorescence due to the production of phenolic compounds (Yu et al., 1998). Therefore, to closely monitor HR induction, leaf discs were excised from the inoculated areas and viewed under a microscope to compare HR-induced autofluorescence. As seen in Figure 3-2B, Col-wt inoculated leaves exhibited a high level of autofluorescence. Interestingly, a marked increase in autofluorescence was also observed in rdd1-1D cngc2-3 inoculated leaves compared to cngc2-3 and dnd1,
though to a lesser degree than Col-wt (Figure 3-2B). Similar results were also observed in plants inoculated with *P. syringae* pv. *tomato* expressing *avrRpt2* (*Pto avrRpt2*) (data not shown). Taken together, the mutation in *rdd1* partially rescues the HR-deficient phenotype observed in *cngc2-3* and *dnd1* plants in response to avirulent pathogen infection.
Figure 3-2. *rdd1-1D* suppresses the HR-deficient phenotype in *cngc2*. A) Col-wt, *rdd1-1D cngc2-3, cngc2-3* and *dnd1* leaves were infiltrated with *P. syringae pv. glycinea* expressing *avrRpt2* (Psg *avrRpt2*) at 2 x 10⁸ cfu/ml and assessed for macroscopic cell death (HR). Col-wt leaves exhibit visible HR at 24 hours while *rdd1-1D cngc2-3, cngc2-3* and *dnd1* did not. MgCl₂ infiltrations served as negative controls. B) Microscopic cell death was monitored using fluorescence microscopy. Inoculated wt leaves displayed significant autofluorescence compared to *cngc2-3* and *dnd1*. *rdd1-1D cngc2-3* leaves also displayed autofluorescence, but to a lesser extent than Col-wt. MgCl₂ infiltrations served as negative controls.
3.4.3. \textit{rdd1-1D cngc2-3} partially lost \textit{cngc2}-conferred autoimmune phenotypes

As previously mentioned, \textit{cngc2} mutant plants display autoimmune phenotypes such as elevated levels of SA and constitutive expression of \textit{PR} genes. They also display enhanced resistance to virulent strains of \textit{P. syringae} and \textit{Hyaloperonospora arabidopsis} (\textit{Hpa}) despite their HR-deficient phenotypes. To examine the effect of the \textit{rdd1-1D} mutation on these phenotypes, the growth of the bacterial pathogen \textit{P. syringae pv. tomato} DC3000 (DC3000) was measured. Leaf bacterial populations were assessed on 5-6 week old plants 3 days after inoculation. As shown in Figure 3-3A, both \textit{dnd1} and \textit{cngc2-3} showed reduced pathogen growth compared to Col-wt, whereas \textit{rdd1-1D cngc2-3} did not. A similar breakdown of \textit{cngc2}-conferred enhanced resistance was also observed after infection with the virulent oomycete pathogen \textit{Hpa}, isolate Noco2. This strain is virulent to the Col ecotype, which is the background of both \textit{dnd1} and \textit{cngc2-3}. Plants were scored 7 days post infection by assessing sporangiophore formation. As shown in Figure 3-3B, a partial breakdown of enhanced resistance in \textit{cngc2} was observed in \textit{rdd1-1D cngc2-3}. As previously reported, \textit{dnd1} plants display auto-induction of \textit{PR-1} gene expression. This was also observed in \textit{cngc2-3}. However, \textit{rdd1} showed a significant reduction in auto-induction of \textit{PR-1} (Figure 3-3C). These data indicate a reduction of \textit{cngc2}-mediated accumulation of SA in \textit{rdd1-1D cngc2-3}; thus the endogenous level of total SA was measured. Similar to \textit{PR-1} gene expression, lower levels of total SA in \textit{rdd1-1D cngc2-3} compared to \textit{dnd1} and \textit{cngc2-3} were detected (Figure 3-3D). Taken together, the \textit{rdd1-1D} mutation causes a significant suppression of the enhanced resistance phenotype conferred by \textit{cngc2}. 
**Figure 3-3.** *rdd1-1D* partially suppresses *cngc2*-mediated constitutive defense response. A) Leaf bacterial populations were assessed 3 days after infiltration with virulent *P. syringae pv. tomato* DC3000 at $5 \times 10^4$ cfu/ml. Col-wt and *rdd1-1D cngc2-3* leaves supported significantly more bacterial growth compared to *cngc2-3* and *dnd1*. Bars marked with the same letter indicate no significant difference (Student’s t-test, $P < 0.05$). Data are representative of 7 experiments. B) Col-wt, Ws-wt and mutant cotyledons (n = 40) were inoculated with virulent *H. arabidopsidis*, isolate Noco2, at a suspension of $8 \times 10^5$ spores/ml, and monitored for sporangiophore formation. At 7 days post inoculation, *rdd1-1D cngc2-3* cotyledons supported an intermediate level of sporangiophore formation between Col-wt and *cngc2-3*. Ws ecotype is resistant against to Noco2 and served as a control. Data are representative of 2 separate experiments. C) *PR-1* transcript levels in 3-4 week old Col-wt and mutant leaves measured by quantitative real-time PCR. *PR-1* expression is shown relative to *EF-1α*. Error bars indicate standard error of the mean of 3 replicates. Bars marked with the same letter indicate no significant difference (Student’s t-test, $P < 0.05$). D) Total SA levels (SA + SAG) in 3-4 week old wt and mutant leaves. Error bars indicate standard error of the mean of 3 replicates. Bars marked with the same letter indicate no significant difference (Student’s t-test, $P < 0.05$).
3.4.4. *cngc2* affects floral transition and *rdd1-1D* suppresses this novel *dnd1*-conferred phenotype

Through characterization of *rdd1-1D cngc2-3*, we discovered a novel phenotype in *cngc2 (dnd1)*. Under our long day (16 hour light / 8 hour dark) and ambient humidity conditions, Col-wt plants flowered 23 days post stratification, while *cngc2-3* and *dnd1* mutant plants flowered significantly later at 29 days post stratification (Figure 3-4A and B). This novel phenotype was also repressed in *rdd1-1D cngc2-3* plants, which flowered at approximately the same time as Col-wt (Figure 3-4B). Delayed flowering can also be reflected by an increased number of rosette leaves prior to bolting. As shown in Figure 3-4C, Col-wt plants had significantly less rosette leaves at the time of flowering compared to *cngc2-3* and *dnd1* plants, confirming the delayed flowering phenotype of *cngc2* and *dnd1* mutants. Similar to Col-wt, the rosette leaf number in *rdd1-1D cngc2-3* was significantly reduced compared to *cngc2-3* and *dnd1* (Figure 3-4C), indicating that the mutation in *rdd1-1D* suppresses the delayed floral transition observed in *cngc2* mutants. Similar but more pronounced results were observed under short day (8 hour light / 16 hour dark) (Table 3-2). Consistent with these results, expression levels of the floral repressor, *FLOWERING LOCUS C (FLC, He and Amasino, 2005)* was significantly elevated in *cngc2-3* and *dnd1* compared to Col-wt, and almost wt expression levels were observed in *rdd1-1D cngc2-3* (Figure 3-4D). Taken together, a novel delayed flowering phenotype was observed in *cngc2* and *dnd1* mutants, which is likely caused by an alteration in *FLC* expression. This phenotype was abolished in *rdd1-1D cngc2-3* under both long and short day conditions.
Figure 3-4. *rdd1-1D* represses *cngc2*-mediated delay in floral transition. A) *cngc2-3* and *dnd1* plants exhibit delayed flowering compared to *Col-wt* and *rdd1-1D cngc2-3*. B) Flowering time was measured in *Col-wt* and mutant plants by determining the emergence of the first bud. Data is a representative of a total of 4 experiments. Error bars indicate standard error of the average flowering time of approximately 30 – 40 plants. Bars marked with the same letter indicate no significant difference (Student’s t-test, P < 0.05). C) Rosette leaf number was measured upon bolting. Values represent the mean of 30 – 40 plants. Error bars indicate standard error of the mean, and bars marked with the same letter indicate no significant difference (Student’s t-test, P<0.05). D) Quantitative real-time PCR analysis of FLC expression. The expression is shown relative to *EF-1α*. Error bars indicate standard error of the mean of 3 replicates. Bars marked with the same letter indicate no significant difference (Student’s t-test, P < 0.05).
It was previously shown that null mutations of \textit{AtCNGC2} lead to hypersensitivity to \textit{Ca}^{2+} that impairs plant growth (Chan et al., 2003). To determine whether \textit{rdd1-1D} also suppresses this phenotype, we compared the size of Col-\textit{wt}, \textit{rdd1-1D cngc2-3}, \textit{cngc2-3} and \textit{dnd1} plants grown in \textit{Ca}^{2+} supplemented media. As seen in Figure 3-5A, although slight chlorosis was observed, Col-\textit{wt} plants did not display any strong suppression in growth. In contrast, \textit{cngc2-3} and \textit{dnd1} plants exhibited severely stunted growth and chlorosis, which was consistent with the previous report (Chan et al., 2003). Interestingly, \textit{rdd1-1D cngc2-3} plants also displayed stunted growth and severe chlorosis similar to \textit{cngc2-3} and \textit{dnd1}. To quantify this observation, the average fresh weight of each genotype was measured. At 4 weeks post stratification, \textit{rdd1-1D cngc2-3}, \textit{cngc2-3} and \textit{dnd1} plants showed a significant size reduction compared to Col-\textit{wt} on media supplemented with 20mM CaCl$_2$ (Figure 3-5B). Taken together, the \textit{rdd1-1D} mutation does not suppress the \textit{Ca}^{2+} hypersensitivity of \textit{cngc2} mutants.
**Figure 3-5.** *rdd1-1D* mutation does not rescue Ca\(^{2+}\) hypersensitivity in *cn gc2*. A) Col-wt and mutant plants grown on both control media and media supplemented with 20mM CaCl\(_2\). At 3 weeks, *rdd1-1D cngc2-3, cngc2-3* and *dnd1* plants are indistinguishable from Col-wt when grown on control media, but exhibit severely stunted growth and chlorosis on 20mM CaCl\(_2\). Bar = 1 cm. B) Average fresh weights of 3 week old Col-wt and mutant plants grown on control media and media supplemented with 20mM CaCl\(_2\). Error bars indicate standard error of the mean of 4 replicate plates (20 plants per plate). Asterisks indicate significant differences between 0 and 20 mM CaCl\(_2\). (Student’s t test, \(p<0.05\)).
3.4.6. **RDD1** is involved in both CNGC2 and CNGC4-mediated signal transduction

It has been reported that null mutants of *AtCNGC4 (hlm1/dnd2)* exhibit strikingly similar phenotypes to *cngc2-3* and *dnd1* plants, such as dwarfism, enhanced pathogen resistance and an impaired HR response to avirulent pathogens (Balagué et al., 2003; Jurkowski et al., 2004). As mentioned, *AtCNGC2* and *4* are the sole members of group IVB and share high sequence similarity (Mäser et al., 2001). This prompted us to investigate whether *rdd1-1D* can also suppress *dnd2*-mediated phenotypes. For this analysis we used an *AtCNGC4* T-DNA insertion line (Salk_081369) (Figure 3-6A). The knockout status of this line (i.e. expression) was confirmed by RT-PCR, and this line was named *cngc4-5* (Figure 3-6B). We crossed this line with *rdd1-1D cngc2-3* plants. Following self pollination of F$_1$ plants, a total of 243 F$_2$ plants were monitored for their morphological phenotype. As shown in Table 3-3, they segregated in a 148:78:15:2 (wt: *rdd1-1D cngc2-3*-like: *dnd*-like: enhanced *dnd* phenotype (termed super *dnd*). This observation fits the expected ratio of 36:21:6:1, which was predicted in the case that *rdd1-1D* suppresses not only *cngc2*-conferred, but also *cngc4*-conferred phenotypes, and even the *cngc2/cngc4* double mutation which was reported to be an enhanced *dnd* phenotype (Table 3-3, Jurkowski et al., 2004). In addition, the data suggest that the single *rdd1-1D* mutation does not cause a significant morphological difference compared to Col-wt. This was also supported by the phenotypes of F$_1$ plants obtained by crossing *rdd1-1D cngc2-3* with Col-wt (Figure 3-6C).

T-DNA insertion status in all 78 individuals exhibiting *rdd1-1D cngc2-3*-like morphology was analyzed, and the repression of *cngc4* mutant as well as *cngc2/cngc4* double mutant phenotypes by *rdd1-1D* was confirmed (Figure 3-6D and E). Taken
together, the data indicate that \textit{rdd1-1D} also suppresses \textit{cngc4 (hlm1/dnd2)}-confferred morphological phenotypes to the same degree as \textit{cngc2}-confferred phenotypes.
Figure 3-6. Analysis of the *rdd1-1D cngc2-3/cngc4-5* cross A) The T-DNA insertion position in *cngc4-5* (Salk_081369) and the primer position used for RT-PCR. B) RT-PCR analysis for *AtCNGC4* in *cng4-5*. “3′” indicates the result using forward primer “3′” and the reverse primer in (as indicated in A). “Full” indicates the result using forward primer “full” and reverse primer in (as indicated in A). β-tubulin was used as the loading
control. C) \textit{rdd1-1D} single mutant does not have \textit{rdd1-1D cngc2-3} phenotype. \textit{F1} plants of Col-wt and \textit{rdd1-1D cngc2-3} are identical to Co-wt in terms of morphology (upper panel) and spontaneous cell death (lower panel). Since the \textit{cngc2-3} mutation is recessive and \textit{rdd1-1D} mutation is dominant, this data suggest that \textit{rdd1-1D} single mutant does not have \textit{rdd1-1D cngc2-3} phenotype. D) Analysis of \textit{rdd1-1D cngc4-5} and \textit{rdd1-1D cngc2-3 cngc4-5}. T-DNA-insertion status was confirmed by PCR using the \textit{CNGC4 LP} and RP (\textit{CNGC4}), and RP and LBb1 primers (T-DNA) for the insertion in \textit{AtCNGC4}. \textit{CNGC2 LP3} and RP2 (\textit{CNGC2}), and RP2 and LBb1 primers (T-DNA) were used to confirm the insertion in \textit{AtCNGC2}. Primer sequences are listed in Appendix 1. E) Morphological phenotype of \textit{rdd1-1D cngc2-3}, \textit{rdd1-1D cngc2-3 cngc4-5}, \textit{cngc2-3} and \textit{cngc4-5}.

\begin{table}[h]
\centering
\begin{tabular}{llllllll}
\hline
Cross & Generation & Total No. & Morphological phenotype & Hypothesis & \(X^{2c}\) & P \\
& & & Wildtype & \textit{rdd1} & \textit{dnd} & \textit{superdnd} & \\
\hline
\textit{rdd1-1D} \textit{x cngc4-5/cngc4-5} & \textit{F2} & 243 & 148 & 78 & 15 & 2 & 36:21:6:1 & 4.49 & 0.95>P>0.9 \\
\hline
\end{tabular}
\caption{Segregation analysis of \textit{rdd1-1D/cngc4-5} cross}
\end{table}

\textbf{a} The pollen acceptor plant is indicated first, and the pollen donor is second

\textbf{b} Hypothesis: \textit{rdd1} is inherited as a dominant mutation. \textit{rdd1} (dominant), \textit{cngc2} (recessive) and \textit{cngc4} (recessive) mutations segregate independently in the \textit{F2} generation. Suppression of \textit{cngc4} by \textit{rdd1} results in a higher ratio of \textit{rdd1}-like to \textit{dnd}-like plants (36:21:6:1; wildtype: \textit{rdd1}: \textit{dnd}: \textit{superdnd}). In contrast, no suppression by \textit{rdd1} results in a higher ratio of \textit{dnd}-like to \textit{rdd1}-like plants (36:9:18:1; wildtype: \textit{rdd1}: \textit{dnd}: \textit{superdnd}). The latter hypothesis was rejected.

\textbf{c} Three degrees of freedom
3.4.7. *rdd1-1D* suppresses *cngc4*-mediated phenotypes

To assess the effect of *rdd1-1D* on *cngc4 (hlm1/dnd2)*, *rdd1-1D* *cngc4-5* double mutants were isolated from the above *F₂* plants. The *rdd1-1D* *cngc4-5* double mutant displayed the same *rdd1-1D* *cngc2-3* phenotype suggesting that *rdd1-1D* also suppresses *cngc4* phenotypes (Figure 3-7). To examine the effect of the *rdd1* mutation on *cngc4 (hlm1/dnd2)*-conferred autoimmune phenotypes, we first monitored HR induction upon infection with a high titer of *Psg avrRpt2* in *rdd1-1D* *cngc4-5* plants. At 24 hours post inoculation, Col-wt leaves displayed visible leaf collapse whereas *rdd1-1D* *cngc4-5*, *cngc4-5* and *dnd2* leaves did not (Figure 3-8A). For further evaluation, HR-induced autofluorescence was monitored. Similar to *rdd1-1D* *cngc2-3*, *rdd1-1D* *cngc4-5* leaves displayed a marked increase in autofluorescence compared to *cngc4-5* and *dnd2*, suggesting that *rdd1* also suppresses *cngc4*-mediated alteration in HR (Figure 3-8B). Next, we tested for enhanced resistance against virulent *Hpa*, isolate Noco2, on ten-day-old seedlings. At 7 days post inoculation, *rdd1-1D* *cngc4-5* showed a partial breakdown of enhanced resistance similar to *rdd1-1D* *cngc2-3* compared to *cngc2-3* and *cngc4-5* single mutants (Figure 3-8C). As expected, qRT-PCR results from 5 week old leaves showed a marked decrease in *PR-1* gene expression in *rdd1-1D* *cngc4-5* compared to *cngc4* and *dnd2* mutants (Figure 3-8D). Delayed flowering was also observed in *cngc4-5* and *dnd2*, and this phenotype was also suppressed in *rdd1-1D* *cngc4-5* (Figure 3-9). Taken together, *rdd1* suppresses *cngc4*-conferred phenotypes to a similar degree as those of *cngc2*. These results demonstrate that *AtCNGC2* and *AtCNGC4* mediate the same signaling pathway involving *RDD1* to regulate immunity and floral transition.
Figure 3-7. *RDD1* is involved in *CNGC4*-mediated signal transduction. Morphological phenotype of 5 week old *Col-wt*, *rdd1-1D cngc2-3*, *cngc2-3*, *rdd1-1D cngc4-5* and *cngc4-5* plants. *rdd1-1D cngc4-5* plants exhibit an intermediate rosette morphology similar to *rdd1-1D cngc2-3*. Bar = 1 cm.
Figure 3-8. *rdd1-1D* suppresses *cngc4*-mediated autoimmune phenotypes. A) Col-wt, *rdd1-1D cngc4-5*, *cngc4-5* and *dnd2* leaves were infiltrated with avirulent *P. syringae* pv. *glycinea* expressing *avrRpt2* (*Psg avrRpt2*) at $2 \times 10^8$ cfu/ml and assessed for HR. Col-wt leaves exhibit visible HR at 24 hours while *rdd1-1D cngc4-5*, *cngc4-5* and *dnd2* did not. MgCl$_2$ infiltrations served as negative controls. (B) *rdd1-1D cngc4-5* leaves
displayed significant autofluorescence 24 hours after infiltration with *Psg avrRpt2*, but to a lesser extent than Col-wt. MgCl₂ infiltrations served as negative controls. C) Col-wt and mutant cotyledons (n = 40) were inoculated with virulent *H. arabidopsidis*, isolate Noco2, at a suspension of 8 x 10⁵ spores/ml, and monitored for sporangiophore formation. At 7 days post inoculation, *rdd1-1D cngc4-5* cotyledons supported similar levels of sporangiophore formation as *rdd1-1D cngc2-3*, and were less resistant than *cngc4-5*. D) PR-1 transcript levels in 3-4 week old Col-wt and mutant leaves were measured by quantitative real time-PCR. PR-1 expression and shown relative to *EF-1α*. Error bars indicate standard error of the mean of 3 replicates. Bars marked with the same letter indicate no significant difference (Student’s t-test, P < 0.05).
Figure 3-9. *rdd1-1D* represses *cngc4 (dnd2/hlm1)*-mediated delay in floral transition. A) *cngc4-5* and *dnd2* plants exhibit delayed floral transition compared to Col-wt and *rdd1-1D cngc4-5*. B) Average flowering time of Col-wt, *rdd1-1D cngc4-5*, *cngc4-5* and *dnd2* plants. Data is a representative of a total of 4 experiments. Error bars indicate standard
error of the mean average flowering time of 40 plants. Bars marked with the same letter indicate no significant difference (Student’s t-test, \( P < 0.05 \)).

3.4.8. CNGC2 and CNGC4 subunits interact in planta

Animal CNGC subunits have been shown to form hetero-tetrameric channels at the plasma membrane, where they mediate ion fluxes into the cell. However, the composition of CNGCs and subunit interactions in plants remain unknown (Moeder et al., 2011). Since the genetic analysis and characterization of \( rdd1-1D cngc4-5 \) indicated that \( RDD1 \) participates in signaling mediated by both \( AtCNGC2 \) and \( AtCNGC4 \), two possible scenarios arise. One scenario is that \( AtCNGC2 \) and \( AtCNGC4 \) subunits form homomeric channels at the plasma membrane to regulate parallel pathways that converge downstream. In this case, \( RDD1 \) functions after the conversion. Alternatively, \( AtCNGC2 \) and \( AtCNGC4 \) subunits might interact to form heteromeric channels that regulate one pathway involving \( RDD1 \). To examine the subunit interactions in planta, full length \( AtCNGC2 \) and \( AtCNGC4 \) were fused C-terminally to the N- and C-terminal portions of yellow fluorescent protein (\( YFP^N \) and \( YFP^C \), respectively), and transiently co-expressed in \( N. benthamiana \) leaves for bimolecular fluorescence complementation (BiFC). C-terminal fusions of \( AtCNGCs \) were previously shown to not disturb channel function when transiently expressed in \( N. benthamiana \) leaves (Urquhart et al., 2007). Furthermore, BiFC was recently used to evaluate the in planta subunit interactions of \( AtCNGCs \) (Abdel-Hamid et al., 2013). At 48 hours post infiltration, weak but steady fluorescence was detected with \( AtCNGC2-YFP^N + AtCNGC4-YFP^C \) co-expression, indicating that \( AtCNGC2 \) and \( AtCNGC4 \) subunits are able to form heteromeric channels (Figure 3-10A). Reciprocal co-expression showed similar results (Figure 3-11A).
Interestingly, AtCNGC2-YFP\textsuperscript{N} + AtCNGC2-YFP\textsuperscript{C} and AtCNGC4-YFP\textsuperscript{N} + AtCNGC4-YFP\textsuperscript{C} combinations also produced weak but detectable fluorescence, indicating that AtCNGC2 and AtCNGC4 subunits are able to form homomeric channels (Figure 3-10B and C). YFP signals were not observed in single infiltrations of each construct or co-infiltration with an unrelated subunit, AtCNGC11/12 (Abdel-Hamid et al., 2013, Yoshioka et al., 2006) confirming the specificity of the interaction between AtCNGC2 and AtCNGC4 (Figure 3-10D-G, 3-11B-G). The expression of full length YFP-tagged AtCNGC2 and 4 is shown in Figure 7H and I. Taken together, these results demonstrate the ability of AtCNGC2 and AtCNGC4 subunits to form both homo- and heteromeric channels \textit{in planta}. 
Figure 3-10. AtCNGC2 and AtCNGC4 subunits interact in planta. (Panels A-G) Bimolecular fluorescence complementation (BiFC) with full length AtCNGC2 and AtCNGC4 C-terminally fused to the N- and C-terminal portions of YFP (YFP\textsuperscript{N} and YFP\textsuperscript{C} respectively). Co-expression of AtCNGC2-YFP\textsuperscript{N} and AtCNGC4-YFP\textsuperscript{C} (Panel A), AtCNGC2-YFP\textsuperscript{N} and AtCNGC2-YFP\textsuperscript{C} (Panel B), and AtCNGC4-YFP\textsuperscript{N} and AtCNGC4-YFP\textsuperscript{C} (Panel C) show steady YFP fluorescence. Single expressions of each construct served as negative controls (Panel D-G). (Panels H-I) Expression of AtCNGC2 and AtCNGC4 fused to full length YFP. The columns show YFP fluorescence (left), chlorophyll autofluorescence (middle left), DIC (middle right), and an overlay of all 3 images (right). Bars = 100µm.
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Figure 3-11. Co-expression of AtCNGC4-YFP\textsuperscript{N} and AtCNGC2-YFP\textsuperscript{C}. Panel A shows steady YFP fluorescence detected 48 hours after co-expression of AtCNGC4-YFP\textsuperscript{N} and AtCNGC2-YFP\textsuperscript{C}. Single expressions of each construct served as negative controls (Panel B and C). Co-expression of AtCNGC2-YFP\textsuperscript{N} and AtCNGC11/22-YFP\textsuperscript{C} (Panel D), AtCNGC2-YFP\textsuperscript{C} and AtCNGC11/22-YFP\textsuperscript{N} (Panel E), AtCNGC4-YFP\textsuperscript{N} and AtCNGC11/22-YFP\textsuperscript{C} (Panel F), AtCNGC4-YFP\textsuperscript{C} and AtCNGC11/22-YFP\textsuperscript{N} (Panel G). The columns show YFP fluorescence (left), chlorophyll autofluorescence (middle left), DIC (middle right), and an overlay of all 3 images (right). Bars = 100\textmu m.
3.5. Discussion

Our understanding of the role of Ca$^{2+}$ based signaling in plants has significantly improved over the last 20 years and it is now clear that Ca$^{2+}$ plays crucial roles in the signal transduction by which a wide variety of abiotic, biotic and developmental stimuli are transmitted (McAinsh and Schroeder, 2009). The influx of Ca$^{2+}$ into the cytosol is accomplished by plasma membrane and endomembrane localized cation transport proteins. Genomic sequence data indicated that Arabidopsis contains over 150 cation transport proteins (Mäser et al., 2001). Among them, cyclic nucleotide-gated ion channels (CNGCs) were suggested to be involved in Ca$^{2+}$ signaling (Ali et al., 2007; Urquhart et al., 2007; Chin et al., 2010; Guo et al., 2010; Finka et al., 2012; Gao et al., 2012). Arabidopsis CNGCs form a large gene family consisting of 20 members that have been implicated in a diverse range of biological phenomena such as defense responses, gravitropism, pollen tube growth, ion homeostasis and very recently, thermotolerance (Talke et al., 2003; Kaplan et al., 2007; Frietsch et al., 2007; Chin et al., 2009; Urquhart et al., 2011; Finka et al., 2012; Tunc-Ozdemir et al., 2013).

CNGCs were first discovered in retinal photoreceptors and olfactory sensory neurons, and so far, six CNGC genes have been found in mammalian genomes (Zagotta and Siegelbaum, 1996; Zufall et al., 1994). Important functional features of CNGCs were extensively studied in animal systems, and it has been suggested that the subunit composition of the respective channel complex is an important determinant of functional features such as ligand sensitivity, selectivity and gating (Kaupp and Seifert, 2002). For instance, native CNGCs in rod cells consist of two types of subunits, A1 and B1a. On the other hand, the CNGCs in the chemosensitive cilia of olfactory sensory
neurons consist of three different subunits: A2, A4, and B1b. Thus, it is generally believed that CNGCs form hetero-tetrameric complexes consisting of distinct subunits in order to create specific channels (Kaupp and Seifert, 2002).

Plant CNGCs on the other hand, have only been investigated much more recently. Based on animal CNGC research, plant CNGCs are also suggested to form tetrameric channels. Functional complementation assays in heterologous expression systems, such as Saccharomyces cerevisiae and Escherichia coli as well as electrophysiological analysis in Xenopus laevis oocytes and human embryonic kidney cells indicated that some subunits are also able to form functional homomeric channels (Kaplan et al., 2007, Chin et al., 2009). However, the nature of the subunit composition of CNGCs, i.e. homo- or hetero-tetramer formation in planta has not yet been revealed.

In this study, in order to understand the CNGC-mediated signal transduction in plant defense responses, we have conducted a suppressor screen using the Arabidopsis defense-no death (dnd1) mutant, which is a null mutant of AtCNGC2. Prior to this study, our group conducted a similar suppressor screen using constitutive expresser of PR genes22 (cpr22) mutant that resulted from the fusion of AtCNGC11 and AtCNGC12 (Yoshioka et al, 2006). This mutant shows similar autoimmune phenotypes to dnd1. The suppressor screen using cpr22 however, has so far yielded only intragenic mutants, and thus has not yet revealed novel CNGC-mediated signaling components (Baxter, et al., 2008; Chin et al., 2010; Abdel-Hamid., 2013). This is mainly due to the gain-of-function nature of cpr22. On the other hand, dnd1 is a loss-of-function mutant, thus making the likelihood of obtaining intragenic mutants very low. Furthermore, we have used a T-DNA insertion line to further increase the probability of
obtaining intergenic mutants. This led us to successfully isolate the first dnd1 suppressor, repressor of dnd1, rdd1 that is described in this study.

AtCNGC2 has been relatively well studied among the Arabidopsis CNGCs and has been reported to be involved in pathogen defense responses. Since dnd1 has a reduced hypersensitive response (HR) upon pathogen infection, AtCNGC2 has been proposed to be a positive regulator of defense responses. This notion was demonstrated by Ali et al. (2007), who showed that AtCNGC2 mediates pathogen associated molecular pattern (PAMP)-triggered activation of defense responses through a cAMP-stimulated Ca$^{2+}$ influx, which leads to increased nitric oxide (NO) production. Additionally, they indicated that AtCNGC2-mediated NO production is also required for R-gene mediated HR development. At the same time, dnd1 is known to exhibit typical autoimmune phenotypes, such as elevated levels of salicylic acid (SA), constitutive expression of PR genes and conditional HR-like spontaneous lesion formation without pathogen infection (Yu et al., 1998, Clough et al., 2000). Thus, the precise role of AtCNGC2 in defense responses is still elusive. Nevertheless, rdd1 partially lost all of these dnd1-mediated phenotypes except Ca$^{2+}$ hypersensitivity. It has been shown that the alteration of HR phenotypes upon pathogen infection is independent from SA accumulation and enhanced resistance in dnd1 (Clough et al., 2000; Genger et al., 2008). Collectively, these observations suggest that RDD1 locates relatively upstream in dnd1-mediated signaling: i.e. upstream in the signaling pathway before it branches out to cause the pleiotropic phenotypes of dnd1 (i.e. loss of HR and SA-dependent enhanced pathogen resistance responses), but after the signal for Ca$^{2+}$ sensitivity branches out. Positional cloning strategies have so far mapped the rdd1 mutation to a region containing about 200 genes at the upper arm of chromosome 5 (Chapter 4). This
region does not contain any known genes expected to suppress CNGC-mediated resistance (Genger et al., 2008, Yoshioka et al., 2006, Chin and Yoshioka, unpublished data), thus *RDD1* is likely a novel component in the CNGC-mediated pathogen resistance signaling pathway.

In addition, two significant findings were made in this study: the involvement of AtCNGC2 in flowering time, and the possibility of hetero-tetramerization of AtCNGC2 and AtCNGC4. Recently, several reports connecting pathogen defense and flowering timing have been published (Tsuchiya and Eulgem, 2010; Martínez et al., 2004; Li et al., 2012; Wang et al., 2011; Quesada et al., 2013; Palma et al., 2010). Under normal conditions, flowering time is tightly regulated by signaling networks that integrate endogenous and external cues to follow seasonal changes (Davis, 2009). However, particular environmental stress conditions, such as ultraviolet-C (UV-C) radiation, pathogen infection and extreme temperatures can promote flowering (Raskin, 1992). Martínez et al. (2004) have reported that SA positively regulates flowering timing in Arabidopsis. SA was suggested to act as a negative regulator of the central floral repressor, *FLC*, in a photoperiod-dependent manner as SA deficient mutants, such as *nahG*, *sid2* and *eds5/sid1*, exhibit late flowering phenotypes and increased levels of *FLC* transcript under short day conditions (Martínez et al., 2004). Consistent with this observation, defense mutants with elevated SA levels, such as *pub13* or *siz1*, display earlier SA-dependent flowering phenotypes (Jin et al., 2008; Li et al., 2012). In our study however, *dnd1* shows delayed flowering phenotypes in spite of elevated levels of SA, and this phenotype was observed under both long and short day conditions, although enhanced in the latter condition. Thus, it is opposite to what was observed by Martínez et al. (2004) as well as for *pub13* or *siz1*. These observations together with our
preliminary analysis using dnd1/sid2 double mutants indicate that SA is unlikely the cause of the alteration in flowering timing in dnd1 (Chin and Yoshioka unpublished data). Interestingly, the Arabidopsis lesion mimic mutant, rugosa1 (rug1), showed a delayed flowering phenotype with enhanced disease resistance responses, such as accumulation of H₂O₂, elevation of PR-1 and the SA biosynthetic gene, SID2, which is reminiscent of dnd1 (Quesada et al., 2013). rug1 is a loss-of-function mutant of porphobilinogen deaminase which is a tetrapyrrole biosynthetic enzyme. The precise molecular mechanisms of rug1 that induce delayed flowering and autoimmune phenotypes much like dnd1 are however, currently unclear. Autoimmune mutants, like rug1 and dnd1, typically show severe developmental defects and some phenotypes could be attributable to indirect effects of physiological perturbations. However, a clear crosstalk between defense and floral transition has been reported in several cases. For instance, the Arabidopsis mutant edm2 (enhanced downy mildew 2) is compromised in disease resistance mediated by the R gene, RPP7. However, the role of EDM2 in defense is rather limited and its role in flowering in a FLC-dependent manner was reported (Tsuchiya and Eulgem, 2010). EDM2 physically interacts with, and is phosphorylated by the protein kinase WNK8, which is known to affect the photoperiodic pathways in floral transition (Wang et al., 2008). Thus, this example provides convincing evidence of the crosstalk between defense responses and flowering time. Further analysis of floral transition in dnd1 is in progress.

Another significant finding is the relationship between the downstream signaling pathways mediated by AtCNGC2 and AtCNGC4. As shown, rdd1-1D not only suppresses cngc2-mediated, but also cngc4-conferred phenotypes. Both hlm1 and dnd2 are loss of function mutants of AtCNGC4, which is the closest paralog of
AtCNGC2 and the only other member of group IVB in the AtCNGC family (Mäser et al., 2001). These cngc4 mutants display almost identical phenotypes to those seen in cngc2 mutants (Genger et al., 2008, Balagué et al., 2003; Figure 3-7). Furthermore, the aforementioned delayed flowering phenotype is also found in cngc4 (Figure 3-9). Therefore, the relationship between these two CNGCs is of interest and it could be hypothesized that they are both part of the same hetero-tetrameric channel. It has, however, been reported that the loss of both AtCNGC2 and AtCNGC4 shows synergistic effects, as dnd1/dnd2 double mutants display enhanced dnd phenotypes (termed super-dnd phenotype in Table 3-3, Jurkowski et al., 2004). This suggests the existence of homo-tetramers or parallel signaling pathways mediated by AtCNGC2 and 4.

In this study, our data showing the suppression of both mutant phenotypes by rdd1 provides compelling genetic evidence that AtCNGC2- and AtCNGC4-mediated signal transduction pathways are at least overlapping or merge at some point. In addition, our BiFC data demonstrated in planta interaction between these two subunits as well as with themselves, suggesting the existence of both homo- and hetero-tetramers in planta. Together with prior data showing the enhancement of the phenotypes in the double mutant (Jurkowski et al., 2004), we propose that AtCNGC2 and 4 form hetero- as well as homo-tetrameric channels in planta, and that they share the same biological function. At this moment, it is not known whether both types of channels exist in wild type plants, but at least in the single dnd1 mutant, only AtCNGC4 homo-tetrameric channels could be formed. This would also be the case for hlm1/dnd2, which could have only AtCNGC2 homo-tetramers (Figure 3-12A). In the case that wild type plants have both homo- and hetero-tetrameric channels, they would have at least
three different channels with these two subunits; AtCNGC2 homo-, AtCNGC4 homo- and AtCNGC2 and 4 hetero-tetrameric channels, and they work synergistically. In this scenario, single mutants would have lost two of the three different channels, and consequently display the reported phenotypes. Alternatively, the homo-tetrameric versions may not exist in wild type plants possibly due to a lower affinity of homo-tetramerization compared to hetero-tetramerization. In this case, homo-tetrameric channels would form abnormally when one of the subunits does not exist (i.e. single mutants) (Figure 3-12B). This is likely, since stronger BiFC signals were observed in two out of three experiments when we co-expressed AtCNGC2 and 4 compared to single gene expression. In this case, although the function of homo-and hetero-tetrameric channels might be the same, the efficacy could be different, i.e. hetero-tetrameric channels may function more effectively than homo-tetrameric ones. This could lead to the reported phenotypes in cngc2 and cngc4 mutants, and the enhanced dnd phenotype in the double mutant (Jurkowski et al., 2004). This model can explain our observations as well as previously reported data. Another possibility that cannot be excluded is that AtCNGC2 and 4 form a channel with an additional unidentified subunit(s). In this case, the absence of AtCNGC2, 4, or both, may create an increasingly aberrant channel that would lead to an uncontrolled influx of Ca$^{2+}$ into the cell (Figure 3-12C). This model would explain the increased [$Ca^{2+}]_{cyt}$ levels in cngc2 plants (Chan et al., 2008). Thus, further studies regarding the subunit composition of CNGCS are required.

The precise mechanisms by which the lack of these CNGCs can induce autoimmunity, delayed flowering and hypersensitivity to Ca$^{2+}$ under normal conditions without specific stimulations is still elusive, but it can be explained by their
housekeeping roles to maintain stable physiology in plant cells. Recently, Finka et al. (2012) reported that AtCNGC2 and its Physcomitrella patens ortholog, CNGCb, act as the primary thermosensors of land plant cells. Interestingly, CNGCb loss-of-function caused a hyper-thermoresponsive Ca$^{2+}$ influx and a systemic higher background of [Ca$^{2+}$]$_{cyt}$. A similar observation was made by Chan et al. (2008), who demonstrated that cngc2 plants displayed a gene expression profile similar to that of wild type plants grown in high external Ca$^{2+}$. Such abnormally high levels of Ca$^{2+}$ ions could cause the alterations in defense and developmental signal transduction that are observed in dnd1 and hlm1/dnd2. In addition, CNGCs may have multiple biological functions, as AtCNGC11 and 12 have been reported to mediate multiple Ca$^{2+}$-dependent physiological responses (Urquhart et al., 2011). AtCNGC2 has previously been shown to play an additional role in senescence (Köhler et al., 2001; Ma et al., 2010). To further understand the role of plant CNGCs, the identification of more downstream signaling components is required. Thus, the identity of RDD1 and its involvement in CNGC2 and 4-mediated phenotypes will provide new insight to this aspect. The cloning of RDD1 is currently in progress, and preliminary results are presented in the following chapter.
A. $cnga2 (dnd1)$, Wild type, $cnga2/cnga4$

- $cnga2 (dnd1)$: 4
- Wild type: 4, 2
- $cnga2/cnga4$: 2

Single mutant phenotype: Wild type phenotype

Enhanced phenotype (super $dnd$)

B. $cnga2 (dnd1)$, Wild type, $cnga2/cnga4$

- $cnga2 (dnd1)$: 4
- Wild type: 2, 4
- $cnga2/cnga4$: 4

Single mutant phenotype: Wild type phenotype

Enhanced phenotype (super $dnd$)

C. $cnga2 (dnd1)$, Wild type, $cnga2/cnga4$

- $cnga2 (dnd1)$: 4
- Wild type: 4, 2
- $cnga2/cnga4$: 4, 2

Ca$_2^+$

Generating abnormal signals that lead to single mutant phenotypes

Stronger abnormal signals lead to enhanced (super $dnd$) phenotypes
**Figure 3-12.** Proposed CNGC compositions involving AtCNGC2 and 4 subunits. A) In the case that wild type plants have three different channels with these two subunits: AtCNGC2 homo-, AtCNGC4 homo- and AtCNGC2 and 4 hetero-tetrameric channels, and they work synergistically. In this scenario, single mutants would have lost two of the three different channels, and consequently display the reported phenotypes. B) In the case that wild type plants have only hetero-tetrameric channels, single mutants have abnormal homo-tetrameric channels. In this case, although the function of homo- and hetero-tetrameric channels may be the same, the efficacy could be different, since double mutants have an enhanced phenotype. C) In the case unidentified subunit(s) is/are involved to form a channel complex with AtCNGC2 and 4 in wild type plants. In this case, the absence of AtCNGC2, 4 or both may create an aberrant channel that would lead to an uncontrolled influx of Ca$^{2+}$ into the cell. Unidentified subunit (U) is functionally redundant since no dnd-like phenotype has so far been observed in other AtCNGC knockout mutants.

### 3.6. Acknowledgements

We greatly appreciate Dr. Andrew Bent for providing the seeds of various double mutants of *dnd1* and *dnd2*. We also thank Henry Hong for his assistance in confocal microscopy during the BiFC analysis.
Chapter 4

Identification of *RDD1*, a novel signaling component of AtCNGC2 and AtCNGC4-mediated signal transduction

Unpublished data by K Chin with the following exceptions:

Whole-genome sequencing and sequence analysis performed by Dr. Marc Champigny and Christine King at the McMaster Institute for Molecular Biology and Biotechnology (MOBIX)
4.1. Abstract

We previously reported the identification of the Arabidopsis mutant, *repressor of defense no death1* (*rdd1*), obtained from a suppressor screen of a T-DNA insertion knockout mutant of *AtCNGC2* (Chapter 3). *rdd1* was identified on the basis of suppression of *dn1*-conferring dwarfism. Characterization of *rdd1* plants revealed suppression of the majority of *dn1*- and *dn2*- mediated phenotypes including autoimmunity, Ca\(^{2+}\) hypersensitivity and delayed floral transition. BiFC analysis also demonstrated *in planta* interactions between *AtCNGC2* and 4 subunits that implicate these two proteins in possibly forming homo- or heteromeric channels that regulate Ca\(^{2+}\) signaling, pathogen defense and floral transition through *RDD1*. Here, we report our attempts to clone the *RDD1* gene using conventional map based cloning methods in combination with whole-genome sequencing. Rough mapping analyses indicated that *RDD1* locates to an 800kb genetic interval in the upper arm of chromosome 5, and whole-genome sequencing identified four potential causative mutations within this interval (AT5G24680, AT5G25590, AT5G25620 and AT5G26050). While the mutations in AT5G24680, AT5G25590 and AT5G26050 are located in the non-coding regions, one was identified in AT5G25620 to cause a synonymous change in the second exon. Preliminary gene expression analyses so far eliminated the three mutations in the non-coding regions, thus indicating that the causative mutation of *rdd1* is likely that of AT5G24620 which encodes the flavin-containing monooxygenase-like protein, called *YUCCA6*. 
4.2. Introduction

A number of CNGCs have been implicated in mediating pathogen defense responses in plants. Subsequent work following the identification of the Arabidopsis mutant cpr22 implicated AtCNGC11 and AtCNGC12 in positively regulating defense responses (Yoshioka et al., 2006). AtCNGC11 and AtCNGC12 null mutant plants exhibit partial suppression of R-gene mediated resistance to avirulent pathogens compared to Col-wt (Moeder et al., 2011; Yoshioka et al., 2006). Arabidopsis mutants of the “dnd” (defense, no death) class named dnd1 and dnd2/hlm1 are reported to exhibit broad spectrum disease resistance without induction of HR (Clough et al., 2000; Jurkowski et al., 2004). The DND1 and DND2 (HLM1) genes encode AtCNGC2 and AtCNGC4, respectively, in which null mutations result in constitutive PR gene expression, elevated levels of SA, and enhanced disease resistance (Clough et al., 2000; Balague et al., 2003; Jurkowski et al., 2004). Although the characterization of dnd mutants have suggested important roles for AtCNGC2 and 4 in plant defense, the molecular mechanisms by which immune responses are thought to be regulated by these channels have been met with some controversy. Ali et al. (2007) demonstrated that AtCNGC2 transduces a Ca²⁺ signal that stimulates an increase in nitric oxide (NO) production that lead to the induction of HR and innate immune signaling, thereby implicating AtCNGC2 to be a positive regulator of defense. In contrast, later studies including microarray data showing the down regulation of both AtCNGC2 and 4 in cpr22 as well as pathogen infection in wild type plants, implicate these genes in negatively regulating defense signaling (Moeder et al., 2011). In addition, Zhu et al. (2010) more recently identified the transcriptional co-repressor TPR1 that associates with the Toll-like/interleukin-1 receptor (TIR)-NB-LRR R protein, suppressor of npr1-1, constitutive 1
(SNC1) to suppress transcription of AtCNGC2 and AtCNGC4 upon virulent and avirulent pathogen challenge, further strengthening the notion that these genes act to negatively regulate defense signaling. Thus, the molecular mechanism(s) by which AtCNGC2 and 4 mediates pathogen defense remains unclear.

Recently, we reported the identification of the Arabidopsis mutant, rdd1 (repressor of defense no death1) as a suppressor of the dnd1-mediated signal transduction (Chapter 3). rdd1 (rdd1-1D cngc2-3) was isolated from a suppressor screen of a T-DNA knockout mutant of AtCNGC2 based on the partial suppression of dnd1-conferring dwarfism. Characterization of rdd1-1D cngc2-3 plants revealed partially impaired dnd1-conferring autoimmune phenotypes including the suppression of constitutive PR-1 gene expression, elevated levels of SA and enhanced resistance to oomycete and bacterial pathogens (Chapter 3). Additionally, the rdd1 mutation suppressed the delayed floral transition phenotype observed in dnd1 plants by altering expression of the central flowering repressor, Flowering Locus C (FLC). Furthermore, rdd1-1D cngc2-3 plants displayed partial hypersensitivity to elevated Ca$^{2+}$ compared to dnd1 plants. Interestingly, genetic analysis of the rdd1-1D cngc4-5 double mutant showed that rdd1 also suppressed dnd2 (cngc4)-mediated signaling (Chapter 3).

It has been reported that the alteration in HR induction in dnd1 is independent of SA accumulation and enhanced disease resistance (Clough et al., 2000, Genger et al., 2008). Therefore, it is hypothesized that RDD1 is located relatively upstream in the signaling pathway, at least before the signal branches out to cause the pleiotrophic phenotypes of dnd1, and Ca$^{2+}$ sensitivity. Lastly, BiFC experiments demonstrated potential interactions between AtCNGC2 and AtCNGC4 subunits, to form homo- and
heteromers in planta. Therefore, it was proposed that AtCNGC2 and AtCNGC4 can form either hetero- and/or homo- tetrameric channels that mediate signaling pathway(s) involving RDD1 in regulating defense responses, Ca\textsuperscript{2+} homeostasis and floral transition.

Here we report the preliminary identification of RDD1 using conventional map based cloning strategies together with whole-genome sequencing. RDD1 was roughly mapped to an 800kb region in the upper arm of chromosome 5, and whole genome sequencing identified four potential causative mutations within that region.

4.3. Materials and Methods

4.3.1. Plant materials and growth conditions

The T-DNA insertion knockout line of AtCNGC2 (Salk_066908) cngc2-3 was used. The seeds were planted on Sunshine Mix #1 (Sun Gro Horticulture Canada Ltd.) and stratified at 4\degree C for 4 days. Plants for the mapping populations were either grown in a growth chamber with a 9 hour photoperiod (9 hour light/ 15 hour dark) and a day/night temperature regime of 22\degree C/18\degree C. Plants for whole-genome sequencing were grown under a 9 hour photoperiod and harvested 4 weeks post stratification.

For map based cloning, mapping populations were generated by crossing rdd1-1D cngc2-3 to cngc2-1 (Chan et al, 2003; Ws ecotype), and selected on the basis of cngc2-like morphology in the F\textsubscript{2} generation.

4.3.2. DNA and RNA extraction

High quality DNA extraction for whole-genome sequencing was carried out using 4 week old leaves. 100mg leaf samples were frozen in liquid N\textsubscript{2} and homogenized in
700ul extraction buffer (200mM Tris pH 7.5, 250mM NaCl, 25mM EDTA pH 8, 0.5% SDS). Leaf homogenates were pelleted at 10000 x g for 5 minutes in 4°C, and nucleic acids were precipitated by adding 700ul isopropanol to the supernatant. Nucleic acids were then pelleted at 12000 x g for 20 mins in 4°C, and resuspended in 400ul nuclease-free water. After resuspension, small scale phenol:chloroform extraction was performed by adding 400ul phenol:chloroform (1:1) and centrifuged at 12000 x g for 10 minutes at 4°C for phase separation. For DNA purification, Ambion® RNAs cocktail (Life Technologies, Burlington, ON, CA) was added to the aqueous layer, and incubated at 37°C for 30 minutes followed by small scale phenol:chloroform (1:1) extraction. DNA was then precipitated following the addition of 200mM NaOAC and 2.5 volumes of 95% EtOH, and incubation at -20°C for 1 hour. DNA was then pelleted and washed with 75% EtOH, and resuspended in nuclease-free TE buffer.

Small-scale RNA extraction was carried out using the TRIlzol reagent (Invitrogen, Carlsbad, MO, CA) according to the manufacturer's instructions.

4.3.3. Quantitative real-time PCR (qRT-PCR)

Quantitative real time-PCR (qRT-PCR) was performed as previously described (Mosher et al., 2010) using Elongation Factor-1α (EF-1α) gene expression for normalization. Primer sequences are listed in Appendix 1.

4.3.4. Whole genome sequencing

Genomic DNA was extracted from 4 week old leaves of rdd1-1D cngc2-3 and cngc2-3 plants, and then sequenced on an Illumina® HiSeq 1500 system (Illumina, Inc., San Diego, CA, USA) according to the manufacturer’s protocol (Christine King and Marc
Champigny, McMaster Institute for Molecular Biology and Biotechnology (MOBIX), Hamilton, ON, Canada). Sequence assembly and analysis were also performed at MOBIX (Dr. Marc Champigny)

4.4. Results

4.4.1. Identification of rdd1-1D

As previously reported, a T-DNA insertion homozygous line (Salk_066908) for AtCNGC2 (At5g15410) was obtained from TAIR (www.arabidopsis.org), and named cngc2-3 (Figure 3-1A). Approximately 10,000 cngc2-3 seeds were mutagenized with EMS, and screened for plants suppressing the morphological phenotype of cngc2 in the M_2 generation. rdd1-1D cngc2-3 was identified in the M_2 generation based on its intermediate morphology between Col-wt and cngc2-3 plants (Figure 3-1C, upper panel). dnd1 has been reported to exhibit conditional spontaneous lesion formation (Clough et al., 2000). Under our growth conditions, we observed this lesion formation in dnd1 as well as cngc2-3, while this phenotype was suppressed in rdd1-1D cngc2-3 (Figure 3-1C, lower panel). rdd1-1D cngc2-3 plants were backcrossed to cngc2-3 to determine the genetic nature of the suppressor mutation. As seen in Table 3-1, all B_1 (backcrossed, first generation) plants displayed rdd1-1D morphology, suggesting that rdd1-1D is a dominant mutation (Table 3-1). The subsequent self-pollinated B_2 generation (backcrossed, selfed) segregated with a 3:1 (rdd1:cngc2) phenotypic ratio, confirming that the morphological phenotype of rdd1 segregates as a single locus that is dominant to the wild type RDD1 allele (Table 3-1).
4.4.2. *rdd1-1D* maps to the upper arm of chromosome 5

To determine the chromosomal location of *rdd1-1D*, homozygous *rdd1-1D cngc2-3* plants were crossed to *cngc2-1* plants (Ws ecotype) to generate a mapping population in the F2 generation for map based cloning. Approximately 50 plants from two homozygous F2 lines were selected on the basis of having *cngc2*-like morphology, and analyzed with a well dispersed grid of CAPS and SSLP markers that spanned the upper and lower arms of all five chromosomes (Appendix 1). Analysis of all markers established tight linkage to NGA151 and NGA158 in the upper arm of chromosome 5 (Figure 4-1, Table 4-1). For rough mapping, a total of 705 F2 mapping population comprised of 2 lines were analyzed with dCAPS markers generated from the *19 Genomes of Arabidopsis thaliana* database [http://mus.well.ox.ac.uk/19genomes/](http://mus.well.ox.ac.uk/19genomes/) (Gan et al, 2011, Appendix 1). The *RDD1* locus was mapped to an approximate 800kb region that contains 193 coding sequences (Figure 4-2). It should be noted that further mapping, including analysis of the F3 lines became increasingly complex due to phenotypic plasticity observed amongst the populations, likely related to the Ws ecotype effect. As such, whole-genome sequencing was employed to identify the causative mutation within this mapping interval.
Figure 4-1. Position of the markers used for linkage analysis. Approximately 50 $F_2$ *rdd1-1D/cngc2-1* (Ws ecotype) plants were tested with genetic markers (Appendix 1) that spanned the upper and lower arms of all 5 chromosomes. Linkage was established to NGA151 and NGA158 in the upper arm of chromosome 5 (red box).
Table 4-1. Genetic linkage analysis of \textit{rdd1-1D/cngc2-1} (Ws) \textit{F}_2 generation

<table>
<thead>
<tr>
<th>Genetic Marker</th>
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<th>Segregation</th>
<th>Hypothesis(^a)</th>
<th>(\chi^2) (^b)</th>
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<td>10</td>
<td>31</td>
<td>8</td>
<td>1:2:1</td>
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</tbody>
</table>

\(^a\) Hypothesis: No genetic linkage of \textit{RDD1} will result in a 1:2:1 (Ws:Heterozygous:Col) segregation of Ws and Col alleles. In the case of NGA158 and NGA151 (in bold), the 1:2:1 segregation is rejected and the number of alleles increases towards Ws, thereby indicating genetic linkage of \textit{RDD1} to this region of the chromosome.

\(^b\) 2 degrees of freedom
Figure 4-2. Genetic and physical map of the rdd1-1D-containing region on chromosome 5. The top line and bottom lines represent chromosome 5 and a larger scale map of the rdd1-containing region respectively, with various markers indicated below the line and the number of recombination events for each marker indicated above it. The location of the 800kb interval is indicated as a black box.
4.4.3. Whole genome sequencing identifies four candidate mutations

As mentioned above, the phenotypic plasticity of the F$_2$ mapping population and subsequent F$_3$ lines rendered our conventional map based approach to be increasingly difficult. To this end, whole-genome sequencing was employed to identify the causative mutation within the 800kb interval of chromosome 5 in which the RDD1 allele was roughly mapped to. High quality genomic DNA was extracted from 4 week old leaves of rdd1-1D cngc2-3 and cngc2-3 plants, and then sequenced on an Illumina® HiSeq 1500 system (Illumina, Inc., San Diego, CA, USA). 107bp long reads were generated for both rdd1-1D cngc2-3 and cngc2-3 totaling 78774306 and 100117138 paired reads respectively, and compared to the TAIR10 Col-0 reference genome sequence. Within the mapping interval, 11 single nucleotide polymorphisms (SNPs) and 2 InDels were identified in rdd1-1D cngc2-3, while 3 SNPs and 1 InDel was identified in cngc2-3 compared to Col-0. Of these, 10 SNPs and 1 InDel were identified to be unique to rdd1-1D cngc2-3. Further analysis narrowed the causative mutations related to four candidate genes: At5G24680, At5G25590, At5G25620 and At5G26050, each containing one putative SNP. Analysis of At5G24680, At5G25590 and At5G26050 revealed one point mutation within the non-coding regions including the 5'-UTR, potential promoter region and 3'-UTR, respectively. Lastly, analysis of At5G25620 identified a point mutation that caused a non-synonymous change in the second exon, which resulted in an amino acid sequence change from proline (P) to leucine (L) at position 289. In agreement with the mutagenizing effect of EMS, all four mutations are G/C to A/T conversions (summarized in Table 4-2). All mutations were individually re-sequenced in rdd1-1D cngc2-3, and confirmed for their respective mutations. Therefore,
taking together the results of the rough mapping and whole-genome sequencing, it is likely that one of these four candidates will be the causative mutation of \textit{rdd1-1D}.

\textbf{Table 4-2. Summary of mutations identified in \textit{rdd1}}

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Residue No.</th>
<th>Original Seq.</th>
<th>Mutated Seq.</th>
<th>Residue Change</th>
<th>Location</th>
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<td>-</td>
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<td>A</td>
<td>-</td>
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<td>Promoter</td>
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<td>CTG</td>
<td>P:L</td>
<td>Exon 2</td>
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<td>-</td>
<td>G</td>
<td>A</td>
<td>-</td>
<td>3'-UTR</td>
</tr>
</tbody>
</table>
4.4.4. Gene expression analysis of candidate genes in *rdd1-1D cngc2-3*

The identification of three causative mutations within the non-coding regions of At5G24680, At5G25590 and At5G26050 prompted us to assess any effects on gene expression. Since these mutations identified within the non-coding regions could alter transcriptional regulation of the aforementioned genes, no changes in their expression levels between *rdd1-1D cngc2-3* and *cngc2-3* would eliminate them as candidates for *RDD1*. Therefore, quantitative RT-PCR was conducted on four week old *rdd1-1D cngc2-3* and *cngc2-3* leaves to compare expression levels of At5G24680, At5G25590, At5G25629 and At5G26050 between both genotypes. As seen in Figure 4-3A and B, there are no differences in expression of At5G24680 and At5G26050 measured between *rdd1-1D cngc2-3* and *cngc2-3*, indicating that the mutations in the untranslated regions of At5G24680 and At5G26050 cause no alterations in gene expression, and are unlikely candidates for *RDD1*. In contrast, *rdd1-1D cngc2-3* showed a slight increase in At5G25590 expression compared to *cngc2-3* (Figure 4-3C). Additionally, *rdd1-1D cngc2-3* showed an increase in At5G25620 expression as well compared to *cngc2-3* (Figure 4-3D). This was not expected since the mutation is in the coding region. The results shown here indicate that either mutations in At5G25590 or At5G25620 could be the causative mutation of *rdd1-1D*. However, since the difference in expression level of At5G25590 is much more minimal than that of At5G25620, we hypothesize that the causative mutation is contained within the latter gene. Expression analysis of At5G25590 and At5G25620 will be repeated for confirmation.
**Figure 4-3.** Quantitative real-time PCR analysis of At5G24680, At5G26050, At5G25590 and At5G25620. (A-D) The expression is shown relative to EF-1α in *rdd1-1D cngc2-3* and *cngc2-3*. Error bars indicate standard error of the mean of 3 technical replicates.
4.5. Discussion

Although pathogen defense signaling in Arabidopsis has been well studied, the signal transduction of CNGCs in mediating these pathway(s) remains to be elucidated. The family of CNGCs in Arabidopsis is comprised of twenty members that are divided phylogenetically into 4 groups, with 2 subgroups in group IV. Of these CNGCs, only four – AtCNGC2, 4 (Group IVB), and 11, 12 (Group I) have been characterized to be involved in pathogen defense signaling. The identification and characterization of the Arabidopsis mutant cpr22 implicated AtCNGC11 and AtCNGC12 as positive regulators of pathogen defense (Yoshioka et al, 2006; Moeder et al 2011), while the characterization of the dnd class of mutants identified AtCNGC2 and 4 to be involved in regulating broad spectrum disease resistance in both a positive and negative manner (Moeder et al, 2011). As previously mentioned, forward genetic screens have been carried out to identify suppressor mutants of cpr22. However, this screen so far only discovered intragenic mutants (Baxter et al., 2008, Chin et al., 2010 and Abdel-Hamid, 2013). More recently, a second suppressor screen aimed to identify mutants of dnd1-mediated signaling identified the Arabidopsis mutant rdd1 (Chapter 3). Characterization of rdd1 (rdd1-1D) showed suppression of the majority of dnd1- and dnd2-conferred phenotypes, and BiFC analysis demonstrated that AtCNGC2 and 4 interact in planta to possibly form a channel complex that induces a Ca^{2+} signal to mediate immunity and floral transition through RDD1.

In this study, we identified four potential causative mutations of rdd1-1D. Of these, two are in the non-coding regions of At5G24680 and At5G25590, and one in the potential promoter region of At5G26050. The expression of each was compared
between *rdd1-1D cngc2-3* and *cngc2-3* plants. Preliminary data show comparable levels of At5G24680 and At5G26050 expression between *rdd1-1D cngc2-3* and *cng2-3*, and are unlikely to be the causative mutation of *rdd1-1D*. Thus, either the third or fourth mutations in the putative promoter region of At5G25590 and the coding sequence of At5G25620, respectively is the most likely candidate. However, since the difference in At5G25590 expression it is minimal compared to that of At5G25620, it is tempting to speculate that the causative mutation lies within At5G25620. In this case, the expression of At5G25620 is not necessarily altered in *rdd1-1D* since the mutation causes an amino acid change in the coding sequence. Gene expression analysis of these two genes will have to be repeated for confirmation, and complementation analyses of At5G25590 and At5G25620 into *dnd1* to recapitulate the *rdd1-1D* phenotype will be required as well.

At5G25620 encodes a flavin-containing mono-oxygenase (FMO)-like protein belonging to the 11-member YUCCA family in Arabidopsis, designated YUCCA6 (Zhao et al, 2001; Cheng et al, 2006; Kim et al, 2007). FMOs are hypothesized to exist in all kingdoms of life as they have been detected in bacteria, fungi, plants and animals (Schlenk, 1998). They have been most extensively studied in animals, and are reported to be involved in detoxifying xenobiotic compounds (Schlaich, 2007). The roles of FMOs in plants however, have only recently been demonstrated to be involved in auxin biosynthesis, glucosinolate metabolism and pathogen defense (Bartsch et al, 2006; Mishina and Zeier, 2006; Koch et al, 2006; Hansen et al, 2007; Zhang et al, 2008; Li et al, 2008). The YUCCA family of FMO-like proteins in Arabidopsis has been shown to be a critical component of *de novo* auxin biosynthesis (Zhao, 2001; Cheng et al, 2006, Kim et al, 2007; Kim et al, 2011). Over-expression of *YUCCA6* in gain-of-function and
activation mutants lead to increased induction of IAA-responsive genes, and elevated auxin accumulation culminating in altered leaf morphologies, increased inflorescence heights, and delayed senescence phenotypes (Kim et al, 2007, 2011). Whether YUCCA6 is directly involved in defense signaling remains unknown, but it has been reported that alterations in auxin signaling are involved in promoting resistance to pathogens (Navarro et al, 2006; Chen et al, 2007; Spaepen and Vanderleyden, 2010). Navarro et al (2006) demonstrated rapid down-regulation of auxin signaling by the bacterial PAMP, flg22, through the augmentation of auxin receptor transcripts. In addition, it was shown that exogenous applications of IAA enhanced bacterial virulence. Chen et al (2007) later demonstrated that free IAA levels increase upon P. syringae infection, and that the type III effector, AvrRpt2, specifically promotes elevations in IAA to promote susceptibility in host tissues. We hypothesize that dnd1-mediated signal transduction likely involves to some extent, the down-regulation of YUCCA6, and that the suppressor phenotypes observed in rdd1-1D cngc2-3 could be attributable to the up-regulation of auxin biosynthesis. This hypothesis agrees well with the observation by Chan et al. (2008) that several auxin-related genes are down-regulated in dnd1. Microbial synthesis of auxin has been one of the most well studied aspects of plant-microbe interactions. However, the role of auxin in plant defense responses is a fairly new avenue that remains to be elucidated especially in the context of CNGC-mediated signaling. The preliminary identification of YUCCA6 as a putative downstream component of the dnd1-signal transduction cascade provides further insight into auxin physiology during plant pathogen defense responses. Future work aimed to confirm YUCCA6 as RDD1 using complementation analyses is in progress.
4.6. Acknowledgements

We thank Dr. Marc Champigny and Christine King for whole-genome sequencing and sequence analysis.
Chapter 5

General Discussion
5.1. Discussion

Ca\(^{2+}\) signal transduction plays crucial roles in many physiological and cellular processes. It is this signal that allows for the integration of endogenous and environmental cues for the plant to generate appropriate adaptive responses. These cues that are recognized by the plant elicit stimulus-specific Ca\(^{2+}\) oscillations that change spatial and temporal Ca\(^{2+}\) fluxes within the cell (Jammes et al, 2011). While electrophysiological studies have identified many distinct Ca\(^{2+}\) channel activities at the plasma membrane, the identity of these channels together with the structure-function relationships that underlie the mechanism of Ca\(^{2+}\) conductance are not yet fully understood (Jammes et al, 2011; Ward et al, 2009; Pottosin and Schonknecht, 2007; Qi et al, 2006; Hua et al, 2003a; Leng et al, 2002, Leng et al 1999). Furthermore, the identity of such channels in plants that can bind signaling intermediates such as cyclic nucleotides remain unclear. One such family of Ca\(^{2+}\) channels proposed to do so is the Arabidopsis CNGCs. CNGCs are non-selective cation channels that were first identified in animal olfactory neurons and photoreceptors, and are directly gated by cyclic nucleotides. The Arabidopsis genome encodes a large family of CNGCs comprised of 20 members that have been implicated in mediating a variety of physiological responses including abiotic stress, pathogen defense, plant development, thermal sensing, ion homeostasis and uptake (reviewed in Chapter 1). Apart from these advances, only a handful studies regarding the structure-function relationships and components involved in the pathways mediated by this family of CNGCs have been reported (Kohler and Neuhaus, 2000; Baxter et al., 2008; Chin et al., 2010; Fischer et al., 2013; Abdel-Hamid et al., 2013).
Previous forward genetic studies using suppressor mutants of AtCNGC11/12 identified key residues for overall channel function and regulation. Baxter et al (2008) first identified a functionally important residue (E519) in the C-linker of the CNBD that was crucial for cAMP binding. In addition, E519 was likely counterpart of W573 of the human CNGA3, in which mutations at this position cause complete achromatopsia suggesting that this residue plays a functionally important role for CNGCs in general. This study was later extended and identified two more residues (R381 and G459) that are important for subunit interactions within AtCNGC11/12 (Abdel-Hamid et al., 2013).

In this study, we identified a fourth residue, R557 that serves an important regulatory role involving CaM binding (Chin et al., 2010; Chapter 2). Our study also investigated the functional importance of the αC- and αB-helices, in which the loss of both resulted in impaired channel function. Interestingly, Ali et al (2006) demonstrated that the elimination of the both helices in AtCNGC1, the region hypothesized to contain the CaMBD, demonstrated enhanced channel function. They attributed this result to the inability for CaM binding to occur, thereby alleviating any negative regulation on the channel protein. This however, is contrary to what we observed in AtCNGC11/12 (same as AtCNGC12). This discrepancy in observation can be attributable to the differences in modes of regulation occurring between different CNGCs. The recent discovery of putative CaMBDs residing in both the N- and C-terminal regions of Arabidopsis CNGCs (Abdel-Hamid Ph.D. thesis, 2013) lends to the hypothesis that many of these channels are regulated differently. The Calmodulin Target Database CaMBD prediction tool identified one putative C-terminal CaMBD in AtCNGC1 in contrast to two putative CaMBDs, one in each of the N- and C-termini of AtCNGC12. Although it remains to be investigated in plants, CaMBDs in animal CNGCs are also located in the N- and C-
terminal regions that directly mediate subunit interactions and ligand sensitivity (Weitz et al., 1998; Trudeau and Zagotta, 2002, 2003; Pifferi et al., 2006). For both olfactory CNG2A and rod CNGA1/CNB1 channels, Ca\(^{2+}\)-CaM binds to an N-terminal CaMBD to directly disrupt subunit interactions between this region and the C-terminal region (Varnum and Zagotta, 1997; Trudeau and Zagotta, 2003). Therefore, it is likely that the aforementioned differences in CaM-regulation and channel function observed between AtCNGC1 and AtCNGC11/12 (AtCNGC12) is attributable to the variability in CaMBDs – one in AtCNGC1 and two in AtCNGC12. To date, IQ-like CaMBDs in the C-terminal regions of AtCNGC20 and AtCNGC12 have been shown to bind to CaM (Fischer et al, 2013; DeFalco and Yoshioka, unpublished data), but the predicted N-terminal CaMBDs remain to be tested. Further analyses of these CaMBDs will be required to dissect the molecular mechanisms that govern CNGC regulation in Arabidopsis.

Aside from the structure-function relationship of AtCNGC11/12 that was investigated in Chapter 2, we also report the identification of a putative novel component in dnd- mediated signal transduction (Chapter 3 and 4). dnd1 and dnd2/hlm1, which are the null mutants of AtCNGC2 and 4, respectively, exhibit autoimmune phenotypes that are suppressed in the rdd1-1D mutant. Our preliminary data indicates that RDD1 is the FMO-like protein involved in de novo auxin biosynthesis named YUCCA6. It has been shown that SA represses auxin responses, and the down-regulation of auxin levels contribute to resistance signaling indicating a specific antagonism that occurs between these two signaling molecules (Navarro et al., 2006; Chen et al., 2007; Wang et al., 2007; Cui et al., 2013). Thus, it can be hypothesized that the mutation in YUCCA6 in rdd1-1D causes an up-regulation of auxin that suppresses dnd-mediated autoimmunity likely through auxin-SA antagonism. This hypothesis
sounds likely since Chan et al. (2008) conducted genome-wide transcriptional analysis of *dnd1* that showed the down-regulation of auxin-related genes in *dnd1*. However, interestingly, our very recent preliminary data indicates that the *rdd1-1D* mutation does not suppress *cpr22*-conferred phenotypes (Chin and Yoshioka, unpublished data).

Although the dwarfed stature is significantly suppressed, *rdd1-1D/cpr22* double mutants exhibit strong curly leaves and spontaneous cell death similar to *cpr22* mutants (Figure 5-1). Furthermore, overall morphology of *rdd1-1D/cpr22* does not resemble *rdd1-1D cngc2-3* (Figure 5-1). This was unexpected since if *rdd1-1D* suppresses the phenotypes of *dnd1* and *dnd2* through auxin-SA antagonism, then it would likely suppress the SA-dependent phenotypes of *cpr22* as well. These results indicate two possibilities: 1) the molecular mechanisms and/or signaling pathways that induce autoimmunity in *dnd1* and *dnd2*, and *cpr22* are different, and 2) the suppression of *dnd1* and *dnd2* phenotypes by *rdd1-1D* may not be due to auxin-SA antagonism. Although there is some controversy regarding the role of *AtCNGC2* and 4 in defense responses, increasing evidence implicates both genes in negatively regulating immunity. Besides null mutations resulting in broad spectrum autoimmune phenotypes, genome wide transcript profiling of *cpr22* and another autoimmune mutant *ssi4*, showed suppression of *AtCNGC2* and 4 (Mosher et al., 2010; Moeder et al., 2011). This observation was corroborated in publicly available microarray data that showed a repression of *AtCNGC2* and 4 after SA treatment and pathogen infection (Moeder et al; 2011). Furthermore, Zhu et al. (2010) identified the transcriptional co-repressor TPR1 that associates with SNC1 to suppress *AtCNGC2* and *AtCNGC4* transcription upon pathogen challenge. In contrast, *AtCNGC11* and 12 are implicated in positively regulating defense responses where null mutants of *AtCNGC11* and 12 display partial breakdown of *R*-gene mediated resistance.
(Yoshioka et al, 2006, Moeder et al., 2011). Another line of evidence that places the aforementioned CNGCs in separate signaling pathways is the involvement of JA/ET. The *cpr22* mutation is reported to activate SA- and JA/ET-dependent pathways simultaneously, while the latter pathway was found to be induced in *dnd1* only when SA-signaling is impaired (Jirage et al., 2001; Yoshioka et al., 2006; Genger et al., 2008). All these reports support the notion that the signal transduction and/or molecular mechanisms behind the autoimmunity in *dnd1* and *dnd2*, and *cpr22* are different. If this is the case, it is still possible that the suppression of *dnd1* and *dnd2* phenotypes is due to auxin-SA antagonism. Alternatively, it is possible that the suppression of *dnd1* and *dnd2* by *rdd1-1D* may not be related to auxin-SA antagonism at all. In any case, our preliminary data with *rdd1-1D cngc2-3/cpr22* strongly supports the notion that the pathways mediated by *AtCNGC2* and 4 are fundamentally different from those mediated by *AtCNGC11* and 12. Whether these differences lie in the molecular mechanisms that govern Ca\(^{2+}\) conductance however, is for most part unknown. All four CNGCs have been shown to be permeable to a selected number of cations including Ca\(^{2+}\) (reviewed in Chapter 1). Although the precise regulatory properties of these channel proteins remain to be identified, there is supporting evidence that identifies certain differences amongst them. For example, *AtCNGC11/12* (same as *AtCNGC12*) was shown to be preferentially activated by cAMP over cGMP in K\(^{+}\)-deficient yeast (Yoshioka et al., 2006) while electrophysiological studies indicate that *AtCNGC2* is activated with similar efficacies by cAMP and cGMP (Leng et al., 1999; Leng et al., 2002). Additionally, *AtCNGC4* was shown to be activated more strongly by cGMP than cAMP (Balague et al., 2003). Moreover, as mentioned above, *AtCNGC12* is predicted to have two CaMBDs, with one in each cytosolic region. In the same analysis, *AtCNGC2* and 4 are
predicted to have only one CaMBD that resides in the C-terminal domain (Abdel-Hamid Ph.D. thesis, 2013). AtCNGC2 and 4 make up Group IVB, which is the most evolutionarily divergent group in the family. As such, it could be possible that the differences in regulatory properties observed in AtCNGC2 and 4, together with their evolutionary divergence identifies them as fundamentally different channel proteins. One piece of evidence that lends to this hypothesis is the recent identification of a salt bridge between E450 and R381 required for intra-subunit interactions in AtCNGC2 and 4 (Abdel-Hamid et al., 2013). Interestingly, E450 does not exist in the other 18 members of the CNGC family, and the interaction between E450 and R381 was found to more closely resemble those occurring in animal CNGCs. Additionally, a recent bioinformatics analysis using moss and Arabidopsis CNGCs revealed a diversification in biological function in AtCNGC2 and 4 together with AtCNGC19 and 20 from the rest of the CNGC family (Finka et al., 2012). Thus, there is increasing evidence that indicates a potential diversification of AtCNGC2 and 4 from AtCNGC11 and 12. Future work to further elucidate the regulatory and structural differences amongst the 20 members, together with the mechanisms by which they oligomerize will be worthwhile.
Figure 5-1. *rdd1-1D* does not suppress *cpr22*-conferred curly leaf morphology and cell death. (Upper panel) *rdd1-1D/cpr22* plants (red box) retain curly leaves and partial dwarfed stature compared to *rdd1-1D cngc2-3* and *cpr22* plants (black box). (Lower panel) *rdd1-1D/cpr22* leaves display spontaneous cell death comparable to *cpr22*. 
5.2. Future Directions

5.2.1. CNGC structure-function studies

The structure-function study (Chapter 2) and suppressor screen of *dnd1* (Chapter 3 and 4) provides further insight into the regulation, and subunit interactions of CNGCs. What is evident from our findings presented in this study is the lack of knowledge of the regulatory domains in each of the 20 members. In animals, CaMBDs within the N- and C-terminal domains of CNGC hetero-tetramers contribute to the stable interaction of subunits that promote the open conformation of the channel complex. To date, the CaMBD of a selected few CNGCs have been studied. The R557C mutation in AtCNGC11/12:S58 confirms at least in part, a CaMBD within the cytosolic C-terminal domain of AtCNGC11/12 (AtCNGC12). A bioinformatics approach has since identified a second CaMBD residing in the N-terminal region of AtCNGC12. Interestingly, AtCNGC11 is predicted to have one only one CaMBD in the N-terminus in contrast to AtCNGC12 in the Col ecotype. Thus, it is clear that stark differences in domain architecture exist even within closely related members of CNGCs, which supports the hypothesis that many of these CNGCs are regulated differently, and can form different channel complexes either as homomers or heteromers that mediate different physiological responses. In animal CNGCs, the stoichiometry of the interacting subunits that make up the channel complex was found to dictate cation permeability (Shuart et al., 2011). Furthermore, specific domains within the protein dictate the stoichiometry of the interacting subunits. For instance, the carboxy-terminal leucine zipper (CLZ) domain of the animal CNGA3 provides the constraint for the channel complex to contain three CNGA3 subunits, and one CNGB1 subunit. Moreover, the N-terminal CaMBD of
CNGB1 was shown to directly interact with the CLZ domain in CNGA1. \( \text{Ca}^{2+} \)-calmodulin binding in this case, disrupts the interaction and separates the CNGB1 N-terminus from the CNGA1 C-terminus to promote channel closure (Trudeau and Zagotta, 2003, 2004). To date, such molecular determinants of CNGC assembly in plants have not been identified. Functional analyses of these predicted domains in AtCNGCs will be required to not only determine how these channels are regulated, but also how these channel subunits hetero-oligomerize. Furthermore, determining \textit{in planta} interactions of all 20 CNGC subunits will be worthwhile. Not only will this identify interacting partners within the CNGC family, it will allow for the precise delineation of the structure-function relationship between these partnering subunits. In light of the large number of CNGCs in plants, it is possible that some functions of these CNGCs may be difficult to uncover due to potential redundancy. Moreover, it is not clear whether the loss of one subunit will render any partnering subunits inactive, or if the remaining subunits will form novel channels with new partners. The latter scenario would thus create alternate phenotypes that are not directly related to the function of the missing subunit. Therefore, the key to understanding CNGC function, as well as regulation, is to find out which subunits interact with each other, and whether these interactions are “permanent” or whether the subunits rearrange depending on the stimulus or the developmental stage. For such analyses, further technological breakthroughs that allow the monitoring of spatial and temporal interactions between subunits will be required. The identification of these subunit interactions will therefore provide a good start. Previous work using yeast two hybrid analyses have proven to be difficult (Chin and Yoshioka, unpublished results), and although we were able to demonstrate \textit{in planta} interactions between AtCNGC2 and 4 subunits, much work remains to be done in addressing these perspectives.
5.2.2. CNGC-mediated signal transduction

The preliminary identification of the putative downstream component of *dnd*-mediated signal transduction, *YUCCA6* may open up a new avenue of CNGC research involving auxin signaling. *YUCCA6* is a crucial component of *de novo* auxin synthesis, and this finding may provide further evidence that supports the role of auxin in pathogen defense signaling. Future work will involve confirming that *RDD1* is in fact *YUCCA6*. This will be performed using a variety of complementation analyses in *dnd1* plants. The elimination or over-expression of *YUCCA6* in the *dnd1* background may identify the suppressor mutation as either a loss or gain-of-function. Measuring auxin/IAA levels in both *dnd* and *rdd1-1D* mutants may also provide further evidence of altered auxin physiology during *dnd*-mediated signal transduction. Especially since the stunted morphology of *dnd* mutants are reported to be largely SA-independent (Genger et al., 2008), it could be possible that this morphological phenotype is attributable to the alterations in auxin/IAA signaling. Additionally, it is worthwhile to pursue further characterization of *rdd1-1D/cpr22* double mutants that extend to pathogen resistance. As mentioned above, *rdd1-1D/cpr22* double mutants show no suppression of *cpr22*-conferred morphology and spontaneous cell death by *rdd1-1D*. Further characterization will provide insight to the involvement of auxin signaling in *AtCNGC11* and 12-mediated signal transduction as well. Long term perspectives will involve the characterization of defense responses in *yucca6* mutants.
5.3. Conclusions

The forward genetic approach taken in this project has opened a new avenue for CNGC research. First, the identification of R557C confirmed at least the existence of a CaMBD in the C-terminal region of AtCNGC11/12 (same as AtCNGC12). This finding extends our knowledge of CaM-binding in Arabidopsis CNGCs, which will be important for not only identifying differential regulation, but also for elucidating subunit interactions amongst these channel proteins. Secondly, we were able to show for the first time, in planta interactions between AtCNGC2 and AtCNGC4 subunits to form homo- and hetero-oligomers. Our findings also suggest that both AtCNGC2 and AtCNGC4 subunits form channel complexes that mediate a signaling pathway involving RDD1 to regulate pathogen defense, Ca\(^{2+}\) homeostasis and floral transition.

In addition, the preliminary identification of YUCCA6 as a putative downstream component of dnd-mediated signaling may provide a new view on CNGC-mediated pathogen defense. Lastly, the findings of our research lend to the hypothesis that AtCNGC2 and 4 are fundamentally different from AtCNGC11 and 12. Therefore, the findings outlined in this thesis provide a new foundation for an extensive amount of work to further elucidate the physiological roles and biophysical properties of CNGCs in Arabidopsis.
References


Abdel-Hamid H., Chin K., Moeder W., Shahinas D., Gupta D., Yoshioka K. (2013) A suppressor screen of the chimeric AtCNGC11/12 reveals residues important for inter-subunit interactions of cyclic-nucleotide gated channels. Plant Physiol. (Epub ahead of print)


Ausbuel F.M., Bent R., Kingston R.E., Moore D.D., Seidman J.G.,


odor adaptation by cAMP-gated channel subunits. Science 294: 2176-2178


**Chen T.Y. and Yau, K.W.** (1994) Direct modulation by Ca^{2+}-calmodulin of
cyclic nucleotide-activated channel of rat olfactory receptor neurons. Nature 368: 545-548


Chin K., Moeder W., Yoshioka K. (2009) Biological roles of cyclic-nucleotide-gated ion channels in plants: What we know and don’t know about this 20 member ion channel family. Botany 87: 668-677


GMP of cationic conductance in plasma membrane of retinal rod outer segment. Nature **313**: 310-313

**Finka A., Cuendet A.F., Maathuis F.M.J, Saidi Y., Goloubinoff P.** (2012)

Plasma membrane cyclic nucleotide gated calcium channels control land plant thermal sensing and acquired thermotolerance. Plant Cell **24**: 3333-3348

**Fischer C., Kugler A., Hoth S., Deitrich P.** (2013) An IQ-domain mediates the interaction with calmodulin in a plant cyclic nucleotide-gated channel. Plant Cell Physiol. **54**: 573-584

**Fischer M., Schnell N., Chattaway J., Davies P., Dixon G., Sanders D.** (1997)

The Saccharomyces cerevisiae CCH1 gene is involved in calcium influx and mating. FEBS Letters **419**: 259-262


Structure and rearrangements in the carboxyterminal region of SpIH channels. Structure **15**: 671-82


Goulding E.H., Ngai J., Kramer R.H, Colicos S., Axel R., Siegelbaum S.A.,


Kleene S.J. (1999) Both external and internal calcium reduce the sensitivity of
the olfactory cyclic-nucleotide-gated channel to cAMP. J. Neurophysiol. 81: 2675-2682


Identification of a putative voltage-gated Ca$^{2+}$ permeable channel (OsTPC1) involved in Ca$^{2+}$ influx and regulation of growth and development in rice. Plant Cell Physiol. 45: 693-702


Electrophysiological analysis of cloned cyclic nucleotide-gated ion channels. Plant Physiol. 128: 400-410


Li X., Borsics T., Harrington H.M., Christopher D.A. (2005) Arabidopsis
AtCNGC10 rescues potassium channel mutants of *E. coli*, yeast, and *Arabidopsis* and is regulated by calcium/calmodulin and cyclic GMP in *E. coli*.

Funct. Plant Biol. **32**: 643-653


Liman E.R. and Buck L.B. (1994) A second subunit of the olfactory cyclic nucleotide-gated channel confers high sensitivity to cAMP. Neuron **13**: 611-621


Mäser P., Thomine S., Schroeder J.I., Ward J.M., Hirschi K., Sze H., Talke


Moeder W. and Yoshioka K. (2009) Environmental sensitivity in pathogen

Molday R.S., Molday L.L., Dose A., Clark-Lewis I., Illing M. Cook M.J.,


Mosher S., Moeder W., Nishimura N., Jikumaru Y., Joo S.H., Urquhart W.


Munger S.D., Lane A.P., Zhong H., Leinders-Zufall T., Yau K.W., Zufall F.,


**Rostoks N., Schmierer D., Mudie S., Drader T., Brueggeman R., Caldwell**


Trends Plant Sci. 12: 412-418


PatchDock and SymmDock: servers for rigid and symmetric docking. Nucl. Acids Res. 33: 363-367


Trudeau M.C. and Zagotta W.N. (2004) Dynamics of Ca$^{2+}$-calmodulin-
dependent inhibition of rod cyclic nucleotide-gated channels measured by patch-clamp fluorometry. J. Gen. Physiol. 124: 211-223


Urquhart W., Chin K., Ung H., Moeder W., Yoshioka K. (2011) The cyclic nucleotide-gated channels, AtCNGC11 and 12, are involved in multiple Ca$^{2+}$ dependent physiological responses and act in a synergistic manner. J. Ex. Bot. 62: 3671-3682


Varnum M.D. and Zagotta W.N. (1997) Interdomain interactions underlying activation of cyclic nucleotide-gated channels. Science 278: 110-113


Zagotta W.N., Olivier N.B., Black K.D., Young E.C., Rolson R., Gouaux E.


Zhang Z., Lenk A., Andersson M.X., Gjetting G., Pedersen C., Nielsen M.E.,


Zhao Y., Christensen S.K., Fankhauser C., Cashman J.R., Cohen J.D.,


# Appendix 1

## Table A1-1. Chapter 2 Primer Sequences

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## Appendix 2

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