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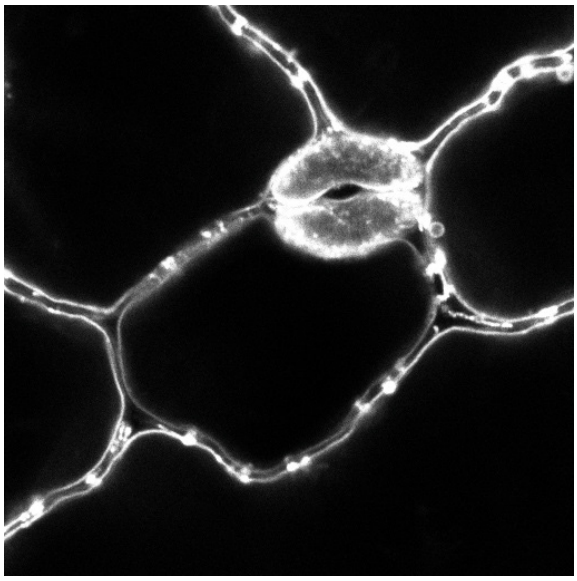
KERRI HUNTER

THE CYSTEINE-RICH RECEPTOR-LIKE KINASE CRK2 DURING STRESS RESPONSES IN ARABIDOPSIS THALIANA



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Faculty of Biological and Environmental Sciences
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Finland

THE CYSTEINE-RICH RECEPTOR-LIKE KINASE CRK2 DURING STRESS RESPONSES IN *ARABIDOPSIS THALIANA*

Kerri Alyssa Hunter

ACADEMIC DISSERTATION

To be presented, with the permission of the Faculty of Biological and Environmental Sciences of the University of Helsinki, for public examination in lecture hall B5, Forest Sciences Building (Latokartanonkaari 7), on Friday December 13th at 12 o'clock noon.

Helsinki 2019

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“For what it’s worth, it was worth all the while.”

Green Day – Good Riddance

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ORIGINAL PUBLICATIONS

I. Bourdais, G., Burdiak, P., Gauthier, A., Nitsch, L., Salojärvi, J., Rayapuram, C., Idänheimo, N., **Hunter, K.**, Kimura, S., Merilo, E., Vaattovaara, A., Oracz, K., Kaufholdt, D., Pallon, A., Anggoro, D.T., Glów, D., Lowe, J., Zhou, J., Mohammadi, O., Puukko, T., Albert, A., Lang, H., Ernst, D., Kollist, H., Brosché, M., Durner, J., Borst, J.W., Collinge, D.B., Karpiński, S., Lyngkjær, M.F., Robatzek, S., Wrzaczek, M., Kangasjärvi, J., on behalf of the CRK Consortium, 2015. Large-Scale Phenomics Identifies Primary and Fine-Tuning Roles for CRKs in Responses Related to Oxidative Stress. PLOS Genet. 11, e1005373. https://doi.org/10.1371/journal.pgen.1005373

II. **Hunter, K.**, Kimura, S., Rokka, A., Tran, H.C., Toyota, M., Kukkonen, J.P., Wrzaczek, M., 2019. CRK2 Enhances Salt Tolerance by Regulating Callose Deposition in Connection with PLDα1. Plant Physiol. 180, 2004. https://doi.org/10.1104/pp.19.00560

III. Kimura, S., **Hunter, K.**, Vaahtera, L., Tran, H.C., Vaattovaara, A., Rokka, A., Stolze, S.C., Harzen, A., Meißner, L., Wilkens, M., Hamann, T., Toyota, M., Nakagami, H., Wrzaczek, M., 2019. CRK2 and C-terminal phosphorylation of NADPH oxidase RBOHD regulate ROS production in Arabidopsis. Submitted manuscript; pre-print at bioRxiv 618819. https://doi.org/10.1101/618819

Author's contributions:

I. KH participated in the creation of complementation lines for CRK2, CRK5, CRK10, CRK31, CRK40, CRK42, and CRK45 (cloning, Arabidopsis transformation and selection), plant size and water loss complementation assays, and editing of the manuscript.

II. KH participated in experimental design, performed experiments (cloning, Arabidopsis transformation and selection, genotyping, RT-PCR, transient transformations and experiments with *Nicotiana benthamiana* and Arabidopsis seedlings, immunnoprecipitations for proteomics analysis, germination assays, root length assays, western blots, microscopy, callose assays, plasmodesmata permeability assays, fluo-4-AM calcium imaging), analyzed the data, and wrote the manuscript.

III. KH participated in experimental design, performed experiments (cloning, Arabidopsis transformation and selection, microscopy, callose assays), participated in data analysis, and participated in the writing and editing of the manuscript.

ABBREVIATIONS

ABA	abscisic acid
Arabidopsis	*Arabidopsis thaliana*
ATP	adenosine triphosphate
CALS	callose synthase
CDK	cyclin-dependent kinase
CDPK	calcium-dependent kinase
CRK	cysteine-rich receptor-like kinase
DAMP	damage-associate molecular pattern
DUF26	domain of unknown function 26
ETI	effector-triggered immunity
HEK293T	human embryonic kidney 293T cell line
MAMP	microbe-associated molecular pattern
MAPK	mitogen activated protein kinase
PA	phosphatidic acid
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
PH	plekstrin homology
PLD	phospholipase D
PRR	pattern recognition receptor
PTI	pattern-triggered immunity
PX	phox homology
RBOH	respiratory burst oxidase homolog
RLCK	receptor-like cytoplasmic kinase
RLK	receptor-like kinase
ROS	reactive oxygen species
YFP	yellow fluorescent protein

ABSTRACT

In order to maintain health, growth, and productivity, plants must be able to adapt to increasingly variable environmental conditions. Plants are continuously flooded with information from their surrounding environment, which must be sensed, incorporated, and responded to accordingly. Much of the communication between plant cells and the extracellular environment is carried out by the receptor-like protein kinases (RLKs), including the cysteine-rich receptor-like kinase (CRK) subfamily. Despite the large size of the CRK gene family, their physiological roles and functions on a biochemical and cellular level remain largely uncharacterized. We performed large scale phenotyping of a *crk* T-DNA mutant collection in *Arabidopsis thaliana* (Arabidopsis), which suggested roles for the CRKs in several developmental processes, as well as during abiotic and biotic stress responses. CRK2 emerged as an important CRK, with several strong loss-of-function phenotypes and a notable phylogenetic position. We established that CRK2 enhances salt tolerance through the regulation of callose synthase 1 (CALS1) dependent callose deposition at plasmodesmata. This revealed a previously uncharacterized role for callose deposition in response to high salinity. We showed that this callose deposition has an effect on plasmodesmal permeability, and therefore a potential impact on intercellular signalling. Additionally, CRK2 was found to regulate the formation of an unknown vesicle type during salt stress, which could possibly be involved in cell-to-cell signalling as well. We have described how CRK2 regulates ROS production during immunity by regulation of RBOHD via C-terminal phosphorylation. We observed highly specific changes in the subcellular localization of CRK2 in response to various stress treatments, and demonstrated that these localization patterns are critical for protein function and interactions. The subcellular localization and many of the cellular functions of CRK2 were dependent on phospholipase D alpha 1 (PLDα1) activity, and PLDα1 was consistently identified as one of the top proteins to interact with CRK2. Thus, we propose that CRK2 is a fundamental CRK, which acts in connection with PLDα1 to regulate several cellular processes during the response to environmental stimuli.

1. INTRODUCTION

1.1 Plants in a changing environment

Plant life comprises approximately 80% of Earth's total biomass (Bar-On et al., 2018) and is essential for supporting life in numerous ways. Plants provide a means for carbon fixation and oxygen production through photosynthesis, they affect landscape and soil architecture, and they provide habitats and nutrition for organisms throughout the biosphere. From a human perspective, plants also represent vast economic interests, including agriculture, forestry, horticulture, pharmaceuticals, cosmetics, and more recently biofuels. However, due to a growing human population and the expansion of urban and industrialized areas, combined with environmental shifts due in part to a changing climate, plants are increasingly exposed to less than ideal growth conditions (Fedoroff et al., 2010; World Meteorological Organization, 2019). As sessile organisms, there is no option for plants to move away from causes of stress or seek out more favorable conditions. Therefore, in order to survive, plants must be able to adapt to their environment, including both short term immediate responses and long term evolutionary changes.

Individual plant cells are continuously inundated with information from the surrounding micro and macro environment. Chemical changes, osmotic pressure, water availability, light levels, temperature, pH, physical wounding, and invading pathogens are all examples of environmental factors which, if not optimal, can provoke stress in the plant. Thus, in order to maintain health and growth, these changes must be sensed and responded to accordingly. In addition, since plants are multicellular organisms, signals from individual cells need to be integrated and communicated to the rest of the plant. Plants exposed to suboptimal conditions may be unable to germinate or survive, or may require more resources, due to increased energy expenditure devoted to stress responses instead of growth, all leading to decreased plant health, growth, and overall agricultural productivity (Machado and Serralheiro, 2017; Shrivastava and Kumar, 2015). If the goal is to produce plants with increased stress tolerance, either through traditional breeding or genetic engineering, it is imperative to first develop a more thorough understanding of the factors causing these stresses, as well as the underlying mechanisms and molecular pathways leading to tolerance.

1.1.1 Abiotic stress

Abiotic stress refers to stress-inducing stimuli of a non-living origin. This includes such things as salt, drought, flooding, heat, cold, heavy metals, and nutrient deficiency. These factors may be present to varying degrees in an environment, and may be dependent on the long term geographical and climate constraints, as well as shorter term weather patterns and human impact. Accordingly, different plant

species and populations are likely to have differential inherent tolerances to these various environmental conditions. Temperature, water availability, and salt content are considered the main abiotic factors affecting the global distribution of plants, agricultural sustainability, and food security (Zhu, 2016).

1.1.1.1 Salt stress

One major category of abiotic stress is salt stress, referring specifically to NaCl. Soil with high salt content is becoming increasingly more prevalent, especially across irrigated agricultural land. Recent estimates have categorized at least 20% of the total cultivatable land as affected by high salinity (FAO and ITPS, 2015). This is problematic due to the fact that the majority of crop species are not inherently salt tolerant, thus high salinity poses a widespread threat to agricultural productivity (Yang and Guo, 2017).

An environment with high salt concentrations imposes various ecological, physical, and physiological stresses on the life it supports. Salt content affects soil composition and erosion, as well as nutrient and water uptake by the plant (Shrivastava and Kumar, 2015). On a cellular level, high salinity exerts both an osmotic and ionic stress, and can disturb membrane integrity, ionic balances and membrane potentials, and the transport and balance of water, nutrients, and solutes (Machado and Serralheiro, 2017). Thus, there is a multitude of ways in which high salinity can affect plant health and growth, and accordingly, salt tolerance is a complex and multifaceted process. The cellular responses to increased salt concentrations are currently known to include: calcium influx (Choi et al., 2014; Knight et al., 1997; Tracy et al., 2008), activation of NADPH oxidase RESPIRATORY BURST OXIDASE HOMOLOGS (RBOHs) and reactive oxygen species (ROS) production (Ma et al., 2012), activation of phospholipase D (PLD) and phosphatidic acid (PA) production (Hong et al., 2010; Li et al., 2009), changes in plasma membrane composition and formation of microdomains (Elkahoui et al., 2004; Hao et al., 2014; López-Pérez et al., 2009; Wu et al., 1998), cell wall modifications (Tenhaken, 2015), and increased endocytosis of various receptors and channels (Baral et al., 2015), notably aquaporins to regulate water transport (Li et al., 2011; Luu et al., 2011; Ueda et al., 2016). However, a complete understanding of the integration and regulation of these processes is still lacking.

1.1.2 Biotic stress

As the name suggests, biotic stress encompasses all stress-inducing stimuli of a biological origin. Pathogen infection is one extensively studied area of biotic stress, and includes bacterial, fungal, and viral pathogens. Plant-pathogen interactions are complex and continuously evolving, and pathogens have been of considerable concern to agriculture throughout history, and remain so today (Wirthmueller et al., 2013). Agricultural practices of planting large areas of uniform crops, or

monocultures, can further increase the risk of disease outbreaks (Newton, 2016). Another major source of biotic stress comes from herbivory, whereby animals or insects cause physical damage to the plant by feeding on it (War et al., 2018, 2012).

The immune response in plants can be divided into two stages: pattern-triggered immunity (PTI) and effector-triggered immunity (ETI). PTI acts as the first layer of defence; it is triggered when pattern recognition receptors (PRRs) perceive specific microbe-associated molecular patterns (MAMPs) or damage-associated molecular patterns (DAMPs) (Boller and Felix, 2009; Boller and He, 2009). Some pathogens are able to suppress PTI using effector proteins which are transferred into the cell. Therefore, the second layer of defence, ETI, is aimed at recognition of these effector proteins (Jones and Dangl, 2006). In many cases, some signalling components and mechanisms overlap between PTI and ETI; therefore, the specificity of outcomes may be derived instead from the timing, amplitude, or interaction of the various responses elicited by the pathogen (Tsuda and Katagiri, 2010).

1.1.3 Interaction of stresses

Stress impacts plants not only in the elicitation of a direct response to that stimulus, but also through its negative effect on growth, development, and symbiotic interactions (Atkinson and Urwin, 2012; de Souza et al., 2016; Mittler, 2006; Pandey et al., 2017). Various stress stimuli, while quite different in origin, share some of the same signalling network components and cellular responses, and therefore may interact and produce different outcomes than each singular stress alone. Some stresses have been shown to have additive effects, such as drought and heat (Mittler, 2006), whereas others may have protective effects, such as ozone and UV light (Mittler, 2006). Global warming has caused an increase in the prevalence of multiple stress combinations experienced concurrently (Pandey et al., 2017). Abiotic stress, on both a short time scale and long term climate change, can also influence the prevalence, severity, and outcomes of biotic stresses (Atkinson and Urwin, 2012).

1.1.4 Arabidopsis as a model species

Arabidopsis thaliana (Arabidopsis) is the most commonly used model species in plant research. Arabidopsis makes for a good model species due to its relatively short life cycle, capacity for both cross- and self-pollination, small size, ease of growth in laboratory and other controlled environments, a fully sequenced genome, and the availability of tools and resources for genetic manipulation (Koornneef and Meinke, 2010). Generating transgenic Arabidopsis plants is now relatively easy and reproducible using the *Agrobacterium tumefaciens* (Agrobacteria) based transformation method. This has enabled: directed expression (including overexpression, ectopic expression, and inducible expression) of specific genes; production of tagged proteins for biochemical or microscopy applications; and expression of reporter genes, to monitor specific conditions, such as pH (Moseyko

and Feldman, 2001; Schulte et al., 2006) or Ca^{2+} (Allen et al., 1999; Horikawa et al., 2010; Mithöfer and Mazars, 2002; Vincent et al., 2017), or localizations, at the tissue, cellular, or subcellular level. It has also enabled the production of T-DNA insertion mutant lines, which are now available for the majority of Arabidopsis genes and are maintained in large public databases (T-DNA Express, http://signal.salk.edu/cgi-bin/tdnaexpress) and stock centers (NASC, http://arabidopsis.info/; ABRC, https://abrc.osu.edu/). Homologs of many Arabidopsis genes are present in other plant species, highlighting the potential for transfer and application of the information gained from Arabidopsis to more commercially-oriented plant species.

1.2 Perception of environmental stimuli

In order for an organism to properly adapt to suboptimal conditions, it must first become aware of the situation, and then initiate the necessary steps to respond to the stress. Changes in the extracellular environment must therefore be sensed, and that information transmitted into the intracellular environment. Protein kinases are often vital components of signal transduction, and are necessary for the perception of numerous environmental stimuli.

1.2.1 Protein kinases

Protein kinases catalyze the transfer of the γ-phosphate from adenosine-tri-phosphate (ATP) on to a substrate. Serine, threonine, tyrosine, and histidine amino acids are all potential acceptors of this phosphate, and different types of kinases have differential phosphorylation site preferences and capabilities. The specificity of the kinase-phosphorylation site interaction can also depend on the surrounding amino acids. The histidine kinases generally differ in structure and phosphorylation mechanism from the more typical serine/threonine and tyrosine kinases (Wolanin et al., 2002). Protein phosphorylation is a reversible process, with the de-phosphorylation reaction catalyzed by phosphatases. Together, the dynamics of protein phosphorylation and de-phosphorylation provide a rapid and potentially highly regulated means of controlling enzyme activation, protein interactions, and conformational changes. Phosphorylation is one of the most common post-translational modifications found in eukaryotes, and accordingly, kinases are crucial components of a wide range of cellular processes and signalling networks (Kornev et al., 2006).

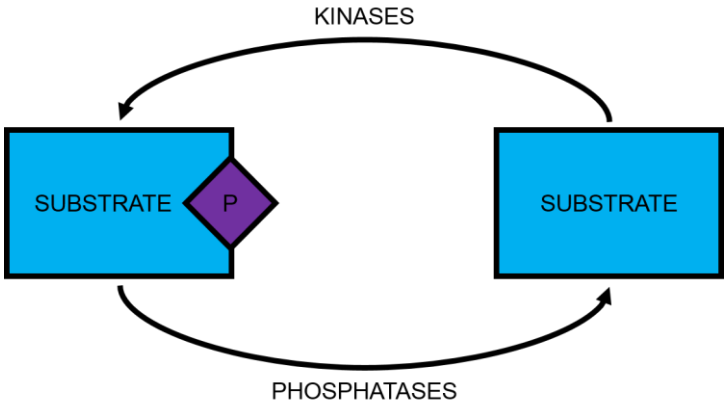

Figure 1. Actions of kinases and phosphatases on substrate proteins.

1.2.1.1 Kinase domains and activation

The typical kinase core structure consists of a smaller N-terminal lobe and a larger C-terminal lobe, in between which resides the ATP binding site. When the kinase is in an active state this site is exposed, whereas in the inactive conformation it is not accessible (Kornev et al., 2006). The majority of kinases found in eukaryotes contain several conserved protein domains which are important for their activity and regulation (Hanks et al., 1988; Stone and Walker, 1995). The first of these is a conserved lysine (K) in the N-lobe, which is required for ATP binding, as well as structural stabilization (Iyer et al., 2005; Kornev et al., 2006). The aspartic acid – phenylalanine – glycine (DFG) domain is important for orienting the ATP (Kornev et al., 2006). The histidine – arginine – aspartic acid (HRD) domain is required for catalysis and the proper orientation of the acceptor site on the substrate protein (Adams, 2003; Kannan and Neuwald, 2005; Kornev et al., 2006). Mutations disrupting either the conserved K or HRD domains are commonly used to create kinase-dead protein variants, which are incapable of carrying out the phosphorylation reaction (Iyer et al., 2005; Strong et al., 2011; Zhang et al., 2015).

1.2.1.2 Kinases in plants

Plant genomes contain a large number of kinases; in Arabidopsis, kinases make up approximately 4% of the total encoded genes (Champion et al., 2004). These can be divided into either membrane-bound kinases, which account for approximately two thirds of the total kinases in Arabidopsis, or soluble kinases, which account for approximately one third of the total kinases in Arabidopsis (Zulawski et al., 2014). Kinases are further classified within these groups based on their function, structure, and phylogeny. The membrane-bound kinases are discussed in detail in the next section. The soluble kinases include such notable groups as the mitogen-activated protein kinases (MAPK, MAPKK, MAPKKK), the calcium-dependent protein kinases (CDPKs), and the cell cycle regulating cyclin-dependent kinases (CDKs) (Zulawski et al., 2014).

1.2.1.3 Receptor-like kinases

The receptor-like protein kinases (RLKs) are largely responsible for communication between plant cells and the extracellular environment. This protein family is widely represented across plant lineages and highly expanded, comprising approximately 60% of the total kinases (Zulawski et al., 2014). In Arabidopsis, more than 600 different RLKs exist (Shiu and Bleecker, 2003). RLKs are transmembrane proteins typically located at the plasma membrane, with the N-terminal signal perception domain residing in the apoplast and the C-terminal kinase domain extending into the cytoplasm. This orientation permits the sensing of extracellular stimuli and subsequent transmission of the signal into the cell, via the kinase activity or other protein interactions.

The RLKs can be further divided into subgroups based on the composition of their extracellular domain and kinase domain phylogeny (Shiu and Bleecker, 2001). Some well-known examples of RLK subfamilies related to stress responses are: the leucine-rich repeat (LRR) RLKs which typically bind protein or peptide ligands (Couto and Zipfel, 2016), including the MAMP receptors FLAGELLIN-SENSITIVE 2 (FLS2) (Chinchilla et al., 2006; Felix et al., 1999; Gómez-Gómez and Boller, 2000) and EF-TU RECEPTOR (EFR) (Zipfel et al., 2006); the lysin motif (LysM) RLKs which bind carbohydrate-based ligands (Couto and Zipfel, 2016), including LysM-CONTAINING RECEPTOR KINASE 5 (LYK5)(Cao et al., 2014; Wan et al., 2008) and CHITIN ELICITOR RECEPTOR KINASE 1 (CERK1) (Miya et al., 2007); and the lectin RLKs (LecRLKs) which bind carbohydrate ligands (Bellande et al., 2017; Liu et al., 2018). The RLK family also includes the receptor-like cytoplasmic kinases (RLCKs), most notably BOTRITIS-INDUCED KINASE 1 (BIK1) (Couto and Zipfel, 2016), which lack the extracellular and transmembrane domains (Shiu and Bleecker, 2001).

The large numbers of RLKs allows for diverse receptor functions. This diversity, combined with the potential for interaction and signalling crosstalk, suggests RLKs could facilitate responses to a vast range of stimuli. Accordingly, RLKs are known to regulate various aspects of growth, development, and stress responses (Kimura et al., 2017).

1.2.1.4 Cysteine-rich receptor-like kinases

The cysteine-rich receptor-like kinases (CRKs) comprise a subgroup of RLKs, which has 44 members in Arabidopsis (Wrzaczek et al., 2010). CRKs are defined by their extracellular domain, which contains two copies of the domain of unknown function 26 (DUF26) configuration of conserved cysteines C-X8-C-X2-C (Chen, 2001; Vaattovaara et al., 2019). The CRKs can be further classified as belonging to either the basal or variable groups of DUF26-containing proteins (Vaattovaara et al., 2019). The basal group proteins show higher conservation amongst each other, and

representative proteins from all vasculature plants can be found in this group. In contrast, the variable group proteins show a lower degree of conservation and contain many lineage specific expansions (Vaattovaara et al., 2019). Therefore, identification of orthologs and transfer of knowledge between species is more promising with basal group CRKs.

Figure 2. Schematic representation of the general domain structure of CRKs.

Evidence from CRK expression profiles (Lehti-Shiu et al., 2009; Wrzaczek et al., 2010) suggests that the CRKs are likely involved in the signalling responses to both abiotic and biotic stress. Some CRKs have been linked to ROS signalling (Idänheimo et al., 2014) and cell death (Burdiak et al., 2015; Yadeta et al., 2017), and also more broadly to salt stress (Zhang et al., 2013b), immunity (Yeh et al., 2015; Zhang et al., 2013a), and ABA signalling (Tanaka et al., 2012; Zhang et al., 2013b). However, the functions on a cellular and biochemical level remain largely uncharacterized for the majority of CRKs.

1.3 Cellular responses to stimuli perception

Following the initial perception event of a stimulus, cells may then undergo a number of changes in order to properly respond and adapt to that stimulus. The earliest cellular responses often include small molecule messengers, such as Ca^{2+}, ROS, or lipids, which can be induced rapidly and coupled to various downstream signalling events. Since these messengers are common to numerous organisms and responses, the specificity likely arises from the precise timing, duration, amplitude, location, and cellular context, including cell type, priming, or developmental stage (Bickerton et al., 2016; Lodish et al., 2000; McAinsh and Hetherington, 1998; McAinsh and Pittman, 2009; Vaahtera et al., 2014). Further downstream signalling events can include activation of kinases and other enzymes; these may directly lead to physiological changes in the cell, or to transcriptional changes. Many stresses affecting plants induce the same or similar symptoms and responses, despite differences in the initial stimulus, and they may share some of the same signalling components, networks, and cellular responses (Tsuda and Katagiri, 2010).

1.3.1 Ca^{2+} elevation

Calcium signalling is critical for a large number of processes in eukaryotic cells, and has recently been recognized as important also in prokaryotic cells (Domínguez, Buchholz, and Behringer, 2018; Domínguez et al., 2015). The Ca^{2+} ion is a rapidly induced second messenger capable of regulating numerous downstream proteins and signalling events. Ca^{2+}concentrations are tightly controlled in various compartments

of the cell, with changes usually indicating perception of a stimulus or other need for a cellular response (Lecourieux et al., 2006; Maintz et al., 2014; McAinsh and Pittman, 2009). In plants, cytoplasmic concentrations of Ca^{2+} are usually kept low under normal conditions. Increased cytoplasmic Ca^{2+} concentrations are triggered following perception of a stimulus, due to either influx from the apoplast or release from internal stores such as the vacuole or endoplasmic reticulum (McAinsh and Pittman, 2009). Current knowledge indicates that Ca^{2+} increases follow perception of various abiotic and biotic stresses, including salt (Kiegle et al., 2000; Knight et al., 1997; Tracy et al., 2008), drought (Kiegle et al., 2000; Knight et al., 1998, 1997), cold (Allen et al., 2000; Kiegle et al., 2000; Knight et al., 1991), pathogens (Blume et al., 2000; Keinath et al., 2015; Lecourieux et al., 2005; Thor and Peiter, 2014), wounding (Kiep et al., 2015), and herbivory (Kiep et al., 2015; Maffei et al., 2004; Verrillo et al., 2014; Vincent et al., 2017).

1.3.2 ROS production

ROS are another class of molecules which have diverse roles as signalling molecules, and can be produced rapidly in response to stimuli. The term ROS includes singlet oxygen ($^1O_2$), the superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($HO^{\bullet}$). ROS signalling is important for several developmental processes, such as cell expansion (Mangano et al., 2016; Schmidt et al., 2016), casparian strip formation (Lee et al., 2013), pollen tube growth (Potocký et al., 2007), and root hair elongation (Foreman et al., 2003). ROS production has also been associated with various abiotic and biotic stress responses, including salt, drought, wounding, and pathogens (Choudhury et al., 2017; Kovtun et al., 2000; Lamb and Dixon, 1997; Noctor et al., 2014; Nxele et al., 2017; Orozco-Cardenas and Ryan, 1999).

ROS are produced in several subcellular compartments, including chloroplasts, mitochondria, peroxisomes, and the apoplast (Waszczak et al., 2018). Intracellular ROS production is largely due to photorespiration and metabolic processes, whereas extracellular ROS is produced in the apoplast through the action of cell wall peroxidases and the RBOH plant plasma membrane NADPH oxidases (Kärkönen and Kuchitsu, 2015; Kimura et al., 2017; Waszczak et al., 2018). Ten RBOHs have been identified in Arabidopsis, with differences in expression, location, and ROS-producing activity (Kaya et al., 2019; Morales et al., 2016). RBOHD and RBOHF in particular are known to be involved in stress responses (Kimura et al., 2017). RBOHs contain several transmembrane regions with regulatory domains in the N- and C- termini, both of which reside in the cytoplasm (Kärkönen and Kuchitsu, 2015). Regulation of RBOH activity can involve both Ca^{2+}, through the two EF hand motifs, and phosphorylation (Kaya et al., 2014; Kimura et al., 2012; Ogasawara et al., 2008), as well as G-proteins (Liang et al., 2016), S-nitrosylation (Yun et al., 2011), and phosphatidic acid (Zhang et al., 2009).

As mentioned earlier, RLKs are primarily responsible for sensing extracellular cues in plant cells. A ROS burst has been observed following signal perception for numerous RLKs, indicating that ROS production is likely an important component of the general RLK signalling mechanism (Couto and Zipfel, 2016; Kimura et al., 2017). In addition to upstream regulation, RLKs, and especially the subgroup of RLCKs, could potentially directly regulate RBOHs by phosphorylation (Kimura et al., 2017); this has been shown in the case of the RLCK BIK1, which is known to phosphorylate and activate RBOHD (Kadota et al., 2014).

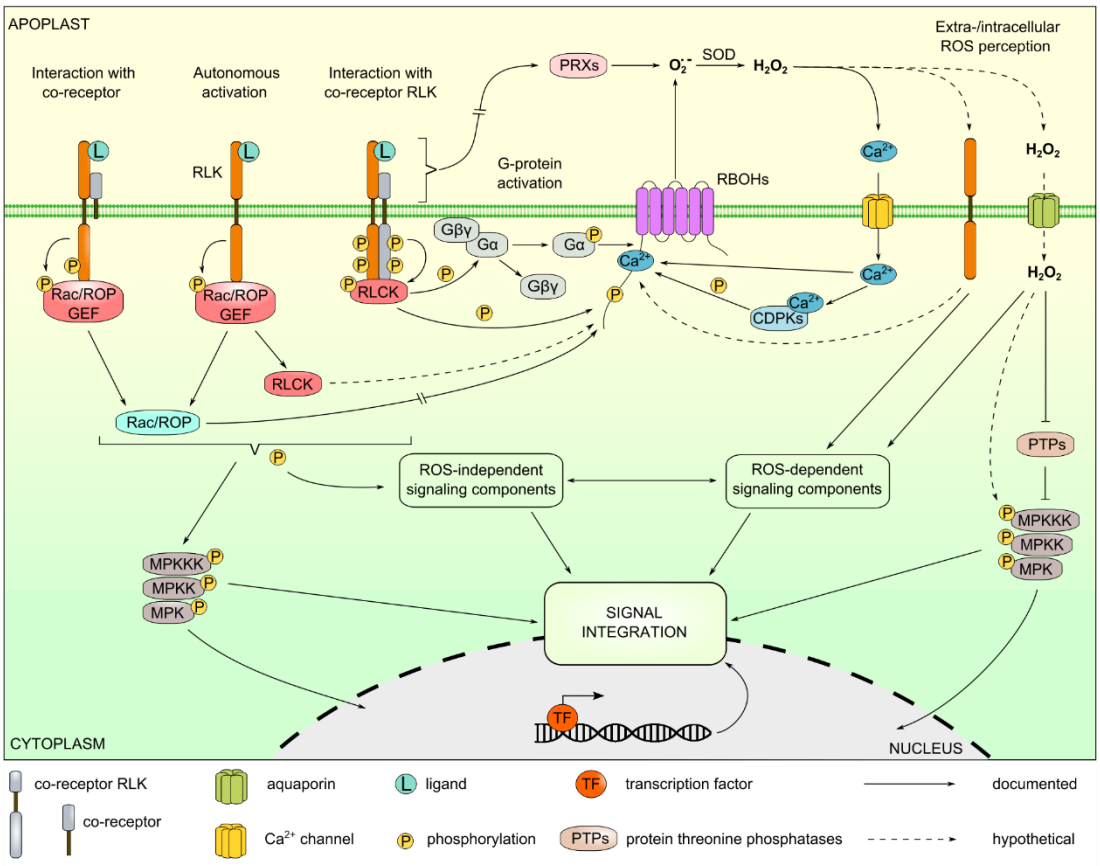

Figure 3. Involvement of ROS and Ca^{2+} during the perception of environmental stimuli and subsequent signal transmission. Modified from Kimura et al., 2017.

1.3.3 Ca^{2+} and ROS in long distance signalling

In addition to its local role in individual cells, Ca^{2+} is also important for long distance systemic signalling in plants. While a stress may first be perceived by or affect only a small number of cells, in many cases multiple cells or even the whole plant may require an appropriate reaction in order to properly adapt to the stress. Changes in membrane potential, ion concentrations, and ROS levels have been proposed as long-distance signals in plants, in addition to hormones and other small molecules (Choi et al., 2016). It was shown that salt-induced Ca^{2+} increases were elicited not only local to the site of application, but also in more distal parts of the

plant. The spread of Ca^{2+} signal propagated away from the site of stress in a wave-like manner and at a rapid rate, crossing several cells per second (Choi et al., 2014). ROS signalling is closely intertwined with Ca^{2+} signalling, and often a ROS burst and cytoplasmic Ca^{2+} elevation occur concurrently in response to a stimulus. Ca^{2+} is required for RBOH activation, and apoplastic ROS triggers Ca^{2+} influx, in a positive feedback loop relationship (Choi et al., 2016). Ca^{2+} and ROS have also been proposed to work together during long distance systemic signalling, where a symplastic Ca^{2+} signal and apoplastic ROS signal would propagate together from cell-to-cell in a wave-like pattern (Choi et al., 2016, 2014).

1.3.4 Lipid signalling

Lipids form the structural basis of cellular membranes, and can also act as signalling molecules. In the basal state, without stimulation from either environmental or developmental cues, cells generally have low amounts of signalling lipids. Upon stimulation, these molecules are produced from pre-existing membrane lipids, or other intermediates, through the action of phospholipases and esterases (Okazaki and Saito, 2014). Phospholipases can be classified based on the site of cleavage in their phospholipid substrates: phospholipase A_1 and A_2 cleave at the SN-1 or SN-2 acyl chain respectively, producing arachidonic acid; phospholipase B cleaves both acyl chains; phospholipase C cleaves before the head group phosphate, producing diacylglycerol and inositol triphosphate; phospholipase D cleaves after the head group phosphate, producing phosphatidic acid and an alcohol (Munnik and Testerink, 2009).

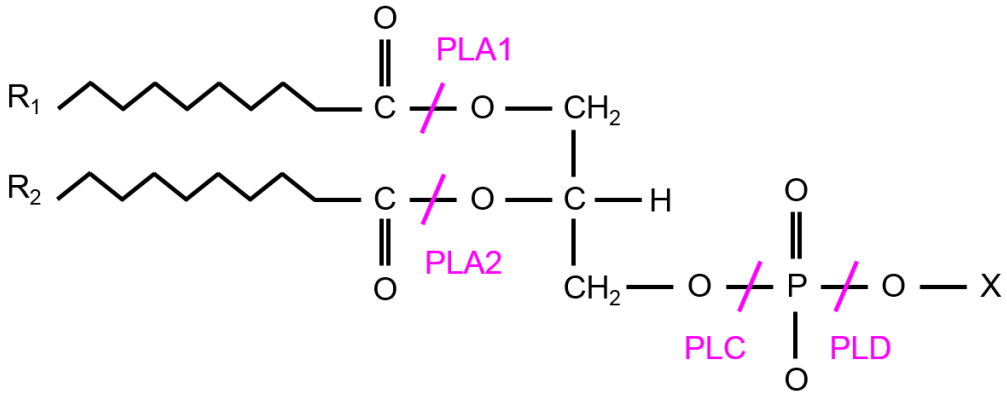

Figure 4. Hydrolysis of phospholipids by phospholipases. Enzymes and their respective cleavage sites are shown in pink.

The following lipid classes have been identified as signalling lipids in response to stimuli: lysophospholipid, fatty acid, phosphatidic acid, inositol phosphate, diacylglycerol, oxylipin, sphingolipid, and N-acylethanolamine (Kang and Weylandt, 2008; Kilaru et al., 2011; Markham et al., 2013; Munnik and Testerink, 2009; Okazaki and Saito, 2014; Wang et al., 2006). In addition to acting as

signalling molecules themselves, lipids also play a role in stress tolerance by modulating cellular responses through their structural properties. Lipid remodelling occurs during the response to various stresses and is essential for maintaining membrane integrity, as well as contributing to microdomain formation and the function and localization of membrane proteins (Faraudo and Travesset, 2007; Okazaki and Saito, 2014; Zhao et al., 2017). The deposition of wax and cutin at plant surfaces is another example of a stress tolerance mechanism involving lipids (Okazaki and Saito, 2014).

1.3.4.1 Phospholipase D

The phospholipase D (PLD) enzymes hydrolyze phospholipids to produce phosphatidic acid (PA) and a free head group. Plant PLDs can be divided into two subfamilies based on their domain structure: C2-PLDs contain the C2 Ca^{2+}-binding domain and require Ca^{2+} for their activity; PX/PH-PLDs contain the phox homology (PX) and plekstrin homology (PH) phosphoinositide-interacting domains, and are Ca^{2+}-independent (Wang, 2005). Arabidopsis contains twelve PLD genes, with PLDα1 being the major isoform (Pappan et al., 1997a, 1997b; Qin and Wang, 2002; Qin et al., 1997; Wang and Wang, 2001)). PLDα(1-3), β(1,2), γ(1-3), δ, and ε proteins belong to the C2 subfamily, and PLDζ(1-2) proteins belongs to the PX/PH subfamily (Hong et al., 2016; Wang, 2005). In addition to differences in Ca^{2+} requirements, the PLDs also have differential requirements for phosphatidylinositol 4,5-bisphosphate (PIP_2), oleate, and pH (Hong et al., 2016). In contrast to many PLDs, PLDα does not require PIP_2 for activity (Hong et al., 2016; Qin and Wang, 2002). C2-PLDs, including PLDα1, can use phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylglycerol (PG) as substrates, whereas PX/PH-PLDs utilize only PC (Hong et al., 2016).

On a cellular level, PLD and its product PA have been linked to a wide range of cellular processes in eukaryotic cells. These include endocytosis and vesicle trafficking (Koch et al., 2003; Lee et al., 2006; Shen et al., 2001; Thakur et al., 2016), membrane composition and microdomains (Faraudo and Travesset, 2007), and microtubule and cytoskeletal dynamics (Zhang et al., 2017, 2012). PLD plays a role in multiple plant stress responses (Hong et al., 2016; Testerink and Munnik, 2005), including those to salt (Bargmann et al., 2009; Hong et al., 2010; Katagiri et al., 2001), drought (Bargmann et al., 2009; Hong et al., 2010), hyperosmotic (Hong et al., 2008), cold (Li et al., 2004; Wang et al., 2006), wounding (Bargmann et al., 2009; Wang et al., 2000), and pathogens (Hong et al., 2016; Pinosa et al., 2013; Zhao et al., 2013). However, while PLD activity was identified as important for these responses, in many cases the downstream targets and effectors are still unknown.

1.4 Plasmodesmata and intercellular signalling

Signalling between plant cells can occur either via extracellular signals in the apoplast, or through symplastic intercellular connections called plasmodesmata. Plasmodesmata are specialized channels between neighboring cells, where the plasma membrane, cytoplasm, and endoplasmic reticulum extend through from cell-to-cell. The degree to which these channels are open or closed can be dynamically regulated by the addition or removal of callose deposits in the surrounding cell wall (De Storme and Geelen, 2014). Most small soluble molecules, such as water, ions, sugars, and metabolites, are expected to able to cross through plasmodesmata passively (Cheval and Faulkner, 2018; De Storme and Geelen, 2014). Proteins, RNA, and other large molecules can also move between cells through plasmodesmata, however this likely involves some kind of active transport mechanism (Cheval and Faulkner, 2018; Kragler, 2013).

Due to the vast range of molecules potentially able to pass through plasmodesmata, plasmodesmal trafficking must be tightly controlled to ensure proper communication (Tilsner et al., 2016). Plasmodesmata are essential for plant development and growth, and current knowledge indicates that mutants lacking functional plasmodesmata cannot survive (Kobayashi et al., 2007; Stonebloom et al., 2009; Tilsner et al., 2016). Plasmodesmal regulation is also important during abiotic and biotic stress responses (Tilsner et al., 2016).

1.4.1 Callose deposition

Callose deposition provides the major mechanism for controlling plasmodesmal aperture, and therefore the size and type of molecules which can pass through (De Storme and Geelen, 2014; Guseman et al., 2010; Levy et al., 2007a, 2007b; Vatén et al., 2011). When callose is deposited at the neck region of plasmodesmata it causes the inner size and permeability of the channel to decrease (De Storme and Geelen, 2014). Callose (β-1,3-glucan) is a polysaccharide derived from glucose found only in embryophytes, or land plants (De Storme and Geelen, 2014). The deposition of callose at plasmodesmata is a dynamic process which is regulated by callose synthases (CALS), which synthesize callose, and β-1,3-glucanases, which degrade callose (De Storme and Geelen, 2014). The Arabidopsis genome encodes twelve known callose synthases (CALS1-12) and fifty β-1,3-glucanases (Tilsner et al., 2016). There may exist some degree of specificity as to which callose synthases are expressed and/or active in different cells and tissues, or under different stress conditions (Amsbury et al., 2017).

1.4.2 Plasmodesmata and stress

While plasmodesmata are recognized as an important part of various stress responses, the regulation of callose deposition in response to stress stimuli is not

fully understood. However, it is generally thought that cells tend to close their plasmodesmata in response to stress, restricting the flow of molecules and isolating cells (Cui and Lee, 2016; O'Lexy et al., 2018; Tilsner et al., 2016). Changes in plasmodesmal permeability or callose deposition have been observed in response to pathogens (Faulkner et al., 2013; Lee et al., 2011), osmotic stress (Xie et al., 2012), and heavy metal stress (O'Lexy et al., 2018). In some cases, ROS and salicylic acid (SA) have been identified as regulatory components (Cui and Lee, 2016). Plasmodesmata are important for immunity, from both the perspective of the plant and of the pathogen. Upon infection, plants can notify and initiate defense responses in distant cells via this intercellular communication system. On the other hand, viruses can use plasmodesmata to move between cells, as can effector molecules from bacteria or fungi, spreading the infection to new cells (Cheval and Faulkner, 2018; Tilsner et al., 2016). Therefore, the involvement of plasmodesmata during immunity is complicated and may involve both closure and opening of plasmodesmata, depending on the stage of infection and other factors (Cheval and Faulkner, 2018).

It has been proposed that plasmodesmata-localized receptor proteins are involved in the regulation of stimulus-dependent callose deposition (Amsbury et al., 2017; Faulkner, 2013). One example where this has already been described is the salicylic acid (SA)-dependent regulation of CALS1 by PLASMODESMATA-LOCALIZED PROTEIN 5 (PDLP5) (Cui and Lee, 2016). Further characterization of other plasmodesmata-localized proteins is necessary in order to fully understand the regulation of stimulus-dependent callose deposition, and its significance for stress responses and tolerance.

2. AIMS OF THE STUDY

This dissertation explores the significance of the CRK gene family during stress responses in *Arabidopsis thaliana*, with a focus on CRK2. The specific aims were:

1) Investigation of the overall roles of the CRK gene family in *Arabidopsis thaliana* by large scale phenotyping of a T-DNA mutant collection.
2) Characterization of CRK2 functions during abiotic and biotic stress, including protein interactions, cellular responses, and biochemical activities.
3) Characterization of CRK2 subcellular localization in relation to protein function, and potential changes in localization in response to stress stimuli.

3. MATERIALS AND METHODS

The materials and methods used in this dissertation are described in detail in publications I, II, and III. A summary of the methods used and the publication(s) in which they appear is provided in Table 1. Methods for the unpublished work included in this dissertation are described below.

Table 1. Methods used in this dissertation. Brackets indicate experiments performed by co-authors in the respective publication.

Method	**Publication**
Growth conditions	I, II, III
Plant lines and constructs	I, II, III
Genotyping and RT-PCR	I, II
Transient expression in *Nicotiana benthamiana*	II
Transient transformation of Arabidopsis seedlings	II
Plant size and weight	I, (III)
Water loss	I
Immunoprecipitation of protein complexes	II
Mass spectrometry	(II)
Germination assay	II
Root length assay	II
Western blot	II, III
In vitro kinase assay	II, (III)
Subcellular protein localization	II
Callose staining	II, III
DANS assay for plasmodesmata permeability	II
Calcium imaging, cell level	II
Calcium imaging, tissue level	(II), (III)
HEK293T cell culture and transfection	(III)
ROS measurements	(III)
Protein extraction and co-immunoprecipitation	(III)
In vitro phosphorylation site identification	(III)

Vesicle visualization

Seven-day-old seedlings were transferred to 12 well plates for treatments. The conditions for all treatments are described in detail in paper II. Cells were loaded with 5 µM fluorescein diacetate (Thermo Fisher Scientific) for 15 min in darkness, washed, and mounted in water for immediate imaging. Fluorescent images were obtained with Leica TCS SP5 II HCS confocal microscope using standard GFP settings of 488 nm excitation and a detection range of 500–600 nm. Vesicles were quantified from each image area (150 µm2) with ImageJ using the Analyze Particles function with the following parameters: binary image, minimum area 2.0 µm2, circularity 0.20–1.00, exclude on edges. In the NaCl-treated *crk2* samples the number of vesicles exceeded what could be separated visually; therefore these counts

were set to 100 to represent the maximum. Statistical significance was determined by one-way ANOVA with post hoc Tukey HSD using JMP Pro 13. Replicates are as indicated in the figure legends.

FM4-64 staining

Seven-day-old seedlings were transferred to 12 well plates for treatments, either to untreated water or 150 mM NaCl for 30 min. Cells were loaded with 5μM FM4-64 for 20 min in darkness. Fluorescent images were obtained with Leica TCS SP5 II HCS confocal microscope using standard RFP settings of 561 nm excitation and a detection range of 560–600 nm.

Localization of endocytosis markers

Endocytosis markers were transiently expressed in Arabidopsis seedlings via co-cultivation with Agrobacteria, as described in paper II. Plasmids for the endocytosis markers RabF2b–YFP (W2Y) and VTI12–YFP (W13Y) have been previously described (Geldner et al., 2009) and were obtained from the authors. Fluorescent images were obtained with Leica TCS SP5 II HCS confocal microscope using standard YFP settings of 514 nm excitation and a detection range of 525–590 nm.

4. RESULTS AND DISCUSSION

4.1 The CRKs are involved in stress responses and development

The CRKs comprise one of the largest groups of RLKs in plants; however, until recently, they remained largely uncharacterized. Previous studies revealed changes in several *CRK* expression profiles in response to ozone, with the majority of the *CRKs* showing increased expression (Wrzaczek et al., 2010). Ozone induces apoplastic ROS production, in a similar manner to the situation triggered by various abiotic and biotic stress stimuli, thereby suggesting that the CRKs may be involved in stress responses and ROS signalling (Wrzaczek et al., 2010).

A collection of loss-of-function T-DNA mutants was obtained for all available CRKs, and large-scale phenotyping of this mutant collection was carried out by several research groups across various areas of expertise to determine the physiological roles of this protein family (I). This suggested physiological roles for the CRKs in development, abiotic stress, biotic stress, and stomatal function (I). On a molecular level, it suggests the CRKs are involved in ROS signalling. CRKs may themselves be either regulated by ROS or involved in the perception of ROS, or they may regulate downstream ROS production in response to stress stimuli (I). Many of the CRKs had different or even contrasting phenotypes, suggesting that individual CRKs may be relevant for specific processes (I). It also highlights a potential role for the CRK family to provide regulatory fine tuning across a wide range of cellular events involved in ROS signalling and stress responses (I).

4.1.1 CRK2 is an evolutionarily conserved CRK with central signalling functions

During the analysis of the CRK mutant collection, CRK2 emerged as a potentially interesting and important CRK for both development and stress responses. The *crk2* mutant has multiple strong phenotypes (I), and was also identified as a member of the basal group of CRKs (I) (Vaattovaara et al., 2019), making it valuable for evolutionary analysis.

One of the most striking phenotypes of the *crk2* mutant is its small size and delayed development (I). This dwarf phenotype can be restored by complementation with CRK2–YFP expressed under its native promoter or overexpressed under the 35S promoter (I, III). Expression of kinase-dead CRK2 protein variants cannot complement the dwarf phenotype, indicating that CRK2 kinase activity is important for proper plant development (III). Besides the small size, there are several other aspects of development altered in *crk2*. These include delayed bolting and flowering, and early senescence (I). The *crk2* mutant also has abnormal stomatal density, stomatal length, stomatal aperture ratios, and darkness-induced stomatal closure (I). Additionally, larger changes in fresh weight were observed when compared to Col-0

in water loss assays, indicating a higher degree of water loss in *crk2* (I); this could be restored to wild type by complementation with CRK2−YFP expressed under the 35S promoter (I).

In addition to its roles in plant development, CRK2 is also important for stress tolerance. Loss of CRK2 affects the ability of plants to respond to various abiotic and biotic stimuli. The *crk2* mutant exhibits decreased germination under high salt concentrations, increased susceptibility to ultraviolet light (UV-AB), light stress, and ozone (assessed by electrolyte leakage, as a measure of cell death), decreased flg22-induced ROS production, and decreased chitin-induced stomatal closure (I). Conversely, *crk2* showed increased resistance to the powdery mildew fungal pathogen *Golovinomyces orontii* (I).

CRK2 was identified as a member of the basal group of CRKs, which show a higher degree of conservation across species (I) (Vaattovaara et al., 2019). Therefore, CRK2 likely has more ancestral functions, whereas CRKs belonging to the variable group may be more involved in the specificity and fine-tuning of responses (Vaattovaara et al., 2019). Combined with its strong loss-of-function phenotypes, this further supports the hypothesis that CRK2 is one of the more essential CRKs, and that it could be an important regulator during multiple stress responses and developmental processes.

4.1.1.1 CRK2 is an active kinase

CRK2 contains all of the conserved motifs found in a typical kinase domain (Kornev et al., 2006; Stone and Walker, 1995) and was therefore predicted to be an active kinase. By using an *in vitro* kinase assay we were able to demonstrate that the cytosolic region of CRK2 has kinase activity and is capable of both autophosphorylation and trans-phosphorylation of a generic kinase substrate (II). We also produced two kinase-dead versions of CRK2, harbouring point mutations designed to disrupt the motifs required for kinase activity. The K353E mutation disables the ATP-binding site, and the D450N mutation disables the catalytic core. Neither of the kinase-dead versions of CRK2 exhibited kinase activity *in vitro* (II). Having now established that CRK2 is an active kinase, and that this activity is absent in the kinase-dead versions, these tools were then used in future experiments to assess the requirement of kinase activity for each of CRK2's cellular functions.

4.2 CRK2 enhances salt tolerance

The results of the CRK mutant collection phenotyping indicated that CRK2 is involved in the response to salt stress (I), and we chose this aspect of CRK2 for further investigation. We assessed salt tolerance by percentage of germination on salt-containing media, and confirmed that CRK2 enhances salt tolerance (II). The *crk2* mutant is more salt-sensitive at the germination stage, whereas plants

overexpressing CRK2−YFP (35S::CRK2−YFP_9-3, in the Col-0 background) have a higher salt tolerance when compared to Col-0 (II). The germination defect of *crk2* can be restored to wild type by complementation with CRK2−YFP expressed under its native promoter (pCRK2::CRK2−YFP_1-22 and 1-17, in the *crk2* background) (II). Expression of kinase-dead variants of CRK2−YFP (35S::CRK2^{K353E}−YFP and 35S::CRK2^{D450N}−YFP, in the *crk2* background) could not complement the germination defect of *crk2*, indicating that the salt tolerance conferred by CRK2 is dependent on kinase activity (II).

Root length and morphology is another feature which, in addition to germination rate, is commonly studied in the context of salt stress (Bayazid et al., 2016; Julkowska et al., 2014; Kawa et al., 2016). We found that CRK2 has an effect on root length, under both normal and high salt conditions, and that this is dependent on kinase activity (II). Both kinase-dead CRK2−YFP lines had significantly shorter roots compared to Col-0 when transferred to salt-containing media; normal root length was restored by expression of CRK2−YFP under its native promoter or the 35S promoter (II). Thus, CRK2 likely acts on cellular processes involved in multiple aspects of salt tolerance.

4.2.1 CRK2 interacts with other proteins involved in salt tolerance

Having ascertained that CRK2 is involved in conferring salt tolerance, the next step was to characterize its specific functions and protein interactions on a cellular and biochemical level. RLKs frequently carry out their signalling functions as part of protein complexes (Kimura et al., 2017). Therefore, as a starting point for the characterization of CRK2 protein function, we carried out a proteomics screen to identify proteins interacting with CRK2 (II). Several of the top identified interactors have been previously linked to salt stress, including aquaporins (Bhardwaj et al., 2013), ATPases (Janicka-Russak and Kabała, 2015), and PLDα1 (Bargmann et al., 2009) (II). Three callose synthases were also identified (II), raising an interesting question about the involvement of callose and plasmodesmata during salt stress. Callose deposition has not yet been explicitly documented as a response to high salinity, however it is a common feature of several other stress responses, including osmotic stress (Xie et al., 2012), pathogen infection (Cui and Lee, 2016; Felix et al., 1999; Gómez-Gómez and Boller, 2000; Jacobs et al., 2003), wounding (Cui and Lee, 2016), and heavy metal toxicity (O'Lexy et al., 2018), and it is therefore conceivable that it might also be important during salt stress.

4.2.2 CRK2 regulates salt-induced callose deposition and plasmodesmal permeability

We revealed a novel role for callose by demonstrating that callose deposition occurs in response to acute salt stress and is important for salt tolerance (II). This is mediated at least in part by CALS1 (II), which is one of the callose synthases found

to interact with CRK2 (II). In further support of this interaction, functional CRK2 is required for the salt-induced callose response (II). The *crk2* and kinase-dead lines do not show increased callose deposition following salt treatment, whereas overexpression of CRK2–YFP results in an amplified callose response to salt, suggesting that CRK2 is a positive regulator of salt-induced callose deposition and highlighting CRK2 kinase activity as an important aspect of this response (II). We also showed that the observed callose deposition correlated with changes in plasmodesmal permeability, and thus has a potential relevance for intercellular communication (II). CRK2 is able to phosphorylate CALS1 *in vitro* and therefore could possibly directly regulate CALS1 by phosphorylation (II). Further investigation of the interaction between CRK2 and CALS1, including the conditions under which is occurs, as well as identification of phosphorylation sites is a promising topic for future work.

CRK2 also had an effect on callose deposition induced by flg22, a MAMP from bacterial flagella, however the effect was opposite to that observed under salt stress (III). Callose deposition in response to pathogens, or elicitors such as flg22, has already been characterized (Cheval and Faulkner, 2018). Here we showed that following flg22 treatment, *crk2* responded with excessive callose deposition (III). By contrast, the overexpression of CRK2–YFP resulted in little to no callose deposition in response to flg22 (unpublished results). These opposing results suggest that CRK2 might interact with different callose synthases during abiotic and biotic stress conditions, and that CRK2's regulation of callose deposition is specific to the type of stimulus.

4.2.3 CRK2 regulates formation of an unknown vesicle type

Increased endocytosis and vesicle trafficking is a well characterized aspect of the cellular response to salt stress (Baral et al., 2015). Several receptors and other membrane proteins are known to internalize following treatment with NaCl, including multiple aquaporins and RBOHD (Hao et al., 2014; Li et al., 2011; Luu et al., 2011; Ueda et al., 2016). Based on the Dyngo-4a results it appears CRK2–YFP itself does not undergo internalization (II), however it could still be involved in regulating this process. Thus we investigated whether CRK2 has an effect on vesicle trafficking during salt stress.

Fluorescein diacetate is a non-fluorescent membrane permeable esterase substrate, which upon entry into cells is converted to the membrane-impermeable fluorescent derivative fluorescein. Using this approach enabled visualization of total dye uptake by the cells, as well as vesicle formation within cells. In untreated seedlings, CRK2–YFP overexpression resulted in a significant decrease in the number of vesicles compared to Col-0, whereas the *crk2* mutant showed an increased number of vesicles (Fig. 5A and B). Overexpression of both kinase-dead variants of CRK2 did not yield a significantly different phenotype from overexpression of the native

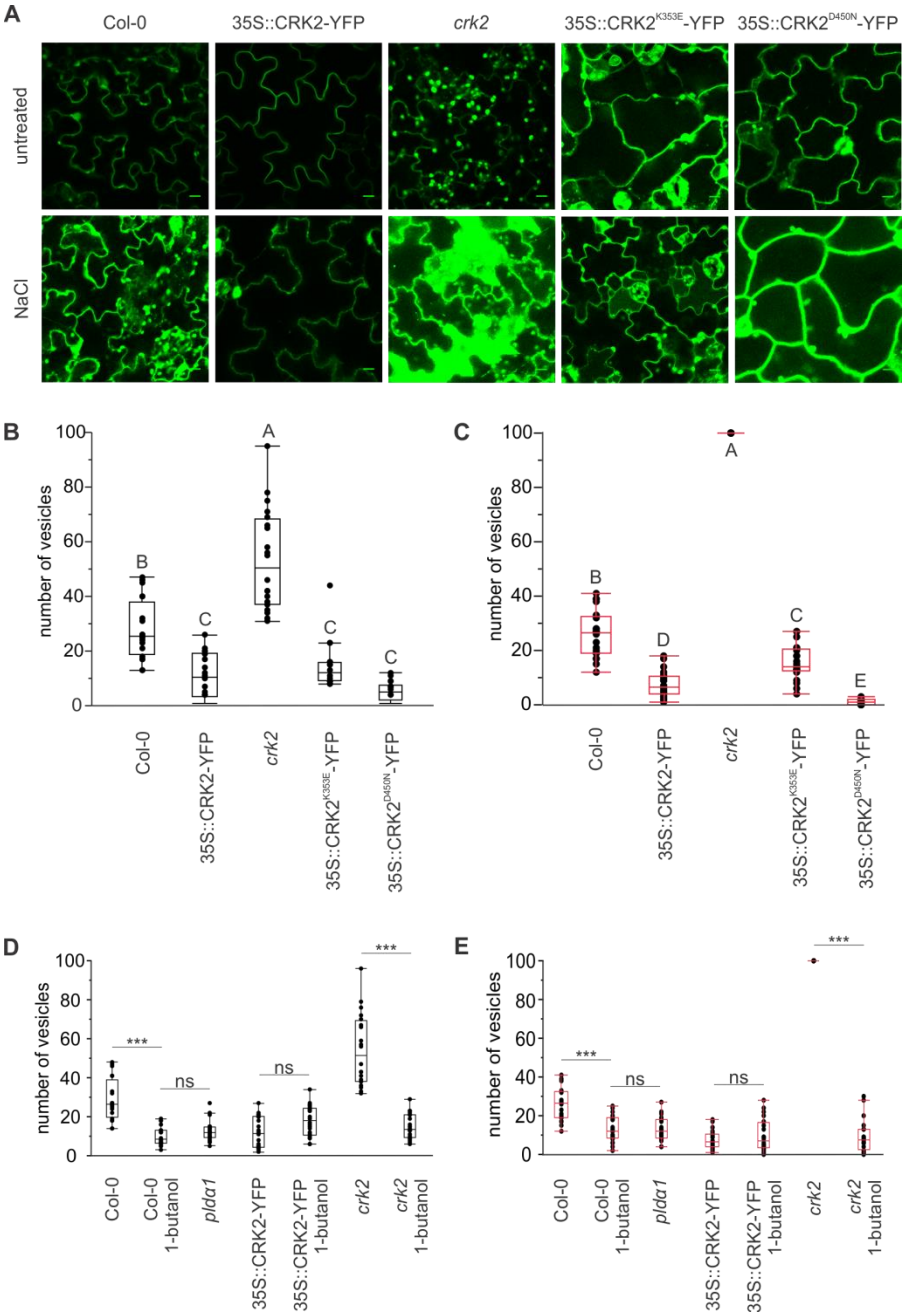

Figure 5. CRK2 negatively regulates formation of an unknown vesicle type. (A) CRK2 expression levels are inversely related to the presence of large intracellular vesicles. Visualization of vesicles using fluorescein diacetate. Scale bar = 10 µm. (B-C) Quantification of number of vesicles in untreated (B) and NaCl treated (C) samples; increased CRK2 results in decreased vesicle formation. Comparisons are made among all lines; different letters indicate significant differences at $P < 0.05$ (one-way ANOVA, post hoc Tukey HSD). (D-E) Impact of PLD activity on vesicle formation in CRK2 lines; quantification of number of vesicles in untreated (D) and NaCl-treated (E) samples, when pre-treated with 1-butanol. Comparisons are between untreated and 1-butanol-treated samples for each line; ns not significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (one-way ANOVA, post hoc Tukey HSD). Seven-day-old seedlings; n = at least 18.

protein (Fig. 5A and B). In NaCl-treated seedlings, the same overall pattern was observed as in the untreated seedlings, with CRK2 overexpression yielding significantly less vesicles than Col-0, and *crk2* having significantly more vesicles

(Fig. 5A and C). The amplitude of the differences between lines was more obvious after salt treatment and now all five lines significantly differed from each other. However, the kinase-dead lines still resembled more closely the native CRK2 overexpression line rather than Col-0 or *crk2* (Fig. 5A and C). These observations suggest that CRK2 is a negative regulator of vesicle formation under both basal and salt stress conditions, and that kinase activity is not required for this function. Thus, we speculate that CRK2 has a phosphorylation-independent function in the vesicle formation machinery, possibly related to scaffolding or protein sequestration.

The vesicles were initially considered to be products of endocytosis. However, FM4-64 staining (Fig. 6A) and co-localization with endocytosis markers (Fig. 6B) showed that this was not the case, and no differences were observed between Col-0 and *crk2* in either of these experiments. It is also possible that some of the structures observed may be plastids. However, Dyngo-4a treatment decreased and nearly eliminated the vesicles in all three lines, indicating these vesicles are likely still mediated by a clathrin-dependent process (Fig. 6C). There was also no significant difference between the Dyngo-4a-treated lines and the untreated CRK2 overexpression line (Fig 6C), further supporting a role for CRK2 as a negative regulator of vesicle formation.

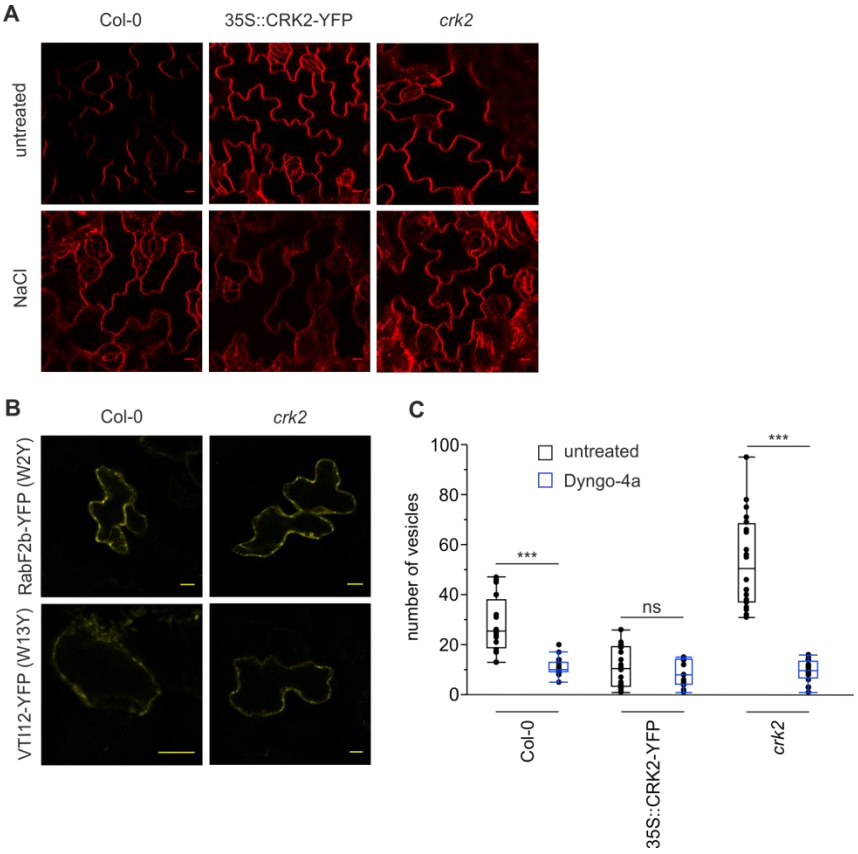

Figure 6. The unknown vesicles are not derived from endocytosis, but are clathrin-dependent. (A) Visualization of the plasma membrane and endocytotic vesicles with FM4-64. (B) Expression of endocytosis markers does not show the presence of the large vesicles observed in Figure 5. (A-B) Seven-day-old seedlings; n = at least 3. Scale bar = 10 μm. (C) Dyngo-4a strongly reduces the number

of vesicles. Quantification of number of vesicles; comparisons are between untreated and Dyngo-4a-treated samples for each line; ns not significant, * P < 0.05, ** P < 0.01, *** P < 0.001 (one-way ANOVA, post hoc Tukey HSD). Seven-day-old seedlings; n = at least 18.

We also investigated if PLD was required for vesicle formation, since PLD is a known regulator of clathrin-mediated endocytosis and vesicle trafficking in eukaryotes (Koch et al., 2003; Lee et al., 2006; Shen et al., 2001; Thakur et al., 2016). We assessed the involvement of PLD in relation to CRK2 using 1-butanol to inhibit PA production. We first confirmed that 1-butanol inhibition is an appropriate alternative to eliminating PLD protein, by demonstrating that Col-0 treated with 1-butanol shows the same phenotype as the *pldα1* mutant, under both basal conditions and following NaCl treatment (Fig. 5D and E). Inhibition of PLD greatly reduced the number of vesicles observed under both basal (Fig. 5D) and NaCl-treated (Fig. 5E) conditions in all lines, reinforcing that PLD is a positive regulator of vesicle formation. There was no significant difference between the 1-butanol-treated lines and the untreated CRK2 overexpression line (Fig. 5D and E), which suggests that PLD and CRK2 have opposite effects on the regulation of vesicle formation. However, loss of CRK2 cannot compensate for inhibition of PLD activity. This would suggest that either CRK2 is upstream of PLD in this pathway, or that they are acting in parallel. In both scenarios, the requirement for PLD activity appears to overrule the effects of CRK2.

CRK2 appears to be important for the regulation of vesicle formation not only during salt stress, but also under standard growth conditions. Accordingly, the dependency of this phenotype on PLD was also observed under both standard and salt conditions. Thus, we propose CRK2 is a constitutive negative regulator of formation of this vesicle type. While this regulation is also important for salt tolerance, it may not be linked directly with the other CRK2 functions and salt-induced localization changes.

4.3 CRK2 regulates RBOHD and ROS production

The phenotyping of CRK2 suggested that this protein is involved in ROS signalling (I). The results further indicated that CRK2 has an effect on ROS production in response to biotic stress, as the *crk2* mutant shows reduced flg22-induced ROS production (I, III). This was further investigated in paper III, which focuses on the interaction of CRK2 and RBOHD. RBOHD is the most notable RBOH with regards to stress-induced apoplastic ROS production (Couto and Zipfel, 2016; Kimura et al., 2017) and is known to be regulated by both phosphorylation and Ca^{2+} binding (Kadota et al., 2014; Kaya et al., 2019; Kimura et al., 2012; Ogasawara et al., 2008).

Co-immunoprecipitation assays demonstrated that CRK2 and RBOHD associate with each other *in planta*, under both untreated and flg22-treated conditions, and that they can directly interact with each other *in vitro* (III). We used the human embryonic kidney cell line HEK293T as an *in vitro* cellular system in which to measure ROS production. HEK293T cells have low endogenous extracellular ROS

production (Ogasawara et al., 2008), and are therefore uniquely suited for this purpose. Expression of RBOHD + CRK2 in HEK293T cells yielded elevated basal ROS production compared to the control RBOHD + GFP cells (III). The kinase-dead variants of CRK2 did not enhance ROS production when expressed with RBOHD, indicating that this effect is kinase-dependent (III). We also demonstrated that the effect of CRK2 on basal ROS production is independent of Ca^{2+} (III).

Since the kinase activity of CRK2 was important for its effect on ROS production, we tested the potential for direct phosphorylation. CRK2 was able to phosphorylate both the N- and C- termini of RBOHD *in vitro* (III). This is an intriguing result, given that previous research has focused predominantly on phosphorylation of the N-terminus for regulation of RBOHs. Regulation via the C-terminus has, however, been reported in human NADPH oxidases (Jagnandan et al., 2007; Raad et al., 2009), and therefore could also be important in plants. Two of the identified phosphorylation sites in the C-terminal region, S703 and S862, had an effect on ROS production in HEK293T cells (III). RBOHD protein containing a mutated version of S703 which is unable to be phosphorylated had lower basal ROS production in HEK293T cells, and phosphorylation of this site *in planta* was increased upon treatment with flg22, suggesting S703 as a positive regulatory site (III). Mutation of the S862 site on the other hand resulted in higher basal ROS production in HEK293T cells, suggesting it is a negative regulatory site (III). CRK2 may therefore regulate RBOHD differentially through multiple phosphorylation sites, to provide fine tuning of ROS production in response to various environmental stimuli.

4.4 CRK2 exhibits stress-specific patterns of subcellular localization

One means by which protein function can be regulated post-translationally is by modulation of subcellular localization within specific cellular compartments or domains. Several RLKs localize to specific plasma membrane microdomains, including FLS2 and BRI1 (Bücherl et al., 2017). We found that the subcellular localization of CRK2 is dependent upon environmental conditions, and observed highly specific localization patterns in response to both abiotic and biotic stimuli (II). CRK2 localizes evenly along the plasma membrane in epidermal pavement cells under normal growth conditions (II, III). Following osmotic or salt stress, CRK2 re-localizes into concentrated spots along the plasma membrane (II). The salt-triggered localization was confirmed to be at plasmodesmata, as assessed by co-localization of the CRK2 spots with the plasmodesmata marker protein PDLP5 (II). A different pattern of smaller, more frequent spots was observed following treatment with flg22, to mimic biotic stress, or H_2O_2, to raise the extracellular ROS concentration (II). These spots do not resemble plasmodesmata and instead likely represent some form of microdomain.

The kinase-dead CRK2 protein variants localize to the plasma membrane similarly to wild type CRK2 under control conditions, indicating that kinase activity is not

required for stable protein expression or localization to the plasma membrane (II). However, the stress-induced localization patterns of CRK2 require an active kinase domain (II). Using an inhibitor-based approach, we determined that the re-localization of CRK2 is also dependent on elevated cytosolic Ca^{2+}, enhanced by extracellular ROS production, and does not require clathrin-mediated endocytosis (II). While Ca^{2+} was required in all cases, and likely serves as the primary signal, extracellular ROS production was also required for re-localization upon flg22 treatment (II). Thus, in addition to the differences in localization patterns induced by abiotic and biotic stress, there appears to also be some differences in the mechanism. We therefore propose that CRK2 adopts stimulus-specific localization patterns, which influence its protein interactions and cellular functions during stress responses.

4.5 The subcellular localization and cellular functions of CRK2 are dependent on PLDα1 activity

PLDα1 was consistently identified as one of the top proteins interacting with CRK2 (II), and several of the cellular functions in which CRK2 was found to play a role have also been linked to PLDα1. The regulation of salt-induced callose deposition and vesicle formation by CRK2 requires PLD activity (II; Figure 4D and E), and the *crk2* and *plda1* mutants have similar phenotypes with regards to salt tolerance (II). In addition, the stress-induced re-localization of CRK2 is dependent on PLD activity (II).

Microdomains with specific plasma membrane properties offer a means of localizing proteins to a specific area or grouping proteins together, for example during formation of a signalling complex. Plasmodesmata contain specialized microdomains, which are necessary for the proper localization of several plasmodesmata-localized proteins (Grison et al., 2019; Nicolas et al., 2018). PLD can alter membrane properties and create microdomains through the formation of PA-rich areas. PA can affect membrane properties in several ways: it is negatively charged, has an increased binding capacity for divalent cations (Faraudo and Travesset, 2007), can directly act as a localization signal for PA binding proteins (Delon et al., 2004), and can induce membrane curvature (Kooijman et al., 2003), which is important for membrane budding and vesicle formation as well as protein localization (Zhao et al., 2017). PLD can also influence microtubules and cytoskeletal structure in response to stress (Jiang et al., 2014; Zhang et al., 2012). This could also be important for plasmodesmal permeability, plasmodesmal transport specificity, and distribution of molecules to plasmodesmata (White and Barton, 2011).

Therefore, PLDα1 could provide a mechanism for bringing together various components, such as CRK2 and CALS1, and for directing the stress-induced re-localization patterns of CRK2. The placement of PLDα1 upstream of CRK2 is

further supported by the observation that NaCl-induced callose deposition – and the effect of CRK2 on this process – is dependent on PLD activity, but basal callose deposition is not (II). We propose a model in which increased salinity triggers Ca^{2+} influx, and ROS production, as early initial responses. The resulting increase in cytosolic Ca^{2+} activates PLDα1 and prompts its translocation to the plasma membrane, where PA production alters the membrane composition (Fig. 7). This shift in membrane properties serves as a scaffold for the re-localization of CRK2 from uniformly along the plasma membrane to concentrated domains at plasmodesmata. There, CRK2 interacts with CALS1 to promote callose deposition and decrease plasmodesmal permeability, which ultimately leads to enhanced salt tolerance (Fig. 7). The roles of CRK2 for vesicle formation and ROS production during immune responses likely follow separate signalling cascades, which may also involve PLDα1-dependent subcellular localization patterns.

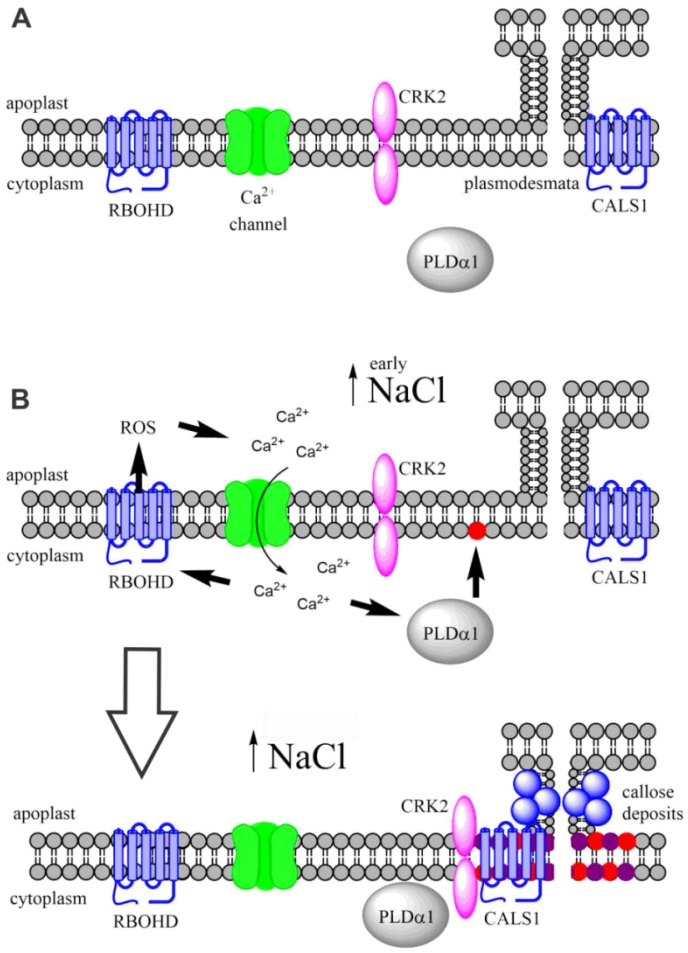

Figure 7. Schematic of proposed pathway for CRK2 regulation of callose deposition at plasmodesmata during salt stress. (A) Resting state. (B) Early responses to salt stress. Increased extracellular NaCl triggers Ca^{2+} influx, as well as ROS production by RBOHD. Cytoplasmic Ca^{2+} elevation activates PLDα1 leading to PA production and a shift in membrane properties; this serves as a scaffold for changes in CRK2 localization from uniformly along the plasma membrane to specific domains concentrated at plasmodesmata. Once localized at plasmodesmata, CRK2 interacts with CALS1 to promote callose deposition, ultimately leading to enhanced salt tolerance. Modified from Hunter et al., 2019.

5. CONCLUSIONS AND FUTURE PERSPECTIVES

The innate ability of plants to respond and adapt to their environment is a pre-requisite for survival, due their inability to re-locate in the face of adverse conditions. Climate change, allocation of land for urbanization and industrialized purposes, and even some agricultural practices themselves have contributed to the current situation where plants are increasingly exposed to more variable and unpredictable environmental conditions. The deleterious effects of abiotic and biotic stress on plant health, growth, and productivity can disturb the balance of natural ecosystems, as well as threaten food security and economic interests in agriculture, forestry, and other commercial plants. It is therefore desirable to produce plants which would be more sustainable and tolerant to such stresses, either through traditional breeding or genetic engineering. Any attempts at increasing stress tolerance, however, must first be preceded by a more thorough understanding of the molecular mechanisms and cellular pathways underlying the sensing of and responses to environmental stimuli.

The RLKs are central to the communication between plant cells and the extracellular environment, and as such are often fundamental components of stress response signalling networks. However, despite this, the functions and protein interactions of many RLKs remain largely uncharacterized on a biochemical and cellular level. This dissertation explores the significance of the CRK subfamily of RLKs during stress responses, with a focus on CRK2.

Publication (I) demonstrates that the CRK gene family is involved in several processes during development as well as abiotic and biotic stress responses. Some of the CRKs appear to have roles in multiple processes and could have central signalling functions, whereas others may be more involved in the fine tuning of responses. CRK2 in particular exhibits several strong loss-of-function phenotypes, and is therefore proposed as a fundamental member of the CRK gene family.

Publication (II) establishes that CRK2 enhances salt tolerance through the regulation of callose deposition by CALS1, in connection with PLDα1. This publication also demonstrates the highly specific stress-induced subcellular localization patterns of CRK2. These unique localization patterns are essential for CRK2 protein functions and interactions, as exemplified in the case of salt stress: upon exposure to high salinity, CRK2 re-localizes to plasmodesmata, where it interacts with CALS1 to regulate callose deposition and plasmodesmal permeability. Also presented are unpublished results detailing the involvement of CRK2 as a negative regulator of the formation of an unknown vesicle type during salt stress. Together, this work identifies a novel role for callose deposition in response to salt stress, and demonstrates its importance for salt tolerance. It supports the view that regulation of plasmodesmal permeability and symplastic signalling is important not only during biotic stress, but also in response to abiotic stress. The unknown vesicles could also

play a role in intercellular communication, as plasmodesmata are known to be hubs for vesicle trafficking and transport of molecules between cells. It will be interesting in the future to fully characterize the nature of these vesicles and their association with salt stress. Another important area for further research is to expand on the CRK2 and CALS1 interaction by identifying target phosphosites, and other potential proteins involved in the regulation. CRK2 was also found to interact with two other callose synthases; whether these are also involved in salt stress remains to be seen. Additionally, there is still a lack of complete understanding of the functional purpose behind salt-induced callose deposition and exactly how this response leads to improved salt tolerance.

Publication (III) focuses on the action of CRK2 during biotic stress, and shows that CRK2 can interact with and phosphorylate RBOHD, contributing to the regulation of stress-induced ROS production. While other kinases have been shown to regulate RBOHD via phosphorylation of the N-terminus, CRK2 is unique in that it phosphorylated several sites on the C-terminus. Phylogenetic analysis indicated that the identified C-terminal phosphosites are conserved across both plant and animal species. This opens new possibilities for investigation of the regulation of stress-induced ROS production in plants through C-terminal phosphorylation of RBOHs.

As a whole, the results presented in this dissertation suggest that CRK2 is a highly important CRK, with multiple cellular functions essential to stress response signalling. The placement of CRK2 within the basal group of CRKs suggests more ancestral functions (Vaattovaara et al., 2019), and highlights the potential for the transfer and application of information gained from model organisms to other species. CRK2 is therefore a promising candidate for further research into understanding stress response mechanisms in plants, and a potential target for improving tolerance to both abiotic and biotic stresses.

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