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The Role of Cysteine-rich Receptor-like Protein Kinases in ROS Signaling in *Arabidopsis thaliana*

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The Role of Cysteine-rich Receptor-like Protein Kinases in ROS Signaling in *Arabidopsis thaliana*

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Academic dissertation

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One child,
one teacher,
one book and
one pen can change the world.
Education is the only solution.

Malala Yousafzai

CONTENTS

ORIGINAL PUBLICATIONS

This thesis is based on the following articles, referred to in the text by their Roman numerals.

I) Wrzaczek M, Brosché M, Salojärvi J, Kangasjärvi S, **Idänheimo N**, Mersmann S, Robatzek S, Karpinski S, Karpinska B, Kangasjärvi J. Transcriptional regulation of the CRK/DUF26 group of Receptor-like protein kinases by ozone and plant hormones in *Arabidopsis*. BMC Plant Biology 2010, 10:95.

II) **Idänheimo N**[#], Gauthier A[#], Salojärvi J, Siligato R, Brosché M, Kollist H, Mähönen AP, Kangasjärvi J[&], Wrzaczek M[&]. The Arabidopsis thaliana cysteine-rich receptor-like kinases CRK6 and CRK7 protect cells against apoplastic oxidative stress. Biochem. Biophys. Res. Commun. 2014, 445(2): 457-462.

III) 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 DT, Glow D, Lowe J, Zhou J, Mohammadi O, Puukko T, Albert A, Lang H, Ernst D, Kollist H, Brosché M, Durner J, Borst JW[&], Collinge DB[&], Karpiński S[&], Lyngkjær MF[&], Robatzek S[&], Wrzaczek M[&], Kangasjärvi J[&] (2015). Large-scale phenomics identifies primary and fine-tuning roles for CRKs in responses related to oxidative stress. PLoS Genetics 2015, 7(11): e1005373

Author's contribution:

I) NI participated in the analysis of transcriptional regulation of *CRKs* in response to O_3.

II) NI designed and performed the experiments to address the role of CRK6 and CRK7 in apoplastic and chloroplastic ROS responses and analyzed the obtained results. NI wrote the manuscript with AG, MW and JK.
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III) NI and AG designed and performed the experiments to address the role of CRKs in apoplastic ROS signaling and analyzed the obtained results.
[#] These authors contributed equally to the work.
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[&] These authors contributed equally to the manuscript.

ABBREVIATIONS

ABA	abscisic acid
Ami-RNA	artificial microRNA
Bgh	*Blumeria graminis* f. sp. *hordei*, a non-host powdery mildew fungus
C	cysteine residue
CL	complementation line
Col-0	Columbia-0
CRK	cysteine-rich receptor-like protein kinase
crk	T-DNA insertion mutant (loss-of-function)
Cys	cysteine residue
DAB	3,3'-diaminobenzidine
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea
DUF	domain of unknown function
Go	*Glovinomyces orontii*, a biotrofic virulent powdery mildew fungus
GUS	β-glucoronidase
H_2O_2	hydrogen peroxide
LRR-RLK	leucine-rich repeat receptor-like protein kinases
MV	methyl viologen
NADPH	nicotinamide adenine dinucleotide phosphate
NBT	nitro blue tetrazolium
$^1O_2$	singlet oxygen
O_2^-	superoxide
O_3	ozone
OE	overexpression line
$OH^\bullet$	hydroxyl radical
PCD	programmed cell death
ppb	parts per billion
PTI	pattern-triggered immunity
PQ	paraquat
Pto DC3000	*Pseudomonas syringae* pv. *tomato* DC3000, a hemi-biotrophic bacterial pathogen
qPCR	quantitative real time PCR
RLK	receptor-like protein kinase
RLCK	receptor-like cytoplasmic protein kinase
ROS	reactive oxygen species
T	threonine residue
TB	trypan blue
Thr	threonine residue
Tyr	tyrosine residue
WT	wild type
X+XO	xanthine + xanthine oxidase
Y	tyrosine residue
35S	cauliflower mosaic virus 35S promoter

ABSTRACT

Responses to environmental changes are mediated via complex signaling networks in plants. Overlapping signaling pathways guarantee information flow from many simultaneous stress factors leading to both synergistic and antagonistic responses in order to maintain the most optimal conditions for growth under non- or suboptimal conditions. Adaptation to stressful environmental conditions is based on flexible interactions between hormone and reactive oxygen species (ROS) signaling. During the recent years researchers have started to understand the complexity of the crosstalk needed for stress tolerance. However, there are still many fundamental questions unanswered. For example, how are the intertwined signal transduction networks regulated, and how are ROS sensed and signaling specificity achieved?

Receptor-like protein kinases (RLKs) are plasma membrane proteins which have a role in signal sensing. RLKs have been linked to many different physiological processes, such as plant development, pathogen defense and abiotic stress response. RLKs are involved in ROS signaling and it has been suggested that members of the cysteine-rich protein kinase (CRK) subfamily could be involved in direct ROS sensing due to the redox regulation possibilities in their extracellular protein domain. The large number of CRKs and their protein similarity suggests partly overlapping functions and possibilities for fine-tuning the stress responses. In this study, Arabidopsis CRKs, especially CRK6 and CRK7, have been characterized and their involvement in ROS signaling studied.

Based on the presence of conserved kinase subdomains, this study suggests that Arabidopsis CRKs are active kinases and verifies kinase activity for CRK6 and CRK7 *in vitro*. This study shows that in addition to stress responses, as previously suggested, CRKs are involved also in many important developmental processes, such as germination and senescence. This novel finding broadens our understanding of the role of CRKs' in plants. Despite the observed redundancy in *crk* phenotypes due to sequence similarity, some *crk* mutants, such as *crk2* and *crk5*, showed clear individual phenotypes suggesting specific functions for these CRKs. *crk6* and *crk7* phenotypes were partly disguised by redundancy effect. Based on the obtained results and proposed redox regulation possibilities of CRK ectodomain, it can be proposed that CRKs are essential regulatory elements of cellular redox circuits that relay environmental information to the cell. Therefore the role of CRKs in cellular crosstalk is essential for maintaining the delicate balance between growth and defense. The loss of CRK function disrupts the information flow and leads to impaired stress tolerance. Thus, the obtained results suggest protective roles for CRKs. Furthermore, the large number of CRKs and their specific yet partly overlapping functions could bring operational reliability to the signal transduction and suggests specificity and fine tuning opportunities for signal transduction.

1 INTRODUCTION

Why study plants?

Plants are fundamental to all life on Earth and their importance cannot be neglected. Plants are important not only because of the oxygen they produce but also for food, fuel, fiber and medicines they provide us. Plants affect also our mental wellbeing. Being in the nature, or even watching pictures of it, has a calming and relaxing effect on us. Unfortunately, too often humans take plants and the benefits they provide for granted. Especially during the 20th century plants and nature have been used for industrial benefits with huge negative, even catastrophic, environmental effects. In the 21st century climate change is creating new challenges for food and wood production when at the same time fossil fuel reserves are declining. Raising the awareness of importance of plants for our past and for our future is one of the major goals for the 21st century as plants will provide solutions to many of the coming grand challenges facing the Earth e.g. food and energy crises.

How do we feed our children and their children is not the only question concerning the food crisis. Secure food production and food quality are important questions too. The potential of optimizing the nutritional content of foods is an important and relevant research topic but the use of GMO plants for food production raises many environmental and health related concerns in customers. Can we develop stress tolerant and disease resistant crops to increase yields without creating invasive plants or harming biodiversity? And to what extent do epigenetic changes affect heritable characteristics of plants? Can we optimize photosynthesis to better harness the energy of the sun? How can we use our knowledge of plants and their properties to improve human health? For a long time, the most effective medicinal molecules have been identified from plants. With GMO technologies we can develop new medicinal molecules which mimic the originals at lower cost. Using plants as chemical factories for drug production may not be a futuristic idea anymore. Furthermore, many new novel medicinal molecules might be still found from plants. These are only few of the important questions that the plant science research has to face in the near future (Grierson et al., 2011).

Raising awareness of the potential of plant science and its solutions and applications for solving the future environmental challenges will hopefully attract many young talented scientists to the field. Thinking beyond the limits of plant science combined with integrated efforts of scientists with diverse expertise will be required for creating new scientific methods for resolving the coming crises (Allahverdiyeva et al., 2015). Priorities of research challenges will change as new needs arise but the need for solid understanding of plant biology will exist as long as plants and people co-exist on Earth.

1.1 How to survive under ever-changing conditions?

Plant life is full of compromises as growth conditions in nature are rarely optimal. Indeed, most plants grow under suboptimal conditions and have to adapt to survive. Adaptation to the changing environmental conditions costs energy and requires compromises in metabolism and growth which is why it is considered as stress. Traditionally stress is defined as any adverse environmental parameter that limits plant growth and productivity (Boyer, 1982). The most important criterion of plant life is reproduction. Stress induced early flowering is a common phenomenon which aims only to earlier seed production and thus successful reproduction (Wada and Takeno, 2010; Xu et al., 2014). If the ability to reproduce fails, all other criteria of productivity e.g., growth and yield, become meaningless. From the economical point of view yield is the most important criterion. As environmental stress often decreases growth and yield the research focus has been on improving the stress tolerance of economically important crop plants (Baena-Gonzalez and Sheen, 2008; Bohra, 2013; Collinge et al., 2010; Manavalan et al., 2009; Mittler and Blumwald, 2010; Nemali et al, 2014; Prado et al., 2014; Ronald, 2014; Tran and Mochida, 2010).

Plants have developed many sophisticated ways to optimize growth in response to changing conditions. For example, plants can gather water and nutrients in roots and stems and regulate their use; they have ways to adapt to changes in light intensity and temperature; they also have developed sophisticated methods to defend against many pathogens (Dodds and Rathjen, 2010; Jones and Dangl, 2006). In life-threatening situations, programmed cell death (PCD) is employed to limit entry and growth of pathogens in the plant (Bruggeman et al., 2015; Coll et al., 2011; Jones and Dangl, 2006; Williams and Dicman, 2008). For example, by leaf abscission plants can eliminate the infected leaves to save the mother plant (González-Carranza et al., 1998; Taylor and Whitelaw, 2001). In addition, plant hormones have an important role in the coordination of stress responses to improve stress tolerance, survival and growth (Chouldhary et al., 2012a; Ha et al., 2012; Lozano-Durán and Zipfel, 2015; Peleg and Blumwald, 2011). Noteworthy, plants have not only evolved methods to protect themselves but also to protect other plants growing nearby. For example, under dangerous circumstances e.g. herbivore attack, plants can emit volatile organic compounds (VOCs) to warn other plants of impending danger (Baldwin et al., 2006; Ueda et al., 2012). Nearby plants respond to VOCs by strengthening their own defense system before being attacked themselves. VOCs also help predators to locate feeding herbivores and flowers to attract pollinator to ensure reproduction (Baldwin et al., 2006; Schuman et al., 2012; Ueda et al., 2012).

Guard cells, surrounding the stomatal pores in the leaf epidermis, are considered to be one of the most important sensors of environmental conditions. Multiple receptors in their plasma membrane can respond to different stimuli. Depending on

the stimuli, guard cells can open or close stomatal pores by regulating their osmotic pressure. Like their name suggests, guard cells are like gatekeepers controlling what goes in and out though the pore. Noteworthy, by controlling their primary processes i.e. the exchange of water vapor and carbon dioxide (CO_2), the tiny stomatal pores play a central role in the regulation of the Earth's water and carbon cycles (Outlaw, 2003). In addition to gas exchange, guard cells respond to air pollutants, plant hormones, water status, light intensity, reactive oxygen species (ROS) and pathogens (Kollist et al., 2014). As natural openings to the plant, pathogens and gaseous substances try to enter plants through stomatal pores. By closing the pores plants can limit the entry of pathogens, as well as air pollutants e.g., ozone (O_3).

Similar to guard cells, plasma membrane of all cells contains a variety of receptors for environmental parameters. Receptors sense various environmental (and endogenous) signals with their extracellular domain. Signal recognition activates or inhibits the corresponding biochemical pathway. Receptor kinases deliver the signal downstream *via* their intracellular kinase domain (Cock et al., 2002; Lehti-Shiu and Shiu, 2012; Stone and Walker, 1995; Tichtinsky et al., 2003). The biggest group of receptor kinases in plants is receptor-like protein kinases (RLKs) and histidine kinases (HKs) (Nongpiur et al., 2012; Shiu and Bleecker, 2001a, b, 2003). Protein kinases are capable of transferring a phosphate group (PO_4^{3-}) to a substrate protein and thus capable of regulating the function, localization and stability of their target proteins and interactions with other proteins. Reversible phosphorylation is the key mechanism in signal transduction. In general, by phosphorylation kinases activate or inactivate their target proteins and phosphatases do the process *vice versa* through dephosphorylation (Luan, 1998).

Compared to animals the number of kinases in land plants is huge. The human genome contains about 500 protein kinases, whereas *Arabidopsis thaliana* contains over 1000 protein kinases and rice over 1400 (The Arabidopsis Genome Initiative, 2000; Dardick et al., 2007; Lehti-Shiu and Shiu, 2012; Lehti-Shiu et al., 2009; Mannig et al., 2002; Shiu and Bleecker, 2001a, b, 2003). The large number of protein kinases in land plants is a consequence of tandem and whole genome duplications and could be an evolutionary result of a need to adapt to changing environmental conditions (Hanada et al., 2008; Lehti-Shiu and Shiu, 2012). Adaptation and survival is based on extensive intra- and intercellular signaling and most of the plant responses are based on regulation of phosphorylation status of the proteins. Different environmental signals, including drought, light intensity, temperature changes, air pollutants and pathogens, lead to the activation or inactivation of separate signal transduction pathways for delivering the message to the nucleus for decision making. The readout of all messages aims to maintain the best possible cellular conditions for growth.

Membrane-localized RLKs play a central role in sensing the environmental stress signals (Morris and Walker, 2003; Osakabe et al., 2013). RLKs can bind different kind of ligands, such as carbohydrates, polypeptides, cell wall components,

hormones, etc., depending on their extracellular domain (Shiu and Bleecker, 2001b). RLKs can be activated by ligand binding or conformational change induced by receptor complex formation upon ligand binding (Han et al., 2014; Shiu and Bleecker, 2001b). Resent research has suggested that ROS might have an important role in receptor activation (Kangasjärvi and Kangasjärvi, 2014; II). ROS molecules which are released during stressful conditions can damage cell wall components and the breakdown products might act as ligands for receptors. It has been suggested that ROS molecules could activate receptors also by direct redox modification (Kangasjärvi and Kangasjärvi, 2014; Shapiguzov et al, 2012). In addition, ROS have been shown to deliver both local stress signals to the neighboring cells and long-distance signals throughout the plant (Gilroy et al., 2014; Miller et al., 2009; Mittler et al., 2011; Suzuki et al., 2013). Through intra- and intercellular ROS signaling plants are able to coordinate the responses induced by environmental changes and maintain the cellular balance for optimal growth. These new findings have broadened our understanding of processes coordinating adaptation to changing environmental conditions and opened up an exciting new research field.

1.2 "ROS-talk" coordinates adaptation to the changing environmental conditions

ROS are highly reactive oxygen-based molecules that are constantly produced as by-products of cellular metabolism in plants, animals and microbes. The main sources of intracellular ROS in plants are chloroplasts, peroxisomes and mitochondria, nucleus, cytosol and endoplasmic reticulum being minor production sites. ROS molecules, i.e. hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^{\bullet}$), singlet oxygen ($^1O_2$) and superoxide ($O_2^{\cdot-}$), have ability to oxidize a wide range of biomolecules which is why ROS levels are tightly controlled to avoid cellular damage (Correa-Aragunde et al., 2015; Møller et al., 2007; Tripathy and Oelmüller, 2012; Wrzaczek et al., 2011, 2013). Especially hydroxyl radical and superoxide are very reactive due to their radical nature. Antioxidants e.g., ascorbic acid and glutathione, and scavenging enzymes e.g., superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT), have important roles in keeping ROS levels low under normal growth conditions (Foyer and Noctor, 2005, 2009, 2011; Mittler et al., 2004; Noctor et al., 2012; Potters, 2010; Shigeoka and Maruta, 2014; Wrzaczek et al., 2011) as high ROS levels can cause oxidative damage to proteins, lipids and nucleic acids and ultimately cell death (Møller et al., 2007; Wrzaczek et al., 2011).

In addition to the metabolic by-products, ROS are important messenger molecules in intra- and intercellular signaling. ROS signaling is highly conserved and controls a wide range of biological processes such as growth and development, and responses to environmental stress such as drought, salinity, heavy metals, strong light, heat and cold, nutrient deprivation, pathogen attack, mechanical stress and air

pollutants (Figure 1). Changes in ROS levels lead to changes in the cellular redox homeostasis. The redox state of the cell plays an important role in plant life defining the physiological status of a plant. Tightly regulated ROS production guides plant development by regulating for example cell division and cell expansion, stem cell renewal and differentiation, and cell cycle (Considine and Foyer, 2014, Potters et al., 2010; Swanson and Gilroy, 2010). In addition, it guides stress acclimation, programmed cell death (PCD), metabolism and hormonal response (De Pinto et al., 2012; Dietz, 2014; Shigeoka and Maruta, 2014; Torres, 2010; Van Breusegem and Dat, 2006). Environmental stress leads to redox imbalances in different subcellular compartments and leads to local and even systemic acclimation processes involving coordinated changes in gene expression and optimization of metabolic pathways. ROS play important roles also in transcriptional regulation (Gadjev et al., 2006; Geisler at al., 2006; Vaahtera et al., 2014; Wang et al., 2013). Information flow *via* inter-compartmental ROS signaling is highly important for cellular homeostasis, hormonal balance and coordinated stress responses (Baxter et al., 2014; Considine and Foyer, 2014; Kangasjärvi and Kangasjärvi, 2014; Mittler and Blumwald, 2015; Shapiguzov et al., 2012; Shigeoka and Maruta, 2014; Sierla et al., 2013; Suzuki et al., 2012; Vaahtera et al., 2014).

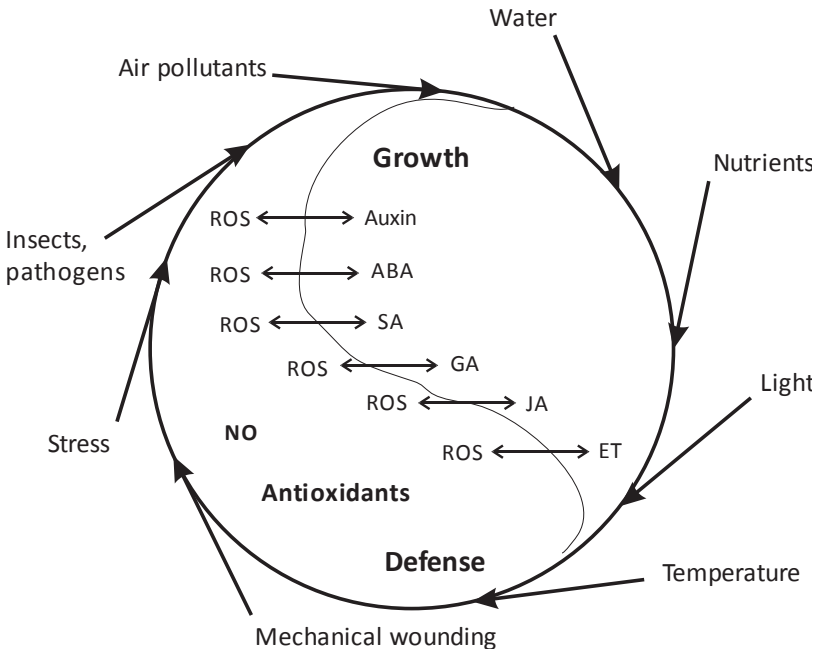

Figure 1. Adaptation is based on the delicate balance between growth and defense. The flexible interactions between hormone and ROS signaling pathways control growth and defense responses. Environmental stresses disturb the delicate balance and lead to metabolic changes needed for rebalancing the system. The flexibility guarantee high buffering capacity against environmental stresses but in the case of a strong stress system overload leads to the collapse of vital processes and cell death. ABA: abscisic acid; SA: salicylic acid; GA: gibberellic acid; JA: jasmonic acid; ET: ethylene, NO: nitric oxide

ROS signaling shares similarities with nitric oxide (NO) signaling. The free radical NO is a secondary messenger which controls essential biological processes in animals, plants and microbes. In plants, NO has important role in seed germination, primary and lateral root growth, stomatal closure, flowering, pollen tube growth, fruit ripening, senescence and programmed cell death (Belenghi et al., 2007; Delledonne, 2005; Bethke et al, 2006; Corpas et al., 2004; Correa-Aragunde et al., 2004; He et al., 2004; Leshem et al., 1998; Neil et al., 2002; Prado et al., 2004; Sanz et al., 2015; Serrano et al., 2015; Zhao et al., 2007). In addition, NO controls plant immune responses (Delledonne et al., 1998; Durner et al., 1998; Yu et al., 2012) and abiotic stress responses (Corpas et al., 2011). Based on their reactivity and involvement in the same biological processes, ROS and NO are suggested to function together, at least in plant biotic interactions (del Río, 2015; Scheler at al., 2013; Wendehenne et al., 2014; Yun et al., 2011). NO improves plant's antioxidant capacity to counteract the ROS-generated oxidative environment (Correa-Aragunde et al., 2015). For example, NO reduces H_2O_2 levels, ROS accumulation and cell damage during salt stress (Bai et al., 2011), photo-oxidative stress (Beligni and Lamattina, 2002) and UV exposure (Tossi et al., 2011). Thus, together ROS, NO and antioxidant molecules and enzymes regulate cellular redox balance which is crucial for plant growth and survival (Correa-Aragunde et al., 2015).

1.2.1 ROS in stress responses

ROS production, especially extracellular ROS production, increases dramatically under environmental stress and stress-induced ROS signaling has an important role in stress responses and stress tolerance (Baxter et al., 2014; Jaspers and Kangasjärvi, 2010; Miller et al., 2008, 2010; Mittler et al., 2011; Suzuki et al., 2012; Tripathy and Oelmüller, 2012; Wendehenne et al., 2014). When the antioxidant capacity of the apoplast is exceeded, ROS can accumulate and activate stress-specific signaling pathways thereby "alarming" cells leading to fast and proper stress responses. ROS signaling leads to a local response and later to a systemic whole plant response. Apoplastic ROS production by plasma membrane respiratory burst oxidases (RBOH) induces a rapid long distance auto-propagating "ROS wave" signal which is transmitted throughout the plant (Baxter et al., 2014; Miller et al., 2009; Mittler et al., 2011. It is suggested that superoxide, produced by NADPH oxidase (respiratory burst oxidase D, RBOHD), is dismutated by superoxide dismutase to H_2O_2 which would be the primary ROS mediating the rapid systemic signal (Miller et al., 2009). The ROS wave which spreads relatively fast, about 8 cm/min, transmits the "alarm" signal from affected parts of the plant to the unaffected parts, which have not received the primary stimulus and plays an important role in defense and acclimation processes (Fu and Dong, 2013; Mittler and Blumwald, 2015; Suzuki et al, 2013).

During plant-pathogen interactions, extracellular ROS production is involved in the regulation of the hypersensitive response (HR), which is a well-documented

defense mechanism used in the battle against biotrophic pathogens (Lamb and Dixon, 1997; Morel and Dangl, 1997; Torres, 2010). Plants respond to the non-host microorganisms by inducing pathogenesis-related (PR) genes and by strictly controlled localized programmed cell death (PCD) (Dodds and Rathjen, 2010; Fu and Dong, 2013). Bi-phasic ROS production is characteristic for the hypersensitive response. Apoplastic ROS production leads to the pathogen recognition which induces ROS production in various sub-cellular compartments and activates defense mechanisms. Intercellular ROS production combined with apolastic ROS production by plasma membrane NADPH oxidases induces "the ROS wave" which activates adjacent cells and alarms also the non-infected plant parts. Such pre-resistance mechanism is termed systemic acquired resistance (SAR) (Dempsey and Klessig, 2012; Kachroo and Robin, 2013; Shah and Zeier, 2013). In the infection site ROS signaling leads to controlled cell death of infected cells to limit the pathogen growth and thus the infection.

ROS have an important role also in the regulation of guard cell responses (Song et al., 2014). ROS mediate stomatal responses to both external and internal factors. Stress induced apoplastic ROS production by NADPH oxidases leads to the rapid closure of the stomatal pore thus protecting cells from more injury. Guard cells have been used as a single cell model to study ROS signaling mechanisms. Recent research has revealed a conserved sequence of events leading to stomatal closure: stress induced apoplastic ROS production by plasma membrane NADPH oxidases in guard cells is sensed in the apoplast and in the cytoplasm which leads to the Ca^{2+} - flux through the plasma membrane. Ca^{2+}- flux leads to Ca^{2+} sensing in cytoplasm and simultaneous anion fluxes through the SLOW ANION CHANNEL 1 (SLAC1) in the plasma membrane which leads to rapid stomatal closure and ROS production in the chloroplasts (Kollist et al., 2014; Sierla et al., 2013 and Song et al., 2013; Vahisalu et al., 2010).

Environmental stress leads to elevated ROS levels not only in the apoplast but also in chloroplasts (Kangasjärvi and Kangasjärvi, 2014; Shapiguzov et al., 2012; Sierla et al., 2013; Song et al., 2013; Suzuki et al., 2012). ROS are produced continuously in chloroplasts due to numerous electron transfer reactions during photosynthesis (Tripathy and Oelmüller, 2012). Due to constantly changing environmental conditions e.g. light, wind and temperature, the photosynthetic machinery is continuously adjusted in order to maintain the delicate physiological equilibrium (Dietz, 2015; Foyer et al., 2012). The flexibility of the photosynthetic machinery combined with efficient regulatory mechanisms helps to maintain the balance between production and consumption of reducing equivalents. Accumulation of ROS leads to inter-organellar signaling which leads to appropriate stress response including local and systemic acquired acclimation processes (SAA), systemic acquired resistance (SAR) and in the extreme programmed cell death (PCD). It has been shown that chloroplastic ROS signaling plays a crucial role at least in O_3, light, pathogen and wounding stress response (Chang et al., 2004; Dietz, 2015; Fryer et al., 2002, 2003; Joo et al., 2005).

Chloroplasts respond also to ROS signals from other cellular compartments. For example, when apoplastic ROS levels are elevated in response to pathogen attack, the information is delivered to the chloroplasts by so far unknown mechanism. Together with other subcellular compartments, chloroplasts contribute to ROS production during the hypersensitive response (HR) and induce the second phase of ROS production in the battle against pathogens (Liu et al., 2007; Stael et al., 2015; Yao and Greenberg, 2006; Zurbriggen et al., 2009, 2010). The involvement of chloroplast function in plant immunity has been increasingly recognized in the last few years (Kangasjärvi et al., 2012; Shapiguzov et al., 2012; Sierla et al., 2013). The role of chloroplasts in ROS signaling in response to pathogen attack is central to defense and survival e.g. it has been shown that the pathogen response differs between light and dark (Lozano-Durán and Zipfel, 2015; Roden and Ingle, 2009) and that some bacterial and viral elicitors interact with chloroplast-targeted proteins or are imported into chloroplasts (Padmanabhan and Dinesh-Kumar, 2010).

It has been also suggested that chloroplastic ROS production is needed for systemic ROS signaling i.e. chloroplastic ROS signals would precede the ROS wave and not *vice versa* (Joo et al., 2005, Zurbriggen et al,, 2009, 2010). The complex organellar crosstalk *via* ROS molecules is a challenging research topic with many open questions at the moment. In spite of complexity, it is clear that chloroplasts have a crucial role in the inter-compartmental ROS signaling. Chloroplasts are able to amplify ROS signals and transmit them to the apoplast and nucleus *via* cytosolic signaling pathways (Galvez-Valdivieso and Mullineaux, 2010; Joo et al, 2005; Kangasjärvi and Kangasjärvi, 2014, Shapiguzov et al., 2012, Sierla et al., 2013). The short distance between the plasma membrane and the chloroplast allow efficient and rapid signal transduction for inter-compartmental communication. As a result, ROS signals from apoplast and chloroplasts are decoded in the nucleus by various transcription factors leading to appropriate response in order to maintain the most optimal physiological conditions for growth.

1.2.2 ROS in development

ROS signaling relays information about environmental conditions and plays a central role in maintaining optimized metabolic homeostasis for growth. The delicate balance between growth and defense is based on flexible interactions between hormone and ROS signaling which is regulated by antioxidants (Figure 1). Due to this flexibility plants display an enormous capacity to adapt to changing environmental conditions and adjust growth accordingly. Under unfavorable conditions the growth rate decreases, or even ceases, due to changes in energy allocations. However, when conditions become again favorable for growth, plant continues to grow according to the genetically defined program. Plant architecture is based on genetically defined structural units, such as roots, branches, leaves, flowers and fruits, whose number and size depend on available resources and environmental conditions for growth. Stress-induced alterations in plant growth

and architecture are called stress-induced morphogenetic response (SIMR) (Potters et al., 2007). SIMR is a well-documented phenomenon but its molecular mechanism and connection to ROS signaling is not well-understood (Potters et al., 2007). In addition to visible stress symptoms such as stunted growth and early flowering, stress adaptation requires rapid changes in metabolism including transcriptional and translational adjustments, and precise timing of cell defense activation and developmental transitions (Dietz, 2014).

Tightly regulated ROS production guides plant development and growth by regulating for example cell division and cell expansion, stem cell renewal and differentiation, and cell cycle (Considine and Foyer, 2014, Potters et al., 2010; Swanson and Gilroy, 2010). Also the transition from cell proliferation to cell differentiation is under ROS regulation (Tsukagoshi et al., 2010). One of the most extensively studied developmental processes is root growth. Almost every stage of root development, starting from the breaking of the seed dormancy to the development of lateral root and root hairs, is under redox control (Considine and Foyer, 2014; De Tullio et al., 2010; Dunand et al., 2007; El-Maarouf-Bouteau and Bailly, 2008; El-Maarouf-Bouteau et al., 2013; Foreman et al., 2003). For example, the *A. thaliana* mutant, *root hair defective2* (*rhd2)*, defective in NADPH oxidase, displays stunted root growth and short root hairs due to impaired ROS production (Foreman et al., 2003). Impaired ROS production leads to a defective Ca^{2+} influx and thus impaired cell expansion highlighting the role of NADPH oxidase in the control of development (Foreman et al., 2003). In *A. thaliana rootmeristemless (rml*) mutants, the cell cycle is arrested in the primary root at an early stage due to glutathione (GSH) deficiency highlighting the importance of GSH in activating and maintaining the cell division cycle in the root apical cells (Cheng et al., 1995; Vanstraelen and Benková, 2012; Vernoux et al., 2000). In plants, the NADPH-dependent glutathione/glutaredoxin (GSH/GRX) and thioredoxin (TRX) systems are the two major systems maintaining the cellular redox homeostasis and controlling developmental processes. Overlapping function highlights the importance of these two pathways as it has been shown that the corresponding single knock-out mutants of the members of GRX and TRX multigene families display unaltered phenotypes (Marty et al., 2009; Meyer et al., 2008; Reichheld et al., 2007). The two pathways are intertwined and have common targets in developmental processes (Reichheld et al., 2007; Potters et al, 2010). Observing the phenotypes of mutants involved in these two pathways has revealed new functions and connections between different hormonal pathways (Benitez-Alfonso et al., 2009; Reichheld et al., 2007). For example, there are many reports which suggest tight connection to auxin, which is an essential regulator for plant growth and development (Benjamins and Scheres, 2008; Salehin et al., 2015; Schaller et al., 2015). The flowerless triple mutant *ntra ntrb cad2*, generated by crossing the TRX reductase mutation (*ntra ntrb)* with GHS biosynthesis mutation (*cad2*), is reminiscent of polarized auxin transport (PAT) mutants linking NADPH-dependent GRX and TRX pathways to auxin signaling (Bashandy et al., 2011). The triple mutant has disturbed auxin levels and displays pin-like phenotype, which is typical for mutants deficient in auxin transport (Adamowski and Friml, 2015; Gälweiler et al., 1998). The results suggest that the

pin-like phenotype of the triple mutant is due to a disturbed auxin biosynthesis and transport while the stunned root growth is due to disturbance of polar auxin transport (Bashandy et al., 2011). Moreover, crossing the viable TRX double mutant *ntra ntrb* with *rml1*, a mutant blocked in root growth due to glutathione (GSH) deficiency, led to inhibition of both shoot and root growth (Reichheld et al., 2007). Another well-studied example of redox regulated developmental process leading to environment-responsive developmental plasticity is root apical meristem (RAM) growth. Unfavorable conditions lead to reorganization of RAM leading to cessation of new cell production in root tip but enhanced lateral root growth (De Tullio et al., 2009). RAM growth is regulated by flexible interactions of redox/hormone crosstalk, where auxins, gibberellins and cytokinins play a central role (De Tullio et al., 2009). These results highlight the role of redox/hormone/antioxidant crosstalk in the developmental processes. In conclusion, the crosstalk between hormone and ROS signaling pathways is crucial for maintaining the cellular balance needed for the proper development and stress adaptation in plants (Mittler and Blumwald, 2015; Palsternak et al., 2005; Tognetti et al., 2010, 2012; Xia et al., 2015).

1.2.3 ROS crosstalk

ROS signaling between the different cellular compartments is only a part of the overall cellular control over plant life. In addition to ROS, calcium-mediated signaling, hormonal and circadian regulation, nitrogen oxide species, antioxidants, nutritional status, growth phase, etc., affect how plants respond to environmental stresses (Akpinar et al., 2012; Baena-González and Sheen, 2008; Bartoli et al., 2012; Dodd et al., 2010; Du and Poovaiah, 2005; Mazars et al., 2010; Poovaiah et al., 2013; Ramon et al., 2008; Steinhorst and Kudla, 2013; Tognetti et al., 2012; Xia et al., 2015; Yang and Poovaiah, 2003). Crosstalk between different signaling pathways is crucial for information flow and the fitness and the survival of a plant is the result of a multilayer regulation. The synergistic and antagonistic interactions of multiple signaling pathways are thought to be important in the fine-tuning of plant responses to abiotic and biotic stress (Shigeoka and Maruta, 2014). However, it is not well understood how environmentally induced stress signals are transmitted through the plasma membrane to the cytosol, chloroplasts and eventually to the nucleus and how this complex signaling network is regulated. Nevertheless, it has been shown that ROS production, ion fluxes across the plasma membrane, increased cytoplasmic Ca^{2+} levels and activation of cytoplasmic mitogen-activated protein kinase (MAPK) signaling cascades are part of early signaling events in response to different environmental stimuli (Baxter et al., 2014; Miller et al., 2009).

The MAPK cascade is a well-documented three-layered cytoplasmic signal transduction unit where reversible protein phosphorylation plays a crucial role (Pitzschke, 2015; Rodriguez et al., 2010; Taj et al., 2010; Xu and Zhang, 2015). The first layer of the unit consists of a MAP kinase kinase kinase (MAPKKK or MAP3K) which is activated by signals transmitted by the plasma membrane proteins. The

MAPKKK transfers the signals to the second layer of the unit consisting of a MAP kinase kinase (MAPKK or MAP2K). The MAPKK activates a MAP kinase (MAPK) which finally activates its target proteins by phosphorylation. As Arabidopsis has at least 60 MAPKKKs, 10 MAPKKs and 20 MAPKs, the number of possible combinations of this signal transduction units is high which brings specificity to the responses (Jaspers and Kangasjärvi, 2010; Taj et al., 2010). Crosstalk between MAPKs and calcium dependent protein kinases (CDPKs) is known to be involved in plant stress responses (Wurzinger et al., 2011). CDPKs are plasma membrane proteins involved in Ca^{2+} sensing (Steinhorst and Kudla, 2013). Ca^{2+} is an important second messenger involved in many central signaling cascades in plants and animals. For example, NADPH oxidase activity and the production of apoplastic ROS is controlled via Ca^{2+}-dependent conformational changes i.e. increased cytosolic Ca^{2+} concentration leads to the activation of NADPH oxidase and ROS production (Baxter et al., 2014; Dubiella et al., 2013; Kimura et al., 2012; Ogasawara et al., 2008; Sierla et al., 2013).

ROS signaling via MAPK pathways seems to be crucial to plant stress responses and many new signaling components have been characterized in recent years (Dodds and Rathjen, 2010; Taj et al., 2010; Teige et al., 2004; Pitzschke and Hirt, 2006, 2009; Vainonen and Kangasjärvi, 2014). In addition to CDPKs, plasma membrane located RLKs could be another link between the apoplastic signals and the cytoplasmic MAPK signaling pathways, as RLKs have the ability to transmit signals through plasma membrane and regulate cytoplasmic proteins by phosphorylation.

The large number of RLKs could explain also the specificity of the responses. In the nature plants grow under ever-changing conditions and have to respond to multiple simultaneous stress factors which can lead to synergistic or antagonistic responses. Albeit the complexity, somehow the plants are able to sort out the stress signals to maintain the most optimal physiological status for growth (Vaahtera and Brosché, 2011). ROS, hormone, Ca^{2+} and MAPK signaling cascades are intertwined and there are many open questions concerning this crosstalk. In addition, despite the recent advances in ROS research the specificity of ROS signals is still an open question (Møller and Sweetlove, 2010; Shigeoka and Maruta, 2014; Vaahtera et al., 2014). Another intriguing open question concerns ROS sensing: How are ROS molecules sensed and how can this sensory system make distinctions between different types of ROS?

1.2.4 ROS sensing

The apoplast is the intercellular space outside plasma membrane formed by the continuum of cell walls and the extracellular spaces. Diffusion through apoplast is much faster than through cytosol and therefore a place through which information can flow rapidly from cell to cell. The apoplast has few special features affecting its redox properties (Potters et al., 2010). Apoplastic ROS production is an active process controlled by a plasma membrane-bound NADPH oxidase and cell wall

peroxidases. Compared to the cytosol the apoplast has lower levels of low molecular weight antioxidants, such as glutathione and ascorbate, and thus a lower antioxidant buffering capacity due to which ROS are able to accumulate in the apoplast thus enabling the activation of ROS signaling pathways. In addition, a large number of apoplastic proteins contain thiol groups which could be redox regulated. Despite the recent research interest towards ROS sensing in apoplast the question of ROS sensing mechanism has remained unsolved. However, several distinct systems have been proposed to be involved in ROS sensing in the apoplast (Kangasjärvi and Kangasjärvi, 2014; Mittler et al., 2011; Møller et al., 2010; Shapiguzov et al., 2012; Wrzaczek et al., 2010, 2011, 2013). For example, changes in apoplastic ROS levels affect the ascorbate gradient and lead to changes in cellular redox homeostasis (Foyer et al., 2009; Munné-Bosch et al., 2013). Superoxide, produced by NADPH oxidases, is dismutated to H_2O_2 which can cross plasma membranes through aquaporins (Bienert et al., 2007). In addition, ROS can also cause direct oxidative modifications to secreted and membrane-localized proteins and lipids. These changes could be sensed through conformational changes or breakdown products, which might act as ligands for receptors. The recent results suggest that a significant number of RLKs is involved in the response to environmental cues (Chae et al., 2009; Kangasjärvi and Kangasjärvi, 2014; Osakabe et al., 2013; Sierla et al., 2013). However, despite the many proposed options, ROS perception and specificity and the role of RLKs in ROS signaling are still intriguing questions waiting to be answered.

1.3 The role of receptor-like protein kinases in ROS-mediated adaptation

The largest group of kinases in plants is RLKs, which are plasma membrane proteins involved in many important signaling processes (Shiu and Bleecker, 2001a, 2003). *Arabidopsis thaliana* contains more than 610 RLKs which represents nearly 2.5 % of its protein coding genes and 60% of all protein kinases (Shiu and Bleecker, 2001a, 2003). RLKs contain a variable extracellular domain responsible for signal perception, a transmembrane domain and a conserved intracellular kinase domain responsible of signal transduction similar to animal receptor tyrosine kinases (Figure 2). More than 20% of RLKs are cytoplasmic proteins (receptor-like cytoplastic kinases, RLCKs) without extracellular and transmembrane domain (Figure 2; Shiu and Bleecker, 2001b, 2003). RLKs can make homo- and heterodimers with other RLKs and receptor complexes with different kind of membrane proteins, apoplastic proteins, cytoplasmic proteins, etc. (Han et al., 2014). Receptor specific stimulus leads to the receptor complex formation and signal transduction across the plasma membrane and leads to the activation of otherwise inhibited phosphorylation events on the cytoplasmic side of the plasma membrane (Clouse et al., 2012). Ligand or stimulus induced conformational changes of the kinase domain

reveal the active site for phosphorylation and thereby activate the cytoplasmic signal transduction events (Clouse et al., 2012; Hubbard, 2004; Pawson, 2004).

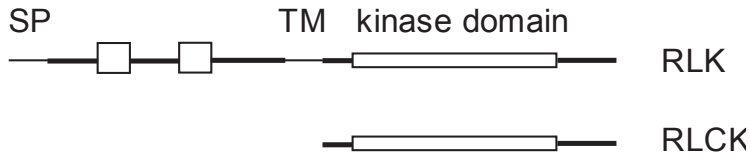

Figure 2. The general RLK protein structure: N-terminal signal peptide (SP) followed by a variable extracellular domain connected by a single transmembrane domain (TM) to an intracellular protein kinase domain. RLCKs contain only the intracellular protein kinase domain.

The RLK family is divided into 46 subfamilies according to the presence or absence of the variable extracellular domain, kinase domain phylogeny and the intron locations (Shiu and Bleecker, 2003). In most cases, the number of exons and intron locations are conserved between the subfamily members. This means that in general, members of the same subfamily have identical or similar number of exons and intron locations (Shiu and Bleecker, 2003). There are at least 15 different extracellular protein domains which are used to classify RLK subfamilies (Figure 3). Some of the biggest RLK subfamilies are divided into subgroups such as leucine-rich repeat (LRR) subfamily (LRRI I-XIV), RLCK (RLCK I-XII) and S-domain RLKs.

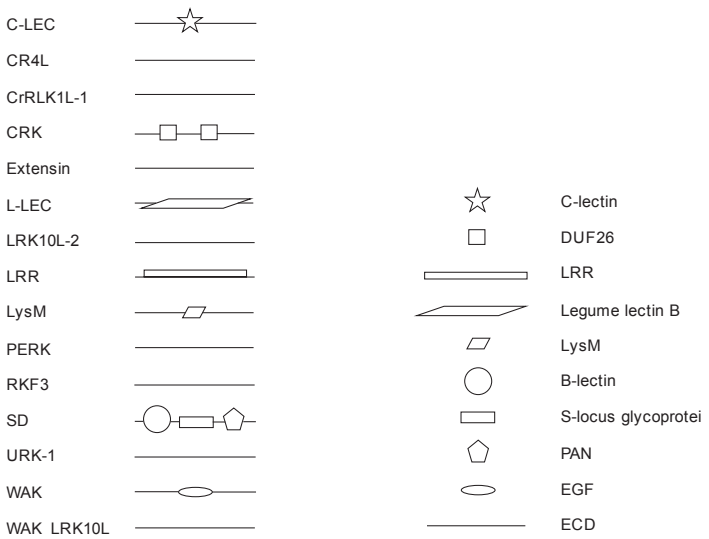

Figure 3. Extracellular protein domains found from Arabidopsis RLK subfamilies.

The variability of extracellular domains has been suggested to guarantee a wide coverage of different signals perceived by RLKs (Lehti-Shiu et al., 2009; Shiu and Bleecker, 2003). According to gene expression studies RLKs are involved in many

different physiological processes including development, pathogen defense and abiotic stress responses (Chae et al., 2009; Haffani et al., 2004; Lehti-Shiu et al., 2009; Morillo and Tax, 2006; Morris and Walker, 2003; Munné-Bosch et al., 2013; Wrzaczek et al., 2010). Subfamilies such as CRK, L-LEC, LRR-I, LRR-VIII-2, LRR-Xb, RLCK-VIIa, SD1, SD-2b, WAK and WAK_LRK10L-1 show overrepresentation of genes that have been implicated in biotic stress compared to other RLK subfamilies (Lehti-Shiu et al., 2009). Recent research suggests that a significant number of RLKs is involved in the response to environmental cues and has important functions in stress acclimation and responses (II; III; Kangasjärvi and Kangasjärvi, 2014; Lehti-Shiu et al., 2009; Osakabe et al., 2013; Sierla et al., 2013).

Phylogenetic analyses suggest that animal *Pelle* kinases are the animal homologs of Arabidopsis RLKs, thus RLKs are usually defined as RLK/Pelle kinases (Klaus-Heisen et al., 2011; Lehti-Shiu et al., 2012; Shiu and Bleecker, 2001). RLKs have frequently been described as serine/threonine (Ser/Thr) kinases in contrast to animal receptor protein kinases which usually are tyrosine (Tyr) kinases (Afzal et al., 2008; Shiu and Bleecker, 2001). However, according to phosphoproteomic assays tyrosine phosphorylation is more abundant in plants than generally recognized and many RLKs possess dual-specificity kinase activity (Bojar et al, 2014; de la Fuente Van Bentem and Hirt, 2009; Clouse et al., 2012; Sugiyama et al., 2008). Other closely related kinases are animal receptor tyrosine kinases (RTK) and Raf kinases which have diverged from the plant RLKs after an ancient duplication event (Shiu and Bleecker, 2001). After the divergence of plant and animal lineages, the plant RLK family has expanded dramatically compared to animal receptor kinases (Lehti-Shiu et al., 2009, 2012; Lehti-Shiu and Shiu, 2012)). The expansion has been lineage specific and based on whole genome and tandem duplication (Lehti-Shiu et al., 2009; 2012). Today, this can be observed in a high sequence similarity between RLKs and organization of RLKs in tandem duplications where many RLK genes are organized in back-to-back repeats, some of which have become pseudogenes (Lehti-Shiu et al., 2009; 2012). The functionally important cytoplasmic kinase domain is highly conserved between RLKs whereas the signal perceiving extracellular domain shows great variability, most likely to maximize the coverage of signals that can be sensed and thus to provide fine-tuning opportunities in a plant's response to environmental cues. As many RLKs are implicated in the response to environmental cues, the driving force behind expansion could be the need to deliver environmental signals to guarantee acclimation and survival under changing conditions (Hanada et al., 2008; Lehti-Shiu et al., 2009, 2012; Lehti-Shiu and Shiu, 2012).

In general, there are only few if any RLKs in most eukaryotes except embryophytes (land plants) (Lehti-Shiu et al., 2009, 2012). For example, there are no RLK genes in yeast (*Saccharomyces cerevisiae* or *Candida albicans)* or in *Ostreococcus tauri*, the smallest known free-living eukaryote (green alga), only one RLCK gene in common fruit fly (*Drosophila melanogaster)*, two RLCKs in *Chlamydomonas reinhardtii* (green alga) and four RLCK genes in humans (*Homo sapiens)* (Lehti-Shiu et al., 2009). Compared to these, the number of RLKs and RLCKs in land plants is huge. In

addition, differential expansion of the RLK family in land plants has resulted in huge differences in the number of RLK genes between land plants. For example, the number of RLKs in liverworth (*Marchantia polymorpha*) is 29 and in *Physcomitrella patens* (moss) 329 while *Arabidopsis thaliana* has 610 RLKs and rice (*Oryza sativa*) and poplar (*populus trichocarpa*) over thousand RLKs (Lehti-Shiu et al., 2009, 2012; Sasaki et al, 2007; Shiu et al., 2004). Differences in expansion can also be seen at the RLK subfamily level. During evolution some subfamilies have remained relatively constant in size while other has expanded dramatically. For example, the rice WAK subfamily is six times larger than in *Arabidopsis thaliana* and the SD1 subfamily in rice and poplar is more than ten times larger than in *Arabidopsis thaliana*. On contrast, the number of CRKs in rice, poplar and *A. thaliana* is similar (Lehti-Shiu et al., 2012).

1.3.1 RLKs are involved in many central signaling pathways

Since the identification of the first RLK more than two decades ago (Walker and Zhang, 1990) only about 10% of Arabidopsis RLKs has been characterized. The most extensively studied RLKs belong to LRR-RLK subfamily and are involved in important signaling processes such as growth and development such as brassinosteroid signaling *via* BRASSINOSTEROID INSENSITIVE 1 (BRI1), meristem development control by CLAVATA1 (CLV1) (Li and Chory, 1997; Clark et al., 1997; De Smet et al., 2009) and resistance to bacteria such as the perception of flagellin by FLAGELLIN SENSING 2 (FLS2) (Gomez-Gomez and Boller, 2000). Interestingly, most of the RLK complexes identified in Arabidopsis seem to be complexes between the same RLK subfamily members, especially between LRR-RLK subfamily members (Gou et al., 2010; Han et al., 2014). For example, FLS2 and EF-Tu receptor (EFR) are LRR-RLKs involved in innate immunity. FLS2 and EFR are pattern recognition receptors (PRRs) for different pathogen-associated molecular patters (PAMPs), whose perception leads to PAMP-triggered immunity (PTI) restricting pathogen growth (Albert et al., 2010; Nürnberger and Kemmerling, 2006; Postel and Kemmerling, 2009; Schwessinger and Zipfel, 2008). The extracellular domains of FLS2 and EFR bind flagellin (or the surrogate flg22 peptide) and elongation factor Tu (EF-Tu, or the surrogate elf18/elf26 peptides), respectively (Chinchilla et al., 2006; Gomez-Gomez and Boller, 2000; Kunze et al., 2004; Zipfel et al., 2006). Interestingly, in both cases ligand binding leads to complex formation with the BRI1-associated receptor kinase (BAK1) (Chinchilla et al., 2007; Heese et al., 2007; Roux et al. 2011). BAK1 is a comparatively small LRR-RLK identified originally to interact with BRI1, which is a LRR-RLK receptor for the growth-promoting plant steroid hormones brassinosteroids (BR) (Kinoshita et al., 2005; Li et al., 2002; Nam and Li, 2002; Li and Chory, 1997; Wang et al., 2001). As it has been reported that BAK1 interacts also with LRR-RLKs PEP1 receptor 1 (PEPR1) and PEPR2, BAK1 seems to be an important partner in many different signaling pathways and more LRR-RLK/BAK1 receptor pairs are likely to be revealed in the future (Chinchilla et al., 2009; Postel et al., 2010). The latest LRR-RLK identified to interact with BAK1 was the enzymatically

inactive BAK1-interacting receptor-like kinase 2 (BIR2) (Blaum et al., 2014; Halter et al., 2014a, b). This interaction negatively regulates PAMP-triggered immunity (PTI) by limiting BAK1-receptor complex formation in the absence of ligands (Blaum et al., 2014; Halter et al., 2014a, b).

Signaling pathways *via* the multifunctional BAK1 are well-characterized and especially the BRI1/BAK1 pair has become one of the best-studied plant receptor models. Results cover the whole signaling pathway from the ligand binding and receptor activation to the endocytosis of the receptor complex, cytoplasmic phosphorylation events, BR-related pathways until the regulation of BR-responsive genes (Chinchilla et al., 2009; Han et al., 2014; He et al., 2007, 2008; Jiang et al., 2015; Karlova and de Vries, 2006; Kemmerling et al., 2007; Kemmerling and Nürnberger, 2008; Shimada et al., 2015; Vert, 2008; Zhang et al., 2014). Other well-characterized LRR-RLK receptor complexes are the plant immunity related FLS2/EFR and BAK1 pairs (Han et al., 2014; Kadota et al., 2014 Li et al., 2014; Postel and Kemmerling, 2009; Schwessinger and Zipfel, 2008), and development and growth related CLAVATA and ERECTA complexes (Bleckmann et al., 2010; Clark et al., 1993, 1997; Guseman et al., 2010; Lee et al., 2012; Pillitterri and Torii, 2012; Zhu et al., 2010). The obtained results from LRR-RLK receptor complex formation and activation mechanism provide valuable information which will facilitate identification of other RLK receptor complexes and their function in the near future (Han et al., 2014; Roux and Zipfel, 2012).

During the recent years a huge amount of information has been gained about the function of RLKs in *Arabidopsis thaliana* but less is known about their role in crop plants. Despite the fact that research focus has been on improving the stress tolerance of economically important crop plants, only few RLKs have been characterized in crop plants. However, the few RLKs characterized from crop plants e.g., barley (Hordeum vulgare), maize (*Zea mays*) and wheat (*Triticum aestivum*), are involved in similar biological processes as in *Arabidopsis thaliana*. For example, rice XA21, barley CRK1 and wheat TaRLKs, are involved in ROS mediated disease resistance (Lee et al., 2009; Rayapuram et al., 2011; Song et al., 1995; Zhou et al., 2007). A tomato LysM-RLK, *AvrPtoB tomato-interacting 9* (*Bti9*), is involved in plant immunity and a rice LRR-RLK, *defective in outer cell layer specification 1* (*Docs1*), is involved in the proper development of root outer cell layers (Zeng et al., 2012 and Huang et al., 2012, respectively). In addition, RLKs are also involved in developmental processes controlling symbiosis between plant roots and bacteria and fungi (Berrabah et al., 2014; Yoshida and Parniske, 2005). The obtained results suggest that RLKs have as important functions in crop plants as in model plant *Arabidopsis thaliana*. Better understanding of RLK function in crop plants should provide new clues for improving crop plants' tolerance mechanisms against environmental challenges.

According to the obtained results, RLKs are activated by ligand binding or conformational change induced by receptor complex formation upon ligand binding. RLKs can bind different kind of ligands, such as carbohydrates,

polypeptides, cell wall components, hormones, etc., depending on their extracellular domain (Gish and Clark, 2011; Morris and Walker, 2003). For example, a recent study by Ranf et al. showed that the bulb-type (B-type) lectin S-domain (SD) -1 receptor-like kinase LORE (SD1-29) mediates lipopolysaccharide (LPS) sensing in *Arabidopsis thaliana (*Ranf et al., 2015). SD1-RLKs have different protein motifs on their extracellular domain for possible signal perception: an amino-terminal B-type lectin domain followed by an S-locus glycoprotein domain or epidermal growth factor-like domain and a plasminogen-apple-nematode domain (PAN) (Figure 3). Arabidopsis LORE mutants, *lore1*, *lore2*, *lore3* and *lore4*, which had mutations in their extracellular protein domains and in the kinase domain, were hypersusceptible to *Pseudomonas syringae* infection (Ranf et al., 2015). The study showed that LORE specifically sensed the lipid A moiety of *Pseudomonas* and *Xanthomonas* LPS and that lipid A moiety was enough for recognition (Ranf et al., 2015).

A significant number of RLKs is involved in the response to environmental cues (Kangasjärvi and Kangasjärvi, 2014; Osakabe et al., 2013; Sierla et al., 2013). Thus the role of RLKs in ROS sensing and signaling has become an interesting research topic. Resent research has suggested that ROS have important roles in receptor activation. Apolastic ROS which are released during stressful conditions can damage cell wall components and apoplastic proteins and the breakdown products might act as ligands for receptors. For example, a short peptide cleaved of from the extracellular GRIM REAPER (GRI) protein by ROS acts as a ligand for pollen-specific receptor-like kinase 5 (PRK5) protein, which is a LRR-RLK involved in ROS-induced cell death (Wrzaczek et al., 2009, 2015). It has also been suggested that ROS molecules could activate receptors by direct redox modifications on their extracellular domain e.g. cysteine residues leading to conformational changes and thus receptor activation and signal transduction. Especially, it has been proposed that cysteine-rich RLKs such as CRKs could be activated by such redox modification and thus act as ROS sensors (II; Kangasjärvi and Kangasjärvi, 2014; Wrzaczek et al., 2013).

1.4 The role of cysteine-rich protein kinases in ROS signaling

The cysteine-rich protein kinases (CRKs), also known as DUF26 RLKs, are one subfamily of RLKs. CRKs have a typical RLK domain structure i.e. they contain an extracellular domain responsible for signal perception, a transmembrane domain and a conserved intracellular protein kinase domain responsible for signal transduction (Figure 2). Three CRKs, CRK43, CRK44 and CRK45, consist only of the cytoplasmic domain, thus they are considered to be receptor-like cytoplasmic kinases (RLCKs) (Shiu et al., 2004). The subfamily contains 44 members in *Arabidopsis thaliana* (I; II; III). The CRKs are classified by one to three copies of the extracellular DUF26-domain (Domain of Unknown Function, PF01657; stress-

antifungal domain; http://pfam.sanger.ac.uk/family/PF01657), which contain three conserved cysteine (Cys; C) residues in C-X_8-C-X_2-C configuration (Chen, 2001; Shiu and Bleecker, 2001). The function of the domain is unknown but it is suggested to have a function in redox regulation and protein-protein interaction (I; Chen et al., 2004; Wrzaczek et al., 2013). The redox modification possibilities of the extracellular Cys residues and comprehensive transcriptional and phenotypic analyses of *crk* mutants suggest that the CRKs could be involved in apoplastic ROS sensing (I; II; Chen et al., 2004; Czernic et al., 1999; Kangasjärvi and Kangasjärvi, 2014; Munné-Bosch et al., 2013; Wrzaczek et al., 2013).

1.4.1 CRKs are involved in development and stress responses

Overlapping functions due to sequence similarity has caused difficulties in analyzing the phenotypes of the T-DNA insertion mutants in *Arabidopsis thaliana*. Nevertheless, few loss-of-function phenotypes have been observed. Results of Burdiak et al. (2015) show that development is altered in *crk5* as it shows early senescence compared to Col-0 wild type (Burdiak et al., 2015). In addition, *crk5* is sensitive to ultraviolet radiation and has impaired stomatal conductance (Burdiak et al., 2015). Ederli et al. have reported that *crk20* showed a slight reduction in *Pseudomonas syringae* pv. *tomato (Pst)* growth (Ederli et al., 2011), and Tanaka et al. reported that *crk36* showed increased sensitivity to abscisic acid (ABA) and osmotic stress (Tanaka et al., 2012). CRK45 is involved in ABA signaling by positively regulating ABA responses in seed germination, early seedling development and abiotic stress responses (Zhang et al., 2013). In contrast to *crk36*, *crk45* was less sensitive to ABA than wild type (Zhang et al., 2013). Plants overexpressing *CRK45* were more sensitive to ABA and hypersensitive to salt and glucose inhibition of seed germination, whereas the knockout mutants showed the opposite phenotypes (Zhang et al., 2013). Furthermore, *CRK45* overexpression plants showed enhanced drought tolerance (Zhang et al., 2013). In addition to abiotic stress responses, few overexpression studies have suggested that CRKs have a role in cell death and disease resistance. Chen et al. reported that overexpression of *CRK4*, *CRK5*, *CRK19* and *CRK20* leads to induced hypersensitive response (HR) –like cell death (Chen et al., 2003, 2004) and Acharya et al. reported that overexpression of *CRK13* leads to enhanced tolerance to the bacterial pathogen *Pseudomonas syringae* pv. *tomato* (*Pst*) (Acharya et al., 2007). Recent results of Yeh et al. (2015) show that overexpression of *CRK4*, *CRK6* and *CRK36* leads to enhanced pattern-triggered immunity (PTI) and suggests that CRK4, CRK6 and CRK36 can associate with the well-characterized pattern-recognition receptor (PRR) FLS2 (Yeh et al., 2015). Appendix 1 lists Arabidopsis CRKs and the publications concerning their function.

Despite the recent interest in the roles of CRKs in Arabidopsis, relatively few CRKs have been characterized from other species. However, some CRKs have been connected to ROS signaling also in crop plants. For example, barley (*Hordeum vulgare*) HvCRK1 is involved in ROS-mediated basal resistance against powdery

mildew infection and wheat (*Triticum aestivum*) TaCRK1 against *Rhizoctonia cerealis* infection (Rayapuram et al., 2011; Yang et al., 2013). *TaCRK1* transcript abundance increased after *R. cerealis* infection and exogenous abscisic acid (ABA) treatment (Yang et al., 2013). However, silencing of *TaCRK1* transcript did not lead to susceptibility to *R. cerealis* (Yang et al., 2013). In rice, an apoplastic protein, *O.sativa* root meander curling, OsRMC, with DUF26 domains in its extacellular domain is reported to be involved in salt stress response and in the development of rice roots (Zhang et al., 2009 and Jiang et al., 2007, respectively). In *Medicago truncatula*, SymCRK is involved in symbiotic interactions preventing early senescence and defense responses (Berrabah et al., 2014). These examples suggest that CRKs in other species are involved in similar ROS-mediated processes as in *Arabidopsis thaliana*. Conserved biological functions suggest high biological importance for CRKs in plants.

1.4.2 CRKs in ROS perception

CRKs have been suggested to be involved in ROS perception (Kangasjärvi and Kangasjärvi, 2014; Shapiguzov et al., 2012; Vainonen and Kangasjärvi, 2014; Wrzaczek et al., 2010, 2011, 2013).The suggestion is based on structural properties of the CRK extracellular domain, plasma membrane localization and gene expression analyses which show that several members of the family display elevated transcript levels in response to pathogen infection or treatment with reactive oxygen species, salicylic acid and ozone (O_3) (Du and Chen, 2000; Chen, 2011; Czernic et al., 1999; Lehti-Shiu et al., 2009; Ohtake et al., 2000; Wrzaczek et al., 2010). Most of the *CRKs* display elevated transcript levels in response to ROS and many CRKs are involved in ROS signaling pathways but could CRKs really act as ROS sensors? In their extracellular domain CRKs have a distinct set of conserved Cys residues. Cysteines have special roles in protein chemistry. They are important not only to the catalytic activity of many enzymes (e.g., thiol enzymes) but also in the formation of disulphide bonds between two thiol groups of Cys residues. Disulphide bonds (i.e. disulphide bridges) can be internal or they can form between two proteins (i.e. protein-protein interaction) and they have an important role in protein folding and stability (e.g., secondary, tertiary and quaternary protein structure). Disulphide bonds can be redox regulated i.e. bonds can form under oxidative conditions or break under reducing conditions which can lead to conformational changes as disulphide bonds rearrange according to the redox conditions (Akter et al., 2015; Foyer and Noctor, 2005; Finkel, 2011; Joeng et al., 2011; Møller et al., 2007; Spadaro et al., 2010; Wang et al., 2012; Waszczak et al., 2015). A well-characterized example of the redox regulation of Cys residues in Arabidopsis is the NONEXPRESSOR OF THE PATHOGENESIS RELATED 1 (NPR1), which is the master regulator of SAR (Mou et al., 2003). SA induced redox changes lead to the opening of the intermolecular disulphide bonds and to the monomerisation of the oligomeric NPR1 protein complex. The monomerisation leads to the translocation of the protein from the cytosol to the nucleus, interaction

with redox-sensitive TGA transcription factors and defense gene induction (Mou et al., 2003). Potentially, the Cys residues in the CRK extracellular domain could react to changes in the redox conditions leading to conformational change and thus receptor activation (D'Autréaux and Toledano, 2007; Spadaro et al., 2010).

1.4.3 DUF26 domain

The extracellular DUF26 domain is plant specific and contains three conserved Cys residues in C-X_8-C-X_2-C configuration. Most of the CRKs, including CRK6 and CRK7, have two copies of DUF26 domains in their extracellular domain. CRK15 and CRK27 are predicted to have only a single DUF26 domain while CRK23 has three DUF26 domains. CRK43, CRK44 and CRK45 lack DUF26 domains as they lack the extracellular domain. In most cases the first DUF26 domain is located approximately between amino acids 80 and 110 and the second from amino acids 195 to 220. CRK23's third motif is located between amino acids 310 and 330.The DUF26 domain is found in at least 50 secreted Arabidopsis proteins and in eight *Arabidopsis* PLASMODESMATA-LOCATED PROTEINs (PDLPs) (Amari et al., 2010; Thomas et al., 2008; Vaattovaara et al., in preparation). PDLPs resemble CRKs but they lack the intracellular kinase domain resembling the receptor-like proteins (RLPs). The function of the DUF26 domain is unknown but the structural analysis of the DUF26 domain of ginkbilobin-2 (Gnk2) from *Ginkgo biloba* has provided first evidence for intramolecular disulphide bridges between Cys residues (Miyakawa et al., 2009). Three internal disulphide bridges are formed between: Cys10-Cys86, Cys62-Cys71, and Cys74-Cys99 (Miyakawa et al., 2009). Every cysteine in the Gnk2 DUF26 motif seems to be critical for the 3-dimensional structure as they all are involved in the formation of disulphide bonds.

1.4.4 CRK kinase domain

Arabidopsis RLKs resemble animal receptor tyrosine kinases (RTKs). RLKs and animal RTKs share the same domain organization: extracellular ligand-binding domain, a single-pass transmembrane domain and an intracellular kinase domain (Figure 2; Cock et al., 2002; Shiu and Bleecker, 2001). The conserved domain structure between animal and plant receptor kinases suggests similar functional mechanism for signal transduction including ligand binding, receptor complex formation and phosphorylation events (Cock et al., 2002; Hubbard, 2004; Hubbard and Till, 2000; Johnson and Ingram, 2005; Lemmon and Schlessinger 2010; Morris and Walker, 2003; Oh et al., 2009b; Pawson and Nash, 2000; Shiu and Bleecker, 2001a; Ullrich and Schlessinger, 1990; Wang et al., 2005a, b, 2008). Generally speaking, protein kinases are molecular switches whose activity can be either "on" or "off" depending on their three-dimensional conformation (Huse and Kuriyan,

2002). However, protein kinases can adopt many distinct conformations depending on their functional state and this conformational plasticity is important for kinase regulation (Huse and Kuriyan, 2002; Tong and Seeliger, 2015). In general, protein kinases are kept in "off" state by various regulation mechanisms (Huse and Kuriyan, 2002).

The general RLK and RTK receptor activation mechanism is phosphorylation dependent and reversible phosphorylation plays a key role in downstream signal transduction. Protein kinases transfer the γ-phosphate from ATP (ATP -> ADP) to the hydroxyl group of serine (Ser; S), threonine (Thr; T), or tyrosine (Tyr; Y) and are thus able to regulate the function, localization, stability and protein-protein interactions of their target proteins. The active "on" conformation is structurally very similar in all protein kinases while the inactive "off" conformation varies among protein kinases (Huse and Kuriyan, 2002; Johnson et al., 1996; Noble et al., 2004; Tong and Seeliger, 2015). The catalytic cleft is formed between the small and large lobes (N-lobe and C-lobe, respectively), where the small lobe binds ATP and the large lobe binds the protein substrate. Phosphorylation can be counteracted by phosphatases which dephosphorylate their target proteins (ADP -> ATP). Albeit the structural and functional similarity, RLKs and RTKs display differences in their kinase specificity towards substrates. According to the results, Ser/Thr kinase specificity is more common in plant RLKs, whereas animal RTKs show mostly Tyr kinase specificity (Cock et al., 2002; Shiu and Bleecker, 2001).

Nevertheless, some RLKs have also Tyr kinase activity (de la Fuente van Bentem and Hirt, 2009; Mithoe and Menke, 2011; Nakagami et al., 2010; Oh et al., 2009b; Sugiyama et al., 2008). For example, in addition to Ser/Thr activity, the recently found Tyr phosphorylation of BRI1 and BAK1 seems to play essential role in BR-signaling (Figure 4; Macho et al., 2015). The exact order of events is not known but it is suggested that release of BRI1 KINASE INHIBITOR 1 (BKI1) from BRI1 precedes formation of the BRI1-BAK1 heterodimer (Clouse et al., 2012). BL binding to BRI1 leads to the several phosphorylation events, e.g. BAK1 is phosphorylated at the TYR610 (Y610) site and BRI1 at the Tyr831 (Y831), Tyr956 (Y956) and Thr1049 (T1049), and to the release of BKI1 and formation of BRI1-BL-BAK1 receptor complex (Oh et al., 2009b, 2010). Phosphorylation of BAK1 at the Tyr610 (Y610) and BRI1 at Thr1049 (T1049) are essential for BR signaling (Oh et al., 2010 and Wang et al., 2005a, respectively). According to the structural studies of BRI1-BL-BAK1 complex, BRI1 and BAK1 together form the hormone binding pocket and interact with BL (Santiago et al., 2013). Thus BL acts as a "molecular clue" promoting association of BRI1 with BAK1 (Santiago et al., 2013). The formation of BRI1-BL-BAK1 complex induces interaction of their cytoplasmic kinase domains and activation of the downstream signaling pathway (Santiago et al., 2013). The formation of BRI1-BL-BAK1 signaling complex activates the PP1-related protein phosphatase BRI SUPPRESSOR 1 (BSU1) (Jaillais et al., 2011), which leads to the inactivation of BRASSINOSTEROID INSENSITIVE 2 (BIN2) by dephosphorylation at the Tyr200 site (Tang et al., 2010). Inactivation of BIN2 leads to the dephosphorylation of the transcription factors BRASSINAZOLE-RESISTANT1/2

(BZR1/2) whose activation leads to the BR-responsive gene expression (Tang et al., 2011; Wang et al., 2002). The very detailed information of BR-signaling pathway starting from the ligand binding to the essential phosphorylation events provides a valuable model for studying other RLK signaling complexes and their functional mechanisms (Figure 4; Belkhadir and Chory, 2006; Bojar et al., 2014; Han et al., 2014; Hothorn et al., 2011; Oh et al., 2009b; Santiago et al., 2013; Wang et al., 2005a, b, 2008; Wang and Chory, 2006).

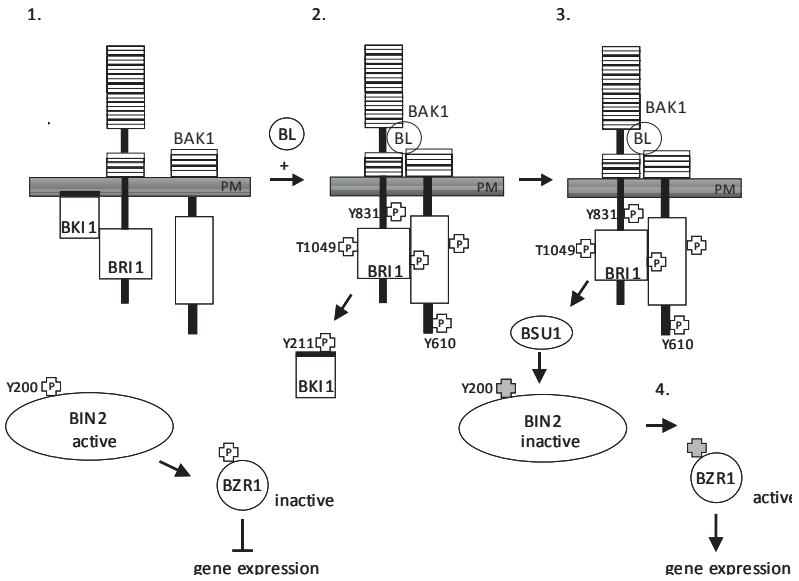

Figure 4. Simplified schematic representation of some of the essential Ser/Thr and Tyr phosphorylation events regulating the BR signaling pathway.

1. In the absence of BL ligand, BRI1 and BAK1 kinases are separated and inactive in the plasma membrane (PM). The cytoplasmic BIN2 kinase is active due to phosphorylation on Tyr-200 (Y200) and inactivates BZR1 by phosphorylation. Inactive BZR1 repress BL-regulated gene expression (Wang et al., 2002)
2. BL binding to the BRI1 leads to the phosphorylation of several Ser (S) and Thr (T) residues including the essential T1049 site and thereby BRI1 kinase activation. The BRI1 phosphorylates BKI1 Y211 site, located within the membrane targeting motif, which leads to the release of inhibitor BKI1 from BRI1. Release of BKI1 inhibition allows BRI1 association with BAK1.
3. The formation of BRI1-BL-BAK1 receptor complex activates auto- and/or trans-phosphorylation events and leads to the activation of the PP1-related protein phosphatase BSU1 which dephosphorylates soluble BIN2 kinase at Y200 site and thereby inactivates BIN2.
4. Inactivation of BIN2 activates transcription factor BZR1 through dephosphorylation by PP2A family members which leads to the activation of BL-regulated gene expression.

Other BRI1 interacting proteins, such as transthyretin-like (TTL) protein, TGF-β receptor interacting protein 1 (TRIP-1) and kinase-associated protein phosphatase (KAPP), are not included in the figure. Modified from Clouse et al., 2012.

Predictions of kinase function can be done also by analyzing the amino acid sequence of the kinase domain (Johnson et al., 1996; Nolen et al., 2004; Zhou et al, 2007). The importance of the kinase function is reflected in the conservation of the amino acid sequence between eukaryotic protein kinases. As the three-dimensional structure is based on the amino acid sequence, the structurally important residues are highly conserved among protein kinases and mutations in these residues leads to the changes in kinase activity. The catalytic domain contains 250-300 amino acids. This area contains 11 conserved subdomains with highly conserved amino acids which are essential for the kinase function (Figure 5; Hanks and Hunter, 1995; Hanks et al., 1988; Walker, 1994). Protein structures which have functional importance usually contain residues from more than one subdomain. In general, the juxtamembrane domain (JM) is important for dimerization and thereby for kinase activity (Jura et al., 2009). Phosphorylation of amino acid residues in the JM and carboxyterminal (CT) region is also important as phosphorylation-induced conformational changes may release the kinase activity inhibition (Hubbard, 2004; Pawson 2004). In addition, phosphorylation of JM and CT residues generates docking sites for kinase interacting substrates (Clouse et al., 2012). Other essential residues for kinase activity are located in the activation loop formed between the kinase subdomains VII and VIII and the catalytic loop of subdomain VIb (Figure 5; Hubbard, 2004; Pawson 2004).

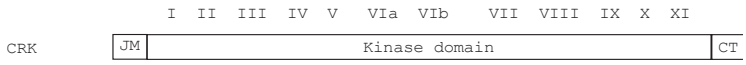

Figure 5. Schematic representation of the cytoplasmic CRK juxtamembrane (JM), kinase domain and carboxy terminus (CT) organization and the localization of the conserved subdomains (I-XI).

Definition of the conserved kinase subdomains I-XI:
Subdomain I forms two beta strands connected by the glycine-rich ATP-binding loop with the conserved GxGxxG motif.
Subdomain II contains a highly conserved lysine (K) residue that interacts with the phosphates of ATP.
Subdomain III forms an α-helix structure whose orientation is central for kinase activity. ATP is coupled to subdomain III by the salt bridge that is formed between the highly conserved glutamate (E) of the subdomain III and the lysine (K) from the subdomain II.
Subdomain IV forms a structurally important β-strand of the small lobe (N-lobe).
Subdomain V is structurally very important. It links together the small and large lobes and contributes residues to the ATP binding pocket and for peptide substrate binding.
Subdomain VIa forms a long α-helix that parallels another α-helix from subdomain IX.
Subdomain VIb contains the catalytic loop with the conserved HRDLKxxN motif where D is highly conserved in active kinases.
Subdomain VII forms Mg-binding loop with the DFG motif. Activation loop is formed between the DFG motif of the subdomain VII and the APE motif of the subdomain VIII. D and G of the DFG motif are conserved in active kinases.
Subdomain VIII contains structurally essential amino acids. In addition to the activation loop that it forms together with subdomain VII, it forms a salt bridge (between the glutamate (E) of the subdomain VIII and the arginine (R) in the subdomain XI) that is critical for forming the stable kinase core. P+1 loop starts from APE motif until the phosphorylated residue. P+1 residue is the next residue in the sequence.
Subdomain IX forms a very hydrophobic α-helix and contains an invariant aspartate (D) residue in the DxWxxG motif.
Subdomain X and subdomain XI forms three α-helices that form the kinase core and which are involved in substrate binding. Subdomain XI contains a conserved arginine (R).

Kinases can be divided into two classes, RD or non-RD kinases, according to the presence or absence of an arginine (R) residue before the conserved aspartate (D) residue in the catalytic loop of subdomain VIb (Figure 5). Most of the plant and animal kinases, including RLKs, are RD-kinases. In RD kinases, the positively charged R residue resides in a charged cluster and inhibits catalysis by the neighboring negatively charged D residue keeping the kinases in inactive state. Phosphorylation of the activation loop removes inhibition by introducing negative charges which neutralize the positively charged R residue leading to kinases activation (Adams, 2003; Johnson et al., 1996). Non-RD kinases, which lack the R residue, usually have an uncharged residue such as Cys, Gly, Phe, or Leu in its place (Dardick et al., 2012; Krupa et al., 2004). The lack of a positive charge leads to the possible mechanistic differences in the mode of action of non-RD kinases, such as the lack of auto-phosphorylation of the activation loop leading to either constitutively active or inactive mode (Dardick et al., 2012). Most of the characterized non-RD kinases in plants are pattern recognition receptors (PRRs), such as FLS2 and EFR, which recognize conserved microbial signatures leading to innate immunity *via* PTI (Pathogen Associated Molecular Pattern (PAMP) Triggered Immunity) pathway (Dardick et al., 2012; Dardick and Ronald, 2006; Jones and Dangl, 2006; Ronald and Beutler, 2010; Schwessinger and Ronald, 2012). Recently, it was reported that in *Medicago truncatula*, the non-RD kinase symCRK is involved in symbiotic interactions, which are connected to immunity, preventing defense responses during symbiosis (Berrabah et al., 2014). Thus the absence of RD-motif in a kinase could suggest a role in innate immunity signaling but not *vice versa* as there are kinases with RD-motif which are involved in innate immunity, such as the ones involved in DAMP (Danger-Associated Molecular Patterns) perception (Schwessinger and Ronald, 2012). In conclusion, by analyzing the absence or presence of the conserved motifs or amino acids of the kinase domain it is possible to make predictions about the kinase domain function e.g., activity and kinase specificity (Ser/Thr vs. Tyr phosphorylation) or the possible biological role in signaling.

According to such predictions, roughly 13% of all Arabidopsis kinases are kinase-defective i.e. pseudokinases, whereas the level in humans and yeast is lower, 10% and 6%, respectively (Castells and Casacuberta, 2007). Interestingly, in the *Arabidopsis* RLK family the level of kinase-defective RLKs is 20% (Castells and Casacuberta, 2007). The high level of pseudokinases in plants, especially among RLKs, could be a result of duplication based expansion of the kinase family and might be on their way to becoming pseudogenes (Lehti-Shiu and Shiu, 2012). However, during the evolution, pseudokinases might have been also maintained due to new functions gained in signal transduction (Stein and Staros, 2000). Most of the observed kinase-defective RLKs belong to the LRR-RLK subfamilies or to the receptor-like cytoplasmic kinase (RLCK) subfamilies (Blaum et al., 2014; Castells and Casacuberta, 2007; Chevalier et al., 2005; Halter et al., 2013). For example, a small peptide derived from apoplastic GRI protein is recognized by PRK5 which is a kinase deficient LRR-RLK (Wrzaczek et al., 2015). Recognition triggers ROS-dependent cell death response in Arabidopsis though yet uncharacterized pathway (Wrzaczek et

al., 2009, 2015). This observation suggests that phosphorylation independent signaling mechanism, such as protein-protein interaction based mechanisms, are also important in plant signal transduction as it has been shown for animal RTKs (Castells and Casacuberta, 2007; Kroiher et al., 2001). Also STRUBBELIG kinase, which is central for proper *A. thaliana* development, lacks enzymatic phospho-transfer activity (Chevalier et al., 2005). Moreover, the number of predicted pseudokinases might be overestimated as some of these pseudokinases possess a kinase activity (Kannan and Taylor, 2008). For example, the human pseudokinase CASK, which lacks the essential DGF motif, possesses a kinase activity (Mukherjee et al., 2008). The result suggests that also other pseudokinases might be active in physiologically relevant environments and opens a new vision into the phospho-transfer reactions and kinase activation mechanisms (Kannan and Taylor, 2008; Mukherjee et al., 2008).

Interestingly, the results obtained from LRR-RLKs suggests that *in vitro* kinase domain autophosphorylation sites are highly predictive of *in vivo* phosphorylation sites (Clouse et al., 2012; Oh et al., 2000; Wang et al., 2005a, 2008). Thus comparing the *in vitro* phosphorylation sites with the *in vivo* phosphorylation sites is a valuable *in silico* analysis in pre-examining the kinase domain function (Clouse et al., 2012; Oh et al., 2000; Wang et al., 2005a, 2008). Advances in technology, such as in mass spectrometry and phosphopeptide enrichment, have facilitated the analysis of the kinase domain function and understanding of RLK phosphorylation mechanism and its role in plant signal transduction. Unravelling the mechanisms of CRK phosphorylation and its role in signal transduction will provide new clues for plants' adaptation mechanisms and hopefully one day possibilities for enhancing plants' tolerance mechanisms against future challenges.

2 AIMS OF THE STUDY

The topic of this PhD study is the role of CRKs in ROS signaling in *Arabidopsis thaliana*. As sessile organism plants cannot "walk away" from stressful conditions, instead they have to face the conditions on the very spot they are growing. Adaptation to continuously changing, even stressful, environmental conditions demands extensive amount of signaling. ROS are universal messenger molecules in plants known to deliver signals in growth, development and environmental related processes. Adaptation is based on flexible interactions between hormone and ROS signaling which controls growth and defense responses. Despite the extensive research on ROS field during the last two decades, some fundamental questions still remain: how are ROS sensed and how is ROS signaling specificity achieved? The cysteine-rich CRK ectodomain can potentially sense apoplastic redox changes due to which CRKs are interesting candidates for ROS sensors. In addition, large number of CRKS and overlapping functions due to sequence similarity between the family members could provide specificity and security for signal transduction in essential biological pathways.

To gain understanding of the role of CRKs in ROS signaling the transcriptional responses of the whole *CRK* family members and the phenotypical responses of the *crk* family mutants to ROS-inducing conditions were analyzed with special interest in CRK6 and CRK7. For thorough analysis of CRK6 and CRK7, overexpression, complementation, multi-knockdown lines and promoter-GUS lines were created. In addition to stress related studies, some growth and development related studies were performed to analyze the role of CRKs in growth and development.

The specific aims of this project were:

1. To study the role of CRKs in ROS mediated stress processes in *Arabidopsis thaliana* by characterizing the phenotypes of existing and novel CRK mutant lines under control and different ROS inducing conditions.
2. To study the role of CRKs in growth and development related processes.
3. To analyze the conservation and organization of ectodomain cysteine residues and their possible role in ROS sensing.
4. To analyze the kinase domain properties and predict kinase activity based on the presence or absence of the conserved kinase domains.
5. To examine CRK6 and CRK7 kinase activity *in vitro*.

3 MATERIAL AND METHODS

The materials and methods used in the articles I-III are described within each publication and only the procedures concerning unpublished results and the work in general are described here.

Plant material and growth conditions	I, II, III
Gene expression analyses	I
Transgenic plant lines	I, II, III
Plasmid transformation to *Agrobacterium tumefaciens*	II
Floral dip transformation	II
Ozone exposures	I, II, III
Ion leakage measurements	I, II, III
qPCR	I, III
Trypan blue staining	II
DAB staining	II
GUS staining	II
Xanthine + xanthine oxidase experiments	II, III
Methyl viologen assays	II, III
Pathogen treatments	II, III
in vitro kinase assay	II
Light stress experiments	III
UV-A and UV-B treatments	III
Analysis of fluorescence transients	III
Germination assay	III
Root length	III
Bolting, flowering and senescence assays	III
Salt stress	III
Water loss	III
Gas exchange	III

Transgenic plant lines used in this study

For comprehensive phenotypic characterization of the CRK family, T-DNA insertion mutants were ordered from the Nottingham Arabidopsis Stock Centre (NASC; http://nasc.life.nott.ac.uk/) (III: Fig. 1B; S3; Table S1). For CRK7, a second independent T-DNA insertion allele (*crk7-1*) was obtained. Two genes were excluded from this collection: the putative pseudogene CRK35 (At4g11500) and CRK9 (At4g23170) which has a truncated kinase domain. An age-matched seed collection was generated for homozygous *crk* mutant lines. However, no homozygous T-DNA insertion lines for *crk27*, *crk34* and *crk44* were obtained; consequently these lines were excluded from analyses. This could suggest a critical role for *crk27*, *crk34* and *crk44* in plant development. However, expression analysis did not reveal any clues towards their function (III: Table S5) thus further analyses

are needed to reveal their biological roles. Expression levels of obtained homozygous *crk* mutants were determined by RT-PCR and qPCR (III: Fig. 1B; S4). Most of *crk* mutants, including *crk6*, *crk7-1* and *crk7-2,* and the most heavily affected *crk2, crk5* and *crk31*, displayed absent or reduced transcript levels of the corresponding *CRK* (III: Fig. 1B). In *crk18* and *crk36*, the T-DNA insertion did not lead to reduction of transcription levels and in *crk15*, *crk26* and *crk30* transcript levels were even increased due to which these *crk* mutants were not included in the phenotype analyses (III). No transcript was detected for *CRK1*, *CRK32*, *CRK33* and *CRK46* in Col-0 wild type due to which their expression status is unclear (III: 1B). In total, 39 *crk* mutants were included in this study. Overexpression/complementation constructs were created only for few heavily affected *crk* mutants, such as *crk2*, *crk5* and *crk45* (III).

crk T-DNA insertion lines used in the phenotype studies:

*crk1-1**, *-2**, *crk2*, *crk3*, *crk4*, *crk5*, *crk6*, *crk7-1*, *-2*, *crk8*, *crk10-2*, *-4*, *crk11*, *crk12*, *crk13*, *crk14*, *crk16*, *crk17*, *crk19 -2*, *crk20*, *crk21-1*, *crk22*, *crk23-1*, *-2*, *crk24*, *crk25*, *crk28*, *crk29*, *crk31*, *crk32**, *crk33**, *crk37*, *crk38*, *crk39*, *crk40*, *crk41*, *crk42*, *crk45*, *crk46**

* Transcript not detected in wild type, expression status unclear

Not included:
Expression level similar to Col-0 wild type: *crk18*, *crk19-1*, *crk21-2*, *crk36*, *crk43*
Increased expression: *crk15*, *crk26*, *crk30*
No homozygous lines obtained: *crk27*, *crk34*, *crk44*
Putative pseudogenes: CRK9, CRK35

For CRK6 and CRK7, artificial microRNA (ami-RNA) lines were created where the five closely related *CRK6*, *CRK7*, *CRK8*, *CRK10* and *CRK15* showed reduced transcript abundance (II: Fig. 1A and S4). Ami-RNA lines were included in the experiments to overcome the problems caused by phenotype redundancy. For CRK6 and CRK7, in addition to the loss-of-function mutants, overexpression (*35S::CRK6* and *35S::CRK7*), genomic complementation lines (*CRK6::CRK6 and CRK7::CRK7*) and promoter::GUS lines (*CRK6::uidA* and *CRK7::uidA*) were created to verify the loss-of-function phenotypes and to visualize the stress induced spatiotemporal expression of the GUS reporter gene under the control of CRK6 and CRK7 promoters (II).

Methods used to induce ROS production in this study

Different artificial systems have been developed to study apoplastic ROS signaling (Kangasjärvi and Kangsjärvi, 2014). In this study ozone (O_3), xanthine oxidase (XO) and pathogen treatments were used to induce apoplastic ROS production in order to study ROS induced responses in different mutant plant lines. O_3 induces

extracellular ROS production and has been used to study ROS signaling events and to identify novel components in ROS signaling (Baier et al., 2005; Kangasjärvi et al., 2005; Kangasjärvi and Kangasjärvi, 2014; Overmyer et al., 2000; Vaahtera et al., 2013; Vainonen and Kangasjärvi, 2014; Wrzaczek et al., 2009; Wrzaczek et al., 2010). O_3 is a gentle and convenient tool to produce apoplastic ROS in non-invasive way. O_3 enters the cells through stomata, it degrades into various ROS in the apoplast and reacts with plasma membrane components causing oxidative damage which leads to downstream responses and ultimately cell death in sensitive plants (Kangasjärvi et al., 2005; Vaultier and Jolivet, 2015). In contrast to the rather gentle O_3 treatment, xanthine oxidase treatment is a harsh method to produce apoplastic ROS. Xanthine-xanthine oxidase (X+XO) is an enzymatic system that produces apoplastic ROS (O_2^- and H_2O_2) similar to O_3 but in comparison to O_3 treatment X+XO treatment is not controlled by the stomatal aperture. Instead, X+XO is vacuum-infiltrated to the cells leading to a more dramatic effect on ROS production. In addition, as increased ROS production in apoplast is a well-documented component of pathogen defense in plants, also the responses of *crk*s to different pathogen treatments were analyzed. UV-B treatment was used to produce ROS both in apoplast and in the chloroplasts. Chloroplastic ROS production was induced by light stress, methyl viologen (MV, also known as paraquat) and 3-(3, 4-dichlorophenyl)-1,1-dimethylurea (DCMU).

Microarray experiments

Microarray experiments were performed with ozone exposed wild-type Columbia to identify new regulators in the ozone induced stress response. Plants were grown for three weeks and then exposed to ozone (250 ppb) for seven hours. Samples were taken for RNA isolation and expression analysis after 30 min, 1 h, 2 h, 4.5 h and 8 h. About 50 of the highly ozone inducible genes and about 10 of the down-regulated genes were obtained as T-DNA knock-out mutants from SALK (http://signal.salk.edu/cgibin/tdnaexpress). Preselected knock-outs were screened with ozone and sensitive plants were identified. These include amongst others various proteins of unknown function and several RLKs. One of the RLK families identified was the CRKs.

Flowering time

For analyzing the flowering time differences between age-matched Col-0 wild type and the *crk* mutants the plants were grown in the short day (12 h photoperiod) and long day (16 h photoperiod) conditions in controlled growth chambers (Sanyo, Sakata, Japan) with 130 µmol m^{-2} s^{-1} and at 23 °C / 19 °C (day / night) under 70 % relative humidity, or in the growth rooms or green house conditions . The following developmental milestones were recorded: bolting time when the plants change from the vegetative growth state into flowering state, the day when the stem was 1cm long and the day when the first flower opened.

4 RESULTS

This PhD project focuses on ROS signaling, sensing and ROS-induced cell death. In a broader context it studies how plants sense stress and transmit stress signals at the cellular level leading to a proper response in order to survive under changing environmental conditions. Plants' enormous flexibility to respond to different environmental stresses is based on finely balanced interactions between hormonal growth regulators and ROS molecules. Information about environmental changes and cellular physiology is sensed as changes in redox balance. Through ROS-mediated crosstalk plants can regulate their responses to different stresses in order to maintain the delicate cellular balance crucial for optimal growth, reproduction and adaptation.

4.1 Localization and grouping of *CRK* genes (II, III)

Most of the *CRKs* in *Arabidopsis thaliana* are arranged in several clusters (III: Fig. S2). Chromosome IV contains the majority of *CRKs* and the biggest *CRK* gene cluster with 19 genes, including *CRK6* and *CRK7*, arranged one after the other (II: Fig. 1A; III: Fig. S2; Shiu and Bleecker, 2001). Smaller clusters are located in chromosome I and IV (III: Fig. S2). One separated *CRK* is located in both chromosome III and V (III: Fig. S2). In a cluster, CRKs are organized in back-to-back repeats separated only by short promoter areas (II: Fig. 1A). This tight genetic linkage makes traditional double/triple/quadruple mutant approaches difficult which is why other silencing techniques, such as artificial microRNA (ami-RNA)-lines, have been used to overcome the problem (II).

In general, 33% of *RLKs* in *Arabidopsis thaliana* are organized in tandem clusters, but among *CRKs* the extent of clustering is as high as 84% (Lehti-Shiu et al., 2009; Shiu and Bleecker, 2003). The *CRK* distribution into several clusters suggests that tandem duplications and an internal chromosomal duplication are behind the expansion of this subfamily (Shiu and Bleecker, 2001). CRK evolution by tandem duplications is somewhat reflected in the new grouping of CRKs. According to phylogenetic clustering based on amino acid sequence of the extracellular or the intracellular kinase domain of *Arabidopsis thaliana* CRKs can be divided into five distinct groups (Figure 8; III: Fig. S1A and B). The phylogenetic analysis suggests tightly linked evolution of both extra-and intracellular domains as division into groups according to both extra- and intracellular domain gives the same result. According to the above mentioned grouping, CRK6 and CRK7, together with their closest homologs CRK8, CRK10 and CRK15, belong to the group V which is the group most distant from the basal group I. Group I represents an ancestral clade that is common to most plant species (Vaattovaara et al., in preparation). Group I CRKs are distributed over different chromosomes, with the exception of *CRK2* and *CRK3,*

which are located next to each other on chromosome I (III: Fig. S2). T-DNA insertion mutants representing group I CRKs display the greatest difference and the most striking phenotypes compared to each other and other *crk* mutants. For example, in many cases *crk2* shows opposite phenotype compared to other *crk* mutants (III).

4.2 Transcriptional profiling of *CRKs* (I)

This study was initiated with O_3 screens. A microarray-based gene expression profiling approach was performed to identify new elements in ROS signaling (described in material and methods). Genes, whose expression was changed in response to O_3-induced increased extracellular ROS production, were identified by comparing gene expression profiles from treated and control samples. Microarray experiments were performed using O_3 exposed Col-0 wild type plants. *CRK6* and *CRK7* were among the 50 genes whose transcript abundance levels increased after O_3 treatment (Mikael Brosché, Nina Federoff and Jaakko Kangasjärvi, unpublished microarray data). CRKs were interesting ROS signaling components based on their plasma membrane localization and proposed redox regulation possibility of their extracellular thiol groups. In addition, supporting transcriptional studies had shown that CRK5, CRK6, CRK10 and CRK11, were involved in ROS mediated signaling processes as their transcript abundance increased after treatment with SA, pathogens and ROS (Du and Chen, 2000; Chen, 2001; Chen et al., 2003, 2004; Czernic et al., 1999; Ohtake et al., 2000). Later, with the help of more sensitive technology, the transcriptional responses of the *CRK* family to O_3- and pathogen/elicitor –induced extracellular and high light –induced chloroplastic ROS production were analyzed by quantitative real-time PCR (qRT-PCR) (I). In addition, the role of hormone signaling on transcriptional regulation of *CRKs* was addressed by analyzing the transcript abundance levels of *CRKs* in several mutants impaired in hormone biosynthesis and/or signaling, including the *salicylic acid induction deficient 2* (*sid2*), *non-expressor of pathogenesis-related genes 1* (*npr1*), *defense, no death 1* (*dnd1*), *ethylene insensitive 2* (*ein2*), and *fatty acid desaturase 3/7/8* (*fad3/7/8*) (I). Especially the role of SA was addressed.

Approximately 60% of the *CRKs,* including *CRK6* and *CRK7*, displayed elevated transcript abundance levels after O_3 fumigation (25 out of 42 *CRKs* at 1 h time point and 26 out of 42 *CRKs* at 8 h time point (6 h fumigation followed by 2 h recovery under clean air conditions) (I: Fig. 1). Eight *CRKs* displayed fast response i.e. their transcript levels were higher at 1h time point than at 8 h time point and 15 *CRKs* displayed late response i.e. they displayed higher transcript abundance level at 8 h time point than at 1 h time point (I: Fig.1). *CRK21*, *CRK22*, *CRK30*, *CRK33* and *CRK46* displayed reduced transcript abundance levels in response to O_3 treatment at both time points (I: Fig. 1). Sequence similarity of CRK kinase domains could not explain the transcriptional response of *CRKs* to O_3 treatment, instead O_3-induced genes were distributed across the phylogenetic tree (I: Fig.2). However, there was a

correlation with the closest family members. For example, *CRK36*, *CRK37*, *CRK38*, *CRK39* and *CRK40* were all significantly induced by O_3 (I: Fig. 2).

Transcriptional response of CRKs to high light induced chloroplastic ROS production led to transcriptional downregulation of most *CRKs*, only eight *CRKs* displayed increased transcript abundance levels (I: Fig. 3). Transcriptional response of *CRKs* to other ROS inducers, such as chloroplastic ROS producing norflurazon and paraquat and mitochondrial ROS producing rotenone, was analyzed by examining the publicly available microarray data. The study revealed that O_3-triggered expression profile was not related to the expression profiles triggered by chloroplastic or mitochondrial ROS inducers. Instead, the O_3-triggered expression profile was similar to the expression profiles triggered by several pathogen and PAMP treatment, such as as *Blumeria graminis* var. *hordei* (*Bgh*), hairpin Z (HrpZ), and flg22 (I: Fig. 4).

To study the role of plant hormones SA, ET and JA in ROS signaling, the O_3-triggered changes in transcript abundance levels of *CRKs* in Col-0 wild type plants and in the SA signaling deficient *sid2* and *npr1*, ET signaling deficient *ein2*, JA-deficient *fad3/7/8* and Ca^{2+}-transport deficient *dnd1* mutants were analyzed by qPCR (I). In general, O_3-induced transcriptional activation pattern was similar to Col-0 in *sid2* and *npr1* mutants, but stronger (I: Fig. 6). In contrast, in *ein2* and *fad3/7/8* mutants *CRKs* displayed reduced transcript abundance levels and in *dnd1* there was hardly any induction compared to Col-0 wild type plants (I: Fig. 6). The results suggest that SA plays more important role in CRK-mediated ROS signaling than ET and JA. The lack of O_3 response of *CRKs* in *dnd1* mutant could be due to increased SA levels of the *dnd1*mutant leading to stronger SA signaling compared to ROS signaling. Other reason could be the Ca^{2+}-transport deficiency of the *dnd1* mutant leading to abolished apoplastic ROS production by NADPH and O_3 response.

4.3 CRKs - possible candidates for ROS sensors?

No ROS sensor has yet been identified in plants but CRKs have been suggested as candidate ROS sensors. Based on their proposed redox regulation possibility of their extracellular thiol groups and highly conserved intracellular kinase domain they could be involved in ROS sensing.

As the name “cysteine-rich” receptor-like protein kinases suggests there are many Cys residues in the CRK extracellular domain. The DUF26 domain contains three conserved Cys residues. It has been published that the fourth Cys residue relies in the C-terminal side of the domain but its position varies slightly among subfamily members (Chen, 2001). Actually, the level of conservation is much higher. Every CRK has more Cys residues in common than just the three conserved Cys residues in the DUF26 domain. CRKs can be organized into four different categories according to the level of conserved Cys residues in the following way:

10 Cys CRKs: **C**-$X_{(45-53)}$-**C**-X_8-**C**-X_2-**C**-$X_{(10-13)}$-**C**-$X_{(12-18)}$-**C**-$X_{(48-96)}$-**C**-X_8-**C**-X_2-**C**-$X_{(3-24)}$-**C**

CRK4, CRK5, CRK6, CRK7, CRK8, CRK10, CRK15, CRK16, CRK17, CRK19, CRK20, CRK36, CRK37, CRK39, CRK40, CRK41, CRK46

11 Cys CRKs: **C**-$X_{(45-50)}$-**C**-X_8-**C**-X_2-**C**-$X_{(10-11)}$-**C**-$X_{(8-16)}$-**C**-$X_{(9-22)}$-**C**-$X_{(41-82)}$-**C**-X_8-**C**-X_2-**C**-$X_{(10-20)}$-**C**

CRK1, CRK2, CRK3, CRK38, CRK42

12 Cys CRKs: **C**-$X_{(49-68)}$-**C**-X_8-**C**-X_2-**C**-X_{11}-**C**-$X_{(12-18)}$-**C**-$X_{(74-96)}$-**C**-X_8-**C**-X_2-**C**-X($_{10-12)}$-**C**-**C**-$X_{(12-63)}$-**C**

CRK11, CRK12, CRK13 CRK14, CRK18, CRK21, CRK22, CRK24, CRK25, CRK26, CRK28 CRK29, CRK30, CRK31, CRK32, CRK33

Uncategorized: CRK23, CRK27, CRK34 and CRKs without extracellular domain: CRK43, CRK44 and CRK45.

Figure 6. The organization of the conserved Cys residues in the CRK extracellular domains. The conserved core unit (consensus sequence) with 10 Cys residues is marked with grey color. DUF26 domain is underlined. Conserved Cys residues are marked with bold letters (**C**). There are small differences in the number of amino acids between Cys residues in the core unit. CRK9 has truncated kinase domain and CRK35 is a putative pseudogene which is why they are not included in the list. In addition to these organized Cys residues some CRKs have a variable number of extra Cys residues located in the N-terminal or C-terminal side of the extracellular domain (Appendix 2.)

The core unit consists of 10 Cys residues (group 10 Cys-CRKs) and CRK6 and CRK7 together with their closest homologues belong to this group (Figure 6). In the group 11 Cys-CRKs, the core unit is divided by one extra Cys residue between the DUF26 domains (total of 11 Cys residues) (Figure 6). Group 12 Cys-CRKs have two extra Cys residues in C-$X_{(12-63)}$-C configuration after the core unit (total of 12 Cys residues) (Figure 6). Czernic et al. reported that RLK3 (CRK11) contains 12 Cys residues and that eight of these Cys residues are found within two stretches where they are organized as C-X_8-C-X_2-C-X_{11}-C configuration (Czernic et al., 1999). Only few CRKs cannot be classified into these three categories. CRK27 and CRK34 resemble the core unit configuration except CRK27 has C-X_9-C-X_2-C configuration in the place of the first DUF26 domain and CRK34 has two Cys residues in a row (C-C) between the two DUF26 domains (Appendix 2). CRK23 has three DUF26 domains: a DUF26 domain plus the 11 Cys unit which includes two DUF26 domains (Appendix 2). In addition to these organized Cys residues some CRKs have a variable number of extra Cys residues located in the N-terminal or C-terminal side of the extracellular domain (Appendix 2.) CRK43, CRK44 and CRK45 cannot be classified as they lack extracellular domain.

The sequence similarity between ginkbilobin-2 (Gnk2) from *Ginkgo biloba* and CRK extracellular domains is approx. 30% (Miyakawa et al., 2009). Interestingly, Gnk2, CRK6 and CRK7 have identical organization of Cys residues in the first DUF26 motif

(Figure 7). Three internal disulphide bridges (Cys10-Cys86, Cys62-Cys71, and Cys74-Cys99) have been identified for Gnk2 (Miyakawa et al., 2009). The structure of CRK6 and CRK7 is not determined but it could be postulated that three internal disulphide bonds are formed similarly in CRK6 (Cys34-Cys110, Cys86-Cys95, and Cys98-Cys123) and in CRK7 (Cys33-Cys109, Cys85-Cys94, and Cys97-Cys122). Noteworthy, as CRK6 and CRK7 have two copies of DUF26 domain in their extracellular domain, whereas Gnk2 has only one, the 3-dimensional domain structure might not form similarly in CRKs. However, the conserved organization of Cys residues suggests that they are important for the three-dimensional structure of the DUF26 domain while some of the extracellular Cys residues could have a role in ROS signaling.

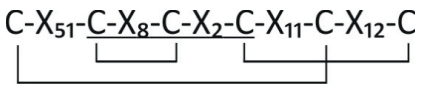

Figure 7. The organization of Cys residues in the first DUF26 motif of CRK6, CRK7 and Gnk2 is identical. The DUF26 domain is underlined. The three disulphide bridges (Cys10-Cys86, Cys62-CYs71, Cys74-Cys99) have been identified for Gnk2 (Miyakawa et al., 2009).

The level of Cys conservation correlates well with the phylogenetic grouping done in the publication III (III: Fig. S1A and B) i.e. the members of the phylogenetic groups I-V have similar number of conserved Cys residues in their ectodomain with few exceptions in the group I, II and III. Group III and V CRKs have a 10 Cys configuration, group I CRKs have a 11 Cys configuration and group II and IV CRKs have a 12 Cys configuration (Figure 8).

In conclusion, the conserved organization of Cys residues suggests that they are important for the three-dimensional structure of the DUF26 domain and some of the extracellular Cys residues could have a role in ROS signaling. Potentially, the Cys residues could react to changes in the redox conditions leading to conformational change and thus receptor activation (D'Autréaux and Toledano, 2007; Spadaro et al., 2010).

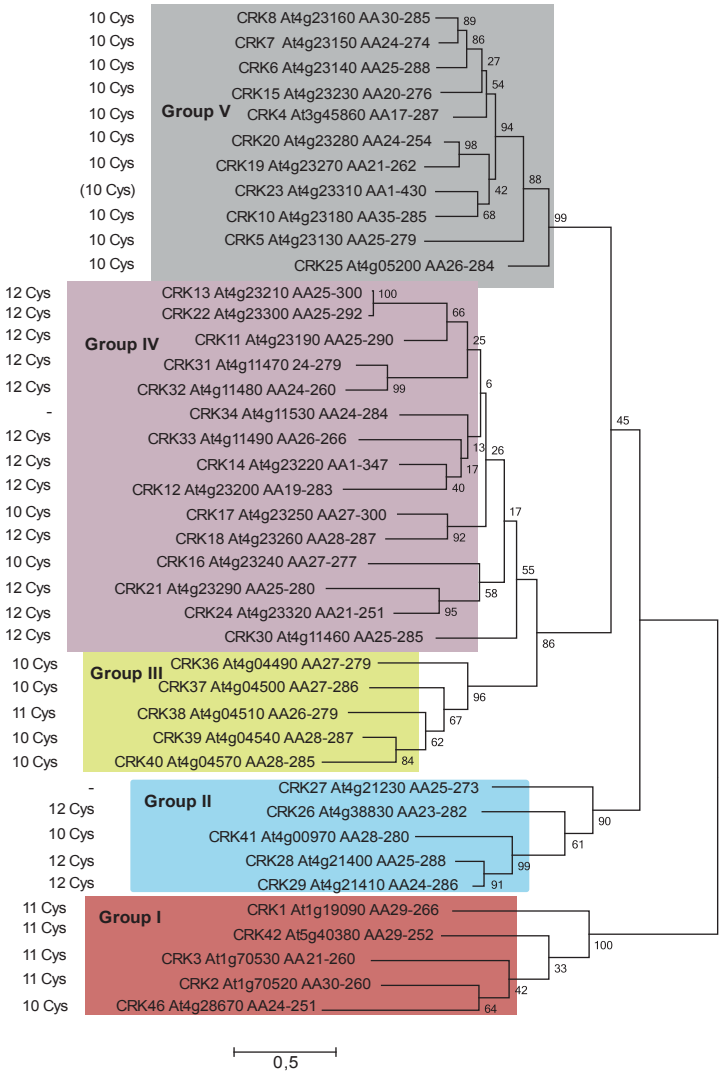

Figure 8. Correlation of phylogenetic clustering according of the CRK ectodomain and Cys residue organization in CRKs. CRK43, CRK44 and CRK45 are cytoplasmic CRKs (RLCKs) without extracellular domain and CRK9 (truncated kinase domain) and CRK35 (putative pseudogene) are not included in the figure.

4.4 The role of CRKs in apoplastic ROS signaling

4.4.1 One for all and all for one – the effect of redundancy in *crk6* and *crk7* responses to ozone and xanthine oxidase (II, III)

Oxidative stress causes plasma membrane damage and electrolyte leakage is one sign of such damage. Therefore, an easy way to quantify O_3 and X+XO induced cell damage is to measure the amount of electrolyte leakage with a conductivity meter. Sensitive plant lines can be identified by comparing electrolyte leakage of treated samples to that of untreated samples. To study the role of CRKs in apoplastic ROS signaling, the level of O_3 induced cell damage in the T-DNA insertion mutants were analyzed after fumigation with 350 ppb O_3 for six hours. Col-0 wild type plants were used as an O_3-tolerant control line. Although approximately 60% of *CRKs* showed elevated transcript abundance levels in response to O_3 (I: Fig. 1), only one fourth of *crk* mutants displayed increased electrolyte leakage levels (i.e. sensitivity to O_3) compared to Col-0 wild type plants after O_3 fumigation (III: Fig. 4D, Fig. S10). *crk1-2, crk2* and *crk31*, responded fast to O_3 treatment displaying significantly increased electrolyte leakage at the early time points (8h and 9.5h after the onset of O_3 experiment) while *crk3*, *crk13*, *crk20*, *crk25*, *crk28*, *crk41*, *crk42* displayed increased ion leakage at the later time points (9.5h, 24h and 32h) (III: Fig. 4D, Fig. S10). Three fourths of the *crk* mutants, including *crk6*, *crk7-1, crk7-2*, *crk8* and *crk10,* did not show significantly elevated electrolyte leakage levels after treatment with O_3 for 6h (II: Fig. 4A; III: Fig. S10). Also *crk6/crk7/crk8/crk10/crk15-1/2* ami-RNA plants showed only slightly elevated electrolyte leakage levels compared to *crk6* and *crk7-2* (II: Fig. 4A). In conclusion, even though O_3 fumigation is inducing *CRK* gene expression, most of the single knockout mutants did not display great sensitivity to O_3 suggesting that absence of the corresponding CRK protein is not detrimental to *Arabidopsis thaliana*.

To further study the role of CRKs in apoplastic ROS signaling, xanthine-xanthine oxidase (X + XO) system was used to generate extracellular ROS (O_2^- and H_2O_2) similar to O_3. However, X+XO treatment is not controlled by the stomatal aperture. Instead, it is vacuum-infiltrated to the cells leading to a more dramatic effect on ROS production. X+XO-induced cell damage was quantified with electrolyte leakage measurements. Most of *crk* mutants did not differ from Col-0 wild type plants in their response to X+XO (III: S12). The responses of *crk6* and *crk7* to X+XO were weak, only *crk7-2* showed slightly increased electrolyte leakage after the treatment (II: Fig 4B). *crk6* and *crk6/7/8/10/15 -1/2* ami-RNA lines did not show significantly increased electrolyte leakage compared to Col-0 wild type plants (II: Fig. 4B). Several *crks,* especially *crk1-2*, *crk4*, *crk11*, *crk21-1*, *crk38* and *crk46* displayed greater tolerance, showing reduced electrolyte leakage compared to Col-0 wild type plants (III: Fig. 4E, S12). Only *crk19-2* was significantly more sensitive to X+XO than Col-0 wild type plants displaying significantly increased electrolyte leakage (III: Fig. 4E, S12).

Generally, most *crk* mutants did not show altered response to O_3 or X+XO treatments although more than half of *CRKs* displayed elevated transcript abundance levels in response to treatment with O_3. As the CRKs are very similar to each other at the amino acid level, the responses of the single knockout mutants could be disguised by the redundancy effect i.e. the highly similar sister gene could compensate the loss of one gene leading to unaltered response. However, silencing the five closest homologues did not lead to elevated sensitivity or stronger responses of *crk6/crk7/crk8/crk10/crk15-1/2* ami-RNA plants. This suggests that more than these five CRKs are involved in the signal transduction processes during the apoplastic oxidative stress or that the role of the CRKs in extracellular ROS signaling is more complex than anticipated.

4.4.2 Ozone induces visible signs of cell damage in *crk6* and *crk7-2* mutant plants (II)

Different staining methods have been developed to visualize cell damage and cell death in plants. Trypan blue (TB) stains only dead cells and it can be used to visualize localization and the amount of cell death. DAB (3,3'-diaminobenzidine) staining generates dark brown precipitate in the presence of H_2O_2 and it can be used to visualize the location and the amount of H_2O_2 produced in plants. To measure the amount of O_3-induced H_2O_2 production and cell death in *crk6*, *crk7-2* and *crk6/crk7/crk8/crk10/crk15* -1/2 ami-RNA plants, the plants were exposed to 350 ppb O_3 for six hours. *crk7-1* was not included in the staining experiments. Leaf samples were collected for DAB and TB staining after an additional two hour recovery time. *crk6*, *crk7-2* and particularly *crk6/crk7/crk8/crk10/crk15* plants displayed increased H_2O_2 accumulation compared to Col-0 wild type plants (II: Fig. S5A and B). Similar results were obtained from TB staining: *crk6* and especially *crk6/crk7/crk8/crk10/crk15* -1/2 ami-RNA plants displayed more TB stained dead cells than Col-0 wild type plants while *crk7-2* plants displayed only slightly more dead cells compared to Col-0 wild type plants (II: Fig. 3B).

O_3 is clearly inducing cell death and ROS accumulation in *crk6*, *crk7-2* and in particular in *crk6/crk7/crk8/crk10/crk15* ami-RNA plants but the quantification of O_3 induced damage using electrolyte leakage as a measure might not be sensitive enough for such small changes. Microscopic analyses of TB and DAB stained leaf samples in turn seems to be sensitive method for analyzing the strength of O_3 response in mutant lines.

DAB and TB staining results support the suggested strong redundancy between CRK6 and CRK7. The stronger staining of ami-RNA plants indicates that silencing the five homologous *CRKs* leads to greater O_3 sensitivity compared to the single knockout mutants (II: Fig. 3B). In addition to CRK6 and CRK7, CRK8, CRK10 and CRK15 all together or just some of them might be involved in O_3 induced ROS

signaling adding cumulative effect to O_3 stress response leading to stronger phenotypes.

4.4.3 Call of duty - Ozone treatment induces CRK6 and CRK7 promoter activation (II)

In addition to analyzing the phenotypes of the individual T-DNA insertion mutants, the O_3-induced spatiotemporal changes in *CRK6* and *CRK7* promoter activity were analyzed in wild type plants using promoter-GUS lines. The promoters of *CRK6* and *CRK7* were fused to the β–glucuronidase (*uidA*) reporter gene, whose activity can be detected as a blue color with GUS staining (II). *CRK6::uidA* and *CRK7::uidA* plants were exposed to 350 ppb O_3 for six hours and samples were harvested after a two hour recovery time for GUS staining. *CRK7::uidA* expression was induced strongly by O_3 whereas only a slight induction of *CRK6::uidA* was observed (II: Fig. 3A and B). Interestingly, in mature leaves expression was localized to the leaf areas typical for O_3 induced cell damage i.e. lesions (III: Fig. S12) and in addition to tissues surrounding stomata and leaf vasculature (II: Fig. 3A-B; S3A-B). Notably, the expression was stronger in the young leaves where O_3 induced cell damage is rarely observed (II: Fig. 3A).

Taken together, O_3 experiments indicate that O_3 fumigation leads to increased H_2O_2 production and cell death in the single knockout mutant plants and especially in *crk6/crk7/crk8/crk10/crk15* ami-RNA plants. O_3 also induces *CRK6* and *CRK7* promoter activation in *CRK6::uidA* and *CRK7::uidA* plants. Rapid induction of *CRK6* and *CRK7* promoters suggests that the *CRK6* and *CRK7* genes are activated in response to acute O_3 stress to prevent cell damage. Leaf areas in mature leaves, where promoter activation was detected as GUS-induced color change, commonly display signs of O_3-induced cell damage (II: Fig. 3A and B; III: Fig. S12). This result suggests a protective role for CRK6 and CRK7. Cell damage can be observed in the absence of CRK6 and CRK7 but in their presence *Arabidopsis thaliana* can cope with increased apoplastic ROS levels induced by O_3. Strong GUS-induced color change in young leaves suggests higher CRK promoter activity during development which could explain why young leaves seem to be better protected against oxidative stress as they rarely display signs of cell damage.

It could be postulated that overexpression of *CRK6* and *CRK7* could protect against O_3 damage. However, enhanced O_3 tolerance was not observed in *35S:CRK6* and *35S:CRK7* overexpression plants (II: Fig. S8). Plants overexpressing *CRK6* and *CRK7* responded to O_3 and X+XO treatments like Col-0 wild type (II: Fig. S8). However, genomic complementation rescued the X+XO sensitive phenotype of *crk7-2* (II: S6). It has to be taken into account that the obtained overexpression results might be affected by the general difficulties in the gain-of function approaches or that overexpression is limited by the components needed for CRK6 and CRK7 kinase activity.

4.4.4 Few *crk* mutants responded to pathogen treatments (II, III)

Increased extracellular ROS production, frequently referred to as oxidative burst, is a component of pathogen defense in plants (Schwessinger and Zipfel, 2008; Wrzaczek et al., 2013). Treatments with specific components of pathogen- and microbe-associated molecular patterns (PAMPs and MAMPs, respectively) trigger similar ROS production. Pathogen-, PAMP- and MAMP-induced responses have similarities with O_3 responses, such as apoplastic oxidative burst and production of salicylic acid (SA) and ethylene (ET) (Overmyer et al., 2003), due to which the role of CRKs in pathogen induced ROS signaling was analyzed. More similarities were found in the transcriptional responses of CRKs to pathogen and O_3 treatment (I: Fig. 4). For example, the O_3-induced expression profile of *CRKs* clustered together with several pathogen and PAMP treatments, such as *Blumeria graminis* var. *hordei* (*Bgh*), hairpin Z (HrpZ), and flg22 (I: Fig. 4).

To study the responses of *crk* mutants to pathogen/PAMP/MAMP-induced ROS production different treatments were used. ROS production levels in *crk* mutants were analyzed after treatment with flg22, which is a peptide derived from flagellin, a well-documented bacterial component known to induce bacterial response (Albert et al., 2010). Pathogen growth was analyzed in response to infection with the hemi-biotrophic bacterial pathogen *Pseudomonas syringae* pv. tomato DC3000 (*Pto* DC3000) and in response to two types of powdery mildew infection: to the biotrophic virulent powdery mildew fungus *Glovinomyces orontii* (*Go*) and the non-host powdery mildew fungus *Blumeria graminis* f. sp. *hordei* (*Bgh*). *Pto* DC3000 infects plants by entering the leaves through stomata (Melotto et al., 2006) whereas powdery mildew fungus strains *Go* and *Bgh* penetrate epidermal cells (Jensen et al., 2008; Micali et al., 2008; Rayapuram et al., 2012). In addition, the response of *crk6* and *crk7-2* to flg22 and cellulase which are bacterial and fungal PAMPs, respectively, was analyzed by following the root growth of the mutant plants (II).

In general, about 70% (27 out of 38) of *crk* mutants, including *crk6*, *crk7-1*, *crk7-2*, *crk8* and *crk10*, behaved like Col-0 wild type plants but *crk1*, *crk17*, *crk20*, *crk23*, *crk25*, *crk28*, *crk32* and *crk38* were significantly more susceptible to the biotrophic virulent powdery mildew fungus *Go* which does not need natural openings like stomata or accidental wounds to enter plant tissues, but rather infects plants by penetration though epidermal pavement cells (III: S24B). In contrast, only *crk2* and *crk5* were more resistant to *Go* infection compared to Col-0 wild type plants (III: 9A, S24B). None of the *crk* mutants were susceptible to *Bgh* infection but *crk5, crk20, crk28 and crk29* were susceptible to *Pto* DC3000 infection (III: Fig. 7B; S22; S25). Other *crk* mutants, including *crk6* and *crk7*, responded to *Pto* DC3000 infection like the Col-0 wild type plants (II: Fig. S7 and III: Fig. 7B; S22; S25). In addition, no differences in root growth in response to the presence of flg22 or cellulase in the growth medium were observed in *crk6* and *crk7-2* mutant plants compared to Col-0 wild type plants (II: Fig. S7). About one fourth (11 out of 38) of *crk* mutant plants,

including *crk6,* showed significantly increased ROS production after flg22 treatment but the result did not correlate well with any of the studied bacterial responses (III: Fig. S22A; S25). While most of the *crk* mutants responded with increased ROS production or similar to Col-0 wild type plants, *crk2, crk3, crk13* and *crk31* showed strikingly decreased ROS production in response to flg22 treatment (III: Fig. S22A).

While several studies have linked CRKs to pathogen responses, the actual role of CRKs in pathogen responses is still relatively unclear. It has been shown that *CRK6* is induced by pathogen attack and its promoter area contains W-box sequences i.e. (T/A)TGAC(T/A) cis-elements, the binding sites for WRKY transcription factors, whose presence is connected to pathogen responses (Chen et al., 2004; Czernic et al., 1999; Du and Chen, 2000). The transcript abundance levels of several *CRKs*, including *CRK6* and *CRK7* were increased in Arabidopsis overexpressing *LecRK-VI.2*, a positive regulator of pattern-triggered immunity (PTI) (Singh et al., 2012). In order to study the role of these *LecRK-VI.2* responsive CRKs (*CRK4*, *CRK6*, *CRK7*, *CRK13*, *CRK23*, *CRK36*, *CRK37*) in pathogen response, Yeh et al. analyzed the responses of T-DNA insertion lines and corresponding overexpression lines to *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) infection (Yeh et al., 2015). Similar to the results obtained in this PhD-study and in previous study by Acharya et al. (2007) with *crk13*, none of the tested T-DNA insertion lines displayed altered resistance phenotype (Yeh et al., 2015). However, the overexpression lines *CRK4*, *CRK6* and *CRK36* under the control of the CaMV 35S promoter displayed enhanced resistance to *Pst* DC3000 infection suggesting a role in innate immunity (Yeh et al., 2015). In contrast, lines overexpressing *CRK13*, *CRK23* and *CRK37* displayed a wild type phenotype (Yeh et al., 2015). In addition, CRK4, CRK6 and CRK36 could interact with FLS2 suggesting a role for these CRKs in innate immunity (Yeh et al. 2015).

Notably, in contrast to the results obtained by Yeh et al., Acharya et al. have shown that overexpression of *CRK13* under the control of the strong constitutive 35S promoter led to stunned growth and death before maturity but steroid-inducible *Gal4* promoter led to enhanced tolerance to *Pst* DC3000 infection (Acharya et al., 2007; Yeh et al., 2015). Despite the presence of W-box sequences and pathogen-induced gene expression, phenotype studies with *crk* mutants do not support their proposed role in pathogen response. However, the contrasting results obtained from overexpression studies and the lack of phenotype of T-DNA insertion lines most likely due to strong functional overlap disguising the effect of gene losses calls for further analyses with multi-knockout mutants to reveal the specific role of CRKs in pathogen response.

4.5 The role CRKs in chloroplastic ROS signaling (I, II, III)

In addition to extracellular ROS production, intracellular ROS production plays a critical role in stress responses. The chloroplasts and peroxisomes are the main ROS producing organelles in the plant cell. Increased chloroplastic ROS production induced by high light led to the decreased transcript abundance levels of most *CRK* genes (I: Fig. 3). As extracellular and chloroplastic ROS production are not isolated but can influence each other (Shapiguzov et al., 2012, Sierla et al, 2013) *crk* mutants' response to elevated ROS accumulation in the chloroplast was analyzed. ROS production was induced by light stress, methyl viologen (MV, also known as paraquat) and 3-(3, 4-dichlorophenyl)-1,1-dimethylurea (DCMU).

Light stress did not induce severe damage in most *crk* mutants including *crk6*, *crk7-1* and *-2* (III: Fig. 10; S8; S25). Only four *crks*, *crk16*, *crk40*, *crk42* and *crk45,* displayed increased electrolyte leakage levels and only one, *crk2*, displayed decreased electrolyte levels after high light treatment compared to Col-0 wild type plants (III: Fig. 10; S8; S25). Treatment of *crk* mutants with MV or DCMU which cause increased production of superoxide and singlet oxygen, respectively, led to increased photoinhibition i.e. reduced photosynthesis in about two thirds of *crk* mutants (24/34) in response to MV and in half of the *crk* mutants (17/34) in response to DCMU compared to Col-0 wild type plants (III: Fig. 4A-C; 10; S9; S25). Especially *crk5*, *crk8*, *crk17*, *crk20*, *crk21*, *crk22*, *crk42* and *crk45* showed stronger photoinhibition in response to MV treatment and *crk2*, *crk5*, *crk31*, *crk37*, *crk38*, *crk40*, *crk42*, *crk45* and *crk46* in response to DCMU (III: Fig. 4A-C; 10; S9; S25). Photoinhibition of *crk5*, *crk42* and *crk45* was strongly increased by both treatments. *crk6*, *crk7-1, -2* and *crk10* responded like Col-0 wild type plants to MV and DCMU treatment (III: Fig. S9). In response to MV *crk8* displayed increased photoinhibition, but its response to DCMU was similar to Col-0 wild type plants (III: Fig. 4A-B; Fig. S9). The obtained results suggest that while specific *crk* mutants responded to ROS production in the chloroplasts CRK6 and CRK7 are not crucially involved in the chloroplastic ROS signaling. However, the role of redundancy disguising the *crk* phenotypes has to be taken into account.

In addition to the tests described above, the effect of MV to germination efficiency and fresh weight was analyzed. No altered response to MV was observed in *crk6* or *crk7-2* mutants compared to Col-0 wild type (II: Fig. 4C). However, plants overexpressing *CRK7* showed slightly increased tolerance to MV (II: Fig. 4C) indicating that chloroplastic ROS production could affect extracellular ROS signaling. *crk7-1* mutant was not included in the experiment.

UV-B treatment induces ROS production and signaling in plants (A-H-Mackerness et al., 2001; Ballaré, 2003; Brosché and Strid, 2003; Jenkins, 2009). The site of UV-B-induced cellular ROS production is not known but the most likely sources are the photosynthetic machinery in the chloroplasts and NADPH oxidases at the plasma membrane (Kalbina and Strid, 2006; Jenkins, 2009; Mittler et al., 2004). To study

the role of CRKs in UV light induced ROS signaling the responses of *crk* mutants to UV-A/B light was analyzed. The level of UV-A/B induced cellular damage was quantified by measuring the level of electrolyte leakage. Most of the mutants were tolerant and responded to UV-A/B treatment similar to Col-0 wild type but *crk2*, *crk5*, *crk40* and *crk42* displayed significantly elevated electrolyte leakage indicating more UV-A/B induced cell damage (III: Fig. 4F; S13A). Complementation of *crk5* rescued the hypersensitivity to UV A/B radiation (Burdiak et al., 2015). *crk6* and *crk7-1* and *-2* displayed slightly increased electrolyte leakage compared to Col-0 wild type plants (III: Fig. S13A).

In conclusion, the obtained results from the experiments mentioned above suggest that CRKs are involved more in O_3- and X+XO-induced than in pathogen-induced apoplastic ROS signaling. Nevertheless, few specific CRKs are involved in chloroplastic ROS signaling. In general, CRKs seem to have a protective role i.e. they are needed for proper response to ROS. Several CRKs, especially CRK2, CRK5 and CRK31, have a specific role in ROS response as the corresponding T-DNA insertion mutants, *crk2*, *crk5* and *crk31*, displayed strong phenotypes under most of the studied ROS-inducing conditions (Table 1). However, responses of the majority of *crk* mutants, including *crk6* and *crk7*, were relatively subtle. *crk7-1* and *-2* displayed similar responses compared to each other. Table 1 (below) summarizes the ROS-phenotypes of *crk2*, *crk5*, *crk6*, *crk7-2* and *crk31*.

Table 1. The observed responses of *crk2*, *crk5*, *crk6*, *crk7-2* and *crk31* to the tested abiotic and biotic stresses. Responses to O_3, X+XO, high light and UV-A/B were measured as a change in electrolyte leakage, response to MV and DCMU were measured as a change in photoinhibition and bacterial responses were measured as pathogen growth on leaves. Increased tolerance is marked with "↓", increased sensitivity with "↑", and response similar to Col-0 wild type plants with "–".

	O_3	X+XO	High light	MV	DCMU	*Pto* DC3000	*Go*	UV-A/B
crk2	↑	–	↑	↓	↓	–	↓	↑
crk5	–	↑	–	↓	↓	↑	↓	↑
crk6	–	↑	↑	↓	–	–	–	↑
crk7-2	–	↑	–	↓	↓	–	–	↑
crk31	↑	–	–	↓	↓	–	–	–

4.6 Few CRKs are involved in stomatal regulation (III)

Guard cells surrounding stomata are important regulators of plant responses to environmental stresses such as pathogen infection, air pollutants and drought stress (Serna, 2014). By closing stomata, plants can limit the entry of pathogens, air pollutants and minimize water loss during drought stress. Thus, for example O_3 sensitivity can be a result of stomatal miss-regulation (Brosché et al., 2010). If

stomata are not able to close properly in response to O_3, more O_3 gets into cells leading to more severe cell damage.

Rapid stomatal closure by elevated CO_2 levels, ABA, darkness or acute pulse of O_3 leads to immediate rapid decrease in stomatal conductance in wild type Col-0 plants (Kollist et al., 2007, 2014; Vahisalu et al., 2010). Mutants with altered stomatal function can be thus identified by analyzing the stomatal conductance levels in response to CO_2, darkness and acute pulse of O_3 (Kollist et al., 2007, 2014; Vahisalu et al., 2010). Several CRKs, such as *crk6*, *crk8* and *crk40*, displayed slightly increased basal steady-state stomatal conductance under control conditions but the phenomenon did not correlate with any other defects (III: Fig. S16A). In addition, while most *crk* mutants closed their stomata similar to Col-0 wild type plants in response to ABA, few mutants, including *crk22*, *crk24*, *crk37* and *crk46*, exhibited increased sensitivity to ABA displaying stronger stomatal closure (III: Fig. 5E; S15A). In contrast to this, *crk21-1*, *crk39*, *crk42* and *crk45* did not display ABA-induced stomatal closure (III: Fig. 5E; S15A).

In order to analyze the role of the CRKs in stomatal response, water loss and stomatal conductance in response to induced stomatal closure were analyzed for the *crk* mutant collection. The results revealed that stomatal development and regulation is altered in only a few *crk* mutants while most of the *crk* mutants, including *crk6, crk7-1* and *-2*, responded like Col-0 wild type (III: Fig. 5; S25). The few strongly affected *crk* mutants, *crk2*, *crk5* and *crk31*, were not able to close stomata properly in the water loss experiment leading to more rapid and greater loss of fresh weight compared Col-0 wild type (III: Fig. 5A, Table S4). Complementation rescued the water loss phenotype of *crk2* and *crk5* (III: Fig. 5B-C). In response to ABA *crk5* and *crk31* mutants did not show altered response suggesting that CRK5 and CRK31 are not involved in ABA-dependent control of stomatal closure (III: Fig. S15A). *crk2* mutant displayed slightly stronger stomatal closure in response to ABA (III: Fig. S15A). The ABA sensitive mutants which displayed stronger stomatal closure, *crk22*, *crk24*, *crk37* and *crk46*, displayed also smaller fresh weight loss in response to water loss suggesting that that they are involved in ABA-mediated stomatal closure (III: Fig. 5E; S15A; Table S4). Also *crk12*, *crk20*, *crk21*, *crk23*, *crk42* and *crk45* displayed smaller fresh weight loss in response to water loss even though they did not show ABA-induced stomatal closure (III: Fig. 5A;S15A; Table S4) suggesting that they are not involved in ABA-mediated stomatal closure. Overexpression of CRK45 in *crk45* mutant background showed an opposite phenotype from crk45 leading to even greater water loss compared to Col-0 wild type plants (III: Fig. 5D).

The *crk* mutants, *crk5* and *crk31*, which showed increased water loss, did not show the typical decrease in stomatal conductance in response to elevated CO_2, darkness and short impulse of O_3 (III: Fig. 5G-I). Complementation of *crk5* mutation rescued the stomatal conductance responses (III: Fig. 5K). However, basal stomatal conductance levels were decreased significantly in complementation lines compared to Col-0 wild type plants and *crk5* mutant plants (III: Fig. 5J).

Interestingly, *crk2* which displayed greater water loss than Col-0 wild type plants, responded to elevated CO_2, darkness and short impulse of O_3 similar to Col-0 wild type plants (III: Fig. 5G-I; S16B-D; S17-19). Few mutants, such as *crk3*, *crk20* and *crk42* responded to darkness and CO_2 with stronger decrease in stomatal conductance than Col-0 wild type plants (III: Fig. 5H-I; 16C-D; S18-19). In response to O_3 exposure, *crk2* and *crk31* were the only *crk* mutants which showed significantly increased electrolyte leakage levels as a sign of O_3 induced cell damage, especially at the early time points (III: Fig. 4D, S10). Thus O_3 sensitivity of *crk2* and *crk31* could be related to their inability to close stomata properly in response to O_3 and water loss.

Pathogen treatments induce apoplastic ROS production by plasma membrane NADPH oxidase which leads to stomatal closure (Kadota et al., 2014; Li et al., 2014; Schwessinger and Zipfel, 2008). The recent study shows that receptor-like cytoplasmic kinase botrytis-induced kinase 1 (BIK1), which is involved in FLS2 mediated pathogen recognition, directly phosphorylates the NAPDH oxidase to enhance ROS production leading to stomatal closure (Kadota et al., 2014; Li et al., 2014). To study the role of CRKs in pathogen induced stomatal closure, stomatal responses of *crk* mutants to bacterial and fungal pathogens were tested by treating the mutants with flg22 and chitin, respectively. Stomatal closure was measured as ratio between stomata width and length.

Most *crk* mutants, including *crk7-1* and *-2,* closed their stomata similar to Col-0 wild type plants in response to flg22 treatment (III: Fig. 8A and C; S23A). Stomatal closure was impaired especially in *crk5*, *crk10-2*, *crk17*, *crk20* and *crk28* (III: Fig. 8A and C; 7C; S23A). Even though ROS production in *crk6* mutant plants was increased in response to flg22 treatment, stomatal closure in response to flg22 treatment was only slightly impaired in *crk6* (III: Fig. S22A; S23A). Only *crk23-1* and *crk46* displayed greater flg22-induced stomatal closure compared to Col-0 wild type plants (III: Fig. 8A and C; S23A). Notably, the flg22-induced stomatal response in *crk2* and *crk31* did not differ significantly from Col-0 wild type plants but *crk5* displayed slightly reduced stomatal closure (III: Fig. 8A and C; S23A).

In response to chitin-induced stomatal closure one third of *crk* mutants, including *crk7-1, -2* and *crk8*, behaved like Col-0 wild type plants (III: 8B-C; S23B). Several mutants, including *crk2*, *crk6, crk10, crk12, crk19* and *crk28*, could not close their stomata as much as Col-0 wild type plants and few mutants, such as *crk23-2*, *crk37*, *crk41* and *crk46*, displayed greater chitin-induced stomatal closure compared to Col-0 wild type plants (III: Fig. 8B-C; S23B).

Taken together, even though many *crk* mutants displayed impaired stomatal responses, only few *crk* mutants, such as in *crk2*, *crk5* and *crk31*, displayed severely impaired stomatal regulation suggesting that CRK2, CRK5 and CRK31 are involved in the regulation of stomatal closure (Table 2; III: Fig. 6; 8;10; S25). In general, most *crk* mutants responded to water loss similar to Col-0 wild type plants and were tolerant to pathogen infections and could close their stomata similar to Col-0 wild

type plants after pathogen treatments (III: Fig. 8; 10; S25). In addition, most *crk* mutants showed similar stomatal conductance levels leading to stomatal closure in responses to O_3, CO_2 and darkness treatments (III: Fig. 5G-I; 6; S16B-D; S17-19). Even though ROS production was enhanced in *crk6* after flg22 treatment and *crk6* showed slightly impaired stomatal closure after chitin treatment the otherwise unaltered phenotype results suggests that CRK6 and CRK7 alone do not have a central role in pathogen, O_3, CO_2 or darkness induced rapid stomatal closure. However, redundancy effect might disguise the *crk6*, *crk7-1* and *-2* phenotypes leading to unaltered phenotype.

Table 2. Stomatal responses of *crk2*, *crk5*, *cr6*, *crk7-2* and *crk31* to different stress treatments. Responses to CO_2, O_3 and darkness were measured as a change in stomatal conductance, responses to chitin, flg22 and ABA were measured as changes in the stomatal closure (ratio between stomata width and length), response to flg22-induced ROS production was measured as a change in light units (RLU), and water loss as a relative water loss after cutting rosettes. Increased sensitivity to CO_2, O_3, darkness, chitin, flg22, ABA, and increased flg22-induced ROS production and water loss is marked with "↑", and decreased sensitivity, decreased ROS production and decreased water loss with "↓",and response similar to Col-0 wild type plants with "–".

	CO_2	O_3	Darkness	Chitin	flg22	ABA	flg-induced ROS	Water loss
crk2	↑	–	↓	↓	–	↑	↓	↑
crk5	↓	↓	↓	–	–	–	–	↑
crk6	–	↑	–	↓	–	–	↑	–
crk7-2	↑	↑	–	–	–	–	–	–
crk31	–	↓	↓	–	–	–	–	↑

4.7 Many CRKs are involved in growth and developmental processes (III)

Many growth and developmental processes, such as germination, root growth, cell expansion, cell cycle control, flowering and senescence, are regulated by ROS (Barth et al., 2006; Foreman et al., 2003; Foyer et al., 2008; Kranner et al, 2010; Lai et al., 2012; Lee et al., 2012; McInnis et al., 2006; Rodriquez et al, 2002). Environmental stresses lead to changes in energy allocation and thus have negative effects on growth and development. The delicate balance between environmental stresses and growth is based on crosstalk between hormone and ROS signaling. RLKs regulate such crosstalk in many developmental processes such as meristem development, cell fate determination, cell expansion and proliferation. For example, members of the Arabidopsis ERECTA (ER)-family, which belong to the LRR-RLK subfamily of RLKs, regulate inflorescence architecture, organ shape, epidermal stomatal pattering, floral meristem organization and organ identity (Bemis et al., 2013; Chen et al., 2013; Torii et al., 1996; Uschida et al., 2012). In addition, the RLK

FERONIA mediates ROS-dependent root hair elongation and pollen tube rupture (Duan et al., 2010; Escobar-Restrepo et al., 2007) and the RLK SCRAMBLED/STRUBBELIG regulates specification of epidermal root hairs, floral organ shape, ovule development and leaf pattering (Chevalier et al., 2005;Kwak et al., 2005; Lin et al., 2012). Although many RLKs had been linked to growth and development, there was no published data which would connect CRKs into developmental processes. Therefore, to study the role of CRKs in ROS regulated developmental processes, few developmental phenotypes, such as senescence, germination and flowering, were analyzed for the *crk* family. The obtained results showed that development is altered in a large number of *crk* mutants (III: Fig. 2; 10; S25).

4.7.1 Several *crk* mutants displayed early flowering and most *crk* mutants early senescence compared to Col-0 wild type plants (III)

Under control growth conditions *crk* mutants displayed similar morphology as the Col-0 wild type plants except *crk2* which has a dwarf phenotype (III: Fig. 3A; Fig. S5). Despite the unaltered morphology, many *crk* mutants displayed visible signs of altered physiology, such as early flowering and symptoms of senescence. Several mutants, especially *crk6*, *crk7-1, crk16*, *crk19* and *crk38* flowered earlier than Col-0 wild type plants (Figure 9B, III: Fig. 3C; S6B). For example, under short day conditions half of the *crk6* plants had bolted and opened first flower at 39 and 46 days after stratification (DAS), respectively, while half of the Col-0 wild type plants had bolted at 42 and flowered at 49 DAS (Figure 9A and 9B). The difference in the flowering time between *crk6* and Col-0 wild type plants was even greater under long day conditions (Figure 9D). The early flowering phenotype of *crk6* was very clear and consistent, both under short day and long day conditions and in the growth rooms and growth chambers. Preliminary results of plants overexpressing (OE) *CRK6* showed that *CRK6-OE* plants flowered later than *crk6* but earlier than Col-0 wild type plants (Figure 9C). *crk7-2* plants flowered similar to Col-0 wild type plants but mutant plants overexpressing *CRK7* (CRK7-OE) flowered later than *crk7-2* and Col-0 wild type plants (Figure 9C). Under long day conditions, genetic complementation of *crk6* could not rescue the early flowering phenotype (Figure 9D). Instead, complementation plants flowered earlier than Col-0 wild type plants, some lines even earlier than *crk6* (Figure 9D). The early flowering *rcd1-1* mutant plants were used as early flowering control plant line (Figure 9D; Ahlfors et al., 2004).

Interestingly, *crk2* which displayed a clear dwarf phenotype, was the only *crk* mutant to bolt and flower later than Col-0 wild type plants but showed signs of senescence almost ten days before Col-0 wild type plants (III: Fig. 3A-C; S6A-B). Noteworthy, almost every *crk* mutant including *crk6* and *crk7-2*, but especially *crk1*, *crk2, crk3*, *crk4, crk5*, *crk28*, *crk29* and *crk40*, showed signs of early leaf senescence,

judged by visible yellowing of leaves, under long-day conditions (16h light/8h dark) compared to Col-0 wild type plants (III: Fig. 3B; S6A).

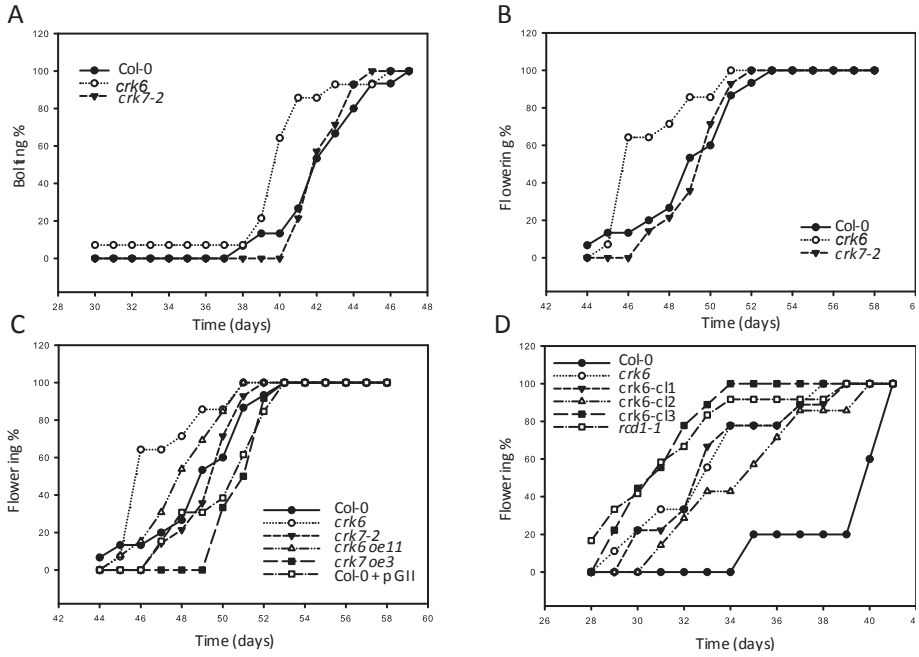

Figure 9. Bolting and flowering time of *crk6* and *crk7-2* mutants. A) Under short day conditions in the growth room half of the *crk6* plants had bolted at day 39 after stratification, while Col-0 and *crk7-2* reached the same level at day 42. Bolting time was recorded when the flower bud formation was detected. B) Under short day conditions in the growth room half of the *crk6* plants had opened their first flower by day 46 while Col-0 and *crk7-2* plants by day 49. Flowering time was recorded when the first flower opened. The experiment (A and B) was repeated 5 times with similar results, using 8-20 plants/line, usually 16 plants/line (4x4 plants/pot). C) Preliminary flowering time results of *CRK6* and *CRK7* overexpression lines (*CRK6-OE* and *CRK7-OE* lines) under short day conditions in the growth rooms. D) Preliminary flowering time results of *crk6* genetic complementation lines (*CRK6-CL* lines) under long day conditions in the growth chamber. *rcd1-1* mutant was used as an early flowering control plant line.

The obtained results suggest that CRKs are involved in flowering and senescence which is a novel finding. However, their role in these processes is not clear. Early flowering and early leaf senescence are common signs of environmental stress. Therefore, based on the results it can be suggested that absence of one CRK gene product in the corresponding *crk* mutant simulates environmental stress conditions leading to earlier flowering and senescence or perhaps it leads to impaired timing of flowering and senescence by still unknown mechanism. Nevertheless, the role of epigenetic modification was minimized by using only age-matched mutant population grown under similar conditions.

4.7.2 Germination was delayed in most *crk* mutants including *crk6* and *crk7* (III)

ROS play a dual role also in seed development. While controlled ROS production guides seed dormancy release and completion of germination the uncontrolled ROS accumulation leads to oxidative damage and ultimately cell death causing seed aging and seed desiccation. RLKs are involved in the ROS crosstalk regulating germination and seed longevity (Pitorre et al., 2010). For example, Arabidopsis RLK7, a leucine-rich repeat receptor-like kinase (LRR-RLK), is required for proper germination speed and tolerance to oxidative stress (Pitorre et al., 2010). ROS treatment delayed the germination of *rlk7* mutants and seed longevity was decreased in *rlk7* mutants suggesting that RLK7 is involved in ROS controlled germination and seed longevity (Pitorre et al., 2010).

In order to study the role of CRKs in germination, the germination phenotype of *crk* mutants was determined by analyzing testa and endosperm rupture over time (III). The obtained results show that germination was delayed in most *crk* mutants, including *crk6* and *crk7-1 and -2*, but especially in *crk11*, *crk40*, *crk41*, *crk45* and *crk46* (III: Fig. 3D; 10; S25; Table S3). *crk1*, *crk37* and interestingly the dwarf *crk2* with late flowering and early senescence where the only *crk* mutants with unaltered germination compared to Col-0 wild type plants (III: Fig. 3D; 10; S25). Nevertheless, almost every *crk* mutant reached 80 % germination i.e. 80% of seeds had germinated in 48 hours (III: Table S3).

High salinity affects germination and causes growth reduction in many areas. To analyze the response of *crk* mutants to NaCl, germination on medium containing 120 mM NaCl was followed for six days. Although germination was delayed significantly on NaCl medium compared to control medium (see results above), less *crk* mutants displayed delayed germination on NaCl medium compared control medium (III: Fig. 10; S13B-C). At day five, twenty *crk* mutants, especially *crk2*, *crk5*, *crk6*, *crk8*, *crk11*, *crk16*, *crk28*, *crk29*, *crk32*, *crk33*, *crk37*, *crk38*, *crk41*, *crk45* and *crk46*, displayed delayed germination (III: Fig. S13B). A day later at day six, 13 *crk* mutants, especially *crk5*, *crk8*, *crk11* and *crk28*, still displayed delayed germination compared to Col-0 wild type plants (III: Fig. S13C). The germination profile of *crk7* plants was similar to Col-0 wild type plants. *crk6* mutant plants displayed significantly delayed germination during the first five days but on the day six germination phase was similar to Col-0 wild type plants (III: Fig. S13B-C). Generally, the same *crk* mutants which displayed delayed germination on NaCl medium displayed delayed germination also on control medium, except *crk1*, *crk2* and *crk37*, which displayed unaltered germination on control medium (III: Fig. 3D; 10; S13B-C; S25). However, only about 50% of *crk* mutants had reached 80% germination level in six days (III: Fig. S13C).

The obtained results suggest that CRKs are involved in seed germination process as most *crk* mutants displayed delayed germination under control conditions.

Presence of NaCl in the growth medium enhanced the delay. Absence of CRK proteins leads to delayed seed germination by unknown mechanism but sensitivity of *crk* mutant seeds to NaCl supports the proposed protective role of CRKs in *Arabidopsis thaliana*.

4.7.3 Root length was affected in specific *crk* mutants (III)

Several RLKs are involved in the regulation of root growth, such as FERONIA (FER) and CLAVATA (Duan et al., 2010; Perilli et al., 2012). FER is an upstream regulator for the NAPDH oxidase-dependent RAC/ROP-signaled pathway, which mediates growth, development, reproduction and environmental related signaling in plants (Duan et al., 2010). RAC/ROP signaling complex acts as a signaling hub which integrates ROS and hormonal signals downstream for transcriptional reprogramming (Duan et al., 2010; Nibau et al., 2006; Yang and Fu, 2007). In order to study the role of CRKs in root growth the root length of the *crk* mutants was measured. Under control conditions at day eight, root growth was affected only in *crk28*, *crk29* and *crk40* which had slightly longer roots compared to Col-0 wild type plants (III: Fig. 3F; S25).

Even though *crk6* and *crk7-2* responded to flg22 and cellulase similar to Col-0 wild type plants i.e. flg22 reduced and cellulase increased root growth similarly in *crk6*, *crk7-2* and Col-0 wild type plants, *crk6* and *crk7-2* displayed longer roots compared to Col-0 wild type plants on control, flg22 and cellulase medium at day 10 DAS (II: Fig. S7). *crk6* root growth was delayed during the first six days after which *crk6* displayed longer roots than Col-0 wild type plants (II: Fig. S7). *crk7-2* displayed longer roots compared to Col-0 wild type plants during the observed 10 day period (II: Fig. S7). *crk-1* was not included in the experiment.

The results obtained from root growth experiment suggest that only a small number of CRKs are involved in root growth in *Arabidopsis thaliana*. In the affected *crk* mutants, absence of the corresponding CRK protein led to enhanced root growth i.e. longer roots at day eight compared to Col-0 wild type under control growth condition. *crk6* displayed delayed root growth until day seven after which it displayed longer roots compared to Col-0 wild type. Delayed *crk6* root growth could be a consequence of delayed *crk6* germination on control growth medium. The growth profile of other *crk* mutants was not recorded therefore it is not known if there is a general correlation between delayed germination and delayed root growth. However, if delayed root growth was a consequence of delayed germination, the delaying effect was over by day seven after which *crk6* displayed longer roots compared to Col-0 wild type plants.

Taken together, the results obtained from the growth and development related experiments, most *crk* mutants displayed growth and development related phenotypes (III: Fig. 2; 10; S25). Germination, flowering and senescence were the

most heavily affected processes. In general, *crk* mutants displayed delayed germination, early flowering and senescence compared to Col-0 wild type plants under control conditions. Most heavily affected mutants were *crk2* and *crk5* suggesting that CRK2 and CRK5 have an important role in the development of *Arabidopsis thaliana*. Table 3 (below) summarizes the observed development related phenotypes of *crk2*, *crk5*, *crk6*, *crk7-2* and *crk31*. Noteworthy, *crk7-1* and *crk7-2* mutants displayed difference in flowering time. While *crk6* and *crk7-1* were early flowering, *crk7-2* flowered similar to Col-0 wild type. In summary, the obtained results suggest that in addition to stress responses the CRKs are involved in the regulation of specific developmental processes, such as germination, flowering and senescence.

Table 3. Summary of the development related phenotypes of *crk2*, *crk5*, *crk6*, *crk7-2* and *crk31*. The most heavily affected processes were germination, flowering and senescence. Early senescence, bolting and flowering and delayed root growth is marked with "↓" sign, delayed germination, bolting and flowering and increased root growth with "↑" sign, and phenotype similar to Col-0 wild type plants with "–".

	Senescence	Germination	NaCl	Bolting	Flowering
crk2	↓	–	↓	↑	↑
crk5	↓	↓	↓	↑	–
crk6	↓	↓	↓	↓	↓
crk7-2	↓	↓	–	–	–
crk31	↓	↓	↓	↓	–

4.7.4 Stomatal development was affected in most *crk* mutants (III)

Like stomatal function stomatal development is regulated by numerous endogenous but also environmental factors (Serna, 2014). Three major phytohormones, brassinosteroids, abscisic acid (ABA) and auxins, negatively regulate stomatal development (Kim et al., 2012; Le et al., 2014; Tanaka et al., 2013). Series of asymmetrical and a final symmetrical cell division that precede stomatal formation are critical for stomatal function and define stomatal density and localization (Nadeau and Sack, 2002). For example, correct orientation of the planes of cell division guarantees that the stomata are not in direct contact with neighboring stomata ensuring stomatal movements and ion flux between guard cells and non-stomatal cells (Geisler et al., 2000; Nadeau and Sack, 2002).

CRKs are universally expressed in *Arabidopsis* but according to the publicly available *Arabidopsis* (ecotype Col-0) microarray data most CRKs displayed lower transcript abundance levels in guard cells compared to total leaf (III: Fig. S14A). However, qPCR analyses revealed the opposite result: most CRKs displayed higher transcript

abundance levels in guard cells compared to total leaf (III: Fig. S14B). Only few *CRKs*, such as *CRK4*, *CRK21*, *CRK22* and *CRK37*, displayed higher levels of transcript abundance in total leaf compared to guard cells (III: Fig. S14B). The mutants with stomatal defects, *crk2* and *crk31*, displayed higher transcript abundance levels in guard cell, except *crk5* which displayed lower levels, compared to total leaf (III: Fig. S14B). *CRK6* and *CRK7* displayed slightly higher transcript levels in guard cells compared to total leaf (III: Fig. S14B).

To analyze the role of CRKs in stomatal development, stomatal density and length was measured. Measurements revealed a novel and interesting feature of *crk* mutants: about half of the *crk* mutants showed increased stomatal length, especially *crk31*, *crk37*, *crk41* and *crk46* (III: Fig. 5F; S15B). Several *crk* mutants with increased stomatal length, for example *crk16*, *crk19-2*, *crk25*, *crk29*, *crk31* and *crk40*, displayed also reduced stomatal density (III: Fig. 5F; S15B). On contrast, several *crk* mutants displayed reduced stomatal length, such as *crk2*, *crk4*, *crk6*, *crk7-2*, *crk8* and *crk12* (III: Fig. 5F; S15B). Of these mutants, stomatal density was increased in *crk4*, *crk7-2* and *crk12* (III: Fig. 5F; S15B) and aperture ratio increased in *crk2*, *crk4*, *crk7-2* and *crk12* (III: Fig. 5F; S15B) which could be natural compensation for reduced stomatal length. The stomatal density and length of *crk7-1* was similar to Col-0 wild type (III: Fig. S15B). Pavement cell density in cotyledons was reduced in *crk1-1*, *ckr3*, *crk12*, *crk13*, *crk16*, *crk19-2*, *crk23-1*, *crk25*, *crk32*, *crk37* and *crk40* (III: Fig. 3E; S6C). The pavement cell density was similar to Col-0 wild type plants in rest the of the *crk* mutants, including *crk2*, crk5, *crk6*, *crk7-2* and *crk31* (III: Fig. 3E; S6C). Reduction of pavement cell density can be a result of increased cell expansion (Gudesblat et al., 2012; Serna, 2014) which could explain the increased stomatal length i.e. stomatal length would positively correlate with pavement cell size based on the fact that pavement cells and guard cells are interdependent, because pavement cells are formed together with stomata (Geisler et al., 2000). Therefore increased stomatal length could be a natural consequence of increased cell expansion (reduced pavement cell density). However, as stomatal density is affected by many environmental factors, such as internal CO_2 concentration, light, humidity, drought, etc., and cell size by cell cycle and developmental phase (Asl et al., 2011; Šantrůček et al., 2014) the correlation might not be so simple. But for example, *crk* mutants with increased stomatal length and decreased stomatal and pavement cell density, such as *crk16*, *crk19-2*, *crk25* and *crk40*, display only minor alterations in stomatal responses compared to Col-0 wild type plants. On contrast, the few *crk* mutants which displayed defects in stomatal function had disturbed ratio/balance between stomatal density and length. For example, *crk5* displayed reduced stomatal density but unaltered stomatal length suggesting decreased capacity for stomatal function due to lower number of stomata in *crk5* leaves. As *crk5* displayed increased water loss and decreased stomatal closure in response to O_3, CO_2 and darkness, some other factors in addition to the ratio of stomatal density and length might be involved in the processes regulating stomatal closure and function.

crk2, the dwarf mutant, which displayed slightly reduced stomatal length but unaltered stomatal density compared to Col-0 wild type plants, displayed increased ratio of stomatal aperture under control conditions which could compensate the slightly reduced stomatal length. Nevertheless, *crk2* displayed increased water loss and slightly reduced darkness induced stomatal closure. In response to ABA *crk2* displayed slightly stronger stomatal closure than Col-0 wild type plants which suggests that CRK2 could be involved in ABA-dependent control of stomatal closure. The connection to ABA is supported by the earlier predictions according to which CRK2 might be involved in growth regulation in response to ABA (Bassel et al., 2011). However, *crk2* displayed unaltered stomatal density which suggests unaltered ABA and brassinosteroid signaling during stomatal development (Serna, 2014; Tanaka et al., 2013). Defects in brassinosteroid signaling are usually observed as growth reduction but the involvement of brassinosteroids in *crk2* dwarfism was not analyzed in this study. Reasons for *crk2* dwarfism will be addressed in the future studies.

The increased stomatal length could compensate the decreased stomatal density of *crk31* but reasons leading to reduced stomatal aperture ratio could limit stomatal functions (III: Fig. 5F; S15B). These unknown reasons could be behind the weaker drop of stomatal conductance levels of *crk31* in response to O_3, CO_2 and darkness even though the basal steady-state stomatal conductance was unaltered under control conditions (III: 5G-I; S16A).

In conclusion, the obtained results suggest that several CRKs, especially CRK2, CRK5 and CRK31, are important regulators of stomatal development and function controlling stomatal responses to environmental stresses. Table 4 (below) summarizes the stomatal development related phenotypes of *crk2*, *crk5*, *crk6*, *crk7-2* and *crk31*. Noteworthy, in contrast to *crk7-2*, *crk7-1* displayed unaltered stomatal density and length compared to Col-0 wild type.

Table 4. Summary of the stomatal development related phenotypes of *crk2*, *crk5*, *crk6*, *crk7-2* and *crk31*. Decreased stomatal length, stomatal and pavement cell density, aperture ratio and conductance is marked with "↓" sign, whereas increased stomatal length, stomatal and pavement cell density, aperture ratio and conductance is marked with "↑" sign, and phenotype similar to Col-0 wild type plants with "–" .

	Length	Density	Aperture ratio	Steady state conductance	Pavement cell density
crk2	↑	–	↓	–	–
crk5	–	↓	–	–	–
crk6	↓	–	↓	↑	–
crk7-2	↓	↑	↑	–	–
crk31	↑	↓	↓	–	–

In addition to stomatal development, the obtained results in this study suggest that other developmental processes, such as flowering and senescence, are regulated by CRK-dependent signaling pathways. As CRKs have not been linked to developmental processes or stomatal function before these results broadens the knowledge of the biological relevance of CRKs in plants and open new research areas for future studies. Figure 10 summarizes the processes in which CRKs are involved according to the observed phenotypes in this study (III: Fig. 2).

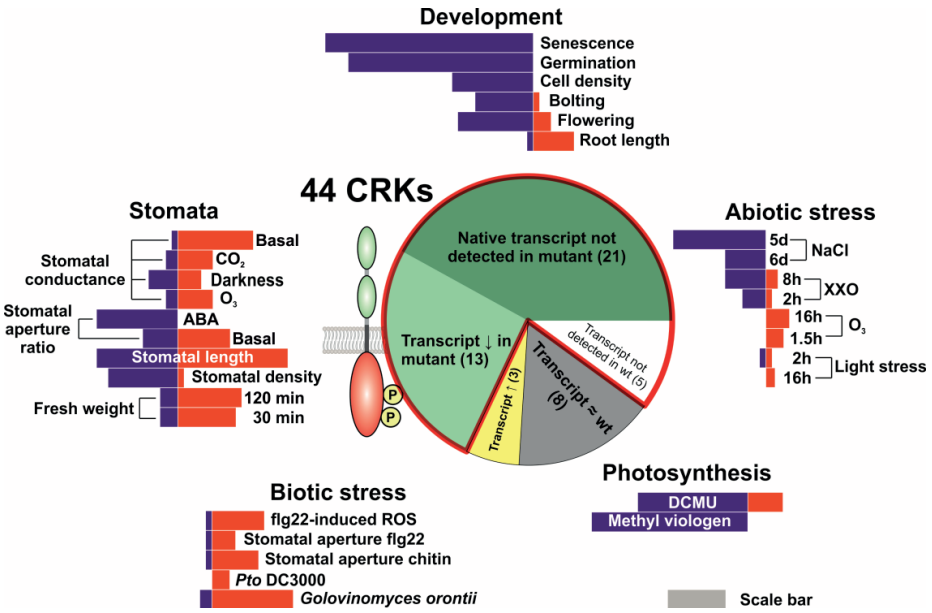

Figure 10. Phenotypic analyses of the *Arabidopsis thaliana* CRK protein family. A T-DNA insertion collection for the CRK family was compiled and subjected to phenotyping addressing aspects of plant development, biotic and abiotic stress responses, photosynthesis as well as stomatal regulation. Length of red and blue bars in the five phenotyping sections is representative of the number of *crk* lines found to have phenotypes in the thematic area. The length of the gray scale bar corresponds to ten lines. The blue color represents earlier developmental milestones or reduced values while red color represents later developmental milestones or increased values compared to Col-0 wild type plants. The red outline in the pie chart highlights the 39 *crk* lines included in the analyses. Information about the sections in the pie chart is described in Material and methods section (p. 35-36) and in publication III: Fig. S1; S3, S4 and Table S1.

4.8 CRK kinase activity (II)

Ligand-induced receptor complex formation and phosphorylation has been characterized as a mode of action for RLKs. RLKs activate many downstream signaling pathways regulating growth and stress adaptation responses. However, no activating ligands have yet published for CRKs but *in vitro* kinase activity has been verified for CRK36 (Tanaka et al., 2012). In addition, Tanaka et al. showed that CRK36 phosphorylates ARCK1 *in vitro* which is the only published data of CRK phosphorylation targets (Tanaka et al., 2012).

Some predictions of the kinase activity can be done by analyzing the protein sequence of the kinase domain. Arabidopsis CRKs are RD kinases based on the presence of an arginine (R) residue before the conserved aspartate (D) residue in the catalytic loop of subdomain VIb (Figure 11B). The kinase domain of CRKs is highly conserved and CRK6 and CRK7 have an almost identical kinase domain (II: Fig. S2). CRK6 and CRK7, and their closest homologs CRK8, CRK10 and CRK15, contain all the conserved amino acids essential for kinase activity (Figure 11).

Figure 11. (A) Schematic representation of the cytoplasmic CRK juxtamembrane (JM), kinase domain and carboxy terminus (CT) organization and the localization of the conserved subdomains (I-XI). (B) The alignment of the kinase domain of the closest homologs CRK6, CRK7, CRK8, CRK10 and CRK15 show high sequence similarity. The line under the amino acids represents identical amino acids. The conserved amino acids of the subdomain I-XI are marked with bold letters, and the amino acids conserved in active kinases are marked with an asterisk on the top. Catalytic and activation loop are marked with broken line.

The predicted kinase activity of CRK6 and CRK7 was confirmed by *in vitro* kinase assays (II: Fig.2). For determining kinase activity CRK6 and CRK7 full cytoplasmic domains were produced as glutathione S-transferase (GST)-tagged recombinant proteins in *E. coli*. Both GST-CRK6 and GST-CRK7 phosphorylated the artificial substrate MBP but only GST-CRK7 displayed autophosphorylation activity (II: Fig. 2). Despite their high sequence identity (75,68%) and similarity (82,51%) CRK6 and CRK7 showed a striking difference in their preference for divalent cation: GST-CRK6 preferred manganese (Mn^{2+}) while GST-CRK7 magnesium (Mg^{2+}) (II: Fig. 2). These results demonstrate that GST-CRK6 and GST-CRK7 are active protein kinases with a preference for Mn^{2+} and Mg^{2+}, respectively. As *in vitro* autophosphorylation sites are highly predictive of *in vivo* phosphorylation sites (Clouse et al., 2012; Oh et al., 2000; Wang et al., 2005a, 2008) it is likely that CRK6 and CRK7 possess kinase activity also in *in vivo* conditions.

The kinase activity for other CRKs has not been verified but according to predictions based on essential amino acids for kinase activity, every CRK should possess kinase activity, except CRK45 which lack the DFG motif (subdomain VII) conserved in active kinases (Figure 11; Appendix 3). However, the DFG motif might not be necessary for kinase activity as shown with the human pseudokinase CASK (Mukherjee et al., 2008).

5 DISCUSSION

Reactive oxygen species (ROS) signaling in plants has been under extensive research for the last two decades. New signaling compounds and pathways have been identified and our understanding of the function of ROS has shifted from harmful side-products to important signaling intermediates with diverse roles in development and stress adaptation. Nevertheless, ROS sensing and signaling specificity are still open questions. This study addressed the role of cysteine-rich receptor-like protein kinases (CRKs) in ROS signaling, sensing and specificity. The role of CRKs in ROS sensing and signaling is intriguing and has been in the focus of recent research. It has been shown that transcript abundance of many *CRKs* is increased by several ROS inducing conditions, e.g. O_3 and pathogen attack. Furthermore, the extracellular properties of CRKs suggest redox regulation possibilities and therefore a possible role in ROS sensing. Several previous overexpression and knockout studies have linked Arabidopsis CRKs to abiotic and biotic stress induced ROS signaling but the actual role of CRKs in signal transduction is unknown.

Arabidopsis thaliana contains 44 highly similar CRKs. Analysis of amino acid conservation of extra- and intracellular domains divided CRKs into five distinctive phylogenetic groups and suggested tightly linked evolution of extra- and intracellular domains (III: Fig. S1). CRKs are characterized by one to three extracellular DUF26 domain(s), which contains three cysteine residues in the C-X_8-C-X_2-C configuration. The DUF26 domain is plant specific and highly conserved. This study revealed that the level of conservation extends beyond the DUF26 domains and that all CRKs have 10-12 conserved cysteine residues in their extracellular domain. Conservation of cysteine residues highlights their importance to the protein function. The conservation of extracellular cysteine residues correlates well with sequence similarity i.e. the members of each phylogenetic group generally has the same level of cysteine residue conservation (Figure 8). Although the function of the conserved cysteines or the DUF26 domain is not known, it has been shown that all three cysteine residues of DUF26-domain of ginkbilobin-2 (Gnk2) from *Ginkgo biloba* are involved in the formation of three structurally important internal disulphide bridges (Miyakawa et al., 2009). The DUF26 domain(s) found in Arabidopsis CRKs could have similar structurally important function. Redox changes can lead to formation or opening of disulphide bridges leading to conformational changes, therefore it has been suggested that such a mechanism could be behind ROS sensing and CRK activation. However, there is yet no experimental evidence available and the question of ROS sensing in plants stays open. Nevertheless, CRKs are still considered as good candidates for ROS sensors.

As every CRK possess the critical amino acids needed for kinase activity this study suggests kinase activity for each CRK and verifies the *in vitro* kinase activity of CRK6 and CRK7. Sequence similarity of CRK6 and CRK7 suggests functional redundancy but the observed difference in their preference for divalent cation for the kinase

function suggests possible difference in signaling specificity, perhaps through different regulation or phosphorylation targets. This observation supports the proposed fine-tuning role of CRKs in plants' response to environmental stresses. For example, differentially regulated kinase activity could bring specificity to the highly conserved kinase activity. The highly conserved amino acid sequence of CRK extra- and intracellular domains and the predicted kinase activity suggests important signaling roles for CRKs as there have been only minor changes during evolution. In addition, the number of CRKs in *Arabidopsis thaliana* has remained relatively constant during evolution (Lehti-Shiu et al., 2012). Only two CRKs out of 44 seems to be non-functional, the putative pseudogene CRK35 (At4g11500) and CRK9 (At4g23170) which has a truncated kinase domain. The collection of 44 CRKs with overlapping functions creates a powerful signal transduction unit on the plasma membrane for cellular crosstalk to maintain cellular balance crucial for plant life.

Most CRKs belonging to the same phylogenetic group are located next to each other in the chromosome 4, except members of group I which are located randomly to chromosomes 1, 4 and 5 (III: Fig. S2). Despite the high amino acid similarity between group members, observed phenotypes did not correlate with the grouping. This was especially clear with group I *crk* mutants, which frequently displayed opposite responses compared to each other and generally the strongest phenotypes compared to other *crk* mutants. The same result was observed for transcriptional responses. For example, half of the *CRKs* displayed increased transcript abundance levels in response to O_3 but the response did not correlate well with sequence similarity (I: Fig. 2). Only the members of group III displayed significantly increased transcript abundance levels in response to O_3 treatment. However, in group V, there was a correlation among the closest group members. For example, highly similar CRK6, CRK7, CRK8 and CRK10, which belong to group V, displayed similar responses to the most of the tested stresses both at transcriptional and phenotypical level (I; II). This could be due to the strong redundancy between the closest homologues i.e. the highly similar sister gene(s) compensates the loss of one gene leading to unaltered response. Redundancy leading to overlapping functions might be a result of evolutionary pressure to guarantee essential signal transduction under changing environmental conditions. In that sense, redundancy could be seen as a nature's backup system for essential signal transduction pathways. *Summa summarum*, the large number of CRKs and their redundant yet specific function provides specificity and fine tuning opportunities for signal transduction and suggests important roles in many essential signal transduction pathways.

To study the ROS induced responses in CRKs, ROS production was induced by different mechanisms, mostly O_3, to simulate abiotic and biotic stresses. O_3 is an air pollutant which causes negative effects on plant growth depending on O_3 concentration and the duration of the exposure. The low dose (<100 ppb) chronic exposure to O_3 causes reduction of growth and premature senescence (Betzelberger et al., 2012; Krupa, 2003; Wilkinson et al., 2012). The high dose (>200 ppb) acute exposure, even a short one, causes visible lesions and cell death

(Kangasjärvi et al., 2005; Rao et al., 2000). Plants respond to O_3, both low and high dose exposures, with increased extracellular ROS production which is why it has been used as a tool to identify proteins involved in ROS-mediated stress signaling and cell death. In addition to this study, O_3 has been used to identify proteins involved in ROS signaling, such as apoplastic protein GRIM REAPER (GRI), the chloroplast envelope membrane protein RETICULATA (RE) and the nuclear proteins RADICAL-INDUCED CELL DEATH 1 (RCD1) and SIMILAR TO RCD-ONE (SROs) (Kangasjärvi and Kangasjärvi, 2014). These proteins are good examples of the successful use of O_3 as a probing tool to find new elements in ROS signaling as their function cover nicely the essential parts of the signal transduction pathway from the apoplast through plasma membrane to cytosol and chloroplasts finally reaching the target, the nucleus. Another good example is the characterization of the O_3-sensitive ascorbic acid –deficient vitamin c (*vtc*) mutants in *Arabidopsis thaliana* that led to the unravelling of the ascorbate biosynthesis pathway in plants (Conklin et al, 1996, 2000).

The obtained results suggest that several CRKs are essentially involved in ROS signaling mediating environmental signals leading to stress tolerance and adaptation. Analyses of the transcriptional responses of *CRKs* revealed that more than half of *CRKs* displayed increased transcript abundance in response to O_3 whereas high light treatment lead to reduction in transcript abundance for most *CRKs* (I). In addition to stress response phenotypes, large-scale phenotyping of the *crk* T-DNA insertion mutants revealed specific growth related phenotypes for most *crk* mutants, especially for *crk2* and *crk5*, despite the strong redundancy in the family (III). Therefore, especially CRK2 and CRK5 seem to have very essential role in stress responses and developmental processes. In general, most of *crk* mutants displayed growth and development related phenotypes while only several *crk* mutants displayed abiotic and biotic stress related phenotypes. Generally, *crk* mutants showed different responses to different stresses, but *crk2* and *crk5* displayed similar responses to most stresses (III: Fig. 10; S28). While RLKs and ROS signaling have been linked to growth and development, in addition to stress responses, the role of CRKs in developmental processes has not been thoroughly addressed before. According to this study, CRKs are essentially involved also in developmental processes and this novel finding highlights the importance of CRKs not only in ROS-mediated stress adaptation but also in plant growth and development.

The most heavily affected growth and development related processes were germination, flowering, senescence and stomatal development. Even though the dwarf *crk2* was the only *crk* mutant which displayed different morphology compared to Col-0 wild type plants under control conditions, about half of the *crk* mutants flowered earlier and about 90% of *crk* mutants displayed delayed germination and early senescence compared to Col-0 wild type plants under control conditions. Flowering time is an essential factor determining reproductive success and thus regulated by many environmental and endogenous factors, such as light, temperature and phytohormones (Blázquez and Weigel, 2000; Domagalska et al.,

2010; Koornneef et al., 1998; Verhage et al., 2014). Environmental stress leads to earlier flowering and thus earlier but typically lesser seed production in order to guarantee reproduction in shorter time under unfavorable conditions (Wada and Takeno, 2010; Xu et al., 2014). Noteworthy, the role of epigenetics has to be kept in mind when analyzing the stress responses, especially flowering time and root growth (Yaish et al., 2011). Through epigenetic modifications, such as DNA methylation, histone modifications and chromatin remodeling, plants can adjust their stress tolerance in timely manner without changing the original DNA sequence (Bonasio et al., 2010; Boyko and Kovalchuk, 2008; Kooke et al., 2015; Yaish et al., 2011). In contrast, epigenetic modifications are heritable. Epigenetic modifications which accumulate during plant life in response to various stress factors are passed into progeny forming epigenetic memory which increases stress tolerance towards the stresses faced in the previous generations (Bonasio et al., 2010; Boyko and Kovalchuk, 2008; Kooke et al., 2015; Yaish et al., 2011). Whether CRKs are involved in the regulation of flowering and senescence or whether the observed early flowering and senescence of *crk* mutants are signs of lowered stress tolerance, artefacts caused by gene silencing technique or epigenetic modifications needs further analyses to be resolved. It has been suggested that the reason behind the early senescence of *crk5* is the elevated ROS levels in the apoplast (Burdiak et al., 2015) i.e. the lack of CRK5 would disrupt the ROS-mediated signal transduction pathway *via* CRK5 leading to defective response and accumulation of ROS in the apoplast. The accumulation of ROS in the apoplast could trigger other stress response pathways leading to general symptoms of stress: early flowering and senescence. However, although *crk5* displayed early senescence it did not flower earlier compared to Col-0 wild type (III: S6A-B; Burdiak et al., 2015).

Stomatal development was affected in several *crk* mutants, especially strongly in *crk2*, *crk5* and *crk31*. In general, stomatal length was increased in half of *crk* mutants and stomatal density reduced and stomatal aperture ratio and basal stomatal conductance levels increased in one third of *crk* mutants. Stomatal conductance levels in response to O_3, CO_2 or darkness were affected in only a few mutants. In addition, stomatal closure in response to pathogens, water loss and ABA was severely altered in few mutants. *crk6* and *crk7* did not display strong alterations in stomatal closure in response to the studied conditions. Proper regulation of stomatal aperture is critical for plants' responses to various stimuli. According to the obtained results several CRKs are involved in the complex hormone and ROS signaling network controlling stomatal aperture. Some CRKs seem to be pathway-specific while some CRKs have overlapping roles in abiotic and biotic stimuli induced ROS signaling leading to stomatal closure (III: Fig. 11A). For example, the obtained results connect several CRKs, such as CRK2, CRK5 and CRK31, to stomatal processes suggesting that these CRKs regulate stomatal development and function. The involvement of CRKs in stomatal regulation and growth and development related processes is a novel finding and opens an interesting new field of study which will provide more information of the biological relevance of CRKs.

Phenotypes of *crk6* and *crk7* were partly disguised by redundancy. However, few phenotypes were identified. *crk6* was early flowering and *crk7-2* was slightly sensitive to apoplastic ROS induced by X+XO treatment. In general, *crk7-1* and *crk7-2* displayed similar phenotypes under the studied stress conditions. However, there were few differences. *crk7-1* flowered earlier and displayed increased stomatal aperture ratio compared to *crk7-2* which behaved like Col-0 wild type. Nevertheless, *crk7-2* mutants displayed higher sensitivity to O_3- and darkness-induced stomatal closure while *crk7-1* behaved like Col-0 wild type. Silencing the most homologues genes, *crk6*, *crk7*, *crk8*, *crk10*, *crk15*, overcame the redundancy effect and revealed some additional consequences of the gene losses in ROS signaling. The ami-RNA lines *crk6/7/8/10/15-1* and *-2* were more sensitive to O_3-induced cell damage than the corresponding single knockout lines. The result suggests that CRK6 and CRK7, together with their closest homologues CRK8, CRK10 and CRK15, are involved in the coordination of a proper response to extracellular ROS caused by O_3 and X+XO treatments in *Arabidopsis thaliana*. Analyses of the GUS expression of the *CRK6::uidA* and *CRK7::uidA* plants revealed that promoters of CRK6 and CRK7 are rapidly activated by O_3 treatment and that expression is localized to the leaf areas typical for O_3-induced damage. According to these results CRK6 and CRK7 are needed in ROS-mediated stress signaling and thus results suggest protective role for CRK6 and CRK7. Several other CRKs were also linked to abiotic stress tolerance, for example *crk2*, *crk5* and *crk19* which displayed increased sensitivity to increased apoplastic ROS production. Noteworthy, only few *crk* mutants, such as *crk1*, *crk2*, *crk5*, *crk20*, *crk23* and *crk25*, displayed altered response to biotic stress even though pathogen infection induces apoplastic ROS production similar to abiotic stress. However, pathogen treatments led to stomatal closure in many *crk* mutants suggesting that CRKs are involved in stomatal regulation.

Apoplastic ROS is produced mainly by the NADPH oxidases which produce superoxide and therefore it is tempting to link CRKs into part of the “ROS wave” (III: Fig. 11B). CRKs could perceive ROS from neighboring cells and transmit the “alarm” signal into the cytosol for proper response. Unfortunately the NBT staining analysis for visualizing and quantifying the level of superoxide production in *crk6* and *crk7* in response to increased apoplastic ROS levels in response to O_3 treatment was not successful due to technical problems. Thus this study could not address the possible role of CRK6 and CRK7 in the “ROS wave”. As the precise mechanism of apoplastic ROS production by NADPH oxidase and other enzymes in response to different stress stimuli is not known, the involvement of CRKs in the “ROS wave” and signal propagation needs further analyses in the future. Nevertheless, it can be proposed that redox-regulated CRKs are essential parts of cellular redox circuits transferring environmental information to cytosol and different cellular compartments in order to maintain cellular balance crucial for optimal plant growth and development.

Last but not least, the analysis of *crk* mutant collection demonstrates that for large gene families, instead of studying individual family members, thorough analysis of comprehensive mutant collections can facilitate the discovery of subtle phenotypic

responses and aspects which might otherwise be missed. As observed in this study, functional redundancy disguising phenotypes can be very strong and multiple knockout mutants are needed to reveal some of the consequences of gene losses. Therefore, when analyzing the loss-of-function phenotypes of large gene families, this observation should be kept in mind.

6 CONCLUSION AND FUTURE PERSPECTIVES

Adaptation of plants to stressful environmental conditions is based on flexible interactions between hormone and ROS signaling which controls growth and defense responses. During the recent years researchers have started to understand the complexity of the crosstalk needed for stress tolerance. Based on transcriptional analyses and the CRK ectodomain properties, it has been suggested that CRKs could be involved in ROS-mediated stress tolerance. The results obtained in this study support that suggestion and suggest a protective role for CRKs against many environmental stresses. According to the obtained results it can proposed that CRKs are essential elements of cellular redox circuits that relay environmental information to the cell (Figure 12). Furthermore, this study suggests that Arabidopsis CRKS are active kinases which in addition to stress responses are essentially involved also in plant growth and development. This novel finding broadens our understanding of the role of CRKs in *Arabidopsis thaliana* and highlights the importance of CRKs in transferring ROS-mediated environmental signals to the cell in order to maintain the balance between growth and defense. Furthermore, the large number of CRKs and their specific yet partly overlapping function suggests specificity and fine tuning opportunities for signal transduction. Together CRKs create a flexible and robust regulatory system aimed to generate the most appropriate output in response to both extra- and intracellular signals.

The ultimate goal of this study beyond characterizing CRKs is to understand how CRKs connect extracellular signals to transcriptional reprogramming to guarantee survival under changing environmental conditions. To achieve this, further studies are needed to reveal the possible ROS sensing mechanism, signaling partners, the role of conserved extracellular cysteine residues, activation mechanism and cytoplasmic phosphorylation targets and induced signaling cascades, etc. For example, CRKs involved in different signaling pathways, such as stress and developmental processes, could possibly make receptor complexes with different signaling partners leading to different responses depending on the activating stimulus. The possible interaction of CRKs with multifunctional BAK1, involvement of CRKs in NADPH oxidase-dependent ROS wave and activation of the most common downstream signaling cascades in stress adaptation and growth are just few of the interesting topics of the future CRK studies. Due to overlapping functions analyses of multi-knockout mutants might be needed to reveal additional phenotypes and thus processes regulated by CRKs. In addition to redundancy effect, the possible role of epigenetics in response to different environmental conditions has to be taken into account. Understanding the role of CRKs in complex ROS and hormone signaling network in *Arabidopsis thaliana* will provide new clues for ROS-mediated endogenous and environmental crosstalk maintaining the cellular balance crucial for plant growth and reproduction. In addition, it could provide new clues for improving crop plants' tolerance mechanisms against the future environmental challenges, such as global warming and intensive farming. Improved tolerance could lead to more optimal growth under non- or sub-optimal growth

conditions which could increase crop yield and thus guarantee food supply for the ever-growing human and animal population.

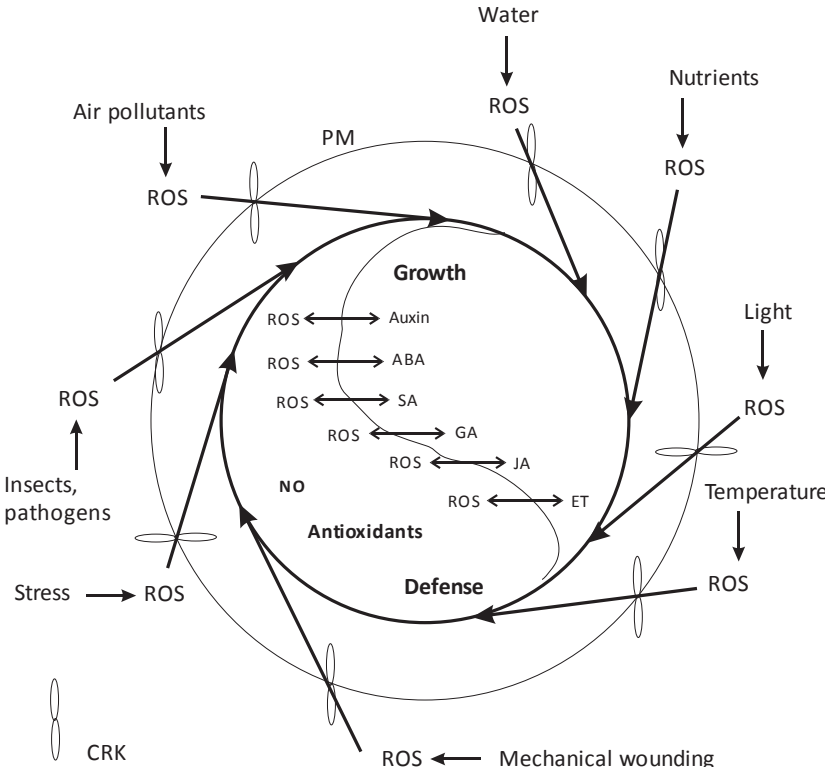

Figure 12. This simplified figure describes the proposed role of plasma membrane (PM) located CRKs in mediating environmental signals to the cell. It suggests that CRKs are important elements of a cellular redox circuit through which information flows from extracellular space to cytosol and different subcellular compartments maintaining the cellular balance crucial for plant growth and defense under continuously changing environmental conditions.

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Appendix 1.

The list of Arabidopsis CRKs with their AGI-code, DUF26 nomenclature, subgroup number and corresponding references. CRK9 has truncated kinase domain and CRK35 is a putative pseudogene which is why they are not included in the table.

CRK	AGI	DUF26	Group	Ref.
1	At1g19090	40	I	I, III
2	At1g70520	41	I	I, III
3	At1g70530	39	I	I, III
4	At3g45860	14	V	Chen et al., 2004; Yeh et al., 2015; I, III
5	At4g23130	13	V	Chen et al., 2003, 2004; Burdiak et al., 2015; I, III
6	At4g23140	6	V	Yeh et al., 2015; I, II, III
7	At4g23150	8	V	I, II,III
8	At4g23160	7	V	I, III
10	At4g23180	9	V	Chen et al., 2003, 2004; I, III
11	At4g23190	4	IV	Chen et al., 2003; I, III
12	At4g23200	1	IV	I, III
13	At4g23210	25	IV	Acharya et al., 2007; I, III
14	At4g23220	2	IV	I, III
15	At4g23230	36	V	I, III
16	At4g23240	22	IV	I, III
17	At4g23250	21	IV	I, III
18	At4g23260	20	IV	I, III
19	At4g23270	15	V	Chen et al., 2004; I, III
20	At4g23280	11	V	Chen et al., 2004; Ederli et al., 2011; I, III
21	At4g23290	23	IV	I, III
22	At4g23300	5	IV	I, III
23	At4g23310	12	V	I, III
24	At4g23320	24	IV	I, III
25	At4g05200	10	V	I, III
26	At4g38830	30	IV	I, III
27	At4g21230	43	II	I, III
28	At4g21400	28	II	I, III
29	At4g21410	29	II	I, III
30	At4g11460	19	IV	I, III
31	At4g11470	17	IV	I, III
32	At4g11480	18	IV	I, III
33	At4g11490	16	IV	I, III
34	At4g11530	3	IV	I, III
36	At4g04490	31	III	Tanaka et al., 2012; Yeh et al., 2015; I, III
37	At4g04500	32	III	I, III
38	At4g04510	35	III	I, III
39	At4g04540	34	III	I, III
40	At4g04570	33	III	I, III
41	At4g00970	26	II	I, III
42	At5g40380	38	I	I, III
43	At1g70740	37	I	I, III
44	At4g00960	27	II	I, III
45	At4g11890	45	II	Zhang et al., 2013; I, III
46	At4g28670	42	I	I, III

Appendix 2

The organization of the cysteine residues in the CRK ectodomains. The conserved Cys residues are marked with bold. The DUF26 motif is underlined. The putative pseudogene CRK35 (At4g11500) and truncated CRK9 (At4g23170) were excluded. CRK43, CRK44 and CRK45 lack ectodomain.

At1g19090: CRK1: C-X_{30}-**C**-X_{50}-**C**-X_8-**C**-X_2-**C**-X_{11}-**C**-X_{11}-**C**-X_{22}-**C**-X_{47}-**C**-X_8-**C**-X_2-**C**-X_{10}-**C**-X_{11}-C-X_{74}-C

At1g70520: CRK2: C-X_{24}-**C**-X_{48}-**C**-X_8-**C**-X_2-**C**-X_{10}-**C**-X_{12}-**C**-X_{21}-**C**-X_{51}-**C**-X_8-**C**-X_2-**C**-X_{11}-**C**-X_{12}-C-X_{47}-C

At1g70530: CRK3: **C**-X_{50}-**C**-X_8-**C**-X_2-**C**-X_{10}-**C**-X_{16}-C-X_{21}-**C**-X_{51}-**C**-X_8-**C**-X_2-**C**-X_{10}-**C**-X_{12}-C

At3g45860: CRK4: **C**-X_{52}-**C**-X_8-**C**-X_2-**C**-X_{11}-**C**-X_{12}-**C**-X_{76}-**C**-X_8-**C**-X_2-**C**-X_{21}-**C**

At4g23130: CRK5: **C**-X_{49}-**C**-X_8-**C**-X_2-**C**-X_{11}-**C**-X_{12}-**C**-X_{76}-**C**-X_8-**C**-X_2-**C**-X_{21}-**C**-X_{61}

At4g23140: CRK6: **C**-X_{51}-**C**-X_8-**C**-X_2-**C**-X_{11}-**C**-X_{12}-**C**-X_{78}-**C**-X_8-**C**-X_2-**C**-X_{21}-**C**

At4g23150: CRK7: **C**-X_{51}-**C**-X_8-**C**-X_2-**C**-X_{11}-**C**-X_{12}-**C**-X_{78}-**C**-X_8-**C**-X_2-**C**-X_{21}-**C**

At4g23160: CRK8: C-X_{19}-C-X_{18}-C-X_{10}-C-X_{41}-C-X_{58}-C-X_{48}-C-X_{10}-C-X_{16}-C-X_{101}-C-X_{69}-C-X_{24}-C-X_{54}-C-X_{26}-**C**-X_{51}-**C**-X_8-**C**-X_2-**C**-X_{11}-**C**-X_{12}-**C**-X_{77}-**C**-X_8-**C**-X_2-**C**-X_{22}-**C**-X_{64}-C

At4g23180:CRK10:**C**-X_{51}-**C**-X_8-**C**-X_2-**C**-X_{11}-**C**-X_{12}-**C**-X_{75}-**C**-X_8-**C**-X_2-**C**-X_{21}-**C**

At4g23190: CRK11: **C**-X_{68}-**C**-X_8-**C**-X_2-**C**-X_{11}-**C**-X_{15}-**C**-X_{81}-**C**-X_8-**C**-X_2-**C**-X_{10}-**C**-**C**-X_{12}-**C**

At4g23200: CRK12: **C**-X_{50}-**C**-X_8-**C**-X_2-**C**-X_{11}-**C**-X_{15}-**C**-X_{81}-**C**-X_8-**C**-X_2-**C**-X_{10}-**C**-**C**-X_{12}-**C**-X_{73}-C-C-

At4g23210: CRK13: C-X_{15}-**C**-X_{51}-**C**-X_8-**C**-X_2-**C**-X_{11}-**C**-X_{15}-**C**-X_{84}-**C**-X_8-**C**-X_2-**C**-X_{12}-**C**-**C**-X_{12}-**C**

At4g23220: CRK14: C-X_{25}-C-$_{X6}$-C-C-X_6-C-X_{29}-**C**-X_{50}-**C**-X_8-**C**-X_2-**C**-X_{11}-**C**-X_{15}-**C**-X_{77}-**C**-X_8-**C**-X_2-**C**-X_{10}-**C**-**C**-X_{12}-**C**

At4g23230: CRK15: **C**-X_{51}-**C**-X_8-**C**-X_2-**C**-X_{11}-**C**-X_{12}-**C**-X_{76}-**C**-X_8-**C**-X_2-**C**-X_{21}-**C**-X_{69}-C

At4g23240: CRK16: C-X_{13}-**C**-X_{50}-**C**-X_8-**C**-X_2-**C**-X_{11}-**C**-X_{17}-**C**-X_{75}- **C**-X_8-**C**-X_2- **C**-X_{11}-**C**-X_{12}-C

At4g23250: CRK17:C-X_4-C-X_{18}-**C**-X_{51}-**C**-X_8-**C**-X_2-**C**-X_{11}-**C**-X_{15}-**C**-X_{96}-**C**-X_8-**C**-X_2-**C**-X_{24}-**C**-X_{77}-C

At4g23260: CRK18: C-X_4-C-X_{18}-**C**-X_{51}-**C**-X_8-**C**-X_2-**C**-X_{11}-**C**-X_{15}-**C**-X_{81}-**C**-X_8-**C**-X_2-**C**-X_{10}-**C**-**C**-X_{12}-**C**

At4g23270: CRK19:**C**-X_{52}-**C**-X_8-**C**-X_2-**C**-X_{11}-**C**-X_{12}-**C**-X_{75}-**C**-X_8-**C**-X_2-**C**-X_{21}-**C**

At4g23280: CRK20:C-X_{26}-**C**-X_{51}-**C**-X_8-**C**-X_2-**C**-X_{11}-**C**-X_{12}-**C**-X_{73}-**C**-X_8-**C**-X_2-**C**-X_{21}-**C**

At4g23290: CRK21: C-X_6-**C**-X_{51}-**C**-X_8-**C**-X_2-**C**-X_{11}-**C**-X_{15}-**C**-X_{79}-**C**-X_8-**C**-X_2-**C**-X_{10}-**C**-**C**-X_{12}-**C**

At4g23330: CRK22: **C**-X_{67}-**C**-X_8-**C**-X_2-**C**-X_{11}-**C**-X_{15}-**C**-X_{74}-**C**-X_8-**C**-X_2-**C**-X_{10}-**C**-**C**-X_{12}-**C**-X_{74}-C

At4g23310: CRK23:C-X_{53}-**C**-X_8-**C**-X_2-**C**-X_{11}-C-X_{12}-C-X_{21}-**C**-X_{51}-**C**-X_8-**C**-X_2-**C**-X_{11}-**C**-X_{12}-**C**-X_{75}-**C**-X_8-**C**-X_2-**C**-X_{21}-**C**-X_6-C-X_{41}-C-X_{18}-C-X_{157}

At4g23320: CRK24: **C**-X_{51}-**C**-X_8-**C**-X_2-**C**-X_{11}-**C**-X_{15}-**C**-X_{73}-**C**-X_8-**C**-X_2-**C**-X_{10}-**C**-**C**-X_{12}-**C**

At4g05200:CRK25:C-X_{29}-**C**-X_{53}-**C**-X_8-**C**-X_2-**C**-X_{11}-**C**-X_{12}-**C**-X_{75}-**C**-X_8-**C**-X_2-**C**-X_{10}-**C**-**C**-X_{12}-**C**-X_{57}-C-X_6-C

At4g38820: CRK26: C-X_{13}-**C**-X_{51}-**C**-X_8-**C**-X_2-**C**-X_{11}-**C**-X_{12}-**C**-X_{77}-**C**-X_8-**C**-X_2-**C**-X_{10}-**C**-**C**-X_{12}-**C**

At4g21230: CRK27: **C**-X_{48}-**C**-X_9-**C**-X_2-**C**-X_{11}-**C**-X_{12}-**C**-X_{72}-**C**-X_8-**C**-X_2-**C**-X_{10}-**C**-**C**-X_{12}-**C**

At4g21400: CRK28:C-X_8-C-X_{14}-**C**-X_{52}-**C**-X_8-**C**-X_2-**C**-X_{11}-**C**-X_{12}-**C**-X_{77}-**C**-X_8-**C**-X_2-**C**-X_{10}-**C**-**C**-X_{63}-**C**-X-C

At4g21410: CRK29: C-X_{23}-**C**-X_{50}-**C**-X_8-**C**-X_2-**C**-X_{11}-**C**-X_{12}-**C**-X_{77}-**C**-X_8-**C**-X_2-**C**-X_{10}-**C**-**C**-X_{12}-**C**

At4g11460: CRK30: **C**-X_{51}-**C**-X_8-**C**-X_2-**C**-X_{11}-**C**-X_{16}-**C**-X_{83}-**C**-X_8-**C**-X_2-**C**-X_{10}-**C**-**C**-X_{12}-**C**-X_{67}

At4g11470: CRK31: C-X_4-C-X_4-C-X_{14}-C-X_{51}-C-X_8-C-X_2-C-X_{11}-C-X_{18}-C-X_{77}-C-X_8-C-X_2-C-X_{10}-C-C-X_{12}-C

At4g11480: CRK32: C-X_8-C-X_{14}-**C**-X_{50}-**C**-X_8-**C**-X_2-**C**-X_{11}-**C**-X_{15}-**C**-X_{74}-**C**-X_8-**C**-X_2-**C**-X_{10}-**C**-**C**-X_{12}-**C**

At4g11490: CRK33: **C**-X_{50}-**C**-X_8-**C**-X_2-**C**-X_{11}-**C**-X_{15}-**C**-X_{81}-**C**-X_8-**C**-X_2-**C**-X_{22}-**C**-**C**-X_{53}-**C**

At4g11530: CRK34: **C**-X_{49}-**C**-X_8-**C**-X_2-**C**-X_{11}-**C**-X_{11}-C-**C**-X_{85}-**C**-X_8-**C**-X_2-**C**-X_{10}-**C**-**C**-X_{12}-**C**-X_{70}-C

At4g04490: CRK36: C-X_{18}-**C**-X_{45}-**C**-X_8-**C**-X_2-**C**-X_{11}-**C**-X_{18}-**C**-X_{80}-**C**-X_8-**C**-X_2-**C**-X_{24}-**C**

At4g04500: CRK37: C-X_{24}-**C**-X_{49}-**C**-X_8-**C**-X_2-**C**-X_{11}-**C**-X_{18}-**C**-X_{82}-**C**-X_8-**C**-X_2-**C**-X_3-**C**-X_{20}-C

At4g04510: CRK38: **C**-X_{45}-**C**-X_8-**C**-X_2-**C**-X_{11}-**C**-X_8-C-X_9-**C**-X_{82}-**C**-X_8-**C**-X_2-**C**-X_{20}-**C**-X_3-C

At4g04540: CRK39: **C**-X_{49}-**C**-X_8-**C**-X_2-**C**-X_{13}-**C**-X_{18}-**C**-X_{81}-**C**-X_8-**C**-X_2-**C**-X_3-**C**-X_{20}-C

At4g04570: CRK40: C-X_{25}-**C**-X_{50}-**C**-X_8-**C**-X_2-**C**-X_{11}-**C**-X_{16}-**C**-X_{81}-**C**-X_8-**C**-X_2-**C**-X_3-**C**-X_{20}-C-X_{33}-C

At4g00970: CRK41: C-X_{41}-**C**-X_{53}-**C**-X_8-**C**-X_2-**C**-X_{12}-**C**-X_{12}-**C**-X_{77}-**C**-X_8-**C**-X_2-**C**-X_{18}-**C**-X_3-C-X_{35}-C

At5g40380: CRK42: C-X_{37}-**C**-X_{48}-**C**-X_8-**C**-X_2-**C**-X_{10}-**C**-X_{12}-C-X_{22}-**C**-X_{41}-**C**-X_8-**C**-X_2-**C**-X_{10}-**C**-X_{12}-C

At4g28670: CRK46: **C**-X_{48}-**C**-X_8-**C**-X_2-**C**-X_{10}-**C**-X_{12}-C-X_{21}-**C**-X_{48}-**C**-X_8-**C**-X_2-**C**-X_{10}-**C**-X_{12}-C-X_{44}-C-X_2-C-X_{34}-C

Appendix 3

The localization of the conserved kinase subdomains in CRKs. The subdomains are marked with gray color and the amino acid residues conserved in active kinases are marked with bold letters and bigger font size: **K** in the subdomain II, **D** and **N** in the subdomain VIb, **D** and **G** in the subdomain VII, and **E** in the subdomain VIII. Subdomains defined in the figure 5.

At1g19090: CRK1

310_MLEKATESFHDSMKLGQGGAV**K**KLFFNTREWADQFFNEVNLISGVQHKNLVRLLGCSIEGPKSLLVYEYVHNRSLDQILFM
KNTVHILSWKQRFNIIIGISEGLEYLHRGSEVKII**H**R**D**IKTS**N**ILLDRNLSPKIA**D**F**G**LIRSMGTDKTQTNTGIAGTLGYLAP**E**
YLIKGQLTEKA**D**VYAF**G**V-

At1g70520: CRK2

332_**G**Q**G**GF**G**TVYKGVLPDGRDIAV**K**RLFFNNRHRATDFYNEVNMISTVEHKNLVRLLGCSCSGPESLLVYEYLQNKSLDRFIFD
VNRGKTLDWQRRYTIIVGTAEGLVYLHEQSSVKII**H**R**D**IKAS**N**ILLDSKLQAKIA**D**F**G**LARSFQDDKSHISTAIAGTLGYMAP**E**
YLAHGQLTEMV**D**VYSF**G**V-

At1g70530: CRK3

330_**G**Q**G**GS**G**SVYKGVLTNGKTVAV**K**RLFFNTKQWVDHFFNEVNLISQVD**H**KNLVKLLGCSITGPESLLVYEYIANQSLHDYLFV
RKDVQPLNWAKRFKIILGTAEGMAYLHEESNLRII**H**R**D**IKLS**N**ILLEDDFTPRIA**D**F**G**LARLFPEDKTHISTAIAGTLGYMAP**E**
YVVRGKLTEKA**D**VYSF**G**V-

At3g45860: CRK4

358_**G**Q**G**GF**G**EVYKGIFPSGVQVAV**K**RLSKTSGQGEREFANEVIVVAKLQHRNLVRLLGFCLERDERILVYEFVPNKSLDYFIFD
STMQSLLDWTRRYKIIGGIARGILYLHQDSRLTII**H**R**D**LKAG**N**ILLGDDMNAKIA**D**F**G**MARIFGMDQTEANTRRIVGTYGYMSP
EYAMYGQFSMKS**D**VYSF**G**V-

At4g23130: CRK5

351_**G**Q**G**GF**G**QVYKGTLPNGVQVAV**K**RLSKTSGQGEKEFKNEVVVVAKLQHRNLVKLLGFCLEREEKILVYEFVSNKSLDYFLFD
SRMQSQLDWTTRYKIIGGIARGILYLHQDSRLTII**H**R**D**LKAG**N**ILLDADMNPKVA**D**F**G**MARIFEIDQTEAHTRRVVGTYGYMSP
EYAMYGQFSMKS**D**VYSF**G**V-

At4g23140:CRK6

358_**G**R**G**GF**G**EVYKGTFSNGKEVAV**K**RLSKNSRQGEAEFKTEVVVVAKLQHRNLVRLLGFSLQGEERILVYEYMPNKSLDCLLFD
PTKQIQLDWMQRYNIIGGIARGILYLHQDSRLTII**H**R**D**LKAS**N**ILLDADINPKIA**D**F**G**MARIFGLDQTQDNTSRIVGTYGYMAP
EYAMHGQFSMKS**D**VYSF**G**V-

At4g23150:CRK7

343_**G**R**G**GF**G**DVYKGTFSNGTEVAV**K**RLSKTSEQGDTEFKNEVVVVANLRHKNLVRILGFSIEREERILVYEYVENKSLDNFLFD
PAKKGQLYWTQRYHIIGGIARGILYLHQDSRLTII**H**R**D**LKAS**N**ILLDADMNPKIA**D**F**G**MARIFGMDQTQQNTSRIVGTYGYMSP
EYAMRGQFSMKS**D**VYSF**G**V-

At4g23160:CRK8

947_**G**R**G**GF**G**EVYKGTFSNGKEVAV**K**RLSKNSRQGEAEFKTEVVVVAKLQHRNLVRLLGFSLQGEERILVYEYMPNKSLDCLLFD
PTKQTQLDWMQRYNIIGGIARGILYLHQDSRLTII**H**R**D**LKAS**N**ILLDADINPKIA**D**F**G**MARIFGLDQTQDNTSRIVGTYGYMAP
EYAMHGQFSMKS**D**VYSF**G**V-

At4g23180:CRK10

355_GQGGFGEVYKGTLSDGTEVAVKRLSKSSGQGEVEFKNEVVLVAKLQHRNLVRLLGFCLDGEERVLVYEYVPNKSLDYFLFD
PAKKGQLDWTRRYKIIGGVARGILYLHQDSRLTIIHRDLKASNILLDADMNPKIADFGMARIFGLDQTEENTSRIVGTYGYMSP
EYAMHGQYSMKSDVYSFGV-

At4g23190: CRK11

357_GEGGFGAVYKGKLSNGTDVAVKRLSKKSGQGTREFRNEAVLVTKLQHRNLVRLLGFCLEREEQILIYEFVHNKSLDYFLFD
PEKQSQLDWTRRYKIIGGIARGILYLHQDSRLKIIHRDLKASNILLDADMNPKIADFGLATIFGVEQTQGNTNRIAGTYAYMSP
EYAMHGQYSMKSDIYSFGV-

At4g23200: CRK12

332_GQGGFGEVYKGTLVNGTEVAVKRLSKTSEQGAQEFKNEVVLVAKLQHRNLVKLLGYCLEPEEKILVYEFVPNKSLDYFLFD
PTKQGQLDWTKRYNIIGGITRGILYLHQDSRLTIIHRDLKASNILLDADMIPKIADFGMARISGIDQSVANTKRIAGTFGYMPP
EYVIHGQFSMKSDVYSFGV-

At4g23210: CRK13

365_GHGGSGHVFKGRLPDGKEIAVKRLSEKTEQSKKEFKNEVVLVAKLQHRNLVRLLGFSVKGEEKIIVYEYLPNRSLDYILFD
PTKQGELDWKKRYKIIGGTARGILYLHQDSQPTIIHRDLKAGNILLDAHMNPKVADFGTARIFGMDQSVAITANAAGTPGYMAP
EYMELGEFSMKSDVYSYGV-

At4g23220: CRK14

414_GRGGFGEVFMGVLNGTEVAIKRLSKASRQGAREFKNEVVVVAKLHHRNLVKLLGFCLEGEEKILVYEFVPNKSLDYFLFDP
TKQGQLDWTKRYNIIRGITRGILYLHQDSRLTIIHRDLKASNILLDADMNPKIADFGMARIFGIDQSGANTKKIAGTRGYMPPE
YVRQGQFSTRSDVYSFGV-

At4g23230: CRK15

224_GQGGFGEVYKGTFSNGTEVAVKRLSKSSGQGDTEFKNEVVVVAKLQHRNLVRLLGFSIGGGERILVYEYMPNKSLDYFLFD
PAKQNQLDWTRRYKVIGGIARGILYLHQDSRLTIIHRDLKASNILLDADMNPKLADFGLARIFGMDQTQENTSRIVGTFGYMAP
EYAIHGQFSVKSDVYSFGV-
At4g23240: CRK16

35_GHGGFGEGTFPNGTEVAVKRLSKISGQGEEEFKNEVLLVAKLQHRNLVRLLGFSVEGEEKILVYEYMPNKSLDYFLFDHRRR
GQLDWRTRYNIIRGVTRGILYLHQDSRLTIIHRDLKAGNILLDVDMNPKIADFGVARNFRVDQTEATTGRVVGTFGYMPPEYVA
NGQFSMKSDVYSFGV-

At4g23250: CRK17

361_GAGGFGEVYKGMLLNGTEIAVKRLSKTSGQGEIEFKNEVVVVAKLQHINLVRLLGFSLQGEEKLLVYEFVPNKSLDYFLFD
PNKRNQLDWTVRRNIIGGITRGILYLHQDSRLKIIHRDLKASNILLDADMNPKIADFGMARIFGVDQTVANTARVVGTFGYMSP
EYVTHGQFSMKSDVYSFGV-

At4g23260: CRK18

346_GKGGFGEVYKGMLMNGTEIAVKRLSKTSGQGEVEFKNEVVVVAKLQHINLVRLLGFSLQGEEKLLVYEFVSNKSLDYFLFD
PTKRNQLDWTMRRNIIGGITRGILYLHQDSRLKIIHRDLKASNILLDADMNPKIADFGMARIFGVDQTVANTGRVVGTFGYMSP
EYVTHGQFSMKSDVYSFGV-

At4g23270: CRK19

333_GQGGFGEVYKGTLSSGLQVAVKRLSKTSGQGEKEFENEVVVVAKLQHRNLVKLLGYCLEGEEKILVYEFVPNKSLDHFLFD
STMKMKLDWTRRYKIIGGIARGILYLHQDSRLTIIHRDLKAGNILLDDDMNPKIADFGMARIFGMDQTEAMTRRVVGTYGYMSP
EYAMYGQFSMKSDVYSFGV-

At4g23280: CRK20

341_GQGGFGEVYKGTFPSGVQVAVKRLSKNSGQGEKEFENEVVVVAKLQHRNLVKLLGYCLEGEEKILVYEFVPNKSLDYFLFD
PTMQGQLDWSRRYKIIGGIARGILYLHQDSRLTIIHRDLKAGNILLDADMNPKVADFGMARIFGMDQTEANTRRVVGTYGYMAP
EYAMYGKFSMKSDVYSFGV-

At4g23290: CRK21

370_GHGGFGAVYKGMFPNGTEVAAKRLSKPSDQGEPEFKNEVLLVARLQHKNLVGLLGFSVEGEEKILVYEFVPNKSLDHFLFD
PIKRVQLDWPRRHNIIEGITRGILYLHQDSRLTIIHRDLKASNILLDAEMNPKIADFGLARNFRVNQTEANTGRVVGTFGYMPP
EYVANGQFSTKSDVYSFGV-

At4g23330: CRK22

360_GEGRFGEVYKGKFSNGTEVAVKRLSKVSGQDTKKFRNEAVLVSKIQHRNLARLLGFCLQGDGKFLIYEFVLNKSLDYFLFD
PEKQGELDWTRRYKIIGGIAQGILHLHQDPQLTIIYRDFKASNILLDADMNPKISDFGMATVFGMEESRGNTNWIAETFVYMSP
EYAVHGKFSMKSDVYSFGI-

At4g23310: CRK23

515_GQGGFGEVYKGTFPSGVQVAVKRLSKTSGQGEREFENEVVVVAKLQHRNLVRLLGYCLEGEEKILVYEFVHNKSLDYFLFD
TTMKRQLDWTRRYKIIGGIARGILYLHQDSRLTIIHRDLKAGNILLDADMNPKVADFGMARIFGMDQTEANTRRVVGTYGYMAP
EYAMYGQFSMKSDVYSFGV-

At4g23320: CRK24

180_GHGGFGEVYKGTFPNGTEVAVKRLSKTSGQGEEEFKNEVFLVAKLQHRNLVKLLGYAVKGDEKILVYEFLPNKSLDHFLFD
PVKKGQLDWTRRYNIINGITRGIVYLHQDSRLTIIHRDLKAGNILLDADMNPKIVDFGVARNFRVDQTEATTARVVGTIGYMPP
EYVTNGQFSTKSDVYSFGV-

At4g05200:CRK25

354_GHGGFGEVYKGQLITGETVAIKRLSQGSTQGAEEFKNEVDVVAKLQHRNLAKLLGYCLDGEEKILVYEFVPNKSLDYFLFD
NEKRRVLDWQRRYKIIEGIARGILYLHRDSRLTIIHRDLKASNILLDADMHPKISDFGMARIFGVDQTQANTKRIVGTYGYMSP
EYAIHGKYSVKSDVYSFGV-

At4g38830: CRK26

351_GEGGFGAVYKGVLSDGQKIAVKRLSKNAQQGETEFKNEFLLVAKLQHRNLVKLLGYSIEGTERLLVYEFLPHTSLDKFIFD
PIQGNELEWEIRYKIIGGVARGLLYLHQDSRLRIIHRDLKASNILLDEEMTPKIADFGMARLFDIDHTTQRYTNRIVGTFGYMA
PEYVMHGQFSFKTDVYSFGV-

At4g21230: CRK27

340_GEGGFGVVYKGHLPDGLEIAVKRLSIHSGQGNAEFKTEVLLMTKLQHKNLVKLFGFSIKESERLLVYEFIPNTSLDRFLFD
PIKQKQLDWEKRYNIIVGVSRGLLYLHEGSEFPIIHRDLKSSNVLLDEQMLPKISDFGMARQFDFDNTQAVTRRVVGTYGYMAP
EYAMHGRFSVKTDVYSFGV-

At4g21400: CRK28

368_GRGGFGSVYKGVFSGGQEIAVKRLSCTSGQGDSEFKNEILLLAKLQHRNLVRLLGFCIEGQERILVYEFIKNASLDNFIFG
NCFPPFSPYDDPTVLFFLLCVDLYAVTDLKKRQLLDWGVRYKMIGGVARGLLYLHEDSRYRIIHRDLKASNILLDQEMNPKIAD
FGLAKLYDTDQTSTHRFTSKIAGTYGYMAPEYAIYGQFSVKTDVFSFGV-

At4g21410: CRK29

364_GRGGFGSVYKGVFPQGQEIAVKRLSGNSGQGDNEFKNEILLLAKLQHRNLVRLIGFCIQGEERLLVYEFIKNASLDQFIFD
TEKRQLLDWVVRYKMIGGIARGLLYLHEDSRFRIIHRDLKASNILLDQEMNPKIADFGLAKLFDSGQTMTHRFTSRIAGTYGYM
APEYAMHGQFSVKTDVFSFGV-

At4g11460: CRK30

353_GQGGFGEVYKGTLSNGTEVAVKRLSRTSDQGELEFKNEVLLVAKLQHRNLVRLLGFALQGEEKILVFEFVPNKSLDYFLFG
STNPTKKGQLDWTRRYNIIGGITRGLLYLHQDSRLTIIHRDIKASNILLDADMNPKIADFGMARNFRDHQTEDSTGRVVGTFGY
MPPEYVAHGQFSTKSDVYSFGV-

At4g11470: CRK31

346_GQGGFGEVYKGMLPNETEIAVKRLSSNSGQGTQEFKNEVVIVAKLQHKNLVRLLGFCIERDEQILVYEFVSNKSLDYFLFD
PKMKSQLDWKRRYNIIGGVTRGLLYLHQDSRLTIIHRDIKASNILLDADMNPKIADFGMARNFRVDQTEDQTGRVVGTFGYMPP
EYVTHGQFSTKSDVYSFGV-

At4g11480: CRK32

328_GKGGFGEVYKGMLPNETEVAVKRLSSNSGQGTQEFKNEVVIVAKLQHKNLVRLLGFCLERDEQILVYEFVPNKSLNYFLFG
NKQKHLLDPTKKSQLDWKRRYNIIGGITRGLLYLHQDSRLTIIHRDIKASNILLDADMNPKIADFGMARNFRVDQTEDNTRRVV
GTFGYMPPEYVTHGQFSTKSDVYSFGV-

At4g11490: CRK33

328_GQGGFGEVFKGVLQDGSEIAVKRLSKESAQGVQEFQNETSLVAKLQHRNLVGVLGFCMEGEEKILVYEFVPNKSLDQFLFE
PTKKGQLDWAKRYKIIVGTARGILYLHHDSPLKIIHRDLKASNILLDAEMEPKVADFGMARIFRVDQSRADTRRVVGTHGYISP
EYLMHGQFSVKSDVYSFGV-

At4g11530: CRK34

352_GRGGFGEVYRGKLSSGPEVAVKRLSKTSGQGAEEFKNEAVLVSKLQHKNLVRLLGFCLEGEEKILVYEFVPNKSLDYFLFD
PAKQGELDWTRRYNIIGGIARGILYLHQDSRLTIIHRDLKASNILLDADMNPKIADFGMARIFGVDQSQANTRRIAGTFGYMSP
EYAMRGHFSMKSDVYSFGV-

At4g04490: CRK36

347_GQGGFGSVYKGILPSGQEIAVKRLAGGSGQGELEFKNEVLLLTRLQHRNLVKLLGFCNEGNEEILVYEHVPNSSLDHFIFD
EDKRWLLTWDVRYRIIEGVARGLLYLHEDSQLRIIHRDLKASNILLDAEMNPKVADFGMARLFNMDETRGETSRVVGTYGYMAP
EYVRHGQFSAKSDVYSFGV-

At4g04500: CRK37

352_GQGGFGSVYKGILPSGQEIAVKRLRKGSGQGGMEFKNEVLLLTRLQHRNLVKLLGFCNEKDEEILVYEFVPNSSLDHFIFD
EEKRRVLTWDVRYTIIEGVARGLLYLHEDSQLRIIHRDLKASNILLDAEMNPKVADFGMARLFDMDETRGQTSRVVGTYGYMAP
EYATYGQFSTKSDVYSFGV-

At4g04510: CRK38

346_GQGGFGSVYKGKLPGGEEIAVKRLTRGSGQGEIEFRNEVLLLTRLQHRNLVKLLGFCNEGDEEILVYEFVPNSSLDHFIFD
EEKRLLLTWDMRARIIEGVARGLVYLHEDSQLRIIHRDLKASNILLDAYMNPKVADFGMARLFNMDQTRAVTRKVVGTFGYMAP
EYVRNRTFSVKTDVYSFGV-

At4g04540: CRK39

360_GQGGFGTVYKGTLLNGQEVAVKRLTKGSGQGDIEFKNEVSLLTRLQHRNLVKLLGFCNEGDEQILVYEFVPNSSLDHFIFD
DEKRSLLTWEMRYRIIEGIARGLLYLHEDSQLKIIHRDLKASNILLDAEMNPKVADFGTARLFDSDETRAETKRIAGTRGYMAP
EYLNHGQISAKSDVYSFGV-

At4g04570: CRK40

355_GQGGFGTVYKGTFPNGQEVAVKRLTKGSGQGDMEFKNEVSLLTRLQHKNLVKLLGFCNEGDEEILVYEFVPNSSLDHFIFD
EDKRSLLTWEVRFRIIEGIARGLLYLHEDSQLKIIHRDLKASNILLDAEMNPKVADFGTARLFDSDETRAETKRIAGTRGYMAP
EYLNHGQISAKSDVYSFGV-

At4g00970: CRK41

351_GEGGFGAVYKGVLDYGEEIAVKRLSMKSGQGDNEFINEVSLVAKLQHRNLVRLLGFCLQGEERILIYEFFKNTSLDHYIFD
SNRRMILDWETRYRIISGVARGLLYLHEDSRFKIVHRDMKASNVLLDDAMNPKIADFGMAKLFDTDQTSQTRFTSKVAGTYGYM
APEYAMSGEFSVKTDVFSFGV-

At5g40380: CRK42

322_GQGGNGTVFLGILPNGKNVAVKRLVFNTRDWVEEFFNEVNLISGIQHKNLVKLLGCSIEGPESLLVYEYVPNKSLDQFLFD
ESQSKVLNWSQRLNIILGTAEGLAYLHGGSPVRIIHRDIKTSNVLLDDQLNPKIADFGLARCFGLDKTHLSTGIAGTLGYMAPE
YVVRGQLTEKADVYSFGV-

At1g70740: CRK43

69_GEGGFGPVFKGRLPDGRDIAVKKLSQVSRQGKNEFVNEAKLLAKVQHRNVVNLWGYCTHGDDKLLVYEYVVNESLDKVLFKS
NRKSEIDWKQRFEIITGIARGLLYLHEDAPNCIIHRDIKAGNILLDEKWVPKIADFGMARLYQEDVTHVNTRVAGTNGYMAPEY
VMHGVLSVKADVFSFGV-

At4g00960: CRK44

63_GEGGFGAVYKGVLDSGEEIAVKRLSMKSGQGDNEFVNEVSLVAKLQHRNLVRLLGFCFKGEERLLIYEFFKNTSLEKRMILD
WEKRYRIISGVARGLLYLHEDSHFKIIHRDMKASNVLLDDAMNPKIADFGMVKLFNTDQTSQTMFTSKVAGTYGYMAPEYAMSG
QFSVKTDVFSFGV-

At4g11890: CRK45

47_GRGGFGFVYKGRLQNGQEIAVKILSTSSIRTERQFHNELIILSKLKHKNLINLLGFCTKRDQHGLVYEFMPNSSLDCFILDP
HRAAQLNWEMCRNIIDGIARGLRYLHEESGLWVVHRDIKPGNILLDSDLKPKIVGFELARTMQQGENAAETTEIVGTVGYLDPE
YIRSGRVSVKSDVYAFGV-

At4g28670: CRK46

338_GVGGYGEVFKGTLSDGREIAIKRLHVSGKKPRDEIHNEIDVISRCQHKNLVRLLGCCFTNMNSFIVYEFLANTSLDHILFN
PEKKKELDWKKRRTIILGTAEGLEYLHETCKIIHRDIKASNILLDLKYKPKISDFGLAKFYPEGGKDIPASSLSPSSIAGTLGY
MAPEYISKGRLSNKIDAYSFGV-

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