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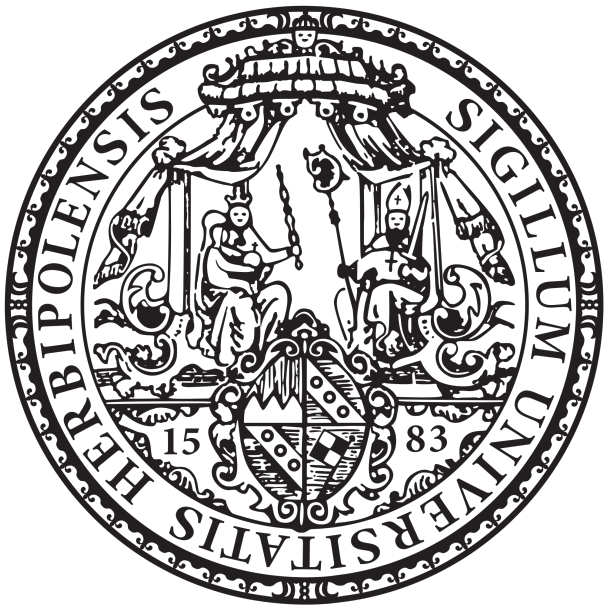

Dissertation zur Erlangung des
naturwissenschaftlichen Doktorgrades
der bayerischen Julius-Maximilians-Universität Würzburg

Vorgelegt von
Eva Kopic
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Würzburg 2023

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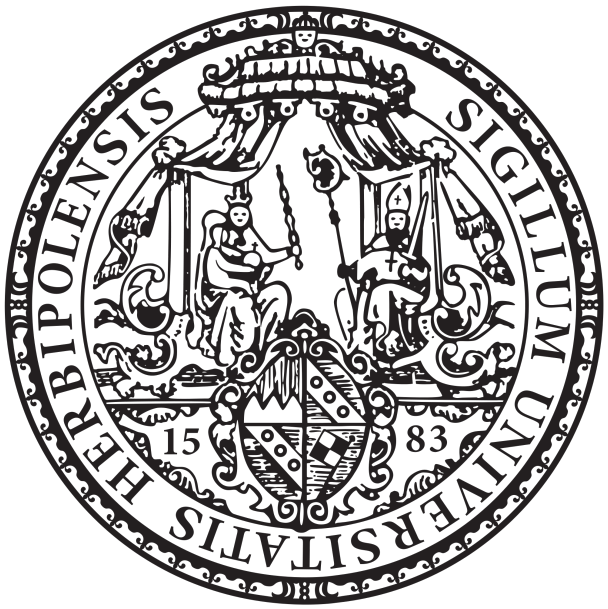

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Eingereicht am:

Mitglieder der Promotionskommission
Vorsitzender:
Gutachter: Prof. Dr. Dirk Becker
Gutachter: Dr. Christoph Weiste

Tag des Promotionskolloquiums: .....

Doktorurkunde ausgehändigt am: .....

Contents

Summary

Plants are incessantly challenged by both biotic and abiotic stresses. Their responses towards those stressors involve complex physiological, cellular, and molecular processes, with stomatal movement as one of the fastest responses. The transfer of stress signals in plants is mediated by various primary and secondary messengers.
As an essential macronutrient K^+ plays crucial roles in diverse physiological processes during plant growth and development. It serves as a major osmoticum regulating turgor pressure and membrane potentials. K^+ channels and transporters regulate K^+ homeostasis in plant cells and have diverse regulatory functions.
A lot of abiotic and biotic stresses act on disturbing the K^+ balance by influencing K^+ channels. This influence can be carried out on transcriptional level, controlling a channel's expression, as well as via post-translational regulation. The latter can appear as e.g. reversible phosphorylation of a channel protein or by binding a signal molecule, like a 14-3-3 dimer.Post-translational modification is essential in the context of rapid (stress) signaling networks. So, it is not suprising that a huge array of (de-)phosphorylation events are involved in the signaling resulting in stomatal movement.

To keep the balance between CO_2 uptake and transpiration is important for plants and relies on tightly controlled turgor changes, which are caused by the activity of different anion and cation channels. Those channels are part of signaling cascades that are set off e.g. via phytohormones, such as ABA (abscisic acid) and JA (jasmonate), both acting during drought stress in guard cells. JA is furthermore known to be involved in a plant's response to pathogen attack or wounding.
GORK (guard cell outward rectifying K^+ channel) is the only outward rectifying K^+ channel known in guard cells, so it is primarily responsible for K^+ efflux during stomatal closure.
In the course of this work it could be demonstrated by stomatal aperture assays, that GORK is an essential part of JA-induced stomatal closure. This is true for both triggers, leaf wounding as well as direct MeJA (methyl jasmonate) application. Patch clamp experiments on guard cell protoplasts backed this finding by revealing GORK K^+_{out} currents as a target of JA signaling in guard cells.

As cytosolic Ca^{2+} signals are known to be involved in both ABA as well as JA signaling, the interaction of GORK with Ca^{2+}-dependent kinases was examined consequently. Protein-protein interaction studies disclosed interaction of CIPK5 (calcineurin B-like interacting protein kinase 5) in combination with CBL1 or CBL9 (calcineurin B-like proteins) with GORK both *in-vitro* and *in-vivo*.
In addition, the PP2C (protein phosphatase type 2C) ABI2 (abscisic acid insensi-

tive 2), a well-known negative regulator of ABA-dependent stomatal closure, was found as another interactor of GORK and potential counteractor of the CIPK5 mediated phosphorylation. This probable, antagonistic regulation of GORK by CIPK5-CBL1/9 complexes and ABI2 could be backed up by DEVC (double electrode voltage clamp) experiments.
Hence, *in-vitro* kinase assays with recombinant proteins were performed and could show, that CIPK5 phosphorylates the N-terminus but not the C-terminus of GORK and that this phosphorylation is directly counteracted by ABI2. Using site-directed mutagenic, recombinant proteins in *in-vitro* kinase assay, it was possible to determine the amino acids T34 and T48 of the GORK N-terminus as probable phosphorylation targets of CIPK5. Thus, patch-clamp recordings on guard cell protoplasts of *cipk5-2* kinase loss-of-function mutant revealed the importance of CIPK5 for JA-triggered stomatal closure via activation of GORK. For the first time these results identify CBL-type Ca^{2+} sensor proteins and their interacting kinase as essential components in fast JA signal transduction in guard cells.

CDPKs (Ca^{2+} dependent protein kinases) are an important group of directly Ca^{2+}-sensing protein kinases that have been demonstrated to be part of numerous signaling pathways and function as positive regulators of abiotic stress responses. Additionally, the interaction of GORK with CDPKs was investigated in the course of this study.
In-vitro phosphorylation assays indicated, that GORK N- and C-terminus are both phosphorylated by CPK3 and CPK21 while CPK6 only phosphorylates the N-terminus. During the further research, only CPK21 could be examined closer and the amino acids T34 and to a certain extent also S24 were identified as potential phosphorylation targets of CPK21 in the GORK N-terminus.
CDPKs had been identified as possible binding partners for the small 14-3-3 proteins in *Arabidopsis thaliana* before and also an effect of 14-3-3 on GORK function had been indicated in previous studies. Therefore, this potential menage-a-trois and its physiological impact on wounding-associated JA signaling was addressed during this work. This way it could be indicated, that GRF2 (aka 14-3-3 omega) enhances the interaction of GORK with CPK21 and most probably CPK6, thus modifying the channel's activity.

Besides Ca^{2+} signaling also ROS (reactive oxygen species) production followed by cytoplasmic alkalization is essential in ABA and MeJA signaling. In DEVC experiments a reversible effect of ROS on GORK channel activity could be demonstrated during this study, which could be one piece in the explanation of those ROS effects in ABA and MeJA signaling.

Finally, a model of jasmonate regulated stomatal closure and crosstalk with the ABA signaling pathway, based on available published evidence, could be deduced as an assembly of this study's results. It converges hormone and Ca^{2+}-mediated activation allowing for efficient fine-tuning of GORK-mediated K^+ efflux and providing the mechanistic flexibility to facilitate stomatal closure in response to diverse stimuli such as drought or wounding.

Zusammenfassung

Pflanzen sind ständig mit biotischen und abiotischen Stressfaktoren konfrontiert. Ihre Reaktionen auf diese Stressoren umfassen komplexe physiologische, zelluläre und molekulare Prozesse, wobei die Bewegung der Schließzellen eine der schnellsten Reaktionen darstellt. Die Übertragung von Stresssignalen wird durch verschiedene primäre und sekundäre Botenstoffe vermittelt.
Als essentieller Makronährstoff spielt Kalium (K^+) eine entscheidende Rolle bei verschiedenen physiologischen Prozessen während des Wachstums und der Entwicklung von Pflanzen. Dies zeigt sich u.a. bei der osmotischen Regulation des Turgordrucks und der Membranpotentiale. K^+-Kanäle und -Transporter steuern die K^+-Homöostase in Pflanzenzellen und haben vielfältige regulatorische Funktionen.
Viele abiotische und biotische Stressfaktoren, stören das K^+-Gleichgewicht, indem sie K^+-Kanäle beeinflussen. Dieser Einfluss kann sowohl auf transkriptioneller Ebene erfolgen, indem die Expression der Kanäle kontrolliert wird, als auch durch posttranslationale Modifikation. Letztere kann z. B. durch reversible Phosphorylierung eines Kanalproteins oder durch Bindung eines Signalmoleküls, wie eines 14-3-3-Dimers, an (phosphorylierte) Proteinsequenzen des Kanals erfolgen. Solche posttranslationalen Modifikationen sind wesentlich beim Ablauf schneller Signalwege. Es ist daher nicht überraschend, dass eine Vielzahl von (De-)Phosphorylierungsvorgängen an der Signalweiterleitung in den Schließzellen beteiligt ist, die in Stomatabewegungen resultieren.

Ein stabiles Gleichgewicht zwischen CO_2-Aufnahme und Transpiration ist für Pflanzen essentiell und wird von streng kontrollierten Turgorveränderungen verursacht, die durch die Aktivität verschiedener Anionen- und Kationenkanäle erfolgen. Diese Kanäle sind auch Teil von Signalkaskaden, die z. B. durch Phytohormone wie Abscisinsäure (ABA) und Jasmonat (JA) ausgelöst werden. Es ist bekannt, dass beide Phytohormone bei Trockenstress in den Schließzellen eine Rolle spielen. Darüber hinaus ist JA an der Reaktion von Pflanzen auf Pathogenbefall oder Verwundung beteiligt.
Als einziger in den Schließzellen bekannter, auswärts gleichrichtender K^+-Kanal, ist GORK (guard cell outward rectifying K^+-Kanal) in erster Linie für den K^+-Ausstrom während des Schließens der Stomata verantwortlich. Seine Regulierung und insbesondere seine posttranslationale Modifikation werden daher seit langem untersucht, sind allerdings noch nicht vollständig geklärt.
Im Rahmen dieser Arbeit konnte durch Analyse von Stomaschlussweiten gezeigt werden, dass GORK ein wesentlicher Bestandteil des JA-induzierten Stomaschlusses ist. Dies gilt sowohl bei Blattverwundung als auch bei direkter Stimulation des Blattes mit Methyljasmonat (MeJA). Dieser Befund konnte durch Patch-Clamp-

Experimente an Schließzellprotoplasten untermauert werden, welche zeigen konnten, dass GORK-K^+_{out}-Ströme Ziel von JA-Signalen in Schließzellen sind.

Da zytosolische Ca^{2+}-Signale bekanntermaßen sowohl in ABA- als auch in JA-Signalwwegen beteiligt sind, wurde die Interaktion von GORK mit Ca^{2+}-abhängigen Kinasen untersucht. Protein-Protein-Interaktionsstudien ergaben, dass die Calcineurin B-ähnliche interagierende Proteinkinase 5 (CIPK5) in Kombination mit den Calcineurin B-ähnlichen Proteinen CBL1 oder CBL9 sowohl *in-vitro* als auch *in-vivo* mit GORK interagiert. Darüber hinaus wurde die PP2C (Proteinphosphatase Typ 2C) ABI2 (Abscisinsäure insensitive 2), ein bekannter negativer Regulator der ABA-abhängigen Stomaschließung, als ein weiterer Interaktionspartner von GORK und somit potenzieller Gegenspieler der CIPK5-vermittelten Phosphorylierung gefunden. Diese vermutete antagonistische Regulierung von GORK durch CIPK5-CBL1/9-Komplexe und ABI2 konnte durch DEVC-Experimente (double electrode voltage clamp) bestätigt werden. Daher wurden *in-vitro*-Kinase-Assays mit rekombinanten Proteinen durchgeführt und konnten zeigen, dass CIPK5 den N-Terminus, nicht aber den C-Terminus von GORK phosphoryliert und dass dieser Phosphorylierung durch ABI2 direkt entgegengewirkt wird. Unter Verwendung von zielgerichtet mutagenen, rekombinanten Proteinen im *in-vitro*-Kinase-Assay konnten die Aminosäuren T34 und T48 des N-Terminus von GORK als wahrscheinliche Phosphorylierungsstellen von CIPK5 bestimmt werden. Mit Hilfe von Patch-Clamp-Messungen an Schließzellprotoplasten der *cipk5-2*-Loss-of-Function-Mutante konnte die Bedeutung von CIPK5 für den JA-ausgelösten Stomataschluss über die Aktivierung von GORK nachgewiesen werden. Diese Ergebnisse identifizieren erstmals Ca^{2+}-Sensorproteine vom CBL-Typ und die mit ihnen interagierende Kinase als wesentliche Komponenten der schnellen JA-Signaltransduktion in Schließzellen.

Darüber hinaus wurde die Interaktion von GORK mit Ca^{2+}-abhängigen Proteinkinasen (CDPKs oder kurz CPKs) untersucht, einer wichtigen Gruppe von direkt Ca^{2+}-bindenden Proteinkinasen, die nachweislich Teil zahlreicher Signalwege sind und als positive Regulatoren von abiotischen Stressreaktionen fungieren. Im Rahmen dieser Studie zeigten *in-vitro*-Phosphorylierungsassays, dass sowohl der N- als auch der C-Terminus von GORK durch CPK3 und CPK21 phosphoryliert werden, während CPK6 nur den N-Terminus phosphoryliert. Im weiteren Verlauf wurde ausschließlich CPK21 näher untersucht und die Aminosäuren T34 und S24 wurden als potentielle Phosphorylierungsziele von CPK21 im GORK N-Terminus identifiziert.
CPKs wurden schon als mögliche Bindungspartner für 14-3-3-Proteine in *Arabidopsis thaliana* identifiziert, und auf ein Zusammenspiel von 14-3-3 und GORK wurde in früheren Studien mehrfach hingewiesen. Daher wurde diese potenzielle Menage-a-trois und ihre physiologische Auswirkung auf die verwundungsassoziierte JA-Signalkette in dieser Studie untersucht. So konnte gezeigt werden, dass GRF2 (auch bekannt als 14-3-3 omega) die Interaktion von GORK mit CPK21 und höchstwahrscheinlich CPK6 verstärkt und dadurch die Aktivität des Kanals beeinflusst. Eindeutige Beweise für diese Hypothese müssen noch gefunden werden.

Neben Ca^{2+}-Signalen ist auch die Produktion von ROS (reactive oxygen species),

gefolgt von einer zytoplasmatischen Alkalisierung, für die ABA- und MeJA-Signalwege wesentlich. In DEVC-Experimenten konnte hier ein reversibler Effekt von ROS auf die Aktivität des GORK-Kanals nachgewiesen werden, was ein Teil der Erklärung für diese ROS-Effekte bei der ABA- und MeJA-Signalweiterleitung sein könnte.

Schließlich wurden die Ergebnisse dieser Studie in einem Modell des JA-gesteuerten Stomatenschlusses basierend auf bereits veröffentlichter Fakten und unter Einbezug des Zusammenspiels mit dem ABA-Signalweg zusammengefasst. Darin wird die Hormon- und Ca^{2+}-vermittelte Aktivierung zusammengeführt, wodurch eine effiziente Feinabstimmung des GORK-vermittelten K^+-Ausflusses ermöglicht wird. Es bietet außerdem die notwendige Flexibilität, die das Schließen der Stomata als Reaktion auf verschiedene Stimuli wie Trockenheit oder Verwundung erleichtert.

1 Introduction

Terrestrial plants are sessile organisms and need to be able to continuously adjust to environmental changes. Molecular analysis, functional characterization of plant proteins and elucidation of networks and crosstalk allows us to recognize the mechanisms that plants have developed in order to react appropriately to their abiotic and biotic environment. Among the most important reactions are reactions of a plant to stress situations and health threats. Ion channels that regulate the osmotic balance of plant cells play a crucial role in these responses and have been in the focus of investigation for years. In addition, the elucidation of possible interactions within networks that control the activity of these channels and coordinate whole signaling cascades, are of major interest.

1.1 The role of potassium in higher plants

Potassium (K^+) is essential for higher plants' nutrition and can comprise up to 10% of total plant dry weight [102]. Cellular functions of K^+ can be classified into two basal aspects. On the one hand, many physiological processes in distinct cellular compartments depend on constantly high K^+ concentrations in distinct cellular compartments, this includes enzyme activation or neutralization of negatively charged proteins. On the other hand, the movement of K^+ ions across membranes causes changes in the osmotic pressure and plays an important role for example in stomatal movement or growth processes.[118, 74]
K^+ is taken up by the plant root through the epidermal and cortical cells and is then transported to the shoot. Already in 1963 Emanuel Epstein discovered that at least two independent systems are involved in K^+ uptake in plants: a high-affinity system ($K_M \simeq 18\mu M$) for very low soil K^+ concentrations in the micromolar range and a low-affinity system ($K_M \simeq 16mM$) for K^+ concentrations in the millimolar range [56]. A resulting biphasic K^+ uptake response has since been observed in a lot of plant species, although some exceptions can be found. Maize for example shows a linear non-saturating response in the low-affinity range [91]. The high-affinity uptake against a steep concentration gradient is most likely energized by K^+/H^+ symporters, whilst in the low-affinity range K^+ uptake seems to be mediated by inwardly rectifying K^+ channels that offer a way of simple diffusion of K^+ ions along its electrochemical gradient across biological membranes [64, 114, 144]. Despite its overall importance, it is currently not known, how plants are able to sense intra-/extracellular K^+ concentrations, how this information is processed and how it leads to a distinct physiological or molecular response.

Under repleted K^+ conditions, plant cells maintain a K^+ concentration of around 80 mM in the cytosol, which is an optimal level to fulfill its metabolic functions [217]. In contrast to this relatively high and stable K^+ concentration, the available K^+ in the surrounding soil is highly variable, ranging between approximately 0.025 to 5 mM [113]. Only a small percentage of the total K^+ in the soil exists in a form available for plant uptake, In addition, K^+ is relatively immobile in the soil solution, limiting its availability to a small cylinder surrounding each root [119]. Agricultural practices attempt to lessen soil K^+ variability using fertilizers, nevertheless intensively cultured land can become K^+ deficient. Plants that experience K^+ deficiency show reduced growth and a higher susceptibility to pathogens [4, 118]. Elucidating the molecular mechanism of root K^+ uptake has been of considerable interest, in order to obtain crop varieties displaying improved K^+ use efficiency [166]. Members of the KT/HAK/KUP family, such as HAK1 in rice ([10]), pepper ([121]) or HAK5 in *Arabidopsis* ([170, 169]) have been identified as candidates for high-affinity K^+ uptake, while for the low-affinity range AKT1 (Arapidopsis K^+ Transporter 1), a K^+ channel of the Shaker family, has been suggested ([98, 169]). Using T-DNA insertion lines Nieves et al. (2010) showed, that AtHAK5 is an important system mediating root K^+ uptake at concentrations below 0.01 mM. In the range between 0.01 and 0.05 mM K^+ AtHAK5 and AtAKT1 both contribute to K^+ acquisition and at higher K^+ concentrations, other systems, probably non-selective cation channels, come into operation and participate together with AtAKT1 in low-affinity K^+ uptake. [143]
Upon K^+ depletion, root cells respond with ROS accumulation and hyperpolarization of the membrane potential [5, 47, 79, 189, 188]. Both reactions have been linked to HAK5 gene expression [145, 189]. Other signal molecules that have been proposed to be involved in plant responses to low-K^+ stress are phytohormones, such as ethylene, auxin and jasmonic acid, microRNAs or calcium [221]. Xu et al. figured out, that in *Arabidopsis* the Ca^{2+} sensors CBL1 and CBL9 via interaction with their interacting kinase CIPK23 activate AKT1 and HAK5 under low-K^+ conditions [162, 230]. The previously proposed low-K^+ induced Ca^{2+} signal was recently observed employing the Ca^{2+} reporter YC3.6 [17]. Behera et al. could show two spatially and temporally defined Ca^{2+} signals in *Arabidopsis* roots upon low-K^+ stress [17].

As a very important nutrient and osmolyte, K^+ regulates turgor pressure of plant cells and therefore guard cells and grass coleoptiles have become important model systems for studying the role of K^+ channels in reversible respectively irreversible plant growth and movements. The cell expansion during monocot coleoptile and dicot hypocotyl growth including gravi- and phototropism depend on the redistribution of the phytohormone auxin, differential expression of K^+ uptake channels and accumulation of K^+ in the expanding cells [16, 59].
K^+ homeostasis is further essential for polar, tip growing cell types like root hairs or pollen tubes, which represent ideal model systems as well [74, 216]. Root hairs are epidermal cells that exhibit K^+ influx at the growing tip and K^+ efflux at the base. Besides AKT1 in *Arabidopsis* they express AtKC1, which functions as modulatory

subunit in heteromeric K^+ channels and GORK (Guard cell Outward Rectifying K^+ channel) [85]. Pollen tubes, emerging from the germinating pollen grain, show expression of SPIK (Shaker Pollen Inward K^+ channel), GORK, SKOR (Stelar K^+ Outward Rectifying channel) and exclusively TPK4 (Tandem Pore K^+ channel 4) [14, 74, 101]. The polar growth of both cell types is achieved by continuous exocytosis at their tip regions - a process, which is sensitive to osmotic changes. These seem to be fine tuned by the activity of K^+ channels. In *Arabidopsis* pollen tubes for example, the calcium-dependent kinases CPK11 and CPK24 play an important role in the regulation of SPIK and consequently modulate pollen tube elongation [242].

1.2 Plant potassium channels

K^+ uptake by plant root cells as well as K^+ transport inside plants are performed by a large number of K^+ channels and transporters. Thus, identification, functional characterization and regulatory mechanisms of K^+ channels and transporters have been in the focus of research for years. This led to the identification of numerous novel K^+ transporters and channels with diverse physiological functions in K^+ uptake, photosynthesis, development, reproduction and stress tolerance. [74, 215]
Plants maintain their cytoplasmic K^+ concentration at approximately 100 mM [118], which is usually far above the surrounding concentration. Consequently, K^+ has to get into the cytoplasm against a steep chemical gradient. Proton gradients together with hyperpolarized membrane potentials make this possible: the resulting electrochemical gradient represents the driving force for ion passage across biological membranes.
In *Arabidopsis* 15 K^+-selective channels are known and are divided into three different groups as seen in figure 1.1. Nine channels belong to the *Shaker* family, five to the tandem pore K^+ channels (TPKs) and one (KCO3) is a KCsA-like inward rectifier [74, 122, 214].

KCO3 has the most simple structure, as it is build of two transmembrane domains that flank a pore region [214] (cp. figure 1.1). These channels are located at the plasma and the vacuolar membrane and activate over a broad voltage range.[14, 43, 74, 176].
Shaker-type channels belong to the best characterized transport proteins in plants so far. They got their name from their similarity to the *Shaker* channels identified in *Drosophila melanogaster*. Defects in these genes lead to trembling legs of the fly. [202, 207]
Shaker channels form tetramers from four α subunits, with six transmembrane domains each (S1-S6). The highly conserved pore loop is located between S5 and S6. The four pore loops of the tetramer extend into the plasma membrane, forming a hydrophilic pore, which is selective for K^+. The transmembrane S4 includes many positively charged amino acids and is considered as the channel's voltage sensor [52, 209]. The movement of S4 in response to voltage changes results in opposite

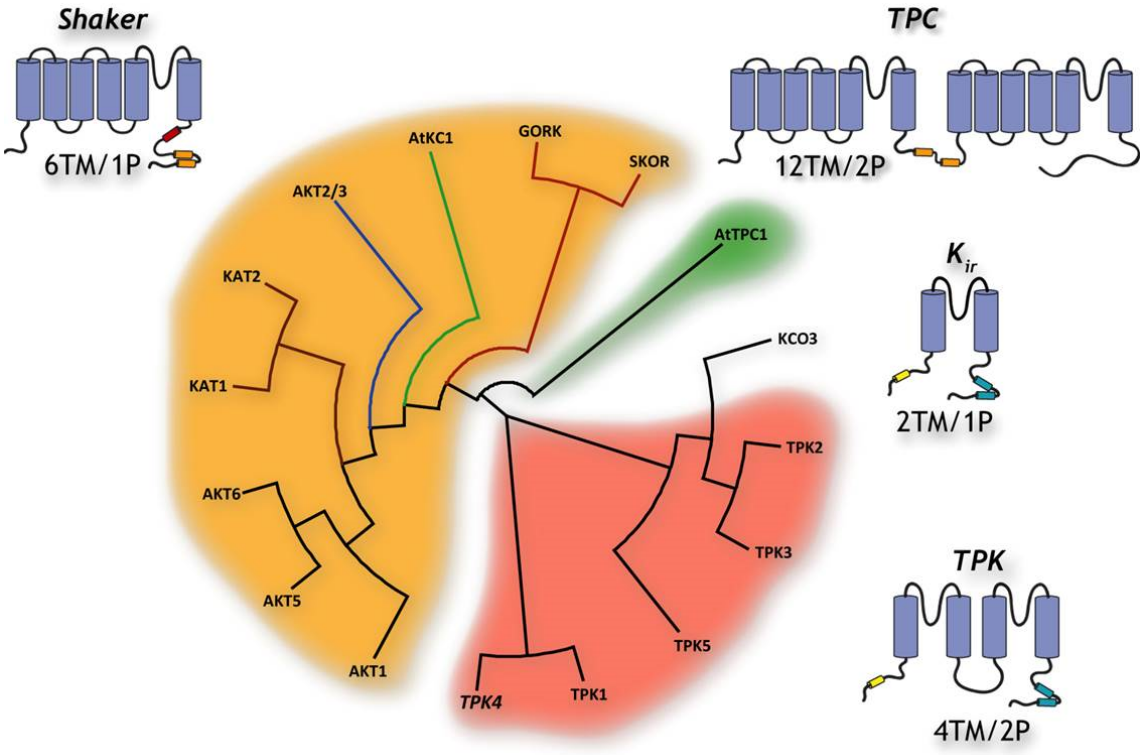

Figure 1.1: Phylogeny and Topology of *Arabidopsis* K^+ channels

Arabidopsis K^+-selective channels can be divided into three families based on sequence similarity. Traditionally known are six *Shaker*-like channels, five TPK channels and KCO3. TPC1 represents a cation channel of unique structure. TM = transmembrane domain, P = pore [74]

effects on the channel open probability in inward and outward rectifiers [238]. At the C-terminus, *Shaker* channels possess a putative cyclic nucleotide binding site and some also contain five to six repeats of an ankyrin sequence, which is proposed to be involved in cytosolic regulation or interaction with other proteins. [2, 214]
Shaker channels are typically localized to the plasma membrane and are activated in a voltage-dependent manner. The nine members of this family in *Arabidopsis* are grouped according to their characteristical opening and closing transitions, i.e. "gating". Inward rectifying channels, like AKT1, open upon hyperpolarization of the plasma membrane and mediate K^+ influx into the cell, while weakly rectifying channels, like AKT2, allow both K^+ influx and efflux and are only slightly dependent on the membrane potential. Depolarisation of the membrane activates the outward rectifying channels GORK and SKOR and K^+ release occurs. [53]
GORK, the channel of interest in this work, belongs to this last class and is thus active upon membrane depolarisation positive of the reversal potential of K^+. This prevents K^+ release under disadvantageous conditions of K^+ supply [2, 74]. The membrane depolarisation can result for example from anion efflux due to the activation of anion channels during stomatal closure. GORK is the only outward rectifying K^+ channel known in guard cells, so it is primarily responsible for K^+ efflux during

stomatal closure, which represents a crucial process for plant survival under conditions of drought [2, 74, 82]. In addition, GORK also takes part in K^+ homeostasis in root hairs and is actually ubiquitously expressed in the *Arabidopsis* plant [85]. The channel responds to the external K^+ concentration by different activation kinetics, offering different membrane K^+ conductances for different K^+ gradients [2]. Stress signals such as the phytohormone abscisic acid (ABA), drought and salt stress increase the expression level of GORK [2, 15, 82, 214]. Cytosolic potassium homeostasis under salt stress conditions appeared as an essential mechanism of salinity stress tolerance [107, 198, 228]. Being confronted with salinity stress, plants have to cope with rapid and massive membrane depolarization upon Na^+ uptake [33, 182]. It has been shown that GORK is involved in K^+ efflux and its resulting programmed cell death after membrane depolarization upon sodium influx into the cell during salinity stress [46, 183, 184]. Furthermore, electrophysiological and genetic studies have shown that GORK is a key player for K^+ efflux from plant cells in salinitiy, oxidative and hypoxia stress tolerance [46, 47, 185, 218]. Activation of the channel by reactive oxygen species (ROS) is in agreement with ROS being a central part of many plant stress responses [46]. The upregulation of GORK expression by ABA requires extracellular calcium, which also is involved in ABA signaling in guard cells [2]. Consequently, calcium-dependent signaling networks are likely to be involved in GORK activation [6]. The regulatory domains in the N- and C-terminus of the protein lead to the proposition, that GORK activity is post-translationally modified by protein-protein interactions or other factors, i.e. superoxide [205]. This highlights an interesting research field that will help understanding the role of GORK in *Arabidopsis*.

Comparison of K^+ channels in different plant species enables to deduce different stages in channel evolution (cp. figure 1.3). From the example of the GORK-family it can be seen, that GORK-like-channels from human (*Homo sapiens*, Hs), fruit fly (*Drosophila melanogaster*, Dme) and the algae *Chara braunii* (Cb) cluster apart from all the rest. This reflects, that algae and fauna split off from plants long ago in evolution. Next *Selaginella moellendorffii* (Sm) branches off, followed first by the mosses (*Marchantia polymorpha*, Mp) and second by the (aquatic) ferns (*Azolla filiculoides*, Af and *Salvinia cucullata*, Sc). Then the higher plants evolved. First the conifera branch off (*Picea abies*, Pa and *Pseudotsuga menziesii*, Pme) and then the last group is build of the dicots (*Nicotiana tabacum*, Nt, *Arabidopsis thaliana*, At and *Dionea muscipula*, Dmu). The GORK-like-channels from the monotypic genus *Amborella trichopoda*, which is considered as the sister group to flowering plants, seperates solely between ferns and higher plants on the one hand (*AmTrORK1*) and together with date palm (*Phoenix dactylifera*, Pda) it appears again as a sidegroup of the dicotyledons. This marks its unique rank in plant evolution.

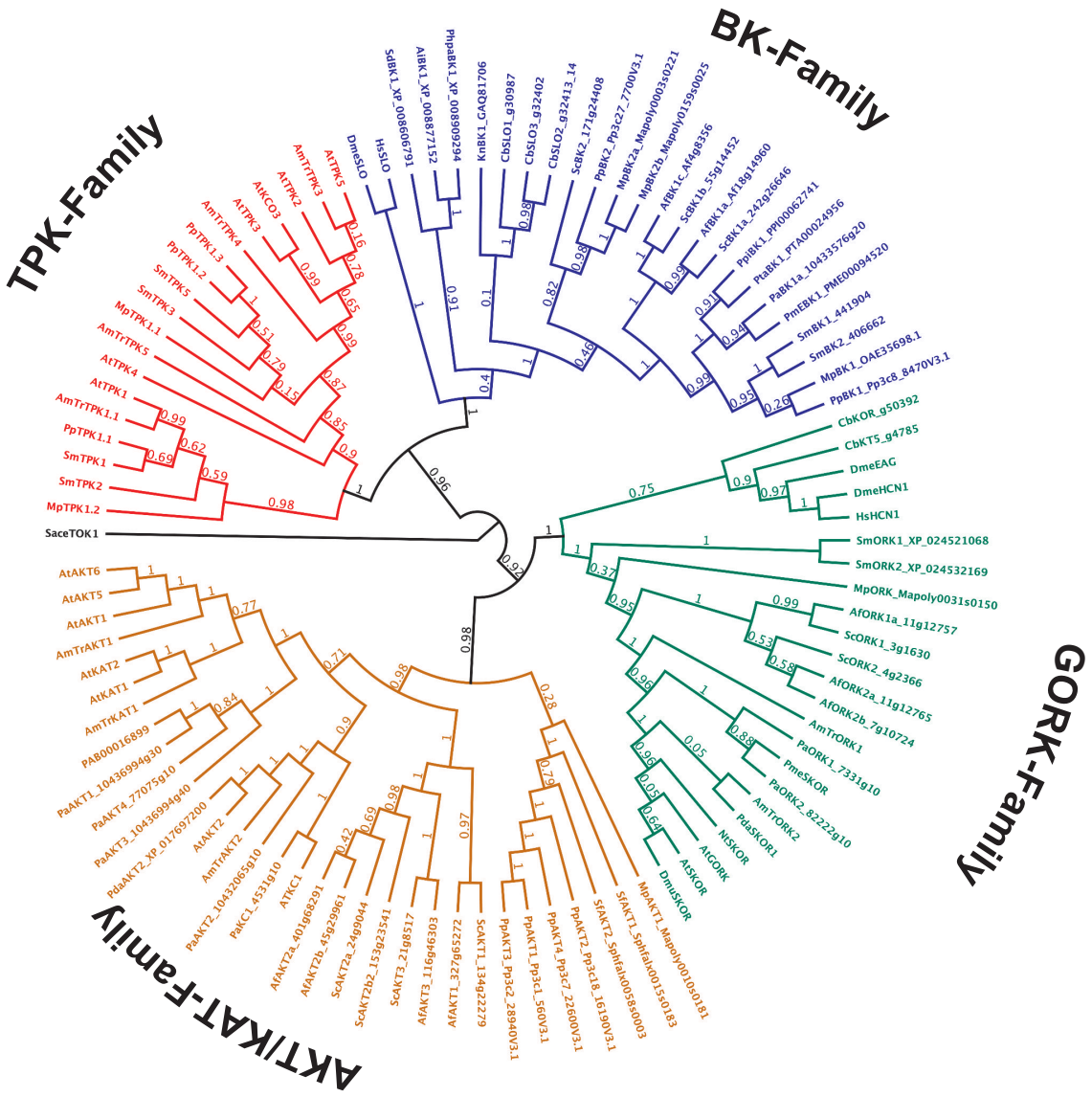

Figure 1.3: Phylogenetic tree of K^+-channels of various plant species

Evolutionary relationship of plant K^+-channels from the GORK-, AKT/KAT-, TPK- and BK-family; prefixes stand for respective species: Af = *Azolla filiculoides*, Ai = *Aphanomyces invadans*, Am = *Amborella trichopoda*, At = *Arabidopsis thaliana*, Cb = *Chara braunii*, Dme = *Drosphila melanogaster*, Dmu = *Dionea muscipula*, Hs = *Homo sapiens*, Kn = *Klebsormidium nitens*, Mp = *Marchantia polymorpha*, Nt = *Nicotiana tabacum*, Pa = *Picea abies*, Pda = *Phoenix dactylifera*, Phpa = *Phytophthora parasitica INRA-310*, Pme = Pseudotsuga menziesii, Pp = *Physcomitrella patens*, Ppi = *Pinus pinaster*, Pta = *Pinus teada*, Sace = *Saccharomyces cerevisiae*, Sc = *Salvinia cucullata*, Sd = *Saprolegnia diclina VS20*, Sf = *Sphagnum fallax*, Sm = *Selaginella moellendorffii*; calculated with IQTree [206] and visualized with Geneious and Affinity Designer; alignments done with MAFFT in Geneious [168]; values are boostrap values

1.3 Regulation of plant ion channels

The control of the activity of distinct proteins in a plant cell is retained by a tight regulatory network of post-translational modifications, ions, metabolites, protein-protein-interactions and so forth. Post-translational modifications are highly monitored in the cells, and often serve as rapid and specific molecular switches to regulate biochemical and physiological processes. [9] Tightly regulated and reversible phosphorylation reactions catalyzed by protein kinases and phosphatases are important in signal transduction pathways, that involve various physiological and pathological processes.

1.3.1 Calcium-(in)dependent phosphorylation

Calcium (Ca^{2+}) signals are core regulators of cell physiology in all organisms. The signaling pathways that use Ca^{2+} as a second messenger integrate information from biotic and abiotic stimuli and impact upon gene expression and general physiological responses. Crucial for the widespread function of this simple ion, and for the generation of specificity in linking signal and response, is the variety of Ca^{2+} signaling in terms of spatio-temporal patterning [3, 13, 171, 224]. Stimulus induced rise in free cytosolic Ca^{2+} in plants often occurs as repetitive oscillations or spikes of different amplitude, frequency and shape [34, 51, 94, 95, 171]. Spatially distinct release of Ca^{2+} from different Ca^{2+} stores, together with the slow cytoplasmic diffusion rate of Ca^{2+} ions ensure locally defined signaling events [13, 39]. The apoplast or several intracellular compartments such as the vacuole, the endoplasmic reticulum, mitochondria or chloroplasts can serve as sources for Ca^{2+} entry into the cytoplasm[13, 148, 171]. Ca^{2+} transport across cell membranes has been studied in detail during the last years. It is a combination of Ca^{2+} flux via several families of ion channels, including cyclic nucleotide-gated channels (cNGCs), glutamate receptors, two-pore channel 1 (TPC1), annexins, mechanosensitive channels, hyperosmolality-gated calcium-permeable channels (OSCAs) and active, energy-dependent transport through Ca^{2+}-ATPases and Ca^{2+}/H^{+} exchangers [48, 95]. In many cases, stress stimuli induce Ca^{2+} signals in plant cells, as reported for ABA- or flg22-induced stomatal closure [142] (see also 1.4.2). Diverse proteins including transcription factors, transporters and kinases represent targets for Ca^{2+} signals [164]. Most Ca^{2+} sensoring proteins recognize Ca^{2+} signals via the elongation factor hand (EF-hand) motif, a characteristic and conserved helix-loop-helix structure capable to coordinate one Ca^{2+} ion [65, 187]. The genome of *Arabidopsis thaliana* encodes over 200 EF-hand containing, Ca^{2+}-binding proteins [45], with many of these genes co-expressed in the same cell types, illustrating the need for specific Ca^{2+} signaling mechanisms [26, 51].

Post-translational modifications are recognized as a major processes accounting for protein structural variation, functional diversity, and the dynamics and complexity of the proteome. They are essential to coordinate signaling networks and to regulate

important physiological processes in eukaryotes. Chemical modifications of specific amino acid residues of a protein and/or cleavage of the translated sequence can occur. They greatly increase the structural and functional diversity of proteins in a proteome by affecting properties of the proteins as charge status and conformation or resulting in changes of activity,binding affinity, localization and stability. [9] Reversible phosphorylation represents an important post-translational modification of proteins, regulating numerous aspects of eukaryotic cell responses. Consequently, phosphorylation cascades are primary downstream interpreters of Ca^{2+} signals.

In plants often Ca^{2+} binding proteins or Ca^{2+} sensors (calmodulin, calmodulin like proteins, calcineurin B-like proteins, and Ca^{2+} dependent protein kinases) recognize the changes in Ca^{2+} concentration [95, 171]. While some channels, as TPKs or TPC1 are directly activated by Ca^{2+} [14, 75, 180], in many cases Ca^{2+} sensors function as transmitters for Ca^{2+} signals to effectors. Ca^{2+} binding to Ca^{2+} sensors elicits a structural change that supports the interaction between the sensor and its target protein(s) or changes the activity of the Ca^{2+} sensor directly [65, 187].
Calcineurin B-like proteins (CBLs, 1.5C) function as membrane-anchored Ca^{2+} sensors that, upon activation, recruit a specific set of serine/threonine (Ser-Thr) kinases, calcineurin B-like protein-interacting protein kinases (CIPKs, 1.5B) to their sites of action [94, 109]. They share very little sequence similarity with calmodulins, thus they seem to have diverged from the calmodulins very early in evolution. [13, 95] CBLs, which are involved in the salt-overly-sensitive (SOS) pathway of salt stress signaling, were first identified in *Arabidopsis thaliana* [106].
CBL proteins contain four EF-hand motifs, of which the first one has an extraordinary and characteristic feature as its Ca^{2+} binding loop consists of 14 instead of 12 amino acids in *Arabidopsis*, but it is still capable to bind Ca^{2+} [138] (see fig. 1.5C). *Arabidopsis thaliana* encodes 10 CBL and 26 CIPK proteins. The CBLs can be classified based on their N-terminal region. CBL10 has a hydrophobic N-terminus transmembrane domain, which is special and unique. A short N-terminal region of 27-32 amino acids characterizes the first class of CBL proteins wich includes CBL1, CBL4, CBL5, CBL8 and CBL9. With exception of CBL8 they all have a MGXXX(S/T) consensus sequence for myristoylation. The second category comprises CBL2, CBL3 and CBL6, which harbor an extended, 41-43 amino acid long N-terminus. According to sequence comparison CBL7 belongs to the second category, as well, even though it may have lost the N-terminal extension during evolution. [11, 12, 225]

Several CBL-CIPK complexes have been identified that connect Ca^{2+} sensing with different physiological responses through a range of target proteins. They are involved in mediating Ca^{2+} signals from different stressors, such as low magnesium, low nitrate, low potassium, high salt, low phosphorus, ABA, high pH and cold. [26, 35, 117, 161, 187, 221] Each CBL, though, preferentially interacts with a defined subset of CIPKs and vice versa to ensure signal specificity. [13, 95] CIPKs show a conserved modular structure consisting of an N-terminal Ser-Thr kinase domain, a junction domain and a C-terminal regulatory domain including the autoregulatory NAF-domain and a phosphatase interaction domain (PPI; see fig. 1.5B)

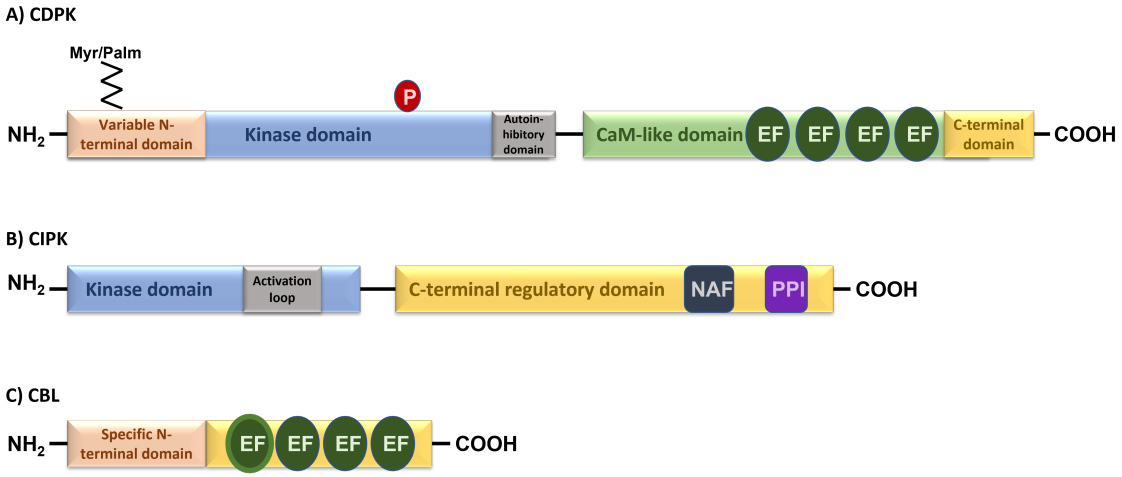

Figure 1.5: Schematic structure of CPKs, CIPKs and CBLs

A) Basic structure of a calcium-dependent protein kinase (CPK) with the variable N-terminus including potential lipid-modification sites, the serine/threonine kinase domain with ATP-binding site, an autoinhibitory domain, a calmodulin-like domain, which includes the EF-hands and the C-terminal domain;
B) The conserved modular structure of calcium-independent protein kinases (CIPKs) with an N-terminal Ser-Thr kinase domain, a junction domain and a C-terminal regulatory domain including the auto-regulatory NAF-domain and a phosphatase interaction site (PPI);
C) Calcineurin B-like proteins (CBLs) contain four EF-hand motifs, of which the first one has an extraordinary and characteristic feature as its Ca^{2+} binding loop consists of 14 instead of 12 amino acids in *Arabidopsis* and can be classified based on their specific N-terminal region.

[95, 151, 186]. CBL-CIPK interaction releases the inhibitory effect of the NAF-domain by structural rearrangements and targets the kinase towards its phosphorylation objective and lipid modification of the CBL N-terminus is responsible for their membrane association [13, 32, 95]. This process allows one CIPK to function at different membranes depending on the interaction partner. More and more regular functions have been assigned to CBL-CIPK complexes that range from channel regulation, abiotic stress tolerance and plant development to immune responses [94, 95]. As an example CIPK23 appears to be multifunctionally important as it has been reported to function in potassium and nitrate as well as iron, magnesium and ammonium homeostasis [63, 81, 116, 195]. Held et al. showed that CBL4 interaction with CIPK6 modulates the activity and plasma membrane targeting of the K^+ channel AKT2 from *Arabidopsis thaliana* by mediating translocation of AKT2 to the plasma membrane and enhancing its activity [78]. Remarkably, no phosphorylation activity of CIPK6 toward AKT2 could be detected *in vitro*. the regulation of AKT2 by CIPK6 seems to be phosphorylation-independent but Ca^{2+}-dependent and the resulting translocation from the endoplasmatic reticulum to the plasma membrane builds the critical step of AKT2 activation. [78]

Another fundamental family of protein kinases in plants are the calcium-dependent protein kinases (CPKs or CDPKs, 1.5A). The first plant CPK was reported in *Pisum sativum* in 1984 [80], while CPK studies in *Arabidopsis thaliana* began with CPK10 and CPK11 ten years later [210]. The *Arabidopsis* CPK family consists of 34 different isoforms, that can be divided into four distinct subgroups, based on their sequence similarities [23, 83]. Because the CPKs possess four C-terminal EF-hands (figure 1.5A), they can be directly regulated by Ca^{2+}. Thus, they act as Ca^{2+} sensors in the cell, which translate a rise in the intracellular Ca^{2+} concentrations provoked by stress signals to target proteins, in order to control downstream processes [34, 51, 83]. For example, CPKs are able to phosphorylate nitrate reductase [99] and sucrose-phosphate synthase [84] *in vitro*. Protein autophosphorylation is commonly observed on serine and threonine residues of most CPKs, occurs in a Ca^{2+}-dependent manner and stimulates kinase activity [77, 187].
The structure of the CPKs is divided into four domains, as shown in figure 1.5A: a variable N-terminus, the serine/threonine kinase domain with ATP-binding site, an autoinhibitory domain, that includes the putative autophosphorylation sites in 16 CPKs, a calmodulin-like domain, which includes the EF-hands and the C-terminal domain [34, 83, 171]. Upon Ca^{2+} binding to the EF-hands, conformational changes detach the autoinhibitory domain from the kinase domain, relieving the active site of the protein, thereby activating the kinase activity [70, 72]. The Ca^{2+}-sensitivity and Ca^{2+}-dependence or Ca^{2+}-binding ability of a CPK is dependent on EF-hand integrity and presence and varies among the 34 *Arabidopsis* CPKs [22, 104, 187]. Most of the CPKs (i.e. CPK6, 21 and 23) have predicted putative myristoylation and palmitoylation sites at the N-terminus, which are responsible for kinase membrane association [34, 71].
CPKs localize in different subcellular compartments, i.e. cytosol, nucleus, plasma membrane [71] and are known to be involved in diverse cellular processes, including stomatal movement, metabolism, response to abiotic stress and pathogen defense [21, 34, 104, 132, 187, 210]. CPK6, CPK21 and CPK23 for example, are known to be part of the ABA signaling pathway (cp. 1.4.3) where they phosphorylate and activate the anion channel SLAC1 (slow anion channel 1), which in turn triggers plasma membrane depolarization as the primary driving force for K^+ efflux from guard cells [25, 62, 175] (compare 1.4.2). CPK3 also functions in ABA regulation of stomatal closure [132], and CPK6 has been reported to promote methyl jasmonate induced stomatal closure [134] (cp. 1.4.3 and 1.4.1). The closest homolog to CPK6, CPK5, is associated with ROS signaling [21]. Disruption of CPK23 expression results in reduced stomatal apertures, while overexpression of CPK23 increased stomatal apertures [111]. In the past, CPKs were often recognized as positive regulators of abiotic stress responses and the over-expression of the respective kinase leads to plants with improved stress tolerance [7, 23], but now various studies have reported that they also behave as negative regulators. CPK3, CPK6 and CPK23 have been shown to play an important role in *Arabidopsis* responses to drought and salt stresses, with implications for their regulatory effect on K^+ uptake [111, 124, 229]. Although the functions of CPKs in plant responses to environmental stress have been demonstrated, the molecular biological mechanisms of CPKs remain widely

unclear.

1.3.2 14-3-3 proteins - small all-rounders in plant signaling

Members of the eukaryotic 14-3-3 family are proteins that regulate distinct biological processes through phosphorylation-dependent protein-protein interactions. The understanding of 14-3-3 function in higher organisms is surprisingly limited, mainly due to the huge spectrum of putative targets and the multigene 14-3-3 family. 14-3-3 proteins are active as dimers, both homo- and heterodimers, and their general role is to complete a signal transduction process by binding to a phosphorylated target. Kinases can serve as target proteins, as well as transcription factors, structural proteins, ion channels and other functional proteins can do [110, 152]. Their name originates from a systematic classification of brain proteins in 1967, and is based on their elution position after DEAE-cellulose chromatography and their mobility in subsequent gel electrophoresis [130]. 14-3-3 proteins have been found in all eukaryotic organisms studied so far and many organisms contain multiple isoforms.
14-3-3 proteins are able to interact with other proteins through canonical binding motifs, other non-canonical binding sites or phosphorylation events [199, 235]. The three identified binding motifs are Mode-1 (K/R xx S_p/T_p x P), Mode-2 (K/R xxx S_p/T_p x P, where x represents any amino acid and S_p/T_p is phosphorylated) and Mode-3 (YT_pV) [123, 136, 231]. Binding kinetics of 14-3-3s to these motifs is suboptimal, which enables them to perform their regulatory role.
Each L-shaped monomer forms a highly conserved amphiphatic groove where binding is mediated. In contrast, the N- and C-terminus of 14-3-3 proteins differ among isoforms and may provide their specificity [199]. Moreover, the N-terminus has been shown to be directly involved in dimerization [86], while the C-terminus is capable of binding divalent cations, a interaction that might influence client protein association [8]. From animal 14-3-3 proteins it is known, that the N-terminus can be acetylated, so the proteins might be N-terminally processed [120].

The *Arabidopsis* genome encodes 15 different 14-3-3 proteins, of which 13 are trancriptionally expressed and are also called general regulating factors 1-13 (GRF1-13) or named with greek letters chosen from the end of the alphabet (i.e. GRF2 = GRFω) [167]. They are expressed ubiquitously and the proteins containing forkhead-associated domains excepted, they are the only known phosphoprotein-binding proteins in plants [36, 199]. The major challenges to understand the functions of 14-3-3s in plants are, to identify their clients and to determine how they alter those structure and function. 14-3-3 proteins are able to regulate the target protein directly or can bring together two different partners, because two binding grooves are present due to dimerization.
It has been suggested that several metabolic enzymes are modified upon 14-3-3 binding, i.e. nitrate reductase [99] and sucrose-phosphate sythase [131], both are also known to be phosphorylated by CPKs (see 1.3.1). Some isoforms may also be able to bind directly to CPK1 and increase its activity [29]. CPK3 is also regulated by

certain 14-3-3 proteins. For example, it phosphorylates At14-3-3ζ, which in turn leads to CPK3 degradation in the end [97].
In addition to their regulation of cytoplasmic localized proteins such as CPKs and other enzymes, 14-3-3 proteins are also regulators of membrane associated proteins. For example, the plasma membrane H^+-ATPase is activated upon 14-3-3 binding, while F-type ATP synthases are inhibited [28]. Channels have also been reported to interact with 14-3-3 proteins. For instance, regulation of K^+-conducting vacuolar channels by Ca^{2+}, CPKs and 14-3-3s has been described [44, 101, 212] and KAT1 (K^+ channel *Arabidopsis thaliana* 1) interacts directly with 14-3-3 proteins in a manner, that shifts its activation curve and maximal conductance, as well as the total number of channels integrated in the plasma membrane [173, 192, 193]. The vacuolar channel TPK1 and the SV channels (Slow Vacuolar channels) are influenced by the same GRF, GRF6, in an opposing way. TPK1 is activated in a Ca^{2+}-dependent manner [101], while SV channel activity is suppressed [212].
Other identified potential interaction partners of 14-3-3 proteins predict the involvement of 14-3-3 proteins in cell cycle regulation and hormone signaling cascades [31, 158]. Schoonheim et al. showed 14-3-3 participation in ABA signal transduction *in vivo* in barley [177].
14-3-3 proteins bind and influence phosphoproteins, but they are also phosphorylated on various residues themselves [139, 165, 196]. Swatek et al. recently identified several CPKs that are responsible for these phosphorylation events [199]. Ser^{244} had earlier been identified as a phosphorylation site in 14-3-3s and is likely to be involved in phosphorylation of 14-3-3s by CPKs [139, 196, 199]. Luo et al. found GRF2 to be up-regulated under drought stress and GRF8 as well as CPK21 up-regulated under salt stress [110]. Furthermore, the Ca^{2+} signal that occurs upon salt stress is amongst others decoded by 14-3-3 proteins followed by modulation of SOS protein and PKS5 kinase activity and regulation of Na^+ homeostasis [233].

In summary, plant 14-3-3 proteins can be considered as key nodes in signaling networks and may participate in establishing crosstalk between diverse pathways, as has already been shown for their animal counterparts.

1.4 Stress physiology in higher plants

In order to control growth and development, an organism needs to be able to perceive, integrate and transduce internal and external signals. Plants as sessile organisms need to respond to continuously varying environmental changes and abiotic (i.e. salt stress or drought) as well as biotic (i.e. pathogens) stress signals. Phytohormones are structurally and biochemically diverse small compounds whose perception and signaling manipulate practically all cellular processes that steer plant growth, development and adaptation in order to rationalize the use of resources for the fitness of a plant. In the past decades breakthrough discoveries in phytohormone sensing and signal transduction were made.

1.4.1 Jasmonate - the wounding hormone

Jasmonates (JAs) are enzymatically formed oxylipins (oxygenated fatty acid derivatives). With their discovery in the 1960s, JAs were thought to be only secondary metabolites in the oil of *Jasminum sp.* flowers [222]. Now it is known, that plants produce JAs to regulate a multitude of different processes and JAs belong to the group of highly investigated phytohormones.
JAs, together with salicylic acid and ethylene, coordinate a plant's immune response to pathogenic attacs. By crosstalk of the different pathways the immune system is being tweaked and JA is presumed to be responsible for defense reactions upon necotrophic pathogens and chewing and biting insects. Increase of JA levels and activation of JA signalling follow wounding by herbivores. [88, 160] 2013 Mousavi et al. linked the membrane potential depolarization upon wounding to the activation of JA signalling in *Arabidopsis thaliana* [133]. Likewise, in carnivorous plants, prey capture triggers JA biosynthesis and signaling in a Ca^{2+}-dependent manner, finally leading to activation of secretory glands and prey digestion [76].
Besides being a stress hormone JA also is involved in developmental processes like flower and pollen development, root hair formation, seed germination or leaf senescence [223]. JAs also induce biosynthesis of many secondary metabolites, like alkaloids or glucosinolates via JA-responsive transcription factors (TFs) [223, 243].

The first steps of JA formation take place in the chloroplasts. There polyunsaturated C_{16}- and C_{18} fatty acids are oxygenated by enzymes like lipoxygenase (LOX) and allene oxide synthase (AOS). The cyclated derivatives are transported to peroxisomes, where several β-oxygenation steps are performed. This leads to formation of jasmonic acid, which is then modified further in the cytoplasm. For studying JA signaling a volatile methyl esther of JA, methyl jasmonate (MeJA), has been widely used. It is generated out of JA by jasmonate methyl transferase (JMT) and was thought to be the preferentially active signal in JA response until the specificity of jasmonoyl-isoleucine (JA-Ile) was discovered. The modification of JA to JA-Ile is catalyzed by the JA-amino acid synthase 1 (JAR1).[66, 194, 203, 223]

Even though phytohormones have a wide variety in both their chemical structure and the processes they regulate, their signalling pathways show strikingly parallel mechanisms. The degradation of repressor proteins through the ubiquitin-proteasome system (UPS) is one appliance that is used by a lot of plant hormones. The most important step in the UPS is ubiquitin (Ub)- conjugation to a target protein, which determines it for degradation by the 26S proteasome. [159, 172]
JA perception and signal transduction are tightly regulated by the formation of protein complexes, out of TFs, repressor proteins and members of the UPS (compare figure 1.7). In the absence of JA the expression of hormone-responsive genes is prevented by blocking the TFs with repressor proteins belonging to the JASMONATE ZIM-domain family (JAZ). In *Arabidopsis* the JAZ family has 12 canonical members that contain two well-conserved domains for protein-protein interaction, the ZIM

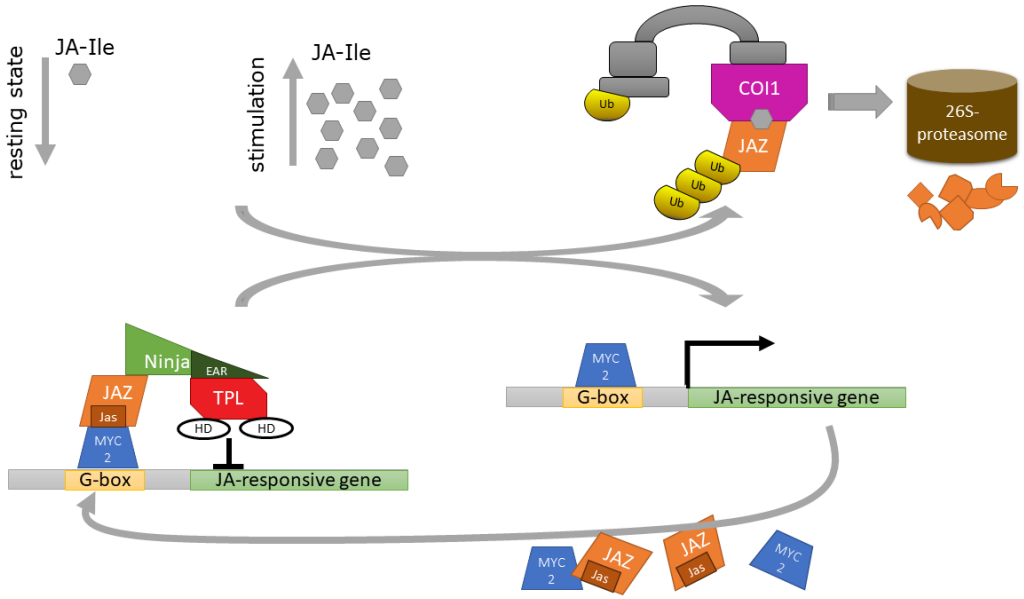

Figure 1.7: Jasmonic acid (JA) perception via the COI1–JAZ co-receptor complex leading to JA-induced gene expression

In the resting state (left, low JA-Ile level), the MYC2 bound to a G-box within the promoter of a JA-responsive gene does not activate transcription due to repression by Jasmonate ZIM domain proteins (JAZs). The co-repressors Novel Interactor of JAZ (NINJA) bound to JAZs, and TOPLESS (TPL) repress transcription via histone deacetylases (HDAs). TPLs is recruited via an ethylen response factor (ERF-)associated amphiphilic repression (EAR) motif. Upon stimulation (right, high JA-Ile level), JAZs are recruited by COI1. This leads to ubiquitinylation of the JAZs and subsequent degradation by the 26S proteasome. Hence, MYC2 can activate transcription of early JA-responsive genes such as those encoding JAZ and MYC2. Illustration adapted from [223] and [159].

and the Jas motif, and the atypical JAZ13 repressor with variant domains. JAZ5 to JAZ8 exhibit there own ethylen response factor (ERF-)associated amphiphilic repression (EAR) motif for the interaction with the co-repressor TOPLESS (TPL), while the other JAZs need to recruit another co-repressor, namely Novel Interactor of JAZ (NINJA), via interaction with the ZIM domain (compare fig. 1.7). The C-terminal Jas motif mediates the interaction of the JAZs with most TFs, for example the master JA-regulator MYC2. [38, 159, 223, 239, 243] MYC2, which belongs to clade IIIe of the basic helix-loop-helix (bHLH) superfamily, was the first identified direct target of JAZ repressors [37, 38]. Several other JAZ-interacting TFs from different families have been identified since then.
Upon wounding JA synthesis is triggered and JA accumulates withing 30 seconds near the injury site [66]. The increase of bioactive JA-Ile stimulates the binding of JAZ to the F-box protein CORONATINE INSENSITIVE1 (COI1) and subsequent formation of the SKP1-Cullin-F-box protein (SCF) E3 ubiquitin-ligase complex. This leads to ubiquitination and consequent degradation of JAZ, which in return releases TFs and induces hormone-dependent transcription of their target genes.

In this way downstream signaling processes and plant defense responses are modulated. Additionally, JA biosynthesis genes are upregulated likewise, giving a positive regulatory feedback loop for JA production. On the other hand JAZ genes are upregulated, too providing for a negative regulatory feedback. [38, 159, 223, 232, 239, 243]

In the field of JA physiology a lot of insight has been gained and it is out of question, that the bioactive forms of JA play a crucial role in systemic plant responses that vary in intensity, time of occurrence and spatial scale [89, 223]. Activation of secondary metabolic pathways, as well as direct signaling may occur, for example in order to enable a plant to reallocate energy from growth to defence mechanisms [88, 93].

1.4.2 Stomata and their distinct role in stress responses

The epidermis of higher plants is covered by a cuticle, a waxy, hydrophobic covering layer, impermeable to most substances, including water and CO_2. To provide for CO_2 uptake during photosynthesis the epidermis of higher plants possesses stomata, microscopic pores, which are bordered and defined by pairs of guard cells. These specialized cells have a defined cell wall structure, which allows them to open and close the pore depending on the cell turgor. Changes in turgor pressure are accomplished by directed transport of K^+ and inorganic anions plus biosynthesis of osmotically active metabolites, followed by passive water flux. So, stomatal movements require the regulation of several membrane ion channels, transporters and pumps. Furthermore guard cells are symplastically isolated from neighbouring cells allowing them to regulate their volume largely independently [227]. Fine control of stomatal aperture is essential to plant survival because they can control the balance of CO_2-uptake for carbon fixation and concurrent water loss by regulating the width of the gap between the two guard cells.

Stomata respond to various environmental stimuli to optimize the transpiration rate and metabolism under fluctuating stress conditions in nature. Detailed studies of different signal transduction processes in guard cells provide important information for understanding the perception and integration of stress signals and the plant's responses towards them.
Bacteria lack the ability to penetrate plantal epidermis directly and need to use natural openings to enter a plant and initiate pathogenesis. Stomatal pores are crucial for gas exchange and thereby for plant survival, but these small holes in the cuticle and epidermis also present a possibility for pathogens to invade plants. To avoid this, plants have evolved mechanisms to close their stomata in response to pathogen-associated molecular patterns (PAMPs) such as flg22, a 22 amino acid long part of a flagellin peptide [126].
On the other hand, some pathogens have evolved effectors to prevent stomatal closure or even to actively open stomata. Melotto et al. (2006) found that several

strains of the bacterium *Pseudomonas syringae* overcome PAMP-induced stomatal closure by employing the nonhost-specific polyketide phytotoxin coronatine (Cor) to reopen stomata for pathogenesis [126, 155]. Cor is a structural and functional mimic of JA-Ile [18]. In low concentrations Cor counteracts PAMP induced stomatal closure and even inhibits ABA responses in guard cells.

A plant's response to both biotic and abiotic stresses involves physiological, cellular and molecular processes with stomatal movement as a very early type of response. Post-translational modifications are essential processes for the functional dynamics of diverse proteins and therefore become important in the context of rapid (stress) signaling networks, too (cp. 1.3). Key players in the signaling pathways directing stomatal opening and closure undergo highly controlled, specific post-translational modifications, like phosphorylation and redox modification [240]. The above mentioned flagellin's signal transduction for example involves phosphorylation of the flg22-receptor Flagelling-Sensitive 2 (FLS2) and association with its co-receptor Brassinosteroid insensitive 1-Associated Kinase (BAK1) within less than 15 s [179]. Further downstream, the botrytis-induced kinase 1 (BIK1) and CPK5 phosphorylate and activate RBOH D (Respiratory Burst Oxidase Homolog D) to produce ROS [55, 103]. The importance of protein phosphorylation in stomatal movements is further supported by transcriptomic studies that identified 689 protein kinases and 113 protein phosphatases in *Arabidopsis thaliana* guard cell protoplasts [220]. This indicates that a huge array of (de-)phosphorylation events are involved in guard cell signaling [240].

1.4.3 ABA and stress signaling in stomata

The phytohormone abscisic acid (ABA) is involved in many plant developmental processes, such as seed dormancy, development and response to abiotic stress [140]. It is also known to play a certain role in early pre-invasive defense mechanism preventing microbe infection [204].
When a plant experiences drought stress or salinity and the consequent demand for saving water, concurrently synthesized ABA stimulates stomatal closure and inhibits stomatal opening by regulating ion fluxes in the guard cells [178]. In that way transpirational water loss can be reduced and CO_2 uptake assured. Stomata are able to close within a few minutes after an ABA stimulus, so all components for sensing the stimulus and achieving a rapid change in the ion balance must be existent in guard cells themselves [115].

Binding of ABA to its receptors, the RCAR/PYR1/PYL (Regulatory Compontents of ABA Receptor/Pyrabactin Resistance Protein1/PYR-Like) proteins in guard cells, results in conformational change and activation of a cascade of signaling events promoting stomatal closure [112, 163]. By this conformational changes, ABA receptors can interact with and inhibit the activity of some PP2Cs (Protein Phosphatases

type 2C) [112, 146, 163], including ABI1 (Abscisic Acid Insensitive 1) and ABI2. These PP2Cs are known to be negative regulators of ABA signalling in guard cells [127]. In contrast, OST1 (Open Stomata 1) is an ABA-activated protein kinase, that belongs to the SnRK (Sucrose Nonfermenting Kinase 1 Related) protein family and acts as a positive regulator of ABA responses [137]. It interacts with ABI1, but not with ABI2 in the way that the PP2C is able to dephosphorylate and inactivate one central node in the ABA network: OST1 [208, 236]. When ABI1 is fixed in the ABA-binding receptor complex, OST1 gets active and impinges on ABA-dependent gene expression and ion channels. Amongst others, slow anion channels as SLAC1 (Slow Anion Channel Associated 1) and SLAH3 (SLAC1 Homolog 3) are activated by OST1. SLAC/SLAH mediated anion efflux plays an essential role in the depolarization of the plasma membrane [62, 211]. Membrane depolarization inactivates guard cell inward-rectifying K^+ channels, such as KAT1 (Potassium channel in *Arabidopsis thaliana* 1) but activates the outward-rectifying K^+ channel GORK (Guard cell Outward Rectifying K^+ channel) [2, 15, 82]. The outward movement of ions results in turgor loss and the resulting guard cell shrinking causes stomatal closure. It has been proposed, that ABA can directly enhance K^+ efflux through GORK, too [153].
Besides the mentioned anion channels, activated OST1 phosphorylates several substrates, including RBOH D and RBOH F [1, 190]. These plasma membrane localized NADPH oxidases generate ROS, what in turn activates Ca^{2+} channels leading to Ca^{2+} release into the cytosol [92] and activation of Ca^{2+} signaling pathways.
CPKs function in parallel to the Ca^{2+}-independent OST1 in stomatal closure (compare chapter 1.3.1), thus representing the Ca^{2+}-dependent major hub in the guard cell ABA signaling network. CBL/CIPK combinations are involved in the Ca^{2+} dependent ABA signaling pathway as well (compare chapter 1.3.1). For example, CBL9 acts together with different CIPKs as negative regulator in ABA signaling, while its cognate homologue CBL1 seems to be not involved [35, 156, 157]. CPKs come also into play regarding the activation of RBOHs by phosphorylation [55, 60] (cp. 1.3.1). Thus, activation of CPKs downstream of the rise in ROS and Ca^{2+} levels could in turn promote ROS production by RBOH phosphorylation and consequently provide an amplification feedback regulation of the signaling pathway.

In addition to Ca^{2+} homeostasis, ROS accumulation enhances synthesis of nitric oxide (NO) by NR1 (nitrate reductase 1) [27]. NO can provide both positive and negative feedback in stomatal closure [61]. On the one hand it induces phophatidic acid (PA) production via activation of phospholipases, which in turn inhibts ABI1 and activates RBOH D and RBOH F [50, 241]. But then, accumulation of NO triggers degradation of ABA receptors via tyrosine nitration and inhibits OST1 and RBOH D activity by S-nitrosylation [237, 219, 30].

1.5 Aims of this work

Stress signaling in guard cells is a complex network that is far from being completely understood. This work wants to clarify new molecular details of signaling pathways involved in stomatal closure in response to biotic and abiotic stress stimuli. Creelman and Mullet (1997) have already shown that jasmonates accumulate in plants during drought stress [41], but in comparison to ABA signaling in guard cells very few studies have focused on the MeJA cascade leading to stomatal response. This thesis aims to identify components of the transduction system for ABA and jasmonates in *Arabidopsis thaliana* guard cell signaling networks. Using electrophysiological techniques in combination with biochemical assays, this study is focusing on the Ca^{2+} dependent regulation of the guard cell K^+ efflux channel GORK within the hormonal pathways.

2 Material and methods

2.1 Plant material and handling

Arabidopsis thaliana has gained big attention as a model system for plant biologists, due to its short generation time and its small genome, whose whole sequence is available. In this work, guard cell protoplasts from this model plant were used for patch-clamp experiments as described below (see 2.1.4, 2.5.3) and excides rosette leaves were used in stoma assays (see 2.1.2).

2.1.1 Growth conditions

Seedlings of *Arabidopsis thaliana* ecotype Col-0 were pricked out after 2 weeks and grown in standard soil EEP (Einheitserde Typ P, Patzer GmbH & Co. KG, Sinntal Jossa, D).
The fix conditions in the used climate chamber were 10/14 h day/night time and 21/16°C day/night temperature, around 60% relative humidity and photon flux density 100 µmol m^{-2} s^{-1}. The plants were watered every third day without any additional fertilizers.

2.1.2 Stoma assays

Stomatal aperture measurements were carried out on leaves of 5 to 6 week old *Arabidopsis thaliana* plants (see 2.1.1). Two excised rosette leaves per experiment were incubated for 2 h in the light in buffer containing 10mM MES-KOH, pH=6.15, 10mM KCl, 50 µM $CaCl_2$ (photon flux density 100 µmol m^{-2} s^{-1}) to promote stomatal opening.
After two hours incubation the solution was changed to incubation buffer containing the respective stimulus. As a control pure buffer was used and every day one set of Col-0 experiments was done, to control for environmental influences. The stimulus mediums were buffer-solutions with coronatine (Sigma Aldrich®, product number: C8115), methyl jasmonate (Sigma Aldrich®, product number: 392707) or ±ABA (Sigma Aldrich® , product number: A1049) in distinct concentrations (0.5-20 µM). Coronatine and ABA were dissolved in methanol as 100mM stock solutions, while methyl jasmonate was used pure (e.g. 2.24 µl per 10ml to obtain 10 µM). The incubation in the stimulus respectively control solution took place for one hour.

Stomatal aperture was determined using a digital microscope (Keyence vhx, Keyence Corporation, Osaka, Japan) at 2,000x magnification. Length and width of 20 stoma per leaf were measured to subsequently get the ratio (width/length) for better comparison of the different results. Average and standard error of stomatal aperture ratios were calculated for each experiment. At least three independent experiments were performed per plant and condition.

2.1.3 Monitoring stomatal movements upon wounding

Detached leaves of Col-0 were incubated in bath solution containing 10mM KCl, 5mM MES-BTP, pH=6.0, 0.1mM $CaCl_2$ for 2-3 h under microscope light. Every 30 s a picture was taken and after ca. 10 min a neighbouring stoma, which was approximately 100µm away from the monitored stoma was wounded using a sharp electrode. The response of the stoma was recorded and the images were put together in a video.

2.1.4 Preparation of guard cell protoplasts

Epidermal peels of rosette leaves from about four weeks old *Arabidopsis* plants were incubated for 16 h at 18 °C in enzyme solution composed of 0.65% (wt/vol) cellulase Onozuka- R10 (Serva), 0.35% (wt/vol) macerozyme Onozuka-R10 (Serva), 0.4 M sorbitol, 0.05 mM KCl, 0.05 mM $CaCl_2$, 5 mM ascorbic acid, 0.05% (wt/vol) kanamycin sulfate (Fluka) and adjusted to pH 5.5 MES/Tris. Then, the suspension was filtered through a 50 µm nylon mesh, washed with washing solution (20 mM $CaCl_2$, 5 mM MES-Tris, pH=5.6, adjusted to an osmolality of 400 mosmol/kg with D-sorbitol) and centrifuged at 600 ×g and 4 °C for 10 min. The supernatant was discarded immediately and the enriched protoplasts were placed on ice in measuring chambers as samples of 50 µl (plus 250 µl washing solution) for minimum 1 h before they were used for patch-clamp experiments.

2.1.5 Tobacco leaf infiltration

Leaves of 6-7 weeks old *Nicotiana benthamiana* plants were transformed using the agroinfiltration method, essentially as described by Jung et al. [87] and Te et al. [201]. Cultures of *Agrobacterium tumefaciens* were grown over night at °C in 50 ml Falcon tubes to an optical density of 1.5 - 2.0, pelleted by centrifugation, washed and resuspended in agromix solution containing 10 mM $MgCl_2$, 10 mM MES (pH 5.6/KOH), 150 μM acetosyringone (Sigma-Aldrich, St. Louis, USA).

2.2 Molecular biology

2.2.1 Cloning strategies

Molecular cloning is used to integrate DNA, in most cases a gene's coding sequence, into a vector suitable for the desired purpose. The generated circular, double-stranded plasmids can then be amplified in bacteria, usually in *E.coli*. Typically, the plasmids carry a minimum of one antibiotic resistance gene in order to select bacteria colonies containing the cloned vector.

2.2.1.1 USER system

The uracil-excision based cloning was introduced in the early 1990s [147, 191], has been commercially available since 2003 (uracil-specific excision reagent, USER™, New England Biolabs, Ipswich, MA) and was established in molecular biology labs by Nour-Eldin *et al.* 2006 [149]. It provides a ligation independent way to generate recombinant DNA molecules at a very high efficiency.
The principle of the USER system relies on the ability of complementary overhangs at the ends of respectively PCR products or linearized vectors, to make stable hybridization products. These eight nucleotide long overhangs are generated in a vector containing a 38 bp long PacI cassette, by digestion with the restriction enzyme PacI (New England Biolabs, Ipswich, MA), with subsequent nicking by Nt.BbvCI (New England Biolabs, Ipswich, MA). This was carried out by digesting 10 µg vector DNA with 70 U PacI and 20 µl buffer overnight at 37°C in a total volume of 200 µl. Additional 20 U PacI, as well as 40 U Nt.BbvCI, were added the next day and the digestion was incubated at 37°C for another 6 h. The linearized vector was then checked on an agarose gel and purified with the QIAquick® PCR Purification Kit (Qiagen, Hilden, D). The required eight nucleotide overhangs on PCR products were generated by placing a single uracil residue in each primer. It is important to use a proof-reading polymerase that is able to read through uracils in the sequence, such as Phusion Cx, that was used for all PCRs performed in this work. The PCR products were then treated with uracil DNA glycosylase and DNA glycosylase-lyase Endo VIII, which are included in the USER™ enzyme mix from New England Biolabs (Ipswich, MA). These enzymes catalyze the removal of the uracil residues and enable the dissociation of the single-stranded fragments upstream from this cleavage site.
For the purposes in this work, the PCR products were purified with the QIAquick® PCR Purification Kit (Qiagen, Hilden, D) and digested with 1.5 µl DpnI and 5 µl 10x Tango buffer (both from Thermo Scientific, Waltham, MA) in a total volume of 50 µl at 37°C for 1 h to remove the template and 20 min at 82°C to inactivate the enzyme. For the actual USER reaction, 4 µl of this PCR product were mixed with 2 µl linearized vector, 1 µl USER™ enzyme mix and 5 µl water, incubated at 37°C for 25 min, followed by 25 min incubation at 25°C, then directly transformed into *E.coli* (see 2.2.2).

The USER™ system can also be used for site-directed mutagenesis. In that case the primers need to be designed with the mutational nucleotide(s) of interest in their middle. The cloning process itself does not change for this purpose.

2.2.1.2 Gateway® and the 2in1 cloning system

The Gateway® recombinational cloning system from Invitrogen utilizes modified site-specific recombination sites of bacteriophage λ for highly efficient cloning. [73] Orientation of the cloned DNA is maintained by the use of two nearly identical but noncompatible versions of the λ *att* recombination sites.
In addition to antibiotic resistance genes, the positive selection gene *ccdB* is also present in the recombinant cassette on the vector backbones, in order to select the required recombinant product. This gene, taken from the *E.coli* F plasmid segregation control system, is able to inhibit *E.coli* DNA gyrase, thus cells are unable to grow. [19]
The gene of interest with flanking *attB* sites, generated by PCR using suitable primers , is recombined into a vector with the corresponding *attP* sites. This reaction is called BP recombination and it is accomplished by the λ Int and the IHF protein. The generated entry clones can then be used for LR recombination with different destination vectors. Both recombination reactions are usually complete within hours but can also be left overnight.
For this work, a 2in1 cloning system, which represents multisite Gateway cloning and was established by Grefen *et al.* 2012 [68], was used. This strategy enables coexpression of fusion proteins from a single plasmid, so ratiometric bimolecular fluorescence complementation assays (rBiFC). Two candidate genes for protein-protein interaction are to be cloned into a single vector backbone containing an internal fluorescence marker for expression control and also ratiometric analysis. Hence, the credibility of the results is increased and different protein pairs can be easily compared.
The vectors for this cloning system were kindly provided by the lab of Prof. Dr. Grefen at the University of Tübingen. Due to the *ccdB* gene (see above), the vectors were amplified in the *E.coli* strain *ccdBsurvival* (see 2.2.2). For BP recombination, 150 ng vector and about the same amount of template was used and incubated with 8 µl TE buffer and 2 µl BP clonase mix (Gateway® BP Clonase® II Enzyme Mix by life technologies, Carlsbad, CA) at room temperature for 3 h for CPKs, and overnight for GORK and then transformed into *MRF'* (see 2.2.2).

2.2.2 Work with bacteria

The bacterium *Escherichia coli* is extensively used in molecular biology laboratories for work with recombinant DNA due to its multiple application possibilities and simplicity of manipulation. In this work, different *E.coli* strains were used. For

amplification of the Gateway vectors (see 2.2.1.2) *ccdB survival* and *DB3.1* (both from Life Technologies, Carlsbad, CA) were used. In all other cases, *MRF'* bacteria (Stratagene, Amsterdam, NL) were used, apart from the insertion of GORK into pDONR221 P1P4, where *CopyCutter™EPI400™* (Epicentre) were used. Bacteria were stored at -80°C and defrosted on ice directly before transformation.

All strains were chemically competent, so plasmids were introduced into the cells via heatshock. For DNA retransformation 1-2 µl, for new cloned constructs the whole batch was added to the 50 µl thawed cells, incubated on ice for ca. 20 min, followed by 40 s at 42°C in a waterbath and again 2 min on ice. Then 450 µl SOC medium was added and the cells were left at 37 °C, gently shaking for ca. 1 h. This step was skipped when the plasmid contained the ampicillin resistance gene. The cells were plated on LB-plates (1.5% agar) containing the antibiotic for selecting for positive transformants and incubated at 37°C overnight. For *CopyCutter™EPI400™*, this step was performed at room temperature for four days.
Single colonies were used for inoculation of LB-medium containing the specific selective antibiotic (concentrations 50 $\frac{\mu g}{ml}$ ampicillin/kanamycin/streptomycin; 100 $\frac{\mu g}{ml}$ spectinomycin) and incubated at 37°C or at 25°C overnight, with shaking. Plasmid DNA was then gained by using QIAprep® Spin Miniprep Kit or QIAGEN® Plasmid *Plus* Midi Kit (both Qiagen, Hilden, D) depending on the amount of present *E.coli* liquid culture, following the kit's manual's instructions and used for further attempts (see also 2.2.1).

2.2.3 In vitro synthesis of RNA

In vitro transcription is used to synthesize cRNA for later use for example, for expression in oocytes (see 2.4.2). Vectors (e.g. pNBI) containing a T7 promotor can be used for transcribing DNA fragments to RNA by the T7 polymerase. Fragments generated by *in vitro* polymerase chain reaction (IVT-PCR) were used as template DNA in this work. The conditions and reaction mix for this PCR reactions are shown in table 2.1. The used IVT-primers bind in the untranslated regions of the pNBI#16-vector (see *HIER CONSTRUCT LISTE EINFUEGEN!*) and linear PCR products containing the desired DNA and the T7 promotor result from this reaction.

Table 2.1: Reaction mix and conditions for an IVT-PCR

Reaction mix	Step	Time	Temperature
36 µl H_2O	1	2 min	98°C
10 µl 5x Phusion Cx-Buffer	2	10 s	98°C
1 µl dNTPs (10 mM)	3	30 s	55°C
1 µl IVT-Primermix (10 µM)	4	1 min	72°C
0.5 µl Phusion polymerase	5	5 min	72°C
1 µl template	steps 2-4 were repeated 30x		

The PCR products were purified with the QIAquick® PCR Purification Kit (Qiagen, Hilden, D) and analysed on an 1% agarose gel for the appropriate size and approximate amount of the products.
For RNA synthesis the AmpliCap-Max T7 High Yield Message Maker kit (Biozym, Hessisch Oldendof, D) was applied according to the kit's manual. 2.75 µl purified IVT-PCR product was added to the 7.25 µl mastermix, which contained 1 µl buffer, 4 µl CAP/NTP mix, 1 µl DTT, 1 µl enzyme and 0.25 µl RNAse inhibitor. The reaction was mixed thoroughly and incubated at 37°C for 1.5 h.
Then the RNA was precipitated by incubation at -20°C overnight after adding 20 µl NH_4Ac to each reaction. Following a centrifugation step of 30 min at 4°C and maximum speed, the pellet was washed with 70% ethanol, dried at 37°C and resuspended in 15 µl RNAse free water (supplied with the kit). 1 µl RNA was then mixed with 1 µl RNA loading buffer and verified by gel electrophoresis for the correct size. RNA yields were then measured by quantitation with 260 nm light absorption using a NanoDrop 2000c spectrometer (Thermo Scientific, Waltham, MA) and the RNA was adjusted to the desired concentration for injection into oocytes (GORK 600 $\frac{ng}{\mu l}$, CPKs and GRFs 200 $\frac{ng}{\mu l}$, see 2.4.2) with RNAse free water.

2.3 Cell culture

2.3.1 Handling of used cell strains

For patch clamp measurements, COS-7 cells were used. This cell line was originally derived from CV-1, a simian cell line (*Cercopithecus aethiops*) by transformation with an origin-defective mutant of SV-40 virus by Gluzman 1981 [67]. The COS-7 cells used in this study, were kindly provided by Dr. Jean-Baptiste Thibaud (Biochimie et Physiologie Moleculaire des Plantes, Montpellier) or ordered from CLS (Cell Lines Service GmbH, Eppelheim, D). They were cultivated in Dulbecco's modified Eagle's medium (DMEM, gibco®, Life Technologies, Carlsbad, CA) containing 10% fetal bovine serum (FBS), previously inactivated for 30 min at 56°C, and 1x penicillin/streptomycin. The cells were kept as adherent monolayers in cell culture bottles and dishes of different sizes (all from Nunc, Wiesbaden, D) in an incubator at 37°C and 5% CO_2.
All handling of COS cells was carried out under sterile conditions (cleanbench, sterile material, etc.) and the condition of the cells in use was microscopically examined on a regular basis, usually daily (magnification 10x; Axiovert10, Carl Zeiss AG, Oberkochen, D). The COS-7 cryostocks were kept at -80°C in FBS containing 10% sterile DMSO. For passaging of the maintenance culture, the approximately 80% confluent monolayer of COS-7 was washed with prewarmed PBS (gibco®, Life Technologies, Carlsbad, CA), then trypsin-EDTA (0.5%, gibco®, Life Technologies, Carlsbad, CA) was gently applied to the cells. Due to this proteolytic enzyme the cells were detached after 3-5 min and could be taken up in fresh medium and carefully resuspended. The cell suspension was seeded into new dishes/flasks con-

taining fresh medium for the desired dilution (for later transfection 1:1 up to 1:16; for maintenance culture 1:25 - 1:30).

2.3.2 Transfection of CHO cells

Cells were transfected 1-3 days before patch clamp experiments. This transient transfections were performed using polyethylenimine (PEI) (1 $\frac{mg}{ml}$, dissolved at 70°C, pH=7.0) [24]. For each transfection in 2 ml medium, 4 µg GORK plasmid and 2 µg YFP (both in pcDNA3.1) were mixed with 12 µl PEI and 100 µl Optimem (gibco®, Life Technologies, Carlsbad, CA) and incubated at room temperature for a minimum of 20 min. Afterwards, this mixture was applied directly to the cells.

2.4 Double electrode voltage clamp

Double electrode voltage clamp (DEVC) is an outstanding method for the electrophysiological investigation of membrane proteins. The technique allows the analysis of currents conducted by, for example, channels over a cellular membrane for a given (clamped) membrane potential and diverse channel characteristics can be monitored and manipulated.

2.4.1 The principle of DEVC and the used setup

For DEVC, large cells with low membrane resistance, such as oocytes from *Xenopus laevis*, are impaled with two electrodes. Cells are clamped to a set membrane potential with these electrode. Transmembrane voltage is recorded through a voltage electrode, relative to ground, and a current electrode applies current to the cell, until the membrane potential equals the set command potential. Using negative feedback, it is possible to maintain the cell membrane at this voltage; the cell is "clamped" at the command potential. If ion channels cause a current through the membrane, the voltage drop is compensated by a current amplied to the membrane by the amplifier. The amplitude of this current corresponds to the current flow through the channels. By systematically changing the command potential, the currents through the membrane caused by the channel of interest, can be investigated. [181]
The oocyte chamber, perfusion system, micromanipulators and binocular microscope used in this work were set up on a vibration-cushioned table, shielded from external disturbances by a Faraday cage. All conducting parts of the setup were grounded. The preamplifier was the only electronical device, that was placed inside the Faraday cage. The current answer of the cell was led to the amplifier, where the signal was low pass filtered and could be monitored on the computer, which was connected to the amplifier via a 16 bit AD/DA converter (ITC16 ST, Instrutech Corporation, Colorado).
Microelectrodes, first described by Ling and Gerard 1949 [105], were pulled from

Kwik-Fil™ borosilicate glass capillaries (World Precision Instruments, Sarasota, FL) using a PC-10 puller (Narishige, Tokyo, Japan). These capillaries were filled with 3 M KCl and fixed to the micromanipulators onto the Ag/AgCl electrodes made of silver wire electrolytically chlorated in a 3 M KCl solution. For the reference electrode the Ag/AgCl wire was inserted into a small plastic tube filled with 3 M KCl and clogged with an agar bridge (2% agar, 3 M KCl). It was connected to the preamplifier and placed in the measuring chamber near the oocyte.
For a measurement, a healthy appearing oocyte was placed in the chamber and the microelectrodes were inserted in the bath solution, using the micromanipulators. Before impaling the oocyte, the electrode resistance was verified as between 0.5-1.5 MΩ and the offset of potential and current electrode was compensated in current clamp mode. Then the oocyte was carefully impaled with the microelectrodes, assisted by an acoustical signal and controlled microscopically. After changing to voltage clamp mode, test pulses were applied to the cell (see 2.4.3).

2.4.2 The heterologous expression system Xenopus oocytes

Oocytes from the African clawed frog *Xenopus laevis* are an ideal tool for the expression and functional analysis of proteins. They have a diameter of about 1mm, a surface of several mm^2 and are able to heterologously translate mRNA into proteins within a few days. In addition, oocytes can readily bear impalement by microelectrodes and have little background activity, therefore offering a high signal-to-noise ratio in electrophysiological experiments.
The oocytes were collected by surgery of the institute's frogs and stored in ND96 solution (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM HEPES pH = 7.4, 50 $\frac{\mu g}{ml}$ gentamycin, π=220 mosml) 16°C.
For microinjection with cRNA (see 2.2.3) capillaries (3 1/2"Drummond # 3-000-203-G/X, Drummond Scientific Company, Broomall, PA) were pulled vertically in a PC-10 puller. The tip was broken using a microforge wire to get a 6-8 µm wide opening, to reduce damage the oocyte membrane. The capillary was filled with incompressible, coloured mineral oil and fixed into a micro injector (Nanoliter 2000 Injector, World Precision Instruments, Sarasota, FL). The RNA was sucked in the capillary and 30 ng GORK RNA was injected into each oocyte. For interaction measurements, the oocytes were subsequently injected with 10 ng CPKdEF RNA, and in some cases, also with 10 ng GRF RNA, 1-2 d before measuring. The oocytes were stored at 16°C in ND96 until the DEVC measurements were performed, 4-5 d after GORK injection.

2.4.3 Solutions and protocols used

All DEVC measurements were performed under perfusion by a bath solution described in table 2.2. The holding potential was -40 mV. The applied test pulse, as well as the activating pulse protocol were compiled with the program PatchMaster

Table 2.2: Bath solution for DEVC experiments

bath solution
10 mM KCl
90 mM LiCl
1 mM $CaCl_2$
1 mM $MgCl_2$
10 mM HEPES pH=7.5

v2.10 (HEKA Elektronik, Lambrecth, D) and generated by the amplifier (Turbo TEC 10CD, npi electronics, Tamm, D). The rectangular shaped pulses have the pivotal advantage, that the capacitive currents, that occur upon depolarisation of the membrane are very short (normally 1-2 ms) and do not have to be compensated. Therefore, the time dependent changes of the currents through the oocyte membrane can be analysed without any interrupting capacitive components. [181]

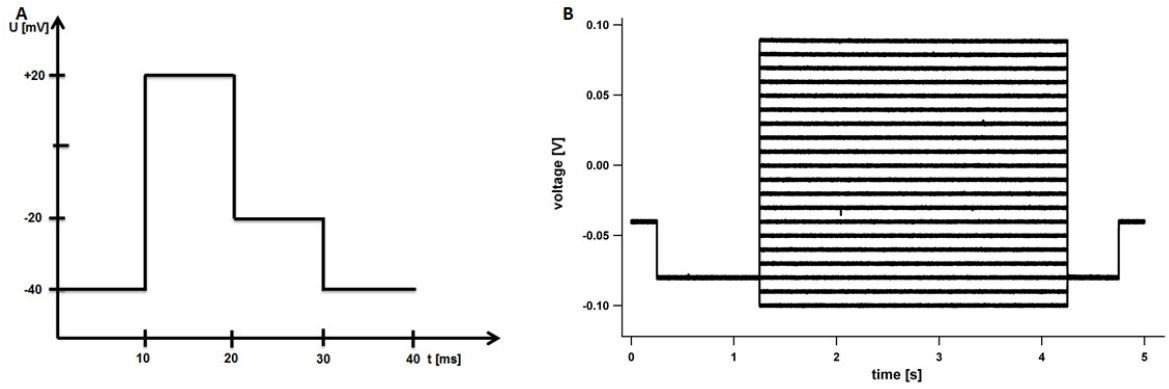

Figure 2.1: Pulse protocols applied in DEVC experiments; A is the test pulse for checking leakage of the oocytes and B the single voltage pulse protocol used for measurements

The test pulse (see figure 2.1 A) was applied to the oocytes in the beginning of a measurement to assess their leak currents and only cells with leak currents $\leq$ 0.08 µA were used for measurements. The remaining leakage was subtracted from the recorded currents during analysis. The currents of the oocyte membrane were investigated by applying the single voltage protocol shown in figure 2.1 B. Based on a holding voltage of -40 mV, followed by a hyperpolarisation to -80 mV for 1 s to deactivate possibly active channels, different test voltages were applied for 3 s (-100 mV to 90 mV, in steps of 10 mV).The clamp was then returned to -80 mV for 0.5 s to deactivate the channels and then back to the holding voltage.

2.5 Patch clamp measurements

The patch clamp technique was developed in 1976 by Neher and Sakmann and is another technique for investigating ion channels electrophysiologically [141]. It allows measurements at a higher resolution and even single channel currents can be measured *in vivo*, for example in mammalian cells, like the COS-7 cells used in this work. In patch clamp experiments, a glass micropipette with a tip diameter of around 1 µm is placed on the cell surface. By applying negative pressure, a membrane piece within the pipette opening is sealed off and a high resistance in the range of GΩ needs to be achieved for successful measurement of currents in the range of pA to nA (= gigaseal). This configuration is called "cell-attached" or "on-cell". The solution outside the cell can be manipulated and the composition of the cytoplasm remains the same. This way, channels in the membrane patch can be investigated under physiological conditions. By applying more vacuum and/or short electric voltage pulses the membrane patch can be ruptured while the gigaseal still remains. Thus, the "whole-cell" configuration is achieved and the currents of the whole cell membrane can be measured under voltage clamp conditions. The pipette solution replaces the cytoplasm and so the inside of the cell has a defined composition. This conformation can be realized for small cells and even carrier provoked currents can be registered. [181]
A biological membrane possesses both ohmic (R_m) and capacitive ($C_m \simeq 1\frac{\mu F}{cm^2}$) electrical features, connected in parallel. With the help of a patch clamp electrode, filled with an electrolyte solution, so access to the cell interior, also filled with an electrolyte, is gained. This access holds another resistance, that can be considered as connected in series to the membrane resistance, the series resistance (R_s). This configuration allows the experimentator to impose defined potentials on the membrane with the use of a proper amplifier. This causes currents, that can influence the membrane polarisation or can flow off by the membrane conductivity. These currents can be analyzed in a patch clamp measurement. [181]

2.5.1 Setup, material and solutions

The setup was build up according to the description in Hamill *et al.* (1981, [69]). An inverse microscope (Axiovert25, Carl Zeiss AG, Oberkochen, D) equipped with an UV-lamp and a micromanipulator (Scientifica, Uckfield, UK) were arranged on an air-suspended table, surrounded by a Faraday cage. The microscope stage had a cavity for holding the measuring cuvette, which was simply a cut-off small petri dish. Polyethylene tubes for perfusion were adjusted as desired on the edges of the cuvette. They were connected via glass olives to peristaltic pumps (Pharmacia LKB Pump P-1, Pfizer, New York, NY), which controlled the flow rate of the bath solution. Inside the Faraday cage, a tube system for controlling the pressure application to the pipette holder, including a mouthpiece for concerted sucking and an u-pipe with a syringe to apply positive pressure, was installed. This system was regulated by a three-way valve. All electronic devices, besides the preamplifier were

located outside the Faraday cage and all conductive elements inside the cage were grounded. The preamplifier was connected to the reference electrode and passed the measured signals to the computer via a filter (LHBF-48x, npi electronics, Tamm, D), an AD/DA converter (ITC 16, Instrutech Corp., Colorada) and the amplifier (PatchClamp-L/M-EPC7, HEKA Elektronik, Lambrecht, D). All operations were carried out with the software Pulse 8.80® 2006 (HEKA Elektronik, Lambrecht, D).

The micropipettes were pulled from borosilicate capillaries (1.5-1.8x100 mm, Kimble Chase) using a vertical puller (Narishige, Tokyo, Japan). Prior pulling, the capillaries' inside was coated with the hydrophobic substance Sigmacote (SL-2, Sigma, St. Lous, MI), to minimize the system's capacity and support sealing the membrane. After pulling, the pipette tip was covered with Sylgard® (184 silicone elastomer kit, Dow Corning Corporation, Midland, MI) also to reduce system capacity and heated in a microforge to produce a smooth surface that would assist in forming a high resistance seal with the cell membrane. [150]
The Ag/AgCl electrodes and reference electrode were made as described in 2.4.1, except instead of 3 M KCl, only a 0.3 mM KCl solution and agar were used for reference electrode preparation.

The used solutions are listed in table 2.3. The solutions were kept at 4°C and heated up to room temperature before usage. The osmolality was verified on a regular basis using a vapor pressure osmometer (5520, Wescor, Logan, UT).

Table 2.3: Contents of the solutions used for patch clamp experiments in whole-cell mode on COS-7 cells

pipette solution	**bath solution**
150 mM KCl	150 mM KCl
3 mM $MgCl_2$	3 mM $MgCl_2$
1 mM $CaCl_2$	1 mM or 5 mM $CaCl_2$
20 mM HEPES/Tris pH = 7.5	20 mM HEPES/Tris pH = 7.5
5 mM EGTA	
π= 340 mosmol (D-sorbitol)	π = 350 mosmol (D-sorbitol)

2.5.2 Performance of patch clamp measurements on mammalian cells

Transfected COS-7 cells, that had been diluted 1:10 before were patched adherent to a coverglass (see 2.3.1). The coverglass was washed in bath solution before placing

it in the measurement cuvette. A good fluorescing and healthy cell, without any contact to another cell was selected for measurements by UV microscopy.
Micropipettes for patch clamp recordings were filled with pipette solution (table 2.3) filtered through a 0.2 µm filter. First, the pipette tip was dipped into a small solution droplet and a small amount of the solution was sucked in by applying vacuum using a syringe. Then the pipette was backfilled with the filtered solution and freed from air bubbles by flicking and shaking. The prepared pipette was installed in the pipette holder of the micromanupulator, pressure was applied over the tube system, the pipette was moved into the bath solution and the offset potential was compensated. The filter was set to 1.3 kHz and the gain was always remained at 500 µV/pA.
A rectangular test pulse was used for measuring the pipette resistance and monitoring the formation of the gigaseal. The pipette was moved slowly towards the cell, placed gently on the membrane and a membrane piece was sucked into the pipette tip by sucking on the mouthpiece. When a gigaseal was achieved, the system's capacity was compensated and rupture of the sealed patch was accomplished by either sucking slightly more or applying the zap pulse or both. If the gigaseal still existed after breaking through, the membrane capacity was compensated as well as the conductance of the electrode and the destroyed membrane and measurements could be performed. The leak current was digitally subtracted before quantitative analysis of the data.

2.5.3 Performance of patch clamp measurements on guard cell protoplasts

The whole-cell patch clamp configuration was established on guard cells isolated from epidermal peels as described in 2.1.4. Micropipettes were prepared analog to the procedure described in 2.5.2. Macroscopic current recordings were performed at a sampling rate of 100 µs and low-pass filtered at 1.3 kHz using an EPC7 patch clamp amplifier (HEKA Electronic, Lambrecht, Germany). From a holding potential of −60 mV, voltage pulses ranging from −180 to +100 mV were applied in 20 mV steps for 1 s. The standard bathing solution and the standard pipette solution, facing the cytosolic side of the plasma membrane, are both listed in the table 2.4. 5 mM EGTA in the pipette solution correspond to 1 µM free calcium. To study the effect of JA on potassium channel activity guard cell protoplasts were preincubated with 0.1-1 µM coronatine in washing solution (20 mM $CaCl_2$, adjusted to pH=5.6 using 5 mM MES/Tris with an osmolality of 400 mOsmol/kg using D-sorbitol) for 15min on ice. Steady state current amplitudes were determined at the end of each voltage pulse and normalized to the membrane capacitance of individual guard cell protoplasts. The clamped voltages were corrected off-line for the liquid junction potential.

Table 2.4: Contents of the solutions used for patch clamp experiments in whole-cell mode on guard cell protoplasts

pipette solution	bath solution
100 mM KGlu	10 mM KCl
5 mM EGTA	2 mM $MgCl_2$
4.5 mM $CaCl_2$	10 mM $CaCl_2$
2 mM Mg_2ATP	0.5 mM $LaCl_3$
10 mM HEPES/Tris pH = 7.4	5 mM MES/Tris pH = 5.6
π= 440 mosmol (D-sorbitol)	π = 400 mosmol (D-sorbitol)

2.6 Biochemical work with proteins

There are several strategies to express proteins recombinantly. Conventional *in vivo* systems are for example bacteria, mostly *E. coli* or yeast. Cell-free *in vitro* expression describes the production of recombinant proteins in solution using cell lysates. It usually gives less amount of protein but has several advantages compared to conventional *in vivo* methods, like quickness and the possibility to express toxic proteins.

2.6.1 Solutions for protein biochemistry

TB medium 1.2 % tryptone; 2.4 % yeast extract; 72 mM K_2HPO_4; 17 mM KH_2PO_4; 0.4 % glycerine; pH 7.4

PBS buffer (10x) 1.4 M NaCl; 27 mM KCl; 100 mM Na_2HPO_4; 18 mM K_2HPO_4; pH 7.3

GST elution buffer 50 mM TRIS/HCl, pH 8; 10 mM reduced glutathione

SDS running buffer (10x) 15.15 g TRIS; 72 g glycerine; 5 g SDS; pH 8.3 (in 500 ml)

Loading dye (6x) 500 mM TRIS/HCl, pH 6.8; 20 % SDS; 24 % glycerine; 0.012 % bromphenolblue; 200 mM DTT

Coomassie 0.1 % Brilliant Blue R250; 50 % MeOH; 10 % acetic acid

Destaining solution 25 % MeOH; 10 % acetic acid

Dialysis buffer 150 mM NaCl; 50 mM HEPES/NaOH, pH 7.4

Strep washing buffer 100 mM Tris/HCl, pH 8; 150 mM NaCl; 1mM EDTA; 0.5 mM Brij 35

Strep elution buffer 100 mM Tris/HCl, pH 8; 150 mM NaCl; 1mM EDTA; 0.5 mM Brij 35; 2.5 mM desthiobiotine

CPK Kinase buffer (5x) 250 mM HEPES (pH 7.4); 50 mM $MgCl_2$; 10 mM DTT; 25 mM EGTA; 5 mM $CaCl_2$

CIPK5 kinase buffer (5x) 25 mM $MnCl_2 \cdot 4\ H_2O$; 5 mM $CaCl_2$; 10 mM DTT; 250 mM HEPES (pH 7.4)

2.6.2 Expression and purification of recombinant proteins

The constructs pIVEX(1.3)-WG:StrepII-CIPK5 and pET-24b(+):GST-GORK_NT were kindly provided by the group Jörg Kudla (Universität Münster). The control SLAC1-N-terminus, as well as CPK3,6,21,23 and both parts of the GORK C-terminus were cloned into pGEX6P1:GST-V5-HIS via USER cloning (cp. 2.2.1.1). The GORK N-terminus consisted of amino acids 1-67 and GST-GORK-NT has a mass of ca. 34 kDa. The GORK C-terminus had to be split into two overlapping parts because it turned out to be unsoluble in *E. coli* and could not be purified in a whole. The first part (P1) cotains the amino acids 301 to 559 and GST-GORK-CT-P1 weighs about 56 kDa, while the second part (P2) includes amino acid 531 to amino acid 820 and GST-GORK-CT-P2 has a mass of approximately 58 kDa.

2.6.2.1 Expression in E. coli

All constructs besides pIVEX(1.3)-WG:StrepII-CIPK5 were transformed into Rosetta (Merck). Out of a fresh 5 ml overnight culture 100 ml TB-medium were inoculated with the distinct clones and incubated ca. 16 hours at 37 °C shaking. Then this culture was transfered into 1 l fresh TB-medium and put back to 37 °C and 130 rpm. Under regular control the expression culture was grown to an OD600 of 0.6 - 0.8. When this density was reached the expression was induced with 0.4 mM IPTG and the culture was now put to 18 °C and 130 rpm for about 18 h.
The cells were pelleted at 6000 xg for 15 min and resuspended in 40 ml cold PBS. Lysis of the cells was achieved by adding 5 mg lysozyme (Sigma) and sonifying four times 20 s at about 100 W (Sonopuls HD 3100 VS 70 T; Bandelin) on ice. The lysate was pelleted 25 min at 16.000 xg and 1 °C (with an Optima™L-100K; Beckmann Coulter; 45 Ti rotor).

2.6.2.2 Purification by GST-tag

Glutathion-S-transferase (GST) has been used widely to tag proteins N- or C-terminally for purification via affinity chromatography. A glutathion matrix binds the tagged protein and by addition of reduced glutathion it can be eluted. GST is a quiet big tag and has a mass of ca. 26 kDa. All proteins used in this work besides

CIPK5 have a N-terminal GST-tag and therefore the supernatant from the ultra-centrifugation step (cp. 2.6.2.1) was used for purification with glutathione sepharose according to the manual (Glutathione Sepharose 4B; GE Healthcare). To bind the tagged protein to the matrix the lysate was incubated for 1-3 h at 4 °C with 750 µl sepharose, then washed 3-5 times with cold PBS, followed by 4-6 elution steps with 600 µl GST elution buffer. The elution fractions were dialyes at 4 °C overnight, analyzed via SDS PAGE (Thermo Fisher Scientific Precise Tris-Glycine Gels 4-12%)and stored at -20 °C. The samples of CPK3, 6, 21, and 23 were stored with 50% glycine as cryo-protectant and 1x proteinase inhibitor (EGTA free, Roche).

2.6.2.3 Cell free expression and purification of StrepII-CIPK5

For expression of StrepII-CIPK5, pIVEX(1.3)-WG:StrepII-CIPK5 was used with the continuous exchange cell-free (CECF) protein *in vitro* expression system RTS 500 Wheat Germ CECF Kit (biotechrabbit) according to the kit's manual. The 1 ml reaction contained 300 µl wheat germ lysate, 300 µl reaction mix, 40 µl amino acid mix, 4.5 µl methionine, 55.5 µl reconstitution buffer, 20 µl template DNA, 24 µl Brij 35 (20.5 mM), 256 µl DEPC water and was incubated in the reaction chamber containing 10 ml feeding mix 24 h at 24 °C and 150 rpm.
The purification of the lysate was executed according to the manufacturer's protocol using Strep-Tactin MacroPrep Matrix (iba lifesciences) and the product was checked via SDS PAGE and dot blot.

2.6.3 In vitro kinase assays

To investigate the phosphorylation reaction of several kinases with the GORK N- and C-terminus *in vitro* phosphorylation assays were performed. SLAC1-N-terminus served as a positive control for the CPKs, as this reaction has been shown before . In general 1-3 µg purified protein in the respective elution buffer and 5mCi γ^{32P} ATP (100 µM ATP; 3.000 Ci/mmol; Hartmann Analytic) were used for each reaction. The reactions were incubated for 30 min at room temperature and stopped by adding SDS loading dye and heating to 90 °C for 5 min.
The samples were then ran on an SDS PAGE (Thermo Fisher Scientific Precise Tris-Glycine Gels 4-12%), fixed by Coomassie staining for 30 min and destained until the background was cleared to get rid of unspecific radioactivity on the gel. The visualization of the phosphorylation was achieved with the help of an imager (BAS 2000, FujiFilm) and the softwares Basread (FujiFilm) and Aida (raytest).

3 Results

3.1 Wounding triggers JA signaling in guard cells

Wounding enhances JA levels in different *Arabidopsis* ecotypes and has also been implicated in promoting stomatal closure [174]. Stomatal aperture assays on wildtype Col-0 leaves (cp. 2.1.2) underline this finding. Figure 3.1 A shows that mechanical wounding with a forceps leads to local, as well as systemic stomatal closure.

The systemic leaf was in position +3 of the wounded local leaf. 15 min after wounding, both the local and systemic stomata exhibit an aperture ratio of lower than 0.2, while in control leaves the aperture ratio is higher than 0.4. Thus, wounding results in a reduction of over 50% of stomatal aperture. The time course of wounding-induced stomatal movement can be monitored easily as shown in figure 3.1 B, where a neighbouring stoma (around 100 µm apart) was damaged with a sharp microelectrode at time point 12:30. Around less than 5 min after wounding (time point 17:00) the closure is already at its maximum and the stoma remains closed until the end of the recording 28 min post wounding.
To follow wounding induced hormonal signaling the recently developed genetically encoded, fluorescent JA-biosensor Jas9-VENUS ([100]) was employed. Jas9-VENUS allows quantification of dynamic changes in JA levels *in planta* with high spatiotemporal sensitivity. The sensor's signal is dependent on bioactive JA-Ile, COI1 as co-receptor, a functional JAZ motif and proteasome activity. While not interfering with endogenous JA perception and signaling responses, Jas9-VENUS is specifically and rapidly degraded in a bioactive JA dose-dependent manner. A nuclear Histone marker fused to a red fluorescent protein (H2B-RFP) is used for ratiometric analysis of the sensor's fluorescent signal. [100]
Figure 3.3 D shows that Jas9-VENUS/H2B-RFP fluorescence ratios in guard cells decline significantly after wounding. Concurrently stomata close fully within 2 hours after wounding (3.3 C). These findings suggest that, in the manner of wounding, activation of the guard cell JA signaling cascade precedes stomatal closure. In unwounded leaves this correlation could not be found (3.3 A and B; Dr. Antoine Larrieu, Université de Lyon). The idea that wounding addresses the JA pathway in guard cells is corroborated by the finding, that both stomatal closure and JA signaling was impaired upon wounding in the *coi1* mutant (3.3 A and B; Dr. Antoine Larrieu, Université de Lyon).

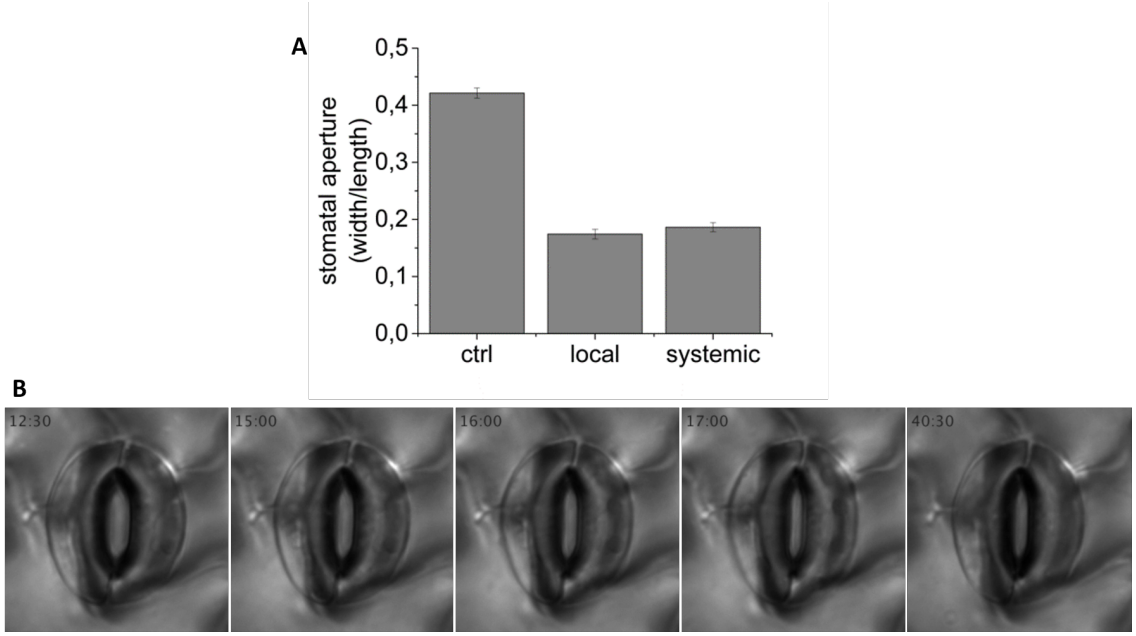

Figure 3.1: Wounding induces local and systemic stomatal closure

(A) Stomatal aperture assays of wildtype Col-0; Guard cells were pre-opened in the light for 2 h, then control apertures were measured and one leaf per plant was mechanically wounded using forceps. Apertures were determined 1h after the wounding as the ratio of stomatal width to length. Systemic leaf was in position +3 of the local leaf. Averages from three or more independent experiments are shown. Data represent mean ± S.E., n $\geq$ 80 stomata
(B) Monitoring wounding induced closing of an open stoma (12:30h right before wounding); a neighbouring stoma (around 100 µm away) was damaged with a sharp electrode and the movement was recorded until 28 min after the wounding; closing is at its maximum less than 5 min after wounding (time point 17:00) and the stoma remains closed until the end of the recording (time point 40:30h); time points reflect minutes after starting the experiment; experiment carried out by Dr. Shouguang Huang (University of Würzburg).

3.2 GORK is an essential part of JA-induced stomatal closure

In 2003 Evans showed in patch clamp studies on *Vicia faba* guard cell protoplasts that MeJA affects K^+ channel activity and promotes potassium efflux and thereby stomatal closure in a concentration-dependent manner [57]. The *Arabidopsis thaliana* genome includes the two xylem parenchyma and guard cell expressed K^+ efflux channels SKOR and the GORK, respectively [6, 2]. Taking advantage of the model plant *Arabidopsis thaliana* and its available mutants, whole-cell patch clamp measurements on guard cell protoplasts isolated from Col-0 wild-type (wt) or *gork1-2* (GABI_865F05) mutants were performed under asymmetric potassium conditions with 100 mM K^+ in the pipette solution and 10 mM K^+ in the bath solution. Starting from a holding potential of -60 mV rectangular-shaped voltage

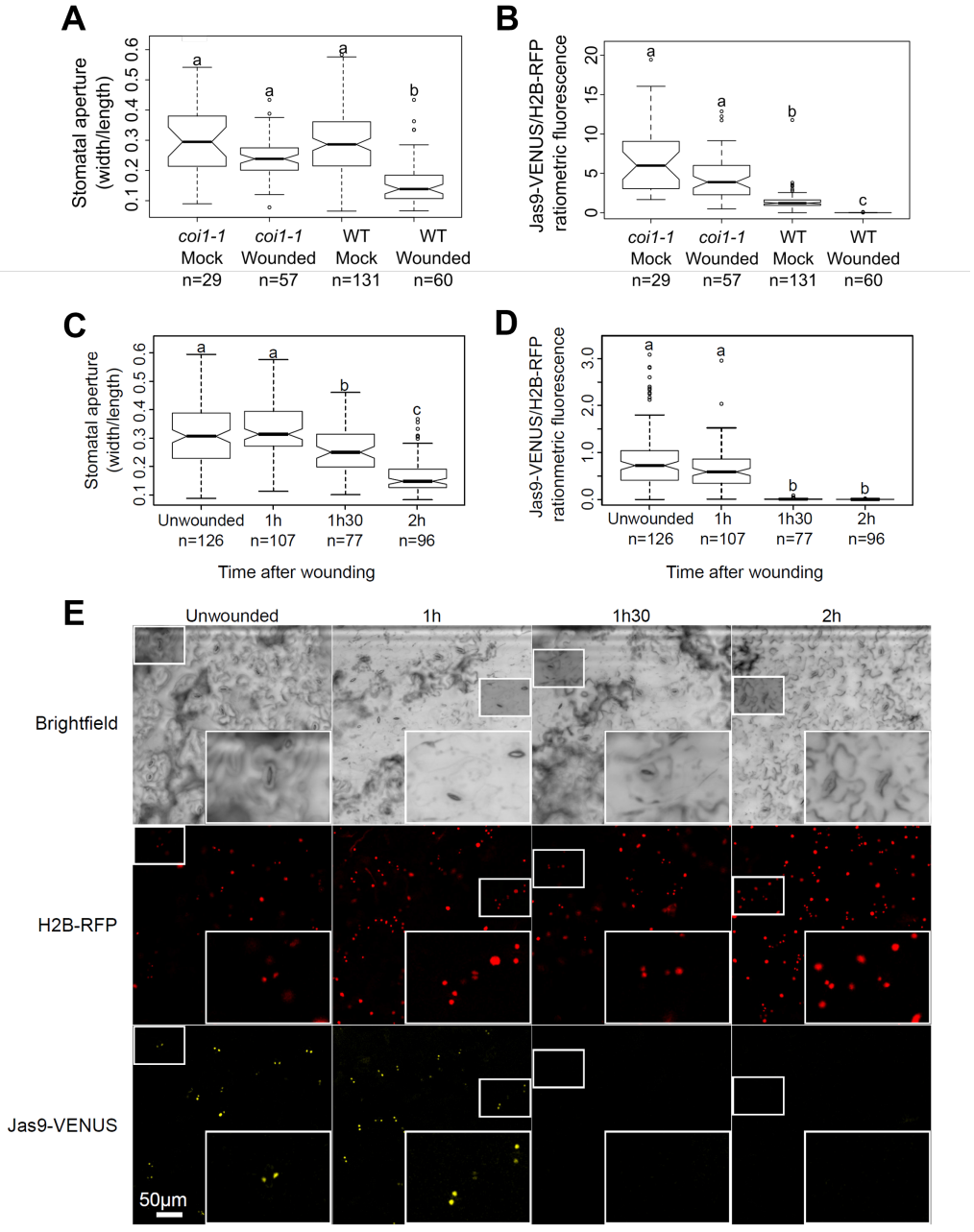

Figure 3.3: JA signaling precedes stomatal closure upon wounding

(A, C) Boxplots showing stomatal apertures in response to wounding of wildtype Col-0 (both) and *coi1-1* homozygous mutants expressing the JA-sensor p35S::Jas9-Venus-N7 (onl A). Epidermal strips were taken from the abaxial side of leaves that were unwounded or wounded for the indicated time (n indicates number of stomata measured).
(B, D) Boxplots showing the ratiometric quantification of Jas9-VENUS/H2B-RFP fluorescence of epidermal strips shown in (A) respectively (C) indicative for JA signaling efficiency.
The confocal images below (E) show representative images including magnifications for the different time points analyzed. The experiments were performed and analyzed by Dr. Antoine Larrieu (Université de Lyon).

pulses ranging from -170 to 100 mV were applied to the patched cell. Representative recordings and statistical analysis are shown in figure 3.5. Under control conditions large inward (-90 ± 9 pA/pF at -160 mV) and small outward (13 ± 1.7 pA/pF at 100 mV) K^+ currents were elicited in wt Col-0 guard cells (figure3.5 A, left traces). The depolarization activated outward currents were absent in the *gork1-2* mutant (1.7 ± 0.6 pA/pF at 100 mV), while K_{in} currents were comparable to wt or even slightly higher (-162 ± 23 pA/pF at -160 mV; figure 3.5 A, right traces).

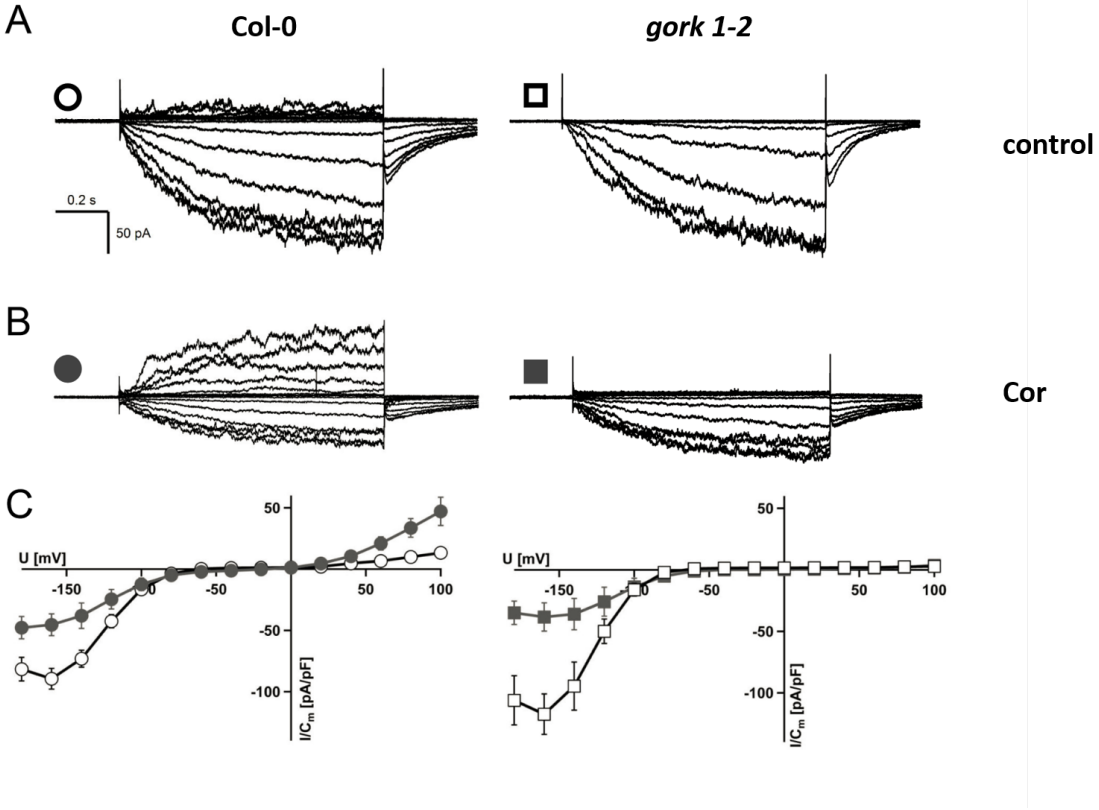

Figure 3.5: GORK K^+_{out} currents are a target of JA signaling in guard cells

Patch-Clamp experiments showing representative whole cell current densities from guard cell protoplasts of wt Col-0 (left panel) and *gork1-2* (GABI_865F05) mutants (right panel) under control conditions (A) or in response to 1 µM Cor (*gork1-2*) or 0.1 µM Cor (Col-0) (B). Starting from a holding potential of -60 mV rectangular-shaped voltage pulses ranging from -180 to 100 mV elicited large inward (-90 ± 9 pA/pF at -160 mV) and small outward (13 ± 1.7 pA/pF at 100 mV) K^+ currents under control conditions in wt Col-0 guard cells. The outward currents appeared absent in *gork1-2* under control as well as under Cor treatment. Col-0 guard cells showed highly increased outward currents upon 15 minutes preincubation with COR.
(C) I/V plots of Col-0 (left panel) and *gork1-2* (right panel) guard cell K^+ current densities under control (white symbols) as well as Cor stimulated (grey symbols) conditions. Averages from three or more independent experiments are shown. Data represent mean ± SE, n ≥ 4

Following a 15 min preincubation with Cor a significant increase of the outward K^+ current density by 3.62 ± 0.85 fold ($p < 0.005$) was observed in the wt Col-0 guard protoplasts (figure 3.5 B and C, left; 47 ± 11.5 pA/pF at 100 mV)). Again, K+ efflux currents were absent in the *gork1-2* knockout mutant (figure 3.5 B and C, right; 3.7 ± 1.5 pA/pF at 100 mV). Opposite to that in both wt and *gork1-2* the activity of inward rectifying K^+ channels appeared significantly inhibited upon Cor treatment (figure 3.5 B and C) with Col-0 showing ca. half of the control currents at -160 mV (-45.5 ± 9 pA/pF) and *gork1-2* even only about a fourth (-38.3 ± 11.5 pA/pF). Taken together the patch clamp measurements suggest that GORK represents a target of JA-signaling in guard cells.

This finding was confirmed by investigation of guard cell responses to MeJA or ABA in stomatal aperture assays (see 2.1.2). As seen in figure 3.7, the *gork1-2* mutant exhibited partially impaired stomatal closure upon externally applied ABA with a stomatal aperture ratio of 0.31 ± 0.005 (in Col-0 background) respectively 0.27 ± 0.009 (in Ws background) after ABA application compared to 0.41 ± 0.004 (Col-0) respectively 0.47 ± 0.010 (Ws) under control conditions.

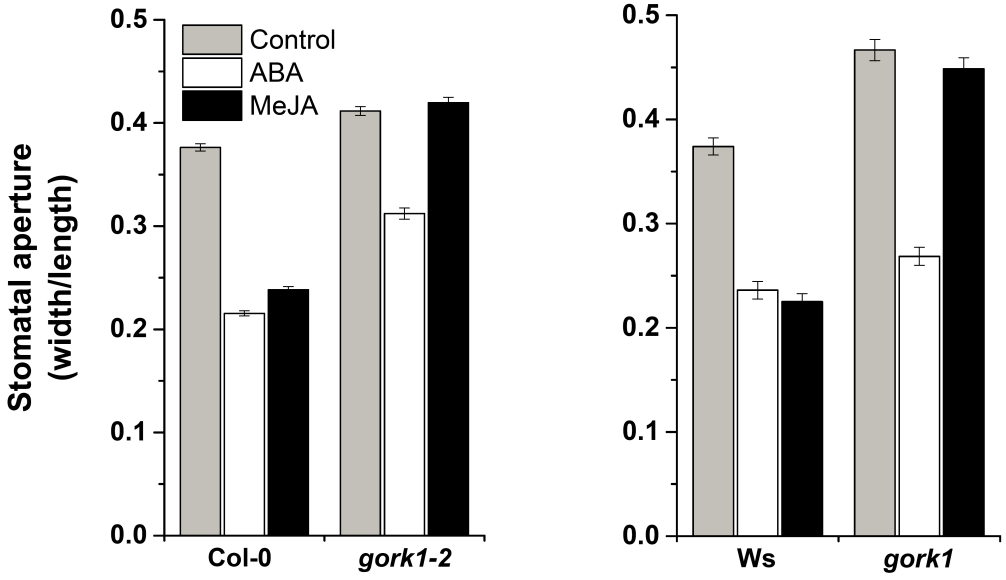

Figure 3.7: GORK functions specifically in JA induced stomatal closure

Stomatal aperture assays of wildtype Col-0 and *gork1-2* (left) or wildtype WS and *gork1* (right). Guard cells of excised leaves were pre-opened in the light for 2 h, then incubated with either 20 µM (±)-ABA or 10 µM MeJA for 1 h. Both wildtypes show the same responses, while both *GORK*-knockouts are partially impaired in ABA-induced closure and do not close at all upon JA stimulus. Apertures were determined as the ratio of stomatal width to length. Averages from three or more independent experiments are shown. Data represent mean ± S.E., n≥ 80 stomata

In comparison wt Col-0 and wt Ws show a maximum closure upon ABA treatment with a stomatal aperture ratio of 0.22 ± 0.002 (Col-0) respectively 0.27 ± 0.009 (in

Ws background) after ABA application compared to 0.22 ± 0.002 (Col-0) respectively 0.24 ± 0.008 (Ws) under control conditions (figure 3.7, white bars). On the other hand stomatal closure in GORK-knockout leaves was completely abolished for the MeJA stimulus with a stomatal aperture ratio of 0.42 ± 0.005 (in Col-0 background) respectively 0.45 ± 0.010 (in Ws background)(figure 3.7, black bars). The stomatal behaviour in both wt ecotypes towards JA (Col-0: 0.24 ± 0.003, WS: 0.23 ± 0.008)and ABA is comparable. Hence, this JA stoma phenotype seems to be independent of the respective *Arabidopsis* ecotype. Together, this results establish GORK function as an essential step during JA-induced guard cell response.

In a physiological context the function of GORK in JA signaling can be shown with the wounding stoma assay, already seen in figure 3.1 for wt Col-0. One hour after wounding *gork1-2* leaves showed very weak stomatal closure response both systemically (stomatal aperture ration 0.33 ± 0.01) and locally (stomatal aperture ration 0.38 ± 0.01) compared to the wildtype (locally 0.17 ± 0.01 and systemically 0.19 ± 0.01; figure 3.9). This indicates an unique role of GORK in (wounding induced) JA signaling in guard cells.

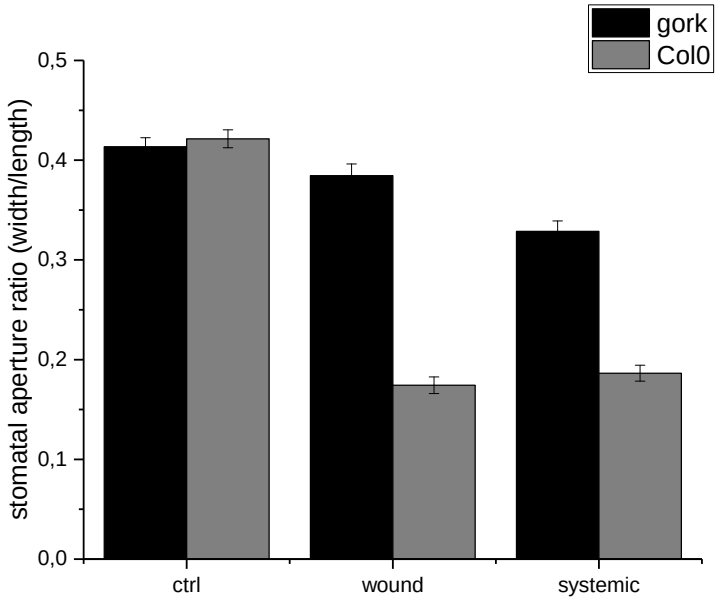

Figure 3.9: GORK is part of JA signaling upon wounding

Stomatal aperture assays of wildtype Col-0 and *gork1-2*. Guard cells were pre-opened in the light for 2 h, then control apertures were measured and one leaf per plant was mechanically wounded using forceps. Apertures were determined on excised leaves 1h after the wounding as the ratio of stomatal width to length. Systemic leaf was in position +3 of the local leaf. The stomatal closing response that appears in the wt upon wounding both locally and systemically is not present in the *gork1-2* leaves. Averages from three or more independent experiments are shown. Data represent mean ± S.E., n ≥ 80 stomata

3.3 Regulation of GORK via calcium-dependent, reversible phosphorylation

Ca^{2+} is a basic second messenger in eukaryotic organisms that often targets membrane transporters and channels via phosphorylation by Ca^{2+}-dependent kinases. Source of Ca^{2+} in plants' second messenger cascades can be either intracellular compartments or the apoplast as well as neighbouring cells.

3.3.1 GORK interaction with CIPK5 and ABI2 provides for channel activity control

Cytosolic Ca^{2+} oscillations are known to be a crucial part of both short-term and long-term regulation of stomatal closure [3]. In 2010 Islam et al. found, that Ca^{2+} oscillations together with cytosolic alkalization function in JA signaling in *Arabidopsis* guard cells. Potentially, this Ca^{2+}-based regulation is carried out via Ca^{2+}-regulated kinases which modulate the activity of the channels involved in stomatal closure, mainly anion channels and the K^+ channel of interest in this work - GORK.

In search for Ca^{2+}-regulated kinases interacting with GORK yeast two-hybrid experiments with guard cell-expressed members of the calcineurin B-like interacting protein kinase (CIPK) family were performed. Thereby, CIPK5 was identified as the only member of this kinase family to associate with the N-terminal (NT) domain of the GORK channel (data not shown, [58]). A combination of CIPK5 with all 10 CBLs from *Arabidopsis* further identified CBL1, CBL4, CBL5 and CBL9 as potential interaction partners of the kinase (data not shown, [58]). Those interactions were confirmed by bimolecular fluorescence complementation (BiFC) assays in transiently transformed *Nicotiana benthamiana* leaves or *Arabidopsis* mesophyll protoplasts (figure 3.11), respectively.

Co-expression of full-length GORK-YFP^C with YFP^N-CIPK5 caused readily detectable fluorescence in contrast to YFP^N-CIPK15 as control (figure 3.11 A). This implies a specificity of the CIPK5-GORK interaction, also observed in BiFC analysis of transiently transfected protoplasts (figure 3.11 C). Efficient interaction was further revealed with YFP^N-CIPK5 and the guard cell-expressed Ca^{2+} sensors CBL1-YFP^C and CBL9-YFP^C but not with CBL7-YFP^C (figure 3.11 B).

To identify regulatory components counteracting the potential CBL-CIPK impact on GORK, the guard cell expressed type 2C (PP2C)-type protein phosphatases ABI1 and ABI2, which are well-known negative regulators of ABA-dependent stomatal closure ([112, 163], cp. 1.4.3), were analyzed in the same way. First yeast two-hybrid analysis provided evidence for interaction of GORK-NT with ABI2 but not with ABI1 (data not shown, [58]). In BiFC analysis in *Arabidopsis* protoplasts this interaction was verified. In addition, an interaction of CIPK5-YFP^N with ABI2-YFP^C was observed as well (figure 3.11 C). These results are suggestive for an antagonistic regulatory phosphorylation/dephosphorylation switch due to specific

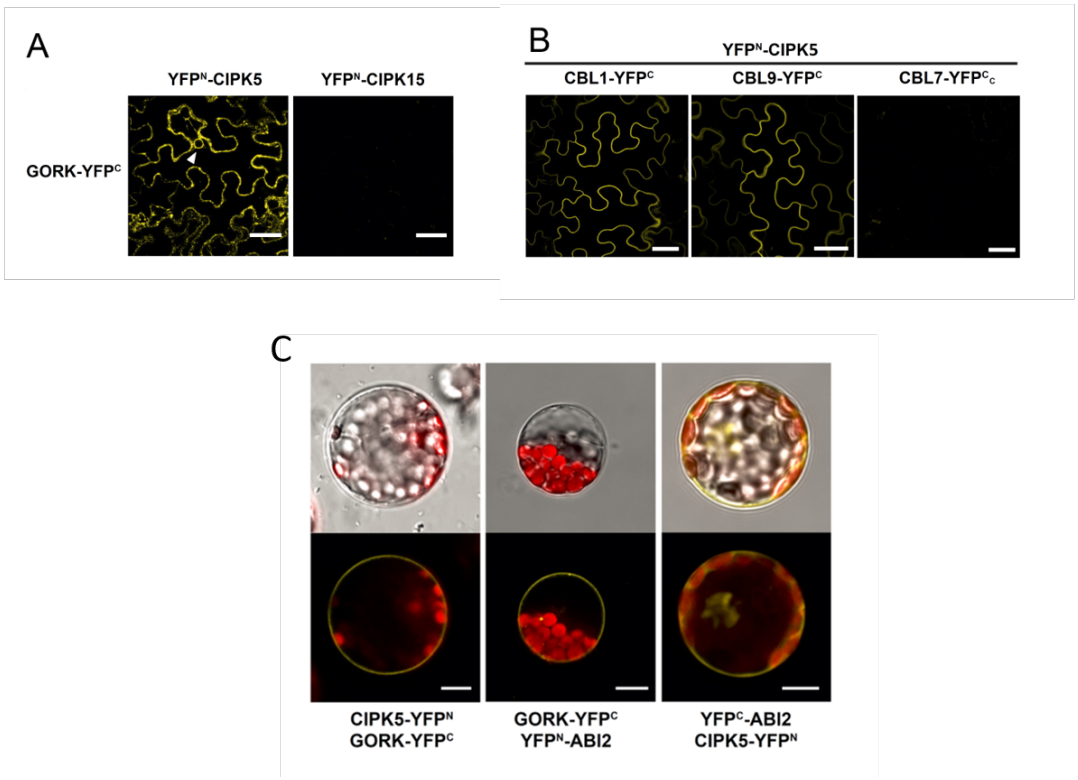

Figure 3.11: BiFC analysis of interaction of CIPKs with GORK, CBLs and ABI2

(A,B) Bimolecular fluorescence complementation (BiFC) analysis in transiently transformed *N. benthamiana* epidermal cells of (A) GORK-YFP^C combined with either YFP^N-CIPK5 or YFP^N-CIPK15 (the nucleus region is marked by a white arrow) and (B) YFP^N-CIPK5 co-expressed CBL1-YFP^C, CBL9-YFP^C or CBL7-YFP^C. Microscopic analysis were performed 2-3 days after infiltration. White bars depict scale bars of 40 µm.
(C) BiFC analysis in transiently transfected *Arabidopsis* mesophyll protoplasts of GORK-YFP^C combined with either CIPK5-YFP^N or YFP^N-ABI2 and CIPK5-YFP^N co-expressed with YFP^C-ABI2. Interaction is indicated by yellow while the red color represents autofluorescence of chloroplasts. White bars depict scale bars of 10 µm.
Representative images of at least three independent experiments are shown. Pictures were taken by Dr. Lena Schmidt (University of Münster).

interaction of GORK with the sensor kinase complex CIPK5-CBL on one hand and the phosphatase ABI2 on the other hand.

To support the potential regulatory phosphorylation-dephosphorylation mechanism for GORK, biochemical *in-vitro* kinase assays were performed using recombinant proteins. Combining the CIPK5 kinase protein with wildtype GORK N-terminal and C-terminal cytoplasmic domains *in vitro* revealed that this kinase only phosphorylates the amino terminus of the channel (figure 3.13 A).

Consistent with the suggested role of ABI2 as counteractor of CIPK5 mediated phosphorylation of GORK, CIPK5 dependent N-terminus phosphorylation was inhibited

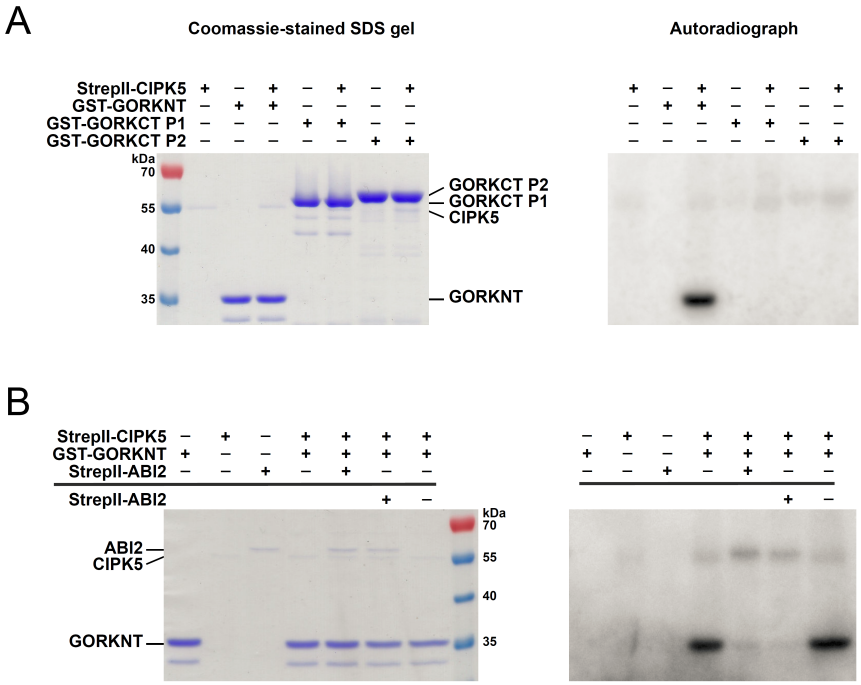

Figure 3.13: The cytoplasmic N-terminal domain of GORK is phosphorylated by CIPK5 and dephosphorylated by ABI2

(A) CIPK5 phosphorylates the N-terminus but not the C-terminus of GORK. *In vitro* phosphorylation assays were performed in a 1:23 molar ratio of kinase to GORK regions by incubating StrepII-tagged kinase CIPK5 (100 ng) with equivalent molar amounts of the GST-tagged proteins GORK N-terminal domain (amino acids 1 to 67), GORK C-terminus peptide 1 (amino acids 301 to 559) or GORK C-terminus peptide 2 (amino acids 531 to 820).
(B) ABI2 directly dephosphorylates the N-terminal domain of GORK. *In vitro* phosphorylation assays were performed with StrepII-tagged CIPK5 (75 ng), an equivalent molar amount of StrepII-tagged ABI2 and GST-tagged GORK N-terminus (1500 ng). CIPK5 - GORKNT in vitro kinase reactions shown in lanes 6 and 7 were performed for 30 min before StrepII-ABI2 protein (lane 6) or buffer control (lane 7) were added, and reactions were incubated for an additional 30 min. Experiments were performed by Dr. Lena Schmidt (University of Münster).

when the ABI2 phosphatase was present in the experiment from the beginning (figure 3.13 B, lane 5) or added 30 min after the reaction had been started (figure 3.13 B, lane 6). This provides clear evidence that ABI2 is not only interacting with CIPK5 and GORK, as seen before (figure 3.11 C), but also dephosphorylates either the N-terminus of the guard cell K^+ efflux channel or it dephosphorylates and inactivates CIPK5 or both. The efficacy of CIPK5 to phosphorylate the GORK N-terminus was unabatedly high irrespective of absence or presence of CBL1 in the *in vitro* reaction (data not shown; Dr. Lena Schmidt, University of Münster).

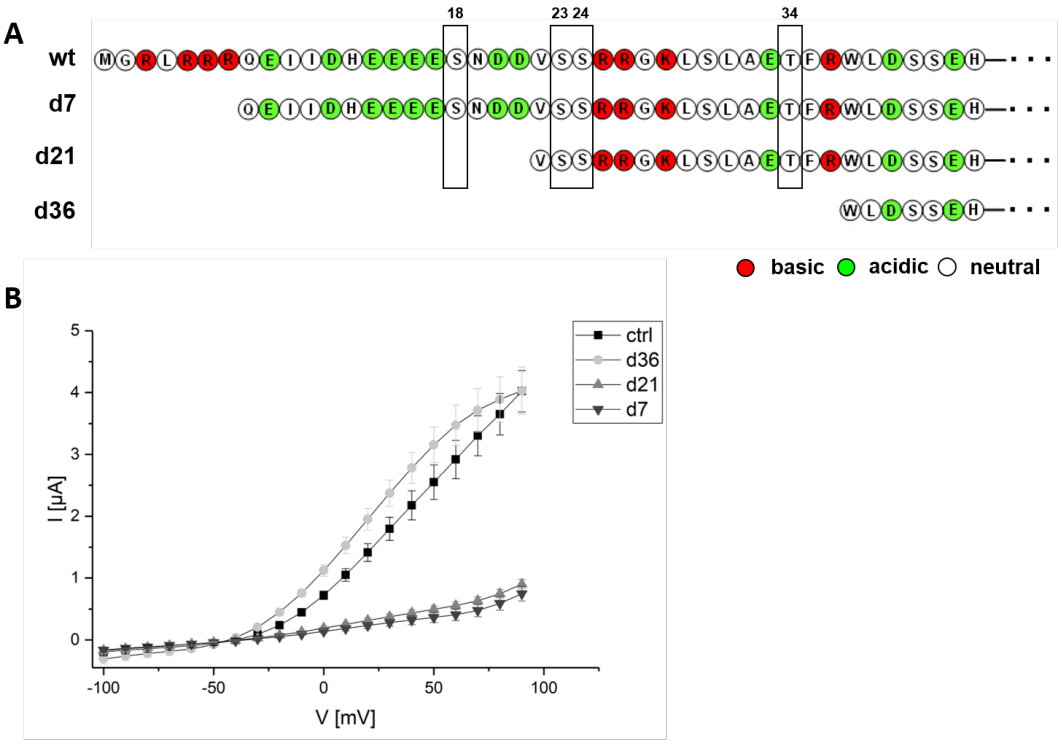

Figure 3.15: Regulatory function of GORK N-terminus

Current-voltage plot (B) of DEVC whole-oocyte experiments with GORK N-terminus deletion mutants without the first 7, 21 or 36 amino acids (A). From a holding potential of 0 mV a 1 s deactivation pulse at -80 mV followed by rectangular shaped, 2 s long pulses from -100 to 90 mV (in 10 mV steps), again 1 s at -80 mV were applied. The steady state currents shown are taken from the end of the rectangular shaped pulses. Deletion of 7 (dark grey, downward triangle) or 21 (grey, upward triangle) amino acids leads to significantly lower currents compared to the control (wt gork; black square). The d36 mutant (light grey circle) shows the same current densities as the control, but different kinetics.
Averages from three or more independent experiments are shown. Data represent mean currents ± SE, n ≥ 7.

The regulatory function of the GORK N-terminus is underlined by the results of DEVC experiments on *Xenopus* oocytes (cp. 2.4), where N-terminal deletions clearly affect the GORK mediated currents, as shown in figure 3.15. In figure 3.15 B the difference between the currents of d7 (0.75 ± 0.12 µA) and d21 (0.90 ± 0.108 µA) mutants at 90 mV compared to the wildtype GORK-expressing oocytes (ctrl; 4.02 ± 0.34 µA) can be clearly seen. The d36 mutant shows wildtype-typical current height of 4.03 ± 0.39 µA at 90 mV but slightly differing kinetics in the I/V plot.

As CIPK5 possesses serine and threonine kinase activity, potential serines (S18, S23, S24 and the double mutation S23/S24, compare figure 3.15 A) and threonines (T34 compare figure 3.15 A and T48) in the GORK N-terminus were mutated to alanine using selective mutagenesis USER cloning (cp. 2.2.1.1). These mutants were analyzed in *in vitro* phosphorylation experiments (figure 3.17). The results show a clear phosphorylation of the wildtype GORK N-terminus by CIPK5, while both T34A and the double mutant (DM) have one-fifth of radiograph intensity relative to the wildtype (0.21 in both cases, average of three independent experiments each). The radiograph intensity of T48A appears half, namely 0.53 (average of three independent experiments) relative to that of the wildtype CIPK5 phosphorylation. All serine mutants show comparable radiograph intensities to the wildtype GORK N-terminus (1.02 for S18A, 0.96 for S23A, 0.94 for the double mutant, 1.09 for S24A; average of three independent experiments each). Together these data suggest that T34 and T48 are phosphorylated by CIPK5 while S18, S23 and S24 are not.

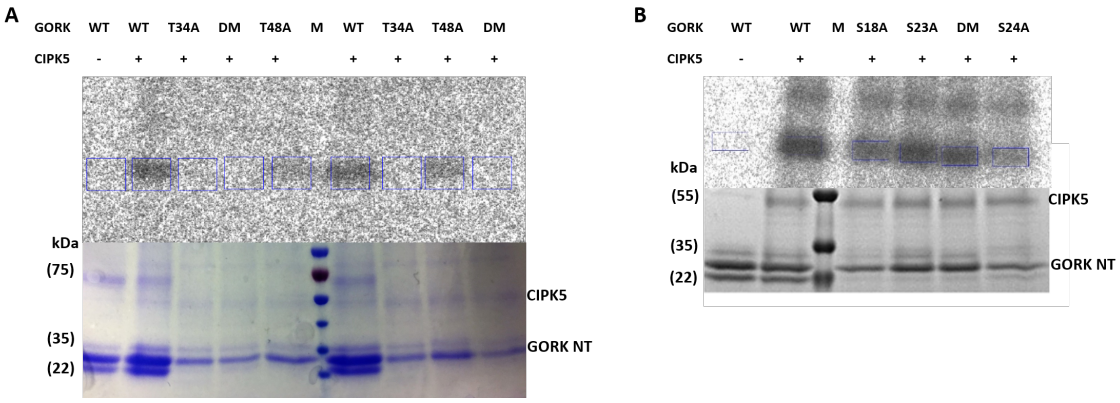

Figure 3.17: CIPK5 phosphorylates the GORK N-terminus at amino acids T34 and T48

(A) Autoradiography (upper half) and Coomassie-stained SDS gel of *in vitro* phosphorylation experiments of two different elutions of CIPK5 and different variants of the GORK N-terminus (wildtype wt, mutant T34A, double mutant DM T34A/T48A and mutant T48A) plus marker (M). It can be stated, that amino acids T34 and T48 of the cytoplasmic N-terminal domain of GORK are phosphorylated by CIPK5.
(B) Autoradiography (upper half) and Coomassie-stained SDS gel of *in vitro* phosphorylation experiments of CIPK5 and different variants of the GORK N-terminus (wildtype wt, mutant S18A, mutant S23A, double mutant DM S23A/S24A and mutant S24A) plus marker (M). The amino acids S18, S23, S24 of the cytoplasmic N-terminal domain of GORK seem not to be phosphorylated by CIPK5.
Experiments were performed together with Fabian Link, Bachelor's Thesis at Departement of plant physiology and biophysics, University of Würzburg

To back this phosphorylation hypothesis deduced from *in vitro* experiments, functional analysis was done using the electrophysiological techniques double electrode voltage clamp (DEVC) and patch-clamp (PC) on *Xenopus laevis* oocytes or *Arabidopsis thaliana* guard cell protoplasts, respectively (cp. 2.4 and 2.5). In the heterologous *Xenopus* oocyte system GORK characteristic steady state currents can

be measured in whole cell configuration three to five days after nanoinjection of GORK-cRNA (cp. figure 3.19 and 2.4.2). As co-expression of CIPK5/CBL1 with GORK does not lead to any detectable effect on channel activity (data not shown), GORK appears to be preactivated by endogenous kinases in oocytes. Co-injection and -expression of ABI2 reduced these currents by 74 ± 1.9% to 26 ± 1.9% , while in line with previous studies (see above) ABI1 had no effect and the currents remained at 88.1 ± 8.6% (cp. figure 3.19).

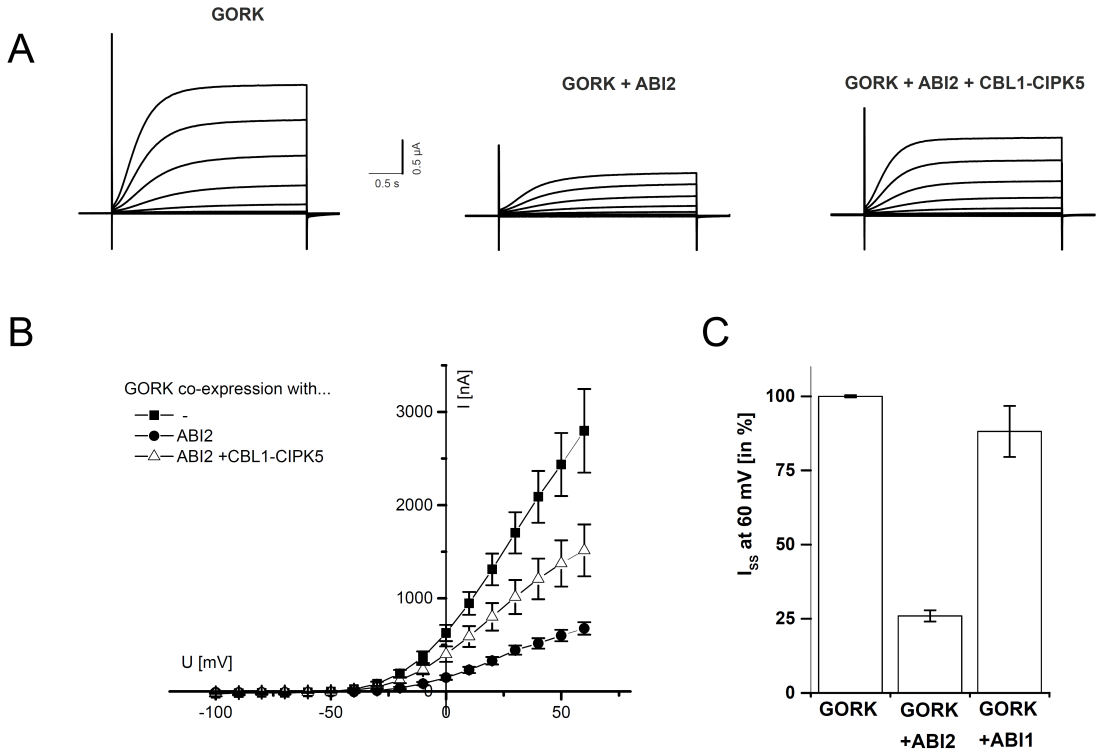

Figure 3.19: Antagonistic regulation of GORK currents by ABI2 and CBL1/CIPK5 in DEVC experiments

(A) DEVC whole-oocyte currents recorded upon 3 s voltage pulses ranging from -100 to +60 mV in 20 mV increments, using a holding potential of -40 mV. Expression of GORK (left trace) resulted in the typical slow activation of the Shaker-type efflux channel. Co-expression with ABI2 reduced GORK currents (middle). Addition of CIPK5 and CBL1 counteracted ABI2-inhibition (right). Current traces of representative cells are shown.
(B) Current-voltage plot of steady-state currents (I_{SS}) of GORK (filled square), GORK co-expressed with ABI2 (filled dot) or GORK co-expressed with ABI2, CIPK5 and CBL1 (empty triangle). Data represent mean currents ± S.E., n ≥ 6
(C) Phosphatase specificity of the inhibition is shown in normalized steady-state currents at 60 mV from oocytes expressing GORK or GORK with either ABI1 or ABI2. ABI2 significantly inhibited GORK activity by 74 ± 1.9 % . ABI1 had no significant effect on GORK (11.9 ± 8.6 %). Results represent mean values ± S.E.,n ≥ 14
Experiments were partially performed by Dr. Sabrina Förster, PhD Thesis at Departement of plant physiology and biophysics, University of Würzburg.

In DECV experiments GORK inhibition by ABI2 could be partially relieved when CLB1/CIPK5 were co-expressed, as well. The sensor-kinase complex was able to restore 55.8 ± 4.3% of the maximum GORK channel activity (cp. figure 3.19 A right and B). CIPK15 did not affect ABI2 inhibition, neither did mutated CIPK5 versions with defective kinase activity (data not shown). Also, in the absence of CBL proteins CIPK5 did not relieve ABI2-mediated inhibition of GORK channel activity as was observed for a CBL1 mutant, disrupted in plasma membrane anchoring ($CBL1^{G2A}$; data not shown). Ca^{2+} binding to the CBL1 protein appeared necessary, as mutations in the Ca^{2+}-coordinating amino acids of the Ca^{2+}-binding EF-hands in CBL1 led to significantly reduced GORK-mediated K^+ currents ($CBL1^{E56QD91NE128QE172Q}$; data not shown).

These results let one assume, that plasma membrane targeting of CBL1 and Ca^{2+} binding to CBL1 represent important steps in activation of GORK by CBL1/CIPK5 complexes. In DEVC experiments, as in *in vitro* phosphorylation studies, ABI2 and CBL1/CIPK5 antagonistically regulate GORK-mediated K^+ currents. The possible impact of this regulation on JA-signaling in guard cells was investigated by patch-clamp recordings on guard cell protoplasts of *cipk5-2* kinase loss-of-function mutant. Those mutant protoplasts exhibited outward and inward K^+ currents comparable to the wildtype (see figure 3.5 A) under control conditions (figure 3.21 A).

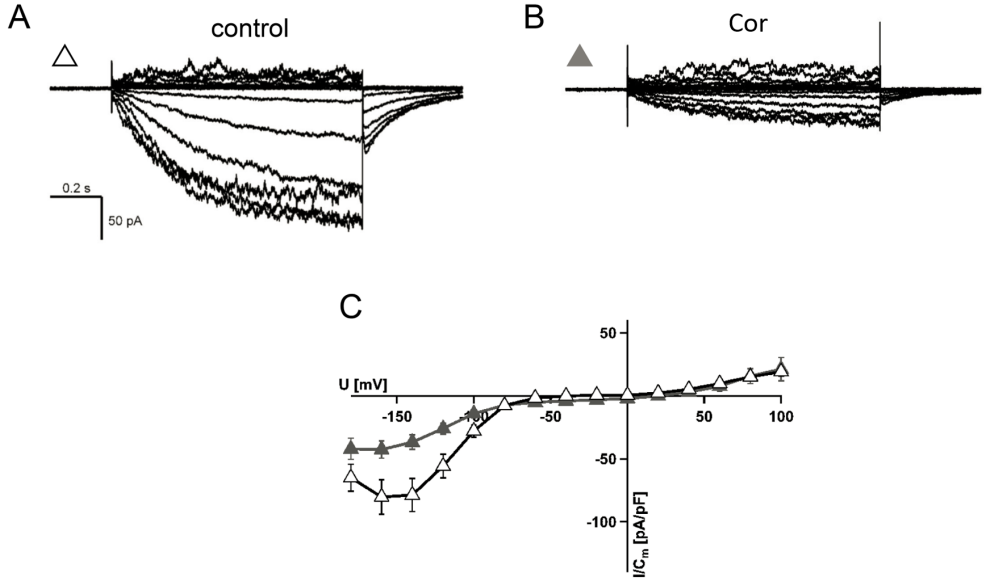

Figure 3.21: CIPK5 is essential for JA-mediated GORK activation

Patch-Clamp experiments showing representative whole cell current densities from guard cell protoplasts of *cipk5-2* mutants (SALK_ 084455) under control conditions (A) or in response to 0.1 µM Cor (Col-0) (B). Experimental procedures were as in figure 3.5.
C) I/V plots of guard cell K^+ current densities under control conditions (open symbols) and after Cor treatment (closed symbols); (n(ctrl)=16, n(Cor)=6; mean ± S.E.). Inhibition of K^+_{in} current density by Cor at -140 mV is 59 ± 12% , $p < 0.005$.

Also, the inhibited K^+_{in} current activity in the kinase mutant was corresponding to the wildtype. In contrast however, Cor failed to stimulate K^+_{out} activity in the mutant (cp. figure 3.5 B, C and figure 3.21 B,C).

Together with phosphorylation and DEVC studies these patch clamp data suggests an important role for CIPK5 in JA-triggered fast stomatal closure.
As a physiological proof for this assumption the stomatal response to JA of several *Arabidopsis thaliana* loss-of-function mutants was analysed as described in 2.1.2. Intact leaves of 5-6 weeks old plants were treated with 20 µM (±)-ABA or 10 µM MeJA and this led to stomatal closure in case of wildtype *Arabidopsis* as seen in figure 3.7. The same figure illustrates a clear phenotype for GORK-knockout plants, where no response upon MeJA treatment and partially impaired stomatal closure upon externally applied ABA can be seen (see also figure 3.7). The same MeJA-phenotype appears in the case of *cipk5-2* and *cbl1/9* mutants as illustrated in figure 3.23. After 1 h, guard cells of both mutants were fully closed in response to ABA, resembling wildtype-like responsiveness. In contrast to wildtype, but reminiscent of the *gork* mutants, guard cells of neither *cipk5-2* nor *cbl1/9* closed in response to exogenously applied MeJA.

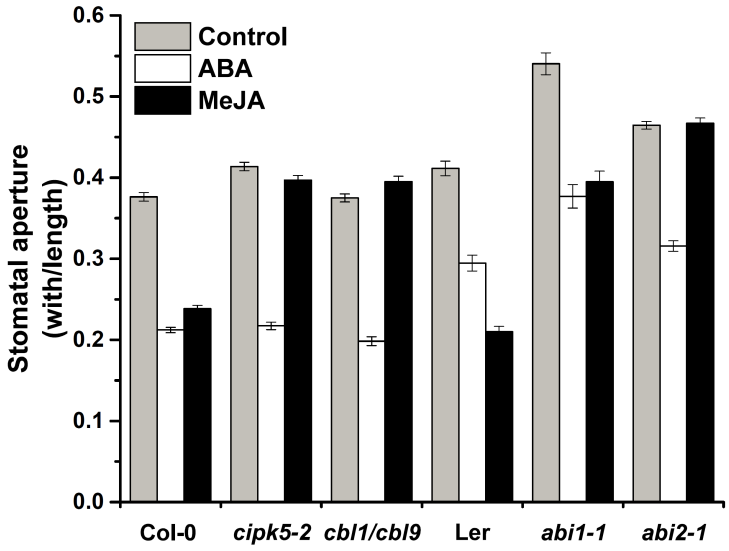

Figure 3.23: Guard cell responses of *cipk5-2*, *cbl1/9*, *abi1-1* and *abi2-1* mutants towards ABA or MeJA

Stomatal aperture assays of wildtype Col-0, *cipk5-2*, *cbl1/9*, wildtype Ler, *abi1-1* and *abi2-1*. Guard cells of excised leaves were pre-opened in the light for 2 h, then incubated with either 20 µM (±)-ABA or 10 µM MeJA for 1 h. Both wildtypes show similar responses, while *abi2-1*, *cipk5-2* and *cbl1/9* mutants respond to ABA wt-like but do not close at all upon JA stimulus. Apertures were determined as the ratio of stomatal width to length. Averages from three or more independent experiments are shown. Data represent mean ± S.E., n ≥ 80 stomata. Experiments were partially performed by Dr. Sabrina Förster, PhD Thesis at Departement of plant physiology and biophysics, University of Würzburg.

For the first time these results identify CBL-type Ca^{2+} sensor proteins and their interacting kinase as essential components in fast JA signal transduction in guard cells. Concrete, these data provide strong evidence, that CBL1/9-CIPK5 complexes are essential for JA-triggered stomatal closure via their activating role for the GORK K^+ efflux channel.
In order to assess the role of PP2Cs in this physiological context, also *abi1-1* and *abi2-1* mutants were tested. Both express mutant phosphatases refractory to inhibition by ABA receptors. In line with this reduced ABA sensitivity, stomata of both lines exhibited greater stomatal apertures under control conditions (0.54 ± 0.01 and 0.47 ± 0.01) compared to their background wildtype Ler (0.41 ± 0.01; see figure 3.23). Guard cell closing response to ABA appeared weaker compared to wildtype plants (0.38 ± 0.01 and 0.32 ± 0.01, Ler wt: 0.29 ± 0.01). While stomata of *abi1-1* responded similarly to ABA and MeJA (0.39 ± 0.01), stomata of *abi2-1* did not close in responae to JA application (0.47 ± 0.01; Ler wt: 0.21 ± 0.01).
These data are in further support of the conclusion that ABI2 but not ABI1 represents a component of JA-induced signaling processes that regulate GORK activity and thereby guard cell aperture. In this context, ABI2 function appears to be different from its role in ABA signaling.

3.3.2 CPKs' and GRFs' impact on GORK activity

Apart from CBL proteins, there are more than 250 Ca^{2+} sensors in *Arabidopsis* [45]. An important group of directly Ca^{2+}-sensing protein kinases is represented by the Calcium Dependent Protein Kinases (CDPKs or short CPKs, cp. 1.3.1) that contain Ca^{2+} binding EF-hands adjacent to their kinase domain. They have been demonstrated to be part of numerous signaling pathways and function as positive regulators of abiotic stress responses [128]. CPK6 functions in ABA signal cascades in guard cells and has been reported to participate in MeJA signaling as well [132, 134]. CPK21 is also known to be part of the ABA signaling pathway where it activates the anion channel SLAC1 [62]. Various studies have reported that the CPK genes not only act as positive regulators of abiotic stress signaling but also as negative regulators. For example, CPK23 improved the drought tolerance in *Arabidopsis* as a negative regulator of abiotic stress signaling [111] and, alike CPK21, regulates SLAC1 [62]. Just a few years ago Corratge-Faillie et al. could show that CPK33 stimulates GORK channel activity in a phosphorylation-dependent manner and that the *cpk33* knockout mutant is impaired in Ca^{2+}-induced stomatal closure [40]. CPK33 action on GORK was specific, since the authors could not detect any impact on GORK for CPK3, CPK6, CPK7, CPK11 or CPK13 [40].

3.3.2.1 Analysis of protein-protein interaction of GORK with CPKs

Hence, CPKs represent interesting candidates involved in Ca^{2+} dependent regulation of ion channels and the interaction of CPK6, CPK21 and CPK23 with GORK was analyzed during the course of this study. Ratiometric BiFC (rBiFC) analysis [68]

showed that GORK interacts with all three CPKs as shown in figure 3.25. In these experiments CIPK23 in combination with CBL1 was used as positive control (relative fluorescence 1.32 ± 0.09) and CIPK23 together with CBL 6 functioned as negative control (relative fluorescence 0.60 ± 0.03).

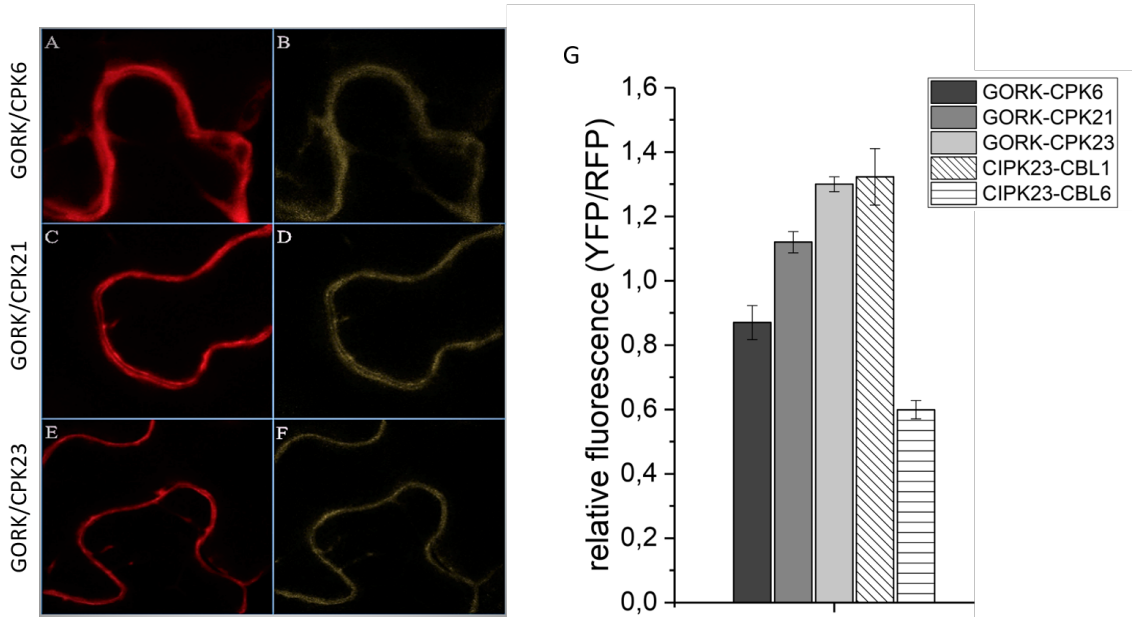

Figure 3.25: Ratiometric BiFC analysis shows interaction of GORK with CPK6, CPK21, CPK23

Epidermal cells of *Nicotiana benthamiana* leaves infiltrated with agrobacteria that carry the respective rBiFC construct. Images were taken at a confocal laser scanning microscope. RFP (left panel, A, C, E) was excited at 561 nm and monitored in the range of 580 - 610 nm. YFP fluorescence (right panel, B, D, F) was excited at 514 nm and monitored in the range of 525 - 589 nm. The pictures were analysed with ImageJ1.48 and the relative fluorescence shown in G indicates interaction of all three CPKs with GORK. CIPK23-CBL1 was used as positive control for an interaction, CIPK23-CBL6 as negative control as shown before by Grefen and Blatt 2012 [68], fluorescence recordings are not shown here; n $\geq$ 10; mean ± SE; Experiments were performed together with Simon Heimann, Bachelor's Thesis at Departement of plant physiology and biophysics, University of Würzburg

According to the ratiometric analysis the weakest interaction could be detected between GORK and CPK6 (relative fluorescence 0.87 ± 0.05), while the interaction was strongest with CPK23 (relative fluorescence 1.30 ± 0.02). With a relative fluorescence of 1.12 ± 0.03 the strength of the detected interaction of GORK and CPK21 was in between the other two.

To fuel the hypothesis, that all tested CPKs interact with GORK *in vitro* phosphorylation assays were performed. As CPK3 is known to be involved in ABA signaling [132] it was also included in this screen. SLAC1 represents the major anion channel mediating S-type anion currents in guard cells and is involved in ABA-induced stomatal closure [25, 211]. Several studies report that CPKs directly phosphorylate and activate the SLAC1 S-type anion channel [25, 26, 62, 116, 175]. For that reason,

SLAC1 N-terminus was chosen as positive control in *in vitro* phosphorylation assay. All four tested CPKs were capable to phoshporylate the SLAC1 N-terminus as seen in figure 3.27 A. CPK3, CPK6 and CPK21 phosphorylate the N-terminus of GORK, as well, while CPK23 did not show detectable phosphorylation.

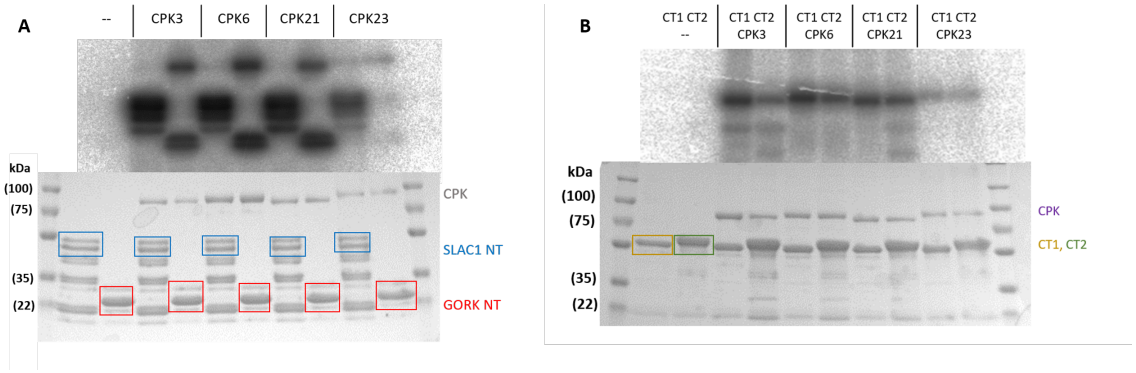

Figure 3.27: GORK N- and C-terminus are both phosphorylated by CPK3 and CPK21 while CPK6 only phosphorylates the N-terminus

(A) Autoradiography (upper half) and Coomassie-stained SDS gel of *in vitro* phosphorylation assays with recombinant, GST-tagged proteins SLAC1 NT (1-186), GORK NT (1-67), CPK3, CPK6, CPK21 and CPK23. Marker on the left and right edge; one representative experiment out of five.
(B) Autoradiography (upper half) and Coomassie-stained SDS gel of *in vitro* phosphorylation assays with recombinant, GST-tagged proteins; the GORK C-terminus is too big to be prepared correctly on the whole, so it is split into two overlapping parts: CT1 (301-559) and CT2 (531-820). Both C-terminual peptides were tested with CPK3, CPK6, CPK21 and CPK23. Marker on the left and right edge; one representative experiment out of three is shown.
The cytoplasmic N-terminal domain of GORK seems to be phosphorylated by CPK3, CPK6 and CPK21 but not by CPK23, while the cytoplasmic C-terminal domain of GORK is phosphorylated by CPK3 and probably by CPK21 but not by CPK6 and CPK23.
Experimtents were performed together with Dr. Sönke Scherzer (University of Würzburg).

The GORK C- and N-terminus are located in the cytoplasm. We thus attempted to test for the interaction of both channel domains with the CPKs. Unfortunately, the GORK C-terminal domain (58 kDa) appeared too big to be correctly expressed recombinantly and purified in total. The C-terminus was thus split into two overlapping peptides, comprising aminoacids 301-559 (CT1) and the aminoacids 531-820 (CT2; cp. also figure 3.13). As illustrated in figure 3.27 B the experiments revealed, that CPK3 clearly phosphorylated both parts of the C-terminus, while CPK6 did not.

CPK21 appeared to phosphorylate the CT2 peptide, that – according to PhosPhAt (https://phosphat.uni-hohenheim.de/) - possesses minimum 12 potential phosphorylation sites. This observation is in line with former findings, of CPK21 dependent phosphorylation of GORK at residue S649 [42, 213]. The second study by Kleeff

et al. also claimed GORK C-terminus residues T344 and S518 to be phosphorylated upon interaction with CPK21 which could not be detected in this work [213]. CPK23 showed only some autophosphorylation, but no detectable interaction with, or phosphorylation of the GORK C-terminus.

The certainty about GORK phosphorylation by CPK21 at certain residues at the C-terminus arises the question which distinct residues are involved in the detected phosphorylation with the GORK N-terminus and CPK21 (cp. figure 3.27 A). To shed some light on this issue, potential residues of the GORK N-terminus were mutated to alanine using selective mutagenesis USER cloning and this recombinant proteins were analyzed *in vitro* with respect to their interaction with CPK21 (cp. figure 3.15 and 2.2.1.1). The mutations S18A, S23A as well as T48A had no influence on the phosphorylation efficiency of GORK N-terminus by CPK21 when compared to the wildtype peptide as seen in figure 3.29 A (relative radiograph intensity compared to the wildtype: 1.06 for S18A, 1.08 for S23A, 1.07 for T48A; average of three independent experiments each).

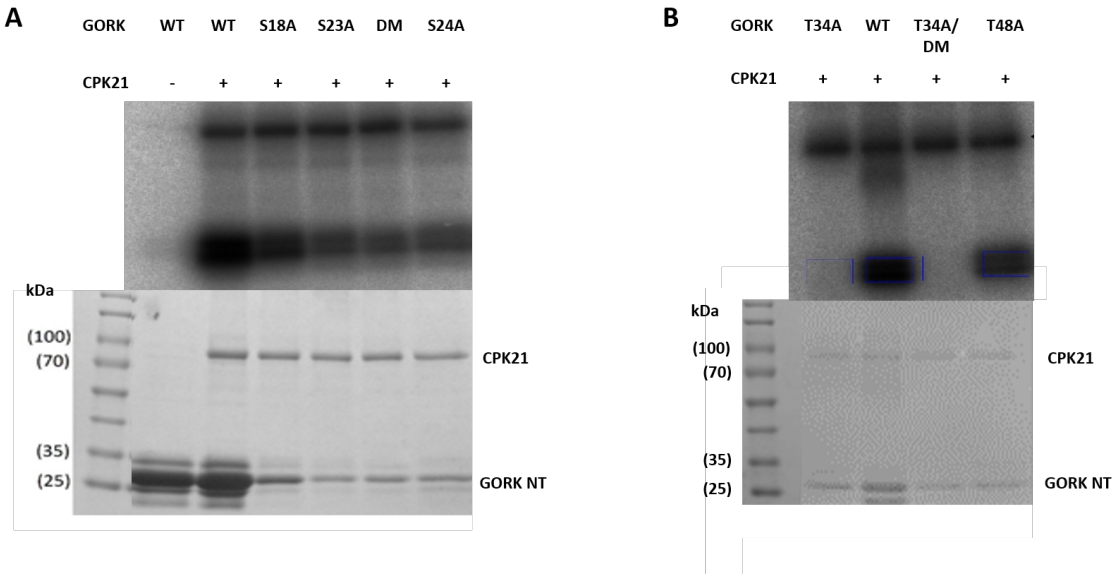

Figure 3.29: T34 and S24 of the cytoplasmic N-terminal domain of GORK are phosphorylated by CPK21

Autoradiography (upper half) and Coomassie-stained SDS gel of *in vitro* phosphorylation experiments of CPK21 and different variants of the GORK N-terminus (A: wildtype wt, mutant S18A, mutant S23A, double mutant DM S23A/S24A, mutant S24A) plus marker; B: mutant T34A, wildtype wt, combined mutant T34A/S23A/S24A, mutant T48A plus marker). S18, S23 and T48 seem to be not phosphorylated by CPK21, while T34 and probably S24 are.
Experiments were performed together with Fabian Link, Bachelor's Thesis at Departement of plant physiology and biophysics, University of Würzburg.

Phoshporylation efficiency of mutant peptides, that included S24A alone or in combination with adjacent S23A (DM in figure 3.29 A) was reduced by approximately 75% in comparison to the wildtype peptide (0.77 for S24A, 0.72 for DM; average of

three independent experiments each). A clear reduction of the radiograph intensity to 11% of the wildtype intensity can be seen for T34A (cp. figure 3.29 B). The mutant peptide containing T34A combined with S23A/S24A also led to a diminished phosphorylation signal and exhibited only 17% of the wildtype radiograph intensity (cp. figure 3.29 B).
Taken together this suggests, that CPK3, CPK6 and CPK21 - but not CPK23 - might be capable to phosphorylate the GORK N-terminus. Phosphorylation by CPK21 seems to occur at residue T34 and probably at S24, too. In addition, the C-terminus of GORK is likely to be phosphorylated by CPK3 and CPK21, but not by CPK6 and CPK23.

3.3.2.2 Add another level of complexity to the interaction: the probable menage-a-trois CPK/GRF/GORK

Apart from their interaction with plant ion channels, pulldown assays have identified CPKs as possible binding partners for so-called 14-3-3 proteins in *Arabidopsis thaliana* [31, 158]. The eukaryotic 14-3-3 family is a highly conserved protein family that regulates the activity of a wide range of target proteins through phosphorylation-dependent protein-protein interactions. The *Arabidopsis* genome encodes 15 different 14-3-3 proteins, of which 13 are trancriptionally expressed and are also called general regulating factors 1-13 (GRF1-13) or named with Greek letters (e.g. GRF2 = GRFω) [167]. They are expressed ubiquitously and the proteins containing forkhead-associated domains excepted, they are the only known phosphoprotein-binding proteins in plants [36, 199]. Some GRF isoforms may increase the activity of kinases upon direct binding [29]. Furthermore, 14-3-3 proteins have been shown to stimulate the autophosphorylation of CPK3 [97]. In addition, 14-3-3 proteins are known to affect both inward and outward rectifying K^+ channels [20, 192, 226]. Despite the absence of a bona fide canonical binding motif for 14-3-3s, GORK was shown in tandem affinity purification as well as in yeast two-hybrid studies to be a possible interaction partner of GRFs [54, 31]. Recently van Kleeff et al. identified 14-3-3 proteins as binding partners of recombinant GORK C-terminus in a phoyphorylation dependent manner [213] but they could not demonstrate a direct interaction. Consequently, they searched for a third involved factor and found it in CPK21 as it phosphorylates GORK C-terminus (cp. above and figure 3.27 B). Kleeff et al. could show that interaction of distinct 14-3-3 proteins and CPK21 stimulates its kinase activity and the same is true for CPK23, as well. Using *in planta* vibrating probe experiments, they also provided evidence for altered GORK activity by 14-3-3 during salt stress in *Arabidopsis* roots. [213]
This findings raise questions about the functional consequences of this menage-a-trois and its physiological impact on other (abiotic) stress signaling pathways including wounding-associated JA signaling.

BiFC experiments confirmed the interaction between CPK3, CPK6, CPK21, CPK23 and several GRFs, which is shown in figure 3.31 for selected examples. GRF1 (chi), GRF2 (omega), GRF4 (phi), GRF5 (upsilon), GRF6 (lambda) and GRF7 (nu) were

tested. All 14-3-3s showed positive BiFC signals in combination with one to four of the CPKs (data not shown).

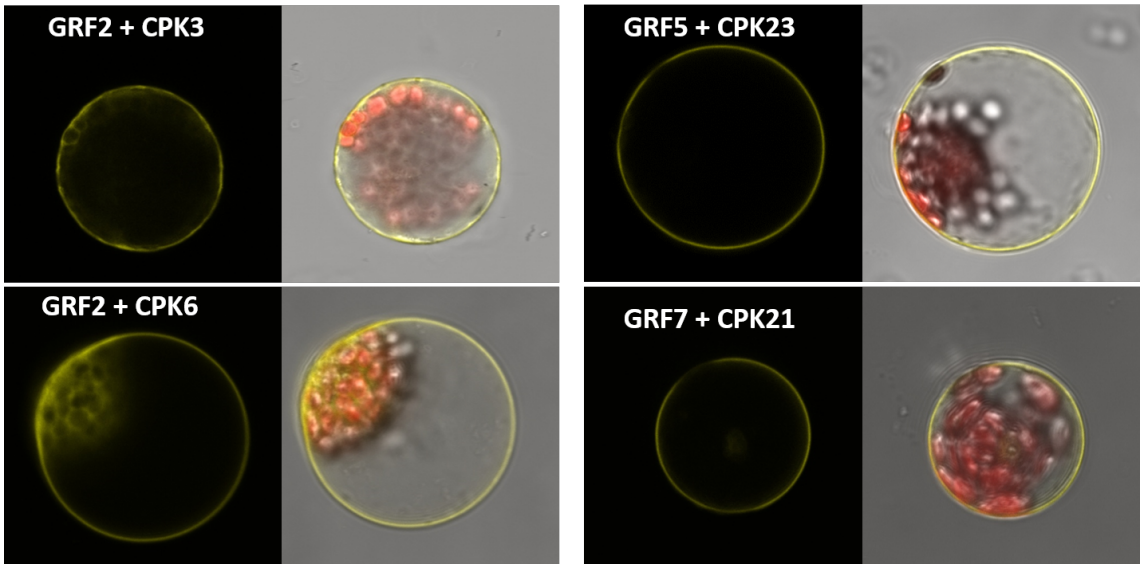

Figure 3.31: BiFC analysis shows interaction of CPKs with several GRFs

Bimolecular fluorescence complementation (BiFC) analysis in transiently transfected *Arabidopsis* mesophyll protoplasts of CPK-YFP^C combined with GRF-YFP^N. Interaction is indicated by yellow while the red color represents autofluorescence of chloroplasts. Representative images of at least three independent experiments are shown.

Among the 14-3-3s tested, GRF2 (omega) exhibited interaction with all four CPKs investigated. Next, rBiFC experiments were performed to probe for a possible impact of GRF2 on the interaction between CPKs and GORK (cp. figure 3.25). For this purpose, Agrobacteria carrying mTurqouise-tagged GRF2 were co-infiltrated with the rBiFC constructs and transiently expressed in tobacco epidermal cells. As depicted in figure 3.33 A, the known interaction of CPK6 or CPK21 with GORK (cp. figure 3.25) seemed to be enhanced in the presence of GRF2 as the relative fluorescence was increased by approximately 16% in both cases (1.00 ± 0.07 for CPK6, 1.31 ± 0.08 for CPK21). In contrast, CPK23-GORK interaction was unaffected in the presence of GRF2, as the mean relative fluorescence in the presence of GRF2 was just 4% less compared to control (without the GRF2).

As an example for the effect of a possible tertiary complex, the interaction of GRF2 and CPK21 with GORK was examined in DEVC experiments in *Xenopus* oocytes (cp. 2.4) and the results are shown in figure 3.33 B.
If only GORK was expressed, currents of 3.33 ± 0.64 µA could be measured at 70 mV. The coexpression of CPK21 resulted in comparable currents (3.16 ± 0.29 µA at 70 mV). In the case of expression of all three possible interaction partners (GRF2, CPK21 and GORK) together, the maximum currents rise by 74% to 5.55 ± 0.73 µA at 70 mV. This indicates that the additional presence of GRF2 in the oocytes promotes GORK activity probably by enhanced phosphorylation of GORK by CPK21.

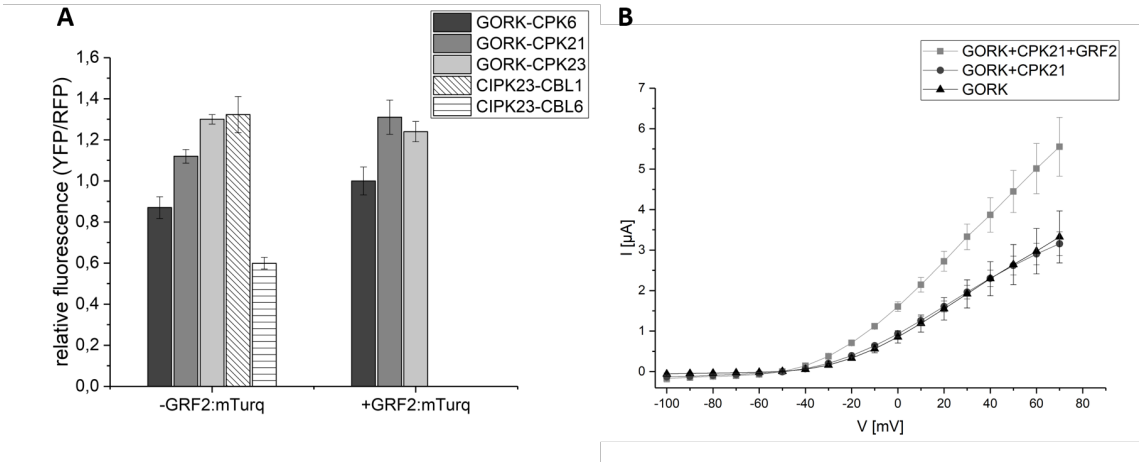

Figure 3.33: GRF2 enhances the interaction of GORK with CPK6 and CPK21 and thereby the channel's activity

(A) Ratiometric BiFC analysis shows GRF2-mediated, enhanced interaction of GORK with CPK6 and CPK21. Epidermal cells of *Nicotiana benthamiana* leaves had been infiltrated with agrobacteria carrying the respective rBiFC construct and in some cases coinfiltrated with mTurquoise-tagged GRF2 transformed agrobacteria. Images were taken at a confocal laser scanning microscope. RFP was excited at 561 nm and monitored in the range of 580 - 610 nm. YFP fluorescence was excited at 514 nm and monitored in the range of 525 - 589 nm. The pictures were analysed with ImageJ 1.48 and the relative fluorescence shown indicates that GRF2 intensifies the interaction of CPK6 and CPK21 with GORK, while it has no influence on the interaction of CPK23 with GORK. CIPK23-CBL1 was used as positive control for an interaction, CIPK23-CBL6 as negative control as shown before by Grefen and Blatt 2012 [68]; n $\geq$ 10; mean $\pm$ SE; Experiments were performed together with Simon Heimann, Bachelor's Thesis at Departement of plant physiology and biophysics, University of Würzburg.
(B) A tertiary complex of GORK, CPK21 and GRF2 enhances GORK activity in DEVC experiments. Current-voltage plot of DEVC experiments on *Xenopus* oocytes expressing only GORK (triangles) as a control, GORK and CPK21 (circles) or GORK, CPK21 and GRF2 (squares). From a holding potential of -40 mV a 1 s deactivation pulse at -80 mV followed by rectangular shaped, 3 s long pulses from -100 to 70 mV (in 10 mV steps), again 1 s at -80 mV were applied. The steady state currents shown are taken from the end of the rectangular shaped pulses. Coexpression of GORK, CPK21 and GRF2 results in considerably higher currents.
Averages from three or more independent experiments are shown. Data represent mean currents $\pm$ SE, n $\geq$ 7.

As physiological approach GRF double or quadruple loss-of-function mutants were screened in stoma assays upon their ABA- and MeJA-stimulus reaction (see figure 3.35). Unfortunately, no clear effect can be seen. All mutants react on ABA and MeJA with closing their stomata as the wildtype (Col-0). One double mutant as well as two quadruple mutants react less on MeJA ("un": upsilon = GRF5, nu = GRF7; "klpc": kappa = GRF8, lambda = GRF6, phi = GRF4, chi = GRF1 and "unpc": upsilon = GRF5, nu = GRF7, phi = GRF4, chi = GRF1 63-77% opening

compared to wildtype 56%). The other mutants closed their stomata to a higher extent (53-58% compared to control).

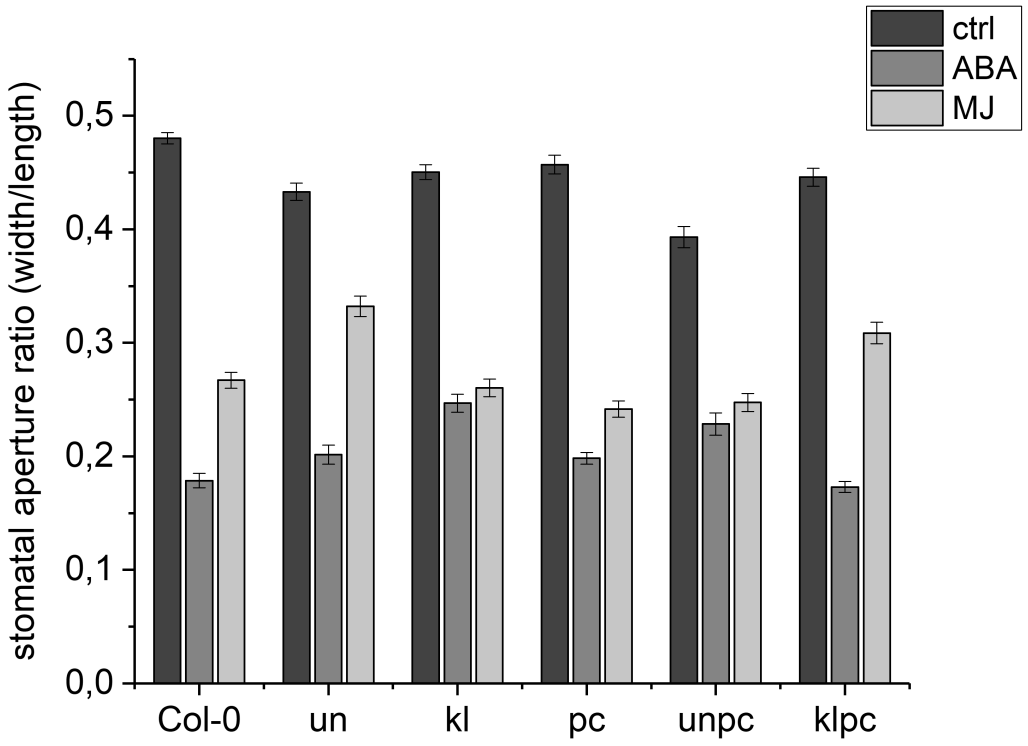

Figure 3.35: Guard cell responses of different 14-3-3 loss-of-function double and quadruple mutants towards ABA or MeJA

Stomatal aperture assays of wildtype Col-0, *grf1* = chi = c, *grf4* = phi = p, *grf5* = upsilon = u, *grf6* = lambda = l, *grf7* = nu = n and *grf8* = kappa = k. Guard cells of excised leaves were pre-opened in the light for 2 h, then incubated with either 20 µM (±)-ABA or 10 µM MeJA for 1 h. Wildtype leaves and all loss-of-function mutants respond to ABA and MeJA by closing their stomata. Apertures were determined as the ratio of stomatal width to length. Averages from three or more independent experiments are shown. Data represent mean ± S.E., n ≥ 120 stomata

Upon ABA treatment two mutants ("kl": kappa = GRF8, lambda = GRF6; "unpc" see above) close their stomata only to 55% respectively 59% compared to the measured controls. Col-0 (37%) and the other mutants (39-47%) show higher reaction to ABA. Taken together the presented results do not provide any real physiological proof for the involvement of GRF1 and GRFs4-8 in MeJA and ABA signaling in guard cells even though interaction between CPKs and 14-3-3 could be demonstrated.

3.4 The role of ROS in GORK-dependent JA signaling

It has previously been shown, that both ABA and MeJA evoke the production of reactive oxygen species (ROS) in guard cells. To investigate the effect of this ROS signal on the signal consequence upon JA or ABA stimulus, its impact on GORK channel activity was tested in DEVC experiments in *Xenopus* oocytes.

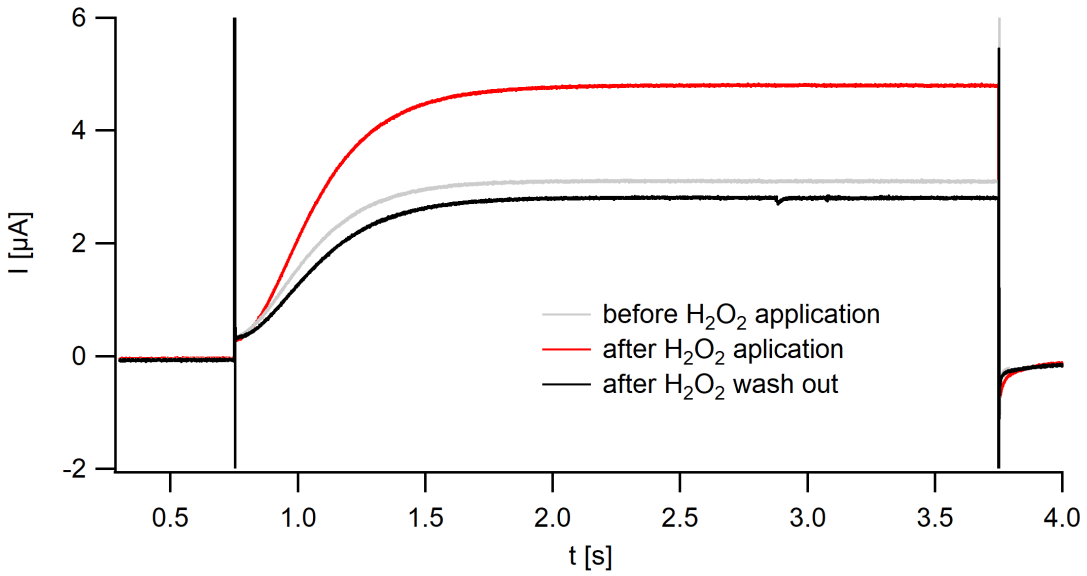

Figure 3.37: Reversible effect of H_2O_2 on GORK channel activity

DEVC experiment on *Xenopus* oocytes expressing GORK showing representative whole cell currents. From a deactivating, holding potential of -80 mV, 6 s 60 mV were applied. Application of 0.06% H_2O_2 results in 70% higher currents. This effect is nearly completely reversible by washing the H_2O_2 out again. Measurements were carried out in ND96 (cp. 2.4.2) with 10mM KCl added extra. Representative traces from one out of three independent experiments are shown.

Less than a minute after application of 0.06% H_2O_2 to the oocyte chamber, the measured currents upon the activation pulse at 60 mV raise by 70% from stable 2.81 µA before to 4.80 µA in the case of the experiment shown in figure 3.37. By washing out H_2O_2 the currents could be nearly completely reset to the level before application (3.10 µA in this example) after a few minutes. This reversible effect of ROS on GORK channel activity could be one piece in the explanation of ROS effects in JA and ABA induced stomatal closure.

Many possible sources of ROS production exist in plant cells, among them integral plasma membrane NAD(P)H oxidases ([197]). Suhita et al. as well as Kwak et al. found that gene disruption of two *Arabidopsis* NAD(P)H oxidases, *At*RbohD and *At*RbohF affected MeJA and ABA induced stomatal closure, respectively [96,

197]. These two NAD(P)H oxidases might therefore be major ROS sources in guard cell MeJA and ABA signaling and are involved in root elongation, too [96]. Alike AtRbohD/F, *At*RbohC is also known to play a fundamental role in root growth and root hair development [129, 200] and might be involved in guard cell signaling as well. As ROS production followed by cytoplasmic alkalization and Ca^{2+} signaling are essential in ABA and MeJA signaling, leading to GORK activation and stomatal closure RbohD, RbohF and RbohC were investgated in the course of this study in stoma assays, too (see figure 3.39).

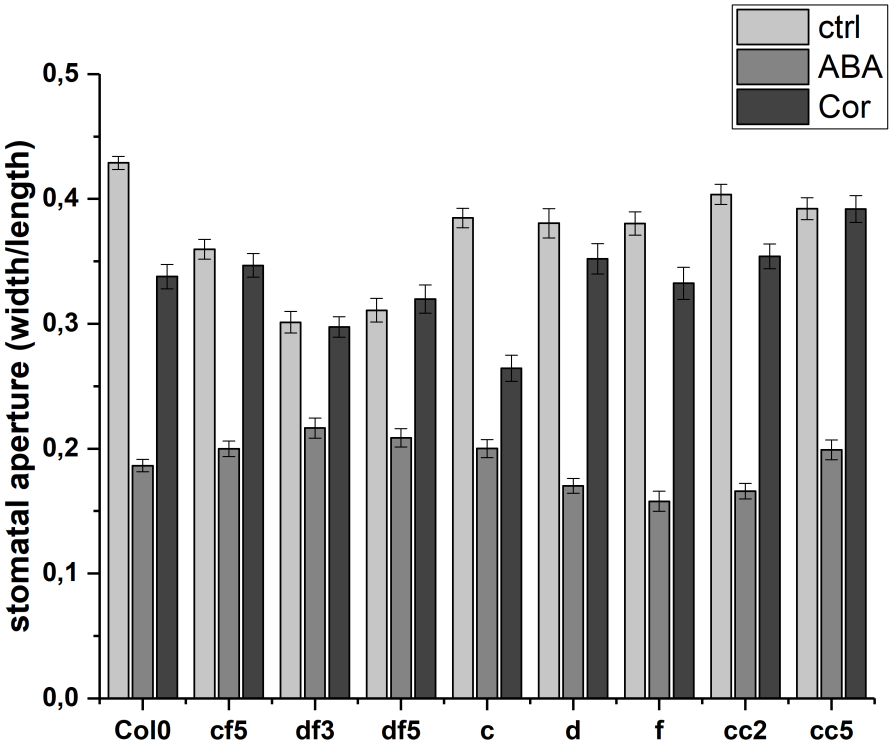

Figure 3.39: Guard cell responses of different Rboh loss-of-function mutants towards ABA or Cor

Stomatal aperture assays of wildtype Col-0, *rbohd* (d), *rbohf* (f), *rbohc* (c) and reconstituted RbohC (cc) mutants. Guard cells of excised leaves were pre-opened in the light for 2 h, then incubated with either 20 µM (±)-ABA or 10 µM Cor for 1 h. Wildtype leaves and all loss-of-function mutants respond to ABA by closing their stomata, while some of the mutants show impaired stomatal closure behaviour upon Cor treatment. Apertures were determined as the ratio of stomatal width to length. Averages from three or more independent experiments are shown. Data represent mean ± S.E., n ≥ 120 stomata

The corresponding wild type plants (Col-0) and all examined Rboh mutants close their stomata upon treatment with 20 µM (±)-ABA (Col-0 43% normalized to the control). To Cor even the wild type does not respond as strong as to ABA, but with closed stomata to only 79% compared to the control. The single mutant *rbohc*

(c) closes comparably by 69% , while the other single mutants (*rbohf*, f, and *rbohd*, d) show less closure with stomatal opening of 87% respectively 93% . Same is the case for the complemented lines of RbohC mutant (cc2 and cc5), which show 88% respectively 100% open stomata. Also for the double mutants (cf and df) no stomatal closure was detected (96-103% open stomata). This is in line with the above mentioned studies, that ROS and its production by Rbohs play an important role in JA signaling in stomata.

4 Discussion

4.1 GORK is essential for wound-induced JA signaling and the effected stomatal closure

Plants are constantly confronted with environmental signals, some of which cause stress. In response to stress signals plants activate defense reactions that are crucial for their survival. Phytohormones are mediators of plant stress and knowledge of the specific pathways in which these phytohormones interact could be used for example in improving crops. ABA and jasmonates are such phytohormones, both acting for example during drought stress in guard cells (see 1.4).
Wounding is another stress signal. It enhances JA levels in different *Arabidopsis* ecotypes and has also been implicated in promoting stomatal closure [174]. Stoma assays within this work could confirm that wounding induces stomatal closure (cp. figure 3.1 A) and monitoring the wounding-induced stomatal closure in the nearby of an injured guard cell underlines how fast this reaction is spread across the leave surface within minutes (cp. figure 3.1 B). Dr. Antoine Larrieu could show with the help of the genetically encoded JA-biosensor Jas9-VENUS ([100]) that in the manner of wounding, activation of the guard cell JA signaling cascade precedes stomatal closure and can therefore be considered to be a fast, phytohormone-based process (see figure 3.3).
During stomatal closure accumulated ions must be released from guard cells to the apoplast. On the one hand this happens via anion channels and on the other hand outward rectifying K^+ channels are activated in response to the depolarized membrane potential following anion efflux (compare figure 4.3). GORK has for a long time been considered the main K^+ efflux actor during stomatal closure and the basic transcript level of GORK is especially high in guard cells compared to other cell types [15, 82].

By whole-cell patch clamp experiments on guard cell protoplasts isolated from Col-0 wild-type (wt) or *gork1-2* (GABI_865F05) mutants under asymmetric potassium conditions with 100 mM K^+ in the pipette solution and 10 mM K^+ in the bath solution, it could be shown, that GORK K^+_{out} currents are a target of JA signaling in guard cells (see figure 3.5). This activation of GORK is already present 15 minutes following JA application, which supports the assumption of the presence of a fast JA-signaling chain in guard cells. The concurrently detected decrease in K^+_{in} currents (see figures 3.5 and 3.21) shows that JA is capable to promote a net loss of K^+ from the guard cell cytoplasm supporting stomatal closure. The changes in the

conductance for K^+_{out} and K^+_{in} of the guard cell plasma membrane are in line with the findings from Evans in 2003, who showed in patch clamp studies on *Vicia faba* guard cell protoplasts that JA-effects on K^+_{in} currents are slower to respond than K^+_{out} currents, but can be clearly detected 20 min after addition of the phytohormone [57]. The mechanism behind this effect still remains unclear. Single channel studies could be facilitated to determine the involved signal transduction processes.
Stomatal aperture assays on the *gork* mutants and their respective background wt plants strengthen the specific role of GORK activity in JA-induced stomatal closure (see figure 3.7). Mutation of GORK and in consequence, lack in GORK channel activity, point out, that GORK is essential for wound-induced, JA-controlled but not for ABA-induced stomatal closure (see figure 3.7 and 3.9). This uncovers that there have to be important differences in the contribution of GORK to different signaling processes in guard cells.

4.2 Calcium and calcium mediating proteins in stomatal signaling pathways control GORK activity

Fast control mechanisms via post-translational regulation are essential for rapid control of ion channel function during stomatal movement. One example for post-translational regulation is phosphorylation or dephosphorylation of a protein by kinases or phosphatases. There are several examples for this kind of regulation in the model plant *Arabidopsis thaliana* and they are often Ca^{2+} signal-dependent (e.g. [62],[111][124],[229]).
It is generally known that Ca^{2+} functions as an important second messenger in stress responses, including those following ABA or jasmonates ([3, 108, 117, 171]). Ca^{2+} signals are transmitted via sensors, for example CBLs or CPKs (cp. 1.3.1). For activity CBLs interact with the protein kinases CIPKs. By yeast two-hybrid experiments this interaction has been shown to be specific due to expression patterns, subcellular localization and the CBLs' affinity [90].
This work aimed at exploring the Ca^{2+}-dependent, post-translational regulation of GORK by both CIPKs, together with CBLs and CPKs, possibly in combination with GRFs, that act regulatory on phosphorylated targets. As possible counteractor the phosphatase ABI2 was examined and identified.

From yeast two-hybrid experiments with guard cell-expressed members of the CIPK family and the N-terminal (NT) domain of the GORK channel or the 10 CBLs from *Arabidopsis* respectively, only CIPK5 showed interaction with GORK-NT and with CBL1, CBL4, CBL5 and CBL9 (data not shown, [58]). These specific interactions were confirmed by BiFC assays (see figure 3.11). The same approaches (Y2H and BiFC) revealed, that the PP2C-type phosphatase ABI2 is interacting with GORK-NT and CIPK5, but not ABI1 (see figure 3.11 and [58]). Furthermore, biochemical and electrophysiological analysis support the antagonistic regulation of the GORK

N-terminus due to specific interaction with the sensor kinase complex CIPK5-CBL promotes channel activity on the one hand and the interaction with the phosphatase ABI2 on the other hand via a phosphorylation/dephosphorylation switch (see figures 3.13, 3.15 and 3.19). From the results of DEVC experiments it can be even concluded, that CBL protein presence and functional Ca^{2+} binding to CBL is necessary for the impact on GORK channel activity.
As CIPK5 posseses serin and threonine kinase activity, relevant site directed mutagenesis of the GORK NT followed by biochemical experiments point out, that threonines T34 and T48 of GORK are possibly phosphorylated by CIPK5 in this specific interaction (see figures 3.17 and 4.1). The impact of this regulation on JA-signaling in guard cells was investigated by patch-clamp recordings on guard cell protoplasts of *cipk5-2* kinase loss-of-function mutant, where coronatine failed to stimulate K^+_{out} activity compared to WT (see figures 3.5 B, C and 3.21 B,C).
Stoma assays on *Arabidopsis thaliana* loss-of-function mutants of either CBL1/9 or CIPK5 show that these mutations lead to completely abolished JA response in guard cells (see figure 3.23). Stomata of the *abi2-1* mutant, leading to reduced ABA sensitivity, did not close upon JA stimulus, while *abi1-1* mutant stomata reacted WT-like (see figure 3.23).

Altogether, these results establish a unique and essential role for CBL1-CIPK5 complexes in fast JA signal transduction in guard cells via their activating role for the GORK K^+ efflux channel by phosphorylation. ABI2 represents another component of JA-induced signaling processes and antagonizes CIPK5 action (cp. figure 4.3). It acts as a negative regulator of GORK K^+_{out} activity in guard cells and thus seems to have a different role in this context, than in ABA signaling (see also 1.4.3). Additionally, direct inhibition of CIPK5 by ABI2 can not be excluded, similar to the reported dual inhibition of a CIPK and its substrate for SLAC1 by Maierhofer et al. in 2014 [116].

Another fundamental family of protein kinases in plants are the CPKs (see also 1.3.1), which are known to be involved in diverse cellular processes, including stomatal movement [34, 132]. They have been shown to play an important role in *Arabidopsis*' responses to drought and salt stresses, with implications for their regulatory effect on K^+ uptake [111, 124, 229]. The CPKs chosen for this study (3, 6, 21, 23) are all present in guard cells of *Arabidopsis thaliana* and have been studied before in different signaling pathways, as well as in combination with GORK (see 1.3.1). The performed rBiFC analysis shows interaction of all used CPKs with GORK (see figure 3.25). The performed *in vitro* phosphorylation assays reveal a more diverse picture of the different interactions in the manner, that the cytoplasmic N-terminal domain of GORK is phosphorylated by CPK3, CPK6 and CPK21 but not by CPK23, while the cytoplasmic C-terminal domain of GORK is phosphorylated by CPK3 and probably by CPK21 but not by CPK6 and CPK23 (see figure 3.27). CPK21 has been claimed before to phosphorylate the GORK C-terminus at S649, T344 and S518 [42, 213]. For S649, which lies on the second half of the C-terminus this can be confirmed according to this study's results, but not for T344 and S518, which would be part of the first half, where no reaction can be seen in figure 3.27 B. Further

investigation of CPK21's interaction with the N-terminus lead to the predication that T34 and S24 are phosphorylation targets in this case (see figures 3.29 and 4.1).

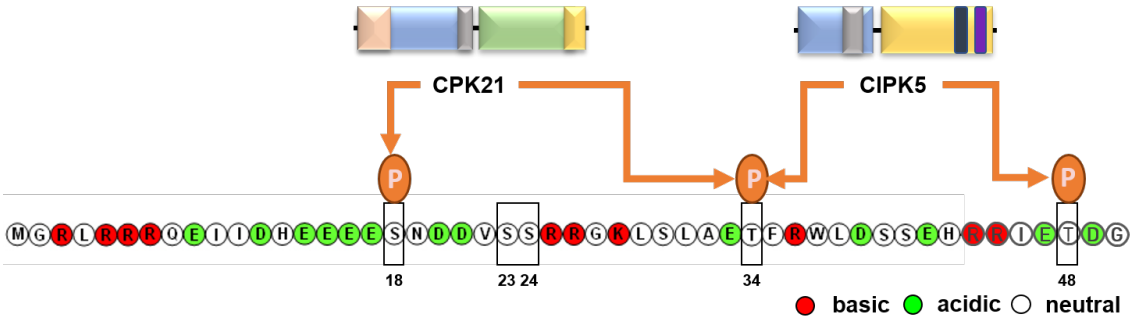

Figure 4.1: Identified phosphorylation sites in the GORK N-terminus

Biochemical experiments on site directed mutagenesis GORK N-terminus variants together with CIPK5 respectively CPK21 protein revealed that threonines T34 and T48 of GORK are possibly phosphorylated by CIPK5 in this specific interaction, while T34 and S24 seem to be phosphorylation targets of CPK21's interaction with the GORK N-terminus.

CPKs have been related to distinct 14-3-3 proteins [29, 97, 199] and there are several hints leading to the assumption of a tertiary complex of CPK, 14-3-3 and GORK (see 1.3.2 and 3.3.2). This adds another level of complexity to the kinase-channel interaction and as GRF2 (omega) had shown potential interaction with all investigated CPKs before (see figure 3.31), its impact in this possible menage-a-trois was investigated further within the course of this study. In rBiFC analysis including mTurqouise-tagged GRF2, enhanced interaction of GORK with CPK6 and CPK21 could be detected (see figure 3.33 A). This could be confirmed by DEVC experiments on *Xenopus* oocytes expressing GORK, CPK21 and GRF2 altogether, which resulted in up to 74% enhanced channel activity (see figure 3.33 B). In the performed stomatal aperture assays no real proof for any physiological effect of the investigated GRF loss-of-function mutants could be detected (see figure 3.35). This might be due to high redundancy in the 14-3-3 proteins' functionality in the way that other isoforms jump in to ensure plant metabolism and functions.
Assuming an effect of at least GRF2 on GORK brings up the question of the site for this interaction as GORK shows none of the three canonical 14-3-3-binding motifs (see 1.3.2). In some CPKs canonical binding motifs are found, the others have at least regions similar to a known binding motif [199]. It might also be, that GRFs get phosphorylated by CPKs upon Ca^{2+} signal perception as reported e.g. for CPK1 or CPK3, which are capable of phosphorylating distinct GRF serines *in vitro* [29, 97, 199]. The menage-a-trois would get an important physiological meaning with its possible role of enhancing or prolonging the activation of GORK, in order to ensure proper response of the guard cells. This is possibly achieved by phosphorylation of the GRFs by CPKs, as reported before [97, 199], which might result in stabilisation of the kinase-channel-complex.
Further investigation, e.g. by directed mutagenesis experiments, is needed to find the real interaction sites, but beforehand the characteristics of the interactions should

be examined to a greater extent.

4.3 Proposed model of JA regulated stomatal closure and crosstalk with the ABA signaling pathway

Based on the data brought together in this thesis and considering the available published evidence, a model of JA function in comparison to and in liaison with ABA function in guard cell closure can be deduced, that is shown in figure 4.3.
Upon binding to their respective receptors, COI1 and the RCAR/PYR/PYLs, both JA and ABA increase cytosolic Ca^{2+} and ROS levels. JA triggers Ca^{2+} signatures [108, 135] and activates ABA signaling, which is in line with the requirement of functional ABA receptors for JA-induced stomatal closure reactions (data not shown here; [234]). Activated ABA receptors recruit the PP2Cs ABI1 and ABI2 to the receptor complex, thereby inactivating phosphatase activity. This enables phosphorylation and release of kinases such as OST1 (Ca^{2+}-independent), or CIPKs and CPKs (Ca^{2+}-dependent). Importantly, ABI2 no longer inhibits the GORK channel and is also involved in JA signaling, while ABI1 is not.
OST1 dependent inhibition of KAT1 and Ca^{2+}-dependent kinase activation of anion channels (SLAC1/SLAH/QUAC) leads to membrane potential depolarization, and in turn, activation of the voltage dependent GORK channel. In parallel, kinase dependent GORK activation further promotes potassium efflux and finally this provokes stomatal closure.
JA affects ABA signaling leading Ca^{2+}-dependent phosphorylation and in turn activation of GORK. While this work shows that GORK is essential in JA signaling, it is known that for ABA also members of the K^+ uptake transporter (KUP) family can contribute to the K^+ efflux [154]. This is most likely not the case for JA signaling due to the clear effects seen in the patch clamp experiments on *gork1-2* guard cell protoplasts (cp. figure 3.5). ABI1 and ABI2 both have different functions in ABA and JA signaling pathways, too, as seen in the course of this study.
ABA/JA-stimulated ROS production via respiratory burst oxidase homologs (RBOHs) further stimulates GORK activity while at the same time inhibiting ABI2 activity [125]. The stoma assays on RBOH mutants (see figure 3.39) are in line with former studies proposing that ROS and its production by RBOHs plays an important role in JA signaling in stomata [96, 197, 234]. The reversible effect of ROS on GORK channel activity in DEVC experiments (see figure 3.37) could be one piece in the explanation of ROS effects in JA and ABA induced stomatal closure.
In this manner, convergence of hormone and Ca^{2+}-mediated activation allows for efficient fine-tuning of GORK-mediated K^+ efflux for tightly regulating stomatal aperture adjustment and provides the mechanistic flexibility to facilitate stomatal closure in response to diverse stimuli such as drought and light stress [49] or wounding.

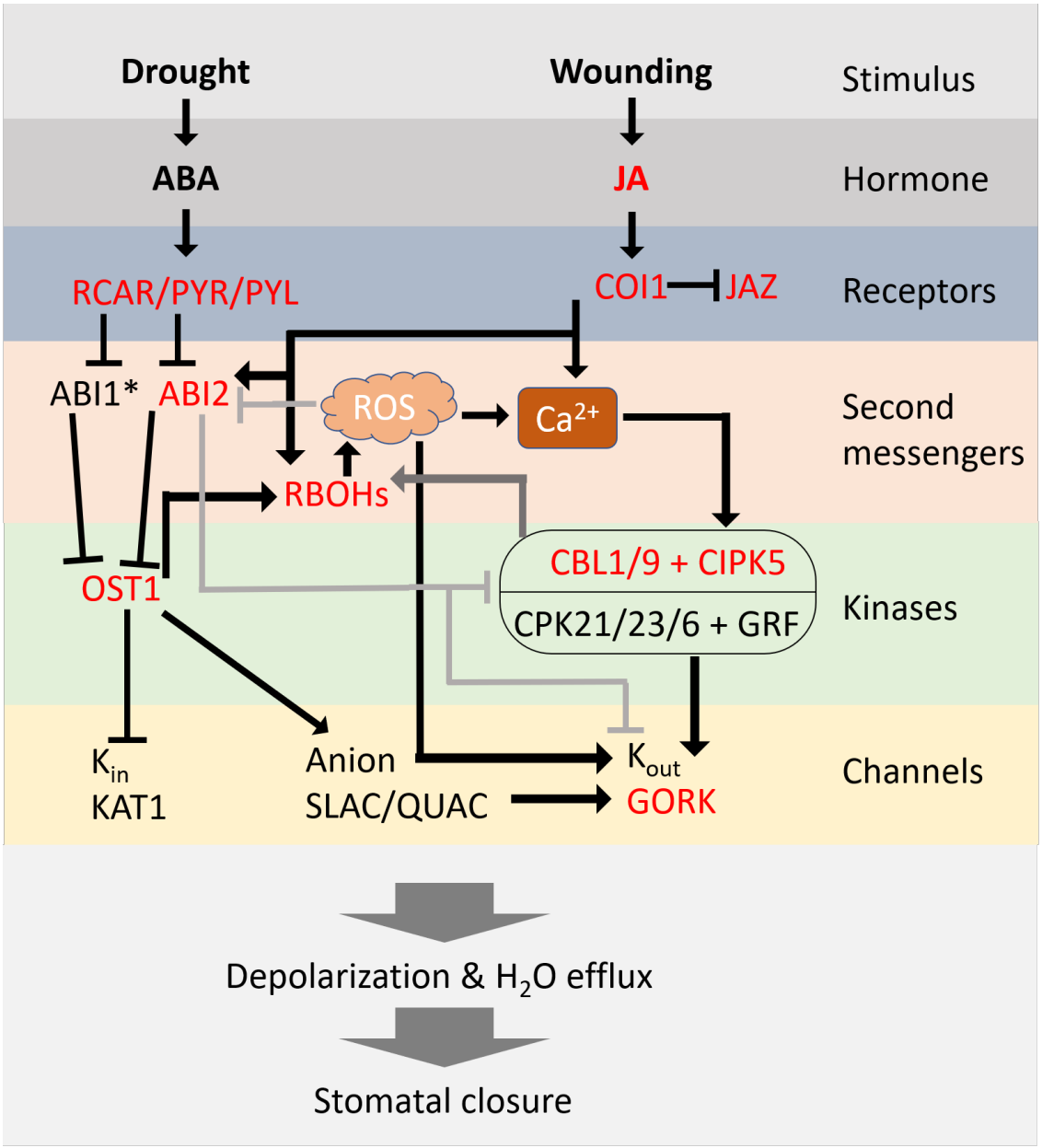

Figure 4.3: Working model of the convergence of JA and ABA-mediated stomatal closure

Hormone binding to their respective receptors causes the release of second messengers as ROS and Ca^{2+} and activation of kinases, both Ca^{2+}-dependent and Ca^{2+}-independent ones. This leads to inhibition of KAT1 and activation of anion channels. The effected membrane depolarization activates GORK and causes K^+ efflux, water efflux and, in turn, stomatal closure.
Red elements are essential in JA signaling. Grey arrows are only for better distinction of single arrows.

4.4 Outlook

Phytohormone signaling leading to stomatal closure as a response to biotic and abiotic stress still remains a secret in many ways. Even though a lot of successful steps have been made amongst others in this study, many insights into stomatal physiology on molecular level are still open. it has been seen that there are important differences in the diverse siganling processes in guard cells, that have to be clarified in future studies.

This study underlines the finding that tightly regulated and reversible phosphorylation reactions catalyzed by protein kinases and phosphatases are important in signal transduction pathways. The identified interaction of CIPK5 with GORK as well as the possible menage-a-trois of GORK, GRF and CPK should be investigated in further detail to clarify its underlying mechanisms and consequences. Phosphorylation sites in the C-terminus claimed before [42, 213] can be confirmed using site directed mutagenesis of possible phosphorylation sites analogue to the experiments carried out with the N-terminus during this study (compare 3.3.1and 3.3.2). This could possibly be used to find the real interaction site for GRF and CPK or GORK, too. Using a different vector system (for example pGEX6) the recombinantly expressed, untagged proteins of GORK termini could be analyzed via mass-spectrometry after *in vitro* phosphorylation. This could confirm and strengthen the results of the radioactive phosphorylation assays.

As GORK is being phosphorylated by some endogenous kinase in *Xenopus* oocytes, the DEVC data shown in this study can not dissolve the effects of the examined kinases on the channel directly. For this purpose, patch clamp experiments on mammalian cells could help out. During the course of this study preliminary data with both COS and CHO cells was achieved (data not shown here). Both cell lines seem to be appropriate for the purpose of measuring GORK responses to phosphorylation events. Nevertheless the expression of GORK leads to unstable cell membranes and high lethality of the cell strains. An (light) inducible, stable GORK expression line could maybe be the key to success in this case.
When stable GORK patch clamp measurements are achieved in a mammalian cell line, this approach could also be used to analyse the impact of the identified phosphorylation sites' (see above) influence on channel activity and kinetics. Coexpression of GRF and CPK or CIPK5 in the cell line would be helpful for that.
It could also be interesting to conduct patch clamp experiments on guard cell protoplasts of other *Arabidopsis* mutants, that lack or are impaired in other parts of the proposed JA signaling pathway (see figure 4.3).

Additionally, stomata have the function to keep the balance between water loss via transpiration and CO_2 uptake for carbon fixation. The role of CO_2 in the context of JA signaling could be analyzed in gas exchange experiments with the *Arabidopsis* mutants used in the stoma assays in this study. Preliminary data and first trials were already conducted in the end of the course of this study but no presentable data nor finding could be gained, yet.

List of Figures

List of Tables

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List of own publications

Förster, S., Schmidt, L. K., Kopic, E., Anschütz, U., Huang, S., Schlücking, K., ... and Becker, D. (2019). Wounding-induced stomatal closure requires jasmonate-mediated activation of GORK K^+ channels by a Ca^{2+} sensor-kinase CBL1-CIPK5 complex. Developmental cell, 48(1), 87-99.

Acknowledgement

This PhD thesis has been carried out at the Department of Molecular Plant Physiology and Biophysics at the University of Würzburg. A number of people deserve thanks for their support and help.

Special thanks belongs to Prof. Dr. Dirk Becker for giving me the possibility to work on this interesting topic, for his big support, for helpful suggestions on how to develop the topic, for letting me work independently and all the good discussions on an equal footing. The other (former) members of the Becker-group deserve a huge thanks for all the help,support, encouragement, ideas and friendship during the different steps of my "Becker-group-career". You did a great job and enabled me to do as good as I could!

Prof. Dr. Rainer Hedrich for allowing me to work in his group at the Julius-von-Sachs Institute at the University of Würzburg.

I would like to thank Prof. Dr. Irene Marten, Dr. Justyna Jaslan and Dr. Dawid Jaslan for all the support regarding patch clamp measurements, setup or software problems and really helpful discussions; Prof. Dr. Thomas Müller for the discussions on biochemistry; Dr. Sönke Scherzer for helping me with kinase assays, Dr. Shouguang Huang for providing the video of monitored wounding assays and all the other colleagues, students and collaborators, who contributed to this work.

I also owe Dr. Christoph Weiste gratitude for his willingness to be the second referee of this thesis and interest in my work.

Moreover, I am thankful to all gardeners, technicians and workmen, as well as the entire group at the institute for a very good working atmosphere, good discussions, a lot of help and advices.

Last but not least, from the bottom of my heart I want to thank my husband, my whole family and all the friends who always loved and patiently supported me along this (long) way.

Eidesstattliche Erklärungen nach §4 Abs. 3 Satz 3, 3a, 3b, 5 der Promotionsordnung der Fakultät für Biologie

Hiermit erkläre ich an Eides statt, die Dissertation: „Die physiologische Rolle der posttranslationalen Regulation des auswärtsgleichrichtenden Kaliumkanals GORK in *Arabidopsis*-Schließzellen“, eigenständig, d. h. insbesondere selbständig und ohne Hilfe einer kommerziellen Promotionsberatung, angefertigt und keine anderen, als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.

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