A moonlighting kinesin modulates abiotic sensing

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Content

A. List of Abbreviations

1-but	1-butanol
2-4 D	2,4-Dichlorophenoxyacetic acid
2-but	2-butanol
AlF^{4-}	Aluminium tetrafluorid
ATT	Anti α tubulin
BY2	Tobacco Bright Yellow 2
CH-domain	Calponin homology domain
CPMCW-continuum	Cytoskeleton-plasma membrane-cell wall continuum
DMSO	Dimethyl sulfoxid
freeGFP	Free green fluorescent protein
G protein	GTP binding protein
GPCR	GTP binding protein coupled receptor
IEM	Electromanipulation
KCH	Kinesin with calponin homology
MI	Mitodic index
MS medium	Murashige-Skoog medium
NP	Nuclear positioning/ Nuclear position
nsPEF	Nano second pulsed electric fields
ntKCH	*Nicotiana tabacum* Kinesin with caplonin homology
osKCH	*Oryca sativa* Kinesin with calponin homology
PA	Phosphatidic acid
PI	Pusling index
PLC	Phospholipase C
PLD	Phospholipase D
PPB	Preprophase band
PTX	Pertussis toxin
ROS	Reactiv oxygen species
TRITC	tetramethylrhodamine isothiocyanate
WT	Wild type

B. List of Tables and Figures

C. Abstract

Due to the indeterminate morphogenesis of plant, patterns are usually formed in an iterative manner, whereby cells or organs are continuously added to the emerging pattern. This requires some kind of integration over the entire pattern that will determine the morphogenetic response of the individual element in the pattern (either a cell or an organ). Mechanic tensegrity provides a mechanism capable for such integration. Proteins that connect the mechanically rigid microtubules with the flexible actin filaments, are key players for tensegrity.

Minus end directed superfamily 14 member kinesins with a calponin-homology domain (KCH) do not only serve as motor proteins, they have the capability to cross-link microtubules and actin filaments. As cytoskeletal cross-linkers, KCHs are suggested to have an important function in processes of plant biological pattern formation. Previous studies had showed that KCH do have two different functions at two different locations, the perinuclear cage and below the plasma membrane (KLOTZ & NICK, 2012). However, the function of KCH at the plasma membrane has remained unknown. To get insight into this elusive role of KCH at the plasma membrane, two different strategies were pursued in the course of the current dissertation:

1. Targeted physical manipulation at the plasma membrane by means of nanosecond pulsed electrical fields (nsPEFs).
2. Genetic and pharmacological manipulation of phospholipase D as membrane-associated signaling hub that is interconnected with the cytoskeleton.

The targeted physical manipulation at the plasma membrane was doene by a specific kind of ultrashort electroporation: nanosecond pulsed electric fields (nsPEFs). Dependent on the surface and interior structure of the cell, nsPEF can be specifically applied to manipulate different membranes in a cell. In animal cells, nsPEFs act directly in the cell center around the nucleus. The explicit target of the electrical pulses in plant cell is still discussed. One hypothesis is, that plant

vacuoles serves as condenser protecting the perinuclear region. Due to this, in plant cells, nsPEFs are suggested to be targeted at the plasma membrane.

Mitotic analyses of pulsed BY2 cells overexpressing KCH showed a synergistic effect of both factors on mitosis. These experiments suggest a site of action in the cell interior. However, it should be kept in mind that the nucleus is tethered, through a radial network of actin filaments and microtubules to the plasma membrane. Therefore one can make use of a second cellular function of KCH that is unequivocally linked to the cell periphery: cell expansion which is controlled by a directional extensibility of the cell wall that, in turn, is regulated by the orientation of cortical microtubules binding a specific subset of KCH subtending the plasma membrane. These experiments showed a stimulated cell expansion of pulsed cells in the KCH overexpressor but not in the wild type. These results support the hypothesis of a site of action at the plasma membrane.

Furthermore KCH is suggested to play a role in changing microtubules from transverse to longitudinal direction to stop the process of cell elongation. Other important forces for reorientation of cortical microtubule from transverse into longitudinal direction are abiotic stimuli like physical and thermal stresses, or salinity (NICK, 2008). The term ‘mechanic tensegrity’ describs the characteristics, that the cytoskeleton is combining rigid microtubules and tense actin filaments providing the ability of the installation of a sensitive system for signalling to abiotic stimuli. Due to KCHs ability of cross-linking actin filaments it is therefore suggested to be part of a cytoskeleton-plasma membrane-cell wall continuum. This cytoskeleton-plasma membrane-cell wall continuum also serves as signalling hub. For clarifying the putative role of KCH in this signalling hub, it is necessary to bring another participant of this continuum into play: phospholipase D the mayor component of PLD signalling pathway. PLD signaling pathway manipulating pharmaceuticals (1-butanol (1-but), 2-butanol (2-but), Aluminium tetrafluorid (AlF^{4-}), and Pertussis toxin (PTX)) are used to study the role of all players in this cytoskeleton-plasma membrane-cell wall continuum. Again elongation studies of KCH overexpression cell lines underlined the involvement of KCH in signaling. Moreover the expansion of the cells under pharmaceutical treatment together with

low concentrations of the microtubule drug Oryzalin suggests an important involvement in plant cell signaling.

Microscopical studies showed that BY2 cells loose their directionality due to PLD overexpression that is caused by a heavy loss of cortical microtubule organization, leading to an inability to conduct cell expansion. Furthermore no explicit co-localisation of PLD and microtubules was found.

As an conclusion this dissertation provides an model for KCH being part of the cytoskeleton-plasma membrane-cell wall continuum, that serves as signaling hub. Taking all results together, this work is providing evidence for a moonlighting function of kinesins in plant sensing to abiotic stressors.

D. Zusammenfassung

Aufgrund der statischen Morphologie von Pflanzen werden die gängigen Muster ihrer Physiologie iterativ geformt. Bei dieser Musterbildungsart werden Schritt für Schritt Zellen oder Organe an das bestehende Muster hinzugefügt. Dies erfordert eine bestimmte Art von Einbindungsprozess, sowie ein Überblick auf das gesamte Muster um die morphogenetische Konfiguration eines neuen Elements zu bestimmen. Das Prinzip der mechanischen Tensegrität des Zytoskeletts kann diese Anforderungen erfüllen. Bestimmte Proteine sind in der Lage die beiden Bestandteile des pflanzlichen Zytoskellets, Mikrotubuli und Aktin Filamente mechanisch zu vernetzen. Deshalb werden sie als Schlüsselspieler der Tensegrität gehandelt.

Dem Minusende von Mikrotubulli zulaufende Kinesine mit einer calponin-homologen Domäne (KCH), aus der Kinesin Superfamilie 14, dienen nicht nur als Motorproteine, sie sind ebenso in der Lage Mikrotubuli und Aktin Filamente zu vernetzen. Mit dieser Eigenschaft steht KCH im Verdacht ein wichtiger Faktor in biologischen Prozessen der pflanzlichen Musterbildung zu sein. Vorangegangene Studien zeigten, dass KCHs verschiedene Funktionen an unterschiedlichen Lokationen der Zelle besitzen (KLOTZ & NICK, 2012). Dennoch ist die Funktion von KCH an der Zellmembran bisher noch ungewiss. Um in die Rolle KCHs an der Zellmembran hinein schauen zu können wurden in dieser Dissertation folgende zwei Strategien verfolgt:

1. gezielte physikalische Manipulation der Zellmembran durch spezifische, elektrische Pulse.
2. Genetische und pharmakologische Manipulation von Phospholipase D als membran-assoziierte Signalschaltstelle externer Stimuli, die mit den Bestandteilen des Zytoskellets verbunden ist.

Die zielgerichtete physikalische Manipulation an der Zellmembran wurde durch spezifische, elektrische Pulse im Nanosekundenbereich (nsPEFs) hervorgerufen. In Abhängigkeit der Oberfläche und auch der inneren Struktur der Zelle können

nsPEFs spezifisch dazu verwendet werden, unterschiedliche Membranen zu beeinflussen. In tierischen Zellen wirken nsPEFs direkt im Zellzentrum um den Nukleus herum. Das explizite Ziel der Pulse in pflanzlichen Zellen wird noch diskutiert. Eine mögliche Hypothese ist, dass pflanzliche Vakuolen den elektrischen Puls kondensieren und so die perinukleare Region der Zelle schützen. Aufgrund dessen wird in pflanzlichen Zellen das Ziel eher unterhalb der Zellmembran vermutet.

In dieser Arbeit zeigen mitotische Analysetechniken von gepulsten BY2-Tabakzellen, die ein KCH Gen überexprimieren, einen synergetischen Effekt beider Faktoren, was auf eine Interaktion von nsPEFs tief im Zellinneren vermuten lässt. Dennoch darf nicht vergessen werden, dass der Nukleus in einem radialen Netzwerk über die Bestandteile des Zytoskellets, Mikrotubuli und Aktin Filamente mit der Zellmembran verbunden ist. Um dies zu klären kann man eine zweite Funktion von KCH, die mit der Zellperipherie verlinkt ist zur Hand nehmen: die Beeinflussung der Zellexpansion durch eine spezifische, hoch dynamische Untereinheit des KCHs, die an kortikalen Mikrotubuli lokalisiert ist. Die Zellexpansion wird durch die Orientierung dieser kortikalen Mikrotubuli und die dadurch direktional ausgerichtete ständige Erweiterung der Zellwand reguliert. Die Experimente zeigten eine stimulierte Zellexpansion bei gepulsten Zellen, die KCH überexprimieren. Dies unterstützt die Hypothese, dass nsPEFs eher an der Zellmembran wirken.

Von KCH wird vermutet, dass sie eine Rolle in dem Orientierungswechsel der kortikalen Miktrotubuli, die die Expansionsphase determinieren, spielen. Andere wichtige Kräfte die diesen Prozess beeinflussen sind abiotische Stimuli wie physikalische oder thermische Kräfte, oder auch Versalzung (NICK, 2008). Um auf das Prinzip der mechanische Tensegrität, das innerhalb des Zytoskeletts rigide Mikrotubuli und flexible Aktin Filamente kombiniert zurückzukommen, bietet es ebenso ideale Vorraussetzungen für eine Installation zur Erkennung abiotischer Signale. Aufgrund der Möglichkeit von KCH Mikrotubuli und Aktin Filamente miteinander zu verbinden, wird vermutet, dass sie ein Teil eines Zytoskeletts-Zellmembran-Zellwand Kontinuum (CPMCW-Kontinuum) zu sein. Um die Rolle von KCH in diesem CPMCW-Kontinuum aufzuklären wurde ein weiter Bestandteil

dessen, ins Spiel gebracht, Phosphilipase D, der Hauptbestandteil des PLD Signalweges. Der PLD Signalweg kann durch Anwendung verschiedener Stoffe (1-Butanol, 2-Butanol, Aluminiumtetrafluorid und Pertussis Toxin) manipuliert werden. Wieder ist die Zellexpansion der wichtige Schlüssel für die Investigation der Einflussnahme von KCH im Bereich der Signaltransduktion. Mit der Zuhilfenahme des Mikrotubuliinhibitors Oryzalin wurde die Beteiligung von KCH an Signalwegen deutlich unterstrichen.

Mikroskopische Studien von PLD überexprimierenten BY2-Tabakzellen haben einen heftigen Verlust der Direktionalität der Zellen gezeigt. Dieser Verlust hat die Unmöglichkeit der Zellexpansion zufolge. Weiterhin wurde durch folgende Immunofluoreszenzfärbung keine explizite Kolokalisation von PLD mit Mikrotubuli gefunden.

Als Schlussfolgerung dieser Dissertation wird ein Model aufgestellt, das KCH als Bestandteil des CPMCW-Kontinuums zeigt. Somit zeichnet diese Arbeit Hinweiße für eine versteckte Funktion von pflanzlichen Kinesinen auf: der Signalverarbeitung abtiotischer Stimuli.

1. Introduction

Structure and behaviour of natural organisms often follow complicated patterns. Since many centuries, scientists have tried to understand the natural laws governing these patterns, in particular, how they are constructed and perpetuated. The Italian mathematician Leonardo Fibonacci (1170 ad – 1250 ad) is famous for introducing the 'Fibonacci sequence' to Europe, described for the first time in early Indian mathematics. By definition, the first numbers of this sequence are 0 and 1. The subsequent numbers are the sum of the previous two (0,1,1,2,3,5,8,13,21,34,55,89,144 aso.). The German mathematician and astrologer Johannes Kepler (1571 ad – 1630 ad) found out, that the division of two adjacent numbers (with increasing Fibonacci sequence) approximates Phi (1.6180339887498948482045868 34...). This number Phi equals also the division of the 'golden ratio'. Due to this, Fibonacci sequence is highly related to the 'golden ratio'. Both mathematical phenomena do actually appear in several biological structures like patterns of snail shells, starfishs, plenty of morphological ratios in animals and humans, but also many constructions in plants like branching of trees, leaf and flower anatomy, or the order of seeds in flowers and fruits follow these mathematical laws.

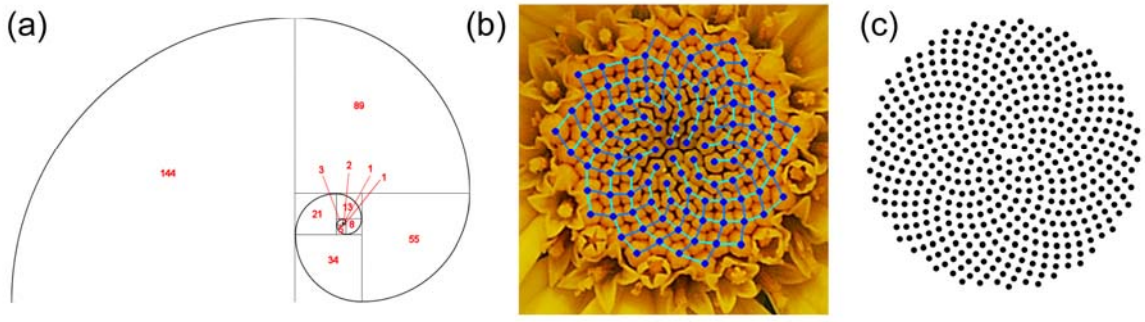

Fig.1: Figural illustration of the 'Fibonacci sequence' in form of a snail shell (a) and sun flower seed anatomy (b and c)

1.1. Why plants need tensegrial sensors?

In animals, specific signals like hormones elicit patterning of the cells after their development (e.g. embryonic development of *Drosophila melanogaster*). In contrast to that, plants contribute to an open pattern formation, where patterns are continuously completed with new elements like cells or organs. This kind of pattern formation is called 'iterative patterning' (NICK, 2006). It undergoes indeterminated morphogenesis, the addition of an element guided by the pre-existent element. This dodgy kind of pattern formation confronts plants with a simple question. Which factor is responsible for guiding the complete collective pattern? This 'supervisor' must be able to meet two specific requirements: first, it must be able to handle differentiated intrinsic and extrinsic signals, with various amplitudes and second, it must be able to react in a relatively exact tempo spatial manner. Obviously the plant cytoskeleton, a system offering tensile as well as rigid structures is able to offer these requirements for iterative pattern formation. Rigid structures in forms of microtubules and on the other side tensile actin is an ideal system to transmit and process internal and external signals for the synchronisation. The complex cytoskeletal system follows the law of 'mechanic tensegrity', a term derived from the combination of 'tensile' and 'rigid'. Cellulosic microfibers integrated into the cell wall matrix are able to maintain cells in shape. Due to this, the tensegrial cytoskeleton is released from its architectural function, as in animial cells and can be optimized for sensory integration of different stimuli (NICK, 2011). Also the second requirement can be met by tensegrity. In contrast to hormones, the signal transmission by the plant cytoskeleton follows a contemporaneous manner.

An important factor for the tensegrity in plant cytoskeleton is a stringent cooperation and also communication of actin filaments and microtubules. Plenty of actin filament – microtubules cross linkers have been identified. In the subsequent years of research one of these cross linkers were focused intensively. KCH (a kinesin with calponin homology) is not only an important motor protein, using microtubules as tracks and is thereby responsible for spatial orientation and tension of microtubules in the cell, furthermore is possess an actin linker domain,

calponin. With these characteristics KCH might be the fundamental requirement of tensegrity of the plant cytoskeleton.

Due to the high diversity of the different subjects in this work that all pursue the goal to investigate the function of plant kinesins, the following introduction is separated into several parts. At the beginning a general overview on the members of the cytoskeleton is shown (**Chapter 1.3.**). Afterwards motor proteins, specifically Kinesins with calponin homology (KCH) that are playing a central role in this work are introduced (**Chapter 1.4.**). This is followed by an overview of the Phospholipase D (PLD) signalling pathway (**Chapter 1.5.**). The importance of the cytoskeleton, Kinesins, as well as the PLD signalling pathway in plant signalling to external stresses are discussed in **Chapter 1.6..** At the end of this introduction an important tool in this work is introduced, called nano second pulsed electric fields (**Chapter 1.7.**). For a better understanding, the scope of this dissertation is in the introduction put into front and follows subsequentially.

1.2 Scope of this study – Investigation of KCHs functions

Todate it is known that KCHs, due to its ability to cross-link microtubules and actin filaments are responsible for tempo spatial orientation of members of the plant cytoskeleton. A detailed description of the functions of KCH is, because of missing investigations not available. This study was started to fill the lack of knowledge in this area partially. The function of KCH, especially the behavior to the cytoskeleton is to be investigated. But also moonlighting functions where challenged. To get insight into this elusive role of KCH in the tensegrial system of the plant cytoskeleton, two different strategies were pursued in the course of the current dissertation:

1. Targeted physical manipulation at the plasma membrane by means of nanosecond pulsed electrical fields (nsPEFs).

2. Genetic and pharmacological manipulation of phospholipase D as membrane-associated signaling hub that is interconnected with the cytoskeleton.

The second strategy arises during work, when surprising results occurred during exertion of the first strategy. Especially these experiments where targeted to clarify the moonlighting function of KCH kinesins.

1.3 The cytoskeleton

Eukaryotic cells possess an extensive compartmentalisation of their organelles and intracellular components. For the spatial arrangement, movement, or fixation of these compartments in the cell, eukaryotic cells have a complex network of filamental and tubular structures of proteins that fills out the entire cell. This complex system is called the cytoskeleton. The cytoskeleton arises in evolution before plant and animal cells were separated. Thus, the main characteristics of plant and animal cytoskeleton are similar. The plant cytoskeleton is a significant requirement for cell division, cell elongation, for differentiation, and cell wall alignment. It is responsible for cytoplasmic streaming and movement of vesicles and organelles. Besides, the cytoskeleton is needed for structural stability of the cytoplasm and anchors multi enzyme complexes in the cytoplasm. In contrast to animals, the plant cytoskeleton is not responsible for consistency of the entire cells. Here interplay of turgor pressure and the cell wall is ensuring the cells stiffness. Recent publications are showing strong evidences, that the cytoskeleton is significantly involved in sensing of extracellular signals, like environmental stresses. Numerous studies and reviews show the enormous scope of function of eukaryotic cytoskeleton, but the state of research is extremely dependent on their year of publication. A selection of studies and reviews on the functions of the cytoskeleton are STAIGER & DOONAN, 1993; GODDARD ET AL. 1994; KOST ET AL., 1999; KOST ET AL. 2002; KOST & CHUA, 2002; HUSSEY ET AL., 2004; NICK, 2008.

Also prokaryotic cells exhibit cytoskeletal structures that show higher plasticity in their composition. The discovery that bacteria possess tubulin (DE BOER ET AL. 1992; MUKHERJEE ET AL. 1993) and actin (BORK ET AL. 1992) encouraged process in evolutionary research of the differences in prokaryotic, archael, and eukaryotic cytoskeleton rapidly (WICKSTEAD AND GULL, 2011).

The cytoskeleton is a non-static, dynamic system that is permanently dissembled, assembled and rearranged. It is moving constantly. In some cellular processes, especially during cell division, the cytoskeleton is moving enormously. The main components of the eukaryotic cytoskeleton are microfilaments made of aggregated G actin, microtubules consisting of the globular proteins α tubulin and β tubulin, and intermediate filaments made of filamental protein. All members of the cytoskeleton are individually introduced below.

1.3.1. Actin filaments

Actin filaments, made of the aggregated G actin (globular actin) are with their 7 nm in diameter the smallest member of the cytoskeleton. Forced by an ATP hydrolase system G actins polymerise to form protofilaments. Two of them congregate by twisting around each other clockwise, to form the double-helical actin filaments (WEGNER, 1976; HOLMES ET AL., 1990). G actin, that has a molecular weight of 42kDa, is highly conserved through all organisms, but in eukaryotic genomes there are several copies of actin genes present. In plants, expression of these genes is tissue dependent (e.g. KOST ET AL., 1998). The actin filament, with its polar structure has two ends that polymerise G actin in different speed rates. At one of these ends, polymerisation of G actin is occurring more rapidly then depolymerising. This growing end is called ‘barbed end’. On the opposite side, at the ‘pointed end’ the depolymerisation is faster then polymerisation. At this end the filament is ‘melting’ (CARLIER, 1991).

The most prominent function of actin filaments is the contraction system of muscles in vertebrates. Together with the motor protein myosin they form myofibrils that spread out through the muscle cell. Myosin converts chemical into

mechanical energy, thus generating contraction force. A similar actin-myosin transport system is responsible for vesicle and organelle movement in the cell. This movement is directed to the barbed end of the filament (KOST & CHUA, 2002).

The assembly and disassembly of actin filaments to form highly ordered structures, their crosslinking to microtubules and the plasma membrane and the nucleus, or the formation of bundles to three-dimensional networks (for review see HUSSEY ET AL., 2006; INSALL AND MACHESKY, 2009), suggests that there are plenty of further functions in animal and plant cells.

1.3.2. Intermediate filaments

Intermediate filaments are made of filamental proteins that form a long helical structure that are flanked from two globular domains at their ends. With its 10-15nm diameter they are thinner then microtubules but thicker then actin filaments. As long as researchers found two classes of intermediate filaments that are part of the nuclear lamina and responsible function as anchors for the chromatin and nuclear pores. Only one genetically proof for intermediate has been found in plants (YANG ET AL., 1992). Nevertheless, intermediate filaments are still suggested to be not existent in plant.

1.3.3. Microtubules

The third members of the cytoskeleton are the cylindrical microtubules. With around 25nm in diameter they form up to 250µm long tubular structures. As well as actin filaments microtubules are constantly dynamic structures and undergo continual assembly and disassembly within the cell. Microtubules are formed of heterodimers of tubulin, that possesses two closely related globular, 50 kDa proteins, α- tubulin and β- tubulin. These tubulin subunits polymerize end-to-end into protofilaments. The cylindrical, filamental shape is formed by parallel alignment of 13 protofilaments, becoming hollow rods, the microtubules. These polar tubules have two distinct ends (for review see MANDELKOW & MANDELKOW,

1989). Likewise to the actin filaments, polymerisation of tubulin is occurring more rapidly at one end then depolymerising. In microtubules this growing end is called ‘plus end’. On the other side, at the ‘minus end’ depolymerisation occurs faster then polymerisation. Steady state conditions between increase on one side and decrease on the other side of the microtubules is called the ‘tread milling’ behaviour (MARGOLIS & WILSON, 1978; MARGOLIS & WILSON, 1998).

The dynamic variation of a microtubule is regulated by GTP binding and hydrolysis. A GTP binding tubulin dimer has a higher affinity to the existent microtubule then GDP bound tubulin. However, hydrolysis of GTP to GDP is not sufficiently needed for direct polymerisation, but for the arbitrary change between polymerisation and depolymerisation. If GTP is bound to the GTP-binding side that is present at every tubulin dimer, a conformational change promotes association with other tubulin dimers. After polymerisation of the new tubulin dimer, GTP is hydrolysed to GDP, that leads to a weaker association and a final release of GDP-bound tubulin at the minus end. Due to the rapid polymerisation process on the opposite plus end of the microtubule, non-hydrolised GTP-tubulin is still more present, which prevents the plus end from depolymerisation. This important type of microtubual variation is called ‘dynamic instability’. It results in alternations between cycles of growth and shrinkage. This dynamic instability is responsible for the continuous and rapid turnover within the cell and microtubules’ half-lives of only several minutes (for review see DESAI AND MITCHISON, 1997; DESAI & MITCHISON, 1997; AKHMANOVA & STEINMETZ, 2008). The dynamical processes of microtubules are regulated by microtubule associated proteins (MAPs), especially microtubule plus end tracking proteins ($^{+}$TIPs) which are responsible for bundling, cross linking, cutting, and anchoring of microtubules (MITCHISON & KIRSCHNER 1984A MITCHISON & KIRSCHNER 1984B; AKHMANOVA & STEINMETZ, 2008).

The most obvious function of microtubules is formation of the spindle apparatus during mitosis. In plant cells a cortex of bundled and parallel microtubules are located right below the plasma membrane during interphase. When a cell prepares for division, this cell cortex is moving to the cell interior to mark the position of the later equatorial division plane. Microtubules at the cell cortex are also responsible for orientation of polar elongation in plant cells. It determines the direction of the

enzyme cellulose synthase during cell wall formation. Like actin filaments, microtubules are also responsible for vesicle and organelle transport. However, in plant cells actin filaments are used for transport systems more intensively.

Current findings showed microtubules influences in sensory signalling. On the one hand microtubules are part of the response machinery that links signalling with cellular morphogenesis. On the other hand it is proven, that they are an important part of signalling itself (NICK, 2012). Due to the importance for this thesis the microtubule functions in pattern formation and signalling the, chapters 'Microtubules in pattern formation' and 'Microtubules as sensors" address these functions explicitly. Microtubule functions in the different fields are collected in several reviews for example WESTENEYS 2004, or NICK 2013.

1.3.4. Microtubules in pattern formation and cell division

Organisms are able to be enlarged by two different mechanisms: cell expansion and cell division. Plants do limit their cell division to individual extension tissues and their specific developmental progress. Plant cells enlargement is triggered by expansion growth that results from the maintained turgor pressure (NICK, 2001 AND NICK, 2008). Also tensibility of the cell wall is constricted, so that continuous attachment of call wall material is required. These cell wall components are delivered by Golgi vesicles and integrated into the cell wall by exocytosis. The cell wall is built from cellulose microfibrils that are embedded into a matrix of polysaccharides. These microfibrils are directed in the cell wall in a transverse manner that is determining the direction of cell elongation (ZIEGENSPECK, 1948). In the interphase, cortical microtubules are arranged in the same perpendicular direction towards the cell axis, indicating a direct involvement of microtubules in elongation direction of plant cells. More precisely, in the year 1974 HEATH postulated and 2006 PAREDEZ has proven the *'Monorail model'* stating that cortical microtubules serve as tracks for the cellulose synthase complex, an annular protein complex with six subunits. Recent studies brought important evidences for a central role of the kinesin motor proteins in cell-wall deposition (reviewed by CAI AND CRESTI, 2012). These kinesins, for example a kinesin-related protein belonging

to the KIF4 family might serve as motors for cellulose synthase tracking along cortical microtubules (**Fig. 2**) (ZHONG ET AL., 2002). During this elongation process cortical microtubules are arranged in a transverse manner. Before the onset of mitosis these microtubules change their direction from a cortical to longitudinal pattern. This process stops cellulose deposition into the cell wall and thereby the entire cell elongation process.

For the onset of the division it is necessary to move the nucleus to the position where chromosome separation and duplication occurs, and where the cell plate is formed. This nuclear positioning is controlled by both, actin filaments and microtubules that are meant to form together with an anchoring protein a cytoskeleton-plasma membrane-cell wal continuum (CPMCW-continuum). This complex continuum tethers the cell nucleus to the plasma membrane (BALUSKA ET AL. 2002; HOHENBERGER ET AL., 2011). When the cell nucleus has reached its final position it will start to organise the formation of the preprophase band (PPB). The division spindle is arranged perpendicular to the PPB. When the chromosomes are separated, the phragmoplast appears at the same side of the PPB. This is the reason why premitotic nuclear positioning is responsible for the entire geometry of the daughter cell (NICK, 2013).

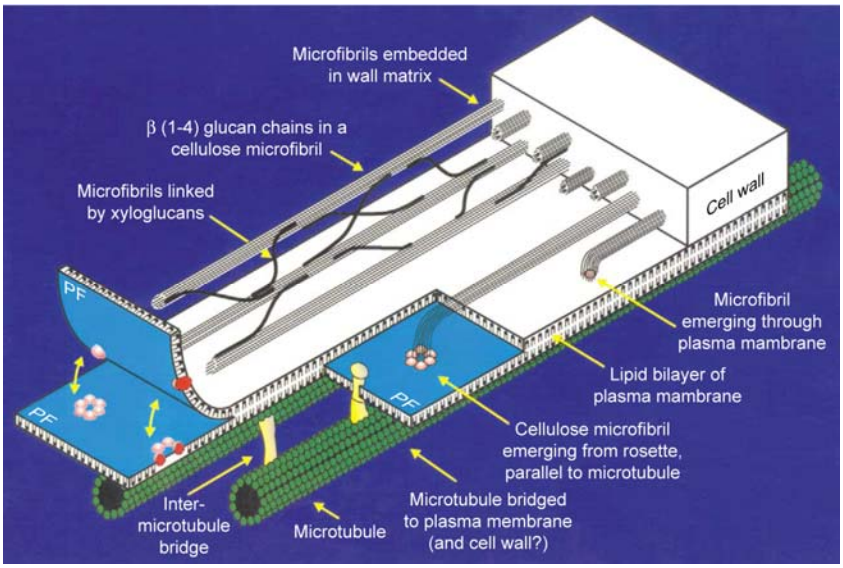

Fig.2: Cellulose synthase – microtubular complex. Cellulose deposition into cell wall emerges from pores formed in parallel to cortical microtubules, suggesting that cellulose synthase using microtubules as tracks. (WASTENEYS, 2004)

1.4. Motor proteins in plant cells

As mentioned above, a cell is not simply a bagful of cytoplasmic fluid with floating organelles like Golgi apparatus, the ER or the nucleus. A healthy cell is a stable but highly dynamic system, lanced by a diverse and complex branch of filamentous and tubular proteins. Furthermore, the cell is not a static system of randomly ordered organelles. In fact, these organelles are tethered to their extinct position in the cell, depending on intrinsic factors like division status and extrinsic factors like pathogen attack. To ensure a rapid reaction on changes of these factors, spatial organisation of the cell interior is sorted and organelles are pulled to the right position. This organelle movement is driven by a wide variety of special motor proteins that move along members of the cytoskeleton, actin filaments and microtubules, serving as tracks. Not only organelle movement is driven by motor proteins, also the cytoskeleton is reorganised and rearranged e.g. during cell division through these protein cargoes. Furthermore, motor proteins in plant cells are responsible for cytoplasmic streaming. They are important factors in cell polarity and cell growth and they are involved in morphogenesis and chromosome segregation during cell division (for review see REDDY, 2001; LIU AND LEE, 2001).

In eukaryotic cells there are three different kinds of motor proteins: the dyneins, the myosins, and the kinesins. All three kinds of motor proteins utilize ATP to convert chemical into mechanical energy and move step by step along actin filaments or microtubules.

1.4.1. Dyneins

Dyneins are microtubule based motors that are directed to the pointed end. For many years, researchers had no evidence of the presence of dyneins in higher plants. As dyneins are important forces for flagella, it was thought that plants lost their dyneins, when they removed their fagella in evolution. The analysis of the *Arabidopsis* genome revealed, that angiosperms do not contain dyneins.

Nowadays it is known that dynein genes are existent in monocotyledons like *Oryca sativa* and *Zea maydis* (KING, 2002).

1.4.2. Myosins

As mentioned above, the molecular motor of the actin filament systems are the myosins. Myosins are barbed end directed. In eukaryotes there are 37 subfamily members, but only two of these families are present in plant cells. Myosins persist of a highly conserved head, a motor domain (around 850 amino acids) that is located at the C-terminus, a relatively stiff neck domain that binds to calmodulin or calmodulin-related proteins (IQ motif), followed by a tail domain that is determining, which kind of cargo is transported (REDDY AND DAY, 2001; YOKOTA ET AL., 1995; YOKOTA ET AL., 1999B).

1.4.3. Kinesins

Conventional kinesins and kinesin-like proteins (KLPs), ordered in the kinesin superfamily are microtubule associates motorproteins, that are present in all eukaryotic cells (HIKROKAWA, 1998). They have important functions in and developmental processes e.g. transport of organelles, vesicles, proteins, DNA, and RNA. They play an important role in mitotic and meiotic spindle formation and chromosome segregation during cell division. Furthermore they are important for cell elongation and microtubule organisation and dynamics (ENDOW 1999, GOLDSTEIN AND PHILIP 1999, HIROKAWA 1998, REDDY 2001B). As the actin associated myosins, kinesins do possess a highly conserved motor domain consisting of 350 amino acids. This motor domain exhibits an ATP- and a microtubule binding site. Furthermore they have a stalk region and a highly variable tail domain. The motor domain of the kinesins is located either in N-terminus, the C-terminus region, or in the middle of the protein. Conventional kinesins are heterotetrameric complexes consisting of two heavy and two light proteins chains. The globular head region is mainly formed by the motor domain. This globular head is linked to the stalk region by a short neck (HIROKAWA &

TAKEMURA, 2004). The stalk region itself forms an α-helical structure with heptapeptical repeats that form a coiled-coil protein chain (FISCHER 2002, ENDOW & BARKER 2003). The two light protein chains bind at the tail region that functions in binding the specific cargo that is moved through the cell. (DIEFENBACH ET AL., 1998).

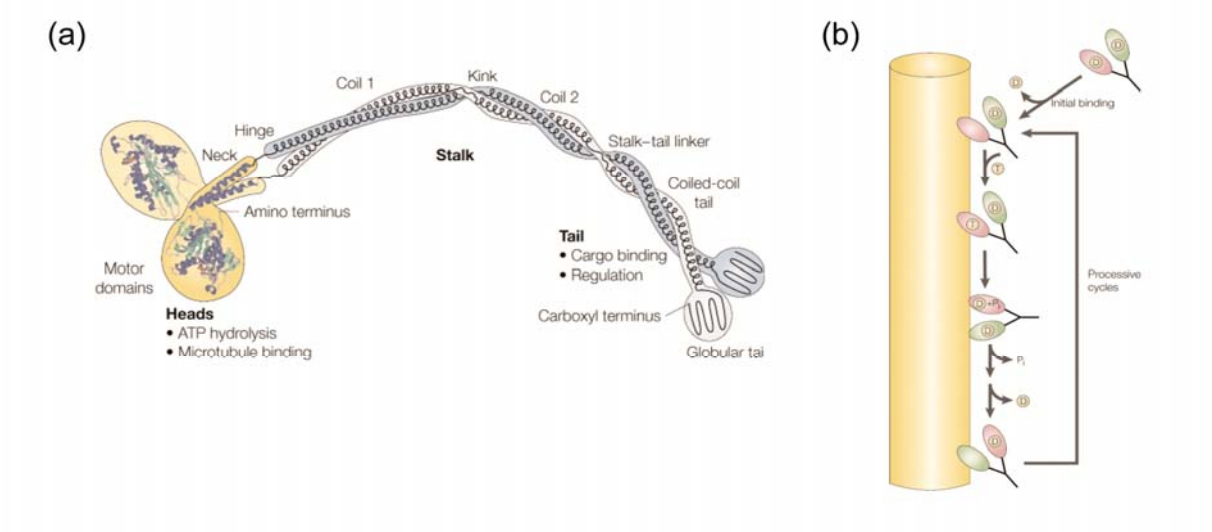

Fig.3: (a) The kinesin structure consisting of the head domain containing the ATP and microtubule binding site at the N terminus, a long helical stalk region and the highly variable C terminal tail domain. **(b)** A model for movement of kinesins at microtubues. Coordinated catalysis of the kinesin heads: D and T inidicate bound ADP and ATP, respectively (WOEHLKE & SCHLIWA 2000).

1.4.4. Kinesins serve as cross-linkers between microtubules and actin filaments

As described above the plant cytoskeleton consists of two complex protein networks: actin filaments and microtubules, that act in distinct ways. However, for many cellular processes, tempo-spatial coordination and organisation of both partners is necessary. Interaction between actin filaments and microtubules must be an important prerequisite for this coordination (COLLINGS, 2008; PETRÁŠEK & SCHWARZEROVÁ, 2009). Plenty of microscopic and biochemical studies gave evidence for this interaction (FRANKE, 1972; LANCELLE & HEPLER, 1991, SEAGULL & HEATH, 1979; HARDHAM *ET AL.*, 1980; TIWARI *ET AL.*, 1984; TOMINAGA *ET AL.*, 1997). During interphase the arrangement of cortical actin filaments and cortical microtubules are congruent (TRAAS ET AL., 1987; SONOBE & SHIBAOKA, 1989;

BLANCAFLOR, 2000). During mitosis actin filaments co-localize with microtubules mainly in the preprophase band and the phragmoplast, while in metaphase actin filaments are anchored at the spindle poles having no contact to spindle microtubules (CLAYTON & LLOYD, 1985; GUNNING & WICK, 1985; TRAAS ET AL., 1987; MINEYUKI, 1999; PANTERIS, 2008).

To date only little is known about the conditions and the players that lead to the actin filament – microtubule cross linkage. Some few plant proteins are known to be responsible for that linkage. Microtubule–associated protein 190 (MAP190) for example has been isolated together with microtubules. While MAP190 keeps its position during interphase around the cell nucleus, in mitosis it is localized at the spindle, suggesting a putative role in spindle formation (IGARASHI ET AL., 2000). Also a protein called SB401 binds and bundles actin as well as microtubules (HUANG ET AL., 2007).

The interaction between actin filaments and microtubules might be also promoted by protein complexes, carrying at least one actin and one microtubule binding side. **Fig. 4** shows the possibilities of motor protein mediated interaction between both members of the cytoskeleton (PETRÁŠEK & SCHWARZAROWÁ, 2009).

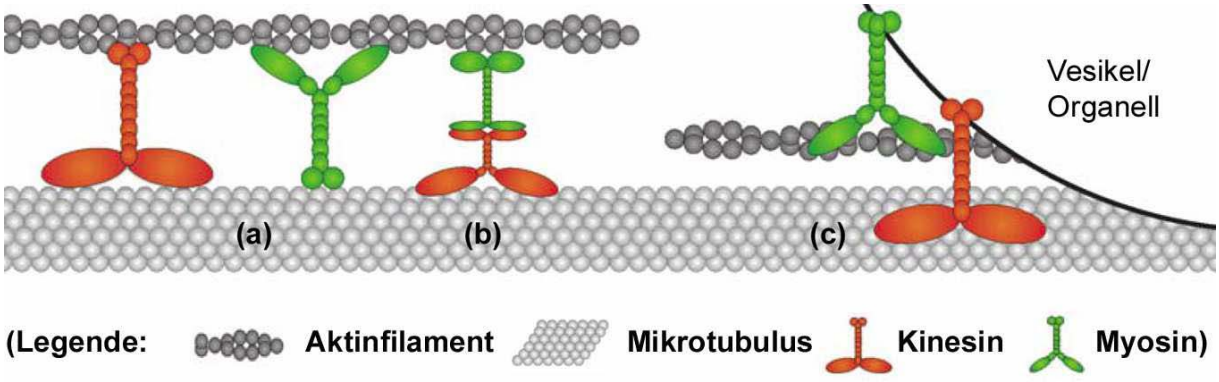

Fig.4: Motorproteins serve as cross linker between microtubules and actin filaments. (a) A first possibility might be one motor protein complex carrying binding domains for both, actin filaments and microtubules. (b) In the second possibility two motor protein complexes, one containing an actin binding side and the other one is carrying a microtubule binding side mediate the interaction indirectly. (c) Another possibility might be a interaction mediated through vesicles or organelles (PETRÁŠEK & SCHWARZAROWÁ, 2009).

1.4.5. Kinesins with Calponin homology domains (KCHs)

Kinesins are able to mediate cross linage between actin filaments and microtubules as well. An important member of these special kinesins, are kinesins with Calponin homology (KCH). The first KCH was isolated from flower cells of the model plant *A. thaliana* (TAMURA ET AL., 1999). Surprisingly, genetic studies revealed an existence of a N-terminal Calponin domain (CH-domain), next to the central motor domain and the typical stalk region. This sequence motif is specific for the protein calponin, which is an actin binding protein of muscle cells in mammals (TAKAHASHI *ET AL.*, 1986; NORTH *ET AL.*, 1994; GIMONA *ET AL.*, 2002). Next to KCHs, CH-domains were found in several actin binding and bundling proteins like β-Spectrin, α-Actinin, or Fimbrin. Recently, researchers were able to identify a KCH homologue from rice (*Oryza sativa* L.) named osKCH1 and they transformed it stably into the tobacco (*Nicotiana tabacum* L.) suspension cell line BY2 (FREY ET AL., 2009). Later, the innate KCH homologue of this tobacco cell line, ntKCH1 could be identified, cloned, and overexpressed (KLOTZ & NICK, 2012). Both cell lines showed a domain-dependent alignment of actin filaments and microtubules (FREY ET AL., 2010; KLOTZ & NICK, 2012). Furthermore osKCH1 expression in BY2 is triggering an increase of cell length. Additionally it causes a one-day delay in nuclear positioning, the premitotic migration of the nucleus into cell centre to the site where the future cell division plate is formed (FREY ET AL., 2010). Nuclear positioning is an important step for the initiation of plant cell division (for a review see NICK, 2008).

Furthermore the analysis of these overexpression lines together with loss-of-function mutants in rice (FREY ET AL., 2010) identified two cellular functions and sites of action, where KCHs are involved: During interphase, they promote cell elongation (KLOTZ & NICK, 2012). In preparation of mitosis, KCH constitutes a central element of the perinuclear cytoskeletal cage that controls the movement of the nucleus towards the cell centre heralding the ensuing cell division (FREY ET AL. 2010) (**Fig.5**).

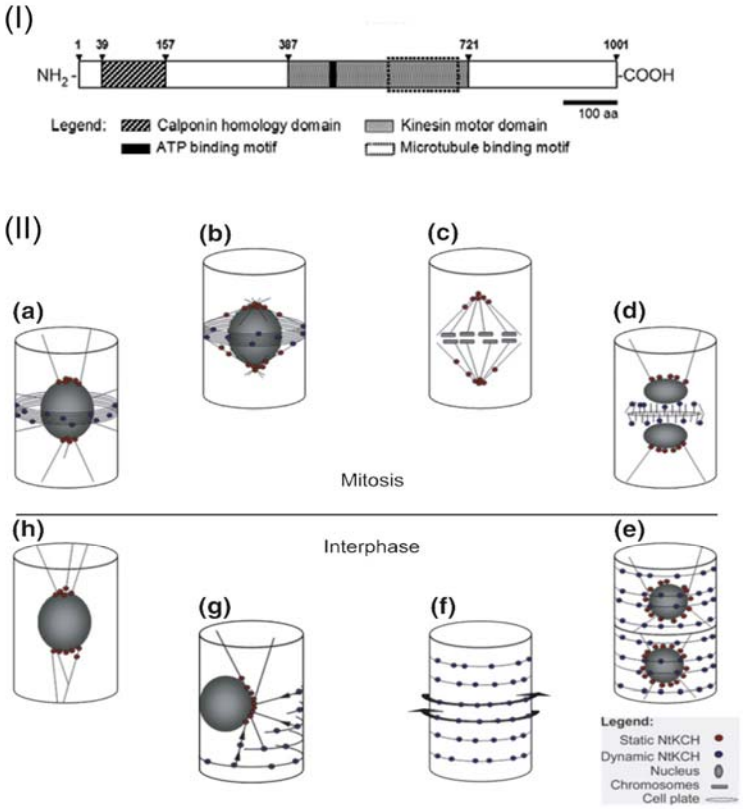

Fig.5: (I) Map of ntKCH1, containing the n-terminal CH-domain and the c-terminal Kinesin motor domain as well as a ATP-binding motif and the microtubule binding motif. **(II)** During interphase, a static fraction of ntKCHs is moving along cortical microtubules, giving the evidence for an involvement in cell elongation. Before onset of mitosis, these KCHs move to the cell centre to form a static fraction in the perinuclear cage, giving evidence for an involvement in mitosis (KLOTZ & NICK, 2012).

1.5. The PLD signalling pathway

Due to invalidity of plants, a major challenge is to find strategies to defend extracellular environmental and biological stressors like hyperosmotic stress, wounding, or pathogen attack. Therefore plants have evolved specific systems in response to these extrinsic factors. As a central element of these strategies, they must have developed broad and specific sensor machinery. Generally transduction starts with reception, often though receptors located in the plasma membrane that perceive the signal and relay the information into the cell. After the signal is transmitted it is received by a specific intracellular signalling pathway or

cascade. These signalling pathways contain for example reactive oxygen species (ROS), calcium or jasmonate, MAP kinases, or Phopholipase D (PLD) signalling.

This work focuses on the *PLD signalling pathway*. PLD activity has been shown for the first time by HANAHAN AND CHAIKOFF in 1947. Extracellular information is received by cell surface receptors called membrane-associated Guaninnucleotide binding Protein Coupled Receptors (GPCR). These receptors are not only playing a role in starting the signal transduction as receptors for external stress signals, like drought, pathogens, or salt. Many hormones and other molecules transmit their action via these GPCRs like Adrenalin, Dopamin or Morphin. This is the reason why GPCRs are known to be a major target for pharmaceuticals like betablockers, antihistaminika and antideprissiva. Consequentially, the Nobel Prize for chemistry in the year 2012 was awarded to Robert Lefkowski and Brian Kobilka for there studies on GPCRs. As the name of these receptors suggest they are associated to Guaninnucleotid binding, i. e. GTP-binding proteins (G proteins). This protein contains three subunits α, β, γ, while the α-subunit owns the GTP/GDP binding domain. The surface receptor with incoming extracellular signals catalyses the exchange of GPF to GTP at the α-subunit. This exchange is responsible for the dissociation of the α-subunit from the βγ-subunit. The βγ-subunit is now the activated form of the G proteins and influences the effector enzymes PLD (MUNNIK ET AL, 1995). The first evidence for the presence of G proteins in plants was published by MA ET AL, 1994. Studies had shown that activation of PLD and PLC are often activated in parallel. It is suggested that PLC is producing a short pulse of second messenger release that lasts only for a few seconds. In contrast, PLD is producing a longer pulse of second messenger release that lasts for some few minutes (LISCOVITCH, 1992; NISHIZUKA, 1995).

PLD is located underneath the plasma membrane. It is highly conserved though all organisms, there is one example in yeast, two in human and twelve in Arabidopsis. It catalyzes the hydrolysis of phosphatidyl cholin and phosphatidy ethanolamin to form phosphatidic acid (PA). PLD cuts the head group of the phosphatidyl molecule to form PA directly. Also PLC is generating phosphatidic acid, but in a quiet complicated pathway with several components and enzymes (MUNNIK ET AL., 1995). Because of its chemical property, PA is a precursor for the biosynthesis of

many other lipids. Due to its physical property, PA is able to influence membrane flexibility. Furthermore PA is a major intracellular signalling messenger. To date plenty modes of actions are known, for example it triggers phosphorylation and dephosphorylation of proteins and lipids, vesicular trafficking and metabolism, membrane tethering, and modulation of enzymatic activities. Additionally it changes physiological properties like structural effects on cell membranes, cell growth, proliferation, and reproduction. The cascade of the PLD signaling pathway is illustrated in **Fig.6**.

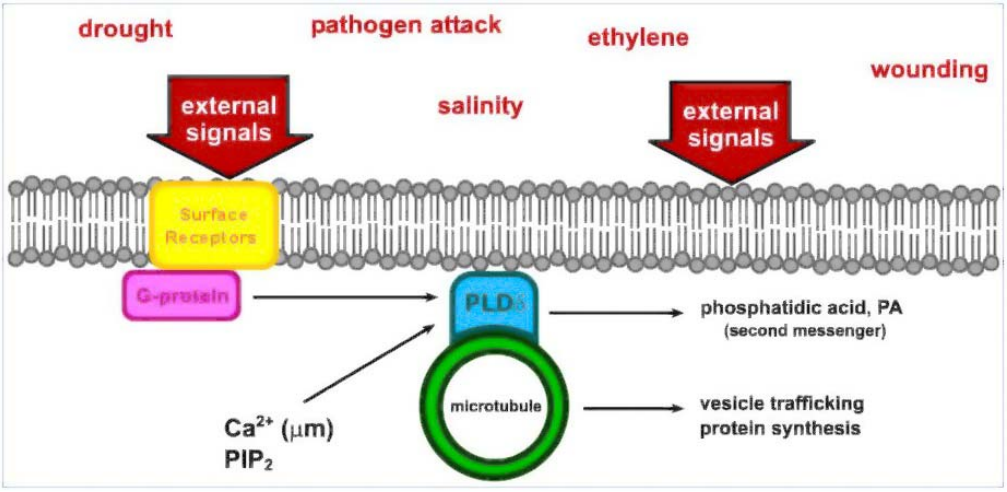

Fig.6: PLD signalling pathway. The external signal is perceived from GPCR in the plasma membrane and transmitted to G proteins in the interior of the cell. G proteins activate PLD that generate the production and release of phosphatidic acid, an important second messenger in plant cells. Furthermore PLD is associated to microtubules and is involved in different cytoskeletal processes like vesicle trafficking (JAN MARC, 2006).

1.5.1. Artificial Activation and Inhibition of the PLD signalling pathway

In the meantime we are able to activate and inhibit PLD signalling pathway by using specific pharmaceuticals. G proteins are activated by substances like mastoparan, different ethanols, and cholera toxin. Inhibition by pertussis toxin was shown in different cell lines in the late 80s and early 90s (MOSS ET AL, 1983, 1994; GILLMAN, 1986; BIRNBAUMER ET AL., 1990; HOEK ET AL. 1992; CARTY, 1994; LAW AND NORTHROP, 1994; ROSS AND HIGASHIJIMA, 1994). Alcohols and mastoparan act in such a way, that G proteins are pushed in the activated GTP state, while Cholera

toxin catalyzes activation of G proteins by ribosylation of the α-G protein subunits, so that they are not able to switch back to the inactive GDP form. Pertussis toxin on the other side prevents reassociation of the βγ-subunit with the α-subunit and holding it in the inactive GDP state (MUNNIK ET AL, 1995). Also the chemicals substance Alumunium tetrafluorid (AlF^{4-}) is able to activate heterotrimetic G proteins. It mimics the γ-phosphate of GTP in the active site of the GDP-bound α-subunit (for reviews see GASPER ET AL., 2009). The first evidence for an activation of the PLD signalling pathway in plants via an activation of G proteins was provided by MUNNIK ET AL. in 1995.

The alcohol butanol is an often used agent for influencing PLD signalling pathways artificially. Based on its special ability of forming different conformations [primary (*n*), secondary (*sec*) and tertiary (*ter*)], this agent seems to be a special case. Due to the fact that n-butanol is able to activate phospholipases, but also act as acceptor for the cleavage product PA and consuming it thereby, it is described in literature as a PLD signalling pathway inhibitor (MUNNIK ET AL., 1995). On the other hand *sec*- and *ter*-butanols do not act as acceptors for PA leading to the conclusion that they seem to be real activators (TESTERINK AND MUNNIK, 2011).

1.5.2. Activation and Inhibition of components of the PLD signalling pathway in plants

Plenty of studies using artificial activation, inhibition, and other interferences of components of the PLD signalling pathway had been published. Also plant cells had been important models for these surveys. Immunochemical studies on a tubulin-binding protein form BY2 membranes were identified as the γ-subunit of PLD in *Arabidopsis*. These finding brought the evidence that PLD binds directly to plant microtubules and the plasma membrane (GARDINER ET AL., 2001). Confocal imaging analyses revealed afterwards that inhibition of PLD by *n*-butanol triggers the release of cortical microtubules from the plasma membrane during interphase. Furthermore, microtubules in the PPB and the phragmoplast are affected by *n*-butanol during mitosis. *Sec*- and *ter*- butanol did not show this effect (DHONUKSHE ET AL., 2003). In addition it was found that *n*-butanol also disrupts cortical

microtubules in *Arabidopsis* and inhibits thereby root elongation. Again *sec*- and *ter*-butanol did not show this effect (GARDINER ET AL., 2003). At the end of cell elongation during interphase transverse cortical microtubules are typically reoriented into longitudinal ones. PLD inhibition agent *n*-butanol treatment triggers rapid detachment of these microtubules from the plasma membrane and accelerated thereby the process of reorientation from transverse into longitudinal microtubules (SAINSBURY ET AL., 2008). Also silencing of a γ-subunit of PLD in *Arabidopsis* revealed the promotion of longitudinal orientation of cortical microtubules (ANDREEVA ET AL., 2008).

Studies on drought and salinity stress showed influences of different PLDs in plants to their responds. *Triticum* cells respond to hyperosmotic stress with the formation of massively bundled microtubules called macrotubules to withstand osmotic pressure (KOMIS ET AL., 2006). Hence PLD could be for example involved in stomatal closure mediating tolerance to drought and salinity stress (HONG ET AL., 2010).

Many plant hormones are involved in PLD signalling pathways. 1-Naphthaleneacetic acid (NAA), an important plant auxin, stimulates cell elongation at lower concentrations than those needed for cell division. AlF^{-4} as a G protein activator stimulates cell division at concentrations that are not permissive for cell division. On the other hand, 2,4-Dichlorophenoxyacetic acid (2,4-D) promotes cell division and Pertussis toxin as an inhibitor of G proteins, inhibits this cell division. Based on these observations CAMPANONI AND NICK (2005) established a model in which one receptor with high affinity for NAA triggers cell elongation and a second receptor with high affinity to 2,4-D activates cell division through trimeric G proteins. These pathways might be somehow linked together. The model is illustrated in **Fig.7**.

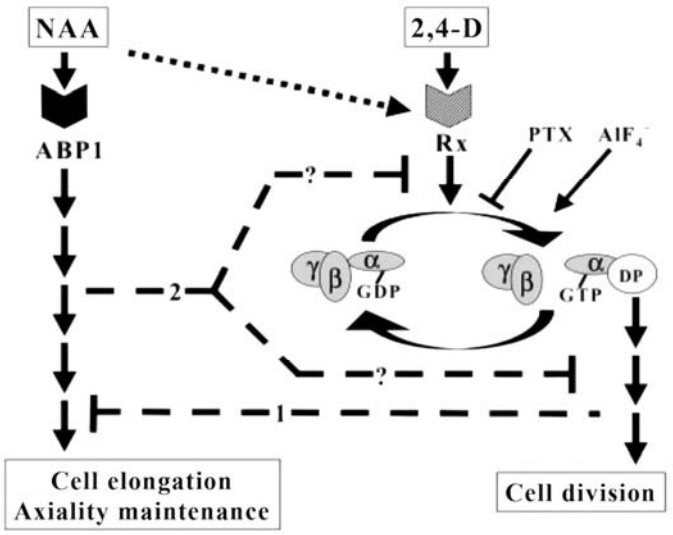

Fig.7: Model of the interaction of plant auxins with components of the PLD signalling pathway. One receptor with high affinity for NAA triggers cell elongation and a second with high affinity to 2,4-D activates cell division through trimeric G proteins. (Campanoni and Nick, 2005).

1.6. Microtubules, kinesins and Phospholipase D as biosensors for external stresses

As already mentioned above, the plant cytoskeleton plays an important role in complex response cascades that are triggered by internal and external signals. Current research on the function of the cytoskeleton suggests an intensive role of microtubules in signalling itself. Two morphological requirements might be responsible for this involvement. The first one is the stiffness and rigidity of microtubules resulting in a maximal robustness that is able to transmit compression forces (GITTES ET AL., 1993). The second requirement for the involvement of the cytoskeleton in sensory systems are tensile elements, in this case actin filaments that are able to transmit traction forces (NICK, 2013). The existence of both morphological characteristics has been later called 'tensegrity' a fusion of the words tension and integrity (ROBBY, 1996). The advantage of the tensegrity system is the ability of mixing maximal mechanical stability with contemporary reaction induced by growth and development. Furthermore, tensegrity provides the ability for a sensory system. Especially in plant cells, where the cell wall is responsible for preservation of the shape, the cytoskeleton has evolved to be an important part of sensory tensegrity. To be able to sweep through

sensory tensegrity a complex device is responsible. In plant cells, this function is carry out by the CPMCW-continuum (BALUŠKA ET AL., 2003; PICKARD & FUJIKI, 2005) which links different structures, like adhesive components, calcium channels, and plant integrin analogues that connect microtubules, actin filaments and the plasma membrane. Also cellulose synthase and Phospholipase D are suggested to play a role be linkers between the members of the CPMCW-continuum (BALUŠKA ET AL., 2003). Circumstanial evidences for intensive interactions between the cytoskeleton, the plasma membrane, and the cell wall, was brought together in an model describing the evidence for a third element of the plant cytoskeleton, the plasmalemma reticulum (PICKARD, 2005). This plasmalemma reticulum is suggested be in cell elongation and cellulose deposition (PICKARD, 2008).

As already mentioned, this cytoskeleton-plasma membrane-cell wall continuum is also suggested to be involved in tethering the nucleus to the cell wall and as driving force for positioning the nucleus into the cell centre initiating the onset of mitosis. In his working model NICK (2013) described the microtubular function of the CPMCW-continuum in terms of mechanical, osmotic, cold stress, and wounding. According to this model stable microtubules act directly to perceive mechanical stress. Plant cells reaction on osmotic, cold stress and wounding interact through stable and dynamic microtubules, resulting in a complex way of response including calcium influx, the onset of PLD signalling pathway, or ROS formation (NICK, 2013).

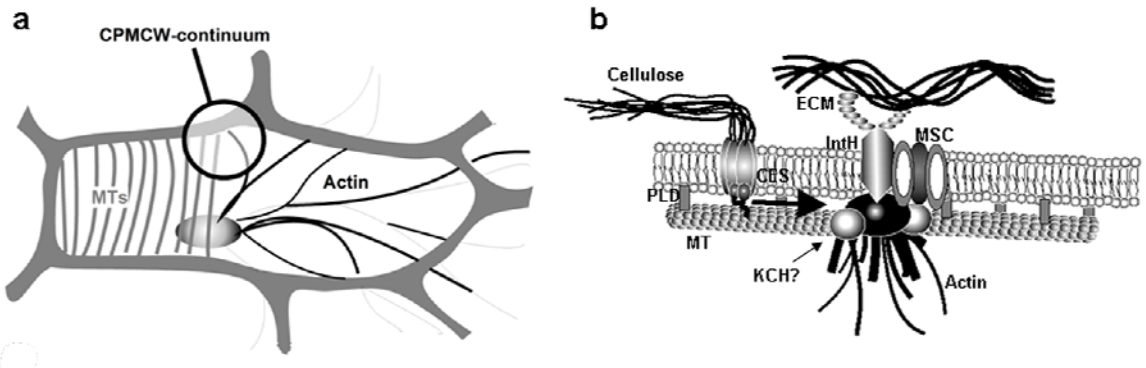

Fig. 8: The cytoskeleton-plasma membrane-cell wall continuum (CPMCW-continuum) in a BY2 cell during interphase **(a)**. The location of this CPMCW-continuum is indicated by the loupe. Transverse cortical microtubules (MTs) and actin filaments spanning the entire cell are forming the tensegrial system of the cytoskeleton. Molecular players of the CPMCW-continuum are indicated in **(b)**. Cellulose synthase (CES) is depositing Cellulose into the extracellular matrix (ECM). Interin Homologues (IntH) and mechanosenstive ion channels are located in the plasma membrane and is anchoring together with Popholipase D (PLD) transverse cortical microtubules to the plasma membrane. Also actin filaments are participating. A possible molecular player for anchoring actin filaments into the CPMCW-continuum are KCH kinesins. (according to NICK, 2011).

An important function of microtubules in sensory mechanisms is their role in mechanosensitivity, particularly as response to osmoadaptation. As already written above, external osmotic signals trigger the formation of macrotubules, massively bundled microtubules, that are necessary for osmotic adaptation (KOMIS ET AL. 2002; KOMIS ET AL. 2006). Interestingly, inhibitors of phopholipase D for example *n*-butanol or N-acetylethonolamine suppress macrotubule formation and osmoadaptation (KOMIS ET AL. 2006) Furthermore decrease of membrane fluidity is a reaction to cold temperatures. This processed is also controlled by microtubules. This reaction can be alternated by pharmacological manipulation (ABDRAKHAMANOVA ET AL, 2003).

A second important external signal that especially plants have to respond is gravity. Gravity-sensing requires 'susception', a term that describes the transformation of physical stimuli into a biological type of energy that can be perceived by sensors (BJÖRKMANN 1988). In gravity-sensing the external force is transformed into a mechanical force. Hereby heavy, mineralised particles called statoliths are used. They assist sensing by acting as susceptors. When statoliths

change their position or direction by gravity they are focussed on small areas to exceed the noise. Therefore, gravitropic forces are transformed into mechanosensitive. Microtubules participate in this bending action by gravitropically stimulated replacement of transverse cortical microtubules into longitudinal in the upper flank, whereas microtubules remain transverse in the lower flank (NICK ET AL. 1980). The microtubules morphological consistence is an ideal level for this perception (NICK, 2012). There are several evidences that microtubules function as these preceptors. Gravitropism for example can be blocked by antimicrotubular drugs (for review see NICK, 2008B.

1.7. Nano second pulsed electric fields (nsPEFs)

In this work a specific kind of electroporation, pulsed electric fields (PEFs), was used to study the involvement of the cytoskeleton in pattern formation and sensing. Research on intense PEFs can be dated back to the late 1950's (STAEMPFLI, 1958) and since then the potential application of PEFs has been explored intensively for various applications, ranging from food pasteurisation (QIN ET AL., 1996) to delivery of macromolecules (Belehradek et al., 1993), including genetic materials (CHANG & REESE, 1990), extraction of cellular contents (BRODELIUS ET AL., 2005), the treatment of certain types of cancer (WEAVER ET AL., 1996), and a dramatic stimulation of mushroom growth via unknown mechanisms (TAKAKI ET AL., 2007). The effect of the PEF treatment depends on environmental factors, innate characteristics of the specimen, and the composition of the media (ALKHAFAJI & FRAID, 2007). However, the mode of application, i.e., the combination of field strength, pulse duration and number of pulses is crucial as summarised by TANG ET AL. (2009):

Longer pulses with lower field strength lead to reversible pore formation and can induce membrane passage of macromolecules, whereas shorter pulses with higher field strength cause IEM (electromanipulation) (BEEBE ET AL. 2002, BUESCHER & SCHOENBACH 2003), and are effective to alter cellular functions. For instance, intermediate combinations of pulse duration and field strength can induce apoptosis which has been exploited for the successful treatment of skin

cancers (NUCCITELLI ET AL., 2006; NUCCITELLI ET AL., 2009). Ultra-short nanosecond pulses (nsPEF) are thought to penetrate into the cell interior and to affect intracellular membranes or organelles before being dissipated by charging of the cell membrane (SCHOENBACH ET AL., 2001; SCHOENBACH ET AL., 2004; CHEN ET AL., 2006; GOWRISHANKAR ET AL., 2006). Therefore it is to be expected that the shorter the duration of the electrical pulse, the more it will act in the cell interior. In fact, rupture of intracellular granules or vacuoles without detectable electroporation to the outer membrane (SCHOENBACH ET AL., 2004; TEKLE ET AL., 2005) or calcium release (BEEBE ET AL., 2003; BUESCHER AND SCHOENBACH, 2003) were observed in mammalian cells exposed to nsPEFs. This stands in stark contrast with changes of plasma membrane permeability explained by the formation of nanopores (HU ET AL., 2005; GOWRISHANKAR ET AL.; 2006; FLICKINGER ET AL., 2010). Recent studies indicate that even with very short pulse durations membrane structures are affected, for instance even nsPEFs of pulse durations from 10 to 60 ns can trigger membrane permeabilization (NESIN ET AL., 2011; IBEY ET AL., 2011; BRETON ET AL. 2012).

1.7.1. Cytoskeletal response to nsPEFs

Since cellular structures adjacent to the plasma membrane respond to ultra-short electrical pulses, the membrane associated cytoskeleton of plant cells could also be a site of action. Cortical microtubules, a plant-specific cytoskeletal array responsible for the directional expansion of plant cells, disorganise and progressively redistribute into mesh-like structures during only three minutes after pulsing. In parallel, actin filaments at the cell cortex disintegrate, and actin bundles tethering the nucleus detach from the periphery and contract towards the nucleus. These early cytoskeletal responses were later followed by a progressive loss of plasma-membrane integrity (BERGHÖFER ET AL., 2009). When actin was stabilised by either phalloidin (BERGHÖFER ET AL., 2009), by transgenic expression of the actin-bundling domain of plant fimbrin, or by inducible expression of the actin bundling LIM domain of the plant-WLIM protein, the plasma membrane became more robust against electric permeabilisation as monitored by the uptake of the membrane impermeable dye Trypan Blue (HOHENBERGER ET AL., 2011). Imaging of

GFP-tagged actin filaments and microtubules by Total Internal Reflection Microscopy (TIRF), a technique based on the very low penetrance (around 50 nm) of an evanescent wave from a total reflected laser beam, has revealed that this part of the plant cytoskeleton is directly adjacent to the membrane (HOHENBERGER ET AL., 2011), which is consistent with the concept of a cytoskeletal-membrane-cell-wall continuum in plants (PICKARD, 2008). It should be mentioned that nsPEFs were reported to disrupt actin also in melanoma cells that are key targets for nsPEF-based curative treatment, followed by activation of caspases as central regulators of apoptosis (FORD ET AL., 2010).

2. Materials and Methods

2.1. Buffers, solutions and ingredients

A list of all buffers, solutions and other chemicals and their producers see below. Buffers and solutions were solved in demineralized water using a Milipore device ((ddH_2O) and autoclaved for 20 minutes and 120°C temperature at a pressure of about 1 bar. Heat sensitive chemicals like antibiotics were filter sterilized using a 0.22µm grid size PVDF filter set. All chemicals were processed and solutions were produced under protection of a laminar flow sterile.

Buffer, solution, chemical	Company
1-butanol	Carl Roth GmbH, Karlsruhe Germany
2,4-Dichlorophenoxyacetic acid	Sigma Aldrich, St. Louis, USA
2-butanol	Carl Roth GmbH, Karlsruhe Germany
Bovine Serum Albumine	Sigma Aldrich, St. Louis, USA
$CaCl_2 \cdot 2H_2O$	Carl Roth GmbH, Karlsruhe Germany
Danish agar	Carl Roth GmbH, Karlsruhe Germany
EGTA	Carl Roth GmbH, Karlsruhe Germany
Evans Blue	Sigma Aldrich, St. Louis, USA
HOECHST 22358	Sigma Aldrich, St. Louis, USA
Inositol	Sigma Aldrich, St. Louis, USA
Kanamycin	Carl Roth GmbH, Karlsruhe Germany
KCl	Merck KGaA, Darmstadt, Germany
KH_2PO_4	Merck KGaA, Darmstadt, Germany
KOH	Carl Roth GmbH, Karlsruhe Germany
Macerozyme	Duchefa, Haarlem, The Netherlands
$MgCl_2 \cdot 6H_2O$	Carl Roth GmbH, Karlsruhe Germany
$MgSO_4$	Carl Roth GmbH, Karlsruhe Germany
MS-medium	Duchefa, Haarlem, The Netherlands
NaCl	Carl Roth GmbH, Karlsruhe Germany
NaF	Carl Roth GmbH, Karlsruhe, Germany
NaH_2PO_4	Carl Roth GmbH, Karlsruhe Germany
Oryzalin	Sigma Aldrich, St. Louis, USA
Paraformaldehyde	Sigma Aldrich, St. Louis, USA
Pectolyase	Dechefa, Haarlem, The Netherlands

Pertussis toxin	Life technologies GmbH, Darmstadt, Germany
PIPES	Carl Roth GmbH, Karlsruhe Germany
Sorbitol	Carl Roth GmbH, Karlsruhe Germany
Sucrose	Carl Roth GmbH, Karlsruhe Germany
Thiamine	Sigma Aldrich, St. Louis, USA
Tris	Carl Roth GmbH, Karlsruhe Germany
Triton-X-100	Carl Roth GmbH, Karlsruhe Germany

Tab.1: List of buffers, solutions, and chemicals used in this work

2.2. Equipment, tools, and electronical devise

A detailed list of all equipments, tools, and electronical devises and their producers see below. Sterile equipment was autoclaved for 20 minutes with 1 bar pressure and 120°C temperature or dry serilized for 4 h at 180°C in a sterilzier.

Equipment, Tools, electronical devise, and Software	**company**
Falcons, Eppendorf tubes and other plastic ware	Greiner Bio-One GmbH, Frickenhaussen, Germany
Any kind of pipettes	Eppendorf AG, Hamburg, Germany or Gilson Inc., Holliston, USA
Autoclave devise	Systec GmbH, Type VE-95, Wettenberg, Germany
Axio-Cam MRm	Carl Zeiss AG, Jena, Germany
AxioImager Z.1	Carl Zeiss AG, Jena Germany
AxioObserver Z.1	Carl Zeiss Ag, Jena Germany
AxioVision Rel. 4.8 Software	Carl Zeiss AG, Jena, Germany
Cleanbench	Product Technical Solution KG, Bötzingen, Germany
CSU-X1 5000 spinning-disc device	Yokogawa, Japan
Flasks and other glass ware	Schott AG, Mainz Germany or VWR International GmbH, Darmstadt, Germany
ImageJ software	http://rsb.info.nih.gov/ij/

Millipore device	SG Wasseraufbereitung und Regenerierstation GmbH, Barsbüttel, Germany
MS Office 2003 Software	Microsoft Cooperation, Redmond, USA
nsPEF Electroporation cuvettes	BTX Instrument Division, Holliston, MA
nsPEF transmission and pulse generator	IHM, KIT, Karlsruhe, Germany
Orbital shaker	IKA Werke GmbH, Stauffen, Germany
Photoshop CS5 Software	Adobe Systems, San Jose, USA
Sterile Filter	Carls Roth, Karlsruhe, Germany
Zen 2012 Software	Carl Zeiss AG, Jena, Germany

Tab.2: List of equipments, tools, and electrical devises used in this work

2.3. Cell lines and cultivation

In this work, the tobacco cell line BY2 (*Nicotinia tabacum* L. cv Bright Yellow 2 (NAGATA ET AL. 1992) has been used for all investigations. Cells were cultivated for a duration of seven days into 30 ml of fresh, autoclaved MS-medium (4.3 g/l Murashige and Skoog salts (Duchefa, Nederland), 30 g/l sucrose, 200 mg/l KH_2PO_4, 100 mg/l inositol, 1 mg/l thiamine, and 0.2 mg/l 2,4-Dichlorophenoxyacetic acid (2,4-D); pH 5.8) in a 100 ml Erlenmeyer flask (Roth, Germany). Dependent on the time point of the cells reaching stationary phase, the volume of the subcultivated inoculums ranged between 1.0 and 1.5 ml depending on the batch. The suspension was incubated at 25°C in the dark on an orbital shaker (KS250 basic, IKA Labortechnik, Staufen, Germany) at 150 rpm. As back-up, calli were subcultured monthly on the same medium solidified with 0.8 % (w/v) Danish agar (Roth, Germany) (FREY ET AL. 2009). The transgenic BY2-osKCH1 cell line has been derived from BY2 by stable transformation with osKCH1 (Kinesin with Calponin Homology domain from *Oryza sativa*) in fusion with GFP under control of a constitutive CaMV 35S promotor (FREY ET AL. 2009), and maintained in MS medium under the same conditions, but supplemented with 50 mg/l kanamycin as selective agent to enforce expression of the transgene. A second transgenic KCH1 overexpressor cell line, representing a GFP fusion to a novel homologue of KCH1 from BY2 *Nicotinia tabacum* L. plants called BY2-ntKCH1 was used (KLOTZ & NICK, 2012). This transgenic cell line was also

cultivated in MS medium under the same conditions as BY2-WT. For maintaining selective pressure 50 mg/l kanamycin was supplemented. A cell line with a Phospholipase D construct from *Nicotina tabacum* was kindly provided by A. Zimmermann (unpublished, BOTANICAL INSTITUTE, KIT) and a cytosolic BY2-freeGFP cell line was provided by K. Schwazerová (CHARLES UNIVERSITY, PRAGUE, CZECH REPUBLIC). Selective pressure in these both cell lines was established with 25mg/l kanamycin.

Name	Genotype	Antibiotics	Source
BY2-WT	*Nicotinia tabacum* L. Bright Yellow 2, wilde type	Non	Nagata et al. 1992
BY2-osKCH1	*Nicotinia tabacum* L. Bright Yellow 2, CaMV-35s, Kinesin with calponin homology 1 (KCH1) from *Oryza sativa*, fused to EGFP with kanamycin resictance	50mg/l kanamycin	Frey ket al. 2009
BY2-ntKCH1	*Nicotinia tabacum* L. Bright Yellow 2, CaMV-35s, Kinesin with calponin homology 1 (KCH1) from Nicotinia tabacum, fused to EGPF with kanamycin resistance	50mg/l kanamycin	Klotz and Nick, 2011
BY2-PLD-GFP	*Nicotinia tabacum* L. Bright Yellow 2, ntPLD	25mg/l kanamycin	Aleksandra Zimmermann, Botanical Institute, KIT, Germany
BY2-freeGFP	*Nicotinia tabacum* L. Bright Yellow 2, CaMV-35s, EGFP with kanamycin resistance	25mg/l kanamycin	K. Schwarzerová, Charles University, Prague, Czech Republic

Tab.3: List of wild type and transgenic tobacco cell line used in this work

2.4. Application of Nanosecond Pulsed Electric Fields

Aliquots of stationary tobacco cells (7 d after subcultivation) were complemented with pulsing buffer (125 mM KCl, 5 mM $CaCl_2 \cdot 2H_2O$, 5 mM $MgCl_2 \cdot 6H_2O$, 150 mM sorbitol, and 1 mM Tris; pH 7.2, conductivity 4 mS/cm) with a ratio of 5 volumes of dense packed cells to 3 volumes of buffer. 1.6 ml of this mixture were used for treatment by nsPEFs. Electroporation cuvettes (BTX Instrument Division, Holliston, USA) with a rated electrode distance of 4 mm and a volume of 800 µl

were used as treatment chambers during all experiments. The field strength (kV/cm) arises from the calculation of 2.5 times the pulse amplitude. Rectangular pulses with corresponding field strengths of 5 kV/cm and 10 kV/cm were applied. The pulse duration of all pulses used in in this work was defined at about 25 ns. This pulse duration was generated by a transmission line pulse generator (EING ET AL. 2009). The rise time of the pules, that is defined of 10 % to 90 % of the entire pulse duration was about T_r = 2.5 ns. The electrical energy density [J/cm^3] delivered to the cell suspension was calculated as product of the square of the field strength, pulse duration, number of pulses, and conductance = 4 mS/cm. For simplicity reasons, it was assumed that 1 ml of cell suspension represents a mass of 1 g. In this study the BY2-WT and the KCH1 overexpression cell line, where pulsed with parameter sets defined as pulsing indices (PI) 1-3 for a general overview of the responds to nsPEFs. For further investigations PI 3 was chosen. **Tab.4** shows the applied parameter sets for these indices. Pulsing index 0 represents control treatment with no nsPEF application.

Pulsing-Index (PI)	0	1	2	**3**
Duration time T [ns]	-	25	25	**25**
Numberof pulses N	-	20	10	**20**
Electric strength E [kV/cm]	-	5	10	**10**
Energy W [J/kg]	-	50	100	**200**

Tab.4: Pulsing parameters including pulse duration, number of pulses and electric strength applied in the experiments using KCH1 overexpressor BY2 cell line. These parameter sets were taken for general overview on the responds to nsPEFs. For further investigations PI 3 was taken. Note that the duration is 25 ns throughout.

Before each batch of experiments, a test sample of the same suspension was prepared to adjust conductivity and working temperature in order to ensure matched load conditions to the transmission line generator. After adjusting the pulse generator to the optimal working conditions for the respective parameter sets and samples, the actual probing samples were treated. After the pulsing, 1.6 ml of cell suspension from two consecutively pulsed cuvettes were transferred into 30 ml of fresh medium in 100-ml Erlenmeyer flasks supplemented with the respective antibiotics, and cultivated as described above. Aliquots of the cells were collected at different time points after pulsing for phenotypical analysis.

2.5. Treatment with PLD signalling cascade manipulating agents

To investigate the sensory responsibilities of microtubules on external signals in plant cells, KCH1 overexpression was combines with an important member of response pathway on external signal: the PLD signalling pathway. In this signalling cascade external stresses are recognized by membrane standing surface receptors that lead the signal to trimeric G proteins located below the plasma membrane. Separation of these G proteins' subunits activates Phospholipase D (PLD). PLD generates the hydrolysis of structural lipids like Phosphatidylcholin or Phosphatidylethanolamin to form Phosphatidic acid (PA), an important intracellular signalling messenger. *In vitro* we are able to influence this process artificially by the addition of G protein activating or inhibition agents. These agents were applied to BY2-WT, BY2-PLD-GFP and BY2-ntKCH cell lines during regular subcultivation. In transgenic cell lines the normal amount of selective antibiotic was used. Cell length, cell width, and mortality were explored at 1 day incubation after application of the chemical. Mitotic index was investigated at the time points 0d, 1d, 2d, 3d, and 4d after treatment with the chemical.

As activating agents 1-butanol and 2-butanol were used as direct application to the BY2 cell lines. The cells were treated with 1- and 2-butanol concentrations of about 0.05%, 0.1%, 0.25%, and 0.5% (all v/v) to investigate the sensitivity to these G protein activating agents. Because the lowest concentration on about 0.05% 1- and 2-butanol has already shown effects, this concentration was used for further

investigations. Aluminium tetrafluorid (AlF^{4-}) was also been used as a G protein activating chemical. It was synthesized in a reaction of Aluminium chloride and sodium fluoride as a 100mM stock solution in a molar ratio of about 4:1 (CAMPANONI & NICK, 2005). At the beginning 50mM Tris-HCl was used as solvent and the solution has been set to the pH value of 8. Using demineralised, sterile water did not show any significant differences compared to the Tris-HCl solvent. BY2 cells were treated with AlF^{4-} in concentrations of 10µM, 50µM, 100µM, and 200µM. Again, the lowest AlF^{4-} of about 10µM was used for a deeper investigation. Because AlF^{4-} is relatively unstable the same amount was applied daily in Mitotic index investigations (CAMPANONI & NICK, 2005)

Pertussis toxin (PTX) is an exotoxin of the bacterium *Bordetella pertussis* and it is inhibiting trimeric G proteins that are involved in the PLD signalling pathway. The solid PTX was solved in demineralised and sterile H_2O and applied in concentrations of about 5ng/ml and 10ng/ml. Again the lowest concentration of about 5ng/ml was used for further investigations.

2.6. Oryzalin Treatments

Drug treatments with herbicides are widely used to explore microtubules behaviour. The drug Oryzalin acts through depolymerisation of Tubulin, thereby blocking microtubule assembly. Oryzalin sequesters tubulin dimers and therefore eliminates microtubules depending on their innate turnover. Different dilutions from a 100 µM stock solution of Oryzalin in Dimethyl sulfoxid (DMSO) were added directly after subcultivation, and the effect on growth assessed by determining the respective measurement one day after treatment. Oryzalin treatment was applied to WT and ntKCH cells as well as in combination with G protein activating or inhibiting agents.

2.7. Cell Treatments with the Microtuble Drug Oryzalin and Packed Cell Volume

Again BY2-WT and BY2-ntKCH cells were treated with different concentrations (0.01µM, 0.02µM, 0.03µM, 0.04µM, 0.05µM, 0.06µM, 0.07µM, 0.08µM, 0.09µM, and 0.1µM) of Oryzalin from a 100µM stock in DMSO. The drug was added directly after subcultivation to the 30ml fresh and autoclaved MS-media in a 100ml Erlenmeyer flask. After cultivation for 4 days, 15ml of the culture has been decant into a 15 ml tube and stored at 4°C to make the cells sink to the ground of the tube. Packed cell volume is taken after 24h storage.

2.8. Quantification of mortality

To determine, how many cells were killed due to the application of nsPEFs, and the application of the different chemicals like PLD signalling pathway manipulating agents and Oryzalin, mortality was measured using an assay based on the membrane-impermeable dye Evans Blue (GAFF & OKONG O'-OGOLA 1971). Aliquots of 300 µl of pulsed cell suspension were drained in custom-made staining chambers using a sieve with a mesh-size of 70 µm, and then transferred into 1 ml of 2.5% Evans Blue (w/v) in MS medium. After incubation for 5 min, the dye was drained, and cells washed three times for 5 min with fresh MS medium. Living cells did not take up the dye, whereas dead cells were readily discriminated by intense colouring as shown in **Fig.9**. At least 500 cells for each data point were imaged in differential interference contrast microscopy (AxioImager Z.1, Carl Zeiss AG, Jena, Germany) using the MosaiX Function to ensure unbiased sampling (Carl Zeiss AG, Jena, Germany). The cell counter algorithm of ImageJ (http://rsb.info.nih.gov/ij/) was used to quantify the incidence of dead cells.

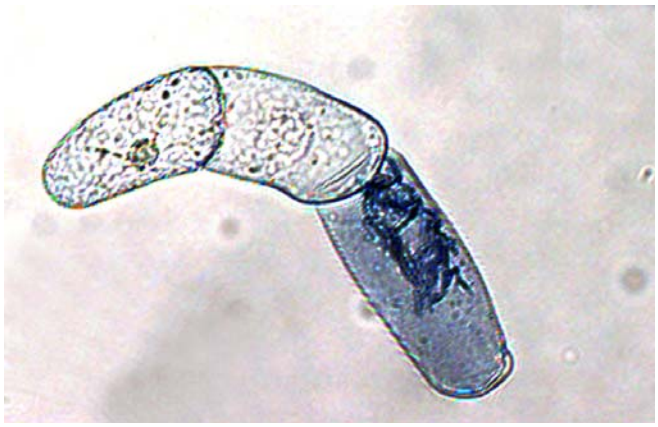

Fig.9: The Evans Blue Staining assay (GAFF & OKONG O'-OGOLA 1971). Living cells did not take up the dye, whereas dead cells were readily discriminated by intense colouring.

2.9. Mitotic Index

To investigate the proliferation status of the pulsed and non-pulsed BY2 cells, the Mitotic Index (MI) was measured as described in (MAISCH & NICK, 2007). The MI is defined as relative frequency of cells in prophase, metaphase, anaphase, and telophase. To discriminate non-mitotic (uncondensed chromatin) from mitotic cells (condensed chromosomes), the fluorescent dye HOECHST 22358 intercalating into the DNA was used. For each data point, a sample of 500 individual cells from three independent experiments was scored.

2.10. Measurement of Cell Length and Width

To detect morphogenetic responses to nsPEFs or chemical treatment, cell length and cell width were measured at days 1 and 5 after subcultivation or pulsing using the measurement function of the AxioVision Imaging software (Carl Zeiss AG, Jena, Germany). At day 1 mitotic activity is maximal, whereas at day 5 cells have completed expansion and enter the stationary phase. Mean values and standard errors (SE) were calculated from about 500 cells per data point. Cell elongation was defined by the division of cell length and cell width. Cell elongation was only consulted when the results showed considerable effects.

2.11. Quantification of Nuclear Positioning

Nuclear positioning is an important step for the preparation of plant cell division (for a review see NICK, 2008). The nuclear position is controlled by both, actin filaments and microtubules that are meant to form together with an anchoring protein a CPMCW-continuum that tether the cell nucleus to the plasma membrane (HOHENBERGER ET AL., 2011). Nuclear Positioning (NP) was quantified from images recorded daily by differential interference contrast microscopy (Carl Zeiss AG, Jena, Germany) through days 0 to 5 after subcultivation per pulsing. The ratio of the shortest distance between nuclear center and the cell wall and cell width in the central plane was determined using the measurement function of the AxioVision software (Carl Zeiss AG, Jena, Germany), which was also used for cell length and cell width measurment. This ratio was calculated by taking the division of the cell wall – cell center distance and the cell wall – cell wall distance (FREY ET AL. 2010). A value of 0.5 represents a central position, whereas a value of 0.25 represents a nucleus adjacent to the side wall. For a simplified understanding see **Fig.10**. For each data point, NP values from 500 cells were recorded to calculate mean values, SE and frequency distributions classifying into the following clusters: 0-0.1, 0.11-0.2, 0.21-0.3, 0.31-0.4, and 0.41-0.5.

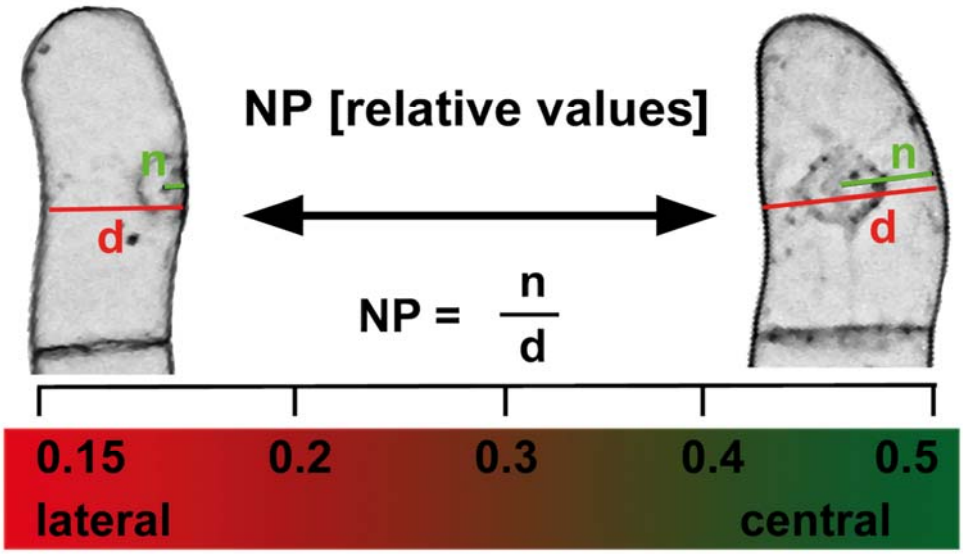

Fig.10: The Nuclear Positioning (NP) assay is the ratio of the shortest distance between nuclear center and the cell wall (n) cell width in the central plane (d). A value of 0.5 represents a central position, whereas a value of 0.25 represents a nucleus adjacent to the side wall.

2.12. Immuno-fluorescence Microtubule staining

For qualitative and morphological investigations of microtubules in BY2-WT and BY2-ntKCH, as well as BY2-PLD-GFP tobacco cell lines a common antibody-based immuno-fluorescence staining method was performed. Furthermore this staining offers the ability of co-localization studies of microtubules with both, the kinesin with calponin homology and Phospholipase D. For the purposes of this study an indirect staining method containing two antibodies was used. In this case the primary antibody was binding to alpha Tubullin. The secondary antibody was binding to the primary and contains Tetramethylrodamine isothiocyanate (TRITC) red fluorescent label.

All staining steps where performed in costumer-made jars containing at one side filter sieve with a grid size of about 70µm. The content of the jar was incubated by placing them into containers with the respective solution for each step. Solution residues where discarded by using capillary force of tissue placed at the filter

sieve side of the jars. For the immuno-fluorescent staining method, an aliquot of 250 µl 3-4 days old BY2 cells were fixed by incubation in 3.70% Paraformaldehyd. Depolymerised Formaldehyde is fixing cells by cross linking proteins on the primary structure level and enables their deformation and degradation. Paraformaldehyde was solved in Microtubule-stabilization buffer (MSB) (0.05M PIPES, 2mM $MgSO_4$, 2mM EGTA, 0.10% Triton-X-100 (w/v), the pH was adjusted to a value of pH 6.9), that contains Magnesia for prevention of microtubule depolymerisation. After fixation, the cells where washed three times for 5 minutes with MSB. To ensure, that the antibodies are able to reach cell interior, the cell wall is partly lysed for 5 min with digestion buffer containing 1% Macerozyme (Cellulases and Pektinases) as well as 0.20% Pectolyase (w/v). The enzyme mixture was also solved in MSB media for microtubule stabilizing. After washing with MSB three times for 5 minutes the cells where incubated in 20min Bovine Serum Albumine (BSA) for 20 min. BSA blocks inactive binding sides and prevents unspecific antibody binding. BSA was solved in Phosphate buffered saline (PBS) (150 mM NaCl, 2,7mM KCl, 1,2mM KH_2PO_4, 6,5mM NaH_2PO_4), a blood substitute for maintaining physiological conditions of BSA and antibodies. After blocking the cell where incubated over night with the primary antibody Anti alpha tubullin (ATT) at 4°C. The incubation of the primary antibody was done surrounding a high humidity to prevent evaporation. After incubation the cells where washed three times for 5 minutes with PBS. The incubation of the secondary antibody was done for 45 min at 37°C. Again the cells are washed 5 times for 5 minutes in PBS and prepared for microscopy.

2.13. Cortical Microtubule Orientation Assay

According to the monorail model, cellulose synthase are using cortical microtubules as tracks to deposit cellulose microfibrills into the plasma membrane (Paredez, 2006). Due to this, the direction of BY2 cells growth is determined by the direction of the transverse cortical microtubules. At the transition from log phase, when the cell is typically elongating and stationary phase, when elongation has already been finished, these transverse cortical microtubules change their direction to form longitudinal ones. By this change, cell elongation is stopped. To

investigate, if orientation of cortical microtubules is responsible for increased cell elongation in BY2-ntKCH cells, a cortical microtubule orientation assay was performed. For this experiment microtubules of BY2-WT and BY2-ntKCH cell where stained by the immunofluorescent assay, that had been described previously. The cells where observed under fluorescent microscopy and the angle between the microtubule and the plasma membrane was measured by angle measrument tool of the AxioVision 4.7 software (Carl Zeiss AG, Jena, Germany).

2.14. Microscopy and Image Analysis

Mortality, mitotic index and division synchrony were determined using an Axioskop 2 FS microscope (Carl Zeiss AG, Jena, Germany) equipped with a CCD (Axio-Cam MRm, Carl Zeiss AG, Jena, Germany) digital imaging system and differential interference-contrast (DIC). Images were acquired by the AxioVision 4.7 software (Carl Zeiss AG, Jena Germany). For the analysis of actin filaments, microtubules and nuclear positioning, an AxioImager.Z.1 microscope (Zeiss, Jena, Germany) was used. This microscope is equipped with an ApoTome microscope slider for optical sectioning and a cooled digital CCD camera (Axio-Cam MRm, Carl Zeiss AG, Jena, Germany). GFP fluorescence was recorded through the filter set 38 HE (excitation at 470 nm, beam splitter at 495 nm and emission at 525 nm). For the evaluation of nuclear position, samples were observed in the differential interference contrast using a 20x objective and the MosaiX imaging software (Carl Zeiss AG, Jena, Germany), which allows automatic imaging of specified small areas of the slide frame by frame, instead of taking individual pictures manually, a procedure that ensures unbiased sampling. Images were analysed by the AxioVision 4.7 software (Zeiss, Jena, Germany) using the length measurement option. Images were processed with Adobe PhotoShop® (Adobe Systems, San Jose, USA).

For visualization of the PLD-GFP cell line, were localisation of PLD was observed and co-localisation studies by immunofluorescence a highly advanced spinning disk confocal fluorescence microscope were used. These images were recorded with an AxioObserver Z1 (Car Zeiss AG, Jena, Germany) using a 63 × LCI-

Neofluar Imm Corr DIC objective (NA 1.3), the 488nm emission line of an Ar-Kr laser, and a CSU-X1 5000spinning-disc device (YOKOGAWA, Japan). Images proceeded and analysed by using the Zeiss microscopy software Zen 2012 (Carl Zeiss AG, Jena, Germany). For further processing of the images, Adobe PhotoShop® (Adobe Systems, San Jose, CA, USA) was used.

3. Results

3.1. Electromanipulation of BY2 cells

3.1.1. Setting parameter for nsPEFs

Electromanipulation is able to cause various reversible but also irreversible changes in confirmation of the different compartments of plant cells. Irreversible damages lead indispensably to the death of the cell. Usually a cell line is able to regenerate by cell division within few days, but especially the first days measurements, could be under the involvement of cell damages and cell death caused by the electric pulses. Crucial for the intensity of the damage are three different modes of application, field strength, pulse duration, and number of pulses. These three sets resulting in an energy level, forced to the cell. Due to this, a set of different Pulsing indices (PI) were taken to treat BY2 cells with electric pulses and several factors like mortality, Nuclear position (NP), and Mitotic Index (MI) were measured (data not shown). Taking the results of these experiments, three different sets of parameters had been taken for all following experiments (**Tab.X**).

Pulsing-Index (PI)	**0**	**1**	**2**	**3**
Duration time T [ns]	-	25	25	25
Numberof pulses N	-	20	10	20
Electric strength E [kV/cm]	-	5	10	10
Energy W [J/kg]	-	50	100	200

Tab.4: Pulsing parameters including pulse duration, number of pulses and electric strength applied in the experiments exploring the potential site of action of nsPEFs in the KCH1 overexpressor line. Note that the duration is 25 ns throughout.

To avoid the described effects of cell damages, the first important view had to be a measurement of mortality via Evans blue stain. The results for untreated BY2-WT and BY2-ntKCH cells as well as WT and ntKCH cells treated taking the PI with highest energy level are shown in **Fig.11**. A generally higher cell death rate of untreated BY2-ntKCH cells has already been shown by FREY ET AL. 2009. The most important information on the experiment is that the difference of untreated to treated cells in the number of dead cells is generally 2% to 3% higher. This increase is negligible for further experiments. It is independent of the time elapsed after pulsing and of the cell line taken for the experiment. Hence, these pulsing effects and the effects resulting from KCH over expression work in an additive way.

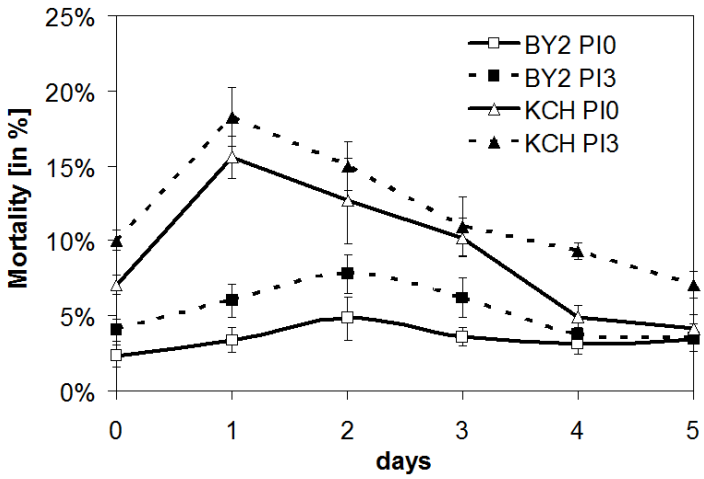

Fig.11.: Mortality of BY2 wild type and BY2-ntKCH cell treated and untreated with nsPEFs. Generally ntKCH seem to have a peak in mortality at d1. The pulsed results (broken lines) are generally 2% - 3% higher and as an additive effect negligible for further experiments.

3.1.2. Synergistic effect of nsPEFs and osKCH1 on mitosis

Microtubules control premitotic nuclear migration in concert with the minus-end directed kinesin osKCH1 (FREY ET AL. 2010). This kinesin exists in two dynamic states, either detached from actin adjacent to the plasma membrane, or linked to actin near the nuclear envelope (KLOTZ & NICK 2012). Therefore, in this study the role of nsPEFs on nuclear positioning (assessed 1 day after pulsing) in the non-transformed BY2 wild type versus a cell line overexpressing osKCH1 for pulses of

increasing energy was investigated, as indicated in **Tab.4**. Hereby, a pulsing index of PI0 represented a mock treatment without pulsing cells, PI1 the lowest (50 J/kg), and PI3 the highest (200 J/kg) energy density. Pulse duration for these treatments was kept constantly to 25 ns. In the non-transformed wild type, the control without pulsing produced a value of ~0.37, which means that most nuclei had reached the cell center. All three pulse treatments reduced nuclear positioning by about 30 % (**Fig.12a**), already for the lowest energy density. Similarly, the PI0 control value for the osKCH1 overexpressor was reduced with respect to nuclear positioning by about 30 % as compared to the wild type without pulsing, consistent with previous results (FREY ET AL. 2010). When the osKCH1 line was challenged by nsPEFs, this impaired nuclear positioning even further culminating in a reduction by about 60 % for 200 J/kg representing a situation, where most nuclei had not moved out from their starting position at the cell periphery. Thus, the nsPEFs and the elevated expression of osKCH1 act synergistically.

To test the persistence of the delay, we followed nuclear positioning through several days after pulsing for the pulsing index PI3 (200 J/kg), where the difference between non-transformed wild type and osKCH1 overexpressor was maximal (**Fig.12b**). When the wild type was not challenged by a pulse, the nucleus had reached a central position within one day and subsequently gradually moved out from the cell center after day 3, when the culture progressed from rapid cycling to a stationary phase. By the pulse treatment, the movement of the nucleus into the cell center was delayed by about one day but eventually the nucleus reached a central position at day 2 after pulsing and then returned to a lateral position at roughly the same time as the unpulsed control. Again, the unpulsed osKCH1 overexpressor resembled the pulsed wild type. Nuclear positioning was not completed before day 2 and the nuclei did not become as central as in the wild type. Upon pulsing the osKCH1 overexpressor, nuclear positioning was even further delayed by an additional day confirming the synergistic effect of pulsing and osKCH1 overexpression.

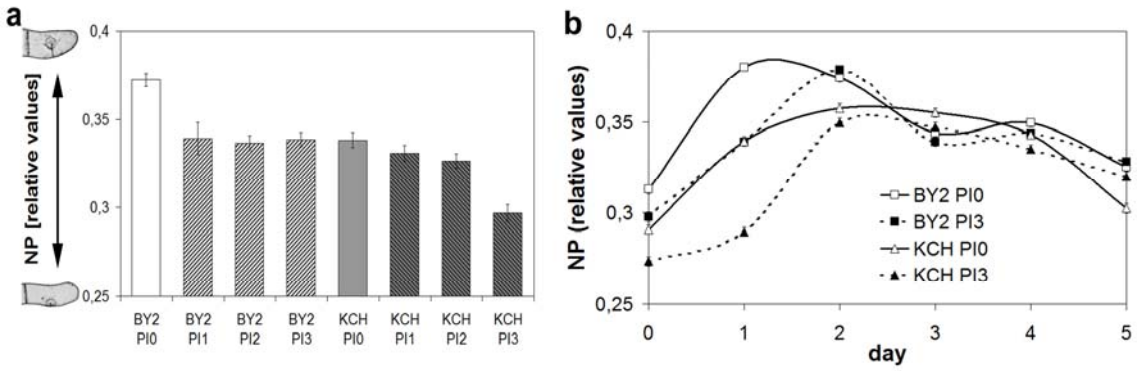

Fig.12: Synergy of nsPEFs and KCH kinesins in the delay of premitotic nuclear positioning and entry into mitosis. **a** Relative nuclear position (NP) values (1 d after pulsing) in relation to nsPEFs of different Pulsing Indices (see **Tab.4** for parameters, duration was 25 ns througout). **b** Time course for nuclear positioning for pulsed and non-pulsed BY2-WT (squares) and BY2-osKCH1 cell lines (triangles). experimental series.

To visualize the shift of the time course of nuclear positioning in a more obvious way, a method of classification had been taken (FREY ET AL. 2009). Eight classes, representing the progress of nuclear positioning were defined and their occurrence of cells been in the specific class was counted 1d after pulsing. In **Fig.13a,b,c,a** and **d** arrows representing the approximated peak of each curve. This peak is shifted to classes representing cell nuclei that positioning to the centre of the cell, for preparing the onset of mitosis had not been forwarded progressively. As already shown before, pulsed BY2 wild type and unpulsed BY2-ntKCH represent a roughly similar progression of nuclear positioning.

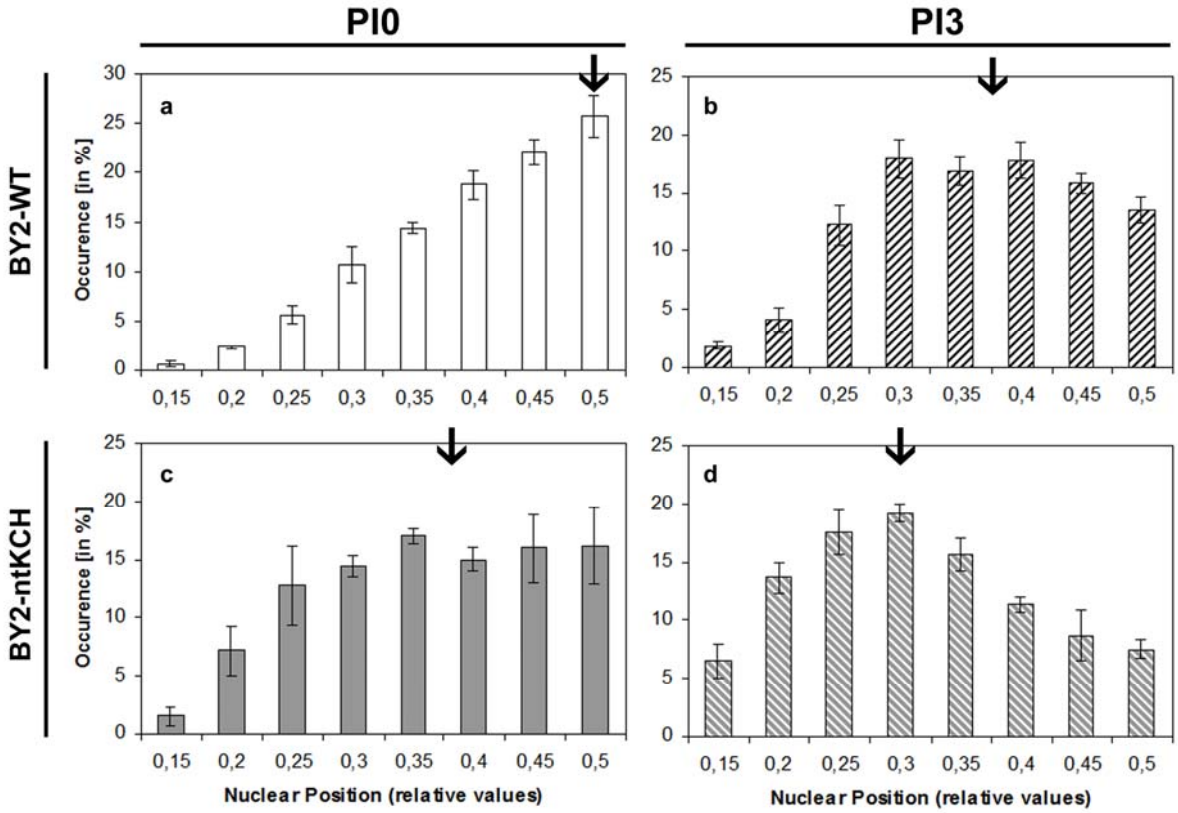

Fig.13. Occurrence of untreated and treated BY2 WT and ntKCH cell had been classified into eight classes depending on their progress of nuclear positioning. The arrow in each chart represents an approximated peak. This peak is shifted to classes representing cell nuclei that positioning to the centre of the cell, for preparing the onset of mitosis had not been forwarded progressively. Results are figured for unpulsed (**a**) and pulsed BY2-WT (**b**), and unpulsed (c) and pulsed (**d**) BY2-ntKCH cells

Since nuclear positioning is a prerequisite for mitosis, in parallel the time course of mitotic index was investigated (**Fig.14**), and we observed a pattern that was almost identical to that found for nuclear positioning. Similarly to the results of the nuclear positioning the first peak of MI in treated WT and untreated ntKCH cell lines is compared the untreated WT cell line shifted from day one to day two after pulsing. Furthermore treated ntKCH cell line is having its first peak of MI even later at day four.

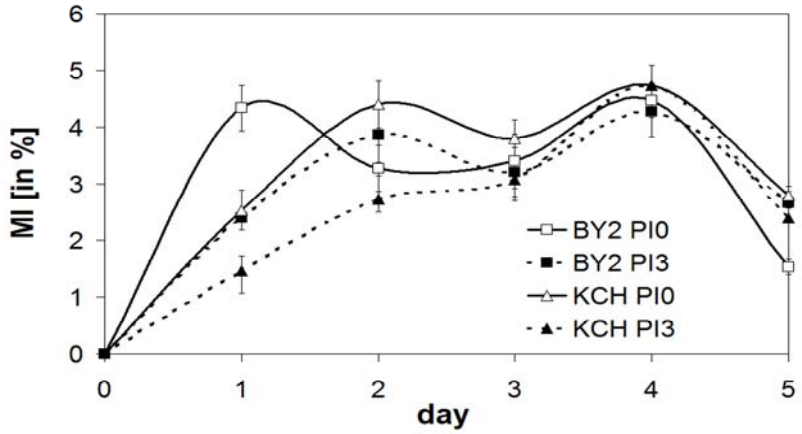

Fig.14: Time course of Mitotic Indices (MI) for pulsed and non-pulsed BY2-WT and BY2-osKCH1 cell lines. Data show mean values and standard errors from n = 1500 individual cells from three independent experimental series.

3.1.3. The response of cell expansion in BY2-osKCH1 to nsPEFs is inverted

Expansion of plant cells is driven by the osmotic gradient between medium and protoplast, but it is limited by the extensibility of the cell wall. The mechanical properties of the cell wall depend on cellulose texture, which in turn is controlled by a plant specific subpopulation of the membrane associated cytoskeleton, the cortical microtubules. Overexpression of the plant-specific KCH kinesin osKCH1 promoted both cell width (**Fig.15a, c**), and cell length (**Fig.15b, d**). Approximating volume by a cylindrical shape, the volume of the osKCH1 overexpressing cells is estimated to excel that of the non-transformed wild type considerably (by a factor of 2.3 at day 1 after inoculation, when mitotic activity ensues, and still a factor of 1.51 after any mitotic activity has ceased at day 5 after inoculation). This difference is even promoted upon pulsing with PI3: Now the osKCH1 overexpressing cells are 3.4 times (day 1) or 2.04 times (day 5) larger as compared to the pulsed wild type. The cause for this volume increase is a pronounced stimulation of both elongation and lateral expansion in the osKCH1 overexpressor during the first day after the pulse (**Fig.15a, b**). In contrast, the non-transformed wild type shows reduced lateral expansion, and constant length under

these conditions. During the subsequent division phase, the length increment caused by cell elongation is counterbalanced by the length reduction in consequence of (axial) cell divisions. Therefore, in both lines, the nsPEF treatment causes a reduction in length (**Fig. 15d**) indicating that cell elongation is more sensitive as compared to cell division. In contrast, lateral expansion (**Fig.15**) follows the same pattern as observed for day 1 (**Fig.15a**) with the general difference that all cells are narrower at day 5 as compared to day 1.

The effect of a mild (PI3) nsPEF treatment on cell expansion can therefore be summarised as follows: (i) cell expansion is inhibited in the non-transformed wild type, but stimulated in the osKCH1 overexpressor, (ii) cell expansion is more sensitive as compared to cell division in both non-transformed wild type and osKCH1 overexpressor.

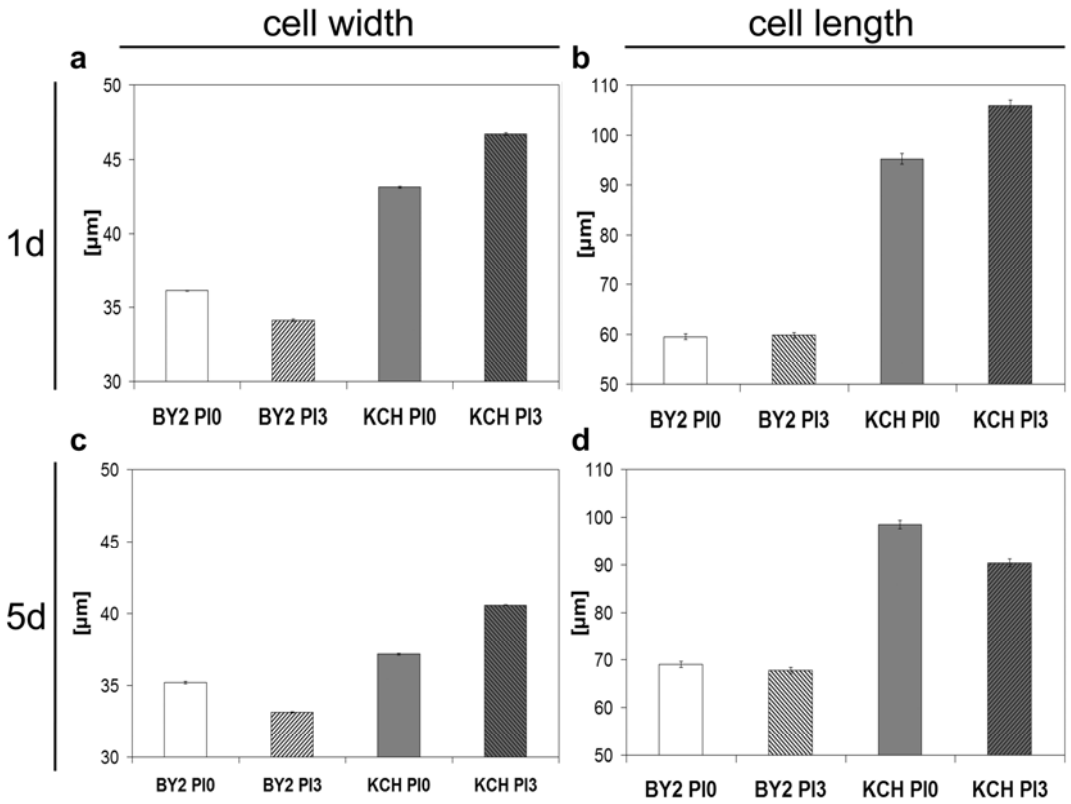

Fig.15: Response of cell expansion to nsPEFs. **a, c** cell width and **b, d** cell length at day 1 (**a, b**) and day 5 (**c, d**) after pulsing in non-transformed (white bars) BY2-WT versus BY2-osKCH1 (grey bars) either without pulsing (blank bars), and with pulsing using a PI3 treatment (duration 25 ns, striped bars). Data represent mean values and standard errors from 500 individual cells from three independent experimental series.

3.2. Pharmacological Analysis of Phospholipase D

3.2.1. Partially high mortality of BY2 cell lines to PLD signalling pathway manipulating pharmaceuticals

An important reaction in response to external stresses in plants is the onset of the PLD signalling pathway, that leads to the formation of phosphatidic acid, an important second messenger that triggers plenty of physiological and biochemical responses. To date we are able to manipulate the cascade of this PLD signalling pathway and its partners involved (membrane standing surface receptors, G proteins and PLD itself) by different pharmaceuticals. In this work two PLD activating agents (2-butanol (2-but) and Aluminium tetrafluorid (AlF^{-4})) and two PLD inhibiting agents (1-butanol (1-but) and Pertussis toxin (PTX)) were taken. A special attention has to be taken with 1-but treated cells. It is actually activating PLD. In the same time it also serves as acceptor for the cleavage product phosphatidic acid and consuming it thereby. This is the reason why it is described in literature as a PLD signalling pathway inhibitor (MUNNIK ET AL., 1995). The concentration of the pharmaceuticals was set in previous experiments (data not shown) and defined as the lowest range of concentrations which effects are clearly visible. A first view on the effects is always a test of the mortality via Evans blue staining on the different cell lines. The results are blotted in **Fig.16**. To all cell lines pharmaceuticals have different toxic effects. Generally the effect of 2-but (up to 20%, **Fig.16b**) is higher than the effects of 2-but, PTX, and AlF^{4-} (all three up to 13%, **Fig.16a, c, d**). The mortality rises in dependence to the concentration of the drugs. Only the curve of 1-but seems to be saturated in a low concentration and mortality is not rising concentration dependently. To verify the evidence, whether these effects are caused by the drugs and not the overexpression of ntKCH respectively, a third cell line was consulted. BY2-freeGFP is expressing a free cytoplasmatic GFP and it is often used as a control for physiological experiments with genetically transformed cell lines. For all drugs, toxicity to the freeGFP cell line is in a same range or only insignificantly higher then in the WT.

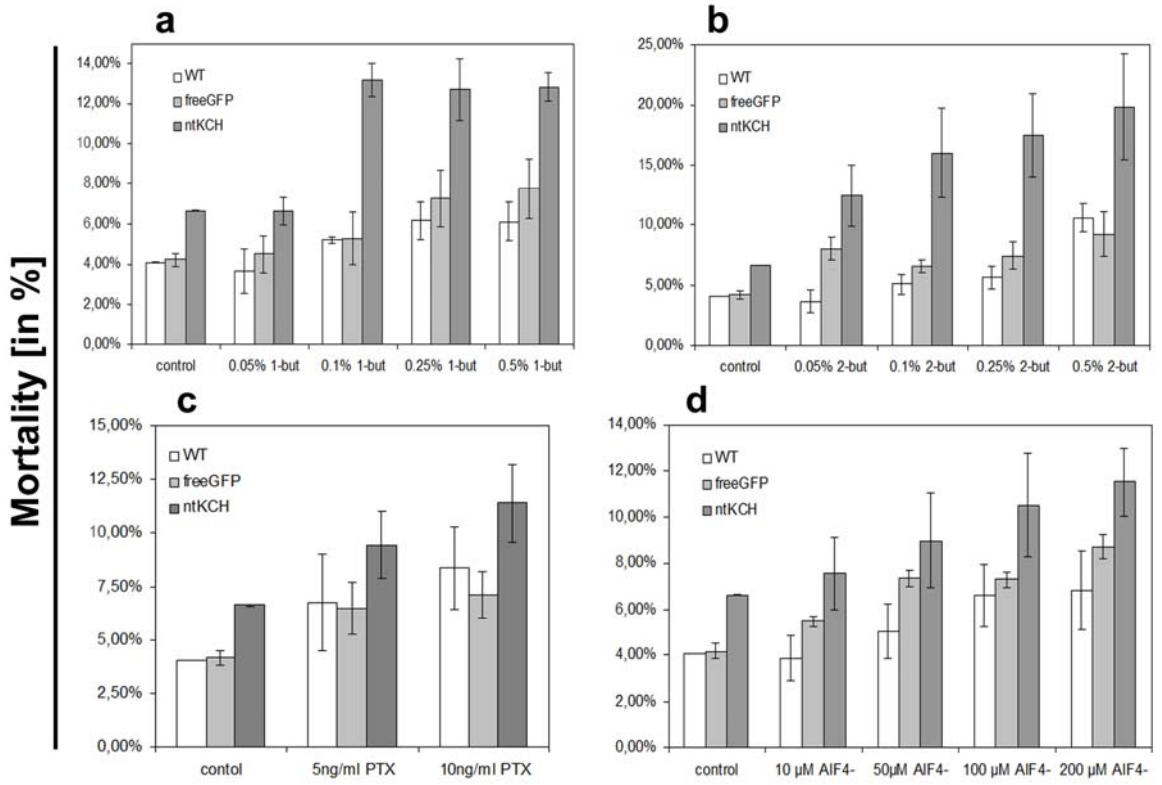

Fig.16: Mortality of BY2-WT, BY2-freeGFP, and BY2-ntKCH in response to the PLD signalling pathway manipulating pharmaceuticals 1-but (**a**), 2-but (b), PTX (**c**), and AlF^{4-}(**d**). Mortality of ntKCH cells treated with 2-but is generally higher than all other treatments. Mortality of a cell line expressing free cytosolic GFP as control is in a same range or insignificantly higher than in WT. This is indicating that these mortality effects are caused by the drug and not by the transformation itself.

3.2.2. Onset of Mitosis decelerated due to PLD signalling pathway manipulating agents

As already mentioned above, microtubules are partially responsible for the onset of mitosis. This is driven by premitotic nuclear migration into the cell centre (FREY ET AL. 2010). A specific fraction of ntKCH kinesins are responsible for this movement of the nucleus (KLOTZ & NICK, ET AL. 2012). As microtubules and ntKCHs are forming together with PLD a CPMCW-continuum that may also serve as senor for the onset of mitosis, the role of PLD and its signalling cascade should be clarified. Therefore the Mitotic index (MI) was measured for four day after

subcultivation in BY2-WT and BY2-ntKCH overexpressing cell lines. To discover the involvement of PLD, all four agents manipulating PLD signalling pathway were added during subcultivation and incubated for the respective time. MI in percentage is representing the amount of cells currently adjudged in a mitotic phase (prophase, anaphase, metaphase, and telophase) (MAISCH & NICK 2007). For all cell treatments with PLD signalling cascade manipulating agents, untreated BY2-WT (about 8% MI) representing at the important day one, have the highest MI. The percentages for treated BY2-WT cells are reduced of about 40% in comparison to the untreated WT cells indicating a delayed onset of mitosis. Both, untreated and treated BY2-ntKCH cells are in the same range of the MI value of treated BY2-WT cells. Surprisingly, treatment with PLD signalling pathway manipulating agents to BY2-ntKCH does not diminish mitosis at day one as in BY2-WT cells. **Fig.17 a,b,c,d** showing the MI for all treatments and cell lines over 4 days after subcultivation.

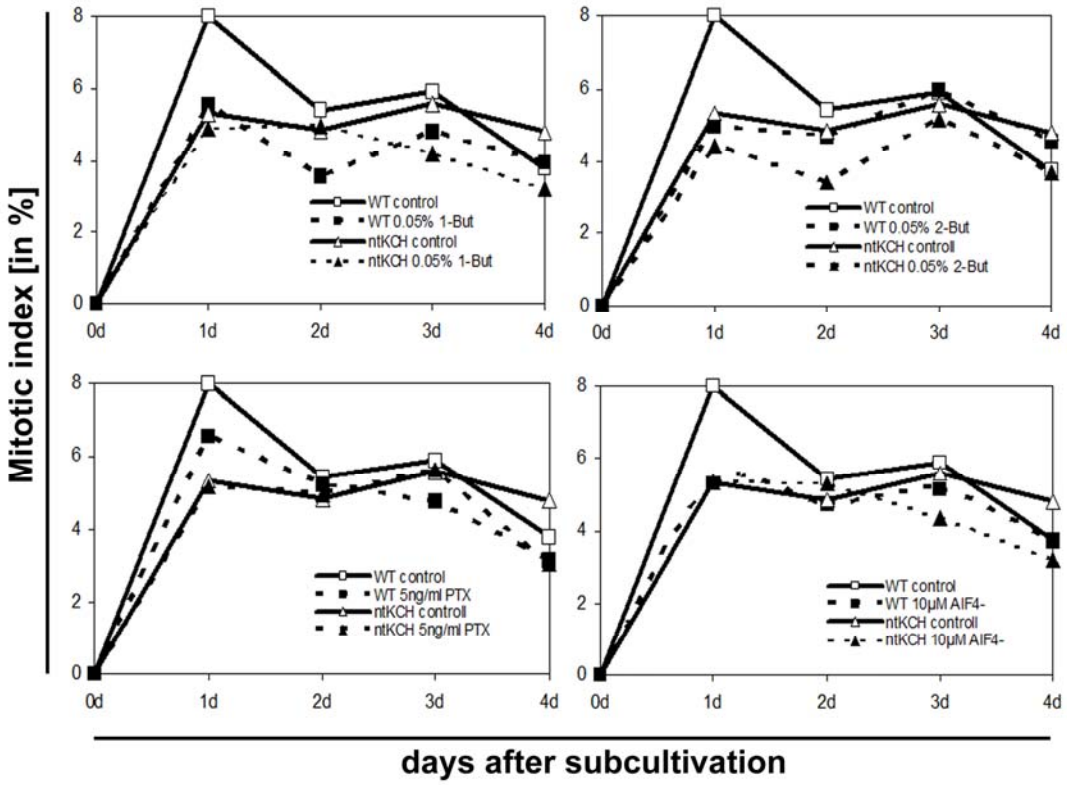

Fig.17: Mitotic index of BY2-WT versus BY2-ntKCH cells treated with PLD signalling pathway manipulating agents. In all treatments untreated BY2-WT cells represent the highest MI at the important first day after subcultivation. Surprisingly, treating PLD signalling pathway with manipulating agents to BY2-ntKCH does not diminish mitosis at day one as in BY2-WT cells.

3.2.3. Pharmaceuticals act on BY2 cell expansion in diverse manners

In the interphase, cortical microtubules are arranged in a perpendicular direction to the cell axis. These cortical microtubules serve as tracks for the cellulose synthase complex. Hence, the arrangements of microtubules are responsible for elongation direction and also cell size. Furthermore different kinesins might serve as motors for this cellulose synthase tracking along cortical microtubules. PLD is also linked to cortical microtubules. As this work is exploring the orchestration of microtubules, kinesins, and PLD in a CPMCW-continuum, an experiment dealing with all players was necessary. Especially the role of PLD is unknown in this field. A simple measurement of cell length and width of BY2-WT and BY2-ntKCH cells with treatment of PLD signalling pathway activating and inhibiting agents was performed. The bar charts in **Fig.18** show the results of these cell length (**a**) and cell width (**b**) measurements. Generally there are massive cell length differences visible (**Fig.18a**). The maximal differences between both single cell lines are around 10 µm, resulting from the treatment of the PLD signalling pathway manipulating agents. As in chapter 3.1.1. already described the general differences between BY2-WT and BY2-ntKCH are resulting from the transformation and ntKCH overexpression (FREY ET AL. 2009). In comparison to the cell length, the differences of the cell width (**Fig.18b**) are minimal and insignificant. In the BY2-WT cell line the cells treated the PLD activating agents AlF^{4-}, 1-but, and 2-but are elongated. In BY2-ntKCH cell line AlF^{4-} and 1-but treated cells are elongated while cells treated with 2-but are truncated. Interestingly, in both cell lines the PLD inhibiting agent is shortened heavily.

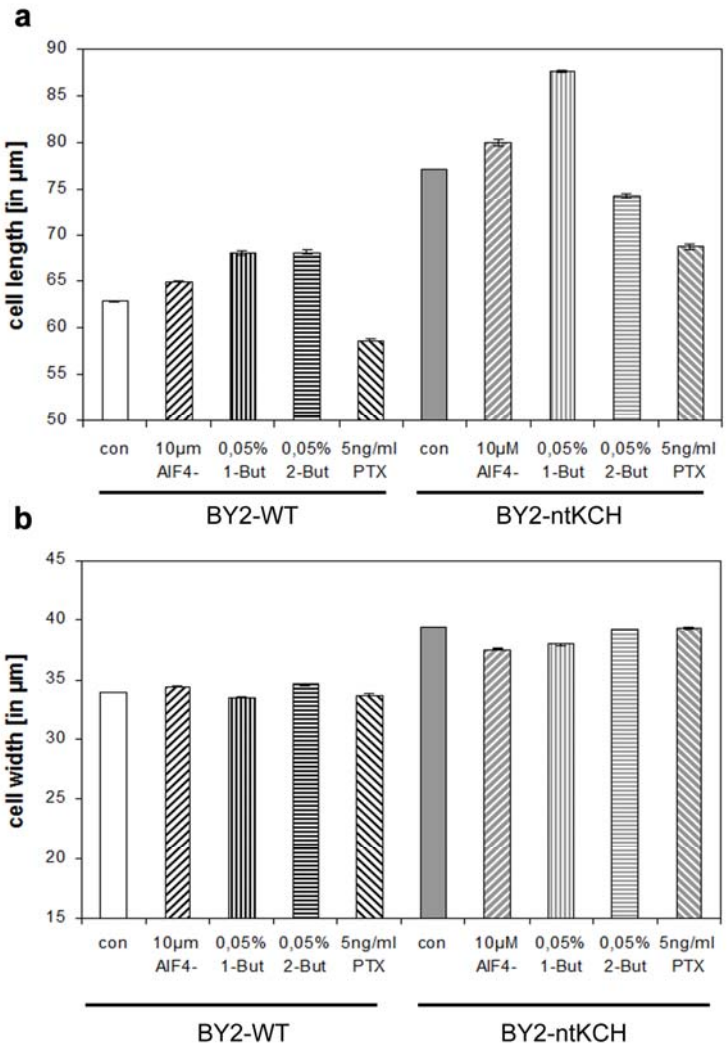

Fig.18: Cell length (**a**) and cell width (**b**) measurement of BY2-WT and BY2-ntKCH cells treated with PLD signalling pathway manipulating agents. In contrast to the cell width, there are massive changes of the cell length due to the interaction with PLD signalling pathway manipulation agents. Especially cells treated with 1- Butanol are highly elongated. The results for cells treated with PTX are inverted. Cell elongation is stopped earlier than for non-treated cells.

3.2.4. Oryzalin dose curve

Drug treatments with herbicides are widely used to explore microtubules behaviour. The drug Oryzalin acts through depolymerisation of Tubulin, thereby blocking microtubule assembly. Here, the role of microtubules in concert with players involved in the CPMCW-continuum are studied. For the investigation of the general reaction on treatment of Oryzalin, the packed cell volume was measured

in BY2-WT and BY2-ntKCH cells. At the beginning (no treatment of Oryzalin) the packed cell volume of ntKCH is higher, than the WT. This expected result is due to the elongated size resulting from ntKCH overexpression (FREY ET AL., 2009). **Fig.19** Is showing the logarithmic curve of both cell lines. Up to a concentration of 0.03 µM for BY2-WT and 0.02 µM for BY2-ntKCH, the treatment of Oryzalin seems to have no toxic effect the cells. The following increase of toxicity is parallel, with only small differences visible. Thus, ntKCH cell line seems to be more susceptible at lower concentrations than the WT. This curve was taken to find a proper concentration for the following experiments. To show an effect, but not total the total toxicity of Oryzalin for these experiments, the lowest concentration level of about 0.05 µM was taken (black arrow).

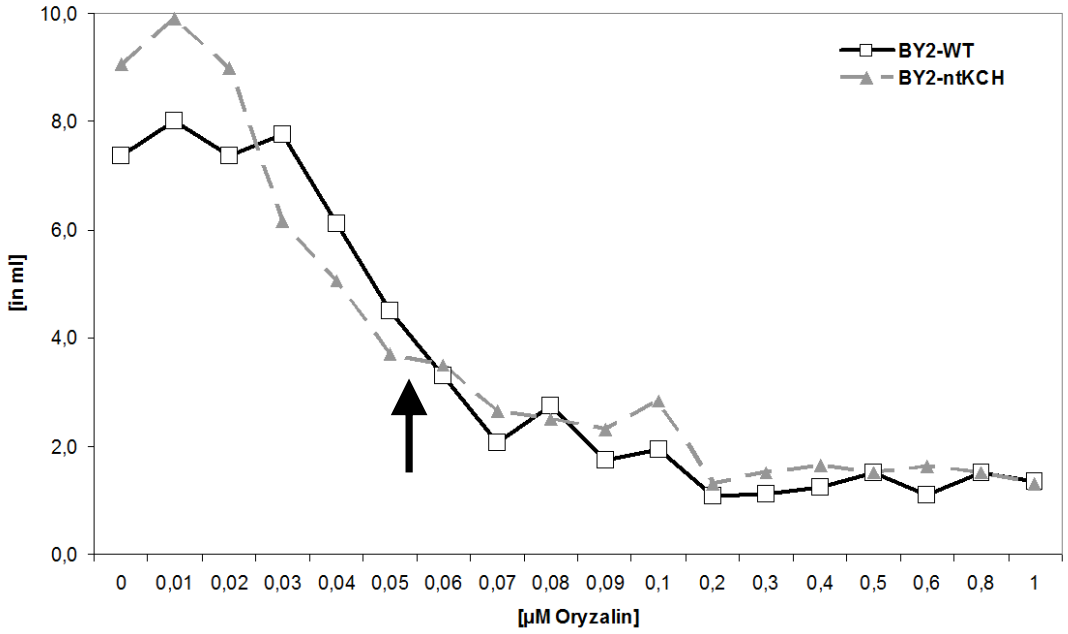

Fig. 19: Oryzalin dose curve via packed cell volume of BY2-WT and BY2-ntKCH cell line. Higher packed cell volume of BY2-ntKCH cells result from ntKCH overexpression. The earlier decrease of the ntKCH curve suggests a higher susceptibility to Oryzalin. These results were also was taken to set the concentration of Oryzalin in the consequent experiments to a value of about 0.05 µM (array).

3.2.5. In low concentrations Oryzalin supports cell elongation

As already mentioned above, cortical microtubules are arranged in perpendicular direction to the cell axis in interphase. The arrangements of microtubules are responsible for elongation direction and also cell size. Furthermore ntKCH might serve as motors for cellulose synthase tracking along cortical microtubules. This work is exploring the orchestration of microtubules, kinesins, and PLD in a cytoskeleton-plasma membrane-cell wall continuum. To explore the important role of this orchestration a cell length measurement of BY2-WT and BY2-ntKCH with treatment of PLD signalling pathway manipulating agent and Oryzalin was performed. For this experiment, 1-but and PTX were selected. The results are blotted in **Fig.20a, b**. As already described about, 1-but treatment without Oryzalin is elongating the cells. In contrast PTX treatment without Oryzalin is shortening the cells. Surprisingly, low concentrations of Oryzalin, in concert with both PLD signalling pathway manipulating agents, support cell elongation. Especially treatment with the PLD inhibiting agent PTX, cells is heavily elongated. This effect is disappearing with the rise of the concentration of Oryzalin. This elongation effect in response to PLD signalling pathway manipulating agents and Oryzalin is present in both, BY2-WT and BY2-ntKCH cell lines. ntKCH cells seems to be highly elongated in response to the treatment of Oryzalin and PTX.

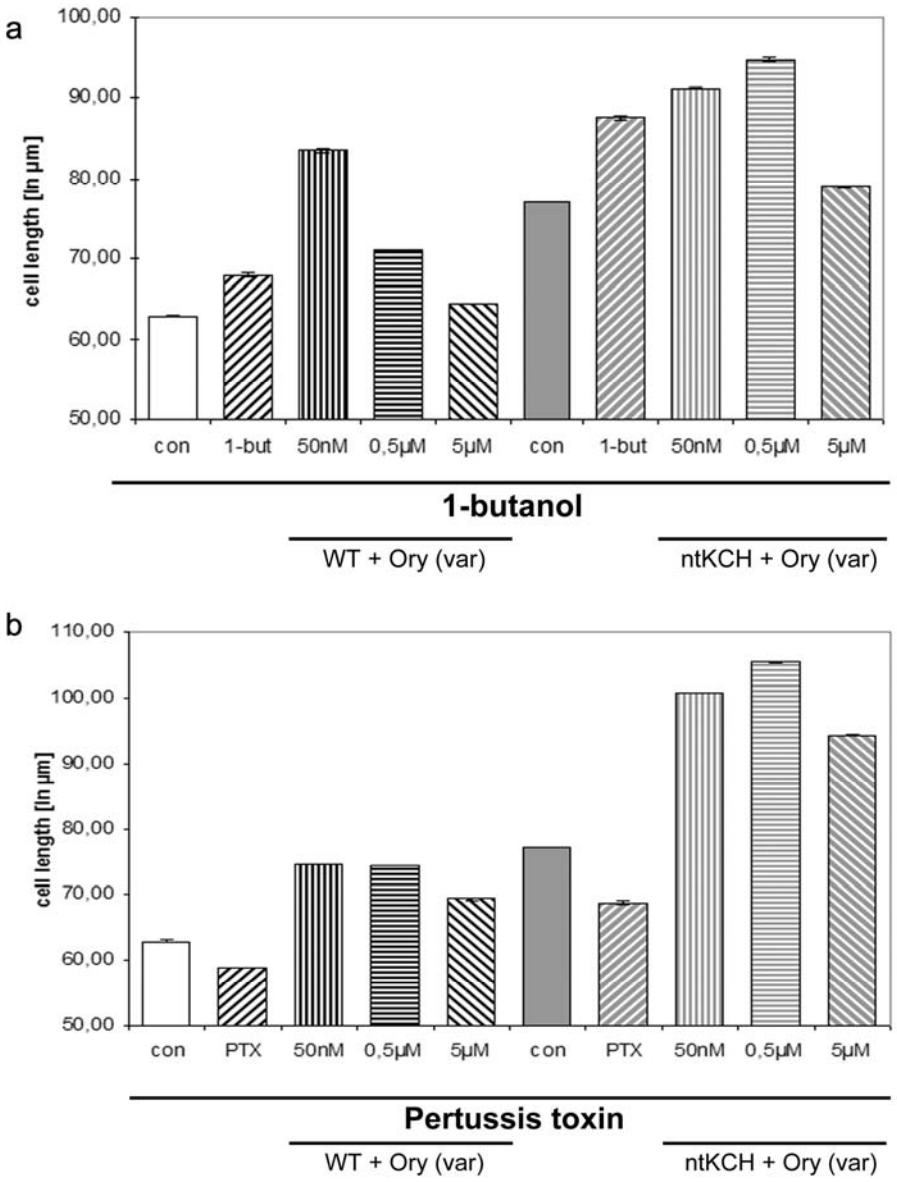

Fig. 20: Cell length measurements in response to the treatment of the PLD signalling pathway manipulating agent 1-butanol (**a**) and PTX (**b**) and Oryzalin in various (var) concentrations. In low concentrations of Oryzalin cell elongation the cells seem to be more elongated. This elongation effect is disappearing with increased concentrations of Oryzalin. BY2-WT and BY2-ntKCH cell lines shows similar effects. Compared to the other measurements, the elongation of higher concentrations of Oryzalin in ntKCH cells treated with PTX is much more elevated.

3.3. Molecular Analysis of a PLD overexpressing BY2 cell line

3.3.1. Microscopic characterisation of BY2-PLD cell line

For the molecular analysis of the enzyme Phospholipase D in purpose of its role in cell biology, a PLD overexpressing BY2 cell line was generated (ALEKSANDRA ZIMMERMANN, BOTANICAL INSTITUTE, KIT, GERMANY). The target gene coding for a Phospholipase D from *Oryza sativa* L. was coupled to GFP to make localisation under the fluorescent microscope possible. Representative samples of pictures in merged light and fluorescent microscopy are shown in **Fig.20a,b,c**, and **d**. As already mentioned above, ntKCH overexpression triggers elongation of the cell (FREY ET AL. 2009). This elongation is clearly visible in comparison of the WT cell line (**a**) and the ntKCH cell line (**b**). In contrast to these two cell lines, the BY2-PLD line possesses massive phenotypical differences. Cells in this BY2-PLD line seem to stick together in clusters. These clusters occur typically in cultures, that cells have lost its elongation direction. Due to this, cells divide in randomized direction leading to the clusters that are shown in the pictures (**c**) and (**d**). Although the fluorescent signal of GFP is present in every cell of the culture, it is expressed in different levels. Especially in those cells that stick together in the clusters, the level of expression seems to be lower than in cells that are released. Also the loss of the directionality seems to be strengthened in clustered cells. For studying defined localisation of PLD, a simple fluorescence microscope with low resolution is not sufficient. A comparison with freely expressed cytosolic GFP was not distinguishable.

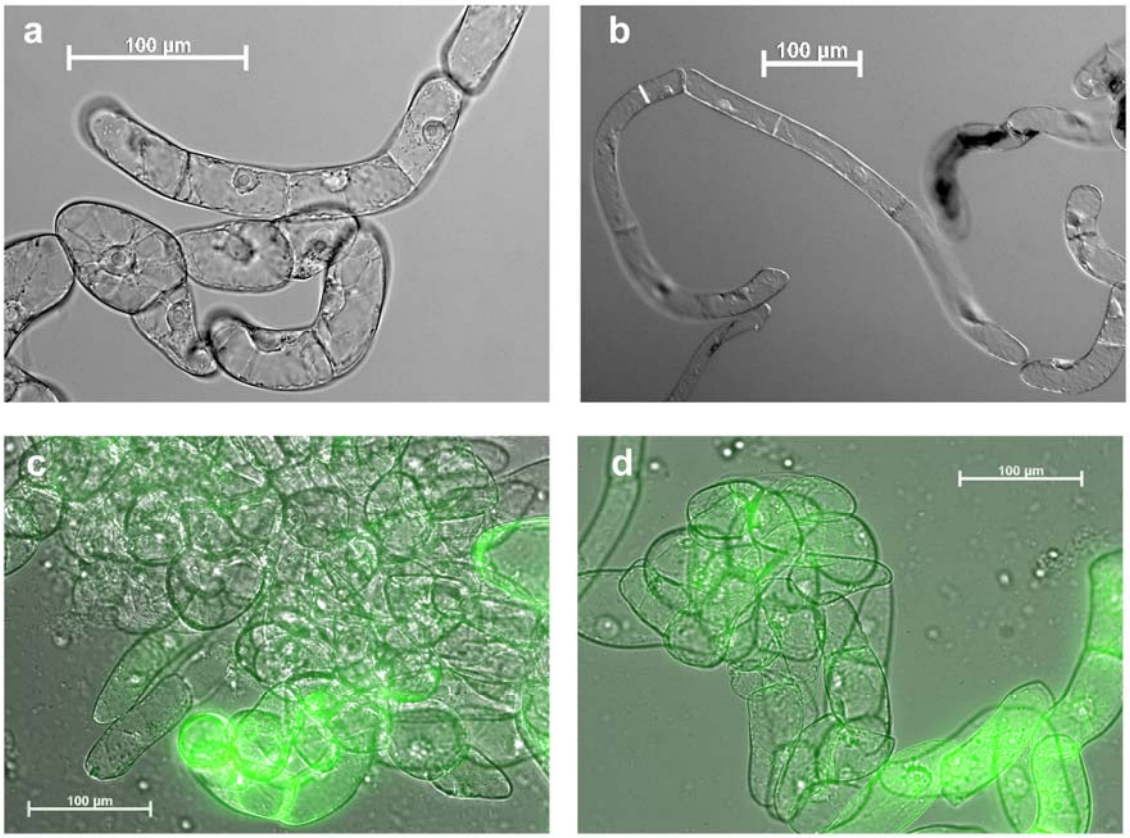

Fig.20: Light and fluorescent microscopically characterisation of BY2-WT (**a**), BY2-ntKCH (b), BY2-PLD (**c+d**). In comparison to BY2-WT and BY2-ntKCH, cell files of BY2-PLD are not that easily comparable, rather they form cell clusters that are typical for cells that lost their directional elongation growth. GFP signal in BY2-PLD is visible with various concentrations, depending on the location of the cells in these clusters.

3.3.2. Spinning disc microscopical characterisation of PLD

As already described above, in a simple fluorescent microscope, an exact interpretation of the localisation of PLD in the cell is not viable. The PLD overexpression cell line BY2-PLD and the BY2-freeGFP, a typical control for microscopical studies, are not capable of being differentiated. In the freeGFP cell line GFP is genetically transformated without a target gene and remains in the cytosol. To solve this problem modern confocal spinning disc microscopy was performed. Here, the differences between BYP-PLD and BYP-freeGFP are clearly visible. Representative pictures are shown in **Fig.21,c,** and **d**. For a better

comparison, the control cell lines cytosolic By2-freeGFP (**Fig.21a)** and BY2-ntKCH (**Fig.21b**) are shown first. BY2-freeGFP shows a green signal in the cytosol, the ntKCH-GPF construct occurs like pearl necklaces directly under the plasma membrane. This arrangement is due to its linkage to cortical microtubules (FREY ET AL. 2010). The PLD signal lies in smearlike structure right underneath the plasma membrane that is especially visible at the cell-to-cell border (white array in (**c**)). Especially at spots where borders of tonoplasts from interior cell touch the plasma membrane, these clusters occur more heavily (white array in (**d**)). When generating a 3D picture of the cell, these smearlike PLD signals are also visible in the cell interior (**e** + **f**; white array in (**f**)). The localisation of this fraction of PLD leads to the assumption, that it is linked to the tonoplast. A closer look to PLD at the plasma membrane shows a clustered distribution.

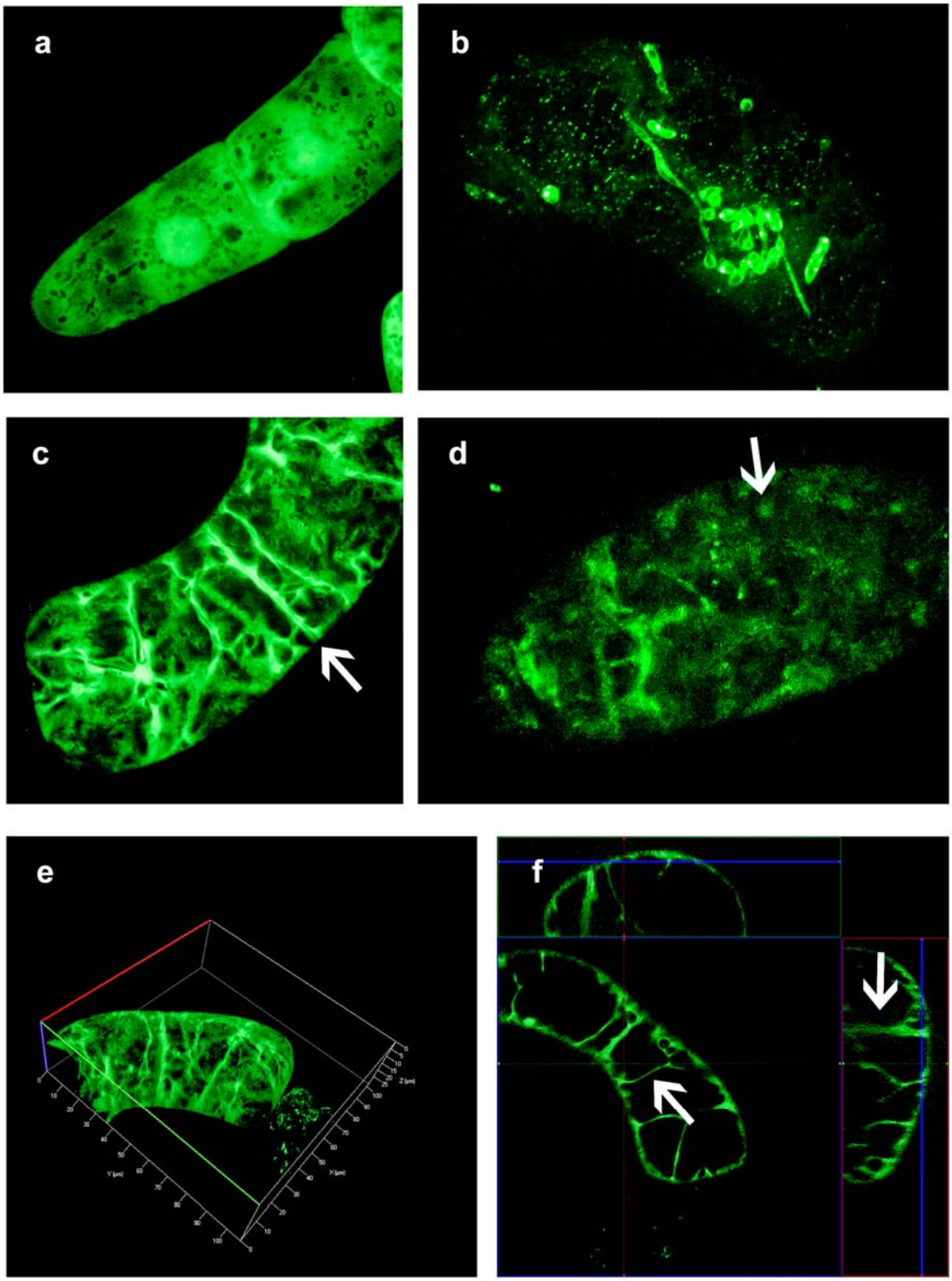

Fig.21 (a,b,c,d,e,f,): Confocal spinning disc microscopical characterisation of freeGFP, ntKCH, and PLD. **(a)** freeGFP signal is constantly expressed and released in the cytoplasm. (**b**) ntKCH appears in pearl necklaces structures right underneath the plasmamembrane. (**c**) PLD signal is showing a smearlike structure, also underneath the plasmamembrane. (**d**) getting closer, single PLD signal seem to be clustered. Also at the cell interior these PLD stuctures are visible (**e**+ with array in (**f**))

3.3.3. Colocalisation studies of PLD-GFP with Microtubules using immunofluorescene staining

To investigate the direct interaction of PLD with microtubules, they were stained with an antibody binding to β Tubullin. The general yield of successfully stained microtubules in PLD overexpressing cells was enormously limited because of the fact, that PLD cells are aggregated in sticky clusters. In the few cells, were microtubules are visible, cortical microtubules are not bundled, but they are heavily disordered. An explicit co-localization of microtubules and PLD is not visible (**Fig.22**).

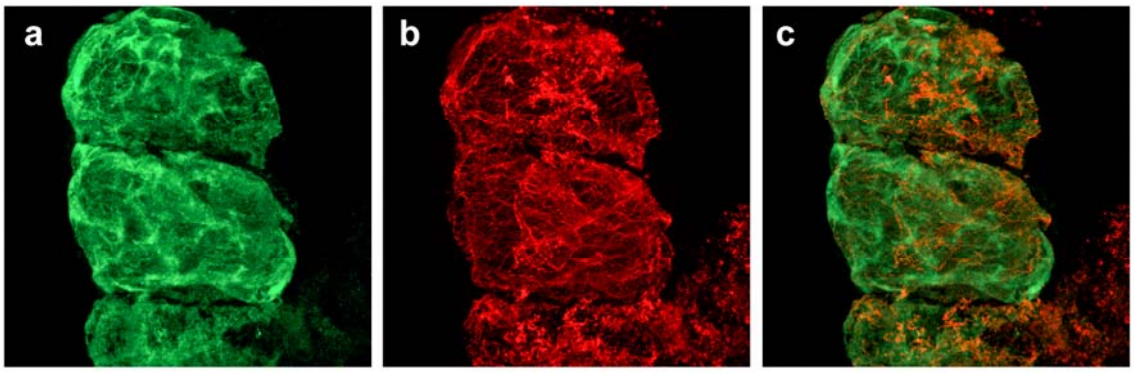

Fig.22. Co-localization studies of PLD and microtubules by immuno fluorescence staining. (a) PLD signal is already disordered after immunofluorescence staining. (b) Microtubules are not bundled but disordered. (c) the merge of both pictures so not allow any colocalisation studies.

3.3.4. Changes of transverse cortical microtubules to longitudinal ones is not responsible for cell elongation during log phase to stationary phase transition

To investigate if the change of orientation of cortical microtubules from transverse to longitudinal ones is responsible for the observed differences in cell elongation in the experiments of this study, another microscopical study was performed. By measuring the angle of cortical microtubules' orientation and the plasma membrane, the progress of the orientation change could be observed. As a

previous experiment non-treated BY2-WT and BY2-ntKCH cell were taken. The time point of 4 and 5 days after subcultivation, was set as the point of transition between log phase were typically cells elongate and stationary phase were cell elongation has already been finished. However, differences between both cell lines are not observable at these time points.

4. Discussion

1.1. KCH possess fundamental function in processes of plant cell biology and pattern formation

As already mentioned above KCHs ability of cross-linking actin filaments and microtubules suggests a function in plant cells' pattern formation. The movement of the nucleus is dependent on a perinuclear cage of actin filaments and microtubules that are linked by KCH kinesins. This linking might be responsible for pulling the nucleus into the cell centre to prepare the onset of microtubules. The first evidence for this function was shown by measuring the nuclear positioning in KCH overexpressing cell lines in 2010 (FREY ET AL. 2010). This study investigates how this cell line reacts when adding another stress factor on the cytoskeleton, nsPEFs a specific ultrashort electroporation. The effects of these both factors on mitosis, the delay of the nuclear positioning for preparing the onset of mitosis and the delayed mitosis itself is supporting this hypothesis enormously. The delay of mitosis in responds to application of nsPEFs alone might be triggered by the bundling and detachment of actin and microtubules from the plasma membrane (BERGHÖFER ET AL, 2010). The results of KCH overexpression cell line are showing a similar pattern. Also the additive affect when taking both factors together underlines this similarity. KCH overexpression might disturb the cross-linkage of actin filaments and microtubules involved in the positioning of the nucleus in such an enormous way, that the onset of mitosis is delayed. The investigation of the mitotic index indicates that in some of the cell the effect is preventing the cell to undergo mitosis. Consequently, these cells die before they can undergo cell division.

The direction of cell elongation is controlled by the arrangement of cortical microtubules. These cortical microtubules are arranged in the same perpendicular direction to the cell axis. The 'monorail model' postulates, that these cortical microtubules serves as tracks for the complex cellulose synthase, that produce cellulose microfibrills embedded into the matric of polysaccharides (PARADEZ,

2006). Furthermore KIF4 kinesin family might serve as motors for cellulose synthase tracking along cortical microtubules (ZHONG ET AL., 2002). Application of nsPEFs probably results in a release of microtubules from actin, which promotes alignment of microtubules in a transverse array of parallel bundles. This alignment requires microtubule detachment of the membrane (SAINSBURY ET AL., 2008). The resulting reinforcement of transverse microtubule alignment is then transduced into a transverse orientation of cellulose microfibrils (the cellulase synthase move along microtubules), which provides the anisotropy of cell-wall extensibility required to sustain elongation of the expanding cell. All these processes driving cell elongation proceed to a certain extent also under control conditions; however, the results indicate that KCH stimulates cell elongation and nPEFs promote this stimulation in the KCH overexpressor but not in the wild type. In contrast to the disturbance of mitosis this cell elongation stimulus can be explained by the increased deposition of cellulose due to enhanced activity of cellulose synthase motor action. The stimulus of cell elongation is only present at the first day after treatment and subcultivation and not the fifth day. This phenomenon is easily explained by the fact, that cell elongation ends approximately at day four, when microtubules changes their arrangement from transverse to longitudinal ones.

1.2. In addition to intracellular targets nsPEFs can specifically affect targets located at the plasma membrane

The debate, whether nsPEFs cause their biological effect by altering targets at the plasma membrane or by directly perturbing targets in the cell interior, has remained difficult, because the experimental evidence derives from different biological systems. Here, this issue is addressed by using a single experimental system, but a target that is localized either in the cell interior or adjacent to the plasma membrane (KLOTZ & NICK, 2012).

As already mentioned above the movement of the nucleus is dependent on a perinuclear cage of actin filaments and microtubules that are linked by KCH kinesins. When this linkage is disturbed by overexpression of KCH, nuclear

movement is delayed. Similarly, a mild nsPEF treatment was able to cause a similar delay. A combination of both factors acted synergistically. At first sight, this experiment suggests a site of action at the nucleus, i.e. in the cell interior. However, it should be kept in mind that the nucleus is tethered, through a radial network of actin filaments and microtubules, to the cell wall by means of transmembrane proteins (PICKARD 2008). If the attachment of this radial network to the plasmamembrane was disrupted by nsPEFs, this should result in a similar delay of nuclear migration. Thus, the analysis of nuclear migration is not sufficient.

Therefore one can make use of a second cellular function that is unequivocally linked to the cell periphery: cell expansion which is controlled by a directional extensibility of the cell wall that, in turn, is regulated by the orientation of cortical microtubules binding a specific subset of KCH subtending the plasma membrane. In fact, we observed that (i) overexpression of KCH promoted cell expansion, (ii) this promotion was stimulated by nsPEFs in the KCH overexpressor, but not in the non-transformed wild type (i.e. in a strict synergy), and (iii) that cell expansion responds more sensitively as compared to cell division. These phenomena are specific for the membrane-associated cytoskeleton. Thus, whereas the observed effect on nuclear migration (and consequently mitotic activity) can be also explained by a site of action at the plasma membrane, it is not possible explain the effect of nsPEFs on cell expansion by a site of action at the perinuclear cytoskeleton. A model for this side result of this work is shown is **Fig.23**.

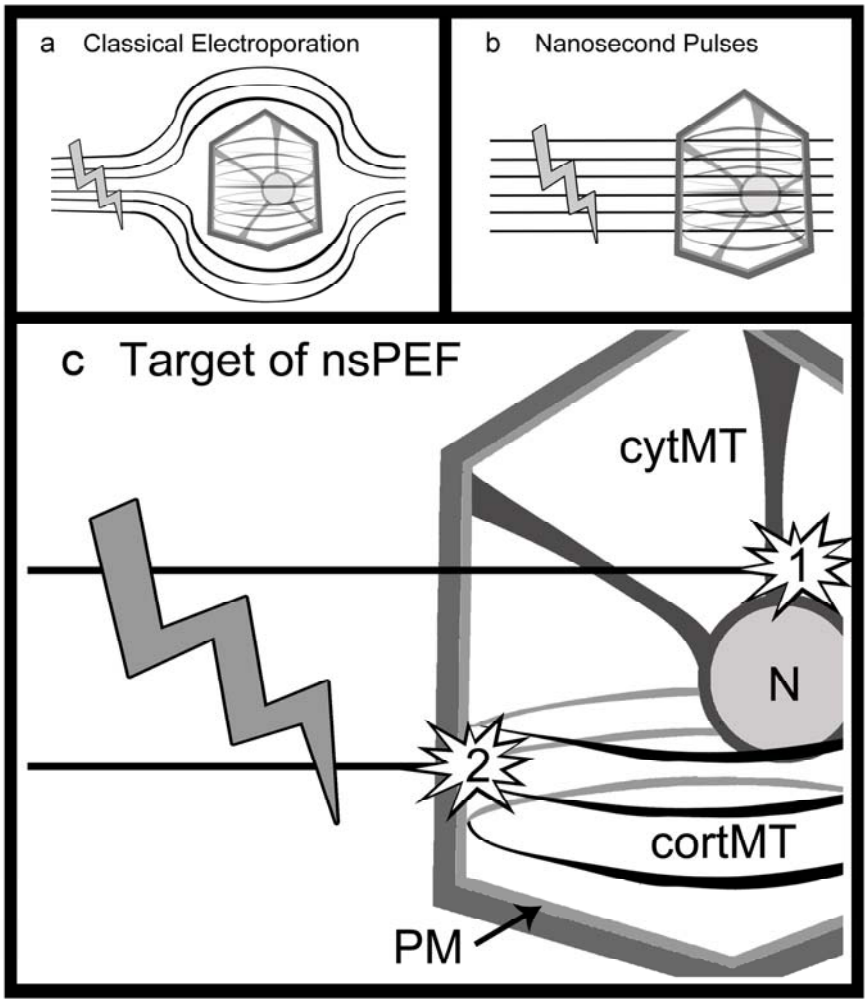

Fig.23: Model for the site of action of pulsed electrical fields in plant cells. Classical electroporation uses pulses with low field strength and high durations. Such pulses are dissipated by charging of the cell membrane **(a).** Ultra-short nanosecond pulses (nsPEF) with high field strengths and low durations are thought to penetrate into the cell interior and to affect intracellular targets before being dissipated by charging of the cell membrane **(b).** In this work we investigate the location of the cellular targets of nsPEFs. We show that in addition to intracellular targets **[1]** nsPEFs can specifically affect targets located at the plasma membrane **[2] (c)** Abbreviations: cytMT cytoplasm microtubules, corMT cortical microtubules, N Nucleus, PM plasma membrane.

The differences in cell expansion of pulsed cells suggest an influence of KCH on cortical microtubules and their time point of changing the direction of transverse microtubules to longitudinal ones, to stop the elongation process. In hypothesis, the orientation change from transverse to longitudinal microtubules must be shifted to a later time point in KCH overexpressing cells. This shift was tried to be

investigated by immunofluorescence stained microtubules in wild type and KCH overexpressing cells. As the chosen time point for this experiment was the transition from log phase, when cells are elongated to stationary phase when cell elongation has already been finished. In BY2 tobacco cells this time point is at day four or five. However the results did not show any differences in BY2-WT and BY2-ntKCH cell lines. One explanation for this fact would be an orientation change of the microtubules at an even later time point of day six or seven. Indeed, former studies showed that already at earlier time points, ntKCH cells are elongated, that does not support this hypothesis. In fact, an explanation of an improved communication of transverse cortical microtubules due to ntKCH is more plausible. Nevertheless the hypothesis of orientation change of cortical microtubules was brought into focus.

Other important forces for reorientation of cortical microtubule from transverse into longitudinal direction are abiotic stimuli like physical and thermal stresses, or salinity (PETER NICK, 2008). The term ‘tensegrity’ describs the characteristics, that the cytoskeleton is combining rigid microtubules and tense actin filaments providing the ability of the installation of a sensitive system for signalling to abiotic stimuli. Due to its calponin-homology domain, KCH1 is cross-linking actin and microtubules as part of a cytoskeleton-plasma membrane-cell wall continuum, that tether the nucleus to the plasma membrane and drives the force for positioning of the nucleus to the center of the cell for onset of mitosis. This cytoskeleton-plasma membrane-cell wall continuum also serves as signalling hub.

For clarifying the putative role of KCH in this signalling hub, it is necessary to bring another player of this continuum into play: phospholipase D the mayor component of PLD signalling pathway. In this signaling cascade G protein coupled surface receptors transmit external signals like pathogen attack, salinity or drought to heterotrimeric G proteins. The incoming external signal is triggering the exchange of GTP to GDP at the heterotrimeric G proteins. This exchange is responsible for the dissociation of the α-subunit. The remaining βγ-subunit is than activating. PLD, that has as its main enzymatic product phosphatidic acid, which is a mayor second messenger in plant cells. The exchange of GTP to GDP is an easy target to manipulate the whole PLD signaling pathway artificially. Aluminium tetrafluorid as

a PLD activator and Pertussis toxin as a PLD inhibitor was used for pharmaceutical studies. Furthermore 1-butanol and 2-butanol are also used. Both butanol confirmations are activating PLD, but as 1-butanol also serves as acceptor for Phosphatidic acid and consuming it thereby, it is described in literature as PLD signaling pathway inhibitor.

1.3. Results giving evidence for an involvement of KCH in a cytoskeleton-plasma membrane-cell wall continuum that serves as biosensors

To investigate the toxicity of the pharmaceuticals, an Evans blue test was performed, that indicated an increased mortality through manipulating PLD signaling artificially in KCH overexpressor line but not in the wild type. Because of this difference, a multiplicative effect of both factors: PLD signaling manipulating and KCH overexpression was found. This is the first small evidence for an involvement of KCH in the cytoskeleton-plasma membrane-cell wall continuum. To exclude that these effect is deriving from preceded genetic transformation generally, also a cytosolic freeGFP cell line was treated with the pharmaceuticals, indicating no or only a negligible effect than in KCH line. With these results one is able to assume, that the effect is only derived from KCH overexpression. Furthermore this study represents a template for finding the correct concentration for subsequent experiments.

The following measurement of the mitotic index is showing a decelerated onset of mitosis after treatment with all PLD signaling pathway manipulating agents. In contrast to nsPEFs, taking both factors together, PLD signaling manipulating and KCH overexpression it does not show any additive effect on mitosis. Again an experiment dealing with players of the sub-membrane area of the cell was needed. Because of the evidence of an involvement in tracking cellulose synthase at cortical microtubules a measurement of the cell size is needed. Here, cells treated with the PLD activating agents Aluminum tetrafluorid stimulate cell elongation of both cell lines slightly. 1-butanol treated cells react, especially in the KCH overexpression cell line with enormous elongation stimulation, while 2-butanol

treated cells react unequally. The difference between 1- and 2-butanol might be triggered by the concluding difference in the concentration of Phosphatidic acid. This concentration difference is triggered by the fact, that 1- butanol serves as accepter for Phoasphatitic acid and consuming it thereby. Thus, one could assume that cell elongation progresses might be influenced by the second messenger Phosphatidic acid. Because no other aspects are in this favor, this hypothesis remains without reply. Cells treated with PLD signaling pathway inhibiting agent PTX stop cell elongation earlier and remain shorter than untreated ones. Especially the results of 1-butanol and KCH overexpressing together show a direct involvement of KCH in response to PLD signaling triggered exchanges. KCH might be in that turn a force for reorientation of microtubules from transverse to longitudinal direction after exception and transmission of PLD signaling mediated stimuli. This experiment is a small, but important proof for KCHs modulation in sensing.

1.4. KCH is involved in sensing to external signals

As discussed above microtubules and actin filament are, with their tensegrial characteristics able to provide ideal conditions for answering external, abiotic stimuli. This ’tensegrity’ is collapsing either when actin filaments or microtubules are bundled for variable reasons, of one of the two members is unable to provide fresh supply of construction material. Because especially microtubules are enormously dynamic structures, this collapse would appear rapidly. To induce this collapse of the cytoskeleton ‘tensegrity’ system, the herbicide Oryzalin that inhibits microtubules polymerization was applied. A packed cell volume experiment integrating diverse concentrations of Oryzalin was preformed to investigate the general differences in the reaction of wild type and KCH overexpressing cell line. Generally the KCH cell lines seem to be slighly more susceptible to Oryzalin, indicating that disturbed construction of the cytoskeletal system due to KCH overexpression lead, under treatment of Oryzalin to an earlier collapse of the cell. However, a direct involvement of KCH in sensing was only possible by performing cell length measurements studies. Furthermore signaling is brought back into play

by treating the cells not only with Oryzalin but also with the PLD signaling pathway manipulating agents 1-butanol and Pertussis toxin.

The cell length measurement experiments combining three factors together, PLD manipulation, KCH overexpression and Microtubule disturbance. It showed surprisingly that treatment of the pharmaceuticals together with low concentrations of the microtubule drug Oryzalin triggers cell expansion in WT cells. There are several publications of signaling involvements of Microtubules on salinity and drought, where low concentrations of Oryzalin were increasing the defense of such stressors. Interestingly this effect is shifted to higher concentrations of Oryzalin in ntKCH cells, suggesting a stabilization effect of the 'tensegrity' system by KCH overexpression. Apparently, cell shrinkage of cells due to PTX treatment is inverted by additional treatment with Oryzalin.

Although the involvement of KCH in sensing is far from been understood it is a first speculative assumption for that hypothesis. A spatial explanation for the involvement for KCH in sensing could again offer the CPMCW-continuum that also serves as signaling hub. Signal sensitive molecular cascades in this CPMCW-continuum like PLD signaling pathway are able to transmit the external signals to the tensegrial system of transverse cortical microtubules and actin filaments via KCH. A specific reaction of this signal, for example verifying of microtubules' compression and actin filaments' tension forces to react in a specific pattern manner, e.g. cell length differences, variation in composition of cellulose beein deposited into the extra cellular matrix, or a variation of the time point for the onset of mitosis. The possible kind of signal is variable. When thinking of tensigrity, one could assume that mechanosensing or gravitropism are the preferential signals for KCH sensing. However, changes in the PLD experiments in this dissertation, provide evidences for other external stressors (e.g. drought, salinity, pathogen attack), that are processed via KCH sensing. A possible spatial arrangement of the involvement of KCH in the CPMCW-continuum is shown in the model in **Fig.24**.
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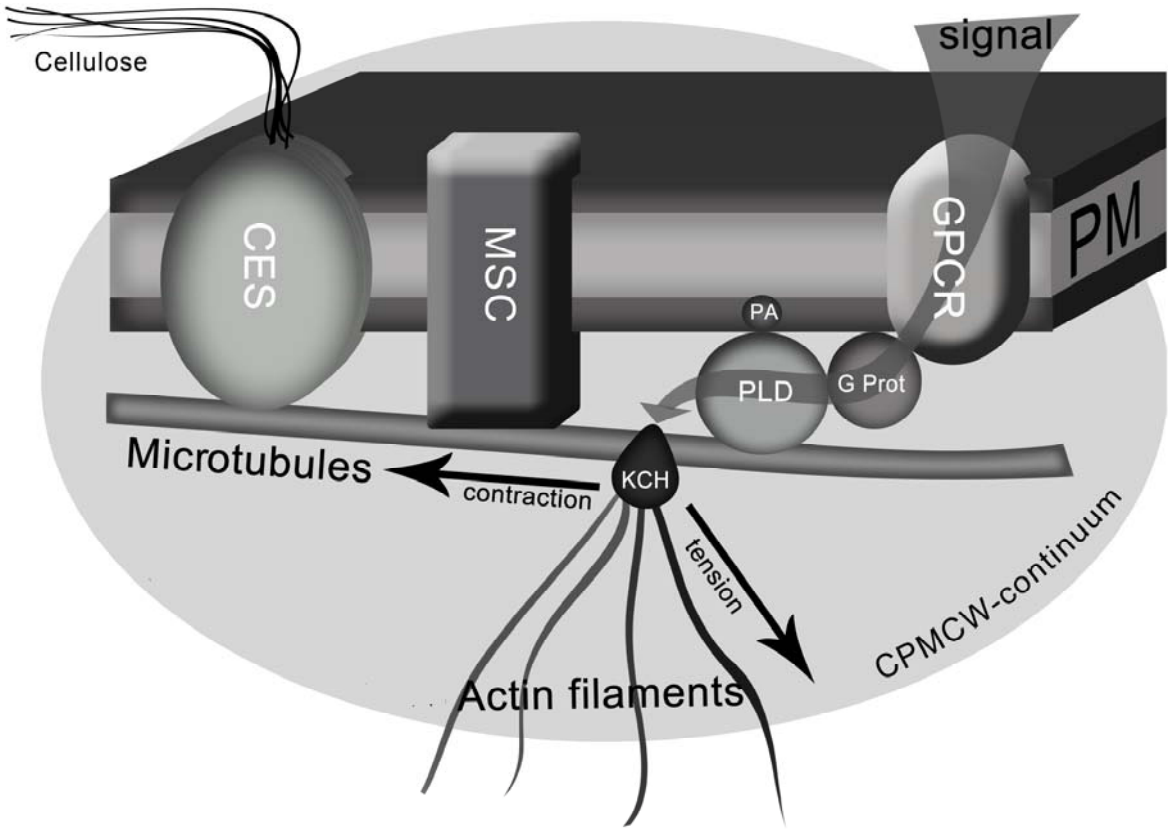

Fig.24: Model for KCHs being involved in the Cytoskeleton-plasma membrane-cell wall continuum. Various signals are being processed via the PLD signaling pathway .Signal perception of G Protein couple resecptors (GPCR), transmission of the signal via G proteins (Gprot) and Phospholipase D (PLD), a main product of this cascade is Phosphatidic acid (PA) that serves as second messenger and construction material for the plasma membrane. The signal reaction is possibly processed via KCH that changes the relation between microtubules' contraction and actin filaments tension forces, leading to a specific reaction of the cells pattern. Also Cellulose synthase or mechanosensitive ion channels could be involved in this process.

1.5. BY2 cell loose directionality due to PLD overexpression

In this work studies of fluorescence microscopy of a BY2 cell line overexpressing a PLD of *Oryza sativa* have brought several interesting findings. The cell line was generated several years ago (Aleksandra Zimmermann, Institute of Botany, Karlsruhe Institute of Technology, not published), but due to the inability of differentiation of this PLD-GFP cell line and a cytosolic freeGFP cell line it sank

into oblivion for some time. Advanced microscopy technology with higher resolutions brought PLD-GFP back to mind and in this work the cell line was investigated phenotypically. When observing the cell line in a normal light microscope, an interesting phenotype is visible. The cells accumulate in clusters and they seem to stick together. Often these cells do not grow in cell files, however they remain single cells or they constrict from their mother cell directly after division. Furthermore they possess often a round structure that indicates a loss of the directionality due to PLD overexpression. Only those cell that are not clustered, grow in cell files and show a directional cell elongation. Because of the direct association of PLD to microtubules, overexpression might lead to a disturbed network construction of cortical microtubules, that unable cellulose synthase to embed cellulose into cell wall matrix for cell elongation. The fact, that PLD is also associated to the cell wall is underlining this hypothesis. However, prior experiments in this work found a direct involvement of microtubules in plant signalling, which was found by cell elongation stimuli of the microtubule drug Oryzalin in very low concentrations. One could also conclude from the loss of the directionality that overexpressed PLD leads to a disturbed progress of signalling processes. As we have heard above, signalling to external stresses could also lead to disturbed cell expansion by alternating the time of exchange of transverse microtubules into longitudinal ones. The exact reason for the phenotype of PLF-GFP cell line remains unknown at this point.

As already mentioned before, PLD overexpressing cell line was not distinguishable to cytosolic freeGFP taking simple fluorescence microscopy. Using highly advanced spinning disk confocal microscopy the differences were clearly visible. PLD is expectedly located at below the plasma membrane. Also at the periphery of plasma streaming of the cells fully packed with vacuoles PLD-GFP signals were observed. These findings suggest an association of PLD not only the plasma membrane but also to the tonoplast. At the tonoplast the PLD from rice which was used in this study could function in phospholipid recycling as already found for a PLD in Arabidopsis (YAMARYO ET AL., 2008).

To investigate the direct interaction of PLD with microtubules they were stained using a immunofluorescence protocols. The general yield of successfully stained

microtubules in PLD overexpressing cells was enormously limited because of the fact that PLD cells are accumulated in sticky clusters and the antibody seem to be unable to reach the cell surface. In the few cells successfully stained are surly those that are released from the sticky cluster. These cells do not represent cell who have lost their directionality. For a co-localization of microtubules and PLD these cells are sufficient. Microtubules in these cells are not bundled, but they are heavily disordered. An explicit co-localization of microtubules and PLD is expectedly not visible.

1.6. Future prospects

Using KCH overexpession BY2 cell lines, this study shows that nsPEFs can cause a specific biological response produced by the cytoskeleton adjacent to the plasma membrane of plant cells. We cannot exclude that nsPEFs are also active at the cell centre (although the observed effects can be understood as secondary effect from cytoskeletal perturbation at the plasma membrane), but our results showed a clear side of action of these ultrashort pulses at the membrane-associated cytoskeleton, even at very short pulse durations. To investigate the explicit target of nsPEFs one could pulse other cell lines to understand especially the role of cytoskeleton in response to ultrashort electrical fields. Some of these other cell lines were already processed and published in KÜHN ET AL. 2013. Especially cell lines visualizing microtubules (BY2-beta-TUB) and actin filaments (BY2-GF11) showed cytoplasmic and cytoskeletal changes.

This study also showed a function of KCH in progresses of pattern formation like cell elongation and nuclear positioning. Furthermore it raises high evidences for an involvement in transduction and processing of external signals through PLD signalling pathway. Both functions of KCH could be underlined by converse results of a BY2 cell line suppressing ntKCH. This cell line could be generated by RNA interference. In this work it was heavily tried to generate such a suppressing line. All successfully transformed cells were showing suppressed expression of ntKCH, dedicated by common expression analysis. The cells were growing normally until they were subcultivated on media containing antibiotics for maintaining selective

pressure. This sudden death is indicating a disturbance in antibiotic resistance through the transferred resistance gene. Generally production of RNAi cell lines in plants is not often successful; due to this the explicit reason of the sudden death remains unclear.

To explain the role of KCH in sensing to abiotic stress, more combinations of all stressors used in this work are needed. PLD and KCH overexpression, PLD signalling manipulation pharmaceuticals, treatment of Oryzalin and even nsPEFs combined with the different experiments could help to underline the findings of this work. Especially immunofluorescence staining and other microscopical experiments are needed. Interesting would be studies of the exchange of transverse microtubule into longitudinal ones at KCH cells treated with PLD manipulating agents. These investigations could help to understand chances in cell expansion and also the role of KCH in PLD mediated signalling. Furthermore even higher advanced microscopy techniques are able to help visualising the partners with even higher resolution. Total Internal Reflection fluorescent microscopy (TIRF) that is visualizing especially regions blow the plasma membrane in size of down to 200nm can be observed. This kind of microscopy could help to co-localisation of cortical microtubules and PLD, right below the plasma membrane.

For a direct interaction of the partners in the CPMCW-continuum especially PLD, Microtubules, and as this work showed also KCH biochemical protein-protein interaction studies are needed. Highly advanced Cytotrap® experiments, the new generation of yeast-two hybrid studies can be one important tool for such direct interaction study.

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