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A role for the phytocalpain DEK1 in plant mechanosensing

Enrique D. Neumann

Supervisor Dr Gwyneth Ingram

Thesis submitted for the Degree of Doctor in Philosophy

The University of Edinburgh

2013

II. Declaration

I declare that all the results presented and statements included in this thesis are my own and that any contribution made by others is clearly cited.

III. Abstract

The effect of mechanical stimulation in plants has been studied in depth for more than a century. This type of stress has been shown to trigger alterations in development such as stunting, thickened stems and differential cell wall deposition. These responses are very likely to be initiated at a subcellular level, but the molecular mechanisms transducing mechanical signals into intracellular responses still remain unknown in plants. In this thesis I test the hypothesis that the membrane anchored protein Defective Kernel 1 (DEK1) could act as a plant-specific mechanosensor in plants.

Constitutive overexpression of the cytoplasmic CALPAIN domain DEK1 causes a phenotype in *Arabidopsis*, that that resembles that of mechanically stressed plants. The CALPAIN domain of DEK1 shows a very high homology with animal calpains; a class of calcium-dependent Cysteine proteases which undergo a calcium-stimulated CALPAIN domain-releasing autolytic cleavage event during activation. A similar autolytic cleavage event has been observed in DEK1 which, together with the fact that the CALPAIN domain alone can rescue the embryo-lethality associated with loss of DEK1 function, has led to the suggestion that this domain represents an activated form of the protein. I show that like mechanically stressed plants, CALPAIN overexpressing plants show a modified call wall composition. Consistent with this, transcriptional analysis of these plants shows a deregulation of genes encoding cell wall modifying enzymes, amongst others. Other characteristics of mechanically stimulated plants which I have characterized in CALPAIN overexpressing lines include late flowering and thickened stems. Therefore, I proposed a model in which the CALPAIN domain of DEK1 acts as an effector which is normally activated by mechanical stimulation. In this model, the transmembrane domains of DEK1 would regulate activation (cleavage) of the CALPAIN domain, potentially in response to mechanical stress.

In order to test this model further, CALPAIN overexpressing lines were generated in a *dek1* mutant background. If the model is correct, these plants should not only behave as if responding constitutively to mechanical stimulation, but should also lack appropriate responses to applied mechanical stimuli due to lack of the mechanosensory integral membrane domain of DEK1. My results confirm that the absence of the transmembrane domains of DEK1 is indeed translated into a lack of some, but not all responses to mechanical stimulation compared to wild-type plants. Furthermore, the lack of the transmembrane domains of DEK1 correlates with the absence of a mechanically-triggered calcium flux in the plant. Thus my work suggests that the transmembrane domains of DEK1 are involved in sensing mechanical stimulation, via the regulation activity of a mechano-sensitive calcium flux at the plasma membrane.

In summary, my proposal is that Defective Kernel 1 (DEK1) acts both as a key mechanosensory cellular component, and as the first effector of the signalling cascade in response to mechanical stimulation, via an autolytic activation in response to mechanical stress.

IV. Acknowledgments

First and foremost I would like to thank Gwyneth Ingram. Without her this thesis would not have been possible. It was a true pleasure having her as a supervisor and sharing together deceptions and excitement about discoveries. Her passion for science was an inspiration throughout the whole project.

I should thank everyone that contributed in this work: Kim Berthet, Roberta Galletti, Pauline Wagnon, Pascale Milani, Steve Fry and his lab, Mike Blatt and his lab, Chris Jeffree and Anne-Aliénor Véry.

Everyone at the big lab in Edinburgh deserves a big thank you as well. There I took my first steps as PhD student and everyone that answered my questions or taught me something made an important contribution to this work. Moving to Lyon was not simple, but everyone at the RDP made it much simpler. Everyone there made the arrival (and the two following years) much easier. They helped to make my terrible French become slightly less terrible. Here I have to give special thanks to Olivier and Benoît for their ideas and for their patience teaching a pure biologist some biophysics!

At a personal level a very special thank you should be addressed to Rita. Apart from a great lab partner I found in her a wonderful friend and partner of adventures.

Adri: Thanks for being there next to me during the whole thesis; I know that living with a PhD student boyfriend was not the easiest thing! Your support gave me the strength to get to the end. Your endless patience bearing all the science talk is the best proof of your love. I love you!

Last but not least, I would like to thank my loving family. They believed in me even when I had doubts. My mamá, my papá (wherever he is) and my brother Nico are the best family one can ask for. Thanks for your support and for being so proud of me!

¡Gracias a todos! Thank you all! Merci à tous!

V. Abbreviations

Ag	Silver
AIR	Alcohol insoluble residue
Arg	Arginine
Asp	Aspartic acid
Ba^{2+}	Barium ion
bp	Base pair
BSA	Bovine serum albumin
Ca^{2+}	Calcium ion
cDNA	complementary DNA
Cl^{-}	Chlorine ion
cm^2	Square centimetre
Cys	Cysteine
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycyidine 5'-triphosphate
dGTP	2'-deoxyguanosine 5'-triphosphate
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphosphate
dTTP	2'-deoxythymidine 5'-triphosphate
EDTA	Ethylenediaminetetraacetic acid
EtOH	Ethanol
g	Gram
Gd^{3+}	Gadolinium ion
GFP	Green Fluorescent Protein
Gly	Glycine
H^+	Proton/Hydrogen ion
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HPLC	High performance liquid chromatography
Hz	Hertz
I	Current
K^+	Potassium ion
kb	Kilobase
l	Litre
La^{3+}	Lanthanum ion
LB	Lauria Broth
MES	2-(*N*-morpholino)ethanesulfonic acid
mg	Milligram
min	Minute
ml	Millilitre
mM	Millimolar
mOsm	Milliosmolar
mPa	Millipascal
mRNA	Messenger RNA
MS	Murashige and Skoog
mV	Millivolt
mΩ	Milliohm
N_2	Nitrogen
Na^+	Sodium ion
ng	Nanogram
nM	Nanomolar
nm	Nanometer
pA	Picoampere
PCR	Polymerase chain reaction
pH	Hydrogen Potential
Q-RT-PCR	Quantitative-RT-PCR
revs	Revolutions
RNA	Ribonucleic acid

RPM	Revolutions per minute
RT-PCR	Real time PCR
SD	Synthetic defined media
SDS	Sodium dodecyl sulphate
SEM	Scanning electron microscopy
TAE	Tris-base acetic acid buffer
TE	Tris-EDTA buffer
Tris	Tris(hydroxymethyl)aminomethane
UTR	Untranslated region
UV	Ultra violet
V	Volt
V/V	Volume-volume
Y2H	Yeast two hybrid
YPAD	YPD plus adenine
YPD	Yeast Extract Peptone Dextrose
μA	Microampere
μg	Microgram
μl	Microliter
μM	Micromolar

VI. Contents

II. Declaration 3

III. Abstract 5

IV. Acknowledgments 7

V. Abbreviations 9

VI. Contents 13

1. Introduction 21

1.1. Mechanosensing in plants 21

1.2. Mechanical design of developing plants 24

1.2.1. Role of the epidermis in growth and mechanosensing 25

1.3. Role of the cell wall in plant mechanics 27

1.3.1. Introduction to plant cell walls 27

1.3.2. Role of cell walls in the determination of plant mechanical properties 30

1.4. Role of mechanical forces in patterning 32

1.5. How are mechanical signals perceived and what responses do they elicit? 33

1.5.1. Mechanoperception in animals 33

1.5.2. Mechanoperception in plants 37

1.5.2.1. Cell walls as mechanosensors 38

1.5.2.2. Mechanosensitive ion channels 41

1.5.2.3. Mechanical stimulation and Ca^{2+} transport 43

1.5.2.3.1. Effects of mechanically activated Ca^{2+} fluxes 45

1.5.2.4. Effect of mechanical stimulation on gene expression 49

1.5.2.5. Role of the cytoskeleton in mechanosensing 51

1.5.2.5.1. Microtubules play a key role in plant mechanosensing 53

1.6. Is DEK1 involved in mechanoperception? 58

1.6.1. Introduction to calpains ... 59
1.6.1.1. Structure of calpains ... 60
1.6.1.2. Localization and activity of calpains ... 62
1.6.1.3. Function and regulation of calpains ... 65
1.6.1.4. Calpain substrates ... 66
1.6.2. Phytocalpains ... 68
1.6.2.1. Activity and function of the CALPAIN domain of DEK1 ... 71
1.7. Aims and objectives ... 73
2. Material and methods ... 79
2.1. Plant culture and plant material ... 79
2.1.1. Cultivation of plant lines ... 79
2.1.2. NPA treated plants ... 81
2.2. DNA techniques ... 81
2.2.1. Genomic DNA extraction ... 81
2.2.2. PCR reactions ... 82
2.2.3. Agarose gel electrophoresis of DNA ... 83
2.2.4. PCR product and gel clean-up ... 84
2.2.5. Digestion and ligation of DNA ... 84
2.2.6. Construction of Gateway® plasmids ... 85
2.2.7. Transformation of plasmids into E. coli ... 85
2.2.8. Preparation of plasmid DNA from bacterial cultures ... 86
2.2.9. Sequencing of plasmid DNA ... 86
2.3. Phenotypic analysis: microscopy techniques ... 87
2.3.1. Fluorescence microscopy ... 87
2.3.2. Cryofracture scanning electronic microscopy ... 88
2.3.3. Atomic force microscope ... 89
2.4. Phenotypic analysis: biochemical analysis ... 90

2.4.1. Cellulose quantification 90

2.4.2. Lignin quantification 91

2.5. Q-RT-PCR 92

2.5.1. Plant material collection 92

2.5.2. RNA extraction and DNAse treatment 93

2.5.3. Determination of RNA concentration and reverse transcription 93

2.5.4. RT-PCR 94

2.6. Ion fluxes and concentrations 97

2.6.1. Flame photometry 97

2.6.2. *In planta* ion flux measurements (impalements) 97

2.6.3. Electrophysiological measurements in *Xenopus* oocytes 98

2.6.3.1. Building of constructs 98

2.6.3.2. Injection in *Xenopus* oocytes and electrophysiological measurements 99

2.7. Yeast techniques 100

2.7.1. Bait and prey construction for yeast 2 hybrid (Y2H) assays 100

2.7.1. Screening of interactions of calpain bait with cDNA library 101

2.7.2. Double transformation of plasmids in to yeast 102

2.7.3. Serial drop test 102

3. Effect of mechanical stress in *Arabidopsis thaliana* plants 105

3.1. Introduction 105

3.2. Mechanically stressed wild-type *Arabidopsis* show an increase in cellulose content 108

3.3. Mechanically stressed wild-type *Arabidopsis* show an increase in lignification…… 109

3.4. Transcriptional changes in osmotically and mechanically stimulated *Arabidopsis* wild-type plants 111

3.4.1. Osmotic stress up-regulates both osmotic and mechanical sensitive genes……. 111

3.4.2. Mechanical stress cannot be used as a proxy for osmotic stress 121

3.5. Summary and conclusions 125

4. Phenotypic effects of calpain deregulation 133

4.1. Introduction 133

4.2. dek1[CALPAIN] plants have thicker epidermal cell walls than wild-type plants…… 135

4.3. dek1[CALPAIN] plants produce more cellulose per unit of dry mass that wild-type plants 136

4.4. *dek1[CALPAIN]* plants have thicker stems with an increased lignification 141

4.5. Wild type cell walls show a different stiffness patterns compared to those of CALPAIN OE plants 143

4.6. Stem apical meristems of OE plants show lower anisotropy in microtubule orientation 150

4.7. Transcriptional effects of the deregulation of the CALPAIN domain of DEK1 . 155

4.8. CALPAIN OE plants may show constitutive mechanical and osmotic stress responses 160

4.9. Summary and conclusions 163

5. Identification of targets of the CALPAIN domain of DEK1 173

5.1. Introduction 173

5.2. Introduction to Yeast two-hybrid (Y2H) experiments 175

5.3. Y2H screening of CALPAIN domain putative interactors against a cDNA library….. 179

5.3.1. Identified potential targets of the CALPAIN domain of DEK1 181

5.3.1.1. Lung seven transmembrane receptor family protein (At3g09570) 183

5.3.1.2. Irregular Xylem 15 Like (At5g67210) 185

5.3.1.3. D-xylose-proton symporter-like 2 (At5g17010) 187

5.3.1.4. Cellulose synthase like A9 (At5g03760) 188
5.3.1.5. NOD-26 Intrinsic Protein 5;1 (At4g10380) 189
5.3.1.6. Membrane Anchored MYB (At5g45420) 190
5.3.1.7. Eceriferum 9 (At1g34100) 193
5.3.1.8. Sodium/hydrogen exchanger 4 (At3g06370) 196
5.3.1.9. Tubulin β chain 2 (At5g62690) 198
5.4. Summary and conclusions 200
6. Role of the transmembrane domains of DEK1 in mechanosensing 209
6.1. Introduction 209
6.2. dek1[CALPAIN] plants show a decreased sensitivity to mechanical stress 211
6.2.1. Effect on flowering time 211
6.2.2. Effect on cellulose content 213
6.2.3. Effect on lignin content 215
6.2.4. Transcriptomic analysis of stressed wild-type and DEK1 mutants 216
6.3. Implication of DEK1 in Ca^{2+} flux through the plasma membrane 220
6.3.1. dek1[CALPAIN] root epidermal cells show decreased Ca^{2+} intake under osmotic stress 220
6.3.2. *Xenopus* oocytes expressing the transmembrane domains of DEK1 show Ca^{2+}-activated Cl^{-}-conductances 225
6.4. Summary and conclusions 232
7. Discussion 241
7.1. The transmembrane domains of DEK1 play a role in the process of sensing mechanical signals at a plasma membrane level 241
7.2. The CALPAIN domain of DEK1 has a role as effector in mechanosensing pathways 245
7.3. Proposed model of action of DEK1 250
8. References 253

1. Introduction

1.1. Mechanosensing in plants

1.2. Mechanical design of developing plants

1.2.1. Role of the epidermis in growth and mechanosensing

1.3. Role of the cell wall in mechanosensing

1.3.1. Introduction to plant cell walls

1.3.2. Role of cell walls in the determination of plant mechanical properties

1.4. Role of mechanical forces in patterning

1.5. How are mechanical signals perceived and what are their responses?

1.5.1. Mechanoperception in animals

1.5.2. Mechanoperception in plants

1.5.2.1. Cell walls as mechanosensors

1.5.2.2. Mechanosensitive ion channels

1.5.2.3. Mechanical stimulation and Ca^{2+} transport

1.5.2.3.1. Effects of mechanically activated Ca^{2+} fluxes

1.5.2.4. Effect of mechanical stimulation on gene expression

1.5.2.5. Role of the cytoskeleton in mechanosensing

1.5.2.5.1. Microtubules play a key role in plant mechanosensing

1.6. Is DEK1 involved in mechanoperception?

1.6.1. Introduction to calpains

1.6.1.1. Structure of calpains

1.6.1.2. Localization and activity of calpains

1.6.1.3. Function and regulation of calpains

1.6.1.4. Calpain substrates

1.6.2. Phytocalpains

1.6.2.1. Activity and function of the CALPAIN domain of DEK1

1.7. Aims and objectives

1. Introduction

1.1. Mechanosensing in plants

Although plants lack specialised sensory organs and cannot sense what surrounds them in the same way as animals do, they are still able to perceive and respond to stress imposed by the environment. In particular, mechanical stress could be potentially harmful if plants did not evolve to respond and acclimate to it. Already in 1881, Charles Darwin reported in detail, the effects of mechanical stimulation in plants. In his book "The Power of Movement in Plants" he described how roots can change their growth direction away from sources of mechanical stimulation (Darwin and Darwin 1880). In 1893 he expressed his amazement at the touch-induced response of the specialised leaves of the carnivorous plants *Drosera rotundifolia* and *Drosera muscifera*, referring to them as 'one of the most wonderful in the world' (Darwin 1893). These plants respond to mechanical perturbations with impressive rapidity and are one of the best examples of plant species that respond within instants after sensing a mechanical stimulus. The very rapid folding up of the leaflets of the compound leaves of *Mimosa pudica* is also very spectacular and has attracted the attention of scientists. In this case the fact that the touch response is not only restricted to the touched leaflet, but propagates to the surrounding leaflets has been a subject of considerable interest (Malone 1994).

Although not all plants have specialized sensory organs that allow them to respond immediately to mechanical stimulation they all still respond to this type of stress. Most species, if exposed to repetitive mechanical stimulation such as wind or touch, can respond with different types of morphological alterations. According to Jaffe and Forbes these touch-induced morphological changes have been recognized since very early, the first document referring to them being the third century B.C. book "De Causis Plantarum", by Theophrastus (Jaffe and Forbes 1993). Some of these

slower morphological responses to mechanical stress include touch sensitive tendril coiling or vine climbing (Darwin 1906).

In 1973 the term "thigmomorphogenesis" was introduced by Mark Jaffe, to describe growth responses that can be induced by mechanical stimulation (Jaffe 1973). Thigmomorphogenesis happens in a vast variety of plants species including both herbaceous species (Biddington 1986; Braam and Davis 1990) and woody species (Coutand et al. 2009; Porter et al. 2009; Telewski and Pruyn 1998). Thigmomorphogenetic responses are thought to confer an evolutionary advantage that allows plants to resist and acclimate to environmental mechanical perturbations and their potentially damaging consequences (Rodriguez et al. 2008). For example, trees grown in windy conditions have a higher root growth rate and root carbon allocation than trees grown in sheltered conditions, as has been demonstrated in the angiosperm *Prunus avium* (Coutand et al. 2008). Dwarfed forms, reduced leaf size and allocation of biomass to below ground organs are also observed as responses in aquatic plants that suffer periodic hydrodynamic forces, for instance plants grown in tide zones or in streams (Boeger and Poulson 2003; Doyle 2001).

In the case of the model plant *Arabidopsis thaliana,* clear developmental alterations are observed in plants undergoing mechanical stimulation. If stressed twice daily they develop shorter petioles, begin to bolt later and have shorter bolts than untouched wild-type plants (Fig. 1-1) (Braam and Davis 1990). Thigmotropic events are also observed in the roots of this model plant. When roots find a glass obstacle in their culture substrate, a thigmotropic response occurs. Instead of growth towards the gravity vector, following normal gravitropism, they change their growth pattern and grow away from the point of contact with the mechanical barrier, thus avoiding it (Kimbrough et al. 2004; Massa and Gilroy 2003). This response is believed to be triggered at a subcellular level, as it is known that mechanical stimulation causes an increase of free Ca^{2+} (Knight et al. 1991; Legue et

al. 1997). This phenomenon is seen in root tips that encounter an obstacle (Monshausen et al. 2009).

Mechanical stress is usually classified into two categories, exogenous or endogenous. There are several ways in which the environment imposes the first type, such as wind or rain. However, mechanical stresses not only depend on exogenous stimuli, but can also be a consequence of the expansive growth of pressurized cells (Boudaoud 2010; Hamant and Traas 2010). Although mechanosensing in plants has been widely studied and physiologically characterised thoroughly, the molecular responses to this second type of stimulus still remains unknown. It is likely that certain molecules could participate in detecting both types of mechanical stress.

Figure 1-1. When stimulated mechanically in a repetitive way, *Arabidopsis thaliana* plants show a delay in flowering and a decreased inflorescence elongation. The group of plants on the right were stimulated mechanically twice daily throughout their development; while the plants on the left are untreated controls. From Braam J., 2005.

1.2. Mechanical design of developing plants

Biological shape is not the product of an intelligent design, but it is, as described by Jacob in 1977, a *bricolage*, and therefore it "oscillates around the state of minimal energy without ever reaching it" (Jacob 1977). As all other living organisms, plants have to follow physical laws when determining their size, shape and structure. In plants, the shape is mainly defined by the rate and direction of growth (Coen et al. 2004; Dumais et al. 2006; Hamant and Traas 2010; Kwiatkowska and Dumais 2003; Silk and Erickson 1979). The mechanical design of plants depends on several factors, that include cell wall properties, supporting tissues, and spatial organization of the body of the plant (Niklas 1992). How biomechanics affect a biochemical substrates may therefore be important in determining the morphological and anatomical characteristics of organisms. In contrast to animals, which have different food sources depending on the species, all plants require basically the same resources (light, water, minerals, space, atmospheric gases), and they are therefore more strongly influenced by abiotic factors than by biotic ones when determining their shape and size, leading to a much higher plasticity in their development (Wojtaszek 2011). This was summarized elegantly by E.J.H. Corner in 1964: "A plant is a living thing that absorbs in microscopic amounts over its surface what it needs for growth. It spreads therefore an exterior whereas the animal develops, through its mouth, an interior." (Corner).

A certain amount is known about the role of mechanical forces in driving the cellular growth of plants. Internal pressure, which is consequence of osmotic pressure, is counterbalanced in plant growing cells by a continuous synthesis and remodelling of the cell wall. It is generally accepted that this pressure is the driving motor of growth (Hamant and Traas 2010). Osmotic pressure was first measured in an indirect way by Pfeffer, by determining the point of plasmolysis of cells (Pfeffer 1877). In the 1960s Paul Green developed, for the first time, a method that could measure the turgor pressure in cells in a direct manner (Green 1968; Green and

Stanton 1967). This technique was developed over time to obtain the modern pressure probes. This original probe consisted of an oil-filled microcapillary, connected to a pressure sensor and a movable plunger, which was introduced into cells of interest. It was observed that the value of internal turgor pressure of plant cells is extremely high, going up to 10 bar. Considering the fact that 0.03 bar corresponds to high blood pressure in humans, it becomes clear why plants are able to grow and push their way through hard materials such as road surfaces (Hamant and Traas 2010).

1.2.1. Role of the epidermis in growth and mechanosensing

The idea of internal pressure as a developmental force is not restricted to the single cell level. The proposition that the epidermis, i.e; a tissue, could work as a biophysical barrier to expansion is very old, existing at least since the 19th century, when it was observed that different tissues present different tensions. This led to the conclusion that certain tissues grow passively pushed by the driving force generated by others. In support of this idea, stems of the houseplant *Solenostemon scutellarioides* sectioned longitudinally and placed in water always bend outwards, suggesting that the expansion of the inner layers is normally constrained by the outer ones (Sachs 1882; Sachs 1865).

Paul Green proposed a model in which the epidermis restricts and controls the rate and direction of plant growth, since this tissue can resist more stress than it generates (Green 1992). Savaldi-Goldstein and Chory recently argued that this argument seems logical since the turgor pressure that is generated and experienced by the inner cells is predicted to generate a force in all directions equally, as they are completely surrounded by other cells. Their internal pressure will thus, to some extent, be balanced by that of their neighbours. On the other hand, epidermal cells interface with environment; therefore they have a free face that does not press

against another tissue. As a result, the wall properties of the epidermis and its ability to counteract internal forces are expected to significantly influence and regulate the extent of growth. In agreement to this, the outer wall of the epidermis is usually at least 5 to 10 fold thicker than internal cell walls (Savaldi-Goldstein and Chory 2008). Furthermore, Kutschera and Niklas presented experimental data that supports this classic theory. Their work added new considerations into the model of the "tensile skin theory". These include, in addition to the presence of thickened outer cell walls, the mechanical constraints that are associated with the presence of a cuticle, and the interactions between cell layers through hormonal signals (Kutschera and Niklas 2007).

Studies carried out recently also support the premise that the epidermis controls growth at the shoot apex. When applied exogenously and locally to stem apical meristems, cell wall loosening enzymes, such as expansins, can induce a primordium-like structure (Fleming et al. 1997). However, expression of expansin in all cell layers of the meristem, and not just application to the L1 of these primordia, is necessary for the production of fully developed leaves (Pien et al. 2001). Laser ablation of cells of the L1 of the meristem showed that the epidermis is essential for organ formation, as leaf primordia are unable to arise in sites of epidermal ablation (Reinhardt et al. 2003).

The fact that the epidermis can resist and control pressure generated from within the plant, as well as within its own cells, suggests that this tissue may have a particularly well developed mechanosensing network. It is to be noted that nearly all the experiments that will be described in this introduction, and thesis, which look for subcellular responses to mechanosensing, have been performed in epidermis. This is almost exclusively for reasons of practicality since the tissue is very well described and is easy to reach and work with. However this fact may somewhat bias our thinking regarding the mechanosensitivity of plant cells. Despite this, it is known that internal tissues also respond to mechanical stimulation, as is

seen in the case of reaction wood, a type of wood formed as a response to mechanical stress. However, very little is known about the molecular mechanisms underlying these reactions. This leaves a gap in current knowledge regarding responses to mechanical stress in underlying cells, and opens the question of whether responses are due to transmitted signals from the epidermis. However, it is more likely that mechanical stimulation is directly sensed in internal tissues, at least in the root pericycle, where there is an elevation in free cytoplasmic Ca^{2+} as a response to bending (Richter et al. 2009).

1.3. Role of the cell wall in plant mechanics

As previously mentioned, in plant cells, the cell wall controls the speed and direction of growth driven by internal turgor pressure. Cell wall behaviour is regulated by molecular effectors located in the cell wall. Cell walls have a very important role in plant cells as, in addition to absolute growth, they control the shape and size of all types of cells, thus shaping tissues and organs.

1.3.1. Introduction to plant cell walls

Classically plant cell walls are divided into two categories: primary and secondary cell walls. Primary cell walls are relatively thin and dynamic and surround cells that are either growing or can potentially grow. Secondary cell walls are laid down after growth has ceased, and provide mechanical stability. They are thicker and can be, lignified such as those present in vessels and fibres. Nowadays it is known that almost all differentiated cells present a characteristic type of cell wall. Therefore, cell walls exist in a spectrum of varieties with primary and thickened, lignified secondary walls at the extremes (Keegstra 2010).

The primary and most abundant component of the plant cell walls is cellulose. It is a polymer of β-1,4-linked glucans. These chains are able to interact with each other through hydrogen bonds giving rise to a crystalline structure, the cellulose microfibril (Somerville 2006). Apart from cellulose, plant cell walls contain other polysaccharides in their matrix. These can be separated into two categories. One of these is the pectic polysaccharides, which include xylogalacturonan, rhamnogalacturonan and homogalacturonans. The different pectins are not present in the wall as independent molecules, but as covalently linked chains. These chains consist of a backbone of D-galacturonic acid, which can be substituted in certain positions by other sugars. From this backbone, complex side chains emerge. These side chains are formed by 12 types of glycosyl residues, bonded by 22 different sorts of bonds (Harholt et al. 2010). The pectin matrix is often described as a hydrated "gel" or "filler" which occupies much of the space between cellulose microfibrils. The other category is the hemicellulosic polysaccharides. They include xylans, xyloglucans, glucomannans and mixed-linkage glucans. These are polysaccharides that present backbone chains of sugars with β-1,4 links. They interact with, and cross-link cellulose microfibrils, and in some cases have been shown to interact with lignin (Scheller and Ulvskov 2010).

Lignins comprise another important saccharidic component of cell walls. They are derived from three main monolignols: p-coumaryl, coniferyl and synaptyl alcohols. These monolignols are present in discrete quantities in certain types of cell walls. There they are polymerised and form cell wall reinforcement components (Davin and Lewis 2005).

Plant cell walls are also rich in proteins and glycoproteins that include both enzymes and structural proteins (Rose and Lee 2010). One example are the arabigalactan proteins. These are structural proteins present on the plasma membrane and in the cell wall. They have been proposed to have a role in cell surface recognition and signalling (Ellis et al. 2010). A very important group of

enzymes present in cell walls are expansins. They are involved in cell wall modifications during cell elongation (McQueen-Mason et al. 1992) and have been proposed to play a role in the regulation of tissue differentiation (Goh et al. 2012; Pien et al. 2001; Reidy et al. 2001)

Many different models have been proposed to explain how cell wall components are organized to form the final structure. One of the first models that described the molecular structure of the cell wall was that presented by Keegstra and collaborators in 1973. Thanks to the action of degradative enzymes, cell wall fragments could be isolated, purified and characterised structurally. Based on results from such experiments they proposed that covalent connections were possible between xyloglucans and pectic polysaccharides. These pectins could also bind to the structural proteins of the cell wall. Due to the existence of strong bonds between cellulose and xyloglucans they proposed that all the components would be arranged in a crosslinked network (Keegstra et al. 1973). Aided by the advances in electron microscopy, Carpita and Gibeaut proposed in 1993 a much more detailed and accurate model of how the polysaccharides are arranged in the cell wall. They proposed a network of cellulose microfibrils interlocked with xyloglucan chains. They stated that this network is embedded in a matrix of pectic saccharides (Carpita and Gibeaut 1993). More recently, the use of molecular biology techniques helped to elucidate roles of specific molecules in the structure and function of cell walls (Somerville et al. 2004).

It is important to note that the cell wall is not a static structure, but a highly dynamic one. New wall material is being continuously synthesized and laid down during cell growth. Some of this new material is intercalated with existing material, and some is laid down directly adjacent to the membrane, pushing “old” material outwards. This process can lead to non-uniformity within the cell wall. One example of this is that of pectins, which are believed to be strongly synthesised soon after cell division. This gives rise to a “middle lamella”, the border between the cell walls of two cells,

which is rich in pectic components, and plays an important role in cell adhesion (Keegstra 2010).

1.3.2. Role of cell walls in the determination of plant mechanical properties

During growth, newly synthesised cellulose is constantly deposited outside the plasma membrane, preventing the wall from becoming thinner and, therefore, weaker. These deposited cellulose microfibrils have a very high tensile strength, and are the principal determinant of cell stiffness (resisting turgor pressure). Cellulose microfibrils have a filamentous structure and are often aligned in parallel arrays, forming bundles. This characteristic means that they are also the main determinants of cell wall anisotropy. This is a term describing the situation when a cell wall has different mechanical properties when stressed in different directions. In situations where cells are surrounded by organized bands of cellulose microfibrils, such as it is the case of internal stem tissues, cells generally show very little growth in the direction parallel to the spirals of cellulose, and grow mainly perpendicular to fibrils (Baskin 2005; Cosgrove 2005; Kutschera 2000; Kutschera and Niklas 2007; Lloyd and Chan 2004; Marga et al. 2005). Expansion driven by turgor pressure is a non-reversible process and it involves a gradual and slow reorganization of microfibrils and their surrounding matrix as the cell grows. This matrix first loosens (a process thought to involve the activity of enzymes such as hydrolases, transglycolases and expansin (Cosgrove 2005)) allowing the microfibrils to slide and help the cell wall to yield to the forces generated by the internal pressure.

Cellulose orientation not only affects the physical properties of primary cell walls. For instance, it has been observed that the orientation of cellulose fibrils relative to the longitudinal axis in the cell wall affects the mechanical properties of wood. This was studied by small-angle-X-ray scattering, and it was seen that the angle was

higher in the case of softwood than in hardwood. It was also noted that the angle changed substantially from one species to another, and correlated with their mechanical properties (Lichtenegger et al. 1999).

Components other than cellulose fibrils also help to determine the mechanical properties of the wall. Amongst them are hemicelluloses. These are complex polysaccharides which are divided in several groups, including xyloglucan, xylans, mannans, and mixed-linkage glucans (Buchanan et al. 2000). It is believed that they are responsible of forming a three-dimensional network, linking cellulose microfibrils. This network has been proposed to be the main load-bearing structure of the cell wall (Fry 1989; Hayashi and Kaida 2011; Somerville et al. 2004; Willats et al. 2001).

Xyloglucan, in particular, has a role of great importance in the mechanical properties of cell walls. *xxt1/xxt2* double mutant plants lack detectable xyloglucan, as the genes affected code for key enzymes for its synthesis; Xyloglucan Xylosyltransferase 1 and 2 (Cavalier and Keegstra 2006; Faik et al. 2002). This double mutant leads to slight dwarfism, and the formation of atypical root hairs, but most importantly the cell walls show significant changes in their mechanical properties, as they present a reduced stiffness and an decrease in resistance to imposed mechanical stress before breaking (Cavalier et al. 2008).

Classically, it has been thought that the mechanical characteristics of the cell wall depended only on the network formed by cellulose fibrils and hemicelluloses. Recently, however it has been shown that pectins also play very important roles (Ryden et al. 2003). Mutants with pectin alterations, such as *murus1* and *quasimodo2*, show a reduced tensile stiffness in their hypocotyls when compared to wild-type plants. Based on the existing mechanical data and the current cell wall models, it is thought that that the hemicellulose xyloglucan, which is generally folded, may unfold between cellulose fibrils during straining of the hypocotyls. Pectins may

resist this unfolding (Abasolo et al. 2009). In fact, pectins have been proposed to play a key role in both the determination of the mechanical properties of organs and on organ initiation. It is believed that processes as fundamental as phyllotactic patterning are under the control of the methyl-esterification of the pectins in the cell wall, and therefore the activity of pectin methylesterases (Braybrook et al. 2012; Höfte et al. 2012; Peaucelle et al. 2011; Peaucelle et al. 2008).

1.4. Role of mechanical forces in patterning

Despite molecular techniques providing a large amount of information on how cells behave; the processes that integrates mechanical signals within single cells, plant tissues and organs still remain unknown. Plants generate their complexity by a process of reiterative growth, meaning that cells, tissues, meristems and organs, are produced in a repeated and predictable arrangement. Patterning is a highly complex process, and understanding its co-ordination necessitates knowledge of how cells interact and communicate (Dupuy et al. 2008). Bending of roots triggers new lateral roots to emerge in the bent area and stress-driven buckling has been proposed as a method for primordium initiation in both the capitula of *Helianthus annuus* (sunflower) and phyllotaxial events on shoot apical meristem (Dumais 2007; Newell et al. 2008; Yin et al. 2008). Thus organogenesis and patterning are determined, in part, by mechanical forces.

Recent studies suggest that patterns of plant organ emergence and formation may depend on a very complex network that links the phytohormone auxin with mechanical signalling (Kuhlemeier 2007). In the *Solanum lycopersica* shoot apical meristem it has been shown that mechanical strain in the plasma membrane regulates the abundance of the auxin transporter PIN1 (Li et al. 2012a; Nakayama et al. 2012). Interestingly, auxin accumulation is directly linked with cell wall physical properties, both through non-transcriptional and transcriptional processes (Murray

et al. 2012). Auxin is thought to trigger the acid growth mechanism, described in a model that proposes that cell growth can be driven by an acidification-dependent cell wall loosening process. In particular, the activity of plasma membrane H^+-ATPases has been shown to be induced by auxin (Takahashi et al. 2012). In addition a transcriptional pathway working over longer periods of growth has been suggested, as auxin deficient mutants, such as *arx3*, have dwarfed phenotypes (Leyser et al. 1996). Transcriptional analyses showed that a set of approximately 100 genes are repressed in this mutant in comparison to wild-type plants (Overvoorde et al. 2005). These genes include several pectin methylesterases, known to be directly associated with organ emergence (Peaucelle et al. 2011; Peaucelle et al. 2008).

Since both cell walls and mechanical signals can be thought of as regulators of cell plant growth, it seems likely that a significant interplay exists between the plant cell wall and the perception of mechanical signals. One possibility is that a feedback loop exists whereby mechanical signals lead to local changes in cell wall deposition, and these changes in the cell wall subsequently alter the capacity of the cell to perceive mechanical signals. Although this idea is appealing it is technically difficult to investigate, especially given the current and extremely frustrating lack of knowledge regarding the nature of mechanoreceptors in plants.

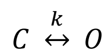
1.5. How are mechanical signals perceived and what responses do they elicit?

1.5.1. Mechanoperception in animals

As in most aspects of molecular biology, much more is known about mechanoperception in the animal field in comparison to what is known in the plant field. The best described mechanisms of mechanosensing in animal cells are the ones

linked to membrane deformation. This deformation is not sensed directly by the membrane, but through stretch deformable proteins embedded in membranes.

The basic concept that any mechanosensitive system has to follow is that it should be able to change between different conformational states. These states should have specific functional properties such as a response to the tension present in the membrane in which the system is present. The topology of proteins depends of several parameters. These parameters are the conformational equilibrium to tension and the functional properties of the relaxed and stimulated states. In the case of mechanosensitive channels, the best described mechanosensitive molecules in animals, the functional property that changes between one state and the other is the capacity to mediate the transport of solutes across the membrane (Haswell et al. 2011). The activity of this type of channel can be described in a very simple and reductionist way, as the mechanical tension-dependent equilibrium between two functionally different states, closed (C) and open (O), that present different conductance properties (Sukharev et al. 1997). This can be expressed by the a kinetic scheme,



$$C \overset{k}{\leftrightarrow} O$$

where k is the equilibrium constant between both states in total absence of tension. The presence of tension in the membrane displaces this equilibrium towards the open side of the equation. This is a consequence of changes in its topology, which can change the energetic balance of embedded proteins, stabilizing, for instance, the open state of a channel and therefore activating a transport of solutes (Phillips et al. 2009).

Several proteins have been described as good models for mechanosensitive channels in yeast and animals. Some of these belong to the transient receptor potential (TRP) channel family and their homolog in yeast, TRPY (Christensen and Corey 2007; Folgering et al. 2008), the TREK K^+ channel family (Folgering et al. 2008) and the

DEG/ENaC voltage-independent Na^+ channel family (Bianchi 2007; Christensen and Corey 2007).

It has been proposed that phenomena other than membrane deformation may also be responsible for perception of mechanical stresses in animals. Ingber proposed a model in which surface adhesion receptors would act like "pegs" and mechanically couple the extracellular matrix with the cytoskeleton. For this reason integrins have been proposed to have a role in mechanoperception (Ingber 1991; Ingber and Jamieson 1985; Wang et al. 1993).

Integrins are transmembrane heterodimers, which in humans have 18 α-subunits and 8 β-subunits. These subunits are known to be combined in at least 24 different ways, giving rise to a multitude of different potential receptors. These different αβ heterodimers perform different functions, with both tissue and ligand specificity. Even though the prototypical ligand of integrins is fibronectin, there are several other binding proteins such as laminin or collagen, all of which are components of the extracellular matrix (Barczyk et al. 2010; Hynes 2002). Nevertheless, all integrins share a common general structure and mechanism of action (Anthis and Campbell 2011).

In order to test the hypothesis that integrins were mechanoreceptors, a very elegant technique was developed by Ingber and his collaborators. They created a magnetic cytometry technique in which they could control the mechanical stress applied directly to the integrins of mammal endothelial adherent cells. Integrins were bound to magnetic microbeads coated with integrin ligands. In this way the mechanical forces applied to single molecules could be controlled by magnetic fields (Alenghat et al. 2004; Matthews et al. 2006; Overby et al. 2005; Wang et al. 1993; Wang and Ingber 1994). Thanks to this technique they observed that when integrins are mechanically stimulated, cells are stiffened, a response that does not occur when

growth factor receptors or transmembrane scavenger receptors are stressed (Wang et al. 1993; Yoshida et al. 1996).

It was observed that this stiffening response involved more players than just integrins. In fact, this process is mediated by a mechanical interplay in which all three types of cytoskeleton elements participate: microfilaments, microtubules and intermediate filaments (Geiger et al. 2009). It was also observed that the level of stiffening could be increased or decreased, depending on the enhancement or dissipation of cytoskeletal tension, known as pre-stress (Pourati et al. 1998; Wang et al. 1993; Wang and Ingber 1994). It was shown that the level of stiffening varies proportionally to the level of pre-stress in the cell (Wang et al. 2001; Wang et al. 2002). This led to the deduction that the cytoskeleton is a key player in the regulation of the response to mechanical stimulation.

In the last few years some light has been shed on how mechanoperception can translate stimulation into a biochemical signal. For instance, it has been shown that the tension present in the cytoskeleton can change the conformation of p130Cas (Sawada et al. 2006), a regulator of cell mobility (Meenderink et al. 2010). This change opens the molecule and a hidden phosphorylation site, target of the Src family of kinases, emerges and is phosphorylated. This phosphorylation event leads to the activation of p130Cas' enzymatic activity, which is a transducer of mechanical stimulation to downstream signalling molecules, that include several types of kinases and tyrosine phosphatases (Geiger 2006; Sawada et al. 2006).

It has also been shown that focal adhesions, which are formed by integrins at sites exposed to high stress, are mechanically reinforced by other proteins, such as talin (del Rio et al. 2009) and α-catenin (Yonemura et al. 2010). When a mechanical force is applied to these adhesion complexes, talin rods stretch and expose binding sites for vinculin (del Rio et al. 2009; Grashoff et al. 2010). The observations gave rise to a new concept, which is that any deformable structure can act as a potential

mechanosensor through conformation changes which are a consequence of strain (Farge 2011).

In addition to mechanosensitive proteins, another primary mechanosensing entity has been proposed to exist, in the form of the plasma membrane itself. It has been experimentally proved that membrane tension is able to block endocytosis and thus reduce the degradation of receptor-ligand complexes within endosomes (Rauch et al. 2002; Raucher and Sheetz 1999). In the presence of receptor ligands, this lack of endocytosis and degradation could increase the number of activated receptors at the membrane and thus enhance the activation of downstream pathways. This mechanism has been shown to be important in regulating the internalization of BMP2, affecting the differentiation processes of bone cells and myoblasts (Rauch et al. 2002). In this case the membrane is acting as the mechanosensor itself, without the need for any embedded protein (Farge 2011).

1.5.2. Mechanoperception in plants

Animal mechanoreceptors, such as integrins, link the cytoskeleton with the extracellular matrix, which has very distinct chemical and structural compositions in animals and plants. In animals the extracellular matrix is mainly composed of modified proteins, whilst in plants it is mainly composed of polysaccharides. Structurally the presence of a cell wall, in addition to allowing the generation of high internal turgor pressures, may also mean that plants and animals perceive mechanical stress differently. These differences could well mean that completely different molecules might have adapted to perform the function of receptors of mechanical stress in animals and plants (Kasprowicz 2011a).

Despite the analysis of several higher plant genomes, no true integrin homologues have been found. Nevertheless, in plants experimental data that suggests the

presence of integrin-like proteins. In animals, integrins bind to an Arg-Gly-Asp (RGD) motif, which is present in extracellular matrix proteins, which are responsible for adhesion. In *Arabidopsis,* proteins have been identified that have high affinity for, and can bind to, RGD motifs. In fact, if peptides containing the RGD sequence are added to plasmolysed *Arabidopsis* cells they are capable of disrupting adhesion between the plasma membrane and the cell wall (Senchou et al. 2004). Amongst the 12 RGD -binding proteins identified in *Arabidopsis*, eight are predicted to encode receptor-like kinases (RLKs). Even though these proteins have been thoroughly described structurally, very little is known about their physiological role (Kasprowicz 2011a). These RLKs, amongst others (described below) might be involved in mechanosensing.

1.5.2.1. Cell walls as mechanosensors

In other walled model organisms, such as yeast, it has been proposed that the cell wall may act as an osmosensor. This process happens through sensing the integrity of the cell wall in response to changes in volume, and thus, the apparition of new tensions and forces in the periphery of the cell (Hohmann 2002). The pathways triggered by this type of stress have as players, a family of plasma membrane proteins containing a periplasmic ectodomain. It has been proposed that this ectodomain functions as a cell surface sensor, which transmits the perceived signals of cell wall integrity to the small G-protein Rho1. Changes in the architecture of the actin cytoskeleton are observed in response to Rho1 activation. Interestingly the actin cytoskeleton controls several subcellular processes, such as cell polarization and synthesis of new cell wall components (Levin 2005).

The detection of cell wall integrity as a means of mechanical stress perception has been proposed for plant cells (Marshall and Dumbroff 1999; Nakagawa and Sakurai 2001). Members of the *Catharantus roseus* RLK1-Line (CrRLK1L) protein-kinase

family of proteins, with 17 members in *Arabidopsis*, have been proposed as putative cell wall integrity sensors (Boisson-Dernier et al. 2011; Hematy and Hofte 2008). One protein member of this family is Theseus1 (THE1), which is expressed in elongating cells and in the vasculature. It has been shown that this protein can mediate growth repression in the presence of perturbations in cellulose synthesis in growing cells. It has also been observed that, in the case of cellulose deficient mutants, THE1 controls the ectopic accumulation of lignin (Hematy and Hofte 2008; Hematy et al. 2007).

Another member of this family is Feronia (FER), which is localized almost ubiquitously but has a known function in the mycropilar pole of synergid cells (Escobar-Restrepo et al. 2007). It has been proposed that this protein can sense cell wall changes when synergids encounter pollen tubes (Rotman et al. 2008). Two very closely related proteins to FER are Anxur1 (ANX1) and Anxur2 (ANX2). These proteins, unlike FER, are present only in the tip of the pollen tube (Boisson-Dernier et al. 2009; Miyazaki et al. 2009). Double mutants for *anx1 anx2* present a phenotype of almost complete male sterility as pollen tubes burst (Boisson-Dernier et al. 2009). This phenotype is very similar to that of *vanguard (vgd)* mutants, which lack a cell wall pectin methylesterase that plays an important role in the rigidification of cell walls (Jiang et al. 2005; Mohnen 2008).

Other *CrRLK1L* family genes, such as *Hercules1* (*HERK1*), have been proposed to act as regulators of cell growth pathways (Boisson-Dernier et al. 2011), as the double *herk1 the1* mutant presents a down-regulation of several cell wall loosening enzymes (Guo et al. 2009).

Apart from being an indicator of the mechanical state of the cell via their integrity, cell walls might also act in the mechanosensing processes in a more direct way. In plants, as in animal cell wall, plasma membrane and cytoskeleton form a continuum. It is believed that this complex is involved in perception and transduction of signals that come from the environment to the inside of cells (Wyatt

and Carpita 1993). A large number of plasma membrane proteins present ectodomains embedded in the cell wall. Some also interact strongly with components of the cell wall. It has been proposed that these proteins may act as linkers within the continuum. The ectodomains interacting with the cell wall are connected to transmembrane domains and, in many cases to cytoplasmic domains that can transmit the signal sensed in the cell wall (Kasprowicz 2011b).

This is a similar scenario to the animal integrin model presented previously. Nevertheless, there are no integrin homologues present in plants, where other molecules have been suggested to play an equivalent role (Baluška et al. 2003). Some examples are of these are lectin receptor kinases (Gouget et al. 2006), arabinogalactan proteins (Pont-Lezica et al. 1993) and wall associated kinases (WAKs) (Anderson et al. 2001; Kohorn 2001; Verica and He 2002). In particular, it has been shown that WAK2 interacts with pectins present in the cell wall. It is likely that the signal generated by this protein is transduced by the Mitogen-Activated Protein Kinase (MAPK3). The latter is capable of activating the vacuolar invertase, which produces fructose and glucose from sucrose. These products could collaborate in the increase of osmotic pressure, thus triggering cell elongation (Kohorn et al. 2009; Kohorn et al. 2006). Indeed, if grown without any available sucrose, *wak1* mutants show a reduced rate of cell elongation. This phenotype can be rescued by the expression of a maize sucrose phosphate synthase (Kohorn et al. 2006). However, although it is tempting to speculate, no concrete links have yet been made between mechanical signals and the function of WAK2.

Although there is still considerable controversy regarding the role of Receptor kinases as mechanosensors in plants, it is widely accepted that membrane stretch-dependent ion currents, especially Ca^{2+} currents, are key players in mechanoperception in plants.

1.5.2.2. Mechanosensitive ion channels

The idea of the presence of mechanosensitive ion channels is not new, and they have been previously described in both prokaryote and animal cells (see above). In plants, action potentials were successfully recorded in the specialised mechanosensitive leaves of *Dionaea muscipula* as early as the 19th century (Burdon-Sanderson and Page 1876; Darwin 1893; Sanderson 1872).

In plants, the activities of multiple mechanosensitive channels have been described. When a patch pipette is attached to the plasma membrane of a plant protoplast their activity can be measured since, when forming a tight seal between the membrane patch encircled by the tip of a glass capillary, the patch tends to be exposed to negative hydrostatic pressure. While establishing seals, most patch clampers have experienced current fluctuations in the picoampere range (Cosgrove and Hedrich 1991; Ding and Pickard 1993; Martinac et al. 1987), which can no longer be seen when the negative pressure is released (Hedrich 2012). These currents are triggered by mechanical forces on the membrane, and are generally attributed to stretch-activated or mechanosensitive channels (Sachs 2010).

Even though several mechanosensitive channels have been proposed in animals, these putative mechanosensitive channels do not have any known homologs in any sequenced plant genome to date (Monshausen and Gilroy 2009b).

In contrast, bacterial mechanosensitive channels have been, so far, a very useful template for the identification of potential plant mechanoperceptors. Bacterial mechanosensitive channels of small (MscS) and large (MscL) conductance work in a similar way to the animal mechanosensitive channels described above. When tension is present in the membrane they open, allowing water to flux out of the cell in the case of hyperosmotic stress and, therefore, they regulate internal osmotic pressure and prevent cells from bursting (Corry and Martinac 2008). These proteins

were first identified from their electrophysiological activities regulated by membrane stretch. They were first described as plasma membrane proteins seen in studies carried out in giant *E. coli* spheroplasts (Cui et al. 1995; Martinac et al. 1987; Sukharev et al. 1993). Their ion transport activities were characterised by heterologous expression in the oocytes of the African frog *Xenopus laevis* and subsequently in patch clamp experiments (Maksaev and Haswell 2011).

In *Arabidopsis* ten MscS-like genes have been identified, but only the single knockouts of two of them (*msl2* and *msl3*) gave a phenotype, which affects plastid division, with especially enlarged plastids found in the case of the double mutant *msl2 msl3* (Haswell and Meyerowitz 2006; Wilson et al. 2011). It has been recently shown that these genes play a key role in the maintenance of osmotic balance in plastids, consistent with their roles in bacterial systems (Veley et al. 2012). The quintuple mutant of all root expressed *MSL* genes (*msl4 msl5 msl6 msl9 msl10*) has been shown to disturb the mechanosensitive channel activity of root cells. Even though there is no published material about the subcellular localization of most of these channels, it has been shown electrophysiologically that MSL9 and MSL10 are plasma membrane tension sensors (Haswell et al. 2008; Peyronnet et al. 2008). MSL9 and MSL10 proteins are likely to be part of a multimeric Cl^{-}-permeable channel (Corry and Martinac 2008; Haswell et al. 2008). However, no developmental perturbations were reported to be associated with this electrophysiological phenotype, and, in addition, no major phenotypes are observed upon osmotic, salt, mechanical, dehydration and rehydration stresses (Haswell et al. 2008), suggesting the likely presence of other, as yet uncharacterized mechanosensitive channels in plants.

A novel class of mechanosensitive channels, only recently discovered in animals, are the members of the Piezo family. Piezo1 and Piezo2 are expressed and have been characterized in animals (Coste et al. 2010). Piezo1 has been shown to be a cation selective channel (Gottlieb and Sachs 2012). Homolog genes of this family have been

found in all kingdoms. In *Arabidopsis* in particular, only a single gene that encodes a Piezo protein (At2g48060) is known. However, whether this protein presents any mechanosensitive channel function or not, remains to be demonstrated (Hedrich 2012).

1.5.2.3. Mechanical stimulation and Ca^{2+} transport

For mechanosensitive Ca^{2+} channels in particular, activities have been recorded and physiologically characterized in various cell types. Nevertheless, the specific function and molecular identity of the molecules responsible for these currents are practically unknown (McAinsh and Pittman 2009).

The MCA1 protein, which is expressed in *Arabidopsis* roots, has been described to be involved in Ca^{2+} influx. This protein was first identified in a screen for plant Ca^{2+}-permeable stretch-activated channels. In order to do this, a cDNA library was constructed in a yeast expression vector and expressed in *mid1* mutants of *Saccharomyces cerevisae*. These lethal mutants lack a Ca^{2+}-permeable stretch-activated channel (Kanzaki et al. 1999). Of all the screened transformants, one was found to be viable and the protein responsible was named *mid1 Complementing Activity 1 (MCA1).* In *Arabidopsis,* loss of MCA1 activity in the *mca1* mutant gives rise to problems in root growth and in responses to mechanical stress. If expressed constitutively, MCA1 gives rise to plants with strong defects in development and an increased basal Ca^{2+} uptake (Nakagawa et al. 2007). This gene was heterologously expressed in Chinese hamster ovary cells and in *Xenopus laevis* oocytes and a spike of free cytoplasmic Ca^{2+} was observed after stretching the membranes of the cells (Furuichi et al. 2012; Nakagawa et al. 2007).

A single paralogue of the *MCA1* gene was found in the *Arabidopsis* genome and named *MCA2*. These two genes are very similar in sequence and encode proteins

similar in their structure. Their N-terminus has a region with high homology to a putative regulatory element present in *Oryza stativa* protein kinases, an EF-hand (free Ca^{2+}-binding structure) and a coiled-coil motif. Their C-terminus consists of several (2 to 4) transmembrane domains and a *cys*-rich domain, of which the function still remains to be described (Nakagawa et al. 2007).

MCA2 was studied and found to be able to complement the *mid1* mutant of yeast, suggesting functional similarities with MCA1. In contrast to MCA1 which is expressed almost exclusively in the root, MCA2 expression is practically ubiquitous. The expression of both genes overlaps in vascular tissues and neither is expressed in root hairs (Yamanaka et al. 2010). At a subcellular level, consistent with their predicted structure, both proteins are plasma membrane anchored (Nakagawa et al. 2007; Yamanaka et al. 2010). MCA2 has been proposed to interact with molecules of the cell wall because if roots of MCA2-GFP plants undergo plasmolysis, part of the fluorescent signal of GFP remains in the cell wall (Yamanaka et al. 2010).

Knockout *mca2* mutants were obtained and they showed normal growth although defects in Ca^{2+} uptake were seen in their roots. In contrast, the double mutant *mca1 mca2* presents important growth defects, with retarded apparition of leaves and late bolting. This phenotype is also seen to a lesser extent in the *mca1* single mutants, but not in the *mca2* single mutant. Double mutants also show shorter roots than the single mutants and wild-type plants. MCA2 overexpressing plants gave no apparent phenotype, suggesting differences in the function of this protein to that of MCA1 (Yamanaka et al. 2010).

More recently the regions responsible for Ca^{2+} uptake in these proteins were identified. It was concluded that the EF-hand and the N-terminus of both proteins were necessary for Ca^{2+} transport. The coiled-coil region appeared to have a regulatory role, which was negative in the case of MCA1 and positive in the case of

MCA2. These genes have been proposed to work as Ca^{2+}-permeable mechanosensitive Ca^{2+} channels (Nakano et al. 2011).

Even though stretch-activated cation channels have been more widely studied, some activity of stretch-activated anion channels has also been described in plants. Mechanosensitive channels with a high affinity for anions have been detected in *Nicotiana tabacum* protoplasts. Pressure steps of 10 mmHg or more were observed to trigger transport through these channels (Falke et al. 1988). Channels with a very similar activity have also been detected in *Arabidopsis* mesophyll cells. These *Arabidopsis* channels have the characteristic that they are activated when positive pressure is applied, but not during application of negative pressure (Qi et al. 2004).

In summary, even though many ion currents have been described to be activated by stretching of membranes in plants, only a few of the proteins mediating this transport have been identified. With the exceptions described above, almost no homologues of mechanosensitive ion channels from other kingdoms are found in plants. Thus, even though the plant electrophysiology field has advanced a great in the last few decades and several currents have been characterized, the molecules responsible for mechanosensitive fluxes, and particularly Ca^{2+} fluxes, still remain to be discovered.

1.5.2.3.1. Effects of mechanically activated Ca^{2+} fluxes

Studies carried out in roots of *Arabidopsis* expressing the free Ca^{2+} reporter Yellow Chameleon showed that mechanical stimulation triggers a transient elevation in cytosolic concentrations of free Ca^{2+} (Monshausen and Gilroy 2009b). Changes in Ca^{2+} concentrations can be triggered in different ways, including by touching single cells, bending whole organs or by endogenous mechanical stresses, such as those present during thigmotropic growth responses. In all these cases, the effect of

mechanical stimulation can be impeded using Ca^{2+} channel blockers, such as lanthanum (La^{3+}) or gadolinium (Gd^{3+}), which suggests the rise of cytosolic Ca^{2+} is initially due to an influx from the extracellular space and not from endomembrane compartments (Monshausen et al. 2009).

In other higher plants, such as *Nicotiana plumbaginifolia*, the relation between mechanoperception and an increase in cytosolic Ca^{2+} was shown by transgenically expressing the bioluminescent protein aequorin, which can bind to free Ca^{2+} and emit a blue signal. When genetically transformed seedlings are stimulated mechanically by wind, there is an immediate and transient increase in Ca^{2+}-dependent luminescence of this protein (Cessna et al. 2001; Knight et al. 1992).

A range of subcellular responses are thought to occur in response to local increase in the cytoplasmic Ca^{2+} concentration. Chloroplast movements, dependent on external Ca^{2+}, were recorded after mechanical stimulation in the fern *Adiantum capillus-veneris* (Sato et al. 1999; Sato et al. 2001) and the moss *Physcomitrella patens* (Sato et al. 2003). For both cases, chloroplast relocation is abolished totally by Ca^{2+}-permeable channel inhibitors. In *Arabidopsis*, after touching one point of the surface of a cotyledon epidermal cell, actin filaments, peroxisomes and endoplasmic reticulum are reorganised and accumulate beneath the point of contact shortly after the stimulation. Around the contact point a depolymerisation of microtubules also occurs. All these reorganization events of subcellular structures are likely to be caused by a local increase in the cytoplasmic Ca^{2+} concentration induced after mechanical stimulation (Hardham et al. 2008).

Changes in the concentration of cytosolic free Ca^{2+} are also thought to be crucial for cell growth events, as has been demonstrated during the elongation of the pollen tube in various species, such as *Lilium longiflorum, Nicotiana tabacum, Petunia hybrida, Gasteria verrucosa* and *Zea mays*. This growth involves the extension of the cell wall and addition of plasma membrane components to the elongating tip. A cytosolic

gradient of Ca^{2+} is necessary for growth of the pollen tube, and any disruption of this gradient causes the cessation of the tip elongation (Franklin-Tong 1999; Messerli et al. 2000; Messerli et al. 1999). Thus, Ca^{2+} signalling seems not only to take part in growth signalling indirectly, but also directly. Stretch-activated Ca^{2+} channels have been identified in *Lilium longiflorum* pollen protoplasts (Dutta and Robinson 2004), so it is formally possible that this growth could be dependent upon mechanical stimuli, although this remains to be demonstrated.

Responses to increased Ca^{2+} in the cytoplasm are likely to be mediated by calcium sensors. In plants the presence of four families of Ca^{2+}-sensor proteins, which are characterized by the presence of EF-hands (Ca^{2+}-binding structures) has been demonstrated. Even though EF-hands were first described in animal proteins, they seem to be much more numerous in plants, with 250 putative proteins identified in the *Arabidopsis* genome in comparison to 132 in *Drosophila* and 83 in humans (Hashimoto and Kudla 2011).

Out of the four protein families characterized as Ca^{2+} sensors in plants only one is highly conserved along eukaryotes, the calmodulin (CaM) family. The other three, the calmodulin-like protein (CML) family, the calcineurin B-like protein (CBL) family and the Ca^{2+}-dependent protein kinase (CDPK) family are only found in plants, with homologues in just a few species of protozoans (Batistič and Kudla 2009; Billker et al. 2004; Weinl and Kudla 2009).

Of all families, only CDPKs present an enzymatic activity, a protein kinase activity. CaMs and CMLs are known to work by interacting with target proteins. In the case of CBLs, although they do not have an enzymatic activity themselves, they interact in a specific manner with a family of protein kinases named CBL-interacting protein kinases (CIPKs). Therefore, CDPKs and CBL-CIPK complexes can be considered "sensor responders", while CaMs and CMLs are "sensor relays" (Hashimoto and Kudla 2011).

CDPKs form a family of 34 proteins in *Arabidopsis*. They present a serine/threonine protein kinase domain, an auto-inhibitory domain and a CaM-Like domain that contains four EF-hands (Hrabak et al. 2003). Activation of these proteins involves the displacement of the auto-inhibitory domain as a consequence of Ca^{2+} binding (Ludwig et al. 2004). This is followed by a self-phosphorylation event that contributes to a state of full kinase activation. Targets of these proteins consist of a wide range of membrane, cytoplasmic and nuclear proteins (Hashimoto and Kudla 2011).

CBL proteins show a partial similarity to animal calcineurin and Ca^{2+}-sensors in neurons. They interact specifically with CIPKs, which are serine/threonine kinases (Kudla et al. 1999; Shi et al. 1999). The *Arabidopsis* genome contains 10 *CBL*-coding genes and 26 *CIPK* genes (Weinl and Kudla 2009). The encoded proteins can be paired in several different combinations which appear to be very stable in *in vivo* conditions and, therefore, can be considered as complexes (Batistič et al. 2008; Batistič et al. 2010). It is believed that the different combinations determine target specificity (Batistic and Kudla 2004). Interestingly, several ion channels have been shown to be targeted by these complexes (Ho et al. 2009; Li et al. 2006; Xu et al. 2006a).

CaMs in plants show a high structural homology to those in animals. In *Arabidopsis* there are 7 genes that encode for CaMs (Hashimoto and Kudla 2011). In addition, 50 genes encode for CMLs, which are thought to have evolved from ancestral CaMs, and show a difference in the number of EF-hands. As in animals, CaM-dependent kinases exist and are known to interact specifically with different CaMs/CMLs (Zhang and Lu 2003). These proteins have been shown to play a key role in regulation of gene expression, as several transcription factors are regulated by phosphorylation mediated by these kinases (Finkler et al. 2007; Galon et al. 2010; Kim et al. 2009; Liu et al. 2008).

In addition to all these relatively direct effects to the presence of free Ca^{2+} in the cell, more indirect effects are also likely to take place. A model of possible plasma membrane signalling events in response to mechanical stimulation has been proposed by Monshausen and Gilroy. Membrane tension leads to a change in structure and opening of a Ca^{2+}-permeable channel or channels (Monshausen and Gilroy 2009a; b). This would cause a peak in free cytosolic Ca^{2+}, amplified locally by release of internal calcium stocks in the ER, which in turn would be sufficient to activate a proton transporter. These transporters may acidify the cytosol and alkalinize the cell wall. In parallel, a NADPH oxidase is thought to be activated by Ca^{2+}, generating an accumulation of ROS in the cell wall, which could leak back to the cytoplasm (Monshausen et al. 2009). Changes in pH and the accumulation of ROS have a profound effect on cell physiology. Apart from their roles as modulators of gene expression (Apel and Hirt 2004; Lapous et al. 1998) many other non-transcriptional responses have been described. For example, it is known that pH is a regulator of many plasma membrane transporters (Blatt 1992; Regenberg et al. 1995), of cytoskeleton associated proteins (Allwood et al. 2002; Frantz et al. 2008) and of aquaporins (Verdoucq et al. 2008). Furthermore, alterations in extracellular ROS and alkalinisation of the cell wall caused by Ca^{2+}-permeable channels (Monshausen and Gilroy 2009b) are likely to cause changes in the rigidity and growth of this structure by facilitating the formation of intermolecular crosslinks (Brady and Fry 1997; Cannon et al. 2008). These changes could underlie at least some of the thigmotropic responses observed in plants stressed mechanically in a periodic way.

1.5.2.4. Effect of mechanical stimulation on gene expression

In addition to cytoplasmic re-organization, mechanical stimulation is known to cause profound changes in gene expression. The first described touch-inducible genes in plants were the *Arabidopsis TCH* genes (Braam and Davis 1990). Of these

genes, three encode for possible Ca^{2+} sensors. Calmodulin 2 (CaM2) is encoded by *TCH1* (Braam and Davis 1990; Lee et al. 2005). *TCH2* and *TCH3* code for Calmodulin-like 24 and 12 (CML24 and CML12), respectively (Braam and Davis 1990; Khan et al. 1997; McCormack et al. 2005; Sistrunk et al. 1994). A genome wide microarray analysis to identify touch-inducible genes in *Arabidopsis* also detected an enrichment for genes encoding Ca^{2+}-binding proteins amongst the most up-regulated genes (Lee et al. 2005). This transcriptional response of genes encoding Ca^{2+} sensors to mechanical stimulation is also seen in species other than *Arabidopsis*. *Solanum tuberosum* and *Vigna radiata* show CaM and CML gene induction after being stimulated mechanically (Botella et al. 1996; Botella and Arteca 1994; Gawienowski et al. 1993; Ling et al. 1991; Oh et al. 1996; Perera and Zielinski 1992). Therefore, the touch-induced expression of genes encoding Ca^{2+} sensors is conserved throughout all higher plants (Chehbab et al. 2011). This could be considered as a possible feedback loop, in which an event of mechanical stimulation would cause an influx of Ca^{2+} into the cell, which in turn enhances the expression of Ca^{2+} sensors. The up-regulation of these sensors would make the cell more sensitive in the case of subsequent mechanical stimulation events.

As discussed previously, responses to mechanosensing lead to many morphological changes throughout the plant. These changes often implicate modifications in the cell wall. Plant size, form, and mechanical properties are determined, to a large extent, by the structural characteristics of the cell wall and its extensibility capabilities (Carpita and Gibeaut 1993; McNeil et al. 1984; Varner and Lin 1989). It is therefore relatively unsurprising that genes encoding enzymes involved in cell wall modification are regulated by mechanical stimulation. A very good example of this is a family of genes encoding endotransglucosylase/hydrolases (XTHs) (Braam and Davis 1990; Lee et al. 2005; Xu et al. 1995). This gene family is thought to be responsible for cell wall modification. In particular they have been proposed to incorporate xyloglucans into the cell wall via a “cut and paste” mechanism which both maintains the strength of the cell wall and facilitates cellular growth (Campbell

and Braam 1998; Liu et al. 2007; Maris et al. 2009; Osato et al. 2006; Shin et al. 2006). *TCH4*, part of the set of *TCH* genes first identified as touch-sensitive, is also known as *XTH22* (Braam and Davis 1990). Lately, other cell wall modifying enzyme encoding-genes were identified as being up-regulated in response to mechanical stimulation, including 12 out of the 33 XTH encoding genes present in *Arabidopsis*, making this family one of the most highly represented amongst touch up-regulated genes (Lee et al. 2005).

In fact a vast number of genes (equating to approximately 2.5% of the *Arabidopsis* genome) respond to mechanostimulation. In addition to Ca^{2+}-binding proteins other genes shown to be to be highly regulated include genes potentially involved in the response to pathogens, suggesting a possible mechanosensitive component in plant defence. Genes that encode transcription factors are also regulated by touch. Indeed 66 out of the 634 transcription factors whose expression was detected in this experiment were found to be regulated. A similar effect was seen in protein kinase encoding genes (Lee et al. 2005).

These drastic changes in transcription levels are likely to present far reaching effects of immense complexity. Primary touch regulated genes can affect the transcription levels of a second tier of genes or have activities as protein modifiers. Thus the genetic response to mechanical stimulation remains obscure as and both the consequences and kinetics of these changes still remain to be described.

1.5.2.5. Role of the cytoskeleton in mechanosensing

In order to respond to mechanical stress, plant, as well as animal cells, need to be capable of sensing it and translating it into a signal. This signal should trigger cascades that end in changes of the cell metabolism. In order to explain this process, a new way of understanding cells was needed, as models of them as elastic balloons

filled with a viscous cytoplasm were not useful. Ingber visualized animal cells in a completely new way, as tents of cytoplasm and membrane outstreched over the cytoskeleton, like muscles and skin over a real skeleton (Ingber 1993; 1998). This idea is actually based on an architectural model, tensegrity. The term "tensegrity" was used for the first time by Buckminster Fuller, an American architect, to explain structures that rely on the balance between tensile and compressive elements, giving rise to a dynamic state in which there is a constant prestress which mechanically stabilizes all its constituents (Fuller 1961).

In the case of animal cells, actomyosin fibrils can generate tension forces that are resisted by the extracellular matrix, neighboring cells and other components of the cytoskeleton. All elements of the system are in a permanent state of isometric tension so that a minor disturbance in only one of the elements will immediately trigger alterations in all the other components. The fact that the system is delicately balanced makes it very responsive to any sort of external stimulation (Ingber 1993; 2003b). In contrast to actomyosin fibrils which are contractile and flexible, microtubules are stiff and rigid elements. They have a much higher rigidity than actin filaments and their behaviour can be compared to that of very thin glass fibers (Gittes et al. 1993).

The tensegral model in cells is based in the assumption that mechanical stress is perceived by specialised receptor proteins spanning the plasma membrane of cells (integrins in the case of animals). The detected signal is then transmitted to the rest of the cells through changes in cortical and internal cytoskeleton networks (Ingber 2003a; 2008; 2010; Wang et al. 2009).

In the case of plants, even before the first description of microtubules was made by Ledbetter and Porter in 1963 (Ledbetter and Porter 1963), a cytoskeleton-like structure was predicted to exist on the basis of biomechanial considerations. The eminent biophysicist Paul Green started from the geometry of growing plant cells

and arrived at the conclusion that growth repartition was following a "reinforcement mechanism". He predicted that cells were establishing and maintaining the mechanical anisotropy of cell walls. This would require a "yet-unknown lattice of tubular elements that are oriented in an ordered fashion" (Green 1962).

Plant cells have a particularity that makes them work in a different way to animal cells, which is the prescence of a cell wall, a composite structure consisting of load-absorbing elements (cellulose microfibers) that are embedded within an amorphous matrix (hemmicelluloses, pectins, proteins). The cell wall is generally described as combining bending flexibility with mechanical stability (Niklas 1992). The existence of this structure fullfils the tensegrity function, replacing the cytoskeleton interface of animals cells by the tensegrity of the cell wall of plant cells. The plant cytoskeleton is therefore not directly required to support cellular architecture, gaining a potential freedom to adopt other roles (Nick 2011).

It has been proposed that this "freedom" in plants may have led microtubules to gain the funtion of being transducers for mechanical integration, even across the borders of cells, thanks to their high degree of rigidity (Nick 2011).

1.5.2.5.1. Microtubules play a key role in plant mechanosensing

One of the most popular recent theories regarding microtubules in plants states that microtubules can respond to mechanical stimuli by reorienting in a parallel fashion to the main axis of the force present in the cell membrane (Hamant et al. 2008; Hardham et al. 1980). This transducing function has been reported specifically to have a strong impact during phyllotactic patterning (Traas 2013). The participation of auxin in the outgrowth of new primordia has been studied in depth (Hamant and

Traas 2010). Local accumulation of this hormone can lead to wall loosening and can increase growth rates. Recently, it has been shown that feedback mechanisms involving mechanical forces, developmental patterning and growth, also affect the localization of the auxin PIN transporters. In fact, a very high correlation is seen between the localization of the PIN transporters and microtubule orientation. Transporters are usually localized in membranes that are parallel to microtubules, and therefore along stress vectors (Heisler et al. 2010). *In silico* simulations showed that auxin fluxes regulated in this way could generate phyllotactic patterns identical to those seen *in vivo* (Heisler et al. 2010; Jonsson et al. 2012). Interestingly however, a mechanistic link between microtubule organization and PIN protein polarity has not yet been made, and it may be that both processes are regulated in parallel within cells rather than depending upon each other.

A clearer and more concrete link has however been established between microtubules and cell wall deposition. Microtubules have long been proposed to guide the deposition of cellulose microfibrils in the cell wall (Heath 1974; Paredez et al. 2006), and therefore, determine the principal axis of force bearing in the tissue. It was observed as early as the 1960s that microtubule disrupting drugs, such as colchicine, lead to abnormal cell elongation (Green 1962). This fact, alongside the observation that microtubules and cellulose microfibrils align, gave rise to a theory proposing that microtubules orient the cellulose deposition process (Heath 1974; Ledbetter and Porter 1963). This theory originally became controversial and received criticisms (Himmelspach et al. 2003; Sugimoto et al. 2003). However, in the last few years it has been shown using fluorescently labelled cellulose synthase (CesA), that this enzyme tracks along microtubules in the cortical area of the cell (Paredez et al. 2006).

The mobility of cesA complexes is proposed to derive from their catalytic activity. The process of cellulose microfibril immobilisation in the cell wall is thought to push the synthesising complex through the membrane (Bringmann et al. 2012a;

Emons et al. 2007; Guerriero et al. 2010). The co-localization of CesA and microtubules has been observed even in cases in which the later are being reoriented. Indeed, the rotation of these two elements, one driven by the other, has been proposed to explain polylamellate structure that outer epidermal cell walls have in the hypocotyl (Chan et al. 2010).

Several models have been proposed to explain how microtubules might guide the self-generated motion of the the CesA complex. One model proposes that the closed space between cortical microtubules and the plasma membrane could form a sort of barrier that traps the synthesising complexes and constrains them to move along the microtubules (Giddings and Staehelin 1991). Another model, known as the "templated-incorporation" model, proposes that the synthesised cellulose microfibrils stick to a scaffolding determined by microtubules. In this case the deposition would be guided by the scaffolding and not directly by the microtubules (Baskin 2001; Wasteneys 2004). This "templated-incorporation" model coincides with the observation by Paredez and colleagues, that the CesA complexes are able to follow their linear path even if microtubules are not present (Paredez et al. 2006).

Recent discoveries showed that the link between the CesA complex and microtubules is, in fact Cellulose Synthase Interacting 1 (CSI1) (Bringmann et al. 2012b; Li et al. 2012b; Mei et al. 2012). CSI1 is a protein that contains 21 Armadillo (ARM)/β-catenin-like repeats (Gu et al. 2010). These ARM repeats are repetitive clusters forming a superhelix structure that acts as a protein-protein interaction site (Conti et al. 1998), through which CSI1 is capable of binding to microtubules (Li et al. 2012b; Mei et al. 2012).

The discovery that CSI1 binds directly to microtubules and to CesA complexes allowed the proposition of more detailed models explaining the observed microtubule-CesA allignment, even though several possibilities have been proposed to explain the role of this linkage protein. It has been proposed that gliding or

sliding of some nature has to exist *in vivo*, even though it has never described under *in vitro* conditions (Li et al. 2012b). One model proposes that continuous and repeated switches may occur between CSI1 binding and a release of microtubules. This would result in a movement similar to that seen for ATP-dependent kinesins. This could be due to shape changes in CSI1, which is thought to form a "V" shape where the vertex binds the CesA complex and the "legs" bind microtubules (shape described in Gu et al. 2010). This motion has been proposed to be fuelled by cycles of phosphorylation and dephosphorylation (Bringmann et al. 2012a). Another proposed model suggests that the CesA complexes could move due to the energy generated by the complex itself during deposition of microfibrils of cellulose. In this case CSI1 would act as a mere link to the microtubule tracks that guide the synthesising complexes (Bringmann et al. 2012a). The main criticism to this is that if CSI1 was only a linker, the *csi1* mutants should not show any change in the speed of movement of the CesA complexes. However, the speed of movement of the CesA complexes in the mutants has been described to be one third of that in wild-type plants (Gu et al. 2010). Therefore, CSI1 seems to be important for both the guidance and the funtion of CesA (Bringmann et al. 2012a).

Through their role in guiding cellulose biosynthesis, microtubules have been proposed to control the growth direction and rate in plants (Chan 2012). Recent experiments performed in hypocotyls showed that microtubule-cellulose microfibril alignment is possibly also related to growth coordination of tissues. These studies found that the alignments of microtubules and cellulose microfibrils in the inner face of epidermal cells is always perpendicular to the growth direction during hypocotyl elongation (Chan et al. 2011; Crowell et al. 2011). This generates a co-ordination in the microtubule alignment in all epidermal cells, independent of the elongation rate. It has been shown that this alignment in the inner face of the epidermis mirrors that of inner tissues. Furthermore, the orientation of these microtubules determines the direction of elongation (Chan et al. 2011; Crowell et al. 2011). Other experiments support this same idea. One example is that if hormones

that affect the direction of growth, such as ethylene, are added to stems, the only microtubules that show changes in orientation are those present in internal tissues (Lang et al. 1982; Sawhney and Srivastava 1975). It is believed that this alignment of microtubules of the internal faces of epidermal cells is therefore a response to the tension generated by the expanding inner tissues (Chan 2012).

The proposal that growth direction is driven by inner tissues came with the suggestion that the outer epidermal cell walls could be the determinant of the rate of growth. This is because, as discussed previously, they impose a mechanical constraint on the underlying tissues. In fact, this scenario was also predicted by previous research showing that the addition of growth rate regulating hormones, such as auxin and gibberelic acid, and phototropic and gravitropic stimulation generated the reorganization only of the microtubules underlying the outer epidermal cell wall (Bergfeld et al. 1988; Ishida and Katsumi 1992; Mayumi and Shibaoka 1995; Nick et al. 1990). Recent experiments in hypocotyls showed that the organization of these microtubules was transverse and persistant in internal epidermis cell walls, but was transverse only during events of rapid growth in outer epidermal cell walls (Chan et al. 2011) (figure 1-2).

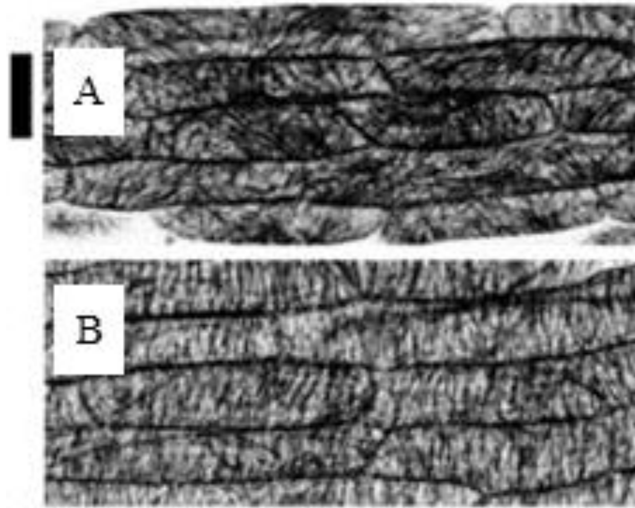


Figure 1-2 Image of epidermal cells of slow-growing hypocotyls of Arabidopsis expressing TUA6-GFP. A non-coordinated microtubule orientation can be appreciated (A). Image of epidermal cells of rapid cell elongation hypocotyls of Arabidopsis expressing TUA6-GFP showing a cross-border transverse arrangement of microtubules. Scale bar: 12 µm. From Chan et al. 2011.

1.6. Is DEK1 involved in mechanoperception?

A highly conserved, plant-specific phytocalpain has been shown to regulate several key aspects of plant growth. This protein is encoded in higher plants by the gene *DEFECTIVE KERNEL1 (DEK1)*.

AtDEK1 is a plasma membrane protein of 2159 amino acids, with a predicted structure of 21 transmembrane stretches and an extracellular loop. At its C-terminus AtDEK1 also contains a predicted intracellular domain with Cys protease activity, which has a high homology with the human m- CALPAIN domains II and III (in figure 1-2) (Lid et al. 2002).

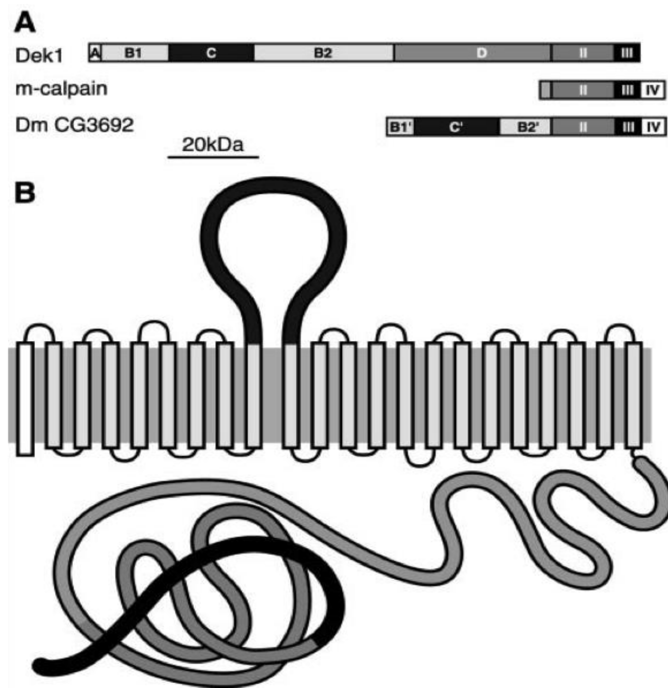


Figure 1-3. A. **Domain structures of different calpains: maize DEK1, human m-calpain and *Drosophila* CG3692 calpain.** B. **Predicted structure of maize DEK1. Taken from Lid et al., 2002.**

1.6.1. Introduction to calpains

Calpains are cytoplasmic, Ca^{2+}-dependent cysteine proteases, with an activity comparable to that of papain. They are characterised by having a highly conserved molecular structure in the catalytic domain (Croall and DeMartino 1991; Murachi et al. 1981). The most thoroughly characterized members of this family of proteases are the human m- and μ-calpains, both of which were first purified in 1976 and described as Ca^{2+}-activated proteases (Dayton et al. 1976a; Dayton et al. 1976b).

These proteins are present in most eukaryotes and in a few eubacteria, but never in archaea (Sorimachi et al. 1997). They have a very high diversity in domain structure and physiological roles (Sorimachi et al. 2012). Single copies of calpain-coding genes have been found in a small number of protozoan genomes, such as those of

Plasmodium falciparum, *Theileria annulata*, *Cryptosporidium parvum* and *Entamoeba histolytica* (Abrahamsen et al. 2004; Gardner et al. 2005; Gardner et al. 2002; Loftus et al. 2005). However, no calpain-like structures could be identified in the genome of the human pathogen *Giardia lamblia*, a protozoan usually considered as the most basal eukaryotic species (McArthur et al. 2000). The calpains of protozoans present some structural similarities to the calpains present in plants and fungi, as they do not have EF-hands, Ca^{2+} binding structures (Croall and Ersfeld 2007). An expansion of calpain genes appears in some parasitic protozoans, such as *Trypanosoma brucei*, with 14 genes encoding calpain-related proteins, *Leishmania major* with 17 and *Trypanosoma cruzi* with 15 (Ersfeld et al. 2005). This tendency of having multiple genes coding for calpains is maintained in most animal genomes, with 14 genes generally present in mammals (Goll et al. 2003). Other vertebrates have at least one orthologue, though generally more, of mammal calpains (Sorimachi et al. 2011a).

1.6.1.1. Structure of calpains

The classical calpain structure, based on that of human m-calpain, is divided into four major domains (Imajoh et al. 1988; Ohno et al. 1986). Domain I (DI) is an N-terminal α-helix that undergoes autocleavage events, either before or in parallel with external proteolytic activity. The conservation of this domain in other members of this protein family is low, and in some cases this region presents a completely different sequence and function (Margis and Margis-Pinheiro 2003). Domain II (DII) contains the catalytic core of the protein and is divided in two subdomains, IIa and IIb (Hata et al. 2001). Even though this domain does not have a known Ca^{2+}-binding site, recombinant human calpains, composed only of DII, show *in vitro* activity only when incubated with Ca^{2+} (Hata et al. 2001). A cysteine residue in DIIa and histidine and asparagine residues in DIIb form the catalytic triad of this enzyme. It has been proposed that Ca^{2+} neutralizes the negatively charged residues in the active domain and causes a three-dimensional reorganization of DII, forming the “closed” active

site conformation (Hata et al. 2001; Moldoveanu et al. 2002). Domain III (DIII) has a β-sandwich tertiary structure. This resembles the C2-like (C2L) structure that can be found in a large number of enzymes, including Protein Kinase C and one phospholipase, both of which show a tendency to bind transiently to membranes (Corbalan-Garcia and Gomez-Fernandez 2010; Rizo and Südhof 1998). The C2L domain presents two regions rich in acidic amino acids, which present a negative charge that can be neutralized by Ca^{2+}, thus becoming potential Ca^{2+} binding sites (Campbell and Davies 2012; Tompa et al. 2001). It has been suggested that the calpain C2L domain may bind to phospholipids in a Ca^{2+} dependent fashion, as seen for other C2 domains.

DIV of m- and μ-calpains carries EF-hands, which are Ca^{2+}-binding structures present in other Ca^{2+}-binding proteins such as calmodulin (Blanchard et al. 1997; Lin et al. 1997). However, this structure is not present in all calpains, even though most are Ca^{2+}-dependent with respect to their protease function (Margis and Margis-Pinheiro 2003). The presence or absence of EF-hand structures in calpains has led to a classification of this family of proteins into two subfamilies. The calpains that present this structure, such as m- and μ-calpain, are considered part of the classical calpain subfamily and are present only in animals and in the protozoan *Tetrahymena thermophyla*. Calpains that lack these Ca^{2+}-binding structures, and which were originally thought to have a Ca^{2+}-independent activity, are considered part of the atypical or non-classical calpain subfamily and can be found in all eukaryotes (Croall and Ersfeld 2007; Sorimachi et al. 2010). All protozoan calpains, apart from that of *Tetrahymena thermophyla*, lack a domain containing EF-hand-type Ca^{2+}-binding sites, as also do plant and fungal calpains, even though it has been proved that their activity depends on Ca^{2+} (Sorimachi et al. 2010). It is therefore likely that the evolutionary origin of "classic" calpains was a cysteine protease-calmodulin gene fusion, that gave place to the "classical" calpain structure, but which occurred exclusively within the animal lineage (Croall and DeMartino 1991; Croall and Ersfeld 2007; Goll et al. 2003; Sorimachi and Suzuki 2001). Interestingly, a

phylogenetic analysis of the active domain of calpains, with a phylogenetic tree rooted to the calpain-related sequence of the prokaryote *Porphyromonas gingivalis,* suggests that the EF-hand-containing calpains from animals (carboxy-terminal EF-hands) and *Tetrahymena* (amino-terminal EF-hands) are well separated evolutionarily. This raises the intriguing possibility that the acquisition of EF-hands may have occurred through two completely independent gene-fusion events. Phylogenetic analysis also reveals a close relationship between this *Tetrahymena* calpain, which contains 21 transmembrane motifs, with the only plant calpain phytocalpain/DEK1, thus raising the possibility of a common evolutionary origin for these unusual calpains. A lateral gene transfer via a green alga-type endosymbiont of ciliates to plants is one of the possible mechanisms proposed and the one most widely accepted (Croall and Ersfeld 2007).

Recently it has been proposed in the calpain community that the different domains should be renamed more descriptively (Ono and Sorimachi 2012; Sorimachi et al. 2011b). The proposal is to rename the Protease Core domains PC1 and PC2, instead of IIa and IIb. Domain III would be renamed C2L, standing for C2-Like domain. As domain IV contains Penta-EF hands it has been proposed to refer to it as PEF domain (Campbell and Davies 2012).

1.6.1.2. Localization and activity of calpains

The subcellular localization of calpains was studied by Hood and colleagues in human lung adenocarcinoma cells. They showed through immunofluorescence techniques that calpains co-localized with the ER marker proteins Calcinexin, a transmembrane chaperone, and the chaperone Grp78/KDEL, present in the lumen of the ER. Their results gave rise to the suggestion that calpains were associated with the cytoplasmic face of the ER, as well as the luminal side. Through very similar experiments they showed that calpains are also co-localized with the vesicle marker

protein COPβ and the Golgi apparatus marker protein P230. Through velocity gradient centrifugation it was shown that calpains also associate with a plasma membrane compartment (Hood et al. 2004). Calpains had been classically thought to be principally cytoplasmic proteins (Sato and Kawashima 2001), but it has been recently proposed that some members of this protease family may also have an activity within mitochondria, constituting the mitochondrial calpain system (Kar et al. 2010).

The crystalline structure of inactive classical calpains shows a topology that suggests that structural changes may occur in the presence of Ca^{2+}, triggering the activation of the protein (Hosfield et al. 1999; Strobl et al. 2000). In fact, later discoveries showed Ca^{2+} to be crucial for activation events in all calpains, independent of the presence or absence of specialised Ca^{2+}-binding structures. This is thought to be because the active domain of calpains includes single Ca^{2+} ion binding sites (Moldoveanu et al. 2002; Moldoveanu et al. 2003). As mentioned above, it has additionally been shown that when the active domain binds to Ca^{2+}, it changes its conformation and closes to form the active catalytic site (Moldoveanu et al. 2004).

In vitro assays showed that the activation of calpains requires concentrations of Ca^{2+} of up to 10 μM, which are very rarely found *in vivo*. However, the concentration of Ca^{2+} needed for activation decreased considerably if phospholipids were added to the media used in the *in vitro* protease activity assay (Saido et al. 1992; Shao et al. 2006; Tompa et al. 2001). This supports the theory that classical calpains need to be associated with membranes, possibly via their C2L domain, which shows a high affinity for phospholipids (Tompa et al. 2001), in order to become active, despite their mainly cytoplasmic localization (Croall and DeMartino 1991; Goll et al. 2003). This, together with the presence of several transmembrane domains in some calpains, including phytocalpains, points to a functional link between membranes and calpain activity (Croall and Ersfeld 2007; Lid et al. 2002).

In 1981 it was shown that m-calpain undergoes an autoproteolytic event in the presence of Ca^{2+} (Suzuki et al. 1981a; Suzuki et al. 1981b), a characteristic that has now also been shown for other calpains, such as the μ-calpain (Cong et al. 1989). This type of autolytic event is common in many different proteolytic enzymes (Goll et al. 2003). Even though the autolysis of μ-calpain is a very rapid process, it happens in several clear steps. First the 14 N-terminal amino acids are removed, reducing the molecular weight of the protein from 80 kD to 78 kD. A second proteolysis then takes place, cutting off 12 further amino acids from the N-terminus. This gives a 76 kD protein (Zimmerman and Schlaepfer 1991). In the case of the m-calpain 9 amino acids are removed in the first event and 10 in the second, giving a final 78 kD protein (Brown and Crawford 1993).

Because of these results, in the early 1990s it was proposed and widely accepted that the calpains were, in fact, pro-enzymes and that the autolytic events were involved in activation. This was because several kinetic studies suggested that prior to detection of the catalytic protease activity, calpains needed to be autolytically cleaved (Baki et al. 1996; Cottin et al. 1991; Hayashi et al. 1992). However, it was subsequently shown that a point mutation in the cleaved amino acid domains could give rise to a non-cleaved but none-the-less active calpain (Elce et al. 1997), suggesting that the cleavage event is likely a consequence of, and not a cause of activation. This autolytic event is very likely to happen in other calpains, even non-classical ones, as they have a very high level of conservation in their sequence and their structure.

Interestingly, in the case of *Drosophila* calpain B, it has been shown that autolysis is necessary for activation. This raises the question of whether the events needed for activation of calpains are always identical or if calpains with longer N-terminus domains, such as calpain B, might undergo a self-cleavage activation event, whereas

calpains with a short N-terminus, such as m- and μ-calpain, do not (Farkas et al. 2004).

1.6.1.3. Function and regulation of calpains

Mammalian calpains have been described as actors in an extensive list of processes, that include cytoskeletal remodelling (Lebart and Benyamin 2006), cell mobility and migration (Franco and Huttenlocher 2005), myofibril maintenance (Goll et al. 2008), signal transduction (Evans and Turner 2007), cell cycle progression (Janossy et al. 2004), regulation of gene expression (Storr et al. 2011) and apoptosis (Johnson et al. 2004) amongst others. Some calpains are of vital importance, as defects in them give rise to lethality in early development of animal embryos (Arthur et al. 2000; Dutt et al. 2006; Takano et al. 2011; Zimmerman et al. 2000). Similarly, calpains have been shown to play a very important role in normal embryonic development in *Xenopus laevis* (Moudilou et al. 2010). This role extends beyond early stages of embryogenesis, as they also seem to play a fundamental role during organogenesis and in the activation of metamorphosis in this species (Moudilou et al. 2010). In mice, m-calpain expression is detected throughout development from the 8-cell stage up to neurulation (Raynaud et al. 2008). Expression of some calpains is even maintained in adult proliferating tissues, such as muscle and bone precursor cells (Raynaud et al. 2004) and in the proliferating cells of adult brains (Konig et al. 2003). In the case of muscle, the most highly expressed calpain is the m-calpain, which is believed to be involved in the cell cycle of proliferating myoblasts. M-calpain is also involved in the permanent regulation of the plasma membrane and the cytoskeleton organization (Raynaud et al. 2004). In adult rat brains calpain 3 is expressed, but restricted to the glial cells, astrocytes. It is believed that this enzyme has a role in protecting these cells against apoptosis, as it cleaves pro-apoptotic proteins. It is also thought that it might have a role in motility control, as it cleaves proteins involved in cellular movement (Konig et al. 2003).

In vertebrates, the regulation of calpains has been widely studied and their best known interactor and activity regulator is calpastatin (Nishimura and Goll 1991; Otsuka and Goll 1987). Calpastatin is the only known protein that inhibits calpains specifically and does not inhibit any other proteases with which it has been tested, including other cysteine proteases such as papain, cathepsin B, bromelin, or ficin (Goll et al. 2003). Neither calpastatin nor calpastatin-like activities have been reported in any invertebrate tissues, and genes having sequence homologies to calpastatin have not been detected in *Drosophila melanogaster* (Friedrich et al. 2004), *Caenorhabditis elegans* (1998; Goll et al. 2003) or *Arabidopsis thaliana* (Margis and Margis-Pinheiro 2003). Thus, the calpastatin genes seem to be restricted to vertebrates (Goll et al. 2003).

1.6.1.4. Calpain substrates

Calpain protease activity has a specific nature, as the same substrates are always proteolized in the same positions, even if there is a variation in the reaction conditions. However, the rules driving this specificity have yet to be fully understood (Sorimachi et al. 2012). Classically, it has been thought that calpains do not recognize specific amino acid sequences in substrate proteins; but rather, they cleave inter-domain linkers (Hayashi et al. 1985; Sakai et al. 1987; Stabach et al. 1997). However, unlike other proteases that do not show high substrate selectivity, calpains cleave only a small number of proteins in a highly selective fashion. This selectivity appears to rely on a complex, and poorly understood substrate-recognition mechanism that depends on the structural features of the target proteins to be cleaved (Croall and Ersfeld 2007). Therefore, calpains perform a limited proteolysis, mainly cleaving inter-domain unstructured regions. Two known exceptions for this are the animal proteins casein (Malik et al. 1983) and myelin basic

protein (Yanagisawa et al. 1983), which are proteolized intensively by many calpains.

Recently, by the analysis of 267 known cleavage sites of numerous calpains through multiple kernel learning, a sequence-based calpain cleavage site predictor was generated. Some of the obtained prediction sites were tested experimentally, confirming the viability of this prediction method (duVerle et al. 2011).

Sorimachi and collaborators have proposed a theory to explain the fact that only inter-domain unstructured regions are cleaved by calpains. It is based on the topology of these proteins. Once activated and restructured, the active site cleft within the domains DIIa-DIIb is narrower and deeper than that in other papain-like cysteine proteases (Moldoveanu et al. 2004). This suggests that the conformation of the substrate appropriate for cleavage has to be "soft" or "malleable" around the cleavage site, helping the enzyme to preferentially proteolyze inter-domain regions (Sorimachi et al. 2012).

Rules that govern the target specificity at an amino acid sequence level are unclear and that is the reason why calpains have been classically thought to have a method of recognition of substrates based on their overall 3D structure and not on their primary structure (Hayashi et al. 1985; Sakai et al. 1987; Stabach et al. 1997). In some cases, however, certain sequence preferences have been seen for different types of calpains (Hirao and Takahashi 1984; Ishiura et al. 1979; Sasaki et al. 1984). However the consensus sequences observed are not strongly enough supported to permit prediction of potential substrates from sequence information alone.

Some of the known substrates for calpains include Ca^{2+}-ATPase, aquaporins, tubulin and several kinases and phosphatases, amongst others (Goll et al. 2003).

1.6.2. Phytocalpains

DEK1 is the only identified member of the calpain gene superfamily in plants (Lid et al. 2002). It is an atypical calpain as it lacks DIV, and in addition, one of its main peculiarities is the presence of an elongated N-terminus, which is subdivided into five domains. Domain A (DA) has predicted ER and plasma membrane targeting signals. Domains B1 (DB1) and B2 (DB2) have 8 and 13, respectively, predicted transmembrane stretches. These domains are interrupted by domain C (DC), which constitutes the predicted extracellular loop. Domain D (DD) is an intracellular region that can be found between DB2 and DII (Lid et al. 2002).

Homologs of the phytocalpain DEK1 have been found even in basal plants such as the moss *Physcomitrella*. DEK1 proteins in different plant species show a very high level of conservation. For example, maize DEK1 shares 70% identity with *Arabidopsis* DEK1. There is an especially high conservation in DII (88%) and DIII (83%) (Lid et al. 2002). This conservation suggests that the arrangement of the residues is essential for the catalysis and binding of substrates of the CALPAIN domain. However, a very high conservation level is also seen in the N-terminus of DEK1, being of 72% identity in DB1 and of 64% in DB2 (Lid et al. 2002). The lowest identity is found in DC, the extracellular loop, where identity is only 57% (Lid et al. 2002). Their high level of conservation suggests that the transmembrane structures of DEK1 presumably also have an important physiological role in the plant.

An independent *in silico* analysis of the rice DEK1 sequence, and topological predictions, has recently been carried out by Kumar and collaborators. Their published material supports the presence of a large transmembrane N-terminus. This structure is predicted to contain 23 transmembrane helices, and contrary to predictions published by Lid and colleagues for maize and *Arabidopsis* DEK1, the "extracellular" loop was found to face the cytoplasm and not the outside of the cell. These researchers furthermore argue that if closely inspected, the sequence of the

transmembrane helices shows that many of them present conserved charged or strongly polar residues and they conclude that there must be a functional relevance for this. Charged and polar residues usually have an important role in the function of transmembrane proteins involved in the transport of charged molecules. Thus, generally, Voltage-gated K^+, Na^+ and Ca^{2+} channels show conservation of polar residues in their TM helices which help in voltage sensing and, therefore, their transport activity (Kumar et al. 2010).

Most published literature about DEK1, is centred on the study of the CALPAIN domain and its physiological role. Nevertheless, due to the high level of conservation, the transmembrane domains of DEK1 are likely to carry out an important an important function. Even though no homologs of this structure are known in any other kingdom, *in silico* analyses suggest that the transmembrane domains of DEK1 could possibly act as an ion channel. Many ion channels are formed from associations of several proteins. However, the large number of transmembrane domains present in DEK1 could suggest that it is capable of forming a monomeric channel. The presence of the loop structure within the transmembrane domains might indicate some form of gating. One intriguing possibility, which will be investigated in this thesis, is that the transmembrane domains of DEK1 act as a stretch activated cation channel.

It has been shown that *AtDEK1* expression is very strong during early embryogenesis, but it is restricted to the embryo and excluded from the suspensor. Expression is also seen in all adult tissues (Johnson et al. 2005). In the case of maize *ZmDEK1* is expressed ubiquitously with peaks of expression in early endosperm, mid-development of embryos and seedling roots (Lid et al. 2002). In the case of *Nicotiana benthamiana NbDEK1* is expressed in all tissues as it is seen in other species (Ahn et al. 2004).

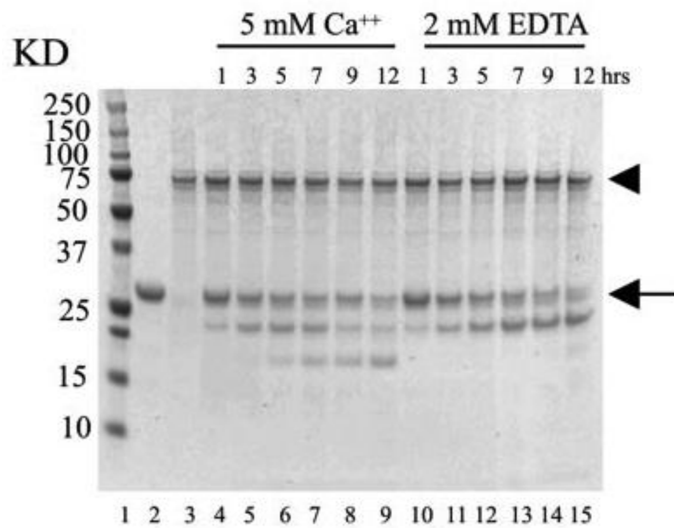


Figure 1-4. *In vitro* activity assay of DEK1 domains IIa and IIb expressed in bacteria. DEK1 domains IIa and IIb were incubated with β-casein and 5 mM Ca^{2+} (lanes 4–9) or 2 mM EDTA (lanes 10–15) to study their Ca^{2+}-dependent protease activity. Samples were incubated at 30°C and were collected in the time points shown in the picture. Lane 1, molecular marker; lane 2, β-casein; lane 3, domains IIa and IIb of DEK1. Arrowhead and arrow indicate the domains IIa and IIb of DEK1 and β-casein, respectively. From Wang et al., 2003

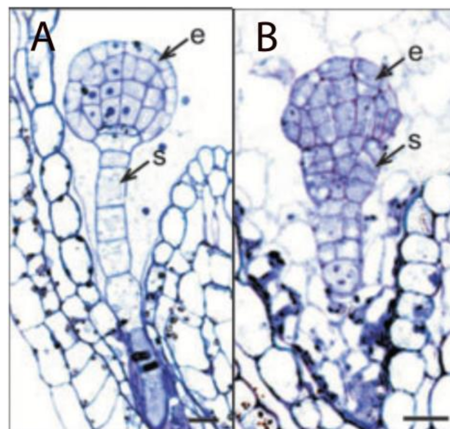


Figure 1-5. Wild-type embryo (A) and *dek1* mutant embryo with a T-DNA insertion in *AtDEK1* (B) at the globular stage. Embryo (e), suspensor (s). From Johnson et al., 2005.

1.6.2.1. Activity and function of the CALPAIN domain of DEK1

In the case of AtDEK1, and as in classic calpains, the CALPAIN domain has been shown to be proteolitically cleaved from the rest of the protein. This cleavage is very likely to be an autocatalytic event (Johnson et al. 2008). Recombinant domains II and III of the CALPAIN domain of maize DEK1 were expressed in *E. coli*. They were purified and shown to be active in *in vitro* assays. The protease activity of this recombinant version of the CALPAIN domain was tested in the presence and absence of Ca^{2+}. Degradation of casein was recorded when the CALPAIN domain of DEK1 was incubated with 5 mM Ca^{2+}, an activity that was very weak and appeared very late in the presence of 2 mM EDTA, a chelating agent that traps free Ca^{2+} ions (figure 1-3). The results obtained suggested an activity is similar to that of m-calpain, and showing that Ca^{2+} enhances the activity of DEK1 (Wang et al. 2003).

A recombinant mutated version of the CALPAIN domain was also generated and purified. This mutant has a single amino acid substitution in position 71, substituting a cysteine of the catalytic domain with a serine residue. Even though this mutation did not cause any structural change in the protein, it was shown that it caused a complete loss of the protease activity (Wang et al. 2003). In *Arabidopsis* this mutation not only makes the DEK1 protein non-functional, but also blocks the self-cleavage event (Johnson et al. 2008). This autocleavage can be considered equivalent to that which occurs in animal calpains and is predicted to release the active form of the CALPAIN domain into the cytoplasm (Johnson et al. 2008).

Knockout and knock-down *dek1* mutants have been obtained in different species. Thanks to these lines it is known that in maize, DEK1 plays a crucial role in early development of the plant embryo; as mutants exhibit slower, abnormal early embryogenesis with arrested embryos and a loss of identity in the aleurone-like layers of the endosperm (Becraft and Asuncion-Crabb 2000; Becraft et al. 2002). In *Arabidopsis*, *atdek1* knockout mutant embryos show defects at the early globular

stage, both in the embryo and in the apical segment of the suspensor, which is thickened. There are irregularities in the surface of the embryo and abnormal division planes can be observed. Embryos arrest their development at the globular stage (Fig.1-4) (Johnson et al. 2005). Endosperm defects have also been observed in comparison with the wild-type (Lid et al. 2002).

Knock-down *dek1* seedlings were obtained in *Arabidopsis* using an RNAi strategy but died as seedlings, showing a profound loss of epidermal identity. In these lines there was an apparent replacement of epidermal cells with mesophyll-like cells and plants showed either a complete absence of meristematic cells or a severe lack of organization in the shoot apical meristem (Johnson et al. 2005). In the case of *Nicotiana benthamiana* a decreased expression on *NbDEK1* leads to defects in epidermal development and arrested flower development (Ahn et al. 2004).

In maize, some weak *dek1* mutants are capable of completing embryogenesis, producing viable plants. These plants show an altered epidermis cell fate , with a proliferation of so-called "bulliform" cells (Becraft et al. 2002). A weak allele of *DEK1* has also recently been described in *Arabidopsis*. This allele is called *dek1-4* and carries a missense mutation, (C to T substitution at base 6816). This change causes a Cys to Arg change at the amino acid 2106 in domain III of the protein. This allele fails to complement the lethality of *dek1-3* mutants. One of the consequences of this mutation is the near absence of giant cells (a specialized epidermal cell-type) in sepals (Roeder et al. 2012).

Complementation of *dek1* mutants is possible not only by reintroduction of the full-length *DEK1* cDNA under a constitutive promoter, but also by the introduction of constructs designed to express synthetic proteins mimicking the smallest self-cleavage product of DEK1 which corresponds to the free cytosolic CALPAIN domain (Johnson et al. 2008). Thus, the CALPAIN domain is essential for plant development and the cleaved form is likely to be the active form of the protein.

During complementation experiments CALPAIN overexpressing (OE) plants were obtained, which should allow uncoupling the activity of the CALPAIN domain from its normal regulation (Johnson et al. 2008). These lines show a phenotype different to that of full-length *DEK1* OE plants, which resemble wild-type individuals. Therefore, these genotypes allow the dissection of the phenotypic characteristics that are a consequence of the OE of the active form of the CALPAIN domain, and of the loss of the transmembrane domains of the protein. CALPAIN OE plants have a darker green colour than the wild-type individuals and show severe rumpling in leaves. This corresponds to an increase in the number and density of mesophyll cell layers. In addition, epidermis cell size appears to be less uniform than in wild-type plants (Johnson et al. 2008). At a more physiological level, it has been observed that CALPAIN OE plants show a late flowering phenotype with shortened stems (Galletti personal communication). A microarray analysis showed that genes up-regulated in CALPAIN OE plants, showed an over-representation in cell wall related functions. Amongst the most highly up-regulated were pectin methyltransferases, expansins and xyloglucan endotransglycosylases (Johnson et al. 2008), supporting a hypothesis that the phytocalpain could be involved in responses to mechanical stimuli.

1.7. Aims and objectives

Aspects of the morphological and transcriptional phenotype of calpain over-expressing plants are reminiscent of those observed during mechanical stimulation. In addition the topology and primary structure of the highly conserved transmembrane domains of DEK1 suggest that they could act as a cation-permeable channel.

Based on these observations I propose a hypothesis that DEFECTIVE KERNEL1 (DEK1) could act as a stretch-activated cation channel. Since the CALPAIN domain

of DEK1 is known to possess a Ca^{2+} activated protease activity one possibility is that DEK1 transports Ca^{2+}, currents of which are the main electrophysiological response seen in cells when undergoing mechanical stimulation events. I further propose that the CALPAIN domain of DEK1 acts as the first effector in the cascade in response to a Ca^{2+} influx into the cell. Thus DEK1 could auto -regulate its activation by self-cleavage of the CALPAIN domain during mechanical stimulation.

In this thesis I investigate these hypotheses. I centre my work on investigating the roles of the different domains of DEK1 in the process of mechanosensing. In the case of the transmembrane domains I propose that they act as a primary mechanosensor, and study their role in sensitivity to mechanical stimulation. This is possible thanks to the knockout mutant lines, expressing the CALPAIN domain alone. These lines allow me to dissect the roles of the different domains of the protein and study the morphological, transcriptional physiological and electrophysiological phenotypes existing in absence of the transmembrane domains. I also studied the possible role of the CALPAIN domain as an effector of mechanical stimulation. This was studied by the characterization of the phenotypes in a variety of lines that show different levels of activity of DEK1. Furthermore, the effects of the CALPAIN domain as a transductor of mechanical stress were studied by hunting for potential interactors, and therefore potential proteolysis targets of this protein.

2. Material and methods

2.1. Plant culture and plant material

2.1.1. Cultivation of plant lines

2.1.2. NPA treated plants

2.2. DNA techniques

2.2.1. Genomic DNA extraction

2.2.2. PCR reactions

2.2.3. Agarose gel electrophoresis of DNA

2.2.4. PCR product and gel clean-up

2.2.5. Digestion and ligation of DNA

2.2.6. Construction of Gateway® plasmids

2.2.7. Transformation of plasmids into E. coli

2.2.8. Preparation of plasmid DNA from bacterial cultures

2.2.9. Sequencing of plasmid DNA

2.3. Phenotypic analysis: microscopy techniques

2.3.1. Fluorescence microscopy

2.3.2. Cryofracture scanning electronic microscopy

2.3.3. Atomic force microscope

2.4. Phenotypic analysis: biochemical analysis

2.4.1. Cellulose quantification

2.4.2. Lignin quantification

2.5. Q-RT-PCR

2.5.1. Plant material collection

2.5.2. RNA extraction and DNAse treatment

2.5.3. Determination of RNA concentration and reverse transcription

2.5.4. RT-PCR

2.6. Ion fluxes and concentrations

2.6.1. Flame photometry

2.6.2. In planta ion flux measurements (impalement)

2.6.3. Electrophysiological measurments in Xenopus oocytes

2.6.3.1. Building of constructs

2.6.3.2. Injection in Xenopus oocytes and electrophysiological measurements

2.7. Yeast techniques

2.7.1. Bait and prey construction for yeast 2 hybrid (Y2H) assays

2.7.1. Screening of interactions of calpain bait with cDNA library

2.7.2. Double transformation of plasmids in to yeast

2.7.3. Serial drop test

2. Material and methods

2.1. Plant culture and plant material

2.1.1. Cultivation of plant lines

Sterilization of seeds was performed using 70% ethanol (EtOH), 0.05% Triton-X-100 for 15 minutes (mins), inverting tubes every 5 mins. They were then rinsed using 96% EtOH in three washes of one min each. Seeds were then pipetted onto sterile 3mm filter paper (Whatman, Maidstone, UK) under a sterile tissue culture hood, to allow the evaporation of the EtOH. As an alternative method, seeds were sterilised in a Cl_2 (gas) atmosphere for 120 mins. This was done by placing the seeds in opened microfuge tubes inside a tightly closed container with a beaker containing 50 ml of bleach (NaClO 2.6%, Oxena, Portes-Lès-Valance, France) and 5 ml of concentrated hydrochloric acid (HCl).

To grow plants in long day conditions (Photoperiod of 16 hours; day: 20.5°C, 50% humidity; night: 19.5°C, 55% humidity), seeds were sprinkled evenly on plates containing Murashige and Skoog (MS) nutrient medium agar (1X MS basal salt mixture (Duchefa, Haarlem, The Netherlands), 0.5% Sucrose (Duchefa, Haarlem, The Netherlands), 0.8% Plant agar (Duchefa, Haarlem, The Netherlands); pH5.7). Plates were sealed with microporous tape and incubated at 4°C for 3 days to stratify. After stratification, the plates were transferred to a growth chamber and incubated at 21°C with a photoperiod of 16 hours. After 10 days of growth in the growth chamber, seedlings were transferred to a soil mixture (Favorit, Lohne-Kroge, Germany) with the insecticide Vectovac® 12-AS (Biobest, Orange, France) and placed in long day growth conditions. The humidity level was increased for early

stages of growth by keeping plants inside clear plastic bags for the first four days. This permits root establishment.

For growth in short day conditions, seeds were suspended in 0.1% agarose (Euromedex, Souffelweyersheim, France) and stratified at 4°C for 3 days covered with aluminium foil. The seeds were then sown directly onto soil (Favorit, Lohne-Kroge, Germany) containing the insecticide Vectovac® 12-AS (Biobest, Orange, France) with a pipette. The humidity level was increased for germination and early stages of development by keeping plants inside clear plastic bags for the first six days. After approximately 10 days of growth seedlings were thinned to leave only one seedling in each pot.

Genetic crosses were made between plant lines between by first emasculating non-dehisced flowers on the lateral shoots of acceptor (mother) plants. 24 hours later, carpels were hand pollinated with pollen obtained from the donor (father) plant. Tweezers used for the whole procedure were sterilised with 96% EtOH between each cross to avoid cross contamination. Once seed was mature it was collected and stored in air-permeable paper envelopes.

Mechanical stimulation of plants was carried out exposing them to a force of 4 g/cm^2 twice daily for one minute. This was carried out by placing a flat tray, adapted to the size of the plant pots used, and carrying appropriate weights gently onto the plants. Treatment was started when the first two true leaves appeared, and continued until flowering.

In order to obtain seedling tissue, plants were grown in liquid culture in 6-well plates (Corning, Corning, USA). Seeds were sterilised as described in section 2.1.1 and transferred into liquid half MS medium (0.5X MS basal salt mixture (Duchefa, Haarlem, The Netherlands), 0.5% Sucrose (Duchefa, Haarlem, The Netherlands); pH5.7). 3 ml of medium and 10 seeds were placed in each well. Seed were stratified

at 4°C for at least three days in darkness. Plates were transferred and incubated at 21°C with a photoperiod of 16 hours. If agitation was required plates were placed on an orbital shaker and agitated at a speed of 180 revs/min. Edge effects were minimised by regularly interchanging the positions of the plates.

2.1.2. NPA treated plants

In order to obtain naked stem apical meristems, plants were grown on plates containing N-1-Naphthylphtalamic Acid (NPA). Seeds were sterilised as described in section 2.1.1. They were sprinkled evenly on plates of *Arabidopsis* medium (47.28g *Arabidopsis* medium powder (Duchefa Haarlem, The Netherlands), 2 mM $Ca(NO_3)_2 \cdot 4H_2O$, 0.5% Agar-Agar (Merk, Darmstadt, Germany); pH5.8; to which NPA was added to a final concentration of 10 μM before plates were poured). Seeds were stratified on plates at 4°C, in darkness, for three days. Plates were transferred and incubated at 21°C with a photoperiod of 16 hours.

2.2. DNA techniques

2.2.1. Genomic DNA extraction

For small sample numbers, leaves were collected and stored in microfuge tubes (Eppendorf, Hamburg, Germany) at -20°C. Frozen leaves were then transferred to liquid N_2 and crushed into a fine powder with the aid of a mini-pestle inside the collection tube. 500 μl of extraction buffer (50 mM EDTA, 0.1M NaCl, 0.1M Tris·HCl, 1% SDS) were added, followed by vortexing thoroughly to mix. Samples were incubated at 65°C for five minutes in a heating block. A phenol/chlorophorm extraction was then performed. 500 μl of phenol/chlorophorm (equilibrated phenol:chlorophorm:isoamylalcohol 25:24:1) were added to the mix. The sample

was mixed thoroughly and incubated at room temperature for five minutes. After centrifugation at a speed of 14,000 RPM for five minutes, the upper (aqueous) phase was recovered to a clean tube and the phenol/chlorophorm extraction repeated. 50 µl of 3M NaAc (pH5.2) and 350 µl of isopropanol were then added to the second recovered aqueous extraction (400 µl). The sample was inverted gently several times and centrifuged at a speed of 14,000 RPM for five minutes. The pellet was washed with 70% EtOH and air-dried before resuspension in 50 µl of R40 buffer (TE (10 mM Tris·HCl pH8, 1 mM EDTA) containing 40 µg/ml ribonuclease A) and stored at a temperature of -20°C.

For large numbers of samples, leaves were collected and stored in 96 format collection tubes with two small metal beads (QIAGEN, Hilden, Germany) at -20°C. Tubes were frozen in liquid N_2 and then subjected to two cycles of 30 seconds at 30 Hz in a TissueLyser II® (QIAGEN, Hilden, Germany). DNA extractions from this powder were performed using a BioSprint 96® (QIAGEN, Hilden, Germany), following the protocol provided by the manufacturer.

2.2.2. PCR reactions

For standard PCR, a mastermix containing: 4 µl 5X Green GoTaq® Flexi Buffer, 1 µl of a 10µM stock of each primer, 0.2 µl of 20 mM dNTPs (20mM of each: dATP, dCTP, dGTP, dTTP (Promega, Madison, USA)), 0.1 µl of GoTaq polymerase (Promega, Madison, USA) and dH_2O up to 20 µl per sample (taking into account DNA sample volume). The mastermix was distributed into PCR tubes/strips/plates and the the plasmid/genomic/cDNA used as a template was then added. Primers were generally designed by eye, assuring that no secondary structures generated. PCR tubes/strips/plates were placed in a 2720 Thermalcycler (Applied Biosystems, Foster City, USA) and the following program was run: 95°C for 2 mins, followed by 30 cycles of 95°C for 30 seconds (denaturing), 55°C to 60°C (depending on primers)

for 30 seconds (annealing), 72°C for 1 minute per expected 1000 base pairs (bp) (extension). This program was finished by a final extension at 72°C for 5 mins.

As an alternative method, for high fidelity PCR reactions Phusion™ Hot Start (Finnzymes, Espoo, Finland) was used. The mastermix contained 4 µl 5X Phusion HF Buffer, 1 µl 10µM forward primer, 1 µl 10µM reverse primer, 0,2 µl 20 mM dNTPs (20mM of each: dATP, dCTP, dGTP, dTTP (Promega, Madison, USA)), 0.2 µl Phusion Hot Start DNA polymerase and dH_2O up to 20 µl (taking in to account the DNA sample volume). After preparing the reaction in PCR tubes, they were placed in a 2720 Thermalcycler (Applied Biosystems, Foster City, USA) and the following program was run: 98°C for 30 seconds, followed by 30 cycles of 98°C for 10 seconds, 60°C -72°C (depending on primers) for 30 seconds and 72°C for 15-30 seconds per expected 1000 base pairs (bp) of product. Reactions were finished by a final extension at 72°C for 5 to 10 mins.

2.2.3. Agarose gel electrophoresis of DNA

DNA fragments were separated by electrophoresis in agarose gel. 1% agarose gels were made dissolving agarose powder (Euromedex, Souffelweyersheim, France) in hot 1X TAE buffer (0.04M Tris acetate, 0.001M EDTA; pH8) in a microwave. 0.1 µg/ml EtBr was added after the gel had cooled and prior to pouring. Gels were poured with a thickness of approximately 7 mm in moulds (Fisher Scientific, Hampton, USA) and run in the corresponding tanks in 1x TAE buffer.

DNA samples were loaded in the gel after the addition of 6X of loading buffer (50% Glycerol, 49.5% TE buffer pH8, 0.5% Orange G (Sigma-Aldrich, St Louis, USA)). 0.5 µg of either 1kb (Smartladder, Eurogentec, Seraing, Belgium) or 100bp (Promega, Madison, USA) ladder were used as a size marker. Migrations were carried out at room temperature at 80 to 160 volts (depending on gel size). Following the

electrophoresis, the separated DNA fragments were viewed and photographed on a transilluminator.

2.2.4. PCR product and gel clean-up

PCR products and gel fragments were purified using a NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany) following the instructions provided by the manufacturer.

2.2.5. Digestion and ligation of DNA

Restriction digestions were carried out following the instructions provided by the manufacturer (Invitrogen, Carlsbad, USA/ New England Biolabs, Ipswich, USA/ Takara, Kyoto, Japan) at optimal suggested temperatures using a mix containing the restriction enzyme, the indicated buffer and bovine serum albumin (BSA) if required. Digestions were incubated for periods of one to three hours. In the case of double digests both enzymes were added simultaneously if they required the same buffer. Sequential digestions were carried out either by adapting the buffer concentrations between enzymes, if possible, or by purifying the first digestion product (as explained in section 2.2.4) followed by the second digestion. If required, the digested DNA was then separated in agarose gels and purified from excised bands as explained in sections 2.2.3 and 2.2.4.

When necessary, calf intestinal alkaline phosphatase (dCIP) (New England Biolabs, Ipswich, USA) was used to dephosphorylate the restricted ends of vectors, preventing re-ligation.

Ligation of insertions into vectors was carried out using T4 DNA ligase (New England Biolabs, Ipswich, USA) following the guidelines provided by the

manufacturer. Reactions were incubated at 16°C overnight and transformed into bacterial cells (as explained in section 2.2.7).

2.2.6. Construction of Gateway® plasmids

Some PCR products were cloned into Gateway® (Invitrogen, Carlsbad, USA) vectors. When designing the primers to generate the PCR products to create entry vectors, Gateway® B sites were added to the 5′ of the forward primer and 3′ of the reverse primer.

Gateway® BP and LR reactions were performed following the guidelines provided by the manufacturer. The resulting products were transformed into bacterial cells (as explained in section 2.2.7).

2.2.7. Transformation of plasmids into E. coli

Vectors were transformed into *Escherichia coli* thermo-competent cells by heat-shock induced transformation. These were either DH5α cells, available at the lab, and prepared using the protocol described by (Inoue et al. 1990), or TOP10 cells (Invitrogen, Carlsbad, USA).

Ligation or Gateway® BP and LR reaction products were transformed into 25 µl of TOP10 cells or 50 µl of DH5α cells, which had been thawed on ice. The tubes were incubated on ice for at least 30 minutes. Cells were heat-shocked at 42°C for one minute and immediately placed on ice for 5 mins. 1 ml of Luria broth (LB) (1% tryptone, 0.5% yeast extract, 0.5% NaCl; pH7) was then added before incubating at 37°C for one hour.

Plates of solid LB (LB Broth (BD, Franklin Lake, USA), 0.8% BactoAgar (BD, Franklin Lake, USA)) containing the appropriate concentrations of the required selective antibiotic were prepared. Ampicillin (Duchefa, Haarlem, The Netherlands) at a final concentration of 100 μg/ml, or kanamycin (Duchefa, Haarlem, The Netherlands) at a final concentration of 50 μg/ml were routinely used. After bacterial transformations had been incubated at 37°C, 100 μl were taken from and plated onto one plate. The remaining culture were centrifuged at a speed of 3,000 RPM, resuspended in a volume of 100 μl of LB and plated onto another plate. Incubation of inverted plates was at a temperature of 37°C, placed upside-down and overnight.

2.2.8. Preparation of plasmid DNA from bacterial cultures

Plasmid DNA was mini-prepped from *E. coli* using NucleoSpin® Plasmid kit (Macherey-Nagel, Düren, Germany), following the protocol provided by the manufacturer.

If large amounts of DNA were required a, NucleoBond® Xtra Midi (Macherey-Nagel, Düren, Germany) kit was used following the guidelines provided by the manufacturer.

2.2.9. Sequencing of plasmid DNA

Plasmids mini-prepped as explained in section 2.2.8 were diluted to a final concentration between 30 and 100 ng/μl in a volume of 30 μl. Samples were processed by GATC Biotech (Konstanz, Germany) using en ABI 3739xl (Applied Biosystems, Foster City, USA) sequencer.

The primers used to sequence full length *DEK1*, and smaller versions were:

Primer	Sequence
DekGW5	CACCATGGAAGGGGATGAGCG
QXCDEK1F1	CTTGAGAGAAGGTTTTCGGAG
QXCDEK1R1	CCTGTTCGAGTTAGATTGTCA
DEKSEQ1	ATCGTCAGTACTTCTGGG
DEKRKpn	CATGATAACTGTGGAACC
DEKXhoF1	GACGATTGTTGGATACTCTCT
DEKSEQ2	AGGATGTCTGTTTCCTGG
DEKFPst	CTCTGCAGCTCTTCTGGTTGG
DEKRBam	CTTTATCACGCATTGCAGAGGG
DEKXcmF1	CTGGACCTGTATGTCTTTTTG
DEKXcmR1	CTCAGAACCAGCAACCAGAATC
DEKFBst	TTGCATGCAGCCATTGGCATGG
DEKRBst	TCTAAGTCAGCCATGCCAATGG
QDEKF1	AAACAAGAGGGGTTCTTACTT
QDEKR1	TTCGAATCTGAACAAGTCTGT
DekSTOP	CTACAAAGCTTCAAGAACAAT

2.3. Phenotypic analysis: microscopy techniques

2.3.1. Fluorescence microscopy

Cross cuts of the basal centimetre of stems were made and fixed in FAA (3.7% Formaldehyde (from 37% solution, VWR International, Fontenay-sous-Bois, France), 50% ethanol, 10% acetic acid) at 4°C. Samples were vacuum infiltrated for three hours and then left overnight in fresh FAA. Samples were dehydrated through one hour baths of ethanol (50%, 70%, 85%, 96%,100%) , infiltrated with Histo-clear (National diagnostics, USA) and then with Paraplast X-tra (Leica Biosystems, Richmond, UK) using an ASP300 S automated tissue processor (Leica Microsystems, Wetzlar, Germany). Following the wax infiltration the stems were taken out of the infiltration cassette cassettes and transferred into a hot wax filled moulds, which were then allowed to solidify.

10 μm sections were made using a RM2025 microtome (Leica Microsystems, Wetzlar, Germany). Sections were floated onto Polysine glass slides (Menzel GmbH + Co., Germany) and allowed to dry overnight. Removal of paraffin was carried out using two sequential baths of 5 mins in Histo-clear (National Dignostics, Atlanta, USA) and two sequential washes of 5 mins in absolute EtOH. Tissue was afterwards rehydrated in successive baths of 30 seconds in 95%, 85%, 70%, 50% and 30% EtOH. A final bath of dH_2O was carried out for 15 minutes, and slides were allowed to air dry.

Samples were treated with 1 mg/ml Fluorescent Brightener 28 (calcofluor) (Sigma-Aldrich, St Louis, USA) for 5 minutes. Pictures were taken using a TE2000-E Motorized Focus microscope (Nikon, Tokio, Japan) using a 10X Nikon lens, under UV illumination using a long-pass filter. Confocal microscopy

All confocal observations were done on LSM700 (Carl Zeiss, Oberkochen, Germany) using either Achroplan® 40X (Carl Zeiss, Oberkochen, Germany) or Neofluar® 5X (Carl Zeiss, Oberkochen, Germany) objectives. The source of laser light was a Laser Rack LSM700 (Lasos, Jena, Germany). A HXP120C Illuminator (Carl Zeiss, Oberkochen, Germany) was used for widefield eyepiece observations.

Projections of stacks of images were made using Merryproj software (Barbier de Reuille et al. 2005). The analysis of microtubule orientation was carried out using the MT plugin for Fiji (Uyttewaal et al. 2012).

2.3.2. Cryofracture scanning electronic microscopy

Stems and fully developed leaves were cryo-fixed using liquid N_2 and afterwards freeze-fractured in a cryo-manipulation chamber. The samples were coated using

gold-sputter in an argon saturated atmosphere. Cryo-SEM images were taken with the aid of an S-4700 Scanning Electronic Microscope (Hitachi High Technologies, Chiyoda, Japan).

2.3.3. Atomic force microscope

AFM experiments were performed using a Catalyst Bioscope (Bruker Nano, Inc.), mounted on an optical fluorescence macroscope (MacroFluoTM, Leica) with an objective (2x plano objective, Leica). Surface topology and maps of apparent modulus were recorded in PeakForce QNMTM AFM mode (Bruker Nano, Billerica, USA / Veeco, Inc., Painview, USA) using a Nanoscope V controller and Nanoscope software versions 8.1. All measurements were carried out using a 0.8μm diameter spherical probe (SD-Sphere-NCH, Nanosensors, Neuchâtel, Switwerland). The spring constant used for cantilevers was quantified using the thermal tuning method ranging between 35-45 N/m. Calibration of deflection sensitivity of cantilevers was done against a clean sapphire wafer.

All recordings were carried out in an aqueous media at room temperature. The standard cantilever holder for experiments in liquid media was used. 30 mm diameter petri-dishes containing the sample were positioned on a XY motorized stage placed on a sample holder designed for the purpose. The AFM head was then mounted on the stage and positioning with respect to cantilever using GFP-signal was performed.

The requested applied force (PeakForce setpoint) throughout the experiments was 1.5 μN. For each sample, the topology and apparent modulus images were collected in areas of 100 x 40 μm2 and using a digital resolution of 128 pixels x 128 pixels. A rate of 0.3 Hz was used.

2.4. Phenotypic analysis: biochemical analysis

2.4.1. Cellulose quantification

This protocol was adapted from Foster el al. 2010b.

Plants were incubated for 24 hours prior material collection in complete darkness, to eliminate starch. Fully developed adult leaves were collected into 2 ml Eppendorf tubes (Eppendorf, Hamburg, Germany) and lyophilised with a Crios lyophilizer (Cryotec, Saint-Gély-du-Fresc, France) equipped with en EMF10 vacuum pump (Edwards, Crawly, UK). Lyophilised tissue was ground into a fine powder using metallic beads and three cycles of 30 seconds at 30 Hz in a TissueLyser II® (QIAGEN, Hilden, Germany).

From this powder the alcohol insoluble residue (AIR) was extracted. This was done by adding 1.5 ml of 70% EtOH to the sample and vortexing thoroughly. The sample was centrifuged at 10,000 RPM for 10 mins and the supernatant was removed. A second wash was done with 1.5 ml chlorophorm/methanol (1:1 v/v) prior to a second centrifugation in the same conditions. After removal of the supernatant the pellet was resuspended in 500 µl of acetone and the left overnight at 35°C to evaporate.

Once dry, between 3 and 6 mg of AIR were weighed in a screw cap 2 ml tube (Starstedt, Nümbrecht, Germany). 1 ml of Updegraff reagent (acetic acid/nitric acid/H_2O 8:1:2 v/v) was added to each sample and they were incubated for 30 minutes in a boiling water bath. Samples were centrifuged at 10,000 RPM for 15 mins and the supernatant was discarded. One wash with dH_2O and one with acetone were performed. The pellet was left overnight at 35°C to dry. 175 µl of 72% H_2SO_4 were added to dry pellets, and samples were incubated at room temperature

for 30 mins, vortexed and incubated for a further 15 mins. 825 µl of H_2O were added and the sample was mixed thoroughly and then centrifuged for 5 minutes at 10,000 RPM to pellet any solid residue.

Glucose content of these samples was quantified by mixing 10 µl of the liquid supernatant and 90 µl of dH_2O in the wells of a microtiter plate (Corning, Corning, USA). A triplicate standard curve of 0, 2, 4, 6, 8 and 10 µg of glucose was included on the same plate. 200 µl of Anthrone reagent (2 mg anthrone (Sigma-Aldrich, St Louis, USA) per 1 ml of pure H_2SO_4) was added to each well and the plate was incubated at 80°C for 30 mins. Glucose containing samples turn from yellow to blue-green, and the absorption of all samples was read at 625 nm using an Infinite 200 PRO microtiter plate reader (Tecan, Mënnedorf, Switzerland).

2.4.2. Lignin quantification

This protocol was adapted from Foster el al. 2010a.

The two basal centimetres of stem were collected into 2 ml Eppendorf tubes (Eppendorf, Hamburg, Germany) and lyophilised with a Crios lyophilizer (Cryotec, Saint-Gély-du-Fresc, France) equipped with en EMF10 vacuum pump (Edwards, Crawly, UK). Lyophilised tissue was ground into a fine powder using metallic beads and three cycles of 30 seconds at 30 Hz in a TissueLyser II® (QIAGEN, Hilden, Germany). AIR was prepared from these samples as described previously.

1 to 1.5 mg of AIR was weighed into a screw cap 2 ml tube (Starstedt, Nümbrecht, Germany). The walls of the tube were rinsed with 250 µl of acetone to collect the cell wall material in the bottom of the tube and the solvent was evaporated. Once dry, 100 µl of a freshly made acetyl bromide solution (25% v/v acetyl bromide (Sigma-Aldrich, St Louis, USA) in glacial acetic acid) was added gently to avoid splashing.

The tubes were capped and incubated at 50°C for 2 hours. They were then heated for another additional hour vortexing every 15 mins. After cooling down to room temperature 400 µl of 2M sodium hydroxide and 70 µl of freshly prepared 0.5M hydroxylamine hydrochloride (Sigma-Aldrich, St Louis, USA) were added. The tubes were filled up to exactly 2 ml with glacial acetic acid. 200 µl of the solution were pipetted into the wells of a UV specific 96 well plate, and read in an Infinite 200 PRO microtiter plate reader (Tecan, Mënnedorf, Switzerland) at 280 nm.

The percentage of acetyl bromide soluble lignin (%ABSL) was determined using the following formula:

$$\%ABSL = \frac{Abs}{C \times PL} \times \frac{2\,ml}{length\,(cm)}$$

Were C is 15.69 and PL is the path length for the used plate. If multiplied by 10 the result gives µg of lignin per mg of cell wall.

2.5. Q-RT-PCR

2.5.1. Plant material collection

All plant samples were generated at least in triplicate. Seedlings were grown under liquid culture conditions as explained in section 2.1.2 and tissue was treated and collected 7 days after germination. At day 4 after germination, the liquid media in all wells was replaced by clean fresh ½ MS.

Plants stimulated osmotically were exposed to an osmotic step of 300 mOsm (reached by the gentle addition of 500µl of a concentrated stock of 1.8M Sorbitol to each well (Sigma-Aldrich, St Louis, USA)).

Plants stimulated mechanically were exposed to a force of 4 g/cm^2. An upside-down 50 ml Falcon tube (BD, Franklin Lake, USA) containing 52g of glass beads, with its bottom end cut off and its lid pierced with small holes (to allow it to sink), was placed gently into each well .

Plant material was collected, blotted briefly, placed into 2ml eppendorf tubes with a large metal bead, (Eppendorf, Hamburg, Germany) and frozen immediately after collection in liquid N_2.

2.5.2. RNA extraction and DNAse treatment

Frozen tissue was ground into a fine powder using two cycles of 30 seconds at 30 Hz in a TissueLyser II® (QIAGEN, Hilden, Germany).

RNA was extracted from collected samples using the Spectrum™ Plant Total RNA Kit (Sigma, St Louis, USA). Guidelines provided by the manufacturer were followed.

The obtained RNA preps were treated using Turbo DNA-Free DNaseI (Ambion, Austin, USA) following to the instructions provided by the manufacturer.

2.5.3. Determination of RNA concentration and reverse transcription

The RNA concentration of the samples was measured with the aid of a NanoDrop ND-1000 UV-Vis spectrophotometer (Thermo Scientific, Wilmington, USA).

1 μg of the extracted RNA was reverse transcribed using a SuperScript® VILO™ cDNA synthesis kit (Invitrogen, Carlsbad, USA) following the instructions provided by the manufacturer.

2.5.4. RT-PCR

To carry out RT-PCR, cDNAs were diluted 1:30 with sterile water, and 5 μl were used in each reaction. The RT-PCR reactions were carried out in optical 96-well plates (Eurogentec, Seraing, Belgium) in a StepOne Plus Real Time PCR System (Applied Biosystems, Foster City, USA). A mastermix was prepared using Platinum® SYBR® Green qPCR SuperMix (Roche Diagnostics, Meylan, France) in a final volume of 20 μl per sample according to provided instructions. The program used in the thermalcycler for these reactions was: 50°C for 2 mins, 95°C for 2 mins, 40 cycles of 95°C for 15 seconds, 60°C for 15 seconds and 72°C for 15 seconds. Dissociation curves were recorded during the reaction after cycle 40 heating gradually from 60°C to 95°C. A ramp speed of 1°C per minute was used. Data were analysed aided by the StepOne Software V2.2 (Applied Biosystems, Foster City, USA). Every RT-PCR reaction was carried out in technical triplicate.

Primers for EIF4 cDNA (G_{hk}) were used as a reference. The PCR efficiency (E) was then estimated from the standard curve amplification obtained data using the equation:

$$E = 10^{-1/slope}$$

The level of expression (R) of the genes of interest (G_{oi}) was calculated using the equation:

$$R = E^{-\Delta Ct}$$

Where

$$\Delta Ct = Ct\ G_{oi} - Ct\ G_{hk}$$

Standard curves were obtained by amplifying the RT-PCR amplicon from cDNA using classic PCR (explained in section 2.2.2). These PCR products were purified as explained in section 2.2.4 and quantified using a NanoDrop ND-1000 UV-Vis spectrophotometer (Thermo Scientific, Wilmington, USA).

A 1:100 dilution was made and used as S_0. The standard curve was prepared as following:

Standard	**S_{n-1}**	**dH_2O**
S_1	50 µl	450 µl
S_2	50 µl	450 µl
S_3	50 µl	450 µl
S_4	50 µl	450 µl
S_5	50 µl	450 µl
S_6	50 µl	450 µl
S_7	50 µl	450 µl
S_8	50 µl	450 µl

The primers used for Q-RT-PCR were:

Primer	**Sequence**
EIF4 FW	GAACTCATCTTGTCCCTCAAGT
EIF4 RV	TTCGCTCTTCTCTTTGCTCTCC
QXCDEK1 FW	CTTGAGAGAAGGTTTTCGGAG
QXCDEK1 RV	CCTGTTCGAGTTAGATTGTCA
QDEK1F1	AAACAAGAGGGGTTCTTACTT
QDEK1R1	TTCGAATCTGAACAAGTCTGT
QDEK1F2	AGTGGACTGATAGGATGAAGC
QDEK1R2	AAGGTGAGTAACACATGAATT
POST3 FW	GGCTAGTTCACATTATGTTCCAAA

POST3 RV	TCTTCTAATCTCAAGCTTCTTGGTG
POST4 FW	TCATTTCCGCCATTAAAGAAG
POST4 RV	GCACTGGTCCTCTCAACGTC
POST5 FW	CATTTCTGGTCTCAACCCAAG
POST5 RV	CGACGTAAGCTTCCATTTCAC
POST8 FW	CTGGTGCTAAACCTTGGAGATT
POST8 RV	CATTGTAAATGTCCCACCATTTC
POST9 FW	AAAGCTGGTGAGACCAGAGG
POST9 RV	AGCCTTGTCCCTCATCGTT
PTT2 FW	TTGAAGAAGATCCACCGACA
PTT2 RV	CGAGAGCTTCTTGTTCTCAGC
PTT3 FW	TCTTCTCAATTTCATTCCAGGAT
PTT3 RV	TGTTAAGGTTCTTGAAGAGCAGAG
TOD1 FW	CGTCACAACATTCTATCTTAAATCTCC
TOD1 RV	AATTGTTGTTCTTTGTCTCCTGAG
TOD2 FW	ACATCCCTCTATCTCTCAAACAACC
TOD2 RV	TTTCATTTCTCTTCCAATGACATTTCG
TOD3 FW	CCTTAAAAACATGGCCAGTACAACC
TOD3 RV	TCATCACCTCACGGTGTTGC
TOD4 FW	AAAGGAGCTTCTGTGGTTATTACAG
TOD4 RV	TTGTAACAGCTTACTCTTTGGAAAACG
TOD5 FW	ATCCACGTTCAGTCTTCCTCC
TOD5 RV	CAAACTACCAATGCTGATGCC
TOD19 FW	AAAGAGAGGATCGAGTGTGG
TOD19 RV	TTGCAAGCATCAATGCAAGGC

2.6. Ion fluxes and concentrations

2.6.1. Flame photometry

Samples were prepared by weighing between 0.5 g and 1 g of fresh tissue. This was placed into containers of aluminium foil and heat dried at an incubator at 60°C for one week. Dried samples were weighed and placed in 50 ml Falcon tubes (BD, Franklin Lake, USA).3 ml of 1.5M HCl per 0.5 g of dry tissue was added. These samples were incubated at 37°C for 10 days and the concentrations of Ca^{2+} and Na^{+} were measured using a Model 410 flame photometer (Sherwood Scientific, Cambridge, UK) after diluting 200 μl of extract in 5ml of dH_2O.

Readings were compared with calibration curves of 250, 125, 67.5, 33.73, 16.875, 8.4575 and 2.218 μM of NaCl or $CaCl_2$.

2.6.2. *In planta* ion flux measurements (impalements)

Double barrelled microelectrodes were made in order to give a tip resistance within the range of 300–500 mΩ when filled with 200 mM of potassium acetate. The microelectrodes built were approximately 2.0 cm-long and tips were shaped with a 1–1.5° angle. These microelectrodes were stored before in a paraffin-coated glass desiccator in order to reduce capacitance before impalement.

A Narashige C2 micromanipulator (Narashige, Tokyo, Japan) containing a brace that had a fixed clamp for one amplifier headstage and an adjustable clamp, was used. Ag|AgCl-KCl type half cells were prepared as follows: a 0.5 mm diameter wire of Ag was soldered into a 2-mm diameter socket and fitted with the aid of a silicon plug behind the tip segment of a 2-ml graduated plastic pipette tip. The

electrode was then filled with electrode buffer (10mM BaHEPES, 33mM CsHEPES, pH7.5) and then fixed to the half-cell with the aid of dental resin.

Impalements were performed by positioning the tip of the microelectrode within the cytoplasm of a root epidermal cell of a seedling grown in liquid culture conditions as explained in section 2.1.2 for 4-5 days. The system was then allowed to rest for a period of 2 to 3 mins in order to stabilize after impalement. Impalements were carried out with the samples immersed in Recovery buffer (10mM KCl, 5mM NaMES; pH6.1).

Voltage clamp data was then recorded with the samples immersed in an osmotic step solution (20mM $BaCl_2$, 5mM NaMES, 30mM mannitol; pH6.1). Buffer change was performed using a gravity-fed system. Data recorded was analysed using Henry's EP Suite software (Blatt 1987; 2004).

2.6.3. Electrophysiological measurements in *Xenopus* oocytes

2.6.3.1. Building of constructs

Full length and the transmembrane domain coding region of *DEK1* were cloned into pGEMXho plasmids. In order to do this I adapted the plasmid to the Gateway® system. For this the Gateway® R_1 and R_2 sites were amplified with a high fidelity PCR (as described in section 2.2.2) from the cDEST vector. This reaction was carried out using the primers TTTGGATCCGGCTAGTTAAGCTATCAACAAGTTTG and AAAACTAGTCGGATAGGCTTACCTTCG. The reaction products were cleaned up (as described in section 2.2.4) and cloned into a pENTR vector (Invitrogen, Carlsbad, USA) following the manufacturer's guidelines. The obtained plasmid was digested using the restriction enzyme SpeI (as described in section 2.2.5).

The expression plasmid was linearized using SpeI and treated with dCIP phosphatase to avoid relinearization (as described in section 2.2.5). The digested PCR product and linearized vector were ligated using T4 DNA ligase (as described in section 2.2.5).

LR Gateway® reactions were then carried out with the genes of interest as described in section 2.2.6.

2.6.3.2. Injection in *Xenopus* oocytes and electrophysiological measurements

Oocytes were obtained from ice-anesthetized *Xenopus laevis* through a surgical process. Once isolated, oocytes were defoliculated by a treatment in a solution containing 82.5 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM Hepes-NaOH pH 7.4 and 2 mg ml^{-1} collagenase for 60 minutes. Oocytes in phases V and VI were selected and placed in a solution containing 96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 2.5 mM Na-pyruvate, 5 mM Hepes-NaOH pH 7.4 and 50 mg ml^{-1} gentamicin.

Injection of oocytes was carried out using a 10-15 μm tip micropipette attached to a pneumatic injector. 30 ng of cRNA or dH_2O were injected. Oocytes were subsequently incubated for 4 days at 19°C.

Electrophysiological recordings were carried out with the voltage-clamp technique using a GeneClamp 500B (Axon Instruments, Sunnyvale, USA). Protocols, acquisition and analysis of data were carried out using pCLAMP10 (Axon Instruments, Sunnyvale, USA) and SigmaPlot (Jandel Scientific, San Rafael, USA). Both current and voltage applied were recorded.

2.7. Yeast techniques

2.7.1. Bait and prey construction for yeast 2 hybrid (Y2H) assays

The gene or gene fragment of interest was amplified using a high fidelity polymerase (Phusion). Specific sequences were added to primers in order to introduce *SfiI* sites. PCR products were purified as detailed in section 2.2.4. Overnight digestions were made using *SfiI* (Takara, Shiga, Japan) at 50°C. The digestion products were run on gels, and purified. Ligations were set up using T4 DNA ligase with SfiI digested vectors; pDHB1 for bait vectors and pPR3N for prey vectors (vectors as sold by Dualsystems Biotech, Schlieren, Switzerland).

The primers used for cloning of genes into Y2H vectors were:

Primer	**Sequence**
Y2H_CALP_FW	GTGGCCATTACGGCCCGGAGAATGCGTTCAGTTGAGTTG
Y2H_CALP_BAIT_RV	CGACATGGCCGAGGCGGCCAACAAAGCTTCAAGAACAATGGATGC
Y2H_CALP_PRAY_RV	AGGCCGAGGCGGCCGAAACAAAGCTTCAAGAACAATGGATGC
IXR15yeastF	AGGCCATTACGGCCATGAAAAGTGGAGGGAACACAAAC
IXR15yeastPREY	AAGGCCGAGGCGGCCGAGCCGATGGAGAAGAAACTG
LUNG7TMyeastF	AGGCCATTACGGCCATGACGAGACTACCCCTCTTC
LUNG7TMyeastPREYr	AAGGCCGAGGCGGCCGGAGTTCAAAATCATCCTCCTTCAAG
TUB2yeastF	AGGCCATTACGGCCATGCGTGAGATTCTTCACATC
TUB2yeastPREYr	AAGGCCGAGGCGGCCGGTACTCTTCCTCCTGTTGATATTC
TUB2yeastBAITr	AGGCCGAGGCGGCCAAGTACTCTTCCTCCTGTTGATATTC
Y2H_FLmaMYB_FW	AACGCGGCCATTACGGCCATGGATTTTTTCGACGAAGAC

Y2H_CLmaMYB_FW	AACGCGGCCATTACGGCCTCTCTCCTTCTTCTACTTCG
Y2H_maMYB_BAIT_RV	CGACATGGCCGAGGCGGCCTTAATTAGCTGGAGTTTTCGA

The vectors were sequenced as described on section 2.2.9 using the following primers:

Primer	Sequence
pDHB1 FW	TTTCTGCACAATATTTCAAGC
pDHB1 RV	GTAAGGTGGACTCCTTCT
pPR3N FW	GTCGAAAATTCAAGACAAGG
pPR3N RV	AAGCGTGACATAACTAATTAC

2.7.1. Screening of interactions of calpain bait with cDNA library

1 mg of silique RNA was extracted as detailed in section 2.5.2 and sent with a bait construct containing the calpain-encoding domain of *DEK1*, to Dualsystems Biotech, where a cDNA library was prepared. Dualsystems Biotech also performed a functional assay for the bait, a pilot screen to test for self-activation and screened the cDNA library following their own protocols. Sequences of potential preys were eventually provided, but a prolonged delay meant that I was unable to follow up as many of these interactors as I would have liked.

Once sequences had been returned, prey vectors were constructed to retest interactions (by co-transformation with the calpain containing bait vector), and to test for false positives (by co-transformation with an empty bait vector).

2.7.2. Double transformation of plasmids in to yeast

Several fresh colonies of yeast strain NMY51 (*Saccharomyces cerevisae*), provided by Dualsystems Biotech, were inoculated into 50 ml of YPAD medium (20g Difco bacto-peptone, 10g Yeast extract, 50mls of 40% (W/V) Dextrose, and water to 1L. 15 ml of filter-sterile Adenine hemisulphate was added after autoclaving). Cutures were incubated overnight with agitation at 28°C. An optical density (OD) at 546 nm between of 0.6 was sought, and if the culture passed this density it was diluted to an OD of 0.2 and left to grow up to 0.6). In order to continue the transformation, the cells were pelleted by centrifuging for 5 mins at 2,500 RPM and resuspended in 2.5 ml of dH_2O. Both plasmids to transform were mixed (1.5 μg of each) with 300 μl of PEG/LiOAc mix (240 μl polyethylene glycol, 36 μl LiOAC) 25 μl DNA carrier (Sonicated Salmon sperm DNA denatured by two baths of 5 mins at 99°C)) and 100 μl of resuspended cells. This mix was mixed thoroughly by vortexing. A heat shock of 42°C for 45 mins was applied. The suspension was centrifuged for 5 mins at 700 RPM, resuspended in 100 μl of dH_2O, and spread on dropout (SD) media (6.8g Difco Yeast Nitrogen base w/o amino acids, 20g glucose, amino acid mix lacking Tryptophan and Leucine to allow selection of bait and prey vectors (as manufacturer's instructions) (TAKARA Bio, Shiga, Japan), pH5.8, 20g Bacto Agar, autoclave 115°C for 15 minutes) and left incubating for 3-4 days at 30°C.

2.7.3. Serial drop test

After transformation and growth in selective SD media, two colonies were taken with a sterile loop and suspended in 1 ml of dH_2O. Serial dilutions of 1:10, 1:100 and 1:1000 were prepared and placed as drops of 11-12 μl on selective SD media as above, but made with amino acid mixes additionally lacking either Histidine (to detect mild interactions), or Histidine and Adenine (to detect strong interactions). Tests were left to grow at 30°C for several days before being photographed.

3. Effect of mechanical stress in Arabidopsis thaliana plants

3.1. Introduction

3.2. Mechanically stressed wild-type Arabidopsis show an increase in cellulose content

3.3. Mechanically stressed wild-type Arabidopsis show an increase in lignification

3.4. Transcriptional changes in osmotically and mechanically stimulated Arabidopsis wild-type plants

3.4.1. Osmotic stress up-regulates both osmotic and mechanical sensitive genes

3.4.2. Mechanical stress cannot be used as a proxy for osmotic stress

3.5. Summary and conclusions

3. Effect of mechanical stress in *Arabidopsis thaliana* plants

3.1. Introduction

Thigmomorphogenetic responses are different from species to species and they generally occur slowly over time. The most common touch triggered alterations reported in the literature include reduction of shoot elongation, but thigmomorphogenesis also correlates with changes in hormone levels, chlorophyll content, stomatal distribution, cell wall structure and composition, leaf size and wax composition (Biddington 1986; Jaffe 1973; Jaffe and Forbes 1993; Mussell et al. 1979; Saidi et al. 2010; Whitehead and Luti 1962).

Changes in the cell walls of mechanically stimulated plants have been reported. In *Solanum lycopersicum* (tomato) subjected to mechanical elicitations, an enhancement in the rate of cell wall lignification was described (Saidi et al. 2010). Similarly, it has been shown that an accumulation of lignin increases the rigidity of the cell wall in mechanically stimulated *Phaseolus vulgaris* (bean plant), leading to plants showing more resistance to herbivorous pests compared to non-stimulated plants (Cipollini 1998; Cipollini Jr 1997).

Although very few studies have been made on the effects of mechanical stimulation in the cell wall of *Arabidopsis*, the effects of altered gravity have been widely described. Plants grown in outer space, and therefore exposed to reduced gravitational forces, show a decrease in cellulose content (Cowles et al. 1984; Nedukha 1996) and in matrix polysaccharides (Hoson et al. 2002). Hypergravity, which is considered to have similar effects on plants to mechanical stimulation, was tested by treating plants for 24 hours at 300g. These plants had shortened and thickened stems, with an increased development of primary xylem and an increase

in lignin deposition (Nakabayashi et al. 2006; Tamaoki et al. 2004; Tamaoki et al. 2006). Similar results were seen in other species, such as *Brassica rapa* (Allen et al. 2009).

Even though not all changes seen in the cell wall are directly due to transcriptional changes, many of these effects could be due to changes in the expression level of touch-induced genes. These may have diverse functions contributing to the diverse morphological and physiological phenotypes associated with mechanical stress. Genes up-regulated by mechano-stimulation have been implicated in biotic resistance (Ma et al. 2008), ion stress responses (Delk et al. 2005), regulation of transition to flowering (Tsai et al. 2007) and cell wall biosynthesis (Xu et al. 1995) amongst others.

In *Arabidopsis thaliana* the effect of direct mechano-stimulation has been relatively poorly described, although it has been shown that it has an effect in flowering time, inflorescence elongation and petiole length (Braam 2005; Braam and Davis 1990; Paul-Victor and Rowe 2011). Until now the effects of this type of stress on the composition of the cell wall are largely unknown.

Osmotic stress has been used for years as an approximation to mechanical stimulation is several organisms, including bacteria (Berrier et al. 1992; Levina et al. 1999). Osmotic stress or shock involves sudden changes in solute concentrations outside the cell. This triggers changes in water transport through the plasma membrane. These changes in water transport are driven by passive forces. Under high osmolarity water flows out of the cell through osmosis. Under low osmolarity it flows into the cell, leading to swelling. Both types of osmotic stress generate membrane stress. This is a consequence of the fluidity of cell membranes. Changes in water activity lead to changes in membrane shape that can alter the mechanically imposed strain of the membrane. Hyper-osmolarity can make the surface of a spherical cell become non-spherical. This requires an in-plane extension and

changes in curvature. Hypo-osmolarity can lead non-spherical cells to become more spherical. This also requires an in-plane extension and changes in curvature (Wood 1999). These changes in the membrane topology have the characteristic of changing the mechanical forces pattern present in the membrane. This is the reason why osmotic stress has been proposed as an approximation for mechanical stress. However, the validity of this proxy remains controversial.

It has been shown that osmotic stress causes characteristic transcriptional changes in several organisms, including yeast (Causton et al. 2001; Gasch et al. 2000), animals (Ho 2006) and plants (Xiong and Zhu 2002). Several datasets studying the effects of osmotic stress in plants are available in literature. It was observed that different tissues show different responses to this type of stress. When treated for 3 hours with an osmotic step very different changes are seen in (483 genes misregulated) and roots (285 genes misregulated) (Kreps et al. 2002). More recent data has been published on the effect of this treatment in seedlings, with significative changes in the transcription level of around 900 genes. This included an important number of transcription factors (around 100) (Zeller et al. 2009). This is useful, as response to osmotic stress can be detected and quantified through transcriptional analysis.

Less is known about touch-regulated genes. In 2005 Lee and collaborators published for the first, and only list to date of genes up- or down-regulated in response to mechanical stimulation. 589 genes were described as showing increased expression and 171 genes with decreased expression in this dataset (Lee et al. 2005).

In this chapter I studied the responses seen in mechanically stressed *Arabidopsis thaliana*. In particular I focused on changes in the cell wall. I also compared transcriptional changes in *Arabidopsis* under mechanical and osmotic stimulation in order to test whether these two types of stress are truly interchangeable and whether osmotic stress represents a valid proxy for mechanical stress in the case of plants.

3.2. Mechanically stressed wild-type *Arabidopsis* show an increase in cellulose content

In order to investigate the possible changes in the cell wall of mechanically stressed *Arabidopsis thaliana* plants, the content in cellulose of the cell wall in mechanically stimulated *vs.* non-stimulated plants was studied.

The contents of the cell wall can be quantified from the alcohol-insoluble residue (AIR) after its extraction from plants which are first incubated in the dark for at least 24 hours to remove starch. During this extraction process all soluble components of the sample are washed out by several rinses with ethanol, leaving mainly the polysaccharides of the cell wall, and lipids. Using a simple technique, cellulose can be isolated from the rest of polysaccharides and quantified by colorimetric assays, such as the anthrone method. This technique assays the coloured products of the reaction of cellulose hydrolysis products with the aromatic molecule anthrone. These products have a maximum absorbance at 620 nm and can be easily quantified by classical colorimetry (Foster et al. 2010b; Trevelyan et al. 1952).

Wild type plants were treated with mechanical stimulation of 4 g/cm^2 twice daily for one minute, starting at the appearance of the first pair of true leaves. Fully expanded adult leaves were collected and their content in cellulose was quantified using the above technique (see materials and methods for details).

Unstressed plants had a content of cellulose of 0.55 μg of glucose equivalents per μg of AIR. Mechanically stimulated plants showed an increase of approximately 50 % in the cellulose content of their cell walls with a value of 0.84 μg of glucose equivalents per μg of cell wall (Table 3.1; Figure 3.1).

Table 3-1 Quantification of the cellulose content of the cell walls of leaves of wild-type mechanically unstressed and stressed plants. Expressed in glucose equivalents per µg of AIR.

	WT
UNSTRESSED	0.55
STRESSED	0.84

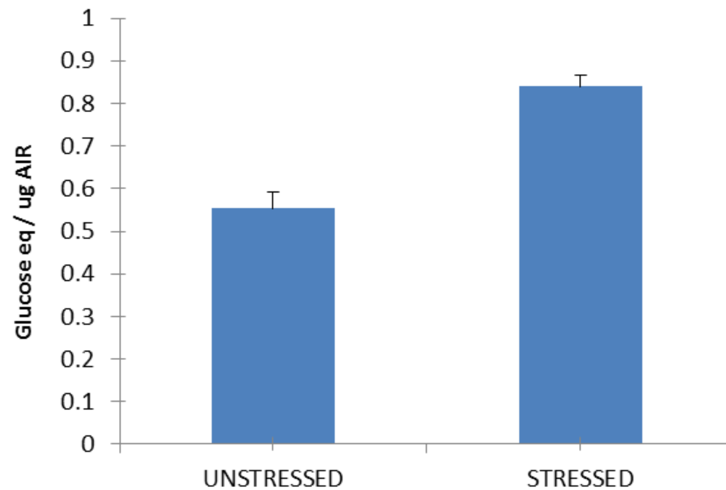


Figure 3-1 Quantification of cellulose content of cell walls of leaves of wild-type mechanically unstressed and stressed wild-type plants. Error bars show standard deviation. Statistical significance tested with a t-test, n=10, p<0.01.

3.3. Mechanically stressed wild-type *Arabidopsis* show an increase in lignification

Several publications suggest that changes in stem lignin content and, in particular, lignification of secondary cell walls, are a characteristic of mechanically stressed plants (Cipollini 1998; Cipollini Jr 1997; Saidi et al. 2010). I therefore tested if this response is present in the stems of wild-type *Arabidopsis* plants under my conditions (treatment with mechanical stimulation of 4 g/cm^2 twice daily for one minute starting at the appearance of the first pair of true leaves).

Lignin can be quantified by its auto-fluorescence after being extracted with acetyl bromide (Foster et al. 2010a) . My results showed that this component of secondary cell wall increases by approximately 55% in mechanically stimulated plants in comparison to non-stimulated individuals (table 3.2, figure 3.2). A value of 121.6 µg of lignin per cm of stem was detected in non-stimulated plants while stimulated individuals showed a value of 213.2 µg of lignin per cm of stem.

Table 3-2 Quantification of content of lignin in µg per cm of stem in mechanically stimulated and non-stimulated wild-type *Arabidopsis*.

	WT
Unstressed	121.64
Stressed	213.17

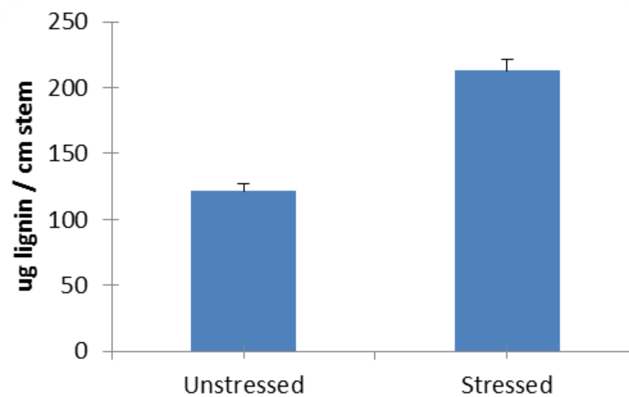


Figure 3-2 Quantification of content of lignin in mechanically stimulated and non-stimulated wild-type *Arabidopsis*. Error bars show standard deviation. Statistical significance tested with a t-test, n=4, p<0.01.

3.4. Transcriptional changes in osmotically and mechanically stimulated *Arabidopsis* wild-type plants

3.4.1. Osmotic stress up-regulates both osmotic and mechanical sensitive genes

All experiments in this section were carried out in collaboration with Kimberly Berthet, a project student who worked under my technical supervision for a 3-month period.

Osmotic steps have long been used as a proxy for mechanical stress in bacteria (Berrier et al. 1992; Levina et al. 1999). In order to develop tools for the analysis of responses to mechanical stresses in *Arabidopsis*, I therefore wanted to test if osmotic stress could be used as an alternative to mechanical stimulation in *Arabidopsis* plants. The rationale for this is that osmotic stresses are much easier to apply to plants in a uniform manner, and without the risk of wounding, than mechanical stimuli.

In order to test this we used an RT-Q-PCR assay. We selected a set of genes to use as transcriptional markers for responses to stress. These genes included both potential targets of osmotic stimulation and potential targets of touch stimulation extracted from the literature and from *in silico* resources. The potential targets of osmotic stimulation were obtained from the *Arabidopsis* eFP browser (Winter et al. 2007), named *POST* (Putative Osmotic Stress Target) and designated by a number. The potential touch targets are a selection of published touch sensitive genes (Lee et al. 2005), were named *PTT* (Putative Touch Target) and designated by a number.

POST3 is *At1G53180*, a gene encoding an expressed protein of unknown function. It is expressed in several structures including leaf, flower, apical meristem and root (Winter et al. 2007).

POST5 is *At5G59320*, a gene encoding for LTP3 or Lipid-Transfer Protein 3. The LTPs genes are a family of lipid-transfer proteins that facilitate the transfer of lipids between membranes (Harryson et al. 1996; Kader 1996; Maxfield and Mondal 2006). They have putative roles in several processes including cutin formation (Sterk et al. 1991), embryogenesis (Sterk et al. 1991; Thoma et al. 1994) and defence reactions against pathogens (Molina et al. 1993), amongst others. They are known to be accumulated in various cellular compartments (Yeats and Rose 2008).

POST8 is *At1G56600*, which encodes GolS2, a Galactinol Synthase. This enzyme is responsible for the catalysis of the first step in the synthesis of Raffinose family oligosaccharides from UDP-galactose (Taji et al. 2002). Expression of this gene is induced by drought and high-salinity stress. It seems to be involved in controlling cytokinin levels (Guo et al. 2010; Taji et al. 2002).

POST9 is *At3G15670*, encoding a Late Embryogenesis Abundant family protein (LEA). These proteins are named after certain family members that are accumulated during the late phases of embryo development (Dure et al. 1981). Expression studies showed that several genes encoded by genes of this protein family respond to abscisic acid, low temperature or drought stress (Hundertmark and Hincha 2008).

The potential touch targets were chosen from a published list of touch sensitive genes (Lee et al. 2005). These genes in particular were chosen on the basis that they should not show strong transcriptional responses to wounding, which can be easily confused with mechanical stimulation. For these targets we found that they show a fairly weak response to wounding and that this response disappears within the first

30 minutes after the wounding event. This was checked in the eFP browser (Winter et al. 2007).

PTT2 is *At1G80840*, which encodes a WRKY40, a member of the WRKY Family of Transcription Factors. It has been reported that this protein interacts with the other pathogen induced proteins WRKY18 and WRKY60 (Xu et al. 2006b). The expression of these transcription factors seems to be responsive to abscisic acid and abiotic stress (Chen et al. 2010a).

PTT3 is *At3G55980*, gene encoding a putative zinc finger transcription factor. This protein seems to be involved in the molecular mechanisms governing the response to salinity stress. Over-expressers of this gene show a reduced response to salt stress detected by Q-RT-PCR (Sun et al. 2007).

In order to test the effects of osmotic stress on gene expression we had to set up a robust experimental procedure. We chose to use seedlings, as they are an accessible and uniform material, which is easy to grow in sterile liquid culture and under controlled conditions. For all these experiments to set up the technique, we decided to test only to targets: *POST5* and *POST9*. These targets were shortlisted from the original list as they gave very good results in the preliminary tests.

The first variable we tested was the means of generating the osmotic stress on the seedlings. Classically these sort of experiments have been carried out using the saccharides mannitol and sorbitol, as they are not metabolizable, thus they do not affect parameters other than those related to osmotic pressure (Flores and Galston 1982; Patching and Rose 1971; Riov and Yang 1982). Increasing concentrations of 50 mM, 150 mM, and 300 mM were used to generate the stress. Treatments were carried out for a time lapse of 3 hours. Defined volume of different concentrated stocks were added to the media in which the seedlings were growing. Controls were

treated with the same volume of liquid media as that administered with the osmotic stress treatment.

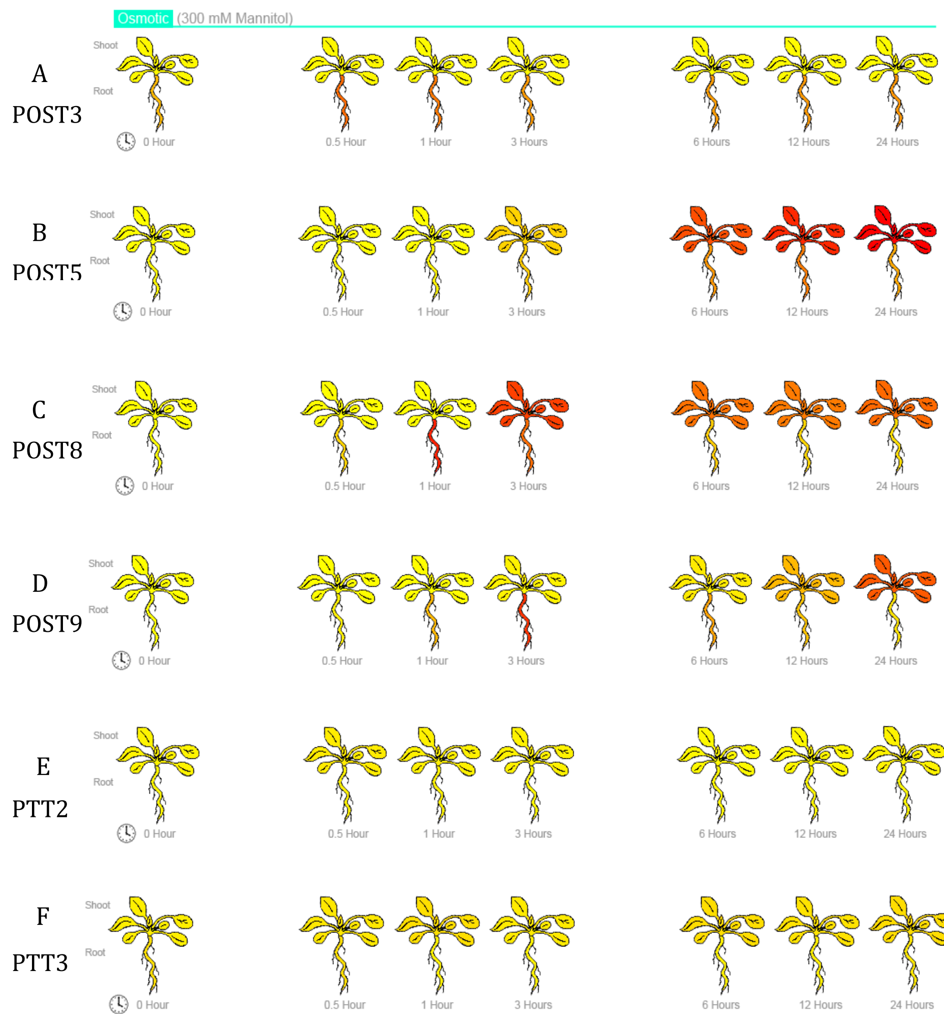


Figure 3-3 Transcriptional responses of osmotic sensitive genes in seedlings treated with 300 mOsm of mannitol in a time lapse of 24 hours. *POST3* (A), *POST5* (B), *POST8* (C) and *POST 9* (D). Taken from eFP Browser (Winter et al. 2007).

A positive correlation was observed between the increasing concentrations of both sorbitol and mannitol and the increased up-regulation of proposed osmotic stress genes. All osmotic stress targets showed the same trend, with the greatest transcriptional response observed at a concentration of 300mM for both sorbitol and mannitol (Figure 3-4). The decision was made to osmotically stimulate the seedlings

with sorbitol at a concentration 300mM, as sorbitol was much more easily dissolved than mannitol. This decision was based purely on practical reasons as mannitol would have been as efficient creating osmotic stress as sorbitol.

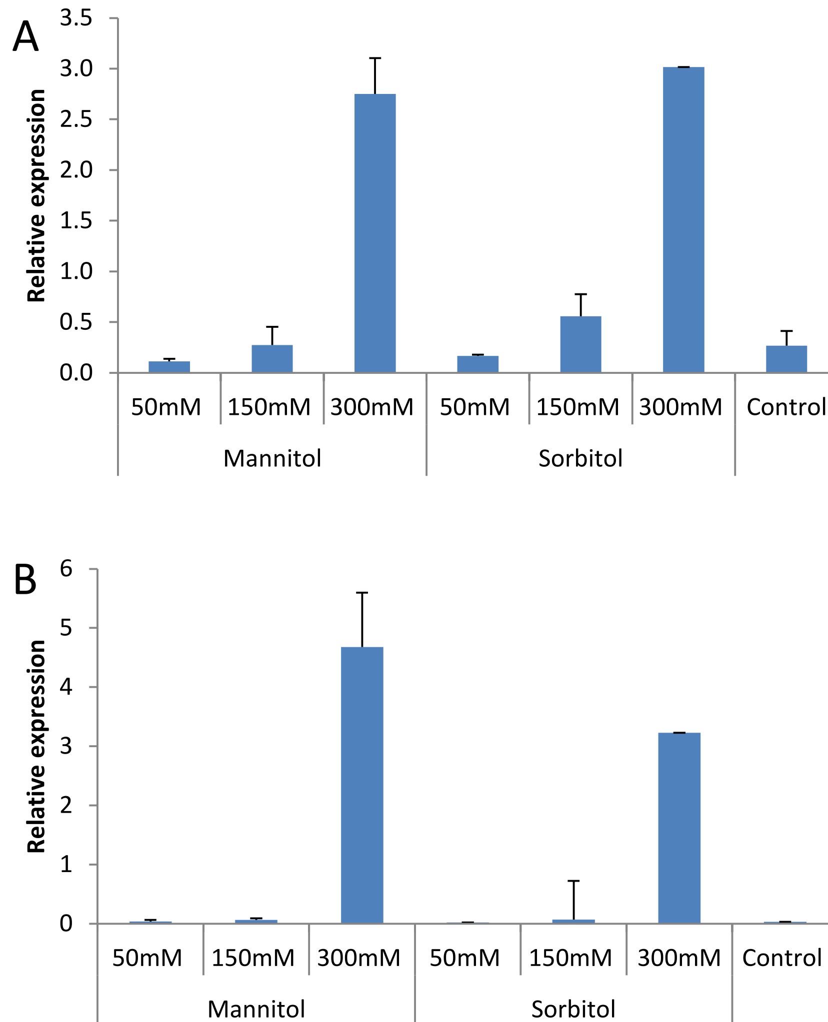


Figure 3-4 Relative expressions of *POST5* (A) and *POST9* (B) under treatments with 50 mM, 150 mM and 300 mM of mannitol and sorbitol. Controls treated with an isosmotic solution. Error bars show standard deviation between three biological replicates. ANOVA test shows a statistical significance between samples treated with 300 mM of mannitol or sorbitoland all others, $p<0.01$.

We next wanted to test the effectiveness of continuous *vs.* transient osmotic stress. An osmotic treatment was administered with 300 mM sorbitol contained in the media. Transient stress was administered for 30 minutes and the media was then replaced by fresh media that no longer contained the osmoticum. Seedlings were left incubating for 3.5 hours longer before collecting the tissue. Continuous stress was administered for 4 hours, replacing the media after 30 minutes with new sorbitol containing media. Controls were treated with the same volume of liquid media as the one administered in the osmotic treatment, with the corresponding change of media after 30 minutes.

A greater transcriptional response was observed in seedlings treated continuously in comparison to the ones treated transiently (Figure 3-5). Therefore continuous treatment with sorbitol at a concentration of 300mM was used in the following tests.

In order to determine the ideal time span of osmotic exposure in order to detect a transcriptional response, sorbitol was administered to create a hypertonic environment of 300 mOsm. Controls were treated in the same way, but using iso-osmotic media. All the seedlings were treated at time points of 30 minutes, 60 minutes, 2 hours and 3 hours.

Proposed osmosensitive genes were up-regulated in the presence of hyperosmotic stress. The length of exposure was positively correlated with the increased transcriptional response in putative osmosensitive targets (Figure 3-6). From this analysis the greatest exposure length of 3 hours was chosen for hyperosmotic stress induction.

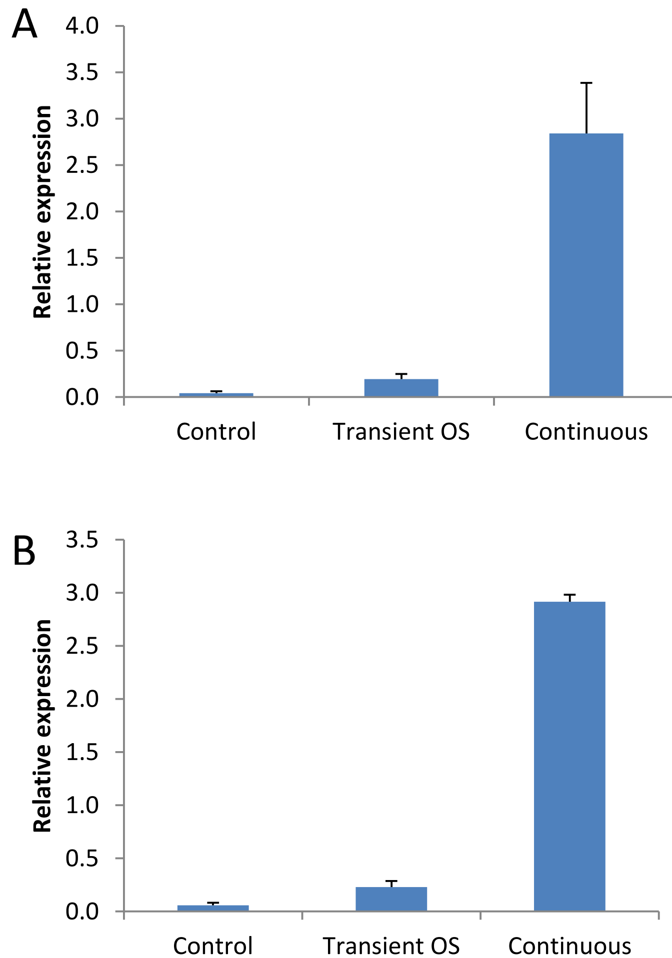


Figure 3-5 Relative expressions of *POST5* (A) and *POST9* (B) under transient or constant treatments with 300 mM of sorbitol. Controls treated with an isosmotic solution. Error bars show standard deviation between three biological replicates. ANOVA test shows a statistical significance between samples treated with continuous stress and all others, $p<0.01$.

Even though we only tested time points up to 3 hours, *in silico* data suggests that longer time points might have also been effective. Different targets can show different kinetics in their response to stress. Response can vary from a robust and constant up-regulation, to a more transient up-regulation that decreases with acclimation. One of the reasons why we chose to test osmotic stress for 3 hours was because we felt that this was more likely to allow us to observe these transient responses than longer time lapses.

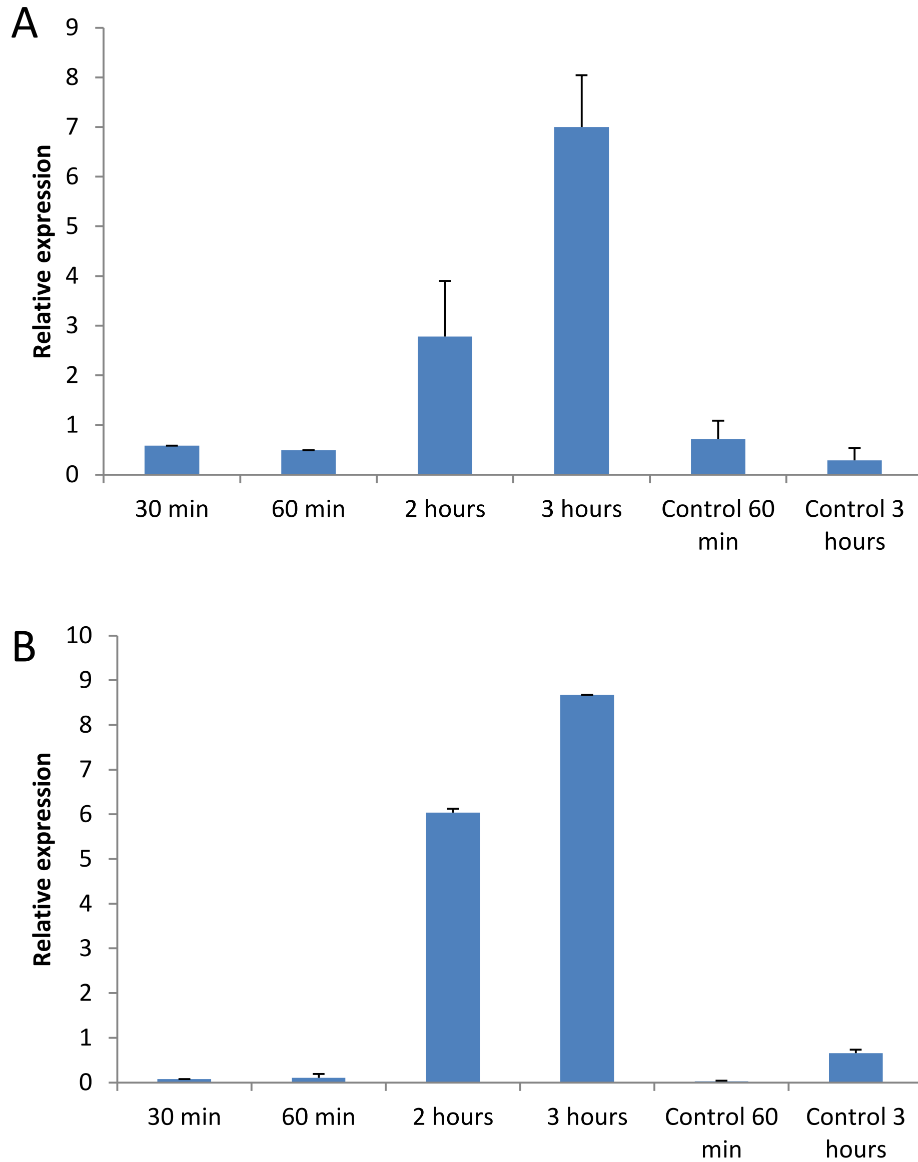


Figure 3-6 Relative expressions of *POST5* (A) and *POST9* (B) under treatment with sorbitol 300 mM for 30 minutes, 60 minutes, 2 hours and 3 hours. Controls treated with an isosmotic solution. Error bars show standard deviation between three biological replicates. ANOVA test shows a statistical significance between samples treated for two and three hours and all others, $p<0.01$.

Having identified the conditions required to give a robust transcriptional response to osmotic stress, we then tested the behaviour of the two other *POST*s and the two *PTT*s which we had chosen to analyse. The experiments were carried out with an osmotic step of 300 mOsm, generated with sorbitol. This was applied in a continuously way for 3 hours immediately before collecting the tissue, from which RNA was obtained in order to do RT-Q-PCRs. Controls were treated in the same way, but using isosmotic liquid media.

For all cases, both targets of osmotic stimulation and "touch genes", we saw an induction of expression as a response to an osmotic step of 300 mOsm. In most cases, these genes are induced at least 5 fold compared to the untreated control (figure 3-7). It should be pointed that there is discrepancy between the experimental data we obtained and the available *in silico* data regarding the over-expression of *PTT2* and *PTT3* under hyper-osmotic stress conditions, since neither gene is detectably induced in this dataset. However the technique we used to obtain our results is different to the one used to build the eFP browser. In particular, in our case we used RT-Q-PCR, while *in silico* data comes from microarray data.

These results suggest that an osmotic step of 300 mOsm, generated with sorbitol, can be used as a good proxy to mechanical stress, as using this kind of osmotic stimulation generates a clear response in both osmotic stimulation and touch stimulation target genes.

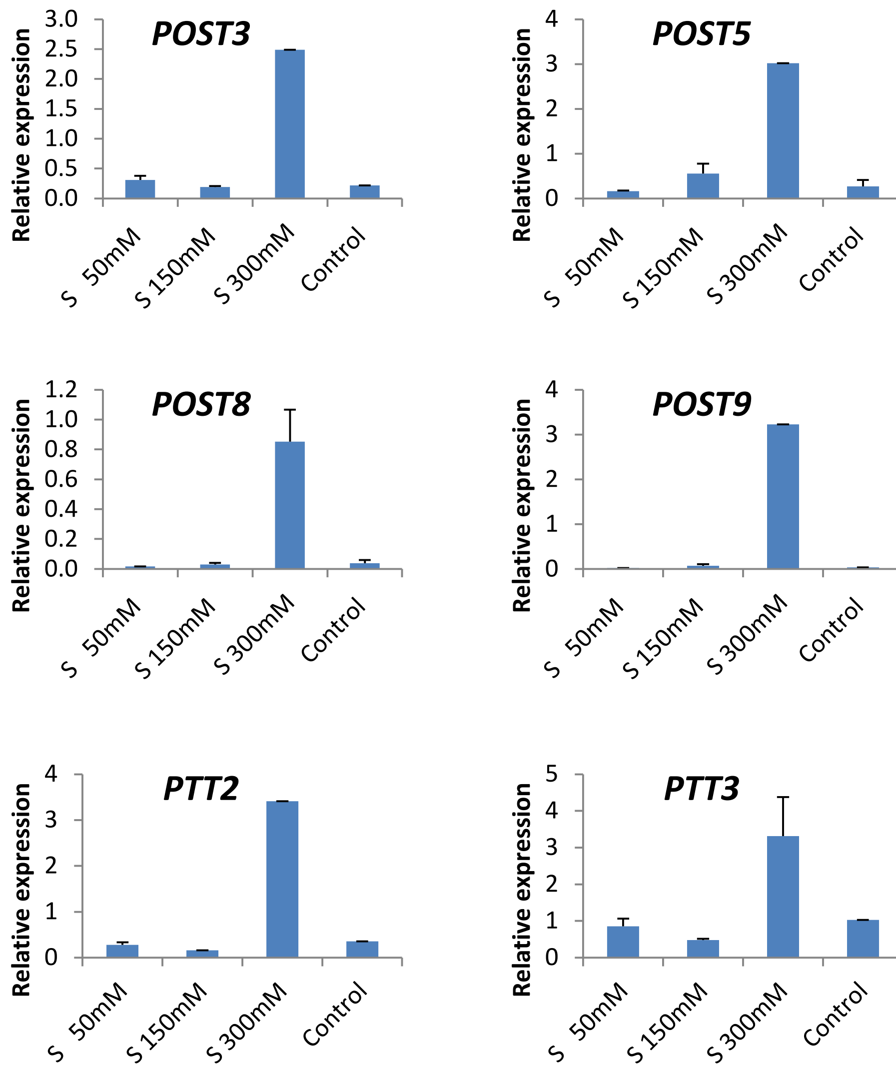


Figure 3-7 Q-RT-PCR recordings of the transcriptional changes in the expression of *POST3, POST5, POST8, POST9, PTT2* and *PTT3* in response to increasing osmotic shocks of sorbitol (S 50 mM, S 150 mM and S 300 mM). Error bars show standard deviation between three biological replicates. ANOVA test shows a statistical significance between samples treated with 300 mM of sorbitol and all others, $p<0.01$ for all except for PTT3, where $p<0.05$.

3.4.2. Mechanical stress cannot be used as a proxy for osmotic stress

We observed that an osmotic step would mimic the effects of mechanical stimulation, seen as the up-regulation of mechanical stress reporter genes. Therefore, it can be used as a proxy for mechanical stress in *Arabidopsis*. We therefore decided to test whether the inverse situation was true, and whether we could consider mechanical and osmotic stimulation as interchangeable.

In order to answer this question we had to develop a mode of administering mechanical stimulation to plants grown in liquid media. To do this we created and tested two different methods. The first method consisted of adding a single round clean glass bead to each well in which the seedlings were growing and placing the plates on a shaker. The second method was to place an inverted 50 ml falcon tube, filled with a constant number of glass beads (52 grams), in each germination well.

After one hour of incubation we could see that the first method did not generate a detectable change in the transcription level of "touch genes" *PTT2* and *PTT3*. This is probably because the glass bead tended to roll around the periphery of the well and hardly touched the plants. The stress induced simply by agitation alone appears to have been insufficient to activate "touch genes". On the other hand, the second method proved to be very effective in generating a transcriptional response (figure 3-8).

Stimulation of full seedlings might not be the ideal method as some tissues might be responding in different way to the applied stress. Nevertheless, I strongly believe that using isolating a certain organ/tissue for this experiment could generate a transcriptional response to wounding, possibly masking the transcriptional effects of the applied mechanical stress.

Therefore, we decided to stimulate the plants mechanically by applying a force to liquid culture grown seedlings using an inverted, glass bead filled, 50 ml falcon tube (figure 3-9). The bottoms of the tubes were removed and the lids were perforated repeatedly with a needle to allow the liquid media flow into the tube and, therefore, reduce buoyancy. This stimulation was carried out for 3 hours. For these experiments we tested *POST5*, *POST9* and *PTT2*.

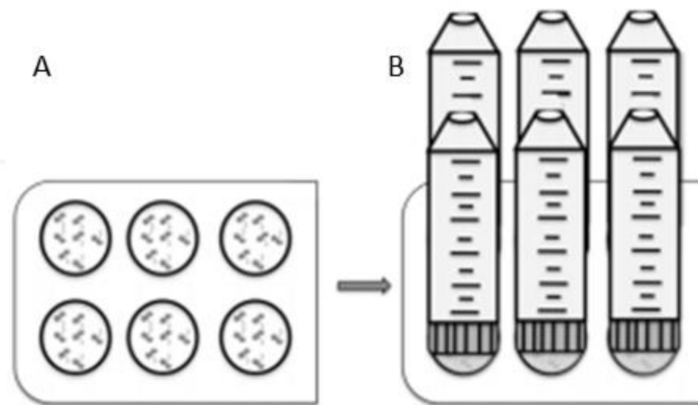


Figure 3-9. Scheme of the mechanical stimulation system. 6-well plate liquid culture grown seedlings (A) were treated with inverted, glass bead-filled falcon tubes (B).

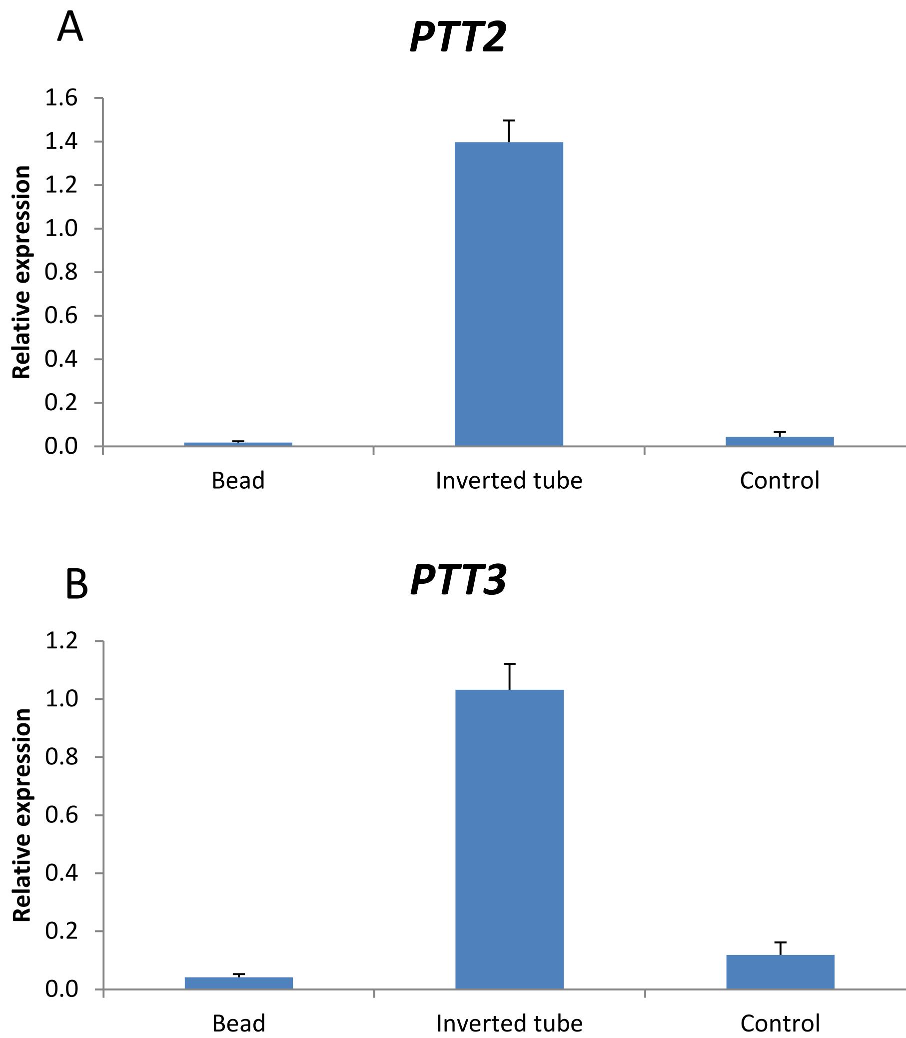


Figure 3-8 Relative expressions of *PTT2* (A) and *PTT3* (B) under mechanical treatments with a glass bead (shaking) or an inverted falcon tube. Controls were untreated samples. Error bars show standard deviation between three biological replicates. ANOVA test shows a statistical significance between samples treated with an inverted tube and all others, $p<0.01$.

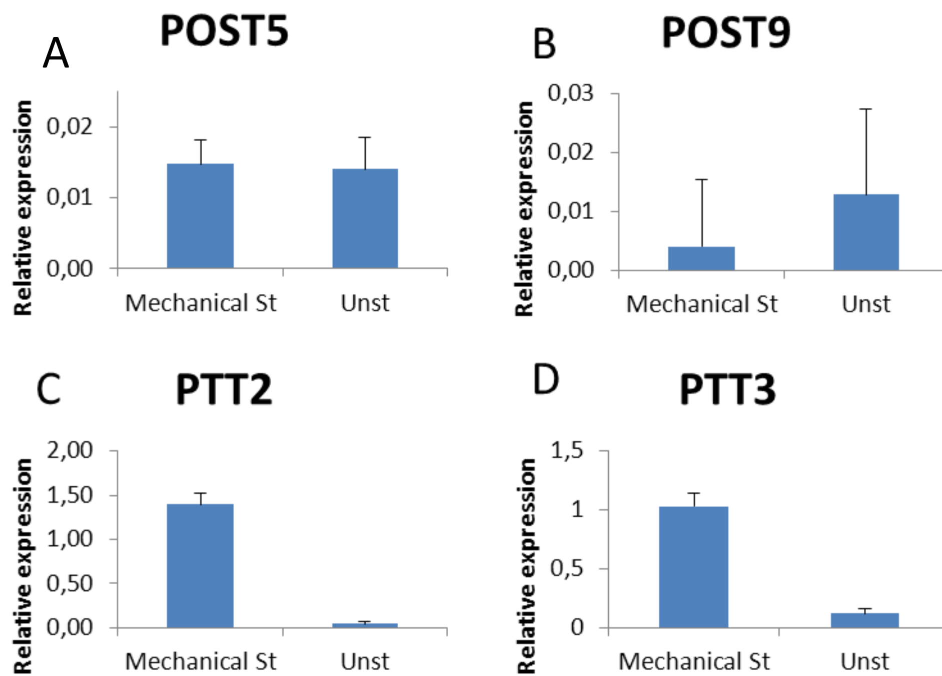


Figure 3-9 Q-RT-PCR recordings of the transcriptional changes in the expression of *POST5, POST9* and *PTT2* in the presence (Mechanical St) or absence (Unst) of a mechanical stimulus. Error bars show standard deviation between three biological replicates. Statistical significance between samples was obtained with a t-test, $p<0.01$ for PTT2 and PPT3. Differences in POST5 and POST9 are not statistically significant.

The presence of mechanical stimulation did not generate any visible change in the expression level of osmotic stress targets, even though it was proven effective by the up-regulation of the control genes *PTT2* and *PTT3,* which are touch targets (figure 3-9). Therefore we can conclude that even though osmotic stimulation can be used as a proxy for mechanical stimulation, the inverse may not be true, at least at the level of the control of the marker genes which we tested in these experiments.

3.5. Summary and conclusions

As discussed in the introduction, plants show specific phenotypes in response to mechanical stress. Many of these responses involve changes in the cell wall composition and mechanics, as well as a lower rate of primary growth.

These changes have been described in a wide variety of species, with relatively little known about responses in the model plant *Arabidopsis*. In addition, much of the previous work carried out in *Arabidopsis* was centred on the effect of mechanic stress on growth of the primary stem, which is retarded in comparison to the one of non-stressed plants (Braam 2005; Braam and Davis 1990; Paul-Victor and Rowe 2011). This aspect in particular corresponds strongly with the phenotypes reported in other species, such as woody plants (Leblanc-Fournier et al. 2008; Telewski and Jaffe 1986; Telewski and Pruyn 1998). However, changes in the cell wall of mechanically stressed *Arabidopsis thaliana* plants remained to be characterized.

In wind stressed *Phaseolus vulgaris* plants an induction of peroxidase activity was detected (Cipollini 1998; Cipollini Jr 1997), which is thought to be responsible for the polysaccharide-polysaccharide cross linking reaction in the cell wall and involved in regulating cell wall extension processes (Ralph et al. 2004) and in lignin synthesis (Barceló et al. 2004). In woody species it has been demonstrated that mechanically stressed trunks generate modifications in their cell walls by a deposition of extra layers of cell wall material (Frankenstein and Schmitt 2006; Melcher et al. 2003). Even though we do not know the nature of this cellulose deposition I observed that mechanically stressed *Arabidopsis* also present a higher amount of cellulose in their cell walls. Therefore the response of this species is consistent with that seen in woody species.

I also detected a modification in the quantity of lignin in the stems of mechanically stressed *Arabidopsis* plants. A similar situation has been reported in other species,

such as *Solanum lycopersicum*, where stems show a higher lignification rate after undergoing a mechanical stress treatment (Saidi et al. 2010). In previous experiments done in *Arabidopsis*, such as the one performed by Paul-Victor and Rowe, no changes in lignification were seen (Paul-Victor and Rowe 2011). I believe that the differences seen between my experiments and those performed by Paul-Victor and Rowe is that they express lignin per unit of AIR, while I express it per cm of stem. It is possible that the increase in lignin I see is, at least in part a reflection of an increase in stem circumference, an effect present in mechanically stressed plants (Telewski and Jaffe 1986). Unfortunately, due to severe black-fly infestations in the growth rooms in Lyon, I was unable to test this hypothesis, despite numerous aborted attempts.

It is interesting to note that all the phenotypes I observed in mechanically stressed plants, fit very well with the phenotypes observed in *Arabidopsis* plants grown in hypergravity conditions, which could be considered to perceive increased mechanical stimulation. Such plants have been described as presenting shortened, thickened stems (Allen et al. 2009; Nakabayashi et al. 2006; Tamaoki et al. 2006) and a higher content of lignin (Tamaoki et al. 2004; Tamaoki et al. 2006). It is also noticeable that they show a phenotype opposed to that of plants grown under microgravity conditions, which could be considered to lack exogenous mechanical stimulation. These are characterized by a decrease in cellulose content (Cowles et al. 1984; Nedukha 1996) and in matrix polysaccharides (Hoson et al. 2002).

Even though my results show quantitative differences in the cell wall composition of mechanically stimulated plants, they do not allow me to describe the qualitative changes in this structure. In the case of cellulose we do not know if the increase is due to a thickening of the wall, to a different architecture or both. In the case of lignin it could be due to an increase in lignin content, to an ectopic lignification of walls or to both. In any case, the obtaining of chemically measurable differences in cell wall composition by the imposition of mechanical stresses requires very long

time-scales, and technically challenging experiments. Measurements of this type of parameter are therefore impracticable as a means of ascertaining sensitivity to mechanical stress as a routine procedure in the laboratory.

Work remains to be done in the characterization of mechanically stressed *Arabidopsis thaliana*. It would be of great interest to study the biomechanical properties of the leaves and stems of stressed and unstressed plants. Leaf strength, toughness and stiffness can be measured by the punch and die technique (Aranwela et al. 1999), that has been successfully used to characterize leaves of *Plantago major* (Onoda et al. 2008). This technique is based in an indentation system that "punches" the tissue with growing forces in order to calculate its mechanical properties.

In the longer term it would be useful to quantify changes in the quantities of components of the cell wall, other than cellulose and lignin in mechanically stressed plants. It has been shown that many other components of the cell wall play a vital role in the micromechanical properties of this structure. Even though, classically, most studies on the mechanical properties of the cell wall were centred on the study on cellulose-xyloglucan networks; recently a model in which non-cellulosic components plays a key role in the determination of physical properties of the wall has been proposed (Höfte et al. 2012). In particular, pectin has been shown to be vital for control of the extensibility of the cell wall (Peaucelle et al. 2011; Peaucelle et al. 2008). When pectin methylesterases, enzymes that modify the properties of pectins (Pelloux et al. 2007), are inhibited, a general stiffening of the walls can be recorded. This has profound effects on the morphogenesis of the plant, as it prevents the apparition of new primordia in the SAM (Braybrook et al. 2012; Höfte et al. 2012; Peaucelle et al. 2008).

It would be of great interest to know as well how these other components of the wall behave in response to mechanical stimulation. This could be done by extracting the saccharides with trifluoroacetic acid followed by an analysis using gas

chromatography or high performance liquid chromatography (HPLC) (Foster et al. 2010c).

Even though not all responses generated by mechanical stress are direct transcription-mediated responses, specific transcriptional phenotypes are known to appear as a response to mechanical stress (Lee et al. 2005). This fact has been known since the early 1990s, when the first touch induced genes (TCH) were identified (Braam and Davis 1990). Part of the aim of this chapter was to develop a transcriptional assay for the perception of mechanical stimuli that would subsequently allow me to assay changes in perception in various mutant and transgenic backgrounds. I therefore investigated various methods of inducing reproducible transcriptional responses to mechanical stimuli.

Mechanical stimulation has been applied in other organisms, such as bacteria, by using an osmotic step as a proxy (Berrier et al. 1992; Levina et al. 1999). In plants it has been known that an osmotic shock generates incipient plasmolysis (Oparka 1994) and that this leads to changes in the ultrastructure of the cell, such as a retraction and compression of the cytoplasm, as described in the green algae *Zignema* (Kaplan et al. 2012). In theory, these changes could generate differences of tension in the plasma membrane, therefore mimicking the effect of mechanical stress. I showed that in plants, using a restricted set of transcriptional reporters, this is a valid approximation. When osmotic stress is applied in our system, not only osmotic stress reporters, but also touch reporter genes, show changes in their transcriptional level. Nevertheless, the converse is not true, as when mechanical stimulation is applied, osmotic stress reporters are not up-regulated, even though touch reporters show changes. One possibility is that even though osmotic stress, like mechanical stress, generates changes in the membrane tension the later does not generate the movement of solutes through the plasma membrane, and the subsequent effects of this, that is triggered by hyperosmotic stress.

Concerning the transcriptional response to mechanical and osmotic stress, I believe that this subject requires considerable further work. The generation of a complete transcriptome of plants grown side by side and stressed with both conditions for a range of times would provide an extremely useful tool, and would allow the direct comparison of the transcriptional behaviour of genes to these two stresses. This would open new paths allowing more extensive analysis of which targets can be used as proper indicators of mechanical stimulation when using osmotic steps as proxies. It would also facilitate the analysis of novel downstream targets of osmotic stress, giving hints about whether they respond to a signal originating from changes in membrane topology (events shared with mechanical stimulation) or from other factors. However, despite the fact that many transcriptomic analyses of the effects of different sources of abiotic stress, including osmotic and mechanical stress have been carried out (Fowler and Thomashow 2002; Kilian et al. 2007; Kreps et al. 2002; Lee et al. 2005; Seki et al. 2001). Osmotic and touch stress have never been analysed together in a comparative manner. I believe that the conditions developed and tested in the work described in this chapter would provide an ideal basis for such a study in the future.

4. Phenotypic effects of calpain deregulation

4.1. Introduction

4.2. *dek1[CALPAIN]* plants have thicker epidermal cell walls than wild-type plants

4.3. *dek1[CALPAIN]* plants produce more cellulose per unit of dry mass that wild-type plants

4.4. *dek1[CALPAIN]* plants have thicker stems with an increased lignification

4.5. Wild type cell walls show a different stiffness patterns compared to those of CALPAIN OE plants

4.6. Stem apical meristems of OE plants show lower anisotropy in microtubule orientation

4.7. Transcriptional effects of the deregulation of the CALPAIN domain of DEK1

4.8. CALPAIN OE plants respond constitutively to mechanical stress

4.9. Summary and conclusions

4. Phenotypic effects of calpain deregulation

4.1. Introduction

As described in the introduction, calpains are cytoplasmic, Ca^{2+}-dependent cysteine proteases with a highly conserved molecular structure in the catalytic domain (Croall and DeMartino 1991).

Conventional calpains, like human m-calpain, are cytosolic enzymes, which are activated by a rise in the intracellular levels of the Ca^{2+}. These proteins are intracellular proteases, expected to function as bio-modulators of cell physiology (Sato and Kawashima 2001).

In the case of DEK1, the CALPAIN domain is proteolitically removed from the rest of the protein. This cleavage is very likely to be an autocatalytic event (Johnson et al. 2008). In maize, substituting the active cysteine by serine eliminates calpain activity against β-casein *in vitro* (Wang et al. 2003). In *Arabidopsis* this mutation blocks the autolytic-cleavage event in GFP-tagged protein expressed *in planta* (Johnson et al. 2008). This autolytic cleavage may be similar to the one that occurs in animal calpains, and is predicted to release the CALPAIN domain into the cytoplasm (Johnson et al. 2008).

Knockout mutants (*dek1*) show an early embryo lethal phenotype. Defects in the embryo can be detected at the globular stage. Wild-type siblings present a normal globular stage with a smooth and round profile and a well-defined protoderm. In *dek1* embryos, defects are observed throughout the embryo proper and in the apical portion of the suspensor. Abnormal cell divisions in the protoderm lead to irregularities on the embryo surface and abnormal division planes are seen in underlying tissues. During later embryogenesis, the morphology of these mutants

does not show strong changes, keeping a shape and size comparable to that of globular stage embryos, and arresting development at this stage. This phenotype can be observed in both available knockout lines, *dek1-2*, with a T-DNA insertion in the fourth intron, and *dek1-3*, with a T-DNA insertion in the 22nd intron (Johnson et al. 2005).

This lethal phenotype can be complemented by the expression of a transgene containing the active version of the CALPAIN domain of DEK1. The majority of complemented lines present a fairly wild-type appearance. This indicates that the cytoplasmic (CALPAIN) domain alone is sufficient for normal plant development, suggesting that this cleavage product may represent the active form of DEK1 (Johnson et al. 2008).

CALPAIN domain over expressing (CALPAIN OE) plants have a distinct phenotype from wild-type plants. They are darker green than the wild-type, show severe rumpling in leaves, have more compact rosettes (Johnson et al. 2008) and show a delay in flowering time (Galletti unpublished results). Multiple aspects of plant development are highly affected by the OE of this cysteine protease domain. 10th leaves from the apex of 8-week old short-day-grown CALPAIN OE plants appear smaller in area and show an increase epidermal cell density compared with the wild-type, especially on the adaxial surface (Johnson et al. 2008). Complementation with the active form of the CALPAIN domain and overexpression are possible in both knockout backgrounds *dek1-2* and *dek1-3*.

The phenotypes shown by CALPAIN OE plants present superficial similarities with those shown by mechanically stressed plants. This similarity in phenotype is best illustrated by comparison with mechanically stressed *Arabidopsis* plants presented by Janet Braam and collaborators (1990, 2005). In particular, both mechanically stressed plants and CALPAIN OE plants present a late flowering phenotype

(Galletti unpublished results) and have more compact rosettes with shorter petioles (Johnson et al. 2008).

Based on these observations I hypothesised that DEK1 might be involved in mechanoperception and that deregulating the CALPAIN domain of DEK1 could alter plant responses to mechanical stimuli. In this chapter I focused on the characterization of the effects of constitutive expression of the active, cleaved CALPAIN domain of DEK1.

4.2. dek1[CALPAIN] plants have thicker epidermal cell walls than wild-type plants

The transcription of several cell wall-related genes is misregulated in CALPAIN OE plants (Johnson et al. 2008), leading to the hypothesis that this structure could be modified in these mutants.

The outer epidermal cell wall is much thicker and less extensible than the walls of the inner tissues. For example, it is 20 fold thicker in the epidermis of hypocotyls of *Helianthus* compared to walls of internal tissues. Moreover, in the outer epidermal wall the amount of cellulose per unit wall mass is considerably greater than in the inner tissues (Kutschera 2008). It has been proposed that this thickening may be a direct response to the perception of endogenously generated mechanical stresses, and for this we reasoned that the outer cell wall of epidermis is the ideal tissue in which to test this hypothesis.

Measuring cell wall thickness in sections of plant cells is notoriously difficult due to variations and artefacts which can be caused by the fixation process. In particular, because the wall is basically a fibrous gel, dehydration steps can cause spurious thickness measurements to be obtained. However, very high quality images of

unfixed cell walls can be obtained with cryo-scanning electron microscopy (SEM), using freeze fractured sections of the desired tissue. In this way, accurate measurements of the thickness of this structure can be made (Derbyshire et al. 2007). Wild type and *dek1[CALPAIN]* plants were analysed in this way. Cell walls of mature adult leaf adaxial surfaces and basal stem epidermal cells were measured. A set of 11 images in the case of leaves and 17 for the case of stems were taken.

Ten measurements were taken in the central zone of each imaged cell. The outer central zone was chosen as we believe that this reduces the measurement error compared to measurements made near cell junctions, as it is much more obvious in which direction measurements perpendicular to the plasma membrane should be made. The average thickness of the cell wall of each cell was calculated and the average thickness of the cell wall for each phenotype was then obtained.

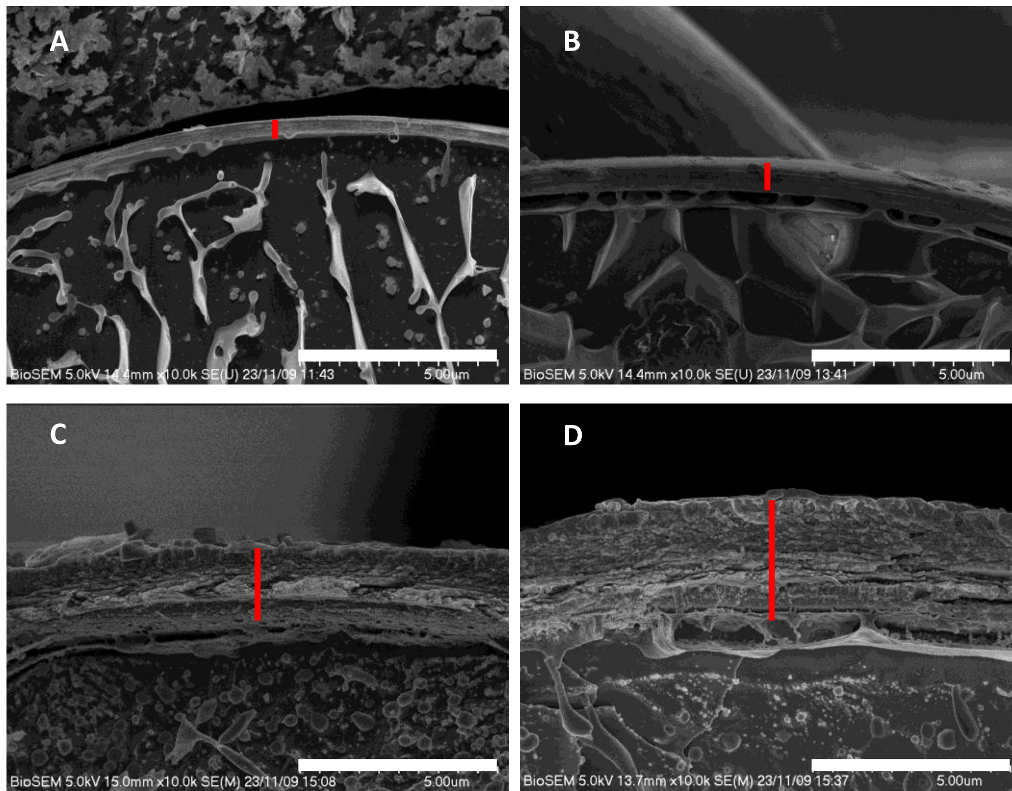
Significant differences in the thickness of the cell wall were observed in both leaf and stem epidermis between Calpain over-expressing and wild-type plants (figure 4-1). Results show that the outer epidermal cell wall is approximately two fold thicker in *dek1[CALPAIN]* than in wild-type in both the adaxial surface of leaves and the stem (Figure 4-1). All the measurements were statistically tested with student's t test, and the difference has a significance level of <0.1%.

4.3. dek1[CALPAIN] plants produce more cellulose per unit of dry mass that wild-type plants

Differences in cell wall thickness can be caused by changes in the architecture of cell wall components, or due to differences in their quantities. In order to investigate the later possibility, the different components of the cell wall can be quantified.

The contents of the cell wall can be quantified by extracting the alcohol-insoluble residue (AIR). By this process, involving several washes with alcohol, most proteins and alcohol-soluble sugars are eliminated. The AIR contains all polysaccharides present in the cell wall as well as lignin, waxes and other lipids. Cellulose can be isolated from the AIR after solubilisation by acid hydrolysis of all other cell wall saccharides, and quantified by colorimetric assays such as the anthrone method (see previous chapter).

Cellulose was quantified using this technique and a difference in content was observed in the CALPAIN OE mutants compared to wild-type plants. There is an increase of approximately 35% in the cellulose content of AIR isolated from CALPAIN OE plants compared to the value seen in wild-type individuals (figure 4-2, table 4-1). 0.55 μg of glucose equivalents per μg of AIR was measured in wild-type fully developed leaves while in different CALPAIN OE lines the values obtained were of 0.69 and 0.68 μg of glucose equivalents per μg of AIR.



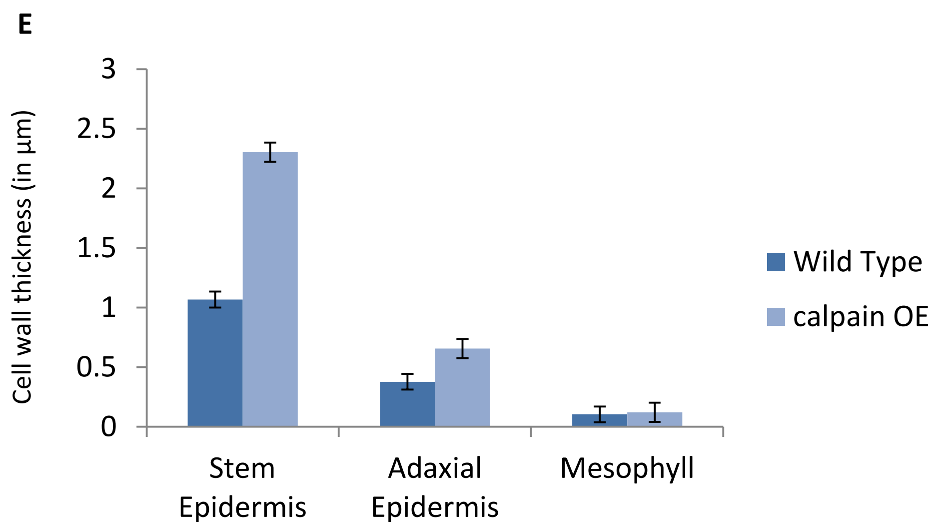


Figure 4-1 Cryofracture scanning electron micrographs of wild-type leaf adaxial epidermis (A), CALPAIN OE leaf adaxial epidermis (B), wild-type stem epidermis (C) and CALPAIN OE stem epidermis (D). Examples of measured distances are marked with red lines. Scale bars of 5µm. Measurement of cell wall thickness of these same tissues and in mesophyll cells from the same tissues (E). Stem and leaf epidermis show statistically significant differences (analysed with an ANOVA test) in thickness ($p<0.01$) while the mesophyll does not. Error bars show standard deviation. N=10.

Table 4.1 Cellulose content of mature leaves of wild-type (Col-0) and CALPAIN OE lines (in µg of glucose equivalents per µg of AIR).

	WT	dek1-2[CALPAIN]	dek1-3[CALPAIN]
Cellulose content (in glucose eq per µg of AIR)	0.55	0.69	0.68

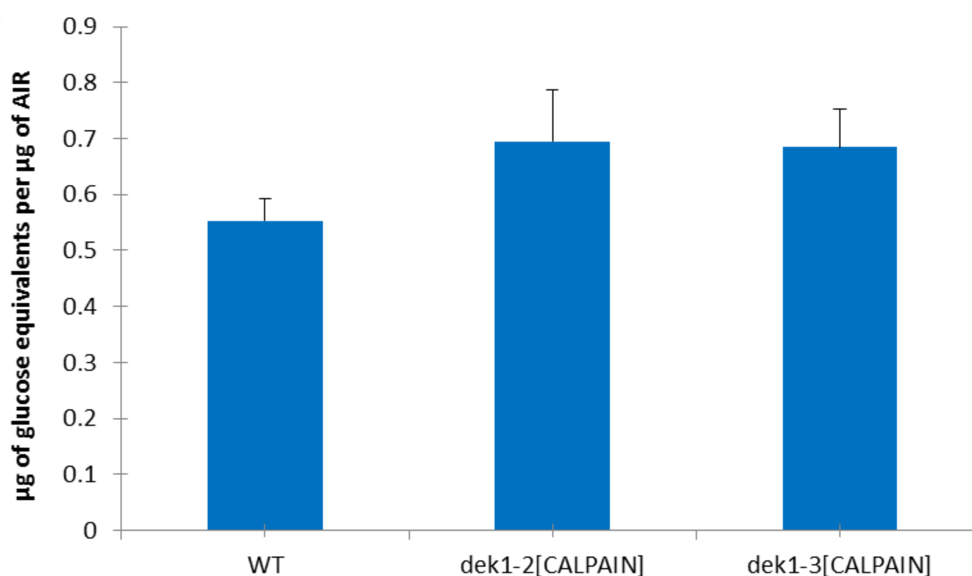


Figure 4-2 Cellulose content of mature leaves of wild-type (Col-0) and CALPAIN OE lines (in µg of glucose equivalents per µg of AIR). Error bars show standard deviation. Statistical significance tested with an ANOVA test show a difference between wild-type and both of the over-expressing lines, n=4, $p<0.05$.

Although cellulose fibres are one of the main structural components of the cell wall other polysaccharides have an important cross-linking role. Glucans can bond with the cellulose microfibrils and link them, forming a network (Albersheim et al. 1994). Of these, the most important are xyloglucans and glucoarabinoxylans which can be broken down with trifluoroacetic acid, to which cellulose is resistant. The product

from this reaction can be characterized and quantified using various modes of chromatography (Foster et al. 2010b).

A high performance liquid chromatography (HPLC) was run and the quantity of non-cellulosic polysaccharides in the AIR of CALPAIN OE plants was found to be 23% greater than in the wild-type (data shown in table 4-2). (S Fry unpublished results).

Table 4-2 Content of non-cellulosic cell wall components of the AIR of fully developed leaves of wild-type (Col-0) and CALPAIN OE (dek1-3[CALP]) plants. N=3.

Averages (mg/ml)	dek1-3[CALP] (µg/ml)	Col-0 (µg/ml)	dek1-3 as % of Col-0
Fucose	2,0	2,0	100
Rhamnose	14,1	12,4	114
Arabinose	18,8	15,5	121
Galactose	30,2	24,2	124
Glucose	8,7	8,1	108
Xylose	10,6	8,3	128
b-Mannose	5,3	4,2	127
GalA	63,5	50,2	126
TOTAL	153,1	124,8	123

4.4. *dek1[CALPAIN]* plants have thicker stems with an increased lignification

CALPAIN OE plants show differences compared to wild-type plants in tissues other than the epidermis. In leaves they have up to three tightly packed layers of palisade mesophyll instead of one palisade mesophyll layer seen in wild-type plants. Additionally extra spongy mesophyll layers can also be observed (Johnson et al. 2008).

We used stems in order to study the changes in response to mechanical stimulation (Chapter 3). This organ responds in a very clear way to mechanical stimulation, as observed in previous studies on the effect of mechanical stress in *Arabidopsis* (Allen et al. 2009; Nakabayashi et al. 2006; Paul-Victor and Rowe 2011; Tamaoki et al. 2006). In addition, we were very interested in recording the changes in lignification, a process which is very visible in the vasculature of stems. For these reasons, cross sections of stems were observed with a fluorescence microscope. Cellulose in the base of the stem was stained with calcofluor white, which emits a blue fluorescence when excited with UV light (Hughes and McCully 1975), to observe the primary cell wall. Lignin exhibits auto-fluorescence (Radotic et al. 2006), which allows the observation of secondary cell wall in vascular bundles.

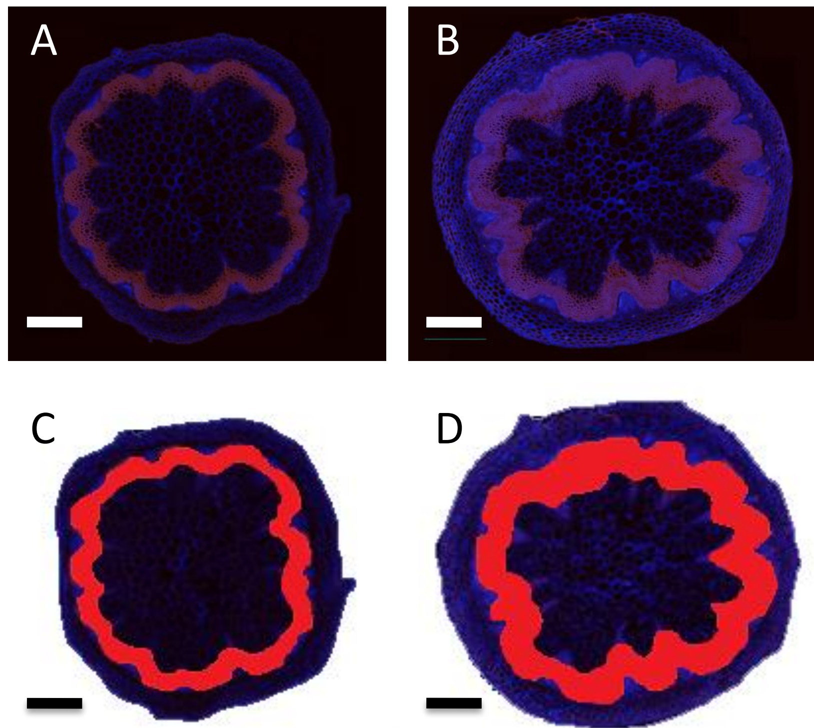


Figure 4-3 8μm cross sections of fixed basal stems of wild-type (A) and CALPAIN OE plants (B) stained with calcofluor white. The blue signal shows cellulose and the red signal is due to the auto-fluorescence of lignin. In the diagrams the lignified zones are marked in red in wild-type (C) and CALPAIN OE (D) stems. Scale bars: 0.2 mm.

CALPAIN OE plants have a thickened stem with a thicker layer of vasculature (figure 4-3). This layer shows an increase in area of approximately 45% (statistical significance of $p<0.01$; $n=3$). It is very likely that, as the vascular bundles are thicker, the stems contain a higher content of lignin per unit of length of the stem.

Lignin can be quantified by its auto-fluorescence after being purified with the acetyl bromide method (Foster et al. 2010a) . This component of cell walls shows an increase of nearly 50% in CALPAIN OE plants in comparison to wild-type individuals (figure 4-4). A value of 180.6 μg of lignin per cm of stem was detected in CALPAIN OE plants while wild-type individuals show a value of 121.6 μg of lignin per cm of stem.

This increase in lignin follows the increase in the thickness of the vasculature; therefore I cannot conclude that what I see is an increase in lignification rate. On the other hand, I can say that the increase in lignin we see per unit of length of the stem is likely to be due to a thickening of the vasculature of the stem. This thickening of stems was previously observed after performing other types of mechanostimulation, such as three-point bending (Paul-Victor and Rowe 2011) or in hypergravity conditions (Allen et al. 2009; Nakabayashi et al. 2006; Tamaoki et al. 2006).

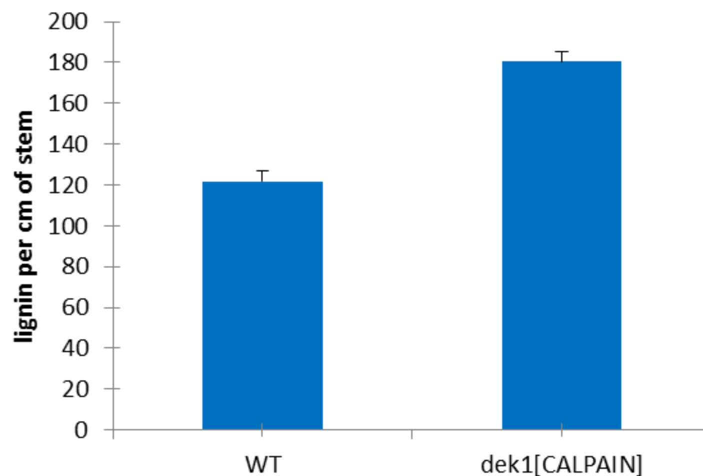


Figure 4-4. Content of lignin per cm of basal stems in wild-type and CALPAIN OE plants. Error bars show standard deviation. Statistical significance tested with a t-test, n=4, p<0.01.

4.5. Wild type cell walls show a different stiffness patterns compared to those of CALPAIN OE plants

All experiments in this section were carried out in collaboration with Dr Pascale Milani.

Cell walls are one of the main factors that determine the mechanical properties of plant cells, being crucial also for shape determination. The internal pressure

generated within of plant cells is an isodiametric force, thus without the presence of mechanical irregularities in this “contention wall” cells would be spherical (Baluška et al. 2003; Mathur 2006). Consistent with this view, the cell walls that surround plant cells do not generally have the same mechanical properties throughout the cell and are composed of several domains (Wojtaszek et al. 2007). This, combined with cell turgor helps to controls anisotropic cell growth (Wojtaszek 2000).

The micromechanical design of cell walls depends mostly on their biochemical composition. Cellulose, its most abundant component, is the polymer the most resistant to tension polymer in the cell wall(Niklas 2000).

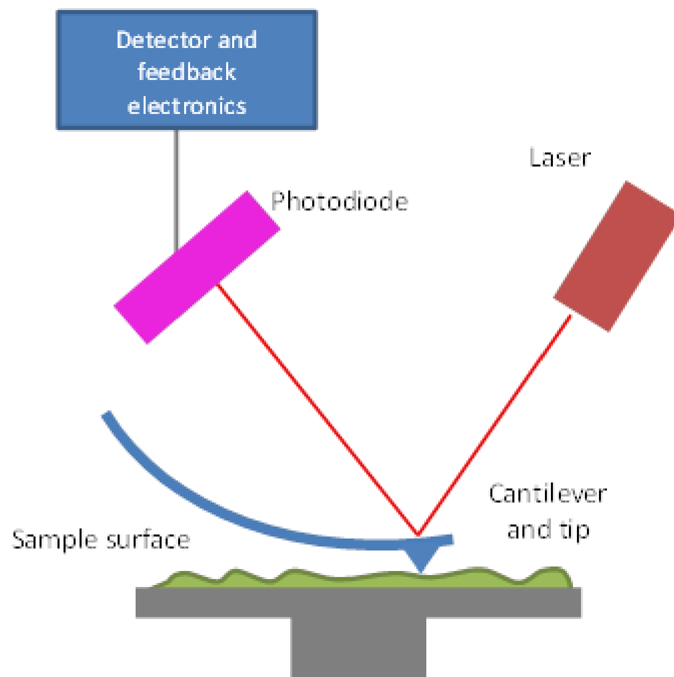


Figure 4-5. Simplified scheme of an atomic force microscope (AFM).

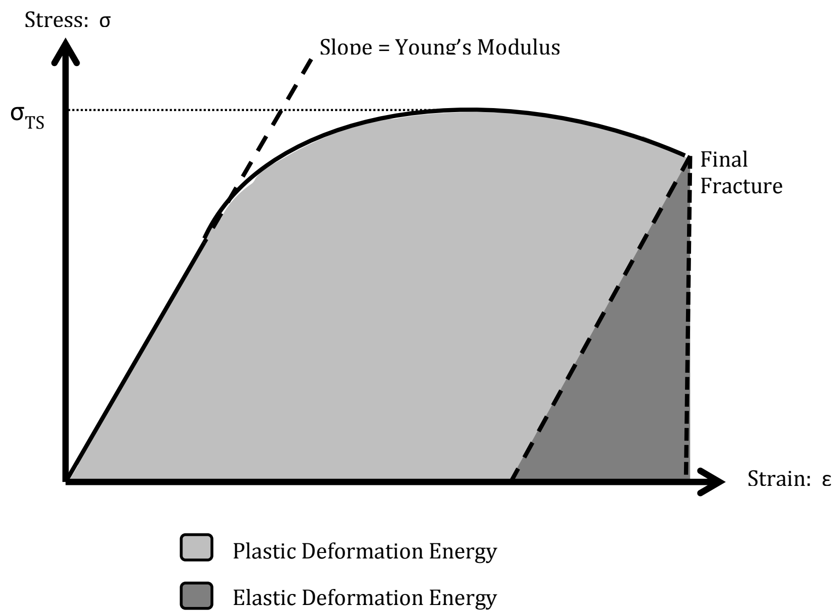


Figure 4-6. Example of a typical stress-strain curve for a ductile metal. The first portion of the curve is linear with a slope (E), corresponding to the Young's or Elastic Modulus. In the linear zone of the graph the sample deforms reversibly or elastically. Above this stress the sample deforms irreversibly or plastically. The maximum stress (σ_{ST}), or tensile strength is the maximum stress that the material can support before failure.

Mechanical properties of samples can be measured using an atomic force microscope (AFM). This microscope uses a nanometric cantilever ending in a sharp tip that is used to scan the surface of the sample. When the tip makes contact with the sample this cantilever is deformed, allowing measurement of forces through laser deflection (figure 4-5). This technique gives measurements both of the elastic modulus of the sample and its topography.

Atomic force microscopy measures the resistance to forces of the material in the sample, i.e. its "mechanical behaviour". The cantilever applies a known force to the sample and the displacement of this cantilever is measured. This force is measured as a stress (σ), which is defined as a force that acts perpendicularly to a surface,

divided by the area to which this force is applied (the area of the tip of the cantilever). Stress is expressed in units of pressure (Newtons per square metre, given in Pascals). The displacement of the cantilever is measured as strain (ε), which is the change in length divided by a reference length and thus has no units. From these direct measurements, the elastic modulus, also known as the Young's modulus, can be deduced. This is deduced from the first portion of the curve relating stress and strain. In the linear portion of this curve, its slope (E) corresponds to the Young's modulus. A higher value of E implies a less elastic tissue; therefore a bigger force, thus more stress, is necessary to generate deformation (figure 4-6).

Although the technique was initially conceived to test the properties of inert samples, AFM has now been used in a large number of biological samples including bacteria, yeast and animal cells. Results have shown differences in elasticity between different tissues and subcellular zones in these organisms, and have also aided in the understanding of how these properties vary in response to mechanical stimuli and in response to chemical or enzymatic treatments (Alonso and Goldmann 2003; Jacot et al. 2010; Kumar and Weaver 2009; Scheuring and Dufrene 2010). Differences at a subcellular level have been described with this technique, such as the difference in stiffness present in the bud of budding yeast cells (Touhami et al. 2003). In plants this technique has been used principally to image the structures of cell walls (Kirby 2011) and to study the mechanical properties of the shoot apical meristem (Braybrook et al. 2012; Milani et al. 2011).

In our case we expected a change in the mechanical properties of the cell wall, as CALPAIN OE plants show variations in the composition of their cell wall compared to those of wild-type individuals. We observed the abaxial surface of cotyledons of 7 day-old seedlings and saw that there is a slight change in the apparent modulus (E) of the tissue of CALPAIN OE compared to wild-type cotyledons. The mutant shows a decreased E, therefore a lower degree of stiffness of the cell walls. No apparent changes in the deformability of the tissue were observed (Figure 4-7 C and D).

The most noticeable difference in the E maps of the samples was that the CALPAIN OE epidermis shows a very different pattern of E values than that of the wild-type sample. In wild-type samples we can see that central bodies of the cells tend to be stiffer than the lobes (figure 4-7). On top of this pattern there is an organization of stiffer and softer "bands", which are very likely to reflect the disposition of cellulose microfibrils (O. Hamant, personal communication). In CALPAIN OE plants, not only does the general stiffness of the cell wall appear to be more homogeneous, but the "microfibril-like" stiffness pattern is lost completely. Therefore, it is very possible that there is a profound difference in architecture of the cell wall. My interpretation of data is that this is due to a difference in the deposition patterns of the cellulose microfibrils. This deposition process in guided by the organization of the microtubules, which I therefore hypothesized might be affected in the CALPAIN OE mutants.

Even though the results in the AFM maps obtained are promising they should be considered very preliminary results, as the data obtained should be tested in a quantitative manner by analysing the curves obtained in the experiment.

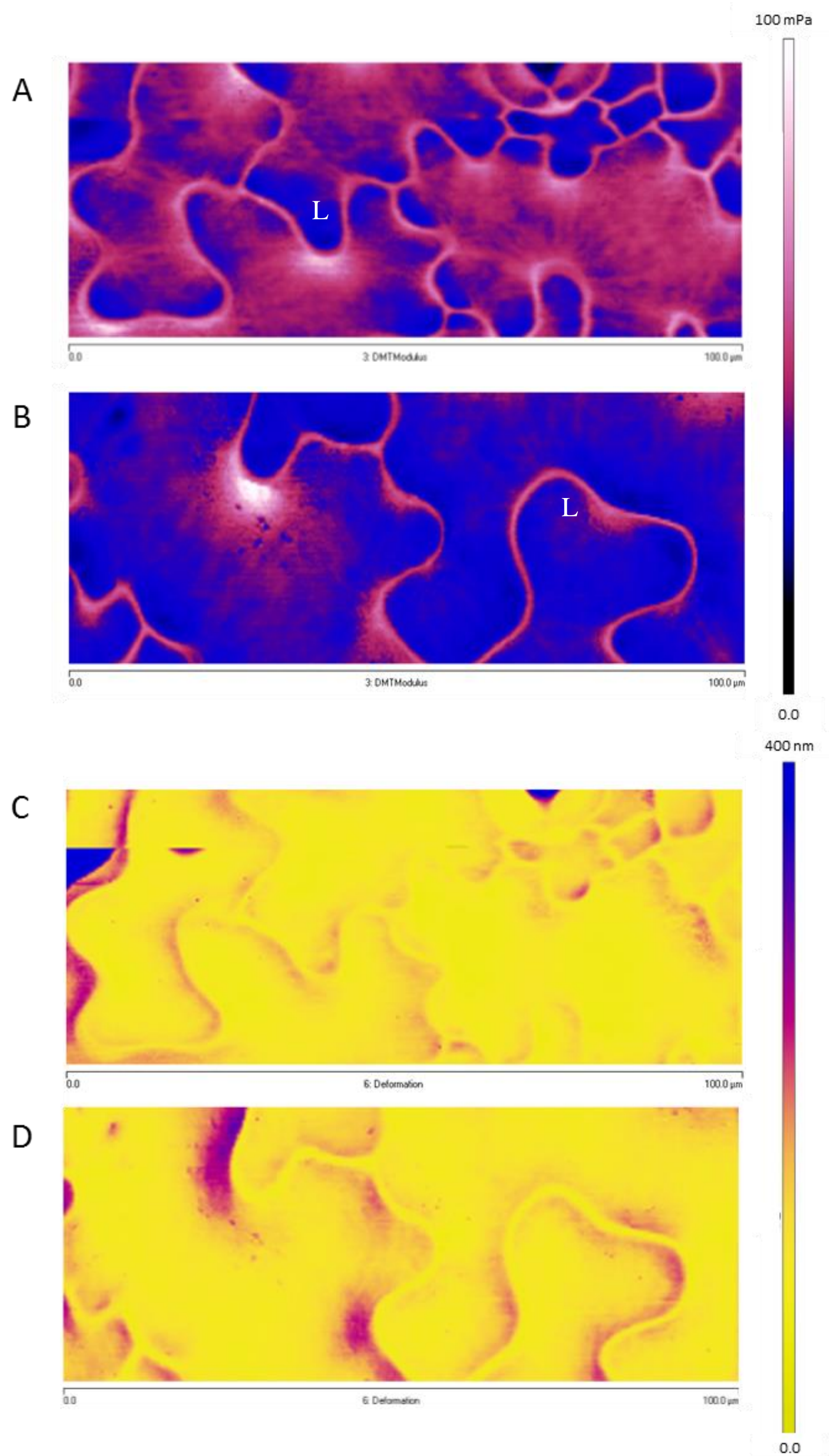


Figure 4-7. Apparent modulus (E) maps of Col-0 (A) and *dek1[CALPAIN]* (B) cotyledon abaxial epidermis. Deformation maps of Col-0 (C) and *dek1[CALPAIN]* (D) for the same tissue. Lobes marked as L.

4.6. Stem apical meristems of OE plants show lower anisotropy in microtubule orientation

A widely accepted theory proposed by Paul Green, and based on experiments carried out by Sachs in the 19th century, states that at organ levels tissue tensions develop due to a higher growth rate in the underlying tissues than in the epidermis. This would generate tensions in the external layer, which would apply continuously a strain to the deeper layers (Green 1962; Sachs 1882; Sachs 1865). Anisotropic mechanical forces exist in the walls of all turgid plant cells. These forces give important directional information and could offer location-specific information (Williamson 1990). This tension may be used for mechanointegration when, for instance, new organs emerge and generate new tension patterns. Such events have been described thoroughly for phyllotaxis in the SAM where Paul Green and co-workers created models of stress-strain that could predict the position of new primordia (Green 1980). Cortical microtubules take part in this process, as they reorient parallel to the main axis of the force in the cell membrane (Cleary and Hardham 1993; Fischer and Schopfer 1998; Hamant et al. 2008; Hardham et al. 1980) and as microtubules guide cellulose deposition (Bringmann et al. 2012b; Li et al. 2012b) the existence of a feedback loop between cytoskeleton and cell wall is very likely to exist (Williamson 1990). Experimental data supported the fact that cell growth responds to external forces and that this response process is microtubule dependent since microtubules need to be intact for this response to take place (Wymer et al. 1996). This phenomenon has been revisited using microtubules marker lines labelled with fluorescent proteins in the SAM of *Arabidopsis thaliana*, where it was again seen that morphogenesis depends on microtubules, which are regulated by mechanical stress. Feedback loops have also been proposed to coordinate tissue morphology, stress patterns and microtubule-mediated cellular properties in this tissue (Hamant et al. 2008). These feedback loops are supported by the fact that the stability and organization of cortical microtubules is affected in mutants for genes involved in cellulose biosynthesis, such as *PROCUSTE1* and

KORRIGAN. A similar effect is seen in plants treated with isoxaben, an inhibitor of cellulose synthase activity (Paredez et al. 2008).

I used transgenic lines provided by Dr Olivier Hamant expressing a GFP-tagged version of the protein MBD (Microtubule Binding Domain) (Hamant et al. 2008). This line was crossed with CALPAIN OE lines (in this case *dek1[CALPAIN:HIS]*, so that GFP fluorescence from microtubules would not be confused with that from the calpain domain). Double homozygous lines were selected and grown on N-1-Naphthylphtalamic Acid (NPA) containing plates. This generates a pinoid phenotype due to the blocking of auxin transport. Pinoid plants are unable to generate primordia on the flanks of their inflorescence meristems, and thus present stems with a naked apical meristems (Casimiro et al. 2001). These NPA grown plants were observed under a confocal microscope to determine the distribution of microtubules.

I observed that the microtubules of CALPAIN OE stem apical meristems follow the force-responsive pattern seen in wild-type plants. This consists in an orthoradial (circular) orientation in the lateral regions and base of the meristem and a random orientation in the apical tip (figure4-8) (Hamant et al. 2008). The main difference in the microtubules of CALPAIN OE SAMs is that they present a lower level of anisotropy in the flanks of the meristem; therefore the microtubules are strongly oriented parallel to the predicted stress pattern if compared to those of wild-type plants (figure 4-9).

The cortical microtubule orientation can be measured using the MT macro (Uyttewaal et al. 2012). This analyses the anisotropy of the microtubule bundles in each cell. I carried out this analysis for three meristems of each analysed phenotype, analysing 10 cells in each. The chosen cells are in the side of the meristem, as this area is the one that presents the higher stress, and therefore, organization of microtubules (Hamant et al. 2008). In the selected cells only the centre was used for

quantification of microtubule orientation, therefore avoiding measuring the cortical microtubules, which could mask results due to their high level of anisotropy. I found that the level of anisotropy was twice as high in the flanks of CALPAIN OE meristems compared to wild-type ones. The average anisotropy value obtained for CALPAIN OE was of 0.623 (±0.016), while for wild-type plants it was of 0.291 (±0.007). A t test was performed on these data sets and these results are different with a significance of < 0.01%.

This observation can be interpreted as a possible influence of the active CALPAIN domain on microtubule dynamics, consistent with the observed interaction of the CALPAIN domain with microtubules. It is possible that the DEK1 CALPAIN domain is involved in the process of microtubule severing which is necessary for their reorganization. The phenotype observed is opposite to that present in the *atktn1 (katanin)* mutants (Uyttewaal et al. 2012). This mutant is known to present decreased microtubule dynamics (Burk et al. 2001; Burk and Ye 2002; Nakamura et al. 2010; Stoppin-Mellet et al. 2006; Wasteneys and Ambrose 2009). Thus, my results support a model in which CALPAIN OE plants respond more strongly to the mechanical stress patterns in tissues.

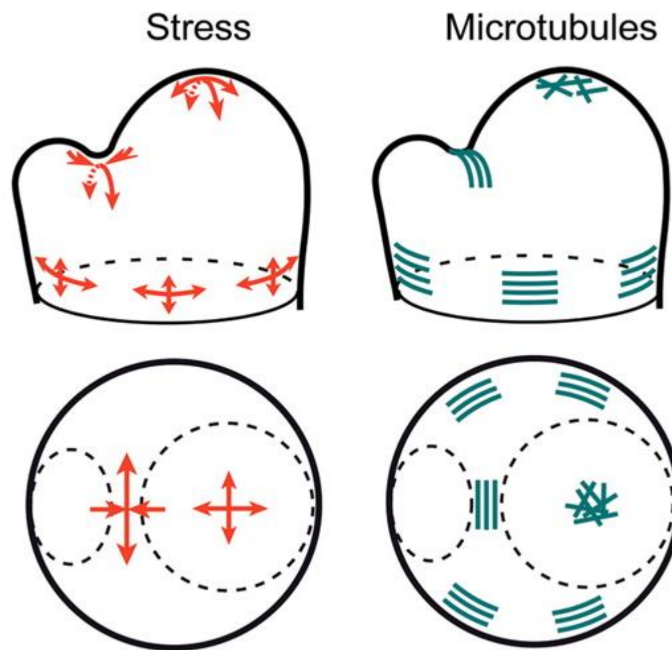


Figure 4-8. Direction of stress patterns in the SAM (in red). The direction of microtubules is in agreement with the highest-stress orientations (in blue). From Hamant et al. 2008.

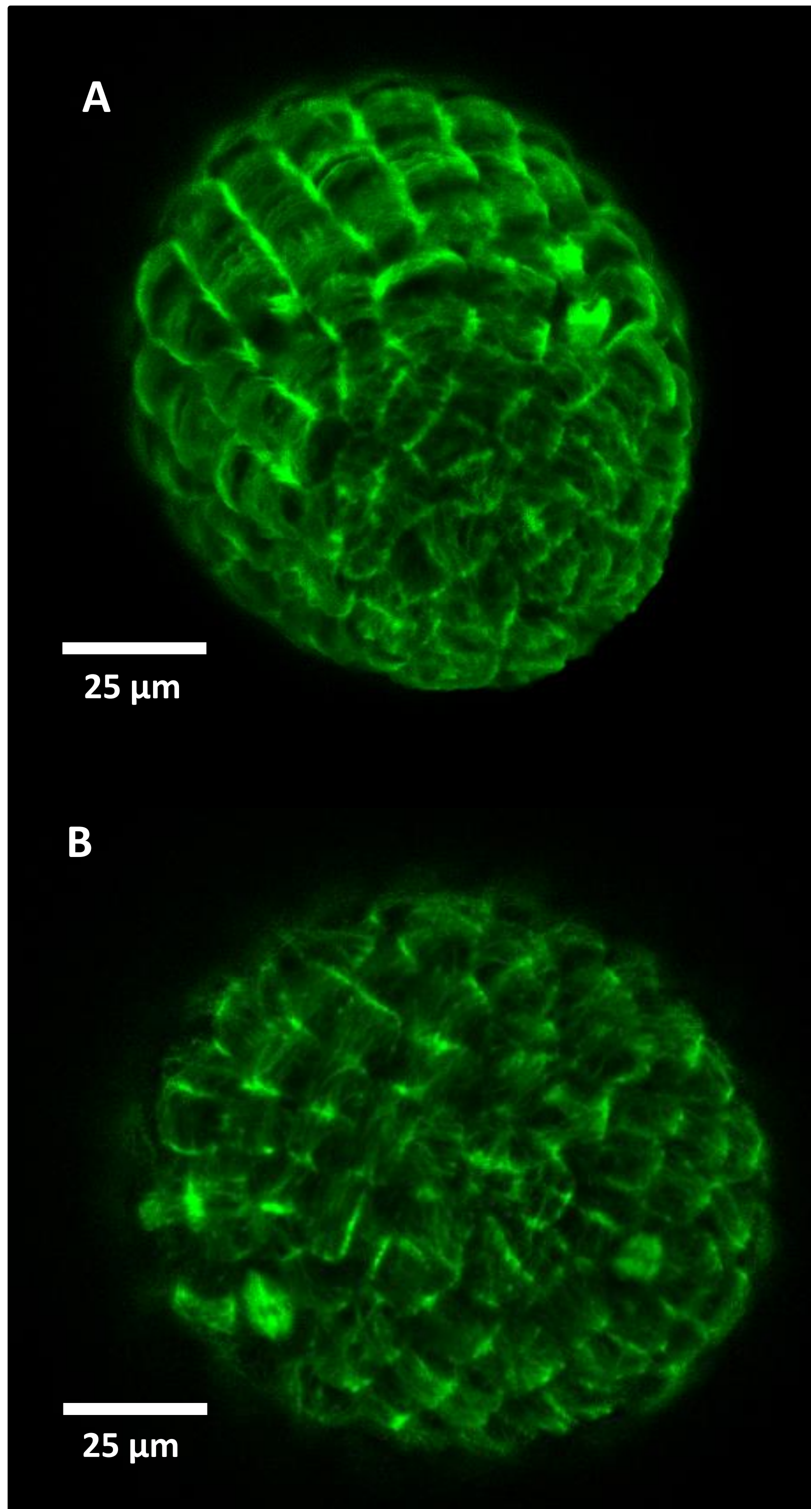


Figure 4-9 Projections of a stacks of images of SAMs of NPA grown MBD:GFP (A) and dek1[CALP:HIS] MBD:GFP (B) plants.

4.7. Transcriptional effects of the deregulation of the CALPAIN domain of DEK1

Microarray analysis of RNA extracted from young leaves of wild-type and CALPAIN OE plants showed a change in the level of transcription of a number of genes in CALPAIN OE plants compared to the wild-type and to CALPAIN-NULL OE plants (Johnson et al. 2008).

In order to further characterize potential targets identified during this analysis, I carried out an experiment to correlate the transcriptional phenotype of CALPAIN OE plants to the level of overexpression of the active CALPAIN domain. Different lines expressing the CALPAIN domain of DEK1 at different levels were used. These lines used were fusion versions of the CALPAIN domain of DEK1, tagged with HIS in the case of lines named KJ79 and MYC for lines named KJ80. This was performed by Q-PCR on inflorescence tip cDNA, as in this organ the levels of expression of DEK1 are higher.

Three pairs of primers within the DEK1 cDNA were designed; two of them target the CALPAIN domain and the third targets the predicted extracellular loop present amongst the transmembrane domains. This last pair, named QXDEK1, was used to confirm the level of endogenous DEK1 expression, as this part of the cDNA is not present in the calpain over-expression construct (figure 4-10). Analysis using this primer pair shows that levels of endogenous *dek1* transcripts are not affected in the transgenic lines used.

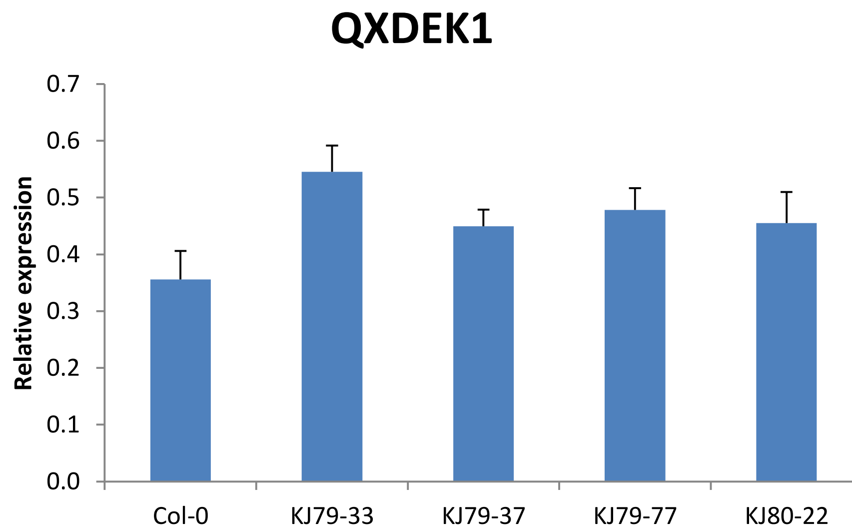


Figure 4-10 Level of expression of endogenous full length DEK1 in leaves of different lines. Tested with the primers QXDEK1, that amplifies a fragment in the extracellular loop. Error bars show standard deviation between three biological replicates. ANOVA test shows a no statistical significant difference between samples.

A set of 30 target genes present in the microarray data from Johnson et al. 2008 were chosen, corresponding to those most strongest supported in the original data, and a final analysis was performed on a shortlist of 3 targets. These last 3 targets were chosen on the basis of a pre-screen carried out by by Yassir Naouli and Dr Nathalie Depege-Fargeix in collaboration with myself (unpublished). The three chosen targets were named *TOD* (target of DEK1) and identified as *TOD4, TOD5* and *TOD19*.

TOD4 is *Expansin11* (*AT1G20190*), a member of the alpha-expansin family gene family, which is characterised by the presence of an "α-insertion" of approximately 14 residues that contains a motif of 4 highly conserved residues (GWCN) at its 3' end (Li et al. 2002). These proteins are wall-loosening enzymes responsible for plant cell wall growth (Sampedro and Cosgrove 2005).

TOD5 is part of the Pectin lyase-like superfamily (*AT2G43870*), and more specifically the polygalacturonase family (Kim et al. 2006a). Polygalacturonases are

an enzyme family known to catalyse pectin modification and disassembly. They are capable of reducing the apparent molecular size of pectic polymers, cleaving neutral side chain residues (De Veau et al. 1993). Although polygalacturonases have been mainly studied in relation to fruit ripening, it is known that they have functions in all developmental stages, and may play roles in cell expansion (Hadfield and Bennett 1998).

TOD19 is *Cor413im* (*AT1G29395*), a protein present at the inner membrane of chloroplasts (Okawa et al. 2008). Structure predictions and comparative genome analysis suggests that the *Cor413* genes encode putative G-protein-coupled receptors (Breton et al. 2003). This protein seems to be related to stress response pathways, in particular to providing freezing tolerance (Breton et al. 2000; Seki et al. 2001; Thomashow 1999).

For all three targets the same trend was observed. Expression levels of these putative targets of DEK1 mirror the OE level of the active CALPAIN domain, although not perfectly. *TOD5* mirrors in the level of expression of the CALPAIN domain of DEK1 well (Figure 4-11 D). On the other hand, *TOD4* and *TOD19* do not show such clear tendency for the samples KJ79-77 and KJ80-22 (Figure 4-11 C and E). This problem could be due to the fact that these are not direct targets of the CALPAIN domain, but are regulated by downstream responses. Therfore their regulation is much less direct.

The transcriptional phenotype of the weak allele *dek1-4* was also characterised. This allele presents a single nucleotide mutation at the position 6316 of the gene (T instead of C), which changes a cysteine residue into an arginine residue. This mutation is located close to the C-terminus of the protein, in domain III of the CALPAIN domain. This missense mutation appears to generate a less active version of DEK1, as the allele is unable to complement strong knockout alleles such as *dek1-3* (Roeder et al. 2012).

In the case of *dek1-4* the level of expression of the CALPAIN domain of DEK1 stays constant compared to wild-type. This is expected as the allele is caused by a point mutation, and no results from our studies support any form of transcriptional feedback on DEK1 expression levels. Changes were observed in the level of expression of downstream potential targets of the CALPAIN domain of DEK1 in *dek1-4* mutants. *TOD4, TOD5* and *TOD19* expression levels were analysed by Q-RT-PCR and a statically significant decrease in expression levels of each gene was observed. This is the opposite result to that observed in CALPAIN OE plants (figure 4-11 F).

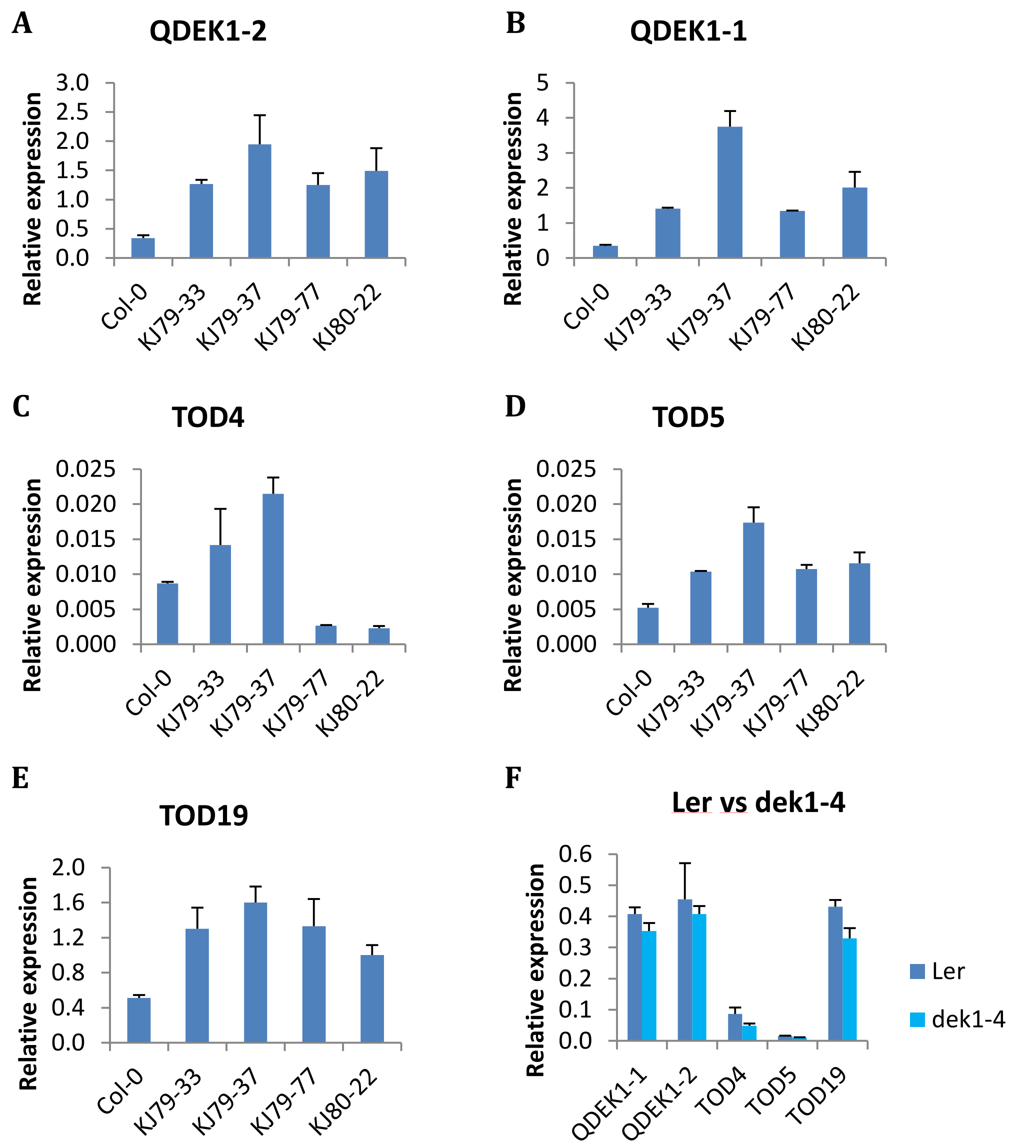


Figure 4-11 RT-PCR analysis of gene expression of the CALPAIN domain of *DEK1* and several of its targets. A-B Level of expression of the CALPAIN domain of *DEK1* in several different OE lines. C Level of expression of *TOD3* in several different CALPAIN OE lines. D Level of expression of *TOD4* in several different CALPAIN OE lines. E Level of expression of *TOD19* in several different CALPAIN OE lines. F Level of expression of the CALPAIN domain and of *TOD4, TOD5* and *TOD19* in wild-type (Ler) and *dek1-4* mutant plants. Error bars show standard deviation between three biological replicates. ANOVA test shows a statistical significant difference between Col-0 and all other lines, p<0.01 (A-E). In F a t-test shows significant differences (p<0.01) between all pais except for Q-DEK1-1 and Q-DEK1-2.

4.8. CALPAIN OE plants may show constitutive mechanical and osmotic stress responses

The phenotypes of the CALPAIN OE lines reminded us of those of mechanically stressed *Arabidopsis*. These lines, as discussed earlier in this chapter, show modified cell walls and a late flowering phenotype. There are very clear effects on growth, including stunting and thickening of stems, that can also be seen as an effect of direct mechanical stimulation (Braam 2005; Braam and Davis 1990; Paul-Victor and Rowe 2011) or of hypergravity conditions (Nakabayashi et al. 2006; Tamaoki et al. 2004; Tamaoki et al. 2006). Effects in the composition of the cell wall can also be seen as a response to both of these types of stimulation (Cipollini Jr 1997; Nakabayashi et al. 2006; Saidi et al. 2010; Tamaoki et al. 2004; Tamaoki et al. 2006).

I, therefore decided to test for the level of expression of mechanical and osmotic stress reporter genes, such as those described in chapter 3, in CALPAIN OE lines. I did this in 7 day old seedlings grown in sterile culture through Q-RT-PCR. The targets used were *POST5*, *POST9*, *PTT2* and *PTT3*.

The level of overexpression of the CALPAIN domain in these seedlings was tested with two different pairs of primers located in the CALPAIN domain, named DEK1-1 and DEK1-2 (figure 4-13). In order to check that the over-expression seen was only due to the expression level of the transgenic CALPAIN domain and not due to changes in the level of expression of the full length protein we used as a control a pair of primers present in the transmembrane domains of DEK1. For this last pair of primers the expression level remained unchanged in the calpain OE lines compared to the wild-type (figure 4-12).

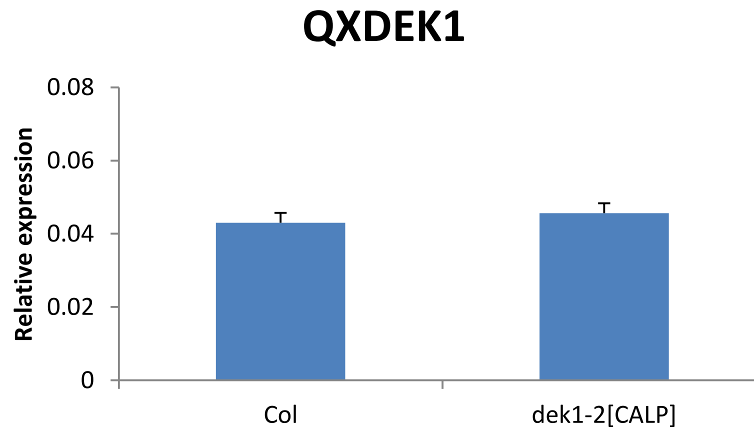


Figure 4-12 Level of expression of endogenous full length *DEK1* in seedlings of wild-type and *dek1-2[CALPAIN]*, a CALPAIN OE line. Tested with the primer pair QXDEK1, which amplifies a fragment in the extracellular loop-encoding region. Error bars show standard deviation between three biological replicates. No statistical differences between the samples were seen when tested with a t-test.

I observed an over-expression of all tested mechanical and osmotic stress reporter genes in the CALPAIN OE line (figure 4-13). This could indicate that the OE of the CALPAIN domain of DEK1 causes a constitutive response to mechanical and osmotic stimulation, even in absence of the stimuli.

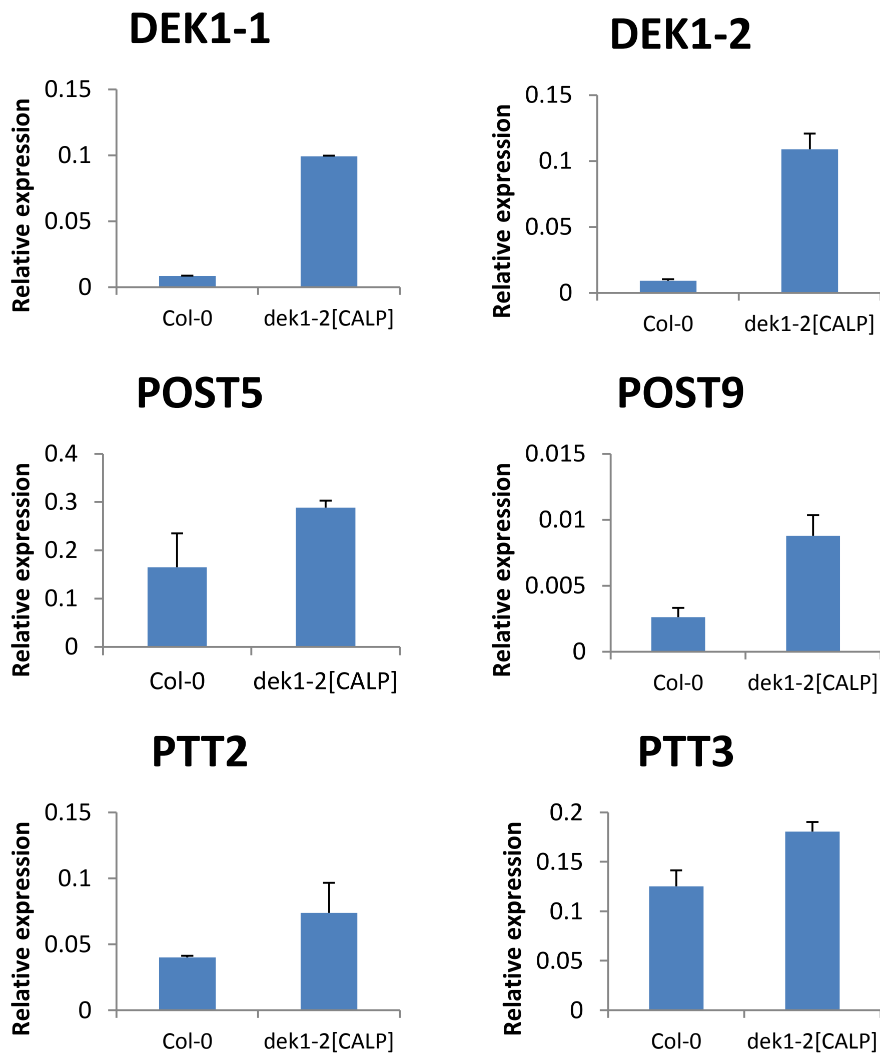


Figure 4-13 Q-RT-PCR analysis of the CALPAIN-encoding domain of *DEK1* (DEK1-1 and DEK1-2), osmotic stress reporter genes (*POST5* and *POST9*) and touch stress reporter genes (*PTT2* and *PTT3*), in wild-type and CALPAIN OE plants. Error bars show standard deviation between three biological replicates. Statistical differences between the samples were seen when tested with a t-test ($p<0.05$).

4.9. Summary and conclusions

Arabidopsis plants that over express the CALPAIN domain of DEK1 were described previously to show a very interesting phenotype of compact rosettes with shorter petioles in their leaves, which are darker and show a rumpled surface (Johnson et al. 2008). It was also shown that these mutants show a delay in flowering time (Galletti unpublished results). Interestingly this phenotype mimics in several aspects that of mechanically stimulated plants. This rosette phenotype of CALPAIN OE described by Johnson and collaborators (2008) and the flowering time recorded by Galletti reminded us of the phenotype of mechanically stressed *Arabidopsis* published by Braam and collaborators (1990; 2005).

The epidermis, and in particular the outer cell wall of this cell layer, is responsible for the mechanical integrity of organs (Kutschera 2008). According to the tensile-skin theory, first proposed in the 19th century (Peters and Tomos 1996; Sachs 1882; Sachs 1865), the expansion of underlying tissues generate a tension in the epidermis, which has a lower expansion rate, posing a mechanical constraint (Kutschera and Niklas 2007; Savaldi-Goldstein and Chory 2008).

I observed a very dramatic increase in the thickness of the outer cell wall of CALPAIN OE plants leaf and stem epidermis. This structural change in the cell wall could be due to several causes. These cell walls could show a change in architecture and different organization of the components (mostly cellulose microfibrils), fact that would lead to a thickening. The other possible explanation would be a change in composition, thus an increase in cell wall polysaccharides.

As I showed in chapter 3 of this thesis, mechanically stimulated *Arabidopsis* have higher quantities of cellulose in their cell walls. I proceeded to quantify the cellulose of CALPAIN OE lines, and showed that they also synthesize more cellulose than wild-type plants, as is the case in mechanically stressed plants. These changes in

synthesis are very probably due to the calpain regulated cell wall modification enzymes being up-regulated constitutively, as a response to the over expression of the CALPAIN domain of DEK1. Although no changes were reported in the expression level in any cellulose synthesis related enzymes, genes encoding enzymes involved in extension of cell wall, such as pectinases and expansins, were found to show altered expression in CALPAIN OE plants. It is possible that the activity of cellulose synthases, rather than their transcription, is altered by DEK1. If this is the case it would not be the first described example of cellulose synthesis regulation where gene transcription is not altered. One of the main enzymes responsible for cellulose synthesis, CesA1, regulates the mobility of the synthesising complex through phosphorylation (Chen et al. 2010b). In wild-type cells these complexes move at a constant speed in opposing directions, following the "tracks" laid by the microtubules (Paredez et al. 2006). When a mutated version of CesA1, lacking phosphorylation sites, is expressed, complexes show an asymmetry in their movement (Chen et al. 2010b). This affects the cell wall properties, as the mutant form cannot recover the cellulose-deficient phenotype of the *rsw1* mutant (Arioli et al. 1998). In the immediate future it would therefore be for great importance in to confirm the expression levels of cellulose synthases in wild-type and CALPAIN OE plants. This would allow us to know if the changes in cellulose content which I have observed are regulated via transcriptional changes, or through a cytoplasmic mechanism.

In leaves, other components of the cell wall are also present in different quantities in CALPAIN OE plants. Other saccharides, including those responsible for the cross linking of cellulose microfibrils, are more abundant than in wild-type plants. On the other hand, even though these quantities are higher, they do not increase by the same proportion as cellulose. Therefore the cell walls of these CALPAIN OE plants are very likely to have a change in their architecture. Even though the effects of different ratios of non-cellulosic polysaccharides and cellulose have not been described in depth, these other saccharides are thought to interact with cellulose to

form a three-dimensional network that functions as the principal load-bearing structure of the primary cell wall (Fry 1989; Hayashi and Kaida 2011; Somerville et al. 2004; Willats et al. 2001). In rice it has been shown that adult plants that have a reduction of 97% in mixed-linkage glucan have weaker cell walls (Vega-Sánchez et al. 2012). Thus the cell walls of CALPAIN OE plants could also show changes in their micromechanical properties, as these components are vital for this character (Abasolo et al. 2009; Cavalier et al. 2008).

Consistent with a change in architecture, AFM apparent modulus maps show changes in the micromechanical properties of cotyledon adaxial epidermis outer cell walls of CALPAIN OE plants measured with AFM, compared to wild-type. In these experiments we see a much more homogeneous pattern of resistance in the surface of cells, which is very likely to be explained by a disorganization of the cellulose microfibrils in the cell wall. As previously discussed this data should be confirmed statistically by the analysis of the indentation curves of the experiment in order to state sound conclusions. Even though it is known that mechanical force patterns guide the position and direction of cellulose microfibrils in meristems, the effects in cotyledons still remain to be studied.

Mechanical forces have an effect on microtubules, which are ideal transducers for mechanical integration in plants, due to their high degree of rigidity, even across the borders if individual cells (Nick 2011). It was proposed in the 1970s' that microtubules could act as a guidance for the deposition of cellulose microfibrils (Heath 1974). Experimental proof that indicating that cellulose synthase has a functional association with microtubules was obtained recently (Paredez et al. 2006). And more recently still, the molecular link between microtubules and cellulose synthase complexes has been pinpointed (Bringmann et al. 2012b; Li et al. 2012b). In order to understand whether the mechanical property maps obtained using AFM are directly related to the position of cellulose microfibrils it would be of great interest to carry out this same experiment using the lines we used to observe

microtubules. MBD:GFP lines would allow us to do sequential observations, first generating a mechanical property map of wild-type and CALPAIN OE lines with AFM. Secondly, the samples could be observed under a confocal microscope in order to record the position and direction of microtubules and see if they can be directly related to the mechanical properties of the cell wall. The problem that arises with this experiment is that if we compare the obtained images of the mechanical properties map and the pictures of the microtubule disposition they might not coincide. This is because with the microtubules observed would predict the orientation of microfibrils in the newly synthesised cell wall (next to the membrane), whilst the AFM would be imaging the properties of "historical" cell walls, synthesised earlier.

Synthesis of non-saccharidic components of the cell wall has been reported to be modified in response to mechanical stimulation. A very good example of this is lignin. Changes in degree of lignification have been observed in species such as *Solanum lycopersica* (Saidi et al. 2010) or *Phaseolus vulgaris* (Cipollini 1998; Cipollini Jr 1997), in response to mechanical stress. In the case of *Arabidopsis*, it was shown that the increase in lignin that could be seen was proportional to the overall increase of cell wall material, extracted as AIR. Therefore, the weight-weight ratio of lignin and other cell wall components remains unchanged (Paul-Victor and Rowe 2011). I detected an increase in lignin in the stems of CALPAIN OE plants. However, I expressed the amount of lignin per unit of length of the stem and not as a weight-weight relation. My results suggest that the observed increase of lignin is proportional to the increase in radius of the vasculature in the stem, suggesting that if I had expressed my data as a proportion of lignin in the AIR, I could expect similar results to those obtained by Paul-Victor and colleagues (2011). As explained previously I was unable to repeat these experiments satisfactorily due to pest problems in the growth rooms in Lyon.

In addition to changes in cell wall composition, I observed a lower level of anisotropy (higher level of orientation) of the microtubules of the stem apical meristem of these plants. Very interestingly this is the opposite phenotype to the one seen in *katanin1* (*ktn1*) mutants which have decreased microtubule dynamics, therefore a lower level of orientation (Nakamura et al. 2010; Stoppin-Mellet et al. 2006). It has been described that this is due to decreased sensitivity to mechanical stress (Uyttewaal et al. 2012). In our case I observed an opposite reaction. A tempting interpretation of this is that it could be due to a constitutive response to mechanical stress in CALPAIN OE plants. This may also be linked to my observations regarding the properties of the cell wall, since microtubules are very clear integrators of mechanical signals, and guide the deposition of cellulose microfibrils, as discussed previously in this chapter and in depth in the general introduction of the thesis. Thus, if the CALPAIN OE plants respond to mechanical stress constitutively they could be involved in enhancing the activity of the cellulose synthase complexes. However, this pinpoints the fact that two of the results of my work seem contradictory, one showing that there is a disorganization in the cell wall in the cotyledons of CALPAIN OE plants, and the other one showing that the cortical microtubules have a higher degree of organization in the meristems of these same plants. This latter observation might lead to the prediction that the cell wall would presents a much more organized structure, as the cellulose synthase complexes are guided by microtubules (Paredez et al. 2006). One explanation for this discrepancy is based on the fact that the observations are made in different tissues. I propose that in the meristems the active calpain can interact with microtubules and it is responsible for cutting them in order to allow their reorganization. There are examples of calpains interacting with microtubules in published literature. One of them is the case of the animal calpain CAPN6, which is a key player in the stabilization of microtubules in mice osteoblasts (Hong et al. 2011). The peculiarity of CAPN6 is that it is very likely, a non-active protease, as it lacks the typical cysteine active site (Dear et al. 1997). In the case of the CALPAIN domain of DEK1, we have an active cysteine protease (Wang et al. 2003), opening

the possibility that this enzyme could be cleaving one of the components of the microtubule. It is possible that such cleavage events in the microtubules could allow other effectors to mediate their reorganization following the existing tension of the cell. Thus an increased cutting activity in CALPAIN OE plants would explain the "over-organization" of microtubules in the meristem. This leaves open the important question of the identity of the effector that reorganizes the microtubules and of how is the directionality of the tension in the tissue sensed. In the cotyledons similar events may occur, with the over-expression of the active calpain leading to the cutting of the microtubules. However, in this tissue, tensions levels are much less anisotropic, especially during early cotyledon growth, when the outer cell wall would have been laid down. Increased cutting of microtubules in such a system might prevent the deposition of a structured cell wall, leading to a stochastic organization, and also affecting the characteristics of the cell wall. This might also explain the decrease in lobbing observed in CALPAIN OE cotyledon cells. .

My transcriptional analysis also supports our theory that CALPAIN OE plants respond, to some extent, in a constitutive manner to mechanical stimulation. Using some of the touch sensitive targets published by Braam in 2005 (Lee et al. 2005) and osmo-sensitive genes found in the eFP browser (Winter et al. 2007) I saw an up-regulation of these genes in CALPAIN OE seedlings. This suggests that at the nuclear level CALPAIN OE plants leads to a continuous stimulation of the expression of these genes.

Most of these experiments indicate that it would be of great utility to generate inducible lines for the OE and silencing of the CALPAIN domain of DEK1. These lines would allow characterising the dynamics of transcriptional responses to the overexpression of the CALPAIN domain allowing the study of the kinetics of the response to overexpression and absence of this protease. These lines have now been generated in the laboratory and will open the way for more detailed studies of both

the transcriptional and cytoplasmic responses to changes in the level of active calpain.

The idea of calpains being effectors to mechanical stimulation is not a new idea. It has been described in animal homologs of the CALPAIN domain of DEK1 that they have a role in mechanosensing. In particular the ubiquitous human m-calpain and μ-calpain have been shown to have a function in integrating the traction forces in migrations of fibroblasts. Mutants for the regulatory domain of these calpains are less adhesive than wild-type cells and they fail to respond to mechanical stimulation (Undyala et al. 2008). Other calpains, such as calpain 3, have been shown to form a complex with mechanosensitive proteins such as the members of the MARP family (Belgrano et al. 2011; Frey et al. 2004; Toko et al. 2002).

In summary, the phenotypes of plants overexpressing the CALPAIN domain of DEK1 coincide in several respects with those of mechanically stressed *Arabidopsis* plants. Substantial changes are seen in the micromechanical characteristics of the cell walls, which is of great interest since these structures are one of the main actors in determining the mechanical properties of plants, as well as being modified in response to mechanical stimulus. Therefore, my results support the hypothesis that the function of the CALPAIN domain of DEK1 is very likely to be related to responses to mechanical stimulation in plants.

5. Identification of targets of the CALPAIN domain of DEK1

5.1. Introduction

5.2. Introduction to Yeast two-hybrid (Y2H) experiments

5.3. Y2H screening of CALPAIN domain putative interactors against a cDNA library

5.3.1. Identified potential targets of the CALPAIN domain of DEK1

5.3.1.1. Lung seven transmembrane receptor family protein (At3g09570)

5.3.1.2. Irregular Xylem 15 Like (At5g67210)

5.3.1.3. D-xylose-proton symporter-like 2 (At5g17010)

5.3.1.4. Cellulose synthase like A9 (At5g03760)

5.3.1.5. NOD-26 Intrinsec Protein 5;1 (At4g10380)

5.3.1.6. Membrane Anchored MYB (At5g45420)

5.3.1.7. Eceriferum 9 (At1g34100)

5.3.1.8. Sodium/hydrogen exchanger 4 (At3g06370)

5.3.1.9. Tubulin β chain 2 (At5g62690)

5.4. Summary and conclusions

5. Identification of targets of the CALPAIN domain of DEK1

5.1. Introduction

As discussed in depth in the general introduction, calpains are specific proteinases. A given calpain substrate is always proteolized at the same position; however, the rules governing this specificity are not yet understood (Sorimachi et al. 2012).

Calpains have been described as having a Ca^{2+}-dependent activity. After binding to Ca^{2+} the topography of the protein changes in such way that the active catalytic site is assembled together (Moldoveanu et al. 2001; Moldoveanu et al. 2002; Moldoveanu et al. 2004). It is believed that this triggers self-cleavage events, either before or in parallel with external proteolysis (Margis and Margis-Pinheiro 2003).

It has been classically believed that calpains cause limited proteolysis; mainly within inter-domain unstructured regions (Sakai et al. 1987; Stabach et al. 1997). However, although the question of whether there are any rules governing specificity at an amino acid level has been raised, the answer still remains quite unclear. Nevertheless, some sequence preferences have been described (Hirao and Takahashi 1984; Ishiura et al. 1979; Sasaki et al. 1984).

This lack of information regarding the characteristics of direct targets for general calpains is acute in the case of the phytocalpain DEK1. Even though an extensive list of indirect transcriptional effects of deregulating the CALPAIN domain of DEK1 has been published by Johnson and colleagues in 2008, nothing is known about its direct targets. Unpublished results of co-immunoprecipitations of calpain-GFP in siliques carried out by Roberta Galletti in the Ingram lab show putative interactions with Heat Shock Protein 70 (HSP70) proteins, several members of the β-tubulin family,

actin 2 and the vesicle related proteins clathrin and coatomer. However it should be borne in mind that interactions detected in this way are not always direct, and could require an intermediate protein or complex.

Similar interactors to the ones detected by Roberta Galletti have been described in animals. It has been shown that several vesicle related proteins interact with animal calpains. For example, calpains are known to regulate clathrin dependent endocytosis in rat neural tissues. In this case the interaction has been described to be indirect, as it is mediated through the hydrolysis of the α- and β-subunits of the tetrameric adaptor complex 2 (Rudinskiy et al. 2009). A similar case has been described for extracts of bovine brain, in which calpains cleave the clathrin assembly molecule (Kim and Kim 2001). Other vesicle-related proteins have been detected as interactors of calpains, such as it is the β-subunit of the coatomer complex, which was found to be cleaved by a stomach-specific rat calpain (Hata et al. 2006).

The interaction between members of the calpain family and cytoskeleton proteins has also been observed in other systems. It has, for example, been shown that the calpain CAPN6 participates in the stabilization of microtubules in mice osteoblasts. CAPN6 is a non-classical calpain that does not present a cysteine active site, being very likely a non-active protease (Dear et al. 1997), even though is a protein relevant for development (Tonami et al. 2007). This protein has been described to be responsible for the acetylation of tubulin, the basic building block of microtubules, a process should promote the stabilization of this structure (Hong et al. 2011).

As discussed into depth in the general introduction of this thesis, calpains are capable of interacting with substrates through their domains IIa and IIb. These domains present the catalytic triad of amino acids that perform the protease activity (Hata et al. 2001). This active catalytic site has a narrow and deep conformation (Moldoveanu et al. 2004), possible requiring the targets to be “malleable”.

In order to shed some light on the possible substrates of the CALPAIN domain of DEK1, and other potential interacting proteins, I performed a Yeast two-hybrid experiment, which it was hoped would provide complementary data to the immunoprecipitation approach, and potentially identify direct calpain substrates.

5.2. Introduction to Yeast two-hybrid (Y2H) experiments

In the last two decades new methods for the study of protein-protein interactions have been developed. In the early '90s it was very difficult to identify any protein-protein interaction, mostly due to technical limitations. The development of two-hybrid systems contributed to making this type of study technically accessible , and ultimately high-throughput (Hamdi and Colas 2012).

The first described two-hybrid system was the classical yeast two-hybrid (Y2H) method. It was published by Fields and Song in 1989 (Fields and Song 1989). This original method, developed in *Saccharomyces cerevisae*, is based in the fact that many transcription factors (including the GAL4 transcription factor used by these researchers) are modular, having domains with independent functions. One such domain can be involved in DNA-binding while another, physically distinct domain in responsible for the regulation of transcription. This classical method involves the expression of two chimeric proteins in yeast cells. One of them is the DNA-binding domain of GAL4 fused to a bait protein; the other is the activation domain of GAL4 fused to a prey protein. If a protein interaction occurs between the bait and the prey, the functional transcription factor is reconstituted. This triggers the transcription of one or more reporter genes, which can be factors that either allow the growth of the cells under selective conditions or encode enzymes that can produce coloured products upon provision of defines substrates. Using this method the bait protein can be co-expressed together with different preys, for example, a whole cDNA

library. This allows the screening of potential interactions with a whole population of characterized and novel proteins (Suter et al. 2008).

A large number of variants of this original classic two-hybrid technique are now available. Some of these systems do not even involve yeast. For example, mammalian two-hybrid systems were developed mainly to study human proteins, as the context in which the interaction is studied is much more realistic (Luo et al. 1997). Bacterial two-hybrid systems were generated by Hochschild and collaborators (Dove et al. 1997; Hu et al. 2000), and can be useful in the avoidance of the classic problem in yeast systems, auto-activation by either bait or prey, giving rise to false positives (Serebriiskii et al. 2005). A modified bacterial system has also been designed to study protein-DNA interactions (Joung et al. 2000).

In this study I used a variant of the Y2H system, based on the properties of split ubiquitin. The reasons for doing this were first that classic Y2H experiments were previously carried out by former members of our group and they showed a very high level of auto-activation of bait constructs containing the CALPAIN domain, and secondly, that the CALPAIN domain is usually located in the cytoplasm rather than the nucleus. The split ubiquitin anchors the bait to the plasma membrane, allowing interactions to be detected in a cytoplasm/membrane context. In our case, we used the DUALhunter system (Dualsystems Biotech, Schlieren, Switzerland).

The split ubiquitin system (Johnsson and Varshavsky 1994; Stagljar et al. 1998) is based in the reconstruction of ubiquitin, a highly conserved small protein that is attached to other proteins, marking them for degradation (Hershko 2005; Mayer 2000). When a protein has to be degraded, a cascade of enzymatic reactions attaches a tail of ubiquitin molecules to it through covalent bonds. The poly-ubiquitin tagged protein is then degraded by the 26s proteosome. In order to recycle the ubiquitin, a group of ubiquitin specific proteases (UBPs) act. These UBPs recognise intact, folded

ubiquitin and cleave multimeric or ligated ubiquitin after the last residues. Therefore, free monomeric ubiquitin is released into the cytosol.

The creators of the split ubiquitin system discovered that ubiquitin can be split into two halves, called Nub (for N-terminal ubiquitin) and Cub (for C-terminal ubiquitin). If they are expressed separately, they remain only partially folded, so they cannot be recognised by the UBPs. However, if both halves are co-expressed in the same cell, they show a very strong affinity for each other. This leads to assembly of Nub and Cub into a "split-ubiquitin" which will assume the native conformation of ubiquitin, and thus becomes recognisable by UBPs (Johnsson and Varshavsky 1994).

The strong affinity between the wild-type Nub and Cub can be avoided. This is done by interchanging isoleucine for glycine in the residue in position 3 of the Nub (turning the NubI into NubG). This way, the two halves of the "split ubiquitin" do not re-assemble automatically when co-expressed, and stay unrecognisable by UBPs.

This modified system can thus be used as the basis of a protein complementation assay. A protein of interest can be fused to Cub and possible interactors fused to NubG. In the case of an interaction between the proteins of interest, NubG and Cub are forced into very close proximity, leading to the reassembly of the split-ubiquitin. This split-ubiquitin can therefore be recognised and cleaved by UBPs.

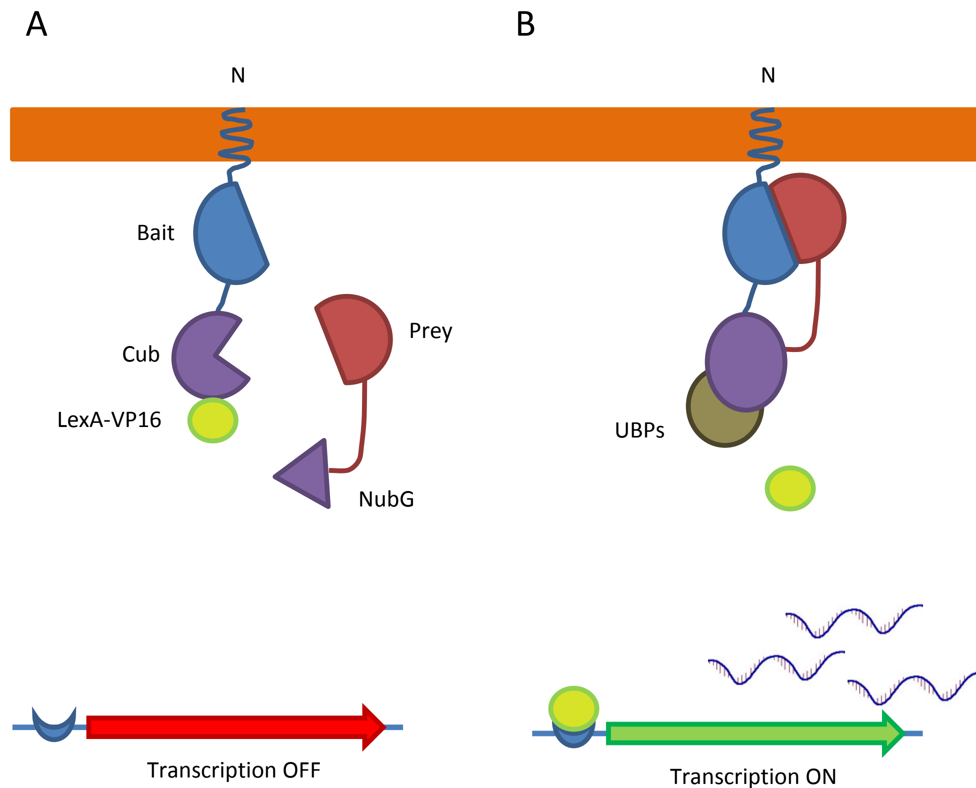


Figure 5-1 Schematized view of how the split ubiquitin –based Y2H system used in this study works in case of a lack of interaction (A) or a positive interaction (B) between bait and prey.

In our experiment (using the Dual Hunter system) we fused our protein of interest (bait) at its N-terminus to a small membrane anchor and at its C-terminus to a reporter cassette composed of the C-terminal half of ubiquitin (Cub) and a transcription factor (LexA-VP16). The prey (a cDNA library from young *Arabidopsis* seeds) is expressed fused to the N-terminal half of ubiquitin (NubG). Any interaction between bait and prey leads to reassembly of the split-ubiquitin and makes it recognisable by UBPs. This leads to a cleavage of LexA-VP16, which would be transported into the nucleus and trigger the transcription of a set of reporter genes (Figure 5-1). In our case the reporter genes used are responsible for the synthesis of histidine and alanine.

5.3. Y2H screening of CALPAIN domain putative interactors against a cDNA library

In order to find putative interactors of the CALPAIN domain of DEK1 we cloned the cleaved version of the CALPAIN domain (domains IIa and IIb) into the bait vector. This allowed us to obtain a fusion protein with a membrane anchor in the N-terminus and Cub and a transcription factor in the C-terminus.

The vector carrying this construct was transformed into yeast cells, and tested for auto-activation of reporter genes, which was found to be minimal (unlike the situation in previous nuclear Yeast-two hybrid screens where the CALPAIN domain was found to activate reporter expression to unacceptably high levels). Transformed strains then underwent a secondary transformation with a normalized Prey-cDNA library produced from siliques containing embryos up to the torpedo stage of embryogenesis.

Around 1.2 million cDNA containing plasmids were screened for interactions with the DEK1 CALPAIN domain, on selective media. Prey plasmids were rescued from positive clones, and sequenced.

This experiment gave a list of putative interactors of the CALPAIN domain of DEK1, which is detailed in table 5-1.

Table 5-1 List of potential target proteins of the CALPAIN domain of DEK1 detected by a Y2H experiment.

Prey-ID	Protein Name (description)	Uniprot code	TAIR code
Prey_077	HR-like lesion-inducing protein-like protein	O22690	At1g04340
Prey_017 Prey_051	ZCF37 protein	Q9SLT9	At1g59590
Prey_018	Lung seven transmembrane receptor family protein	Q9C5T6	At3g09570
Prey_043	Defective Accumulation of Cytochrome B6/F Complex	Q94BY7	At3g17930
Prey_029	Albino or Pale Green Mutant 1 (APG1)	Q9LY74	At3G63410
Prey_070	DNAJ heat shock N-terminal domain-containing protein	Q9FMX6	At5g23240
Prey_019 Prey_033	HR-like lesion-inducing protein-like protein	Q9LSW5	At5g43460
Prey_009 Prey_074	Unknown function protein	Q9LVV4	At5g52980
Prey_062	Irregular Xylem 15 Like (IRX15L)	Q9FH92	At5g67210
Prey_020	Bi-functional inhibitor/lipid-transfer protein/seed storage protein	Q94AQ3	At1g48750
Prey_048	D-xylose-proton symporter-like 2	Q6AWX0	At5g17010
Prey_038 Prey_046	Unknown function protein	Q9MAL3	At1g44920
Prey_040	Cellulose synthase like A9 (CSLA9)	Q9LZR3	At5g03760
Prey_002	Acclimation of Photosynthesis to Environment 2 (APE2)	F4KG18	At5g46110
Prey_021	ORMDL-like protein	Q9C5I0	At1g01230
Prey_045	Proton Gradient Regulation 5-Like 1 (PGRL1)	Q8H112	At4g22890
Prey_005 Prey_066	Reduced Oleate Desaturation 1 (ROD1)	Q9LVZ7	At3g15820
Prey_015	NOD-26 Intrinsic Protein 5;1 (NIP5;1)	Q9SV84	At4g10380
Prey_026	Protein RER1C	Q9ZWI7	At2g23310
Prey_065	Translocation at Inner-membrane of Chloroplasts 21 (TIC21)	Q9SHU7	At2g15290
Prey_012 Prey_031	Putative uncharacterized protein	Q9LPD7	At1g44920
Prey_022	Putative uncharacterized protein	Q9SKQ1	At2g21120
Prey_023	Acclimation of Photosynthesis to Environment 1 (APE1)	Q8H0W8	At5g38660
Prey_028 Prey_059	Membrane Anchored MYB (maMYB)	Q9FHJ4	At5g45420

Prey_041	Rhodanese/Cell cycle control phosphatase-like protein	F4J9G2	At3g59780
Prey_030 Prey_054	Eceriferum 9 (CER9)	F4JKK1	At4g34100
Prey_036	Sodium/hydrogen exchanger 4 (NHX4)	Q8S397	At3g06370

5.3.1. Identified potential targets of the CALPAIN domain of DEK1

Even though the proteins detected as potential interactors of the CALPAIN domain of DEK1 show a great variation regarding their function, we can see that there are certain patterns that are repeated in most of them.

We noticed that most of the putative interactors obtained from this Y2H assay are integral membrane proteins. This is an encouraging result, as calpains have been described to be tightly associated with membranes. Calpains are known to be enzymes with a Ca^{2+}-dependent protease activity and *in vitro* tests showed that they have very high Ca^{2+} concentration requirements in order to activate. These required concentrations are in the order of 10 μM, which is very rarely found *in vivo*. This requirement of very high Ca^{2+} concentrations is reduced dramatically if phospholipids are added to the reaction media in the *in vitro* assay (Saido et al. 1992; Shao et al. 2006; Tompa et al. 2001). This supports the observation that calpains need to be associated with membranes in order to be active (Goll et al. 2003).

Another characteristic found throughout the whole list of potential interactors is that they all present disorganized regions, which have previously been shown to be likely cleavage sites for calpains. As discussed in the general introduction, calpains have a tendency to cleave unstructured inter-domain regions. This is due to the topology of the active calpain molecule. Once active, the catalytic site of this enzyme is assembled and localized in a narrow and deep structure (Moldoveanu et al. 2004). This fact suggests that the substrate, in order to be cleaved by the calpain, should

present a “soft” conformation around the cleavage site, which is usually present in inter-domain regions (Sorimachi et al. 2012). Even though these topology predictions have been made for animal classical calpains, we can extend it to phytocalpains as most described calpains, including the phytocalpains, present a very highly conserved sequence in the active domains. Therefore we can assume that most calpains have very similar requirements when it comes to substrate selectivity.

Using *in silico* resources (Winter et al. 2007) we noted that the detected proteins are predicted to be expressed in multiple different organs of the plant, which vary from seeds to fully developed adult tissues. This is not a problem for co-localization, as DEK1 is present ubiquitously throughout the whole plant and at all developmental stages (Lid et al. 2002).

Despite these positive aspects, some of the putative interactors described in the previous list are very unlikely to be true interactors of the CALPAIN domain of DEK1. We can see that there is an extensive list of integral thylakoid membrane proteins, which, in the plant cell would not co-localize with DEK1, which has never been reported to be found in chloroplasts either using fusion proteins, or in proteomics studies. DEK1:GFP fusion proteins give a florescent signal in the plasma membrane and around the nucleus. The latter is believed to be due to localization in the ER (Johnson et al. 2008).

In order to ascertain which proteins might be true interactors of DEK1, a series of confirmation experiments were carried out in yeast in collaboration with Pauline Wagnon. These experiments aimed to test 1) that prey constructs did not cause positive signals in the presence of the empty bait vector and 2) that we could recapitulate the interaction observed in yeast. They generally involved remaking prey vectors expressing the full-length prey protein detected in the screen, and co-

expressing these with the original bait vector. Unfortunately these experiments are still on-going, due to time constraints caused by the late delivery of the data.

5.3.1.1. Lung seven transmembrane receptor family protein (At3g09570)

This protein is closely related to human GPR107 and murine GPR108, initially cloned from different mammalian lung tissues. The LUNG7 family proteins are predicted to have an amino-terminal hydrophobic signal peptide sequence, an extracellular domain and a C-terminus that consists of a seven transmembrane domain, known also as a LUSTR domain. Human GPR107 has been described to be a G-protein coupled receptor, whose activity is regulated by small peptides (Ben-Shlomo et al. 2003). This receptor binds, in particular, to neurostatin (Yosten et al. 2012), which is a 13-aminoacid peptidic hormone that regulates several cardiovascular and metabolic actions in a whole variety of tissues (Hua et al. 2009; Samson et al. 2008; Yosten et al. 2011).

G protein coupled receptors are responsible for perceiving extracellular signals and transducing them to G proteins. These proteins are capable of sending these signals to downstream effectors. Thus, they play a crucial role in a variety of signalling pathways (Tuteja 2009). G proteins are so-named because of their ability to bind to the guanidine residue of GTP (Temple and Jones 2007). Even though this family has been very well characterized in animals, with more than 1,000 homologues known, they have not been thoroughly characterized in plants (Tuteja 2009). In plants there is only one known regulator of G protein coupled receptors, RGS1. This protein has a positive regulatory activity (Chen et al. 2003).

This protein would be a very interesting potential interactor of the CALPAIN domain of DEK1, as the later could act on these proteins as a regulator. Due to its

proteinase activity, DEK1 could have a negative effect. This process has already been described in animals, where it was described that a G-protein coupled receptor kinase 2 (GPK2) of lymphocytes is degraded through a calpain mediated process (Lombardi et al. 2002; Salim and Eikenburg 2007).

We tried to confirm this interaction by cloning the full length cDNA of At3g09570 into the prey vector. However, we were unable to detect any interaction (figure 5-2). Therefore, it is not possible for us to confirm the interaction between these two proteins. However, the version of the protein identified in the Dual hybrid screen was not a full length version of the protein. We will therefore recreate this version of the protein in future experiments.

Figure 5-2. Y2H test with the CALPAIN domain encoding region of *DEK1* in the bait vector and the Lung seven transmembrane receptor family protein in the prey vector. Transformed cells were grown on media lacking leucine and tryptophan to confirm the presence of both plasmids (-LT), and on media additionally lacking histidine (-LTH) and media additionally lacking histidine and alanine (-LTHA) to test for interactions. From left to right: serial dilutions of 1:10.

5.3.1.2. Irregular Xylem 15 Like (At5g67210)

IRX15-LIKE is closely related to the protein Irregular Xylem 15 (IRX15). They both contain a Domain of Unknown Function 579 (DUF579). Neither of the single mutants for these genes presents phenotypic changes in comparison to wild-type, but the double mutant *irx15 irx15-l* has a moderate reduction in stem xylose (Jensen et al. 2011). Therefore, it is believed that these proteins are very likely involved in the biosynthesis and deposition of xylan (Brown et al. 2011).

Xylan is one of the main hemicellulose components present in the secondary cell wall of eudicots and in the primary cell wall of grasses and cereals. Its content can go up to 30 % of the dry mass of some grasses and woody species (Gibeaut and Carpita 1994; Heinze et al. 2006).

This protein seemed to be a very promising potential interactor of DEK1 as it affects directly the mechanical properties of the cell wall. Xylan, as other hemicelluloses, forms a network with cellulose fibrils that is the main load-bearing structure of the cell wall (Hayashi and Kaida 2011; Somerville et al. 2004) and as we described in previous chapters, the CALPAIN domain of DEK1 may play a role in regulating the mechanical characteristics of the cell wall. Unfortunately, when we tested this protein in the yeast-two hybrid system we found that it was capable of activating reporter expression when expressed with the empty bait vector. Therefore, although we cannot state categorically that this protein does not interact with the DEK1 CALPAIN domain, it seems unlikely.

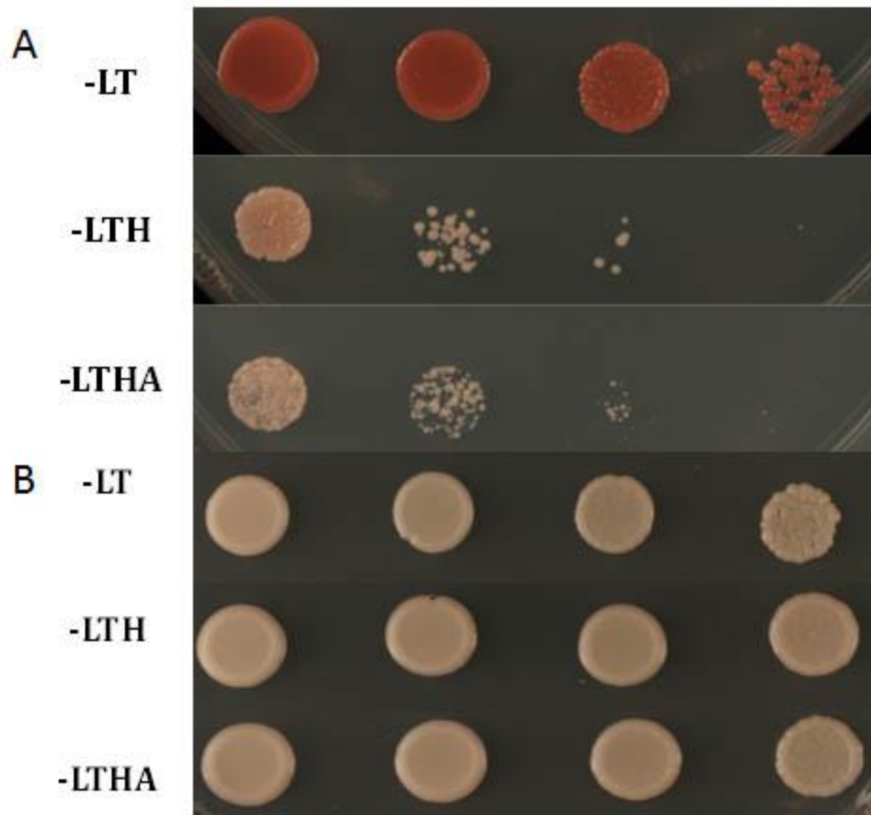


Figure 5-3 Y2H test with full length *IRX15-L* in the prey vector and the CALPAIN encoding domain of *DEK1* in the bait vector (A) and the empty bait vector with *IRX15-L* in the prey (B). Transformed cells were grown in media lacking leucine and tryptophan to confirm the presence of both transgenes (-LT), in media additionally lacking histidine (-LTH) and media additionally lacking histidine and alanine (-LTHA) to test for interactions. From left to right: serial dilutions of 1:10.

5.3.1.3. D-xylose-proton symporter-like 2 (At5g17010)

This protein is also known as VGT2, and it is a member of the sub-family of VGT-like proteins. Originally it was described as a sugar transporter, due to its high homology to the bacterial xylose permease LbXyIT. However, it should be born in mind that this bacterial transporter shows a very high resemblance to most Monosaccharide transporter-like proteins. Nevertheless, when different saccharide transporters are compared, they all show a very high homology, independently of the solute they transport. Therefore, a high homology in these proteins does not necessarily means that it is reflected in a substrate specificity (Büttner 2007).

VGT2 is closely related to another gene of the *VGT* sub-family, *VGT1* (At3g03090), not described as a xylose transporter, but as a glucose transporter with its main activity during germination and flowering (Aluri and Buttner 2007). Both VGT1 and VGT2 have been described as vacuolar membrane proteins (Aluri and Buttner 2007). This VGT-like family is closely related to another sub-family of sugar transporters, the Sugar Transport Protein-like (AtSTP-like) (Büttner 2007). AtSPTs are some of the best characterised hexose transporters in *Arabidopsis*. All members of this family are plasma membrane localized. They catalyse hexose uptake from the apoplast into the cell. To date, all described AtSPTs are found in sink tissues with one exception: AtSTP3. This protein was shown to have very low affinity to glucose and is expressed in photosynthetic tissues. It has been proposed to have a role in sugar retrieval (Büttner et al. 2000).

In this case the version of the protein detected in the Dual hybrid system was not the full length version of the protein. It was a short version that has only 245 amino acids instead of the 503 present in the full length protein. This version of the protein, according to *in silico* structure predictions (Letunic et al. 2012; Schultz et al. 1998), would contain 5 of the 6 most C-terminal transmembrane domains of the protein and most of an unstructured region present in the centre of the protein, which is

likely to be a cytoplasmic domain, and therefore, a potential target of the CALPAIN domain of DEK1.

Confirmation experiments have not yet been performed for this target, but it would be of great interest to test the potential interaction between the CALPAIN domain of DEK1 and VGT2.

5.3.1.4. Cellulose synthase like A9 (At5g03760)

Cellulose synthase like A9 (*CSLA9*) is a member of the *Cellulose Synthase-Like* (*CSL*) gene family. The genes in this family contain conserved motifs that are needed for nucleotide-sugar binding and processive glucosyltransferase activity. This is the synthesis of 1→4)-β-linked polysaccharide backbones, in which residues are turned 180° with respect to their immediate neighbour (Delmer 1999; Richmond and Somerville 2001). The different proteins of this family have different sequences, which has led to speculation that they may actually be involved in the synthesis of several different cell wall polysaccharides (Zhu et al. 2003).

CSLA9 has been described as a Golgi apparatus membrane protein, like several other members of the CSL family (Davis et al. 2010; Sandhu et al. 2009). This protein is predicted to be an integral membrane protein, with very probably five transmembrane domains. Its active site is predicted to face the lumen of the Golgi apparatus (Davis et al. 2010).

The version of the protein detected with the assay is a short version, which contains from amino acids 424 to 511. This fragment of the protein includes the C-terminal two transmembrane domains and a low complexity region. Even though the protein does not have any described cytoplasmic domains, this disorganised region could potentially face the cytoplasm.

Confirmation experiments are currently being carried out for this interaction, which, given my results regarding changes in cell wall composition induced by CALPAIN expression, is an intriguing candidate.

5.3.1.5. NOD-26 Intrinsic Protein 5;1 (At4g10380)

NIP5;1 is part of the major intrinsic protein family (Johanson et al. 2001). Members of this protein family work as water-selective or relatively non-selective channels that transport water and other small size molecules (Tyerman et al. 2002). *NIP5;1* transcription is up-regulated in roots that are grown under boron deficient conditions (Takano et al. 2006). Subsequently, the activity of this plasma membrane localized protein was characterized by expressing it in *Xenopus laevis* oocytes, where its boric acid transport activity was recorded (Takano et al. 2006). T-DNA insertion lines in this gene have problems in boron uptake in roots. Lower biomass production and an increased sensitivity to boron deficiency were seen in the mutants. If grown under boron deficiency conditions, a lower growth rate was recorded in its shoots and roots (Takano et al. 2006). An overexpression of this gene gave rise to an improved elongation of roots under low boron conditions (Kato et al. 2009).

Boron is an element of great importance for cell walls, as it participates in the cross-linking of rhamnogalacturonan-II, a pectic polysaccharide (Kobayashi et al. 1996; O'Neill et al. 1996). This ion forms part of the covalent bridge in the cross-linking of saccharide molecules (O'Neill et al. 2001; O'Neill et al. 1996). In *Arabidopsis*, a reduced degree of cross-linking of rhamnogalacturonan-II gives reduced growth both in aerial tissues and roots (O'Neill et al. 2001).

The fact that this possible interactor of the CALPAIN domain of DEK1 has a direct effect on cell wall properties is very interesting since, as described in previous chapters, the misregulation of the CALPAIN domain gives place to changes in the cell wall, which could have an origin in structural changes in its architecture. If this interaction were confirmed, it would be very interesting to measure boron flux through the plasma membranes of different *dek1* mutants to know more about its possible role in regulating the activity of these channels.

5.3.1.6. Membrane Anchored MYB (At5g45420)

MaMYB is a membrane anchored protein. It is a non-classic member of the R2R3-MYB transcription factor family (Dunkley et al. 2006). The R2R3-type MYB-proteins form a subgroup with 126 members. No homologues of this family have been found outside the plant kingdom, making them plant exclusive (Dubos et al. 2010; Stracke et al. 2001). Transcription factors of this family are characterized by their structure which contains two imperfect MYB repeats. These repeats form helix-turn-helix structures that have the ability to bind to DNA. It has been suggested by bioinformatics analyses that MaMYB is the only membrane-anchored member of the R2R3-MYB family (Kim et al. 2010). Homologs have been identified as far back at the moss *Physcomitrella patens*, but not in *Chlamydomonas reinhardtii*, which suggest that MaMYB is conserved throughout multicellular plants (Altschul et al. 1997).

MaMYB was identified as an ER membrane-anchored protein. The transcription factor domain, its N-terminus, is exposed to the cytosol. Truncated versions of MaMYB containing only the R2R3-MYB transcription factor domain, are retained in the nucleus, where MaMYB presumably regulates gene expression. Silencing of *MaMYB* resulted in the production of significantly shorter root hairs compared to wild-type plants, but no changes in root hair density. This phenotype of could be

rescued using auxin analogues. This suggests a role of *maMYB*, in auxin signalling in root hair elongation of *Arabidopsis* (Slabaugh et al. 2011).

We found this protein to be a very interesting candidate. In fact, the interaction of calpains with membrane-bound transcription factors has been suggested previously. The membrane-anchored transcription factors NTL1 and NTL6, both members of the plant specific NAC transcription factor family have been suggested to interact with the CALPAIN domain of DEK1. According to Park and collaborators, these transcription factors are cleaved, and therefore activated, by a cysteine protease. It has to be noted that even though this interaction was suggested, it has not yet been shown proved by molecular or genetic methods (Kim et al. 2006b; Seo et al. 2010).

On top of this, we also found very interesting the fact that MaMYB may act upstream of auxin in signalling pathways determining root hair length. This could be due to the fact that the active transcription factor could trigger the transcription of an enzyme involved in the auxin synthesis pathway. Therefore we checked for the presence of auxin synthesis-related genes in the published microarray data, finding that *TAA1* is overexpressed in plants overexpressing the CALPAIN domain of DEK1 (Johnson et al. 2008). *TAA1* stands for *Tryptophan Aminotransferase of Arabidopsis 1*, and is the gene encoding a protein involved in tryptophan-dependent auxin biosynthesis (Mashiguchi et al. 2011; Stepanova et al. 2008; Tao et al. 2008).

The MaMYB clones we obtained were truncated, lacking the first out of the three predicted transmembrane domain. We therefore ran tests to confirm this interaction using both the full length and a truncated version of MaMYB fused as bait for an Y2H experiment with the CALPAIN domain of DEK1 as prey. Both tests showed negative results, (figure 5-4 A-B). In addition, the truncated version of MaMYB as prey produces positive results with an empty bait vector (figure 5-4 C), suggesting that this interaction was, very probably, a false positive result.

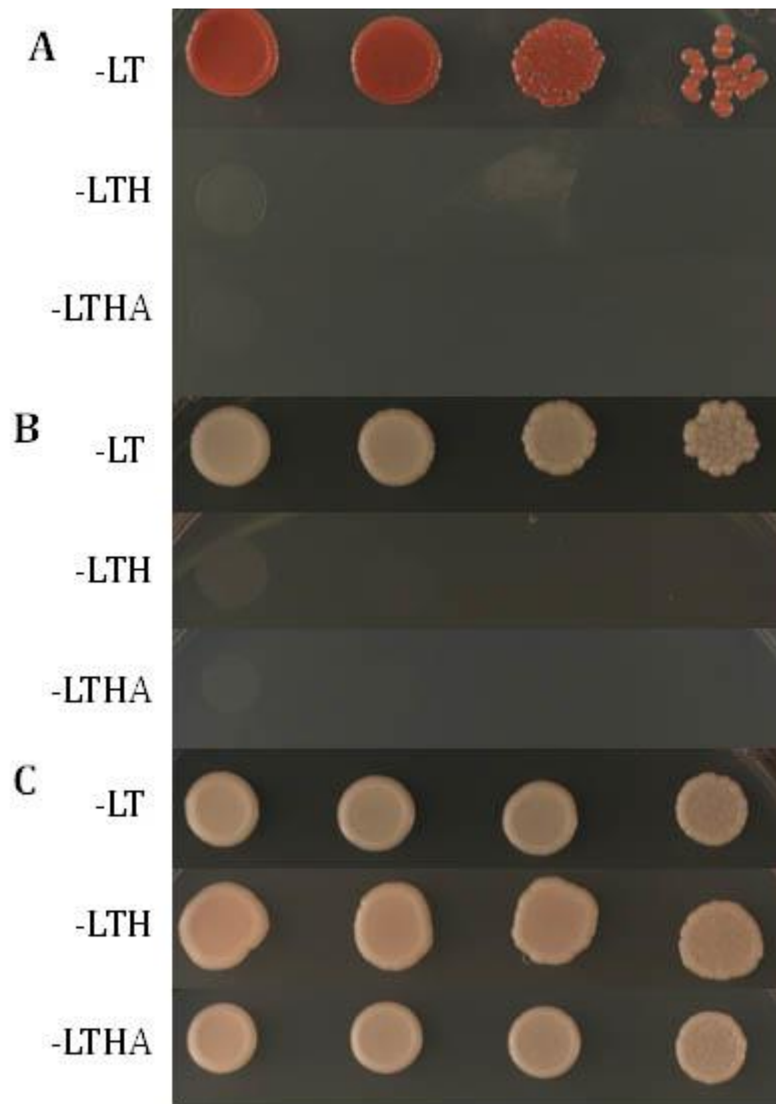


Figure 5-4 Y2H test with full length MaMYB asthe bait and the CALPAIN domain of DEK1 as the prey (A), the truncated version of MaMYB asthe bait and the CALPAIN domain of DEK1 as the prey (B) and the empty bait vector with truncated MaMYB asthe prey (C). Transformed cells were grown in media lacking leucine and tryptophan to confirm the presence of both transgenes (-LT), in media additionally lacking histidine (-LTH) and media additionally lacking histidine and alanine (-LTHA) to test for interactions. From left to right: serial dilutions of 1:10.

5.3.1.7. Eceriferum 9 (At1g34100)

CER9 is a protein described as being involved in wax metabolism. In *Arabidopsis*, the knockout mutant of the *cer9* gene was originally described as having a semi glossy stem as a consequence of wax defects (Koornneef et al. 1989). Mutants for this gene have a very different wax profile in comparison to that of wild-type plants. Mutants showed an elevation in the amounts of very long chain fatty acids (VLCFAs) tetracosanoic acid (C_{24}) and hexaxosanoic acid (C_{26}) compared to wild-type plants (Goodwin et al. 2005; Jenks et al. 1995). Apart from these changes in the VLCA components of cuticular waxes, the *cer9* mutant also presents important changes in cutin monomer composition, leading to a modified cuticle membrane ultrastructure. The amount of the cutin monomer is 1.6-fold higher in the mutant if compared to wild-type individuals. Stem cuticle membrane thickness is increased 2.1-fold in the mutant. These mutants also show physiological changes, such as a delayed wilting of leafs in the case of drought, very probably a consequence of reduced transpiration rates (Lü et al. 2012).

CER9 is expressed in the plant in a constitutive manner (Lü et al. 2012). In roots, the levels of suberin are elevated in comparison to wild-type plants in *cer9* mutants. This is not of great surprise as suberin is chemically very similar to cutin, shown to be over-synthesized in aerial tissues of *cer9* mutants. It has been proposed that this increase in suberin could restrict the uptake of water in the roots, helping to the altered water relations seen in *cer9*. Other mutants with alterations in suberin, such as *esb1*, which also presents high suberin levels, have lower transpiration rates than wild-type plants. This supports the model that suberin works as an extracellular barrier for water and solutes transport in the apoplast (Baxter et al. 2009). However, grafting experiments where *cer9* plants were grafted onto wild-type roots showed that the increase in suberin in the mutant roots does not play an apparent role in the low transpiration rates observed in *cer9*. Therefore the cuticular differences in the

aerial parts are likely to be responsible for the low transpiration rates and the delay in wilting of leafs (Lü et al. 2012).

Recently, it has been shown that the coding sequence of *CER9* encodes a protein that shows very high homology to a yeast protein called Doa10, and its human orthologue, TEB4. Within the sequence there is a particularly high conservation in two domains, a transmembrane region and the RING-CH-type zinc finger domains (Kreft and Hochstrasser 2011; Swanson et al. 2001). In fact, the mutation present in the *cer9-1* allele is a point mutation in the RING-CH domain, which changes a Cys to a Tyr. This domain is very likely to play an important role, as this mutant, which expresses the full length protein with just one amino acid change, shows a phenotype comparable to the one of *cer9-2*, which expresses a highly truncated version of the protein (Lü et al. 2012).

It has been observed that the yeast protein Doa10 has a role as an E4 ubiquitin ligase, which participates in ER-associated degradation of proteins that have been misfolded or unassembled (Vashist and Ng 2004). The active domain of this protein, the RING domain, is cytosolic (Hassink et al. 2005; Swanson et al. 2001). It is has been proposed that CER9 could be involved in the cuticle wax synthesis process as a negative regulator of the wax synthesising enzymes through its putative ubiquitin ligase activity (Lü et al. 2012). However, the fact that CER9 works as an E3 ubiquitin ligase and the processes that lead to the altered wax profile phenotype still remain to be proved.

I believe that this protein could be a good potential interactor of the CALPAIN domain of DEK1, and tests to confirm this interaction are underway in the laboratory. When cryofracture scanning electronic microscopy images of calpain over-expressing plants were generated (figure 5-5), changes in the structure of the wax crystals seen on the surface of the stem were observed (figure 5-5). It would be very interesting to analyse the wax profiles of CALPAIN OE plants and compare

them to those of wild-type plants. This is technically possible thanks to mass spectrometry techniques (McNevin et al. 1993).

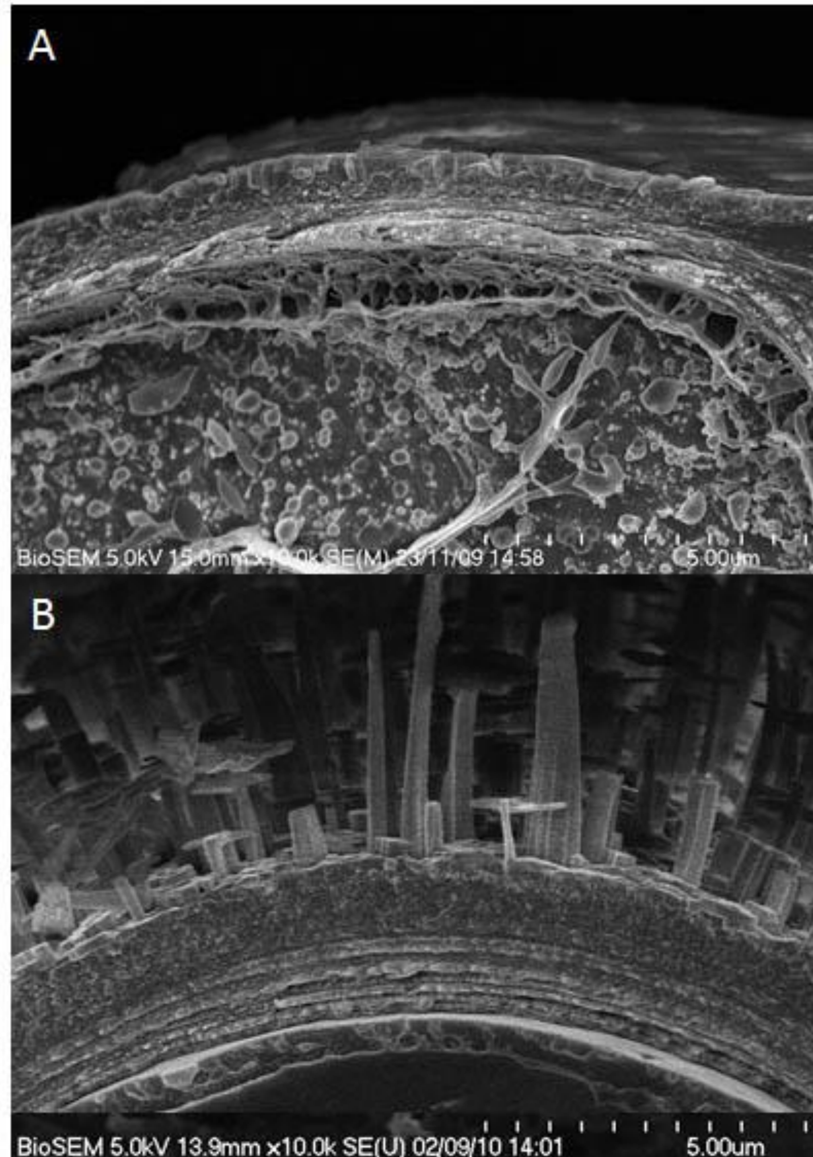


Figure 5-5 Cryofracture scanning electron micrographs of wild-type stem epidermis (A) and CALPAIN OE stem epidermis (B) in which differences in the structure of the epicuticular waxes crystals can be readily observed.

5.3.1.8. Sodium/hydrogen exchanger 4 (At3g06370)

NHX4 is a protein present in the vacuolar membrane. It is part of the NHX subfamily, a group of Na^+/H^+ antiporters (Pardo et al. 2006). This subfamily belongs to a family of monovalent cation/proton antiporters (monovalent cation/proton antiporters (CPA1) (Maser et al. 2001). This type of protein plays key roles in the homeostasis of Na^+, K^+ and pH (Apse and Blumwald 2007; Yokoi et al. 2002). In plants, these antiporters mediate the transport and exchange of monovalent ions and protons. As a motor to drive ion transport, they use the pH gradients generated by the H+-ATPases of the plasma membrane and the H+-ATPase and H+-PPase present in the vacuolar membrane (Brett et al. 2005; Pardo et al. 2006; Rodríguez-Rosales et al. 2009).

The *nhx4* mutant has a very high tolerance to salt stress when compared to wild-type plants, and has decreased internal concentrations of Na^+ if grown under NaCl stress. If *NHX4* is expressed heterologously in *E. coli* it gives rise to salt hypersensitive bacteria (Li et al. 2009).

Exchangers member of the NHX family are predicted to have a classic transporter topology. This consists of 10 to 12 transmembrane domains and a C-terminus that is predicted to face the cytosol (Pardo et al. 2006). This makes this protein a valid potential interactor of calpains, as it is associated with a membrane and moreover it presents an unstructured loop between the transmembrane domains 4 and 5, according to the predictions made by the structure predictor SMART (Letunic et al. 2012; Schultz et al. 1998).

Interestingly, this is not the first clue on potential interaction between calpains and Na^+ channels. Such interactions have previously been described for voltage-gated channels in brain cells (von Reyn et al. 2012; von Reyn et al. 2009). Furthermore, I detected a relation of plant calpains with Na^+ homeostasis, as *dek1[CALPAIN]*

mutants show significantly increased concentrations of Na^+ if compared to wild-type plants.

The technique used for the quantification of Na^+ is AAS (atomic absorption spectroscopy) in which a solution obtained from the sample is atomized as a spray into a flame. The heat of the flame causes an excitation of the electrons in the outer orbital of the element. The lifetime of this excited species is limited and the electron will rapidly return to its ground state emitting a photon of radiation of a particular wavelength. For example, in the case of sodium this photon is of a wavelength of 589 nm, radiation that can be detected and quantified. A calibration curve is used to compare the measurement of the photometer with a set of standards of known concentration (Walsh 1955).

Preliminary tests were run to quantify the concentrations of three different ions: calcium (Ca^{2+}), potassium (K^+) and sodium (Na^+). No significant differences were detected for Ca^{2+} and K^+, but the concentration of Na^+ was significantly higher in the *dek1[CALPAIN]* mutants.

Measurements of Na^+ in a larger number of samples were made in two different complemented *dek1* mutant lines (*dek1-2[CALPAIN]* and *dek1-3[CALPAIN])* and wild-type plants. The two complemented mutant lines show different levels of calpain overexpression. The tissue used for this purpose was full soil-grown rosettes prior to flowering.

An increase of approximately 40% in the sodium concentration was seen in both mutant lines compared to the wild-type rosettes. Values were around 7 mg of Na^+ per g of fresh tissue for wild-type rosettes and of 10 mg in both of the mutants (figure 5-6).

These data could be interpreted as a problem in the exclusion of Na^+ from plants that express the active CALPAIN domain constitutively, or could be due to the lack of the DEK1 transmembrane domains. This remains to be resolved.

Confirmation of the interaction between the DEK1 CALPAIN domain and NHX4 are on-going in the laboratory.

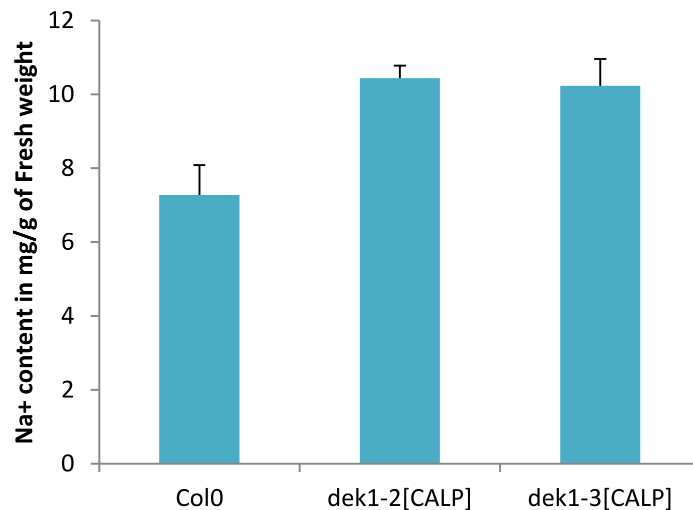


Figure 5-6 Concentrations of Na^+ detected in wild-type (Col0) plants and in *dek1-2[CALPAIN]* and *dek1-3[CALPAIN]* detected by acid extraction and flame photometry. Error bars show standard deviation. Statistical significant differences between the wild-type and CALPAIN over-expresser samples were seen when tested with a t-test ($p<0.01$). N=5.

5.3.1.9. Tubulin β chain 2 (At5g62690)

In order to test if we could detect a direct interaction between the CALPAIN domain of DEK1 and β-tubulin we set up an Y2H experiment using TUB2. This gene was chosen as it is the highest scoring member of the β-tubulin family detected in co-immunoprecipitations (Galletti unpublished results). TUB2 is one of the basic components of microtubules, which consist of heterodimers of globular α- and β-

tubulin subunits. They are arranged in a head-to-tail fashion and form a bundle of 13 protofilaments. These protofilaments form a cylindrical structure, the microtubule. They have an outer diameter of approximately 25 nm (Amos and Schlieper 2005). In the case of *A. thaliana* there are 6 genes encoding for α-tubulin subunits and 9 encoding for β-tubulin subunits.

The *TUB2* gene was cloned into the prey vector and was co-transformed with the bait containing the CALPAIN domain of DEK1. This experiment gave a negative result for the interaction (figure 5-6). This result, however, merely shows that Calpain does not interact with monomeric TUB2 in yeast. We cannot exclude either an interaction with polymerized tubulin (microtubules) or an indirect interaction

Figure 5-6 Y2H test with the CALPAIN-encoding domain of DEK1 as the bait and TUB2 as the prey. Transformed cells were grown in media lacking leucine and tryptophan to confirm the presence of both transgenes (-LT), in media additionally lacking histidine (-LTH) and media additionally lacking histidine and alanine (-LTHA) to test for interactions. From left to right: serial dilutions of 1:10.

5.4. Summary and conclusions

Even though Y2H screens are a simple way to recognise putative protein-protein interactions, their results have to be interpreted in a cautious way as this is a technique that presents numerous drawbacks. The main criticism usually made of Y2H screens, is the possibility of their resulting in a high number of false positive and false negative detections. This could be partially due to the fact that the proteins that are used in these screens are not native proteins, but chimeras, fusion proteins. Fusion of proteins can lead to changes is the topology of both the bait and the prey, affecting their functionality. Misconformation can give place to a limited activity or it can make binding sites either inaccessible, or abnormally accessible.

A common cause for false positives is that for these assays the genes encoding the fusion proteins are driven by strong promoters. This gives rise to a much higher concentrations of the proteins than are usually present in cells. This can have as a consequence interactions that are not seen under the normal concentrations. Moreover, Y2H assays can involve the expression of proteins in cellular compartments other than those in which they are usually localized. This may be exemplified, for example, by the high occurrence of chloroplast-localized proteins as false positive interactors in Y2H screens.

On the other hand, false negatives are seen in many cases as in most Y2H screens only direct binary protein-protein interactions are tested. The consequence of this is that interactions that require a third protein are usually ignored. Nowadays, some commercial kits allow the detection of ternary protein complexes, such as the pBridge™ method developed by Clontech (Mountain View, USA). Through this method, two of the potential interactors are expressed as fusion proteins, similar to the situation in the classic Y2H method. The difference is that a third proteins can be expressed and screened for interactions.

The use of yeast cells for this type of experiment may also affect results, as proteins from heterologous systems may be incorrectly folded in the yeast cell context. Another problem could involve toxicity for yeast cells, leading to the sequestration of fusion proteins in protein bodies where they are inaccessible. In our case, we screened a whole cDNA library, which can also cause problems. It has been shown that single domains often show more and better interactions than full length proteins in yeast (Criekinge and Beyaert 1999). In our case, sequencing of random library clones demonstrated that the vast majority were full length, and this could have caused problems in our screen.

We decided to carry out an Y2H screen as a complement to immunoprecipitation, as DEK1 is a protein which, even though it is ubiquitously expressed, is found in low concentrations in tissues. Furthermore, the levels of expression of tagged calpain in transgenic plants are rarely highly elevated, and this means that immunoprecipitation techniques are likely only to detect a subset of interactions. The fact that only cDNA sequences are needed to launch a yeast-two hybrid screen gives a clear advantage if contrasted to classical biochemical assays, which require very high protein concentrations or highly efficient antibodies. These facts were decisive for us as we considered that a Y2H screen would allow us to detect interactions that would be very hard to see using an *in planta* assay.

Results obtained in this experiment so far have been very disappointing as all the confirmation tests we ran ended up either in the identification of false positives or in the inability to replicate the results seen in the first screen. Another fact was that we found a large number of thylakoid integral membrane proteins, which cannot be interactors of the CALPAIN domain of DEK1 because they are unlikely to be found in the same subcellular compartment. Nevertheless, with this experiment we detected several putative interactions of the CALPAIN domain of DEK1 of great potential interest, which merit further investigation.

One of these is a Lung seven transmembrane receptor family protein, a protein that is closely related to G-protein coupled receptors. Interactions between animal G-protein coupled receptors and calpains have been described in several cases. The G-protein coupled receptor kinase 2 (GPK2) of lymphocytes have been described to be degraded via a calpain mediated process (Lombardi et al., 2002; Salim and Eikenburg, 2007). Another very interesting example of calpains and G-protein coupled receptors interacting involves the mammal calpain Capns1 which binds to a class II G-protein coupled receptor (Juppner et al. 1991) altering its receptor function (Shimada et al. 2005) by causing a partial hydrolysis, at of the receptor.

Unfortunately we were not able to confirm this interaction by cloning the full length gene into the prey vector. However, the version of the protein identified in the first screen was not the full length version. In the future, it would be of great interest to obtain this short version of the transcript used for the initial screen. This would allow us to test and confirm the results and study the possible activity of this shorter transcript.

I detected several proteins that have a channel activity in my experiment. These include VGT2, NIP5;1 and NHX4. Several cases of channels with an activity regulated by a member of the calpain family have been described. In particular, calpains play a key role in the regulation of glutamate receptors. The NMDA receptor, activated by glutamate and glycine as well as membrane depolarization, is a hetero-tetrameric channel of which both main subunits are necessary for functionality. Subunit NR1 contains a glycine-binding site and is encoded by a single gene. Subunit NR2 has four subtypes (NR4A-D) that are encoded by four different genes. NR2 includes a glutamate-binding site (Ishii et al. 1993). A third subunit has been identified (NR3), but its function still remains unknown (Andersson et al. 2001; Sucher et al. 1995). While NR1 and NR3 are not proteolysed by calpains, three of the variants of NR2 (NR2A-C) have been proved to be calpain substrates. They are cleaved at their C-terminus (Guttmann et al. 2001). This

proteolysis seems to lead to further degradation and, therefore, a loss of NMDA receptor function (Guttmann et al. 2002). Another type of glutamate receptor regulated by calpains are AMPA receptors, glutamate-gated ion channels. In particular they act as cation channels when glutamate molecules bind to two of their four glutamate-binding sites (Platt 2007). They can undergo cleavage events that affect channel activity (Bi et al. 1996). Calpains modulate their transport function by cleaving the GluR1 subunit in the C-terminus (Burnashev et al. 1995).

Other channels, such as voltage-gated ion channels are regulated by calpains as well. One example is the voltage-gated Na^+ channels (NaChs), present in rat brain cells. When high Ca^{2+} concentrations are present, a calpain-dependent degradation of their α-subunit is triggered. This α-subunit regulates channel activity (von Reyn et al. 2009). There is also evidence that some calpain-like proteases may be responsible for the cleavage and regulation of Ca^{2+} permeable voltage-gated channels. This proteolysis inactivates the channel, so it has been proposed that, as calpain activity is Ca^{2+} dependent, it could represent a form of feedback loop (Abele and Yang 2012).

I identified several cell wall-related proteins as potential interactors of the DEK1 CALPAIN domain. One of them is CSLA9. This protein is related to the CesA protein, and contains conserved motifs that are needed for nucleotide-sugar binding and for the catalytic activity of processive glycosyltransferases (Delmer, 1999; Richmond and Somerville, 2001). Alongside other members of the CSLA family (CSLA3 and CSLA7), CSLA9 plays a role in the synthesis of the cell wall saccharide glucomannan (Goubet et al. 2009). Glucomannan is involved in the micromechanical properties of the cell wall because glucomannan fibres are arranged in a parallel fashion to cellulose microfibrils (Salmén 2004).

Another potentially interesting interactor of the CALPAIN domain of DEK1 was CER9. CER9 has a role in wax and cutin biosynthesis (Kunst and Samuels 2009).

From Scanning Electron Microscope observations we suspect that the CALPAIN OE plants have a different epicuticular wax composition and/or load compared to wild-type plants. This interaction remains to be confirmed, but nonetheless it would be interesting to do a qualitative and quantitative analysis of the components of the epicuticular waxes, in various backgrounds. No direct roles for calpain activity have been described in animals for any major lipid metabolism pathway. Nevertheless, direct interactions between calpains and lipids have been described repeatedly. Fatty acids have been shown to be key regulators, inactivators in particular, of many different members of the calpain family (Kang et al. 2009; Lee et al. 2012; Park et al. 2012; Perez-Martinez et al. 2011). As discussed in the general introduction of this thesis, calpains also interact with phospholipids through their C2-like domain (Tompa et al. 2001). In this case the lipids are thought to be key regulators of the activity of the protein, as it happens in other proteins that contain C2-like domains (Corbalan-Garcia and Gomez-Fernandez 2010).

Finally, I tested for interaction between the CALPAIN domain of DEK1 and TUB2. I could not detect a positive interaction between the two proteins in yeast, even though a potential interaction was observed in immunoprecipitations, and a microtubule phenotype is observed as a result of CALPAIN overexpression, as discussed in chapter three. As introduced previously in this chapter, it has been observed in animals that members of the calpain family interact with tubulin, the basic component of microtubules (Hong et al. 2011). In the future experiments studying the possible protein-protein interaction between calpains and microtubules, rather than concentrating on monomeric tubulin, should be carried out *in planta*, for example by taxol mediated microtubule isolation. In this technique, microtubules are forced to polymerize in cellular extracts using taxol. Therefore, they can be isolated and their associated proteins can be characterized (Vantard et al. 1991). In addition, it seems probable that a third protein or a complex mediates this interaction. Pulling down tagged TUB and trying to detect calpain in co-immunoprecipitations has already been attempted in our lab, but was unsuccessful.

Again the problem in this case is that, as in the Y2H assays, monomeric TUB is the primary form pulled down in immunoprecipitations.

In summary, the work carried out with the Y2H technique may have provided hints regarding potential interactors of the CALPAIN domain of DEK1. Nevertheless, a lot of work remains to be done regarding the verification of these potential interactors both in yeast and in plants.

6. Role of the transmembrane domains of DEK1 in mechanosensing

6.1. Introduction

6.2. dek1[CALPAIN] plants show a decreased sensitivity to mechanical stress

6.2.1. Effect on flowering time

6.2.2. Effect on cellulose content

6.2.3. Effect on lignin content

6.2.4. Transcriptomic analysis of stressed wild-type and DEK1 mutants

6.3. Implication of DEK1 in Ca^{2+} flux through the plasma membrane

6.3.1. dek1[CALPAIN] root epidermal cells show decreased Ca^{2+} intake under osmotic stress

6.3.2. Xenopus oocytes expressing the transmembrane domains of DEK1 show Ca^{2+}-activated Cl^--conductances

6.4. Summary and conclusions

6. Role of the transmembrane domains of DEK1 in mechanosensing

6.1. Introduction

As discussed in depth in previous chapters, mechanically stressed plants present a very characteristic phenotype that involves stunting, delayed flowering, thickening of stems and changes in cell wall composition, amongst other traits (Braam 2005; Braam and Davis 1990; Cipollini 1998; Paul-Victor and Rowe 2011; Saidi et al. 2010).

Even though not all changes seen in the plants are due to transcriptional changes, many of these effects may be consequences of changes in the expression level of touch-induced genes. These may have diverse functions contributing to the diverse morphological and physiological phenotypes associated with mechanical stress.

Events leading to transduction of mechanical stimulation into a molecular signal that triggers processes at a subcellular level are not yet completely understood. The mechanosensor or mechanosensors that lead to the phenotype of mechanically stressed plant still remain to be described. As a consequence of this obscurity in the field, most ideas about receptors for physical signals in plants are based on comparisons and possible homologies with sensors present in other organisms which include ion channels, osmotic sensors and cell-wall associated kinases (Decreux and Messiaen 2005; Guo et al. 2009; Hedrich 2012; Hematy and Hofte 2008; Hematy et al. 2007; Kohorn et al. 2012; Monshausen and Gilroy 2009b).

There is wide evidence from patch clamp analyses that the plasma membranes of plant cells contain an important diversity of mechanosensitive ion channels. However, few of the effectors for these mechanosensitive conductances, which have been characterized electrophysiologically, have yet been identified (Haswell et al. 2011; Monshausen and Gilroy 2009b). There is strong evidence linking changes in

ion fluxes, mainly Ca^{2+} fluxes, to mechanical stimulation events both at the whole plant and at the cellular level, with stimulation ranging from touch to wind disturbance leading to a transient increase in cytosolic Ca^{2+} levels (Haley et al. 1995; Knight et al. 1992; Monshausen et al. 2008; Trewavas and Knight 1994).

Changes in membrane potential have been proposed to have an early role in response to touch stimuli (Shimmen 2006). Membranes in plant cells show different resting potential values, ranging from -110 to -150 mV in the case of the plasma membrane (Roelfsema et al. 2001; Thiel et al. 1992) and of 0 to -30 mV in vacuolar membranes (Bethmann et al. 1995; Walker et al. 1996). Therefore, plants operate with a trans-cytoplasmic potential that is close to a value of -100 mV, due mainly to an asymmetry in the distribution and nature of ion transport proteins present in the plasma and vacuolar membranes (Hedrich 2012). As discussed in the general introduction of this thesis, fluxes of ions lead to numerous cellular effects, including changes in these resting potentials, thus triggering the generation of action potentials. Action potentials are very likely the cause of changes in the gating of voltage-sensitive channels (Grabov and Blatt 1998; 1999; Hodick and Sievers 1988; Iijima and Sibaoka 1981; Schroeder and Keller 1992; Shimmen 2006). A very good example that shows that action potentials are a consequence of mechanical stimulation and of mechanosensitive Ca^{2+}-permeable channels in particular, is the case of the green algae *Chara corallina*. When this alga undergoes strong mechanical stimulation, mechanosensitive Ca^{2+}-permeable channels trigger an immediate increase in the concentration of cytoplasmic free Ca^{2+}. This current is capable of activating Ca^{2+}-sensitive Cl^--permeable channels, generating a Cl^- outflow that depolarizes the membrane. If this depolarization is strong enough to reach a certain threshold it is propagated to the neighbouring cells (Shepherd et al. 2001).

In many plant species, such as *Arabidopsis* and *Nicotiana plumbaginifolia*, cells show rapid and transient fluctuations of intracellular Ca^{2+}, under mechanical stimulation (Allen et al. 1999; Monshausen et al. 2009; Trewavas and Knight 1994). The

amplitude and duration of this presence of free cytosolic Ca^{2+} depends both on the stimulated tissue and intensity of stimulus (Allen et al. 1999; Haley et al. 1995; Knight et al. 1991; Legue et al. 1997). It has been proposed that the elevation of free cytosolic Ca^{2+} is, in fact, a process more complex that a simple influx of Ca^{2+} across the plasma membrane of the cell. It is believed that small increases of Ca^{2+}, mediated by the plasma membrane and hardly detectable, triggers a large wave of release of internal Ca^{2+}. This model, known as Ca^{2+}-induced Ca^{2+} release (CICR), was described in plants in guard cells (Ward and Schroeder 1994). The channel responsible for this secondary release , described in red beet root cells, is the family of slow activating vacuolar (SV) channels, which are both activated by, and permeable to, Ca^{2+} (Bewell et al. 1999).

In this chapter I try to elucidate the role that the transmembrane domains of DEK1 might play in mechanosensing. I also test as well the potential role they might have in Ca^{2+} transport across the plasma membrane as the first molecular response to stretching of the plasma membrane.

6.2. dek1[CALPAIN] plants show a decreased sensitivity to mechanical stress

6.2.1. Effect on flowering time

As discussed in previous chapters, *Arabidopsis* plants are sensitive to mechanical stimulation, showing shorter petioles, later bolting and shorter bolts in mechanically stressed plants than untouched wild-type plants (Braam and Davis 1990). *dek1[CALPAIN]* mutants lack the transmembrane domain of DEK1, which we propose that could work as a mechanosensor in the plasma membrane in *Arabidopsis* cells. We believed that the absence of this structure could lead to a reduced sensitivity to externally applied mechanical stress. In order to investigate this

hypothesis, duplicate trays of wild-type and *dek1[CALPAIN]* mutant plants expressing the active CALPAIN domain at different levels were grown under long day conditions. One of the duplicate trays was mechanically stimulated twice every day for one minute applying a pressure of approximately 4 g/cm^2 on the rosettes. This mechanical stimulation treatment was begun after the emergence of the first pair of true leaves and carried out throughout the whole developmental process.

Bolting time was recorded as number of rosette leaves at the time of flowering for wild-type and two different lines of CALPAIN OE plants, *dek1-2[CALPAIN]* and *dek1-3[CALPAIN]*. Both of these lines, as introduced in chapter 4, lack the full length wild-type DEK1 protein and the resulting lethal phenotype is complemented by the expression of transgenic versions of the CALPAIN domain. In wild-type plants a statistically significant difference ($p<0.01$) in bolting time was observed. In untouched plants an average of 9.66 leaves were produced prior to bolting and in touched plants, 12.56 leaves. In the case of mutants that lack the transmembrane domain of DEK1 the variation in flowering time was not significantly different. 10.05 leaves were produced prior to flowering in untouched and 10.17 leaves for touched dek1-2[CALPAIN] plants and for dek1-3[CALPAIN] plants flowering occurred with 11.21 leaves for untouched plants and 10.67 leaves for touched plants.

We conclude from this experiment that adult plants that lack the transmembrane domain of DEK1 are less sensitive to mechanical stimulation than wild-type plants that undergo the same treatment. This lack of sensitivity is likely to be due to the lack of the transmembrane domains of DEK1, rather than to the calpain expression level, as both CALPAIN OE lines, which have differing levels of Calpain expression, show similar results (Galletti unpublished results).

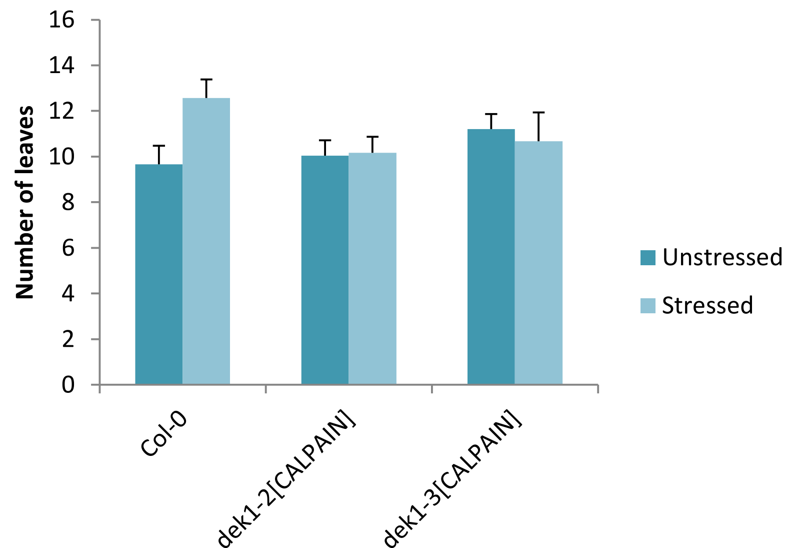


Figure 6-1 Flowering time in stressed and unstressed wild-type and in two different lines of *dek1*[CALPAIN] plants expressed as number of leaves by the time of flowering. Error bars show standard deviation. Statistical differences ($p<0.01$) where seen with a Mann-Whitney U test between wild type unstressed and stressed plants, but not in the mutants. N=15.

6.2.2. Effect on cellulose content

Cell wall components were quantified by extracting the alcohol-insoluble residue (AIR) of plants after they had been kept in the dark for 24h to eliminate starch. Cellulose was isolated from the rest of the polysaccharides in the AIR and quantified by the anthrone method as described in depth in chapter 3 (Foster et al. 2010b; Trevelyan et al. 1952).

A difference in cellulose content was observed in the wild-type plants, with or without mechanical stimulation (figure 6-2, table 6-1). A value of 0.55 µg of glucose equivalents per µg of AIR was seen in wild-type fully developed leaves whilst in mechanically stimulated plants the corresponding value was of 0.84 µg of glucose equivalents per µg of AIR. This difference was statistically significant ($p<0.1$).

In the case of *dek1-2[CALPAIN]* mutants a value of 0.69 μg of glucose equivalents per μg of AIR was measured in unstressed plants. In stressed plants the value was of 0.68 μg of glucose equivalents per μg of AIR. For *dek1-3[CALPAIN]* mutants the measured value in non-stressed plants was of 0.68 μg of glucose equivalents per μg of AIR. In mechanically stressed plants the value was of 0.67 μg of glucose equivalents per μg of AIR. Thus, unlike the situation in wild-type plants, mechanically stressing *dek1[CALPAIN]* plants results in no detectable increase in cellulose content.

Table 6-1. Quantification of the cellulose content of the cell walls of leaves of wild-type, *dek1-2[CALPAIN]* and *dek1-3[CALPAIN]* mechanically unstressed and stressed plants. Expressed in glucose equivalents per μg of AIR.

	WT	*dek1-2[CALPAIN]*	*dek1-3[CALPAIN]*
UNSTRESSED	0.55	0.69	0.68
STRESSED	0.84	0.67	0.66

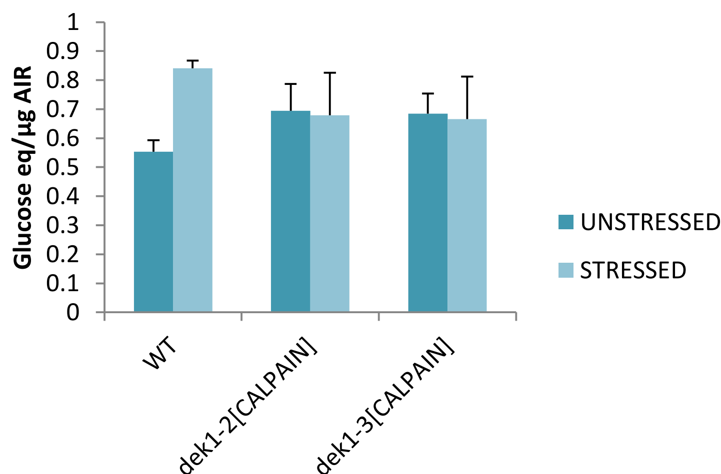


Figure 6-2 Quantification of cellulose content of cell walls of leaves of wild-type *dek1-2[CALPAIN]* and *dek1-3[CALPAIN]* mechanically unstressed and stressed wild-type plants. Error bars show standard deviation. Statistical differences ($p<0.01$) where seen with an ANOVA test between wild type unstressed and stressed plants, but not in the mutants. N=10.

6.2.3. Effect on lignin content

In other species, such as the common bean (*Phaseolus vulgaris*), when stimulated mechanically, an increase in the lignification of tissues has been recorded. This is thought to be due to an induction of enzymes such as phenylalanine ammonialyase and peroxidase, which leads to an accumulation of phenolic compounds such as lignin (Cipollini 1998; Cipollini Jr 1997).

Lignin was quantified by its auto-fluorescence after being purified using the acetyl bromide method (Foster et al. 2010a). Measurements were carried out on the base of the inflorescence stems of mechanically perturbed and non-perturbed plants. A strong increase in lignin content was observed in mechanically stimulated wild-type plants in comparison to non-stimulated individuals (figure 6-3, table 6-2). A value of 213.2 μg of lignin per cm of stem was detected in mechanically stimulated plants while non-stimulated show a value of 121.6 μg of lignin per cm of stem.

In the case of *dek1[CALPAIN]* plants the value of lignin for non-stimulated plants was of 180.6 μg of lignin per cm of stem. Stimulated plants showed a value of 203.3 μg of lignin per cm of stem. Thus, the response of *dek1[CALPAIN]* plants to mechanical stimulation in terms of lignin production was less strong than in wild-type plants, although the treatment still produced a statistically significant increase in lignin content in this background.

Table 6-2 Quantification of content of lignin in μg per cm of stem in mechanically stimulated and non-stimulated wild-type and *dek1[CALPAIN]* plants.

	WT	dek1[CALPAIN]
Unstressed	121.6	180.5
Stressed	213.1	203.3

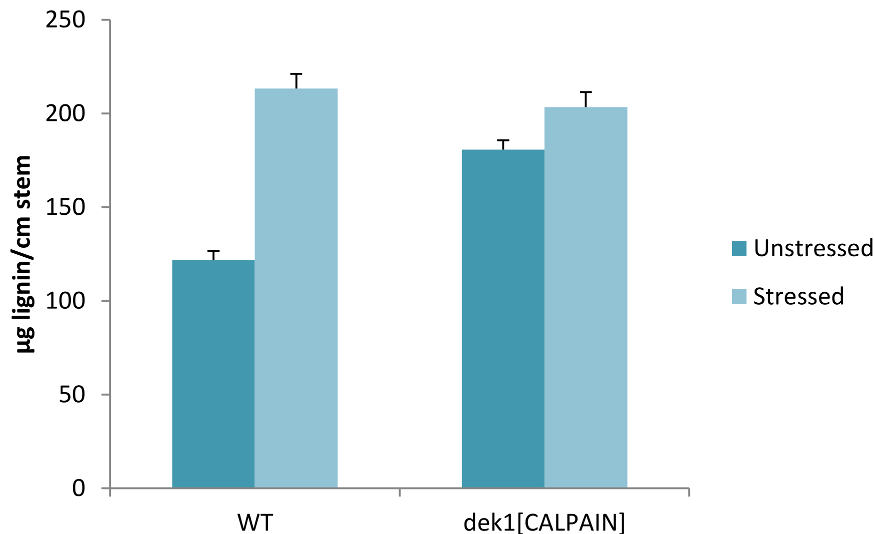


Figure 6-3 Quantification of content of lignin in mechanically stimulated and non-stimulated wild-type and *dek1[CALPAIN] Arabidopsis*. Error bars show standard deviation. Statistical differences ($p<0.01$) where seen with an ANOVA test between wild type unstressed and stressed plants, but not in the mutant. N=4.

6.2.4. Transcriptomic analysis of stressed wild-type and DEK1 mutants

In order to understand the role of the transmembrane domains of DEK1 in the perception of mechanical and osmotic stress we decided to assess the transcriptional phenotype of wild-type and *dek1[CALPAIN]* plants. We chose the same downstream targets of the CALPAIN domain as we used in other transcriptomic analysis experiments throughout the thesis. These genes are *TOD4*, which is *Expansin 11* (*AT1G20190*) and *TOD5*, which is part of the *Pectin lyase-like* superfamily (*AT2G43870*). Both of these targets show a positive correlation with the expression level of the CALPAIN domain of DEK1.

In these experiments we used the method of stressing seedlings mechanically and osmotically that was described in chapter 3. Seedlings were grown in sterile liquid

culture. Osmotic stress consisted of a shock of 300 mOsm sorbitol, and samples were collected at different time points (0, 30, 90 and 180 minutes). Plants were stimulated mechanically by applying a force to liquid culture grown seedlings using an inverted, glass bead filled, 50 ml falcon tube and samples were collected at the same time points as for the osmotic stress treatment.

In order to detect if the plants showed a transcriptional response to being stressed osmotically and mechanically, the transcript levels of the osmotic and mechanical stress marker genes *POST5* and *PTT2* were measured. The use of these genes for this purpose has been previously discussed in chapter 3. In the case of *POST5* we see that this osmotic stress target is up-regulated in response to osmotic stress but not in response to mechanical stress in wild-type plants (figure 6-4 A-B), an effect previously described in chapter 3. As previously observed, *PTT2* shows a response to both mechanical and osmotic stress (figure 6-4 C-D).

Interestingly, the responses of *dek1[CALPAIN]* plants and wild-type plants to osmotic and mechanical stress as determined by the expression of these genes appears very similar to that of wild-type, suggesting that the transmembrane domains of DEK1 are not necessary for the transcriptional responses of these genes to either osmotic or mechanical stresses.

In order to take this analysis further we analysed the expression of the CALPAIN-regulated genes *TOD4* and *TOD5*. As previously shown these genes respond to both osmotic and mechanical stress in wild-type plants, and are over-expressed in untreated CALPAIN over-expressing plants. Interestingly, in the case of these genes, *dek1[CALPAIN]* mutants that lack the transmembrane domains of DEK1 do not show coherent transcriptional changes in response to either osmotic or mechanical stress (figure 6-5 B and D). This supports the hypothesis that the transmembrane domains of DEK1 may be important for the transcriptional responses of some genes to mechanical stimulation, but not others.

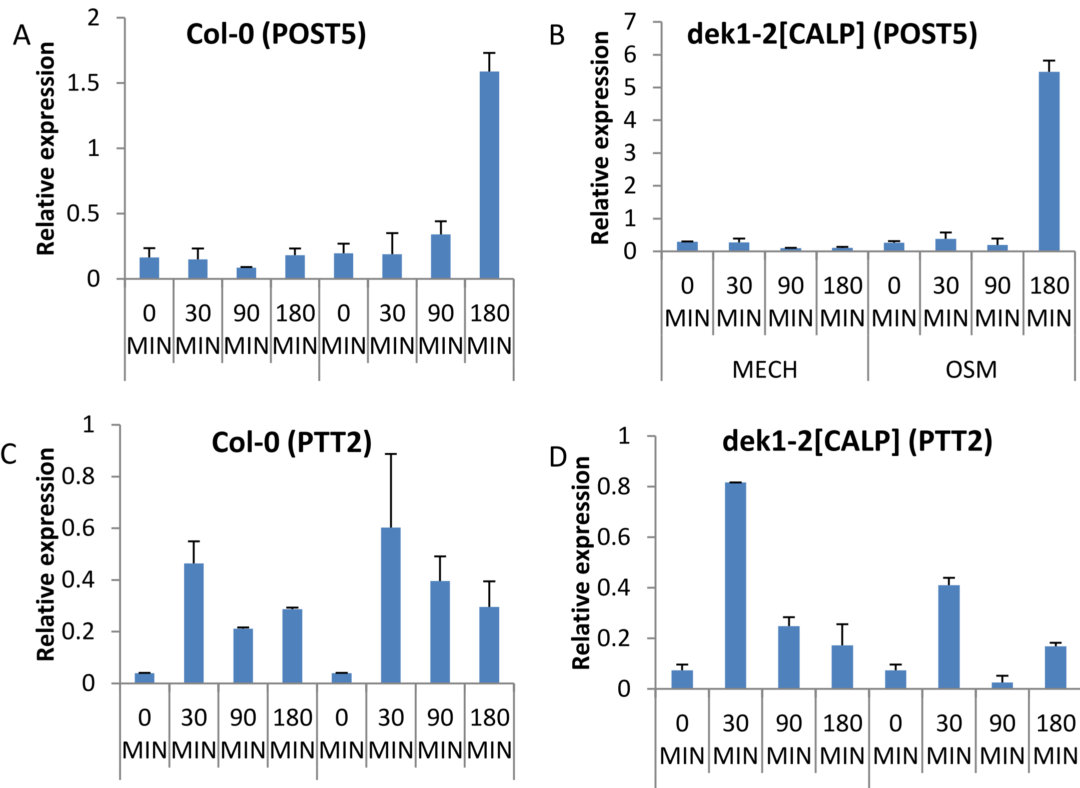


Figure 6-4 RT-PCR analysis of the expression level of *POST5* (A and B) and *PTT2* (C and D) in wild-type plants (A and C) and *dek1-2[CALPAIN]* mutants (B and D). All of them were treated with mechanical stress and an osmotic step of 300 mOsm. Samples collected at 0, 30, 90 and 180 minutes. Error bars show standard deviation between three biological replicates. ANOVA test shows a statistical significant difference between time point 180 min and all others for osmotic stress, $p<0.01$ (A-B). In C and D also shows statistical significant differences between time point 30 min and time 0 for mechanical and osmotic stress ($p<0.05$).

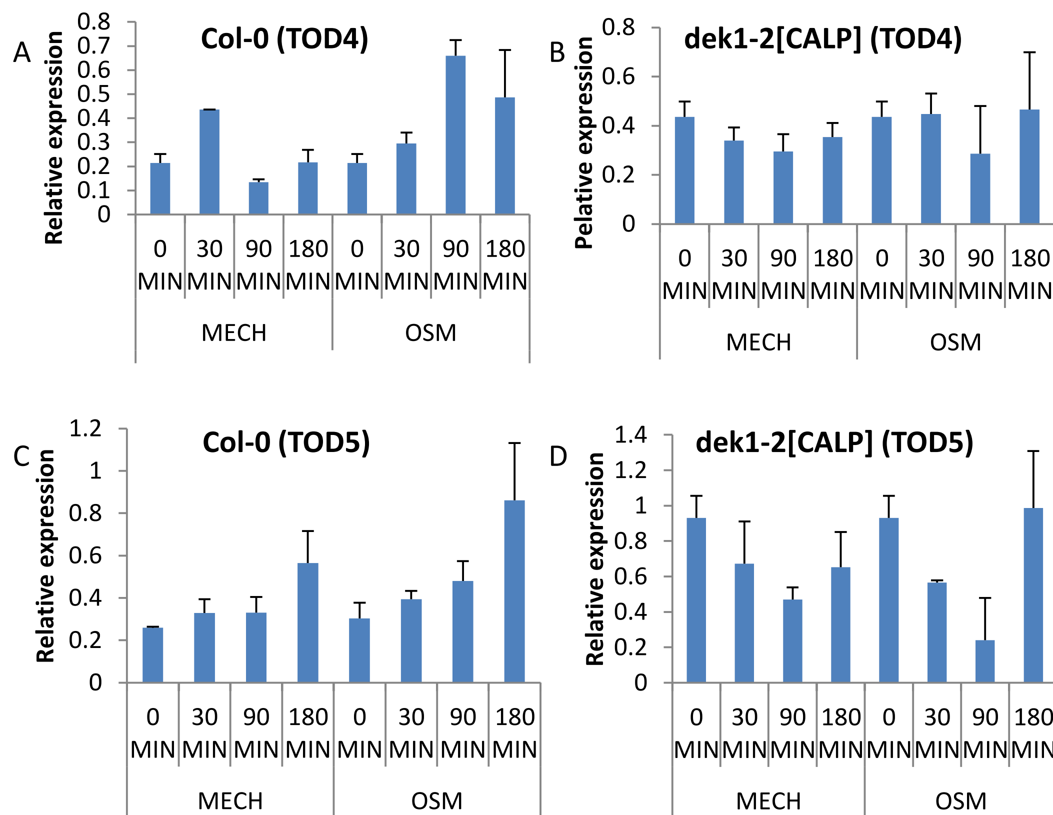


Figure 6-5 RT-PCR analysis of the expression level of *TOD4* (A and B) and *TOD5* (C and D) in wild-type plants (A and C) and *dek1-2[CALPAIN]* mutants (B and D). All of them were treated with mechanical stress and an osmotic step of 300 mOsm. Samples collected at 0, 30, 90 and 180 minutes. Error bars show standard deviation between three biological replicates. ANOVA test shows a statistical significant difference between time point 30 min for mechanical stress and 90 min for osmotic stress and time point 0 min in A ($p<0.01$), whether no statistically significant differences are seen in B. In C statistical significant difference between time point 180 min for mechanical and osmotic stress and time point 0 min in A ($p<0.05$), whether no statistically significant differences are seen in D.

6.3. Implication of DEK1 in Ca^{2+} flux through the plasma membrane

6.3.1. dek1[CALPAIN] root epidermal cells show decreased Ca^{2+} intake under osmotic stress

The activity of ionic channels within membranes can be studied thanks to a variety of electrophysiological techniques. Classical voltage clamp techniques implying the application of two or three electrodes to large plant cells, particularly giant algal cells, provided the foundations, to a large extent, of the current field of plant electrophysiology (Beilby et al. 1982; Findlay 1961; Gradmann et al. 1978; Kishimoto 1964; Lunevsky et al. 1983). Technical developments applied to voltage and patch clamp techniques have allowed more wide-ranging studies. A key advance in conventional two-electrode voltage clamp studies has been the use of double-barrelled electrodes to impale single small cells, such as those present in higher plants. This opened the possibility of performing *in planta* analysis of membrane fluxes. In this configuration, one of the barrels of the electrode is used to measure voltage while current is applied through the other barrel. One of the best examples of the use of this technique is the work carried out by Blatt and his co-workers on the stomatal guard cell (Blatt 1987; 1991; 1992; Chen et al. 2010c; Wang et al. 2012). This type of plant cell, with its dramatic ion fluxes, is probably the most intensively studied and has acted as a model for the description of higher plant cells from an electrophysiological point of view. In the studies of Blatt and co-workers, electrodes were filled with 200 mM K^+-acetate in order to minimise the effects of Cl^- leakage into the relatively small cytoplasmic volume, therefore avoiding the masking other currents with this leakage.

The double-barrelled voltage clamp method presents one major advantages over patch clamping of plant protoplasts, another extensively used technique. This is that

it can be used *in situ* on cells that are still inside their cell walls, and thus probably reflects a situation closer to physiological conditions. Nevertheless, this technique presents drawbacks. Complications are seen if the cells studied are electrically coupled to other cells, which is the case for many types of plant cells (but not stomatal guard cells). The other main disadvantages of this method is that it is a technically challenging as it is difficult to impale the cytoplasm of highly vacuolated small plant cells without causing any damage to the membranes (Brownlee 1994).

In order to test the electrophysiological consequences of loss of the DEK1 transmembrane domains, *in vivo* tests were run, measuring ion currents using electrodes implanted in cells of the root epidermis of *Arabidopsis* seedlings of wild-type and *dek1[CALPAIN]* mutant plants. The method used was an adaptation of the double barrelled electrode technique described before. All experiments were done in collaboration with Dr Zhong-Hua Chen and Yizhou Wang at Dr Michael Blatt's laboratory at the University of Glasgow.

In wild-type plants, steady-state current-voltage relations studies were carried out with and without an osmotic step. As discussed previously, osmotic steps are often considered as proxies for mechanical stimulation of cells, as the tension of the plasma membrane can be altered by variations in the osmotic pressure. We observed that in cells that underwent the osmotic step, an activation of the influx of Ca^{2+} to the cytoplasm was detectable. In cells without the osmotic step the influx of this cation is substantially smaller. This lets us confirm the existence of Ca^{2+}-permeable channels sensitive to osmotic stimuli in the plasma membrane of *Arabidopsis* root cells (Chen unpublished results; figure 6-6). These currents do not resemble fluxes previously reported from protoplasts, although the fact that our measurements were carried out *in planta*, and in electrically coupled cells, may have affected the characteristics of this flux.

It should be noted that in these experiments Ca^{2+} is substituted by Ba^{2+} in the solutions used. The reason for this is that channels with selectivity for Ca^{2+} are also permeable to Ba^{2+} which is a divalent cation with similar properties. However, the use of Ba^{2+} presents significant advantages over Ca^{2+} in electrophysiological studies. The first advantage is that it avoids the triggering of all the processes, including activation of secondary ion currents, which would be caused by increases in free cytoplasmic Ca^{2+}. The other is that this ion is capable of blocking monovalent cation channels, such as K^+ channels, therefore blocking currents that could mask the movements of divalent cations (Sabirov et al. 1997; Syeda et al. 2008).

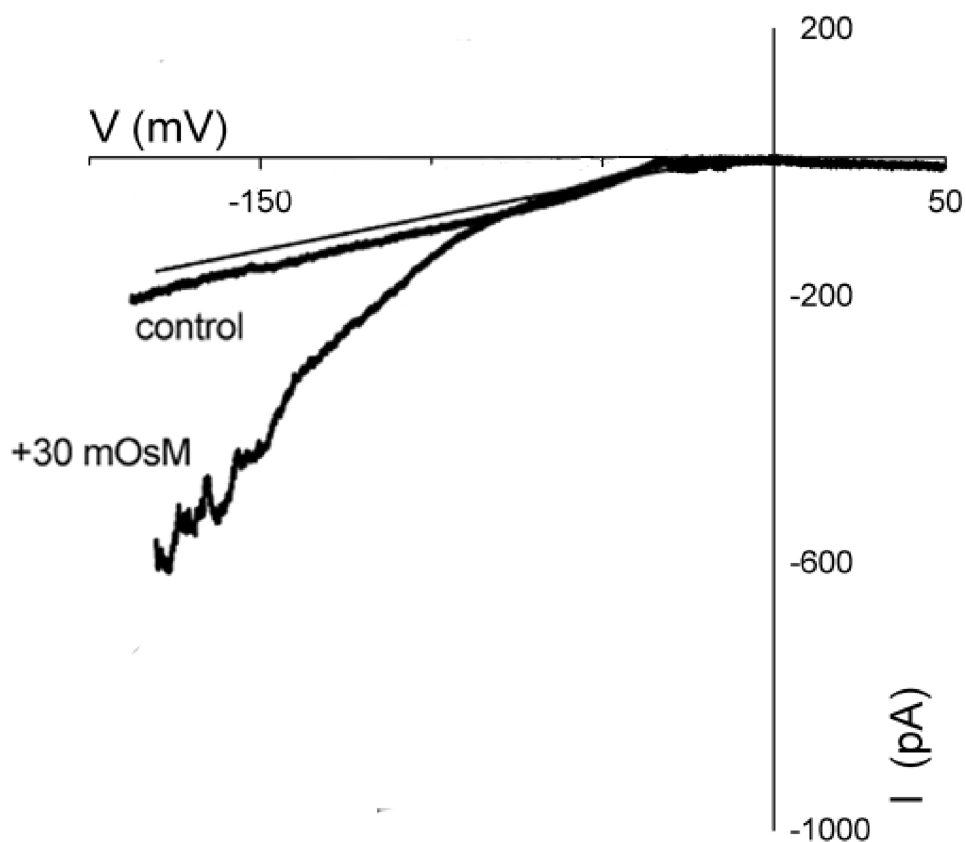
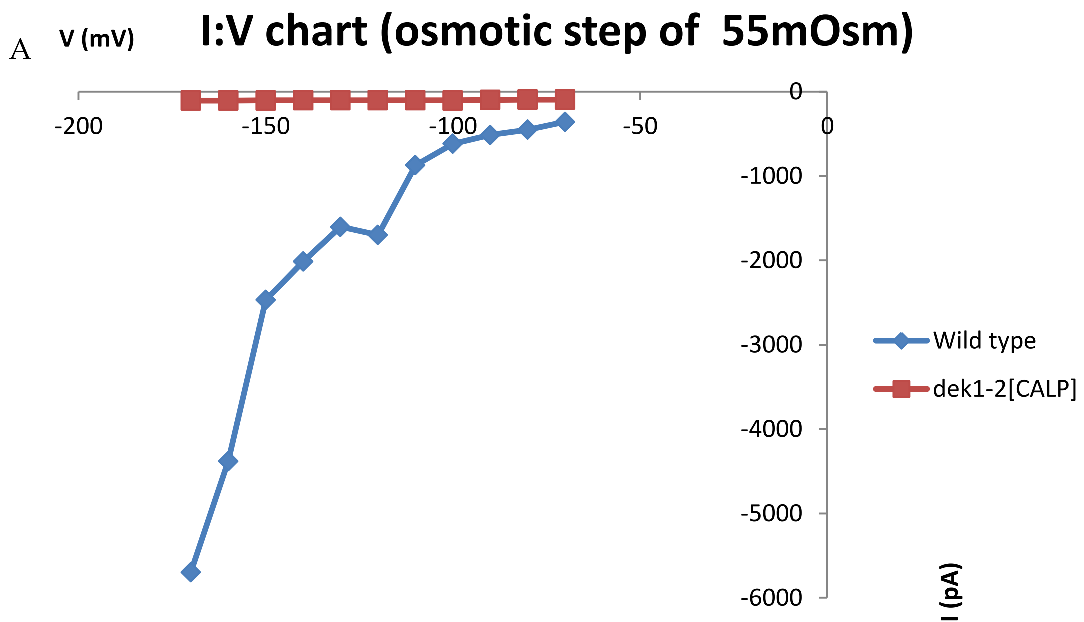


Figure 6-6 Voltage ramp and currents at iso-osmotic potential and with a 30 mOsM differential imposed between bath and pipette solutions. Measurements carried out in root epidermal cells.

I then carried out the same experiments but comparing the behaviour of wild-type plants and *dek1[CALPAIN]* mutants. Mechanical stimuli were mimicked by an osmotic step of 30 mOsM. In wild-type cells an increase in the influx of Ca^{2+} to the cytoplasm was recorded as previously described. In *dek1[CALPAIN]* mutants that lack the transmembrane domain of DEK1, the calcium influx is practically inexistent. If these currents are quantified a very significant difference is seen, with currents being more than 20 fold bigger in wild-type plants than in *dek1[CALPAIN]* mutants (figure 6-7)

My results led me to conclude that *dek1[CALPAIN]* mutants which lack the transmembrane domains of DEK1 also lack a Ca^{2+}-permeable channel activity sensitive to osmotic stimulation. It seems likely, therefore that the activity of DEK1 is closely related that of a Ca^{2+}-permeable channel, either being the channel itself or a regulator of a channel.

A



B

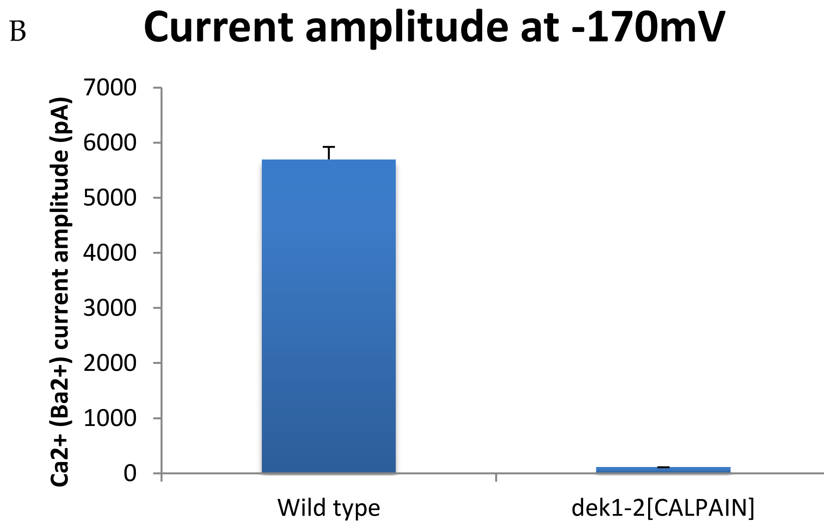


Figure 6-7 Steady-state current-voltage relations from wild-type and *dek1[CALPAIN]* complemented *Arabidopsis*. Bath and pipette solutions with 3 and 10 mM Ba^{2+}, respectively, as the charge carrying ion (A). Current amplitude means ±SE taken at -150 mV from six independent experiments in wild-type and *dek1[CALPAIN]* epidermal root cells. Error bars show standard deviation. A t-test showed a statistically significant difference ($p<0.01$) (B).

6.3.2. *Xenopus* oocytes expressing the transmembrane domains of DEK1 show Ca^{2+}-activated Cl^--conductances

Molecular cloning techniques have made possible the functional study of ion channels and transporters encoded by identified genes in a variety of heterologous systems, which should be done in order to prove a certain protein presents an ionic transport activity. This approach generally makes use of certain cell types that do not express endogenous proteins which could generate currents that could potentially confound results. The cell types used should also be responsive to genetic transfection or permit the direct injection of genetic material. For those reasons we chose *Xenopus laevis* oocytes to carry out our experiments.

The *Xenopus laevis* oocyte is one of the most commonly used cell types for electrophysiological studies. These cells are a very popular model as they present a very large size, they express and faithfully insert channel proteins in their cell membrane, and they have a relatively low number of endogenous channels which could complicate the analysis of electrophysiology measurements (Papke and Smith-Maxwell 2009). All these characteristics make them very attractive as models for electrophysiological experiments. Oocytes are the precursors of mature egg cells in amphibians. They are harvested from the ovarian lobes present in the adult female frog by survival surgery. They can be classified into several developmental stages (named stage I to stage VI), of which stages V and VI are those that can be used for electrophysiological experiments.

Xenopus oocytes were first used by Miledi and co-workers. In 1982 they used this cell type to study nicotinic acetylcholine receptors. This experiment was carried out by injecting mRNA obtained from cat muscle (Miledi et al. 1982). Within the last 20 years *Xenopus* oocytes have been used as a tool to study a large number of ion channels and receptors. In the few years following the invention of this technique

very important discoveries were made in the field of neurotransmitter receptors that were not themselves ion channels (e.g., G-protein coupled receptors). *Xenopus* oocytes contained the components needed to transduce the activation of receptors into a current mediated by Ca^{2+}-dependent Cl^- channels (Gundersen et al. 1983; Miledi and Parker 1984). In the late 1980s, cloned receptors and channels became available thanks to the developing molecular biology techniques and the *Xenopus* oocyte system became a tool of great utility. (Papke and Smith-Maxwell 2009).

Membrane proteins from yeast (Yu et al. 1989), bacteria (Calamita et al. 1995) and plants have been expressed in oocytes with full functionality, showing that there is no major kingdom or codon usage limitation for this technique. The first plant membrane proteins expressed in this system were a hexose carrier (Boorer et al. 1992) and K^+ channels (Cao et al. 1992). Since then, a large number of different types of membrane proteins have been characterised including several transporters (Miller and Zhou 2000).

In our case, we wanted to test if the transmembrane domains of DEK1 presented an ion transporter activity, by expressing the protein in *Xenopus* oocytes and characterizing their electrophysiological activity across the plasma membrane using the voltage clamp technique. In order to do this we had to clone the protein into a vector containing a promoter for vertebrates. Our first choice was to clone out DNA fragment into a Gateway® vector. For this purpose we used the vectors cDEST and NEO. This did not work as we could not obtain bacterial colonies carrying the right clone. Finally, we chose pGEMHXho, which contains a T7 promoter located between the untranslated regions of the 5′ and 3′ *Xenopus* β-globin gene (figure 6-8 A) (Zhang et al. 2011). This vector was injected as DNA into *Xenopus* oocytes, but the expression of full length DEK1 in these cells resulted in lethality before any measurement could be carried out.

To avoid this problem we decided to inject the RNA of DEK1 into oocytes and carry out the electrophysiological measurements in the following days. RNA was obtained by transcription with the transcriptase SP6 from the linearized vector pGEMXho containing the *DEK1* gene (figure 6-8 B). Several experiments involving the full length *DEK1* gene were carried out, but gave irreproducible results, probably because the gene is too long to be transcribed fully *in vitro*, resulting in the production of a variety of shorter RNAs which may be unstable. Therefore, we prepared a new clone that encoded only the transmembrane domains of DEK1 (figure 6-8 C) and which was transcribed successfully and injected into oocytes. Control oocytes were injected with water.

The transcription of *DEK1*, injection of oocytes and following electrophysiological measurements were carried out in collaboration with Dr Anne Aliénor Very in the laboratory of Dr Hervé Sentenac, INRA Montpellier. I carried out the majority of the electrophysiological measurements on the oocytes, as well as being responsible for the design and construction of all the vectors

The oocytes injected with the DEK1 RNA were incubated for 4 days and impaled with two glass electrodes. After the introduction of the electrodes into the cell the oocytes were treated with a set of solutions containing different solutes and with different osmolarities. Three different combinations of solutes were used: one was 30mM $BaCl_2$, another one 10mM $CaCl_2$/ 20mM $MgCl_2$ and the other 40mM NaCl/ 10 mM KCl/ 20.5 mM $CaCl_2$. All of them were prepared at two different osmolarities: 140 mOsm and 220 mOsm. Osmolarities were adjusted with mannitol. Control oocytes were injected with distilled water.

In the DEK1 injected oocytes treated with the high Ca^{2+} containing solutions, specific Ca^{2+}-activated Cl^- currents were seen, even though Ca^{2+} currents were not reproducibly detectable, probably they are below the sensitivity limit of the method used. These Ca^{2+}-activated Cl^- currents were not observed in water-injected oocytes.

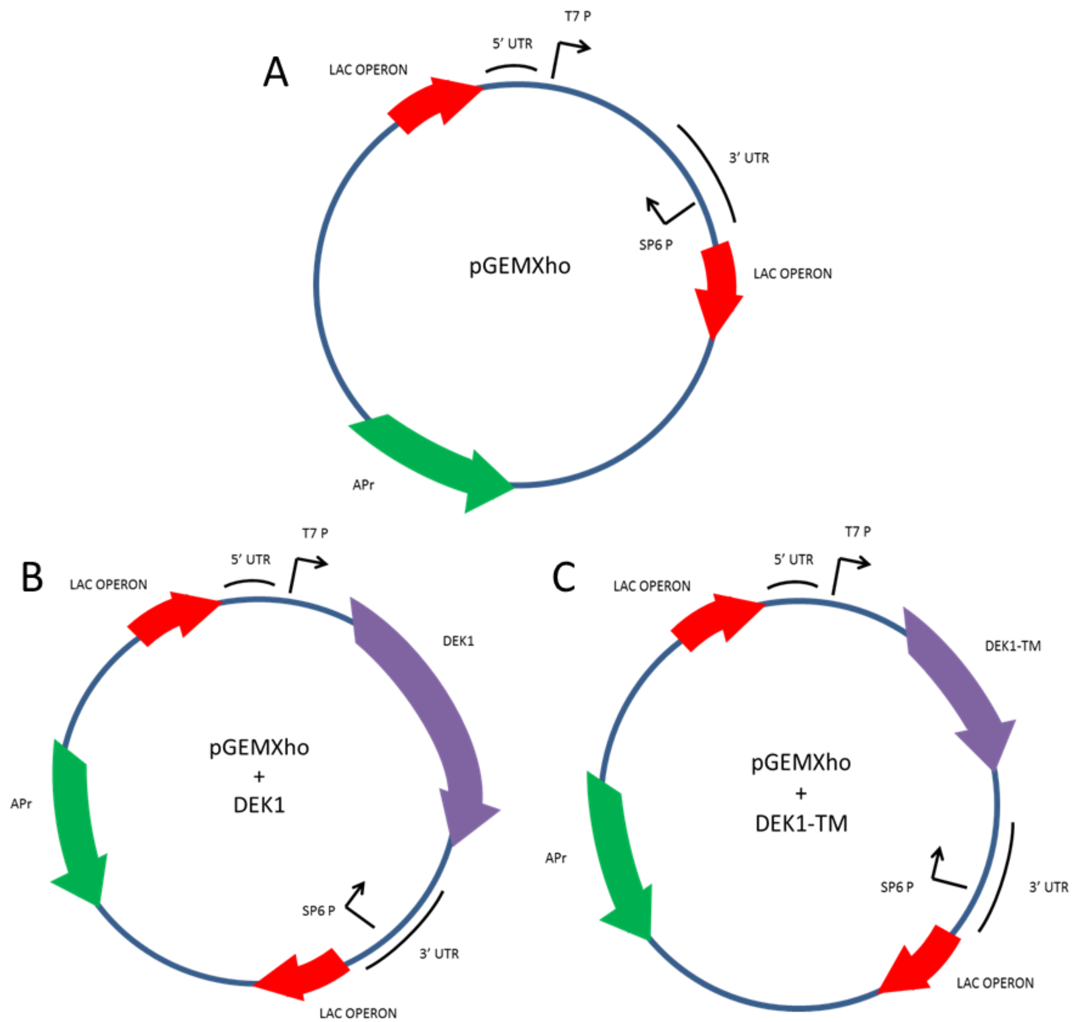


Figure 6-8 pGEMXho plasmid, Ampicillin resistant (APr) and containing the β-globin 5'UTR (5'UTR) and 3'UTR (3'UTR) (A). pGEMXho plasmid carrying full length *DEK1* (B) and the region coding for the transmembrane domains of *DEK1* (C).

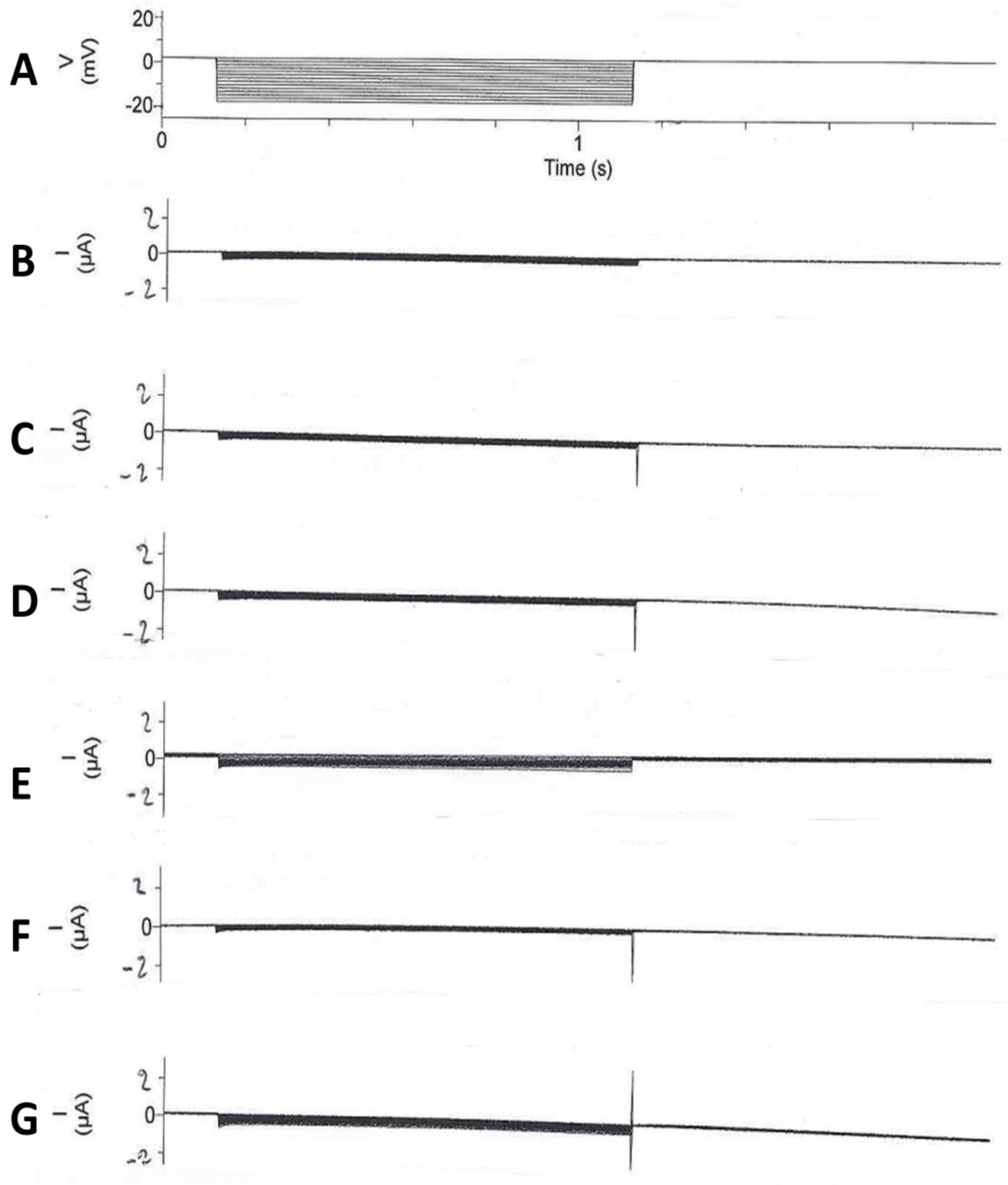


Figure 6-9 Voltages applied to oocytes injected with water (A). Whole currents recorded by voltage-clamp in oocytes undergoing a treatment of 40mM NaCl/ 10 mM KCl/ 20,5 mM $CaCl_2$ (B and E); 30mM $BaCl_2$ (C and F) or 10mM $CaCl_2$/ 20mM $MgCl_2$ (D and G). They were treated with an osmotic step of 140 mOsm (B to D) or 220 mOsm (E to G).

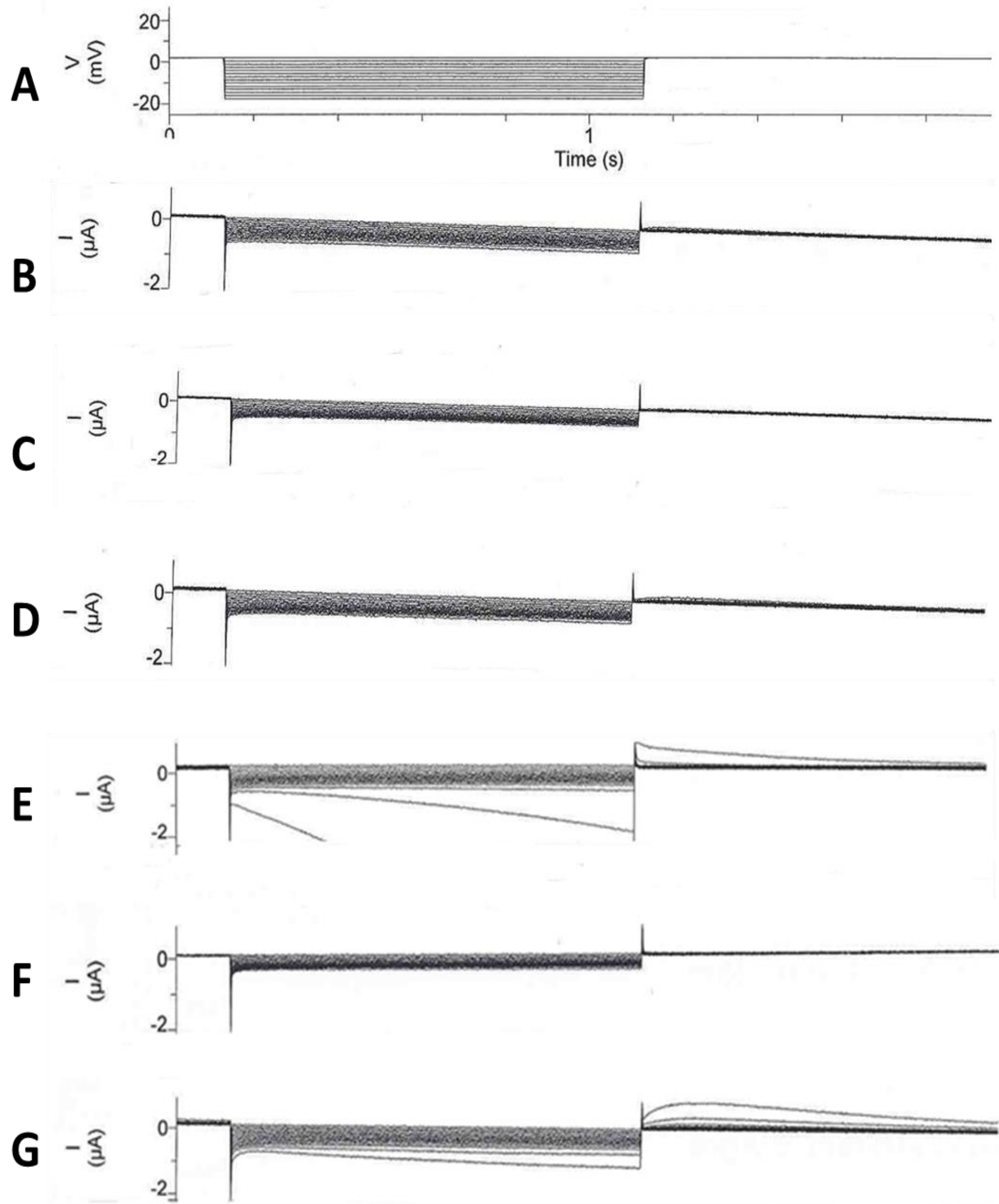


Figure 6-10 Voltages applied to oocytes injected with 30 ng of RNA coding for the transmembrane domains of DEK1 (A). Whole currents recorded by voltage-clamp in oocytes undergoing a treatment of 40mM NaCl/ 10 mM KCl/ 20,5 mM $CaCl_2$ (B and E); 30mM $BaCl_2$ (C and F) or 10mM $CaCl_2$/ 20mM $MgCl_2$ (D and G). They were treated with an osmotic step of 140 mOsm (B to D) or 220 mOsm (E to G).

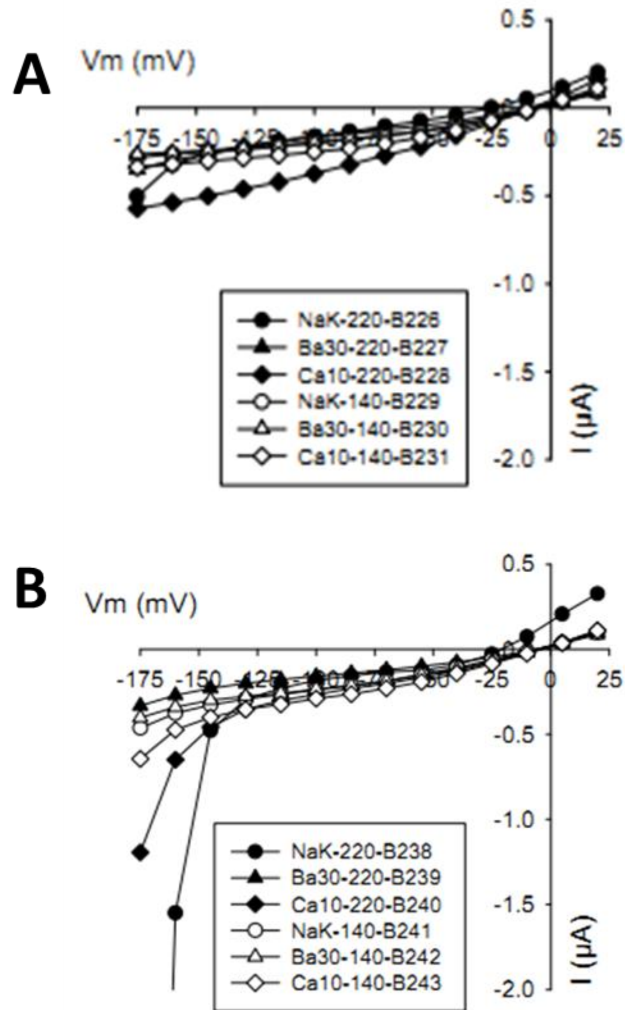


Figure 6-11 I:V (current:voltage) diagrams of oocytes injected with water (A) or with 30 ng of RNA encoding for the transmembrane domains of DEK1 (B). They were treated with 40mM NaCl/ 10 mM KCl/ 20,5 mM $CaCl_2$; 30mM $BaCl_2$ and 10mM $CaCl_2$/ 20mM $MgCl_2$ and an osmotic step of 140 mOsm or 220 mOsm.

In our experiments, changes in osmolarity did not cause changes in the Cl^- currents. This could be interpreted in several different ways. One is that Ca^{2+} currents provoked by the transmembrane domains of DEK1 give rise to a background that reaches a threshold current enough to activate completely the Cl^- channels. Another and probably more likely possibility, as the putative Ca^{2+} fluxes are small, is that the

placing of the electrodes used might cause a stretch force in the membrane that activates mechanosensitive Ca^{2+} currents, probably mediated by the transmembrane domains of DEK1.

6.4. Summary and conclusions

In animal cells it has been proposed that, rather than sensing mechanical signals directly through plasma membrane deformation, cell surface adhesion receptors mechanically couple the cytoskeleton to the extracellular matrix. Therefore, extracellular matrix receptors such as integrins and associated molecules are likely to be amongst the first molecules on the membrane to sense physical forces, and could act as primary mechanoreceptors (del Rio et al. 2009; Ingber 1991; Ingber and Jamieson 1985; Wang et al. 1993; Yonemura et al. 2010). It has also been proposed that deformation of plasma membrane proteins can directly influence biochemical activities, providing another mechanism of stress-sensing (Ingber 1997; 2006). Examples of this phenomenon include stress-activated ion channels (Ingber 2008). In other kingdoms, such as fungi, mechanosensitive ion channels have been described (Watts et al. 1998; Zhou et al. 1991) and integrin-like molecules have been predicted (Kumamoto 2008; Pelling et al. 2004).

In plants, no role for integrins or integrin-like molecules in mechanoperception has yet been proven and it has been suggested that ion channels play a major role in mechanosensing (Haswell et al. 2011; Monshausen and Gilroy 2009b). Therefore I was interested in the link between the transmembrane domains of DEK1 and their potential role as a mechanosensor. In particular I was interested in the possible link between this structure and Ca^{2+}-permeable channel activities that have been described electrophysiologically, but for which the effector molecules still remain to be determined.

I was able to observe with the experiments in this chapter that the presence of the transmembrane domains of DEK1 is important for mechanosensing at the whole plant level. I stressed plants mechanically from early in their development up to late stages in the life cycle. This allowed me to observe a major difference between the response to this type of stress in wild-type plants and mutants lacking the transmembrane domains of DEK1. While the wild-type plants presented a delay in flowering and an increase in cell wall components in response to mechanical stress, none of these effects could be seen in DEK1 transmembrane-domain-lacking-mutants when compared to the unstressed control.

The response to mechanical stress regarding flowering time has been widely described in *Arabidopsis* (Braam 2005; Braam and Davis 1990) and it is known to exist in other plant species (Chehbab et al. 2011). However, the effects of mechanical stress on cell wall composition, even though they have been described in depth for several species (Biddington 1986; Chehbab et al. 2011; Telewski and Jaffe 1986; Telewski and Pruyn 1998) were practically unknown for *Arabidopsis*, with the exception of the effects of three-point bending in stems (Paul-Victor and Rowe 2011).

I described the effects of mechanical stress on cellulose and lignin content in depth in chapter 3. I saw coherence between the results in this chapter and those in chapter 3, regarding the effect of mechanical stress on wild-type plants, but this effect was totally lost in the case of plants that lacked the transmembrane domain of DEK1. It would be very interesting, for future work, to compare the responsiveness of plants lacking the transmembrane domains of DEK1 regarding the physical properties of their organs. This could be studied using the same system proposed in chapter 3. This would allow the measurement of tissue strength, toughness and stiffness using the punch and die technique (Aranwela et al. 1999).

In addition to whole plant responses to mechanical stress I tested responses to mechanical stress at a transcriptional level. Even though not all the phenotypic responses to mechanical stress can be linked to changes in gene transcription, it has been shown that specific transcriptional phenotypes are present in mechanically stressed plants (Lee et al. 2005). Unlike whole plant responses to mechanical stress, transcriptional responses can be detected almost immediately after the stimulus is applied. Interestingly in my experiments, I showed that plants lacking the transmembrane domains of DEK1 were still able to respond normally to both mechanical and osmotic stress, as gauged by the expression of the marker genes *POST5* and *PTT2*. This is very interesting, as it may indicate that transcriptional responses to stress do not require the DEK1 transmembrane domains in the presence of the cleaved CALPAIN domain. One possible explanation for this observation is that other proteins present in the cell membrane can compensate for the role of the DEK1 transmembrane domains in transmitting mechanical stress signals to the nucleus. If this were the case then it might suggest that the lack of growth responses seen in *dek1[CALPAIN]* plants are largely attributable to cytoplasmic functions of DEK1, which could include spatially localized (at the subcellular level) growth modifications possibly mediated by microtubule regulation and/or modification of cell wall properties.

Interestingly, though mechanical and osmotic stress transcriptional reporters responded normally in *dek1[CALPAIN]* plants, genes identified as being regulated by calpain over expression, and which respond to mechanical and osmotic stress in wild-type backgrounds no longer appeared responsive to either osmotic or mechanical stress in the absence of the transmembrane domains of DEK1 . However, it should be noted that these genes are markedly over-expressed in the *dek1[CALPAIN]* backgrounds used in this study, which may mean that they have reached an expression maximum above which further stress-responsiveness is not possible. Alternatively, DEK1 could regulate gene expression via two different, as yet unknown mechanisms.

Studies carried out in *Arabidopsis* roots showed that mechanical stimulation triggers an elevation in cytosolic concentrations of calcium ion (Ca^{2+}). Changes in Ca^{2+} concentration can be triggered either by touching individual root cells, bending the whole organ or by endogenous mechanical stress, as observed during thigmotropic growth responses. In all cases the effect can be impeded using calcium channel blockers, such as lanthanum (La^{3+}) or gadolinium (Gd^{3+}), which suggests the rise of cytosolic Ca^{2+} is due to a flux from the extracellular space (Monshausen et al. 2009; Monshausen and Gilroy 2009a; Monshausen et al. 2008).

We were able to document the existence of mechanically activated Ca^{2+} currents in root epidermal cells using an *in planta* voltage-clamp technique. This technique, as previously discussed, allows the measurement of ion fluxes in close to normal physiological conditions, as it is carried out impaling single cells within intact plant tissues. In this experiment we observed that inward Ca^{2+} currents were provoked by an osmotic step, which is thought to mimic mechanical stress. This current disappeared completely in the absence of the transmembrane domains of DEK1. This is a tantalising and important result supporting the idea that transmembrane domains of DEK1 sense mechanical stress and are involve in its transduction into an ion flux. It would be of great interest to test this technique in more lines that lack the transmembrane domains of DEK1 and express the CALPAIN domain at different levels. In addition, it would be interesting to test the effects of overexpressing the CALPAIN domain of DEK1 in a wild-type background on ion fluxes. This would allow us to completely dissect the ion transport activity from the presence of the active version of this cysteine protease.

The *in planta* results suggested the possibility that the transmembrane domains of DEK1 could act as a channel themselves. In order to test this hypothesis I carried out experiments in *Xenopus* oocytes.

Even though we were not able to detect Ca^{2+} currents, we found Ca^{2+}-dependent Cl^- current activities specifically in DEK1 injected oocytes. Ca^{2+}-activated Cl^- channels (CaCCs) are one of the best described endogenous ion transporters in *Xenopus* oocytes. They are ligand-activated Cl^- channels that have the characteristic of being activated by intracellular Ca^{2+} (Weber et al. 1995). These channels have been proposed to present physiological role in the oocyte maintaining a potential of ~+20mV across the plasma membrane. This potential is known as the fertility potential and is an amphibian specific method to avoid polyspermia, the fecundation by more than one spermatozoid (Hartzell et al. 2005; Webb and Nuccitelli 1985).

An increase in cytosolic Ca^{2+} is enough to activate CaCCs. This can happen due to two different phenomena. One is the influx of Ca^{2+} into the cell and the other is its release from internal membrane compartments, such as the ER. Activation appears to be direct. This was proved by the activation of CaCCs present in isolated membrane patches of oocytes by the sole addition of Ca^{2+} (Kuruma and Hartzell 2000).

The presence of this Ca^{2+}-dependent Cl^- current has been used in published material as an indirect proof of the presence of heterologous Ca^{2+} channels in the membrane of *Xenopus* oocytes, even if their current is not detectable. One example is an experiment performed by Limon and collaborators, in which they transplanted patches of plasma membrane of human brain cells to *Xenopus* oocytes in order to measure electrophysiological activities (Limon et al. 2008).

A possible Ca^{2+} current mediated by DEK1 might not have been observed because it was too small and under the threshold of detection of the technique. A low conductance of Ca^{2+} could have several explanations. It is possible that in *Xenopus* oocytes, DEK1 lacks an interactor that helps to enhance the current. This enhancer could be the cell wall, present in the *in planta* experiments but not in the

heterologous system. The idea of the cell wall being involved in mechanosensing processes is not new. In yeast it has been proposed that it could be involved in osmosensing processes by enhancing the perception of shape changes (Hohmann 2002). In plants several plasma membrane proteins that interact with the cell wall and could be involved in the mechanosensing process have been described, such as lectin receptor kinases (Gouget et al. 2006) and wall associated kinases (Anderson et al. 2001; Kohorn 2001; Kohorn et al. 2006; Verica and He 2002). Interestingly, proteomics experiments have led to the detection of phosphorylated DEK1 peptides from the juxta- CALPAIN domain, suggesting that DEK1 activity might be regulated by phosphorylation (Kumar et al. 2010).

Another possible enhancer of the Ca^{2+} currents could be DEK1 itself through the protease activity of its CALPAIN domain. However, calpains have never been described as activators of channels. Indeed, although they have been proposed to interact with channels on several occasions, this was always described in the context of negative regulation (Abele and Yang 2012; Guttmann et al. 2001). Furthermore, initial tests with full length DEK1 in oocytes, whilst giving very irreproducible results, tended to suggest that the presence of the CALPAIN domain had a negative, rather than a positive effect on ion fluxes. However, this regulation could be easily tested by expressing the active version of the CALPAIN domain of DEK1 alongside the transmembrane domains in *Xenopus* oocytes in order to observe whether the presence of the CALPAIN domain affects the putative conductance of the transmembrane domains.

Summing up, in this chapter I was able to observe the role that the transmembrane domains of DEK1 play in mechanosensing at the phenotypic and elecrophysiological level. I also noted an interesting disparity in the fact that some transcriptional responses to mechanical and osmotic stresses were not abolished in plants lacking the DEK1 transmembrane domains. I have provided strong evidence that the transmembrane domains of DEK1 are involved in a mechanosensitive

process of ion transport, in particular the transport of Ca^{2+}. It is therefore possible that plants lacking the DEK1 transmembrane domains are impaired in local cytoplasmic responses to stress at the membrane, which can effect growth, but that other, as yet unidentified mechanosensitive channels at the membrane can compensate for the lack of the DEK1 transmembrane domains in terms of transcriptional regulation, in the presence of the cleaved CALPAIN domain.

7. Discussion

7.1. The transmembrane domains of DEK1 play a role in the process of sensing mechanical signals at a plasma membrane level

7.2. The CALPAIN domain of DEK1 has a role as effector in mechanosensing pathways

7.3. Proposed model of action of DEK1

7. Discussion

7.1. The transmembrane domains of DEK1 play a role in the process of sensing mechanical signals at a plasma membrane level

As thoroughly discussed throughout this thesis, mechanosensing processes in general, and in plants in particular, are mainly unknown. In the last few decades a number of publications have been released on this field and several mechanosensors have been identified, especially in animal cells.

In plants, several candidates have been proposed as mechanosensors. These proteins include kinases involved in sensing changes in the structure and integrity of the cell wall (Baluška et al. 2003; Boisson-Dernier et al. 2011; Hematy and Hofte 2008; Kohorn et al. 2009; Kohorn et al. 2006). However the relevance of these proteins to mechanosensing, in the strictest sense of the term, remains controversial. My results, especially those presented in chapter 6, support the idea that DEK1 is involved in a mechanosensing process. One of the main characteristics of the phenotype of mechanically stressed *Arabidopsis* is a delay in flowering time compared to non-stressed plants (Braam and Davis 1990). In my case, although I succeeded in recording this delay in flowering time in mechanically stressed wild-type plants; I could not record any statistically significant difference for this parameter in plants that lack the transmembrane domains of DEK1, between stressed plants and non-stressed controls.

I wanted to test this insensitivity using other parameters, at a molecular level. It has been described for several other species that mechanical stimulation causes changes in the composition of the cell wall (Biddington 1986; Chehbab et al. 2011; Saidi et al. 2010; Telewski and Jaffe 1986; Telewski and Pruyn 1998). This type of response had

not been characterized in *Arabidopsis*, with the sole exception of one recent publication that analyses responses to mechanical stimulation in terms of changes in lignin quantities (Paul-Victor and Rowe 2011). Therefore, in order to test the responsiveness of cell walls of mutants to mechanical stress I had to first describe the behaviour of wild-type *Arabidopsis* plants. For this reason I carried out quantifications of cellulose and lignin in stressed plants and non-stressed controls. In these experiments I was able to show that both cellulose and lignin showed quantitative differences in mechanically stressed plants.

Having established this fact, I could proceed to analyse the responses of mechanically stressed mutants lacking the transmembrane domains of DEK1, where the results were comparable to those seen for flowering time. The loss of the transmembrane domains of DEK1 correlates a loss of responsiveness to mechanical stimulation, probably due to a lack of sensitivity.

Interestingly, contrary to what I observed in whole plants, the transcriptional analysis of stressed plants showed that *dek1[CALPAIN]* mutants, lacking the transmembrane domains, were still responsive to mechanical and osmotic stress, the latter being considered as a proxy for the former. My interpretation of this is that it is that possibly there are one, or of several proteins in the plasma membrane of cells, which can compensate for the loss of the transmembrane domains of DEK1. This would point the existence of a redundancy in the mechanosensing role of DEK1 at the plasma membrane. This hypothesis could explain the fact that the CALPAIN domain alone can compensate for loss of *DEK1* function, and give rise to relatively normal looking plants. This compensation appears to act at the transcriptional level, but may be less efficient at the cytoplasmic level, explaining why plants lacking the DEK1 transmembrane appear unresponsive to mechanical stress at the level of morphology.

It has been proposed that ion channels play key roles in mechanosensing events in plants and the electrophysiological activity of some of these has been characterized (Cosgrove and Hedrich 1991; Ding and Pickard 1993; Martinac et al. 1987; Sachs 2010). In particular, mechanosensitive Ca^{2+}-permeable channels have been proposed to represent one of the main mechanosensing elements present in plant cells (Hedrich 2012; McAinsh and Pittman 2009; Monshausen and Gilroy 2009b). Even though the currents generated by these channels have been electrophysiologically described the molecules responsible for them still remain to be identified. Indeed, the identification of the molecules responsible for these currents is held, by some, to be one of the "Holy Grails" of electrophysiology.

My results strongly support the hypothesis that the transmembrane domains of DEK1 are responsible for Ca^{2+} mechanosensitive currents through the plasma membranes of cells. Our collaborators were able to identify the presence of mechanically-activated Ca^{2+} currents across the plasma membrane of root epidermal cells using voltage-clamp experiments. I was subsequently able to show, by comparing wild-type and *dek1[CALPAIN]* plants that mutants lacking the transmembrane domains of DEK1 a completely lack these currents. This strongly suggested that these currents are somehow mediated by the transmembrane domains of DEK1.

Because one possibility was that the transmembrane domains of DEK1 could act as channels themselves, as suggested by *in silico* predictions (Kumar et al. 2010), I generated constructs to express the transmembrane domains of DEK1 in a heterologous system (*Xenopus* oocytes) and, in collaboration with researchers in Montpellier, recorded the currents in these cells compared to control oocytes. Even though Ca^{2+} currents could not be measured directly, I demonstrated the activity of presence of Ca^{2+}-activated Cl^- currents (CaCCs) specifically in the oocytes expressing the DEK1 protein. These currents are an indirect indication of the presence of an elevated level of Ca^{2+} in the cytoplasm of these cells, which could be caused by the

presence of Ca^{2+} currents, which themselves are of too low magnitude to be detected in a direct manner.

One reason why the Ca^{2+} flux mediated by the DEK1 transmembrane domains might be too weak to be detected, it is that the transmembrane domains need an interactor, or modifier, in order to enhance their channel activity. This interactor could be the cell wall, as has been shown for several wall associated kinases (Anderson et al. 2001; Kohorn et al. 2006; Verica and He 2002). *In silico* analysis led to the proposal that phosphorylation could be as well responsible for regulating the activity of DEK1 (Kumar et al. 2010). Unfortunately attempts to identify interactors of the DEK1 CALPAIN domain by Yeast two hybrid approaches (this work), and interactors of either the CALPAIN domain or the full length DEK1 protein using Immunoprecipitation (Roberta Galletti unpublished results), have not yet identified a kinase as a potential interactor. Having said this, interactions of kinases with their substrates are notoriously fleeting, and are rarely detected using these approaches.

The idea that the transmembrane domains of DEK1 act as a mechanosensitive calcium channel is consistent with the observation that *dek1*[CALPAIN] plants lack mechanosensitive calcium fluxes, as I was able to observe *in planta.* However, this later finding conflicts somewhat with the hypothesis that there is redundancy within the cell for the activity of the DEK1 transmembrane domains. Taken to its logical conclusion this hypothesis would suggest that in the absence of DEK1, other mechanosensitive calcium channels should still be detected. However, although background calcium fluxes were still apparent in *dek1*[CALPAIN] plants, they were very reduced. It is however, possible that the channels giving this background flux are sufficient to permit the activity of the CALPAIN domain. Another possibility is that mechanosensitive channels permeable to other ions can cause membrane depolarization in response to mechanical stress, which is sufficient to activate voltage-gated calcium channels either in the plasma membrane or in other compartments. These types of currents have been described for several plant cell

types, but no molecules have been identified as effectors (Demidchik et al. 2002; Stoelzle et al. 2003; Very and Davies 2000). These fluxes would not have been observed under our conditions as we used a barium containing solution, which can block the activity of monovalent ion channels (Sabirov et al. 1997; Syeda et al. 2008). As a result, this question remains to be elucidated.

7.2. The CALPAIN domain of DEK1 has a putative role as effector in mechanosensing pathways

Previous studies on *At*DEK1 and its homologues in other species have been centred in on its C-terminal domain, which shows a very high homology with animal calpains (Lid et al. 2002), a class of Ca^{2+}-dependent *cys*-protease.

In our group, previous members had generated a variety of calpain overexpressing lines (Johnson et al. 2008), which show a phenotype characterized by compact rosettes, short petioles, dark colour, rumpled leaf surface (Johnson et al. 2008) and a delay in flowering time (Galletti unpublished results). Interestingly this phenotype coincides with that of mechanically stressed plants (Braam 2005; Braam and Davis 1990). This phenotype is the opposite of those associated with plants showing reduced DEK1 activity due to either co-suppression or incomplete complementation (Johnson et al. 2005), constitutive or inducible expression of artificial microRNAs targeted against *DEK1* (Galletti unpublished results), or in *dek1-4* mutants (Roeder et al. 2012).

In this project I probed more deeply the resemblance to mechanically stressed plants shown by CALPAIN OE lines, and focused on the differential deposition of cell wall components. In order to record these changes I quantified cellulose and lignin present in the cell walls of CALPAIN OE plants. Through these experiments I could see that both cell wall components showed a quantitative increase in response to

CALPAIN over-expression. I was also able to confirm that this phenotype is similar to that of mechanically stressed *Arabidopsis, as* previously discussed.

I additionally showed that CALPAIN OE plants present a thicker outer epidermal cell wall than wild-type plants. The outer epidermal cell wall is thought to be thickened in response to mechanical signals perceived during growth (Kutschera and Niklas 2007), and this phenotype is therefore consistent with our hypothesis that CALPAIN OE plants act as if they are constitutively stressed. As microtubules are responsible for laying the "tracks" needed by cellulose synthase complexes for oriented cellulose microfibril deposition (Bringmann et al. 2012a; Chan 2012; Paredez et al. 2006), I decided to study microtubule deposition in CALPAIN OE lines. With the aid of fluorescent microtubule markers, I could observe microtubule deposition in the stem apical meristem, where recent work has shown their exquisite responsiveness to mechanical forces in the cell surface, and where they have been shown to align parallel to the main axis of the force perceived (Hamant et al. 2008; Hardham et al. 1980). In my case I saw a higher degree of organization of microtubules in CALPAIN OE mutants than in wild-type plants. This could be, once again, interpreted as a higher responsiveness to mechanical stress in plants that present augmented levels of the active CALPAIN domain. Interestingly however, recent results from cell ablation studies in the meristems of CALPAIN OE plants, carried out by a project student in the lab, have shown that the microtubules of CALPAIN OE lines, although they appear very organized DO NOT realign in response to exogenous stresses. This is true both in rescued *dek1* mutant backgrounds, and wild-type backgrounds. This is intriguing in the light of measurements which I made on the cell walls of *dek1[CALPAIN]* lines which suggested that although the walls of these plants are thicker than those of wild-type, they are also softer, and possibly more disorganised. One interpretation is that deregulated CALPAIN activity may uncouple microtubule reorientation from the perception of "real" mechanical stress, and possibly also affect the microtubule-mediated regulation of cell wall deposition by microtubules. Analysis of the genetic

interactions between lines with different activities of *DEK1* and lines lacking players involved in some of these processes such as *csi1* (Gu et al. 2010) or cellulose synthesis complexes, such as *procuste1* (*prc1*) (Fagard et al. 2000) would help elucidate these possibilities.

Through transcriptional analysis of CALPAIN OE plants I could demonstrate that these plants are, as proposed, likely responding to mechanical stimulation in a constitutive fashion. I recorded the transcriptional levels of several mechanical and osmotic stress reporter genes. All of them showed an over-expression in the mutant if compared to wild-type levels.

It has to be pointed that one of the most obscure aspects of plant calpains is the identities of their direct targets. Even though I performed a yeast two-hybrid assay in order to find putative interactors of the CALPAIN domain there is still a long way to go in this direction. Results of this experiment were disappointing, but a few putative interactors of the CALPAIN domain of DEK1 were found, which remain to be tested. These include cellulose synthesising proteins and membrane channels, amongst others.

Immunoprecipitation results from other members of the laboratory (Galletti, unpublished results) are strongly indicative of interactions between DEK1 and both the microtubule and secretory networks within cells. Since both microtubules and secretion have been shown to be mechanoresponsive in plants (Hamant et al. 2008; Hardham et al. 1980; Jaffe et al. 2002), and similar interactions have been seen in animals (Farge 2011; Rauch et al. 2002; Raucher and Sheetz 1999), it appears that this type of approach may be more promising than yeast-two hybrid studies. However, it remains to be proved whether candidates identified by immunoprecipitation are direct interactors, and whether they include true targets of DEK1.

Although this discussion is centred on the possible role of the CALPAIN domain in mechanosensing is should not be forgotten that this protease potentially plays a much wider role in plant physiology.

One of the phenotypes observed in CALPAIN over-expresser lines is the presence of a thicker and visually different epicuticular wax layer. *A priori* this phenotyope cannot be related to that of mechanically stressed plants, and therefore could therefore involve pathways not related to mechanical stimulation and mechanoperception. However, it should be pointed out that cuticular changes in response to mechanical stress have not been studied, and thus a link cannot be officially excluded. This phenotype, in my opinion, deserves further study, as Cer9 is a putative proteolytic target of the CALPAIN domain.

The phytocalpain DEK1 has been proposed as a regulator of the cell cycle. As observed by Roeder and collaborators in their 2012 paper, *dek1-4* plants present an absence of giant cells in sepals. The lack of these cells is interpreted as a defect in cell cycle regulation, and in particular in the switch from proliferative to endoreduplication cell cycles, which are needed for the formation of giant cells. In the weak-allele *DEK1* allele *dek1-4*, giant cells are not found as cell cycles are completed by cell division. Again, this phenotype cannot be directly connected with mechanosensing events in plants since direct links between mechanical stresses and the regulation of cell division have not been studied. However, indirect connections are starting to become apparent. For example mechanical stress has recently been shown to regulate auxin transport (Nakayama et al. 2012), and auxin accumulation is known to influence cell proliferation (Perrot-Rechenmann 2010). In addition, Integrin-mediated perception of mechanical stresses in animals has long been known to affect proliferation (Streuli 2009). Thus, although changes in the regulation of the cell cycle associated with DEK1 activity cannot be definitively linked to mechanical signalling, a possible link cannot, to date, be excluded.

It is known that in other systems, such as animals, calpains are key regulators of cell death events. It has been shown that BAX activation, one of the early and crucial steps in apoptosis, can be activated by calpains. This activation happens as a Ca^{2+}-dependent cleavage of BAX, but has the particularity that it takes place within the mitochondria (Smith and Schnellmann 2012; Sobhan et al. 2013; Wood et al. 1998). In animals calpain-mediated apoptosis has been described to have a crucial role in neural development (Momeni 2011). Apoptosis regulation by calpains is unlikely to happen in plants for two reasons. Firstly the CALPAIN domain of DEK1 was never shown to be present in mitochondria, despite the growing number of proteomics studies carried out on this organelle. Furthermore neither CALPAIN over-expressers nor the weak *DEK1* alleles (*dek1-4*) show any apparent phenotype affecting the hypersensitive response or developmental cell death processes (Galletti personal communication).

Finally, Calpains have been described in animals as key regulators in signal transduction pathways (Sorimachi et al. 2010). For instance, mammal Calpain 1 has been described as a regulator of phosphorylation activities through the proteolysis of ezrins (McRobert et al. 2012). More classically, calpains have been described as regulators of tumor supressors such as p53 (Gonen et al. 1997), of phospholipid-dependent kinases (Kishimoto et al. 1983) or of cytokine receptors (Noguchi et al. 1997). These processes represent only a small fraction of those in which calpains have been implicated in animals. Although some animal calpain substrates have no obvious homologues in plants, many are, at least partially conserved; although whether they are *bona fide* substrates for DEK1 activity remains to be tested. At another level the results in this study show that DEK1 regulates calcium influx into plant cells. Calcium regulates innumerable signalling pathways in plant cells, as extensively discussed in the introduction to this thesis. Thus, although I believe that role of DEK1 activity in mechanoperception investigated in this thesis is real; it seems more than possible that this is only one of multiple signalling pathways in which phytocalpains could potentially participate.

7.3. Proposed model of action of DEK1

As a summary for my thesis I am now in a position to propose a mechanism of action mechanism for the *Arabidopsis thaliana* DEK1 protein.

I have provided evidence that the transmembrane domains of DEK1, present in the plasma membrane, act as a mechanosensor. In particular, they appear to control, or form, a mechanosensitive (stretch activated) Ca^{2+}-permeable channel. Therefore, in the event of presence of tension in the membrane this structure would open to allow a local Ca^{2+} flux into the cell. Once inside the cell, I propose, based on parallels with animal systems, that free Ca^{2+} would activate the CALPAIN domain, triggering an autolytic cleavage event, and subsequently permitting the action of calpain on targets that remain to be described (figure 7-1). Based on animal systems it seems likely that the activity of CALPAIN, even after autolytic cleavage, requires elevated cytoplasmic calcium, explaining the maintenance of transcriptional responses to mechanical stress in CALPAIN over expressing plants.

This would not be the first described case of a calpain activated by the activity of a mechanosensitive Ca^{2+}-channel. A similar case, necessary for the normal development of *Xenopus* nervous system has recently been described (Kerstein et al. 2013). It is therefore possible that calpains form part of mechanosensitive cascades in many other organisms. The major difference and apparently plant-specific innovation of DEK1 is that the CALPAIN domain (effector) is covalently associated with its proposed activator (the transmembrane domains). One possibility is that, in plant cells, where in most cases the cytoplasm is present as a thin layer covering a large vacuole, the physical association of the mechanosensor and the effector permits accurate and finely spatially controlled local subcellular responses to mechanical stress, without necessitating massive calcium influx into the cell cytoplasm. The loss of this coupling, and thus of the subcellular localization of responses in *dek1*[CALPAIN], or CALPAIN OE plants may explain many of the

observed differences in development, in these lines, including changes in the organization of cell walls.

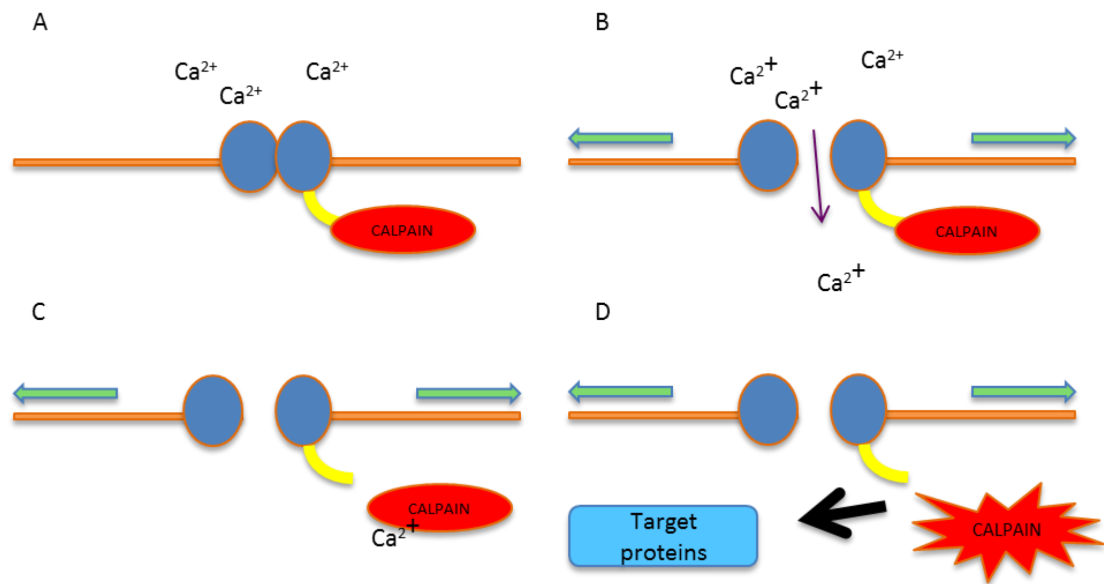


Figure 7-1 Inactive DEK1 (A). When tension is sensed in the membrane the transmembrane domain opens generating a Ca^{2+} influx into the cell (B). Ca^{2+} binds to the calpain (intracellular) domain, which is activated, cleaved and released into the cytoplasm (C). The active CALPAIN domain acts on unknown target proteins (D).

In addition to the need to identify the substrates of DEK1, further studies are required to elucidate the requirements for calcium binding, and potentially for phospholipid association in the regulation of CALPAIN activity. To this end, current experiments within the laboratory are focussed on the analysis of the activity of the CALPAIN domain, in vitro, as well as understanding in more detail the behaviour of the DEK1 protein *in planta*, including the continuation of studies, which I initiated but was unable to complete, regarding changes in sub-cellular localization of the CALPAIN domain of DEK1 in response to mechanical and osmotic stress.

8. References

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