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Molecular basis for regulation of cell wall pH in *Arabidopsis thaliana*

Molekulární mechanismy regulace pH buněčných stěn v modelové rostlině *Arabidopsis thaliana*

Bachelor´s thesis

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Declaration

I declare that I carried out this bachelor thesis independently, and only with the cited sources, literature and other professional sources. I declare that this thesis has not been used to gain any other academic title.

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Abstract:

The cell wall is one of the defining parts of the plant cell. Cell walls resist turgor pressure, give plants their rigidity and still allow the cell to grow despite their stiffness. The plant cell wall is a composite material that consists of different elements; thus, the knowledge of the plant cell wall composition helps us understand the impact of the mentioned pH changes. Alterations of plant cell wall properties result in a change of plant cell growth rate. A prime example of this is the modification of wall properties by pH change, termed the acid growth. Although acid growth has been studied for a long time, we still do not fully understand the underlying mechanisms, as we have not yet identified all the agents involved in acid growth and our ability to determine apoplastic pH is limited. Local cell wall pH fluctuations arise also due to plant organs having different roles and being affected by a diverse range of stimuli. Despite the fact that new pH measurement techniques, such as genetically encoded fluorescent probes, were developed in the last two decades, there is still a need to provide a higher spatiotemporal resolution of pH analysis.

Key words: *Arabidopsis*, apoplastic pH, cell growth, acid growth theory, cell wall, auxin, apoplastic pH measurement

Abstrakt:

Stěny rostlinných buněk jsou jednou z definujících částí rostlinné buňky. Vyznačují se schopností odolávat tlaku turgoru, dávat rostlinám jejich tuhost a zároveň dovolit rostlinným buňkám růst. Rostlinná buněčná stěna je kompozitní materiál, který se skládá z různých prvků, a proto nám znalost složení rostlinných buněčných stěn pomáhá pochopit dopad zmíněných změn pH. Změny vlastností buněčných stěn rostlin vedou ke změně rychlosti růstu rostlinných buněk. Hlavním příkladem změn vlastností stěny vyvolanými změnou pH je kyselý růst. Ačkoliv byl kyselý růst dlouhodobě studován, stále ještě zcela nerozumíme jeho mechanismům, protože jsme zatím neidentifikovali všechny činitele podílející se na kyselém růstu a naše schopnosti určovat pH apoplastu jsou omezené. Lokální fluktuace pH buněčné stěny vznikají v důsledku toho, že rostlinné orgány mají různé role a jsou ovlivňovány různorodými podněty. Navzdory skutečnosti, že v posledních dvou desetiletích byly vyvinuty nové techniky měření pH, jako jsou například geneticky kódované fluorescenční sondy, stále existuje potřeba vyššího časového a prostorového rozlišení analýzy pH.

Klíčová slova: *Arabidopsis*, pH apoplastu, buněčný růst, teorie kyselého růstu, buněčná stěna, auxin, měření pH apoplastu

Contents:

List of used abbreviations

ABA	Abscisic acid
ABP1	AUXIN BINDING PROTEIN 1
BL	Brassinosteroid
BLUS1	BLUE LIGHT SIGNALING1
BRI1	BRASSINOSTEROID INSENSITIVE 1
CesA	Cellulose synthetase protein gene
CMF	Cellulose microfibril
COR	Cordycepin
EGFP	Enhanced Green Fluorescent Protein
FC	Fusicoccin
GFP	Green Fluorescent Protein
HDA19	HISTONE DEACETYLASE19
HPTS	8-hydroxypyrene-1,3,6-trisulfonic acid trisodium salt
CHX	Cycloheximide
IAA	Indole-3-acetic acid
mRFP	monomeric Red Fluorescent Protein
PME	pectin methylesterase
PMEI	pectin methylesterase inhibitor
PP1	PROTEIN PHOSPHATASE 1
RALF	Rapid alkalization factor
SAUR	SMALL AUXIN UP-RNA
TIR1/AFB-Aux/IAA	TRANSPORT INHIBITOR RESPONSE1/AUXIN SIGNALING F-BOX–AUXIN/INDOLE-3-ACETIC ACID
TPL	TOPLESS
XG	Xyloglucan

1. Introduction

Plant physiology and development is highly influenced by the plant cell wall. Without plant cell walls, plants, as such, would not exist. The attributes of plant cell walls can be altered by changes in their pH. The understanding of the plant cell wall composition greatly helps with the comprehension of cell wall pH effects on its individual components. The plant hormone – auxin – induces shifts in apoplastic pH. These pH modifications lead to changes in cell growth rate. Auxin-induced changes of apoplastic pH that induce growth are commonly referred to as acid growth. Throughout the decades of acid growth and cell wall pH research, key players for apoplastic pH changes were identified. As the research of cell wall pH progressed, so did the pH measurement and detection techniques – from simple dyes through electrodes to genetically-encoded sensors. These tools have proven to be irreplaceable in the research of apoplastic pH.

In my thesis, I focus on apoplastic pH changes during plant cell growth and auxin induction of growth. I am going to discuss key players of apoplastic pH shifts such as AHA ATPases, auxin signalling, the effect of fusicoccin, SAUR proteins and RALF peptides. I have included a chapter about cell walls, as the understanding of plant cell wall composition greatly helps with the comprehension of the effects of pH alterations on its individual components, eventually creating a bigger picture. I have also incorporated a chapter about pH measuring and visualisation as the methods described provide the information necessary for the research of apoplastic pH. Future advancements of these methods have a great potential to advance our understanding of pH changes. The focal point of my last chapter are organ specific pH changes which include blue light stimulation, sugar accumulation and gravistimulation.

2. Cell wall

The plasma membrane of the plant cell is surrounded by a cell wall which together with turgor enables the plant to take form, shape and grow. Because of the support of cell walls, plants can grow great heights without collapsing (Braidwood, Breuer and Sugimoto, 2014). Cell walls also provide an important surface for interactions between the cells, defence responses against potential pathogens (Keegstra, 2010).

The cell wall essentially controls the cell growth because it counterbalances the turgor pressure and thus preventing the cell from bursting. Cell growth is essential for plant morphogenesis - formation of tissues and organs. The ability of plant cells to grown and shape are gained thanks to turgor pressure, the cell wall and its dynamics during cell growth and division (Wolf, Hématy and Höfte, 2012).

2.1 Composition

Plant cell walls are largely diverse in their composition. These differences exist not only among species, but even within individual domains of the same cell wall (Wolf, Hématy and Höfte, 2012).

There are two major types of cell walls - primary and secondary. The primary cell walls surround plant cells which are still growing, while the secondary cell walls are formed after the cell ceased growing (Keegstra, 2010). The composition of the secondary cell wall is linked to the specialized function of cells such as transport tissues or defence plant structures (Taiz and Zeiger, 2002). In this thesis, I will focus mainly on the characteristics of the primary cell wall.

2.1.1 Primary cell wall building blocks

Cellulose is a homopolysaccharide consisting of β-1,4 linked glucan chains (Li *et al.*, 2014). Cellulose chains are approximately 30 – 100 molecules long (Heredia, Jiménez and Guillén, 1995). Cellulose forms crystalline microfibrils (CMFs) which are key components of the cell wall (Braidwood, Breuer and Sugimoto, 2014). Cellulose is produced at the plasma membrane by multimeric complexes known as cellulose synthetase complex forming a characteristic six-fold symmetry called rosette (Mueller and Brown, 1980). The *Arabidopsis* family of cellulose synthase proteins (CESA) consists of 10 proteins (Richmond, 2000). Current evidence suggests that cellulose synthetase complexes of the primary and secondary cell wall are composed of three CESA proteins – CESA 1, CESA 3 and CESA 6 in the primary cell wall and CESA 4, CESA 7 and CESA 8 in the secondary cell wall (Desprez *et al.*, 2007; Persson *et al.*, 2007). The ratio of CESA proteins is 1:1:1 in both cases (Gonneau *et al.*, 2014; Hill, Hammudi and Tien, 2014). Cellulose contains both hydrophobic and hydrophilic regions defined by the presence of $-OH^-$ and -CH groups (Cosgrove, 2018).

Callose is a biopolymer comprising of glucose residues linked by β-1,3-linkages with some β-1,6-branches (Chen and Kim, 2009). In contrast to cellulose, callose is less abundant. Higher amounts of callose are produced in certain cell walls, such as the walls of a growing pollen tube (Schneider *et al.*, 2016).

Pectins are diverse polysaccharides of the cell wall – they contain galacturonic acid and are presumably synthetized in the Golgi apparatus (Harholt, Suttangkakul and Vibe Scheller, 2010). Their prime example is homogalacturonan. These polysaccharides form a component of the matrix that is cross-linked by bonds between the polysaccharides of the cell wall (Caffall and Mohnen, 2009; Braidwood, Breuer and Sugimoto, 2014). Homogalacturonans are transported to the cell wall in their highly esterified forms and they are selectively de-esterified by pectin methylesterases (PMEs), reviewed in Peaucelle, Braybrook and Höfte (2012). Ions like Ca2+ and B3+ between pectin chains strengthen their bond, therefore they stiffen the cell wall (Shi et al., 2017). Because of these links and their structure, they not only contribute to the strength of the cell wall, but also to its flexibility and functionality (Caffall and Mohnen, 2009).

Hemicelluloses are plant cell polysaccharides which are usually defined as having β-1-4 linked glucose backbones (Scheller and Ulvskov, 2010). Hemicelluloses include xylans, xyloglucans (XGs), mannans and glucomannans. These polysaccharides (mainly XGs) interact extensively with pectins. XG-cellulose interactions are scarce and limited to the cellulose microfibril surface (Dick-Pérez *et al.*, 2011). The above mentioned backbones of xylans can be decorated with α-1,2-linked glucuronic acid, reviewed in (Pauly *et al.*, 2013). This is not the only modification hemicelluloses can undergo, as both xylans and XGs can be acetylated, resulting in altered cross-linking capacity of other cell components (Zhang *et al.*, 2017).

2.1.2 Plant cell wall structure model

The main components of the primary cell wall are well known (Burton, Gidley and Fincher, 2010), but we lack knowledge regarding their interactions. Current views on the cell wall structure suggest that the plant cell wall is an assembly of polymers which interact with each other primarily through non-covalent bonds, forming a strong structure (Cosgrove, 2014). The main evidence for the existence of these bonds are chromatographic analyses of cell wall components, in which cross-links may be found (Cornuault *et al.*, 2015; Cornuault, Posé and Knox, 2018). For instance, the binding of mucilage of *Arabidopsis* seeds through pectin-xylan cellulose binding is an example of these cross-links (Voiniciuc *et al.*, 2015; Ralet *et al.*, 2016). Current evidence suggests that cellulose forms ordered (crystalline) and disordered regions, while the precise location of the disordered regions is unknown (Jarvis, 2018). It is proposed that xylans bind to the hydrophilic parts of the CMFs (Simmons *et al.*, 2016), while the site

CMF-CMF interactions is argued about (Ding, Zhao and Zeng, 2014; Zhang, Zheng and Cosgrove, 2016). XG deficient mutants prove that XG is needed for correct disturbance of CMF-CMF interactions (Xiao *et al.*, 2016). Pectins, as suggested by the studies of Jarvis, (1992) and Zhang, Zheng and Cosgrove, (2016) seem to fill interlamellar spaces while extensively interacting with cellulose (Wang *et al.*, 2015; Phyo *et al.*, 2017).

2.1.3 Cell wall modification proteins

In order for the cell to grow in the desired manner control over cell wall modifying agents must be maintained. For this, plants maintain control over the cell wall by altering its composition. These post-depositional modifications are what allows plants to react to new stimuli. The most notable cell wall modification proteins are expansins and pectin methylesterases (Braidwood, Breuer and Sugimoto, 2014).

Expansins can induce growth and tissue expansion both *in vitro* and *in vivo* (McQueen-Mason *et al.*, 1993; Cosgrove and Durachko, 1994; Fleming *et al.*, 1997). Expansins were first identified as "wall loosening proteins" in cucumber hypocotyls nearly three decades ago (McQueen-Mason, 1992). Expansins are small extracellular proteins with no apparent enzymatic activity that disrupt the non-covalent interactions between the polysaccharides of the cell wall. As a consequence, the cell wall loosens and its extensibility is increased, allowing cell growth (Braidwood, Breuer and Sugimoto, 2014). They have been shown to be effective in monocots as well as in dicots (McQueen-Mason, 1992).

In *A. thaliana*, thirty-six different expansin genes were identified (Cosgrove, 2015). These sequences have been divided into four subfamilies, α-, β-, expansin-like A and expansin-like B (Cosgrove, 2015). The α-expansin family of *A. thaliana* and consists of twenty-six genes (Cosgrove, 2015). They highly resemble the sequences of the first cloned cucumber expansins that were mentioned (Li, Jones and McQueen-Mason, 2003). β-expansins are a subfamily which consists of six genes. These have some conserved features from the α subfamily, and include group-1 pollen allergens (Sampedro *et al.*, 2015). The function of the last two subfamilies, expansin-like A with three genes and expansin-like B with one gene in *A. thaliana*, is unknown (Cosgrove, 2015).

Another example of cell wall modifying proteins are the pectin methylesterases (PMEs). PMEs, as their name suggests, catalyse the removal of methyl esters from pectin. The removal of methyl esters leaves negatively charged pectin chains, reviewed in (Braidwood, Breuer and Sugimoto, 2014). These pectin chains are then cross-linked and calcium ions are chelated between them. This is called the "egg-box" model (Morris *et al.*, 1982). Although PMEs degrade pectin, which would result in the extensibility of the cell wall, they do this in a

particular manner – either linearly on a chain or randomly on a group of chains, reviewed in (Braidwood, Breuer and Sugimoto, 2014). The involvement of PMEs has been suggested in growing and emerging new organs, reviewed in (Braidwood, Breuer and Sugimoto, 2014).

The activity of PMEs and pectin methylesterase inhibitors is highly regulated. The organ specificity of two PMEIs seem to be regulated at a translational level by alternative splicing (Rocchi *et al.*, 2012). Type-I pectin methylesterase inhibitors can be posttranslationally modified during their maturation (Wolf, Rausch and Greiner, 2009). Subtilisin-serine proteases, such as Subtilisin-like protease SBT3.5, which is produced alongside PME 17, seem to cleave off the PRO region (Wolf, Rausch and Greiner, 2009; Sénéchal *et al.*, 2014). A RING E3 ubiquitin ligase called FLYING SAUCER 1 was suggested to have a role in the recyclation of pectin methylesterase enzymes in the endomembrane system of seed coat epidermal cells (Voiniciuc *et al.*, 2013).

PME activity is highly pH dependant, as the de-methylesterification patterns made by them vary depending on the environmental pH, while different isoforms of PMEs have different pH optimum (Catoire *et al.*, 1998; Denès *et al.*, 2000). The activity of PMEs is increased by cell wall cations. This activity enhancement depends on the type of cations, as well as their concentration (Moustacas *et al.*, 1991; Schmohl *et al.*, 2000).

2.2 Plant cell wall growth

The plant cells need to take on many different shapes in order to fulfil their function. This is why most of the growth is anisotropic. Anisotropic growth can be classified into two main growth modes - diffuse growth and tip growth (Braidwood, Breuer and Sugimoto, 2014).

These two types of growth differ. Diffuse growth happens over the majority of the cell, while tip growth defined by a local growth, like in the case of pollen tubes (Braidwood, Breuer and Sugimoto, 2014).

2.2.1 Turgor

Turgor, alongside the properties of the cell wall, plays a crucial role in cell growth. Turgor is the pressure produced by water inside the cell. The origin of water is extracellular. The influx of water is controlled by osmosis.

The turgor of a typical plant cell ranges from 0.3 to 1.0 Mpa (Wei, 2002) - the pressure the plant cell wall has to equilibrate in order to maintain its shape and size. At equilibrium, a turgid cell balances this inner pressure by stress within the cell wall. This produces a zero-water potential difference.

The first equation to describe diffuse growth was the Lockhart equation (Lockhart, 1965). Here is a simplified version of the equation adapted from Braidwood, Breuer and Sugimoto (2014):

$$\frac{dV}{dt} = \theta(P - Y)$$

Where $\frac{dV}{dt}$ is the rate of cell volume increase, θ is the irreversible wall extensibility, P is pressure and Y is the yield threshold. The yield threshold is a point where the growth of the cell wall begins due to increased turgor pressure (P). The irreversible wall extensibility tells us how much will the wall grow as it describes its stiffness. The variables described above greatly depend on the composition of the cell wall and their relations are currently not understood well according to Braidwood, Breuer and Sugimoto (2014). This equation has many variations that include different factors; however, this simplistic form captures the core idea of diffuse growth.

This simple equation raises an important question – does the cell wall grow first and the water pressure follows or does the pressure increase first and through this it expands the cell wall?

2.2.2 "Water first" theory

An example of a "water first" theory is the loss of stability theory proposed in Wei and Lintilhac (2003) and further expanded upon in Wei, Lintilhac and Lintilhac (2006) and Wei and Lintilhac (2007). The loss of stability theory states that growing cells have walls with a steadily rising turgor. The turgor grows until the critical point is reached. This critical point is defined by cell geometry and properties of the wall. When the critical point is reached, the cell wall yields to the pressure resulting in an immediate decrease of turgor and slight extension of the wall in its weakest point (Wei and Lintilhac, 2003). However, this model has been opposed by Schopfer (2008), stating that in order to generate water influx, the turgor has to decrease first, which is exactly the opposite of what the loss of stability theory predicts as it states that growth occurs when the critical point of turgor pressure is reached. The other weak point of this theory is discussed in Geitmann and Ortega (2009), stating that the loss of stability model cannot predict the where and how much will the cell wall yield to the pressure after reaching the critical point.

2.2.3 "Cell wall first" theory

On the other hand, there is the "cell wall first" category of theories. One of these theories focuses on the chemistry of calcium and pectins. Based on the research of (Proseus and Boyer, 2005, 2006a, 2006b, 2007), the theory states that the growth rate of the cell wall is governed by the rate of unsubstituted polygalacturonic acid supplied to the cell wall and the chemistry of

calcium pectates (Geitmann and Ortega, 2009). Calcium seems to be one of the main keys to the loosening of the cell wall alongside with magnesium, as these ions are crucial in cell wall integrity and hardening. It has been shown in Proseus and J. S. Boyer (2006a), that a massive removal of calcium from the cell wall coupled with a total removal of magnesium ions results in bursting of the cell wall. According to the findings in Proseus and Boyer (2007) and Kroeger, Zerzour and Geitmann (2011) stress or tension is needed in order to control the cell growth. This has been modelled on the growing tip of a pollen tube (Fig.1).

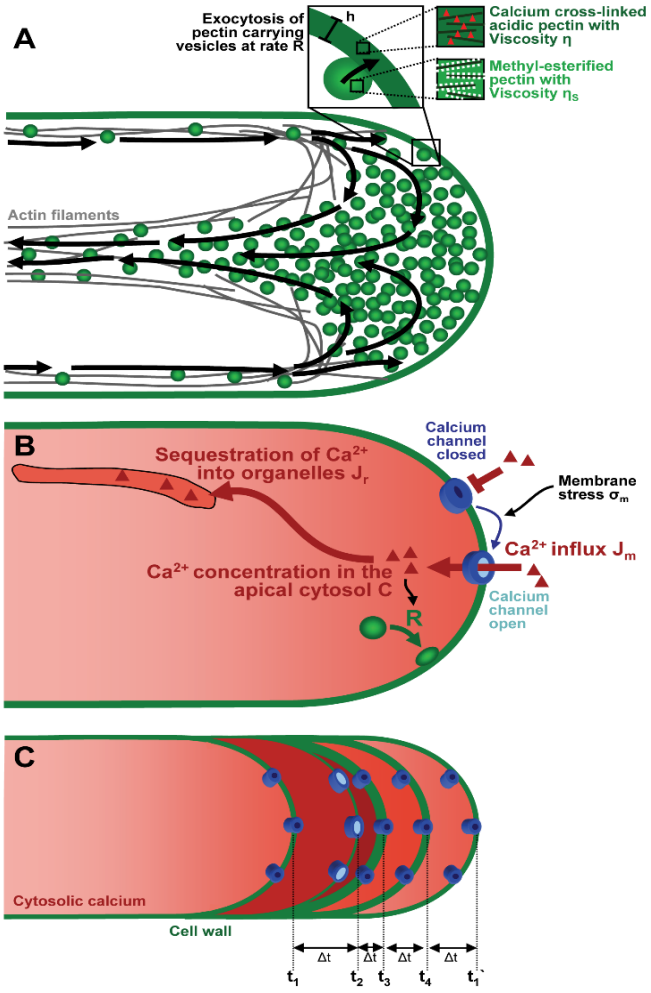

Figure 1 - Schematic representation of pollen tube growth - adapted from Kroeger, Zerzour, and Geitmann (2011).

At first, cell elongation occurs, but the tension of the plasma membrane is not high enough to open the stretch-activated calcium ion channels which are located on the plasma membrane (Fig. 1C- t_1). When the cell wall stress and growth rate is maximal, the calcium ion channels open as a response to the stress on the plasma membrane and enable the influx of calcium to the apical cytosol (Fig. 1C- t_2) (Kroeger, Zerzour and Geitmann, 2011). Because of

this calcium influx, the wall thickens as the result of soft cell wall structures being secreted in vesicles to the wall, reinforcing the wall (Fig. 1C- t_3). Because of the decreased turgor pressure and the thickness of the wall, the plasma membrane is under low stress and the calcium channels close. After that the wall stretches as a result of rapid extension until the cell wall viscosity returns to normal (Fig. 1C- t_4) (Kroeger, Zerzour and Geitmann, 2011).

This model of growth is currently more favoured, but regardless of the initiating mechanism behind plant cell growth , it is apparent that wall modifications are necessary for growth.

3. Measurement and visualisation of apoplastic pH

Apoplast is the space outside of the symplast, including cell walls and intercellular space which contain gas or water with solutes as well as lumen of xylem (Canny, 1995). Measurements of pH in these spaces can tell us a lot about the pH values which influence the cell wall as well as how the tissues can influence their environment, for example how roots can influence the rhizosphere (Zhao *et al.*, 2016). There are several types of methods available; each of them is useful in different plant organs with different spatial and temporal resolutions.

3.1 Apoplastic fluid extraction

There are two main ways of apoplastic fluid extraction. The first is via a modified Scholander pressure bomb (Hartung, Weiler and Radin, 1992), exuding the sap from the desired organ. The second method involves an intercellular washing fluid which is then recovered through vacuum infiltration and centrifugation (Aked and Hall, 1993; Mühling and Sattelmacher, 1995; O'Leary *et al.*, 2014). The pH of the acquired apoplastic sap can be either tested using dyes (Villiers and Kwak, 2013) or microelectrodes (Hartung, Weiler and Radin, 1992). In the Scholander pressure bomb method, the desired organ, be it leaf, stem or root, is excised and then put into the pressure chamber, where a balancing pressure is reached and then slowly increased in order to extrude the apoplastic fluid (Hartung, Weiler and Radin, 1992). The effect of this infiltration method lasts about 30 minutes, during which the apoplastic pH increases by approximately 1.5 to 1.7 units and then retains values similar to those at the start of the experiment (Felle, 2006), so this method is unreliable when measuring pH.

3.2 Influx of carbon labelled organic acids

Weak, organic ^{14}C-labelled acids like acetic acid (Thibaud *et al.*, 1988; Toulon *et al.*, 1989), butyric acid and isobutyric acid (Yu, Tang and Kuo, 2000) can be used as probes to measure pH of the plasma membrane surface (Thibaud *et al.*, 1988). This pH measurement is based on two assumptions. The first one is that these acids, like the indole-3-acetic acid (IAA) and auxin, can diffuse freely through the plasma membrane only in their undissociated forms (Marigo *et al.*, 1983; Marrè *et al.*, 1986). The second assumption is that the uptake of undissociated acids relies only on their concentration gradient across the plasma membrane (Yu, Tang and Kuo, 2000). This means that when the pH of the apoplast decreases, more of these labelled acids are taken up as there are more protons which can associate with dissociated acid forms making them undissociated again, enabling their uptake through the plasma membrane. In case of high pH in the apoplast, weak acids dissociate more which prevents their uptake as their dissociated parts are charged and cannot traverse through the plasma membrane. However, this method is not very reliable as it has been proven that apoplast composition and organic acid

metabolization are very dynamic as seen in interactions between *Phaseolus vulgaris* and the halo blight pathogen *Pseudomonas syringae* Pv. *phaseolicola* (O'Leary *et al.*, 2016). Another drawback of this technique is the need to work with radioactive material.

3.3 H^+-selective microelectrodes

This pH measuring technique has been in use since the 1970s (Bowling, 1973; Penny and Bowling, 1975) in plant studies. One of the main usages of these microelectrodes is to measure the apoplastic pH in the close vicinity of the electrode with very high precision and sensitivity (Yu, Tang and Kuo, 2000), providing valuable data. Surface pH measurements of cells can be measured by placing the electrode on the desired surface area using a micromanipulator (Felle *et al.*, 2009), which enables precise manipulation. Using this measurement technique, G. B. Monshausen et al. (2007) found that the reactive oxygen species-related growth of *Arabidopsis* root hairs is coupled with distinct regional oscillatory changes in extracellular pH. Another use of this technique is the non-invasive insertion of microelectrodes in the substomatal cavity of plants (Hanstein and Felle, 1999) which led to greater understanding of the relationship between substomatal pH and stress factors of *Vicia faba* (Felle and Hanstein, 2002). The largest drawbacks of this measurement method are its long and difficult preparation (Yu, Tang and Kuo, 2000) and low success of insertion, which was 10% in the case of H. H. Felle (1998).

3.4 pH indicator dyes

Bromosecol purple is a pH indicator which changes its colours depending on the pH conditions. Bromosecol purple is yellow at pH 5.2 and it becomes purple at pH 6.8 (Weisenseel, Dorn and Jaffe, 1979). Either the roots of a the plant can be embedded in an agar gel containing this pH indicator and a nutrient solution (Marschner, Römheld and Ossenberg-Neuhaus, 1982) or seedlings can be placed on top of the agar gel, so the roots grow on the surface of the gel (Weisenseel, Dorn and Jaffe, 1979). Unfortunately, the pH has to be estimated based on a colour scale made by fixed pH via buffers in bromosecol purple agar gel (Yu, Tang and Kuo, 2000). This method was for example used by Spartz *et al.*, (2017) to show that the *SAUR* gene activates the plasma membrane H^+-ATPase to induce acidic growth in the roots of *Solanum lycopersicum*.

3.5. pH-sensitive fluorescent dyes

pH fluorescent dyes are usually visualised with confocal fluorescence microscopy. This combination enables for high spatial and temporal resolution pH monitoring. The fluorophores must be attached to a bulky and cell-impermeable molecule, e.g. dextran used in GEILFUS and MÜHLING (2012), in order to prevent the dye from entering the cytoplasm. Second, the

fluorophores should be sensitive only to the free H^+ concentration, ignoring other redox-changing substances (Geilfus, 2017). More precise measurements of pH via fluorescence intensity are achieved by ratio imaging, which requires at least two emission or excitation wavelengths of the probe to be sensitive to pH (Bright *et al.*, 1987). During ratio imaging, the fluorescence is measured at two different wavelengths – the relationship of the resulting excitation or emission spectrums can vary – one wavelength can be pH non-sensitive, largely pH insensitive, or sensitive in the opposite direction to the other wavelength (Bright *et al.*, 1987). The ratio between these intensities provides pH values with a correction for a large set of possible local variations like dye concentration, light scattering, dye bleaching and other issues (Bright *et al.*, 1987). The dyes used in pH monitoring are usually fluorescein derivates such as fluorescein isothiocyanate (Hoffmann and Kosegarten, 1995), fluorescein tetramethylrhodamine (Pitann, Kranz and Mühling, 2009) and Oregon Green 488 (McLachlan *et al.*, 2016). A calibration with pH buffered solutions containing the dyes is required in order to use these probes *in vivo* (Geilfus *et al.*, 2014) and achieve measurements of absolute pH values. The dyes need to be loaded into the apoplast in a non-destructive manner. In the case of Monshausen *et al.* (2011), it was done via a 30-minute immersion of four to five-days old seedlings into a liquid growth medium which contained the dye. By using this method, the authors were able to analyse the pH cell walls and apoplast, and reveal a calcium signal which regulates pH in *Arabidopsis* roots. Another example of loading method is the infiltration of Oregon Green into the open stomata by Geilfus and Mühling (2011).

More recently, 8-hydroxypyrene-1,3,6-trisulfonic acid trisodium salt (HPTS) were used to determine apoplastic pH changes in *Arabidopsis* root, investigating the acid growth theory. It was revealed that auxin induces a 2-phase response: at first, it induced a rapid rise in pH of root apoplast which is thought to inhibit cell elongation but enable the bending of roots. After a few hours apoplast acidification occurred (Barbez *et al.*, 2017). HPTS is suitable for pH imagining in roots because it is in-expensive, soluble in water (Zhujun and Seitz, 1984) and it has very low toxicity (Wolfbeis *et al.*, 1983).

3.6. Genetically encoded pH sensors

Except the pH measurement and visualisation methods mentioned above, there are genetically encoded fluorescent pH probes available. These probes include pHusion (Gjetting *et al.*, 2012), pHluorin (Gao *et al.*, 2004) and *Pt*-GFP (Geilfus *et al.*, 2014). The main advantage of these genetically encoded protein sensors is that there is no need to load the dye in the organs which reduces stress. By fusing a signal peptide to the N-terminus of the protein, it can be targeted to the apoplast. pHluorin is a ratiometric pH-sensitive derivative of the Green Fluorescent Protein

(GFP) (Gao *et al.*, 2004). It was designed to reveal changes in apoplastic pH during abiotic stress such as salinity, cold, drought and oxidative stress.

Another pH sensor protein – apo-pHusion – was expressed in *Arabidopsis* plants using the same targeting method. It was created by fusing the monomeric Red Fluorescent Protein (mRFP) with the enhanced Green Fluorescent Protein (eGFP) (Gjetting *et al.*, 2012). In the range of pH values that occur in the plant apoplast, the fluorescence of mRFP is pH independent while the fluorescence of eGFP quenches in low pH conditions (Gjetting *et al.*, 2012). This means that high apoplastic pH causes higher fluorescence of eGFP resulting in a green and red signal, while low pH causes lower eGFP fluorescence, resulting in a red signal caused by the largely unaffected mRFP. Using this targeted pHusion sensor (apo-pHusion), Fendrych, Leung, and Friml (2016) revealed that auxin induces acidification and cell growth in etiolated *Arabidopsis* hypocotyls, through the auxin-induced transcriptional response.. These changes occur approximately 20 minutes after the application of auxin. It was also used to examine gravitropic response of hypocotyls.

The use of the pH visualisation and measurement techniques has improved our understanding of apoplastic pH and its shifts. Although this is true, further advancements in these methods is needed in order to achieve high organ-wide spatial and temporal resolution. In Fig.2, you can see an example of a genetically encoded pH sensor and *Arabidopsis* roots growing on a medium that contains bromosecol purple.

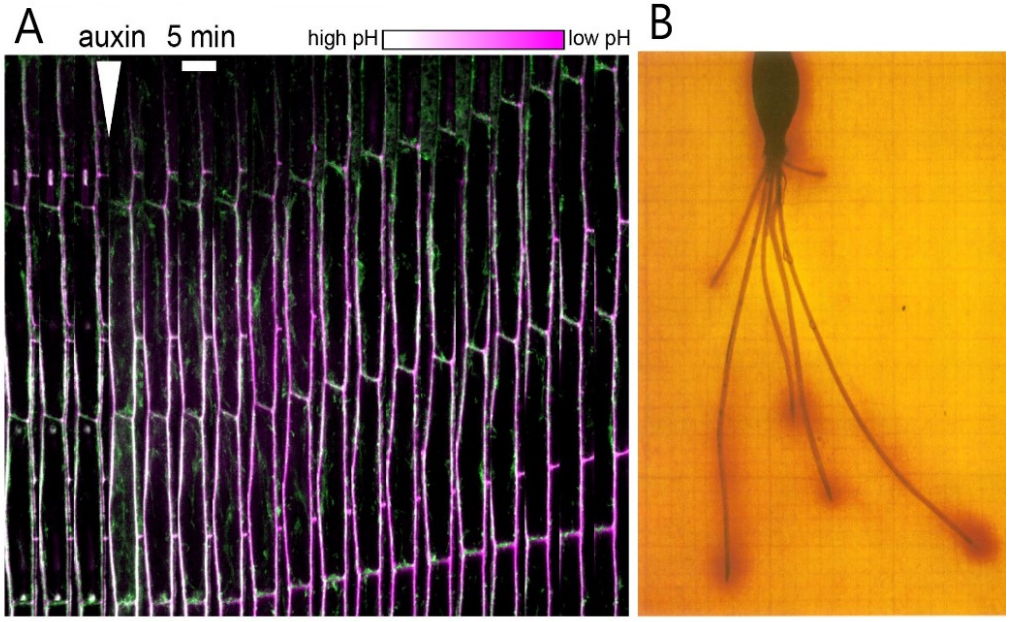

Figure 2 – A) *Arabidopsis* hypocotyl segment with apo-pHluorin visualised pH – adapted from (Fendrych, Leung and Friml, 2016). B) *Hordeum vulgare* roots growing on a 3-mm agar medium which contains bromosecol purple – adapted from (Weisenseel, Dorn and Jaffe, 1979).

4. Acid growth theory

It was long known that auxin induces growth. Fifty years ago, elongation growth caused by acidic pH was observed by Hager, Menzel and Krauss, (1971) in buffers with pH values approximately 4. When it comes to growth rate, it was very similar to auxin induced growth (Hager, Menzel and Krauss, 1971). It was found that auxin stimulates a rapid (horizon of 20-30 min) secretion of protons post its application in *Avena* coleoptiles which had their cuticle physically removed (Rayle, 1973) thus linking auxin and acid growth.

It was not until the experiments in the 1970s that the acidification of the apoplast was considered as a factor in plant cell growth. The experiments of Rayle (Rayle and Cleland, 1970) have shown that the growth rates proportionally increase with a pH decreasing up to the pH level of approximately 2.6. The same experiments found that the level of pH must be maintained in order to induce rapid growth. If the pH is raised from 3 to 7, the growth stops. This stop indicates that the low pH level does not serve just as a growth trigger**.**

4.1 Protons excreted by plant vs. externally supplied protons

Plant growth can be triggered by means other than the usage of auxin. Fusicoccin (FC) has been used as an effective growth stimulant in vitro. FC is a fungal toxin produced by *Fussicoccum amygdali* (Ballio *et al.*, 1964) . This toxin stimulates the discharge of protons alongside with the uptake of potassium or other monovalent ions depending on the conditions (Marre, 1979). Later experiments of (Rayle and Cleland, 1980) show that the application of FC induces rapid excretion of protons quantitatively correlating with fast cell elongation after a short lag phase of approximately 2 minutes with the processes reaching a constant rate approximately 30 to 60 minutes after application (Kutschera and Schopfer, 1985b). The measured proton extrusion and growth was far greater than that of auxin application. The response to FC is greatly dependent on its concentration and when it is in suboptimal concentration alongside with IAA, their effects are additive (Lado *et al.*, 1973). FC induces acidification by turning on the plasma membrane auto inhibitory proton pumps (Hager, Menzel and Krauss, 1971; Marre, 1979)by binding to the preformed 14-3-3 protein – AHA2 H^+-ATPase complex (Baunsgaard *et al.*, 1998; Fuglsang *et al.*, 1999; Camoni *et al.*, 2000), stabilising their interaction resulting in permanently active H^+-ATPase.

Experiments on auxin and buffers conducted by (Kutschera and Schopfer, 1985b) show that the application of buffers with neutral pH completely inhibit the growth response produced by FC. The higher the buffering capacity of the buffer the longer is the inhibition effect. This implies that if we maintain a neutral pH in the cell walls, the FC response will be inhibited while on the contrary, no such effect was observed with auxin mediated growth (Kutschera and

Schopfer, 1985a, 1985b). Acidic buffers were found to mimic the elongation effects of FC. Cycloheximide (CHX) has inhibitory effects on both the buffer-induced and FC-induced growth. This inhibition happens after a 40-45 min lag period in case of buffers and 2 hour lag period in the case of FC (Kutschera and Schopfer, 1985b). CHX has immediate inhibitory effects on IAA induced growth (Kutschera and Schopfer, 1985b). When FC is added to the acidic buffer, no further increase in growth response was measured (Kutschera and Schopfer, 1985b). These experiments imply that acidic growth is not driven by the proton extrusion itself but by the acidic environment in the cell wall created by them.

4.2 Acid growth and its effect on mechanical properties of the cell wall

Upon the discovery of the acidic growth phenomenon, its mechanical basis was immediately being investigated. This investigation was started by the discovery that organs killed via plasmolysis or freezing-thawing can grow in acidic medium while being mechanically pulled by an instron TM-S linear extensometer (Rayle, Haughton and Cleland, 1970) or by applying external weight (Hager, Menzel and Krauss, 1971) to simulate turgor pressure in living cells. To check to what extend do the mechanical properties of the cell wall change, both dead and living organs have been used alongside each other to measure growth (Hager, Menzel and Krauss, 1971; Rayle, 1973; Rayle and Cleland, 1980; McQueen-Mason, 1992).

The growth caused by neutral pH buffers and the application of external weight to dead organs such as plasmolysed hypocotyls or frozen-thawed coleoptiles decreases rapidly after 30 minutes (Rayle, Haughton and Cleland, 1970). However, after the application of low pH buffers, there is an onset of quick elongation after a lag phase of approximately 1-15 minutes and continues on for at least an hour (Rayle, Haughton and Cleland, 1970). It has been also found, that this extension increases proportionally with the decrease of pH to the optimum of pH around 4 in intact hypocotyl sections (Hager, Menzel and Krauss, 1971). This means that pH affects the mechanical properties of plant cell walls and the lower the pH value, the greater is the effect.

4.3 Regulation of AHA ATPases

The acidification of the plant cell wall is caused by protons being pumped by plasma membrane H^+-ATPases. Plasma membrane H^+-ATPases activate many transporters by forming a membrane potential (Palmgren, 2001). In *Arabidopsis* there are 11 H^+-ATPase isoforms named *AHA1* to *AHA11* (Merlot *et al.*, 2007; Haruta and Sussman, 2012). These isoforms are active in different tissues, developmental phases and environmental conditions (Merlot *et al.*, 2007), for example *AHA3* is essential for male gametophyte development (Robertson *et al.*, 2004) and *AHA4* seems to play a role in salt resistance (Vitart *et al.*, 2001). In *Arabidopsis* seedlings and

adults, *AHA1* and *AHA2* are expressed the most (Haruta and Sussman, 2012). Double mutants in both of these genes are embryonic lethal (Haruta and Sussman, 2012). Single homozygous knockdown mutants in these genes are able to live without any growth alterations in ideal conditions, however transcriptomes revealed that these plants have altered gene expression (Haruta and Sussman, 2012). The transcriptome of *aha1-6* mutants displays a change of expression of 12 genes with genes related to lipid metabolism (Haruta and Sussman, 2012). The *aha2-4* mutant showed a significant transcriptional change in 27 genes related to various nutrient stresses (Haruta and Sussman, 2012). Other *aha1* and *aha2* mutants showed similar results as the *aha1-6* respectively the *aha2-4* mutants (Haruta and Sussman, 2012).

The regulation of AHA H^+-ATPases is based on posttranslational phosphorylation of serine and threonine in their C-terminus binding site which acts like a regulatory domain (Haruta, Gray and Sussman, 2015). AHA H^+-ATPases are activated by phosphorylation of their penultimate threonine (Fuglsang *et al.*, 1999) and subsequent binding of the 14-3-3 protein (Korthout and de Boer, 1994) into the regulatory domain (Baunsgaard *et al.*, 1998). The dephosphorylated state of the AHA H^+-ATPases is ensured by the activity of PP2C.D phosphatases as the overexpression of these phosphatases results in dwarf phenotypes and their decrease results in higher AHA H^+-ATPase activity (Regenberg *et al.*, 1995; Ren *et al.*, 2018).

The phosphorylation of the AHA H^+-ATPases is regulated by proteins and peptides. SMALL AUXIN UP-RNA (SAUR) are a group of proteins which are expressed in response to auxin application (Hagen and Guilfoyle, 2002). The SAUR proteins are a family of proteins which are able to activate plasma membrane AHA H^+-ATPases and induce rapid apoplast acidification (Spartz *et al.*, 2014) by inhibiting PP2C.D family protein phosphatases. This induced acidification promotes expansin activity which loosens the cell wall. The SAUR19 protein can be stabilized by fusing with GFP, creating GFP-SAUR19 which results in bypassing the need for auxin and inducing cell wall elongation accompanied by apoplast acidification (Spartz *et al.*, 2012; Spartz *et al.* 2017; Fendrych, Leung and Friml, 2016). The full function of SAUR proteins is unknown, as they may have other targets on which they action (Fendrych, Leung and Friml, 2016).

Phosphorylation may also cause the deactivation of AHA H^+-ATPases. A good example of PM proton pump deactivation by phosphorylation are the rapid alkalization factors (RALFs) that were found to inhibit root growth (Pearce *et al.*, 2001). FERONIA, which is a receptor kinase from the CrRLKL1 family localised on the plasma membrane (Feng *et al.*, 2018), is expressed alongside RALFs in the mature parts of roots in seedlings (Haruta *et al.*, 2014). The binding of the RALF peptide to FERONIA triggers a rapid phosphorylation of the

AHA2 H^+-ATPase at Ser^{899}, causing its inactivation, inhibiting H^+ extrusion as a consequence (Haruta *et al.*, 2014).

An example of AHA H^+-ATPase activation via phosphorylation is the effect of growth-promoting brassinosteroids (BLs). BLs bind to the brassinosteroid receptor BRASSINOSTEROID INSENSITIVE 1 (BRI1) which is located on the plasma membrane and has kinase activity (Caesar *et al.*, 2011). After the BL-mediated activation, the BRI1 directly interacts with the AHA1 H^+-ATPase. However, it is unknown, whether a direct phosphorylation of AHA1 by BRI1 is involved in its activation as an *Arabidopsis* mutant showed that the phosphorylation of the AHA1 penultimate threonine (Witthöft *et al.*, 2011).

4.4 RNA and protein synthesis as growth limiting processes

As shown above, proteins, peptides and steroids play a crucial role in apoplastic pH regulation. This fact raises an important question – is transcription and translation needed to initiate and maintain cell growth? CHX application was found to block auxin induced growth (Cleland, 1970; Kutschera and Schopfer, 1985b) alongside with proton extrusion and increased wall extensibility after auxin application (Cleland, 1970; Rayle, Haughton and Cleland, 1970; Rayle, 1973; Rayle and Cleland, 1980; Kutschera and Schopfer, 1985b). When FC was used instead of auxin, the acidification via proton extrusion was not blocked immediately, but had a lag phase of about 2 hours (Kutschera and Schopfer, 1985b). These experiments imply that auxin-induced protein synthesis is needed for initiation and maintenance of auxin induced growth and apoplast acidification, while acidic growth requires only apoplast acidification as it is in case of FC application.

In another experiment, (Edelmann and Schopfer, 1989) an RNA synthesis inhibitor – cordycepin (COR) – was used to test the relationship between the effects of auxin treatment and RNA synthesis. It was found that COR can completely inhibit auxin action if applied 10 minutes before IAA application (Edelmann and Schopfer, 1989). If COR was added when the IAA induced elongation rate reached a steady state, there was a period of approximately 24 minutes after the elongation rate reached 50% inhibition and it took more than an hour for the inhibition to become total (Edelmann and Schopfer, 1989). All these findings considered, it seems that auxin induced growth needs mRNA synthesis in order to induce growth, proton extrusion and mechanical changes in the plant cell wall.

4.5 The TIR1/AFB-Aux/IAA pathway and ABP1

I have established that transcriptional changes need to occur in order to induce growth via auxin. Currently, there is one known co-receptor family for auxin perception in plants (Bargmann and Estelle, 2014) – the TRANSPORT INHIBITOR RESPONSE1/AUXIN SIGNALING F-BOX–

AUXIN/INDOLE-3-ACETIC ACID (TIR1/AFB-Aux/IAA) nuclear co-receptor (Kim, Harter and Theologis, 1997; Dharmasiri *et al.*, 2005; Kepinski and Leyser, 2005). TIR1 is an F-box protein, meaning it has an F-box domain which mediates protein-protein interactions (Bai *et al.*, 1996; Kipreos and Pagano, 2000). The TIR1 works as a substrate recognition protein for the ubiquitin ligase complex SCF (Wang *et al.*, 2016). Proteins like the COP9 signalosome complex, ubiquitin-like protein Nedd8 and the cullin-associated Nedd8-disassociated protein have been shown to play key roles in the regulation of SCF complexes (Gray *et al.*, 1999; Schwechheimer *et al.*, 2001; Hellmann *et al.*, 2003; Chuang, Zhang and Gray, 2004; Hua *et al.*, 2011). Auxin concentration plays a key role in interactions between the parts of transcription pathways.

At low levels of auxin, AUXIN RESPONSE FACTOR transcription factors recruit a transcription co-repressor called TOPLESS (TPL) that interacts with Aux/IAAs through the ETHYLENE RESPONSE FACTOR associated motif domains (Szemenyei, Hannon and Long, 2008). TPL then interacts with HISTONE DEACETYLASE19 (HDA19), resulting in DNA condensation and transcriptional repression (Long *et al.*, 2006; Kagale and Rozwadowski, 2011).

High enough concentrations of auxin trigger an interaction between the F-box domain of TIR1/AFB and the Aux/IAA repressors, leading to the repressor degradation via its ubiquitination by $SCF^{TIR1/AFB}$ ubiquitin ligase and degradation by 26S proteasome (Salehin, Bagchi and Estelle, 2015; Strader and Zhao, 2016). The degradation leads to the corepressor and HDA19 disassociation, enabling the expression of auxin induced genes (Wu *et al.*, 2015). The experiments of Leyser *et al.* (1996) and Swarup *et al.* (2005) on *axr3-1* which has altered auxin response, clearly showed that a nuclear auxin response is needed for gravitropism, as these mutants are agravitropic and have short, slow growing roots.

There is also a protein which binds auxin - the AUXIN BINDING PROTEIN 1 (ABP1) which is located mainly in the endoplasmic reticulum and at the cell surface (Löbler and Klämbt, 1985; Grones and Friml, 2015; Grones *et al.*, 2015).

The ABP1 interaction with auxin was proven to induce rapid protoplast swelling in response to auxin (Dahlke *et al.*, 2017) which can be blocked by these ABP1 antibodies (Leblanc *et al.*, 1999; Yamagami *et al.*, 2004). It is unknown what is the biological relevance of this swelling. The importance of the ABP1 protein in plant growth has been discredited when the *Arabidopsis abp1* knockout mutants were investigated as they lacked obvious developmental phenotypes (Gao *et al.*, 2015; Grones *et al.*, 2015; Michalko *et al.*, 2015, Michalko *et al.,* 2016; Fendrych, Leung and Friml, 2016).

4.6 Other modifiers of apoplastic pH

The TIR1/AFB-Aux/IAA pathway is surely not the only modifier of apoplastic pH in *Arabidopsis*. One of the modifying agents are the Ca^{2+}/H^{+} antiporters of tonoplasts and their *CAX* genes. *Arabidopsis cax1/cax3* mutants show significantly reduced levels of AHAs and a threefold greater Ca^{2+} concentration in the apoplast which results in reduced cell wall extensibility due to higher pH, reduced transpiration and leaf growth rate, as well as altered expression of cell wall modifying proteins, including expansins (Conn *et al.*, 2011; Cho *et al.*, 2012).

Another of the before unanticipated apoplastic pH modifying agents seems to be the type I vacuolar proton-pumping pyrophosphatase and its gene *AVP1.* The tonoplast localized AVP1 is hypothesised to upregulate the plasma membrane H^{+}-ATPase activity by increasing the trafficking of proteins associated with this H^{+}-ATPase, resulting in lower apoplastic pH (Li *et al.*, 2005). The role of *AVP1* in apoplast acidification has been questioned by (Schilling *et al.*, 2017) and it has been implied that further research is needed.

These are just two examples of apoplastic pH modifiers in *Arabidopsis.* Apoplastic pH shifts caused by H^{+}-ATPases and pH modifiers that influence the levels of H^{+}-ATPases or upregulate their action have significant impact on the properties and activities of plant cell wall components.

4.7 Action of expansins

The activity of expansins depends on the pH of the cell wall (McQueen-Mason, 1992). Their activity is significantly increased by lowering the wall pH. At first, the mechanism of expansins action was thought to be enzymatic (McQueen-Mason, 1992). However, no proof was found for this to be the case. Instead, a new model of expansin action was proposed. In this model expansins weaken non-covalent polysaccharide bonds (Cosgrove, 2000). This model proposes that expansins use mechanical strain energy to induce an inchworm-like movement in wall polymers, specifically XG slipping (Cosgrove, 2000; Arsuffi and Braybrook, 2018). The proposed mechanism seems to be supported by the fact that expansins induce lesser growth in grasses and related plants (McQueen-Mason, 1992) where arabinoxylans are dominant (Cosgrove, 2015). Another supporting fact for this theory is that in XG deficient *xxt1/xxt2 Arabidopsis* mutants the extensibility of the cell wall was bigger, but these walls were less extensible in processes that are mediated by α-expansins (Park and Cosgrove, 2012). At the time when the expansin-mediated cell wall loosening hypothesis was formed (Cosgrove, 2000), the plant cell wall model assumed that the cellulose microfibrils made no contact with each other, but were connected via XGs. However, the cell wall model was revised and now we think

of XG-cellulose binding sites as "biomechanical hotspots" which are limited to the sites of close contact between cellulose microfibrils (Cosgrove, 2014). In these hotspots, cellulose – cellulose contacts are made and are mediated by small amounts of XG (Cosgrove, 2014). These "hotspot" sites are now hypothesized to be the sites of action for expansins. Because the number of these "hotspots" is limited, it is likely that expansins are not the only modification proteins involved in growth stimulation. There is a need to further characterize the action of expansins in order to strengthen our knowledge of expansin action and its dependency upon the decrease of pH which induces cell wall remodelling and changes in the mechanical properties of the wall (Arsuffi and Braybrook, 2018).

4.8 A revised model of acid growth

Based on current knowledge, a revised acid growth model (Fig.3) was proposed by Arsuffi and Braybrook (2018) in their review.

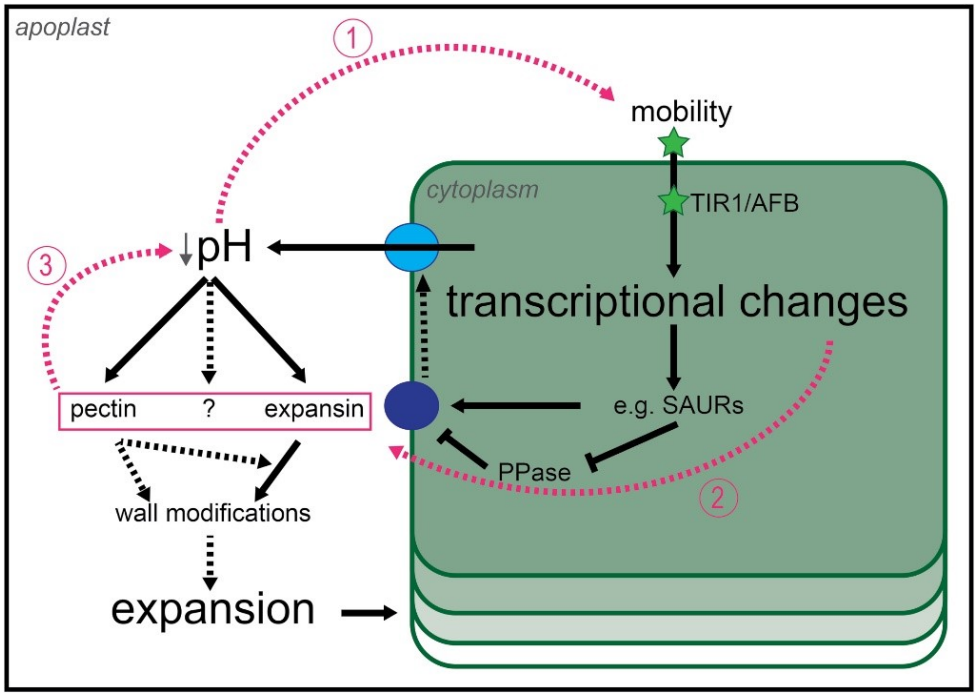

Figure 3- The revised model of auxin-driven cell growth. Solid black arrows indicate proven reactions; dashed black arrows indicate reactions that need more investigation; dashed pink arrows indicate the hypothesised positive feedback loop; dark blue circle indicates inactive proton pumps; light blue circle indicates active proton pumps; green stars indicate auxin. Adapted from Arsuffi and Braybrook (2018).

This model is based on a positive feedback loop which is hypothesised to maintain growth. In this model, apoplastic pH affects the mobility of auxin which, when in high concentrations, activates TIR1/AFB driven transcriptional changes. These changes then stimulate the production of SAUR proteins, activating the plasma membrane H^+-ATPases. Auxin induced transcription also regulates other cell wall modifying agents. This leads to

apoplast acidification which has an effect on expansins and pectins via PMEs that may lead to cell wall extension and local pH changes. These pH changes then may give positive feedback onto the loop. If the environment remains strongly acidic, the protonation status of auxin changes, which inhibits its dissociation, thus closing the loop.

5. Apoplastic pH and organs of *Arabidopsis thaliana*

Apoplast acidification and acid growth theory need to be tested in all plant organs and a wide range of plant species. This is crucial for our understanding of molecular mechanisms behind plant cell wall pH changes and its effects on individual organs in different species as there might be some key differences. *Arabidopsis* a widely used model plant and it became a focus of the acid growth theory (Arsuffi and Braybrook, 2018). Individual organs are under different stress conditions and stimuli that reflect on their apoplastic pH.

5.1 Roots

The acid growth model for roots is a topic of research and debate (Barbez *et al.*, 2017), as the acid growth research has been predominantly focused on organs above the ground. On one hand, on the short time scale, auxin inhibits root cell growth (Chadwick and Burg, 1967; Fendrych *et al.*, 2018) and this effect is the basis for the root gravitropic response (Evans, Ishikawa and Estelle, 1994). On the other hand, studies have concluded that apoplast acidification and functional plasma membrane H^+-ATPases are needed for root elongation (Lado *et al.*, 1976; Moloney, Elliott and Cleland, 1981; Haruta *et al.*, 2010). Evans, Mulkey and Vesper (1980), Barbez *et al.* (2017) and others found, that apoplast acidification in roots of *A. thaliana* occurs only in elongating cells. Because of this observation of apoplast acidification, exogenous root acidification with a pH 4.6 medium and alkalization with a pH 6.4 medium was tested for 2.5 hours and yielded results which suggest that apoplast acidification plays a part in epidermal root cell elongation (Barbez *et al.*, 2017). By the induction of the *GRETCHEN HAGEN 3.6* gene expression in mutants which reduces endogenous auxin levels (Woodward and Bartel, 2005), cell growth was stopped and higher apoplastic pH levels were measured compared to the wild type plants. Drought and salinity induce stress, elevating the levels of Abscisic acid (ABA) (Fernando and Schroeder, 2016). ABA in roots inhibits the activity of AHA2 ATPases by activating the SnRK2.2 protein kinase, which was found to phosphorylate the AHA2 C-terminal domain *in vitro* (Planes *et al.*, 2015).

An essential aspect of root growth is root gravitropism. This gravity-responsive bending (Fig. 4) is accompanied by different pH levels on each side of the root. The apoplastic pH of the lower side of the root increases as a response to gravistimulation, stiffening the cell walls and stopping their growth, resulting in the bending of the root (Fasano *et al.*, 2001; Monshausen *et al.*, 2011).

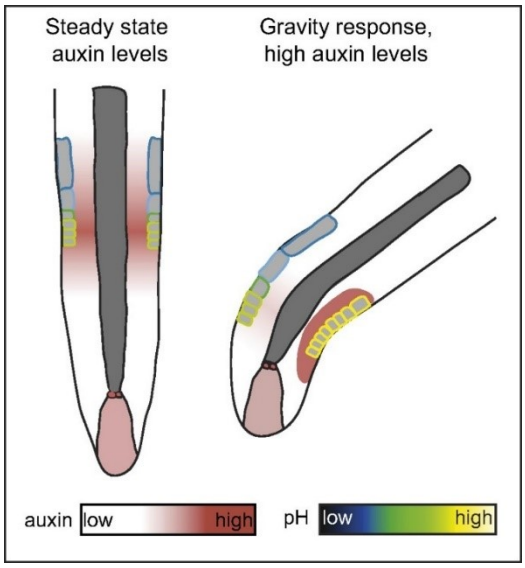

Figure 4 – Scheme of apoplastic pH in *Arabidopsis* roots during steady state auxin levels (*left*) and after gravistimulation (*right*). Adapted from Barbez et al., (2017).

5.2 Hypocotyl

Hypocotyls are used as a model for auxin-induced growth because of their rapid growth induced by auxin (Schenck *et al.*, 2010; Takahashi, Hayashi and Kinoshita, 2012). In auxin-deprived hypocotyls, the application of auxin induces growth and rapid apoplast acidification with a delay of approximately 20 minutes (Fendrych, Leung and Friml, 2016). This time aligns well with times reported in other experiments (Rayle, 1973; Kutschera, 1994). Hypocotyl FC-induced growth is triggered 6-8 minutes after its application (Fendrych, Leung and Friml, 2016). As in maize coleoptiles (Cleland, 1970; Kutschera and Schopfer, 1985b), the use of CHX prevents auxin-induced apoplast acidification (Fendrych, Leung and Friml, 2016). The *Arabidopsis tir1-1/afb2-1/afb3-1* triple mutants had their auxin-induced signalling pathway largely unaffected in the hands of Fendrych, Leung and Friml, (2016) while these mutants had the previously mentioned pathway visibly disturbed in the experiments of Dharmasiri *et al.*, (2005). Nonetheless, both Fendrych, Leung and Friml, (2016) and Dharmasiri *et al.*, (2005) report serious phenotype defects in quadruple mutants. The presumed cause why a such high mutation order is needed to achieve the elimination of the TIR1/AFB- Aux/IAA pathway is the excess of 6 TIR1/AFB receptors (Fendrych, Leung and Friml, 2016). This clearly shows that TIR1/AFB-Aux/IAA signalling pathway is needed to achieve apoplast acidification. When mutant *Arabidopsis 35S::GFP-SAUR19* and mutant *Solanum lycopersicum* overexpress the stabilized SAUR protein (Fendrych, Leung and Friml, 2016; Spartz *et al.*, 2017) AHA PM H^+-ATPases become activated (Spartz *et al.*, 2014) resulting in acidification. SAUR proteins might

be able to activate other PM H^+-ATPases or target other proteins to trigger growth (Fendrych, Leung and Friml, 2016). The mutant *ost2-2D/aha2-5* hypocotyl segments showed auxin independent growth (Fendrych, Leung and Friml, 2016). This implies that the acidification of the apoplast through the activation of PM H^+-ATPases might be enough to cause growth. ABA application to hypocotyls reduces Thr-947 phosphorylation of AHA ATPases through ABA-INSENSITIVE1 (ABI1) protein, resulting in their reduced activity (Hayashi *et al.*, 2014).

Gravitropic growth of hypocotyls is based on auxin asymmetry (Harrison and Pickard, 1989; Friml *et al.*, 2002) which is established quickly after gravistimulation (Harrison and Pickard, 1989). This asymmetry results in different pH levels and bending approximately 20–40 min after gravistimulation (Fendrych, Leung and Friml, 2016). It was shown that in the lower part of the hypocotyl, the apoplast is visibly more acidified than that of the upper part, resulting in bending (Fendrych, Leung and Friml, 2016) (Fig. 5). This is in line with the observations of tropisms made on *Helianthus annuus* L and *Zea mays* L (Mulkey, Kuzmanoff and Evans, 1981).

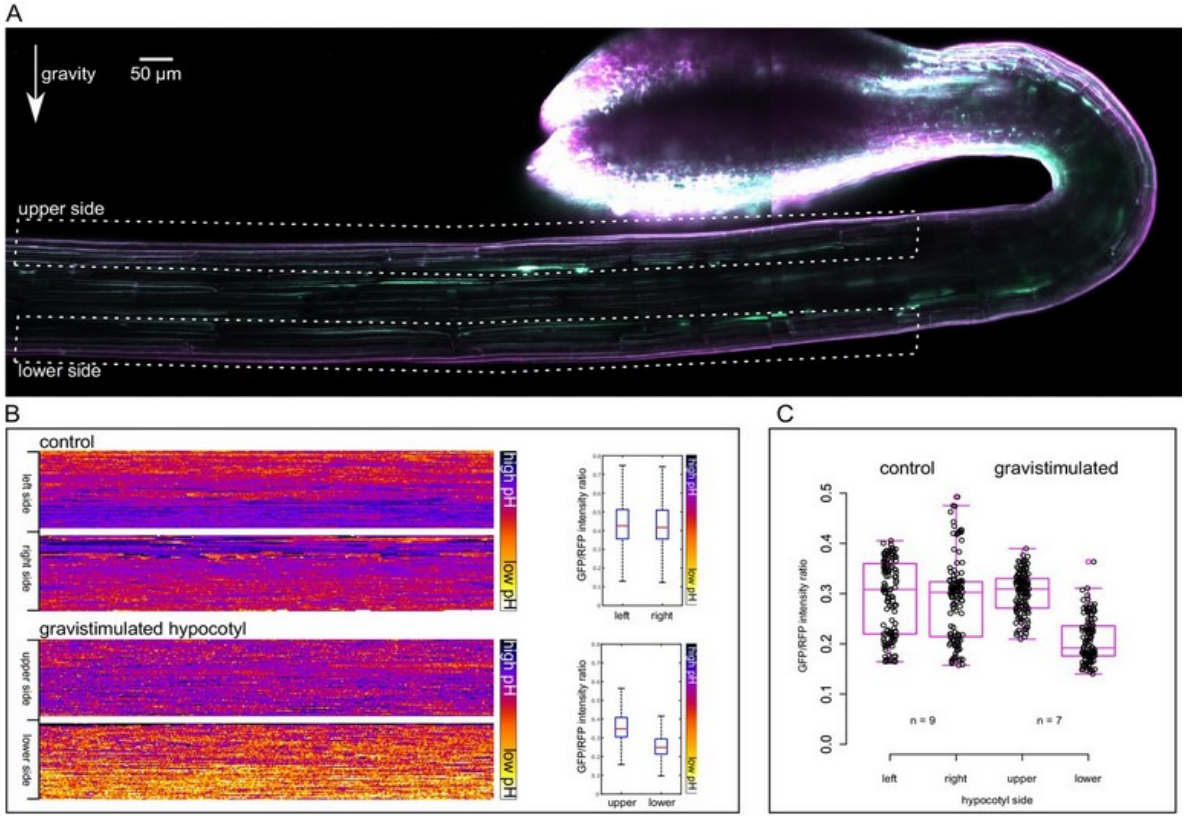

Figure 5 – A) Apoplastic pH in gravistimulated (1h) *Arabidposis* hypocotyl visualised by apo-pHusion. B) AreaKymo representation of lower and upper side apoplastic pH in control and gravistimulated (1h) hypocotyls C) Apoplastic pH quantification in the upper and lower sides of control and gravistimulated (1h) hypocotyls. Adapted from Fendrych, Leung and Friml, (2016).

5.3 Leaves

Leaves, as the main site of photosynthesis, display an interesting reaction between the plasma membrane H^{+-}ATPase and sugar which accumulates as a result of photosynthesis (Okumura *et al.*, 2016). Dark-adapted leaves of *Arabidopsis* which are illuminated with white light show activation of plasma membrane H^+-ATPase via phosphorylation (Okumura *et al.*, 2016). Tests on photoreceptor mutants *phyA-201 phyB-1, cry1-304 cry2-1* and *phot1-5 phot2-1* as well as *var2-2* which has leaves without photosynthetic activity in its white sections proved that the phosphorylation of PM H^+-ATPase is not induced by these photoreceptors, but instead by photosynthesis (Okumura *et al.*, 2016). With the use of immunohistochemical detection, Okumura *et al.*, (2016) found that the leaf tissue in which the phosphorylation of the H^+-ATPase occurs is the mesophyll cell layer. By mutagenizing *Arabidopsis* plants using ethyl methanesulfonate, Okumura *et al.*, (2016) identified plants with high amounts of PM H^+-ATPase phosphorylated in darkness. Sequencing revealed a *SUC2* point mutation which resulted in the inability of these plants to load sucrose from leaf apoplast into phloem (Gottwald *et al.*, 2000) and subsequent accumulation of sucrose inside the leaves (Okumura *et al.*, 2016). This, alongside with the discovery that in *suc2-7* mutants the low sugar saturation in new leaves and high saturation in mature leaves corresponded with the level of plasma membrane H^+-ATPase phosphorylation lead to the theory that endogenous sugars mediate light induced H^+-ATPase phosphorylation in leaves (Okumura *et al.*, 2016). The activation of H^+-ATPases in mesophyll cells needs to be further researched as there are currently no data on how this activation reflects on apoplastic pH.

Activating plasma membrane H^+-ATPases in stomatal guard cells provides a stimulus for opening stomata in leaves, securing gas exchange (Kinoshita and Shimazaki, 1999). Blue light is known to induce this response by activating AHA ATPases (Kinoshita and Shimazaki, 1999). Blue light phototropins, PHOTOTROPIN 1 and PHOTOTROPIN 2, which are protein kinases, were identified as receptors mediating the blue light signal (Kinoshita *et al.*, 2001). After activation, they phosphorylate a Ser/Thr kinase called BLUE LIGHT SIGNALING1 (BLUS1) (Takemiya *et al.*, 2013). The phosphatase PROTEIN PHOSPHATASE 1 (PP1) (Takemiya *et al.*, 2006) seems to act downstream of BLUS1 as the inhibition of PP1 suppresses H^+-ATPase phosphorylation while it had no effect on BLUS1 phosphorylation (Takemiya *et al.*, 2013). PP1 signal transduction most likely involves the activation of a protein kinase that phosphorylates Thr^{947} (Kinoshita and Hayashi, 2011) or the deactivation of a protein phosphatase that keeps Thr^{947} dephosphorylated (Falhof *et al.*, 2016).

In conclusion, different levels of plant hormones, such as auxin, are present in different plant organs as they have specific effects on certain plant organs. These organs are exposed to different stimuli and need to react to them in an organ-specific way. The reactions include the activation or deactivation of the plasma membrane H^+-ATPases, resulting in local apoplastic pH shifts which then mediate stimuli-induced changes in plant organs.

6. Conclusion

In this thesis, I discussed the influence of apoplastic pH on plant cell growth, current views on plant cell wall structure, possible plant cell growth mechanisms, pH measurement and visualisation methods, proteins and signalling pathways that play role in apoplastic pH alteration and growth, current views on acid growth and influences of plant hormones and some abiotic stimuli on apoplastic pH. My main objective in this thesis was to summarize current knowledge of apoplastic pH in regards to auxin response and acid growth, as well as the molecular basis of apoplastic pH regulation.

As of now, the mechanisms of apoplastic pH regulation are not fully understood as there are numerous factors involved in governing said mechanisms, some of which are unknown. Further advancements in pH measuring and imaging, such as apoplastic pH with the spatial and temporal resolution of genetically encoded pH reporters on an organ-wide level, are needed for better understanding of apoplastic pH shifts.

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