Role of callose in pollen tube invasive growth

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List of Abbreviations

CalS callose synthase

PT pollen tube

LatB latrunculin B

CytB cytochalasin B

PD plasmodesmata

MT microtubule

WT wild type

CFM cellular force microscope

GSL glucan synthase like

CBM cellulose binding module

Abstract

A distinct feature of plant cells differentiating them from animal cells is the presence of the cell wall. The cell wall in plants plays a critical role in protecting the cell protoplast, serves as key location for many biochemical reactions involved in regulation of cell metabolism and cell biomechanics. The shape and geometry of plant cells are primarily defined by mechanical properties of different cell wall polysaccharides that are strategically deposited during cell differentiation and morphogenesis. The main objective of my thesis is to examine the mechanical role of callose, a ß-glucan, accumulated in significant amounts during pollen grain and pollen tube (male gametophyte) development. The pollen tube, a tubular structure produced by the pollen grain acts as a 'vehicle' to transport the male gametes (sperm cells) to a receptive ovule (female gametophyte) with the goal to achieve successful fertilization, eventually leading to seed formation. These tip-growing unique cells are exposed to different types of stresses such as turgor induced tension stresses in the cell wall and compressive stresses exerted by the growth matrix. Pollen tubes display a characteristic deposition of callose in the cell wall lining as well as distinct callose plugs that separate the active portion of the pollen tube cytoplasm from the degenerating segments. Although not a *conditio sine qua non* for pollen tube growth, callose plugs are suggested to be beneficial for successful fertilization. To understand the role played by callose in regulating pollen tube growth, in the first chapter I investigated the effects of mutations and enzymatic treatments on pollen tube growth behavior using mechanical assays. Combining growth assays, turgor measurement by incipient plasmolysis and high-resolution fluorescence microscopy, I was able to show that the reduction in callose deposition resulted in

tubes with lower invading capacity and higher turgor pressure when exposed to a mechanical obstacle. I also investigated the association of the actin cytoskeleton with callose deposition by exposing the tubes to pharmacological treatments and showed that actin seems to be involved in positioning and transporting callose synthases (enzymes producing callose) at the apex, sub-apex, and distal region of the pollen tube. The second chapter of my thesis is a review of the current state-of-the art of invasive capacity of a wide range of walled cells including those in plants, fungi, and oomycetes. The chapter elaborates on the role of turgor pressure and regulation of cell wall mechanical properties as well as the cytoskeleton in maintaining the invasive lifestyle of plants and fungi. The chapter also contains a mini review on various experimental approaches developed by these specialised cells to characterize this cellular behavior and to quantify the forces generated when invading a matrix.

Résumé de la thèse

Les cellules végétales se caractérisent des cellules animales par la présence de parois cellulaires qui les entourent. Chez les plantes, la paroi cellulaire joue un rôle essentiel de protection du protoplaste et est le lieu de nombreuses réactions biochimiques impliquées dans la régulation du métabolisme et de la biomécanique cellulaires. La forme et la géométrie des cellules végétales sont principalement définies par les propriétés mécaniques des différents polysaccharides de la paroi cellulaire, stratégiquement déposés lors de la différenciation et de la morphogenèse cellulaires. L'objectif principal de ma thèse est d'étudier le rôle mécanique de la callose, un ß-glucane s'accumulant en quantités importantes au cours du développement des grains de pollen et des tubes polliniques. Les tubes polliniques, structures tubulaires produites par les grains de

pollen, agissent essentiellement comme "véhicule" de transport des gamètes mâles pour la fécondation d'un ovule réceptif et la formation subséquente de graines. Ces cellules uniques caractérisées par leur croissance apicale sont exposées à différents types de contraintes, telles que les contraintes de tension induites par la turgescence de la paroi cellulaire et les contraintes de compression exercées par le milieu de croissance extracellulaire. La paroi cellulaire des tubes pollinique se caractérise d'autres parois végétales par le dépôt continu de callose formant à certains endroits des bouchons distincts qui séparent la partie cytoplasmique apicale métaboliquement très active, des segments distaux moins actifs du tube pollinique. Bien qu'il ne s'agisse d'une condition sine qua non à la croissance du tube pollinique, la présence de bouchons de callose est avantageuse pour une fécondation réussie. Afin de comprendre le rôle joué par la callose dans la régulation de la croissance du tube pollinique, j'ai, dans le premier chapitre, étudié les effets des mutations et des traitements enzymatiques sur la croissance du tube pollinique en utilisant des tests mécaniques. En combinant tests de croissance, mesures de turgescence en début de plasmolyse induite et microscopie à fluorescence à haute résolution, j'ai pu montrer que la réduction du dépôt de callose se traduit par des tubes ayant une capacité d'invasion plus faible et une pression de turgescence plus élevée lorsqu'ils sont exposés à un obstacle mécanique. J'ai également étudié la relation entre le cytosquelette d'actine et le dépôt de callose en exposant les tubes à des traitements pharmacologiques. J'ai ainsi démontré que l'actine semble être impliquée dans le positionnement et le transport des callose synthases (enzymes produisant la callose) dans les régions apicale, sous-apicale et distale du tube pollinique. Le deuxième chapitre de ma thèse est une revue de l'état actuel des connaissances sur la capacité invasive d'un large éventail de cellules entourées de parois, telles que les cellules de plantes, de champignons et d'oomycètes. Le chapitre traite des rôles du cytosquelette, de la turgescence et de la régulation des propriétés

mécaniques des cellules dans le maintien du mode de vie invasif des plantes et des champignons. Une revue des différentes approches chimiques, enzymatiques et biomécaniques développées par des cellules spécialisées est aussi détaillée. Enfin, des approches expérimentales permettant de quantifier les forces générées lors de l'invasion d'une matrice par des cellules à croissance invasive sont fournies à la fin du chapitre.

सा विद्या या विमुक्तये

Knowledge is what that liberates

-Vishnu Purana (1.19.41)

To Shrimati Radharani Adiparashakti

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Contribution to original knowledge

The growth of a plant is determined at the cellular level that ultimately has a direct impact on agricultural yield. The first part of the research contributes to predicting the role of cell wall mechanics in manipulating cellular behavior of a pollen tube's journey to an ovule (for a successful fertilization) leading to seed production, with potential agricultural impacts. The second part of the thesis expands our knowledge on plant cell morphogenesis of diverse group of walled cells in plants, fungi and bacteria displaying a characteristic' invasive growth' behavior that serves a diverse range of functions.

Introduction

The pollen tube is the fastest tip-growing cell in the plant kingdom. The cell carries the male gametes to the female gametophyte resulting in double fertilization and seed setting. To accomplish its task, the pollen tube has to elongate in polar manner and to penetrate the tissues of the pistil on its way to the ovary. Successful pathfinding requires a tight control of the spatial and temporal pattern of pollen tube growth. This involves deposition of cell wall material in large amounts to promote fast pollen tube growth and regulation of cell wall remodeling to control mechanical properties and cell shape (Mollet et al. 2013). Because of the rapid growth pattern and the ephemeral functionality of the cell, the pollen tube cell wall differs from most somatic plant cell walls both functionally and structurally. The pollen tube cell wall has various important functions which include protecting the generative cell or sperm cells from any mechanical damage, controlling the physical shape of a cell, resistance against the turgor pressure and adhesion to the transmitting tissue (Geitmann and Steer 2006). The pollen tube cell wall is a complex structure consisting of polysaccharides, proteins, glycoproteins, and several other small molecules that define its structure and function. The polarized and cylindrical growth profile is ensured by a mechanical gradient in the pollen tube cell wall characterized by a softer tip and progressively stiffer wall material at the subapical and distal region (Geitmann and Parre 2004, Fayant et al. 2010). The growth process is sustained by the continuous deposition of new cell wall material at the apex (Mollet et al. 2013). To support the high level of polysaccharide

synthesis, the pollen tube utilizes stored reserves such as sucrose, starch, lipids (Jackson and Linskens 1982, Nakamura et al. 1984) and external sources of sugar (Geitmann and Steer 2006). One of the main components forming the cell wall at the growing pollen tube tip are methyl esterified pectins (Ferguson et al. 1998). While polymers such as cellulose and callose are produced by membrane localized synthases along the tube, the pectic layer is produced through exocytosis occurring at the tube tip (Fig. 1). Methyl esterified pectin (a soft material) is transported to the tip by secretory vesicles (Parre and Geitmann 2005a). Once *in muro*, a gradual de-esterification of methyl esterified pectin takes place as the wall matures to form the cylindrical shank. This modification is mediated by the enzyme pectin methyl–esterase. These enzymes convert the methoxyl group of galacturonan to a carboxyl group that becomes negatively charged and thus facilitates cross linking of the polymers by calcium ions. This gelation enhances the stiffness of the pectins located at the sub apical area of the growing pollen tube. Esterified pectins present at the tip of the tube provide an area with low mechanical stability that yields under the turgor pressure, which results in elongation of the pollen tube in only one direction thus forming a perfect cylinder (Geitmann and Steer 2006, Geitmann and Dumais 2009, Fayant et al. 2010). The inner cell wall of a pollen tube consists of callose and cellulose, materials that are known to be load bearing (Parre and Geitmann 2005a, Aouar et al. 2010). Callose is absent from the tip of the growing pollen tube (Heslop-Harrison 1987, Parre and Geitmann 2005a), whereas the presence of cellulose at the tip varies between species (Fig. 1). Overall, the abundance of cellulose in the pollen tube cell wall is relatively low compared to other primary cell walls, but it nevertheless plays a role in controlling cell shape (Schlupmann et al. 1994, Aouar et al. 2010). In addition to the cell wall polymers, the pollen tube wall also contains proteins, arabinogalactan proteins and pollen extensin-like proteins. The mature pollen

tube cell wall in the cylindrical portion of the cell resists turgor pressure and is stronger than the wall at the apex due not only to the gelation of pectin, but also to the presence of the callosic lining. The pollen tube cell wall is therefore characterized by a very specific spatial profile of polymers which likely also influences the degree of permeability that controls entry of various ions and sporophytic proteins that determine compatibility in several species. Previous research on the pollen tube cell wall has focused on the biochemistry and localization of various cell wall polymers such as pectin, hemicellulose, cellulose and callose and a wide range of pollen tube species has been investigated. Several studies have provided transcriptomic and genomic data suggesting the involvement of specific enzymes participating in pollen tube cell wall remodeling and the regulation of cell wall mechanics. Additionally, mutant analyses have provided critical insight on important genes regulating the pollen tube cell wall biosynthesis and their function during pollen tube growth. However, despite its abundance, the role of callose, one of the main components of the pollen tube cell wall, is poorly understood. I addressed this lack of information by investigating how its deposition is regulated and what its role is for the functionality of the pollen tube. Callose is a peculiar polysaccharide that only occurs in relatively specific situations (specific tissue types, specific developmental stages) and its abundance in pollen must, therefore, have an important role for the specific pollen tube functioning.

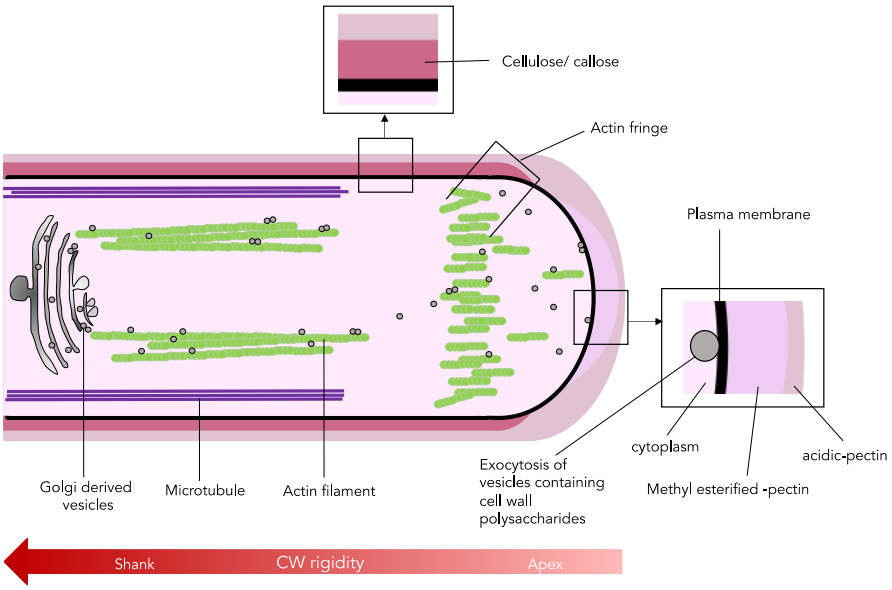

Fig 1: Pollen tube cell wall dynamics. Diagram summarizing the intracellular organization of an elongating pollen tube displaying different cell wall components and cytoskeleton.

2. Background

2.1 History of pollen research

Pollen grains are the microscopic male gametophytes that are produced by flowers and are carried by wind or insects. Pollen transfers the male gametes to the female counterparts, thus resulting in fertilization which will eventually produce a new plant generation. Pollen grains, because of their microscopic size, were not morphologically described until the seventeenth century. Two pioneers, Nehemiah Grew and Marcello Malpighi, were amongst the first to morphologically describe several pollen species with the aid of a microscope during the late 1600 (Malpighi 1687, Grew 1965). The explicit role of the pollen tube during reproduction to

carry the ‘embryonal globule’ that enters the embryo sac which forms the embryo, was proposed by Jacob Matthias Schleiden later in the nineteenth century (Schleiden 1837). In 1849, Hofmeister described specific functions of the male and female gametophytes during plant reproduction and suggested that the ovum located inside the embryo sac later forms the embryo upon fusion with the male gamete (Hofmeister 1849).

2.2 Significance of pollen

Flowers with their ornamental beauty and majestic smell are responsible for ensuring the efficient transfer of the pollen grain from an anther to the stigma of the same species. There are, however, many important economical crops and trees like wheat, barley, oats, pines, spruce that are pollinated by wind and these plants do not invest in resources that attract pollinating organs such as fancy flowers, nectar, and scent. The evolution and development of flowers have resulted in attraction of many types of pollinators such as birds, bats, insects, arthropods, that participate in plant reproduction by ensuring the transfer of male gametophyte to the female counterpart and allowing fertilization and seed setting. Thus, pollen plays an indispensable role in agronomy and horticulture. Pollen has also gained attention in various other scientific fields as the biochemical and architectural properties of pollen exine wall is very resistant to chemical, biological and mechanical stress (Scott 1994). The characteristics of the pollen exine wall—a material that in addition to cellulose, hemicellulose, lignin, pectins contains a strong almost indestructible material called ‘sporopollenin’—is exploited by archaeologist and geologists to study the presence of fossil pollen which can provide critical information about plant survival and colonization (Petit et al. 2002, Tollefsrud et al. 2008). Further, it allows retracing species origin and their evolution including extinct species (Muller 1981, Gaillard et al. 2008) and various other pieces of biologically relevant information such as plant-pollinator coevolution (Hu et al. 2012).

Melissopalynology is a branch of science where pollen is used to confirm the authenticity and quality of commercial honey, as pollen is found in abundance in honey. Pollen allergies and other health related issues have also made pollen an interesting research topic for medical research (Shahali et al. 2009, Xie et al. 2019). Pollen is also an important tool during forensic investigation as it is readily and unnoticeably attached to clothes and objects and provides hints as to the location at which these objects must have been, on the basis of species composition (Ezegbogu 2021). Pollen grains can be of varying shapes and sizes ranging from a 2 μm diameter in the Boraginaceae family to a 350 μm diameter in the Annonaceae family (Walker 1971).

2.3 Pollination and fertilization

Pollen grains are formed in the anther from sporocytes which undergo meiosis and produce microspores that develop into a mature pollen grains or male gametophytes (one male gametophyte consists of one vegetative cell and either one generative cell or two sperm cells) (Twell et al. 1998). A mature pollen is characterized by water loss and dehydrates before being shed as this enhances its ability to survive during transfer from one flower to the next. Upon compatible pollination and presence of favorable environmental conditions, the pollen rehydrates and forms a tube that elongates through the stylar tissue, enters the ovule through the micropylar end, and delivers the sperm cells resulting in double fertilization (Fig. 2) (Lord and Russell 2002). This process of sperm delivery through a cellular catheter is known as siphonogamy and is necessitated by the fact the plant sperm cells are not motile, unlike their animal analogues. The adhesion of the pollen grain to the stigmatic surface is followed by germination and penetration of the elongating pollen tube through the stigmatic, stylar and ovarian tissues to finally enter the embryo sac. This well guided series of fertilizing events involves recognition and close communication between the pollen tube and the pistillar tissues (Cheung 1996,

Geitmann and Palanivelu 2007, Dresselhaus and Márton 2009). Recognition begins as soon as a pollen lands on the stigma. Stigmas can be categorized as wet (e.g. Solanaceae) or dry (e.g. Brassicaceae) (Edlund et al. 2004). In case of a dry stigma, the pollen must go through an important hydrating step with the help of certain lipid compounds which may create a water gradient along which the tube is guided into the stigma (Wolters-Arts et al. 1998). In case of wet stigmas, hydration is a passive process supported by stigma exudates that are readily available (Swanson et al. 2004). Upon pollen germination, the elongating pollen tube penetrates the papilla cell wall in case of a dry stigma or through intercellular spaces of the wet stigmas, a process that is assisted by cell wall loosening enzymes (Fig. 2) (Cosgrove et al. 1997, Nieuwland et al. 2005). Elongation of the pollen tube following stigmatic invasion depends on the anatomy of the adjacent style which can be hollow or solid or a combination thereof. Different types of guidance cues aid the pollen tube to find its way through the style. For instance, in the hollow style of lily, pollen tube growth is guided by a gradient of chemocyanin, a pistillar protein (Kim et al. 2003). Ge et al. (2009) observed a calcium gradient in the style of tobacco following pollination, that may direct the growth of the pollen tube. Once the tube reaches the ovary, female gametophyte guidance based on production of attractants or repellents directs the pollen tubes towards an unfertilized embryo sac and prompts it to deliver the sperm cells (Dresselhaus and Márton 2009, Higashiyama 2010).

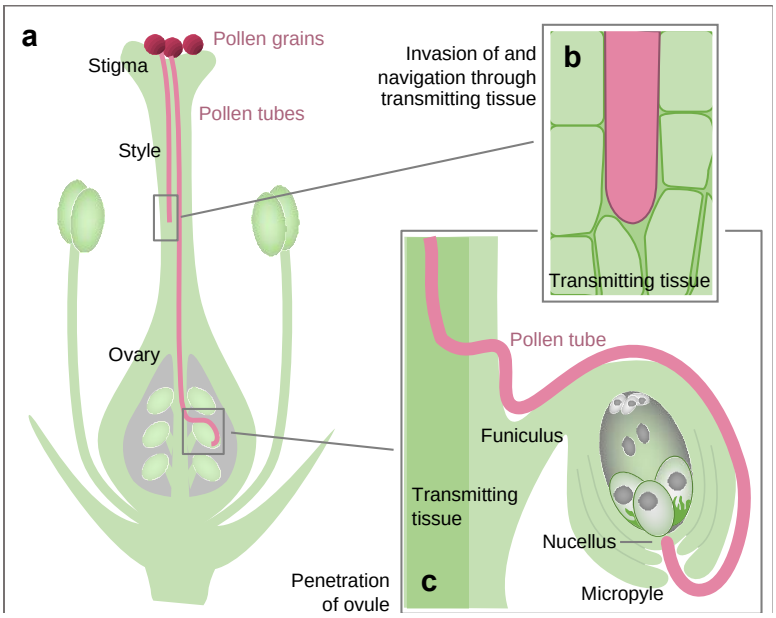

Fig 2: Pollen tube invasive growth: a) Mechanical obstacles in the pathway of pollen tube towards the ovary include the stigmatic cuticle, the apoplast of the transmitting tissue, the micropyle and the nucellus. b) Pollen tube making its way through the apoplast of the stylar transmitting tract. c) Pollen tube emerging from the transmitting tissue elongating on the surface of the funiculus, turning into the micropyle of the ovule. Adapted from (Kapoor and Geitmann 2023a).

During pollen tube elongation through the pistillar tissue, an invasive force is produced that helps the growing tube to elongate within the transmitting tissue and provides resistance against the mechanical compression forces to maintain the cylindrical shape (Sanati Nezhad et al. 2014b, Reimann et al. 2020). Generation of turgor pressure along with continuous supply of cell wall material at the tube apex are the driving forces for this growth process. Unlike the commonly observed ‘diffuse’ type of plant cell growth, the pollen tube elongates only from the tip of the cell - a characteristic shared with a few other cell types with invasive capacity such as root hairs, fungal hyphae, and to a certain degree, animal nerve axons (Sanati Nezhad and Geitmann 2013).

2.4 Pollen tube growth

The pollen tube represents a remarkable example of polarized cell expansion in plants. In lily, a mature pollen tube reaching the ovary has a length to width ratio exceeding 5000:1. Our

understanding of the morphogenetic process leading to this kind of cell shape has been largely enhanced through *in vitro* experiments which are possible since the pollen tube is perfectly able to germinate and elongate in absence of a pistil (Hepler et al. 2001b, Chebli and Geitmann 2007). Pollen tubes can grow very rapidly, for example, *Colchicum autumnale* can grow up to 2.75 cm h^{-1} which is 9000 times the diameter of the pollen grain per hour (Schleiden 1837). However, pollen tube growth rates are much faster inside the pistil when compared to the *in vitro* conditions. Since pollen tubes lack chloroplasts there is a continuous requirement of carbohydrates to support the ongoing assembly of the elongating pollen tube cell wall and to regulate the osmotic potential. Hence, when growing the pollen tubes in *in vitro* conditions, it is important to provide carbohydrates, usually in the form of sucrose, to the growth medium in addition to important micro elements including boron, calcium, magnesium, nitrogen and sulfur (Brewbaker and Kwack 1963). A pollen tube consists of hemi-spheroidal tip followed by cylindrical shank region. The cytoplasm at the tip of the pollen tube is characterized by a 'clear zone' as larger organelles such as amyloplasts, starch grains, Golgi bodies and vacuoles are restricted from moving into this region (Heslop-Harrison and Heslop-Harrison 1990, Lovy-Wheeler et al. 2007). Smaller organelles such as mitochondria, components of the endoplasmic reticulum, and vesicles on the other hand can be found in this apical region of the growing pollen tube and in particular vesicles are abundant in this part of the cell (Lancelle and Hepler 1992). These vesicles supply newly synthesized cell wall material which is deposited at the apical plasma membrane through exocytosis (Franklin-Tong 1999, Bove et al. 2008, Zonia and Munnik 2008) (Fig 1). Also excluded from the clear zone is the male germ unit, comprised of the vegetative nucleus and the two sperm cells. This aggregate moves forward in the tube at the same speed as the tip grows and stays at a constant distance from it (Rounds et al. 2010, Bou

Daher et al. 2011). In pollen tubes, organelle transport is mediated by both actin filaments and microtubules (Lovy-Wheeler et al. 2007, Romagnoli et al. 2007). Inhibiting microtubule polymerization using oryzalin does not affect cytoplasmic streaming, however, whereas interfering with actin functioning using cytochalasin or latrunculin B disrupts both pollen tube growth and cytoplasmic streaming (Gibbon et al. 1999, Vidali and Hepler 2001). At the growing end of the tube, it is primarily an actin-myosin motor system that delivers secretory vesicles to the apex (Bou Daher et al. 2011, Cai et al. 2011), but the motion through the tip region may be driven by diffusion or convection (Kroeger et al. 2009). Cai and coworkers have proposed a model explaining the function of microtubule-based motor in transporting vesicles and organelles slowly and precisely over short distances (Cai et al. 2017).

Pollen tubes share their polar and radially symmetric growth pattern with root hairs, fungal hyphae, and neurons, which has made this cell type ideal to study a variety of aspects of cell growth, including the mechanical aspects of the growth process. Given its intriguing growth pattern and mechanical function, the pollen tube has become a research model for interdisciplinary investigation combining biological approaches with physics and mathematical sciences.

2.5 Pollen tube tropism

On its way through the pistil, the pollen tube must respond to the external cues offered by the female tissues, and it has to change direction when required to attain its target (Cheung and Wu 2001, Geitmann and Palanivelu 2007). Genetic and physiological studies have suggested that directional cues are synthesized by the pistil (Cheung et al. 1995, Palanivelu et al. 2003) and by the female gametophyte (Higashiyama et al. 2003, Palanivelu and Preuss 2006, Márton and Dresselhaus 2010) to assist the pollen tube to reach its target, the embryo sac. Some of the

molecules that have been identified to play a role in reorientation of pollen tube include receptor kinases, cell membrane associated G proteins along with fluctuation in ion fluxes (Hepler et al. 2001b). Additionally, nitric oxide has also been suggested to regulate pollen tube guidance in species like lily and Arabidopsis by influencing calcium influx to the pollen tube (Prado et al. 2004, Prado et al. 2008).

Comprehensive molecular research has been done to identify the key players involved in determining the polarity of the growing pollen tubes, however the mechanism behind the unidirectional growth behavior in response to external cues needs to be determined keeping in mind the mechanical aspect of the growing pollen tube. The biosynthesis and the targeted delivery of soft cell wall material to the tip of pollen tube is ensured to confine surface expansion to the apical part of the pollen tube. Change in the directionality of growing pollen tube, hence, must be achieved via vesicle trafficking towards an area that is located off the center axis of the pollen tube. Thus, it is very important to recognize and comprehend the critical mechanism of vesicular trafficking to understand the tropic growth response of a pollen tube (Bove et al. 2008). During vesicular delivery, myosin is involved in mediating the motion of these cellular organelles along actin filaments and together both myosin and actin are potential regulators of the spatial targeting. Binding of calmodulin activates the plant myosin and dissociation of calmodulin triggered by Ca^{2+} deactivates them (Vidali and Hepler 2001). The deactivation is suggested to be crucial during the final step of vesicle delivery - their detachment from the actin cytoskeleton close to the apical plasma membrane. Since the cytosolic calcium concentration is elevated near the region of exocytosis, a critical role of this ion is suggested to assist vesicles to be released from the actin array into the actin filament free region (apex of the tube) or directly onto the plasma membrane (Bou Daher et al. 2011). To unravel the relationship between actin

dynamics, vesicular movements, and directional growth control it is beneficial to be able to experimentally induce change in direction of pollen tubes growing in *in vitro*. These *in vitro* experiments can be performed by exposing the pollen tubes to chemical signals (Higashiyama and Hamamura 2008) or electric fields (Malhó et al. 1992, Bou Daher and Geitmann 2011). The reorientation observed was suggested to be regulated by ion fluxes in the medium (Malhó et al. 1992). The intensity of this reorientation response was also found to be dependent on calcium concentration in the medium (Nakamura et al. 1991). Ion fluxes are important for pollen tube growth and orientation; however, they alone cannot act directly on change in direction and orientation of pollen tube as their effect is regulated by structural features such as the cytoskeleton and polysaccharides that are synthesized and delivered continuously to form new cell wall material.

2.6 Pollen tube cell wall synthesis and deposition

The organization of cell wall structure is regulated by systematic deposition of different polysaccharides which must be precisely organized to achieve characteristic rapid growth with geometrically correct and efficient cellular morphogenesis. Like other plant cells, cell wall synthesis in pollen tubes involves secretion of specific polysaccharides and glycoproteins along with the local synthesis of polysaccharides at the plasma membrane level. The two processes are regulated by the cytoskeleton although by partially different processes.

The pollen tube apex is characterized by the deposition of methyl-esterified pectins (Li et al. 1994, Li et al. 1995, Bosch and Hepler 2005) (Fig. 1). Pectins are soon converted into their de-esterified (acidic) form to allow tight cross-linking with calcium ions resulting in progressive strengthening of cell wall and maintaining the cylindrical shape of the pollen tubes (Zerzour et al. 2009). Since the secretion of pectin takes place at the apex, it is proposed that pectin

containing vesicles are transported along the actin filaments. Parre and Geitmann (2005b) have suggested that the distribution of pectin and thus the level of cell wall stiffness are closely associated with cytoskeleton organization. This proposition was later supported by using proteasome inhibitors such as MG132 and epoxomicin. The use of these inhibitors caused disruption of the cytoskeleton which resulted in reduced cytoplasmic streaming and consequently in accumulation of acidic pectin and esterified pectins in the sub apical and apical regions respectively, and a reduction of cellulose in the sub apical region of the tube (Sheng et al. 2006).

Localized synthesis of polysaccharides serves many functions. For instance, polysaccharides can be produced in a very systematic and localized manner which is achieved by insertion of synthesizing enzymes at specific sites on the plasma membrane. In case of pollen tubes, the insertion site is the apical plasma membrane (Brownfield et al. 2008, Cai et al. 2011). Secondly, the enzymes that are inserted into the plasma membrane are modulated by various internal (substrate availability) and external factors (signals from other cells) that can influence the pattern of polysaccharide deposition (Lei et al. 2017).

Cellulose and callose are two important polysaccharides synthesized in the pollen tubes but with some remarkable differences. The main difference is the amount being produced with the level of callose being much more abundant than that of cellulose (Geitmann and Steer 2006). Callose accounts for almost 80% of the inner cell wall layer in most pollen tubes. In most other plant tissues, callose is produced in very small quantities and transiently, for example during cell division. In some tissue, callose synthesis is triggered under special conditions such as biotic or abiotic stress, dormancy, or during microsporogenesis (Ellinger and Voigt 2014).

In addition to inner cell wall lining of a pollen tube, callose is also deposited in the form of plugs in fast growing pollen tubes (Snow and Spira 1991, Chebli et al. 2012). The deposition of

amorphous callose in the cell wall lining is known to provide the resistance against tension/ compression stress. This mechanical role of callose was suggested by degrading callose using lyticase (callose degrading enzyme) that resulted in tubes with increased diameter, reduced cellular stiffness and increased viscoelasticity of the distal segment of the pollen tubes (Parre and Geitmann 2005a). On the other hand, the role of callose plugs is thought to maintain the cytosolic volume of the active part of the cell constant and separating the growing tip from the old non turgescent part. This may be important to keep the volume of active cytoplasm within a certain range for the tube to quickly regulate its turgor pressure upon disturbance, but evidence for this is lacking. In theory, a higher turgor pressure could relate to increased growth rate but in liquid medium, the turgor does not correlate with growth rate (Benkert et al 1997).

Cellulose, on the other hand is present in relatively low amounts but fulfills an important function. The pharmacological inhibition of cellulose synthesis has resulted in deformed geometry and reduced growth of the pollen tube (Aouar et al. 2010). Hence, the two polysaccharides have different functions along with different deposition pattern. Where cellulose can be present in apical and distal parts of the pollen tube, callose is only produced 10-20 μm away from the apex (Chebli et al. 2012). As callose is not present in the subapical region, the mechanical role of cellulose microfibrils might be particularly relevant here. The transition region between the apex and the shank is important for pollen morphology as this region essentially determines the diameter of the cell and the tensile stress in the distal part reaches its maximum values. Thus, cellulose is suggested to play an important role in determining the diameter of the growing cell. This is crucial, because the diameter of the pollen tubes needs to be maintained to (a) provide a large enough diameter for the male germ unit to pass through the tube (b) to maintain the smallest possible diameter for easy penetration through the transmitting

tissue of the style. Hence, the diameter of the pollen tube helps to achieve the two mechanical requirements and thus must be tightly controlled.

2.7 Dynamics of the cytoskeleton

Pollen tubes, like other plant cells, display an extensive cytoskeletal network which is highly organized and dynamic and plays a crucial role during pollen tube growth (Gossot and Geitmann 2007, Fu 2015, Xu and Huang 2020, Chebli et al. 2021b). Both actin and microtubules are present along the length of pollen tube displaying some contrasting features. Extensive research both *in vivo* and *in vitro* has been done on actin filaments and microtubules using selectively labeled probes, reporter proteins and antibodies. Research done so far on these two components has characterized the organization of actin filaments and microtubules in pollen tubes of many species such as lilly, tobacco (Cheung et al. 2008). Microtubules are arranged longitudinally along the growth axis, but are largely absent from the tip. Actin filaments occur in thicker bundles in the shank and in a distinct array known as the actin fringe, located close to the apex of the pollen tube (Bou Daher and Geitmann 2011). While the role of actin cytoskeleton in vesicular trafficking with the pollen tube cytoplasm is well defined (Lovy-Wheeler et al. 2007, Bove et al. 2008), its role in the mechanics of the pollen tube tip growth is elusive. Pharmacological interference, for example using Latrunculin B, blocks actin polymerization during pollen tube elongation and results in inhibition of cytoplasmic streaming (Geitmann et al. 2000, Vidali and Hepler 2001). This implies that actin polymerization is closely linked with the mechanical process of pollen tube elongation, as interference with cytoplasmic streaming perturbs the vesicular trafficking to the plasma membrane and may hinder the fusion and delivery of cell wall components through exocytosis at the growing site of the tube (Geitmann 2010). Pharmacological treatments to stabilize or destabilize microtubules have highlighted their

indirect role in short distance trafficking and positioning of cell wall synthesizing enzymes during pollen tube growth (Cai et al. 2011) and establishing directionality of pollen tube growth (Gossot and Geitmann 2007).

2.7.1 Microtubules

Despite comprehensive knowledge on the role of microtubule in various plant stages such as membrane trafficking, somatic cell division, growth and morphogenesis, their role in cells displaying polarized growth such as pollen tubes is still poorly understood. The localization of microtubules somewhat depends on the technique used to visualize them. For instance, immunolocalization techniques may result in disrupted organization and dynamics of microtubules especially in the area where they are delicate. Hence, it is beneficial to observe them in *in vivo* conditions. Our knowledge of the molecular regulation of microtubule dynamics is still poor regarding pollen tube growth. The scenario is even more complicated by the fact that inhibitors of microtubule polymerization do not have visible effects on the pollen tube growth rate (Lovy-Wheeler et al. 2007).

Pharmacological inhibition of microtubule polymerization results in falling behind of male germ unit or no forward movement at all, thus suggesting their crucial role in transporting of male germ unit during pollen tube elongation (Chebli et al. 2013). However, knowledge about the proteins that interact with microtubules is still quite limited. Until now, most of information that is available relates to microtubule associated proteins with motor capabilities. Biochemical approaches have identified two out of three kinesins weighing about 90 and 100 kDa associated with Golgi vesicles of the pollen tube. They are involved in the transport of secretory vesicles (Cai and Cresti 2010). In *in vitro* assays, kinesins work at a comparatively slower speed than the actual velocity observed in pollen tubes, suggesting that the molecular motors are associated with

processes that are perhaps slower but require more accuracy. A recognized model but not yet sufficiently established has proposed the role of microtubule-based motor transport for vesicles and organelles for shorter distances, slowly and rather more accurate. Also, few of these motors may be associated with transportation of enzyme complexes that produce cellulose and may even carry molecules essential for cellulose microfibril assembly (Cai et al. 2017).

Even though several microtubule-associated proteins have been identified in plant cells, their characterization in pollen tubes is limited. Microtubules present in the cortical region of pollen tubes are structured in bundles and aligned along the pollen tube growth axis suggesting the presence of proteins assisting in their bundling (Cai et al. 2017). Although microtubules play an important role in maintaining the male germ unit in the apical region of the elongating pollen tube, the role of this cytoskeletal array in the context of cell wall mechanics remains an unanswered question, since the use of microtubule depolymerizing agents had no significant effect on pollen tube growth.

2.7.2 Actin filaments

Structural organization of actin is very important in order to fulfill its role in tip growing cells. For instance, in the distal area of a pollen tube, actin bundles are arranged in a parallel fashion, aligning themselves next to each other to perform long-distance vesicle trafficking. However, the actin fringe in the apical region consists of individual rather than bundled filaments that polymerize continuously to keep up with the elongating tip (Xu and Huang 2020). In addition to the actin fringe, a group of short, highly dynamic, actin filaments is localized below the pollen tube tip. It is suggested that this population is in between monomeric actin and newly organized actin filaments that finally transforms into the actin fringe (Qu et al. 2015). Therefore, structural

organization of actin can be roughly categorized into three subgroups: longitudinal cables, short actin filaments and actin fringe.

Several factors such as ions, reactive oxygen species (ROS) and proteins are known to regulate the assembly and disassembly of actin filaments (Cai et al. 2017). This molecular system enables the cell to mediate internal and external cues to regulate the speed and growth direction of the pollen tube (Dong et al. 2012). For instance, overall extension of the actin fringe is known to positively influence the growth rate, whereas the lateral extension of the actin fringe is associated with growth direction (Cai et al. 2017). Pectin, one of the main polysaccharides that is inserted into the expanding cell wall at the pollen tube tip, is transported in the vesicles transported on the actin array. Since the spatial distribution of their delivery and exocytosis is crucial for the regulation of the morphogenetic process, the actin fringe fulfills an important regulatory role. Using pharmacological agents that inhibit the polymerization of actin and reduce pollen tube growth, researchers observed pectin accumulation in a wide arc in the pollen tube tip, rather than at a small polar region, implying that the actin fringe participates in the targeted secretion of pectin in the pollen tube cell wall and therefore, regulates the polarized growth of the pollen tubes (Rounds et al. 2014).

The organization of actin filaments is an elaborate process that relies on the activity of several accessory proteins, also known as ’actin binding proteins’ (ABP) (Staiger et al. 2010). ABP are responsible for a variety of functions regulating the dynamics of actin arrays and mediating organelle transport along them. Additionally, many proteins, such as Rho-of-plant (ROP) proteins are known to be important regulators that interact with RIC3 proteins. The latter protein further determines a local increase in calcium levels (Zhao and Ren 2006) and is recognized as a modulator of actin filament assembly (Cárdenas et al. 2008). These ions and specific proteins

with different and localized concentration participate in organizing the actin structures each with a defined role.

2.8 Cytoskeleton based delivery of callose synthase

The mechanism behind the insertion of callose synthase into the pollen tube plasma membrane is still unclear. Molecular research suggests that callose synthase is transported from the Golgi via secretory vesicles, which deliver the enzyme to the cell surface (Brownfield et al. 2008). Since Golgi derived secretory vesicles are transported along actin filaments, it is hypothesized that actin filaments play a crucial role in transporting the callose synthase to the plasma membrane of the pollen tube. However, the insertion site is not yet identified properly. Immunolocalization studies have suggested that the insertion of callose synthase occurs in the apical plasma membrane. This localization pattern of callose synthase is associated with the actin filaments since application of both actin and myosin inhibitors can significantly alter the apical distribution of CalS (Cai et al. 2011). If we assume that callose synthase is inserted at the pollen tube apex, then we must propose that the enzyme inserted is in 'inactive' form given the absence of callose in the tip of growing tubes. As mentioned, callose deposition at the apex of the pollen tube tip would lead to stiffening of the cell wall, thereby disrupting the elongation of pollen tube and ultimately leading to growth arrest. Additionally, proteolytic enzyme assays have also suggested that callose synthases are inserted in dormant form at the plasma membrane and to activate the enzyme a peptide segment is removed once the insertion of CalS into the plasma membrane has taken place (Li et al. 1999). Other evidence for activation of callose synthase was obtained by localizing a GFP-fused Cals5 in BY-2 cells of tobacco (callose synthase 5 is highly expressed in growing pollen tubes) revealing its presence in Golgi membranes and the plasma membrane.

However, the deposition of callose was only detected outside the plasma membrane (Xie and Hong 2011).

The regulation of callose synthase activity once inserted into the plasma membrane is still not clear as there is not enough evidence to conclude whether the enzyme operates exclusively between sub apical and apical region (where peptide removal takes place) or whether its activity is also required at the distal region. Using fluorescence microscopy, imaging for specific callose synthase probes suggests that callose is accumulated in relatively homogeneous thickness along the axis of the pollen tube (Parre and Geitmann 2005a, Chebli et al. 2012). Taking this into account, we can assume that callose synthase is present in its active form and starts synthesizing callose once the plasma membrane it is associated with becomes part of the subapical portion of the growing pollen tube. Since the thickening of the callose layer does not continue indefinitely, it is thought, that the enzyme is subsequently inactivated or removed to prevent excess callose production. Immunolocalization data have demonstrated the accumulation of callose synthase in the apex and sub-apical region of the pollen tube, but it is scarcely present in the more distal region of the cell, thus suggesting a removal rather than inactivation mechanism (Cai et al. 2011). The application of both actin and microtubule depolymerizing agents had no effect on thickness of callose suggesting that cytoskeletal filaments have no role in regulating the thickness of callose once it is synthesized (Laitiainen et al. 2002, Cai et al. 2011). While the deposition of callose seems relatively uniform along the pollen tubes, images obtained using scanning electron microscopy indicate that callose is accumulated in a pattern that resembles the ring like deposition pattern of pectins (Derksen et al. 2011, Cai et al. 2017), which could behave like a ‘primer’ on which callose is deposited.

Callose is also deposited in the form of ‘callose plugs’, central septum-like invaginations of the plasma membrane driven by massive callose deposition that is spatially confined (Cresti and Van Went 1976). The process results in physical isolation of cytoplasmic compartments, dividing the older region of a growing pollen tube from its active portions. This likely helps the growing cell to save energy and facilitates maintaining the turgor pressure in the growing portion of the cell. The development of callose plugs is thought to represent a critical evolutionary step which is most likely associated with the increase in the maximum growth rate that is typical for angiosperms (Williams 2008, Qin et al. 2012). The pollen tube wall of gymnosperms, such as in *Pinus* sp., shows similarity with the pollen tube wall of many angiosperm species in terms of the gradual differentiation of the primary cell wall into fibrillar layer, but the distribution of callose and pectin is different and no callose lining or plugs are observed. In pine tubes, callose was transiently present in young pollen tube tips and absent in older tubes (Derksen et al. 1999). Callose plug synthesis is probably regulated by the cytoskeleton. The use of a microtubule depolymerizing agent (oryzalin) results in temporary incorrect deposition pattern, but not in complete absence of callose plugs (Laitiainen et al. 2002). These results indicate that the microtubules may play a role in either regulating the timing or the site of callose plug formation rather than activating the callose synthase enzymes. H^+-ATPase in the plasma membrane are also known to regulate correct deposition of callose plugs (Certal et al. 2008), although the mechanism in which the proton flow rate may affect the enzymatic activity of callose synthase remains to be elucidated. Based on immunolocalization, it was proposed that in the distal region of the pollen tube, actin filaments distribute Golgi bodies and that CalS is inserted into the plasma membrane via microtubules (Cai et al. 2011). This notion is supported by the fact that oryzalin (microtubule depolymerizing agent) alters the number and position of callose plugs

(Laitiainen et al. 2002). The insertion area is large enough to progressively separate the active part of the pollen tube from the older segment and seems to rely on microtubule alignment. Hence, it is presumed that microtubules assist in the insertion of callose synthase in larger areas which result in formation of callose plugs.

2.9 Role of callose during somatic plant growth and development

Callose is not a component present in all types of cell walls, but it is ubiquitous in higher plants, where it is a key component of specialized cell walls or related structures at distinct phases of growth and development (Stone and Clarke 1992). Callose is deposited during many growth and differentiation events ranging from pollen development (Stone and Clarke 1992, McCormick 1993), cell plate formation during cytokinesis (Samuels et al. 1995), plasmodesmata connections to regulate cell movements (Zavaliev et al. 2011, Wu et al. 2018). Accumulation of callose can also be triggered by wounding, pathogen attack, aluminum, abscisic acid and several other physiological stress (Stone and Clarke 1992).

2.9.1 Callose in cell plate formation

In higher plants, cell plate formation is the first visible evidence of cell wall formation between the two daughter nuclei following mitosis. Immunolocalization studies suggest that callose is the luminal component of cell plate formation (Samuels et al. 1995). Deposition of callose is followed by accumulation and organization of cellulose and several other cell wall polymers. The callose deposited in the cell plate is later degraded by enzyme β 1,3-glucanase (Levy et al. 2007). The transient deposition of callose is hypothesized to provide mechanical support and to stabilize the delicate tubular network during the different stages of cell plate formation, until the deposition of cellulose increases its rigidity (Samuels et al. 1995). Callose synthases must be inserted directly into the plasma membrane enveloping the growing plate since callose was not

detected in cell plate targeting vesicles. Experiments using BY-2 cells demonstrated that callose synthase *AtGSL6* is present at developing cell plates and the enzyme interacts with two other cell plate associated proteins: UDP-glucose transferase and phragmoplastin. The three proteins together form a large protein complex on the cell plate (Hong et al. 2001). In contrast, Chen and Kim (2009) observed T-DNA knockout mutants of *gsl6* with no detectable cell plate phenotype as callose was detected in the cell plate of the mutants suggesting that more than one callose synthase gene may be involved during cell plate formation. Additionally, Thiele et al. (2009) established *GSL8/MASSUE* mutants and observed seedlings to be lethal displaying cytokinesis deformed phenotypes. The above results imply that callose is indeed critical for plant cytokinesis by producing callose at the cell plate.

2.9.2 Plasmodesmata regulation by callose

In higher plants, plasmodesmata (PD) connect all the cells symplasmically which further controls the transport of nutrients, signals, and molecules. The movement of this wide range of molecules through these symplasmic junctions is regulated by both callose dependent and callose independent mechanisms (Lucas et al. 2009, Sager and Lee 2014, Iswanto and Kim 2017). Callose independent mechanisms involve the regulation of the frequency and the structure of the PD, for example the change from a simple to complex branched form. Callose independent PD regulation may also involve the actin cytoskeleton and the permeability can be regulated by PD gating proteins (Lucas et al. 2009, Sager and Lee 2014). Callose dependent regulation of PD is associated with the deposition of callose in the PD neck zone. High accumulation of callose in the neck closes the PD channels and low levels of callose deposition correspond to their open state (Wu et al. 2018). The functional state of PD in a given cell or tissue depends on the external

cues received by the plant. Callose deposition can also be triggered in PD in response to abiotic and/or biotic stresses.

Callose deposits at the PD are shown to have a function in maintenance of dormancy by confining the meristem from symplasmic transport with surrounding tissues. Research done by Rinne et al. (2005) states that providing a short photoperiod can trigger the transition of two concentric symplasmic domains in the shoot apical meristem of birch into exclusively symplasmically confined cells through the synthesis of callose plug formation at the associated PD. Interestingly, on giving a chilling treatment, the shoot apical meristem can restore its symplasmic domains and can break its bud dormancy. This restoration is mediated by the enzyme β-1,3 glucanase because β-1,3 glucan was degraded from PD during chilling treatment.

2.9.3 Callose deposition during stress response

Callose as described above plays critical roles in many aspects of plant growth and development. Callose is also deposited in the plasma membrane and cell wall interface as a response to wound stresses. Callose deposition can be detected within minutes of damage by mechanical, chemical, or ultrasonic treatments and during abiotic and biotic stress responses such as high or low temperature, or pathogen infection. Independent research has reported GSL5 to be involved in callose production in sporophytic tissues in response to wounding or pathogenic attack. The *gsl5* loss of function mutant is unable to produce callose at stigmatic papillae. *Gsl5* mutants result in plants prone to pathogenic attack. These data suggest that callose deposition exhibits a positive effect on plant defense against pathogenic infection as the removal of callose in the *gsl5* mutant can activate the defense system (Jacobs et al. 2003).

2.9.4 Callose deposition during trichome development

The surface layers of land plants have undergone dynamic evolution of their epidermal layer that resulted in formation of various derived structure such as trichomes, across species. Even within the same species, these trichomes can be of multiple forms. Trichomes have in recent years become a popular model to study genetic developmental analyses, cell wall patterning and various other morphogenetic studies. They have been linked to play important role in providing resistance to the plants against various abiotic (Ehleringer 1982, Pérez-Estrada et al. 2000) and biotic stress response (For review,Riddick and Simmons 2014). In Arabidopsis, trichomes are unicellular, that undergo four endo reduplication cycles (Schnittger and Hülskamp 2002), polarized growth consisting of a bulged stalk and three to four branches. In mature Arabidopsis trichomes, the cell wall display thickenings at the base of the stalk in the formation of a ring which is rich in callose, also known as the Ortmannian ring (Kulich et al. 2015). The Ortmannian ring apparently divides the trichome into two segments: the basal and the apical (consisting of the stalk and branches) and is proposed to play structural role (Kulich et al. 2015).

2.10 Callose during male gametophyte development

Callose is a linear polymer made up of β-1,3 glucose residue in addition to β-1,6 branches. The biosynthesis of callose requires UDP-glucose as a substrate. Biochemical and molecular analyses (Verma and Hong 2001, Brownfield et al. 2008) in many plant species have provided evidence for the synthesis of callose by callose synthase enzyme. In the model plant *Arabidopsis thaliana* twelve genes have been identified that encode for putative callose synthase enzymes (Verma and Hong 2001, Brownfield et al. 2008). Somewhat inconsistently, two different nomenclatures have been established for Arabidopsis genes. Desh Verma and coworkers use callose synthase system (*CalS*) to name the twelve genes *AtCalS1-AtCalS12* (Verma and Hong 2001) whereas the

Somerville group refers to them as glucan synthase like (*AtGSL1-AtGSL12*)(Richmond and Somerville 2000).

Past studies have proposed the role of several *GSLs* during *Arabidopsis* pollen development. According to Enns et al. (2005) *GSL1* and *GSL5* are important during pollen development. These two genes are responsible for callose wall synthesis during microsporogenesis and are also expressed during pollen grain germination. Two contradictory results have been put forward about the role of *GSL2* during exine formation and patterning of microgametogenesis and pollen grain viability. Dong et al. (2005) established two T-DNA knock-out mutants of *cals5* (*cals5-1* and *cals5-2*) and reported that the *cals5* mutants exhibited male sterility and displayed absence of callose wall which influenced the exine wall patterning of the developing microspore. In addition, the mutant microspore also had an extracellular pollen coat of tryphine, which was deposited irregularly on the surface of the microspore. As a result, the microspores did not survive, and the pollen wall was collapsed. In contrast, Nishikawa et al. (2005) also identified three additional T-DNA alleles of *cals5*: *cals5-4*, *cals5-5* and *cals5-3* with similar altered exine pattern but with production of viable pollen. Additionally, mutation of *cals5-3* produced pollen tubes that lacked the inner callose lining and callose plugs. However, these tubes were able to fertilize and produce viable seed set, demonstrating that neither the structured exine nor the callose lining in the pollen tubes or the plugs are critical for pollen development and viability. On the other hand, a study by Töller et al. (2008) established both *gsl8* and *gsl10* mutants to be lethal and were not able to recover homozygous mutants for either genes. The researchers further observed specific malfunctions associated with division of microspores as well as unsuccessful entry of mutant microspores into mitosis. The authors further proposed that the two gene products may interact indirectly with other proteins rather than acting alone. Interestingly, Dong

et al. (2005) performed yeast two hybrid screening and demonstrated that the amino terminus of *AtGSL6* may interact with a lectin containing receptor kinase (*LecRLK1*) (Richmond and Somerville 2000). It will be fascinating to see similar interactions for *GSL8* and *GSL10* with RLKs which may regulate microspore undergoing mitosis.

Callose plugs are known to act as mechanical barriers that prevent the cytoplasm with the male germ unit from flowing backwards into the distal portion of the elongating tube or into the grain (Fig 3). Jensen and Fisher (1970) further suggested that the callose plug accumulation prevents the growing tube from shrinking. Tsinger and TP (1961) have also implicated callose plugs in providing the pollen tube cell wall the resilience towards factors like pressure, dehiscence, and mechanical tension. The callose present in the tube wall also participates in maintaining the osmotic balance (Cresti and Van Went 1976). Callose plug deposition is known to be associated with pollen tube growth rate in species such as *Hibiscus* (Snow and Spira 1991), where pollen tubes that are longer because of their faster growth rate feature have a higher number of callose plugs. The causality relationship between growth rate and pollen tube length was not clear from this study, but evidence was provided by Zhang et al. (2008) who observed pollen tube performance in anti-sense mutant lines for pollen receptor kinase (*LePRK2*) of tomato. The tubes in these mutant lines display abnormal accumulation of callose plugs and reduced pollen tube growth rate supporting the notion of a correlation. Inversely, Petunia anti-sense lines for *SHY* (protein interacting with *LePRK2*) lack periodic callose plug deposits and instead accumulated dense streaky callose throughout the length of the tubes and at the tip of the tubes and while the pollen tubes grow, the majority fails to target the ovules (Guyon et al. 2004). While these observations suggest a role for callose plugs in supporting pollen tube growth, the evidence is not necessarily conclusive since for example the mutant of callose synthase 5-3 *(cals5-3)* displays

normal pollen growth both *in vivo* and *in vitro* in Arabidopsis (Nishikawa et al. 2005). Whether the lack of a dramatic effect on pollen tube performance is due to genetic redundance remains to be investigated.

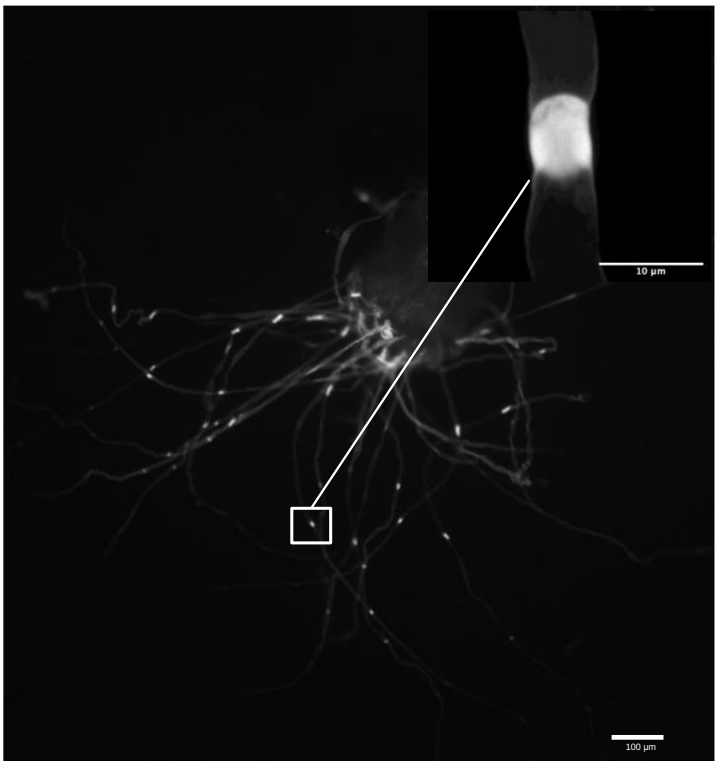

Figure 3: Semi *invivo* set up for Arabidopsis pollen tube germination. Pollinated stigma head is cut and placed on the surface of a layer of agarose – stiffened media placed on a microscopic slide. Callose plugs and callose cell wall lining labeled with aniline blue (white) in 6 hour growing pollen tubes.

Connecting statement 1

To address the lack of knowledge on the role of callose in the pollen tube's growth and mechanics, I used *Arabidopsis* callose synthase mutants in my study. Arabidopsis callose synthase mutants had reduced callose abundance in their cell wall and lacked callose plugs at the distal segment.

The third chapter of my thesis is broadly divided in two parts: the first part provides insight on the role of callose in pollen tube's cell mechanics. Several biomechanical and cell biology approaches are employed on callose synthase mutants such as stiffened media assays and incipient plasmolysis. These experiments support our hypothesis that callose abundance is indeed beneficial for the pollen tube's invasive capacity and that pollen tube's performance is compromised as a result of lack of callose. The second part of this chapter focusses on regulatory mechanism of callose deposition in pollen tubes. Various biochemical and cell biology approach in addition to different microscopy techniques along with application of highly specific antibodies against callose synthases are used to observe the localization of callose and its regulation at single cell level. I also investigate the role of actin cytoskeleton in trafficking and positioning of callose synthases along the plasma membrane (apex and sub apex region of pollen tube) and near the callose plug deposition area. To support our results from immunolocalization experiments we expose the pollen tubes to different actin polymerizing drugs and have observed changes in callose deposition pattern in elongating pollen tubes. Thus, further confirming the role of actin cytoskeleton in callose regulation.

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3. Pollen tube invasive growth is promoted by callose

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Arabidopsis thaliana, Pollen tube, Cell wall, Callose, Callose synthases, Invasive growth, Actin

3.1 Abstract

Callose, a β-1,3-glucan, lines the pollen tube cell wall except for the apical growing region, and it constitutes the main polysaccharide in pollen tube plugs. These regularly deposited plugs separate the active portion of the pollen tube cytoplasm from the degenerating cell segments. They have been hypothesized to reduce the total amount of cell volume requiring turgor regulation, thus aiding the invasive growth mechanism. To test this, we characterized the growth pattern of *Arabidopsis* callose synthase mutants with altered callose deposition patterns. Mutant pollen tubes without callose wall lining or plugs had a wider diameter but grew slower compared to their respective wildtype. To probe the pollen tube's ability to perform durotropism in the absence of callose, we performed mechanical assays such as growth in stiffened media and assessed turgor through incipient plasmolysis. We found that mutants lacking plugs had lower invading capacity and higher turgor pressure when faced with a mechanically challenging substrate. To explain this unexpected elevation in turgor pressure in the callose synthase mutants we suspected that it is enabled by feedback-driven increased levels of de-esterified pectin and/or cellulose in the tube cell wall. Through immunolabeling we tested this hypothesis and found that the content and spatial distribution of these cell wall polysaccharides was altered in callose-deficient mutant pollen tubes. Combined, the results reveal how callose contributes to the pollen tube's invasive capacity and thus plays an important role in fertilization. In order to understand how the pollen tube deposits callose, we examined the involvement of the actin cytoskeleton in the spatial targeting of callose synthases to the cell surface. The spatial proximity of actin with locations of callose deposition and the dramatic effect of pharmacological interference with actin

polymerization suggest a potential role for the cytoskeleton in the spatial control of the characteristic wall assembly process in pollen tubes.

3.2 Introduction

A common characteristic in the cell walls of plants, bacteria and fungi is the presence of ß-glucans which play a crucial role during various developmental processes and adaptation to environmental challenges (Brown and Gordon 2003, Brownfield et al. 2009, Abou-Saleh et al. 2018). Due to their physical and mechanical properties, this diverse group of polysaccharides is exploited by various industries. For example, ß-glucan-based cell wall biopolymers are used as raw material for paper, textiles, various pharmaceutical products, in tissue engineering, prosthetics and nanostructured materials (Zhang and Edgar 2014, Ferreira et al. 2016, Carreño et al. 2017). Although it is well known that *in planta*, ß-glucans play crucial roles in plant growth and development, many aspects related to these polysaccharides are poorly understood. One of the reasons for this knowledge gap is the complex structure of these polymers which makes the investigation of their synthesis, regulatory functions and mechanical properties challenging.

A prominent member of the ß-glucans is callose, a ß-1,3 glucan that features ß-1,6 branches. Callose is not abundant in most plant cell walls, but it does play important regulatory roles in cell-to-cell signalling, dormancy, defence and wound response (Thiele et al. 2009, Xie and Hong 2011, Wang et al. 2021). Many occurrences of callose are transient. For example, callose is deposited during cell division at the time of cell plate formation and, during phloem development, in the sieve pores that connect the phloem vascular system (Chen and Kim 2009, Barratt et al. 2011). Callose is also deposited at plasmodesmata—symplasmic nanochannels that connect the plasma membranes and cytoplasm of neighbouring cells—and are crucial for

intercellular communication and molecular exchanges (Wu et al. 2018). Callose plays a key role in the defense against various abiotic and biotic stresses, as it is deposited in response to wounding and pathogen attack (Xie et al. 2011, Wang et al. 2021). In addition to its role in various vegetative stages, callose features prominently during the development of the male gametophyte, the pollen grain.

Callose is a homopolymer that uses UDP glucose as a substrate and is synthesized by a class of enzymes called callose synthases (Cals) (Chen and Kim 2009). In *Arabidopsis thaliana*, 12 genes (*AtCalS1-At CalS12* or *AtGSL1- ATGSL12*) have been identified that encode for callose synthase enzymes (Richmond and Somerville 2000, Verma and Hong 2001). *AtGSL1* and *AtGSL5* play key roles during microsporogenesis by producing a callose wall that separates the microspores of the tetrad (Enns et al. 2005). The analysis of many callose synthase mutants has highlighted the involvement of callose in both early and later stages of pollen grain and pollen tube development (Dong et al. 2005, Nishikawa et al. 2005, Töller et al. 2008). The prominence of callose in the pollen tube is particularly puzzling since the benefit of this cell material for this fast growing cell remains elusive (Geitmann 1999). The pollen tube is a cellular protuberance emitted by hydrating pollen grain following pollination of a receptive flower. It elongates through the stylar tissue of the receptive pistil, enters an ovule through the micropylar end, and delivers the sperm cells resulting in double fertilization. With approximately 150 nm, the cell wall of the pollen tube is rather thin, a characteristic that is consistent with the ephemeral nature of the cell. As the pollen tube accomplishes its task—the delivery of the sperm cells—it dies, and its lifespan is therefore as short as a few hours. The investment of polysaccharide material into the construction of the pollen tube cell wall is therefore as minimal as possible while allowing rapid elongation and sufficient stability until the mission is accomplished. Does this explain the difference in

biochemical composition between pollen tubes and other primary plant cells, characterized by a low content in cellulose and high contents in callose, in addition to pectin?

Callose is located in the inner layer of the multilayered pollen tube cell wall with the outer layer being dominated by pectin and moderate amounts of cellulose (Chebli et al. 2012). The walls of plant cells have to resist a multitude of mechanical stresses and in a rapidly growing cell like the pollen tube, the stresses are highly complex since they vary both in space and time. A main source of stress on the pollen tube wall is the cell's turgor pressure that keeps the wall under continuous tension. This hydrostatic pressure does not only keep the cell inflated against the pistil's transmitting tissue, but it also constitutes the force that drives the apical expansion of the apical wall to generate cell elongation (Ghanbari et al. 2018, Kapoor and Geitmann 2023b). The cell wall yields at the apical end despite the fact that the tensile stress in this region is only half of the tension generated in circumferential orientation in the cylindrical portion of the cell (Green 1962, Lockhart 1965). This apical yielding does not only generate the elongating cell, but also enables the cell to overcome mechanical obstacles in its path (Sanati Nezhad and Geitmann 2015, Kapoor and Geitmann 2023b). The apical cell wall thus has to not only withstand the cell's own turgor, but also the stress caused by external mechanical obstacles. The same applies to the cylindrical portion of the pollen tube that must withstand any compressive forces exerted by surrounding tissues lest the tube be compressed preventing the passage of the male germ unit (Sanati Nezhad et al. 2013, Kapoor and Geitmann 2023b). Since callose is absent from the growing pollen tube tip but abundant in the lining of the distal cylindrical portion of the wall, it is thought that this robustness of the distal portion of the pollen tube cell wall is in part supported by this polymer (Parre and Geitmann 2005a).

Callose in pollen tubes occurs not only in the cell wall lining, but also in form of highly distinctive feature—the callosic plugs. A plug represents a locally confined thickening of the cell wall that constricts the cytoplasm in septum-like manner. Eventually, it completely separates the cytoplasm creating a cross wall between the apical portion containing the male germ unit and a distal region that gradually loses turgor and degenerates. It has been hypothesized that the repeated formation of callosic plugs as the tube elongates maintains the volume of the active portion of the cytoplasm constant. This has been speculated to allow the cell to avoid having to control the turgor in a cell that can be many centimeters long thus facilitating the rapid adaptation to changes in environmental conditions (Chebli et al. 2012).

Here we wanted to test the hypothesis that callose plays a role in the pollen tube's ability to perform invasive growth and related to this, we endeavored to determine how intracellular processes might regulate the enrichment of callose at specific locations in the cell. To address these questions, we exposed *in vitro* growing pollen tubes of *Arabidopsis* mutants with reduced callose contents to a combination of cytomechanical assays and cytochemical localization of cell wall components.

3.3 Material and methods

3.3.1 Plant material and growth conditions

Sterilized seeds were sown in 0.5× Murashige and Skoog (1962) medium solidified with 0.8% agar. After plating on MS media seeds were cold stratified for 2 days in the dark at 4°C and were germinated and grown for 10 days at 22°C under continuous light. Seedlings were transferred to soil or peat, mixed with vermiculite and perlite (2:1:1), and grown in a growth chamber at 22°C under long day (16/8 h light/dark) photoperiod and 40% relative humidity. Pollen was collected every day from the time flowers bloomed. All experiments used freshly harvested pollen.

3.3.2 Semi-in vitro pollen culture

The pollen tubes analysed for all experiments were germinated using the *semi-in vitro* germination protocol. For *semi-in vitro* pollen germination, stigmas of male sterile *Arabidopsis* plants were cut and placed on a solidified germination medium (2 mM $MgSO_4$, 1 mM $CaCl_2$, 0.01% boric acid, 18% sucrose, pH 7, 0.5% agarose) (Palanivelu et al. 2003). Pollen grains were brushed on the stigma and left to germinate for 12 hours. Pollen tubes emerged from the cut end of stigma about 1 hour after pollination and continued growing on the agarose surface. For measuring the distance between callose plugs, pollen tubes were left to grow for 18 hours.

For mechanical assay experiments, pollen was germinated using the same protocol, but the pollinated stigma heads were pushed into the stiffened agarose (0.5%-3%) using a fine forceps. Pollen tubes emerging from the cut end of the stigma therefore grew within the agarose-stiffened medium.

3.3.3 Genotyping mutants

Mutant seeds were obtained from TAIR (https://www.arabidopsis.org/). The T-DNA mutagenized lines *callose synthase 5-3* (CS68974; WS-2 background) and *callose synthase 5-5* (CS68976; Col background) were genotyped to acquire homozygous lines as described by (Nishikawa et al. 2005).

3.3.4 Incipient plasmolysis

Pollen tubes were germinated in semi-in vitro conditions on 0.8% of agarose. The samples were observed and imaged after 5 h germination. Liquid pollen germination medium with different mannitol (0.5%-5%) concentration was added (100 µl) onto the slide with the pollinated stigma. Pollen tubes were observed and imaged under brightfield optics and experiments were repeated

with different concentrations of mannitol until 50% of cells displayed plasmolysis (retraction of plasma membrane from the cell wall). Germination medium without mannitol was used as control. The mannitol concentration inducing 50% of tubes to display plasmolysis was used for turgor pressure calculations.

3.3.5 Fluorescence labeling and Immunohistochemistry

For callose labelling using aniline blue dye, the pollen tubes were fixed by microwave-assisted fixation using 3.5% freshly prepared formaldehyde in PIPES buffer (50 mM PIPES, 1 mM EGTA, 5 mM $MgSO_4$, 0.5 mM $CaCl_2$, pH 7). The samples were fixed using a microwave oven (PELCO Cold Spot® Biowave 34700) at 150 W for 40s, followed by 2 mins on the bench and a second 40s exposure to 150 W. Unbound aldehydes were removed by washing 5 times using PIPES buffer (pH 7) for 40s @150 W. Aniline blue was used at 0.1% in 0.15 M K_2HPO_4 and incubation with the dye was done for 10 min at 150 W under vacuum. Excess stain was removed by rinsing with 0.15 M K_2HPO_4.

For callose labeling in trichomes using aniline blue dye, the 21-day old rosette (both treated and control, described in pharmacological section, below) were incubated in a clearing solution (1:4 acetic acid: ethanol) for 2 hours. Cleared rosettes were then incubated in 0.1% aniline blue dye (dissolved in KH_2PO_4, pH 8.5) overnight. Samples were observed using a Zeiss epifluorescence microscope with Apotome attachment (Axio Imager Z1 stand) with an excitation of 514 nm and a 550-585 nm bandpass emission filter.

To locate callose synthases, monoclonal antibodies (mAbs) were used (Cai et al. 2011). Pollen tubes were fixed in 3% freshly prepared formaldehyde in PIPES buffer (50 mM PIPES, 1 mM EGTA 0.5 mM $MgCl_2$, pH 6.9 and 12% sucrose) for 30 min; after washing, the cell wall was digested with 1.5% cellulysin for 6 min (in the dark) followed by rinses using PIPES buffer.

Anti-callose synthase mAbs (diluted in PIPES buffer) were used at a concentration of 1:40 overnight at 4°C, followed by washing steps (5 times 8 minutes) the following day. Samples were then incubated with Alexa Fluor 488 -conjugated goat anti rabbit IgG, diluted at 1:100 for 45 minutes at 37°C, followed by washing steps.

For actin cytoskeleton labeling, samples were fixed for 40 s in the microwave oven under 150 W in 3% formaldehyde, 0.5% glutaraldehyde and 0.05% Triton X-100 solution in a buffer composed of 100 mM PIPES, 5 mM $MgSO_4$ and 0.5 mM $CaCl_2$ at pH 8. Pollen tubes were then washed three times for 1 min each in the same buffer, followed by overnight incubation at 4°C in rhodamine–phalloidin (Molecular Probes) in a buffer composed of 100 mM PIPES, 5 mM $MgSO_4$, 0.5 mM $CaCl_2$ and 10 mM EGTA at pH 7. All steps outside of the microwave were performed on ice. On the following day, samples were washed five times for 1 min each in the same buffer. All washing steps were conducted in the microwave at 150 W. Samples were then mounted on glass slides in a drop of Citifluor (Electron Microscopy Sciences), covered with a cover slip, sealed, and immediately observed in the microscope.

For immunolabeling of cellulose, methyl esterified pectin and de-methyl esterified pectin, pollen tubes were fixed in 3.5% (w/v) freshly prepared formaldehyde in PIPES buffer (50 mM PIPES, 1 mM EGTA, 5 mM $MgSO_4$, 0.5 mM $CaCl_2$, pH 7) for 40 s followed by three washes in PIPES buffer. Pollen tubes were then washed three times with 3.5% (w/v) bovine serum albumin (BSA) in phosphate buffered saline (PBS; 135 mM NaCl, 6.5 mM Na_2HPO_4, 2.7 mM KCl, 1.5 mM KH_2PO_4, pH 7.3). All subsequent washes were done with PBS buffer with 3.5% (w/v) BSA for 40 s. All antibodies were diluted in PBS buffer with 3.5% (w/v) BSA, and incubations were done for 10 min (in vacuum) followed by three washes in buffer. Controls were performed by omitting incubation with the primary or the secondary antibody.

Crystalline cellulose was labelled with CBM3a (Plant Probes) at 1:200, followed by a monoclonal mouse anti polyhistidine at 1:12 (Sigma) incubation, and subsequently incubated with Alexa Fluor 594 anti-mouse IgG (diluted 1:100; Molecular Probes). Methyl-esterified pectin was labeled with LM20 (Plant Probes, 1:25) followed by incubation with Alexa Fluor 488 goat IgM anti-rat (1:100, Molecular Probes). Demethyl-esterified pectin was labelled with LM19 (Plant Probes, diluted 1:25) and subsequently incubated with Alexa Fluor 488 goat IgM anti rat (1:100, Molecular Probes).

Following the labeling procedure and final washes, all samples were mounted on glass slides in a drop of Citifluor (Electron Microscopy Sciences) for microscopical observations. Each experiment was repeated at least four times. For Alexa Fluor 488, excitation wavelength and emission window of 488 nm and 493-630 nm were used, respectively. For Alexa Fluor 595, excitation wavelength and emission window 594 nm and 599-734 nm were used, respectively.

3.3.6 Pharmacological treatments

To interfere with actin polymerization, Latrunculin B (LatB, 0.5 nM and 1 nM) or Cytochalasin B (CytB, 100 nM and 500 nM) were administered to the pollen tubes after 3 hours of *semi-in vivo* germination. For plug frequency and to measure plug deposition pattern the tubes were grown in the presence of the drugs for 10 hours (n=50 per sample; 3 technical repeats). Dimethyl sulfoxide (DMSO) diluted at the corresponding concentration in germination medium was used as control.

For drug treatment of trichomes, 21-day old rosettes of *Arabidopsis thaliana* were gently brushed with 5 µM LatB two times a day for a period of 48 hours. The control rosettes were treated with the corresponding concentration of DMSO diluted in ½ MS.

3.3.7 Image processing and analysis

Image processing and analysis were done with Fiji (Image J) software (https://imagej.net). Where images were to be compared for fluorescence intensity, acquisition was done at identical microscope settings and brightness and contrast adjustments were done in identical manner. Any quantification was done on the raw image material. For the quantification of fluorescence intensity of cell wall labels, fluorescence intensity was traced along the periphery of each pollen tube starting from the vertex of the tube to a meridional distance of 15 µm on both sides. Values were normalized to the highest value present on an individual tube before averaging over all tubes and plotting against the meridional distance from the pole of the cell.

For pollen tube length measurements, the segmented line tool of Image J was used. Pollen tube length was measured between the point of emergence from the neck of the stigma and the tip of the tube. For the pollen tube diameter, the segmented line tool was used. Tube width was measured at about 100 µm from the tube tip or around an area close to the most recent callose plug formed. To measure the distance between the most recently formed plug and the second plug, the segmented line tool was used. The distance was measured starting from the apical end of the second plug until the distal end of the most recently formed plug. The cross-sectional area of a callose plug was measured using the segmented line tool. The periphery of the plug was marked, and the surface determined.

3.3.8 Statistical analysis

Statistical analyses were performed using ‘Prism Graph-pad software’ (Lord et al. 2020), unless otherwise stated. For pollen tube length, pollen tubes diameter, and the distance between two

most recently formed plugs, ten to twelve images per slide with at least three pollinated stigmas were imaged. The experiments were repeated at least three times with n > 100 (number of pollen tubes measured using different plants of the same line and same seed set). Student T-test with Welch's correction was performed on the raw data. For analysing the difference in cross-sectional area of callose plugs deposition pattern after several drug treatments, one way ANOVA was applied (n = 20 plugs, for each Arabidopsis line). For incipient plasmolysis data a simple linear regression analysis was performed (n = 30 tubes per sample, 3 technical repeats). To analyze normalised fluorescence intensity for cellulose, methyl, and de-methyl esterified pectin both average and standard error mean were plotted using the software (n = 30 pollen tubes).

3.4 Results

3.4.1 Altered callose content in the pollen tube cell wall is associated with changes in cell dimensions, growth performance, and plug frequency

To assess the role of callose in pollen tube performance, we compared the growth behavior of two mutants with altered callose synthase expression with that of the respective wildtype. *Arabidopsis* mutants *cals5-3* and *cals5-5* were previously shown to have no detectable or reduced amounts of callose in the pollen tube cell wall, respectively, but retained fertility (Nishikawa et al. 2005). Under *semi-in vivo* growth conditions used here, pollen tubes of *cals5-3* lack any visible callose deposition in the cell wall lining and they have no callose plugs (Fig. 1). Pollen tubes of *cals5-5* display some callose in cell wall lining and they do form callose plugs. To quantify morphological differences between mutant pollen tubes and wildtype, tubes were analysed at 12 hours after pollination, or approximately 10-11 hours after emerging from the cut end of the stigma onto the agarose medium. To determine any possible effect of the sporophytic

growth environment, mutant pollen was either grown on their respective mutant stigma or on their respective wildtype background stigma. Pollen tube growth of both mutants was reduced as their length at 12h was 29.5% (*cals5-3*) and 29.8% (*cals5-5*) shorter than that of pollen tubes from the respective wildtype (Figs. 2 a,b). The diameter of the *cals5-3* tubes lacking callose was 26.9% wider than its wildtype (Fig. 2c), but that of the callose containing *cals5-5* was 17.9% narrower (Fig. 2d). To ascertain that the results were not influenced by the stigmatic tissues, the mutant pollen grains were also germinated on their respective wildtype stigmas (Supplemental Fig. S1). These cross pollinations showed the same trends as the self-pollinated stigmas with regards to pollen tube length and diameter.

To assess whether callose plug formation was altered in *cals5-5*, we measured the distance between the two most recently formed plugs (Fig. 2e). The distance between the most recent plugs was higher in *cals5-5* than in the wildtype by 11.7%, but only in self-pollinations. When *cals5-5* tubes were crossed on wildtype stigmas, the distance was slightly reduced (by 4.3%). This suggests that the frequency of callose plug formation is affected in the mutant, but that the genotype of the stigma influences this behavior.

3.4.2 The presence of callose affects pollen tube invasive growth

The finding that lack of callose in the cell wall lining and absence of plug formation resulted in tubes with slower growth and increased diameter, raises questions relating to the role of callose in tip-focused cell elongation and invasive growth capacity. We wondered whether callose plays any role in withstanding the circumferential tension stress in the cell wall created by internal turgor pressure and whether the presence of callose plugs might enable the tubes to generate the force required to invade stiffened media. To answer both questions, we examined how the reduction in or lack of callose affects the behavior of pollen tubes as they invade agarose-

stiffened media. Self-fertilized, *semi-in vivo* growing pollen tubes were exposed to media stiffened with various concentrations of agarose to determine their performance when faced with a mechanical obstacle.

In *Arabidopsis*, *in vivo* pollen tube growth occurs in a solid style pistil, and recent studies have shown that the pollen tubes of this species prefer to grow in stiffer media compared with liquid medium (Reimann et al. 2020). Our data are consistent with these findings since the length of wildtype pollen tubes at 12h after germination increased with increasing agarose concentration (Figs. 3a,b). The ecotypes of *Arabidopsis* behaved slightly differently, however, as WS showed an optimum growth at 2% agarose, whereas the length of Col-0 pollen tubes was longest at the highest agarose concentration tested, 3% (Figs. 3a,b). Consistent with their behavior in absence of a mechanical impedance, the pollen tubes of *cals5-3* displayed a reduced growth rate overall compared to its wildtype. Remarkably, when faced with stiffened medium, this difference was larger than without mechanical impedance. In the presence of agarose, mutant pollen tube length was reduced by more than 40% compared to the wildtype (Figs. 3a,b) whereas without impedance the mutant pollen tubes were only 30% shorter (Figs. 2a,b). This suggests that the absence of callose plugs limits the tubes' ability to perform invasive growth. Consistent with this, the invasive growth capacity of *cals5-5* pollen tubes was much less affected. Only at 3% agarose was pollen tube length reduced in *cals5-5* compared to its wildtype (Fig. 3b).

The agarose-stiffening also influenced pollen tube width consistent with the concept that stress in the circumferential direction resulting from turgor can partly be countered by the constricting effect of the stiffened medium. In *cals5-3* pollen tubes which in the absence of physical obstacles had shown an increase in diameter of 26.9% compared to the wildtype (Fig. 3c), the stiffened environment had a moderating effect on pollen tube width which if wider at all, was limited to

less than 15% (Fig. 3c). In the callose containing *cals5-5* pollen tubes pollen tube, width was virtually identical to the wildtype when exposed to agarose.

3.4.3 Altered callose content in the cell wall leads to altered turgor pressure

Tip elongation in the pollen tube involves the spatial and temporal regulation of a plethora of components and processes such as the cytoskeleton (Cai et al. 1996, Cai et al. 2011), intracellular ion concentrations (Obermeyer and Weisenseel 1991), ion transporters (Malhó et al. 1995), intracellular transport of cell wall material (Geitmann et al. 1996) and turgor (Zerzour et al. 2009). Turgor must be maintained for cell enlargement to occur. We suspected that callose plugs serve to facilitate turgor maintenance by reducing the total active cytoplasmic volume to that of the front segment of tube carrying the sperm cells. Consequently, we hypothesized that a lack of callose plugs would lead to tubes with lower turgor. To test this hypothesis, we estimated pollen tube turgor by performing incipient plasmolysis. 50% of wildtype tubes initiated plasmolysis at the tip upon addition of 4% mannitol for WS, but for *cals5-3*, 5% mannitol was required for the same effect. Contrarily, while 4.5% mannitol caused 50% of tubes to plasmolyse in the Col ecotype, the same effect was achieved in *cals5-5* already at 4% mannitol. As hypothesized, the turgor pressure for *cals5-5* was therefore lower than its wildtype, but surprisingly, the callose-less tubes of *cals5-3* displayed higher turgor than its wildtype. The higher turgor in *cals5-3* could mean that either turgor regulation operates differently in these cells, or that the cell wall has been stiffened through a compensatory mechanism triggered by the reduction in callose content.

3.4.4 Callose synthase mutants display compensatory changes in pollen tube cell wall architecture

To address the puzzling finding that callose-less mutant pollen tubes displayed higher turgor, we investigated whether the tubes might compensate the loss of this cell wall component by altering

the deposition pattern of other cell wall components. We suspected that this compensation could be achieved either by producing more methyl esterified pectins, stiffening pectin through gelation, depositing more cellulose, or a combination of these. To test this, methyl and de-methyl esterified pectin were labeled using LM20 and LM19 antibodies, respectively. In all pollen tubes, the amount of methyl-esterified pectin was highest in the apex (at 0-1.5 μm from the tip) and dropped off gradually along the meridional axis of the tube to be virtually absent at 15 μm from the tip (Figs. 4b,e). In *cals5-3* this drop was significantly more gradual than in its wildtype ecotype where the drop in methyl-esterified pectin abundance was sudden at 8 μm (Fig. 4b). The absolute label intensity of LM20 at the pollen tube tip in the mutants was higher than the respective wildtype (Figs. 4 i-j and o-p). Inversely, the spatial profile of de-methyl esterified pectin showed an increase from tip to distal region in the WS wildtype, whereas the distribution was uniform in the *cals5-3* tubes (Fig. 4c). The more gradual conversion of methyl esterified to de-esterified pectin in *cals5-3* is consistent with the wider diameter of the mutant pollen tubes. In the callose containing *cals5-5* mutant, the difference in the pectin profiles between mutant and wildtype was minimal (Fig. 4f). The absolute label intensity with LM19 in mutants and wildtypes showed minimal differences (Figs. 4 k-l and q-r).

To determine any change in spatial profile of crystalline cellulose, labeling was done using Cellulose Binding Module (CBM3a) combined with anti-poly-His-antibody and a tertiary antibody coupled to Alexa Fluor 594 (Blake et al. 2008, Chebli et al. 2012). In both WS and *cals5-3*, CBM3a label showed a higher signal at the tip than in the rest of the tube (Fig. 4a), but in the mutant this difference between tip and shank was less marked. In Col and *cals5-5* CBM3a was relatively constant throughout the length of the tube (Fig. 4d). The absolute label intensity with CBM3a in the mutants was higher than in the respective wildtypes (Figs. 4 g-h and m-n)

suggesting a compensatory mechanism that leads to the production of an alternate stiffening material (cellulose) to replace the missing callose.

3.4.5 The actin cytoskeleton may be involved in targeting callose synthase enzymes to the plasma membrane

To understand the regulation of callose deposition in the different regions of the pollen tube, we immunolocated callose synthases to interrogate how the subcellular locations of these enzymes correlate with enriched abundance of the polymer. In both wildtype ecotypes, callose synthases were localised at the cell periphery. At the pollen tube apex abundance was high. Compared to the apex, label intensity in the shank was weaker (Fig. 5a), except for the periphery of callose plugs where signal was locally elevated (Fig. 5b,c).

The intracellular transport, positioning, and activation of callose synthases involve the endomembrane system (Brownfield et al. 2008) and are suspected to involve the cytoskeleton but details on the regulatory mechanism are elusive. Recently, the role of microtubules in positioning of the callose synthases was highlighted using biochemical assays and ultra-structural investigations (Cai et al. 2011, Parrotta et al. 2022). However, given that pollen tubes grow unimpeded in the presence of microtubule inhibitors (Gossot and Geitmann 2007) and given that vesicular transport in plant cells is largely associated with actin, we wanted to investigate how the actin cytoskeleton associates with the deposition pattern of callose synthases.

To this end, we labeled actin filaments using rhodamine-phalloidin. As shown in other species' pollen tubes (Lovy-Wheeler et al. 2005), an apical fringe was observed near the *Arabidopsis* pollen tube apex consistent with an apical deposition pattern of both cell wall components and proteins such as callose synthase (Figs. 6a,b). Long actin bundles were also visible in the distal region, both in the central cytoplasm and particularly prominent near callose plug deposits (Figs.

6c-f). Long actin bundles were observed passing through incompletely formed callosic plugs (Figs. 6d,f), and near fully formed plugs the actin filaments pointed to the plug (Figs. 6c,e). This proximity of actin filaments with locations of elevated presence of callose synthase supports the notion that the enzyme may be transported to its destination at the cell surface on the actin cytoskeleton.

3.4.6 Actin depolymerization alters the callose deposition pattern

To further investigate the putative involvement of actin in the regulation of callose deposition, pollen tubes were treated with LatB or CytB, agents that interfere with actin polymerization but allow the tubes to elongate when used at low concentrations. Concentrations chosen here for both LatB and CytB resulted in apical swelling in 40% of the tubes and tip bursting in approximately 20% of the tubes after 3 hour of drug exposure, although the length of the longest tubes was similar to the untreated control tubes. The tubes treated with CytB displayed an increased accumulation of callose at the tip (Fig S3). Additionally, callose plugs were clearly longer when compared to the controls, reflected in an increase in longitudinal cross sectional surface area (Figs. 7a-l). These dramatic effects on the deposition pattern of callose support the hypothesis that actin is involved in the regulation of callose deposition, likely through the precisely targeted deposition of callose synthases both in the apex and in distal segments of the tube. Additionally, we also observed changes in the plug frequency pattern upon treatment with actin depolymerizing drugs. Contrasting results were observed when using LatB and CytB. Although the length of the longest tubes was unaltered by the drug treatment, LatB resulted in 60% of tubes displaying 2 plugs/tube and about 30% of the tubes displaying 1 plug/tube compared to the control in which only about 40% of tubes displayed 2 plugs/tube and about 50% of the tubes displaying 1 plug/tube. CytB treatment on the other hand significantly reduced the

number of plugs in visible tube portions with fewer than 5% tubes displaying 2 plugs/ tube and about 6% tubes displaying 1 plug/tube (Fig. 7m).

To illustrate that the involvement of the actin cytoskeleton in callose deposition is not unique to pollen tubes but operates similarly in somatic tissues, we treated developing leaves in *Arabidopsis thaliana* rosettes with LatB. After 48 hours, this treatment resulted in a significantly lower abundance of callose in the stalk and the tips of the trichomes compared to the untreated control (Supplemental Fig. S2).

3.5 Discussion

3.5.1 Spatial distribution of callose influences pollen tube growth rate and diameter

Tip-focused cell expansion, just like all plant cell growth, is thought to be driven by the osmotic uptake of water that maintains the turgor pressure (Geitmann and Steer 2006). Despite the importance of turgor, its magnitude does not correlate with pollen tube growth rate (Benkert et al. 1997a). Rather, the growth rate is likely determined by the way in which the apical cell wall yields to the turgor (Zerzour et al. 2009). Furthermore, while turgor drives growth, it is unable to force cell expansion into a particular direction. Instead, the unidirectional pollen tube growth pattern is confined to the tip through precise control of cell wall mechanical properties over the cell surface (Geitmann 2010). A key element of this control is a spatial gradient of cell wall stiffness (Fayant et al. 2010) that maintains the diameter of the tubular portion of the cell constant despite the relatively higher tensile stress in the cylindrical portion of the cell envelope (Green 1962, Lockhart 1965, Derksen 1996, Geitmann and Cresti 1998, Hepler et al. 2001b). The lower yielding threshold of the cell wall at the apex is maintained by a constant supply of new, softer cell wall material. Some of the elevated stiffness in the cylindrical portion of the tube is conferred by the callose lining in the wall that is only present here and absent at the tip. In

Arabidopsis pollen tubes, callose deposition in the cell wall lining begins approximately 10 μm from the tip and distal of that area its abundance is evenly distributed in the cell wall lining (Chebli et al. 2012). Evidence for the role of callose in resisting circumferential tensile test stems from treatment of living tubes with lyticase, a callose digesting enzyme. Moderate enzymatic digestion of callose caused the tubes to be wider and to display reduced stiffness and increased viscoelasticity when measured using micro-indentation (Parre and Geitmann 2005a). Here we show that similar effects can be obtained through genetic interference with callose synthase activities. The increase in pollen tube diameter in *cal5-3* confirms that resistance to circumferential tensile stress is reduced in the absence of the callose lining. It is particularly intriguing to observe that this effect is rescued by a stiffened growth medium confirming that the structures withstanding the turgor pressure are a combination of the cell's own wall and the stiffened growth matrix.

The absence of visible callose deposition in the pollen tube apex forms a very different spatial distribution profile from that of callose synthase which is enriched in the apex. Clearly, although the enzyme is delivered to the cell surface at the tip, it is only activated in the subapical region. The comparison between the gradient in abundance of the polymer and that of its synthetic enzyme suggests that the primary region of callose assembly into the cell wall lining seems to be a relative narrow sleeve in the proximal region of the cylindrical portion of the pollen tube.

3.5.2 Lack of callose stimulates the excessive deposition of other polysaccharides

We had hypothesized that the lack of callose in the wall would reduce the cell's capacity to effectively establish turgor, but our incipient plasmolysis approach suggests that turgor is actually more elevated in mutants with reduced amount of callose. Based on this we speculated that either turgor regulation operates differently in the mutants, that unused carbohydrate

precursors accumulate in the cytoplasm increasing the osmotic potential, or that the cell wall was stiffened by other polysaccharides enabling higher pressure. Support for the latter was provided by the observation of an increase in absolute CBM3a label intensity in the mutant pollen tubes when compared to their respective wildtypes reflecting an overall enrichment of cellulose both at the tip and the distal region of the tube. The spatial gradient in pectin methyl esterification was less steep in both mutants compared to their respective wildtype resulting from more elevated levels of de-esterified pectin in the shank compared to the wildtype. We conclude that a decrease in callose content triggers compensatory mechanisms resulting in increased deposition of pectins and cellulose in both apical and sub apical segments of the tube. These in turn allow the cell to establish higher turgor pressure than in the wildtype.

3.5.3 Callose plug deposition is beneficial but not necessary for pollen tube elongation

Callose plugs have been hypothesised to support pollen tube growth efficiency (Brownfield et al. 2008), corroborated by the observation that anti-sense lines for *SHY* (protein interacting with *LePRK2*) in *Petunia* lack callose plugs and the majority of pollen tubes are unable to target ovules (Guyon et al. 2004). Zhang et al. (2008) suggested that the observed elevated frequency of callose plugs in pollen tubes of antisense lines of *LePRK2* (*Solanum lycopersicum* pollen receptor kinase 2) are a compensatory mechanism to compensate for reduced turgor in these cells. Nevertheless, the mutant lines displayed reduced pollen tube growth rate. Importantly, however, turgor was not measured in this study and the reasoning for turgor-callose plug relation remains speculation.

Direct interference with callose deposition tends to have a strong phenotype that completely prevents pollen formation, germination and tube growth. T-DNA knockout lines for CalS5

(*cals5-1* and *cals5-2*) display severe reduction in fertility due to degeneration of microspores as a result of deformed exine wall patterning during microsporogenesis (Dong et al. 2005). *cals5-3* on the other hand, the mutant line used in the present study, displayed normal pollen tube growth both *in vivo* and *in vitro* conditions, but reduced seed set when placed in competition with wildtype pollen, suggesting that fertilization efficiency is reduced (Nishikawa et al. 2005). In the *semi-in vivo* setup used here both *cals5-3* and *cals5-5* displayed moderately reduced growth rates suggesting that the pollen tube's ability to elongate is affected. The exact cause of this behavior remains to be elucidated, but the fact that both the callose plug containing mutant and the mutant devoid of plugs display similar degrees of growth reduction point at an indirect effect rather than a direct effect of the presence or absence of plugs. We postulate that the observed overproduction of pectin and cellulose stiffen the wall at the apex and reduce its yielding capacity. The immunohistochemical data are consistent with this notion as the mutants showed a clearly elevated abundance of these polysaccharides at the pollen tube tip. The deposition of callose plugs in the pollen tube therefore does not seem to be essential for pollen tube elongation *per se*, but inversely, callose might allow pollen tubes to grow optimally by reducing the amount of pectin and cellulose in the cell wall to the minimum required to maintain cell integrity.

3.5.4 Lack of callose reduces the invasive capacity of the pollen tube

To reach the female gametophyte the pollen tube penetrates through different pistillar tissues forcing it to cope with numerous mechanical obstacles. To a certain degree, pollen tubes are able to soften the pistillar tissues either by inducing programmed cell death or by dissolving the pistillar apoplast through enzymatic action (Greenberg 1996, Herrero and Hormaza 1996, Hiscock et al. 2002). However, these processes are unlikely to completely liquify the growth matrix and therefore, the elongating pollen tube tip must exert sufficient penetration force and

withstand the compressive forces exerted by the turgid cells of the pistillar tissues (Kapoor and Geitmann 2023b). Intriguingly, pollen tubes from plant species with solid style are not only able to cope with mechanical obstacles but prefer growing in stiffer media when given a choice. They increase their growth rates and diameter when facing a matrix with increased stiffness (Reimann et al. 2020). This phenomenon hints at the mechanical strategy that is employed to overcome mechanical resistance. Assuming identical turgor, an increase in tube diameter increases the effective invasive force at the interaction surface with the invaded substrate even if the cell's capacity to squeeze between narrow openings may be compromised (Sanati Nezhad and Geitmann 2013). Here we show that growth through a stiffened medium was significantly more affected in the callose-less mutant *cals5-3* compared to the callose-reduced mutant *cals5-5* whose growth rate was largely similar to its wildtype ecotype except at the highest agarose concentration used. Both mutants displayed the agarose-induced widening of the tube, however. From this we conclude that the pollen tube's ability to increase its overall growth force by widening the tube is unaffected by the lack of callose, but that the invasive force is reduced in the absence of callose. Given the previously established ability of the mutant tubes to maintain elevated turgor, we therefore speculate that the reduced growth force is related to the increased deposition of pectin and cellulose in the growing apex of these tubes that limit the turgor's effectiveness against the external growth matrix.

3.5.5 The callose deposition pattern depends on a functional actin cytoskeleton

Pollen tube growth is fueled by the strategic deposition of polysaccharides and polysaccharide-synthesizing enzymes at the growing apex (Chebli et al. 2012). Delivery of these building materials occurs in secretory vesicles originating from the endoplasmic reticulum and Golgi. Callose synthases are delivered to the apical plasma membrane in this manner (Turner et al.

1998, Li et al. 1999). Enzyme activation only occurs after their insertion into the plasma membrane and, likely, only in the maturing portion of the cell—in the subapex (Chebli et al. 2012). The trafficking of the callose synthases in particular to the plasma membrane is still not clear but is hypothesised to be associated with endoplasmic reticulum, Golgi bodies and intracellular vesicles via cytoskeleton (Brownfield et al. 2008, Cai et al. 2011, Çetinbaş-Genç et al. 2022, Parrotta et al. 2022).

The molecular association of callose synthases with microtubule cytoskeleton has been highlighted (Cai et al. 2011, Parrotta et al. 2022). Biochemical and ultrastructural analyses revealed that microtubules interact with callose synthases and might thus be involved in mediating the insertion or activation of callose synthases into the plasma membrane. On the other hand, the role of actin cytoskeleton in transporting and inserting callose synthase is not understood (Cai et al. 2011). Our data indicate that the actin cytoskeleton appears to be critical for callose synthase distribution, localization, and insertion at the apical cell membrane as well as at the callose plug areas. Evidence was provided by proximity of the actin cytoskeleton with callose synthases and the effect of pharmacological interference with actin functioning. The administration of low doses of actin depolymerizing drugs resulted in a reduction of plug frequency and plugs that were less precisely shaped. We expanded the notion of a role of actin cytoskeleton in callose deposition beyond the male gametophyte by exposing *Arabidopsis* leaf trichomes to LatB. A marked reduction in callose abundance in the trichome stalks clearly confirmed that the regulation of callose deposition is closely associated with the actin cytoskeleton in somatic cells as well.

3.6 Acknowledgements

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3.7 Figures

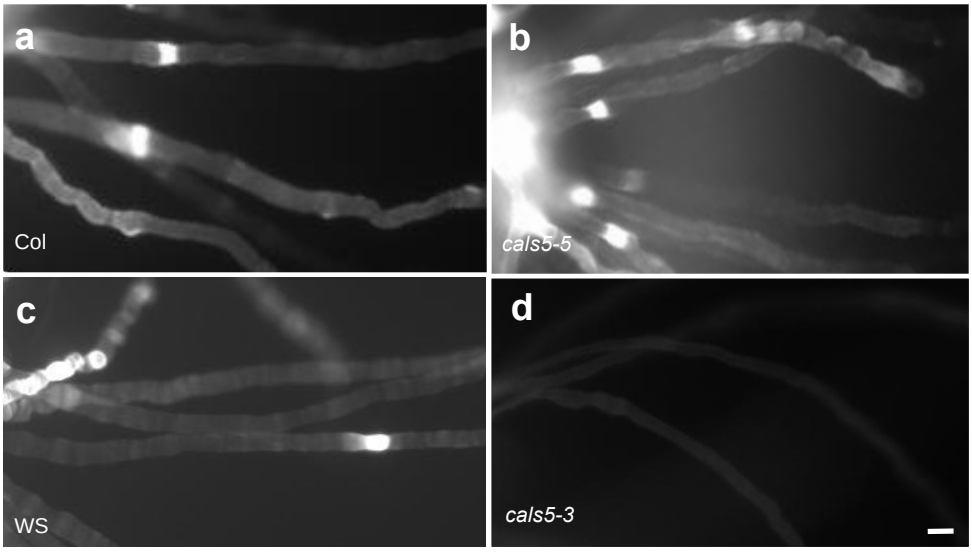


Figure 3.7.1 Fluorescence micrographs using epifluorescence microscopy of pollen tubes of Col-0 **(a)**, *cals5-5* **(b)**, WS **(c)** and *cals5-3* **(d)** labeled with aniline blue at 12 h after pollination through their respective stigmas in a *semi-in vivo* setup. Scale bar = 10 µm.

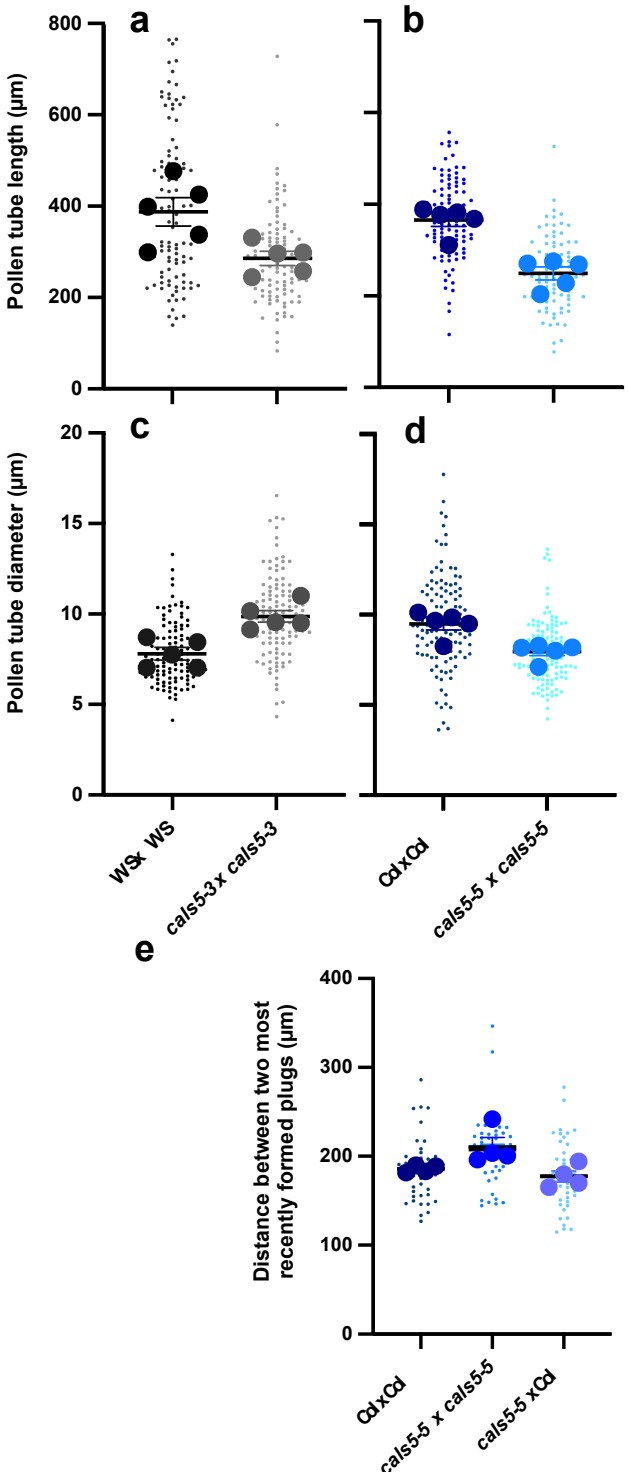


Figure 3.7.2 Pollen tube dimensions in *semi-in vivo* conditions at 12 h after self-pollination. **(a,b)** Pollen tube length measured from the cut neck of the stigma. **(c,d)** Pollen tube diameter of the same tubes shown in a,b. **(e)** Distance between two most recently formed callose plugs. Experiments were repeated 5 times. Bigger dots represent the average values of each experiment and smaller dots represent each pollen tube measured. Graphs depict difference between mutant and respective wildtype pollen tubes with $p<0.05$ (n = 120 tubes per sample).

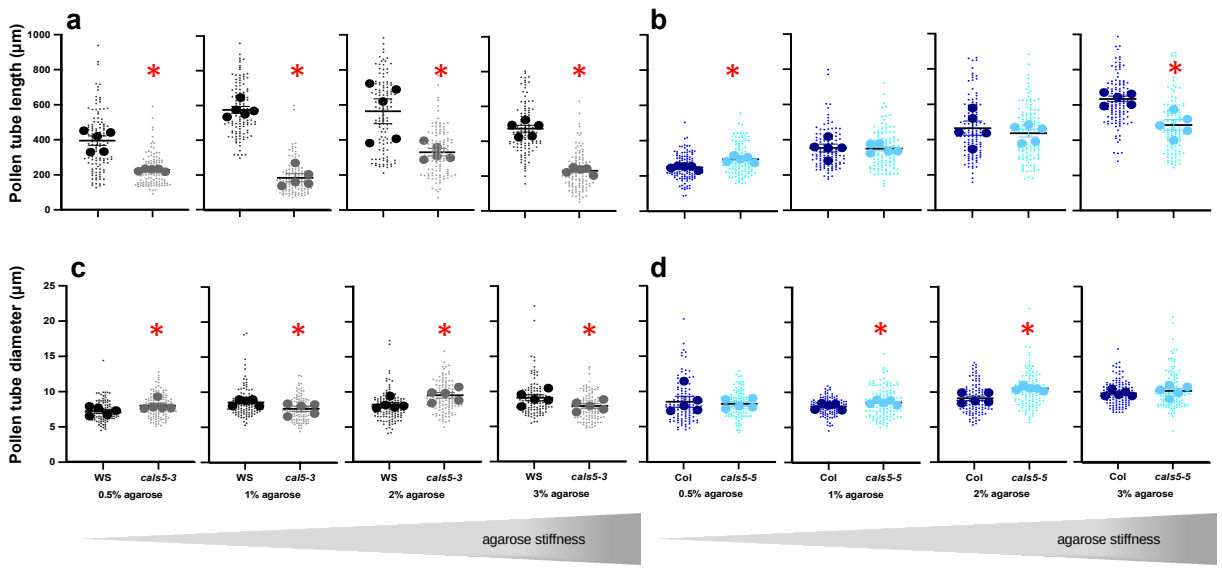


Figure 3.7.3 Pollen tube growth in stiffened media. In the *semi-in vivo* setup, pollinated stigmas are pushed into the agarose to force pollen tubes emerging from the cut neck to grow through media stiffened with various concentrations of agarose. **(a-b)** Pollen tube length of WS and *cals5-3* (a) and Col-0 and *cals5-5* (b) measured from the cut neck of the stigma at 12 h after pollination. **(c-d)** Pollen tube diameter of same tubes shown in a,b ($p<0.05$). Experiments were repeated 5 times. Bigger dots represent the average values of each experiment and smaller dots represent each pollen tube measured (n=100 tubes per sample).

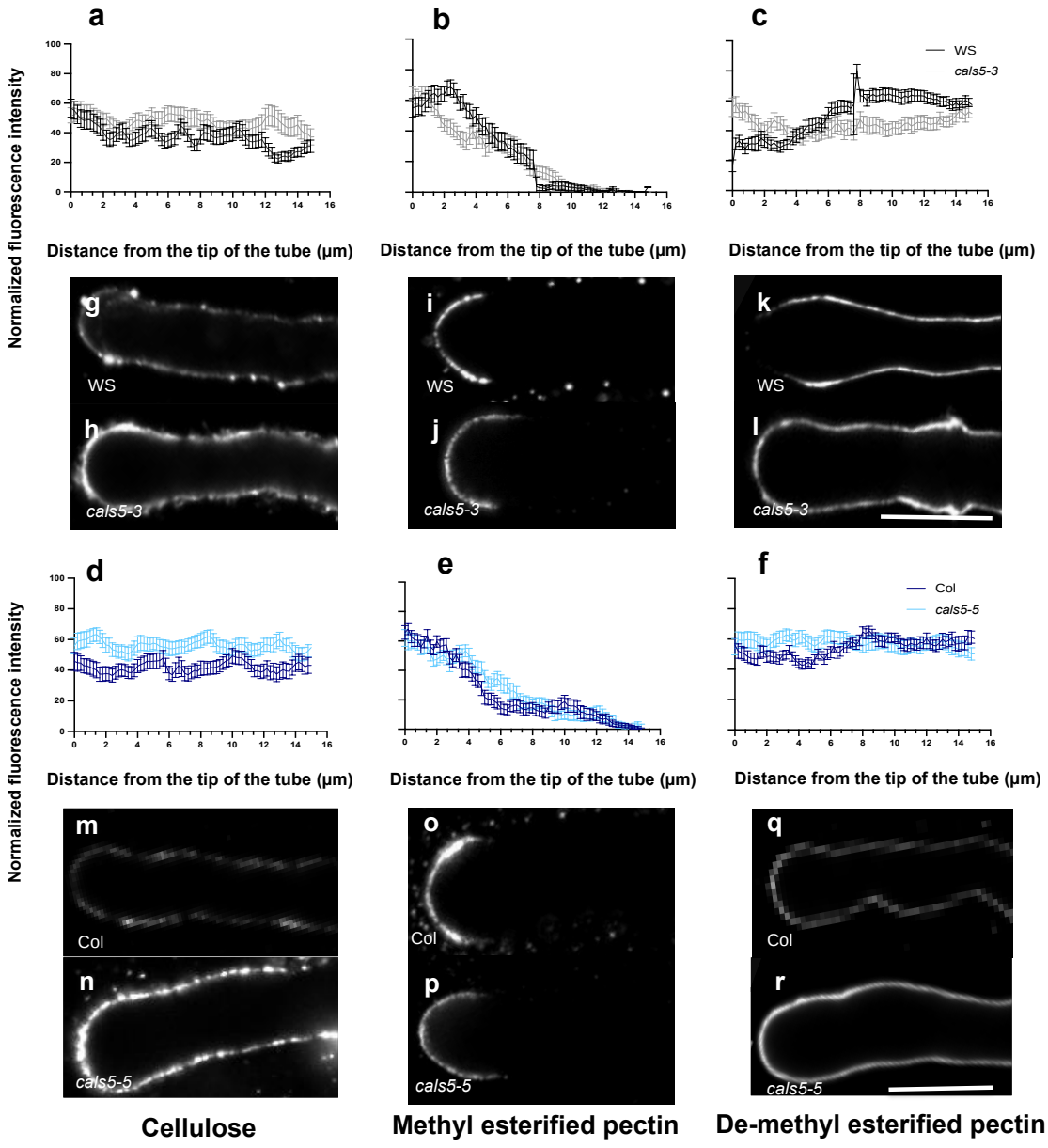


Figure 3.7.4 Spatial distribution of cellulose and pectin in wildtype and mutant pollen tubes grown *semi-in vivo*. (**g-h** and **m-n**) Cellulose labeled with CBM3a, (**i-j** and **o-p**) Methyl-esterified pectin labeled with LM20, (**k-l** and **q-r**) De-methyl esterified pectin labeled with LM19. Graphs show mean of fluorescence intensity along the meridional cell surface plotted against the axial distance from the pollen tube tip. Before averaging, for each image, fluorescence intensity values were normalized to the pixel with the highest value. Micrographs are single optical sections of confocal stacks. Acquisition for the same label was performed at identical microscope settings to allow for comparison of absolute signal intensity. Scale bar = 10 µm)

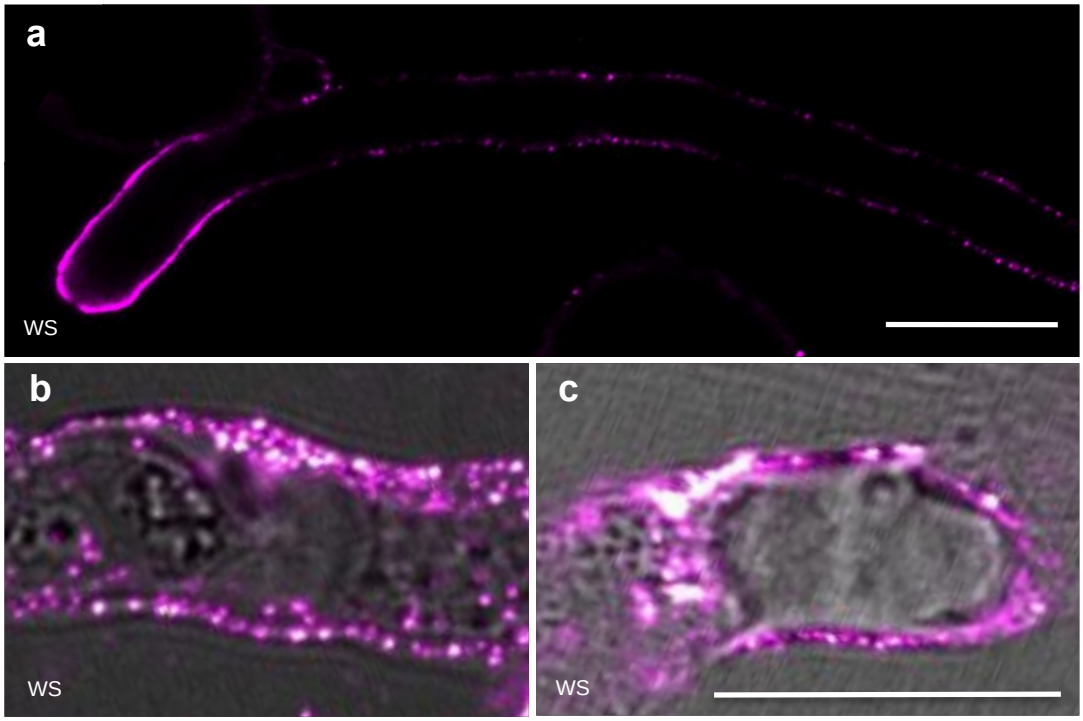


Figure 3.7.5 Distribution of callose synthases labeled with anti callose synthase antibody (magenta pseudo color) in *Arabidopsis thaliana* (WS ecotype) pollen tubes germinated in *semi-in vivo* conditions. Pollen tubes display strong labeling in the apex and sub apex of the tube, weak labeling in the distal region **(a),** and intense labeling at the periphery of callose plugs **(b,c)**. (a) is a fluorescence micrograph (single optical section); (b-c) are overlays of fluorescence micrographs (single optical sections) and corresponding DIC images. Scale bars = 10 μm.

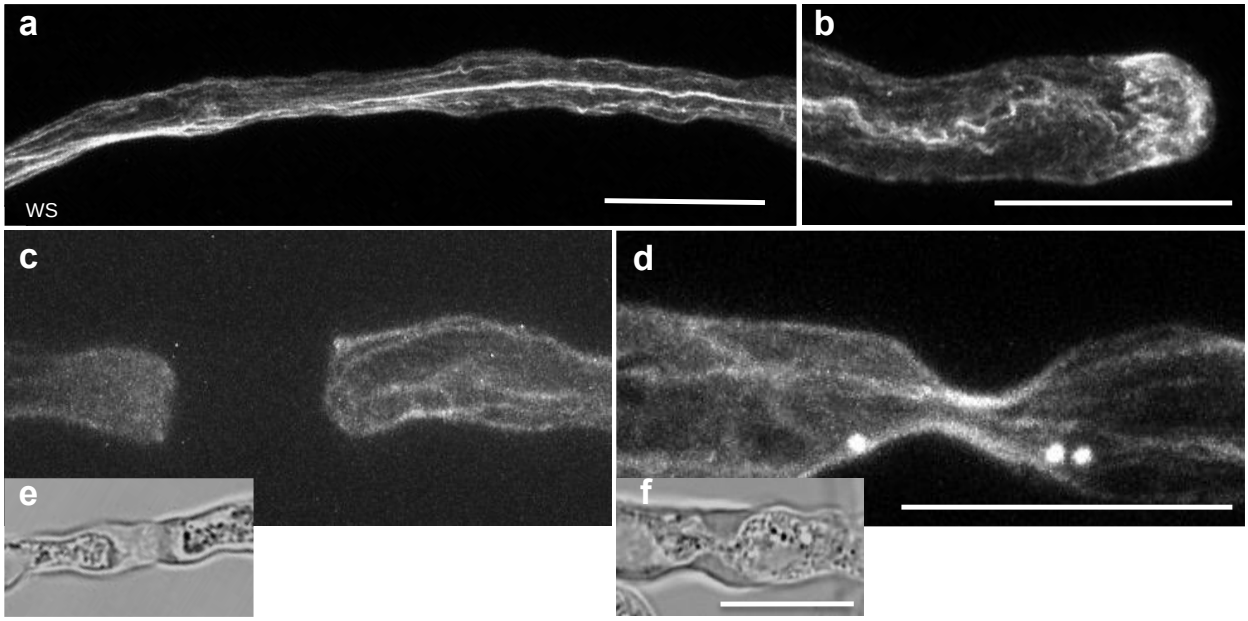


Figure 3.7.6 Localization of actin cytoskeleton in *Arabidopsis thaliana* pollen tubes of WS ecotype germinated in *semi-in vivo* conditions. Actin labeled with Rhodamine-phalloidin. **(a)** Fluorescence micrograph showing long actin bundles aligned parallel to the long axis of the tube. **(b)** Subapical fringe of individual actin filaments and thinner bundles. (**c**) Long actin bundles pointing towards fully formed callose plugs. (**d**) Long actin bundles passing through most recent, incompletely formed callose plug. (a-d) Single optical section of confocal stacks **(e,f)** Differential interference contrast (DIC) images of c and d, respectively. Scale bars = 10 µm

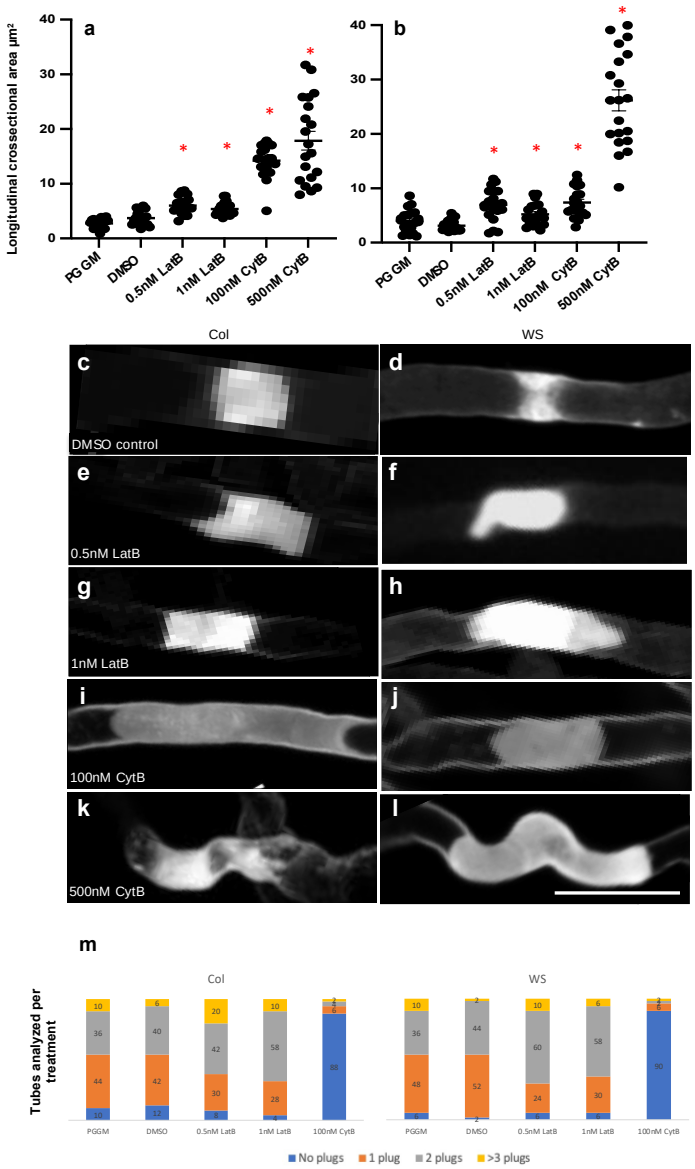
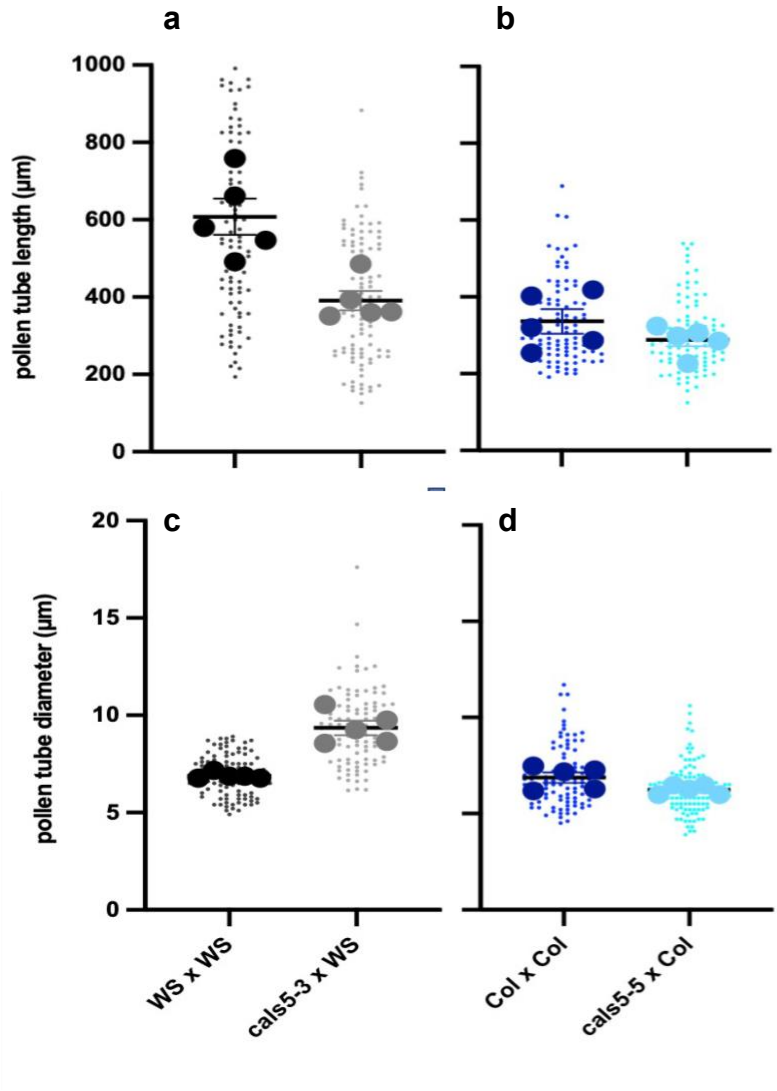
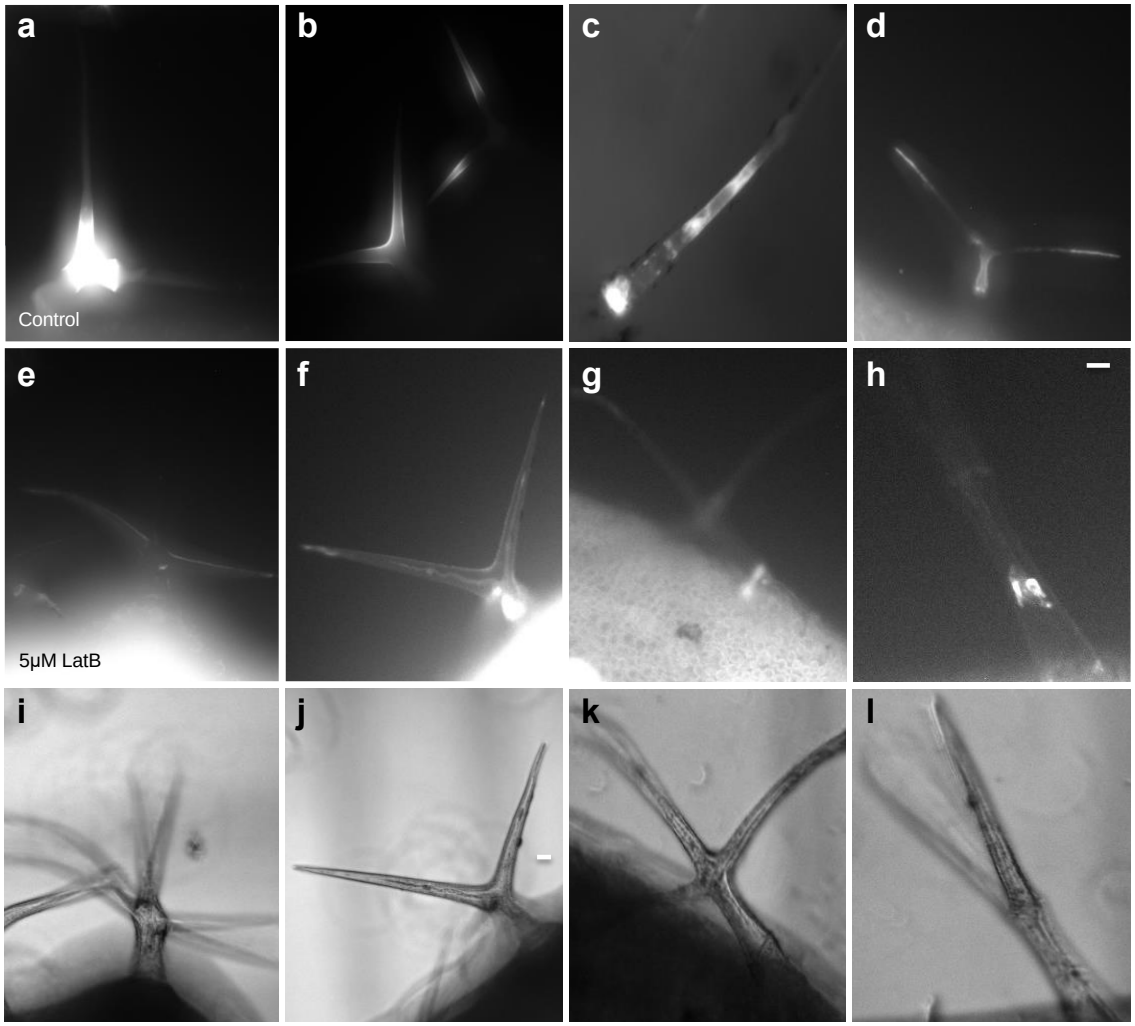


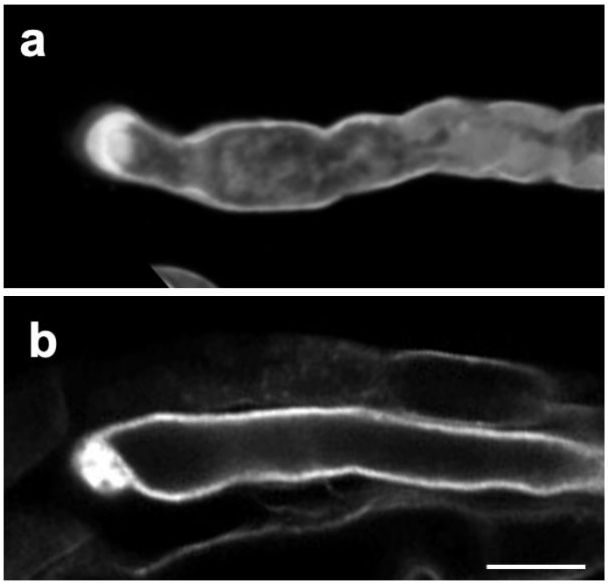
Figure 3.7.7 Effect of actin depolymerizing drugs on callose plug anatomy. (**a,b)** Longitudinal crossectional area of callose plugs deposited in presence of LatB or CytB. Asterisk indicates significant difference compared to control (pollen grain germination media (PGGM) or corresponding concentration of DMSO). Analyses were done using one way ANOVA. n = 20 (**c-l**) Single optical section of confocal stacks of aniline blue labeled *Arabidopsis* pollen tubes grown in presence or absence of LatB or CytB. Left column wildtype Col; right column wildtype WS. Scale bar = 10 µm. **(m)** Number of callose plugs per tube protruding from the cut neck of the stigma. Data collected at 12h after germination. n = 50 tubes/sample, 3 technical repeats.



Supplementary Fig 3.8.1
Pollen tube dimensions in semi-in vivo conditions at 12 h after pollinating mutant pollen or wildtype on stigmas of the respective wildtype. (a,b) Pollen tube length measured from the cut neck of the stigma. (c-d) Pollen tube diameter of the same tubes used for a,b. Graphs plotted depicts relative difference between mutant and respective wildtype pollen tubes with $p<0.05$. (n = 100 tubes)



Supplementary Fig 3.8.2
Effect of actin depolymerizing drug LatB on callose deposition in trichomes of 21-day old *Arabidopsis* leaves. Fluorescence micrographs of aniline blue labeled leaf surface after 48 hours of leaf development in presence or absence of the drug. **(a-d**) DMSO control, (**e-h**) LatB treatment, (**i-l**) Brightfield micrographs corresponding to e-h. Scale bar = 20 μm



Supplementary Fig 3.8.3
Effect of actin depolymerizing drugs on callose deposition pattern in pollen tubes. (a-b) Single optical sections of confocal stacks of aniline blue labeled *Arabidopsis* pollen tubes grown in presence of Cyt B (100 nM in a, 500 nM in b) displaying callose deposition at the tip of the tube. Scale bar = 10 µm

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Connecting statement 2

The third chapter of my thesis provides insight into how cell wall components and cytoskeleton play a critical role in characterizing the invasive capacity of the pollen tube cell as it makes its way to reach its target (the ovule) by invading transmitting tissue matrix for a successful fertilization event. This motivated us to assemble and analyze our knowledge on a variety of other invasive cell types in the plant kingdom (root hairs, sclerenchyma fibres, pollen tubes), fungi (hyphae, mycorrhiza formation) and bacteria that display such characteristic 'invasive growth behavior' with diverse range of functions. Thus, the fourth chapter of my thesis elaborates on structural parameters such as turgor pressure and regulation of cell wall mechanical properties that are involved in maintaining intrusive behavior in walled cells. We discuss the regulatory role of the cytoskeleton in these cells as it pertains to intracellular trafficking and cell wall assembly. We also address how these invasive cells facilitate their activities by secreting softening agents such as chemicals and enzymes to invade a substrate and we discuss experimental strategies and numerical methods to measure the invasive force generated by these invading cells.

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4 Invasive processes in the life cycle of plants and fungi

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4.1 Abstract

Invasive growth is a common characteristic of a variety of cell types in all kingdoms ranging from animals to plants, fungi and bacteria. Invasion in a biological context can be commonly defined as penetration of a substrate by an actively elongating ‘invader’ (single cell or multicellular structure). Invasion requires force which in the case of single cells is produced by cell mechanical features such as turgor pressure or the cytoskeleton. Invasion is often facilitated by agents employed to soften the invaded matrix such as lytic enzymes. This review provides an overview of experimental strategies that have been developed to characterize this particular cellular behavior and to measure the invasive forces generated by tip growing cells in plants and fungi.

4.2 Introduction

The cells of multicellular organisms generally occupy specific locations within tissues and organs to serve specialized metabolic or structural functions. In animal bodies, there are numerous exceptions to this spatial constancy: Blood and lymph cells are transported through the entire body by the respective circulatory systems, neurons elongate their axons through other tissues to innervate them, and cancer cells migrate through tissues and change location via the circulatory system. In plants, no circulation of cells occurs although the position of individual nuclei may change across substantial distances, in particular in large, multinucleate syncytia or coenocytes. The nuclear migration is similar to that occurring in many filamentous fungi and slime molds where nuclei can move over large distances within multinucleate cells(Roper et al. 2015). Because of the absence of cell migration proper, however, spatial constancy of cells within the plant body is more pronounced than in animals. This is even more so since plant cells

are encased in an extracellular matrix and glued to each other by the middle lamella, dramatically limiting movement of cells relative to each other(Zamil and Geitmann 2017). Collectively, the middle lamella and the encasing cell walls in the plant body are called the apoplast, a scaffold that confers structural stability to plant organs and cements cells in space. However, even within the plant body, certain cells produce extensions to reach either distant regions in the same organism or explore the external environment. Just like in animal organisms, these motile activities in plants often require the cells to invade or squeeze between their neighbors and through narrow spaces.

Invasive cell types in plants include root hairs, pollen tubes, sclerenchyma fibers, and laticifers. The invasive lifestyle and associated elongated cell shape can serve a variety of purposes. Elongated cells can provide structural stability to the organ, analogous to the steel rods reinforcing a concrete structure (sclerenchyma fibers), facilitate procurement of nutrients and water from distant sources (root hairs)(Carminati et al. 2017), or transport cargo (pollen tubes). A second type of invasion to which plant bodies are subjected is that by symbionts and pathogens of fungal and bacterial origin. Some of the invasive activities performed by other organisms exploit structural openings in the plant body such as stomata or intercellular air spaces, whereas others see the invader drill into the apoplast or even into the lumen of individual cells. As long as cellular extension or invasion exploits openings in the tissue traversing gas or liquid spaces, the invading cell does not encounter any mechanical obstacle. However, when invasion or extension occurs against or through a solid or viscous matrix, typically the apoplast, the invading cell has to overcome mechanical obstacles.

The forces required for the invasive and migratory behavior of animal cells such as neurons, cancer cells and fibroblasts are generated by the cytoskeletal system and have been covered in numerous reviews (Fife et al. 2014, Lasser et al. 2018). In the following we focus on the invasive lifestyle of walled cells in plants, fungi, and oomycetes. We elaborate on the key structural parameters involved in maintaining an intrusive activity in walled cells—the turgor pressure and the regulation of cell wall mechanical properties (Sanati Nezhad and Geitmann 2013). The cytoskeleton in these cells is important in its role as regulator of intracellular trafficking and cell wall assembly (Chebli et al. 2021a). We discuss how invasive cells may facilitate their activities by secreting agents that soften the invaded substrate and we review experimental techniques and numerical methods developed to measure the invasive force.

4.3 Invasive growth serves a diverse range of functions

4.3.1 Elongated cells can confer structural stability

Among the longest cells in the plant body are fibers with lengths up to 120 mm in *Boehmeria nivea*, 33 mm in *Linum usitatissimum* (Han 1998). These sclerenchymatous cells (cells with thick lignified secondary cell wall and typically dead at maturity) serve to stabilize the plant body against mechanical stress(Snegireva et al. 2010, Sanati Nezhad and Geitmann 2013). This mechanical role depends on structural and geometrical parameters that involve the accumulation of cellulose and lignin in significant amounts at mechanically critical positions within organs(Sanati Nezhad and Geitmann 2013). Fibers are formed in various plant organs including roots, shoots, leaves(Esau 1965, Gorshkova et al. 2018) and are particularly abundant in the phloem or secondary xylem of eudicotyledon plants and surrounding the vascular bundles in the leaves of monocotyledons (Lev-Yadun 2010, Sanati Nezhad and Geitmann 2013). The structural

function of fibers relies on their resistance to compressive, bending and tensile forces all of which may occur in the same organ. Fibers have also been considered to act akin to animal muscles as in some situations they are thought to have contractile properties (Clair et al. 2006, Mellerowicz et al. 2008, Gorshkova et al. 2018). This action is displayed, for instance, during gravitropic responses, climbing and underground positioning of geophytes (Gorshkova et al. 2018). Because of their mechanical, structural and biochemical properties, sclerenchyma fibers are key for the mechanical properties of timber-based construction material and constitute an energy-rich component for the fuel wood industry. The flexibility combined with tensile resistance of bast fibers in flax, ramie, hemp, jute, kenaf are also exploited in the textile industry (Hayward 1938, Hill 1952, Esau 1965, Gorshkova et al. 2018).

The extreme length of sclerenchyma fibers is generated starting from relatively short precursor cells formed in meristems—the stem cell niches in plants. Fiber differentiation and morphogenesis, therefore, require the cells to expand in highly anisotropic manner and through surrounding, slower-growing tissues (Fig. 4.9.1a). This occurs through intrusive growth that penetrates the apoplast connecting neighboring cells (Esau 1965, Fahn 1979, Sanati Nezhad and Geitmann 2013, Gorshkova et al. 2018). Plant fibers are thus, in principle, an excellent model to study plant invasive growth, cell wall formation and cell wall mechanics. However, despite their structural properties and economic importance the mechanics underlying their intrusive behavior is still poorly understood. This is mainly due to the fact that fibers are formed within the depth of and surrounded by other tissues which renders their isolation or *in situ* live cell observation difficult (Sanati Nezhad and Geitmann 2013, Gorshkova et al. 2018).

4.3.2 Invasion for cargo delivery across tissues

Elongated cells provide an excellent catheter-like system that enables the transport of cargo from one region of the organ to another, even across tissue boundaries. One such trans-tissue transfer is required for successful fertilization in flowering plants. The sperm cells must be shuttled from the pollen grains upon their arrival on the landing platform of the pistil—the stigma—through the stylar transmitting tissue to the female gametophyte located in the ovary. This transfer is accomplished by a cellular protuberance formed by the germinating pollen grain, the pollen tube. This protuberance emerges from an opening in the hard outer wall of the pollen grain and undergoes localized elongation at the very apex. This tip growth process is reflected in the extremely polar organization of the cytoplasm (Geitmann 2010, Chebli et al. 2013) and the spatially confined expansion of the cell surface (Fayant et al. 2010, Geitmann 2011). Depending on plant species, the diameter of a pollen tube varies between 5 μm and 20 μm and the length can extend up to tens of centimeters depending on the length of the pistil. While the entire pollen tube consists of a single vegetative cell, the active portion of the pollen tube cytoplasm is restricted to the apical portion of the cell only, and the distal region is plugged off and eventually degenerates (Chebli and Geitmann 2007). When invading the stigma and transmitting tissue of the style, pollen tubes have to overcome multiple mechanical constraints (Figures 4.9.1b-d). The elongating tip of the tube, therefore, has to exert sufficient penetration force in order to withstand the external compressive stress generated by transmitting tissue while maintaining the direction towards its target, the ovule (Gossot and Geitmann 2007, Reimann et al. 2020).

4.3.3 Spreading out for procurement of nutrients and water

Cells elongating beyond the perimeter of the organism, into the external substrate, confer the ability to explore a larger space on the search for nutrients or water. In order to fulfill their nutritional needs from diverse biological and synthetic substrates, filamentous fungi extend colonies by forming branched hyphae that have the ability to penetrate solid substrates of considerable mechanical stiffness, including rocks. Hyphae are commonly formed by yeast, mushroom forming fungi, and also by oomycete water molds (Money 2007, Sanati Nezhad and Geitmann 2013, Lew 2019). Hyphae are tube shaped cells with a diameter ranging from 2-20 μm, elongating at the tip region, similar to pollen tubes. The hyphae of a fungal organism often grow in a direction centrifugal with regards to the center of the mycelium to ensure most efficient exploration of the substrate (Sanati Nezhad and Geitmann 2013). Although hypha usually elongate individually, they sometimes aggregate in parallel to form a structure called rhizomorph. These rhizomorphs can have a diameter of several mm and can elongate several meters in length (Sanati Nezhad and Geitmann 2013). They have the invading capacity to penetrate through soil or wood in search of nutrients which are transferred to developing fruiting bodies (Shaw III and Kile 1991, Yafetto et al. 2009). When penetrating and elongating, fungal hyphae secrete enzymes that digest polymers to sugars and other molecules that can be taken up through the plasma membrane (Money 2007). The enzymatic digestion is also thought to soften the physical impedance of the surrounding substrate (Money 2007).

Certain fungal hyphae establish a symbiotic relationship with plant root tissues, where the plant provides organic molecules such as sugar to the fungus and the fungal partner provides water and minerals absorbed from the soil to the plant (Bolan 1991). This association is highly intimate since it involves the invasion of the fungal hyphae into the apoplast of the root epidermal layer

(ectomycorrhiza) or the root cortex (endomycorrhiza) (Bolan 1991, Sanati Nezhad and Geitmann 2013) (Figures 4.9.1e,f). Endomycorrhiza invade not only the root apoplast but actually grow into the root cell lumen where they form arbuscular structures that augment the interaction surface between the fungus and the protoplast of the plant cell.

The plant root is not only subject to invasion, it is an active invader and exerts this action at different scales. At cell scale, roots produce their own extensions that explore the substrate beyond the surface of the organ. This is done by root hairs—tube shaped, tip growing extensions emerging from the epidermal cells of the root that range from 5-17 μm in diameter and about 0.1-1.5 mm in length (Dittmer 1949, Jungk 2001). Similar to fungal symbionts, root hairs serve to increase the interaction surface between the absorbant region of the root and the soil thus facilitating nutrient and water uptake (Farr 1928, Bengough et al. 2016, Carminati et al. 2017).

Root invasion also occurs at the scale of the organ. In their interaction with the growth substrate, roots grow against and interact mechanically with soil particles. The soil is not the first or only obstacle for root growth, as newly forming roots (radicles) encounter mechanical impedance in form of the seed coat (Creff et al. 2015) or, in the case of lateral roots, the outer layers of the primary root (Lucas et al. 2013). Since lateral roots are formed from the pericycle—a cell layer in the central stele of the primary root—reaching the outside requires the exertion of invasive forces for the young lateral root primordium to break through the outer tissue layers of the primary root (Figure 4.9.2). The mechanical interaction of the root with an external matrix influences distinct morphological and developmental changes in the root system (Dupuy et al. 2018). Studies done on the roots of cereal crop species have revealed irregular cortical cell growth, increased root diameter and bending and buckling of the root tip as a result of

mechanical resistance from the soil particles (Lipiec et al. 2012, Dupuy et al. 2018). This mechanical impedence created as a result of compacted soil layers or soil drying can be a major limiting factor to root elongation and hence nutrient uptake for the plant. Increased soil strength requires roots to exert higher forces to ensure successful soil penetration (Colombi et al. 2017). To minimize the effect of soil mechanical impedence, roots of maize (*Zea mays)* and soybean (*Glycine max*) use natural or artificial macropores in the soil or growing matrix. Invading these openings in the otherwise compact material allows following the path of least resistance (Stirzaker et al. 1996, White and Kirkegaard 2010, Colombi et al. 2017). Another common response to increased soil strength is the thickening of roots which decreases penetrative stresses and stabilizes the root (Materechera et al. 1992, Chimungu et al. 2015). Root hairs have been suggested to play a supportive role during root penetration by anchoring the root to the surrounding soil (Stolzy and Barley 1968, Bengough et al. 2016).

Given the fundamental importance of root growth through various soil types for yield and drought resistance, numerous biomechanical frameworks have been established for soil penetration mechanics. Root growth forces have been quantified with the help of technologies such as photoelastic discs (Kolb et al. 2012), or cantilever sensors (Bizet et al. 2016) and 3D living imaging of the roots in transparent soil (O'Callaghan et al. 2018) as well as x-ray based imaging (Helliwell et al. 2017) have greatly advanced the field. Since roots are multicellular tissues of macroscopic size, we refer to excellent reviews on the topic of invasive root growth (Dupuy et al. 2018, Stubbs et al. 2019) and focus on the single-celled growth of root hairs in the present chapter and focus on the single-celled growth of root hairs in the present chapter.

4.4 Cell mechanics of intrusive growth

4.4.1 Highly polarized cell extension directs force generation

The growth pattern of invasive cells is quite unique as the cells are highly polarized and typically extend exclusively at the very apex of the cell (Geitmann et al. 2001, Fayant et al. 2010, Geitmann 2010, Geitmann 2011). The morphogenetic process generating these cylindrical cells has been subject to multiple efforts to characterize the cell-mechanical underpinnings through modeling (Kroeger and Geitmann 2012, Bidhendi and Geitmann 2018). The apical cytoplasm of tip growing cells is often densely populated by vesicles, both exocytotic and endocytotic (Figure 4.9.3). Exocytotic vesicles deliver the material required for cell expansion, notably cell wall precursors and membrane material. In pollen tubes, the apical vesicle population forms an inverted cone-shaped region that is fed by Golgi-derived vesicles delivered to the apex by an array of actin filaments (Kroeger et al. 2009) (Figure 4.9.3b), whereas fungal hyphae typically feature a structured vesicle aggregate called Spitzenkörper organized by microtubules (Bartnicki-Garcia et al. 1995) (Figure 4.9.3a). This interaction of Spitzenkörper and microtubule is also known to function as ‘compass’ that serves in preserving the ‘directional memory’ of the fungal hyphae when they navigate around the obstacles (Held et al. 2019). Bigger organelles such as mitochondria and endoplasmic reticulum sometimes share the apical space but more typically remain in more distal regions of the tubular cell. Vesicle movement in hyphae occurs towards the Spitzenkörper and from there radiates to the apical plasma membrane, whereas in pollen tubes, vesicles are delivered through a circular movement which in angiosperm pollen tubes occurs in

an inverse fountain-shaped pattern controlled by the actin cytoskeleton (Hepler et al. 2001a, Bove et al. 2008, Geitmann and Nebenführ 2015). In all tip growing cell types, the spatial organization of the cytoplasm ensures that both delivery of new cell wall material and the expansion of the existing cell wall are confined to the apical region of the elongating cell. This extreme polarization of tip growing cells is very different from the growth behavior of most other plant cells which display more global deformations across the cell surface, also termed diffuse growth (Geitmann and Ortega 2009). The continuous addition of new cell wall material is controlled by cellular feedback mechanisms (Kroeger and Geitmann 2012) and involves breaking and forming crosslinks between newly added and existing cell wall polymers (Geitmann and Steer 2006, Kroeger et al. 2011). Once the cell wall material at the tip is excreted it starts to stiffen which locks in the cylindrical diameter. In pollen tubes, this stiffening is caused by the gelation of pectin polymers whose secreted methyl esterified configuration becomes de-esterified *in muro* (in the wall) through the action of pectin methylesterase (Chen et al. 2003, Chebli et al. 2012). In fungal hyphae, the main wall components are chitin and β-glucans and the distal stiffening involves hydrogen and covalent bonds (De Groot et al. 2005, Gomes et al. 2018). The tip-focused maintenance of the cylindrical geometry is a self-similar morphogenetic process that is regulated through a finetuning between internal turgor pressure and biochemical cell wall properties at the apical growing region (Fayant et al. 2010, Sanati Nezhad and Geitmann 2013).

While pollen tubes, fungal hyphae and root hairs display a clearly discernable pattern of tip growth, the growth pattern of sclerenchyma fibers may be more complex. In secondary xylem fibers, true tip growth seems to prevail as these fibers develop in the portions of the stem in

which the tissues surrounding the fibers ceased elongation (Gorshkova et al. 2018). The initial elongation of primary flax phloem fibers on the other hand, seems to occur through diffuse growth as the neighboring cells continue elongating in lockstep at least during the initial developmental phase of the organ. During this early developmental phase, the entire cell surface enlarges, (Ageeva et al. 2005) followed by an intrusive elongation of the fiber tips once growth in the neighboring cells has ceased (Figure 4.9.1a). Distinguishing the different growth patterns is aided by monitoring strain patterns of the cell surface and by profiling the fiber cell wall and fiber tips via biochemical and mechanical parameters (Mellerowicz et al. 2008, Siedlecka et al. 2008, Gorshkova et al. 2012).

4.4.2 Turgor pressure generates the invasive force in walled cells

Cell growth in plants involves the expansion of the existing cell wall driven by the turgor pressure,(Cosgrove 2000) but whether the exertion of invasive forces is equally dependent on turgor or whether other cellular features are involved has been a matter of discussion. Extension of sclerenchyma fibers has been proposed to rely on elevated turgor based on the assumption that the soft and thin walled growing fibers would be squished if they were less turgescent than the adjacent cells but experimental quantification of turgor is elusive (Snegireva et al. 2010). The increase in turgor pressure at the initiation of intrusive growth and the maintenance of turgor during the fiber elongation require the movement of water into the developing fiber. Gene expression patterns suggest that regulation of this water movement during fiber growth involves aquaporins—protein channels facilitating water movements across membranes (Hertzberg et al. 2001, Israelsson et al. 2003, Snegireva et al. 2010).

The turgor pressure of growing pollen tubes has been measured in lily and was found to range between 0.1 and 0.4 mPa (Benkert et al. 1997b). Consistent with this, the maximum force that a pollen tube can produce when overcoming a mechanical barrier has been measured to be approximately 10 μN for lily (Burri et al. 2018) for 1.5 μN for *Camellia japonica* (Ghanbari et al. 2018). Since force is the product of the pressure and the interaction surface between the pressurized vessel and the substrate (Sanati Nezhad and Geitmann 2013), the latter value was calculated to correspond to a pressure of 0.19 mPa, consistent with the magnitude of turgor. While the measured invasive forces in pollen tubes are consistent with the notion that turgor is the driving force of their invasive activity, this does not necessarily mean that the turgor pressure is also the parameter that is tuned to regulate the magnitude of growth speed or invasive force. In fact, different growth rates do not seem to be correlated with different turgor values, and even non-growing pollen tubes can have a turgor similar to that of growing pollen tubes (Benkert et al. 1997b). Variations in growth rate seem to rely instead on a modulation of the biomechanical properties of the cell wall which in turn can oscillate through the effect of exocytosis of new cell wall material and cell wall modifying enzymes (Zerzour et al. 2009). This modulation takes place at the apical cell wall which is substantially more compliant than the cylindrical portion (Fayant et al. 2010) thus enabling the exertion of forces against an outside substrate at this site of the cell surface (Figure 4.8.3b) (Sanati Nezhad and Geitmann 2013). Even if the apical wall is relatively compliant, a threshold turgor is necessary for pollen tube elongation and invasion as the plasmolyzed pollen tubes are unable to grow (Kroeger et al. 2011). On the other hand, excessive turgor pressure can result in tube bursting (Beauzamy et al. 2014) and turgor clearly must be carefully calibrated to be within a particular range (Hill et al. 2012).

The maintenance and rapid regulation of turgor in pollen tubes is likely facilitated by the segmentation of the protoplast that separates the continuously elongating growing region from degenerating distal regions where turgor is gradually lost. This segmentation is achieved by the deposition of plugs made through localized centripetal invagination of the cell wall built from callosic wall material (Figure 4.9.3b). These plugs are produced repeatedly once the male germ unit has moved forward through a particular tubular segment and ascertains that the volume of the living portion of the cytoplasm remains within a relatively constant range (Parre and Geitmann 2005a, Beauzamy et al. 2014).

The values of maximum force generated by pollen tubes in *in vitro* setups does not necessarily allow to deduce the actual penetration force exerted inside the pistil as this force is dependent on both growth rate and the stiffness and texture of the surrounding matrix (Sanati Nezhad and Geitmann 2013, Reimann et al. 2020). The use of differently stiffened growth matrices allows determining whether the penetrative ability in a given cell type is influenced by the substrate and what the optimal stiffness is (Bastmeyer et al. 2002, Gossot and Geitmann 2007, Reimann et al. 2020). Pollen tube species such as *Arabidopsis* actually grow better in a stiffened medium compared to a liquid medium, in line with the tissue architecture of the pistil in this species (Reimann et al. 2020). A systematic comparison of plant species with solid style (transmitting tissue consisting of densily packed cells) or hollow style (transmitting tissue lining a cell-free canal filled with a viscous extracellular matrix) revealed a consistently different behavior of the respective pollen tubes when confronted with a stiff artificial matrix *in vitro* (Reimann et al. 2020). Tubes adapted to a solid style do not only display greater ability to penetrate a stiffer matrix when compared with the pollen tubes from species with hollow style, but also prefer a

stiffer medium when presented with a choice, a phenomenon termed durotropism (Reimann et al. 2020). The growth matrix influences pollen tube growth not only through its stiffness properties, but a structural anisotropy can cause pollen tubes to display significantly altered growth directions. In *Arabidopsis* mutants with altered cellulose orientation in the stigmatic papilla, pollen tubes formed coils around the papillae rather than growing straight along these longitudinal cells (Riglet et al. 2020).

Fungal hyphae are exposed to highly variable substrates and, therefore, have to be able to efficiently adjust to changing external osmolyte concentration. Certain substrates that hyphae are able to penetrate are phenomally stiff such as rock. Some fungal species can create specialized structures able to produce the pressures that are required to invade particularly resistant surfaces. One such example is the plant pathogen *Magnaporthe grisea* which produces infection pegs from flattened, enlarged hyphal tips called appressoria. Appressoria can establish a turgor pressure of up to 8 mPa that enable the invasive structure to penetrate the plant epidermis (Bechinger et al. 1999). A tight control of the turgor pressure therefore seems to be crucial for fungal organisms. Upon hyperosmotic shock, fungal hyphae of *Neurospora crassa* display reduced turgor pressure, hyphal growth and decrease in hyphal volume, but all parameters were found to rapidly return to their original values through regulatory mechanisms (Lew and Nasserifar 2009). The second crucial element in addition to high turgor is the capacity of the appressorium to firmly adhere to the surface of the structure to be invaded. This adherence is key to prevent pushback caused by the invading infection peg. While the invasion angle of the *Magnaporthe grisea* infection peg is normal to the plant leaf surface, a different strategy is employed by *Phytophthora*, a plant pathogenic oomycete. *Phytophthora* hyphae do not form

appressoria, but they do adhere to the surface of the plant orgen to then assume an oblique angle to breach the surface. The angled approach has been likened to the slicing principle of single-beveled Japanese kitchen knifes and was accordingly named 'naifu-mechanism' (Bronkhorst et al. 2021). Whatever the angle of attack, it is generally assumed that the infection peg or invading hypha requires substantial turgor pressure, although whether this truly applies to all types of hyphae and under all conditions remains unclear since certain oomycete hyphae seemingly grow even in the absence of any measurable turgor pressure (Harold et al. 1996, Lew et al. 2004).

4.4.3 Cytoskeletal elements regulate tip growth and invasion through cell wall assembly

Force generation in animal cells involves cytoskeleton-based actions such as polymerization of cytoskeletal arrays and contractile mechanisms. Similar principles have been proposed for some walled cells (Gay and Greenwood 1966, Li and Heath 1994) since they were observed to elongate under low or absent turgor pressure (Li and Heath 1994). In pollen tubes, pharmacological interference with actin polymerization reduces the cell's ability to invade and penetrate a stiffened medium,(Chebli and Geitmann 2007, Gossot and Geitmann 2007) however, whether this effect can be ascribed directly to any force generation by the cytoskeletal arrays is unclear. Interestingly, moderate interference with the actin cytoskeleton also abolishes growth oscillations in these tubes(Geitmann 1999). This indicates that the role of actin in regulating the invasive force of pollen tube growth may be mediated by the delivery of cell wall material to the expanding apex and thus the dynamics of the supply of the building material that is required for the cell to elongate (Gibbon et al. 1999, Vidali et al. 2001, Chen et al. 2003, Chebli and Geitmann 2007).

Studies in oomycetes *Achlya bisexualis* and *Phytophthora cinnamomi* provide similar evidence for such an indirect role of actin. Comparing hyphae growing either invasively (through agar) or non-invasively (on the agar surface) revealed an ‘actin-free zone' at the tip region of the former but not the latter (Walker and Garrill 2006) (Vaškovičová et al. 2013). The authors suggest that the depletion of actin at the tip region of invasive cells would ensure a greater yielding capacity of the cell wall thus allowing for higher invasive force to be exerted (Walker and Garrill 2006, Suei and Garrill 2008). Similar evidence for actin functioning as a ‘restraint’ for tip expansion has been found in zygomycete *Gilbertella persicaria* (Grove and Sweigard 1980) and ascomycete *Geotrichum candidum* (Heath et al. 2003). In other words, actin is suggested to be involved in tip yielding and thus the invasive growth in fungal hyphae, through its effect on the cell wall.

Microtubules, on the other hand, do not have a significant effect on the pollen tubes' ability to invade a stiffened medium, but they seem to be involved in enabling pollen tubes to change growth orientation as demonstrated by pharmacological treatment (Gossot and Geitmann 2007). Mutation in the microtubule-severing enzyme KATANIN1 confirms that invasive pollen tube growth is not affected by interfering with microtubule dynamics (Riglet et al. 2020). The helically bundled cortical microtubules localized at the sub apical region of a tip growing cell are, however, suggested to be involved in developing a ’structural memory’ for the cell to enable to restore growth direction after an encounter with an obstacles (Sieberer et al. 2005, Vaškovičová et al. 2013).

4.5 Chemical and enzymatic tools facilitating invasion

The pollen tube encounters multiple types of obstacles on its way through the stigmatic and stylar tissues, starting with the stigmatic cuticle layer (if present), followed by the transmitting tissue, the micropyle of the ovule, the nucellus enveloping the female gametophyte and the synergid cells adjacent to the egg cell. In species with solid transmitting tissue, the intercellular spaces are typically narrower than the tube diameter,(Erbar 2003) several additional mechanisms such as enzymatic lysis and chemical digestion are thought to be employed by the tube to facilitate invasion. Enzymes can help break down the molecules involved in cell adhesion or can degrade the invading tissue completely. Some pollen tubes, for example those of *Brassica napus* L., produce cutinase, an enzyme that digests the cuticle of the stigmatic papillae (Hiscock et al. 1994). Agents with potential to affect the apoplast are expansins, (Cosgrove et al. 1997, Pezzotti et al. 2002) polygalacturonase, glucanase, endoxylanase, (Bih et al. 1999) and pectin esterase (Konar and Stanley 1969). While gene expression profiles show that these proteins are expressed by pollen tubes, it has been difficult to tease apart whether they serve to modulate the pollen tubes own wall or are targeted at the transmitting tissue. Another elegant way to soften the solid matrix is autodigestion via programmed cell death (Greenberg 1996). This has been shown to occur upon pollination in plant species such as petunia, (Herrero and Dickinson 1979) where the transmitting tissue undergoes cell death leading to turgor loss and tissue softening. This both softens the pollen tubes path and may also provide additional nutrients for the elongating cell. Enzymatic lysis is a crucial tool for fungal species to enable infection of other organisms. Plants use their external cuticle layer and the polysaccharide rich cell wall as the ‘first line of defense’ against invaders (De Lorenzo et al. 1997). Fungi, therefore, produce a wide variety of enzymes

that have the ability to depolymerize the plant cell wall polysaccharides (De Lorenzo et al. 1997). Among these are pectinase, (Linhardt et al. 1986, Blanchette et al. 1989) cellulase, (Blanchette et al. 1989) arabinase, (Flipphi et al. 1993) xylanase, (Apel et al. 1993) and galactanase (Urbanek and Zalewska-Sobczak 1986). Enzyme deficient fungal mutants display reduced ability to cause infection (Rogers et al. 1994, Tonukari et al. 2000). Cutinase is formed in particular by fungal species that do not form appressoria and hence are unable to generate similarly high physical forces (Mendgen et al. 1996, Bastmeyer et al. 2002). The secondary cell walls of wood tissues are more resistant to degradation both physically and chemically, but some fungal species such as white rot and brown rot produce enzymes that degrade hemicellulose, lignin and cellulose, core polysaccharides forming the compound middle lamella in wood (Daniel 2016). Enzymatic digestion of cell walls is also often employed by invasive organisms that trigger the development of plant tumors (Ullrich et al. 2019). For instance, in the case of corn smut disease, the infection by the biotrophic fungus *Ustilago maydis* alters the level of hemicelluloses in the infected plant cell wall, modifying the abundance of xylose and arabinose, a process that presumably facilitates piercing the organ surface (Matei et al. 2018). Subsequent tumor formation results from a local plant cell enlargement and cell division triggered by the fungus. Contrary to animals, tumors are rarely fatal for plants since metastasis is impossible due to the lack of circulatory system (Ullrich et al. 2019).

4.6 Biomechanical approaches to quantify invasive forces

Invasive growth clearly relies on cell mechanical features and the cells ability to generate forces. To better understand invasive growth, it is, therefore, essential to quantitatively characterize these features. Significant effort has been put into designing experimental devices that are able to

determine the mechanics of the cell wall, to quantify turgor, and to measure the invasive force of individual cells. Advancement in micro-measurement technology in recent years has been instrumental, notably microfluidics (microdevices with controlled fluid flow) and microelectromechanical system (MEMS)- based platforms (Agudelo et al. 2013, Horade et al. 2013, Sanati Nezhad 2014, Geitmann 2017, Yanagisawa et al. 2017). These Lab-on-Chip (LoC) devices allow creating micro-environments that mimic aspects of the natural growth environment such as varying degrees of mechanical resistance, chemical gradients, or patterned physical obstacles (Figures 4.9.4a-c). Importantly, LoC allow observation and manipulation of individual cells confining their growth to a single focus plane thus enabling high resolution microscopy and extended time-lapse imaging (Yanagisawa et al. 2021). The fabrication of microfluidic and MEMS devices allows for micrometer precision but requires engineering expertise (e.g. direct-write lithography and cleanroom facilities) (Agudelo et al. 2012, Agudelo et al. 2014), but technically simpler and more affordable alternatives can be used if spatial resolution of the design is less critical (Bertrand-Rakusová et al. 2020). Exploiting LoC technology, a platform called the TipChip was developed to study the growth pattern of pollen tubes, for example to characterize their chemotropic behavior and response to electrical fields (Agudelo et al. 2013, Agudelo et al. 2016) (Figure 4.9.4a). These studies are conducted with the aim to understand how the pollen tube orients its growth in the maze of the female tissues (Geitmann and Palanivelu 2007). The electric LoC is fabricated from two separate bondable modules: a PDMS-based microfluidic module for accommodating the suspension of cells in liquid medium and a micro electrode module with a metallic layer that serves to apply the electric field (Agudelo et al. 2013). As in all variations of the TipChip, the height of the microfluidic channel network is

determined by the size of the pollen grains (80 µm for *Camellia japonica*) (Agudelo et al. 2013), whereas other microfluidic platforms place the grains outside of the microchannel network proper allowing for a smaller vertical dimension to fit the growing pollen tubes more snugly (Yanagisawa et al. 2017). Depending on the experimental design and the needs for continuous fluid flow, the channel design must allow fluid-flow mediated placement of pollen grains to locations or traps where they are immobilized and from where the tubes grow into or towards the testing setup (Ghanbari et al. 2014, Sanati Nezhad et al. 2014a). The microchannel architecture must also be designed to avoid clogging to allow for effective liquid exchange.

To quantify the pollen tubes invasive forces, LoC have been employed to expose them to a variety of narrow spaces and complex mazes (Sanati Nezhad 2014) with sophisticated microscopic design features such as elastic cantilevers serving as strain gauges (Figures 4.9.b-c, 4.9.5c&d) (Ghanbari et al. 2018). Microchannels featuring consecutive narrow gaps were designed to mimic the microarchitecture of the pistillar tissue (Figures 4.9.4b, 4.9.5b) (Sanati Nezhad et al. 2013, Sanati Nezhad 2014, Yanagisawa et al. 2017). An elongating pollen tube deformed the PDMS sidewalls of the gap allowing for the calculation of the force exerted to maintain its diameter against compressive stress (Sanati Nezhad et al. 2013). Intriguingly, the pollen tube diameter changed transiently after it made its way through the gap (Figure 4.9.4b), suggesting the existence of a feedback mechanism that calibrates the invasive force through modulation of cell wall mechanical properties (Sanati Nezhad et al. 2013). It was also shown that the vegetative nucleus and sperm cells were able to move forward through the tube while significantly constricted by the gap demonstrating substantial elastic deformability (Figure

4.9.5b). Similar observations were made for root hairs and moss protonemata (Yanagisawa et al. 2017).

The strain gauge principle was also used to measure the invasive force exerted by the hyphal apices (Wright et al. 2005, Money 2007). In both pollen tubes and fungal hyphae, a technical difficulty consisted in stabilizing the longitudinal cell sufficiently to enable measurement of reliable data for the elongation force. Strain gauges and cantilevers have been combined with kinked microchannels (Figure 4.9.5c) or agarose to stabilize the base of the tubular cell. This is a critical element of the experimental design as both pushback and buckling must be prevented to enable reliable and reproducible quantitative measurements. Because of their ability to strongly adhere to surfaces, the problem of stabilizing the base of the cell was less of a challenge in the case of the infection pegs formed from fungal appressoria. This allowed the use of a waveguide whose deformation by the emerging peg could be monitored optically and used to calculate the force (Bechinger et al. 1999, Geitmann 2006) (Figure 4.9.4d).

Other attempts to measure the invasive force of tip growing cells used optical tweezers. Fungal hyphae of *N. crassa* were faced with obstacles in form of polystyrene beads trapped in a beam of laser light (Figure 4.9.4e). The force required to push a bead from its trapped position is directly proportional to the bead's size implicating that more force is required to dislocate a larger bead (Wright et al. 2005). Regular hyphae displaced all the beads, because the strength of the optical trap is limited only to a few piconewton range, (Wright et al. 2005, Money 2007) but the force of a conidial germ tube was determined to be within the dynamic range of this assay revealing that its invasive force is much lower than that of leading fungal hypha tips (Wright et al. 2007).

Since the invasive force of tip growing walled cells is the turgor pressure, establishing values for this parameter is an important component when characterizing the cell mechanical underpinnings of invasion. If the cell wall is completely pliable, the 'entire' internal pressure generated can be exerted to an outside substrate, but if the apical cell wall poses substantial resistance to deformation, the invasive force exerted by the tip growing cell can be expected to be lower than its turgor (Wright et al. 2005, Money 2007). The invasive force therefore does not equate turgor. In order to measure the turgor pressure, several techniques have been employed such as incipient plasmolysis or the pressure probe—an oil filled microcapillary that is injected into the cell (Green 1968, Benkert et al. 1997b, Tomos and Leigh 1999). Since these methods are invasive in nature, they cannot be used repeatedly or over longer periods of time (Burri et al. 2019). As a minimally invasive method, ball tonometry and other indentation techniques (Figure 4.9.4f) have been used to estimate turgor. In ball tonometry, a large spherical probe with a controlled load is applied to the cell and the contact area is measured to deduce turgor. Other indentation techniques are based on pressing a cylindrical probe into the cell and measuring the applied force and the indentation depth. Recently, Burri et al. (2019) coupled a modified ball tonometry approach with microindentation technique to calculate turgor pressure and measure cell wall elasticity. The authors employed a non-invasive microrobotic system based on cellular force microscopy (CFM) in combination with two force sensors with different geometries and force ranges for simultaneous biomechanical measurements on elongation pollen tubes (Burri et al. 2019).

Cell wall mechanical properties are important parameters and atomic force microscopy (AFM), an indentation technique with smaller indentation depth and higher spatial resolution than CFM,

has been used to assess plant cells (Milani et al. 2011, Fernandes et al. 2012, Radotić et al. 2012, Majda et al. 2017). Depending on tip size and indentation depth, the deformation that is applied normal to the cell surface may be influenced by the turgor pressure and/or by the geometry of the tissue structure and the extraction of absolute mechanical values from indentation measurements is not trivial, (Peaucelle et al. 2011, Beauzamy et al. 2015) but has delivered insights on the viscoelastic nature of the fungal hyphae cell wall (Ma et al. 2005, Money 2007).

Exposing a tip growing cell to a mechanical cue has the potential to trigger the cell to modulate its invasive force or other cellular parameter—a consideration that must be made when making force measurements. Evidence for this ability of invading cells to modulate their force stems from the observation that pollen tubes growing through increasingly narrow openings continue at a constant speed despite the increasing impedance (Sanati Nezhad et al. 2013). This suggests that they may increase their invasive force during the process. This adaptive dynamic behavior was corroborated by the finding that once the obstacle is passed, the tube widens (Figure 4.9.4b) indicating that its cell wall had softened while it was pressing against the obstacle.

In addition to the absolute value of the invasive force, a comparative approach is therefore warranted. Pollen tubes exposed to an interface between two different concentrations of agarose were scored for their behavior and ability to penetrate into the stiffer medium thus offering a test assay to assess the effect of pharmacological interference with specific cell features on invasive growth (Figure 4.9.4g). To put the measured values for the penetrative behavior of tip growing cells in context, it will be valuable to measure the stiffness of the invaded matrix *in situ* (Reimann et al. 2020). In case of pollen tubes, it will therefore be crucial to quantify the stiffness of the pistil transmitting tissue. Determining the stiffness of a uniform material is relatively

straightforward and can be done by microindentation (Reimann et al. 2020). Results obtained with larger indenters can be extrapolated to calculate the invasion required by the microscopic invasive cell. This extrapolation is less obvious for matrices with complex micron-scale architecture such as that generated by the cellularity of the transmitting tissue and the presence of the middle lamella. Here, indenter size will matter, and the interpretation of such measurements will require careful consideration of geometrical features.

4.7 Conclusion and Perspective

Quantifying physical and mechanical properties at cellular and subcellular levels is a challenge as practical experimentation on these structures is difficult owing to the small size of the specimens. Despite these challenges, the parameters characterizing invasion and penetration events such as turgor driven growth and the dynamic regulation of cell wall mechanical properties have been assessed successfully in a range of cell types. Recent developments in micromechanics technologies such as LoC and MEMS-based force sensors will enhance our ability to directly quantify the role of osmolyte concentration and turgor pressure and will also provide insight into the cellular regulation of biomechanical properties. Quantitative micromanipulation will increasingly be coupled with powerful image analysis software, for example those that enable the detection of the subpixel resolution in order to do quantitative analysis of cell behavior (Damineli et al. 2017).

One of the major challenges associated with studying cellular invasive growth is the observation of this behavior *in situ*, as the invasive growth events occur several layers deep within the

invaded tissues. Two-photon excitation microscopy allows deeper penetration of plant tissues compared to conventional epi-fluorescence microscopy and confocal laser scanning microscopy and may thus be one of several possible avenues (Cheung et al. 2010). Novel high-resolution microscopy techniques such as lightsheet fluorescence microscopy are other options for deep tissue imaging and the continuous improvement in spatial resolution is promising. Since lightsheet imaging requires only low doses of light in order to acquire an image in a single plane, this technique will be crucial to allow long-term time-lapse imaging (Candeo et al. 2017). The combination of micromechanics with powerful microscopy technology will open exciting avenues for single cell analysis.

4.8 Acknowledgments

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4.9 Figures

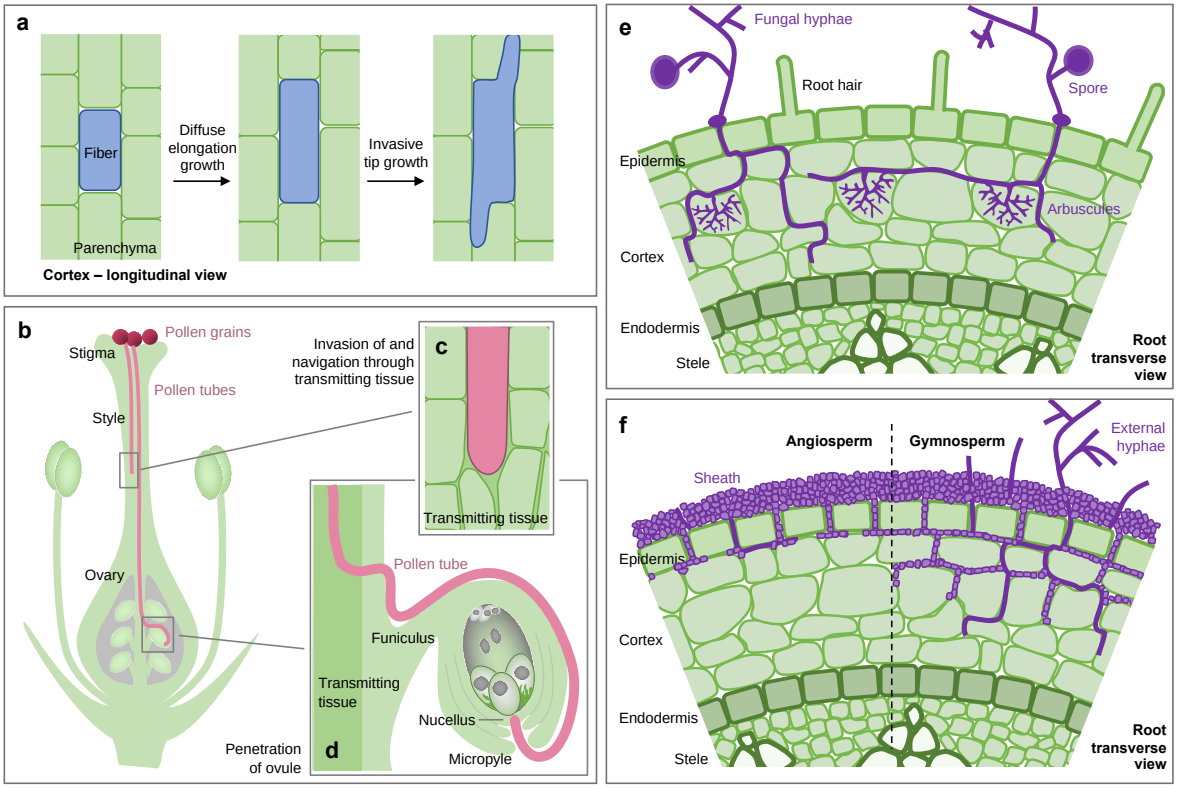

Figure 4.9.1 Various types of invasive cells: a) Development of sclerenchyma fiber cell from meristem precursor. Initial elongation occurs by diffuse growth in lockstep with surrounding parenchyma cells, but once these cease elongation the continuation of fiber growth occurs by tip growth. b) Mechanical obstacles in the pathway of pollen tube towards the ovary include the stigmatic cuticle, the apoplast of the transmitting tissue, the micropyle and the nucellus. c) Pollen tube making its way through the apoplast of the stylar transmitting tract. d) Pollen tube emerging from the transmitting tissue elongating on the surface of the funiculus, turning into the micropyle of the ovule. e) Fungal hyphae of arbuscular mycorrhiza, a type of endomycorrhiza, invade the epidermis and cortex of the plant root by penetrating the apoplast and building branched structures inside the lumen of root cortex cells. d) Fungal hyphae of ectomycorrhiza cover the root surface forming a sheath and penetrate the root epidermis forming a Hartig net. Occasionally, in particular in contact with gymnosperms, the hyphae of ectomycorrhiza reach external cortex layers.

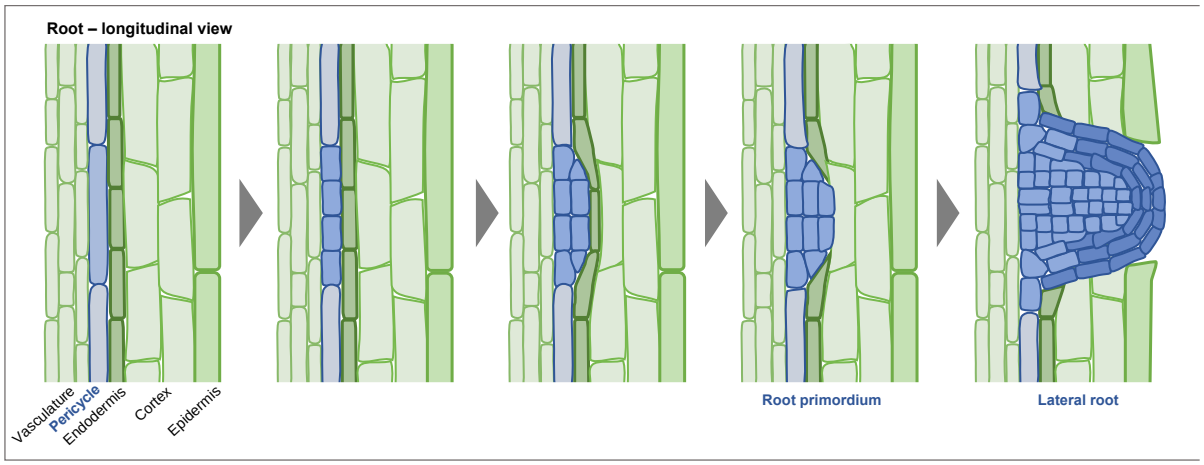

Figure 4.9.2 Lateral root formation initiates with cell divisions in a spatially confined region of the pericycle located in the central steele of the primary root. The resulting lateral root primordium develops a new root apical meristem whose continuous cell divisions produce the elongating new root which in turn breaks through the cortex and epidermis of the primary root to continue growing through the soil.

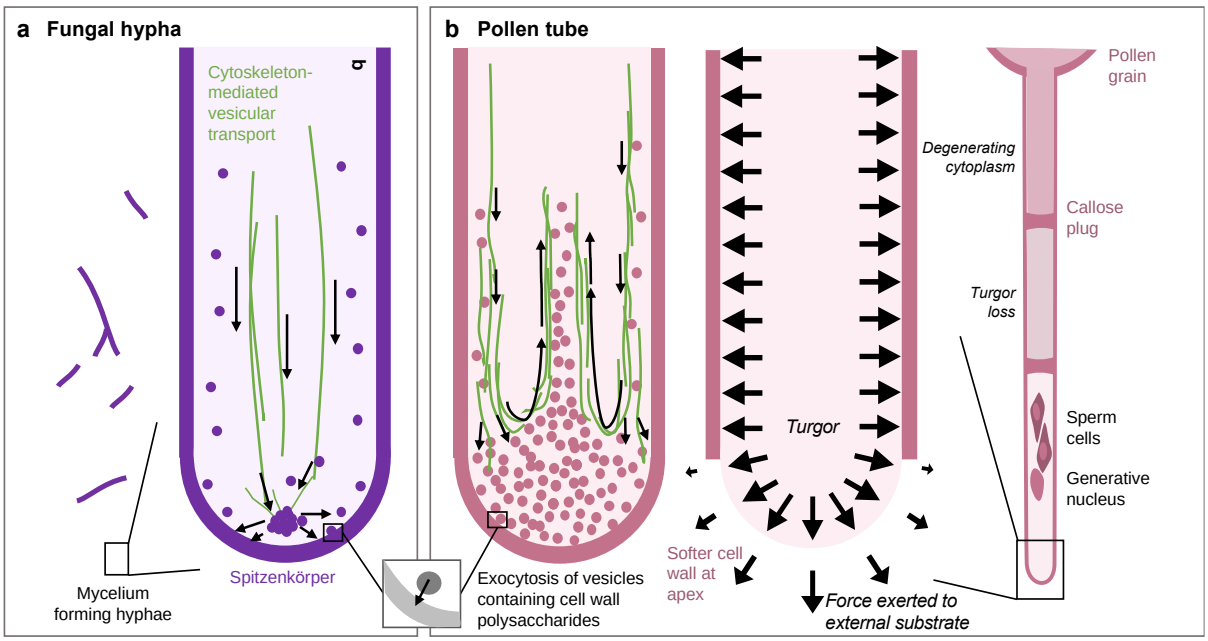

Figure 4.9.3 Cellular tip growth is fuelled by a continuous supply of secretory vesicles delivered by cytoskeleton-mediated transport. a) Many fungal hyphae feature a distinct vesicle aggregate, the Spitzenkörper, which regulates vesicle transport to the apical plasma membrane. b) In angiosperm pollen tubes, vesicles and other organelles move in a reverse fountain shaped pattern. The apical cell wall is softer than the wall in the distal region. Yielding of the cell wall to the turgor pressure in the apical region enables the cell to exert forces onto an external substrate. The cytoplasm of the pollen tube is segmented by callose plugs into the apical, viable region containing the sperm cells and generative nucleus, and distal regions that lose turgor and degenerate.

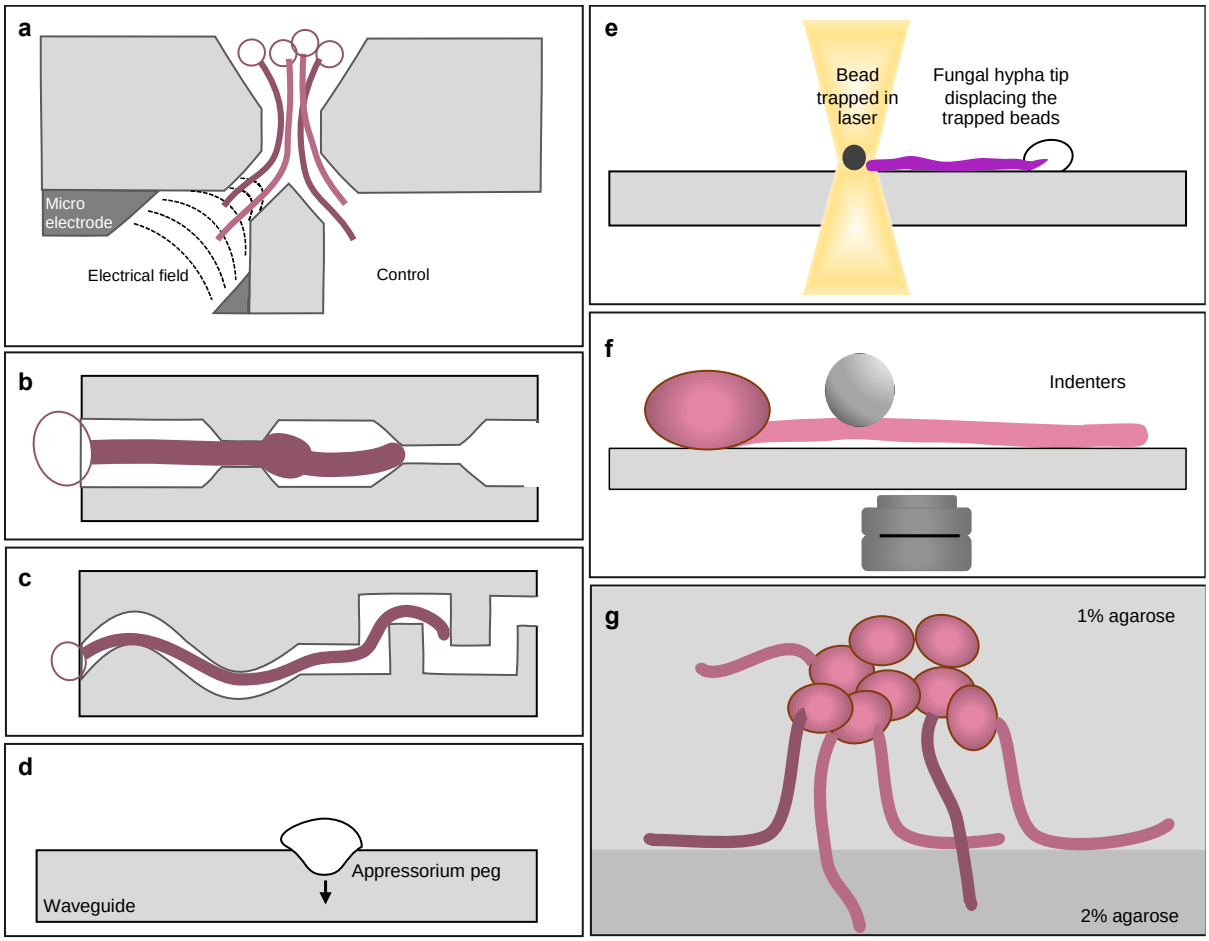

Figure 4.9.4 Biomechanical approaches to quantifying invasive and oriented tip growth behavior. a) Lab-on-chip device offering pollen tubes a choice to grow toward or away from an electrical field. b) Microfluidic design to challenge elongating pollen tubes with narrow gaps. The pollen tube diameter transiently widens following gap passage. c) Microchannels with complex geometrical patterns designed to investigate the pollen tube's ability to cope with mechanical obstacles and reorient growth. d) Measuring penetrative forces of fungal appressorium using waveguide deformation. e) Optical tweezers to measure the force exerted by hyphal tips by way of displacement of trapped beads. f) Microindentation using indenters of different shape and size used to determine cellular stiffness. The indenters are attached to cantilevers the deflection of which is monitored by optical sensors. g) Assay exposing growing pollen tubes to media stiffened to different degrees by varying the concentration of agarose.

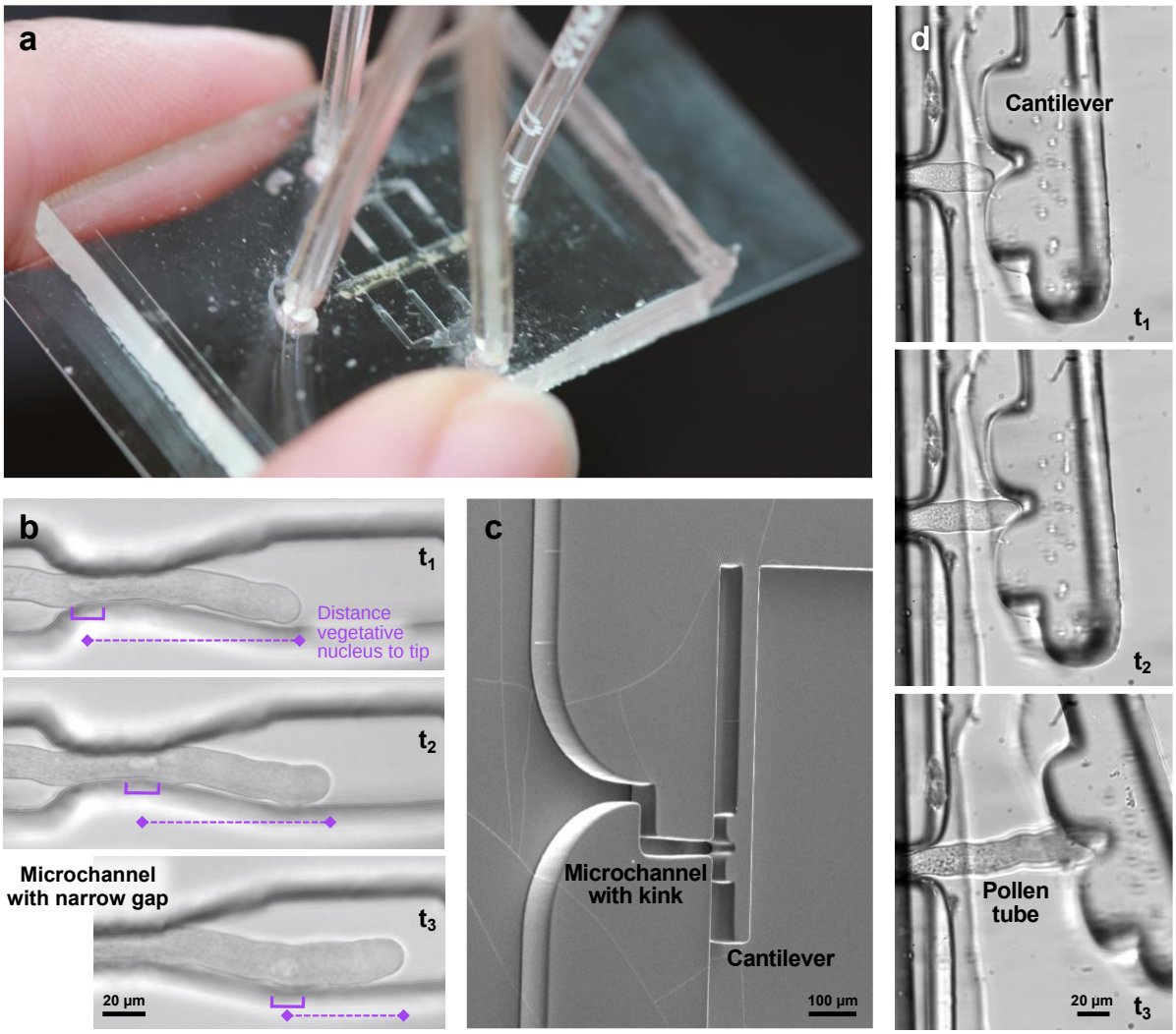

Figure 4.9.5 Applications of Lab-on-chip devices for the assessment of invasive growth behavior. a) General design of the TipChip with PDMS layer containing microfluidic network adherent to cover slip. Tubes serve as inlets and outlets for liquids and for injection of pollen suspension. b) *Camellia* pollen tube traversing a narrow gap. At t_1, the vegetative nucleus (purple bracket) has fallen behind its default distance from the growing tip because it got trapped in the narrow passage created by the gap. At t_2, the vegetative nucleus has almost made its way through the gap thanks to its elastic deformability. At t_3, the vegetative nucleus has reached its default distance from the pollen tube tip (60 µm) by accelerating its forward movement following gap passage. Image series provided by Amir Sanati Nezhad. Related data in Sanati Nezhad et al.(Sanati Nezhad et al. 2013) c) Scanning electron micrograph of PDMS cantilever used to measure the growth force of pollen tubes in Ghanbari et al.(Ghanbari et al. 2018) d) Brightfield micrographs of *Camellia* pollen tube growing against cantilever shown in c). Images in d) reproduced from Ghanbari et al.(Ghanbari et al. 2018) with permissions.

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5 General discussion

Plants, bacteria, and fungi have their cells encased in a rigid and semipermeable structure, the cell wall. The presence of this layer also represents the major difference between a plant and an animal cell. The plant cell wall is known to perform many diverse functions such as controlling and directing cell growth, providing mechanical support and strength for the plants, cell to cell communication, defense against biotic and abiotic stress. It also serves as a apoplastic conduit for entry and exit of molecules and storage site for carbohydrates and other molecules (Zhang and Zhang 2020). To perform these functions, plants have evolved a complex cell wall that is constituted of polysaccharides classified into three groups: cellulose, hemicellulose, and pectin. Cellulose is organized into crystalline microfibrils that are embedded in gel like matrix that consists of pectin, hemicellulose, and proteins.

For a cell to grow, assembly of cell wall material is required that may directly influence the direction and velocity of the growth. Moreover, during cell growth, the cell morphogenesis is a primarily governed by the material properties of cell wall components which is influenced by the assembly of new cell wall material (Chebli et al. 2012, Bidhendi and Geitmann 2016). Therefore, in walled cells growth and morphogenesis are tightly linked, and the two processes cannot be understood well without understanding the properties of cell wall, its composition, mechanics, and regulation. An interesting cellular model to study the direct links between wall properties, elongation and shape determination are the tip growing walled cells. These cells display extremely polarized growth that typically yields tube-like structures. Tip-growing cells such as pollen tubes and root hairs display common features such as tip-focused active metabolism and

many display oscillatory growth patterns (Rounds and Bezanilla 2013, Takeshita 2018), but the biological purpose of these different cell types varies significantly (Kapoor and Geitmann 2023a).

5.1 Callose abundance makes the pollen tube cell wall unique

One of the distinguishable features between primary cell wall of a somatic and pollen tube cell is the low abundance of cellulose in the latter. Instead, callose is the main cell wall polysaccharide in angiosperm pollen tubes (Parre and Geitmann 2005a, Derksen et al. 2011).

Given the spatial gradient of pectin esterification at the apex and sub apex region and the substantive abundance of callose in the inner cell wall lining starting from subapical segment (Cai et al. 2011, Chebli et al. 2012), the role of callose the pollen tube cell wall mechanics had to be addressed. Therefore, I investigated the role of callose abundance by observing the growth rate and diameter of the mutants lacking callose in their cell wall lining. I found out that the lack of callose in the cell wall lining and in form of plugs resulted in tubes with slower growth and increased diameter. These results thus complement our previous findings that callose has a role in resisting circumferential stress at the distal segment of the tube (Parre and Geitmann 2005a).

During the cell wall assembly of a pollen tube pectins and hemicelluloses are synthesized in the Golgi and delivered at the apex by exocytosis, often passing through the trans-Golgi network (Cameron and Geitmann 2018). Cellulose and callose on the other hand are synthesized directly at the surface of the cell by enzymes that are delivered to the plasma membrane via vesicular trafficking from the endoplasmic reticulum or Golgi bodies and inserted by exocytosis (Cameron and Geitmann 2018). Studies on tobacco pollen tubes provided insights on regulatory mechanism of cell wall biosynthesis where multiple enzymes such as cellulose synthase, callose synthases

and sucrose synthase complexes are carried by the dictyosomes along the actin bundles to the sub apex, which further gets transported along the actin fringe to get incorporated into the plasma membrane near the tip (Cai et al. 2011).

In pollen tubes, callose synthase complexes produce callose and are constituted of *CALLOSE SYNTHASES5* (*CALS5*) carrying two clusters of transmembrane domains that flank a long hydrophobic domain (Abercrombie et al. 2011). Verma and Hong (2001) proposed a model that consists of UDP-Glucose transferase -1 (UGT1) as a subunit of callose synthase complex which interacts with membrane bound phragmoplastin (cell plate associated protein). Additionally, UGT-1 also interacts with Rho-like GTPase (ROP1) that regulates callose biosynthesis (Hong et al. 2001). Once the sucrose synthase synthesizes UDP glucose, the ROP1-activated callose synthase complex converts UDP-glucose to callose and releases it across the plasma membrane. Cell culture studies in *Arabidopsis* suggested a critical role of Ca^{2+} ions and Mg^{2+} for callose synthase activity (Aidemark et al. 2009). Additionally, studies on cotton fibers showed potential interaction of annexins (*ANN*) with callose synthase in calcium-dependent manner thereby inhibiting callose synthase activity.

The β-1,3 -glucan hydrolase genes (*GH17* family), conserved across land plant species, are known to be involved in callose hydrolysis and recycling, hence, putatively involved in pollen tube remodeling (Tucker et al. 2018). Exogenous application of callose degrading enzymes such as lyticase on pollen tubes resulted in increased tube diameter, decreased cell stiffness, and increased viscoelasticity in the distal segment of the tube (Parre and Geitmann 2005a). No such effects were observed at the tip of the tube consistent with the absence of callose in this region of the cell (Parre and Geitmann 2005a). In lily pollen tubes, exo-glucanases were shown to play an

important role in the regulation of pollen tube elongation (Takeda et al. 2004) as application of glucanase inhibitors led to reduced pollen tube growth.

5.2 Callose plug septum formation in the pollen tube cell

A characteristic feature of fast-growing pollen tubes is the deposition of callose plugs formed at regular intervals. The presence of callose plugs in angiosperm pollen tube has puzzled pollen tube researchers for a long time. Since mutational absence of plugs does not prevent fertilization, clearly, these features are not necessary for the tubes to grow. However, given that various mutants with altered plug formation displayed altered pollen tube growth rates or fertilization efficiency, there was reason to investigate this phenomenon closer. I found that tubes with altered callose plugs deposition displayed slower growth when compared to their respective WT, when germinated with and without mechanical obstacles. The presence of plugs is hypothesized to prevent the cytoplasmic streaming to the distal segment of the pollen tube, with the newest plug formed close to the large vacuole. This unique feature has been proposed to help the tube maintain its cellular function while focusing its energy and machinery on tip-focused elongation. Thus our results are consistent with the notion that plugs have enabled pollen tubes to become extremely fast growing cells, whereas other tip growing cells are much slower, such as root hairs (Galway et al. 1997, Hepler et al. 2013).

The plugs have also been hypothesized to prevent backward movement of cytoplasmic content thereby maintaining the tube pressure and integrity (Li et al. 1997, Parre and Geitmann 2005a). Callose plugs feature only in angiosperm pollen tubes and are regarded as a marker for evolutionary development as they are not deposited in pollen tubes of gymnosperm (Williams 2008). The interval and frequency of plugs formed in a pollen tube may vary among different

species and even among different ecotypes of the same species (Laitiainen et al. 2002, Mogami et al. 2006, Cai et al. 2011). Using tobacco pollen tubes, Cai et al (2011) highlighted the role of microtubules in determining the site of plug formation. The authors hypothesize that microtubules incorporate callose synthase complex in the plasma membrane around the site of plug formation in the distal segment of the tube. If this is confirmed, it would mean a significant addition to our understanding of the role of microtubules in pollen tubes. So far, the involvement of microtubules in tip growth was thought to be rather minimal. Pharmacological inhibition of microtubules polymerization showed that pollen tubes continue elongating almost unimpeded although the movement of vegetative nucleus and generative cells towards the direction of the growing tip is affected (Laitiainen et al. 2002). Given the absence of an effect on pollen tube growth, it has not been clear whether pollen tube microtubules exert the role they typically play in other primary plant cells - the spatial guidance of cellulose synthases. However, if microtubules are confirmed to contribute to the position of callose synthases, they clearly have a potential direct effect on the assembly of the apoplast.

What triggers the regulatory mechanism that initiates the plug formation is still not known, however. A plausible hypothesis could be the internal turgor pressure of the tube, provided that it changes with increasing length of the apical tube segment. However, the plugs are also deposited in the tubes that display constant turgor pressure (Hill et al. 2012). The formation of callose plugs in tubes with constant turgor pressure could possibly be due to concentration gradient of enzymes that are associated with microtubule dynamics and /or callose synthase complex incorporation in the plasma membrane at the putative plug formation site (Adhikari et al. 2020).

These unknown enzymes may be controlled by a negative feedback loop in such a way that its function/expression would be impaired at its higher concentration (Adhikari et al. 2020).

5.3 Callose plugs as evolutionary markers

As mentioned above, callose represents about 80% of the wall mass of the tube distal segment (Schlupmann et al., 1994) and the bulk of wall volume in most angiosperm pollen tubes (Williams et al., 2016). Producing amorphous callose-based wall found in angiosperms is faster and more energy efficient than building a cellulose microfibril-based wall produced by gymnosperms (Kudlicka and Brown Jr, 1997; Williams, 2012). Some early-divergent angiosperm species like *A. trichopoda*, *Nuphar polysepala* or *Austrobaileya scandens* have pollen tubes with callose wall lining and produce callose plugs (Williams 2009, Dehors et al 2019). The presence of these two features in early divergent angiosperms along with novel secretory carpel tissues (Williams, 2009) are probably the reason for the difference of growth rates between angiosperms and gymnosperms. The fast growth rate of pollen tube is accomplished in angiosperms by efficient exocytosis of new wall material at the apex or the sub apex segment of the tube (Williams et al., 2016) along with endocytosis of cell wall components such as esterified pectin and cellulose (Malhó et al. 2005, Chebli et al. 2012).

The inner callosic layer at the shank of the pollen tube ensures an efficient polarized tip-growth and resistance to internal turgor pressure. In the present study the reduced growth rates and the reduced invasive capacity of callose synthase mutants pollen tubes further corroborate the notion that the short-lived and fast-growing pollen tubes associated with the presence of an inner callose wall and callose plugs deposition are the novel features that have instigated the ecological success of angiosperms.

5.4 Pollen tube elongation

The specialized male gametophyte (pollen tube) in higher plants contains two sperm cells but expands as a single cell since the tube's growth is governed by its haploid vegetative nuclear genome. The pollen tube invades and navigates through several female sporophytic tissues to reach its target (ovule) and delivers the two sperm cells. This directional growth towards the female gametophyte is guided by attractants produced in the ovule (Okuda et al. 2009, Palanivelu and Tsukamoto 2012, Johnson et al. 2019). In case of an *in vitro* germination set up, the pollen grain germinates, and tubes elongation occurs in random direction, but the tubes expand unidirectionally keeping their shape intact, suggesting a self-organizing mechanism with precise growth polarity even in absence of a guiding signal.

An elongating pollen tube displays an extreme form of polar growth in which exocytosis is targeted to the apical region for apical cell surface expansion (Kroeger et al. 2009, Luo et al. 2017, Bibeau et al. 2018). The intracellular trafficking of organelles and vesicles in pollen tubes involves redistribution of membrane bound structures that also allows the specific accumulation of secretory vesicles at the tube tip, giving rise to a highly polarized cell (Kroeger et al. 2009). The transport and accumulation of the vesicles is balanced by endocytosis through the internalization of excess plasma membrane, cell wall materials, and associated signaling molecules to regulate pollen tube tip growth (Hepler et al., 2001). Because of pollen tube's geometry, its cellular envelope (cell wall and plasma membrane) – and the vesicular delivery have a different ratio of the two materials (polysaccharide/ phospholipids). Hence, the relative amount of membrane material delivered to the outside is in excess and must be internalized (Bove et al. 2008). Since the tip growth requires a large volume of cell wall material to keep up

with the fast-paced growth of the cell, the apical region of the growing pollen tube sustains high rates of both endo and exocytosis essentially at the same location (Bove et al. 2008, Cai et al. 2015).

The transport of organelles and vesicles rely on the cytoskeleton and is driven by motor proteins (Rogers and Gelfand 2000). The vesicular trafficking occurs along specific tracks defined by the organization of the cytoskeleton elements (reversed fountain streaming in angiosperm pollen tubes) and hence studying the dynamics of the cytoskeleton provides important insight on the machinery supporting and regulating the wall assembly process (Chebli et al. 2013). Although studies in the past have highlighted the role of actin cytoskeleton in vesicular delivery of different cell wall material, its association with callose regulation was unknown. The present study provides insights on the potential role of the actin cytoskeleton in regulation of callose deposition both at the subapex and the distal segment of the pollen tube. The results from my immunolabeling and microscopy experiment show that the short actin fringe is localized near the tube apex where high callose synthase abundance was also observed. Long actin bundles were observed passing through the incomplete callosic plugs and pointing to fully formed plugs implicating the potential role of actin cytoskeleton in transporting, delivering, or positioning the callose synthases at these specific areas. Additionally, the pharmacological treatment experiments using actin depolymerizing drugs (Latrunculin B and Cytochalasin B) also confirmed the role of actin's involvement in callose regulation, since a significant increase in longitudinal cross sectional surface area of callose plugs were observed after the drug treatment.

5.5 Durotropic growth of pollen tubes

A pollen tube must invade and navigate different sporophytic tissues of the stigma and style to reach its target, the ovule. Plants like *Arabidopsis thaliana* and *Nicotiana tabacum* have solid styles that require the pollen tube to invade through cell extracellular matrices, whereas plants like *Lilium longiflorum* and *Eschscholzia californica* have hollow styles filled with a viscous extracellular matrix or mucilage that the pollen tube can pass through and over, respectively (Lord and Sanders 1992, Becker et al. 2005). Pollen from plants with different style composition grow better on appropriately stiff or soft agarose gel media (Gossot and Geitmann 2007, Ghanbari et al. 2018) but the reason behind this growth behavior is not known. To address this knowledge gap various attempts have been made to understand the interaction of the pollen tube with the external matrix.

Recently Reimann et al. (2020) showed that the pollen tubes growing in species with solid and semi solid transmitting tissue display an increase in elongation rate when exposed to stiffer artificial growth matrices and the tubes from species with hollow styles display opposite growth behavior. Additionally, the force exerted by the tubes invading solid style transmitting tissue was recorded considerably lower (<11μN for tobacco pollen tube) than that of pollen tubes invading hollow style transmitting tissue (36 μN for lily). The growth of both types of pollen tubes was arrested upon exposure to very stiff matrix of 12 % agarose. Similar results were also observed in the present research where *Arabidopsis* pollen tubes growth rate increased on exposing the tubes to stiffer environment, thus confirming the durotropic behavior of these tip growing cells. However, contradictory observations have also been reported by Haduch-Sendecka et al. (2014) where pollen tubes of *Nicotiana tabacum* grew fastest in the least stiff medium (0.3% agarose).

The differences could potentially be due to different properties of agarose used in the two studies. Since most of the pollen tube cellular studies are performed in an *in vitro* set-up their behavior may not reflect that under *in vivo* conditions. The sporophytic tissue is a nutrient rich extracellular matrix that provides a path for the pollen tube elongation and actively supports and augments the process (Lord 2003, Qin and Yang 2011). The presence of several stigma-specific genes and molecules participates in a precisely orchestrated manner to drive the pollen tube growth towards the female gametophyte (Cheung et al. 1995, Okuda et al. 2009, Takeuchi and Higashiyama 2016). An alternative method to study the tube growth could be using the *semi-in vitro* set up where the pollen grains are germinated on the excised stigma head and the pollen tube protruding out of stigma neck are then exposed to germination media. Tubes growing in *semi in vivo* are faster than the tubes growing in *in vitro* set up and they also become competent to perceive and respond to navigation cues secreted by the pistil (Qin et al. 2009). Hence the *semi in vivo* set up work as an excellent assay to understand pollen germination processes and post germination development (Dickinson et al. 2018).

5.6 Mechanosensing in pollen tubes

Mechanosensing in plants is not researched as much in depth as that in the animal kingdom (Hamant and Haswell 2017). In plants, a crucial component in the response to mechanical triggers is the microtubule. Microtubules are known to direct the deposition of cellulose microfibrils at the plasma membrane undergoing maximal stress by regulating the positioning and incorporation of cellulose synthase complexes (Kesten et al. 2017). It is now well known that the tip of an elongating pollen tube is under lowest membrane tension that sharply increases towards the sub apex and distal segment (Hepler et al. 2013). Resistance against this stress is

achieved by the gradual pectin de-esterification at the sub apex segment and microtubule assisted incorporation of callose synthase complexes, sucrose synthase and cellulose synthase complexes at the site near the apex (Cai et al. 2011), the membrane site apparently with highest membrane tension.

The plasma membrane localized FERONIA senses the cell wall tension by interacting with LRX extension protein and pectin (also localized in the plasma membrane) that triggers the cell expansion (Doblin et al. 2014, Du and Jiao 2020). In pollen tubes, LRX proteins interact with RALF4/19 that in turn interacts with ANXUR1/2 (a pollen tube specific FERONIA homolog)-BUPS1/2 (CrRLK1L members)–LLG2/3 (LORELEI-like-GPI anchored proteins) complex at the plasma membrane which is crucial for maintaining pollen tube tip integrity (Adhikari et al. 2020). The actin cytoskeleton is also involved in pollen tube oscillatory behavior (a dynamic process that displays growth rate fluctuations at regular intervals) and is regulated by ROP1 (Rho GTPase member) that activates Rho GTPase effectors: RIC3 and RIC4 ((Fu et al. 2001, Gu et al. 2005). Additionally, ROP1 participates in regulation of callose synthesis at the tip-shank junction of an elongating PT, which is crucial for cushioning the structure against the external compression (Hong et al. 2001).

Recent advances have been made by discovering candidates for mechanosensory (MS) channels such as MscS-like (MSL) proteins, that act as mechanical sensors during pollen grain and pollen tube growth (Toyota et al. 2018). For instance, in Arabidopsis pollen, *MSL 8* was found to be localized on the plasma membrane and endomembrane (Hamilton et al. 2015). The study showed that mature pollen grains in *msl8* mutants had lower viability when exposed to hypo osmotic treatment during rehydration and a higher germination rate than the wild type, implying a crucial

role of *MSL8* in responding to changes in membrane tension during pollen hydration and germination process. Another study highlighted the role of MS channel activities in lily pollen tube by exposing the tubes to venom of the spider, *Grammostola spatulate* (inhibits MS channel activity in animals), and observed inhibition of pollen tube elongation, disruption of vesicular trafficking at the tip, loss of cytoplasmic streaming leading to plasmolysis (Dutta and Robinson 2004). These mechanosensors are localized on the plasma membrane connected to the cell wall. It is hence reasonable to assume that MS channels can convert the external mechanical stimuli rapidly into intracellular signals (Toyota et al. 2018). A pollen tube exhibits an active mechanosensory mechanism also known as 'durotropism' that directs its growth through stiff transmitting tissues but why and how do they exhibit such behavior is still not known in depth. To address the above question, the second chapter of my thesis provides an overview on strategies employed by tip growing cells such as pollen tubes and fungi to invade different matrices and the experimental strategies to measure the invasive force generated by these cells while invading these mechanical obstacles.

6 Conclusions and Perspective

The pollen tube must maintain its cylindrical shape to allow polarized, rapid, and directional growth, to achieve its mission: delivery of sperm cells to the ovule. Various factors such as Ca^{2+}ions, pH gradient, dynamics of cytoskeleton elements, balance between exocytosis and endocytosis and turgor pressure play crucial roles in accomplishing that purpose by facilitating the growth process. The close association of above components is essentially a feedback mechanism that receives input from signal transduction system that consists of receptors,

GTPases and membrane phospholipids. This feedback loop in turn regulates the dynamics of the actin cytoskeleton and the trafficking of vesicles that contain new cell wall materials. The vesicles transported to the tip of the tube fuse with the apical plasma membrane by exocytosis which then release molecules that determine the elongation and shape of the growing pollen tube. Subsequently, strategic deposition of cell wall material in the cell wall stabilizes the tube shape and growth directionality. On the other hand, endocytosis recovers excess membrane vesicles and plays a crucial role in remodelling of the tube shape. Any alteration of these multicomponent mechanism alters the pollen morphology thereby stunting the tube elongation process and hence the rate of reproductive success.

Although in depth research has helped us decipher the regulatory mechanism behind pollen tube growth, we still need clarification on certain points. First, how is the synthesis of callose regulated, given that callose synthases are localized at the tip but the accumulation of callose begins just behind the tip region. What is the molecular mechanism behind the activation of callose synthases at the sites of plug formation? Second, interplay between actin and microtubule cytoskeleton needs to be elucidated in depth. Do cytoskeletal elements stop or slow down organelles or vesicles at specific sites? Answering these questions will contribute to our understanding of this cell model which can also be extended to other tip focused cell growth.

7 References

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