

Characterization of regulatory factors
controlling tip growth in *Nicotiana
tabacum* pollen tubes

Jia Sun

*Faculty of Natural Resources and Agricultural Sciences
Department of Plant Biology
Uppsala*

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Abstract

Rho GTPases constitute a family of highly conserved signaling proteins with key functions in the regulation of cellular polarization and polar cell growth. In plants, Rho GTPases are represented by a plant specific subfamily referred to as Rac/Rop GTPases. Some members of this subfamily associate with the plasma membrane specifically at the tip of pollen tubes and are important regulators of polar cell expansion. The aim of my study has been to improve our understanding of the molecular mechanisms that control the activity of pollen tube Rac/Rop, and the signaling downstream of this activity.

BiFC (Bimolecular Fluorescence Complementation) is a technique used to validate protein-protein interactions. My studies on the interactions between Nt-RhoGAP1/RhoGDI2 and Nt-Rac5 show that 35S promoter regulated BiFC works in normally growing *N. tabacum* pollen tubes and that the intracellular interaction sites between Nt-RhoGAP1/RhoGDI2 and Nt-Rac5 are found at the subapical plasma membrane and apical cytoplasm, respectively. This technique was consequently used to confirm potential interacting protein partners involved in Nt-Rac5 signaling networks (i.e. Nt-Risap and Nt-Ric), and to detect the subcellular localization of these interactions in pollen tubes.

Nt-Risap (Rac5 interacting sub-apical protein) and Ric (Rho interacting CRIB containing) were isolated as effectors of Nt-Rac5 in yeast two-hybrid screens. The two proteins were confirmed to be highly and specifically expressed in *N. tabacum* pollen tubes and to interact with Nt-Rac5 both *in vitro* and *in vivo*. Functional studies of the two proteins suggest that they behave as downstream effectors of Nt-Rac5 and are involved in the Nt-Rac5 signaling pathways that regulate pollen tube tip growth by activating specific downstream events.

Keywords: tip growth, Rac/Rop GTPases, *Nicotiana tabacum*, pollen tubes, BiFC, Nt-RhoGAP1, Nt-RhoGDI2, signaling pathway, Nt-Risap, Nt-Ric

Author's address: Jia Sun, SLU, Department of Plant Biology,
P.O. Box 7080, 750 07 Uppsala, Sweden
E-mail: Jia.Sun@slu.se

Dedication

To my parents.

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List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

- I **Sun, J.**, Eklund, D.M., Montes-Rodriguez, A & Kost, B. (2015). *In vivo* Rac/Rop localization as well as interaction with RhoGAP and RhoGDI in tobacco pollen tubes: analysis by low-level expression of fluorescent fusion proteins and bimolecular fluorescence complementation. *The Plant Journal* 84, 83-98.
- II Stephan, O., Cottier, S., Fahlén, S., Montes-Rodriguez, A., **Sun, J.**, Eklund, D.M., Klahre, U & Kost, B. (2014). RISAP is a TGN-associated RAC5 effector regulating membrane traffic during polar cell growth in tobacco. *The Plant Cell* 26, 4426–4447.
- III **Sun, J.**, Stephan, O., Fahlén, S., Eklund, D.M., Klahre, U & Kost, B. Functional characterization of Nt-Ric, an effector of Nt-Rac5 in tobacco pollen tubes. (Manuscript).

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The contribution of Jia Sun (JS) to the papers included in this thesis was as follows:

- I JS was highly involved in planning the work and performed the majority of laboratory work, except for constructs cloning. JS analysed all the results and wrote the first draft of the paper.
- II JS established an essential analytical method (BiFC) and employed it to generate key data on *in vivo* protein-protein interactions, which are presented in this publication.
- III JS was highly involved in planning the work and performed the majority of laboratory work except for phylogenetic analysis and *in vitro* pull down assays. JS produced a majority of the data, analysed a majority of the data and wrote the first draft of the manuscript.

1 Introduction

1.1 Cell polarity and tip growth

Regulation of cell polarity is a fundamental process in biological systems. The major characteristic of cell polarity is the asymmetric distribution of intracellular components and of the cytoskeleton along a particular axis within a cell, making one end different from the other (Asnacios & Hamant, 2012; Xu & Scheres, 2005). Cell polarity occurs in all eukaryotic organisms during developmental processes and is important for various functions, such as intracellular communication, cell migration, asymmetric cell division and cell shape formation (Dettmer & Friml, 2011). It is relevant to single cell systems such as budding yeast (Pruyne *et al.*, 2004), animal neuronal axons (Wiggin *et al.*, 2005), plant pollen tubes and root hairs (Hossain *et al.*, 2015; Cole & Fowler, 2006), as well as to multicellular systems e.g. cell migration during wound repair and development in animals (Muñoz-Soriano *et al.*, 2012) or apico-basal polarity in epithelial cells (Mack & Georgiou, 2014).

1.1.1 Polar growth in plants

Unlike animal cells, plant cells are constrained by spatially organized cell walls and are unable to rapidly change shape or to move. During plant development, the orientation and the direction of cell expansion must be specified and strictly controlled (Kost *et al.*, 1999; Sachs, 1991).

Polarized growth is a form of cell growth where expansion occurs in a unidirectional manner. Tip growth, an extreme form of polarized cell growth, results in highly elongated tubular cell shape with a rounded tip at which all growth activity takes place (Campàs *et al.*, 2012; Lee & Yang, 2008). However, the mechanisms mediating cell polarity at cellular and molecular level are not well understood. In order to study this, readily accessible tip-growing cells including pollen tubes, root hairs, moss filaments and algae

rhizoids are widely used as model systems (Nakamura *et al.*, 2012; Grebe, 2004; Hepler *et al.*, 2001).

Tip growth of a plant cell begins with the specification of a growth site in the cell to which vesicles are targeted and fused with the plasma membrane, resulting in a polar extension of the plasma membrane and the cell wall at that site, leading to the formation of an elongated cell (Yang, 1998). Both internal and external signals are known to determine the initiation site, and the orientation of tip growth (Brand *et al.*, 2008; Ovecka *et al.*, 2005). A signaling network coordinating polarized actin-mediated organelle movements and the fusion of Golgi-derived secretory vesicles with specific domains of the plasma membrane is required (Porat-Shliom *et al.*, 2013; Eitzen, 2003). Moreover, regulation of microtubule and actin dynamics appears to be involved in this process (Fu, 2010; Joos *et al.*, 1994; Doonan *et al.*, 1988).

Although the general principle for polarity control appears to be conserved during plant evolution, the signaling machinery mediating tip growth and how it contributes to rapid cell expansion needs further studies (Yang, 2008; Uhrig & Hülskamp, 2001).

1.1.2 The pollen tube: a model system for polarized cell growth in plant

Unlike yeast cells or cultured mammalian cell lines, cell polarity in land plants is generally expressed in a multi-cellular context and not normally in cultured cells. This also contributes to the difficulties in studying the molecular mechanism of cell polarity control in land plants, which remained mysterious until the functions of Rac/Rop GTPases began to be understood.

Plant sexual reproduction depends on interactions between male gametophytes and female reproductive organs, pollen and pistils, respectively (Mascarenhas, 1993). During compatible pollination, once pollen grains land on the surface of the stigma, they hydrate, germinate and form pollen tubes that penetrate the female reproductive organ (pistil) (Chapman & Goring, 2010). The vegetative pollen tube cell is a longitudinally expanding single cell that carries all cellular contents, elongates rapidly in a strictly polarized manner as it grows through pistil tissue, and transports and delivers male sperm cells enclosed in its cytoplasm to the egg apparatus located within ovules. Pollen tube mediated fertilization is essential for sexual plant reproduction as well as for seed production (Boavida *et al.*, 2005).

Pollen tubes are haploid and have the capacity to grow synchronously in *in vitro* cultures (Suwińska *et al.*, 2015). They thus constitute an attractive model system to understand the mechanism and regulation of polarized cell growth, a process essential for cell differentiation and organ morphogenesis throughout plant development (Guimil & Dunand, 2007). In addition to their extraordinary

rapid cell elongation and strictly polarized expansion in *in vitro* cultures, they are also easily accessible and effectively transformable.

The pollen tube displays a uniform cylindrical shape and is organized into distinct zones. The tip growing region, a 10 to 15 μm long clear zone at the extreme apex, is a secretory vesicle rich region free of other organelles (Hepler & Winship, 2015; Franklin-Tong, 1999). Tip growth of a pollen tube depends on massive tip-directed targeted secretion of cell wall material, which is synthesized in Golgi stacks and transported to the apex within Golgi derived secretory vesicle (Franklin-Tong, 1999)(Figure 1). Fine F-actin (filamentous actin) present in the CZ region were proposed to promote short-distance transport of secretory vesicles as well as vesicles fusion with the apical plasma membrane (Fu *et al.*, 2001). Behind the CZ is a region with densely packed cytoplasm containing all organelle types including ER, Golgi stacks, F-actin cables, microtubules and the nucleus (Hepler *et al.*, 2001). Longitudinally oriented F-actin cables in this region are thought to mediate the rapid movement of Golgi stacks, secretory vesicles and other organelles through the pollen tube cytoplasm (cytoplasmic streaming) (Hepler *et al.*, 2001). The actin-myosin dependent cytoplasmic streaming occurs in a reverse fountain pattern, with organelles moving towards the apex along the cortex, changing direction and moving backward in the centre of the cell right behind the CZ (Figure 1) (Onelli & Moscatelli, 2013; Cheung & Wu, 2007; Hepler *et al.*, 2001). While the cytoplasm accumulates at the apex, the vacuole fills a large area in the centre of the basal part of the pollen tube. Older and more distant parts of the tube are blocked by callose plugs, keeping the concentration and the volume of the cytoplasm constant during pollen tube elongation (Franklin-Tong *et al.*, 1996; Mascarenhas, 1993).

Quantitative analysis by electron microscopy has indicated that during pollen tube growth, plasma membrane extension requires fusion of secretory vesicles with the plasma membrane at a significantly lower rate (400 vesicles/min) than cell wall biogenesis (3450 vesicles /min) (Derksen *et al.*, 1995). To balance membrane trafficking, excess plasma membrane materials are transported backwards and must be retrieved by endocytosis at the flanks of the pollen tube tip (Kienzle & von Blume, 2014; Campanoni & Blatt, 2007; Helling *et al.*, 2006). In plant cells, material destined for secretion is believed to move through the Trans-Golgi-Network (TGN), a poorly defined endomembrane compartment, on its way from Golgi apparatus to the cell surface via secretion pathways (Gendre *et al.*, 2015; Gu *et al.*, 2001). Compelling recent evidence indicates that the TGN is not only a late compartment of the secretory system in plant cells, but also the first organelle to which endocytosed plasma membrane material is transported (Dettmer *et al.*, 2006).

However, the cellular mechanisms defining the tip growth domain, and how signaling cascades are coupling cytoskeletal regulation, vesicle trafficking and cytoplasmic polarity is unclear and an area of ongoing research.

Evidence suggests that active Rac/Rop GTPase signaling defines the tip growth domain and controls exocytosis through both the regulation of actin dynamics and of cytosolic calcium accumulation at the tip (Qin & Yang, 2011; Li *et al.*, 1999). Multiple studies have demonstrated that tip-localized Rac/Rop GTPases are a central component of these mechanisms, and have lead to the development of a model for a Rac/Rop-dependent signaling network in the control of tip growth (Gu *et al.*, 2005; Lin *et al.*, 1996).

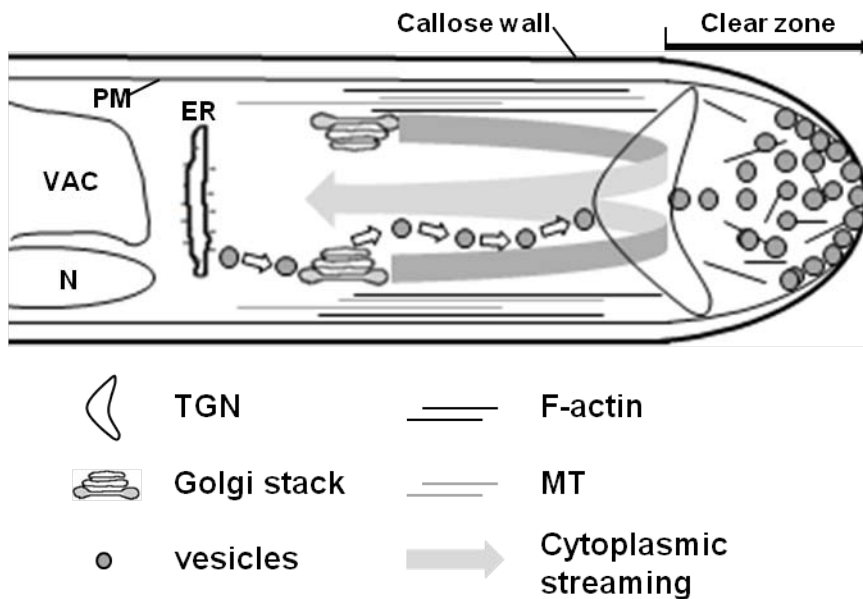


Figure 1. Pollen tube model shows major structures and molecules known to be critical for tip growth in pollen tubes.

N. tabacum pollen tubes are used in my study as an experimental system, because they have several advantages: 1) The growth of these cells is fast (5 to 10 $\mu\text{m}/\text{min}$) and restricted to the tip, similar to other tip growing systems. 2) They are easier to culture *in vitro* than e.g. *Arabidopsis* pollen tubes (Chebli *et al.*, 2012). In contrast to *Arabidopsis* pollen tubes, *N. tabacum* pollen tubes can grow in culture long enough to enable efficient transient gene transformation. 3) Their size is suitable for *in vivo* microscopy. 4) It is easy to obtain enough material for different experimental procedures including biochemical analysis.

1.1.3 Cytoskeletal regulation of pollen tube tip growth

To maintain tip growth, secretory vesicles have to be actively transported to the tip via the actin cytoskeleton (Stamnes, 2002; Cai *et al.*, 1997). Microtubules appear to be involved in this process as well (Agouzal & Quyou, 2012).

Actin microfilaments

Actin microfilaments in pollen tubes are essential for cytoplasmic streaming and cell expansion (Hepler *et al.*, 2001), presumably because they mediate myosin motor protein-dependent organelle and vesicle motility (Park & Nebenfuhr, 2013). Actin filaments (F-actin) are thought to be responsible for the transport of Golgi-derived secretory vesicles containing membrane and cell wall material to a defined area at the apex (Hepler *et al.*, 2001). At least two forms of actin have been observed in tip growing cells: F-actin cables and dynamic fine F-actin (Fu *et al.*, 2002). Fine F-actin in the apical region as well as a subapical cortical F-actin cable located at the interface between the apical CZ and the granular cytoplasm (Kost *et al.*, 1998), are thought to have important function in apical membrane traffic. Longitudinally oriented F-actin cables in the pollen tube shank are thought to serve as tracks for myosin mediated organelle motility, the process known as cytoplasmic streaming (Cheung & Wu, 2007; Hepler *et al.*, 2001). Although different concepts of the role of actin filaments in tip growth have been proposed, the role of F-actin in polarization is still unclear. F-actin may contribute to polarized pollen tube growth by the polarized elongation of microfilament bundles. Alternatively, actin filaments may provoke a bidirectional cytoplasmic streaming and thereby might support the establishment of cell polarity.

Microtubules (MTs)

Microtubules (MTs) present in pollen tubes are longitudinally oriented and tend to be slightly helically arranged at least in the cell cortex (Geitmann & Emons, 2000). MT are absent from the CZ (Lancelle & Hepler, 1992). Application of microtubule inhibitors does not have a major effect on angiosperm pollen germination or tube growth (He *et al.*, 1996; Heslop-Harrison *et al.*, 1988). In diffusely growing cells, cortical microtubules have been shown to modulate the direction of expansion (Whittington *et al.*, 2001). It has also been reported that microtubules maintain the directionality of tip growth in root hairs (Bibikova *et al.*, 1999). Although microtubules co-align and co-localize with actin microfilaments in pollen tubes (Geitmann & Emons, 2000), and are associated with the endoplasmic reticulum and the plasma membrane, the function of microtubules in polarized growth seems less essential as compared to actin filaments (Heath & Geitmann, 2000).

1.2 Rho GTPases

The research of small GTPases started more than fifty years ago with the identification of the Ras oncogene (Harvey, 1964), which was soon followed by a series of discoveries of related proteins now forming the superfamily of small GTPases (Cherfils & Zeghouf, 2013). Small GTPases function as molecular switches that regulate a wide range of signal transduction pathways in all eukaryotic cells. They alternate between GDP-bound and GTP-bound forms. The GDP-bound form is considered inactive, while the GTP-bound form activates downstream pathways by binding to effector proteins (Etienne-Manneville & Hall, 2002; Van Aelst & D'Souza-Schorey, 1997).

Small GTPases are known for their pivotal role in regulating cell polarity, cytoskeletal rearrangements and membrane trafficking (Delprato, 2012). Underlying this biological complexity is a simple biochemical strategy; multiple signaling pathways can be co-ordinately activated by switching on a single small GTPase. As the activity of small GTPases can also be tightly spatially and temporally controlled, it is not surprising to find that small GTPases have such an important role in eukaryotic cell biology (Kost, 2008; Etienne-Manneville & Hall, 2002).

1.2.1 Small GTPases in animals and yeast

In animals, the Ras superfamily of small GTPases can be divided into five subfamilies, Ras, Rab, Arf, Ran and Rho, according to sequence homology, which largely correlates with cellular function (Rojas *et al.*, 2012; Brembu *et al.*, 2006).

The Ras GTPase subfamily is known to be involved in a wide range of cellular processes responsible for cell proliferation, differentiation, and migration (Montalbano *et al.*, 2009). The Rab and Arf subfamilies of GTPases regulate intracellular membrane trafficking, with roles in regulating the formation of vesicles, their transport and their fusion with the membranes of target organelles (Hutagalung & Novick, 2011; Nielsen *et al.*, 2008; Behnia & Munro, 2005). Ran is best known for its function in nucleocytoplasmic transport through the nuclear pore complex. Ran also regulates spindle assembly during mitosis as well as DNA replication (Stewart, 2007; Wennerberg *et al.*, 2005; Li *et al.*, 2003). Rho GTPases behave as regulators of membrane trafficking, cytoskeletal dynamics, and cell polarity, in part by regulating the dynamics of actin filaments (Yalovsky *et al.*, 2008). In mammals, the Rho GTPase family can be divided into 8 subgroups: Cdc42, Rac, Rho, Rnd, RhoD, RhoH, RhoBTB and Miro (Brembu *et al.*, 2006).

In the yeast *Saccharomyces cerevisiae*, Cdc42 was the first identified GTPase of the Rho family (Fujimura-Kamada *et al.*, 2012; Adams *et al.*, 1990).

Cdc42 controls the establishment of cell polarity associated with the asymmetrical distribution of cortical actin filaments, which is responsible for the establishment of the budding site (Bi & Park, 2012; Perez & Rincon, 2010; Qadota *et al.*, 1996). Cdc42 also appears to regulate the activity of Rho subfamily proteins that are required for subsequent bud growth (Kang *et al.*, 2014).

The polar growth of animal and yeast cells is controlled by Ras and Rho subfamily small GTPases. Rho GTPases are further divided in three related subgroups Cdc42, Rac and Rho, which are represented in most eukaryotes (Yang, 2002). Rho GTPases function as molecular switches that regulate fundamental cellular events, including cytoskeleton dynamics, polarized vesicle trafficking, transcriptional activation, protein kinase cascades and cell cycle transitions (Nagawa *et al.*, 2010; AE & CJ, 2000). Rho GTPases are activated at specific plasma membrane domains and regulate F-actin organization as well as membrane trafficking (Etienne-Manneville & Hall, 2002). Although sets of conserved families of regulatory proteins have been identified, which directly interact with Rho GTPases and alter their activity, the molecular mechanisms responsible for the control of Rho activity in intact animal and yeast cells are not well understood.

1.2.2 Rho GTPases in plants

No specific orthologs of Cdc42, Rac or Rho subfamily GTPases are found in plants. Instead, plants possess a unique subfamily known as Rops (Rho of plants), which are sometimes called Racs because they are most closely related to the animal Rac subfamily GTPases (Nagawa *et al.*, 2010). Plant Rac/Rop GTPases are highly similar to Rho subfamily GTPases found in other eukaryotic organisms, but diverge somewhat from these proteins with respect to their regulation and downstream signaling.

In the *Arabidopsis thaliana* genome, 93 genes apparently coding for small GTPases have been identified. These GTPases belong to four different families: 57 Rab GTPases, 21 Arf GTPases, 4 Ran GTPases, and 11 Rac/Rops. The Rab, Arf, and Ran family proteins appear to have conserved functions and are thought to regulate similar processes in plants and in other organisms (Vernoud *et al.*, 2003; Yang, 2002). Rabs have a role in vesicle trafficking (Vernoud *et al.*, 2003), Arfs control vesicle budding, Rans have functions in nuclear transport, and Rac/Rops have been shown to transmit extracellular signals and to regulate processes involved in polar cell growth (Gu *et al.*, 2004). Some of the 11 *Arabidopsis* Rac/Rops are specifically expressed in pollen tubes, whereas others are not expressed in these cells (Li *et al.*, 1998). The functions of *Arabidopsis* Rac/Rops are diverse and complex. An individual

member can have a specific function, or functionally overlap with other Rac/Rops (Craddock *et al.*, 2012).

Plants do not have representatives of the Ras subfamily of small GTPases. Rac/Rops are therefore expected to participate in a wide variety of signaling events in plants (Yang, 2002). Like their counterparts in animals and in yeast, Rac/Rops participate in signaling pathways that regulate cytoskeletal organization, vesicular trafficking and cell polarization (Nagawa *et al.*, 2010). Most knowledge about Rac/Rop signaling is derived from the study of a few model systems, which include the tip growth of pollen tubes or root hairs, and the morphogenesis of leaf epidermal pavement cells (Yang, 2008). Another area of intensive study is the role of Rac/Rops in plant defence responses (Agrawal *et al.*, 2003). Rac/Rops have also been reported to contribute to hormone signaling, more specifically to pathways stimulated by abscisic acid or auxin, and to be required for responses to abiotic stresses (Nibau *et al.*, 2013; Tao *et al.*, 2002; Lemichez *et al.*, 2001).

As previously described, Rac/Rop GTPases are the only Rho GTPase in plants. Although the investigation of Rac/Rop-dependent signaling networks in plants lags behind the corresponding work in animals and yeast, important observations have recently been made, which provide insights into how plants regulate so many fundamental cellular processes through only a limited number of Rac/Rops. Both conserved and plant-specific functions of Rac/Rops are beginning to be understood (Craddock *et al.*, 2012; Vernoud *et al.*, 2003).

In order to learn more about the functions of Rac/Rops in plants, it is important to investigate how the activity of Rac/Rops is regulated and to characterize the signaling network that is stimulated by this activity.

1.2.3 Rac/Rop function in pollen tubes

It is known that Rac/Rop GTPases are key regulators of polar cell growth in several plant cell systems. The importance of Rac/Rop GTPases in cell polarity and tip growth has been best established by studies of pollen tube growth (Zheng & Yang, 2000).

The *Nicotiana tabacum* Rac/Rop protein Nt-Rac5 controls tip growth in cultured *N. tabacum* pollen tubes and is associated with the plasma membrane at the apex of these cells (Figure 2a) (Klahre & Kost, 2006). Over-expression of Nt-Rac5 dramatically affects the growth of *N. tabacum* pollen tubes. Nt-Rac5 is thought to control tip growth by regulating actin organization and membrane trafficking (Kost, 2008). Transient over-expression of constitutively active (CA) Nt-Rac5 (Nt-Rac5^{G15V}) depolarizes pollen tube growth and causes massive ballooning at the tip. By contrast, over-expression of dominant negative (DN) Nt-Rac5 (Nt-Rac5^{T20N}) strongly reduces pollen tube length

presumably by inhibiting Nt-Rac5 signaling activity and thereby blocking secretion (Figure 2b) (Hwang *et al.*, 2005; Kost *et al.*, 1999).

Mutant forms of Nt-Rac5 with altered ability to interact with regulators or effectors are considered important molecular tools to investigate signaling and biological functions of Nt-Rac5. Besides CA and DN form of Nt-Rac5, other Nt-Rac5 mutants can be employed for such studies. Nt-Rac5^{R69A} is disrupted in its ability to bind to Nt-RhoGDI2, but not to other regulators or effectors (Klahre *et al.*, 2006). Nt-Rac5^{C21/158S} is unable to undergo transient S-acylation, which has been reported to result in reduced, but not abolished, stimulation of downstream signaling (Sorek *et al.*, 2010; Sorek *et al.*, 2007).

Effects of over-expressing wild type or mutant Nt-Rac5 on *N. tabacum* pollen tube growth, as well as the intracellular localization of these different forms of Nt-Rac5 fused to GFP, are similar to those observed with other pollen tube Rac/Rops (Hwang *et al.*, 2010; Klahre *et al.*, 2006; Li *et al.*, 1999).

Although plants Rac/Rops have been demonstrated to be important factors in the control of tip growth, the signaling networks into which they are integrated are only understood to a limit extent.

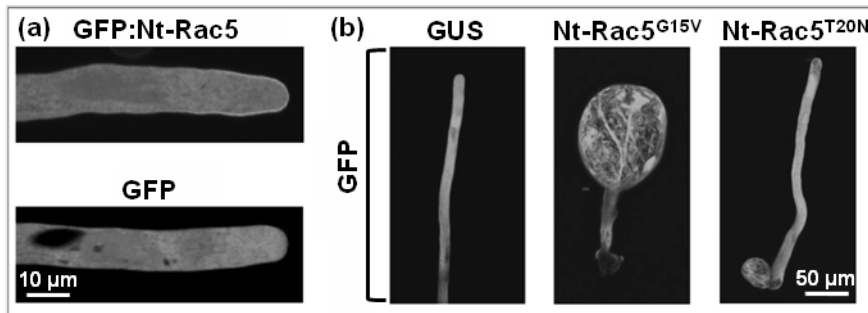


Figure 2. Control of tip growth by transient over-expression of Nt-Rac5. (a) Green fluorescent protein (GFP) fused to Nt-Rac5 accumulates at the plasma membrane specifically at the tip of growing pollen tube (upper lane), whereas free GFP displays a diffuse cytoplasmic distribution (lower lane). Scale 10 μm . (b) Over-expression of free GFP with β -glucuronidase (GUS), with Nt-Rac5^{G15V} or with Nt-Rac5^{T20N}: effects on pollen tube growth. Modified from (Kost, 2008).

1.3 Regulation of Rho GTPases in plants

Rho GTPases are known to act as molecular switches that transmit extracellular signals and activate multiple intracellular signaling cascades (Gu *et al.*, 2004). Rho GTPases are shuttling between an active GTP-bound form and an inactive GDP-bound form (Figure 3) (Zou *et al.*, 2011). Most Rho GTPases are post-translationally modified by prenylation, a process that results in the attachment of a lipid tail to the C-terminus of target Rho GTPases, which

is required for their biological activities (Ahearn *et al.*, 2012; Berzat *et al.*, 2006). Prenylation is responsible for the correct subcellular targeting of Rho GTPases to specific domains of plasma membranes, and is therefore essential for the function of these proteins (Gao *et al.*, 2009; Roberts *et al.*, 2008).

The balance between the active and inactive states of Rho GTPases is tightly controlled by three classes of regulatory proteins (Ota *et al.*, 2015). GTPase activating proteins (GAPs) inactivate the signaling functions of Rho GTPases by stimulating hydrolysis of GTP to GDP. Guanine dissociation inhibitors (GDIs) interact with the GDP bound form of Rho GTPases, extract them from the plasma membrane and transfer them to the cytoplasm. Guanine nucleotide exchange factors (GEFs) are usually associated with the plasma membrane and activate Rho GTPases by promoting the exchange of GDP for GTP (Figure 3) (Cherfils & Zeghouf, 2013).

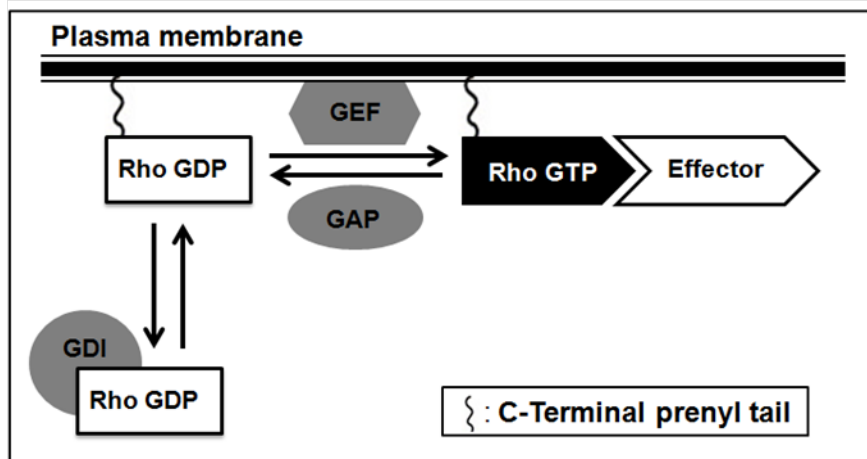


Figure 3. Regulation of Rho GTPase activity by regulatory proteins that directly interact with Rho GTPases. Modified from (Eklund *et al.*, 2010).

Rac/Rop GTPases appear to be regulated by a plant specific set of regulators (Nagawa *et al.*, 2010). A detailed functional characterization of these regulators is required for an improved understanding of the molecular mechanisms that are responsible for the polarized accumulation and activation of Rac/Rop GTPases at the plasma membrane at the apex of tip growing cells, which determines the direction of cell expansion. Under the control of upstream regulators, Rac/Rop GTPases act as signaling nodes to stimulate multiple downstream signaling pathways, thereby regulating and coordinating cellular processes essential for tip growth. Several regulators and effectors of

plant Rac/Rop GTPases have been identified, which play a role in the control of polar cell growth (Nagawa *et al.*, 2010).

1.3.1 GAPs

Animal and yeast RhoGAP families generally appear to be two to three times larger than the corresponding Rho GTPase families (Kost, 2010). In plants, the situation is thought to be less complex than in animals. The *Arabidopsis* genome encodes 11 Rac/Rops, but contains only nine genes coding for predicted RhoGAP proteins with a RhoGAP domain (Wu *et al.*, 2000). Plant RhoGAPs contain a single recognizable functional domain in addition to the conserved GAP domain, and can be divided into two subfamilies: one subfamily of plant RhoGAPs, which all contain a CRIB (Cdc42/Rac interactive binding) domain upstream of the GAP domain, are called RopGAPs. The second subfamily consists of plant RhoGAPs containing a PH domain near the N-terminus of the GAP domain, which are called RENs (Rop enhancer). Members of both subfamilies display RhoGAP activity towards Rac/Rop GTPases *in vivo* and *in vitro* (Hwang *et al.*, 2008; Klahre & Kost, 2006).

A *N. tabacum* pollen tube RhoGAP (Nt-RhoGAP1) that contains a CRIB domain was isolated in a yeast two-hybrid screen for proteins interacting with the active form of the Rac/Rop GTPase Nt-Rac5. CRIB domains are found exclusively in downstream effectors of small GTPases in animals and yeast, but never in non-plant RhoGAPs (Schaefer *et al.*, 2011). The function of the CRIB domain in plant RhoGAPs is not entirely clear. However, the CRIB domain in Nt-RhoGAP1 has been proposed to modulate interactions with Nt-Rac5 and to enhance GAP activity (Klahre & Kost, 2006).

Nt-RhoGAP1 shows sequence homology to GAP domain containing RhoGAPs from animals (Schaefer *et al.*, 2011). Nt-RhoGAP1 also enhances the Nt-Rac5 GTPase activity *in vitro* and strongly inhibits the growth of *N. tabacum* pollen tubes when over-expressed. YFP tagged Nt-RhoGAP1 accumulates at the plasma membrane of *N. tabacum* pollen tubes at the flanks of the tip, but not at the apex, where active Nt-Rac5 presumably accumulates (Klahre & Kost, 2006). These observations strongly suggest that Nt-RhoGAP1 plays a key role in maintaining Nt-Rac5 activity at the apex by promoting its GTPase activity at the flanks of the pollen tube tip and thereby inactivating its signaling function. Unfortunately, *in vitro* interaction studies between Nt-RhoGAP1 and Nt-Rac5 are difficult to perform because recombinant Nt-RhoGAP1 is unstable when expressed in bacteria as a full-length protein (Klahre & Kost, 2006).

1.3.2 GDIs

Rho guanine dissociation inhibitors (RhoGDIs) are highly conserved and appear to have similar functions in all organisms (Nagawa *et al.*, 2010; Berken & Wittinghofer, 2008). RhoGDI mediated recycling of Rac/Rops appears to play important roles in regulating polar growth of pollen tubes and root hairs (Zhang & McCormick, 2007). RhoGDIs were reported to have different biochemical activities towards Rho GTPases. RhoGDIs are generally thought to act as negative regulator of Rho GTPases based on their ability to transfer inactive GDP bound Rho GTPases from the plasma membrane to the cytoplasm, where the two proteins can form inactive heterodimers. Furthermore, RhoGDIs also appear to be able to interact with active GTP bound forms of Rho GTPases and maintain these proteins in this form (Ota *et al.*, 2015; Hoffman & Cerione, 2000)

Nt-RhoGDI2 was identified in a yeast two-hybrid screen for proteins interacting with active Nt-Rac5 (Klahre *et al.*, 2006). Nt-RhoGDI2 is highly and specifically expressed in *N. tabacum* pollen and pollen tubes, and is closely related to previously characterized plant RhoGDIs. Over-expressed YFP-fused Nt-RhoGDI2 accumulates in the pollen tube cytoplasm and strongly inhibits pollen tube elongation. Nt-RhoGDI2 was shown to effectively transfer co-expressed YFP labelled Nt-Rac5 from the plasma membrane to the apical cytoplasm (Klahre *et al.*, 2006).

In yeast two-hybrid assays, Nt-RhoGDI2 shows strong interaction with the wild type version of Nt-Rac5, weaker interaction with the constitutively active (CA) form of this protein (Nt-Rac5^{G15V}) and no interaction with the dominant negative (DN) form (Nt-Rac5^{T20N}). This suggests that Nt-RhoGDI2 preferentially interacts with Nt-Rac5 in its inactive GDP bound form. Generally, RhoGDIs do not strongly interact with DN forms of Rho GTPases, which display low affinity for both GDP and GTP, and are therefore considered to be nucleotide-free. Interestingly, the Nt-Rac5 mutant Nt-Rac5^{R69A} is specifically unable to bind to Nt-RhoGDI2, but normally interacts with other regulators and effectors. Both DN and R69A mutants of Nt-Rac5, which do not interact with Nt-RhoGDI2, accumulate at plasma membrane at the flank of the pollen tube tip and are completely absent from the apex where Nt-Rac5 accumulates. Hence, Nt-RhoGDI2 has been proposed to mediate recycling of Nt-Rac5 from the flanks of the pollen tube tip to the apex, which is necessary for the maintenance of apical Nt-Rac5 activity *in vivo* (Klahre *et al.*, 2006).

1.3.3 GEFs

Rho Guanine nucleotide exchange factors (RhoGEFs) are a large family of proteins that have the ability to promote the exchange of GDP for GTP in Rho GTPases (Rossman *et al.*, 2005). Two types of RhoGEFs have been identified in animal and in yeast, namely DH domain and CZH domain containing proteins. Both types have been investigated in various animal systems (Meller *et al.*, 2005). Recently, both types of RhoGEFs were found to be present in plants (Yamaguchi *et al.*, 2012; Basu *et al.*, 2008) as well, although plant Rac/Rops seem to predominately use a plant-specific family of GEFs called RopGEFs for their activation (Berken *et al.*, 2005). RopGEFs share a highly conserved domain, called “plant-specific Rop nucleotide exchanger” (PRONE) domain, which is responsible for GEF activity, and flanked by variable N- and C- terminal regions (Gu *et al.*, 2006).

The *Arabidopsis* genome encodes 1 RhoGEF named At-SPK1 which is distantly related to CZH RhoGEFs (Qiu *et al.*, 2002) and 14 RopGEFs that share the central PRONE domain (Berken *et al.*, 2005). The PRONE domain was demonstrated to have GEF activity toward several *Arabidopsis* Rac/Rops. When pollen-specific members of the *Arabidopsis* RopGEF family are expressed in *N. tabacum* pollen tubes, these proteins accumulate at the plasma membrane specifically at the tip and can cause depolarized cell growth (Gu *et al.*, 2006). However, the roles of RopGEFs in plant development and in polar cell growth are not well understood to date. The identification of endogenous RopGEFs from *N. tabacum* pollen tubes will enable further investigation of how these proteins coordinate with other regulatory factors (e.g. Nt-RhoGAP1 or Nt-RhoGDI2) to control Nt-Rac5 signaling pathways.

1.4 Effectors of Rho GTPases in plants

A wide variety of animal and fungal Rho GTPase effector proteins have been identified and were found to regulate various cellular processes (Bishop & Hall, 2000). However, most of those effector proteins are missing in plants. In those cases where animal or fungal homologs are present in plants (e.g., formins), no direct evidence is available supporting their function as direct Rac/Rop downstream targets (Nagawa *et al.*, 2010). Instead, a number of plant specific proteins have been identified to act as effectors of active Rac/Rops. Although the majority of these proteins have not been fully functionally characterized, it is evident that plants have evolved a unique Rac/Rop mediated signaling network. ICRs (interactors of constitutively active Rop) (Li *et al.*, 2008) and RICs (Rop interacting CRIB domain containing) (Wu *et al.*, 2001) were reported as plant specific Rac/Rop GTPases effectors. Available data

suggest that members of these two families of effectors directly interact with Rac/Rop GTPases, and have functions in the control of pollen tube growth downstream of the activation of these proteins (Lavy *et al.*, 2007; Wu *et al.*, 2001).

1.4.1 ICRs

The Rac/Rop effector protein ICR1 (interactor of constitutively active ROP 1), was identified in two independent studies (Li *et al.*, 2008; Lavy *et al.*, 2007). The ICR family, composed of 5 members in *Arabidopsis*, is conserved in plants (Nagawa *et al.*, 2010). At-ICR1 was reported to bind to activated Rac/Rop GTPases both *in vitro* and *in vivo* (Lavy *et al.*, 2007). In addition, At-ICR1 binds to At-SEC3, a component of the exocyst complex, which controls polarized secretion. Interestingly, SEC3 was reported to interact directly with Rho GTPase in yeast, and to function as an adaptor that links Rho-family GTPases to the exocyst in these cells (Hala *et al.*, 2008; Guo *et al.*, 2001).

The results shown by Li *et al.* (2008) support the notion that ICR1 is involved in exocytosis. ICR1 was shown to localize to the cell cortex at potential germination sites before pollen tube emergence, as well as to the apical region of growing pollen tubes, where At-ROP1 dependent exocytosis occurs (Li *et al.*, 2008). Furthermore, At-ICR1 over-expression depolarizes root hair and pollen tube expansion, and enhances accumulation of GFP fused At-ROP1 at the PM (Nagawa *et al.*, 2010; Lavy *et al.*, 2007). Thus, At-ROP1 and At-ICR1 appear to be components of a positive feedback loop that maintains localized Rac/Rop GTPase activity at the tip of elongating pollen tubes. Additionally, ICR1 appears to play a role in polar auxin transport in leaf pavement cells (Wu *et al.*, 2011; Hazak *et al.*, 2010). However, whether ICR functions are controlled by Rac/Rop GTPases also in cell types other than pollen tubes and root hairs needs to be determined.

1.4.2 RICs

Another family of Rac/Rop GTPase effector proteins is a class of plant specific proteins named RICs (Rop interacting CRIB domain containing). All plant CRIB domain containing proteins show higher similarities with each other than with CRIB domain containing proteins from other kingdoms.

The *Arabidopsis* genome encodes 11 RICs all containing a CRIB domain. Outside of the CRIB domain these proteins share little detectable sequence similarity with each other, or with other CRIB domain containing proteins (Wu *et al.*, 2001). The CRIB domain is responsible for the specific interaction of RICs with active form of Rac/Rop GTPases (Pirone *et al.*, 2001). The CRIB

domain is located near the N-terminus of the majority At-RIC proteins, with the exception of At-RIC2 and At-RIC4, which have a CRIB domain closer to the C-terminus (Eklund *et al.*, 2010). Currently, nothing is known about the functions of the highly variable regions of At-RICs outside of the CRIB domain, and no downstream effector of RICs has been identified. RICs were proposed to serve as scaffold proteins, which promote the formation of signaling complexes containing regulatory proteins after CRIB domain binding to active Rac/Rop GTPases (Berken & Wittinghofer, 2008).

At-RIC3 and 4 were suggested to act as At-ROP1 effectors in pollen tubes based on the observation that these two proteins are the only members of the At-RIC family that depolarize *N. tabacum* pollen tube growth when over-expressed (Gu *et al.*, 2005). Furthermore, At-ROP1 was shown to regulate pollen tube tip growth by activating two counteracting downstream pathways mediated by At-RIC3 and 4, respectively, with opposite effects on F-actin organization (Lee *et al.*, 2008; Gu *et al.*, 2005). At-RIC3 appears to promote F-actin disassembly through the stimulation of a calcium gradient at the tip, whereas At-RIC4 promotes F-actin assembly by an unknown mechanism. At-RIC3 and At-RIC4 dependent F-actin dynamics are required for pollen tube tip growth, and the coordination of these two cellular processes was proposed to be mediated by At-ROP1 (Gu *et al.*, 2005).

At-RIC1 is another member of the RIC family (Wu *et al.*, 2001). Studies in leaf pavement cells revealed that At-RIC1 is a microtubule-associated protein that can be activated by At-ROP6 to reorder microtubules by promoting the microtubule severing activity of katanin, a microtubule severing protein (Lin *et al.*, 2013; Fu *et al.*, 2009; Fu *et al.*, 2005). A recent study indicates that At-RIC1 may function differently in various cell types by modulating either microtubules or F-actin (Zhou *et al.*, 2015). At-RIC1, which appears to be highly expressed in pollen grains based on microarray analysis, regulates pollen tube growth through its F-actin severing and capping activities. The apical PM localized At-RIC1 helps to release F-actin from the apical/subapical PM into the cytoplasm. Additionally, At-RIC1 also severs F-actin in the cytoplasm at the pollen tube tip and then caps the barbed ends of the F-actin fragments to prevent further elongation (Zhou *et al.*, 2015).

1.5 Bimolecular fluorescence complementation (BiFC)

Studying protein-protein interactions can provide important information on the functions of proteins in the regulation of biological processes, because protein complexes composed of distinct combinations of proteins control different cellular functions at different times, in different subcellular localizations and in

different cell types. Therefore, the identification and characterization of protein-protein interactions is important for all living systems (Rao *et al.*, 2014; Hudry *et al.*, 2011; Kerppola, 2006a). Interactions between protein partners visualized in individual living cells enables the analysis of different cellular pathways in cells in their normal environment. Hence, the direct visualization of protein interactions in living cells provides valuable information complementing results obtained with other methods allowing the analysis of protein-protein interactions (Kerppola, 2008; Kerppola, 2006b).

Nowadays, different methods are available to investigate protein-protein interactions *in vivo* and *in vitro*, each with advantages and limitations. Bimolecular fluorescent complementation (BiFC), a non-invasive and sensitive fluorescence based method, enables direct visualization of protein-protein interactions in living cells or organisms (Citovsky *et al.*, 2006; Hu & Kerppola, 2003). BiFC is based on the formation of a functional fluorophore when two proteins fused to non-fluorescent fragments of a split fluorescent protein interact with each other (Figure 4). Binding of the two interaction partners facilitates the association between the non-fluorescence fragments to reconstruct a functional fluorescence protein (Ohad *et al.*, 2007).

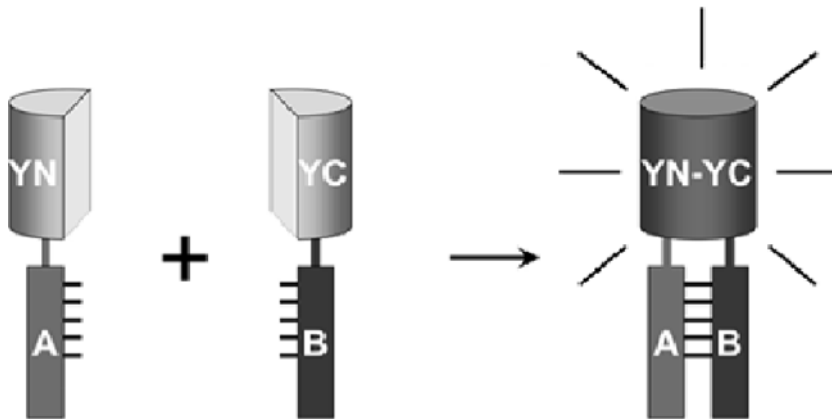


Figure 4. Principle of bimolecular fluorescence complementation (BiFC). Two non-fluorescent fragments (YN and YC) of the yellow fluorescent protein (YFP) are fused to putative interaction partners (A and B). The association of the interaction partners allows formation of a bimolecular fluorescent complex. Modified from (Kerppola, 2008).

Several characteristics of the BiFC approach make it valuable for many protein-protein interaction studies. BiFC enables direct visualization of protein-protein interactions in living cells and does not depend on detection of secondary effects. BiFC is a sensitive assay, which can be used to detect weak and transient interactions and to determine the subcellular localization of

protein complexes. The observation of BiFC in living cells is simple and does not require dedicated equipment, apart from an epifluorescence microscope equipped with objectives and relevant filters that allow fluorescence imaging in living cells (Ohad *et al.*, 2007; Hu *et al.*, 2002).

Although BiFC has become more and more popular as a method to detect and confirm protein-protein interactions in living plant cells, application of this technique is not without problems (Bracha-Drori *et al.*, 2004; Walter *et al.*, 2004). A major drawback is that split fluorescence protein fragments have a substantial tendency to self-assemble independently of protein-protein interaction. Therefore, careful and appropriate control experiments need to be performed. Furthermore, fluorescence fragment attached to interaction partners may affect the biological activities of these proteins.

During the last couple of years, a wide range of novel vectors and improved protocols have been developed, with the goal to improve the robustness of BiFC experiments and to reduce the risk of false positive results. This technique is now widely employed to investigate protein-protein interactions in living cells of different organisms, including plants.

2 Aims of this study

The key goal of this study has been to improve our understanding of the molecular mechanisms by which regulators and effectors of Rac/Rop GTPases control cellular processes required for pollen tube growth.

Specific aims:

- Analysis of *in vivo* interactions of Nt-Rac5 with the upstream regulators Nt-RhoGAP1 and Nt-RhoGDI2.
- Functional characterization of the Nt-Rac5 effector Nt-Risap.
- Functional characterization of the Nt-Rac5 effector Nt-Ric.

3 Results and Discussion

3.1 Analysis of the interactions of Nt-Rac5 with regulators or effectors, in normally elongating pollen tubes based on low level transgene expression driven by the 35S promoter and on BiFC (I, II, III)

BiFC as a non-invasive fluorophore-based method is commonly used to detect protein-protein interactions and to determine the subcellular localization of proteins complexes in living cells. This method is currently frequently applied in different plant systems, but has some weaknesses just like other techniques that can be used for protein-protein interaction studies (Bhat *et al.*, 2006). In order to optimize and apply BiFC in pollen tubes, this technique was used to study interactions of Nt-Rac5 with Nt-RhoGAP1 or with Nt-RhoGDI2 in living *N. tabacum* pollen tubes.

3.1.1 The 35S promoter is more suitable for BiFC assays in pollen tubes than the Lat52 promoter (I)

The pollen-specific Lat52 promoter is routinely used for transient gene expression in pollen tubes (Van der Leede-Plegt *et al.*, 1992; Twell *et al.*, 1991). However, we found that because the Lat52 promoter confers high-level gene expression in pollen tubes, which is toxic to the cells and tends to cause false-positive signals, this promoter is not optimal for BiFC studies to investigate protein-protein interactions in these cells.

The cauliflower mosaic virus (CaMV) 35S promoter is generally used for transgene expression as well as for BiFC assays in most plant cell types (Walter *et al.*, 2004; Benfey & Chua, 1990). For BiFC experiments in pollen tubes, promoters such as the 35S promoter, which are conferring low-level expression in these cells, are preferable. We have shown that a tandem CaMV

35S promoter (2x35S) (Citovsky *et al.*, 2006) works well for this purpose. Moreover, the expression of fluorescent Rac/Rop signaling fusion proteins under the control of this tandem 35S promoter affected pollen tube growth less dramatically, and generated more reliable data with regards to protein localization, as compared to Lat52 driven expression.

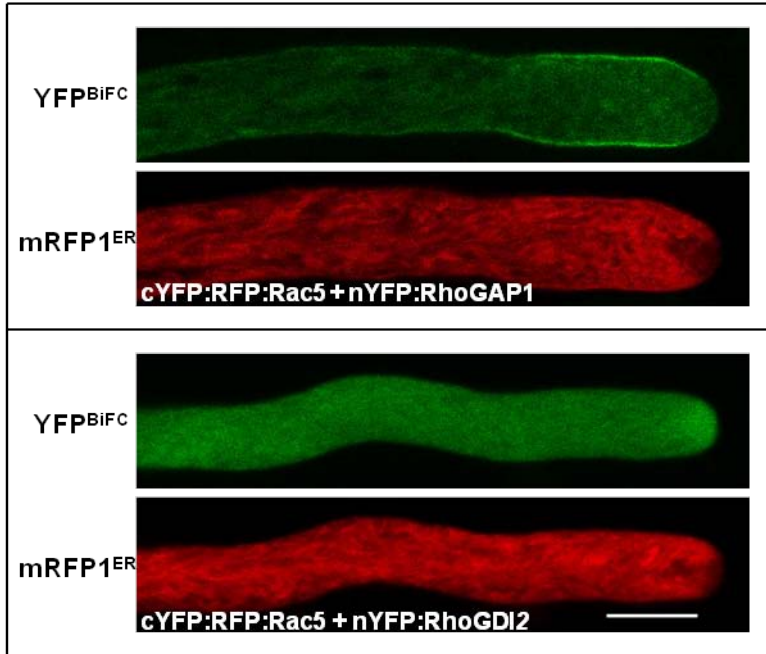


Figure 5. Bimolecular fluorescence complementation (BiFC) analysis of *in vivo* interactions between Nt-Rac5 and Nt-RhoGAP1 (upper part) or Nt-RhoGDI2 (lower part). Single confocal sections are shown through weakly fluorescent *N. tabacum* pollen tubes, which transiently expressed the indicated proteins fused to YFP fragment under the control of the 2x35S promoter (YFP^{BiFC} channel). Transformed pollen tubes were identified by co-expression of ER targeted mRFP1 driven by a regular CaMV 35S promoter (mRFP1^{ER} channel). Images were taken 6 hours after gene transfer. Scale bar: 10µm.

3.1.2 BiFC experiments confirmed predict interactions between Nt-Rac5 and regulatory proteins *in vivo* and allowed determination of the subcellular localization of these interactions (I)

Nt-RhoGAP1 and Nt-RhoGDI2 were shown to act as upstream regulators of Nt-Rac5 in pollen tubes. The functional characterization of these proteins suggested that Nt-RhoGDI2 recycles Nt-Rac5 from the plasma membrane at the flanks of the pollen tube tip, where this protein is inactivated by Nt-RhoGAP1, back to apex (Klahre *et al.*, 2006; Klahre & Kost, 2006). However, interactions predicted by this hypothesis between Nt-Rac5 and Nt-RhoGAP1 at

the lateral plasma membrane, and between Nt-Rac5 and Nt-RhoGDI2 in the apical cytoplasm, have not been confirmed by *in vivo* experiments.

Based on BiFC experiments performed under optimized conditions and including appropriate negative controls, we have now been able to confirm that Nt-Rac5 in fact interacts with Nt-RhoGAP1 and with Nt-RhoGDI2 in normally elongating *N. tabacum* pollen tubes. Moreover, the subcellular localization of the complexes formed between these proteins was identified (Figure 5). cYFP:RFP:Rac5 was transiently co-expressed with nYFP:RhoGAP1 or nYFP:RhoGDI2 under the control of 2x35S promoter. Obtained results show that Nt-Rac5 interacts with Nt-RhoGAP1 specifically at the lateral plasma membrane at the pollen tube tip, and that Nt-Rac5 interacts with Nt-RhoGDI2 specifically in the apical cytoplasm of pollen tubes. These observations are in agreement with the previously proposed hypothesis, that at the plasma membrane at the flanks of the tip Nt-RhoGAP1 inactivates Nt-Rac5, which is transported there along with a retrograde membrane flow that is generated by apical membrane trafficking, and that subsequently cytoplasmic Nt-RhoGDI2 recycles GDP bound inactive Nt-Rac5 from lateral back to apical domains of the pollen tube plasma membrane (Klahre *et al.*, 2006 Figure 8; Klahre & Kost, 2006 Figure 10).

3.1.3 BiFC experiments confirmed interactions of Nt-Rac5 with downstream effectors and allowed the identification of the intracellular sites at which these interactions occur in living pollen tubes (II, III)

The optimized BiFC assay we have developed was also employed to investigate interactions of Nt-Rac5 with Nt-Risap or with Nt-Ric, downstream effectors that preferentially interact with active Nt-Rac5, in transiently transformed normally growing *N. tabacum* pollen tubes.

Nt-Rac5 interacts with its effector Nt-Risap at the subapical TGN compartment (II)

Nt-Rac5 accumulates at apical plasma membrane of pollen tubes. By contrast, Nt-Risap (Rac5 interacting subapical pollen tube protein), an effector of Nt-Rac5, is found at a subapical trans-golgi-network compartment, and perhaps at vesicles within the apical clear zone. In order to observe if and where interaction between Nt-Rac5 and Nt-Risap occurs, we performed BiFC experiments. Interestingly, the results of these experiments establish interactions between these two proteins specifically at a subapical TGN compartment.

Nt-Rac5 interacts with its effector Nt-Ric at the apical plasma membrane (III)

Like Nt-Rac5, another effector of this protein called Nt-Ric also accumulates to high levels at the apical pollen tube plasma membrane.

In order to investigate interactions between Nt-Rac5 and Nt-Ric *in vivo*, cYFP:Ric and nYFP:RFP:Rac5 were transiently co-expressed in *N. tabacum* pollen tubes under the control of the 2xCaMV 35S promoter. These experiments established that Nt-Rac5 interacts with Nt-Ric at the apical plasma membrane of pollen tubes, where these two proteins co-localized.

Nt-Ric interacts with Nt-Risap in the subapical cytoplasm (III)

BiFC experiments were also performed to analyze *in vivo* interactions between the two Nt-Rac5 effectors Nt-Ric and Nt-Risap. Interestingly, co-expression of cYFP:Ric and nYFP:Risap demonstrated that these two proteins interact with each other in normally elongating pollen tubes in the subapical cytoplasm, directly behind the clear zone, exactly where we have found a Nt-Risap associated subapical TGN compartments to be localized.

3.2 Identification and functional characterization of effectors of pollen tube Nt-Rac5 (II, III)

In pollen tubes, apically localized Rac/Rop GTPases are essential for the control of tip growth. In order to enhance our understanding of the Rac/Rop dependent signaling network that regulates pollen tube growth, it is important to identify and characterize proteins interacting with apical Rac/Rop GTPases, in particular Nt-Rac5 effectors.

Rho, Cdc42 or Rac interact with more than 10 effectors each in animals, most of which contain a CRIB (Cdc42/Rac interactive binding) domain. Effectors of these GTPases include P21 activated protein kinase (Kumar *et al.*, 2006), tyrosine kinases ACK-1/2 (Mahajan & Mahajan, 2015) and Cdc42 effector proteins (CEPs) (Hirsch *et al.*, 2001). However, phylogenetic analysis shows that there are no homologs of effectors of animal Rho GTPases in plants (Wu *et al.*, 2001). This suggests that plant Rac/Rop GTPases use a unique set of target proteins as effectors. The identification and functional characterization of such effectors is required to improve our knowledge concerning the molecular mechanisms underlying the control of pollen tube tip by Rac/Rop signaling.

In yeast two-hybrid screens, a number of proteins that specifically or preferentially interact with active Nt-Rac5, and are therefore likely to act as effectors of this protein, were identified. Two of these putative Nt-Rac5 effectors, Nt-Risap and Nt-Ric, were functionally characterized in this study.

3.2.1 Functional characterization of Nt-Risap (II)

Nt-Risap, a novel Nt-Rac5 effector, was identified in a yeast two-hybrid screen for proteins that interact with active Nt-Rac5. The interaction between Nt-Rac5 and Nt-Risap was demonstrated and further characterized using yeast two-hybrid assays, *in vitro* pull down assays and *in vivo* BiFC assays.

RNA gel blot analysis showed that Nt-Risap is highly and specifically expressed in *N. tabacum* pollen and pollen tubes. Sequence analysis showed that Nt-Risap contains a DUF (domain of unknown function) 593 domain (amino acid 327-427) at the C-terminus and belongs to a plant specific family of DUF593 containing proteins. Although the DUF593 protein family is represented in all land plants, the functions of the members of this family have not been well characterized. A recent study has established that three *Arabidopsis* DUF593 proteins function as myosin receptors on the surface on unidentified cytoplasmic organelles, which recruit the C-terminal globular cargo binding tail domain (GTD) of myosin motor proteins. These three DUF593 proteins were therefore called myosin binding proteins 1-3 (MYOB1-3) (Peremyslov *et al.*, 2013).

Phylogenetic analysis revealed that of the at least 35 classes of myosins known, only classes VIII and XI are represented in land plants (Odrionitz & Kollmar, 2007). In a yeast two-hybrid screen for *N. tabacum* pollen tube proteins interacting with a truncated form of Nt-Risap missing an N-terminal hydrophobic region, a 608 amino acids protein fragment was identified. This fragment shared 59.6% identity with the C-terminus of MYA-1, the *Arabidopsis* class XI myosin that was first identified and that has been most extensively studied to date (Li & Nebenfuhr, 2008; Kinkema *et al.*, 1994). The identified Nt-Risap interacting *N. tabacum* pollen tube MYA-1 homolog was named Nt-MYOXIpt. Interactions between Nt-Risap and Nt-MYOXIpt were confirmed by *in vitro* pull down assays, which were also employed to demonstrate that Nt-Risap binds to Nt-MYOXIpt via its DUF593 domain.

To further investigate the molecular and cellular functions of Nt-Risap, effects of up- or down-regulation of Nt-Risap expression on the growth of *N. tabacum* pollen tubes were analysed. Three independent transgenic *N. tabacum* lines containing an Nt-Risap RNA interference (RNAi) construct did not display defects in pollen tube growth, suggesting that DUF593 proteins may have redundant functions in pollen tubes. By contrast, Nt-Risap over-expression reduced pollen tube length and induced tip swelling. YFP fused to Nt-Risap was found to accumulate in the sub-apical cytoplasm right behind the apical clear zone in normally growing pollen tubes, where it appears to be associated with a subapical trans-golgi-network (TGN) compartment.

Nt-Risap was also shown to interact with F-actin, consistent with a function of this protein as a myosin receptor, which mediates and maintains subapical positioning of the associated TGN compartment by interacting with the actin cytoskeleton.

3.2.2 Functional characterization of Nt-Ric (III)

Nt-Ric (Rho interacting CRIB containing protein) was also identified in a yeast two-hybrid screen for proteins interacting with active Nt-Rac5. Nt-Ric has a short (25 amino acids) N-terminus, followed by a CRIB (Cdc42/Rac interactive binding) domain and a long (166 amino acids) C-terminus, which presumably binds to downstream targets. Nt-Ric belongs to the RIC-family of putative Rac/Rop effectors, is most closely related to the subfamily II of the *Arabidopsis* RIC protein family based on sequence homology within the CRIB domain, and displays little homology to other characterized proteins.

The interaction between Nt-Rac5 and Nt-Ric was confirmed by yeast two-hybrid assays, by *in vitro* pull-down assay and *in vivo* by BiFC experiments. In yeast two-hybrid and pull-down assays, Nt-Ric was found to interact with wild type and with constitutively active Nt-Rac5, but not with essentially nucleotide-free dominant negative versions of this proteins, suggesting that Nt-Ric preferentially interacts with GTP bound, active Nt-Rac5. This supports the view that Nt-Ric functions as an effector of Nt-Rac5 in pollen tubes.

Quantitative real time PCR was employed to determine the expression pattern of Nt-Ric. The results of this analysis established that Nt-Ric transcripts specifically accumulate to high levels in *N. tabacum* pollen and pollen tubes.

In order to investigate the molecular and cellular function of Nt-Ric, effects of loss and gain of Nt-Ric function on pollen tube growth were analyzed. Two independent transgenic *N. tabacum* lines containing an Nt-Ric RNA interference (RNAi) construct were phenotypically characterized. Based on qPCR analysis, Nt-Ric expression was shown to be essentially abolished in both lines. However, none of the characterized Nt-Ric RNAi lines displayed detectable defects in pollen tube growth *in vitro* or *in situ* as determined by segregation analysis after reciprocal backcrossing. Apparently, an Nt-Ric homolog with redundant functions is expressed in *N. tabacum* pollen tubes. By contrast, over-expression of Nt-Ric dramatically reduced pollen tube length and induced tip swelling.

The intracellular distributions of full-length and different truncation forms of Nt-Ric, as well as over-expressing effects of these different proteins, were examined in transiently transformed *N. tabacum* pollen tubes. Confocal imaging established that YFP fused Nt-Ric accumulated at the apical plasma membrane of normally growing pollen tubes, where it co-localized with Nt-

Rac5. BiFC experiments were employed to demonstrate that these two proteins not only co-localize, but also interact with each other, at this location. Truncated Nt-Ric¹⁻⁶⁰ (N-terminus with CRIB domain) displayed the same intracellular distribution as full length Nt-Ric, whereas the isolated CRIB domain alone was essentially cytoplasmic. This suggests that the small N-terminus upstream of the CRIB domain has a role in the apical PM localization. Pull-down data also showed that Nt-Rac5 interacts with truncated Nt-Ric¹⁻⁶⁰, consistent with the notion that Nt-Ric accumulation at the apical pollen tube plasma membrane is likely to be mediated by the binding of this protein to activated Nt-Rac5.

In addition, yeast-two hybrid experiments were performed to investigate the function of the long Nt-Ric C-terminus. The results of these experiments suggested that the C-terminus of Nt-Ric is not required for binding to Nt-Rac5, but may mediate downstream signaling by specifically recruiting downstream effectors when Nt-Ric is bound via its CRIB domain to active Nt-Rac5.

3.3 Model of Nt-Rac5 signaling network (I, II, III)

Based on observations discussed above, a model is proposed of possible physical and functional interactions between Nt-Rac5 and its regulators and effectors at the pollen tube tip (figure 6). Newly synthesized and prenylated Nt-Rac5 may be transported on the surface of the secretory endomembrane system to the subapical TGN (Lane & Beese, 2006; Michaelson *et al.*, 2001), where this protein can bind to the N-terminus of the myosin receptor Nt-Risap. Nt-Risap, still in complex with Nt-Rac5, may then mediate the transport of TGN derived secretory vesicle along fine apical actin filaments (Geron *et al.*, 2013) to the apical plasma membrane. At this location, Nt-Rac5 is presumably activated by RhoGEFs and consequently binds to Nt-Ric, which may promote Nt-Ric binding to the C-terminus of Nt-Risap. Binding of complexed Nt-Rac5 and Nt-Ric to both the N- and C-termini of Nt-Risap may release this protein from the surface of secretory vesicles. This could result in the detachment of these vesicles from actin filaments, which may allow them to fuse with the plasma membrane.

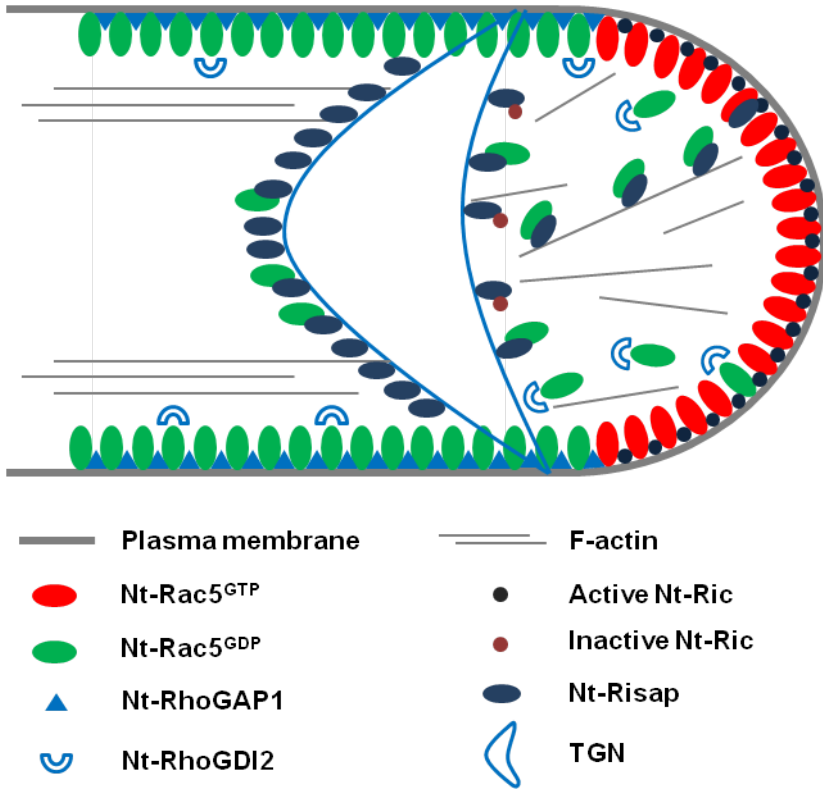


Figure 6. Model of physical and functional interactions between Nt-Rac5 and its regulators and effectors at the pollen tube tip.

As discussed above, after arrival at the apical plasma membrane, active Nt-Rac5 is eventually inactivated by Nt-RhoGAP1 at the flanks of pollen tube tip, and subsequently shuttled back to the apical plasma membrane by Nt-RhoGDI2 for reactivation.

4 Conclusions

Bimolecular fluorescence complementation (BiFC) is an excellent method to investigate protein-protein interactions in normally growing *N. tabacum* pollen tubes, in particular when analysed interaction partners are expressed under the control of the 35S promoter. BiFC analysis of interactions between Nt-Rac5 and Nt-RhoGAP1/RhoGDI2 confirmed and supported a previously proposed model predicting that functional interactions between Nt-RhoGAP1 and Nt-RhoGDI2 are essential for the apical polarization of Nt-Rac5 activity at the tip of *N. tabacum* pollen tubes.

The functional characterization of Nt-Risap, an Nt-Rac5 effector identified by yeast two-hybrid screening, showed that this protein functions as a myosin receptor and is associated with a subapical TGN compartment. Nt-Risap binds to MYOXIpt via its DUF593 domain and interacts with F-actin. This protein also interacts with Nt-Rac5 via its N-terminus and has a function in controlling apical membrane trafficking during pollen tube tip growth.

Nt-Ric, another Nt-Rac5 effector identified by yeast two-hybrid screening, was demonstrated to be specifically expressed in pollen and pollen tubes, and to accumulate at the apical plasma membrane during tip growth. Nt-Ric belongs to the RIC family of Rac/Rop effectors, and interacts with active Nt-Rac5 via its N-terminus and CRIB domain. Apical localization and activation of Nt-Ric is Nt-Rac5 dependent. After Nt-Ric activated by Nt-Rac5, the C-terminus of Nt-Ric is proposed to bind to downstream effectors and possibly to Nt-Risap. Nt-Ric binding to Nt-Risap may release this myosin receptor from secretory vesicles, allowing them to fuse with the apical pollen tube plasma membrane.

5 Future perspectives

- *In situ* detection of protein-protein interactions in normally growing *N. tabacum* pollen tubes by BiFC

In this thesis Bimolecular Fluorescence Complementation (BiFC) was demonstrated to be a powerful technique to study and visualize protein-protein interactions in normally growing *N. tabacum* pollen tubes, in particular when interaction partners are expressed under the control of the 35S promoter. This useful tool should be further exploited to investigate additional protein-protein interactions within the Nt-Rac5 signaling network that have not been studied to date, and in particular to determine where exactly within the pollen tube cell these interactions occur.

- More detailed characterization of Nt-Ric functions during pollen tube growth

Based on work presented in this thesis, we are beginning to understand the molecular function of Nt-Ric as an effector of Nt-Rac5. However, further work is required to determine the role of Nt-Ric in the control of cellular processes required for polar cell expansion, e.g. actin filaments dynamics or membrane trafficking.

In addition, Nt-Ric was shown to interact with Nt-Risap, another Nt-Rac5 effector that also binds to F-actin. The role of interplay between these two Nt-Rac5 effectors in the control of tip growth needs to be clarified.

- Further characterization of the Nt-Rac5 dependent signaling network that controls pollen tube growth

To fully understand the molecular mechanisms responsible for the regulation of polar cell growth, in particular of pollen tube tip growth, more information needs to be collected about Rac/Rop GTPase dependent signaling cascades. This can be achieved through the identification and characterization of additional factors involved in the Nt-Rac5 dependent signaling network. Several putative interactors of Nt-Ric and of Nt-Risap have been identified by

yeast two-hybrid screening. The further functional characterization of these interactors promises to contribute to the advancement of this line of research.

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