DISSERTATION

ISOLATION AND CHARACTERIZATION OF PROTEINS THAT INTERACT WITH A POLLEN-SPECIFIC CALMODULIN-BINDING PROTEIN

Submitted by
Sung-Bong Shin
Graduate Degree Program in Cell and Molecular Biology

In partial fulfillment of the requirements
For the Degree of Doctor of Philosophy
Colorado State University
Fort Collins, Colorado
Fall 2008

COLORADO STATE UNIVERSITY

Nov 11th 2008

WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY SUNG-BONG SHIN ENTITLED ISOLATION AND CHARACTERIZATION OF PROTEINS THAT INTERACT WITH A POLLEN-SPECIFIC CALMODULIN-BINDING PROTEIN BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

Committee on Graduate work

(Dr. Patricia A. Bedinger)

(Dr. Michael H. Fox)

(Dr. Stephen M. Stack)

Advisor (Dr. A.S.N Reddy)

Department Head/Director/Chair (Dr. Paul J. Laybourn)

ABSTRACT OF DISSERTATION

ISOLATION AND CHARACTERIZATION OF PROTEINS THAT INTERACT WITH A POLLEN-SPECIFIC CALMODULIN-BINDING PROTIEN

Calcium and calmodulin, a calcium sensor, are implicated in pollen germination and tube growth. However, the mechanisms by which calcium and calmodulin regulate these processes are largely unknown. Calcium bound calmodulin regulates diverse cellular processes by modulating the activity of other proteins called calmodulin-binding proteins. Maize pollen-specific calmodulin-binding protein (MPCBP) and its homolog (NPG1, no pollen germination) from Arabidopsis were isolated previously. Studies with a knockout mutant have shown that *AtNPG1* is not necessary for pollen development but is essential for pollen germination. Analysis of the Arabidopsis genome sequence with AtNPG1 revealed the presence of two other proteins (AtNPGR1, NPG-Related1; AtNPGR2, NPG-Related 2) that are closely related to AtNPG1. To gain insights into the function of AtNPG1 and AtNPGRs, I focused my research on characterization of these proteins. Specifically, my research focused on *in vivo* localization of AtNPG1 in pollen grain and tube, interaction between AtNPGs, isolation and characterization of AtNPG1 interacting proteins, and functional analysis of AtNPGR1 in plant development.

Transgenic plants containing GFP fused to *AtNPG1* promoter showed GFP expression only in mature and germinating pollen, suggesting that the promoter is active only in pollen. Localization of GFP-AtNPG1, driven by *AtNPG1* promoter, during different stages of pollen germination revealed uniform cytosolic distribution of GFP-AtNPG1 in the growing pollen tube that was similar to GFP alone. However, the observed uniform localization of GFP-AtNPG1 is not due to degraded fusion protein. AtNPGRs, like AtNPG1, bind calmodulin in a calcium-dependent manner. The calmodulin-binding domain in AtNPGs was mapped to a short region.

AtNPG1 and AtNPGRs have several tetratricopeptide repeats (TPRs) that are known to be involved in protein-protein interaction. I tested the interaction among AtNPGs using the yeast two-hybrid analysis. AtNPG1-BD interacted with itself-AD and AtNPGR1-AD and AtNPGR2-AD. AtNPGR1-BD interacted with itself-AD, AtNPG1-AD and AtNPGR2-AD. However, AtNPGR2-BD did not interact with AtNPG1-AD or

AtNPGR1-AD and showed a very weak interaction with itself-AD. To study the role of AtNPG1, AtNPG1 interacting proteins from a petunia pollen library were isolated in a yeast two-hybrid screen and identified as pectate lyase-like proteins. Using *in vivo* and *in vitro* protein-protein interaction assays, I show that AtNPGs interacts with four Arabidopsis pectate lyase-like (PLL) proteins with the highest similarity to petunia PLLs. Truncated AtNPG1 lacking the TPR 1 did not interact with most of partners or showed drastically decreased interaction with some proteins, suggesting that the TPR 1 domain is essential for this interaction. To understand the role of Arabidopsis PLL proteins, we characterized these using molecular and biochemical tools. Of the 26 Arabidopsis PLLs, fourteen were expressed in pollen and four *AtPLLs* were highly expressed. These four *AtPLLs* showed expression in other tissues also. Analysis of pectate lyase activity in Arabidopsis tissues (flower, root, stem, and leaf) revealed enzyme activity in all four tissues and the activity varied depending on the buffer pH. To see if AtNPG1 interacting AtPLLs have enzyme activity, four AtPLLs were expressed in bacteria or yeast and assayed for their enzyme activity under different conditions with different substrates. None of the AtPLLs expressed by bacterial or yeast showed pectate lyase activity. To discover the role of AtPLL in Arabidopsis development, one AtPLL mutant, *atpll8*, was isolated. Phenotypic analysis of *atpll8* under different growth condition showed no significant differences as compared to wild type.

AtNPGR1, unlike *AtNPG1*, is expressed in tissues other than pollen. To understand the role of AtNPGR1 in plant development, I isolated an *atnpgr1* knockout mutant and characterized its phenotype under different growth conditions. The *atnpgr1* showed a sugar resistance phenotype, suggesting that it might be involved in sugar sensing and/or signaling pathway. Expression of *hexokinase (Hxk)*, an important component in sugar signaling in plants, and other genes in the Hxk pathway, revealed that NPGR1 might be involved in an Hxk independent pathway.

Sung-Bong Shin
Graduate Program in Cell and Molecular Biology
Colorado State University
Fort Collins, CO 80523
Fall 2008

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

Pollination

Pollination and fertilization are essential processes for sexual reproduction in flowering plants (Lord, 2000). In general, pollination and fertilization include several steps: pollen landing on stigma, pollen recognition by stigma, pollen hydration, pollen germination, pollen tube growth in female tissue and delivery of sperm cells to the embryo sac (Figure 1.1). After pollen germination, pollen tubes grow through the transmitting tract to reach the ovule where one of sperm cells is released in a degenerating synergid to fuse with the egg cell and form a diploid zygote (Lord, 2000; Edlund et al., 2004). In Arabidopsis, pollen contains two sperm cells and one vegetative cell. The pollen grain surface is composed of three layers: inner intine, outer exine, and pollen coat (Edlund et al., 2004). The stigma is the part of female tissue on which pollen lands and there are two different types of stigmas: wet and dry stigmas (Edlund et al., 2004). Wet stigmas have a viscous surface because surface cells of the wet stigmas easily lyse and release substances that cause abundant viscous secretions containing sugar and glycoproteins (Heslop-Harrison and Shivannah, 1977; Luu et al., 1999; Edlund et al., 2004). Since wet stigmas have a sticky surface, pollen grains easily adhere to the surface without any discrimination between proper and improper pollen (Luu et al., 1999). Dry stigma surfaces are composed of surface cells that have a primary cell wall, a waxy cuticle and a proteinaceous pellicle. These stigmas are considered as more evolutionarily advanced than wet stigmas, because only the pollen grains that have cell wall surface complementary to the surface of the stigmas can adhere to dry stigmas (Luu et al., 1999; Edlund et al., 2004). After pollen lands on a stigma, it adheres to the stigma, hydrates, and finally germinates (Lord and Russell, 2002). The cell signaling between male and

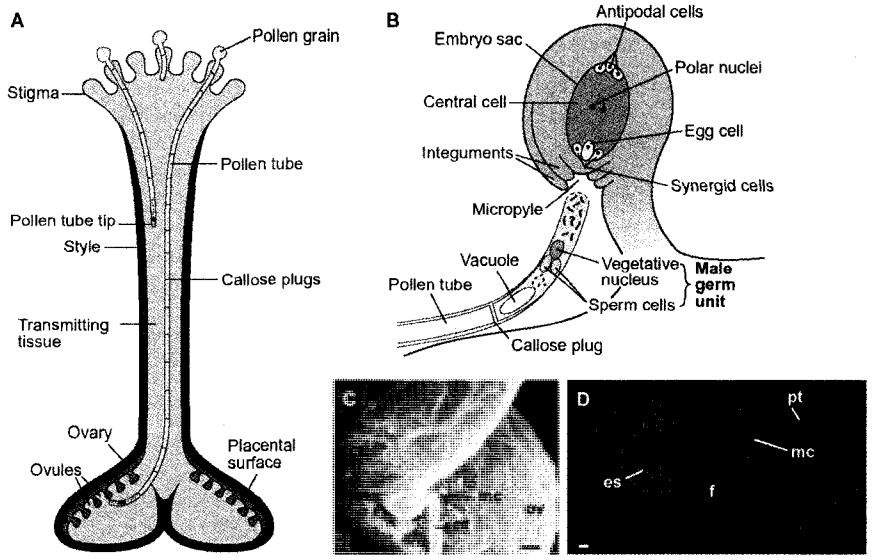


Figure 1.1. The lure of the pistil.
a) During fertilization, the pollen tube grows through the transmitting tract in the style. To successfully guide the pollen tube to ovules, female tissue structure and chemical cues are involved in the guidance mechanism. b) In the vincinity of an ovule, signals from synergid cells guide the pollen tube to micropyle. c) A scanning electron micrograph of a pollen tube entering micropyle. d) Image of a GFP-expressing pollen tube entering micropyle. pt, pollen tube; mc, micropyle; ov, ovule; es, embryo sac; f, funiculus.
(from Cheung AY and Wu H (2001) SCIENCE VOL 293 1441-1442).

female cells is known to control pollination processes for successful reproduction (Wilhelmi and Preuss, 1999).

Pollen adhesion and hydration

In the pollen-stigma interaction of Arabidopsis, lipophilic molecules in the pollen exine wall, the pollen coat, have been known to play a role in the initial step of pollen adhesion to the dry stigma (Zinkl et al., 1999). After initial contact on dry stigmas, a lipid-rich pollen coat forms "a foot" like interface between the pollen and stigma cells that contributes to adhesion (Edlund et al., 2004). Here pollen coat proteins interact with the S-locus related protein (SLR1) on the stigma (Luu et al., 1999; Takayama et al., 2000).

After pollen is captured by the stigma, the pollen must hydrate, for germination to occur (Bedinger et al., 1994). During pollination, the stigma recognizes and hydrates only compatible pollen (Wilhelmi and Preuss, 1999; Edlund et al., 2004). It is important that the pollen-stigma recognition occurs when the pollen lands on the stigma surface because stigma-level defense mechanisms prevent inappropriate pollen germination and growth by regulating water and nutrient transfer to the pollen grain (Wilhelmi and Preuss, 1999). For example, self-incompatible Brassica prevents self-pollination on its stigma surface. This self-incompatibility (SI) is due to a complex polymorphic locus that includes male and female specific genes (Nasrallah, 1997). These S locus genes code S-locus cysteine-rich protein (SCR) in pollen and S-locus receptor kinase (SRK) and S-locus glycoprotein (SLG) in the stigma (Franklin-Tong and Franklin, 2003; Hiscock and McInnis, 2003). These S-locus gene products are involved in cell-to-cell communication to determine compatibility (Franklin-Tong and Franklin, 2003; Hiscock and McInnis,

2003), So only compatible pollen is captured and hydrated for germination (Bedinger et al., 1994) (Nasrallah, 1997).

After adhesion, water channels in the plasma membrane of stigma cells promote pollen hydration (Lord, 2000). On dry stigmas, an aquaporin-like protein controls pollen hydration, whereas lipids (trilinoleins) are involved in pollen hydration on wet stigmas (Lord and Russell, 2002). Hydration triggers pollen germination and then the pollen tube grow through the transmitting tract to the ovary (Lord and Russell, 2002; Edlund et al., 2004). Following hydration, pollen grains become polarized cells. These processes include reorganizing filamentous actin cytoskeleton in germinating pollen (Heslop-Harrison and Heslop-Harrison, 1992), orienting the vegetative nucleus, establishing a calcium gradient at the pollen tube tip, accumulation of mitochondria at the tip of the pollen tube (Cresti et al., 1985; Heslop-Harrison and Heslop-Harrison, 1992; Lalanne and Twell, 2002; Mazina et al., 2002), and depositing callose behind the growing pollen tube (Johnson and McCormick, 2001).

Water, lipids and ions have been implicated as the possible polarization signals (Feijo et al., 1995; Lush et al., 1998; Wolters-Arts et al., 1998), and these signals are known to recruit RHO OF PLANTS1 (ROP1), a GTP binding protein involved in F-actin dynamics. These signals also establish the pollen tube tip-focused calcium gradients (Gu et al., 2003). ROP1 GTPase has been shown to have a pollen-specific expression pattern. Active ROP1 localizes to the apical dome area of the pollen tube and it functions as a central regulator of F-actin dynamics and tip-focused calcium gradient oscillations in the pollen tube (Gu et al., 2003). Annexin also has been suggested as a major regulator of tip-oriented exocytosis (Clark et al., 1995). After polarity is established inside the pollen

grain, a pollen tube starts to emerge. In Arabidopsis, pollen has three distinct apertures and the pollen tube grows out by breaking through the inter-aperture exine walls where one of the apertures is contact with the stigma (Edlund et al., 2004). Exine remodeling in pollen walls (Gherardini and Healey, 1969; Dickinson and Lewis, 1974) and turgor pressure (Fan et al., 2001; Zonia et al., 2001; Mouline et al., 2002) are known to play important roles in pollen tube emergence.

Pollen tubes show a high rate (Lord and Russell, 2002) polar tip growth (Bedinger et al., 1994). During this process, pollen tubes produce new membrane and wall only at the tip (Lord and Russell, 2002). During tube growth, cell structures such as the vegetative nucleus, sperm cells, and other organelles follow tip growth so they are maintained at the distal end of pollen tube, and callose plug are formed periodically to separate the tip content from the empty proximal pollen tube (Bedinger et al., 1994).

Pollen tube growth and guidance in female tissue

Pollen tubes grow through the extra-cellular matrix (ECM) of the transmitting tract in the style. The ECM has adhesion molecules for tube guidance in the style and provides nutrients required for tube growth (Bedinger et al., 1994; Wilhelmi and Preuss, 1997; Lord and Russell, 2002). Besides the ECM, chemotropic ions and molecules, such as calcium, glucose,γ-amino butyric acid (GABA), TTS (Transmitting Tissue-Specific) protein and various amino acids, are also thought to play important roles in guiding the pollen tube to the embryo sac (Bedinger et al., 1994; Cheung et al., 1995; Wu et al., 2000; Palanivelu et al., 2003). GABA has been shown to stimulate pollen tube growth *in vitro*, even though super-extra amount inhibits tube growth and a GABA gradient appears to be required for pollen tube growth and guidance in the Arabidopsis pistil (Palanivelu et

al., 2003). GABA transaminase, an enzyme that degrades GABA, is implicated in the GABA gradient. Pollen tube growth was inhibited, and pollen tubes were mis-guided in a GABA-transaminase mutant, *pollen-pistil interaction 2* (*pop 2*) (Palanivelu et al., 2003). TTS protein, a member of the arabinogalactan protein family, in the extracellular matrix of the transmitting track appears to be involved in pollen tube adhesion in the transmitting tract (Cheung et al., 1995). Using *in vitro* and *in vivo* experiments, it was demonstrated that TTS protein stimulates pollen tube growth and is involved in attracting the growing pollen tube (Cheung et al., 1995; Wu et al., 2000). During the early stages of pollen tube growth in the female tissue, it seems that the structure of the female organ, mostly the ECM, has a major role in pollen tube guidance. At later stages in pollen tube growth, chemo-attractants most likely guide the pollen tube to the ovules (Wilhelmi and Preuss, 1997).

Pollen tube elongation mechanisms

During pollen tube elongation, a calcium ion gradient is observed at the pollen tube tip area and actin filaments are organized for polar growth. Cell wall structure is controlled by cell wall modifying enzymes to support pollen tube growth. In addtion, small GTPase, motor proteins (e.g. kinesins and myosins), and second messengers have been studied for their essential roles in pollen tube growth (Figure 1.2). By blocking pollen tube calcium channels, it was shown that a calcium ion gradient at the pollen tip is essential for pollen tube growth (Rathore et al., 1991; Pierson et al., 1994). Actin filaments with myosin motors are involved in vesicle transportation during pollen tube growth, and the disruption of actin polymerization using drugs caused inhibition of pollen tube growth (Gibbon et al., 1999).

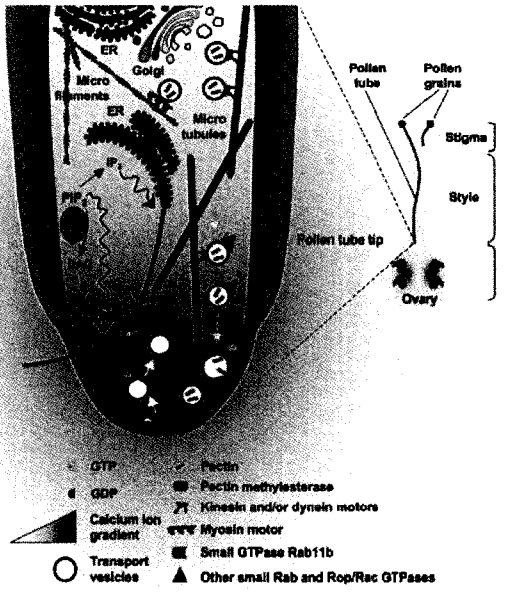


Figure 1-2. Major mechanisms involved in pollen tube growth. Cell wall modifying enzyme, PME, localizes at tip area. Actin microfilaments and microtubules provide structual support and foundation for vesicle transportation. Molecular motors transport vesicles to the tip. Rab and Rop/Rac GTPase control membrane fusion. Calcium and phospholipase C provide signals for pollen tube growth and direction. (From Krichevsky, A., Kozlovsky, S.V., Tian, G.W., Chen, M.H., Zaltsman, A., Citovsky, V., Developmental Bilology 303 (2007) 405-420)

Pollen tube cell wall modification

The pollen tube cell wall has two layers, a callose and cellulose inner layer and an outer layer composed of pectin, cellulose and hemicellulose (Taylor and Hepler, 1997; Ferguson et al., 1998). Since pollen tube growth is limited to the tip area, a single pectin layer without callose or celluose forms the tip cell wall and this structure may allow directional of their growth (Steer and Steer, 1989). Pectin is produced in the Golgi apparatus as a methyl esterified form before it is transported to the pollen tip area (Sterling et al., 2001). On the cell wall, pectin methyl esterase (PME) removes the methyl ester group to de-esterify pectin (Catoire et al., 1998). Calcium interacts with carboxyl groups resulting from de-esterification to act as bridges to interconnect pectins, which results in cell wall stiffening (Catoire et al., 1998). The location of methyl esterified pectin and de-esterified pectin on the pollen tube was studied by using a immunochemical method, and it was shown that de-esterified pectin is located in the pollen tube cell wall except in the tip area, while esterified pectin exists in cytoplasm and pollen tip area, as well as in the pollen tube wall (Abreu and Oliveira, 2004).

One Arabidopsis PME gene, *VANGUARD1 (VGD1)* that is specifically expressed in pollen and pollen tubes (Jiang et al., 2005). VGD1 localizes to the plasma membrane and pollen cell wall (Jiang et al., 2005). In a study of the knockout mutant, *vgd1* pollen tubes grew slower than wild type and ruptured very easily (Jiang et al., 2005). This indicated that the pollen tube cell wall structure of *vgd1* was changed because of loss of VGD1 function and VGD1 might be involved in maintaining the pollen tube cell wall structure (Jiang et al., 2005). Another pollen-specific PME, AtPPME1, has been studied by using the technique of Fluorescent Tagging of Full-Length Proteins (FTFLP) (Tian et

al., 2006). With FTFLP, it was shown that AtPPME1 is located around the cell periphery and in intracellular vesicles, such as the ER and Golgi apparatus in the pollen grains (Tian et al., 2006). In pollen tubes, AtPPME1 is distributed in the whole tube area like VGD1 (Jiang et al., 2005; Tian et al., 2006). In mutant *atppme1* plants pollen tube grow slower, their pollen tubes have abnormal shape, and the pollen showed 20% decreased PME activity. However, the mutation does not affect pollen tube stability or fertility (Tian et al., 2006). Since both exogenously applied and endogenously (over-expression) produced PME makes pollen tubes cell wall thicker and inhibits tube growth, it is suggested that PMEs have a critical role in controlling pollen tube growth by modifying cell wall mechanical structure. Compared to PME, pectate lyase has been known to have a cell wall loosening role by cutting linkages within pectin polymers. Twenty-six pectate lyase-like genes (PLLs) were identified in Arabidopsis and 14 of them are expressed in pollen, but their enzyme activity and roles in pollen have not been identified yet (Palusa et al., 2007).

Small GTPase

Small GTPases have been known to act as biological switches by interacting with either GTP or GDP and activating downstream partners. During pollen tube growth, small GTPases are involved in regulating vesicle traffic and actin filament organization (Steer and Steer, 1989; Hepler et al., 2001). For pollen tubes to grow rapidly, it is essential to transport cell wall materials to the tip where exocytosis occurs and to recycle excessive materials (Picton and Steer, 1983; Derksen et al., 1995). Plants have membrane fusion machinery that is similar to the SNARE complex in animal systems and have homologs of Rab and Ypt that are members of Ras-related small GTPases

(Rutherford and Moore, 2002; Vernoud et al., 2003). Although it is not clear how vesicle trafficking is regulated in pollen tubes, a recent study showed that small Rab GTPases are involved in pollen tube growth (Molendijk et al., 2004; Cole and Fowler, 2006). There are 57 Rab homologs in Arabidopsis (Rutherford and Moore, 2002; Vernoud et al., 2003). One Rab GTPase, NtRab11b, was isolated from tobacco pollen and was localized mainly to the transport vesicles around the apical clear zone area (Haizel et al., 1995). With an actin depolymerization drugs, the normal location of NtRab11b was disrupted, and with its inhibited function, it was observed that apical accumulate extracellular fluorescent dye became disappeared, suggesting that NtRab11b might play a role in endocytosis (Haizel et al., 1995; Hepler et al., 2001). By studying constitutively active and dominant negative mutants of Rab11b, it was suggested that the correct ratio between active and inactive forms of Rab11b might be essential for targeting transport vesicles to the pollen tip area and pollen tube growth (de Graaf et al., 2005). Another small GTPase, NtRab2, was isolated from tobacco pollen and fluorescent-NtRab 2 localized between the ER and the Golgi (Cheung et al., 2002). NtRab 2 showed high expression in not only growing pollen tubes, but also in other tissues that grow rapidly (Cheung et al., 2002). A dominant negative mutant form of NtRab2 GTPase inhibited vesicle transport between ER and Golgi (Cheung et al., 2002).

Rho GTPase has been known to regulate a number of actin-based cellular processes in eukaryotes (Franklin-Tong, 1999; Gu et al., 2003). In plants, Rop 1, a Rho GTPase, appears to play a key role in pollen tube polar growth by regulating the dynamics of F-actin in the tip (Lin et al., 1996; Lin and Yang, 1997; Fu et al., 2001; Gu et al., 2003). Interestingly, Rop1 protein localizes to the apical region in a tip-based

gradient, and it co-localizes with myosin, which implies that Rop1 plays a role in the actin-myosin motor system in the pollen tube apical region (Lin et al., 1996). In an anti-Rop antibody microinjection experiment, Rop appeared to control Ca^{2+}-dependent vesicle docking or the fusion process (Lin and Yang, 1997). Expression of constitutively active Rop1 in pollen caused severe depolarized growth (Gu et al., 2003).

Dyneins and Kinesins

Dyneins and kinesins are microtubule motor proteins that connect vesicles or organelles to microtubules for transport (Moscatelli et al., 2003). Since pollen tube growth requires rapid transport of materials and organelles, motor protein involvement has been suspected (Moscatelli et al., 2003). Dynein heavy chain-like proteins were found in tobacco pollen tubes and bound to microtubules in an ATP dependent manner (Moscatelli et al., 1995). It was suggested that their function might be the same as the dynein heavy chain (Moscatelli et al., 1995). A kinesin-like calmodulin binding protein (KCBP), ZWI, was discovered in a trichome development study, and it was shown that ZWI might be involved in pollen tube growth (Reddy et al., 1996; Oppenheimer et al., 1997).

Calcium in pollen germination and tube growth

Calcium has been known as a major second messenger in plant signaling: it mediates a number of biotic and abiotic signals in producing various appropriate cellular responses and is involved in regulating a number of basic cellular processes, such as cell growth, development and defense responses (Bush, 1995; Reddy, 2001a). Calcium is one of the key regulators of pollen germination, tube growth, and tube guidance (Bedinger et al., 1994; Taylor and Hepler, 1997; Franklin-Tong, 1999; Hepler et al., 2001). Recently,

by direct imaging of cytosolic free calcium concentration ($[Ca^{2+}]_i$), it was shown that $[Ca^{2+}]_i$ change is closely correlated with pollen tube growth rate and direction (Malho and Trewavas, 1996; Pierson et al., 1996; Holdaway-Clarke et al., 1997; Taylor and Hepler, 1997). A steep tip-focused $[Ca^{2+}]_i$ apical gradient is commonly observed in growing pollen tubes by several ratiometric calcium imaging systems (Figure 1.3) and pollen tube growth can be inhibited when the apical $[Ca^{2+}]_i$ gradient is disturbed by blocking calcium uptake (Malho and Trewavas, 1996; Pierson et al., 1996; Holdaway-Clarke et al., 1997). Studies indicate that active calcium channels at the pollen tube tip area cause a localized calcium influx within the narrow tip apex area, and this small restricted area of calcium influx makes apical $[Ca^{2+}]_i$ gradients (Pierson et al., 1996; Holdaway-Clarke et al., 1997). Although calcium channels on pollen tube membranes are not well characterized, it is generally accepted that apical $[Ca^{2+}]_i$ gradients are observed only in growing pollen tubes because of calcium channel activity whereas in non-growing pollen tubes dissipated gradients are observed (Pierson et al., 1994; Malho et al., 1995). The calcium concentration in the pollen tube apex is more than 3μM at the tip area, and it falls to less than 200nM within 20μm, which is similar to basal levels of cytosolic calcium (Malho and Trewavas, 1996; Pierson et al., 1996). This steep tip-focused calcium gradient is established because Ca^{2+}-ATPases on the ER membrane sequester calcium and lower $[Ca^{2+}]_i$ until basal level are reached in the sub-apex area (Obermeyer and Weisenseel, 1991; Lancelle and Hepler, 1992; Sze et al., 1999). With ratiometric imaging and aequorin photon counting, it was observed that apical calcium concentration oscillates and this oscillation coincides with growth rate in growing pollen tubes (Figure 1.3) (Pierson et al., 1996; Holdaway-Clarke et al., 1997). Although it is thought that there is a

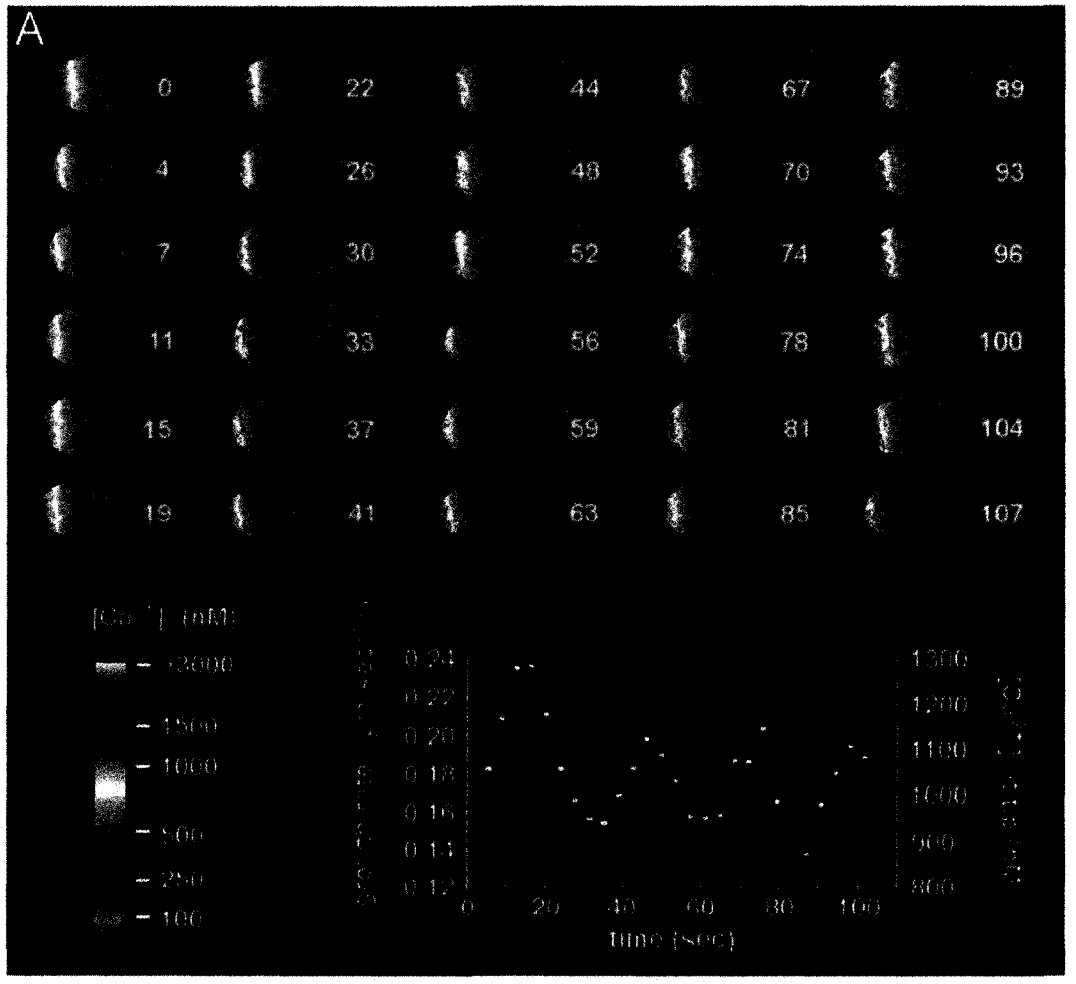


Figure 1.3. Pollen tube growth and free calcium at the tip oscillate in phase. Cytosolic calcium concentration gradient changes at the tip of growing pollen tubes and this change is correlated with pollen tube growth rate oscillation. Top panel shows pseudocolor image of calcium levels in growing pollen tubes. Fura-2-dextran was used to measure cytosolic calcium concentration. Number in each image indicates time in seconds. The graph shows growth rate and cytosolic calcium changes in a single tube as a function of time.
(from Holdaway-Clarke TL, Feijo JA, Hackett GR, Kunkel JG and Hepler PK (1997) Plant Cell 9: 1999-2010).

direct link between calcium oscillation and pollen tube growth, it is still debatable whether calcium concentration changes stimulate growth rate changes or whether growth rate changes cause calcium oscillation (Franklin-Tong, 1999). Work by Calder and colleagues has supported the first possibility, in that increased calcium can stimulate calcium oscillation in growth-arrested pollen tubes and this oscillation can trigger tube growth (Calder et al., 1997). In support of the second possibility, Holdaway-Clark et al. (Holdaway-Clarke et al., 1997) have shown that oscillation and growth rate are coincidental and Ca^{2+}influx lags by 11sec, which means Ca^{2+}influx comes after growth. To explain this phenomenon, two models were proposed (Figure 1.4). In the first model, a Ca^{2+} channel briefly opens by stretch activation and allows a small Ca^{2+} influx, and this Ca^{2+} triggers calcium-induced calcium release from the ER, which results in an increased $[Ca^{2+}]_i$ gradient (Holdaway-Clarke et al., 1997). In the second model, the cell wall, instead of ER, works as a Ca^{2+}store. Ion binding affinity to pectin in cell walls can change over time, alternating cell wall properties, which can regulate Ca^{2+} influx and cause oscillation (Holdaway-Clarke et al., 1997).

Although the detailed functions of apical $[Ca^{2+}]_i$ remain unknown, it appears to be involved in regulation of vesicle secretion at the apex of the pollen tube, and it is expected that high Ca^{2+} concentration at the tip can stimulate vesicle exocytosis and promote cell elongation (Franklin-Tong, 1999). Annexin, a Ca^{2+} binding protein, is known to be involved in secretion and localizes to the same area as Ca^{2+} (Franklin-Tong, 1999). These studies imply that annexin, Ca^{2+} and vesicle fusion have some relationship (Franklin-Tong, 1999). The apical Ca^{2+}gradient is also considered to affect pollen tube cytoskeleton organization because Ca^{2+} and Ca^{2+}-dependent enzymes regulate

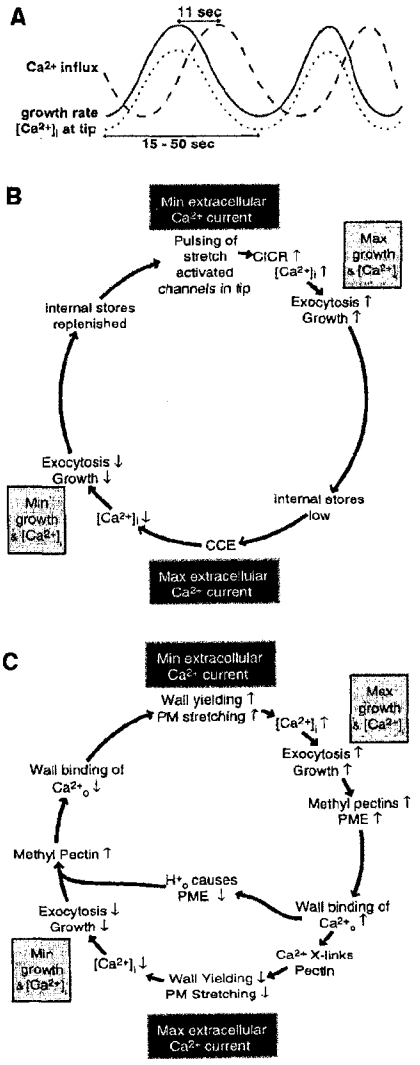


Figure 1.4. Two models that explain oscillatory growth in pollen tubes.
A) Relationship among growth rate oscillations, calcium influx, and the calcium gradient with time. B) Internal stores model, ER plays a major role for calcium uptake and release to regulate cytosolic calcium concentration at the tip. C) External stores model, cell wall acts as a growth regulator.
(from Holdaway-Clarke TL, Feijo JA, Hackett GR, Kunkel JG and Hepler PK (1997) Plant Cell 9: 1999-2010).

microfilament dynamics (Franklin-Tong, 1999).

For successful fertilization, it is necessary that pollen tubes change their direction and grow toward the synergids in the female tissue. Identification of signals, elucidation of signal pathways, and the mechanism involved in this reorientation process are fundamental to understanding pollen tube guidance (Franklin-Tong, 1999). Using Ca^{2+}imaging studies, Malho et al provided evidence to support the idea that $[Ca^{2+}]_i$ plays a role in pollen tube reorientation (Malho et al., 1994; Malho et al., 1995; Malho and Trewavas, 1996). To simulate reorientation, localized release of caged Ca^{2+} was used to locally increase $[Ca^{2+}]_i$, which perturbed the polarity in pollen tubes and showed that reorientation occurs (Malho et al., 1994; Malho et al., 1995). After polarity disappeared, pollen tube growth is temporarily arrested and the apical $[Ca^{2+}]_i$ gradient is lost. Subsequently a new apical gradient is established and tube growth is started in the new direction (Malho et al., 1994; Malho et al., 1995). Based on these observations, it was concluded that "locally increasing $[Ca^{2+}]_i$ within the pollen tube" or "locally altering external Ca^{2+} concentration" can reorient the direction of pollen tube growth (Malho and Trewavas, 1996). In this reorientation processes, local Ca^{2+} channel activity in the apical dome can be a key player in determining the direction of elongation (Malho and Trewavas, 1996; Pierson et al., 1996). Overall, based on these data, pollen tube growth and guidance can be controlled by a Ca^{2+}-mediated signal transduction pathway, even though there are some other components in pistil tissue that can also affect direction change (Franklin-Tong, 1999).

The ER and cell wall have been known as Ca^{2+} storage sites in plant cells, but there are still some questions as to what signals allow Ca^{2+} release from these storage

sites (Franklin-Tong, 1999). Recently, some evidence supports the idea that a phosphoinositide signal transducing system may have a role in regulating pollen tube growth (Franklin-Tong, 1999). In the plant phosphoinositide signal transducing system, phospholipase C (PLC) is activated by a signal from outside resulting in hydrolysis of phosphatidylinositol-4, 5-bisphosphate (PIP_2). As a result of this hydrolysis, two messengers, inositol-1, 4, 5-triphosphate (IP_3) and diacylglycerol (DAG) are produced (Franklin-Tong et al., 1996; Reddy, 2001a). IP_3 induces calcium release from the endoplasmic reticulum (ER) or vacuole by binding the IP_3 receptor on the membrane and DAG activates protein kinase C on the plasma membrane (Franklin-Tong et al., 1996; Reddy, 2001a). Helsper et al. (Helsper et al., 1987) showed the existence of phosphoinositides and phosphatidylinositol phospholipase C activity in the *Lilium longiflorum* pollen tube. Franklin-Tong and colleagues (Franklin-Tong et al., 1996) confirmed this by first demonstrating that calcium-dependent PIP_2 specific phospholipase C activity exists in *Papaver rhoeas* pollen. Increase in IP_3 concentration, using caged IP_3, causes an increase in calcium concentration in pollen tube cytosol and the high calcium concentration initiated from an 'unclear region' moves to the tip area like a wave (Franklin-Tong et al., 1996). Additionally, a series of IP_3-generating and calcium-mobilizing events, release calcium and stimulate another production of IP_3, which has been known to contribute to this 'calcium wave' (Franklin-Tong et al., 1996). This study has shown that calcium waves generated by IP_3 inhibit tube growth in *Papaver rhoeas* pollen. Interestingly, inhibition of phosphoinositide (PI) turnover, and inhibition of IP_3 binding to its receptor also resulted in inhibition of tube growth. Based on these results, it is suggested that a two-tier regulation is involved in PI mediated pollen tube growth

control (Franklin-Tong et al., 1996).

In the self-incompatibility (SI) response in *Papaver*, it is proposed that Ca^{2+}, as a second messenger, mediates the pollen tube growth inhibition process (Franklin-Tong et al., 1993; Franklin-Tong et al., 1995; Franklin-Tong et al., 1997). It is considered that S-protein triggers Ca^{2+}-dependent signal transduction by binding pollen-receptors on the pollen membrane (Franklin-Tong, 1999). SI response specific $[Ca^{2+}]_i$ increase occurs in the sub-apical region of the pollen tube, and this $[Ca^{2+}]_i$ change inhibits pollen tube growth (Franklin-Tong et al., 1993; Franklin-Tong et al., 1995; Drobak et al., 1997; Franklin-Tong et al., 1997). With these observations, it can be concluded that not only alternating $[Ca^{2+}]_i$ in apical regions has an important role for regulating pollen tube growth, but $[Ca^{2+}]_i$ in other regions also has a role (Franklin-Tong, 1999). Pollen protein kinases also appear to have an important role in pollination. Ca^{2+}-dependent calmodulin-independent protein kinase (CDPK) is thought to have an essential role in pollen germination and growth, and the CDPK may be involved in cytoskeleton dynamics (Estruch et al., 1994; Franklin-Tong, 1999).

Role of calmodulin in pollen germination and tube growth

The signal induced Ca^{2+} elevation in pollen tubes is likely recognized by Ca^{2+} sensors or Ca^{2+} binding proteins, which mediate Ca^{2+} signals (Reddy, 2001a; Yang and Poovaiah, 2003). Ca^{2+} sensors are broadly classified into four groups according to their structural and functional features (Reddy, 2001a). These four groups are calmodulin (CaM), CaM-like and other EF-hand containing Ca^{2+}-binding proteins, Ca^{2+}-regulated protein kinases, and Ca^{2+} binding proteins without EF-hand motifs (Reddy, 2001a). The first three groups of Ca^{2+} sensors have helix-loop-helix containing EF-hand motifs that

are known Ca^{2+} binding domains (Roberts and Harmon, 1992; Zielinski, 1998). Ca^{2+} sensors have different numbers of EF-hand motifs and because of that, the Ca^{2+} affinity of these sensors might be different (Reddy, 2001a). Ca^{2+} binding can cause conformational changes in Ca^{2+} sensors and this can modulate the sensor's own activity or modulate binding affinity to their target proteins, whose function or activity can be changed by interaction with Ca^{2+} sensors (Reddy, 2001a).

Among Ca^{2+} sensors, CaM is the most well-known and common Ca^{2+} binding protein in eukaryotes (Reddy, 2001a; Luan et al., 2002; Yang and Poovaiah, 2003). CaM is a small (148aa) acidic protein that has 4 Ca^{2+} binding EF-hand motifs. The crystal structure of CaM shows that CaM has two globular domains at the N- and C-termini with two EF-hand motifs in each domain. The two globular domains are connected by a flexible central helix (Babu et al., 1988). Arabidopsis has 11 CaM genes and at least seven isoforms (Yang and Poovaiah, 2003). It has been shown that different stimuli can induce differential expression of CaM genes. Furthermore, CaM gene expression also differs in different developmental stages, cell types, and tissues (Yang and Poovaiah, 2003). Plant CaM isoforms have different affinities to their target proteins (Yang and Poovaiah, 2003). The pattern of CaM gene expression and the specificity to target protein interactions suggest that each isoform has its own functional significance (Yang and Poovaiah, 2003).

CaM has no enzymatic activity, but it can interact with proteins and regulate their activity/function (Zielinski, 1998; Reddy, 2001a; Yang and Poovaiah, 2003). Because of this, it is critical to identify and characterize CaM-binding proteins to further understand the Ca^{2+}-CaM signal transduction pathway in plants. Several CaM target

proteins have been identified and these proteins play roles in diverse processes, such as ion transport, gene regulation, cytoskeleton organization, disease resistance, metabolism and stress tolerance (Yang and Poovaiah, 2003; Reddy and Reddy, 2004a; Popescu et al., 2007).

CaM is known to be involved in pollen germination and tube growth (Tirlapur et al., 1994; Moutinho et al., 1998; Ma et al., 1999). Artificial blocking of CaM by antiserum or CaM antagonists causes cessation of pollen tube growth, and pure exogenous CaM can enhance pollen germination and tube growth (Ma and Sun, 1997). In hydrated pollen, high levels of CaM localized to the germinal apertures, to the plasma membrane of the germination bubble and to the cytoplasm near the bubble (Tirlapur et al., 1994). Although CaM distributes uniformly in pollen tubes, a higher level of activated CaM was observed in the tip region, and the activated CaM forms a tip-focused gradient, like Ca^{2+} in the pollen apex (Figure 1.5) (Tirlapur et al., 1994; Rato et al., 2004). Moreover, activated CaM oscillates in growing pollen tubes, and it correlates with Ca^{2+} oscillation (Rato et al., 2004). Localized release of CaM inhibitor also indicated a role for CaM in pollen tube reorientation (Rato et al., 2004). Although the Ca^{2+}-CaM signal pathway is implicated in pollen germination and pollen tube growth, the CaM target proteins that mediate signals have not been well studied.

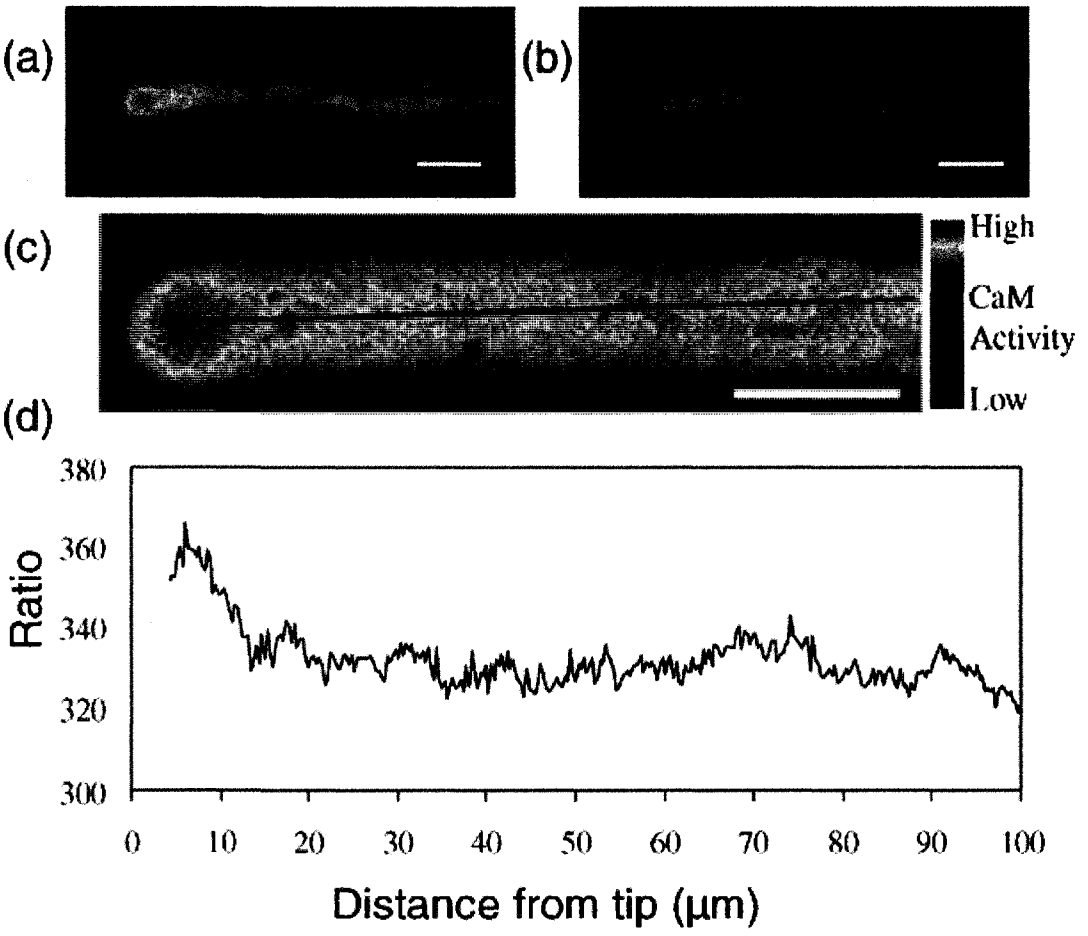


Figure 1.5. CaM activity in a growing pollen tube of *Lilium*.
a) Distribution of TA-CaM which measures active CaM. b) Distribution of FL-CaM whose fluorescence is insensitive to CaM activation. c) Tip-focused CaM activity gradient was shown by ratio image. d) Line profile from CaM-activity in pollen tube. CaM activity is similar to calcium gradient, reaching basal level within 10-15 micrometer.
(from Rato et al. (2004) The Plant Journal 38;887-897)

CaM target proteins in pollen

To understand the functions of calmodulin target proteins in pollen, Safadi et al. (2000) isolated a maize Ca^{2+}-dependent CaM-binding protein using a protein- protein interaction screening of a maize pollen cDNA library with labeled CaM. The Maize CaM-binding protein is called ZmMPCBP (Maize Pollen-specific CaM Binding Protein). The *ZmMPCBP* gene is composed of 5 exons and 4 introns (Figure 1.6 A, B). The *ZmMPCBP* cDNA codes for 659 amino acids (72 kD). ZmMPCBP has three TPR (tetratricopeptide repeats) domains and its CaM-binding domain was mapped to a region spanning amino acids 421-438 (Figure 1.6C). Northern and western analyses have shown that ZmMPCBP is expressed only in pollen (Safadi et al., 2000). During germination and pollen tube growth, ZmMPCBP protein remained at a constant level. ZmMPCBP protein binds CaM isoforms in a calcium-dependent manner.

In Arabidopsis, there are three proteins that are similar to ZmMPCBP and these were named APCBP1 (Arabidopsis Pollen CaM-Binding Protein1), APCBP2 and APCBP3 at that time. After recent functional characterization of APCBP1, the name of APCBP1 was changed to AtNPG1 (No Pollen Germination) and APCBP2 and APCBP3 were also renamed to AtNPGR1 (NPG1 Related Protein1) and AtNPGR2 (Golovkin and Reddy, 2003) (Figures 1.7 to 1.12). AtNPG1 is 704 amino acids long and contains 5 TPR domains and a calmodulin-binding domain (Figures 1.7 and 1.8). AtNPG1, AtNPGR1 and AtNPGR2 have similar exon/intron organization (Figures 1.7-1.12). AtNPG1, AtNPGR1, and AtNPGR2 share high sequence similarity among themselves and with ZmMPCBP (Figure 1.13). The amino acid sequence of AtNPG1 and ZmMPCBP share 56% identity and 70% similarity, and all AtNPGs and ZmMPCBP share 39-56% identity

Figure 1.6. Sequence of *MPCBP*.

a) Nucleotide and deduced amino acid sequence of *MPCBP*. In nucleotide sequence, uppercase letter indicate exon; lowercase letter indicate introns; CaM-binding domain is underlined. b) Gene structure of *MPCBP*. lines, introns; boxes, exons. C) Domains in MPCBP. Three tetratricopeptide repeats (TPRs) were found using SMART. CaM binding domain (CBD) is from amino acids 421-438.

(from Safadi et al. (2000) The J Biol Chem 275; 35457-35470).

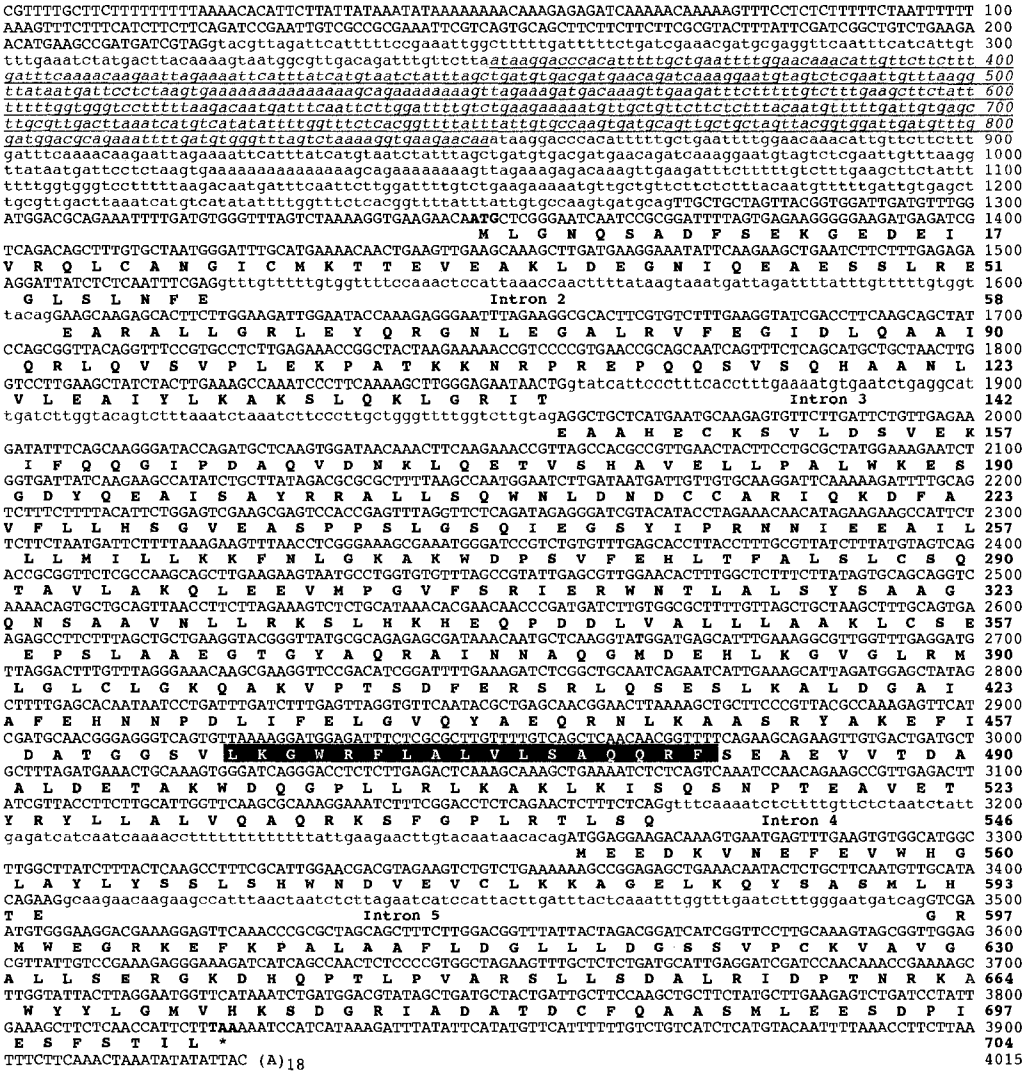


Figure 1.7. Nucleotide (exons in uppercase letters; introns in lowercase letters) and deduced amino acid sequence (under nucleotide sequence) of *NPG1*. Shaded region indicates CaM-binding domain (CBD).
(from Golovkin M and Reddy ASN (2003) Proc. Natl. Acad. Sci USA 100:10558-10563).

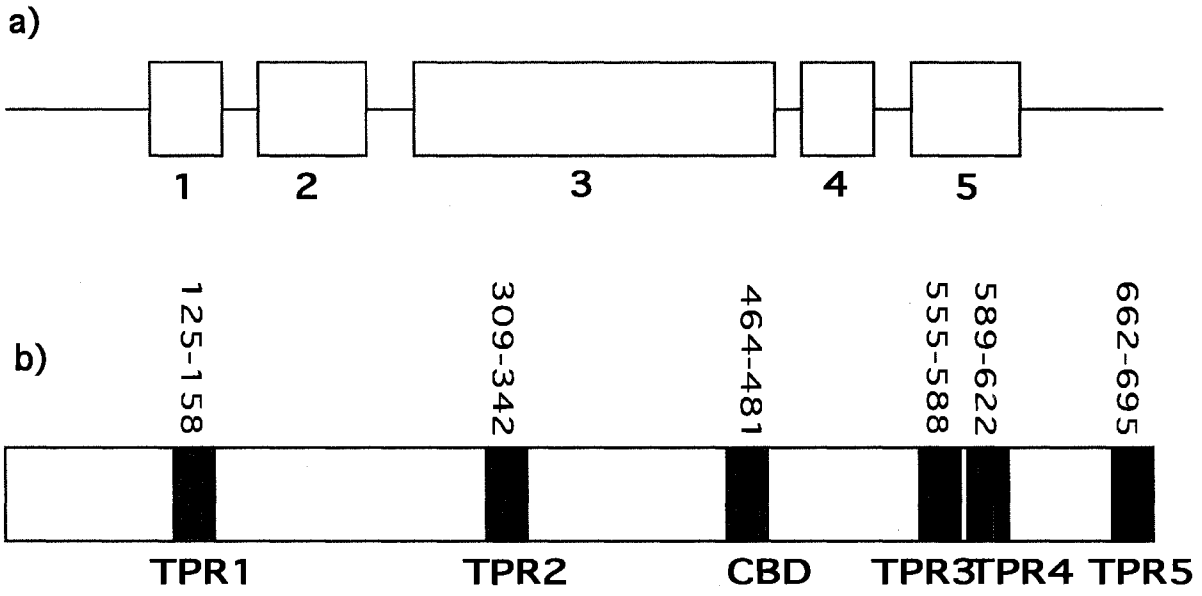


Figure 1.8. Gene structure and domain organization of *NPG1*.
a) *NPG1* has 5 exons (lines, introns; boxes, exons). b) Schematic representation of *NPG1*. Five TPR domains were identified by SMART and putative CaM-binding domain (CBD) was identified by comparing with MPCBP CBD sequence.

```
   1 ataaacaaaa agaaaatcac agcgtggttg ctgctggctt gttgcttaca ttgacatgaa
  61 caagacacac agtgcaaaat gacaaagagc ccttcatcga tttcgaatct tctctctgag
 121 taaatcaatt tcacaaattc gaggtttttg gtttttagct tactacacta ctccaagaag
 181 aaaatctcaa gttaaagtca tttcctatca aatctctttt catggcggct gctattctcg
 241 tttctcctgt tcgctaaatt gcgtcatttt catgtaagct tctttcattt tgcttaccaa
 301 agctcgtttt cactatgctt tagcaacaac attattgtag aggttaaagg cagcaaaatc
 361 tataggttaa ggatttcttg tggttaattt ctgaaattca tgtttctgtg tgttttctta
 421 gtgaagctta ttataaaagc ggcgtagatc tgtgattcta ctcattgcat ttccgcgttt
 481 gtgaggagaa agaggagaag ATGTTGTGTG CTTGTTCAGG CGAACAATTC CGGTTCGAGG
   1                       M  L  C   A  C  S  G   E  Q  F   R  F  E
 541 ATCAGCCCGG TTCGCCGGAA TCCCTAGCTA CAAGAGATTT CTCAGCTAGT GGACTTTCTT
  14 D  Q  P  G   S  P  E   S  L  A   T  R  D  F   S  A  S   G  L  S
 601 CTCGAAACGG AGGAGGCGAT TGGGATTCCA AATTGGAAGA TATTCAAGTC GATGAAGCTG
  34 S  R  N  G   G  G  D   W  D  S   K  L  E  D   I  Q  V   D  E  A
 661 AATCGACTTT AAAGGAAGCT CTCTCACTCA ACTATGAGgt tggttttagt tactctgatg
  54 E  S  T  L   K  E  A   L  S  L   N  Y  E
 721 catcaatttt ttggttttaa ctggaatcat cctgatgctg agttttgttt ttgcttcctt
 781 atgtgtgtta gGAAGCTAGG GCTTTGTTGG GGAGACTTGA ATATCAGAGA GGTAATTTTG
  67              E  A  R   A  L  L   G  R  L  E   Y  Q  R   G  N  F
 841 ATGCAGCACT TCAAGTGTTT AAAGGGATTG ACATTAAGGT CTTAACTCCA AGGATAATCA
  83 D  A  A  L   Q  V  F   K  G  I   D  I  K  V   L  T  P   R  I  I
 901 AAGCCATTGT TGAGAAAACT CTTCCTTGCA AACCACGCTC TAAAGCTGTT ATTGTGCCTC
 103 K  A  I  V   E  K  T   L  P  C   K  P  R  S   K  A  V   I  V  P
 961 CCACCACAAT GTCAATGCAT TCAGTCAGTT TACTTCTTGA AGCAATCTTA CTTAAAGCTA
 123 P  T  T  M   S  M  H   S  V  S   L  L  L  E   A  I  L   L  K  A
1021 GATCACTTGA AGAACTTGGC TCTTACAAAG gtatgtatgc atgaatttga atattacctg
 143 R  S  L  E   E  L  G   S  Y  K
1081 tgttgctctc cagatttttg cattggaatc gacttacatt accctgctgt ctattgtttt
1141 accagAGGCT GCAGAAGAAT GCAAAATAAT CCTGGACGTG GTTGAAAATG CACTGCCAAG
 153      E  A   A  E  E   C  K  I  I   L  D  V   V  E  N   A  L  P  S
1201 CGGCATGCCT GACGGAATCA GCGGATTCGC TAAACTGCAG GATATCTTTC AGAAGGCCCT
 172   G  M  P   D  G  I   S  G  F  A   K  L  Q   D  I  F   Q  K  A  L
1261 GGAATTGCTT CCTTTACTAT GGAAAAAAGC GGGGAATCAT CATGAAACTA TTGCTTCATA
 192   E  L  L   P  L  L   W  K  K  A   G  N  H   H  E  T   I  A  S  Y
1321 TCGCCGTGCT TTATCCAGAC CATGGAATTT GGATCCCCAG AGGTTAGCTG TTACGCAGAA
 212   R  R  A   L  S  R   P  W  N  L   D  P  Q   R  L  A   V  T  Q  K
1381 ATCCTTAGCT TTGGTTTTAC TTTACGGGAG TGTTGAGGCA TGTCCAAAAG ACAACATCGA
 232   S  L  A   L  V  L   L  Y  G  S   V  E  A   C  P  K   D  N  I  E
1441 GGAAGCAATT GTGTTGCTAA TGTTACTTGT AAAGAAGATG GTGGTTGGGG ACATACAGTG
 252   E  A  I   V  L  L   M  L  L  V   K  K  M   V  V  G   D  I  Q  W
1501 GGATCCAGAA CTCATGGATC ATCTCACTTA CGCCCTTTCC ATGACTGGAC AGTTTGAAGT
 272   D  P  E   L  M  D   H  L  T  Y   A  L  S   M  T  G   Q  F  E  V
1561 GTTAGCGAAC TATCTAGAGC AGACTCTTCC GGGTGTTTAC ACCCGCGGGG AGAGATGGTA
 292   L  A  N   Y  L  E   Q  T  L  P   G  V  Y   T  R  G   E  R  W  Y
1621 CCTTCTCTCC CTTTGTTACA GTGCTGCTGG GATCGATAAA GCGGCCATTA ATCTATTAAA
 312   L  L  S   L  C  Y   S  A  A  G   I  D  K   A  A  I   N  L  L  K
1681 GATGGCCCTA GGCCCATCTG AATCAAGACA AATACCACAC ATTCCGTTGT TATTGTTTGG
 332   M  A  L   G  P  S   E  S  R  Q   I  P  H   I  P  L   L  L  F  G
1741 AGCAAAGCTG TGCTCTAAAG ATCCAAAACA CTCAAGGGAC GGCATAAATT TTGCTCATAG
 352   A  K  L   C  S  K   D  P  K  H   S  R  D   G  I  N   F  A  H  R
1801 GCTGCTCGAC TTGGGAAACA GTCAGAGTGA ACATCTTTTG AGCCAAGCAC ACAAGTTCCT
 372   L  L  D   L  G  N   S  Q  S  E   H  L  L   S  Q  A   H  K  F  L
1861 TGGTGTATGC TATGGAAACG CTGCAAGAAG TTCTAAATTA GACTCTGAAC GGGTTTTTCT
 392   G  V  C   Y  G  N   A  A  R  S   S  K  L   D  S  E   R  V  F  L
1921 TCAGAAAAAA TCTCTGTTCT CTTTAAATGA AGCAGCAAAG AGGGGTAAGG CTGATCCGGA
 412   Q  K  K   S  L  F   S  L  N  E   A  A  K   R  G  K   A  D  P  E
1981 ACTAGACGTT ATATTTAACC TAAGCGTTGA GAATGCAGTT CAAAGAAATG TTCAGGCGGC
 432   L  D  V   I  F  N   L  S  V  E   N  A  V   Q  R  N   V  Q  A  A
2041 TTTGGATGGT GCGGTTGAGT ATTCTAGTAT GGTGGGAGGA GTTTCAACTA AAGGATGGAA
 452   L  D  G   A  V  E   Y  S  S  M   V  G  G   V  S  T   K  G  W  K
2101 ACATTTAGCT ATTGTCCTTT CAGCGGAGAA ACGGCTCAAG GATGCTGAAT CAATACTGGA
 472   H  L  A   I  V  L   S  A  E  K   R  L  K   D  A  E   S  I  L  D
2161 CTTCACCATG GAAGAGGCTG GTGACATTGA AAAGATAGAG CTTCTGAGGT TAAAAGCTGT
 492   F  T  M   E  E  A   G  D  I  E   K  I  E   L  L  R   L  K  A  V
```

Continued

```
2221 GCTTCAGATG GCTCAAGAAC AACCTAAAAA GGCAATGAAG ACATGCAGCA GCTTACTGGG
 512   L  Q  M   A  Q  E   Q  P  K  K   A  M  K   T  C  S   S  L  L  G
2281 ACTAATTCGA GCGCAAGAGA AATCTGAACA ATCCGAGgta cctagtttgg atgtcacaat
 532   L  I  R   A  Q  E   K  S  E  Q   S  E
2341 atgaaagaaa catattctgt gaataatctt attctttgtt tccagAGTCT GCTACAGAAA
 544                                                  S  L   L  Q  K
2401 TTTGAAACGG AAGCTTGGCA AGACCTGGCC TCTGTCTATG GAAAGCTAGG TTCATGGTCA
 549  F  E  T   E  A  W  Q   D  L  A   S  V  Y   G  K  L  G   S  W  S
2461 GATGCAGAAA CATGTCTCGA GAAGGCAAGA TCCATGTGCT ATTATTCTCC CAGAGGCTGG
 569  D  A  E   T  C  L  E   K  A  R   S  M  C   Y  Y  S  P   R  G  W
2521 AATGAAACAG gtatggagaa aaggcttcag tcttaagcca actcatcaag ttttccacat
 589  N  E  T
2581 tcataatttt ccgtgtttat aacaagcttc atacatttgc ttctatggct aatacagGTT
 592                                                             G
2641 TGTGTCTAGA AGCTAAATCA CTTCATGAAG AGGCTTTAAT ATCCTTCTTC CTTTCGCTCT
 593 L  C  L  E   A  K  S   L  H  E   E  A  L  I   S  F  F   L  S  L
2701 CGATCGAACC AGATCACGTA CCTAGCATTG TTTCTATAGC AGAGGTCATG ATGAAATCTG
 613 S  I  E  P   D  H  V   P  S  I   V  S  I  A   E  V  M   M  K  S
2761 GAGATGAATC ACTTCCAACC GCTAAAAGTT TTCTGATGAA TGCCTTAAGG TTAGACCCGA
 633 G  D  E  S   L  P  T   A  K  S   F  L  M  N   A  L  R   L  D  P
2821 GAAATCATGA TGCATGGATG AAGCTAGGGC ACGTTGCAAA GAAGCAAGGC TTGTCACAGC
 653 R  N  H  D   A  W  M   K  L  G   H  V  A  K   K  Q  G   L  S  Q
2881 AAGCTGCAGA GTTTTACCAA GCTGCATATG AACTAGAACT ATCCGCTCCG GTACAGAGTT
 673 Q  A  A  E   F  Y  Q   A  A  Y   E  L  E  L   S  A  P   V  Q  S
2941 TCATTTGAcg aataatatga gttgaatagt gcacaattct ttttctttta agccttttt
 693 F  I  *
3001 tctttaatat atgatttcgg tactactaaa cttgcttaag ataataacac tcgagtatta
3061 tatattccac attaccattt tttatagcga caaacagatt cagatacggg ttaaatttcc
3121 tccagctctt gaaatatgtg tgtagtttaa gcacttaaca tttgagttct ctaagagatt
3181 ctccttctac gatcgcttcc agcccaagtt gtgaagacaa caacgttaag tagacattta
3241 ccaacgaaat ttataagttc tgtatttcaa gagattatta aaccattcta ctataagaaa
3301 gaacttgttc ttcttaactc atactgtatt tcaacagcgt aattatatat atgactaacg
3361 acatttacat atttgctata gaggatatat ataattacgc taaacactac tagaaagggt
3421 aacacacata ctccaaaacc taatctaa
```

Figure 1.9. Nucleotide and deduced amino acid sequence of *NPGR1*. In nucleotide sequence, uppercase indicates exon regions and lowercase indicates intron regions. Deduced amino acid sequence is under nucleotide sequence. Box indicates putative CaM-binding domain (CBD).

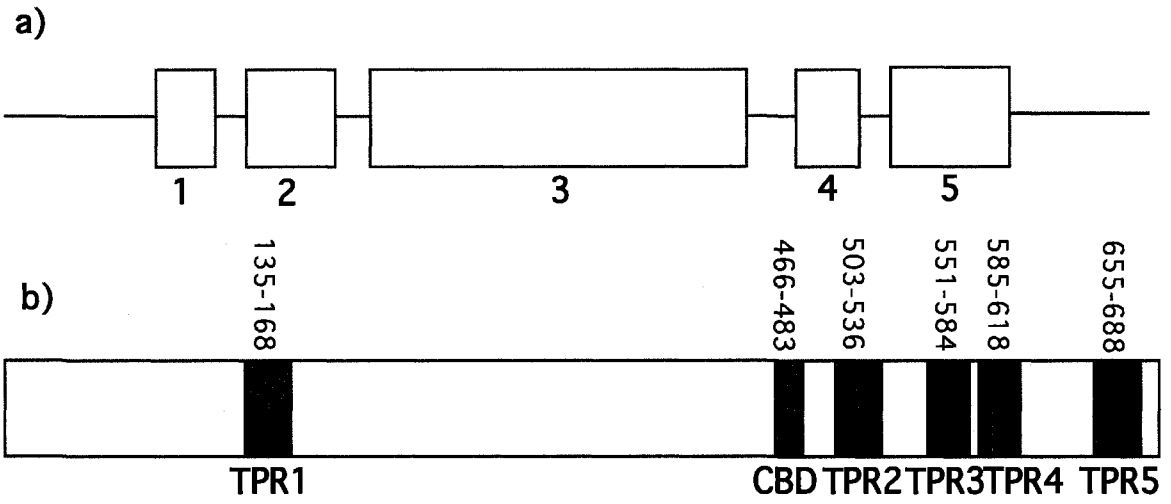


Figure 1.10. Gene structure and domain organization of *NPGR1*.
a) *NPGR1* gene has 5 exons. Lines indicate introns and boxes indicate exons.
b) Schematic diagram of *NPGR1*. Five TPR domains were identified by SMART and putative CaM-binding domain (CBD) was identified by comparing it with MPCBP CBD sequence.

```
   1 ttcgaggcgg ccatggctgt cggctttctc tttttctctt cttctttctc tgattctcaa
  61 aggtctcttc tttatccctc tctgtttctt gatcttcctt attacttcaa ttgggtttgt
 121 gattcgatct gattttaggg ttttgaattg tttaatttgt caatttcgat tctgaaattt
 181 cgagccttttt tggttactgt acttagttag aatccccaaa gttctcgact ttctcgcgga
 241 ttcacttttt cttgaggggg ggtgagttct taaagttgaa atgctgaaat tattgtcact
 301 gttctttctc gaaatatgtt caatgatttt tgagttctcg gcgttagttt ttaggaattg
 361 gatacatcaa gattttgatc atataatgtt ctgttatatt ttgcattatg tgaatatttc
 421 ttatctgttt ctgttatgtt tgatccaaat cttaatatca ttattgtctc tgatgtcatt
 481 ttgaagatca ttgagaagtt ATGAAGAACA GCGAGATTAG GCCTGAGAAG TTGCATTTAC
   1                       M  K  N   S  E  I  R   P  E  K   L  H  L
 541 GTAAACTAAG AAAAAGTTTG AGGAAGATCA GAATGAAGTG TTTGTGTTCT GGTGAACAAA
  14 R  K  L  R   K  S  L   R  K  I   R  M  K  C   L  C  S   G  E  Q
 601 TGAGGCATAG AGAAGAAGAA GACAAGAAAT CTGAGGTTGG AGTTGGTAGA GACTACAATG
  34 M  R  H  R   E  E  E   D  K  K   S  E  V  G   V  G  R   D  Y  N
 661 GAAGCTCAGC TTTATCGACC GCAGAAAGCG AGAATGCTAA GAAACTAGAT AATGGAAATA
  54 G  S  S  A   L  S  T   A  E  S   E  N  A  K   K  L  D   N  G  N
 721 TCGAAGAAGC CGAGTTATCT CTGCGTGAGA CGAGTTCATT GAACTACGAG gtttgatttc
  74 I  E  E  A   E  L  S   L  R  E   T  S  S  L   N  Y  E
 781 ttggtcactc ttgagctttg tttgattgga caagcttagc cattgtgttg ttgtttcagG
 841 AGGCGAGAGC GCTTTTGGGA AGAATTGAGT ATCAGAAAGG AAATATAGAG GCGGCATTGC
  91 E  A  R  A   L  L  G   R  I  E   Y  Q  K  G   N  I  E   A  A  L
 901 GAGTCTTTGA AGGGATAGAT ATCAATGGTA TCACAGTAAA GATGAAAACC GCTCTAACCG
 111 R  V  F  E   G  I  D   I  N  G   I  T  V  K   M  K  T   A  L  T
 961 TTAGAGAAGA CCGGAAACAT AGAAGACGGT CTAAAGGCGG CTTTTCTACT GCTCCTTCAC
 131 V  R  E  D   R  K  H   R  R  R   S  K  G  G   F  S  T   A  P  S
1021 CTGCCATGTC TAAACATGCT GTTAGTTTAC TTTTCGAAGC TATTTTTCTC AAAGCCAAGT
 151 P  A  M  S   K  H  A   V  S  L   L  F  E  A   I  F  L   K  A  K
1081 CTCTCCAGCG TCTTGGAAGG TTCCAAGgta gtaacagtca tttctcttaa tccgtgtttg
 171 S  L  Q  R   L  G  R   F  Q
1141 gttttgtttt tggctaaaca gaaatatcat gtgcttcttc ttgcagAAGC TGCGGAGTCT
 180                                                   E  A   A  E  S
1201 TGCAGAGTTA TTCTTGATAT AGTCGAGACT TCTTTAGCAG AAGGTGCGTC GGATAATGTG
 185  C  R  V   I  L  D  I   V  E  T   S  L  A   E  G  A  S   D  N  V
1261 ACTGGAGATA TTAAGTTGCA GGAGACACTG ACAAAAGCGG TTGAGCTGCT TCCTGAGTTG
 205  T  G  D   I  K  L  Q   E  T  L   T  K  A   V  E  L  L   P  E  L
1321 TGGAAGCTTG CTGATTCACC TCGTGATGCT ATTTTGTCGT ATAGAAGAGC GCTTCTCAAT
 225  W  K  L   A  D  S  P   R  D  A   I  L  S   Y  R  R  A   L  L  N
1381 CACTGGAAAC TCGATCCAGA AACCACGGCG AGGATACAGA AAGAGTACGC AGTGTTTCTC
 245  H  W  K   L  D  P  E   T  T  A   R  I  Q   K  E  Y  A   V  F  L
1441 CTCTATTCCG GGGAAGAAGC AGTGCCGCCA AACCTACGTT CTCAGACTGA GGGCTCATTC
 265  L  Y  S   G  E  E  A   V  P  P   N  L  R   S  Q  T  E   G  S  F
1501 ATTCCGAGGA ACAATGTGGA AGAAGCTATT CTTCTTCTGA TGTTACTTCT CAGGAAAGTT
 285  I  P  R   N  N  V  E   E  A  I   L  L  L   M  L  L  L   R  K  V
1561 AATCTGAAAA GAATCTCATG GGATGCGGCA ATCTTGGACC ATCTCTCGTT CGCTCTTACA
 305  N  L  K   R  I  S  W   D  A  A   I  L  D   H  L  S  F   A  L  T
1621 ATCGCAGGCG ATCTAACCGC TCTGGCTAAG CAGTTTGAAG AGCTTAGTCC TGAGCTCTTG
 325  I  A  G   D  L  T  A   L  A  K   Q  F  E   E  L  S  P   E  L  L
1681 GATCAGAGGG AACTTTATCA TACATTGTCT TTGTGTTACC AAGGTGCAGG AGAAGGTCTT
 345  D  Q  R   E  L  Y  H   T  L  S   L  C  Y   Q  G  A  G   E  G  L
1741 GTTGCGTTAG GTTTACTGAG GAAGCTATTT TCAGAACGAG AGGATCCAAA CAGGACTTCG
 365  V  A  L   G  L  L  R   K  L  F   S  E  R   E  D  P  N   R  T  S
1801 GGTTTGCTGA TGGCTTCCAA AATATGCGGT GAGAGGTCTG GTCTTGCTGA GGAAGGGTTA
 385  G  L  L   M  A  S  K   I  C  G   E  R  S   G  L  A  E   E  G  L
1861 GATTATGCCC GGAAAGCTAT CGGAAACTTG GGGAAGGAAT GCAGTCAATT AGATGGAGCA
 405  D  Y  A   R  K  A  I   G  N  L   G  K  E   C  S  Q  L   D  G  A
1921 GCGCGTTTCG TTTTAGGGAT CACGCTCACG GAAAGCTCTA GAATGGCTGT TACAGAGACT
 425  A  R  F   V  L  G  I   T  L  T   E  S  S   R  M  A  V   T  E  T
1981 GAGAGGATAG CTAGGCAATC CGAGGGGATT CAGGCCCTGG AATCTGCAGA TATGACAAAC
 445  E  R  I   A  R  Q  S   E  G  I   Q  A  L   E  S  A  D   M  T  N
2041 CCGAGGGTTG TGCACCGTCT TGCGTTGGAG AATGCAGAAC AGAGGAAACT GGATTCTGCA
 465  P  R  V   V  H  R  L   A  L  E   N  A  E   Q  R  K  L   D  S  A
```

Continued

```
2101 TTAGCATATG CTAAAGAGGC GTTGAAACTT GGAGCAGAGT CTGATCTTGA AGTATGGTTA
 485  L  A  Y   A  K  E  A   L  K  L   G  A  E   S  D  L  E  V  W  L
2161 CTTTTGGCTC GGGTTTTATC TGCACAGAAG CGGTTTTCAG ACGCGGAGAC CATTGTGGAT
 505  L  L  A  R  V  L  S  A  Q  K  R  F  S   D  A  E  T   I  V  D
2221 GCAGCGCTTA ACGAGACAGG GAAATGGGAA CAGGGGAAGC TGTTACGTTT GAAGGCAAAG
 525  A  A  L   N  E  T  G   K  W  E   Q  G  K   L  L  R  L   K  A  K
2281 CTTCGTTTAG CTAAAGGAGA AGTGAAAGAT GCGATTAAGA CTTACACACA ACTTCTAGCA
 545  L  R  L   A  K  G  E   V  K  D   A  I  K   T  Y  T  Q   L  L  A
2341 CTCCTTCAGG TTCAAAGCAA AAGCTTCAAT TCTGCAAAGA AGCTGCCTAA Ggtaacattt
 565  L  L  Q   V  Q  S  K   S  F  N   S  A  K   K  L  P  K
2401 tcgtatgttc tgatttctct accaaagttg tgagttgtgc agaattaaca ctgaaacaga
2461 tttattcatt acagGGATAT GTAAAGGAAT TGATGAGTCT GGAGCTTGGG ACGTGGCACG
 582                 G  Y   V  K  E   L  M  S  L   E  L  G   T  W  H
2521 ATCTGGCTCA TATTTACATA AACCTCTCGC AATGGCGTGA CGCAGAGTCA TGTCTCTCGA
 597  D  L  A  H   I  Y  I   N  L  S   Q  W  R  D   A  E  S   C  L  S
2581 GATCAAGACT CATTGCACCT TACTCTTCTG TTAGATACCA CATTGAAGgt attaccttct
 617  R  S  R  L   I  A  P   Y  S  S   V  R  Y  H   I  E
2641 tctctacttc catttagact tggagtgttt gtttgtcttt ttgaaacaag caaggcggta
2701 atattgatag cttattcgta ccgattggca gGTGTACTGT ACAATAGACG GGGGCAATTA
 633                                   G  V  L   Y  N  R  R   G  Q  L
2761 GAAGAAGCAA TGGAGGCGTT CACAACCGCT CTTGACATAG ATCCGATGCA CGTCCCAAGC
 643  E  E  A   M  E  A  F   T  T  A   L  D  I   D  P  M  H   V  P  S
2821 TTAACATCGA AGGCTGAGAT ACTGCTGGAG GTCGGTAACC GATCAGGCAT AGCGGTTGTT
 663  L  T  S   K  A  E  I   L  L  E   V  G  N   R  S  G  I   A  V  V
2881 AGAAGCTTTC TAATGGAGGC TCTTAGGATT GATAGGCTGA ACCACTCGGC TTGGTACAAT
 683  R  S  F   L  M  E  A   L  R  I   D  R  L   N  H  S  A   W  Y  N
2941 CTTGGAAAGA TGTTCAAAGC TGAAGGGTCC GTCTCGTCGA TGCAAGAAGC CGTTGAGTGT
 703  L  G  K   M  F  K  A   E  G  S   V  S  S   M  Q  E  A   V  E  C
3001 TTTCAAGCCG CCGTTACTTT GGAGGAAACA ATGCCAGTGG AGCCATTCAG ATGAttttgc
 723  F  Q  A   A  V  T  L   E  E  T   M  P  V   E  P  F  R   *
3061 cctctcaaat catatttttt ttgtctttgg ttatgatttc tccttttctg attctttttt
3121 tttgtgtttg tttttgatag atgaatataa gaagatataa agatgtatgt gtacgagtat
3181 atcatttgat gtcaagttta taaacatttt gtaatacaca gttaaacaaa aattaatata
3241 ggttttggtg aggtaaattt tataagccag gtttactatt tactcataaa agattataaa
3301 tttttcggag aaaacttttc aggtttgata ttctaaaaaa gattaacata agaaatctgt
3361 agctgtaaaa ctaatgatga gttaagcatt tagattacac tgaatgatga agattctccc
3421 aaactaactt tttatttttg ttttgtttct tccttgggga tctgttgttt gtctacacag
3481 ctgagttgag agtcttccat cagaaaaata gttaaacagt aaagagagaa gtcctgaaga
3541 acctcaagat aaga
```

Figure 1.11. Nucleotide and deduced amino acid sequence of *NPGR2*.
In nucleotide sequence, uppercase letters indicate exon regions and lowercase letters indicate intron regions. Deduced amino acid sequence is under the nucleotide sequence. Boxed area indicates putative CaM- binding domain (CBD).

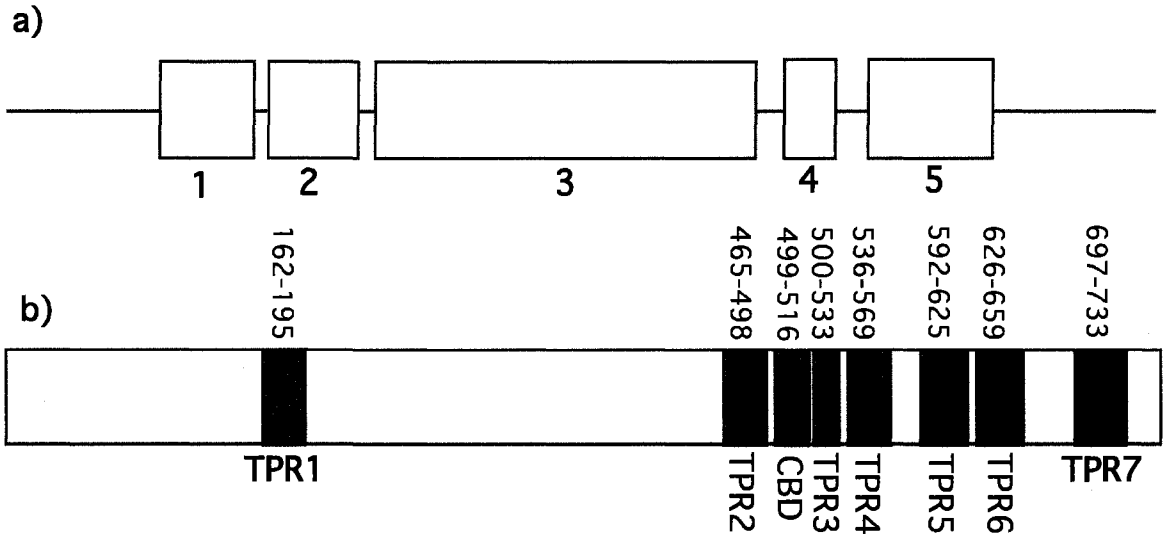


Figure 1.12. Gene structure and domain organization of *NPGR2*.
a) *NPGR2* has five exons. Lines indicate introns and boxes indicate exons. b) Diagam of NPGR2. Five TPR domains were identified by SMART and putative CaM-binding domain (CBD) was identified by comparing with MPCBP CBD sequence.

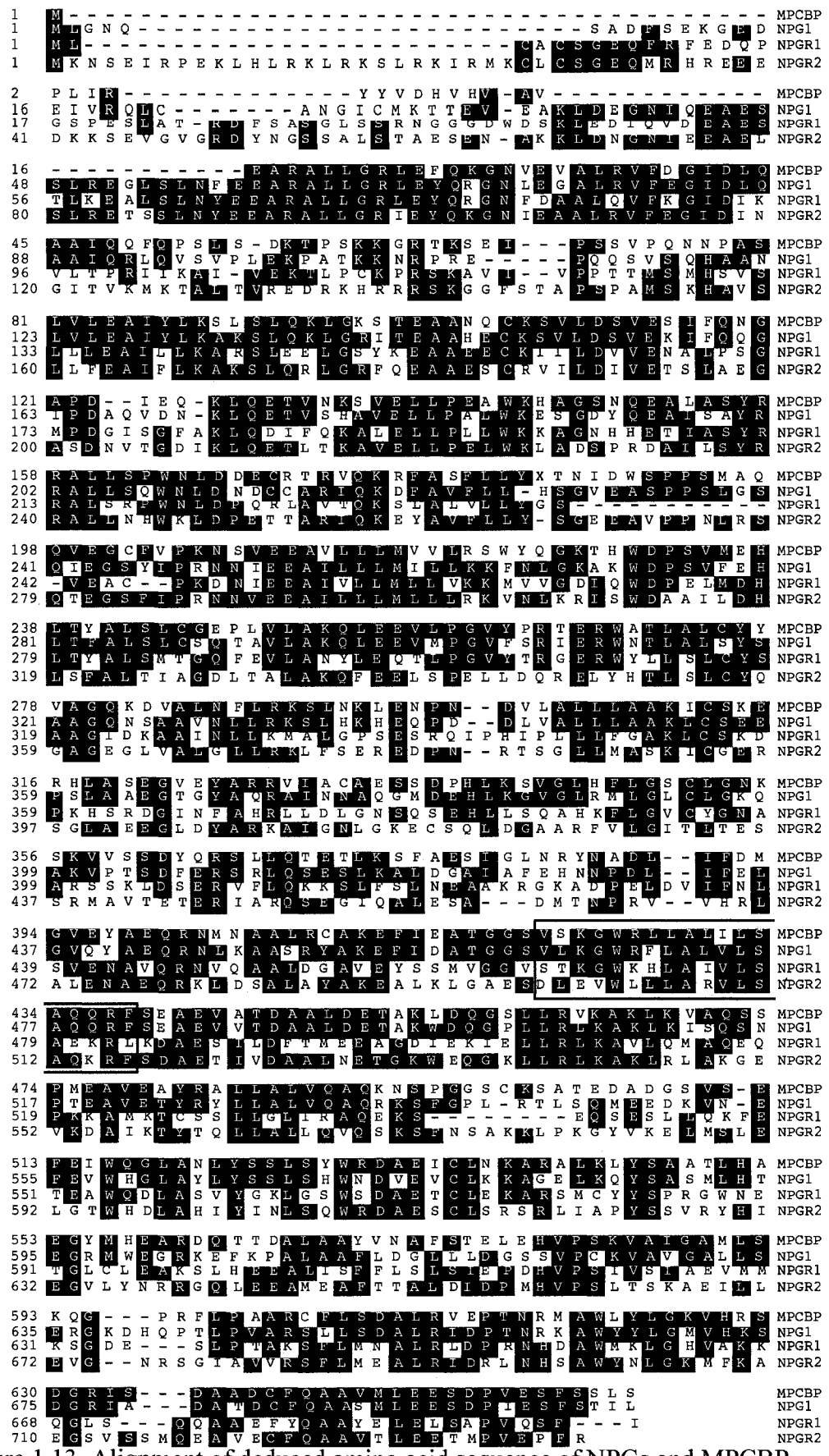


Figure 1.13. Alignment of deduced amino acid sequence of NPGs and MPCBP. Identical amino acid are indicated by reverse lettering. Boxed area indicates the conserved CaM-binding doamin (CBD).

and 56-70% similarity. AtNPGs have 5 - 7 TPRs and the second TPR overlaps with the putative CaM binding domain (Golovkin and Reddy, 2003). Beside Maize and Arabidopsis, the homologs of AtNPGs were identified in *Vitis vinifera* and *Oryza sativa.* In *Oryza sativa,* there are five AtNPG-like proteins and they showed 40-66 % identity and 58-81 % similarity (Appendices 1 and 2). There are no homologues of the AtNPGs in any non-plant systems, such as yeast, *Drosophila melanogaster, Caenorhabditis elegans*, and humans. Therefore, AtNPGs might represent a small plant-specific CaM-binding protein family.

Although *AtNPG1,* like ZmMPCBP, showed pollen-specific expression, *AtNPGR1* and *AtNPGR2* mRNA were found in other tissues also, such as suspension culture cells, pollen, flowers, and fruits (Figure 1.14) (Golovkin and Reddy, 2003). To analyze the function of AtNPG1, a TDNA-insertion mutant in *AtNPG1* was isolated in a reverse genetic screening. Mutant *atnpg1* pollen can develop normally (Golovkin and Reddy, 2003), but segregation studies with this mutant have revealed that the mutant allele is not transmitted through pollen. A cross between *AtNPG1/atnpg1* and a male sterile mutant (*cer6-2*), confirmed that the *atnpg1* allele is not transmitted through pollen. With light microscopy, it was found that pollen development in the *AtNPG1/atnpg1* plant is normal. Analysis of pollen from *AtNPG1/atnpg1* in a *quartet* background has further confirmed that pollen development is normal in the *AtNPG1/atnpg1* heterozygote. *In vitro* germination of *quartet* pollen from *AtNPG1/atnpg1* indicated that the pollen containing the *atnpg1* allele does not germinate. To study *AtNPG1* expression, transgenic plants containing GFP fused to the *AtNPG1* promoter were used (Figure 1.15) and the reporter gene was expressed only in mature and germinating pollen. To

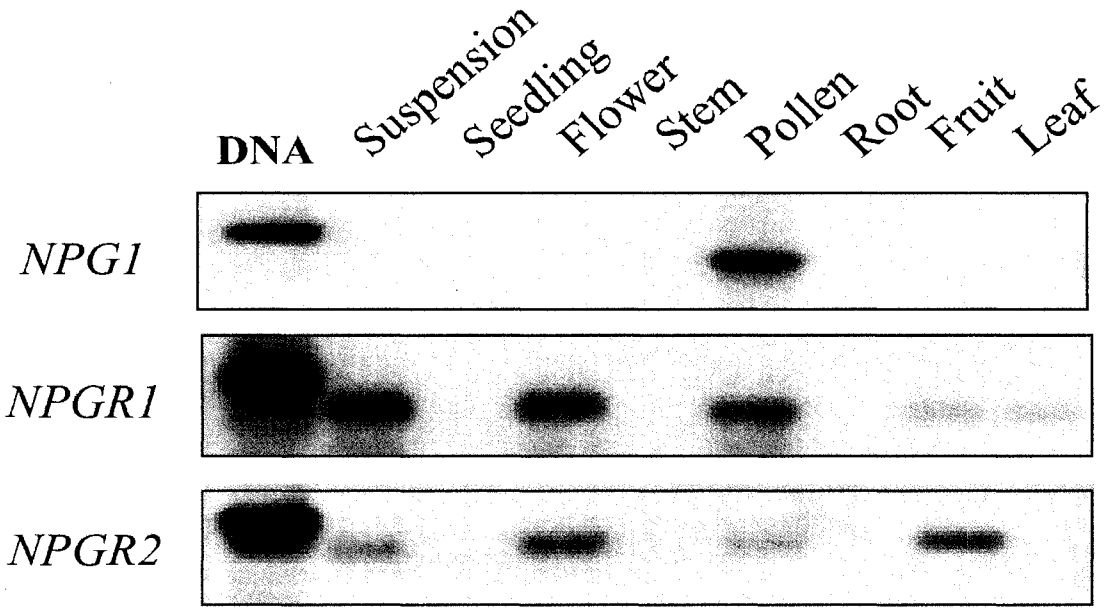


Figure 1.14. Expression analysis of *NPGs* by RT-PCR. Each gene transcript was amplified with gene specific primers. The amplified product was analyzed by electrophoresis, boltting and hybridization with labeled cDNA. Although all *NPGs* are expressed in pollen, only NPG1 is pollen-specific.
(from Golovkin M and Reddy ASN (2003) Proc. Natl. Acad. Sci USA 100:10558-10563).

determine the localization of AtNPG1, transgenic plants expressing AtNPG1 fused to GFP under the control of the *AtNPG1* promoter were used (Figure 1.15). Localization of GFP-AtNPG1 during different stages of pollen germination revealed uniform cytosolic distribution of GFP along the growing pollen tube (Figure 1.15). The presence of intact GFP-AtNPG1 fusion protein in pollen tube was confirmed by western blot (Figure 1.16). The cDNAs encoding the two AtNPG1-related proteins (AtNPGR1 and AtNPGR2) were cloned by RT-PCR using Arabidopsis pollen cDNA as a template. Using bacterially expressed protein and *in vitro* interaction assays, it was shown that all three proteins bind calmodulin in a calcium-dependent manner (Figure 1.17).

To further understand the calcium-calmodulin signaling pathway in pollen function, I focused my studies on AtNPG1 and AtNPGRs. Specifically, my research was focused on: i) Characterizing the interaction between AtNPG1 and AtNPGRs, ii) Identifying AtNPG1 interacting proteins, iii) Characterizing AtNPG1 interacting proteins by biochemical and molecular approaches, and iv) Determining the role of AtNPGR1 in plant development using a loss of function mutant.

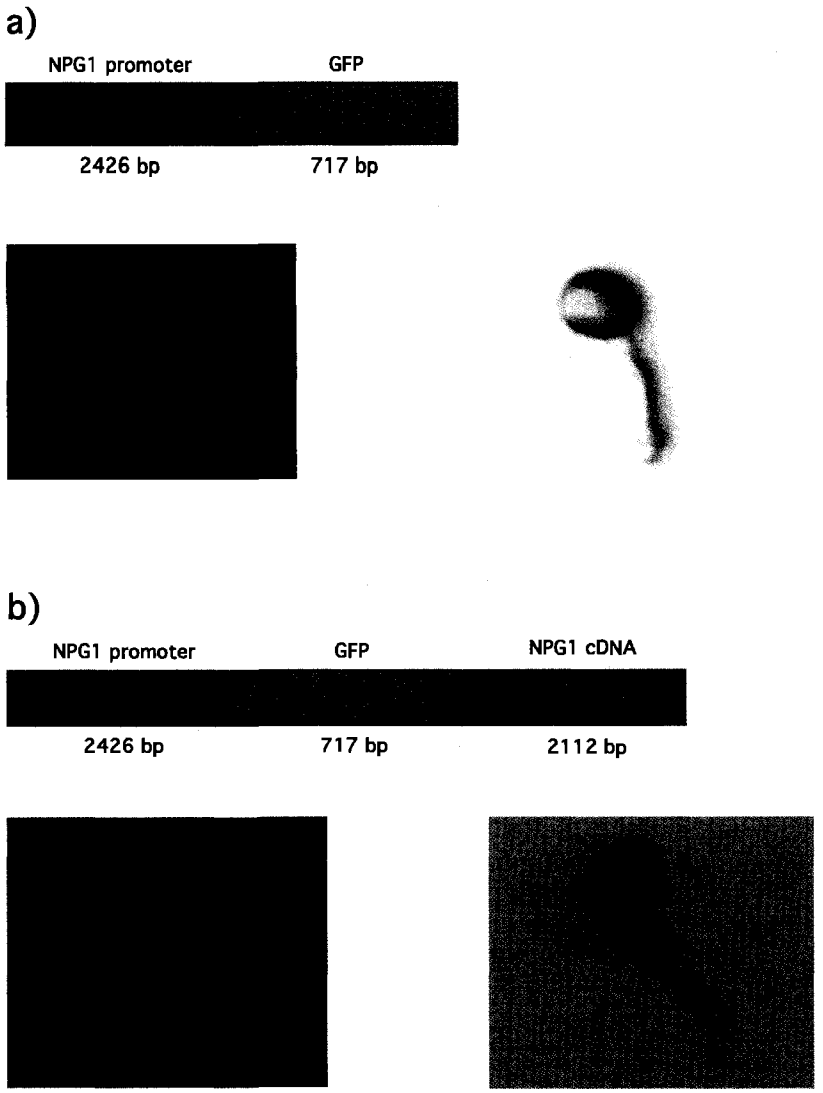


Figure 1.15. Localization of NPG1. a) Schematic diagrams of *NPG1* promoter-GFP and Fluorescence and bright fild picture of geminating pollen from transgenic plant. *NPG1* promoter-GFP fusion construct has 2.4kb *NPG1* promoter and GFP (green fluorescent protein) reporter. b) Schematic diagrams of *NPG1* promoter -GFP-*NPG1* fusions and confocal micrograph of GFP-NPG1 fusion protein in pollen and pollen tube of transgenic plant. *NPG1* promoter-GFP -*NPG1* cDNA fusion construct is a fusion of NPG1promter-GFP and NPG1 cDNA . GFP-NPG1 fusion protein localizes uniformally in pollen grain and pollen tube.

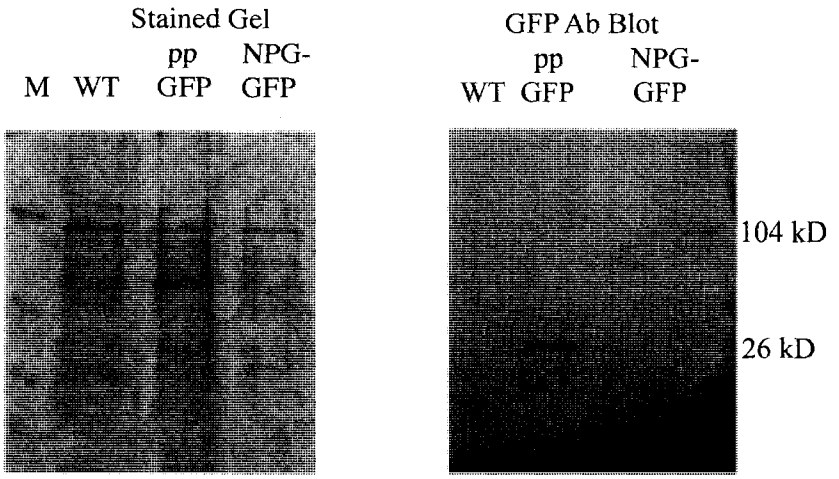


Figure 1.16. Immunodetection of GFP in *NPG1* promoter-GFP (ppGFP) plant pollen and GFP-NPG1 fusion protein from *NPG1*promoter-GFP-*NPG1* (NPG-GFP)plant pollen. Anti-GFP antibody detected 26 kD GFP and 104 kD GFP-NPG1 fusion protein. WT (Wild Type)

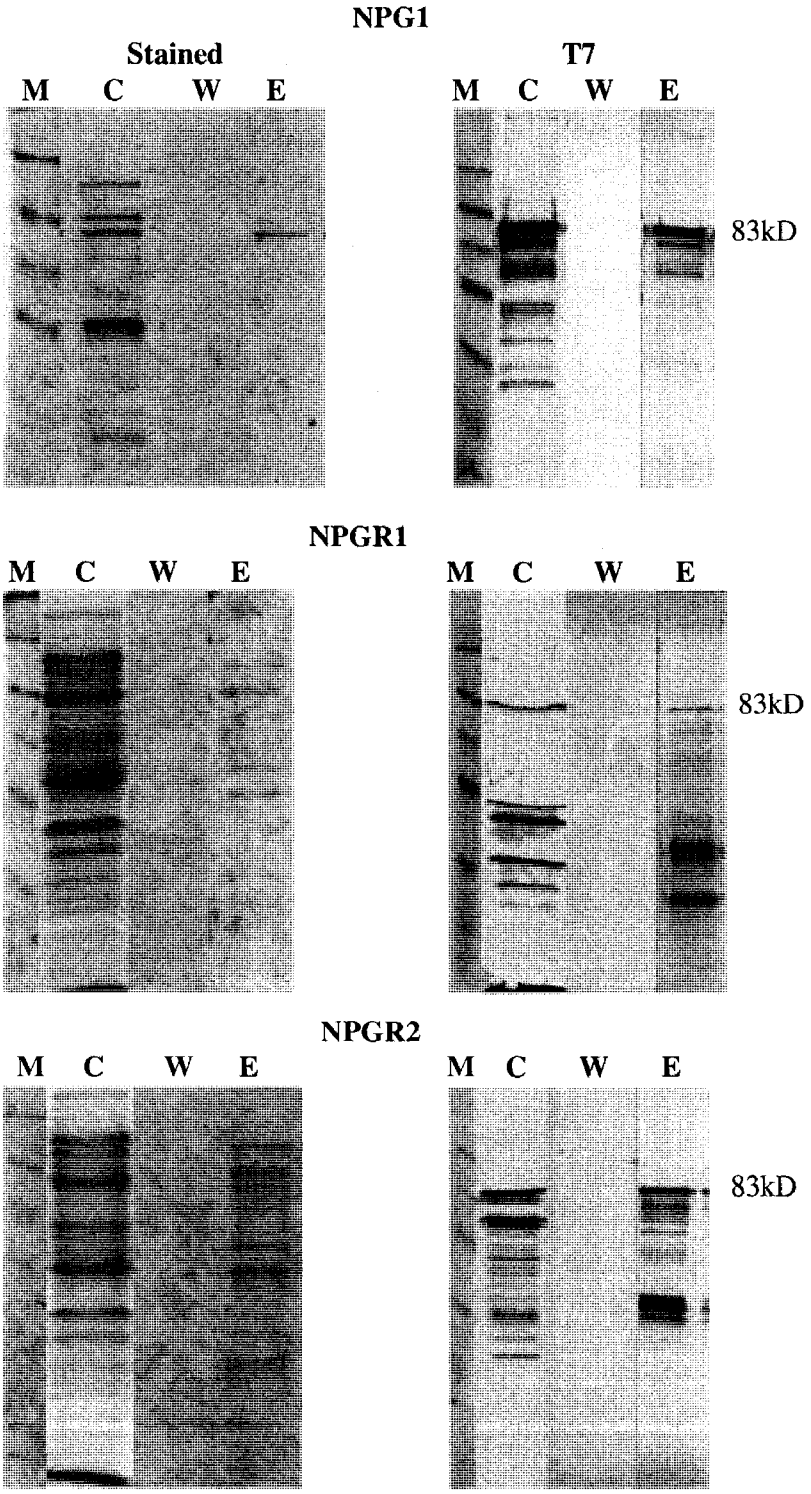


Figure 1.17. NPGs purification with CaM sepharose beads. Bacterially expressed full-length NPGs were applied to CaM-sepharose 4B affinity column. Crude extract and washing buffer contained calcium and elution buffer contained EGTA. NPG (83kD) protein was detected by T7tag antibody. C, crude extract; W, final wash fraction; E, elution.

CHAPTER 2

Analysis of interactions among AtNPG proteins and identification of AtNPG1 interacting proteins

Abstract

Tetratrico peptide repeats (TPRs) are 34 amino acid long helical structures known to be involved in protein-protein interactions. All three AtNPGs contain 5 to 7 TPR domains, suggesting that AtNPGs may form homo- or hetero- dimmers. cDNAs of *AtNPG1, AtNPGR1* and *AtNPGR2* were fused to coding regions of the yeast *GAL4* DNA binding domain (BD) or activation domain (AD). These constructs were used to study the interaction among AtNPGs using the yeast-two hybrid (Y2H) system. AtNPG1-BD interacted with itself-AD, AtNPGR1-AD and AtNPGR2-AD. AtNPGR1-BD interacted with itself-AD, AtNPG1-AD and AtNPGR2-AD. However, AtNPGR2-BD did not interact with AtNPG1-AD or AtNPGR1-AD and showed a very weak interaction with itself. To gain insights into the mechanisms by which AtNPG1 functions, I used AtNPG1 as a bait in an Y2H screen to isolate its interacting proteins from a petunia pollen library. This screen resulted in isolation of pectate lyase-like proteins as AtNPG1-interacting proteins. Using *in vivo* (Y2H) and *in vitro* protein-protein interaction assays, I have shown that AtNPGs interacted with four Arabidopsis pectate lyase like (PLL) proteins that showed high similarity to petunia PLLs. To study the role of TPR domains in these protein-protein interaction, truncated AtNPG1 lacking the TPR1 domains was used to test for interaction with AtNPG1 interacting partners. The truncated AtNPG1 either did not interact with most AtPLLs or drastically decreased its interaction with some PLLs, suggesting that the TPR 1 domain is essential for this interaction.

Introduction

Tetratrico peptide repeat (TPR) is a helical structure composed of a degenerate 34 amino acid sequence that is involved in protein-protein interaction (Blatch and Lassle, 1999). TPRs were first discovered in a cell division cycle (CDC) protein in yeast that acted as a protein interacting module (Hirano et al., 1990; Sikorski et al., 1990). The proteins containing TPR domains often have other protein-protein interaction motifs, such as J domains and SH2-binding domains (Blatch and Lassle, 1999). The proteins containing TPR domains have been discovered in various organisms from bacteria to humans and in various sub-cellular locations, such as the cytosol, nuclei, mitochondria, and peroxisomes (Blatch and Lassle, 1999). The number and position of TPR motifs vary in different proteins, and which there is evolutionary conservation in the function of TPR motifs, there are some exceptions (Das et al., 1998; Schlegel et al., 2007).

The degenerate 34 amino acids of the TPR has a conserved pattern in terms of amino acid size, hydrophobicity, and spacing (Sikorski et al., 1990), especially at eight positions, 4 (W/L/F), 7 (L/I/M), 8 (G/A/S), 11 (Y/L/F), 20 (A/S/E), 24 (F/Y/L), 27 (A/S/L), and 32 (P/K/E) (Sikorski et al., 1990). Even functionally different TPR motifs share the most conserved 4 amino acids at position 8, 20, 24, and 27 (Blatch and Lassle, 1999). One TPR motif forms two alpha-helices, called helix domains A and B, and these helices are antiparallel and amphipathic (Hirano et al., 1990; Sikorski et al., 1990). Most TPR-containing proteins have more than one TPR motif, and these TPR motifs form a tandem array that creates a right-handed super-helix (Das et al., 1998).

It has been shown that most proteins containing TPR motifs are in multi-protein complexes and might have an essential role in cell cycle, transcription, and protein

transport complexes (Blatch and Lassle, 1999). In a molecular chaperone complex, Co-chaperone STI1 interacts with Hsp70 and Hsp90, and its TPR motifs are essential for this assembly (Smith et al., 1993; Chang and Lindquist, 1994; Chen et al., 1996; Lassle et al., 1997). In an anaphase promoting complex (APC), there are proteins containing TPR motifs, such as CDC16, CDC23, and CDC27 (Lamb et al., 1994). When the TPR motif was mutated, these proteins showed reduced ability to interact, and the APC function in the cell cycle was disrupted (Lamb et al., 1994). TPR motifs are also involved in protein-protein interaction in the transcription repression complex (Smith et al., 1995; Tzamarias and Struhl, 1995) and protein import receptor complex in peroxisomes and mitochondria (Riezman et al., 1983; Dodt et al., 1995).

The presence of 5 to 7 TPR motifs in all three AtNPGs suggests that they may form homo- or hetero- dimers. To study the interaction among AtNPGs, *AtNPGs* were cloned into a yeast expression vector, and the yeast two-hybrid method was used. Although genetic studies indicate an essential role for AtNPG1 in pollen germination and tube growth, the mechanism by which AtNPG1 regulates this process is not known. To gain some insights into the role of AtNPG1, I used AtNPG1 as bait in a yeast two hybrid (Y2H) screen to isolate interacting proteins from petunia pollen library. This screen resulted in isolation of interacting pectate lyase-like proteins. Using *in vivo* (Y2H) and *in vitro* protein-protein interaction assays, I showed that AtNPGs interacted with four Arabidopsis pectate lyase like (PLL) proteins that showed the highest similarity to petunia clones. To study the role of TPR domains in these protein-protein interactions, truncated AtNPG1 proteins lacking the TPR1 domain was used to test interaction of AtNPG1 with its interacting partners including AtNPGs, two petunia clones (#5 and #30),

and four AtPLLs. Without the TPR1 domain, truncated AtNPG1 did not interact with most of partners or drastically decreased interaction with some partners. These results indicate that AtNPG1 interacts with AtPLLs and that the TPR1 domain is essential for this interaction.

Materials and Methods

Cloning of *AtNPG1* into yeast two hybrid vectors

The *AtNPG1* gene was cloned into two yeast expression vectors to test the interaction with itself and for screening the petunia pollen library. For ligation with pACT2J plasmid, the *Nde*I-*Xho*I fragment from the *AtNPG1*-pET28a clone was prepared and inserted into pACT2J that was digested with the same enzymes. *AtNPG1*-pET28a and pACT2J plasmids were digested with *Nde*I. The digested fragments were isolated by agarose gel electrophoresis and a QIAgen gel extraction kit. The *Nde*I digested DNA was digested at 37°C overnight with 30U of *Xho*I before it was used for ligation. In the ligation mixture consisted of about 400-500 ng of the *Nde*I-*NPG1*-*Xho*I fragment, about 500-600 ng of the linearized pACT2J plasmid, 1 μl of T4 ligase (5 U/μl) and 4 μl 5X T4 ligase buffer (with ATP). The ligated plasmid was transformed into DH5α *E. coli* electrocompetent cells, and the colonies were grown on an LB/ampicillin plate. Colony hybridization with the DIG-labeled *AtNPG1* probe using the DIG DNA labeling and detection kit from Roche was used to identify positive clones. For colony hybridization, colonies were transferred to Hybond nylon membranes. Lysis, denaturation and neutralization were performed as follows. The membranes were pre-treated in 20 ml of hybridization solution (5X SSC, 0.1% N-Lauroylsarcosine, 0.02% SDS, 1% Blocking reagent) at 55°C for 6 hours with shaking. DIG-labeled *AtNPG1* probe was prepared by PCR. The 50 μl PCR reaction mixture included 2 μl of *AtNPG1* 5' and 3'-end primers (50 ng/μl), 2 μl of *NPG1*–pET28a template (20 ng/μl), 1 μl of Taq polymerase (1 U/μl), 5 μl of 5X Taq polymerase buffer (without $MgCl_2$), 3 μl of $MgCl_2$, 10 μl of dNTP mixture (including DIG-11-dUTP) and 27 μl of distilled water. The mixture was preheated at

94°C for 2 min, and then 30 cycles of amplification were performed. Each amplification cycle consisted of denaturation at 94°C for 30 sec, annealing at 48°C for 30 sec and extension at 72°C for 1 min. A final extension was done at 72°C for 10 min. The PCR amplified DIG-labeled *AtNPG1* DNA probe was denatured in boiling water for 5 min and rapidly cooled on ice and water mixture. The membrane was hybridized with the DIG-labeled *AtNPG1* DNA probe at 55°C overnight with shaking. The membrane was washed twice with 2X SSC and 0.1% SDS solution at room temperature for 5 min each. The filter was washed another two times with the 0.5X SSC and 0.1% SDS solution at 55°C for 5 min each. The membrane was rinsed with the washing buffer (0.1 M Maleic acid, 0.15 M NaCl, pH 7.5, 0.03% Tween 20) for 3 min. The membrane was incubated with the blocking solution (0.01 M Maleic acid, 0.015 M NaCl, pH 7.5, 1% block reagent from kit) for 30 min and then incubated with the antibody solution [(1:5,000 dilution anti DIG (digoxigenin)-AP (alkaline phosphatase) conjugated antibody in the blocking solution)] for 30 min. The membrane was washed with the washing buffer twice for 15 min each and presoaked with AP buffer (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$). The membrane was placed in the color development solution - 10 ml AP buffer, 45 μl NBT (nitroblue tetrazolium chloride, 75 mg/ml) and 30 μl BCIP (5-bromo-4-chloro-3-indolylphosphate p-toluidine salt, 50 mg/ml). DNA from positive colonies was prepared and analyzed by *Nde*I or *Nde*I/*Xho*I restriction enzyme digestion. The positive clones were verified by sequencing.

For ligation with the pAS1-CYH2 plasmid, the *Nde*I-*Sal*I fragment from *AtNPG1*-pET28a clone was prepared, and the *AtNPG1* fragment was inserted into the pAS1-CYH2 plasmid that was digested with the same enzymes. The pAS1-CYH2

plasmid was digested first with the *Nde*I and isolated by agarose gel electrophoresis and the QIAgen gel extraction kit. The *Nde*I cut fragment was digested with *Sal*I enzyme and isolated by agarose gel electrophoresis and a QIAgen gel extraction kit. The enzyme digestion and the ligation were done in the same manner described above. To confirm *AtNPG1*-pAS1-CYH2 construct, colony hybridization and enzyme digestion mapping with *Eco*RI enzyme were used. The positive clones were confirmed by sequencing.

Cloning of *AtNPGRs* into yeast two hybrid vectors

For *AtNPGR1* cloning into pAS1-CYH2 and pACT2J, 10 μg of *AtNPGR1*-pET28a was digested with *Nhe*I enzyme overnight and 5μg of pAS1-CYH2 and 5μg of pACT2J were digested with *Nde*I enzyme. These enzyme-digested fragments and vectors were isolated using the QIAgen gel extraction kit. The ends were filled to generate blunt ends. To make blunt ends, the enzyme reaction mixture containing 25 μl of the DNA fragment in water, 3 μl of buffer M from Roche, 1 μl of 50X dNTP (PCR grade), 1 μl of klenow enzyme (2 U/μl) was incubated at 37°C for 1 hour, and the reaction was stopped by heating the sample at 65°C for 10 min. The blunt end fragments and vectors were purified by the QIAgen minprep kit and used for the second digestion with *Sal*I (*AtNPGR1*, pAS1-CHY2) or *Xho*I (pACT2J). The fragments for ligation were isolated by agarose gel electrophoresis and the QIAgen gel extraction kit. The fragments, blunt-*AtNPGR1-Sal*I, were inserted into blunt-pAS1-CYH2-*Sal*I or blunt-pACT2J–*Xho*I plasmid. In the ligation, about 400-500 ng of insert, about 500-600 ng of the plasmid, 1 μl of T4 ligase (5 U/μl) and 4 μl 5X T4 ligase buffer (with ATP) were used. The ligation mixture was transformed into DH5α *E. coli* electrocompetent cells, and the colonies grown on LB/ampicillin plates. To confirm the construct, colony hybridization was done

with a DIG-labeled *AtNPGR1* probe as described above. *Hin*dIII digestion mapping was used to verify the *AtNPGR1*- pACT2J construct, and *Bam*HI digestion mapping was used to verify the *AtNPGR1*- pAS1-CYH2 construct. The positive constructs were verified by sequencing.

For *AtNPGR2* cloning into pAS1-CYH2 and pACT2J, 10 μg of *AtNPGR2*-pET28b, 5 μg of pAS1-CYH2 and 5 μg of pACT2J were digested with *Nco*I enzyme at 37°C overnight. The digested fragments were purified with the QIAgen miniprep kit. The *Nco*I digested *AtNPGR2*-pET28a and the pAS1-CYH2 fragments were digested with *Sal*I enzyme, and the pACT2J fragment was digested with *Xho*I. These 5'*Nco*I–*AtNPGR2-Sal*I3' fragments were inserted into 5'*Nco*I – pAS1-CYH2-*Sal*I3' or 5'*Nco*I–pACT2J-*Xho*I3' using the same ligation method described above. The ligation mixture was transformed into DH5α *E. coli* electrocompetent cells, and the colonies were grown on LB/ampicillin plates. To confirm the constructs, *Nde*I digestion mapping was used for both constructs. The positive constructs were sequenced and confirmed by BLAST searches against the Arabidopsis genome data bank.

Analysis of interaction among AtNPGs

To determine the interaction among the AtNPGs, the combinations shown below were tested.

1) *AtNPG1*-pAS1-CYH2 (BD) + *AtNPG1*-pACT2J (AD)

2) *AtNPG1*-pAS1-CYH2 (BD) + *AtNPGR1*-pACT2J (AD)

3) *AtNPG1*-pAS1-CYH2 (BD) + *AtNPGR2*-pACT2J (AD)

4) *AtNPGR1*-pAS1-CYH2 (BD) + *AtNPG1*-pACT2J (AD)

5) *AtNPGR1*-pAS1-CYH2 (BD) + *AtNPGR1*-pACT2J (AD)

6) *AtNPGR1*-pAS1-CYH2 (BD) + *AtNPGR2*-pACT2J (AD)

7) AtNPGR2-pAS1-CYH2 (BD) + *AtNPG1*-pACT2J (AD)

8) AtNPGR2-pAS1-CYH2 (BD) + *AtNPGR1*-pACT2J (AD)

9) AtNPGR2-pAS1-CYH2 (BD) + *AtNPGR2*-pACT2J (AD)

The constructs with binding domain (BD) fusions were introduced into Y190 yeast cells. The yeast cells with the BD construct grown on Trp-SD plates were used as competent cells for transformation with the construct containing the activation domain (AD). The yeast cells with both BD and AD constructs grew on Trp-, Leu- SD plate and the colonies on Trp-, Leu- SD plate were transferred to Trp-, Leu-, His- and 25mM AT SD plate to determine the interaction between the proteins fused to BD and AD.

To confirm the interaction between proteins in the BD and AD construct, the β-galactosidase filter and liquid culture assays were done for each combination. In the β-galactosidase filter assay, Whatman #5 or VWR grade 410 filter paper was soaked with Z buffer/X-gal solution and placed on the plates to transfer yeast cells. Z buffer solution was made by mixing 16.1 g $Na_2HPO_47H_2O$, 5.5 g $NaH_2PO_4H_2O$, 0.75 g KCl, 0.246 g $MgSO_47H_2O$, pH 7.0 with enough water to make a liter. Z buffer/X-gal solution contained 0.135 ml β-mercaptoethanol, 0.5 ml X-gal (100 mg/ml) and 50 ml Z buffer. The filter was lifted and transferred into a pool of liquid nitrogen, submerged for 10 sec. The filter was placed on another presoaked filter paper and incubated at room temperature. The Y190 yeast cells with no constructs and the Y190 yeast cells with the BD or AD construct were used as negative controls.

Liquid culture β-galactosidase assay with CPRG (Chlorophenol red-β-D-galactopyranoside) as substrate was used for studying the interaction among NPGs. Each

of the yeast colonies was inoculated into 5 ml Trp-, Leu-, SD medium and the cultures were grown overnight at 30°C with 250 rpm shaking. Two ml of the overnight culture was inoculated into 8 ml YPD medium, and the cultures were grown for 5 hours at 30°C with 250 rpm shaking, until an OD_{600} of 1 ml = 0.5-0.8. Then each culture was aliquoted into three 1.5 ml tubes and spun down at 14,000 rpm for 30 sec. The pellet was resuspended in 1.0 ml of buffer 1 (2.38 g HEPES, 0.9 g NaCl, 0.06 5g hemimagnesium salt of L-aspartate, 1.0 g BSA, 50.0 μl Tween 20, pH 7.3 in 100 ml sterile water) and was spun at 14,000 rpm for 30 sec. The medium was removed and the pellet was resuspended in residual liquid that was about 100 μl of buffer 1. The tube was frozen in liquid nitrogen and thawed at 37°C for 1 min. This freezing/thawing process was repeated once. To this, 900 μl of buffer 2 [0.02709 g Chlorophenol red-β-D-galactopyranoside (CPRG) in 20 ml of buffer 1] was added and vortexed. The suspension was spun down at 14,000 rpm for 1 min, and supernatant was decanted into a cuvet. The supernatant was incubated at room temperature for 60 min, and then 0.5 ml of 3 mM $ZnCl_2$ was added to stop the color development reaction. The sample was measured for its absorbance at 578 nm using a mixture of 1ml of buffer 1 and 0.5 ml of 3 mM $ZnCl_2$, as a blank. The β-galactosidase units were calculated as follows:

$$\text{Units} = (1000 \times OD_{578})/(\text{elapsed min} \times 1.5\text{ml culture} \times OD_{600})$$

The suspensions from the Y190 yeast cells with no plasmids and the Y190 yeast cells with the BD or AD constructs were used as negative controls. The suspension from the Y190 yeast cells with *AtNPG1*-pAS1-CYH2 and clone *#5/#30* (petunia pectin lyase)-pGAD424 from the petunia library screening were used as positive controls.

Screening petunia yeast two hybrid library

The petunia yeast two-hybrid library in pGAD424 was kindly provided by Teh-Hui Kao (Penn. State Univ). Screening of the petunia pollen library was done using *AtNPG1*-pAS1-CYH2 as "bait". The *AtNPG1*-pAS1-CYH2 construct was transformed into Y190 yeast cells. The Y190 cells were grown at 30°C for 18 hours with 250 rpm shaking. About 50 ml culture was transferred to 300 ml YPD medium and grown at 30°C for 3 hours. The cells were then collected by centrifugation at 1,000xg for 5 min at room temperature. The cell pellet was resuspended in 30 ml of distilled water and collected by centrifugation at 1,000xg for 5 min at room temperature. The cell pellet was resuspended in 1.5 ml of sterile 1X TE/LiAc solution. The yeast transformation mixture contained 0.1 μg of the *AtNPG1*-pAS1-CYH2 construct, 0.1 mg salmon sperm carrier DNA, and 0.1 ml of the yeast competent cells in 1X TE/LiAc solution. 0.6 ml of 1X PEG/LiAc solution (40% PEG 4000, 1X TE, 1X LiAc) was added to the transformation mixture and mixed well with vortexing. The mixture was incubated at 30°C for 30 min with 200 rpm shaking and 70 μl of DMSO was added and mixed with gentle shaking. Then the mixture was incubated in a 42°C waterbath for 15 min for heat shock and chilled on ice for 2 min. The transformed yeast cells were collected by centrifugation at 14,000 rpm for 5 sec and resuspended in 0.5 ml of 1X TE solution. The Y190 yeast cells with the *AtNPG1*-pAS1-CYH2 construct grew on a Trp- SD plate at 30°C. For petunia library transformation, Y190 yeast cells with *AtNPG1*-pAS1-CYH2 were used as competent cells. Y190 yeast cells with *AtNPG1*-pAS1-CYH2 in 150 ml Trp- SD medium were grown at 30°C for 18 hours with 250 rpm shaking. The culture was then transferred to

1000 ml YPD medium and incubated at 30°C for 3 hours with 250 rpm shaking. The yeast cells were placed in two 250 ml tubes and collected by centrifugation at 1,000g for 5 min at room temperature. The cell pellet was resuspended in 200 ml of distilled water for each 250 ml tube and collected by centrifugation at 1,000g for 5 min at room temperature. Finally the cell pellet was resuspended in 8 ml sterile 1X TE/LiAc solution. The yeast transformation mixture contained 0.5 mg of the petunia pollen library, 20 mg salmon sperm carrier DNA and 8 ml yeast competent cells in 1X TE/LiAc solution. 60 ml of 1X PEG/LiAc solution (40% PEG 4000, 1X TE, 1X LiAc) was added to the transformation mixture and mixed well by vortexing. The mixture was incubated at 30°C for 30 min with shaking (200 rpm), and 7 ml of DMSO was added and mixed with gentle shaking. The cells were incubated in a 42°C water bath for 15 min and chilled on ice for 2 min. The cells were collected by centrifugation at 1,000xg for 5 min and resuspended in 10 ml of 1X TE solution and plated on Trp-and Leu- SD plates.

The yeast cell colonies that grew on Trp-and Leu- SD plate were transferred to Trp-, Leu-, His- and 25mM AT (3-aminotrizole) SD plates. The colonies that grew on Trp-, Leu-, His- and 25mM AT (3-aminotrizole) SD plates were duplicated and one of the duplicated plates was used for a β-galactosidase filter assay. In the β-galactosidase filter assay, Whatman #5 or VWR grade 410 filter paper was soaked with Z buffer/X-gal solution and placed on the plates to transfer yeast cells. Z buffer solution was made by mixing 16.1 g/L $Na_2HPO_47H_2O$, 5.5 g/L $NaH_2PO_4H_2O$, 0.75 g/L KCl, 0.246 g/L $MgSO_47H_2O$, pH 7.0. Z buffer/X-gal solution contained 0.135 ml β-mercaptoethanol, 0.5 ml X-gal (100 mg/ml) and 50 ml Z buffer. The filter was lifted and transferred into a pool of liquid nitrogen, submerged for 10 sec. The filter was placed on another

presoaked filter paper and incubated at room temperature.

The blue colonies from the β-galactosidase filter assay were selected and grown in Trp-and Leu- SD medium at 30°C overnight with 250 rpm shaking. The cells were aliquoted into 1.5ml tubes and spun down at 600g for 2 min. The cell pellet was suspended in 150 μl of solution I (1.2 M sorbitol, 1 mM EDTA, 20 mM Tris pH8.0) and 2 μl of Zymolyase. The mixture was incubated at 37°C for 15-60 min. One hundred fifty μl of solution II (0.2 M NaOH, 1% SDS) was added to the mixture and mixed well, and then 150 μl of solution III (3 M KoAc pH 5.7) was added and mixed. The mixture was spun down in a microfuge at maximum speed for 2 min, then the supernatant was transferred to a new tube and mixed well with 400 μl of isopropanol. Finally, DNA was collected from the mixture by centrifugation at maximum speed for 8 min. The supernatant was removed and the DNA pellet was suspended in 35 μl of water.

The DNA from the yeast cells was transformed into DH5α *E. coli* electro-competent cells and the transformed DH5α cells were grown on LB/ampicillin plates. The colonies on LB/ampicillin plates were inoculated into 10 ml of LB/ampicillin liquid medium and grown at 37°C overnight. The DNA was collected from the overnight culture using a QIAgen miniprep kit, then sequenced and analyzed.

Cloning of 4 Arabidopsis *PLL (pectate lyase-like)* genes into pACT2J

For *At1g14420* and *At2g02720* cloning into pACT2J, 10 μg of *At1g14420* or *At2g02720* in pGEM-T and 5 μg of pACT2J were digested with *Nde*I enzyme at 37°C overnight and run on an agarose gel. A QIAgen gel extraction kit was used to purify the digested fragments. The fragments were then digested with *EcoR*I. The 5' *Nde*I - *At1g14420- EcoR*I3' or 5' *Nde*I *-At2g02720- EcoR*I3' fragments were inserted into

5'*Nde*I–pACT2J-*EcoR*I3' using the same ligation method described above. The ligation mixture was transformed into DH5α *E. coli* electro competent cells, and the colonies were grown on LB/ampicillin plates. To confirm the constructs, *Nde*I and *EcoR*I digestion mapping was used for both constructs. The positive constructs were sequenced and confirmed by BLAST searches against the Arabidopsis genome data bank.

For *At3g01270* and *At5g15110* cloning into pACT2J, 10 μg of *At3g01270* or *At5g15110* in pGEM-T and 5 μg of pACT2J were digested with *Xma*I enzyme and then digested with *Xho*I. The 5'*Xma*I -*At3g01270*- *Xho*I3'or 5'*Xma*I -*At5g15110* - *Xho*I3' fragments were inserted into 5'*Xma*I–pACT2J-*Xho*I3' using the same ligation method described above. The ligation mixture was transformed into DH5α *E. coli* electro competent cells and the colonies were grown on LB/ampicillin plates. To confirm the constructs, *Xma*I and *Xho*I digestion mapping was used for both constructs. The positive constructs were sequenced and confirmed by BLAST searches against the Arabidopsis genome data bank.

Detection of AtPLLs expressed in yeast

For AtPLLs (At1g14420, At2g02720, At3g01270 and At5g15110) isolation, AtPLL transformed yeast were grown in 10 ml of Trp^-, Leu^-, His^- SD media overnight at 30°C. The overnight culture was used to inoculate 40 ml of YPD media, and the new culture was grown at 30°C until OD_{600} 0.5-0.8. The cell pellet was collected by spinning at 5000 rpm for 5 min at 4°C and was transferred to flat-bottom, O-ring screw cap microcentrifuge tubes. The cell pellet was resuspended in 50 μl of Laemmli buffer, and 500 μl of acid washed beads (425-600 microns) was added. The mixture was vortexed at maximum speed for 1 minute twice. The tube was spun at maximum speed for 5 min at

4°C, and the supernatant was used for SDS-PAGE gel electrophoresis.

The AtPLL protein expressed in yeast was identified by Western blot with a Gal 4 AD antibody. The supernatant was mixed with 3X sample buffer (0.1875 M Tris –HCl pH 6.8, 6% SDS, 15% mercaptoethanol, 30% glycerol, and 0.0225% bromophenol blue) and electrophoresed for 45 min at 200V on two 10% denaturing gel. One of two gels was stained with coomassie blue (0.25% Coomassie blue R250, 7.5% acetic acid, 42.5% methanol), and the other gel was blotted to a nitrocellulose membrane and probed with Gal4 AD antibody. Proteins were transferred to membranes at 100V for 75 min in a transblot buffer (25 mM Tris-HCl, 192 mM glycine, and 20% methanol). For detection with Gal4 AD antibody, the blot was incubated in 5% non-fat dry milk in TBST for 1 hour at room temperature with gentle shaking. The membrane was washed with TBST 3 times, 5 min each, and incubated for 1 hour at room temperature in TBST containing 0.4 μg/ml Gal4 AD antibody. The membrane was washed with TBST 3 times, 5 min each and incubated with 1:50 dilution goat normal serum in 5% non-fat dry milk in TBST for 30 min at room temperature. The membrane was washed with TBST 3 times, 5 min each, and incubated for 30 min at room temperature in TBST containing 1:5,000 dilution of goat anti-mouse IgG antibody alkaline phosphatases conjugate. The membrane was washed with TBST 4 times, 5 min each, and presoaked with AP buffer (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$). Then the blot was placed in color development solution (10 ml AP buffer, 45 μl NBT (nitroblue tetrazolium chloride, 75 mg/ml) and 30 μl BCIP (5-bromo-4-chloro-3-indolylphosphate p-toluidine salt, 50 mg/ml)).

Analysis of interaction between AtNPGs and AtPLLs

To analyze the interaction between AtNPGs and AtPLLs including petunia putative pectate lyase (clone #5 and #30) and four Arabidopsis putative pectate lyases (At1g14420, At2g02720, At3g01270 and At5g15110), the combinations shown below were tested.

1) *AtNPG1* (BD) + *#5* (AD)

2) *AtNPG1* (BD) + *#30* (AD)

3) *AtNPG1* (BD) + *At1g14420* (AD)

4) *AtNPG1* (BD) + *At2g02720* (AD)

5) *AtNPG1* (BD) + *At3g01270* (AD)

6) *AtNPG1* (BD) + *At5g15110* (AD)

7) *AtNPGR1* (BD) + *#5* (AD)

8) *AtNPGR1* (BD) + *#30* (AD)

9) *AtNPGR1* (BD) + *At1g14420* (AD)

10) *AtNPGR1* (BD) + *At2g02720* (AD)

11) *AtNPGR1* (BD) + *At3g01270* (AD)

12) *AtNPGR1* (BD) + *At5g15110* (AD)

13) *AtNPGR2* (BD) + *#5* (AD)

14) *AtNPGR2* (BD) + *#30* (AD)

15) *AtNPGR2* (BD) + *At1g14420* (AD)

16) *AtNPGR2* (BD) + *At2g02720* (AD)

17) *AtNPGR2* (BD) + *At3g01270* (AD)

18) *AtNPGR2* (BD) + *At5g15110* (AD)

The constructs with binding domains (BD) were introduced into Y190 yeast cells. The yeast cells with the BD constructs grew on Trp-SD plates and were used as competent cells for transformation of the constructs with activation domains (AD). The yeast cells with both the BD and AD constructs grew on Trp-, Leu- SD plates and the colonies on Trp-, Leu- SD plates were transferred to Trp-, Leu-, His- and 25mM AT SD plates to test the interaction between the proteins.

To confirm the interaction between proteins in the BD and AD construct, the β-galactosidase filter and liquid culture assays were done for each combination. The β-galactosidase filter assay was done as described earlier, and the Y190 yeast cells with no constructs and the Y190 yeast cells with the BD or AD constructs were used as a negative control. The Y190 yeast cells with *NPG1*-pAS1-CYH2 and clone *#5/#30* (petunia putative pectin lyase)-pGAD424 from petunia library screening were used as positive controls. Liquid culture β-galactosidase assay with CPRG as substrate method was used as described earlier. The suspension from the Y190 yeast cells with no construct and the Y190 yeast cell with the BD or AD construct were used as negative controls.

Cloning AtPLLs in pET32b

For cloning *At1g14420* and *At2g02720* into pET32b, *At1g14420* or *At2g02720* in pACT2J and pET32b were digested with *Bgl*II enzyme and then digested with *Eco*RI. The 5'*Bgl*II-*At1g14420-Eco*RI3' or 5'*Bgl*II-*At2g02720-Eco*RI3' fragment and *Bgl*II-pET32b-*Eco*RI fragments were used for ligation.

For cloning *At3g01270* into pET32b, *At3g01270* in pACT2J and pET32b were digested with *Bgl*II enzyme and then digested with *Xho*I. The 5'*Bgl*II-*At3g01270-Xho*I3'

fragment and *Bgl*II-pET32b-*Xho*I fragments were used for ligation.

For cloning *At5g15110* into pET32b, *At5g15110* in pACT2J and pET32b were digested with *EcoR*I enzyme and then digested with *Xho*I. The 5' *EcoR*I –*At5g15110-Xho*I3' fragment and *EcoR*I-pET32b-*Xho*I fragments were used for ligation. The ligation mixture was transformed into DH5α *E. coli* electro competent cells and the colonies grown on LB/ampicillin plates. To confirm the structure of the construct, appropriate enzyme digestion mapping was used to verify the constructs. The positive constructs were verified by sequencing.

Protein induction

The sense clones of AtPLLs-pET32b were transformed by electrophoresis to BL21 (DE3) *E. coli* cells containing a chaperone plasmid pG-KJE8. Colonies growing on LB plates containing 50 μg/ml ampicillin and 20 μg/ml chloramphenicol were used for protein induction. The BL21 (DE3) colonies containing At3g01279-pET32b/pG-KJE8 or At5g15110-pET32b/pG-KJE8 were inoculated into 5 ml LB containing ampicillin and chloramphenicol and incubated at 37 °C with shaking. The next morning, 0.5 ml overnight culture was inoculated into 50 ml LB containing 5 μg/ml tetracycline and 200 mg/ml L-arabinose and incubated at 37 °C with shaking until O.D $_{600}$ of 0.5. The culture was induced by adding 0.4 mM IPTG (isopropyl-1-thio-β-D-galactopyranoside) and incubated at 37 °C with shaking for 4 hours. The cells were collected by centrifuging at 4000g for 10 min.

AtNPG1-pET28a, AtNPGR1-pET28a and AtNPGR2-pET28b in BL21 (DE3) were used for AtNPG protein induction. The BL21 (DE3) *E. coli* cells containing AtNPG1-pET28a, AtNPGR1-pET28a or AtNPGR2-pET28b were inoculated into 5 ml

LB/kanamycin (30 μg/ml) medium and incubated at 37 °C with shaking. The next morning, 0.5 ml overnight culture was inoculated into 50 ml LB/kanamycin and incubated at 37 °C with shaking until O.D $_{600}$ of 0.5. The culture was induced by adding 0.4 mM IPTG (isopropyl-1-thio-β-D-galactopyranoside) and incubated at 37 °C with shaking for 4 hours. The cells were collected by centrifuging at 4000g for 10 min.

The cell pellets were resuspended in one-tenth of the original volume of lysis solution (200 mM Tris-HCl pH 7.5, 1.5 M NaCl, 1% Triton X-100, 1X protease inhibitor, 100 μg/ml lysozyme) and incubated at 4 °C for 30 min. Samples were sonicated 4 times for 15 sec each and centrifuged at 15,000 rpm in a Sorvall SS-34 for 30 min at 4 °C. Twenty μl supernatant (soluble) were mixed with 3X sample buffer (0.1875 M Tris-HCl pH 6.8, 6% SDS, 15% mercaptoethanol, 30% glycerol, and 0.0225% bromophenol blue). Pellets (insoluble) were mixed with 1X sample buffer and sonicated 4 times for 15 sec each. Both soluble and insoluble fractions of proteins were electrophoresed for 45 min at 200 V on three 10% denaturing gels. One gel was stained with Coomassie blue (0.25% Coomassie blue R250, 7.5% acetic acid, 42.5% methanol) and the other two gels were blotted to nitrocellulose membranes and probed with either S-protein or T7-tag antibody. Proteins were transferred to membranes at 100V for 75 min in transblot buffer (25 mM Tris-HCl, 192 mM glycine, and 20% methanol). For detection, the blots were incubated in 3% gelatin in TBST for 30 min at 30°C with gentle shaking. Membranes were washed with TBST (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1% Tween 20) 3 times, 5 min each, and incubated for 30 min at 30 °C in TBST containing 1% gelatin, and 1:10,000 dilution of T7-tag antibody alkaline phosphatase conjugate or 1:5,000 dilution of S protein alkaline phosphatase conjugate. Membranes were washed with TBST 3 times, 5

min each, and presoaked with AP buffer (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$). Then the blot was placed in color development solution (10 mM AP buffer, 45 μl NBT (nitroblue tetrazolium chloride, 75 mg/ml) and 30 μl BCIP (5-bromo-4-chloro-3-indolylphosphate p-toluidine salt, 50 mg/ml).

Pull down assay

Bacterially expressed soluble fractions of AtPLL10 (At3g01270) or AtPLL11 (At5g15110) were incubated with 200 μl of S-protein agarose beads at 4°C for 3 hours. After incubation, the mixtures were centrifuged 500g for 10 min at 4°C and the supernatant was removed. The beads were resuspended in 1.5 ml of bind/wash buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Triton X-100) and incubated at 4°C for 10 min. The beads were centrifuged at 500g for 10 min at 4°C, and the supernatant was removed. Bacterially expressed soluble fractions of AtNPGs were incubated with the beads at 4°C for 3 hours. The combination of incubation is below:

1) AtPLL10 – AtNPG1

2) AtPLL10 – AtNPGR1

3) AtPLL10 – AtNPGR2

4) AtPLL11 – AtNPG1

5) AtPLL11 – AtNPGR1

6) AtPLL11 – AtNPGR2

After incubation, the mixtures were centrifuged at 500g for 10 min at 4°C and the supernatant was removed. The beads were resuspended in 1.5 ml of bind/wash buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Triton X-100) and incubated at 4°C for 10 min. The beads were centrifuged at 500g for 10 min at 4°C, and the supernatant was

removed. Seventy-five micro liter of 1X SDS loading buffer were added to beads and incubated at boiling temperature for 5 min. The mixtures were centrifuged at maximum speed for 5 min, and the supernatant was electrophoresed for 45 min at 200V on three 10% denaturing gels. The gels were blotted, probed and detected as mentioned above.

AtNPG1 bacterial induction

BL21 (DE3) colonies containing AtNPG1-pET28a were inoculated into 5 ml LB/kanamyacin (30 μg/ml) medium and incubated at 37 °C with shaking. The next morning, 5 ml overnight culture was inoculated into 400 ml LB/kanamyacin medium and incubated at 37 °C with shaking until it reached $O.D_{600}$ of 0.5-0.8. At this time, the culture was induced by adding 1 mM IPTG (isopropyl-1-thio-β-D-galactopyranoside). Induction was done at 30 °C with shaking for 4 hours. Cells were collected by centrifuging at 4000g for 10 min. The cell pellet was resuspended in 25 ml His binding buffer (20 mM Tris-HCl pH 7.9, 500 mM NaCl, 5 mM imidazole) and 1X protein inhibitor cocktail from Roche. The cells were lysed by the French press method and the lysed sample was centrifuged at 17,000 rpm in a Sorval SS-34 for 30 min. The supernatant was filtered with a 0.45 micron filter.

AtNPG1 purification with His-bind affinity column

The protein extract was applied to a His-bind affinity column. The column was washed with 10 volumes of 1X Binding Buffer (20 mM Tris-HCl pH 7.9, 500 mM NaCl, 5 mM imidazole) and then washed with 6 volumes of 1X Wash Buffer (20 mM Tris-HCl pH 7.9, 500 mM NaCl, 60 mM imidazole). The proteins bound to the column were eluted with 10 ml of 1X Elute Buffer (20 mM Tris-HCl pH 7.9, 500 mM NaCl, 1 M imidazole).

Purified AtNPG1 was identified by electrophoresis and western blot. Protein samples were mixed with 3X sample buffer (0.1875 M Tris-HCl pH 6.8, 6% SDS, 15% mercaptoethanol, 30% glycerol, and 0.0225% bromophenol blue) and electrophoresed for 45 min at 200V on two 10% denaturing gels. One gel was stained with coomasie blue (0.25% coomassie blue R250, 7.5% acetic acid, 42.5% methanol) and the other gel was blotted to a nitrocellulose membrane and probed with T7-tag antibody. Proteins were transferred to a membrane at 100V for 75 min in a transblot buffer (25 mM Tris-HCl, 192 mM glycine, and 20% methanol). For detection with T7-tag antibody, the blot was incubated in 3% gelatin in TBST for 30 min at 30 °C with gentle shaking. The membrane was washed with TBST 3 times, 5 min each, and incubated for 30 min at 30 °C in TBST containing 1% gelatin and 1:10,000 dilution of T7-tag antibody alkaline phosphatases conjugate. The membrane was washed with TBST 3 times, 5 min each, and presoaked with AP buffer (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$). Then the blot was placed in color development solution (10 ml AP buffer, 45 μl NBT (nitroblue tetrazolium chloride, 75 mg/ml) and 30 μl BCIP (5-bromo-4-chloro-3-indolylphosphate p-toluidine salt, 50 mg/ml)).

AtNPG1 Sepharose 4B column preparation

AtNPG1 affinity column was prepared by immobilizing purified AtNPG1 to CNBr-activated Sepharose 4B (GE Healthcare). Two grams of CNBr-activated Sepharose 4B powder was suspended in 10 ml of 1 mM HCl. The beads were washed for 15 minutes with 1 mM HCl on a sintered glass filter (porosity G3) and kept in coupling buffer (0.1 M $NaHCO_3$ pH 8.3, 0.5 M NaCl).

AtNPG1 eluted from the His column was prepared by dialysis against coupling buffer. AtNPG1 and CNBr-activated Sepharose 4B were mixed and incubated, rotating the mixture end-over-end for overnight at 4 °C. Excess ligand was washed away with 5 medium volumes of coupling buffer on a sintered glass filter (porosity G3). Remaining active groups were blocked by incubating in 0.1 M Tris-HCl buffer pH 8.0 for 2 hours. The beads were washed with at least three cycles of alternating pH with at least 5 medium volumes of buffer (0.1 M acetic acid/sodium acetate pH 4.0 containing 0.5 M NaCl and 0.1 M Tris-HCl pH 8 containing 0.5 M NaCl).

Maize pollen protein preparation

One gram of maize pollen and 1.2 g of sand were added to a chilled mortar and ground by pestle for 1 hour. Ten ml of protein extraction buffer on ice (50 mM TrisHCl pH 7.5, 250 mM sucrose, 5 mM DTT, 1X protease inhibitor cocktail from Roch) was added. The sample was ground one more hour and centrifuged at 17,000 rpm in a Sorval SS-34 for 30 min. The supernatant was centrifuged in an ultracentrifuge at 50,000 rpm for 1 hour. The supernatant was filtered with a 0.22-micron filter.

Purification and identification of maize pollen AtNPG1 binding protein

AtNPG1-CNBr-Sepharose 4B column was prepared by washing with 5 volumes of binding buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl). Maize pollen protein extract was applied to the column and washed with binding buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl). The column was washed with 10 mM sodium phosphate buffer pH 7.2. The proteins bound to the column were eluted by elution buffer (3.5 M $MgCl_2$, 10 mM sodium phosphate buffer pH 7.2).

The eluted proteins were detected by electrophoresis and silver staining. Protein samples were mixed with 3X sample buffer (0.1875 M Tris-HCl pH 6.8, 6% SDS, 15% mercaptoethanol, 30% glycerol, and 0.0225% bromophenol blue) and electrophoresed for 45 min at 200V on 10% denaturing gels. The gel was washed with 100 ml of 50% methanol and 10% acetic acid for 15 min, and washed with 100 ml of 10% methanol and 10% acetic acid for 15 min. The gel was washed with deionized water briefly twice and incubated 0.04 mM DTT for 10 min. The gel was incubated with 0.1% $AgNO_3$ for 10 min and rinsed with deionized water. The gel was incubated with a solution containing 3% $NaCO_3$ monohydrate and 0.017% formaldehyde and rinsed with 3% $NaCO_3$ monohydrate. The reaction was stopped by adding 2.5 ml of 2.3 M citric acid.

The eluted proteins were digested by trypsin enzyme and the amino acid sequences of digested fragments were identified by LC-MS/MS. The protein identification was done by W. M. Keck foundation biotechnology resource laboratory at Yale University. The amino acid sequences of identified fragments were used for BLAST search against the Arabidopsis database.

Results

Interaction between AtNPGs

All AtNPGs have five to seven TPR (tetratrico peptide repeat) domains. The TPR domain is a degenerate 34 amino acid sequence, which is known to be involved in protein-protein interaction (Lamb et al., 1994; Das et al., 1998). Since the AtNPG proteins have TPR domains, it is possible that AtNPGs can interact either to form homodimers and/or interact with one another to form heterodimers. To test this possibility, a yeast two-hybrid assay was used with the combinations described in Methods. To study interaction among AtNPGs (AtNPG 1, AtNPGR 1 and AtNPGR 2), the *AtNPG* cDNAs were cloned into the yeast expression vector, pAS1-CYH2 or pACT2J as GAL4 DNA binding domain or activation domain, respectively. These constructs were confirmed by hybridizations with DIG-*NPGR1* or *2* probes, by restriction enzyme digestion mapping (Figure 2.1-2.3) and by sequencing. For restriction digestion mapping, *AtNPG1*-pAS1-CYH2 with *EcoRI* digestion produce an insert 2.0 kb size fragment and *AtNPG1*-pACT2J with *NdeI* was expected to be linearized and *NdeI/XhoI* digestion was expected to produce a 2.4 kb insert fragment (Figure 2.1). A sense *AtNPGR1*-pAS1-CYH2 construct with *BamHI* released 9.7 kb and 0.66 kb size fragments and a sense *AtNPGR1*- pACT2J with *HindIII* released 7.3 kb, 2.0 kb, and 0.63 kb size fragments (Figure 2.2). Sense clones of *AtNPGR2*-pAS1-CYH2 and *AtNPGR2*- pACT2J were digested with *NdeI* and yielding two fragments of the expected sizes 9.6 kb and 1.3 kb for *AtNPGR2*-pAS1-CYH2, and 9.2 kb and 1.3 kb for *AtNPGR2*- pACT2J (Figure 2.3). The constructs were introduced into Y190 yeast cells. Colonies that contained both plasmids grew on Trp- and Leu- plates indicating the presence of both plasmids.

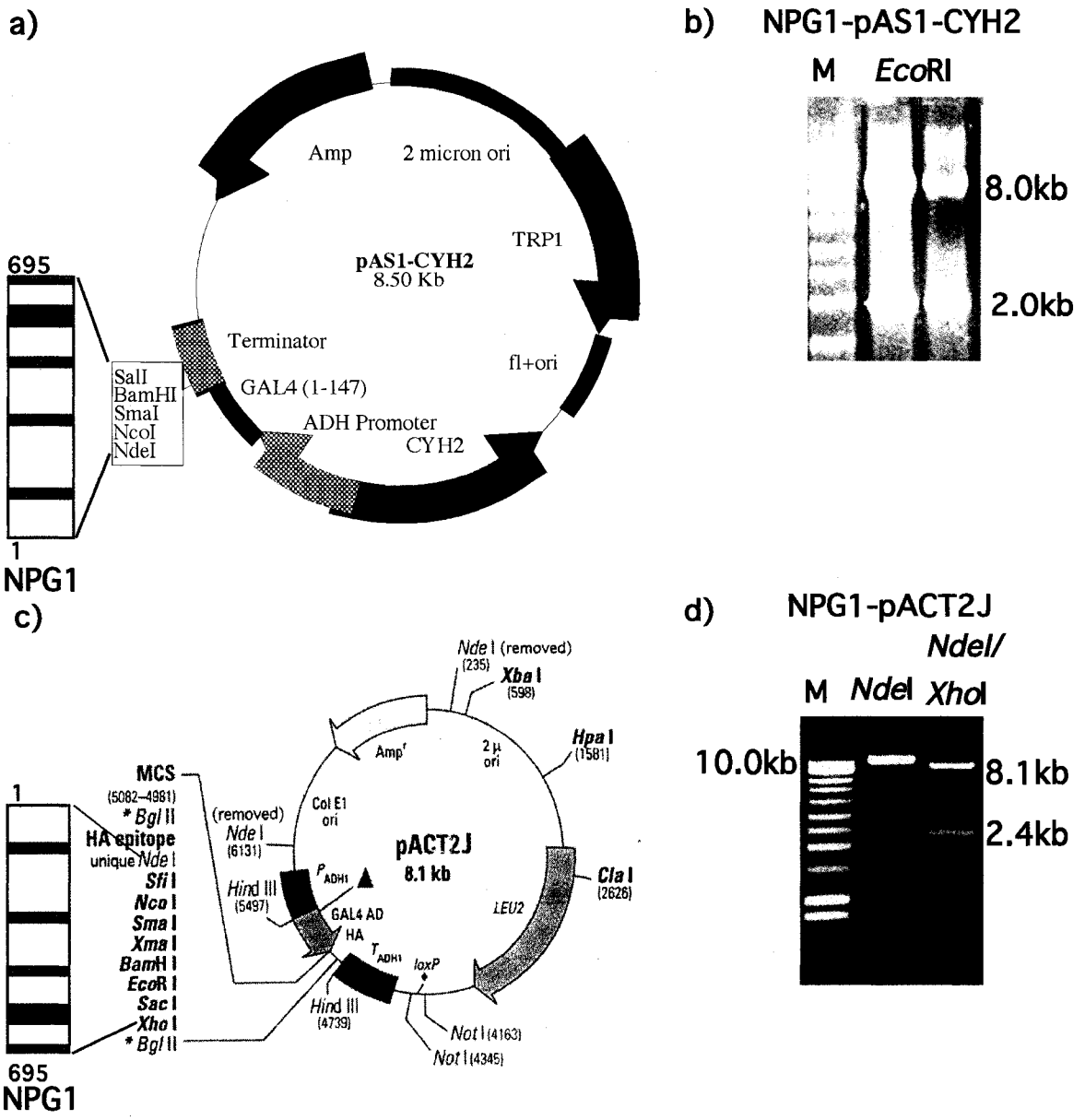


Figure 2.1. Cloning of *NPG1* into the yeast two-hybrid vectors pAS1-CYH2 and pACT2J. a) Schematic diagram of cloning of *NPG1* into pAS1-CYH2. *NPG1* fragment and pAS1-CYH2 plasmid were prepared by *NdeI* and *Sal*I digestion, and *NPG1* was inserted at *Nde*I and *Sal*I site of pAS1-CYH2. b) *Eco*RI digestion of *NPG1*-pAS1-CYH2 releases a 2kb fragment. c) Schematic diagram of cloning of *NPG1* into pACT2J. *NPG1* fragment and pACT2J plasmid were prepared by *NdeI* and *Xho*I digestion, and *NPG1* inserted at *Nde*I and *Xho*I site of pACT2J. d) Digestion of *NPG1*-pACT2J with *Nde*I or *NdeI*/*Xho*I. With NdeI digestion, NPG1-pACT2J was expected to be linearized and with *Nde*IXhoI digestion, *NPG1* insert was expected to be released.

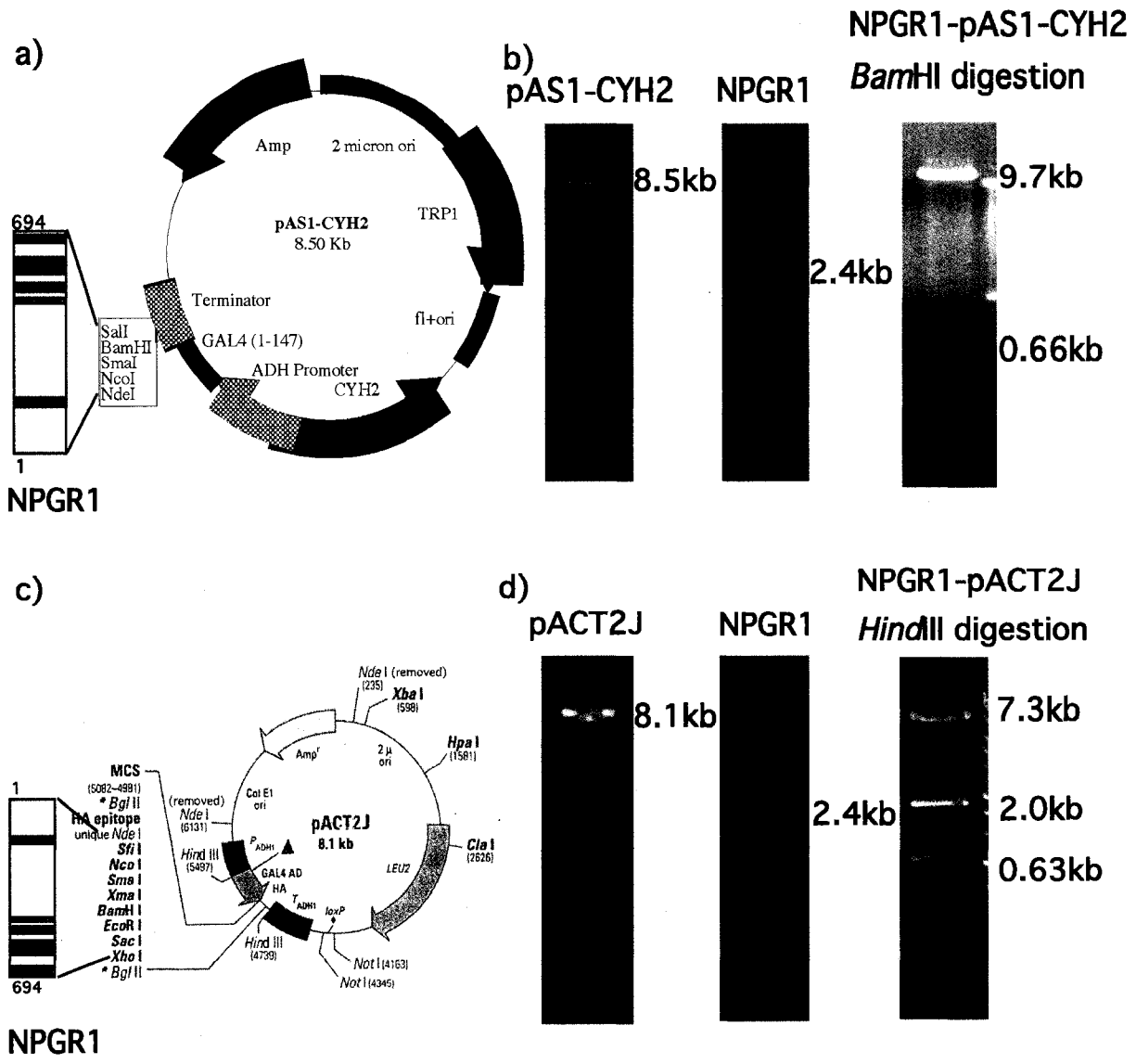


FIgure 2.2. Cloning of *NPGR1* into yeast two-hybrid vectors pAS1-CYH2 and pACT2J. [*Nhe*I]-*NPGR1*-*Sal*I fragment ligated with [*Nde*I]-pAS1-CYH2-*Sal*I or [*Nde*I]-pACT2J-*Xho*I. a)Schematic diagram of cloning of NPGR1 into pAS1-CYH2. b) NPGR1 and pAS1-CYH2 fragments and *Bam*HI digestion to determine orientation. c) Schematic diagram of cloning of NPGR1into pACT2J. d) NPGR1 and pACT2J fragments and HindIII digestion to release the expected size fragments for a sense construct. [*Nde*I], *Nde*I site was blunted; [*Nhe*I], *Nhe*I cut site was also blunted.

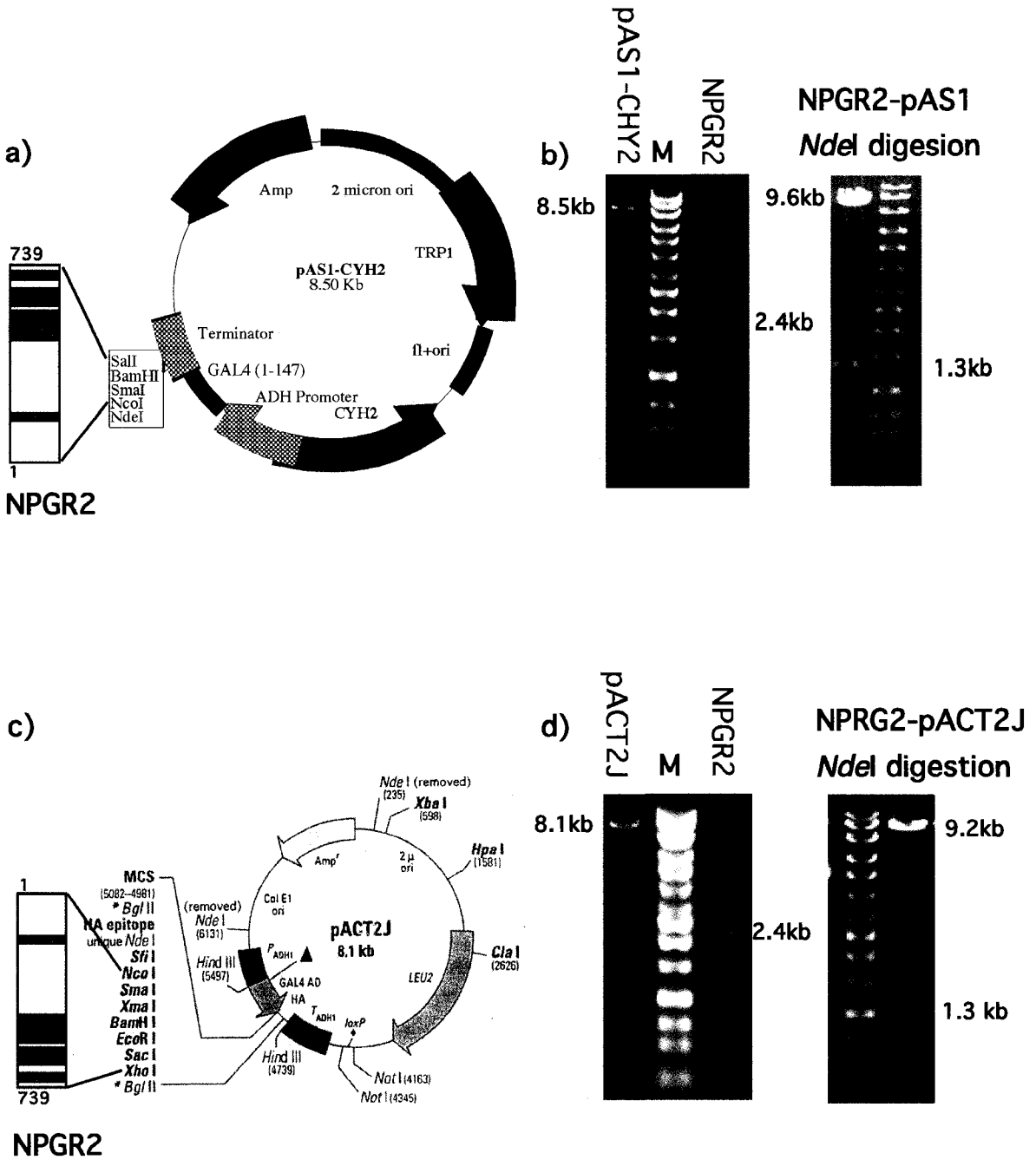


Figure 2.3. Cloning of *NPGR2* into yeast two-hybrid vectors pAS1-CYH2 and pACT2J. *Nco*I-*NPGR2*-*Sal*I fragments were inserted into *Nco*I- pAS1-CYH2-*Sal*I or *Nco*I-pACT2J-*Xho*I sites. a) Schematic diagram of cloning of *NPGR2* into pAS1-CYH2. b) Gel picture of *NPGR2* and pAS1-CYH2 fragments and *Nde*I digestion to determined orientation. c) Schematic diagram of cloning *NPGR2* into pACT2J. d) Gel picture of *NPGR2* and pACT2J fragments and *Nde*I digestion to determined orientation.

All combinations, except *AtNPGR2* (BD)+ *AtNPG1* (AD), grew on Trp-, Leu-, His-, 25mM AT plates, indicating that they interact. To further confirm this, we measured the β-galactosidase activity of yeast colonies selected on Trp- and Leu- plates using filter (Figure 2.4) as well as liquid culture assays (Table 2.1). Liquid β-galactosidase assays, which permit quantification of interaction, were performed to detect any weak interactions. Table 2.1 shows the protein-protein interactions as β-galactosidase units. The background activity observed with Y190 alone was subtracted. AtNPG1-BD interacted with AtNPG1-AD, AtNPGR1-AD and AtNPGR2-AD. AtNPGR1-BD interacted with itself-AD, AtNPG1-AD and AtNPGR2-AD. However, AtNPGR2-BD did not interact with AtNPG1-AD or AtNPGR1-AD and showed a very weak interaction with itself-AD (Table 2.1).

Petunia library screening

To find proteins that interact with AtNPG1, a petunia pollen yeast two-hybrid library screen was performed using AtNPG1 as a bait. The *AtNPG1* gene was cloned into the pAS1-CYH2 plasmid as a fusion to the GAL4 DNA binding domain and the petunia pollen library had been cloned into pGAD424 as fusions to the GAL4 activation domain. Yeast Y190 cells containing *AtNPG1*-pAS1-CYH2 were transformed with the petunia pollen library and the transformants were grown on Trp-, Leu-, His-, 25mM AT plates. The colonies that grew on these selection plates were assayed for β-galactosidase activity. Transformants that grew on selection plates and showed β-galactosidase activity were selected for further analysis (Figure 2.5). The cDNAs in the activation domain vector were isolated by transforming bacterial cells (*E. coli* DH5α) with the DNA from positive yeast colonies. The cDNAs were then sequenced. Screening has resulted in isolation of

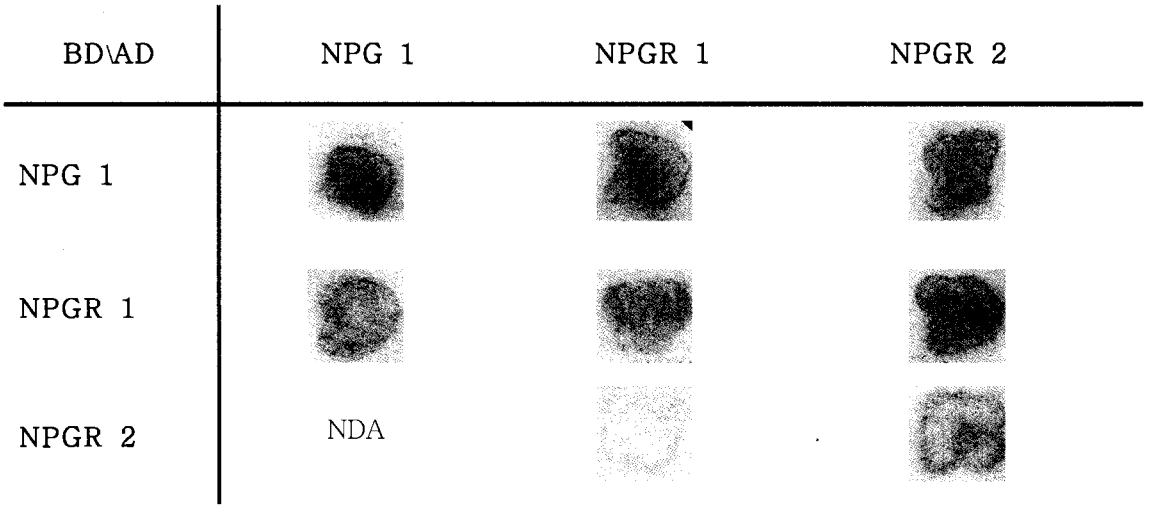


Figure 2.4. Interaction between NPGs using the yeast two-hybrid system. β-galactosidase assays with yeast cells containing different combinations of NPGs. NDA, No Detectable Activity.

Table 2.1. Liquid β-galactosidase assays with yeast cells containing different combinations of NPGs.

BD\AD	NPG 1	NPGR 1	NPGR 2
NPG 1	111.3	80.7	50.0
NPGR 1	476.7	165.0	1062.4
NPGR 2	NDA	NDA	14.5

β-galactosidase units: (1000 X OD_{578})/(elapsed min X 1.5ml X OD_{600}); NDA, No Detectable Activity.

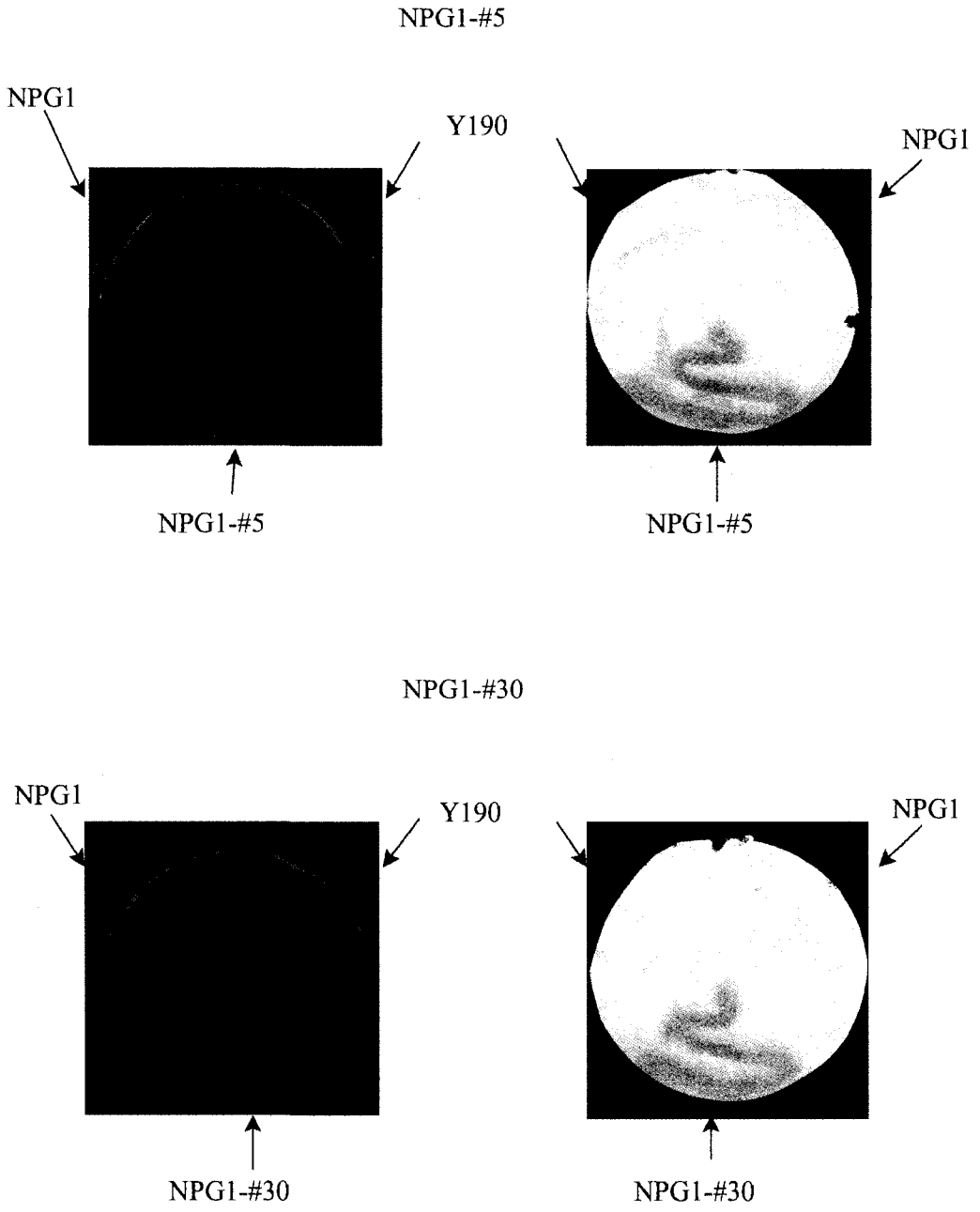


Figure 2.5. Protein-protein interaction assay between NPG1 and two clones (#5 and #30) from petunia pollen library. Y190 yeast, Y190 containing BD-*NPG1* only (NPG1), and Y190 containing both BD-*NPG1* and AD-#5 or#30 were grown on YPD. Only Y190 containing both BD-*NPG1* and AD-#5 or#30 showed beta-galactosidase activity.

two clones (#5 and #30) with very strong sequence similarity to pectate lyase-like proteins (Figure 2.6). Both cDNAs are partial. The deduced amino acid sequence of the petunia clones was aligned with similar proteins from Arabidopsis, tobacco and tomato as shown in Figure 2.6c. The partial sequence of petunia pectate lyase-like proteins showed up to 93% similarity with tobacco and tomato pectate lyase-like proteins.

Putative pectate lyase interacts with AtNPGs

Two proteins that interacted strongly with Arabidopsis AtNPG1 in the petunia pollen library screening were putative pectate lyases (Figure 2.5 and 2.6). Although these proteins are very similar to pectate lyase-like genes, they were partial genes. To see if full-length Arabidopsis pectate lyase-like (PLL) proteins can interact with AtNPGs, four Arabidopsis pectate lyase like (PLL) genes that showed the highest sequence similarity at the amino acid level to petunia PLLs were cloned into yeast expression vector, pACT2J (Figure 2.7). Those AtPLLs are AtPLL8 (At1g14420), AtPLL9 (At2g02720), AtPLL10 (At3g01270), and AtPLL11 (At5g15110). AtPLLs expression in yeast cells was confirmed by western blot (Figure 2.8). To see if these pectate lyase-like proteins can interact with NPGs, a yeast two-hybrid assay was used. *AtNPG1*-pAS1-CYH2, *AtNPGR1*-pAS1-CYH2 and *AtNPGR2*-pAS1-CYH2 were used for interaction assays with the two putative pectate lyase clones, *#5* or *#30*, from the petunia pollen library and four PLLs from Arabidopsis. All colonies containing *AtNPG1*-pAS1-CYH2 and *#5*, *#30*, or one of the *AtPLLs* grew on Trp-, Leu- plates and on Trp-, Leu-, His- and 25mM AT plates. Similarly all colonies containing *AtNPGR1*-pAS1-CYH2 and *#5*, *#30*, or one of the AtPLLs grew on Trp-, Leu- plates and on Trp-, Leu-, His- and 25mM AT plates.

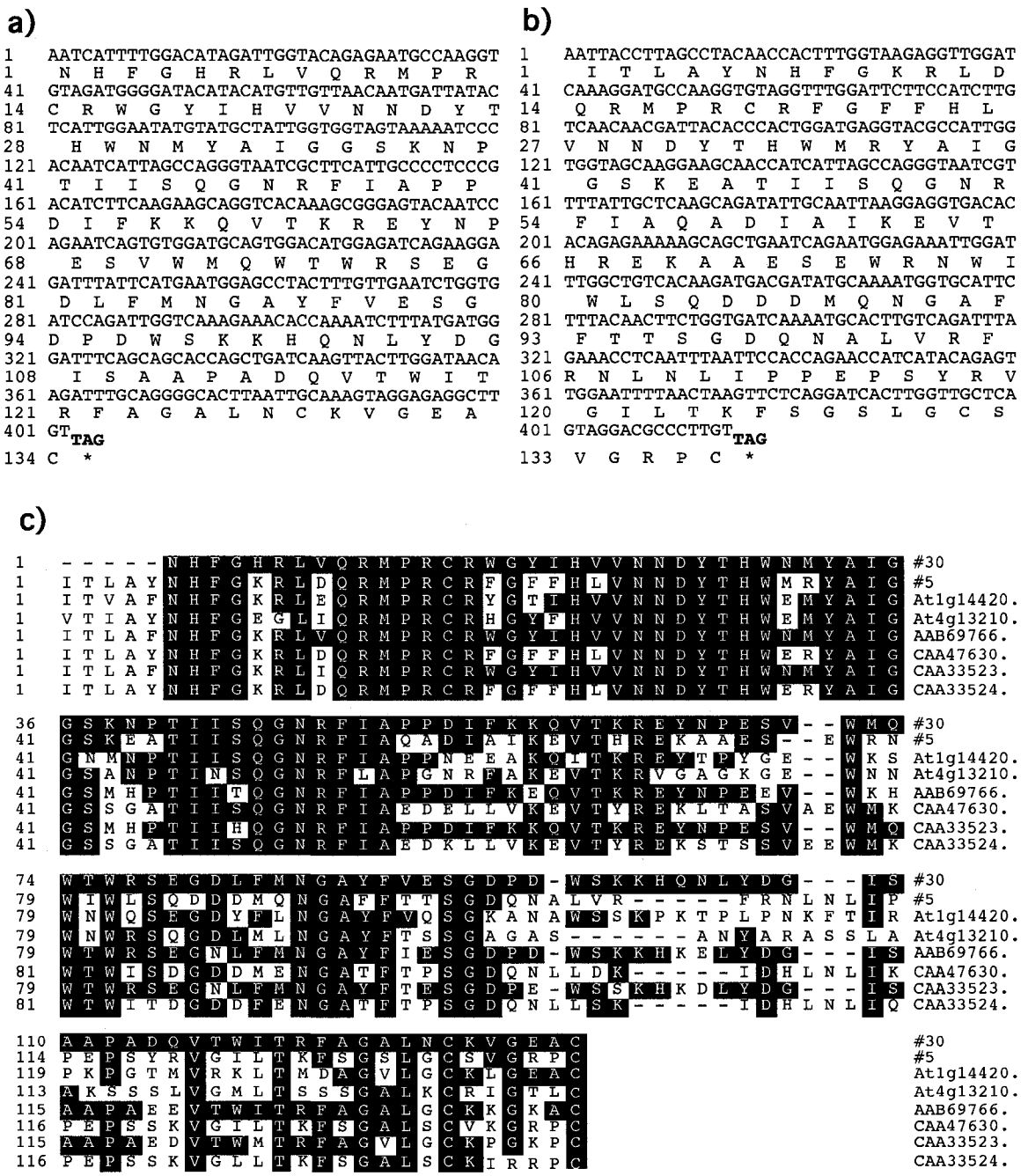


Figure 2.6. Sequence analysis of two NPG1 binding proteins.
a) Nucleotide and deduced amino acid sequence of #30. b) Nucleotide and deduced amino acid sequence of #5. c)Alignment of deduced amino acid sequence of #30, #5, with putative pectate lyases from Arabidopsis (At1g14420 and At4g13210), tobacco (AAB69766 Nt59 and CAA47630), and tomato (CAA33523, p59 and CAA33524, p56).

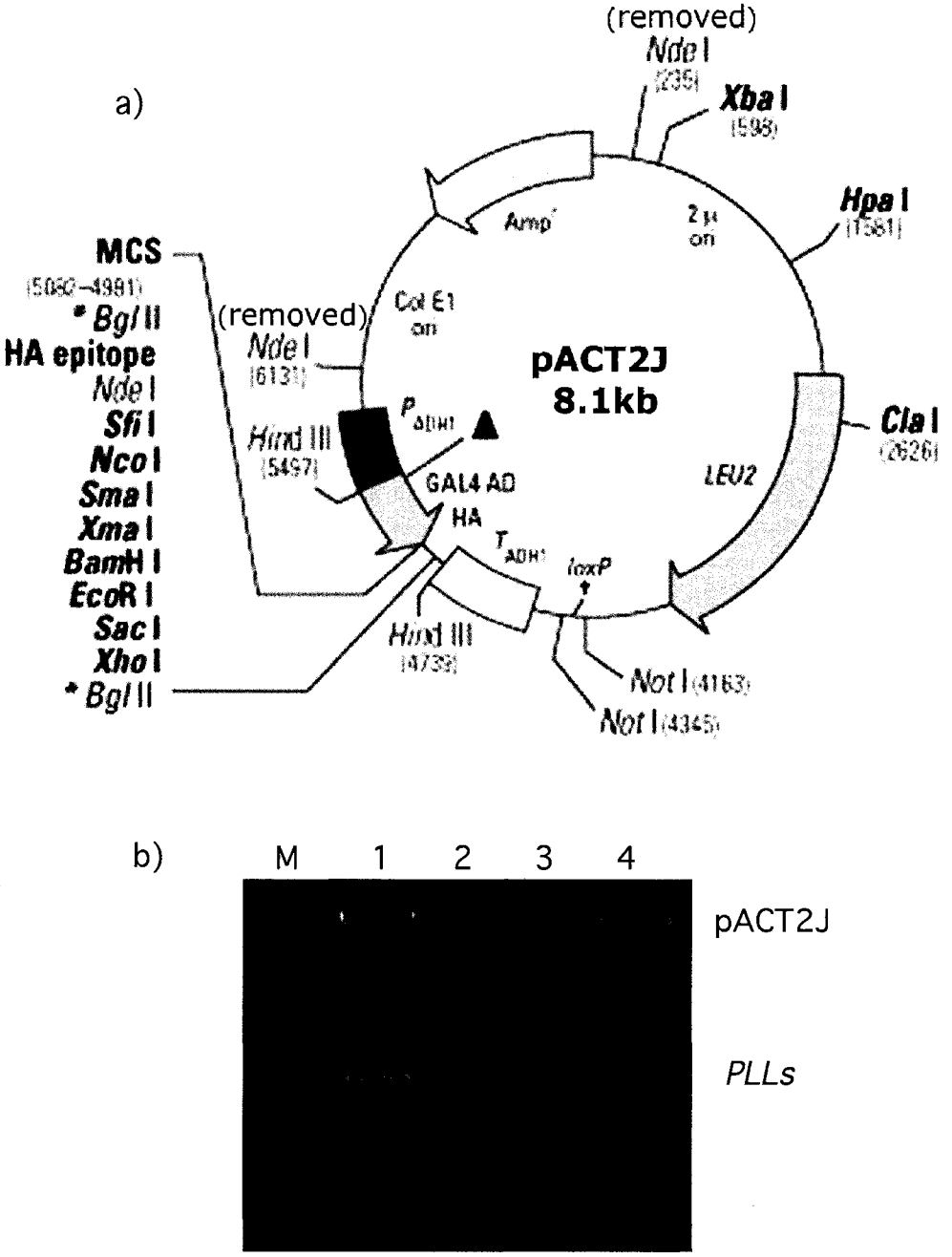


Figure 2.7. Cloning of four Arabidopsis pectate lyase like (*PLL*) genes into the yeast two hybrid vector, pACT2J. a) Plasmid map of pACT2J. b) The *PLLs*-pACT2J constructs were confirmed by restriction enzyme digestion mapping. 1, *PLL8* (1.38 kb) and pACT2J; 2, *PLL9* (1.368 kb) and pACT2J; 3, *PLL10* (1.428 kb) and pACT2J; 4, *PLL11* (1.419 kb) and pACT2J; 1 and 2 digested with Nde I and EcoR I; 3 and 4 digested with Xma I and Xho I.

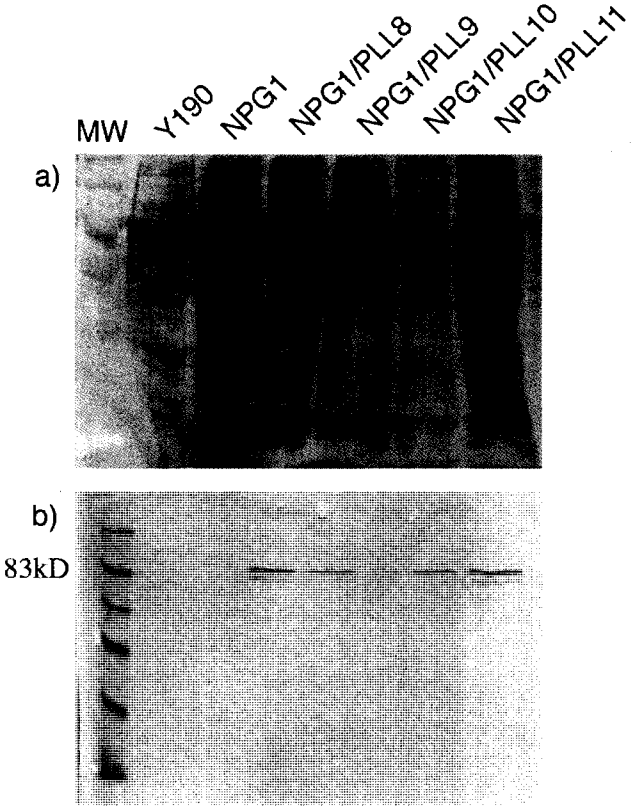


Figure 2.8. Pectate lyase expression in Y190 yeast cell extracted with Laemmli buffer. a) Coomassie blue stain. b) Western blot probed with Gal 4 AD antibody. 1, Y190; 2, Y190 with NPG1; 3, Y190 with NPG1 and PLL8; 4, Y190 with NPG1 and PLL9; 5, Y190 with NPG1 and PLL10; 6, Y190 with NPG1 and PLL11.

Yeast cells containing the *AtNPGR2*-BD + *AtPLL9*-AD grew on Trp-, Leu-, His- and 25mM AT plates, except *AtNPGR2*-BD + *AtPLL9*-AD, whereas cells with other *AtPLLs* did not grow. The transformants from Trp-, Leu- plates were grown on Trp-, Leu-, His- and 25mM AT plates and assayed for β-galactosidase activity (Figure 2.9). The results of liquid culture β-galactosidase assay confirmed the data obtained with the filter assay (Table 2.2). AtNPG1 and AtNPGR1 interacted with all four of the putative pectate lyases and the two clones from petunia pollen library (*#5*, *#30)*, but AtNPGR2 showed weak interaction only with AtPLL9 and did not interact with the other three putative pectate lyase (Table 2.2).

AtNPG1 without AtTPR1 did not interact with its partners

To study the role of TPR domains in these protein-protein interactions, truncated AtNPG1 [TPR1 domain was removed] was constructed (Figure 2.10). *AtNPG1w/oTPR1* (*AtNPG1* without *TPR1* domain) was cloned into pAS1-CYH2 vector and used in yeast two-hybrid assays to test interaction with AtNPGs, two petunia pollen library clones (#5 and #30), and four AtPLLs (Figure 2.10). All colonies containing *AtNPG1w/oTPR1*-BD and NPGs, *#5*, *#30*, or one of AtPLLs grew on Trp-, Leu- plates and not on Trp-, Leu-, His- and 25mM AT plates, except *AtNPG1w/oTPR1*-BD + *AtNPGR2*-AD and *AtNPG1w/oTPR1* + *AtPLL9*-AD. The transformants from Trp-, Leu- plates were grown on Trp-, Leu-, His- and 25mM AT plates and assayed for β-galactosidase activity (Figure 2.10). The results of the liquid culture β-galactosidase assay confirmed the data obtained with the filter assay (Figure 2.10). To quantify the interaction, liquid culture β-galactosidase assay with AtNPG1 and AtNPG1w/oTPR1 was compared. It showed that AtNPG1w/oTPR1 did not interact with most of AtNPG1 interacting partners and

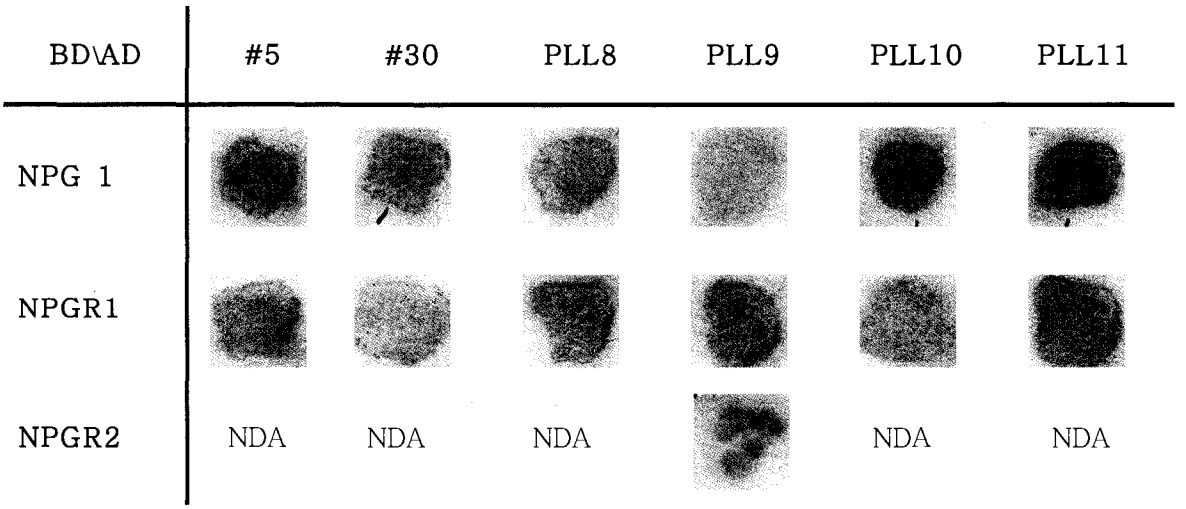
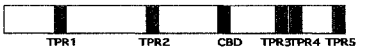


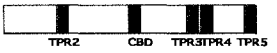
Figure 2.9. Protein-protein interaction assay between NPGs and pectate lyase-like (PLL) proteins including two petunia clones (#5 and #30) and four *Arabidopsis* PLLs. NDA, No Detectable Activity; PLL8, At1g14420; PLL9, At2g02720; PLL10, At3g01270; PLL11, At5g15110.

Table 2.2. Liquid β-galactosidase assays to analyze interaction between NPGs and pectate lyase-like proteins.

BD\AD	#5	#30	PLL8	PLL9	PLL10	PLL11
NPG 1	36.1	87.3	46.1	104.5	130.5	162.9
NPGR1	182.9	335.5	1270.2	956.3	608.8	987.4
NPGR2	NDA	NDA	NDA	1.1	NDA	NDA

β-galactosidase units: (1000 X OD_{578})/(elapsed min X 1.5ml X OD_{600}); NDA, No Detectable Activity; PLL8, At1g14420; PLL9, At2g02720; PLL10, At3g01270; PLL11, At5g15110.





AD\BD	NPG1	NPG1w/oTPR
NPG 1	111.3	NDA
NPGR1	80.7	NDA
NPGR2	50.0	40.1
#5	36.1	NDA
#30	87.3	NDA
PLL8	46.1	NDA
PLL9	104.5	16.0
PLL10	130.5	NDA
PLL11	162.9	NDA

Figure 2.10. Interaction between NPGw/oTPR1 and NPG1 interacting partners using yeast two-hybrid system. β-galactosidase units: (1000 X OD_{578})/(elapsed min X 1.5ml X OD_{600}); NDA, No Detectable Activity; PLL8, At1g14420; PLL9, At2g02720; PLL10, At3g01270; PLL11, At5g15110.

interacted very weakly with two proteins (Figure 2.10).

In vitro interaction of AtNPGs with Arabidopsis putative pectate lyase

To further confirm the interaction between AtNPGs and Arabidopsis putative pectate lyase *in vitro*, pull down assays were performed. AtNPG1, AtNPGR1 and AtNPGR2 were expressed in pET28a as T7 tag fusions. For the putative pectate lyases, *AtPLL8*, *AtPLL9*, *AtPLL10* and *AtPLL11* were expressed in pET32b as S-tag fusions (Figure 2.12). AtPLL8 and AtPLL9 had too low expression to use for pull down assay. Interaction between AtNPGs and two AtPLLs (AtPLL10 and AtPLL11) was analyzed by incubating S-protein beads bound to a AtPLL with AtNPG protein. The unbound protein was removed by washing with binding buffer and centrifugation. The protein bound on the S protein tag beads was analyzed by SDS-PAGE and identified by T7 tag antibody for AtNPGs and S protein for AtPLL. As shown in Figure 2.13, AtPLL10 and AtPLL11 interacted with AtNPGs (AtNPG1, AtNPGR1 and AtNPGR2) in these pull-down assays.

AtNPG1 binding proteins in maize pollen

To gain a better understanding of the role of AtNPG1 in pollen, AtNPG1-binding proteins in maize pollen were isolated and identified. To isolate AtNPG1- binding protein, an AtNPG1 affinity column was used. To prepare an AtNPG1 affinity column, AtNPG1was purified on a His-binding affinity column, and the purified AtNPG1 protein was visualized on an SDS-PAGE gel with Coomassie Blue stain and a Western blot with T7 antibody (Figure 2.14). The purified AtNPG1 was immobilized on Sepharose 4B beads as described in methods. AtNPG1 binding proteins in maize pollen were isolated by applying maize pollen total soluble proteins to the AtNPG1 Sepharose 4B column. The column was washed with two washing buffers, and the AtNPG1 binding proteins

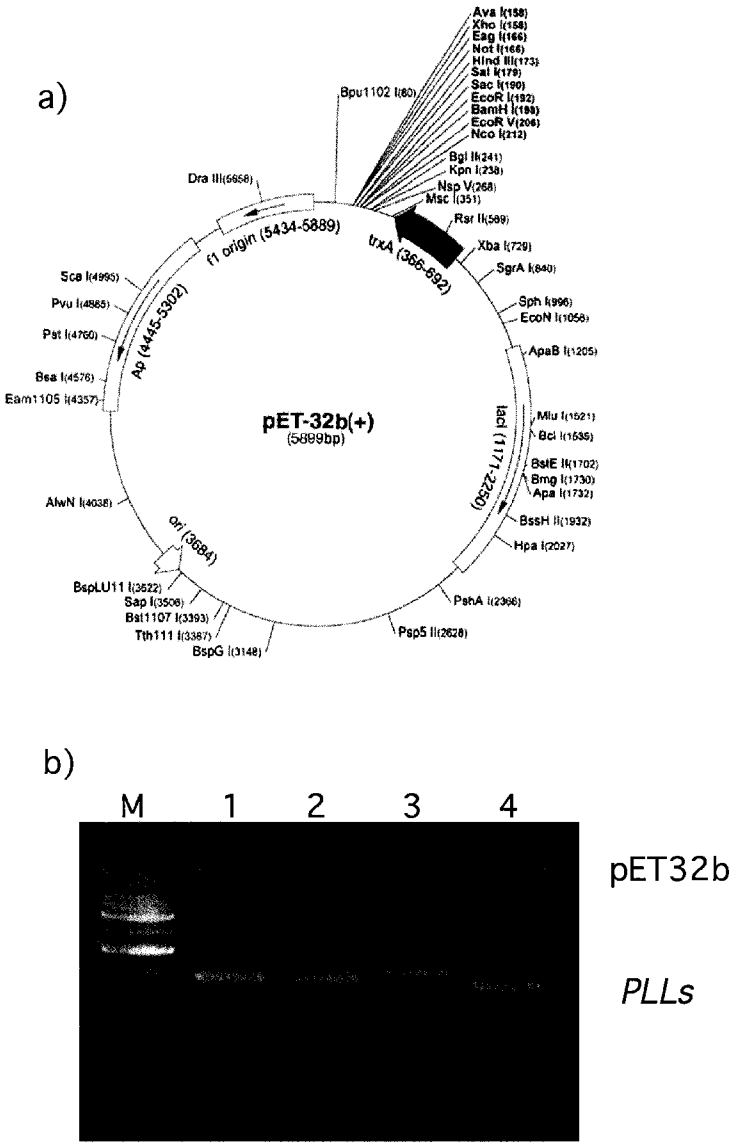


Figure 2.11. Cloning of four Arabidopsis pectate lyase like (PLL) genes into pET32b bacterial expression vector. a) Plasmid map of pET32b; PLLs were inserted into polyclonal site in pET32b according to the positions mentioned in methods. b) PLLs-pET32b constructs were confirmed by appropriate restriction enzyme digestion. 1, PLL8-At1g14420 (1.38 kb); 2, PLL9-At2g02720 (1.368kb); 3, PLL10-At3g01270 (1.428 kb); 4, PLL11-At5g15110 (1.2 kb); 1 and 2, Bgl II and EcoRI digestion; 3, Bgl II and Xho I digestion; 4, Bgl II digestion.

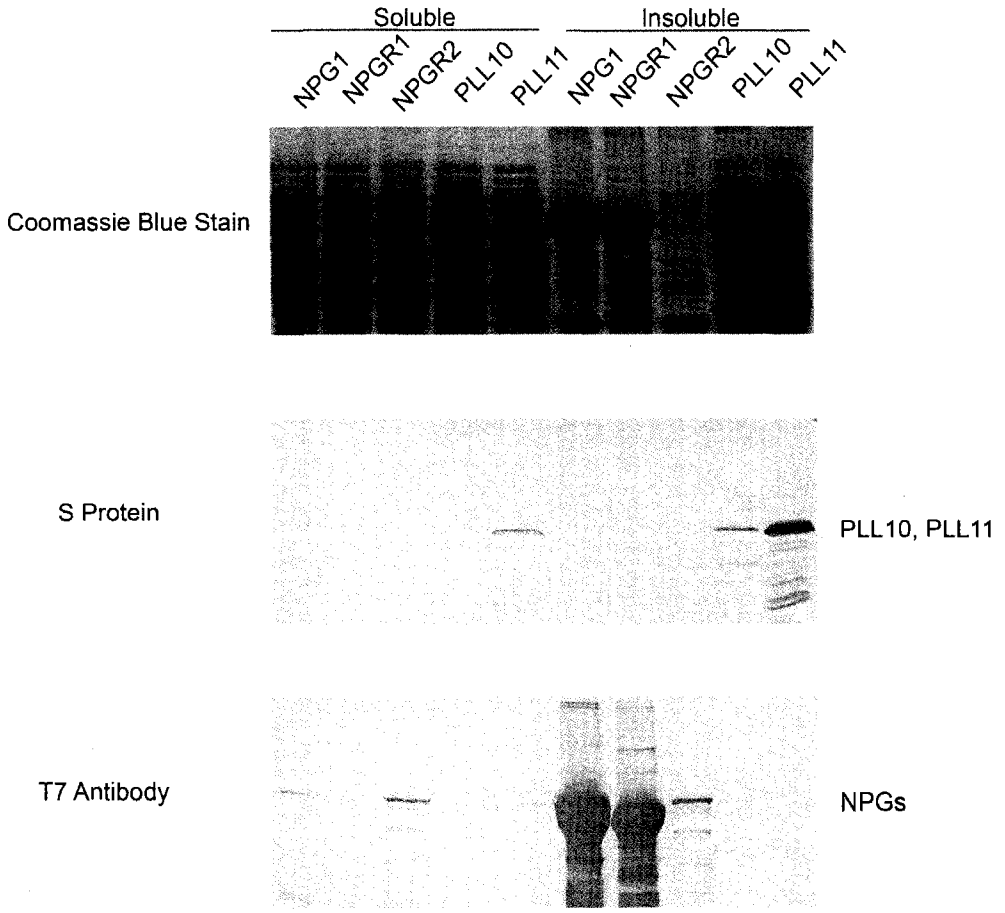


Figure 2.12. Bacterially expressed NPGs and two Arabidopsis pectate lyase like (PLL) proteins. Soluble and insoluble proteins were seperated on three SDS-PAGE gels, one gel was stained and the other two were blotted. One blot was probed with S protein and the other blot was probed with T7 tag antibody.

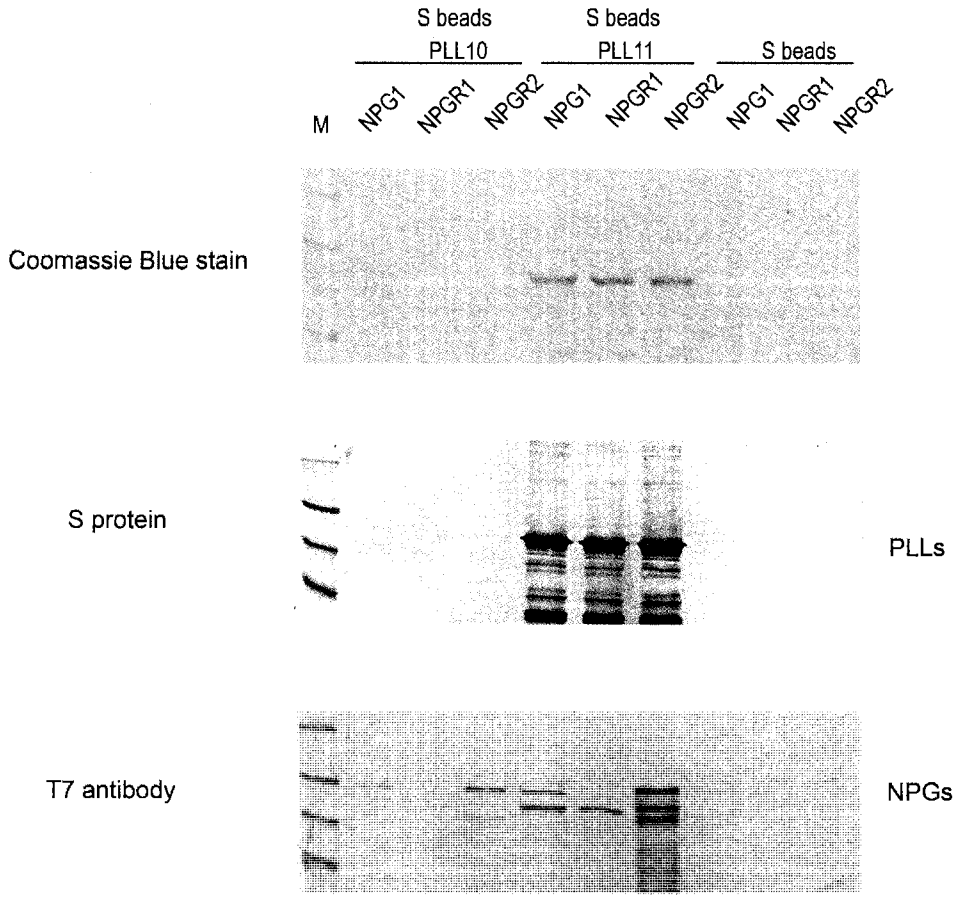


Figure 2.13. In vitro interaction between NPGs and two Arabidopsis pectate lyase-like (PLL) proteins using pulldown assay. S protein bead bound proteins were seperated on SDS-PAGE gels and identified by S protein for PLLs and T7 tag antibody for NPGs.

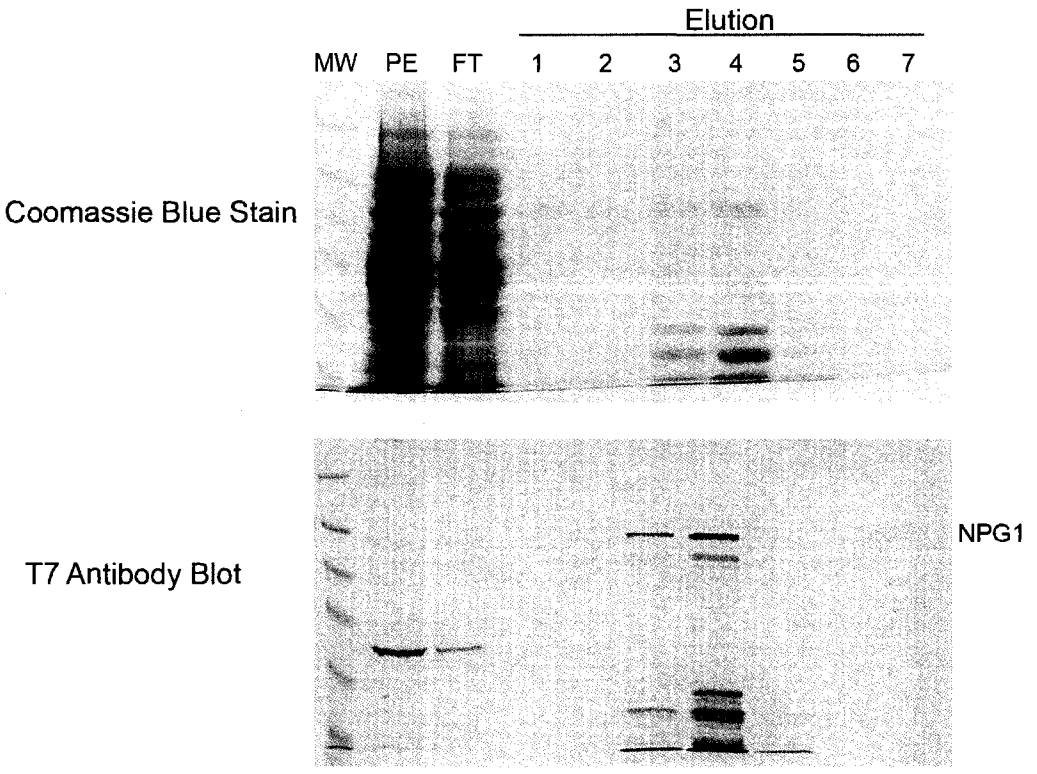


Figure 2.14. Bacterially expressed NPG1 was purified by His bind affinity column; PE, total protein extract from NPG1-pET BL21; FT, flow through of His colum; Elution with fractions 1 to 7

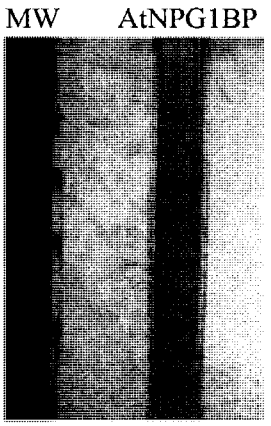


Figure 2.15. AtNPG1 interacting proteins were separated by SDS-PAGE gel and identified by silver staining. MW, Molecular Weight Markers; AtNPG1BP, AtNPG1 binding proteins.

were eluted with an elution buffer. The eluted proteins were visualized on SDS-PAGE gel with silver staining (Figure 2.15). Proteins in the eluted buffer were subjected to trypsin digestion and the sequences of the peptides were identified by LC-MS/MS. The amino acid sequences from LC-MS/MS were identified by BLAST analysis. Interestingly, one of the proteins identified in this analysis is a pectate lyase. In addition, sequences similar to glycosyl hydrolase, pectinesterase, SKU5 protein, and actin were present in the eluted fractions (Figure 2.16).

KLANVDLTGGYYDAGDNVKY	NPG1BP1
KLVNVDLSGGYYDAGDNVKY	Glycosyl Hydrolase (At3g43860)
KNWVWHTEDDLFMNGAIFNPSGGA	NPG1BP2
KKWNWRSEGDLFLNGAFFTPSGGG	Pectate lyase (At1g67750)
RVIFADTYLSKT	NPG1BP3
RVIFAKTYLSKT	Pectinesterase (At3g17060)
RTFIEVVFENPEKS	NPG1BP4
RTFIEVVFENHEKS	SKU5 (At3g13390)
KSYELPDGQVITIGAERF	NPG1BP5
KNYELPDGQVITIGAERF	ACTIN (At3g18780)

Figure 2.16. NPG1 interacting proteins from maize pollen. Proteins were identified by using LC-MS/MS and doing BLAST against Arabidopsis database. Upper sequences are NPG1 interacting peptides 1-5 (NPG1BP1-5).

Discussion

Pollen germination and pollen tube growth are important for sexual reproduction in plants. Successful germination of pollen, tube growth and delivery of sperm cells to the embryo sac are dependent on intricate signaling between pollen and the female pistil. A number of pollen and/or pistil-specific proteins have been shown or implicated to be involved in recognition of appropriate pollen (Cheung et al., 1995; Rubinstein et al., 1995a; Rubinstein et al., 1995b; Lord and Russell, 2002; Nasrallah, 2002; Tang et al., 2002; Edlund et al., 2004; Guyon et al., 2004; Tang et al., 2004). To fully understand sexual reproduction in plants it is necessary to understand the mechanisms that control pollen germination, pollen tube growth and guidance. Pollen tubes show rapid (e.g., maize pollen tubes grow 1 cm/hour) tip-focused growth (Gu et al., 2003). Hence, pollen tubes have been used extensively as a model system to understand cell growth and cell polarity (Bedinger, 1992; Bedinger et al., 1994; Lord and Russell, 2002; Gu et al., 2003).

Calcium, which is known to play a key role in transducing a variety of signals, has been shown to be involved in pollen germination, tube growth and guidance. A number of studies showed that the establishment and maintenance of a precise tip-focused intracellular Ca^{2+} gradient is essential for pollen tube elongation and directional growth (Pierson et al., 1994; Malho and Trewavas, 1996; Pierson et al., 1996; Holdaway-Clarke et al., 1997; Franklin-Tong, 1999; Camacho, 2000; Messerli and Robinson, 2003). How the tip-focused Ca^{2+} gradient at the tip regulates pollen tube growth and direction is poorly understood. It has also been shown that calmodulin, a major calcium sensor, plays a role in calcium-mediated signaling in pollen (Ma and Sun, 1997; Ma et al., 1999; Rato et al., 2004). Despite considerable evidence for the involvement of calcium and

calmodulin in pollen germination, tube growth and guidance, the information on downstream targets of calmodulin and their functions is limited. Here, I have characterized three calmodulin-binding proteins (AtNPG1, AtNPGR1 and AtNPGR2, collectively called AtNPGs) expressed in Arabidopsis pollen to further our understanding of the calcium/calmodulin-mediated signal network in Arabidopsis pollen.

AtNPG1 interacts with itself and AtNPGRs

The presence of several tetratricopeptide repeats (TPRs) in AtNPG1 and AtNPGRs suggests that they are members of the TPR family of proteins (Lamb et al., 1995). The TPR motif typically contains 34-amino acids. This motif was described first in CDC23, a cell cycle regulator in yeast. Since then the TPR motif was identified in a large number of proteins that perform diverse functions (Lamb et al., 1995). A number of studies indicate that TPR-containing proteins function through protein-protein interaction. This family of proteins has been shown to modulate many cellular processes including cell cycle (Hirano et al., 1990; Lamb et al., 1995), transcription (Schultz et al., 1990; Sikorski et al., 1990; Lamb et al., 1995; Tzamarias and Struhl, 1995), protein transport across mitochondria and peroxisomes (Botella and Arteca, 1994; Kragler et al., 1998), dephosphorylation of proteins (Albertazzi et al., 1998), and muscle development (Venolia et al., 1999). The presence of variable number of TPRs in proteins with a wide array of functions has led researchers to assume that they function as a scaffold in binding to specific substrates depending upon the secondary structure assumed by the individual or combination of TPRs (Lamb et al., 1995; Albertazzi et al., 1998; Venolia et al., 1999). This notion is supported by the observation that TPR-proteins have been shown to interact with different target proteins (Albertazzi et al., 1998; Prodromou et al.,

1999; Venolia et al., 1999). The loss of function of TPR-containing proteins by a mutation or deletion in the TPRs also suggests the importance of this domain in protein function (Lamb et al., 1995; Venolia et al., 1999). The functions reported for the TPR-containing proteins require that they form a complex with other proteins (Sikorski et al., 1990; Hernandez Torres et al., 1995; Lamb et al., 1995; Vucich and Gasser, 1996; Kragler et al., 1998; Prodromou et al., 1999; Venolia et al., 1999). Since all three AtNPGs are expressed in pollen (Figure 1.14) and have TPRs (Figure 1.8, 1.10 and 1.12) it is possible that AtNPGs might interact among themselves to form either homo- or heterodimers and/or interact with other proteins in pollen. To test this, I studied the interactions between AtNPGs using the yeast two-hybrid system. AtNPG1 interacted with itself or AtNPGRs (AtNPGR1 and AtNPGR2) (Figure 2.4, Table 2.1). AtNPGR1 showed interaction with AtNPG1, AtNPGR1 and AtNPGR2 (Table 2.1). AtNPGR2 did interact only with itself. Based on these results, we conclude that AtNPGs might form homo- or heterodimers.

AtNPGs interact with Petunia pectate lyase-like proteins

The absence of any enzymatic domains in AtNPG1 suggests that identification of the interacting proteins of AtNPG1 might be the key element for understanding the role of AtNPG1. To identify AtNPG1 interacting proteins, a petunia pollen yeast two-hybrid library was screened using AtNPG1 as bait. Two AtNPG1-interacting proteins were identified in this screen. Both showed very strong interaction with AtNPG1 (Figure 2.5). Sequence analysis of these clones revealed that they are very similar to late anther specific proteins (LAT56 and LAT59), both of which are putative pectate lyases and are preferentially expressed in anthers and pollen (Twell et al., 1991; Kulikauskas and

McCormick, 1997).

AtNPGs interact with Arabidopsis pectin lyase-like proteins

Although two petunia clones were very similar to pectate lyase, they were partial clones. To see if AtNPGs interact with full-length Arabidopsis pectate lyases, four Arabidopsis pectate lyase like (PLL) genes that are most closely related AtPLLs to the petunia clones were cloned into a yeast expression vector. These four AtPLLs are expressed highly in pollen tissue and it is thought that they might have a role in pollen. To study the interaction between AtNPG1 and AtPLLs, Arabidopsis PLLs were cloned into a yeast two-hybrid vector and their expression in the yeast cell was confirmed by western blot with Gal 4 AD antibody. Using the yeast two-hybrid assay, I showed that AtNPG1 interacts with all four of AtPLLs (Figure 2.9, Table 2.2). AtNPG1 is closely related to AtNPGRs, which, like AtNPG1, have tetratricopeptide repeat motifs and a calmodulin-binding domain. Since AtNPGRs share strong similarity with AtNPG1, I tested the interaction between AtNPGRs and AtPLLs. AtNPGR1, like AtNPG1, did interact with two isolated petunia clones and all four AtPLLs (Figure 2.9, Table 2.2), but AtNPGR2 showed no interaction, except very weak interaction with AtPLL9 (Figure 2.9, Table 2.2). To see if TPR domain is involved in these interactions, a truncated version of AtNPG1 that does not have the N-terminal TPR1 domain was used (Figure 2.10). Without this TPR1 domain, the interaction ability of truncated AtNPG1 was either abolished or decreased dramatically (Figure 2.10). This suggests that the TPR1 domain is essential for AtNPG1 interaction with its partners. Using a pull-down assay, I further demonstrated that AtNPG1 interacts with AtPLLS (Figure 2.13). The results from yeast two-hybrid assays and pull down assays suggest that AtNPGs interact with AtPLLs under

in vivo and in vitro conditions. The possibility of the interaction between AtNPG1 and pectate lyase-like protein was more significant, since one of the proteins from the AtNPG1 affinity column is identified as a pectate lyase protein (Figure 2.16). Together these results suggest that AtNPG1 function in pollen germination and tube growth might involve its interaction with AtPLLs.

Pectate lyases were initially characterized as pathogen-secreted extracellular enzymes that help the pathogen to invade the host by degrading pectins. These enzymes cleave α 1,4-glycosidic linkages of galacturonosyl residues of demethylated pectin. This enzymatic cleavage of glycosidic bonds occurs by a β-elimination reaction and produces 4,5 unsaturated oligogalacouronates (Yoder et al., 1993; Barras, 1994). Calcium is required for the activity of the enzyme, and it was found that calcium binds to this enzyme (Barras, 1994; Domingo et al., 1998; Centanni et al., 2001). In pollen, pectate lyase has been implicated in pollen tube emergence and growth by initiating loosening of the pollen cell wall and also in pollen tube penetration into transmitting tissue by breaking down the cell wall (Taniguchi et al., 1995; Wu et al., 1996). Although some pectate lyase-like proteins (LAT56 and LAT59) are preferentially expressed in pollen their function is not known (Twell et al., 1991; Kulikauskas and McCormick, 1997). Despite the similarity of LAT56 and LAT59 to pectate lyases, the proteins expressed using baculovirus did not show pectate lyase activity (Dirckse, 1996). Although it is difficult to interpret negative results, it is possible that i) they are not true pectate lyases, ii) they have the specificity for some other pectic substrates, or iii) the activity of these proteins may be modulated by other factors such as calcium/calmodulin. In the next chapter I have described out efforts to test the enzyme activity of AtNPG1 interacting

AtPLLs.

In Arabidopsis there are at least twenty-six predicted pectate lyase-like proteins (Palusa et al. 2008) and the functions of most of these are not known. Two predicted proteins (encoded by At1g14420 and At4g13210) that are very similar to the petunia putative pectate lyases are shown in Figure 2.6. Whether all of them are true pectate lyases or they have other activities is not known at this time. A number of AtPLLs are expressed only in pollen (see next chapter). Although they all contain a pectate lyase-like catalytic domain, the length of the proteins vary greatly, from 307 to 580 amino acids, suggesting that members of this family may have different regulation and/or localization. For example, one of the putative pectate lyases in Arabidopsis (PMR6, see below) has a long C-terminal extension as compared to most known pectate lyases, which contains a plasma membrane anchoring domain (Vogel et al., 2002). Using mutant analysis, it was shown that one of the pectate lyase-like genes (*PMR6*) is necessary for powdery mildew susceptibility in Arabidopsis (Vogel et al., 2002). In addition, mutation of *PMR6* alters leaf morphology and decreases leaf size due to decrease in cell expansion. Unlike LAT 56 and LAT59, PMR6 is expressed in all tissues, suggesting that different members of this family are expressed differentially. Expression analysis of all Arabidopsis PLLs also confirmed differential expression of these genes in different tissues and in response to various hormones and stresses (Palusa et al., 2007).

The interaction of AtNPG1 with the pectate lyase-like proteins is intriguing and raises many questions. Since the pectate lyases are extracellular enzymes or thought to be attached to the extracellular surface of the plasma membrane, the significance of the observed interaction is not clear. Due to the fact that the pectate lyase-like protein family

is poorly characterized we do not know if any of the 26 putative pectate lyases have different cellular localization or are bound to the plasma membrane with a regulatory region facing the cytosol. It would be interesting to test the localization of the AtNPG1 interacting pectate lyases. It may be that some of the pectate lyase-like proteins are performing some unexpected roles.

CHAPTER 3

Analysis of AtPLLs expression, enzyme activity and characterization of a putative pectate lyase knockout mutant

Abstract

Pectate lyase cleaves α-1,4 glycosidic linkages in homogalacturonan of pectate by β elimination and produces unsaturated oligogalacturonides. Pectin, together with cellulose and hemicellulose, is important for maintaining the cell wall integrity. Pectate lyases have been implicated in cell wall loosening during cell growth and plant pathogen interactions. Pectate lyases have been well studied in fungal and bacterial pathogens. Although plants contain putative pectate lyase genes, little progress has been made in biochemical and functional characterization of these enzymes. Since AtNPG1 interacted with putative pectate lyases (AtPLLs) I have studied the expression of all 26 Arabidopsis AtPLLs in pollen, analyzed pectate lyase activity in different tissues and tested if the AtNPG1 interacting AtPLLs have pectate lyase activity. My results show that 14 of the 26 AtPLLs are expressed in pollen and four of these [AtPLLs including 8, 9, 10 and 11 that interacted with AtNPG1] are highly expressed in pollen. However, these four AtPLLs are expressed in other tissues also. Pectate lyase activity of *Arabidopsis* tissue extracts showed the presence of pectate lyase activity in all tested tissues. To see if AtNPG1 interacting AtPLLs have pectate lyase activity, AtPLLs expressed in bacteria or yeast were used to monitor their enzyme activity. However, no enzyme activity was detected with these AtPLLs with different substrates in the presence or absence of AtNPG1. To understand the role of AtPLLs in plant development, a mutant of one of the AtNPG1 interacting AtPLLs (*atpll8*) was isolated and characterized.

Introduction

Since the cell wall can determine the shape of the plant cell and control the direction and rate of cell wall growth, it has been thought to have an important role in generating different cell types during plant development (Carpita and Gibeaut, 1993; Carpita, 1996). Many different polysaccharides, proteins and other aromatic substances are the major components of the cell wall. The primary cell wall in plants is composed of three matrices: the matrix of cellulose and cross-linking glycan (hemicellulose), the matrix of pectin, and the matrix of structural proteins (Carpita and Gibeaut, 1993; Carpita, 1996). Low-molecular-weight compounds and ions are also found in the primary cell wall (McNeil et al., 1984; Willats et al., 2001). Since these components have a major structural role in the plant cell wall, it has been considered that the deposition and modification of those components are the key processes of plant cell growth and development (Carpita and Gibeaut, 1993; Carpita, 1996). When plant cells expand and grow, materials are transported and deposited to the cell wall and the cellulose-glycan mesh network is loosened in the primary cell wall matrix (Cosgrove, 2000).

Pectins are a group of polysaccharides that contain 1,4-linked α-D-galactosyluronic acid (GalA), and they are a major component in the primary cell wall and middle lamella (Carpita and Gibeaut, 1993; Willats et al., 2001). There are three polysaccharides in pectin, homogalacturonan (HGA), rhamnogalaturonan-I (RG-I) and rhamnogalacturonan-II (RG-II) (Willats et al., 2001). HGA has a linear structure with a homopolymer of α(1-4) linked D-galacturonic acid and RG-II has the same backbone structure as HGA except it has an additional branched structure (Willats et al., 2001). RG-I has α(1-2) L-rhamnose -α(1-4)-D-galacturonic acid backbone structure and

additional branch structures (Willats et al., 2001). Pectin is synthesized in the golgi and transported to the cell wall as a form of high methyl ester pectin (Willats et al., 2001). In the cell wall matrix, the high methyl ester pectin loses its methyl ester by pectin methyl esterase action (Willats et al., 2001). Pectin has various roles in cell wall development. It has recently been found that pectin plays an important role in the deposition of major cell wall components e.g., pectin makes cellulose microfibrils deposit correctly on the cell wall matrix for proper structural function (Chanliaud and Gidley, 1999). After the methyl ester group is removed, pectin creates a junction zone by de-esterified residues interacting with Ca^{2+} ions, which allows pectin to regulate cell wall porosity, matrix pH and ion balance (Willats et al., 2001). In the middle lamella, pectin is involved in regulating cell-cell adhesion (Crookes and Grierson, 1983). Oligosaccharides from HGA can serve as a signal molecule that can activate the plant defense system (Willats et al., 2001). Pectin is also involved in cell wall swelling and fruit ripening (Marin-Rodriguez et al., 2002), leaf and fruit abscission, pod dehiscence and root cap cell differentiation (Wen et al., 1999).

Pectate lyase ((PL, EC 4.2.2.2)) is one of the pectin degrading enzymes that have a role in disassembling pectin polymers in the cell wall when plant pathogens infect plants and in modifying expanding the cell walls (Collmer and Keen, 1986). Pectate lyase cleaves α(1-4) linked galacturonosyl residues in pectin polymers by β-elimination (Carpita and Gibeaut, 1993; Willats et al., 2001) (Figure 3.1). PL activity requires calcium ions. Its enzymatic cleavage breaks the glycosidic bonds of a galacturonide chain and results in unsaturated C4-C5 bond in galacturonosyl residues at the non-reducing end (Hansen et al., 2001). Pectate lyase was first found in plant pathogenic

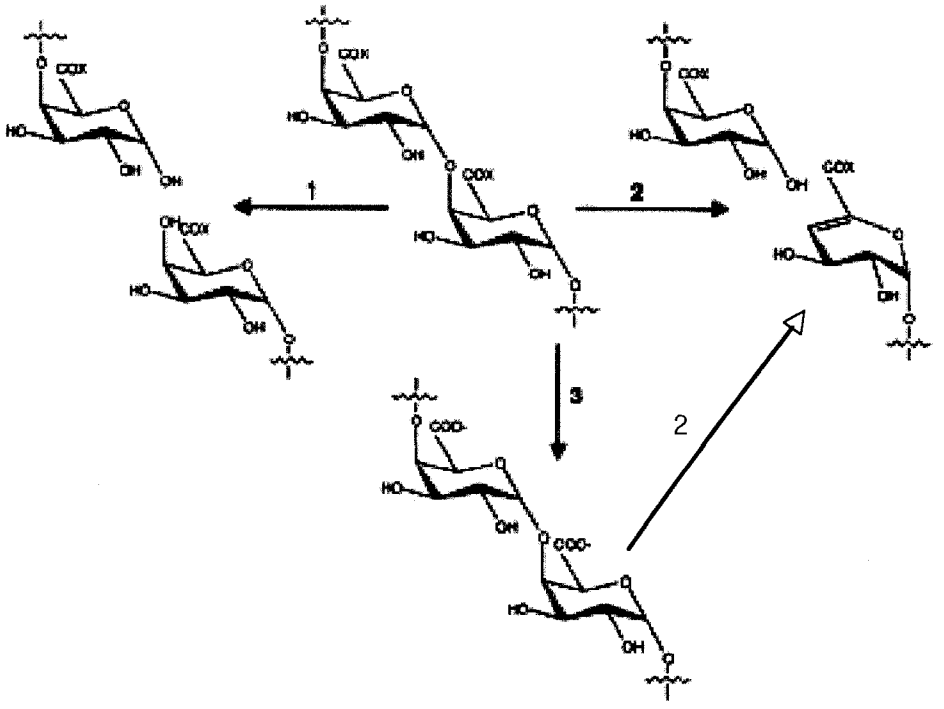


Figure 3.1. Pectin degradation enzymes; 1: pectinases and exo-polygalacturonase; 2: pectin lyase, exo-polygalactronate lyase, and pectin lyase; 3: pectin methyl esterase. (From Solbak et. al. J. Biol. Chem. (2005) 280; 9431-9438)

bacteria, *Erwinia carotovara* and *Bacillus sp.* (Starr and Moran, 1962) where its pectate lyase activity was well studied (Collmer and Keen, 1986). Pectate lyase in *Erwinia chrysanthemi* is the best-studied pathogen pectate lyase. *Erwinia chrysanthemi* causes diseases that destroy fundamental structural supporting tissue in plants (Collmer and Keen, 1986; Barras, 1994). During the destructive processes, pectate lyase cleaves polygalacturonides in the cell wall and disintegrates plant tissues (Collmer and Keen, 1986; Barras, 1994). It has been shown that Erwinia has five isoforms of pectate lyase, namely PelA, PelB, PelC, PelD and PelE, and the genes that encode those pectate lyase isomers are expressed and regulated independently (Lietzke et al., 1994; Lietzke et al., 1996). By combining different isoforms, Erwinia pectate lyase can degrade more pectin than a single isoform (Bartling et al., 1995), because combined pectate lyase isoforms degraded more high ester pectin than when they acted individually, even though combinations of isoforms showed no difference with low ester pectin (Bartling et al., 1995). The synergism of pectate lyase isoforms makes pectate lyase more destructive to plant tissue by extending the range of pectin substrates (Bartling et al., 1995). Pectate lyases not only degrade the plant cell wall, but the resulting oligogalacturonides trigger the plant defense system.

Two plant pectate lyase-like genes that are homologus to bacterial and fungal pectate lyase were first discovered in tomato anthers (Wing et al., 1990). Those two genes, LAT56 and LAT59, are expressed highly in mature anthers and pollen (Wing et al., 1990). The similarity to the bacterial pectate lyase and high pollen expression of LAT56 and 59 suggested their role in pectin degradation during pollen tube growth (Wing et al., 1990). Japanese cedar pollen allergen, *Cry* I, which is similar to the tomato pollen

proteins (LAT 56 and 59) and bacterial pectate lyase, shows pectate lyase activity in a calcium-dependent manner (Taniguchi et al., 1995). The pectate lyase studies in pollen suggest that pectate lyases function in pollen cell wall loosening during pollen tube emergence, growth and penetrating female tissue (Taniguchi et al., 1995; Wu et al., 1996).

Two pectate lyase-like genes were found from *Zinnia elegans* in the group of genes that are involved in cell wall architecture modification during cell differentiation (Domingo et al., 1998; Milioni et al., 2001). The pectate lyase-like gene, *ZePel1*, is expressed strongly during early tracheary element induction, and its expression is auxin inducible (Domingo et al., 1998). Bacterial expressed recombinant protein showed calcium dependent pectate lyase activity (Domingo et al., 1998). Since pectate lyase activity was discovered in an elongating and differentiating *in vitro* cell population, it has been suggested that pectate lyase enzyme may control the deposition of newly synthesized cell wall materials by remodeling of the existing pectin matrix (Domingo et al., 1998). Moreover, since purified pectic enzyme could digest the cell wall in a manner similar to plant pathogens, it has been assumed that pectic enzyme may be involved in disassembly of the cell wall structure (Collmer and Keen, 1986; Domingo et al., 1998). Genes involved in cell wall metabolism are found in a cDNA library prepared from opium poppy latex. Homologs to pectin methylesterase (PME), pectin acetylesterase (PAE) and pectin lyase (PL) were expressed highly in latex (Pilatzke-Wunderlich and Nessler, 2001). The high expression of genes encoding pectin-degrading enzymes in latex suggests that these enzymes might be involved in laticifer development that is achieved by gradual degradation of adjacent cell walls (Pilatzke-Wunderlich and Nessler, 2001). In a study of powdery mildew-resistant mutants, one of the genes involved in

resistance is a pectate lyase-like gene named *PMR 6* (*powdery mildew resistant 6*) (Vogel et al., 2002). Unlike other pectate lyases, *PMR 6* has a glycosylphosphatidylinositol anchor motif on its C-terminal domain (Vogel et al., 2002). The cell wall of the *pmr 6* mutant has a different composition than wild type, such as increased pectin content and reduced pectin esterification (Vogel et al., 2002). Moreover, the mutant has a smaller size than wild type (Vogel et al., 2002). Since it is believed that pectin plays an important role in plant cell wall expansion and growth, the difference in pectin content and plant growth in *pmr 6* suggests that the pectate lyase-like gene might be involved in plant development and growth (Vogel et al., 2002).

There are 26 pectate lyase-like genes in Arabidopsis and they are differentially expressed in various tissues (Palusa et al., 2007). Using an RT-PCR assay, I identified fourteen PLLs that were expressed in pollen and 4 PLLs that were expressed at a high level in pollen were characterized for their expression in other tissues, such as flower, stem, leaf, and root. All 26 Arabidopsis PLLs have a highly conserved active site and a calcium-binding site as in pectate lyase (Domingo et al., 1998; Palusa et al., 2007). This similarity suggested that those gene products could have pectate lyase activity (Palusa et al., 2007). Using purified pectin and a spectrophotometeric method, I analyzed pectate lyase activity in four different Arabidopsis tissues, including stem, leaf, flower and root. To see if Arabidopsis PLLs have pectate lyase activity, enzyme activity of bacterially expressed and yeast expressed PLLs was measured. For this pectate lyase assay, two kinds of purified pectin (high ester pectin and low ester pectin) were used. The pectate lyase *trans*-elimination of hydrogen at the C4 and C5 position of the galacturonosyl moiety produce an unsaturated polysaccharide, and the double bond of the polysaccharide

has a maximum absorption at 235 nm (Hasegawa and Nagel, 1962; Hansen et al., 2001). Pectate lyase activity in the plant tissue was analyzed by measuring unsaturated product amount at 235 nm. Since the cell wall determines the shape and size of plant cell, it has been suspected that pectate lyase plays an essential role in plant cell growth and development. To study the role of pectate lyase like (AtPLL) genes in plant development in Arabidopsis, a PLL knockout mutant (*atpll8*) was identified and characterized.

Material and Methods

Plant material

Flower, stem and leaf tissues were collected from flowering *Arabidopsis thaliana* ecotype Columbia grown on soil at 22°C with 16/8h light/dark cycles. Root tissue was collected from 2 weeks old seedlings. Root tissue was prepared by growing *Arabidopsis thaliana* ecotype Columbia in Murashige and Skoog medium (Gibco BRL, Grand Island, NY) with 1% sucrose at room temperature with 70 rpm shaking and 16/8h light/dark cycles. For pollen RNA extraction, Arabidopsis pollen was collected by the method described by Huang et al. (Huang et al., 1997). Arabidopsis flowers were collected in TE buffer (10 mM Tris-HCl pH 7.6, 1 mM EDTA) and shaken vigorously until pollen was released from the flower. Four layers of cheesecloth were used for filtering the pollen suspension and the pollen was concentrated by centrifugation at 10,000xg for 10 min.

RNA extraction and RT-PCR assay

To extract RNA, 80-120 mg of tissues were collected and ground to powder in liquid nitrogen. Total RNA was extracted using Qiagen RNeasy plant mini kit and the amount measured by spectrophotometry at 260 nm. About 1.5 μg of RNA was treated with 1 μl (1u/μl) DNase 1 (Fermentas) at room temperature for 10 min. The reaction was stopped by adding 1 μl of 25 mM EDTA and incubating the mixture at 65 °C for 10 min. To synthesize first-strand cDNA, DNase treated RNA was mixed with 1 μl 100 mM oligo (dT) primer and incubated at 65 °C for 10 min. For 20 μl of first-strand cDNA synthesis reaction mixture, 2 μl 100 mM DTT, 1 μl 10 mM dNTP, 1 μl (40u/μl) RNase out enzyme (Invitrogen), and 1 μl (200u/μl) SuperScript II RNase H-reverse transcriptase (Invitrogen) were added, and the mixture was incubated at 42 °C for 1 hour. After 1 hour

incubation, the mixture was incubated at 65 °C for 10 min and then transferred to ice. Twenty μl PCR reactions contained 1 μl of cDNA, 2.5 mM NTP, 0.75 μM primers, and 0.25 μl (5u/μl) Takara EX Taq TM polymerase. To amplify the 26 *PLLs,* specific primers for each *PLL* were prepared (sequences are in Table 3.1). Actin was used as a loading control for pollen tissue, and cyclophilin was used in the case of other tissues. For PCR reaction conditions, initial denaturation was done at 94 °C for 2 min followed by 30 cycles of reaction at 94 °C for 30 sec, 56 °C for 30 sec, and 72 °C for 1 min. Final extension was done at 72 °C for 10 min. The PCR products were separated by electrophoresis in 1% agarose gels.

Protein extraction preparation

Total protein extract from Arabidopsis tissues, including root, leaf, stem and flower, was used for pectin lyase assays. To extract proteins from Arabidopsis tissue, plant tissue was ground with mortar and pestle in liquid nitrogen for 20 min and extraction buffer (50mM Tris pH 8.0, 250mM sucrose, 5mM DTT, and 1X protease inhibitor) was added. The mixture was vortexed for 30 sec and rotated at 4°C for 15min. Plant extract was collected by centrifuging at 16,000g for 15 min at 4°C.

Substrate preparation

Two different kinds of pectin, high ester pectin and low ester pectin, from Megazyme (Bray, Ireland) were used for pectin lyase assay. Each kind of pectin was prepared by dissolving 50 mg of pectin in 100 ml of deionized water with 2 drops of 2-propanol. Dissolved pectin was adjusted to pH 12 by adding 0.5M NaOH and incubated for 15 min at room temperature. Then the pH of the pectin solution was lowered to 8 by addition of 0.5M HCl.

Table 3.1. Sequences of gene-specific primers of Arabidopsis *PLL* genes used in RT-PCR.

	##	Gene	cDNA	Introns	Primers
PLL 1	At3g09540	2059	1137	4	**F** 5'-CAT ATG GGA AAC TTA CAT GGC AT-3' **R** 5'-GAA TTC TTA TGC GGT GGT CGG -3'
PLL 2	At3g55140	1407	996	4	**F** 5'-CAT ATG ACG TCG CTA CCG TAC-3' **R** 5'-GAA TTC TTA CTT AGG AAC TCC ATG A-3'
PLL 3	At5g09280	1175	894	2	**F** 5'-CAT ATG ACG GGA AAT ATC GGT AAA-3' **R** 5'-GAA TTC TAA AAA GTC ATG TTA AGC G-3'
PLL 4	At4g22080	1820	1185	3	**F** 5'-CAT ATG ACT CTT TTC ACC GTT TCG-3' **R** 5'-GAA TTC TAA TAA CAA GGG CCG TT-3'
PLL 5	At4g22090	2040	1185	3	**F** 5'-CAT ATG ACT CAT TTC ACC GTT TCG-3' **R** 5'-GAA TTC TAA TAA CAA GGA CCG TTG C-3'
PLL 6	At1g11920	1431	1155	3	**F** 5'-CAT ATG GCT TCT CTT TTC TTA ACA ATT-3' **R** 5'-GAA TTC TTA GCA AAT TCT GCC GG-3'
PLL 7	At1g30350	1471	1107	3	**F** 5'-CAT ATG GCT TCT CTC GTG GTA AT-3' **R** 5'-GAA TTC TTA GCA AAT CCT GCC GA-3'
PLL 8	At1g14420	1562	1380	2	**F** 5'-CAT ATG GCA GCA GCT TTC TTG-3' **R** 5'-GAA TTC AGC AAG CTT CAC CAA GTT-3'
PLL 9	At2g02720	1662	1368	3	**F** 5'-CAT ATG GTG AAT CTT GGG AGC TAC-3' **R** 5'-GAA TTC AGC AGG ACT TGC CAG GTT-3'
PLL 10	At3g01270	1829	1428	3	**F** 5'-CCG GGT ATG GAG ACG GGC TAG GCT TTT-3' **R** 5'-CTC GAG TTA GCA TGC TTT TCC GAC CCG-3'
PLL 11	At5g15110	1717	1419	3	**F** 5'-CCC GGG TAT GGA GAT GGT TAG GCT GTC-3' **R** 5'-CTC GAG CTA ACA GCG TCT CCC GAC TCT-3'
PLL 12	At5g04310	2223	1428	3	**F** 5'-GAG CAT CAT CAA CCT CAG CA -3' **R** 5'-TCA ATG TGA AAA GCC CAT CA -3'
PLL 13	At3g54920	3167	1506	3	**F** 5'-CAT ATG CTT CTT CAA AAC TTC TCC A-3' **R** 5'-GAA TTC ACA ATA ATA GAG TTG ATA ACG-3'
PLL 14	At5g55720	1450	1179	2	**F** 5'-CAT ATG TCA ATT GTA TGT ACG TTT TTC-3' **R** 5'-GAA TTC TTA ACA AGC TTG GTC AA-3'
PLL 15	At5g63180	2008	1299	3	**F** 5'-CCC GGG TAT GTT TCG TCC CAA TT-3' **R** 5'-CTC GAG TCA ACA ATG AGA ACC CC-3'
PLL 16	At1g67750	1506	1227	3	**F** 5'-CAT ATG AGA ATG ACA CTT GTT CAC T-3' **R** 5'-GAA TTC TTA ACA TCG TGA TCC TTT TC-3'
PLL 17	At3g53190	3367	1452	6	**F** 5'-CAT ATG ATG CTT CAA AGA AGC TG-3' **R** 5'-GAA TTC TTA CAA CAT AAA CAT TTG GG-3'
PLL 18	At3g27400	2701	1239	4	**F** 5'-CCC GGG TAT GGT TTC GTA TTC TAA-3' **R** 5'-GAA TTC TTA ACA GCG ACG GCC TC-3'
PLL 19	At4g24780	1597	1227	2	**F** 5'-CCC GGG TAT GAA AAT GCA GAC GA-3' **R** 5'-CTC GAG TCA GCA ACG GGA ACC TT-3'
PLL 20	At3g07010	3334	1251	5	**F** 5'-CAT ATG GCT GTC ACA AAA CTT ATT C-3' **R** 5'-GAA TTC TTA ACA TCG ACG ACC GG-3'
PLL 21	At5g48900	3670	1254	5	**F** 5'-CAT ATG GCT GTT ACA CAA ATA CTT G-3' **R** 5'-GAA TTC TTA ACA CCG GCG AC-3'
PLL 22	At3g24670	2597	1323	6	**F** 5'-CCC GGG TAT GGT GAT CTT TAG CA-3' **R** 5'-CTC GAG CTA ACA CCG CAT ACC GA-3'
PLL 23	At4g13210	3096	1257	5	**F** 5'-ATG GTG GTC GCT AGA ACA TTG-3' **R** 5'-TTA ACA AAG TGT ACC GAT CCT GC-3'
PLL 24	At3g24230	2670	1359	6	**F** 5'-CAT ATG GCG ACG TCA TCT CTG-3' **R** 5'-GAA TTC TAG TAG CAA GGT TTG CC-3'
PLL 25	At4g13710	3021	1266	6	**F** 5'-CAT ATG GTT GCT GAT GAA GTC G-3' **R** 5'-GAA TTC TAG CAA GGT CTT CCC TT-3'
PLL 26	At1g04680	3729	1296	5	**F** 5'-CAT ATG GCG GTT CTT CCG ACA T-3' **R** 5'-GAA TTC TAT GAG GAA CAT TGA CGT C-3'

Enzyme assay

Pectate lyase activity was measured with five different pectin substrates and at three different pHs, 6, 8, and 10. The enzyme blank contained 0.5ml of 50 mM Tris-HCl, 1 mM $CaCl_2$ pH 8.0, 1.0 ml pectin substrate, and 1.0 ml of deionized water. The substrate blank contained 0.5ml of 50 mM Tris-HCl, 1 mM $CaCl_2$ pH 8.0, 0.5 ml of protein extract and 1.5 ml of deionized water. The reaction mixture contained 0.5ml of 50 mM Tris-HCl, 1 mM $CaCl_2$ pH 8.0, 1.0 ml of substrate, 0.5 ml of deionized water, and 0.5 ml of total protein extract. The absorbance at 235 nm was measured before incubation, i.e., 0 min absorbance. Samples were incubated at 37 °C for 1 hour. The absorbance value at 235 nm of the samples was measured after a 1 hour incubation. The absorbance of the enzyme/substrate mixture at 0 min or 60 min was determined by subtracting the enzyme blank and substrate blank from the mixture of protein extract and substrate. The increase of enzyme activity after 1 hour incubation was determined by subtracting the 0 min absorbance from the 60 min absorbance (Hansen et al., 2001). The activity from tissues was normalized by calculating activity per mg of protein extract.

PLL protein induction in bacteria

The sense clones of AtPLLs-pET32b (see chapter 2) were introduced into *E. coli* BL21 (DE3) cells containing a chaperone plasmid pG-KJE8. Colonies growing on LB plates containing 50 μg/ml ampicillin and 20 μg/ml chloramphenicol were used for protein induction. The BL21 (DE3) colonies containing *AtPLL10*-pET32b/pG-KJE8 or *AtPLL11*-pET32b/pG-KJE8 were inoculated into 5 ml LB containing ampicillin and chloramphenicol and incubated at 37 °C with shaking. The next morning, 0.5 ml overnight culture was inoculated into 50 ml LB containing 5 μg/ml tetracycline and 200

mg/ml L-arabinose and incubated at 37 °C with shaking until O.D $_{600}$ of 0.5 was reached. The culture was induced by adding 0.4 mM IPTG (isopropyl-1-thio-β-D-galactopyranoside) and incubated at 37 °C with shaking for 4 hours. The cells were collected by centrifuging at 4000g for 10 min.

The cell pellets were resuspended in 2 ml of the original volume of lysis solution (50 mM Tris-HCl pH 8.0, 1X protease inhibitor, 100 μg/ml lysozyme) and incubated at 4 °C for 30 min. Samples were sonicated 6 times for 15 sec each and centrifuged at 15,000 rpm in a Sorvall SS-34 for 30 min at 4 °C. Protein amount in the soluble fraction was measured by using the Bradford method, and this protein was used for enzyme assay. Bacterially expressed AtPLL protein was separated by SDS-PAGE gel electrophoresis and identified in western blots with S protein. Twenty μl of the supernatant (soluble) were mixed with 10 μl of the 3X sample buffer (0.1875 M Tris-HCl pH 6.8, 6% SDS, 15% mercaptoethanol, 30% glycerol, and 0.0225% bromophenol blue). The soluble fractions of proteins were electrophoresed for 45 min at 200 V on two 10% denaturing gels. One gel was stained with Coomassie blue (0.25% Coomassie blue R250, 7.5% acetic acid, 42.5% methanol) while one other gel was blotted to nitrocellulose membranes and probed with S-protein (Novagen). Proteins were transfered to membranes at 100V for 75 min in transblot buffer (25 mM Tris-HCl, 192 mM glycine, and 20% methanol). For detection, the blot was incubated in 3% gelatin in TBST for 30 min at 30°C with gentle shaking. Membrane was washed with TBST (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1% Tween 20) 3 times, 5 min each, and incubated for 30 min at 30 °C in TBST containing 1% gelatin, and 1:5,000 dilution of S protein alkaline phosphatase conjugate. Membranes were washed with TBST 3 times, 5 min each, and presoaked with AP buffer

(100 mM Tris-HCl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$). Then the blot was placed in color development solution (10 mM AP buffer, 45 μl NBT (nitroblue tetrazolium chloride, 75 mg/ml) and 30 μl BCIP (5-bromo-4-chloro-3-indolylphosphate p-toluidine salt, 50 mg/ml). The soluble fraction of protein was used for the pectate lyase enzyme assay mentioned earlier. During the assay, the reaction mixture was incubated at 30 °C for 1 hour.

AtPLL and AtNPG1 bacterial expression and His purification

AtPLL protein was expressed bacterially as above. AtNPG1-pET28a in BL21 (DE3) was used for AtNPG protein induction. The BL21 (DE3) *E. coli* cells containing AtNPG1-pET28a were inoculated into 5 ml LB/kanamycin (30 μg/ml) medium and incubated at 37 °C with shaking. The next morning, 0.5 ml overnight culture was inoculated into 50 ml LB/kanamycin and incubated at 37 °C with shaking until $O.D_{600}$ of 0.5 was reached. The culture was induced by adding 0.4 mM IPTG (isopropyl-1-thio-β-D-galactopyranoside) and incubated at 37 °C with shaking for 4 hours. The cells were collected by centrifuging at 4000g for 10 min.

The cell pellets were resuspended in His binding buffer (20 mM Tris-HCl pH 7.9, 5 mM imidazole, 500 mM NaCl) with 1X protease inhibitor and 100 μg/ml lysozyme. The soluble fraction of protein was extracted as above and applied to a His tag affinity column (Novagen). The column was washed with 10 volumes of 1X Binding Buffer (20 mM Tris-HCl pH 7.9, 500 mM NaCl, 5 mM imidazole) and then washed with 6 volumes of 1X Wash Buffer (20 mM Tris-HCl pH 7.9, 500 mM NaCl, 60 mM imidazole). The proteins bound to the column were eluted with 10 ml of 1X Elute Buffer (20 mM Tris-HCl pH 7.9, 500 mM NaCl, 1 M imidazole). His-tag system purified AtNPG1 and

AtPLL were separated on SDS-PAGE gel and visualized by coomasie blue staining. The purified proteins were used in PL enzyme assays.

AtPLLs isolation from yeast

For isolation of AtPLLs (AtPLL8, AtPLL9, AtPLL10 and AtPLL11) from yeast, *AtPLL* transformed yeast were grown in 10 ml of Trp^-, Leu^-, His^- SD media overnight at 30°C. The overnight culture was used to inoculate 40 ml of YPD media and was grown at 30°C until an OD_{600} 0.5-0.8 was reached. The cell pellet was collected by spinning at 5000 rpm for 5 min at 4°C. The cell pellet was transferred to flat-bottom, O-ring screw cap microcentrifuge tubes. The cell pellet was resuspended in 200 µl of extraction buffer (50 mM Tris-HCl pH 8.0, 250 mM sucrose, 5 mM DTT, 1X protease inhibitor) and 200 µl of acid washed glass beads (425-600 microns). The mixture was vortexed at maximum speed for 1 minute 5 times. The tube was spun at maximum speed for 5 min at 4°C, and the supernatant was saved. Fresh 200 µl of extraction buffer was added to the tube. The mixture was vortexed at maximum speed for 1 minute 5 times. The tube was spun at maximum speed for 5 min at 4°C, and the supernatant was saved. Fresh 100 µl of extraction buffer was added to the tube. The mixture was vortexed at maximum speed for 1 minute 5 times. The tube was spun at maximum speed for 5 min at 4°C, and the supernatant was saved. All three supernatants were mixed together and used for the PL assay as above. During the assay, the reaction mixture was incubated at 37 °C for 1 hour.

The AtPLL protein expressed in yeast was identified by western blot with the Gal 4 AD antibody. The supernatant was mixed with 3X sample buffer (0.1875 M Tris –HCl pH 6.8, 6% SDS, 15% mercaptoethanol, 30% glycerol, and 0.0225% bromophenol blue) and electrophoresed for 45 min at 200V on two 10% denaturing gels. One of two gels

was stained with coomassie blue staining (0.25% Coomassie blue R250, 7.5% acetic acid, 42.5% methanol), and the other gel was blotted to a nitrocellulose membrane and probed with Gal4 AD antibody. Proteins were transferred to membranes at 100V for 75 min in a transblot buffer (25 mM Tris-HCl, 192 mM glycine, and 20% methanol). For detection with Gal4 AD antibody, the blot was incubated in 5% non-fat dry milk in TBST (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1% Tween 20) for 1 hour at room temperature with gentle shaking. The membrane was washed with TBST 3 times, 5 min each, and incubated for 1 hour at room temperature in TBST containing 0.4 μg/ml Gal4 AD antibody. The membrane was washed with TBST 3 times, 5 min each and incubated with 1:50 dilution goat normal serum in 5% non-fat dry milk in TBST for 30 min at room temperature. The membrane was washed with TBST 3 times, 5 min each, and incubated for 30 min at room temperature in TBST containing 1:5,000 dilution of goat anti-mouse IgG antibody conjugated to alkaline phosphatase. The membrane was washed with TBST 4 times, 5 min each, and presoaked with AP buffer (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$). Then the blot was placed in color development solution (10 ml AP buffer, 45 μl NBT (nitroblue tetrazolium chloride, 75 mg/ml) and 30 μl BCIP (5-bromo-4-chloro-3-indolylphosphate p-toluidine salt, 50 mg/ml)).

Isolation of homozygous *atpll8* mutant

Insertion line (CS871253) seeds from ABRC were grown on MS (Murashige and Skoog from Sigma) with 5 μg/ml basta plates to select T-DNA inserted lines. The T-DNA insertion in this line contains a GUS reporter gene and is in a pollen tetrad defect mutant (*qrt1-2*) background. To identify a homozygous T-DNA insertion line, RT-PCR assay and a GUS reporter gene were used. The mRNA was extracted from *atpll8* plant

leaves, and cDNA was synthesized using methods mentioned above. The cDNA was used for PCR with *AtPLL8* specific primer (Table V), and PCR reaction conditions described above were used. GUS activity on the pollen tetrad of *atpll8* was measured by using a reagent mix containing GUS staining buffer (50 mM NaPO4 pH 7.0, 10 mM beta-mercaptoethanol, 10 mM Na2EDTA pH 8.0, 0.1 % sarcosyl, 0.1 % triton X-100) and 1.0 mM X-glu (5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid). The reagent mix was added to *atpll8* pollen tetrad and incubated at room temperature for 2 hours. The GUS staining of pollen was observed using a Nikon SMZ 1500 stereoscopic microscope. The confirmed *atpll8* homozygous T-DNA inserted line was used for further studies.

Phenotype assay in plate and soil

To see the mutation effect on germination and seedling growth, homozygous seeds (*atpll8)* were plated under the following conditions: MS, MS with 0.5 μM ABA (abscisic acid), 1 μM ABA, 0.5 μM BA (6-benzylaminopurine), 1μM BA, 1 μM IAA (indoleacetic acid), 3% glucose, 5%glucose, 100 mM NaCl, or 150 mM NaCl. The plates were incubated at 22 °C and 16 hours light/ 8 hours dark and monitored for 2 weeks. Germination and seedling growth of *atpll8* was monitored by comparing with quartet 1-2 as a control. The *atpll8* effect on plant development was studied by growing *atpll8* on soil. Plants were grown at 22 °C and 16 hours light/ 8 hours dark and were observed from seed to seed stage. The *quartet 1-2* mutant line was used as a control.

Result

Fourteen *AtPLLs* are expressed in pollen

There are 26 pectate lyase-like (*AtPLL*) genes in Arabidopsis and it has been shown that some *AtPLLs* are expressed highly in pollen (Wing et al., 1990; McCormick et al., 1991). To study how many *AtPLLs* are expressed in pollen, RNA was extracted from Arabidopsis pollen and an RT-PCR assay was performed. Gene specific primers for Arabidopsis *PLLs* were designed (Table 3.1). To remove genomic DNA contamination, extracted RNA was treated by DNase and tested by performing PCR with the *AtPLL* primer set. Constitutively expressed actin was used as control. Although none of *AtPLLs* were expressed exclusively in pollen, fourteen genes (*AtPLL 2, 5, 8, 9, 10, 11, 13, 16, 19, 20, 21, 22, 24*, and *25*) out of 26 Arabidopsis *AtPLLs* are expressed in pollen and four genes (*AtPLL 8, 9, 10*, and *11*) expressed at a high level (Figure 3.2).

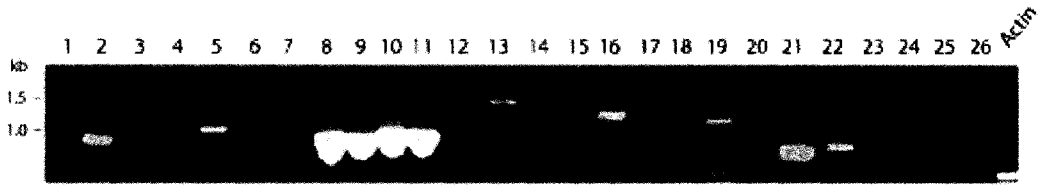
Four *AtPLLs* that are highly expressed in pollen are also expressed in other tissues

To characterize expression of the four *AtPLLs* expressed highly in pollen, their expression in other tissues was studied. RNA from roots, stems, leaves, and flowers was extracted and treated with DNase to synthesize cDNA. Amplification of *AtPLL* transcripts was done by PCR using the equal amount of cDNA with gene specific primers (Table 3.1). Constitutively expressed cyclophilin was used as a control. All four genes are more highly expressed in flowers than in other tissues, and the size of the transcripts are same as the expected from the size of cDNAs (Figure 3.2). However, these four AtPLLs expressed differentially in different tissues.

Aspergillus sp. pectate lyase activity

To develop an assay method to measure pectate lyase activity, highly purified

a)



b)

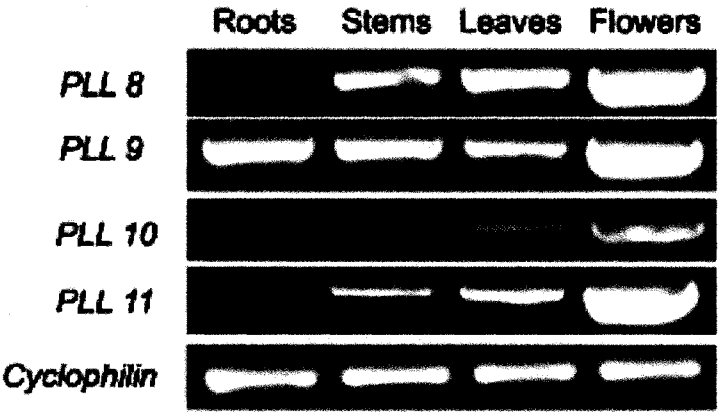


Figure 3.2. Expression analysis of *Arabidopsis PLLs*. a) Expression of 26 *Arabidopsis PLLs* in pollen. Numbers 1-26 corresponds to *PLL1* to *PLL26*, respectively. Amplification of actin was used as a positive control. b) Expression of four *Arabidopsis PLLs* in roots, stems, leaves and flowers. DNase-treated RNA from different organs was used to prepare first strand cDNA. An equal amount of first strand cDNA was used in polymerase chain reaction (PCR) with primers specific to each *PLL* gene. An equal amount of template in each reaction was verified by amplifying a constitutively expressed cyclophilin. The names of the *PLL* genes are shown on the left of each panel.

fungus pectate lyase (PL) from *Aspergillus sp.* was used as a positive control. To assay the fungal pectate lyase, two different pectin substrates, high ester pectin and low ester pectin, were used, Those pectin derivatives were prepared as described in Methods. The reaction mixture was composed of *Aspergillus sp.* pectate lyase, substrate and calcium-containing buffer. After incubation at 37 °C for 1 hour, the product, 4,5 unsaturated oilgogalacouronates was measured at 235 nm.

The enzyme assay was done in three different pH conditions - pH 6, pH 8, and pH 10. To measure enzyme activity, the amount of product (4,5 unsaturated oligogalacturonates) generated from the reaction was measured spectrophotometrically at 235 nm. The amount of 4,5 unsaturated oligogalacturonates was much higher with low ester pectin than high ester pectin, and pH 8 was found to be optimum (Figure 3.3a). To see the calcium requirement of PL enzyme activity, EGTA, a calcium chelator, was added to the buffer solution instead of $CaCl_2$. With EGTA, the pectate lyase activity decreased drastically with both substrates (Figure 3.3b).

Pectate lyase activity in *Arabidopsis* tissues

To measure pectate lyase (PL) activity in different Arabidopsis tissues, total protein extract from Arabidopsis tissues, including root, flower, stem, and leaf, was used for the assays. Root tissue showed the highest activity among the four tissues (Figure 3.4b). PL activity of the root tissue with both pectin derivatives increased with pH, and much higher activity was observed at pH 10 compared to pH 6 or pH 8 (Figure 3.4b). No PL activity with either of the pectin derivatives was seen at pH 6 in flowers and leaf and at pH 10 in stems (Figure 3.4). At certain pHs, flower and root tissue showed little higher activity with high ester pectin than with low ester pectin while stem and leaf showed

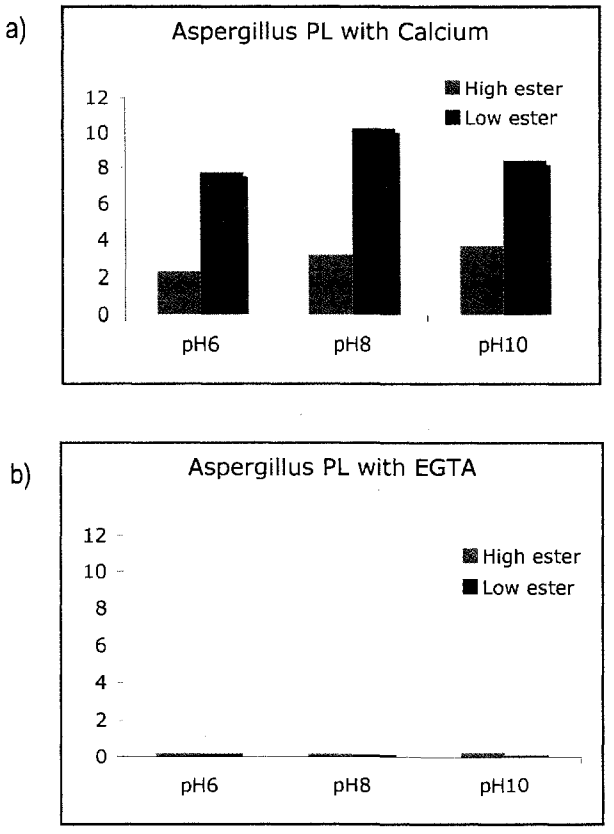


Figure 3.3. Pectate Lyase activity of Aspergillus sp. Two different kinds of substrates, high ester pectin and low ester pectin, were used. The assay was done at three different pHs (pH 6, 8,and 10). a) PL activity with 1 mM calcium, b) PL activity with 3 mM EGTA.

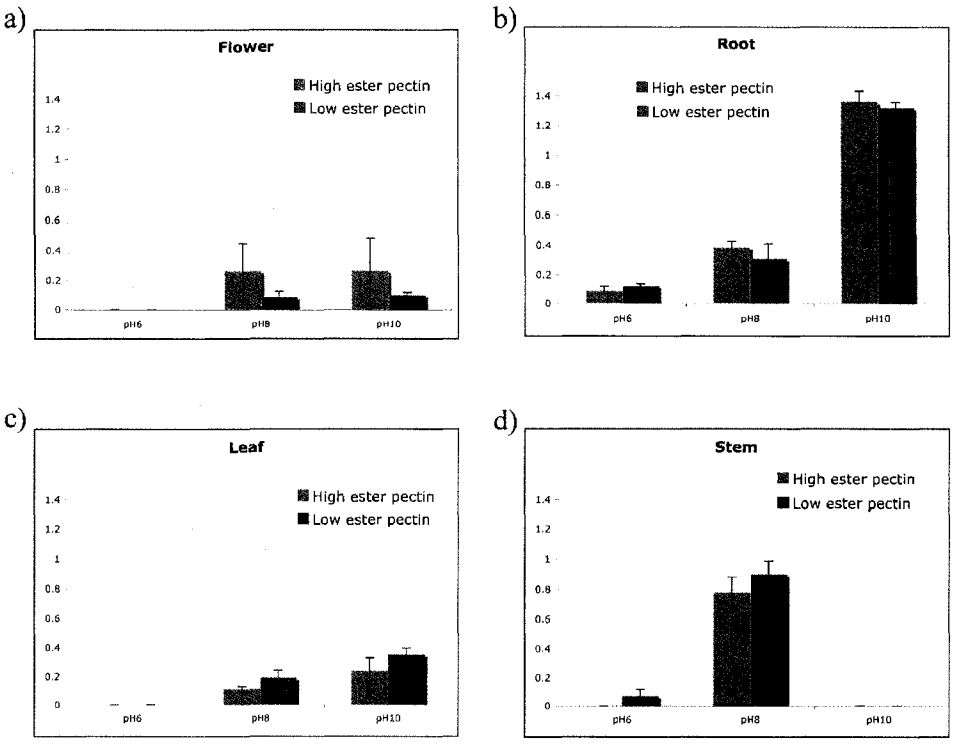


Figure 3.4. Pectate Lyase activity in protein extract of Arabidopsis flowers, roots, leaves, and stems. Assay was done for 1 hour incubation at 37 °C with 1 mM calcium.

better activity with low ester pectin (Figure 3.4), suggesting a difference in substrate preference in PLs from different tissue. Over all, these results show that different tissues have PL activity, but the activity varies depending on pH, the type of tissue and the type of substrate.

PL activity of bacterially expressed AtPLLs

Although total protein extract from Arabidopsis tissues showed pectate lyase activity, it is not clear that the products of *AtPLL* genes that interact with AtNPG1 have pectate lyase activity or are responsible for the enzyme activity shown in the above experiment. To see if AtPLLs are real pectate lyases and have enzyme activity, two Arabidopsis *PLLs* (*AtPLL 10* and*At PLL 11*) were expressed in bacteria (Figure 3.5). and used for a pectate lyase assay. Pectate lyase activity of AtPLL 10 and 11 was measured using the method described above. As shown in Table 3.2, no enzyme activity was detected with either of these AtPLLs.

Bacterially expressed AtPLLs also did not show pectate lyase activity, and it might be that i) other bacterial proteins inhibit enzyme activity, ii) proteins expressed in bacteria do not fold properly, iii) proteins expressed in bacteria lack posttranslational modification needed for the enzyme activity or iv) another protein such as AtNPG1 might be needed to activate the enzyme. To address the first possibility, bacterially expressed AtPLLs were purified using a His affinity column. AtPLLs bound to the His column were eluted and confirmed by western blotting (Figure 3.6). Purified AtPLLs were then used for the pectate lyase assay as above. No enzyme activity showed with both types of pectin and at all three different pHs (Table 3.3). To see if AtNPG1 is necessary for enzyme activity, bacterially expressed AtNPG1 was purified with a His affinity column

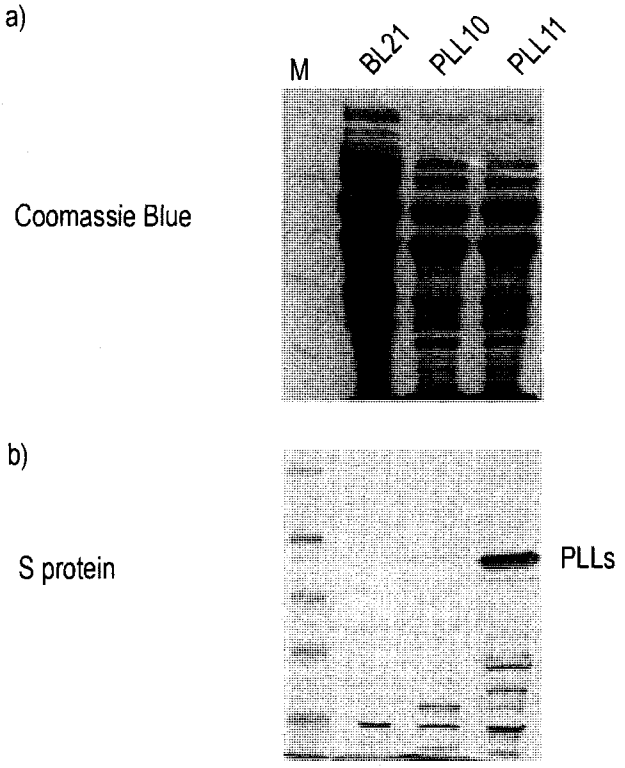


Figure 3.5. Bacterially expressd PLL10 and PLL11 were separated on SDS-PAGE gel. a) coomassie blue staining b) Western blot with S protein. M, Molecular Weight; BL21, BL21 without PLL was used as a negative control.

Table 3.2. Pectate lyase assay of bacterially expressed PLL10 and PLL11

		pH 6	pH 8	pH 10
PLL10	High ester pectin	-	-	-
	Low ester pectin	-	-	-
PLL11	High ester pectin	-	-	-
	Low ester pectin	-	-	-

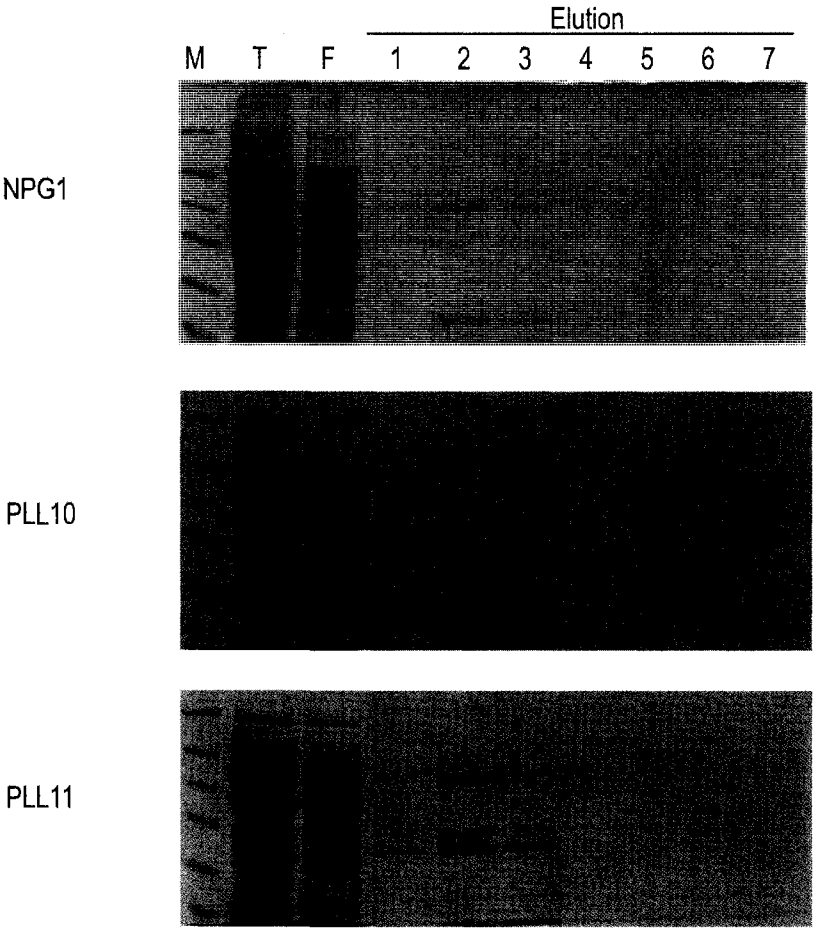


Figure 3.6. Bacterially expressed NPG1, PLL10 and PLL11 were purified with His affinity column. Names of proteins are on the left. M, Molecular Weight; T, total protein extract; F, flow through.

Table 3.3. Pectate lyase assay of His purified NPG1, PLL10 and PLL11

		pH 6	pH 8	pH 10
PLL10	High ester pectin	-	-	-
	Low ester pectin	-	-	-
PLL11	High ester pectin	-	-	-
	Low ester pectin	-	-	-
PLL10 /NPG1	High ester pectin	-	-	-
	Low ester pectin	-	-	-
PLL11 /NPG1	High ester pectin	-	-	-
	Low ester pectin	-	-	-

(Figure 3.6) and added to His purified AtPLLs. Again, no enzyme activity was detected with both kinds of pectin and in all three pHs (Table 3.3).

PL activity of yeast expressed PLLs

Since eukaryotic proteins might not be correctly expressed and processed in bacteria, *Arabidopsis PLLs* (*AtPLL8, 9, 10,* and *11*) were cloned into a yeast expression vector and expressed in yeast cells. AtPLLs were expressed in yeast and extracted. The extracted proteins were identified by western blot with Gal 4 AD antibody (Figure 3.7b). Three of these AtPLLs were expressed well in yeast. However, there is considerable degradation of expressed proteins. AtPLLs expressed in yeast were used for pectate lyase enzyme assay as mentioned before. Two different kinds of pectin, high ester and low ester pectin, were used for assay at three different pH conditions (pH 6, 8, and 10). All four Arabidopsis PLLs showed no enzyme activity (Table 3.4).

Isolation of a homozygous line of *AtPLL8* T-DNA mutant

AtPLL8 codes for a putative pectate lyase in *Arabidopsis*. Using a RT-PCR assay (Figure 3.2b) and in microarray analysis (https://iii.genevestigator.ethz.ch), it has been shown that *AtPLL8* is expressed in root, stem, and leaf tissues with high expression in flowers and pollen. *AtPLL8* has 3 exons, and the T-DNA insertion was in the 2nd exon (Figure 3.8a). To study its role in plant growth and development, the T-DNA insertion mutant of AtPLL8 (CS871253 *atpll8*) was analyzed for germination rate and growth under various hormonal and chemical conditions on plates as well as plant growth and development on soil.

To identify a homozygous T-DNA insertion line, mRNA was extracted from plant leaves from line CS871253 and used to synthesize cDNA. The cDNA was used as a

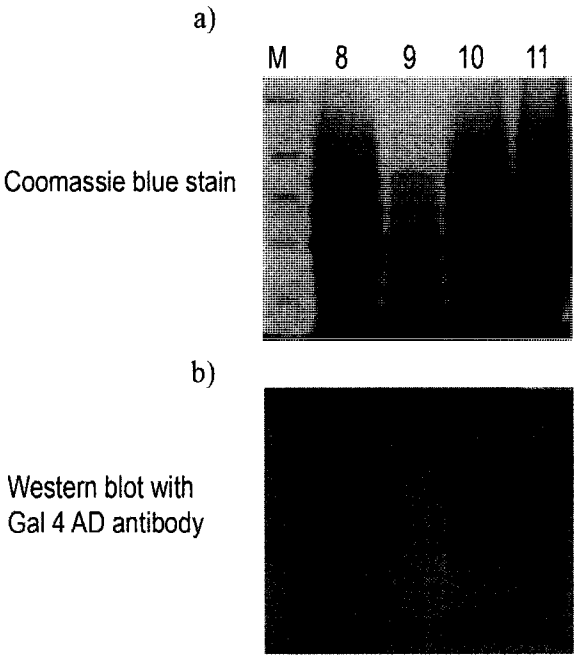


Figure 3.7. Yeast expressed *Arabidopsis* PLLs (PLL8, 9, 10, and 11) were separated on SDS-PAGE gel. a) Coomassie blue staining b) Western blot with Gal4 AD antibody. Numbers 8-11 correspond to PLLs 8-11, respectively. M, Molecular Weight.

Table 3.4. Pectate lyase assay of yeast expressed PLLs

		pH 6	pH 8	pH 10
PLL8	High ester pectin	-	-	-
	Low ester pectin	-	-	-
PLL9	High ester pectin	-	-	-
	Low ester pectin	-	-	-
PLL10	High ester pectin	-	-	-
	Low ester pectin	-	-	-
PLL11	High ester pectin	-	-	-
	Low ester pectin	-	-	-

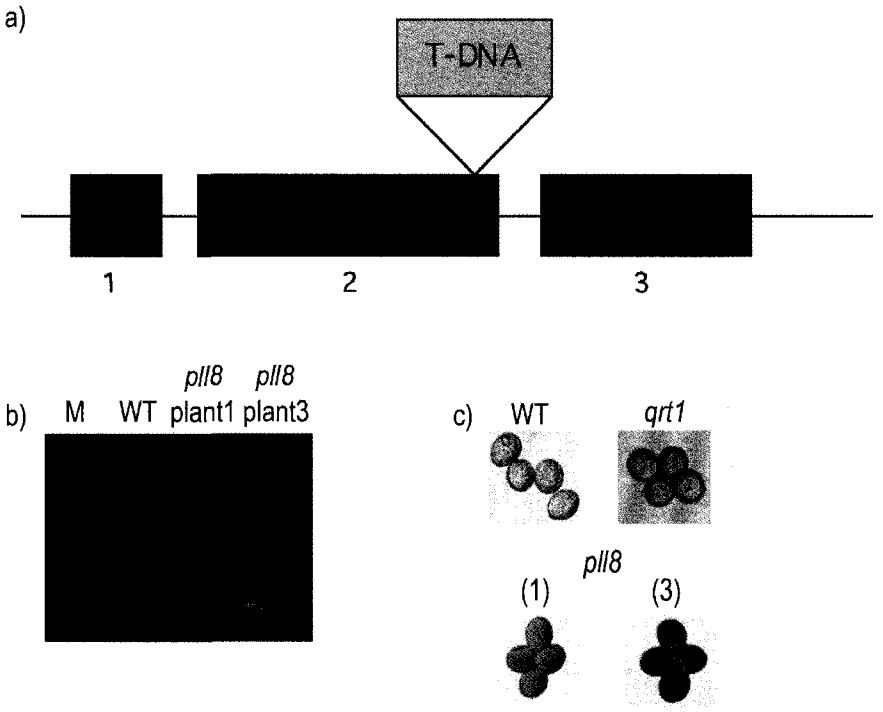


Figure 3.8. Homozygous line of *PLL8* T-DNA inserted mutant, CS871253. a) Schematic diagram showing the site of T-DNA insertion of *pll8* (CS871253), b)RT-PCR analysis to identify *pll8* homozygous line, c) GUS activity in quartet pollen of CS871253.
WT, *Arabidopsis thaliana* wild type Columbia; *qrt1*, quartet1-2 mutant; *npg1/qrt1*, *npg1* and *qrt1* double mutant

template to amplify *AtPLL8* transcript using gene-specific primers. The homozygous line did not show *AtPLL8* transcript (Figure 3.8b), indicating that it is a null mutation. Since the inserted T-DNA contains a GUS reporter gene driven by a pollen promoter and is in pollen tetrad defect mutant (*qrt1-2*) background, GUS activity on tetrad pollen was monitored to identify the homozygous mutant line. Mature pollen was collected from open flowers and submerged in GUS staining buffer and X-glu solution. Pollen from wild type, *qrt1-2*, and *atnpg1/qrt1*, were used as negative controls. All four pollen grains in the tetrad from *atpll8* showed GUS activity (Figure 3.8c). The homozygous mutant line confirmed by both RT-PCR and GUS activity was used for phenotypic characterization.

Phenotypic characterization of *atpll8*

To see the mutation effect on germination and seedling growth, homozygous seeds were plated under the following conditions: MS, MS with 0.5 μM ABA, 1 μM ABA, 0.5 μM BA, 1μM BA, 1 μM IAA, 3% glucose, 5%glucose, 100 mM NaCl, or 150 mM NaCl. The plates were incubated at 22 °C and 16 hours light/ 8 hours dark. The *atpll8* mutant plant development was monitored and compared with *quartet 1-2* control. There was no significant difference in germination rate, leaf and root development and plant size under all the tested conditions (Figure 3.9 to 3.13).

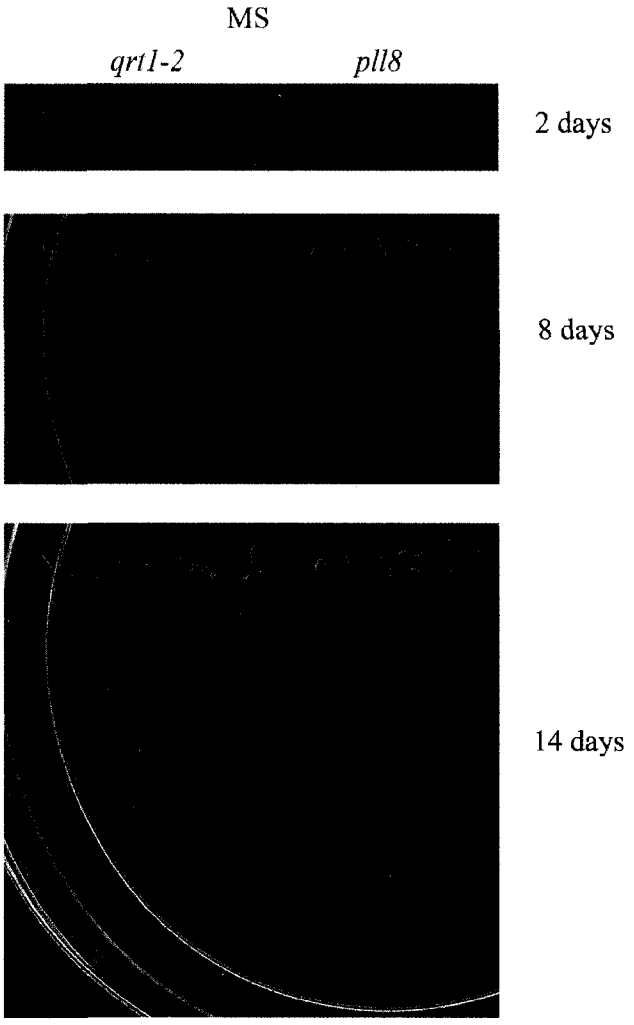


Figure 3.9. *pll8* (CS871253) on MS plate. The days of seedling growth are indicated on the right. *qrt1-2, quartet1-2* mutant as control.

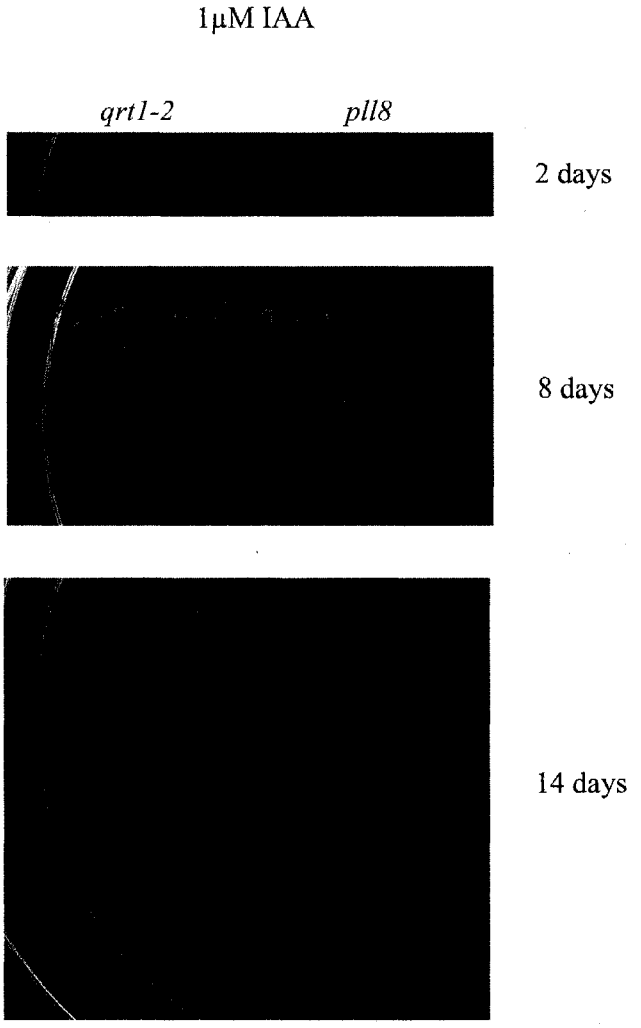


Figure 3.10. *pll8* (CS871253) on MS and 1 μM IAA (indoleacetic acid) plate. The days of seedling growth are indicated on the right. *qrt1-2, quartet1-2* mutant as control.

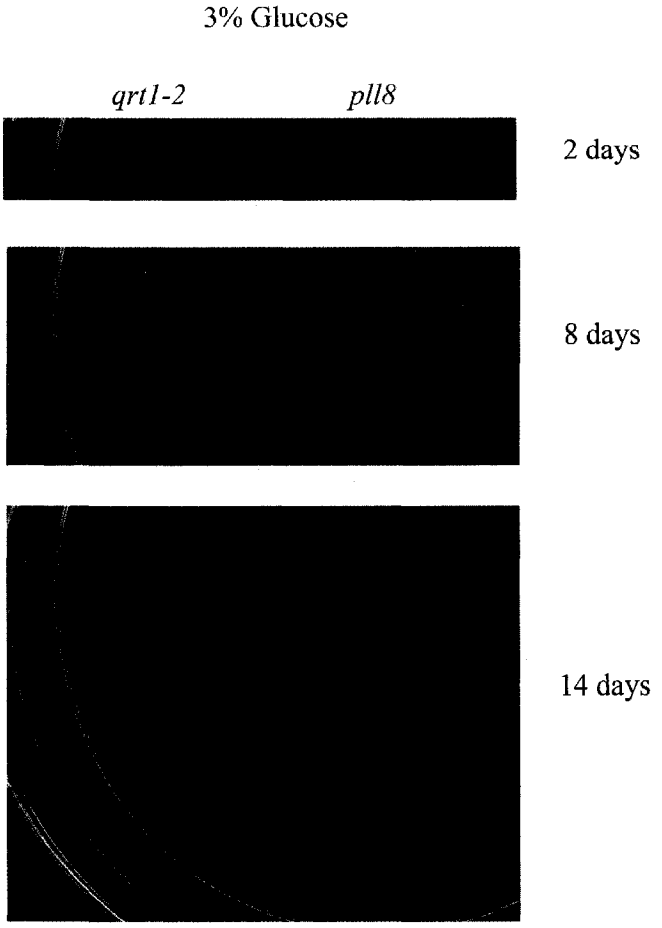


Figure 3.11. *pll8* (CS871253) on MS and 3% glucose plate. The days of seedling growth are indicated on the right. *qrt1-2, quartet1-2* mutant as control.

Figure 3.12. *pll8* (CS871253) on MS and 5% glucose plate. The days of seedling growth are indicated on the right. *qrt1-2, quartet1-2* mutant as control.

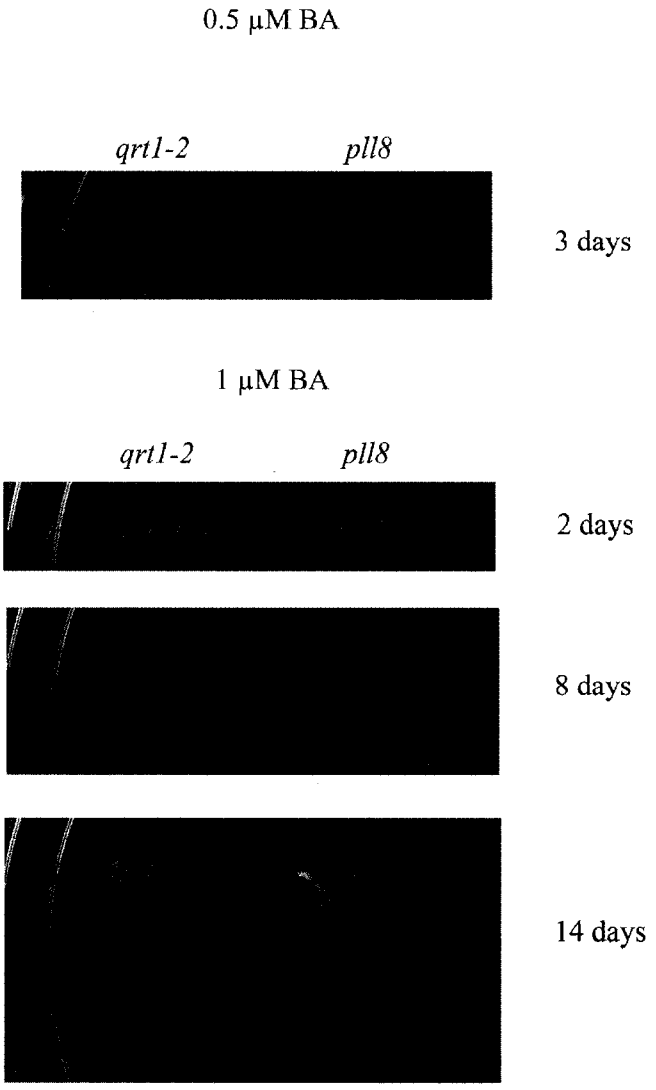


Figure 3.13. *pll8* (CS871253) on MS and 0.5 or 1 μM BA (6-benzylamino purine) plate. The days of seedling growth are indicated on the right. *qrt1-2, quartet1-2* mutant as control.

Discussion

Pectate lyase is a cell wall modifying enzyme and is thought to be involved in cell wall expansion during cell growth by degrading pectin. Pectate lyases have been well studied in fungal and bacterial pathogens (Collmer and Keen, 1986). During the pathogen invasion of plants, pectate lyase is involved in degrading the cell wall structure and allowing pathogens to penetrate the plant (Collmer and Keen, 1986; Barras, 1994). There are 26 genes encoding pectate lyase-like (PLLs) proteins in Arabidopsis, and all AtPLLs have pectate lyase active site sequence (RMPRPCR) (Domingo et al., 1998; Palusa et al., 2007). Besides pectate lyase (PL) active site, a calcium-binding site containing three Asp residues is conserved in all AtPLLs (Domingo et al., 1998; Palusa et al., 2007). The similarity in sequence suggested that AtPLLs in Arabidopsis might have a pectate lyase activity and be involved in plant development by modifying cell wall structure.

Since pollen tubes are fast growing, it has been thought that the pollen tube cell wall reorganizes its structure in a dynamic manner. Since AtNPG1, which is essential for pollen germination, interacts with AtPLLs I analyzed the expression of all AtPLL encoding genes in pollen. Using RT-PCR, fourteen of the 26 *AtPLLs* were found to be expressed in pollen and four (*AtPLL 8, 9,10* and *11*) expressed highly in pollen (Figure 3.2), suggesting that they might have a role in pollen tube growth and development. Those four *AtPLLs* have a high sequence similarity to tomato and tobacco *LAT 59*. *LAT 56* and *LAT 59* are *PLL* genes with very high expression in anthers and pollen (Budelier et al., 1990; Wing et al., 1990; Kulikauskas and McCormick, 1997). In pollen, it is proposed that pectate lyase might be involved in pollen tube emergence and growth by

loosening the pollen tube cell wall and in pollen tube penetration into female tissue by depolymerizing pectin in the cell wall and extracellular matrix in transmitting tissue (Taniguchi et al., 1995; Wu et al., 1996). The four *AtPLLs* that are highly expressed in pollen are also expressed in other tissues (Figure 3.2). So far, one of the Arabidopsis pectate lyase-like proteins role in plant development has been studied. *PMR6* (*AtPLL13*) was shown to play a role in powdery mildew pathogen invasion and in control of plant cell size by modify cell wall composition (Vogel et al., 2002). Although pectate lyase enzyme activity of PMR6 could not be measured, the cell wall structure of *pmr6* showed high levels of pectin with a lower degree of esterification and a modification in the H-bonding environment of cellulose microfibrils (Vogel et al., 2002). This suggested that PMR6 might have a role in pectin metabolism. Previous studies showed PLLs might play a role in various stages of plant growth and development including vascular differentiation (Domingo et al., 1998; Milioni et al., 2001), cell-to-cell adhesion (Rhee and Somerville, 1998; Mollet et al., 2000), lateral root development (Laskowski et al., 2006), and fruit ripening (Dominguez-Puigjaner et al., 1997; Medina-Escobar et al., 1997; Medina-Suarez et al., 1997; Nunan et al., 2001; Marin-Rodriguez et al., 2002; Benitez-Burraco et al., 2003; Marin-Rodriguez et al., 2003).

Although 26 *AtPLLs* showed a high sequence similarity with PL, enzyme activity of these AtPLLs has not yet been demonstrated. Fungi, such as *Aspergillus*, *Penicillium*, and *Fusarium* sp, are known to be major producers of pectate lyase, and one bacterium, *Erwinia*, also produces pectate lyase (Gummadi and Kumar, 2005). Aspergillus pectate lyase has been used widely to study pectate lyase, since it can be expressed as a recombinant protein. Purified Aspergillus pectate lyase was used as a positive control to

show the activity of Aspergillus pectate lyase with different substrates. To assay pectin lyase activity in Arabidopsis tissues, two kinds of pectin were used. Both types of pectins share a common structure of a linear chain of α(1-4)-D-galacturonic acid residues, but they have different degrees of methylation and have other side chains. High ester pectin has more than 50% methylated side chains, and it is thought that the golgi apparatus produces the high ester form of pectin and the pectin, is transported to the cell wall and gets de-methylated. Low ester pectin has less than 50% of methylated side chains, and it is the de-methylated pectin that composes the plant cell wall. With these two different substrates, the Aspergillus pectate lyase showed a preference for low ester pectin, i.e., Aspergillus pectate lyase showed a much higher activity with low ester pectin than with high ester pectin (Figure 3.3a). Bacterial and fungal pathogen studies also showed 2 or 3 times higher activity with low ester pectin than high methyl ester pectin (Hansen et al., 2001; Gummadi and Kumar, 2005; Solbak et al., 2005). As previously reported, PL activity requires calcium (Figure 3.3b) (Barras, 1994; Domingo et al., 1998; Centanni et al., 2001).

Protein extract from different Arabidopsis tissues showed pectate lyase activity (Figure 3.4). In comparing the activities of Aspergillus pectate lyase and plant protein extracts, two differences were found. First, Aspergillus pectate lyase showed much higher activity than plant protein extract with the same amount of protein. This is expected since Aspergillus PL used in the assay was purified. Second, some of the plant protein extracts, such as flower and root, have a different activity profile than Aspergillus pectate lyase in that the Arabidopsis extract showed higher activity with high ester pectin than with low ester pectin. It has been suggested that the role of plant pectate lyase might

be in regulation of the hardness of the cell wall (Willats et al., 2001). Newly synthesized methyl ester pectin is transported to the cell wall, and it becomes de-methylated by pectin methyl esterase (PME) (Willats et al., 2001). At the de-methylated region, pectin can form a cross-link, mediated by calcium, to provide a harder cell wall (Willats et al., 2001). Cell wall hardness has been known to be affected by not just PME, but also pectate lyase enzyme (Willats et al., 2001). Some of the plant protein extracts show a different digestion profile than Aspergillus pectate lyase. Also, some of plant protein extracts prefer to digest high ester pectin rather than low ester pectin. It is known that pectin lyase digests high methyl ester pectin derivatives preferably; pectin lyase has a different structure in its oligosaccharide-binding loop than pectate lyase (Vitali et al., 1998; Gummadi and Kumar, 2005; Solbak et al., 2005). In a study of three Erwinia pectate lyase isoforms, it was shown that combined pectate lyase isoforms have a much higher activity with high ester pectin than any individual isoenzyme, but combined pectate lyase isoforms have no difference with low ester pectin or pectate (Bartling et al., 1995). Based on their research, Bartling et al. (1995) suggest a synergism in the action of pectin lyases. With a combination of pectate lyase isoforms, the range of pectic substrates that could be digested was extended. It has been shown that Arabidopsis has 26 pectate lyase-like genes, and they are expressed differentially in different tissues (Palusa et al., 2007). The pectate lyase-like gene expression analysis in Arabidopsis tissues showed that 22 genes are expressed in roots, 16 in stems, 15 in leaves and all in flowers (Palusa et al., 2007). To understand the function of each of the pectate lyase-like proteins, it is necessary to establish a heterologus expression system in bacteria, yeast or baculovirus, but so far there has been little success with the expression of PLLs in heterologus

expression systems for biochemical studies.

It has been shown that pectate lyase has a high pH optimum. Pectate lyase in fungal pathogens has an optimum of pH 9.4, and pectate lyase in Japanese cedar pollen and Zinnia has the highest activity at pH 10 (Crawford and Kolattukudy, 1987; Dean and Timberlake, 1989; Taniguchi et al., 1995; Domingo et al., 1998). Although pectate lyase is suspected to be involved in cell wall expansion during plant development, plant pectate lyase and plant protein extract (Figure 3.4) showed low activity at low pH (Taniguchi et al., 1995; Domingo et al., 1998). According to the "acid–growth hypothesis", H^+-ATPase, regulated by auxin, transports protons into the cell wall. This causes the cell wall to be acidic, and this results in a loosening the cell wall that result in cell expansion due to turgor pressure (Cleland, 1976; Jacobs and Ray, 1976; Rayle and Cleland, 1977). During acidification of the cell wall, the pH of the cell wall drops from pH 7 to pH 4.5. Since cell wall modification enzymes including pectate lyase show no activity in low pH conditions where acid growth is supposed to happen, it is unclear how pectate lyase can be involved in cell wall growth.

Although PL activity in Arabidopsis tissues was shown and *AtPLLs* genes has been identified, it is not known if any of these *AtPLLs* are bonafide pectate lyase enzymes and how they affect plant development. Unfortunately, our attempt to measure the AtPLL enzyme activity expressed in bacteria and yeast was not successful (Table 3.2-3.4). Although the AtPLLs were expressed, enzyme activity was not detected (Figure 3.5-7). This might be because the plant PLLs were not correctly folded and/or modified posttranslationally. *atpll8*, a T-DNA insertion mutant, showed no difference in plant development compared to its parental background of the mutant (Figure 3.9-13). Since

several AtPLLs are expressed in pollen and other tissues, it is likely that there is some functional redundancy.

There are several problems in studying plant pectate lyases. First, a heterologus expression system for plant pectate lyase is not available, and this prevents biochemical characterization of these enzymes. Second, there are few tools available to monitor how the cell wall modification enzymes affect cell wall structure and integrity. To study the effect of pectin degrading enzymes on the cell wall, one of the most promising methods might be to study the mutant of putative plant pectate lyase genes. Dr. MaCann's group at Purdue University suggests Fourier transform infrared (FTIR) micro-spectroscopy could be the best way to study the cell wall pectin composition change in pectin lyase mutants (Chen et al., 1998). Since biochemical methods have many obstacles for studying plant pectate lyase and other pectin modification enzymes, the phenotype of loss of function single or double mutants together with cell wall composition analysis using the new methods such as FTIR might be the best answer for future PLLs research.

CHAPTER 4

Analysis of interaction between AtNPGs and ZmProfilin

Abstract

The actin component of the cytoskeleton plays an important role in many cellular processes including cell polarity and tip growth. Actin binding proteins the organization and dynamics of F-actin microfilaments. Profilin is one of the many actin binding proteins that is implicated in microfilament organization and dynamics. During pollen tube growth and guidance, extracelluar as well as intracellular signals reorganize actin filaments. Recently, Dr. Chris Staiger's group at Purdue University isolated the maize NPG1 homolog using a profilin affinity column. This raised the possibility that NPG1 might directly interact with profilin and regulate pollen germination and/or tube growth by modulating actin organization. To test if NPGs directly interact with profilin, I used ZmPRO1 (a *Maize* pollen specific profilin) and NPGs in pull down and yeast two-hybrid assays. My results showed no direct interaction between profilin and NPG1 or NPGRs, suggesting that NPG1 may interact with NPG1 through other proteins in a complex.

Introduction

Actin is a major component of the cytoskeleton in eukaryotic cells and actin filaments play an essential role in various cellular processes, including cytoplasmic streaming, organelle movement, cytokinesis, establishment of polarity and tip growth (Kost et al., 1999; Staiger, 2000b, a; Wasteneys, 2000). Actin filaments can be reorganized by polymerization and de-polymerization, and this reorganization is controlled by actin binding proteins (Staiger, 2000a). There are more than 70 families of actin binding proteins (Pollard, 2001), which can be separated into several groups. Changes in calcium and pH affect the activity of these proteins. The actin binding proteins are involved in signaling transduction pathways that transfer extracelluar signals or endogenous cues to cytoskeleton reorganization (Schmidt and Hall, 1998; Staiger, 2000b).

Profilin is a 12 to 15 kD cytoplasmic actin-binding protein that is ubiquitous in various organisms among eukaryotes (Staiger, 2000a). Based on Arabidopsis profilin under-expression and in vivo expression studies, it has been suggested that it is involved in various cellular functions in plants, such as cell elongation, cell shape maintenance, and flowering (Ramachandran et al., 2000). Profilin binds not just to the actin monomer, but it can also interact with some other proteins: phosphatidylionositol-4,5-bisphosphate (Sohn et al., 1995), poly-L-proline (Bjorkegren et al., 1993; Gibbon et al., 1998), a proline-rich protein called vasodialator-stimulated phosphoprotein (Haffner et al., 1995; Reinhard et al., 1995), formin homology domain-containing proteins (Frazier and Field, 1997; Kamei et al., 1998), Arp2/3 complex (Mullins et al., 1998; Loisel et al., 1999), and annexins (Alvarez-Martinez et al., 1996; Alvarez-Martinez et al., 1997). In regard to

actin filaments, profilin is likely to be involved in both polymerization and de-polymerization. Although it is still in debate, the role of profilin in actin polymerization is dependent on the presence of other actin binding proteins or the molecular ratio between profilin and actin molecules (Kovar et al., 2000).

Plant profilin was originally isolated from birch pollen (Valenta et al., 1991; Valenta et al., 1993). Plants have multiple isoforms, and it is estimated that both Arabidopsis and Maize have six isoforms of profilin (Staiger et al., 1993; Christensen et al., 1996; Huang et al., 1996; Staiger et al., 1997; Gibbon et al., 1998). These isoforms differ in their biochemical properties and can be classified as two groups depending on their expression: constitutive (vegetative) and pollen-specific (reproductive). (Kovar et al., 2000; Kandasamy et al., 2002). ZmPRO1 is a maize profilin isoform, which is 131 amino acids long and is expressed specifically in anther/pollen (Staiger et al., 1993). Although actin filament polymerization/de-polymerization and calcium signaling are essential for pollen germination and tube growth, it is still not clear as to how they are functionally connected to each other. Recent work of Dr. Chris Staiger's lab at Purdue University revealed a possibility that AtNPG1 through profilin (ZmPRO1) may affect actin organization (personal communication). They isolated AtNPG1 homolog from maize using profilin affinity column chromatography. In this chapter, I tested the interaction between AtNPG1 and ZmPRO1. My data show that there is no direct interaction between profilin and AtNPGs, suggesting that it may interact with profiling indirectly through other proteins.

Materials and Methods

Pull down assays with AtNPG1 and ZmProfilin

AtNPG1 was expressed in BL21 as a T7-tag fusion (See chapter 2). ZmPRO1, purified Maize profilin, was kindly provided by Dr. Chris Staiger (Purdue University). The bacterially expressed soluble fraction of AtNPG1 was incubated with 800 µl of T7-tagged antibody agarose beads at 4°C for 1 hour. After incubation, the mixture was centrifuged at 500g for 10 min at 4°C, and the supernatant was removed. The beads were resuspended in 1 ml of bind/wash buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM $CaCl_2$) and incubated at 4°C for 10 min with rotating. The beads were centrifuged at 500xg for 10 min at 4°C, and the supernatant was removed. The 800 µl of T7-tagged antibody agarose beads was split into four tubes with 200 µl in each. ZmPRO1 were incubated with the beads at 4°C for 1 hour. The conditions of incubation are given below:

1) T7-tag bead +AtNPG1 + ZmPRO1

2) T7-tag bead +AtNPG1 + ZmPRO1 with 1 mM EGTA

3) T7-tag bead +AtNPG1 + ZmPRO1 with 1 mM $CaCl_2$

4) T7-tag bead +AtNPG1 + ZmPRO1 with 100 µM $CaCl_2$ and 15 µM CaM

After incubation, the mixtures were centrifuged at 500g for 10 min at 4°C, and the supernatant was removed. The beads (1, 3, and 4) were resuspended in 1 ml of bind/wash buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM $CaCl_2$) and incubated at 4°C for 10 min. The beads from the 2nd treatment (with EGTA) were resuspended in 1 ml of bind/wash buffer (20 mM Tris-HCl pH 7.5 and 150 mM NaCl) and incubated at 4°C for 10 min. The beads were centrifuged at 500g for 10 min at 4°C, and the

supernatant was removed. The beads were washed with bind/wash buffer for five times. One hundred microliter of 1X SDS loading buffer was added to beads and boiled for 5 min. The mixtures were centrifuged at maximum speed with microcentrifuge for 5 min, and the supernatant was electrophoresed for 45 min at 200V on three 10% denaturing gels. One gel was stained with coomassie blue. The other two were blotted, and probed with either T7 tagged antibody (see chapter 2) or anti-profilin antibody.

To probe with anti-profilin antibody, the blot was incubated in 3% gelatin in TBST for 2 hours at 30°C with gentle shaking. Membranes were washed with TBST (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1% Tween 20) 3 times, 5 min each, and incubated for 2 hours at 30 °C in TBST containing 1% gelatin, and 1:10,000 dilution of anti-profilin antibody. Membranes were washed with TBST (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1% Tween 20) 4 times, 5 min each, and incubated for 2 hours at 30 °C in TBST containing 1% gelatin, and 1:5,000 dilution of anti-rabbit alkaline phosphatase conjugated antibody. Membranes were washed with TBST 3 times, 5 min each, and presoaked with AP buffer (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$). Then the blot was placed in color development solution [(10 mM AP buffer, 45 μl NBT (nitroblue tetrazolium chloride, 75 mg/ml) and 30 μl BCIP (5-bromo-4-chloro-3-indolylphosphate p-toluidine salt, 50 mg/ml)].

Yeast two-hybrid assay

To test interaction between AtNPGs and ZmPRO1 (a Maize profilin), *AtNPGs*-pACT2J clones (see chapter 2) were fused to the GAL4 activation domain and *ZmPRO1*-5 Gly linker-p426GPD or *ZmPRO1*-p426GPD were fused to the GAL4 DNA binding domain. The *ZmPRO1*-5 Gly linker-p426GPD and *ZmPRO1*-p426GPD clones were

provided by Dr. Cris Staiger lab at Purdue University. To determine the interaction, the combinations shown below were tested.

1) *ZmPRO1*-p426GPD (BD) + *AtNPG1*-pACT2J (AD)

2) *ZmPRO1*-p426GPD (BD) + *AtNPGR1*-pACT2J (AD)

3 *ZmPRO1*-p426GPD (BD) + *AtNPGR2*-pACT2J (AD)

4) *ZmPRO1*-5 Gly linker-p426GPD (BD) + *AtNPG1*-pACT2J (AD)

5) *ZmPRO1*-5 Gly linker-p426GPD (BD) + *AtNPGR1*-pACT2J (AD)

6) *ZmPRO1*-5 Gly linker-p426GPD (BD) + *AtNPGR2*-pACT2J (AD)

The constructs with binding domain (BD) fusions were introduced into PJ69-4A yeast cells. The yeast cells with the BD construct grew on Ura-SD plates and were used as competent cells for transformation of the construct with the activation domain (AD). The yeast cells with both BD and AD constructs grew on Ura-, Leu- SD, plate and the colonies on Ura-, Leu- SD plate were transferred to Ura-, Leu-, His- and 25mM AT SD plate to determine the interaction between the proteins fused to BD and AD. The following combinations were tested:

To confirm the interaction between proteins in the BD and AD construct, the β-galactosidase filter and liquid culture assays were done for each combination. In the β-galactosidase filter assay, Whatman #5 or VWR grade 410 filter paper was soaked with Z buffer/X-gal solution and placed on the plates to transfer yeast cells. Z buffer solution was made by mixing 16.1 g/L $Na_2HPO_47H_2O$, 5.5 g/L $NaH_2PO_4H_2O$, 0.75 g/L KCl, 0.246 g/L $MgSO_47H_2O$, pH 7.0. Z buffer/X-gal solution contained 0.135 ml β-mercaptoethanol, 0.5 ml X-gal (100 mg/ml) and 50 ml Z buffer. The filter was transferred into a pool of liquid nitrogen, where it was submerged for 10 sec. The filter was then placed on another

presoaked filter paper and incubated at room temperature. The PJ69-4A yeast cells with no constructs and the PJ69-4A yeast cells with the BD or AD construct were used as negative controls.

β-galactosidase activity was measured with CPRG (Chlorophenol red-β-D-galactopyranoside) as the substrate to quantify the interaction between ZmPRO1 and the NPGs. Each yeast colony was inoculated into 5 ml Ura-, Leu-, SD medium, and the cultures were grown overnight at 30°C with 250 rpm shaking. Two ml of the overnight culture was inoculated into 8 ml YPD medium, and the cultures were grown for 5 hours at 30°C with 250 rpm shaking, until an OD_{600} of 1 ml = 0.5-0.8 was reached. Then each culture was aliquoted into three 1.5 ml tubes and spun down at 14,000 rpm for 30 sec. Each of the pellets was resuspended in 1.0 ml of buffer 1 (2.38 g HEPES, 0.9 g NaCl, 0.06 5g hemimagnesium salt of L-aspartate, 1.0 g BSA, 50.0 μl Tween 20, pH 7.3 in 100 ml sterile water) and then spun at 14,000 rpm for 30 sec. The medium was poured off, and the pellet was resuspended in residual liquid that was about 100 μl of buffer 1. The tube was frozen in liquid nitrogen and thawed at 37°C for 1 min. This freezing/thawing process was repeated once. The sample was resuspended in 900 μl of buffer 2 (0.02709 g Chlorophenol red-β-D-galactopyranoside (CPRG) in 20 ml of buffer 1) with vortexing. The suspension was spun down at 14,000 rpm for 1 min, and the supernatant was decanted into a cuvet. The supernatant was incubated at room temperature for 60 min, and then 0.5 ml of 3 mM $ZnCl_2$ was added to stop the color development reaction. The sample was measured for its absorbance at 578 nm using a mixture of 1ml of buffer 1 and 0.5 ml of 3 mM $ZnCl_2$ as a blank. The β-galactosidase units were calculated as follows:

$$\text{Units} = (1000 \times OD_{578})/(\text{elapsed min} \times 1.5\text{ml culture} \times OD_{600})$$

The suspensions from the Y190 yeast cells with no constructs and the Y190 yeast cells with the BD or AD constructs were used as a negative control. The suspension from the Y190 yeast cells with *AtNPG1*-pAS1-CYH2 and clone *#5/#30* (petunia pectin lyase)-pGAD424 from the petunia library screening were used as positive controls.

Results

Dr. Chris Staiger's lab at Purdue University have been studying the role of maize profilins in actin organization as well as in pollen biology. In their efforts to isolate ZmProfilin binding proteins, they used ZmProfilin affinity column chromatography. Sequence analysis of proteins bound to ZmProfilin column revealed the presence of AtNPG1. Although this result suggests a possible interaction between AtNPG1 and ZmProfilin, it does not tell if profilin interacted with AtNPG1 directly or indirectly. To test a direct interaction, I performed a pull down assay. Bacterially expressed AtNPG1-T7 tag fusion protein and a Maize profilin, ZmPRO1, were used to test the possible interaction. AtNPG1-T7 tag fusion protein was incubated with T7 tag antibody agarose beads and unbound proteins were removed. The beads were then incubated with ZmPRO1 and washed to remove unbound proteins. During the incubation with ZmPRO1, the interaction was tested under four different conditions: ZmPRO1 only, ZmPRO1 with EGTA, ZmPRO1 with calcium, and ZmPRO1 with calcium and calmodulin. The proteins bound to beads were analyzed by using SDS-PAGE gel electrophoresis and western blotting. The beads bound AtNPG1 as show by western blot with T7 tag antibody, but no ZmPRO1 was detected on the blot with anti-profilin antibody (Figure 4.1). AtNPG1 and purified ZmPRO1 were used as positive controls (Figure 4.1).

To further study the interaction between AtNPGs and ZmPRO1 in vivo, the yeast two-hybrid assay was used. ZmPRO1 fused to DNA binding domain (BD) and AtNPGs fused to the activation domain (AD) were used. Beside ZmPRO1/BD domain, ZmPRO1-5Gly/BD that has a 5 glycine linker between ZmPRO1 and the BD was also used to provide fusion protein structural flexibility. The combinations tested for their interaction

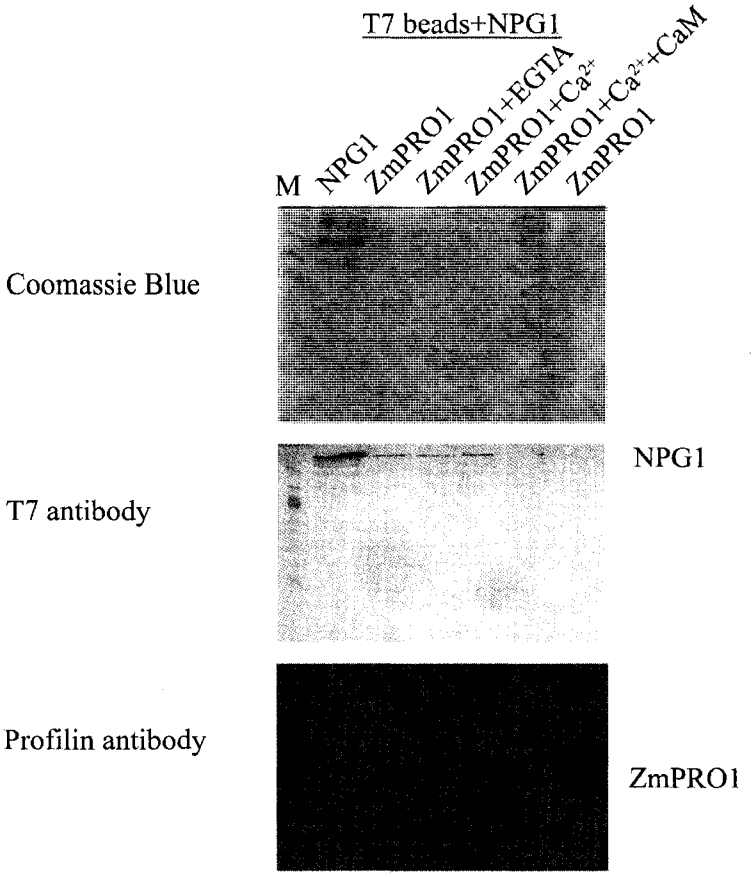


Figure 4.1. In vitro interaction between ZmPRO1 and NPG1 was studied using a pull-down assay. Bacterially expressed NPG1 was incubated with T7 tag antibody agarose beads and followed by incubation with ZmPRO1. Four conditions were used, ZmPRO1 alone, ZmPRO1+EGTA, ZmPRO1+Ca^{2+}, and ZmPRO1+Ca^{2+}+CaM. Proteins bound to beads were analyzed with coomassie blue stain and western blot by probing with T7 tag antibody or anti-profilin antibody. NPG1 in crude proteins (1st lane) and purified ZmPR01 (last lane) were used as controls.

between the two ZmPRO1 constructs and three AtNPGs are listed in the Methods section. Colonies that contained both plasmids grew on Ura- and Leu- plates indicating the presence of both plasmids. All combinations did not grow on Ura-, Leu-, His-, 25mM AT plates, indicating that they do not interact. To further confirm this, we measured the β-galactosidase activity of yeast colonies selected on Ura- and Leu- plates using filter as well as liquid culture assays. Liquid β-galactosidase assays, which permit quantification of interaction, were performed to detect any weak interactions. No activity was detected in all combinations tested (Table 4.1), indicating no interaction between AtNPGs and ZmProfilin.

Table 4.1. Yeast two-hybrid assay between NPGs and Profilin

AD\BD	ZmPRO1	ZmPRO1-linker
NPG 1	-	-
NPGR 1	-	-
NPGR 2	-	-

Discussion

The differences in expression and biochemical properties between plant profilin isoforms might be translated into functional distinctions. By studying maize and Arabidopsis profilin isoforms, it has been shown that they can be separated into two classes based on their expression: constitutive (vegetative) and pollen-specific (reproductive) (Staiger et al., 1993; Kovar et al., 2000; Kandasamy et al., 2002). Not only do they have a distinct regulation in expression, but they differ in their biochemical properties and functions (Staiger et al., 1993; Kovar et al., 2000). It has been suggested that the pollen-specific isoforms play a unique role by interacting with pollen-specific reproductive actin or other pollen-specific signaling pathways.

Since AtNPG1 has a pollen-specific role and is involved in calcium signaling, I thought that it might have a role in connecting calcium signals to response machinery, such as actin polymerization or cell wall modification. ZmPRO1 is a Maize profilin isoform and belongs to the reproductive class (Staiger et al., 1993). Because one of the proteins bound to a Maize profilin affinity column was identical to maize homolog of AtNPG1, it was thought that AtNPG1 might link the calcium signal to actin reorganization in pollen. To study this possibility, direct interaction between AtNPG1 and ZmPRO1 was studied *in vivo* in a yeast two-hybrid system and *in vitro*. Since it is known that calcium affects the affinity of profilin to actin (Staiger, 2000b) and AtNPG1 is a calmodulin-binding protein, pull down assays were done in four different conditions to see if the calcium and calmodulin effect on the interaction. With calcium and EGTA, there is no difference and ZmPRO1 did not bind AtNPG1. With calcium and calmodulin also no interaction was observed. For the yeast two-hybrid assay, AtNPG1 and two

AtNPGRs were also used to test the interaction with ZmPRO1. To eliminate the possibility that the ZmPRO1-BD fusion might interfere with the interaction between AtNPG1 and ZmPRO1 in yeast cells, ZmPRO1-5Gly linker-BD fusion, which allows structural flexibility, was also used. All combinations tested did not grow on Ura-, Leu-, His-, 25mM AT plates, indicating that they do not interact. In addition, with liquid β-galactosidase assays, no activity was detected in all combinations tested (Table 4.1). In conclusion, no direct interaction between AtNPG1 and ZmPRO1 *in vivo* or *in vitro* was observed, suggesting an other protein partner might mediate the interaction between AtNPG1 and ZmProfilin.

Although not all of the profilin genes in Arabidopsis and Maize have been characterized, amino acid sequence and nucleotide sequence similarity between three Arabidopsis profilins (PRF1, 2, and 3) and three Maize profilins (Zma 1, 2, and 3) were reported (Huang et al., 1996). It was shown that there is 68.4-74.5% identity in nucleotide sequence and 69.5-79.4% identity in amino acid sequence (Huang et al., 1996). The Maize pollen calmodulin binding protein (ZmMPCBP) was originally isolated from Maize pollen expression library and it was shown that the amino acid sequence of AtNPG1 and ZmMPCBP share 56% identity and 70% similarity, and all AtNPGs and ZmMPCBP share 39-56% identity and 56-70% similarity. Even though they are similar, the difference may account for the non-interaction of AtNPG1 and ZmPRO1. This can be tested by using the AtNPG1 homolog as well as profilin from maize.

CHAPTER 5

Functional analysis of AtNPGR1 role in plant development

Abstract

AtNPGR1, a paralog of AtNPG1, is expressed in pollen as well as in other tissues. In addition, AtNPGR1 interacts with AtNPG1. Since AtNPG1 has a role in pollen germination, I analyzed the function of AtNPGR1 using a loss-of-function mutant. I isolated an *npgr1* T-DNA insertion mutant from the Salk collection. Expression analysis of leaf tissue revealed no *AtNPGR1* transcript in the homozygous mutant, indicating that it is a loss-of-function mutant. Surprisingly, no pollen phenotype was observed in the *npgr1* mutant. I performed phenotypic analysis of the mutant in the presence of various hormones and chemicals (BA, ABA, IAA, NaCl, glucose and mannitol). The mutant showed no significant differences in root and shoot growth as compared to wild type in the presence of ABA, IAA, NaCl and mannitol. However, in the presence of BA, the mutant showed better growth in dark and increased shoot growth in light. In addition, germination and seedling development of the mutant was insensitive to glucose, suggesting that it might be involved in sugar sensing and/or signaling. Recent studies have shown that hexokinase1 is a sensor of a sugar signal, and it transduces the signal to a downstream sugar signaling pathway. To see if AtNPGR1 is in the hexokinase pathway, I analyzed the expression of five genes implicated in hexokinase pathway in the mutant. My results show that the expression of these genes is not changed in *npgr1* in the presence or absence of glucose, suggesting that the hexokinase pathway may not be affected in the mutant. Further genetic studies are needed to place AtNPGR1 in the known sugar signaling pathway.

Introduction

Recent studies have shown that sugars can act as a signaling molecule and regulate plant growth and development (Rolland et al., 2002). It has been known that sugar, the end product of photosynthesis, acts as a negative feedback regulator for photosynthesis by repressing photosynthetic gene expression (Koch, 1996). Moreover, it has been discovered that sugars are involved in regulation of plant growth during the whole life cycle. For example, high sugar level can repress seed germination and early seedling development and control both floral transition and leaf senescence (Rolland et al., 2002). Sugar signaling has been studied in various living organisms including bacteria, yeast, animals and plants (Stulke and Hillen, 1999; Johnston and Kim, 2005; Moreno et al., 2005; Santangelo, 2006). Recent studies have shown that a transporter like glucose sensor (Snf3 and Rgt2), G protein-coupled receptor (Gpr1) or hexokinase2 are involved in sensing glucose and translating glucose signals to regulate transcription of genes and growth (Entian, 1980; Lemaire et al., 2004; Johnston and Kim, 2005; Moreno et al., 2005). Among these sugar sensors, it has been shown that hexokinases are the most evolutionary conserved in yeasts, mammals and plants (Moore et al., 2003; Wilson, 2003; Moreno et al., 2005).

The role of Hxk in Arabidopsis was studied by using transgenic lines that either do not produce Hxk or overproduce it (Jang et al., 1997). With anti-sense Hxk, transgenic plants were sugar insensitive, whereas over-expressing Hxk plants showed hypersensitivity to sugar (Jang et al., 1997). The sugar-mediated gene regulation of chlorophyll a/b binding protein (*CAB1*), ribulose-1, 5-bisphosphate carboxylase small subunit (*RBCS*), and nitrate reductase (*NR1*) was also affected by Hxk expression.

Furthermore, it was shown that sugar signaling is not linked to sugar metabolism (Jang et al., 1997). By studying an Hxk1 mutants (*gin2*), it was shown that Hxk1 is involved in light and hormonal signaling as well and Hxk1 coordinates light signal to intrinsic signals to control cell expansion in roots, leaves, and inflorescences (Moore et al., 2003). Hxk1 might also play a role to connecting sugar to light and hormonal signal networks (Moore et al., 2003). To answer how Hxk1 mediates glucose and hormone signaling to control sugar related gene expression, Hxk interacting partners were identified (Cho et al., 2006). Three proteins [vacuolar H+-ATPase B1 (VHA-B1) and 19S regulatory particle of proteasome subunit (RPT5B)] were found to interact with Hxk1 (Cho et al., 2006). It was suggested that nuclear Hxk1 and those two partners form a glucose-signaling complex that regulates target gene expression (Cho et al., 2006).

AtNPGR1 is an AtNPG1 like protein (see chapter 1) that is expressed in suspension cells, flowers, pollen, fruits, and leaves. To study possible role for AtNPGR1 in pollen as well as in plant growth and development, I isolated a T-DNA insertion mutant line (*atnpgr1*) and used it for phenotype analyses under various hormonal and chemical conditions. With two different BA concentrations, *atnpgr1* showed a better germination rate and bigger size than wild type. With glucose, *atnpgr1* showed insensitivity to sugar during germination, and seedling growth and sugar insensitivity were more drastic with increased sugar concentration. The sugar insensitivity of *atnpgr1* suggests AtNPGR1 might play a role in sugar sensing and signaling. To see if AtNPGR1 is involved in the Hxk sugar signaling pathway, the expression of genes that are in Hxk pathway was analyzed in *atnpgr1*.

Materials and Methods

Isolation of a T-DNA insertion line

SALK090514 is an *AtNPGR1* T-DNA insertion mutant where the gene is interrupted in its #3rd exon. SALK090514 seeds from ABRC were grown on MS (Murashige and Skoog from Sigma) and 30 μg/ml kanamycin plates to select for plants with T-DNA insertions. To identify a homozygous *atnpgr1* line, RT-PCR assays with mRNA from *atnpgr1* plant leaves were performed. *AtNPGR1*-specific primers (5' *NPGR1*: 5'-GCA GAA TTC ATG TTG TGT GCT TGT TCA GGC G-3' and 3' *NPGR1*: 5'-GCA GGA TTC AAA TGA AAC TCT GTA CCG GAG-3') were used in PCR as described in chapter 3. The PCR product was visualized by using agarose gel electrophoresis.

Phenotype analyses of *npgr1*

To see how the mutation affected germination and seedling growth, *atnpgr1* seeds were plated under the following conditions: MS, MS with 0.5 μM ABA (abscisic acid), 1 μM ABA, 0.5 μM BA (6-benzylaminopurine), 1μM BA, 1 μM IAA (indoleacetic acid), 3% glucose, 5%glucose, 5% mannitol, 100 mM NaCl, or 150 mM NaCl. The plates were incubated at 22 °C and 16 hours light/ 8 hours dark and monitored for 2 weeks. The germination and seedling growth of *atnpgr1* was compared to wild type *Arabidopsis thaliana* Columbia. In addition, wild type and *atnpgr1* plants were grown in soil, and their growth and development was followed until seed set. Plants were grown at 22 °C and 16 hours light/ 8 hours dark and were observed from seed to seed stage. Plant development stages were observed according to Boyes et al (Boyes et al., 2001).

Expression of genes in Hxk signaling pathway.

Gene expression of five genes (hexokinase 1, carbonic anhydrase, vacuolar H^+-ATPase, sedoheptulose bisphosphatase and chlorophyll a/b –binding protein) in the hexokinase pathway was studied in *npgr1* mutant using RT-PCR. Two-week old seedlings grown under 22 °C and 16 hours light/ 8 hours dark condition were used for this assay. Seedlings were grown under three different conditions including MS, MS containing 3% glucose, and MS containing 3% mannitol. Total RNA was extracted from seedlings and cDNA was synthesized using methods described in chapter 3. The cDNA was used for PCR reaction with gene specific primers: hexokinase 1 (Forward: GTG TCA TGC GTG TGC TTC TT, Reverse: TTC TGC ACC TCC TCG TCT TT), carbonic anhydrase (Forward: TGA ATA CGC TGT CTT GCA CC, Reverse: TGT GAT GGT GGT GGT AGC GA), vacuolar H^+-ATPase (Forward: TGC TCA GAT TTG TCG TCA GG, Reverse: ATA CGG AGC AAT GTC CAA GC), sedoheptulose Bisphosphatase (Forward: ATG GAG ACC AGC ATC GCG TG, Reverse: CTT CCA CTG GAC CTC CCA T), and chlorophyl a/b –binding protein (Forward: ATG GCC ACT TCA GCA ATC CAA, Reverse: CAC AAC TTG ACA CGC CCC ATA T). PCR reaction condition described in chapter 3 were used. The PCR product was visualized on agarose gel. Cyclophilin was used as loading control.

Results

Isolation of *atnpgr1* mutant

AtNPGR1 is related to AtNPG1. Unlike *AtNPG1*, *AtNPGR1* has been shown to be expressed in a number of vegetative tissues. *AtNPGR1* has 5 exons with similar intron/exon organization to *AtNPG1*. AtNPGR1 also has a calmodulin-binding domain and 5 TPR domains. Although AtNPGR1 is similar to AtNPG1, it is still unknown if it has a role in plant development. To study the role of AtNPGR1 in Arabidopsis, a T-DNA insertion mutant of *AtNPGR1* was used (Figure 5.1a). I studied its phenotype under various hormonal and chemical conditions.

I identified the homozygous *atnpgr1* mutant by RT-PCR using *AtNPGR1* specific primers. Six SALK090514 plants grown on MS/Kan plate were used for RT-PCR, and five of them showed no *AtNPGR1* transcript in leaf tissue (Figure 5.1b). These homozygous lines were used for further analysis.

Phenotypic characterization of *atnpgr1*

To see if the mutation affected plant growth and development, homozygous seeds were plated under various conditions: MS, MS with 0.5 μM ABA, 1 μM ABA, 0.5 μM BA, 1μM BA, 1 μM IAA, 3% glucose, 5%glucose, 5% mannitol, 100 mM NaCl, or 150 mM NaCl. With 0.5 μM BA, *atnpgr1* was grown for 3 days under dark condition. As shown in Figure 5.3, *atnpgr1* plants were much bigger than wild type, and the germination rate of *atnpgr1* seeds was also higher than wild type seeds (Figure 5.3). Except for BA, IAA and glucose, no significant differences in seedling growth were observed between wild type and the mutant (Figures 5.2 and 5.7; Appendices 5, 6 and 7).

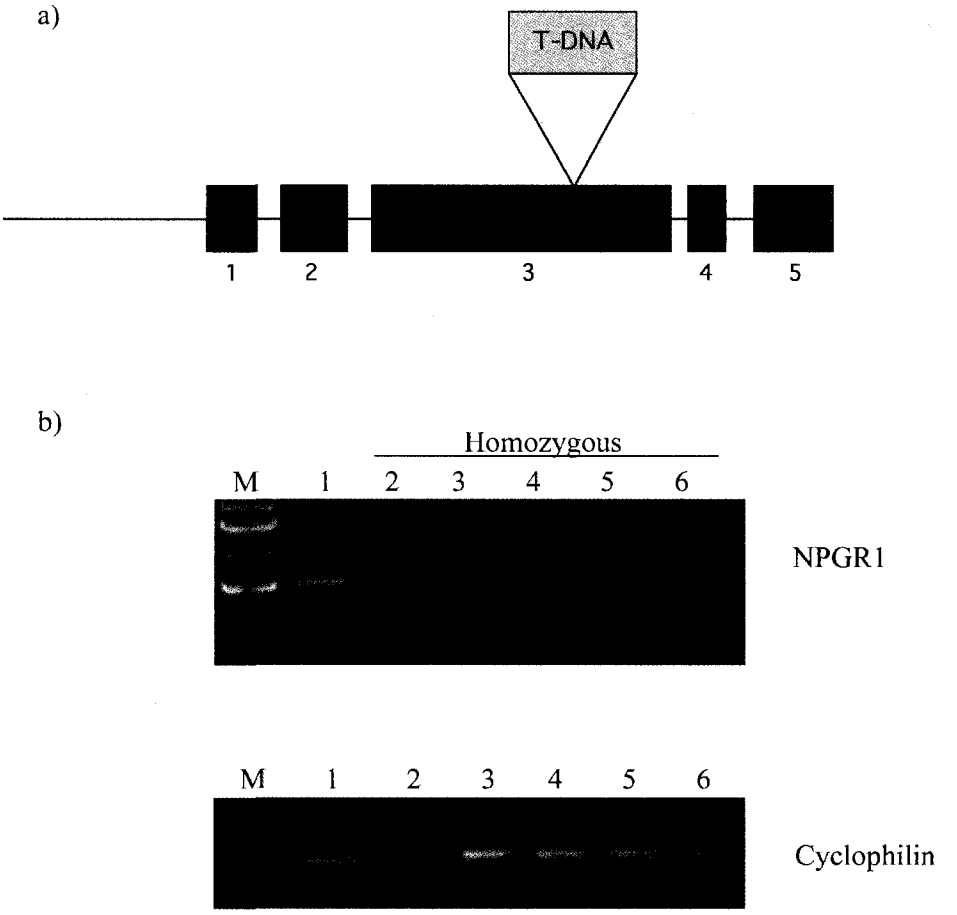


Figure 5.1. Homozygous line of *NPGR1* T-DNA inserted mutant, SALK090514.
a) Schematic diagram showing the site of T-DNA insertion of *npgr1* (SALK090514).
b) RT-PCR analysis to identify *npgr1* T-DNA homozygous line. Cyclophilin was used as loading control.

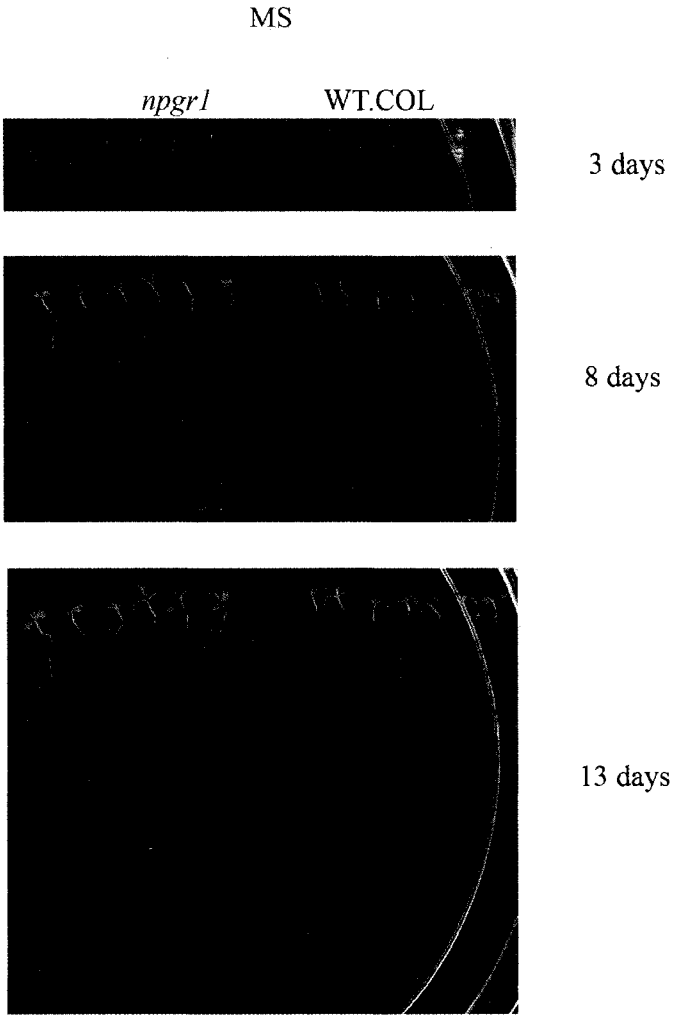
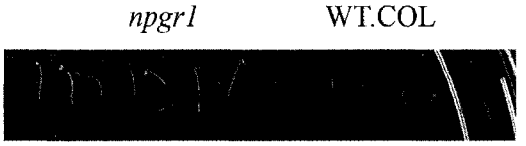


Figure 5.2. *npgr1* (SALK090514) on MS plate. The days of seedling growth are indicated on the right. WT.COL, *Arabidopsis thaliana* wild type Columbia as control.

a) 0.5 μM BA



3 days

b)

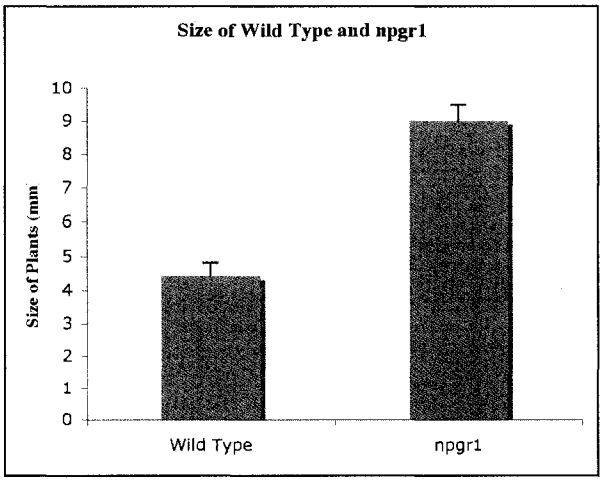


Figure 5.3. *npgr1* (SALK090514) on 0.5 μM BA/MS plate. The days of seedling growth are indicated on the right. WT.COL, *Arabidopsis thaliana* wild type Columbia as control.

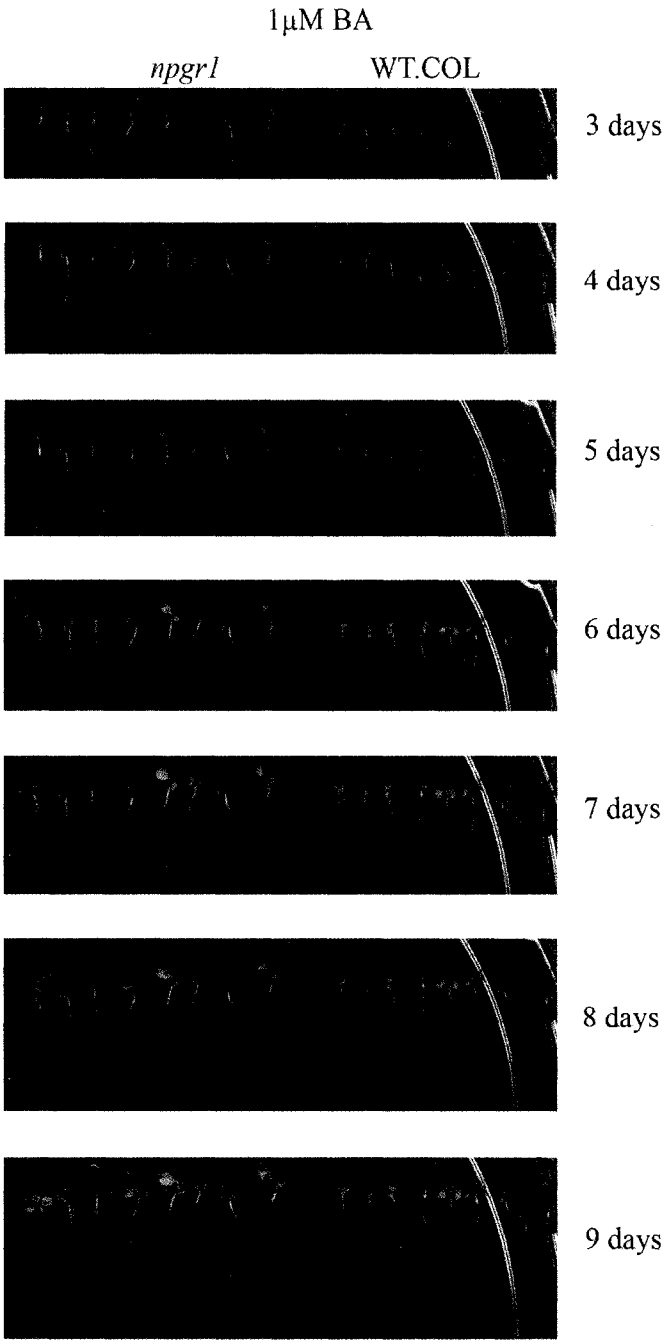


Figure 5.4a. *npgr1* (SALK090514) on 1μM BA/MS plate. The days of seedling growth are indicated on the right. WT.COL, *Arabidopsis thaliana* wild type Columbia as control.

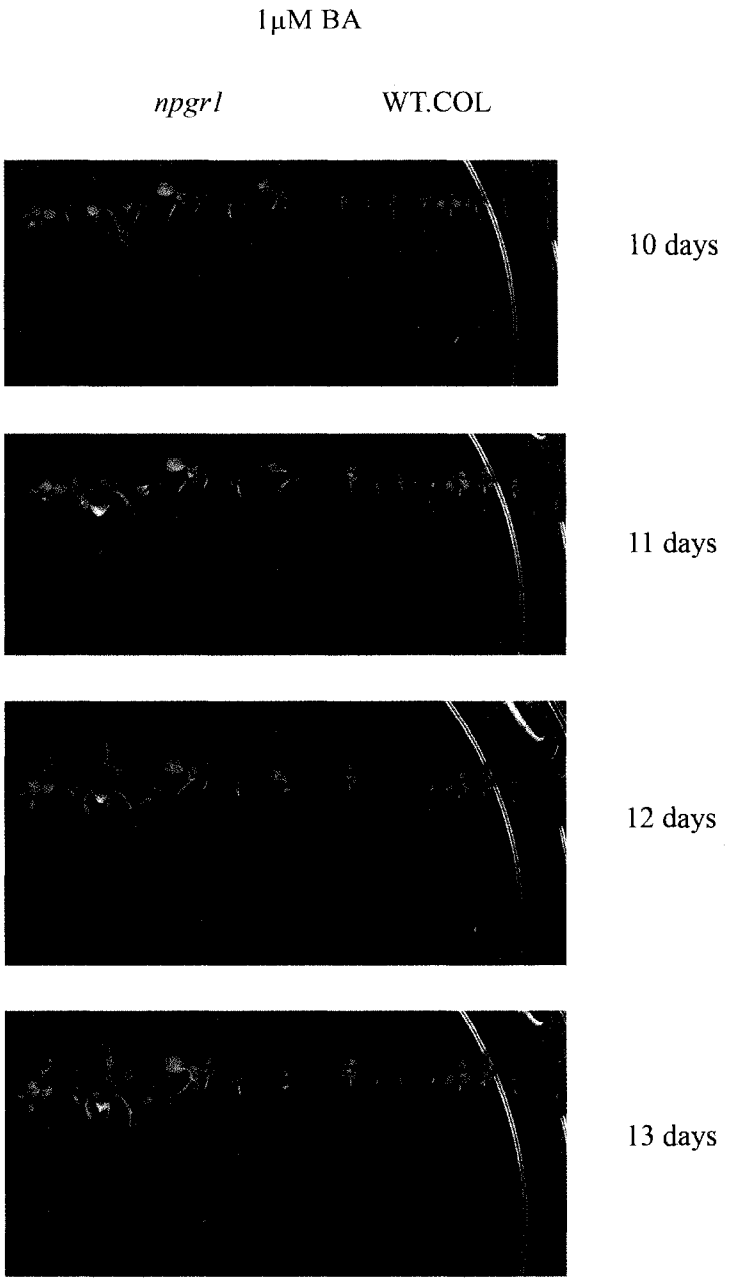


Figure 5.4b. *npgr1* (SALK090514) on 1μM BA /MS plate. The days of seedling growth are indicated on the right. WT.COL, *Arabidopsis thaliana* wild type Columbia as control.

However, *npgr1* germination was better in glucose, With 1μM BA in light, *atnpgr1* showed increased plant size as compared to wild type (Figure 5.4). Germination of *atnpgr1* was reduced in the presence of 150 mM sodium chloride (Appendix 8). *atnpgr1* plants showed better growth than wild type on 3% glucose in its size and germination rate (Figure 5.5). The difference between *atnpgr1* and wild type on glucose was more significant on 5% glucose condition. With 5% glucose, almost no wild type plant showed germination and growth, whereas most of *atnpgr1* seeds were germinated (Figure 5.6 and 5.10). Compare to wild type, *atnpgr1* showed significant sugar insensitivity at 3% and 5% glucose. This might be because of osmotic pressure or sugar signal from glucose. To see if it is because of osmotic pressure, 5% mannitol was used instead of glucose and no difference between *atnpgr1* and wild type was observed (Figure 5.7). The *atnpgr1* plants were a little bigger in size than wild type plant in soil. However no significant differences were observed in developmental stages, flowering time, silique size and shape, and seed number (Figure 5.9).

Hexokinase signaling pathway was not affected by *atnpgr1*

The glucose insensitivity phenotype of *atnpgr1* suggests that AtNPGR1 might be involved in sugar sensing and sugar signaling processes in Arabidopsis. Hexokinase dependent sugar signaling in Arabidopsis has been well studied, and the mutants in the hexokinase pathway showed sugar insensitive phenotype. It has been shown the plants with reduced or no expression of hexokinase 1 exhibit glucose insensitivity. To test the possibility that AtNPGR1 might be involved in the hexokinase pathway, expression of five genes in the hexokinase pathway was studied in the *atnpgr1* mutant. These genes are hexokinase 1, carbonic anhydrase, vacuolar H^+-ATPase, sedoheptulose bisphosphatase

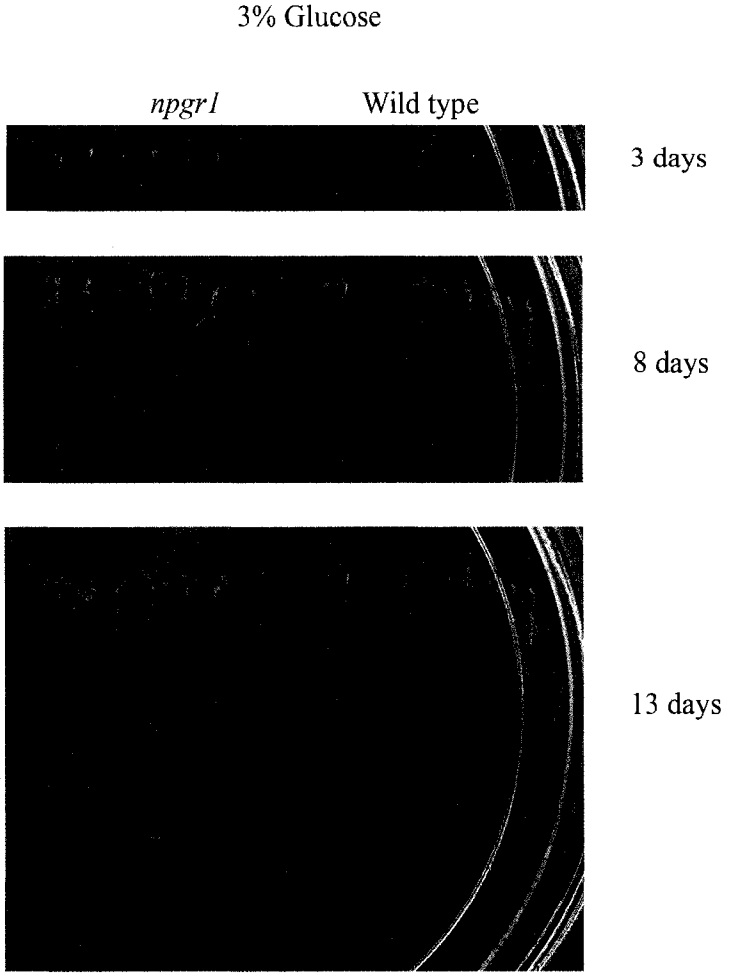


Figure 5.5. *npgr1* (SALK090514) on 3% glucose/MS plate. The days of seedling growth are indicated on the right. WT.COL, *Arabidopsis thaliana* wild type Columbia as control.

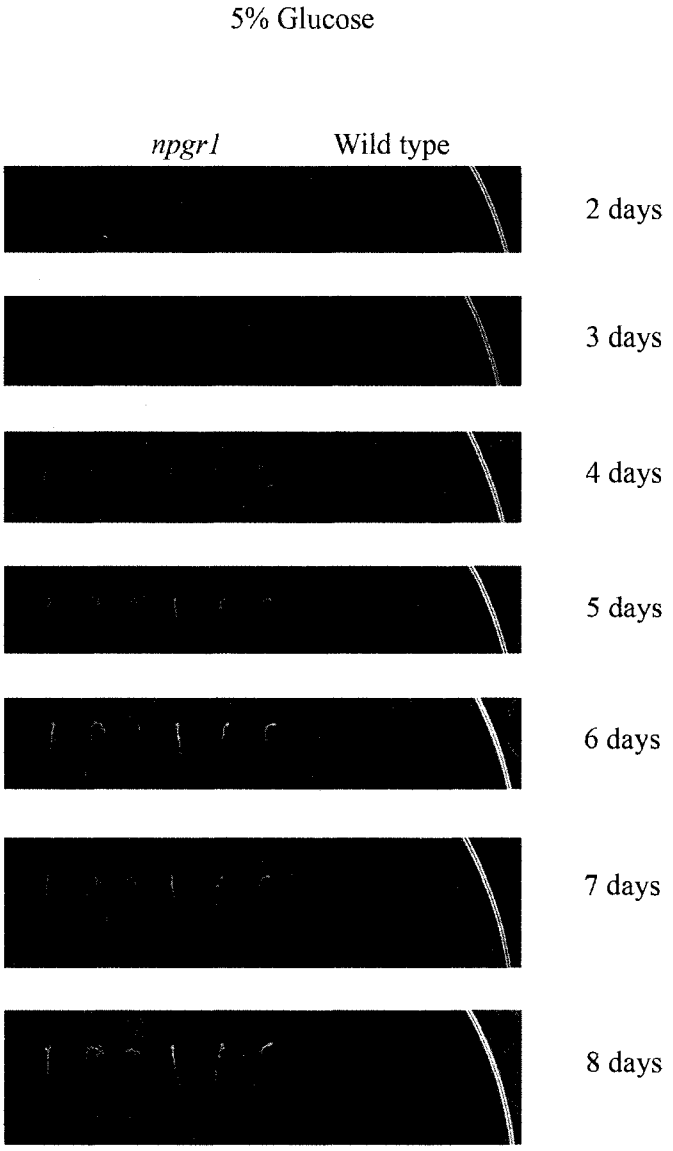


Figure 5.6. *npgr1* (SALK090514) on 5% glucose/MS plate. The days of seedling growth are indicated on the right. WT.COL, *Arabidopsis thaliana* wild type Columbia as control.

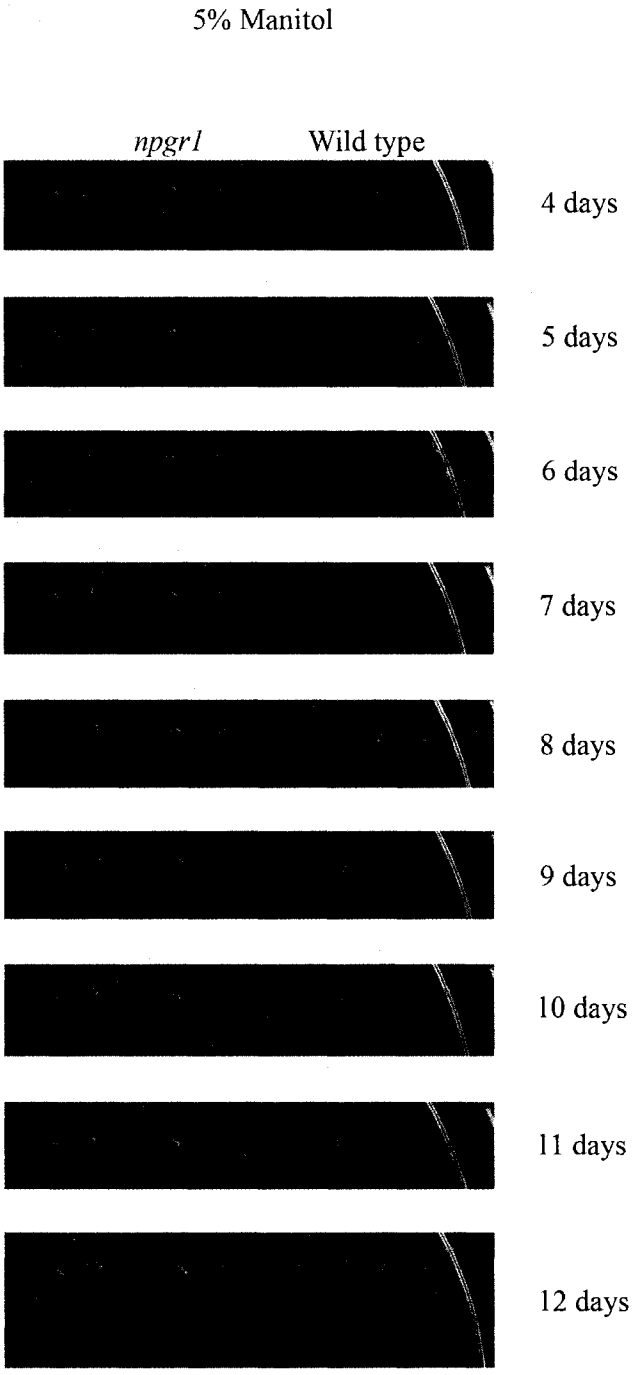


Figure 5.7. *npgr1* (SALK090514) on 5% manitol/MS plate. The days of seedling growth are indicated on the right. WT.COL, *Arabidopsis thaliana* wild type Columbia as control.

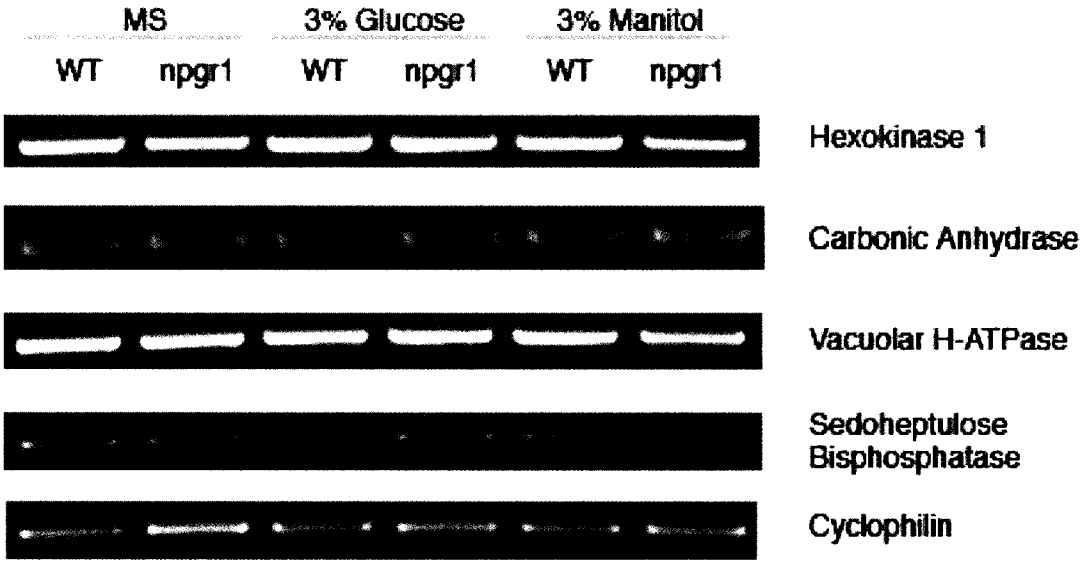


Figure 5.8. Genes in hexokinase glucose signaling pathway were not affected by *npgr1*. Gene expression in npgr1 was compared with WT (Arabidopsis thaliana wild type columbia). Cyclophilin was used as loading control.

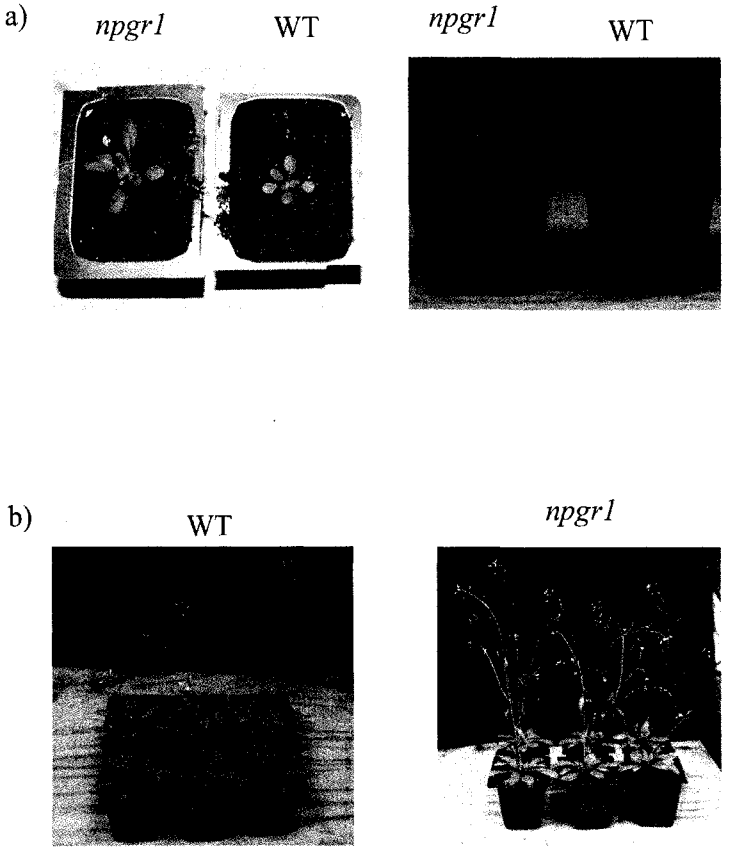


Figure 5.9. *npgr1* growth on soil. a) 2-weeks old *npgr1* bigger than wild type. b) 4 weeks old WT and *npgr1* plants.

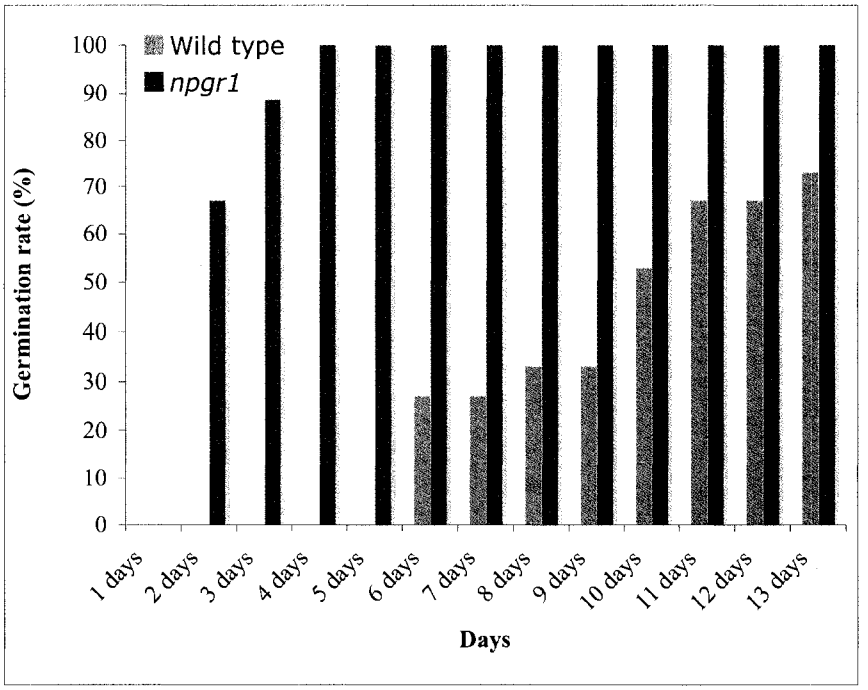


Figure 5.10. Germination of WT and *npgr1* on 5% glucose. On 5% glucose, *npgr1* is insensitive to glucose.

and chlorophyll a/b –binding protein. Expression of these was analyzed in *atnpgr1* and wild type under three different conditions: MS, 3% glucose/MS and 3% mannitol/MS conditions. The expression of five genes in the hexokinase signaling pathway under these three conditions showed no difference between *atnpgr1* and wild type (Figure 5.8) suggesting that *NPGR1* does not affect the expression of those five genes.

Discussion

To study the role of AtNPGR1 in plant growth and development, *atnpgr1*, a T-DNA mutant line, was isolated, and phenotype analysis was performed. With 3% and 5% glucose, the *atnpgr1* mutant showed sugar insensitive phenotype during germination and seedling growth, suggesting that AtNPGR1 might be involved in the sugar signaling pathway. Sugar signaling can regulate germination and early seedling development by inhibiting nutrient transportation, hypocotyl elongation, cotyledon greening and expansion, and shoot development (Yu et al., 1996; Dijkwel et al., 1997; Jang et al., 1997; Perata et al., 1997; Kurata and Yamamoto, 1998; Arenas-Huertero et al., 2000; Gibson, 2000; Smeekens, 2000; Gazzarrini and McCourt, 2001). High sugar levels during early seedling development might be considered as a feedback signal to arrest plant growth to prevent undesirable growth (Lopez-Molina et al., 2001)

In Arabidopsis, Hxk has been well studied as a sugar sensor. Furthermore, several recent studies have shown clear cross-talk between sugar signaling and hormonal signaling. Glucose has been shown to affect ethylene, ABA, cytokinin as well as auxin signaling (Yanagisawa et al., 2003). To see if AtNPGR1 is involved in the Hxk mediated sugar sensing and signaling pathway, gene expression of five genes (hexokinase 1, carbonic anhydrase, vacuolar H^+-ATPase, sedoheptulose bisphosphatase and chlorophyll a/b –binding protein) in the hexokinase pathway was monitored in the *atnpgr1* mutant. Mutant and over-expression studies of Hxk1 have shown that transcriptional and translational level of Hxk1 is correlated with sugar sensing (Jang et al., 1997; Moore et al., 2003). Carbonic anhydrase, sedoheptulose bisphosphatase, and chlorophyll a/b – binding protein are photosynthesis genes that are thought to be repressed by a Hxk1

dependent glucose signal (Moore et al., 2003). Vacuolar H^+-ATPase is a partner of Hxk1 and forms a nuclear Hxk1complex to control glucose signal response genes (Cho et al., 2006). Expression of these genes was not different between wild type and *atnpgr1* and with or without glucose (Figure 5.8). Therefore, there is a possibility AtNPGR1 might be involved in an Hxk-independent sugar signaling pathway.

An Hxk independent glucose pathway was discovered in yeast, and it was shown that two glucose transporters, Rgt2p and Snf3p, act as glucose sensors (Ozcan et al., 1996). Glucose signaling by glucose transporters has not yet been discovered in plants. Although Hxk has a critical role in sugar signaling in plants, recent genetic studies showed close interconnection between Hxk pathway and other hormonal signaling pathways including ABA, auxin, cytokinin, ethylene (Zhou et al., 1998; Cheng et al., 2002; Rolland et al., 2002; Moore et al., 2003; Rolland and Sheen, 2005). Interestingly, one glucose insensitive mutant in the Hxk pathway, *gin4*, showed the same phenotypic features as a constitutive ethylene mutants, while an ethylene insensitive mutant showed glucose hypersensitivity (Zhou et al., 1998). GIN4 might function downstream of both the glucose sensor and the ethylene sensor by connecting both signal pathways (Zhou et al., 1998). The *atnpgr1* mutant showed not just a glucose insensitive phenotype but was also larger with a long hypocotyl phenotype with 0.5 μM BA under dark condition (Figure 5.3), suggesting AtNPGR1 might be involved in a cytokinin signaling pathway. Since AtNPGR1 is a calmodulin binding protein, it might be involved in sugar signaling through a calcium signaling pathway. It was shown that a sugar signal induces calcium-dependent protein kinase in tobacco leaves (Iwata et al., 1998), and a calcium signal is also involved in other sugar induced gene expression. Sugar also causes calcium levels

to increase in the cytosol (Ohto and Nakamura, 1995). It was shown that increased calcium level inside the cell might facilitate sugar transport (Allen and Schroeder, 2001). In conclusion, my mutant studies implicate AtNPGR1 in sugar signaling. Crosses between *atnpgr1* and various known sugar signaling mutants should further our understanding of the function of AtNPGR1 in sugar signaling.

CHAPTER 6

Discussion

Sexual reproduction in angiosperms depends on pollen germination and pollen tube growth. Pollen tubes grow through female tissue and deliver two sperm cells to the embryo sac. This involves complex communication events between pollen and pistil to successfully complete this process. There are a number of pollen and/or pistil-specific proteins that function in male and female tissues to recognize appropriate pollen and permit germination, assist tube growth and guide the tube to the ovary (Cheung et al., 1995; Rubinstein et al., 1995a; Rubinstein et al., 1995b; Lord and Russell, 2002; Nasrallah, 2002; Tang et al., 2002; Edlund et al., 2004; Guyon et al., 2004; Tang et al., 2004). Pollen tube growth, besides its importance in sexual reproduction, is used as an important model system to study polar growth because of its rapid (e.g., maize pollen tubes grow 1 cm/hour) tip-focused growth (Bedinger, 1992; Bedinger et al., 1994; Lord and Russell, 2002; Gu et al., 2003). Consequently, understanding the mechanisms that control pollen germination, pollen tube growth and guidance are not only important for sexual reproduction in plants, but also for understanding the mechanisms that control tip growth.

Calcium and calmodulin signaling pathways are implicated in pollen functions during reproduction. Calcium is a key messenger in plants and mediates a number of diverse signals in regulating a number of cellular processes (Bush, 1995; Reddy, 2001a). It is known that establishment and maintenance of a tip-focused intracellular Ca^{2+} gradient is essential for pollen tube elongation and directional growth (Pierson et al., 1994; Malho and Trewavas, 1996; Pierson et al., 1996; Holdaway-Clarke et al., 1997;

Franklin-Tong, 1999; Camacho, 2000; Messerli and Robinson, 2003). How the tip-focused Ca^{2+} gradient regulates pollen tube growth and direction is poorly understood. It has also been shown that calmodulin, a major calcium sensor, plays a role in calcium-mediated signaling in pollen (Ma and Sun, 1997; Ma et al., 1999; Rato et al., 2004). However, the mechanisms by which calcium/calmodulin regulate pollen germination and tube growth are not well understood. Since calmodulin has no enzymatic activity but interacts with other proteins and regulates their activity/function (Zielinski, 1998; Reddy, 2001a; Yang and Poovaiah, 2003), identification of calmodulin target proteins and their functional characterization is essential to elucidate the function of calcium/calmodulin signaling pathways in pollen. Here, I have characterized three calmodulin-binding proteins from Arabidopsis (AtNPG1, AtNPGR1 and AtNPGR2, collectively called AtNPGs) that are expressed in pollen and their interacting proteins, AtPLLs (Pectate Lyase Like) to further our understanding of the calcium/calmodulin-mediated signaling pathway in pollen.

AtNPG1 is a pollen-specific calmodulin binding protein

To understand the roles of calcium-calmodulin in pollen, a calmodulin binding protein, (MPCBP) that is expressed specifically in pollen was isolated from maize. A homolog of ZmMPCBP was identified in Arabidopsis, and it was named as AtNPG1 (no pollen germination1). Northern and RT-PCR analyses have shown that *AtNPG1* is expressed only in pollen. Transgenic plants expressing a reporter gene (GFP) fused to the *AtNPG1* promoter (2.4 Kb) showed GFP expression only in pollen and pollen tubes. Although it was previously shown that pollen carrying the mutant allele of *AtNPG1* does not germinate (Golovkin and Reddy, 2003), the mechanisms by which AtNPG1 regulates

pollen germination are not known. The fact the *AtNPG1* promoter is active during pollen germination indicates that it may have a role in pollen tube growth also. To gain further insight into the function of AtNPG1, I localized the protein in mature and germinating pollen. Since AtNPG1 binds calmodulin in a calcium-dependent manner, it is of interest to see if its localization is similar to calcium gradients in the pollen tube. For this analysis I expressed AtNPG1 fused to GFP so that AtNPG1 localization can be followed in live cells at different stages of pollen germination. The CaMV 35S promoter that is commonly used to express introduced genes in plants is not active in pollen (Twell et al., 1991; Eyal et al., 1995; Reddy and Reddy, 2004b). Also, overexpression of a protein using non-native promoters sometimes results in abnormal localization of protein. Hence, I used the *AtNPG1* promoter that is active only in pollen. Transgenic plants expressing the AtNPG1-GFP fusion were used for AtNPG1 localization analysis. Based on AtNPG1's role in germination we expected that it might localize to the region of germinal aperture and/or at the tip of pollen tube. Mature pollen showed strong uniform fluorescence (Figures 1.15). Analysis of GFP-AtNPG1 at different time points of pollen germination showed uniform localization in the tube (Figures 1.15). Analysis of GFP fluorescence using confocal microscopy confirmed that the NGP1 is in the cytoplasm and its localization is uniform (Figure 1.15). The observed pattern of GFP-AtNPG1 was similar to that of GFP alone, raising the possibility that the fusion protein may be degraded and the observed localization reflects free GFP. However, immunoblot analysis indicated that the observed localization of GFP-AtNPG1 is not due to degradation of the fusion protein into GFP and AtNPG1 (Figure 1.16).

The uniform localization of AtNPG1 does not preclude its role in germination

and tip growth. For example, total calmodulin is uniformly distributed in the pollen tube. However, activated calmodulin, like calcium, showed a tip-focused gradient (Rato et al., 2004). Since calcium and active calmodulin exhibit a tip-focused gradient, it is possible that AtNPG1 interacts with the calcium/calmodulin complex only at the tip. Fluorescence resonance energy transfer (FRET) assay is an *in vivo* method to measure protein-protein interaction using a pair of fluorescent proteins, such as CFP (cyan fluorescent protein) and YFP (yellow fluorescent protein) (He et al., 2003). Co-expression of calmodulin fused to cyan fluorescent protein (CFP) and YFP-AtNPG1 in pollen and analysis of their *in vivo* interaction using FRET should help determine whether the AtNPG1 interaction with calmodulin is restricted to the tip.

AtNPGs represent a small family of plant-specific calmodulin-binding proteins

The AtNPGs were identified by searching the Arabidopsis genome sequence database with the sequence of the maize pollen-specific calmodulin-binding protein (ZmMPCBP). *AtNPGs* (*AtNPG1, AtNPGR1* and *AtNPGR2*) were cloned from pollen cDNA by RT-PCR and sequenced. The deduced sequence of the AtNPGs showed high sequence similarity and organization with ZmMPCBP. For example, they all contain several tetratricopeptide repeat domains. The similarity between ZmMPCBP and the AtNPGs implies that the AtNPGs might be calmodulin-binding proteins and function in the calcium/calmodulin signaling pathway. RT-PCR analysis with RNA from pollen and other tissues has shown that all three genes are expressed in pollen. However, of the three, AtNPG1 is expressed only in pollen whereas the other two are expressed in pollen as well as several other tissues. *AtNPGR1* and *AtNPGR2* are expressed in suspension culture cells, flower, pollen and fruits. In addition, *AtNPGR1* expression was detected in

leaf also. These results suggest that AtNPG1 functions only in pollen whereas the other two (AtNPGR1 and AtNPGR2) may function in pollen as well as in other tissues.

I have demonstrated that all AtNPGs bind calmodulin in a calcium-dependent manner. Although the calmodulin-binding domain is not conserved among different calmodulin-binding proteins (Reddy, 2001a), it is highly conserved among the three AtNPGs. Together these results suggest that all three AtNPGs are calcium-dependent calmodulin-binding proteins and are likely to be involved in the Ca^{2+}-CaM signaling pathway. Beside Maize and Arabidopsis, the homologs of AtNPGs were identified in *Vitis vinifera,* and *Oryza sativa.* In *Oryza sativa,* there are five AtNPGs homologs, and they showed 40-66% identity and 58-81% similarity to Arabidopsis proteins. There are no homologs of AtNPGs in any non-plant systems, such as yeast, *Drosophila melanogaster, Caenorhabditis elegans*, and humans, suggesting that these proteins are specific to plants. However, these proteins are found in phylogenetically divergent flowering plants (dicots and monocots), suggesting that they may be ubiquitous in plants.

The binding of AtNPGs to calmodulin in a calcium-dependent manner suggests that the function of AtNPGs is likely to be modulated by calcium/calmodulin. Activated calmodulin (calcium/calmodulin complex) has been shown to activate the interacting proteins in most cases (Reddy, 2001a; Luan et al., 2002). However, activated calmodulin has been shown to negatively regulate the activity of certain enzymes (Reddy and Reddy, 2004a). In some cases, the binding of calmodulin to its target protein has been shown to regulate the interaction of the target protein with other proteins (Reddy, 2001b). The AtNPG1 has no known enzymatic domain. Hence, it is not possible to evaluate the physiological significance of this interaction biochemically. Since AtNPG1 interacts

with other proteins (see below) calcium/calmodulin may modulate this interaction.

Interactions among AtNPGs

Since AtNPGs have TPR domains that are known to be involved in protein-protein interactions, I hypothesized that AtNPGs might interact among themselves to form homo- or hetero-dimers and/or with other proteins. It is known that TPR-containing proteins through protein-protein interactions function in various cellular processes including the cell cycle (Hirano et al., 1990; Lamb et al., 1995), transcription (Schultz et al., 1990; Sikorski et al., 1990; Lamb et al., 1995; Tzamarias and Struhl, 1995), protein transport across mitochondria and peroxisomes (Botella and Arteca, 1994; Kragler et al., 1998), dephosphorylation of proteins (Albertazzi et al., 1998), and muscle development (Venolia et al., 1999). The number of TPR domains in TPR-containing protein varies and it is assumed that unique secondary structure created by different number of TPR domains may decide specificity of target proteins (Lamb et al., 1995; Albertazzi et al., 1998; Venolia et al., 1999). It has been shown that TPR domain is important for function of TPR containing proteins (Lamb et al., 1995; Venolia et al., 1999); (Sikorski et al., 1990; Hernandez Torres et al., 1995; Lamb et al., 1995; Vucich and Gasser, 1996; Kragler et al., 1998; Prodromou et al., 1999; Venolia et al., 1999). I tested the possibility of interaction among AtNPGs using a yeast two-hybrid assay. AtNPG1 interacted with itself and the other two related proteins and AtNPGR1 also interacted with itself, AtNPG1 and AtNPGR1. However, AtNPGR2 showed interaction with itself and very weakly with AtNPGR1. Although we detected interaction between two AtNPGs-BD (AtNPG1 and AtNPGR1) fusion and AtNPGR2-AD fusion, AtNPGR2-BD fusion did not show interaction with AtNPGs. This is could be due to BD fusion to AtNPGR2, which

may have adversely affected protein folding resulting no interaction.

AtNPGs interact with pectate lyase like (PLLs) proteins

Although it was shown that AtNPG1 has a role in pollen germination, the mechanistic basis for this is not known. Since AtNPG1 has no known enzymatic domains but contains protein interaction domains (TPRs), I screened for Arabidopsis AtNPG1 interacting proteins in pollen using a petunia yeast two-hybrid library with the hope that identification of such proteins could provide insights into the role of AtNPG1. Two clones that showed a strong interaction with AtNPG1 were isolated and sequence analysis of these revealed that they are very similar to late anther specific proteins (LAT56 and LAT59), both of which are putative pectate lyases and are preferentially expressed in anthers and pollen of tomato (Twell et al., 1991; Kulikauskas and McCormick, 1997). To test if Arabidopsis AtNPG1 interacts with putative pectate lyase from Arabidopsis, four pectate lyase like (*PLL*) genes that showed high amino acid sequence similarity to petunia *PLLs* were tested for their interaction with AtNPG1 using the yeast two-hybrid assay. Since AtNPGRs share strong similarity with AtNPG1, AtNPGRs interaction with two petunia PLLs was tested. AtNPGR1, like AtNPG1, interacted with two petunia and Arabidopsis PLLs, but AtNPGR2 showed no interaction, except for very weak interaction with AtPLL9 (Figure 2.9). I verified the interaction between AtNPGs and AtPLLs (PLL10 and PLL11) using pull-down assay with bacterially expressed AtNPGs and AtPLLs. My results have also shown that that the most N-terminal TPR1 domain in AtNPG1 is important for its interactions with its protein partners. To support AtNPG1 interaction with AtPLLs, one of the maize proteins bound to an Arabidopsis NPG1 affinity was identified as a ZmPLL. The evidence from

these interaction assays suggest a possibility that AtNPGs might be involved in pollen cell wall function by regulating pectate lyase activity. Although AtPLLs showed highly conserved sequence in catalytic domain with pectate lyase, enzyme activity of none of the Arabidopsis PLLs has been demonstrated. Using protein sub-cellular localization prediction programs including PSORT and Target P, potential signaling peptide sequences were identified from all four AtPLLs, suggesting that these four AtPLLs might be localized to cell wall (Appendices 3 an 4). GFP-AtNPG1 fusion showed that it localizes to cytoplasm in pollen and pollen tube. These observations raised physiolocial relevance of this interaction since they are localized in two different cellular locations. One possibility is that localization of AtNPG1 from GFP-AtNPG1 fusion might be not accurate. To address this possibility, we will localze ZmMPCBP using an antibody (Safadi et al., 1999) specific to this protein.

What is the role of AtNPG1 interacting pectate lyase like (PLLs) proteins in pollen?

Although it has been shown that AtNPGs interact with AtPLLs, the role of AtPLLs as pectate lyases is still in question. To understand how AtNPGs are involved in pollen cell wall metabolism, it is essential to know the role of the AtPLLs that showed interaction with AtNPGs. The pollen tube cell wall has a unique structure compared to other plant cell walls. Pollen tube cell wall, except in the tip area, is composed of two layers: an inner callose/cellulose layer and outer layer composed of pectin (Taylor and Hepler, 1997; Ferguson et al., 1998). At the pollen tip area where tube growth occurs, the cell wall is composed of only a single layer of pectin without callose or cellulose (Steer and Steer, 1989). Pectin is produced in Golgi and transported to the pollen tip area in vesicles (Sterling et al., 2001). Cell wall modifying enzymes, such as pectin methyl

esterase (PME), pectin lyase and polygalacturonase, are involved in processing pectin during pollen tube growth. Pectin methyl esterase removes the methyl ester group and pectin becomes de-esterified, and then carboxyl groups resulting from de-esterification act as a bridge with calcium to link pectins tightly, resulting in cell wall stiffening (Catoire et al., 1998). Compared to PME, pectate lyase ((PL, EC 4.2.2.2) is one of the pectic degrading enzymes that have a role in disassembling pectin polymers on the cell wall when plant pathogens infect plants and to modify the cell wall when the cell expands (Collmer and Keen, 1986). My research has shown that fourteen out of 26 *AtPLLs* in Arabidopsis are expressed in pollen and four AtPLLs that showed interaction with AtNPGs are highly expressed in pollen. These four *AtPLLs* have a high sequence similarity with two petunia PLLs, tomato and tobacco *LAT 59*. *LAT 56* and *LAT 59* which encode *PLLs* and all of them are highly expressed pollen (Budelier et al., 1990; Wing et al., 1990; Kulikauskas and McCormick, 1997), suggesting that they might have a role in pollen development and reproduction processes. In pollen, it is suggested that pectate lyase might be involved in pollen tube emergence and growth by loosening the pollen tube cell wall and in pollen tube penetration into female tissue by depolymerizing pectin in the cell wall and extracellular matrix in the stigma transmitting tissue of the style (Taniguchi et al., 1995; Wu et al., 1996). Although Arabidopsis PLLs have a highly conserved catalytic domain in pectate lyases, enzyme activity was not demonstrated in expressed proteins. To test the activity of AtNPG1 interacting AtPLLS, I first developed a PLL assay using the Arabidopsis tissues and a fungal enzyme as a positive control. Pectate lyase activity in Arabidopsis tissues varied and was dependent on calcium and buffer pH. To determine if AtNPG1 interacting AtPLLs have pectate lyase activity,

bacterially or yeast expressed AtPLLs were used for enzyme activity. Unfortunately, I was not able to detect any enzyme activity with these proteins under different assay conditions with different substrates. It might be that either bacterially and yeast expressed AtPLLs are not correctly folded or lack certain posttranslational modifications. Expressing them as tagged fusions in plants and using these for pectate lyase enzyme assays could address this. The other possibility is that they function on a specific type of pectin that we did not use in our assay. It is also possible that they do not have pectate lyase activity, although they share significant similarity with bacterial and fungal AtPLLs. Further research on these AtPLLs is needed to answer these questions.

The function of one (AtPLL13, also called PMR6) of the 26 Arabidopsis PLLs was studied using a mutant, which showed an interesting phenotype. The *pmr6* mutant showed resistance to powdery mildew pathogen and reduced plant size (Vogel et al., 2002). The cell wall structure of *pmr6* showed high levels of pectin with a lower degree of esterification and a modification in the H-bonding environment of cellulose microfibrils (Vogel et al., 2002). This suggested that PMR6 might have a role in pectin metabolism. To study role of the AtNPGs interacting AtPLLs in plant development, I isolated a loss-of-function mutant of one of the four AtNPGs interacting with AtPLLs, AtPLL8. The *atpll8* showed no difference in plant development under various chemical and hormonal conditions. It might be because there is redundancy between AtPLLs function as there are a number of AtPLLs with overlapping expression. Generation of double/triple mutants for genes that have similar expression pattern should reveal functions of at lease some AtPLLs.

Based on the interaction between AtNPGs and AtPLLs, a model that can provide

possible relationship of calcium-calmodulin signaling and pollen germination/tube growth is proposed. High calcium level activated calmodulin, activated calmodulin regulates its target protein, AtNPG1, which then interacts with AtPLLs. Since AtPLLs have a high sequence similarity to pectate lyases, a cell wall modifying enzyme, it is possible that the signaling pathway of Ca^{2+}-CaM-AtNPGs may play a role in Pollen germination/ pollen tube growth via controlling pollen tube cell wall architeture. However, this model requires us to demonstrate the localization of NPG1 and PLLs at the same location.

High $[Ca^{2+}]$	→	Ca^{2+}-CaM	→	Ca^{2+}-CaM-AtNPG1	→	AtPLLs (8, 9, 10, and 11)	- - - →	Pollen germination/ pollen tube growth

AtNPGR1 might be involved in sugar signaling

Although AtNPGR1 has high similarity in sequence and domain structure to AtNPG1, it is expressed in other tissues as well as in pollen, suggesting that AtNPGR1 might have, in addition to a role in pollen, some other functions in plant development. I isolated a homozygous *atnpgr1* mutant and characterized its growth and development in the presence of different hormones and other chemicals. Since AtNPGs interact with each other, it was expected that *atnpgr1* might show a pollen phenotype. However, *atnpgr1* did not have any pollen phenotype or fertilization defect. The reason for this is that AtNPGR1 may not be essential for pollen function and may has some other functions in plant development.

With 3% glucose, the *atnpgr1* showed insensitivity to glucose and this phenotype was even more obvious with 5% glucose. The glucose insensitive phenotype suggests

that AtNPGR1 might be involved in sugar sensing and signaling pathway. Sugar is not just an important nutrient for most living organisms but also acts as a signal molecule (Rolland et al., 2002). In plants, it has been discovered that sugars are involved in regulation of plant growth during its whole life cycle. High sugar levels can repress seed germination and early seedling development, and affect floral transition and leaf senescence (Rolland et al., 2002). In Arabidopsis, one sugar sensor has been well studied. Hxk (hexokinase), the first enzyme in glycolysis, has been shown to function as a sugar sensor and as an inter-connector between sugar signaling and other hormonal signaling. With anti-sense Hxk, transgenic plants showed a sugar insensitive phenotype, whereas over-expressed Hxk caused plants to be hypersensitive to sugar (Jang et al., 1997). The sugar-regulated genes (e.g, chlorophyll a/b binding protein (*CAB1*), ribulose-1, 5-bisphosphate carboxylase small subunit (*RBCS*), and nitrate reductase (*NR1*)], expression levels were also affected by Hxk expression. Using site-directed mutant of Hxk, it was shown that enzyme activity is not necessary for its role in sugar signaling. The phenotypes of *glucose insensitive 2* (*gin2),* an Hxk1 mutant, are affected by other plant hormones such as auxin and cytokinin, suggesting that Hxk1 might play a role in connecting sugar, light, and hormonal signal networks (Moore et al., 2003). Recently, vacuolar H+-ATPase B1 (VHA-B1) and 19S regulatory particle of proteasome subunit (RPT5B) were identified as Hxk interacting partners (Cho et al., 2006). It was suggested that nuclear Hxk1 and those two partners form a glucose signaling complex that regulates target gene expression (Cho et al., 2006).

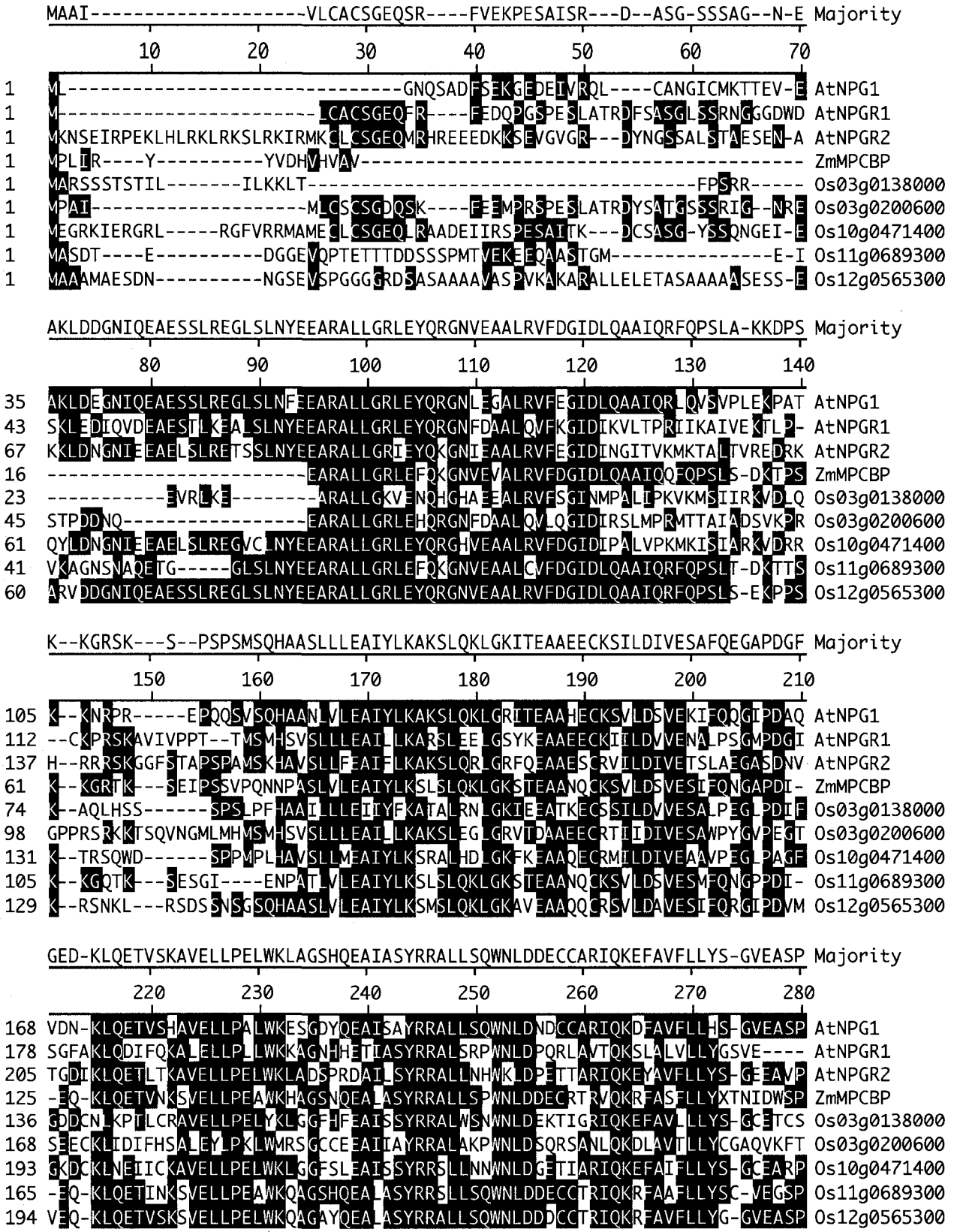
I have tested if AtNPGR1 is involved in the Hxk pathway by analyzing gene expression of five genes (hexokinase 1, carbonic anhydrase, vacuolar H^+-ATPase,

sedoheptulose bisphosphatase and chlorophyll a/b –binding protein) in the hexokinase pathway in the *atnpgr1* mutant. Previously, mutant and over-expression studies of Hxk1 showed that transcriptional and translational level of Hxk1 is correlated with sugar sensing (Jang et al., 1997; Moore et al., 2003). Decreased expression of Hxk1 leads to sugar insensitive phenotype. Carbonic anhydrase, sedoheptulose bisphosphatase, and chlorophyll a/b –binding protein Chlorophyll a/b –binding protein were photosynthesis genes and were thought to be repressed by an Hxk1 dependent glucose signal (Moore et al., 2003). Vacuolar H^+-ATPase is a partner of Hxk1 to form a nuclear Hxk1 complex to control glucose signal response genes (Cho et al., 2006). The expression of these genes was similar in *atnpgr1* and wild type in the presence or absence of glucose, suggesting that AtNPGR1 might not be involved in Hxk-dependent sugar signaling and is likely to function in Hxk independent pathway.

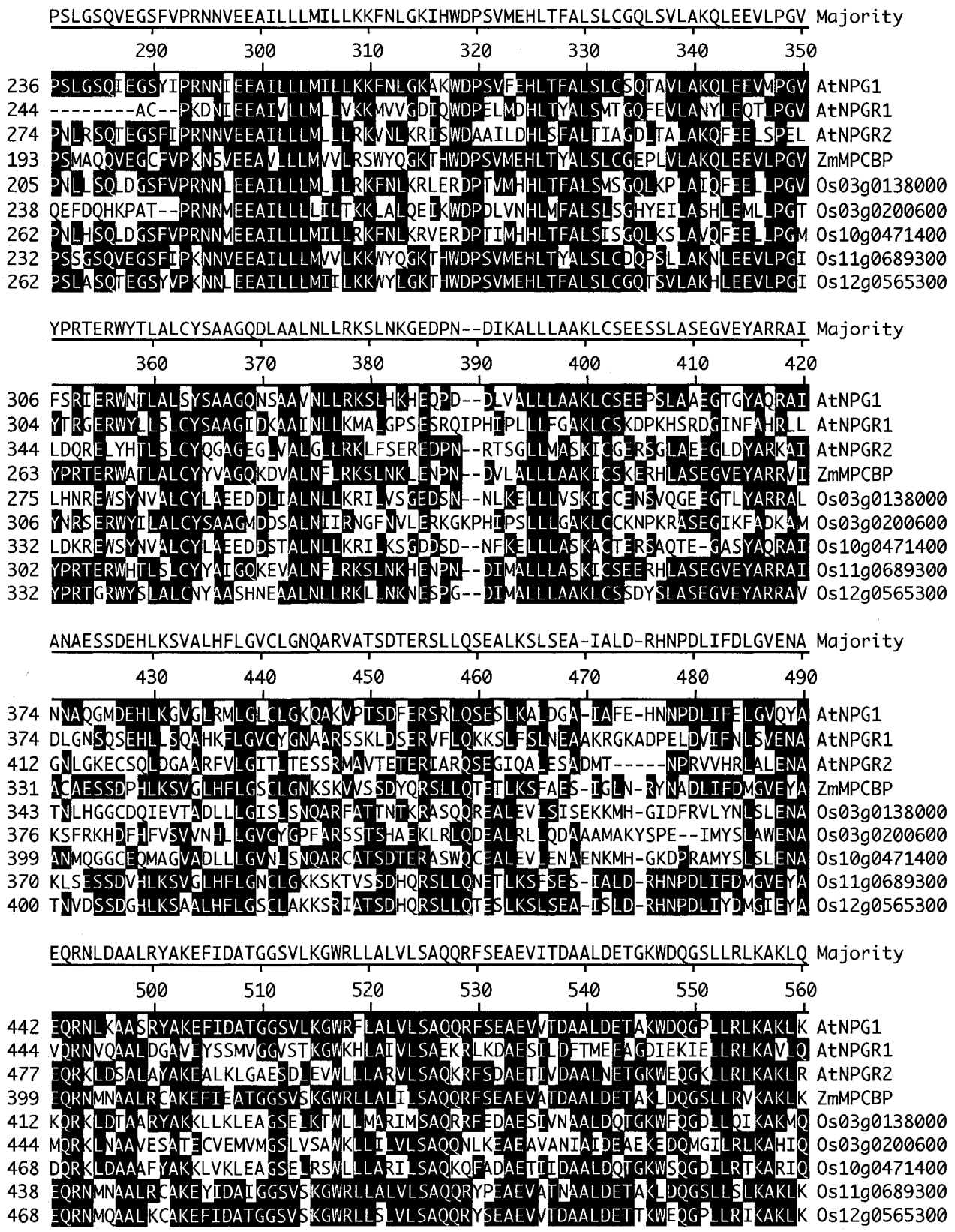
An Hxk independent glucose pathway was discovered in yeast and it was shown that two glucose transporters, Rgt2p and Snf3p, act as glucose sensors and initiate sugar signaling pathways (Ozcan et al., 1996). However, in plants glucose signaling by glucose transporters has not been reported yet. Although Hxk has a critical role in sugar signaling in plants, recent genetic studies showed extensive cross-talk between the Hxk pathway and other hormonal signaling pathways including ABA, auxin, cytokinin, ethylene (Zhou et al., 1998; Cheng et al., 2002; Rolland et al., 2002; Moore et al., 2003; Rolland and Sheen, 2005). Several hormonal signaling mutants were also isolated as glucose signaling mutants. For example, *ABA2, ABA3, ABI4* are the same as *glucose insenstive1* (*GIN1*), *GIN5* and GIN6 respectively. Similarly GIN4 is the same as constitutive triple response 1 (*CTR1*), which is involved in ethylene signaling (Rolland et al., 2006). The

apnpgr1 showed not just a glucose insensitive phenotype but also showed altered responses to cytokinin (Figure 5.3) suggesting the AtNPGR1 might be involved in a hormonal signaling pathway also. Elevation of cellular calcium by sugar has been reported (Ohto and Nakamura, 1995). Further, it was shown that increased cellular calcium levels might facilitate sugar transport (Allen and Schroeder, 2001), and sugar signals induced calcium-dependent protein kinase in tobacco leaf (Iwata et al., 1998). Calcium is also involved in other sugar induced gene expression. These results suggest a connection between sugar and calcium signaling pathways. AtNPGR1, a calmodulin-binding protein, might be involved in sugar signaling through the calcium signaling pathway. Many well characterized sugar signaling mutants are available (Rolland et al., 2006). Genetic crosses between *atnpgr1* and these sugar signaling mutants should further our understanding of AtNPGR1 function in sugar signaling.

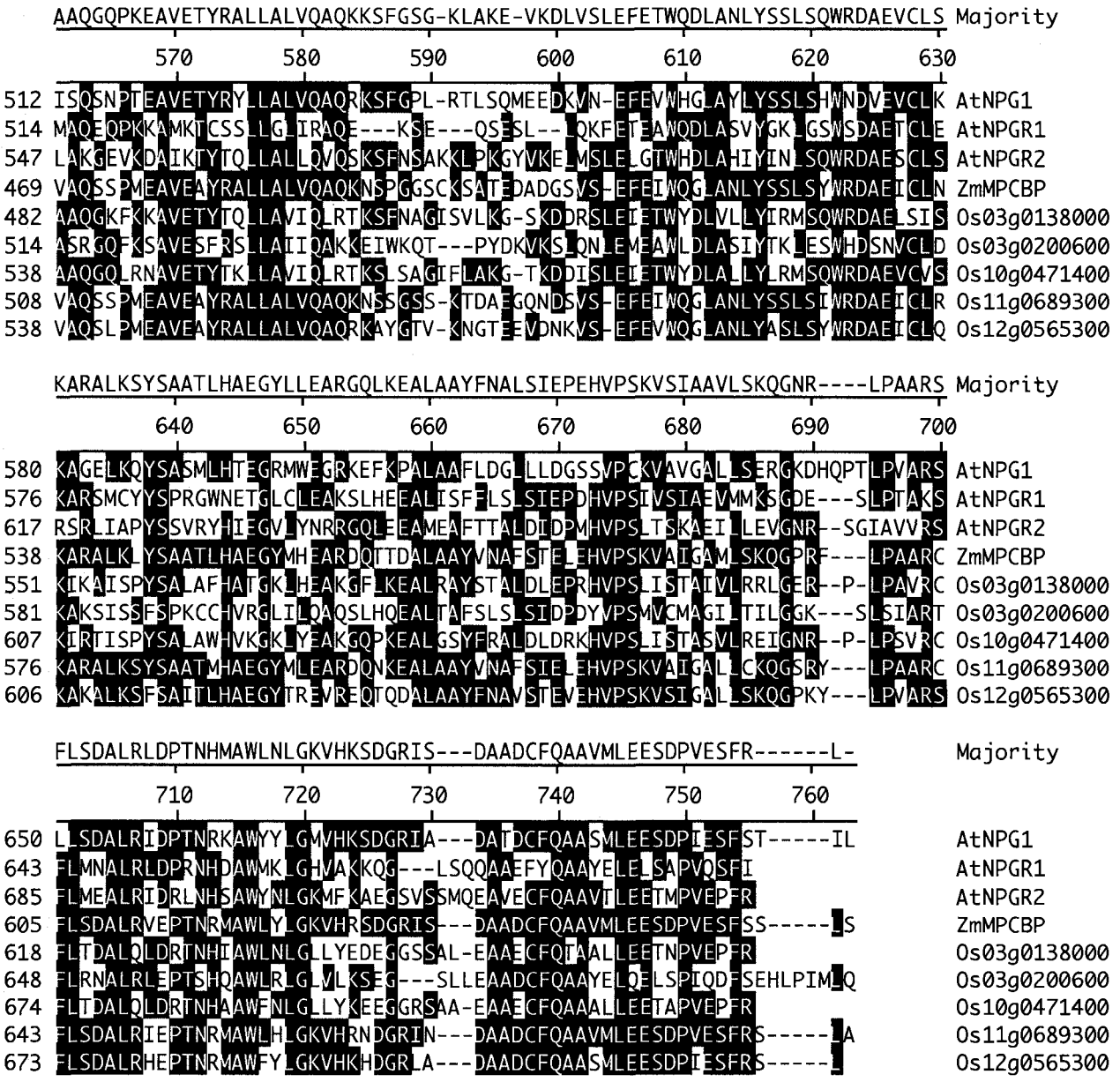
Appendices



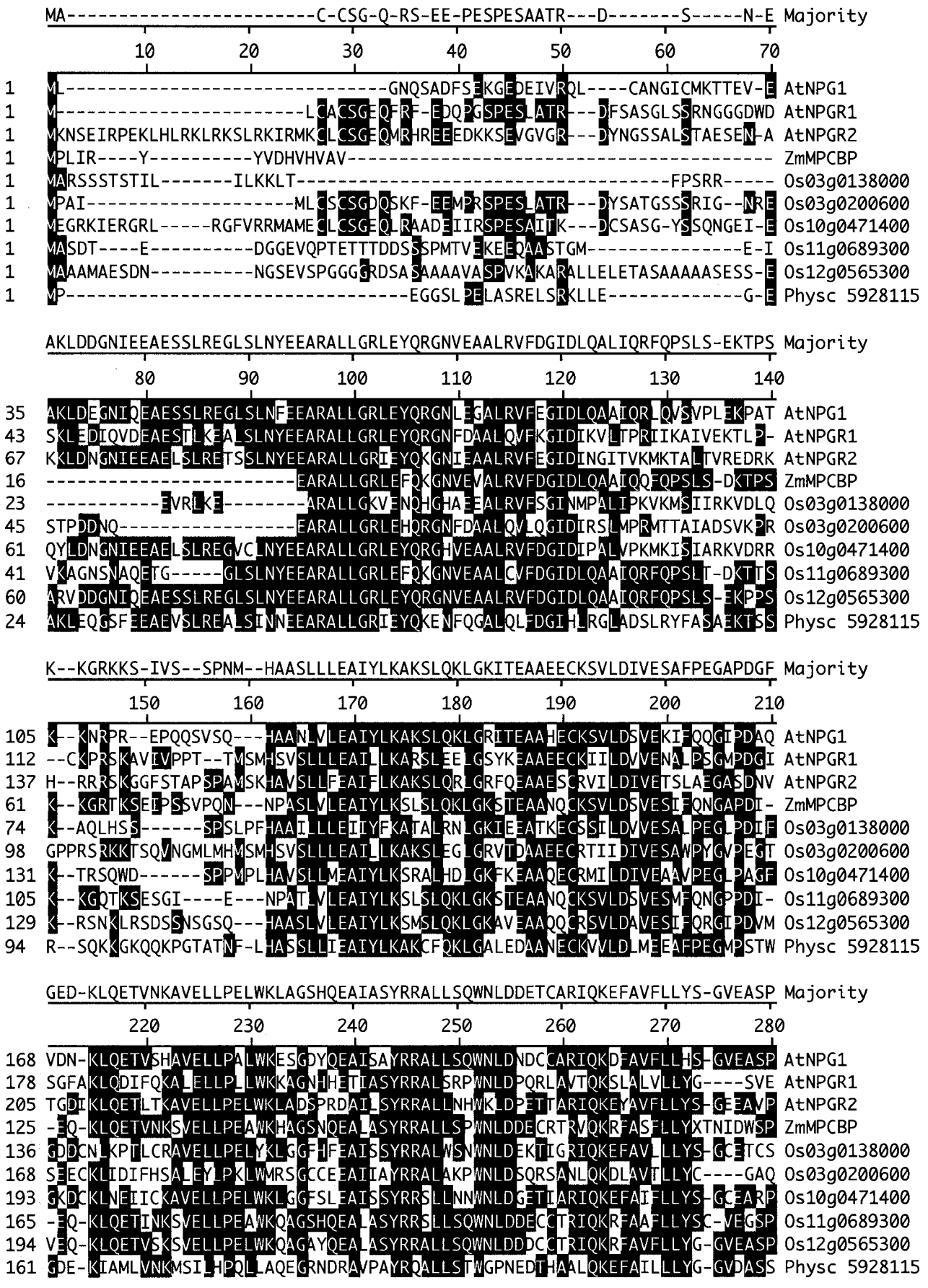
Appendix 1a. Alignment NPG and NPG-related sequence from different plants. At, Arabidopsi; , Zm, Maize; Os, Rice.



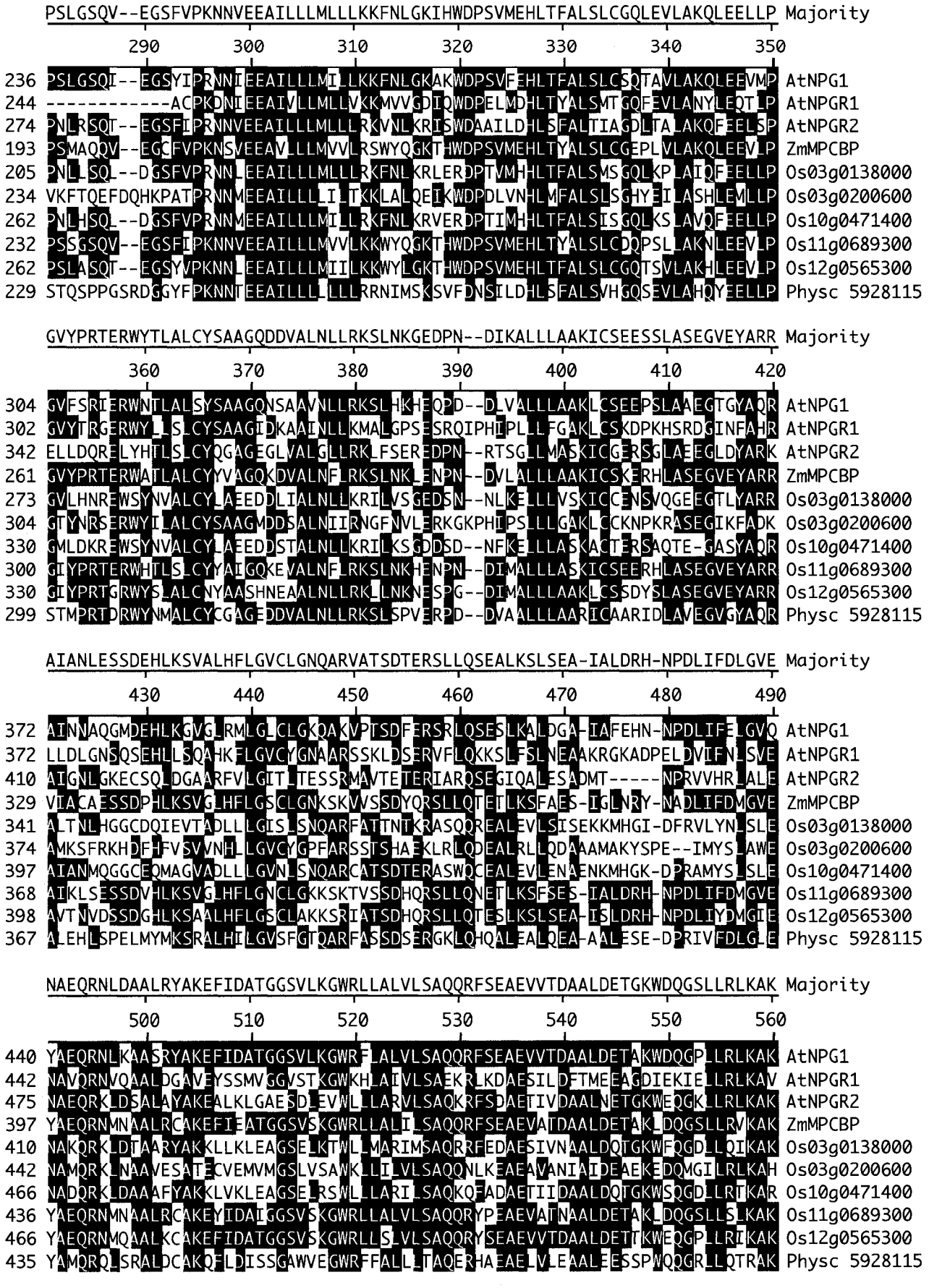
Appendix 1b. Alignment NPG and NPG-related sequence from different plants. At, Arabidopsi; Zm, Maize; Os, Rice



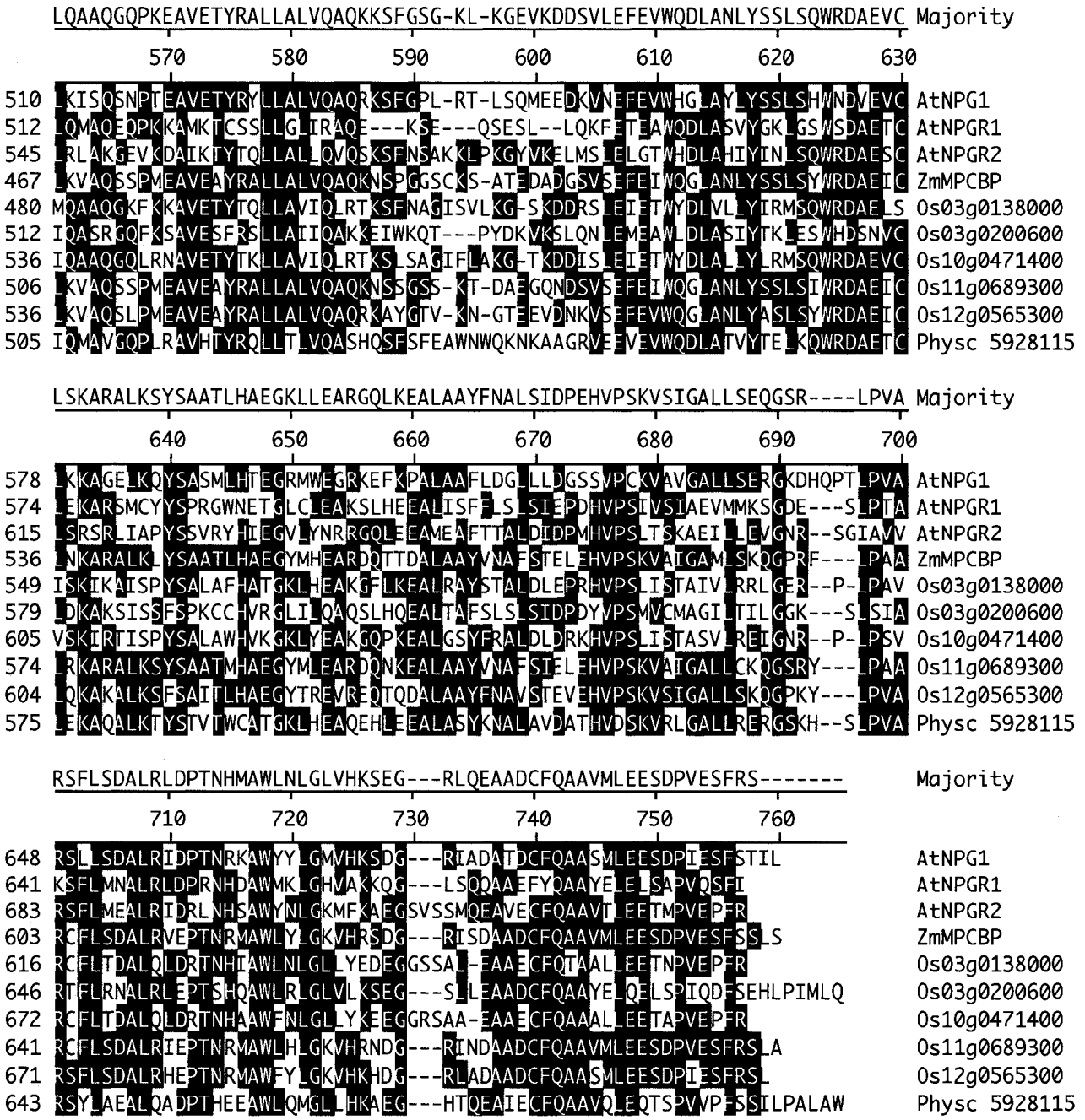
Appendix 1c. Alignment NPG and NPG-related sequence from different plants. At, Arabidopsi; , Zm, Maize; Os, Rice.



Appendix 2a. Alignment NPG and NPG-related sequence from different plants. At, Arabidopsi; , Zm, Maize; Os, Rice; Physc, Moss.



Appendix 2b. Alignment NPG and NPG-related sequence from different plants. At, Arabidopsi;



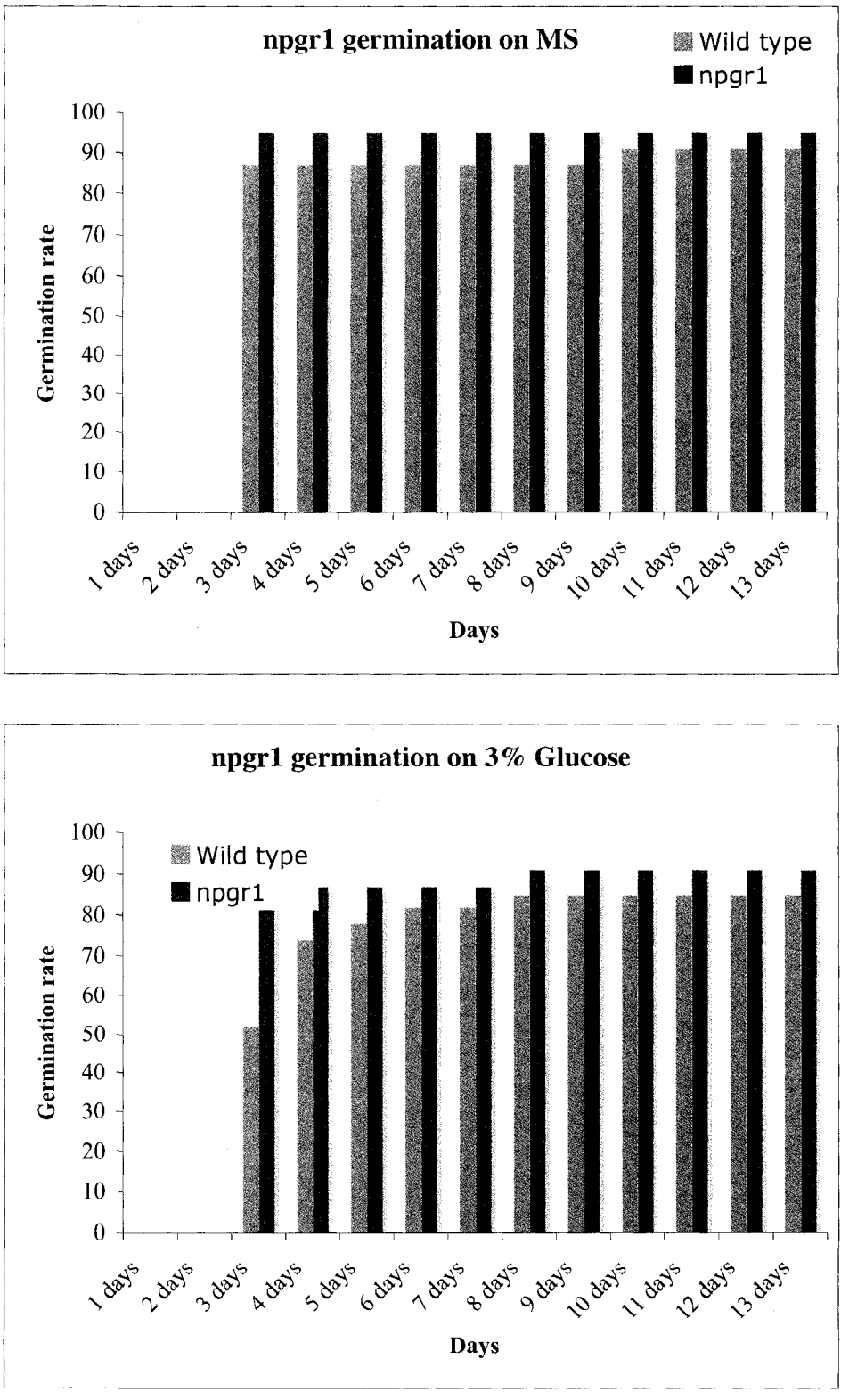
Appendix 2c. Alignment NPG and NPG-related sequence from different plants. At, Arabidopsi; , Zm, Maize; Os, Rice; Physc, Moss.

Appendix 3. The final results of PSORT of AtNPGs and AtPLLs: (Prediction scale is from 0 to 1 where 1 indicates the strongest prediction).

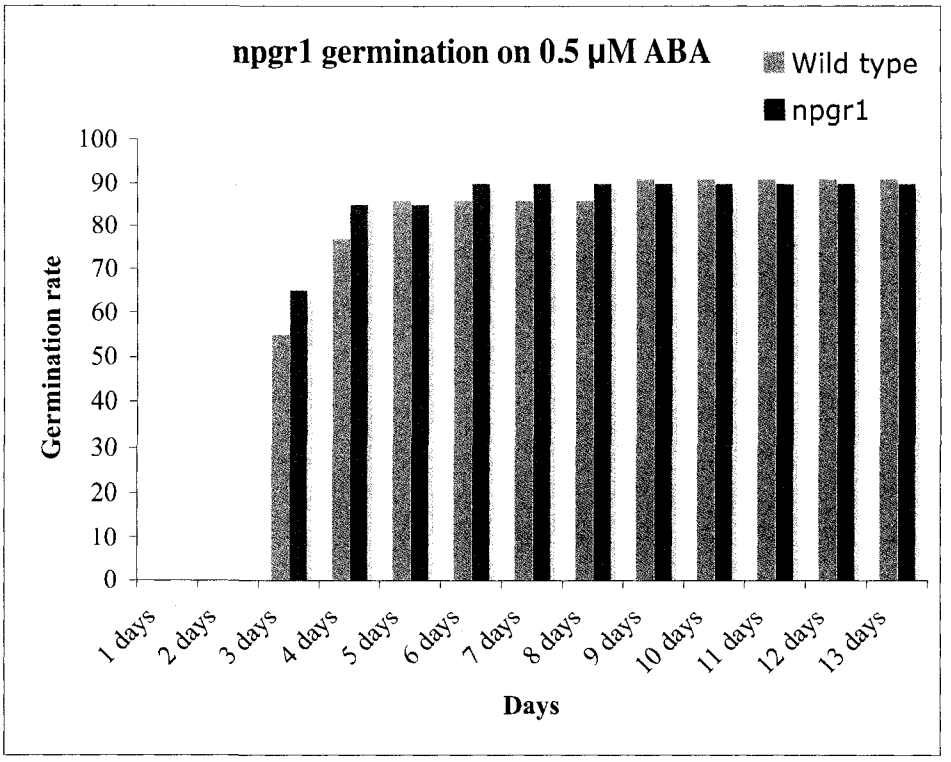
	AtNPG1	AtNPGR1	AtNPGR2	AtPLL8	AtPLL9	AtPLL10	AtPLL11
Cytoplasm	0.450						
Peroxisome	0.3	0.3	0.3		0.127	0.205	0.215
Mito	0.1		0.1				
Matrix	0.1	0.802	0.1				
Chlor Thyl		0.585					
(m)		0.585					
Chlor			0.7		0.108		
Strom				0.795	0.82	0.82	0.671
Chlor Thyl				0.1	0.1	0.1	0.1
(s)				0.1		0.1	0.1
Nucleus				0.1			
Outside							
ER memb							
ER lumen							
Golgi							

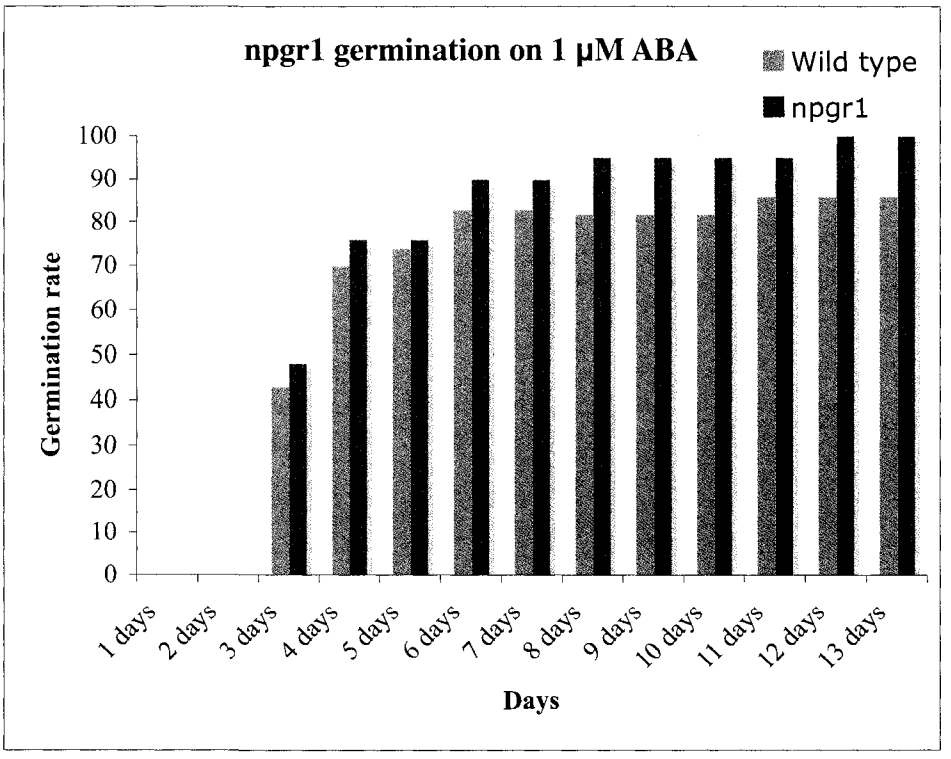
Appendix 4. The final results of Target P of AtNPGs and AtPLLs

	AtNPG1	AtNPGR1	AtNPGR2	AtPLL8	AtPLL9	AtPLL10	AtPLL11
cTP	0.142	0.270	0.118	0.002	0.003	0.008	0.009
mTP	0.1	0.134	0.286	0.133	0.043	0.038	0.043
SP	0.071	0.119	0.032	0.983	0.990	0.966	0.966
other	0.886	0.751	0.645	0.014	0.027	0.124	0.087

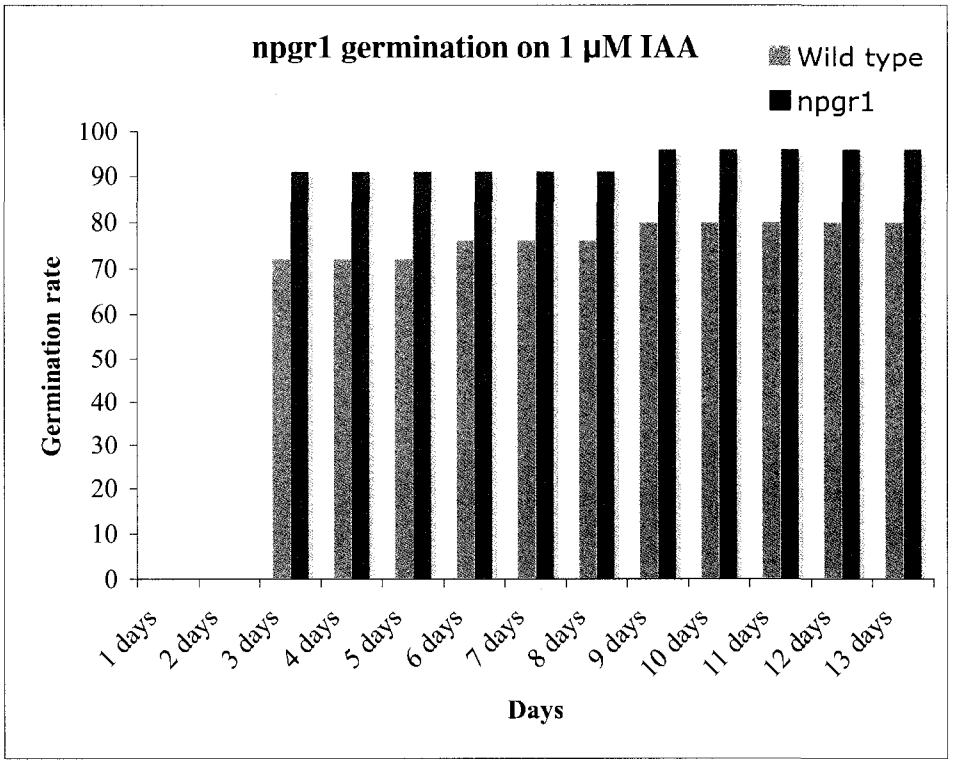


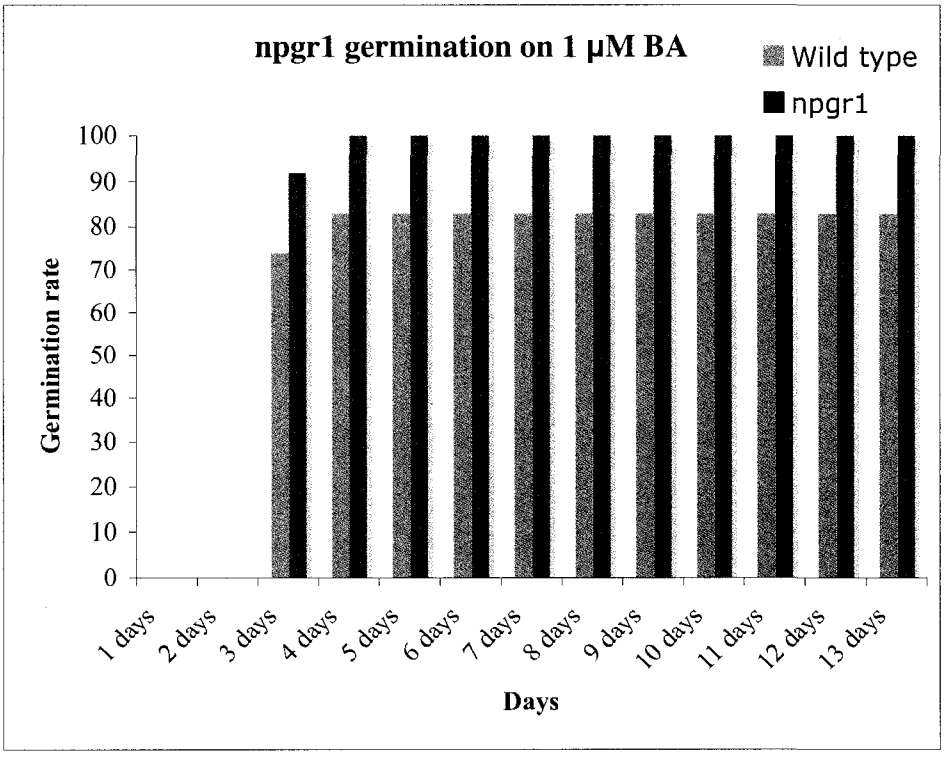
Appendix 5: Germination of wild type and *npgr1* seeds on MS and 3% glucose plates.



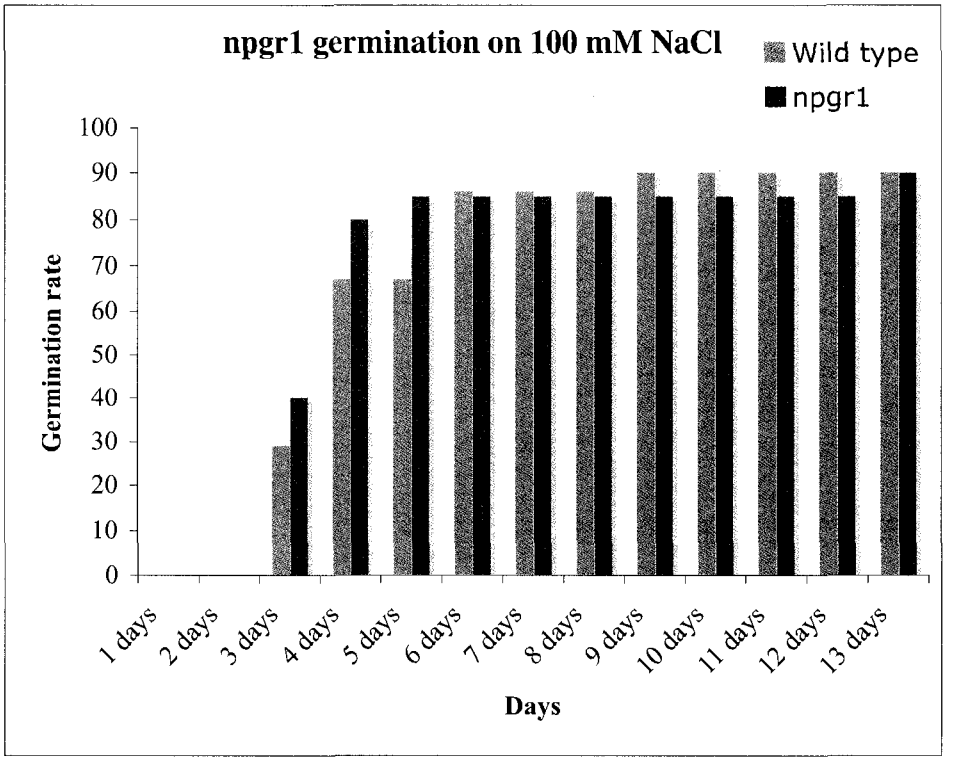


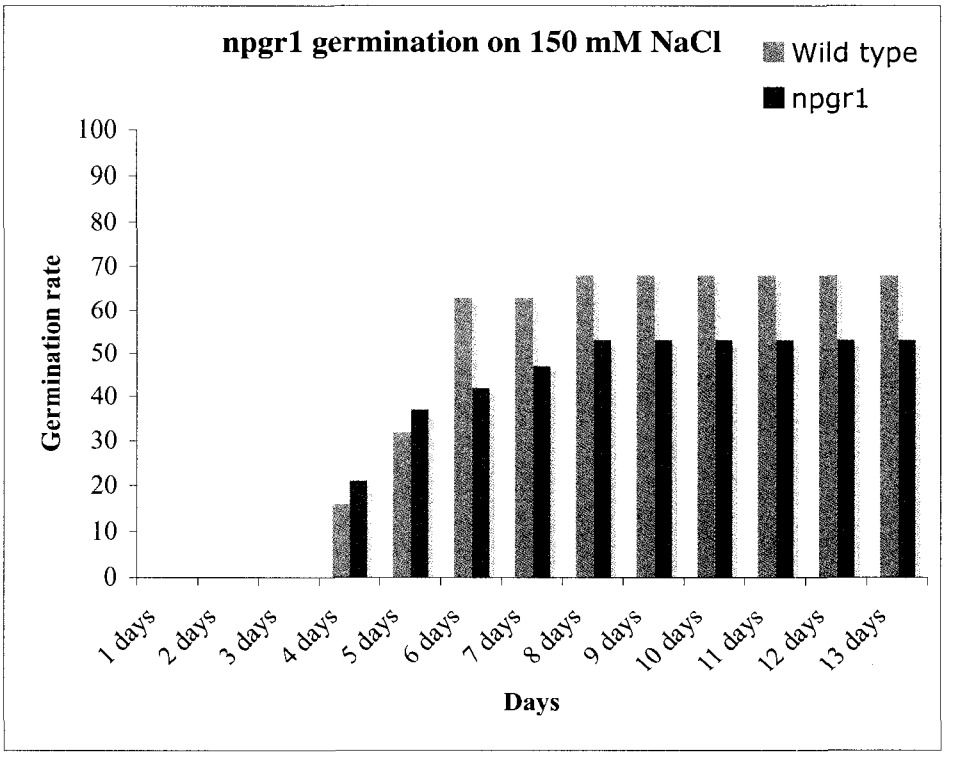
Appendix 6: Germination of wild type and *npgr1* seeds on ABA plates.





Appendix 7: Germination of wild type and *npgr1* seeds on IAA and BA plates.





Appendix 8: Germination of wild type and *npgr1* seeds in the presence of NaCl..

References

Abreu, I., and Oliveira, M. (2004). Immunolocalisation of arabinogalactan proteins and pectins in Actinidia deliciosa pollen. Short communication. Protoplasma 224, 123-128.

Albertazzi, F.J., Kudla, J., and Bock, R. (1998). The cox2 locus of the primitive angiosperm plant Acorus calamus: molecular structure, transcript processing and RNA editing. Mol Gen Genet 259, 591-600.

Allen, G.J., and Schroeder, J.I. (2001). Combining genetics and cell biology to crack the code of plant cell calcium signaling. Sci STKE 2001, RE13.

Alvarez-Martinez, M.T., Porte, F., Liautard, J.P., and Sri Widada, J. (1997). Effects of profilin-annexin I association on some properties of both profilin and annexin I: modification of the inhibitory activity of profilin on actin polymerization and inhibition of the self-association of annexin I and its interactions with liposomes. Biochim Biophys Acta 1339, 331-340.

Alvarez-Martinez, M.T., Mani, J.C., Porte, F., Faivre-Sarrailh, C., Liautard, J.P., and Sri Widada, J. (1996). Characterization of the interaction between annexin I and profilin. Eur J Biochem 238, 777-784.

Arenas-Huertero, F., Arroyo, A., Zhou, L., Sheen, J., and Leon, P. (2000). Analysis of Arabidopsis glucose insensitive mutants, gin5 and gin6, reveals a central role of the plant hormone ABA in the regulation of plant vegetative development by sugar. Genes Dev 14, 2085-2096.

Babu, Y.S., Bugg, C.E., and Cook, W.J. (1988). Structure of calmodulin refined at 2.2 Å resolution. J. Mol. Biol. 204, 191-204.

Barras, F.V.G., F. and Chatterjee, AK. (1994). Extracellular enzymes and pathogenesis of soft-rot Erwinia. Annu. Rev. Phytopathol. 32, 201-234.

Bartling, S., Wegener, C., and Olsen, O. (1995). Synergism between Erwinia pectate lyase isoenzymes that depolymerize both pectate and pectin. Microbiology 141 (Pt 4), 873-881.

Bedinger, P. (1992). The remarkable biology of pollen. Plant Cell 4, 879-887.

Bedinger, P., Hardeman, K.J., and Loukides, C.A. (1994). Travelling in style: the cell biology of pollen. Trends Cell Biol. 4, 132-138.

Benitez-Burraco, A., Blanco-Portales, R., Redondo-Nevado, J., Bellido, M.L., Moyano, E., Caballero, J.L., and Munoz-Blanco, J. (2003). Cloning and characterization of two

ripening-related strawberry (Fragaria x ananassa cv. Chandler) pectate lyase genes. J Exp Bot 54, 633-645.

Bjorkegren, C., Rozycki, M., Schutt, C.E., Lindberg, U., and Karlsson, R. (1993). Mutagenesis of human profilin locates its poly(L-proline)-binding site to a hydrophobic patch of aromatic amino acids. FEBS Lett 333, 123-126.

Blatch, G.L., and Lassle, M. (1999). The tetratricopeptide repeat: a structural motif mediating protein-protein interactions. Bioessays 21, 932-939.

Botella, J.R., and Arteca, R.N. (1994). Differential expression of two calmodulin genes in response to physical and chemical stimuli. Plant Mol Biol 24, 757-766.

Boyes, D.C., Zayed, A.M., Ascenzi, R., McCaskill, A.J., Hoffman, N.E., Davis, K.R., and Gorlach, J. (2001). Growth stage-based phenotypic analysis of Arabidopsis: a model for high throughput functional genomics in plants. Plant Cell 13, 1499-1510.

Budelier, K.A., Smith, A.G., and Gasser, C.S. (1990). Regulation of a stylar transmitting tissue-specific gene in wild-type and transgenic tomato and tobacco. Mol Gen Genet 224, 183-192.

Bush, D.S. (1995). Calcium regulation in plant cells and its role in signaling. Ann. Rev. Plant Physiol. Plant Mol. Biol. 46, 95-122.

Calder, G.M., Franklin-Tong, V.E., Shaw, P.J., and Drobak, B.K. (1997). Ca2+ oscillations in plant cells: initiation by rapid elevation in cytosolic free Ca2+ levels. Biochem Biophys Res Commun 234, 690-694.

Camacho, L.P., R.Trewavas, A.J. and Malho, R. (2000). Image cytosolic free-calcium distribution and oscillations in pollen tubes with confocal microscopy: a comparison of different dyes and loading methods. Protoplasma 212, 162-173.

Carpita, N.C. (1996). Structure and Biogenesis of the Cell Walls of Grasses. Annu Rev Plant Physiol Plant Mol Biol 47, 445-476.

Carpita, N.C., and Gibeaut, D.M. (1993). Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. Plant J 3, 1-30.

Catoire, L., Pierron, M., Morvan, C., du Penhoat, C.H., and Goldberg, R. (1998). Investigation of the action patterns of pectinmethylesterase isoforms through kinetic analyses and NMR spectroscopy. Implications In cell wall expansion. J Biol Chem 273, 33150-33156.

Centanni, S., Di Marco, F., Castagna, F., Santus, P., Guarnieri, R., and Allegra, L. (2001). Atopy prevalence and spirometric performance in asymptomatic schoolchildren exposed

to air pollution. Monaldi Arch Chest Dis 56, 304-308.

Chang, H.C., and Lindquist, S. (1994). Conservation of Hsp90 macromolecular complexes in Saccharomyces cerevisiae. J Biol Chem 269, 24983-24988.

Chanliaud, E., and Gidley, M.J. (1999). In vitro synthesis and properties of pectin/Acetobacter xylinus cellulose composites. Plant J 20, 25-35.

Chen, L., Carpita, N.C., Reiter, W.D., Wilson, R.H., Jeffries, C., and McCann, M.C. (1998). A rapid method to screen for cell-wall mutants using discriminant analysis of Fourier transform infrared spectra. Plant J 16, 385-392.

Chen, S., Prapapanich, V., Rimerman, R.A., Honore, B., and Smith, D.F. (1996). Interactions of p60, a mediator of progesterone receptor assembly, with heat shock proteins hsp90 and hsp70. Mol Endocrinol 10, 682-693.

Cheng, W.H., Endo, A., Zhou, L., Penney, J., Chen, H.C., Arroyo, A., Leon, P., Nambara, E., Asami, T., Seo, M., Koshiba, T., and Sheen, J. (2002). A unique short-chain dehydrogenase/reductase in Arabidopsis glucose signaling and abscisic acid biosynthesis and functions. Plant Cell 14, 2723-2743.

Cheung, A.Y., Wang, H., and Wu, H.M. (1995). A floral transmitting tissue-specific glycoprotein attracts pollen tubes and stimulates their growth. Cell 82, 383-393.

Cheung, A.Y., Chen, C.Y., Glaven, R.H., de Graaf, B.H., Vidali, L., Hepler, P.K., and Wu Hm, H. (2002). Rab2 GTPase Regulates Vesicle Trafficking between the Endoplasmic Reticulum and the Golgi Bodies and Is Important to Pollen Tube Growth. Plant Cell 14, 945-962.

Cho, Y.H., Yoo, S.D., and Sheen, J. (2006). Regulatory functions of nuclear hexokinase1 complex in glucose signaling. Cell 127, 579-589.

Christensen, H.E., Ramachandran, S., Tan, C.T., Surana, U., Dong, C.H., and Chua, N.H. (1996). Arabidopsis profilins are functionally similar to yeast profilins: identification of a vascular bundle-specific profilin and a pollen-specific profilin. Plant J 10, 269-279.

Clark, G.B., Turnwald, S., Tirlapur, U.K., Haas, C.J., von der Mark, K., Roux, S.J., and Scheuerlein, R. (1995). Polar distribution of annexin-like proteins during phytochrome-mediated initiation and growth of rhizoids in the ferns Dryopteris and Anemia. Planta 197, 376-384.

Cleland, R.E. (1976). Kinetics of Hormone-induced H Excretion. Plant Physiol 58, 210-213.

Cole, R.A., and Fowler, J.E. (2006). Polarized growth: maintaining focus on the tip. Curr Opin Plant Biol 9, 579-588.

Collmer, A., and Keen, N.T. (1986). The role of pectic enzymes in plant pathogenesis. Ann. Rev. Phytopathol. 24, 383-409.

Cosgrove, D.J. (2000). Expansive growth of plant cell walls. Plant Physiol Biochem 38, 109-124.

Crawford, M.S., and Kolattukudy, P.E. (1987). Pectate lyase from Fusarium solani f. sp. pisi: purification, characterization, in vitro translation of the mRNA, and involvement in pathogenicity. Arch Biochem Biophys 258, 196-205.

Cresti, M., Ciampolini, D., Mulcahy, D., and Mulcahy, G. (1985). Ultrastructure of Nicotiana alata pollen its germination and early tube growth. Am. J. Bot. 72, 719 - 727.

Crookes, P.R., and Grierson, D. (1983). Ultrastructure of Tomato Fruit Ripening and the Role of Polygalacturonase Isoenzymes in Cell Wall Degradation. Plant Physiol 72, 1088-1093.

Das, A.K., Cohen, P.W., and Barford, D. (1998). The structure of the tetratricopeptide repeats of protein phosphatase 5: implications for TPR-mediated protein-protein interactions. EMBO J 17, 1192-1199.

de Graaf, B.H., Cheung, A.Y., Andreyeva, T., Levasseur, K., Kieliszewski, M., and Wu, H.M. (2005). Rab11 GTPase-regulated membrane trafficking is crucial for tip-focused pollen tube growth in tobacco. Plant Cell 17, 2564-2579.

Dean, R.A., and Timberlake, W.E. (1989). Production of cell wall-degrading enzymes by Aspergillus nidulans: a model system for fungal pathogenesis of plants. Plant Cell 1, 265-273.

Derksen, J., Ruttens, T., van Amstel, T., de Win, A., Doris, F., and Steer, M. (1995). Regulation of pollen tube growth. Acta Bot. Neerl. 44, 93-119.

Dickinson, H., and Lewis, D. (1974). Changes in the pollen grain wall of Linum grandiflorum following compatible and incompatible intraspecific pollinations. Ann. Bot. 38, 23 - 29.

Dijkwel, P.P., Huijser, C., Weisbeek, P.J., Chua, N.H., and Smeekens, S.C. (1997). Sucrose control of phytochrome A signaling in Arabidopsis. Plant Cell 9, 583-595.

Dirckse, L., Vancanneyt, G.U.Y., and McCormick,s. (1996). Biochemical characterization and baculovirus expression of the pectate lyase-like LAT56 and LAT59 pollen proteins of tomato. Plant Physiol. Biochem. 34, 509-520.

Dodt, G., Braverman, N., Wong, C., Moser, A., Moser, H.W., Watkins, P., Valle, D., and Gould, S.J. (1995). Mutations in the PTS1 receptor gene, PXR1, define complementation

group 2 of the peroxisome biogenesis disorders. Nat Genet 9, 115-125.

Domingo, C., Roberts, K., Stacey, N.J., Connerton, I., Ruiz-Teran, F., and McCann, M.C. (1998). A pectate lyase from Zinnia elegans is auxin inducible. Plant J 13, 17-28.

Dominguez-Puigjaner, E., I, L.L., Vendrell, M., and Prat, S. (1997). A cDNA clone highly expressed in ripe banana fruit shows homology to pectate lyases. Plant Physiol 114, 1071-1076.

Drobak, B., Franklin, F., Shaw, P., Calder, G., Trewavas, A., Hepler, P., and Franklin-Tong, V. (1997). Second messenger-induced signaling events in pollen tubes of Papaver rhoeas. Exp. Biol. Online 2, 10.

Edlund, A.F., Swanson, R., and Preuss, D. (2004). Pollen and stigma structure and function: the role of diversity in pollination. Plant Cell 16 Suppl, S84-97.

Entian, K.D. (1980). Genetic and biochemical evidence for hexokinase PII as a key enzyme involved in carbon catabolite repression in yeast. Mol Gen Genet 178, 633-637.

Estruch, J.J., Kadwell, S., Merlin, E., and Crossland, L. (1994). Cloning and characterization of a maize pollen-specific calcium- dependent calmodulin-independent protein kinase. Proc Natl Acad Sci U S A 91, 8837-8841.

Eyal, Y., Curie, C., and McCormick, S. (1995). Pollen specificity elements reside in 30 bp of the proximal promoters of two pollen-expressed genes. Plant Cell 7, 373-384.

Fan, L.M., Wang, Y.F., Wang, H., and Wu, W.H. (2001). In vitro Arabidopsis pollen germination and characterization of the inward potassium currents in Arabidopsis pollen grain protoplasts. J. Exp. Bot. 52, 1603-1614.

Feijo, A., Malho, R., and Obermeyer, G. (1995). Ion dynamics and its possible role during in vitro pollen germination and tube growth. Protoplasma 187, 155 - 167.

Ferguson, C., Teeri, T.T., Siika-aho, M., Read, S.M., and Bacic, A. (1998). Location of cellulose and callose in pollen tubes and grains of Nicotiana tabacum. Planta 206, 452-460.

Franklin-Tong, N.V., and Franklin, F.C. (2003). Gametophytic self-incompatibility inhibits pollen tube growth using different mechanisms. Trends Plant Sci 8, 598-605.

Franklin-Tong, V., Ride, J., and Franklin, F. (1995). Recombinant stigmatic self-incompatibility (S) protein elicits a Ca2+ transient in pollen of Papaver rhoeas. Plant J. 8, 299-307.

Franklin-Tong, V., Hackett, G., and Hepler, P. (1997). Ratio-imaging of Ca2+i in the self-incompatibility response in pollen tubes of Papaver rhoeas. Plant J. 12, 1375-1386.

Franklin-Tong, V., Ride, J., Read, N., Trewavas, A., and Franklin, F. (1993). The self-incompatibility response in Papaver rhoeas is mediated by cytosolic free calcium. Plant J. 4, 163-177.

Franklin-Tong, V.E. (1999). Signaling and the modulation of pollen tube growth. Plant Cell 11, 727-738.

Franklin-Tong, V.E., Drobak, B.K., Allan, A.C., Watkins, P., and Trewavas, A.J. (1996). Growth of Pollen Tubes of Papaver rhoeas Is Regulated by a Slow-Moving Calcium Wave Propagated by Inositol 1,4,5-Trisphosphate. Plant Cell 8, 1305-1321.

Frazier, J.A., and Field, C.M. (1997). Actin cytoskeleton: are FH proteins local organizers? Curr Biol 7, R414-417.

Fu, Y., Wu, G., and Yang, Z. (2001). Rop GTPase-dependent dynamics of tip-localized F-actin controls tip growth in pollen tubes. J Cell Biol 152, 1019-1032.

Gazzarrini, S., and McCourt, P. (2001). Genetic interactions between ABA, ethylene and sugar signaling pathways. Curr Opin Plant Biol 4, 387-391.

Gherardini, G., and Healey, P. (1969). Dissolution of outer wall of pollen grain during pollination. Nature 224, 718 - 719.

Gibbon, B.C., Kovar, D.R., and Staiger, C.J. (1999). Latrunculin B Has Different Effects on Pollen Germination and Tube Growth. Plant Cell 11, 2349-2364.

Gibbon, B.C., Zonia, L.E., Kovar, D.R., Hussey, P.J., and Staiger, C.J. (1998). Pollen profilin function depends on interaction with proline-rich motifs. Plant Cell 10, 981-993.

Gibson, S.I. (2000). Plant sugar-response pathways. Part of a complex regulatory web. Plant Physiol 124, 1532-1539.

Golovkin, M., and Reddy, A.S. (2003). A calmodulin-binding protein from Arabidopsis has an essential role in pollen germination. Proc. Natl. Acad. Sci. USA 100, 10558-10563.

Gu, Y., Vernoud, V., Fu, Y., and Yang, Z. (2003). ROP GTPase regulation of pollen tube growth through the dynamics of tip-localized F-actin. J Exp Bot 54, 93-101.

Gummadi, S.N., and Kumar, D.S. (2005). Microbial pectic transeliminases. Biotechnol Lett 27, 451-458.

Guyon, V., Tang, W.H., Monti, M.M., Raiola, A., Lorenzo, G.D., McCormick, S., and Taylor, L.P. (2004). Antisense phenotypes reveal a role for SHY, a pollen-specific leucine-rich repeat protein, in pollen tube growth. Plant J 39, 643-654.

Haffner, C., Jarchau, T., Reinhard, M., Hoppe, J., Lohmann, S.M., and Walter, U. (1995). Molecular cloning, structural analysis and functional expression of the proline-rich focal adhesion and microfilament-associated protein VASP. Embo J 14, 19-27.

Haizel, T., Merkle, T., Turck, F., and Nagy, F. (1995). Characterization of membrane-bound small GTP-binding proteins from Nicotiana tabacum. Plant Physiol 108, 59-67.

Hansen, K.M., Truesen, A.B., and Soderberg, J.R. (2001). Enzyme assay for identification of pectin and pectin derivatives, based on recombinant pectate lyase. J AOAC Int 84, 1851-1854.

Hasegawa, S., and Nagel, C.W. (1962). The characterization of an alpha, beta-unsaturated digalacturonic acid. J Biol Chem 237, 619-621.

He, L., Bradrick, T.D., Karpova, T.S., Wu, X., Fox, M.H., Fischer, R., McNally, J.G., Knutson, J.R., Grammer, A.C., and Lipsky, P.E. (2003). Flow cytometric measurement of fluorescence (Forster) resonance energy transfer from cyan fluorescent protein to yellow fluorescent protein using single-laser excitation at 458 nm. Cytometry 53A, 39-54.

Helsper, J., Heemskerk, J., and Veerkamp, J. (1987). Cytosolic and particulate phosphatidylinositol phospholipase C activities in pollen tubes of Lilium longiflorum. Plant Physiology. 71, 120-126.

Hepler, P.K., Vidali, L., and Cheung, A.Y. (2001). Polarized cell growth in higher plants. Annu. Rev. Cell Dev. Biol. 17, 159-187.

Hernandez Torres, J., Chatellard, P., and Stutz, E. (1995). Isolation and characterization of gmsti, a stress-inducible gene from soybean (Glycine max) coding for a protein belonging to the TPR (tetratricopeptide repeats) family. Plant Mol Biol 27, 1221-1226.

Heslop-Harrison, J., and Heslop-Harrison, Y. (1992). Germination of monocolpate angiosperm pollen: evolution of actin cytoskeleton and wall during hydration, activation and tube emergence. Ann. Bot. 69, 385-394.

Heslop-Harrison, y., and Shivannah, K. (1977). The receptive surface of the angiosperm stigma. Ann. Bot. 42, 1233-1258.

Hirano, T., Kinoshita, N., Morikawa, K., and Yanagida, M. (1990). Snap helix with knob and hole: essential repeats in S. pombe nuclear protein nuc2+. Cell 60, 319-328.

Hiscock, S.J., and McInnis, S.M. (2003). Pollen recognition and rejection during the sporophytic self-incompatibility response: Brassica and beyond. Trends Plant Sci 8, 606-613.

Holdaway-Clarke, T.L., Feijo, J.A., Hacket, G.R., Kunkel, J.G., and Hepler, P.K. (1997). Pollen tube growth and intracellular cytoplasmic calcium gradient oscillate in phase

while extracellular calcium influx is delayed. Plant Cell 9, 1999-2010.

Huang, S., McDowell, J.M., Weise, M.J., and Meagher, R.B. (1996). The Arabidopsis profilin gene family. Evidence for an ancient split between constitutive and pollen-specific profilin genes. Plant Physiol 111, 115-126.

Huang, S., An, Y.Q., McDowell, J.M., McKinney, E.C., and Meagher, R.B. (1997). The Arabidopsis ACT11 actin gene is strongly expressed in tissues of the emerging inflorescence, pollen, and developing ovules. Plant Mol. Biol. 33, 125-139.

Iwata, Y., Kuriyama, M., Nakakita, M., Kojima, H., Ohto, M., and Nakamura, K. (1998). Characterization of a calcium-dependent protein kinase of tobacco leaves that is associated with the plasma membrane and is inducible by sucrose. Plant Cell Physiol 39, 1176-1183.

Jacobs, M., and Ray, P.M. (1976). Rapid Auxin-induced Decrease in Free Space pH and Its Relationship to Auxin-induced Growth in Maize and Pea. Plant Physiol 58, 203-209.

Jang, J.C., Leon, P., Zhou, L., and Sheen, J. (1997). Hexokinase as a sugar sensor in higher plants. Plant Cell 9, 5-19.

Jiang, L., Yang, S.L., Xie, L.F., Puah, C.S., Zhang, X.Q., Yang, W.C., Sundaresan, V., and Ye, D. (2005). VANGUARD1 encodes a pectin methylesterase that enhances pollen tube growth in the Arabidopsis style and transmitting tract. Plant Cell 17, 584-596.

Johnson, S.A., and McCormick, S. (2001). Pollen germinates precociously in the anthers of raring-to-go, an Arabidopsis gametophytic mutant. Plant Physiol 126, 685-695.

Johnston, M., and Kim, J.H. (2005). Glucose as a hormone: receptor-mediated glucose sensing in the yeast Saccharomyces cerevisiae. Biochem Soc Trans 33, 247-252.

Kamei, T., Tanaka, K., Hihara, T., Umikawa, M., Imamura, H., Kikyo, M., Ozaki, K., and Takai, Y. (1998). Interaction of Bnr1p with a novel Src homology 3 domain-containing Hof1p. Implication in cytokinesis in Saccharomyces cerevisiae. J Biol Chem 273, 28341-28345.

Kandasamy, M.K., McKinney, E.C., and Meagher, R.B. (2002). Plant profilin isovariants are distinctly regulated in vegetative and reproductive tissues. Cell Motil Cytoskeleton 52, 22-32.

Koch, K.E. (1996). Carbohydrate-Modulated Gene Expression in Plants. Annu Rev Plant Physiol Plant Mol Biol 47, 509-540.

Kost, B., Mathur, J., and Chua, N.-H. (1999). Cytoskeleton in plant development. Curr. Opi. Plant Biol. 2, 462-470.

Kovar, D.R., Drobak, B.K., and Staiger, C.J. (2000). Maize profilin isoforms are functionally distinct. Plant Cell 12, 583-598.

Kragler, F., Lametschwandtner, G., Christmann, J., Hartig, A., and Harada, J.J. (1998). Identification and analysis of the plant peroxisomal targeting signal 1 receptor NtPEX5. Proc Natl Acad Sci U S A 95, 13336-13341.

Kulikauskas, R., and McCormick, S. (1997). Identification of the tobacco and Arabidopsis homologues of the pollen-expressed LAT59 gene of tomato. Plant Mol Biol 34, 809-814.

Kurata, T., and Yamamoto, K.T. (1998). petit1, a conditional growth mutant of Arabidopsis defective in sucrose-dependent elongation growth. Plant Physiol 118, 793-801.

Lalanne, E., and Twell, D. (2002). Genetic control of male germ unit organization in Arabidopsis. Plant Physiol 129, 865-875.

Lamb, J.R., Tugendreichs, S., and Hieter, P. (1995). Tetratricopeptide repeat interactions: to TPR or not to TPR. Trends Biochem. Sci. 20, 257-259.

Lamb, J.R., Michaud, W.A., Sikorski, R.S., and Hieter, P.A. (1994). Cdc16p, Cdc23p and Cdc27p form a complex essential for mitosis. Embo J 13, 4321-4328.

Lancelle, S.A., and Hepler, P.K. (1992). Ultrastructure of freeze-substituted pollen tubes of *Lilium longiflorum*. Protoplasma 167, 215-230.

Laskowski, M., Biller, S., Stanley, K., Kajstura, T., and Prusty, R. (2006). Expression profiling of auxin-treated Arabidopsis roots: toward a molecular analysis of lateral root emergence. Plant Cell Physiol 47, 788-792.

Lassle, M., Blatch, G.L., Kundra, V., Takatori, T., and Zetter, B.R. (1997). Stress-inducible, murine protein mSTI1. Characterization of binding domains for heat shock proteins and in vitro phosphorylation by different kinases. J Biol Chem 272, 1876-1884.

Lemaire, K., Van de Velde, S., Van Dijck, P., and Thevelein, J.M. (2004). Glucose and sucrose act as agonist and mannose as antagonist ligands of the G protein-coupled receptor Gpr1 in the yeast Saccharomyces cerevisiae. Mol Cell 16, 293-299.

Lietzke, S.E., Yoder, M.D., Keen, N.T., and Jurnak, F. (1994). The Three-Dimensional Structure of Pectate Lyase E, a Plant Virulence Factor from Erwinia chrysanthemi. Plant Physiol 106, 849-862.

Lietzke, S.E., Scavetta, R.D., Yoder, M.D., and Jurnak, F. (1996). The Refined Three-Dimensional Structure of Pectate Lyase E from Erwinia chrysanthemi at 2.2 A Resolution. Plant Physiol 111, 73-92.

Lin, Y., and Yang, Z. (1997). Inhibition of Pollen Tube Elongation by Microinjected Anti-Rop1Ps Antibodies Suggests a Crucial Role for Rho-Type GTPases in the Control of Tip Growth. Plant Cell 9, 1647-1659.

Lin, Y., Wang, Y., Zhu, J.K., and Yang, Z. (1996). Localization of a Rho GTPase Implies a Role in Tip Growth and Movement of the Generative Cell in Pollen Tubes. Plant Cell 8, 293-303.

Loisel, T.P., Boujemaa, R., Pantaloni, D., and Carlier, M.F. (1999). Reconstitution of actin-based motility of Listeria and Shigella using pure proteins. Nature 401, 613-616.

Lopez-Molina, L., Mongrand, S., and Chua, N.H. (2001). A postgermination developmental arrest checkpoint is mediated by abscisic acid and requires the ABI5 transcription factor in Arabidopsis. Proc Natl Acad Sci U S A 98, 4782-4787.

Lord, E. (2000). Adhesion and cell movement during pollination: cherchez la femme. Trends Plant Sci 5, 368-373.

Lord, E.M., and Russell, S.D. (2002). The Mechanisms of Pollination and Fertilization in Plants. Annu Rev Cell Dev Biol 18, 81-105.

Luan, S., Kudla, J., Rodriguez-Concepcion, M., Yalovsky, S., and Gruissem, W. (2002). Calmodulins and calcineurin B-like proteins: calcium sensors for specific signal response coupling in plants. Plant Cell 14 Suppl, S389-S400.

Lush, W.M., Grieser, F., and Wolters-Arts, M. (1998). Directional guidance of nicotiana alata pollen tubes in vitro and on the stigma. Plant Physiol 118, 733-741.

Luu, D.T., Marty-Mazars, D., Trick, M., Dumas, C., and Heizmann, P. (1999). Pollen-stigma adhesion in Brassica spp involves SLG and SLR1 glycoproteins. Plant Cell 11, 251-262.

Ma, L., and Sun, D. (1997). The effects of extracellular calmodulin on initiation of Hippeastrum rutilum pollen germination and tube growth. Planta 202, 336-340.

Ma, L., Xu, X., Cui, S., and Sun, D. (1999). The presence of a heterotrimeric G protein and its role in signal transduction of extracellular calmodulin in pollen germination and tube growth. Plant cell 11, 1351-1363.

Malho, R., and Trewavas, A.J. (1996). Localized apical increases of cytosolic free calcium control pollen tube orientation. Plant Cell 8, 1935-1949.

Malho, R., Read, N., Pais, M.S., and Trewavas, A.J. (1994). Role of cytosolic free calcium in the reorientation of pollen tube growth. Plant J. 5, 331-341.

Malho, R., Read, N., Trewavas, A.J., and Pais, M.S. (1995). Calcium channel activity during pollen tube growth and reorientation. Plant Cell 7, 1173-1184.

Marin-Rodriguez, M.C., Orchard, J., and Seymour, G.B. (2002). Pectate lyases, cell wall degradation and fruit softening. J Exp Bot 53, 2115-2119.

Marin-Rodriguez, M.C., Smith, D.L., Manning, K., Orchard, J., and Seymour, G.B. (2003). Pectate lyase gene expression and enzyme activity in ripening banana fruit. Plant Mol Biol 51, 851-857.

Mazina, S.E., Matveeva, N.P., and Ermakov, I.P. (2002). [Determination of a position of a functional pore in the tobacco pollen]. Tsitologiia 44, 33-39.

McCormick, S., Twell, D., Vancanneyt, G., and Yamaguchi, J. (1991). Molecular analysis of gene regulation and function during male gametophyte development. Symp Soc Exp Biol 45, 229-244.

McNeil, M., Darvill, A.G., Fry, S.C., and Albersheim, P. (1984). Structure and function of the primary cell walls of plants. Annu Rev Biochem 53, 625-663.

Medina-Escobar, N., Cardenas, J., Moyano, E., Caballero, J.L., and Munoz-Blanco, J. (1997). Cloning, molecular characterization and expression pattern of a strawberry ripening-specific cDNA with sequence homology to pectate lyase from higher plants. Plant Mol Biol 34, 867-877.

Medina-Suarez, R., Manning, K., Fletcher, J., Aked, J., Bird, C.R., and Seymour, G.B. (1997). Gene expression in the pulp of ripening bananas. Two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis of in vitro translation products and cDNA cloning of 25 different ripening-related mRNAs. Plant Physiol 115, 453-461.

Messerli, M.A., and Robinson, K.R. (2003). Ionic and osmotic disruptions of the lily pollen tube oscillator: testing proposed models. Planta 217, 147-157.

Milioni, D., Sado, P.E., Stacey, N.J., Domingo, C., Roberts, K., and McCann, M.C. (2001). Differential expression of cell-wall-related genes during the formation of tracheary elements in the Zinnia mesophyll cell system. Plant Mol Biol 47, 221-238.

Molendijk, A.J., Ruperti, B., and Palme, K. (2004). Small GTPases in vesicle trafficking. Curr Opin Plant Biol 7, 694-700.

Mollet, J.C., Park, S.Y., Nothnagel, E.A., and Lord, E.M. (2000). A lily stylar pectin is necessary for pollen tube adhesion to an in vitro stylar matrix. Plant Cell 12, 1737-1750.

Moore, B., Zhou, L., Rolland, F., Hall, Q., Cheng, W.H., Liu, Y.X., Hwang, I., Jones, T., and Sheen, J. (2003). Role of the Arabidopsis glucose sensor HXK1 in nutrient, light, and hormonal signaling. Science 300, 332-336.

Moreno, F., Ahuatzi, D., Riera, A., Palomino, C.A., and Herrero, P. (2005). Glucose sensing through the Hxk2-dependent signalling pathway. Biochem Soc Trans 33, 265-268.

Moscatelli, A., Scali, M., Vignani, R., Onelli, E., and Cresti, M. (2003). Dynein heavy chain (DHC)-related polypeptides during pollen tube growth. Cell Biol Int 27, 237-238.

Moscatelli, A., Del Casino, C., Lozzi, L., Cai, G., Scali, M., Tiezzi, A., and Cresti, M. (1995). High molecular weight polypeptides related to dynein heavy chains in Nicotiana tabacum pollen tubes. J. Cell Sci 108, 1117-1125.

Mouline, K., Very, A.A., Gaymard, F., Boucherez, J., Pilot, G., Devic, M., Bouchez, D., Thibaud, J.B., and Sentenac, H. (2002). Pollen tube development and competitive ability are impaired by disruption of a Shaker K(+) channel in Arabidopsis. Genes Dev 16, 339-350.

Moutinho, A., Love, J., Trewavas, A.J., and Malho, R. (1998). Distribution of calmodulin protein and mRNA in growing pollen tubes. Sex. Plant Reprod. 11, 131-139.

Mullins, R.D., Kelleher, J.F., Xu, J., and Pollard, T.D. (1998). Arp2/3 complex from Acanthamoeba binds profilin and cross-links actin filaments. Mol Biol Cell 9, 841-852.

Nasrallah, J.B. (1997). Signal perception and response in the interactions of self-incompatibility in Brassica. Essays Biochem 32, 143-160.

Nasrallah, J.B. (2002). Recognition and rejection of self in plant reproduction. Science 296, 305-308.

Nunan, K.J., Davies, C., Robinson, S.P., and Fincher, G.B. (2001). Expression patterns of cell wall-modifying enzymes during grape berry development. Planta 214, 257-264.

Obermeyer, G., and Weisenseel, M.H. (1991). Calcium channel blocker and calmodulin antagonists affect the gradient of free calcium ions in lily pollen tubes. Eur. J. Cell Biol. 56, 319-327.

Ohto, M., and Nakamura, K. (1995). Sugar-Induced Increase of Calcium-Dependent Protein Kinases Associated with the Plasma Membrane in Leaf Tissues of Tobacco. Plant Physiol 109, 973-981.

Oppenheimer, D.G., Pollock, M.A., Vacik, J., Szymanski, D.B., Ericson, B., Feldmann, K., and Marks, D. (1997). Essential role of a kinesin-like protein in Arabidopsis trichome morphogenesis. Proc. Natl. Acad. Sci. USA 94, 6261-6266.

Ozcan, S., Dover, J., Rosenwald, A.G., Wolfl, S., and Johnston, M. (1996). Two glucose transporters in Saccharomyces cerevisiae are glucose sensors that generate a signal for

induction of gene expression. Proc Natl Acad Sci U S A 93, 12428-12432.

Palanivelu, R., Brass, L., Edlund, A.F., and Preuss, D. (2003). Pollen tube growth and guidance is regulated by POP2, an Arabidopsis gene that controls GABA levels. Cell 114, 47-59.

Palusa, S.G., Golovkin, M., Shin, S.B., Richardson, D.N., and Reddy, A.S. (2007). Organ-specific, developmental, hormonal and stress regulation of expression of putative pectate lyase genes in Arabidopsis. New Phytol 174, 537-550.

Perata, P., Matsukura, C., Vernieri, P., and Yamaguchi, J. (1997). Sugar Repression of a Gibberellin-Dependent Signaling Pathway in Barley Embryos. Plant Cell 9, 2197-2208.

Picton, J.M., and Steer, M.W. (1983). Membrane recycling and the control of secretory activity in pollen tubes. J Cell Sci 63, 303-310.

Pierson, E.S., Miller, D.D., Callaham, D.A., van Aken, J., Hackett, G., and Hepler, P.K. (1996). Tip-localized calcium entry fluctuates during pollen tube growth. Dev. Biol. 174, 160-173.

Pierson, E.S., Miller, D.D., Callaham, D.A., Shipley, A.M., Rivers, B.A., Cresti, M., and Hepler, P.K. (1994). Pollen tube growth is coupled to the extracellular calcium ion flux and the intracellular calcium gradient: effect of BAPTA-type buffers and hypertonic media. Plant Cell 6, 1815-1828.

Pilatzke-Wunderlich, I., and Nessler, C.L. (2001). Expression and activity of cell-wall-degrading enzymes in the latex of opium poppy, Papaver somniferum L. Plant Mol Biol 45, 567-576.

Pollard, T.D. (2001). Genomics, the cytoskeleton and motility. Nature 409, 842-843.

Popescu, S.C., Popescu, G.V., Bachan, S., Zhang, Z., Seay, M., Gerstein, M., Snyder, M., and Dinesh-Kumar, S.P. (2007). Differential binding of calmodulin-related proteins to their targets revealed through high-density Arabidopsis protein microarrays. Proc Natl Acad Sci U S A 104, 4730-4735.

Prodromou, C., Siligardi, G., O'Brien, R., Woolfson, D.N., Regan, L., Panaretou, B., Ladbury, J.E., Piper, P.W., and Pearl, L.H. (1999). Regulation of Hsp90 ATPase activity by tetratricopeptide repeat (TPR)- domain co-chaperones. EMBO J. 18, 754-762.

Ramachandran, S., Christensen, H.E., Ishimaru, Y., Dong, C.H., Chao-Ming, W., Cleary, A.L., and Chua, N.H. (2000). Profilin plays a role in cell elongation, cell shape maintenance, and flowering in Arabidopsis. Plant Physiol 124, 1637-1647.

Rathore, K.S., Cork, R.J., and Robinson, K.R. (1991). A cytoplasmic gradient of Ca2+ is correlated with the growth of lily pollen tubes. Dev Biol 148, 612-619.

Rato, C., Monteiro, D., Hepler, P.K., and Malho, R. (2004). Calmodulin activity and cAMP signalling modulate growth and apical secretion in pollen tubes. Plant J 38, 887-897.

Rayle, D.L., and Cleland, R. (1977). Control of plant cell enlargement by hydrogen ions. Curr Top Dev Biol 11, 187-214.

Reddy, A.S. (2001a). Calcium: silver bullet in signaling. Plant Sci. 160, 381-404.

Reddy, A.S.N. (2001b). Molecular motors and their functions in plants. Int. Rev. Cytol. & Cell Bio. 204, 97-178.

Reddy, A.S.N., Narasimhulu, S.B., Safadi, F., and Golovkin, M. (1996). A plant kinesin heavy chain-like protein is a calmodulin-binding protein. Plant J. 10, 9-21.

Reddy, V.S., and Reddy, A.S. (2004a). Proteomics of calcium-signaling components in plants. Phytochemistry 65, 1745-1776.

Reddy, V.S., and Reddy, A.S. (2004b). Developmental and cell-specific expression of ZWICHEL is regulated by the intron and exon sequences of its gene. Plant Mol Biol 54, 273-293.

Reinhard, M., Giehl, K., Abel, K., Haffner, C., Jarchau, T., Hoppe, V., Jockusch, B.M., and Walter, U. (1995). The proline-rich focal adhesion and microfilament protein VASP is a ligand for profilins. Embo J 14, 1583-1589.

Rhee, S.Y., and Somerville, C.R. (1998). Tetrad pollen formation in quartet mutants of Arabidopsis thaliana is associated with persistence of pectic polysaccharides of the pollen mother cell wall. Plant J. 15, 79-88.

Riezman, H., Hase, T., van Loon, A.P., Grivell, L.A., Suda, K., and Schatz, G. (1983). Import of proteins into mitochondria: a 70 kilodalton outer membrane protein with a large carboxy-terminal deletion is still transported to the outer membrane. Embo J 2, 2161-2168.

Roberts, D.M., and Harmon, A.C. (1992). Calcium modulated proteins: targets of intracellular calcium signals in higher plants. Ann. Rev. Plant Physiol. Plant Mol. Biol. 43, 375-414.

Rolland, F., and Sheen, J. (2005). Sugar sensing and signalling networks in plants. Biochem Soc Trans 33, 269-271.

Rolland, F., Moore, B., and Sheen, J. (2002). Sugar sensing and signaling in plants. Plant Cell 14 Suppl, S185-205.

Rolland, F., Baena-Gonzalez, E., and Sheen, J. (2006). Sugar sensing and signaling in plants: conserved and novel mechanisms. Annu Rev Plant Biol 57, 675-709.

Rubinstein, A.L., Broadwater, A.H., Lowrey, K.B., and Bedinger, P.A. (1995a). Pex1, a pollen-specific gene with an extensin-like domain. Proc Natl Acad Sci U S A 92, 3086-3090.

Rubinstein, A.L., Marquez, J., Suarez-Cervera, M., and Bedinger, P.A. (1995b). Extensin-like Glycoproteins in the Maize Pollen Tube Wall. Plant Cell 7, 2211-2225.

Rutherford, S., and Moore, I. (2002). The Arabidopsis Rab GTPase family: another enigma variation. Curr Opin Plant Biol 5, 518-528.

Safadi, F., Reddy, V., and Reddy, A.S.N. (2000). A pollen-specific novel calmodulin-binding protein with tetratricopeptide repeats. J. Biol. Chem., In Press.

Santangelo, G.M. (2006). Glucose signaling in Saccharomyces cerevisiae. Microbiol Mol Biol Rev 70, 253-282.

Schlegel, T., Mirus, O., von Haeseler, A., and Schleiff, E. (2007). The tetratricopeptide repeats of receptors involved in protein translocation across membranes. Mol Biol Evol 24, 2763-2774.

Schmidt, A., and Hall, M.N. (1998). Signaling to the actin cytoskeleton. Annu Rev Cell Dev Biol 14, 305-338.

Schultz, J., Marshall-Carlson, L., and Carlson, M. (1990). The N-terminal TPR region is the functional domain of SSN6, a nuclear phosphoprotein of Saccharomyces cerevisiae [published erratum appears in Mol Cell Biol 1992 Jun;12(6):2909]. Mol Cell Biol 10, 4744-4756.

Sikorski, R.S., Boguski, M.S., Goebl, M., and Hieter, P. (1990). A repeating amino acid motif in CDC23 defines a family of proteins and a new relationship among genes required for mitosis and RNA synthesis. Cell 60, 307-317.

Smeekens, S. (2000). Sugar-Induced Signal Transduction in Plants. Annu Rev Plant Physiol Plant Mol Biol 51, 49-81.

Smith, D.F., Sullivan, W.P., Marion, T.N., Zaitsu, K., Madden, B., McCormick, D.J., and Toft, D.O. (1993). Identification of a 60-kilodalton stress-related protein, p60, which interacts with hsp90 and hsp70. Mol Cell Biol 13, 869-876.

Smith, R.L., Redd, M.J., and Johnson, A.D. (1995). The tetratricopeptide repeats of Ssn6 interact with the homeo domain of alpha 2. Genes Dev 9, 2903-2910.

Sohn, R.H., Chen, J., Koblan, K.S., Bray, P.F., and Goldschmidt-Clermont, P.J. (1995).

Localization of a binding site for phosphatidylinositol 4,5-bisphosphate on human profilin. J Biol Chem 270, 21114-21120.

Solbak, A.I., Richardson, T.H., McCann, R.T., Kline, K.A., Bartnek, F., Tomlinson, G., Tan, X., Parra-Gessert, L., Frey, G.J., Podar, M., Luginbuhl, P., Gray, K.A., Mathur, E.J., Robertson, D.E., Burk, M.J., Hazlewood, G.P., Short, J.M., and Kerovuo, J. (2005). Discovery of pectin-degrading enzymes and directed evolution of a novel pectate lyase for processing cotton fabric. J Biol Chem 280, 9431-9438.

Staiger, C., Gibbon, C., Kovar, D.R., and Zonia, L.E. (1997). Profilin and actin-depolymerizing factor: modulators of acting organization in plants. Trends Plant Sci. 2, 275-281.

Staiger, C.J. (2000a). Actin : a dynamic framework for multiple plant cell functions. (Dordrecht ; Boston: Kluwer Academic Publishers).

Staiger, C.J. (2000b). Signaling to the Actin Cytoskeleton in Plants. Annu Rev Plant Physiol Plant Mol Biol 51, 257-288.

Staiger, C.J., Goodbody, K.C., Hussey, P.J., Valenta, R., Drobak, B.K., and Lloyd, C.W. (1993). The profilin multigene family of maize: differential expression of three isoforms. Plant J 4, 631-641.

Starr, M.P., and Moran, F. (1962). Eliminative split of pectic substances by phytopathogenic soft-rot bacteria. Science 135, 920-921.

Steer, M.W., and Steer, J.M. (1989). Pollen tube tip growth. New Phytol. 111, 323-358.

Sterling, J.D., Quigley, H.F., Orellana, A., and Mohnen, D. (2001). The catalytic site of the pectin biosynthetic enzyme alpha-1,4-galacturonosyltransferase is located in the lumen of the Golgi. Plant Physiol 127, 360-371.

Stulke, J., and Hillen, W. (1999). Carbon catabolite repression in bacteria. Curr Opin Microbiol 2, 195-201.

Sze, H., Li, X., and Palmgren, M.G. (1999). Energization of plant cell membranes by H+-pumping ATPases. Regulation and biosynthesis. Plant Cell 11, 677-690.

Takayama, S., Shiba, H., Iwano, M., Asano, K., Hara, M., Che, F.S., Watanabe, M., Hinata, K., and Isogai, A. (2000). Isolation and characterization of pollen coat proteins of Brassica campestris that interact with S locus-related glycoprotein 1 involved in pollen-stigma adhesion. Proc Natl Acad Sci U S A 97, 3765-3770.

Tang, W., Ezcurra, I., Muschietti, J., and McCormick, S. (2002). A cysteine-rich extracellular protein, LAT52, interacts with the extracellular domain of the pollen receptor kinase LePRK2. Plant Cell 14, 2277-2287.

Tang, W., Kelley, D., Ezcurra, I., Cotter, R., and McCormick, S. (2004). LeSTIG1, an extracellular binding partner for the pollen receptor kinases LePRK1 and LePRK2, promotes pollen tube growth in vitro. Plant J 39, 343-353.

Taniguchi, Y., Ono, A., Sawatani, M., Nanba, M., Kohno, K., Usui, M., Kurimoto, M., and Matuhasi, T. (1995). Cry j I, a major allergen of Japanese cedar pollen, has pectate lyase enzyme activity. Allergy 50, 90-93.

Taylor, L.P., and Hepler, P.K. (1997). Pollen germination and tube growth. Annu. Rev. Plant Physiol.Plant Mol. Biol. 48, 461-491.

Tian, G.W., Chen, M.H., Zaltsman, A., and Citovsky, V. (2006). Pollen-specific pectin methylesterase involved in pollen tube growth. Dev Biol 294, 83-91.

Tirlapur, U.K., Scali, M., Moscatelli, A., Del Casino, C., Cai, G., Tiezzi, A., and Cresti, M. (1994). Confocal image analysis of spatial variations in immunocytochemically identified calmodulin during pollen hydration, germination and pollen tube tip growth in *Nicotiana tabacum* L. Zygote 2, 63-68.

Twell, D., Yamaguchi, J., Wing, R.A., Ushiba, J., and McCormick, S. (1991). Promoter analysis of genes that are coordinately expressed during pollen development reveals pollen-specific enhancer sequences and shared regulatory elements. Genes Dev 5, 496-507.

Tzamarias, D., and Struhl, K. (1995). Distinct TPR motifs of Cyc8 are involved in recruiting the Cyc8-Tup1 corepressor complex to differentially regulated promoters. Genes & Dev. 9, 821-831.

Valenta, R., Duchene, M., Pettenburger, K., Sillaber, C., Valent, P., Bettelheim, P., Breitenbach, M., Rumpold, H., Kraft, D., and Scheiner, O. (1991). Identification of profilin as a novel pollen allergen; IgE autoreactivity in sensitized individuals. Science 253, 557-560.

Valenta, R., Ferreira, F., Grote, M., Swoboda, I., Vrtala, S., Duchene, M., Deviller, P., Meagher, R.B., McKinney, E., Heberle-Bors, E., and et al. (1993). Identification of profilin as an actin-binding protein in higher plants. J Biol Chem 268, 22777-22781.

Venolia, L., Ao, W., Kim, S., Kim, C., and Pilgrim, D. (1999). unc-45 gene of *Caenorhabditis elegans* encodes a muscle-specific tetratricopeptide repeat-containing protein. Cell Motil. Cytoskeleton 42, 163-177.

Vernoud, V., Horton, A.C., Yang, Z., and Nielsen, E. (2003). Analysis of the small GTPase gene superfamily of Arabidopsis. Plant Physiol 131, 1191-1208.

Vitali, J., Schick, B., Kester, H.C., Visser, J., and Jurnak, F. (1998). The tree-dimensional

structure of aspergillus niger pectin lyase B at 1.7-A resolution. Plant Physiol 116, 69-80.
Vogel, J.P., Raab, T.K., Schiff, C., and Somerville, S.C. (2002). PMR6, a pectate lyase-like gene required for powdery mildew susceptibility in Arabidopsis. Plant Cell 14, 2095-2106.

Vucich, V.A., and Gasser, C.S. (1996). Novel structure of a high molecular weight FK506 binding protein from Arabidopsis thaliana. Mol Gen Genet 252, 510-517.

Wasteneys, G.O. (2000). The cytoskeleton and growth polarity. Curr Opin Plant Biol 3, 503-511.

Wen, F., Zhu, Y., and Hawes, M.C. (1999). Effect of pectin methylesterase gene expression on pea root development. Plant Cell 11, 1129-1140.

Wilhelmi, L.K., and Preuss, D. (1997). Blazing New Trails (Pollen Tube Guidance in Flowering Plants). Plant Physiol 113, 307-312.

Wilhelmi, L.K., and Preuss, D. (1999). The mating game: pollination and fertilization in flowering plants. Curr Opin Plant Biol 2, 18-22.

Willats, W.G., McCartney, L., Mackie, W., and Knox, J.P. (2001). Pectin: cell biology and prospects for functional analysis. Plant Mol Biol 47, 9-27.

Wilson, J.E. (2003). Isozymes of mammalian hexokinase: structure, subcellular localization and metabolic function. J Exp Biol 206, 2049-2057.

Wing, R.A., Yamaguchi, J., Larabell, S.K., Ursin, V.M., and McCormick, S. (1990). Molecular and genetic characterization of two pollen-expressed genes that have sequence similarity to pectate lyases of the plant pathogen Erwinia. Plant Mol Biol 14, 17-28.

Wolters-Arts, M., Lush, W.M., and Mariani, C. (1998). Lipids are required for directional pollen-tube growth. Nature 392, 818-821.

Wu, H.M., Wong, E., Ogdahl, J., and Cheung, A.Y. (2000). A pollen tube growth-promoting arabinogalactan protein from nicotiana alata is similar to the tobacco TTS protein. Plant J 22, 165-176.

Wu, Y., Qiu, X., Du, S., and Erickson, L. (1996). PO149, a new member of pollen pectate lyase-like gene family from alfalfa. Plant Mol Biol 32, 1037-1042.

Yanagisawa, S., Yoo, S.D., and Sheen, J. (2003). Differential regulation of EIN3 stability by glucose and ethylene signalling in plants. Nature 425, 521-525.

Yang, T., and Poovaiah, B.W. (2003). Calcium/calmodulin-mediated signal network in plants. Trends Plant Sci. 8, 505-512.

Yoder, M.D., Keen, N.T., and Jurnak, F. (1993). New domain motif: the structure of pectate lyase C, a secreted plant virulence factor. Science 260, 1503-1507.

Yu, S.M., Lee, Y.C., Fang, S.C., Chan, M.T., Hwa, S.F., and Liu, L.F. (1996). Sugars act as signal molecules and osmotica to regulate the expression of alpha-amylase genes and metabolic activities in germinating cereal grains. Plant Mol Biol 30, 1277-1289.

Zhou, L., Jang, J.C., Jones, T.L., and Sheen, J. (1998). Glucose and ethylene signal transduction crosstalk revealed by an Arabidopsis glucose-insensitive mutant. Proc Natl Acad Sci U S A 95, 10294-10299.

Zielinski, R.E. (1998). Calmodulin and Calmodulin-Binding Proteins in Plants. Annu Rev Plant Physiol Plant Mol Biol 49, 697-725.

Zinkl, G.M., Zwiebel, B.I., Grier, D.G., and Preuss, D. (1999). Pollen-stigma adhesion in Arabidopsis: a species-specific interaction mediated by lipophilic molecules in the pollen exine. Development 126, 5431-5440.

Zonia, L., Cordeiro, S., and Feijo, J. (2001). Ion dynamics and hydrodymanics in the regulation of pollen tube growth. Sexual Plant Reproduction 14, 111 - 116.