G Proteins and Plant Innate Immunity

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Abstract Under the peaceful appearance of lovely green meadow, the different plant communities are engaged in a continuous struggle for life. Plants use every imaginable mechanism to enhance their defenses in order to survive attacks from an enormous number of pathogens. Plant innate immunity strongly relies on signal transduction pathways connecting pathogen recognition with the establishment of specific defense responses. Heterotrimeric and small GTP-binding proteins provide such signaling between plasma membrane receptors and cytoplasm localized effector molecules. Recent studies, mostly in *Arabidopsis* and rice, have revealed very important roles for G proteins in plant resistance to fungal pathogens. Experimental evidence implicating G proteins in plant innate immunity and putative signaling mechanisms is presented and discussed in this chapter.

1 Introduction

Members of the superfamily of GTP hydrolyzing proteins (G proteins) are present in most living organisms. Their functions are extremely divergent as are the cellular processes in which they are involved. In this chapter, we review the involvement of G proteins in plant innate immunity.

The connection between G proteins and disease resistance was first established in medical research. Defects in G proteins or their associated receptors leading to dysfunctional signal transduction pathways result in an impressive variety of

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diseases in humans (Farfel et al. 1999; Spiegel 1996). Specific mutations in $G\alpha_s$, an alpha subunit of the heterotrimeric G proteins, are established molecular basis for Albright hereditary osteodystrophy, acromegaly, and hyperfunctional thyroid nodules (Spiegel 1996). Cholera is a devastating disease caused by the bacterium *Vibrio cholera*. An enterotoxin secreted by the harmful strains of the bacteria, known as cholera toxin (CTX), is responsible for the infectious gastroenteritis characteristic of cholera infection (Sharp and Hynie 1971). CTX exerts its action by ribosylating Gα subunits and hence locking the G proteins in a permanently active state. Other medical conditions caused by the malfunction of G-protein-mediated pathways are color blindness, mental retardation, cancer, familial male precocious puberty, Nephrogenic diabetes insipidus, and congenital bleeding (Bos 1989, Farfel et al. 1999; Ropers and Hamel 2005; Spiegel 1996).

2 Heterotrimeric G Proteins and Plant Innate Immunity

Plants have faced their arch nemeses – plant pathogens for millions of years. It is impossible to trace when this war begun, but it is certainly far from over. Plants constantly develop new defense mechanisms while pathogens continuously search for ways to overcome the plant defense. As a result, innate immunity of modern plants possesses an enormous arsenal of defense pathways including multiple receptors recognizing bacterial and fungal elicitors, signal transduction pathways conveying information to transcription machinery, and a multitude of protein and metabolic compounds providing an appropriate response to the attacker. The quicker the response, the more chances to survive. Heterotrimeric GTP-binding proteins are well known in animal systems as one of the fastest signal transducing elements and soon after their discovery in plants (Ma et al. 1990) their involvement in plant innate immunity was also studied (Legendre et al. 1992).

2.1 Pharmacological Studies

Initially, the absence of G proteins mutants prevented the use of genetic approaches to study heterotrimeric G proteins in plants. However, a broad assortment of compounds able to modulate heterotrimeric G protein activity was well known from animal and medical studies. Pharmacological approaches, modulating heterotrimeric G protein activity with different chemical and biochemical agents had been widely used in animal systems to study their function. The most common G protein modulators used in such experiments were mastoporan, cholera toxin, pertussis toxin, GTPγS, and suramin. Mastoparan is a 14-amino acid peptide present in wasp venom. When bound to the phospholipid membrane, mastoparan mimics two basic intracellular loops of the G protein-coupled receptors; it interacts with and activates the Gα subunit (Higashijima et al. 1988). Cholera toxin is a protein complex

secreted by the bacterium *V. cholerae*. It modifies the Gα subunit by disabling its intrinsic GTPase activity, and thus, locks Gα in a constant active state. Pertussis toxin is a protein produced by the human pathogenic bacterium *Bordetella pertussis*. It inactivates G proteins by catalyzing the ADP-ribosylation of the Gα subunit (Burns 1988). GTPγS is a nonhydrolysable GTP analog, once bound to Gα GTPγS retains it in the active state (Seifert et al. 1986). Suramin, polysulfonated naphthylurea, prevents binding between Gα and GTP, thus keeping the heterotrimer in the inactive form (Beindl et al. 1996).

2.1.1 Modulation of G Proteins Activity Results in Altered Resistance to Various Pathogens and/or Deregulation of Defense Pathways

The first report describing the possible involvement of G proteins in plant defense dates back to 1992 (Legendre et al. 1992). The authors studied a specific defense related process – the oxidative burst. In general, the defense- or pathogenesis-related oxidative burst is a part of the plant response to a pathogen invasion manifested by the rapid release of reactive oxygen species such as peroxides, superoxide, and free radicals. Upon infection, bacterial and fungal pathogens produce cell wall degrading enzymes to gain access inside the host cells. Degradation of cell wall pectin with pectinases results in the production of polygalacturonic acid which acts as a signal of pathogen intrusion. It has been shown that application of polygalacturonic acid almost linearly correlates with production of hydrogen peroxide molecules in cultured soybean cells. Using purified polygalacturonic acid and elicitors extracted directly from the pathogenic fungus *Verticillium dahliae* (Legendre et al. 1992) demonstrated that antigen-Gα-binding fragments were able to facilitate hydrogen peroxide production. In concert with this observation, the G protein activator mastoparan induced defense-related oxidative burst even without elicitor stimulation. On the contrary, other G protein modulators such as pertussis and cholera toxins had very small or no effect on the induced hydrogen peroxide production (Legendre et al. 1992). Follow-up studies by the same group established that phosphaditylinositol-directed phospholipase C (PI-PLC) might be involved in the defense pathway connecting G proteins and peroxide production (Legendre et al. 1993a, b). Importantly, most members of the PI–PLC family were shown to be direct downstream effectors of G proteins in animals (Suh et al. 2008).

A number of pharmacological studies by different research groups have repeatedly confirmed the involvement of G-proteins in plant defense (Beffa et al. 1995; Beindl et al. 1996; Han and Yuan 2004; Higashijima et al. 1988; Mahady et al. 1998; Perekhod et al. 1998; Rajasekhar et al. 1999; Roos et al. 1999; Vera-Estrella et al. 1994b). Collectively, most of those experiments were organized as follows: a pathogen or an elicitor/s was introduced to a plant or cultured plant cells either pretreated or not pretreated with a particular pharmacological agent; thereafter, the effect of the agent on the specific process was estimated and interpreted. These experiments encompass a wide range of plant species, numerous pathogens and elicitors, and a number of important biological processes such as activation/

inactivation of specific elements in signaling and biosynthetic pathways, production of reactive oxygen species and phytoalexins, and direct disease progress evaluation. It has been suggested that G proteins could be involved in the activation of plasma membrane-localized H^+-ATPase, Ca^{2+}-permeable channels, and redox reactions in tomato plants treated with elicitors from *Cladosporium fulvum* (Gelli et al. 1997; Vera-Estrella et al. 1994a, b). Transgenic tobacco plants expressing cholera toxin show accumulation of salicylic acid resulting in upregulation of PR genes and increased resistance to *Pseudomonas tabaci* (Beffa et al. 1995). Using pharmacological agents, G proteins have been linked to resistance to *Phytophthora infestans* in potato (Perekhod et al. 1998), *Pseudomonas syringae*-induced oxidative burst in soybean cell cultures (Rajasekhar et al. 1999), stimulation of phospholipase A (PLA) activity in response to yeast elicitors in Californian poppy (Roos et al. 1999), production of phytoalexin 6-methoxymellein in carrot cell cultures treated with oligogalacturonide elicitor (Kurosaki et al. 2001), generation of active oxidative species induced by shear stress in suspension cultures of *Taxus cuspidata* (Han and Yuan 2004), fungal-induced benzophenanthridine alkaloid biosynthesis in *Sanguinaria canadensis* suspension cell cultures (Mahady et al. 1998), production of phytoalexin scoparone as part of the hypersensitive response against *Alternaria alternata* in lemon (Ortega et al. 2002), and mediation of jasmonic acid pathway leading to biosynthesis of the phytoalexin, β-thujaplicin, in *Cupressus lusitanica* cell cultures (Zhao and Sakai 2003).

2.1.2 Limits of the Pharmacological Approach in Plants

The unquestionable advantage of the pharmacological approach in heterotrimeric G protein research is that a modulating agent can be easily applied to any plant species of interest and the effect can be readily analyzed. However, it also has substantial pitfalls. First, although most of the G protein modulators are directed to the Gα subunit, they inescapably affect the Gβγ dimer. In plants, just like in animals, Gα and Gβγ act as two functional subunits transmitting signals to their distinct pathways (Chen et al. 2006; Joo et al. 2005; Trusov et al. 2008; Ullah et al. 2003). As a consequence, it is hard, if not impossible, to interpret the specific involvement of each G protein subunit in an especific process. Second, application of a modulator to an entire plant cannot be uniform and even for all cells, for that reason most of the pharmacological studies were carried out on suspension cell cultures. Therefore, the role of G protein in processes such as plant development could not be studied. Finally, despite the wide use and acceptance of the pharmacological agents, their specificity for modulation of canonical heterotrimeric G-proteins in plants has been questioned (Fujisawa et al. 2001; Miles et al. 2004). A demonstrative example is the amphiphilic tetradecapeptide mastoparan, used as a G-protein activator in many of the studies mentioned above. In animals, mastoparan is capable of direct stimulation of the Gα-subunit, which in turn activates multiple downstream effectors, including mitogen-activated protein kinases (MAPK). However, in plants, it has been shown that mastoparan-mediated induction of MAPK signaling does not

require the participation of either the Gα or Gβ subunits of the plant heterotrimeric G-proteins (Miles et al. 2004). Thus, final conclusions about the involvement of heterotrimeric G-proteins in specific process should be entirely based on pharmacological studies, but need to be supported by comprehensive genetic analyzes.

2.2 Genetic Studies

The production and discovery of mutants lacking G protein subunits in rice and *Arabidopsis* have provided an invaluable tool for plant heterotrimeric G protein research. The study of G protein mutants has provided conclusive evidence of the involvement of heterotrimeric G proteins in plant development, physiology, and defense.

2.2.1 The Gα Subunit Facilitates Resistance to Pathogens in Rice

The first heterotrimeric G protein mutant was found in rice. The Daikoku *d1* mutant is a dwarf that produces small seeds (Ashikari et al. 1999). Several *d1* alleles were shown to be defective in the only canonical Gα subunit present in rice (*RGA1*) (Ashikari et al. 1999). The involvement of *RGA1* in plant defense system and the establishment of the corresponding signaling pathway was elegantly documented by the Shimamoto's group (Lieberherr et al. 2005; Suharsono et al. 2002). As a plant–pathogen model, the authors used the rice–*Magnaporthe grisea* interaction. This fungal pathogen is responsible for rice blast – one of the most important rice diseases worldwide. The authors found that *M. grisea* infection as well as treatment with sphingolipid elicitors extracted from *M. grisea* membranes caused alterations in *RGA1* transcription. At an early stage, both avirulent and virulent races of the fungus suppressed *RGA1* expression. Later, however, from day 1 to day 3 after infection, *RGA1* transcript levels increased when using the avirulent race, while the virulent race kept *RGA1* expression suppressed. When transgenic plants expressing the β galacturonidase (GUS) gene under the control of the native *RGA1* promoter were subjected to the same treatments, the increased expression was localized at the infection spots.

To test if Gα has a role in resistance against avirulent races of this pathogen, four Daikoku *d1* alleles were exposed to avirulent *M. grisea* and the intensity of hypersensitive response quantified. All the four *d1* mutants showed greatly reduced hypersensitive response, indicating reduced resistance to the avirulent race of rice blast. In agreement with these observations, the induction of pathogenesis related genes *PR1* and *PBZ1* in response to *M. grisea* infection was significantly delayed in all *d1* mutants compared with wild-type control (Suharsono et al. 2002).

Further analysis of the rice Gα subunit-mediated defense signaling was performed in suspension cell cultures generated from embryo-derived calli from four *d1* mutant alleles (Suharsono et al. 2002). It was shown that production of hydrogen

peroxide in response to *M. grisea* sphingolipid elicitors was dramatically decreased or even abolished in *d1* cell cultures, depending on the allele tested. Moreover, induction of the *PBZ1* gene in response to the elicitors did not occur in any of the *d1* cell cultures.

Once Gα's involvement in plant defense and peroxide production was established, it was important to investigate the signal transduction pathway used. OsRac1, a rice homolog of the small GTPase Rac, plays an important regulatory role in the production reactive oxygen species and disease resistance in rice (Kawasaki et al. 1999). To test if heterotrimeric G proteins and OsRac1 operate in the same pathway, three independent *d1* mutants were transformed with a constitutively active form of OsRac1 (Suharsono et al. 2002). Cell cultures were subsequently generated and hydrogen peroxide production as well as *PBZ1* expression in response to elicitors treatment were analyzed. Interestingly, sphingolipid elicitors-induced peroxide production was restored in all three *d1* mutants expressing active OsRac1 to the same levels than the WT plants expressing active OsRac1, suggesting that OsRac1 acts downstream of Gα in the peroxide production pathway (Suharsono et al. 2002). Very different observations were reported when *PBZ1* expression was studied. While this gene was completely suppressed in *d1* mutants regardless of induction, in transgenic *d1* expressing active OsRac1 the *PBZ1* mRNA was clearly detectable even without induction. However, upon sphingolipid elicitors treatment, *PBZ1* was induced in WT and in WT expressing active OsRac1, but remained in steady state level in *d1* mutants expressing active OsRac1. The authors, thus, hypothesized that Gα could control *PBZ1* induction independently from OsRac1 (Suharsono et al. 2002). Importantly, the transgenic *d1* plants expressing active OsRac1 were fully resistant to avirulent races of *M. grisea*, but they were still dwarf and set small round seeds. This observation provides further evidence that Gα is involved in different pathways and OsRac1 does not operates in all of them.

At least three other downstream elements playing a part in defense-related Gα–OsRac1 signaling in rice have been reported: a mitogen-activated protein kinase, OsMAPK6 (Lieberherr et al. 2005); cinnamoyl-CoA reductase 1 (OsCCR1), an enzyme involved in lignin biosynthesis (Kawasaki et al. 2006); and metallothionein (OsMT2b) acting as ROS scavenger (Wong et al. 2004). Homologs of OsMAPK6 in *Arabidopsis*, tomato, and tobacco have a well-recorded involvement in plant innate immunity (Mayrose et al. 2004; Menke et al. 2004; Yang et al. 2001; Zhang and Liu 2001). In WT plants, OsMAPK6 is posttranslationally activated in response to pathogen attack or elicitor application (Lieberherr et al. 2005). In *d1* mutants and in transgenic rice plants with OsRac1 silenced by RNAi, OsMAPK6 protein levels were substantially reduced. Moreover, coimmunoprecipitation experiments showed that OsMAPK6 assembles a complex with active OsRac1, but not with the inactive form of OsRac1. Nevertheless, yeast two-hybrid assays failed to detect direct interaction between these proteins, suggesting the involvement of other proteins in the complex (Lieberherr et al. 2005). Direct interaction between OsRac1 and OsCCR1 has been demonstrated. This interaction led to the enzymatic activation of OsCCR1, which resulted in stimulation of lignin

biosynthesis. The increase in lignin production strengthens cell walls, providing a mechanical barrier for the fungus (Kawasaki et al. 2006). OsRac1 suppresses the expression of metallothionein (OsMT2b), a potent ROS scavenger, thus increasing the ROS pool in the infected cells (Wong et al. 2004).

The above mentioned studies have demonstrated the complexity of the Gα-mediated defense signaling with bifurcating pathways. However, since there are no Gβ or Gγ mutants available in rice, a complete picture of the role of heterotrimeric G proteins in rice innate immunity is still missing.

2.2.2 Gβγ1 Signaling is Involved in *Arabidopsis* Defense Against Necrotrophic Fungi

Arabidopsis boasts with a complete set of mutants lacking each of the heterotrimeric G protein subunits, allowing a comprehensive analysis of their role in a particular biological process. Similarly to rice, *Arabidopsis* has a single canonical Gα subunit gene, GPA1 (Ma 2001; Ma et al. 1990). The amino acid sequences of the rice RGA1 and the *Arabidopsis* GPA1 are approximately 80% identical. Such high level of similarity would usually imply functional resemblance of the proteins. However, a mere look at rice *d1* (RGA1 deficient) "dwarves" and *Arabidopsis gpa1* (GPA1 deficient) "giants" with statistically significantly enlarged rosettes contradict such hypothesis. Furthermore, *gpa1* mutants displayed increased resistance to fungal pathogens compared with WT controls (Llorente et al. 2005; Trusov et al. 2006; Trusov et al. 2007). Importantly, mutants lacking the single canonical Gβ subunit (*agb1* mutants) showed dramatically increased susceptibility to these pathogens. Analysis of double mutants lacking both Gα and Gβ subunits revealed that Gβ-mediated signaling plays the leading role in *Arabidopsis* defense, while the observed Gα effect on resistance is probably due to its ability to impound Gβ into the inactive heterotrimer (Trusov et al. 2006). This does not necessarily preclude participation of the Gα subunit in defense role in *Arabidopsis*, especially considering that Gα is required for the late, cell death-associated oxidative burst (Joo et al. 2005).

The *Arabidopsis* genome contains only one gene encoding a canonical Gβ subunit, *AGB1* (Weiss et al. 1994). Two Gβ-deficient mutants have been identified (Lease et al. 2001; Ullah et al. 2003). Genetic studies implicating Gβ signaling in plant innate immunity were recently reported by two independent groups (Llorente et al. 2005; Trusov et al. 2006). Both reports described straightforward experiments in which disease progression was quantified in WT control plants and *agb1* mutants after pathogen infection. Collectively, five fungal species *Botrytis cinerea, Fusarium oxysporum, Plectosphaerella cucumerina, Alternaria brassicicola*, and *Peronospora parasitica* and one bacterium *P. syringae* were tested. Disease symptoms were significantly more severe in *agb1* mutants infected with the necrotrophic *B. cinerea, P. cucumerina, A. brassicicola* and the vascular fungus *F. oxysporum*. In contrast, no differences between WT and mutants were observed for the oomycete *P. parasitica* and the bacterium *P. syringae*, even though in both cases virulent

and avirulent races were tested. Expression analysis of pathogenesis-related genes revealed that induction of *PDF1.2, OPR3*, and *PAD3* in response to *A. brassicicola* and methyl jasmonate (MeJA) treatments was significantly delayed (Trusov et al. 2006). At the same time, expression of the salicylic acid dependent gene *PR1* was increased upon *P. cucumerina* infection (Llorente et al. 2005). These observations, together with the necrotrophic nature of the fungi studied, suggest a possible interaction between G proteins and the JA/ethylene pathways. Indeed, MeJA-dependent inhibition of seed germination, postgermination development and root elongation were, to some extent, weakened in the *agb1* mutants compared with WT, suggesting a role for Gβ signaling promoting several JA-mediated processes (Trusov et al. 2006). On the other hand, it was found that *agb1* mutants were incapable of accumulating callose – a high-molecular weight β-1,3-glucan depositing in plant tissues challenged with a pathogen. Interestingly, the callose deposition was only arrested in the *agb1* mutants in response to necrotrophic pathogen *P. cucumerina*, while the oomycete *P. parasitica* induced callose deposition similarly in WT and *agb1* mutants. This indicates that the *agb1* mutation affected a rather specific signaling pathway triggering callose deposition, but not its biosynthesis pathway (Llorente et al. 2005).

The *Arabidopsis* genome contains two genes encoding Gγ subunits, *AGG1* and *AGG2* (Mason and Botella 2000, 2001). Gβ and Gγ subunits form a tightly bound dimer that acts as a single functional unit and can only be dissociated using denaturing conditions (Gautam et al. 1998). Despite possessing a similar domain structure, AGG1 and AGG2 are quite different in primary sequence (only about 55% identity), tissue-specific expression patterns (Trusov et al. 2007, 2008), and in their ability to target the plasma membrane (Adjobo-Hermans et al. 2006; Zeng et al. 2007), and consequently they mediate different cellular processes (Trusov et al. 2007, 2008). Infection with *F. oxysporum* or *A. brassicicola* induced *AGG1*, but not *AGG2* gene expression (Trusov et al. 2007). Consequently, when mutants lacking Gγ1 (*agg1*), Gγ2 (*agg2*), or both proteins simultaneously (*agg1 agg2*) were infected with either *F. oxysporum* or *A. brassicicola*, they showed very different behaviors. When plant decay and rosette growth inhibition for *F. oxysporum* inoculation and lesion expansion for *A. brassicicola* infection were monitored, *agg1* and double *agg1 agg2* mutants were more susceptible to pathogen attack than WT plants, while *agg2* mutants showed WT behavior. These results suggested that Gβγ1 is the specific subunit involved in plant defense (Trusov et al. 2007). However, subsequent large-scale analysis using a more reliable quantitative Fusarium wilt monitoring technique – counts of yellow leaves per plant, revealed that both Gγ subunits additively contribute to the resistance. Interestingly, at an early stage of symptom development (5–9 days) both *agg1* and *agg2* mutants displayed similar levels of susceptibility – somewhat intermediate between levels of *agb1* or double *agg1 agg2* mutants and WT. In contrast, at a later infection stage (10 days to plant decay), *agg1* mutants accumulated yellow leaves significantly faster than *agg2* mutants, resulting in similar values to *agb1* and double *agg1*/agg2 mutants at the end of the experiment, while *agg2* mutants ended up with values similar to WT (Trusov and Botella unpublished data).

The presented data, from both pharmacological and genetic studies, provide unequivocal evidence of heterotrimeric G proteins involvement in plant innate immunity. It is also clear that distinct roles of the subunits can vary depending on the plant species and the pathogen studied. It is, therefore, not possible to predict with certainty the role which G proteins will play in a particular plant–pathogen interaction.

3 Heterotrimeric G Protein and Plant Cell Death

In multicellular organisms, programmed cell death (PCD) plays an essential role in the control of several biological processes, including immunity, development, and senescence (Patel et al. 2006). In plants, the most studied form of PCD is the localized cell death associated with the hypersensitive response (HR), which is a constituent of a significant number of immune responses against different type of pathogens. Although the biological relevance of HR-PCD is not fully understood, its timely activation is thought to be essential for containment of the pathogen at the penetration site (Patel et al. 2006). Several studies have implicated a number of signaling molecules in HR-PCD. Among these molecules are nitric oxide (NO), salicylic acid (SA), and reactive oxygen species (ROS) (Torres and Dangl 2005).

The production of ROS is one of the earliest events in the plant immune response. It occurs shortly after pathogen recognition and involves the transient increase of, predominantly, superoxide (O_2^-) and hydrogen peroxide (H_2O_2) in the apoplast leading to initiation of the oxidative burst. ROS were initially thought to function just as executioners of PCD. However, a broader role of ROS in cell signalling and regulation of gene expression in response to biotic and abiotic stresses and to developmental cues has emerged recently. ROS production can be also elicited by phytohormones such as auxin or ABA (Joo et al. 2001; Pei et al. 2000; Schopfer et al. 2002; Zhang et al. 2001), and by a large variety of abiotic stresses, including drought, high temperatures, high light intensity and ozone (Joo et al. 2005; Overmyer et al. 2003). Moreover, ROS can be produced inside the plant cell, in chloroplasts, mitochondria, and peroxisomes as by-products of processes such as photosynthesis and respiration (Apel and Hirt 2004). The molecular basis of these novel ROS functions is still poorly understood.

Heterotrimeric G proteins have been involved in the regulation of cell death and ROS generation during defense responses in rice (Suharsono et al. 2002). In *Arabidopsis*, G proteins were shown to control ROS production stimulated by extracellular calmodulin in guard cells (Chen et al. 2004) and upon plant exposure to ozone (Joo et al. 2005). Together, these studies suggest that heterotrimeric G proteins might be a common component of ROS and PCD signaling in response to different stresses or developmental cues.

3.1 Function of G Protein in Activation of Oxidative Stress

ROS were initially proposed to orchestrate the establishment of plant defense response and the HR-PCD associated to pathogen recognition, and latter on have been implicated in the regulation of molecular responses associated with some abiotic stresses. The production of ROS in these processes has been explained by several biochemical mechanisms. In apoplast, H_2O_2 can be produced by the coordinated action of cell-wall peroxidases, amino oxidases, or germin-like oxalate oxidases (Apel and Hirt 2004). Also, ROS can be generated by plant NADPH oxidases upon pathogen challenge or ABA signaling during stomata closure. Plant NADPH oxidases are similar to those producing superoxide during the respiratory burst in mammalian phagocytes (Groom et al. 1996). The mammalian NADPH oxidases are also known as respiratory burst oxidases (RBOs), a protein complex comprised of a membrane-bound NADPH-binding flavocytochrome b558 and cytosolic regulatory proteins. The activity of mammalian NADPH oxidases is regulated by additional proteins, such as RAC GTPases. Plant NADPH oxidase homologs were first identified in rice and named respiratory burst oxidase homologs (Rboh) (Groom et al. 1996). In *Arabidopsis*, there are ten *AtRboh* genes, and among them *AtRbohC, D*, and *F* are the best characterized (Torres and Dangl 2005).

The first evidence of the putative involvement of heterotrimeric G proteins in the regulation of ROS production and cell death came from the demonstration that the Dikoku *dwarf1* (*d1*) rice mutant, lacking a functional Gα subunit, showed a significant reduction in HR response upon infection with avirulent races of the blast fungus *Magnaporthe grisea* (Suharsono et al. 2002). This attenuation of HR in *d1* was accompained by 24-h delayed induction of *PR* genes expression and compromised resistance to blast fungus compared to wild-type plants. The H_2O_2 production was also compromised in *d1* cell cultures upon treatment with elicitors, such as sphingolipid derivatives. This phenotype was restored to wild-type levels by expression of the constitutively active OsRAC1 (CA-OsRAC1), a ROP protein (see Chapter "ROP GTPases and the Cytoskeleton" and Section "Small G proteins in plant innate immunity"), which can induce H_2O_2 production in the absence of pathogen infection or an elicitor treatment. These results indicated that *d1*-enhanced susceptibility was due to a H_2O_2 deficient production and that OsRAC1 function downstream from Gα in the rice heterotrimeric G-protein signaling pathway (Suharsono et al. 2002). In *Arabidopsis*, production of ROS upon pathogen infection does not seem to be altered in mutants defective in heterotrimeric G-protein subunits. For example, the *Arabidopsis* Gβ-deficient mutant *agb1* shows enhanced susceptibility to necrotrophic fungi but the production of ROS upon inoculation with *P. cucumerina* does not differ from that of wild-type plants (Llorente et al. 2005). However, a detailed characterization of ROS production in heterotrimeric G protein mutants upon infection with different types of pathogens is required to determine the function of the GPA1, AGB1, and AGG1/AGG2 subunits in the regulation of ROS production and signaling in plant innate immunity.

Ozone (O_3) causes oxidative stress in living organisms and is a major pollutant affecting plants. Plant exposure to ozone (O_3) induces a bimodal oxidative burst that has some similarities to the pathogen-induced oxidative burst during plant immune response (Overmyer et al. 2003). In *Arabidopsis*, ozone exposure leads to production of ROS and activation of ROS-mediated signaling. Interestingly, heterotrimeric G proteins control this ROS production and signaling, as well as the sensitivity to ozone. Gα- and Gβ-deficient mutants are, respectively, less and more sensitive to O_3 damage than wild-type plants. In *gpa1* and *agb1* mutants, the first peak of the oxidative burst elicited by O_3 is missing, whereas the late peak is missing in *gpa1*, but not in *agb1* mutants. ROS production is primarily detectable in chloroplast of leaf epidermal guard cells, and expands to adjacent cells by the action of the membrane-bound AtRbohD and AtRbohF NADPH oxidases. The ROS produced by the action of these NADPH oxidases act on guard cell plasma membrane calcium channels, stimulate ROS production in adjacent cells, and influence intercellular ROS signaling (Kwak et al. 2003). ROS signaling from the chloroplast is central to the O_3-induced oxidative stress response, as it has been shown to mediate light and signaling (Chang et al. 2004; Fryer et al. 2003, 2002). AtRbohD and AtRbohF also participate in the O_3-induced oxidative stress response that leads to intercellular signaling and ultimate to PCD. Interestingly, the observed cell death- associated with ozone exposure requires only the Gα protein (Joo et al. 2005). These results indicate that *Arabidopsis* heterotrimeric G proteins act both synergistically and separately in activating different intracellular ROS-generating systems.

Heterotrimeric G proteins have also been implicated in the regulation of ROS production through activation of extracellular calmodulin (ExtCaM) during stomatal closure (Chen et al. 2004). ExtCaM activation triggers a significant increase in NO levels associated with stomatal closure in wild-type plants. This effect is abolished in the *Arabidopsis atnoa1* (nitric oxide associated 1) mutant, which is defective in NO production. Interestingly, ExtCAM-mediated production of NO is regulated by GPA1, as it is blocked in *gpa1* mutants and enhanced by overexpression of a constitutive active form of GPA1 (cGα) (Li et al. 2009). In line with these results, the defect in NO production in *gpa1* was rescued by overexpression of *AtNOA1*. G protein-mediated activation of NO production depends on H_2O_2 accumulation, which is impaired in the *atrbohD atrbohF* double mutant. Previous results demonstrated that stomatal closure and plasma membrane calcium channel activation are reduced in *atrbohD atrbohF* double mutants, but can be restored by treatment with H_2O_2, indicating that ROS serve as second messengers in ABA signaling (Kwak et al. 2003). The data discussed above suggest the existence of a signaling pathway leading to ExtCaM-induced stomatal closure, which involves GPA1-dependent activation of H_2O_2 production and subsequent AtNOA1-dependent NO accumulation (Li et al. 2009). This signaling pathway provides an additional link between G proteins and Rboh NADPH oxidases that deserves an additional investigation.

3.2 Regulation of Cell Death by G Proteins

After O_3 stress, the PCD signaling system is intact in Gβ-deficient mutants. The increased resistance to O_3 observed in the *gpa1-4* mutant is consistent with the suggested role for the Gα subunit in activating the membrane-bound NADPH oxidases to produce damaging levels of ROS (Suharsono et al. 2002). The association of cell death with ROS production by membrane-bound NADPH oxidases is further supported by the observation that mutants lacking either the AtrbohD, or the AtrbohF, or both NADPH oxidase proteins were deficient in the late oxidative burst and were more resistant to O_3 than wild-type plants (Joo et al. 2005).

Another oxidative stress that leads to cell death is produced when hypocotyls of far red (FR) grown seedlings are exposed to white light. ROS are again critical mediators of the cell death. This type of cell death is phytochrome A-mediated but heterotrimeric G proteins are also involved (Wei et al. 2008). The *gpa1* mutant plants exhibit reduced cell death, while the *agb1* mutant shows enhanced death, indicating that GPA1 and AGB1 play antagonistic roles in the phyA-mediated cell death pathway.

Heterotrimeric G proteins have been recently involved in the cell death process associated with the unfolded protein response (UPR) in *Arabidopsis* (Wang et al. 2007). The UPR is a protective response that takes place in the cells whose protein folding and modification machineries at the endoplasmic reticulum (ER) are disrupted. This complex response is quite well characterized in yeast and mammalian cells, where transcriptional and translational changes occur to promote protein folding and disposal of misfolded proteins. When these mechanisms fail, the UPR activates apoptotic cell death (Schroder and Kaufman 2005). In plants, mutations that affected ER chaperone and secretory protein genes also compromised defense responses to pathogen attack, suggesting that the folding and secretory machinery are critical for plant immunity (Bilgin et al. 2003; Jelitto-Van Dooren et al. 1999; Wang et al. 2005). Interestingly, Gβ-deficient mutants are more resistant than wild-type plants to the cell death induced by the antibiotic tunicamycin (Tm), an inhibitor of the *N*-linked protein glycosylation (Wang et al. 2007). Moreover, the majority of Gβ protein localizes to the ER, cofractionates with ER lumenal chaperone protein and is degraded during the UPR, whereas Gα is not (Wang et al. 2007). It can be concluded that the Gβγ dimer mediates cell death signaling in the UPR and that ER stress-related proteins are probable downstream targets of Gβγ signaling. These observations have lead to the hypothesis that heterotrimeric G signaling is not exclusively confined to the plasma membrane but can also occur in cellular compartments, where hormone receptors have been identified (Wang et al. 2007). Further research is needed to establish the role of ER in heterotrimeric G protein signaling.

A putative connection between the plant heterotrimeric G protein and PCD through the regulation of Ca^{2+} ion channel remains to be elucidated. In mammals, Gβγ complex is known to interact with calcium channels, and calcium signaling

plays a pivotal role in plant stress responses (Klusener et al. 2002). Interestingly, it has been suggested that the chloroplast-localized Ca^{2+} ion channel PPF1 functions as an inhibitor of programmed cell death in apical meristems (Li et al. 2004). Moreover, as indicated above, plasma membrane calcium channel activation are reduced in *atrbohD atrbohF* double mutants, but can be restored by treatment with H_2O_2 (Kwak et al. 2003). Together these data suggest a putative connection between calcium channels, ROS production, and PCD that could be regulated by G proteins through Rboh oxidases, but this model needs further characterization.

4 Innate Immunity Networks Regulated by Heterotrimeric G Proteins

Plant innate immunity encompasses an enormous variety of molecules conferring resistance to a not lesser variety of plant pathogens. These molecules are organized in an intricate network of pathways starting from pathogen recognition, following activation of signal transduction, leading to transcription induction, protein synthesis, and metabolite production, and eventually resulting in formation of a protective response. Heterotrimeric G proteins are apparently involved in the signal transduction part of the response. It is generally accepted that most of the plant defense responses are governed by several parallel signaling pathways. The most important of them are regulated by low-molecular weight signaling molecules, such as SA, jasmonic acid (JA), and ethylene (ET) (Ausubel 2005; Kachroo and Kachroo 2007). Brassinosteroids, and more recently abscisic acid (ABA) and auxin, have also been added to this list of immune signaling molecules (Adie et al. 2007; Asselbergh et al. 2008; Hernandez-Blanco et al. 2007; Llorente et al. 2008; Navarro et al. 2006; Robert-Seilaniantz et al. 2007; Wang et al. 2007). These distinct parallel pathways nevertheless interact or intersect at certain crucial regulatory steps (Kunkel and Brooks 2002; Pieterse and Van Loon 2004). Importantly, either acting independently or as a part of the network, these signaling pathways contribute differently to the resistance against a particular pathogen. It has been hypothesized that fungal life style could be used as a predictor of the induced plant defense response (Dangl and Jones 2001; Mcdowell and Dangl 2000). For instance, plants usually activate JA/ET-mediated signaling pathways in response to necrotrophic fungi, while biotrophs and bacteria generally provoke SA signaling (Glazebrook 2005; Schenk et al. 2000; Thomma et al. 1998, 1999, 2001b; Van Wees et al. 2003). The activation of an appropriate signaling pathway with external application of the hormones prior to an infection is an effective method to protect plants from pathogens. On the other hand, mutations disrupting a particular pathway can compromise resistance to the corresponding group of pathogens, while they may have no effect or even a beneficial effect on resistance against a different group of pathogens (Grant and Lamb 2006; Kunkel and Brooks 2002; Maleck and Dietrich 1999; Schenk et al. 2003; Takahashi et al. 2004; Thomma et al. 2001a). However, exceptions to this

general rule have been increasingly reported over the last few years, indicating the complexity of the innate immunity network controlling plant resistance to a particular type of pathogen (Berrocal-Lobo and Molina 2008; Edgar et al. 2006; Glazebrook 2005; Thatcher et al. 2009; Van Wees et al. 2003). The effectiveness of a signaling pathway for plant protection also depends on the especific growth conditions and inoculation technique used (Trusov and Botella, unpublished). Therefore, it is important to keep in mind that the specific inputs from the pathways involved in resistance for a specific plant–pathogen system may vary between reported experimental data.

Heterotrimeric G proteins have been implicated in plant resistance to necrotrophic and vascular fungi (Llorente et al. 2005; Trusov et al. 2006), but their place and interactions in the innate immunity network remains largely unknown. Recently, an attempt has been made to establish relations between Gβ defense signaling and SA-, JA-, ET-, and ABA-mediated pathways (Trusov et al. 2009). It has been shown that mutants lacking the Gβ subunits, *agb1-2*, were extremely susceptible to *F. oxysporum* compared with wild type, but displayed only mildly decreased resistance to *A. brassicicola*. On the other hand, when a number of mutants defective in SA-, JA-, ET-, and ABA-mediated signaling pathways were tested against these pathogens in the same conditions, a range of responses was found as follows. Mutants deficient in SA biosynthesis (*NahG* expressing transgenic plants and *eds5-1* mutant) were susceptible to *F. oxysporum* at levels similar to *agb1-2* mutants, while *npr1-2* and *eds1-22* mutants impaired in SA signaling were similar to wild-type control. Neither of these SA-related mutations had a measurable effect on resistance against *A. brassicicola*. On the contrary, mutants deficient in ABA (*aba1-6*), insensitive to ethylene (*ein2-1*) or JA (*coi1-21* and *jin1-9*) were more resistant to *F. oxysporum* compared with wild-type plants; at the same time, when challenged with *A. brassicicola aba1-6* and *coi1-21*, but not *ein2-1* or *jin1-9*, displayed high levels of susceptibility. Interestingly, another mutant impaired in JA response, *jar1-1*, was to some extent more susceptible to *F. oxysporum* than wild type and fully resistant to *A. brassicicola*, showing an opposite behavior compared to *coi1-21* and suggesting that JA mediate at least two different pathways involved in plant defense (Trusov et al. 2009). Pretreatment of wild-type *Arabidopsis* with SA, ethylene, methyl jasmonate, or ABA and subsequent resistance analysis confirmed the genetic results obtained for *F. oxysporum*. The effect of hormonal pretreatment on *A. brassicicola* infection was not tested. To establish whether G proteins act as a part of these pathways or interact with their components, the *agb1-2* mutant was crossed with all mutants mentioned above. The study of double mutants is proven to be useful for establishing genetic interactions between two elements in a particular response. If two elements are agonistically involved in the same pathway, the double mutant should behave like one of the single mutants, while if they control different mechanisms, the double mutant should display an enhanced phenotype compared with the single mutants, if such an increase is possible. This last scenario was observed for *agb1NahG, agb1eds5*, and *agb1jar1* when infected with *F. oxysporum*. Similarly, the double mutants *agb1coi1* and *agb1aba1* showed additive effect after infection with

A. brassicicola, indicating that Gβ subunit signaling is independent of the tested pathways. The double mutant approach is also useful to analyze the interaction between elements involved in the same process in antagonistic manner. In this case one mutation should suppress the effect of the other. Since *agb1-2* influenced resistance to *F. oxysporum* in an opposite way than *coi1-21, jin1-9, ein2-1*, and *aba1-6*, the suppression effect was analyzed on the corresponding double mutants infected with *F. oxysporum*. It was found that the *agb1coi1, agb1aba1, agb1jin1* and *agb1ein2* double mutants had intermediate resistance levels (quantified as number of yellow leaves) between the *agb1-2* and all the single mutants, suggesting that Gβ acts independently from the pathways in which these proteins are involved. On the other hand, when percentage of decayed plants was counted, it was observed that *agb1coi1* and *agb1jin1* plants eventually survived the infection, while *agb1aba1* and *agb1ein2* perished. Development of yellow leaves' counts and percentage of decayed plants are two methods to evaluate disease progression in plants infected with *F. oxysporum*. Both parameters usually show a good correlation, but it should be noted that yellow leaves' counts reflect early stages of infection, while plant decay indicates the final outcome of the disease. The described results, therefore, suggested that Gβ might act upstream of COI1 and ATMYC2 at the later stages of infection. It has been recently established that COI1 is a receptor for several JA derivatives (Katsir et al. 2008), which activate the transcription factor ATMYC2 which in turn regulates expression of several pathogenesis-related genes. ATMYC2 plays a central role in switching between drought- and pathogenesis-related signals (Anderson et al. 2004; Lorenzo et al. 2004). *ATMYC2* expression is originally increased in response to *F. oxysporum* intrusion, but then quickly returns to the steady-state level (Trusov et al. 2009) or could even be suppressed (Anderson et al. 2004). In the *agb1-2* mutant, *ATMYC2* expression was significantly higher compared with that of wild-type levels during infection, but not in uninfected plants, suggesting that Gβ signaling suppresses *ATMYC2* during *F. oxysporum* attack. Expression analysis of the pathogenesis-related genes *PDF1.2, CHITINASE*, and *PR4* in single *agb1-2, jin1-9*, and double *agb1jin1* mutants revealed a complex interaction between Gβ and ATMYC2 during *F. oxysporum* infection. It was observed that Gβ and ATMYC2 suppressed *CHITINASE* independently, while the positive effect of Gβ signaling on *PDF1.2* and *PR4* is due to Gβ suppression of ATMYC2 (Trusov et al. 2009). Taken together, these observations point out that Gβ signaling apparently intersect with one of the JA-mediated pathways at ATMYC2; it is possible that this interaction accounts for part of the Gβ immune response. However, the nature of the other part of the Gβ-facilitated defense remains unknown.

The next logical question to ask is how plasma membrane-localized heterotrimeric G proteins regulate gene expression in the nucleus? One candidate to fill this gap is the oxidative burst-induced signaling. Involvement of oxidative burst in plant innate immunity has been established, although it is not fully understood (Berrocal-Lobo and Molina 2008; Galletti et al. 2008; Lamb and Dixon 1997; Torres and Dangl 2005). It has been demonstrated that Gβ signaling is required for an early component of the oxidative burst triggered by ozone (Joo et al. 2005).

On the other hand, accumulation of ROS caused by *P. cucumerina* inoculation was independent of Gβ signaling (Llorente et al. 2005). To reconcile these contradicting facts, one may assume that during pathogen attack, as opposite to ozone treatment, Gβ signaling and oxidative burst interact downstream of ROS production. Such scenario converges a possible G protein signaling pathways with pathogen-induced ROS at oxidative signal inducible kinase OXI1 has been proposed (Anthony et al. 2004, 2006). The protein kinase OXI1 (also known as AGC2-1) was independently discovered and studied by two groups (Anthony et al. 2004; Rentel et al. 2004). It was shown that expression of *OXI1* gene is strongly induced by hydrogen peroxide, cellulose, the fungus *P. parasitica*, and wounding (Rentel et al. 2004). At the same time, activation of OXI1 kinase activity depends on binding to activated 3-phosphoinositide-dependent protein kinase PDK1 (Anthony et al. 2004). Activation of PDK1 occurs upon binding to phosphatidic acid (PA), which relies on phospholipases C (PLC) and D (PLD) for its production. Importantly, there is pharmacological evidence implicating G proteins in PA production (Munnik et al. 1995). Moreover, it was observed that the heterotrimeric G proteins agonist mastoparan activates OXI1 in a PDK1-dependent manner (Anthony et al. 2004). Further analysis revealed that activated OXI1 directly binds and phosphorylates serine/threonine protein kinase PTI1 (Anthony et al. 2006). PTI1 is a homolog of a tomato PTI1 kinase implicated in hypersensitive response and resistance to bacterial speck disease (Sessa et al. 1998; Zhou et al. 1995). On the other hand, OXI1 is required for full activation of the MAPK MPK3 and MPK6 (Rentel et al. 2004). Involvement of MPK3 and MPK6 in plant innate immunity is well documented (Asai et al. 2002; Menke et al. 2004; Ren et al. 2008). Finally, the potato ortholog of *Arabidopsis* MPK6, StMPK1, has been shown to phosphorylate a number of nuclear proteins (Katou et al. 2005), including two jasmonate ZIM-domain (JAZ) proteins with closest homology to tomato and *Arabidopsis* JAZ3 and tobacco JAZ2 (Katsir et al. 2008; Shoji et al. 2008) and a transcription factor highly similar to *Arabidopsis* WRKY33. Importantly, JAZ proteins, and JAZ3 in particular, were shown to be a missing link between COI1 and ATMYC2 (Chini et al. 2007). During JA signaling, the activated receptor COI1 as a part of SCF^{COI1} complex targets JAZ3 for degradation; this in turn releases ATMYC2 from the JAZ3–ATMYC2 complex allowing transcription of the corresponding genes (Chini et al. 2007). Phosphorylation of the JAZ proteins has not yet been studied, but it was shown for other proteins targeted by cullin-containing E3 ubiquitin ligases similar to SCF^{COI1} that phosphorylation effectively prevented their degradation (Schwechheimer and Calderon Villalobos 2004).

The following scenario connecting all the steps could be envisioned. Pathogen intrusion activates yet-to-discover G protein coupled receptors, resulting in release of the Gβγ1 dimer, which induces PLC/PLD dependent PA production and activation of PDK1. Simultaneous and independent ROS accumulation also takes place. PDK1 and ROS synergistically activate OXI1. Activated OXI1 binds to and phosporylates PTI1 and through its activation or by yet another way activates MPK6. Upon activation, MPK6 moves into the nucleus and phosphorilates JAZ proteins, protecting them from degradation and hence preventing ATMYC2

transcriptional activity. Bear in mind that ATMYC2 plays a negative role in resistance to *F. oxysporum*, and covers only a part of the Gβ defense response (Trusov et al. 2009). On the other hand, MPK6 could possibly phosphorylate WRKY33, as well stabilizing it. WRKY33 plays a positive role in resistance to necrotrophic fungi (Zheng et al. 2006), including *F. oxysporum* (Trusov and Botella, unpublished data) and could be accountable for the second part of the Gβ-mediated defense response. Despite the existence of indirect evidence supporting this hypothesis, the direct test is yet to be done for all components in a single plant–pathogen model. Also, pharmacological data involving Gβ signaling in PA production and subsequent activation of the serine/threonine kinases should be confirmed by genetic analysis.

A potential interconnection between heterotrimeric G proteins and the ERECTA receptor-like kinase (RLK) has been also described (Lease et al. 2001; Llorente et al. 2005). RLKs are the predominant cell surface receptors in plants (Shiu et al. 2004) and are involved in perception of pathogen-associated molecular patterns (PAMPs) and activation of innate immunity signaling cascades (Chinchilla et al. 2007; Kemmerling et al. 2007). *Arabidopsis* mutants lacking the Gβ subunit of the heterotrimeric G protein exhibit an erecta-like developmental phenotype similar to plants impaired in the ERECTA RLK (*er*), suggesting that they may form a part of the same pathway (Lease et al. 2001; Llorente et al. 2005). In line with this hypothesis, *er* and *agb1* mutants showed an enhanced susceptibility to the necrotrophic fungus *P. cucumerina* (Llorente et al. 2005). In contrast, no enhanced susceptibility to *F. oxysporum* and *B. cinerea* was observed in *er* mutants, whereas *agb1* mutants showed an enhanced susceptibility to these fungal pathogens (Llorente et al. 2005). Interestingly, ROP GTPases have been shown to interact with several RLKs (see "Small G proteins in plant innate immunity"). Based on these published data, potential direct or indirect (through ROP proteins) interactions of G proteins with different RLKs can be hypothesized (Fig. 1).

5 Small G Proteins in Plant Innate Immunity

ROP (from Rho-related GTPases from plants) proteins are plant-specific small GTPases that act as signaling transducers connecting extracellular inputs/stimuli to the cell (see Chapter "ROP GTPases and the Cytoskeleton"). These monomers are activated by a wide range of upstream signals that trigger the conversion of the guanosine diphosphate (GDP)-bound inactive form of ROP to the active guanosine triphosphate (GTP)-bound form. This active form is able to interact with one or more specific downstream effector proteins and therefore exert its biological function. Activation can be catalyzed by guanine nucleotide exchange factors (GEFs) (see chapter "Structure and Function of ROPs and Their GEFs"), while GTPase activating proteins (GAPs) promote GTP cleavage to return the switch back to the inactive GDP state and guanine nucleotide dissociation inhibitors (GDIs) prevent the activation process (see chapter "Regulatory and Cellular

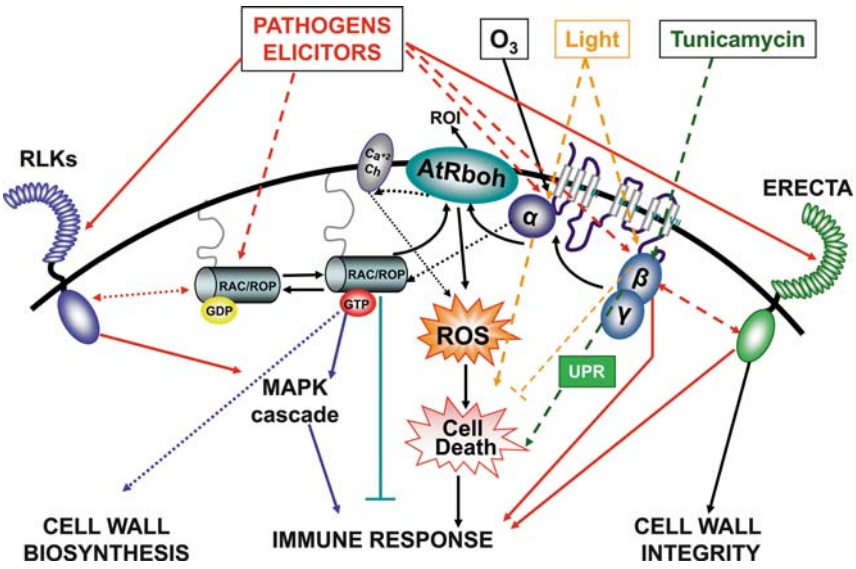

Fig. 1 *G and ROP proteins regulatory network controlling the production of ROS and the activation of plant cell death in response to different external treatments*. The different external stimuli/treatments that have been described to induce the production of ROS or/and the development of cell death through the activation/inactivation of G and ROP proteins are indicated in different colors. In some cases, immune responses have been associated to ROS production and cell death activation. *Dotted arrows* indicate biochemical/genetic interactions or regulatory effects not fully demonstrated or just suggested by the published data. Ca^{+2} *Ch* calcium channel. See the text for more details.

Functions of Plant RhoGAPs and RhoGDIs"). Since the first plant cDNA encoding a Rho-related GTPase (Rho1Ps) from pea was identified in 1993 (Yang and Watson 1993), a large number of Rho GTPases have been identified in mosses and higher plants (Delmer et al. 1995; Li et al. 1998; Winge et al. 1997; Yang and Watson 1993). The *Arabidopsis* genome encodes a gene family of 11 ROP Small GTPases, while rice comprise seven members and barley appears to express six ROP genes (Nibau et al. 2006; Schultheiss et al. 2008).

ROPs are the key switches that regulate many pathways important for development, such as cell polarity establishment and cell growth and differentiation, but also some are required for plant immunity (Yang and Fu 2007; Zheng and Yang 2000). The involvement of ROPs in plant defense responses was first demonstrated by the extensive work done on rice OsRAC1 (Kawasaki et al. 1999; Ono et al. 2001; Suharsono et al. 2002). Latter on, the studies performed on species as diverse as soybean, tobacco, maize, barley, and *Arabidopsis* have corroborated the function of ROPs in plant innate immunity (Agrawal et al. 2003).

In rice, constitutively active forms of OsRac1 (CA-OsRac1) activate H_2O_2 production and induces spontaneous PCD in leaves. The HR-like responses triggered by the overexpression of CA-OsRac1 also induced an enhanced resistance against a virulent race of rice blast fungus (*Magnaporthe grisea*, race 007) and the

bacterial blight (*Xanthomonas oryzae* pv. *oryzae*, race 1). This resistance was also associated to the higher accumulation of the phytoalexin momilactone A, and altered gene expression of some defense/stress-related genes, such as the *D9* and *POX22.3* encoding, respectively, a terpenoid cyclase and a peroxidase. By contrast, the dominant negative form of OsRac1 (DN-OsRac1) inhibited H_2O_2 production and lesion formation induced by the avirulent race of blast fungus (*M. Grisea*, race 031). (Kawasaki et al. 1999). CA-OsRac1-induced H_2O_2 production was inhibited by the NADPH oxidase inhibitor diphenylene iodonium (DPI). Similar results were seen when mutant forms of cotton ROP (GhRac13) and human HsRac1 were manipulated, respectively, in the heterologous *Arabidopsis* and soybean cultured cells (Agrawal et al. 2003). OsRAC1 also suppresses expression of a metallothionein, a reactive oxygen scavenger, further enhancing the ROS-signaled defense response (Wong et al. 2004). In mammals, Rac2 GTPase interacts with the p67phox regulatory subunit of NADPH oxidase; however, plants, apparently, do not contain p67phox and p47phox regulatory subunits, and just the p91phox homologues are present in plants (Sagi and Fluhr 2001). Interestingly, it has been suggested that OsRAC1 activates the NADPH-mediated production of H_2O_2 by directly binding to the N-terminus of Rboh protein (the catalytic subunit of NADPH oxidase) (Wong et al. 2007). From the large series of studies performed in rice, it can be concluded that OsRAC1 activates defense responses in rice acting as a positive regulator of the NADPH oxidase complex, transiently stimulating ROS production, and enhancing pathogen resistance (Kawasaki et al. 1999; Ono et al. 2001).

Recent studies support the proposed function of plant ROPs in mediating plant immunity, mainly through modulating ROS production and accumulation, PCD, and defense signalling cascades (Jung et al. 2006; Molendijk et al. 2008; Opalski et al. 2005; Pathuri et al. 2008; Schultheiss et al. 2008; Thao et al. 2007). Tobacco NtRAC5 downregulates NADPH oxidase in tobacco cells in response to elicitors (Morel et al. 2004). Also, tobacco transgenic plants expressing heterologous *Medicago sativa*, *MsRac1* gene, results in development of necrotic lesions and cell death, while antisense expression have no effect or any other visible defense reaction (Schiene et al. 2000). In tomato, a Rac2-specific antibody detects a 21-kDa tomato protein that could be translocated to microsomal membranes in response to elicitor treatments (Agrawal et al. 2003).

A connection between ROPs and G proteins in the regulation of pant innate immunity has been established. As explained before, the rice *d1* mutant impaired in the Gα subunits of the heterotrimeric G protein exhibited reduced resistance to *M. grisea* and H_2O_2 production, and delayed *PR* gene expression, that were restored to wild-type phenotype by the constitutive overexpression in *d1* plants and cell cultures of CA-OsRac1 (Suharsono et al. 2002). All these data indicated that OsRac1 operates downstream of Gα as a positive regulator for defense pathway (Suharsono et al. 2002). It is believed that pathogen/elicitor-derived signals are likely to be received by as yet unknown receptor(s), and transmitted to OsRac1 through Gα. OsRac1 could also regulate defense response by additional molecular mechanisms. Thus, a recent study has revealed that OsRac1 forms a complex with RAR1, HSP90, and HSP70 in vivo, and coordinates the activity of these key

components of the plant innate immunity (Thao et al. 2007). The putative connection of these immunity regulators with Gα subunit is currently unknown.

In contrast to rice OsRac1 that is functioning in the defensive responses to the hemibiotrophic fungus *Magnaporthe grisea*, barley HvRacB appears to be involved in susceptibility to the biotrophic fungi *Blumeria graminis* f. sp. *hordei* (*Bgh*). Transient knocking-down of barley *HvRacB* gene renders epidermal cells more resistant to penetration by *Bgh*, indicating that HvRacB negatively regulates the defense pathway (Schultheiss et al. 2008). Barley susceptibility to *Bgh* is mediated by the host seven transmembrane domain receptor MLO protein, as mutants that do not express functional MLO are completely resistant to penetration by *Bgh,* and by *ROR1* and *ROR2* genes (Assaad et al. 2004; Bhat et al. 2005; Collins et al. 2003). The role of HvRacB on susceptibility is dependent on functional *MLO* and *ROR1* genes. The function of HvRacB appears to be linked to the actin cytoskeleton, as overactivation of HvRacB in single-cell hampers actin-filament reorganization in *Bgh* attacked cells, while lack of HvRacB promotes polarization (Schultheiss et al. 2008). HvRacB interacts *in planta* with RIC171, a protein whose overexpression leads to more susceptible epidermal cells. Additionally, RIC171 accumulates at sites of fungal attack, suggesting enhanced ROP activity at sites of attempted fungal penetration (Schultheiss et al. 2008). The accumulation of ROP at the infection sites has also been observed in parsley cells infected with the oomycete *P. infestans,* exactly to the membrane subtending the oomycetous appressorium, where the actin cables focus (Schutz et al. 2006). The accumulation of ROP small GTPases at the infection sites can be explained because, some pathogens direct the plant to follow a tip growth mimicking program by hijacking host signalling via ROP and RIC proteins for accommodation of the fungal haustorium (Schultheiss et al. 2008). Recent studies have showed that constitutively activated expression of other barley ROP members of the family, such as HvRac1 and HvRac3, also lead to enhanced susceptibility to *Bgh*. However, from these barley ROPs, only HvRac1 overexpression promoted H_2O_2 accumulation. Interestingly, HvRac1 is also involved in callose deposition and resistance to *Magnaporthe oryzae,* hence, barley ROPs might have overlapping and specific roles in plant–microbe interactions (Pathuri et al. 2008). The isolation of rice closest ortholog to HvRacB and OsRacB confirmed the existence of specific functions of the different members of the small GTPases family, since OsRacB functions as a negative regulator for basal disease resistance (Jung et al. 2006).

The use of yeast two-hybrid strategies has allowed the identification of potential ROP interactors involved in plant defense signaling and responses. These assays have confirmed the existence of cross-talk between ROP and specific receptor-like kinases (RLKs) through direct molecular interaction (Molendijk et al. 2008). Coimmunoprecipitation of ROPs with RLKs, such as the *Arabidopsis* CLAVATA1 (Trotochaud et al. 1999) or the tomato LePRKs (Wengier et al. 2003), have been described. Also, in vitro and in vivo interactions of *Arabidopsis* NRCK, a receptor kinase belonging to the RLCK Class VIII, with AtROP4 and AtROP11 GTPases have been found (Molendijk et al. 2008). RLKs are the predominant cell surface receptors in plants. Receptor-like serine/threonine kinase family encompasses

more than 400 members in *Arabidopsis* (Shiu et al. 2004). The role of RLKs in plant immune responses is an exciting field that has attracted attention of many research groups in recent years, as RLKs are involved in perception of pathogen-associated molecular patterns (PAMPs), regulation of PCD upon pathogen infection, and activation of innate immunity signaling cascades (Chinchilla et al. 2007; Kemmerling et al. 2007; Llorente et al. 2005). The interaction of ROPs with RLKs suggests that ROPs could mediate cell surface-associated signal perception and transductions. All ROPs contain two putative serine/threonine phosphorylation sites, SYR and SSK, which might be the targets of the RLKs identified. A connection between RLK ERECTA and heterotrimeric G proteins has been also hypothesized (Lease et al. 2001; Llorente et al. 2005). *Arabidopsis* mutant plants lacking Gβ subunit of the heterotrimeric G protein exhibit an erecta-like developmental phenotype similar to plants impaired in ERECTA RLK, suggesting that they may form a part of the same pathway (Lease et al. 2001; Llorente et al. 2005). In line with this hypothesis, *er* and *agb1* mutants showed an enhanced susceptibility to the necrotrophic fungus *P. cucumerina* (Llorente et al. 2005).

Recent studies have revealed the interaction between the small GTPases and downstream kinase-mediated immune signaling pathways. Silencing of the small GTPase OsRac1 by RNA interference prevents the elicitor-induced accumulation and posttranslational activation of the rice kinase OsMAPK6, although the mechanism of OsRAC1-dependent OsMAPK6 activation is still not known (Lieberherr et al. 2005). As indicated above, AtROP4 interacts, in vitro and *in planta,* with several kinases, including RBK1 and RBK2 – RLCK VIb family members (Molendijk et al. 2008). The expression of *RBK1* gene is upregulated in leaves inoculated with *P. infestans* and *B. cinerea* (Molendijk et al. 2008). Two other plant RLCK VI family members, the *Medicago truncatula* MtRRK1 and MtRRK2, as well as the *Arabidopsis* homolog AtRLCK VI_A2 kinase (AtRRK1), can also directly interact with ROP GTPases in yeast two-hybrid. In vitro kinase activation experiments showed that their kinase activity is specifically and strongly increased in the presence of active GTP-bound, but not of inactive GDP-bound, ROP GTPases, supporting a hypothesis that these kinases are potential downstream ROP GTPase effectors (Dorjgotov et al. 2009). However, further *in planta* experimentation are necessary to confirm these interactions and to verify the possible role in the activation of plant defense responses.

Plant cell wall integrity has emerged in the last years as a key modulator of some defense responses in *Arabidopsis* (Hematy and Hofte 2008; Hernandez-Blanco et al. 2007). Currently, it is not well understood how wall integrity changes are perceived by the plant cells. Like it has been described in yeast, some receptor (e.g., RLKs) or cell wall sensors may perceive the change produced in the wall by environmental stresses, such as pathogen attack (Philip and Levin 2001). Several studies linked ROPs with the biosynthesis of secondary cell walls (Delmer et al. 1995), as well as with some modifications in the cell walls, such as callose and lignin depositions, that take place after pathogen attack. Thus, GTP–AtROP1, but not GDP–AtROP1, interacts with a putative subunit of *Arabidopsis* callose synthase, and OsRac1 stimulates cinnamoyl-CoA reductase 1 (CCR1), a key

enzyme required for the biosynthesis of lignin polymers, which guard off pathogens attack (Hong et al. 2001, Kawasaki et al. 2006). Also, OsRac1 coordinately activates the production of two intermediates (monolignol and hydrogen peroxide) required for lignin biosynthesis. Therefore, it cannot be ruled out a possible new interconnection between ROPs function and some plant cell wall mediated-defense responses. Interestingly, a specific function of ERECTA RLK in regulating cell wall-mediated disease resistance has recently been proposed (Sánchez-Rodríguez et al. 2009). Mutations in *ERECTA* gene led to altered cell wall composition and increased disease susceptibility to the necrotrophic pathogen *P. cucumerina*. Mutations suppressing the *erecta* phenotype and thereby restoring the resistance to *P. cucumerina* up to wild-type levels were identified (Sánchez-Rodríguez et al. 2009). The potential interaction among ROPs, RLKs (e.g., ERECTA), and heterotrimeric G proteins in the regulation of cell wall integrity is a research area that deserves additional attention in the future.

Studies on auxin signaling have revealed a previously uncharacterized cellular role of ROPs positively modulating the ubiquitin/26S proteasome-regulated proteolysis (Tao et al. 2005). In addition, ROP GTPases are important negative regulators of abscisic acid (ABA) signaling, and ABA has been shown to inactivate RAC/ROPs interaction (Lemichez et al. 2001; Xin et al. 2005; Zheng et al. 2002). The signaling pathways regulated by these hormones have been recently involved in modulating some plant defense responses to particular pathogens (Hernandez-Blanco et al. 2007; Llorente et al. 2008; Navarro et al. 2006), thus, again it is mandatory to gain a deeper understanding of the functionality of these small GTPases, and to determine the interconnection between their function in hormone regulation and their putative function in resistance to pathogens.

In summary, we can conclude that plant ROPs small GTPases have emerged as relevant signaling modules in plant immunity, as they regulate plant response to cellular oxidative environments that could lead to host PCD. Our current data indicate that ROPs roles in plant immunity responses are specific and different depending on the plant–pathogen interaction analyzed, since ROPs may act as positive or negative regulators of plant immunity. Additionally, the interaction of ROPs with cell surface-associated signal perception apparatus (e.g., RLKs receptors) to perceive a broad range of extracellular stimuli, including pathogen insult, may directly regulate downstream kinase signaling and activation of immune responses. Further experiments are necessary to determine whether these promising interactions between some RLKs/kinases and ROP observed using the in vitro yeast two-hybrid system also occur in vivo, and also to clarify the genetic and biochemical interaction of ROPs with G proteins in plant innate immunity.

6 Future Directions

A plethora of data is now available linking G proteins, small and large, with plant defense. Our present knowledge emphasizes that, although there is bound to be common themes, the roles played by G proteins can vary depending on the specific

plant–pathogen system studied. The next challenge will be to establish the signaling pathways used by G proteins to mediate plant innate immunity; some components of these pathways are now emerging but many more need to be identified. Interactions identified using the yeast two hybrid system need to be confirmed *in planta*. Functional studies need to be complemented with genetic studies to determine the position of each component in the overall signal transduction cascade. Arguably, the most important components of these cascades will be the receptors, either seven transmembrane G-protein-coupled receptors, RLKs, or other as yet unidentified receptors.

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