FUNCTIONAL ANALYSIS AND BIOCHEMICAL CHARACTERIZATION OF HETEROTRIMERIC G PROTEIN IN *ARABIDOPSIS*

A Thesis in

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by

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Heterotrimeric G proteins are recognized as important signal transduction molecules in all eukaryotic organisms from yeasts to humans. Compared to mammals and invertebrates, simplicity of G protein gene family in *Arabidopsis* provides a unique advantage for investigating its functions in various aspects of different physiological processes. Extensive studies in the last 10 years have shown that the *Arabidopsis* heterotrimeric G protein is involved in a transducing a variety of signals, including light, hormones, biotic and abiotic stressors.

First, we analyzed the functional roles of heterotrimeric G protein in *Arabidopsis* oxidative stress responses to ozone (O$_3$). *Arabidopsis* thaliana plants of homozygous *gpa1-4* (G$\alpha$ null mutation) are less sensitive to O$_3$ damage than wild-type Columbia-0 plants, whereas *agb1-2* (G$\beta$ null mutation) plants are more sensitive to O$_3$ damage than wild-type Columbia-0 plants. The genes encoding the $\alpha$ and $\beta$ subunits of the *Arabidopsis* heterotrimeric G protein are differentially expressed in the course of the oxidative stress response to O$_3$, suggesting that they play different roles in the plant’s O$_3$ responses. The first peak of the biphasic oxidative burst elicited by O$_3$ in wild-type plants is absent in both mutant plants, suggesting that the first peak of the oxidative burst requires both $\alpha$ and $\beta$ subunits or the intact heterotrimeric G protein. The second peak is missing in *gpa1-4* mutant plants, but is normal in *agb1-2* mutant plants, suggesting that the second peak of the oxidative burst requires only the $\alpha$ subunit but not the $\beta$ subunit.
Thus, the opposing O₃ phenotypes and the differential ROS production patterns between gpa1-4 and agb1-2 mutant plants indicate that the α and β subunits play separable roles in cell death associated oxidative stress response to O₃.

Second, we analyzed the functional role of heterotrimeric G protein of Arabidopsis unfolded protein responses to Tm. Seedlings of agb1-2 mutant plants with a null mutation in the gene coding for the β subunit of the heterotrimeric G protein are more resistant to growth inhibition by the protein glycosylation inhibitor tunicamycin (Tm) than wildtype plants and gpa1-4 plants with a null mutation in the gene encoding the α subunit of the heterotrimeric G protein. Leaves of agb1-2 mutant plants exhibit markedly less cell death after Tm treatment than those of wildtype plants. The transcriptional response of agb1-2 mutant plants to Tm is less pronounced than that of wildtype plants. On the other hand, AtrbohD (AtrbohD null mutation) and AtrbohD/F (AtrbohD and AtrbohF null mutation) mutant plants show more cell death in response to treatment with Tm than wildtype plants and AtrbohF (AtrbohF null mutation) mutant plants. The protective role of the endogenous ROS elicited by Tm treatment against Tm-induced cell death is also seen using ROS scavengers. Moreover, the transcriptional responses are delayed by the pretreatment with ROS scavengers.

A majority of the Arabidopsis Gβ protein is associated with the endoplasmic reticulum (ER) and is degraded after Tm treatment, while Gα protein is not. Collectively, these observations indicate that the Gβγ complex, not Gα, plays an important role in cell death associated with the unfolded protein response in Arabidopsis. Moreover these
observations imply a direct role for both ROS and G protein signaling in the UPR.

To elucidate the mechanism of G protein signaling transduction, we characterized the *Arabidopsis* heterotrimeric G protein complex. Using fluorescence resonance energy transfer (FRET) and co-immunoprecipitation, we show interaction between the $\beta_1$ and $\gamma_1$ subunits and between the $\alpha_1$ and $\beta_1\gamma_1$ subunits of the *Arabidopsis* heterotrimeric G proteins in plant protoplasts. We report that both the $G\alpha_1$ and the $G\beta_1$ subunits are associated with macromolecular complexes in plasma membrane fractions. We estimate the size of the G-protein-containing plasma membrane complex to be approximately 669 kD based on blue native polyacrylamide gel electrophoresis, suggesting that the heterotrimeric G protein is present in a complex with other proteins. We find that $G\alpha_1$ is present in both the large ca. 669 kD complex and in smaller complexes in plants homozygous for the *agb1-2* $G\beta_1$ null allele. Deletion of the $G\beta_1$ interacting domain in $G\alpha_1$, ah$G\alpha_1$, abolishes normal function of $G\alpha_1$ in the oxidative stress response to ozone, suggesting the interaction between $G\alpha_1$ and $G\beta_1\gamma$ complex may be required for transmitting the O$_3$ signal in plant cells. Treatment of the plasma membrane fraction with hydrogen peroxide (H$_2$O$_2$), an important signaling molecule, results in the partial dissociation of the $G\alpha_1$ complex. This observation suggests direct or indirect activation of G protein signaling by ROS in the oxidative stress response.
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Chapter 1

INTRODUCTION

Heterotrimeric G proteins are recognized as important signal transduction molecules in all eukaryotic organisms from yeasts to humans (Neer, 1995). Compared to mammals and invertebrates, simplicity of G protein gene family in *Arabidopsis* provides a unique advantage for investigating its functions in various aspects of different physiological processes (Jones and Assmann, 2004; Assmann, 2005a). Extensive studies in the last 10 years have shown that the *Arabidopsis* heterotrimeric G protein is involved in transducing a variety of signals, including light, hormones, biotic and abiotic stressors (Perfus-Barbeoch et al., 2004). However, the mechanism of G protein signaling transduction in *Arabidopsis* is still under investigation (Assmann, 2005b).

Overview of the *Arabidopsis* Heterotrimeric G Protein Genes and Mutants

Heterotrimeric G Proteins in Mammals

G proteins are guanine-nucleotide binding proteins found in all eukaryotes (Pennington, 1994). G proteins are classically divided into heterotrimeric G proteins and small G proteins. Heterotrimeric G proteins consist of α, β and γ subunits, whereas small G proteins are monomeric and similar in function to the α subunit of heterotrimeric G
proteins (Neer, 1995). In mammals, all three heterotrimeric G protein subunits belong to multigene families (Wilkie and Yokoyama, 1994). At least 20 different Gα (Strathmann and Simon, 1990), five or six Gβ (Seack et al., 1998) and 12 Gγ subunits (Cook et al., 2001) have been identified in humans. All Gαs contain several important conserved structural domains: a GTPase domain, a GTP binding domain and two switch domains, which change conformation with binding of GTP or GDP (Bourne et al., 1991). The important characteristic of Gβ subunits is a seven-bladed β-propeller structure (Lambright et al., 1996; Sondek et al., 1996), which comprises seven or eight tandem repeats with a conserved Trp-Asp (WD) domain. Gβ and Gγ subunits can interact strongly through the coiled-coil structure formed between them (Sondek et al., 1996). All Gγs (Song and Dohlman, 1996), as well as some species of Gα (Casey, 1994), can be modified by lipids that attach proteins to membranes.

The classic heterotrimeric G protein functions as follows (Bourne et al., 1991). In the inactive state, the GDP-bound α subunit associates with the Gβγ dimer to form a heterotrimeric G protein complex. The heterotrimer interacts with a family of plasma membrane receptors containing seven transmembrane domains (7-TM) known as G protein coupled receptors (GPCRs). In metazoans, signal perception by the GPCR catalyzes the exchange of GDP for GTP on an α subunit, causing Gα to dissociate from the GPCR and Gβγ dimer. In the GTP-bound form, Gα regulates downstream target proteins, as does the free Gβγ dimer. Gα has intrinsic GTPase activity and ultimately causes Gα to return to the inactive state, where it can re-associate with Gβγ subunits. In a
few cases, the dissociation of Ga from Gβγ dimer is not necessary for the activation of G protein signaling (Klein et al., 2000; Bunemann et al., 2003).

G protein activity can be manipulated using nonhydrolyzable GTP analogs and ADP-ribosylating bacterial toxins. GTPγS is a nonhydrolyzable GTP analog that locks mammalian Ga subunits in their active state. Conversely, GDPβS is a nonhydrolyzable GDP analog that locks mammalian Ga subunits in their GDP-bound, inactive form. ADP ribosylation by cholera toxin (CTX) modifies a conserved Arg residue found in the mammalian Ga subunit, locking the protein into a conformation that inhibits its GTPase activity, resulting in its prolonged activity (Gillison and Sharp, 1994). Pertussis toxin (PTX) ribosylates a Cys with ADP near the carboxyl terminus of Ga and inactivates signaling from GPCR to Ga (Carty, 1994). Mastoparan, a short peptide from wasp venom, is able to activate G proteins by interfering with Ga-GPCR interactions, mimicking GPCR activation (Klinker et al., 1994).

Heterotrimeric G Protein in Arabidopsis

In contrast to the large number of different G protein subunits in animals, Arabidopsis has only one canonical Ga protein (AtGPA1) (Ma et al., 1990), one canonical Gβ protein (AtAGB1) (Weiss et al., 1994), and two Gγ proteins (AtAGG1 and AtAGG2) (Mason and Botella, 2000, 2001). All of these subunits have limited homology with their animal counterparts. The AtGPA1 protein shows roughly 30% identity with the mammalian Ga subfamily protein (Ma et al., 1990). It possesses the sites for CTX, N-myristoylation and
N-palmitoylation but lacks the site for PTX. Although the biochemical properties of the Arabidopsis AtGPA1 protein have not been characterized as extensively as those of the rice GPA1 (RGA1) (Seo et al., 1997), recombinant AtGPA1 has been shown to bind GTPγS and have GTPase activity, which can be enhanced by the regulator of G protein signaling (AtRGS1) (Chen et al., 2003). AtAGB1 has about 42% similarity to mammalian Gβ subunits (Weiss et al., 1994), and AtAGGs display 24%-31% identity with certain mammalian Gγ subunits. Both AGG1 and AGG2 proteins contain C-terminal CaaX motifs for farnesylation and geranylgeranylation (Mason and Botella, 2000, 2001). At the cellular level, Gα1 has been immunolocalized in the plasma membrane and endoplasmic reticulum (Weiss et al., 1997), while Gβ1 has been detected in the plasma membrane, endoplasmic reticulum and Golgi structure (Obrdlik et al., 2000; Wang et al., 2007). Gγ1 and Gγ2 are both observed in the plasma membrane using fluorescent fusion proteins in cowpea protoplasts (Adjobo-Hermans et al., 2006).

Structural modeling of Gα1, Gβ1, and Gγ1 shows that the functionally important regions of the G protein heterotrimer structure are highly conserved in the Arabidopsis subunits (Ullah et al., 2003), suggesting that they can form a heterotrimer similar to that formed by the mammalian G protein subunits. Interactions between Gβ1, Gγ1 and Gγ2 have been detected by yeast two-hybrid and in vitro binding assays (Mason and Botella, 2000, 2001), and there is similar evidence from yeast two-hybrid and co-immunoprecipitation assays for interactions between the rice Gα subunit and Gβ subunits (Kato et al., 2004). The heterotrimerization of Arabidopsis G protein subunits in
cowpea protoplasts was recently reported.

Whole genome sequences of *Arabidopsis* are available. Mutant alleles of AtGPA1 (Ullah et al., 2001; Wang et al., 2001; Ullah et al., 2003) and AtAGB1 (Lease et al., 2001; Ullah et al., 2003) by screening a T-DNA insertion library or a genetic screen of ethyl methanesulfonic acid (EMS)-mutagenized plants were identified, which all facilitates the functional study of the heterotrimeric G protein in *Arabidopsis*.

### Important Roles of Heterotrimeric G Protein in *Arabidopsis*

Although pharmacological experiments suggested that plant heterotrimeric G proteins mediate diverse signals in plants such as blue light (Warpeha et al., 1991), red light (Romero et al., 1991), auxin, and abscisic acid (ABA) (Ritchie and Gilroy, 2000), recent genetic studies provide direct evidence that *Arabidopsis* heterotrimeric G protein is involved in the transmission of different kinds of signals, including hormones, biotic and abiotic stress.

*AtGPA1* and *AtAGB1* mutant plants show both similar and opposite phenotypes at various developmental stages (Ullah et al., 2003). Both mutants have rounded lamina in their leaves and reduced cell division in their hypocotyls. Compared with those of wild-type plants, fruit and seed weights are greater for both mutants. However, *gpa1-1* sepalas are longer than wild-type sepalas, whereas *agb1-1* sepalas are shorter than wild-type. *gpa1-1* seedlings are smaller than wild-type, whereas *agb1-1* seedlings are larger than wild-type. *agb1-1* and *agb1-2* have more roots mass than wild-type, whereas *gpa1-1* and
gpa1-2 have less root mass than wild-type. Moreover, agb1-1 mutant plants have increased apical dominance and gpa1-1 mutant plants have decreased apical dominance. There are also some phenotypes unique to each mutant. Pedicels in gpa1-1 mutant are uniquely long and agb1-1 mutant leaves are uniquely curly. The divergent alterations in developmental phenotypes of G protein mutants suggest that functions of Arabidopsis Ga and Gβγ can differ in specific cell types and different developmental processes (Ullah et al., 2003).

Likewise, phenotypes are altered differentially when G protein mutants are exposed to varieties of hormones, biotic and abiotic stresses. gpa1-1 and gpa1-2 mutant seeds are not only less sensitive to GA, but completely insensitive to brassinosteroids (BR) (Ullah et al., 2002). However, the gpa1-4 mutant shows moderately enhanced sensitivity to ABA inhibition in seed germination (Ullah et al., 2002; Pandey and Assmann, 2004), while the agb1-2 mutant exhibits hypersensitivity to ABA inhibition in seed germination. Moreover, only the agb1-2 mutant exhibits decreased sensitivity in a number of jasmonic acid (JA)-induced responses such as inhibition of root elongation and seed germination (Trusov et al., 2006). In biotic stress responses, agb1-1 shows the enhanced susceptibility to the fungal pathogen Plectosphaerella cucumerina, and necrotrophic pathogens Alternaria brassicicola and Fusarium oxysporum, whereas the gpa1-4 mutant shows enhanced resistance to these pathogens compared to wild-type (Llorente et al., 2005). In abiotic stress responses, the agb1-2 and agb1-1 mutants are more sensitive to O₃ damage than wild-type plants, whereas the gpa1-4 mutant is more resistant to O₃ damage than
wild-type (Joo et al., 2005). Moreover, only the \textit{agb1-2} mutant shows a more resistant phenotype to tunicamycin \textit{(Tm, protein glycosylation inhibitor)}-induced leaf cell death (Wang et al., 2007). This summary of G protein mutant phenotypes in response to hormones and stressors pinpoints the important roles of the \textit{Arabidopsis} heterotrimeric G protein in a variety of signal transduction events.

The functions of \textit{Arabidopsis} Ga and G\textit{βγ} are well understood in some biological processes. Ozone induces a bimodal oxidative burst \textit{(reactive oxygen species, ROS)} in wild-type plants (Joo et al., 2005). The first peak is almost entirely missing in both \textit{gpa1-4} and \textit{agb1-2} mutant plants. The late peak is normal in \textit{agb1-2} but missing in \textit{gpa1-4} mutant plants. This observation indicates that only Ga is required for the production of the late peak, but both Ga and G\textit{βγ}, or the entire heterotrimeric G protein, are required for the first peak. Further characterization of ROS production induced by ozone shows that the production of ROS in the late peak is mainly attributable to its ability to activate the NADPH oxidase signaling pathway, which is intact in \textit{agb1-2}, but not in \textit{gpa1-4} mutant plants. This result indicates that Ga subunit can mediate activation of membrane-bound NADPH oxidases in Gβ protein independent of the formation of the heterotrimer.

The \textit{Arabidopsis} Ga and G\textit{βγ} proteins have also been reported to play different roles in modulating cell division in roots (Chen et al., 2006). By comparing root growth rate and lateral root formation in \textit{gpa1-4} and \textit{agb1-2} single and double mutants as well as in transgenic lines overexpressing AtGPA1 in \textit{agb1-2} and overexpressing AtAGB1 in


*gpαl*-4 mutant backgrounds, the functions of Ga, Gβγ and heterotrimeric G protein in cell proliferation in roots were determined. Results show that the heterotrimeric complex is a negative regulator of cell proliferation in root growth, whereas the GTP-bound Ga subunit is a positive regulator in this process. On the other hand, Gβγ dimer has a function independent of Ga in attenuating cell division during the formation of lateral roots. These comparisons also clearly suggest that *Arabidopsis* Ga and Gβγ have both common and independent roles in the modulation of cell division in roots.

**Proteins that Interact with the *Arabidopsis* Heterotrimeric G Protein**

The different functions of *Arabidopsis* Ga or Gβγ may result from their interaction with different proteins, which perform functions in a variety of development-related events and stress-associated processes in response to G protein signals. A number of *Arabidopsis* Ga-interacting proteins have been identified, although no downstream targets of Gβγ signaling have yet been identified in plants. By yeast two-hybrid analysis and in vitro pulldown assays, AtPirin1, a presumed transcription factor, and Ga1 have been shown to physically interact. An atpirin1 null mutant is hypersensitive to ABA in inhibition of seed germination and early seedling development (Lapik and Kaufman, 2003). Given the enhanced sensitivity of *gpαl*-1 and *gpαl*-2 mutants to ABA in inhibition of seed germination (Pandey et al., 2006) and the interaction between AtPirin1 and Ga1, it was inferred that the enhanced sensitivity of *gpαl*-4 mutant to ABA could be due to loss of an activating signal from Ga1 to AtPirin1, thought to be a negative regulator of ABA
action (Assmann, 2005b).

Another well-known $\alpha_1$ interacting protein is *Arabidopsis* phospholipase $\alpha_1$ (AtPLD$\alpha_1$). $\alpha_1$ was shown to interact with AtPLD$\alpha_1$ using pulldown assays in bacteria and plant extracts (Zhao and Wang, 2004). The DRY motif in AtPLD$\alpha_1$, which is conserved in G-protein coupled receptors, was identified as the interaction site with $\alpha_1$ using site-directed mutagenesis (Zhao and Wang, 2004). The biological significance of their interactions was determined by introducing a mutant PLD$\alpha_1$ (AtPLD$\alpha_{1k564A}$), which decreases the binding of $\alpha_1$ to PL$\alpha_1$ by 90%, into the PLD$\alpha_1$ mutant plants. The result shows that AtPLD$\alpha_{1k564A}$ can’t replace the function of PLD$\alpha_1$ in the ABA response to inhibit stomatal opening, suggesting that $\alpha_1$ acts downstream of PLD$\alpha_1$ and phosphatidic acid (PA) to regulate the ABA inhibitory effect on stomatal opening (Mishra et al., 2006). Additional components of G protein signaling have also been identified as $\alpha_1$ interacting proteins such as AtRGS1 (regulator of G protein signaling) (Chen et al., 2003), AtGCR1 (G protein coupled receptor 1) (Chen et al., 2004; Pandey and Assmann, 2004), AtGCR2 (G protein coupled receptor 2) (Liu et al., 2007). AtRGS1 accelerates the GTPase activity of $\alpha_1$ and acts as a negative regulator of $\alpha_1$ in modulating cell proliferation in roots and hypocotyls (Chen et al., 2003). AtGCR1 and AtGCR2 both have a predicted 7 transmembrane (TM) structure, which is conserved in mammalian GPCRs (Pandey and Assmann, 2004; Liu et al., 2007). Although they share similar structural properties, they play different regulatory roles in $\alpha_1$ functions. AtGCR1 acts in concert with $\alpha_1$ and $\beta_1$ in ABA signaling during germination and early seedling development.
(Pandey et al., 2006), whereas AtGCR2 has an opposite effect on this process. AtGCR2 and \( \alpha_1 \) function together to mediate ABA signal in guard cells, whereas AtGCR1 may act as a negative regulator in this response. Since a large pool of predicted 7TM containing proteins is present in *Arabidopsis*, it is likely that more AtGCRs could be identified (Pandey and Assmann, 2004; Liu et al., 2007). The more components in the G protein signaling pathway that are identified, the more complicated are the emerging picture of G protein regulatory mechanisms in *Arabidopsis*. Nevertheless, these efforts will eventually contribute to dissecting the mechanism of G protein signaling transduction.

**Redox Signaling in the *Arabidopsis* Oxidative Stress Response to Ozone**

**Ozone Effects on Plant Defense**

Ozone (\( O_3 \)) has been recognized as a major component of photochemical air pollution responsible for causing significant damage in both nature and cultivated plants since 1958 (Darley et al., 1959). \( O_3 \) generally inhibits plant photosynthesis and growth to influence the likelihood of biotic plant disease (Ordin, 1965; Coulson and Heath, 1974). \( O_3 \) is taken up from leaf stomata and is apparently destroyed rapidly in the apoplast compartment. Ozone dissociates in aqueous solutions and produces reactive oxygen species (ROS) such as superoxide (\( O_2^- \)) and hydrogen peroxide (\( H_2O_2 \)), which can further react with transition metals generating hydroxyl radicals (\( OH^- \)) and singlet oxygen (Kanofsky and Sima, 1995).
Superoxide can also react with amine, phenolic compounds and extracellular ascorbate to exacerbate the production of hydroxyl radicals (Kanofsky and Sima, 1995). These ROS, especially singlet oxygen and hydroxyl radicals, are harmful to all living organisms due to their strong oxidizing effects on biologically important macromolecules. Thus, O3 is widely used to study the plant’s defense response to the oxidative stress.

O3 triggers plant defense responses including the production of an oxidative burst, the biosynthesis of antimicrobial compounds, cell wall proteins, antioxidants, and signaling molecules, as well as the apoptotic hypersensitive response (HR) and the systemic acquired resistance (SAR) (Mahalingam et al., 2003). The plant’s response to this oxidative stress shares some commonalties with the pathogen defense response, including the production of ROS and induction of the HR and SAR (Lamb and Dixon, 1994; Lamb and Dixon, 1997; Baker and Orlandi, 1999; Inze and Van Montagu, 2002; Scheel, 2002; Apel and Hirt, 2004).

Production of the Oxidative Burst

A variety of biotic and abiotic stressors trigger a transient increase in endogenous reactive oxygen species, predominantly •O2 and H2O2 in plant cells (Levine et al., 1994; Schraudner et al., 1998; Joo et al., 2005). This phenomenon is termed “oxidative burst”. The oxidative burst was first reported in plants challenged with an incompatible pathogen Phytophthora infestans. Subsequently, the oxidative burst has also been observed in plants challenged with fungal, bacterial and viral pathogens, as well as with many other
abiotic stressors including high temperature, intense light, drought, cold and air pollutants such as O$_3$. In most cases, the plant oxidative burst is a biphasic response, comprising a primary peak 1-2h, followed by a secondary peak with greater magnitude 3-6 h after infection (Lamb and Dixon, 1994). However, the occurrence, intensity and duration of the oxidative burst in plants vary depending on the plant system studied and the elicitor used (Allan and Fluhr, 1997; Lamb and Dixon, 1997; Tenhaken and Rubel, 1998). The oxidative burst triggered by ozone is also dependent on treated plants, intensity and duration of ozone (Schraudner et al., 1998; Joo et al., 2005).

Detailed studies on ROS production in response to different stressors have shown that the ROS can be distinguished enzymatically and spatially (Suharsono et al., 2002; Apel and Hirt, 2004; Joo et al., 2005; Fedoroff, 2006). There are many enzymatic sources of ROS in plants, both extra- and intracellular, including varieties of oxidases and peroxidases, such as cell-wall peroxidases and amine oxidases, plasma membrane-bound NADPH oxidases, and intracellular oxidases and peroxidases in mitochondria, chloroplasts, peroxisomes and nuclei (Foyer and Noctor, 2005). There are also various metabolic pathways to continuously produce ROS as byproducts in different cellular compartments. While different mechanisms have been proposed for the ROS increase triggered by either biotic or abiotic stressors, how various cellular ROS sources are activated and propagated to produce the transient burst is not well understood (Apel and Hirt, 2004; Fedoroff, 2006).

Recently we reported that *Arabidopsis* heterotrimeric G protein signaling is required
to activate the intracellular sources of ROS that contribute to the first component of the biphasic, O$_3$-elicited oxidative burst (Joo et al., 2005). ROS are produced rapidly in guard cell chloroplasts and peripheral membranes after O$_3$ treatment and these two sources of ROS act as signals to elicit production of more ROS in the adjacent cells, resulting in the first oxidative burst. However, this oxidative burst is absent in both gpa1-4 and agb1-2 mutant plants. This result further suggests that ROS production in response to O$_3$ may require not only ROS-generating enzymes but other signaling molecules to finely control the initiation of ROS production. The small GTPase, one of monomeric G proeins, has been implicated in the activation of membrane bound NADPH oxidase in the pathogen response in rice (Suharsono et al., 2002).

**Signaling Roles of the Oxidative Burst**

The hazards of reactive oxygen species have long been recognized, although the essential roles for them in signaling are still under investigation (Sauer et al., 2001; Cormack et al., 2002; Droge, 2002). Nevertheless, increasing evidence shows that ROS are produced locally or systemically and act specifically for signaling in both plant stress responses and developmental processes (Joo et al., 2001; Schopfer et al., 2002; Overmyer et al., 2003). The oxidative burst can induce programmed cell death (PCD) in various systems. Genetic studies support this concept by showing that *Arabidopsis atrbohD* or *atrbohF* mutant plants lacking functional rbohD or rbohF genes (i.e., respiratory burst oxidase homolog genes) exhibit reduced ROS generation and PCD following bacterial
challenge (Torres et al., 2002). ABA stimulates ROS production, which induces stomatal closure via activation of plasma membrane calcium channels (Kwak et al., 2003). Moreover, stomatal closure and plasma membrane calcium channel activation are reduced in atrbohD atrbohF double mutants but can be restored with H₂O₂, suggesting that ROS serve as second messengers in the ABA response in Arabidopsis guard cells (Kwak et al., 2003). H₂O₂ is also involved in auxin signaling and gravitropism in maize roots (Joo et al., 2001). Arabidopsis atrbohC mutant plants lacking a functional rbohC gene show a root development defect phenotype, suggesting that ROS function as local signaling molecules in Arabidopsis root development (Carol et al., 2005; Jones et al., 2007).

ROS react with proteins directly in many different ways, such as oxidizing thiol residues like Cys, Met, attacking Lys, Pro, Arg and Thr to result in the formation of protein carbonyl derivatives (Johansson et al., 2004). Thus, ROS act on some ROS sensor protein, transcription factors and components of signaling pathways to change their conformation and regulate their functions, suggesting that ROS serve as signaling molecules (Fedoroff, 2006). ABI1 is a member of protein phosphatase 2C family (PP2C) in Arabidopsis and plays a negative role in ABA signaling (Leung et al., 1997). An enzymological study shows that H₂O₂ has a strong inactivating effect on ABI1 activity, which may be caused by oxidation of cysteine residues in the active site (Meinhard and Grill, 2001; Meinhard et al., 2002). Thus H₂O₂, as a second messenger, could mediate ABA signaling by inactivating the negative regulator ABI1. Similarly, Arabidopsis protein tyrosine phosphatase (AtPTP), which de-phosphorylates AtMAPK6, can also be
inactivated by H$_2$O$_2$ (Gupta and Luan, 2003). Thus H$_2$O$_2$ activates AtMAPK6 and concomitantly inhibits the activity of AtPTP (Moon et al., 2003).

Unlike OxyR protein in bacteria and Gpx3 protein in yeast, ROS sensor proteins have not yet been identified in *Arabidopsis* (Zheng et al., 1998; Lee et al., 2004). However, an *Arabidopsis* protein, AtOXS2, has been reported to have properties of an ROS sensor (Branvillain et al., 2006). Yeast cells expressing AtOXS2 cDNA show an enhanced ability to tolerate diamide-induced oxidative stress. AtOXS2 protein belongs to a zinc-finger transcription factor family but with C-terminal nuclear export sequence (NES). Under non-stress condition, AtOXS2 protein stays in the cytoplasm, whereas under stress conditions, the protein moves into the nucleus and activates flowering gene expression to promote early reproduction. Although how this shuttling mechanism is triggered by stress is not clear yet, it is likely that its nuclear export sequence is shielded or modified by ROS produced in response to stress (Branvillain et al., 2006).

The transcriptional regulatory factor NPR1 (Nonexpressor of Pathogenesis-Related genes) is activated in a redox regulated manner (Pieterse and Van Loon, 2004). Under unstressed conditions, NPR1 is locked in the cytoplasm in disulfide-bonded intermolecular oligomeric form. With the application of salicylic acid (SA), the intermolecular disulfide bonds are reduced, resulting in the release of monomeric NPR1, which can move into the nucleus and activate expression of defense genes such as PR (Rairdan and Delaney, 2002). NPR1 becomes constitutively monomeric and active for promoting defense gene expression under unstressed condition when it is mutated at the
Cys82 or Cys216 residues (Despres et al., 2003; Moon et al., 2003). SA, as a mandatory hormone of the SAR, can induce ROS production, which in turn can enhance Cu-Zu superoxide dismutase (SOD), peroxidase, glutathione reductase and ascorbate peroxidase activities or their transcript level in plants to counteract the toxic effects of ROS (Mahalingam et al., 2003). As a result of the accumulation of antioxidants, oligomeric NPR1 could be reduced and activated to be monomeric NPR1 (Fobert and Despres, 2005).

In mammals, some signaling pathways activated by H$_2$O$_2$ show similarities with those activated by Gi protein coupled receptors. For example, two mammalian G$\alpha$ proteins, G$\alpha_i$ and G$\alpha_o$, are ROS targets and directly regulated by H$_2$O$_2$ (Nishida et al., 2000). H$_2$O$_2$ treatment promotes increased affinity of GTP for the G$\alpha$ subunit in dose-dependent manner and activates G$\beta\gamma$ signaling as well as its downstream effectors (Nishida et al., 2000). Moreover, Cys$^{287}$ in G$\alpha_i$ and G$\alpha_o$ required for their ROS activation (Nishida et al., 2002) is conserved in AtG$\alpha_1$ (Ma et al., 1990). This raises the intriguing possibility that Arabidopsis G protein activity is redox regulated.

ROS can also play its signaling role in an indirect way (Orozco-Cardenas and Ryan, 1999). ROS induces production of the intercellular messengers such as salicylic acid (SA), jasmonic acid (JA) and ethylene, which act in concert to regulate O$_3$-induced protective and adaptive responses such as defense gene expression and hypersensitive cell death (Sharma et al., 1996; Klessig et al., 2000; Orozco-Cardenas et al., 2001; Tamaoki et al., 2003). Since these messengers have a longer life than ROS, some function of them may
be beyond the direct control of ROS in O$_3$ response (Apel and Hirt, 2004). Genetic studies about mutant plants lacking SA, JA or ethylene signaling have established their essential roles in O$_3$ response (Nawrath and Metraux, 1999; Rao and Davis, 1999; Rate et al., 1999; Morris et al., 2000; Overmyer et al., 2000; Shah et al., 2001). However, their interconnected relationship with ROS is still not well understood (Torres et al., 2005). The ROS production can alter the redox environment in cells, which itself can cause numbers of biological responses like modulating enzyme activity and altering gene expression level, especially in chloroplasts (Creissen et al., 1999; Morris et al., 2000). Although redox regulatory processes are emerging to be central for activating the stress responses, given the multiple sources of ROS and the complexity of cell redox environments, identification of ROS target and ROS action is still challenging for the future research (Fedoroff, 2006).

Unfolded Protein Responses in Arabidopsis

ER Stress and Unfolded Protein Responses in Mammals

The endoplasmic reticulum (ER) is a primary organelle in which secretory proteins are synthesized, modified, and delivered to their target sites (Schroder and Kaufman, 2005). ER stress occurs when protein folding, modification and transportation are perturbed (Schroder and Kaufman, 2005). To protect cells from this stress, a series of signaling cascade, termed the unfolded protein response (UPR), is activated to re-balance
the folding demand and the folding capacity in the ER (Wu and Kaufman, 2006).

UPR can be induced experimentally and physiologically (Rutkowski and Kaufman, 2004). Chemicals which prevent proteins from folding properly can activate UPR. Tunicamycin, which inhibits N-linked glycosylation in the ER (Duksin and Mahoney, 1982), thapsigargin (Tg), which depletes the cell’s energy stores for protein folding (Thastrup et al., 1990) and dithiothreitol (DTT), which creates reductive stress in ER (Jamsa et al., 1994), are all widely used in ER stress and UPR studies in mammalian cells. Some physiological responses can also activate the UPR including certain pathogen infections, such as hepatitis C, differentiation of secretory cells and nutrient deprivation (Rutkowski and Kaufman, 2004).

To increase the capacity of protein folding and the disposal of misfolded proteins in the ER, the array of biochemical and physiological processes believed to be activated in the UPR includes 1) induction of ER resident chaperone synthesis, 2) attenuation of translation of most proteins, 3) up-regulation of ER-associated degradation pathways (ERAD), and 4) initiation of apoptotic cell death, which occurs when the three prosurvival responses have failed (Wu and Kaufman, 2006). Perturbation of any of these processes can cause cells or organisms to be susceptible to the ER stress. Furthermore, the ER stress is thought to be involved in certain diseases such as diabetes, Alzheimer’s, atherosclerosis, and cancer (Zhao and Ackerman, 2006).

In mammalian cells, the three distinct protective responses of the UPR are believed to be mediated by ER-resident IRE1 (inositol-requiring transmembrane kinase and
endonuclease 1), ATF6 (activation of transcription factor 6) and PERK (protein kinase–like ER kinase) respectively (Bertolotti et al., 2000; Liu et al., 2000). These three proteins remain bound by ER-resident luminal binding protein (BiP) under resting conditions. When the ER is stressed, BiP preferentially binds to the unfolded proteins, effectively being titrated away from IRE1, ATF6 and PERK (Bertolotti et al., 2000). The dissociation from BiP activates PERK, which phosphorylates the translation elongation factor eIF2α and furthermore inhibits translation of new proteins (Harding et al., 1999). After the dissociation from BiP, ATF6 is translocated to the Golgi and cleaved by S1/S2 protease to form the active ATF6, which is a transcription factor promoting chaperone gene expression (Li et al., 2000; Wang et al., 2000). Upon its release from BiP, IRE is activated by dimerization and auto phosphorylation. The active IRE cleaves the precursor of XBP1u mRNA to mature XBP1s mRNA encoding X-box binding protein, by the effective translation which enters the nucleus and functions as a transcription factor inducing the expression of genes involved in unfolded protein degradation (Yoshida et al., 2001).

Compared with these adaptive pathways, apoptotic cell death induced by the ER stress is less well understood (Schroder and Kaufman, 2005). Apoptosis is mainly initiated by two pathways. One is an intrinsic pathway; the other is an extrinsic pathway (Rutkowski and Kaufman, 2004). The intrinsic pathway is activated by intracellular stresses like DNA damages. The extrinsic pathway is activated by extracellular stimuli and mediated by cell surface receptors. The ER stress is more like an intrinsic than an
extrinsic apoptotic signal, although components of both pathways appear to be involved in ER-induced apoptotic cell death.

The difference in the concentration of Ca\(^{2+}\) between the ER, cytosol, and mitochondria is thought to control the initiation of apoptotic cell death (Boya et al., 2002; Nguyen et al., 2002). Transient elevation of the cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)\(_{\text{cyt}}\)]\(_{\text{cyt}}\) released from the ER by the insertion of BH family proteins like Bak and Bax into the ER membrane activates the Ca\(^{2+}\)-dependent caspase calpain (Breckenridge et al., 2003; Reimertz et al., 2003). Activated calpain cleaves and activates procaspase-9, which eventually activates the executioner caspase, caspase-3. Mitochondria can also take up Ca\(^{2+}\) released from the ER, triggering cytochrome \(c\) release into the cytoplasm and formation of the apoptosome complex. The apoptosome can activate caspase-9, which activates procaspase-3 to initiate cell death (Crompton, 1999).

The ER stress can enhance the interaction between c-Jun N-terminal inhibitory kinase (JIK) and tumor necrosis factor receptor-associated factor 2 (TRAF2), which also interacts with IRE1\(\alpha\). Formation of this trimeric complex mediates the signal to apoptosis signal-regulating kinase 1 (ASK1), which leads to cell death (Yoneda et al., 2001; Nishitoh et al., 2002). This signaling cascade represents an extrinsic pathway of apoptosis induced by the ER stress. Although some essential molecules in UPR induced apoptotic pathways have been identified, the detailed molecular mechanism of the cellular decision to commit cells to apoptosis in the UPR remains poorly understood (Rutkowski and Kaufman, 2004; Schroder and Kaufman, 2005; Wu and Kaufman,
Although the study of the UPR and ER stress in plants is not extensive, it has been shown that plants have a protective transcriptional response when exposed to ER stress, including induction of genes encoding protein-folding enzyme and chaperones (Martinez and Chrispeels, 2003; Kamauchi et al., 2005). Both tunicamycin (Tm), an inhibitor of N-linked protein glycosylation, and CPA cyclopiazonic acid (CPA), an inhibitor of the ER-type II Ca\(^{2+}\), can trigger the UPR and programmed cell death in cultured plant cells (Zuppini et al., 2004). However, only a few orthologs of mammalian UPR proteins have been identified so far in plant UPRs. These are Ire1 (Noh et al., 2002), eIF2\(\alpha\) (Chang et al., 1999; Chang et al., 2000) and p58\(^{IPK}\) (Bilgin et al., 2003). The Arabidopsis genome has no orthologs of the ER stress-induced XBP1 or ATF6 genes, although other transcription factors like bZIP60 (Iwata and Koizumi, 2005) and NTM1 (Kim et al., 2006) contain a trans-membrane domain, which can be cleaved by a peptidase and hence may function in similar manner as ATF6.

Recently we reported that ER stress induced by Tm can cause leaf senescence in Arabidopsis (Wang et al., 2007). Senescence is the aging process in the last developmental stage of plants (Gepstein, 2004). In mammalian cells, unfolded proteins also accumulate with aging, thus it seems that the ER stress accelerates the aging of plants. Senescence is defined as a specific type of programmed cell death (PCD) in plants.
As described above, the ER stress can activate PCD, which suggests that PCD induced by the ER stress is conserved in plant and mammals. However, the mechanism of PCD in plants is not as well understood as PCD in mammals.

It was reported that cyclopiazonic acid (CPA), an inhibitor of the ER-type II Ca\(^{2+}\) pump, induces cell death resembling PCD in cultured soybean cells (Zuppini et al., 2004). Moreover, CPA also elicits the activity of a caspase 3-like protease. CPA-treated cultured soybean cells exhibit a bi-phasic increase in \([\text{Ca}^{2+}]_{\text{cyt}}\), with an initial transient peak at about 2 min after administration of CPA and a longer subsequent sustained rise in \([\text{Ca}^{2+}]_{\text{cyt}}\), with a peak at about 10 min. Although the \([\text{Ca}^{2+}]_{\text{cyt}}\) burst was demonstrated in response to treatment by CPA, the causal connection between PCD and \([\text{Ca}^{2+}]_{\text{cyt}}\) has not been established yet. The intracellular Ca\(^{2+}\) chelator BAPTA-AM cannot prevent cell death caused by CPA.

Plants lack caspases, which play a central role in PCD in animal cells (Thornberry and Lazebnik, 1998), but contain a family with 9 related proteins termed metacaspases, which have a different cleavage specificity (Arg/Lys) than caspases (Vercammen et al., 2004; Watanabe and Lam, 2005). Whether they contribute to ER stress-induced cell death has not been determined, although it has been reported that the metacaspase mcII-Pa is critical for PCD during Norway spruce embryogenesis (Bozhkov et al., 2005). Plants also lack BH family protein like Bax and Bak, however, plants contain the Bax inhibitor-1 (BI-1), which can suppress the H\(_2\)O\(_2\)-induced apoptosis in Arabidopsis. Thus, it is likely that BI-1 is involved in ER stress-induced cell death (Kawai et al., 1999; Sanchez et al., 2005).
As important a signaling molecule as calcium (Sanders et al., 1999), ROS can play critical roles in Tm mediated plant ER stress response. The ER strictly requires a reducing environment for protein folding, disulfide bond formation and some post-translational protein modifications (Fewell et al., 2001). Any disturbance of the reducing environment will inhibit the activity of protein foldase and protein disulfide isomerase (PDI), which will consequently result in the ER stress or aggravate the ER stress (Fewell et al., 2001). On the other hand, the ER stress can disrupt the electron transfer chain in protein disulfide bond formation, which results in the generation of ROS (Noiva, 1999). This is how oxidative stress and ER stress are interconnected by redox mechanisms.

ROS could act on chaperone proteins to regulate their biological activity. The heat shock protein (hsp70), a eukaryotic chaperone, provides a remarkable example of a delicate redox regulation mechanism for altering its chaperone activity upon oxidative stress. Hsp70 can be oxidized by S-glutathionylation and the glutationylated form is more effective in preventing protein aggregation than the reduced form (Hoppe et al., 2004; Shelton et al., 2005). Heat shock factor (Hsf1) belongs to a highly conserved eukaryotic transcription factor family, which induces the expression of a variety of heat shock proteins in response to heat, oxidative stress and a variety of other stressors (Young et al., 2004). The DNA binding activity of Hsf1 is directly promoted by oxidation with H$_2$O$_2$. *Arabidopsis* contains both hsp70 and heat shock factors, some of which have been
identified as potentially redox-regulated (Ahn and Thiele, 2003).

ROS production is also involved in the UPR associated apoptotic pathway. CPA elicits an oxidative burst at 25 minutes after treatment of soybean cells prior to the occurrence of PCD (Zuppini et al., 2004). The relationship between the oxidative burst and cell death in the UPR-associated apoptotic pathway remains unclear. Nonetheless, Arabidopsis oxidase mutants like rbohD and rbohF together with the application of ROS generation inhibitors and scavengers will help to provide insight into the role of ROS in UPR-associated cell death.


glutathione biosynthetic capacity in the chloroplasts of transgenic tobacco plants paradoxically causes increased oxidative stress. Plant Cell 11, 1277-1292.


Chapter 2

DIFFERENT SIGNALING AND CELL DEATH ROLES OF HETEROTRIMERIC
G PROTEIN OF α AND β SUBUNITS IN THE ARABIDOPSIS OXIDATIVE
STRESS RESPONSE TO OZONE*

Abstract

*Arabidopsis* thaliana plants of homozygous *gpa1-4* (Gα null mutation) are less sensitive to O₃ damage than wild-type Columbia-0 plants, whereas *agb1-2* (Gβ null mutation) are more sensitive to O₃ damage than wild-type Columbia-0 plants. The genes encoding the α and β subunits of the *Arabidopsis* heterotrimeric G protein are differentially expressed in the course of the oxidative stress response to O₃ and play different roles in the plant’s response. The first peak of the biphasic oxidative burst elicited by O₃ in wild-type plants is absent in both mutant plants, suggesting that the first peak of the oxidative burst requires both the α and β subunits or the intact heterotrimeric G protein. The second peak is missing in *gpa1-4* mutant plants, but is normal in *agb1-2* mutant plants, suggesting that the second peak of the oxidative burst requires only the α subunit but not the β subunit. Thus, the opposing O₃ phenotypes and the differential ROS production pattern between *gpa1-4* and *agb1-2* mutant plants indicate that the α and β subunits play separable roles in cell death associated oxidative stress response to O₃.

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Introduction

Unlike yeast and mammals, plants have a small number of heterotrimeric G proteins (Assmann, 2002). The *Arabidopsis* genome encodes a single canonical α (AtGPA1) (Ma et al., 1990) and a single canonical β subunit (AtAGB1) (Weiss et al., 1994) and at most 2 γ subunits (AtAGG1 and AtAGG2) (Mason and Botella, 2000, 2001). Although there is no experimental evidence to show the interaction among *Arabidopsis* G protein subunits, structural predictions for Gα₁, Gβ₁, and Gγ₁ suggest that they can form a heterotrimer similar to that formed by the mammalian G protein subunits (Ullah et al., 2003). The results of studies in *Arabidopsis* plants with null mutations in the genes encoding the α and β subunits show that the plant heterotrimeric G protein is involved in the transmission of different kinds of signals, including light, hormones and external stresses (Perfus-Barbeoch et al., 2004). Some opposite phenotypes in phenotypic profiling of the gpa1 and agb1 mutants suggest positive and negative regulatory roles for the α and β subunits in different biological processes throughout development (Ullah et al., 2003). Here we address the role of the heterotrimeric G protein in the oxidative stress response to ozone (O₃).

O₃, a component of photochemical smog, represents an oxidative stress to living organisms and is a major atmospheric pollutant, damaging crops and forests (Runeckles et al., 1990). Like other stresses, O₃ triggers a series of changes in plants to adapt to this external stimulus, such as the production of reactive oxygen species (ROS), the
biosynthesis of antioxidants, cell wall proteins and signaling molecules, as well as the hypersensitive response (HR) and systemic acquired resistance (SAR) (Lamb and Dixon, 1994; Levine et al., 1994; Lamb and Dixon, 1997; Baker and Orlandi, 1999; Droge, 2002; Inze and Van Montagu, 2002). Plants prevent pathogens from invading and further propagating from the infection site by activating a programmed cell death called HR (Scheel, 2002). Signaling through the α subunit of the heterotrimeric G protein to activate membrane-bound NADPH oxidase has been implicated in the development of disease resistance and the apoptotic HR in rice (Suharsono et al., 2002).

A variety of biotic and abiotic stressors trigger a transient increase in endogenous reactive oxygen species, predominantly superoxide and \( \text{H}_2\text{O}_2 \) in plant cells, termed oxidative burst (Baker and Orlandi, 1999; Overmyer et al., 2003; Apel and Hirt, 2004). In plants, ROS are mainly generated by enzymes and metabolic reactions in chloroplasts, mitochondria and peroxisomes (Baker and Orlandi, 1999; Overmyer et al., 2003; Apel and Hirt, 2004). In addition to that, oxidases and peroxidases in the apoplast and plasma membrane have also been implicated in the ROS production during pathogen and ABA responses (Torres et al., 2002; Kwak et al., 2003). The hazards of reactive oxygen species have long been recognized, although the essential roles of them for signaling are still under investigation. Evidence increasingly shows that ROS are essential for auxin and ABA signaling, as well as in activating stress and defense responses (Lamb and Dixon, 1997; Joo et al., 2001; Sauer et al., 2001; Inze and Van Montagu, 2002). However, the oxidative burst elicited by O₃ has not been analyzed with respect to its cellular sources.
and its role for O₃ responses has not been determined yet (Schraudner et al., 1998; Rao and Davis, 1999; Inze and Van Montagu, 2002; Mahalingam et al., 2003).

I show here that the α and β subunits of the Arabidopsis heterotrimeric G protein play different roles in the plant’s response to O₃. First, I show that plants lacking the α subunit are more resistant to O₃ damage than Col-0 plants, while plants lacking β subunit are more sensitive to O₃ damage than Col-0 plants. The genes encoding AtGPA1 and AtAGB1 are differentially expressed during the time course of the O₃ response. I show that both Gα and β proteins are required for the first peak and only Gα is required for the second peak of the biphasic oxidative burst elicited by O₃.

**Materials and Methods**

**Plant Materials, Growth Conditions, O₃ Treatment**

Gα null mutant plants (gpa1-4), Gβ null mutant (agb1-2) plants, and double mutant plants (gpa1-4, agb1-2) in the Columbia background were kindly provided by Dr. Jones (Ullah et al., 2003). Plants were grown in MetroMix 200 (Scotts-Sierra Horticultural Products Company, Marysville, OH) in 5 cm pots (50 per flat) at 65% humidity under fluorescent light at 30 W/m²/s with a 14 h light/10 h dark photoperiod for 4-4.5 weeks. For O₃ treatments, 4 week-old plants were transferred to the O₃ fumigation chamber and exposed to 350± 50 ppb O₃ for 6 hrs or to 500±50 ppb or 700±50 ppb ozone for 3 hrs. Ozone was generated using an Ozone Generator (Model No. 2000, Jelight Company,
Inc.), and monitored with an Ozone Monitor (Model 450, Advanced Pollution Instrumentation, Inc., San Diego, CA). Control plants were transferred to an adjacent chamber under identical growth conditions except for the O$_3$ treatment. For pretreatment experiments, 4 week-old plants were transferred to the O$_3$ fumigation chamber and exposed to 350±50 ppb O$_3$ for 2 hrs; then were taken out to an adjacent chamber without O$_3$ for 2 hrs and finally transferred to the O$_3$ fumigation chamber and exposed to 700±50 ppb ozone for 3 hrs. Entire rosettes were harvested at different times after ozone treatment by cutting them from the roots and freezing them at -80°C for RNA analysis and ROS measurement.

Quantification of Tissue Damage

To assess the formation of visible lesions, wildtype, agbl-2, and gpa1-4 plants were exposed to 500 or 700 ppb O$_3$ for 3 hrs, then transferred to O$_3$-free air. At 24 hours after the onset of O$_3$ exposure, plants were examined for visible lesions; plants with lesions were photographed with a digital camera (Progres 3012, Kontron elektronik, Germany) and the number of plants showing visible lesions was recorded. To measure tissue damage by ion leakage, 18 leaves from 2 plants were collected at the time indicated in each figure, rinsed with distilled water, then shaken in 25 ml distilled water on a rotary shaker at 100 rpm for 4 hrs at room temperature. The conductivity of the wash solution (μS/cm) was determined using a Corning 316 conductivity meter (Corning Inc. Big Flats, NY). The total ion content was obtained by determining the conductivity of the same
leaf-containing solution after autoclaving. The relative ion leakage was obtained by dividing the conductivity of solution before and after autoclaving and the values further normalized by dividing them by the value obtained with a control sample of leaves from plants not exposed to O₃.

**ROS Assays***

Frozen plant tissue was hand-ground with liquid nitrogen, the powder were weighed and immediately taken up in 10 mM Tris-HCl buffer (pH 7.3). The extract was centrifuged twice at 15000 rpm for 5min. ROS production was assayed at each time point after the addition of 100 mM 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA) in dimethyl sulfoxide (DMSO) to a final concentration of 10 μM. Fluorescence was quantified using a VersaFluor fluorometer (Bio-Rad, Hercules, CA). Total protein was quantified using a BioRad DC protein assay kit (Bio-Rad, Hercules, CA). The average fluorescence values obtained from three successive measurements were divided by the protein content and expressed as relative fluorescence units (RFU) per mg protein. These values were then expressed as a ratio of RFU obtained with O₃-exposed and control plants.

**RNA Preparation and Analysis**

Leaf tissues were flash-frozen in liquid N₂ immediately after removal from the plants and stored at –80°C. Total RNA was isolated using RNEasy plant RNA isolation

*: ROS assay experiments in this part were done by Junghee Joo.
kit (Qiagen, Valencia, USA). Ten micrograms of total RNA from control and O3-treated leaf tissue was fractionated on a 1.2% agarose/0.4 M formaldehyde RNA gel and transferred to Hybond N+ nylon membrane (Amersham-Pharmacia, Buckinghamshire, England). Membranes were stained with methylene blue to visualize the rRNA to confirm equal loading. Probes were cloned from Arabidopsis cDNA made as described before with primers GPA1 5’-ATGGGCTTACTCTGAGTA-3’ and 5’-CATAAAAGGC CAGCCTCCAGT3’; AGB1 5’-TCAAATCACTCTCCTGTGTCCTCC-3’ and 5’-TGTCTGTCTCCGAGCTCAAAGACG-3’; and labeled using the ReadyPrime DNA labeling kit (Amersham-Pharmacia, Buckinghamshire, England) with [α-32P]dCTP (MP Biomedicals Inc., Irvine, USA). Blots were hybridized and washed according to standard procedures.

Protein Analysis

Total proteins were extracted from frozen tissues using a buffer containing 50 mM Tris-HCl, pH 7.6, 1 mM EDTA, 1% Triton, 1% SDS, 5 mM DTT, 1 mM PMSF and quantified using the BioRad’s DC Protein Assay kit, which is compatible with Triton and SDS (Bio-Rad, Hercules, CA). Equal amounts of total membrane protein were loaded on a 12% polyacrylamide discontinuous gel (Biorad Mini electrophoresis system). After electrophoresis, proteins were transferred to Hybond-P PVDF membrane (Amersham, Piscataway, NJ) using a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad, Hercules, CA). Immunoblotting was performed with rabbit polyclonal anti-Gα antibody (kindly
provided by Dr. Alan Jones). After incubation with horseradish peroxidase-conjugated anti-rabbit IgG antibodies, proteins were detected using ECL Plus Western Blotting Detection Reagents (Amersham, Piscataway, NJ) according to the manufacture’s instruction.

Results

Altered Ozone Sensitivity in G protein Mutant Plants

The absence of gene transcript for AtGPA1 and AtAGB1 was confirmed by reverse transcriptase-mediated PCR in *gpa1-4, agb1-2* and *agb1-2/gpa1-4* double mutant plants, as shown in Fig.1A. We exposed wildtype and mutant plants to either 500 ppb (parts per billion) or 700 ppb (parts per billion) O$_3$ for 3 hours and then checked plants for visible lesions 24 hours after the onset of O$_3$ exposure. Selected plants were photographed (Fig. 1B) and the number of plants showing visible lesions was counted (Fig. 1C). The *gpa1-4* mutant plants showed less tissue damage than wildtype plants, while *agb1-2* mutant and double mutant plants showed more extensive tissue damage after either O$_3$ treatment. To quantify cell death after O$_3$ treatment, we monitored ion leakage, an indicator of tissue damage, immediately after O$_3$ treatment in wildtype and mutant plants (Fig. 1D). Thus, *agb1-2* mutant and double mutant plants are more sensitive to O$_3$ damage than wildtype plants; while *gpa1-4* mutant plants are less sensitive to O$_3$ damage.
Figure 1. Altered ozone sensitivity in G protein mutant plants
A. Reverse transcriptase–mediated PCR analysis of the AtAGB1 and AtGPA1 transcript in wildtype (wt), gpa1-4, agb1-2 and gpa1-4/agb1-2 mutant plants. B. Photographs of plants with and without leaf damage (red circle) after indicated O₃ treatment. C. The percentage of 4-week-old plants with visible lesions 24 h after a 3-h exposure to O₃ at the indicated concentrations. D. Ion leakage (see Methods and Materials) was assayed 24 h after a 3-h exposure of 4-week-old plants to indicated O₃ treatment (n=6).
Differential Expression Profile of GPA1 and AGB1 Gene after Ozone Treatment

Different functions of Gα and Gβ subunits in the oxidative stress response are suggested by the different transcription profiles of *AtGPA1* and *AtAGB1* genes over the course of the oxidative stress response to O₃. Following O₃ treatment for 6h at 350ppb, we monitored the *AtGPA1* and *AtAGB1* transcript level by Northern blotting and the AtGPA1 protein level by Western blotting. Blots were quantified using NIH Image software. Transcript levels of both the *AtGPA1* and *AtAGB1* genes increased rapidly in response to O₃, peaking at about 1 hr after the onset of O₃ exposure (Fig. 2A and 2B). The *AtGPA1* gene showed a second later increase in transcript level, which was not observed for the *AtAGB1* gene (Fig. 2A and 2B). Fig. 2C further showed that both the first and second peaks of *AtGPA1* transcript abundance were followed by increases in GPA1 protein levels, as detected by Western blotting with anti-GPA1 antibodies. Given the opposite phenotypes of the gpa1-4 and agb1-2 mutant plants with respect to O₃ damage, the differential expression profile of *AtGPA1* and *AtAGB1* gene after ozone treatment supported the concept that Gα and Gβ subunits play separable roles in the oxidative stress response to O₃.
Perturbed O₂-Induced Oxidative Burst in G protein Mutants

The oxidative burst is believed to be an important signal mediating gene expression, cell death and adaptive responses to protect plants from biotic and abiotic stress damage (Lamb and Dixon, 1994; Levine et al., 1994; Lamb and Dixon, 1997; Baker and Orlandi, 1995).

Figure 2. Different expression profiles of GPA1 and AGB1 genes after ozone treatment

Gα (A) and Gβ (B) transcript levels were measured by Northern blotting and blots were scanned and quantified using Image J. Gα protein (C) was detected by protein gel blotting with Gα antibodies; the blots were scanned, quantified using Image J and the values normalized by the amount present at the initial time point.
We measured the oxidative burst, predominantly H$_2$O$_2$, as catalase-inhibitable fluorescence using the dye 2′,7′-dichlorodihydrofluorescein diacetate (H$_2$DCFDA) as described in Materials and Methods. A 6-hr exposure of wildtype plants to 350 ppb O$_3$ evokes a biphasic oxidative burst with a first peak at about 1 hour of exposure, followed by a second possibly bimodal peak between 12 and 24 hrs after the onset of exposure (Fig. 3A). The first peak is markedly reduced and the late peak is totally absent in O$_3$-exposed $gpa1-4$ mutant plants (Fig. 3B). By contrast, while the first peak is missing in ozone-exposed $agb1-2$ mutant plants (Fig. 3C), the second peak is similar to that in wildtype plants. These observations imply that the early peak requires the intact heterotrimeric G protein or both G$\alpha$ and G$\beta$ subunits, whereas the late peak requires G$\alpha$ subunit but not G$\beta$ subunit or formation of the heterotrimer.
Enhanced Resistance to O₃ Damage by Lower Dose O₃ Pretreatment

It has been observed that ozone exposure induces resistance of *A. thaliana* to infection with virulent phytopathogenic *Pseudomonas syringae* strains (Sharma et al., 1996). This phenomenon is referred to as “cross protection” or “cross tolerance”. To determine if G protein signaling is attributed to enhanced O₃ tolerance by cross protection with lower dose O₃ pretreatment, we briefly exposed plants to 350 ppb O₃ (2 hours) and let them recover in the air for 2 hrs. We then exposed plants to 700 ppb O₃, which causes tissue damage, to examine whether they showed a higher tolerance to O₃. As shown in Fig. 3D, the pretreatment rendered wildtype plants, *agb1-2* and double mutant plants less sensitive to tissue damage compared with plants without pretreatment, although *gpa1-4* mutant plants exhibited little tissue damage with or without pretreatment because of their marked enhanced O₃ resistance (Figure 4). Thus, pretreatment with lower dose of O₃...
increased their ability to withstand higher dose of $O_3$, which suggested that biochemical or molecular changes that occurred within the first few hours after 350 ppb $O_3$ exposure play a role in eliciting protective responses from cell death. However, the $agb1-2$ and double mutant plants remained more sensitive to $O_3$ damage than wildtype plants even with pre-treatment, which suggested that $G\beta$ protein or the $G\beta\gamma$ complex contributes to the rapid enhancement of ozone tolerance observed following $O_3$ pretreatment. Given that $agb1-2$ and double mutant plants still showed some enhancement of $O_3$ tolerance after pretreatment, $G\beta$ protein or the $G\beta\gamma$ complex was not solely responsible for the development of $O_3$ tolerance.

![Graph showing relative ion leakage](image)

**Figure 4.** Enhanced resistance to $O_3$ damage by lower does $O_3$ pretreatment

Mutant and wild-type plants (4 weeks old) were exposed to $O_3$ 350ppb for 2 h, transferred to $O_3$-free air for 2 h, and then exposed to 700 ppb ozone for 3 h, following which leaves were collected and assayed for ion leakage as described in Methods and Materials ($n=6$).
Discussion

The results show that Ga and Gβ subunits of the *Arabidopsis* heterotrimeric G protein are required for development of the first component of the oxidative burst. On the other hand, the absence of the early ROS peak, which is present in O3-exposed wildtype plants, in the gpa1-4 and the agb1-2 mutant plants indicates that ROS derived from O3 and dissolved in the apoplast is not the source of ROS burst and it can not activate endogenous ROS production without the heterotrimeric G protein. Thus, exposure to external ROS requires G protein to transduce or amplify signals to further activate the endogenous ROS-generating system. This raises an intriguing question about how the heterotrimeric G protein is activated by extracellular ROS and how it activates the intracellular ROS-generating systems.

The extracellular ROS can activate *Arabidopsis* heterotrimeric G protein either directly or indirectly. Two mammalian Ga proteins, Gai and Gao, are ROS targets and directly regulated by H2O2 (Nishida et al., 2000; Nishida et al., 2002). H2O2 treatment increases the GTP affinity of the Ga subunit in a dose-dependent manner and activates Gβγ signaling and its downstream effectors (Nishida et al., 2000). Cys287 in Gai and Gao (Nishida et al., 2002), which is required for their ROS activation, is conserved in the AtGa1 protein (Ma et al., 1990). Thus it is possible that the *Arabidopsis* G protein itself is redox regulated. However, we can not exclude the possibility of the indirect activation of
G protein by the extracellular ROS. It could be a G protein receptor or another interacting protein that is oxidized to activate G protein signaling for the ROS-generating system.

A small GTPase has been implicated in the downstream of the heterotrimeric G protein in the activation of membrane bound NADPH oxidase in the pathogen response of rice (Suharsono et al., 2002). Phospholipase Dα (PLDα), which hydrolyzes phospholipids into free head groups and phosphatidic acid (PA), is another potential intermediate molecule for ROS production mediated by G protein. Phosphatidic acid (PA) as a lipid messenger has been shown to promote the production of superoxide, which is decreased in the PLDα mutant plant (Sang et al., 2001). Moreover, the direct effect of Gα on the activity of PLDα has been demonstrated by their physical interaction (Zhao and Wang, 2004). Since no downstream targets of Gβγ signaling have yet been identified in plants, it is hard to rationalize the role of Gβγ in activating the component of ROS burst.

In addition to their role in ROS production in the oxidative stress response, Gα and Gβ subunits of the heterotrimeric G protein may serve different functions in cell death associated oxidative stress response to O₃. The observation that the late peak of ROS occurs at 20 h after the onset of O₃ exposure and is coincident with cell death as well as the development of large lesions in plants, allows us to hypothesize that the late peak mediated by Gα/NADPH-oxidase mainly contributes to cell death.

Although the resistance of gpa1-4 mutant plants to O₃ damage may be attributable to the absence of the late ROS peak, what causes the hypersensitive phenotype of the agb1-2 mutant and double mutant to O₃ damages is still unclear. Nonetheless, the
phenotype of the double mutant is similar to that of the *agb1-2* single mutant, suggesting that Gβ act predominantly in cell death associated oxidative stress response to O₃. The opposite phenotypes of the *gpa1-4* and *agb1-2* mutants have been seen in other biological process. For example, *agb1-1* mutant plants have increased apical dominance whereas *gpa1-1* mutant plants have decreased apical dominance. Moreover, *agb1-1* and *agb1-2* have more root mass than wild-type, whereas *gpa1-1* and *gpa1-2* have less root mass than wild-type (Ullah et al., 2003). However, similar phenotypes of the *gpa1-4* and *agb1-2* mutants at various developmental stages are also found. For example, fruit and seed weights are greater for both mutants than wildtype plants. Since we know little about the action of heterotrimeric G protein in *Arabidopsis*, it is hard to predict how Ga and Gβγ subunits play common and separable roles in their heterotrimeric complex or what causes them dissociate from complex to interact with their downstream effectors.
References


Chapter 3

BOTH HETERO TRIMERIC G PROTEIN SIGNALING AND ROSIGNALING ARE IMPLICATED IN THE ARABIDOPSIS UNFOLDED PROTEIN RESPONSE

Abstract

We present evidence that heterotrimeric G protein signaling and ROS signaling are involved in UPR-associated cell death in Arabidopsis. Seedlings of agb1-2 mutant plants with a null mutation in the gene coding for the β subunit of the heterotrimeric G protein are more resistant to growth inhibition by the protein glycosylation inhibitor tunicamycin (Tm) than wildtype plants and gpa1-4 plants with a null mutation in the gene encoding the α subunit of the heterotrimeric G protein. Leaves of agb1-2 mutant plants exhibit markedly less cell death after Tm treatment than those of wildtype plants. The transcriptional response of agb1-2 mutant plants to Tm is less pronounced than that of wildtype plants. On the other hand, AtrbohD (AtrbohD null mutation) and AtrbohD/F (AtrbohD and AtrbohF null mutation) mutant plants show more cell death in response to treatment with Tm than wildtype plants and AtrbohF (AtrbohF null mutation) mutant plants. The protective role of the endogenous ROS elicited by Tm treatment against Tm-induced cell death is also seen using ROS scavengers. Moreover, the transcriptional response is delayed by the pretreatment with ROS scavengers.

A majority of the Arabidopsis Gβ protein is associated with the endoplasmic
reticulum (ER) and is degraded after Tm treatment, while Gα protein is not. Collectively, these observations indicate that the Gβγ complex, not Gα, plays an important role in cell death associated with the unfolded protein response in Arabidopsis. Moreover these observations imply a direct role for both ROS and G protein signaling in the UPR.

**Introduction**

The endoplasmic reticulum (ER) is the organelle in which secretory proteins are synthesized, modified and delivered to their target sites. ER stress occurs when protein folding, modification and transportation are perturbed. Cells from ER stress activate a number of biochemical changes termed the unfolded protein response (UPR) (Schroder and Kaufman, 2005; Wu and Kaufman, 2006).

In mammalian cells, the three distinct protective responses of the UPR are believed to be activated by ER-resident IRE1 (inositol-requiring transmembrane kinase and endonuclease 1), ATF6 (activation of transcription factor 6) and PERK (protein kinase–like ER kinase) respectively (Schroder and Kaufman, 2005). These three proteins are bound to the ER-resident luminal binding protein (BiP) under resting conditions. When the ER is stressed, BiP preferentially binds to the unfolded proteins, effectively being titrated away from IRE1, ATF6 and PERK. The dissociation from BiP activates PERK, which phosphorylates the translation elongation factor eIF2α and furthermore inhibits translation of most new proteins (Bertolotti et al., 2000; Liu et al.,
After dissociation from BiP, ATF6 is translocated to the Golgi and cleaved by S1/S2 protease to form active ATF6, which is a transcription factor promoting chaperone gene expression (Li et al., 2000; Wang et al., 2000). Upon its release from BiP, IRE is activated by dimerization and auto-phosphorylation. The active IRE cleaves the precursor of X-box binding protein from XBP1u mRNA to mature XBP1s mRNA, which is then translated into a transcription factor to induce the expression of genes involved in degradation of unfolded proteins (Yoshida et al., 2001). In addition to these pathways, the UPR also enhance ER-associated protein degradation (ERAD) to remove unfolded or misfolded proteins from their target sites (Kruse et al., 2006). Perturbation of any of these processes in the UPR can cause cells or organisms to be susceptible to ER stress and cell death, which is involved in certain diseases such as diabetes, Alzheimer’s, atherosclerosis, and cancer (Wu and Kaufman, 2006).

Although the study of the UPR and ER stress in plants is not as extensive as that in animals, it has been shown that plants have a protective transcriptional response when exposed to ER stress, including induction of genes encoding protein-folding enzymes and chaperones (Martinez and Chrispeels, 2003; Kamauchi et al., 2005). Both tunicamycin (Tm), an inhibitor of N-linked protein glycosylation and cyclopiazonic acid (CPA), an inhibitor of the ER-type II Ca\(^{2+}\) pump, can trigger the UPR and programmed cell death in cultured plant cells (Zuppini et al., 2004). However, only a few orthologs of mammalian UPR proteins have been identified so far in plants. These are Ire1 (Noh et al., 2002), eIF2\(\alpha\) (Chang et al., 1999; Chang et al., 2000) and p58\(^{IPK}\) (Bilgin et al., 2003). The
Arabidopsis genome has no orthologs of the ER stress-induced XBP1 or ATF6 genes, although other transcription factors like bZIP60 (Iwata and Koizumi, 2005) and NTM1 (Kim et al., 2006) contain a trans-membrane domain, which can be cleaved by a peptidase and hence may function in similar manner as ATF6.

Plants respond to both biotic and abiotic stress by producing ROS (reactive oxygen species), which are believed to be a fundamental part of intracellular signal transduction and intercellular cell communication (Fedoroff, 2006). The redox regulation of the transcriptional regulatory factor NPR1, which promotes UPR gene expression in systemic acquired resistance (SAR), appears to suggest an interconnection between ROS homeostasis and UPR induction in plants (Mou et al., 2003; Wang et al., 2005). Moreover, both ROS signaling and UPR have been demonstrated to be a critical part of the plant’s defense response (Fedoroff, 2006). However, the functional interaction between them has not yet been investigated.

We have reported that the Gα and Gβ subunits serve both separable and synergistic functions in signaling by reactive oxygen species (ROS) in the oxidative stress response to O₃ (Joo et al., 2005). The Arabidopsis genome encodes a single canonical heterotrimeric G protein and one α subunit (Ma et al., 1990), one β subunit (Weiss et al., 1994), and two γ subunits (Mason and Botella, 2000, 2001). Here we show that signaling through the Gβ subunit of heterotrimeric G protein plays a direct role in ER-stress associated cell death and the biphasic oxidative burst induced by Tm exerts a protective effect against ER-stress associated cell death. We show that plants homozygous for a null
mutation of the Gβ subunit of heterotrimeric G protein are substantially more resistant to Tm-induced cell death than either wildtype plants or plants homozygous for a null mutation of the Ga subunit. We also show that the transcriptional response of the agb1-2 mutant plants (Gβ null mutation) is reduced and delayed compared with that of wildtype plants and that the large protein aggregates characteristic of the ER stress are not observed in mutant plants. We show that interference with ROS production by a flavin oxidase inhibitor, a ROS scavenger or mutations that affect the AtrbohD and AtrbohF genes increases the sensitivity of Arabidopsis plants to Tm-induced cell death and delays the expression of UPR chaperones and other markers. A majority of the Gβ protein is detected in the ER, judged by ER marker protein BiP. Moreover, the Gβ protein is degraded during the UPR. These observations strongly suggest the Gβ subunit of the heterotrimeric G protein plays a direct role in cell death signaling in the UPR.

**Methods and Materials**

**Plant Materials, Growth Conditions, and Tm Treatment**

We used *Arabidopsis thaliana* Col-0 plants, homozygous *agb1-1* (Lease et al., 2001), *agb1-2*, *gpa1-4*, and *agb1-2/gpa1-4* double mutants (Ullah et al., 2003), all characterized as transcript-null mutants in the Col-0 background and *atrbohD*, *atrbohF*, and *atrbohD/F* double mutants in the Col-0 background (Torres et al., 2002). Plants were grown in MetroMix 200 (Scotts-Sierra Horticultural Products Company, Marysville, OH)
in 5-cm pots at 65% humidity under fluorescent light at 30 W/m²/s with 14-h light/10-h dark photoperiod for 5 weeks. Five rosette leaves of similar size were infiltrated sub-epidermally on lower surface with 15-30 μg/ml Tm (Sigma-Aldrich, St. Louis, MO) in 1.6% DMSO using a needleless syringe. Controls were infiltrated with the same DMSO solution lacking Tm. Where indicated, plants were pre-treated with 20 μM diphenyleneiodonium sulfate (DPI) (Sigma-Aldrich), a flavin oxidase inhibitor, or 2 mM N-acetylcysteine (NAC) (Sigma-Aldrich), reactive oxygen species (ROS) scavenger, for 4 hours prior to Tm injection either by watering.

Quantification of Leaf Senescence and Cell Death.

Chlorophyll degradation, an indicator of leaf senescence, was measured 8 days after Tm injection as described (Arnon, 1949). The chlorophyll measurement was normalized to the wet leaf weight. Release of electrolytes was used to quantify cell damage and death. To measure electrolyte release, 5 injected leaves from a single plant were collected each day after inoculation and shaken in 15 ml distilled water at 100 rpm for 4 h at room temperature. The conductivity of the wash solution (mS/cm) was determined using a portable conductivity meter (Control Company, Friendswood, TX). The total electrolyte content was obtained by measuring the same leaf-containing solution after autoclaving. Six replicates were averaged to calculate the final fraction of electrolytes released as a fraction of the total electrolyte content. Relative electrolyte leakage is the ratio of the fraction obtained with leaves of treated plants to that obtained with leaves from untreated
control plants. Cell death was detected histochemically by trypan blue staining as described (Pasqualini et al., 2003).

RNA Isolation and Northern Blot Hybridization

Leaves were fresh frozen in liquid N\textsubscript{2}. Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA). Ten micrograms of total RNA from control and Tm-injected leaf tissue were fractionated on a 1.2% agarose/0.4 M formaldehyde gel and transferred to Hybond N\textsuperscript{+} nylon membrane (Amersham-Pharmacia, Piscataway, NJ). Membranes were stained with methylene blue to visualize rRNA as loading controls. Probes were cloned from Arabidopsis thaliana Col-0 cDNA, which was made as previously described with the following gene-specific primers Bip3: 5'-ATGATTTTTATCAAGGAAAACACAGCG-3 and 5'-TTACCAAGGGCTTTGTGATCC; Bip1&2: 5'-GGCTCGCTCGTTTGGACAA-3 and 5'-CCGTTATCAATGGTCAAGACACT-3; PDI: 5'-ATGGCGAAATCTCAGATCTGGTT-3 and 5'-GTCCCTGCTGGTCCCAGATTT-3. The probes were labeled using the ReadyPrime DNA labeling kit (Amersham-Pharmacia) with [$\alpha^{32}$P]dCTP (MP Biomedicals Inc., Irvine, CA). Blots were hybridized and washed according to manufacture’s instructions (Sigma-Aldrich).

ROS Assays

Frozen plant tissues were hand-ground with liquid nitrogen immediately after Tm treatment, the powder were weighed and immediately taken up in 10 mM Trs-HCl buffer
(pH 7.2). The extract was centrifuged twice at 15,000 rpm for 5 min. ROS production was assayed at each time point after the addition of 100 mM 2',7'-dichlorodihydrofluorescein diacetate (H$_2$DCFDA) in dimethyl sulfoxide (DMSO) to a final concentration of 10 μM. Fluorescence was quantified using a VersaFluor fluorometer (Bio-Rad, Hercules, CA). Total protein was quantified using a BioRad DC protein assay kit (Bio-Rad, Hercules, CA). The average fluorescence values obtained from three successive measurements were divided by the protein content and expressed as relative fluorescence units (RFU) per mg protein. These values were then expressed as a ratio of RFU obtained with Tm infiltrated and control plants.

**Cellular Membrane Fraction**

Five week-old transgenic plants containing a CFP-Gβ fusion protein expressed from the AGB1 promoter were homogenized using buffer containing 50 mM Tris-MES, pH 7.5, 1 mM dithiothreitol, 5 mM EDTA, 0.3 M sucrose and protease inhibitors. The homogenate was filtered through a double layer of Miracloth (EMD Biosciences, Inc. La Jolla, CA) and centrifuged at 3,200xg for 10 min (JA 20, Beckman Coulter, Inc. Fullerton CA). The supernatant was further pelleted by centrifugation at 100,000xg (TY70 Ti, Beckman) to obtain microsomal membranes, which were then subjected to aqueous two-phase partitioning (Chen et al., 2002; Kato et al., 2004). The microsomal pellet was suspended in a solution containing 0.3 M sucrose, 3 mM KCl, and 5 mM KH$_2$PO$_4$ and adjusted to 6.4% (w/w) each of Dextran T500 and PEG3350. The phases were separated...
by centrifugation at 750xg (Thermo IEC 243) and the phase partitioning was repeated 3 times. The upper and lower phases were diluted separately with buffer containing 0.3 M sucrose, 3 mM KCl, 1 mM EDTA and centrifuged at 100,000g (TY70 Ti, Beckman) to obtain plasma membrane (Pm) and intracellular membrane (Im) fractions. The Pm fraction was dissolved in SDS-PAGE sample buffer for analysis and Im fraction was re-suspended in buffer containing 25 mM Tris (pH 7.5), 10% sucrose, 1 mM dithiothreitol, 2 mM EDTA, and protease inhibitors and sedimented through 10-50% sucrose density gradient at 100,000xg for 16 h (SW28 3707, Beckman). Fractions (0.8 ml) were collected, concentrated using StrataClean Resin (Stratagene, Cedar Creek, TX) and dissolved in SDS-PAGE sample buffer for analysis. Isolated membrane fractions were loaded on a 10% polyacrylamide discontinuous gel (Bio-Radmini Electrophoresis System). After electrophoresis, proteins were transferred to Hybond-P PVDF membrane (Amersham, Piscataway, NJ) using a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad). Immunoblotting was performed as described below.

Protein Analysis

Total proteins were extracted using extraction buffer (50mM Tris-HCl, pH 7.6, 1 mM EDTA, 1% Triton, 2% SDS, 5 mM DTT, and 1 mM PMSF) and quantified using Bio-Rad’s DC protein assay kit, which was compatible with Triton and SDS. Equal amounts of total membrane protein were loaded on a 12% polyacrylamide discontinuous gel (Bio-Radmini electrophoresis system). After electrophoresis, proteins were
transferred to Hybond-P PVDF membrane (Amersham, Piscataway, NJ) using a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad). Immunoblotting was performed with goat polyclonal anti-BiP antibodies (1:250, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and horseradish peroxidase–conjugated anti-goat IgG antibodies (Santa Cruz Biotechnology) for BiP protein analysis. The immunoblot was incubated with mouse monoclonal anti-CFP antibodies (1:2000, BD Bioscience, Mountain View, CA) and horseradish peroxidase–conjugated anti-mouse IgG antibodies for AtAGB1 protein level measurement. The above blot was striped and probed with rabbit polyclonal anti-Gα antibodies and horseradish peroxidase–conjugated anti-rabbit IgG antibodies (Invitrogen). Proteins were detected with ECL Plus protein gel blotting detection reagent (Amersham) according to the manufacturer’s instructions.

Results

Plants Lacking the Heterotrimeric Gβ Subunit are Tm-Resistant.

To compare the Tm sensitivity of G-protein mutants with Arabidopsis wildtype plants, seeds of plants homozygous for null mutations in the genes encoding the Gα and Gβ subunits, as well as doubly mutant plants, were plated on Tm-containing medium for 6 days, then transferred to tunicamycin-free agar medium for 10 days. Tm inhibited the growth in gpa1-4 (Gα null mutation) seedlings and wildtype seedlings, while homozygous agb1-2 (Gβ null mutation) seedlings and doubly mutant gpa1-4/agb1-2
seedlings were much less sensitive than either wildtype or \textit{gpa1-4} seedlings (Fig. 5).

To observe the effect of Tm on adult plants, rosette leaves of 5 weeks plants were infiltrated sub-epidermally on the lower surface by Tm. Wildtype plants infiltrated with Tm showed large areas of leaf senescence and cell death by 6 days after injection (Fig. 6A), whereas, leaves of \textit{agb1-2} plants showed small areas of damage (Fig. 6A). Close-up views also showed larger areas of tissue collapse and cell death in injected wildtype leaves than \textit{agb1-2} mutant leaves, which have restricted damaged areas around the injection site (Fig. 6B). Vital dye staining with Trypan blue also revealed much larger areas of dead cells in wildtype than in \textit{agb1-2} mutant leaves (Fig. 6B). Similar results were obtained with leaves of plants homozygous for a second mutant allele, \textit{agb1-1}, of the gene coding for the Gβ subunit of the heterotrimeric G protein (data not shown).

\textbf{Figure 5.} Plants homozygous for null mutations in genes coding for the \(\alpha\) and \(\beta\) subunits of the heterotrimeric G protein exhibited different sensitivities to Tm induced growth inhibition. Homozygous \textit{gpa1-4} (G\(\alpha\) null mutation), \textit{agb1-2} (G\(\beta\) null mutation), and \textit{gpa1-4/agb1-2} double mutants were germinated on agar medium containing 0.3 \(\mu\text{g/ml}\) Tm for 6 days, then transferred to medium without Tm, allowed to recover for 10 days, and photographed.
A. Figure 6. Leaf senescence and cell death in wildtype and agb1-2 mutant plants
A 15 μg/ml Tm solution in 1.6% DMSO was infiltrated into leaves as described in Methods. Plants were photographed at 6 days after infiltration (A) and leaves were photographed at 8 days (B, left panel) or cleared and stained with trypan blue (B, right panel); leaves were harvested for daily to determine electrolyte release as described (C); for determination of chlorophyll content at 8 days (D).
Cell damage was also quantified by measuring the release of intracellular electrolytes to the extracellular space and leaf senescence was measured by loss of chlorophyll. Wildtype leaves showed a transient increase in the electrolyte release a day after Tm treatment, recovered transiently, then showed a progressive increase in the release of intracellular electrolytes as the area of leaf damages enlarges (Fig. 6C), while homozygous *agb1-2* mutant plants only exhibited a little enhanced ion leakage after Tm treatment. Similarly, wildtype leaves exhibited much more extensive loss of chlorophyll than leaves of *agb1-2* mutant plants (Fig. 6D). Thus, plants lacking β subunit of the heterotrimeric G protein are more resistant to Tm-induced cell death than wildtype plants, suggesting that Gβ subunit plays a role in cell death response induced by ER stress.

The Transcriptional and Translational Response to Tm is Muted in Gβ Mutants

The induction of several characteristic ER stress markers occurred in wildtype and *agb1-2* mutant plants after Tm treatment. The abundance of BiP transcripts, particularly for BiP3, increased rapidly, peaking at about 3 hrs after Tm infiltration. The abundance of PDI transcripts also increased, but peaks later at 6 hrs after Tm infiltration (Fig. 7A). Both BiP and PDI mRNA levels declined at 9 hrs and then increased again at 12 hrs. Although BiP and PDI transcript levels increased in *agb1-2* mutant plants after Tm infiltration, they increased more slowly than in wildtype plants and did not reach the levels observed in wildtype plants. Moreover, BiP proteins did not accumulate to the same levels in the *agb1-2* mutants as in wildtype plants (Fig. 7B). Large aggregates
migrating more slowly than the BiP monomers can be detected in extracts from wildtype plants, but are much less prominent in *agb1-2* mutant plants. Thus the transcriptional and translational responses to Tm-induced ER stress were much less marked in *agb1-2* mutant plants than in wildtype plants.

![Figure 7. The Tm-induced UPR is attenuated in *agb1-2* mutant plants.](image)

A. Northern blot analyses of Col-0 and *agb1-2* mutant plants treated with 15 μg/ml Tm. Total RNA was extracted at the indicated times and 10 μg total RNA was loaded into each lane. UPR marker probes are indicated on the right. 25s rRNA was used as internal loading control. B. Western blot analysis of Col-0 and *agb1-2* mutant plants treated as in A. Total protein was extracted at the indicated times and 30 μg of protein was loaded into each lane. Anti-AtBiP1&2 was used to probe the membrane.
Tm Induces an Oxidative Burst, Perturbation of which Delays Expression of UPR Marker Genes

The production of ROS is induced by a variety of biotic and abiotic stressors. To determine whether Tm-induced ER stress can trigger ROS production, we measured H$_2$O$_2$ level after Tm treatment. Our results showed that Tm induced a biphasic oxidative burst in Arabidopsis leaves (Fig. 8A). The Tm treatment promoted a rapid transient increase in H$_2$O$_2$ level which subsided by 3 hours, then increased again at 6 hrs and declined at 9 hrs after the onset of treatment.

To further evaluate the role of ROS in UPR-associated gene expression, we inhibited ROS production with the flavin oxidase inhibitor diphenylene iodonium (DPI) by pretreatment before Tm application and examined the effect of the inhibitor on gene expression in the UPR. Our results showed that inhibition of ROS production delayed and attenuated the increase in the expression level UPR marker genes (Fig. 8B). Early induction of BiP1&2, BiP3 and PDI mRNA levels at 3 hrs after the Tm treatment was delayed until 6 hrs after Tm treatment in plants treated with the inhibitor. These observations indicated that ROS are required for the activation of UPR-associated gene expression in response to Tm.
**Figure 8.** Tm induces an oxidative burst and DPI delays UPR marker gene expression

A. ROS production was measured by H\textsubscript{2}DCFDA fluorescence in plants treated with 15 \(\mu\)g/ml tunicamycin (Tm). Fluorescence values were normalized to the protein content of the leaf extracts and expressed as a ratio (RFU) of the values obtained at the indicated times to the control value at 0 time. B. Northern analysis of UPR marker genes in response to Tm treatment with and without DPI pre-treatment. Wildtype (Col-O) plants were treated with buffer alone (control), or buffer containing 20 \(\mu\)M diphenylene iodonium (DPI), 15 \(\mu\)g/ml Tm (Tm); or both (DPI+Tm) 4 hrs. After pre-treatment, leaves of plants were infiltrated with Tm (15 \(\mu\)g/ml).

**Endogenous ROS Protect against Tm-Induced Tissue Damage and Cell Death.**

Endogenous ROS serve as both intra- and inter-cellular signals, as well as triggering cell death (Torres et al., 2002; Joo et al., 2005). To assess the role of ROS in the
UPR-associated cell death, we pre-treated plants with diphenylene iodonium (DPI), a flavin oxidase inhibitor, or N-acetyl cysteine (NAC), a ROS scavenger, for four hours before Tm injection and observed the phenotype after 3 days. We found the area of cell death around the site of Tm infiltration was substantially greater in leaves from plants pre-treated with either DPI or NAC (Fig. 9A). We quantified Tm-induced cell death by measuring electrolyte release. Electrolyte release from wildtype plants pretreated with either DPI or NAC was roughly four-fold greater after Tm treatment than from plants pretreated with the same buffer alone (Fig. 9B). Thus either inhibiting endogenous ROS production or suppressing ROS exacerbated Tm-induced cell death in wildtype plants, indicating that ROS serve a protective role in the UPR-associated cell death.

Figure 9. Suppressing ROS production increases Tm-induced cell damage and death
A. 5 week old plants were pre-treated either with buffer alone or buffer containing 20 μM DPI or 2 mM NAC, then leaves were injected with 15 μg/ml Tm. Control plants were pretreated with either buffer alone or with buffer containing either NAC or DPI, but not injected with Tm. After 3 days of treatment, photographs were taken. B. Electrolyte leakage was measured from leaves treated as above. The values reported are the ratio of the fraction of total ion content released by treated and control plants.
We have previously reported that both AtrbohD and AtrbohF contribute to cell death signaling mediated by ROS between cells in response to oxidative stress (Torres et al., 2002; Joo et al., 2005). To determine the role of ROS generated by AtrbohD and AtrbohF oxidase in the UPR-associated cell death, plants homozygous for the atrbohD mutation, the atrbohF mutation or doubly mutant atrbohD/F plants were infiltrated by Tm and cell death induced by Tm was quantified by electrolyte release. The atrbohD plants and doubly mutant atrbohD/F plants show much larger areas of cell death around the Tm injection site than wildtype plants and atrbohF single mutants (Fig. 10A). Electrolyte release from atrbohD mutant and doubly mutant atrbohD/F plants was only slightly higher than that from wildtype plants and atrbohF single mutants 3 days after Tm treatment. It then increased to a value about two-fold greater in atrbohD mutant and doubly mutant atrbohD/F plants than wildtype plants and atrbohF single mutants 7 days after Tm treatment (Fig. 10B). The susceptible phenotype of atrbohD mutant and doubly mutant atrbohD/F plants to Tm induced cell death indicated that intercellular ROS signaling prevents the over proliferation of UPR-associated cell death. Moreover, the major contributor to such signaling is the AtrbohD NADPH oxidase, not AtrbohF NADPH oxidase. Thus, both inhibitor studies and genetic evidence from mutants showed intracellular and intercellular ROS signaling play protective roles in the UPR-associated cell death.
β Protein is in the ER and Co-Fractionates with BiP.

Gα1 has been immunolocalized to the plasma membrane and endoplasmic reticulum before (Weiss et al., 1997). As shown in Chapter 4, the interaction between Gα1 and Gβ1γ1 complex in Arabidopsis mesophyll protoplasts were identified using fluorescence resonance energy transfer (FRET) and co-immunoprecipitation. Given the evidence that the Gβ protein is involved in the ER stress response, we asked whether the cellular localization is consistent with such a role. A CFP-Gβ fusion construct was transformed into agb1-2 mutant plants and found to complement its mutant phenotype, as judged by leaf morphology. Expression of the CFP-Gβ was controlled by its native promoter to

Figure 10. Tm-induced cell death was accelerated in atrobhD and atrobhD/F double mutant plants. A. Leaves of 5 week old wildtype (Col-O) plants, atrobhD, atrobhF and atrobhD/F mutant plants were injected with 15 μg/ml Tm and photographs were taken after 5 days. B. Cell damage was monitored by the release of electrolytes from leaves 3 days to 7 days following the injection.
ensure the same expression level of Gβ in transgenic plants as wildtype plants. A leaf extract was prepared and subjected to ultracentrifugation to obtain the microsomal fraction, which was then further purified by aqueous two-phase partitioning into plasma membrane and intracellular membrane fractions. The intracellular membrane fraction was further subjected to sucrose density gradient centrifugation to fractionate the Gβ protein. Both Ga and Gβ proteins were enriched in the microsomal fraction (Fig. 11A). The Ga protein was present at a slightly higher level in the plasma membrane than in the intracellular membrane fraction. In contrast, a majority of the Gβ protein was in intracellular membranes, although a significant fraction of it co-fractionated with the Ga protein in the plasma membrane (Fig. 11A). Thus, the Gβ protein was mainly associated with the ER, as judged by its co-sedimentation with the luminal marker protein BiP in the sucrose gradient, while the Ga protein equally distributed throughout the sucrose gradient (Fig. 11B). The accumulation of the Arabdiopsis Gβ protein in ER suggested that it may have a signaling function in the ER.

Gβ Protein is Degraded during the UPR.

ER-associated protein degradation is another hallmark of the UPR. To evaluate the degradation of Gβ protein during the UPR, we monitored the amount of Gβ protein over the course of Tm treatment. The abundance of Gβ protein started to decline on the first day after Tm treatment and continuously decreased to roughly 25% of its initial level by 4 days after Tm treatment (Fig. 12). In contrast, the amount of Ga protein did not decrease,
but rather showed a slight increase over the course of the 3 days following Tm treatment (Fig. 12). Hence, the decreased level of Gβ protein in the course of Tm treatment suggested that Gβ protein underwent ER-associated degradation during the Tm-induced UPR. The differential changes in Ga and Gβ protein levels in response to Tm further suggest that the Arabidopsis Ga and Gβ proteins have functions separable from those of the heterotrimeric G protein.

**Figure 11.** Gβ protein is localized in the ER and co-fractions with BiP.  
A. Aliquots of total cell extracts (T), soluble (S) supernatant and pelleted microsomal (M) fractions, as well as lower, intracellular membrane (Im) fractions and the upper, plasma membrane fraction (Pm) after aqueous two-phase partitioning were analyzed together by SDS-PAGE and Western blotting using anti-GPA antibodies to detect Gα protein, anti-CFP antibodies to detect the CFP-Gβ fusion protein in extracts of transgenic plants expressing a Gβ-CFP fusion protein from the Gβ promoter and anti-BiP antibodies for the ER markers BiP1. B. The Im fraction was sedimented through a 10-50% sucrose density gradient and aliquots of each fraction were analyzed by SDS-PAGE and Western blotting as in A.
Discussion

We have presented evidence that both signaling by the Gβ subunit of the *Arabidopsis* heterotrimeric G protein and ROS signaling are implicated in the UPR-associated cell death in response to Tm treatment. Gβ null mutant plants are much more resistant to Tm-induced cell death than either wildtype or Gα null mutant plants. This observation indicates that the Gβ subunit, but not the Gα subunit, plays a direct role in UPR-associated cell death. The cell death induced by Tm is accelerated by the application of DPI and NAC. Moreover, a mutation in the *AtrbohD* gene also exacerbates Tm induced cell death, which indicates that intracellular and intercellular ROS signaling play a protective role in UPR-associated cell death. The transcriptional response of Gβ mutant plants to Tm is much less pronounced than that of wildtype plants, which indicates that the Gβ subunit is directly involved in the UPR-associated gene expression. The administration of DPI delays the activation of the UPR-associated gene expression,
which indicates that ROS is required for the rapid activation of UPR-associated gene expression.

A significant fraction of the *Arabidopsis* Gβ protein is associated with ER membranes, indicating that the Gβ subunit may function directly in ER stress. Gβ protein is degraded during the UPR, presumably by ER-associated protein degradation, while Gα protein is not. Given the resistant phenotype of agb1-2 mutants to Tm induced cell death and the presence of a majority of the Gβ subunit in the ER, we conclude that signaling events mediated by Gβγ subunit, not Gα subunit, trigger UPR-associated cell death, which further supports the concept that Gβγ and Gα subunits serve independent functions in different biological processes.

Tm has been used widely to study the UPR in mammalian cells (Shen et al., 2004). Using Tm, we have demonstrated that ER stress can cause leaf senescence in plants. Senescence, as the last developmental stage of plants, is defined as a specialized plant programmed cell death (PCD) (Gepstein, 2004). It is well documented that ER stress can activate PCD in mammals to eliminate severely damaged cells (Shen et al., 2004; Wu and Kaufman, 2006), which suggests that PCD induced by ER stress is conserved in plant and animal systems. The phenotypic characterization of cell death in *Arabidopsis* plant leaves during Tm-induced ER stress could be used as a tool to further evaluate and identify the essential components in plant UPR.

Thapsigargin (Tg), which depletes the cell’s energy stores for protein folding (Thastrup et al., 1990), and dithiothreitol (DTT) (Jamsa et al., 1994), which creates
reductive stress in the ER, were also tried to induce plant ER stress. We found that their effects are different from that of Tm with respect to leaf senescence. DTT caused leaf necrosis quickly after the injection (data not shown) and Tg induced only slight leaf senescence (data not shown) at hundreds of times higher concentrations than used in mammalian cells. This may be due to the impermeability of plant cells to this compound.

Previous studies have shown that the *Arabidopsis* Gβ protein plays important roles in root growth (Ullah et al., 2003), leaf development (Lease et al., 2001), ozone stress response (Joo et al., 2005) and pathogen response (Llorente et al., 2005). The results of the present study establish a connection between signaling by the Gβγ complex and the UPR. Although several downstream effectors of the Ga subunit have been identified, such as phospholipase Da in *Arabidopsis* (Zhao and Wang, 2004) and the small GTPase Rac1 in *Rice* (Suharsono et al., 2002), no downstream targets of Gβγ signaling have yet been identified in plants. Moreover, there are no other proteins or signaling identified to be involved in plant UPR. Thus, it is hard to predict how Gβγ signaling mediates the UPR in *Arabidopsis*.

Gβγ signaling is better understood in animals than in plants. Some signaling pathways mediated by Gβγ are conserved in animals and plants (Assmann, 2002). Hence knowledge for animals may give some hints for the role of *Arabidopsis* Gβγ signaling in the UPR. In animal cells, Gβγ is a central component of phosphoinositide signaling through its ability to activate phospholipase Cβ2 (PLCβ2) and PLCβ3 to generate the second messengers diacylglycerol and inositol (1,4,5) triphosphate (IP3), which further
interact with receptors to trigger Ca\(^{2+}\) release from the ER (Feng et al., 2005; Bonacci et al., 2006). This signaling pathway which originates from G\(\beta\gamma\) to IP\(_3\), resulting in Ca\(^{2+}\) release is involved in many physiological responses, such as ER stress responses, apoptotic and necrotic cell death (Lin et al., 1997; Kim et al., 2001; Xu et al., 2001). Nonetheless, whether this signaling pathway is involved in UPR-associated cell death in animal cells has not been directly investigated. Although the connection between the G\(\beta\gamma\) complex, IP\(_3\) production and Ca\(^{2+}\) release has been established in Arabidopsis, PLC activation, IP\(_3\) production, and Ca\(^{2+}\) spikes are all rapid responses to some biotic and abiotic stresses in Arabidopsis (Price et al., 1994; Knight et al., 1996, 1997). Moreover, Ca\(^{2+}\) release is unequivocally involved in programmed cell death triggered by different stresses in Arabidopsis (Davies et al., 2006; Ali et al., 2007). Hence, it is possible that PLC or perhaps ER IP\(_3\) receptors are G\(\beta\gamma\) complex effectors that contribute to Ca\(^{2+}\) releases to mediate UPR-associated cell death in Arabidopsis.

As important a signaling molecule as calcium, ROS also plays critical roles in Tm-mediated plant ER stress response. Oxidative stress and ER stress have been investigated separately, although there is increasing evidence that they are interconnected with respect to cellular environment and stress responses (Fedoroff, 2006). ROS may function in one of the following ways to regulate ER stress response. First, the ER strictly requires a reducing environment for protein folding, disulfide bond formation and some post-translational protein modifications. Any perturbation of this reducing environment in the ER prevents proper folding of new synthesized proteins. Thus, excess ROS could
cause ER stress or aggravate ER stress (Schroder and Kaufman, 2005). However, our results show that elimination of ROS exacerbates Tm-induced cell death, indicating that ROS serve a protective role in the normal UPR in *Arabidopsis*.

On the other hand, ER stress can disrupt the electron transfer chain in protein disulfide bond formation, which results in the generation of ROS (Schroder and Kaufman, 2005). ROS could act on chaperone proteins to regulate their biological activity (Jakob et al., 1999; Papp et al., 2003). Heat shock factor (Hsf1) belongs to a highly conserved eukaryotic transcription factor family, which induces the expression of a variety of heat shock proteins in response to heat, oxidative stress and a variety of other stresses. The DNA binding activity of Hsf1 is directly promoted by oxidation with H$_2$O$_2$ (Jacquier-Sarlin and Polla, 1996; Mishra et al., 2002). *Arabidopsis* contains heat shock factors, some of which have been identified as potentially redox-regulated (Baniwal et al., 2004). Activation of chaperone proteins by ROS could explain the effect of ROS scavengers on the acceleration of cell death induced by Tm because suppressing ROS concurrently deactivates chaperone proteins, resulting in less protective responses to ER stress. However, some ER stress related proteins, such as ATF6, are activated by reduction instead of oxidation (Nadanaka et al., 2007). It is hard to conclude that total activity of chaperones is higher in oxidative condition or reducing condition. Thus, ROS may also act on other signaling pathways to manipulate cell death-associated UPR in *Arabidopsis*.

There is an apparent paradox between the protective role of ROS in cell death
associated UPR based on our observation and long-held belief that ROS initiated or spreaded cell death in the pathogen and ozone response. In previous studies, either in the oxidative stress response or pathogen response in *Arabidopsis*, extracellular ROS produced by the *AtrbohD*- and *AtrbohF*-encoded NADPH oxidases contributed to cell death, since mutation of *AtrbohD* and *AtrbohF* rendered resistance to both responses (Torres et al., 2002; Joo et al., 2005). However, a recent study showed that cell death in null mutant plants with *lsd1* mutation, which was identified by its “runaway cell death,” phenotype in oxidative stress to superoxide (Jabs et al., 1996), was exacerbated by the mutation of *AtrbohD* and curtailed by overexpression of *AtrbohD* in pathogen response (Torres et al., 2005), suggesting ROS can play a protective role, limiting the spread of cell death in the pathogen response. However, how ROS signals are integrated and able to commit cells to survival or death individually is not clear yet.
References


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Chapter 4

CHARACTERIZATION OF THE ARABIDOPSIS HETEROTRIMERIC G PROTEIN COMPLEX

Abstract

Using fluorescence resonance energy transfer (FRET) and co-immunoprecipitation, we show interaction between the β1 and γ1 subunits and between the α1 and β1γ1 subunits of the Arabidopsis heterotrimeric G proteins in plant mesophyll protoplasts. We report that both the Gα1 and the Gβ1 subunits are associated with macromolecular complexes in plasma membrane fractions. We estimate the size of the G protein containing plasma membrane complex to be approximately 669 kD based on blue native polyacrylamide gel electrophoresis, suggesting that the heterotrimeric G protein is present in a complex with other proteins. In plants homozygous the agb1-2 Gβ1 null allele, Gα1 is present in both the large ca. 669 kD complex and in smaller complexes. Deletion of the Gβ1 interacting domain in Gα1, ahGα1, abolishes normal function of Gα1 in the oxidative stress response to ozone, suggesting the interaction between Gα1 and Gβ1γ complex may be required for transmitting the ozone signal in plant cells. Activation of Gα1 subunit by GTPγS does not result in complete dissociation Gα1 subunit from its complex. Treatment of the plasma...
membrane fraction with hydrogen peroxide (H$_2$O$_2$), an important signaling molecule, results in the partial dissociation of the G$\alpha_1$ complex, suggesting direct or indirect activation of G-protein signaling by ROS in the oxidative stress response.

**Introduction**

The results of both pharmacological and genetic studies indicate that the plant heterotrimeric G protein is involved in the transmission of different kinds of signals, including light (Warpeha et al., 1991) and hormones (Bowler et al., 1994), as well as in the regulation of ion channels (Wu and Assmann, 1994). The Arabidopsis gpa1 mutant, which lacks the G$\alpha_1$ protein (AtGPA1), exhibits reduced cell division during hypocotyl and leaf formation (Ullah et al., 2001), while overexpression of AtGPA1 causes ectopic cell division, including massive meristem proliferation (Okamoto et al., 2001). Homozygous gpa1 mutant plants show reduced sensitivity than wildtype plants to ABA inhibition of stomatal opening and inward K$^+$ channel regulation (Wang et al. 2001), although gpa1 mutant seeds exhibit wild-type sensitivity to ABA inhibition in germination (Ullah et al., 2002; Pandey and Assmann, 2004). In addition, gpa1 mutant plants are not only less sensitive to gibberellic acid, but completely insensitive to brassinosteroids (BR) (Ullah et al., 2002). Arabidopsis agb1-2 mutant plants, which lack the G$\beta_1$ protein (AtAGB1), show alterations in leaf, flower and fruit development as well as cell division in the hypocotyl and are D-glucose hypersensitive (Lease et al., 2001;
Evidence is also accumulating that the heterotrimeric G protein plays a role in the plant response to bacterial and fungal pathogens, as well as abiotic stress. Heterotrimeric G protein signaling to membrane-bound NADPH oxidase has been implicated in the development of disease resistance and in the apoptotic hypersensitive response in rice (Suharsono et al., 2002). A signal perception role of heterotrimeric G protein in necrotrophic fungus responses is also suggested by the enhanced susceptibility phenotype of agb1-1 mutant plant to Plectosphaerella cucumerina (Llorente et al., 2005). Moreover, it was reported that the Gα1 and Gβ1 subunits serve both separable and synergistic functions in signaling by reactive oxygen species (ROS) in the oxidative stress response (Joo et al., 2005). An independent role for the Gβ1γ complex of the heterotrimeric G protein in cell death signaling in the unfolded protein response has been reported in Arabidopsis.

Contrary to the large number of different G protein subunits in animals, Arabidopsis has only one Gα protein (Ma et al., 1990), one Gβ protein (Weiss et al., 1994), and at most two Gγ proteins (AtAGG1 and AtAGG2) (Mason and Botella, 2000, 2001). All of them exhibit limited homology with their animal counterparts. The AtGPA1 protein is roughly 30% identical with the mammalian Gα subfamily protein, AtAGB1 shows about 42% similarity to mammalian Gβ subunits, and AtAGGs display 24%-31% identity with certain mammalian Gγ subunits (Mason and Botella, 2001). At the cellular level, Gα1 has been immunolocalized to the plasma membrane and endoplasmic reticulum (Weiss et al.,
1997), while Gβ₁ has been detected in the plasma membrane, endoplasmic reticulum and Golgi apparatus (Obrdlik et al., 2000, Adjobo-Hermans et al., 2006). Interactions between Gβ₁ and both Gγ₁ and Gγ₂ have been detected by yeast two-hybrid and in vitro binding assays (Mason and Botella, 2000, 2001), and there is evidence from yeast two-hybrid and co-immunoprecipitation assays for interactions between the rice Gα and Gβ subunits (Kato et al., 2004). Structural predictions for Gα₁, Gβ₁ and Gγ₁ suggest that they can form a heterotrimer similar to that formed by mammalian G protein subunits (Ullah et al., 2003). Although the heterotrimerization of *Arabidopsis* G protein subunits in cowpea protoplasts was recently reported (Adjobo-Hermans et al., 2006), there is still limited information available about the structural and biochemical characteristics of *Arabidopsis* heterotrimeric G protein.

Here we provide structural and biochemical evidence that further supports the concept of common and separate signaling functions of Gα₁ and the Gβ₁γ₁ complex. First, we show interactions between Gβ₁ and Gγ₁, as well as between Gα₁ and Gβ₁γ₁ complex in *Arabidopsis* mesophyll protoplasts using fluorescence resonance energy transfer (FRET) and co-immunoprecipitation, presenting direct evidence that the plant G protein subunits form a heterotrimer in vivo. Then, we show that the heterotrimeric G protein is part of a large complex using blue native gel electrophoresis. We also show that Gα₁ is presenting a complex that is more than 669 kD and found in the plasma membrane fraction in wild type and *agb1*-2 mutant plants, although some smaller complexes are also detected in the *agb1*-2 mutant. We further show that a mutant ahGα₁ lacking Gβ₁
interacting domain in Gα1 is not functional in the oxidative stress response to ozone. Although activation by GTPγS can not completely dissociate Gα from the complex, the Gα complex dissociates upon treatment with hydrogen peroxide (H₂O₂), suggesting a mechanism by which G protein signaling can be directly or indirectly activated in response to oxidative stress.

**Methods and Materials**

**Plant Materials**

We used *Arabidopsis thaliana* Col-0 plants and *agb1-2* and *gpa1-4* null mutant homozygotes in the Col-0 background (Ullah et al., 2003). Plants used to isolate plasma membrane fractions were grown in MetroMix 200 (Scotts-Sierra Horticultural Products Company, Marysville, OH) in 5-cm pots (51 per flat) at 65% humidity under fluorescent light at 30 W/m²/s with a 14-h-light/10-h-dark photoperiod for 5 weeks.

**Generation of Vectors for Expression of Green and Yellow Fluorescent Protein-G Protein Fusions**

*AtAGB1* and *AtAGG1* were cloned by PCR from an *Arabidopsis thaliana* Col-0 cDNA library, which was made as previously described (Han et al., 2004). According to the sequence information in the *Arabidopsis* database (http://www.arabidopsis.org), primers were designed as follows, with the underlined sequences adding restriction sites and the bold faced sequences adding a spacer (purchased from Integrated DNA
Technologies, Coralville, IA). The forward primer for *AtAGB1* is:

(5′-AGATCTGGAGGTGGAGGTAAGTAGATGTCTGTCTCCGAGCTCAA-3′). The reverse primer for *AtAGB1* is: (5′-TCTAGATCAAATCACTCTCCTGTGTCC-3′). The forward primer for *AtAGG1* is: (5′-AGATCTGGAGGTGGAGGTAGTGCGAGAGGAAACTGTGGTT-3′). The reverse primer for *AtAGG1* is: (5′-TCTAGATCAAATGATTAAAGCATCTGCA-3′). To insert CFP into *AtGPA1*, the binary vector of loop CFP-*AtGPA1* was requested from Dr. Alan Jones (University of North Carolina, Chapel Hill, North Carolina). The full length sequence of *AtGPA1*(l)CFP was cloned from the binary vector. The amplified PCR fragments were cloned into pGEM-Teasy (Promega, Madison, WI) and sequences were verified. To make constructs of pVAV321-35S-(C/Y)FP-*AtAGB1* and pVAV321-35S-YFP-*AtAGG1*, PCR products were excised from pGEM-Teasy (Promega) and further cloned into pVAV321-35S-C/YFP (a kind gift of Dr. Xuemei Chen, University of California, Riverside, California) using the BglII and XbaI sites. To make constructs pVAV321-35S-*AtGPA1*(l)CFP, pVAV321-35S-*AtAGB1* and pVAV321-35S-*AtAGG1*, PCR fragments of *AtGPA1*(l)CFP, *AtAGB1* or *AtAGG1* were excised from corresponding pGEM-Teasy constructs and cloned into pVAV321 with the removal of C/YFP via NcoI and XbaI. Then, the entire expression cassette of 35S-*AtAGG1* or 35S-*AtAGB1* was ligated into pVAV321-35S-YFP-*AtAGB1* or pVAV321-35S-YFP-*AtAGG1* individually using the SmaI and ClaI sites, which were modified by Klenow large fragment, resulting in pVAV321-35S-*AtAGG1*-35S-YFP-*AtAGB1* and pVAV321-35S-*AtAGB1*-35S-
YFP-AtAGG1. Finally, the entire expression cassette of 35S-AtGPA1(l)CFP was cloned into pVAV321-35S-AtAGG1-35S-YFP-AtAGB1 or pVAV321-35S-AtAGB1-35S-YFP-AtAGG1 via Smal and SacI sites, which were modified by Klenow large fragment, resulting in pVAV321-35S-AtGPA1(l)CFP-35S-AtAGG1-35S-YFP-AtAGB1 and pVAV321-35S-AtGPA1(l)CFP-35S-AtAGB1-35S-YFP-AtAGG1. The 35S-AtGPA1(l)CFP and 35S-YFP-AtAGB1 expression cassettes were further cloned into the pCAMBIA3300 Agrobacterium binary vector and transformed into homozygous agb1-2 and gpa1-4 mutant plant individually. Transformed plants that exhibited the wildtype phenotype in terms of leaf morphology on 10 g/ml glufosinate MS plates were used for further study (Ullah et al., 2003).

Protoplast Isolation and Transfection, Confocal Microscopy and FRET

Arabidopsis mesophyll protoplasts were prepared from fully expanded leaves of 4 to 5 week old plants and transfected by a polyethylene glycol method as described by J. Sheen’s laboratory http://genetics.mgh.harvard.edu/sheenweb/protocols. An additional 21% sucrose gradient was applied to the isolated protoplasts to improve the quality of transfected protoplasts, followed by the transfection with plasmids containing the FRET pair to be tested. Plasmids were isolated using a Plasmid Maxi Kit (Qiagen, Chatsworth, CA). A total of \( \sim 1 \times 10^5 \) cells were transfected with 50 µg plasmid for each microscopy observation, FRET measurement or immunoprecipitation experiment. The transfected protoplasts were incubated at 22°C for 12-16 h before being mounted in chambers
(Molecular Probes, Eugene, OR) for microscopy. The transfected protoplasts were imaged using a Zeiss laser scanning microscope (Carl Zeiss, Thornwood, NY), LSM 510 META with a 40×NA 1.2 water objective. To monitor fusion protein expression and localization, protoplasts were excited with two Argon laser lines, 458 nm for CFP and 514 nm for YFP and emission images were collected simultaneously with 480-520 nm filter for CFP and 530-590 nm filter for YFP.

FRET is a widely used method to identify interaction of two proteins tagged with a FRET donor (CFP) and acceptor (YFP) fluorophore pair in vivo. FRET between CFP and YFP fusion G protein subunits was measured with a Zeiss LSM510 Meta spectral confocal fluorescence microscope (Carl Zeiss). The transfected protoplasts were excited with a chameleon multiphoton laser (Coherent MRU 1000), which was modulated to 820 nm wavelength and set at 4-5% laser intensity ideal for cross-talk free FRET analysis. The emission spectra from selected regions (ROI) were recorded by a connected multi-channel-spectrometer (MCS) in twelve channels, each with a 10 nm band width, from 464 to 584 nm, using a 650 KP dichroic mirror in the lambda stack acquisition mode. Emission spectra were recorded from 20 individual protoplasts transfected with each G protein FRET pair tested. Spectral analysis with automatic peak detection and spectral color encoding, were done with the software package for the LSM 510. Final fluorescence spectra of G protein subunits CFP and YFP fusion protein were corrected for background fluorescence. The reliability test in spectrum-based FRET measurement with 2-photon excitation was performed using published negative and positive FRET pair
fusion protein in mesophyll protoplasts (Kato et al., 2002). FRET tested pairs of CFP and YFP fusion protein constructs were kindly provided by Dr. Lam Eric.

Immunoprecipitation and Western blotting

The catch and release reversible immunoprecipitation system (Upstate, Lake Placid, NY) was used for immunoprecipitation experiments. The transfected protoplasts were lysed with 1×wash buffer provided by the kit and then centrifuged at 15,000×g for 10 min. The clear extract was incubated with the rabbit polyclonal anti-Gα antibody (1:250) and the affinity ligand (1:50) in the kit column at 4°C for 10-12 h. The column was washed three times by centrifugation with 1×wash buffer. 1×Denaturing elution buffer in the kit was used to elute proteins binding in the column, which were subjected to further electrophoresis and Western blotting. Total extract, flow through that was concentrated with Strataclean resin (Stratagene, La Jolla, CA), and eluted proteins were loaded on a 12% polyacrylamide discontinuous gel (Bio-Rad mini electrophoresis system, Hercules, CA). After electrophoresis, proteins were transferred to Hybond-P PVDF membrane (Amersham, Piscataway, NJ) using a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad). Immunoblotting was performed with mouse monoclonal anti-CFP antibodies (1:2000, BD Bioscience, Mountain View, CA). After incubation with horseradish peroxidase–conjugated anti-rabbit IgG antibodies, proteins were detected using ECL Plus protein gel blotting detection reagents (Amersham) according to the manufacturer’s instructions.
Blue Native (BN)-PAGE

Plasma membrane fraction for BN-PAGE was prepared in an aqueous two-phase partitioning system as described (Thomson et al., 1993; Yan et al., 1998; Sagi and Fluhr, 2001). Blue Native/SDS-PAGE 2-dimensional gel electrophoresis was performed as described previously with some modification (Rivas et al., 2002; Han et al., 2004; Karlova et al., 2006). Plasma membrane proteins were incubated with solubilization buffer (20 mM Bis-Tris-HCl, pH 7.0, 250 mM ε-aminocaproic acid, 2 mM EDTA, 1.0% Triton X-100, 0.25% Coomassie blue G 250 and 10% glycerol) for 30 mins and centrifuged twice at 15,000×g for 5 mins. Solubilization buffer containing 100 µM GTPγS or 20µM H2O2 was used for GTPγS or H2O2 treatment experiments. The supernatant was separated in 5.5%-16% polyacrylamide gradient gel and albumin bovine monomer (66 kD), lactate dehydrogenase (140 kD), catalase (232 kD), ferritin (440 kD), porcine thyroid (669kD) from Amersham, were loaded alongside as marker proteins. 1st dimension BN-PAGE gel was transferred to Hybond-P PVDF membrane (Amersham) and immunoblotted with anti-G/C/Y antibodies (BD Bioscience) to detect CFP tagged Gβ1 complex (anti-G/C/Y antibodies detect GFP, CFP and YFP proteins). The remaining first dimension lanes of the BN-PAGE gel were cut and incubated in denaturing solution (1% SDS, 1% β-mercaptoethanol) for 2h and mounted on 10% SDS-PAGE gel. After electrophoresis, the gel was subjected to immunoblotting analysis with anti-Gα antibody, which can not detect the protein complex in 1st dimension BN-PAGE gel.
Mutagenesis Study in AtGα₁

We amplified *ahAtGPA1* and *AtGPA1* by PCR from an *Arabidopsis* cDNA library as described above. For *ahAtGPA1*, the forward primer is (5′-CACCATGGGCTTACTCTGCAGTAGAA-3′) and the reverse primer is (5′-TCATAAAAGGCCAGCCTCCAGTAA-3′). For *AtGPA1*, the forward primer is (5′-CACCATGGGCTTACTCTGCAGTAGAA-3′) and the reverse primer is (5′-TCATAAAAGGCCAGCCTCCAGTAA-3′). PCR products were subcloned into the pENTR/D-TOPO vector (Invitrogen, Carlsbad, CA), then cloned into Gateway plant transformation destination binary vector pGWB2 (Karimi et al., 2002) by LR recombination reactions. Both 35S:GPA1 and 35S:ahGPA1 constructs were transformed into *gpa1-4* mutant backgrounds. The transformants were compared with wildtype plants for their sensitivity to ozone.

Recombinant AtGPA1 Expression, Purification and [35S]GTPγS Binding Assay

We amplified *AtGPA1* by PCR from an *Arabidopsis* cDNA library as described above with forward primer (5′-GAGCTCATGGGCTTACTCTGCAGTAGAA-3′) and reverse primer (5′-GGTACCTCATAAAAGGCCAGCCTCCAGTAA-3′), cloned it into QIAexpress pQE-30 RGS•His tag vector (Qiagen) at the SacI and KpnI restriction sites (underlined in primer sequences) and expressed it in *BL21-CodonPlus (DE3)-RIPL* strain (Statagene). The recombinant 6×His tagged *Gα₁* was purified according to the
manufacturer’s instructions (The QIAexpressionist, Qiagen). Purification was confirmed by PAGE and the purified recombinant AtGP A1 protein was dialysed against GTPγS binding assay buffer (50 mM Tris•HCl, pH 8.0, 5 μM GDP and 3 mM MgCl, 1 mM EDTA, 100 mM NaCl). The recombinant Gα1 was incubated with 20 nM [35S]GTPγS in the above buffer for different times and the reaction was diluted with stop solution (25 mM Tris•HCl, pH 8.0, 25 mM MgCl, 100 mM NaCl). The reaction was further applied to a 0.45 μm nitrocellulose membrane filter (Whatman, Florham Park, NJ), which was rinsed 3 times with stop solution. The amount of recombinant Gα1 bound [35S]GTPγS was then quantified by liquid scintillation spectrometry. The specific binding of [35S]GTPγS with recombinant Gα1 was evaluated by nucleotide competition experiments. The effect of H2O2 on the binding of [35S]GTPγS to recombinant Gα1 was tested as described (Nishida et al., 2002).

Results

FRET Detection of In Vivo Interactions between the β1 and γ1 Subunits of the Arabidopsis Heterotrimeric G protein.

First, we used FRET to determine the interaction between Gβ1 and Gγ1 in vivo. Modeling predictions suggested the existence of the heterotrimeric form of G protein subunits in Arabidopsis (Fig. 1A) (Ullah et al., 2003). Moreover, interaction between Gβ1 and Gγ1 has been demonstrated in yeast two-hybrid and in vitro pull-down assays (Mason
and Botella, 2000). In yeast and leukocytes, FRET has been used to monitor the association of the subunits of the heterotrimeric G protein and the dissociation of the Ga subunit from Gβ1γ1 dimer in response to external stimuli (Janetopoulos et al., 2001; Ruiz-Velasco and Ikeda, 2001; Humrich et al., 2005). Therefore, we used FRET to determine whether the *Arabidopsis* G protein subunits form the heterotrimer in vivo.

![Arabidopsis diagram](image)

**A.**

**B.**

<table>
<thead>
<tr>
<th>Construct</th>
<th>35S</th>
<th>TL</th>
<th>AtGPA1(l)CFP</th>
<th>NOS</th>
</tr>
</thead>
<tbody>
<tr>
<td>35S</td>
<td>TL</td>
<td>YFP-AtAGB1</td>
<td>NOS</td>
<td></td>
</tr>
<tr>
<td>35S</td>
<td>TL</td>
<td>YFP-AtAGG1</td>
<td>NOS</td>
<td></td>
</tr>
</tbody>
</table>

**C.**

**D.**

![Fluorescence intensity graph](image)

Fluorescence Intensity (arbitrary units)
**Figure 13.** Spectral characteristics and cellular localization of CFP and YFP of Arabidopsis G protein subunits

A, The predicted structure of Arabidopsis heterotrimeric G protein is depicted (Ullah et al., 2003) B, Constructs of CFP and YFP of Arabidopsis G protein subunits were used in this study for FRET measurement. C, Cellular localization images of CFP and YFP of Arabidopsis G protein subunits were taken. CFP was excited by chameleon multiphoton laser modulated to 820 nm wavelength and YFP was excited by Ar laser lines at 514 nm. D, Fluorescent emission spectra from selected regions of C (crossing marks) were recorded by Zeiss LSM510 Meta spectral confocal fluorescence microscope.

Based on the *Arabidopsis* G protein structural predictions, we constructed genes encoding N-terminal fusions of enhanced C/YFP (yellow/cyan fluorescent proteins) with Gβ1 and Gγ1 and expressed them from a CaMV 35S promoter (Fig. 13A and 13B). We transfected *Arabidopsis* mesophyll protoplasts with YFP-Gβ1 and CFP-Gγ1 and determined the cellular localization by microscopy and their interaction by FRET. We observed the fluorescence of CFP-Gβ1 in small bodies (Fig. 13C) in the cytoplasm. The fluorescence of YFP-Gγ1 was observed at the very periphery of the protoplast, which indicates that Gγ1 is predominantly localized in the plasma membrane (Fig. 13C). Upon co-transfection of CFP-Gβ1 and YFP-Gγ1, we observed signal from CFP-Gβ1 at the very periphery of the protoplast along with YFP-Gγ1 (Fig. 14A.1), suggesting that plasma membrane localization of Gβ1 requires the co-expression of Gγ1. We also used the co-transfected protoplasts with CFP-Gβ1 and YFP-Gγ1 to determine FRET by spectral recording microscopy with crosstalk-free chameleon multiphoton laser excitation. FRET
is detected as the occurrence of an emission spectral shift from CFP to YFP (Fig. 14B). In protoplasts co-transfected with CFP-Gβ1 and YFP-Gγ1, we detected a strong increase from 520 to 530 nm, representing the typical YFP spectrum (Fig. 13D), opposed to CFP spectrum itself (Fig. 13D), which has a broad peak from 475 to 500 nm, indicating that Gβ1 and Gγ1 interact in plant cells.

**FRET Detection of the Interaction between the α1 Subunit and the βγ1 Complex.**

Next, we used FRET to determine the interaction between Gα1 and Gβ1γ1 in vivo. To minimize the possibility of interference of CFP with the structure and function of the Gα1 (Humrich et al., 2005), we inserted CFP into the putative αAB-loop within the α helical domain of Gα1 as described previously (Fig. 13A) (Chen et al., 2003). We co-transfected *Arabidopsis* mesophyll protoplasts with combinations of plasmids encoding the following: CFP(I)-Gα1 and YFP-Gβ1; CFP(I)-Gα1 and YFP-Gγ1; and CFP(I)-Gα1, YFP-Gβ1 and YFP-Gγ1. We were able to detect the spectral shift only when we co-transfected the protoplasts with constructs containing the coding sequences for all three subunits, not just two of the three (Fig. 14A. 2, 3 & 4). This observation indicates that although FRET pairs are transfected into wildtype protoplasts which contain endogenous Gγ1 or Gβ1, for the FRET detection, overexpression of Gγ1 or Gβ1 is still needed to ensure plasma membrane distribution of the Gβ1 subunit by Gγ1 or closeness of Gγ1 to Gα1 by Gβ1. This observation further suggests that the α subunit does not interact with either the β or the γ subunit alone in *Arabidopsis* protoplasts (Fig. 14A. 4 and 14B).
To further determine whether the energy transfer is from CFP(l)-Gα₁ to YFP-Gβ₁ or from CFP(l)-Gα₁ to YFP-Gγ₁ in the G protein complex formed in protoplasts, we created a single construct expressing all three genes with two as a FRET pair and the third one untagged as follows: 35S-CFP(l)-Gα₁-35S-YFP-Gβ₁-35S-Gγ₁ and 35S-CFP(l)-Gα₁-35S-Gβ₁-35S-YFP-Gγ₁ (Fig. 13B). We detected the emission spectral shift in protoplasts transfected with 35S-CFP(l)-Gα-35S-Gβ₁-35S-YFP-Gγ₁ (Fig. 14A. 5, 14A. 6 and 14B), but not in protoplasts transfected with the 35S-CFP(l)-Gα₁- 35S-YFP-Gβ₁-35S-Gγ₁ construct. Thus, energy transfer occurred between CFP(l)-Gα₁ and YFP-Gγ₁, but not between CFP(l)-Gα₁ and YFP-Gβ₁, indicating that Gα₁ subunit is in closer proximity to the Gγ₁ subunit than to Gβ₁ subunit in the G protein complex formed in Arabidopsis protoplasts. However, energy transfer between Gα₁ and Gγ₁ does not occur in the absence of the Gβ₁, suggesting that the subunits of Arabidopsis heterotrimeric G protein form a complex in vivo.
Figure 14: FRET images of CFP and YFP of Arabidopsis G protein subunits.
A, Spectral encoded images of FRET between CFP and YFP of Arabidopsis G protein subunits. B, Fluorescent emission spectra from selected regions of observed cells were recorded by Zeiss LSM510 Meta spectral confocal fluorescence microscope with the excitation of chameleon multiphoton laser modulated to 820 nm.

To examine the formation of the complex among the subunits of the Arabidopsis heterotrimeric G protein in plant cells, we co-immunoprecipitated CFP(l)-Gα₁, Gβ₁, and YFP-Gγ₁ proteins transiently expressed in protoplasts. YFP-Gγ₁ can be co-immunoprecipitated with CFP(l)-Gα₁ using Gα₁ antibody from protoplasts transfected with a 35S-CFP(l)-Gα₁-35S-Gβ₁-35S-YFP-Gγ₁ construct, but not from protoplasts co-transfected with 35S-CFP and 35S-YFP-Gγ₁ (Fig. 15A). CFP(l)-Gα₁ and YFP-Gγ₁ differ in size and can be detected together in the same gel to evaluate the efficiency of immunoprecipitation. Thus, we conclude that the subunits of the Arabidopsis
heterotrimeric G protein form a complex in vivo.

The *Arabidopsis* Heterotrimeric G Protein Is in a ~700 kD Complex

To analyze the plasma membrane-bound complex of the heterotrimeric G protein, we used blue native polyacrylamide gel electrophoresis (BN-PAGE) to resolve the complex followi by the plasma membrane purification. We isolated the plasma membrane fraction using the aqueous two-phase partitioning system (Serrano, 1984; Basboa et al., 1987), solubilized that with NP-40 and fractionated them by BN-PAGE. BN-PAGE was followed by a 2nd dimension denaturing SDS-PAGE gel and the proteins were detected by Western blotting with G\(_\alpha_1\) antibody. In plasma membrane fraction, the antibody detected the G\(_\alpha_1\) monomer (42 kD) in a complex of approximately 700 kD, as well as in a low-molecular weight apparently monomeric form. Thus, G\(_\alpha_1\) protein appeared to be part of a complex larger than anticipated for the heterotrimer, whose molecular mass was expected to be about 100 kD (Fig. 15B). A complex of about the same size was detected by Western blotting with anti-CFP antibodies of a membrane fraction purified from *Arabidopsis* plants expressing a CFP-G\(_\beta_1\) fusion protein (Fig. 15C). However, in contrast to what was observed with G\(_\alpha_1\), G\(_\beta_1\) protein was not observed at the expected mobility of either the heterotrimer or the heterodimer. This observation further confirms that the *Arabidopsis* heterotrimeric G protein is part of a large complex in the plasma membrane.

To determine whether the G\(_\beta_1\) subunit is required for the formation of the large complex containing the G\(_\alpha_1\) subunit, we analyze the G\(_\alpha_1\)-containing complexes in the
plasma membrane fractions of *agb1-2* mutant plant. Much less of the Gα1 protein is associated with the ca. 700 kD complex in *agb1-2* mutant plant than in wildtype plants, as judged by the detection of Gα1 with anti-Gα1 on SDS-PAGE after BN-PAGE. Complexes of several different sizes are detected with anti-Gα1 after BN-PAGE and SDS-PAGE. The approximate sizes of the complexes are approximately 700, 400 and 150 kD (Fig. 15D). Gα1 protein is detected in association with several smaller complexes in the 140-400 kD range. Such Gα1-containing complexes are not observed in wildtype plants (Fig. 15B). These observations suggest that Gβ1 participates the association of Gα1 with the large complex, for its formation or its stability, but not for the plasma membrane localization of Gα1. Alternatively, other proteins may bind to Gα1 in the absence of Gβ1.

**Heterotrimeric G protein Signaling in the Oxidative Stress Response Requires the Interaction of Gα1 with Gβ1**

To further determine the requirement of Gβ1-Gα1 interaction for the normal function of Gα1, we perturbed their interaction by deleting the domain from N-AtGPA6 to N-AtGPA32 (ahGα1), which was predicted to form the first alpha-helix of Gα1 and mediate the interaction between Gβ1 and Gα1 (Ullah et al., 2003). We kept first five residues, which contained lipid modification sites of Gα1 and are required for the proper localization of Gα1 (Adjobo-Hermans et al., 2006). Plants homozygous for the *gpa1-4* mutations were transformed with a construct expressing Gα1 (*gpa1-4* WT) or ahGα1 (*gpa1-4 *ahGα1) and the transformants were compared with wildtype plants for their
sensitivity to ozone. The *gpa1-4*WT plants exhibited the wildtype leaf morphology phenotype, while the *gpa1-4*ahGa1 plants did not, indicating that the shortened protein cannot complement the mutation (Fig. 16A). Similarly, the wildtype construct restored the ozone-sensitivity phenotype of mutant, while the deletion construct did not, indicating that the mutant protein cannot replace the normal protein with respect to its signaling function in oxidative stress (Fig. 16B).
Figure 15. Detection of heterotrimeric G protein complexes in Arabidopsis

A, Protein extract of protoplasts transfecting 35S-CFP and 35S-N-YFP-Gγ1 as negative control (1-4) or 35S-L-CFP-Gα1-35S-Gβ1-35S-YFP-Gγ1 (5-8) were immunoprecipitated with anti-Gα1. Samples (1, 5: 10% total extract; 2, 6 concentrated flow through from the column; 3, 7: 1st elute by denaturing buffer; 4, 8: 2nd elute by denaturing buffer) were loaded in the SDS-PAGE gel and the blot was incubated with anti C/YFP antibody. B, The plasma membrane fraction from Col-0 plants and gpa1-4 mutant plants was separated in BN-PAGE gel in the 1st dimension and SDS-PAGE in the 2nd dimension. The blot was incubated with Gα1 antibody. Protein mass standards were indicated by the arrow on the top of the blot. C, The total extract of agb1-2 overexpressing CFP and CFP-AtGβ1 were separated in BN-PAGE gel and the blot was detected with anti C/YFP antibody. Protein mass standards were indicated by the arrow on the right side of the blot. D. The plasma membrane fraction from the agb1-2 was separated in BN-PAGE gel in the 1st dimension and SDS-PAGE in the 2nd dimension. The blots were detected with Gα1 antibody. Protein mass standards were indicated by the arrow on the top of the blot.

The G protein Complex Dissociates upon the Treatment of GTPγS or H2O2

To examine whether the activation of G protein causes the dissociation of Gα1 from the complex, we used BN-PAGE to evaluate the ratio changes of complex over monomer of plasma membrane bound Gα1 upon the treatment of GTPγS. It has been reported that Gα subunit of rice is fully dissociated from the complex in the presence of GTPγS (Kato et al., 2004), which binds Gα and makes it active. To test the occurrence of this reaction in Arabidopsis, we isolated the plasma membrane fraction from wildtype plants, incubated it with 100 μM GTPγS and separated it in BN-PAGE followed by SDS-PAGE. We compared the ratio of complex over monomer with and without GTPγS treatment as judged by the detection of Gα1 with anti-Gα1 on SDS-PAGE. We found that GTPγS only caused ~30% of Gα1 to dissociate from the complex (Fig. 17A). This result is different from that reported in rice (Kato et al., 2004). However, this result supports the
observation of FRET experiments performed between constitutive active Gα1 and Gβ1γ, which shows that activation of Gα1 can not completely promote the dissociation of it from Gβ1γ (Adjobo-Hermans et al., 2006). Taken together by those results, Gα1 subunit of Arabidopsis may not be completely dissociated from the complex upon its activation.

To determine the direct effect of H2O2 on the activity of Gα1, we measured the GTP binding activity of recombinant Gα1 after treatment of H2O2. The GTP binding activity of two mammalian Gα proteins, Gαi and Gαo, can be directly regulated by reactive oxygen species (ROS) (Nishida et al., 2000; Nishida et al., 2002; Nishida et al., 2005). To test this possibility for Arabidopsis Gα1, we purified recombinant 6×His-Gα1 (Fig. 17C) and assayed its GTP-binding activity before and after treatment with H2O2 (Nishida et al., 2000; Nishida et al., 2002). However, we were unable to detect an increase in the GTP-binding activity of recombinant Gα1 in response to H2O2 treatment (Fig. 17D, 17E), suggesting that Arabidopsis Gα1 may not be directly activated by ROS.

To further determine whether ROS could affect G protein signaling by altering or destabilizing G protein complex instead of directly activating it, we analyzed the ratio complex to monomer in plasma membrane bound Gα1 upon the treatment with H2O2. We isolated plasma membrane fractions from wildtype plants and treated them with physiological levels of hydrogen peroxide (20 μM) prior to BN-PAGE followed by SDS-PAGE. We found that H2O2 treatment could promote the dissociation of monomeric Gα protein (70%) from the ~700 kD plasma membrane complex (Fig. 17B), as judged by the detection of Gα1 with anti-Gα1 on SDS-PAGE. This observation suggests a
A. Growth phenotypes of 4 week-old light-grown plants of the indicated genotypes constitution;
B. Electrolyte release was assayed after a 3-hr exposure of 4 week-old plants to the indicated amount of ozone by measuring the conductivity of water in which leaves were shaken and expressed as a fraction of the total electrolytes measured.

Figure 16: Phenotypes of 4 week-old light-grown plants of the indicated genotypes constitution

mechanism by which G protein signaling could be directly regulated by ROS and destabilizes its complex through G protein coupled receptor or other interacting protein/s.
Figure 17. Redox regulation of heterotrimeric G protein complexes in Arabisopsis A and B, The isolated plasma membrane fraction from Col-0 plants was treated with buffer and 100μM GTPγS (A); 20 μM H$_2$O$_2$ and H$_2$O as control (B), dissolved in the solubilization buffer and then separated in BN-PAGE gel followed by SDS-PAGE gel. The blots were detected with anti-Gα antibody. Protein mass standards were indicated by the arrow on the top of the blot.

C. The purification of recombinant RGS-6xHisAtGPA1 was examined in 10% SDS-PAGE gel. The lane 1 is the lysate of bacteria expressing RGS-6xHisAtGPA1 and the lane 2 is the flowthrough from the His-tag affinity beads. The lane 3 is the wash solution from the His-tag affinity beads and the lane 4 is the eluted from beads. Bench protein mark was loaded alongside of protein samples to indicate the size of the purified proteins. D, [35S]GTPγS binding to the recombinant RGS-6xHisAtGPA1 protein at different concentration of ATP and GTP. E, [35S]GTPγS binding to the recombinant RGS-6xHisAtGPA1 incubated with 20 μM H2O2 and H2O as control.
Discussion

We have reported that G\(\alpha\) and G\(\beta\gamma\) subunits of the Arabidopsis heterotrimeric G protein act both synergistically and separately in signaling by reactive oxygen species in the oxidative stress response (Joo et al., 2005). Here, we provide structural and biochemical evidence that further supports the concept that the G\(\alpha_1\) and G\(\beta_1\gamma\) signals can play both common and separate roles in varieties of development-related events and stress-associated processes in *Arabidopsis*. Using FRET and immunoprecipitation, we provide evidence that the Arabidopsis G protein subunits interact in the plasma membrane in plant cells. Using BN-PAGE, we make a comparison of the plasma membrane-bound G\(\alpha_1\) complex between the wild type and agb1-2 mutant plant, which suggests the concurrence of dependent and independent manner that G protein subunits function in *Arabidopsis*.

Previous studies have shown that the extracellular ROS activated the heterotrimeric G protein either directly or indirectly in the oxidative stress response (Joo et al., 2005). Here we show that the treatment of the plasma membrane fraction with hydrogen peroxide (\(H_2O_2\)) results in the partial dissociation of the G\(\alpha_1\) complex; however, \(H_2O_2\) can not promote the increase in GTP-binding of recombinant G\(\alpha_1\), which indicates that the G protein signaling is not regulated by ROS through the direct activation of G\(\alpha_1\), but likely to be mediated by some other interacting proteins in the complex.
The Interaction of $G\beta_1$ and $G\gamma_1$

The FRET is detected between N-terminal CFP tagged $G\beta_1$ (CFP-$G\beta_1$) and N-terminal tagged YFP-$G\gamma_1$ (YFP-$G\gamma_1$) in the plasma membrane of *Arabidopsis* mesophyll protoplasts, indicating the functional interaction of $G\beta_1$ and $G\gamma_1$. This FRET pair, as a positive control, also makes our FRET assay for all G protein subunits more reliable. We also attempted to test the FRET in pairs of C-terminal CFP tagged $G\beta_1$ ($G\beta_1$-CFP) and YFP-$G\gamma_1$; CFP-$G\beta_1$ and C-terminal YFP tagged $G\gamma_1$ ($G\gamma_1$-YFP); and $G\beta_1$-CFP and $G\gamma_1$-YFP. No FRET are detected in these FRET tested pairs (data not shown), although both CFP-$G\beta_1$ and $G\beta_1$-CFP work as functional $G\beta_1$ subunit with respect to the phenotype recovery after their introduction into *agb1-2* mutant plant. This observation clearly demonstrates that the close proximity of fluorophores for FRET tested pairs is required for FRET detection, even for the physically interacting proteins. This result indicates that the distance between CFP-$G\beta_1$ and YFP-$G\gamma_1$ is closer than the other FRET tested pairs for $G\beta_1$ and $G\gamma_1$ and proves the molecular prediction for the structure of the *Arabidopsis* heterotrimeric G protein (Ullah et al., 2003), which shows that N-terminal $G\beta_1$ and N-terminal $G\gamma_1$ are the closest pair compared to the other combinations.

In addition, the subcellular localization of the expressed fluorescent $G\beta_1$ and $G\gamma_1$ was examined in *Arabidopsis* mesophyll protoplasts. As expected, YFP-$G\gamma_1$ is mainly localized in the plasma membrane, where it is speculated to be anchored by its C-terminal palmitoylated cysteine residue (Mason and Botella, 2000, 2001; Adjobo-Hermans et al.,
2006). Surprisingly, transiently expressed CFP-Gβ₁ forms small bodies in cells and the pattern varies from cell to cell, unlike plasma membrane localization as published before (Obrdlik et al., 2000). Once co-expressed with YFP-Gγ₁, CFP-Gβ₁ is also observed in the plasma membrane, which indicates that the plasma membrane localization of Gβ₁ requires the Gγ₁, although there are fewer small bodies still left in the cytoplasm, which was reported to be localized in Golgi (Adjobo-Hermans et al., 2006). The small bodies of CFP-Gβ₁ could also be explained by the physical aggregation of transient expressing proteins, which were not folded efficiently without enough chaperones. It has been reported that the phosducin-like protein is an essential chaperone, required for the proper folding of Gβ subunits in HEK-293 cells and Dictyostelium (Loew et al., 1998; Blaauw et al., 2003; Lukov et al., 2005). Moreover, two subgroups of phosducin-like proteins have been identified in Arabidopsis (Blaauw et al., 2003). The function of phosducin-like proteins in Arabidopsis G protein signaling is being investigated in our laboratory.

The Interaction of Ga₁ and Gβ₁,Gγ₁

The FRET is detected between CFP(l)-Ga₁ and YFP-Gγ₁ with co-expression of Gβ₁ in the plasma membrane of Arabidopsis mesophyll protoplasts, indicating the presence of heterotrimeric G protein complex in Arabidopsis. However, FRET is not detected between CFP(l)-Ga₁ and YFP-Gβ₁ with co-expression of Gγ₁ in the plasma membrane of Arabidopsis mesophyll protoplasts. This result is distinct from mammalian studies where FRET can be detected between between CFP(l)-Ga and YFP-Gβ and between between
CFP(l)-Ga$_1$ and YFP-G$_\gamma$ (Bunemann et al., 2003). This difference may suggest the slight structure difference between mammalian heterotrimeric G protein and *Arabidopsis* heterotrimeric G protein, caused by additional residue inserts in Ga$_1$ and Gb$_1$, although the structure prediction of heterotrimeric G protein shows high conservation between *Arabidopsis* and mammals (Ullah et al., 2003). On the other hand, FRET is not detected in all transfected cells even with CFP(l)-Ga$_1$ and YFP-G$_\gamma_1$ pair, which may suggest that the interaction of *Arabidopsis* heterotrimeric G protein subunits have transient and spatial properties like mammalian heterotrimeric G protein (Janetopoulos et al., 2001; Ruiz-Velasco and Ikeda, 2001; Janetopoulos and Devreotes, 2002). The result of immunoprecipitation coincides with our FRET results that not all of the YFP-G$_\gamma_1$ is co-immunoprecipitated with CFP(l)-Ga$_1$, raising the other possibility that not all of G protein subunits are present in the same complex.

**The Complex of *Arabidopsis* Heterotrimeric G Protein**

Indeed, BN-PAGE/SDS gel results show that some of Ga$_1$ still remains in the same size complex even in the absence of Gb$_1$, indicating some independent functions of Ga$_1$ on Gb$_1$, which was shown in the roles of Ga$_1$ in the modulation of cell division in roots (Chen et al., 2006). This notion is also consistent with our previous report that the Ga$_1$ protein can mediate activation of membrane-bound NADPH oxidases in the absence of the Gb$_1$ protein (Joo et al., 2005). Conversely, we have also reported that a significant fraction of the intracellular Gb$_1$ protein, not Ga$_1$ protein, is ER-associated in *Arabidopsis*,

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which is coincident with an independent role for the G$\beta_1$$\gamma$ complex of the heterotrimeric 
G protein in cell death signaling in the unfolded protein response in *Arabidopsis*. Collectively, we provide the biochemical evidence to interpret the independent function of Ga$_1$ and G$\beta_1$ in numbers of physiological phenomena in *Arabidopsis*.

On the other hand, our mutagenesis study in Ga$_1$ addresses the synergistic action of 
G$\beta_1$ on Ga$_1$ because ahGa$_1$ lacking G$\beta_1$ interacting domain is not functional as Ga$_1$ in the oxidative stress response to ozone, suggesting certain signal transduction events through Ga$_1$ may require the entire heterotrimeric G protein complex. Although this result could be explained by the effect of deleting the first alpha-helix on the proper folding of Ga$_1$, the less importance of the first 25 residues on the whole structure or folding of Ga subunits has been formulated in the structural study of Ga subunits in mammalian cells. It was reported that the amino-terminus of Ga$_i$ and Ga$_i$ is dynamically disordered in the GDP bound state and becomes $\alpha$-helical only upon interaction with G$\beta_\gamma$ (Medkova et al., 2002). Moreover, crystal structures of Ga$_t$-GDP and Ga$_t$-GTP$\gamma$S were successfully analyzed even without the first 25 residues (Noel et al., 1993; Lambright et al., 1994). Thus, our results could provide another line of evidence to demonstrate the synergistic effects of G$\beta_1$ on some signaling event mediated by Ga$_1$.

**The Redox Regulation of Ga Complex**

The dissociation of Ga from G$\beta_\gamma$ complex upon the action by the GTP binding to Ga has been well established in mammalian and yeast (Joo et al., 2005). In rice, this
phenomenon was observed (Kato et al., 2004), although in Arabidopsis this reaction is not obviously detected by FRET and complex isolation (Adjobo-Hermans et al., 2006). Instead, we find H$_2$O$_2$ promotes G$\alpha_1$ to dissociate from its complex. The G$\alpha$ subunit of the heterotrimeric G protein is required for the activation of the membrane-bound NADPH oxidase system in Arabidopsis and rice (Suharsono et al., 2002, Joo et al., 2005). Thus, the redox regulation of G protein complex may infer a common feedback in G protein signaling by ROS production. Despite the dissociation of G$\alpha_1$ complex by H$_2$O$_2$ treatment, the mechanism for the dissociation of G protein complex in plants has not been explored. Moreover, the role of signaling transduction mediated by the dissociation of G protein complex also put an intriguing challenge for further study in plants.
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Chapter 5

CONCLUSION

Function of Heterotrimeric G Protein in *Arabidopsis*

Functions of heterotrimeric G protein were revealed by mutant analysis in *Arabidopsis*. In this study, I also used G protein mutant: *gpa1-4* lacking Gα subunit and *agb1-2* lacking Gβ subunit to analyze the functional role of heterotrimeric G protein in *Arabidopsis* oxidative stress to O₃ and UPR to Tm. I found that upon the O₃ treatment, G protein mutant plants have altered ozone sensitivity: *gpa1-4* mutant plants are more resistant to O₃ induced tissue damages, while *agb1-2* mutant plants are more sensitive to O₃ induced tissue damages. This observation suggests that Gα subunit and and Gβ subunit play different roles in cell death-associated O₃ responses. To further understand the specific function of Gα subunit and and Gβ subunit, I monitored the expression level of Gα subunit and and Gβ subunit in the course of O₃ treatment. I found that the transcripts of Gα subunit and and Gβ subunit were differently induced by O₃, which reinforced the concept that Gα subunit and and Gβ subunit serve different functions in O₃ responses. The oxidative burst induced by O₃ was also measured in *gpa1-4* mutant, *agb1-2* mutant and wildtype plants. Results show that Gα subunit is required for the production of both peaks of the oxidative burst and Gβ subunit is only needed for the first peak of the oxidative burst induced by O₃. This observation indicates that Gα subunit and and Gβ
subunit can act separately in O₃ responses.

Up-regulation of UPR is essential in plant defense system. Using Tm injection method, we demonstrated that ER stress induced by Tm can cause leaf senescence in wildtype and gpa1-4 mutant plants; however, this toxic effect was much attenuated in agb1-2 mutant plants. This observation indicates that independent role for the Gβ subunit of heterotrimeric G protein in cell death signaling in the UPR. Further measurement of induction of UPR marker gene in wildtype and agb1-2 mutant plants show that UPR is less extensive in agb1-2 mutant than that in wildtype plants. Thus, the Gβ subunit subunit may be directly involved in the transcriptional response and cell death signaling in the UPR.

As in other stress responses, ROS are also implicated in the ER stress. Tm can trigger the oxidative burst in wildtype plant, which exerts a protective role in UPR induced cell death. Suppresing the endogenous ROS level or preventing the production of ROS can exacerbate the cell death induced by Tm. This protective effect of ROS was confirmed by the atrbohD and atrbohD/F double mutant analysis. We find that the area of dead tissue that develops in response to local subepidermal infiltration of tunicamycin is much larger in ROS-inhibited plants than in untreated plants and in AtrbohD and atrbohD/F double mutant than in wildtype plants. The protective effects of ROS are also reflected by the fact that inhibition of ROS production by diphenylene iodonium (DPI), a flavin oxidase inhibitor, delays the expression of UPR marker genes. Thus ROS signaling evokes a protective response that curtails the spread of UPR-induced cell death and
triggers the early induction of UPR gene expression.

**Heterotrimeric G Protein Complex in *Arabidopsis***

To further investigate the underlying mechanism, I characterized the heterotrimeric G protein complex in *Arabidopsis*. Using fluorescence resonance energy transfer (FRET) and co-immunoprecipitation, I show interactions between the β and γ1 subunits and between the α and βγ1 subunits of the *Arabidopsis* heterotrimeric G protein in protoplasts. I report that both the Gα and the Gβ subunits are associated with macromolecular complexes in plasma membrane fractions. I estimate the size of the G-protein containing plasma membrane complex to be approximately 700 kD based on blue native polyacrylamide gel electrophoresis, suggesting that the heterotrimeric G is complexed with many other proteins. I find that Gα is present in both the large ca. 700 kD complex in wildtype plants and in smaller complexes in plants homozygous for the *agb1-2* Gβ null allele. Activation of the Gα subunit with GTPγS results in partial dissociation of the Gα subunit from the complex. Hydrogen peroxide (H2O2) promotes the extensive dissociation of the Gα complex, but does not interfere with binding of GTPγS to purified recombinant Gα, suggesting that reactive oxygen species affect the stability of the complex, but not the activity of Gα itself.
Significance of the Present Findings

The present study has revealed two novel functions of *Arabidopsis* heterotrimeric G protein in oxidative stress to O$_3$ and ER stress to Tm. It is the first demonstration that O$_3$ signaling perception is not from extracellular spaces in plants but mediated by an intracellular signaling molecule, the heterotrimeric G protein. Moreover, oxidative burst can’t be produced by dissolution of O$_3$ in the apoplastic fluid directly, but requires signaling through the heterotrimeric G protein. Thus a connection between O$_3$ signal perception and the production of oxidative burst is established by *Arabidopsis* heterotrimeric G protein.

The mechanism was further explored by which the heterotrimeric G protein mediates ozone signaling to induce plant responses to ozone. While heterotrimeric G protein signaling is involved in stress-associated physiological processes, information about the structure and biochemistry of heterotrimeric G protein was not available. The knowledge gap was filled between the structure and function of the *Arabidopsis* heterotrimeric G protein. The interaction between G protein subunits using the fluorescence resonance energy transfer technique was identified. The large complex with other proteins of which the heterotrimeric G protein is a component was analyzed. Surprisingly, it was found that G protein complex can be dissociated by hydrogen peroxide treatment. This is of particular importance because this dissociation suggests that the heterotrimeric G protein can be activated by O$_3$-derived hydrogen peroxide.
directly or indirectly, supporting the essential roles of G protein in plant ozone responses. This finding significantly helps scientists to understand the mechanism of G protein activation in the O$_3$ stress response and to develop new strategies to derive plants with higher O$_3$ resistance in the long run.

Activation of UPR is not only the central part of ER stress, also critical for other plant defense response, in which a large amount of secretory or vacuolar proteins are induced like pathogen stress. In contrast to its extensive study in animal systems, ER stress is an almost unexplored field in plant biology. This study links the important signaling molecule, heterotrimeric G protein with a common biological process, ER stress response. This signaling pathway was not even reported in animal ER stress responses (Schroder and Kaufman, 2005). ER stress can induce cell death in plants, as it does in animal cells. ER stress can kill cells and it is involved in a number of human diseases, including diabetes, Gaucher’s disease, and Huntington’s disease. Given the similar toxic effect on animal and plants, the investigation of UPR-signaling machinery in plant system brings new insight to animal UPR study. Furthermore, it was found that the G$\beta$ subunit of heterotrimeric G protein is localized in the ER, which supports the notion that G protein signaling is directly involved in ER stress.

The significance of research on the plant ER stress response lies in its connection with other biotic and abiotic stress response. The ER is central to the cell’s ability to coordinate its protein biosynthetic and secretory activities with calcium storage and signaling functions. The exploration of the relationship between ER stress and oxidative...
stress was performed. A similar oxidative burst in ER stress and oxidative stress was demonstrated. Moreover, the protective role of the oxidative burst in UPR induced cell death was demonstrated. Hence, the ability of plants to produce ROS in response to ER stress curtails the spread of cell death. It has been difficult to determine whether such ROS signaling plays a positive role in the stress response because ROS production also contributes to cell death. However, a recent study suggests that the ability to produce ROS curtails pathogen-induced cell death in plants homozygous for the \textit{lsd1} mutation, which was identified through its propensity to produce “runaway cell death,” (Weymann et al., 1995) a phenomenon in which the HR lesions observed in response to pathogens and ROS expand to much larger sizes than observed in wildtype plants. The study reports the paradoxical observation that cell death is even more extensive when the \textit{lsd1} null mutation is combined with an \textit{atbbohD} mutation and can be curtailed by overexpression of the \textit{AtrbohD} gene (Torres et al., 2005). These observations suggest that endogenous ROS play a protective role, limiting the spread of cell death. The parallels between what we observe in UPR-induced cell death and the control of HR in the pathogen response suggests that the plant cell death response has a common physiological and molecular basis irrespective of the causative agent. The interconnection between ER stress and oxidative stress is of particular significance because this expands the knowledge of how plant stress responses are interconnected.
Future Direction

The main question that remains to be answered in future studies is what downstream targets of heterotrimeric G protein are. Although several Gα interacting proteins have been identified, there is no report about Gβ protein interacting protein. Our preliminary analysis has shown that Gβ protein is associated with a very large complex of about 700 kD, which may contain other proteins. Thus, to dissect the specific function of Gβ protein in the ER stress, purification of Gβ-interacting protein under normal condition and ER stress condition is needed.

Downstream targets of Gβ protein also can be hypothesized based on the conservation of that between animal and plant system. One of the best known downstream signaling targets of the Gβγ complex in animal cells are phospholipases PLCβ2 and PLCβ3 (Bonacci et al., 2005; Feng et al., 2005). Moreover, accumulating evidence shows that a plant cell’s response to pathogens and many abiotic stresses is mediated by activation of PLC (Cessna et al., 1998; Blume et al., 2000; Andersson et al., 2006). Hence, the activity of PLC can be directly tested in wildtype and agb1-2 mutant plants upon Tm treatment to determine whether PLC is the downstream target of Gβγ complex or not under the ER stress.

It was also showed that Gβγ complex is involved in the regulation of secretory processes in both neuronal cells and pancreatic cells (Shajahan et al., 2004; Blackmer et al., 2005; Gerachshenko et al., 2005). In *Arabidopsis*, we found that Gβγ complex is
localized in both intracellular membrane and plasma membrane. Thus, a careful analysis of the change in subcellular localization of the Gβγ complex in response to Tm may also provide insight of its function in UPR.
References

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