Heterotrimeric G Protein Signaling Is Required for Epidermal Cell Death in Rice

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In rice (Oryza sativa) adventitious root primordia are formed at the nodes as part of normal development. Upon submergence of rice plants, adventitious roots emerge from the nodes preceded by death of epidermal cells above the root primordia. Cell death is induced by ethylene and mediated by hydrogen peroxide (H₂O₂). Pharmacological experiments indicated that epidermal cell death was dependent on signaling through G proteins. Treatment with GTP-γ-S induced epidermal cell death, whereas GDP-β-S partially inhibited ethylene-induced cell death. The dwarf1 (d1) mutant of rice has repressed expression of the Ga subunit RGA1 of heterotrimeric G protein. In d1 plants, cell death in response to ethylene and H₂O₂ was nearly completely abolished, indicating that signaling through Ga is essential. Ethylene and H₂O₂ were previously shown to alter gene expression in epidermal cells that undergo cell death. Transcriptional regulation was not generally affected in the d1 mutant, indicating that altered gene expression is not sufficient to trigger cell death in the absence of Ga. Analysis of genes encoding proteins related to G protein signaling revealed that four small GTPase genes, two GTPase-activating protein genes, and one GDP dissociation inhibitor gene but not RGA1 were differentially expressed in epidermal cells above adventitious roots, indicating that Ga activity is regulated posttranscriptionally.

G proteins act as signaling molecules that are activated by binding of GTP (Perfus-Barbeoch et al., 2004). G proteins can be classified into heterotrimeric G proteins comprised of α-, β-, and γ-subunits, small GTPases, and other unconventional G proteins such as the extra-large GTP-binding protein AtXLG1 from Arabidopsis (Arabidopsis thaliana; Ding et al., 2008) or OsYchF1 from rice (Oryza sativa; Cheung et al., 2008). Plasma membrane localized G protein-coupled receptors (GPCRs) transduce an extracellular signal to a heterotrimeric G protein complex. Heterotrimeric G proteins are localized at the plasma membrane where they interact with downstream effectors (Temple and Jones, 2007). Rice possesses one Ga protein, one Gβ protein, and two Gγ proteins, all of which were shown to be localized at the plasma membrane (Kato et al., 2004). The Ga subunit was present in a large 400-kD complex containing Gβ, Gγ, and likely some other proteins as the Gaβγ trimer has an apparent molecular mass of 100 kD. The dwarf1 (d1) mutant of rice is defective in Ga (RGA1; Fujisawa et al., 1999). In extracts from d1 plants lacking Ga, the Gβγ dimer was freed from the larger complex, indicating that Ga is required for recruiting the Gβγ dimer to the membrane-bound complex (Kato et al., 2004).

G protein subunits in plants are generally encoded by single or few genes. In Arabidopsis and rice the Ga and Gβ subunits are each encoded by one gene and the Gγ subunit is encoded by two genes (Perfus-Barbeoch et al., 2004). Nonetheless, heterotrimeric G proteins are involved in the regulation of diverse processes. The Ga subunit GPA1 of Arabidopsis was shown to take part in the regulation of stomatal closure (Wang et al., 2001) and in modulation of cell proliferation (Ullah et al., 2001). The gpa1 null mutant of Arabidopsis had reduced cell division activity in aerial parts while overexpression promoted cell division, an effect that was shown to be due to altered sensitivity toward auxin (Ullah et al., 2001, 2003). Analysis of the d1 mutant showed that Ga in rice is required for normal shoot elongation and seed growth (Fujisawa et al., 1999). The d1 mutant has a nearly 1,000-fold lower sensitivity toward GA3 (Ueguchi-Tanaka et al., 2000). Likewise, induction of α-amylase activity in the aleurone of rice seeds required a 1,000-fold higher GA3 concentration as compared to wild type, and α-amylase transcripts accumulated to much lower levels in d1 than in the wild type. These phenotypes implicated a role for a heterotrimeric G protein in GA signaling. In addition, d1 displayed reduced sensitivity toward 24-epi-brassinolide in the inhibition of root growth, and coleoptile elongation in rice, indicating that Ga may be linked to brassinosteroid signaling or activity (Wang et al., 2006; Oki et al., 2009).

Heterotrimeric G proteins are also involved in stress-related processes such as in oxidative stress induced by ozone (O₃) treatment (Joo et al., 2005). Null
mutations of the single Ga and Gβ subunits of Arabidopsis showed different responses. gpa1 mutants lacking Ga were more resistant to O₂-induced damage, whereas mutants lacking the Gβ subunit were more susceptible. O₂ is known to trigger an oxidative burst, i.e. production of high levels of hydrogen peroxide (H₂O₂) similar to that observed in the biotic hypersensitive defense response. Wild-type plants showed biphasic H₂O₂ production when treated with O₂. gpa1 plants no longer produced elevated H₂O₂ in the presence of O₂. The agb1 null mutant, which is defective in the Gβ subunit, showed the late but not the early peak of H₂O₂ production, indicating that the early phase of H₂O₂ production required both Ga and Gβ while the later phase required only Ga (Joo et al., 2005).

G proteins are involved in the plant defense response. agg1 mutants affected in one of the two Arabidopsis Gy subunit genes had reduced resistance to necrotrophic pathogens (Trusov et al., 2007). In rice, Ga likely participates in disease resistance. Transcript levels of RGA1 were elevated in response to an avirulent rice blast strain. Similar induction of RGA1 was observed after application of a sphingolipid elicitor (Suharsono et al., 2002). In accord with the known fact that reactive oxygen species (ROS) act as signals in disease resistance, sphingolipid elicitor treatment caused production of H₂O₂ in suspension-cultured rice cells. d1 mutant lines with reduced RGA1 mRNA levels displayed a dwarf phenotype and showed reduced resistance to the avirulent rice blast strain, albeit responses to a virulent strain were not different between the wild type and d1. Suspension cells of d1 showed strongly reduced H₂O₂ levels when treated with sphingolipid elicitor (Suharsono et al., 2002), supporting the view that H₂O₂ levels are controlled through Ga activity. Constitutive activation of the small GTPase OsRac1 restored H₂O₂ production in d1 suspension cells. OsRac1 was shown to signal resistance to pathogen infection, indicating that two different G proteins play a role in H₂O₂-mediated disease resistance in rice (Ono et al., 2001).

Heterotrimeric G proteins cycle between an active, GTP-bound state and an inactive, GDP-bound state. GTPase-activating proteins (GAPs) activate the intrinsic GTPase activities of G proteins, resulting in hydrolysis of GTP. In rice, 85 putative GAP genes were identified (Jiang and Ramachandran, 2006). Guanine-nucleotide exchange factors (GEFs) catalyze the exchange of GDP with GTP thereby restoring the active state of G proteins (Sprang, 2001).

Flooding is a frequent environmental stress to which plants get exposed to (Bailey-Serres and Voesenek, 2008). It results in a suboptimal supply of oxygen that affects growth and development, and ultimately threatens survival of plants. Rice is a semiaquatic plant and as such well adapted to survive hypoxic conditions. In rice, adventitious root initials develop at the nodes of the rice stem as part of regular development (Bleecker et al., 1986). Upon submergence, adventitious roots emerge from the stem to support or replace the primary root system in the soil that becomes dysfunctional at anaerobic conditions. Epidermal cells that are located at the nodes above adventitious root primordia undergo cell death prior to onset of adventitious root growth, likely to prevent mechanical damage to the growing root tip (Mergemann and Sauter, 2000). Epidermal cell death as well as adventitious root growth is controlled by ethylene. Ga₃ promotes ethylene-induced cell death while abscisic acid acts as a strong inhibitor (Steffens and Sauter, 2005). Ethylene was shown previously to down-regulate the ROS scavenger gene OsMT2b (Steffens and Sauter, 2009a). Constitutive genetic down-regulation of OsMT2b through OsMT2b-RNAi was shown to promote H₂O₂ accumulation in rice cells (Wong et al., 2004). Reduced OsMT2b transcript levels in rice OsMT2b::Tos17 and in OsMT2b-RNAi lines caused epidermal cell death in the absence of ethylene. When stems were treated with ethylene, cell death rates exceeded those observed in the wild type, indicating that H₂O₂ promotes epidermal cell death.

Based on previous findings, we hypothesized that Ga might participate in the regulation of H₂O₂ abundance and consequently in epidermal cell death in rice. To test this hypothesis, effector studies and mutant analysis of the Ga-deficient d1 mutant were performed. The results indicate a key role for Ga signaling in epidermal cell death downstream of ethylene and downstream of H₂O₂.

RESULTS

Epidermal Cell Death in Rice Is Mediated by a G Protein

Death of epidermal cells above adventitious roots is regulated by ethylene. To understand how the cell death response is mediated, a possible role of G proteins in cell death signaling was analyzed using a pharmacological approach. GTP-γ-S irreversibly binds to G proteins thus locking them in a permanently activated state. Treatment of rice cv Pin Gaew 56 (PG56) stem sections with 0.1 mM or 1 mM GTP-γ-S for 24 h resulted in a doubling of the cell death rate from 16.1% to 33.3% or 31.4%, respectively (Fig. 1A). In comparison, treatment with 150 μM ethephon resulted in a cell death rate of 83.6%. Cell death induced by GTP-γ-S was observed exclusively in epidermal cells above adventitious root initials. Treatment of stem sections of the rice cv Kinmaze with 1 mM GTP-γ-S induced cell death rates of 41.1% after 26 h and of 47.9% after 48 h (Table I). Treatment with 150 μM ethephon resulted in a cell death rate of 52.6% after 26 h and of 87.9% after 48 h (Table I).

In a complementary experiment, stem sections were treated with GDP-β-S that locks G proteins in an inactive state. Treatment of rice cv PG56 stem sections with 0.1 mM GDP-β-S did not alter the basal cell death rate (Fig. 1B). Treatment with 150 μM ethephon resulted in a cell death rate of 38.3% after 24 h.
were preincubated for 3 h with water or 0.1 mM GDP-
subsequent treatment using Evans blue staining. Prior to treatment, stem sections
above adventitious roots were determined subse-

d1

Figure 1. Ethylene-induced cell death is mediated by G protein in rice
cv PG56. A, Stem sections were treated for 24 h without or with 0.1 mM
GTP-γ-S or 150 μM ethephon (E). Rates of epidermal cell death
above adventitious roots were determined using Evans blue staining. Bars indicate averages ± se from 23 to 24 stem sections per treatment.
Different letters above bars indicate significantly different values (P < 0.05). B, Stem sections were treated for 24 h with or without 0.1 mM
GDP-β-S in the absence or presence of 150 μM ethephon, and rates of
epidermal cell death above adventitious roots were determined subse-

Treatment with 150 μM ethephon in the presence of 0.1
mM GTP-β-S lowered the ethylene-induced cell death
rate significantly to 64%. Albeit the G protein effectors
did not fully induce or completely abolish the cell
death response, the results nonetheless pointed to the
involvement of a G protein in cell death signaling.

The d1 Mutant Is Impaired in Submergence Signaling,
Ethylene Signaling, and H2O2 Signaling of Epidermal
Cell Death

RGA1 (D1) encodes the sole Gα subunit of hetero-
trimeric G proteins in rice. The three allelic d1 mutant
lines d1-248, d1-1232, and d1-1361 were shown to
have reduced RGA1/D1 mRNA levels and display a
dwarfed phenotype (Suharsono et al., 2002; Fig. 2A).
The dwarfed shoot phenotype results from reduced
internodal elongation as shown for d1-1361 (Fig. 2B).

Table I. Average cell death rates (%) in cv Kinmaze after treatment with or without 150 μM ethephon (E) or 1 mM GTP-γ-S for 26 or 48 h
Results are averages (± se) from five to eight stem sections analyzed per treatment. Values with different superscript letters are significantly
different from each other at P < 0.05.

| Time | Control | 150 μM E | 1 mM GTP-γ-S |
|------|---------|----------|--------------|
| 26   | 8.4 (±4.5)b | 52.6 (±14.4)b | 41.1 (±12.5)b |
| 48   | 25.2 (±11.3)b | 87.9 (±9.7)b | 47.9 (±12)b |

While shoot growth is strongly inhibited in d1, the
formation of adventitious root primordia at the nodes
of the stem is not affected (Fig. 2C). Both the wild type
and all three d1 lines possessed an average of 17 root
primordia per node.

Since d1 is a mutant of the cv Kinmaze background,
cell death was further analyzed in this genotype. To
study epidermal cell death in response to submer-

d1-248 plants, the basal cell death rate was lower than
in the wild type and submergence did not promote cell
death, indicating that a Gα-containing G protein may
be required for cell death signaling.

To further study the level at which Gα may act in cell
death signaling, stem sections of wild-type and d1
plants were treated with 150 μM ethephon and cell
death rates were determined after 26 h (Table II) and
48 h (Fig. 3B). In the wild type, cell death rates rose
from about 20.9% in untreated stems to 48.5% after
26 h and to 88.2% after 48 h of ethylene treatment. In
d1-248, the basal cell death rate was zero and only
minor induction by ethylene to 1.1% after 26 h and to
22.5% after 48 h was observed. Similar results were
obtained for the allelic lines d1-1232 and d1-1361 (Fig.
3B, Table II). Weak albeit not significant induction of
cell death in the d1 lines may be due to residual RGA1/
D1 expression (Suharsono et al., 2002).

It was shown previously that H2O2 acts downstream
or independent of ethylene as a pro-death signal in
epidermal cells (Steffens and Sauter, 2009a). In wild-
type cv Kinmaze, epidermal cell death rates rose from
23% in controls to more than 50% after treatment with
0.01% (v/v) or 0.1% (v/v) H2O2 (Fig. 3C). In the wild
type, H2O2 had no significant effect on cell death rates,
which did not exceed 20% after treatment with H2O2
for 48 h. In conclusion, neither submergence, nor
ethylene, nor H2O2 promoted epidermal cell death in
the Gα-deficient d1 lines to a degree comparable to the
wild type, supporting the view that perception or
transmission of these signals is dependent on a Gα-
containing G protein.
Regulation of Genes Encoding G Proteins, Small GTPases, GAPs, GEFs, GDP Dissociation Inhibitors, and GPCRs in Epidermal Cells Above Adventitious Root Primordia

To evaluate a general role of G protein signaling in cell death induction, genes encoding G proteins, small G proteins, GAPs, GEFs, GDP dissociation inhibitors (GDIs), and GPCRs were identified from rice and their expression was analyzed using previously described microarray data (Steffens and Sauter, 2009a). Previously, 61 genes were found to be up- or down-regulated in epidermal cells above adventitious roots after treatment with either ethylene or H$_2$O$_2$ ($P < 0.001$). None of these genes encoded for a G protein or for a G protein-regulating protein, indicating that these are not regulated by pro-death signals at the transcriptional level.

To analyze if G protein genes were differentially expressed in epidermal cells above adventitious roots prior to cell death induction we analyzed about 2,600 genes that were previously found to be differentially expressed in epidermal cells above adventitious root primordia as compared to other epidermal cells above adventitious root primordia.

**Figure 2.** The $d1$ mutant of rice has shorter internodes but is not impaired in the formation of adventitious root primordia. A, Shoots of 20-week-old wild-type (wt) rice plants cv Kinmaze and of three alleles of the $d1$ mutant, $d1$-248, $d1$-1232, and $d1$-1361. On average, shoots of wild-type plants were about 115 cm in length, $d1$-248 shoots were about 60 cm long, and shoots of lines $d1$-1232 and $d1$-1361 were about 65 cm in length as determined from 12 plants for each line. B, The distance between the second (2.) and third (3.) youngest node indicates the length of the internodes in the wild type and $d1$-1361. C, Cross sections through the second node show adventitious root primordia in the wild type and $d1$-1361.

**Figure 3.** Epidermal cell death induced by submergence, ethylene, or H$_2$O$_2$ is mediated by Ga. A, Submergence-induced epidermal cell death was analyzed in the wild type (wt) and in $d1$-248 rice cv Kinmaze plants. The plants were partially submerged for 26 or 48 h, or kept in air as a control. Values indicate averages ± s.e from 12 to 25 stem sections analyzed per treatment. The asterisk indicates a significantly different value ($P < 0.001$). B, Stem sections were isolated from wild-type, $d1$-248, $d1$-1232, and $d1$-1361 rice cv Kinmaze plants and treated with or without 150 $\mu$M ethephon for 48 h. Bars indicate averages ± s.e from 15 to 30 stem sections analyzed per treatment. The asterisk indicates a significantly different value ($P < 0.001$). C, Stem sections were isolated from wild-type, $d1$-248, $d1$-1232, and $d1$-1361 rice cv Kinmaze plants and treated for 48 h with H$_2$O$_2$ at the concentrations indicated. Values are averages ± s.e from 15 to 18 wild-type, 23 to 34 $d1$-1361, five to 10 $d1$-248, and three to six $d1$-1232 stem sections analyzed. Asterisks indicate significantly different values ($P < 0.001$).
cells (Steffens and Sauter, 2009a, 2009b). Rice has four genes encoding heterotrimeric G protein subunits, RGA1, RGB1, RGG1, and RGG2, none of which were differentially expressed in epidermal cells. The 107 small GTPases from rice can be classified into six families, Rop, Rab, Ras, Arf, Ran, and other GTPase genes (Supplemental Table S1; Jiang and Ramachandran, 2006). Of these, one Rab and two Rop genes were down-regulated in epidermal cells above adventitious root primordia as compared to other epidermal cells, and a GTP-binding protein synthesis factor of the family of other GTPase genes was up-regulated (Supplemental Table S1). The 60 known GAP genes of rice divide into the subgroups RopGAP, RabGAP, ArfGAP, RanGAP, and other GAPS (Supplemental Table S2). Of these, RopGAP10 and a member of the family of other GAPS were down-regulated in epidermal cells above root primordia (Supplemental Table S2). One of the three GDI genes found in the rice genome was down-regulated in epidermal cells above adventitious roots (Supplemental Table S3), whereas none of the five GEF genes were differentially expressed in epidermal cells above adventitious roots (Supplemental Table S4). Eleven of the 13 GPCR genes that were bioinformatically predicted for rice (Gookin et al., 2008) were identified in the microarray analysis (Supplemental Table S5). None of these were regulated.

Table II. Average cell death rates ± se (%) of three d1 lines after treatment with 150 μM ethephon (E) or without ethephon (control) for 26 h

| Genotype | Control | 150 μM E |
|----------|---------|----------|
| Wild type | 20.9 (±8.6)a | 48.5 (±2.3)b |
| d1-248 | 0° | 1.1 (±1.1)b |
| d1-1232 | 0.4 (±0.4)a | 2.0 (±1.4)a |
| d1-1361 | 0° | 2.0 (±2.0)a |

Values with different superscript letters are significantly different from each other at P < 0.001.

Taken together, the results showed that defined G protein genes were differentially expressed in epidermal cells above root primordia prior to cell death induction, possibly enabling these cells to initiate or execute cell death.

Transcriptional Regulation in Response to Ethylene and H₂O₂ Is Not Dependent on Gα

We previously reported for the rice cv PG56 that nodal epidermal cells above adventitious root primordia are covered by a cuticle that has a distinct surface structure as compared to the cuticle of epidermal cell that do not cover a root. To find out if this morphological distinction was conserved and thus possibly relevant to cell death signaling we performed scanning electron microscopy (SEM) studies on cv Kinmaze. Nodal epidermal surfaces were scanned in areas above adventitious roots and in areas that did not cover adventitious roots (Fig. 4). Unlike what was observed in cv PG56, epidermal cells of cv Kinmaze did not display differences in epicuticular structures irrespective of their localization, indicating that it was not a hallmark of epidermal cell death fate (Fig. 4, A–C). Analysis of d1-1361 plants revealed that these had the same surface structures as the wild type with no morphological differences between epidermal areas above adventitious roots and other epidermal areas (Fig. 4, D–F).

It was previously reported for indica rice cv PG56 that epidermal cells that undergo cell death are molecularly distinct from those that do not (Steffens and Sauter, 2009a). To test if differential gene expression was conserved in japonica cv Kinmaze we selected 12 genes representative of the major categories identified, i.e. signal transduction, stress response, and ethylene synthesis (Supplemental Fig. S1). MT2b is an H₂O₂ scavenger and PAP16-like a purple acid phosphatase16-like protein. CDC6 contains a FAR1 DNA-binding

Figure 4. Nodal epidermal surfaces in cv Kinmaze do not differ between the wild type (wt) and d1. SEM pictures from the second node of rice cv Kinmaze. A and D, Bulges (arrows) develop above underlying adventitious root initials. B and E, Surface of epidermal cells above an adventitious root. C and F, Surface of epidermal cells that do not cover an adventitious root.
domain and could be a transcriptional regulator as are HOX9, MYB, ARF2, ARF3, and ANT-like. HT1-like is a Ser/Thr kinase, and BBI3-3 a Bowman-Birk-type Ser protease inhibitor. Finally, ACC oxidase1 and ethylene overproducer-like are involved in ethylene biosynthesis. Reverse transcription (RT)-PCR results indicated that these genes were differentially expressed in epidermal cells above adventitious root primordia of cv Kinmaze as was observed for cv PG56. Next, regulation of these genes by ethylene and H$_2$O$_2$ was analyzed. The lag phase for induction of cell death in cv Kinmaze is longer than in cv PG56. Therefore, cv Kinmaze stem sections were treated for 20 h. Furthermore rice cv Kinmaze is more sensitive to H$_2$O$_2$ with significant induction of cell death by 0.01% (v/v) H$_2$O$_2$ rather than 3% (v/v) H$_2$O$_2$ as was required for cv PG56. Taking these differences into consideration, treatment with 150 $\mu$M ethephon or 0.01% (v/v) H$_2$O$_2$ for 20 h resulted in similar gene regulation as previously observed for cv PG56. MT2b, for instance, was down-regulated by ethylene and H$_2$O$_2$ in both cultivars.

To find out if gene regulation was dependent on G$\alpha$ signaling, we next analyzed expression of selected genes in the d1-1361 mutant. When relative transcript levels were compared between the wild type and d1-1361, highly similar expression patterns were observed. These results indicated that signaling through G$\alpha$ was not generally required for transcriptional regulation by ethylene or H$_2$O$_2$. In summary, our data support the view that G$\alpha$ acts downstream of ethylene and H$_2$O$_2$ and is indispensable for signaling of cell death. However, gene regulation by ethylene or H$_2$O$_2$ can occur through a G$\alpha$-independent signaling pathway.

**DISCUSSION**

Heterotrimeric G proteins, small G proteins, and Ran-type GAPs were shown to regulate disease resistance in rice (Tameling and Baulcombe, 2007). d1 plants deficient in the G$\alpha$ subunit of heterotrimeric G proteins displayed enhanced susceptibility to the avirulent rice blast fungus. G$\alpha$ was furthermore shown to be required for induction of the small G protein gene Rac1 and of the disease resistance gene PBZ1 in rice. Rac1 is a member of the Rop family of small GTPases that directly interacts with the respiratory burst oxidase homolog (RBOH) protein, which functions as NADPH oxidase in plants (Wong et al., 2007). Constitutively activated OsRac1 promoted H$_2$O$_2$ synthesis in rice suspension cells and plants. The enhanced susceptibility to avirulent rice blast fungus observed in d1 was rescued by constitutively activated Rac1. G$\alpha$ was therefore proposed to act upstream of OsRac1 and upstream of ROS producing RBOH (Kawasaki et al., 1999; Suharsono et al., 2002). Disease resistance is brought about by various cellular responses, one of which is the hypersensitive response. Constitutive activation of Rac1 promoted disease resistance, but it was not sufficient to promote a hypersensitive cell death response. Thus, disease resistance conferred by G$\alpha$ and OsRac1 in rice does not involve induction of cell death. By contrast, G$\alpha$ was shown here to play a central role in the epidermal cell death response. Down-regulation of the single G$\alpha$ gene RGA1 (D1) resulted in near-complete inhibition of cell death induced by submergence, treatment with ethylene, or treatment with H$_2$O$_2$. These observations suggested that G$\alpha$ acts downstream of H$_2$O$_2$. It is also possible that G$\alpha$ acts in a dual way, for one downstream of RBOH and, in addition, through regulation of RBOH by way of Rac1 as described in disease resistance (Wong et al., 2007).

Binding of Rac1 to RBOH is facilitated by a Ca$^{2+}$- and phosphorylation-induced conformational change of the N-terminal cytoplasmic loop of RBOH (Wong et al., 2007; Ogasawara et al., 2008), indicating that Rac1 induces RBOH-dependent H$_2$O$_2$ production through posttranscriptional regulatory mechanisms. Neither RGA1 nor Rac1 genes were regulated in epidermal cells above adventitious roots prior to cell death, or post-induction of cell death. This finding supports the view that G$\alpha$ protein signaling of epidermal cell death is regulated posttranscriptionally.

Heterotrimeric G proteins were identified as an early mediator of stress signaling (Suharsono et al., 2002; Joo et al., 2005). Arabidopsis plants deficient in the G$\beta$ subunit of heterotrimeric G protein were more susceptible to damage by O$_3$ and displayed higher rates of leaf cell death than plants deficient in G$\alpha$ (Joo et al., 2005). By contrast, Arabidopsis plants lacking the G$\beta$ subunit displayed greater resistance to leaf cell death triggered by tunicamycin, whereas mutants lacking the G$\gamma$ subunit were as susceptible as the wild type (Wang et al., 2007). This difference in response may be due to the differential localization and to unique functions of the G$\alpha$ and G$\beta$ subunits. While the G$\alpha$ protein was detected at about equal amounts in the plasma membrane and in the endoplasmic reticulum (ER), the G$\beta$ protein was more abundant in the ER. Tunicamycin induces ER stress, and G$\beta$ may specifically participate in the control of the ER stress response (Wang et al., 2007).

In the ER stress response, knockout of the G$\beta$ subunit resulted in altered transcriptional regulation in response to tunicamycin treatment. In rice, expression levels of nearly 3,000 genes differed between epidermal cells that are located above adventitious root primordia and other epidermal cells, indicating that these cells possess different molecular cell identities which, in turn, may be related to their different fates (Steffens and Sauter, 2009b). Epidermal cells above root primordia undergo cell death when triggered by ethylene or H$_2$O$_2$, whereas other epidermal cells will not. Formation of adventitious root primordia at the nodes was not impaired in d1 plants. Despite the presence of adventitious root primordia, epidermal cells above these did not respond to pro-death
signals with cell death. Interestingly, differential gene expression between the two epidermal cell types, or in response to pro-death signals was not generally altered in d1 as compared to the wild type as exemplified for 12 selected genes.

The ROS scavenging capacity in d1 has not been described to date. According to this study, transcript levels of OsMT2b (Os05g0111300) were not altered in d1. Furthermore, previously published microarray data did not reveal regulation of other genes encoding for known ROS scavengers or ROS detoxifying enzymes in epidermal cells that undergo cell death (Steffens and Sauter, 2009a), supporting the view that d1 was not impaired in ROS scavenging. It is, however, possible that ROS scavenging activities are posttranslationally regulated.

It is conceivable that residual RGA1/D1 activity in d1 was sufficient to regulate gene expression but was insufficient to promote cell death. Ethylene-induced epidermal cell death is promoted in the presence of GA3 (Ueguchi-Tanaka et al., 2000). It is also conceivable that the cell death-promoting activity of Ga depends on brassinosteroid signaling as brassinosteroid sensitivity is also reduced in d1 (Wang et al., 2006; Oki et al., 2009). Contributions of these hormones to Ga-mediated epidermal cell death signaling will have to be resolved in future studies. It is further possible that two signaling pathways exist. One pathway leading from ethylene or H2O2 to cell death is strictly dependent on Ga signaling. A second pathway that is initiated by ethylene or H2O2 is independent of Ga and results in differential expression of at least some genes. The results further showed that regulation in response to pro-death signals of the subset of genes analyzed was not sufficient to trigger cell death in the absence of Ga.

In Arabidopsis seedlings, phytochrome-dependent cell death is mediated by heterotrimeric G protein. In hypocotyls of far-red grown seedlings that were subsequently exposed to white light, a heterotrimeric G protein was shown to take part in phytochrome A-mediated signaling leading to far-red irradiation-preconditioned cell death (Wei et al., 2008). The gpa1 mutant showed extenuated cell death in comparison to the wild type, while in the Gβ mutant agb1, cell death was intensified, indicating an antagonistic role of Ga and Gβ in this cell death pathway. In addition, ROS mediated this type of cell death. Interestingly, agb1 was more sensitive to H2O2 than wild-type seedlings, indicating that the G protein may modify the sensitivity of the seedlings to H2O2 stress. Whereas heterotrimeric G proteins have been shown to regulate cell death and their diverse roles have been studied in various plant species, no information is available on the functions of those G protein genes that were found to be differentially expressed in epidermal cells above adventitious roots. Their cellular function in cell death, cell type specification, or other cellular process has yet to be elucidated.

CONCLUSION

Epidermal cell death in rice is induced by ethylene and H2O2 and is accompanied by transcriptional regulation in response to these pro-death signals. The work presented here identified G protein signaling through Ga (D1) as an essential step in epidermal cell death signaling. Since no genes encoding G proteins or G protein regulatory proteins were transcriptionally controlled in dying epidermal cells after treatment with ethylene or H2O2, we conclude that heterotrimeric G protein activity is regulated posttranscriptionally. While cell death rates were strongly reduced in d1, some gene regulation was still observed in response to ethylene or H2O2 in d1, indicating that D1 may act downstream of transcriptional regulation.

MATERIAL AND METHODS

Plant Materials and Growth Conditions

Seeds of rice (Oryza sativa indica ‘PG56’) were cultivated according to Sauter (1997). Internodal stem sections were prepared from 12- to 14-week-old plants. They were excised 2 cm below the third youngest node. Seeds of rice japonica cv Kinnmaze were imbibed in 50 μM S-nitroso-N-acetylpenicillamine (Molecular Probes) to promote germination. Rice cv Kinnmaze wild type and d1 mutant lines were provided by Dr. Hann Ling Wong and Prof. Dr. Ko Shimamoto (Plant Molecular Genetics, Nara Institute of Science and Technology, Ikoma, Nara, Japan). Stem sections were prepared from 18- to 23-week-old plants (Sauter, 1997). They were excised 5 mm below the second node. Unless stated otherwise, stem sections had a total length of 20 cm. Up to eight stem sections were placed in a 150-mL beaker containing 20 mL of aqueous solutions of GTP-γ-S (Sigma Aldrich), GDP-β-S (Sigma Aldrich), ethephon (2-chloroethanephosphoric acid; Sigma Aldrich), or H2O2 (Roth) at the concentrations indicated. Plastic cylinders covered the beakers to assure high humidity. For submergence treatment, intact plants were partially submerged in a 600-L plastic tank filled with tap water with approximately 30 cm of the leaf tips remaining above the surface. Plants and stem sections were incubated in a growth chamber at 27°C in continuous light with 150 μE m-2 s-1.

Evans Blue Staining

After treatment of plants or stem sections, the node was excised and stained with 2% (w/v) Evans blue for 3 min (Mergemann and Sauter, 2000). Subsequently, nodes were washed twice with water. Staining was observed with a binocular (Olympus). Each epidermal patch above a root initial that showed blue staining was counted as one cell death event. Nodes of cv Kinnmaze contained approximately 17 root primordia. Cell death events were calculated as percentage of the total number of epidermal patches above root primordia analyzed.

SEM

Nodes from 12-week-old rice cv Kinnmaze wild type and d1-T361 plants were harvested and prepared for SEM. SEM was performed on a Zeiss DSM 940.

Microarray Analysis

Microarray experiments were performed according to Steffens and Sauter (2009a). Briefly, stem sections were isolated from 12-week-old rice cv PG56 plants. The stem sections were treated with 150 μM ethephon, with 3% (v/v)
RT-PCR

Eighteen- to 23-week-old rice cv Kinmaze and d1-1361 plants were used to obtain epidermal patches above adventitious roots of the second node and epidermal patches from the epidermis approximately 5 mm above the ring of adventitious root primordia. Two biological repeats were performed using tissues obtained from stem sections that were treated for 20 h with 150 μM ethephon or 0.01% (v/v) H2O2. RNA was isolated using Tri-reagent (Sigma Aldrich) according to manufacturer's instructions. cDNA was synthesized from 100 ng of total RNA with oligo dT as primer. For amplification of the cDNA fragment, the primers and cycle numbers indicated in Supplemental legends. The reproducibility of the chip hybridization in microarray analysis was confirmed by components analysis of the robust multichip average value for the Pearson product moment correlation is indicated in the figure legends. The reproducibility of the chip hybridization in microarray analysis was confirmed by components analysis of the robust multichip average expression values (Steffens and Sauter, 2009a).

Statistical Analysis

Statistical analyses of cell death rates were performed with Minitab (Minitab Inc.). Rates in percent were transformed with arcsine(x/100) to obtain normal distributed data. Comparison of means was analyzed for statistical significance with an ANOVA and Tukey test. Constant variance and normal distribution of data were verified before statistical analysis and the P value was set to P < 0.001 if one of both conditions was not achieved. The P value for the Pearson product moment correlation is indicated in the figure legends. The reproducibility of the chip hybridization in microarray analysis was confirmed by components analysis of the robust multichip average expression values (Steffens and Sauter, 2009a).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Ca is not generally required for gene regulation by ethylene or H2O2, in epidermal cells that undergo cell death.

Supplemental Table S1. Rep, Rar, Rar, Arf, Ran, and other small GTPase genes (Jiang and Ramachandran, 2006) that are up-regulated, down-regulated, or not differentially expressed in epidermal cells above adventitious roots as compared to other epidermal cells.

Supplemental Table S2. RepGAP, RadGAP, ArfGAP, RanGAP, and other small GAP gene families (Jiang and Ramachandran, 2006) are down-regulated or not differentially expressed in epidermal cells above adventitious roots.

Supplemental Table S3. One of three GDI genes is regulated.

Supplemental Table S4. GEF genes (Jiang and Ramachandran, 2006) are not differentially expressed in epidermal cells above adventitious roots.

Supplemental Table S5. GPCR genes (Gookin et al., 2008) are not differentially expressed in epidermal cells above adventitious roots.

Supplemental Table S6. Primers and cycle numbers used for semiquantitative RT-PCR, and expected sizes of amplified cDNA fragments.

H2O2 or without effector for 4 h in the light. Epidermal patches above adventitious roots and epidermal patches from the epidermis approximately 5 mm above adventitious roots were harvested. RNA was isolated using Tri-reagent (Sigma Aldrich) according to manufacturer's instructions. Experiments were performed with three independent biological replicates resulting in 18 samples used for microarray hybridization. GeneChip rice genome arrays (Affymetrix) containing approximately 46,564 japonica transcripts and 1,260 transcripts representing the indica cultivar were used for transcriptome analysis. Analysis of RNA quality, chip hybridization, and data processing were performed at the MicroArray Facility (MAF, VIB) and were described elsewhere (Steffens and Sauter, 2009a). A cutoff at P values of 0.05 was used to indicate differentially expressed genes combined with a cutoff at a fold-change of 2.

ACKNOWLEDGMENTS

We gratefully acknowledge Dr. Hann Ling Wong and Prof. Dr. Ko Shimamoto for generously providing d1 mutant lines and Timo Staffel for excellent technical assistance.

Received May 27, 2009; accepted July 31, 2009; published August 5, 2009.

LITERATURE CITED

Bailey-Serres J, Voesenek LA (2008) Flooding stress: acclimations and genetic diversity. Annu Rev Plant Biol 59: 313–339

Bleecker AB, Schuette JL, Kende H (1986) Anatomical analysis of growth and developmental patterns in the internode of deepwater rice. Planta 169: 490–497

Cheung MY, Zeng NY, Tong SW, Li WYE, Xue Y, Zhao KJ, Wang C, Zhang Q, Fu Y, Sun Z, et al (2008) Constitutive expression of a rice GT-Pase-activating protein induces defense responses. New Phytol 179: 530–545

Ding L, Pandey S, Assmann SM (2008) Arabidopsis extra-large G proteins (XLGs) regulate root morphogenesis. Plant J 53: 248–263

Fujisawa Y, Kato T, Ohki S, Ishikawa A, Kitano H, Sasaki T, Asahi T, Iwatsuki Y (1999) Suppression of the heterotrimeric G protein causes abnormal morphology, including dwarfism, in rice. Proc Natl Acad Sci USA 96: 7575–7580

Gookin TE, Kim J, Assmann SM (2008) Whole proteome identification of plant candidate G-protein coupled receptors in Arabidopsis, rice, and poplar: computational prediction and in-vivo protein coupling. Genome Biol 9: R120

Jiang SY, Ramachandran S (2006) Comparative and evolutionary analysis of genes encoding small GTPases and their activation proteins in eukaryotic genomes. Physiol Genomics 24: 235–251

Joo JH, Wang S, Chen JG, Jones AM, Fedoroff NV (2005) Different signaling and cell death roles of heterotrimeric G protein alpha and beta subunits in the Arabidopsis oxidative stress response to ozone. Plant Cell 17: 957–970

Kato C, Mizutani T, Tamaki H, Kumagai H, Kiami T, Hirobe A, Fujisawa Y, Kato H, Iwasa Y (2004) Characterization of heterotrimeric G protein complexes in rice plasma membrane. Plant J 38: 320–331

Kawasaki T, Hemmi K, Ono E, Hatakayama S, Iwano M, Satoh H, Shimamoto K (1999) The small GTP-binding protein rac is a regulator of cell death in plants. Proc Natl Acad Sci USA 96: 10922–10926

Mengenmann H, Sauter M (2000) Ethylene induces epidermal cell death at the site of adventitious root emergence in rice. Plant Physiol 124: 609–614

Ogasawara Y, Kaya H, Hiraoka G, Yumoto F, Kimura S, Kadota Y, Hishinuma H, Senzaki E, Yamagoe S, Nagata K, et al (2008) Synergistic activation of the Arabidopsis NADPH oxidase AtRbohD by Ca2+ and phosphorylation. J Biol Chem 283: 8885–8892

Oki K, Inaba N, Kitagawa F, Fujioka S, Kitano H, Fujisawa Y, Kato H, Iwasa Y (2009) Function of the alpha subunit of rice heterotrimeric G protein in brassinosteroid signaling. Plant Cell Physiol 50: 161–172

Ono E, Wong HL, Kawasaki T, Hasegawa M, Kodama O, Shimamoto K (2001) Essential role of the small GT-Pase Rac in disease resistance of rice. Proc Natl Acad Sci USA 98: 759–764

Perfus-Barbeoch L, Jones AM, Assmann SM (2004) Plant heterotrimeric G protein function: insights from Arabidopsis and rice mutants. Curr Opin Plant Biol 7: 719–731

Sauter M (1997) Differential expression of a CCK (cck2-activating kinase)-like protein kinase, cyclins and cdc2 genes from rice during the cell cycle and in response to gibberellin. Plant J 11: 181–190

Sprang SR (2001) Conformational display: a role for switch polymorphism in the superfamily of regulatory GT-Pases. Sci STKE 2000: 50

Steffens B, Sauter M (2005) Epidermal cell death in rice (Oryza sativa L.) is regulated by ethylene, gibberellin and abscisic acid. Plant Physiol 139: 713–721

Steffens B, Sauter M (2009a) Epidermal cell death in rice is confined to cells with a distinct molecular identity and is mediated by ethylene and H2O2 through an auto-amplified signal pathway. Plant Cell 21: 184–196

Steffens B, Sauter M (2009b) Epidermal cells that undergo cell death differentially express cell identity genes. Plant Signal Behav 4: 247–248

Suharsono U, Fujisawa Y, Kawasaki T, Iwashiki Y, Satoh H, Shimamoto K (2002) The heterotrimeric G protein α subunit acts upstream of the small
Tameling WI, Baulcombe DC (2007) Physical association of the NB-LRR resistance protein Rx with a Ran GTPase-activating protein is required for extreme resistance to Potato virus X. Plant Cell 19: 1682–1694
Temple BR, Jones AM (2007) The plant heterotrimeric G-protein complex. Annu Rev Plant Biol 58: 249–266
Trusov Y, Rookes JE, Tilbrook K, Chakravorty D, Mason MG, Anderson D, Chen JG, Jones AM, Botella JR (2007) Heterotrimeric G protein gamma subunits provide functional selectivity in Gβγ dimer signaling in Arabidopsis. Plant Cell 19: 1235–1250
Ueguchi-Tanaka M, Fujisawa Y, Kobayashi M, Ashikari M, Iwasaki Y, Kitano H, Matsuoka M (2000) Rice dwarf mutant d1, which is defective in the alpha subunit of the heterotrimeric G protein, affects gibberellin signal transduction. Proc Natl Acad Sci USA 97: 11638–11643
Ullah H, Chen JG, Temple B, Boyes DC, Alonso JM, Davis KR, Ecker JR, Jones AM (2003) The β-subunit of the Arabidopsis G protein negatively regulates auxin-induced cell division and affects multiple developmental processes. Plant Cell 15: 393–409
Ullah H, Chen JG, Young JC, Im KH, Sussman MR, Jones AM (2001) Modulation of cell proliferation by heterotrimeric G protein in Arabidopsis. Science 292: 2070–2072
Wei Q, Zhou W, Hu G, Wei J, Yang H, Huang J (2008) Heterotrimeric G-protein is involved in phytochrome A-mediated cell death of Arabidopsis hypocotyls. Cell Res 18: 949–960
Wong HL, Pinolioan R, Hayashi K, Tabata R, Yaeno T, Hasegawa K, Kojima C, Yoshioka H, Iba K, Kawasaki T, et al (2007) Regulation of rice NADPH oxidase by binding of Rac GTPase to its N-terminal extension. Plant Cell 19: 4022–4034
Wong HL, Sakamoto T, Kawasaki T, Umemura K, Shimamoto K (2004) Down-regulation of metallothionein, a reactive oxygen scavenger, by the small GTPase OsRac1 in rice. Plant Physiol 135: 1447–1456

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GTPase Rac in disease resistance in rice. Proc Natl Acad Sci USA 99: 13307–13312
Wang L, Xu YY, Ma QB, Li D, Xu ZH, Chong K (2006) Heterotrimeric G protein α subunit is involved in rice brassinosteroid response. Cell Res 16: 916–922
Wang S, Narendra S, Federoff N (2007) Heterotrimeric G protein signaling in the Arabidopsis unfolded protein response. Proc Natl Acad Sci USA 104: 3817–3822
Wang XQ, Ullah H, Jones AM, Assmann SM (2001) G protein regulation of ion channels and abscisic acid signaling in Arabidopsis guard cells. Science 292: 2070–2072
Wong HL, Pinolioan R, Hayashi K, Tabata R, Yaeno T, Hasegawa K, Kojima C, Yoshioka H, Iba K, Kawasaki T, et al (2007) Regulation of rice NADPH oxidase by binding of Rac GTPase to its N-terminal extension. Plant Cell 19: 4022–4034
Wong HL, Sakamoto T, Kawasaki T, Umemura K, Shimamoto K (2004) Down-regulation of metallothionein, a reactive oxygen scavenger, by the small GTPase OsRac1 in rice. Plant Physiol 135: 1447–1456