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Function of Heterotrimeric G Protein in Gibberellin Signaling

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Abstract

The importance of plant heterotrimeric G protein functions has recently been recognized. Rice and *Arabidopsis* mutants of genes coding the subunits of the G proteins have been isolated and physiological studies on these mutants have suggested that plant heterotrimeric G proteins are involved in several intra-signaling pathways driven by external signals, such as gibberellin, auxin, abscisic acid, brassinolide, ethylene, light, and elicitor. The possible functions of rice heterotrimeric G proteins in gibberellin signaling are discussed here.

Introduction

Gibberellins (GAs) are a family of compounds with the ent-gibberellane as the basic structure, and more than 100 GAs have been identified in the plant kingdom. Some are biologically active, others are inactive. Studies on the biosynthesis of GAs, such as identification of enzymes involved in GA synthesis, isolation of genes encoding these enzymes, analysis of expression patterns of the genes and isolation of mutants for the genes, are making great progress. However, the biochemical processes of cell responses to GAs remain unknown. Hooley (1994) has proposed that the types of responses of plant cells and tissues to GAs are classified into three categories: cell growth in vegetative tissues, flower and fruit development, and seed reserve mobilization by aleurone cells. Research to understand the perception and transduction of GA signals has been conducted mainly in the last category, namely, the responses of aleurone cells in the Gramineae to GAs (Lovegrove and Hooley 2000). Here we focus on molecules involved in GA signaling, as proposed from studies of aleurone cells.

Gibberellin Perception at the Plasma Membranes in Plants

GA Perception Occurs on the Surface of the Plasma Membrane

GA perception is believed to occur on the surface of plasma membranes on the basis of results reported by Hooley and others (1991).

First a 17-thiol derivative of GA_4 was immobilized to Sepharose 6B beads (GA_4-Sepharose). The GA_4-Sepharose stimulated the expression of the α-amylase gene and the secretion of α-amylase in oat (*Avena fatua*) aleurone protoplasts, although the biological activity of the GA_4 was reduced by approximately two orders of magnitude through the immobilization. Because GA_4-Sepharose is membrane-impermeable, perception must occur at the surface of oat aleurone protoplasts.

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GA Perception Occurs Through a GA Receptor(s) on the Surface of the Plasma Membrane

A microinjection of GAs into barley (*Hordeum vulgare* L.) protoplasts resulted in no stimulation of the expression of theα-amylase gene (Gilroy and Jones 1994). The possibility that the protoplasts may be damaged during the microinjection procedure is excluded, because the microinjected protoplasts still had the ability to respond to externally applied GA_3. This result suggests that GA_3 could stimulate the expression of the α-amylase gene through its perception by a putative receptor(s) present on the surface of the plasma membrane and not inside the cell.

GA-Binding Proteins are Present in the Plasma Membrane Fraction

Two polypeptides (68 and 18 kDa) photoaffinity-labeled with GAs were found to be present in a plasma membrane fraction from oat (*Avena fatua*) aleurone protoplasts (Lovegrove and others 1998). In the experiments, a photoaffinity reagent (GAPR) with a biologically active and radioiodinated GA was used. The labeling is completed with biologically active, but not inactive, GAs. The amino acid sequence of the N-terminal part is GQVDPEIGDK for the photoaffinity-labelled 18 kDa polypeptide, but remains unknown for the 68 kDa polypeptide because the N-terminal is blocked. No information on the function of the 18 kDa polypeptide is available although its N-terminal amino acid sequence has been determined, because proteins with similar amino acid sequences are not found in the database. As Scatchard plots of GAPR binding to plasma membrane proteins have not been tested, the number of receptors is not clear. When there is only one specific binding site of GAPR, two polypeptides with 68 and 18 kDa may be the components of a complex. Although the two polypeptides bind to GAs, it remains to be investigated whether they are truly a receptor(s) or transporter(s). They are, however, important candidates for a GA receptor(s) or transporter(s) localized in the plasma membrane of oat (*Avena fatua*) aleurone protoplasts.

A Heterotrimeric G Protein May be Involved in GA Signaling

Mastoparan and Mastoparan 7 are activators of heterotrimeric G proteins in mammals and seem to be convenient chemicals for studying downstream components in the G protein-mediated signaling, of which the true signals are not identified. Mastoparan 7 stimulates the expression of the α-amylase gene in oat (*Avena fatua*) aleurone protoplasts (Jones and others 1998) and rice (*Oriza sativa* L.) embryoless half-seeds (Fujisawa and others 2001b), which raises two concepts concerning GA signaling. First, plant heterotrimeric G proteins may be involved in GA signaling. Second, the fact is reconfirmation that GAs are perceived at the surface of the plasma membrane because plant heterotrimeric G proteins are localized in the plasma membranes. Mastoparan and its analogs, however, are known to activate not only heterotrimeric G proteins but also some other proteins including calmodulin and phospholipase. Additional experiments are required to determine whether plant heterotrimeric G proteins actually participate in GA signaling.

A Heterotrimeric G Protein-Deficient mutant, *d1*, Results in Partial Defect in GA Signaling

A rice dwarf mutant, *d1*, has a mutation in the *α-subunit* gene of the heterotrimeric G protein (Ashikari and others 1999; Fujisawa and others 1999). The *α-subunit* gene is a single copy gene in rice and *Arabidopsis*. Western blot analysis shows that no α subunit exists in a plasma membrane fraction from *d1*, and so *d1* is a null mutant for the α subunit of heterotrimeric G protein (Fujisawa and others 2001b). The enzymatic activity and mRNA amount of α-amylase in embryoless half seeds of *d1* and normal cultivar were assayed after application of low concentrations of GA_3 (10^{-9} M to 10^{-7} M). Increases in the activity and amount, of a response to GA_3 for *d1* were repressed, but not completely, compared with those for the normal cultivar (Ueguchi-Tanaka and others 2000). The response of *d1* to high concentrations of GA_3 (more than 10^{-6} M GA), however, was similar to that observed with normal cultivar.

The results suggest the presence of at least two different pathways for GA signaling in plants. Plant heterotrimeric G proteins seem to be involved in a signaling pathway of GAs at low but not at high concentrations. Because heterotrimeric G proteins are known to be signal transducers and signal amplifiers in mammals, it seems likely that plant G proteins may also amplify week signals such as low concentrations of GAs. There is another interpretation for partial impairment of GA signaling in *d1*. On the assumption that there is only one GA signaling pathway, the heterotrimeric G protein may enchance GA sensitivity. In this case, the similar level of α-amylase induced at high concentrations of GA in *d1* and the normal cultivar may be influenced by the unusual condition of excess GA concentrations.

Transgenic Rice Plants Expressing Constitutively Active α Subunit of Heterotrimeric G Protein Produce Small Amounts of α-Amylase Without GAs

Substitution of an amino acid residue for a conserved glutamine one in the GTPase domains of the α-subunits of various G proteins has been shown to result in a constitutively active form (Kaziro and others 1991). The glutamine at position 223 of the α subunit of rice heterotrimeric G protein was substituted by a leucine one through site-directed mutagenesis. A recombinant protein synthesized in *E. coli* with the mutated cDNA (RGA1Q223L) was constitutively active. When the embryoless half seeds of rice transformants expressing mRNA for RGA1Q223L were incubated in acetate-Ca^{2+} buffer, activity of α-amylase, though a small amount, was induced even in the absence of GAs (Y. Iwasaki unpublished results). The induced amount was about the same as that when induced by 10^{-9} M GA_3 in the embryoless half seeds of the normal cultivar. This result suggests that the constitutively active α subunit drives the signaling pathway with low concentrations of GAs even when no GAs are supplied.

Slender (*slr*) Overcomes *d1*

A rice *slender* mutant (*slr1-1*) is produced by loss-of-function mutation of the *SLR1* gene, which is an ortholog of *GAI, RGA, RHT,* and *D8* (Ikeda and others 2001). The gene acts as a negative regulator of GA signaling in wild-type plants. The *slr1-1* is a constitutively GA-responsible phenotype. A double mutant generated by crossing *d1* and *slr1-1* shows *slr1-1* phenotype (Ueguchi-Tanaka and others 2000), a result that raises two possibilities: the SLR1 may be a downstream component of the G protein or it may be a component of the pathway functioning with high concentrations of GAs. The pathway with low concentrations of GAs, which involves the heterotrimeric G protein, appears to function weakly and negligibly when the pathway with high concentrations of GAs is driving. Because rice transformants expressing mRNA for RGA1Q223L show the normal phenotype and not *slr1-1* phenotype, the heterotrimeric G protein seems to be involved in the GA-signaling pathway with low concentrations of GAs, whereas SLR1 may be involved in the pathway with high concentrations of GAs. As discussed above, we cannot rule out the possibility that there is only one GA-signaling pathway. According to the assumption, RGA1Q223L protein (constitutively active α subunit of rice heterotrimeric G protein) seems to activate SLR1 only a little.

Second Messengers after GA Perception

On the basis of the results of the α-amylase induction pattern in *d1*, we have proposed the presence of two GA-signaling pathways in plants: the pathways operating at both low (10^{-9} to 10^{-7} M) and high concentrations of GAs (more than 10^{-6} M). In this section, we discuss possible downstream molecules in GA signaling, which may be regulated by a heterotrimeric G protein. Some second messengers—Ca^{2+}, cGMP and inositol-1,4,5-triphosphate—are important components in heterotrimeric G-protein-mediated signaling pathways in mammals (Kaziro and others 1991; Clapham and Neer 1993; Neves and others 2002). In plants, too, the second messengers have been proposed to be important signaling molecules in the downstream pathways of GA signaling, although most of them do not seem to be linked to one another and it remains obscure as to whether all the second messengers are produced through activation of the G proteins.

Plant Heterotrimeric G Proteins Activate Ca^{2+} Channels on the Plasma Membranes

A constitutively active α subunit of tomato heterotrimeric G protein (TGα1Q223L) was produced by substituting a leucine residue for the glutamine residue at position 223 by site-directed mutagenesis. Experiments with the patch-clamp technique showed that a Ca^{2+} channel in tomato plasma membrane was activated by TGα1-Q223L (Aharon and others 1998). This is a strong piece of evidence that plant heterotrimeric G proteins have the ability to regulate cytosolic Ca^{2+} concentrations via regulation of the activity of Ca^{2+} channels in the plasma membranes. Elevation in Ca^{2+} concentrations by the G proteins may be an important event in GA signaling. Experiments with plant material should be conducted to examine whether a GA receptor activates the heterotrimeric G protein and activated G protein stimulates Ca^{2+} channels in the plasma membrane.

Inositol-1, 4,5-Triphosphate in Plant Cells Increased with GAs at μM-Order Concentrations

The level of inositol 1,4,5-triphosphate increases about 3 times within 25 min after application of 5

μM GA_3 in embryoless half seeds of rice (Chen and others 1997). The increase is transient and not maintained for long. Inositol-1,4,5-triphosphate is considered to be an activator of Ca^{2+} channels localized in endoplasmic reticulum and to increase cytosolic Ca^{2+} concentrations. This may be one of the events in the early stages of signaling after GA perception.

External Ca^{2+} in the Medium May Enter Aleurone Cells Via Putative Calcium Channel or Transporter After Application of GAs at μM-Order Concentrations

GAs induces an increase in cytosolic Ca^{2+} concentration in the aleurone protoplasts of barley (Gilroy and Jones 1992). Treatment of the protoplasts with an incubation medium containing 10 mM Ca^{2+} and 5 μM of GA_3 for 4–6 h results in an increase from 50 to 150 nM in cytosolic Ca^{2+} concentration. The increase precedes an increase in the synthesis of α-amylase. When Ca^{2+} concentration in the incubation medium is below 500 μM, the cytosolic Ca^{2+} concentration and α-amylase synthesis do not increase even in the presence of GA_3. Consequently, the source of increased cytosolic Ca^{2+} is considered to be the medium. The increase of cytosolic Ca^{2+} may be attributable to an increase in Ca^{2+} influx at the plasma membrane. It is unknown whether Ca^{2+} influx in aleurone cells is regulated through activation of the G proteins.

Ca^{2+} ATPase in Plant Endoplasmic Reticulum is Induced by GAs of μM-Order Concentrations

The expression of the gene of endoplasmic reticulum membrane Ca^{2+} ATPase is induced when 5 μM of GAs is applied to rice aleurone cells (Chen and others 1997). The gene expression started within 1 h after GA_3 application and precedes an increase in α-amylase which occurs at 6 h after application. In transient assay in rice aleurone cells using a particle gun, the expression of introduced Ca^{2+} ATPase cDNA is observed to result in induction of the expression of α-amylase gene without GAs. The expression profile of a major gene in GA signaling, *α-amylase c gene* (*Osamy-c*), is studied using a chimera gene consisting of an *Osamy-c* promoter and luciferase cDNA. The level of luciferase activity induced by overexpression of Ca^{2+} ATPase cDNA seems to be the same as that induced when GA_3 is applied. This study suggests that changes in Ca^{2+} levels in intracellular compartments (in this case, an increase in the Ca^{2+} concentration in the lumen side of the endoplasmic reticulum through the action of overexpressed Ca^{2+} ATPase) play an important role in the induction of *Osamy-c* gene. There is also a possibility that the activity of α-amylase secretion may be stimulated by Ca^{2+} ATPase, because there is little preexisting transcripts of *GAMyb* and *α-amylase* genes.

The amount of Ca^{2+} ATPase mRNA is not induced in *d1* under low concentrations of GA_3 (Ueguchi-Tanaka and others 2000) but is under high concentrations. The reason *d1* does not induce α-amylase at low concentrations of GAs may be that it lacks Ca^{2+} ATPase. Because Ca^{2+} ATPase mRNA is induced in *d1* at high concentrations of GAs, *d1* has the ability to respond to high, but not low, concentrations of GAs for induction of *α-amylase* gene.

Calmodulin is Induced by GAs of μM-Order Concentrations

Calmodulin (CaM) mRNA is induced after application of GAs in barley (*Hordeum vulgare* L. cv Himaraya) within 1 h (Schuurink and others 1996). The time for induction of CaM mRNA is similar to that for Ca^{2+} ATPase. Barley CaM has been shown to be localized in the cytosol, the vacuolar membrane and the nucleus. It is supposed to activate ER-Ca^{2+} ATPase and vacuole Ca^{2+} channels. Activation of the former may accelerate the secretion of α-amylase, as discussed before, and that of the latter may lead to an increase in cytosolic Ca^{2+} concentrations which causes stimulation of Ca^{2+} signaling. Thus, CaM appears to play an important role as a second messenger in GA signaling.

When Ca^{2+} and CaM were microinjected into barley aleurone protoplasts without GAs, the protoplasts did not secrete α-amylase (Gilroy 1996), suggesting that the set, Ca^{2+} and CaM, alone is not sufficient to mimick GA signaling. In other words, Ca^{2+} and CaM may be necessary for the secretion pathway at late stages of GA signaling, but not for early stages.

cGMP Increases with GAs at μM-Order Concentrations

The levels of cGMP increases transiently about fivefold at 2 h after incubation of barley aleurone protoplasts with 5 μM GA (Penson and others 1996). The time of induction tends to be later than that for the expression of *Ca^{2+} ATPase* and *CaM* genes. LY83583, an inhibitor of guanynyl cyclase, prevents the GA-induced increase in cGMP and the GA-induced synthesis of α-amylase. Membrane-

permeable analogs of cGMP did not induce α-amylase in barley aleurone cells without GAs, but experiments with LY83583 have confirmed that cGMP is necessary for stimulation of the expression of *GAMyb* and *α-amylase* gene. The observations indicate that cGMP is an important second messenger in GA signaling.

It has been pointed out that Ca^{2+} and cGMP are important signal mediators in phytochrome signal transduction pathways. Nam-Hai Chua has suggested that high cGMP concentrations cause negative regulation of the Ca^{2+}-dependent pathway, and stimulation of the Ca^{2+}-dependent pathway negatively regulates the cGMP-dependent one (Bowler and others 1994). Undoubtedly Ca^{2+} and cGMP are important second messengers in plants.

In mammals, some kinds of heterotrimeric G proteins activate cGMP phosphodiesterase (cGMP PDE) (Fung and others 1981). Thus, the level of cGMP is reduced by activation of the G proteins. If this is the case in plant heterotrimeric G proteins, the regulation of cGMP levels in GA signaling may be *independent* of the G proteins, because a transient increase of cGMP is necessary for GA signaling.

GAMyb, a Transcription Factor of α-Amylase Gene

The *GAMyb* gene, which activates the promoter of *α-amylase* gene, has been isolated from barley (Gubler and others 1995) and rice (Gubler and others 1997). Induction of the *GAMyb* gene occurs at about 2 h after application of GAs at μM-order concentrations in both barley and rice. Although nobody doubts the importance of the *GAMyb* gene in GA signaling, little attention has been paid to the presence of GAMyb mRNA in the seeds of barley and rice before application of GAs. We would take note of existing small amounts of the mRNA in the seeds before GA treatment. We suppose that the transcript may direct some steps of GA signaling, though slightly, in plant cells before GA supplement. We caution that a small amount of GAMyb mRNA and GAMYB before induction with GAs may cause confused interpretations about GA-signaling pathways.

Plant Heterotrimeric G Protein Functions

Studies on heterotrimeric G proteins have recently progressed in the plant kingdoms. The progress is attributable to identification of mutants, which have defects in the subunit genes. The *α-subunit* gene of heterotrimeric G protein is a single copy gene for rice (Ishikawa and others 1995) and *Arabidopsis* (Ma and others 1990). The mutants for the *α subunit* gene have been isolated from rice (Ashikari and others 1999; Fujisawa and others 1999) and *Arabidopsis* (Ullah and others 2001). The β-*subunit* gene of heterotrimeric G protein was also a single copy gene in rice (Ishikawa and others 1996) and *Arabidopsis* (Weiss and others 1994). A mutant for the β-*subunit* gene was isolated from *Arabidopsis* (Lease and others 2001). There are two γ-*subunit* genes in each genome of rice and *Arabidopsis* (Mason and Botella 2000, 2001). So far, no mutant for the γ-*subunit* gene has been isolated from any plants. Analysis of the mutants for the *α-subunit* gene has presented some interesting but complicated results. Here, we briefly summarize the results of mutant analysis for the *α-subunit* gene.

Analysis of *gpa1*, a Mutant for the *α-subunit* Gene in *Arabidopsis*

Studies with *gpa1* have suggested that the signaling pathways regulated by the heterotrimeric G protein may consist of more than seven types, as described below in *Arabidopsis* (Ellis and Miles 2001; Fujisawa and others 2001a; Ma 2001).

1. The heterotrimeric G protein participates in modulation of cell proliferation (Ullah and others 2001), suggesting that the heterotrimeric G protein regulates at least one mode of auxin signaling.
2. The G protein participants in abscisic acid signaling and regulates ion channels in guard cells (Wang and others 2001).
3. The sensitivity to gibberellin, brassinolide and ACC is weak in the *gpa1* mutant compared with the wild-type (Ullah and others 2001; Ullah and others 2002).
4. *gpa1* mutant shows the hypersensitivity to sugar (Ullah and others 2001).

When the α subunit of the heterotrimeric G protein is overexpressed in the wild-type of *Arabidopsis*, the transformants enhance phytochrome-mediated inhibition of hypocotyl elongation (Okamoto and others 2001), indicating that the heterotrimeric G protein is also involved in light signaling.

Analysis of *d1*, a Mutant for the *α-Subunit* Gene in Rice

The heterotrimeric G protein of rice, like the *Arabidopsis* G protein, is involved in many signaling

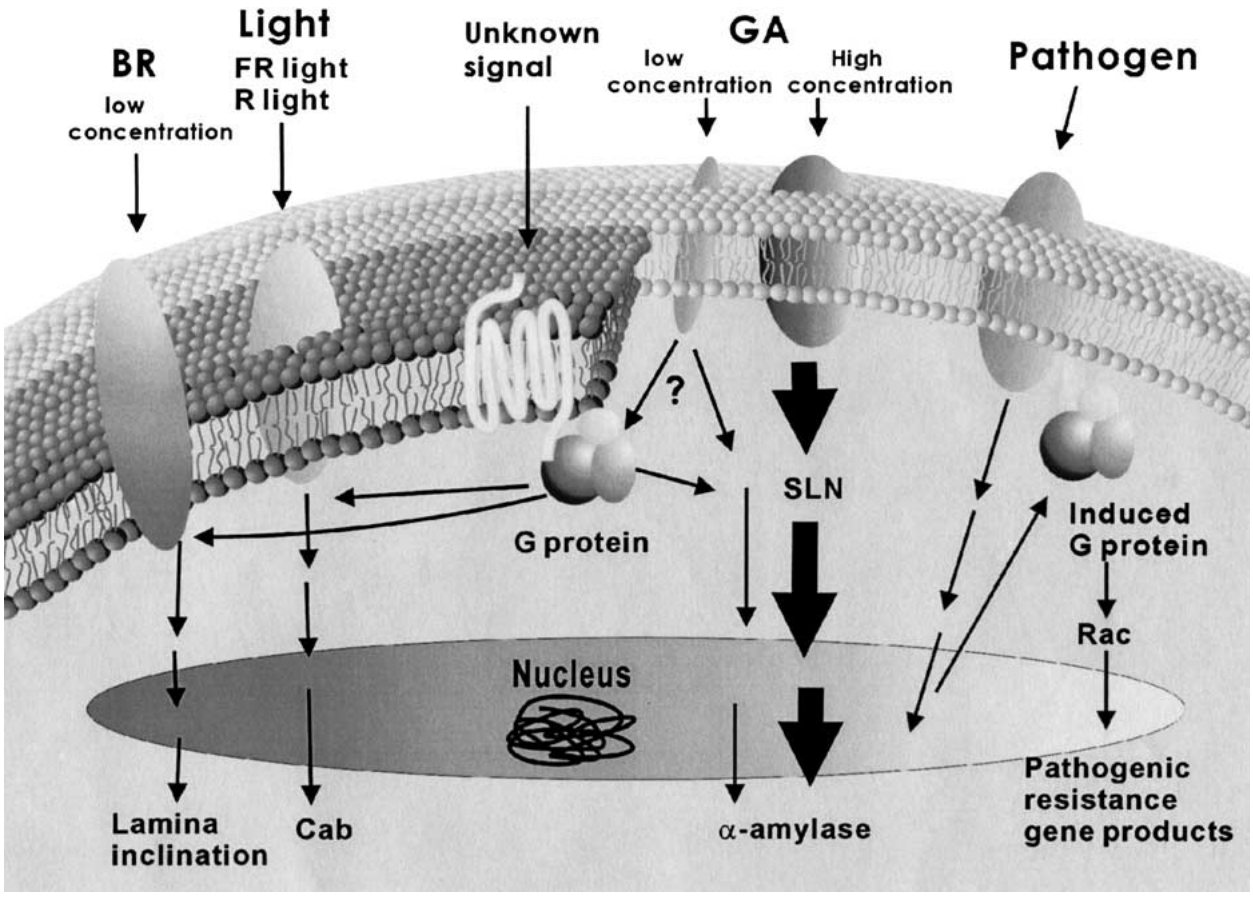

Figure 1. Putative heterotrimeric G-protein-mediated signaling pathways in rice. Rice heterotrimeric G protein may be involved, at least in signal transduction pathways for gibberellin, brassinosteroide, light, and pathogen infection. No upstream and downstream signaling molecules connected to the G protein have yet been identified.

pathways. We show a tentative model for the rice G-protein-mediated signaling in Figure 1.

1. *d1* has a partial defect for GA signaling as described before (Mitsunaga and others 1994; Ueguchi-Tanaka and others 2000).
2. *d1* shows a reduced hypersensitive response to infection by an avirulent race of rice blast (Suharsono and others 2002). The α subunit has been suggested to affect an upstream part in a small G-protein (Rac)-mediated signaling pathway in disease resistance of rice.
3. *d1* shows a partial defect in light signaling (Inagaki personal communication). The accumulation of mRNAs for light-inducible genes after exposure to red or far-red light is reduced in *d*1, as compared with the normal cultivar.
4. Lamina-inchnation tests of *d1* and the normal cultivar have shown that *d1* exerts little response at 10^{-9} M of brassinolide, but does respond at more than 10^{-6} M of brassinolide. The observation suggests that the G protein may be involved in brassinosteroid signaling at low concentrations of brassinosteroid (Fujisawa personal communication). The manner in brassinosteroid signaling seems to be the same as that in GA signaling.

Further Prospects

The mutants for the *α-subunit* genes of heterotrimeric G protein, *gpa1* and *d1*, show defects in many kinds of signals, although the gene for the α subunit is single in each plant species. The signaling pathways seem to be too numerous; a single heterotrimeric G protein may be unable to perceive so many signals. In addition, the mutants show partial, not complete, defects in the characters described above, so far as we know. When we study the function of a heterotrimeric G protein in GA signaling, we should pay attention to these backgrounds and to the possibility of crosstalkings.

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