Rice receptor for chitin oligosaccharide elicitor does not couple to heterotrimeric G-protein: Elicitor responses of suspension cultured rice cells from Daikoku dwarf (*d1*) mutants lacking a functional G-protein α-subunit

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We present evidence that the rice receptor for *N*-acetylchitooligosaccharide elicitor does not couple to heterotrimeric G-protein (G-protein), one of the most important signal transducers from the cell surface to down-stream effectors in various cellular responses of many organisms. Using mutant rice cells lacking functional G-protein α-subunit, cellular responses of suspension-cultured rice cells derived from Daikoku dwarf (*d1*) mutants that were shown to contain mutations in the coding region of the G-protein α-subunit (Fujisawa et al. 1999) to *N*-acetylchitooligosaccharide (oligochitin) elicitor were compared with those of the corresponding parent cell lines. All the elicitor-induced cellular responses, such as medium alkalinization, generation of reactive oxygen species, expression of early responsive genes, PAL and *PR* genes, and production of phytoalexin, were basically identical in the mutant and wild type cell lines. Considering the reported presence of a single copy gene for the G-protein α-subunit in many plant species including rice, these results strongly support the above conclusion.

Introduction

Heterotrimeric GTP-binding protein (G-protein, hereafter) is one of the most highly conserved and important signal transducers connecting cell surface G-protein-coupled receptors (GPCRs) to down-stream effectors (Gilman 1987, Ross 1989, Bourne et al. 1990, 1991, Simon et al. 1991, Strader et al. 1994, Neer 1995). G-proteins are composed of three functional subunits, α, β and γ (Gα, Gβ and Gγ, respectively). Activation by a ligand of a cell surface GPCR causes replacement of GDP by GTP and dissociation of the GTP-Gα complex from the βγ dimer. Gα is thought to confer the specificity of the interaction with the GPCR and the effector protein(s).

GPCRs are primary components of this pathway and constitute a large and functionally diverse superfamily of integral membrane proteins. The GPCRs specifically recognize a diverse range of ligands, including visual signals (Stryer 1986), olfactants (Jones and Reed 1989) and taste stimuli (McLaughlin et al. 1992), biogenic amines and other neurotransmitters like acetylcholine and neuropeptides (Strader et al. 1994), hormones, yeast mating factors (Dietzel and Kurjan 1987, Miyajima et al. 1987, Obata et al. 1991) and act as sensors for nutrition (Isshiki et al. 1992). The DNA and deduced amino acid sequences of more than 700 GPCRs are known and all of them have stretches of between 20 and 28 hydrophobic amino acids capable of forming transmembrane α-helices (Dohlman 1991, Hooley 1999). GPCRs are thought to traverse the membrane seven times, forming a helical

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Abbreviations – Gα, α-subunit of heterotrimeric G-protein; GPCR, G-protein-coupled receptor; MoA, momilactone A; ROS, reactive oxygen species

cluster with associated extracellular and intracellular loops (Dohlman et al. 1991, Strader et al. 1994). Receptors with these characteristics have been identified in vertebrates, invertebrates, arthropods, insects, nematodes, fungi, plants and viruses.

In plants, genes encoding putative Gα subunit homologues have been cloned and sequenced in *Arabidopsis* (Ma et al. 1990), tomato (Ma et al. 1991), barley (Wang et al. 1993), *Lotus japonicus* (Poulsen et al. 1994), soybean (Kim et al. 1995) and rice (Ishikawa et al. 1995, Seo et al. 1995). Moreover, antisera against synthetic peptides from *Arabidopsis* GPα1 (GPA1) have been extensively used to probe Gα proteins in *Arabidopsis* (Clarkson et al. 1991, Weiss et al. 1993), carrot (Drøbak et al. 1995) and *Sorghum bicolor* (Ricart et al. 1993).

Although the complete amino acid sequences predicted for the putative Gα of higher plants share rather low identities of 36–42% with those of mammalian Gα, high sequence identities are observed in the regions that bind GTP and βγ dimers (Ishikawa et al. 1995). Another significant difference between the mammalian and higher plant Gα is that the higher plant genomes each contain only a single (or at most, a few) genes for the putative Gα's (Ma et al. 1990, 1991, Poulsen et al. 1994, Ishikawa et al. 1995, Kim et al. 1995, Seo et al. 1995) in contrast with the existence of a large number of genes for Gα's in mammalian genomes (Gilman 1987, Kaziro et al. 1991, Simon et al. 1991, Neer 1995). Since there are more known GPCRs or effectors than known G-proteins in mammals, different receptors or effectors likely interact with the same G-protein (Firtel et al. 1989, Conklin and Bourne 1993).

Probably the best way to assess the possibility of the involvement of a signalling component in a given signal transduction cascade is the use of mutants for such a component. Recently, Fujisawa et al. (1999) reported that dwarf rice mutants known as Daikoku dwarf (*d1*) have a single mutation in the coding region of the G-protein α-subunit (RGA1). Genomic DNA blot hybridization of rice has shown that even under low-stringency conditions, there is only one DNA fragment that hybridizes with the Gα cDNA clone (Ishikawa et al. 1995), indicating that *RGA1* has a single copy in the genome. Moreover, the *d1* gene in the Daikoku mutant cells produces a truncated product that lacks some domains essential for its function. The fact that the Daikoku mutant cells would have no functional Gα indicates the great value of these mutants for the study of the role of trimeric G-protein in a given signalling pathway. Indeed, by using these Daikoku mutant cells, Ueguchi-Tanaka et al. (2000) showed a partial impairment in gibberellin (GA) signal transduction, indicating the involvement of the heterotrimeric G-protein in the GA signalling pathway.

In the present work, we used the advantage of this mutant for the analysis of the signalling pathways for defense responses induced by a chemically well defined elicitor, *N*-acetylchitooligosaccharides. We have reported that chitin fragments, *N*-acetylchitooligosaccharides (degree of polymerization = 6–8), can induce various defense-related cellular responses in suspension-cultured rice cells. These include transient membrane depolarization (Kuchitsu et al. 1993, Kikuyama et al. 1997), ion flux (Kuchitsu et al. 1997, Kikuyama et al. 1997), reactive oxygen generation (Kuchitsu et al. 1995), expression of typical PR genes as well as novel 'early genes' (Minami et al. 1996, He et al. 1998, Nishizawa et al. 1999, Takai et al. 2001), and biosynthesis of jasmonic acid and phytoalexins (Yamada et al. 1993, Nojiri et al. 1996). Most of these responses seem to be a part of a complicated signal transduction cascade or regulated through the cascade. Identical specificity for the size and structure of *N*-acetylchitooligosaccharides shown by these responses indicated that all these responses are mediated by a single class of receptor molecule for this elicitor. We already reported the presence of a putative receptor molecule, a high-affinity binding protein for this elicitor in the plasma membrane of rice cells by binding assay and affinity labelling (Shibuya et al. 1993, 1996, Ito et al. 1997). We believe that the combination of this model system for elicitor responses with the mutant lines for Gα will provide a clear indication about the role of G-protein in the corresponding signalling cascade.

In this study, we used suspension-cultured cells of three lines of Daikoku dwarf (*d1*) rice (DK22, Taichung 65d1 and CM1361-1) and their parent varieties (Nipponbare, Taichung 65 and Kinmaze, respectively) (Fujisawa et al. 1999). To assess the possibility of RGA1 coupling to the receptor for *N*-acetylchitooligosaccharides, we analysed most of the cellular responses induced by the elicitor in these cells. The results from all the mutant lines were very similar and showed no significant differences between the responses of Daikoku mutant cells and the corresponding parent cells to the elicitor. We conclude from these results that the rice receptor for *N*-acetylchitooligosaccharide elicitor does not couple to Gα, and thus not to heterotrimeric G-protein.

Materials and methods

Cell culture

Suspension-cultured cells from three mutant lines of Daikoku dwarf (*d1*) rice (DK22, Taichung 65d1 and CM1361-1) and their parent line (*Oryza sativa* L. cv. Nipponbare, Taichung 65 and Kinmaze, respectively) were used in this study. All the cells were maintained using a modified N-6 medium with rotary shaking at 120 r.p.m and 25°C in the dark (Kuchitsu et al. 1993, Yamada et al. 1993). Cells were subcultured weekly with filtering through a 20-mesh stainless steel filter to make fine aggregates. Usually, 3- to 4-day-old cells were used for the experiments. The cell suspension was adjusted to 100 mg cells ml^{-1} with a fresh medium and pre-incubated for 12–24 h before use.

Elicitor treatment and application of activator/inhibitor of G-protein

N-Acetylchitoheptaose (GlcNAc)$_7$, was used as an elicitor at a final concentration of 1 μM to induce the defense responses of rice cells. Chitosan heptamer (GlcNH$_2$)$_7$, was also used for some experiments. Sterilized water was used instead of the elicitor solution in the control experiments. The elicitor solution was sterilized by autoclaving at 121°C for 20 min and applied to the cells aseptically in a clean bench except for the analysis of extracellular pH. In the latter case, the elicitor solution was directly applied to the cell suspension ready for pH measurement (see below).

In some experiments, 5–10 μM mastoparan (Wako pure chemicals, Japan) was applied to the suspension-cultured rice cells of Taichung 65 and Taichung 65d1 instead of N-acetylchitooligosaccharide elicitor. One μg ml^{-1} of cholera toxin (CTX) or pertussis toxin (PTX) (Calbiochem, CA, USA) was also applied to the cell suspension prior to elicitor treatment.

Measurement of extracellular pH

Extracellular pH changes were monitored with a TOA pH meter HM-5S equipped with a glass electrode GS-5015C (TOA Electronics Ltd, Tokyo, Japan). Approximately 200 mg of the cells were transferred into a glass vial, then, 2 ml of fresh medium was added to the cells and stirred gently with a magnetic stirrer bar at 25°C for about 30 min to stabilize the conditions of the cells. After the pH of the medium became stable (in most experiments, the initial pH before elicitor treatment was 5.3–5.5), elicitor solution was added to the cell suspension and stirred continuously during measurement. Before and after every measurement, the electrode was calibrated with standard buffers. Real-time pH monitoring described above might damage the cells by mechanical stirring, or affect the physiological conditions of the cells because of the relatively short pre-incubation with a fresh medium and also the treatment under nonaseptic conditions. To assess such a possibility, another set of experiments under different conditions were designed as follows. Approximately 500 mg of cells were suspended in 5 ml of fresh medium under sterile conditions, then, pre-incubated for 12–24 h on a shaker. An aliquot (800 μl) was taken before and 30 min after the elicitor treatment. The pH value of each aliquot was measured using a pHI-34 pH meter (Beckman Instruments Inc., Fullerton, CA, USA) equipped with 39849 glass electrode (Beckman Instruments Inc.). The difference of the pH of the medium between 30 min after the elicitor addition ([pH]$_{30}$) and the initial pH ([pH]$_i$) was taken as ΔpH (ΔpH = [pH]$_{30}$ − [pH]$_i$) and used as an index of the magnitude of alkalinization. The results in Fig. 1 were obtained by the former method and those in Table 2 by the latter method.
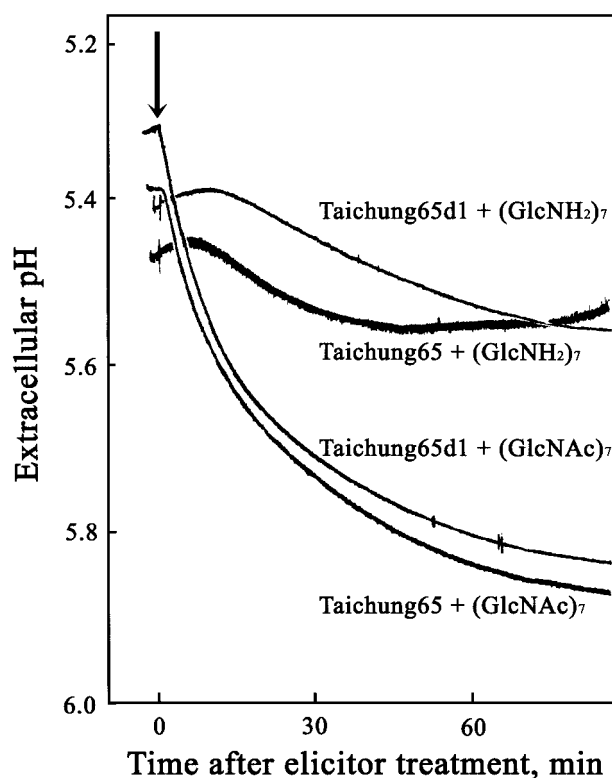

Fig. 1. Extracellular alkalinization induced by N-acetylchitoheptaose (GlcNAc)$_7$, in the cultured cells from Taichung 65 and Taichung 65d1 rice. The elicitor (GlcNAc)$_7$, or its structural analogue (GlcNH$_2$)$_7$, was added to the suspension cultured cells at a final concentration of 1 μM. All other pairs of cell lines (Nipponbare and DK 22; Kinmaze and CM1361-1) gave basically the same result.

Analysis of reactive oxygen generation

Reactive oxygen generation induced by the elicitor was analysed as the amount of hydrogen peroxide (H$_2$O$_2$) in the culture medium by the ferricyanide-catalysed oxidation of luminol (Schwacke and Hager 1992). Chemiluminescence was measured using a model TD-20/20 photon counting luminometer (Turner Design, Sunnyvael, CA, USA). The concentration of H$_2$O$_2$ was determined using a standard curve obtained from standard H$_2$O$_2$ solutions.

RNA isolation and Northern blot analysis

Total RNA was isolated from the rice cells by the sodium dodecyl sulphate (SDS)-phenol method. Ten micrograms of total RNA was denatured with glyoxal, separated by electrophoresis in 1.4% (w/v) agarose gel, and blotted to nylon membranes (Biodyne A, Pole Co. Ltd, Washington, NY, USA) according to the method of Thomas (1983). Membranes were hybridized with ^{32}P-labelled cDNAs for *EL2*, *EL3* (Minami et al. 1996), *CP-1* for rice PAL (Minami et al. 1989), *RCC-1* for rice chitinase (Nishizawa and Hibi 1991), *RβG* for rice β-1,3-

Table 1. Sequences of the mutated regions of Daikoku dwarf (d-1), DK22, Taichung 65, and CM1361-1, found in the coding region of the G-protein α-subunit (RGA1). The number at the left side of each sequence indicates the position of the first base in the *RGA1* coding region. The mutations are indicated by bold letters (replacement or insertion) or underlines (deletion).

Daikoku mutant	Normal cultivar	Alignment of *RGA1* found in Daikoku mutant	Type of mutation
DK22	Nipponbare	593 TGT**A**AAATTTGGATCGATTAG	single base replacement (T for G)
Taichung 65d1	Taichung 65	997 TG_ _AGTGGTTTAAAGACTAC	deletion of two bases (CG)
CM1361-1	Kinmaze	351 TAAA**GTATGCAATACTGGAAAAG**A	insertion

glucanase (K. Yamada, R. Takai, H. Kaku, N. Shibuya, and E. Minami, *unpublished results*). Membranes were washed twice with $2 \times SSC$ ($1 \times SSC$ is the mixture of 0.15 M sodium chloride and 0.015 M sodium citrate), 0.1% (w/v) SDS for 5 min at room temperature then twice with $0.1 \times SSC$, 0.1% (w/v) SDS for 15 min at 60°C.

Analysis of phytoalexin accumulation

Approximately 500 mg of cultured cells (3–5 days after subculture) were pre-incubated in 5-ml of fresh medium for 12–24 h before elicitor treatment. After elicitor treatment, the cells were incubated for 72 h at 25°C with reciprocal shaking at 120 r.p.m. Two ml of the culture medium was applied onto a Bond Elute ODS column (Varian Analytical Instruments, Harbor City, CA, USA) equilibrated with pure water. After washing with 5 ml of water, metabolites were eluted with 1 ml of pure methanol. The alcoholic eluent was dried up at 70°C, dissolved in 50 μl of 100% methanol, and stored at 4°C until analysis. Momilactone A (MoA) was quantified by liquid chromatography-tandem mass spectrometry (HPLC/MS/MS). The conditions for the separation of MoA were as follows: HPLC system, 1090 SeriesII/L (Hewlett-Packard Co., Palo alto, CA, USA); column, Inertsil ODS-3, 5 μm, $\phi 2.1 \times 150$ mm (GL Sciences Inc., Tokyo, Japan); guard column, OPTI-GUARD C18 $\phi 1.0 \times 15$ mm (Optimize Technologies Inc., Oregon City, OR, USA); oven temperature, 40°C; eluent, 75% (v/v) methanol containing 5 mM acetic acid; and flow rate, 0.2 ml min^{-1}. Tandem mass spectrometry was carried out with an API 300 triple quadrupole mass spectrometer equipped with a TurboIonSpray^{TM} electrospray ionization inlet (Perkin-Elmer Sciex Instruments, Ontario, Canada) under multiple ion monitoring (MRM) mode. The MS operation for quantification of MoA were as follows: ion spray voltage, 5 kV; orifice, 21 V; focusing ring, 190 V; quadrupole ion guide (Q0) offset, -4 V; interquad lens 1 (IQ1), -5 V; stubby rod, -9 V; quadrupole 1 (Q1) offset, -4.6 V; IQ2, -19 V; Q2 offset, -23 V; IQ3, -63 V; Q3 offset, -28 V; collisionally activated dissociation gas, nitrogen; nebulizer gas flow, 1.23 l min^{-1}; heated gas temperature, 400°C. The monitored MS/MS transition of the MoA was m/z 315 ($[M + H]^+$) $\rightarrow$ 271. The concentrations of MoA in the medium were calculated based on the peak area of the product ion derived from known amount of standard MoA and expressed as μg g^{-1} of fresh cell weight. The limit of detection based on the amount injected was approximately 1 pg.

Results

Characteristics of suspension-cultured cells of Gα-lacking mutant rice, Daikoku Dwarf (d1), and of the corresponding wild-type rice

Fujisawa et al. (1999) reported that Daikoku dwarf (*d1*) rice cells have a single mutation on the coding region of rice Gα (RGA1). Because the *RGA1* gene is believed to be the only gene encoding a G-protein α-subunit in rice (Ishikawa et al. 1995), these mutant cells should have no functional G-protein. If the receptor for *N*-acetylchitooligosaccharide elicitor couples with trimeric G-protein, Daikoku mutant cells will not respond to chitin treatment.

For this purpose, callus of three cell lines of Daikoku dwarf (*d1*) and their corresponding wild-types (Table 1) were made from grains and maintained as suspension-cultures. Although these Daikoku dwarf (*d1*) plants showed an abnormal phenotype, no significant difference was observed in the growth of these mutant cell lines compared to those of the parent varieties.

Because the results obtained for all Daikoku mutant cells were quite similar, hereafter we only show typical results for a pair of cell lines: Taichung 65 (wild-type) and its Daikoku dwarf (*d1*) mutant, Taichung 65d1. For some experiments, results from other cell lines of Daikoku mutant and corresponding wild-type rice are also shown.

Table 2. Extracellular alkalinization induced by N-acetylchitoheptaose in Daikoku mutant and parent cell lines. The pH of the culture medium was measured before and 30 min after the elicitor treatment (1 μM N-acetylchitoheptaose). The pH changes in the 30 min (ΔpH_{30}) are shown as an indicator for the extracellular alkalinization induced by the elicitor. For control experiments, 1 μM of chitosan heptamer (GlcNH$_2$)$_7$, was applied to the cell suspensions. The data are the mean value ($\pm$ sD) from three independent experiments. The alkalinization of the extracellular medium is expressed as a positive value.

Cells	(GlcNAc)$_7$	(GlcNH$_2$)$_7$
Nipponbare	0.605 ± 0.021	0.12 ± 0.014
DK22	0.70 ± 0.071	0.17 ± 0.014
Taichung 65	0.69 ± 0.021	0.095 ± 0.064
Taichung 65d1	0.715 ± 0.021	0.115 ± 0.007
Kinmaze	0.47 ± 0.028	0.15 ± 0.042
CM1361-1	0.49 ± 0.017	0.13 ± 0.014

Extracellular alkalinization induced by N-acetylchitoheptaose in Daikoku mutant and parent cell lines

Alkalinization of the medium was reported by Felix et al. (1991, 1993) as an early cellular response to N-acetylchitooligosaccharide elicitor in tomato cells. Transmembrane ion fluxes were also observed in suspension-cultured rice cells treated with N-acetylchitooligosaccharide elicitor (Kuchitsu et al. 1997). To assess whether the elicitor-induced ion flux is mediated by G-protein we analysed the elicitor-induced pH change of extracellular medium of suspension-cultured Daikoku mutant cells.

Figure 1 shows a representative pH profile for Taichung 65 and Taichung 65d1 suspension-cultured cells treated with the elicitor. Rapid alkalinization was induced in both Taichung 65 and Taichung 65d1 cells by the addition of 1 μM of N-acetylchitoheptaose (GlcNAc)$_7$. No such response was observed by the addition of 1 μM chitosan heptamer (GlcNH$_2$)$_7$ (Fig. 1).

Extracellular pH changes after 30 min of elicitor treatment ([ΔpH]$_{30}$) for all sets of mutant and parent cell lines are summarized in Table 2. Extracellular alkalinization elicited by (GlcNAc)$_7$ was observed in all Daikoku mutant cells (Table 2). Moreover, there were no significant differences in the magnitude of pH change between Daikoku mutant and corresponding parent cell lines.

Generation of reactive oxygen species (ROS) induced by N-Acetylchitoheptaose in Daikoku mutant and parent cell lines

N-Acetylchitooligosaccharide elicitor was reported to induce the generation of ROS in suspension-cultured rice cells (Kuchitsu et al. 1995). We analysed the profile and extent of elicitor-induced ROS generation in Daiko-ku mutant as well as parent cell lines. When 1 μM of (GlcNAc)$_7$ was applied to the cell suspension, characteristic biphasic generation of H$_2$O$_2$ was observed both in Taichung 65 and Taichung 65d1 (Fig. 2A) cells. Maximum levels and kinetics of H$_2$O$_2$ accumulation were quite similar for Taichung 65 and Taichung 65d1, with a first peak at 0.25–0.5 h and a second peak at 2–2.5 h. The addition of 1 μM (GlcNH$_2$)$_7$ induced neither phase I nor phase II ROS generation.

In the case of GA$_3$-dependent α-amylase induction in Taichung 65d1 mutant cells, it was found that the maximum amylase activity induced by GA$_3$ was the same for mutant and parent lines but that the GA$_3$ concentration required for the induction was 1000 times higher in the mutant cells, indicating a drastic change in the sensitivity to GA$_3$ in the mutant line (Ueguchi-Tanaka et al. 2000). Thus, we compared the dependency of induction of ROS generation on the concentration of (GlcNAc)$_7$ for mutant and parent cells. The dose-response curves for the ROS generation in Taichung 65 and Taichung 65d1 rice cells were basically the same (Fig. 2B). The concentration which gave half-maximum values for the induction of ROS generation were also the same (1 nM) for

mutant and parent cells, indicating that the $d1$ mutation does not affect the sensitivity for the N-acetylchitooligosaccharide elicitor.

Regulation of elicitor responsive genes induced by N-acetylchitoheptaose in daikoku mutant and parent Cell Lines

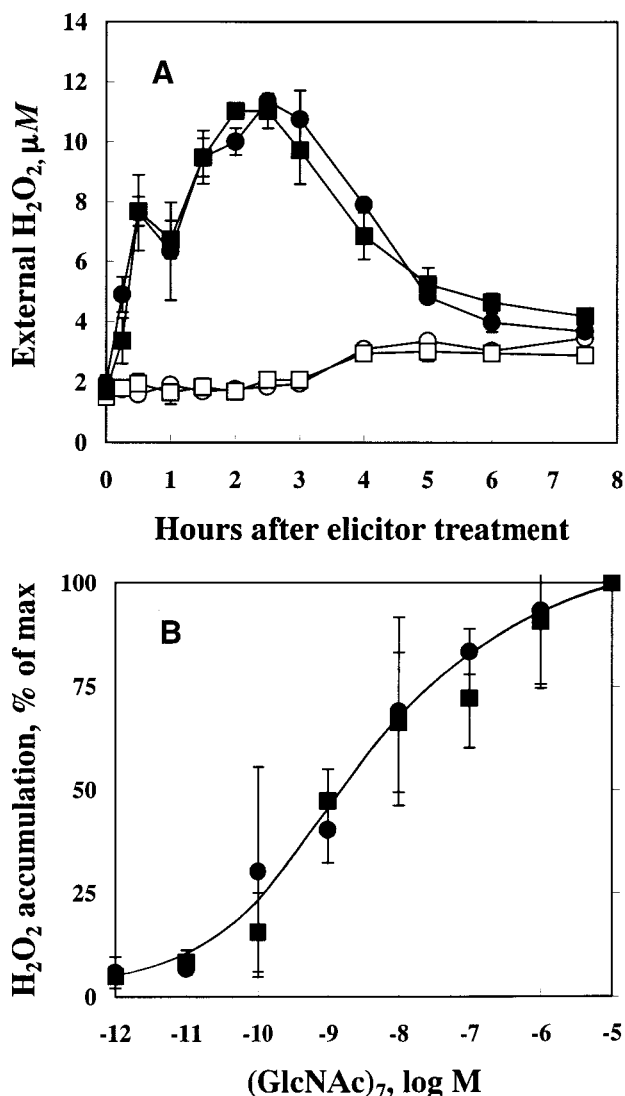Although the N-acetylchitooligosaccharide elicitor induces expression of various defense-related genes in rice

Fig. 2. H$_2$O$_2$ generation induced by (GlcNAc)$_7$ in the cultured cells from Taichung 65 and Taichung 65d1 rice. (A) 100 mg of the suspension-cultured cells of Taichung 65 and Taichung 65d1, were incubated in 1 ml of the medium containing the tested oligosaccharide at 25°C. Each data point represents the average ($\pm$ SD) of three independent experiments. Symbols are: Taichung 65 (GlcNH$_2$)$_7$, 1 μM (○); Taichung 65 (GlcNAc)$_7$, 1 μM (●); Taichung 65d1 (GlcNH$_2$)$_7$, 1 μM (□); Taichung 65d1 (GlcNAc)$_7$, 1 μM (■) (B) Dose-response curves for H$_2$O$_2$ generation by (GlcNAc)$_7$. Suspension-cultured cells (100 mg ml^{-1}) of Taichung 65 (●) or Taichung 65d1 (■) were incubated in the presence of differing concentration of (GlcNAc)$_7$. The amount of H$_2$O$_2$ after 2 h of the elicitor treatment was measured. Each data point is expressed as the percentage of the highest level of H$_2$O$_2$.

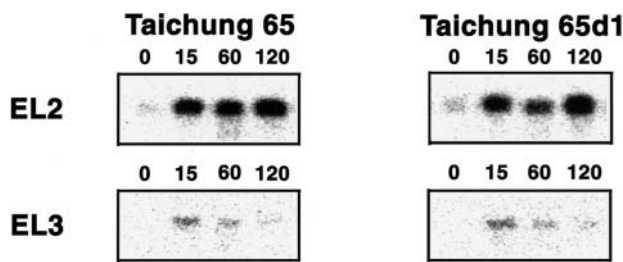**Taichung 65**   **Taichung 65d1**

EL2 — 0 15 60 120

EL3 — 0 15 60 120

Fig. 3. Expression of EL2 and EL3 induced by *N*-acetylchitooligoheptaose in the cultured cells from Taichung 65 and Taichung 65d1 rice. 10 μg of total RNA was applied to each lane. Similar expression patterns were also observed for the other pairs of cell lines.

Table 3. Accumulation of momilactone A (MoA) induced by *N*-acetylchitoheptaose in Daikoku mutant and parent cell lines. MoA ($\mu g\ g^{-1}$ FW cell) was extracted from the culture medium 72 h after the elicitor treatment. For control experiments, 1 μM of $(GlcNH_2)_7$ was applied to the cell suspensions. The data are the mean value ($\pm$ SD) from three to five independent experiments.

Cell	$(GlcNAc)_7$	$(GlcNH_2)_7$
Nipponbare	5.5 ± 1.9	0.025 ± 0.003
DK22	3.9 ± 1.1	0.022 ± 0.012
Taichung 65	1.9 ± 0.3	0.0039 ± 0.0007
Taichung 65d1	2.3 ± 0.3	0.027 ± 0.022
Kinmaze	1.9 ± 0.3	0.00058 ± 0.00023
CM1361-1	2.1 ± 0.3	0.00073 ± 0.00013

cells, these genes seem to be regulated by different signal transduction pathways depending on group (He et al. 1998, Nishizawa et al. 1999, Takai et al. 2001). Thus, we analysed whether such elicitor-responsive genes behave similarly in the mutant rice cells lacking functional Gα protein. Figure 3 shows the expression pattern of *EL2* and *EL3*, early genes that can be induced by both the elicitor and cytoplasmic acidification (Minami et al. 1996, He et al. 1998), induced by 1 μM of $(GlcNAc)_7$ in Taichung 65 and Taichung 65d1 cells. EL2 and EL3 showed a similar rapid and transient expression pattern comparable to the previously reported one (Minami et al. 1996) in both cell lines. The expression patterns of three other genes, PAL gene (*CP-1*), responsive to both the elicitor and cytoplasmic acidification, and the genes encoding chitinase (*RCC-1*) and *β*-glucanase (*RβG*), responsive to the elicitor but not to cytoplasmic acidification, were also compared for the Gα-lacking mutant and parent cell lines treated with 1 μM of $(GlcNAc)_7$. As shown in Fig. 4, all three genes were equally induced in Taichung 65 and Taichung 65d1 cells.

Accumulation of momilactone A Induced by N-acetylchitoheptaose in Daikoku mutant and parent cell lines

Momilactone A (MoA), which was initially isolated as a dormancy factor from rice husks by Kato et al. (1973),

is an effective antifungal agent and a major phytoalexin produced by rice (Cartwright et al. 1981). *N*-Acetylchitooligosaccharides can elicit the biosynthesis of MoA in suspension-cultured rice cells (Yamada et al. 1993). Nojiri et al. (1996) reported the involvement of jasmonic acid in the induction of MoA biosynthesis. LC/MS/MS analysis of MoA induced by the elicitor in mutant and parent cell lines showed that the amount of MoA increased hundreds to thousands-fold compared to the control cells but that the levels of the increment were comparable for each set of mutant and parent cells (Table 3). Typical brownish colouring induced by the elicitor was also observed for all cell lines treated with the elicitor (data not shown). Thus, metabolic changes related to defense reactions seemed to occur similarly in all the elicitor-treated cells.

Cholera toxin and Pertussis toxin did not affect cellular responses to the elicitor in wild-type and Daikoku mutant cell lines, but more complex responses were obtained with the mammalian G-protein activator, mastoparan

Cholera toxin (CTX) and pertussis toxin (PTX) are known as inhibitors of mammalian G-protein-dependent signal transduction. When Taichung 65 and Taichung 65d1 cells were treated with 1 $\mu g\ ml^{-1}$ of CTX or PTX before elicitor addition, the biphasic pattern of
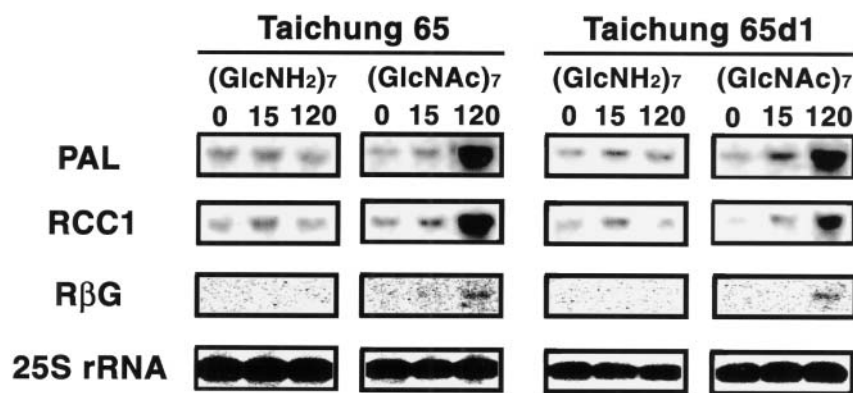

Taichung 65   **Taichung 65d1**

$(GlcNH_2)_7$ $(GlcNAc)_7$ $(GlcNH_2)_7$ $(GlcNAc)_7$

0 15 120   0 15 120   0 15 120   0 15 120

PAL

RCC1

RβG

25S rRNA

Fig. 4. Expression of PAL (*CP-1*), chitinase (*RCC-1*) and *β*-glucanase (*RβG*) induced by *N*-acetylchitooligoheptaose in the cultured cells from Taichung 65 and Taichung 65d1 rice. 10 μg of total RNA was applied to each lane. Similar expression patterns were also observed for the other pairs of cell lines.
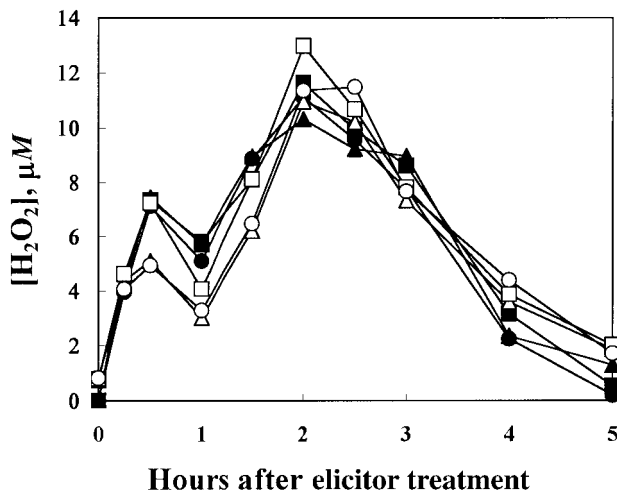
Fig. 5. Effects of cholera toxin and pertussis toxin on H_2O_2 generation induced by $(GlcNAc)_7$ in the cultured cells from Taichung 65 and Taichung 65d1 rice. 100 mg of the suspension-cultured cells of Taichung 65 and Taichung 65d1, were incubated in 1 ml of the medium containing the tested oligosaccharide at 25°C. Inhibitors (1 μg ml^{-1}) were applied to cell suspensions prior to elicitor treatment (1 μM). Each data point represents the mean value of three independent experiments. Symbols are: Taichung 65 $(GlcNAc)_7$ (●); Taichung 65, PTX + $(GlcNAc)_7$ (■); Taichung 65, CTX + $(GlcNAc)_7$ (▲); Taichung 65d1 $(GlcNAc)_7$ (○); Taichung 65d1, PTX + $(GlcNAc)_7$ (□); Taichung 65d1, CTX + $(GlcNAc)_7$ (Δ).

Fig. 6. Extracellular alkalinization induced by mastoparan in cultured cells of Taichung 65 and Taichung 65d1 rice. Mastoparan was added to the suspension-cultured cells at a final concentration of 5 μM.

ROS generation induced by chitin elicitor was not affected (Fig. 5). Mastoparan is the most used activator of mammalian G-protein. Mastoparan did not induce any ROS burst in any of the cell lines (data not shown). However, mastoparan (5–10 μM) did produce an extracellular pH alkalinization similar to the elicitor response (Fig. 6). Furthermore, mastoparan had only a weak effect on the expression of PAL and chitinase genes (data not shown). Thus there were no essential differences in mastoparan-induced responses of the wild-type and *d1* mutant rice cells. Fujisawa et al. reported that rather high concentration of mastoparan 7 (Mas 7) will cause rapid cell death in suspension-cultured rice cells and they claimed that mastoparan should be used with care in plant biochemistry (Fujisawa et al. 2001). Our results also indicate the complicated nature of mastoparan effects on plant responses.

Discussion

There have been a number of papers that indicate the involvement of G-proteins in the signalling pathways for plant responses to external stimuli such as phytohormones, light and elicitors (Legendre et al. 1992, 1993, Wang et al. 1993, Mehdy 1994, Hebe et al. 1999, Rajasekhar et al. 1999). Most of the papers are based on the results from various types of pharmacological studies with the use of known activators or inhibitors of G-protein, or antibodies against mammalian G-protein. However, the results from such pharmacological studies
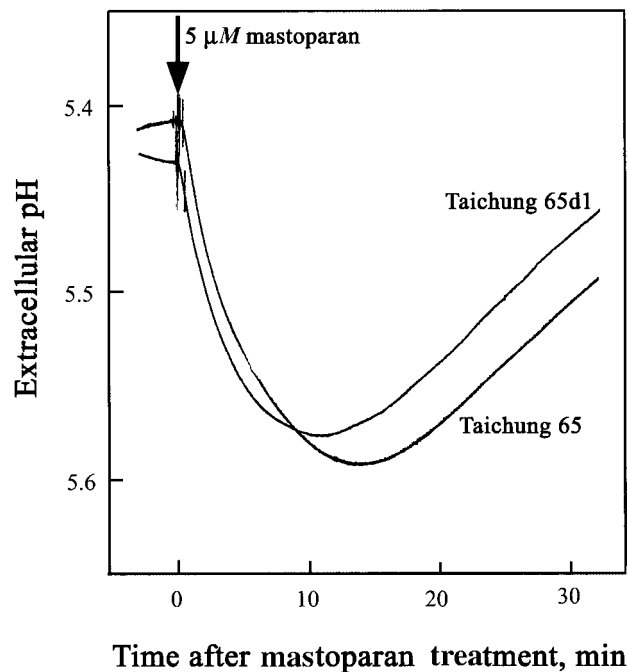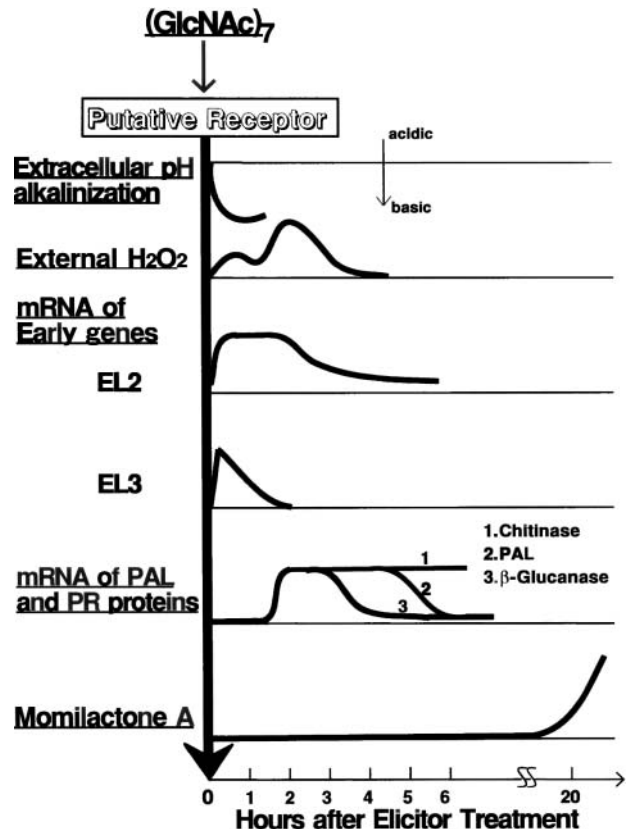

Fig. 7. Schematic drawing of the cellular responses induced by $(GlcNAc)_7$.

should be examined carefully because the specificity of the reagents may not be the same for mammalian and plant G-proteins, as indicated from the low sequence homology between plant and mammalian Gα's. For example, the well-known G-protein inhibitor, pertussis toxin, ADP-ribosylates a C-terminal cysteine residue in mammalian Gα's but no corresponding cysteine residue is found in plant Gα's (Neer 1995, Hooley 1999). Possible confusion of trimeric G-proteins and small G-proteins is another problem. Conditions used for ADP ribosylation by cholera toxin also allow the modification of small G-proteins, and GTP-binding assays using a non-hydrolysable GTP analogue, such as GTPγS, detect both heterotrimeric and small G-proteins (Ephritikhine et al. 1993). Mastoparan (Ross and Higashijima 1994), a well-known G-protein activating peptide, has rather low specificity. Finally, the anti-G-protein antibodies that have been used for the detection, or sometimes for the inhibition, of plant G-protein may cross-react with small G-proteins (Zaina et al. 1994).

The approach used in this study was more straightforward than the traditional pharmacological studies discussed above, which inevitably suffer from the uncertainty of the interpretation of experimental results. By using cell lines from Daikoku dwarf (*d1*) mutants that lack the functional RGA1 (Fujisawa et al. 1999), we got the evidence that the rice receptor for *N*-acetylchitooligosaccharide elicitor does not couple with RGA1, an α-subunit of rice heterotrimeric G-protein (Gα).

Because the signal transduction pathway downstream of the *N*-acetylchitooligosaccharide receptor, which leads to various cellular responses as shown in Fig. 7, seems to be multiply branched (He et al. 1998, Nishizawa et al. 1999, Takai et al. 2001), we analysed several cellular responses to the elicitor in both mutant and parent cells. This enabled us to investigate whether each response requires the presence of RGA1.

So far as we analysed, all the cellular responses induced by the elicitor occurred similarly in the mutant and parent cell lines. The extracellular alkalinization, a typical cellular response to various elicitors reflecting the change in ion flux through the plasma membrane, was induced similarly for the time course and the extent of pH change (Fig. 1, Table 2). The elicitor-induced ROS generation was also similar in all cell lines (Fig. 2A). Interestingly, the ROS generation induced by *N*-acetylchitooligosaccharide elicitor showed a clear biphasic profile, which mimics the ROS generation induced by the infection of pathogenic microbes in potato (Doke 1983) and soybean (Baker and Orlandi 1995, Lamb and Dixon 1997). The biphasic ROS generation was observed both in mutant and parent cell lines (Fig. 2A). The sensitivity to the elicitor in mutant cells was also the same for the parent cells (Fig. 2B), contrasting to the case of GA_3 signalling in *d1* mutants (Ueguchi-Tanaka et al. 2000). The expression pattern of elicitor-responsive genes, which can be classified into several groups according to the signal transduction pathways leading to their expression (He et al. 1998, Nishizawa et al. 1999, Takai et al. 2001), was the same for the mutant

and parent cells (Figs 3 and 4). Finally, a cellular response in the later phase of defense reaction, phytoalexin biosynthesis, was also induced similarly in each set of cell lines (Table 3).

As *RGA1* seems to be the only gene encoding a G-protein α-subunit in rice (Fujisawa et al. 1999), these results strongly suggest the receptor for *N*-acetylchitooligosaccharide elicitor does not couple to G-protein itself. In other words, the rice receptor for chitin oligosaccharide elicitor does not belong to the family of GPCR, although it is possible that unknown G-protein(s) having quite different molecular structure from mammalian counterparts exist.

Because there has been no pharmacological analysis for the involvement of the G-protein signalling pathway in elicitor responses in plants, we tried the pharmacological approach to determine whether G-protein coupling was involved in the defense responses induced by the elicitor.

Cholera toxin (CTX) and pertussis toxin (PTX) did not affect ROS burst induced by the chitin elicitor in rice cells of wild-type and Daikoku mutant (Fig. 5). Mastoparan has been reported to act as an activator of G-protein signalling via GTPase activity of heterotrimeric G protein in rice (Iwasaki et al. 1997). When mastoparan (5–10 μ*M*) was applied to suspension-cultured rice cells, there was no ROS burst observed and no induction of PAL (data not shown). On the other hand, medium alkalinization was induced by mastoparan (5–10 μ*M*) alone without elicitor treatment in both wild-type and Daikoku *d1* mutant rice cells (Fig. 6). Fujisawa et al. (2001) reported a rapid death of suspension-cultured rice cells of Nipponbare and its *d1* mutant DK22 induced by more than 5 μ*M* Mas 7 (active mastoparan) treatment. In the case of Mas 17 (inactive mastoparan analogue), more than 10–100 μ*M* was needed to induce cell death (Fujisawa et al. 2001). They suggested that high concentration of mastoparan might disrupt the membrane structure of suspension-cultured rice cells.

It must be noticed that these effects of drugs for G-protein were observed similarly in both wild-type and its corresponding *d1* mutant rice cells. Thus, as discussed above, we could not make any conclusion from these pharmacological experiments.

Our conclusion does not mean that all the receptor molecules for various types of elicitors belong to the same family. It is well known that the elicitors for plant defense reactions include various types of signal molecules (Boller 1995, Ito and Shibuya 2000). Some elicitors induce hypersensitive cell death, yet others do not (Ebel and Cosio 1994). Some elicitor such as *N*-acetyl-chitooligosaccharide can be recognized by a wide range of plant cells (Okada et al. 2002), yet others, such as AVR9 from *Cladosporium fulvum* can only be recognized by tomato cells carrying the *Cf-9* resistance gene (Joosten and De Wit 1999). These observations indicate the presence of a diverse class of receptor molecules for these elicitors, probably reflecting the co-evolution of these molecules in plants and microbes. It will not be

surprising if some of these molecules require trimeric G-protein for the downstream signalling.

Even suspension-cultured rice cells can respond to multiple elicitor molecules. A glucopentaose derived from the cell wall glucan of rice blast fungus (*Pyricularia oryzae*/*Magnaporthe grisea*) was found to be a potent elicitor for the rice cells (Yamaguchi et al. 2000). The structure of this glucopentaose was quite contrasting to the hepta-*β*-glucoside elicitor for soybean (Sharp et al. 1984), indicating the difference in the specificity of the corresponding receptors. Although the cellular responses induced by this elicitor are not well studied, at least some cellular response, such as ROS generation, seems to be different from that induced by *N*-acetylchitooligosaccharide (T. Yamaguchi, unpublished results). Although these oligosaccharide elicitors seem not to induce hypersensitive cell death, Che et al. (2000) recently showed that a flagellin from rice-incompatible strain of *Pseudomonas avenae* induced hypersensitive cell death, as well as the expression of defense-related genes. These results indicate that elicitor molecules that differ in the mode of action and the coverage of plant species act on a single plant, rice cells. It will be very interesting to compare the characteristics of the corresponding receptor molecules as well as the downstream signalling cascades. Daikoku dwarf mutants should be a powerful tool for such studies.

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