Flow cytometry and surface plasmon resonance analyses demonstrate that the monoclonal antibody JIM19 interacts with a rice cell surface component involved in abscisic acid signalling in protoplasts

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Received 17 May 1999; received in revised form 2 July 1999

Abstract **Abscisic acid (ABA) is a plant hormone involved in many developmental and physiological processes, but as yet, no ABA receptor has been identified. Flow cytometry of rice protoplasts and immunoblotting of purified plasma membranes (PMs) have been used to demonstrate that the monoclonal antibody JIM19 recognizes carbohydrate epitopes of cell surface glycoproteins. Using surface plasmon resonance technology specific binding of PMs to JIM19 was observed. Such interaction was antagonized significantly by ABA, but not by the biologically inactive ABA catabolite phaseic acid. These in vitro interactions were correlated with the biological activities of JIM19, ABA and phaseic acid on activation of the ABA-inducible *Em* promoter using two different transient reporter gene assays, β-glucuronidase/luciferase and quantitative flow cytometry of *Aequoria* green fluorescent protein. Pre-treatment with JIM19 resulted in significant inhibition of ABA-inducible gene expression. Taken together, these data suggest that JIM19 interacts with a functional PM complex involved in ABA signalling.**

Key words: Abscisic acid; Biosensor; Flow cytometry; Glycoprotein; Green fluorescent protein; Plasma membrane; Surface plasmon resonance

1. Introduction

Abscisic acid (ABA) is a sequiterpene plant hormone that modulates seed development, dormancy, cell division and responses to environmental stresses such as drought, cold, salt, pathogen attack and UV light [1–3]. It is ubiquitous in higher plants and acts via multiple pathways, inducing rapid closure of stomatal pores by ion efflux from guard cells and slower changes in gene expression [4]. Molecular genetic studies in the model organisms maize and *Arabidopsis* have resulted in the identification of downstream components of ABA and stress signalling, including protein phosphatases and kinases, transcription factors and a subunit of farnesyl transferase [4–10]. Reverse genetic and biochemical approaches have led to the characterization of ABA- and stress-responsive *cis*-promoter elements and transcription factors, the functional cloning of a syntaxin and demonstration of the involvement of secondary messengers such as cyclic ADP ribose and calcium [4,11–15].

Despite this rapid progress in understanding the molecular details of ABA signalling, little is known of the mechanisms of ABA perception. Indirect evidence suggests the existence of multiple ABA receptors [16,17], but with the exception of an unconfirmed report in 1984 [18] no such receptors have yet been described. Although ABA binding proteins [19,20] and carrier- mediated uptake of ABA have been reported [21,22], there is no evidence to link these to the physiological effects of ABA.

In this report, we describe the innovative uses of surface plasmon resonance (SPR) and flow cytometry to demonstrate biochemical and functional interactions of the monoclonal antibody JIM19 with plasma membranes (PMs) and protoplasts derived from rice embryonic suspension cultures. JIM19 is one of a panel of monoclonal antibodies previously generated against pea guard cell protoplasts [23] that was shown to modulate ABA responses in barley aleurone protoplasts, another target cell for ABA [24]. Our data show that JIM19 binds to glycoproteins (GPs) in rice PMs and that such binding is antagonized by ABA but not by the structurally related, biologically inactive ABA catabolite, phaseic acid (PA). Transient gene expression assays using β-glucuronidase (GUS)/luciferase and novel flow cytometric quantitation of green fluorescent protein (GFP) demonstrate that ABA but not PA induces *Em* promoter activity; such ABA-induced promoter activity is antagonized by JIM19. These results support a model of ABA signalling through a PM complex that includes an ABA receptor and JIM19 epitope-presenting GP.

2. Materials and methods

2.1. Materials

Embryonic rice (*Oryza sativa*) callus cultures (Radon 6 from the International Rice Research Institute, Los Baños, Phillipines) were obtained from Dr. Tom Hodges, Purdue University, West Lafayette, IN 47909, USA. Cultures were propagated and digested for making protoplasts as previously described [25] except that 10 mM HEPES (Sigma, St. Louis, MO, USA), pH 5.6, was substituted for phosphate in the Krens' F medium and 2% weight/volume (w/v) cellulase YC, 0.35% (w/v) macerozyme and 0.1% (w/v) pectolyase Y23 were used for overnight digestion (Karlan Research Products, Santa Rosa, CA, USA). Phaseic acid was obtained from Dr. Sue Abrams, Institute of Plant Biotechnology, Saskatoon, Canada. (±)*Cis*, *trans*-ABA (Sigma) and phaseic acid were quantified by spectrometry [26] and their purity and relative quantities confirmed by mass spectrometry using a Finnegan (San Jose, CA, USA) GCQ ion trap [27].

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Abbreviations: ABA, abscisic acid; FITC, fluorescein isothiocyanate; GFP, green fluorescent protein; GP, glycoprotein; GUS, β-glucuronidase; LUC, firefly luciferase; PBS, phosphate-buffered saline; PM, plasma membrane; SPR, surface plasmon resonance; w/v, weight/volume

PII: S0014-5793(99)00972-2

2.2. Immunological studies

JIM19 and JIM20 [23] were obtained as hybridoma supernatants from Ms. Jan Peart, John Innes Centre, Norwich, UK. For functional assays the supernatants were desalted by passing over NAP-10 columns (Amersham-Pharmacia Biotech., Uppsala, Sweden) equilibrated with 0.1 M phosphate-buffered saline (PBS), pH 7.4, or dialyzed against PBS to remove sodium azide and low molecular weight impurities. Immunoblotting to nitrocellulose (Schleicher and Schull, Dassel, Germany) of PM proteins separated by SDS-PAGE was by standard methods [28]. JIM19 hybridoma supernatant was used to probe immunoblots at 1/100 dilution; JIM20 was used at 1/10 dilution. Rabbit anti-rat IgM conjugated to alkaline phosphatase (Zymed, South San Francisco, CA, USA) and the chromogenic substrates nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate (Sigma) were used to visualize JIM-bound antigens. The titer of JIM19 was estimated to be 2-fold higher than JIM20 by immunoblot analysis (data not shown). Sodium periodate (25 mM solution in 50 mM NaOAc, pH 4.3) was applied overnight to blotted PM proteins to oxidize GP carbohydrates before immunodetection with JIM antibodies.

Flow cytometry of rice protoplasts was performed on a Becton-Dickinson (San Jose, CA, USA) FACS Vantage dual beam instrument equipped with a 200 μm nozzle and Lysis II acquisition and analysis software. The excitation wavelength for flow cytometry was 488 nm. Fluorescence emission detection was with a fluorescein isothiocyanate (FITC) 530/30 nm filter set. For *Aequoria victoria* GFP studies, live cells were analyzed. Cell viability was determined by flow cytometry of an aliquot of live protoplasts treated five min with 0.01% (w/v) fluorescein diacetate (Molecular Probes, Eugene, OR, USA). For immunofluorescence studies, protoplasts at a density of 10^6/0.5 ml were incubated for 30 min in Krens-HEPES with or without JIM19 or JIM20 hybridoma supernatant (1/10 dilution). After two washes with Krens-HEPES, the protoplasts were incubated for 20 min with goat anti-rat IgG-conjugated FITC (1/20 dilution, Sigma), washed two times with Krens-HEPES and fixed in 1% paraformaldehyde in 0.1 M PBS, pH 7.4 for 30 min.

2.3. Plasma membrane preparation by aqueous two phase partitioning

PMs were prepared from fresh or frozen callus cultures according to Larsson [29] with modifications. The tissue was homogenized by sonication in 50 mM Tris, 0.25 M sucrose, 3 mM EDTA (Sigma), 1 mM $MgSO_4$, 2 mM dithiothreitol, pH 7.8. PEG-1540 (Polysciences, Warrington, PA, USA) was used instead of PEG-3350 as the upper phase. Membrane marker enzyme assays [30] for the PM (vanadate-sensitive ATPase), endoplasmic reticulum and mitochondria (antimycin A-resistant and -sensitive cytochrome c reductases, respectively) indicated a PM-enrichment over total membranes of 7- to 20-fold (data not shown). Protein concentations were determined by the dye binding method [28] (Bio-Rad, Hercules, CA, USA).

2.4. Surface plasmon resonance biosensor analysis

The immunoaffinity purification of JIM19 was as follows. Alkaline phosphatase-conjugated rabbit anti-rat IgM (15 mg; Zymed), dialyzed against 10 mM HEPES, pH 6.5, was covalently bound to 0.5 ml Affigel-Gel 10 support (Bio-Rad) according to the manufacturer's protocol. Active esters were blocked with 1 M ethanolamide, pH 8. The column was equilibrated with PBS and 5 ml JIM19 hybridoma supernatant passed over the column. After washing with 10 bed volumes of PBS, the JIM19 antibody was eluted with 2 ml 100 mM Gly, pH 2.5 and collected in 0.1 volume of 1 M KH_2P_i, pH 8.0. The purified JIM19 was dialyzed against PBS and frozen at −20°C until further use.

Approximately 3 ng immunoaffinity-purified JIM19 was immobilized on a CM5 carboxymethylated dextran chip (Pharmacia Biotech, Uppsala, Sweden) by amine coupling at pH 4.6 in 10 mM NaOAc using the manufacturer's provided kit. Detection of SPR was with a BIAcore 2000 (Pharmacia). The JIM19 chip was regenerated after PM binding by passing 0.1 N NaOH over the chip for 60 s. The concentration of immunoaffinity-purified JIM19 used in solution competition experiments was estimated to be 1 ng/μl by NanoOrange (Molecular Probes) fluorescent dye binding and detection with a Cytofluor II microplate spectrofluorometer (Molecular Dynamics, Sunnyvale, CA, USA). Rabbit anti-GUS antibody was from Molecular Probes.

2.5. Functional studies

Transformation of protoplasts was according to [31], using 25 μg each of pBM207 and pAHC18 and 3×10^6 protoplasts/transformation, which were split into six paired samples. pBM207 [32] contains the 650 bp wheat *Em* promoter driving *uidA* expression (GUS). pAHC18 contains the 2.0 kb maize ubiquitin promoter driving firefly luciferase (LUC; [33]) and was included as an internal reference for non-ABA-inducible transient transcription. pCR559 contains the *Em* promoter driving a modified GFP (sGFP) with preferred codons and the S65T mutation that gives enhanced fluorescence, faster chromophore formation and slower photobleaching [34]. It was made by ligation of the 3.3 kb. *Nco*I–*Eco*RI fragment of pBM207 and the 1.0 kb. *Nco*I–*Eco*RI fragment of p35SC4PPDK-sGFP(S65T) containing the nos 3′ terminator [34]. Transformed, washed protoplasts were first treated with 1/10, 1/20, 1/40, or 1/80 volumes of JIM antibody and after 15 min, various concentrations of ABA added to give a final volume of 0.5 ml Krens-HEPES and 0.05% ethanol. For enzyme-based reporter assays, after 18 h incubation, protoplasts were lysed in Reporter Lysis Buffer plus 10 mM 2-mercaptoethanol and LUC activity measured according to the manufacturer's protocol (Promega, Madison, WI, USA) on a Monolight 2010 bioluminescence luminometer (Analytical Luminescence, San Diego, CA, USA). The LUC limit of detection for a linear response of the detector was approximately 7000 relative light units and the sample activities were between 40 000 and 160 000 relative light units/20 μl extract. GUS activities of the samples were determined according to [35], using 4-methylumbelliferone glucuronide (Rose Scientific, Edmonton, Alta., Canada) as substrate dissolved in 50 mM NaP_i, pH 7.0, 10 mM EDTA, 0.1% (v/v) Triton X-100, 3.4 mM *N*-lauryl sarcosine and 10 mM 2-mercaptoethanol. Activities ranged between 4 and 140 pmol 4-methylumbelliferone/h/40 μl extract. Data presented are of experiments that were repeated once with similar results.

3. Results

3.1. JIM19 and JIM20 recognize cell surface glycoproteins in rice

Flow cytometry is a powerful tool that is relatively unexploited in plant biology. The application of this technique to embryonic rice protoplasts immunodecorated with the JIM19 antibody demonstrated substantial binding to the cell surface, i.e. the PM (Fig. 1). Fig. 1B shows the extent of JIM19 binding to a population of rice protoplasts measured as relative fluorescence intensity of FITC-labelled anti-rat IgG secondary antibody. Approximately 70% of the protoplasts are labelled by JIM19, based on a threshold of background fluorescence of control protoplasts labelled with secondary antibody alone (Fig. 1A). Similar results were obtained with JIM20 (Fig. 1C), except the intensity of the fluorescence signal was less than half that of JIM19. JIM20 is related to JIM19 [23] but had no biological activity [24]. Varying the concentration of JIM antibodies over the range of 1/10 to 1/80 dilution did not affect significantly the percentage of cells labelled (data not shown). These data suggest that the affinity of JIM19 for rice PM epitopes is higher than that of JIM20.

In order to characterize further the surface antigens recognized by JIM19 and JIM20, SDS-PAGE and immunoblot analysis were performed on PMs purified by aqueous two phase partitioning. The lability of the rice PM JIM epitopes to sodium periodate, which oxidizes carbohydrates was tested, as previous work suggested that JIM19 and JIM20 recognize the carbohydrate moieties of GPs [23]. JIM19 and JIM20 recognized exclusively the carbohydrate moieties of multiple PM GPs, evidenced by loss of all immunoreactive signals after treatment of immunoblots with sodium periodate (Fig. 2). Similar treatment of an electroblotted non-GP control (β-glu-

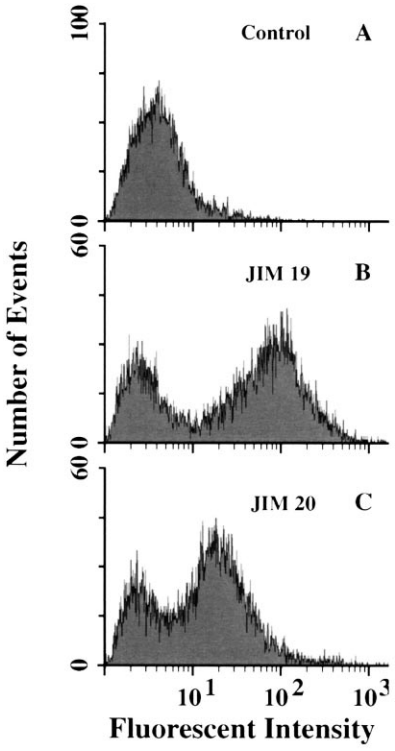

Fig. 1. JIM19 binds to the PM of protoplasts. Flow cytometry histogram of 10 000 rice protoplasts stained with (A) FITC-conjugated secondary antibody against rat IgG alone, or (B) stained after labeling with 1/10 dilution of JIM19 or (C) JIM20. Abscissa is relative fluorescence intensity.

curonidase) had no effect on immunodetection (data not shown). At least five GPs ranging in relative size from 25 kDa to more than 175 kDa were recognized by JIM19. JIM20 also recognized GPs larger than approximately 100 kDa and of similar size to the proteins recognized by JIM19. However, there are also unique PM proteins in the range of 25–60 kDa which react only with JIM19 (Fig. 2). A 10-fold higher concentration of JIM20 than JIM19 did not result in a stronger signal or detection of other epitopes (Fig. 2), consistent with the flow cytometry data (Fig. 1) indicating that the affinity of JIM19 for PM epitopes is higher than that of JIM20.

3.2. Binding of plasma membranes to JIM19 is specifically antagonized by abscisic acid

We used an SPR biosensor [36] to characterize the binding of PM epitopes to JIM19. SPR technology allows monitoring of specific biomolecular interactions between a ligand (e.g. antibody) and an analyte (e.g. antigen), resulting in a change in refractive index measured as arbitrary resonance output units. A major strength of this technology is the sensitive, quantitative, direct determination of real time binding activity in crude, complex mixtures without labelling of components. Three ng (65 fmol of IgM binding sites, assuming 10 binding sites/IgM) of immunoaffinity-purified JIM19 was covalently bound to a carboxymethyl dextran-coated biosensor chip, giving a baseline SPR signal of 21 600 units (Fig. 3, stage 1). A non-saturating amount of PMs (5 μg of protein, or 100 pmol of 'average' [50 kDa] sized proteins) was then bound to the JIM19 chip over a period of 400 s, after which time the binding reaction approached steady state (Fig. 3, stage 2). The amount of PM bound to the chip was linearly dependent on the amount injected (correlation coefficient $r = 0.98$, $n = 7$; data not shown), as expected for concentrations of analyte far below the equilibrium dissociation constant for independent ligand sites. The detection limit of the biosensor for PM binding was calculated as three times the standard deviation of the baseline SPR signal between sample injections; the PM binding signal was on average more than six times higher than the detection limit (data not shown). About 400 SPR units of specific PM binding were observed under the standard binding conditions (Table 1). The specificity of PM binding to JIM19 was investigated using three solution competition assays for JIM antibody specificity. Binding of PMs to the JIM19 chip could be prevented by pre-incubation of PMs with increasing amounts of JIM19 (Table 1). JIM20 also antagonized PM binding to JIM19, suggesting that JIM19 and JIM20 do indeed share specificity for the high molecular weight epitopes observed in Fig. 2. Non-specific interference with PM binding was low, as measured by pre-incubation of PMs with an unrelated antibody (anti-GUS, Table 1). Furthermore, JIM19 did not bind to bovine serum albumin (Table 1) or to human

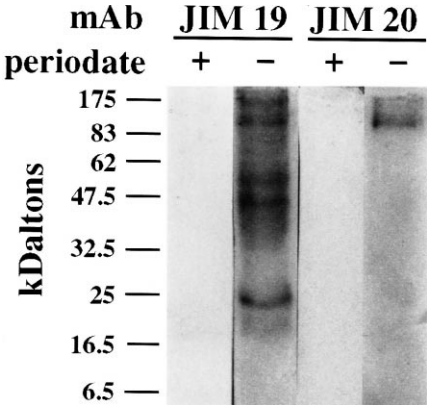

Fig. 2. JIM19 and JIM20 recognize multiple GPs in PMs. Immunoblot of PM proteins separated by SDS-PAGE and probed with either JIM19 (1/100 dilution) or JIM20 (1/10 dilution) with or without pre-treatment of the blot with sodium periodate to remove carbohydrates from GPs.

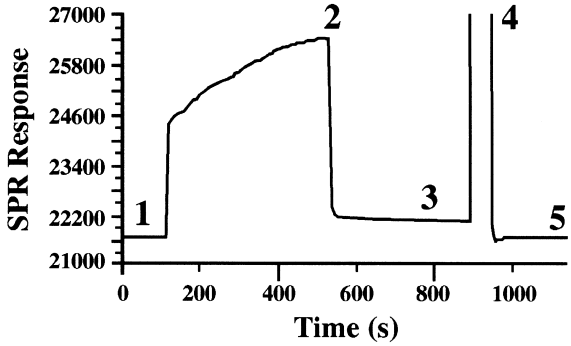

Fig. 3. SPR sensorgram of PM binding to the JIM19 carboxymethyldextran chip. Stage 1: baseline signal in HBS buffer (Pharmacia), followed by injection of 5 μg purified PMs; stage 2: steady state binding of PMs; stage 3: measurement of bound PMs in HBS buffer; stage 4: regeneration of JIM19 chip with 0.1 N NaOH; stage 5: baseline. Discontinuities in the response signal at stages 1, 2 and 4 are due to refractive index mismatch during buffer change.

Table 1
Rice PMs bind specifically to JIM19 monoclonal antibody

Pre-treatments	SPR units (±S.E.M.)	*n*	% Change from control
Control, untreated PM	395(±17)	14	–
PM plus 4 ng JIM19	194(±19)	5	−51
PM plus 8 ng JIM19	169(±34)	4	−57
PM plus 16 ng JIM19	83(±14)	5	−79
PM plus 24 ng JIM20	93	2	−73[a]
PM plus 20 ng non-specific IgG	303	1	−3[a]
50 ng bovine serum albumin	24	1	+6

PMs (5 μg protein in 100 μl HBS buffer, either untreated or pre-treated with various amounts of immunoaffinity purified antibody) were bound in 7 min to a JIM19-immobilized chip.
[a]Denotes effect relative to control samples run on the same day.

neutrophil membrane proteins as detected by immunoblotting (data not shown). Assuming the SPR signal due to binding of PM vesicles (average diameter, 100 nm [29]) is similar to that of other cellular ligands (Pharmacia BIAtechnology Note #105; Uppsala, Sweden), the observed amount of specific binding corresponds to a maximum of 28 fmol of average-sized 50 kDa proteins [36], or less than 0.03% of the total PM proteins applied.

Because previous work suggested that JIM19 may interact with ABA signalling mechanisms [24], we tested in a solution competition assay the effect of ABA on binding of PMs to the JIM19 chip. A dose-dependent partial inhibition by ABA of PM binding to JIM19 was observed (Fig. 4). Statistical analysis of the ABA treatments showed that taken together, the three highest ABA concentrations (>10 μM) resulted in significantly higher inhibition of JIM19 binding to PMs than the three lowest ABA concentrations (<0.1 μM; $P<0.04$, one-sided *t*-test, equal variance). The specificity of this inhibition by ABA was demonstrated by control experiments with phaseic acid (PA), an oxidized catabolite of ABA that is biologically inactive in most, but not all, physiological assays [17]. Equivalent concentrations of PA did not affect PM binding to JIM19 (Fig. 4). A statistical analysis of the data set indicates that ABA was significantly more active than PA in antagonizing the binding of PMs to JIM19 (one-tailed paired Student's *t*-test, $P<0.0008$, $n=16$).

3.3. Functional analysis of JIM19 effects on ABA-inducible gene expression

In order to explore the biological relevance of the in vitro JIM19 interaction with PMs, ABA and phaseic acid, we quantified by two independent methods ABA-inducible transient expression of the *Em* promoter [25,32] in rice protoplasts. Fig. 5 shows the results of an enzyme-based reporter gene assay in transiently transformed rice protoplasts. Exogenous ABA activated the *Em* promoter in a dose-dependent manner, whereas phaseic acid was not active in triggering *Em-GUS* expression (Fig. 5). Hill et al. [17] also found phaseic acid to be inactive in inducing *Em* gene expression in cultured barley embryos. Thus, the in vivo specificity and sensitivity for ABA perception leading to *Em* gene expression (Fig. 5) correlate well with the in vitro specificity for ABA antagonism of PM binding to JIM19 (Fig. 4).

Because protoplasts are a heterogeneous population with potentially different characteristics that might complicate analysis of signalling pathways, we developed a novel, quantitative, ABA-inducible reporter gene expression system based

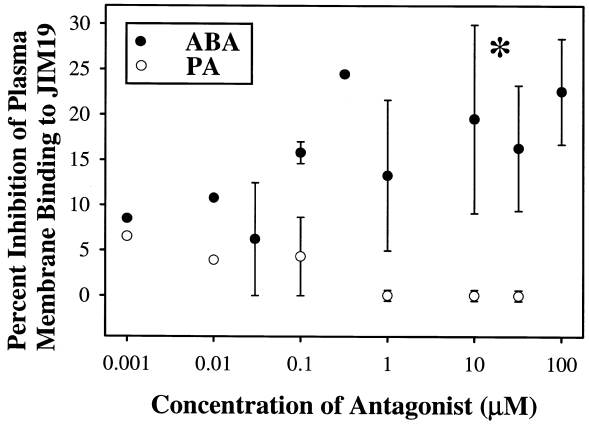

Fig. 4. ABA specifically antagonizes PM binding to JIM19. Various concentrations of ABA (●) or phaseic acid (○) were pre-incubated for 15 min with PMs (5 μg protein equivalent) in 100–200 μl HBS buffer and passed over the JIM19 chip at a rate of 5–10 μl/min. Data points except at 0.01 μM antagonists are the average of two or three independent experiments. Asterisk (*) indicates 10–100 μM ABA treatments are significantly more inhibited than 0.001–0.03 μM ABA treatments, taken together ($P<0.04$, one-sided *t*-test, equal variance). Error bars are ±S.E.M.

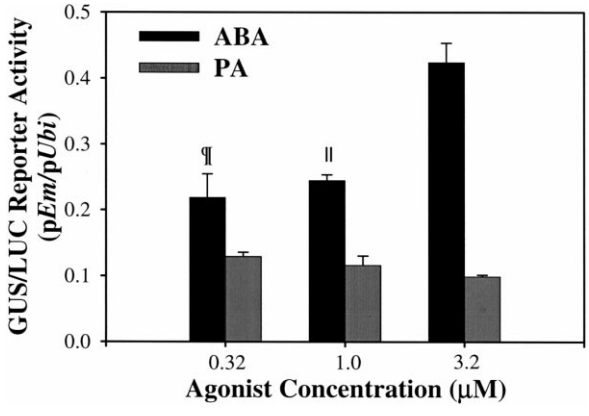

Fig. 5. Phaseic acid does not transactivate the *Em-GUS* reporter. Rice protoplasts were co-transformed with ABA-inducible (*Em*-GUS) and non-ABA-inducible (*Ubiquitin*-LUC) promoter-reporter constructs and treated for 18 h with various concentrations of ABA (dark bars) or phaseic acid (light bars). The ratio of GUS to LUC reporter activities of paired samples is shown. Results are the average of three replicates ±S.E.M. The experiment was performed twice with similar results. ¶: Significantly higher than phaseic acid, $P<0.1$; ıı: $P<0.03$ (Student's one-sided paired *t*-tests).

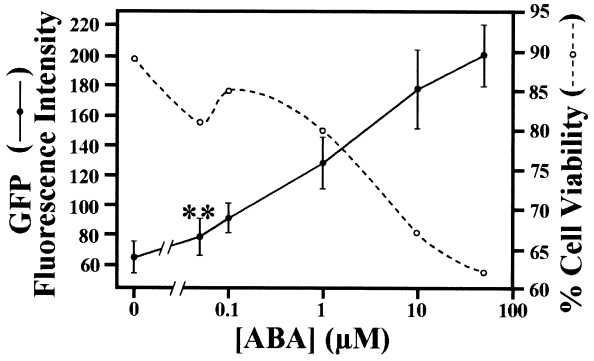

Fig. 6. ABA dose *Em*-GFP intensity response (●) and cell viability (○) curves of transiently transformed rice protoplasts measured by flow cytometry. Treatments were as in Fig. 5 except *Em*-GFP was the reporter construct in transformations. Results are the weighted mean of two independent experiments ± S.E.M. ($n = 77$–154 per datum). ** Indicates 0.05 μM ABA response is significantly higher than untreated control, $P < 0.05$ (one-sided Student's *t*-test, equal variance, $n = 162$).

on GFP and flow cytometry. An ABA dose-GFP intensity response curve for populations of protoplasts transiently expressing GFP driven by the *Em* promoter is shown in Fig. 6. Transformation efficiency was approximately one percent (data not shown). Cell viability of the population was affected negatively by high ABA concentrations (Fig. 6). Nonetheless, a typical log-linear relationship between ABA dose and GFP abundance in individual cells was observed (Fig. 6). Therefore, flow cytometry allows quantitative analysis of *Em*: GFP expression on a per cell basis. The concentrations of ABA required for transactivation of the *Em* promoter in these assays (Figs. 5 and 6) are in the physiological range found in plant tissues [3] and correlate well with concentrations required for ABA-inhibition of PM binding to JIM19 (Fig. 4).

In an attempt to correlate further the in vitro and in vivo effects of JIM19 on ABA signalling, the ability of JIM19 to antagonize ABA-inducible transient *Em*: GUS and *Em*: GFP expression was measured. Initial experiments demonstrated that JIM19 inhibited ABA effects, but that JIM20 had no activity, as reported previously [24]. Consequently, we used JIM20 as a control to account for any non-specific antibody effects. The inhibition of ABA-inducible *Em* promoter activation by co-incubation of protoplasts with JIM19, relative to JIM20 treatment, was low but highly significant (Table 2; one-tailed paired Student's *t*-test, $0.0003 < P < 0.001$). About 45% inhibition of the ABA-inducible expression of the *Em-GUS* reporter gene was achieved by treatment of protoplasts with JIM19 at various low ABA concentrations (Table 2). The inhibition was consistently 25% when measured by flow cytometry of GFP (Table 2). These results on the interaction of JIM19 with ABA perception leading to *Em* promoter activation correlate well with the ABA/PM interaction observed in vitro (Fig. 4).

4. Discussion

The techniques of SPR and flow cytometry have allowed us to probe the mechanisms of JIM19 bioactivity in rice protoplasts. Our working hypothesis and model, based on results presented here, is that the JIM19 antibody recognizes an epitope in rice PMs that interacts with an ABA receptor. It may be that interaction of JIM19 with a surface GP adjacent to, or interactive with, an ABA receptor, particularly masks an ABA binding site and thereby inhibits ABA responses. We speculate that the binding of ABA to a receptor complex in PMs may induce a conformational change that alters the affinity of an *O*-linked GP epitope for JIM19. In this context it is interesting to note that a putative *O*-glucosyl transferase (SPY), when overexpressed in barley aleurone cells, can transactivate the ABA-inducible *DHN* promoter [37].

There are at least five different GPs that are bound by JIM19 (Fig. 2). Solution competition assays with JIM20 showed a high degree of antagonism of PM binding to JIM19 (Table 2), suggesting that JIM19 and JIM20 both recognize the high molecular weight epitopes visualized by immunoblots of PM proteins (Fig. 2). Although such a heterogeneous system complicates biochemical analyses by SPR, these results do suggest that JIM19 interacts, possibly indirectly, with ABA signalling mechanisms.

The semi-quantitative capability of the SPR biosensor allows an assessment of traditional saturable binding assays applied to the postulated ABA receptor, assuming that the calibration of the SPR signal for protein also holds for vesicles. Assuming the observed ABA-antagonized binding of PMs to JIM19 is due to stoichiometric binding of ABA to an ABA receptor which interacts with one of the GPs, then the observed SPR signal corresponds to 50 pCi ^{3}H-ABA, or approximately 2 Bq (120 dpm)/μg PM protein. This amount of label is near the limit of detection of binding assays. The SPR PM-JIM19 biosensor assay described here provides a novel and sensitive method for characterization of an activity associated with ABA perception.

There are conserved heterodimeric GPs in plants and animals, viz. integrins, that function in cell surface recognition, adhesion and signal transduction [38,39]. The affinities and conformation of integrins are modulated by cations and receptor activation [40]. Consistent with an integrin model of ABA signalling is the agonistic effect of multivalent cations on ABA-regulated gene expression [15,41]. In addition, recent reports have demonstrated the existence in plants of glycosylphosphatidylinositol (GPI)-anchored arabinogalactan proteins [42], with exciting implications for signal transduction pathways.

Table 2
JIM19 partially inhibits ABA-inducible transient expression of the *Em* promoter measured by two independent reporter gene assays

Reporter genes	Percent inhibition (± S.E.M.)	*n*	*P* value
GUS/LUC	41(± 4)	9	0.0003
GFP	25(± 4)	6	0.001

For *Em*-GUS experiments, samples were treated with 1/10 dilution desalted JIM19 or JIM20 hybridoma supernatant and either 0.32, 1.0 or 3.2 μM ABA to induce expression. For *Em*-GFP experiments, samples were treated with 1.0 μM ABA and either 1/20, 1/40, or 1/80 dilutions of dialyzed JIM19 or JIM20. Results are expressed as a percentage of the ABA induction of JIM20-treated paired samples. *P* values were calculated by one-sided paired *t*-test; *n* = number of independent samples.

Acknowledgements: The authors would like to thank Dr. Jen Sheen for p35SC4PPDK-sGFP(S65T), Patrick Po Fai Ng, Leigh Brian and Regina Kit Fong Chak for assistance with rice tissue culture and protoplasts, Kim Kam-Chi Li for assistance with BIAcore instrumentation, Frances Shuk-Lan Chan for assistance with flow cytometry, Drs. Tom Schultz and Ralph Quatrano for sharing unpublished results and Drs. Yung Hou Wong and Kevin Lee for critical reading of the manuscript and helpful discussions. This work was supported by Grant #JRS96/33 from the United Kingdom/Hong Kong Joint Research Scheme to C.D.R. and S.J.N. and by Competitive Earmarked Research Grant #HKUST 6173/97M from the Hong Kong Research Grants Council to C.D.R.

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