Quantitative Analysis by Flow Cytometry of Abscisic Acid-Inducible Gene Expression in Transiently Transformed Rice Protoplasts

Dik Hagenbeek and Christopher D. Rock*

Department of Biology, Hong Kong University of Science and Technology, Kowloon, Hong Kong, China

Background: Quantifying plant gene expression by flow cytometry (FCM) would allow multidimensional cell-parameter analysis on a per-cell basis, thereby providing insight into the cellular mechanisms of plant gene regulation. Here we sought to establish quantitation by FCM of plant hormone (abscisic acid, ABA)-inducible green fluorescent protein (GFP) expression and to compare the method directly with traditional reporter enzyme assays.
Materials and Methods: GFP, β-glucuronidase, and luciferase reporter genes driven by ABA-inducible or constitutive promoter constructs were expressed in transiently cotransformed rice protoplasts and reporter activities quantified by FCM (for GFP) or traditional enzyme assays. Treatments included cotransformations with specific ABA signaling effector cDNA constructs (encoding *VIVIPAROUS-1*, an ABA transcription factor, and *ABA-INSENSITIVE1-1*, a dominant-negative protein phosphatase regulator) and the ABA agonist lanthanum chloride. Dual-color FCM was also performed on GFP-expressing cells immunodecorated with an mAb recognizing a rice cell surface epitope.
Results: Quantitative analysis of ABA-inducible gene expression by FCM using GFP as reporter gave comparable results to traditional reporter enzyme assays, although the signal-to-noise ratio was less for FCM, which can be a limitation of the method at low promoter strengths. Multiparameter-correlated analysis of ABA-inducible GFP expression with a plasma membrane marker showed no apparent correlation between ABA sensitivity, marked by GFP, and presence of a cell surface arabinogalactan glycoprotein.
Conclusions: Quantitative FCM of GFP-expressing plant cells is a rapid, robust, reproducible, and value-added method relative to traditional enzymatic reporter gene assays. Cytometry 45:170–179, 2001.
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Quantification of reporter gene expression of transiently transformed cells is a powerful method to characterize *cis*- and *trans*-acting elements and to screen for novel pharmacological agents affecting signaling pathways. Plant protoplasts (spherical cells devoid of the cell wall) are especially suitable to perform gene regulation studies because they are easily prepared in vast numbers (1). The most commonly used reporter gene in plants is the bacterial *uidA* gene (β-glucuronidase; GUS). It is very stable and can be precisely measured in low quantities (2). However, quantitative analysis requires extracting the transformed samples and performing an enzymatic assay. This gives reporter activities for whole-cell extracts rather than for the transformed population only, since there is no easy method to separate transformed cells from untransformed cells. The reporter enzyme activities are usually measured in vitro, and introduction of substrate into live cell assays can also produce artifacts (3). Furthermore, a second internal reference reporter gene construct is desirable to normalize transcription activity, but also adds experimental variables. The use of green fluorescent protein (GFP) and luciferase (LUC) reporters in plants has the advantages of noninvasive visualization and quantitation (4–9). The development of GFP as an intracellular vital reporter has made flow cytometry (FCM) of gene expression feasible (10–13). The fluorescence intensity of GFP is a more direct measure of the promoter activity that drives

its expression than are assays for enzymes or protein binding, since there is no amplification of signal with substrates or multiple fluorophores. However, despite the advantages of multidimensional cell parameter analysis and population sorting by FCM, to date only a few reports of FCM and gene reporter detection in plant tissue have appeared (4,14,15), and their focus has not been on quantitation of GFP.

Abscisic acid (ABA) is a major plant hormone that has a vital role in seed development, germination, and physiological adaptations to environmental stresses (16,17). Most of the physiological responses of plants to ABA are through ABA-inducible or ABA-repressible gene regulation (18–20). A major group of ABA-inducible genes is the large family of late embryogenesis-abundant (*LEA*) genes, which includes the *Em* gene of wheat (*Triticum aestivum*) (21). Two-component ABA-responsive *cis*-elements have been defined for *Em* and other cereal promoters (21–23). Our understanding of the ABA signal transduction pathways leading to the activation or repression of genes is far from complete (20), and would benefit from the application of cell biological methods.

Two genes that have been shown genetically to be involved in ABA-regulated gene expression are *ABA-IN-SENSITIVE-1* (*ABI1*) of *Arabidopsis* (24,25), and *VIVIPA-ROUS-1* (*Vp1*) of maize (26). The *ABI1* gene encodes a serine/threonine protein phosphatase with a negative regulatory role in ABA signaling (27). Mutation of the glycine to aspartic acid at position 180 was shown to result in a dominant-negative phenotype in vitro and in vivo (27,28). *Vp1* is a transcriptional regulator that has both activation and repression activities on hormone-responsive genes and binds to various proteins (29–32). Lanthanide ions have been shown to act as specific agonists of ABA-inducible gene expression upstream of *ABI1* and *Vp1* in the signal transduction pathway; however, the molecular target of lanthanum is unknown (33,34).

The work reported here was initiated to critically compare FCM to traditional enzyme assays for quantitation of gene expression. We demonstrated that ABA-inducible GFP expression quantified by FCM reliably measures ABA-regulated and constitutive gene activity. Furthermore, we performed multiparameter-correlated analysis of ABA-inducible GFP expression and epitope abundance of an arabinogalactan-containing cell surface glycoprotein. Our results establish FCM of GFP as a robust, stable, and value-added method for the quantification of gene regulation and characterization of the cell biology of plant signal transduction.

MATERIALS AND METHODS
Plant Materials

Rice suspension cultures (*Oryza sativa* L., cultivar IR54 from the International Rice Research Institute, Los Baños, Philippines), initiated from germinating embryos, were propagated and digested for making protoplasts, as previously described (21,35).

Transient Transformation and Enzymatic Reporter Assays

Protoplasts were transiently transformed by polyethylene glycol, as described by Maas et al. (1) with modifications (35). For the experiment presented in Figure 2, increasing amounts of pCR559 (*Em*-GFP (35), modified GFP with the S65T mutation (5)) were used to transform aliquots of 3×10^6 rice protoplasts. Each transformation was split into four paired samples in a final volume of 1 ml Krens solution (21), with two of them supplemented with 100 μM ABA. After 17-h incubation, cells were fixed in 2% paraformaldehyde/Krens, and GFP was expression analyzed by FCM.

For the experiment presented in Figure 3, aliquots of 3×10^6 protoplasts were transformed with 40 μg of pCR559 (*Em*-GFP), alone or in combination with 20 μg pAHC18 (encoding the *Zea mays* ubiquitin (*Ubi*) promoter driving LUC (6)) and increasing amounts (10, 30, 50, or 70 μg) of pBM207 encoding *Em*-GUS (36). Each transformation was split into four paired samples in a final volume of 1 ml Krens solution with or without 100 μM ABA. Live cells (i.e., not fixed cells) were analyzed by FCM for GFP expression after 15-h incubation.

For the experiment presented in Figure 4, aliquots of 3×10^6 rice protoplasts were transformed with 80 μg pCR559 (*Em*-GFP) or pDH559 (*Ubi*-GFP (34)). Each transformation was split into four paired samples in a final volume of 1 ml Krens solution containing Krens only, 100 μM ABA, and 1 mM lanthanum chloride, or 100 μM ABA plus 1 mM lanthanum chloride, respectively. Live cells were analyzed by FCM for GFP expression after 16-h incubation.

For the experiment presented in Figure 5, 40 μg of the reporter plasmids pCR559 (*Em*-GFP) and pBM207 (*Em*-GUS), 20 μg of pAHC18 (*Ubi*-LUC), and a total of 60 μg of effector plasmids were combined and used to transform 2.5×10^6 rice protoplasts. The amounts of effector plasmids were: 40 μg pG2 (*Ppdk35S*-abi1-1; "4x" (28)), 10 μg pG2 ("1x"), 20 μg pCR349.13S (*35S-Vp1* (36)), and pDirect2.6, which contains the *Ubi* promoter alone. Transformed cells were split into four paired samples and treated for 15.5 h in a final volume of 1 ml Krens solution containing 0, 1, 10, or 100 μM ABA. Live cells were then analyzed by FCM, and the remainder of the samples was frozen in liquid nitrogen (after 45 min) for later extraction and analysis of GUS and LUC activities. When comparing the FCM and reporter enzyme results by ANOVA, all values were converted to unitless "z scores" ($z = (x - x_{mean})/\sigma$).

Transformations for dual-color FCM received 80 μg of pCR559 (*Em*-GFP) or no DNA as control per 3×10^6 rice protoplasts in a fixed volume of 300 μl. Transformed samples were split and treated with or without 100 μM ABA/Krens for 15 h. Protoplasts were then fixed in 2% paraformaldehyde/Krens for 1 h at room temperature and immunostained (see below).

The experiments (Exp.) presented in Table 1 were performed as follows. In Exp. I, aliquots of 2.5×10^6 rice

protoplasts were cotransformed with 40 μg pBM207 (*Em*-GUS) or pBM314 (Cauliflower Mosaic Virus 35S promoter (*35S*) driving GUS) plus 40 μg pCR559 (*Em*-GFP) and 20 μg pAHC18 (*Ubi*-LUC). Transformed samples were split and treated with or without 100 μM ABA/Krens (1 ml/ 0.6×10^6 protoplasts) for 15 h. Half of the protoplasts from each of the samples were fixed in 2% paraformaldehyde/Krens and analyzed by FCM for GFP expression, while the remaining half were extracted, and GUS and LUC activities were measured. Exp. II was as described above for Figure 5. In Exp. III, aliquots of 3.5×10^6 rice protoplasts were transformed with 80 μg pCR559 (*Em*-GFP) or pCR522 (*35S*-GFP). Transformed samples were split and treated with or without 100 μM ABA/Krens for 18 h. Protoplasts were then fixed in 2% paraformaldehyde/Krens and analyzed by FCM for GFP expression. Exp. IV was as described above for Figure 4. In Exp. V, aliquots of 2×10^6 rice protoplasts were cotransformed with 40 μg pCR559 (*Em*-GFP) or pDH559 (*Ubi*-GFP) and 100 μg of other DNA constructs that were included as controls for unrelated parameters. After 15 h treatment with or without 100 μM ABA/Krens, GFP expression in live cells was analyzed by FCM.

For reporter enzyme assays, protoplasts were lysed in 250 μl lysis buffer (Luciferase Analysis Kit, Promega, Madison, WI) and spun at maximum speed for 1 min in a microcentrifuge. One hundred microliters of substrate (luciferin) were mixed with 10 μl supernatant by autoinjection of the substrate, and luciferase activity was measured by a Zylux FB15-luminometer (Fisher Scientific, Pittsburgh, PA). GUS activities were determined by fluorimetry with 4-methylumbelliferyl-glucuronide as substrate according to Desikan et al. (35), detected with a Cytofluor II microplate fluorimeter (Biosearch, Inc., Framingham, MA). The relative reporter gene activity was represented as the ratio of GUS to LUC activities per unit volume of cell extract.

Immunostaining

Transformed protoplasts (3×10^6) were fixed for 1 h with 2% (w/v) paraformaldehyde in Krens solution at room temperature, followed by three washes with 1 ml Krens to remove fixative, and then immunostained. Primary Ab used was a 1:10 dilution of JIM13, a rat mAb that recognizes an arabinogalactan epitope on the cell membrane of protoplasts from several plant species (37). Secondary Ab was a 1:100 dilution of Rabbit Anti-Rat (catalog no. 61-9822, Zymed, San Fransisco, CA), and the tertiary Ab was a 1:200 dilution of R-PE conjugated Goat Anti-Rabbit (catalog no. P-2771, R-Phycoerythrin-conjugated IgG, Molecular Probes, Eugene, OR). All Ab treatments were in 0.4 ml Krens, 1.25% (w/v) BSA, and 2 mM sodium azide overnight at 4°C, followed by two washes with 1 ml Krens after each staining step. Fixed protoplasts were resuspended in 1 ml Krens and analyzed by FCM.

Plasmid Constructions

The plasmid constructs pCR522, pCR559 (35), and pDH559 (34) contain the modified S65T *Aequoria victo-ria* green fluorescent protein (GFP) gene with enhanced codon usage (sGFP (5)) driven by the *35S*, *Em*, and *Ubi* promoters, respectively. The plasmid pCR522 was generated by ligation of the 3.5-kbp *Bam*HI/*Pst*I fragment of pAG32 to the 0.78-kbp *Bam*HI/*Pst*I fragment of psGFP. The plasmid pAG32 was generated by ligation of the 2.9 kbp *Sma*I/*Nco*I fragment of pDH51 (38) to the 0.66-kbp *Nco*I fragment of pBM314, followed by a fill-in reaction with Klenow fragment and a second ligation step. The plasmid constructs pBM314 and pBM207 (36) were the kind gift of Dr. William Marcotte (Clemson University, Clemson, SC). Plasmid pAHC18 contains the *Ubi* promoter driving LUC (6), and was included in transformations as an internal reference for non-ABA-inducible transient transcription. Plasmid pG2 encodes the chimaeric maize C_4 pyruvate orthophosphate dikinase (Ppdk)-*35S* promoter driving the coding region of the *Arabidopsis thaliana abi1-1* dominant-negative G180D mutant cDNA (28). Plasmid pCR349.13S contains the *35S* promoter driving *Vp1* cDNA (36). Plasmid pDirect2.6 contains only the *Ubi* promoter (no reporter gene), and was used as a control construct to balance the total amount of input plasmid DNA between various treatments.

Flow Cytometric Analysis

FCM of live (i.e., not fixed) protoplasts expressing GFP was performed on a Becton-Dickinson (San Jose, CA) FACS Vantage instrument equipped with a 200-μm nozzle, Lysis II acquisition and analysis software, and a water-cooled Enterprise coherent argon-ion laser (1.3-W output) tuned to 488 nm. The sheet-fluid used was Krens (21). GFP fluorescence was detected with an FITC 530/30-nm band-pass filter. For each sample, 10,000 or 20,000 protoplasts were gated on forward light scatter, and the weighted GFP fluorescence per population of cells was calculated as the product of the average fluorescence intensity of the population of cells above the background threshold (set arbitrarily based on a zero DNA transformed control, so that all control cells fall below this threshold), times the number of individual cells above the same threshold (33,34). The filter configuration for dual-color FCM with single-laser (488-nm) excitation was an FITC 530/30-nm band-pass filter for GFP detection and a 575/26-nm band-pass filter for R-PE-conjugated Ab detection. Single-color control circuits were set up to compensate for spectral overlap between the GFP and R-PE signals. As an indication, the compensation applied to FL2 for Exp. 6 was 16.4% of FL1 (for GFP crossover into R-PE signal), and for FL1, 2% of FL2 (for R-PE crossover into the R-PE signal).

Three-dimensional scatter plots/histograms were generated with the Windows Multiple Document Interface Flow Cytometry Application (WinMDI, © 1997 by Joseph Trotter, Scripps Research Institute, La Jolla, CA), available from http://facs.scripps.edu/software.html. A single level of smoothing interation was performed on the raw data, to render the contour lines shown in Figure 1.
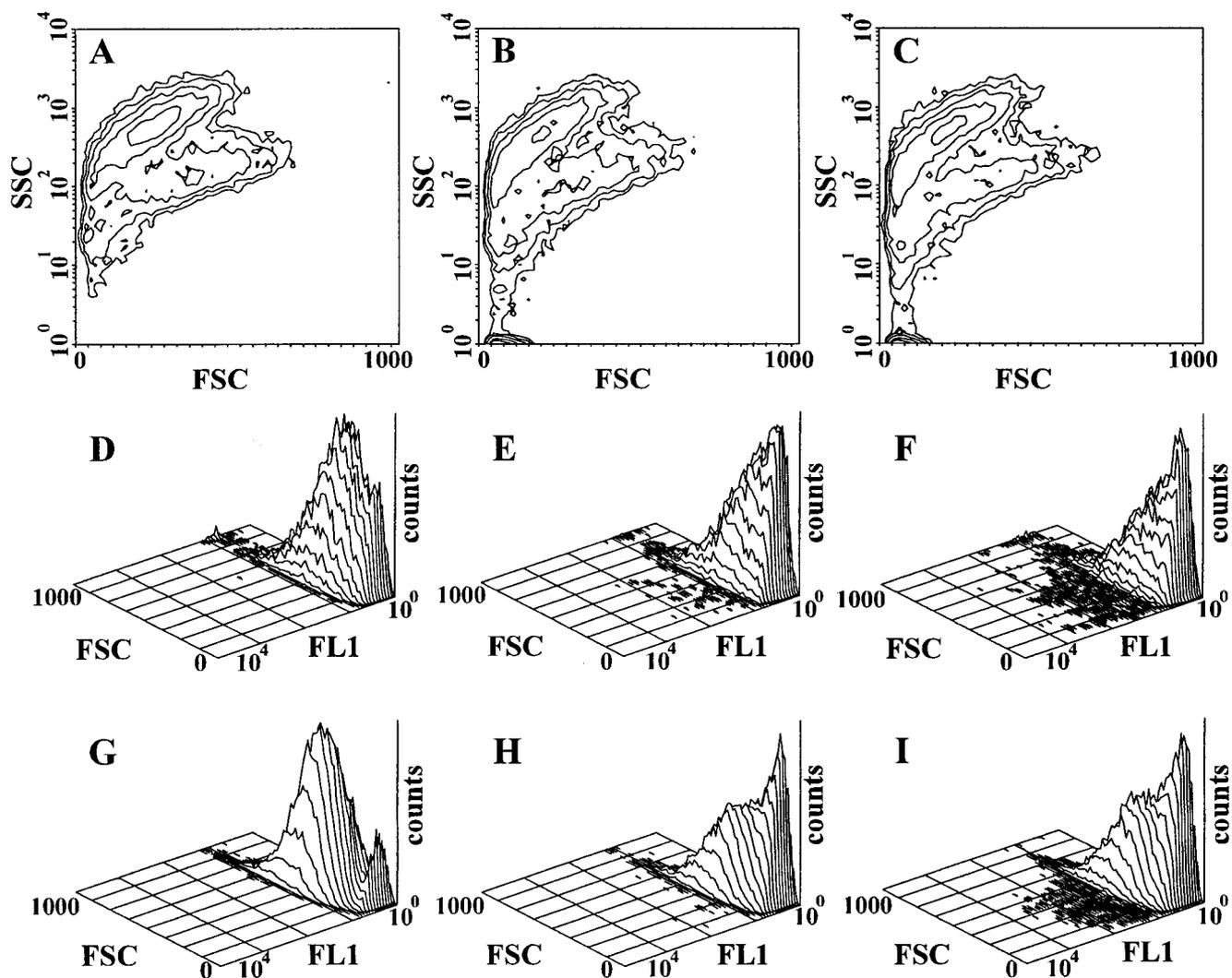
FIG. 1. Three-dimensional scatter plots/histograms of representative data from FCM of rice protoplasts. **A–F:** Fresh (live) protoplasts. **G–I:** Protoplasts fixed with 2% paraformaldehyde. A, D, G: Protoplasts transformed without *Em*-GFP reporter construct. C, D, E, F, H, I: Protoplasts transformed with *Em*-GFP. A, B, D, E, G, H: Protoplasts untreated with ABA. C, F, I: Protoplasts treated with 100 μM ABA. A–C: Side scatter (SSC) versus forward scatter (FSC). D–I: GFP fluorescence (FL1) versus FSC.

RESULTS

Because the heterogeneous nature of protoplasts (39) raises questions about the interpretation of transient gene expression studies that rely on cotransformation of effector and reporter constructs, we sought to employ FCM to characterize the cell biology of ABA signaling on a single-cell level in transiently transformed rice protoplasts expressing GFP. In several experiments, rice protoplasts were transformed with a construct (pCR559) (35) encoding an enhanced GFP cDNA driven by the ABA-inducible *Em* promoter and incubated overnight in the presence or absence of ABA. Protoplasts were then directly analyzed (live protos) by FCM or analyzed after being fixed in 2% paraformaldehyde/Krens. The viability of protoplasts varies from experiment to experiment as determined by FDA staining and FCM analysis (35), but equal viability of pro-

toplasts given different treatments within one experiment, and an overall viability of at least 15%, were observed (data not shown). Representative FCM plots, including controls for these experiments, are presented in Figure 1. Figure 1A–C shows three-dimensional contour plots of live cells, with side scatter (SSC) plotted against forward scatter (FSC) of a zero DNA control sample (Fig. 1A), and *Em*-GFP- transformed samples in the absence (Fig. 1B) and presence (Fig. 1C) of 100 μM ABA. Interestingly, the cells constitute two subpopulations: one with low SSC, and the other with higher SSC (Fig. 1A–C). Upon fixation of cells, the two subpopulations became indistinguishable by SSC (data not shown), an artifact that has been observed with fixation of different cell types (40,41). The nature of the subpopulations is unknown, but observed SSC intensities presumably relate to organellar den-
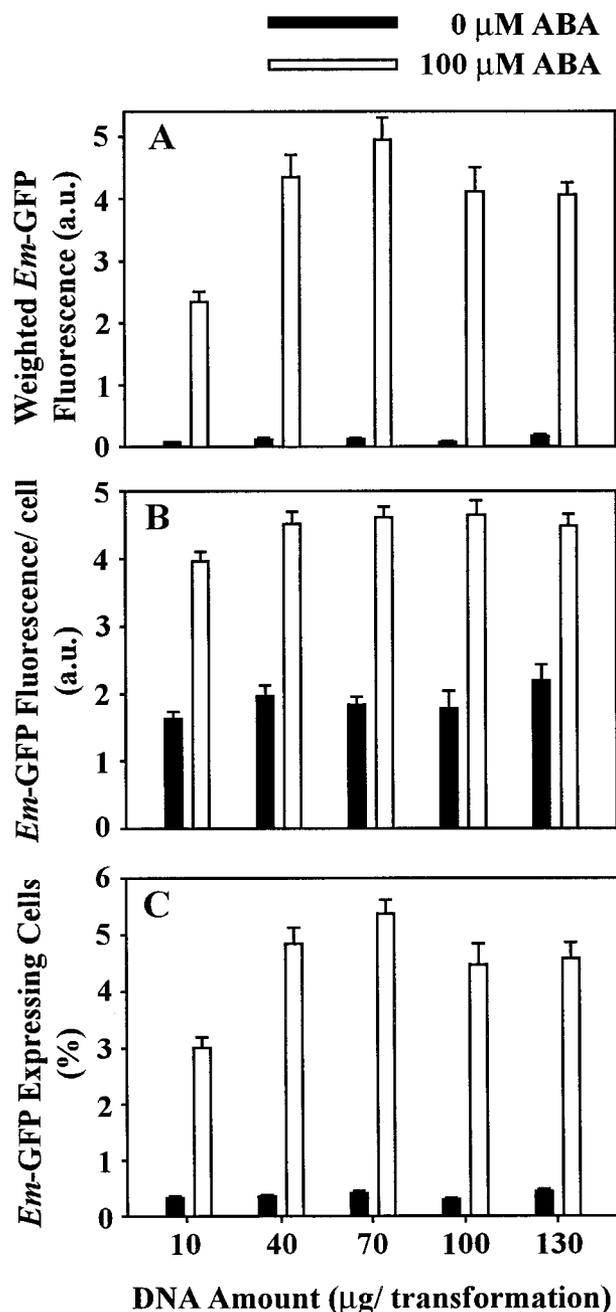
FIG. 2. Three different FCM measurements of ABA-inducible *Em*-GFP expression compared as a function of input DNA. Rice protoplasts were transformed with increasing amounts of *Em*-GFP, and transformed cells were split into two treatments: 0 or 100 μM ABA. After 17-h incubation, cells were fixed and GFP expression was analyzed by FCM. Results are presented in a.u. as: (**A**) weighted GFP Fluorescence, which is the product of (**B**) GFP fluorescence per cell times (**C**) the percentage of cells above the gating threshold. Transformations were performed in triplicate; variance bars are ±SEM.

FIG. 3. Dependence of ABA-inducible *Em*-GFP expression on total DNA input. Aliquots of rice protoplasts were transformed with 40 μg of *Em*-GFP alone or in combination with increasing amounts of other reporter construct-DNAs. Transformations were split into two treatments: 0 or 100 μM ABA. After 15-h incubation, live cells were analyzed by FCM for GFP expression. Numbers in parentheses indicate ABA-fold response relative to untreated, paired samples. *Significantly different than 40 μg treatment, *P* < 0.05 (Student's two-sided *t*-test, equal variance assumed). Transformations were performed in triplicate; variance bars are ±SEM.

sity differences. The SSC of cell populations did not change in response to treatment with ABA or expression of *Em*-GFP (Fig. 1A–C). Arbitrary minimum thresholds were set for gating of fluorescing cells, based on autofluo-
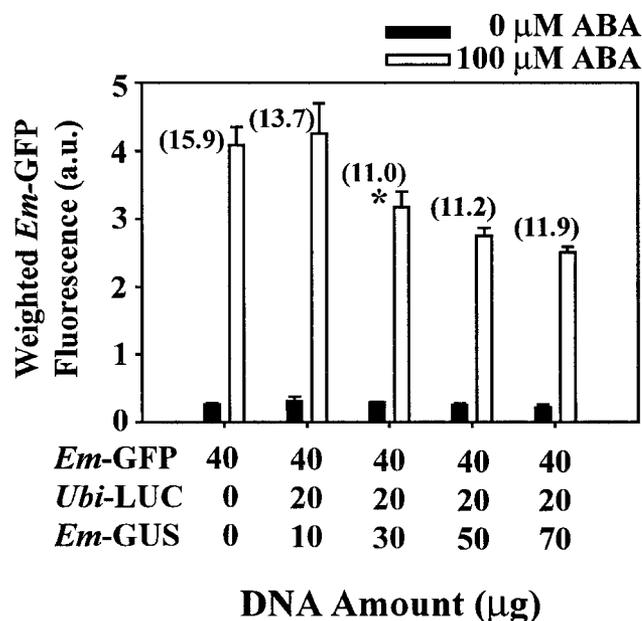
rescence and minimum size (FSC) of zero DNA-transformed cells (data not shown). Three-dimensional contour plots of raw FCM data revealed that fixation of protoplasts resulted in slightly higher autofluorescence (Fig. 1G–I) relative to live cells (Fig. 1D–F). A small population of GFP-expressing cells (approximately 1% of total cells) was gated in uninduced, *Em*-GFP-transformed cells (Fig. 1E,H). Upon induction with 100 μM ABA, an increase in the percentage of gated *Em*-GFP-expressing cells (to approximately 6%), as well as an increase in the fluorescence intensity of the gated population, was observed in both fresh (live) and fixed protoplasts (Fig. 1F,I). Both small (low FSC) and large (high FSC) cells expressed GFP to a similar extent (Fig. 1F,I), and were thus equally transformed. It was indicated from the increase in gated cell number in response to ABA (Fig. 1F,I) that the limit of detection of GFP expression by FCM was near the gating threshold for background autofluorescence.

Previous studies on DNA uptake in plant protoplasts showed that only a subset of cells is competent to take up DNA, and the amount of uptake is limited (42–45). Therefore, the effect of the amount of input DNA on ABA-inducible gene expression was determined. Rice protoplasts were transformed with increasing amounts of a construct (pCR559) (35) encoding an enhanced GFP cDNA driven by the ABA-inducible *Em* promoter and incubated overnight in the presence or absence of ABA. The results of three different calculations of GFP fluores-
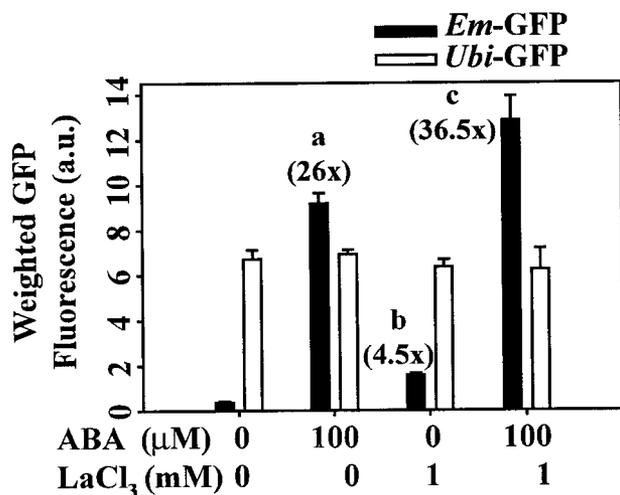
FIG. 4. Specificity of ABA- and lanthanum-induced *Em*-GFP gene expression measured by FCM. Rice protoplasts were transformed with either *Em*-GFP or *Ubi*-GFP, and then aliquots were incubated for 16 h with: Krens only, 100 μM ABA, 1 mM lanthanum chloride, and ABA plus lanthanum chloride. GFP expression was analyzed by FCM of live cells. a and b indicate significantly higher than Krens only ($P < 0.005$); c indicates significantly higher than 100 μM ABA ($P < 0.03$) (Student's one-sided *t*-test, equal variance assumed). Transformations were performed in triplicate (*Em*-GFP) or quadruplicate (*Ubi*-GFP); variance bars are ±SEM.
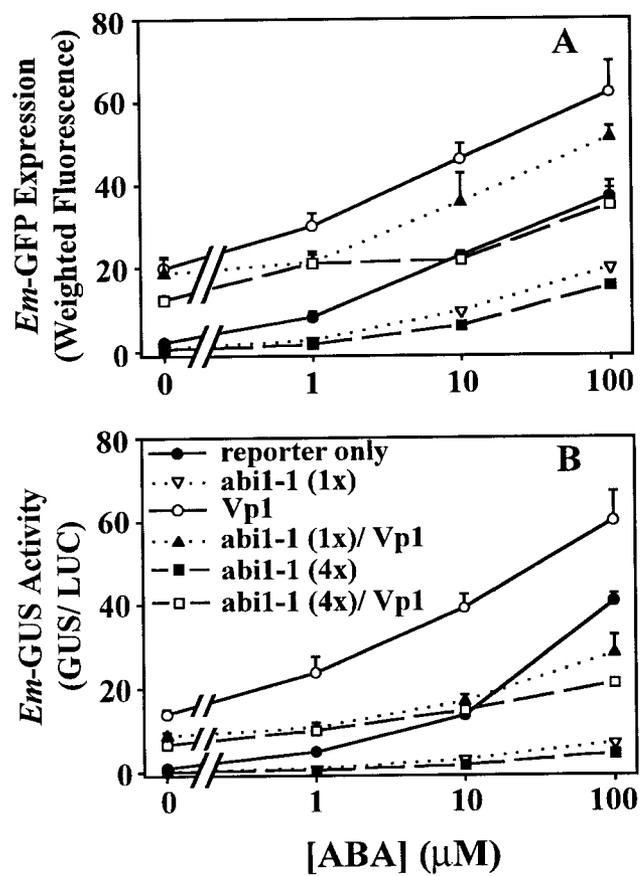

FIG. 5. Direct comparison of FCM (*Em*-GFP) to enzymatic assay (*Em*-GUS/*Ubi*-LUC) in cotransformed protoplasts expressing ABA effector constructs. Rice protoplasts were transformed with equal amounts of *Em*-GUS and *Em*-GFP. The non-ABA-inducible *Ubi*-LUC reporter construct was cotransformed as an internal control for transcription activity. Cotransformed effectors were: *35S*-Vp1 and *Ppdk35S*-abi1-1 alone, or combined (with two input concentrations for *Ppdk35S*-abi1-1). **A:** Aliquots of transformants were treated for 15.5 h with: Krens solution only, 1 μM ABA, 10 μM ABA, and 100 μM ABA. **B:** FCM was performed on aliquots, and the remainder of the samples were extracted (after 45 min) for GUS and LUC enzyme assays. Transformations were performed in quadruplicate; variance bars are ±SEM.

cence from FCM data are shown in Figure 2. FCM of GFP fluorescence marked a DNA dose-dependence of ABA-inducible *Em* promoter activity at low (10–40 μg) DNA input, whether calculated as the average fluorescence per cell (Fig. 2B), the percent of GFP-fluorescence expressing cells (Fig. 2C), or the product of these two measurements (the "weighted fluorescence;" Fig. 2A, data not shown). However, the DNA dose-dependence of ABA-inducible GFP expression was not strongly evident on a per-cell basis (Fig. 2B). The weighted GFP fluorescence of ABA-treated cells increased with increasing DNA input, reaching a maximum at 70 μg input DNA (Fig. 2A), and decreasing at higher DNA inputs. The maximum percentage of cells in the population that expressed GFP in response to 100 μM ABA was 5.3%, whereas in the absence of ABA the percentage of cells expressing GFP was only 0.5% (Fig. 2C). The average GFP fluorescence intensity per cell was about two times higher in the presence of ABA than in the absence of ABA at all DNA input concentrations (Fig. 2B). Taken together, these observations suggest a threshold effect of ABA for GFP fluorescence measurement by FCM, since transformed cells in the absence of ABA did not exhibit DNA dose-dependent GFP expression (Fig. 2A–C).

The results of Figure 2 suggested that high DNA inputs could affect the quantitation of GFP in transient assays. Therefore, the relationship of ABA-inducible *Em*-GFP expression measured by FCM was analyzed as a function of input DNA. When the GFP-encoding input DNA was kept constant at 40 μg per transformation and the total amount of DNA input was increased with various promoter-reporter constructs, a significant drop in ABA-inducible *Em*-GFP expression was observed at 90 μg total DNA input

(Fig. 3, $P < 0.05$, Student's two-sided *t*-test, equal variance assumed). When the dynamic range (fold-induction) of the ABA response was calculated as a function of input DNA, a similar DNA-dependent drop in ABA-inducible GFP fluorescence (fold-response relative to the without ABA control) was observed (Fig. 3). These results demonstrate that maximum GFP expression is a function of the total, rather than specific, DNA input. Consistent with this interpretation was the observation that the LUC activity was negatively correlated with increasing total DNA input over 90 μg (data not shown). The optimal amount of total input DNA for GFP detection by FCM was between 40–70 μg per transformation of 3×10^6 protoplasts. We observed that the time-courses for detection of reporter gene activity by FCM and enzyme assays are similar (S. Gampala and C. Rock, unpublished observations). Furthermore, although the microplate fluorimeter used in GUS enzyme

Table 1
*Signal-to-Noise Ratios for GFP and GUS Reporters Driven
by Different Strength Promoters in Rice Protoplasts**

Construct	Ratios of signal-to-noise for treatments					
	Zero ABA	(SEM)	n	100 μM ABA	(SEM)	n
Exp. I						
Em-GFP	2.1	(0.2)	8	13.0	(1.5)	8
Em-GUS	49.0	(3.6)	4	670.0	(39.0)	4
35S-GUS	150.0	(11.0)	4	140.0	(9.1)	4
Exp. II						
Em-GFP	3.2	(0.6)	4	35.0	(2.2)	4
Em-GUS	66.0	(12.0)	4	2,800.0	(120.0)	4
Exp. III						
Em-GFP	5.1	(1.4)	3	80.0	(4.5)	3
35S-GFP	6.2	(0.7)	3	4.1	(1.4)	3
Exp. IV						
Em-GFP	2.1	(0.4)	3	54.0	(2.6)	3
Ubi-GFP	39.0	(2.3)	4	40.0	(1.0)	4
Exp. V						
Em-GFP	1.5	(0.1)	3	86.0	(20.0)	3
Ubi-GFP	23.0	(4.3)	3	27.0	(2.1)	3

*Signal-to-noise ratios from five independent experiments (Exp.) were calculated for the respective promoter reporter-construct sample activities relative to gene activity measured for "zero DNA" transformed controls (noise). Results are the average (±SEM) of "n" samples.

assays was not sensitive enough to detect GFP expression in transiently transformed rice protoplasts, it could detect GFP in mammalian cells which are transformed at about 10-fold higher efficiencies than rice (S. Gampala and C. Rock, unpublished observations).

In order to directly demonstrate that *Em*-GFP gene expression quantified by FCM reflects ABA signaling and not indirect effects of ABA on transformation efficiency or GFP stability, protoplasts were transformed in parallel experiments with *Em*-GFP or the non-ABA-inducible *Ubi*-GFP construct and treated with ABA, the ABA signaling agonist lanthanum chloride (33,34), or both. The results of FCM of transformed protoplasts are shown in Figure 4. The weighted *Ubi*-GFP expression was not affected by ABA or lanthanum treatments, in contrast to *Em*-GFP, which showed a 26-fold increase in response to 100 μM ABA, a 4.5-fold increase in response to 1 mM lanthanum, and a 36.5-fold synergistic response to both ABA plus lanthanum (Fig. 4), as previously shown (33,34).

In order to directly compare GFP quantitation by FCM to traditional reporter enzyme assays, protoplasts were cotransformed with three or more reporter constructs. Each cotransformation included *Em*-GUS (reference) and *Em*-GFP (test) reporter genes, as well as the non-ABA-inducible *Ubi*-LUC as an internal control for transformation efficiency and nonspecific transcriptional effects. The parallel expression of the GFP, GUS, and LUC reporter genes in response to exogenous ABA treatment, transactivation, or transrepression by cotransformed ABA-signaling effector constructs (*35S*-Vp1 and *Ppdk-35S*-abi1-1, respectively) was quantified both by FCM (for GFP fluorescence) and GUS/LUC enzyme assays. The results are shown in Figure 5. Transactivation by the Vp1 transcription factor, transrepression by abi1-1 dominant-negative

protein phosphatase, and ABA-dose responsiveness of the *Em* promoter, measured either by FCM (Fig. 5A) or traditional reporter enzyme assays (Fig. 5B), showed qualitatively similar results. Both the FCM and enzyme assays marked a significant synergistic activation by ABA and Vp1, and a dose-dependent antagonism of ABA-inducible and Vp1 trans-activation by abi1-1 dominant-negative protein phosphatase, as previously observed (33,34). Analysis of variance (ANOVA) was performed to ascertain whether the two methods gave comparable results. There was no significant difference ($P > 0.39$) between the results obtained by FCM versus enzyme assays, with the exception of a reduced effect of abi1-1 inhibition of Vp1 transactivation of *Em*-GFP at all concentrations of ABA (Fig. 5). ABA/Vp1 synergy was antagonized by *Ppdk-35S*-abi1-1 (1x) and (4x) doses by 50.5% and 59.3%, respectively, when reported by *Em*-GUS, but only 19.0% and 41.1%, respectively, when reported by *Em*-GFP (Fig. 5; data not shown). Further work is needed to understand the apparent interaction of *Em*-GFP and *Em*-GUS with combined effector genes *Vp1* and *abi1-1*.

The reporter enzyme assays employed in these experiments also use photons as the signal output and PMTs as detectors. It was therefore of interest to directly compare the dynamic range exhibited by FCM versus reporter enzyme assays on paired samples. The signal-to-noise ratios calculated from several transient expression experiments that used three different promoters (non-ABA-inducible, *Ubi* and *35S*; ABA-inducible, *Em*) driving GUS or GFP are shown in Table 1. The signal-to-noise ratios ranged from 1.5–5.1 for non-ABA-induced *Em*-GFP (Table 1, Exp. I–V), and from 49–66 for non-ABA-induced *Em*-GUS (Table 1, Exp. I–II). The non-ABA-inducible promoter *35S* gave a signal-to-noise ratio between 4.1–6.2 by GFP (Exp. III)
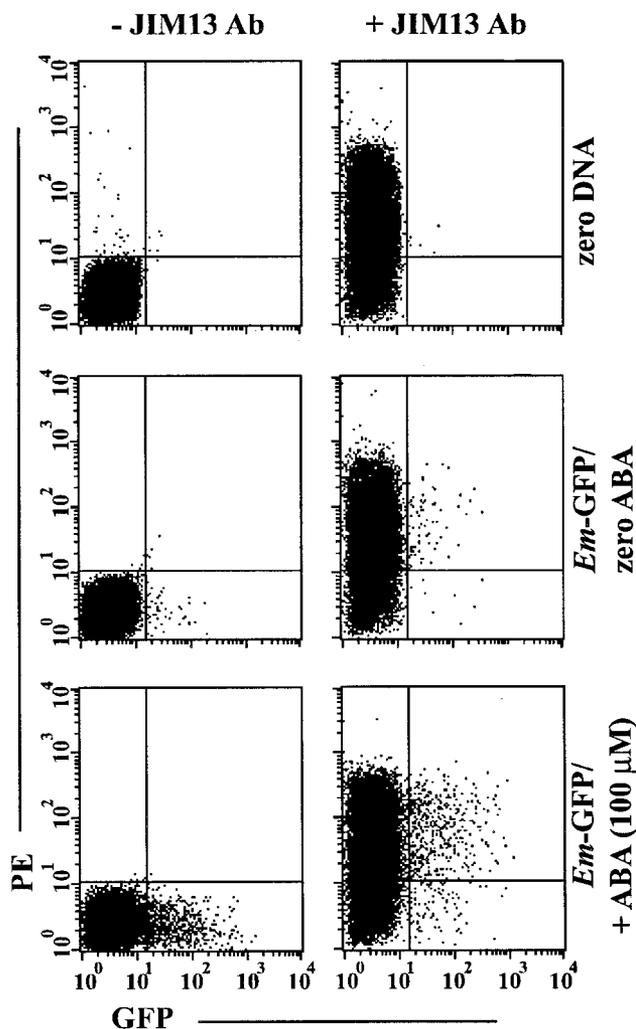
FIG. 6. Dual-color FCM to correlate *Em*-GFP expression with presence of a plasma membrane marker. Rice protoplasts transformed with *Em*-GFP (or no DNA control) were treated with 0 or 100 μM ABA for 15 h, and then were fixed and immunostained with JIM13, followed by a PE-conjugated secondary Ab.

versus 140–150 by GUS (Exp. I). The stronger non-ABA-inducible *Ubi* promoter gave a signal to noise of 23–39-fold when reported by GFP (Exp. IV, V). The observed signal-to-noise ratios for the ABA-induced *Em* promoter were between 13–86 and 670–2,800 for ABA-induced *Em*-GFP and *Em*-GUS, respectively (Table 1, Exp. I–V and I–II, respectively). In these experiments, LUC activity was defined experimentally as unity in reference to measured GUS activity. The signal-to-noise ratio for LUC measurement with PMT in these experiments was typically greater than 800 (data not shown).

A major feature of FCM is dual- or multiple-color analysis that allows correlation of biological parameters on a per-cell basis. *Em*-GFP-expressing cells treated with or without 100 μM ABA were immunostained with an mAb (JIM13) that recognizes a cell surface arabinogalactan glycoprotein (37) and were analyzed by dual-color, single-

beam FCM. The results are shown in Figure 6. When induced with 100 μM ABA, 3.5% of cells expressed *Em*-GFP. JIM13 treatment resulted in immunodecoration of 75.0% of rice protoplasts. There was no significant difference in JIM13 immunodecoration of ABA-treated versus untreated cells (data not shown), indicating that supplementing protoplasts with ABA and DNA plasmids does not significantly affect the cellular processes associated with JIM13 epitope abundance (Fig. 6). JIM13 binding showed significant preference for GFP-expressing cells (84% of total ± 1.6% SEM) compared to non-GFP-expressing cells (74% ± 0.8% SEM; $P < 0.001$, Student's two-sided *t*-test, equal variance assumed). The ABA response, measured as fold-induction of *Em*-GFP plus 100 μM ABA, was not significantly different between non-JIM13-epitope carrying cells (15.5-fold, ± 10.5 SEM) versus JIM13-epitope-carrying cells (12.1-fold, ± 2.9 SEM; $P > 0.78$, Student's two-sided *t*-test, equal variance assumed; data not shown).

DISCUSSION

More than 5 years ago, analysis of transgene expression in plants by FCM utilizing GFP as reporter was reported, and the potential for this method was discussed (4,5). Although the use of GFP as vital reporter in plants has accelerated since then, there are limited reports of quantitative GFP analysis by FCM in plants. We previously employed FCM of GFP to quantify gene expression (33,34), but a direct comparison of FCM to traditional reporter enzyme assays has not been reported. Here we critically compare our quantitative gene expression assay, which uses a novel "weighted fluorescence" unit, to the traditional enzymatic GUS/LUC assay and demonstrate that the FCM method is valid (Figs. 4, 5). The ABA response of the *Em* promoter, as reported by GFP and analyzed by FCM, is not only represented by an increase in average fluorescence per expressing protoplast (which would ideally be the case), but also by an increase in the percentage of GFP-expressing cells (Figs. 2, 3). Therefore, to capture the maximum response in one unit, we introduced "weighted fluorescence," which utilizes both the percentage of GFP-expressing cells as well as their above-background average fluorescence. FCM is less sensitive than reporter enzymes when driven by low-activity promoters such as uninduced *Em* and *35S* promoters (Fig. 2, Table 1). The signal-to-noise ratio for *Em*-GFP by FCM is on average 20–30-fold less than that of *Em*-GUS detection by fluorometric enzyme assay (Table 1; data not shown). Weak promoters might therefore not be detected efficiently by FCM. Engineered GFP variants with higher fluorescence efficiencies and spectral shifts (46) may overcome this drawback and provide additional advantages for FCM of gene expression. A yellow fluorescent variant of GFP has a similar emission intensity when excited at 488 nm, despite its excitation efficiency at 488 nm being only about 40% that of GFP (12). Engineering two spectrally distinct GFP reporter cDNAs on one reporter plasmid construct also could exponentially enhance the signal-to-noise ratio when analyzed by dual-color FCM. Adding an enhancer element to the promoter of the reporter con-

struct could extend the sensitivity range. A good example of these two points in animal cells is the chimeric reporter based on the genes for enhanced fluorescent protein and *Photinus* luciferase, which allows clonal selection based on green fluorescence and consecutive high-throughput screening by luminescence with improved signal-to-noise ratios for reporter cell lines expressing G-protein-coupled receptors (47). Kar-Roy et al. showed that an enhanced GFP (EGFP) was a more sensitive reporter than chloramphenicol acetyl transferase (CAT) in HeLa cells at low DNA concentrations, while CAT became more dominant at higher DNA doses, which they explained by possible enzymatic amplification of the signal (48). Alternatively, stable transformation of GFP-expressing inducible cassettes could enhance the signal-to-noise ratio and allow, for example, further analysis of the heterogeneous nature of plant hormone sensitivity (39).

Here we have demonstrated the feasibility of dual-color FCM of GFP and a cell surface epitope in rice protoplasts (Fig. 6), which paves the way for cell biological studies of ABA signaling by multiparameter-correlated analysis. We employed a second laser with ultraviolet (351–364 nm) emission in order to obviate the need for spectral compensation circuits; however, approximately 5% of the protoplasts exhibited autofluorescence when detected with a blue 424/44 band-pass filter (data not shown), which probably emanated from the vacuoles (4). This observation is an example of the value of FCM to characterize a subpopulation of cells. The fixing of protoplasts preserved the cell shape (data not shown) and GFP fluorescence, giving similar FCM results for live (fresh) and dead (fixed) cells (Fig. 1D–I). Further work is needed to establish methods for permeabilization of fixed rice protoplasts to quantify cytoplasmic or organellar-localized gene products by immunostaining.

Analysis by FCM of dose dependence of DNA input to GFP expression in rice protoplasts showed that a subset of cells is competent to take up DNA, and the amount of uptake is limited (Figs. 2, 3), as reported for other species (42–45). This result highlights an advantage of FCM over biochemical methods, namely, the ability to quantify gene expression on a per-cell basis in a small (<6%; Fig. 1F,I) subpopulation of transformed cells, rather than measuring the average of the population. We interpret our results to indicate that the subpopulation of transformed cells responds uniformly to ABA signaling effectors. The homogeneous nature of the rice protoplast response to ABA revealed by FCM is in contrast to gibberellin response in barley protoplasts (39), and stands as an example of the value of FCM over traditional biochemical methods. By utilizing the non-ABA-inducible *Ubi*-GFP construct, we directly demonstrated the specificity and magnitude of ABA induction of gene expression (Fig. 4). However, caution should always be exercised when performing and interpreting transient assays, since DNA inputs affect the expression of GFP (Figs. 2, 3) and other reporters and effectors (data not shown). For example, overexpression of transcription factors, or titration by input DNA of endogenous transcription factors, could distort the intracel-

lular dynamics of the regulatory components under study and result in artifactual or erroneous readouts. Sorting was successfully performed on *Em*-GFP-expressing rice cells (data not shown). This gives the advantage of physically separating cells of interest which can be used for further investigation or, for example, generation of stably transformed cell lines. Since transformation efficiencies are typically low in rice tissue culture protoplasts, cell sorting provides practical advantages.

The abundance of JIM13-binding cell surface epitope was not altered by ABA treatment (Fig. 6). The higher percentage of JIM13 binding to GFP-expressing cells (84%) versus non-GFP-expressing cells (74%) may be an artifact caused by a higher percentage of dead cells in the non-GFP-expressing population that may have lost JIM13 epitopes due to degradation. We are currently examining by multiparameter FCM the relationship between JIM19 epitope abundance and ABA sensitivity marked by *Em*-GFP expression. JIM19 recognizes a unique plasma-membrane glycoprotein that may be part of an ABA receptor complex, since JIM19 antagonizes ABA-inducible gene expression (35,49) and, conversely, ABA can antagonize JIM19 binding to plasma membranes in vitro (35). Multiparameter FCM could elucidate the cellular mechanisms of ABA perception.

In conclusion, quantitation by FCM of inducible gene expression in rice protoplasts is a valid method with the advantages over biochemical assays of live cell analysis, sorting of populations, and multiparameter correlative analysis. These attributes have the potential to bring together the fields of cell biology and gene regulation to elucidate the mechanisms of signal transduction from the cell surface to the nucleus.

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