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Aequorin-based measurements of intracellular Ca^{2+}-signatures in plant cells

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ABSTRACT

Due to the involvement of calcium as a main second messenger in the plant signaling pathway, increasing interest has been focused on the calcium signatures supposed to be involved in the patterning of the specific response associated to a given stimulus. In order to follow these signatures we described here the practical approach to use the non-invasive method based on the aequorin technology. Besides reviewing the advantages and disadvantages of this method we report on results showing the usefulness of aequorin to study the calcium response to biotic (elicitors) and abiotic stimuli (osmotic shocks) in various compartments of plant cells such as cytosol and nucleus.

INTRODUCTION

As in animal cells, intracellular free Ca^{2+} in plant cells, is a key actor in regulatory functions, playing a major role in triggering various endogenous and exogenous signals to cellular responses. The range of Ca^{2+} effects is quite extraordinary. An involvement of Ca^{2+} has been described, for example, on growth, differentiation, mitosis, cytosolic streaming, stomata regulation, induction of defense responses, and stress adaptation (1,2). Among the Ca^{2+}-mediated signals are biotic stimuli such as phytohormones and different external signaling compounds, as well as abiotic stimuli, such as phytochrome-mediated red-light and environmental stress factors (2). Thus, in the plant cell Ca^{2+} signals are ubiquitous and present at all times. Since plants are immobile, they face a myriad of possible deleterious stimuli without any possibility to depart. To survive they have to discriminate both the nature and the strength of the stimulus to mount an appropriate adaptive response. It has been established now that the calcium variation does not proceed in a stereotypical manner. This observation led to the concept of "calcium signature" (3,4) whose specificity has been illustrated (5-8). Calcium signature has been clearly correlated with the final response on the basis of its constitutive parameters which are the shape, the amplitude, the duration and, in the case of oscillations, the frequency (8). However, this signature has been mainly evaluated only in one compartment, the cytosol, without simultaneously considering the putative variation in other compartments which are probably implicated in the generation of specific cellular signatures. The recombinant aequorin technology allows to address a calcium reporter-protein to a given sub-cellular compartment at will. It then becomes possible to measure and compare calcium fluctuations occurring simultaneously in different organelles or compartments and, consequently, to evaluate their relative contribution to the response elicited by a stimulus. In this way, in addition to the cytosol, the nucleus is certainly one of the most important compartments to consider because knowing specific changes in nuclear calcium concentration opens up new promising research areas: (I) How nuclear calcium might control gene expression in plants and thus participate in the processing of the adaptive plant response; (II) How the cytosol and nucleus might be interconnected in terms of calcium signals for encoding specificity; (III) The putative homeostasis machinery of the nucleus.

How to monitor intracellular [Ca^{2+}] changes

To make sure that intracellular Ca^{2+} and/or the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) has a role in the signal transduction of a certain stimulus, in 1980 Jaffe (9) postulated three criteria to be all fulfilled on the experimental level: (I) an inhibition of the $[Ca^{2+}]_{cyt}$ increase inhibits the physiological response, (II) an artificial increase of the $[Ca^{2+}]_{cyt}$ induces the response in the absence of the stimulus, and (III) an increase of the $[Ca^{2+}]_{cyt}$

precedes or accompanies the response. Whereas the first two claims could be proven by the use of chelators and ionophores, meeting the last criterion is much more difficult to approach, because it is necessary to measure the intracellular resting level as well as any raise upon treatment with the various stimuli. Besides monitoring the quality of the signal, the quantification of the $[Ca^{2+}]_{cyt}$ during the signaling event must be realized. Ca^{2+}-selective microelectrodes and dyes have been used as efficient tools for analyzing changes of the intracellular Ca^{2+} concentration (10,11). However, because microelectrodes are not easy to handle and need strong technical equipment this technique is not widely distributed. Making use of Ca^{2+}-sensitive dyes is more convenient. A great number of fluorescent calcium indicators are commercially available now (www.probes.com). However, some dyes have to be injected into the cells raising difficulties concerning their localization in plant cells. Other Ca^{2+}-sensitive dyes are impermeable unless their carboxyl group(s) were modified to acetoxymethyl esters. Moreover, a few dyes e.g. quin-2 and indo-1 bleach in the light (12). Nowadays, most of the calcium signaling studies on plant cells are performed using the aequorin technology that is based on bioluminescence.

Principle of aequorin-bioluminescence

Aequorin is a Ca^{2+}-binding photoprotein composed of an apoprotein (apoaequorin) which has an approximate molecular weight of 22 kDa and a prosthetic group, a luciferin molecule, coelenterazine (MW 432). In presence of molecular oxygen the functional holoprotein aequorin reconstitutes spontaneously. The protein contains three EF-hand Ca^{2+}-binding sites. When these sites are occupied by Ca^{2+}, aequorin undergoes a conformational change and behaves as an oxygenase that converts coelenterazine into excited coelenteramide which is set free together with carbon dioxide. As the excited coelenteramide relax to the ground state, blue light (λ = 469 nm) is emitted (Fig. 1) (13). This emitted light can be easily detected with a luminometer.

Here we report on the localization of the heterologously expressed apoaequorin that was directed to a sub-cellular compartment of tobacco suspension cells, and on measurements of transient $[Ca^{2+}]_{cyt}$ and nuclear Ca^{2+} concentrations ($[Ca^{2+}]_{nuc}$) based on the usage of the Ca^{2+}-sensitive aequorin system. We challenged transgenic cell cultures of soybean and tobacco with a biotic stimulus, a phytopathogen-derived elicitor, and an abiotic stimulus, osmotic stress, respectively. We discuss the advantages and disadvantages of the aequorin technique compared to Ca^{2+}-sensitive fluorescent dyes with respect to the analyses of Ca^{2+} signaling in plant cells.

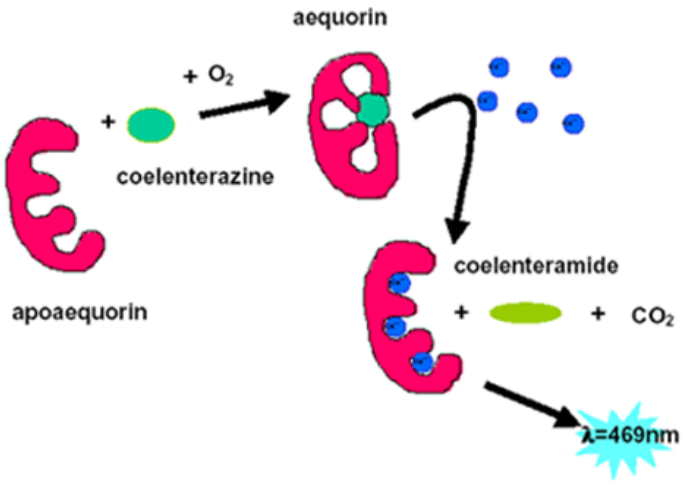

Fig. 1: Mechanism of light emission by aequorin upon Ca^{2+}-binding.

MATERIALS AND METHODS

Cell cultures

The cell suspension cultures of soybean (*Glycine max* L., line 6.6.12) expressing apoaequorin were grown at 22°C under constant light conditions (3,000 lux) on a rotary shaker (125 rpm) in Murashige & Skoog medium supplemented with 5 g l^{-1} sucrose, 1 mg l^{-1} α-naphthylacetic acid, and 0.2 mg l^{-1} kinetin, pH 5.8 (5). The transformed tobacco (*Nicotiana tabacum* L. cv BY-2) suspension cells were grown under agitation (130 rpm) at 25°C in darkness in Linsmaier & Skoog (LS) medium supplemented with 30 g l^{-1} of sucrose and 1 mg ml^{-1} of 2,4-dichlorophenylacetic acid, pH 5.8 (14). Subculturing was done every 2 weeks with a 2% inoculum of a 14 days old culture.

Construction of nucleus-targeted apoaequorin

The chimeric construct including the CaMV 35S promoter that controls the nucleoplasmin coding region placed in frame with the coding region of apoaequorin (15) was obtained as an *EcoR1* fragment from the pCon3 vector kindly provided by A. van der Luit (Amsterdam, The Netherlands). The whole chimeric gene was inserted into the *EcoR1* site of the *Agrobacterium tumefaciens* binary vector pBIN19. The plasmid was then mobilized from *Escherichia coli* to *A. tumefaciens* LBA4404 strain. Fig. 2 illustrates the construction steps of the pBin19 vector carrying the nucleoplasmin-aequorin cDNA obtained from the pCon3 vector.

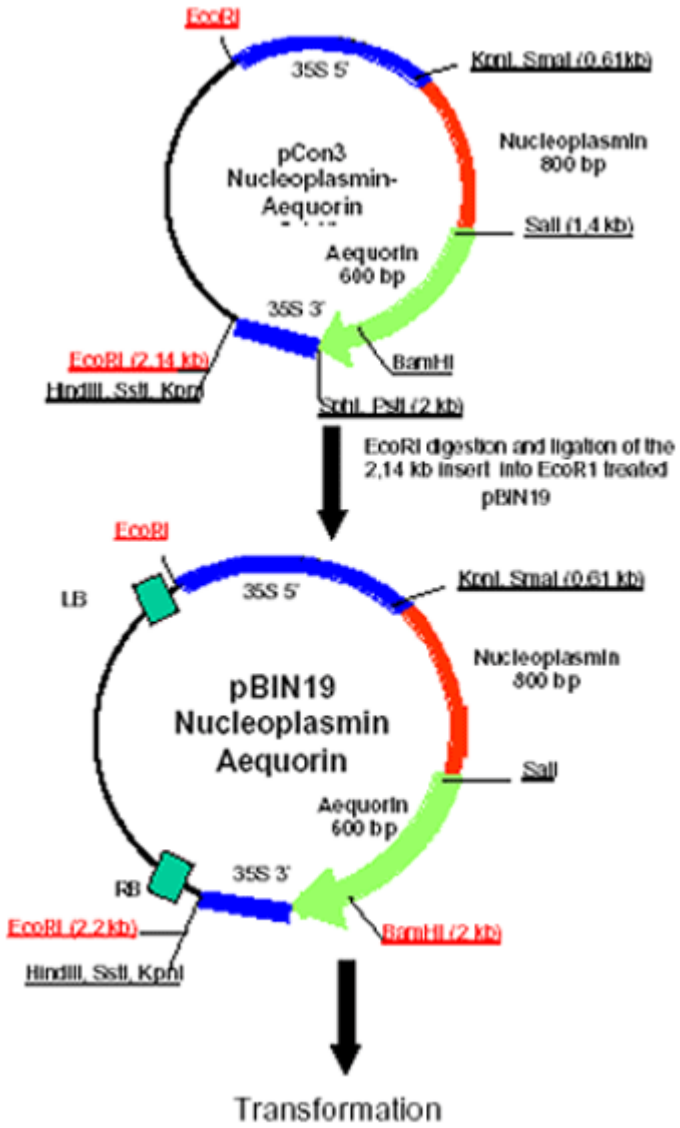

Fig. 2: Construction of pBin19 vector carrying the nucleoplasmin-apoaequorin cDNA derived from the pCon3 vector.

Tobacco BY-2 cell transformation and transgenic soybean

Tobacco BY-2 cells expressing apoaequorin in the nucleoplasm were established by transformation using the above strain according to (16). Briefly, 4 ml of 8 d-old exponentially growing suspension-cell culture were transferred to a 90 mm Petri dish and incubated at 28°C with 100 µl of fresh overnight culture of *A. tumefaciens* LBA4404 containing the pBIN19 binary vector including the chimeric cassette. After 48 hrs of co-cultivation, the tobacco cells were washed and plated on LS agar medium (1%) containing 500 µg ml^{-1} of carbenicillin and 250 µg ml^{-1} of kanamycin. After 4 weeks of selection, transformed calli with a size of about 2 mm were collected and subcultured on the same medium for two more weeks before introducing them in the LS medium supplemented with the antibiotics.

For cytosolic Ca^{2+} measurements in soybean, we used the transgenic cell line 6.6.12 carrying the stably integrated plasmid pGNAequ/neo2. This plasmid includes the apoaequorin cDNA under the control of a double CaMV 35S promoter fused to the *omega* element of tobacco mosaic virus (yielding cytosolic apoaequorin) and as a selectable marker, the neomycin phosphotransferase II cDNA controlled by the CaMV 35S promoter which was introduced to the cells by particle bombardment.

Immunolocalization of apoaequorin in the nucleus

Protoplasts were prepared from the BY-2 cells expressing apoaequorin in the nucleus, fixed and mounted in agarose gel as previously described (17). Immunolocalization was performed using a 1/500 dilution of a crude rabbit anti-apoaequorin serum provided by M. T. Nicolas (INSERM U432, Montpellier, France) and a Texas-red-labeled goat anti-rabbit IgG-conjugate from ICN as the secondary antibody. Control experiments were performed by omission of the first antibody. Samples were observed with an inverted microscope (DMIRBE, Leica, Heidelberg, Germany). Fluorescence was visualized using a green excitation range (excitation filter BP 530-560 nm, suppression filter LP 580 nm). Images were acquired with a CCD camera (Color Coolview, Photonic Sciences, Milham, UK). Image processing was performed using Image-Pro Plus software (Media Cybernetics, Maryland, USA) in order to eliminate the background of the epifluorescent images and to overlap images. The merging of images acquired in bright field and in fluorescent microscopy was useful to show the exact localization of apoaequorin within the nucleus.

Luminescence measurements

Aequorin light emission was measured using a digital luminometer (Bio Orbit 1250, Turku, Finland). BY-2 cells were collected by filtration during the exponential growth phase, washed with fresh medium and resuspended at a 20% packed cell volume in fresh medium. *In vivo* reconstitution of the aequorin was performed by incubating an appropriate volume of washed cells with 2.5 µM of coelenterazine (Calbiochem, Bad Soden, Germany) under agitation for at least 3 hrs. A variable amount (50 µl to 100 µl) of reconstituted cells were transferred to a luminometer cuvette and luminescence was recorded every second during the experiment. Typically, the luminescence was monitored until the base-line luminescence was reached. At the end of the experiment, the remaining reconstituted aequorin was estimated by discharging by addition of an equal volume of 100 mM $CaCl_2$ containing 10% ethanol (v/v) and 2% Nonidet P-40(v/v) . The emitted light expressed as RLUs (Relative Luminescence Units) was calibrated into Ca^{2+} concentrations by a method based on the calibration curve of Allen *et al*. (18):

$$[Ca^{2+}] = \{(L_0/L_{max})^{1/3} + [KTR(L_0/L_{max})^{1/3}] - 1\}/\{KR - [KR(L_0/L_{max})^{1/3}]\},$$

where L_0 is the luminescence intensity per second and L_{max} is the total amount of luminescence present in the entire sample over the course of the experiment. $[Ca^{2+}]$ is the calculated Ca^{2+} concentration, KR is the dissociation constant for the first Ca^{2+} ion to bind, and KTR is the binding constant of the second Ca^{2+} ion to bind to aequorin. The luminescence data were determined using the KR and KTR values of 2 x 10^6 M^{-1} and 55 M^{-1}, respectively, calculated by van der Luit *et al*. (19) using native coelenterazine and the specific aequorin isoform that we have used in these experiments. For soybean, transgenic 6.6.12 cell lines were used to reconstitute aequorin *in vivo* with 10 µM synthetic coelenterazine on a shaker (125 rpm) in the dark for up to 24 hrs. The Ca^{2+}-specific luminescence measurements were performed at room temperature in a final volume of 100 µl containing 5-10 mg (fresh mass) of reconstituted cell suspension culture. Again, the residual aequorin was completely discharged and the resulting luminescence was used to estimate the total amount of aequorin present in various experiments in order to determine the rate of aequorin consumption. This enabled us to calculate the cytosolic Ca^{2+} concentrations. According to Moyen *et al.* (20) we used the equation:

$$pCa = 0.332588(-\log k) + 5.5593$$

where k is a rate constant equal to luminescence at any time point divided by total remaining luminescence counts. In each experiment, the concentration of reconstituted aequorin was not limiting under any of the experimental conditions, with a maximal consumption not exceeding 10%.

Osmotic shock and elicitor treatments

For hyper-osmotic challenges, a known volume of BY-2 medium supplemented with various concentrations of mannitol was added to the cells in the luminometer tube. The variation in osmolality was calculated by subtracting the osmolality value of culture medium (240 mosmol) to the value measured at the end of the experiment in the luminometer cuvette using a osmometer (VAPRO 5520 WESCOR, Fisher Bioblock Scientific, Illkirch, France). For elicitation of soybean cells we used β-(1,3)-β-(1,6)-glucans prepared from cell walls of the phytopathogenic oomycete *Phytophthora sojae* (5). Treatments with β-glucan elicitors was performed by adding 1-10 µl of stock solutions to the cell suspension culture. Mixing time for the addition of any compound was 5-7 s.

RESULTS AND DISCUSSION

After cloning the cDNA encoding apoaequorin from jellyfish (21), Knight and co-workers did a pioneer work in the development of the aequorin system for the first time in plants (22). The numerousness following investigations making use of the recombinant aequorin technique strongly emphasizes the importance of this method in studies on intracellular Ca^{2+} changes induced by both, biotic or abiotic stimuli (Table 1).

Induction of $[Ca^{2+}]_{cyt}$ increase in soybean cells upon elicitor treatment

In soybean suspension cells, indirect evidence obtained by Ca^{2+} channel-blockers, chelators and ionophores indicated a requirement of Ca^{2+} for the induction of defence responses including phytoalexin production (23). However, to really understand the site of suspected changes in intracellular Ca^{2+} concentrations, its kinetics, and its position in the signal transduction chain towards cellular responses, a detailed study of the reactions triggered by Ca^{2+} is required and prompted us to investigate an elicitor-mediated Ca^{2+} response in soybean cells expressing aequorin. The addition of the elicitor derived from the soybean pathogen, the oomycete *P. sojae*, to these soybean cells resulted in a rapid increase in luminescence indicating a rise in the $[Ca^{2+}]_{cyt}$ (Fig. 3).

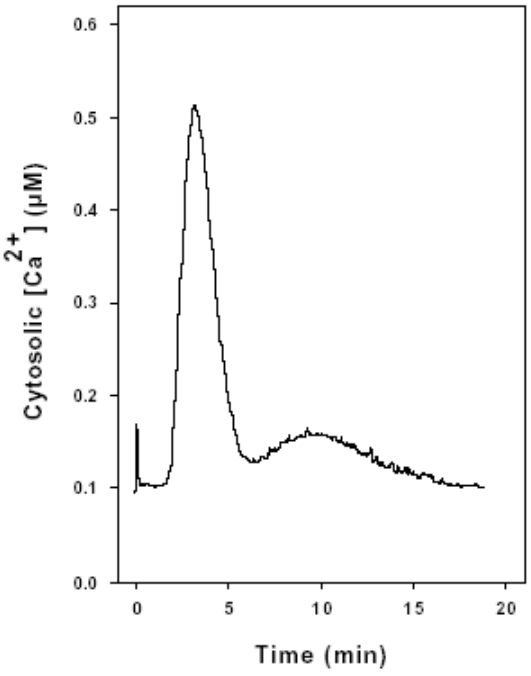

Fig. 3: Kinetics of the β-glucan elicitor-induced enhancement of the $[Ca^{2+}]_{cyt}$ in soybean cells. β-Glucan fragments with the degree of polymerization of 7-15 (DP 7-15, 60 mM) were added at time = 0.

The concentration of the $[Ca^{2+}]_{cyt}$ increased from its basal level of about 100 nM, starting after 90-120 s. While the initial rise in concentration was similar to that obtained with other biotic stimuli, such as chitotetraose (CH4) (5), a second increase in

Ca^{2+} concentration was observed before the resting level of the $[Ca^{2+}]_{cyt}$ was reached after 18 to 20 min. This second peak in $[Ca^{2+}]_{cyt}$ had its maximum at about 8-10 min. The biphasic response to the *P. sojae* elicitor correlated with phytoalexin production, whereas the CH4-induced monophasic response did not (5). The magnitude of the Ca^{2+} response was dependent on the elicitor concentration with a concentration causing a half maximal effect (EC_{50}) of 72 µM for the β-glucans used (DP 7-15) (5). When the Ca^{2+} antagonist La^{3+} was added to the cell culture medium, the elicitor-mediated elevation of the $[Ca^{2+}]_{cyt}$ was inhibited (Fig. 4). In this case, the apparent lag phase of elicitation was prolonged with increasing concentrations shifting the maximum of the Ca^{2+} response to at least 4-6 min (Fig. 4). As La^{3+} is not taken up by the cells, it appears likely that extracellular Ca^{2+} which enters the cells through Ca^{2+} channels is at least in part an important source for the $[Ca^{2+}]_{cyt}$ increase.

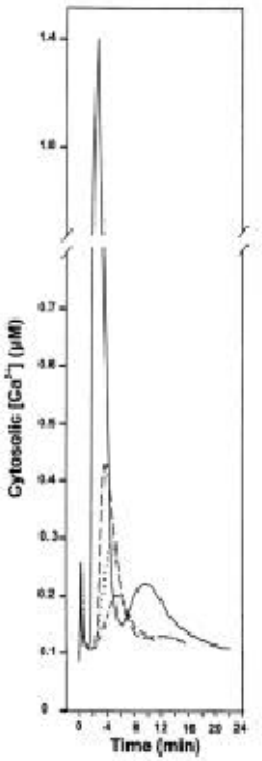

Fig. 4: Effect of the Ca^{2+} channel inhibitor La^{3+} on the β-glucan-induced $[Ca^{2+}]_{cyt}$ response. β-Glucan was added at time = 0 (DP 7-15, 250 mM) alone (—) or after 2 min of preincubation with 2 mM La^{3+} (— —), 4 mM La^{3+} (···) or 6 mM La^{3+} (- -).

Cytosolic and nuclear $[Ca^{2+}]$ changes in tobacco BY-2 cells challenged with hyperosmotic shock

Besides the kinetic parameters, a growing body of evidence suggests that spatial components, such as various subcellular compartments, are involved in the fine tuning of Ca^{2+} signature and Ca^{2+} homeostasis as well. For example, the vacuole and the endoplasmic reticulum are considered as internal sources and buffer for $[Ca^{2+}]_{cyt}$ regulation (24). Moreover, the nucleus also shows an autonomously regulated Ca^{2+} pool involved in certain signaling processes (14,25). These different compartments might contribute *per se* or in combination with the cytoplasm to a specific Ca^{2+} signature preceding specific cellular responses to specific stimuli. To address this question, a nucleus-targeted apoaequorin was expressed in transgenic tobacco cells. The nuclear localization of the chimeric apoaequorin in tobacco BY-2 cells was proved by using an anti-apoaequorin serum from rabbit that mainly localized the transgenic apoaequorin within the nucleus (Fig. 5) as it was reported for *N. plumbaginifolia* cells before (19).

Thus, these tobacco BY-2 cells became an interesting tool to study the calcium signaling in this particular compartment in response to various stimuli. Here, we present evidence showing that plant cell suspension cultures respond to an abiotic stress factor, the variation of their osmotic environment by either a binary or a graduated changes in cellular free calcium at least in the cytosolic and the nuclear compartments.

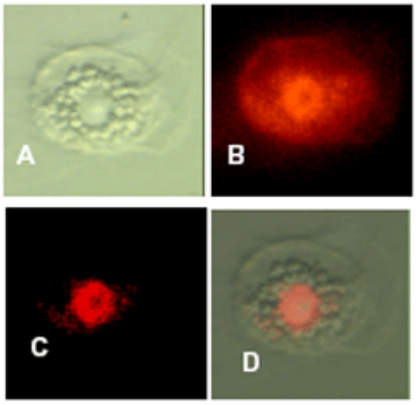

Fig. 5: Targeting of apoaequorin to BY-2 tobacco cell nuclei. (A) Phase contrast image of a typical BY-2 protoplast. (B) Epifluorescence image of the same protoplast treated with antiapoaequorin and Texas-red labeled secondary antibody. (C) Processed image of the same labeled protoplast in order to eliminate the lowest fluorescent pixels. (D) Co-localization of the nucleus and the aequorin by merging fluorescent image.

Fig. 6A displays typical calcium responses in both compartments, the cytosol and the nucleus, after challenging the tobacco BY-2 cells with a neutral osmoticum (mannitol, traces a-c) or an ionic osmoticum (NaCl, traces d-f).

By considering the cytosolic calcium responses, it clearly appears that the tobacco cells are able to sense the strength of the stimulus independently on the nature of the stimulus. The $[Ca^{2+}]_{cyt}$ increased in a bimodal manner both in the presence of mannitol or NaCl. When the osmolality of the external medium containing mannitol as osmoticum was ≤ 520 mosmol, the ratio r of the first peak (peak 1) over the second peak (peak 2) was <1, (r= 0.48, trace a) and r= 0.7 (trace b), respectively.

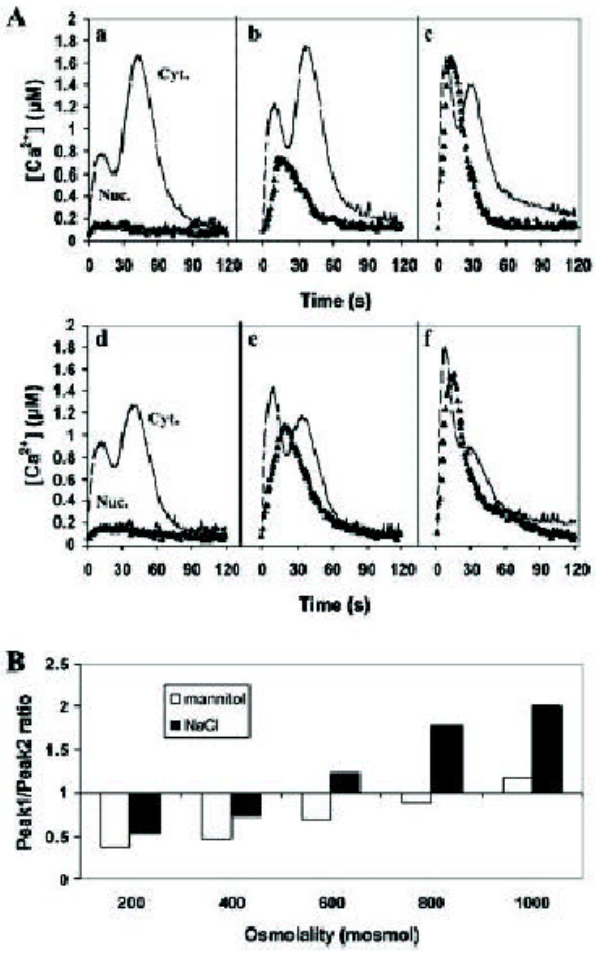

Fig. 6: Effect of the nature of the osmoticum and the strength of the hyper-osmotic shocks on the changes in nuclear and cytosolic calcium in tobacco BY-2 cells. Panel A: Calcium signatures monitored in BY-2 cells expressing apoaequorin either in the nucleus (▲) or in the cytosol (-) in response to various hyper-osmotic treatments challenged with neutral (mannitol; traces a-c) or with saline osmoticum (NaCl; traces d-f). The variations of osmolality were 300 mosmol (traces a,d), 520 mosmol (traces b,e) and 880 mosmol (traces c,f). Panel B: Changes observed in the ratio of peak1/peak2 according to the strength and the nature of the osmoticum in independent experiments (n=3). Reprinted from (14): Pauly et al. (2001), Cell Calcium 30:413-421 with permission of the publisher Churchill Livingstone.

This ratio became progressively >1 (r= 1.18) when the strength of the stimulus was around 880 mosmol or higher (trace c). When the cells were challenged with NaCl using the same differences in osmolality, the ratio of peak 1 over peak 2 was <1 with an osmolality of around 300 mosmol but as soon as the osmolality was equal or higher than 520 mosmol, the ratio r became greater than 1 (compare Fig. 6A traces b,c and traces e,f). The differential behavior of the two cytosolic peaks is illustrated in Fig. 6B where a ratio r >1 indicates that peak 1 is the major peak. These results clearly indicate that the cytosol discriminates both the strength and the nature of the osmotic stimulus in terms of calcium response. The major differences observed between the cytosolic and nuclear calcium response after challenging the cells either with a neutral or saline osmoticum, deal with the calcium pools mobilized as discussed extensively (14). Considering the nuclear calcium response, the nuclear compartment is non-responsive below 300 mosmol either after challenging the cells with NaCl or mannitol (compare Fig. 6A, traces a and d). At higher concentrations of osmoticum the response is displayed as a single peak whose amplitude is related to the strength of the stimulus. In contrast to the cytosol, the nucleus clearly did not modulate the calcium responses in function of the nature of the stimulus (ionic or neutral). In addition, these data clearly demonstrate that $[Ca^{2+}]_{cyt}$ variation are not systematically followed by a $[Ca^{2+}]_{nuc}$ variation, arguing against a free passive diffusion of cytosolic calcium through the nuclear pores. The resulting overall calcium response in the cell, deduced from the combination of the nuclear and the cytosolic calcium response might contribute to the specificity of the final response to a specific stimulus. We have reported in this paper two examples of calcium responses using different kind of stimuli, abiotic (osmotic stress) and biotic (elicitors). The calcium signals obtained are different and specific due to the nature of the stimulus and linked to a certain adaptive response. This specificity of the calcium response has led to the concept of calcium signature where the compartmentation, amplitude, duration and frequency of a given signal are the characteristic parameters.

Why is aequorin so useful in plant Ca^{2+}-signaling studies?

The intensive use of aequorin in plants is very probably due to its versatility, its high dynamic range from 100 nM up to 10 µM, the lack of side-effects encountered with the fluorescent probes such as buffering capacity or toxicity, and its ability for a clear compartmentalization within the cell due to the facility for a specific targeting to an organelle or a sub-cellular compartment (26). The compartmentalization probably represents one of the main problems using the Ca^{2+}-fluorescent dyes which depends on the cell type, the indicator, and the loading conditions. In the latter case when loading of the plant cells is made by microinjection with impermeable dyes it is sometimes difficult to restrict the probe to the cytosol due to the large vacuole of plant cells. To circumvent the injection process, permeable derivatives of the Ca^{2+}-dyes have been developed which are more suitable for plant cells. After uptake, the acetoxymethyl esters of these compounds are cleaved off by endogenous plant esterases which, however, not always exhibit the corresponding specificity. Moreover, an incomplete hydrolysis might allow them to enter other compartments such as ER or mitochondria where the esters will be hydrolyzed (12). Since this enzymatic activity is temperature dependent and because indicator sequestration is sometimes mediated also by organic anion transporters, the cytosolic dye concentration will be influenced by the temperature (27). Another problem encountered with fluorescent dyes is photodamage of the indicator under constant illumination generating less fluorescent species and a decreasing signal over time. A comparative study has been performed with various calcium indicators under a continuous

exposure to a 20 μW laser illumination, the energy close to the one used during confocal imaging. The half time life was between 73 s for the most susceptible dye, Oregon green 488, and 339 s for the most resistant dye, Fluo-4 (28). The major advantage of Ca^{2+}-sensitive fluorescent indicators is brightness. This parameter became the major limitation of aequorin emitting only one photon per molecule. In a transformed cell we can expect only 1 out of 10^2 to 1 out of 10^7 molecules emitting a single photon from the total pool in comparison to up to 10^4 photons emitted by a single fluorescent molecule (26). Thus, the light emission is very low and requires a photomultiplier tube for photon detection prohibiting spatial resolution and investigations on the single-cell level. In the case of fluorescent probes this brightness is much higher and responses can be detected under fluorescence microscopy using for instance a charged–coupled device (CCD) camera adding spatial information to the study. However, fusion of aequorin and green fluorescent proteins (GFP) increases the intensity of emitted light taking advantage of an intramolecular chemiluminescence resonance energy transfer between aequorin and GFP. Combining the Ca^{2+} sensitivity of aequorin and the fluorescence of GFP, the fusion protein enables to visualize calcium signals in a single neuroblastoma cell using an intensified CCD camera (29). Genetically improved aequorin in combination with the possibilities for a specific targeting within the cells or a specific cell type expression will keep the aequorin-based technique in the Ca^{2+} signaling business in plants.

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Table 1: Studies on intracellular [Ca^{2+}] changes induced by biotic and abiotic stimuli and monitored using the aequorin technology.

Stimulus	Organism	Reference
Mechanical stress (touch,wind)	N. plumbaginifolia (cyt)	22,30
Cold	N. plumbaginifolia (cyt)	22
	A. thaliana (cyt)	31
Heat	N. plumbaginifolia (cyt)	32
Blue light	A. thaliana (cyt)	33
	N. plumbaginifolia (cyt)	34
Darkness	N. plumbaginifolia (chl, cyt)	35
Drought	A. thaliana (cyt)	36
Osmotic shocks	N. tabacum (cyt)	16
	A. thaliana (cyt)	36
	N. plumbaginifolia (cyt)	37
	N. tabacum (nuc)	25
Circadian rhythms	N. plumbaginifolia (chl, cyt)	38
Anoria	A. thaliana	39
Oxidative stress	N. plumbaginifolia (cyt)	40
	A. thaliana (cyt)	41
Gravity	A. thaliana (cyt)	42
Acidification of external medium	A. thaliana (cyt)	43,44
Alkalinisation of external medium	A. thaliana (cyt)	45
Elicitors	N. plumbaginifolia (cyt)	22
	N. plumbaginifolia (cyt)	46
	L. esculentum (cyt)	20
	G. max (cyt)	5,47
	P. crispum (cyt)	6
Nod factors	G. max (cyt)	48
Phytohormones (ABA)	N. plumbaginifolia (cyt)	49
(auxin)	A. thaliana (cyt)	44
Sucrose	A. thaliana (cyt)	50
Glutamate	A. thaliana (cyt)	51
cAMP, cGMP	N. plumbaginifolia (cyt)	52
Lanthanum	A. thaliana (cyt)	44
Mastoparan	N. tabacum (cyt)	53
	N. plumbaginifolia (cyt, nuc)	14

Intracellular localization of aequorin expression: cytosol (cyt); chloroplast (chl); nucleus (nuc).

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PROTOCOLS

In the following protocol a procedure is exemplarily described how to create transgenic plant cells expressing nucleus-targeted aequorin and to monitor any changes of Ca^{2+} concentrations in this particular compartment. By using different or no targeting signals, different subcellular compartmentation of aequorin is feasible at will based on the present protocol.

Equipment

- Digital luminometer (BioOrbit 1250, Turku, Finland) equipped with a side-window type photomultiplier tube with a dynamic range up to 6 decades and a sensitivity of 0.05 fmoles of ATP per assay.
- Epifluorescence microscope (DMIRBE, Leica, Heidelberg, Germany).
- CCD camera (Color Coolview, Photonic Sciences, Milham, UK).

A. Transformation of plant suspension culture cells with the apoaequorin-nucleoplasmin construct

The chimeric construct including the 35S promoter controlling the nucleoplasmin coding region which was placed in frame with the coding region of apoaequorin was originally prepared by Badminton et al. (15) and was subcloned in the pCon3 vector kindly provided by A. van der Luit (Amsterdam, The Netherlands). The pBIN19 vector was purchased from Clontech, (Heidelberg, Germany).

All media must be autoclaved.

Reagents and Solutions

- LB (Luria-Bertani)-medium: 1% trypton, 0.5% yeast extract,1% NaCl (adjust pH to 7.0 with 5 N NaOH).
- LB(amp): LB-medium containing 100 µg ml^{-1} ampicillin.
- LB(kan): LB-medium containing 50 µg ml^{-1} kanamycin.
- LB(rif): LB-medium containing 50 µg ml^{-1} rifampycin.
- Plant growth medium (PGM), e.g. LS (Linsmaier & Skoog)-medium: 4.3 g of micro and macro elements including vitamins (ref L0230 DUCHEFA: http://www.duchefa.com), 1 mg of 2,4 dichlorophenylacetic acid in 1 liter U.H.Q water, adjust the pH to 5.8 using 1 M KOH.
- PGM(car): PGM-medium containing 500 µg ml-1 carbenicillin.
- TBE: Tris-Borate buffer: 45 mM Tris/borate pH 8.0, 1 mM EDTA.
- TAE: Tris-Acetate buffer: 40 mM Tris/acetate pH 8.0, 1 mM EDTA.
- Agarose-EtBr (agarose containing 0.1µg ml^{-1} of ethidium bromide.
- High Pure Isolation Plasmid Kit (Boehringer, Mannheim, Germany).
- Concert Rapid PCR Purification System (Life-Technologies, Invitrogen, Karlsruhe, Germany).
- Minipreps according to the High Pure Plasmid Isolation kit from Boehringer (Mannheim, Germany).
- Midiprep according to the Plasmid Midi kit from Qiagen (Hilden, Germany).

Methods

Preparation of the chimeric construct from a bacterial strain containing the pCon3 vector

1. Remove a single colony of the strain containing the pCon3 vector and culture in 1.5 ml of LB(amp) under agitation at 37°C overnight.
2. From this overnight culture prepare the pCon3 plasmid with the Boehringer Kit according to the manufacturer's protocol and check its purity by an electrophoresis of 5 µl of the eluate on 1% agarose-EtBr gel in 1x TBE at 100 V.
3. Digest 3.5 µg of purified pCon3 plasmid for 2 hrs at 37°C in the presence of 24 units of *EcoR1* within the recommended buffer supplied with the enzyme. Digest 2 µg of pBIN19 binary plasmid with 12 units of *EcoR1* as described above.
4. Separate the pCon3 digested products by electrophoresis in a 1.2% agarose-EtBr gel in TAE buffer at 50 V.
5. Under a UV lamp , cut out the segment of the gel containing the 2 kb fragment corresponding to the apoaequorin-nucleoplasmin construct. Extract and purify the 2 kb fragment from the agarose-EtBr gel using the Life Technologies kit.

6. Similarly, check the pBIN19 digestion as above in the presence of 200 ng of non-digested pBin19. If linearization is obtained, purify it by using the Concert Rapid PCR Purification System.
7. Withdraw an aliquot of the purified fragment and quantify it using the optical density at 260 nm (OD_{260}) and store the whole preparation at –20°C.
8. Treat about 1 µg of EcoR1-digested pBin19 plasmid with 200 units of alkaline phosphatase (CIP) from Appligene Oncor (Illkirch, France) at 37°C for 30 min, in order to remove 5' phosphate to prevent self-ligation.
9. Perform a phenol-chloroform extraction of the above treated plasmid.
10. Ligate the 2 kb fragment into the *EcoR1* digested pBIN19 plasmid previously treated with CIP. To 1 µl (50 ng) *of EcoR1*-digested and CIP-treated pBIN19, mix 4 µl (35 ng) of the 2 kb fragment in the presence of 1 µl ligase, 1 µl of 10x ligation buffer and 3 µl of water and incubate at 15°C overnight according the DNA ligation system kit procedure from Amersham Biosciences (Orsay, France).

Transformation of competent E. coli DH5α strain with the pBin19 aequorin-nucleoplasmin construct

1. Thaw 200 µl of frozen competent DH5α cells on ice. Mix the thawed cells with the total ligation reaction sample (10 µl) by inverting a 1.5 ml Eppendorf tube and incubate during 30 sec on ice, 90 sec at 42°C and again 5 min on ice. To this transformation medium, add 400 µl of LB-medium without ampicillin and incubate for 75 min at 37°C with shaking.
2. Plate different dilutions (10 µl, 50 µl, 100 µl) of this transformation reaction mixture onto LB(kan) plates and culture for at least 15 hrs at 37°C.

Screening of transformed colonies of E. coli DH5α cells

1. Subculture the grown colonies in a 3 ml LB(kan) medium during 15 hrs and perform minipreps according to the Boehringer kit.
2. Digest 17 µl of each plasmid preparation with *EcoR1* as described above and separate the products by agarose gel electrophoresis (1.2%) in TBE buffer at 100 V.
3. Visualize the products after ethidium bromide staining under UV light. Detect the positive colony carrying the 2 kb construct.
4. Add 100 µl of the overnight grown positive colony to 100 ml of LB(kan) medium and culture overnight at 37°C.
5. Prepare a Midiprep according to Qiagen kit to purify a stock of pBIN19 carrying the nucleoplasmin-aequorin construct.
6. Quantify the plasmid and check the presence of the construct by digesting 1 µg of the purified plasmid as described above.

Agrobacterium tumefaciens transformation

1. Inoculate 5 ml of LB(rif) with a single colony of *A. tumefaciens* LBA4404 strain and culture overnight at 28°C under strong agitation (>250 rpm). When the OD_{660} = 1.0 centrifuge the suspension 6 min at 1500*g* and suspend the pellet in 300 µl of a sterile and cold 20 mM $CaCl_2$ solution.
2. Place 100 µl of this mixture in a cold and sterile 2 ml Eppendorf tube in the presence of 2 µg of the above purified pBIN19 plasmid harboring the aequorin construct.
3. Freeze the tube in liquid nitrogen and then thaw it during 4 min at 37°C.
4. Withdraw the content of the Eppendorf tube and place it in a bacterial culture tube containing 1 ml of LB-medium without (kan) and incubate in a tilted position during 4 hrs at 28°C at 150 rpm.
5. Spin down the cells for 10 sec in a microcentrifuge, dilute the pellet in 200 µl of LB(kan), plate aliquots of 10 µl,50 µl,150 µl onto LB(kan) plates and culture for 48 hrs at 28°C
6. Pick each colony and subculture it in 3 ml of LB containing 50 µg ml^{-1} kanamycin and 50 µg ml^{-1} rifampycin.

Plant suspension culture cell transformation

1. Withdraw 30 µl of the overnight culture of one isolated colony of transformed *A. tumefaciens* and inoculate 3 ml of LB medium without antibiotics. Grow the culture until OD_{660} = 0.1.
2. Mix 4 ml of exponentially growing suspension-cell culture (approximately 40% of packed cell volume) with 100 µl of the above culture of *A. tumefaciens* LBA4404 containing the pBIN19 binary vector harboring the chimera cassette. Transfer the whole mixture onto 90 mm Petri dishes containing PGM solidified with 1% agar.
3. Following a culture at 28°C during 48 hrs in the darkness, collect the cells by scraping the plates and wash them 3 times in a 50 ml Falcon tube with 50 ml of fresh PGM. Perform the washes by a 10 min centrifugation at 100*g* in order to eliminate most of free contaminant *A. tumefaciens*.
4. Suspend the washed pellet in 4 ml of PGM(car) and plate 500 µl or 1 ml aliquots onto solidified PGM medium supplemented

with 500 µg ml^{-1} carbenicillin and 250 µg ml^{-1} kanamycin. Then place the plates at 28°C for 4 to 5 weeks until the formation of a callus.
5. Subculture the calli on the same medium for two more weeks before diluting them in small volumes (10 to 50 ml) of PGM containing the antibiotics mentioned above lowering the concentration of kanamycin to 50 µg ml^{-1}
6. Stabilize the transformed cells by subculturing in presence of antibiotics during at least five cultures progressively increasing the volume of the culture.

B. Immunolocalization of aequorin in the nucleus of plant cells

Reagents and Solutions

- Digestion buffer (DB): 10 mM Mes/KOH pH 5.5, 0.6 M mannitol, Cellulase RS 0.1%; pectolyase 0.01%, BSA 0.1% .
- Washing Buffer (WB1): 25 mM Hepes/KOH pH 6.7, 0.6 M sorbitol.
- Fixation buffer (FB): 50 mM PIPES/KOH pH 6.9, 5 mM EGTA, 5 mM $MgSO_4$, 0.6 M mannitol, *p*-formaldehyde 4%.
- Washing buffer2 (WB2): 50 mM PIPES/KOH pH 6.9, 5 mM EGTA, 5 mM $MgSO_4$, 0.6 M mannitol.
- Permeabilizing buffer: (PB): 50 mM PIPES/KOH pH 6.9, 5 mM EGTA, 5 mM $MgSO_4$, 0.6 M mannitol, 0.25% of reduced Triton X100.
- Reductive buffer (RB): $NaBH_3CN$ (1 mg ml^{-1}) solution in 10 mM phosphate buffered saline pH 8.0 (PBS), prepare extemporaneously.
- TBS-Mg buffer: 10 mM Tris/HCl pH 7.6, 150 mM NaCl, 30 mM $MgCl_2$.
- TBS-BSA buffer: 10 mM Tris/HCl pH 7.6, 150 mM NaCl, 0.1% BSA fractionV (Sigma).
- REC buffer: 10mM Tris/HCl pH7.4, 5mM EDTA, 5mM β-mercaptoethanol, 0.5M NaCl, 0.1% gelatin.
- Normal goat serum (Amersham).
- Rabbit anti-aequorin antiserum (provided by M.T. Nicolas; I.N.S.E.R.M. U432, Montpellier, France).
- Texas-Red-conjugated goat affinity purified antibody to rabbit IGG (ICN, Frankfurt, Germany).
- Poly-L-lysine; Ficoll 400; agarose, NYTAL cloth .

Methods

Preparation of protoplasts

1. Collect exponentially growing plant suspension culture cells harboring the apoaequorin-nucleoplasmin construct by filtration through a 25 µm nytal cloth.
2. Weight 1 g of compacted cells and dilute them in 10 ml of DB.
3. Digest the cell wall with DB during 90 min at 40 strokes min^{-1} and 37°C.
4. Collect the protoplast by centrifugation to 200*g* for 5 min.
5. Wash the pellet once with 10 ml of WB1 by slowly inverting the tube, spin down the digested cells and discard the supernatant.
6. Resuspend the pellet in 2 ml of WB1-20% Ficoll 400, add carefully a 2 ml layer of a WB1-10% Ficoll 400 and a 2 ml layer of WB1 on the top.
7. Centrifuge the discontinuous gradient at 200*g* for 30 min.
8. Collect the purified protoplasts at the WB1–Ficoll 10% interface.
9. Wash twice the protoplasts in 10 ml of WB1 and store at 4°C.
10. Prepare an aqueous solution of 1 mg ml^{-1} poly-L-lysine (Sigma, München, Germany) and coat a glass microscope slide with 10 µl and allow to dry up. Adjust the protoplast concentration to about 10^6 ml^{-1} in FB.
11. Fix 100 µl of the protoplast suspension for 15 min at room temperature.
12. Wash the protoplasts twice with WB2.
13. Embed the protoplasts by carefully adding 1 volume of 1% melted agarose solution at 55°C and immediately withdraw 50 µl of the mixture and spread it over the poly-L-lysine pre-coated microscope slide with the tip of a pasteur pipette.
14. Put the microscope slides in a plastic box saturated with water (100% humidity).
15. Permeabilize the protoplast adding onto agarose 200 µl of RB during 30 min.
16. Wash out the Triton X100 rinsing with 200 µl of FB.
17. Reduce the remaining aldehydes with 3 successive 10 min washing steps with RB
18. Rinse with 2 successive washing steps (200 µl each) in 10 mM TBS-Mg buffer for 10 min each.

19. Incubate the preparation with 200 µl of 5% Normal Goat Serum (Amersham) during 30 min at room temperature.
20. Wash once with 200 µl of TBS-Mg.
21. Apply a 1/500 dilution of a crude rabbit anti-aequorin serum in TBS-Mg for 2 hrs at room temperature.
22. Wash off the excess of this primary antibody with two washing steps (10 min) with TBS-Mg and a 10 min washing in TBS-BSA buffer.
23. Apply a 1/500 dilution of Texas-Red-X anti-rabbit IgG-conjugate as the secondary antibody for 2 hrs in TBS-BSA in the dark.
24. Wash the microscope slide twice for 10 min with TBS-BSA.
25. Cover the specimen with a small amount of Citifluor (Leicester U.K.**)** and add the coverslip.
26. Visualize the labeled aequorin under an epifluoresence microscope at 590 nm.

C. Aequorin-based intracellular [Ca^{2+}] measurements

Reagents and Solutions

- Reconstitution buffer (RB): 10 mM Tris/HCl pH7.4, 5 mM EDTA, 0.5 M NaCl, 5 mM β-mercaptoethanol, 0.1% gelatin.
- Discharging solution (DS): 100 mM $CaCl_2$ containing 10% of ethanol (v/v) and 2% of Nonidet P40 (v/v).
- Control solution (CS): 200 mM Tris/HCl pH 7.0, 5 mM EDTA.
- Coelenterazine (Calbiochem, Bad Soden, Germany), 5 mM stock solution in methanol, store at –20°C.

Methods

In vivo aequorin reconstitution and in vitro aequorin reconstitution to control putative side-effects of pharmacological compounds on aequorin luminescence.

In vivo reconstitution

1. At least 3 hrs before luminescence measurements, prepare a 20% packed cell volume of an exponentially growing plant suspension culture by filtration and dilution of the culture in fresh PGM.
2. Reconstitute the aequorin by adding 2.5 µl of the coelenterazine stock solution to 5 ml of the washed cell suspension in a 50 ml Falcon tube and agitate at room temperature in the dark for at least 3 hrs.
3. Remove the excess of coelenterazine by slowly washing the cells with 50 ml of fresh PGM. Cells are ready for calcium measurements.

In vitro reconstitution

1. Grind a known amount of plant cells in liquid nitrogen and resuspend in RB.
2. Collect the supernatant obtained after centrifugation at 12000*g* for 10 min.
3. Reconstitute aequorin by adding coelenterazine (2.5 µM) in the dark for 2 hrs.
4. Dilute a known volume of the supernatant in 0.5 ml of CS in the presence or absence of the compound which have to be checked in a luminometer tube.
5. Insert the tube in the luminometer and add 0.5 ml of 50 mM $CaCl_2$. Compare the luminescence response in the presence and absence of the compound.

Luminescence measurements

1. Add aliquots of 50 to 100 µl of coelenterazine treated cells in a adequate plastic tube placed in the chamber of the luminometer.
2. Stimulate the cells while recording the light every second or fraction of second depending of the amount of light emitted.
3. After the calcium response, discharge the remaining aequorin in the sample by adding 0.5 ml of DS.