

Quantum Dot-Mediated Detection of γ -Aminobutyric Acid Binding Sites on the Surface of Living Pollen Protoplasts in Tobacco

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Summary

γ -Aminobutyric acid (GABA) is an inhibitory transmitter in the central nervous system of mammals. Recent investigations showed that it also plays an important role in regulating pollen tube growth and orientation in plants. To determine whether GABA receptors are also present on the membrane of pollen protoplasts, a fluorescence probe of quantum dots (QDs) was constructed and applied. The water-soluble CdSe-ZnS (core-shell) QDs were first synthesized and verified to possess good optical properties. GABA was then bioconjugated to the QDs in the presence of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS) to make the fluorescence probe. Using the probe, GABA binding sites were detected on the protoplast membrane of both pollen and somatic cells. Both the fluorescent signals on the surface of the protoplasts and the Ca^{2+} oscillation assayed via the Ca^{2+} probe Fluo-3/AM inside the protoplasts provided evidence that the potential GABA_B receptors are present on the plant protoplast membrane.

Introduction

γ -Aminobutyric acid (GABA) is a four-carbon, nonprotein amino acid. It is omnipresent in all organisms, from prokaryotes to eukaryotes. Studies in animals indicate that its action is mediated via both ionotropic (mainly GABA_A) and metabotropic (GABA_B) receptors [1, 2]. The GABA_A receptor predominantly regulates Cl^- , whereas the GABA_B receptor mainly regulates K^+ and Ca^{2+} because it is a G protein-coupled receptor that can regulate Ca^{2+} and K^+ channels. In mammals, GABA acts as a neurotransmitter to control the excitation and inhibition of the central nervous system (CNS) [3]. It can regulate the sex glands to secrete and release hormones by stimulating the secretion of gonadotropin releasing hormone (GnRH) from the subdienecephalon pituitary [4]. In the rat, the concentration of GABA in the oviduct is ten times higher than that in the cerebrum; thus, it is considered to be involved in fertilization in the rat [5], although its role is poorly understood. In 1993, Wistrom and Meizel [6] reported the presence of GABA receptors on the membrane of human sperm, which may be involved in

the acrosome reaction. In 1997, the GABA_B receptor was first cloned from the brain [3]. The mechanism underlying its function in fertilization remains enigmatic. It has been speculated that GABA is involved in the regulation of Ca^{2+} [7], which activates a second messenger to regulate additional physiological reactions.

Extensive investigations of GABA in plants have mainly focused on its function in osmotic adaptation to various stresses, including hypoxia, acidosis, mechanical stress, or cold stress [8]. A recent study has shown that the gradient of GABA in the pistils of *Arabidopsis* flowers can regulate pollen growth and orientation [9]. This finding has broadened our understanding of the role of GABA [10–12]. However, the mechanism underlying the function of GABA in regulating the growth and orientation of pollen tubes remains unknown. It has been proposed that this regulation is related to GABA receptors [11, 12]. However, the corresponding genes have not yet been cloned, which has greatly hindered the functional analysis of GABA receptors. Although glutamate (the precursor of GABA biosynthesis) receptors are present in plant genomes [13], there is no evidence that GABA functions by binding to glutamate receptors. Therefore, determining whether GABA receptors are present in plants, especially on the membrane surface of pollen protoplasts, is of great importance in understanding the mechanism of GABA function. However, few available techniques can be used to localize unknown receptors. A recently developed fluorescence probe with semiconductor quantum dots (QDs) has shown powerful potential to provide a solution to this problem [14–21].

QDs have been widely used to study protein localization [14, 15]. This new, to our knowledge, class of potential fluorescence probes has advantages over traditional fluorescence dyes. QDs have broad excitation spectra, but narrow, symmetrical, and tunable emission spectra because of their quantum confinement effects, making it possible to excite all colors of QDs simultaneously by using a single-excitation light source. In addition, QDs exhibit a high photobleaching threshold and excellent photostability [14–16]. Over the past decades, improvement of the technology used for QD synthesis and cellular imaging has enabled nanocrystals to be a useful tool in biological and biomedical applications. They are extensively used in real-time imaging of living cells [16, 17]. A specific use is to synthesize the ligand-modified nanocrystals as probes to target cell membrane proteins, such as receptors, ion channels, and transporters. The transport of serotonin can be regulated by QDs modified with the neurotransmitter serotonin [18]. The dynamic diffusion of glycine receptors can be tracked by using QDs [19]. Peptide-conjugated QDs can activate neuronal receptors and initiate downstream signaling of neurite growth [20]. Bharali and coworkers used folate-conjugated QDs to observe the folate receptor-mediated delivery of QDs [21]. The application of QDs in animals, especially for the detection of receptors, suggests the possibility of detecting GABA receptors in plants. Here, we report the successful synthesis of a

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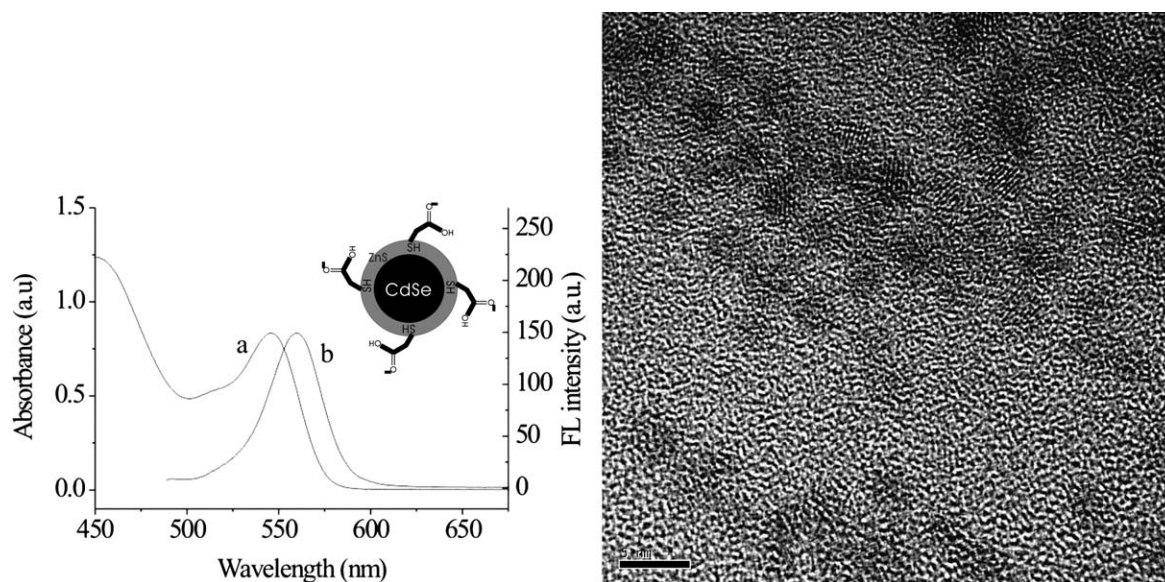


Figure 1. Characterization of the Water-Soluble CdSe-ZnS Quantum Dots

Left: Schematic diagram of the water-soluble CdSe-ZnS (core-shell) nanoparticle with mercaptoacetic acid surface capping groups (insert) and its (a) UV-vis absorption and (b) fluorescence spectra. Right: Transmission electron microscopy image of water-soluble CdSe-ZnS (core-shell) QDs. QDs are ~5 nm in diameter. The scale bar is 5 nm.

specific QD probe to detect GABA binding sites on the membrane of tobacco pollen protoplasts. To our knowledge, we provide the first evidence that potential GABA_B receptors are present on the membrane of plant protoplasts.

Results

Characterization of the Synthesized QDs

Thermolysis of organometallic precursors in TOP/TOPO medium is one of the most extensively applied methods for the synthesis of CdS or CdSe QDs [22]. To improve the emission quantum yield, it is necessary to conjugate the ZnS shell to the surface of CdSe core QDs. After coating the surface of CdSe QDs with a ZnS shell, the emission peak usually shifted to red (5–20 nm) because of the increased size of the CdSe core QDs. The optical properties of the CdSe-ZnS QDs synthesized in this work were characterized by using UV-vis absorption spectrometry and fluorescence spectroscopy (Figure 1). The emission spectrum was sharp, with a full width at half-maximum (FWHM) at about 30 nm, and it had a broad absorption, which enabled the excitation of the QDs at wavelengths ranging from 350–500 nm. This feature provided flexibility in choosing a suitable wavelength to excite the QDs and minimize the interference from background emission.

The advantage of this method is that the size of the QDs can be controlled by varying the heating temperature and reaction time. In this study, the diameter of the CdSe core was about 3.2 nm. The CdSe core, synthesized in TOPO medium, showed strong band-edge emission. This allowed us to observe QD-labeled cells by using blue-light excitation, thereby both minimizing the autofluorescence of cells and avoiding the possible toxic effects of semiconductor QDs on living cells that occur under UV illumination.

The QDs synthesized in the TOPO medium were capped with a surfactant. As a result, they were hydrophobic and only dispersible in organic solvent, such as chloroform or hexane. For biologically compatible applications, hydrophilic ligands containing a thiol group were used to change the surface bound surfactant to make the CdSe-ZnS QDs water-soluble. Mercaptoacetic acid has been widely used to prepare water-soluble QDs [15]; thus, we used mercaptoacetic acid as the ligand to envelop the QDs. The mercapto binds to the Zn atom, and the polar carboxylic acid group renders the QDs water-soluble (Figure 1, left, insertion). The free carboxyl group is also available for covalent coupling to various biomolecules by crosslinking to a reactive amine group. In our experiments, the prepared water-soluble QDs had good stability and could be kept stable for at least 7 days.

The narrow emission lines were consistent with the nearly monodispersed size distribution of the nanocrystals, as demonstrated with transmission electron microscopy (TEM). The synthesized water-soluble QDs were ~5 nm in diameter (Figure 1, right).

Synthesis of the QD-GABA Luminescent Probe and Characterization

Carbodiimide is used to mediate the formation of amide linkages between a carboxylate and an amine [23]. We used the crosslinking reagent ethyl-3-(dimethylamino) carbodiimide (EDC) to synthesize the QD-GABA luminescent probe, which was catalyzed by N-hydroxysuccinimide (NHS). In this method, a good yield of luminescent probe was acquired with 2 hr of reaction at room temperature. After bioconjugation under EDC initiation, β-mercaptoethanol was added to disperse the bioconjugates. This not only neutralized the extra unreacted EDC, but it also made the QDs more stable in a water solution (Figure 2).

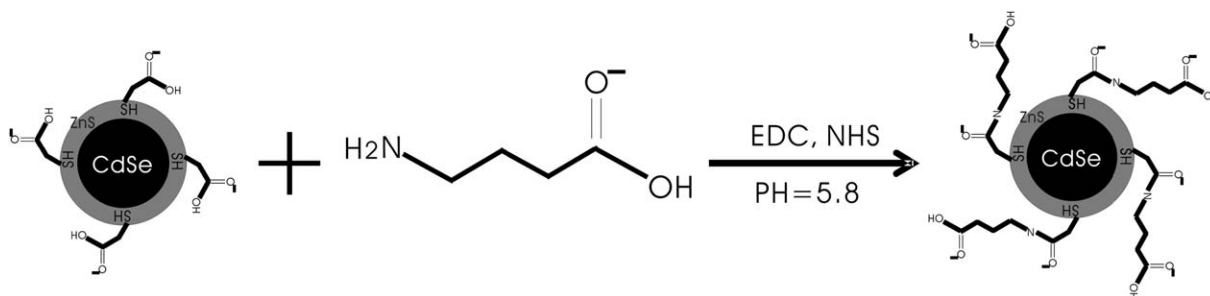


Figure 2. Schematic Diagram Showing the Preparation of QD-GABA

To detect whether GABA was bioconjugated to the QD nanoparticles, a mobility shift assay was conducted on a 0.5% agarose gel [24]. Although the absorption and emission spectra of QDs were unchanged after GABA coating (data not shown), when the QDs were separated by electrophoresis on agarose gel, the GABA-capped QDs were less mobile than the uncapped controls (see Figure S1 in the Supplemental Data available with this article online). This lag was clearly caused by the increase in molecular weight resulting from the conjugation of GABA, which indicated that this bioconjugation is specific and that GABA is indeed covalently bound to the nanocrystal surface.

Protoplast Isolation and Imaging of GABA Binding Sites by Using QD-GABA Probes

Mature pollen protoplasts were isolated by using the germination-enzymatic method established in our lab. To monitor the enzymatic condition of the cell wall during the course of isolation and possible regeneration of the cell wall during incubation, the specific cell wall fluorescence Calcofluor White ST was employed. In hydrated mature pollen, the fluorescence was mainly distributed on the germination pores (Figures 3A and 3A1). The fluorescence was weakly distributed around the pollen. After 3 hr of enzymatic digestion, the cell wall was completely removed, which was indicated by the lack of fluorescence around the pollen protoplasts (Figures 3B and B1). During the 3 hr incubation of protoplasts in the medium (the same medium used to incubate the probes), no fluorescence was observed on the membrane, indicating that no cell wall was regenerated during this period (Figures 3C and C1). As a control, mesophyll protoplasts after cultivation were well labeled by Calcofluor White ST (see Figure S2). Our result confirms that pollen protoplasts isolated by following the procedure were true protoplasts and excludes the possibility of autofluorescence or fluorescent signals from the regeneration of cell wall materials.

The viability of such pollen protoplasts was further checked by using fluorescein diacetate (FDA), which has long been used as an effective and convenient method for testing cell viability. The pollen protoplasts emitted bright green fluorescence, indicating that they are normal living cells (see Figure S3) and are suitable for further investigations.

The luminescent probe QD-GABA was used to determine whether GABA receptors are present on the membrane of tobacco pollen protoplasts. As a control, we first determined whether QDs could permeate and

adhere to the cell membrane. After 3 hr of incubation with 1 $\mu\text{mol/l}$ QD, the cells were individually fully rinsed with the incubation medium by micromanipulation and were observed under a fluorescent microscope. The results showed that QDs could not diffuse into or adhere to the living cell after washing. However, in dying cells, QD could easily diffuse into the whole cell, even when the cell wall was partially present; this diffusion is indicated by strong green fluorescence throughout the cell (see Figure S4).

After a 3 hr incubation of living pollen protoplasts with 1 $\mu\text{mol/l}$ QD-GABA and complete washing of the cells in the incubation medium, a circle of fluorescence was observed on the membrane (Figure 4A). Compared to QD staining alone (see Figure S4), this fluorescent circle indicates that GABA binding sites were present on the membrane of tobacco pollen protoplasts.

To further test the specificity of the binding sites, we first incubated the cells in incubation medium containing a series concentration of GABA, from 10^{-6} mol/l to 10^{-3} mol/l, for 30 min and then transferred the cells to incubation medium containing 1 $\mu\text{mol/l}$ QD-GABA for another 2.5 hr. After washing, the cells were observed under a fluorescence microscope. The fluorescence intensity was analyzed according to a previous method [25]. Four lines that crossed the cell membrane at eight points were drawn through the center of the cell (Figure 4B). The fluorescent intensities were measured in the way shown in Figure 4C for each line. The average fluorescence intensity at all eight of the sites was calculated as the fluorescence intensity of each cell. The relative fluorescence intensity of the fluorescent circle on the membrane obviously decreased as the concentration of GABA increased for pretreatment (Figure 4D). This indicates that GABA can compete with QD-GABA to bind to the GABA binding sites.

To further confirm whether GABA binding sites are specifically present on the membrane of pollen protoplasts, somatic protoplasts were also tested in the experiments. The results showed that both mesophyll protoplasts of *Arabidopsis* and root protoplast of tobacco were clearly labeled by QD-GABA. This indicates that the GABA binding sites are widely distributed on the membrane of plant protoplasts (Figure 5).

Bioactivity Assay of QD-GABA

To test the biological function of QD-GABA, we monitored the level of Ca^{2+} in tobacco pollen protoplasts. After loading the Ca^{2+} probe Fluo-3, the protoplasts were cultivated in medium containing QD-GABA. The

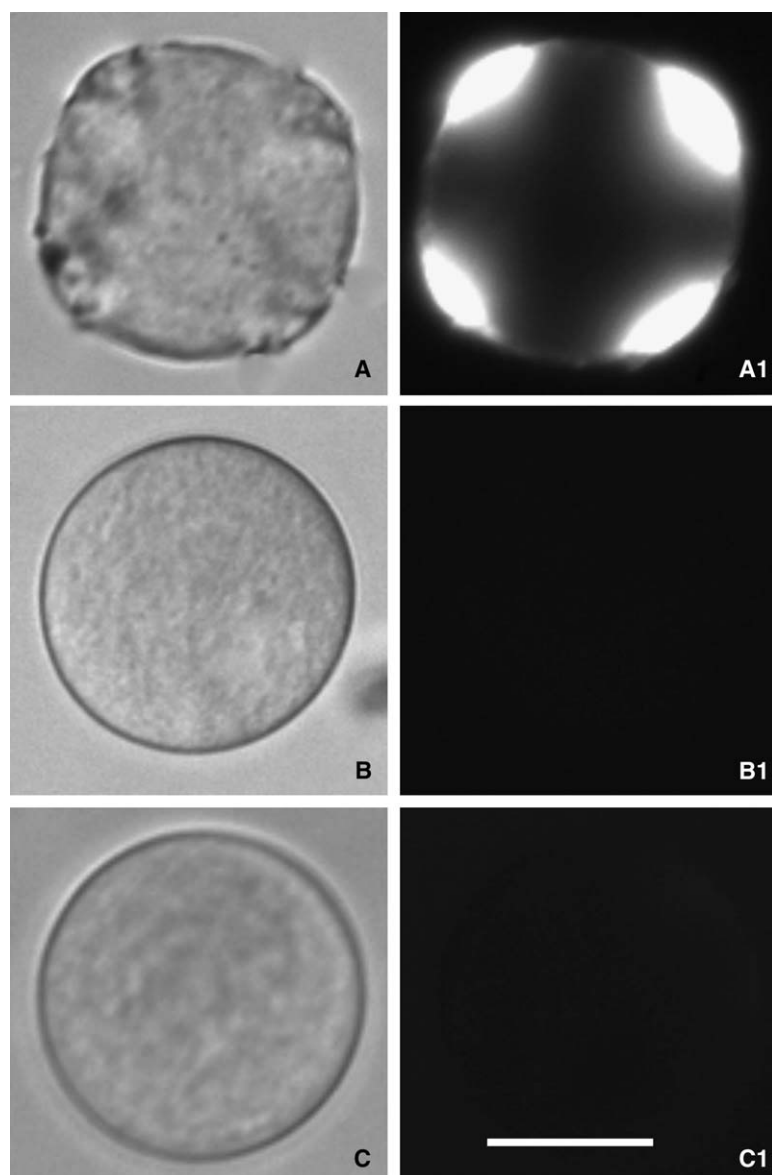


Figure 3. Detection of Cell Wall Materials on Pollen Protoplasts

Pollen and pollen protoplasts were incubated with Calcofluor White (CW) for 3 hr. After washing, the protoplasts were observed under a bright field (left panel) and UV light (right panel). The scale bar is 5 μm .

(A) Pollen grain.

(A1) The same pollen grain stained with CW.

(B) Pollen protoplast.

(B1) The same pollen protoplast stained with CW.

(C) Pollen protoplast incubated in QD medium for 3 hr.

(C1) The same pollen protoplast stained with CW.

level of Ca^{2+} was significantly increased, compared to the resting concentration of Ca^{2+} (Figure 6A, control), with the addition of 1 $\mu\text{mol/l}$ QD-GABA; this increase lasted for about 30 min and then decreased gradually to the normal level (Figure 6A). However, incubation with QD alone did not significantly alter the resting concentration of Ca^{2+} . The effect of GABA on the Ca^{2+} concentration is the most marked among the four treatments (Figures 6A and 6B).

To analyze possible different effects of GABA and QD-GABA on the cells, the transient Ca^{2+} oscillation was monitored immediately after the addition of both reagents. Compared with the resting status of Ca^{2+} , the effect of 1 $\mu\text{mol/l}$ QD did not show much difference on the calcium oscillation pattern. Whereas, both 1 $\mu\text{mol/l}$ GABA and QD-GABA triggered calcium transient increase (Figure 6C), their oscillation patterns were obviously different. This indicates that different mechanisms might be involved in the response of the cell to the two treatments.

Discussion

Since Bruchez and Chan [14, 15] used fluorescent nanocrystals as biological labels, luminescent QDs have been widely used for multiplexed biological detection and imaging. QDs provided a promising approach for targeting and detecting receptors on cell surfaces. Here, we used GABA-labeled QDs to detect GABA binding sites on the membranes of tobacco protoplasts. To our knowledge, this is the first report to confirm GABA binding sites on the plant cell membrane and to reveal the presence of a potential GABA receptor in plant cells.

The preparation of biocompatible water-soluble QDs is a key step in the use of semiconductor QDs for live cell imaging. We first synthesized CdSe-ZnS (core-shell) QDs according to routine methods of synthesis in TOP/TOPO medium via thermolysis, which can produce the crystalline quality well and can readily control QD size. However, the hydrophobic surfactant-capped nanoparticles could only disperse in organic solvents. Because

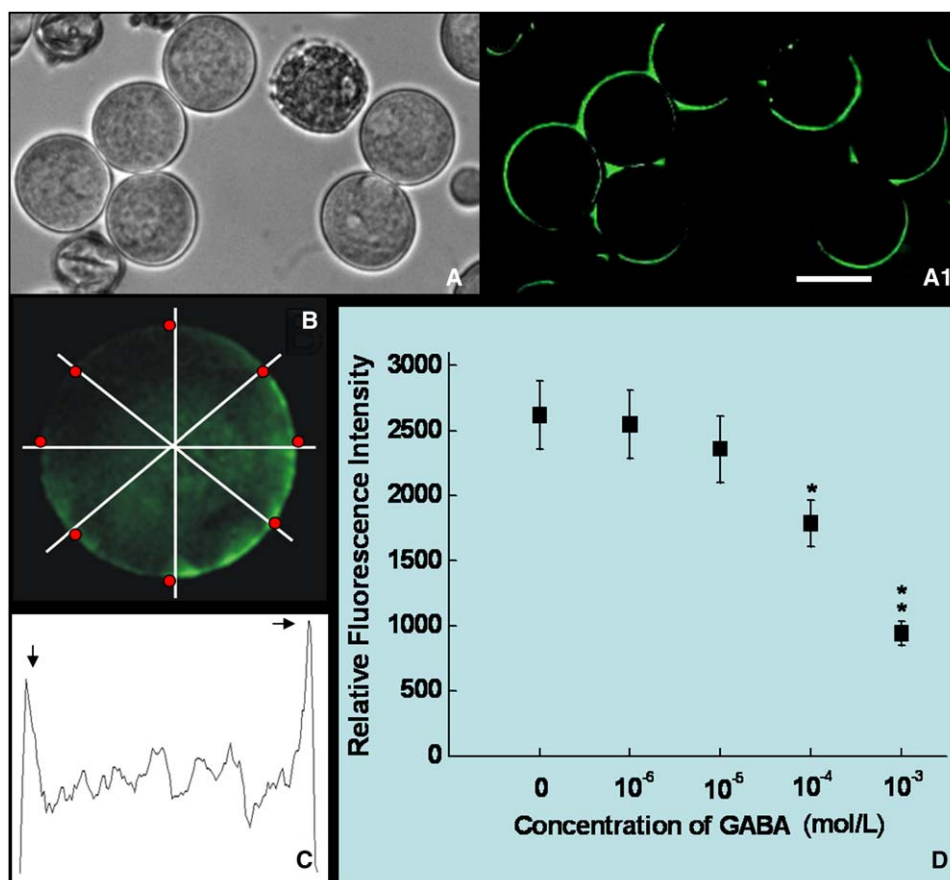


Figure 4. Detection of GABA Binding Sites on the Pollen Protoplast Membrane of Tobacco

(A) Pollen protoplasts.

(A1) The same pollen protoplast in (A), but incubated with 1 $\mu\text{mol/l}$ QD-GABA. Green fluorescence was observed on the membrane of protoplasts. The scale bar is 7 μm .

(B) One of the protoplasts labeled with the probes. The fluorescence intensities at eight points where the lines cross the cell membrane were measured by using Image J software. The average fluorescence intensity of all eight of the sites was calculated as the fluorescence intensity of each cell.

(C) Fluorescent intensities along one of the lines in (B). Two peak values (arrows) indicating the fluorescent intensities at two crossing points were only collected for calculation to delete background interference.

(D) Competition assay of GABA binding sites with different concentrations of GABA. A total of 100 cells were measured in each treatment. A one-way ANOVA assay was conducted via SPSS.10.0 software. *, the difference significance ($p < 0.05$). **, $p < 0.01$.

aqueous medium is required for QDs to conjugate with biological molecules, such as proteins, peptides, and amino acids [14, 15, 18–21], a hydrophilic ligand containing a thiol group, mercaptopropionic acid (MPA), was used to exchange the surfactant, making CdSe-ZnS water-soluble. This method shows good bioconjugation and has little effect on the size of the QDs, although the QDs have a short period of stability [15].

The growth of pollen tubes through a path to their target (ovule) to achieve fertilization is a central event in plant production. Recent studies have revealed that GABA plays an important role in pollen tube guidance [9–12]. The mechanism underlying the role of GABA is still enigmatic. We propose that GABA receptors may occur on the membrane of pollen protoplasts, and that GABA triggers a series of downstream activities, including calcium oscillation to regulate pollen tube growth, by binding to the receptor. Obviously, whether such a receptor occurs on the pollen protoplast surface is the first question that must be answered. This requires a sensitive and reliable technique to detect

the receptors. Therefore, GABA QDs were designed to solve this problem.

To synthesize the luminescent probes, we introduced the standard water-soluble bioconjugate coupling reagent EDC to initiate the formation of covalent bonds between the carboxylic groups of MPA on the CdSe-ZnS QDs and the amino groups of GABA [23]. This luminescent probe released the carboxylic groups to the hydrophilic medium. The carboxylic groups were considered functional groups to bind the GABA receptors [2]. This strategy in synthesizing the luminescent probe is similar to the protocol of Bharali and coworkers, who modified QDs with folate to observe the receptor-mediated delivery of QDs [21]. Our results confirmed that the synthesized probes are effective and suitable tools for membrane surface protein labeling.

Our results showed that the synthesized GABA QDs could specifically bind to GABA binding sites on the plant protoplast surface, which was confirmed by the fact that QDs alone could not bind to the membrane, and that pretreatment with GABA could significantly

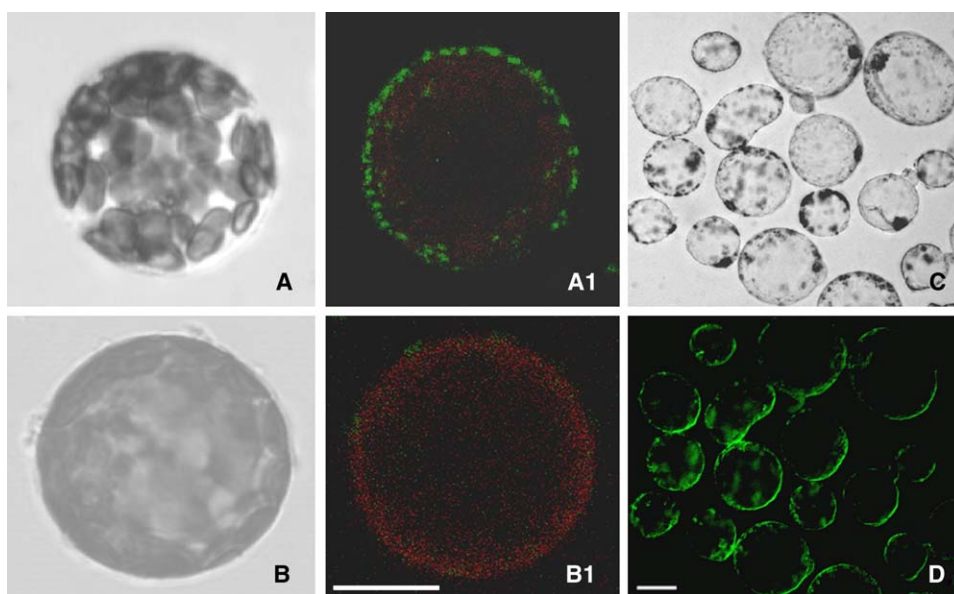


Figure 5. Detection of GABA Binding Sites on Nonpollen Protoplasts

(A) Bright field image of an *Arabidopsis* mesophyll protoplast.
 (A1) CLSM image of the same protoplast in (A). The green circle indicates the QD-GABA binding sites.
 (B) *Arabidopsis* mesophyll protoplast as control.
 (B1) CLSM image of the same protoplast in (B), but incubated with QD only.
 (C) Population of protoplasts from tobacco roots (bright field).
 (D) Fluorescent image of the same protoplasts in (C). The green circles show the QD-GABA binding sites in the protoplasts from tobacco roots.
 The scale bar is 30 μm in (A) and (B) and 50 μm in (C) and (D).

reduce the relative fluorescence intensity of QD-GABA. Although we could not distinguish the GABA receptors and transporters on the protoplasts at present, we showed that GABA QDs have obvious bioactivity that can regulate the Ca^{2+} level in the protoplasts as soon as they bind to the protoplast surface. Ca^{2+} is a significant second messenger and plays a critical role in the growth of pollen tubes. Thus, the GABA binding sites are probably GABA_B receptors.

We noticed that when GABA QDs bind to the pollen protoplasts, Ca^{2+} increases rapidly in a specific location in the cell. This may indicate the orientation of Ca^{2+} influx. It has been suggested that the growth of pollen tubes is comparable to that of axons [26]. This mechanism, which is found in animals, may also work in plants. We also found that different patterns of GABA and QD-GABA induced Ca^{2+} oscillation. One possible reason is that although both of them can bind to the cell membrane, GABA can also act by permeating into the cell, but QD-GABA cannot. This suggests that different mechanism might be involved in the cell's response to the treatments. In addition, GABA binding sites were found on the membrane of both pollen protoplasts and somatic protoplasts isolated from *Arabidopsis* and tobacco. This finding suggests that the binding sites may not specifically function in pollen/pollen tube development. Extensive investigation is required to reveal its multifunctional role in plant development.

Significance

To detect GABA receptors on the pollen protoplast membrane, water-soluble QDs 5 nm in diameter were

synthesized. When the QDs were conjugated with GABA, they were confirmed to be a useful luminescent probe. Using this probe, GABA binding sites distributed in a polar manner were detected on the membrane of pollen protoplasts. We confirmed that GABA QDs have obvious biological activity in regulating Ca^{2+} in the cell. To our knowledge, our results offer the first evidence showing that GABA binding sites, which may be GABA_B receptors, occur on the plant cell membrane.

Experimental Procedures

Preparation of CdSe-ZnS QDs

Except where otherwise noted, all chemicals and biochemicals were purchased from Sigma-Aldrich and were used without further purification. Synthesis of the CdSe-ZnS core was based on the method of Peng et al. [22], with a small modification. A mild and simple synthesis condition was used that was considered to be a more environmentally friendly approach. Briefly, the mixture of CdO (0.0127 g) and stearic acid (0.1140 g) was heated to 150°C under a flow of Ar until the CdO completely dissolved. The mixture was then cooled to room temperature. Then, 1.94 g trioctylphosphine oxide (TOPO) and 1.94 g hexadecylamine were added, and the mixture was heated to 310°C under a flow of Ar. At this temperature, the Se solution (0.079 g Se dissolved in 0.238 g tributylphosphine [TBP] and 1.681 g dioctylamine) was quickly injected into the reaction flask. The reaction was kept at this temperature for 3 min, after which the heating mantle was immediately removed and the mixture was allowed to cool to room temperature. After purification by precipitation, centrifugation, and decantation, the vacuum-dried CdSe QDs were redispersed in hexane.

Synthesis of the ZnS shell was performed according the method developed by Hines and Dabbousi and coworkers [27, 28]. The S precursor was prepared by dissolving 0.0128 g sulfur in 10 ml octadecene (ODE) at 200°C. The Zn precursor was prepared by dissolving 0.0728 g $\text{Zn}(\text{Ac})_2$ in 10 ml ODE and 1.4 g TOPO at 200°C. The

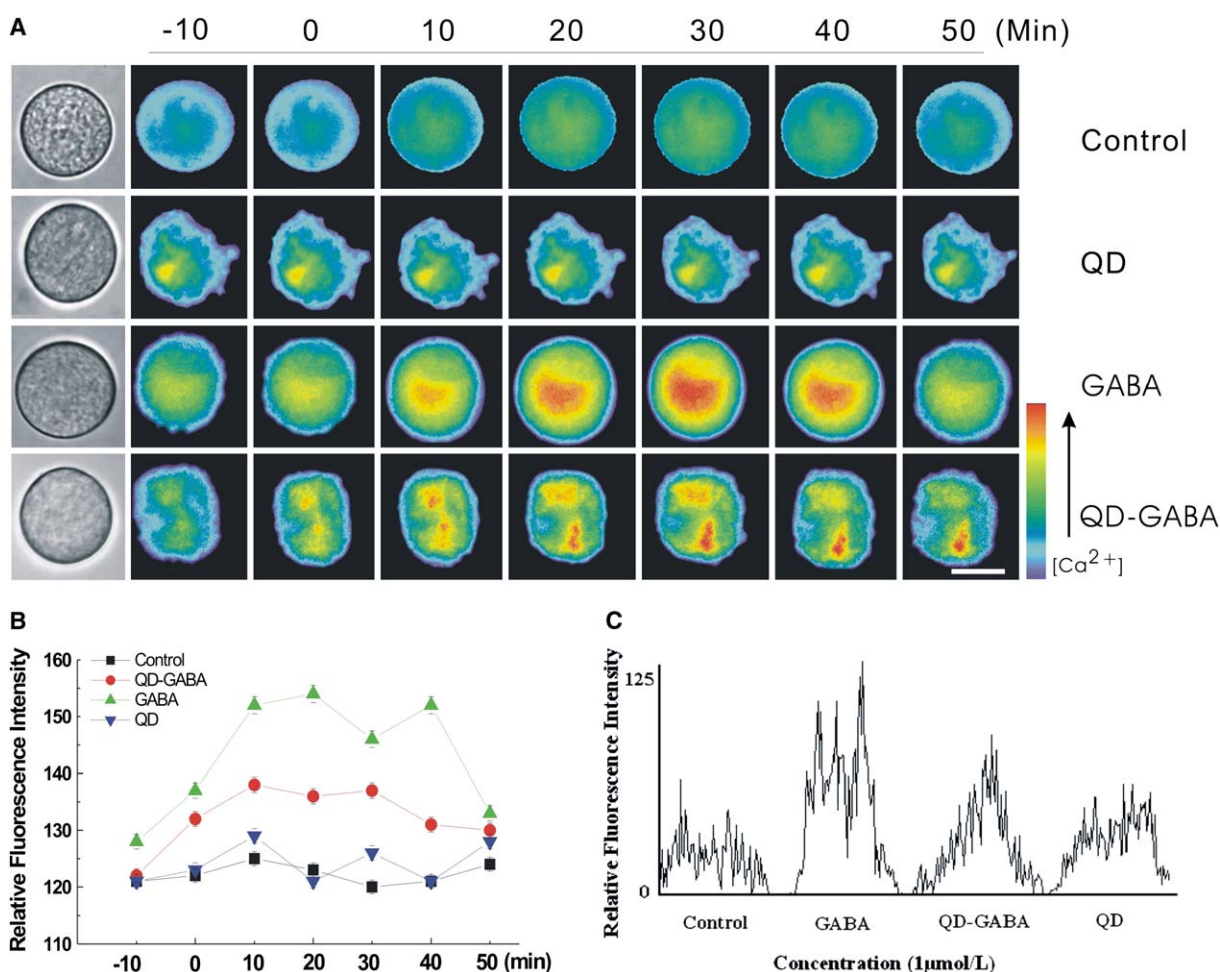


Figure 6. Time Course of the Relative Ca²⁺ Level in Tobacco Pollen Protoplasts after the Addition of Luminescent QD-GABA (A and B) (A) The bar on the right shows the correlation between Ca²⁺ levels and cell pseudocolor, whose quantification is shown in (B). Errors are derived from three replicate assays, each performed on five cells. (C) Transient Ca²⁺ oscillation pattern after addition of the reagents.

CdSe QDs dissolved in hexane were mixed with 1.94 g TOPO and 1.94 g hexadecylamine in a 25 ml three-necked flask. The flask was then pumped down at room temperature for 30 min by using a mechanical pump to remove the hexane. The system was then switched to Ar flow, and the reaction mixture was further heated to 220°C. The S and Zn precursors (4.0 ml of each) were added to the system dropwise. The final product, CdSe-ZnS QDs, was precipitated with anhydrous methanol, separated by centrifugation, and redispersed in chloroform to obtain an optically clear solution.

Preparation of Water-Soluble CdSe-ZnS QDs

To obtain biocompatible QDs for the experiment, water-soluble CdSe-ZnS QDs were synthesized according to the method reported by Chan et al. [15, 29]. Glacial mercaptoacetic acid was added to chloroform-solvated CdSe-ZnS QDs prepared as described above until the solution became cloudy. The QDs were then centrifuged. Double deionized water was added to the pellet, and the pH was adjusted to 10–11 by the dropwise addition of 1 mol/l NaOH. Next, acetone (50/50% v/v) was added to the aqueous solution to precipitate the mercapto-conjugated QDs. This solution was centrifuged, and the pellet was then air dried until ready for use.

The absorption spectra were acquired on a TU-1900 UV-vis spectrometer (Beijing, China). All fluorescence measurements were made with a Perkin Elmer Model LS-55 luminescence spectrometer equipped with a 20 KW xenon discharge lamp as a light source. Autofluorescence spectra and QD spectra were manually selected

from the spectral image by using the computer mouse to select appropriate regions. The size of QDs was characterized by using a JEOL-JEM 2010 high-resolution transmission electron microscope operated at 200 kV; to do so, samples were dropped onto a copper grid covered with a thin film of amorphous carbon.

Synthesis of QD-Labeled GABA and Mobility Shift Assay

QD-bioconjugated GABA was synthesized in accordance with reported methods [15, 23]. The reaction volume was 0.2 ml MES-NaOH buffer (pH 5.8) in which the concentration of QDs was 1×10^{-6} mol/l and that of GABA was 1×10^{-4} mol/l. The concentrations of 1-ethyl-3-(3)-dimethylaminopropyl carbodiimide (EDC) and N-hydroxysuccinimide (NHS) were 1 and 0.1 mmol/l, respectively. The solution was incubated and vortexed at room temperature for 2 hr. The GABA QDs were separated from free GABA by adding 0.6 ml acetone to cause their precipitation, and they were then centrifuged at 10,000 rpm for 10 min. The pellet was left to dry completely in the dark, and it was then resuspended with 10 μl 0.1% (v/v) β-mercaptoethanol. The solution was used for protoplast membrane staining by medium dilution.

Gel electrophoresis was performed according to the procedure of Pinaud et al. [24] on 0.5% and 1% agarose gels in TBE buffer (89 mM Tris, 89 mM borate, and 2 mM EDTA [pH 8.3]) for 10 min at 120 V. The bands were detected by using a gel scanner (Biorad, Gel 2000) with UV light excitation and were transformed to fluorescence bands by using Image J software.

Manipulation of Protoplasts

Nicotiana tabacum cv. SR1 and *Arabidopsis* (Columbia ecotype) were grown in a greenhouse at Wuhan University, Wuhan, China.

The isolation of pollen protoplasts was based on the method of Wang with modifications [30]. Mature pollen was collected and purified by using a 50-mesh nylon membrane once, followed by the use of a 10-mesh nylon membrane twice more. The purified pollen was germinated in the medium containing major salts of Brewbaker and Kwack medium (1 mmol/l KNO₃, 1 mmol/l Ca(NO₃)₂·4H₂O, 1 mmol/l MgSO₄·7H₂O, 1 mmol/l H₃BO₃) and 20% sucrose (pH 5.8). When germinated pollen tubes were as long as the pollen grain diameter, they were transferred into enzymatic solution containing 1% cellulase (Onozuka R-10), 1% pectinase (Merck reagent), 1 mol/l mannitol, 0.4 mol/l sorbitol, and D₂ medium major salts (270 mg/l NH₄NO₃, 1480 mg/l KNO₃, 900 mg/l CaCl₂·2H₂O, 900 mg/l MgSO₄·4H₂O, 800 mg/l KH₂PO₄, 27.8 mg/l FeSO₄·7H₂O, 37.3 mg/l Na₂·EDTA [pH 5.8]). The intact pollen protoplasts were released after 1–3 hr of enzymatic digestion at 30°C in the dark. The protoplasts were collected and washed individually for later use. Pollen protoplast viability was detected by using fluorescein diacetate (FDA) according to the method of Yang [31]. After staining with 10 μg/ml FDA for 20 min, the protoplasts were washed with FDA-free medium and observed with a Leica Dmire 2 fluorescence microscope.

Mesophyll protoplasts of *Arabidopsis* were isolated according to the procedure of Abel et al. [32]. Root protoplasts of tobacco were isolated according to the procedure described by Sun et al. [33]. The released protoplasts were collected by a micropipette and washed with W5 solution (154 mM NaCl, 125 mM CaCl₂, 5 mM KCl, 5 mM glucose, and 1.5 mM Mes-KOH [pH 5.6]).

Labeling of Cells by Using QD Conjugates and Image Acquisition

To label cells with the QD-GABA probe, the protoplasts were collected and cultivated in germination medium containing 1 μmol/l QD-GABA or QD for only 3 hr at 25°C. After 3 hr, the cells were carefully washed 2–3 times in microdrops of the germination medium with a micropipette. The images were collected with a Leica Dmire 2 fluorescence microscope equipped with a charge-coupled device (CCD) camera. Image processing and analysis were done by using MetaMorph (Universal Imaging) and Photoshop software.

Somatic protoplasts were incubated with 1 μmol/l QD-GABA dispersed in W5 for 1 hr. The protoplasts were then fully washed in W5 solution before observation. Images were acquired with a Leica TCS 4D confocal laser scanning microscope (CLSM).

Competition Assay of GABA Binding Sites with a Different Concentration of GABA

Pollen protoplasts were incubated in medium containing 1 μmol/l QD-GABA or were preincubated with 10⁻⁶, 10⁻⁵, 10⁻⁴, and 10⁻³ mol/l GABA and then transferred to medium containing 1 μmol/l probes. Signal detection and image processing were carried out according to the method described above.

Cytosolic Ca²⁺ Measurement

The Ca²⁺ fluorescent probe Fluo-3 (Sigma Reagent) was loaded into pollen protoplasts according to the method of Wang [34], with a small modification. Equal volumes of Fluo-3 (1 mmol/l in DMSO) and Pluronic F-127 (20% in DMSO, Molecular Probes reagent) were mixed and then diluted with the loading buffer (1 mmol/l KNO₃, 0.2 mmol/l KH₂PO₄, 5 mmol/l CaCl₂, 1 mmol/l MgSO₄, 1 mmol/l KI, 0.1 mmol/l CuSO₄, 1.0 mol/l mannitol, 5 mmol/l MES, pH adjusted to 5.8 by using Tris). The final concentration of Fluo-3/AM was 10 μmol/l. The isolated pollen protoplasts were incubated in the solution at 20°C for 30 min before Ca²⁺ imaging. After cells were washed with Fluo-3/AM-free loading buffer, 1 μmol/l QD, GABA, and QD-GABA were added to cells in the loading buffer. Sequencing images were acquired at 10 min intervals by using a Leica TCS 4D confocal laser scanning microscope (CLSM). The excitation wavelength was 488 nm, and emission signals between 515 and 550 nm were collected.

Supplemental Data

Supplemental Data include Figures S1–S4 and are available at <http://www.chembiol.com/cgi/content/full/13/7/723/DC1/>.

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