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# Response to Comment on “A G Protein–Coupled Receptor Is a Plasma Membrane Receptor for the Plant Hormone Abscisic Acid”

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Our study provided experimental evidence that GCR2 is a membrane-associated abscisic acid receptor that interacts with the G protein  $\alpha$  subunit GPA1 in *Arabidopsis*. Although we cannot rule out GCR2 as a lanthionine synthetase homolog, our data indicate that it may define a new type of nonclassical G protein–coupled receptor.

Based entirely on in silico modeling and bioinformatic prediction, Johnston *et al.* (1) suggest that *Arabidopsis* GCR2 is neither a transmembrane protein nor a G protein–coupled receptor (GPCR), but rather a plant homolog of bacterial lanthionine synthetase (LanC) proteins. We provide experimental evidence to support our proposal that GCR2 may be a new type of GPCR.

We initially predicted that GCR2 was a seven-transmembrane protein using TMpred and DAS software programs (2). We further used 12 distinct software programs to predict the topological structure of GCR2 and found that 9 of them (TMHMM, SOSUI, and DAS TMfilter excluded) showed that GCR2 is a transmembrane protein with various numbers of transmembrane domains. TMHMM has underpredicted transmembrane domains in many instances (3), and the only other reported GPCR in *Arabidopsis*, GCR1 (4), was predicted to be a three-transmembrane protein by SOSUI. In addition, about 14% of known transmembrane proteins (established by crystal structure or biochemical evidence) cannot be correctly predicted by available software (3). Thus, computational prediction of membrane proteins is not yet a mature science and mainly serves to generate hypotheses for experimental testing.

We therefore focused our effort on characterizing biochemical properties of GCR2. We found that a GCR2-YFP fusion protein was only localized in the plasma membrane, even with a high expression level driven by an inducible promoter (Fig. 1A), and was associated with the membrane fraction in cell fractionation and Western blot analysis (Fig. 1B). Despite washing with detergent (0.1% or 0.5% Triton at pH7.5) or a higher pH buffer (pH 10), conditions known to completely remove

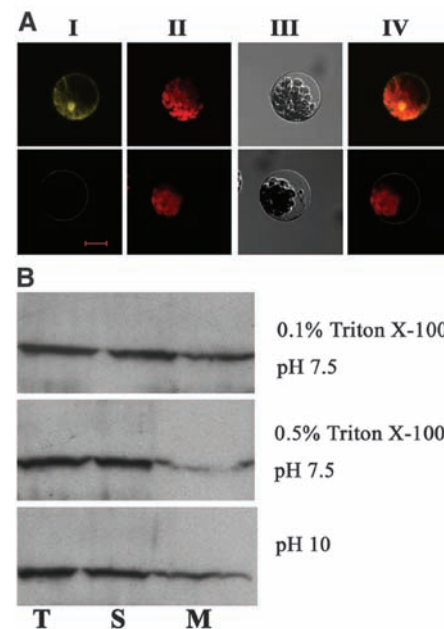
human LanC homolog LANCL1 (5), a substantial amount of GCR2 was retained within the membrane fraction (Fig. 1B).

It is well established that the intrinsic GTPase activity of the G protein  $\alpha$  subunit modulates its binding to GPCRs (6). Indeed, the GTPase activity of *Arabidopsis* G protein  $\alpha$  subunit GPA1 modulates its interaction with GCR2 and the likely plant GPCR GCR1 as shown by a split-ubiquitin assay (Fig. 2A). In addition, and similar to some other GPCRs, GCR2 can interact with both  $G\alpha$  and  $G\beta$  subunits independently (7) (Fig. 2B).

Johnston *et al.* (1) assume that the SPR signal reported in (2) is due to a lack of negative control subtraction. However, the interaction between GCR2 and GPA1 after negative control subtraction is still evident (Fig. 3D). As we indicated in (2), the rate constants were calculated based on 0, 20, 40, 60, 80, 100, and 120 nM of GPA1 flowing through an immobilized GCR2 chip. The GPA1 concentrations used in figure S3A in (2) are near saturating and thus cannot be used to determine the rate constants. Therefore, the simulated SPR sensorgrams of Johnston *et al.* (1), which are based on SPR signals obtained from high concentrations of GPA1 but use rate constants obtained with low and linear-range concentrations of GPA1, are problematic. Experimental and simulated SPR binding curves based on our raw data (from which the rate constants were calculated) are very similar (Fig. 3, G and H), which suggests that our SPR data are robust. The purpose of figure S3A in (2) was simply to show the qualitative nature of the interaction between GCR2 and GPA1. The surface regeneration step is necessary for each interaction assay, and it was always done after each SPR signal detection step in our experiments (Fig. 3I). It is true that the dissociation rate of GCR2 and GPA1 is slow in the SPR assay (Fig. 3D). However, our data are very similar to and consistent with the reported interaction between rhodopsin (a typical GPCR) and  $G\alpha$  in the SPR assay, in which the dissociation rate was increased obviously by the addition of  $G\beta\gamma$  subunits (8). The lower dissociation rate between GCR2 and

GPA1 in the SPR assay may be due to a lack of synergistic interaction with  $G\beta\gamma$  subunits.

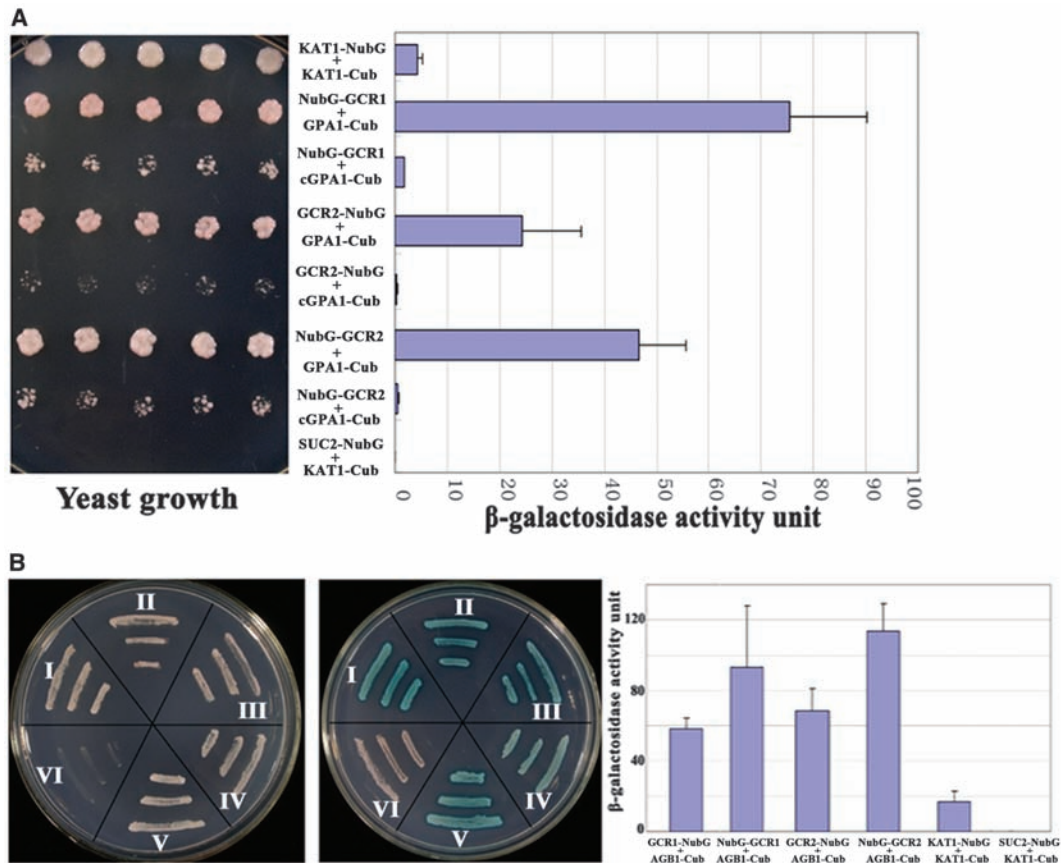
Based on a low degree of sequence similarity to bacterial LanC proteins (~17% identity of the full-length sequence; figure 1B in (1) shows the percentage identity using alignment of partial sequences of NisC and GCR2), Johnston *et al.* suggest that GCR2 is a plant homolog of bacterial lanthionine synthetases and thus can not be a GPCR (1). However, we have several lines of evidence supporting the conclusion that GCR2 is biochemically distinct from human LanC-like (LANCL) proteins, including the difference in membrane association properties between GCR2 and LANCL1 [Fig. 1B and (5)] and the difference in subcellular localization between GCR2 and LANCL1. LANCL1-GFP was exclusively localized to the cytosol and nucleus but was absent from the plasma membrane (9), whereas GCR2-YFP was only localized to the plasma membrane (Fig. 1A). Finally, there are also differences between GCR2 and LANCL1 in terms of their interactions with  $G\alpha$  and  $G\beta$  subunits. There is no evidence for the interaction between LANCL1 and G protein,



**Fig. 1.** GCR2 subcellular localization. (A) GCR2 is localized in the plasma membrane in the protoplast expressing *35S::GFP* (top) and *dex-inductive promoter::GCR2-YFP* (bottom). I, YFP fluorescence; II, chloroplast fluorescence; III, bright field; IV, merge of I and II. (B) GCR2 is predominantly associated with the membrane fraction. Total protein (T) was isolated from transgenic plants expressing *dex-inductive promoter::GCR2-YFP*. Proteins were fractionated into soluble (S) or membrane (M) fractions. Equal amounts of protein were separated on SDS-PAGE and subjected to immunoblotting using antibodies to GFP. The samples were isolated from the transgenic plant under dex-inductive conditions.

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**Fig. 2.** Physical interaction between GCR2 and GPA1, and between GCR2 and G $\beta$  subunit. **(A)** The dependence of intrinsic GTPase activity for physical interaction between GPA1 and GCR2 or GCR1 shown by Yeast growth assay (left) and *LacZ* activity assay (right). KAT1-NubG + KAT1-Cub, positive control; SUC2-NubG + KAT1-Cub, negative control; cGPA1, Q222L-mutated GPA1 defect in GTPase activity. **(B)** GCR2 and GCR1 interact with the G $\beta$  subunit

(AGB1) by split-ubiquitin assay in yeast. (Left) Yeast growth assay. (Middle) X-gal overlay assay. (Right) Corresponding *LacZ* activity for each yeast strain. I, GCR1-NubG + AGB1-Cub; II, NubG-GCR1 + AGB1-Cub; III, GCR2-NubG + AGB1-Cub; IV, NubG-GCR2 + AGB1-Cub; V, NubG + KAT1-Cub, positive control; VI, SUC2-NubG + KAT1-Cub, negative control.  $\beta$ -galactosidase activity unit: Miller unit.

whereas GCR2 could interact with G $\alpha$  and G $\beta$  subunits.

Johnston *et al.* (1) argue that the structural similarity between GCR2 and the *Lactococcus lactis* LanC protein, nisin cyclase (NisC), provides evidence that GCR2 is a member of the LanC protein superfamily. GCR2 could be a LANC homolog. However, there are several examples of homologous proteins with comparable similarity that exhibit distinct functions and biochemical properties in plants and bacteria (e.g., cryptochromes and photolases, phytochromes, and two-component histidine kinases). In addition, the ERF/AP2 transcription factor gene family (144 members in *Arabidopsis*) evolved from bacterial HNH endonuclease (10), whereas the B3

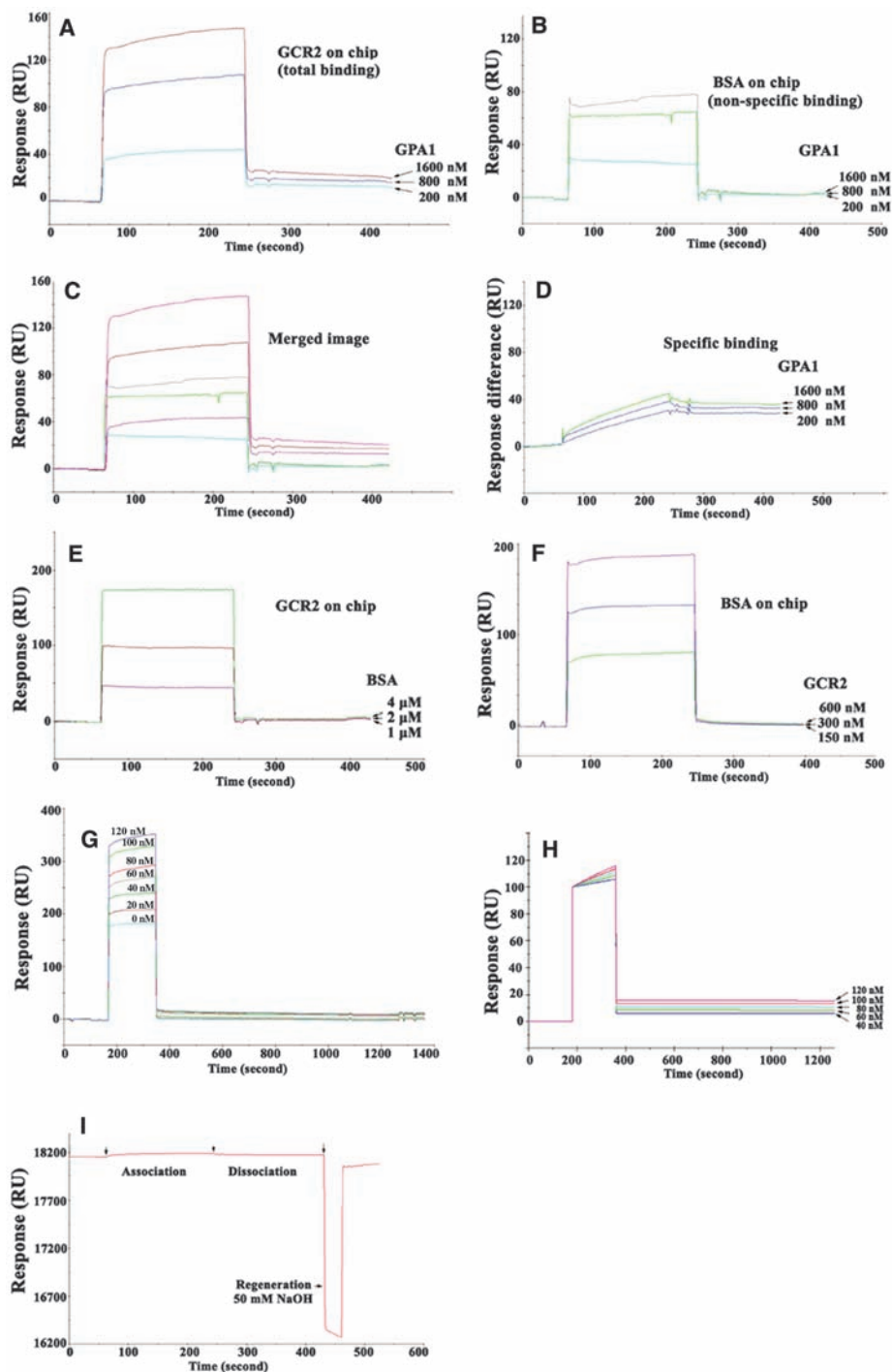
domain transcription factor gene family (37 members in *Arabidopsis*) exhibits sequence similarity to *E. coli* EcoRII (11). Thus, it is difficult to assess biochemical function based only on computational modeling. We have provided multiple lines of biochemical evidence that support the role of GCR2 as an ABA receptor and its interaction with G protein  $\alpha$  and  $\beta$  subunits in *Arabidopsis*. Other properties of GCR2 may not be consistent with classic GPCRs and suggest that this protein may define a new type of GPCR.

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**Fig. 3.** Physical interaction between GCR2 and GPA1 by SPR assay. Representative SPR experiments show (A) the binding of GPA1 to immobilized GCR2, (B) nonbinding of GPA1 to immobilized BSA, (C) merged image from (A) and (B), (D) specific binding of GPA1 to GCR2, (E) nonbinding of BSA to immobilized GCR2, and (F) nonbinding of GCR2 to immobilized BSA. Representative SPR experiments show the binding of series concentrations of GPA1 to immobilized GCR2 (G). (H) Simulated SPR binding curves for a 2.1 nM affinity interaction between GPA1 and GCR2 using the data from (G) (the maximum inputs for the simulation in the software is 5). (I) Representative image for the surface regeneration of the sensor chip.