Trans-plasma membrane electron transfer is achieved by $b$-type cytochromes of different families, and plays a fundamental role in diverse cellular processes involving two interacting redox couples that are physically separated by a phospholipid bilayer, such as iron uptake and redox signaling. Despite their importance, no direct recordings of trans-plasma membrane electron currents have been described in plants. In this work, we provide robust electrophysiological evidence of trans-plasma membrane electron flow mediated by a soybean ($Glycine max$) cytochrome $b_{561}$ associated with a dopamine $\beta$-monooxygenase redox domain (CYBDOM), which localizes to the plasma membrane in transgenic Arabidopsis ($Arabidopsis thaliana$) plants and CYBDOM complementary RNA-injected Xenopus laevis oocytes. In oocytes, two-electrode voltage clamp experiments showed that CYBDOM-mediated currents were activated by extracellular electron acceptors in a concentration- and type-specific manner. Current amplitudes were voltage dependent, strongly potentiated in oocytes preinjected with ascorbate (the canonical electron donor for cytochrome $b_{561}$), and abolished by mutating a highly conserved His residue (H292L) predicted to coordinate the cytoplasmic heme $b$ group. We believe that this unique approach opens new perspectives in plant transmembrane electron transport and beyond.

In biological membranes, the presence of ion channels and transporters enables the selective movement of ions and charged/polar metabolites across phospholipid bilayers. The development of electrophysiological techniques allowed the direct study of the functional properties of these proteins. After the first recordings of ionic currents from protoplasts enzymatically isolated from wheat ($Triticum aestivum$) and Vicia faba leaves more than 30 years ago (Moran et al., 1984; Schroeder et al., 1984), there was an incredible progress in the understanding of the biophysical structures and the physiological roles of a plethora of plant channels and transporters (Hedrich, 2012). However, for the intriguing class of plant transmembrane electron transporters, no direct current recordings are available.

Cells and sealed plasma membrane (PM) vesicles isolated from different plant species and organs have long been known to constitutively reduce externally added, hydrosoluble electron acceptors such as ferricyanide (FeCN) or other suitable artificial dyes, often in the absence of any inductive stimulus (Lüthje et al., 2013, and references therein). Reduction of the external electron acceptors was typically associated with PM depolarization, supporting the view that the electrons required for the redox activity were provided by a cytoplasmic reductant via a transmembrane electron transport system whose molecular identity remained elusive (Lüthje et al., 2013). By their activity, these electron transport systems provide a connection between cytoplasmic and apoplastic redox couples that are physically separated by the PM.

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Cytochrome b561 (CYB561) and related proteins containing a CYB561 core domain are membrane-spanning proteins encoded in angiosperms by gene families of approximately 15 members (Tsubaki et al., 2005; Preger et al., 2009; Asard et al., 2013). The CYB561 core domain is constituted by a bundle of four transmembrane α-helices with two heme b groups facing opposite sides of the membrane (Asard et al., 2013). The simplest members of the CYB561 family consist of a single CYB561 core domain plus two additional transmembrane helices with no obvious catalytic role (Asard et al., 2013). Plants often contain four isoforms of these CYB561 sensu stricto; one of these (CYB561B1; for a systematic nomenclature, see Asard et al., 2013) has been localized on the tonoplast (Griesen et al., 2004; Preger et al., 2005) or the PM, depending on the plant species investigated (Nanasato et al., 2005). A second isoform from Arabidopsis (Arabidopsis thaliana; At_CYB561B2), for which the crystal structure has recently been solved (Lu et al., 2014), is apparently chloroplastic (Dutta et al., 2014). The few CYB561 proteins characterized to date catalyze transmembrane electron transport between cytoplasmic ascorbate (ASC) and extracytoplasmic electron acceptors that can be either monodehydroascorbate (MDHA), for extracytoplasmic ASC regeneration, or a ferrichelate, for ferrous ion mobilization (Asard et al., 2013). In animal systems as well, MDHA and ferrichelates are suitable electron acceptors for different CYB561 isoforms. CYB561 of chromaffin granules in mammals reduces intravesicular MDHA to sustain ASC-dependent catecholamine biosynthesis (Njus and Kelley, 1993), and a similar MDHA reductase activity was demonstrated in several CYB561 plant isoforms (Horemans et al., 1994; Nakaniishi et al., 2009; Asard et al., 2013). On the other hand, the activity of plant CYB561 with ferrichelates (Bérczi et al., 2007) recalls the activity of a duodenal CYB561 isoform (duodenal cytochrome b564 [Dcytb]) of mammals that performs the ASC-dependent reduction of ferric chelates derived from the diet, allowing the absorption of ferrous iron by divalent metal transporters (McKie et al., 2001). In several instances, both in plants and animals, single CYB561 isoforms proved capable of reducing both MDHA and ferrichelates, leaving some uncertainty about their specific role in vivo (Asard et al., 2013).

Besides CYB561 sensu stricto, the CYB561 protein family includes CYB561-related proteins in which an additional domain, known as dopamine β-monoxygenase rebox domain (DOMON; Iyer et al., 2007) and sometimes present in two copies, extends toward the extracytoplasmic space. These proteins have been named cytochrome b56l associated with a dopamine β-monoxygenase rebox domains (CYBDOMs; Asard et al., 2013). The hydrophilic DOMON domain binds an additional heme b group (Preger et al., 2009), such that all CYBDOMs contain two hemes in the CYB561 core plus one heme for each extracellular DOMON (Asard et al., 2013). Very little is known on the physiological role of CYBDOMs, for which angiosperms often contain around 10 encoding genes.

Here, we show the electron transport capability of a soybean (Glycine max) CYBDOM localized at the PM (systematic name Gm_CYBDOMF21; Asard et al., 2013), the first plant CYBDOM ever described, and demonstrate the feasibility of electrophysiological techniques to measure electron currents generated by transmembrane electron transport systems expressed in Xenopus laevis oocytes. The electron currents mediated by soybean CYBDOM proved to be stimulated by the membrane potential and to depend on both internal ASC concentration and external FeCN concentration. Albeit less efficiently, extracellular FeCN could be substituted by a ferrichelate (ferric nitrilotriacetate [FeNTA]). This work paves the way for further experiments, aimed at elucidating the molecular mechanism underlying these elusive electron transport systems present in particularly large numbers in plants.

RESULTS

PM Localization of CYBDOM in Leaves of Transgenic Arabidopsis Plants

Localization of soybean CYBDOM was studied in Arabidopsis following different strategies. First, Arabidopsis plants were stably transformed with a CYBDOM::Enhanced Green Fluorescent Protein (EGFP) construct under the control of a double 35S promoter. The EGFP fluorescence signal detected in leaves of CYBDM-EGFP transgenic plants nicely matched the boundaries of leaf epidermal cells (Fig. 1, A, C, and D) and stomatal guard cells (Fig. 1, E, G, and H). Spectral analysis confirmed that the observed fluorescence was due to EGFP and not to cell wall autofluorescence (data not shown). Moreover, the spatial distribution of the EGFP signal matched the PM marker FM4-64 (Fig. 1, I–L).

In an alternative experimental strategy, a CYBDM-EGFP fusion construct was used for transient transformation of isolated Arabidopsis mesophyll protoplasts (Supplemental Fig. S1). Again, the CYBDM-EGFP signal perfectly matched the FM4-64 signal at the PM. The EGFP fusion of the tonoplast protein Arabidopsis Two-Pore Channel1 (AtTPC1; Boccaccio et al., 2014), used as a negative control, did not match the FM4-64 distribution (Supplemental Fig. S1), despite the close proximity of tonoplast and PM in these cells.

PM Localization and Ferric Reductase Activity of CYBDOM in X. laevis Oocytes

For functional studies, we used X. laevis oocytes microinjected with CYBDOM or CYBDM-EGFP complementary RNA (cRNA). Confocal imaging showed strong EGFP signals at the surface of oocytes expressing a CYBDM-EGFP fusion protein (Fig. 2A). Colocalization studies with the PM marker FM4-64 confirmed that the fusion protein was also targeted to the PM in this heterologous expression system (Fig. 2, B and C). A colorimetric assay was used to probe the capability
of CYBDOM to reduce an external electron acceptor like FeNTA in the presence of ferrozine as an Fe$^{2+}$ chelator. In CYBDOM cRNA-injected oocytes, a 27-fold increase of the reductase activity was observed compared with control oocytes (Fig. 2D).

**ASC-Dependent Electron Transfer Mediated by CYBDOM in X. laevis Oocytes**

We used two-electrode voltage clamp recordings for direct measurements of CYBDOM-mediated transmembrane electron transport in X. laevis oocytes. Exposure of CYBDOM cRNA-injected oocytes to the electron acceptor FeCN (100 μM) reversibly elicited small inward currents at −20 mV (Fig. 2E, bottom trace), whereas none of the control oocytes showed downward deflections of the current level (Fig. 2E, top trace). Similar experiments were performed at different voltages between +40 and −100 mV. FeCN-dependent currents were completely absent in control oocytes, whereas CYBDOM cRNA-injected oocytes showed an increase of the current amplitude with negative-going potentials (Fig. 2F).

To investigate the responsiveness of soybean CYBDOM to cytosolic ASC, the canonical electron donor of the CYB561 family, FeCN-elicited currents were measured twice on the same oocyte (Fig. 3A): first, in baseline conditions using the endogenous cytosolic ASC pool (black traces) and successively, after an ASC injection raising the intracellular ASC concentration by 10 mM (red traces). The increase of cytosolic ASC strongly affected CYBDOM-dependent inward currents, in particular at elevated FeCN concentrations. Plots of the current amplitudes against the applied FeCN concentration revealed Michaelis-Menten-like behavior in both experimental
conditions (Fig. 3B). Notably, raising the intracellular ASC concentration caused a 7-fold increase of the apparent maximum current at saturating [FeCN] (Fig. 3C). In parallel, the apparent affinity constant $K_{FeCN}$ changed from 4.3 $\mu$M under baseline conditions to 26.0 $\mu$M after ASC injection (Fig. 3D). Taking advantage of the higher current amplitudes recorded after raising the intracellular ASC concentration, experiments described hereafter were conducted on oocytes preinjected with 10 mM ASC.

First, we investigated the current responses of CYBDOM-expressing oocytes challenged with alternative electron acceptors. Applied at a concentration of 500 $\mu$M, FeNTA evoked an approximately 8-fold-smaller response than FeCN, whereas exposure to ferric citrate (FeCIT) and ferric EDTA (FeEDTA) had no significant effect on the current level (Fig. 4, A and B). Since reduced electron acceptors produced during the reaction were rapidly washed away by the perfusion system, differences in electron currents could not be ascribed to differences in the redox potential of the iron compounds. Moreover, none of the electron acceptors elicited measurable current deflections in control oocytes (Fig. 4A). As a negative control, we also tested the effect of ferrocyanide in oocytes preinjected with ASC.

Figure 2. External FeCN elicits inward currents in CYBDOM-expressing X. laevis oocytes. A, Expression of a CYBDOM-EGFP fusion construct in X. laevis oocytes. Confocal microscopy images taken in transmission light (left) and in the EGFP fluorescence channel (right; green signal). B, Confocal images showing the border of an oocyte expressing CYBDOM-EGFP (left; green signal) and stained with the PM marker FM4-64 (middle; red signal); right, merged signals. C, Fluorescence intensity profiles of the EGFP (green) and FM4-64 signals (red) along the z axis of the oocyte border (as in A). au, Arbitrary unit. D, Ferric reductase activity in noninjected ($n = 22$) and CYBDOM cRNA-injected oocytes ($n = 17$) determined by the ferrozine-based colorimetric assay (see “Materials and Methods” for details). The experiment was repeated twice with similar results. E, Membrane current traces recorded in a water-injected oocyte (top trace) and in a CYBDOM cRNA-injected oocyte (bottom trace), in response to exposure (horizontal bar) to 100 $\mu$M FeCN in standard bath solution (pH 5.5), at a holding potential of −20 mV. F, Current-voltage relationships of steady-state membrane currents elicited by 100 $\mu$M FeCN in control ($n = 7$; open symbols) and CYBDOM-expressing oocytes ($n = 7$; black symbols). Membrane currents were recorded in response to 25-ms voltage pulses ranging from +40 to −100 mV in steps of 10 mV, from a holding potential of −20 mV.
As shown in Figure 4C, 100 μM ferrocyanide did not elicit measurable currents.

The characteristics of inward currents in CYBDOM-expressing oocytes (i.e. strong potentiation by intracellular injection of the electron donor ASC, strict dependence on an extracellular electron acceptor such as FeCN, and no detectable signal induced by ferrocyanide) were fully compatible with transmembrane electron transport mediated by CYBDOM. Further experiments using bath solutions with altered ionic composition were conducted to exclude the possible contribution of alternative ion fluxes. FeCN-induced current amplitudes recorded in a modified bath solution, containing only the large, membrane-impermeable ions BTP and MES, were identical to those recorded in standard bath solution (Fig. 4D). Raising the extracellular pH from 5.5 to 7.5 determined smaller FeCN-evoked currents over a range of [FeCN] (Fig. 4E). A closer inspection using dose response analyses (Fig. 4F) revealed that the apparent maximum current at saturating [FeCN] was approximately 36% higher at external pH (pH$_{ext}$) 5.5 compared with pH$_{ext}$ 7.5, whereas the apparent $K_D$ for FeCN was not significantly affected (Fig. 4G). The observed effects likely reflect a pH$_{ext}$-dependent modulation of CYBDOM activity.

The Conserved His Residue H249 Is Necessary for CYBDOM-Mediated Electron Transfer

The two heme $b$ groups of CYB561 core domains are coordinated by four conserved histidines located at the N-terminal end of four consecutive $a$-helices (Asard et al., 2013), as shown in Figure 5A. Based on multiple protein sequence alignments (Fig. 5B), H249 of CYBDOM was identified as a putative ligand of the cytoplasmic heme of the protein. The orthologous His of other CYB561 isoforms was demonstrated to be
essential for proper heme ligation, although dispensable for correct protein folding (Bérczi and Zimányi, 2014, and references therein). We used site-directed mutagenesis to target H249 in CYBDOM. Confocal imaging of oocytes expressing a CYBDOM-H249L-EGFP fusion, together with FM4-64 staining, confirmed that the H249L mutation affected neither the PM targeting (Fig. 5C) nor the surface expression level of the protein (Fig. 5D). Strikingly, however, the mutation completely abolished FeCN-evoked inward currents in *X. laevis* oocytes, both under baseline conditions using the endogenous cytosolic ASC pool and after an ASC

![Diagram](https://example.com/diagram.png)

**Figure 4.** CYBDOM transport activity depends on the type of electron acceptor and is driven by electron transmembrane movement. A, Current recordings in a control oocyte (top traces) and in a CYBDOM-expressing oocyte (bottom traces), pre-injected with 10 mM ASC, upon application (arrow) of FeCN, FeNTA, FeCIT, and FeEDTA in standard bath solution (each at 500 μM). Holding potential at −20 mV. B, Current amplitudes recorded in CYBDOM-expressing oocytes in response to alternative electron acceptors (as shown in A) were normalized to the FeCN amplitude. Values are the mean ± SEM (n = 8 different oocytes for FeNTA, n = 7 for FeCIT, n = 5 for FeEDTA). C, Left: Current recordings in a CYBDOM-expressing oocyte (pre-injected with 10 mM ASC) upon exposure (horizontal bar) to 100 μM FeCN (top traces) and 100 μM ferrocyanide (bottom traces). Holding potential at −20 mV. Right: Mean current amplitudes (± SEM) recorded from five different oocytes. D, Left: Membrane current recordings in a CYBDOM-expressing oocyte (pre-injected with 10 mM ASC) upon exposure to 100 μM FeCN (horizontal bar) in standard bath solution (Std) and successively in a modified bath solution (1,3-bis(tris[hydroxymethyl]methylamino) propane [BTP]/MES). Holding potential at −20 mV. Right: Summary plot of current amplitudes in standard and modified bath solution. Values are the mean ± SEM (n = 5 different oocytes). E, Current responses of a CYBDOM-expressing oocyte (pre-injected with 10 mM ASC) upon exposure to FeCN (horizontal bar) at concentrations of 3, 10, 30, 100, and 500 μM (as in Fig. 3A), recorded in standard bath solution adjusted to pH 5.5 (top traces) or pH 7.5 (bottom traces). Holding potential at −20 mV. F, Dose-response curves showing the dependence of current amplitudes on the applied [FeCN] at pH 5.5 (black symbols) or pH 7.5 (white symbols). Data points were fitted with a Michaelis-Menten function (continuous lines). G, Summary plot of the ratio between the values determined at pH 5.5 and pH 7.5 for the apparent maximum current at saturating [FeCN] (I_{max}) and the apparent K_D for FeCN (K_F), both derived from Michaelis-Menten fits (see F). Values are the mean ± SEM (n = 5 different oocytes).
injection raising the intracellular ASC concentration (Fig. 5, E and F), strongly suggesting that the cytoplasmic heme group coordinated by the H249 residue is necessary for transmembrane electron transport mediated by CYBDOM.

**DISCUSSION**

In the current study, we provide evidence that the soybean family of CYB561 proteins contains at least one PM-associated isoform, named CYBDOM. Heterologous expression in *X. laevis* oocytes confirmed the PM localization of CYBDOM and disclosed its transmembrane electron transport activity.

*CYBDOM* cRNA-injected oocytes acquired the capability to reduce an external electron acceptor, as determined by a simple colorimetric assay. Under two-electrode voltage clamp, extracellular application of the electron acceptor elicited inward currents, consistent with transmembrane electron transport. In support of this hypothesis, we provide multiple lines of evidence:
first, activation of inward currents was dependent on extracellular application of the electron acceptor FeCN in a concentration-dependent manner and was fully reversible upon washout; second, inward current amplitudes increased strongly in CYBDOM-expressing oocytes preinjected with ASC, the canonical electron donor for CYB561 proteins; third, inward current amplitudes were similar in bath solutions containing only membrane-impermeable ions; fourth, inward currents, but not CYBDOM expression on the PM, were totally abolished in the mutant H249L, predicted to lack the cytoplasmic heme (Bérczi and Zimányi, 2014). This latter result also represents the first experimental demonstration that electron transport in CYBDOM proteins depends on the hemes of their CYB561 core domain. We therefore conclude that inward currents reflected the CYBDOM-mediated movement of electrons from the intracellular electron donor ASC to the extracellular electron acceptor FeCN or, less efficiently, the ferrichelate FeNTA. Moreover, electron transport was stimulated by the membrane potential and by acidic external pH.

This work presents the first direct recordings of the activity of a plant trans-PM redox protein (CYBDOM). Prior to soybean CYBDOM, there were only two examples of transmembrane electron transport studied by electrophysiological techniques. The first was the characterization of NADPH oxidase in human phagocytes (Schrenzel et al., 1998; DeCourcey et al., 2003; Petheo and Demaurex, 2005; Ahluwalia, 2008), which took advantage of the high activity of this electron transport system in specialized blood cells such as eosinophils and neutrophils. Current amplitudes in the picocoulomb range were recorded in human eosinophils applying the patch clamp technique in the inside out patch configuration and were unambiguously associated with electrons moving from cytoplasmic NADPH to extracellular oxygen (Petheo and Demaurex, 2005). The activity of a D. melanogaster CYBDOM named stromal cell-derived receptor2 (SDR2) represents the second example of electron current recordings in X. laevis oocytes (Picco et al., 2014). Comparing the functional properties of the two transporters, we found that soybean CYBDOM, although expressed at similar levels in oocytes (Supplemental Fig. S2), presented significantly lower current amplitudes (Supplemental Fig. S2) and higher FeCN affinity (Supplemental Fig. S2) than D. melanogaster SDR2. Interestingly, the lower current amplitude is consistent with the negative shift of CYBDOM voltage dependence (Supplemental Fig. S2), which, in turn, nicely correlates with the more negative resting voltage in plant cells, −110/−150 mV (Hedrich, 2012), compared with animal cells, −40/−100 mV (Hille, 2001).

All features of soybean CYBDOM are consistent with this protein being a component of a constitutive redox system of plant cells and may be related to different physiological functions that need to be tested in planta. The existence of constitutive redox systems in plant PMs was proposed more than 30 years ago (Craig and Crane, 1982), but their molecular nature remained obscure. Trans-PM redox activity in plants was typically revealed by the reduction of external FeCN and found to be associated with membrane potential depolarization and acidification of the external medium, the latter resulting from the activation of the PM proton-translocating adenosine triphosphatase (H+-ATPase; Sijmons et al., 1984; Marrè et al., 1988; Lüthje et al., 2013). In some cases, constitutive reduction of external FeCN was based on NAD(P)H as an internal reductant (Menckhoff and Lüthje, 2004); in others, ASC was shown to be a suitable electron donor, and cytochrome b2 to be involved in the transmembrane electron transfer (Asard et al., 1992). Interestingly, CYBDOM is an ASC-dependent cytochrome b2 localized on the PM, and its activity is electrogenic and may thus depolarize the membrane (see Supplemental Fig. S3 for membrane depolarization in X. laevis oocytes). In the context of a plant cell, CYBDOM-dependent membrane depolarization would stimulate the activity of the H+-ATPase, whereas pump activation hyperpolarizes the PM and acidifies the apoplasm, both of which are conditions that would in turn activate the depolarizing activity of CYBDOM. Such reciprocal interplay between H+ pump and PM redox systems has long been hypothesized, based on the observations that reduction of external FeCN by plant cells activated the H+ pump, and that H+ pump activation by fusicoccin stimulated FeCN reduction (Marrè et al., 1988 and references therein).

Besides regulating the membrane potential, a cooperation between proton pump and PM redox systems would in principle result in energy dissipation. This possibility was investigated in wild watermelon (Citrullus lanatus) where a PM isoform of CYB561 was proposed to form, together with apoplastic ASC and ASC oxidase, a unique pathway for excess light energy dissipation (Nanasato et al., 2005). On the other hand, based on its reducing activity toward ferrichelates, CYBDOM may also play a role in iron homeostasis, reminiscent of duodenal cytochrome b in animal systems (McKie et al., 2001). In any case, whichever the physiological electron acceptors, CYBDOM is clearly a strong candidate as a component of the constitutive redox system of plant PMs.

The technical approach proposed in our work can be applied to the functional characterization of further members of the large family of CYB561 proteins and generally to any other transmembrane electron transporter, opening new perspectives in this fundamental field of plant life.

**MATERIALS AND METHODS**

**DNA Constructs and Plant Expression**

The complementary DNA of soybean (Glycine max) CYBDOM (Glyma09g01390.1) was subcloned into the commercial pICOS vector (GE Healthcare) by PCR using the following pair of primers, both containing BambH restriction sites (underlined): 5′-GTAACGATCCACGGAAGATG-3′ and 5′-AGCCAAATCCATCTATGAG5′−. The complementary DNA sequence was PCR amplified and cloned into pGEMHE (Liman et al., 1992) for oocyte expression and into pSAT6-EGFP-N1 (Tazira et al., 2005) for transient expression in X. laevis oocytes and D. melanogaster SDR2.
Arabidopsis (Arabidopsis thaliana) protoplast transformation. The CYBDOM:EGFP fragment was subcloned into the pTLN plasmid (Lorenz et al., 1996) for oocyte expression of the fusion protein. The single point mutation H249L was generated in the pTLN-CYBDOM:EGFP plasmid using specific primers and the QuickChange site-directed mutagenesis kit (Agilent Technologies Italia). Mutagenesis was confirmed by subsequent DNA sequencing.

For the generation of Arabidopsis plants (Columbia ecotype) stably expressing the CYBDOM:EGFP fusion, the CYBDOM coding sequence was cloned upstream of the EGFP coding sequence in a modified pBI121 vector (Bregante et al., 2008) under the control of a double Cauliflower mosaic virus 35S promoter. The EGFP coding sequence was inserted into the BamHI and SacI sites of the modified pBI121 vector. Subsequently, the CYBDOM sequence, excised from the pGEMHE-CYBDOM plasmid using BamHI, was transferred into the BamHI-linearized pBI121-EGFP plasmid. The obtained construct was inserted into Agrobacterium tumefaciens strain GV3101, which was used to generate transgenic Arabidopsis plants using the floral-dip method (Clough and Bent, 1998). Ten independent antibiotic-resistant transgenic Arabidopsis lines were analyzed for CYBDOM-EGFP localization. The CYBDOM::EGFP chimeric proteins were performed using a Leica SP2 (http://www.leica-microsystems.com) laser-scanning confocal imaging system. For EGFP detection, excitation was set at 488 nm and detection between 650 to 750 nm and 575 to 625 nm, respectively.

After staining, the leaves were immediately imaged. The lambda spectrum of nuclease-free water, using a Drummond Nanoject microinjector. Oocytes were then incubated at 18°C in standard solution supplemented with 0.1 mg mL$^{-1}$ of gentamicin. Isolation of Arabidopsis mesophyll protoplasts and transient expression assays were performed as described previously (Costa et al., 2012).

Oocyte Expression

In vitro transcription of Nhel-linearized pGEMHE-CYBDOM and MluI-linearized pTLN-CYBDOM:EGFP DNA templates was done using mMESSAGE mMACHINE T7 or SP6 kit (Life Technologies), respectively, and the integrity of the transcribed cRNA was confirmed by agarose gel analysis. Oocytes were surgically removed from adult female Xenopus laevis frogs, treated with collagenase (1 mg mL$^{-1}$) for 30 min at room temperature under constant shaking, and washed twice with standard solution (in mM): 96 NaCl, 2 KCl, 1 CaCl$_2$, 1 MgCl$_2$, and 5 HEPES, pH 7.5. Isolated oocytes were injected with transcribed cRNA at 40 ng per oocyte or with an equivalent volume of nuclease-free water, using a Drummond Nanoject microinjector. Oocytes were then incubated at 18°C in standard solution supplemented with 0.1 mg mL$^{-1}$ gentamicin.

Confocal Fluorescence Microscopy

Confocal microscopy analyses of leaves of Arabidopsis transgenic plants expressing the CYBDOM:EGFP chimeric proteins were performed using a Leica SP2 (http://www.leica-microsystems.com) laser-scanning confocal imaging system. For EGFP detection, excitation was set at 488 nm and detection between 515 and 530 nm. For chlorophyll and FM4-64 detection, excitation was set at 488 nm, and detection ranges were 650 to 750 nm and 575 to 625 nm, respectively. For FM4-64 staining, leaf discs were dipped for 5 min in a water-based solution in which FM4-64 was diluted to a final concentration of 17 μM (Bolte et al., 2004). After staining, the leaves were immediately imaged. The lambda spectrum records were carried out following the application notes of the Leica SP2 microscope. Image postacquisition analyses were performed with the ImageJ bundle software (National Institutes of Health).

Confocal microscopy analyses of X. laevis oocytes expressing CYBDOM:EGFP chimeric proteins were performed using a Leica SP2 equipped with a 40× oil-immersion objective. Both EGFP and FM4-64 were excited at 488 nm, and emissions were monitored at 505 to 512 nm and 645 to 655 nm, respectively. Confocal images were obtained by scanning the laser scan line axially (z scan) along a 12-μm section (0.5-μm steps) within the oocyte (for details, see Naso et al., 2009). All measurements were averaged from more than six independent scans in several oocytes (n = 8) harvested from more than two donor frogs.

Colorimetric Ferric Reductase Activity Assay

Ferric reductase activity assays (McKie et al., 2001; Vargas et al., 2003) were conducted on individual X. laevis oocytes placed in 96-well plates. Oocytes were incubated for 15 h in the dark in 50 μL incubation buffer (standard solution containing 100 μM FeNTA and 1 μM terrozone). Formation of ferrous iron was calculated from the optical density of the incubation buffer at 562 nm. Optical density was measured using an EpochMax multi-mode microplate reader (Molecular Devices). Experiments were performed three times using at least 15 oocytes harvested from different frogs.

Two-Electrode Voltage Clamp Recordings

Whole-cell membrane currents were measured at room temperature using a homemade two-electrode voltage clamp amplifier. In brief, oocytes are impaled with two glass microelectrodes, one to measure the membrane potential (termed voltage electrode), the second (termed the current electrode) to pass sufficient current to maintain the membrane voltage at the desired value (voltage clamp), using a feedback circuit. The amount of current is determined from the difference between the actual membrane potential and the desired value (command voltage). Microelectrodes filled with 3 M KCl solution had a pipette resistance of 0.2 to 0.4 MΩ, corresponding to a tip diameter of a few micrometers. Membrane currents elicited by exposure to extracellular electron acceptors were generally recorded at a holding potential of −20 mV. Current-voltage relationships were determined from steady-state membrane currents recorded in response to 25-ms voltage pulses ranging from +40 mV to −100 mV in steps of 10 mV, from a holding potential of −20 mV. By convention, negative currents are inward currents due to either the influx of cations or the efflux of anions.

The ionic bath solution was the standard solution adjusted to pH 5.5 substituting 10 mM HEPES with an equimolar concentration of MES. The BTP/MES solution contained 200 mM MES and 20 mM BTP, pH 5.5. A gravity-driven perfusion system was used for continuous superfusion of oocytes during voltage clamp recordings and for switching between different bath solutions. Ferric compounds were prepared as stock solutions and diluted appropriately in bath solution, potassium FeCN at 100 mM, FeNTA, FeCIT, and FeEDTA at 30 mM. Ferrocyanide was prepared as a stock solution at 100 mM and diluted at 100 μM in the standard solution at pH 5.6; 20 μM dithiothreitol was added in the final solution to avoid production of FeCN from ferrocyanide.

For ASC injection experiments, ASC stock solution (100 μM) was made fresh and adjusted to pH 7.0 with KOH. After the baseline recording series, the oocyte was removed from the voltage clamp setup and injected with 50 μL of ASC stock solution using the microinjector, resulting in a final concentration of injected ASC of 10 μM (considering a volume of approximately 500 μL for a mean oocyte diameter of 1 mm). Upon injection, the oocyte volume transiently increased, but recovered to its original size within minutes. Injected oocytes were allowed to recover for 30 min at room temperature before voltage clamp measurements were resumed.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers GmCYBDOM, XP_003584961.6; Atb561, AAM61586.1; Zmb561, XP_008673872.1, Osj561, BAD05824.1; Tab561, CBH32553.1; Ceb561, NP_001023900.1; Dmb561, NP_00110792.2; Bb561, NP_0777262.1; Mmb561, NP_0013813.2; and Hsb561, NP_001017917.1.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. CYBDOM:EGFP localizes to the plasma membrane in transiently transformed Arabidopsis mesophyll protoplasts.

Supplemental Figure S2. Major differences in electron transport between soybean CYBDOM and D. melanogaster SDR2.

Supplemental Figure S3. CYBDOM-mediated electron transport causes membrane depolarization in X. laevis oocytes.

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LITERATURE CITED


