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Tonoplast cytochrome b561
is a transmembrane ascorbate-dependent monodehydroascorbate reductase:
functional characterisation of electron currents in plant vacuoles

Antonella Gradogna¹, Laura Lagostena¹, Sara Beltrami², Edoardo Tosato², Cristiana Picco¹,
Joachim Scholz-Starke¹, Francesca Sparla², Paolo Trost^{2*} and Armando Carpaneto^{1,3*}

1. *Institute of Biophysics - CNR, Via De Marini 6, 16149 Genova, Italy*
2. *Department of Pharmacy and Biotechnology (FaBiT) - University of Bologna, Via Irnerio 42, 40126 Bologna, Italy*
3. *Department of Earth, Environment and Life Sciences (DISTAV) - University of Genoa, Viale Benedetto XV 5, 16132 Genova, Italy*

*Corresponding authors: Paolo Trost and Armando Carpaneto
Email: paolo.trost@unibo.it ; phone: +390512091329; fax: +39051242576 ;
Email: armando.carpaneto@unige.it ; phone: +390103538570; fax: +39010352169.

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Abstract

- Ascorbate (Asc) is a major redox buffer of plant cells, whose antioxidant activity depends on the ratio with its one-electron oxidation product monodehydroascorbate (MDHA). The cytoplasm contains millimolar concentrations of Asc and soluble enzymes that can regenerate Asc from MDHA or fully oxidized dehydroascorbate. Also vacuoles contain Asc, but no soluble Asc-regenerating enzymes.
- Here we show that vacuoles isolated from *Arabidopsis* mesophyll cells contain a tonoplast electron transport system that works as a reversible, Asc-dependent transmembrane MDHA oxidoreductase.
- Electron currents were measured by patch-clamp on isolated vacuoles and found to depend on the availability of Asc (electron donor) and ferricyanide or MDHA (electron acceptors) on opposite sides of the tonoplast.
- Electron currents were catalyzed by cytochrome b561 isoform A (CYB561A), a tonoplast redox protein with cytoplasmic and luminal Asc binding sites. The K_m for Asc of the luminal (4.5 mM) and cytoplasmic site (51 mM) reflected the physiological Asc concentrations in these compartments. The maximal current amplitude was similar in both directions.
- Mutant plants with impaired CYB561A expression showed no detectable trans-tonoplast electron currents and strong accumulation of leaf anthocyanins under excessive illumination, suggesting a redox-modulation exerted by CYB561A on the typical anthocyanin response to high-light stress.

Introduction

Ascorbate (Asc) is a versatile antioxidant of plant cells that can reduce non-enzymatically different types of radicals and transition metals, and serve as a substrate of several enzymes including Asc peroxidase, Asc oxidase (Foyer *et al.*, 2020) and cytochrome *b*-561 (CYB561) (Asard *et al.*, 2013). Asc is also a cofactor for 2-oxoglutarate-dependent dioxygenases whose catalytic iron is maintained in the reduced/active state by Asc (Smirnoff, 2018). Non-enzymatically, Asc can also reduce protein

53 sulfenic acids thereby potentially interfering with thiol-based redox regulatory systems (Zaffagnini *et al.*, 2019; Anschau *et al.*, 2020). In all redox reactions in which **it** is involved, Asc donates a hydrogen
 54 atom (one electron and one proton) and is converted to monodehydroascorbate (MDHA; Njus *et al.*,
 55 2020). As a radical, MDHA is relatively stable but can be reduced back to Asc by NADH-
 56 monodehydroascorbate reductase (MDHAR; Hossain & Asada, 1985; Park *et al.*, 2016) or ferredoxin
 57 (Foyer *et al.*, 2020), or react with other radicals, including itself (Njus *et al.*, 2020). Spontaneous
 58 disproportionation of MDHA radicals gives rise to Asc and fully oxidized dehydroascorbate (DHA)
 59 that is chemically unstable. DHA can be reconverted to Asc by glutathione-dependent DHA
 60 reductase (DHAR) or alternative systems relying on different electron sources (Potters *et al.*, 2004;
 61 Terai *et al.*, 2020). All these reactions and properties are relevant for the current study dealing with
 62 an Asc-dependent MDHA oxidoreductase activity that connects cytoplasmic and vacuolar Asc pools
 63 across the tonoplast membrane.
 64

65
 66 The concentration of Asc in plant cells is generally very high. In leaves, compartments such as the
 67 cytosol, chloroplast stroma, mitochondria and peroxisomes contain Asc in the order of tens of
 68 millimolar (Heyneke *et al.*, 2013; Zechmann, 2018; Foyer *et al.*, 2020). These compartments are
 69 characterized by neutral pH and contain NADH-dependent MDHARs that regenerate Asc from
 70 MDHA, but also Asc peroxidases that convert Asc to MDHA in the presence of H₂O₂ (Noctor *et al.*,
 71 2018; Smith *et al.*, 2021). Although the *in vivo* determination of MDHA is impractical, the combined
 72 effects of MDHA spontaneous dismutation and MDHAR activity are believed to keep MDHA at very
 73 low levels in these cell compartments (Smirnoff, 2018). Indeed, a high local Asc/MDHA ratio is what
 74 makes Asc a good reductant *in vivo*, in spite of its very positive standard redox potential ($E^{\circ'}_{\text{Asc/MDHA}}$
 75 +280 mV) (Njus *et al.*, 2020). Differently from Asc/MDHA, Asc/DHA ratios are routinely measured in
 76 leaf extracts and values found are typically above 10, but most of the DHA is likely to derive from the
 77 apoplast (Noctor, 2006). Anyway, DHA as such does not directly influence the antioxidant properties
 78 of Asc, that essentially depend on the Asc/MDHA ratio and on pH.
 79

80 Acidic compartments of plant cells, like the apoplast and the vacuole, do also contain Asc but lack
 81 MDHARs and DHARs (Noctor & Foyer, 2016; Smirnov, 2018). In the apoplast, the concentration of
 82 Asc is in the millimolar range (Zechmann, 2018) and typically lower than DHA (Foyer *et al.*, 2020).
 83 The apoplast is also the site where Asc can be oxidized by Asc oxidase and DHA degraded by
 84 specific pathways (Smirnov, 2018; Foyer *et al.*, 2020).

85
 86 In the vacuole, Asc concentration is also millimolar but the Asc/DHA ratio can be higher than in the
 87 apoplast (Ferrerres *et al.*, 2011). Since most living plant cells contain a large central vacuole that can
 88 occupy more than 80% of the cellular volume (Shitan & Yazaki, 2020), the vacuolar pool of Asc can
 89 be as large as the cytosolic pool in a typical photosynthetic plant cell (Zechmann, 2018). Vacuoles
 90 neither contain Asc oxidase nor other soluble enzymes that specifically use Asc as a reductant, but
 91 contain phenolic compounds, including anthocyanins that can reduce hydrogen peroxide either
 92 chemically (Csepregi & Hideg, 2018) or enzymatically via membrane bound class III peroxidases
 93 (Ferrerres *et al.*, 2011; Zipor *et al.*, 2015; L  thje & Martinez-Cortes, 2018; Smirnov & Arnaud, 2019).
 94 In both cases anthocyanin oxidation generates phenoxyl radicals which can rapidly oxidize Asc to
 95 MDHA, and MDHA to DHA (Takahama, 2004; Ferrerres *et al.*, 2011; Smirnov & Arnaud, 2019). In
 96 leaves, the biosynthesis of both Asc and anthocyanins is strongly stimulated under excessive
 97 illumination, the same conditions that drive the production of hydrogen peroxide by photosystem I
 98 (Foyer *et al.*, 2020). However, besides the antioxidant function that connects anthocyanins with Asc,
 99 anthocyanins play also a photoprotective role, independently from Asc, based on the efficient
 100 absorption of green and yellow components of the photosynthetically active radiation (Zheng *et al.*,
 101 2021).

102
 103 Asc is synthesized in the cytosol, with the last enzyme of the pathway being associated to the internal
 104 membrane of mitochondria (Smirnov, 2018). How Asc is distributed in different cell compartments is
 105 little known. An intracellular Asc transporter is known to be located on the inner membrane of
 106 chloroplasts (Miyaji *et al.*, 2015) and a member of the multidrug and toxic compound extrusion family
 107 that works as an ascorbate/proton antiporter (AtDTX25; Hoang *et al.*, 2021) was recently described

108 in the vacuolar membrane of Arabidopsis. The vacuolar Asc transporter AtDTX25, is highly
 109 expressed in seedlings where it promotes the reduction and mobilization of Fe³⁺ stored in vacuoles.
 110 AtDTX25 is also expressed in flowers, with still unknown function, but not in leaves (Hoang *et al.*,
 111 2021). On the other hand, vacuoles isolated from barley leaves were shown to slowly uptake DHA
 112 and Asc following the concentration gradient, with no evidence for any detectable transport system
 113 (Rautenkranz *et al.*, 1994).

114

115 The tonoplast membrane of species like *Arabidopsis thaliana* and *Phaseolus vulgaris* also contains
 116 at least one member of the cytochrome b561 family of di-heme proteins that, as a general property,
 117 catalyze transmembrane electron transports using Asc as electron donor and MDHA or metal
 118 compounds as electron acceptors (Asard *et al.*, 2013). Arabidopsis contains four CYB561
 119 orthologues and several related cytochromes in which CYB561 domains are combined with one or
 120 more extracellular DOMON domains, each binding an additional heme (CYBDOM; Preger *et al.*,
 121 2005; Tsubaki *et al.*, 2005; Asard *et al.*, 2013). One CYBDOM isoform was experimentally localized
 122 on the plasma membrane (Picco *et al.*, 2015). Among CYB561s (also known as CYBASC for Asc-
 123 reducible cytochromes b; Preger *et al.*, 2005; Klein *et al.*, 2020), the isoform A (CYB561A) was
 124 localized on the tonoplast in Arabidopsis leaves (Griesen *et al.*, 2004) and in etiolated bean
 125 hypocotyls (Preger *et al.*, 2005), but on the plasma membrane in wild watermelon (*Citrullus lanatus*;
 126 Nanasato *et al.*, 2005). The intracellular location of the remaining three CYB561 isoforms (B-D) is
 127 unknown but the crystal structure of CYB561B from *Arabidopsis thaliana* was solved (Lu *et al.*, 2014).
 128 Arabidopsis CYB561B is a homodimer and each monomer is made of six transmembrane α -helices
 129 with two heme groups coordinated by two histidine pairs. In each monomer, two cavities, each
 130 located in close proximity to a heme and facing different sides of the membrane, are the Asc binding
 131 sites (Lu *et al.*, 2014). Tonoplast CYB561A is predicted to share with CYB561B of Arabidopsis and
 132 other CYB561 members of the family the same overall structure (Asard *et al.*, 2013; Lu *et al.*, 2014;
 133 Ganasen *et al.*, 2018). The residues involved in heme coordination and Asc binding are also largely
 134 conserved among plant and animal CYB561 orthologues (Lu *et al.*, 2014).

135

In this work we show that CYB561A catalyzes a trans-membrane electron transport from Asc on one side of the tonoplast membrane to MDHA or an artificial electron acceptor like ferricyanide on the other side of the membrane. Electron currents, detected 38 years after the first patch-clamp potassium current recordings in plant cells (Moran *et al.*, 1984; Schroeder *et al.*, 1984), are reversible and can be measured by whole-vacuole patch clamp experiments with vacuoles isolated from mesophyll *Arabidopsis* protoplasts. Steady state kinetic analyses allowed to determine kinetic parameters associated to each of the two Asc binding sites, indicating the role that CYB561A may play in connecting the redox states of different Asc pools separated by the tonoplast membrane. CYB561A appears to be the major protein responsible for the Asc-dependent electron transport detected in *Arabidopsis* vacuoles and plants with lower expression of the *CYB561A* gene are shown to over-accumulate anthocyanins in response to high light, pinpointing a plausible redox role played by CYB561A in modulating this photoprotective response.

Results

Cytosolic ascorbate and vacuolar ferricyanide elicit cytosol-to-lumen directed electron currents in *Arabidopsis* vacuoles

With the aim of testing whether a trans-tonoplast electron transport could be detected *ex vivo*, whole-vacuole patch-clamp experiments were performed on intact vacuoles isolated from *Arabidopsis thaliana* mesophyll protoplasts. Prolonged whole-vacuole patch-clamp recordings were carried out at 0 mV, a tonoplast voltage in the physiological range (-30 to 0 mV) (Bethmann *et al.*, 1995; Walker *et al.*, 1996; Hedrich, 2012). Addition of 10 mM Asc to the bath medium corresponding to the cytosolic side of the vacuole, did not elicit any trans-membrane current. Clear inward currents were instead elicited when the vacuolar lumen was pre-equilibrated with a generic electron acceptor like ferricyanide (FeCN; 1 mM). The effect was reversible and the current returned to the basal level upon Asc wash-out (Fig. 1a).

164 In principle, the observed inward currents could result from an electron transport system connecting
 165 cytosolic Asc to luminal FeCN, or alternatively from the activation of ion fluxes, either anions moving
 166 into the vacuole or cations moving from the vacuole to the cytosol. The currents observed in the
 167 presence of cytosolic Asc and vacuolar FeCN, however, were not affected by substituting the major
 168 ions of the bath solution (K^+ , Cl^-) with large, membrane impermeable charged molecules (BTP^+ ,
 169 MES^-). This indicated that potassium and chloride were not involved in the Asc/FeCN-dependent
 170 currents (Fig. 1b). Moreover, Asc/FeCN-dependent currents were not affected by a vacuolar pH shift
 171 from 5.5 to 6.5 (Fig. 1b) suggesting that proton-coupled transport activities were not involved. The
 172 apparent insensitivity to the vacuolar pH was thus consistent with an electron transport activity
 173 resulting in the reduction of luminal ferricyanide (FeCN), considering that no protons are involved in
 174 the reduction of ferri to ferrocyanide ($[Fe^{3+}(CN)_6]^{3-} + 1 e^- \rightarrow [Fe^{2+}(CN)_6]^{4-}$). Cytosolic Asc to luminal
 175 FeCN electron currents were measured at different membrane potentials and were active at
 176 physiological voltages (Suppl. Fig.1). In eliciting the electron transfer to FeCN, Asc could not be
 177 substituted by a different electron donor like dithiothreitol (DTT) (Fig. 1cd). Overall, these results are
 178 fully consistent with Asc-evoked currents mediated by an electron transport system that connects
 179 cytosolic Asc to luminal FeCN, and exclude concomitant ion transports.

180

181 Currents could be further characterized by varying the concentration of Asc in the bath (cytosolic)
 182 solution, at different concentrations of FeCN in the vacuole. All experiments were performed at 0 mV
 183 and absence of proton gradient (pH 7.5 in both cytosol and lumen). Current densities, i.e. currents
 184 divided by the capacitance of the vacuole, were used to compare data obtained from vacuoles of
 185 different sizes. For the sake of simplicity, we will use the term current instead of current density
 186 throughout the text. Fig. 2ad shows that currents increased in amplitude in both Asc- and FeCN-
 187 dependent manner. The apparent kinetic parameters, i.e. the apparent Michaelis-Menten constants
 188 for cytosolic Asc (K_{Ac}^{app}) and vacuolar FeCN (K_{Fv}^{app}) and the maximal currents interpolated at
 189 saturating Asc (I_{Acmax}^{app}) or FeCN concentrations (I_{Fvmax}^{app}) allowed estimating the limiting kinetic
 190 parameters by secondary plots (Fig. 2bcef). Continuous lines in Fig. 2bcef are obtained by a global
 191 fitting procedure using an extended Michaelis-Menten equation (see Mathematical model in

Methods). In this way the electron transport system is kinetically described as a trans-tonoplast oxidoreductase with a K_m for cytosolic Asc (K_{Ac}) of 51 ± 8 mM, a K_m for vacuolar FeCN (K_{Fv}) of 1.0 ± 0.2 mM and a maximal electron transport capacity (I_{cvmax}) of 3.7 ± 0.5 pA/pF (Table 1).

Vacuolar ascorbate and cytosolic FeCN elicited lumen-to-cytosol directed electron currents in Arabidopsis vacuoles

The reversibility of the electron transport system was tested by loading the vacuole with 5 mM Asc and adding 100 μ M FeCN to the bath medium, as shown in Fig. 3. In this inverted configuration, the application of cytosolic FeCN evoked outward currents that disappeared upon FeCN wash-out (Fig. 3a, middle trace). In contrast, FeCN did not alter the currents in control vacuolar solution (without Asc, Fig. 3a, top trace) or when Asc was substituted by DTT (Fig. 3a, bottom trace).

The combined results of Fig. 1 and Fig. 3 clearly showed that Asc oxidation, and symmetrically FeCN reduction, could occur on both sides of the vacuolar membrane. When the electron donor Asc was in the vacuole, and the electron acceptor FeCN was cytosolic, outward currents were thus compatible with a vacuole-to-cytosol electron transport. Influence of the membrane potential was again measured during a series of voltage pulses ranging from -60 to 60 mV. The electron transport was active at physiological tonoplast voltages (Suppl. Fig. 2)..

Dose-response experiments were performed at 0 mV and 0 Δ pH to estimate the dependence of electron currents on vacuolar Asc and cytosolic FeCN concentrations. Patch-clamp experiments were carried out with different Asc concentrations in the pipette (1 to 40 mM, vacuole) and different FeCN concentrations in the bath solution (3.3 to 100 μ M, cytosol). Positive currents normalized to membrane capacitance (i.e. current density), increased at increasing concentrations of either FeCN or Asc. Current amplitudes (Fig. 4a) were plotted as a function of the varied substrate (Fig. 4be). Data fitting by parametric hyperbolae returned the apparent Michaelis-Menten constants for both

vacuolar Asc (K_{Av}^{app}) and cytosolic FeCN (K_{Fc}^{app}), and the maximal currents interpolated at saturating Asc (I_{Avmax}^{app}) or FeCN concentrations (I_{Fcmax}^{app}). Limiting kinetic parameters were obtained following the same procedure of Fig. 2 (Figure 4cdfg). Limiting K_m values for vacuolar Asc (K_{Avac}) and cytosolic FeCN (K_{Fcyt}) were 4.5 ± 1.0 mM and 24 ± 2 μ M, respectively, with the maximal current (I_{vcmax}) being 3.1 ± 0.9 pA/pF (Table 1). The oxidoreductase system transferring electrons from ascorbate to ferricyanide across the tonoplast is thus fully reversible and has similar maximal electron current capacities in either direction, though kinetic affinities for the substrates on either side of the membrane are very different (Table 1).

228

229 **Detecting the vacuolar acidification associated to ascorbate oxidation**

230

The trans-tonoplast electron transport so far characterized relies on the presence of an electron donor (Asc) and an electron acceptor (FeCN) on opposite sides of the membrane. The first step of this process is expected to involve a one-electron oxidation of Asc to MDHA with concomitant release of one proton in the medium ($Asc^- \rightarrow MDHA^- + 1e^- + 1H^+$). Whether Asc oxidation was associated to a detectable pH change was investigated with the ratiometric fluorescent pH indicator 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF) that was loaded in the lumen of the vacuole from the patch pipette. Whole-vacuole patch-clamp experiments were performed with 5 mM Asc and 10 μ M BCECF in the vacuole upon increase of cytosolic FeCN concentrations. Conversion of BCECF fluorescence ratios (490/440 nm) into pH values was based on a pH calibration procedure previously described (Carpaneto *et al.*, 2017; Gradogna *et al.*, 2021). Positive currents were recorded simultaneously with the BCECF fluorescence ratio, at a holding potential of 0 mV (Fig. 5a). Interestingly, currents increased with increasing cytosolic FeCN and nicely matched with the reversible increase of vacuolar lumen acidification (Fig. 5ab). In the absence of Asc in the pipette, only a slight acidification was observed at the highest concentration of cytosolic FeCN (100 μ M) (Fig. 5b). Results are hence consistent with vacuolar Asc being the source of both electrons for cytosolic FeCN reduction and protons for luminal acidification.

247

248 **Ascorbate-dependent trans-tonoplast electron currents detected in isolated vacuoles of**
 249 **Arabidopsis mesophyll protoplasts depend on cytochrome b561 isoform A (CYB561A)**

250

251 The Asc-dependent, trans-tonoplast electron transport characterized so far is compatible with the
 252 activity of cytochrome b-561, possibly cytochrome b561 isoform A (CYB561A) previously associated
 253 to vacuolar (Preger *et al.*, 2005) or plasma membranes (Nanasato *et al.*, 2005) in different plant
 254 species including Arabidopsis (Griesen *et al.*, 2004). The targeting of Arabidopsis CYB561A to the
 255 tonoplast was confirmed here by the confocal analysis of Arabidopsis protoplasts transformed with
 256 a CYB561A-EGFP construct (Suppl. Fig. 3).

257

258 Two homozygous T-DNA insertional lines for At4g25570 were isolated. Line *cyb561a-1* carries an
 259 insertion in the first intron while line *cyb561a-2* shows an insertion in the third exon of the CYB561A
 260 gene. The level of CYB561A transcripts was analysed in rosette leaves and found to be much lower,
 261 albeit not absent, in both mutant lines in respect to wild type (Suppl. Fig. 4).

262

263 Whole-vacuole patch-clamp experiments were performed on vacuoles isolated from either mutant
 264 line. Addition of 10 mM cytosolic Asc in the presence of 1 mM vacuolar FeCN did not yield to any
 265 detectable inward current (Fig. 6ab). Symmetrically, addition of 100 μ M cytosolic FeCN in the
 266 presence of 5 mM vacuolar Asc did not induce any detectable outward current (Fig. 6cd) or vacuolar
 267 acidification (Suppl. Fig. 5). CYB561A is thus concluded to be the responsible for the bi-directional
 268 Asc-to-FeCN electron transport detected in isolated vacuoles.

269

270 **CYB561A mediates electron transport from vacuolar ascorbate to cytosolic**
 271 **monodehydroascorbate**

272

273 Given the high structural similarity between Asc and its oxidation product MDHA, CYB561A is
 274 expected to bind also MDHA in both its Asc binding sites. Once bound, MDHA could act as an
 275 electron acceptor as shown for cytochromes b561 *in vitro* (Nakanishi *et al.*, 2009; Bérczi & Zimányi,

276 2014). Here we tested whether the electron transport from luminal Asc to cytosolic MDHA could also
 277 be detected in isolated vacuoles.

278

279 To this aim, Asc was included in the luminal pipette solution and the vacuole was perfused with an
 280 ionic solution containing Asc oxidase (AO-solution). The AO-solution was then changed with an ionic
 281 solution containing Asc (Asc-solution) (Figure 7a). At the interface between the two solutions, AO
 282 interacts with Asc and generates MDHA that should locally reach a finite concentration before
 283 dismutating to Asc and DHA. In the perfusing chamber, freshly produced MDHA could possibly act
 284 as an electron acceptor for CYB561A, itself reduced by Asc from the inside of the vacuole. Indeed,
 285 positive currents were clearly elicited when the solution interface entered into the perfusing chamber
 286 and bathed the vacuole. The current was transient as long as the Asc/AO solution was washed out
 287 by the perfusing flow (Figure 7ab). Electron currents were again completely abolished in vacuoles
 288 isolated from both *cyb561a* mutant lines (Figure 7bc).

289

290 In wild type vacuoles, electron currents could be modulated by varying the concentration of Asc used
 291 by AO to generate MDHA. Variations were observed in the sub-millimolar range (0.03-1 mM)
 292 according with the K_m for Asc of AO (0.2 mM; Wimalasena & Dharmasena, 1994) (Fig. 7d). At Asc
 293 concentrations higher than 1 mM, AO was saturated and so was the electron current stimulation.
 294 Interestingly, Asc concentrations up to 20 mM did not compete with the capability of MDHA to bind
 295 the cytosolic site of CYB561A and act as electron acceptor (Fig. 7d), a result that is consistent with
 296 the high K_m for Asc of the cytosolic site of CYB561A (51 mM; Table 1).

297

298 The electron currents generated by Asc/AO were voltage-dependent as shown by voltage ramps
 299 (Suppl. Fig. 6). Currents were enhanced by positive cytosolic voltages which would actually facilitate
 300 the movement of electrons from vacuolar Asc to cytosolic MDHA; again, similar to all other conditions
 301 investigated in this study (Suppl. Fig. 1 and 2), the trans-tonoplast electron transport is active at
 302 physiological tonoplast voltages between -30 and 0 mV. The same experiments performed with
 303 vacuoles isolated from the two *cyb561a* mutant lines gave no response, strongly suggesting that

MDHA was the cytosolic acceptor of electrons which moved from vacuolar Asc and crossed the vacuolar membrane by means of CYB561A.

CYB561A mutant plants show an exaggerated response to high light in terms of anthocyanin accumulation in leaves

Mutants of *CYB561A* were analysed for Asc content in total leaf extracts from 14-day plants grown on agar plates under standard illumination ($100 \mu\text{E m}^{-2} \text{s}^{-1}$). Compared to wild type plants, Asc and Asc+DHA were slightly higher in one of the two mutant lines (*cyb561a-2*) (Fig. 8a), although the Asc/(Asc+DHA) ratio was similar in all genotypes (0.89 on average with no significant differences among genotypes; Suppl. Fig. 7). Nitroblue tetrazolium (NBT) reactive compounds, which are expected to be inversely related to Asc, were lower in both mutant lines with respect to wild type plants (Fig. 8b).

In mature 7-week old plants, the small differences in Asc content observed in young plants were not apparent anymore (Fig. 8c, control). Neither Asc levels nor Asc/(Asc+DHA) ratios were significantly different in leaves of the three genotypes grown under standard light intensity ($100 \mu\text{E m}^{-2} \text{s}^{-1}$). As a typical response of mature plants to excessive illumination, Asc content increased in both wild type and mutants during a prolonged high light treatment (HL; $700 \mu\text{E m}^{-2} \text{s}^{-1}$). At the end of HL experiments (11 days), leaf Asc content was similarly increased in all genotypes (from 4.3-fold in wild type to 5.3-fold in *cyb561a-1*; Fig. 8c). The Asc/(Asc+DHA) ratio was unaffected by the HL treatment throughout the experiment (0.96 on average with no significant differences among genotypes and time points; Suppl. Fig. 7).

Rosette leaves of all genotypes contained no detectable anthocyanins under standard illumination but turned purple because of anthocyanin accumulation during the HL treatment (Suppl. Fig. 8). In wild type plants, anthocyanins were detected at constant levels starting from day 4 of strong illumination. In mutant lines the response was amplified and anthocyanin concentration in mutant

332 leaves exceeded that of wild type by a factor of 2.1-2.7 at day 4, and by a factor of 4.5-4.6 at the end
333 of the treatment (Fig. 8d). The overaccumulation of anthocyanins under HL was overall the most
334 clear and quantifiable phenotypic effect of the diminished *CYB561A* expression in Arabidopsis
335 mutants.

336

337

For Peer Review

338 Discussion

339

340 Isolated vacuoles of Arabidopsis leaves are here shown to possess an electron transport system
 341 that is able to transfer electrons from cytosolic Asc to a soluble electron acceptor inside the vacuole
 342 and vice versa. The electron acceptor can be FeCN, an artificial and stable compound that is reduced
 343 to the stable product ferrocyanide, or MDHA, a physiological short living radical produced by Asc
 344 oxidation. MDHA reduction by the electron transport system gives rise to Asc again on the other side
 345 of the membrane. Overall, the activity of the electron transport system can be defined as trans-
 346 tonoplast, Asc-dependent MDHA oxidoreductase and here is shown to depend on cytochrome b561
 347 isoform A (CYB561A). Among the four CYB561s of Arabidopsis, CYB561A appears to be the only
 348 functional isoform in vacuoles of mesophyll cells or at least, the only one whose activity could be
 349 electrophysiologically detected under tested conditions. Currents were in fact undetectable in
 350 mutants with strongly reduced expression of the CYB561A gene.

351

352 Studying the activity of an electron transporter like CYB561A in intact vacuoles with patch-clamp has
 353 several advantages. With this technique, the electron transport activity is detected with the protein
 354 inserted in its native lipid environment, with the membrane bilayer separating the two aqueous
 355 compartments in which the substrates are dissolved. Moreover, the tonoplast voltage is fixed. This
 356 condition allows measuring transmembrane currents evoked by any chosen combination of electron
 357 donors and acceptors, present on opposite sides of the membrane. This is essential for proteins like
 358 CYB561s with two active sites for Asc that are separated by the membrane in native conditions, but
 359 freely accessible from the medium in *in vitro* studies performed with the purified protein kept in a
 360 soluble state by interacting detergents.

361

362 By measuring the electron transport activity catalysed by CYB561A in intact vacuoles we could
 363 perform steady-state kinetic analyses and derive all kinetic parameters required to define the kinetic
 364 model, i.e. the maximal electron transport activity in either direction (I_{cvmax} , I_{vcmax}) and the K_m for Asc
 365 and FeCN for both cytosolic and luminal binding sites (K_{Ac} , K_{Fv} , K_{Av} , K_{Fc}). The maximal rates of

electron transport ($I_{\max}$) that CYB561A can catalyse in the two directions are similar, suggesting that the energy barriers the electrons have to overcome to travel from the cytosol to the vacuole and vice versa are symmetric. Double reciprocal plots of the primary kinetic data show parallel patterns consistent with a ping pong reaction mechanism and a simple equation to model the activity of CYB561A at any given concentration of electron donor and acceptor could be derived, see Methods and (Picco *et al.*, 2014).

372

Our measures allowed an unprecedented determination of specific K_m values for CYB561A substrates, with the cytoplasmic site showing higher K_m values for both Asc and FeCN in respect to the luminal site (Table 1). Interestingly, in the case of Asc as the physiological electron donor, the cytoplasmic and luminal K_m values (51 and 4.5 mM, respectively) are well suited to the cytosolic and luminal concentrations of Asc determined by quantitative immunoelectron microscopy in mesophyll Arabidopsis cells (22 mM and 2 mM, respectively; Zechmann, 2018), thereby further supporting a bidirectional electron transport system.

380

The prevalent direction of the electron transport catalysed by CYB561A *in vivo* depends in theory on the difference in redox potential of the Asc/MDHA couple between cytoplasm and vacuole. Where the redox potential is lower, Asc acts as an electron donor; where the potential is higher, MDHA is the electron acceptor. This condition sets the net direction of the electron transfer. The difference in redox potential depends on Asc/MDHA ratios and on ΔpH , because one proton is involved in the reaction ($MDHA + 1e^- + 1H^+ \rightarrow Asc$). Unfortunately, the intracellular concentration of MDHA, and hence the redox potential of Asc, is unknown and is likely to remain so until a genetically coded, Asc-specific biosensor will be available. The effect of pH is also difficult to predict because on one side the low pH of the lumen favours MDHA reduction by CYB561A, but on the other it speeds up MDHA dismutation ($MDHA + MDHA + 1H^+ \rightarrow Asc + DHA$) which is also pH-dependent and produces DHA besides Asc, thus lowering the total concentration of Asc in compartments like the vacuole that lack DHAR activities. So, no strong predictions on the prevailing direction of CYB561 activity can be made based on solid available data.

394

395 In spite of this lack of knowledge, it is widely accepted that cytochromes b561 are redox systems
 396 that both in animals and plants transfer electrons from cytoplasmic Asc to extracytoplasmic MDHA
 397 (or alternative electron acceptors as iron chelates; Asard *et al.*, 2013). The main reason supporting
 398 this view is that the cytoplasm is a compartment where Asc is kept reduced by MDHARs and DHARs,
 399 while extra-cytoplasmic compartments like the vacuole or the apoplast do not contain these activities
 400 and therefore may require the MDHA reductase activity of CYB561.

401

402 **Vacuoles** do not contain enzymes that oxidize Asc directly, but phenolic compounds **that may** form
 403 phenoxy radicals **by reacting with H₂O₂ (Csepregi & Hideg, 2018), directly or enzymatically via**
 404 **vacuolar class III peroxidases (Takahama, 2004; Ferreres *et al.*, 2011; Smirnov, 2018). Phenolic**
 405 **compounds include anthocyanins that, together with Asc, are increasingly accumulated in vacuoles**
 406 **under conditions of excessive illumination that concomitantly leads to H₂O₂ production (Zipor *et al.*,**
 407 **2015; Zechmann, 2018; Smirnov & Arnaud, 2019; Zheng *et al.*, 2021). Since Asc is a good** reductant
 408 of phenoxy radicals (Takahama, 2004; Smirnov, 2018), CYB561A may be a system to restore the
 409 redox state of vacuolar Asc at the expense of cytosolic Asc under **high light** conditions. This
 410 hypothesis reminds a similar hypothesis put forward to explain the role of CYB561 in *Citrullus lanatus*
 411 in high light, provided that in this species CYB561 is localized on the plasma membrane and is
 412 coexpressed with apoplastic Asc oxidase under HL stress (Nanasato *et al.*, 2005; Foyer *et al.*, 2020).

413

414 **The phenotypic analysis of CYB561A mutants provides new insights into the inter-relationship**
 415 **between Asc and anthocyanin in HL conditions. Mutants with diminished expression of CYB561A**
 416 **show similar Asc levels and similar Asc redox state (Asc/(Asc+DHA)) as wild type plants, but**
 417 **accumulate many more anthocyanins in high light conditions. The lack of control exerted by**
 418 **CYB561A on Asc levels of whole leaf extracts is not surprising since CYB561A may symmetrically**
 419 **control the redox states of cytosolic and vacuolar Asc without changing its total concentration nor its**
 420 **redox state in whole cells.** A positive correlation between Asc and anthocyanins in HL was already
 421 observed in different Arabidopsis ecotypes and Asc biosynthetic mutants (Page *et al.*, 2012; Plumb

422 *et al.*, 2018) and explained by the positive effect of Asc on the expression of several anthocyanin
423 biosynthetic genes and transcription factors that control the pathway (Munné-Bosch & Müller, 2013;
424 Smirnov, 2018). In this framework, the over accumulation of anthocyanins induced by HL in plants
425 with diminished *CYB561A* expression might be related to altered Asc levels or redox states in
426 specific subcellular compartments that still need to be investigated.

427

428 In conclusion, here we show that it is possible to record electron currents in plant vacuoles by
429 applying the patch-clamp technique and that *CYB561A* represents a redox connection between the
430 cytoplasmic and the vacuolar pools of Asc in *Arabidopsis* mesophyll cells. Under conditions in which
431 vacuolar Asc is oxidized to MDHA, *CYB561A* can regenerate it by oxidizing cytoplasmic Asc. The
432 reverse reaction is also possible and fully sustained by kinetic parameters, determined here for the
433 first time with an electrophysiological approach. Mutants with lower *CYB561A* expression have no
434 altered Asc levels in total leaf extracts, but overaccumulate leaf anthocyanins under prolonged high
435 light stress, suggesting a new unexplored connection between anthocyanin biosynthesis and an Asc-
436 dependent electron transport system of the tonoplast.

437

438

439 **Methods**

440 *Selection of homozygous insertional mutants of Arabidopsis thaliana*

441 Stock seeds of two independent CYB561-A (At4g25570) T-DNA lines were purchased from the
 442 European Arabidopsis Stock Center (NASC, Nottingham, U.K.). Five plants from each line were
 443 analysed by PCR for homozygous selection. Two independent PCR amplifications were performed
 444 on genomic DNA extracted from leaves. The two pairs of primers for SALK_069822 (cytb561-a1)
 445 were cyb561-a1 fw (5'- AATAGTTTCCATTGCGCAATG-3') plus cyb561-a1 rev (5'-
 446 TCAGAACACAAACACAGACGG-3') and cyb561-a1 rev plus LBb1.3 (5'-
 447 ATTTTGCCGATTTTCGGAAC-3'). The two pairs of primers for SALK_058385C (cytb561-a2) were
 448 cyb561-a2 fw (5'- CACACCAGAAGTAGCAAAGCC-3') plus cyb561-a2 rev (5'-
 449 TTGTGTTACCAAAGCAAAGGG-3') and cyb561-a2 rev plus LBb1.3. A Biometra T-gradient
 450 thermocycler was used for PCR amplifications with the following parameters: 5 min at 94 °C followed
 451 by 35 cycles of 30 s at 93 °C, 30 s at 58 °C and 1 min at 72 °C. PCR products were analysed on
 452 0.8% (w/v) agarose gel in TAE buffer and visualized with GelRed (Suppl. Fig. 4).

453 Homozygous T-DNA lines were checked for residual expression level of the gene At4g25570; three
 454 independent total RNA extractions were performed on wild type and T-DNA adult plants using
 455 Spectrum™ Plant Total RNA Kit (Merck). Reverse transcription was performed on 1 µg of total RNA
 456 using OneScript® Plus cDNA Synthesis Kit (abm®) with Oligo(dT) primer. CYB561-A specific PCR-
 457 primers were CYB561-A fw 5'-TCCTGGATTGGTATTGGAGT-3' and CYB561-A rev 5'-
 458 ATGCTTCGGATCCATACTTG-3' and PCR amplification was performed using the following
 459 conditions: 30 s at 95 °C followed by 30 cycles of 30 s at 95 °C, 30 s at 48 °C and 20 s at 68 °C and
 460 a final extension at 68 °C for 5 min. As control the expression level of the gene ACT8 (At1g49240)
 461 was used. The following pair of primers ACT8 fw 5'- CTCAGGTATTGCAGACCGTATGAG-3' and
 462 ACT8 rev 5'- CTGGACCTGCTTCATCATACTCTG-3' was used and the following amplification
 463 conditions were applied: 30 s at 95 °C followed by 30 cycles of 30 s at 95 °C, 30 s at 53 °C and 20
 464 s at 68 °C and ending with 5 min at 68 °C.

465 After amplification, the amount of PCR products was visualized using 1% (w/v) agarose gel
 466 electrophoresis with GelRed and analysed by ImageJ (Suppl. Fig. 4).

467

468 *Plant growth conditions*

469 *Arabidopsis thaliana* plants, both wild type and mutants, were grown on soil at 22 °C, under 12/12 h
 470 light/dark cycle with a photosynthetic photon flux density of 100 $\mu\text{mol m}^{-2} \text{sec}^{-1}$.

471 Stress condition was imposed by transferring 48-day old plants to high light (HL; photosynthetic
 472 photon flux density of 700 $\mu\text{mol m}^{-2} \text{sec}^{-1}$). Plants were collected after 4, 7 and 11 days of HL
 473 treatment (52-, 55- and 59-day old plants, respectively). Both control and treated samples were
 474 immediately frozen, ground in liquid nitrogen and stored at -80 °C before analysis.

475 For NBT-staining wild type and mutant plants were grown on square Petri dishes (10 x 10 cm). Each
 476 Petri dish contained 100 seeds, organized in two contiguous rows (20 seeds) for each genotype.
 477 Half-strength Murashige Skoog (MS $\frac{1}{2}$) medium containing 1 % (w/v) agar was used. Seeds were
 478 sterilized with chlorine smoke for 5 h before being transferred to the Petri dish with the help of a
 479 sterilized toothpick. Petri dishes were stored at 4 °C for 4 days in the dark and then transferred to
 480 the growth chamber for 14 days with 12/12 h light/dark cycle and photosynthetic photon flux density
 481 of 100 $\mu\text{E m}^{-2} \text{sec}^{-1}$.

482

483 *Quantification of ascorbate and anthocyanins*

484 Asc was quantified by adapting the protocol of Queval & Noctor (2007). Briefly, plant powders were
 485 suspended in 200 mM HCl by applying a 1:100 ratio between HCl volume and fresh weight. Samples
 486 were vortexed and centrifuged at 15,000 rpm for 10 minutes at 4 °C. The resulting cell extracts were
 487 neutralized with 200 mM NaOH and 200 mM NaH_2PO_4 , pH 5.6. Neutralized samples (120 μl), either
 488 reduced with 5 μl of 25 mM DTT for assaying Asc+DHA or used as such for Asc assays, were mixed
 489 with 600 μl of NaH_2PO_4 (200 mM, pH 5.6) and 450 μl of MilliQ water. The absorbance at 265 nm
 490 was measured spectrophotometrically (Shimadzu UV-1900) before and after the addition of 0.1 units
 491 of Asc oxidase from *Cucurbita* sp. (Merck). The assay was performed on three (n=3) or four (n=4)
 492 biological replicates for each genotype, with three technical replicates per sample.

493 Anthocyanin content was measured by adapting the method of Giacomelli et al. (2006). The assay
 494 was performed on four biological replicates (n=4) for each genotype, with two technical replicates

per sample. Statistical analysis was performed by ANOVA using a 1-way completely randomized model and Tukey's HSD test with 0.05 significance level (CoHort Software, USA).

497

498 *NBT staining*

499 The superoxide accumulation was qualitatively evaluated in 14 days-old plants grown in agarized
500 MS½. Plants were soaked for 30 min at room temperature in NBT solution (50 mM K-phosphate
501 buffer, pH 7.5; 0.5% (v/v) Triton X-100; 0.01% (w/v) NBT). After staining, plants were bleached in
502 80% (v/v) ethanol.

503

504 *Vacuole isolation*

505 Plants of *Arabidopsis thaliana* Columbia-0 wild type and *CYB561A* mutants (see above) were grown
506 on soil in a growth chamber at 22 °C and 8 h light/16 h dark regime. For isolation of mesophyll
507 protoplasts, rosette leaves from 3-10 week-old plants were used. Leaves were cut in strips and
508 incubated for 1 h in enzyme solution containing 0.5% (w/v) Cellulase Onozuka R-10 (Yakult), 0.05%
509 (w/v) Pectolyase Y-23 (Seishin), 0.25% (w/v) Macerozyme R-10, 1 mM CaCl₂, 500 mM D-sorbitol,
510 10 mM MES, pH 5.5. The protoplasts were washed twice, resuspended and maintained in W5
511 solution (in mM: 125 CaCl₂, 154 NaCl, 5 KCl, 2 MES-KOH, 5 glucose, pH 5.6). An efficient vacuole
512 release was obtained just before patch-clamp experiments by perfusion of protoplasts with vacuolar
513 release solution (VRS) containing (in mM): 100 malic acid, 160 Bis-tris-Propane, 5 EGTA, 3 MgCl₂,
514 200 D-sorbitol, pH 7.3.

515

516 *Patch-clamp recordings*

517 Patch-clamp experiments were performed as described elsewhere (Costa *et al.*, 2012; Boccaccio *et*
518 *al.*, 2014). Protoplasts were placed in the recording chamber and exposed to vacuolar release
519 solution. Patch-clamp experiments were performed on isolated vacuoles in the whole-vacuole
520 configuration. Standard pipette (vacuolar) solution contained (in mM): 100 KCl, 2 MgCl₂, 1 CaCl₂, 10
521 MES, 240 D-sorbitol, pH 5.5 (with KOH); 10 HEPES instead of MES was used in experiments
522 performed at pH 7.5. Standard bath (cytosolic) solution contained (in mM): 100 KCl, 2 MgCl₂, 10

HEPES, 290 D-sorbitol, pH 7.5 (with KOH). Potassium hexacyano-ferrate (III) (ferricyanide, FeCN, throughout the paper) was prepared as 100 mM stock and diluted appropriately in luminal or cytosolic standard solutions. L-Ascorbic acid was dissolved at a concentration of 40 mM in luminal or cytosolic standard solutions and pH was adjusted to 7.5 with BTP. The final solution was aliquoted and stored at -20 °C. Asc aliquots were diluted further in standard solution at the desired concentration just before use and kept on ice. Asc oxidase from *Cucurbita* sp. was dissolved in water at a stock concentration of 100 units/ml, aliquoted and stored at -20 °C. Aliquots were diluted in standard solution at a concentration of 2 units/ml just before use. All chemicals were purchased from Merck (Italy, Germany).

Patch pipettes were pulled from borosilicate glass capillaries (Harvard Apparatus, Holliston, MA, USA) and had resistances of 3-5 MΩ. Currents were recorded with a List EPC-7 amplifier (Darmstadt, Germany), filtered with a low pass filter and controlled by the acquisition program Pulse (Heka Elektronik, Lambrecht, Germany). Bath solution changes were performed by a gravity perfusion system for inflow and a peristaltic pump for outflow (Gilson Inc., Middleton, WI, USA); the perfusion system was optimized to change the bath solution in approx. 1 min (Festa *et al.*, 2016). All experiments were performed in continuous perfusion to minimise mechanical interferences. Kinetics of the current traces upon addition or removal of donor/acceptor reflects the speed of perfusion and has no physiological meaning. Membrane capacitance (C_v) could be estimated by the current transient elicited by a 10 mV voltage pulse (Sakmann & Neher, 1995; Carpaneto *et al.*, 2010). By convention, positive currents correspond to electrons/anions moving from the vacuolar lumen to the cytosolic side or to cations moving in the opposite direction.

544

545 BCECF fluorescence

Experiments designed to estimate pH changes associated with Asc oxidation were performed as described in (Carpaneto *et al.*, 2017; Gradogna *et al.*, 2021). A concentration of 10 μM BCECF was added to the pipette (vacuolar) solution containing (in mM): 100 KCl, 2 MgCl₂, 240 D-sorbitol, pH 7 (with KOH), in the presence or absence (control) of 5 mM Asc. After reaching the whole-vacuole configuration, the process of BCECF loading into the vacuole usually took about 20 min. To increase

551 the success of the experiment, we started the application of cytosolic FeCN when the pH trace
 552 approached its steady-state value. Inside the vacuole, we chose a circular region of interest (ROI),
 553 which was excited alternatively at 490 and 440 nm in 100 ms excitation cycles. Excitation light,
 554 selected by a monochromator (Cairn Research, Faversham, UK) was generated by a 75-Watt Xenon
 555 arc lamp (Osram, Italy). Fluorescence emission was detected using a 515-nm bandpass emission
 556 filter by a CCD camera (Roper Scientific, Germany) mounted on an inverted microscope (Axiovert,
 557 Zeiss, Germany) and recorded every 300 ms. The fluorescence ratio (490/440) was calculated online
 558 (background fluorescence was negligible). Conversion of fluorescence ratios to pH units was
 559 performed as in (Carpaneto *et al.*, 2017; Gradogna *et al.*, 2021).

560

561 *Protoplast transformation*

562 The CYB561A coding sequence was PCR amplified from the U15108 clone, purchased from the
 563 Arabidopsis Biological Resources Stock Centre (Columbus, Ohio, USA). The primers used for the
 564 PCR reaction (U15108LXHOI: 5'-ACTCGAGATGGCTGTCCGGATAAAC-3'; U15108RECORI: 5'-
 565 GAATTCTATAGCAGAATAACTGAAGTCAAC-3') carried the restriction sites for XhoI and EcoRI,
 566 respectively, and allowed the insertion of the coding sequence into the pSAT vector, in frame with
 567 the sequence of a downstream EGFP. The pSAT-CYB561A:EGFP construct was finally validated
 568 by Sanger sequencing.

569 Mesophyll protoplasts were isolated from well expanded leaves from four weeks old plants and
 570 transiently transformed using the polyethylene glycol method as described elsewhere (Yoo *et al.*,
 571 2007; Festa *et al.*, 2022; Gradogna & Carpaneto, 2022). Confocal microscopy analysis of vacuoles
 572 expressing the CYB561A-EGFP protein was performed using a Leica SP2 ([http://www.leica-](http://www.leica-microsystems.com)
 573 [microsystems.com](http://www.leica-microsystems.com)) laser-scanning confocal imaging system. For EGFP and FM4-64 fluorescence,
 574 excitation was set at 488 nm and detection ranges were 515 to 530 nm and 575 to 625 nm,
 575 respectively.

576

577 *Data analysis*

578 The effects of Asc or FeCN were estimated by subtracting the current in control condition from the
 579 steady-state current elicited by the presence of the reductant or oxidant. Currents were divided by
 580 the tonoplast capacitance (current densities) to compare data obtained from vacuoles of different
 581 sizes.

582 Unless otherwise indicated, data are shown as mean $\pm$ standard error, with n indicating the number
 583 of independent experiments. Data analysis, figures and models were done with Igor software
 584 (Wavemetrics, Lake Oswego, OR, USA).

585

586 *Mathematical model*

587 The following extended Michaelis-Menten equation was used to fit experimental currents upon
 588 variation of both cytosolic/vacuolar Asc and FeCN at a fixed tonoplast voltage of 0 mV:

$$589 \quad I = I_{\max} / (K_A/[A] + K_F/[F] + 1) \quad \text{equation 1}$$

590 I is the experimental current, $[A]$ and $[F]$ are the concentrations of Asc and FeCN respectively.
 591 Equation 1, which was developed by a simple kinetic model (Picco *et al.*, 2014), has only three free
 592 parameters i.e. the maximum current $I_{\max}$, the two constants K_A and K_F , which can be interpreted as
 593 the affinity constants for Asc and FeCN, respectively.

594

595

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602

603

604 **Author contributions**

605 PT and AC designed the research, AG, LL, SB, ET, JSS, FS performed the research, AG, ET, JSS,

606 CP, FS, PT and AC analysed the data, AG and PT wrote the manuscript, LL, ET, JSS, CP, FS and
607 AC revised the manuscript.
608

For Peer Review

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771 Tables

772

773 **Table 1** – Steady state kinetic parameters of the Asc-dependent trans-tonoplast electron transport
 774 in isolated vacuoles from Arabidopsis mesophyll protoplasts in the presence of ferricyanide as
 775 electron acceptor

776

recording mode	Electron donor Ascorbate K_A (mM)	Electron acceptor Ferricyanide K_F (mM)	Maximal electron transport rate $I_{\max}$ (pA/pF)
cytosolic Asc/vacuolar FeCN	$K_{Ac}=51\pm8$	$K_{Fv}=1.0\pm0.2$	$I_{cvmax}=3.7\pm0.5$
vacuolar Asc/cytosolic FeCN	$K_{Av}=4.5\pm1.0$	$K_{Fc}=0.024\pm0.002$	$I_{vcmax}=3.1\pm0.5$

777

778

779

780 Figure legends

781

782 Figure 1: Cytosolic ascorbate elicits electron currents in the presence of vacuolar FeCN.

783 (a) Typical membrane currents elicited by the application of 10 mM Asc in the absence (upper trace)
 784 and in the presence (lower trace) of 1 mM vacuolar FeCN. (b) Varying cytosolic ions and vacuolar
 785 pH doesn't change the response to Asc. Current amplitudes elicited by cytosolic 10 mM Asc in
 786 modified bath solutions and vacuolar pH (condition A: control, cytosolic KCl 100 mM / vacuolar pH
 787 5.5 (standard pipette solution at pH 5.5), condition B: cytosolic BTP-MES 100 mM replaces KCl /
 788 vacuolar pH 5.5, condition C: cytosolic BTP-MES 100 mM / vacuolar pH 6.5), in the absence and
 789 presence (+) of 1 mM vacuolar FeCN. **Standard bath solution at pH 7.5.** Current amplitudes elicited
 790 by ascorbate were normalised to membrane capacitance (A: n=6, B: n=6, C: n=3, A+: n=5, B+: n=6,
 791 C+: n=5). (c and d) Specificity of ascorbate effect. (c) Current recording from a vacuole loaded with
 792 1 mM vacuolar FeCN upon exposure (horizontal bars) **to cytosolic DTT and Asc both at 10 mM. The**
 793 **letters above the bars indicate significant differences (one-way ANOVA followed by post hoc Tukey's**
 794 **test, $P < 0.05$).** (d) Current amplitudes elicited by 10 mM DTT and 10 mM Asc normalised to
 795 membrane capacitance (**DTT: n=6; Asc: n=6**). **Statistical significance (according to Student's t-test):**
 796 ***** $P < 0.001$.**

797

798 Figure 2: Electron currents depend on cytosolic ascorbate and vacuolar FeCN concentrations.

799 (a) Dependence of electron currents on cytosolic Asc ($[Asc]_{cyt}$) at three different vacuolar FeCN
 800 concentrations (FeCN 0.3 mM: Asc from 2.5 to 20 mM n=4, Asc 40 mM n=3; FeCN 1 mM: Asc 2.5
 801 mM n=8, Asc from 10 to 40 mM n=9; FeCN 3 mM: Asc 2.5 and 10 mM n=11, Asc 5, 20 and 40 mM
 802 n=12); **pH was 7.5 and 5.5 in the standard bath and pipette solutions, respectively.** At each FeCN
 803 concentration, data points were fitted (continuous lines) by the Michaelis–Menten function:
 804 $I = I_{Acmax}^{app} / (K_{Ac}^{app} / [Asc]_{cyt} + 1)$. (b and c) The apparent maximum current at saturating cytosolic Asc,
 805 I_{Acmax}^{app} (b), and the apparent affinity constant for Asc, K_{Ac}^{app} (c), both derived from the data fit in (a),
 806 are plotted as a function of external FeCN. (d) Dependence of electron currents on vacuolar FeCN
 807 ($[FeCN]_{vac}$) at different cytosolic Asc. At each Asc concentration, data points were fitted the

808 Michaelis–Menten function $I = I_{Fvmax}^{app} / (K_{Fv}^{app} / [FeCN]_{vac} + 1)$, continuous lines. (e and f) The apparent
 809 maximum current at saturating FeCN, I_{Fvmax}^{app} (f), and the apparent affinity constant for FeCN, K_{Fv}^{app}
 810 (g), both derived from the data fit in (e), are plotted as a function of the cytosolic Asc. Continuous
 811 lines in (b and c) and (e and f) were obtained by a global fit of the data using Equation 1, see Methods
 812 and Table 1.

813

814 **Figure 3: Vacuolar ascorbate elicits electron currents in the presence of cytosolic FeCN.**

815 (a) Examples of current recordings upon the addition of 100 μ M cytosolic FeCN in different pipette
 816 (vacuolar) solutions (top: control, middle: control with 5 mM Asc, bottom: control with 5 mM DTT);
 817 standard bath and pipette solutions with symmetrical pH of 7.5. (b) Histogram of the current
 818 amplitudes evoked by 100 μ M cytosolic FeCN in the same vacuolar conditions shown in (a). Current
 819 amplitudes were normalised to membrane capacitance (control: n=6, Asc: n=7, DTT: n=4). Statistical
 820 significance (according to Student's t-test): ***P < 0.001.

821

822 **Figure 4 Electron currents depend on vacuolar ascorbate and cytosolic FeCN concentrations.**

823 (a) Current trace recorded in the presence of 5 mM vacuolar Asc in response to increasing cytosolic
 824 FeCN (in micromolar). (b) Dependence of electron currents on cytosolic FeCN at different vacuolar
 825 Asc concentrations (1 mM: n=3 (FeCN 3.3 and 100 μ M) and n=4 (FeCN 10 and 33 μ M); 5 mM: n=5
 826 (FeCN 3.3), n=6 (FeCN 10 and 33 μ M) and n=7 (FeCN 100 μ M); 20 mM: n=4; 40 mM: n=4 (FeCN
 827 3.3 and 10 μ M) and n=6 (FeCN 33 and 100 μ M)); pH in the standard bath and pipette solutions was
 828 symmetric and equal to 7.5. At each FeCN concentration, data points were fitted (continuous lines)
 829 by the Michaelis–Menten function: $I = I_{Fcmax}^{app} / (K_{Fc}^{app} / [FeCN]_{cyt} + 1)$. (c and d) The apparent maximum
 830 current at saturating cytosolic FeCN, I_{Fcmax}^{app} (c), and the apparent affinity constant for FeCN, K_{Fc}^{app}
 831 (d), both derived from the data fit in (b), are displayed as a function of cytosolic FeCN. (e)
 832 Dependence of electron currents on vacuolar Asc at different cytosolic FeCN. At each FeCN
 833 concentration, data points were fitted (continuous lines) by the Michaelis–Menten function
 834 $I = I_{Avmax}^{app} / (K_{Av}^{app} / [Asc]_{vac} + 1)$. (f and g) The apparent maximum current at saturating Asc, I_{Avmax}^{app} (f),
 835 and the apparent affinity constant for vacuolar Asc, K_{Av}^{app} (g), both derived from the data fit in (E),

are plotted as a function of cytosolic FeCN. Continuous lines in (c and d) and (e and f) were obtained by a global fit of the data using Equation 1, see Methods and Table 1.

838

Figure 5: Vacuolar acidification is associated with electron transport elicited by vacuolar ascorbate and cytosolic FeCN.

(a) Increase of cytosolic FeCN induced a significant drop of vacuolar pH, which was recorded by the proton-sensitive probe BCECF added at 10 μ M in the standard pipette (vacuolar) solution (upper panel, see Methods). Vacuolar Asc was 5 mM; standard bath solution at pH 7.5. The lower panel shows synchronised patch-clamp recordings at a holding potential of 0 mV. Currents increased upon addition of cytosolic FeCN. (b) Histogram of pH changes elicited by FeCN in the presence and absence of vacuolar Asc (Asc 5 mM: n=3 (FeCN 3.3 μ M), n=4 (FeCN 10 μ M), n=6 (FeCN 100 μ M); without vacuolar Asc: n=4 (FeCN 100 μ M). Statistical significance (according to Student's t-test): ***P < 0.001.

849

Figure 6: CYB561A mutant plants lack the electron currents elicited by ascorbate and FeCN.

(a) Both *cyb561a-1* and *cyb561a-2* mutant lines, upon application of 10 mM cytosolic Asc (horizontal bar), in the presence of 1 mM vacuolar FeCN, did not show any current change. (b) Current amplitudes, normalized to membrane capacitance, elicited by 10 mM cytosolic Asc in vacuoles from wild-type (WT: n=9) and mutant plants (*cyb561a-1*: n=9, *cyb561a-2*: n=8); pH was 7.5 and 5.5 in standard bath and pipette solutions, respectively.

(c) In the opposite experimental configuration, addition of 100 μ M cytosolic FeCN (horizontal bar), with vacuolar Asc from 5 to 40 mM, did not induce electron currents in mutant lines. (d) Mean current amplitudes in vacuoles from wild-type (WT: n=24) and mutant plants (*cyb561a-1*: n=18, *cyb561a-2*: n=9); standard bath and pipette solutions with symmetrical pH equal to 7.5. Statistical significance (according to Student's t-test): ***P < 0.001.

861

Figure 7: MDHA is an electron acceptor of vacuolar CYB561A.

863 a) Scheme of the experiment: Asc and AO solutions indicate the cytosolic solutions containing
 864 Ascorbate and Ascorbate Oxidase, respectively. At the interface of the two solutions, the interaction
 865 between Asc and AO generated MDHA, which subsequently dismutates to Asc and
 866 dehydroascorbate (DHA). MDHA, whose concentration is predicted to depend on both the reaction
 867 rate of AO and the dismutation rate of MDHA, could reach the vacuole with the perfusion flow. (b)
 868 Currents elicited by application of cytosolic MDHA (horizontal bars) in vacuoles from wild type (WT)
 869 and mutant (*cyb561a-1* and *cyb561a-2*) Arabidopsis plants. Holding voltage of 0 mV. Gaps in the
 870 current traces correspond to the application of 200 ms voltage ramps from -80 to +80 mV (not shown
 871 for the sake of clarity; see Suppl. Fig. 6). The vacuole was consecutively perfused with a bath solution
 872 containing Ascorbate oxidase at a concentration of 2 u/ml and then changed with a solution
 873 containing 0.5 mM Asc. MDHA was generated at the interface of the two solutions. (c) Current
 874 amplitudes, normalized to membrane capacitance, elicited by MDHA in vacuoles from wild-type (WT:
 875 $n=7$) and mutant plants (*cyb561a-1*: $n=4$, *cyb561a-2*: $n=5$). (d) Electron currents versus Asc used to
 876 generate MDHA in the cytosolic bath solution. Vacuoles were consecutively perfused with a bath
 877 solution containing 2 u/ml of Ascorbate oxidase (AO-solution) and then changed with bath solution
 878 containing different concentrations (Asc 0.03 mM $n=3$, Asc from 0.1 to 5 mM $n=7$ and Asc 20 mM;
 879 $n=4$) of Asc (Asc-solution); standard bath and pipette solutions with symmetrical pH equal to 7.5.

880

881 **Fig. 8. Phenotyping of wild-type and *cyb561a* mutant plants.**

882 (a and b) Plants ($n=45$) were grown for 14 days on agarized MS1/2 medium plates in control
 883 conditions ($100 \mu\text{E m}^{-2} \text{ s}^{-1}$). Asc (colored bars) and DHA (white bars) were measured
 884 spectrophotometrically (a), and ROS were qualitatively assessed with the NBT staining (b). (c and
 885 e) Plants were grown for 48 days on soil in control conditions and then exposed to HL ($700 \mu\text{E m}^{-2}$
 886 s^{-1}) for 4, 7 and 11 days. Asc (colored bars), DHA (white bars) (c) and anthocyanins (d) were
 887 determined spectrophotometrically. (a, c, d) Values are reported as means $\pm$ SE obtained from $n=3$
 888 (panel a) or $n=4$ (panel c) independent biological replicates. Statistical analyses performed with 1-
 889 way completely randomized ANOVA, using the Tukey's HSD test with significance level of 0.05. In

890 panels a and c, ANOVA was run on Asc and Asc+DHA data independently. Letters in the bars refer
891 to Asc data, letters above the bars refer to Asc+DHA data.
892

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893 Supplemental Figure legends

894

895 Supplemental Figure 1 Voltage dependence of electron currents elicited by cytosolic 896 ascorbate and vacuolar FeCN.

897 (a) Membrane currents elicited by a series of voltage steps ranging from -60 to +60 mV in 20 mV
898 steps were recorded in control condition (con, red traces), in the presence of 10 mM cytosolic Asc
899 (+Asc, blue traces) and after Asc washout (rec, green traces). The pH of cytosolic solution was 7.5.
900 The pipette solution, which is present inside the vacuole, was without FeCN at pH 5.5 in the top
901 panel, with FeCN 1 mM at pH 5.5 in the middle panel and with FeCN 1 mM at pH 7.5 in the bottom
902 panel. (b) I-V relationships of steady-state currents of data shown in a. (c) Current density versus
903 voltage elicited by 10 mM cytosolic Asc (cytosolic pH was 7.5) in different vacuolar solutions: at pH
904 5.5 (light red bars, n=4), after addition of 1 mM FeCN at pH 5.5 (dark blue bars, n=7) and after
905 addition of 1 mM FeCN at pH 7.5 (grey bars, n=4). Current density at a fixed voltage was obtained
906 by subtracting to the current elicited at 10 mM cytosolic Asc the control current recorded without Asc
907 and normalising by the vacuole capacitance. Significance (according to Student's t-test): * $P < 0.05$;
908 ** $P < 0.01$; *** $P < 0.001$. In the presence of 1 mM vacuolar FeCN and 10 mM cytosolic Asc, at luminal
909 pH 5.5 and 7.5 there was no significant difference in current densities, indicating that the activity of
910 a possible ascorbate/proton antiporter was negligible in our experimental conditions.

911

912 Supplemental Figure 2 Voltage dependence of electron currents elicited by cytosolic FeCN 913 and vacuolar ascorbate.

914 (a) Membrane currents elicited by a series of voltage steps ranging from -60 to 60 mV in 20 mV
915 steps were recorded in control condition (control, red traces), in the presence of 33 μ M FeCN (+FeCN,
916 violet traces) and after FeCN washout (recovery, green traces). Currents were recorded in the
917 absence, top panel, and presence of 5 mM vacuolar Asc. The pH of both cytosolic and vacuolar
918 solutions was 7.5. (b) I-V relationships of steady-state currents of data shown in a. (c) Current density
919 versus voltage elicited by 33 μ M cytosolic FeCN in vacuolar solutions without (light red bars, n=3),
920 or with 5 mM Asc (dark violet bars, n=3). Current density at a fixed voltage was obtained by

921 subtracting the control current recorded in the absence of cytosolic FeCN from the current elicited
 922 by 33 μ M FeCN. The difference was divided by the vacuole capacitance. Statistical significance
 923 (according to Student's t-test): ***P < 0.001.

924

925 **Supplemental Figure 3 Localization of CYB561-A by confocal microscopy.**

926 Confocal images of a representative vacuole isolated by lysis of a CYB561A:EGFP-expressing
 927 protoplast. (a) Bright field. (b) Green fluorescence of CYB561A:EGFP at the vacuolar membrane.
 928 (c) Staining of the tonoplast with the membrane marker FM4–64 applied at a final concentration of 5
 929 μ M. (d) Overlay. Scale bar: 20 μ m.

930

931 **Supplemental Figure 4 Genetic characterization of *cyb561a-1* and *cyb561a-2* T-DNA lines.**

932 (a) Map of the two T-DNA insertions (*cyb561a-1* and *cyb561a-2*) in *CYB561A* (*At4g25570*) gene.
 933 Black boxes represent exons, black lines represent introns and triangles indicate the insertion site
 934 of the T-DNA in each mutant line.

935 (b) PCR analyses performed to select homozygous T-DNA lines. Genomic DNA amplifications were
 936 carried out with the following pairs of primers: lane 1, *cyb561a-1* fw + *cyb561a-1* rev; lane 2,
 937 *cyb561a-1* fw + LBb1.3; lane 3, *cyb561a-2* fw + *cyb561a-2* rev; lane 4, *cyb561a-2* fw + LBb1.3.

938 (c) RT-PCR detection of the residual expression of *CYB561A* in both T-DNA insertion mutants and
 939 wild-type plants. Expression levels of *CYB561A* were normalized over the expression levels of *ACT8*.
 940 Quantification was performed with ImageJ. Values are reported as means $\pm$ SD obtained from three
 941 independent replicates for each genotype. Statistical analyses were performed using Student's t-
 942 test. * $p \leq 0.05$.

943

944 **Supplemental Figure 5 Vacuolar ascorbate and cytosolic FeCN did not induce vacuolar** 945 **acidification in *cyb561a-2* mutant plant.**

946 (a) The presence of 100 μ M FeCN in the cytosolic solution did not induce a significant luminal pH
 947 change in vacuoles isolated from *cyb561a-2* mutant plants (top panel). The pH was detected by 10
 948 μ M BCECF added in the pipette (vacuolar) solution (see Methods). Vacuolar Asc was 5 mM.

949 Synchronised patch-clamp recordings were shown in the bottom panel (holding potential of 0 mV).
 950 Currents were not modified upon addition of cytosolic FeCN. (b) Histogram of pH changes elicited
 951 by FeCN in the presence of 5 mM vacuolar Asc, in wild-type (WT: n=7) and *cyb561a-2* (*cyb561a-2*:
 952 n=3) plants. Statistical significance (according to Student's t-test): ***P < 0.001.

953

954 **Supplemental Figure 6 Voltage dependence of electron currents elicited by cytosolic MDHA**
 955 **and vacuolar Asc.**

956 (a) Currents elicited by voltage ramps of 200 ms from -80 to +80 mV in the presence (pink traces)
 957 and absence (black traces) of MDHA. Currents were recorded in vacuoles from wild-type (WT, left
 958 panel) and mutant (*cyb561a-1*, middle panel and *cyb561a-2*, right panel) plants. The green trace in
 959 the left panel was the recovery current recorded at the end of the experiment in the absence of
 960 MDHA. Smooth pink and black traces in the left panel were obtained by fitting the experimental
 961 currents with polynomial functions and were used to calculate the value of the current at a definite
 962 voltage. Cytosolic MDHA was generated as described in the legend of Fig. 7a; the concentration of
 963 Asc in the pipette (luminal side) was 5 mM; symmetrical pH equal to 7.5. (b) I-V relationships of
 964 currents activated by MDHA after subtraction of currents recorded in the absence of MDHA in wild-
 965 type (WT: n=7, pink bars) and mutant (*cyb561a-1*: n=4, and *cyb561a-2*: n=5, respectively red and
 966 black bars) plant. Significance (according to Student's t-test): * P<0.05; ** P<0.01; ***P < 0.001.
 967 These experiments showed that currents mediated by CYB561A due to the movement of electrons
 968 from vacuolar Asc to cytosolic MDHA were active at physiological tonoplast voltages and increased
 969 upon voltage increase.

970

971 **Supplemental Figure 7 - Asc redox state in whole leaf extracts of different genotypes and**
 972 **different growth conditions**

973 (a) The Asc redox state, expressed as Asc/(Asc+DHA) ratio, was determined in 14 days old plants
 974 grown on agarized MS1/2 medium plates in control conditions (100 $\mu\text{E m}^{-2} \text{ s}^{-1}$, 12/12 h light/dark
 975 cycle). Same experiment of Fig. 8a. Statistical analyses performed with 1-way completely

976 randomized ANOVA, using the Tukey's HSD test with significance level of 0.05 identified no
977 significant differences among genotypes.

978 (b) The Asc redox state, expressed as Asc/(Asc+DHA) ratio, was determined in plants grown for 48
979 days on soil in control conditions and then exposed to HL ($700 \mu\text{E m}^{-2} \text{s}^{-1}$) for 4, 7 and 11 days.
980 Same experiment of Fig. 8c. Statistical analyses performed with 1-way completely randomized
981 ANOVA, using the Tukey's HSD test with significance level of 0.05 identified no significant
982 differences among average values for any genotype and time point.

983
984 **Supplemental Figure 8 – Anthocyanin accumulation in high light**

985 Plants grown for 48 days on soil in control conditions and then exposed to HL ($700 \mu\text{E m}^{-2} \text{s}^{-1}$) for
986 11 days. Same experiment of Fig. 8d. Picture taken after 4 days of HL treatment already show a
987 stronger pigmentation in *cyb561a* mutant lines in respect to wild type plants.

988

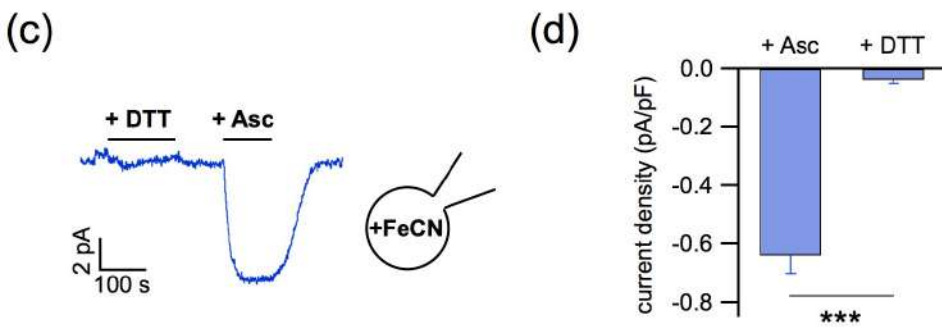
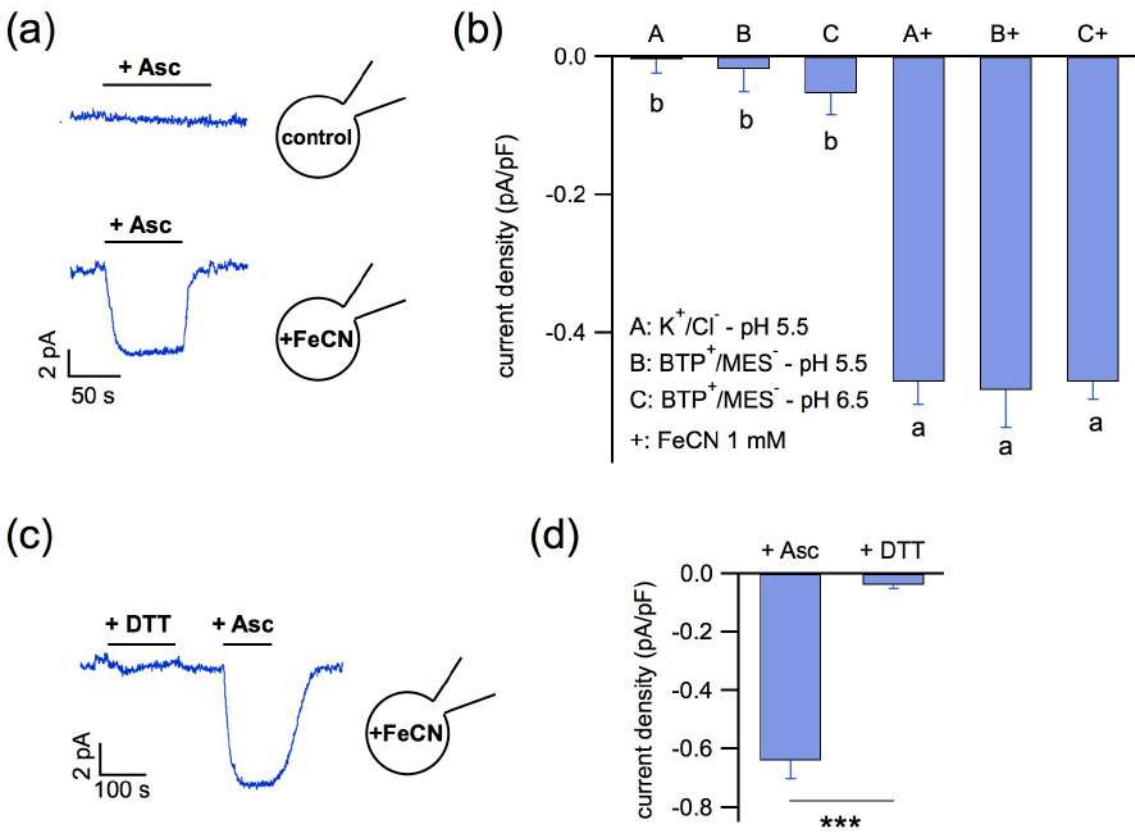


Figure 1

Review

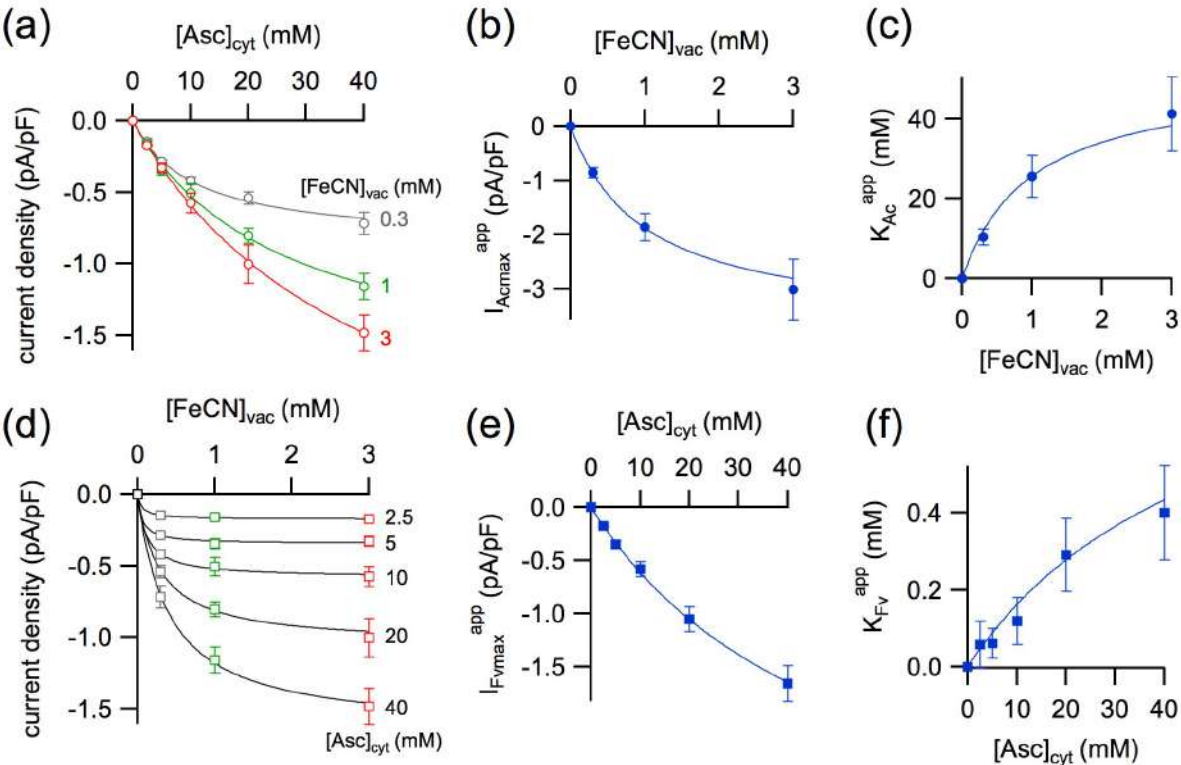


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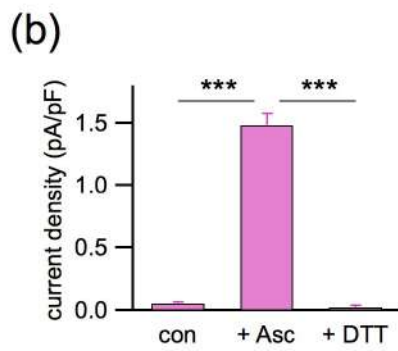
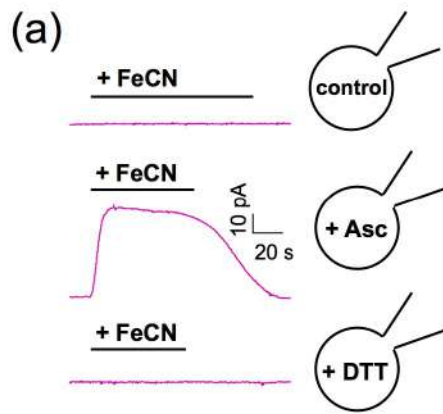


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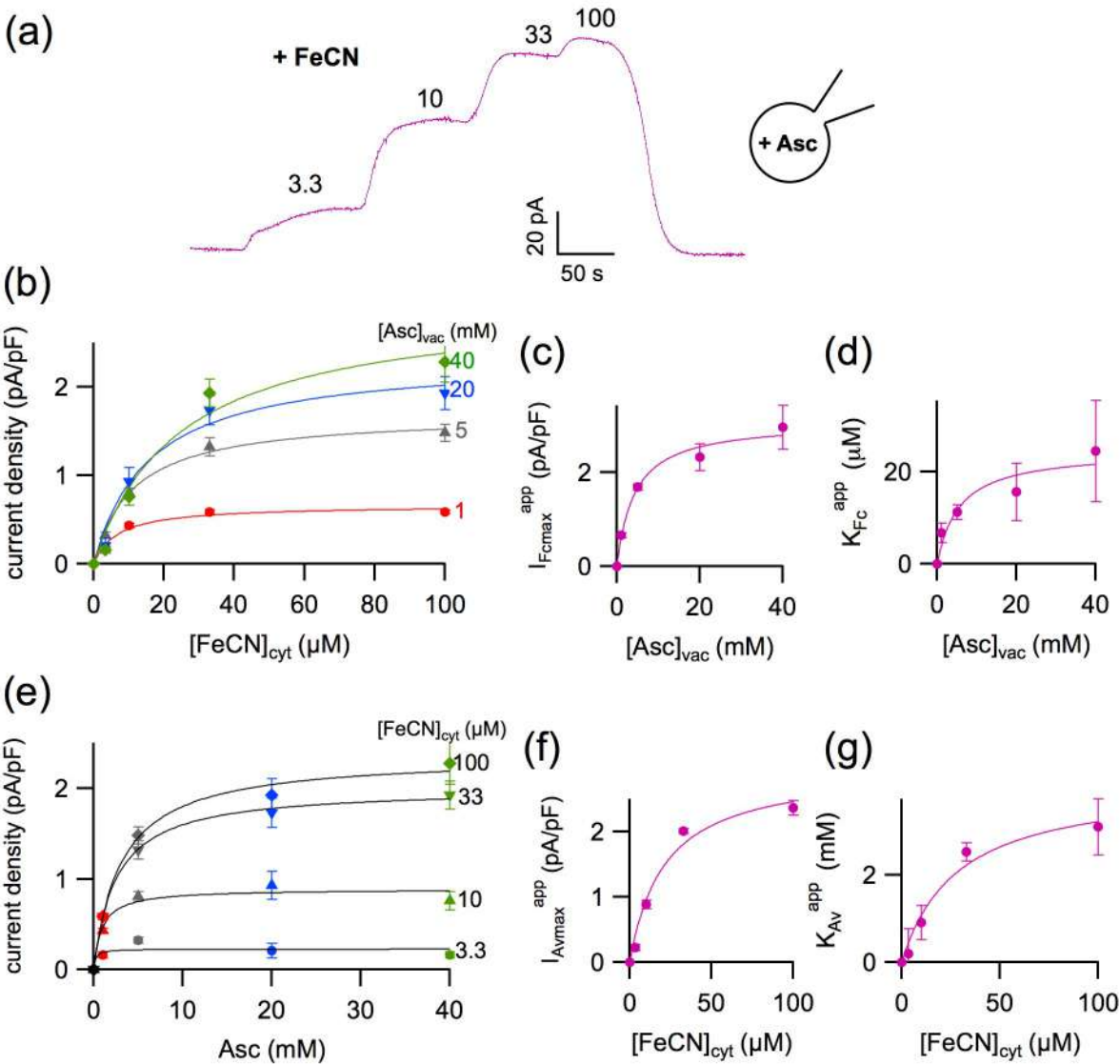


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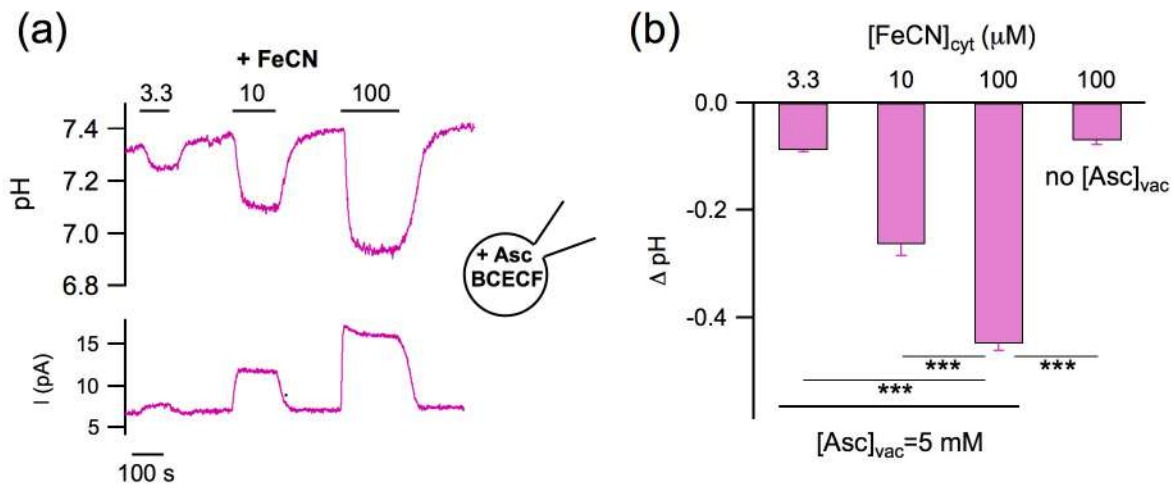


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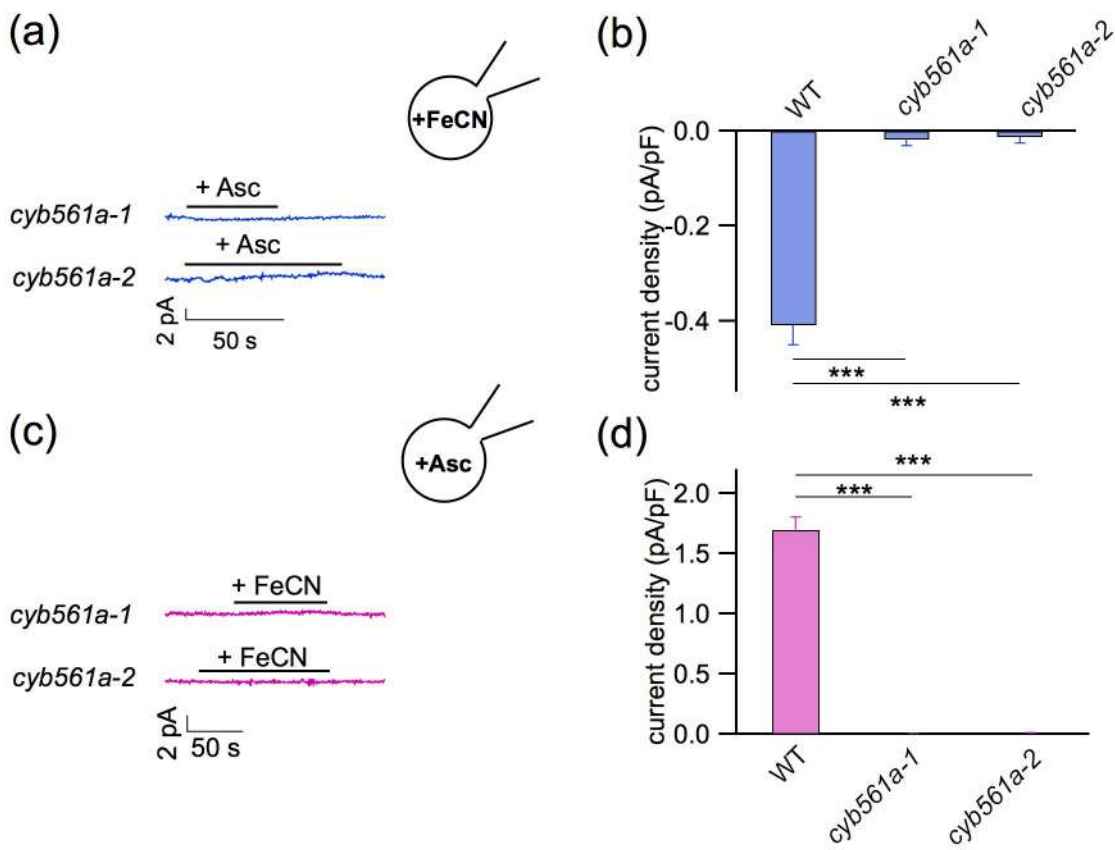


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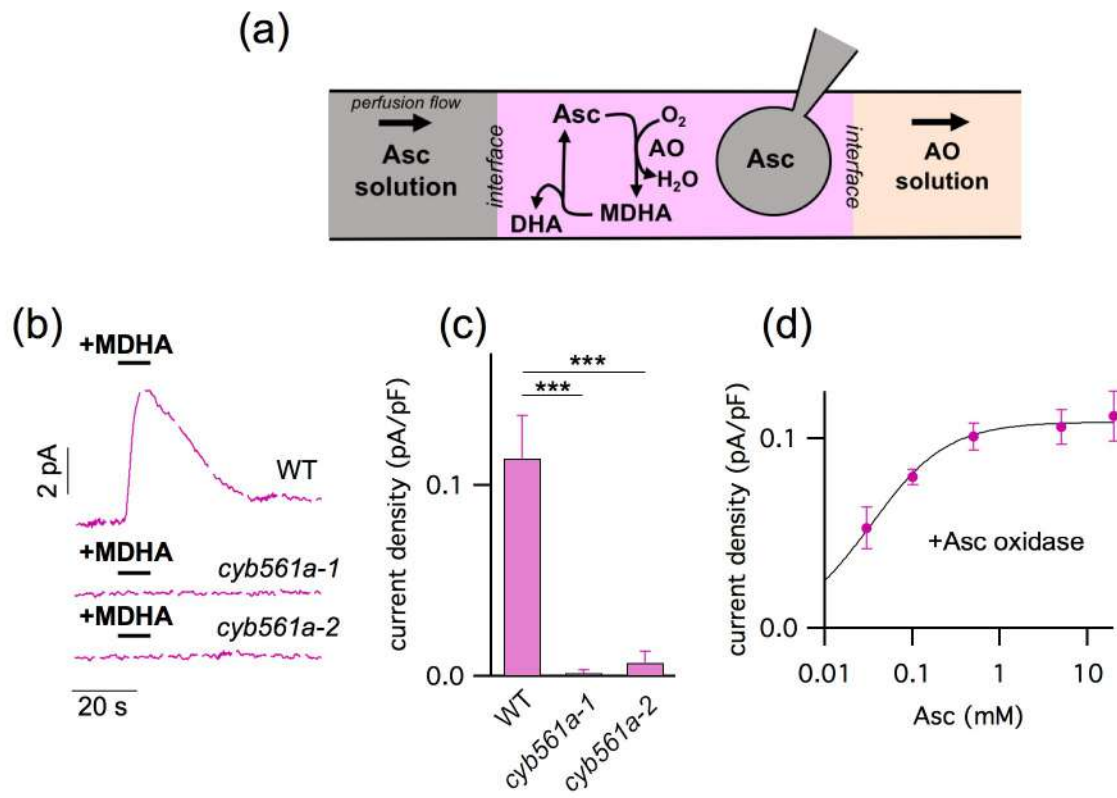


Figure 7

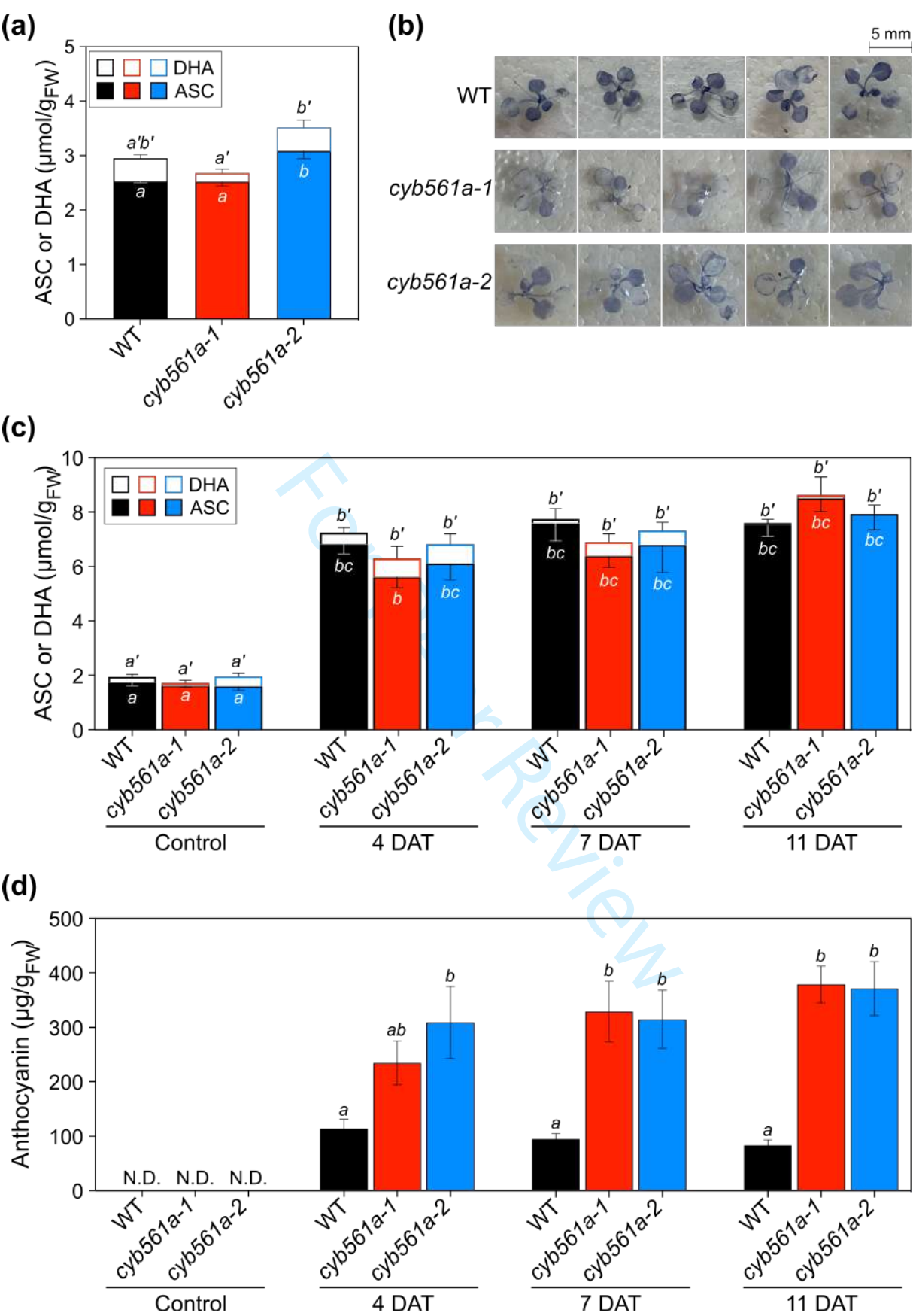
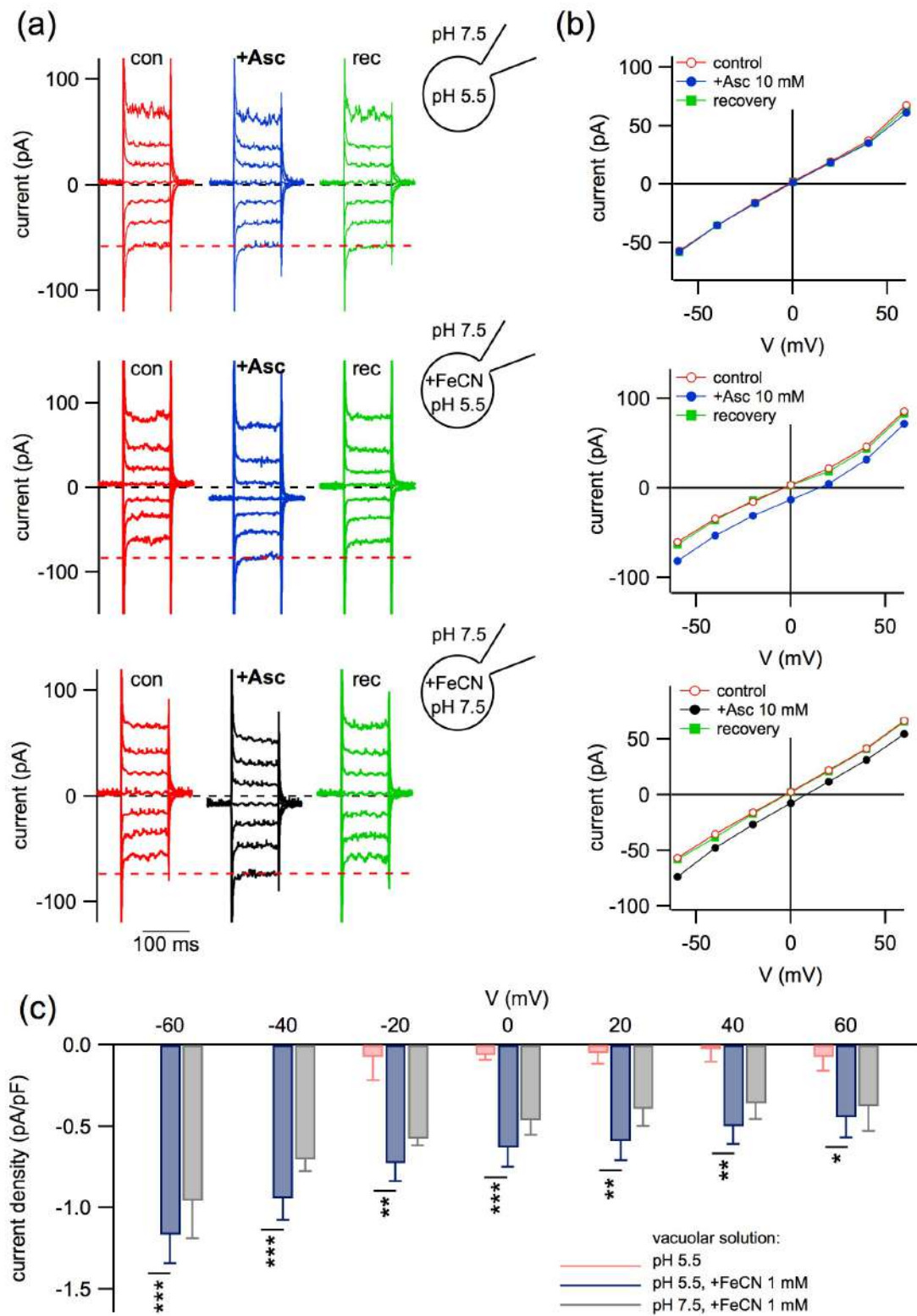
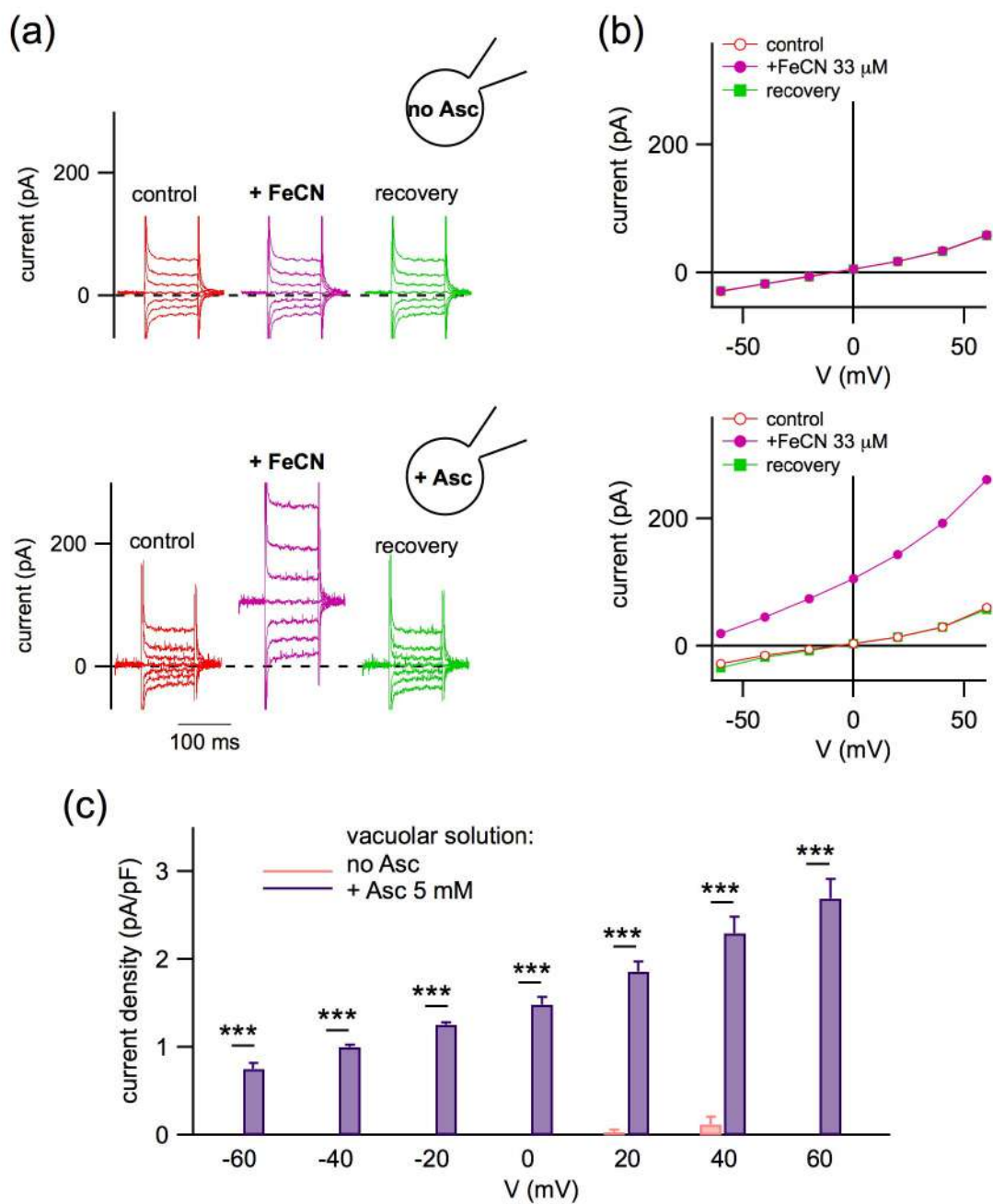


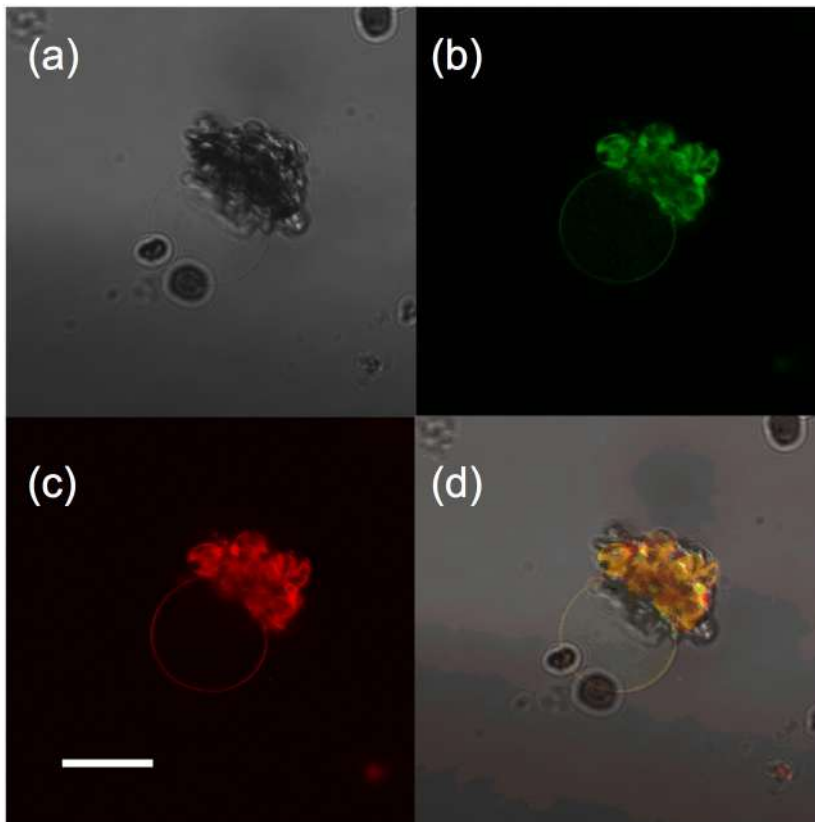
Figure 8



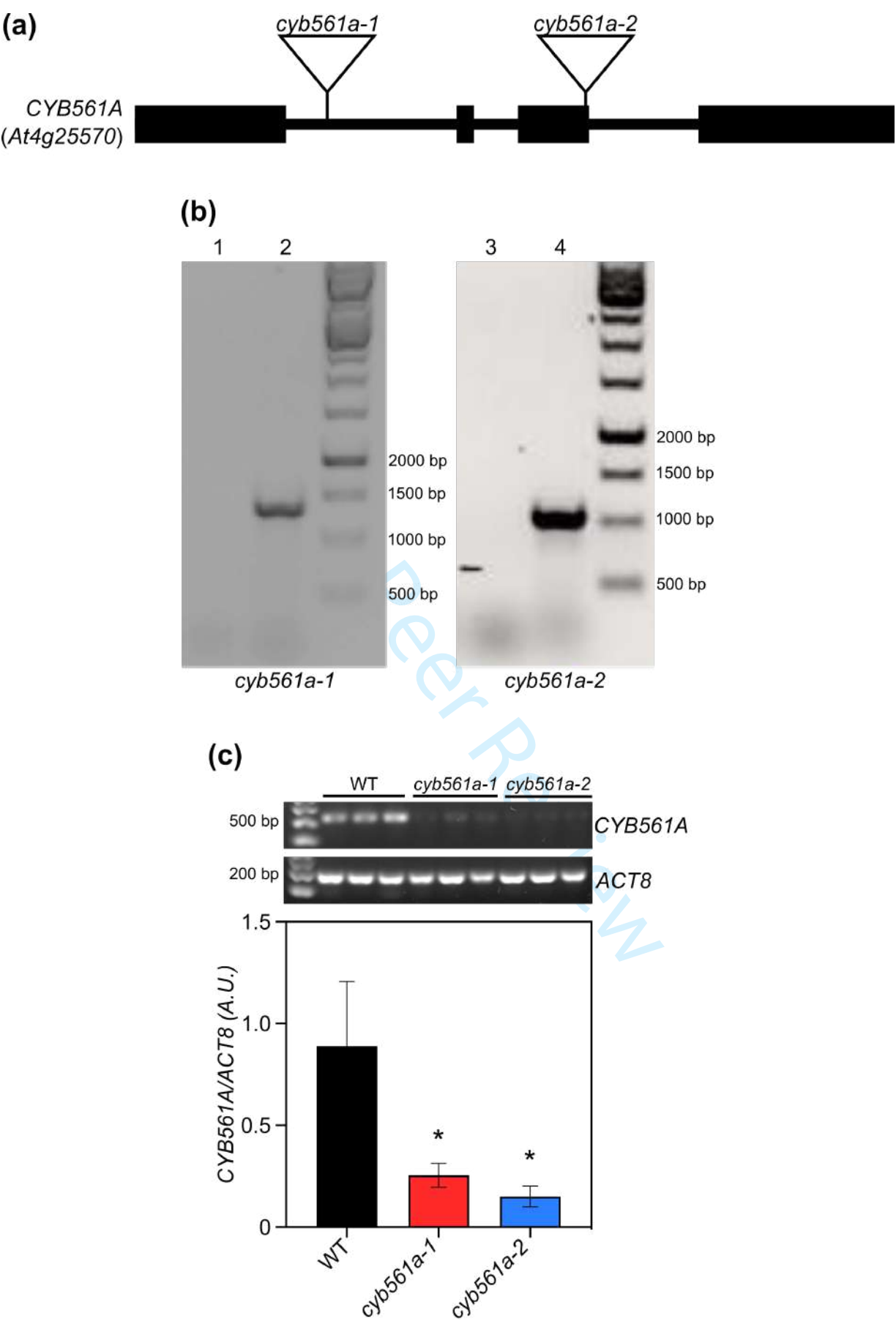
Supplemental Figure 1



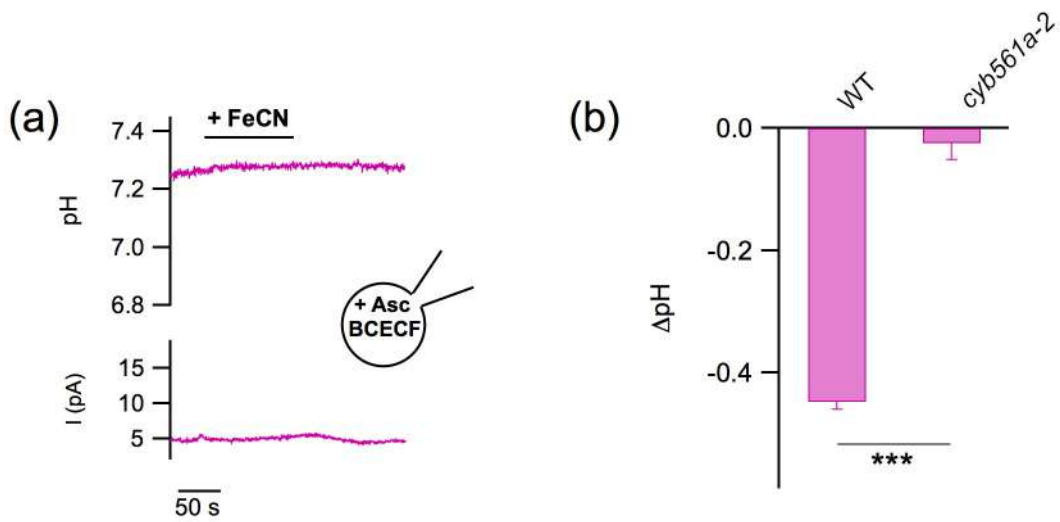
Supplemental Figure 2



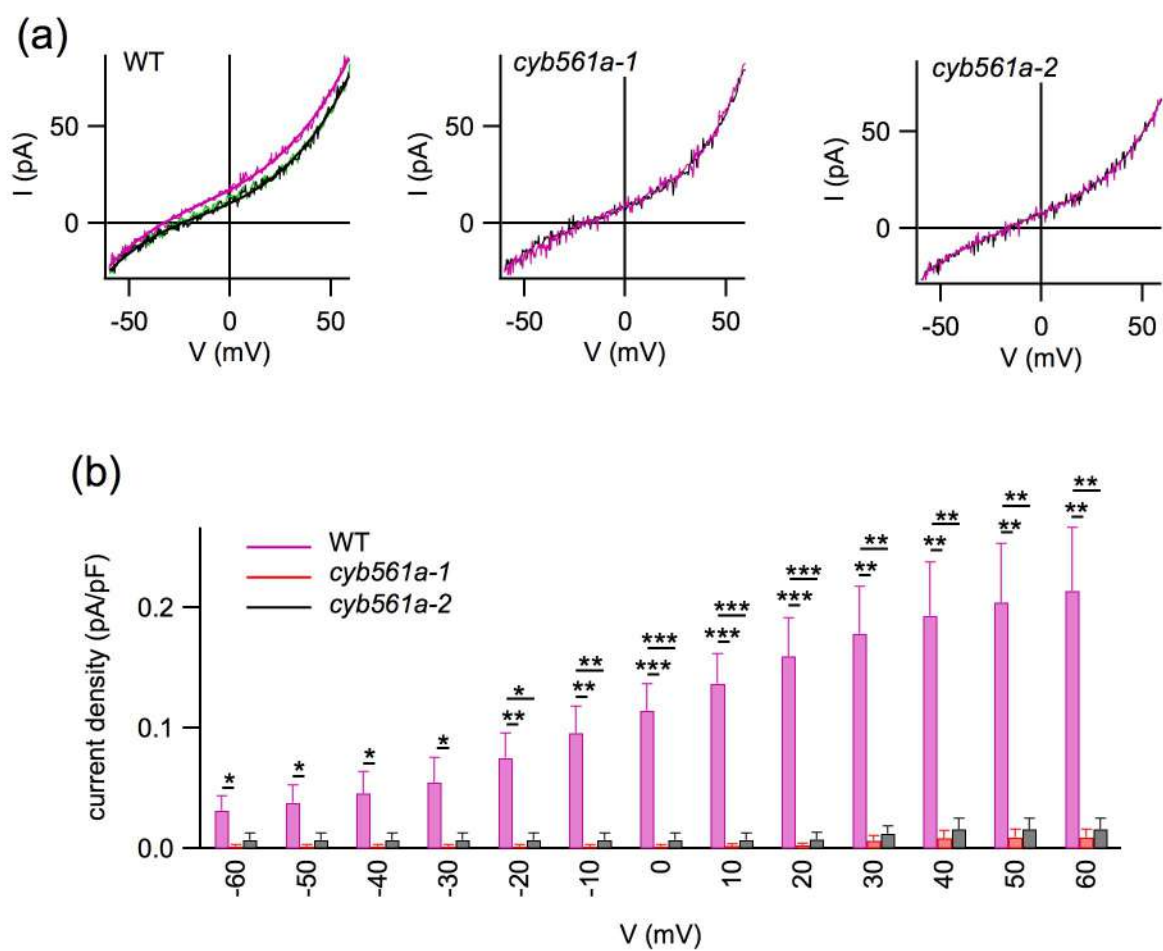
Supplemental Figure 3



Supplemental Figure 4

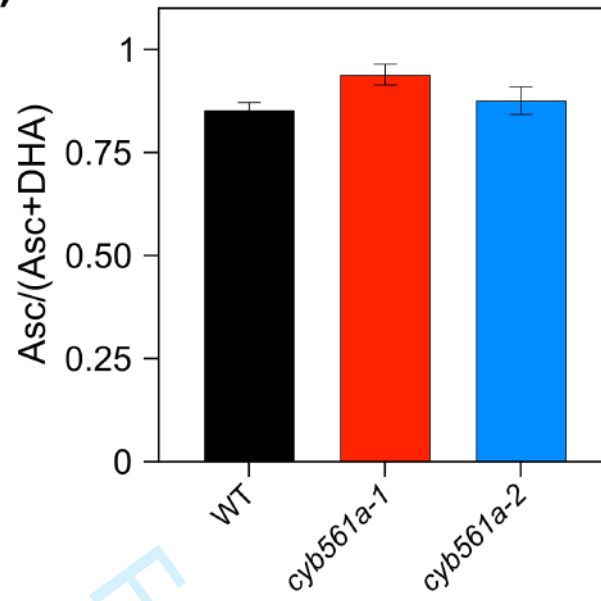


Supplemental Figure 5

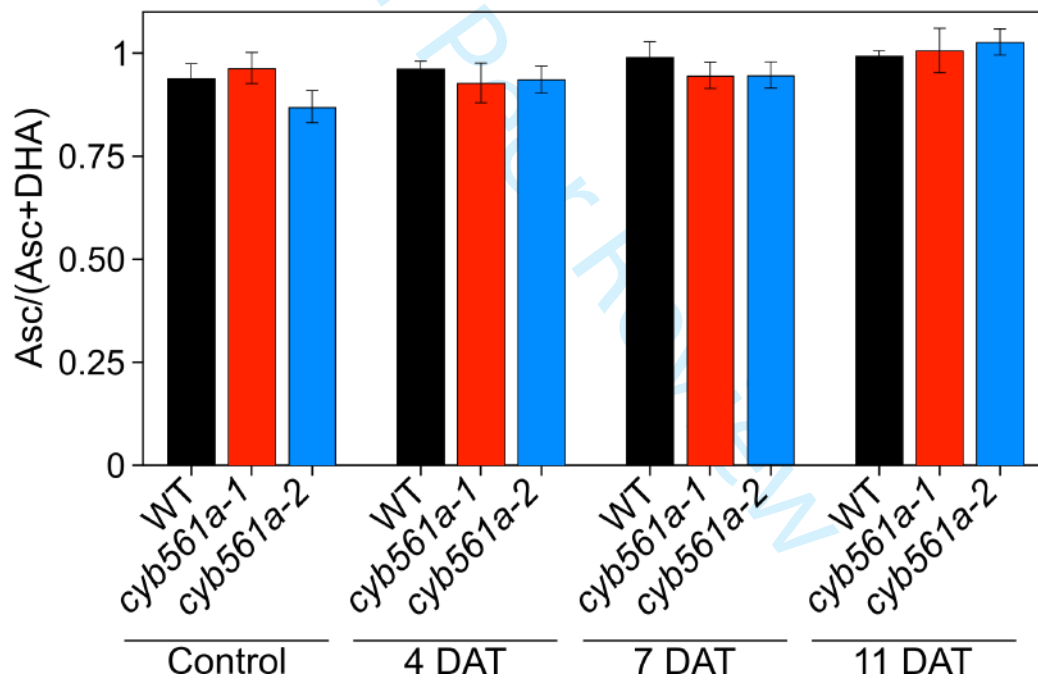


Supplemental Figure 6

(a)



(b)



Supplemental Figure 7



WT



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1035 **Supplemental Figure 8**