Alternative Splicing-Mediated Targeting of the Arabidopsis GLUTAMATE RECEPTOR3.5 to Mitochondria Affects Organelle Morphology

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Since the discovery of 20 genes encoding for putative ionotropic glutamate receptors in the Arabidopsis (Arabidopsis thaliana) genome, there has been considerable interest in uncovering their physiological functions. For many of these receptors, neither their channel formation nor their localization within the plant cells is known. Here, we provide, to our knowledge, new information about in vivo protein localization and give insight into the biological roles of the so far uncharacterized Arabidopsis GLUTAMATE RECEPTOR3.5 (AtGLR3.5), a member of subfamily 3 of plant glutamate receptors. Using the pGREAT vector designed for the expression of fusion proteins in plants, we show that a splicing variant of AtGLR3.5 targets the inner mitochondrial membrane, while the other variant localizes to chloroplasts. Mitochondria of knockout or silenced plants showed a strikingly altered ultrastructure, lack of cristae, and swelling. Furthermore, using a genetically encoded mitochondrial-targeted calcium probe, we measured a slightly reduced mitochondrial calcium uptake capacity in the knockout mutant. These observations indicate a functional expression of AtGLR3.5 in this organelle. Furthermore, AtGLR3.5-less mutant plants undergo anticipated senescence. Our data thus represent, to our knowledge, the first evidence of splicing-regulated organellar targeting of a plant ion channel and identify the first cation channel in plant mitochondria from a molecular point of view.

In vertebrates, ionotropic glutamate receptors (iGluRs in animals) are ligand-gated cation channels that mediate the majority of the excitatory neurotransmission in the central nervous system (Dingledine et al., 1999). In the model plant Arabidopsis (Arabidopsis thaliana), 20 genes encoding homologs of animal iGluRs have been identified (Lam et al., 1998). According to phylogenetic analyses, the Arabidopsis GLUTAMATE RECEPTOR (AtGLR) homologs can be subdivided into three separate subgroups (Chiu et al., 1999, 2002). Some evidence for the channel-forming ability by plant ionotropic glutamate receptors (iGLRs) has been obtained only recently, and only for AtGLR3.4 and AtGLR1.4 expressed in heterologous systems (Vincill et al., 2012; Tapken et al., 2013). Studies with transgenic plants suggested roles of members of the plant GLR family in Ca2+ fluxes (AtGLR2; Kim et al., 2001), coordination of mitotic activity in the root apical meristem (Li et al., 2006), regulation of abscisic acid biosynthesis and water balance (AtGLR1.1; Kang and Turano, 2003; Kang et al., 2004), carbon/nitrogen sensing (AtGLR1.1; Kang and Turano, 2003), resistance against fungal infection (Kang et al., 2006), leaf-to-leaf wound signaling (Mousavi et al., 2013), and lateral root initiation (Vincill et al., 2013).

Application of antagonists and agonists of animal iGluRs revealed that plant GLRs might be involved in the regulation of root growth and branching (Walch-Liu et al., 2006), in light signal transduction (Lam et al., 1998), and in the response to aluminum (Sivaguru et al., 2003). In various plant cell types, the agonists Glu- and Gly-induced plasma membrane depolarization and a rise in intracellular Ca2+ concentration that were inhibited by blockers of nonselective cation channels (NSCCs) and by antagonists of animal iGluRs (Dennison and Spalding, 2000; Dubos et al., 2003; Meyerhoff et al., 2005; Krol et al., 2007; Kwaaitaal et al., 2011; Michard et al., 2011). Furthermore, Glu-activated cation currents in patch-clamped root protoplasts were inhibited by NSCC blockers such as La3+ and Gd3+ (Demidchik et al., 2004). Therefore, it was proposed that plant iGLRs can form Ca2+-permeable NSCCs, are inhibited by animal iGluR antagonists, and might contribute to the shaping of plant Ca2+ signaling (McAinsh and Pittman, 2009).

Studies using AtGLR3.3 mutant plants showed that intracellular Ca2+ rise and membrane depolarization induced by Glu in Arabidopsis hypocotyls and root cells are correlated with the presence of AtGLR3.3 (Qi et al., 2006; Stephens et al., 2008).
However, most plant iGLRs, when expressed in heterologous systems, do not give rise to any current (e.g., in *Xenopus* spp. oocytes) or are toxic to host cells (e.g., in mammalian cells; Davenport, 2002). Recently, to examine whether AtGLR homologs possess functional ion channel domains, Tapken and Hollmann (2008) transplanted the pore loop together with two adjacent intracellular loops of 17 AtGLR subunits into two rat iGLR subunits and tested the resulting chimeric receptors for ion channel activity in the heterologous expression system *Xenopus* spp. oocyte. They showed that AtGLR1.1 and AtGLR1.4 have functional ion pore domains. The AtGLR1.1 pores are permeable to Na\(^+\), K\(^+\), and Ca\(^{2+}\) and are blocked by the nonspecific cation channel blocker La\(^3+\) (Tapken and Hollmann, 2008). Recent work has demonstrated that the expression of full-length AtGLR1.4 in oocytes gives rise to an amino acid-activated, nonselective, calcium-permeable channel that was found to be inhibited by the animal iGluR modulators 6,7-dinitroquinoxaline-2,3-dione and 6-cyano-7-nitroquinoxaline-2,3-dione (Tapken et al., 2013).

The study of these channels has so far been restricted to those members that are located in the plasma membrane and were proved to be functional in the expression systems used. Instead, various localization prediction tools suggest that some of the plant GLRs might have chloroplast and mitochondriod targeting. In general, determining the subcellular localization of a protein is an important step toward understanding its function. We recently reported the localization of GLR3.4 to the inner chloroplast membrane (Teardo et al., 2011), which was also shown to harbor a 6,7-dinitroquinoxaline-2,3-dione-sensitive, calcium-permeable channel activity (Teardo et al., 2010). No other studies have addressed the eventual subcellular localization of other putative Glu receptors.

In this work, we show that an isoform of GLR3.5 is efficiently targeted to the mitochondria. Functional expression of the channel in this organelle is indicated by the fact that its absence in knockout plants leads to a dramatically altered ultrastructure of mitochondria that impacts the plant physiology, ultimately leading to an anticipated senescence.

**RESULTS**

**Cloning of the Two Splicing Variants of AtGLR3.5**

The Arabidopsis Glu receptor AtGLR3.5 is encoded by the At2g32390 gene that is transcribed in two splicing variants, NM_128798 (isoform 1) and NM_001036387 (isoform 2), corresponding to the gene models At2g32390.1 and At2g32390.2, respectively. The 5\’ sequence is affected by the splicing, with the consequent modification of the putative targeting peptide between long (isoform 1) and short (isoform 2) translated proteins (Fig. 1A). Although a third gene model has been generated in The Arabidopsis Information Resource, only two isoforms have been demonstrated to be expressed so far. Their mRNA sequences correspond to accession numbers AF170494 and AY495449.

At the protein level, isoform 1 (NP_565743.1) shows a putative signal peptide for the localization to the mitochondria that is missing in isoform 2 (NP_00103464.1; Aramemnon database [http://aramemnon.uni-koeln.de] and ChloroP [Emanuelsson et al., 2007]). To confirm the predicted localization of the two isoforms to the respective organelles, we isolated and cloned the complementary DNAs (cDNAs) corresponding to transcripts NM_128798 and NM_001036387 from leaf RNA by reverse transcription (RT)-PCR using the primers listed in Supplemental Table S1. As the sequence recognized by the primers, corresponding to the beginning of the coding sequence of isoform 1, is also present at the 5\’ untranslated region of the isoform 2 transcript, the PCR product comprised both cDNAs. We designed a primer spanning the nine nucleotides in positions 150 to 158 of isoform 2 that are missing in isoform 1 to discriminate between the two isoforms. Thus, the *Escherichia coli* clones harboring the two different isoforms have been separated by PCR.

AtGLR3.5 Isoform 1 Is Located in the Mitochondria and Isoform 2 Targets Chloroplasts

The coding sequences of the two isoforms have been cloned into binary vectors developed in our laboratory (Carraretto et al., 2013; pGREAT-2x35S-EGFP and pGREAT-2x35S-DsRed2) and transformed into *Agrobacterium tumefaciens* strain GV3101 for subsequent Arabidopsis leaf agroinfiltration. Figure 1B shows the targeting of isoform 1 (GLR3.5v1) to highly motile structures in the cytoplasm resembling mitochondria visualized using mitochondria-targeted mCherry (Nelson et al., 2007), both for size and motility (Fig. 1B; Supplemental Movies S1 and S2). The fluorescence of the GFP-fused short isoform (GLR3.5v2) instead colocalizes with the autofluorescence of chlorophyll in the chloroplasts (with a Pearson coefficient greater than 0.8 at the chloroplast level; Fig. 1B). The relative expression of the mitochondrial isoform over the plastidial one was assessed in several tissues (Fig. 2A), showing an overall lower expression level for the mitochondrial GLR3.5.

Knocking Out and Silencing of the At2g32390 Gene Alters the Mitochondria Ultrastructure and Dimension

Two knockout homozygous mutant lines have been obtained from the Nottingham Arabidopsis Stock Centre: N656359 (SALK_035264C, atglr3.5-1) and N661846 (SALK_023880C, atglr3.5-2). Both lines have been tested for kanamycin resistance, transfer DNA insertion, and the absence of At2g32390 gene expression. Using different primer combinations to discriminate between the two isoforms, we observed that only the atglr3.5-1 mutant lacks the transcripts (Fig. 2B).

Given that isoform 1 is the first GLR located in the mitochondria, we focused our attention on elucidating the physiological role of AtGLR3.5 in this organelle. First, we selected a homozygous knockout line (N656359, N661846).
SALK_035264C, atglr3.5-1) as the unique line not expressing the gene (Fig. 2B). We then checked the presence or absence of the protein in the mitochondria isolated from wild-type or knockout plants using an antibody, which is able to specifically recognize the recombinant AtGLR3.5 (Fig. 2C) with an apparent molecular mass of approximately 90 kD, which is close to the predicted apparent molecular mass. We clearly detected an approximately 88-kD band in the isolated mitochondria (enriched in cytochrome c; Supplemental Fig. S1) from wild-type Columbia-0 (Col-0) plants, while the protein, as expected, was completely missing in the mutant mitochondria (Fig. 2D).

In order to assess the localization of GLR3.5 within the mitochondria and its membrane topology, we used GLR3.5v1 in fusion with a pH-sensitive ratiometric probe, SypHer. The mitochondria-targeted version of this probe has been reported to monitor dynamic changes in the mitochondrial pH gradient in HeLa cells (Poburko et al., 2011). In mitochondria, the proton...
motive force comprises an electrical component and a chemical component, the transmembrane pH gradient (approximately 0.8, alkaline inside). Thus, the observation that SypHer fused to the C terminus of GLR3.5v1 was able to sense matrix acidification induced by H+ ionophores (Fig. 3A) indicated that the C-terminal part of the protein is facing the matrix, meaning also that GLR3.5 is inserted into the inner mitochondrial membrane.

Next, the number, dimension, and motility of mitochondria were analyzed using the cationic membrane-potential sensitive dye tetramethylrhodamine methyl ester (TMRE) in roots of 10-d-old plants. No significant differences were seen in either the number or motility of these organelles when comparing wild-type (Fig. 3B; Supplemental Movie S3) and knockout (Fig. 3C; Supplemental Movie S4) or silenced (Fig. 3, D and E) plants. Posttranscriptional silencing was performed as described by Carraretto et al. (2013; Supplemental Fig. S2). Moreover, the clear TMRE detection in wild-type and knockout or silenced plants suggests that, in both cases, the mitochondria in these young plants were sufficiently energized to take up the potentiometric dye. Interestingly, in all lines lacking GLR3.5, we observed a significantly higher percentage of mitochondria with an increased size, which was found by measuring the area of each single organelle. In particular, mutant plants displayed a higher percentage of organelles with a size larger than 4 μm² (Fig. 3F).

When we investigated the ultrastructure of these lines at the cellular level by transmission electron microscopy (TEM), we observed that in 3-week-old knockout plants, part of the mitochondria displayed enlargement and partial loss of cristae (Fig. 4A). This result is in agreement with the larger size of part of the mitochondria observed with TMRE in 10-d-old root cells. At 6 weeks, this phenotype became even more pronounced: most mitochondria were less electron dense and swollen, and lacked cristae (Fig. 4B; Supplemental Fig. S3). Altered mitochondrial ultrastructure was evident also in the silenced plants (Fig. 4C; Supplemental Fig. S3). In contrast to mitochondria, no dramatic alterations were noticeable with respect to the wild type for chloroplasts, where isoform 2 is located (Supplemental Fig. S3), possibly due to the presence in these organelles of other iGLRs such as AtGLR3.4 (Teardo et al., 2010, 2011), which might compensate for the loss of function of AtGLR3.5.

**AtGLR3.5 Mutants Show Accelerated Senescence**

An altered mitochondrial size/structure is one of the characteristics of plants subjected to stress and may lead to cell death and senescence (Gan, 2007; Scott and Logan, 2008). Therefore, we investigated whether this aspect is affected in knockout plants. Mutant plants grow similarly to the wild-type plants up to the bolting stage, undergoing accelerated senescence in the subsequent phases. In fact, 5 to 6 weeks after germination, the knockout mutants showed a more pronounced decrease in chlorophyll content in older leaves compared with the wild-type plants (Fig. 5, A and B). Interestingly, we also observed an increase in the expression of the **AtGLR3.5** gene in 5-week-old wild-type plants (Fig. 5C). To prove that the observed changes were associated with senescence, the expression level of the senescence-associated gene **SAG12**, a well-known senescence marker, was assessed. In 6-week-old plants,
the expression level of SAG12 was remarkably higher in atglr3.5-1 mutants than in the wild-type plants (Fig. 5D). Both these results strongly suggest an earlier onset of senescence in knockout plants.

**Mitochondrial Calcium Uptake in atglr3.5-1 Mutants**

Given that some members of the family 3 iGLRs have been proven to allow calcium flux and that mitochondria are known to behave as calcium sinks in eu-karyotic cells (De Stefani et al., 2011; Loro et al., 2012; Rizzuto et al., 2012), our next purpose was to gain evidence that calcium fluxes take place across AtGLR3.5. Our attempts to perform patch-clamp experiments directly on mitochondrial membranes (mitoplasts [i.e. swollen mitochondria]) of Arabidopsis, however, failed because of the very small size of these mitoplasts when compared with mammalian ones (with a diameter of 2–3 μm), where this technique, although demanding, is applicable. Next, we investigated the channel activity of the cell-free expressed AtGLR3.5 protein (Fig. 2C) following its incorporation to the planar lipid bilayer according to the procedure already successfully applied in the case of the mammalian mitochondrial calcium uniporter (De Stefani et al., 2011). The fact that no channel activity could be observed in this in vitro system (data not shown) clearly suggests either the need of an accessory protein to ensure correct folding.
or the need of an auxiliary, unknown partner for the reconstitution of the channel activity in vitro.

Therefore, we directly compared the mitochondrial calcium uptake kinetics in root seedlings from 7-d-old wild-type and mutant plants. Given the large negative membrane potential on the matrix side, we expected calcium influx into the mitochondria through GLR3.5. We crossed wild-type and atglr3.5-1 plants with Arabidopsis Col-0 plants harboring the mitochondria-targeted, genetically encoded calcium probe Cameleon YC3.6 (Loro et al., 2012). To induce mitochondrial Ca\(^2+\) uptake, wild-type and knockout seedling roots were treated with external ATP as in previous studies (Loro et al., 2012) or auxin, known to induce cytosolic calcium increase in transition and elongation root tip cells (Monshausen et al., 2011). The ATP or auxin treatment produced mitochondrial Ca\(^2+\) uptake with peculiar dynamics for each treatment (Supplemental Fig. S4, A and E) in both genotypes, with no significant differences in the maximum mitochondrial calcium concentration ([Ca\(^2+\)]\text{mit}) reached and the kinetics of accumulation and release (Supplemental Fig. S4). Moreover, a comparison of resting [Ca\(^2+\)]\text{mit} in both genotypes did not show substantial differences (data not shown). Overall, in these young plants, no significant differences could be observed in mitochondrial calcium uptake or release. However, in 4-week-old plants, a significant reduction in the maximum [Ca\(^2+\)]\text{mit} was observed in the mutant leaves compared with wild-type

Figure 4. Mitochondria from atglr3.5-1 and silenced plants show altered ultrastructure. A and B, TEM scans of mitochondria from 3-week-old (A) and 6-week-old (B) wild-type (wt) Col-0 and knockout plants. Representative images from wild-type (top rows) and knockout plants (atglr3.5; bottom rows) are shown. C, Mitochondria from nonsilenced and two independent silenced lines.
plants challenged with wounding stress (Supplemental Fig. S5, A and B), a stimulus known to induce steep cytosolic calcium increases in the cells surrounding the damaged area (Beneloujaephajri et al., 2013; Steinhorst and Kudla, 2013). Nevertheless, it has to be pointed out that even if the maximum calcium peak was different, this was the only observable phenotype, since no differences were observed in the calcium recovery phase (Supplemental Fig. S5).

**DISCUSSION**

We present, to our knowledge, the first known findings that one of the members of the iGLRs, AtGLR3.5, is located in the bioenergetic organelle mitochondria in plants. AtGLR3.5 is transcribed into two different splicing variants, one encoding for a protein displaying a clear N-terminal mitochondrial targeting peptide (see Aramemnon database [http://aramemnon.uni-koeln.de]) and the other harboring a predicted chloroplast-targeting peptide (ChloroP). Indeed, localization experiments show a dual targeting of AtGLR3.5 when expressed in a homologous system (i.e. agroinfiltrated Arabidopsis leaves). Several proteins are targeted to both mitochondria and chloroplasts because they have an ambiguous signal that can be recognized by both import systems (Silva-Filho, 2003). Dual targeting by ambiguous signals has been proposed to occur by combining distinct targeting instructions in a
single N-terminal peptide (Karniely and Pines, 2005). Instead, we show that dual targeting of AtGLR3.5 is guided by alternative splicing. Please note that, due to the low abundance and high hydrophobicity, localization of ion channels in subcellular membranes by proteomic analysis proved to be feasible in only very few cases. To our knowledge, this is the first example of dual targeting achieved by this mechanism in the case of a plant ion channel. Among membrane proteins, a mitochondrial and chloroplastic dual-targeted ATP/ADP transporter of the mitochondrial carrier family in maize (Zea mays) and Arabidopsis, AtBT1 (for BRITTLE1), was shown to be localized to the inner plastidial envelope and mitochondria (Kirchberger et al., 2008; Bahaji et al., 2011). AtBT1, localized to the mitochondria, was found to be important for development and growth (Bahaji et al., 2011), but the function of the protein localized to the inner plastidial envelope remains unclear. Recently, a unique dual-targeted mitochondrial carrier protein, 3'-PHOSPHO-ADENOSINE 5'-PHOSPHOSULFATE TRANSPORTER1, was identified in rice (Oryza sativa) and its role in chloroplast development was proven (Xu et al., 2013). In our case, while the lack of AtGLR3.5 leads to clear changes in mitochondrial physiology, chloroplasts of knockout plants are normally developed with intact and stacked thylakoids present, even in the plants (3 weeks old) where mitochondrial changes were already visible. Chloroplasts have been shown previously to harbor AtGLR3.4 (Teardo et al., 2011); therefore, this latter iGLR likely overacts the function of the chloroplast isoform of AtGLR3.5. In mammalian systems, several ion channels are located in multiple membranes within the cell (Köttgen et al., 2005; Shoshan-Barmatz and Israelson, 2005; Szabó and Zoratti, 2014). Furthermore, it is important to note that an N-terminal 5-phosphate Glu receptor has been found in the mitochondrial inner membrane in a hypothalamic neuronal cell line (Korde and Maragos, 2012).

Our results, using knockout and silenced Arabidopsis, provide evidence for the role of AtGLR3.5 in plant physiology. In particular, mitochondria lacking this putative channel show a strikingly compromised ultrastructure with altered cristae morphology and an overall increase in volume. Ion channels in the inner mitochondrial membrane are known to impact mitochondrial volume (Szabó and Zoratti, 2014). Therefore, the observed mitochondrial shape in the knockout plants suggests a localization of AtGLR3.5 in the inner membrane. Experiments using GLR3.5-SypHer are also indicative of such localization. These experiments also suggest that the Glu-recognition part of the channel is oriented versus the cytosol. In both organelles, solute pores in the outer membranes are likely to mediate the flux of Glu, an important metabolite, thereby allowing the binding of Glu to inner mitochondrial membrane GLR3.5 and regulation of its activity. Our data suggest that the lack of this channel causes impairment in ion homeostasis that leads to a stress-like response and mitochondrial swelling. A similar mitochondrial ultrastructure has been observed in different plant species subjected to anaerobic treatment (Vartapetian et al., 2003). In this case, the authors interpreted their findings as the consequence of decreased metabolism (i.e. oxidative phosphorylation). Swelling was observed also upon treatment of Arabidopsis leaves with agents known to cause oxidative stress (hydrogen peroxide, paraquat, and menadione; Yoshinaga et al., 2005). Similarly, swollen mitochondria with altered cristae structure have been observed in chilled cucumber (Cucumis sativus) roots (Lee et al., 2002). Chill-sensitive plants undergo excessive oxidative stress, which, according to the interpretation of the authors, causes the ultrastructural changes observed in mitochondria. In this context, the observed accelerated senescence in 6-week-old plants might be a consequence of impaired mitochondrial integrity. The observation that GLR3.5 transcript level increases in older wild-type plants might indicate that AtGLR3.5 is required to maintain mitochondrial integrity and ATP production until late phases of senescence. It will be possible to clarify the exact link between the lack of AtGLR3.5, the mitochondrial changes, and the anticipated senescence only once the nature of the ions flowing through this protein and the regulation of channel activity are understood. Unfortunately, as mentioned, our attempts to perform patch-clamp experiments on isolated Arabidopsis mitochondria using a protocol that was previously successfully applied for wheat (Triticum aestivum) germ mitochondria (De Marchi et al., 2010) failed. In addition, attempts to record channel activity using the cell-free expressed protein incorporated into an artificial membrane performed as described previously (De Stefani et al., 2011) suggest that an additional, unknown component is necessary to reconstitute the functional AtGLR3.5 channel.

Because the previously studied members of the plant iGLRs of family 3 are permeable to Ca$$^{2+}$$, we measured calcium dynamics with the genetically encoded calcium probe Cameleon targeted to mitochondria in wild-type and knockout plants. A significant difference in the maximum mitochondrial calcium accumulation could be observed only in 4-week-old plants challenged with wounding (used here just as a stimulus known to induce a steep cytosolic calcium increase in intact unstressed leaves). Nevertheless, the maximum peak was indeed the only difference, since the calcium recovery phase was not affected by the absence of GLR3.5. This might be a priori directly related to the lack of AtGLR3.5 but also might be due to an indirect effect (e.g. to a decreased driving force for the flux of calcium toward the matrix). Please note that other calcium-uptake pathways are likely to exist in mitochondria (Stael et al., 2012), including several isoforms of the mitochondrial calcium uniporter. Unfortunately, no experimental evidence is available about these alternative calcium-uptake pathways. However, it cannot be excluded that, in the early stages of plant development, the lack of AtGLR3.5 is counterbalanced by mitochondrial calcium uniporter function. Alternatively, the function of AtGLR3.5 for mitochondrial physiology might be decisive in a more
advanced state of development and/or under stress conditions like those linked to aging or wounding. Swollen mitochondria, however, cannot be isolated, due to high fragility, in order to perform classical bioenergetics to assess mitochondrial physiology parameters. The observation that the expression of AtGLR3.5 increases with aging would be in accordance with the above hypothesis. In this respect, it is interesting to mention the case of stress-induced yeast (Saccharomyces cerevisiae) ortholog of the mammalian kidney disease gene Mpro17 (Sym1), a channel-forming protein (Reinhold et al., 2012) that is the yeast homolog of MPV17, a mammalian mitochondrial inner membrane protein, whose mutations are associated with and inherited autosomal mitochondrial disease characterized by neuronal disorders, growth retardation, liver failure, and hypoglycemia (e.g., Szabo and Zoratti, 2014). Sym1 knockout yeast cells show swollen mitochondria and lack of cristae especially under stress conditions, and stress in turn induces the expression of Sym1 in wild-type cells (Dallabona et al., 2010). While further work is warranted to identify the primary role of AtGLR3.5, our results indicate that, similar to Sym1, this iGLR is involved in the structural and functional stability of the inner mitochondrial membrane, thus controlling crucial mechanisms related to this compartment, including the morphology of mitochondria and the maintenance of its integrity. Furthermore, it is clear that the lack of this protein significantly impacts the physiology of the whole plant on a long-term scale, causing mitochondrial impairment and, possibly as a consequence, an anticipated senescence.

In summary, to our knowledge, this work provides evidence for the first molecular identification of a mitochondrial ion channel and provides insight into its physiological role.

MATERIALS AND METHODS

Plant Material

Arabidopsis (Arabidopsis thaliana) plants used for the experiments were grown in a growth room with controlled climatic conditions (16-h-light/8-h-dark photoperiod; relative humidity, 70%; night temperature, 18°C; and day temperature, 20°C). Seeds of Arabidopsis ecotype Col-0 were sterilized, incubated for 3 d at 4°C in the dark, and allowed to germinate in Murashige and Skoog (MS) (medium (Duchefa) and solid medium (0.8% plant agar; Duchefa) supplemented with 30 g L⁻¹ Suc in a growth chamber (20°C, 16-h-light/8-h-dark photoperiod, and 70% relative humidity). Seedlings were picked and supplemented with 30 g L⁻¹ Suc. In this respect, it is interesting to mention the case of aging would be in accordance with the above hypothesis that the expression of AtGLR3.5 increases with

Mitochondria of Putative Mammalian Ortholog

In summary, to our knowledge, this work provides evidence for the first molecular identification of a mitochondrial ion channel and provides insight into its physiological role.
Seeding Preparation for Ca\textsuperscript{2+} Imaging

Arabidopsis seeds were surface sterilized by vapor-phase sterilization and plated on one-half-strength MS medium (MS salts, M0222 elements including vitamins [Duchefa; http://www.duchefa-biochemie.nl/] supplemented with 0.1%/v/v Suc and 2.34 mM MES, pH 6.0, and solidified with 0.8%/v/v plant agar (Duchefa). After stratification at 4°C in the dark for 3 d, seeds were transferred to the growth chamber with 16-h-light/8-h-dark cycles (70 mmol m\textsuperscript{-2} s\textsuperscript{-1}) at 20°C. The plates were kept vertically. Seedlings used for the analyses were 7 to 8 d old, which corresponds to an average root length of 3 cm. For root cell imaging, the seedlings were prepared according to Behera and Kudla (2013) in dedicated chambers and overlaid with wet cotton to continuously perfuse the root with the imaging solution (5 mM KCl, 10 mM MES, 10 mM Ca\textsuperscript{2+}, pH 5.8, adjusted with Tris). The shoot was not submerged in the solution. ATP (final concentration of 0.1 mM) was added as disodium salt to the chamber by perfusion with the same solution. Similarly, the synthetic auxin naphthaleneacetic acid was added to the perfusion chamber with a final concentration of 0.01 mM.

Time-Lapse Ca\textsuperscript{2+} Imaging Analyses

Whole leaves of 4-week-old plants and seeding roots of both genotypes (wild-type–4mt-YC3.6 and AtGLR3.5–4mt-YC3.6) expressing the mitochondria-localized Cameleon (Loro et al., 2012) were imaged in vivo by an inverted fluorescence microscope (Nikon Ti-E; Nikon; http://www.nikon.com/) with a CFI PlanApo 4×, numerical aperture 0.13, objective for entire leaves and CFI PLAN APO 20×, violet-corrected objective for roots. Excitation light was produced by a fluorescent lamp (Prior Lumen 200 PRO; Prior Scientific; http://www.prior.com) at 440 nm (436/20 nm) set to 50% (for leaves) or 20% (for roots). Images were collected with a Hamamatsu Dual CCD Camera (ORCA-D2; Hamamatsu Photonics; http://www.hamamatsu.com/). For Cameleon analysis, the fluorescence resonance energy transfer (FRET) cyan fluorescent protein (CFP)/yellow fluorescent protein optical block A11400-03 (emission 1, 483/32 nm for CFP; emission 2, 542/27 nm for FRET) with a dichroic 510-nm mirror (Hamamatsu Photonics) was used for the simultaneous CFP and cVenius acquisitions. Exposure times were 500 ms for leaves and 100 ms for roots with a four-by-four CCD binning. Images were acquired every 2 and 5 s for leaves and roots, respectively. Filter and dichroic mirrors were purchased from Chroma Technology (http://www.chroma.com/). The NIS-Element (Nikon; http://www.nis-elements.com/) was used as a platform to control microscope, illuminator, camera, and postacquisition analyses. We used forceps for wounding as described in previous studies (Benelouajehpajri et al., 2013). Regarding time-course experiments, fluorescence intensity was determined over ROIs, which correspond to the root tip zone or the region surrounding the wound area. cVenius and CFP emissions of the analyzed ROIs were used for the ratio (\textit{R}) calculation (cVenius/CFP) and normalized to the initial ratio (\textit{R}o) and plotted versus time (ΔR/\textit{R}o). Background subtraction was performed in each channel before FRET ratio calculation by selecting a ROI outside the sample, in the case of root analyses. In the case of analyses performed on leaves because imaged areas do not contain any background, the latter was not subtracted.

Semiquantitative RT-PCR

\textit{SAG12} (At5g45890) and \textit{AtGLR3.5} (At2g32390) gene expression was determined by semiquantitative RT-PCR using \textit{ACTIN2} (At3g18780) as an internal control. The PCR conditions were as follows: 10 s at 95°C, 30 s at 61°C, and 1 min at 72°C for 21 and 26 cycles for \textit{ACTIN2}, \textit{SAG12}, and \textit{AtGLR3.5} (both isoforms), respectively. Gel images were analyzed using the Geldoc system (Bio-Rad).

TMRM Staining

Sterilized seeds were sown on one-half-strength MS salts, 3% (w/v) Suc, 10 mM MES-KOH (pH 5.6), and 0.08% (w/v) plant agar (Micropoli) on sealed square plates. After 2 d at 4°C, the seeds were incubated vertically in a growth chamber at 20°C and a 16-h-light/8-h-dark photoperiod for 8 d. Ten-day-old seedlings were incubated for 15 min in the imaging solution (10 mM MES, 5 mM KCl, and 10 mM CaCl\textsubscript{2}, pH 5.8) with 500 nM TMRM. After two washing steps in the imaging solution, the roots were viewed using a Leica TCS SPS II confocal microscope (excitation, 543 nm; emission, 580–620 nm).

Chlorophyll Quantification and SDS-PAGE

Every measurement was performed on single leaves stored for 2 d in dimethylformamide at 4°C. Absorbance was measured at 647 and 664 nm, and chlorophyll was calculated as described by Carraretto et al. (2013). SDS-PAGE was performed in the presence of 6 M urea (Carraretto et al., 2013), while western blotting was carried out as described by Teardo et al. (2011). In vitro expression of GLR3.5 was performed as described by Carraretto et al. (2013). In Figure 2B, a 1-µl sample containing a maximum of 100 ng of the expressed protein was loaded; therefore, approximately 100 nmol (or less) of GLR3.5 protein was used.

Supplemental Data

The following supplemental materials are available.

**Supplemental Figure S1.** The mitochondria isolated from Arabidopsis are enriched in cytochrome c.

**Supplemental Figure S2.** Transcript analysis of four putative silenced plants.

**Supplemental Figure S3.** Ultrastructure of organelles in wild-type, atglr3.5-1, and silenced plants.

**Supplemental Figure S4.** [Ca\textsuperscript{2+}]\textsubscript{mit} dynamics in root tip cells of wild-type and atglr3.5-1 Arabidopsis seedlings expressing the 4mt-YC3.6 (mitochondrial Cameleon) probe.

**Supplemental Figure S5.** Mitochondrial calcium dynamics in leaf cells of wild-type and atglr3.5-1 plants.

**Supplemental Table S1.** Primers used for RT-PCR.

**Supplemental Table S2.** Primers and TaqMan probes used for real-time PCR.

**Supplemental Movie S1.** Arabidopsis leaves agroinfiltrated with \textit{A. tumefaciens} strain harboring the pGREAT-2×c35S-G35V1-DsRed2 construct.

**Supplemental Movie S2.** Arabidopsis leaves agroinfiltrated with \textit{A. tumefaciens} strain harboring the mCherry reporter construct that targets the mCherry reporter to the mitochondria.

**Supplemental Movie S3.** Ten-day-old Arabidopsis wild-type roots stained with TMRM.

**Supplemental Movie S4.** Ten-day-old Arabidopsis mutant (atglr3.5-1) roots stained with TMRM.

**ACKNOWLEDGMENTS**

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**LITERATURE CITED**


Behera S, Kudla J (July 1, 2013) High-resolution imaging of cytoplasmic Ca\textsuperscript{2+} dynamics in \textit{Arabidopsis} roots. BMC Plant Biol 13:160


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Table I. Primers used for RT-PCR.

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Supplementary figure legends:

Movie 1. *Arabidopsis* leaves agroinfiltrated with *A. tumefaciens* strain harboring the pGREAT-2x35S-G35v1::DsRed2 construct.

Movie 2. *Arabidopsis* leaves agroinfiltrated with *A. tumefaciens* strain harboring the mito-mCherry construct that target the mCherry reporter to the mitochondria.

**Figure S1. The mitochondria isolated from Arabidopsis are enriched in cytochrome c.**
The chloroplast preparation was not contaminated by mitochondria (no cytochrome c was present). The mitochondria preparation contained a slight contamination by PsaA of Photosystem I. The 85 kDa band in the mitochondria could not be merely due to contamination by the chloroplasts, given the relative intensity of the bands recognized by the anti-GLR3.5 antibody (upper panel).

Movie 3. Ten-day-old Arabidopsis WT roots stained with TMRM.

Movie 4. Ten-day-old *Arabidopsis* mutant (*atglr3.5-1*) roots stained with TMRM

**Figure S2. Transcript analysis of 4 putative silenced plants.** PCR were performed with primers reported in Table I. TEM micrographs of mitochondria from GLR3.5-silenced (*glr3.5 #1 and #2*) and not-silenced (*glr3.5 #3 and #4*) *Arabidopsis* plants.

**Figure S3. Ultrastructure of organelles in WT, *atglr3.5-1* and silenced plants.** TEM micrographs from 3 week-old (A) or 6 week-old (B) WT Col0 and knockout plants. Upper and lower rows show representative images from WT and knockout plants, respectively. C) Representative images from non-silenced (*glr3.5 #4*) and silenced plants (*glr3.5 #1 at 3 and 6 weeks*).
Figure S4. \([\text{Ca}^{2+}]_{\text{mit}}\) dynamics in root tip cells of WT and \textit{atglr3.5-1} Arabidopsis seedlings expressing the 4mt-YC3.6 (mitochondrial Cameleon) probe, challenged with 0.01 mM of NAA or 0.1 mM ATP.

A-D, Root tip cells of seedlings were treated with 0.01 mM of NAA for the indicated time (black rectangles above traces) and the response of cells from the transition and elongation zones analyzed with a widefield fluorescence microscope. A, Depicted \(\text{Ca}^{2+}\) kinetics, of WT (black) and \textit{atglr3.5-1} (grey), have been obtained averaging traces from 17 and 21 independent experiments respectively for each genotype. B, Statistical analysis of maximum \(\Delta R/R_0\) variations, occurred in both genotypes in response to the applied stimulus. C, Statistical analysis of \(\Delta R/R_0\) variations measured in both genotypes 500 sec after the sensing of the NAA. D, Statistical analysis of times at which the maximum \(\Delta R/R_0\) variations are measured in both genotypes in response to NAA.

E-H, Root tip cells of seedlings were treated with 0.1 mM of ATP for the indicated time (black rectangles above traces) and the response of cells from the meristematic zone analyzed with a widefield fluorescence microscope. E, Depicted \(\text{Ca}^{2+}\) kinetics, for WT (black) and \textit{atglr3.5-1} (grey), have been obtained averaging traces from 8 independent experiments for each genotype. F, Statistical analysis of maximum \(\Delta R/R_0\) variations measured in both genotypes in response to the applied stimulus. G, Statistical analysis of \(\Delta R/R_0\) variations measured in both genotypes 300 sec after the sensing of the applied stimulus. H, Statistical analysis of times at which the maximum \(\Delta R/R_0\) variations are measured in both genotypes in response to applied stimulus.

Data are reported as averages ± SD (standard deviation) (p-values calculated by Student T-test.* 0.111; ** 0.193; *** 0.011; ● 0.691; ●● 0.6; ●●● 0.895).

Figure S5. \([\text{Ca}^{2+}]_{\text{mit}}\) dynamics in leaf cells of WT and \textit{atglr3.5-1} 4-week-old Arabidopsis plants expressing the 4mt-YC3.6 (mitochondrial Cameleon) probe challenged with wounding and analyzed with a widefield fluorescence microscope. A, Depicted \(\text{Ca}^{2+}\) kinetics, for WT (black) and \textit{atglr3.5-1} (grey), have been obtained averaging traces from 6 independent experiments for each genotype. B, Statistical analysis of maximum \(\Delta R/R_0\) variations measured in both genotypes in response to wounding. C, Statistical analysis of \(\Delta R/R_0\) variations measured in both genotypes 200 sec after wounding. D, Statistical analysis of times at which the maximum \(\Delta R/R_0\) variations are measured in both genotypes in response to wounding. E, Series of representative Ratio Images (false colours) of leaf from WT and \textit{atglr3.5-1} subjected to wounding. Data are reported as averages ± SD (standard deviation) (p-values calculated by Student T-test.* 0.016; ** 0.306; *** 0.094).
Figure S1.
Figure S\textsubscript{n2}
**FIGURE S3**

3 weeks-old plants

3 weeks-old plants

6 weeks-old plants

**wt**

**atg lr3.5**

non-silenced

silenced at 3 weeks

silenced at 6 weeks
**Figure S3.** [Ca$$^{2+}$$]$_{in}$ dynamics in root tip cells of WT and atglr3.5-1 Arabidopsis seedlings expressing the 4mt-YC3.6 (mitochondrial Cameleon) probe, challenged with 0.01 mM of NAA or 0.1 mM ATP.

A-D, Root tip cells of seedlings were treated with 0.01 mM of NAA for the indicated time (black rectangles above traces) and the response of cells from the transition and elongation zones analyzed with a widefield fluorescence microscope. A, Depicted Ca$$^{2+}$$ kinetics, of WT (black) and atglr3.5-1 (grey), have been obtained averaging traces from 17 and 21 independent experiments respectively for each genotype. B, Statistical analysis of maximum ΔR/Ro variations, occurred in both genotypes in response to the applied stimulus. C, Statistical analysis of ΔR/Ro variations measured in both genotypes 500 sec after the sensing of the NAA. D, Statistical analysis of times at which the maximum ΔR/Ro variations are measured in both genotypes in response to NAA.

E-H, Root tip cells of seedlings were treated with 0.1 mM of ATP for the indicated time (black rectangles above traces) and the response of cells from the meristematic zone analyzed with a widefield fluorescence microscope. E, Depicted Ca$$^{2+}$$ kinetics, for WT (black) and atglr3.5-1 (grey), have been obtained averaging traces from 8 independent experiments for each genotype. F, Statistical analysis of maximum ΔR/Ro variations measured in both genotypes in response to the applied stimulus. G, Statistical analysis of ΔR/Ro variations measured in both genotypes 300 sec after the sensing of the applied stimulus. H, Statistical analysis of times at which the maximum ΔR/Ro variations are measured in both genotypes in response to applied stimulus.

Data are reported as averages ± SD (standard deviation) (p-values calculated by Student T-test.* 0.111; ** 0.193; *** 0.011; • 0.691; •• 0.6; ••• 0.895).
Figure S4. [Ca\(^{2+}\)]\(_{cyt}\) dynamics in leaf cells of WT and *atglr3.5-1* 4-week-old Arabidopsis plants expressing the 4mt-YC3.6 (mitochondrial Cameleon) probe challenged with wounding and analyzed with a widefield fluorescence microscope. A, Depicted Ca\(^{2+}\) kinetics, for WT (black) and *atglr3.5-1* (grey), have been obtained averaging traces from 6 independent experiments for each genotype. B, Statistical analysis of maximum ΔR/R\(_{0}\) variations measured in both genotypes in response to wounding. C, Statistical analysis of ΔR/R\(_{0}\) variations measured in both genotypes 200 sec after wounding. D, Statistical analysis of times at which the maximum ΔR/R\(_{0}\) variations are measured in both genotypes in response to wounding. E, Series of representative Ratio Images (false colours) of leaf from WT and *atglr3.5-1* subjected to wounding. Data are reported as average ± SD (standard deviation) (p-values calculated by Student T-test.* 0.016; ** 0.306; *** 0.094).
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