Biochimica et Biophysica Acta 1807 (2011) 359–367

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbabio

Dual localization of plant glutamate receptor AtGLR3.4 to plastids and plasmamembrane

Enrico Teardo [a,1], Elide Formentin [a,1], Anna Segalla [a], Giorgio Mario Giacometti [a], Oriano Marin [b], Manuela Zanetti [a,2], Fiorella Lo Schiavo [a,b], Mario Zoratti [c], Ildikò Szabò [a,*]

[a] Department of Biology, University of Padova, Italy
[b] CRIBI Biotechnology Center, University of Padova, Italy
[c] CNR Institute of Neuroscience and Department of Biomedical Sciences, University of Padova, Italy

ARTICLE INFO

Article history:
Received 21 June 2010
Received in revised form 21 October 2010
Accepted 17 November 2010
Available online 23 November 2010

Keywords:
Glutamate receptor
Chloroplast
Arabidopsis
Confocal microscopy
Photosynthesis

ABSTRACT

Bioinformatic approaches have allowed the identification in *Arabidopsis thaliana* of twenty genes encoding for homologues of animal ionotropic glutamate receptors (iGLRs). Some of these putative receptor proteins, grouped into three subfamilies, have been located to the plasmamembrane, but their possible location in organelles has not been investigated so far. In the present work we provide multiple evidence for the plastid localization of a glutamate receptor, AtGLR3.4, in *Arabidopsis* and tobacco. Biochemical analysis was performed using an antibody shown to specifically recognize both the native protein in *Arabidopsis* and the recombinant AtGLR3.4 fused to YFP expressed in tobacco. Western blots indicate the presence of AtGLR3.4 in both the plasmamembrane and in chloroplasts. In agreement, in transformed *Arabidopsis* cultured cells as well as in agroinfiltrated tobacco leaves, AtGLR3.4::YFP is detected both at the plasmamembrane and at the plastid level by confocal microscopy. The photosynthetic phenotype of mutant plants lacking AtGLR3.4 was also investigated. These results identify for the first time a dual localization of a glutamate receptor, revealing its presence in plastids and chloroplasts and opening the way to functional studies.

1. Introduction

During the last decade, many ion channels and transporters have been identified in the outer and inner membranes of chloroplast envelope as well as in thylakoids [1,2]. In general, the outer membrane is considered to be quite permeable, while highly regulated ion channels and transporters determine the selective permeability of the inner membrane. It has been hypothesized that at least some of the chloroplast channels may play an essential role by modulating rapid ion fluxes across envelope membranes and thus indirectly regulating photosynthesis [1]. Despite numerous indications of physiological variations in the concentration of different ions (for calcium see, e.g., [3]), in most cases the molecular entities that mediate ion fluxes have not been identified, and their pharmacology is not well developed. These facts have been an obstacle for the determination of the physiological tasks of these channels.

In the model plant *Arabidopsis thaliana*, 20 genes encoding homologues of animal iGluRs have been identified [4]. In vertebrates, ionotropic glutamate receptors (iGluRs) are ligand-gated cation channels that mediate most of the excitatory neurotransmission in the central nervous system. Mammalian iGluRs are grouped into four subfamilies according to pharmacological properties and sequence similarities: α-amino-3-hydroxy-5-methyl-4 isoxazole propionate (AMPA) receptors, kainate (KA) receptors, N-methyl-D-aspartate (NMDA) receptors, and delta receptors.

According to phylogenetic analyses, the *A. thaliana* glutamate receptor homologues can be subdivided into three separate subgroups [5,6]. It has been proposed that plant iGLRs can form Ca^{2+}-permeable non-selective cation channels (NSCCs), are inhibited by animal iGLR antagonists and contribute to the shaping of plant Ca^{2+} signalling (e.g. [7,8]). However, a clear-cut proof of their function as *bona fide* glutamate-activated, antagonist-sensitive ion channels is still missing. Studies with transgenic plants have suggested roles of members of the plant GLR family in Ca^{2+} fluxes (AtGLR2) [9], coordination of mitotic activity in the root apical meristem [10], regulation of abscisic acid biosynthesis and water balance (AtGLR1.1) [11,12], carbon/nitrogen sensing (AtGLR1.1) [11] and resistance against fungal infection [13]. Application of antagonists and agonists of animal iGluRs revealed that plant GLRs might be involved in regulation of root growth and branching [14], in light signal transduction [4,15] and in the response to aluminium [16].

Various localization prediction tools suggest that some of the plant iGLRs might have chloroplast and mitochondrial targeting sequences. The presence of iGLRs in these organelles would be expected to be

* Corresponding author. Department of Biology, University of Padova, 35121 Padova, Italy. Tel.: +39 049 8276324; fax: +39 049 8276300.
E-mail address: ildiko.szabo@unipd.it (I. Szabò).
[1] Equal contribution.
[2] Present address: Fondazione Bruno Kessler, Consiglio Nazionale delle Ricerche – Istituto di Biofisica 38123 Povo, Italy.

0005-2728/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbabio.2010.11.008

relevant for energy transduction. In general, determination of the distribution of a protein in the cell is an important step toward understanding its function. However, no experimental evidence has been presented up to now concerning a possible intracellular localization of iGLRs.

In the present paper, using specific antibodies and confocal microscopy, we demonstrate that a member of the AtGLR subgroup 3, i.e. AtGLR3.4, is present in the chloroplast in *Arabidopsis* and in agroinfiltrated tobacco leaves.

2. Experimental procedures

2.1. Plant material, cell cultures and mutant genotyping

Seeds of *Arabidopsis thaliana* ecotype Columbia were sterilized, incubated for 3 days at 4 °C in the dark and allowed to germinate in Murashige and Skoog medium [17] (Duchefa, The Netherlands) solid medium (0.8% plant agar, Duchefa) supplemented with 30 g/l sucrose in a growth chamber (24 °C, 8/16 h light/dark photoperiod, 70% RH). Seedlings were picked and grown in soil in a growth chamber (22 °C, 8/16 h light/dark photoperiod, 70% RH). Rosette leaves from two weeks old plants were harvested and immediately powdered in liquid nitrogen for total RNA extraction. Shoots of 5 days old seedlings were transferred into MSR2 medium (MS salts, 4.5 mg/l nicotinic acid, 10.9 mg/l thiamine HCl, 9.5 mg/l pyridoxal HCl, 200 mg/l KH_2PO_4, 0.5 g/l malt extract, 30 g/l sucrose, 1 mg/l 6-benzyl-aminopurine, and 2 mg/l 2,4-dichlorophenoxyacetic acid, at pH 5.7) for callus induction and cell cultures preparation. Cell lines were maintained in a growth chamber shaking at 80 rpm and sub-cultured weekly at 1.5/50 (v/v) in MSR2 medium.

For genotyping, *A. thaliana* wt (Col-0) and mutant plants were grown in a controlled growth chamber (23/18 °C, 10/14 h light/dark photoperiod; 100 μmol photons m^{-2} s^{-1} light, 85% RH). Mutant plants are T-DNA insertion lines from the SALK collection: *atglr3.4-1* (SALK_079842), and *atglr3.4-2* (SALK_016904). After genomic DNA extraction by using standard protocol, mutants were genotyped by PCR using the following forward and reverse primers: *atglr3.4-1*-specific, 5′-AACCCGCCTGGCGTTAGAGG-3′ and 5′-GAAACCCCAGCCTGTGCGTG-3′; *atglr3.4-2*-specific, 5′-GCAACTG-CAAATTCCGTACA-3′ and 5′-CAGAGAGGAGCCAACAGAGC-3′; T-DNA-specific, 5′-CGATGGCCCACTACGTGAACCA-3′ and 5′-TGGTTCACG-TAGTGGGCCATCG-3′.

Seeds of *Nicotiana tabacum* cv SR1 were sown in soil and allowed to grow in a growth chamber (25 °C, 16/8 h light/dark photoperiod). Four week old leaves have been used for agroinfiltration.

2.2. Purification of chloroplasts and thylakoids

Chloroplasts were isolated on a discontinuous (10/40/75%) Percoll gradient [18] in order to separate intact chloroplast. Chlorophyll concentration was measured after washing out Percoll. *Arabidopsis* plasma membrane fraction was obtained from total extract, after removing chloroplasts and mitochondria, by centrifugating at 30,000 *g* for 50′ at 4 °C. Thylakoids were isolated from *Arabidopsis* as described in [28].

2.3. Chloroform/methanol precipitation

Membrane proteins were precipitated with different mixtures of chloroform/methanol according to [19]. 1 mg of protein from total extract was mixed with a different chloroform/methanol ratio: after centrifugation, pellets were resuspended in 200 μl of sample buffer. Forty μl of each sample was loaded on SDS-PAGE.

2.4. Gel electrophoresis and Western blotting

Proteins were separated in SDS/7.5% PAGE in the presence of 6 M urea [18]. Gels were transblotted onto polyvinylidene difluoride (PVDF) membranes and decorated with protein A-purified sera against AtGLR3.4, applied at 1:1000 dilution for 2 h. Anti-AtGLR3.4 antibody against the indicated peptide (a.a. 937–947) was obtained in rabbit following standard immunisation protocols. Horseradish peroxidase-coupled anti-rabbit IgG (Kirkegaard & Perry Laboratories) was used as a secondary antibody, and blots were developed by using the enhanced chemiluminescence system (Pierce). Anti-PsaA was purchased from Agrisera, anti-GFP from Invitrogen while the anti-H^+-ATP-ase and anti-BiP antibodies were generous gifts from Profs. De Michelis and Vitale, respectively.

2.5. Genetic material

Total RNA was extracted from 100 mg of powdered leaves using TriZol reagent (Gibco brl, Germany). After treatment with RNase-free DNase I (Ambion Ltd, UK), first strand cDNA was synthesized starting from 5 μg of total RNA using the PowerScript™ Reverse Transcriptase (Clontech, USA).

AtGLR3.4 full-length cDNA was isolated by RT-PCR with primers designed on the *Arabidopsis* gene *At1g05200* coding sequence (GI:79316806; region 276…3155), cloned in the pBlueScriptII vector (Stratagene, USA) between *Sal*I and *Pst*I sites using the following forward 5′-ACGCGTCGACGATGGGATTTTTGGTGATGATAAG-3′ and reverse primer 5′-AAAACTGCAGCCTTAAGTAATTTCGCCATGTTGTG-3′ (restriction sites are underlined).

For subcellular localization experiments, the *AtGLR3.4* full-length cDNA with modified stop codon was fused to the N-terminal of YFP and cloned into the pGreen0029 vector [20] downstream to the CaMV35S dual promoter (*AtGLR3.4::YFP* construct). *AtGLR3.4::YFP* and *TR-BAMY::GFP* [21] constructs were transformed into *Agrobacterium tumefaciens* GV3101 strain carrying the pSoup helper plasmid [20] using the freeze–thaw method [22].

All constructs were sequenced at BMR-Genomics (www.bmr-genomics.it, Padova, Italy).

2.6. Agroinfiltration of tobacco leaves

Transformed *A. tumefaciens* were grown O/N at 28 °C in the dark in YEP medium with 50 μg/ml kanamycin, 50 μg/ml gentamycin, 5 μg/ml tetracycline and 100 μg/ml rifampicin for plasmid selection. Cells were collected by centrifugation, washed twice with infiltration medium (10 mM MES, pH 5.6, 10 mM $MgCl_2$, and 100 μM acetosyringone) and resuspended in the same medium to an $OD_{600} = 0.1$ before infiltration into the abaxial surface of tobacco leaves using a 1 ml syringe with no needle.

2.7. Sequence analysis

Bioinformatics analyses were performed using ChloroP and TargetP tools (http://www.cbs.dtu.dk/services), PCLRv_0.9 (http://andrewschein.com/pclr/) as well as BlastP.

2.8. Protoplast isolation and transformation

Protoplasts of cultured *Arabidopsis* cells were isolated and transformed following the Bregante et al. [23] protocol with some modifications. Briefly, 2 ml (packed volume) of 5 day old suspension cultured cells was washed once in K3 medium (3/1 v/v) (0.4 M sucrose; 1.67 mM xylose; 5 mM $CaCl_2$; 3 mM NH_4NO_3; 1× Gamborg B5 salts; 1× Kao and Michayluk organic acids; 5 μM α-naphtalene-acetic acid; and 4,4 μM 6-benzyl-aminopurine; pH 5.8; filter sterilized) and incubated 4 h at 25 °C in the dark at 80 rpm in plasmolyzation buffer

(4/1 v/v) (MS½; 0.5 M mannitol; 13.5 mM $CaCl_2$; and 4,4 μM 6-benzylaminopurine; pH 5.8; filter sterilized) containing 0.5% macerozyme R10 (Yakult Pharmaceutical, Japan) and 1% cellulase Onozuka R10 (Yakult Pharmaceutical, Japan) (filter sterilized). Protoplasts were then filtered through 170 μm and 50 μm nylon meshes and centrifuged 5 min at 100×*g* at RT. The filtered protoplasts were washed three times with W5 solution (4/1 v/v) (154 mM NaCl; 5 mM KCl; 125 mM $CaCl_2$; and 5 mM glucose; pH 5.8; filter sterilized) and centrifuged 5 min at 100×*g* and resuspended in an appropriate volume of MaCa buffer (0.5 M mannitol; 20 mM $CaCl_2$; and 0.1% w/v 2-[N-morpholino] ethanesulfonic acid (MES); pH 5.8; filter sterilized) to obtain a final concentration of 10^6 pps/ml.

For Polyethylene glycol (PEG) mediated transformation, 15 μg of plasmid DNA (7 μg for positive control) was added to $3.0 \cdot 10^5$ protoplasts and mixed with an equal volume of PEG solution (40% w/v PEG4000; 0.4 M mannitol; and 0.1 M $Ca(NO_3)_2$; pH 8; filter sterilized). The protoplast suspension was gently mixed and left in the dark for 30 min at RT. The PEG was washed out with 5 ml of W5 solution, and then protoplasts were collected by 5 min centrifugation at 100×*g* and resuspended in 2 ml of K3 solution. The protoplasts were incubated at 20 °C in the dark for at least 16 h before microscopy analysis.

2.9. Confocal microscopy

Confocal microscope analyses were performed using either a Nikon PCM2000 (Bio-Rad, Germany) or a TCS SP2 (Leica, Germany) laser scanning confocal imaging system with an excitation wavelength of 488 nm for GFP and 514 nm for YFP, and detection at 530/560 nm for YFP, 515/530 nm for GFP and 600LP (Nikon PCM2000) or 660/720 nm (TCS SP2) for chlorophyll.

ImageJ (http://rsb.info.nih.gov/ij/index.html) was used for images handling and its JaCoP interface [24] for co-localization analysis.

2.10. Chlorophyll fluorescence measurements

Chlorophyll fluorescence induction and recovery curves recorded with Dual-PAM 100 (Walz, Germany). Photochemical quantum yield of Photosystem II (Y(II)) was determined as follows: $Y(II) = 1 - Y(NPQ) - Y(NO)$ where Y(NPQ) corresponds to the quantum yield of regulated energy dissipation of PSII while Y(NO) stands for the quantum yield of non-regulated energy dissipation of PSII. For experiments under light stress conditions (not shown), *Arabidopsis* leaves were illuminated with 2200 μE m^{-2} s^{-1} for 20 min before PAM measurements.

3. Results

The exact localization of most plant glutamate receptors still needs to be clarified. Among the members of subgroup 3 in *Arabidopsis*, according to the Aramemnon database (http://aramemnon.botanik.uni-koeln.de/), GLR3.1/2/6/7 have very strong consensus sequences for the secretory pathway, while GLR3.3/4/5 show multiple targeting prediction. AtGLR3.4 is predicted to locate in chloroplasts by the targeting prediction algorithms ChloroP_v1.1 (probability of 0.541; a score above 0.55 is strongly predictive [25]) and PCLRv_0.9 (probability of 0.957), although other algorithms predict targeting to the secretory pathway. AtGLR3.4 also shows a high degree of homology with glutamate receptors GluR0 of *Synechocystis* and GluRG2 and GluRG3 of cyanobacteria (Table 1), considered to be the precursor of chloroplasts.

Recently, the presence of functional AtGLR3.4 in plasmamembrane has been reported in transiently transformed onion epidermal cells [26]. To investigate the localization of this protein in a homologous system, i.e. that of *Arabidopsis* GLR3.4 (AtGLR3.4) in *Arabidopsis* leaves, we developed a polyclonal antibody against a synthetic peptide which corresponds to a non-conserved region of the C-terminus of AtGLR3.4 (LKQKSSKKLKSTQSA—a.a. 933/947) (Fig. 1A). The antibody efficiently recognized as little as 10 ng of the synthetic peptide in dot blots (not shown). Furthermore, the specificity of the anti-AtGLR3.4 antibody was assayed by expressing full-length AtGLR3.4 with His-tag in *E. coli*. As shown in Fig. S1, the specific anti-His-tag and the anti-AtGLR3.4 antibodies recognized the same band of approx. 105 kDa (the predicted MW for AtGLR3.4 is 107 kDa) exclusively in the sample where expression was induced. In *Arabidopsis* total leaf extract, enriched in hydrophobic proteins by chloroform/methanol extraction [19], the anti-AtGLR3.4 antibody clearly recognized a band at approximately 85 kDa (Fig. 1B). The protein can be found in green tissues but not in the root (Fig. 1B), in agreement with the highest gene expression levels observed in leaves and stem [26]. The predicted molecular weight for AtGLR3.4 is higher, as mentioned above, than that observed in *Arabidopsis*. However, plant channel proteins often migrate at a substantially different apparent MW from that predicted (see e.g. [27–30]). The apparent MW of the protein in *Arabidopsis* total extract did not change by heating the sample in SDS at 55 to 95 °C in 10 °C apart (not shown). In accordance with the previously shown localization of AtGLR3.4 in the plasmamembrane of transformed onion epidermal cells [26], a protein band with an apparent MW of 85 kDa was recognized by the anti-AtGLR3.4 in the plasmamembrane-enriched microsomal fraction (Fig. 1C left panel). The protein was detected also in isolated chloroplasts, but not in purified thylakoids (Fig. 1C right panel). To test for the specificity of the antibody and for the plastid targeting of AtGLR3.4, chloroplasts isolated from wild-type as well as from two independent *atglr3.4* knock-out lines were used (Fig. 1D; for control genetic analysis of mutants see Fig. S2 and reference [30]). Our antibody clearly recognizes an 85 kDa band in the wild-type chloroplasts, while the same band is absent in the *atglr3.4* knock-outs. Comparable total protein quantities were loaded, as indicated by the similar signal intensity of the beta-chain of ATP-ase (Fig. 1D). Although the protein, as expected, has low abundance in the chloroplasts, the difference between the signal of WT and knock-out chloroplasts is evident. To check whether the presence of AtGLR3.4 in the chloroplasts may be due to cross-contamination by microsomal fraction, the chloroplast preparation was assessed for purity. As illustrated in Fig. 1E, while the microsomal fraction (MI : plasmamembrane + endomembranes) is slightly contaminated by chloroplast membranes and contains endoplasmatic reticulum (ER) in our preparation (see the markers PsaA and BiP, respectively, in the MI lane), the chloroplasts do not contain either the ER marker BiP [31], the H^+/ATP-ase, a marker of plasma membrane [32] (Fig. 1E) or mitochondrial cytochrome c (not shown). Therefore the presence of AtGLR3.4 is not due to cross-contamination of chloroplasts by plasmamembrane and/or ER. In turn, the presence of AtGLR3.4 in the PM fraction cannot be fully ascribed to contamination by chloroplasts, given the relative intensity of the bands (see Fig. 1C and E). The above experiments indicate that anti-AtGLR3.4 specifically recognizes the protein in chloroplasts and point to a dual localization of the protein in *Arabidopsis* leaves. The fact that both in total extract and in isolated chloroplasts the apparent molecular weight of the AtGLR3.4 band is approximately 85 kDa suggests that the protein size is not substantially modified upon import into chloroplast. We would

Table 1
AtGluR3.4 and cyanobacterial glutamate receptors share sequence similarity.

	AtGluR3.4 (At1g05200)
Synechocystis sp. PCC 6803 GluR0, slr 1257 (gi 1652933)	22%
Nostoc punctiforme PCC 73102 GluRG2 (gi 23126603)	24%
Magnetospirillum magnetotacticum MS-1 GluRG3 (gi 46202027)	21%

Aminoacid sequences of GLRs channels (GI: accession nos. in NCBI data bank) were compared pairwise by BLASTP algorithm. % of identity is shown.

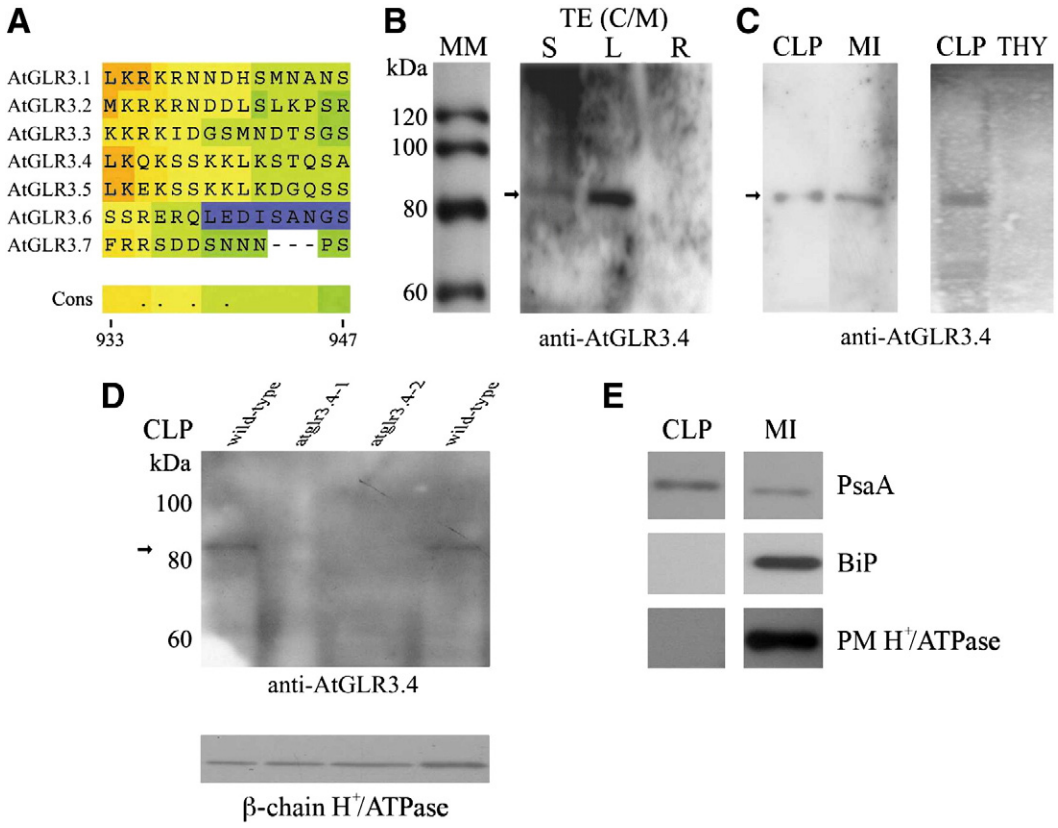

Fig. 1. AtGLR3.4 is present in chloroplasts isolated from *Arabidopsis*. (A) The peptide chosen for the antibody production, corresponding to aminoacids 933/947, is specific for *Arabidopsis* AtGLR3.4. T-Coffee algorithm was used for multiple alignment. "*" : residues which are identical in all sequences in alignment; ":": conserved, ".":semi-conserved substitutions. Colours are indicative of degree of homology (red for highest and blue for lowest homology). (B) Mass marker (Magic Mark - Invitrogen) and chloroform/methanol precipitated total extract (TE C/M) from *A. thaliana* stem (S), leaf (L) and root (R) were loaded (50 μg) on SDS-PAGE in 6 M urea; the Western blot was developed with anti-AtGLR3.4 antibody. (C) Left panel: Western blot of purified chloroplasts (CLP) and microsomal fraction (MI) consisting of plasma membrane and endomembranes contains the 85 kDa protein. Right panel: Equal protein quantity (30 μg) of purified chloroplasts and isolated thylakoids were loaded and Western blot was developed by using alkaline phosphatase-coupled secondary antibody. (D) Purified chloroplasts from wild-types and two atglr3.4 knock-out mutants (pooled leafs for each mutant line) were loaded and assayed with AtGLR3.4-specific antibody. As a control for equal loading, the same blot was stripped and re-blotted with anti-H^+-ATP-ase. (E) *Arabidopsis* chloroplast and microsomal membrane fractions (MI) loaded (30 μg each) on the same gel and processed together, were assayed with antibodies against thylakoid PsaA, ER BiP and plasma membrane H^+/ATP-ase to check for cross-contamination. The same blot was stripped and re-blotted with the various antibodies. Protein concentrations were determined by BCA assay for each sample of this figure. All Western blots are representative of at least 3 experiments.

like to point out that by using the above biochemical analysis, we observed AtGLR3.4 in the chloroplasts of genetically non-modified, wild-type plants, thus avoiding possible mistargeting artifacts.

To confirm these results also by another method in a related system, we expressed the fusion construct *AtGLR3.4::YFP* in a homologous system, i.e. in protoplasts obtained from *Arabidopsis* (ecotype Columbia) cells cultured in suspension. After transient transformation of protoplasts, the following subcellular localization pattern has been observed: (i) a clear YFP signal in plastids and stromules (stroma-filled tubules, see arrows), and a uniform signal at the plasmamembrane in most cells (Fig. 2A–C; three-dimensional reconstruction); (ii) the YFP signal in plastids and stromules (Fig. 2D–F; three-dimensional reconstruction) (iii) in accordance with previous data [25], the fusion protein was visible in the plasmamembrane (Fig. 2G–I). Importantly, all images were analyzed by spectral unmixing, avoiding misidentification as YFP signal of the bleed-through of the chlorophyll fluorescence emission. The thioredoxin-regulated β-amylase (TR-BAMY::GFP, [21]), a stromal enzyme, was used as a positive control to mark all plastids and stromules (arrows) (Fig. 2 J–O). Stromal GFP-fused β-amylase, similarly to AtGLR3.4::YFP, gives a GFP signal also in plastids that do not contain chlorophyll, indicating that both β-amylase and GLR3.4 are expressed in general in plastids and not only in mature chloroplasts. Please note that photosynthesis is not compulsory for these cells since they grow in the presence of sucrose as carbon source. So, protoplasts obtained from these cultured cells have green chlorophyll-containing chloroplasts (with red autofluorescence of chlorophyll) and non-green plastids.

In order to exclude any overexpression artefact and/or mistargeting due to transient expression, we also studied the subcellular localization of the AtGLR3.4::YFP fusion protein in agroinfiltrated tobacco leaves (a nearly-stable transformation) by using a full spectral confocal microscope. Again, in this experimental set-up, fluorescence of chlorophyll and YFP are well-separated by the analyzing software. We observed an evident dual targeting to the chloroplasts and to the plasmamembrane (Fig. 3A–F, see also three-dimensional reconstruction in Fig. S3). A dual localization can be observed also in mesophyll cells, however due to the quenching of the YFP fluorescence by the chlorophyll, the AtGLR3.4::YFP signal is less visible in the chloroplasts (Fig. 3G–I). The JaCoP interface of ImageJ [24] has been used for a quantitative analysis of co-localization of chlorophyll and YFP in chloroplasts of transformed tobacco leaves. Fig. S4 shows an example of the output obtained for the mesophyll cell presented in Fig. 3G.

To further confirm the results of the confocal microscopy, total extracts and isolated chloroplasts, obtained from control and agroinfiltrated tobacco leaves were assayed by Western blot. The same band of approx. 115 kDa (of AtGLR3.4::YFP fusion protein) was recognized by anti-GFP and the anti-AtGLR3.4 antibodies in the total extracts of the transformed plants only (Fig. 3J). Furthermore, this band was visible also in isolated contamination-free (not shown) chloroplasts (Fig. 3J, right panel). The 115 kDa apparent molecular weight is compatible with the sum of the MW of C-terminal fused YFP

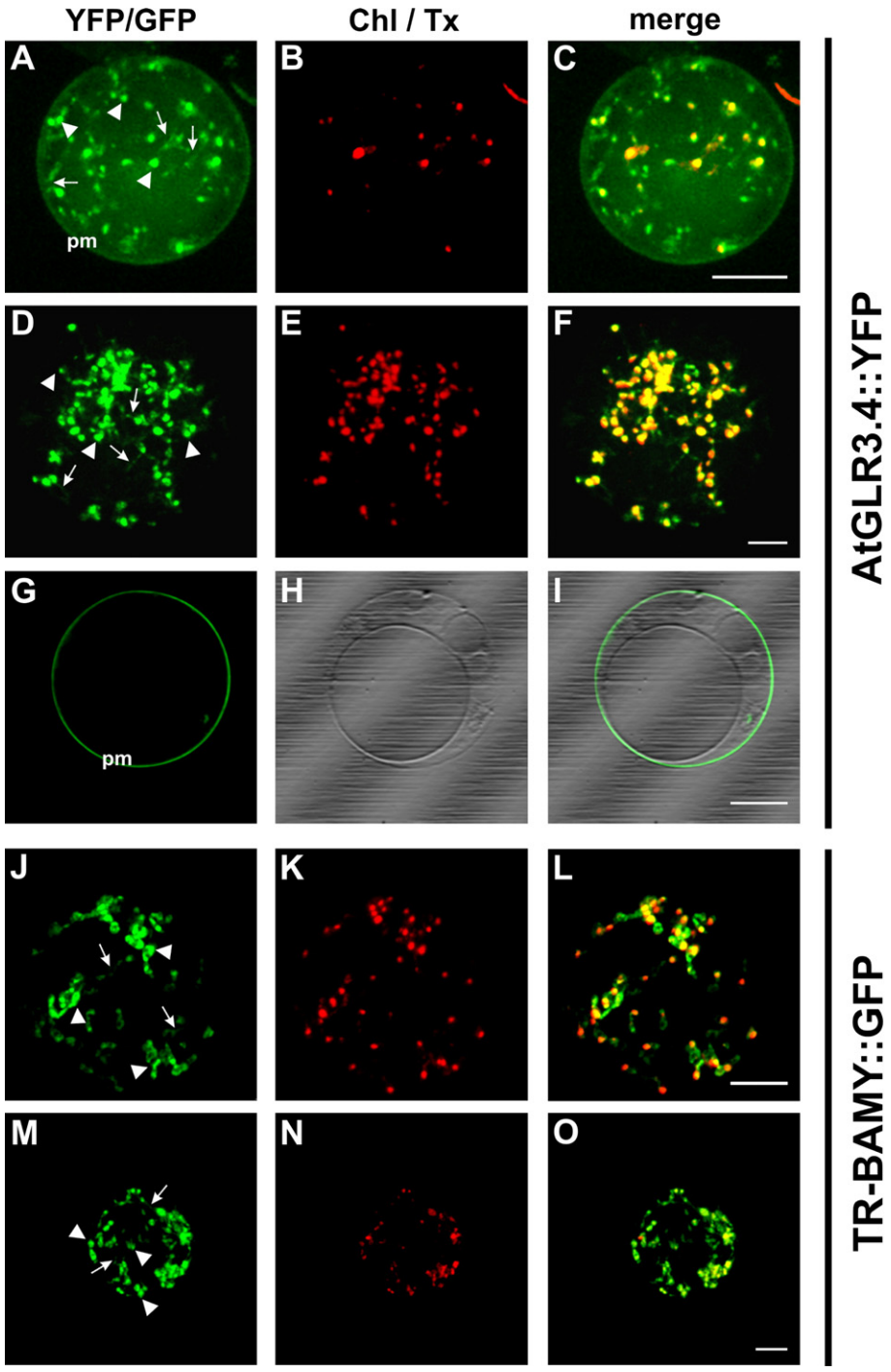

Fig. 2. Subcellular localization of AtGLR3.4 and TR-BAMY in *Arabidopsis* cell protoplasts. Subcellular localization of AtGLR3.4::YFP and TR-BAMY::GFP (plastid marker) fusion proteins expressed in transiently transformed *Arabidopsis* (ecotype Columbia) suspension cell protoplasts. Images are representative of 3 independent experiments. Protoplasts have been imaged by a Nikon PCM2000 laser scanning confocal microscope and then dyes have been separated by the Spectral unmixing tool of ImageJ v.1.43o. Green fluorescence belongs to GFP or YFP; red fluorescence belongs to chlorophyll in chloroplasts, merged fluorescences are in yellow. (A–C) The YFP signal is well visible at the plasmamembrane and plastid (arrowheads) and stromules (arrows) level, and co-localizes with chlorophyll in mature chloroplasts, indicating a dual localization for AtGLR3.4::YFP. (D–F) In some protoplasts the fusion protein is clearly visible in plastids only. (G–I) The targeting to the plasmamembrane is well visible in protoplasts lacking auto-fluorescent plastids. (J–O) The stromal marker TR-BAMY::GFP has been used to identify both mature and immature plastids. Please note the similar size and form of the organelles positive for AtGLR3.4::YFP and TR-BAMY::GFP. Images in A–F are 3D reconstruction of 30 sections (1 μm pass). Chl, chlorophyll; Tx, transmitted light; pm, plasma membrane. Bar is 10 μm. Microscope settings: objective 60×/1.40 NA oil; iris is 2/12 for chlorophyll and 3/12 for YFP/GFP; gain is 21/100 for all; offset is 0; pinhole size is 87 μm.

(28 kDa) and of the apparent MW of AtGLR3.4 migrating in the PAGE slab (85–87 kDa). These data suggest that AtGLR3.4 expressed either in *Arabidopsis* or in tobacco, probably undergoes a posttranslational modification which alters its electrophoretic mobility. Our results indicate a dual localization for AtGLR3.4 in two different systems, i.e. in *Arabidopsis* plants and cells and in transformed tobacco plants. The presence of the fluorescent signal in stromules and the lack of protein in thylakoids (Fig. 1C) suggest the localization of the protein in the inner or outer envelope, as stromules are made of these two membranes but do not contain thylakoids [33]. To further establish

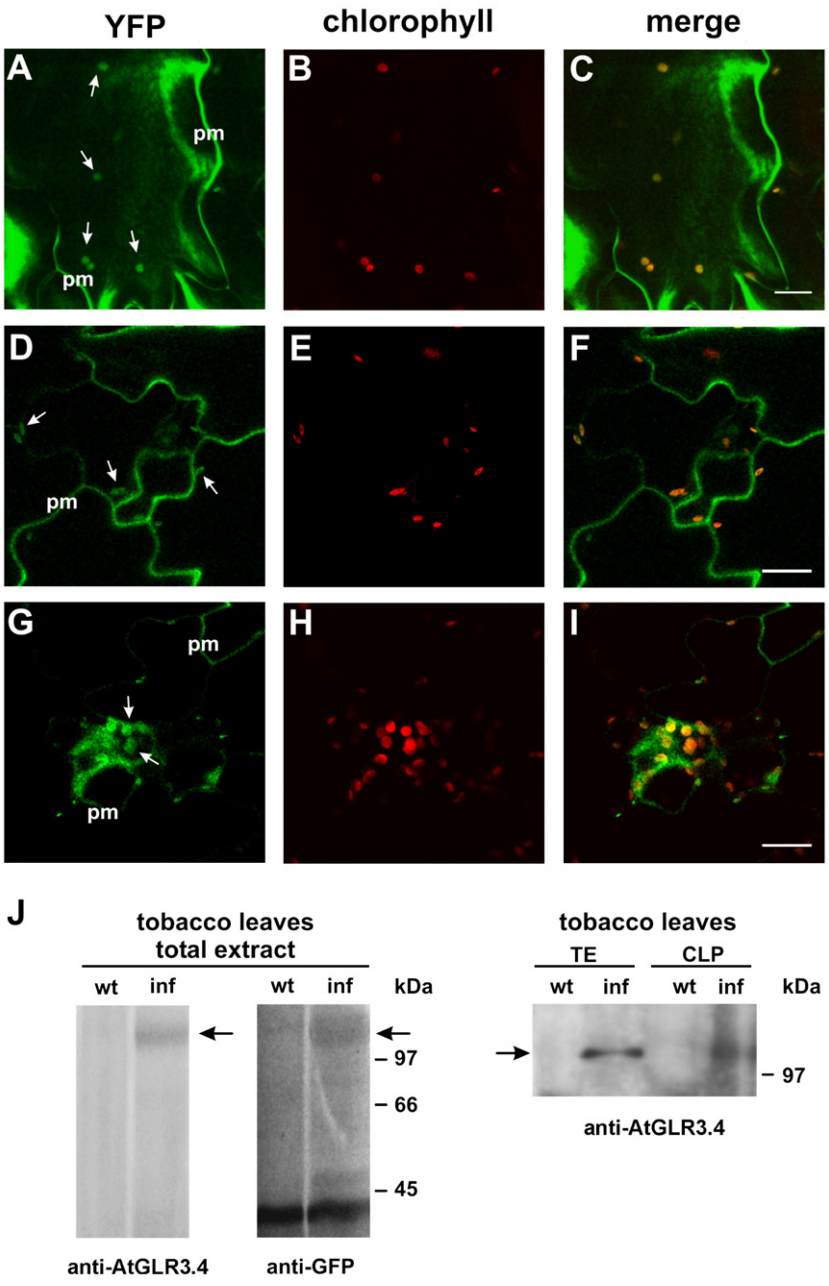

Fig. 3. Subcellular localization of AtGLR3.4 in agroinfiltrated tobacco leaves. Subcellular localization of AtGLR3.4::YFP in agroinfiltrated tobacco (*N. tabacum* cv SR1) leaves. Images are representative of 3 independent experiments. Leaves have been imaged 8 days after agroinfiltration by the use of the Leica TCS SP2 confocal system and chlorophyll and YFP channels have been separated by using the Leica Dye Finder tool. Images represent false coloured outputs of the software. AtGLR3.4::YFP is located in the plasmamembrane and chloroplasts (arrows) in epidermal (A–F) and mesophyll (G–I) cells. Diffuse green fluorescence signals can be observed where the PM is oriented parallel to and coincides with the focal plane. For three-dimensional reconstruction see Fig. S2. pm, plasma membrane. Bar is 30 μm. Microscope settings: objective 40×/1.25–0.75 NA oil; gain is 670–770 for YFP and 570–670 for chlorophyll; offset is 0; pinhole size is 115 μm. (J) Anti-AtGLR3.4 antibody recognizes the protein expressed in tobacco. Left panel: Total cell extracts from non-treated tobacco leaves (wt) and from agro-infiltrated tobacco leaves (inf) were loaded (200 μg total protein/lane) and developed with the indicated antibodies. In this case nitrocellulose membrane and the BCIP technique were used to visualize antibody recognition in order to reveal reaction of the anti-GFP antibody. Right panel: Total extracts (TE) (100 μg total protein/lane) and isolated chloroplasts (CLP, 30 μg/lane) from wild-type and agroinfiltrated (inf) tobacco plants were assayed by using the ECL chemiluminescence system.

the exact location of AtGLR3.4 within the chloroplast, outer and inner envelope membranes should be separately purified from *Arabidopsis* chloroplasts. To our knowledge, suitable preparations of this type cannot be obtained from this model plant.

An indirect role in the regulation of photosynthesis can be envisioned for several ion channels of the envelope membrane [1,2,34]. Glutamate receptor AtGLR3.4 has been shown to mediate calcium fluxes (as shown by using aequorin) [26] and glutamate receptors contribute to light signalling by an unclarified mechanism [4]. Calcium is known to be required for efficient photosynthesis, e.g. for the function of the oxygen evolving complex [35], and for regulation of key metabolic enzymes of CO_2 fixation [36]. We therefore performed an initial investigation of the photosynthetic phenotype of wild-type and of two independent mutant lines. Chlorophyll fluorescence induction and recovery curves were recorded from intact leaves of wild-type and the mutant plants, grown under standard conditions (Fig. 4A–C). Analysis of these data reveals that mutants lacking AtGLR3.4 had a slight, but statistically significant (t-test, $p<0.05$) reduction of photosynthetic yield of Photosystem II (Fig. 4D). Furthermore, experiments under high light stress suggest a slightly reduced capacity of non-photochemical quenching in the mutants (not shown).

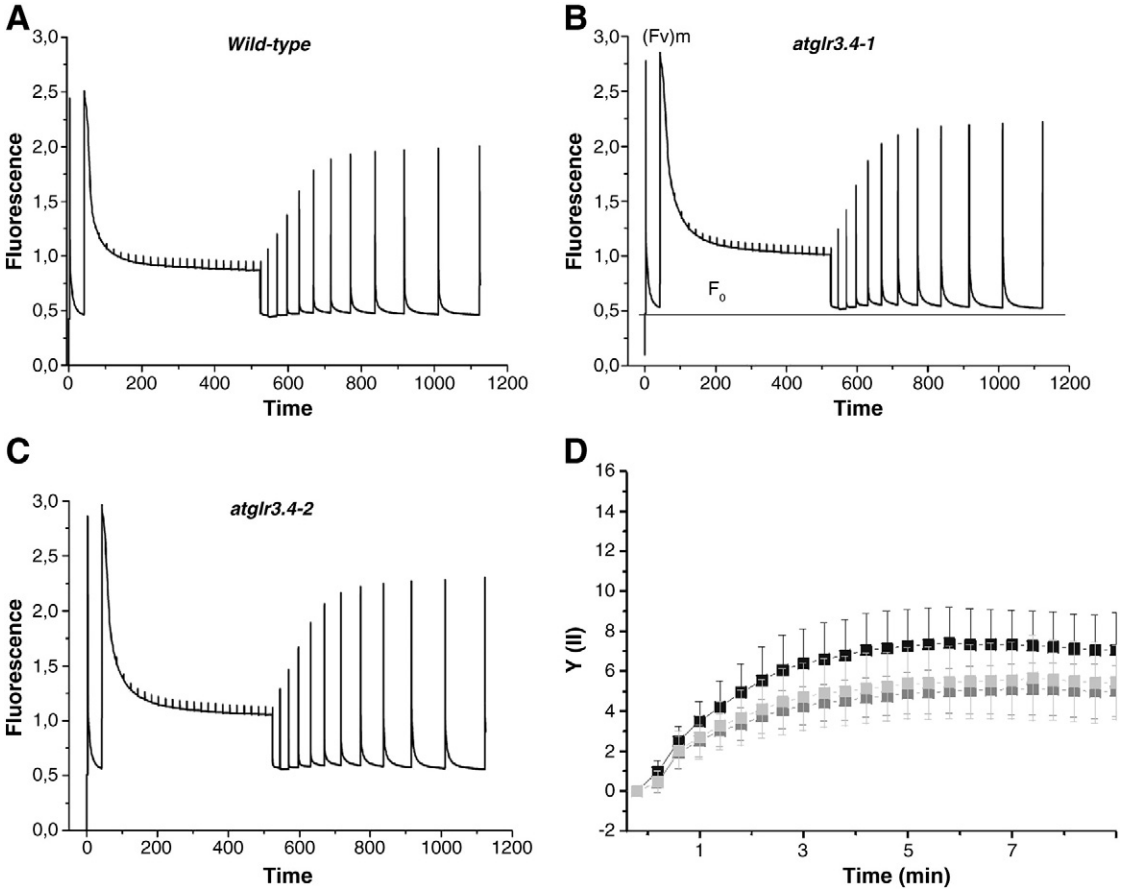

Fig. 4. *Arabidopsis* plants lacking AtGRL3.4 display a subtle photosynthetic phenotype. (A–C) Chlorophyll fluorescence induction and recovery curves recorded from wild type and two independent mutant lines. Fluorescence (a.u.) and time (s) are reported. The curves shown are representative of at least 5 experiments giving similar results. Maximal fluorescence of dark-adapted (for 20 min) leaf was obtained by a saturating flash of 8000 μmol photons $m^{-2}s^{-1}$ of 400 ms duration (Fv)m. Measuring light of 46 μmol photons $m^{-2}s^{-1}$ was used to record basic fluorescence (F_0). Continuous actinic light of 1287 μmol photons $m^{-2}s^{-1}$ was turned on and after the peak of the fluorescence curve, saturation pulses were triggered every 20 s. After 500 s, actinic light was switched off and recovery could be recorded. (D) Analysis by the Dual-PAM 100 software revealed a slight, but significant difference in the photochemical quantum yield of Photosystem II measured in the presence of actinic light ($n = 5$ for each point, $p < 0.05$) WT: black; dark and light grey indicate values obtained in the two mutant lines. Mean values ± SD are reported ($n = 5$).

4. Discussion

In the present work we show that AtGLR3.4 in wild-type *Arabidopsis* and in agroinfiltrated tobacco leaves has a dual localization, being present also in the chloroplasts in addition to the plasmamembrane.

The results shown in Figs. 1–3 point to a dual targeting of AtGLR3.4. Please note that in the present work dual targeting of AtGLR3.4 is observed not only in transiently transfected cells but also in agroinfiltrated tobacco and in wild-type *Arabidopsis* plants. Examples of dual targeting in animal cells abound, and although we do not know the mechanism of dual targeting for AtGLR3.4, plausible hypotheses can be put forward (for review on dual targeting see [37]). For example, it has been reported that several members of the cytochrome P450 family (CYP) are targeted to both the ER and mitochondrial compartments [38,39]. Dual targeting of CYP apoproteins to the ER and to mitochondria is modulated by an amino-terminal bipartite signal, which includes an ER targeting sequence followed by a cryptic mitochondrial targeting sequence. Activation of the cryptic signal is mediated through post-translational modification depending on cellular cAMP levels. By analogy, it can be hypothesized that effective targeting of AtGLR3.4 depends on e.g. cAMP levels, which may vary from cell to cell in *Arabidopsis* cell cultures. In fact, in some cultured cells AtGLR3.4 is targeted to both membranes, in others mainly to chloroplasts, and still in others mainly to the plasma membrane (Fig. 2). This targeting pattern may depend on a different metabolic state of the cultured cells, and/or on the transient nature of the expression. Targeting may also depend on other kind of post-translational modifications or partnership. The chloroplast proteome has been shown to contain N-glycosylated proteins that are transported through the endoplasmatic reticulum (ER) to chloroplasts [40] and direct contact as well as biochemical interactions between ER and chloroplast membrane systems have been demonstrated [41,42]. Such mechanism may *a priori* also be responsible for AtGLR3.4 targeting. Whether the recently described interaction between 14-3-3 proteins and AtGLR3.4 [43] may help targeting of the protein to the chloroplasts remains to be determined. It is important to note, that depending on the transient expression system used, subcellular targeting of channel proteins may be altered, as illustrated by the case of Castor and Pollux (located to chloroplasts or nuclear membrane depending on the system used [44]). Interestingly, CAS, a novel calcium sensor protein was located to the PM when the protein was transiently expressed in onion epidermal cells [45], however a later study demonstrated its presence in thylakoids [46]. Therefore, when using non-homologous, transient expression systems for determination of GFP-tagged protein localization, a biochemical additional proof can be useful [44,46]. In any case, our results are not in contrast with previous reports showing plasmamembrane localization of transiently expressed AtGLR3.4 in onion cells [26] and change in agonist-induced PM currents in *atglr3.4* mutant hypocotil [30], but point to the presence of the protein in plastids as well.

In mammalian system, several ion channels are located in multiple membranes within the cell (e.g. [47–50]). For example different types of potassium channels have been located to both the plasmamembrane and the mitochondria [51]. A classical, N-terminal mitochondrial targeting sequence cannot be revealed in these proteins, and the mechanism for their dual targeting is unknown. A recent work indicates that even small changes in the length of the transmembrane domain of the viral potassium channel Kesv alter its localization between the plasmamembrane and the mitochondria in mammalian cells [52]. The apparent molecular weight of AtGL3.4 is comparable in the plasmamembrane and in the chloroplasts, indicating either a rather short cleavable targeting sequence or an internal, non-cleavable signal. Further future work is required to clarify this point and to understand how AtGLR3.4 is imported into the chloroplasts.

Whether AtGLR3.4 is the only iGLR in chloroplast is currently unknown. In a revised version of Aramemnon released in July 2008, AtGLR2.5 is predicted to be located in chloroplasts as well (0.48 probability of chloroplast targeting with ChloroP_v1.1). No experimental data is available on the localization of this glutamate receptor. In a recent paper Roy and colleagues [53] studied the expression profile of *AtGLR*s in different cell types in single cells by using the MicroExpression amplification (MEX) method. *AtGLR2.5* was detected in mesophyll cells of only one out of six plants, while various AtGLR3 members were present in mesophyll cells of each examined plant. It cannot be excluded therefore that various members of the AtGLR3 subgroup may have redundant functions in forming plastid iGLRs. In accordance with this hypothesis, experiments failed to reveal a strong photosynthetic phenotype in *atglr3.4* knock-out plants grown under standard conditions. A slight decrease of the photochemical quantum yield of Photosystem II could be observed in two independent mutant lines with respect to wild-type plants. Similarly, a chloroplast-located chloride channel, ClC-e, has recently been shown to contribute to the regulation of photosynthesis, although to a rather small extent [18,54]. The mechanism by which glutamate receptor AtGLR3.4 may influence the physiology of chloroplasts is difficult to predict until the *bona fide* ion channel function of this protein is demonstrated. Interestingly, the ATTED II algorithm reveals a high co-expression pattern of AtGLR3.4 with two chloroplast-located proteins: 1) CSP41A (At3g63140), encoding a protein with ribonuclease activity that is involved in plastid rRNA maturation; and 2) a putative MATE-related efflux carrier (AtDTX46) (At2g21340), suggested to be involved in chlorophyll metabolic pathway.

It has been hypothesized that iGLRs may contribute to calcium signalling [8]. Studies using AtGLR3.3 mutant plants showed that intracellular Ca^{2+} rise and membrane depolarisation induced by glutamate in *Arabidopsis* hypocotyls and root cells are correlated with the presence of AtGLR3.3 [55]. However, most plant iGLRs, when expressed in heterologous systems, do not give rise to any current (oocytes), are toxic for host cells (e.g. in mammalian cells) [56], or are retained in the endoplasmatic reticulum [10]. Recently, in order to examine whether AtGLR homologues possess functional ion channel domains, Tapken and Hollmann [57] transplanted the pore loop together with the two adjacent intracellular loops of 17 AtGLR subunits into two rat iGluR subunits and tested the resulting chimaeric receptors for ion channel activity in the heterologous expression system *Xenopus* oocytes. 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 non-specific cation channel blocker La^{3+} [57]. For AtGLR3.4, to our knowledge, there is no information available as to whether it allows the flux of only calcium and divalent cations or also of other, monovalent cations. A fast-activating cation channel has been described in the chloroplast envelope, although it is not known for the moment whether it may correspond to an iGLR [58].

In conclusion, the present work provides evidence for a chloroplast location of AtGLR3.4 in *Arabidopsis* and agroinfiltrated tobacco leaves. The localization of this putative glutamate receptor to this energy-producing organelle may open the way to further bioenergetic and functional studies.

Acknowledgements

The authors thank G. Rabito for help with antibody production and A. Costa for useful pieces of advice. Furthermore we thank all laboratories who provided us with various antibodies (Profs. Vitale and De Michelis). The authors are grateful to EMBO Young Investigator Program (to I.S.), to the Italian Ministry (PRIN to I.S., F. L.S. and G.M.G.), to the University of Padova (progetto di Ateneo to I. S.) and to FIRB (M.Z.) for financial support. E.T. is a post-doc fellowship holder of the University of Padova.

Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.bbabio.2010.11.008.

References

[1] H.E. Neuhaus, R. Wagner, Solute pores, ion channels, and metabolite transporters in the outer and inner envelope membranes of higher plant plastids, Biochim. Biophys. Acta 1465 (2000) 307–323.
[2] A.P. Weber, R. Schwacke, U.I. Flugge, Solute transporters of the plastid envelope membrane, Annu. Rev. Plant Biol. 56 (2005) 133–164.
[3] J. Sai, C.H. Johnson, Dark-stimulated calcium ion fluxes in the chloroplast stroma and cytosol, Plant Cell 14 (2002) 1279–1291.
[4] H.M. Lam, J. Chiu, M.H. Hsieh, L. Meisel, I.C. Oliveira, M. Shin, G. Coruzzi, Glutamate-receptor genes in plants, Nature 396 (1998) 125–126.
[5] J. Chiu, R. DeSalle, H.M. Lam, L. Meisel, G. Coruzzi, Molecular evolution of glutamate receptors: a primitive signaling mechanism that existed before plants and animals diverged, Mol. Biol. Evol. 16 (1999) 826–838.
[6] J.C. Chiu, E.D. Brenner, R. DeSalle, M.N. Nitabach, T.C. Holmes, G.M. Coruzzi, Phylogenetic and expression analysis of the glutamate-receptor-like gene family in *Arabidopsis thaliana*, Mol. Biol. Evol. 19 (2002) 1066–1082.
[7] D. Sanders, J. Pelloux, C. Brownlee, J.F. Harper, Calcium at the crossroads of signaling, Plant Cell 14 (2002) S401–S417.
[8] M.R. McAinsh, J.K. Pittman, Shaping the calcium signature, New Phytol. 181 (2009) 275–294.
[9] S.A. Kim, J.M. Kwak, S.K. Jae, M.H. Wang, H.G. Nam, Overexpression of the AtGluR2 gene encoding an *Arabidopsis* homolog of mammalian glutamate receptors impairs calcium utilization and sensitivity to ionic stress in transgenic plants, Plant Cell Physiol. 42 (2001) 74–84.
[10] J. Li, S. Zhu, X. Song, Y. Shen, H. Chen, J. Yu, K. Yi, Y. Liu, V.J. Karplus, P. Wu, X.W. Deng, A rice glutamate receptor-like gene is critical for the division and survival of individual cells in the root apical meristem, Plant Cell 18 (2006) 340–349.
[11] J. Kang, F.J. Turano, The putative glutamate receptor 1.1 (AtGLR1.1) functions as a regulator of carbon and nitrogen metabolism in *Arabidopsis thaliana*, Proc. Natl Acad. Sci. USA 100 (2003) 6872–6877.
[12] J. Kang, S. Mehta, F.J. Turano, The putative glutamate receptor 1.1 (AtGLR1.1) in *Arabidopsis thaliana* regulates abscisic acid biosynthesis and signaling to control development and water loss, Plant Cell Physiol. 45 (2004) 1380–1389.
[13] S. Kang, H.B. Kim, H. Lee, J.Y. Choi, S. Heu, C.J. Oh, S.I. Kwon, C.S. An, Overexpression in *Arabidopsis* of a plasma membrane-targeting glutamate receptor from small radish increases glutamate-mediated Ca^{2+} influx and delays fungal infection, Mol. Cells 21 (2006) 418–427.
[14] P. Walch-Liu, L.H. Liu, T. Remans, M. Tester, B.G. Forde, Evidence that L-glutamate can act as an exogenous signal to modulate root growth and branching in *Arabidopsis thaliana*, Plant Cell Physiol. 47 (2006) 1045–1057.
[15] E.D. Brenner, N. Martinez-Barboza, A.P. Clark, Q.S. Liang, D.W. Stevenson, G.M. Coruzzi, *Arabidopsis* mutants resistant to S(+)-β-methyl-α, β-diaminopropionic acid, a cycad-derived glutamate receptor agonist, Plant Physiol. 124 (2000) 1615–1624.
[16] M. Sivaguru, S. Pike, W. Gassmann, T.I. Baskin, Aluminum rapidly depolymerizes cortical microtubules and depolarizes the plasma membrane: evidence that these responses are mediated by a glutamate receptor, Plant Cell Physiol. 44 (2003) 667–675.
[17] T. Murashige, F. Skoog, A revised medium for rapid growth and bioassays with tobacco tissue cultures, Physiol. Plant. 15 (1962) 473–497.
[18] E. Teardo, E. Frare, A. Segalla, V. DeMarco, G.M. Giacometti, I. Szabò, Localization of a putative ClC chloride channel in spinach chloroplasts, FEBS Lett. 579 (2005) 4991–4996.
[19] M. Ferro, D. Seigneurin-Berny, N. Rolland, A. Chapel, D. Salvi, J. Garin, J. Joyard, Organic solvent extraction as a versatile procedure to identify hydrophobic chloroplast membrane proteins, Electrophoresis 21 (2000) 3517–3526.
[20] R.P. Hellens, A.E. Edwards, N.R. Leyland, S. Bean, P.M. Mullineaux, pGreen: a versatile and flexible binary Ti vector for *Agrobacterium*-mediated plant transformation, Plant Mol. Biol. 42 (2000) 819–832.

[21] F. Sparla, A. Costa, F. Lo Schiavo, P. Pupillo, P. Trost, Redox regulation of a novel plastid-targeted beta-amylase of *Arabidopsis*, Plant Physiol. 141 (2006) 840–850.
[22] H. Chen, R.S. Nelson, J.L. Sherwood, Enhanced recovery of transformants of *Agrobacterium tumefaciens* after freeze–thaw transformation and drug selection, Biotechniques 16 (1994) 664–668.
[23] M. Bregante, Y. Yang, E. Formentin, A. Carpaneto, J. Schroeder, F. Gambale, F. Lo Schiavo, A. Costa, KDC1, a carrot *Shaker*-like potassium channel, reveals its role as a silent regulatory subunit when expressed in plant cells, Plant Mol. Biol. 66 (2008) 61–72.
[24] S. Bolte, F.P. Cordelières, A guided tour into subcellular colocalization analysis in light microscopy, J. Microsc. 224 (2006) 213–232.
[25] O. Emanuelsson, S. Brunak, G. von Heijne, H. Nielsen, Locating proteins in the cell using TargetP, SignalP and related tools, Nat. Protoc. 2 (2007) 953–971.
[26] O. Meyerhoff, K. Müller, M.R.G. Roelfsema, A. Latz, B. Lacombe, R. Hedrich, P. Dietrich, D. Becker, AtGLR3.4, a glutamate receptor channel-like gene is sensitive to touch and cold, Planta 222 (2005) 418–427.
[27] A. Honsbein, S.C. Sokolovski, P. Campanoni, R. Pratelli, M. Paneque, Z. Chen, I. Johansson, M.R. Blatt, A tripartite SNARE-K^+ channel complex mediates in channel-dependent K^+ nutrition in *Arabidopsis*, Plant Cell 21 (2009) 2859–2877.
[28] M. Zanetti, E. Teardo, N. La Rocca, L. Zulkifli, V. Checchetto, T. Shijuku, Y. Sato, G.M. Giacometti, N. Uozumi, E. Bergatino, I. Szabò, A novel potassium channel in photosynthetic cyanobacteria, PLoS ONE 5 (2010) e10118.
[29] F.J. Turano, M.J. Muhitch, F.C. Felker, M.B. MacMahon, The putative glutamate receptor3.2 from *Arabidopsis thaliana* (AtGLR3.2) is an integral membrane peptide that accumulates in rapidly growing tissues and persists in vascular-associated tissues, Plant Sci. 163 (2002) 45–51.
[30] N.R. Stephens, Z. Qi, E.P. Spalding, Glutamate receptor subtypes evidenced by differences in desensitization and dependence on the GLR3.3 and GLR3.4 genes, Plant Physiol. 146 (2008) 529–538.
[31] E. Pedrazzini, G. Giovinazzo, A. Bielli, M. de Virgilio, L. Frigerio, M. Pesca, F. Faoro, R. Bollini, A. Ceriotti, A. Vitale, Protein quality control along the route to the plant vacuole, Plant Cell 9 (1997) 1869–1880.
[32] C. Olivari, C. Albumi, M.C. Pugliarello, I. De Michelis, Phenylarsine oxide inhibits the fusicoccin-induced activation of plasma membrane H(+)-ATPase, Plant Physiol. 122 (2000) 463–470.
[33] R.H. Köhler, M.R. Hanson, Plastid tubules of higher plants are tissue-specific and developmentally regulated, J. Cell Sci. 113 (2000) 81–89.
[34] A. Segalla, I. Szabò, P. Costantini, G.M. Giacometti, Study of the effect of ion channel modulators on photosynthetic oxygen evolution, J. Chem. Inf. Model. 45 (2005) 1691–1700.
[35] K.A. Vander Meulen, A. Hobson, C.F. Yocum, Calcium depletion modifies the structure of the photosystem II O_2-evolving complex, Biochemistry 41 (2002) 958–966.
[36] G. Kreimer, M. Melkonian, J.A.M. Holtum, E. Latczko, Stromal free calcium concentration and light-mediated activation of chloroplast fructose-1,6-bisphosphatase, Plant Physiol. 86 (1988) 423–428.
[37] S. Karniely, O. Pines, Single translation—dual destination, EMBO Rep. 6 (2005) 420–425.
[38] H.K. Anandatheerthavarada, G. Biswas, J. Mullick, N.B. Sepuri, L. Otvos, D. Pain, N.G. Avadhani, Dual targeting of cytochrome P4502B1 to endoplasmic reticulum and mitochondria involves a novel signal activation by cyclic AMP-dependent phosphorylation at ser128, EMBO J. 18 (1999) 5494–5504.
[39] M.A. Robin, H.K. Anandatheerthavarada, J.K. Fang, M. Cudic, L. Otvos, N.G. Avadhani, Mitochondrial targeted cytochrome P450 2E1 (P450 MT5) contains an intact N terminus and requires mitochondrial specific electron transfer proteins for activity, J. Biol. Chem. 276 (2001) 24680–24689.
[40] A. Villajero, S. Buren, S. Larsson, A. Dejardin, M. Monne, C. Rudhe, J. Karlsson, S. Jansson, P. Lerouge, N. Rolland, G. von Heijne, M. Grebe, L. Bako, G. Samuelsson, Evidence for a protein transported through the secretory pathway en route to the higher plant chloroplast, Nat. Cell Biol. 7 (2005) 1224–1231.
[41] P. Moreau, J.J. Bessoule, S. Mongrand, E. Testet, P. Vincent, C. Cassagne, Lipid trafficking in plant cells, Prog. Lipid Res. 37 (1998) 371–391.
[42] C. Xu, J. Fan, W. Riekhof, J.E. Froehlich, C. Benning, A permease-like protein involved in ER to thylakoid transfer in *Arabidopsis*, EMBO J. 22 (2003) 2370–2379.
[43] I.F. Chang, A. Curran, R. Woolsey, D. Quilici, J.C. Cushman, R. Mittler, A. Harmon, J.F. Harper, Proteomic profiling of tandem affinity purified 14-3-3 protein complexes in *Arabidopsis thaliana*, Proteomics 9 (2009) 2967–2985.
[44] M. Charpentier, R. Bredemeier, G. Wanner, N. Takeda, E. Schleiff, M. Parniske, *Lotus japonicus* CASTOR and POLLUX are ion channels essential for perinuclear calcium spiking in legume root endosymbiosis, Plant Cell 20 (2008) 3467–3479.
[45] S. Han, R. Tang, L.K. Anderson, T.E. Woerner, Z. Pei, A cell surface receptor mediates extracellular Ca^{2+} sensing in guard cells, Nature 425 (2003) 196–200.
[46] S. Weinl, K. Held, K. Schlucking, L. Steinhorst, S. Kuhlgert, M. Hippler, J. Kudla, A plastid protein crucial for Ca^{2+}-regulated stomatal responses, New Phytol. 176 (2008) 675–686.
[47] D. Siemen, C. Loupatatzis, J. Borecky, E. Gulbins, F. Lang, Ca^{2+}-activated K^+ channel of the BK-type in the inner mitochondrial membrane of a human glioma cell line, Biochem. Biophys. Res. Commun. 257 (1999) 549–554.
[48] V. Shoshan-Barmatz, A. Israelson, The voltage-dependent anion channel in endoplasmic/sarcoplasmic reticulum: characterization, modulation and possible function, J. Membr. Biol. 204 (2005) 57–66.
[49] M. Köttgen, T. Benzing, T. Simmen, R. Tauber, B. Buchholz, S. Feliciangeli, T.B. Huber, B. Schermer, A. Kramer-Zucker, K. Hopker, K.C. Simmen, C.C. Tschucke, R. Sandford, E. Kim, G. Thomas, G. Walz, Trafficking of TRPP2 by PACS proteins represents a novel mechanism of ion channel regulation, EMBO J. 24 (2005) 705–716.
[50] I. Szabò, J. Bock, H. Grassmè, M. Soddemann, B. Wilker, F. Lang, M. Zoratti, E. Gulbins, Mitochondrial potassium channel Kv1.3 mediates Bax-induced apoptosis in lymphocytes, Proc. Natl Acad. Sci. USA 105 (2008) 14861–14866.
[51] A. Szewczyk, W. Jarmuszkiewicz, W.S. Kunz, Mitochondrial potassium channels, IUBMB Life 61 (2009) 134–143.
[52] J. Balss, P. Papatheodorou, M. Mehmel, D. Baumesiter, B. Hertel, N. Delaroque, F.C. Chatelain, D.L. Minor, J.L. Van Etten, J. Rassow, A. Moroni, G. Thiel, Transmembrane domain length of viral K^+ channels is a signal for mitochondria targeting, Proc. Natl Acad. Sci. USA 105 (2008) 12313–12318.
[53] S.J. Roy, M. Gilliham, B. Berger, P.A. Essah, C. Cheffings, A.J. Miller, R.J. Davenport, L.H. Liu, M.J. Skynner, J.M. Davies, P. Richardson, R.A. Leigh, M. Tester, Investigating glutamate receptor-like gene co-expression in *Arabidopsis thaliana*, Plant Cell Environ. 31 (2008) 861–871.
[54] A. Marmagne, M. Vinauger-Douard, D. Monachello, A.F. de Longevialle, C. Charon, M. Allot, F. Rappaport, F.A. Wollman, H. Barbier-Brygoo, G. Ephritikhine, Two members of the *Arabidopsis* CLC (chloride channel) family, AtCLCe and AtCLCf, are associated with thylakoid and Golgi membranes, respectively, J. Exp. Bot. 58 (2007) 3385–3393.
[55] Z. Qi, N.R. Stephens, E.P. Spalding, Calcium entry mediated by GLR3.3, an *Arabidopsis* glutamate receptor with a broad agonist profile, Plant Physiol. 142 (2006) 963–971.
[56] R. Davenport, Glutamate receptors in plants, Ann. Bot. 90 (2002) 549–557.
[57] D. Tapken, M. Hollmann, *Arabidopsis thaliana* glutamate receptor ion channel function demonstrated by ion pore transplantation, J. Mol. Biol. 383 (2008) 36–48.
[58] I.I. Pottosin, J. Muniz, S. Shabala, Fast-activating cation channel controls cation fluxes across the native chloroplast envelope, J. Membr. Biol. 204 (2005) 145–156.