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# Characterization of a Plant Glutamate Receptor Activity

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**Key Words**

Glutamate receptor • Chloroplast membrane vesicle • Black lipid membrane • Oxygen evolution

**Abstract**

Bioinformatic approaches have allowed the identification of twenty genes, grouped into three subfamilies, encoding for homologues of animal ionotropic glutamate receptors (iGLRs) in the *Arabidopsis thaliana* model plant. Indirect evidence suggests that plant iGLRs function as non-selective cation channels. In the present work we provide biochemical and electrophysiological evidences for the chloroplast localization of glutamate receptor(s) of family 3 (iGLR3) in spinach. A specific antibody, recognizing putative receptors of family 3 locates iGLR3 to the inner envelope membrane of chloroplasts. In planar lipid bilayer experiments, purified inner envelope vesicles from spinach display a cation-selective electrophysiological activity which is inhibited by DNQX (6,7-dinitroquinoxaline-2,3-dione), considered to act as an inhibitor on both animal and plant iGLRs. These results identify for the first time the intracellular localization of plant glutamate receptor(s) and a DNQX-sensitive, glutamate-gated activity at single channel level in native membrane with properties compatible with those predicted for plant glutamate receptors.

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## Introduction

In vertebrates, ionotropic glutamate receptors (iGluRs) are ligand-gated cation channels that mediate the majority 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.

In the model plant *Arabidopsis thaliana*, 20 genes encoding homologues of animal iGluRs have been identified [1]. According to phylogenetic analyses, the *A. thaliana* glutamate receptor homologues can be subdivided into three separate subgroups [2, 3]. Studies with transgenic plants suggested involvement of members of the plant GLR family in $Ca^{2+}$ fluxes (e.g. [4]). In various cell types of plants, the agonists glutamate and glycine

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induced depolarisation and a rise in intracellular $Ca^{2+}$ concentration that were inhibited by blockers of non-selective cation channels (NSCCs) and by antagonists of animal iGluRs, including DNQX and CNQX (6-cyano-7-nitro-quinoxaline-2,3-dione) [5-8]. Furthermore, glutamate-activated cation currents in patch-clamped root protoplasts were inhibited by NSCC blockers such as $La^{3+}$ and $Gd^{3+}$ [9]. Therefore, it was proposed that plant iGLRs can form $Ca^{2+}$-permeable NSCCs, are inhibited by animal iGLR antagonists and contribute to the shaping of plant $Ca^{2+}$ signaling [e.g. 10, 11]. However, a direct demonstration that these putative iGLRs function as such, is still missing.

Most plant iGLRs, when expressed in heterologous systems, do not give rise to any current (in oocytes), are toxic for host cells (e.g. in mammalian cells) [12] or are retained in the endoplasmatic reticulum [13]. In the few cases of successful channel expression, the observed activity was not sensitive to agonists and/or antagonists. The electrophysiological characterization in the plasmamembrane (PM) of plants is rendered difficult by the redundancy of iGLRs and by numerous channel activities present in the PM. Recently, in order to examine whether AtGLR homologues possess functional ion channel domains, Tapken and Hollmann [14] 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 $La^{3+}$ [14].

Various localization prediction tools suggest that some of the plant iGLRs might have chloroplast or mitochondrial targeting sequences. However, no experimental evidence has been presented up to now concerning a possible intracellular localization of iGLRs. During the last decade,several ion channels and transporters have been identified in the outer and inner membranes of chloroplast envelope as well as in thylakoids [15, 16], but the pharmacology and molecular identity of these channels is still largely unknown. This fact mostly prevented also the determination of the physiological role of these channels. Some of the exception concerning molecular identification regards the presence of two proteins of the ClC chloride channel family, ClC-e and ClC-f in the outer envelope of chloroplasts [17] and outer envelope channels OEP37 and OEP16 [18, 19]. It has previously been hypothesized that at least a part of the chloroplast channels may play an essential role in the physiology of this organelle, e.g. by regulating photosynthesis. Beside thylakoid channels, others are expected to allow rapid ion fluxes across envelope membranes. Despite numerous indications in the literature concerning physiological variations in the concentration of different ions (for example for calcium see e.g. [20]), in most cases the molecular entities that mediate ion fluxes have not been identified.

In the present paper, using a specific antibody raised against putative glutamate receptors of family 3, we demonstrate the localization of member(s) of the subgroup 3 in the inner envelope of spinach leave chloroplasts, where electrophysiological experiments indicate the presence of an activity that is compatible with that of glutamate receptor(s) being cation-selective, sensitive to glutamate and inhibited by DNQX. Furthermore, oxygen evolution measurements suggest that chloroplast-located glutamate receptor(s) may play a role in the regulation of photosynthesis.

## Materials and Methods

*Plant material*

Freshly harvested spinach leaves were purchased at the local market.

*Purification of chloroplasts*

Chloroplasts were isolated on a discontinous (10/40/75 %) Percoll gradient [21] in order to separate intact chloroplast. Chlorophyll concentration was measured after washing out Percoll.

*Purification of outer, inner and thylakoid membrane vesicles*

Vesicles were prepared following the procedures described in [17]. Briefly, Percoll-purified chloroplasts were incubated in 0.7 M sucrose, broken by 50 strokes and diluted to 0.3 M sucrose. Membranes were separated on a discontinuous sucrose gradient at 120000 g for 3 h. PMSF was added throughout the procedure and all steps were performed at 4 °C.

*Chloroform/Methanol precipitation*

Membrane proteins were precipitated with different mixtures of chloroform/methanol according to [22]. 1 mg of protein from total extract was mixed with different chloroform/methanol ratio: after centrifugation, pellets were resuspended in 200 µL of sample buffer. 40 µL of each sample were loaded on SDS-PAGE.

*Gel Electrophoresis and Western Blotting*

Proteins were separated in SDS/7.5% PAGE in the presence of 6 M urea [17]. Gels were transblotted onto poly-



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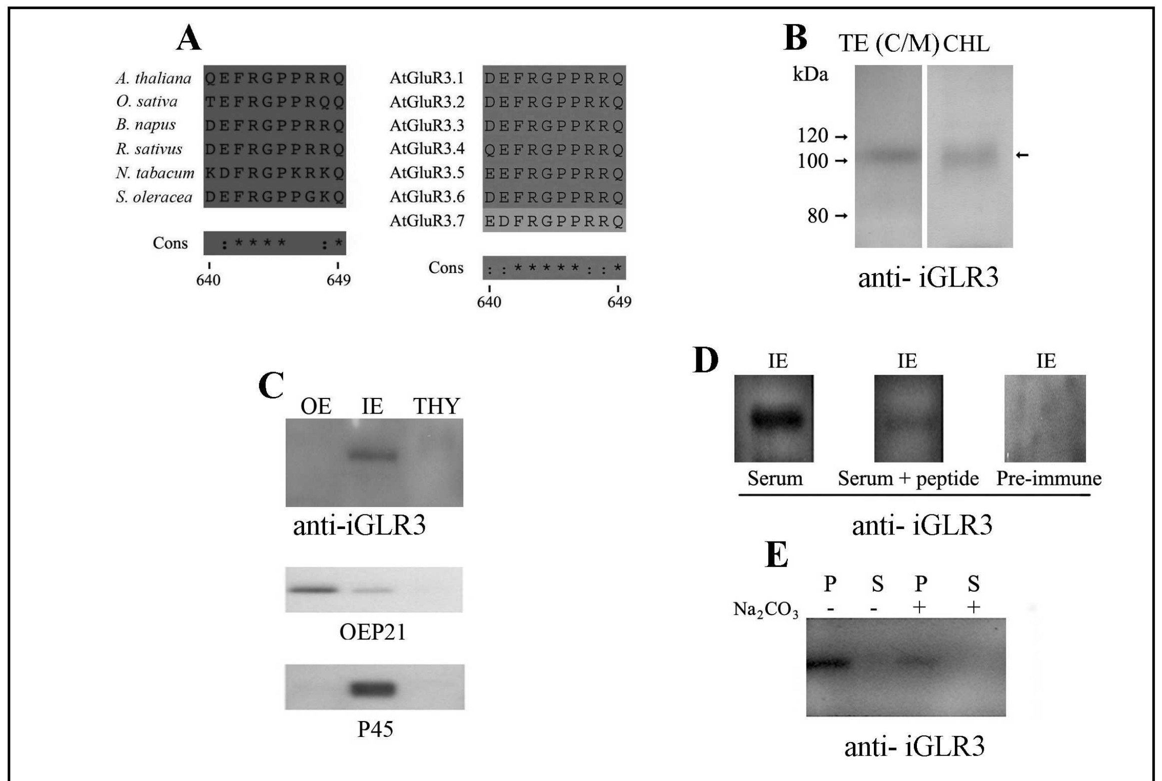


**Fig. 1.** Glutamate receptor(s) of family 3 are located in the chloroplast inner membrane in spinach. A) A peptide, corresponding to aminoacids 640/649 in AtGLRs of family 3 was chosen for the antibody production (QEFRGPPRRQ). This sequence is highly conserved in glutamate receptors of family 3 in *Arabidopsis* (right panel) (but not in members of family 1 and 2) and is conserved in iGLRs from different plants (left panel; *Arabidopsis thaliana* gi:30679161; *Oryza sativa* gi:125600477; *Brassica napus* gi:6650552; *Raphanus sativus* gi:33304542; *Nicotiana tabacum* gi:30013669). T-Coffee algorithm was used for multiple alignment. "*" : residues which are identical in all sequences in alignment; ":": conserved, ".":semi-conserved substitutions. Shading is indicative of degree of homology (dark grey for highest and light grey for lower homology). B) Western blot of spinach total extract treated with chloroform/methanol (TE(C/M)) and chloroplasts (CHL), developed with anti-iGLR3 antibody (30 µg of chloroplasts). C) equal protein quantity (15 µg) of vesicles from outer (OE), inner (IE) and thylakoid (THY) membranes assayed (upper panel). Antibodies against outer-membrane marker OEP21 and inner-membrane marker P45 were used to check for cross-contamination of inner and outer membrane vesicle preparations (lower panel). D) Western blot of inner membrane (IE) assayed with anti- iGLR3 antibody (from the left: serum, serum pre-incubated with its immunogenic peptide at 300 µM for 1h and the pre-immune serum). E) alkaline extraction of inner membrane vesicles; after $Na_2CO_3$ (0.1 M) treatment, the vesicles (50 µg) were centrifuged. P: pellets (recovered vesicles); S: supernatants.

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vinylidene difluoride (PVDF) membranes and decorated with protein A-purified sera against a.a. 640/649 of iGLR3 (see Fig. 1) applied at 1:1.000 dilution for 2 h. Antibody against the indicated peptide 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).

*Genetic material*

The spinach GLR fragment was isolated by RT-PCR and 3' RACE (5'/3' RACE Kit, 2nd Generation, Roche Diagnostics) using the following forward primer 5'-AGA AGT CAT TGC GAT ATT CGT TGA TGA TGA-3' and reverse primer 5'-ACG GTC AGG AAT GAA GTC AAA CTA GCT G-3' and has been directly cloned into the pGEM-T Easy vector (Promega, USA). All constructs were sequenced at BMR-Genomics (Padova, Italy).

*Sequence analysis*

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

*Planar Lipid Bilayer*

Use was made of a Warner Instruments (Hamden, CT, USA) electrophysiological planar bilayer apparatus. Bilayers of approx. 150-200 pF capacity were prepared by painting a chloroform or decane solution of soybean azolectin (Sigma, St. Louis, Mo.) partially purified by precipitation with cold acetone from a chloroform solution, across a 250-µm hole in a polystirene cuvette. The inside of the cuvette constituted the trans compartment. The standard experimental medium was 100 or 300 mM $BaCl_2$ (or $CaCl_2$ gradient in some cases) 10 mM Hepes/$Ba^{2+}$, pH 7.2. 5 mM glutamate and 10 mM glycine were included if not specified otherwise. The lipid membrane was built under symmetric ionic conditions and the gradient was created afterwards, by adding a concentrated salt solution on one side. The contents of both chambers were stirred by magnetic bars when desired. Connections to the electrodes were provided by agar bridges. Vesicles were added to the *cis* side. All voltages reported are those of the *cis* chamber, zero being assigned by convention to the *trans* (grounded) side. Currents are considered as positive when carried by cations flowing from the cis to the trans compartment. A BC-525C unit and headstage were used to control parameters and amplify signals. Output was recorded with a 10 kHz bandwidth on videotape using a Medical Systems (New York) PCM-2 interface and later processed offline using the pClamp program set (Axon Instruments, Union City, CA, USA).

*Oxygen evolution*

Leaves were homogenized in 340 mM sorbitol, 0.4 mM KCl, 0.04 mM EDTA, 2 mM HEPES, pH 7.8, filtered twice, and centrifuged for 10 s at 3500 g [23]. Pellets were resuspended in the assay medium. Experiments were performed using a Clark electrode (Hansatech) as described in [24]. Briefly, the assay medium for intact chloroplasts contained 330 mM sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 50 mM HEPES–KOH (pH 7.6), 0.2% bovine serum albumin and 10 mM D,L-glyceraldehyde (included to inhibit $CO_2$ fixation). The intactness of chloroplasts was assayed by the Hill reaction [24]. $K_3Fe(CN)_6$ (2.7 mM) was added as electron acceptor. Thylakoids were obtained by osmotic shock of intact chloroplasts (corresponding to 78 µg Chlorophyll, diluted in 620 µl sterile water) and then assayed in the same final medium as above. Chorophyll concentration was 60 µg/ml in all experiments and oxygen evolution was measured at 25 °C in the dark, followed by application of light with an intensity of 2000 µE $m^{-2}$ $s^{-1}$. Oxygen evolution in control condition in intact chloroplasts was 15.11 ± 0.99 (SEM) µmol $O_2$/(mg Chl h) (n = 20) while in osmotically shocked chloroplasts (thylakoids) it was 49.6 ± 2.8 µmol $O_2$/(mg Chl h) (n = 20). Chloroplasts or thylakoids were pre-incubated with inhibitors for 10 min in the dark on the ice prior to measurement. Controls were performed to exclude any effect of the solvent added with the inhibitors. Oxygen evolution was determined by using the FIP program (Finland) and expressed as µmol $O_2$/(mg Chl h). The absolute values measured under control conditions varied slightly among preparations depending on spinach sample, and oxygen evolution values in the presence of the inhibitor were normalized accordingly.

## Results

The exact localization of most plant glutamate receptors still needs to be clarified. Among the members of subgroup 3 (iGLR3) 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.

To investigate the possible chloroplast localization of iGLR3, we developed a polyclonal antibody against a synthetic peptide corresponding to a highly conserved region of glutamate receptors of family 3 from different plants (Fig. 1A, left panel) and in those belonging to *Arabidopsis* subgroup 3 (Fig. 1A, right panel). The antibody anti-iGLR3 efficiently recognized the synthetic peptide in dot blots (not shown).

First we checked whether our antibody could recognize any protein in spinach (*Spinacia oleracea*) chloroplasts. As shown in Fig. 1B, this was indeed the case. The anti-iGLR3 antibody recognized a band at around 103 kDa in total extract treated with chloroform/methanol extraction as well as in Percoll-purified spinach chloroplasts. The aminoacid sequences and predicted molecular weights of iGLRs in spinach are not known and may well be slightly different from those predicted for *Arabidopsis* (between 85 to 115 kDa for iGLR3). Therefore we determined the sequence of an iGLR subgroup 3 member from spinach, including the region corresponding to the epitope QEFRGPPRRQ (Fig. 1A and not shown for the nucleotide sequence ). Given that this sequence in spinach is DEFRGPPGKQ (the sequence EFRGPP is identical in *Arabidopsis* iGLRs of subgroup 3), it is more than reasonable to assume that the anti-iGLR3 antibody indeed recognizes iGLRs of subgroup 3 in spinach.

To establish the exact location of iGLR3s within the chloroplast and for further functional studies, outer, inner and thylakoid membranes were isolated from spinach leaves, given that to our knowledge, suitable preparations of this type cannot be obtained from *Arabidopsis*. When assaying the subchloroplast location in purified membrane vesicles, the antibody recognized a 103 kDa band exclusively in the inner membrane vesicles (Fig. 1C). Cross-contamination of these vesicles by other membranes was negligible as shown in Figure 1C. The specificity of the antigen recognition is indicated by a marked decrease in the intensity of the bands when using antibody preincubated with its immunogenic peptide (Fig. 1D). As

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a further control, alkaline extraction was performed, indicating that the 103 kDa protein is indeed an integral membrane protein (Fig. 1E). Data of Fig. 1 indicate the presence of at least one member of iGLR(s) of subgroup 3 in spinach, suggesting that spinach inner membrane vesicles are suitable for functional studies.

Whether plant, non-chimaeric glutamate receptors form functional ion channels or not is still an open question. Furthermore, in order to find out the physiological role(s) of these proteins in the chloroplasts, it is important to discover whether they function as ion channels or not. In plasma membrane of mesophyll cells expressing AtGLR3.4, a DNQX (6,7-dinitroquinoxaline-2,3-dione), MNQX (5,7-Dinitro-1,4-dihydro-2,3-quinoxalinedione) and CNQX (6-cyano-7-nitro-quinoxaline-2,3-dione)-sensitive, glutamate induced inward calcium ion flux (as shown by using aequorin) was described [7]. However, current carried by glutamate receptor(s) was not measured directly in that work. Although the patch clamp technique has successfully been applied to chloroplast membranes (see e.g. [25, 26]), it is technically demanding and does not allow to easily exclude other cationic activities known to be present in the inner envelope membrane [15], due to the presence of potassium in the stroma. An alternative, widely used approach is that of incorporating isolated proteins or inner membrane vesicles in a planar lipid bilayer [27-30]. This experimental setup allows direct access to the solution bathing the channel and complete control of the ionic conditions. If working with a physiological solution, i.e. one containing KCl, one would expect to observe glutamate receptor activity, but also currents carried by potassium (and chloride) channels. Ionic conditions were chosen considering that iGLRs are not expected to be the only proteins giving rise to channel activity: given numerous indications in the literature concerning the capability of GLRs to allow calcium ion flux, in some experiments calcium was used as the only cation. Fig. 2A. reports a representative activity recorded in such a medium. The low open probability in the absence of glutamate and glycine (upper two traces) significantly increased upon addition of these two agonists (lower traces). Glutamate and glycine are considered to act as agonists of various plant glutamate receptors [5-7]. The sustained increase of the current was not due to an aspecific leak (gating is observable in the lower right panel). In most cases barium was used as the only cation, since it is an inhibitor of potassium channels ((known to be present in the chloroplast inner membrane) [15]) but permeates animal and some plant iGLRs [31]. A cation gradient (300-100 mM *cis:trans)* was applied, glutamate

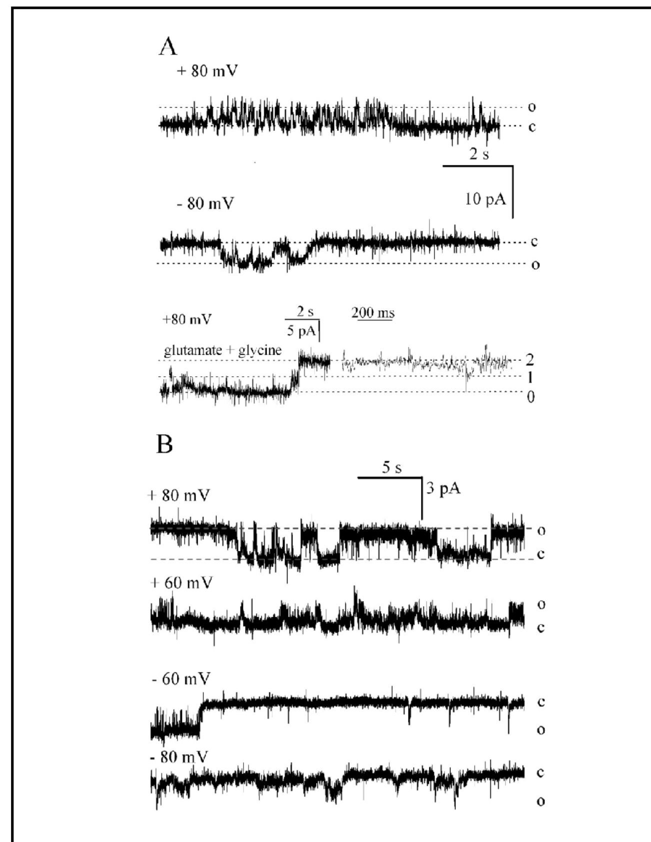


**Fig. 2.** Chloroplast inner envelope vesicles from spinach display a divalent cation-conducting channel activity. A) Channel activity recorded at the indicated voltages in a medium containing calcium as the only charge carrier. The conductance was 35 pS in this medium. The upper two traces were recorded in the absence of added glutamate and glycine. The third current trace was recorded few seconds after addition of 5 mM glutamate and of 10 mM glycine to both chambers (contents were mixed by stirring for about 30 seconds). Right current trace in the third row illustrates gating of the channel, recorded 5 minutes following addition of agonists, on an expanded time scale. B) Ion channel activity recorded at the indicated voltages in a planar lipid bilayer setup in barium chloride medium in the presence of a gradient (300 mM: 100 mM).

and glycine were added to both sides of the artificial membrane and inner membrane vesicles prepared from spinach chloroplasts were added directly to the *cis* chamber (about 5 µg of total protein). In barium-based medium a cation-selective (Fig. 2B, ) as well as an anion-selective (not shown) activity could be observed, but only rarely, in accordance with low abundance observed by Western blots. Fig. 2B. shows an example of cation-selective barium-conducting currents recorded at various holding potentials. The conductance was relatively small, 25 pS in this system. According to Nernst's equation, the

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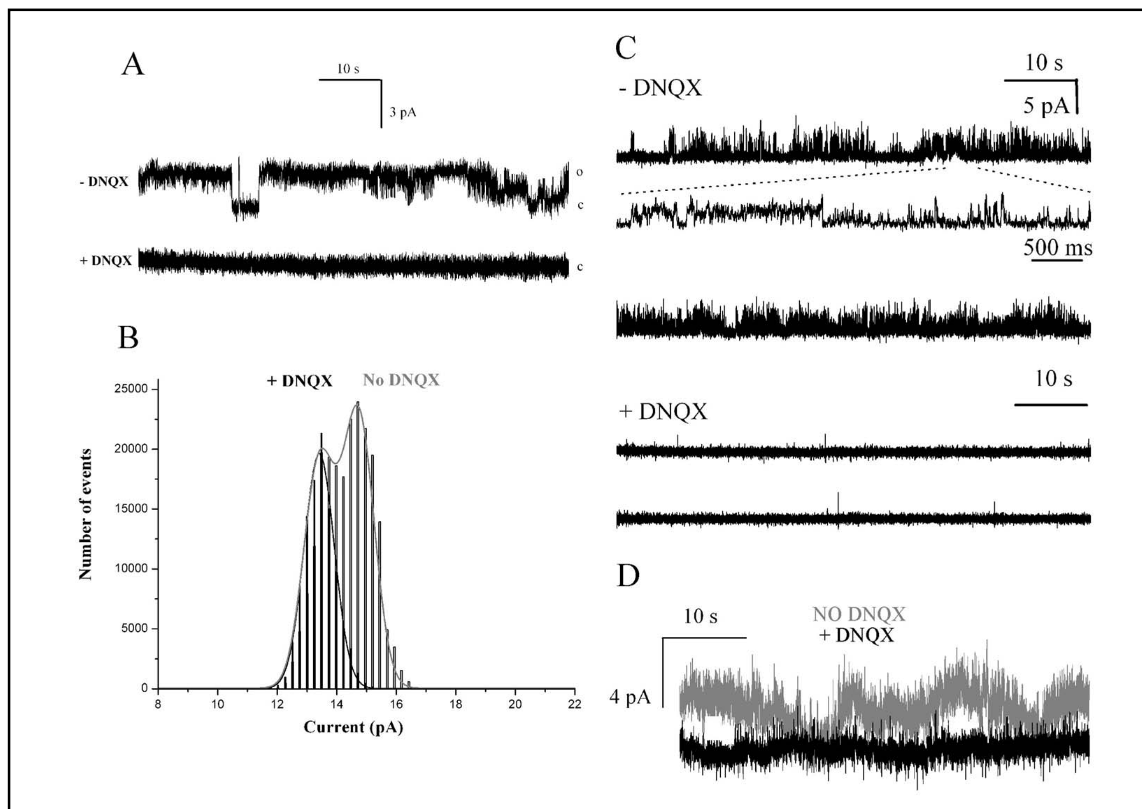


**Fig. 3.** DNQX, an antagonist of animal ionotropic glutamate receptors inhibits channel activity. A) Representative current traces recorded at +80 mV before (upper trace) and after (lower trace) addition of 100 µM DNQX to both chambers. B) Amplitude histograms obtained from the same experiment shown in A). Without inhibitor (gray), the open and closed states of the ion channel are visible; after the addition of DNQX only the closed state remains (black). Leak current (of 13 pA) was not subtracted. Amplitude histograms were obtained from 6.5 (without DNQX) and 4 (with DNQX) minutes of recording respectively, were fitted with the Origin program and the closed state histogram event number was scaled down by a factor of 4. C) Representative current traces recorded at + 90 mV from another experiment before (upper 3 traces) and after (lower 2 traces) addition of 100 µM DNQX to both chambers. 6.5 minutes elapsed between the first and the third trace (the second trace illustrates activity of the indicated region on an expanded time scale), showing that channel activity was stable for a long period in the absence of the inhibitor and no spontaneous rundown occurred. After inhibition, 7.1 minutes elapsed between the two lower traces illustrating that inhibition was not transient. D) Averaged current from 4 different experiments before (gray current trace) and 20 seconds to 4 minutes after (black trace) addition of the inhibitor. In C) and D) openings are upward deflections.

theoretical reversal potential for barium is -14 mV, while for anions a reversal potential of +28 mV is predicted under the ionic conditions used here. Although the small conductance and the high noise level typical of bilayer experiments did not allow us an accurate determination of the reversal potential, the $E_{rev}$ values were negative (-5 to -10 mV) indicating a cation selectivity of the observed ohmic activity (not shown).

DNQX is considered to act as an inhibitor of both animal and plant glutamate receptors [1, 7, 32, 33]. Since the orientation of the putative iGLRs in the inner vesicles is not known, 100 µM DNQX was added to both chambers. Fig. 3A and 3C show representative traces from two different experiments in control conditions and after addition of DNQX. The amplitude histograms reported in Fig. 3B before (gray) and after (black) addition of DNQX were obtained from the experiment shown in Fig. 3A. These data demonstrate that the 25 pS activity was fully inhibited by DNQX. Fig. 3D reports the current averaged over a 50 sec period from 4 different experiments before (gray trace) and after (black trace) the addition of the drug. DNQX had no effect on the anionic chan-



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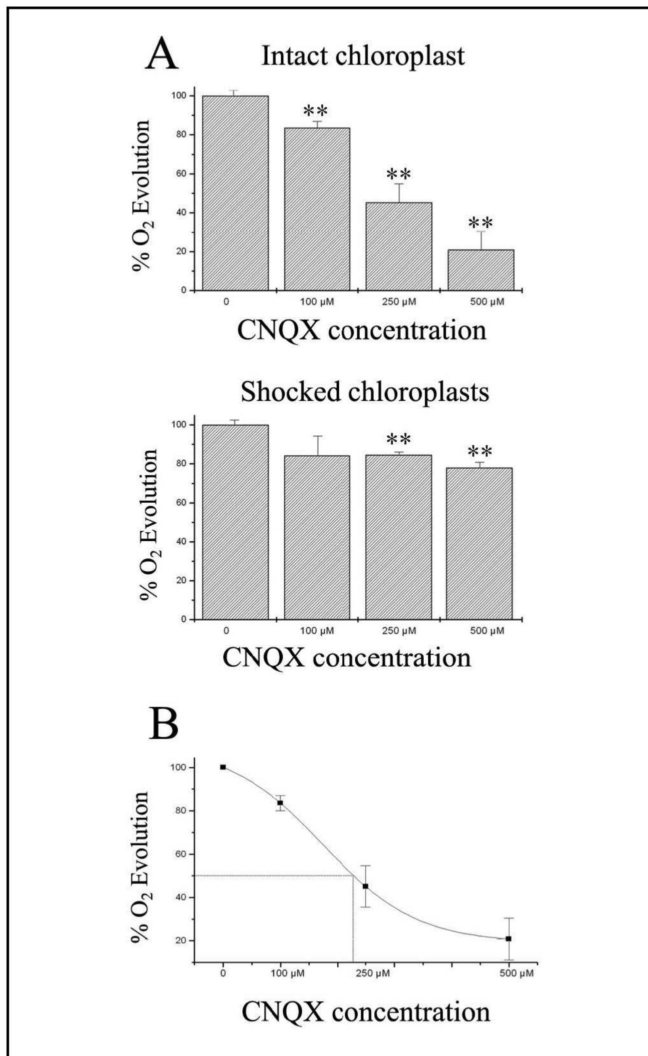


**Fig. 4.** Oxygen evolution by spinach chloroplasts is reduced in the presence of CNQX. Oxygen evolution was measured in intact and shocked (thylakoid) chloroplasts. Oxygen evolution measured in the absence of the inhibitor was considered as 100% and the values measured in the presence of CNQX were normalized (expressed as % of the initial value). (A) With CNQX, the oxygen evolution decreased in a dose-dependent manner in intact chloroplasts (upper panel), while only a slight decrease was observable in thylakoids (lower panel). Mean ± S.D. values are reported for independent experiments (n=17 for 100 µM, n= 5 for 250 µM and n= 9 for 500 µM CNQX). Independent unpaired t-tests were performed. Significant differences with respect to control are marked by asterisks (** $p < 0.05$). B) The $IC_{50}$ value for CNQX is 230 µM.

nels that were observed (not shown). The above data indicate that a glutamate-sensitive, DNQX-inhibited, divalent cation-permeable ion channel is present in the inner envelope membrane of spinach chloroplasts.

Considering that the channel activity we observed is able to conduct $Ca^{2+}$, a physiological role of this channel in regulating oxygen evolution seemed possible. Calcium is known to be required for the function of the oxygen evolving complex [34]. To test our hypothesis, oxygen evolution measurements were performed to evaluate the effect of proposed glutamate receptor inhibitors on photosynthesis. DNQX however had a clear uncoupling effect when added to isolated thylakoids (not shown), therefore a related compound, CNQX was used. CNQX has also been proposed to act on plant glutamate receptors and is expected to permeate the outer membrane. Fig. 4 shows the effect of these inhibitors on intact and osmotically shocked chloroplasts (thylakoids). Inhibition values were calculated with respect to control measurements (100% of oxygen evolution) (see experimental procedures and [35]). Increasing concentrations (100-500 µM) of CNQX induced a dose-dependent inhibition up to 90% in intact chloroplast (Fig. 4A, upper panel). CNQX had only a slight effect on thylakoids (Fig. 4A, lower panel) suggesting that it acts on a protein located in the envelope membrane. The $IC_{50}$ value has been calculated (Fig. 4B) for CNQX: 50% inhibition of oxygen evolution can be observed at about 230 µM CNQX.

## Discussion

In the present work we show the presence of putative glutamate receptor(s) protein belonging to family 3 (iGLR3) in chloroplasts of spinach, in particular in the inner envelope membrane. This is the first indication on the presence of an iGLR in an organelle. Furthermore, a DNQX-sensitive activity, displaying characteristics compatible with those predicted for iGLRs could be observed in purified inner membrane vesicles from spinach by planar bilayer studies. CNQX, another proposed modulator of iGLRs impaired oxygen evolution during photosynthesis when isolated chloroplasts were treated with this substance. This work thus provides the first experimental evidence for the electrophysiological activity of a DNQX-sensitive channel in native membranes at the single channel level and identifies a chloroplast inner envelope component from a biochemical point of view.

The mechanims of targeting to the chloroplasts is not known. In mammalian system, several ion channels are located in multiple membranes within the cell (e.g. [35-39]). In a recent paper Roy and colleagues [31] studied the expression profile of *AtGLR*s in different cell types in single cells by using the MicroExpression amplification

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(MEX) method. Various AtGLR3 members were present in chloroplast-containing mesophyll cells but only *AtGLR3.7* transcripts were detectable in every cell. It cannot be excluded that *Arabidopsis* as well as in spinach various members of the iGLR3 subgroup may have redundant functions in forming chloroplast iGLRs. Further work is required to identify which member(s) of the iGLR3 family is(are) located to the chloroplasts.

In the inner envelope membrane (IE), a 160 pS (in 150/25 mM KCl) potassium-conducting channel has been found. 10 mM tetraethylammonium ($TEA^+$), a general blocker of $K^+$ channels, and 5 mM $Mg^{2+}$ and $Ba^{2+}$ [40] reduced the magnitude of Rb-tracer fluxes. A 100 pS (250/20 mM KCl) cation-selective channel has been shown to be activated upon the addition of 1 mM $MgCl_2$ and to be inhibited by 1 mM ATP [41]. A fast-activating cation channel is also present in chloroplast envelope [26]. None of these activities match the one described here and attributed to iGLRs considering biophysical properties like conductance, kinetics and pharmacology. The channel described by Pottosin and co-workers has a conductance compatible with that observed by us, however the effect of iGLR inhibitors was not investigated in that work. No *bona fide* calcium channel protein has been identified at the molecular level in the inner envelope. A ΔpH and voltage-dependent calcium uniporter, found in inner envelope membrane of pea, has been proposed to mediate influx of calcium into the stroma [42]. In addition, the putative CaM-regulated $Ca^{2+}$-ATPase AtACA1 has been reported to be located at the inner envelope [43], although in another study AtACA1 has been located to ER by proteomic analysis [44] and no detectable $Ca^{2+}$-ATPase activity can be detected at the inner envelope membrane [42].

AtGLR3.7 as well as AtGLR3.4 have been shown to give rise to an agonist/antagonist insensitive, voltage-independent cation conductance permeable to $Na^+$, $Ca^{2+}$ and $Ba^{2+}$ when expressed in *Xenopus* oocytes [7, 31]. We recorded a channel behaving as a $Ba^{2+}$-permeable voltage-independent channel in inner membrane vesicles. The fact that we observe channel activity in a medium containing exclusively divalent (barium or calcium) but not monovalent cations as well as the fact that this activity is clearly inhibited by DNQX, considered to be an inhibitor of plant iGLRs [1, 7, 32, 33], suggests that the channel recorded in our system is a divalent cation-permeable iGLR. It has to be mentioned however, that DNQX has been reported to inhibit glutamate-induced calcium fluxes in plants but direct evidence for the action of this drug on iGLR channels is still missing. A glutamate-induced current was shown to be blocked only by $La^{3+}$ and $Gd^{3+}$ which are general blockers of NSCCs, calcium and mechanosensitive channels. No data is available in the literature concerning the single channel properties of plant iGLRs (for a recent review see [45]), however a conductance of 25 pS is compatible with the observed conductances of animal iGLRs [46]. In summary, our data provide evidence that a glutamate-sensitive, DNQX-inhibited, divalent cation permeable channel is present in the inner envelope membrane vesicles of spinach chloroplasts. These properties are compatible with those predictable for iGLRs, and we detect by biochemical tools an iGLR of family 3 in these membrane vesicles. However a definitive identification of the observed channel activity as iGLR will be only possible when the effect of DNQX and CNQX on recombinant iGLRs is proven.

Calcium ions are essential for oxygen evolution, since calcium depletion modifies the structure of the PSII oxygen evolving complex and results in an almost complete abolition of $O_2$ evolution [34]. In our experiments CNQX caused an inhibition by 50 % of oxygen evolution at 230 μM. This value is in good agreement with that observed for AtGLR3.4 by Meyerhoff and colleagues [7]. In the oxygen evolution experiments no glutamate and glycine were added to isolated chloroplasts given that glutamate is expected to be present in both stroma and inter-envelope space, due to the release of glutamate in exchange with external malate via a dicarboxylate translocator has been described [46]. In addition to this, a translocator catalyzing the exchange of glutamine against glutamate has been found [47]. Whether the inner-envelope located iGLRs are effectively gated or not by physiological concentrations of glutamate remains to be confirmed. In our experiments glutamate and glycine increased the open probability of the observed channel. While several reports describe the presence of glutamate-induced cationic currents in the plasmamembrane of various cells, AtGLR3.7 and AtGLR3.4 have been reported to give rise to a glutamate-independent activity when expressed in *Xenopus oocytes* [7, 31].

In conclusion, the present work provides evidence for a chloroplast location of iGLR(s) of subgroup 3 in spinach and indicates the functional expression of a DNQX-sensitive and glutamate-activated channel activity in the inner envelope membrane.



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