Loop A Is Critical for the Functional Interaction of Two Beta vulgaris PIP Aquaporins

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Abstract

Research done in the last years strongly support the hypothesis that PIP aquaporin can form heterooligomeric assemblies, specially combining PIP2 monomers with PIP1 monomers. Nevertheless, the structural elements involved in the ruling of homo versus heterooligomeric organization are not completely elucidated. In this work we unveil some features of monomer-monomer interaction in Beta vulgaris PIP aquaporins. Our results show that while BvPIP2;2 is able to interact with BvPIP1;1, BvPIP2;1 shows no functional interaction. The lack of functional interaction between BvPIP2;1 and BvPIP1;1 was further corroborated by dose-response curves of water permeability due to aquaporin activity exposed to different acidic conditions. We also found that BvPIP2;1 is unable to translocate BvPIP1;1-ECFP from an intracellular position to the plasma membrane when co-expressed, as BvPIP2;2 does. Moreover we postulate that the first extracellular loop (loop A) of BvPIP2;1, could be relevant for the functional interaction with BvPIP1;1. Thus, we investigate BvPIP2;1 loop A at an atomic level by Molecular Dynamics Simulation (MDS) and by direct mutagenesis. We found that, within the tetramer, each loop A presents a dissimilar behavior. Besides, BvPIP2;1 loop A mutants restore functional interaction with BvPIP1;1. This work is a contribution to unravel how PIP2 and PIP1 interact to form functional heterooligomeric assemblies. We postulate that BvPIP2;1 loop A is relevant for the lack of functional interaction with BvPIP1;1 and that the monomer composition of PIP assemblies determines their functional properties.

Introduction

Plant water channels are a huge family of proteins; among them, PIP (plasma membrane intrinsic proteins) aquaporins are important water transporters. Traditionally, it is considered that PIP aquaporins cluster in two groups: PIP1 and PIP2, but recently it was reported that the common ancestor of mono and dicot plant aquaporins could have three types of PIPs that originated three PIP-like clusters instead of two: i- PIP Cluster I (PIPCLI), which corresponds to the classical PIP1 group, ii- PIP Cluster II (PIPCLII) and iii- PIP Cluster III (PIPCLIII), these last two correspond to most PIP2 aquaporins [1]. Regardless the phylogenetic organization, it is considered that all aquaporins are tetramers [2–5]. Notwithstanding, it was demonstrated that each monomer in the tetramer is a functional unit [6,7].

Likewise, as other multimeric proteins, aquaporins oligomers composition could be homooligomeric or heterooligomeric. Among mammal aquaporins, AQP1 was reported to be homotetrameric [8] while AQP4 exists in two splicing isoforms AQP4M1 (starting at Met1) and AQP4M23 (starting at Met23), which could assemble in the plasma membrane as homo or heterotetramers [9] and are also able to be organized in orthogonal arrays of particles (OAPs) [10]. Regarding plant aquaporins, major research about tetramer composition has been performed for the PIP family. Experimental evidence points that PIP aquaporins can be organized as heterooligomers under particular, not yet fully determined, circumstances. Although the physical and functional interaction among different PIPs has been probed, it is still not elucidated if the protein complex formed is heterooligomeric (homotetramers of different PIPs in contact) or heterotetramers (different PIP monomers organized in a single tetramer). The functional interaction was mainly studied by means of co-injection of PIP cRNA in Xenopus laevis oocytes and many results show that an interaction between different PIPs occurs. For instance, this interaction was reported among maize PIPs, for ZmPIP1;2 with several ZmPIP2; for tobacco NtPIP1;1 with NtPIP2;1, for grape berry VvPIP1;1 with VvPIP2;2 and for wheat TdPIP1;1 with TdPIP2;1 confirming that this behavior occurs
among all PIP groups [11–15]. Moreover, functional and physical interaction was reported between members of a same group [11,16].

Many relevant evidences about PIP interaction can be mentioned. First, a positive cooperation resulting in an increase in the osmotic water permeability (P) of oocyte plasma membrane is seen when ZmPIP2;2 is co-expressed with ZmPIP1;2 [11]. Moreover it was shown for Zea mays PIPs that PIP1;1 are retained in the endoplasmic reticulum (ER) while PIP2 are targeted to the plasma membrane, but they are co-localized in the plasma membrane, as result of their interaction, when co-expressed in the same cell [13]. A second evidence is the modification of pH sensing of oocytes co-expressing BvPIP2;2 and BvPIP1;1 in comparison with oocytes expressing BvPIP2;2 alone [17]. Also co-expression of tobacco PIPs, mAQP1 and mAPIP2;1, studied by means of bimolecular fluorescence complementation (BiFC) experiments, size exclusion chromatography and gel electrophoresis points to the formation of heterotetramers; furthermore functional analysis of artificial tobacco PIP tetramers with a defined proportion of mAQP1 and mAPIP2;1 indicate that membrane permeability was modified by tetramer composition [18]. Finally, a recent work states that the conformational arrangement of maize PIP2 monomers in PIP1-PIP2 heterooligomers is different from that in PIP2 homooligomers [19].

It is interesting to remark that PIPs have a great evolutionary constraint in comparison with other plant aquaporin subfamilies, this high evolutionary constraint may be due to functional constraint [1]. It has been described that proteins that are part of complexes tend to evolve at a relatively slow rate in order to improve the co-evolution with their interacting partners [20]. The high evolutionary constraint found for PIPs can be related to the above-mentioned physical interaction that occurs among different members of the subfamily and modulate their activity.

Among the biological phenomena regulating protein function, control of homooligomerization vs heterooligomerization is an important one. In this context, PIP oligomerization is still emerging as an area of investigation. Aquaporin tetramerization must be studied as a crosstalk mechanism between extracellular signals and water or solute transport, as response. Here, we focused our study in Beta vulgaris BvPIP2;1 which seems to be unable to functionally interact with BvPIP1;1. BvPIP2;1 were studied in terms of functional interaction by means of cRNA co-injection in Xenopus laevis oocytes, pH inhibition experiments and confocal fluorescent microscopy. Furthermore, on the basis of differences found in BvPIP2;1 primary sequence at the first extracellular loop when compared with the same loop of most PIP2, we investigate BvPIP2;1 loop A by direct mutagenesis. We also characterized BvPIP2;1 loop A at an atomic level by Molecular Dynamics Simulation (MDS). From the results obtained in this work we proposed that BvPIP2;1 loop A is involved in the failure of this aquaporin to interact with BvPIP1;1.

We believe this work is a contribution to unravel how PIPs interact to form functional heterologomeric assemblies.

**Materials and Methods**

**Plasmid Constructions**

The coding regions of BvPIP1;1, BvPIP2;1 and BvPIP2;2 (GenBank sequences GQ227845.1, U60148.1 and GQ227846.1, respectively) and the mutants (BvPIP2;1N64H/E65Q and BvPIP2;1N64H/E65Q) were cloned into the BglII and SpeI sites of a pT7Ts derived vector containing T7 RNA polymerase (Ambion, Austin, Texas, USA) by using anti T7 polymerase (Amersham, Piscataway, NJ). All constructs were confirmed by DNA sequencing (Macrogen Inc, USA).

**Phylogenetic Analyses**

Phylogenetic and molecular evolutionary analyses were performed as detailed in [1]. Briefly, the analyses were conducted by using MEGA version 4.0 [21]; history reconstruction of PIPs was restricted to protein sequences with high amino acid identity (>25%). Phylogenetic trees were constructed using the neighbor-joining (NJ) method with genetic distances computed using Poisson correction model. This analysis was developed by setting the following parameters: substitutions to include = all, gaps/missing data = pair wise deletion, phylogeny test = bootstrap 500 replicates and root on midpoint.

**Sequence Analysis**

Sequence search was performed using BLASTP tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Only the following sequences, with reported interaction with PIP1, were selected: ApPIP2;3 (Arabidopsis thaliana GI: 15228096), BvPIP2;2 (Beta vulgaris GI: 1402833), BvPIP1;2 (Momosa pudica GI: 60496864), NtPIP2;1 (Nicotiana Tabacum GI: 17017257), OtPIP2;2 (Oryza sativa GI: 75291011), OvPIP2;4 (Oryza sativa GI: 75299345), TtPIP2;1 (Triticum turgidum GI: 158324048), VvPIP2;2 (Vitis vinifera GI: 124702519), ZmPIP2;1 (Zea mays GI: 29650727), ZnPIP2;4 (Zea mays GI: 29650729). Multiple sequence alignment were performed with ClustalW version 2 [22] and retrieved to WebLogo (http://weblogo.berkeley.edu/logo.cgi) to generate a graphical representation of the patterns within the multiple sequence alignment [23,24].

**Mutations of BvPIP2;1 cDNA**

Mutated cDNA encoding BvPIP2;1 N64H/E65Q and BvPIP2;1 N64H/E65Q were obtained by site direct mutagenesis (Quick-change, Stratagene, USA) following the manufacturer’s recommendations using custom-made oligonucleotides primers ([Eurofins MW Operton, USA], 5'-CAGTTGCAACTGGTTATGGTTACAAACATCAGTTGACCCTTGTG-3' and 5'-GCAAAAGGTCACGTTCAGTTGATGTGTCACCAATACAGTGCACAGTGCAGT-3'. 5'-GCAAAAGGTCACGTTCAGTTGATGTGTCACCAATACAGTGCACAGTGCAGT-3'. 5'-GCAAAAGGTCACGTTCAGTTGATGTGTCACCAATACAGTGCACAGTGCAGT-3'. 5'-GCAAAAGGTCACGTTCAGTTGATGTGTCACCAATACAGTGCACAGTGCAGT-3'. 5'-GCAAAAGGTCACGTTCAGTTGATGTGTCACCAATACAGTGCACAGTGCAGT-3'. 5'-GCAAAAGGTCACGTTCAGTTGATGTGTCACCAATACAGTGCACAGTGCAGT-3'. 5'-GCAAAAGGTCACGTTCAGTTGATGTGTCACCAATACAGTGCACAGTGCAGT-3'. 5'-GCAAAAGGTCACGTTCAGTTGATGTGTCACCAATACAGTGCACAGTGCAGT-3'). The capped complementary RNAs (cRNA) encoding BvPIP1;1 and BvPIP2;2 were synthesized in vitro using the mMESSAGE mMACHINE T7 High Yield Capped RNA Transcription Kit (Ambion, Austin, Texas, USA) as described previously [17]. cRNA encoding BvPIP2;1 and its mutants were synthesized in vitro with mMES-SAGE mMACHINE T7 Ultra Kit (Ambion, USA) by using anti reversed cap analog (ARGA) and poly (A) tailing reagents as previously detailed in [17]. The synthesized products were suspended at a final concentration of 0.1 μg μL⁻¹ in RNase-
free water supplemented with Recombinant RNasin (Ribonuclease inhibitor, Promega, USA) and stored at –20°C until used [25]. The cRNA was quantified by fluorescence using Quant-iT RNA Assay Kit (Invitrogen, UK). Agarose gel electrophoresis and GelRed (BioAmerica Biotech Inc., USA) staining were used to check the absence of unincorporated nucleotides in the cRNA. At least four independent cRNA syntheses were assayed. Results from experiments performed with different oocytes batches were not pooled; therefore all the experiments shown in this work are representative for at least three different experiments.

Before injecting, cRNA was diluted in order to inject a proper amount per oocyte. Then, all masses of cRNA injected in the same experiment were considered as relative to an arbitrary unit of measure. For example, if 1 is 1.25 ng of cRNA injected per oocyte, 3 is three times this quantity.

Oocyte Water Transport Assays

Osmotic water permeability ($P_f$) of oocytes injected with cRNA or non-injected, was determined by measuring the rate of oocyte swelling as explained before [26]. Briefly, osmotic water permeability ($P_f$) was determined by measuring the rate of oocyte swelling induced by a hypo-osmotic shock of 160 mMol kg$^{-1}$ H$_2$O. Changes in cell volume were video-monitored by a VX-6000 color video-camera (Microsoft, CA, USA) attached to a zoom stereomicroscope (Olympus SZ40, Olympus Co., Japan). The cell swelling was video-captured in still images (each 10 s during 70 s) using the AMCap version 9.20 (http://noeld.com/programs.asp?cat=AMCaP) and then the images were analyzed by treating each oocyte image as a growing sphere whose volume could be inferred from its cross-sectional area (software Image J version 1.37, http://rsb.info.nih.gov/ij/).

The osmotic water permeability ($P_f$) was calculated according to [27] and [28]. Non-injected oocytes were used as negative controls because no significant differences were found between this condition and water injected oocytes. All osmolarities were determined using a vapor pressure osmometer (Wescor, USA).

pH Inhibition Assays

For pH inhibition experiments the oocyte internal (cytosolic) pH was modified following an already described protocol in [29] and the modifications made in [17]. Briefly, the oocyte internal pH was acidic by pre-incubating them for 15 min in different pH solutions (NaAc Solution: 50 mM NaAc, 20 mM MES for 5.8–6.7 pH interval or HEPES for the 7.0–7.6 pH interval), acidified by pre-incubating them for 15 min in different pH solutions (NaAc Solution: 50 mM NaAc, 20 mM MES for 5.8–6.7 pH interval or HEPES for the 7.0–7.6 pH interval), supplemented with mannitol 1 M until the desired osmolarity was achieved (~200 mMol kg$^{-1}$ H$_2$O). To calculate final oocyte intracellular pH a calibration curve described previously in our laboratory was used [17]. The swelling response was performed by transferring the oocyte to the same solution diluted 5-fold with distilled water in order to induce the osmotic shock. In all treatments, negative controls were performed by submitting non-injected oocytes to the same protocol and the percentage of inhibition was calculated using the formula: $Inh \% = [1 - (P_f \text{ pH 6.4} / P_f \text{ pH 7.0})] \times 100$, where $P_f$ states non-injected.

Data were fitted to a sigmoidal dose-response curve $P_f = P_f_{max} \times \text{pH}_{min}^b / (EC50^b + \text{pH}_{min}^h) + P_f_{min}$, using Graph Pad Prism (version 3.02).

Assessment of Fluorescent PIP Proteins Expression in Oocyte Plasma Membrane

Confocal fluorescence microscopy was used to localize the respective PIP isoforms tagged with ECFP or EYFP in Xenopus laevis oocytes. As a marker of the interior of the oocyte we used tetramethylrhodamine (TMR) dextran (10,000 MW; Invitrogen-Molecular Probes, USA) an unconjugated non-specific fluorochrome marker that stays in the area of the cortical granules and allows distinguishing plasma membrane from cytosol [30]. Briefly, 3–4 days after cRNA injection and 40 minutes prior to imaging, oocytes were microinjected with 50 nl of a 33 mM aqueous solution of TMR-dextran.

Fluorescence images of ECFP or EYFP distribution together with TMR were obtained with a Fluoview1000 spectral confocal scanning microscope (Olympus Co., Japan), employing a 60X UPlanSapo oil immersion objective lens NA1.35. In order to avoid crosstalk, images were recorded line by line in a sequential order. In the case of ECFP and TMR, they were excited using the 450 nm and 515 nm line of a multilime Argon laser and the emitted fluorescence was detected in the 475–500 nm and 555–655 nm range, respectively. When EYFP and TMR were employed, 488 nm line of the Argon laser and 543 nm He-Ne were used and the emitted fluorescence was detected in the 500–555 nm and 570–670 nm range, respectively. Except for z-stacks, images were obtained using Kalman filtering.

Autofluorescence (monitored in control oocytes) was negligible in comparison to cells expressing the fluorescent PIP. We analyzed 3–4 oocytes for each condition from at least 7 donor frogs.

For reconstructing 3D views, confocal ($x$-$y$) images were collected at various focal depths into the oocyte with 100 nm steps. The stack of images was deconvoluted employing the classic maximum likelihood estimation as a restoration method, then iso-sampled and surface rendered for 3D visualization employing Huygens Professional Software (Scientific Volume Imaging, Hilversum, The Netherlands).

Statistics

Results are reported in the form of means ± SEM. Significant differences between treatments were calculated using the Student’s $t$-test.

Homology Modeling (HM)

In order to explore the structure and dynamics of loop A we built an homology model (HM) of BvPIP2;1 sequence. The sequence was submitted to the Swiss-Model [31] public server and the model was stirred to superimpose over the chain A of the crystal of the SoPIP2;1 (2B3f pdb code) [5]. The sequence identity calculated was 86.6% and the retrieved model was very well superimposed over the chain A, especially in the alpha helix regions. In order to explore the homotrameter geometry, four identical models were superimposed over each monomer of the crystal structure.

Molecular Dynamics Simulation (MDS) Methodology

In order to characterize BvPIP2;1 loop A dynamics, we built the topology file based on the homotrameter that was fully solvated in an octahedral box of TIP3P water model with periodic boundary conditions. The MDS was performed using AMBER 11 [32]. The model was prepared with a 3-steps protocol. First, we ran 2000 steps of side chains optimization, second a 50000 steps of thermalization at 300 K with a Berendsen thermostat at constant volume, and third, a 50000 steps at the same temperature with constant pressure (isotropic position scaling) of 1 atm. Finally, 30 ns of MDS under the NPT ensemble condition were performed. This system remained stably structured along the whole MDS.
Results

**PIP1-PIP2 Interaction**

Three PIP aquaporins from *Beta vulgaris* are known, *Bv*PIP2;1 (GenBank: U60148.1, GI:1402834) (corresponding to CLI; Figure S1), *Bv*PIP2;2 (GenBank: U60147.1, GI:1402832) (corresponding to CLII; Figure S1), and *Bv*PIP1;1 (GenBank: U60149.1, GI:1402836) (corresponding to CLI; Figure S2). Previously, it was shown that *Bv*PIP2;2 is able to functionally interact with *Bv*PIP1;1 [17].

Here, we focused on the functional features of *Bv*PIP2;1. Figure 1 shows that the expression of *Bv*PIP2;1 in *Xenopus laevis* oocytes lead an increase in plasma membrane osmotic water permeability coefficient compatible with an active water channel. Oocyte co-expressing *Bv*PIP2;1 with *Bv*PIP1;1 show not significantly different *P* values in comparison with *Bv*PIP2;1 expressed alone, even at different cRNA ratios of *Bv*PIP2;1:*Bv*PIP1;1 injected. Therefore, *Bv*PIP2;1 is an active aquaporin which is unable to functionally interact with *Bv*PIP1;1.

In order to correlate the previous water transport parameters of co-expressing systems (*Bv*PIP2;1:*Bv*PIP1;1) with the cellular localization of *Bv*PIP1;1, we designed the following fusion proteins: *Bv*PIP2;2-EYFP (as a control of plasma membrane localization) and *Bv*PIP1;1-ECFP. When these fusion proteins are injected in oocytes, the analysis made by confocal fluorescence microscopy show that the fluorescent signal of *Bv*PIP1;1-ECFP is localized in the same area of TMR-dextran (marker of the interior of the cell) (Figure 2 A and B) and that fluorescence due to *Bv*PIP2;2-EYFP (our control for plasma membrane localization) is found in the limit of the cell (Figure 2 C and D). This result indicates that *Bv*PIP1;1-ECFP is retained in the interior of the cell while *Bv*PIP2;2-EYFP is located at the plasma membrane. The localization of *Bv*PIP2;2-EYFP is consistent with the high *P* reported for oocytes injected with this aquaporin [17]. On the other hand, when cRNA of *Bv*PIP1;1 is injected in oocytes, *P* is not different from non-injected oocytes (as shown in Figure 1); this is in accordance with the lack of *Bv*PIP1;1-ECFP in oocyte plasma membrane.

Knowing that *Bv*PIP1;1-ECFP is retained in the interior of the cell, we proceed to test its localization when co-expressed with *Bv*PIP2;1 and *Bv*PIP2;2. Interestingly, when the oocytes are co-injected with cRNAs of *Bv*PIP1;1-ECFP and *Bv*PIP2;2, the fluorescence signal is mainly located at the plasma membrane (Figure 3 A, B and C). However, fluorescent signal is still located at the interior of the oocyte when *Bv*PIP1;1-ECFP is co-expressed with *Bv*PIP2;1 (Figure 3 D, E and F). This result shows that the localization of *Bv*PIP1;1-ECFP is modified from the interior of the oocyte to the plasma membrane only in the presence of *Bv*PIP2;2, suggesting that *Bv*PIP2;1 does not promote the trafficking of *Bv*PIP1;1-ECFP to the plasma membrane. Again, the localization of *Bv*PIP2;1 is in accordance with the functional results shown in Figure 1.

The *P* values of oocytes expressing fluorescent-tagged PIPs are in accordance with the data obtained for the corresponding wild type aquaporins corroborating that the tags are not affecting aquaporin activity (Figure S3).

**BvPIP2;1 pH Response**

It is well known that most PIPs are inhibited by cytosolic acidification. The gating mechanism was explained by the protonation of a conserved histidine residue located on the intracellular loop D [29]. In the case of *Bv*PIP2;1 the conserved histidine responsible of cytosolic pH sensing seems to be H193 regarding its alignment with others PIP2.

To further analyze *Bv*PIP2;1 in terms of its capacity to modulate water permeation under cytosolic acidification, we performed swelling assays under different acidic (intracellular) conditions. Figure 4 shows that the pattern of *P* versus pH is sigmoidal,
suggesting that BvPIP2;1 could be an allosteric channel. The characterizing parameters of BvPIP2;1 pH response are a EC50 equal to 6.41 ± 0.05 and a Pmaximal inhibition of (88 ± 3)% at pHint 6.3 (media ± SEM, n = 3 independent experiments). The reduction of Pmaximal suffered by the oocyte membranes expressing BvPIP2;1 at acidic pH is not partial as the previously reported for the expression of other PIP2. For instance, BvPIP2;2 and FaPIP2;1 show a partial inhibition at acidic pH of 70% and 52% respectively [17,26].

When the pH effect is tested on oocytes co-injected with cRNA encoding for BvPIP2;1 and BvPIP1;1, the maximal inhibitory response found was (87 ± 4)%, which means that there is no significant difference with the inhibition found for BvPIP2;1 alone (Figure 4). Moreover, the EC50 of the dose-response curve of oocytes injected only with cRNA of BvPIP2;1 in comparison with the EC50 corresponding to curves of oocytes co-injected with BvPIP2;1 and BvPIP1;1 (EC50 equal to 6.47 ± 0.01) are also not significantly different (p>0.05) (Figure 4). This result reinforces the hypothesis that BvPIP2;1 does not promote the incorporation of BvPIP1;1 in oocytes plasma membrane and that the inhibition measured corresponds to the blockage of BvPIP2;1 alone.

BvPIP2;1 Loop A

Recently, PIP loop A has been described as important in the stabilization of PIP dimer formation [19]. Here, we focus our study on the characteristics of BvPIP2;1 loop A. First, we found some differences in comparison with the primary sequence of other PIP2 previously reported to be able to interact with PIP1. BvPIP2;1, as other PIP belonging to PIP2, present a gap in their loop A and two low frequency residues, N64 and E65 (Figure 5).

It is unknown if the gap found in loop A is relevant for PIP2-PIP1 functional interaction. Some PIP2 with a short loop A have been reported to functionally interact with some PIP1, as can be the case of VvPIP2;2-VvPIP1;1 [14] and NtPIP2;1-NtPIP1;1 [12]. Since the findings of PIP2-PIP1 interaction are still singular reports, a generalization about the relevance of the gap in some PIP2 cannot be stated. On the other hand, the concernment of N64 and E65 in the lack of functional interaction found for BvPIP2;1 and BvPIP1;1 will be analyzed in the next section (See BvPIP2;1 mutants).

In order to explore the structure and dynamics of BvPIP2;1 loop A we built an homology model (HM) based on the chain A of SsPIP2;1 crystal and performed MDS. After HM observation, it was seen that loop A is defined by residues from position 65 to 72...
of the primary sequence and is oriented towards the center of the tetramer. Interestingly, the residues N64 and E65 are the last residue of the first alpha helix and the first residue of the loop A, respectively.

The MDS showed that each of the four loops A in the tetramer developed a non-equivalent movement, since when starting in identical positions they reached different final conformations after 30 ns of simulation (Figure 6). Regarding the putative fluctuation of the four monomeric loop A positions, RMSF shows that these loops are the most flexible elements of the external face of each monomeric BvPIP2;1 along the whole MDS (Figure 7). Our results also confirm that loops D are the most flexible parts of the inner face of the channel in accordance with functional features proposed for this loop [5,29,33].

MDS points out that the loops A of some monomers could be in contact; indeed the analysis of the distances of all possible pairs of loop A Cys residues (Figure S4) suggest the possibility of S-S bridges formation between contiguous monomers in accordance with previous works [19,34].

A further characterization of loop A behavior in each monomer was assessed by root mean square deviation (RMSD), radial distribution function (g(r)) and Ramachandran plot. RMSD of each loop was calculated along the whole MDS. The result obtained reinforced that loop A belonging to a particular
monomer has different movements and visits different conformational spaces along the whole MDS since the dynamic convergence is reached in their particular ways (Figure S5).

The g(r) functions are in agreement with full hydration of loop A aminoacids polar groups since in all cases a peak corresponding to the first solvation sphere is observed (Figure S6). It is worth noting that some identical polar groups in each monomer show different solvation patterns, as is the case of T66, C69 and T71. On the other hand, the atoms of D67 residues of the four chains have very similar solvation patterns may be due to the distance of this residue from the anchoring extremes of the loop. Considering loop A orientation, this high and even differential exposure of some residues in the loops A may be important to facilitate intermonomer interactions within the tetramer.

Lastly, Ramachandran plots of each loop A residues show that the backbone conformational space is differently explored for some particular residues depending on the monomer they belong to (Figure S7), enhancing the idea of four loops A having different movements.

All these results together point to the possibility of loops A mediating contacts between monomers and let us speculate that aminoacid substitutions may have functional significance.

**Bv**<sub>PIP2;1</sub> Mutants

The above mentioned two non-conserved residues N64 and E65 resulted interesting due to their location: N64 is the last residue of the first transmembrane helix and E65 is the first residue of loop A. So, these residues may play a role in the flexibility of the loop. These characteristics make those residues attractive to be mutated. Considering the conserved residues in other PIP2 with reported interaction with PIP1 (Figure 5), we replace N64 and E65 of **Bv**<sub>PIP2;1</sub> in order to obtain the following mutants: **Bv**<sub>PIP2;1</sub> N64H/E65Q and **Bv**<sub>PIP2;1</sub> N64I/E65Q.

When expressed in Xenopus oocytes the two mutants give rise to a lower P<sub>f</sub> than the developed by **Bv**<sub>PIP2;1</sub> expression, notwithstanding they promote a significant increase in oocyte water permeability showing they are functional aquaporins (Figure 8). Interestingly, the co-injection of cRNA encoding **Bv**<sub>PIP1;1</sub> and **Bv**<sub>PIP2;1</sub> N64I/E65Q triggered the membrane P<sub>f</sub> to a value 4.1 times higher than the one promoted by the mutant **Bv**<sub>PIP2;1</sub> N64I/E65Q alone. A similar result was found for the other mutant, as the co-injection of cRNA encoding **Bv**<sub>PIP1;1</sub> and **Bv**<sub>PIP2;1</sub> N64H/E65Q results in an 4.3 fold increase of P<sub>f</sub>. In contrast with **Bv**<sub>PIP2;1</sub>, both mutants seem to functionally interact with **Bv**<sub>PIP1;1</sub> (Figure 8).
When the activity of the mutant under acidification was assessed we found that even when the response profile of \( \text{BvPIP2;1 N64I/E65Q} \) expressed alone or co-expressed with \( \text{BvPIP1;1} \) remained both sigmoidal, their parameters are different: while \( \text{BvPIP2;1 N64I/E65Q} \) presents a partial pH maximal inhibition at \( \text{pH}_{\text{int}} \) 6.3 and a EC50 equal to 6.49 ± 0.02 (media ± SEM, \( n = 3 \) independent experiments), the co-expression suffered a total blockade at the same \( \text{pH}_{\text{int}} \) and the EC50 was 6.61 ± 0.03 (media ± SEM, \( n = 3 \) independent experiments) (Figure 9). This strong modification of pH response further support the idea of \( \text{BvPIP2;1 N64I/E65Q} \) mutant functionally interacting with \( \text{BvPIP1;1} \).

**Discussion**

\( \text{BvPIP1;1}, \text{BvPIP2;1} \) and \( \text{BvPIP2;2} \) are the only three \( \text{Beta vulgaris} \) PIP aquaporins described and they have been found to be expressed in different red beet tissues [17]. It was previously shown that \( \text{BvPIP2;2} \) functionally interact with \( \text{BvPIP1;1} \) increasing oocytes membrane \( P_f \) and modifying the pH response, from partial to complete shutdown, after cytosolic pH acidification [17]. In the present work we found that \( \text{BvPIP2;1} \) does not behave as \( \text{BvPIP2;2} \), neither concerning its functional interaction with \( \text{BvPIP1;1} \), nor about its inhibition under cytosolic acidification. With regard to this last feature we found that \( \text{BvPIP2;2} \) is partially blocked at \( \text{pH}_{\text{int}} \) 6.4 while \( \text{BvPIP2;1} \) is completely inhibited. Regarding \( \text{BvPIP2;1} \) ability to interact with a PIP1, the complete set of results presented in this work, i.e. water transport measurements and localization pattern studied by confocal fluorescence microscopy, shows that \( \text{BvPIP2;1} \) do not interact with \( \text{BvPIP1;1} \). These results are in accordance with previous findings on \( \text{Beta vulgaris} \) water channels which, by means of biochemical approaches, suggested that the PMIP31 and PMIP27 (now known as \( \text{BvPIP2;1} \) and \( \text{BvPIP1;1} \)) do not form mixed heterodimeric species [34].

Aquaporin heterooligomerization is emerging as an important point of regulation and fine-tuning of water transport. Many evidences in the literature point to the existence of PIP aquaporins as oligomers formed by a combination of PIP1 and PIP2: increased water transport rates due to functional interaction between PIP1 and PIP2 channels have been detected in several
plant species [11], ii- FRET studies show that PIP1 and PIP2 aquaporins are near enough to form heterooligomers [11], iii- pH$_{in}$ sensing in co-expressions of PIP1 and PIP2 systems is different from pH$_{in}$ sensing of PIP2 expressed alone [17,26], iv- changes in transport selectivity have been reported as modified when heterotetramers of PIP1 and PIP2 are constructed [18], v- modification of PIP1 localization from ER to plasma membrane was detected only when PIP2 is also expressed in the same cell [11,13].

Along with all this strong evidence, in the last years it has been widespread that PIP2 and PIP1 can form heterooligomeric assembles. However, there are reports showing some PIP2 that do not functionally interact with PIP1 [11,35,36]. In this subset of plant water channels should be included BvPIP2;1.

We proposed that BvPIP2;1 loop A could be the key element for the lack of functional interaction with BvPIP1;1 after BvPIP2;1 primary sequence comparison with other PIP2, HM and MDS evaluation and the consideration of loops as relevant elements mediating protein-protein interaction [37,38]. Indeed, Hayward and Kitaos [39] stressed the importance of fixed ends in the configuration of protein loops. The authors remark that this constraint might influence the dynamical behavior of the loop. Interestingly, when we mutated the C-terminal of BvPIP2;1 helix 1 and the N-terminal of BvPIP2;1 loop A for two residues highly conserved in PIP2 that have been reported as able to functionally interact with PIP1, i.e. H or I for position 64 and Q for position 65 (Figure 5), the mutated channels acquired the capacity to interact with BvPIP1;1.

The functional interaction between BvPIP2;1 mutants and BvPIP1;1 is demonstrated by an enhanced of fourfold oocyte plasma membrane P$_f$ in comparison with BvPIP2;1 mutants alone (Figure 8 and Figure 9) and also by a change in the inhibitory pH response pattern (Figure 9).

With regard to plant aquaporin loops, main attention was focused on intracellular loop D, which has been described as responsible for the gating of PIPs [5,29,33]. Also, loop A has previously received some consideration. For example, a highly conserved cysteine residue located in PIP loop A has been suggested as having a relevant function in tetrameric organization.

It was reported that due to a particular configuration of SoPIP2;1 loop A, the four cysteines (one in each monomer) may play a role in the stabilization of the tetramer since according to their nearness they can form hydrogen bonds or complex metal ions [40]. Moreover, in a recent work, it was shown that those cysteines could play a role in dimer stabilization of ZmPIPs [19].

When the overall architecture of SoPIP2;1 was studied, it revealed considerable similarity to the mammal AQPI [3,41]. Notwithstanding, differences in the two first transmembrane helices render a different position of the connecting loop A [40]. In AQPI, loop A seems to have a position parallel with the side of the monomer, while in SoPIP2;1 the positions of the C-terminus of helix 1 and N-terminus of helix 2 suggest that loop A is oriented.
towards the center of the tetramer [40]. In the present work, the MDS of BvPIP2;1 shows that the position of loop A is also oriented towards the center of the tetramer (Figure 6) in accordance with results found for SoPIP2;1. Furthermore, these results indicate that the four loops A are flexible parts of the monomers each having a different solvation pattern and different movement along the MDS.

Our functional experiments show the importance of loop A as involved in BvPIP2;1 mutants and BvPIP1;1 functional interaction. This result in combination with the MDS data about flexibility and orientation of loop A facing towards the center of the tetramer (Figure 6) in accordance with results found for BvIP2;1. Furthermore, these results indicate that the four loops A are flexible parts of the monomers each having a different solvation pattern and different movement along the MDS.

Resuming, Beta vulgaris aquaporins present a specific pattern of activity and functional interaction, where BvPIP2;1 is organized as homooligomers (due to, at least, its loop A structure) while BvPIP2;2 and BvPIP1;1 are able to assemble as heterooligomers.

In previous works we studied the water transport characteristics of Beta vulgaris plasma membrane vesicles (PMV) [42,43]. We reported that, under acidification, water transport through PMV was completely shut down. So, all water channels present in those PMV should be completely inhibited at low pH. This result obtained on plant material is consistent with the presence of BvPIP2;1 homotetramers and BvPIP2;2-BvPIP1;1 heterooligomers responding in a concerted way when pH drops to acidic values.

As a general consideration, we would like to stress that the assembly of water channels as heterooligomers is frequently studied by straightforward functional assessment, generally by means of cRNA co-injection of different PIP isoforms in an heterologous system like Xenopus oocytes followed by water transport measurements, i.e. P_f determination. Many results show that P_f of oocytes co-expressing PIP1 and PIP2 is higher than the obtained for PIP2 expression alone. However, on the basis of published results, the increment of P_f does not always happen at the same extent and the threshold of P_f representing an authentic PIP interaction is not well established. At this respect some PIP1 interacting with PIP2 triggered an increase in the membrane P_f of seven fold [17], four fold [26] or even three fold [14], while other PIP2 interacting with PIP1 only leads to a 1.4 fold increment [15]. Additionally, the increase in the membrane P_f sometimes seems to be dependent of the ratio of injected PIP1:PIP2 cRNA in the oocyte [11,12], while in other cases seems to be independent [14]. Under this complex scenario we suggest that, to further confirm that a PIP2 is interacting with a PIP1, it becomes important to take into account different experimental approaches, not only the

Figure 9. pH dose-response curve of BvPIP2;1 N64I/E65Q mutant and its co-injection with BvPIP1;1. The effect of cytosolic acidification on P_f was tested on oocytes injected with BvPIP2;1 N64I/E65Q cRNA alone or co-injected with BvPIP1;1 cRNA in a 1:1 mass ratio. Co-expression of BvPIP2;1 N64I/E65Q:BvPIP1;1 account for a different pH sensitivity in comparison with BvPIP2;1 N64I/E65Q alone. In the main figure values are representative data from three independent experiments using different oocyte batches. For each condition mean values are shown as P_f ± SEM, n = 7–10. Data were fitted to a sigmoidal dose-response curve using Graph Pad Prism (version 3.02). The inset shows mean EC50±SEM, n = 3 independent experiments, EC50 are significantly different (*p<0.05).

doi:10.1371/journal.pone.0057993.g009
increment of membrane P₀. It can be considered, together with water transport assays, the localization pattern of the different PIP isoforms studied by confocal fluorescence microscopy or the pH inhibitory response observed after oocyte cytosolic pH acidification changes when the plasma membrane expresses only one type of PIP in a homotetrameric way or a mix of two different PIP. Therefore, a combination of experimental approaches could overcome the uncertainty about a genuine PIP2–PIP1 interaction that could arise from the lack of a confident cut-off value for water transport increases when comparing P₀ resulting from PIP2 expressing systems with PIP2–PIP1 co-expressing ones.

In summary, in this work we found that: i- not all PIP2 are able to interact with any PIP1, since at least BvPIP2;1 is unable to promote the incorporation of BvPIP1;1 in the plasma membrane, and ii- loop A is relevant for PIP1–PIP2 functional interaction since mutations in this loop modify the behavior of BvPIP2;1–BvPIP1;1 co-expression.

The puzzle of PIP aquaporins interaction and localization is being unveiled in these last years, however despite all this detailed information, the structural elements that can be involved in the formation of heterooligomeric, or heterotetrameric, assemblies are still under investigation. Our results broaden previous findings on the significant role of loop A in monomer–monomer contacts in PIP aquaporins showing that loop A not only may play a role in stabilizing contacts among monomers, but also can be a controller of heterooligomerization.

Supporting Information

Figure S1  Phylogeny of PIPs2 in plants. Phylogenetic trees of PIPCLII and PIPCLIII protein sequences from representative taxa based on NJ method are shown. Bootstrap percentages are indicated at the branch points. Orthologous gene clusters (CL) are found on right. Tree topology obtained using NJ method, Minimum evolution and Maximum parsimony methods were identical.

Figure S2  Phylogeny of PIPs1 in plants. Phylogenetic tree of PIPCL1 protein sequences from representative taxa based on NJ method is shown. Bootstrap percentages are indicated at the branch points. Tree topology obtained using NJ method, Minimum evolution and Maximum parsimony methods were identical.

Figure S3  Osmotic permeability (P₀) of oocytes membranes expressing fluorescent tagged-PIPs. All fluorescent-tagged Bv:PIPs and Bv:PIP1;1–ECFP co-expressions show similar water transport activity than their corresponding wild types, indicating that the fluorescent tag do not modify their activities or functional interaction. NI are non-injected oocytes. Values are representative data of three independent experiments using different oocyte batches. For each condition mean values are shown as mean P₀ ± SEM, n = 7–10.

Figure S4  Putative S–S bridges formed along the MDS between different loops A in the tetramer. The figure shows the distances among all the possible pairs of sulfur atoms, corresponding to conserved cysteines residues in loop A, that might be involved in S–S bridges among the four chains along the MDS. The inset on top of the right establishes the color references for each pair of sulfur atoms (that correspond to A, B, C or D chains) shown in the figure. The inset on top of the left indicates a particular frame of the model where loops A are highlighted and yellow spheres (in CPK style) represent sulfur atoms. The inset in the center shows a schematic representation of the S–S bridges, between Cys of different monomers that can be formed at least during certain time window of the MDS.

Figure S5  BvPIP2;1 loops A root mean square deviation (RMSD). The figure shows the RMSD for BvPIP2;1 loops A calculated for the 30 ns of the MDS. The RMSD for each monomeric loop A is shown in a different color: loop A corresponding to chain A, B, C and D are in yellow, green, blue and red, respectively. It can be observed that a rather stable conformation is reached for the four loops at different times.

Figure S6  BvPIP2;1 loop A polar residues radial distribution function (g(r)). The radial distribution function for each polar residue of BvPIP2;1 loops A is shown up to 7 angstroms from the atomic position. In yellow, green, blue and red are represented the monomeric BvPIP2;1 A, B, C and D chains respectively. Panel A and B correspond to the oxygen/and/of E65 residue, panel C to oxygen/of T66, panel D and E to oxygen/and/of D67, panel F to sulphur/of C69 and panel G to oxygen/of T71. In each panel it can be observed a peak that corresponds to the first solvation sphere for each atom; different solvation patterns are found in panels C, F, G.

Figure S7  Ramachandran plot of BvPIP2;1 loop A residues. Ramachandran plots for each residue of BvPIP2;1 loops A are shown. Loop A of chains A, B, C, D are organized in consecutive rows distinguished by yellow, green, blue and red labels respectively. As can be seen residues E65, T66, C69, A70, T71 and V72 explore the backbone conformational space in different ways depending on the chain they are part of, revealing that loops A are non-equivalent in BvPIP2;1 tetramer.

Author Contributions

Conceived and designed the experiments: CJ PR LS LI GA KA. Performed the experiments: CJ PR LS GS. Analyzed the data: CJ PR LS GS LI KA. Contributed reagents/materials/analysis tools: PR LS GA KA. Wrote the paper: CJ PR LS GS LI KA.

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