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### **► To cite this version:**

Véronique Santoni, Lionel Verdoucq, Nicolas Sommerer, Joelle Vinh, Delphine Pflieger, et al.. Methylation of aquaporins in plant plasma membrane. *Biochemical Journal*, 2006, 400 (1), pp.189-197. <10.1042/BJ20060569>. <hal-00478579>

**HAL Id: hal-00478579**

**<https://hal.science/hal-00478579v1>**

Submitted on 30 Apr 2010

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## Title

Methylation of aquaporins in plant plasma membrane

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## Synopsis

A thorough analysis, using mass spectrometry, of aquaporins expressed in plant root plasma membrane was performed, with the objective of revealing novel post-translational regulations. Here we show that the N-terminal tail of Plasma membrane Intrinsic Protein (PIP) aquaporins can exhibit multiple modifications and is differentially matured between members of the PIP1 and PIP2 subclasses. Thus, the initiating methionine was acetylated or cleaved in native PIP1 and PIP2 isoforms, respectively. In addition, several residues were detected to be methylated in PIP2 aquaporins. Lys3 and Glu6 of PIP2;1, one of the most abundant aquaporins in the plasma membrane occurred as di- and mono-methylated residues, respectively. Ectopic expression in *Arabidopsis* suspension cells of PIP2;1, either wild-type or with altered methylation sites revealed an interplay between methylation at the two sites. Measurements of water transport in plasma membrane vesicles purified from these cells suggested that PIP2;1 methylation does not interfere with the aquaporin intrinsic water permeability. In conclusion, this study identifies methylation as a novel post-translational modification of aquaporins, and even plant membrane proteins and may represent a critical advance towards the identification of new regulatory mechanisms of membrane transport.

Short title

Methylation of plant aquaporins

### **Key words**

Aquaporin, methylation, N-terminal maturation, plasma membrane protein, post-translational modification, root

### **Abbreviations**

<sup>1</sup><sub>me</sub>Glu6: monomethyl Glu6

<sup>2</sup><sub>me</sub>Lys3: dimethyl Lys3

ESI: electrospray ionization

FW: fresh weight

MALDI-TOF: matrix-assisted laser desorption/ionization time of flight

MS: mass spectrometry

MS/MS: tandem mass spectrometry

PIP: plasma membrane intrinsic protein

PM: plasma membrane

PSD : post-source decay

SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis

WT : wild-type

## INTRODUCTION

Aquaporins define a large family of ubiquitous integral membrane proteins [1] which mediate the transport across membranes of water, small neutral solutes, and occasionally ions. In plants, aquaporins critically contribute to plant water homeostasis during development and in response to challenging environmental conditions [2]. Most of aquaporins consist of small (25-34 kDa) hydrophobic proteins assembling in tetramers. All aquaporin monomers share a typical organization with six transmembrane  $\alpha$ -helices, the N- and C-terminus being located in the cytoplasm. The extra-membrane parts of the proteins, which include membrane connecting loops and the N- and C-termini, are the most divergent regions among aquaporin homologues. A large body of data shows that these regions play a critical role in aquaporin expression and function and that these regulations are mediated in part by various post-translational modifications. For instance, the phosphorylation of Ser residues at multiple sites was shown to modulate the gating of plant aquaporins [3-5] and the subcellular trafficking of animal aquaporins [6, 7]. *N*-linked glycosylation of mammalian aquaporin-2 (AQP2) in extra-cellular loop C was recently shown to regulate cell surface expression of the protein [8]. Intracellular redistribution of the plant vacuolar membrane aquaporin McTIP1;1 in response to osmotic stress also requires glycosylation [9]. Mammalian AQP1 undergoes ubiquitination in fibroblasts, a process which controls the differential stability of the protein between normal and osmotic stress conditions [10]. Finally, controlled proteolysis of the N- and C-terminal tails of AQP0 occurs in lens but the functional significance of this processing remains as yet unknown [11-13].

Proteins are made of 20 main amino acids but over 150 post-translationally modified amino acid forms have thus far been reported. Thus, a tremendous repertoire of modified proteins remain to be uncovered in both prokaryotic and eukaryotic organisms. For instance, protein methylation can occur as either *N*-methylation of residues such as Lys, Arg, His, Ala, Pro, Gln, Phe, Asn and Met or carboxymethylation *i.e.* *O*-methylesterification of Glu and Asp residues. *S*-adenosyl methionine is used as a universal methyl donor and the extent of methylation varies from partial to complete. Whereas methylation (at Glu residues) of bacterial chemotaxis receptors has been characterized as typically reversible [14], *N*-methylation of eukaryotic proteins has long been considered as irreversible. Yet, histone lysine demethylases have recently been identified [15, 16]. Protein methylation affects various cell processes, including protein-protein and protein-nucleic acid interactions, chromatin remodeling, and cellular signalling [17, 18]. These processes mainly involve the methylation

of soluble proteins. There have been only a few reports on methylation of eukaryotic transmembrane proteins and its role has remained elusive [19, 20]. Recently, co-expression studies in *Xenopus* oocytes and mouse cortical collecting duct cells have shown that a novel protein *O*-methyltransferase can enhance the activity of an epithelial sodium channel but the methylation site(s) was (were) not identified on the protein [21].

With the objective of revealing novel post-translational regulations of plant aquaporins, we have recently engaged a thorough analysis of the modification status of aquaporins expressed in root plasma membrane (PM) using mass spectrometry (MS). In a previous study we have shown that the *Arabidopsis* root PM contains various Plasma membrane Intrinsic Proteins (PIP) aquaporin isoforms including members of both the PIP1 and PIP2 subclasses [22]. Here we show that the N-terminal tail of PIP aquaporins can exhibit multiple modifications and is differentially matured between the two PIP subclasses. More interestingly, we observed that several residues can be methylated in PIP2 aquaporins. *Arabidopsis* suspension cells were used to overexpress and characterize methylated wild-type (WT) PIP2;1 and mutants with altered methylation sites.

## EXPERIMENTAL

### Plant materials

*Arabidopsis thaliana* L. (Heynh.), ecotype Wassilewskija (WS), plants were cultivated in hydroponic conditions as described [22]. *A. thaliana* suspension cells, ecotype Columbia, were cultured at 24°C under continuous light according to Gerbeau *et al.* [23].

### Gene constructs

Mutated PIP2;1 cDNAs were constructed by using a sense primer which introduces a *Xho*I restriction site upstream the start codon and contains the desired mutation, and an antisense primer, which introduces a *Xba*I restriction site downstream of the stop codon. The oligonucleotides used in this study are described in Table 1. The resulting PCR products were digested with *Xba*I and *Xho*I and cloned into a pBlueScript derivative, downstream of a doubled *CaMV35S* promoter (*CaMV70S*) and upstream of the 3' untranslated transcribed region of a nopaline synthase gene (*nos*). The resulting plasmid was then digested with both *Eco*RI and *Cla*I, and the cDNA-containing inserts were subcloned into the T-DNA region of a pGreenII 0179 binary vector (<http://www.pgreen.ac.uk/>). This vector contains a *nptII* gene for kanamycin selection in *E. coli* and a *aphIII* gene for hygromycin selection in plant.

For ectopic expression of WT PIP2;1, we used a similar construct in pGreenII 0179. For water transport measurements, ectopic expression of WT-PIP2;1 was also obtained using pCAMBIA 1301 plasmid vector (Cambia, Canberra, Australia) carrying the PIP2;1 cDNA under the control of a *CaMV35S* promoter.

### Biolistic transformation of suspension cells

Transformation of five days-old suspension cells was performed essentially as described in [24]. After bombardment, cells (1.5 g fresh weight [FW]) were transferred onto a culture medium containing 3 g/l of phytigel (Sigma) for 2 days and further transferred in 30 ml of liquid culture medium containing 50 mg/l hygromycin. When the percentage of dead cells reached 99%, the cells were resuspended in a fresh medium without hygromycin. The green calli, which eventually appeared after 5-30 days, were transferred in a medium containing 20 mg/l hygromycin and further amplified after two weeks in the presence of 40 mg/l

hygromycin. Genomic DNA was extracted from suspension cells using the CTAB extraction procedure [25] and subjected to PCR to probe for T-DNA insertion.

### **Protein extraction**

Total proteins were extracted from suspension cells (5-10 g FW) as described [26], with the following modifications. The vacuum-dried pellet was resuspended in 0.1 ml/mg FW of a lysis buffer (9 M urea, 4% CHAPS, 0.5% Triton X-100, 65 mM DTT) for 30 min under agitation. The extract was then centrifuged for 10 min at 10,000 g and the supernatant containing total proteins was recovered. Protein concentration was measured using a modified Bradford procedure [22].

### **Plasma membrane purification**

A microsomal fraction was obtained from roots [22] and suspension cells [23]. In the latter case, cells were first homogenized with a Waring Blender for 10 sec and then fully disrupted in a cell disrupter (Constant System Ltd, Warwick, U. K.) at a pressure of 0.54 kBar. PM vesicles were purified by aqueous two-phase partitioning of a microsomal fraction, in a mixture of polyethylene glycol 3350/Dextran T-500, 6.4% (w/w) each in the presence of 5 mM KCl, as described [22]. For both roots and suspension cells, the mean yield of PM extraction was 25 µg protein/g FW. Extrinsic membrane proteins were stripped with an urea and NaOH treatment according to a previously described procedure [22].

### **Immunodetection**

For western-blots, proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 11% acrylamide gels [22] and probed with a primary antibody raised against a 17-amino acid C-terminal peptide of AtPIP2;1, as described [22].

For ELISA assays, serial two-fold dilutions in a carbonate buffer (30 mM Na<sub>2</sub>CO<sub>3</sub>, 60 mM NaHCO<sub>3</sub>, pH 9.5) of 2 µg of total protein extracts or 0.1 µg of PM proteins were loaded in duplicate on Maxisorp immunoplates (Nunc, Denmark) overnight at 4°C. The immunodetection was performed according to the manufacturer's instructions with 0.1% Tween 20 and 1% BSA when required. A 1:2000 dilution and a 1:2500 dilution of primary anti-PIP2;1 antibody and of secondary peroxidase-coupled anti-rabbit antibody, respectively,



were successively applied for 2 h at 37°C. A linear regression between the absorbance signal due to oxidized 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid diammonium salt, as read with a multiplate-reader (Victor, Perkin Elmer, USA), and the amount of total proteins was obtained for each ample and used for relative comparison between samples.

## MS methods

Protein digestion and matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) MS analysis were performed as described [22]. Post-translationally modified peptides were searched using a FindMod software (<http://us.expasy.org/tools/findmod/>) [27] which systematically compares experimental peptide masses that did not match with native protein sequences, with those obtained by addition of a putative modification. Mono-, di- and tri-methylation, and acetylation typically correspond to an increment in monoisotopic mass of 14.01 Da, 28.03 Da, 42.04 Da, and 42.01 Da, respectively.

Post-source decay (PSD) was performed on a BiFlex III mass spectrometer. Peptide parent ions were isolated with a resolution  $M/\Delta M \sim 100$ . A FAST<sup>TM</sup> procedure (Bruker, Bremen, Germany) was used to step scan the reflector tension and to reconstruct the daughter ion spectra. Two hundred spectra were summed for each reflector tension. A MS-BioTools<sup>TM</sup> software (Bruker) was used to confirm peptide sequence and the final interpretation of spectra was done manually. Electrospray ionisation-(ESI)-MS/MS analyses were performed as described [22].

## Stopped-flow light scattering

Water transport measurements were performed at 20°C essentially as previously described [23]. Briefly, membranes were diluted 100-fold into an equilibration solution which contained 20 mM KCl, 30 mM Tris-HCl, pH 8.3. The shrinking of vesicles was initiated by mixing, in a SFM300 stopped-flow spectrophotometer (Biologic, Claix, France), the vesicles with an equal volume of the same buffer containing 500 mM mannitol. The subsequent kinetics of PM vesicles volume adjustment were followed by 90° light scattering at  $\lambda_{\text{ex}} = 510$  nm. The exponential rate constant,  $k_{\text{exp}}$ , fitted from the recorded data was used to estimate the osmotic water permeability of the vesicles [23]. It was assumed that because they were prepared in parallel, membrane vesicles from independent cell lines had similar size repartition.

## RESULTS

### The N-terminus of root PM aquaporins shows isoform-specific maturation

A PM fraction was purified from *Arabidopsis* roots by aqueous two-phase partitioning and enriched in hydrophobic proteins with an urea and NaOH treatment. In previous studies, the proteomic analysis of a major band at 28 kDa identified the presence of at least six PIP isoforms in this fraction (PIP1;1 and/or PIP1;2, PIP1;5, PIP2;1, PIP2;2, PIP2;4, PIP2;7) [22, 28]. These studies relied on the tandem MS sequencing of twelve tryptic peptides. Here, a more thorough analysis allowed to identify additional tryptic peptides that cover new sequence regions of the previously identified PIP1 (Table 2) and PIP2 (Table 3). We also performed a systematic investigation of covalent modifications carried by the N-terminal tail of PIPs. For this, a FindMod software was used, which predicts from MALDI-TOF MS fingerprints, the presence of modifications based on the resulting mass shifts [27]. This study pointed to eleven new peptides that possibly map to the N-terminal region of PIPs and carry putative modifications, that were for most of them confirmed by tandem MS (Tables 2 and 3). In a more general context, FindMod predictions and subsequent sequence analyses allowed us to cover a substantial part of the N-terminus of all PIPs. This corresponded to the entire N-terminal tail of PIP2;1 (Ala2-Arg39) and of PIP1;1 or PIP1;2 (Met1-Arg52). The N-termini of PIP2;2 and PIP2;4 were covered at 80 % whereas that of PIP2;7 was covered at 42 % and those of PIP1;3 (or PIP1;4) and PIP1;5 were covered at 30 % (Tables 2 and 3).

The peptide with a mass  $[M+H]^+$  of 1134.52 Da (Table 2) corresponded to the perfectly conserved N-terminus of the PIP1;1, PIP1;2, PIP1;3 and PIP1;4 isoforms, with the initiating Met being acetylated (Table 2; Figure 1). The corresponding peptide in PIP1;5 whether acetylated or not, was too small to fall into the detection range of the MALDI instrument.

Tandem MS further established that, by contrast to PIP1s, excision of the initiating Met occurred in PIP2;1 and PIP2;2 (Table 3, Figure 2). PIP2;4 was also found in the PM fraction [22] and the MALDI detection of a peptide with a mass  $[M+H]^+$  of 1539.78 Da strongly suggested that the initiating Met was also cleaved (Table 3). One peptide specific for PIP2;7 was also detected in the PM fraction (Table 3) but the N-terminal sequence next to the initiating Met was not covered.

In summary, the initiating Met was co-translationally processed in all PIP isoforms studied. Whereas it was N<sup>α</sup>-acetylated in members of the PIP1 subclass, Met was cleaved in PIP2 isoforms.

### Modification of aquaporins by methylation

Fingerprint analysis of the tryptic digest also revealed putative PIP2;1 and PIP2;2 peptides with mass increments of ~ 14, 28, and 42 Da (Tables 3 and 4). These can be attributed to methylation, dimethylation, and trimethylation or acetylation, respectively. The fragmentation by ESI-MS/MS of a PIP2;1 peptide with a mass  $[M+H]^+$  of 1631.80 Da induced N-terminal (b-type), C-terminal (y-type) and internal fragments (Figure 2A). Analysis of these fragments revealed that Lys3 was dimethylated (<sub>2me</sub>Lys3) (Tables 3 and 4, Figure 2A). Note that this modification can be unambiguously identified, due to the high resolution of MS. The fragmentation of a PIP2;1 peptide with a mass  $[M+H]^+$  of 1645.80 Da revealed that in addition to being dimethylated at Lys3, the peptide was monomethylated at Glu6 (<sub>1me</sub>Glu6) (Tables 3 and 4; Figure 2B). Sequence analysis of an homologous N-terminal peptide from PIP2;2 with  $[M+H]^+ = 1475.70$  Da showed that in this isoform also, Lys3 was dimethylated (data not shown). An additional monomethylation site was detected but could not be unambiguously positioned (Table 3). Because of the presence of a trypsin miscleavage after Lys3, the N-terminal peptides of PIP2;1 and PIP2;2 with the highest signal were identified as Ala2-Arg16 and Ala2-Arg14 peptides, respectively. However, trypsin cleavage occurred occasionally and the Asp4-Arg16 and the Asp4-Arg14 peptides of PIP2;1 and PIP2;2, respectively, could also be detected (Table 3). Overall, the N-terminal extremity (Ala2-Arg16) of PIP2;1 occurred in the PM fraction under four distinct molecular forms (Tables 3 and 4): unmodified, dimethylated on Lys3 (<sub>2me</sub>Lys3), monomethylated on Glu6 (<sub>1me</sub>Glu6), and trimethylated (<sub>2me</sub>Lys3<sub>1me</sub>Glu6).

### Ectopic expression of PIP2;1 in suspension cells

PIP2;1 is one of the most abundant PIPs in the *Arabidopsis* root PM [22] and supposedly plays a central role in water uptake by roots. By contrast to root cells, *Arabidopsis* suspension cells show a low endogenous expression level of PIP aquaporins ([23]; Santoni *et al.* unpublished results). With the prospect of studying N-terminal methylation of PIP2;1 in a simplified system, we evaluated the ability of these cells to ectopically express this aquaporin.

Biolistic transformation using a *CaMV70S::PIP2;1* expression cassette yielded seven independent transformed cell lines. Western blot analysis of total protein extracts using an anti-PIP2;1 peptide antibody revealed a significant overexpression of PIP2 aquaporins in these cells, as compared to untransformed (Untr.) cells or cells transformed with an empty vector (PG) (Figure 3A). The 28 kDa band of a PM extract from cells overexpressing PIP2;1 was further analysed by MS. Of the ~ 20 most abundant peptides of the spectrum, eleven were shown to derive from PIP2;1 (Figure 3B). Further tandem MS analysis of the PIP2;1 peptides revealed a modification status similar to that of endogenous PIP2;1 expressed in the root PM (Table 4): the initiating Met was cleaved, Lys3 and Glu6 were either native or present in dimethylated and monomethylated forms, respectively.

A semi-quantitative MS characterization of the methylation status of PIP2;1 was performed, based on the peak intensity of the detected peptides assuming equal ionisation efficiencies for peptides with identical primary sequences (Table 4). In both roots and suspension cells, the fully unmodified form was the most abundant. The proportion of peptides containing <sup>1</sup>meGlu6 was slightly more abundant in suspension cells (42 %) than in roots (29 %) as indicated by the addition of their respective peak intensities in the MALDI spectrum (Table 4). By contrast, the proportion of peptides carrying <sup>2</sup>meLys3 was similar in suspension cells (34 %) and root cells (33 %) (Table 4). Thus, suspension and root cells appear to have comparable PIP2;1 methylation patterns and the former cells can be used as an expression system to further investigate N-terminal PIP2;1 methylation.

### Modification profile of PIP2;1 mutants with altered methylation sites

With the prospect of dissecting the respective roles of PIP2;1 methylation at positions 3 and 6, we introduced punctual changes at these positions, the corresponding residues being in particular substituted to Ala (E6A-PIP2;1 and K3A-PIP2;1 mutants). The resulting mutants were then overexpressed in *Arabidopsis* suspension cells and their modification profile in purified PM was characterized as described above for WT-PIP2;1 (Table 4).

PM of cells expressing the E6A-PIP2;1 mutant revealed a unique N-terminal peptide. As in the WT form, the initiating Met was cleaved (Table 4). In addition, the peptide carried no decoration. Thus, substitution of Glu6 by an Ala residue resulted in a complete lack of methylation at position 6 but also at position 3 (Table 4).

All N-terminal peptides of K3A-PIP2;1 typically showed Met excision but with a 42 Da mass increment in 86 % of the peptides, which was due to an additional co-translational

N<sup>α</sup>-terminal acetylation [29] (Table 4). Tandem MS also showed a residual mono-methylation on 4 % of the Ala2-Arg16 K3A-PIP2;1 peptide. Curiously, methylation occurred on Ala3 and not on the non-mutated methylation site at position 6 (Table 4). Thus, the K3A mutation was unable to fully prevent methylation at this position whereas it strongly interfered with methylation of the adjacent site. The complex alteration of the PIP2;1 N-terminus modification pattern induced by the K3A mutation prompted us to investigate the effects of a more conservative substitution of Lys3 by an Arg residue. Similar to WT-PIP2;1, the PIP2;1-K3R mutant showed a typical Met excision (Table 4). We also observed mono-methylation and acetylation, but on very minor fractions (2-4 %) of the N-terminal peptides (Table 4). Thus, the K3R mutation resulted in an almost complete lack of methylations at position 3 and 6. Overall, these and the data obtained with the E6A-PIP2;1 and K3A-PIP2;1 mutants show mutual interactions of methylation at the two sites.

### **Intrinsic water transport activity of PIP2;1 mutants with altered methylation sites**

Methylation of PIP2;1 N-terminus may modulate the intrinsic water transport activity of the aquaporin. To explore this possibility, we investigated the water transport activity of the WT and mutant PIP2;1 forms. PM vesicles were purified from at least two representative cell lines of each of the PG, WT-PIP2;1, E6A-PIP2;1, K3A-PIP2;1 and K3R-PIP2;1 genotypes, and water transport in these cells was characterized by means of stopped-flow spectrophotometry. Figure 4A shows typical kinetic changes in light scattering as induced by a sudden hyperosmotic challenge on purified vesicles from PG and WT-PIP2;1 genotypes. The exponential rate constant ( $k_{\text{exp}}$ ) of these changes is indicative of the membrane osmotic water permeability. Membranes purified from WT-PIP2;1 and all other transformed cell lines investigated showed a water transport activity that was about two-fold higher than in membranes from PG cells (Fig. 4A, data not shown). These results suggest that both WT and mutant PIP2;1 can be expressed as active forms in the PM of suspension cells. The relationship between the abundance of PIP2;1 forms in purified PM (as assayed by ELISA) and the water permeability of these membranes was used to evaluate the intrinsic transport activity of each form. Figure 4B shows that PIP2;1-E6A and PIP2;1-K3R mutants had an intrinsic water transport activity similar to that of PIP2;1-WT. By contrast, the PIP2;1-K3A mutant showed a tendency towards a smaller activity (Figure 4B). Altogether, the data show that mutation of PIP2;1 at Lys3 or Glu6 and/or reduced methylation had no major effect on PIP2;1 water transport activity.

## DISCUSSION

Because of their relatively high abundance, aquaporins provide a unique model to address general aspects of membrane protein expression and regulation. In the present work, we have made use of the power and resolution of MS techniques to reveal, in the natural context of plant root membranes, novel co- and post-translational modifications of PM aquaporins. In roots, distinct maturation profiles of the initiating Met, through acetylation or excision were exhibited by native members of the PIP1 and PIP2 subclasses, respectively. In suspension cells, WT-PIP2;1 and its K3A, K3R and E6A derivatives also showed N-terminal Met excision. Excision of the N-terminal Met has been extensively studied in soluble proteins [30, 31] and to a much lesser extent in membrane proteins. This process was shown to depend on the nature of penultimate residues, and occurs in the presence of residues with side chains of reduced steric hindrance [30, 31]. In agreement with this rule, the three *Arabidopsis* PIP2s studied (PIP2;1, PIP2;2, PIP2;4), which exhibit an Ala residue at position 2, all had their Met excised whereas PIP1s, which have a perfectly conserved Glu residue, did not. N-terminal acetylation, which has been thoroughly studied in soluble mammalian and yeast proteins depends on N-terminal sequences [29]. For instance, of 64 eukaryotic proteins examined, which similar to PIP1s exhibited a Met-Glu (or a Met-Asp) N-terminal end, all were acetylated, as were PIP1s [29]. Similar analyses suggested that, although the presence of a Ser or Ala residue at the N-terminus as in PIP2s may permit acetylation, the presence of an adjacent basic residue usually prevents this process. Accordingly, WT-PIP2;1 and the K3R-PIP2;1 mutant were not or almost not acetylated (Table 4). By contrast, the substitution of Lys3 by an Ala yielded a K3A-PIP2;1 mutant with very significant N-terminal acetylation. Curiously, we found that a minor proportion (~13 %) of the mutant was devoid of acetylation (Table 4). This form could reflect an incomplete modification or be indicative of an as yet unknown deacetylase activity.

In the present work, we also observed that PIP2;1 and PIP2;2 isoforms carried methylated residues, a yet discrete but less commonly described decoration. Lys3 and Glu6 of PIP2;1 although they existed in their native form, occasionally carried di-methyl (Lys3) or mono-methyl (Glu6) moieties (Tables 3 and 4). The methylation of Lys residues has been observed in soluble proteins and recently described in a few mammalian membrane intrinsic

or membrane associated proteins, including subunit c of mitochondrial ATP synthase [19] or cytochrome c [20]. Also, a proteomic analysis of rat brain membranes uncovered 15 proteins which possibly carried mono-, di- or tri-methylated Lys [32], suggesting that Lys modification might be common in membrane proteins.

Glu methylesterification is by far less common than Lys methylation and its observation in PIP2s represents a novel modification for eukaryotic membrane proteins. In bacteria, chemotaxis receptors undergo reversible methylation at glutamyl residues, a process which modulates their signalling output [33-35]. We were aware that artefactual carboxymethylation of proteins can take place, when these ones are simultaneously treated by trichloroacetic acid and alcohols (methanol), as during gel Coomassie blue staining [36]. These treatments were, however, carefully avoided in the present work.

Additional analysis indicated that alanine can also be methylated in the K3A-PIP2;1 mutant (Table 4). In that case, we assume that methylation occurs at the peptide bond. Such N-methylation has been described in several biologically active natural peptides and was shown to improve their lipophilicity and proteolytic stability [37]. Its role in membrane proteins is to our knowledge as yet unknown.

The significance of observations made on PIP2;1 N-terminal tail surely goes beyond the context of the present study since putative methylated residues could be identified in other PIPs such as PIP1;1 (Table 2) and other PM proteins (Santoni *et al.* unpublished results). These are, to our knowledge, the first observed methylations in aquaporins and even plant membrane proteins.

Methylation of PIP2;1 was examined in details since this aquaporin is one of the most abundantly expressed aquaporins in plants. Although MALDI-TOF analyses do not allow an absolute quantification of the modified *vs.* unmodified forms of a peptide, their relative peak intensities can be indicative of their relative abundance. Such analyses revealed an interplay between adjacent methylations of PIP2;1 at Lys3 and Glu6 (Table 4). In WT-PIP2;1, the apparent degree of methylation at one position was enhanced by methylation at the other site. For instance, <sub>1me</sub>Glu6 was found in 26 % and 73 % of peptides carrying unmodified Lys3 or <sub>2me</sub>Lys3, respectively. Conversely, <sub>2me</sub>Lys3 was found in 15 % and 59 % of peptides carrying unmodified Glu6 or <sub>1me</sub>Glu6, respectively. We also observed that a PIP2;1 mutant with a punctual E6A substitution did not display any methylation on Lys3 (Table 4). Conversely, the punctual K3A or K3R substitutions and possibly the altered N-terminal maturation of the former mutant protein prevented any methylation of Glu6 (Table 4). Such interactions between modifications at adjacent sites are reminiscent of observations made in histones. In

histone H3 for instance, methylation of Lys4 prevents methylation of Lys9 (for review, see [38]). Our findings also provide primary information on the specificity of protein methyltransferases involved in modifying the PIP2;1 N-terminal end. They conform to the idea that these enzymes have local recognition sequences that encompass a few residues [39]. Enzymes involved in Lys and Glu methylation of PIP2;1 are very likely distinct in nature, and both remain to be identified.

In recent years, the significance of histone methylation in epigenetic control of gene expression has been extensively studied [40] and the effects of methylation on the activity or the subcellular trafficking of a few other soluble proteins have been elucidated [39, 41-43]. Although recent data points to a role of methylation in controlling the activity of an epithelial sodium channel [21], the functional significance of specific methylation events on eukaryotic membrane proteins remains to be elucidated. PIP aquaporins can be gated by phosphorylation of their cytosolic loop B or C-terminal tail [4] or protonation of their cytosolic loop D [44]. To investigate whether methylation of PIP2;1 N-terminal tail plays a similar role, we coupled water transport measurements to aquaporin immunoquantification in PM vesicles expressing WT or mutant PIP2;1 forms. The E6A-PIP2;1 and K3R-PIP2;1 mutants which both are not methylated at Lys3 and Glu6 (Table 4) exhibited a water transport activity similar to that of WT-PIP2;1. These results suggest that methylation at Lys3 and Glu6 does not interfere with intrinsic water permeability. By contrast, the K3A-PIP2;1 showed a tendency to a lower water transport activity. We suppose that the non conservative Lys3 to Ala substitution and /or the resulting alteration of the protein maturation profile, including an abnormal N<sup>α</sup>-acetylation, may result in a partially inactive protein.

As an alternative approach for investigating the functional significance of aquaporin methylation, we also searched for physiological conditions that would induce changes in the modification profile of root PM aquaporins. We observed that salinity that down regulates root water transport by inhibiting aquaporins [45] does not alter their methylation profile (Prak S. *et al.*, unpublished). Since methylation of PIP2;1 seems not to control the gating of this aquaporin, we now investigate the role of this modification in the protein stability and sub-cellular localization.

In conclusion, the present work identifies methylation as a new post-translational modification of aquaporins, and plant membrane proteins in general, and pinpoints to interactions between adjacent methylated sites. Although the functional role of this modification remains as yet unclear, our work may represent a critical advance in identifying new regulatory mechanisms of membrane water transport.



## **ACKNOWLEDGMENT**

Authors are grateful to Dr Giglione, Dr Meinnel and Dr Sentenac for critical reading of the manuscript. Some tandem MS analyses were performed in the Plateau de Protéomique Fonctionnelle, IGF UPR 2580 CNRS, of the Montpellier Languedoc-Roussillon Genopole<sup>®</sup>. This work was supported in part by Genoplante (AF 2001093).

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## TABLES

**Table 1 Oligonucleotide primer sequences**

Primer name	Orientation	Sequence
K3A-XhoI	sense	<b>GACTCGAG</b> <u>ATGGCAGCGGATGTGGAAGCCGTTCC</u>
K3R-XhoI	sense	<b>CGACTCGAG</b> <u>ATGGCAAGGAATGTGGAAGCCGTTCC</u>
E6A-XhoI	sense	<b>GACTCGAG</b> <u>ATGGCAAAGGATGTGGCAGCCGTTCC</u>
PIP2;1-XhoI	sense	<b>GACTCGAG</b> <u>ATGGCAAAGGATGTGGAAGCCGTTCC</u>
PIP2;1-XbaI	antisense	<b>GATCTAG</b> <u>ATTAGACGTTGGCAGCACTTC</u>

The primers were used for the mutation and cloning of WT and mutated forms of PIP2;1 in a pGreenII 0179 vector, as described in the experimental section. All primers are given in the 5' to 3' orientation. Cloning sites are indicated in bold characters, mutated/inserted residues are indicated in italics and the start/stop codons are underlined.

**Table 2 Characterization by MALDI-TOF MS and ESI-MS/MS of N-terminal PIP1 peptides from a root PM extract**

Measured mass (Da) [M+H] <sup>+</sup>	Predicted peptide sequence	Determined peptide sequence	Modification	Isoform	Position in the sequence
<i>1017.55</i>	VGANKFPER		none	PIP1;1/PIP1;2/ PIP1;3/PIP1;4	10-18
<b>1031.55</b>	<b>VGANKFPER</b>		<b>1me</b>	<b>PIP1;1/PIP1;2/ PIP1;3/PIP1;4</b>	<b>10-18</b>
<b>1134.52*</b>	<b>MEGKEEDVR + 42 Da</b>	<b>MEGKEEDVR</b>	<b>acM1</b>	<b>PIP1;1/PIP1;2/ PIP1;3/PIP1;4</b>	<b>1-9</b>
1537.75	QPIGTSAQSDKDYK		none	PIP1;1/PIP1;2	19-32
1638.80	QPIGTSAQSTDKDYK		none	PIP1;4	19-33
1660.82	FPERQPIGTSAQSDK		none	PIP1;1/PIP1;2	15-29
<i>1960.97</i>	DYKEPPAPFFEPGELK	tag APFFE	none	PIP1;5	31-47
2313.14	EPPPAPLFEPGELASWSFWR		none	PIP1;2	33-52
<i>2363.12</i>	EPPPAPFFEPGELSSWSFWR	EPPPAPFFEPGELSSWSFWR	none	PIP1;1	33-52

The table shows peptides that were identified from the 28 kDa band of a root PM fraction enriched in hydrophobic proteins [22] and that corresponded to putative PIP1 tryptic digests. The masses [M+H]<sup>+</sup> of peptides are indicated in the first column. Masses in italics correspond to peptides that were described previously [22].

The corresponding sequence predicted by mass fingerprinting, the sequence determined by MS/MS analysis, the type of modification, the isoform name and position inside the sequence are indicated in the following columns. Peptides with modified residues are indicated in bold characters.

Conventions for post-translational modifications are as follows:

acM1: acetyl Met1; 1me: monomethyl;

\*: peptide the ESI-MS/MS spectrum of which is shown in Figure 1

**Table 3 Characterization by MALDI-TOF MS and ESI-MS/MS of N-terminal PIP2 peptides from a root PM extract**

Measured mass (Da) [M+H <sup>+</sup> ]	Predicted peptide sequence	Determined peptide sequence	Modification	Isoform	Position in the sequence
886.46	KWSFYR		none	PIP2;1 / PIP2;6	34-39 / 33-38
1234.56	DVEGPEGFQTR		none	PIP2;2	4-14
1340.65	DLDEVNESGPPAAR		none	PIP2;4	4-16
1404.70	DVEAVPGEFGFQTR	DVEAVPGEFGFQTR	none	PIP2;1	4-16
<b>1418.70</b>	<b>DVEAVPGEFGFQTR + 14 Da</b>		<b>1me</b>	<b>PIP2;1</b>	<b>4-16</b>
1433.70	AKDVEGPEGFQTR	tag PEGFQTR	M1 cleaved	PIP2;2	2-14
<b>1447.70</b>	<b>AKDVEGPEGFQTR +14 Da</b>		<b>M1 cleaved + 1me</b>	<b>PIP2 ;2</b>	<b>2-14</b>
<b>1461.70</b>	<b>AKDVEGPEGFQTR +28 Da</b>		<b>M1 cleaved + 2me</b>	<b>PIP2 ;2</b>	<b>2-14</b>
<b>1475.70**</b>	<b>AKDVEGPEGFQTR + 42 Da</b>	AKDVEGPEGFQTR	<b>M1 cleaved + <sub>2me</sub>K3 + 1me</b>	<b>PIP2;2</b>	<b>2-14</b>
1539.78	AKDLDEVNESGPPAAR		M1 cleaved	PIP2;4	2-16
1603.80	AKDVEAVPGEFGFQTR	AKDVEGPEGFQTR	M1 cleaved	PIP2;1	2-16
<b>1617.80</b>	<b>AKDVEAVPGEFGFQTR + 14 Da</b>		<b>M1 cleaved + 1me</b>	<b>PIP2;1</b>	<b>2-16</b>
<b>1631.80*</b>	<b>AKDVEAVPGEFGFQTR + 28 Da</b>	AKDVEAVPGEFGFQTR	<b>M1 cleaved + <sub>2me</sub>K3</b>	<b>PIP2;1</b>	<b>2-16</b>
<b>1645.80*</b>	<b>AKDVEAVPGEFGFQTR + 42 Da</b>	AKDVEAVPGEFGFQTR	<b>M1 cleaved + <sub>2me</sub>K3 + <sub>1me</sub>E6</b>	<b>PIP2;1</b>	<b>2-16</b>
1869.93	DYVDPPPAPLLDMGELK		none	PIP2;7	16-32
1872.90	DYQDPPPAPFIDGAELK	DYQDPPPAPFI/LDGAELK	none	PIP2;1	17-33
2000.99	DYQDPPPAPFIDGAELKK	DYQDPPPAPFI/LDGAEL/IKK	none	PIP2;1	17-34
2066.96	DYKDPPPAPFFDMEELR		none	PIP2;4	17-33
2096.94	DYEDPPPTPFFDADELTK	DYEDPPPTPFFDADEL/ITK	none	PIP2;2	15-32

The table shows putative PIP2 peptides that were identified from the 28 kDa band of a root PM fraction as described in Table 2. Peptides with modified residues are indicated in bold characters.

Conventions for post-translational modifications are as follows:

1me: monomethyl; 2me: dimethyl; <sub>1me</sub>E6: monomethyl Glu6; <sub>2me</sub>K3: dimethyl Lys3.

\*: peptide the ESI-MS/MS spectrum of which is shown in Figure 2.

\*\*: the peptide sequence was deduced from PSD fragmentation. Lys3 was shown to be dimethylated (<sub>2me</sub>K3). The additionnal 14 Da mass increment indicates the presence of a methyl that must be positionned within the following sequence DVEGPEGFQTR.



**Table 4 Characterization by MS of N-terminal peptides of WT and mutated forms of PIP2;1**

Form expressed	Measured	Predicted sequence	Abundance (%)	
	peptide mass (Da)		roots	Suspension cells
WT	1603.80*	AKDVEAVPGEGFQTR	64 ± 9	49 ± 27
	1617.80	AKDV <sub>1me</sub> EAVPGEGFQTR	3 ± 2	17 ± 3
	1631.80*	A <sub>2me</sub> KDVEAVPGEGFQTR	7 ± 4	9 ± 6
	1645.80*	A <sub>2me</sub> KDV <sub>1me</sub> EAVPGEGFQTR	26 ± 6	25 ± 15
			<i>n</i> = 9	<i>n</i> = 4
E6A	1545.80*	AKDVAAVPGEGFQTR		100
				<i>n</i> = 2
K3A	1546.75	AADVEAVPGEGFQTR		13 ± 5
	1588.75*	<sup>ac</sup> AADVEAVPGEGFQTR		82 ± 1
	1602.75*	<sup>ac</sup> A <sub>1me</sub> ADVEAVPGEGFQTR		4 ± 4
				<i>n</i> = 3
K3R	1631.81	ARDVEAVPGEGFQTR		94 ± 6
	1645.81 <sup>£</sup>	ARDVEAVPGEGFQTR + 1me		4 ± 4
	1673.81*	<sup>ac</sup> ARDVEAVPGEGFQTR		2 ± 4
				<i>n</i> = 4

The first column refers to the form of PIP2;1, either WT or mutated. The second column refers to the measured peptide mass. The indicated peptides were identified after trypsin digestion of the proteins. They correspond to all identified forms of the 2-16 N-terminal peptide. Note that, because of an inefficient trypsin cleavage after Lys3, the Asp4-Arg16 peptide represents, with respect to Ala2-Arg16, a minor digestion product almost undetectable in the MALDI-TOF fingerprint. The predicted sequence is indicated in the third column. The last column refers to the relative abundance ( $\pm$  SE) in PM fractions from either roots or suspension cells. *n* refers to the number of MS spectra from at least two independent transformed cell lines or plant cultures.

\*: sequence determined by tandem MS.

<sup>£</sup>: the position of the methyl group was not determined

<sup>ac</sup>A: acetyl Ala; <sub>1me</sub>E: monomethyl Glu; <sub>2me</sub>K: dimethyl Lys.

## FIGURE LEGENDS

### Figure 1 Representative sequence analysis of a N-terminal PIP1 tryptic peptide from root PM.

A Met1-Arg9 peptide of PIP1 was analyzed by ESI-MS/MS and both N-terminal (b-type) and C-terminal (y-type) fragments were used to interpret the spectra. Note that PIP1;1, PIP1;2, PIP1;3 and PIP1;4 isoforms share a perfectly conserved Met1-Arg9 sequence. Analysis was performed on the  $[M+2H]^{2+}$  species of 567.76 Da that corresponds to the  $[M+H]^+$  species of 1134.52 Da, as described in Table 2. The whole sequence can be deduced from the spectrum. <sub>Ac</sub>M: acetylated Met.

### Figure 2 Representative sequence analysis of N-terminal PIP2;1 tryptic peptides from root PM.

Ala2-Arg16 peptides of PIP2;1 were analyzed by ESI-MS/MS and N-terminal (b-type), C-terminal (y-type) and internal fragments were used to interpret the spectra. Fragmented peptides are indicated according to either b and y series, or by their presumed sequence in the case of internal fragments.

(A) Analysis was performed on the  $[M+2H]^{2+}$  species of 816.40 Da that corresponds to the  $[M+H]^+$  species of 1631.80 Da, as described in Table 3. The whole sequence can be deduced from the spectrum. The initiating Met is cleaved. Lys3 is dimethylated (<sub>2me</sub>K).  $Z = K + 28$  Da.

(B) Analysis was performed on the  $[M+3H]^{3+}$  species of 549.27 Da that corresponds to the  $[M+H]^+$  species of 1645.80 Da, as described in Table 3. The whole sequence can be deduced from the spectrum. The initiating Met is cleaved. Lys3 is dimethylated (<sub>2me</sub>K) whereas Glu6 is monomethylated (<sub>1me</sub>E).  $Z = K + 28$  Da;  $J = E + 14$  Da.

### Figure 3 Ectopic expression of PIP2;1 in suspension cells

(A) Western blot analysis, using a anti-PIP2;1 peptide antibody, of total protein extracts from independent cell lines, either untransformed (Untr.) or transformed with an empty vector (PG) or with a *CaMV70S::PIP2;1* construct (lines 1-5). Equal amounts of proteins (5 µg) were loaded onto each lane.

(B) MALDI-TOF analysis of the trypsin-cleaved 28 kDa band from PM of suspension cells overexpressing PIP2;1. Peptides derived from PIP2;1 are described by their monoisotopic mass, sequence and position inside the protein sequence.

Three peptides were assigned to endogenous PIP aquaporins:

(•):  $[M+H]^+ = 1017.55$  Da, attributed to sequence VGANKFPER of PIP1 isoforms.

(••):  $[M+H]^+ = 1134.52$  Da, attributed to sequence  $_{ac}MEGKEEDVR$  of PIP1;1/ PIP1;2/ PIP1;3/ PIP1;4 isoforms.

(•••):  $[M+H]^+ = 1312.65$  Da, attributed to sequence SFGAAVIYNNEK of PIP2;4/ PIP2;7/ PIP2;8 isoforms.

The trypsin autolysis peptides are also indicated (\*).  $_{ac}M$ : acetylated Met;  $_{1me}E$ : monomethylated Glu;  $_{2me}K$ : dimethylated Lys;

#### **Figure 4 Relationship between water transport activity and PIP2 abundance in purified PM vesicles.**

(A) Representative time course of stopped-flow scattered light intensity following imposition of an inwardly directed osmotic gradient on PM vesicles purified from PG (blue) and WT-PIP2;1 (green) cells. The data were fitted with a single exponential function with a rate constant of  $k_{exp} = 5.90 \text{ s}^{-1}$  and  $9.39 \text{ s}^{-1}$  for PG and WT-PIP2;1 cells, respectively.

(B) PM vesicles were prepared from at least two representative cell lines of each genotypes, *i.e.* from cells containing an empty vector (PG) or overexpressing the WT, E6A, K3A or K3R form of PIP2;1. PM vesicles were probed for their water transport activity by stopped flow measurements ( $k_{exp}$ ) and for PIP2 abundance by an ELISA assay. For each cell line, water transport activity was calculated as the ratio of  $k_{exp}$  to PIP2. The specific activity of overexpressed PIP2;1 was deduced by substitution of the mean water transport activity measured in PG cells and was expressed in % of the specific activity of WT form.

Each measurement was performed in triplicate from the indicated number ( $n$ ) of independent cell lines.

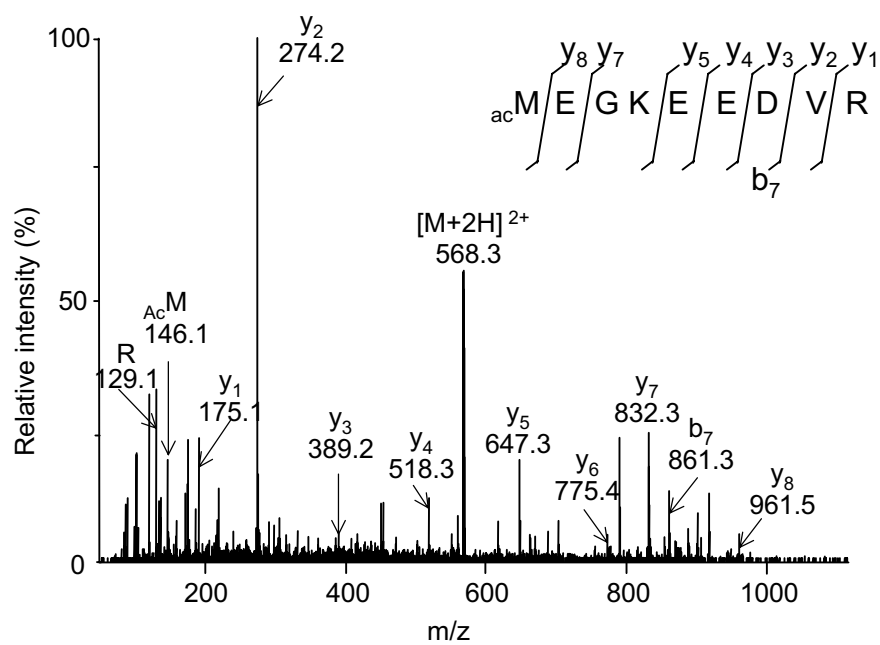


Fig 1

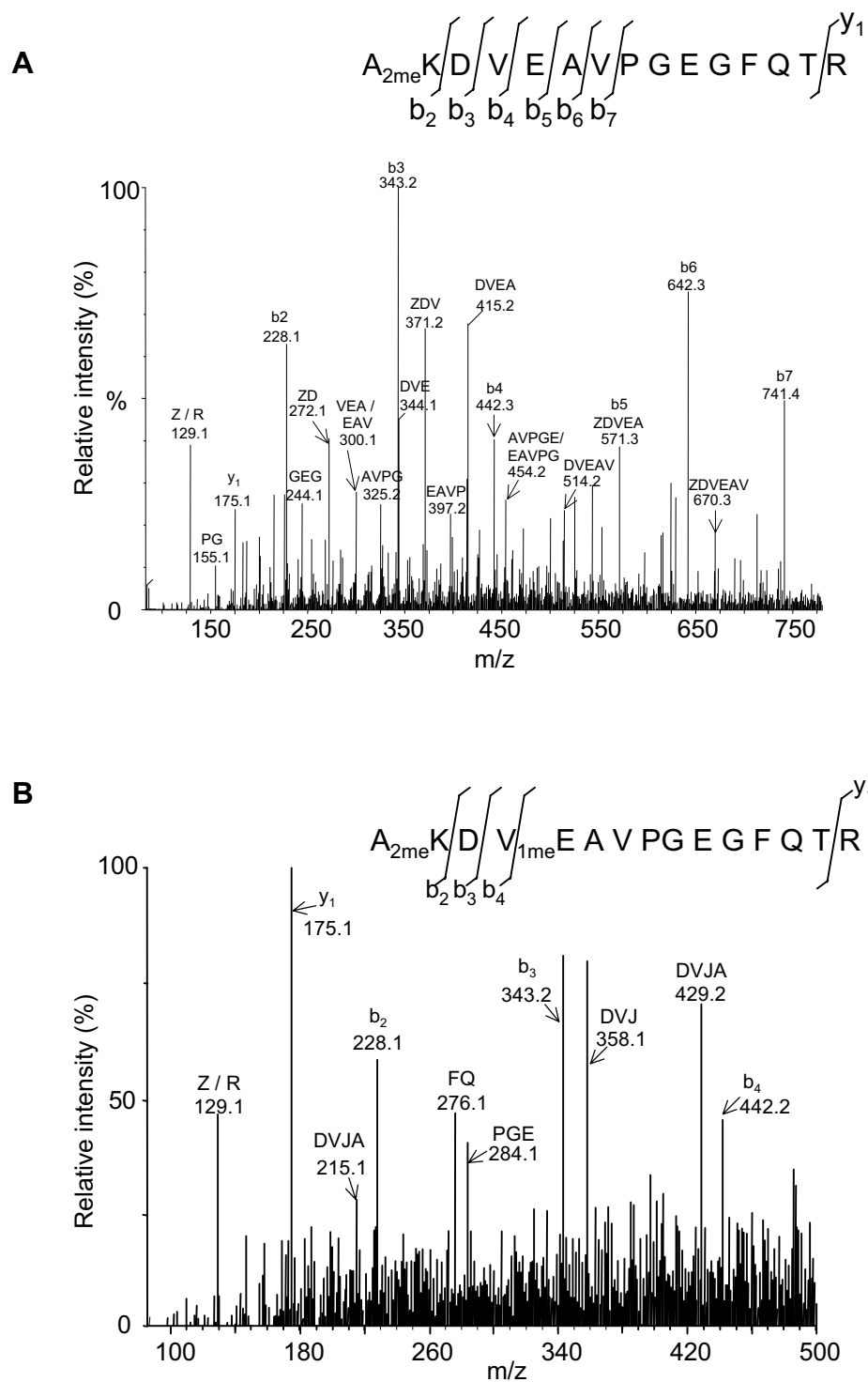


Fig 2

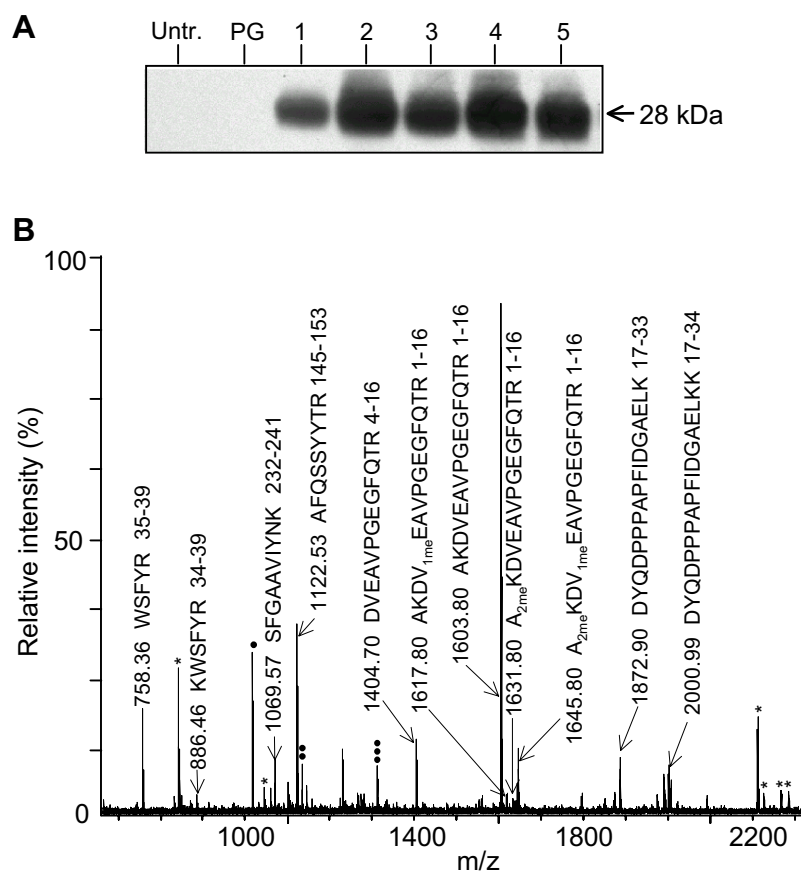


Fig 3

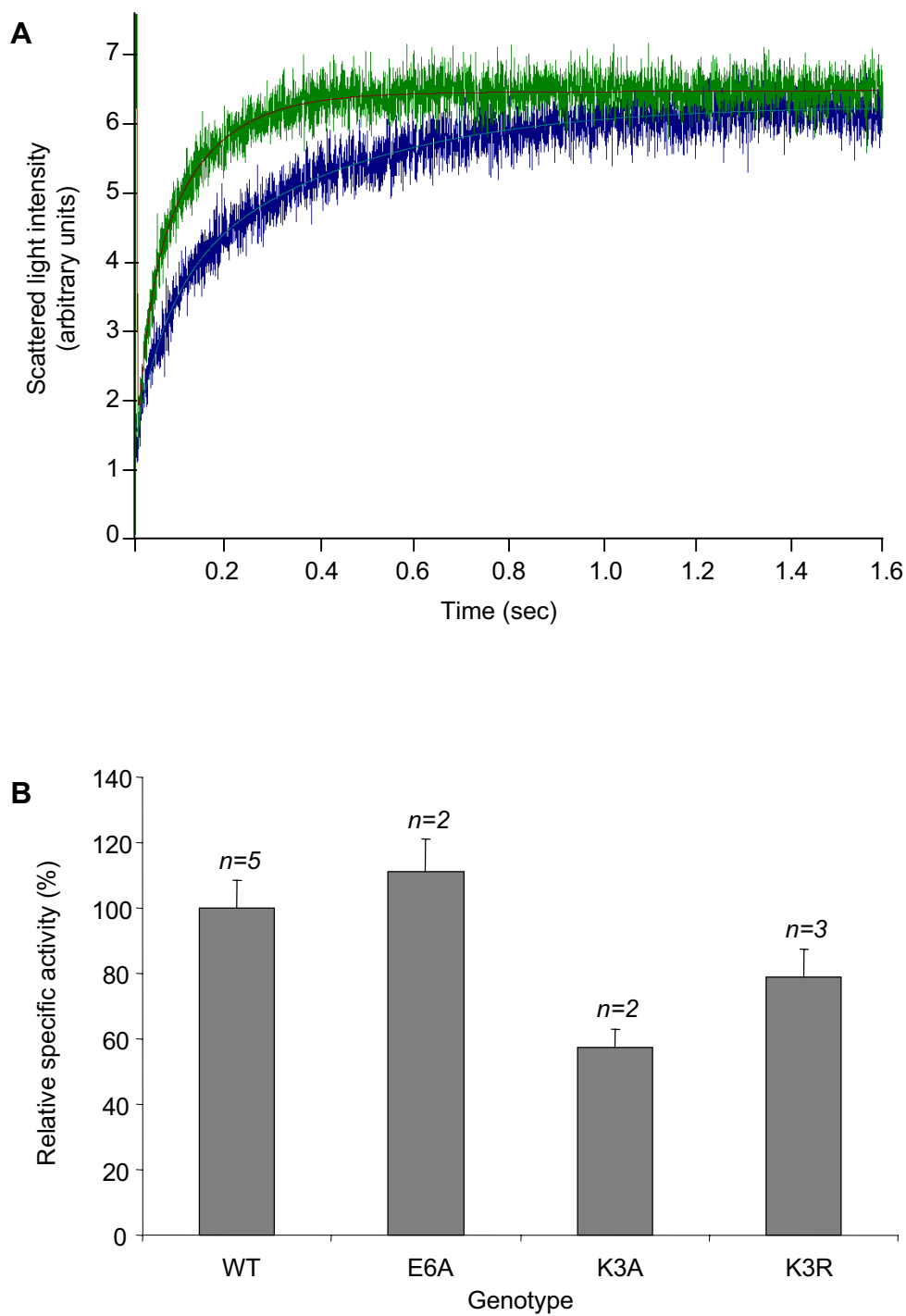


Fig 4