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Research article

# T-homoeolog specific plasma membrane protein 3 [*Nt(t)PMP3-2*] in polyploid *Nicotiana tabacum* shows conserved alternative splicing, derived from extant *Nicotiana tomentosiformis* parent

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ABSTRACT

Abiotic stress induced plasma membrane protein 3 (*PMP3*) genes occur as multigene families in plants, coding for hydrophobic proteins. Group I *PMP3s* code for shorter ORFs while Group II *PMP3s* code for proteins with C-terminal extensions. Allotetraploid *Nicotiana tabacum* (SSTT; 2n = 48) derives its parentage from extant ancestors related to *Nicotiana sylvestris* (SS) and *Nicotiana tomentosiformis* (TT). Polyploidization triggers complex genetic and epigenetic changes, often leading to homoeolog-specific retention or loss of function, sub-functionalization or neo-functionalization. Genomic sequences of *Nt(t)PMP3-1*/*Nt(t)PMP3-2* cloned from *N. tabacum* show near identity with *N. tomentosiformis NtoPMP3-1*/*NtoPMP3-2* genomic sequences respectively (distinct from *N. sylvestris NsPMP3-1*/*NsPMP3-2* genomic regions). RT-PCR with exon 1,2 primer pairs amplified only single fragments for *Nt(t)PMP3-1* and *Nt(t)PMP3-2*. In contrast, for *Nt(t)PMP3-2*, three variants were detected using exon 2,3 primers by RT-PCR. Cloning revealed (i) a transcript coding for a Group I *PMP3* [*Nt(t)PMP3-2CS*], (ii) a transcript with complete retention of the second intron [*Nt(t)PMP3-2IR*] and (iii) a transcript with an alternative (exon 2) 5′ splice site [Nt(t)PMP3-2AS], coding for a longer protein, similar to ORFs of Group II PMP3 genes. All three *Nt(t)PMP3-2* variants have conserved counterparts in the *N. tomentosiformis* transcriptome, suggesting the transcriptional machinery governing alternative splicing of *Nt(t)PMP3-2* in *N. tabacum* has conserved origins, derived from a *N. tomenosiformis* lineage. The above data shows alternative splicing of *PMP3* genes contributes to transcript and ORF diversity in plants. All three *Nt(t)PMP3-2* splice variants show increased root-specific expression. Implications of *Nt(t)PMP3-2* alternative splicing on transcript stability and ORF features are discussed.



## 1. Introduction

Polyploidization is an important mechanism contributing to plant evolution, speciation and adaptation and has contributed to domestication of numerous crop species (Bombarely et al., 2012). Polyploidization involves complex genetic and epigenetic processes, with genome duplication often accompanied by changes in gene expression and/or gene loss. Following polyploidization, homeologs may show retention of previous function, homoeolog-specific loss of function, sub-functionalization or neo-functionalization. *Nicotiana tabacum* is an allotetraploid species (SSTT; 2n = 4x = 48), evolved through interspecific hybridization of ancestors of *Nicotiana sylvestris* (SS; 2n = 24; maternal donor) and *Nicotiana tomentosiformis* (TT; 2n = 24; paternal donor) less than 2,00,000 years ago (Leitch et al., 2008; Fulneček and Matyášek, 2016). The draft genome and transcriptomes of *N. tomentosiformis* and *N. sylvestris* (Sierro et al., 2013) and three tobacco varieties (Sierro et al., 2014) has allowed comparison of the relative expression of homoeologous genes in *N. tabacum*. Analysis of the complex leaf transcriptome of *N. tabacum* suggests little evidence for either preferential maintenance of gene expression from T- or S-genomes or for neo-functionalization (Bombarely et al., 2012).

*Abbreviations:* nr, non-redundant; PMP3, Plasma membrane protein 3; PAD, Pre-amplification denaturation; Semi-QT, Semi quantitative; RACE, Rapid amplification of cDNA ends.

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Plasma membrane proteins 3 (PMP3) are a class of conserved hydrophobic proteins found in bacteria, yeast, invertebrates and plants (Chidambaram et al., 2015). First purified from yeast, strains deleted for $Na^+$ efflux systems Ena1p, Nha1p and also deleted for *PMP3/SNA1*, show $Na^+$ accumulation, salt sensitivity and membrane hyperpolarization (Navarre and Goffeau, 2000). Deletion of *PMP3* in a strain deficient in $K^+$ uptake restores growth at low pH. *PMP3*-like genes occur as multigene families in plants and are variously referred to as *PMP3*, *Lti6* or *RCI2* genes (Rocha, 2016). *PMP3*-like genes were first isolated and cloned from abiotic stress (cold, salinity) induced tissues (Capel et al., 1997; Gulick et al., 1994; Morsy et al., 2005). Expression of PMP3-like genes is induced in response to a wide variety of abiotic stress conditions in plants (Chidambaram et al., 2015). While they are mostly localized to the plasma membrane (Chidambaram et al., 2015; Fu et al., 2012; Imai et al., 2005; Inada et al., 2005; Rocha, 2016), some plant *PMP3s* show localization to other subcellular organelles (Khurana et al., 2015; Medina et al., 2007). PMP3 genes can be grouped into two categories depending on their exon, intron organization and the length of the deduced proteins they code for (Medina et al., 2007; Rocha, 2016). Group I PMP3 genes show the presence of three exons and two introns and code for 50–60 amino acid proteins. Group II PMP3 genes show the presence of two exons and one intron and code for proteins longer than 60 amino acids (usually 60–90 amino acids; Rocha, 2016).

Over-expression and knockout data from plants show that PMP3 proteins participate in cell ion homeostasis and in regulation of membrane stability. *Arabidopsis AtRCI2a* mutants show growth inhibition and increased shoot $Na^+$ accumulation under salinity (Mitsuya et al., 2005), reversed by over-expressing *AtRCI2a* (Mitsuya et al., 2006). *Arabidopsis* plants constitutively over-expressing *AtRCI2a* or *Musa paradisiaca* RCI show lowered shoot $Na^+$ levels and enhanced plant performance under stress (Liu et al., 2012). Transgenic tobacco plants expressing *Aeluropus littoralis PMP3* (*AlTMP1*) show abiotic stress tolerance due to improved water status and cation homoeostasis (Ben Romdhane et al., 2017). PMP3 proteins, being small polypeptides, are unlikely to possess transporter activity (Rocha, 2016). They may, however, contribute indirectly to cation homeostasis within cells by interaction with ion transporters. *ZmPMP3-1* overexpressing *Arabidopsis* lines show up-regulation in expression of genes concerned with vacuolar ion storage under salinity (Fu et al., 2012). *AlTMP1* overexpressing tobacco lines show upregulation of stress regulated transcripts (*NHX1*, *CAT1*, *APX1* and *DREB1A*) and downregulation of $Na^+$ uptake related transcripts (*HKT1* and *KT1*; Ben Romdhane et al., 2017). Recent studies in yeast suggest that Pmp3p is involved in a phosphoinositide regulatory system between the plasma membrane and the vacuole (De Block et al., 2015). Pmp3p also binds to sphingomyelin, with *PMP3* showing genetic interactions with genes involved in sphingolipid synthesis (Bari et al., 2015). *PMP3* might thus be part of a regulatory system between phosphoinositides and sphingolipids. We report here genomic sequences of two *PMP3* genes from *N. tabacum cv.* Petit Havana [*Nt(t)PMP3-1* and *Nt(t)PMP3-2*] that show near identity with *Nicotiana tomentosiformis PMP3* genes (*NtoPMP3-1 and NtoPMP3-2*) respectively. We also show that the three *Nt(t)PMP3-2* transcripts generated by alternative splicing in *N. tabacum* show increased root specific expression and have conserved counterparts in the *N. tomentosiformis* transcriptome.

## 2. Materials and methods

### 2.1. Cloning of PCR products

All genomic, RT-PCR, semi-QT, RACE-PCR amplification products mentioned below were agarose gel eluted using a kit (Favorgen Biotech Corp, Taiwan), cloned in a T-vector (pTZ57R/T; Fermentas, ThermoFisher Scientific, USA) and sequenced using universal M13 Reverse and Forward primers (ABI 3730, ThermoFisher Scientific, USA).

### 2.2. Isolation of Nt(t)PMP3-1and Nt(t)PMP3-2 genomic sequences

*Nt(t)PMP3-1* and *Nt(t)PMP3-2* genomic clones were amplified from *N. tabacum cv.* Petit Havana genomic DNA (50 ng) using primer pairs NtPMP3-1 Fwd A/NtPMP3-1 Rev A and NtPMP3-2 Fwd1/NtPMP3-2 Rev 3 respectively (Supplementary Table 1; final concentration - 200 nM each) in a 20 μL reaction volume using XT-20 Taq Polymerase (proof-reading polymerase; Bangalore Genei, India) and PCR cycling conditions [PAD at 94 °C (5 min); 30 cycles 94 °C (30s); 55 °C (30s) sec; 72 °C (1min)], final extension at 72 °C (7 min). Three genomic clones each for *Nt(t)PMP3-1* and *Nt(t)PMP3-2* were fully sequenced (ABI 3730, ThermoFisher Scientific, USA).

### 2.3. Plant growth conditions

*Nicotiana tabacum cv.* Petit Havana seeds were surface sterilized with 70% ethanol for 1 min, 2% sodium hypochlorite for 30 min and plated on 0.5X Murashige Skoog medium (MS, HiMedia, India) with 2% sucrose (8 g/L agar) and grown at 25 ± 1 °C (8 h dark/16 h light). Two week old seedlings were transferred to jars containing 0.5X MS with 2% sucrose (8 g/L agar) and grown at 25 ± 1 °C (8 h dark/16 h light) for one month. Leaf and root tissues were harvested and frozen in liquid nitrogen for RNA isolation.

### 2.4. Alignment of N. tomentosiformis and N. sylvestris PMP3-1/PMP3-2 genomic and mRNA sequences

Genomic and mRNA sequences of *N. sylvestris* and *N. tomentosiformis PMP3* sequences corresponding to *Nt(t)PMP3-1* and *Nt(t)PMP3-2* were retrieved from NCBI. For *PMP3-1*, these correspond to LOC104108691 (*N. tomentosiformis*) and LOC104245249 (*N. sylvestris*) respectively. For *PMP3-2*, these correspond to LOC104086602 (*N. tomentosiformis*) and LOC104224491 (*N. sylvestris*) respectively. The retrieved *PMP3* genomic sequences were aligned with the isolated *Nt(t)PMP3-1* and *Nt(t)PMP3-2* genomic sequences (Supplementary Figs. S1A and S1B). Primers used for RT-PCR amplification of *Nt(t)PMP3-1* and *Nt(t)PMP3-2* are indicated in Supplementary Table 1 (also see Supplementary Fig. S2A and S2B).

### 2.5. RT-PCR amplification of Nt(t)PMP3-1 and Nt(t)PMP3-2 transcripts

Total RNA was isolated from leaf and root samples using RNAiso Plus (Takara, Japan). RNA integrity and concentration was estimated (Multiskan™ GO Microplate Spectrophotometer, ThermoScientific, USA) and total RNA (2 μg) was treated with DNase I (1U; Fermentas; 37 °C; 40 min), followed by addition of EDTA and heat inactivation (65 °C; 10 min). A 'minus (-RT) PCR' reaction was included to rule out genomic DNA contamination. cDNA synthesis was performed in a 20 μL reaction volume [1 μg (DNase I treated total RNA), (M-MLV Reverse Transcriptase-200U; Invitrogen)] at 37 °C (50 min), followed by heat inactivation at 70 °C (15 min). Diluted cDNA (1:10; 2 μL) was used as template for PCR amplification: 20 μL reaction volume [2X Taq DNA polymerase Master Mix Red (Ampliqon), given primer pair (200 nM each final concentration; Supplementary Table1)]. PCR cycling conditions: PAD- 94 °C (5 min); 35 cycles [94 °C (30s); 55 °C (30s); 72 °C (30s)]; final extension at 72 °C (7 min). Three *Nt(t)PMP3-2* transcript variants