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Article Addendum

Pollen aquaporins: What are they there for?

Juliana Andrea Pérez Di Giorgio^{1,¶}, María Laura Barberini¹, Gabriela Amodeo^{2,3} and Jorge Prometeo Muschietti^{1,3,*}

¹ Instituto de Investigaciones en Ingeniería Genética y Biología Molecular, Dr. Héctor Torres (INGEBI-CONICET), Vuelta de Obligado 2490, C1428ADN Buenos Aires, Argentina.

² Instituto de Biodiversidad y Biología Experimental y Aplicada (IBBEA-UBA-CONICET), Intendente Güiraldes 2160, Ciudad Universitaria, Pabellón II, C1428EGA Buenos Aires, Argentina.

³ Departamento de Biodiversidad y Biología Experimental, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Intendente Güiraldes 2160, Ciudad Universitaria, Pabellón II, C1428EGA Buenos Aires, Argentina.

Current address: Department of Biochemistry and Molecular Medicine, Université de Montréal, Montréal, Québec, H3C 3J7, Canada.

*Correspondence to: Jorge Muschietti; Email: prometeo@dna.uba.ar

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Addendum to: Pérez Di Giorgio J, Bienert GP, Ayub N, Yaneff A, Barberini ML, Mecchia MA, Amodeo G, Soto G, Muschietti JP. Pollen-specific aquaporins NIP4;1 and NIP4;2 are required for pollen development and pollination in *Arabidopsis thaliana*. The Plant Cell 2016, May;28 (5):1053-77.

Abstract

In order to provide more insight into the function of aquaporins during pollination, we characterized NIP4;1 and NIP4;2, two pollen-specific aquaporins of *Arabidopsis thaliana*. NIP4;1 and NIP4;2 displayed high amino acid identity. RT-PCR and GUS promoter analysis showed that they have different expression patterns. *NIP4;1* is expressed at low levels in mature pollen, while *NIP4;2* is highly expressed only during pollen tube growth. Single T-

DNA *nip4;1* and *nip4;2* mutants and double amiRNA *nip4;1 nip4;2* knockdowns showed reduced male fertility due to deficient pollen germination and pollen tube length. Functional assays in oocytes showed that NIP4;1 and NIP4;2 transport water and nonionic solutes. Here, the participation of the different pollen aquaporins in pollen hydration and pollen tube growth is discussed.

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A successful fertilization in plants starts when the pollen grain contacts the surface of the stigma and hydrates. After hydration, the internal hydrostatic pressure of the pollen grain increases and a pollen tube is developed. Then, the pollen tube grows through the papilla cell wall, penetrates the transmitting tract of the style, elongates and reaches the embryo sac where double fertilization takes place.

In dry stigmas, pollen hydration is a highly regulated process and takes place only if a compatible communication between the pollen and the stigma cells is established. It is the checkpoint where self-incompatible and incongruous pollen grains are not hydrated and finally rejected due to the lack of a compatible recognition. This specificity is based on controlling the movement of water and solutes from stigma to the pollen grains. Later, during pollen tube growth, water and osmotic solutes have to move very fast into the pollen tube to maintain the required turgor pressure¹. Under these scenarios, aquaporins become good candidates in regulating the flow of water and osmotic solutes across the pollen membranes²⁻⁴.

We decide to study how *Arabidopsis thaliana* pollen specific aquaporins would contribute to transport water and/or solutes during pollination and pollen tube growth. We first characterized two Arabidopsis pollen aquaporins, TIP1;3 and TIP5;1, which belong to the tonoplast intrinsic proteins (TIPs) subclass. Both TIPs transported not only water but also urea at high permeability levels⁵. Single *tip1;3* and *tip5;1* mutant pollen showed normal growth rate⁶ while double mutant *tip1;3 tip5;1* showed high numbers of sterile pods, especially under water- or nutrient-deficient conditions⁷.

We then study the other two remaining pollen specific aquaporin genes, *NIP4;1* and *NIP4;2*⁸. They both belong to the nodulin26-like intrinsic proteins (NIPs) subclass. Our molecular genetic and functional analysis demonstrated that *NIP4;1* and *NIP4;2* are important players in pollen development and pollination. *NIP4;1* and *NIP4;2* are paralog genes found exclusively in Angiosperms. Even though they have high amino acid identity (84%), they showed different expression patterns. RT-PCR and GUS promoter analysis showed that while *NIP4;1* is expressed at low levels only in mature pollen, *NIP4;2* is highly expressed exclusively

during pollen tube growth.

We found that pollen from single T-DNA *nip4;1* and *nip4;2* mutants and double amiRNA *nip4;1 nip4;2* knockdowns had reduced fertility and lower pollen germination and pollen tube length compared to wild type pollen. When we analyzed the kinetics of in vitro pollen germination, we found that wild type pollen germinated faster than T-DNA *nip4;1* and *nip4;2* mutant pollen grains (Figure 1). This result is in agreement with the defects found in pollination for the NIP mutants. Coincidentally to their different expression pattern, we showed that NIP4;1 functions during pollen development and germination, while NIP4;2 does it exclusively during pollen tube growth.

Related to their water and solutes transport activity, while both NIP4;1 and NIP4;2 have moderate water transport, they both showed high osmotic solute (glycerol) permeability when expressed in *Xenopus* oocytes. Survival analysis in yeast confirmed water transport *Xenopus* results and also showed that NIP4;1 has the capacity of transporting ammonia, urea, boric acid and H₂O₂. Using mass spectrometry, we also found that a specific serine (Ser267) of the C-termini of NIP4;1 and NIP4;2 was phosphorylated in vitro by CPK34, a pollen-specific calcium-dependent protein kinase essential for pollen tube growth⁹. Mutants where Ser267 was replaced by an alanine showed lower levels of water permeability when compared to their wild type versions, suggesting a potential role for S267-phosphorylation in the regulation of the water transport of NIP4;1 and NIP4;2. This result agrees with previous studies in which phosphorylation of soybean NOD26 at Ser-262 enhanced water transport and appeared with the establishment of the symbiosomes involved in nitrogen fixation¹⁰.

The main question is why *Arabidopsis* expresses four specific aquaporin genes during pollen development and pollen tube growth, but none of them seems to be totally essential for pollen life. Both combination of double mutants, *tip1;3 tip5;1* and *nip4;1 nip4;2*, did not show a fully sterile male phenotype. Gene redundancy is always a good explanation for that, but in this case it seems to be unlikely because TIP1;3, TIP5;1, NIP4;1 and NIP4;2 belong to two different subclasses (two TIPs and two NIPs) that are also differentially expressed: while

TIP1;3, TIP5;1 and NIP4;1 are expressed in mature pollen, NIP4;2 is exclusively expressed after pollen germination. Lastly, according to our *Xenopus* results, they transport different solutes.

Despite the fact that the double amiRNA *nip4;1 nip4;2* knockdown showed affected pollen fitness, the lack of a completely infertile phenotype could be explained by the presence of other pollen -not specific- aquaporins that indeed would control the flux of water. SIP1;1 and SIP2;1 are good candidates because they are expressed in pollen although also in most of the vegetative tissues¹¹. SIP1;1, but not SIP2;1, showed water transport activity in yeast membrane vesicles by stopped-flow light scattering assays¹². However, the fact that SIP1;1 and SIP2;1 are both expressed in the ER of stems of *Arabidopsis* plants, makes it difficult to conceive how they would transport water from the female tissue into the pollen¹³.

Perhaps the simplest hypothesis is that these four pollen specific aquaporins transport different solutes propelling the flux of water from female tissues thus increasing the water potential of pollen grains and pollen tubes turgor pressure, necessary for pollen hydration and pollen tube tip growth, respectively^{1,14}. In this direction, it has been suggested that plasma membrane aquaporins could act as osmosensors controlling turgor pressure of pollen tubes while they grow towards the ovary¹⁴.

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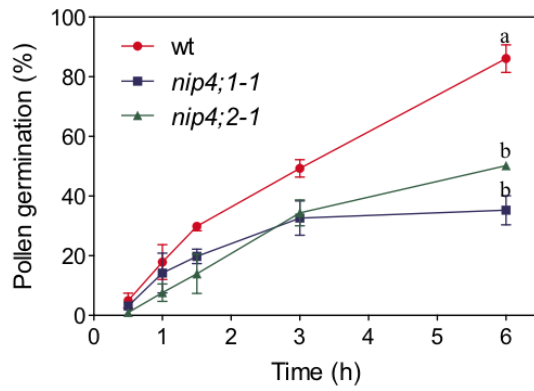


Figure 1. In vitro pollen germination of *wild type* (wt) and single mutants *nip4;1-1* and *nip4;2-1* plants. Assays were performed in three replicates and 100 pollen grains were measured for each replicate. Different letters indicate statistically significant differences ($P < 0.05$, two-way ANOVA, Tukey's test).