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# Functional characterization of *FaNIP1;1* gene, a ripening-related and receptacle-specific aquaporin in strawberry fruit

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ABSTRACT

Strawberry fruit (*Fragaria* × *ananassa*) is a soft fruit with high water content at ripe stage (more than 90% of its fresh weight). Aquaporins play an important role in plant water homeostasis, through the facilitation of water transport and solutes. We report the role played by FaNIP1;1 in the receptacle ripening process. The analysis by qRT-PCR of *FaNIP1;1* showed that this gene is mainly expressed in fruit receptacle and has a ripening-related expression pattern that was accompanied by an increase in both the abscisic acid and water content of the receptacle throughout fruit ripening. Moreover, *FaNIP1;1* was induced in situations of water deficit. Additionally, we show that *FaNIP1;1* expression was positively regulated by abscisic acid and negatively regulated by auxins. The water transport capacity of FaNIP1;1 was determined by a stopped-flow spectroscopy in yeast over-expressing *FaNIP1;1*. Glycerol, $H_2O_2$ and boron transport were also demonstrated in yeast. On the other hand, GFP-FaNIP1;1 fusion protein was located in plasma membrane. In conclusion, *FaNIP1;1* seems to play an important role increasing the plasma membrane permeability, that allows the water accumulation in the strawberry fruit receptacle throughout the ripening process.



## 1. Introduction

Strawberry fruit (*Fragaria* × *ananassa*) is a non-climacteric soft fruit in which water represents more than 90% of its fresh weight at ripe stage. During ripening process, the strawberry fruit undergoes a series of physiological and biochemical changes that are determined by gene expression modifications and influenced by the endogenous biosynthesis of abscisic acid (ABA) and which influence its organoleptic properties (flavour, texture, color and aroma) and determine its final quality [1–3]. In this sense, the ABA biosynthesis increases drastically in the last stages of fruit ripening in strawberry fruit receptacle [1,3,4].

In the strawberry fruit, water movements are crucial since they allow for the rapid expansion of fruit [5]. Previous studies reported that berry size correlates positively with the amount of irrigation water applied during flowering and fruit development [6]. Water

*Abbreviations:* 2,4-D, 2,4-dichlorophenoxyacetic acid; ABA, abscisic acid; dABA, deuterated abscisic acid; CF, carboxyfluorescein fluorescent; CFDA, carboxyfluorescein diacetate; GFP, green fluorescent protein; GIPs, GlpF-like intrinsic protein; G1, green 1stage; G3, green 3 stage; $E_a$, activation energy; HIP, hybrid intrinsic protein; HPLC, MS; LB, Luria-Bertani medium; MIPs, major intrinsic proteins; MC, moisture content; MS, Murashige & Skoog medium; NCED, 9-cis-epoxycarotenoid dioxygenase; NDGA, nordihydroguaiaretic acid; NIP, nodulin 26-like intrinsic protein; OR, overripe stage; PAR, photosynthetically active radiation; PIPs, plasma membrane intrinsic proteins; $P_f$, permeability coefficient; PM, plasma membrane; R, red stage; SIPs, small and basic intrinsic proteins; TIPs, tonoplast intrinsic proteins; VWC, volumetric water content; W, white stage; XIP, X intrinsic protein; YNB, yeast nitrogen base medium.

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balances are always accompanied by water movements across biological membranes by means of diffusion or through water channels [7]. Thus, the presence of water channels, known as aquaporins, in plasma membranes is necessary when high rates and low resistance of water transport across membranes are required [8].

Aquaporins belong to the family of integral membrane channel proteins known as Major Intrinsic Proteins (MIPs). MIPs are particularly abundant in plants and exhibit high multiplicity and diversity, which is likely due to the necessity of a fine-tuned water control that allows the plant to adapt to changing environmental conditions [9]. According to intracellular locations and sequence similarities, they are divided into seven subfamilies: the plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), NOD26-like intrinsic proteins (NIPs), small and basic intrinsic proteins (SIPs), the GlpF-like intrinsic protein (GIPs), hybrid intrinsic protein (HIP) and the uncategorized X intrinsic protein (XIP) [9]. Plant MIPs not only transport water, but they also facilitate the transport of small neutral solutes such as glycerol [10] and boron [11–15].

The relationship between water movement and fruit ripening has been widely studied [16–19]. In apples, two putative *PIP* genes were related to fruit development and osmotic stress [17]. Similarly, in grape and strawberry, two PIP1 aquaporins were associated with the ripening process [18–21]. In addition, several *SlNIP* genes were detected in different development stages in tomato fruits [22].

Changes in aquaporins gene expression during fruit growth and the ripening process have been associated with the increase of fruit softening. In fact, softening has also been claimed to be a physical consequence of a reduction in cell turgor [23–26]. In strawberry fruits, where retaining firmness is so important, the amount of *FaPIP1* and *FaPIP2* mRNAs was higher in ripen fruits of a firmer cultivar (Camarosa) as compared to that found in a softer cultivar (Toyonaka) [21]. Additionally, the co-expression of *FaPIP1;1* and *FaPIP2;1* genes resulted in an increase of water permeability in the strawberry fruit [21,27]. These data seem to indicate that both *FaPIP* genes might be responsible for the control of cell water homoeostasis and the accumulation that coincides with strawberry fruit ripening that can be related to maintaining fruit turgor [20,21]. Thus, the transcription of *PIP* aquaporins can be fine-tuned with the environment in response to declining water availability [28].

In plants, ABA biosynthesis is strongly stimulated by water deficit [29]. It has been proposed that ABA increase in drought stress is due to the perception of a putative hydraulic signal by yet unknown sensor(s) that would lead to conversion of the physical signal into the hormonal ABA signal, which would determine the adaptive responses related with changes in cell turgor [30]. Both ABA biosynthesis and the generation of this hydraulic signal are strongly induced in drought stress.

Additionally, banana transgenic plants over-expressing *MusaPIP1;2* presented an increase of tolerance to different abiotic stresses including drought [31]. A similar behavior was observed in *Arabidopsis thaliana* plants over-expressing the heterologous *MaPIP1;1* gene, which enhanced the tolerance to osmotic and drought stresses and lead to the down-regulation of *RD29a*, *RD29b*, *RAB18* and *KIN2* ABA-responsive genes during dehydration and salt stresses indicating that the *MaPIP1;1* over-expression improved the physiological status of plants under drought and salt stress conditions [32].

In this work, we report the isolation and functional characterization of the receptacle-specific *FaNIP1;1* gene during the strawberry fruit growth and ripening (cv. Camarosa). The expression of this gene is strongly induced in receptacle ripening process in fruit, where an increase in ABA biosynthesis is produced, as well as during drought stress. Besides, FaNIP1;1 presents water transport properties and was located in plasma membrane. Our results strongly support the possibility that the FaNIP1;1 protein could play an important role in the control of the strawberry fruit hydration that could influence fruit size and firmness in normal water conditions, as well as in keeping the fruit healthy in situations of water deficit.

## 2. Materials and methods

### 2.1. Plant material

Strawberry plants (*F.* × *ananassa* Duch. cv. Camarosa, an octoploid cultivar) used in this work were grown under field conditions in Huelva, Spain. Fruits at different developmental stages were harvested: small-sized green fruit (green 1; 2–3 g), full-sized green fruit (green 3; 4–7 g), white fruit (5–8 g), full-ripe red fruit (6–10 g) and overripe fruit (6–10 g). Selected overripe fruits were harder and dark redder than red ones. Other tissues were harvested as well, such - runners, roots, crowns, expanding leaves, flowers and petals. All the collected tissues were frozen in liquid nitrogen and stored at −80 °C. *Nicotiana benthamiana* plants used for agroinfiltration were grown in plant chambers at 25 °C, 300 μmol photons $m^{-2}$ $s^{-1}$ (PAR), 80% humidity and a photoperiod of 16 h light and 8 h dark.

### 2.2. Yeast and bacterial strains and growth conditions

*Saccharomyces cerevisiae* YSH1172 (*MATa leu2*::hisG *trp1*::hisG *his3*::hisG *ura3*-52 *aqy1Δ*::kanMX4 *aqy2Δ*::*HIS3*) was used in this work. Yeast strain was maintained in YPD medium (5 g $L^{-1}$ yeast extract, 10 g $L^{-1}$ peptone, 20 g $L^{-1}$ glucose and 20 g $L^{-1}$ agar). Transformant strain was maintained and grown in a YNB medium without amino acids (DIFCO), with 20 g $L^{-1}$ glucose or galactose and 20 g $L^{-1}$ agar supplemented for prototrophic growth with the appropriate requirements.

*Escherichia coli* DH5α was used for routine propagation of plasmids. It was routinely maintained in a Luria-Bertani medium (LB) with ampicilin (100 mg $mL^{-1}$) at 37 °C.

### 2.3. Determination of water content

The moisture content (MC) of fresh strawberry fruit was obtained from 10 fruit samples at different development stages (Green 1, Green 3, White, Red, Overripe) that were dried in a hot oven at 75 °C for 48 h until the mass did not change between the two weighing intervals. It was performed three replicates for each analyzed fruit stage in order to obtain an average MC value for each sample.

Weight loss on drying to a constant final weight was recorded as moisture content based on the developed method AOAC [33] and using the following equation: $MC = ((M_o - M_s)/M_o) \times 100$; where MC is moisture content, $M_o$ is initial mass and $M_s$ in the final mass of strawberry fruit (g).

### 2.4. Auxin treatment

Achenes of two sets of 50 full-sized green fruits (Green 3) each were carefully removed from their receptacles, still attached to the plant, in accordance with Medina-Puche et al. [3]. One set of deachenes Green 3 fruits was treated with 2,4-dichlorophenoxyacetic acid (2,4-D) in lanolin paste (1 mL) containing 1 mM of auxin 2,4-D in 1% (w/v) dimethyl sulphoxide. The other set of Green 3 deachenes fruits (control situation) was treated with the same paste, but without 2,4-D. The auxin treatment, collection and analysis of samples were performed following the instructions by Medina-Puche et al. [3].