



Research article

Physiological responses and aquaporin expression upon drought and osmotic stress in a conservative vs prodigal *Fragaria x ananassa* cultivar

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ABSTRACT

In order to improve the understanding of plant water relations under drought stress, the water use behavior of two *Fragaria x ananassa* Duch. cultivars, contrasting in their drought stress phenotype, is identified. Under drought, stomatal closure is gradual in Figaro. Based on this, we associate Figaro with conservative water use behavior. Contrarily, drought stress causes a sudden and steep decrease in stomatal conductance in Flair, leading to the identification of Flair as a prodigal water use behavior cultivar. Responses to progressive drought on the one hand and an osmotic shock on the other hand are compared between these two cultivars. Tonoplast intrinsic protein mRNA levels are shown to be upregulated under progressive drought in the roots of Figaro only. Otherwise, aquaporin expression upon drought or osmotic stress is similar between both cultivars, i.e. plasma membrane intrinsic proteins are downregulated under progressive drought in leaves and under short term osmotic shock in roots. In response to osmotic shock, root hydraulic conductivity did not change significantly and stomatal closure is equal in both cultivars. *De novo* abscisic acid biosynthesis is upregulated in the roots of both cultivars under progressive drought.

1. Introduction

Drought stress causes yield losses of up to 50% (Cattivelli et al., 2008; Vij and Tyagi, 2007; Wood, 2005). Worldwide, drought has become more intense and occurs more frequently, a trend that is expected to continue (FAOLand&Water, 2013). Also Europe faces scenarios with lower rainfall and elevated temperatures, increasing the water demand of crops in the open field and in greenhouses (European Environment Agency, 2017). Particularly in strawberry (*Fragaria x ananassa* Duch.), drought stress causes an important yield reduction, due to the shallow root system, large leaf area and high fruit water content (Klamkowski and Treder, 2008; Li et al., 2010; Liu et al., 2007; Nezhadahmadi et al., 2015).

Plants can adopt different strategies to cope with drought stress. The

conservative water use behavior strategy results in slow growth because water consumption is slowed down. Conservative plants react incrementally to stress. This strategy is beneficial when drought is prolonged and severe. The conservative water use behavior displays strict stomatal control with gradual stomatal closure early upon the onset of drought (Passioura, 1982; Sojka, 1985). These characteristics have also been associated with isohydric water use behavior, which is defined by a tightly maintained and stable leaf water potential (ψ_L) (Martínez-Vilalta and Garcia-Forner, 2017; Vilagrosa et al., 2012). In isohydric plants, stomatal conductance (g_s) is said to be sensitive and the response of g_s to abscisic acid (ABA) to be dependent on the leaf water status (Blum, 2015; Tardieu and Simonneau, 1998), allowing gradual, fine-tuned stomatal closure. The prodigal water use behavior, on the other hand, is associated with high g_s and an unchanged water

Abbreviations: ψ_L , leaf water potential; ABA, abscisic acid; BG, β -glucosidases; cDNA, complementary DNA; CNRQ, calibrated normalized relative quantities; gDNA, genomic DNA; g_s , stomatal conductance; HCFM, Hydraulic Conductance Flow Meter; $L_{p,r}$, root hydraulic conductivity; NCED, 9-cis-epoxycarotenoid dioxygenase; PCR, polymerase chain reaction; PEG, polyethyleneglycol; PIPs, plasma membrane intrinsic proteins; RT-qPCR, everse Transcription quantitative PCR; S_R , root surface area; TIPs, tonoplast intrinsic proteins

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consumption, allowing photosynthesis and growth, up until a critical stress level. This strategy is beneficial provided that the drought stress is moderate and not long-lasting. Contrary to the conservative water use behavior, these plants operate in an on-off fashion, where stomatal closure is not triggered until a critical drought stress level (Bacelar et al., 2012; Passioura, 1982; Sojka, 1985). These stomatal responses have also been linked to anisohydric behavior, defined by a non-tightly regulated ψ_L , which is not maintained above a minimum value (Martínez-Vilalta and García-Forner, 2017; Vilagrosa et al., 2012). In anisohydric plants, stomatal closure has been said to be regulated hydraulically and to display a single relationship with ABA, irrespective of the stress level (Blum, 2015; Tardieu and Simonneau, 1998). Martínez-Vilalta and García-Forner (2017) argue, however, that (an)isohydry is well defined in terms of ψ_L and is not necessarily associated with the degree of stomatal control. Conservative and prodigal water use behavior, contrarily, are non-strictly defined concepts that can be associated with stomatal behavior and other characteristics.

The composite water transport model states that water is transported at the cellular level via three main ways: apoplastically (around protoplasts), symplastically (through plasmodesmata) and transcellular (crossing membranes) (Steudle, 1994). The contribution of each path to the total hydraulic conductivity is variable and depending on, among other factors, external conditions and tissue anatomy (Kreszies et al., 2019; Steudle, 2000). When the root endodermis develops Casparian bands, the apoplastic pathway is blocked, making the aquaporin-controlled transcellular pathway very important (Barberon et al., 2016; Hachez et al., 2012). Aquaporins are channels that form pores in plasma- and intracellular membranes that facilitate passive water transport across them. From several species, it is known that root hydraulic conductivity generally decreases upon drought stress (Aroca et al., 2006, 2007; Hachez et al., 2012; Mahdieh et al., 2008; Martre et al., 2001; Maurel et al., 2010; Secchi et al., 2007a; Silva et al., 2004; Sutka et al., 2016; Tyerman et al., 2002). This decrease in conductivity is thought to be at least partially due to the regulatory effects of aquaporins (Chaumont and Tyerman, 2014; Martre et al., 2001; Maurel et al., 2010; North et al., 2004; Secchi et al., 2007b; Tyerman et al., 2002; Vandeleur et al., 2009). When suberin lamellae are deposited in endodermal cells, also the transcellular pathway is blocked, and, consequently, only symplastic transport is possible into the xylem (Barberon et al., 2016).

The use of aquaporin water transport inhibitors revealed that under drought stress conditions, aquaporins can account for 60–80% of root hydraulic conductivity (L_p) (reviewed by Martre et al. (2002)). To compare, under well-watered conditions, they were shown to contribute for only 30–90% (reviewed by Javot and Maurel (2002)). Aquaporins are responsible for most of the water transport across membranes, hence they determine the contribution of the transcellular path to the total hydraulic conductivity (Steudle, 2000). They can modulate the membrane water permeability up to 20 fold (Farooq et al., 2012). Aquaporin activity can be regulated rapidly, through transcriptional regulation, translational regulation or posttranslational modification (Chaumont and Tyerman, 2014). When hydraulic conductivity needs to be modified in the short term, for example in response to acute stress, controlling the aquaporins appears to be the best option, as the apoplastic and symplastic routes are largely determined by tissue anatomy and hence less dynamic.

Aquaporins have often been hypothesized to be downregulated under (severe) drought stress conditions in order to maintain the water inside the plant instead of letting it flow to the drying environment (Alexandersson et al., 2005; Boursiac et al., 2005; Liu et al., 2013; Martre et al., 2001; North et al., 2004; Secchi et al., 2007a; Siemens and Zwiazek, 2003; Šurbanovski et al., 2013). Maintaining the internal water balance is essential to maintain turgor pressure (Galmés et al., 2007). It has been shown that regulation of aquaporins may differ per aquaporin type, plant species, tissue as well as stress level (Alexandersson et al., 2010; Aroca et al., 2007, 2006; Boursiac et al.,

2008; Galmés et al., 2007; Guo et al., 2006; Hachez et al., 2012; Mahdieh et al., 2008; Porcel et al., 2006; Ruiz-Lozano et al., 2009; Secchi et al., 2007a; Šurbanovski et al., 2013; Sutka et al., 2016). An overview of several studies describing expression under drought stress in leaf and root tissue is provided in table A.1, in addition to the studies that are listed by Tyerman et al. (2002) and Aroca (2012). The diversity of the responses among isoforms is likely to be linked to functional diversification. Even isoforms that belong to the same subfamily may play different roles in drought response. Individual aquaporins are increasingly believed to contribute in their own way to concentrate the water there where it is most critical in the water-stressed plant (Šurbanovski and Grant, 2014). This implies that in some cells or tissues increased water transport is desirable, while at the same time decreased water transport is beneficial in others. For example, *Vitis* PIP1; 2 is downregulated in aerial parts and upregulated in roots under moderate water stress (Galmés et al., 2007; Jang et al., 2004). Maurel et al. (2010) state that not only the contribution to the primary drought response but also to the recovery after rewetting of the substrate, should be considered to attribute a role in drought response to specific aquaporins. Additionally, some aquaporins transport small solutes such as ammonium or silicon, which can play a significant role under drought stress (Zargar et al., 2017).

Among the five subfamilies of aquaporins known today in vascular plants (Chaumont and Tyerman, 2017), the plasma membrane intrinsic proteins (PIPs) and tonoplast intrinsic proteins (TIPs) are thought to be most important with respect to the plant water status. This is due to their subcellular location, i.e. the plasma membrane and tonoplast respectively, and their abundance (Chaumont and Tyerman, 2017; Maurel, 2007). Šurbanovski et al. (2013) have underlined the potential value of PIPs in altering *Fragaria* water relations upon drought stress by demonstrating a clear correlation between the severity of drought stress and the expression levels of PIPs in leaves and roots. Groups of PIP and TIP coding sequences have been identified previously in *Fragaria x ananassa* (Merlaen et al., 2018).

Here, we hypothesize that *Fragaria x ananassa* cultivars with contrasting drought stress phenotypes, employ a different water use behavior strategy. The cultivars selected for this study are Figaro and Flair (Merlaen, 2019). Secondly, we hypothesize that cultivars with a different water use behavior respond differently in terms of g_s , L_p , and ABA metabolism and manage their aquaporin expression differently under stress. Effects of two fundamentally different types of stress, i.e. progressive drought and osmotic shock, are evaluated. This knowledge contributes to our understanding of plant water relations management under stress in an economically important and water demanding crop.

2. Materials and methods

2.1. Drought stress experiment

Young plants (8 week old rooted cuttings) of cultivars Figaro and Flair were purchased from Neessen N.V. (The Netherlands). Soil was washed off the roots and plants were potted in fine perlite (0–4 mm) and kept in the growth chamber (200 $\mu\text{mol m}^{-2} \text{s}^{-1}$, day/night 16h/8h, 30% relative humidity, 21 °C (day), 18 °C (night)) to acclimatize. Plants were watered with a nutrient solution (Solufeed 19-8-16-(4), EC: 1.45). Figaro and Flair plants were respectively 11 and 14 weeks old at the start of the experiment.

The experiment was set up with 36 plants per cultivar, each of them randomly assigned to a control or drought stress treatment. All plants were watered to field capacity 9 h after the start of the photoperiod, for three days in a row. After that, watering continued in this way for the control plants. Watering was withheld for the drought treated plants for 7 days and was restarted thereafter. Samples were collected 2 h after the start of the photoperiod (corresponding to 64h, 112h and 160h after the last watering for the drought treatment group). Samples were also collected 64h after the drought treated plants were rewatered.

The substrate moisture level was measured using an HH2 Moisture Meter connected to a WET sensor (Delta T services, United Kingdom). A calibration curve for fine perlite was generated according to the manufacturer's instructions. The substrate moisture level was determined on a fixed subset of 9 plants each day, hence, these are not the plants sampled for expression analysis. Substrate water content data for Flair are not available at 160 h after the last watering due to a technical issue with the device memory. The Flair data point at 15 vol% is based on measurements one day before the plants were sampled for expression analysis and this mean is based on 3 replicates only.

2.2. Osmotic stress experiment

Fragaria x ananassa cv. Figaro and cv. Flair were purchased as cold-stored bare root plants from Neessen N.V. (The Netherlands) and grown in a hydroponic system for 16 weeks in order to allow easy access to undamaged roots, in a growth chamber ($150 \mu\text{mol m}^{-2} \text{s}^{-1}$ at canopy level; day/night 16h/8h; 75% relative humidity; 16°C). The aerated nutrient solution consisted of 7.5 mM NO_3^- , $1.125 \text{ mM H}_2\text{PO}_4^-$, $0.75 \text{ mM SO}_4^{2+}$, 3.75 mM K^+ , 2.44 mM Ca^{2+} and 0.75 mM Mg^{2+} (pH 5.7).

Plants were randomly transferred to a control or polyethyleneglycol (PEG) solution 4 h before the L_p measurement. L_p measurements and sampling happened between 4 and 9 h after the start of the photoperiod, with the same average sampling time for both cultivars. The control solution was a freshly prepared nutrient solution ($\psi_s = -0.06 \text{ MPa}$). The PEG solution was freshly prepared by supplementing the control solution with 8% (w/v) PEG 6000 (Alfa Aesar) (pH 5.7) ($\psi_s = -0.22 \text{ MPa}$). Osmolarities of the solutions were measured using a Fiske one-ten osmometer and converted to osmotic potential using $\psi_s = -\text{osmolarity} \cdot R \cdot T$ with $T = 291.15 \text{ K} = 18^\circ\text{C}$ and $R = 8.3143 \text{ J K}^{-1} \text{ mol}^{-1}$.

2.3. Root anatomy

Feeder rootlets of similar visual appearance (Fig. A1) were sampled in hydroponically grown plants (as described above) of both cultivars. The root anatomy was investigated in two different root types: main feeder rootlets (at 1, 2 and 3 cm from the root tip) and branched rootlets (at 3 mm from the root tip) (Fig. A1). To this end, excised root segments were embedded in 6% (w/v) agarose and sectioned at a thickness of $50 \mu\text{m}$ using a vibrating microtome (HM650V, fisher Scientific) (speed 35, frequency 80, amplitude 0.8). Unstained sections were mounted in water. Lignin and suberin autofluorescence was observed under UV-light using a Nikon Ni-U epifluorescence microscope and a DAPI filter cube (excitation 387/11 BP; emission 447/60 BP), and photographed with a Nikon DS-Fi1c camera using similar exposure settings.

2.4. Physiological measurements

Stomatal conductance (g_s) was measured with an AP4 Porometer (Delta T services, United Kingdom), according to the manufacturer's instructions. It was measured on nine plants, 2 h after the start of the photoperiod (at the time of sample collection) (drought experiment) or on six plants, just before root hydraulic conductivity (L_p) measurements (osmotic stress experiment).

Measurements of root hydraulic conductivity (L_p) were performed with a Hydraulic Conductance Flow Meter (HCFM) (Dynamax) between 4 and 9 h after the start of the photoperiod. For each of 6 plants per cultivar/treatment combination, three roots were measured. Perennial roots bearing many young feeder rootlets were recut at the base under water and connected to the HCFM. After the connection was found to be adequate by a few measurements, three transient measurements were taken with raising pressure from 40 to 450 kPa at a rate of approximately $8\text{--}10 \text{ kPa s}^{-1}$. The slope of the linear regression of the water flow versus the measured pressure was normalized by the temperature

(25°C) (K_r). The root surface area (S_R) was analyzed from a photograph by ImageJ software (<https://imagej.nih.gov/ij/>). L_p ($\text{Kg.s}^{-1} \text{ MPa}^{-1} \text{ m}^{-2}$) was calculated as K_r/S_R .

The leaf water potential (ψ_l) was measured with a Pressure bomb (PMS instrument company Model 1000) according to the manufacturer's instructions on 5 plants, one leaf per plant, 2 h after the start of the photoperiod (at the time of sample collection) (drought experiment).

2.5. Abscisic acid determination

Three biological replicates were sampled in the drought stress experiment. Each replicate was pooled from three plants. Leaves were excised, separated from the petioles and flash frozen in liquid nitrogen. Samples were stored at -80°C until grinding in liquid nitrogen using an IKA A11 basic analytical mill. Ground tissue was stored at -80°C until ABA extraction following Chen et al. (1997). See Appendix B for details. ABA levels were quantified by ELISA with a phytodetek-ABA kit (Ardia-Emea, Grigny, France).

2.6. RNA extraction and reverse transcription

For the drought stress experiment, sampling of leaves and roots was performed in the same way as for ABA determination. Roots were briefly washed under running tap water to remove all perlite, dabbed dry, then flash frozen in liquid nitrogen. Ground tissue was stored at -80°C until RNA extraction following Merlaen et al. (2018). For the osmotic stress experiment, the entire root system of each plant was cut off, immediately frozen in liquid nitrogen and stored at -80°C until grinding. Root tissue was ground with liquid nitrogen using mortar and pestle and stored at -80°C until RNA extraction, which was performed as described in Luypaert et al. (2017). The RNA concentration and quality were assessed using the Nanodrop spectrophotometer (Isogen). The RNA integrity was assessed for a subset of the samples using the Experion microfluidic capillary electrophoresis system (Bio-Rad) in combination with the RNA StdSens Chips (Bio-Rad). For detailed RNA extraction, DNase treatment, cDNA synthesis and quality control protocols and quality control results on both experiments, refer to Appendix B.

2.7. RT-qPCR

Reference gene stability was assessed using geNorm (Appendix B). *PTB* and *ENPI* were used as reference genes for roots (M-value = 0.131 and 0.378; CV-value = 0.110 and 0.120 for the drought stress and the osmotic stress experiment respectively) and *EF1* and *RPS1* for leaf tissue (M-value = 0.418; CV-value = 0.146). RT-qPCR primers and gene specific amplification efficiencies are listed in Appendix B. Aquaporin primers designed by Merlaen et al. (2018) and 9-cis-epoxycarotenoid dioxygenase (NCED) primers amplify groups of transcripts (Table B.5). Gene specific amplification efficiencies were determined by LinRegPCR (Ramakers et al., 2003; Ruijter et al., 2009) for both target genes and reference genes. For details on RT-qPCR, data analysis procedures and missing data (lost samples and genomic DNA (gDNA) contamination), refer to Appendix B.

2.8. Statistical analysis and graphs

All statistical tests were performed in SPSS Statistics version 24 at the 5% significance level, unless indicated differently. For L_p , a boxplot of all conditions was created to identify outliers. Two outliers were removed from the L_p dataset. One L_p data point was missing. All calibrated normalized relative quantities (CNRQ values) were \log_{10} transformed prior to all statistical analyses. Independent samples t-tests (Levene's statistic $p > 0.01$) were performed separately per cultivar (osmotic stress experiment) or separately per cultivar and per time

point (drought stress experiment). For gene expression data of the drought stress experiment, a Dunnett's test was performed per treatment to compare each data point to the first data point (64 h since start of drought treatment) (Levene's statistic $p > 0.01$ or Browne-Forsythe test). Figures showing gene expression data in function of substrate water content are made in R version 3.3.2 (RStudio 1.0.136). All other figures are made in Sigmaplot version 13.

3. Results

3.1. Stomatal conductance and leaf water potential under drought

Figaro and Flair were subjected to progressive drought. The decline in the volumetric substrate water content is demonstrated in Fig. 1 A-B. Figaro g_s decreased gradually, showing a linear relationship (linear regression $R^2 = 0.50$) with the volumetric water in the substrate (Fig. 2A). Fig. 2B shows that Flair g_s declined sharply and suddenly once a substrate water content of 40 vol% was reached. Flair g_s dropped

suddenly below $150 \text{ mmol m}^{-2} \text{ s}^{-1}$, rather than decreasing gradually. The gradual reduction of g_s in Figaro and the sudden, steep drop in Flair g_s are also depicted in Fig. 1C and D. In both cultivars, recovery data points are separated from the others (Fig. 2A and B), indicating that full recovery did not happen within 3 days of rewatering (Fig. 1C and D).

Figaro ψ_L decreases non-significantly ($p = 0.740$ and $p = 0.101$ at 37% and 26% substrate volumetric water content, respectively). Nevertheless, a drop of about 1 MPa in comparison with control values can be observed at an average volumetric substrate water content of 26%, in spite of the large standard error (Fig. 2C). Fig. 2D shows a clear decrease in Flair ψ_L as the substrate became drier. Flair ψ_L dropped significantly lower than the control at a substrate volumetric water content of 27% ($p = 0.008$) and under the most severe drought stress ($p = 0.001$), here plotted at a substrate volumetric water content of 15% (based on $n = 3$ replicates measured 24 h earlier than the leaf water potential). ψ_L recovered within three days (Fig. 2C and D).

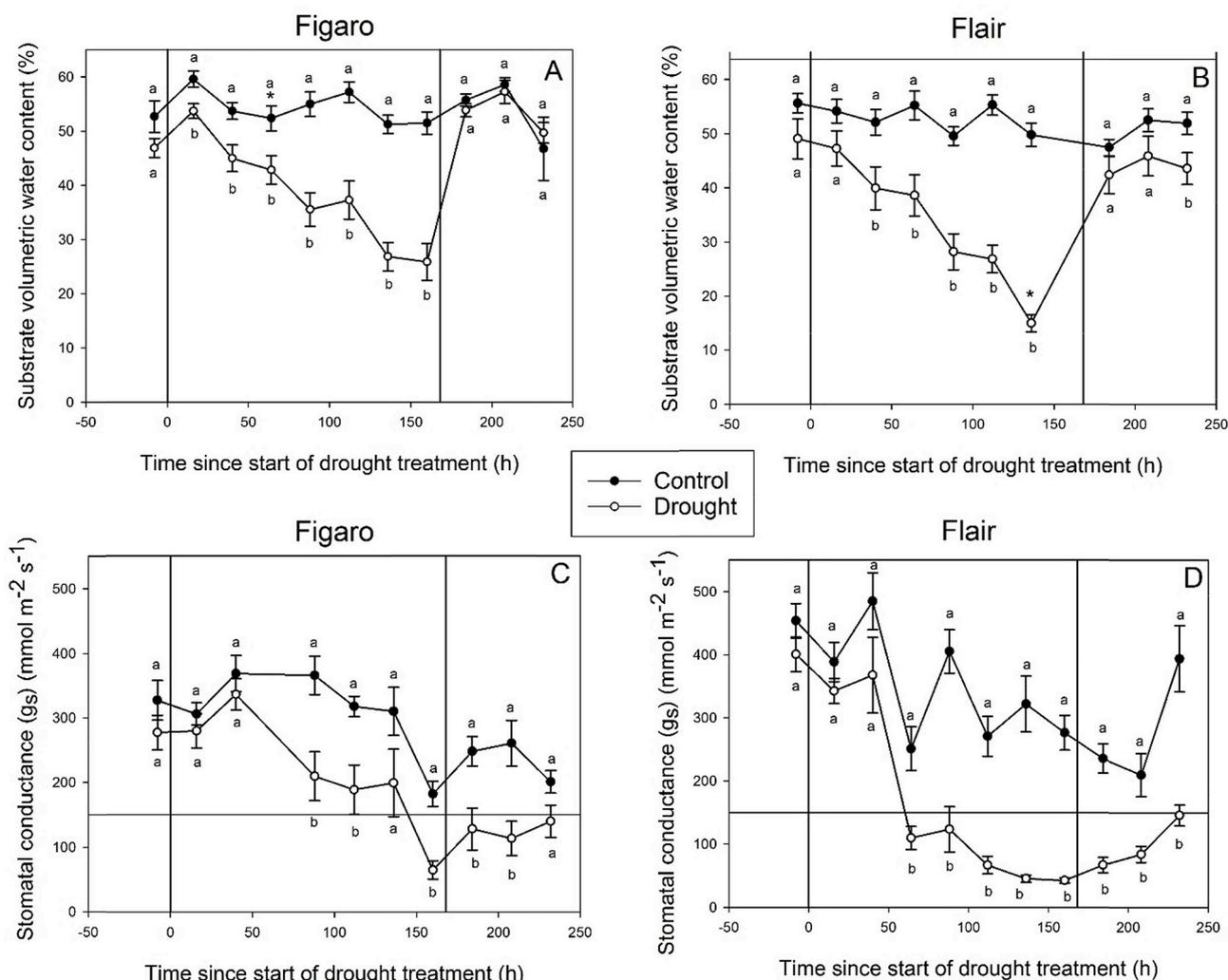


Fig. 1. (A-B) Substrate volumetric water content (vol%), (C-D) Stomatal conductance (g_s) ($\text{mmol m}^{-2} \text{ s}^{-1}$), of *Fragaria x ananassa* cv. Figaro (A,C) and Flair (B,D) under control and drought conditions. 168 h after the start of the drought treatment, watering was restarted (= recovery) (indicated by a vertical line). Values are means \pm SE. $n = 9$ for substrate volumetric water content and stomatal conductance (*means substrate volumetric water content is based on $n = 3$ replicates and leaf water potential is plotted against the substrate volumetric water content of 24 h earlier). Letters indicate significant differences as determined by a t -test ($p < 0.05$) per time point and per cultivar.

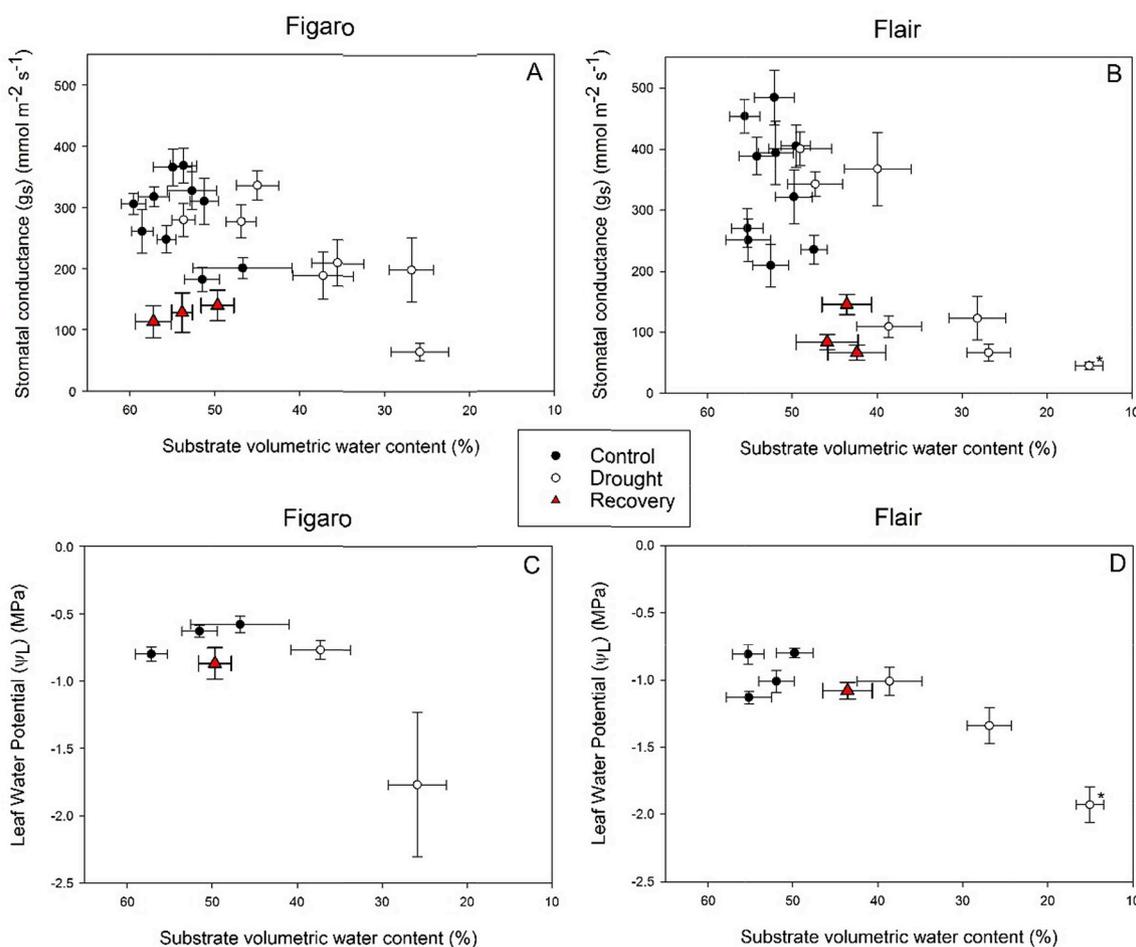


Fig. 2. (A-B) Stomatal conductance (g_s) ($\text{mmol m}^{-2} \text{s}^{-1}$), (C-D) Leaf water potential (ψ_L) (MPa) of *Fragaria x ananassa* cv. Figaro (A,C) and Flair (B,D) under control and drought conditions. Values are means \pm SE. $n = 9$ for substrate volumetric water content and stomatal conductance (*means substrate volumetric water content is based on $n = 3$ replicates and leaf water potential is plotted against the substrate volumetric water content of 24 h earlier). $n = 5$ for ψ_L .

3.2. ABA metabolism under drought and after rewatering

The expression of two types of key enzymes in ABA biosynthesis was quantified: two groups of 9-cis-epoxycarotenoid dioxygenases (NCED), including three and four genes per group and three genes encoding β -glucosidases (BG) (Table B.5).

In roots, expression of groups FaNCED (1) and FaNCED (2) significantly increased during the drought episode for both Flair and Figaro (Fig. 3A–F). Upon rewatering, the effect of the drought treatment disappeared, showing that recovery established within 64h after rewatering (232h after the start of the drought treatment) (Fig. 3A–F). *FaBG2* and *FaBG3* showed a rather stable expression in relation to the substrate water content (Fig. A2 A; D). The relative increase in expression in roots during drought is far higher for the *FaNCED* genes than for the *FaBG* genes tested here. Under control as well as drought treatment, expression levels of groups FaNCED (1) and FaNCED (2) were higher in Figaro compared to Flair ($p < 0.001$ for both groups) (Fig. 3 A; D).

FaNCED transcripts could not be amplified in the leaf. *FaBG2* was the only ABA metabolism gene that was expressed above the detection limit in leaves. In both cultivars, *FaBG2* expression did not change significantly during drought stress (Fig. A3).

ABA determinations in Figaro leaves confirmed significant ABA accumulation under drought stress (Fig. A4). 160 h after the last watering, the difference between control and treatment leaf ABA levels is significant ($p = 0.018$, independent samples *t*-test) (Fig. A4A). 64h after rehydration (i.e. 232h after the start of the treatment), leaf ABA

levels were the highest measured in this experiment (independent samples *t*-test comparing rewatered ABA leaf levels with the control levels at that time point: $p = 0.050$) (Fig. A4). Leaf ABA content is negatively correlated with stomatal conductance ($R^2 = 0.8$) (Fig. A4B).

3.3. Aquaporin expression under drought and after rewatering

Expression levels were assessed for groups of aquaporin genes, according to Merlaen et al. (2018). An overall tendency for downregulation of PIP genes in leaves was observed as the substrate became drier, especially for PIP2 aquaporins, even though this was not always significant (Fig. 4 A,D,G and Fig. A5 A,D,G). The downregulation was significant for group FaPIP2; 1(b) in both cultivars (Fig. 4E and F) and for groups FaPIP1; 2 and FaPIP2; 2 in Flair only (Fig. 4 C,I). Also, PIP expression in leaf tissue recovered quickly. The expression upon rewatering was in accordance with the substrate water content, showing no residual effects of the drought that was experienced earlier (Fig. 4 and Fig. A5). TIP expression was too low to quantify accurately in leaf tissue under control and drought conditions (data not shown).

In roots, group FaTIP(a) is strongly upregulated under severe drought stress in Figaro, while this response is completely lacking in Flair (Fig. 5). The upregulation upon rewatering is significant in both cultivars (Fig. 5B and C). Groups FaPIP1; 1 and FaPIP1; 2 are upregulated at 112 h after the start of the drought treatment in both cultivars (Fig. A6). This effect is significant, except for group FaPIP1; 2 in Figaro. At the most severe drought stress levels, this effect is no longer present (Fig. A6 B-C,E-F). Figaro and Flair show opposite responses upon

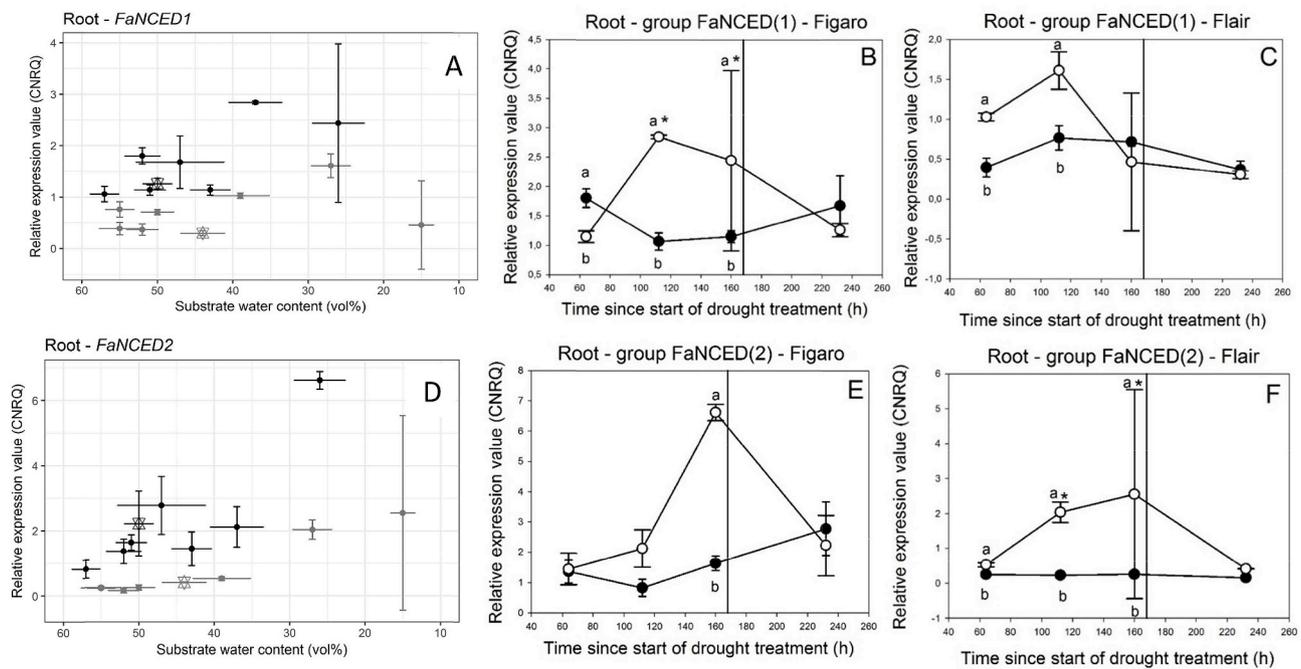


Fig. 3. (A,D) Relative expression values (mean + \pm SE; n=3 (exceptions in Appendix B)) of *NCED* genes in relation to substrate water content (vol%) (mean \pm SE; n=9) in roots of cultivars Figaro (black) and Flair (grey). (B-C,E-F) Relative expression values (mean + \pm SE; n=3 (exceptions in Appendix B)) of *NCED* genes in roots under control (closed circles) and progressive drought (open circles) treatments. The x-axis indicates hours since last watering (the vertical line indicates the start of rewatering). Independent samples *t*-test significant differences between drought and control at one time point are indicated with letters. * indicates Dunnett's test (Levene's statistic $p > 0.01$) significant differences with the first time point of that treatment.

rewatering, i.e. non-significant downregulation in Figaro and non-significant upregulation in Flair (Fig. A6 B-C,E-F). Group FaPIP1; 3 is non-significantly downregulated in both cultivars (Fig. A6 G-I). The more severe drought stress experienced by Flair results in stronger downregulation at 160 h after the last watering and a residual effect upon rewatering (Fig. A6 G). This more severe stress experience by Flair might also explain the upregulation upon rewatering of all FaPIP2 groups in Flair, while in Figaro the expression levels are equal to the time point before rewatering (Fig. A6 K-L; N-O; Q-R). In Flair, there is a trend for downregulation of group FaTIP(b) at the most severe drought stress, which has a significant residual effect upon rewatering (Fig. A6 S; U). From Fig. A6 S, this seems to be due to the lower substrate water content Flair experienced at that time point, compared to Figaro.

3.4. Root anatomy

The presence of Casparian bands and/or suberin lamellae was determined in main feeder rootlets and finer branched rootlets of hydroponically grown plants (Fig. A1). In main feeder rootlets of both cultivars, the presence of Casparian bands was demonstrated at 1, 2 and 3 cm from the root tip (Fig. A7 A). When looking at several roots of the same type and species (biological replicates), the xylem lignification at equal distance from the root tip was not similar, i.e. more xylem vessels are lignified and the xylem autofluorescence is brighter in some roots than in others (Fig. A7 A). The development of suberin lamellae seemed to correlate with the development of secondary xylem in both cultivars, i.e. the development of suberin lamellae relative to the formation of secondary xylem was not markedly different between both cultivars. Nowhere, a fully suberized endodermis was observed. In branched feeder rootlets at 3 mm from the root tip, no lignification or suberization were detected in the endodermis in either of the two cultivars (Fig. A7 A). Altogether, no obvious differences in root anatomy were observed between Figaro and Flair.

3.5. Root hydraulic conductivity and stomatal conductance under osmotic stress

In order to evaluate whether both cultivars respond differently to a short term osmotic shock treatment, L_{pr} was measured in response to 4 h of osmotic stress (8% (w/v) PEG 6000, $\psi_s = -0.22$ MPa). No significant effects were recorded (Fig. 6A). Also the control L_{pr} levels are not significantly different between both cultivars ($p = 0.151$) (Fig. 6A). To further characterize the plant response to osmotic stress, g_s was quantified. PEG caused g_s values in both cultivars to decrease significantly and drop below the $150 \text{ mmol m}^{-2} \text{ s}^{-1}$ threshold (Fig. 6B).

3.6. Root aquaporin expression under osmotic stress

The expression of several groups of *Fragaria x ananassa* aquaporin genes was quantified in roots after 4 h of osmotic stress (8% (w/v) PEG 6000, $\psi_s = -0.22$ MPa) (Fig. 7). All aquaporins showed (a tendency for) downregulation within 4 h of osmotic stress treatment, except for group FaTIP(a) in Flair. No major differences between Figaro and Flair can be observed (Fig. 7).

4. Discussion

Plants apply different strategies in order to cope with drought stress. There are, however, some common responses, such as stomatal closure under the control of ABA, which acts as a signaling molecule that can be produced locally in the leaves or can be transported there from the roots. Also aquaporins are thought to be responsive to drought signaling pathways and to be involved in drought coping mechanisms, such as altering the tissue hydraulic conductivity. Here, we look for differences in these response mechanisms between Figaro and Flair, two *Fragaria x ananassa* cultivars with contrasting drought stress phenotypes (respectively late vs. early wilting in open field production (Merlaen, 2019)). The response of stomatal conductance (g_s), root hydraulic conductivity

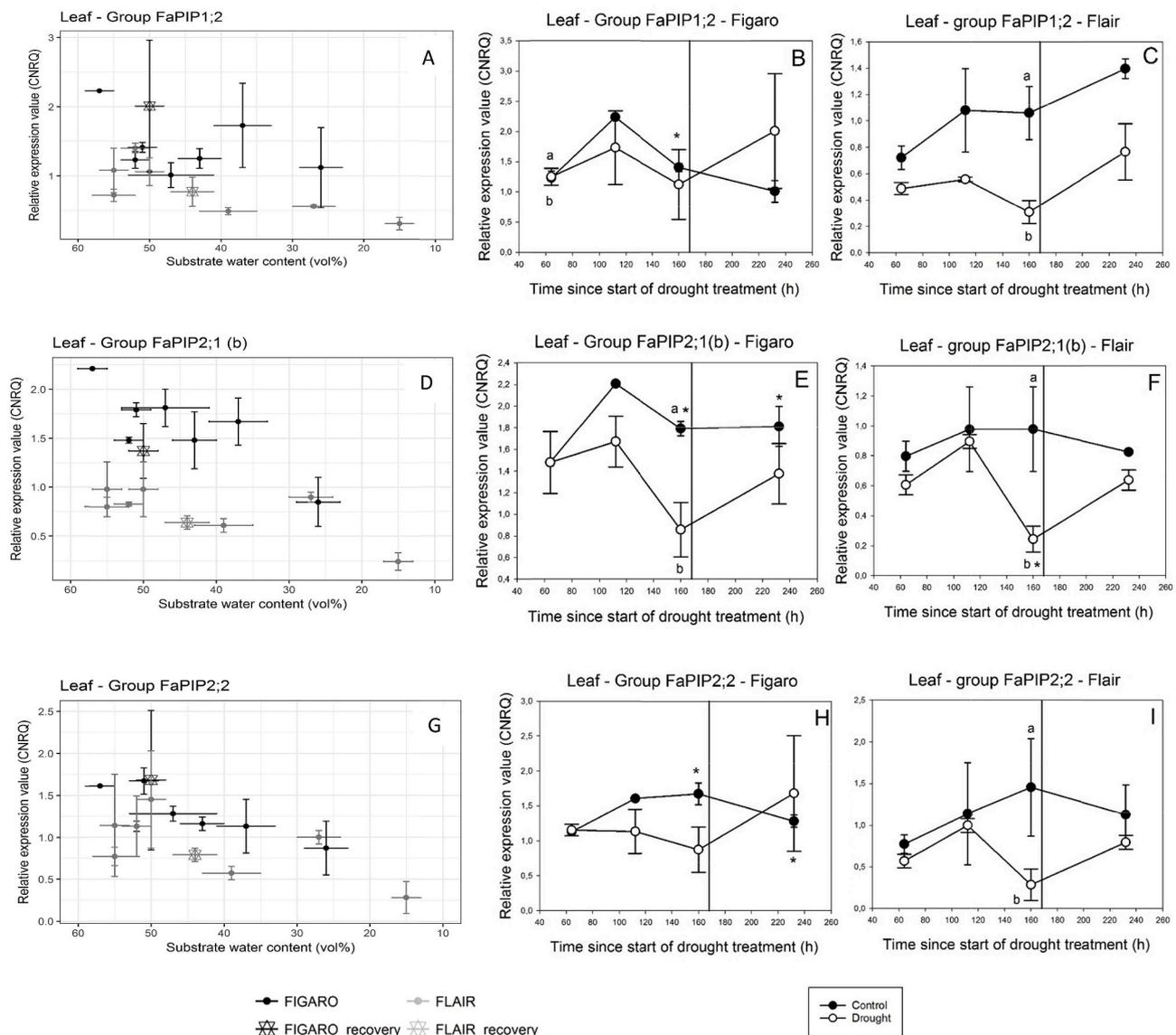


Fig. 4. (A,D,G) Relative expression values (mean + -/SE; n = 3 (exceptions in Appendix B) of PIP groups in leaves in relation to substrate water content (vol %) (mean ± SE; n = 9) in cultivars Figaro (black) and Flair (grey). (B-C,E-F,H-I) Relative expression values (mean + -/SE; n = 3 (exceptions in Appendix B) of PIP groups in leaves under control (closed circles) and progressive drought (open circles) treatments. The x-axis indicates hours since last watering (the vertical line indicates the start of rewatering). Independent samples *t*-test significant differences between drought and control at one time point are indicated with letters. * indicates Dunnett's test (Levene's statistic $p > 0.01$) significant differences with the first time point of that treatment.

(L_p), leaf water potential (ψ_L), ABA accumulation and expression of aquaporins and key enzymes in the ABA biosynthesis in leaves and roots were assessed under progressive drought and/or osmotic shock treatment in Figaro and Flair. The information presented here does not only enable us to identify the water use behavior strategy of each of the cultivars, but the responses to two different types of stress, progressive drought on the one hand vs an osmotic shock on the other hand, also allow us to better understand the behavior of each cultivar.

Stomatal closure under drought stress is gradual in Figaro (Fig. 2A) and correlates with foliar ABA levels (Fig. A4 B). Stomata are considered to be partially closing when g_s drops below $150 \text{ mmol m}^{-2} \text{ s}^{-1}$. Below this value, stomatal limitations gradually impair photosynthesis (Medrano et al., 2002). The pattern relating g_s and different photosynthetic values is independent of the plant species (Flexas and Medrano, 2002; Liu et al., 2005). Gradual, ABA-controlled stomatal closure is characteristic for plants with a conservative water use behavior (Blum, 2015; Sojka, 1985). These plants reduce their water consumption under drought stress by lowering their transpiration rate.

As a consequence of diminished water consumption in Figaro, the substrate retains more water for a longer time (Fig. 1A and B). These data point towards conservative water use behavior for Figaro, a concept that overlaps with so called isohydric behavior, defined by maintenance of ψ_L under drought stress. Isohydric plants indeed maintain a constant ψ_L until a critical soil/substrate moisture threshold is reached, after that ψ_L also becomes more negative, as is shown in the isohydric behavior of potato (*Solanum tuberosum* L.) (Liu et al., 2005). Likewise, if the volumetric water content drops below 30 vol % (Fig. 2C), ψ_L decreases to -1.8 MPa in Figaro.

Flair displays a sudden and steep drop in g_s under increasing drought stress (Fig. 2B), a response that is very different from the one in Figaro. This on-off g_s pattern, or the absence of sensitive stomatal regulation in Flair, indicates prodigal water use behavior, in which stomatal closure is only triggered at a critical level of drought stress (Blum, 2015; Sojka, 1985; Tardieu and Simonneau, 1998). The chore characteristics of this strategy are non-decreased transpiration rates and unchanged water consumption, allowing growth, up to a critical level of

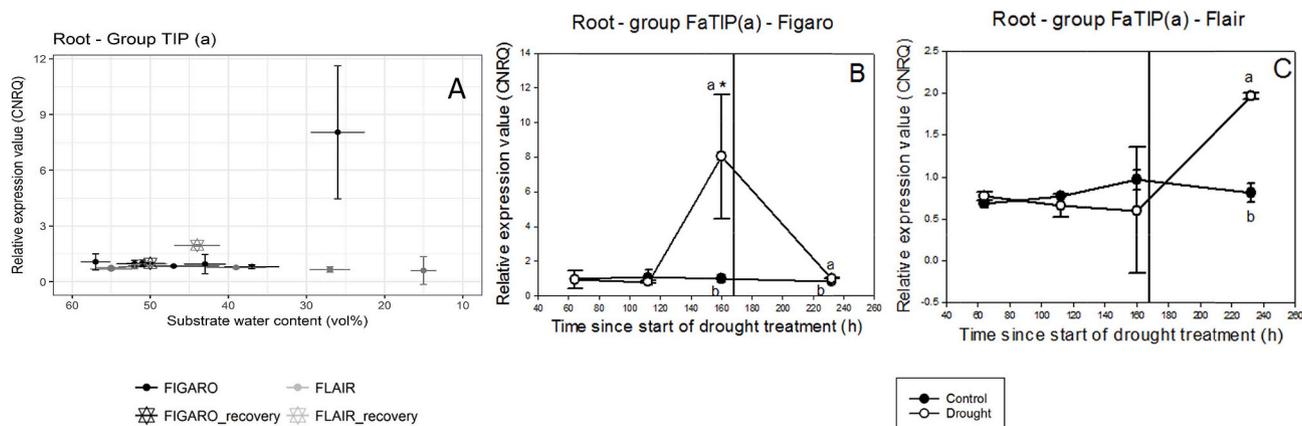


Fig. 5. (A) Relative expression values mean \pm SE; $n = 3$ (exceptions in Appendix B) of group FaTIP(a) in roots in relation to substrate water content (vol %) (mean \pm SE; $n = 9$) in cultivars Figaro (black) and Flair (grey). (B-C) Relative expression values mean \pm SE; $n = 3$ (exceptions in Appendix B) of group FaTIP(a) in roots under control (closed circles) and progressive drought (open circles) treatments. The x-axis indicates hours since last watering (the vertical line indicates the start of rewatering). Independent samples t -test significant differences between drought and control at one time point are indicated with letters. * indicates Dunnett's test (Levene's statistic $p > 0.01$) significant differences with the first time point of that treatment.

water depletion. Additionally, Flair ψ_L is clearly dropping with increasing drought stress (Fig. 2D). This behavior is referred to as anisohydric, a concept that shares many characteristics with prodigal water use behavior. Based on the integration of these physiological parameters, we can conclude that cultivar Flair displays a more prodigal water use behavior, causing it to experience more severe drought stress when watering is withheld for a longer time.

In spite of the distinctly different stomatal behavior, which is known to be regulated by ABA, no differences in root ABA production could be observed. We show enhanced expression of both tested *FaNCED* groups in both cultivars upon drought stress, i.e. *de novo* ABA biosynthesis in roots is not differentially regulated between the conservative and the prodigal water use behavior cultivar (Fig. 3A–F). Also foliar ABA production cannot explain the different stomatal regulation, since no *de novo* ABA synthesis was demonstrated in the leaves: out of the two groups of 9-cis-epoxycarotenoid dioxygenases (NCED), key enzymes in the *de novo* synthesis of ABA and the three β -glucosidases (BG), responsible for the release of ABA from an inactive glycosylated form (Cutler and Krochko, 1999; Li et al., 2013) tested, none are expressed in the leaves of either cultivar, apart from *FaBG2*, whose expression is not responsive to drought. ABA accumulation was confirmed in leaves of Figaro (Fig. A4). After 64h of recovery, no additional ABA is produced in the roots, but the accumulated ABA is still present in the leaves,

causing stomata to remain (partially) closed (Fig. 2A).

Besides stomatal closure, plants also alter the root hydraulic conductivity in response to drought. Even though there are examples of increasing L_p under drought stress as well (Matsuo et al., 2009), a drop in L_p in response to water deprivation is often described for diverse species and explained as an attempt to reduce the amount of water flowing back to the environment (Aroca et al., 2012; Hachez et al., 2012; Martre et al., 2001; Matsuo et al., 2009; Maurel et al., 2005; Meng and Fricke, 2017; Mu et al., 2006; Suga et al., 2002; Sutka et al., 2016; Trillo and Fernández, 2005; Vandeleur et al., 2009), but has in particular been linked to prodigal water use behavior in *Sorghum bicolor* (Sutka et al., 2016). When a sudden stress shock is imposed (osmotic stress experiment), we observe no significant differences in the g_s nor L_p responses of the conservative and the prodigal water use behavior cultivar.

Root hydraulic conductivity is determined by root anatomy (apoplastic and symplastic paths) and by aquaporin activity (transcellular path). In the strawberry root system, the feeder rootlets absorb most water (Tylová et al., 2017; Wilhelm and Nelson, 1970). At least in hydroponics, the apoplastic pathway is blocked by Casparian bands in the main feeder rootlets, within 3 cm from the root tip. The transcellular pathway is not completely blocked, as we did not observe suberin lamellae in all endodermis cells. Water can still reach the xylem via the

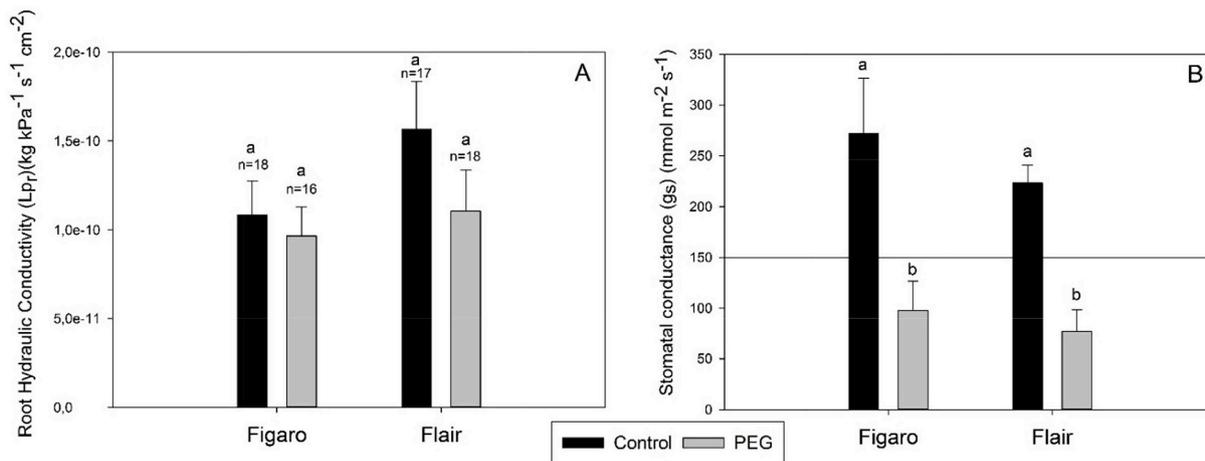


Fig. 6. (A) Root hydraulic conductivity (L_p) ($\text{kg kPa}^{-1} \text{s}^{-1} \text{cm}^{-2}$) and (B) Stomatal conductance (g_s) ($\text{mmol m}^{-2} \text{s}^{-1}$) of *Fragaria x ananassa* cv. Figaro and Flair after control ($\psi_s = -0.06 \text{ MPa}$) or 4h 8% PEG 6000 ($\psi_s = -0.22 \text{ MPa}$) treatment. Values are means \pm standard error (SE). Independent sample t -tests were performed for each cultivar separately. For g_s : $n = 6$; a horizontal line indicates the threshold value.

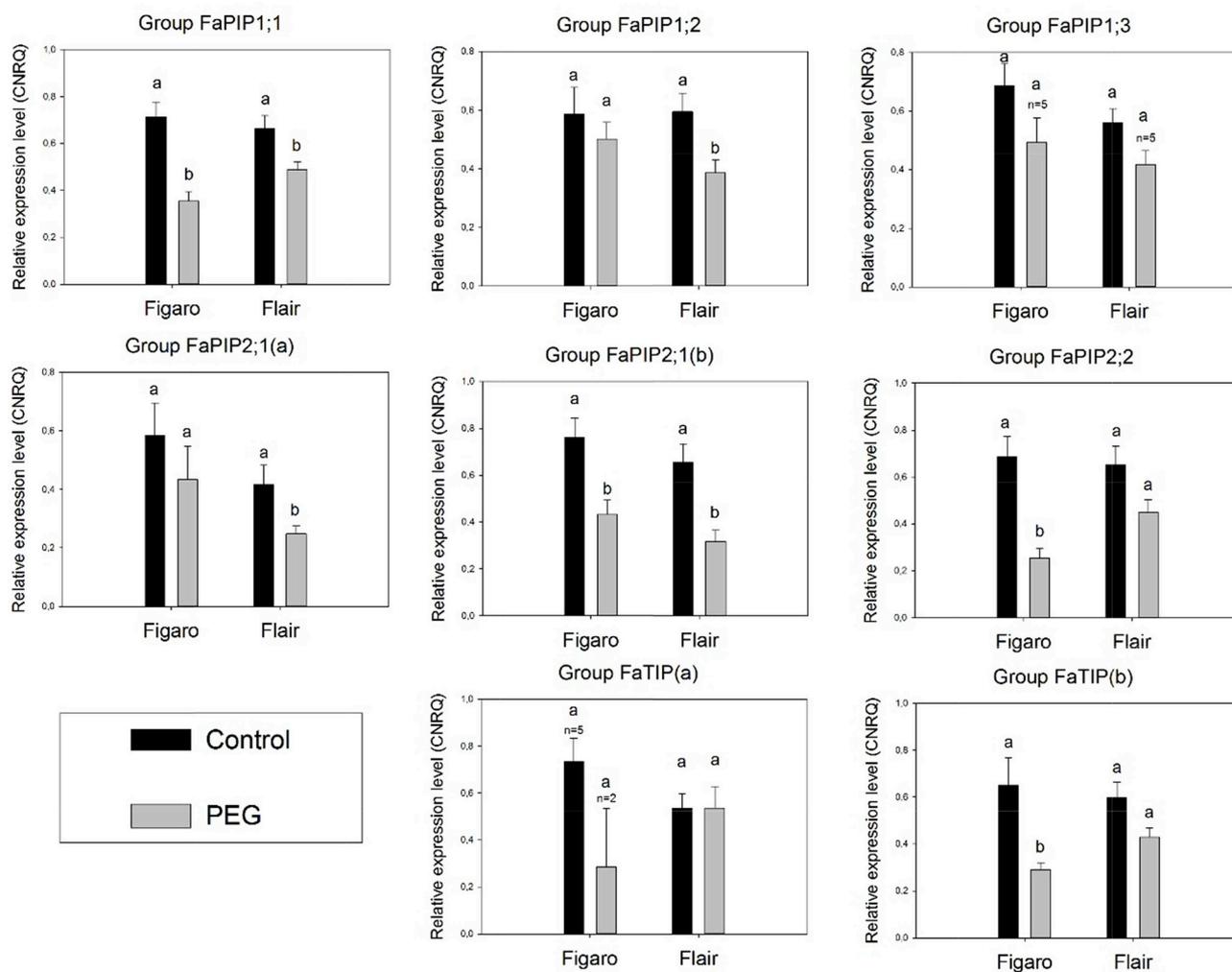


Fig. 7. Relative expression values (geometric mean \pm SE) of aquaporin groups in roots of *Fragaria x ananassa* cultivars Figaro and Flair after control (black) or 4h 8% PEG 6000 ($\psi_s = -0.22$ MPa) treatment (grey). $n = 6$ unless indicated differently in the graph (Appendix B). Significant differences were determined by a t -test ($p < 0.05$) per cultivar.

non-suberized parts of the endodermis (through aquaporins and plasmodesmata). Hence, aquaporins have the potential to greatly influence the radial water flow and, consequently, L_p , either through their abundance at the plasma membrane or through gating (opening and closing of the channels). The apoplastic barriers are, however, absent in the branched feeder rootlets. This implies that the contribution of the aquaporin-controlled transcellular pathway is reduced, although certainly not negligible. By consequence, quantification of aquaporin expression under drought and osmotic stress can contribute to our understanding of how conservative and prodigal water use behavior strategies differ from each other.

Even though there were no significant effects on L_p upon osmotic shock, there were significant effects on root aquaporin expression. The trends were, however, comparable between the conservative and the prodigal cultivar. Under progressive drought, the root PIP responses cannot be generalized (Fig. A6). In *Fragaria vesca* L., contrarily, Šurbanovski et al. (2013) demonstrate a clear correlation between the substrate moisture levels and *FvPIP1;1* and *FvPIP2;1* expression in roots. Also other *FvPIP* aquaporins were downregulated in roots under drought (Šurbanovski et al., 2013), while here, a transient significant upregulation is observed for groups FaPIP1; 1 and FaPIP1; 2, a non-significant downregulation for group FaPIP1; 3 and no responses for the FaPIP2 groups. In *Vitis* sp., no general trend was observed in the aquaporin transcript abundance in roots, while a pattern was found in leaves (Galmés et al., 2007), as is the case here. Contrary to what we

observed, Vandeleur et al. (2009) found significant upregulation of *VvPIP1;1*, in the roots of the anisohydric/prodigal cultivar, as did Lian et al. (2006) for several PIP aquaporins in upland rice. In both cases, the responses were different between plants with different water stress responses. Here, apparent differences in PIP responses are either non-significant or can be attributed to the more severe drought stress experienced by Flair at the last time point before rewatering (Fig. A6). While this was the case for other species, in the two *Fragaria x ananassa* cultivars tested here, no obvious differences in PIP expression upon drought nor osmotic stress were apparent, despite their different water use behavior strategy.

While Figaro upregulates group TIP(a) under the most severe drought conditions and non-significantly downregulates this group upon osmotic shock, both responses are totally lacking in Flair. Cultivar specific TIP expression under drought has been observed in other species (Montalvo-Hernández et al., 2008; Zupin et al., 2017). In tomato (*Solanum lycopersicum* L.), overexpression of *SlTIP2;2* has been shown to substantially improve the water homeostasis under drought stress. Contradictory, this overexpression promoted more prodigal water use behavior (Sade et al., 2009).

As is the case in roots, aquaporins in the leaf also respond to drought in order to contribute to the plant defense against drought stress. At the leaf level, the overall tendency of PIP expression in response to drought was downregulation, with no obvious differences between the conservative and the prodigal cultivar (Fig. 4 and Fig. A5). A higher

number of significant differences could be found for Flair, but these were recorded at very low substrate water content levels, levels that Figaro did not reach during the experiment. Downregulation of aquaporins in response to drought stress has often been explained as an attempt of the plant to preserve the water inside the plant instead of losing it to the drying environment. Even though downregulation upon drought may be the most prevalent response, aquaporin expression can also be upregulated or remain unaffected. It is becoming clear that aquaporin responses cannot be generalized, but rather depend on the species, tissue, organ morphology, aquaporin isoform and severity of the stress (Afzal et al., 2016; Quiroga et al., 2017; Zargar et al., 2017) (Table A.1). In *Phaseolus vulgaris* L., the cultivar displaying conservative water use behavior was the one downregulating a PIP2 gene the most (Zupin et al., 2017). In poplar (*Populus* sp.), several PIP2 aquaporins were non-significantly downregulated in a conservative water use behavior clone, while one of them was upregulated in a prodigal water use behavior clone (Almeida-Rodriguez et al., 2010). These data associate downregulation of aquaporins with conservative water use behavior, while we observed downregulation in the conservative as well as the prodigal one. However, several PIP1 and PIP2 aquaporins were not affected in their expression in the prodigal water use behavior poplar plant, while they were upregulated in the conservative one (Almeida-Rodriguez et al., 2010).

Expression analysis of PIP and TIP aquaporin genes under drought stress conditions is a first important step in characterizing their response, although it is generally recognized that mRNA and protein levels are not necessarily correlated (Aroca et al., 2005; Boursiac et al., 2005; Kammerloher et al., 1994; Lopez et al., 2013; Muries et al., 2011; Suga et al., 2002; Yu et al., 2006). Our data show the cumulative expression level of several grouped genes, hence, the contribution of individual aquaporins is not resolved and we cannot exclude that particular genes behave differently and specifically under the given conditions. Additionally, it must be emphasized that unaltered mRNA or protein levels do not imply unaltered aquaporin activity. Aquaporins can also be (in)activated post-translationally, i.e. through changes at the level of gating, subcellular localization or protein degradation (Chaumont and Tyerman, 2014). These mechanisms allow quick changes in aquaporin activity, which is desirable in a rapidly changing environment, and should be taken into consideration when interpreting expression data.

To conclude, our data point towards conservative water use behavior for cultivar Figaro and prodigal water use behavior for Flair. The identification of the conservative water use behavior in Figaro is based on the tightly controlled stomatal closure, which correlates with foliar ABA accumulation. Prodigal water use behavior was identified based on a sudden and steep drop in stomatal conductance and a decrease in leaf water potential under drought stress in Flair. Certain TIPs were upregulated in roots under drought stress in the conservative water use behavior cultivar only. No other pronounced cultivar differences were observed under progressive drought or osmotic shock.

5. Contributions

B.M. and M.-C. V.L. conceived and planned the experiments. B.M. carried out the experiments. E.D.K. assisted in the RT-qPCR data analysis. L.D. assisted in the HPFM measurements. O.L. assisted in developing the sectioning protocol. F.C. provided valuable feedback regarding HPFM measurements. B.M. and M.-C. V.L. contributed to the interpretation of the results. B.M. wrote the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript. FC research is supported by grants from the Belgian National Fund for Scientific Research (FNRS) and the Belgian French community (ARC16/21–075 project). The funding sources had no involvement in the collection, analysis or interpretation of the data, in the writing of the report, nor in the decision to submit the article for publication.

Author contribution statement

B.M. and M.-C. V.L. conceived and planned the experiments. B.M. carried out the experiments. E.D.K. assisted in the RT-qPCR data analysis. L.D. assisted in the HPFM measurements. O.L. assisted in developing the sectioning protocol. F.C. provided valuable feedback regarding HPFM measurements. B.M. and M.-C. V.L. contributed to the interpretation of the results. B.M. wrote the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

Declaration of competing interest

None.

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Appendix. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.plaphy.2019.10.030>.

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