

# Pearl millet (*Pennisetum glaucum*) contrasting for the transpiration response to vapour pressure deficit also differ in their dependence on the symplastic and apoplastic water transport pathways

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**Abstract.** Genotypic differences in transpiration rate responses to high vapour pressure deficit (VPD) was earlier reported. Here we tested the hypothesis that this limitation could relate to different degrees of dependence on the apoplastic (spaces between cells), and symplastic water transport pathways (through cells via aquaporin-facilitated transport), which are known to have different hydraulic conductivities. The low transpiration rate (Tr) genotype PRLT 2/89/33 either restricted its transpiration under high VPD, or was more sensitive to VPD than H77/833-2, when grown hydroponically or in soil. The slope of the transpiration response to an ascending series of VPD was lower in whole plants than in de-rooted shoots. In addition, the transpiration response of detached leaves to moderately high VPD (2.67 kPa), normalised against leaves exposed to constant VPD (1.27 kPa), was similar in low and high Tr genotypes. This suggested that roots hydraulics were a substantial limitation to water flow in pearl millet, especially under high VPD. The dependence on the apoplastic and symplastic water transport pathways was investigated by assessing the transpiration response of plants treated with inhibitors specific to the AQP-mediated symplastic pathway (AgNO<sub>3</sub> and H<sub>2</sub>O<sub>2</sub>) and to the apoplastic pathway (precipitates of Cu(Fe(CN)<sub>6</sub>) or Cu(CuFe(CN)<sub>6</sub>)). When CuSO<sub>4</sub> alone was used, Cu ions caused an inhibition of transpiration in both genotypes and more so in H77/833-2. The transpiration of high Tr H77/833-2 was decreased more by AQP inhibitors under low VPD (1.8 kPa) than in PRLT 2/89/33, whereas under high VPD (4.2 kPa), the transpiration of PRLT 2/89/33 was decreased more by AQP inhibitors than in H77/833-2. The transpiration rate of detached leaves from H77/833-2 when treated with AgNO<sub>3</sub> decreased more than in PRLT 2/89/33. Although the root hydraulic conductivity of both genotypes was similar, it decreased more upon the application of a symplastic inhibitor in H77/833-2. The transpiration of low Tr PRLT 2/89/33 was decreased more by apoplastic inhibitors under both low and high VPD. Then the hydraulic conductivity decreased more upon the application of an apoplastic inhibitor in PRLT 2/89/33. In conclusion, both pathways contributed to water transport, and their contribution varied with environmental conditions and genotypes. Roots were a main source of hydraulic limitation in these genotypes of pearl millet, although a leaf limitation was not excluded. The similarity between genotypes in root hydraulic conductivity under normal conditions also suggests changes in this conductivity upon changes in the evaporative demand. The low Tr genotype depended more on the apoplastic pathway for water transport, whereas the high Tr genotype depended on both pathway, may be by ‘tuning-up’ the symplastic pathway under high transpiration demand, very likely via the involvement of aquaporins.

**Additional keywords:** apoplastic pathway, aquaporins, aquaporin inhibitors, hydraulic conductance, transpiration, VPD.

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## Introduction

Pearl millet is a well-adapted crop to dry conditions, but water limitation remains the major constraint to its production (Mahalakshmi *et al.* 1987). Previous work has shown that a lower transpiration rate, especially under high vapour pressure deficit (VPD) contributed to small but critical water savings during the

vegetative stage (Kholová *et al.* 2010a, 2010b; Kholová and Vadez 2013) that led to increased yield under terminal drought in pearl millet (Vadez *et al.* 2013a). This mechanism has been described in other species (soybean – Sinclair *et al.* 2008; sorghum – Gholipour *et al.* 2010; peanut – Devi *et al.* 2010; chickpea – Zaman-Allah *et al.* 2011a) and its benefits for crops

production under terminal stress limitations were shown (Sinclair *et al.* 2005, 2010; Zaman-Allah *et al.* 2011b; Vadez *et al.* 2013b, 2014). A review of the different types of transpiration response showed that differences could either be linear with genotypic differences in the slope and intercept of the transpiration response, or with a breakpoint in this response and genotypic differences in the breakpoint value and/or the slope of the response before or after the breakpoint (Vadez *et al.* 2013b), in both cases resulting in transpiration rate ( $T_r$ ) differences allowing water savings. The underlying physiological mechanisms responsible for such plants responses to the atmospheric stimuli are still largely unexplained. In earlier work in pearl millet, we hypothesised that hydraulic signals may be responsible for the rapid stomatal response to an increase in VPD (Vadez 2014). For instance, in soybean, Sinclair and colleagues (2008) showed that hydraulic limitation was indeed the cause for the transpiration restriction, and leaves were interpreted to be the location of this limitation. Similar conclusion was drawn in a study between two sorghum genotypes contrasting for the transpiration response to VPD in sorghum (Choudhary *et al.* 2013). In two studies in wheat roots, hydraulics was shown to be the main cause limiting water movement (Kudoyarova *et al.* 2011; Schoppach *et al.* 2014). In one of these studies, the root hydraulic limitation was explained by smaller meta-xylem vessels, thinner endodermis and a smaller population of mercury-sensitive aquaporins in the roots (Schoppach *et al.* 2014). The other study in wheat also concluded that the mercury-sensitivity of root aquaporin explained the root hydraulic limitation, and further, showed a role of ABA accumulating preferentially in the roots in restoring root hydraulic conductivity (Kudoyarova *et al.* 2011). Therefore, assuming the cause of lower  $T_r$  in PRLT 2/89-33 is a hydraulic limitation, the first objective of this work was to locate this putative hydraulic limitation, either in the root or in the shoot, and determine whether there was any genotypic difference in it. This was addressed by comparing transpiration response across a range of VPD conditions in whole plants, de-rooted shoot, or detached leaves, of low and high  $T_r$  genotypes.

The movement of water through plant tissues to the xylem in the root cylinder, or from the xylem in the leaves, occurs through both the cell-to-cell and apoplastic pathways (Stedle 2000a, 2000b). The dominant pathway has been explored in maize, barley, rice, wheat and lupins, and the conclusion is generally that water flow occurs via a combination of these pathways (see Stedle 2000a; Ranathunge *et al.* 2005; Bramley *et al.* 2009; Knipfer *et al.* 2011). However, whether the predominance of either pathway changes with the evaporative demand is unknown, and is important to address this to understand how plants' transpiration adapts to these changes. The transport in the cell-to-cell pathway can occur via plasmodesmata and/or by crossing membranes. The driving force in the cell-to-cell pathway can be osmotic and/or hydrostatic, the former being in the absence of transpiration, e.g. at night. The cell-to-cell water transport may involve specialised membrane transporters called aquaporins (AQP), wherein the apoplastic water transport involves a water movement in the intercellular space, driven by gradient of hydrostatic pressure. So far the cell-to-cell (hereafter called

'AQP-mediated') water transport pathway has been explored in several crops like wheat (Bramley *et al.* 2009), soybean (Sadok and Sinclair 2010a), barley (Knipfer *et al.* 2011), peanut (Devi *et al.* 2012), sorghum (Choudhary *et al.* 2013), whereas the apoplastic water transport has been studied in maize (Zimmermann and Stedle 1998), rice (Ranathunge *et al.* 2005) and lupin (Bramley *et al.* 2009). The preferred pathway differs among species, organs, developmental stage and environmental parameters (Johansson *et al.* 2000). For instance, a study by Bramley *et al.* (2009) showed that lupin uses predominantly the apoplastic pathway, whereas much of the water movement in the roots of wheat was through the AQP-mediated pathway.

To date there has been no study to assess possible intra-species variation in the dependence on either one pathway, or whether the dependence on either one pathway varies with the evaporative demand. This is an important gap for our research topic because there is still controversy as to which pathway has the highest water conductivity (Stedle 2000a; Javot and Maurel 2002). Assuming there are indeed conductivity differences between the two pathways, differences in the respective proportion of water going through either pathway might then lead to differences in the overall hydraulic conductivity, and could then be linked with how plant transpiration responds to environmental stimuli. Several studies have shown that higher hydraulic conductivity of the root is closely linked to increases in expression of several aquaporin genes (e.g.; Ehlert *et al.* 2009; Hachez and Chaumont 2010; Knipfer *et al.* 2011), indicating that plants have the capacity to increase the hydraulic conductivity of the roots by altering the AQP-mediated pathway of water transport, as it was found in the roots of wheat under ABA control (Kudoyarova *et al.* 2011). By contrast and intuitively, although a recent report concluded to the contrary (Cuneo *et al.* 2016), species, or genotypes, that are more dependent on the apoplastic pathway for water transport would not have this flexibility since the apoplastic space depends on the cell sizes and arrangement between one another, offering a frame that is somewhat fixed once cells are fully developed. Therefore, the main hypothesis of the present work was that genotypes of pearl millet contrasting for the transpiration rate across a range of low to high VPD conditions will vary in their dependence on either of the AQP-mediated or apoplastic pathways. It has been reported that to test the apoplastic pathway inhibition, combination of  $K_4(Fe(CN)_6)$  and  $CuSO_4$  can be used (Ranathunge *et al.* 2005). In contrast, there were also reports where Cu salt is used for the inhibition of AQP-mediated pathway (Ligaba *et al.* 2011). So the effect of  $CuSO_4$  alone was also tested along with the combined effect of  $K_4(Fe(CN)_6)$  and  $CuSO_4$  on plant transpiration response to vapour pressure deficit.

Hence, the main objective of this study was to locate a putative hydraulic limitation to water movement in pearl millet and characterise the water transport pathways in genotypes that have been previously found to contrast for their response to atmospheric stimuli (VPD). Specifically, we aimed to: (i) assess which of the shoot or the root appeared to be responsible for putative hydraulic limitations during the response of transpiration to high VPD, (ii) evaluate plants' transpiration response to water transport pathways inhibitors under low and high VPD regimes; and (iii) evaluate plant organs' conductance

(leaves/roots) under regular conditions and upon action of inhibitors of various water transport pathways.

## Materials and methods

### *Plant material and plant growth*

Two pearl millet (*Pennisetum glaucum* (L.) R.Br.) genotypes; PRLT 2/89-33 (low Tr) and H77/833-2 (high Tr) differing for terminal drought tolerance under field conditions were selected for the study (Yadav *et al.* 2002; Serraj *et al.* 2005). PRLT 2/89-33 has low Tr and shows restriction in transpiration rate to increasing VPD whereas H77/833-2 has high Tr and shows a linear transpiration rate response to raising VPD (Kholová *et al.* 2010b; Vadez *et al.* 2015). PRLT 2/89-33 was derived from the ICRISAT Bold Seeded Early Composite, which is an elite breeding population based largely on Iniadi landrace germplasm from West Africa (Andrews and Anand Kumar 1996). This Iniadi germplasm is known for better grain filling under terminal drought stress conditions. H77/833-2 is the male parent of several thermo tolerant, extra early, high tillering and high yielding pearl millet hybrids including HHB67.

For all experiments (see experimental summaries in Table 1) the plants were grown in glasshouse conditions under natural day-light oscillations and with day/night average temperature around 28/22°C and RH 70/90%. Intact plants were grown in both soil, using 4 L pots and hydroponic solutions, using 350 mL Erlenmeyer flasks. The purpose of growing plants in both conditions was 2-fold: (i) first to confirm that the VPD response of transpiration was similar in hydroponic conditions, where roots develop differently from soil conditions (Peterson 1988; Zimmermann and Steudle 1998; Freundl *et al.* 2000); (ii) to allow for the application of inhibitors. For the soil grown plants, the pot filling used was a mixture of soil, sand and manure (5:3:1) and two seeds per hill sown in three hills. The manure used was approximately 1 year old, processed from cow dung. Di-ammonium phosphate and urea, at a rate of 1 g pot<sup>-1</sup>, was applied after thinning on the second week after sowing and plants were thinned to maintain a single plant per pot. For the hydroponic plants, the seeds were sown in sand irrigated with nutrient solution (modified Hoagland solution; macronutrients: MgSO<sub>4</sub> (2.05 mM), K<sub>2</sub>SO<sub>4</sub> (1.25 mM), CaCl<sub>2</sub>·2H<sub>2</sub>O (3.3 mM), KH<sub>2</sub>PO<sub>4</sub> (0.5 mM), Fe-EDTA (0.04 mM), urea (5 mM) and micronutrients: H<sub>3</sub>BO<sub>3</sub> (4 μM), MnSO<sub>4</sub> (6.6 μM), ZnSO<sub>4</sub> (1.55 μM), CuSO<sub>4</sub> (1.55 μM), CoSO<sub>4</sub> (0.12 μM), Na<sub>2</sub>MoO<sub>4</sub> (0.12 μM)). At the 3rd leaf stage they were transferred to the trays containing nutrient solution. The pH of the nutrient solution was maintained between 6.0 and 6.3. Aeration was continuously provided to enable the nutrient absorption by the roots. The nutrient solution was changed once every 3 days. At 4th leaf stage, plants were transferred into the Erlenmeyer flasks and grown until the end of the experiment (either 6th or 7th leaf stage).

### *Transpiration response to increasing VPD in soil-grown de-rooted shoot and whole plants*

The purpose of this experiment was to compare the transpiration response to increasing VPD in both whole plants and de-rooted shoots, as an attempt to locate a putative source of hydraulic limitation in the root or the shoot. Hypothetically, a limitation

in the roots would lead to a higher transpiration of de-rooted shoots, especially under high VPD conditions. Whole plants were acclimatised for an entire day in the growth chamber, to adapt to the daytime regime (06:30–18:30 hours) at a VPD of 1.8 kPa and night-time regime (19:30–05:30 hours) at a VPD of 0.9 kPa. The 1 hour time between 05:30 and 06:30 and 18:30 to 19:30 hours was used for a gradual transition in the conditions between day/night. The plants were exposed to a light intensity of ~550 μmol at plant canopy level. VPD levels were obtained by simultaneously increasing the temperature and decreasing the relative humidity (see Table S1, available as Supplementary Material to this paper, for details of the ranges that were used). This has the drawback of having two factors involved in changing the VPD. Although this is a natural phenomenon, the caveat was in not being able to distinguish VPD effects from a potential temperature effect. A temperature and relative humidity data logger (Lascar Electronics) was positioned at the plant canopy level to record the temperature and humidity every 5 min inside the chamber until the end of experiment. Pots were brought to field capacity and a layer of plastic beads was added to avoid direct evaporation of water from the soil. For the preparation of de-rooted shoots, whole plants were de-rooted from soil in the following early morning (around 05:00–06:00 hours), on the day of the experiment. The soil particles were washed and root was immersed in deionised water. The de-rooted shoots (Fig. S1, available as Supplementary Material to this paper) were then prepared by severing the shoot from the root at the hypocotyl region. These de-rooted shoots were then rapidly placed in flasks containing 0.1 mM EDTA (Kholová *et al.* 2010a). EDTA was used to preserve the tissues and avoid the oxidative stress (Habiba *et al.* 2015) due to de-rooting injury. The mouths of the flasks were wrapped with aluminium foil to avoid direct evaporation. They were then placed in a dark room for an hour. Later on the de-rooted shoots were gradually exposed to light (ranging from 120 to 550 μmol) at a low VPD (0.60 kPa) for 2 h. Whole plants used for the experiment were exposed to the same pre-experimental conditions. Thereafter, the whole plants and de-rooted shoots were exposed to an ascending series of VPDs ranging from 0.60 (24°C/80% RH) to 4.2 kPa (40°C/43% RH) (temperature was gradually raised to achieve higher VPD; Table S1 for a full detail of T°C and RH% conditions). Each VPD was maintained for an hour and a 15 min transition time was allowed between successive VPD levels. These protocols were used earlier to characterise the transpiration response across a range of VPD conditions (e.g. Kholová *et al.* 2010b). The transpiration values were recorded for 1 hour at each VPD level, by successive weighing of pots/flasks with an analytical balance (KERN 3600-2N, Kern and Sohn GmbH). At the end of the experiment, leaf area was recorded using leaf area meter (LI-3100C area meter, LI-COR BioSciences) to calculate the transpiration rate, Tr (mg water loss cm<sup>-2</sup> min<sup>-1</sup>).

### *Transpiration response to VPD in plants grown in soil and in hydroponic*

Both types of plants (soil, hydroponic) were grown in the glasshouse under natural daylight oscillations and with day/night average temperature around 31/22°C and RH 40/55%.

**Table 1. Overview of the experiments**

Experiment 1 was designed to locate the source of hydraulic limitation. Experiment 2 was to compare the transpiration response of plants grown in two different systems (soil and hydroponics) and exposed to increasing vapour pressure deficit (VPD). In experiment 3, the effect of inhibiting water transport pathways on transpiration were investigated at the beginning (1.27 kPa) of an ascending series of VPDs and at constant low VPD (1.8 kPa). In experiment 4, the effect of inhibiting water transport pathways on transpiration were investigated at the end of an ascending series of VPDs (4.2 kPa). In experiments 5 and 6, plant parts (leaves and roots respectively) were tested to examine the effects of inhibiting AQP mediated and apoplastic pathways in separate plant organs

Experiment no.	Growth conditions	Parts investigated	Traits measured	Inhibitors used	No. of replicates per genotype	No. of replicates of experiments
1	Soil (alfisol : sand : manure -5 : 3 : 1)	Whole plants and de-rooted shoots	Tr response to raising VPD in growth chamber	–	9	1
2	Soil (alfisol : sand : manure -5 : 3 : 1)	Whole plants	Tr response to raising VPD in growth chamber	–	8	2
3	Hydroponics	Whole plants	Effect of Cu alone its combined effect with $K_4[Fe(CN)_6]$ on transpiration at constant low VPD in growth chamber	0.5 mM $CuSO_4$ and 1 mM $K_4[Fe(CN)_6]$	Control, 5; treatment, 5	2
4	Hydroponics	Whole plants	(a) Level of AQP and apoplast inhibitions at the beginning of an ascending series of VPDs in growth chamber (b) Level of AQP inhibition at constant low VPD in growth chamber (c) Level of apoplast inhibition at constant low VPD in growth chamber	1.5 mM $H_2O_2$ (AQP inhibition) 0.5 mM $CuSO_4$ and 1 mM $K_4[Fe(CN)_6]$ (apoplastic inhibition) 400 $\mu M$ $AgNO_3$	Control, 5; treatment, 5	2
5	Hydroponics	Whole plants	Level of AQP and apoplast inhibitions at the end of an ascending series of VPDs in growth chamber	0.5 mM $CuSO_4$ and 1 mM $K_4[Fe(CN)_6]$	Control, 5; treatment, 5	2
6	Hydroponics	Leaf	(a) VPD response (b) Level of AQP inhibition at constant high VPD in growth chamber (c) Level of AQP inhibition at constant high VPD in growth chamber	– 1.5 mM $H_2O_2$ 400 $\mu M$ $AgNO_3$	Control, 3; treatment, 3 Control, 6; treatment, 4	4 7
7	Hydroponics	Root	Root hydraulic conductivity by inhibiting AQP mediated and apoplast pathways in glasshouse	400 $\mu M$ $AgNO_3$ (AQP inhibition) 0.5 mM $CuSO_4$ and 1 mM $K_4[Fe(CN)_6]$ (apoplastic inhibition)	Control, 5; treatment, 5	2

One day before experimenting the transpiration response to increasing VPD, the plants were moved to the growth chambers and acclimatised to the daytime regime (06:30 to 18:30 hours) at a VPD of 1.8 kPa (31°C/60% RH) and night-time regime (19:30 to 05:30 hours) at a VPD of 0.9 kPa (27°C/75% RH) (see Table S1 for a full detail of T°C and RH% conditions). On that day, the soil grown plants were brought to field capacity and a layer of beads was added over the soil to reduce soil evaporation, while the mouths of the flasks with hydroponically grown plants were covered with aluminium foil to avoid direct evaporation. The following morning, on the day of the experiment, a measured amount of fresh solution was given back in case of hydroponic plants. Hereafter, the VPD was gradually increased to several steps maintaining each VPD for an hour (covering a range of VPD values between 1 (31°C/60% RH) and 4 kPa (38°C/40% RH)) (see Table S1 for a full detail of T°C and RH% conditions). Again, changing VPD by changing both RH% and temperature dismissed the possibility to assess possible temperature effects on the transpiration. However, it should be mentioned that pearl millet is commonly exposed to these temperature regimes in nature. Between successive VPD steps, a 15 min transition was allowed to adjust and stabilise the conditions in the growth chamber. Weighing was done during the transition period so that the weight loss due to transpiration was recorded before and after VPD change. At the end of the experiments, leaf area was recorded to calculate the transpiration rate ( $\text{mg water loss cm}^{-2} \text{ min}^{-1}$ ).

#### *Transpiration response to increasing VPD in soil-grown detached leaves*

For detached leaf experiments, single leaves of identical position (6th or 7th leaf) were collected on the day of the experiment, from soil grown plant of both genotypes. At that stage of the plant development there was no or very limited tillering and therefore the leaves from the main stem were used. The initial cut was done with scissors and the cut leaves were rapidly put in deionised water to avoid exposure of the cut part to the air. The cut part was then re-cut under water. To avoid clogging of the vessels, re-cutting was done again under 0.1 mM EDTA ( $\text{Na}_2 + \text{EDTA} \times 2\text{H}_2\text{O}$ ; Kholová *et al.* 2010a), and leaves inserted in test tubes containing 50 mL of 0.1 mM EDTA, earlier prepared with their mouths wrapped with parafilm. The detached leaves were inserted into the test tubes by piercing the parafilm and the mouth of the test tubes was wrapped with aluminium foil to avoid direct evaporation. Each replication consisted of three test tubes, each containing one individual leaf, and these were placed on a 0.01 g precision balance (KERN 3600-2N, Kern and Sohn GmbH) with a 0.01 g precision where the weight loss due to transpiration was recorded every 20 min. The period during which the transpiration of detached leaves (three replicates per genotype) remained constant had been earlier evaluated to be at least 5 h after detaching and the experiments were designed not to exceed this time span. The test tubes containing leaves were placed in two different chambers, one with the VPD of 1.27 kPa (30°C/70% RH) throughout the experiment and the other chamber with 1.27 kPa VPD for a period of 2 h and then with 2.65 kPa (35°C/53%) for 2.5 hours. At the end of the experiment, leaf area

was measured using a LI3000 (LI-COR BioSciences) leaf area meter.

#### *Inhibition of AQP-mediated and apoplastic pathways in hydroponic plants*

For the inhibition of the AQP-mediated water transport pathway of whole plants (Experiments 6 and 7), an initial standardisation had been done to test the optimum stage of the plant. For this, three sets of hydroponically grown plants differing in phenological stages (spanning across 21–27 DAS, 5th–8th leaf stage) had been inhibited with 1.5 mM  $\text{H}_2\text{O}_2$  and 400  $\mu\text{M}$   $\text{AgNO}_3$ . These concentrations of inhibitors had been previously tested on pearl millet (Fig. S2). They were chosen in a way they lead to a transpiration inhibition, in comparison to lower and higher concentrations, but also allowing at least a partial recovery of the transpiration after treatment removal from the solution.  $\text{H}_2\text{O}_2$  was initially used and we shifted to  $\text{AgNO}_3$  for its demonstrated inhibitory effect on transpiration and a relatively easier way to use than  $\text{H}_2\text{O}_2$ . In this standardisation experiment, a non-significant variation in the level of inhibition among the sets had been observed (data not shown). Therefore, we settled for testing after the complete development of the 6th leaf, i.e. usually between 24–27 DAS in our temperature conditions. We are also aware that other inhibitors were used earlier in other works (Devi *et al.* 2012), and settled for  $\text{AgNO}_3$  and  $\text{H}_2\text{O}_2$  since these were able to pinpoint genotypic differences. We chose to not use  $\text{HgCl}_2$  for its unknown effects on other physiological processes. One day before experimenting the inhibition responses, the hydroponic plants were moved to the growth chambers and acclimatised to the daytime regime (06:30 to 18:30 hours) at a VPD of 1.8 kPa (31°C/60% RH) and night-time regime (19:30 to 05:30 hours) at a VPD of 0.9 kPa (27°C/75% RH). On the day of the experiment the acclimatised plants were given a measured amount of fresh nutrient solution and they were positioned on separate balances with 0.01 g accuracy (Kern KB 3600-2N) and their weight loss (due to transpiration) was recorded every 20 min. Inhibitors (1.5 mM  $\text{H}_2\text{O}_2$  and 400  $\mu\text{M}$   $\text{AgNO}_3$ ) were applied after 2 h of initiating the experiment and subsequently transpiration response to treatment was followed up for a minimum of 3 h.

According to previous work (Ranathunge *et al.* 2005) the reaction between 1 mM  $\text{K}_4(\text{Fe}(\text{CN})_6)$  and 0.5 mM  $\text{CuSO}_4$  inside the root tissues produces rusty brown crystals (precipitates) of  $\text{Cu}_2(\text{Fe}(\text{CN})_6)_6$  or  $\text{Cu}(\text{Cu}(\text{Fe}(\text{CN})_6)_6)$  and these restrict the apoplastic water flow. As  $\text{CuSO}_4$  permeates faster than  $\text{K}_4(\text{Fe}(\text{CN})_6)$ , the  $\text{K}_4(\text{Fe}(\text{CN})_6)$  was applied first to the hydroponic solution allowing its slow penetration overnight into the root apoplast followed by exchange with  $\text{CuSO}_4$  solution in the following morning (half molarity of  $\text{CuSO}_4$  was used to assure all molecules react with  $\text{K}_4(\text{Fe}(\text{CN})_6)$  and to avoid a putative poisonous effect of copper on root cells). Longer (maximum of 4 h) exposure of the plants to 1 mM  $\text{K}_4(\text{Fe}(\text{CN})_6)$  didn't affect transpiration and the genotypic difference was not observed (Fig. S3). So the plants were exposed to 1 mM  $\text{K}_4(\text{Fe}(\text{CN})_6)$  overnight that again did not affect the plant transpiration in the following morning hours before the application of  $\text{CuSO}_4$  (Fig. S4). Thereafter, the solution exchange with 0.5 mM  $\text{CuSO}_4$  resulted in the maximum transpiration inhibition and resolution between genotypes (Fig. S4). The experiment then

consisted in assessing transpiration for 2 h before  $\text{CuSO}_4$  application, and then following-up transpiration for at least 3 h after  $\text{CuSO}_4$  application. Here to test a putative effect of  $\text{CuSO}_4$  on transpiration, a set of plants were exposed to  $\text{CuSO}_4$  on the day of experiment without the application of 1 mM  $\text{K}_4(\text{Fe}(\text{CN})_6)$  overnight. Then to test the recovery of plant transpiration, inhibitors were removed and the roots were washed. They were then placed in deionised  $\text{H}_2\text{O}$  and their transpiration was assessed for 3 h. The treated roots appeared darker in comparison with untreated control roots. Free-hand sectioning of root tissues of both H77/833-2 and PRLT 2/89/33 were taken from untreated control and treated plants with  $\text{K}_4(\text{Fe}(\text{CN})_6)$  and  $\text{CuSO}_4$  tested for apoplast inhibition. The sections were cut free-hand, taken from a distance of 4–5 cm from the root apex and stained with acid fuchsin dye. Anatomical structures were viewed under light microscope (Olympus) at  $10 \times 10$  magnification and captured using camera (Fig. S5A, B). Whether different genotypes absorbed different amount of the  $\text{K}_4(\text{Fe}(\text{CN})_6)$  was not tested.

#### *Transpiration response to AQP inhibition on detached leaves*

The transpiration response to AQP inhibition on detached leaves was tested with leaves collected from soil-grown plants, prepared as in the above section describing the transpiration response to VPD in detached leaves. Initially the inhibition was tested at 1.8 kPa (31°C/60% RH) and showed no transpiration inhibition response from the detached leaves (data not shown). Then, the transpiration response to AQP inhibition was tested at 3.5 kPa (38°C/48% RH). The transpiration was measured initially for a period of 2 h and then the inhibitors ( $\text{H}_2\text{O}_2$  and  $\text{AgNO}_3$ ) were injected into the test tubes by piercing the aluminium foil and parafilm with a needle and syringe. Again, the transpiration was monitored for ~3 h. Transpiration was recorded gravimetrically for every 30 min. At the end of the experiment, leaf area was measured and transpiration rate was calculated.

#### *Root hydraulic measurements*

For the measurement of root hydraulic conductivity, hydroponically grown plants were used and the experiment was conducted in the glasshouse, therefore in plants that were exposed to mild VPD conditions, i.e. day/night average temperature around 28/17°C and RH 70/80%. No root hydraulic conductivity measurement was done after exposing the plants to increases in VPD. During hydraulic conductivity measurement, the roots of the plants were immersed in small container with the solution (nutrient solution in case of untreated control or deionised water with inhibitors in case of treatment) and placed in a Scholander pressure chamber (PMS Instruments) and sealed at the hypocotyl level using silicon glue and polyvinylsiloxane (Coltene President Co.). The shoot was then removed with single cut by razor leaving the de-topped roots in the chamber (Miyamoto *et al.* 2001) and the exuded sap was immediately collected using pre-weighed Eppendorf stuffed with tissue paper (Kimtech Science). The sap was collected at three different pressures (0.1, 0.2 and 0.3 MPa). Each pressure was maintained for 15 min and the exuded sap was collected every 5 min. The next pressure was applied once

a constant exudation rate was reached. The average value was taken and the exudation rate was normalised with root surface area and the pressure applied. The root surface area was determined by scanning them with Shimadzu scanner and analysing with Winrhizo software (Winrhizo, Regent Ltd). The measurements were made between 07:00 hours till 18:00 hours. Both the genotypes were measured side by side, which allowed us to compare the root hydraulic conductivity despite the diurnal effect.

#### *Data analysis*

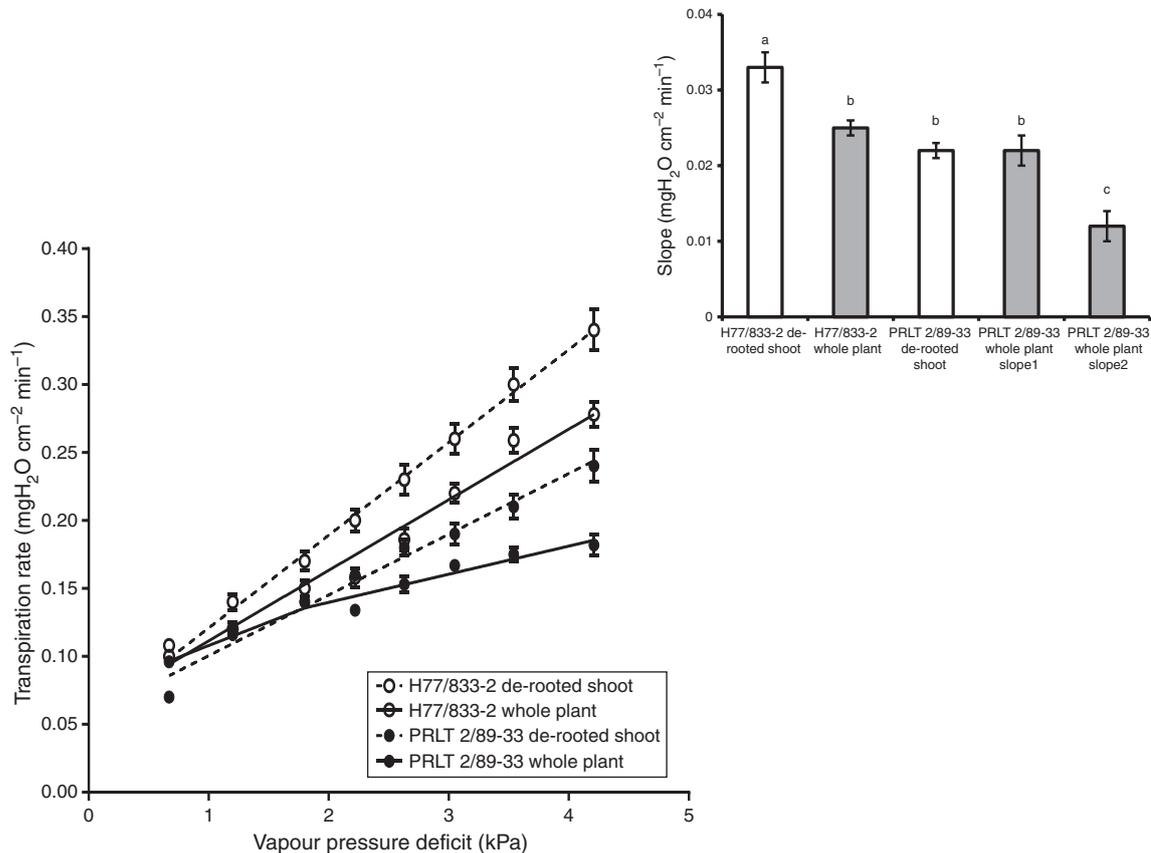
In each experiment, transpiration values (T; g of water loss per unit of time) were divided by leaf area (transpiration rate, Tr; g of water loss per unit of time and leaf area ( $\text{g water loss cm}^{-2} \text{min}^{-1}$ ). In inhibition experiments, Tr data were double normalised to a non-treated control. The first normalisation consisted in dividing the individual Tr data by the mean Tr of the control plants for each genotype (transpiration ratio, TrR). To control possible plant to plant differences in size and therefore transpiration, the values of the Tr ratio before the inhibitor treatment were averaged for each replicated plant and then each individual Tr ratio value was divided by this average (normalised TrR, NTrR). Inhibition effects were therefore compared on the basis of the NTrR differences. Root hydraulic conductivity was calculated by normalising the root exudation to root surface area, time and pressure applied, and expressed in the SI unit of  $\text{ms}^{-1} \text{MPa}^{-1}$ , following similar methodology used earlier in lupin and wheat (Bramley *et al.* 2009), tomato (Bárzana *et al.* 2012) and maize (Zimmermann and Steudle 1998).

Analysis of variance (ANOVA) was done with statistical program package CoStat ver. 6.204 (Cohort Software). Two-way ANOVA was used to evaluate the effect of treatment and genotype and their interaction. One-way ANOVA was conducted to test the effect of treatment within the genotypes. For the statistical analysis of inhibition experiments, the average value of stabilised NTrR after the inhibition treatment were used. Means were analysed using Tukey–Kramer test and l.s.d. Tr response to increasing VPD were analysed with linear and non-linear regressions of Graph pad prism ver. 6 (Graph pad software Inc.).

## **Results**

#### *Transpiration response to VPD of soil-grown whole plants, de-rooted shoots, detached leaves, and plants grown in hydroponics*

The de-rooted shoot, exposed to an ascending series of VPDs (range of 0.67 to 4.2 kPa), had higher Tr than the whole plants in both genotypes (Fig. 1). The de-rooted shoots of H77/833-2 had higher Tr and higher slope value than the de-rooted shoots of PRLT 2/89-33 (Table 2). In the case of H77/833-2, the transpiration response to increasing VPD was linear for both de-rooted shoots and whole plants, although the slope of the linear response (slope=0.07) was ~30% higher in de-rooted shoots than for the whole plant (slope=0.05; see inset in Fig. 1). In the case of PRLT 2/89-33, the transpiration response of the de-rooted shoot was linear while the transpiration response of whole plants showed a breakpoint at 1.8 kPa (Table 3). The slope value of the de-rooted shoot (slope=0.04) was ~100% higher than the slope value beyond



**Fig. 1.** Comparison of the transpiration rate ( $T_r$ ) response in whole plants (solid line) and de-rooted shoots (dashed line) of soil grown plants tested in the growth chamber at vegetative stage in genotypes H77/833-2 (high  $T_r$ , open circle) and PRLT 2/89-33 (low  $T_r$ , closed circle). Each point is the mean of  $T_r$  values ( $n=9$ ) and the error bars indicate  $\pm$  s.e. of the mean. The inset graph represents the slope values of de-rooted shoot (open bar) and whole plant (closed bar) of H77/833-2 and PRLT 2/89-33 (before vapour pressure deficit (VPD) breakpoint (slope 1) and after VPD breakpoint (slope 2)). Bars with different letters are significantly different ( $P < 0.05$ , Tukey–Kramer test).

the breakpoint (1.8 kPa) in whole plants (slope = 0.02; see insert in Fig. 1). The increase in slope caused by de-rooting was then larger in PRLT 2/89-33 (Fig. 1) than in H77/833-2 (Fig. 1). Therefore, de-rooting facilitated water transport to support transpiration, especially under high VPD conditions and then more so in low  $T_r$  PRLT 2/89-33 than in high  $T_r$  H77/833-2.

The transpiration response to increasing VPD was also compared between soil and hydroponics growth conditions. In both growing systems the low  $T_r$  genotype PRLT 2/89-33 had lower  $T_r$  than the high  $T_r$  genotype H77/833-2 over the whole range of VPD conditions (Fig. 2), which was in line with previous results (Kholová *et al.* 2010b). Hence, the genotypic  $T_r$  difference across the range of VPD conditions was consistent across both hydroponics and soil-based growth systems (Fig. 2). The plants grown in hydroponics had about twice the transpiration rate (mg of H<sub>2</sub>O cm<sup>-2</sup> min<sup>-1</sup>; Table 2) of plants grown in soil.

In summary, the genotypic differences in the transpiration response to high VPD of whole plants grown in soil identified earlier (Kholová *et al.* 2010a) were confirmed in plants grown in hydroponics and roots limited water transport to support transpiration in pearl millet, and more so in low  $T_r$  PRLT 2/89-33 than in high  $T_r$  H77/833-2, which seemed to have a higher  $T_r$  capacity than PRLT 2/89-33.

#### Root hydraulic conductivity under non-inhibited control condition

Root hydraulic conductivity measurements were made from control plants grown in the glasshouse, in two experiments described below. No genotypic differences in the root hydraulic conductivity (ms<sup>-1</sup> MPa<sup>-1</sup>) were found under non-inhibited condition (see the ‘control’ bars in Fig. 3a, b; Table 2).

#### Transpiration response to VPD in detached leaves

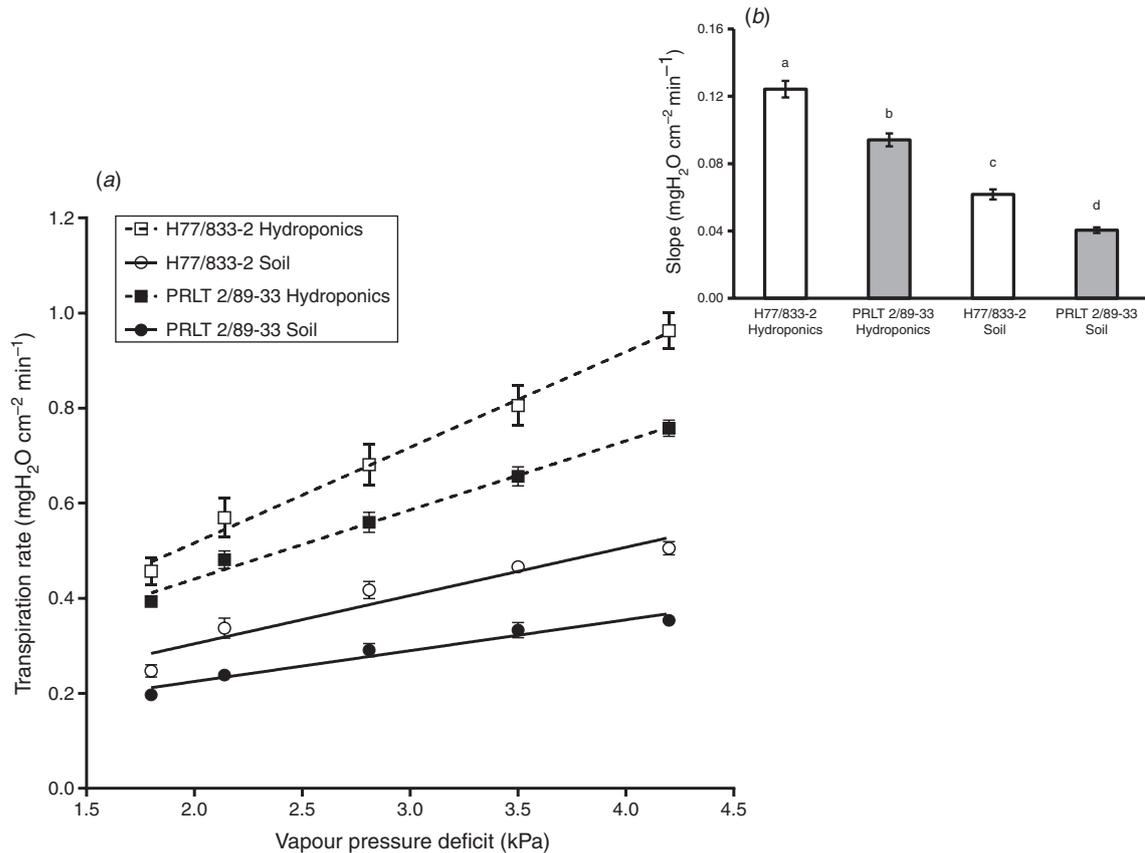
The transpiration rate under varying VPDs was assessed in detached leaves under two VPD conditions (1.27–2.65 kPa). The transpiration was stable and similar in PRLT 2/89-33 and H77/833-2 under the low VPD condition (1.27 kPa) before increasing VPD. Upon increasing VPD conditions, the transpiration of the detached leaves increased by ~30% in the first 40 min following the VPD increase and then somewhat stabilised or further slightly increased another 5–10%. Also, the transpiration of the low VPD control remained stable until ~4 h after harvesting the leaves (Fig. 4). The NTrR (normalised transpiration rate ratio) of detached leaves from both the parents, H77/833-2 and PRLT 2/89-33 showed no genotypic difference (Table 2). The transpiration rate

**Table 2. Means and two-way ANOVA for traits represented in Figs 2–8 (excluding 7a – one-way ANOVA)**  
Mean values followed by different alphabets indicate significant differences obtained through Tukey's test; d.f., degrees of freedom; l.s.d., least significant difference; G × T, genotype by treatment interaction

Fig. no.	d.f.	Genotype/treatment	Mean	l.s.d.	P-value	G × T
Fig. 1	1	H77/833-2	0.03a	0.004	<0.001	Non-significant
		PRLT 2/89-33	0.02b			
	1	De-rooted shoot	0.03a			
Fig. 2		Whole plant	0.02b	0.011	<0.001	Non-significant
	1	H77/833-2	0.11a			
	1	PRLT 2/89-33	0.06b			
Fig. 3a		Hydroponics	0.12a	0.012	<0.001	
		Soil	0.06b			
	1	H77/833-2	21.66a			
Fig. 3b		PRLT 2/89-33	23.24a	4.790	0.5	Non-significant
	1	Control	29.47a			
		AgNO <sub>3</sub>	15.42b			
Fig. 4		H77/833-2	09.89a	2.950	0.52	Non-significant
		PRLT 2/89-33	08.98a			
	1	Control	12.59a			
Fig. 5		Apoplast inhibition	06.28b	2.95	<0.001	
	1	H77/833-2	0.013a			
	1	PRLT 2/89-33	0.007b			
Fig. 6a		30°C/70% RH	0.009b	0.001	<0.05	
		35°C/53% RH	0.011a			
	1	H77/833-2	0.73a			
Fig. 6b		PRLT 2/89-33	0.69a	0.0454	0.10	Significant
	2	Control	1.00a			
		CuSO <sub>4</sub>	0.68b			
Fig. 7a		K <sub>4</sub> (Fe(CN) <sub>6</sub> ) and CuSO <sub>4</sub>	0.44c	0.0556	<0.001	
	1	H77/833-2	0.89b			
	1	PRLT 2/89-33	0.98a			
Fig. 7b		Control	1.00a	0.06	<0.05	Significant
		AgNO <sub>3</sub>	0.91b			
	1	H77/833-2	0.80a			
Fig. 7c		PRLT 2/89-33	0.61b	0.06	<0.001	Significant
	1	Control	1.00a			
		Apoplast inhibition	0.52b			
Fig. 8a		H77/833-2	0.07a	0.01	<0.001	
		PRLT 2/89-33	0.05b			
	1	H77/833-2	0.77a			
Fig. 8b		PRLT 2/89-33	0.72b	0.03	<0.001	Non-significant
	2	Control	1.00a			
		H <sub>2</sub> O <sub>2</sub>	0.83b			
Fig. 8c		Apoplast inhibition	0.40c	0.04	<0.001	
	1	H77/833-2	0.89a			
	1	PRLT 2/89-33	0.84a			
Fig. 8d		Control	1.01a	0.06	0.11	Significant
	2	H <sub>2</sub> O <sub>2</sub>	0.96b			
		Apoplast inhibition	0.63c			
Fig. 8e		H77/833-2	1.07a	0.08	<0.05	Significant
		PRLT 2/89-33	0.95b			
	1	Control	1.00a			
Fig. 8f		H <sub>2</sub> O <sub>2</sub>	1.01a	0.09	0.79	
	1	H77/833-2	0.83b			
	1	PRLT 2/89-33	0.93a			
Fig. 8g		control	1.00a	0.10	<0.001	
		AgNO <sub>3</sub>	0.79b			

**Table 3. Regression results for the comparison of vapour pressure deficit (VPD) response tested in whole plants and de-rooted shoots of H77/833-2 and PRLT 2/89-33**Details include mean transpiration rate (Tr), slope values, VPD breakpoint and R<sup>2</sup> values

Genotype	Tr ± s.e.	Slope 1 ± s.e.	Breakpoint (kPa)	Slope 2 ± s.e.	R <sup>2</sup>
PRLT 2/89-33 whole plant	0.145 ± 0.004	0.035 ± 0.006	1.8	0.021 ± 0.003	0.95
PRLT 2/89-33 de-rooted shoot	0.163 ± 0.006	0.045 ± 0.003	Linear	–	0.97
H77/833-2 whole plant	0.185 ± 0.007	0.052 ± 0.004	Linear	–	0.97
H77/833-2 de-rooted shoot	0.219 ± 0.008	0.068 ± 0.001	Linear	–	0.99



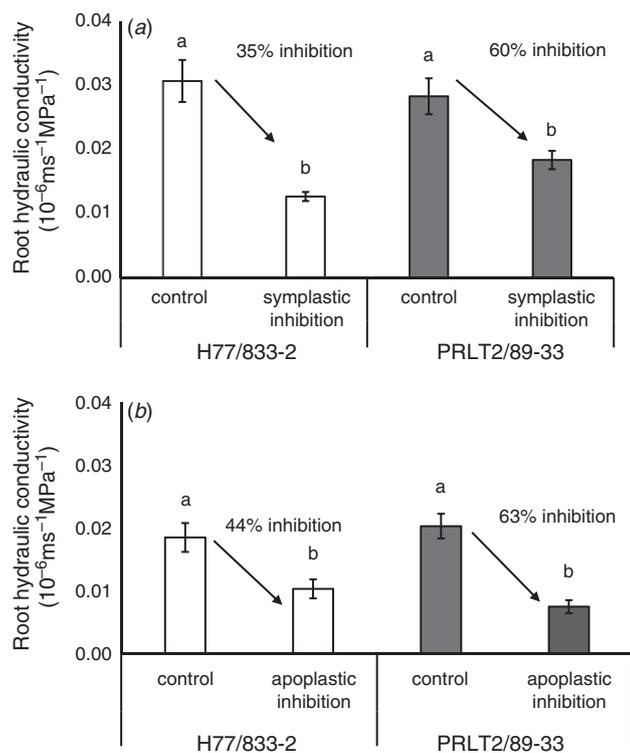
**Fig. 2.** Comparison of vapour pressure deficit (VPD) response in whole plants of (a) of H77/833-2 (high Tr, open symbols) and PRLT 2/89-33 (low Tr closed symbols) grown in soil (solid line) and hydroponic systems (dashed line). Each point is the mean of Tr values ( $n=8$ ) error bars  $\pm$  s.e. of mean. (b) Insert represents the slope values of H77/833-2 (open bar) and PRLT 2/89-33 (closed bar) from both soil and hydroponic systems. Bars with different letters are significantly different ( $P < 0.05$ , Tukey-Kramer test). Experiment was done in growth chamber at vegetative stage.

absolute values at 2.65 kPa also showed no significant genotypic differences (Fig. S6).

#### Transpiration response to CuSO<sub>4</sub> and to apoplastic blockage treatments

Though K<sub>4</sub>(Fe(CN)<sub>6</sub>) and CuSO<sub>4</sub> have been reported to inhibit the apoplastic pathway, there have been reports on AQP inhibition by Cu alone (Ligaba *et al.* 2011). The effect of CuSO<sub>4</sub> on plant transpiration was then compared with its combined effect with K<sub>4</sub>(Fe(CN)<sub>6</sub>). When 0.5 mM CuSO<sub>4</sub> alone was applied, transpiration decreased more in H77/833-2

than in PRLT 2/89-33, i.e. ~25% in PRLT 2/89-33 and 40% in H77/833-2. In contrast, when 0.5 mM CuSO<sub>4</sub> was applied and following prior K<sub>4</sub>(Fe(CN)<sub>6</sub>) infusion, transpiration decreased more in PRLT 2/89-33 than in H77/833-2 (Fig. 5), i.e. ~60% in PRLT 2/89-33 and 40% in H77/833-2. Since CuSO<sub>4</sub> alone decreased transpiration in both genotype, a crude estimate of the degree of transpiration inhibition by the apoplastic blockage would then be the differences in inhibition by CuSO<sub>4</sub> alone from the inhibition by CuSO<sub>4</sub> + K<sub>4</sub>(Fe(CN)<sub>6</sub>). This would indicate that PRLT 2/89-33 had a higher degree of transpiration inhibition from the apoplastic blockage and H77/833-2 a higher degree of transpiration inhibition from the AQP-mediated



**Fig. 3.** Hydraulic conductivity of de-topped roots of hydroponic plants grown in the glasshouse at vegetative stage, quantified after (a) symplastic inhibition (with 400  $\mu\text{M}$   $\text{AgNO}_3$ ) and (b) apoplastic inhibition (with 1 mM  $\text{K}_4(\text{Fe}(\text{CN})_6)$  and 0.5 mM  $\text{CuSO}_4$ ) in H77/833-2 (high Tr, open bar) and PRLT 2/89-33 (low Tr, closed bar). Hydraulic conductivity was also measured in non-treated control plants. Error bars at each column indicate  $\pm$  s.e. of the mean ( $n=5$ ). Bars with different letters are significantly different ( $P<0.05$ ; Tukey-Kramer test).

pathway. Recovery with deionised  $\text{H}_2\text{O}$  showed that H77/833-2 treated with 0.5 mM  $\text{CuSO}_4$  was able to recover its transpiration slightly. Anatomical sections of root segments taken from H77/833-2 and PRLT 2/89-33 did not show any visible evidence of precipitates in extracellular spaces following treatment with  $\text{K}_4(\text{Fe}(\text{CN})_6)$  and  $\text{CuSO}_4$  as reported previously (Ranathunge et al. 2005) (Fig. S5).

#### Transpiration response to aquaporin inhibitor or apoplast blockage treatments in whole plants under low VPD

A comparison of the transpiration response to the inhibitors treatment of the AQP (400  $\mu\text{M}$   $\text{AgNO}_3$ ) and of the apoplastic pathway (1 mM  $\text{K}_4(\text{Fe}(\text{CN})_6)$  + 0.5 mM  $\text{CuSO}_4$ ) was conducted under a constant VPD regime (1.8 kPa). Transpiration of high Tr genotype H77/833-2 was decreased by  $\sim 20\%$  by  $\text{AgNO}_3$ , whereas transpiration of low Tr PRLT 2/89-33 was not significantly affected by  $\text{AgNO}_3$  (Fig. 6a). The genotypic differences for the inhibition treatment were statistically significant (Table 2; Fig. 6a). Overall, the blockage of the apoplast resulted in a larger inhibition of transpiration than the inhibition of the AQP-mediated water transport pathway (Fig. 6; Table 2). The blockage of the apoplastic pathway resulted in more transpiration inhibition in the low Tr genotype

PRLT 2/89-33 (60%) than in the high Tr genotype H77/833-2 (40%) (Table 2) (Fig. 7b) in agreement with Fig. 5.

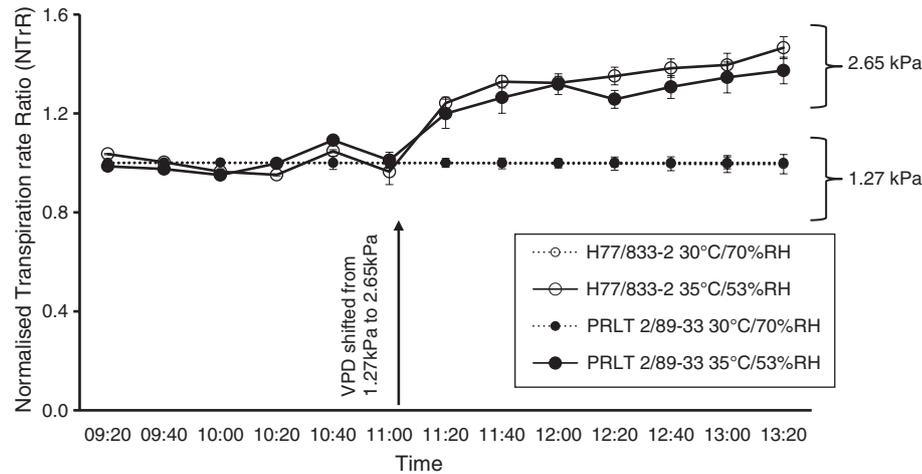
#### Transpiration response to aquaporin inhibitor or apoplast blockage treatments in whole plants under high VPD

The effect of symplast and apoplast inhibition was also tested at the end of an ascending series of VPDs. First, the transpiration response to increasing VPD confirmed the lower Tr of low Tr PRLT 2/89/33 than high Tr H77/833-2 (lower slope; Fig. 7a). At this stage, under high VPD (4.2 kPa), the inhibitors were applied. Again, the apoplastic blockage resulted in a larger inhibition of transpiration than the inhibition of the AQP-mediated pathway (Fig. 7b), and the decrease in the transpiration of PRLT 2/89-33 was comparatively higher than in H77/833-2 (Table 2). However, the AQP inhibition (1.5 mM  $\text{H}_2\text{O}_2$ ) at the end of this ascending series of VPDs (4.2 kPa) differed from the inhibition pattern at low VPD (Fig. 7a). Indeed, the transpiration of PRLT 2/89-33, this time under high VPD, declined more than in H77/833-2 (Fig. 7b; Table 2;  $P<0.001$ ).

A comparison of the transpiration response to the inhibitors treatment of the AQP (1.5 mM  $\text{H}_2\text{O}_2$ ) and of the apoplastic pathway (1 mM  $\text{K}_4(\text{Fe}(\text{CN})_6)$  + 0.5 mM  $\text{CuSO}_4$ ) was also conducted in plants exposed initially to low VPD (1.27 kPa) and where the VPD was increased up to 2.97 kPa during the exposure to the aquaporin inhibitor. Here also, the blockage of the apoplastic pathway led to a larger decrease of the transpiration than the inhibition of the AQP-mediated water transport pathway (Fig. 7c). When the apoplastic pathway was blocked the normalised transpiration rate (NTrR) of low Tr PRLT 2/89-33 dropped significantly more than the high Tr H77/833-2 and the level of apoplastic inhibition were typically between 40 and 50% (Fig. 7c). By contrast, the AQP inhibition by 1.5 mM  $\text{H}_2\text{O}_2$  showed a significantly higher NTrR decline in H77/833-2 than in PRLT 2/89-33, although the level of inhibition was limited to  $\sim 10$ –15% (Fig. 7c).

#### Transpiration response to aquaporin inhibitors treatment in detached leaves

Although the apoplast blockage treatment was targeted to the root, the aquaporin inhibitor treatment could have affected the aquaporins both in the roots and in the leaves. Therefore, the effect of aquaporin inhibitors was tested on the transpiration of detached leaves. The transpiration of detached leaves did not respond to 1.5 mM  $\text{H}_2\text{O}_2$  and even slightly increased in H77/833-2 (Fig. 8a). This contrasted with the whole-plant transpiration response to 1.5 mM  $\text{H}_2\text{O}_2$  treatment applied to the roots of whole plants (Fig. 7c). Similarly, different concentrations of  $\text{AgNO}_3$  ranging from 50 to 800  $\mu\text{M}$  were tested. With low concentration (50  $\mu\text{M}$ ) there was no drop in transpiration (data not shown) and from 400  $\mu\text{M}$  and above concentrations an inhibition in transpiration and genotypic difference was observed. In contrast to  $\text{H}_2\text{O}_2$ , treatment with 400  $\mu\text{M}$   $\text{AgNO}_3$  treatment of detached leaves resulted in a significant decline in the transpiration of H77/833-2 ( $\sim 25\%$ ), whereas the transpiration of low Tr PRLT 2/89-33 either did not decrease in the first 90 min after treatment and decreased only by  $\sim 10\%$  in the following 60 min (Fig. 8b; genotype effect,  $P<0.05$ , Tukey's test; Table 2).



**Fig. 4.** Transpiration rate response in detached leaves from plants grown in soil and tested in growth chamber at vegetative stage. Transpiration data were double normalised, by the mean  $Tr$  of the untreated control first, and then to the initial mean  $Tr$  ratio before increasing vapour pressure deficit (VPD) from 1.27 to 2.65 kPa in H77/833-2 (high  $Tr$ , open circle) and PRLT 2/89/33 (low  $Tr$ , closed circle) tested for VPD response. The solid lines represent NTrR of the increased VPD treatment; the dotted lines represent the NTrR of the untreated control leaves. Each point is the mean of NTrR ( $n=9$ ) and error bar at each time indicates  $\pm$  s.e. of the mean.

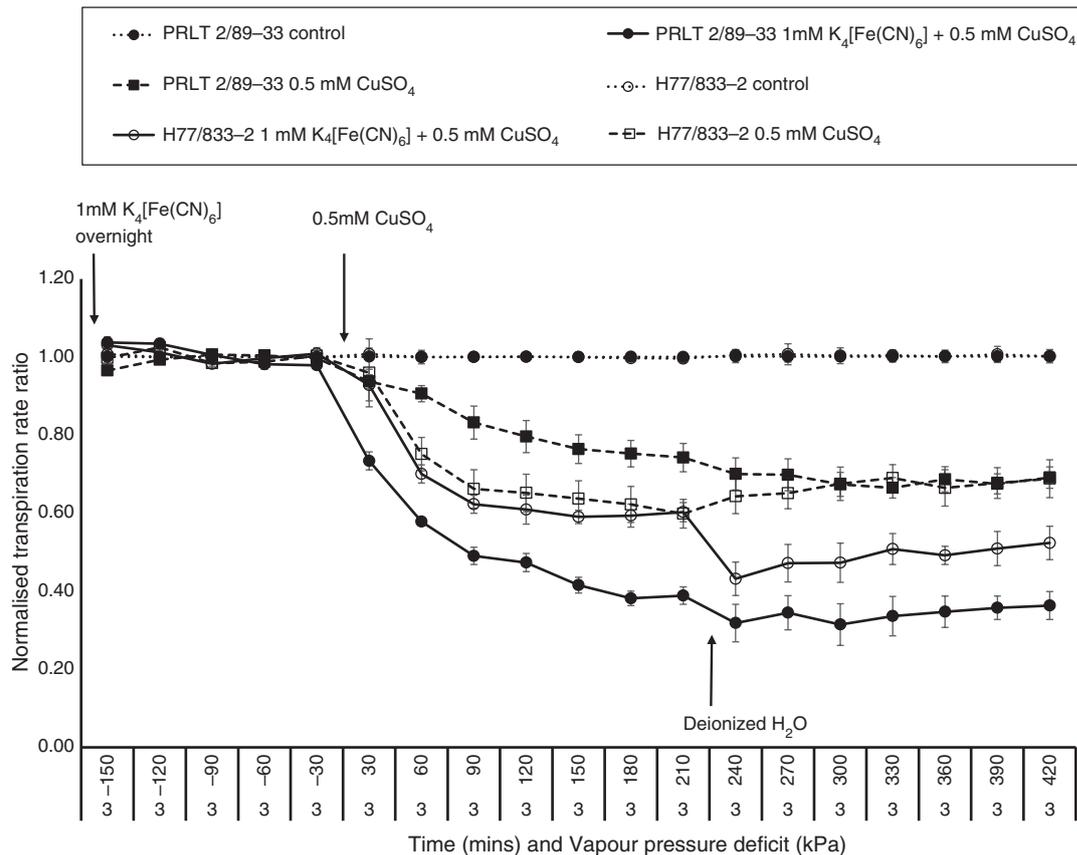
#### Root hydraulic conductance following apoplast or symplast inhibitors treatments

Following the above responses of transpiration to apoplast or symplast inhibitors, the root hydraulic conductivity was measured following various treatments (untreated, apoplastic and AQP inhibition). In the case of root systems treated with inhibitors of either the AQP-mediated or apoplastic pathways for water transport, using the same method as in the case of whole plants inhibitions, there was a clear decrease in the root hydraulic conductivity ( $\text{ms}^{-1} \text{MPa}^{-1}$ ) compared with untreated plants in both the genotypes (Figs 2, 3a; Table 2). Upon the symplast inhibitor treatment, there was a 30–60% decrease in the hydraulic conductivity (Fig. 3a). Upon blockage of the apoplast, the root hydraulic conductivity dropped by ~44–63% (Fig. 3b). The decrease in the root hydraulic conductivity was consistent with the inhibition of the transpiration, i.e. there was a larger root conductivity decrease upon aquaporin inhibitor treatment in H77/833-2 than in PRLT 2/89-33 and, conversely, there was a larger root conductivity decrease upon apoplast blockage in PRLT 2/89-33 than in H77/833-2.

#### Discussion

The main results of these experiments were that: (i) roots limited the transpiration rate in the tested genotypes of pearl millet at all VPD levels and more so in low  $Tr$  PRLT 2/89/33 above 2 kPa; (ii) there was genotypic and environmental variation in the degree to which transpiration responded to the application of a AQP inhibitor or to a blocker of the apoplastic pathway, where high  $Tr$  H77/833-2 suffered AQP inhibition most, except when exposed to high VPD conditions, whereas low  $Tr$  genotype PRLT 2/89-33 suffered apoplastic blockage most; (iii) the transpiration of detached leaves with  $\text{AgNO}_3$  gave a genotypic

pattern of inhibition similar to the whole plants, with a larger inhibition in high  $Tr$  H77/833-2 than in PRLT 2/89-33; and (iv) root hydraulic conductivity did not differ in the two genotypes under untreated conditions. However, there was a larger decrease in root hydraulic conductivity of high  $Tr$  H77/833-2 upon aquaporin inhibitor treatment, and a larger decrease in root hydraulic conductivity of low  $Tr$  PRLT 2/89-33 upon apoplastic blockage. Based on these results, it could be concluded that roots limited water flow to support transpiration under high VPD, although there was still a degree of water flow limitation in the shoot based on the transpiration differences of de-rooted shoots of the two genotypes. Our running hypothesis is that PRLT 2/89-33 depended more on the apoplastic pathway for water movement, whereas H77/833-2 depended on both the apoplastic and symplastic pathway, and probably predominantly on the symplastic pathway. This might explain the lower  $Tr$  capacity of PRLT 2/89-33, especially under high VPD, the apoplast being a rather rigid space between the cell walls with little means to increase the conductivity of this pathway (although see Cuneo *et al.* 2016). In contrast, H77/833-2 had a higher  $Tr$  capacity, especially under high VPD by compensating/adapting via the symplastic pathway, possibly via aquaporin upregulation (Kudoyarova *et al.* 2011). Therefore, so long as the evaporative demand was low, there was no limitation to the water movement in the leaves to fully support transpiration demand. Under higher evaporative demand, there was a need for more water movement to support transpiration and this added demand might have been facilitated by aquaporins, thereby the inhibition effect on transpiration from adding the aquaporin inhibitor. These results need to consider that high VPD was achieved by a combination of higher temperature and lower RH %, which then does not exclude a temperature effect on the responses, independently of the VPD effect that was the target.

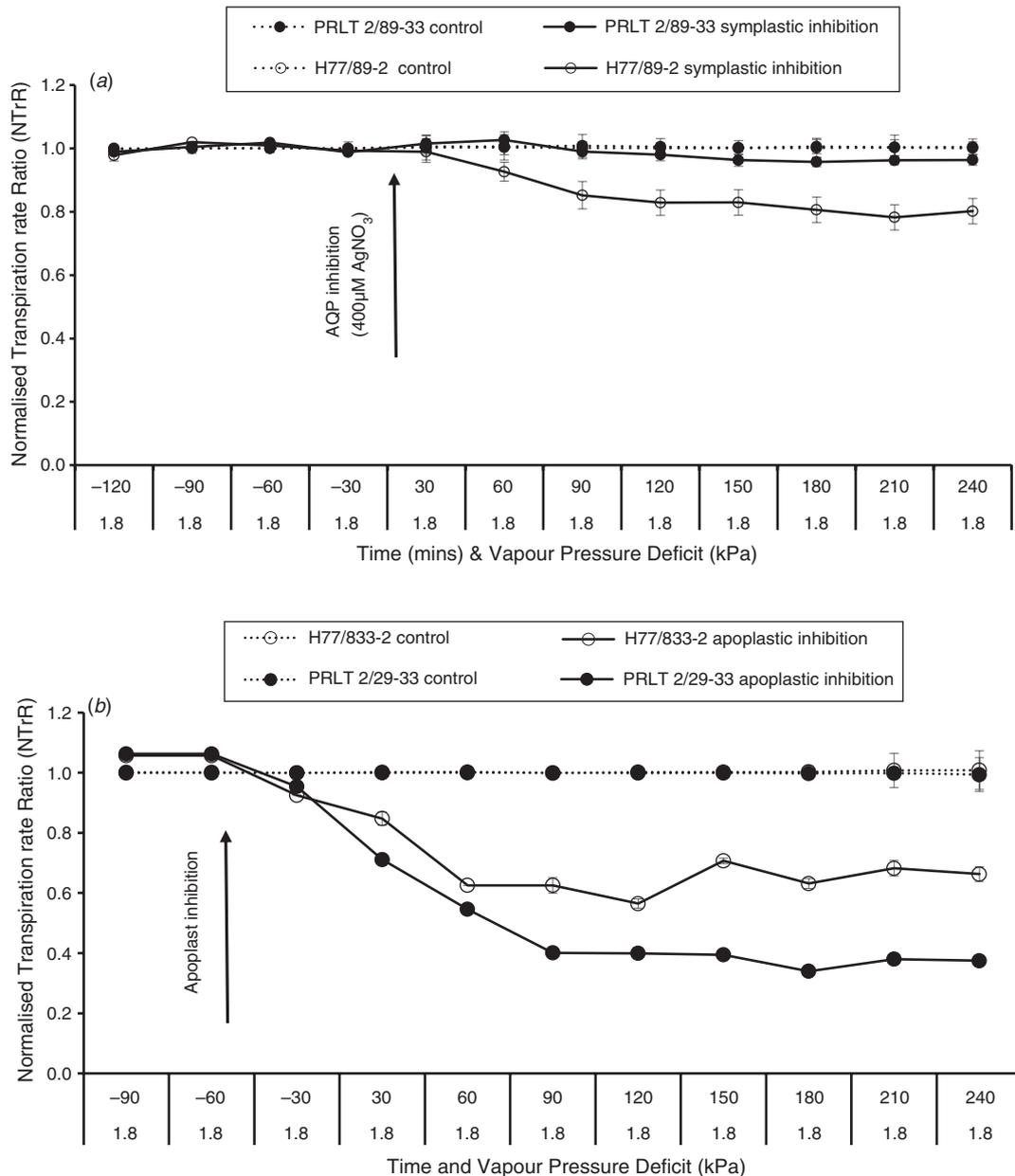


**Fig. 5.** Comparison of  $CuSO_4$  effects, either on its own or in its combined effect with  $K_4[Fe(CN)_6]$  treatment on the transpiration of hydroponically grown whole plants of H77/833-2 (high  $Tr$ , open symbols) and PRLT 2/89-33 (low  $Tr$ , closed symbols). Transpiration inhibition recovery was tested by replacing  $CuSO_4$  with deionized  $H_2O$ . The dotted line represents the response of untreated control plants; dashed lines represent the plants treated with 0.5 mM  $CuSO_4$  alone and the solid lines represent the response of treated plants with initially 1 mM  $K_4[Fe(CN)_6]$  and then with 0.5 mM  $CuSO_4$  and exposed to constant vapour pressure deficit (VPD) (2 kPa). Each point is the mean of NTRR values ( $n=5$ ) and the error bars indicate  $\pm$  s.e. of the mean. The values immediately below the x-axis represent the time series and values further below represent the VPD. The timings with negative symbols refer those before inhibitor application and the timings with positive symbols refer to those after inhibitor application. The experiment was conducted in a growth chamber at vegetative stage.

#### Possible caveats of the study

Experimental data obtained with non-specific inhibitors need to be analysed and interpreted with care. This work has followed the approach of a fairly large number of studies that have used a 'pharmacological' way to inhibit different components of the water transport pathways, where assumptions have been made on the inhibitors (e.g. Zhang and Thermal 1999; Ranathunge *et al.* 2005; Sadok and Sinclair 2010b; Knipfer *et al.* 2011; Devi *et al.* 2012; Choudhary *et al.* 2013). However, the following potential caveats need to be taken into account when interpreting the results: (i) the inhibitors could have been absorbed at different rates leading to differences in the degree of inhibition; (ii) although the recovery of transpiration activity was tested upon removal of the inhibitors in the solution medium, none of the inhibitor tested was specific to the aquaporin and effects on other part of the plant metabolism cannot be excluded; (iii) the molecules involved in the apoplastic blockages could have also penetrated in the roots at different rates between genotypes;

(iv) temperature increases to induce higher VPD conditions could have altered the rate of inhibitor penetration and then the degree of inhibition, whereas lower temperatures could have been limiting their penetration; and finally (v), it was difficult to distinguish the effect of the putative apoplastic blockage from that of an effect on the AQP-mediated pathway since Cu itself decreased transpiration and are known to inhibit the AQP-mediated water transport pathway (Ligaba *et al.* 2011). In parallel work we have shown that blockers of the apoplastic pathway were indeed absorbed and a precipitate was visible in the apoplastic pathway (Sivasakthi *et al.* 2017). Though the precipitates of apoplast blockers were not visible here the combined effect of  $K_4[Fe(CN)_6]$  and  $CuSO_4$  reduced the transpiration of PRLT-2/89-33 more than the effect of  $CuSO_4$  alone, whereas the transpiration of H77/833-2 suffered only slightly more from the addition of  $K_4[Fe(CN)_6]$  after Cu, indicating that the method was still valid to compare dependence on the apoplast and AQP-mediated pathways.

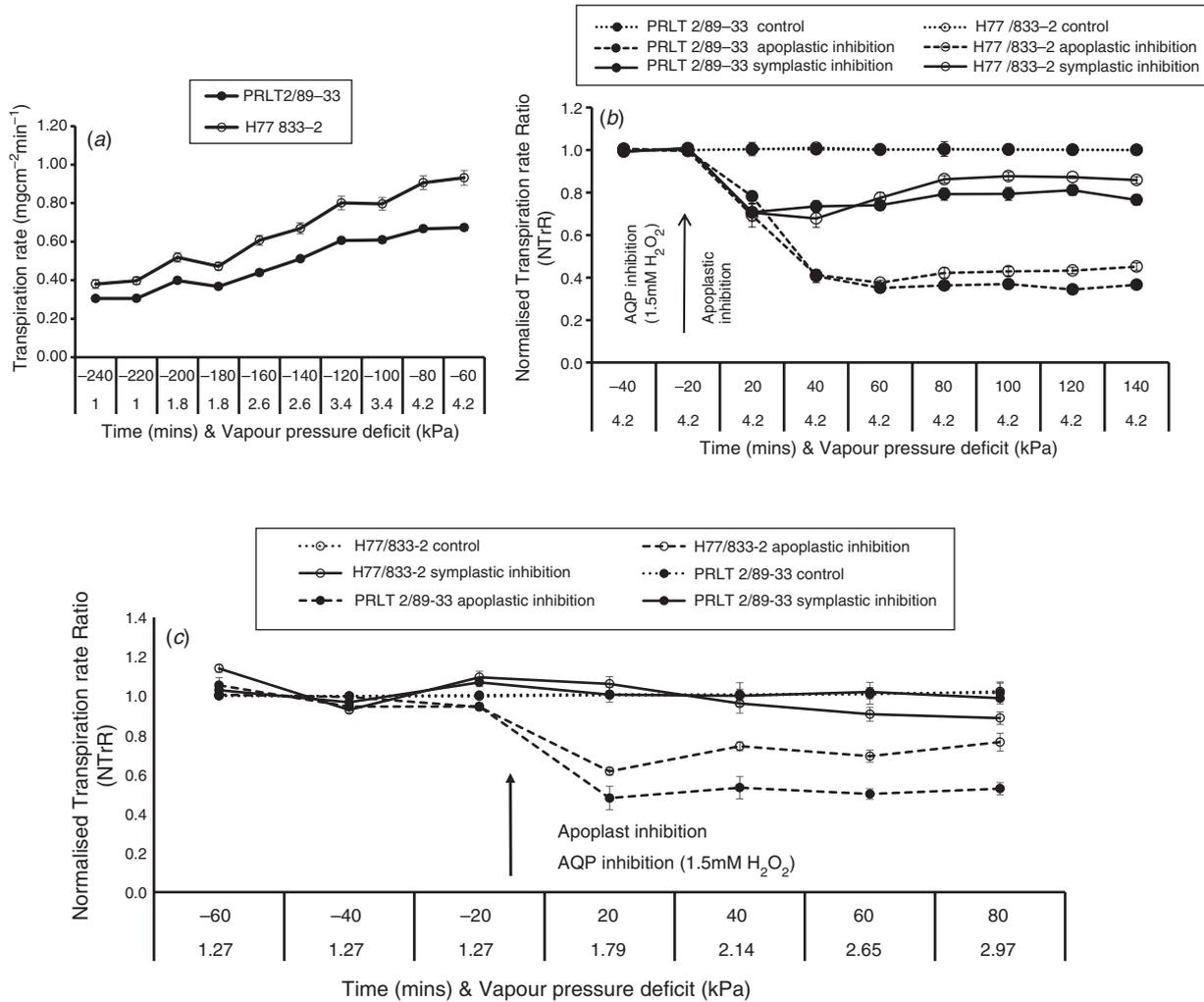


**Fig. 6.** Transpiration rate decline (double normalised by the mean Tr of untreated control and to the mean Tr ratio before the inhibitor application) in hydroponically grown whole plants of H77/833-2 (high Tr, open circle) and PRLT 2/89-33 (low Tr, closed circle) after exposure to (a) symplastic inhibition (with 400  $\mu\text{M}$   $\text{AgNO}_3$ ) and (b) apoplastic blockage (with 1 mM  $\text{K}_4(\text{Fe}(\text{CN})_6) + 0.5$  mM  $\text{CuSO}_4$ ) at constant low VPD (1.8 kPa). The dotted lines represent the NTrR of untreated control plants. Each point is the mean of NTrR ( $n=5$ ) and the error bars indicate  $\pm$  s.e. of the mean. The values immediately below the x-axis represent the time series and the values further below represent the vapour pressure deficits. The timings with negative symbols refer to those before inhibitor application and the timings with positive symbols refer to those after inhibitor application. The experiment was conducted in a growth chamber at the vegetative stage.

### Roots limit the transpiration rate

Although the de-rooting treatment may not provide an absolute test to assess the location of a putative hydraulic limitation in the plant, the increase in the transpiration rates and in the slope of the Tr response to VPD provide strong evidence that roots are a substantial limitation to water flow in both the genotypes

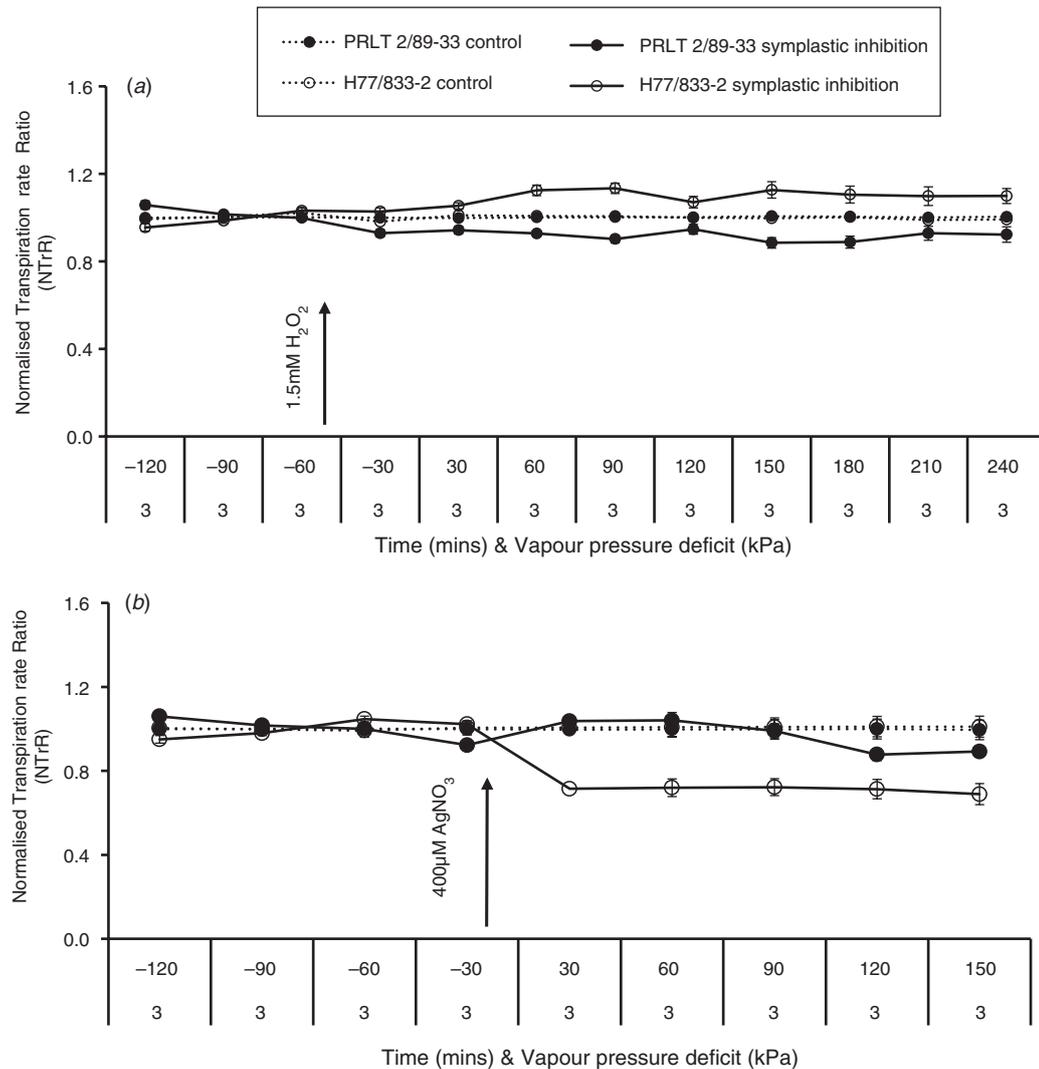
of pearl millet. The Casparian bands in the apoplast of root endodermal cells have deposits of suberin and lignin and this could represent a barrier for water transport (Steudle and Peterson 1998; Zimmermann and Steudle 1998; Steudle 2000b). Similar results have been reported in wheat (Manschadi *et al.* 2006; Bramley *et al.* 2009; Schoppach *et al.* 2014) and chickpea



**Fig. 7.** (a) Transpiration response in hydroponically grown whole plants of H77/833-2 (high Tr, open circle) and PRLT 2/89-33 (low Tr, closed circle) to (a) an ascending series of vapour pressure deficits (VPDs) and subsequent responses to (b) symplastic and apoplastic inhibition at the end of an ascending series of VPDs (high VPD 4.2 kPa). A separate experiment (c) shows the transpiration response of H77/833-2 (high Tr, open circle) and PRLT-2/89-33 (low Tr, closed circle) to AQP and apoplastic inhibition at the beginning of an ascending series of VPDs (starting at 1.27 kPa and ending at 2.97 kPa). The transpiration decline are represented as NTrR (double normalised, first by the mean Tr of the untreated control and then by the mean Tr ratio before the inhibitor application), after exposure to apoplastic blocker (dashed line indicates 1 mM  $K_4(Fe(CN)_6) + 0.5$  mM  $CuSO_4$ ) and symplastic inhibitor treatment (solid line indicates 1.5 mM  $H_2O_2$ ). The dotted lines represent the NTrR of the untreated control plants. Each point is the mean of NTrR ( $n = 5$ ) and the error bars indicate  $\pm$  s.e. of the mean. The values immediately below the x-axis represent the time series and values further below represent the VPD. The timings with negative symbols refer to those before inhibitor application and the timings with positive symbols refer to those after inhibitor application. The experiment was conducted in a growth chamber during the vegetative stage.

(K Sivasakhti, unpubl. data). However, in soybean (Sinclair *et al.* 2008) and sorghum (Choudhary *et al.* 2013) it has been reported that the leaves were the major site of hydraulic limitation. Although the change in the slope of the Tr response to VPD following de-rooting was higher in PRLT, the absence of root hydraulic conductivity differences, measured under low VPD conditions, between the two genotypes suggests the differences between the two genotypes could be related partially to the root surface area (Figs 7a, b, S5). The root surface area of PRLT was indeed significantly higher than in H77. The fact that even in de-rooted shoot there was still a lower transpiration rate in PRLT-2/89-33 than in H77/833-2 also suggests that there

could remain some limitation to water flow in the shoot of PRLT, despite our results with detached leaves implying no Tr capacity difference in the shoot between genotypes. Taken together, our results show that the root at least was an important source of limitation to transpiration in both PRLT-2/89-33 and H77/833-2. However, the limitation was greater in low Tr PRLT. More work would be needed to assess the hydraulic conductivity of the shoot and leaves. Although no root hydraulic measurements were made from plants exposed to high VPD condition, we hypothesise that there could have been an increase in the root hydraulic conductivity in high Tr H77/833-2. Further research is needed to understand the



**Fig. 8.** Transpiration rate response in detached leaves (double normalised, first by the mean Tr of the untreated control and then by the initial mean Tr ratio before the inhibition) of H77/833-2 (high Tr, open circle) and PRLT 2/89/33 (low Tr, closed circle) after symplastic inhibition by exposure to (a) 1.5 mM H<sub>2</sub>O<sub>2</sub> and (b) 400 μM AgNO<sub>3</sub>. The solid lines represent the NTrR response of inhibited plants and the dotted lines represent the NTrR of untreated control plants. Each point is the mean of NTrR ( $n = 9$ ) and error bar at each time point indicates  $\pm$  s.e. of the mean. The values immediately below the x-axis represent the time series and values further below represent the vapour pressure deficit. The timings with negative symbols refer to those before inhibitor application and the timings with positive symbols refer to those after inhibitor application. Plants were grown in soil and tested in a growth chamber at vegetative stage.

signalling to the stomata under high VPD in low Tr genotype, and in particular to understand whether the VPD effect comes indeed via hydraulic signal and xylem tension leading to a chemical signal conveyed to the stomata, or whether the VPD affects the stomata directly. One possible solution to this would be to assess stomatal conductance response to increases in VPD in an isolated part of the leaf, in plants that are kept otherwise under low VPD conditions, or vice versa. Earlier work also showed a higher ABA concentration in the leaves of PRLT-2/89-33 (Kholová *et al.* 2010b). This adds to the finding that the restoration of a higher root hydraulic conductance of wheat roots following an increase in evaporative demand was related to an increase in the root ABA, itself arising from a re-allocation of ABA from the shoot

(Kudoyarova *et al.* 2011). Therefore, the exact role of ABA in increasing root hydraulic conductivity, which has been shown earlier (Thompson *et al.* 2007; Parent *et al.* 2009), needs more research work.

#### Transpiration response to VPD in plants grown in different cultivation systems

Experiment 2 aimed to test whether root-growing conditions could alter the transpiration response to an increase in VPD. This was an important step because the use of inhibitor of water transport pathways is done in a hydroponic system, and it was critical that it be done in a system that also discriminated

genotypes for the transpiration response to high VPD. Results showed that variation in Tr between genotypes was consistent with both systems, so that both hydroponic and soil systems could be used for investigating differences in the plant hydraulic features. The fact that Tr was higher in hydroponics than in soil also suggests that the restriction to transpiration water flow took place at the root–soil interface. Hydroponically grown plants are reported to not fully develop apoplastic barriers, i.e. Casparian bands in the hypodermis and endodermis (Peterson 1988; Zimmermann and Steudle 1998; Freundl *et al.* 2000) but when grown in soil they do develop Casparian bands (Perumalla *et al.* 1990; Damus *et al.* 1997; Enstone and Peterson 1998), and this has consequences for the hydraulic conductivity of the root system. Differences in the root anatomical features between soil-grown and hydroponic-grown plants could have then affected the response to VPD or to the inhibitors. The higher transpiration rate in hydroponics than in soil could also be caused by a smaller leaf area development in hydroponic plants. The absence of a VPD threshold breakpoint in the transpiration response of PRLT-2/89-33 could relate to the fact that the Tr response started at a fairly high VPD (1.8 kPa), whereas the VPD breakpoint response was earlier reported in the range from 1.40 to 1.90 kPa in soil grown PRLT-2/89-33 (Kholová *et al.* 2010b).

#### Water transport pathway

Experiments 3 and 4 were meant to provide a basic insight whether various water transport pathways that could possibly underlay the transpiration variations between genotypes on whole plant level. One of the usual interpretations is that a genotype showing significant transpiration decrease upon the application of an inhibitor might be utilising the inhibited water transport pathway comparatively more than other genotypes showing less inhibition (Sadok and Sinclair 2010b). Alternatively, a genotype maintaining transpiration after inhibition might either have AQP insensitive to the applied inhibitors (Daniels *et al.* 1994; Biela *et al.* 1999) or might be able to compensate the inhibition by enhancing AQP population (Beaudette *et al.* 2007; Tamás *et al.* 2008; Ligaba *et al.* 2011), or by having lower population of inhibitor-sensitive aquaporins (Schoppach *et al.* 2014) or by channelling more water through other water transport pathways (Morillon and Chrispeels 2001). Therefore, even though the effect of inhibitors are not fully understood and may have many side-effects on plant metabolism (Coskun *et al.* 2012), using low concentration for short-duration is one of the common protocols (Maggio and Joly 1995; Sadok and Sinclair 2010a, 2010b; Devi *et al.* 2012; Choudhary *et al.* 2013).

The fact that the inhibition differed between genotypes indicated some genotypic differences in the relative proportion of water channelled through either the AQP-mediated or apoplastic pathway. Low Tr PRLT-2/89-33 depended more on the apoplast pathway for water transport than the high Tr H77/833-2. By contrast, even the inhibition of the apoplastic pathway in the case of H77/833-2 was partially caused by Cu itself, leading to apparently only a small portion of apoplast blockage inhibition (Fig. 3). More research would be needed to confirm such trends using dyes to infer the relative proportion

of water flowing through each of the pathways (Hanson *et al.* 1985; Bárzana *et al.* 2012). Here, the apoplastic pathway might be seen as the predominant and ‘fixed’ part of water transport systems, set by the tissue architecture (i.e. long-term development of plant in particular conditions, Zimmermann and Steudle 1998; Steudle 2000b). This was confirmed by the larger hydraulic conductivity reduction in the low Tr PRLT-2/89-33. In contrast, the inhibition of transpiration by the application of aquaporin inhibitors was more in high Tr H77 under low VPD. The variable range of transpiration responses following AQP inhibition across experimental conditions (low or high VPD) and inhibitor used (AgNO<sub>3</sub> vs H<sub>2</sub>O<sub>2</sub>) also suggests that the AQP-mediated water transport pathway may be a highly flexible, dynamic part of water transport, which depends on environment changes of short- and long term (Steudle 2000a). There are most likely genotypic differences in (i) the portion of water transported through symplast; and (ii) the plasticity of symplast under the environmental fluctuations (here upon changing VPD). There were also slight differences in the response to different inhibitors, indicating that there exist different populations of aquaporins with specific sensitivities to these different inhibitors, as shown earlier (Devi *et al.* 2012). In any case, the higher transpiration inhibition in high Tr H77/833-2 than in PRLT-2/89-33 upon aquaporin inhibitor treatment along with transpiration inhibition upon apoplastic blockage suggests that high Tr H77/833-2 depends on both water transport pathways, and probably predominantly on the AQP-mediated pathway. This is in agreement with similar findings in wheat where high Tr cultivar Kukri had a higher decrease in transpiration following symplastic inhibition than low Tr cultivar RAC875 (Schoppach *et al.* 2014). This was supported by the larger root hydraulic conductivity decrease of H77/833-2 than in PRLT-2/89-33 upon AQP inhibitor treatment. The fact that H77/833-2 had higher transpiration inhibition at low VPD, but lower transpiration inhibition from the application of an aquaporin inhibitor at the end of an ascending series of VPDs allow us to speculate/hypothesise that this genotype could have upregulated the synthesis of the population of aquaporins targeted by the inhibition, and this is currently the object of additional research. Indeed, the decrease in root hydraulic conductivity of PRLT-2/89-33 following apoplastic blockage, i.e. ~60%, was within a close range of the transpiration inhibition. By contrast, the root hydraulic conductivity of H77/833-2 following aquaporin inhibition was also in the order of 60% but the transpiration decline was only 20% or so, which suggest that this genotype has means to compensate transpiration demand via other means, either upregulation of same/other populations of aquaporins, in agreement with earlier report of an increasing in the root hydraulic conductivity of wheat roots following an increase in the evaporative demand (Kudoyarova *et al.* 2011).

#### Conflicts of interest

The authors declare no conflicts of interest.

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