



Variability in temperature-independent transpiration responses to evaporative demand correlate with nighttime water use and its circadian control across diverse wheat populations

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Abstract

Main conclusion Nocturnal transpiration, through its circadian control, plays a role in modulating daytime transpiration response to increasing evaporative demand, to potentially enable drought tolerance in wheat.

Limiting plant transpiration rate (TR) in response to increasing vapor pressure deficit (VPD) has been suggested to enable drought tolerance through water conservation. However, there is very little information on the extent of diversity of TR response curves to “true” VPD (i.e., independent from temperature). Furthermore, new evidence indicate that water-saving could operate by modulating nocturnal TR (TR_N), and that this response might be coupled to daytime gas exchange. Based on 3 years of experimental data on a diverse group of 77 genotypes from 25 countries and 5 continents, a first goal of this study was to characterize the functional diversity in daytime TR responses to VPD and TR_N in wheat. A second objective was to test the hypothesis that these traits could be coupled through the circadian clock. Using a new gravimetric phenotyping platform that allowed for independent temperature and VPD control, we identified three and fourfold variation in daytime and nighttime responses, respectively. In addition, TR_N was found to be positively correlated with slopes of daytime TR responses to VPD, and we identified pre-dawn variation in TR_N that likely mediated this relationship. Furthermore, pre-dawn increase in TR_N positively correlated with the year of release among drought-tolerant Australian cultivars and with the VPD threshold at which they initiated water-saving. Overall, the study indicates a substantial diversity in TR responses to VPD that could be leveraged to enhance fitness under water-limited environments, and that TR_N and its circadian control may play an important role in the expression of water-saving.

Keywords Canopy conductance · Circadian clock · Drought tolerance · Gravimetric phenotyping · Nocturnal transpiration · Stomata conductance · Vapor pressure deficit · Wheat

Abbreviations

TR	Transpiration rate
TR _N	Nighttime transpiration rate
VPD	Vapor pressure deficit
VPD _{Th}	VPD threshold
GraPh	Gravimetric phenotyping

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Introduction

In crops, water-saving traits have promising potential to enhance tolerance to terminal, Mediterranean-type drought conditions where plants typically grow on stored soil moisture after initial rain events. By avoiding unrestricted water use early in the crop growth cycle, such traits

are hypothesized to allow for enhanced availability of soil water during grain-fill, a reproductive phase that is highly sensitive to water deficit, thereby leading to increased yield (Sinclair and Muchow 2001; Passioura 2012; Vadez et al. 2014; Sinclair et al. 2017). Among these traits, reducing canopy transpiration rate (TR) during times of the day where evaporative demand or vapor pressure deficit (VPD) exceeds a certain threshold (VPD_{Th}) has been suggested to be particularly effective in the expression of water-saving (e.g., Sinclair et al. 2005, 2010; Messina et al. 2015).

In wheat, there is evidence suggesting that such water-saving stems from lower root hydraulic conductance arising from smaller metaxylem vessels in the seminal roots, coupled with a radial limitation to water flow putatively mediated by root aquaporins (AQP) and hormonal regulation (Richards and Passioura 1989; Bramley et al. 2009; Schoppach et al. 2014a; Sadok and Schoppach 2019). Given the impracticality of characterizing root hydraulic conductances and any of their proxy traits (e.g., tissue anatomy, AQPs regulation, hormonal status) at a high-throughput level, screening of TR response curves to VPD has been the focus of several pre-breeding research programs including soybean (Sadok and Sinclair 2010), maize (Ryan et al. 2016), cowpea (Belko et al. 2013), pearl millet (Kholová et al. 2016) and wheat (Schoppach et al. 2016). One limiting factor of these approaches is their restricted ability to characterize TR response to VPD independent of substantial co-variation in temperature (with ranges up to 4–45 °C). Particularly, when it comes to dissecting the physiological and genetic basis of water-saving, uncoupling those effects is critical as temperature has been shown to non-linearly interact with TR sensitivity to VPD in a genotype-dependent manner (Yang et al. 2012; Shekoofa et al. 2016). Such effects are likely the result of temperature-induced changes in leaf or root hydraulic conductances through effects on AQPs and/or leaf–root hormonal signaling (e.g., Kudoyarova et al. 2011; Kuwagata et al. 2012).

Recently, Schoppach et al. (2017) documented for the first time on wheat TR response to “true” VPD, that is, response curves that were characterized at a strictly constant temperature of 30 °C. Interestingly, such approach enabled the detection of subtle, year-of-release-dependent differences among south-Australian cultivars released between 1890 and 2008 and the existence of a water-saving TR response to VPD, consisting in a limitation of TR over a VPD threshold (VPD_{Th}) of around 2 kPa for all those lines. This contrasts with previous efforts on wheat where such a response was not found in Australian wheat using experimental setups that did not control for co-variation in temperature (Schoppach and Sadok 2012).

Another major bottleneck to phenotyping and examining the drought tolerance potential arising from temperature-independent TR responses to VPD is the lack of diversity

of germplasm screened so far (e.g., Sinclair et al. 2017). For instance, there is currently little knowledge available about the variability of these responses in wheat, outside of the genotypes from south-Australia. This has important implications since if most genotypes from across the globe tend to express the same response as the Australian lines in Schoppach et al. (2017), then it would be difficult to ascribe drought tolerance necessarily to a limitation in TR response to VPD.

A first goal of this investigation was, therefore, to characterize TR response curves to increasing VPD in a temperature-independent fashion (30 °C) for 2 groups totaling 54 wheat genotypes that were selected to contrast with the group of 23 lines examined in Schoppach et al. (2017). One group consisted in 29 lines from a public breeding program which delivers cultivars adapted to the well-watered environment of Minnesota. A second group was a worldwide diversity panel consisting of 25 landraces assembled from 23 countries and 5 continents. To this end, we used the recently developed Graph platform, a controlled environment phenotyping system which enables to automatically track whole-plant water use under conditions where VPD and temperature could be manipulated independently in the 1–3.3 VPD range (Tamang and Sadok 2018). The platform was designed to enable a high density of datapoints in that range, to facilitate the detection of a change in the slope of TR response to VPD, which is indicative of a water-saving response (i.e., VPD-sensitivity).

In addition to TR sensitivity to daytime VPD, there is evidence suggesting that nocturnal transpiration rate (TR_N) may play a role in modulating drought tolerance in crop plants, potentially via nighttime water-saving mechanisms that maximize water use efficiency (e.g., Coupel-Ledru et al. 2016; Claverie et al. 2018; Rosas-Anderson et al. 2018). On wheat, Rawson and Clarke (1989) theorized that under the drought-prone south-Australian conditions, such nocturnal water losses could amount to over 0.5 mm of evapotranspiration in the field for unstressed plants, based on direct measurements of TR_N on 4 wheat genotypes, suggesting breeding for “thrifty” genotypes. Building on this idea, Schoppach et al. (2014b) confirmed the existence of significant genotypic variability in TR_N in modern wheat and its dependence on nighttime VPD, revealing that a notoriously drought-tolerant Australian cultivar (RAC875) also exhibited the lowest TR_N . In a follow-up study, Claverie et al. (2018) found that under soil water deficit, TR_N could represent an increasing fraction of crop daily water loss. Interestingly, the same genotype (RAC875) was found to significantly limit TR_N in response to soil drying under high nocturnal VPD, in comparison to a check, drought-sensitive cultivar.

Recent evidence suggests that the daytime and nighttime strategies mentioned above could be inter-dependent. For instance, *circadian resonance*, a phenomenon reflecting

a coupling between nighttime and daytime gas exchange, can play a major role in that adaptation and fitness under water-limited conditions (Christman et al. 2008; Drake et al. 2013; Resco de Dios et al. 2016; Resco de Dios and Gessler 2018). The benefit from this phenomenon is thought to result from a circadian regulation of TR_N or stomata conductance (g_s), which primes daytime gas exchange to respond to the environment in a way that enhances water use efficiency in the beginning of the following day (Sadok 2016). Recently, it was shown that genotype-dependent, pre-dawn circadian increase in leaf-level g_s among *Eucalyptus camaldulensis* genotypes positively correlated with faster stomata and higher levels of maximal, early-morning g_s and photosynthetic assimilation (Resco de Dios et al. 2016). However, the universality of this relationship in a given crop species was never examined. If a relationship between pre-dawn TR_N and TR response to evaporative demand existed, this would indicate a more pronounced importance of the circadian clock than previously thought in regulating wheat water use patterns and drought tolerance.

A second objective of this investigation was to characterize TR_N time courses for 77 wheat genotypes and determine whether their variation correlate with daytime TR responses to VPD. This set of lines consisted of the 54 previously mentioned genotypes plus the 23 south-Australian lines of Schoppach et al. (2017) which were characterized for the first time for TR_N in a new independent study. Specifically, we first examined the hypothesis that average nightly TR_N would positively correlate with daytime canopy conductance (G_s) as this would be indirect proof for a coupling between daytime and nighttime water use. Second, we tested whether this relationship was mediated by pre-dawn, endogenous increases in TR_N . Third, we tested the hypothesis that this putative circadian control could be under favorable selection among commercial cultivars released in the drought-prone environment of south-Australia, as this would be indicative of its relevance for breeding for drought tolerance.

Materials and methods

Genetic material

Three groups of (*Triticum aestivum* L.) genotypes, referred to as Group 1, Group 2 and Group 3, were used in this study (Online Resource S1). Group 1 is composed of 23 randomly selected wheat genotypes released in south-Australia between 1890 and 2008 (Schoppach et al. 2017). Group 2 consists of 29 wheat genotypes from different backgrounds, representing randomly selected commercially released cultivars and breeding lines from the University of Minnesota wheat breeding program. Group 3 is comprised of a worldwide diversity panel consisting of a set of 25 wheat

genotypes assembled from 23 countries covering 5 continents, based on genomic diversity (Brian Steffenson, personal communication). Genotypes of each one of these groups were characterized in common experiments.

Plant growth conditions

A total of five independent experiments carried out over 3 years (2015–2017) were conducted in this study (Table 1); two for Group 1 (E1.1 and E1.2), one for Group 2 (E2) and two for Group 3 (E3.1 and E3.2). One additional, sixth experiment was carried out on Group 1 earlier to examine TR responses to VPD (published in Schoppach et al. 2017). Growth conditions and phenology information of these experiments are reported in Table 1.

Group 1

Two experiments were conducted for this group. For each experiment, six replicate plants of each one of the 23 lines were sown at a depth of 2.5 cm in 3-L pots filled with compost garden soil. Ten days after sowing, each pot was thinned to a single plant. Plants were watered every 2–3 days and then daily during the last week prior to measurements. Plants were grown in a glasshouse at the Université catholique de Louvain, Belgium which was regulated for a minimal temperature (T) of 20 °C. Temperature, relative humidity (RH) and vapor pressure deficit (VPD) conditions were recorded continuously (every 5-min) using 5 sensors (EL-USB-2-LCD, Lascar Electronics, Whiteparish, UK) placed at different locations across the setup. The internal resolutions for T and RH offered by this sensor were 0.5 °C and 0.5% RH, respectively. A portable PPFD sensor (Photo/radiometer HD 2102.2; Delta Ohm, Caselle di Selvazzano, Italy) that logged data every 5-min was placed at canopy height to confirm the absence of incident PPFD during nocturnal measurements. The environmental conditions experienced by the plants during the growth period are summarized in Table 1.

Groups 2 and 3

One and two experiments were conducted for Groups 2 and 3, respectively. Three replicate plants for each genotype were sown at a depth of 2.5 cm in 3.8-L pots filled with compost garden soil, which were thinned to single plant 1 week after germination. Plants were grown under well-watered conditions, while pots were watered every 2–3 days and then daily during the last week prior to measurements. The plants were grown in a greenhouse at the University of Minnesota, which was regulated for a minimal temperature of 19 °C. Temperature, RH and VPD conditions inside the greenhouse were recorded every 5 min by means of three pocket sensors of the same type described earlier, placed at different locations.

Table 1 Environmental conditions imposed during the growth period

Group	Experiment	Goal ^a	Sowing date	Measurement date	Daytime conditions		Nighttime conditions		LA (cm ²)	ZS
					T ^b (°C)	VPD ^c (kPa)	T ^b (°C)	VPD ^c (kPa)		
Group 1	E1.1	TR _N	24/07/2015	01/09/2015	32.0±0.6	3.2±0.2	25.8±0.5	1.8±0.1	413±32	51±2.0
	E1.2	TR vs. VPD, TR _N	27/07/2016	09/08/2016	29.7±0.5	2.5±0.5	23.7±0.3	1.3±0.1	388±51	38±2.0
Group 2	E2	TR vs. VPD, TR _N	24/05/2017	26 & 27/06/2017	30.5±0.3	2.9±0.1	21.1±0.2	1.0±0.0	431±21	38±0.7
Group 3	E3.1	TR vs. VPD, TR _N	20/01/2017	21 & 22/02/2017	26.5±0.4	2.4±0.1	19.7±0.1	1.2±0.1	400±29	34±0.7
	E3.2	TR vs. VPD, TR _N	20/02/2017	21 & 23/03/2017	26.4±1.1	2.6±0.2	19.0±0.6	1.3±0.1	556±46	35±0.8

Leaf areas (LA) and Zadok's stages (ZS) are given to indicate average plant size and phenology during each experiment. Numerical values are for averages (±SE)

^aPurpose of the experiment. TR vs. VPD: characterizing transpiration rate (TR) response curves to increasing vapor pressure deficit TR_N; characterizing nocturnal TR

^bAverage temperature

^cAverage atmospheric vapor pressure deficit

Details of environmental conditions experienced by the plants during the growth period are provided in Table 1.

Phenotyping transpiration responses to vapor pressure deficit under constant temperature

Group 1

The procedure for characterizing TR responses to VPD for this group (experiment E1.1) was published and described in Schoppach et al. (2017), where those responses were characterized based on manual weighting of pots. Environmental conditions during TR vs VPD characterization are reported for this group in Table 2.

Groups 2 and 3

The experiments (E2, E3.1 and E3.2) were conducted following the protocol of Schoppach et al. (2017), but inside the GraPh (*Gravimetric Phenotyping*) system, a semi-automated platform developed for high-throughput phenotyping of TR responses to VPD at constant temperatures (Tamang and Sadok 2018). The GraPh platform consists of 54 high-resolution balances that are identical to the one used for Group 1 (Model Fx-3000i, A & D Co. Ltd, Tokyo, Japan). These balances were protected from dust, moisture, vibration and static electricity and connected to dataloggers. The setup allowed for tracking pot mass every 60 s at a resolution of a 1/100 g inside 3 adjacent, identical, walk-in programmable growth chambers of the same model as the one used for Group 1 (Model PGV36, Conviron, Controlled Environments Ltd., Winnipeg, Manitoba, Canada), where identical environmental conditions (PPFD = 600 μmol m⁻² s⁻¹, T ~ 30 °C) could be imposed (Table 2). In addition to the chambers' own sensors, environmental conditions (T, RH and VPD) at canopy level were continuously recorded every 5 min in 3 locations by the same pocket sensors (EL-USB-2-LCD, Lascar Electronics, Whiteparish, UK) used in Schoppach et al. (2017). During TR vs VPD characterizations, environmental conditions experienced by Groups 2 and 3 were very similar to those experienced by Group 1 (Table 2), with the main difference being that in the latter, TR was determined on the basis of manual weightings.

On the day prior to the measurements, the plants were carefully watered to dripping and the soil was covered with aluminum foil to nullify soil evaporation before transfer inside the chambers. The measurements started at the end of nighttime period (i.e., 0700 h for E2 and 0500 h for E3.1. and E3.2, see next section for details about the nighttime treatment), by turning the lights on and progressively rising PPFD and T to the target values of 600 μmol m⁻² s⁻¹ and 30 °C, respectively, at canopy height over the next hour. These conditions were then held constant during the rest of

Table 2 Temperature (T) and vapor pressure deficit (VPD) conditions imposed during the phenotyping of whole-plant transpiration rate (TR) response to VPD and nighttime TR (TR_N) dynamics for the 3 studied groups

Group	Experiment	Daytime conditions		Nighttime conditions	
		T ($^{\circ}\text{C}$)	VPD range (kPa)	T ($^{\circ}\text{C}$)	VPD (kPa)
Group 1	E1.1	N/A	N/A	21.0 ± 0.2	0.9 ± 0.0
	E1.2	30.7 ± 0.2	0.9–3.2	21.3 ± 0.1	1.4 ± 0.0
Group 2	E2	30.0 ± 0.1	1.4–3.0	20.2 ± 0.1	0.8 ± 0.0
Group 3	E3.1	29.9 ± 0.1	1.6–3.3	20.2 ± 0.0	1.0 ± 0.0
	E3.2	30.1 ± 0.1	1.3–3.0	20.3 ± 0.0	0.9 ± 0.0

Values are for averages ($\pm$ SE). TR response curves to VPD were not determined on experiment E1.1

experiment. The well-watered plants were then subjected to a 7-step VPD increase (1.3–3.3 kPa), during which RH was decreased from the highest level ($\sim 90\%$, achieved by means of 4 industrial humidifiers) to progressively lower values (down to $\sim 30\%$), using a programmable industrial dehumidifier. At each step, VPD was held constant for 60 min, resulting in steady-state TR regime (Fletcher et al. 2007; Tamang and Sadok 2018). During the entire procedure, plant water use was constantly and automatically recorded every minute by the balances equipped with the dataloggers. At the end of the sequence, the plants were removed from the growth chambers and leaf areas were destructively measured using a leaf area meter (LI-3100C, Li-Cor, Lincoln, NE, USA). These values were then used to calculate a normalized transpiration rate (TR, $\text{mg H}_2\text{O m}^{-2} \text{s}^{-1}$) to account for genotypic differences in plant leaf areas. Because the capacity of the chambers was 54 pots (18 genotypes replicated 3 times per day), experiments E2 and E3 were carried out over two consecutive days (Table 1).

Phenotyping nighttime transpiration time courses

Group 1

Two experiments (E1.1, E1.2) for characterizing nighttime transpiration rate (TR_N) for this group were conducted. They took place hourly between 2200 h and 0600 h inside the glasshouse, during a period where PPFD was $0 \mu\text{mol m}^{-2} \text{s}^{-1}$, after growing the plants as described for this group in Table 1. Eight hours prior to these measurements, pots were gradually watered until dripping to ensure that they reached field capacity and left to drain excess water before initiating weighting. Before measurements were started, the soil was covered with an aluminum foil to nullify soil water evaporation. Hourly TR_N values were estimated gravimetrically by manual weighing of pots (using the same balance model as described previously) and then normalizing the difference in pot mass between consecutive weightings by total leaf area measured destructively at the end of the experiment using a leaf area meter, as described earlier. The hourly manual weighing for the entire pots lasted about 39 min per measurement period.

Groups 2 and 3

TR_N measurements during E2, E3.1 and E3.2 were conducted inside the GraPh platform, prior to characterizing TR response curves to increasing VPD (Tamang and Sadok 2018). The followed protocol was similar to the one for E1.2. Pots were slowly watered until dripping at around 1100 h and left to drain for approx. 6 h, on the day of experiment. The soil of the pots was then covered with aluminum foil to nullify soil water evaporation before transferring them to the growth chambers at 1800 h. Plants were allowed to acclimate inside the chambers for ~ 3 h while T , RH and PPFD were gradually adjusted to target settings ($T = \sim 20^{\circ} \text{C}$, $\text{VPD} = 0.9 \text{ kPa}$, $\text{PPFD} = 0 \mu\text{mol m}^{-2} \text{s}^{-1}$, Table 2). These conditions were then imposed for the entire nighttime period between 2100 h and 0700 h and between 2100 h and 0500 h, for E2 and E3, respectively, so that durations are consistent with the actual photoperiod experienced by the plants during the growth phase in the greenhouse. TR_N was calculated as previously described for Group 1.

Data analysis

Transpiration response curves to increasing vapor pressure deficit

The approach for characterizing manually measured TR responses to VPD of Group 1 is described in Schoppach et al. (2017). In the case of genotypes from Groups 2 and 3, the higher resolution of the data (every 1 min) enabled by the GraPh platform (i.e., TR time courses) made it possible to develop an automated approach for processing the data as described in Tamang and Sadok (2018). Briefly, prior to averaging TR and VPD values for each VPD step, the first 15 min of each VPD step was excluded from the analysis since some genotypes may be still acclimating to the new regime during that period (Fletcher et al. 2007; Schoppach and Sadok 2012). This was confirmed by testing whether TR was at a steady-state regime during each one of the VPD treatments by examining the slopes of TR regressions against time and testing for significant departure from zero at each VPD step (Tamang and Sadok 2018). Less than 3%

of the examined 1620 time courses exhibited a significant departure from a zero slope, indicating that TR measurements essentially reflected a steady-state regime. This made it possible to average TR and VPD data for each VPD step.

As was the case for Group 1 (Schoppach et al. 2017), two formalisms were compared to fit TR response curved to VPD in Groups 2 and 3. For each genotype, regressing TR against VPD was subjected to two fits: one linear and one segmented. The best fitting model (linear vs. segmental regression line) was automatically determined based on an extra sum-of-squares F test ($P < 0.05$). In the case of Group 3, since TR responses to VPD did not significantly differ between E3.1 and E3.2 for most of the genotypes (ANCOVA, 23 out of 26), data of the 2 experiments were pooled to obtain regression parameters. Segmented regressions consisted in a first slope (Slope 1), a second slope (Slope 2) and a VPD threshold (VPD_{Th}) separating the 2 segments (Fletcher et al. 2007).

Canopy conductance (G_s) was calculated based on a rearrangement of Penman (1948) sink's strength model as detailed in Tanner and Sinclair (1983) and later revisited in Sinclair et al. (2014), i.e., as the slope of TR response to VPD (TR/VPD , $mg\ H_2O\ m^{-2}\ s^{-1}\ kPa^{-1}$). G_s was defined as the slope of TR response to VPD in the VPD range where TR is not restricted. For genotypes exhibiting a linear TR response to VPD, G_s consisted of the slope of that relationship while for those expressing a limitation on TR over a given VPD_{Th} , G_s was proxied as Slope 1 of the segmented regression.

Nighttime transpiration rates and time course analysis

Independent of the group, whole-night TR_N was calculated by averaging values during the considered dark period. In the case of Groups 1 and 3, TR_N values were highly correlated across experiments (Pearson's $r = 0.82$, $P < 0.0001$ for Group 1 and $r = 0.76$, $P < 0.0001$ for Group 2); so they were pooled together to calculate average TR_N .

TR_N time course analyses were carried out to identify patterns indicative of endogenous, circadian control by examining potential pre-dawn increases in TR_N over time, during a period where T , VPD are constant and PPFD is zero (Tamang and Sadok 2018). Because TR_N of Group 1 was measured hourly and manually, while in the case of Groups 2 and 3, TR_N was automatically measured every minute and simultaneously for all genotypes, different approaches were implemented to characterize potential endogenous signatures between Group 1 and Groups 2–3.

In the case of Group 1, since manual weightings took place every 30 s for 39 min during a given hour, a first step was to record the exact time each manual weighting took place. Afterwards, the existence of the pre-dawn increase was examined by regressing consecutive TR_N measurements over the last 4 h of the night, between 0200 h and

0600 h against time and testing for the significance level of the slope (see example on Fig. 4). Since TR_N increase during this period is supposed to be moderate, a systematic outlier detection approach was performed on the 24 data points for each genotype, using the Robust regression and OUTlier removal method (ROUT) developed by Motulsky and Brown (2006) based on a maximum false discovery rate of 0.1%. This analysis excluded one data point from each of three genotypes (Bungulla, Gabo and Mace) and three data points from one genotype (Kingswhite) out of the 24 data points. These outliers were likely the result of errors in the manual recording of the data or resulting from inadequate pot placement on the balance.

In the case of Groups 2 and 3, high-resolution TR_N time courses were first smoothed using moving averages calculated over 3 consecutive observations. Similar to genotypes from Group 1, the existence of the pre-dawn increase in TR_N was tested for the last 4 h of the night, using the same approach as previously described (Tamang and Sadok 2018), except that no outliers (ROUT method applied as described above) were detected in the data of Groups 2 and 3.

Time course data were processed using custom R scripts (R Development Core Team, 2017) and statistical analyses (summary statistics, outlier detection, t test, ANOVA, ANCOVA, correlation analyses, regression fits) were conducted using PRISM 7.0c (GraphPad Software Inc., San Diego, CA, USA, 2017).

Results

Diversity in whole-plant transpiration response curves to increasing VPD independently from temperature

As illustrated in Fig. 1, there was substantial phenotypic diversity in TR responses to increasing VPD in Groups 2 and 3 ($P < 0.0001$). However, this diversity was structured differently within each one of these groups (Fig. 2). In Group 2, virtually all (28 out of 29) of the exhibited a linear, non-restricted rise in TR as VPD increased (Fig. 2), with the only exception being a genotype that displayed the highest Slope 2 among all lines with a segmented response, irrespective of the group (line MN11394-6, Slope 2 = $24.4\ mg\ H_2O\ m^{-2}\ s^{-1}$). In the case of the worldwide diversity panel (Group 3), the outcome was intermediary, with 60% and 40% of the genotypes displaying VPD-insensitive (linear) and VPD-sensitive (segmented) TR responses, respectively. Canopy conductance (G_s) values varied widely within groups (Table 3), with the largest variation observed within the worldwide diversity panel (Group 3). Overall, we observed 1.6-, 1.9- and 2.9-fold variation in G_s among Groups 1, 2 and 3, respectively.

Fig. 1 Examples of transpiration rate (TR) response curves to increasing vapor pressure deficit (VPD) under constant temperature (30 °C) obtained in the GraPh platform. Genotypes in **a**, **d** and **e** are from Group 3, while those in **b**, **c** and **f** are from Group 2 (regressions from Group 1 were published in Schoppach et al. 2017). In each panel, the statistical parameters of the linear or segmented regression analysis are indicated, where Slope is the slope of the linear regression, Slope 1, Slope 2 and VPD_{Th} , respectively, represent the first slope, the second slope and the VPD threshold of the segmented regression

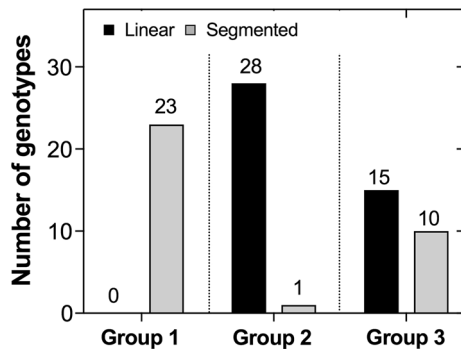
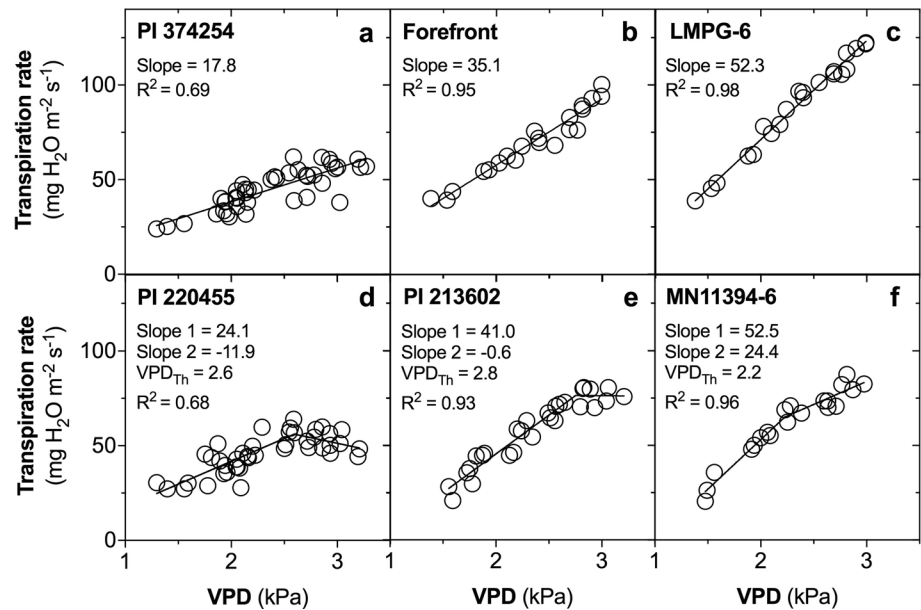


Fig. 2 Variability in the number of genotypes exhibiting a linear (black bars) or a segmented (gray bars) TR response to VPD among the 3 examined groups. Numbers on top of each bar represent the number of genotypes exhibiting the linear or segmented responses

Diversity in nocturnal transpiration, its endogenous control and relationship with daytime canopy conductance

In comparison to TR responses to VPD, the extent of within-group phenotypic diversity in TR_N was larger, with 4.1-, 2.2- and 3.1-fold variation for Groups 1, 2 and 3, respectively (Table 3). As illustrated in Fig. 3, this phenotypic diversity in TR_N was found to be strongly and positively correlated with G_s independently for each of the 3 groups (Fig. 3b–d), an observation that was confirmed when pooling data from all groups together (Fig. 3a, $P < 0.0001$, $R^2 = 0.57$).

As exemplified in Fig. 4, significant pre-dawn increases in TR_N were observed, under near constant T , VPD and PPFD conditions for genotypes from the all groups, with a minimal resolvable value of 0.06 ± 0.0 mg H_2O m $^{-2}$ s $^{-1}$

h $^{-1}$ (Table 3). However, the number of genotypes exhibiting significant pre-dawn increases was different dependently on the group (Table 3). This was the case for 14 genotypes consisting of 6 lines, 7 lines and 1 line from Groups 1, 2 and 3, respectively. Group 1 was the only group where pre-dawn increase in TR_N was found to be positively correlated with TR_N averaged over the entire nighttime period ($P < 0.005$, $R^2 = 0.89$). However, pooling data gathered independently from the 3 groups confirmed the relationship (Fig. 5a, $P < 0.001$, $R^2 = 0.69$). Similarly, Group 1 was the only group for which the pre-dawn increase in TR_N positively correlated with G_s ($P = 0.01$, $R^2 = 0.83$), and this relationship was even more strongly confirmed when pooling data from all groups (Fig. 5b, $P < 0.0001$, $R^2 = 0.90$).

Relationship between circadian control of nocturnal transpiration, year of release, and TR sensitivity to VPD among the drought-adapted Australian cultivars

The drought-adapted south-Australian lines (Group 1) presented the unique attribute of having been released across a range of dates uniformly distributed between 1890 and 2008. Taking advantage of this effect, the analysis revealed that this group exhibited a positive correlation between the pre-dawn variation in TR_N and the year of release (Fig. 6a, $P < 0.01$, $R^2 = 0.31$). In addition, the pre-dawn rate of TR_N increase also correlated positively with Slope 2 (Fig. 6b, $P < 0.01$, $R^2 = 0.36$), while it correlated negatively with VPD_{Th} (Fig. 6c, $P < 0.01$, $R^2 = 0.31$). However, these effects were detected only when pooling significant and non-significant values for pre-dawn TR_N increases.

Table 3 Summary statistics of the regression analysis for daytime transpiration rate (TR) response curves to increasing vapor pressure deficit (VPD) and nighttime transpiration rate (TR_N) time course analysis for Groups 1, 2 and 3

Group	Genotypes	Regression type	Slope or Slope 1 (mg H ₂ O m ⁻² s ⁻¹ kPa ⁻¹ ± SE)	Slope 2 (mg H ₂ O m ⁻² s ⁻¹ kPa ⁻¹ ± SE)	VPD _{Th} (kPa ± SE)	R ²	Average TR _N (mg H ₂ O m ⁻² s ⁻¹ ± SE)	Predawn TR _N rate of increase (mg H ₂ O m ⁻² s ⁻¹ h ⁻¹ ± SE)
Group 1	Heron	Segmented	32.6 ± 6.0	0.5 ± 10.6	2.1 ± 0.3	0.66	5.4 ± 0.3	ns.
	Bencubbin	Segmented	36.9 ± 5.1	4.6 ± 9.1	2.1 ± 0.2	0.80	4.8 ± 0.2	ns.
	Gluyas Early	Segmented	36.7 ± 6.5	0.4 ± 11.1	2.2 ± 0.2	0.72	6.0 ± 0.2	ns.
	Mace	Segmented	39.2 ± 4.0	17.3 ± 3.3	1.9 ± 0.2	0.91	4.2 ± 0.6	0.42 ± 0.2
	Yitpi	Segmented	39.9 ± 4.5	14.5 ± 5.8	2.1 ± 0.2	0.88	5.2 ± 0.5	ns.
	Machete	Segmented	41.9 ± 4.4	19.3 ± 5.8	2.0 ± 0.2	0.90	7.5 ± 0.8	ns.
	Kingswhite	Segmented	41.6 ± 6.4	-0.9 ± 11.4	2.4 ± 0.2	0.80	6.2 ± 0.3	ns.
	Federation	Segmented	42.7 ± 7.1	0.4 ± 12.7	2.1 ± 0.2	0.72	5.3 ± 0.3	ns.
	Warigal	Segmented	44.7 ± 5.5	8.8 ± 5.9	2.0 ± 0.2	0.83	4.1 ± 0.3	0.43 ± 0.1
	Nabawa	Segmented	45.1 ± 5.4	0.8 ± 10.8	2.2 ± 0.2	0.85	7.5 ± 1.2	ns.
	Bungulla	Segmented	43.4 ± 8.4	5.9 ± 10.5	2.1 ± 0.3	0.73	8.8 ± 1.8	ns.
	Koda	Segmented	45.2 ± 9.1	2.8 ± 7.9	2.0 ± 0.2	0.68	7.9 ± 1.3	ns.
	Halberd	Segmented	49.2 ± 5.4	14.8 ± 7.0	2.0 ± 0.2	0.86	5.6 ± 0.2	0.33 ± 0.1
	Frame	Segmented	52.1 ± 5.3	19.3 ± 5.6	2.0 ± 0.2	0.91	4.0 ± 0.2	ns.
	Gamenya	Segmented	51.9 ± 7.1	8.7 ± 10.7	2.1 ± 0.2	0.83	13.0 ± 1.7	ns.
	Trident	Segmented	53.3 ± 6.5	19.5 ± 6.9	2.0 ± 0.2	0.87	4.2 ± 0.2	0.49 ± 0.1
	Ward's Prolific	Segmented	60.7 ± 7.3	1.5 ± 13.1	2.1 ± 0.2	0.81	6.2 ± 1.0	ns.
	Gabo	Segmented	62.1 ± 10.9	-3.2 ± 9.5	2.0 ± 0.2	0.74	12.5 ± 3.4	ns.
	Dundee	Segmented	64.1 ± 9.1	-3.3 ± 12.4	2.1 ± 0.2	0.79	7.0 ± 0.3	0.54 ± 0.2
	Janz	Segmented	69.2 ± 12.5	12.5 ± 13.5	2.0 ± 0.2	0.70	9.9 ± 2.3	ns.
	Kite	Segmented	76.6 ± 8.6	12.9 ± 11.2	2.1 ± 0.2	0.86	16.5 ± 3.6	ns.
	Spear	Segmented	74.9 ± 10.6	22.6 ± 11.2	2.0 ± 0.2	0.82	14.9 ± 3.5	ns.
	Ega Castle Rock	Segmented	83.8 ± 9.7	23.3 ± 11.7	2.0 ± 0.2	0.85	16.3 ± 1.4	1.10 ± 0.5
	P value		< 0.0001	0.74	0.10	—	< 0.0001	
Group 2	Pasa	Linear	27.1 ± 1.4	—	—	0.95	3.2 ± 0.0	ns.
	Popo	Linear	27.9 ± 1.4	—	—	0.96	3.7 ± 0.2	0.07 ± 0.0
	Kulungu	Linear	28.2 ± 1.5	—	—	0.95	3.3 ± 0.2	0.10 ± 0.0
	RB07	Linear	28.8 ± 2.5	—	—	0.77	4.3 ± 0.2	ns.
	LCS Albany	Linear	31.1 ± 1.8	—	—	0.94	3.6 ± 0.3	0.09 ± 0.1
	Gem	Linear	31.7 ± 1.5	—	—	0.96	4.9 ± 0.1	ns.
	Ngiri	Linear	31.6 ± 1.6	—	—	0.95	4.0 ± 0.6	0.06 ± 0.0
	MN06113-8	Linear	32.9 ± 1.7	—	—	0.95	4.7 ± 0.3	ns.
	Forefront	Linear	35.1 ± 1.9	—	—	0.95	6.3 ± 0.4	ns.
	Fahari	Linear	35.0 ± 2.2	—	—	0.93	7.1 ± 0.6	ns.
	MN10201-4-116	Linear	35.8 ± 1.7	—	—	0.96	4.2 ± 0.4	ns.
	Linkert	Linear	36.4 ± 1.2	—	—	0.98	7.3 ± 0.5	ns.
	Tom	Linear	36.3 ± 1.5	—	—	0.97	4.0 ± 0.3	0.07 ± 0.2
	Paka	Linear	36.6 ± 1.9	—	—	0.95	6.8 ± 0.3	ns.
	09FSP3	Linear	36.3 ± 2.7	—	—	0.94	2.7 ± 0.1	0.10 ± 0.0
	Blade	Linear	37.1 ± 2.1	—	—	0.94	5.6 ± 0.1	ns.
	Rollag	Linear	36.6 ± 2.8	—	—	0.91	5.0 ± 0.4	0.15 ± 0.1
	MN99394-1-10	Linear	38.1 ± 2.1	—	—	0.95	5.9 ± 0.6	ns.
	Ada	Linear	39.0 ± 2.6	—	—	0.93	5.7 ± 0.3	ns.
	Kudu	Linear	39.7 ± 2.3	—	—	0.94	5.7 ± 0.5	ns.
	Romany	Linear	40.8 ± 1.5	—	—	0.98	4.2 ± 0.3	ns.

Table 3 (continued)

Group	Genotypes	Regression type	Slope or Slope 1 (mg H ₂ O m ⁻² s ⁻¹ kPa ⁻¹ ± SE)	Slope 2 (mg H ₂ O m ⁻² s ⁻¹ kPa ⁻¹ ± SE)	VPD _{Th} (kPa ± SE)	R ²	Average TR _N (mg H ₂ O m ⁻² s ⁻¹ ± SE)	Predawn TR _N rate of increase (mg H ₂ O m ⁻² s ⁻¹ h ⁻¹ ± SE)
	MN11180-3-2	Linear	40.9 ± 2.2	–	–	0.95	5.9 ± 0.2	ns.
	MN99436-8	Linear	41.7 ± 3.0	–	–	0.91	6.8 ± 0.2	ns.
	Faller	Linear	43.4 ± 1.5	–	–	0.98	8.5 ± 0.1	ns.
	MN98550-5	Linear	43.4 ± 1.9	–	–	0.96	8.1 ± 0.3	ns.
	MN07098-6	Linear	44.7 ± 1.8	–	–	0.97	6.5 ± 0.1	ns.
	Sabin	Linear	43.8 ± 2.9	–	–	0.93	4.2 ± 0.2	ns.
	LMPG-6	Linear	52.3 ± 1.5	–	–	0.98	8.0 ± 0.1	ns.
	MN11394-6	Segmented	52.5 ± 5.8	24.4 ± 5.1	2.2 ± 0.1	0.96	6.1 ± 0.3	ns.
	<i>P</i> value		< 0.0001	–	–		< 0.0001	
Group 3	PI 193938	Linear	14.2 ± 2.1	–	–	0.54	3.6 ± 0.2	ns.
	PI 374254	Linear	17.8 ± 1.9	–	–	0.69	3.3 ± 0.2	ns.
	Cltr 15006	Linear	17.6 ± 2.6	–	–	0.58	4.1 ± 0.2	ns.
	PI 181458	Linear	18.5 ± 2.1	–	–	0.67	4.4 ± 0.2	ns.
	PI 430750	Linear	19.0 ± 2.0	–	–	0.73	3.4 ± 0.3	ns.
	PI 278392	Linear	20.4 ± 2.5	–	–	0.62	4.6 ± 0.2	ns.
	PI 282922	Linear	21.9 ± 1.9	–	–	0.80	3.9 ± 0.2	ns.
	PI 345693	Linear	22.0 ± 2.8	–	–	0.61	4.3 ± 0.4	ns.
	PI 565238	Linear	24.7 ± 2.3	–	–	0.74	4.0 ± 0.3	ns.
	PI 449298	Linear	27.2 ± 2.1	–	–	0.81	4.4 ± 0.2	ns.
	PI 199806	Linear	27.3 ± 2.6	–	–	0.74	4.9 ± 0.2	ns.
	PI 623147	Linear	27.8 ± 3.0	–	–	0.72	5.1 ± 0.9	ns.
	PI 520033	Linear	28.9 ± 2.8	–	–	0.73	4.4 ± 0.3	ns.
	PI 189771	Linear	30.0 ± 4.0	–	–	0.63	4.6 ± 1.0	ns.
	PI 519465	Linear	35.8 ± 3.0	–	–	0.78	5.1 ± 0.2	ns.
	Cltr 14819	Segmented	21.9 ± 4.3	5.8 ± 4.9	2.4 ± 0.2	0.69	4.2 ± 0.2	0.06 ± 0.0
	PI 205714	Segmented	24.1 ± 1.9	8.4 ± 6.5	2.7 ± 0.2	0.90	4.6 ± 0.3	ns.
	PI 220455	Segmented	24.1 ± 3.3	– 11.9 ± 10.3	2.6 ± 0.1	0.68	4.2 ± 0.1	ns.
	PI 344018	Segmented	25.0 ± 2.0	7.7 ± 5.1	2.6 ± 0.1	0.91	3.0 ± 0.2	ns.
	PI 62364	Segmented	26.5 ± 3.2	– 2.7 ± 5.6	2.6 ± 0.1	0.81	5.0 ± 0.1	ns.
	PI 520371	Segmented	28.1 ± 3.1	– 12.4 ± 13.2	2.8 ± 0.1	0.81	3.9 ± 0.3	ns.
	PI 153785	Segmented	29.0 ± 4.9	8.5 ± 5.8	2.4 ± 0.2	0.75	3.3 ± 0.2	ns.
	PI 384403	Segmented	30.4 ± 3.3	– 11.8 ± 12.7	2.8 ± 0.1	0.82	6.5 ± 0.5	ns.
	PI 519580	Segmented	36.8 ± 4.4	11.0 ± 6.3	2.4 ± 0.1	0.84	5.2 ± 0.6	ns.
	PI 213602	Segmented	41.0 ± 3.0	– 0.6 ± 12.6	2.8 ± 0.1	0.93	5.8 ± 0.3	ns.
	<i>P</i> value		< 0.0001	0.37	0.35	–	< 0.0001	

Slope is returned by the linear regression and Slope 1 or Slope 2 are returned by the segmented regression. VPD_{Th} (kPa) is the VPD threshold separating the first and the second segments of the non-linear regression. For each group, *P* values are provided after the last genotype to indicate the significance level of the genotypic variability. Numbers after the symbol (±) represent SE values. ns. indicate a non-significant increase in pre-dawn TR_N. For Group 1, regression data (but not TR_N) was previously published (Schoppach et al. 2017)

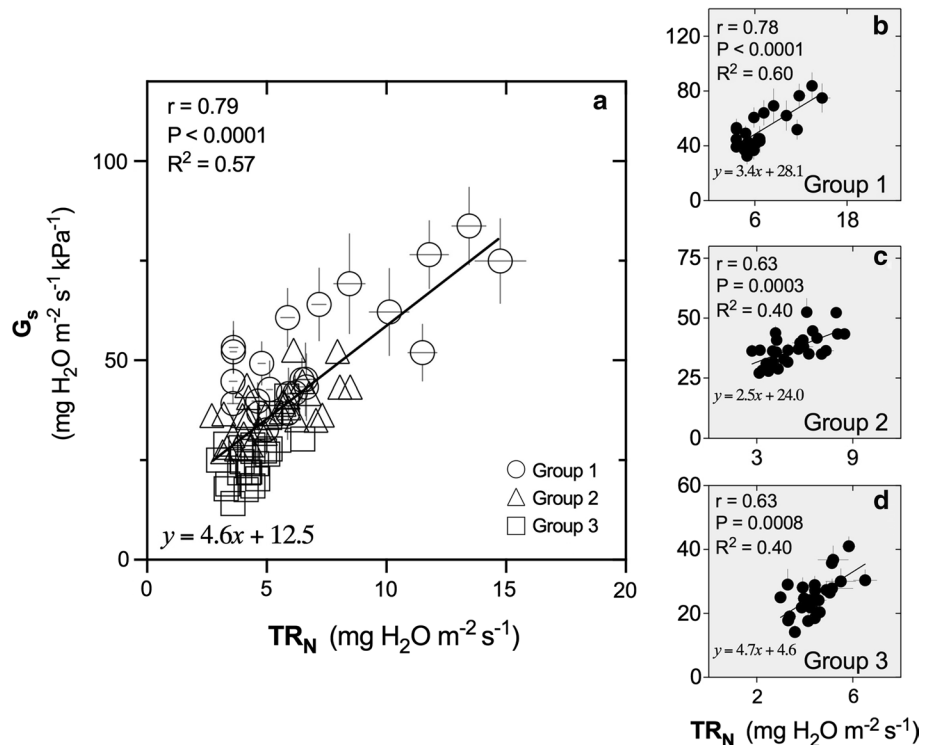
Discussion

There is an extensive variability in wheat transpiration sensitivity to temperature-independent variation in VPD

A first finding of this research was the unexpectedly large diversity in TR response curves to temperature-independent

variation in VPD identified among the genotypes of this study. Recently, Schoppach et al. (2017) characterized those responses in Group 1, but all these exhibited a relatively limited range of potentially water-saving (VPD-sensitive) TR response curves to increasing VPD. With this additional dataset of 54 genotypes, we uncovered the existence of genotypes exhibiting a constant G_s in the 1.3- to 3.3-kPa range (i.e., linear TR response to VPD), with substantial genotypic

Fig. 3 Relationship between canopy conductance (G_s) and average nighttime transpiration rate (TR_N) among the 77 genotypes of the study (**a**) and separately for each group (**b–d**). In **a**, Groups 1 (23 lines), 2 (29 lines) and 3 (25 lines) are represented by circles, triangles and squares, respectively. In each panel, Pearson's r , the linear regression fit (equation), its P value and R^2 are indicated. Each data point is the average of 3–9 replicate plants ($\pm$ SE)



variability, in addition to new modalities of the water-saving response, reflected by variation in VPD_{Th} , G_s (up to ~three-fold) and Slope 2 (Figs. 1 and 2, and Table 3).

To our knowledge, this is the first time such a diverse and large set of genotypes has been examined for TR response to VPD that are not confounded with variations in temperature. Such interaction could be problematic in dissecting the physiological and genetic basis underlying the expression of TR response curves to VPD, since concomitant changes in temperature can interfere in a complex way with physiological processes controlling water movement in the plant, such as AQP regulation or hormonal signaling (e.g., Kuwagata et al. 2012; Lee et al. 2012). Because this variability was found independent of co-variation in temperature or other potentially confounding environmental variables, it indicates that intra-specific variation in VPD-dependent hydraulic properties is likely to be driving this diversity. This is consistent with previous findings where hydraulic traits have been associated with differential water-carrying capabilities expressed by two contrasted (drought-tolerant and drought-sensitive) wheat genotypes (Schoppach et al. 2014a).

Such diversity is not necessarily expected, at least under constant temperature. This is supported by our recent investigation (Tamang and Sadok 2018) where—using the same methodology—we examined TR responses to VPD for a maize diversity panel consisting of 27 lines selected from over 300 worldwide collections of maize inbreds, none of which did express TR sensitivity to VPD. This indicates that in wheat, there is a particularly high potential

to harness this variability and capture its genetic basis in breeding programs. In this regard, based on the apparent differences in modalities of TR responses to VPD across the 3 groups (Fig. 2), one could speculate that breeders in drought-prone south-Australia were favoring the expression of a water-saving response resulting from a limitation on TR under high VPD. Because most of Minnesota lines (Group 2) displayed VPD-insensitive responses (i.e., linear TR response to VPD), an interpretation of Fig. 2 would be that this trait may have been bred out, as it could contribute to photosynthetic limitations to yield arising from restricting gas exchange under high VPD in an environment where soil moisture is not limiting. Consistent with the above, the worldwide diversity panel (Group 3) did not show evidence of this putative selective pressure, as both modalities (segmented and linear TR responses to VPD) were present in nearly similar proportions.

However, despite the fact that all genotypes being characterized under very similar conditions (Table 2), relatively small differences in growth conditions (Table 1) could have contributed to the inter-group differences; so further comparative experiments are needed to examine this hypothesis more closely. Regardless, the observed diversity within Groups 2 and 3 indicates potential to (i) identify modalities for TR responses to VPD that may maximize yield potential under various water availability regimes and (ii) phenotype a large number of lines to identify their genetic basis as part of a pre-breeding program.

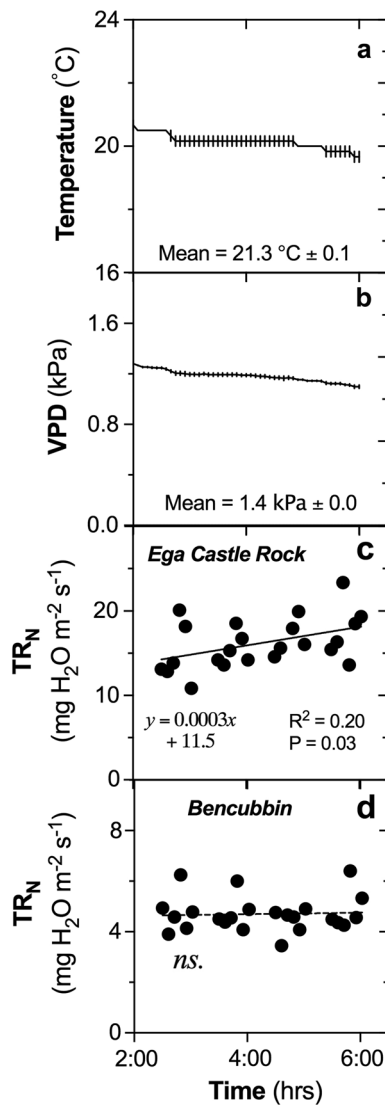


Fig. 4 Example of a genotype-dependent pre-dawn increase in nighttime transpiration rate (TR_N) from Group 1 independent from nighttime temperature (**a**) and vapor pressure deficit (VPD, **b**). PPFD was zero during the entire period (not shown). In **a**, **b**, error bars are for standard errors ($\pm$ SE). TR_N data in **c** and **d** are from Australian lines Ega Castle Rock ($n=6$) and Bencubbin ($n=6$), respectively. When significant (solid line, **c**), the linear regression fit (equation, R^2 and P value) is reported. *ns* non-significant slope (**d**)

Nocturnal transpiration correlates with daytime canopy conductance in wheat and this relationship might be mediated by the circadian clock and beneficial for drought tolerance

Another major finding of this study was the identification of a substantial variability in average nightly TR_N among the genotypes tested (e.g., over fourfold in Group 1). Regardless of the group, this variability was found to be strongly

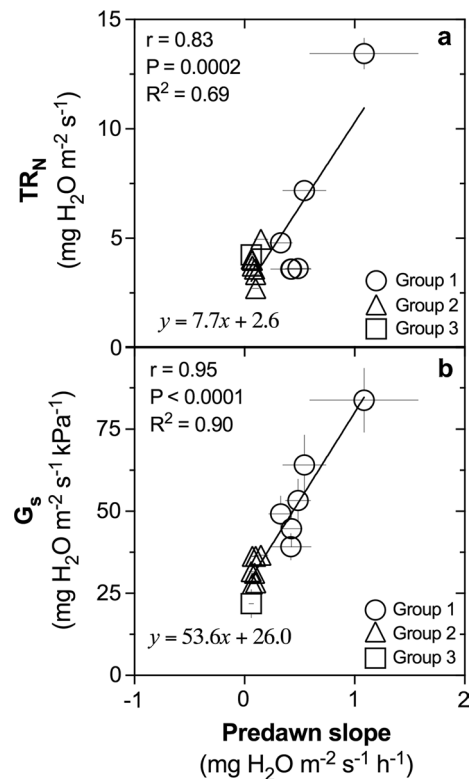


Fig. 5 Relationship between pre-dawn transpiration rate (TR_N) increase and average nightly TR_N (**a**) or canopy conductance (**b**) among the 14 genotypes for which the endogenous TR_N increase was detected (see Table 3 for details). In each panel, Pearson's r , the linear regression fit (equation), its P value and R^2 are indicated. Error bars are for standard errors ($\pm$ SE)

indicative of variation in G_s (Fig. 3). At least for the drought-adapted South-Australian lines, this relationship might be partly driven by the circadian clock, as suggested by the positive correlation between the pre-dawn increase in TR_N and G_s (Fig. 5b). As exemplified in Fig. 4, those pre-dawn increases took place systematically during dark periods where T and VPD were stable, possibly indicating a circadian basis. Such relationships seem to provide support to the hypothesis that the circadian clock may be involved in bridging nighttime and daytime canopy conductances, as previously found on hybrid aspen (Kupper et al. 2012) and Eucalyptus (Resco de Dios et al. 2015, 2016). In this regard, the fact that such relationships were essentially resulting from the drought-tolerant south-Australian cultivars is consistent with the circadian resonance hypothesis, which posits that pre-dawn increase in stomata conductance serves the purpose of priming the plant to maximize water use efficiency early in the day, particularly under dry, high-VPD environments found in south Australia (Resco de Dios et al. 2016).

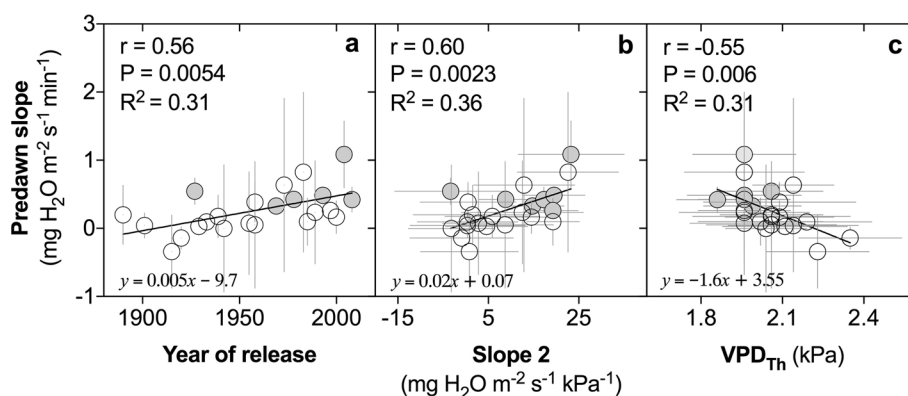


Fig. 6 Relationships between the pre-dawn variation in TR_N observed in the 23 Australia-adapted lines (Group 1) and their year of release (**a**), the second slope of their daytime transpiration rate (TR) response to VPD (Slope 2, **b**), and the VPD breakpoint of the segmented

regression (VPD_{Th} , **c**). In all panels, light gray circle indicate genotypes with statistically significant pre-dawn TR_N increase. Linear regression fits (equation), Pearson's r , their P values and R^2 are indicated in each panel. Error bars are for standard errors ($\pm$ SE)

Finally, although based partly on data reflecting non-significant pre-dawn TR_N increases, findings presented in Fig. 6 provide additional insight into the relevance of this hypothesis. Indeed, these correlations seem to suggest, at least for the drought-adapted south-Australian lines, that the circadian resonance phenomenon might have been an indirect breeding target. This is supported by the observation that pre-dawn TR_N variations in this group positively correlated with the year of release, during a period where wheat yields increased by over 400% (Kirkegaard and Hunt 2010). Surprisingly, such putative circadian control seems to have driven an increase in Slope 2 (Fig. 6b), which would have favored a 'risk-taking' behavior in terms of water use, but which was probably needed to enable fixing more CO_2 needed for additional yield gains. At the same time, such control was also putatively associated with a decrease in VPD_{Th} (Fig. 6c), suggesting that it may also have triggered the water-saving behavior at earlier times of the day. Certainly, further research is needed to examine the mechanistic basis of these relationships.

Combined, the above findings suggest that (i) an endogenous, circadian control of TR_N may play a role in modulating the expression of daytime TR sensitivity to VPD, particularly under water-limited conditions, (ii) this effect is genotype dependent and (iii) it is potentially driven by hydraulic mechanisms that remain to be uncovered. They also suggest that directly screening for variation in TR_N might be a good proxy trait for characterizing whole-plant canopy conductance, which is comparatively more difficult and expensive to phenotype.

Author contribution statement WS conceived the research. WS, JA, BS designed experiments. BT, RS and DM carried out experiments and data analyses. WS, BT and RS wrote the manuscript with input from co-authors.

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