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Plant size directly correlates with water use efficiency in Arabidopsis

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Abstract

Plant transpiration is a fundamental process that determines plant water use efficiency (WUE), thermoregulation, nutrition, and growth. How transpiration impacts on such essential physiological aspects and how the environment modulates these effects are fundamental questions about which little is known. We investigated the genetic and environmental factors underlying natural variation in plant transpiration and water use efficiency in a population of natural *Arabidopsis thaliana* accessions grown under homogeneous conditions. As expected, we observed large variation of total transpiration capacity, transpiration per surface unit, and WUE among *A. thaliana* accessions. Despite the variation of stomatal density and ABA content in the population, WUE did not correlate with any of these parameters. On the contrary, a surprising direct correlation was found between WUE and projected leaf area, with bigger plants displaying a more efficient use of water. Importantly, genome-wide association studies further supported our observations through the identification of several loci involved in WUE variation, mutations in which caused a simultaneous reduction in plant size and a decrease in WUE. Altogether, our results strongly suggest that, although WUE depends on many parameters, plant size is an adaptive trait with respect to water use in *A. thaliana*.

KEYWORDS
Arabidopsis thaliana, climatic variables, ecotypes, GWAS transpiration, WUE

1 | INTRODUCTION

All land plants descend from a single common ancestor and have since diversified into 400 000 species that have colonised every continent on Earth, surviving to extreme environments thanks to evolutionary adaptations to environmental cues (Bowles et al., 2021). Water availability is a major factor in selection of natural plant populations as it is a key factor for plant physiology and a limiting factor for crop yield in agricultural systems (McKay et al., 2008).

Plant adaptation to drought involves phenological and physiological traits leading to three general strategies: tolerance, avoidance and escape. In annual plants, avoidance (keeping water status in dry environments by minimising water loss) and escape (short life cycle allowing plants to reproduce before the dry season, McKay et al., 2008) are more studied as both strategies are more suitable

and commonly exported to agricultural breeding where selected traits are either related to drought avoidance (water use efficiency, WUE) or drought escape (early flowering and fruit maturation).

Among the particular traits that are associated with WUE, transpiration, stomatal conductance, and growth rate have always been considered critical to define plant behaviour with respect to water use, and their variation is mutually interdependent (Arntz & Delph, 2001).

Transpiration is the driving force allowing water uptake from roots to the canopy and it is responsible for maintaining the hydric balance in tissues. Water is mainly released to the environment through stomata, allowing gas exchange and CO_2 fixation. Therefore, transpiration is also a key component of plant nutrition and energetic metabolism (Taiz & Zeiger, 2002) and has a paramount impact on plant ability to grow, survive and successfully reproduce.

The amount of water used to allow gas exchange and produce photoasimilates is variable and, according to each plant genetic potential, there is an optimal rate of water used versus growth (Kang et al., 2021). WUE reflects the ratio of water used in plant metabolism to water lost by transpiration. Two main types of calculation for WUE are used, a photosynthetic (or instantaneous) WUE (iWUE), defined as the net photosynthetic rate (A_n) divided by transpiration rate (E, both measured with an infrared gas analyser) and the productive WUE, defined as the ratio between biomass produced to water transpired (Condon, 2004). However, these two processes (transpiration and growth) are not independent from each other. A low transpiration rate limits both the photosynthetic activity (Eyland et al., 2021) and the nutrient uptake, affecting therefore, plant growth.

Stomatal conductance (g_s) is one of the best indicators for plant transpiration at leaf level. Due to its importance for plant acclimation, g_s is modulated by environmental factors, such as the quality and intensity of light, air temperature, vapour pressure deficit (VPD) and atmospheric CO_2 concentration (Driesen et al., 2020). On the other hand, plant energetic balance, water status, developmental stage, and several metabolic factors can also influence g_s and hence transpiration (Flütsch & Santelia, 2021). These multiple layers of input signals determine the transpiration dynamics throughout plant life. Moreover, transpiration is not only defined by stomatal opening but it can be also influenced by morphological traits such as density and number of stomata, leaf and root architectures, etc (Dittberner et al., 2018). Gravimetric whole plant transpiration normalised to projected leaf area (PLA), from now on TN, integrates transpiration throughout a period of time while allows characterising water consumption per unit of area.

Similar to transpiration, growth rate is a trait that can vary broadly among species and ecotypes even on constant environmental conditions (Clauw et al., 2016). According to White et al. (2016), growth is controlled by physiological and developmental mechanisms surrogated to ecological adaptations and life history traits like flowering time, seed dormancy and growth rate. Those mechanisms arise as strategies for adaptation to a particular ecological environment as a trade-off between growth and defence, energy storage or developmental processes. In this sense, flowering time is a clear example of differential strategies of development as an adaptation to the environment: Arabidopsis summer annuals that must complete their life cycle while temperatures are relatively warm are fast growers and rapidly shift from the vegetative phase to the reproductive stage. However, winter annuals can afford to grow slowly due to milder environmental conditions and their vegetative phase is longer (Shindo et al., 2007). According to this, environmental conditions shape phenology and growth, re-arranging plant metabolism to tune these traits and optimise overall performance.

Previous genetic studies on WUE have mostly focused in the characterisation of mutants affected in stomatal aperture or hormone action (Saez et al., 2004; White & Montes-R, 2005). These studies have been useful as long as they have underscored the interdependence between growth performance and water-related parameters (Lim et al., 2020), but the lack of an integrated view of water use, inherent to these approaches, has resulted in limited success in the design of agronomical strategies outside laboratory environments. An alternative biotechnological strategy stems from the identification of the actual processes that are target for adaptation under natural environments through the exploitation of natural genetic variation. Examples of this approach can be found in the study of developmental and morphological traits (Baird et al., 2021), metabolism (Wu et al., 2018) or stress responses (Deolu-Ajayi et al., 2019), but not so extensively in the case of water use (Bhaskara et al., 2022; Lasky et al., 2014).

Thus, we have undertaken a thorough characterisation of natural genetic variation of physiological traits related to WUE in a large collection of *A. thaliana* accessions, expecting to highlight processes that underlie adaptation to natural environments. Moreover, we have performed genome-wide association studies (GWAS) to identify putative target genes responsible for this adaptation.

2 | MATERIALS AND METHODS

2.1 | Plant material

The *Arabidopsis thaliana* ecotype collection was previously used in (Milhinhos et al., 2019). The rest of genotypes used in this work have been previously characterised: *aba2-1* (González-Guzmán et al., 2002), *abi1-1* and *hab1-1 abi1-2* (Rubio et al., 2009) our laboratory (De Ollas et al., 2019). Loss of function transfer DNA (T-DNA) mutants were purchased from NASC, accession codes and line description are detailed in Table S1. Homozygous plants were selected by PCR (conditions as well as primer design is detailed in Table S2) and seeds for the experiments were bulked from the selected plants. Quantitative gene expression (qRT-PCR) of the genes of interest was monitored in the selected T-DNA lines to assess the degree of downregulation in the gene expression caused by the T-DNA insertion.

Seeds (20–30) of each individual line were sown in peat plugs (Jify-7 peat pellets, Semillas Batlle S.A.) without further stratification.

Five days after germination, individual seedlings were carefully transplanted to plugs with tweezers, and were kept for a week in a growth chamber (Equitec model EGCS 351 3S HR) with a day/night temperature of 23°C/18°C, a 8 h light photoperiod (100 µmol m^{-2} s^{-1}), and a relative humidity of 60%–65%. After 5–7 days growing into the growth chamber plants showing homogeneous growth were selected for the experiment. Plugs were covered with a bottomless cylindric plastic shell to avoid soil evaporation with a hole on top to place the seedling (pot). The bottom of these cylindric plastic shell was sealed with a 4 × 4 cm piece of duct tape exposed to a gentle fame. Pots were randomly distributed in 20 × 20 × 1 cm polystyrene trays (16 plants per tray). Trays were rotated inside the growth chamber every couple of days to avoid any position bias (De Ollas et al., 2019) photographs and area measurements are provided in Figure S4.

2.2 | Transpiration measurements

To calculate plant transpiration, pots ($n = 8$ per genotype) were weighed between 9 and 11 h am and once again after 24 h. Photographs of each plant were taken during the 4 days that weights were scored to calculate PLA) and other morphological parameters using pixel count references according to the Easy Leaf Area software (Easlon & Bloom, 2014). Weight and PLA values were used to calculate daily transpiration for each plant. Results of consecutive days were normalised according to the mean transpiration of the eight Col-0 plants (considered as value one) on well-watered control conditions (>25 g pot weight). Transpiration, area and WUE (calculated as the ratio of rosette projected area to transpiration) of 137 ecotypes, three mutant accessions (*aba2-1*, *abi1-1* and *hab1-1 abi1-2*) and T-DNA accessions were characterised in 23 different experiments, alternatively WUE was calculated in a subset of 65 ecotypes (Figure 4) as the ratio of growth rate and transpiration per unit of area during that period. Growth rate was calculated as the relative difference in PLA after 3 days. All results were normalised to Col-0 (control accession in all experiments) to compare results. Random lines within the collection were characterised multiple times to assess reproducibility.

Instantaneous gas exchange (g_s, A) was measured with a LI-6800 Portable Photosynthesis System (Li-COR Inc.). A single fully developed leaf was used per replication for gas exchange measurements. During measurements, reference CO_2 concentration was equilibrated to 400 µmol mol^{-1} with a CO_2 mixture, and the light adjusted at a PAR of 200 µmol m^{-2} s^{-2}. The block temperature was fixed at 25°C, the leaf-to-air VPD was equilibrated between 1.5 and 2.0 kPa, and the flow was fixed at 300 µmol s^{-1}.

2.3 | ABA quantification

A total of 10 mg of dry material was used for hormone extraction performed according to Durgbanshi et al. (2005) with slight modifications. The dry residue obtained in the extraction was then re-suspended in a 9:1 H_2O:MeOH solution by sonication. The resulting solution was filtered and directly injected into a UPLC system (Waters Acquity SDS, Waters Corp.) interfaced to a TQD triple quadrupole (Micromass Ltd.) mass spectrometer through an orthogonal Z-spray electrospray ion source. Separations were carried out on a Gravity C18 column (50 × 2.1 mm, 1.8 µm, Macherey–Nagel GmbH) using a linear gradient of MeOH and H_2O supplemented with 0.1% acetic acid at a flow rate of 300 µL min^{-1}. Transitions for ABA/d^6-ABA (263 > 153/269 > 159) were monitored in negative ionisation mode. Quantitation of plant hormones was achieved by external calibration with known amounts of pure standards using Masslynx v4.1 software.

2.4 | Stomatal measurements

Stomatal aperture analyses were performed as described by Zandalinas et al. (2016). Briefly, two leaves per plant were cut and their lower surface was immediately stuck to a microspore slide with a medical adhesive (Hollister). The leaf was removed and the slides were washed with distilled water. The lower epidermis of the leaf stuck to the slide was visualised under the microscope and stomatal images acquired. Measurements of stomatal aperture were performed with the imaging software ImageJ (https://imagej.net/). At least 600 stomata were measured in each accession.

2.5 | GWAS and data analysis

Average values were obtained for each trait out of each of the biological replicates of each accession. These values were used to carry out GWAS. All GWAS were performed using GWAP (https://gwas.gmi.oeaw.ac.at/; Seren et al., 2013): a user friendly, interactive and freely available web platform that allows for versatile and precise GWAS analyses. We used a linear mixed model algorithm to correct the confounding effect derived from the population structure and multiple testing was corrected through the Bonferroni test to establish the significance threshold for SNP association at 5% false discovery rate. Genes associated with each hot spot are summarised in Table S3. Climatic data included the 19 Bioclimatic variables from the WorldClim database were downloaded from (https://worldclim.org) with a 2.5 m arc. Data were extracted with the "raster" R package (Supporting Information Data). Correlations and linear models with statistics were calculated with Sigmaplot11, the rest of statistical test (analysis of variance) were performed with the Statgraphics16.1 software. Results were plotted with the OriginPro9.0 software.

2.5.1 | qRT-PCR

RNA was extracted from fresh frozen tissue ground to fine powder with the RNeasy extraction kit from Qiagen according to the

manufacturer's instructions (Qiagen), and the quality of the extracted RNA was measured with a Nanodrop spectrophotometer (Nanodrop 2000, Thermo Scientific) to determine the RNA concentration and the absorbance ratios 260/280 and 260/230 nm to check for contaminations or impurities. A total amount of 5 µg from the extracted RNA was treated with DNase to remove the possibly extracted DNA (DNase I, Fermentas), again measuring the quality with the Nanodrop spectrophotometer. Finally, a total amount of 1 µg of the extracted RNA was retrotranscribed to complementary DNA (cDNA) using the Primescript RT Reagent Kit (Takara). Target gene accessions were obtained by searching the Arabidopsis TAIR database (Berardini et al., 2015). The primers used for the analysis of gene expression are provided in Table S3. Tubulin (TUB2) was used as housekeeping gene to normalise gene expression levels.

The RT-qPCR analysis was performed in an ABI StepOne detection system (Applied Biosystems). Briefly, the amplification was conducted in reactions containing 1 µL of cDNA solution, 5 µL of Maxima SYBR Green/ROX qPCR mix (Thermo Scientific), 1 µL of a 10 µM mix of forward and reverse primers (Table S3), and 3 µL of sterile deionized water to achieve a final volume of 10 µL per reaction. The amplification curve of temperatures consisted in 10 min at 95°C for preincubation and 40 cycles of amplification (each one with 10 s at 95°C for denaturation followed by 10 s at 60°C for annealing and an extension of 20 s at 72°C). The obtained results were processed with StepOne Software v2.3 and Relative Expression Software Tool v2 (Pfaffl et al., 2002).

3 | RESULTS

3.1 | Natural variation of water use physiological parameters

A screening to characterise parameters associated to water use was performed using a collection of 137 *Arabidopsis thaliana* ecotypes including Col-0 as the main reference to properly understand the quantitative variation in the studied parameters. An ecotype is a distinct population or group of organisms within a particular species that has adapted to a specific ecological niche or set of environmental conditions. It is characterised by unique traits, behaviours, or physiological adaptations that enable it to thrive in its specific habitat, ecotypes arise due to natural selection acting on genetic variation within a species. We also have included three mutant accessions in ABA biosynthesis (*aba2-1*) and signalling (*abi1-1* and *hab1-1 abi1-2*) to contextualise the natural variation in TN and WUE. Plants were grown for 4 weeks under short-day and well-watered conditions before multiple rounds of daily gravimetric water use and area measurements. With these data, average total transpiration (T), PLA, normalised transpiration per surface units (TN), and WUE values were determined. To facilitate comparison between ecotypes, parameters were referred to Col-0 values, which were defined as "one".

As previously reported, plants displayed a broad variation in whole plant transpiration values (Supporting Information Data). In fact, this variation was mostly due to differences in plant size (Figure 1), so that smaller plants tend to transpire less. However, more striking was the observation that even after normalisation per plant size, there was still a fivefold variation in TN among the ecotypes tested, with a smooth distribution between extreme behaviours (Figure 1c. Most ecotypes had similar transpiration to Col-0 (98 of 137 ecotypes had a TN between 0.75 and 1.25). Therefore, Col-0 was in the centre of the TN distribution, and it is a good representation of the average transpiration (inset in Figure 1c). Outlier ecotypes with higher TN were more common (30 ecotypes) than those with lower TN (9 ecotypes).

To contextualise this TN range, loss-of-function mutants were included in the study. High transpiration mutants in Col-0 background included *aba2-1*, an ABA deficient genotype (carrying a defective zeaxanthin epoxidase enzyme), with a TN of 1.96. The *aba insensitive 1* (*abi1-2*, with a truncated protein phosphatase type 2C) with a TN of 2.77 was in L*er* background (TN = 1.10). On the other hand, low transpiration mutants as *hab*1-1 *abi1-2* (loss of function in *abi1-1* and HYPERSENSITIVE TO *ABA1* [HAB1]) displayed a significantly lower TN (0.72) compared to Col-0 but higher than some low transpiring ecotypes analysed.

Despite the normalisation by plant size, TN values were appeared inversely correlated with plant size (Figure 1b). Plants with larger rosettes tend to have lower TN and vice versa. According to this correlation, PLA can explain a 29.4% of the variation in TN among the studied ecotypes. Both ABA deficient *aba1-2* and ABA insensitive *abi1-1* had higher TN than those explained by size, probably due to the cumulative effect of a deficient stomatal control (Figure 1b). Values of the hypersensitive *hab*1-1 *abi*1-2 fit within the expected values of low TN due to ABA hypersensitivity.

To relate whole plant transpiration with transpiration at leaf level, the number of leaves was measured in this population. Then, specific projected leaf area (SPLA) or area per leaf were calculated. Variation in leaf number was limited to a narrow range, hence PLA and SPLA were highly correlated (Figure 2a, R^2 = 81.4%). Specific leaf area is also significantly correlated with TN ($p < 0.001$), explaining a 36.7% of TN variation (Figure 2b). In this sense SPLA is a more informative variable than PLA to explain TN variation.

Given the strong correlation between plant leaf area (PLA) and total transpiration per unit of area (TN), we expected that productive WUE would also show a significant correlation with PLA. Since productive WUE depends on biomass (which we approximate using PLA) and we incorporate PLA into the TN calculation, we chose to calculate WUE as the ratio of the growth rate (change in PLA over a specific period) and the transpiration per unit of area during the same period. Our analysis revealed a significant correlation between PLA and WUE ($p < 0.001$), explaining 15.3% of the variation (see Figure 2c). These findings suggest that size not only has a direct relationship with biomass but also exerts an indirect influence on the transpiration rate, making it a crucial determinant of WUE.

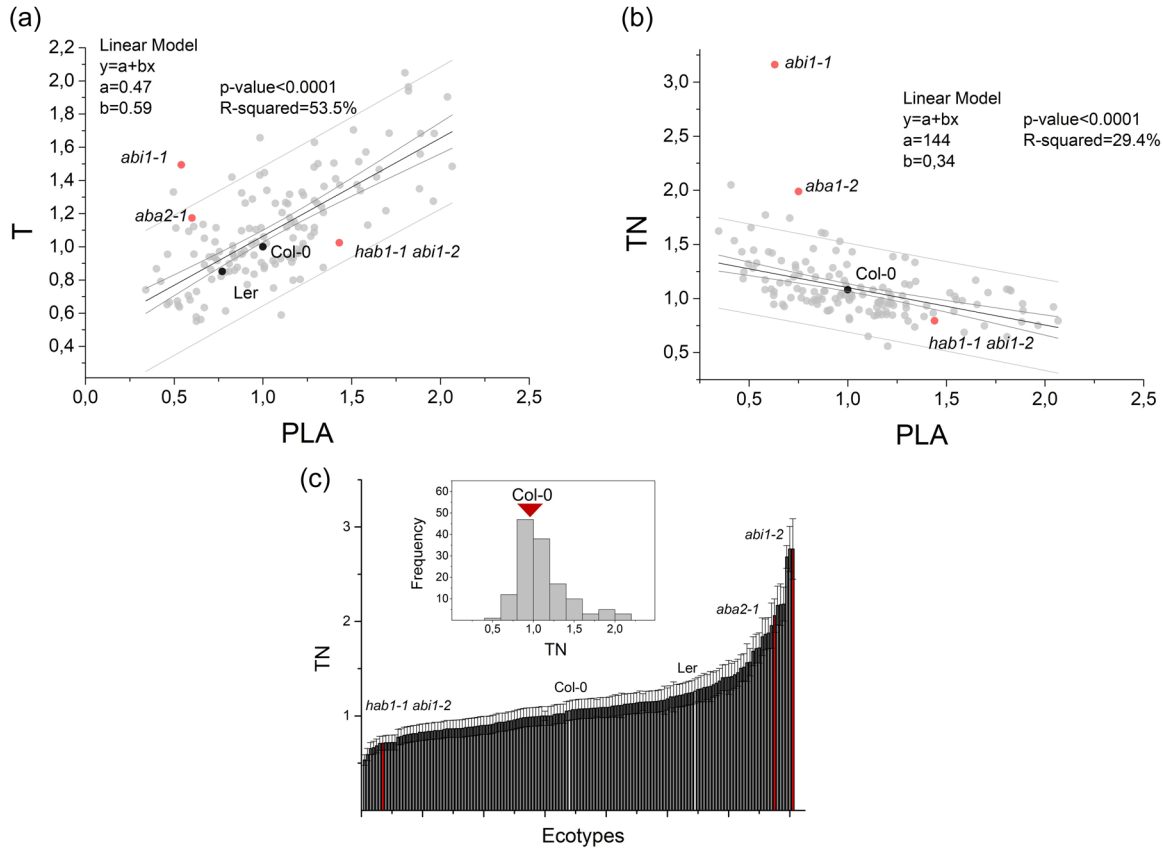

FIGURE 1 (a) Projected leaf area (PLA) versus whole plant transpiration (T). (b) Projected leaf area (PLA) versus whole plant transpiration normalised to rosette area (TN) Both PLA and T are normalised to Col-0 mean values. Grey circles represent results from 137 ecotypes, red circles represent results from ABA deficient (*aba2-1*), ABA insensitive in L*er* background (*abi 1-1*) and ABA hypersensitive (*hab 1-1 abi 1-2*) mutants. Black circles represent results from Col-0 and L*er*, $N = 8$ in all cases. Black line represents linear fitting of all points in the plot, grey lines denote confidence at 95% and light grey lines denote fitting prediction bands. Fitting, parameters and statistical significance are depicted in the table. (c) Transpiration normalised to rosette area (TN) of 137 ecotypes of *Arabidopsis thaliana* (grey bars) relative to Col-0 and L*er* (white bars) and ABA-related mutants *aba* 2-1 (L*er*), *abi* 1-2 (Col-0) and *hab* 1-1 *abi* 1-2 (Col-0, red bars). Values are the mean ± standard deviation of the normalised transpiration of 8 plants. Inset represents the cumulative frequency of transpiration values among the studied accessions.

3.2 | Correlation between physiological and climatic variables

Figure 3 illustrates the most noteworthy correlations observed among TN, PLA, WUE and Bioclimatic variables. Notably, a significant negative correlation ($r = -0.234$, $p = 0.004$) is observed between TN and Temperature seasonality (Figure 3a). This finding suggests that lower TN values are associated with greater variations in temperature throughout the seasons. Additionally, TN shows a positive correlation ($r = 0.245$, $p = 0.003$) with isothermality (Figure 3b). This indicates higher TN are associated with more stable climates with minimal fluctuations in seasonal temperature. The variations in the minimal temperature of the coldest month (Figure 3c, 3f, 3h) are likely the driving factor behind the differences observed in isothermality. High PLA values are positively correlated with seasonality (Figure 3d; $r = 0.244$, $p = 0.003$), indicating that larger leaf areas are associated with greater seasonal changes. On the other hand, PLA shows a negative correlation with isothermality (Figure 3e; $r = 0.174$, $p = 0.034$) and the minimum temperature of the coldest month (Figure 3g; $r = 0.214$, $p = 0.016$). This suggests that smaller rosettes are associated with reduced seasonal variations and isothermal conditions, likely driven by relatively higher minimal winter temperature.

3.3 | Evaluation of selected extreme ecotypes

Physiological characterisation of extreme ecotypes confirmed the positive correlation between WUE and plant size. Those with the highest and lowest WUE values dependent on TN were selected (Figure 4, green and red dots, respectively). Most of the ecotypes selected for low WUE displayed rosette areas (in terms of PLA) 25%–50% smaller than Col-0. Interestingly, most ecotypes selected for high WUE had areas 1.5–2 times larger than Col-0.

Ecotypes with a low WUE displayed values in the range of 0.2–0.7, most of them were in a narrow range between 0.2 and 0.4.

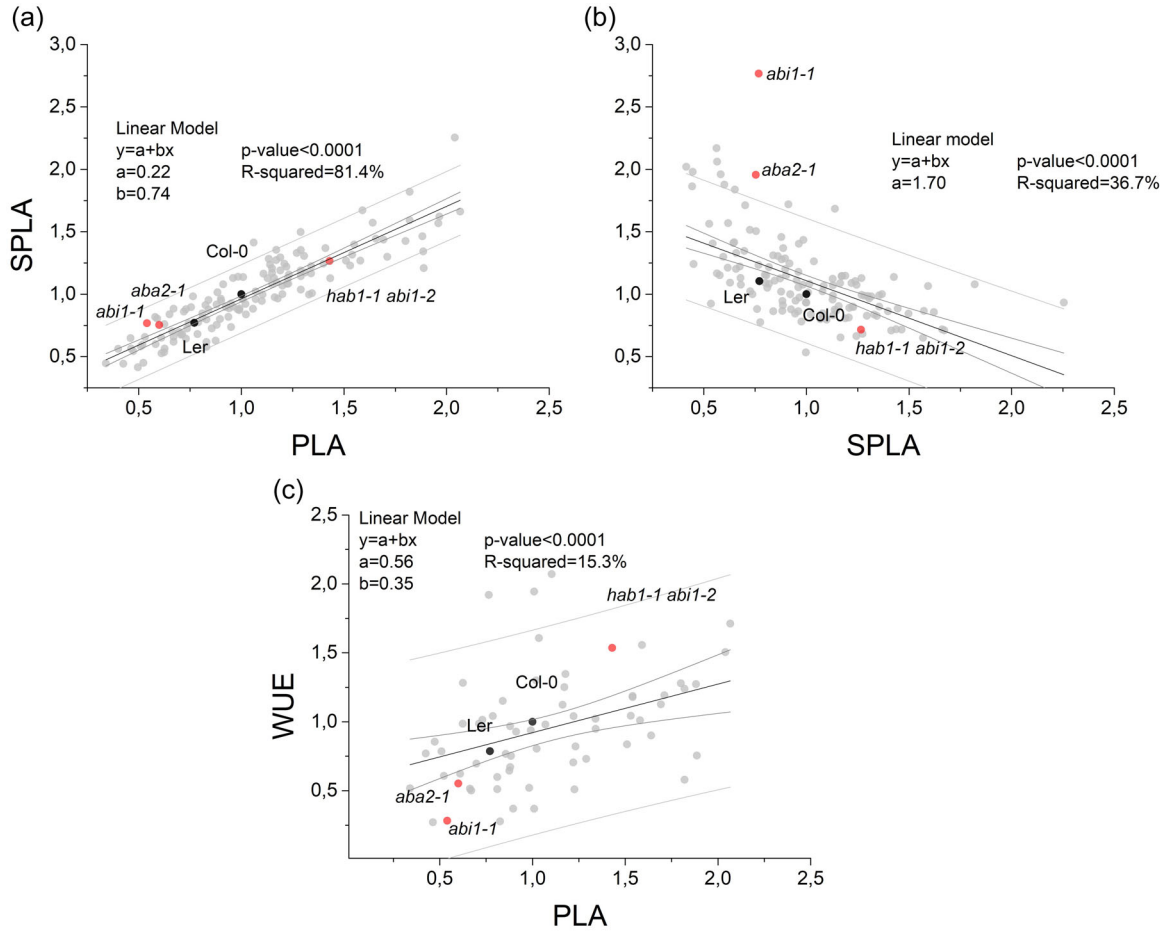

FIGURE 2 (a) Projected leaf area (PLA) versus specific projected leaf area (SPLA). Both PLA and SPLA are normalised to Col-0 mean values (b) Specific projected leaf area (SPLA) versus transpiration normalised to rosette area (TN) (c) Projected leaf area (PLA) versus water use efficiency (WUE). PLA, SPLA, TN and WUE are normalised to Col-0 mean values. Grey circles represent results from 137 ecotypes, red circles represent results from ABA deficient (*aba2-1*), ABA insensitive in Ler background (*abi 1-1*) and ABA hypersensitive (*hab 1-1 abi 1-2*) mutants. Black circles represent results from Col-0 and L*er*, *N* = 8 in all cases. Black line represents linear fitting of all points in the plot, grey lines denote confidence at 95% and light grey lines denote fitting prediction bands. Fitting, parameters and statistical significance are depicted in the table. [Color figure can be viewed at wileyonlinelibrary.com]

On the other hand, WUE in water saving ecotypes was in the range of 1.5–3.8 (Figure 5c). Ecotypes with a high WUE had similar low TN (0.8–0.6); on the contrary, ecotypes selected for low WUE had a broader range of TN (1.4–2.4).

Gas exchange parameters (photosynthetic rate [A], gas exchange [g_s] and iWUE [A/g_s]) were recorded to compare with gravimetric results and to further characterise these ecotypes (Figure 5). Little variation was found in A among selected ecotypes, with values ranging from 2.86 to 1.42 µmol m^{-2} s^{-1}. Accordingly, there was no correlation between PLA and A. Variation in g_s was larger, especially in accessions with low WUE. Therefore, g_s ranged from 0.13 to 0.06 mol H_2O m^{-2} s^{-1}. Ecotypes with high WUE had significantly lower g_s than Col-0 Instantaneous water loss values were significantly correlated with TN ($p < 0.001$ and a $R^2 = 0.87$). These results at leaf level parallels the results of whole plant gas exchange characterisation.

iWUE was similar to previously characterised WUE, with a significant correlation between them ($p < 0.001$) and a $R^2 = 0.69$ (Figure 5i). In contrast with WUE measurements, there was little variation in iWUE among selected high WUE ecotypes, with no significant differences between the top lines. Similarly, low WUE accessions had similar iWUEs, with values in the range of 39.23–14.50 µmol m^{-2} s^{-1}/mol H_2O m^{-2} s^{-1}. Although iWUE results follow the same trend that WUE, differences between ecotypes in the same group are minimal, suggesting that WUE is a more sensitive parameter than iWUE to screen for high and low efficiency in water use.

Stomatal density and ABA levels are traits that have been related to variations in transpiration and WUE in experiments with loss of function mutants and overexpressing lines (Caine et al., 2019; Hepworth et al., 2015; Saez et al., 2004). However, in our ecotype collection there was no correlation between stomatal density and TN

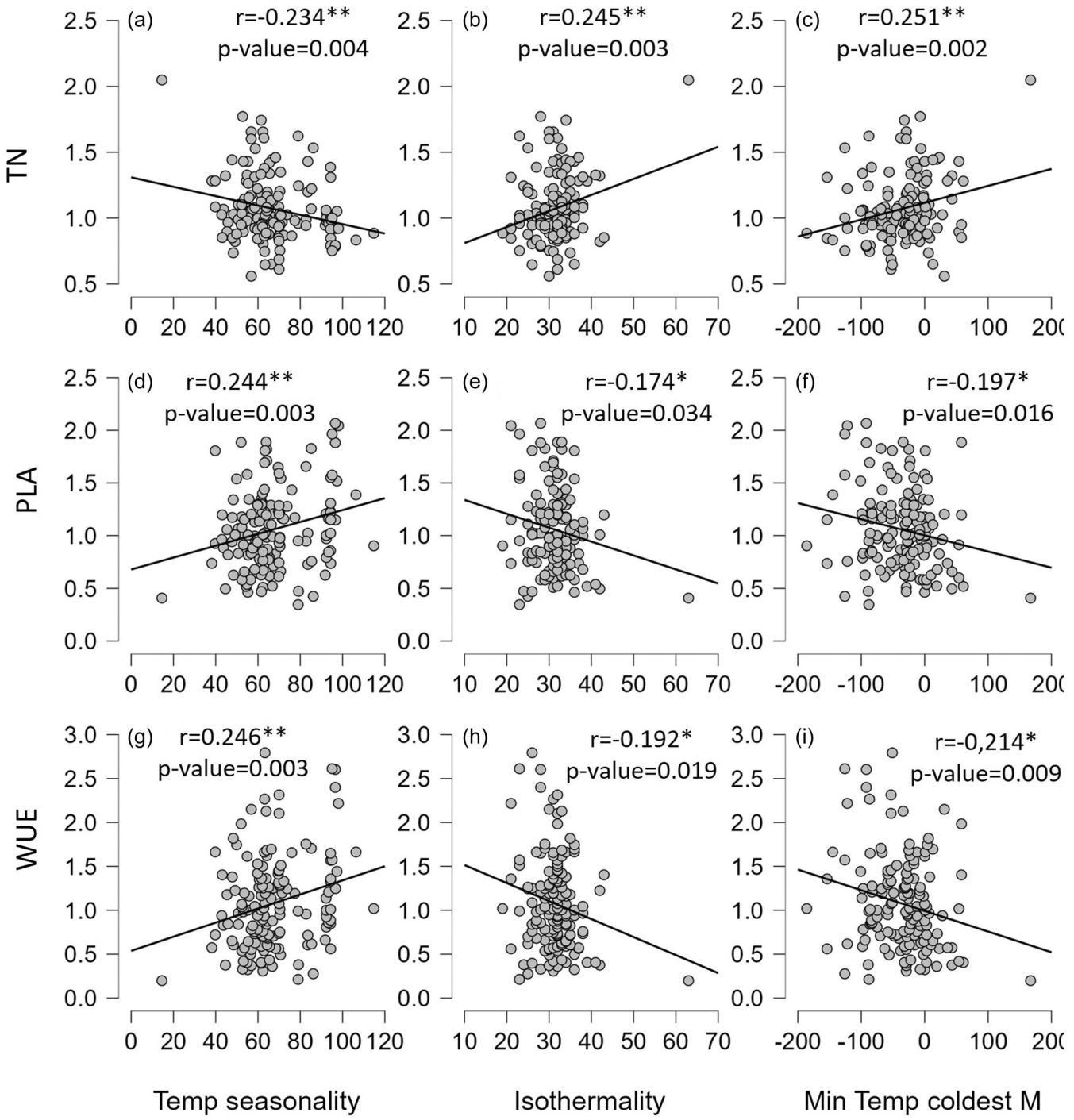

FIGURE 3 Correlation between climatic parameters and Transpiration normalised to rosette area (TN), projected leaf area (PLA) and water use efficiency (WUE) measured across 137 ecotypes and each ecotype local climatic variables. Correlations between [(a), (d), (g)], temperature seasonality (standard deviation × 100) [(b), (e), (h)] isothermality (mean of monthly (max temp-min temp)/maximum temperature warmest month − minimum temperature of coldest month)) [(c), (f), (i)] minimum temperature of the coldest month (°C × 100). Circles represent the genotypic mean of each accession. Fitted black lines are linear regression. Each correlation displays a linear equation with Persons *r* and *p* value. Asterisks denote the value of *r* significance (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

or WUE despite the significant variation in stomatal density found neither among the extreme ecotypes (Figure 6) nor the characterisation of stomatal density and size of 86 ecotypes within our collection (Supporting Information Data). In general, ABA concentrations in the selected ecotypes (Figure 6) had little variation compared to Col-0 except for Nyl-2 and Cvi-0. There was no correlation between ABA levels and transpiration or WUE values under well-watered conditions.

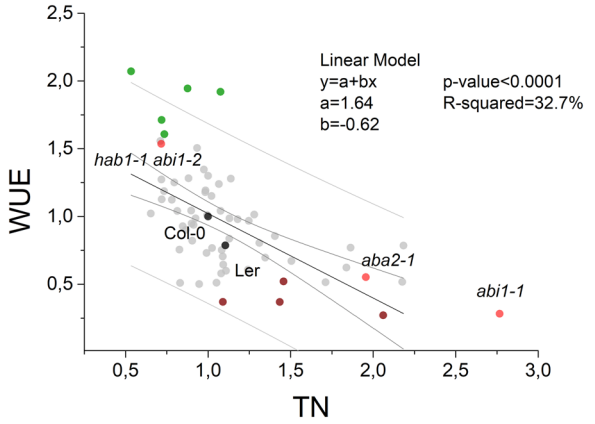

FIGURE 4 Whole plant transpiration normalised to rosette area (TN) versus water use efficiency (WUE). Both TN and WUE are normalised to Col-0 values. Grey circles represent results from 65 ecotypes, red circles represent results from ABA deficient (*aba2-1*), ABA insensitive (*abi 1-1*) and ABA hypersensitive (*hab 1-1 abi 1-2*) mutants. Black circles represent results from Col-0 and L*er*, $N = 8$ in all cases, dark red and green circles represent ecotypes with low and high WUE respectively selected for further analysis. Black line represents linear fitting of all points in the plot, grey lines denote confidence at 95% and light grey lines denote fitting prediction bands. Fitting, parameters and statistical significance are depicted in the table. [Color figure can be viewed at wileyonlinelibrary.com]

3.4 | GWAS identify candidate genes for WUE

A GWAS to find loci associated with WUE was performed. In addition, hot spots associated with TN and PLA were examined as both traits are important to overall WUE. The genome association with the trait WUE gave 9 significant hot spots, 5 of them co-localised with PLA hot spots. The other 4 hot spots were specific (Figure 7a, WUE b, c, d and e) WUE-b is localised in Chr1, WUE-c and WUE-d are in Chr3 and WUE-e is in Chr4 close to a colocalized PLA hot spot, genes within are indicated in Supporting Information Data. The genomic association with the trait TN gave two significant hot spots in chromosomes 2 and 4, labelled as TN-a and b) in Figure 7. TN-a was supported by very few SNPs while TN-b was supported by a greater number of SNPs around position 585125. Within the genomic region contained in this hot spot, a family of Ring/U-box proteins (AT4G09100, AT4G09110, AT4G09120 and AT4G09130) was found. We also found a fifth gene, AT4G09140, encoding a protein similar to Mut1, a mismatch repair protein.

Genomic association with PLA gave 12 significant hot spots, 4 in Chr1, 3 in Chr4 and 5 in Chr5. Positions and genes in the hot spots are summarised in Supporting Information Data.

To explore and validate the results obtained in the GWAS, we selected candidate genes within four of the hot spots regions for WUE, based on the identity and previous information available. WUE-b comprised three genes (AT1G70900, AT1G70910 and AT1G70920). AT1G70900 is classified as a hypothetical protein; AT1G70910 encodes DESPIERTO, a protein involved in ABA sensitivity during seed development that regulates the expression of ABI3 (Barrero et al., 2010); and AT1G70920 encodes the homeobox-leucine zipper protein 18.

When 4-week-old seedlings were analysed, only *despierto* WUE values were significantly lower than those of the other candidates and Col-0 (Figure 8c), this lower WUE was explained due to a significantly smaller PLA (Figure 8a) whereas its TN (Figure 8b) was similar to that of Col-0. Microscopic analysis of the leaves also showed that this loss-of-function mutant (Barrero et al., 2010) had a lower stomatal density and smaller stomata (Figure 8d,e) compared to the other candidates within the hot spot and Col-0.

We also selected AT3G05700 from hot spot WUE-d, AT4G09000, AT4G09020 from hot spot WUE-e and AT4G13180 from WUE-f. AT3G05700 encodes Di19-3, a DNA binding protein with transcription activation activity. It is expressed in response to osmotic, drought and ABA stress (Qin et al., 2014). AT4G09000 and AT4G09020 encode a 14-3-3 protein also named GRF1 chi, and an isoamylase involved in starch breakdown, respectively. Finally, AT4G13180 encodes a NAD(P)-binding Rossmann-fold superfamily protein. Only loss of function in AT4G09000 (14-3-3) produced a significantly reduced WUE, which was associated with a smaller rosette size, TN values were similar to Col-0 (Figure 9). However, stomatal features were only affected in the Di19-3 loss of function, which showed lower stomatal density and larger stomata (Figure 9d,e).

4 | DISCUSSION

In this work, we have identified plant size as one of the main factors controlling TN and WUE after an extensive characterisation of natural variation of whole plant transpiration and WUE in a collection of *Arabidopsis thaliana* ecotypes. At the genetic level, several hot spot driving changes in TN and WUE have been identified supporting the genetic basis for the variation of these traits. Analyses of loss-of-function candidates within those hot spots has allowed confirming their role in WUE. Natural variation associated with these genes have the potential of shaping water use and growth adaptation to environmental conditions. At the ecophysiological level, we have uncovered novel correlations between local climatic parameters and whole plant transpiration, water use, and size. Data suggest that covariation of size and transpiration could be crucial for plant adaptation to different environmental niches.

Transpiration is a transverse trait that has a major influence on plant adaptation to environment and overall plant performance according to any given definition (agronomical, ecological or physiological). In the same way that growth, whole plant transpiration is affected by the genetic background, the physiology and the environment. This context must be accounted to have a good perspective of the key factors shaping its impact on plant physiology.

4.1 | Transpiration variability is limited to a narrow range in most ecotypes

Most ecotypes (including Col-0) had similar TN values. Extreme TN values were asymmetrically distributed and ecotypes with TN values

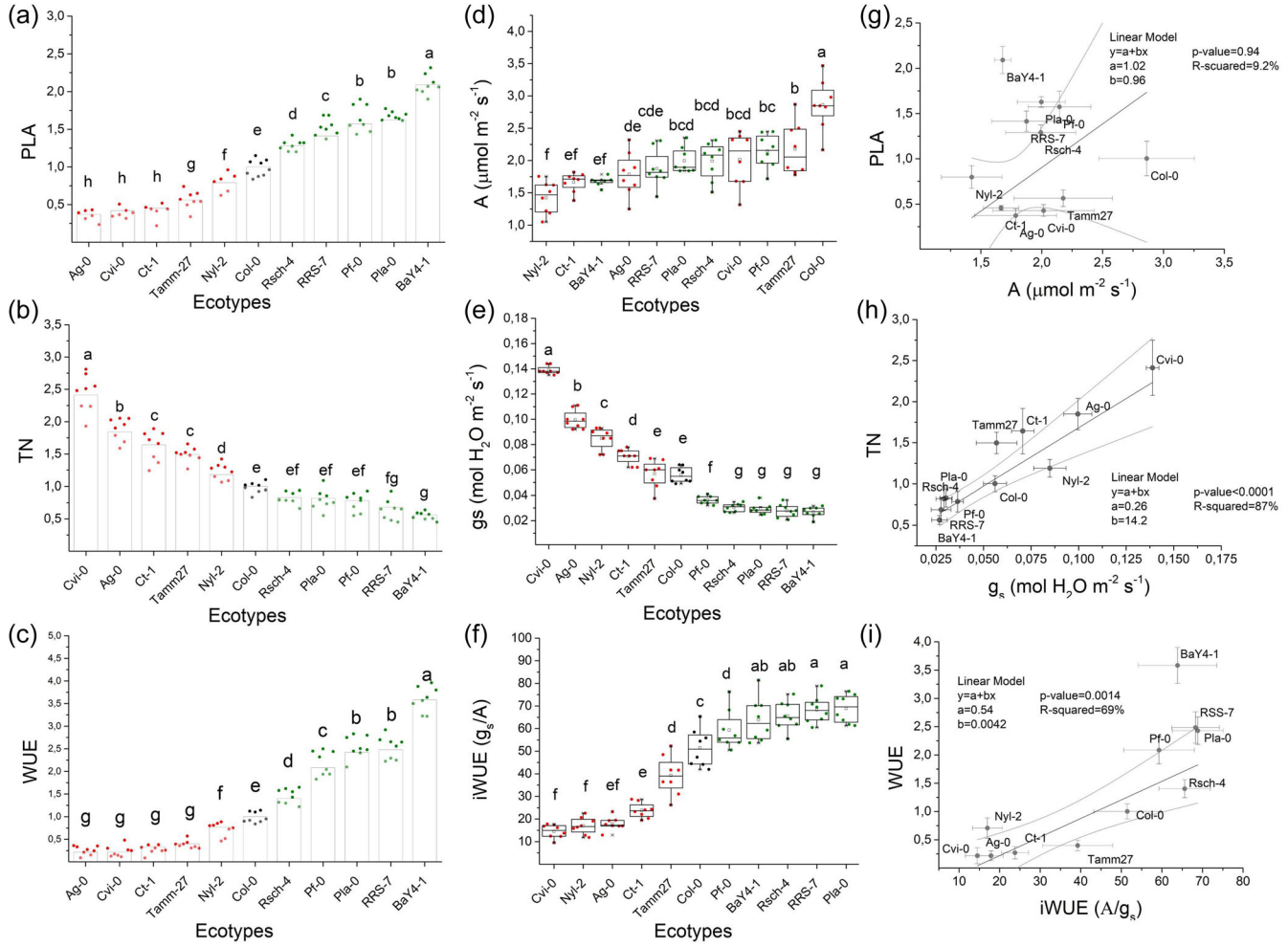

FIGURE 5 Projected leaf area [PLA, (a)], whole plant transpiration normalised to rosette area [TN, (b)] and water use efficiency [WUE, (c)] of selected ecotypes with high/low WUE (green and red scatter points, respectively), bars show the average results of $N = 8$ and points show the dispersion of the values. PLA, TN and WUE are normalised to Col-0 values and are dimensionless. Letters denote significant differences between ecotypes after ANOVA. Photosynthesis rate [A, (d)], stomatal conductance [g_s, (e)] and instantaneous water use efficiency [iWUE, (f)] of selected ecotypes. Box represents median and 25 and 75th percentile, whiskers represent 1.5 per interquartile range. ANOVA, analysis of variance. [Color figure can be viewed at wileyonlinelibrary.com]

higher than Col-0 were more abundant than those with lower values. Thus, in the context of this particular ecotype subset, Col-0 should be classified as water conservative. Low transpiration is considered a desirable trait as it usually parallels high WUE if coupled with a high growth rate (leading to a high biomass). A low water spending phenotype can be crucial for survival under natural conditions although in some environments, leaf cooling and nutrient uptake can be more important than water saving to obtain a good yield (in crops) or to improve reproductive fitness (in wild plants). TN values lower than 0.5 probably imply a costly trade-off in terms of a limited photosynthesis rate or an insufficient cooling under warm conditions. However, some of the low transpiring ecotypes (with TN values in the range of 0.55–0.75) display large rosette areas, which suggests that, in terms of transpiration, there is genetic potential to decrease TN and achieve greater WUE without a yield penalty. Therefore, we hypothesise that breeding programs based on the adaptation mechanisms of natural populations could be successful in present and future climate change scenarios.

4.2 | Rosette size can limit transpiration rate

Plant size is a significant factor affecting T for obvious reasons and explains a 53% of the variation found in whole plant transpiration. By normalising to leaf area, we would shift from an extensive to an intensive variable (TN) that theoretically would be independent of plant size. However, PLA shows an inverse significant correlation with TN, explaining up to a 29% of its variation. Similar correlations have been previously reported in several plant species. The characterisation of black poplar varieties with contrasting leaf surface (Bogeat-Triboulot et al., 2019) showed that genotypes with higher leaf area had a much lower transpiration rate per unit of area,

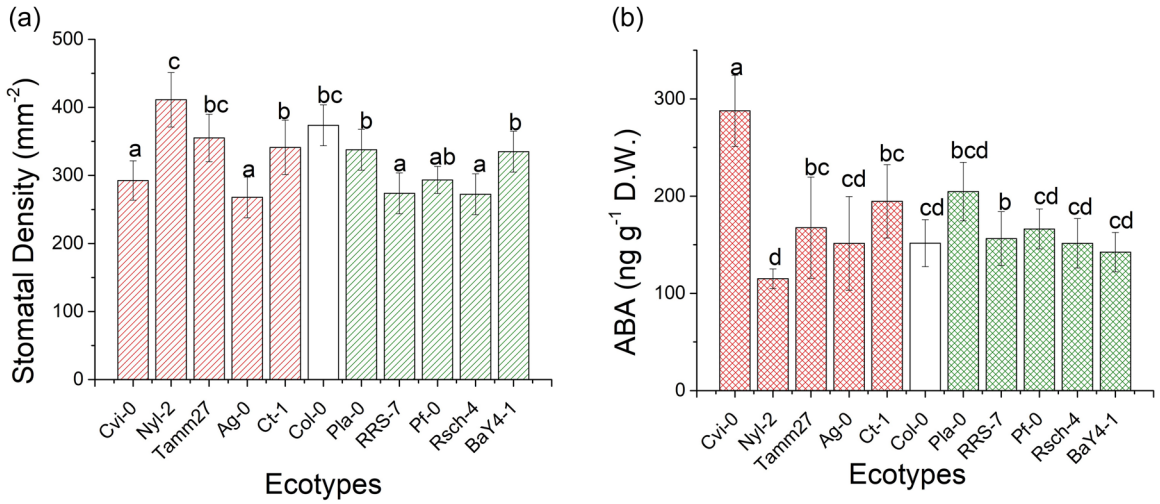

FIGURE 6 (a) Stomatal density of selected ecotypes with high/low WUE (green and red bars, respectively) bars represent the average results of $N = 8$ and with standard deviation. (b) Rosette ABA concentration under well-watered conditions of selected ecotypes with high/low WUE (green and red bars, respectively) bars show the average results of $N = 8$ with standard deviation. Letters denote significant differences between ecotypes after ANOVA. ANOVA, analysis of variance; WUE, water use efficiency. [Color figure can be viewed at wileyonlinelibrary.com]

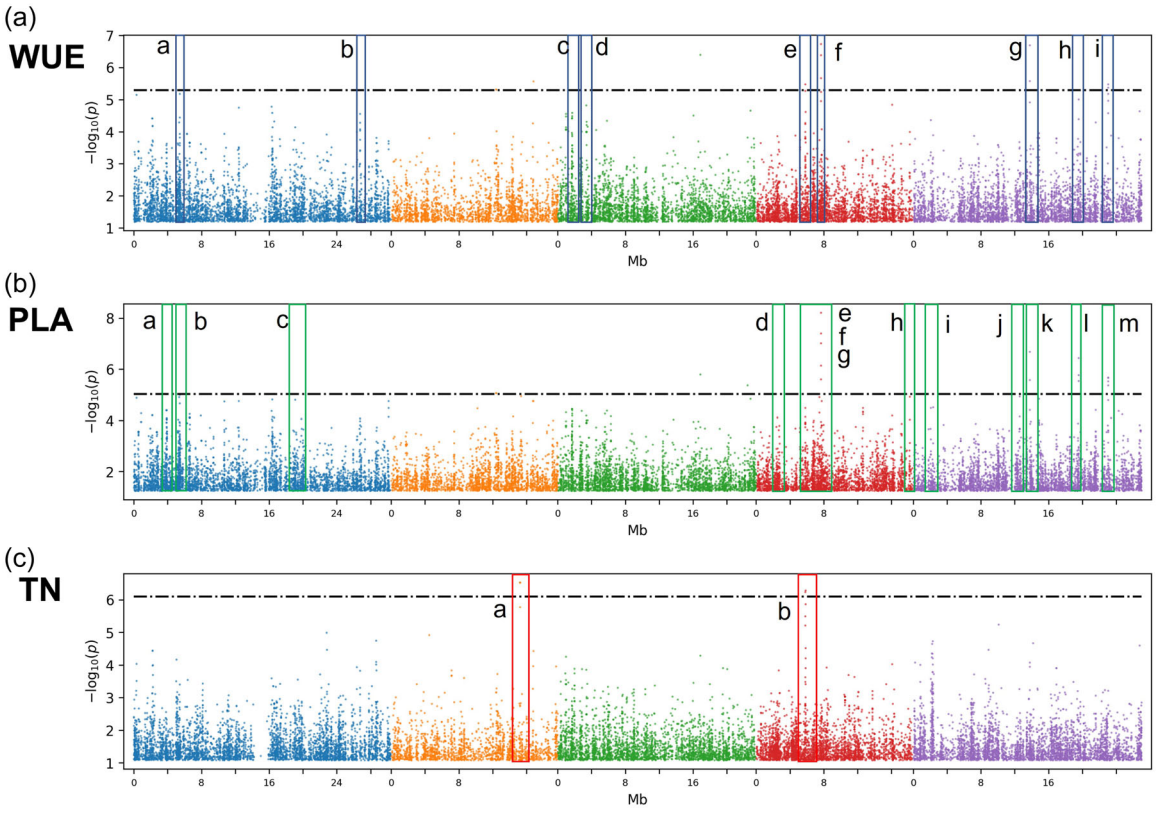

FIGURE 7 Manhattan plot of significant GWAS hot spots of (a) Water use efficiency (WUE), (b) Projected leaf area (PLA) and (c) whole plant transpiration normalised to rosette area (TN). Boxes mark significant hot spots locations labelled with letters for each trait. GWAS, genome-wide association studies. [Color figure can be viewed at wileyonlinelibrary.com]

reducing total water loss, and resulting in a higher WUE. Wang et al. (2019) found that the transpiration rate was inversely proportional to the leaf size in a study with 16 different tree species. Modelling of global leaf size in grasses also shows that small leaves display higher transpiration rates (Baird et al., 2021). In circadian clock mutants of Arabidopsis, biomass and WUE were found to be tightly correlated (Simon et al., 2020). Also, a collection of stomatal mutants showed that low rosette sizes were correlated with high TN and low WUE

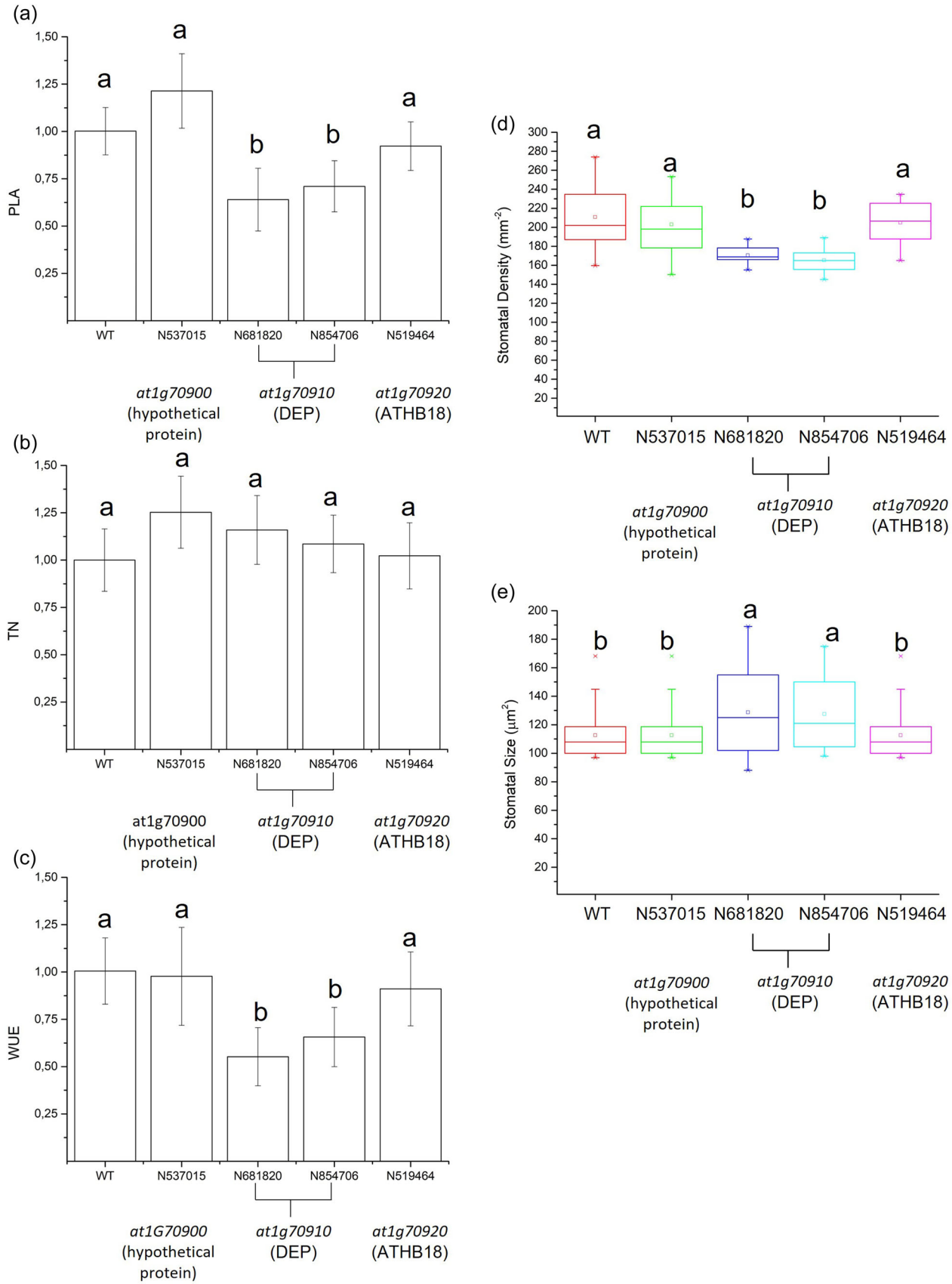

FIGURE 8 Projected leaf area [PLA, (a)], transpiration normalised to leaf area [TN, (b)] and water use efficiency [WUE, (c)] of selected loss-of-function accessions, bars denote standard deviation of $N = 6$. Boxplot of stomatal density (d) and stomatal size (e), box represents median and 25 and 75th percentile, whiskers represent 1.5 per interquartile range and letters denote significant differences between accessions after ANOVA. ANOVA, analysis of variance. [Color figure can be viewed at wileyonlinelibrary.com]

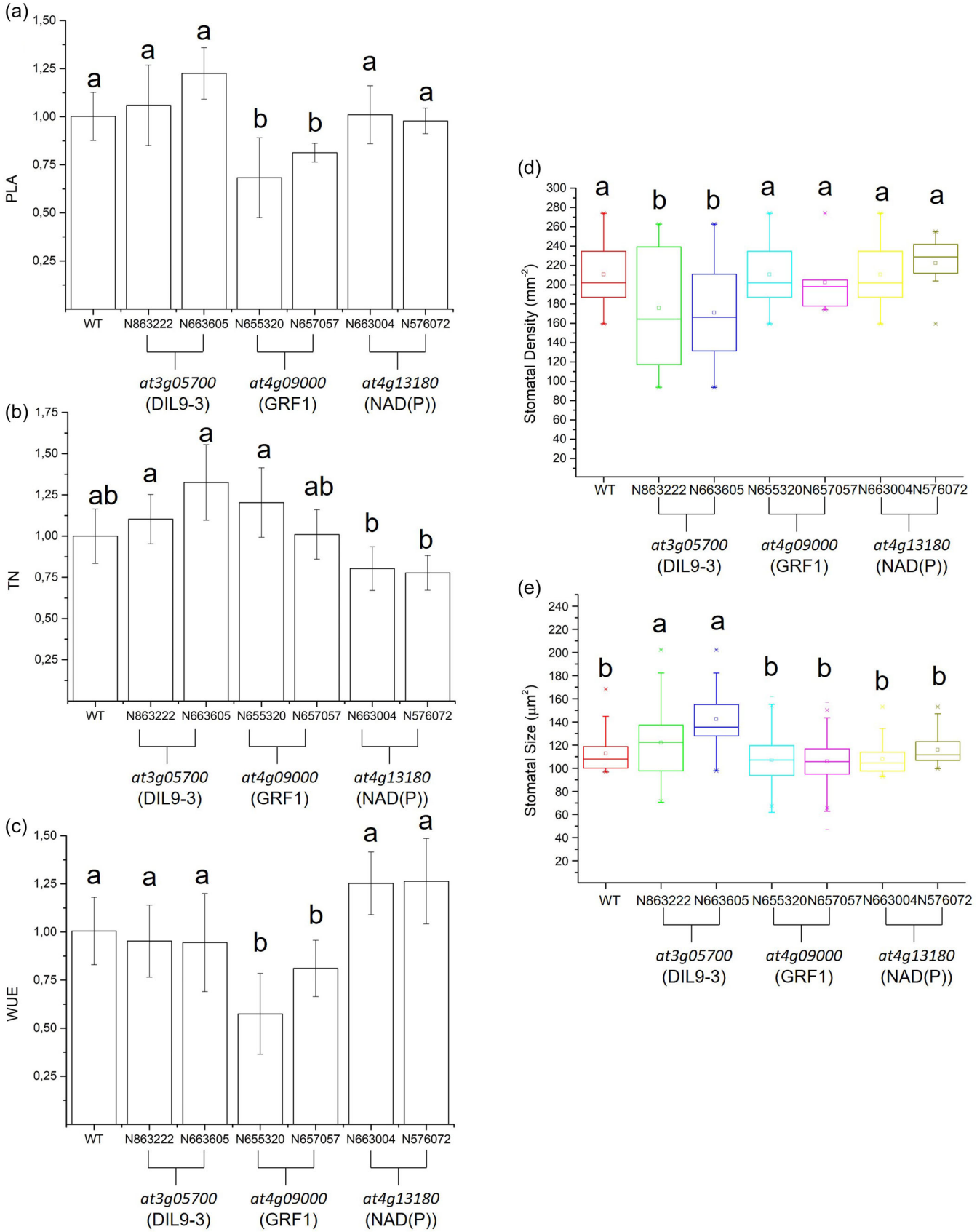

FIGURE 9 Projected leaf area [PLA, (a)], transpiration normalised to leaf area [TN, (b)] and water use efficiency [WUE, (c)] of selected loss-of-function accessions, bars denote standard deviation of $N = 6$. Stomatal density (d) and stomatal size (e), box represents median and 25 and 75th percentile, whiskers represent 1.5 per interquartile range and letters denote significant differences between accessions after ANOVA. ANOVA, analysis of variance. [Color figure can be viewed at wileyonlinelibrary.com]

(Lawson et al., 2014). This inverse correlation between plant size and transpiration, although not new, has been quantified in our work and it is shown to be a limiting factor defining the transpiration ranges for each ecotype. Most of work cited above identify leaf size as the main parameter influencing transpiration. In our population, mean leaf size and total rosette size are highly correlated ($R^2 = 83\%$) so the correlation between PLA and TN is very similar to the correlation between the specific leaf area and TN.

Our data suggest that in small ecotypes, plant size is negatively correlated with TN whereas this dependence is less obvious in bigger plants. This could be mechanistically explained by the leaf boundary layer conductance (Yates et al., 2010). Thickness of the boundary layer influences how quickly gasses and energy are exchanged. Boundary layer conductance works in series with stomatal conductance and is mainly determined by leaf size and morphology (Martin et al., 1999). According to our data the influence of size to limit TN is more obvious in smaller than in bigger plants, were TN reaches a plateau independently of plant size. Other traits such as stomatal density are significant factors regulating transpiration. However, neither in the whole population nor in the extreme ecotypes significant correlation was found between stomatal density and transpiration.

4.3 | Size and WUE are determined by multiple loci with partial colocalization

Genetic analysis of the phenotypic variation in WUE, TN and PLA by GWAS validated the physiological results, obtaining several candidate loci with the potential to explain the genetic basis of the variation in these traits. Moreover, the partial co-localisation of several QTL in WUE and PLA points to a PLA-dependent and -independent variation of WUE and can offer key genetic resources for crop breeding.

Results did not show any strong phenotype in terms of PLA and WUE. It is likely that extreme phenotypes for these traits are polygenic, and the contribution of each gene is relatively small. In any case, the systematic characterisation of all possible contributors to these QTLs is an ongoing effort.

4.4 | Temperature-related parameters drive ecophysiological variation of TN, PLA and WUE

Environmental factors drive plant evolution and define ecotype geographical distribution. Plants must optimise fitness and assure survival and reproduction according to local environments defined by climatic parameters. In that context, TN would be a key trait susceptible to be modified for adaptation to climate conditions. Given the high correlation between TN and size reported in this work, it would be expected that this covariation would impose significant trade-offs to ecotype adaptation to environment.

We have found significant correlations between TN and some temperature derived climatic variables at the geographic origin of the ecotypes (correlations and statistical significance of all 19 Bioclimatic variables and TN, PLA and WUE are available in Supporting Information Data Files)

The most significant correlations observed among TN, PLA, and WUE, along with the bioclimatic variables, suggest a positive correlation between plant size (as measured by PLA) and high seasonality, as well as low isothermality. This relationship is likely driven by the variable of low minimum temperatures in the coldest month. Additionally, considering the high correlation between PLA and TN, it is reasonable to assume that TN is inversely correlated with these parameters. In other words, low TN values are associated with high seasonal temperature changes and low isothermality.

These correlations point towards environments characterised by mild winters and warm summers, similar to the climate found in places like the Canary Islands. On the other hand, small and high transpiring ecotypes are more likely to be associated with areas of high isothermality and low seasonality, such as the southwestern part of England.

The use of homogeneous conditions in all ecotypes can effectively diminish the genotype × environment interaction. Ecotypes are intricately adapted to their respective native environmental conditions, enabling them to occupy specific ecological niches. For instance, an ecotype that exhibits high transpiration in its native habitat may possess a competitive advantage in leaf cooling during elevated temperatures. This interaction is notably complex and intellectually stimulating.

In annual plants, successful strategies also encompass the optimisation of plant-climate interactions through finely tuned germination and flowering times. The genuine exploration of the ecotype's interaction with its native environment provides invaluable insights into the adaptation of life history traits across diverse environments. Consequently, this understanding allows for the reverse engineering of strategies or the identification of desirable traits applicable to crop improvement. Remarkable examples of this approach can be found in Exposito-Alonso et al. (2018, 2019)

It is important to recognise the resource-intensive nature and logistical challenges associated with conducting multisite experiments involving ecotypes and their native environments. As an alternative approach, researchers have employed standardised conditions by homogenising environmental factors to analyze the responses of ecotypes. Although such conditions may not fully represent the native environments to which the ecotypes are adapted, this experimental design allows for the examination of genetic influences on physiological processes, while minimising confounding environmental factors.

There are numerous examples of this strategy. For instance, the works of Milhinhos et al. (2019) and studies focused on hormones and water-use efficiency, such as Kalladan et al. (2017), have demonstrated the efficacy of this approach. These studies have provided valuable insights into the genetic regulation of physiological processes and the underlying mechanisms of plant responses. In our specific case, employing this chosen strategy has enabled the quantification of transpiration rates and characterisation of this

fundamental trait. Furthermore, we have established associations between transpiration and certain anatomical features, such as leaf size, while also identifying the partial influence of specific genes. Notably, we have observed weak but statistically significant correlations between transpiration and the ecotypes native climates. To validate these correlations, a distinct approach involving field-based reciprocal common garden experiments conducted within the targeted range of interest would be necessary. This rigorous validation process would entail the creation (and subsequent validation) of a robust model.

5 | CONCLUSIONS

Here, we have characterised the extent of Arabidopsis natural variation in TN under homogeneous environmental conditions and its dependence on plant size. This will help creating better models explaining plant overall water use and strategies of climate adaptation. It would be interesting to test the impact of transpiration in ecotype local environments with different climatic conditions to fully understand the influence of natural variation of transpiration on plant life history traits. The covariation between size and TN has physiological and ecological implications. At the physiological level, differences in size among transpiration mutants must be taken in consideration when drawing conclusions as significant changes in plant size will be accompanied by changes in transpiration. At the ecological level, the limitation of TN driven by a high PLA can offer a synergistic WUE increase in larger plants but can be a disadvantage under high temperatures if limitation in TN does not allow proper heat dissipation or nutrient uptake.

Given the drastic changes in climatic conditions that the planet is facing, it is necessary to understand the most successful plant strategies for climate adaptation. As previously stated, transpiration is a trait that links WUE, photosynthesis, thermoregulation and nutrition. All these processes are critical for plant fitness and key targets in agriculture. We have provided empirical data on the variation of T, TN, PLA and WUE and proposed a model to explain the significant dependence between transpiration and size. We have also been able to link natural variation of these parameters to climatic data, finding a significant correlation with temperature-derived parameters. Finally, we have characterised some of the candidates obtained from GWAS on these parameters. This work deepens our understanding of the central role of transpiration on plant adaptations to the environment.

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DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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