1 **1. TITLE**

2 Genetic determination of stomatal patterning in winter wheat (*Triticum aestivum* L.)

3 **2. AUTHORS**

4 Steven Yates¹, Andreas Bruun¹, Marius Hodel¹, Christoph Grieder², Andreas Hund³,
5 Achim Walter³ and Bruno Studer¹

6 ¹ Molecular Plant Breeding, Institute of Agricultural Sciences, ETH Zürich, 8092 Zurich,
7 Switzerland

8 ² Agroscope, Reckenholzstrasse 191, 8046 Zurich, Switzerland

9 ³ Crop Science, Institute of Agricultural Sciences, ETH Zürich, 8092 Zurich, Switzerland

10 **3. CORRESPONDING AUTHOR**

11 bruno.studer@usys.ethz.ch

12 **4. SIGNIFICANCE STATEMENT**

13 Stomatal density in European winter wheat germplasm is determined by three QTL
14 on chromosomes 2A, 3A and 7B, closely linked SNP markers provide opportunities to exploit
15 allelic diversity for crop improvement.

16 **5. Key words**

17 Stomata, Stomatal density, Wheat (*Triticum aestivum* L.), Genome-wide association
18 studies (GWAS), *INDUCER OF CBF EXPRESSION 1 (ICE1)*, *STOMATAL CYTOKINESIS-DEFECTIVE*
19 *1 (SCD1)*, Drought tolerance, Water use efficiency (WUE)

20 **6. RUNNING HEAD**

21 Stomatal patterning in winter wheat

22 **7. ABSTRACT**

23 Leaf stomata are microscopic pores mediating plant-environment interactions. Their
24 role in carbon uptake and transpiration make them prime candidates for improving water

use efficiency (WUE). Stomatal density (SD), the number of stomata per unit area, has been shown to be negatively correlated with WUE. However, little is known about the genetic basis of SD in wheat (*Triticum aestivum* L.), and to what extent genetic variation exists in contemporary wheat germplasm. Here, we evaluated stomatal patterning over two growing seasons in a set of 333 wheat lines, representing the European winter wheat germplasm. Stomatal patterning was mainly determined by two underlying traits, the distance between files of stomata and the distance between stomata within a file. By haplotype association mapping, quantitative trait loci for SD were consistently detected in both seasons on wheat chromosomes (CHR) 2A, 3A and 7B. The single nucleotide polymorphism markers most significantly associated with SD coincided with the genes *INDUCER OF CBF EXPRESSION 1 (ICE1)* and *STOMATAL CYTOKINESIS-DEFECTIVE 1 (SCD1)* on CHR 3A, and genes involved in ethylene and auxin signaling on CHR 2A and 7B, respectively. Our study unlocks the phenotypic and genotypic variation for stomatal patterning traits in contemporary wheat germplasm. It provides gene targets for functional validation and practical tools to manipulate SD using marker-assisted selection for crop improvement.

8. INTRODUCTION

Wheat (*Triticum aestivum* L.) is a major cereal crop, annually grown on more than 200 million hectares worldwide (source: <http://www.fao.org/faostat/>). The demand for wheat is expected to increase, however, the current gains in productivity are around half of that required (Fischer *et al.* 2014, Reynolds *et al.* 2012). In previous decades, productivity has been improved by increasing the harvest index (i.e. the biomass partitioned into seed) and light interception efficiency (Hepworth *et al.* 2015). However, these traits have limited room for improvement (Zhu *et al.* 2010). In the future, adverse environmental conditions are a major threat to wheat production (Fischer *et al.* 2014). Notably, average global temperatures are rising and expected to further increase (Long and Ort 2010). Alone the increase in temperature could reduce the yield of wheat in Australia by 50% and have negative repercussions in Europe (Asseng *et al.* 2011). Moreover, elevated temperatures often concur with drought periods (Trenberth *et al.* 2014). The coincidence of heat and drought at the time of flowering compounds yield losses (Semenov and Shewry 2011, Stratonovitch and Semenov 2015). Thus, the adaptation of cultivars to future environments is a means to improve and maintain productivity (Reynolds *et al.* 2012).

56 Given the challenges wrought by elevated temperatures and water shortage,
57 increasing crop productivity with less water is integral to sustainable agriculture.
58 Physiologically, this is defined as water use efficiency (WUE), i.e. the assimilated carbon per
59 unit of water lost (Stanhill 1986). An apparent morphological feature affecting WUE is the
60 number and distribution of leaf stomata. Stomata are microscopic pores, which let CO₂ in
61 and water out, for assimilation and cooling, respectively. A number of studies have shown a
62 positive correlation between stomatal density (SD) and transpiration (Miskin *et al.* 1972,
63 Schoppach *et al.* 2016, Xu and Zhou 2008). Moreover, stomatal conductance, the water and
64 CO₂ exchange capacity of the leaves, has been shown to correlate with WUE (Liu *et al.* 2015,
65 Meng and Yao 2015, Wang *et al.* 2016a, Yoo *et al.* 2010), although some studies did not
66 reach the same conclusions (Lawson *et al.* 2014a). Nevertheless, a number of studies have
67 meanwhile confirmed that decreasing SD improved WUE in dicots (Hepworth *et al.* 2015,
68 Lawson *et al.* 2014b) and in the monocots barley (*Hordeum vulgare* L.) (Hughes *et al.* 2017)
69 and rice (*Oryza sativa* L.) (Caine *et al.* 2018). In addition, SD has been linked with drought
70 and heat stress and is therefore a promising means to improve abiotic stress tolerance
71 (Hepworth *et al.* 2015, Wang *et al.* 2016a).

72 The stomata only account for 5% of the leaf area, yet 70% of water loss is mediated
73 through them (Willmer and Fricker 1996). Their size, frequency and speed of response are all
74 important factors determining WUE (Hetherington and Woodward 2003, Lawson and Blatt
75 2014). In wheat, as with many other grasses, the stomata are arranged in linear files on the
76 adaxial leaf surface either side of a vein. Yet the ordered arrangement of stomata in wheat
77 has received little attention (Raissig *et al.* 2016). Plants can modulate SD in response to
78 environmental conditions such as elevated CO₂, light intensity, temperature and soil
79 moisture (Hetherington and Woodward 2003). Despite this plasticity in response to
80 environment, SD has a strong genetic component (Shahinnia *et al.* 2016, Wang *et al.* 2007).
81 Many genes controlling stomatal development and density have been identified in
82 *Arabidopsis* (*Arabidopsis thaliana* L.) by functional validation (reviewed by Wang *et al.*, 2007
83 and Pillitteri & Torii, 2012). Some of these findings have been transferred to crop plants such
84 as maize (Liu *et al.* 2015) and, most relevant, wheat (Li *et al.* 2016). In wheat, quantitative
85 trait loci (QTL) for SD have been found on chromosomes (CHR) 1A, 1B, 2B, 4B, 7A, 7B
86 (Shahinnia *et al.* 2016, Schoppach *et al.* 2016) and CHR 1A, 1D, 2B, 3D, 4A, 5A, 6A and 7A
87 under water and drought stressed conditions (Wang *et al.* 2016b). Also, CHR substitution

lines revealed a significant effect from chromosome 3A on stomatal resistance (Bobo *et al.* 1992).

Due to its genetic determination, SD is a promising trait to respond to selection. However, its tractability in a breeding program is limited due to laborious phenotyping, restricting high throughput application. Alternatively, marker-trait associations revealed through QTL analyses can be used to select for a trait of interest by linked molecular markers. Already a number of studies have used bi-parental mapping families to uncover genetic loci associated with SD (Shahinnia *et al.* 2016, Schoppach *et al.* 2016, Wang *et al.* 2016b). But the introgression of QTL found in bi-parental studies to other germplasm is not only laborious but also often hampered by unknown epistatic interactions (Kumar *et al.* 2014). In effect, QTL identified in one (bi-parental) population may not have an effect in other recipient genetic backgrounds. A more promising approach to identify genomic regions associated with SD in contemporary breeding germplasm is the use of genome-wide association studies (GWAS). GWAS in large populations has become feasible due to technical advancements in marker technologies, allowing genotyping of tens of thousands of markers simultaneously. A high number of markers is essential to exceed the linkage disequilibrium in a GWAS population. An additional factor contributing to the statistical power and the genetic resolution of GWAS is the structure of the population under study. In a joint effort, the German Plant Genome Research Program (GABI) has established a large set of wheat lines suitable for GWAS. This population, thereafter referred to as the GABI-wheat panel, consists of 358 recent European winter wheat varieties and 14 spring wheat varieties (Kollers *et al.* 2013). The GABI-wheat panel has already been used to study heading date (Zanke *et al.* 2014a), plant height (Zanke *et al.* 2014b) and thousand grain weight (Zanke *et al.* 2015).

The main objective of this study was to understand the genetic architecture of stomatal patterning in winter wheat. Specifically, we aimed at i) assessing the phenotypic variation and heritability of stomatal patterning traits in contemporary wheat germplasm, ii) deconstructing stomatal patterning into constitutive traits underlying observed SD, iii) identifying QTL associated with stomatal patterning traits to get an overview of the genetic architecture and provide markers for selection, iv) assessing epistatic interactions between major QTL and v) identifying candidate genes for SD.

9. RESULTS

In order to phenotypically characterize stomatal patterning in wheat, the GABI-wheat panel was established in randomized field plots of 1 m x 1.5 m at the experimental research station in Lindau-Eschikon, Switzerland, in 2014 and 2015. For each genotype, four adaxial leaf imprints were collected, resulting in 2,633 epidermal images. From these images, the positions of over 387,000 stomata were recorded. Due to the highly ordered patterning of stomata in wheat, the positions of the stomata could be used to quantify the constitutive traits underlying SD. These traits included the distances between veins (BV), across veins (AV) and between stomata along a file (SF), as shown in Figure 1a-b. Two additional traits were calculated based on the above-mentioned parameters: SD estimated (SD_{est}), which is a derived estimate of SD based on AV, BV and SF and the size independent relationship of AV and BV distances (AoB, Across over Between). It was found that SD observed (SD_{ob} , the number of stomata divided by area) was generally lower than SD_{est} and that both SD_{est} and SD_{ob} were lower in 2015 compared to 2014, with a mean of 76 and 71 stomata per mm^2 for 2014 and 2015, respectively. For AV, the mean in 2014 was 117.9 μm and 125.4 μm in 2015. A similar trend was observed for BV, where BV in 2014 was 187.1 μm and 194.5 μm in 2015. SF was relatively consistent across years with a mean distance of 87.7 μm and 90.0 μm for 2014 and 2015, respectively. Also, for the ratio between AV and BV (AoB), the mean was 0.63 and 0.65 for 2014 and 2015, respectively. As a prerequisite to subsequent analyses, the stomatal traits were tested to see if variation existed within the population and between years. A two-way ANOVA found highly significant effects ($P < 0.05$) for all traits for the factors genotype, year and the interaction between genotype and year. Summary metrics of the traits measured are shown in Table 1.

The broad sense heritability (H^2), the genetic contribution to the phenotypic variance of a trait, is an essential measure for breeding and was calculated for the traits determining SD. Generally, H^2 was higher in 2014 compared to 2015. When calculating H^2 within a year, all traits showed H^2 values higher than 0.50, whereas H^2 values over both years ranged from 0.26 to 0.47. Considering both years independently, SD_{est} and SF showed the highest heritability values with 0.60 and 0.74, respectively. The H^2 of SD_{ob} was lower than SD_{est} at 0.58 to 0.61. For AV and BV, H^2 was 0.53 and 0.61, respectively and the H^2 for AoB was 0.68 to 0.72. When testing between years, H^2 for stomatal traits were lower, with the lowest

being 0.26 for SD_{ob} . In contrast, the H^2 of SD_{est} was 0.38, with similar values for AV and AoB. The highest H^2 were found for the traits SF and BV at 0.42 and 0.45, respectively.

To understand the interaction between these traits and to evaluate their contribution to SD, a non-parametric correlation analysis was performed. For all traits within years, highly significant correlations ($P < 0.001$) were found with R^2 values above 0.4. The highest correlation was found between SD_{est} and SD_{ob} , with R^2 values ranging from 0.99 to 0.92 for 2014 and 2015, respectively. All inter file distance traits (AV, BV and AoB) correlated well with each other ($R^2 > 0.42$). Lower, negative correlations were found between inter file distance traits (AV and BV) and SF ($R^2 < 0.27$). However, the inter file distance traits and SF correlated negatively with SD_{ob} and SD_{est} ($R^2 > 0.51$ and $R^2 > 0.54$, respectively). The correlations between years for each trait largely mirrored that of H^2 between years, ranging from 0.39 to 0.49. A detailed summary of the relationships between traits is shown in Figure 1c, the correlation matrixes for 2014, 2015 and between years in Figures S1, S2 and S3, respectively.

Phenotype associations to genomic loci were tested on the basis of haplotypes, determined using a sliding window of three consecutive single nucleotide polymorphism (SNP) markers. Prior analysis revealed no population stratification and all traits under investigation were found to be normally distributed ($P < 0.01$). The mean phenotypic values of 333 and 303 wheat lines were used for GWAS in 2014 and 2015, respectively. Based on overlapping results from both years, 42 significant haplotype associations distributed across eight CHR were detected. The most significant associations were found for SD_{est} on CHR 7B, peaking at the marker Excalibur_rep_c69070_180 with P values of $8.15e^{-10}$ and $3.60e^{-6}$ in 2014 and 2015, respectively. Significant associations were also detected for SD_{est} on CHR 2A, which also coincided with a significant association for AV at the marker Excalibur_c20439_825 ($P < 1.16e^{-05}$). Moreover, highly significant associations were found for SD_{est} and SF on CHR 3A ($P < 4.61e^{-05}$) with two significant haplotypes at 36.27 centiMorgan (cM) for SD_{est} and one significant haplotype at 38.29 cM for SF. On CHR 4D, two groups of associations were identified: AV and BV haplotype associations at 7.80 cM and 9.96 cM, respectively, and two haplotype associations for SF at 44.84 cM. Ten haplotype associations ($P < 1.80e^{-06}$) were found for BV on CHR 6B from 21.89 cM to 22.29 cM. On CHR 7A, a haplotype association (wsnp_Ku_c4615_8326355) was also found for SF ($P < 3.13e^{-5}$). Finally, three groups of associations were located on CHR 7D with one haplotype association

for AoB at 34.21 cM, two haplotype associations for BV at 36.85 cM and another BV association at 68.45 cM.

To test for epistatic QTL effects, the three loci consistently detected for SD_{est} on CHR 2A, 3A and 7B were selected. For these, the haplotype information was extracted, starting with the first SNP of the haplotype block, being Excalibur_c16661_122, Excalibur_c29600_173 and Excalibur_c20439_825 for the QTL on CHR 7B, 3A and 2A, respectively. For CHR 7B, one of the four haplotypes (CAT) significantly ($P < 0.001$) increased SD. For CHR 2A, a total of five haplotypes were found and the haplotype TGG significantly ($P < 0.001$) increased SD. For CHR 3A, three haplotypes were found of which TTA decreased SD ($P < 0.001$) and TTG increased SD ($P < 0.01$). Individually, three loci on CHR 7B, 3A and 2A loci explained 12.3, 9.7 and 7.5 % of the phenotypic variation, quantified as adjusted R^2 , respectively. These loci were further investigated by testing for epistatic QTL interactions (Figure 3). For the interaction between CHR 7B and CHR 2A (Figure 3a), the multi-QTL model significantly ($P < 0.001$) increased the explained variance to $R^2 = 17.4$ %. The positive effect of the CAT haplotype from CHR 7B was consistent with all CHR 2A haplotypes, indicating an additive interaction. The same was found for the interaction between CHR 7B and CHR 3A (Figure 3b), where the multi-QTL model significantly ($P < 0.01$) improved the single haplotype model ($R^2 = 16.5$ %). The allelic interaction here was also additive. For the interaction between CHR 2A and CHR 3A, the combination of the two loci did not significantly improve the single haplotype models ($P < 0.1$). However, this analysis was confounded by some rare haplotypes (TTG on CHR 2A and CGG on CHR 3A), not being present in all combinations (see Figure 3c). Despite this, there is evidence of an additive effect, where the CHR 2A haplotype TGG (which increases SD) and the CHR 3A haplotype TTA (which decreases SD) no longer showed a significant difference in combination with other haplotypes. The triple interaction between CHR 2A, 3A and 7B was not investigated as the number of rare allele combinations (as described for CHR 2A and 3A) made this analysis impossible.

To identify the causal genes underlying the observed QTL, the reference assembly of the International Wheat Genome Sequencing Consortium (IWGSC) was used (Appels *et al.* 2018, https://urgi.versailles.inra.fr/blast_iwgsc/). On CHR 3A, the genome region defined by the five significant markers constituting the haplotypes, plus one million base pairs (Mbp) up and downstream of this interval was searched for annotated genes. This returned 46 gene

models within a total length of 2.2 Mbp. The most interesting of those genes were *TraesCS3A01G442200* and *TraesCS3A01G442300*, corresponding to *INDUCER OF CBF EXPRESSION 1 (ICE1)* and *STOMATAL CYTOKINESIS-DEFECTIVE 1 (SCD1)* orthologs, respectively. Both *ICE1* and *SCD1* are known to be involved in stomatal development (Raissig *et al.* 2016, Falbel *et al.* 2003). On CHR 2A, the same procedure spanned 2.7 Mbp, including 66 annotated gene models. Of particular interest was a putative *Ethylene-responsive transcription factor (TraesCS2A01G542700)*. On CHR 7B, the 66 SNP markers significantly associated with SD defined the target region for candidate gene extraction. With an additional Mbp up and downstream, this corresponded to an interval 3.4 Mbp and contained 214 gene models. Interestingly, three auxin related genes were found: two *SAUR-like auxin-responsive family protein (TraesCS7B01G318100, TraesCS7B01G318200)* and an auxin response factor (*TraesCS7B01G316600*). The complete list of gene models with the annotation is shown in Table S1.

10. DISCUSSION

Here, we report the first GWAS to describe the genetic determination of SD in a collection of historical and contemporary wheat varieties, evaluated in the field over two years. We made use of the highly ordered distribution of stomata in wheat (Shahinnia *et al.* 2016) to dissect SD into its constitutive traits. Broadly, SD could be explained by two independent factors, the inter file distances (AV and BV) and the within file distance (SF). In combination with genome-wide SNP data, three QTL underlying SD were repeatedly identified in two consecutive growing seasons, together explaining 25% of the phenotypic variation. These QTL were located on CHR 2A, 3A and 7B and coincided with genes known to control stomatal development in model plant species (Lake *et al.* 2002, Balcerowicz and Hoecker 2014, Raissig *et al.* 2016). Our results strengthen the findings of previous studies, reporting significant associations for stomatal traits on CHR 3A (Bobo *et al.* 1992, Shahinnia *et al.* 2016) but also provide novel QTL for SD as well as other patterning traits such as SF, AV and BV. On the one hand, these data provide a tantalising insight into the relationship between the genetic control of anatomical arrangement of stomata and intrinsic SD. On the other hand, the SNP and haplotype data reported here are basic prerequisites for manipulating SD in extant germplasm for basic research purposes and applied wheat

breeding. Both is instrumental for the development of ‘climate ready crops’, as described by Hepworth *et al.* (2018).

A decomposition of SD into its constitutive traits and the differentiation of SD_{ob} and SD_{est} was important because SD_{ob}, calculated by dividing the number of stomata per image by the image area, was dependent on the view field of the image. In contrast, SD_{est}, determined by the three parameters AV, BV and SF and calculated as $2/[SF*(AV+BV)]$, provided an SD quantification independent of the view field of the image. This independence improved the quality of the phenotypic data, not only reflected by the higher heritability values for SD_{est} compared to SD_{ob} but also by the more robust associations to genetic markers as revealed by GWAS.

This study focussed specifically on SD of the adaxial flag leaf surface. Despite the positive correlation between SD and the transpiration rate in wheat (Schoppach *et al.* 2016), transpiration in general and the link to WUE may also depend upon other contributing factors, for example the size of stomata (Hetherington and Woodward 2003, Lawson and Blatt 2014). In wheat, stomatal length and width have been shown to correlate negatively with SD, thus demonstrating a certain compensatory effect (Wang *et al.* 2016b). Also, SD of the abaxial surface should be considered, though previous research has shown the correlation between abaxial and adaxial SD to be high ($R^2 = 0.9$, Schoppach *et al.* 2016). Despite such influencing factors, recent research clearly demonstrated the correlation of SD and WUE, not only in wheat but also in barley and rice (Doheny-Adams *et al.* 2012, Liu *et al.* 2015, Hepworth *et al.* 2015, Lawson *et al.* 2014a, Hughes *et al.* 2017, Caine *et al.* 2018).

Despite the strong evidence that low SD improves WUE in wheat, selection for SD in breeding programs has only met with limited success (Jones 1977, Lawson and Blatt 2014). This is in part due to the laborious phenotyping and high genotype x environment interaction of this trait. As a direct outcome of this work, the SNP markers associated with SD could be adopted by plant breeders to decrease SD and thereby improve WUE. Many plant breeders use SNP genotyping data for selection and, by incorporating the SNP markers reported here, could decrease the number of stomata in parallel with marker-assisted selection for other economically important traits. Moreover, the QTL described here were identified in contemporary wheat breeding germplasm, thus introgression of specific alleles would not require extensive backcrossing as being the case with exotic germplasm. In

addition to the three major QTL, there might be other genetic factors modulating SD, for example on CHR 5A and CHR 6B, amongst others. Although the clear detection of such modulating factors was difficult in such a diverse GWAS population as the GABI-wheat panel, targeted crossings of selected genotypes to create bi-parental populations and possibly nested-association mapping families, could resolve this.

A number of promising candidate genes involved in the genetic control of SD and its constitutive traits were identified, known to be functionally associated with SD and stomatal development in Arabidopsis: On CHR 3A, *ICE1*, encoding a basic-helix-loop-helix type transcription factor, is part of a signalling network directing stomatal development (Raissig *et al.* 2016) whereas *SCD1* is involved in guard cell division (Falbel *et al.* 2003). Given that *scd1* mutants result in binucleate miss-formed stomata, we speculate that the *ICE1* is the more plausible candidate gene modulating SD on CHR 3A. Moreover, the role of *ICE1* in stomatal development has been demonstrated in the closely related grass species *Brachypodium distachyon* (Raissig *et al.* 2016) and was recently further discussed for other grass species (Hepworth *et al.* 2018). On CHR 7B, three candidate genes were found related to auxin signaling. This is of interest as the role of auxin in repressing stomatal development is well established (Balcerowicz and Hoecker 2014). On CHR 2A, an ethylene-responsive transcription factor was found. Like auxin, ethylene has also been shown to be involved in stomatal development, where *ein2* (*ethylene-insensitive*) mutants show a significant reduction in SD in Arabidopsis (Lake *et al.* 2002). However, further work will be required to validate the suspected causal genes and polymorphisms attributable to modulated SD. A number of tools such as TILLING (Krasileva *et al.* 2017) or targeted genome modification using the CRISPR-Cas9 system (Shan *et al.* 2013) are nowadays available for this.

Although conducted in multiple replicates over several years, the evaluation of SD presented here was limited to one experimental field site (Lindau-Eschikon, Switzerland). Beyond the current study, a detailed description of SD at multiple sites will enable the quantification of environmental effects on SD and its related traits. Moreover, the combination of SD phenotypes with multi-spectrum image data, thermal imaging, plant height and morphological measurements of the canopy, as for example possible with cable-suspended multi-sensor systems (Kirchgessner *et al.* 2017), might provide new insights into the effect of stomatal development on crop productivity.

Conclusions

The research and data presented here provide a valuable resource for plant breeders to develop and implement SD as a trait to improve WUE in the most important cereal crop worldwide. The genetic resolution of the GWAS, determined by the design and the structure of the GABI-wheat panel, density and accuracy of the SNP marker data as well as the statistical power of haplotype mapping, in combination with the genome sequence information, allowed the identification of candidate genes. This method could be applied to other GWAS populations in wheat, maize or rice, for example, where similar populations with extensive genetic diversity are available.

11. MATERIALS AND METHODS

Plant material

The GABI-wheat panel (Kollers *et al.* 2013) was used to characterize stomatal patterning. Briefly, the population comprises of 14 spring wheat and 358 European winter wheat varieties. The genotypes were grown in 1 m x 1.5 m plots in a partially replicated (p-rep) design in two adjacent fields, allocated using the R package DiGger as described by (Grieder *et al.* 2015). The experiment was conducted at the field research station of ETH Zürich in Lindau-Eschikon (47.449°N, 8.682°E, 520 m above sea level, soil type: gleyic cambisol) in Switzerland over two years (2014 and 2015).

Leaf imprints

Leaf imprints were collected after the ligule of the flag leaf had formed at BBCH rating 39. To produce imprints, the flag leaves were fixed with adhesive tape to a plastic background to avoid leaf curling. Clear nail polish (Manhattan Proshine Basecoat, Coty Inc., New York, USA) was applied in the middle of the flag leaf (second to third quarter of the leaf) on the adaxial leaf side. The polish was air-dried for a minimum of five minutes and then peeled off with adhesive tape. These adhesive tapes with affixed imprints were then stored on a microscope slide (Thermo Fisher Scientific, Waltham, USA) for future processing.

Leaf imprint images

Images of the leaf imprints were taken with a compact 14 M pixel digital camera of the type Samsung ST90 (Samsung Electronics Co., Ltd., Suwon, South Korea). The camera was placed on a tripod in front of the ocular lens of a Leitz Laborlux S microscope (Leitz, Wetzlar, Germany) and pictures were taken through the microscope. In total, the images were magnified 200 fold, whereby the magnification of the ocular was ten, that of the objective four, and the camera used a five times optical zoom. Pixel sizes were determined empirically using a stage micrometer (0.41 μm per pixel). The resulting images covered an area of 2.39 mm² with a resolution of 14 megapixels (4320 pixels x 3240 pixels). Only images where all stomata were identifiable were recorded. The coordinates of all the stomata were recorded manually using the macro record function in ImageJ (Schneider et al. 2012). These coordinates (x, y-axis pixels) of each image were then copied to a separate text file. The imprints were photographed, so that the files of stomata were perpendicular to the bottom of the image.

Image processing

To estimate the stomatal parameters, it was first necessary to allocate the stomata to a distinct cell file within the image, hereafter referred to as 'File Of Stomata' (FOS). All FOS on an image defined the Files Of Stomata' (FsOS). As a prerequisite, all positions (x, y) were incremented by one to avoid future division by zero. All y-axis values (the distribution of stomata within a FOS) were divided by 1000. This was a means to weight future distance matrices in favour of the x-axis (i.e. the distance between FOS). As an initial attempt at allocating stomata to FsOS, the Euclidean distance between all pairs of stomata was calculated and the number of FsOS (or clusters) was estimated using the 'pamk' function from the R package 'fpc', with k-values ranging from 6 to 20. The stomatal coordinates were clustered using the distance matrix and hierarchical clustering (hclust) with the 'ward.D' method as described by Murtagh and Legendre (2014). The resulting tree was used to allocate stomata to a FOS, using the cutree function with the pamk optimal number of clusters. The positions of stomata were plotted as x, y positions and the points coloured based on cluster allocation (tentative FOS allocation) for manual validation.

If the allocation was incorrect, the number of clusters was manually adjusted. We found that a likely cause for the miss-allocation of stomata to a FOS was that the FsOS were not perpendicular to the x-axis, thus rotating the stomatal positions *in silico* was a means to

resolve this. The coordinates were *in silico* rotated from -20 to 20 degrees ($\emptyset$), per degree for the x_1 and y_1 positions. By rotating about the centre of the image (x_0 and y_0) to determine the resulting coordinates x_2 and y_2 , as shown below.

$$x_2 = \cos(\emptyset) \times (x_1 - x_0) - \sin(\emptyset) \times (y_1 - y_0) + x_0$$

$$y_2 = \sin(\emptyset) \times (x_1 - x_0) - \cos(\emptyset) \times (y_1 - y_0) - y_0$$

At every rotation (degree), the clustering process was repeated and linear models (lm) were fitted through each cluster. From these linear models, the residuals of the fits were retained and the mean of all residuals per cluster were recorded. The optimal angle of rotation was the one with the lowest mean residual. These results were then plotted, as described above, and manually validated before further analysis.

Once the stomata were allocated correctly to individual FOS, the distances AV and BV were calculated. The relative x position of each FOS was determined as the mean of all x-axis values of stomata within the FOS. Then the distances between consecutive pairs of FOS were determined. These distances were numbered consecutively, based on distance from the origin. All odd numbered distances were assigned to subset 1, all even numbered distances to subset 2. Since it can be assumed that inter FOS distances alternated between AV and BV, the mean of each distance subset was calculated and the lower value was assigned to AV and the greater to BV, respectively. AoB was calculated by dividing AV by BV, to determine a size independent measure of the two traits. Finally to estimate SF, the distance between the lowest stomata and highest stomata (based on y-axis position) within a FOS was divided by the number of stomata within a FOS minus one. This was calculated for all FOS and the mean of all FOS was used for SF. To convert the pixel coordinate distances into physical distance (μm), the pixel distances were multiplied by the empirically determined conversion factor of 0.41 μm per pixel.

Observed SD was calculated by dividing the number of stomata per image by the image area. With the three parameters AV, BV and SF, SD_{est} was defined. SD_{est} , the number of stomata per unit of area, provides an SD quantification independent of the leaf area used for the image. As there are always two stomata present in the area calculated by the sum of AV and BV, multiplied with SF, SD_{est} was calculated by dividing 2 by the size of this area as shown below. Because SD is normally determined as the number (N) of stomata per square millimetre (N/mm^2), SD_{est} had to be multiplied by 10^6 to scale accordingly.

$$SD_{est} = \frac{2}{SF \times (AV + BV)}$$

Statistical software

For data preparation and statistical analysis, the software environment R (R Development Core Team 2015) was used.

Heritability

Broad sense heritability (H^2) for all traits was calculated (per year) by estimating the genetic (σ_g^2) and the phenotypic ($\sigma_g^2 + \sigma_e^2$) variance components. The variance components were measured by a linear mixed model using restricted maximum likelihood (REML) in the R package “lme4”, with the function “lmer”. From these data the broad sense heritability was calculated using the following formula: $H^2 = \sigma_g^2 / (\sigma_g^2 + (\sigma_e^2 / r))$, where r is the number of replicates. Considering both years, broad sense heritability was calculated with an additional component for year (σ_{ye}^2) following the formula: $H^2 = \sigma_g^2 / (\sigma_g^2 + (\sigma_y^2 / 2) + \sigma_e^2)$.

Genotypic information

The SNP data used in this work was collected prior to this study when the population was established. The GABI-wheat panel was genotyped using a 90k Infinium chip (iSELECT, San Diego, USA), which was developed by Wang *et al.* (2014). This genotyping array includes 90,000 gene-associated and genome-wide distributed SNP markers (Wang *et al.* 2014). The genotypic data were first filtered for a number of criteria, described below, using the GenABEL software in R statistical environment (Aulchenko *et al.* 2007). The following criteria were applied: a call rate of > 95 % per marker, > 5 % minor allele frequency and identity by state (IBS) < 0.975.

Association analysis

The maker-trait associations were calculated using PLINK (Purcell *et al.* 2007). For analysis, three methods were used: i) single SNP analysis, ii) single SNP analysis with population stratification and iii) haplotype mapping with a sliding window of three SNPs using linear regression models. For the latter, the omnibus method, testing all haplotype effects per loci, was applied. After testing for associations, a Bonferroni correction was applied, with 0.05 P significance divided by the number of SNPs (17,308).

Epistasis

To test for epistatic QTL interactions, linear models (Y) were constructed for single effects ($Y_s = \mu + G_a + e$), and double additive ($Y_A = \mu + G_a + G_b + e$) and double dominant ($Y_D = \mu + G_a \times G_b + e$) interactions, where the mean, error term and genetic loci effects are given as μ , G and e , respectively. These models were then compared using ANOVA in R statistical environment. To test for additive effects, the null hypothesis (H_0) was that Y_A did not improve Y_s ($H_0: Y_s = Y_A$). Likewise, the null hypothesis for the dominant model was that $H_0: Y_A = Y_D$. If $H_0 < 0.05$, the null hypothesis was rejected and it was assumed the combination of alleles was either additive or dominant respectively.

Candidate genes

To identify candidate genes within the QTL, the corresponding marker sequences were searched against the IWGSC public database (v1.0, Appels *et al.* 2018) at https://urgi.versailles.inra.fr/blast_iwgsc/. High confidence gene annotations were extracted within the QTL interval, with an additional one million base pairs up and downstream.

12. ACKNOWLEDGMENTS

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13. CONFLICT OF INTEREST

The authors declare no conflicts of interest.

14. TABLES

Table 1. Descriptive statistics for stomatal patterning traits in wheat. The minimum (min), maximum (max), mean ($\bar{x}$), standard deviation (σ) and broad sense heritabilities (H^2) for the years 2014 and 2015 as well as between years (all) are shown. The traits include; estimated and observed stomatal density (SD_{est} and SD_{ob} , respectively), stomatal frequency within a file (SF), across and between vein distances (AV and BV, respectively), and across vein distance divided by between vein distances (AoB).

Trait	2014					2015					All
	min	max	$\bar{x}$	σ	H^2	Min	max	$\bar{x}$	σ	H^2	H^2
SD _{est}	56.9	110.9	76.3	7.8	0.74	56.4	95.9	70.8	6.5	0.72	0.38
SD _{ob}	85.7	142.6	117.9	10.1	0.58	99.8	160.4	125.4	11.6	0.61	0.27
SF	145.0	229.6	187.1	15.8	0.67	156.0	240.0	194.6	16.7	0.60	0.42
AV	0.497	0.812	0.633	0.051	0.61	0.527	0.849	0.649	0.058	0.53	0.38
BV	68.2	107.4	87.7	6.9	0.70	67.6	108.8	90.0	6.6	0.59	0.46
AoB	49.6	106.4	74.3	7.6	0.72	48.2	88.4	62.1	6.2	0.68	0.38

448

449 **Table 2.** Significant haplotype associations to traits determining stomatal density. The
450 table shows the trait, the first out of three SNP markers forming the haplotype block, the
451 chromosome, the position on the chromosome, the number of haplotypes and the
452 significance value (logarithm of the odds, LOD) for 2014 and 2015. The traits include;
453 estimated and observed stomatal density (SD_{est} and SD_{ob}, respectively), stomatal frequency
454 within a file (SF), across and between vein distances (AV and BV respectively), and across
455 vein distance divided by between vein distances (AoB).

Trait	Left most SNP	Linkage group	Position (cM)	Number of haplotypes	LOD (2014)	LOD (2015)
AV	Excalibur_c20439_825	2A	49.78	5	4.94	5.55
SD _{est}	BS00023202_51	2A	49.78	7	5.25	5.59
SD _{est}	Excalibur_c20439_825	2A	49.78	5	6.33	5.62
SD _{est}	RAC875_c1043_258	2A	50.61	5	5.53	5.56
SF	Excalibur_c17655_467	3A	36.27	4	6.08	5.18
SF	GENe-1981_131	3A	36.27	8	5.55	4.34
SD _{est}	Excalibur_c29600_173	3A	38.29	4	5.91	5.36
AV	RAC875_c50278_130	5A	7.801	8	4.64	4.88
BV	TA006001-0725	5A	9.96	4	8.05	5.24
SF	Tdurum_contig55097_601	5A	44.84	3	5.67	4.74
SF	wsnp_Ex_c31799_40545376	5A	44.84	4	5.20	4.57
BV	BobWhite_c15653_484	6B	21.89	3	7.03	5.28
BV	BobWhite_c17750_568	6B	21.89	3	7.03	5.28
BV	BobWhite_c47831_87	6B	21.89	3	7.03	5.28

BV	BS00022370_51	6B	21.89	3	7.06	5.25
BV	Excalibur_c18182_464	6B	21.89	3	7.08	5.67
BV	Kukri_c7972_1529	6B	21.89	4	5.76	5.26
BV	RAC875_c35008_398	6B	21.89	5	5.95	5.74
BV	RAC875_c12879_176	6B	22.05	4	6.49	5.38
BV	Ra_c17448_1214	6B	22.29	6	6.21	5.43
BV	RAC875_c63892_604	6B	22.29	6	6.28	5.32
SF	wsnp_Ku_c4615_8326355	7A	18.43	4	5.91	4.50
SD _{est}	BobWhite_c38957_159	7B	24.43	4	9.60	5.33
SD _{est}	BobWhite_c41328_265	7B	24.43	4	10.08	6.08
SD _{est}	BS00003494_51	7B	24.43	4	9.85	6.08
SD _{est}	BS00022542_51	7B	24.43	4	10.04	5.81
SD _{est}	BS00024065_51	7B	24.43	4	9.66	5.49
SD _{est}	BS00059061_51	7B	24.43	4	9.52	5.49
SD _{est}	BS00060341_51	7B	24.43	4	9.52	5.49
SD _{est}	Excalibur_c16661_122	7B	24.43	4	9.59	6.00
SD _{est}	Excalibur_rep_c111660_89	7B	24.43	4	9.85	5.27
SD _{est}	Excalibur_rep_c69070_180	7B	24.43	4	10.09	5.44
SD _{est}	IAAV1468	7B	24.43	4	10.53	6.15
SD _{est}	Tdurum_contig28176_55	7B	24.43	4	9.66	5.49
SD _{est}	Tdurum_contig93081_162	7B	24.43	4	9.55	5.85
SD _{est}	wsnp_BE498323B_Ta_2_1	7B	24.43	4	9.87	5.88
SD _{est}	wsnp_Ex_c33461_41945399	7B	24.43	5	10.08	5.68
SD _{est}	wsnp_RFL_Contig3405_3533915	7B	24.43	6	9.06	5.29
AoB	IAAV4644	7D	34.21	5	4.59	4.17
BV	JD_c6934_1038	7D	36.85	8	7.79	5.70
BV	JD_c6934_1038	7D	36.85	7	7.79	5.70
BV	Tdurum_contig22897_107	7D	68.45	6	5.41	7.30

Table S1. Table showing SNP markers and gene models on the IWGSC V1.0 genome.

15. FIGURE LEGENDS

Figure 1. Stomatal patterning in wheat. a) shows an image collected from a leaf imprint of a flag leaf. b) Visual display of the leaf parameters calculated from the epidermis, the red arrows show the files of cells which contain the stomata. The two distances indicated between relate to the distance across veins (AV) and between veins (BV). Stomatal frequency (SF) is the distance between two stomata within a file. c) Shows the correlation (R^2) between the stomatal traits, P values are proportional to color intensity. The additional traits shown are stomatal density (SD) and AV over BV (AoB).

Figure 2. Manhattan plots for stomatal traits in wheat. The figure illustrates haplotype associations (combinations of three SNPs) to the five traits AoB, AV, BV, SD_{est} and SF. The y-axis shows the $-\log_{10} P$ -value, the x-axis the relative position across the 21 wheat chromosomes (CHR). CHR 1-7 are numbered on the x-axis (background of odd numbered CHR are shaded gray), the three sub-genomes A, B and D are given in red, green and blue, respectively. Results from 2014 and 2015 are illustrated in the left and right subplots, respectively. Additionally, haplotypes with significant associations in both years are highlighted with yellow circles. For the traits AV and SF, no overlapping significant haplotypes were found across both years. Instead, the overlap (across both years) of the 100 haplotypes with the lowest P values within a year are highlighted with blue circles.

Figure 3. Haplotype interactions for QTL controlling stomata density in wheat. The boxplots show the individual haplotype effects at three loci, on chromosomes (CHR) 2A, 3A and 7B relating to stomatal density (n/mm^2 , y-axis). The x-axis shows one set of haplotype loci (a, CHR 2A; c, CHR 3A; c, CHR 2A) and the colors of the boxplots show the effect of a second loci (a and b, CHR 7B; c, CHR 3A). The haplotype names reflect the concatenation of three underlying SNP variants.

Figure S1. Correlation matrix between traits from 2014. Lower left panels show scatter plots of traits with linear fit shown in black. Diagonal panels show histograms of each trait. The upper right panels show the correlation R^2 . Positive correlations are shown in blue and negative in black.

Figure S2. Correlation matrix between traits from 2015. Lower left panels show scatter plots of traits with linear fit shown in black. Diagonal panels show histograms of each trait. The upper right panels show the correlation R^2 . Positive correlations are shown in blue and negative in black.

Figure S3. Correlation between traits from 2014 and 2015. The scatter plots show each trait with linear fit shown in black. Also shown is the correlation R^2 in the upper left hand corner.

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