Altered stomatal patterning accompanies a trichome dimorphism in a natural population of Arabidopsis

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Keywords: stomata, environmental adaptation, development, herbivory.

Abstract

Trichomes are large epidermal cells on the surface of leaves that are thought to deter herbivores, yet the presence of trichomes can also negatively impact plant growth and reproduction. Stomatal guard cells and trichomes have shared developmental origins, and experimental manipulation of trichome formation can lead to changes in stomatal density. The influence of trichome formation upon stomatal development in natural populations of plants is currently unknown. Here, we show that a natural population of *Arabidopsis halleri* that includes hairy (trichome-bearing) and glabrous (no trichomes) morphs has differences in stomatal density that are associated with this trichome dimorphism. We found that glabrous morphs had significantly greater stomatal density and stomatal index than hairy morphs. One interpretation is that this arises from a trade-off between the proportions of cells that have trichome and guard cell fates during leaf development. The differences in stomatal density between the two morphs might have impacts upon environmental adaptation, in addition to herbivory deterrence caused by trichome development.

Introduction

In *Arabidopsis*, trichomes are large epidermal cells that protrude from the surface of the leaves and petioles. Trichomes play important roles in both biotic defences and abiotic stress tolerance (Levin, 1973; Mauricio and Rausher, 1997; Handley et al., 2005; Dalin et al., 2008; Sletvold et al., 2010; Sletvold and Ågren, 2012; Sato and Kudoh, 2016). However, trichome development appears to impose a fitness cost on growth and reproduction (Mauricio, 1998; Sletvold et al., 2010; Kawagoe et al., 2011; Sletvold and Ågren, 2012; Sato and Kudoh, 2016). In addition to trichomes, stomatal guard cells represent another specialized cell type that is present on the leaf surface. Trichome initiation occurs prior to stomatal meristemoid development, and the patterning of trichomes and guard cells appears to be linked (Larkin et al., 1996; Glover, 2000; Bean et al., 2002; Bird and Gray, 2003). Therefore, there might be a trade-off between trichome and stomatal guard cell development during leaf formation (Glover et al., 1998).

We wished to determine whether trichome formation might be associated with changes in stomatal patterning in natural populations of plants. To achieve this, we investigated stomatal patterning in a naturally-occurring population of *Arabidopsis halleri* subsp. *gemmifera* that includes trichome-forming and glabrous morphs (Kawagoe et al., 2011; Sato and Kudoh, 2016). The glabrous morphs within this population harbour a large transposon-like insertion within the *GLABRA1* (*GL1*) gene (Kawagoe et al., 2011). *GL1* is also required for trichome formation in *A. thaliana*, with homozygous *gl1* mutants being glabrous (Oppenheimer et al., 1991). Our experiments provide new insights into the relationship between stomatal and trichome patterning under natural conditions.

Methods

Study site and experimental model

This investigation used a well-characterized population of *Arabidopsis halleri* subsp. *gemmifera* that is located beside a small stream in central Honshu island, Japan (Fig. 1A) (35°06' N, 134°55' E) (Aikawa et al., 2010; Kudoh et al., 2018). Sampling occurred during September 2016 (photoperiod approximately 12 h, with dawn at 05:40 and dusk at 18:10). During this season, *A. halleri* bore larger rosette leaves that are well-suited for quantification of stomatal density (Fig. 1B).

Stomatal density measurement

Eight plants of each trichome morph (hairy or glabrous) were selected at the study site, with individuals chosen such that the replicate plants were distributed evenly across the site. Glabrous and hairy morphs were identified by visual inspection of the leaf surface. Stomatal density was measured by obtaining impressions from the adaxial surfaces of 3-5 rosette leaves of each plant. Data were obtained from 58 and 62 leaves of hairy and glabrous plants, respectively. We focused on the adaxial surface because this surface also harbours the majority of the trichomes. Between the times of 12:00 and 13:00, President Plus dental impression paste (Coltene) was applied to the adaxial side of each leaf to create a leaf surface impression (Fig. 1C). Solidified impression paste was removed from leaves and transported to the laboratory for further processing. First, each impression was assigned a randomly-generated number to ensure subsequent steps were performed blind. Each leaf impression was painted with transparent nail varnish (60 seconds super shine, Rimmel) that, after drying, was peeled away from the dental impression paste using transparent adhesive tape (Scotch Crystal). Next, the adhesive tape was used to attach the nail varnish impression to a 0.8 mm – 1 mm thick microscope slide. Leaf impressions were examined using an epifluorescence microscope in white light illumination mode. Images were captured from the centre of each leaf half, away from the midrib, using a Hamamatsu camera and

Volocity software set to 20x zoom. Two images were captured from each impression, and the number of stomata and pavement cells was counted in an 800 µm x 800 µm square using the Fiji software to obtain cell density measures. Cell density measures were expressed as per mm^2 (multiplication by 1.56). Stomatal index was calculated according to Equation 1. After all measurements, data were disaggregated according to a blinding/randomization scheme. The differences between hairy and glabrous plants were statistically tested by nested analysis of variance, whereby leaves were nested within the hairy and glabrous morphs. Tests were conducted using the R 3.6.0 software (R Core Team, 2019) and plots generated with the beeswarm R package (v0.2.3) and Inkscape v0.91. No adjustments were applied to images in Fig. 1.

$$SI = \frac{s}{s+p} \times 100$$

Equation 1. Derivation of stomatal index, where *SI* is stomatal index, *s* is the number of stomata in the field of view, and *p* is the number of epidermal pavement cells in the field of view.

Results

We investigated stomatal patterning in naturally-occurring hairy and glabrous morphs of *A. halleri* (Sato and Kudoh, 2016). Approximately half of the *A. halleri* population at this study site is glabrous, whilst remaining plants have trichomes (Kawagoe et al., 2011). As trichome initiation occurs prior to stomatal meristemoid formation (Larkin et al., 1996; Glover, 2000), it is likely that trichome and stomatal patterning are linked (Bean et al., 2002), so we hypothesized that this might produce a difference in stomatal density between the two trichome morphs of *A. halleri* under natural conditions.

We found that the trichome formation dimorphism was accompanied by a difference in stomatal density (Fig. 2A; Supplemental Dataset S1). Glabrous morphs had significantly greater stomatal density compared with hairy-leaved morphs (glabrous: 31.4 ± 1.5 stomata

104 mm^{-2}; hairy: 23.7 ± 1.1 stomata mm^{-2}; ± s.e.m) (Fig. 2A; Table S1A; Supplemental Dataset
105 S1). Furthermore, the stomatal index was significantly greater in glabrous morphs (18.13
106 ± 0.41) compared with hairy morphs (16.11 ± 0.46) (Fig. 2B; Table S1B). The pavement cell
107 density did not differ significantly between the morphs (Table S1C). Stomatal density ranged
108 from 17 – 87 stomata mm^{-2} for hairy morphs and 27 – 119 stomata mm^{-2} for glabrous
109 morphs (Fig. 2A). This stomatal density was lower than for *Arabidopsis thaliana*, which has
110 reported stomatal densities of 180 – 350 stomata mm^{-2} depending on background accession
111 and growth conditions (Gray et al., 2000; Zhang et al., 2008; Franks et al., 2015).

112 **Discussion**

113 Glabrous plants had significantly greater stomatal density and stomatal index compared with
114 hairy plants (Fig. 2A; Fig. 2B). As the density of surrounding pavement cells did not vary
115 between the morphs, these differences in stomatal density and index are due to the greater
116 density of stomata in glabrous morphs compared with hairy morphs (Fig. 2B). Our field data
117 are consistent with a laboratory-based study in which transgenic tobacco plants expressing
118 an *Antirrhinum myb*-like transcription factor, which caused an excess of trichomes, also had
119 significantly reduced stomatal density (Glover et al., 1998). Similarly, the trichome-bearing
120 Col-0 accession of *A. thaliana* has lower stomatal density than the glabrous C24 accession
121 (e.g. about 115 mm^{-2} for Col-0 and 180 mm^{-2} for C24) (Perazza et al., 1998; Lake and
122 Woodward, 2008). This suggests that in natural populations of *A. halleri*, there could be a
123 trade-off between trichome and stomatal development. Since the glabrous *gl1* mutant of *A.*
124 *thaliana* has a significantly greater density of stomatal units compared with the wild type
125 (Berger et al., 1998) and the glabrous phenotype of *A. halleri* at this study site is associated
126 with an insertion within *GL1* (Kawagoe et al., 2011), it is possible that the *GL1* haplotype
127 influences the stomatal density within this population of *A. halleri*.

128 In some cases, there does not appear to be a tradeoff between stomatal and trichome
129 density. For example, elevated CO_2 decreases stomatal density (Woodward and Kelly,

1995), but might also reduces trichome density (Bidart-Bouzat et al., 2005). Therefore, in future, it could be informative to examine the relationship between stomatal and trichome density under a range of different experimental conditions that apply different types of selection pressure.

Interestingly, trichome production appears to impose a fitness cost. For example, glabrous *A. halleri* plants have 10% greater biomass than hairy plants when grown in the absence of herbivores (Sato and Kudoh, 2016). This cost of herbicide resistance arising from trichome formation also occurs in glabrous and hairy *A. lyrata* (Løe et al., 2007; Sletvold et al., 2010) and *A. thaliana* (Mauricio and Rausher, 1997; Mauricio, 1998) under experimental conditions excluding herbivores. Whilst this fitness advantage of glabrous over hairy leaves in the absence of herbivory might be due to trichome production (Mauricio and Rausher, 1997; Mauricio, 1998; Kawagoe and Kudoh, 2010; Sletvold et al., 2010; Kawagoe et al., 2011; Sletvold and Ågren, 2012), we suggest that glabrous morphs might also gain an advantage by having a greater density or number of stomata. It has been proposed that increasing the number of stomata could increase carbon assimilation (Lawson and Blatt, 2014). For example, Arabidopsis overexpressing STOMAGEN has greater stomatal density and a 30% increase in carbon assimilation compared with the wild type. However, these lines also have a higher transpiration rates and consequently lower water use efficiency (Tanaka et al., 2013).

Optimal stomatal density is important to achieve high photosynthetic rates. A low stomatal density restricts CO_2 vertical diffusion through the leaf and reduces photosynthetic rates, whilst high-density stomatal clustering diminishes CO_2 diffusion and causes low carbon assimilation (Lawson and Blatt, 2014). Both *A. halleri* morphs examined are likely to be within an optimal range of stomatal densities, having evolved and survived under natural conditions. However, the higher stomatal density in the glabrous morph might contribute to its faster growth in absence of herbivory (Sato and Kudoh, 2016). In future, it would be interesting to explore this by measuring the CO_2 assimilation rate of these trichome morphs

under laboratory and/or natural conditions. It would also be informative to determine whether the stomatal density difference between the two trichome morphs confers any advantages within microenvironments characterized by differences in water or light availability. The lower stomatal density of *A. halleri* compared with *A. thaliana* (Gray et al., 2000; Zhang et al., 2008; Franks et al., 2015) might reflect differences in growth conditions. An alternative explanation might relate to genome size, because there appears to be a negative correlation between genome size and stomatal density (Beaulieu et al., 2008), and the genome of *A. halleri* (250 Mb) is approximately double the size of the *A. thaliana* genome (125 Mb) (The Arabidopsis Genome Initiative, 2000; Briskine et al., 2017).

In summary, we found that glabrous morphs of *A. halleri* growing under natural conditions had higher stomatal density and stomatal index than a hairy morph. This might contribute to the reported fitness advantage of glabrous plants over hairy plants in absence of herbivores (Sato and Kudoh, 2017). This differing stomatal density phenotype might derive from the common upstream components in the pathways leading to trichome and guard cell development.

Acknowledgements

We thank Dora Cano-Ramirez, Haruki Nishio and Tasuku Ito for experimental assistance. This research was funded by the UK Biotechnology and Biological Sciences Research Council (BBSRC; grant BB/J014400/1), The Royal Society (grant IE140501), and the Japan Society for Promotion of Science (JSPS; CREST no. JPMJCR15O1). This research was conducted using Joint Usage of the Center for Ecological Research, Kyoto University.

Conflict of Interests

The authors declare no competing financial interests.

Author contributions

NMLS, JS, MNH, SAT, GT, HK and AND performed experimentation and/or analysed data, and NMLS, HK and AND wrote the paper.

Data availability

All data generated during this study are included in the published article and Supplementary Information files.

Figure legends

Figure 1. Field sampling of *Arabidopsis halleri* for stomatal density. (A) Overview of field site; (B) Rosette form of *A. halleri* plants during September sampling season; (C) Leaf surface impression acquisition using impression paste. The impression paste is green-coloured and occupies the surface of three rosette leaves.

Figure 2. Stomatal density differs between hairy and glabrous morphs within a natural population of *Arabidopsis halleri*. (A) Stomatal density and (B) stomatal index for hairy and glabrous morphs. Each red point represents one measurement and the centre line of the boxplot indicates the median. Data are mean +/- s.e.m (n = 58 (hairy plants) and n = 62 (glabrous plants); analysed by one-way nested ANOVA. * indicates $p < 0.05$; ** indicates $p < 0.01$.

Table S1. Nested ANOVA analysis of (a) stomatal density, (b) stomatal index and (c) pavement cell density. Df is degree of freedom; *, ** and *** indicates significant at $p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively; NS, not significant at $p > 0.05$.

Dataset S1. Complete stomatal density data collected during experimentation.

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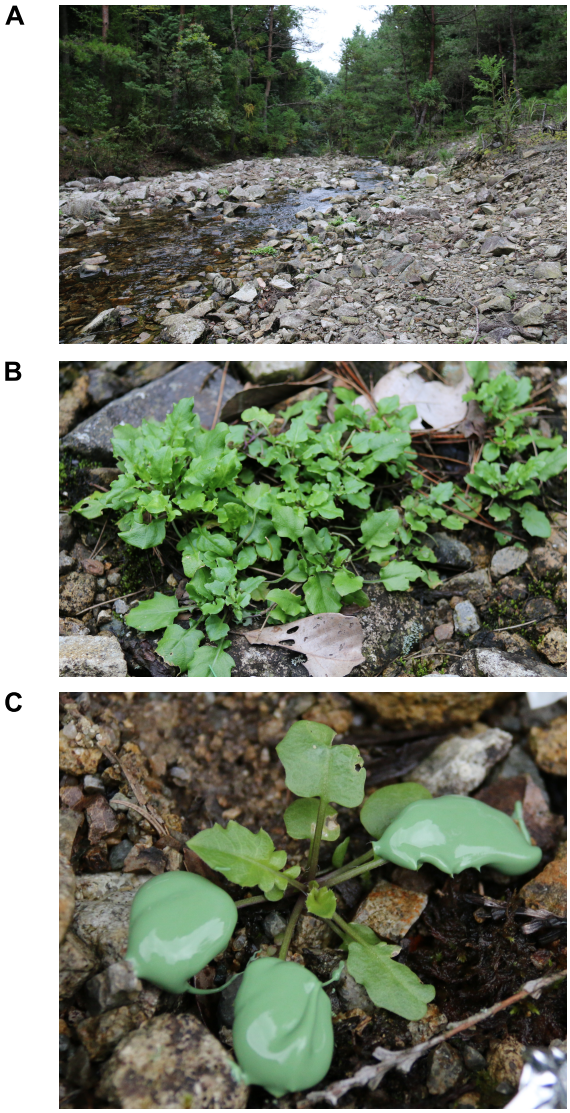
Simon et al. Fig. 1



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