

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

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5 Noriane M. L. Simon¹, Jiro Sugisaka², Mie N. Honjo², Sverre Aarseth Tunstad¹, George
6 Tunna¹, Hiroshi Kudoh², Antony N. Dodd^{1*}

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9 1. School of Biological Sciences, University of Bristol, Bristol BS8 1TQ, U.K.

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11 2. Center for Ecological Research, Kyoto University, Otsu, Shiga, Japan.

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13 * Corresponding author; antony.dodd@bristol.ac.uk

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16

17 **Abstract**

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

31

32 Introduction

33 In *Arabidopsis*, trichomes are large epidermal cells that protrude from the surface of the
 34 leaves and petioles. Trichomes play important roles in both biotic defences and abiotic
 35 stress tolerance (Levin, 1973; Mauricio and Rausher, 1997; Handley et al., 2005; Dalin et al.,
 36 2008; Sletvold et al., 2010; Sletvold and Ågren, 2012; Sato and Kudoh, 2016). However,
 37 trichome development appears to impose a fitness cost on growth and reproduction
 38 (Mauricio, 1998; Sletvold et al., 2010; Kawagoe et al., 2011; Sletvold and Ågren, 2012; Sato
 39 and Kudoh, 2016). In addition to trichomes, stomatal guard cells represent another
 40 specialized cell type that is present on the leaf surface. Trichome initiation occurs prior to
 41 stomatal meristemoid development, and the patterning of trichomes and guard cells appears
 42 to be linked (Larkin et al., 1996; Glover, 2000; Bean et al., 2002; Bird and Gray, 2003).
 43 Therefore, there might be a trade-off between trichome and stomatal guard cell development
 44 during leaf formation (Glover et al., 1998).

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

54 **Methods**

55 *Study site and experimental model*

56 This investigation used a well-characterized population of *Arabidopsis halleri* subsp.
57 *gemmifera* that is located beside a small stream in central Honshu island, Japan (Fig. 1A)
58 (35°06' N, 134°55' E) (Aikawa et al., 2010; Kudoh et al., 2018). Sampling occurred during
59 September 2016 (photoperiod approximately 12 h, with dawn at 05:40 and dusk at 18:10).
60 During this season, *A. halleri* bore larger rosette leaves that are well-suited for quantification
61 of stomatal density (Fig. 1B).

62 *Stomatal density measurement*

63 Eight plants of each trichome morph (hairy or glabrous) were selected at the study site, with
64 individuals chosen such that the replicate plants were distributed evenly across the site.
65 Glabrous and hairy morphs were identified by visual inspection of the leaf surface. Stomatal
66 density was measured by obtaining impressions from the adaxial surfaces of 3-5 rosette
67 leaves of each plant. Data were obtained from 58 and 62 leaves of hairy and glabrous
68 plants, respectively. We focused on the adaxial surface because this surface also harbours
69 the majority of the trichomes. Between the times of 12:00 and 13:00, President Plus dental
70 impression paste (Coltene) was applied to the adaxial side of each leaf to create a leaf
71 surface impression (Fig. 1C). Solidified impression paste was removed from leaves and
72 transported to the laboratory for further processing. First, each impression was assigned a
73 randomly-generated number to ensure subsequent steps were performed blind. Each leaf
74 impression was painted with transparent nail varnish (60 seconds super shine, Rimmel) that,
75 after drying, was peeled away from the dental impression paste using transparent adhesive
76 tape (Scotch Crystal). Next, the adhesive tape was used to attach the nail varnish
77 impression to a 0.8 mm – 1 mm thick microscope slide. Leaf impressions were examined
78 using an epifluorescence microscope in white light illumination mode. Images were captured
79 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 mm² (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

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

Discussion

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

In some cases, there does not appear to be a tradeoff between stomatal and trichome density. For example, elevated CO₂ 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 CO₂ vertical diffusion through the leaf and reduces photosynthetic rates, whilst high-density stomatal clustering diminishes CO₂ 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 CO₂ 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.

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Conflict of Interests

The authors declare no competing financial interests.

180 **Author contributions**

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

183 **Data availability**

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

186

187 **Figure legends**

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

192 **Figure 2.** Stomatal density differs between hairy and glabrous morphs within a natural
193 population of *Arabidopsis halleri*. (A) Stomatal density and (B) stomatal index for hairy and
194 glabrous morphs. Each red point represents one measurement and the centre line of the
195 boxplot indicates the median. Data are mean \pm s.e.m (n = 58 (hairy plants) and n = 62
196 (glabrous plants); analysed by one-way nested ANOVA. * indicates $p < 0.05$; ** indicates $p <$
197 0.01.

198 **Table S1.** Nested ANOVA analysis of (a) stomatal density, (b) stomatal index and (c)
199 pavement cell density. Df is degree of freedom; *, ** and *** indicates significant at $p < 0.05$,
200 $p < 0.01$ and $p < 0.001$ respectively; NS, not significant at $p > 0.05$.

201 **Dataset S1.** Complete stomatal density data collected during experimentation.

References

- Aikawa S, Kobayashi MJ, Satake A, Shimizu KK, Kudoh H (2010) Robust control of the seasonal expression of the *Arabidopsis FLC* gene in a fluctuating environment. *Proceedings of the National Academy of Sciences* 107: 11632-11637
- Bean GJ, Marks MD, Hulskamp M, Clayton M, Croxdale JL (2002) Tissue patterning of *Arabidopsis* cotyledons. *New Phytologist* 153: 461-467
- Beaulieu JM, Leitch IJ, Patel S, Pendharkar A, Knight CA (2008) Genome size is a strong predictor of cell size and stomatal density in angiosperms. *New Phytologist* 179: 975-986
- Berger F, Linstead P, Dolan L, Haseloff J (1998) Stomata patterning on the hypocotyl of *Arabidopsis thaliana* is controlled by genes involved in the control of root epidermis patterning. *Developmental Biology* 194: 226-234
- Bidart-Bouzat MG, Mithen R, Berenbaum MR (2005) Elevated CO₂ influences herbivory-induced defense responses of *Arabidopsis thaliana*. *Oecologia* 145: 415-424
- Bird SM, Gray JE (2003) Signals from the cuticle affect epidermal cell differentiation. *New Phytologist* 157: 9-23
- Briskine RV, Paape T, Shimizu-Inatsugi R, Nishiyama T, Akama S, Sese J, Shimizu KK (2017) Genome assembly and annotation of *Arabidopsis halleri*, a model for heavy metal hyperaccumulation and evolutionary ecology. *Molecular Ecology Resources* 17: 1025-1036
- Dalin P, Agren J, Bjorkman C, Huttunen P, Karkkainen K (2008) Leaf trichome formation and plant resistance to herbivory. In A Schaller, ed, *Induced plant resistance to herbivory*. Springer, pp 89-105
- Franks PJ, W. Doheny-Adams T, Britton-Harper ZJ, Gray JE (2015) Increasing water-use efficiency directly through genetic manipulation of stomatal density. *New Phytologist* 207: 188-195
- Glover BJ (2000) Differentiation in plant epidermal cells. *Journal of Experimental Botany* 51: 497-505
- Glover BJ, Perez-Rodriguez M, Martin C (1998) Development of several epidermal cell types can be specified by the same MYB-related plant transcription factor. *Development* 125: 3497
- Gray JE, Holroyd GH, van der Lee FM, Bahrami AR, Sijmons PC, Woodward FI, Schuch W, Hetherington AM (2000) The HIC signalling pathway links CO₂ perception to stomatal development. *Nature* 408: 713-716
- Handley R, Ekblom B, Ågren J (2005) Variation in trichome density and resistance against a specialist insect herbivore in natural populations of *Arabidopsis thaliana*. *Ecological Entomology* 30: 284-292

- 241 Kawagoe T, Kudoh H (2010) Escape from floral herbivory by early flowering in
242 *Arabidopsis halleri* subsp. *gemmifera*. *Oecologia* 164: 713-720
- 243 Kawagoe T, Shimizu KK, Kakutani T, Kudoh H (2011) Coexistence of trichome
244 variation in a natural plant population: A combined study using ecological and
245 candidate gene approaches. *PLOS ONE* 6: e22184
- 246 Kudoh H, Honjo MN, Nishio H, Sugisaka J (2018) The long-term "in natura" study sites
247 of *Arabidopsis halleri* for plant transcription and epigenetic modification
248 analyses in natural environments. *In Plant Transcription Factors*. Springer, pp
249 41-57
- 250 Lake JA, Woodward FI (2008) Response of stomatal numbers to CO₂ and humidity:
251 control by transpiration rate and abscisic acid. *New Phytologist* 179: 397-404
- 252 Larkin JC, Young N, Prigge M, Marks MD (1996) The control of trichome spacing and
253 number in *Arabidopsis*. *Development* 122: 997-1005
- 254 Lawson T, Blatt MR (2014) Stomatal size, speed, and responsiveness impact on
255 photosynthesis and water use efficiency. *Plant Physiology* 164: 1556-1570
- 256 Levin DA (1973) The role of trichomes in plant defense. *The Quarterly Review of*
257 *Biology* 48: 3-15
- 258 Løe G, Toräng P, Gaudeul M, Ågren J (2007) Trichome production and spatiotemporal
259 variation in herbivory in the perennial herb *Arabidopsis lyrata*. *Oikos* 116: 134-
260 142
- 261 Mauricio R (1998) Costs of resistance to natural enemies in field populations of the
262 annual plant *Arabidopsis thaliana*. *The American Naturalist* 151: 20-28
- 263 Mauricio R, Rausher MD (1997) Experimental manipulation of putative selective
264 agents provides evidence for the role of natural enemies in the evolution of
265 plant defense. *Evolution* 51: 1435-1444
- 266 Oppenheimer DG, Herman PL, Sivakumaran S, Esch J, Marks MD (1991) A *myb* gene
267 required for leaf trichome differentiation in *Arabidopsis* is expressed in
268 stipules. *Cell* 67: 483-493
- 269 Perazza D, Vachon G, Herzog M (1998) Gibberellins promote trichome formation by
270 up-regulating *GLABROUS1* in *Arabidopsis*. *Plant Physiology* 117: 375-383
- 271 Sato Y, Kudoh H (2016) Associational effects against a leaf beetle mediate a minority
272 advantage in defense and growth between hairy and glabrous plants.
273 *Evolutionary Ecology* 30: 137-154
- 274 Sato Y, Kudoh H (2017) Fine-scale frequency differentiation along a herbivory
275 gradient in the trichome dimorphism of a wild *Arabidopsis*. *Ecology and*
276 *Evolution* 7: 2133-2141
- 277 Sletvold N, Ågren J (2012) Variation in tolerance to drought among Scandinavian
278 populations of *Arabidopsis lyrata*. *Evolutionary Ecology* 26: 559-577

279 **Sletvold N, Huttunen P, Handley R, Kärkkäinen K, Ågren J (2010) Cost of trichome**
280 **production and resistance to a specialist insect herbivore in *Arabidopsis***
281 ***lyrata*. Evolutionary Ecology 24: 1307-1319**

282 **Tanaka Y, Sugano SS, Shimada T, Hara-Nishimura I (2013) Enhancement of leaf**
283 **photosynthetic capacity through increased stomatal density in *Arabidopsis*.**
284 **New Phytologist 198: 757-764**

285 **R Core Team (2019) R: A language and environment for statistical computing. R**
286 **Foundation for Statistical Computing, Vienna, Austria. URL [https://http://www.R-](https://http://www.R-project.org/)**
287 **[project.org/](https://http://www.R-project.org/).**

288 **The Arabidopsis Genome Initiative (2000) Analysis of the genome sequence of the**
289 **flowering plant *Arabidopsis thaliana*. Nature 408: 796-815**

290 **Woodward FI, Kelly CK (1995) The influence of CO₂ concentration on stomatal**
291 **density. New Phytologist 131: 311-327**

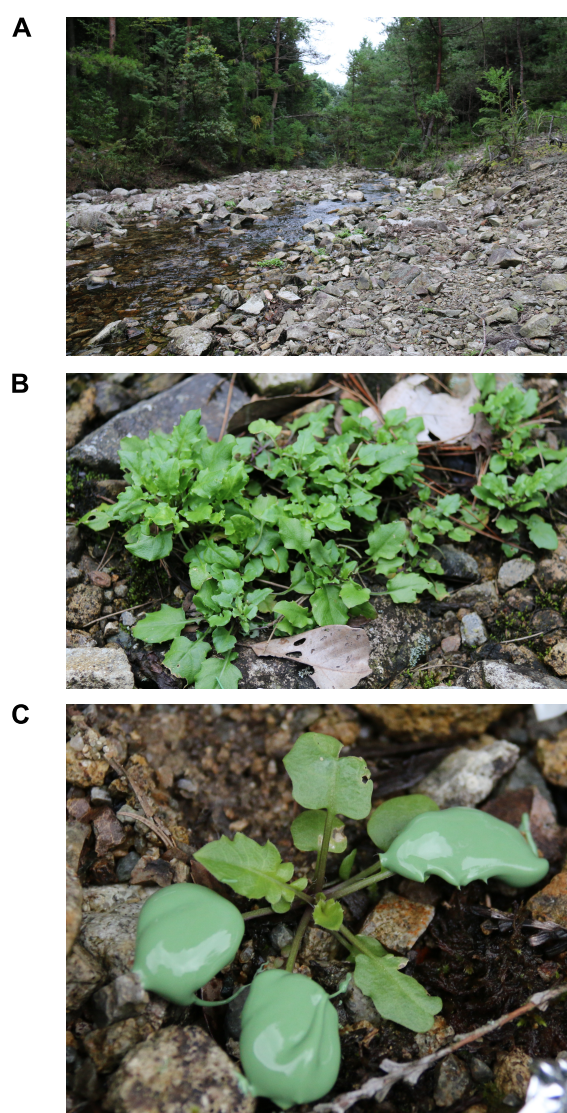
292 **Zhang L, Hu G, Cheng Y, Huang J (2008) Heterotrimeric G protein α and β subunits**
293 **antagonistically modulate stomatal density in *Arabidopsis thaliana*.**
294 **Developmental Biology 324: 68-75**

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Simon et al. Fig. 1



Simon et al. Fig. 2

