1 **Title:**

2 **Activation of ACC synthase 2/6 increases stomatal density and cluster on the**
 3 ***Arabidopsis* leaf epidermis during drought**

4 **Running title:**

5 ACS2/6 integrates stomatal developing with spacing

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28

29 **Highlight**

30 ACC synthase ACS2/6 activation integrated stomatal individual development with

31 space setting between stomata by mediating Ca^{2+} levels in stomatal lineage on the

32 leaf epidermis in response to drought.

33

34 Abstract

35 It is known that the transcription factor SPEECHLESS (SPCH) drives entry of
 36 epidermal cells into stomatal lineage, and that the activation of subtilisin-like
 37 protease SDD1 reduces stomatal density and cluster on the epidermis. However,
 38 there is still a big gap in our understanding of the relationship between stomatal
 39 development and the establishment of stomatal density and pattern, especially
 40 during drought. Interestingly, 1-aminocyclopropane-1-carboxylic acid (ACC) not
 41 only promotes stomatal development, but also is involved in the establishment of
 42 stomatal density and pattern. ACC generation comes from the activity of ACC
 43 synthase (ACS), while ACS activity could be mediated by drought. This work
 44 showed that the Arabidopsis SPCH activated ACS2/6 expression and
 45 ACC-dependent stomatal generation with an increase of stomatal density and
 46 cluster under drought conditions; and the possible mechanisms were that
 47 ACC-induced Ca²⁺ shortage in stomatal lineage reduced the inhibition of the
 48 transcription factor GT-2 Like 1 (GTL1) on *SDD1* expression. These suggest that
 49 ACS2/6-dependent ACC accumulation integrated stomatal development with the
 50 establishment of stomatal density and pattern by mediating Ca²⁺ levels in stomatal
 51 lineage cells on the leaf epidermis, and this integration is directly related to the
 52 growth or survival of plants under escalated drought stress.

53 **Key words:** ACC, ACS2, ACS6, Ca²⁺, SDD1, SPCH, stomatal density, stomatal
 54 cluster, drought.

55

56 Introduction

57 Stomata are pore structures surrounded by guard cells (GCs) on the leaf epidermis
 58 that regulate the exchange of gases (i.e., H₂O, CO₂, or O₂) between plants and the
 59 environment (Acharya and Assmann, 2009; Zoulas *et al.*, 2018). In evolution
 60 from aquatic to terrestrial, plants had generated stomata on epidermis of aerial part
 61 to facilitate transpiration (pulling water to shoot), and to guarantee plant survival
 62 and life on land (Croxdale, 2000; van Veen and Sasidharan, 2021). For terrestrial
 63 plant, stomata can sense environmental water status, especially water deficit or
 64 drought, and regulate water loss from plant (Acharya and Assmann, 2009; Zoulas
 65 *et al.*, 2018). The ability of stomata to regulate water loss is generally estimated
 66 from stomatal density (number of stomata per unit leaf area) and pattern (whether
 67 stomata are distributed singly or in clusters). Stomatal density and pattern are the
 68 consequences of stomatal space setting; for example, stomatal cluster is formed by
 69 directly contacting stomata with no intervening epidermal cell, or, alternatively,
 70 zero-space establishment (Von Groll *et al.*, 2002; Acharya and Assmann, 2009;
 71 Zoulas *et al.*, 2018). The establishment of stomatal space is, therefore, an
 72 important aspect of plant growth and survival under drought conditions
 73 (Hepworth *et al.*, 2015).

74 The regulation of stomatal development, which undergirds stomatal spacing,
 75 has been extensively investigated. Studies on the model plant *Arabidopsis*
 76 *thaliana* (L.) have shown that stomatal development includes a series of epidermal
 77 cell divisions, in which several basic helix-loop-helix transcription factors, such as
 78 SPCH, MUTE and FAMA, are involved in this process. SPCH initiates stomatal
 79 development to transform meristemoid mother cells (MMCs) into a meristemoids
 80 and a sister cell; MUTE converts a sister cell into the guard mother cells (GMCs);
 81 then FAMA drives GMCs to form a stoma with differentiated GCs (Hamanishi *et*
 82 *al.*, 2012; Zoulas *et al.*, 2018). These findings show clearly that SPCH dominates
 83 stomatal development. This view is better interpreted, for example, the
 84 loss-of-function *spch-1* or *spch-3* homozygous mutant do not produce any stomata
 85 (MacAlister *et al.*, 2007; Pillitteri *et al.*, 2007; Han and Torii, 2016). Furthermore,
 86 SPCH expression is known to implicate the establishment of stomatal density
 87 (Tripathi *et al.*, 2016; Zoulas *et al.*, 2018). However, it is still unclear how SPCH
 88 integrates stomatal developing with space setting.

89 Evidences suggest that the subtilisin-like protease Stomatal Density and
 90 Distribution 1 (SDD1) participates in space setting between stomata on leaf
 91 epidermis, or in the establishment of stomatal density and pattern (Berger and
 92 Altmann, 2000; Casson and Gray, 2008; Serna, 2009; Zoulas *et al.*, 2018), as
 93 evidenced by the following: a loss-of-function *sdd1-1* mutant showed a 2- to
 94 4-fold increase in stomatal density and stomatal clustering in all aerial parts,
 95 whereas transgenic *SDD1*-overexpressing plants exhibited a 2- to 3-fold decrease
 96 in stomatal density and arrested stomata (Berger and Altmann, 2000; Von Groll *et*
 97 *al.*, 2002). In line with these findings, *SDD1*-overexpressing plants displayed
 98 diminished transpiration because of a ~25% reduction in abaxial stomatal density
 99 or clustering (Yoo *et al.*, 2010). Evidently, SDD1 activity is negatively correlated
 100 with stomatal density and stomatal clustering ratio. The regulatory mechanism of
 101 SDD1 activity has been uncovered. Significantly, the trihelix transcription factor
 102 GT-2 LIKE 1 (GTL1) binds to the promoter of the *SDD1* gene and inhibits its
 103 expression (Yoo *et al.*, 2010; Weng *et al.*, 2012; Viridi *et al.*, 2015). This
 104 inhibition can be relieved by Ca^{2+} increase because Ca^{2+} -loaded calmodulin
 105 (Ca^{2+} -CaM) destabilizes the docking of GTL1 protein to the *SDD1* promoter (Yoo
 106 *et al.*, 2019). These findings suggest that local Ca^{2+} levels are positively correlated
 107 with SDD1 activity as well as stomatal density and the rate of stomatal clustering.
 108 Nevertheless, the manner by which SPCH signals information on stomatal
 109 development to GTL1-controlled *SDD1* expression or stomatal space setting
 110 remains unclear.

111 The non-proteinogenic amino acid 1-aminocyclopropane-1-carboxylate (ACC)
 112 has recently been shown to independently promote stomatal generation by
 113 facilitating the differentiation of GMCs into GCs in *Arabidopsis* leaves (Yin *et al.*,
 114 2019). Unexpectedly, ethylene is not involved in this process (Yin *et al.*, 2019)
 115 even though ACC is the precursor of ethylene (Bleecker and Kende, 2000). In fact,
 116 ACC is known to be involved in stomatal development and spacing (Acharya and
 117 Assmann, 2009). For example, ACC treatments increased the number of stomata
 118 by ~33% on the hypocotyl or cotyledon epidermis in *Arabidopsis* (Saibo *et al.*,
 119 2003), and also induced stomatal clustering (Serna and Fenoll 1997; Berger and
 120 Altmann, 2000). The production of ACC *in vivo* depends on the activity of ACC
 121 synthase (ACS), which converts S-adenosylmethionine to ACC (Bleecker and

122 Kende, 2000). Various pieces of experimental evidence strongly suggest that ACS
123 activity is an important mediator of stomata formation. For example, inhibitors of
124 ACS activity, such as aminoethoxyvinylglycine (AVG) and paclobutrazol (PAC),
125 were shown to abolish stomatal appearance (Serna and Fenoll, 1996; Saibo *et al.*,
126 2003; Yin *et al.*, 2019). It is known that ACS is encoded by a multi-gene family
127 (Bleecker and Kende, 2000), and that the activity of ACS family members is
128 unique, overlapping, and spatiotemporally specific (Tsuchisaka and Theologis,
129 2004; Tsuchisaka *et al.*, 2009). The *Arabidopsis* genome contains nine ACS genes
130 (*ACS1*, *ACS2*, *ACS4–9*, and *ACS11*) that encode authentic enzymes (Tsuchisaka *et*
131 *al.*, 2009). Interestingly, the expression of ACS genes is induced by drought
132 (Dubois *et al.*, 2017; Dubois *et al.*, 2018), which suggests that ACS activity may
133 be involved in the stomata-based drought response in *Arabidopsis*. Strikingly,
134 chromatin immune-precipitation assays have indicated that SPCH may regulate
135 the transcription activity of *ACS2* and *ACS6* genes (Lau *et al.*, 2014). Nevertheless,
136 further evidence is needed to clarify how SPCH directs ACS2/6 activity during
137 stomatal development.

138 In this study, we explored the specific involvement of ACS2/6 activity in the
139 drought tolerance of *Arabidopsis* seedlings. Our results revealed that the T-DNA
140 insertion mutants *acs2-1*, *acs6-1*, and *acs2-lacs6-1* are more tolerant to drought
141 than is the wild-type (WT) control. Subsequent research on the underlying
142 mechanism indicated that SPCH activates the expression of *ACS2*, *ACS6*, and
143 *GTL1* by directly binding to their promoters. ACS2/6-dependent ACC
144 accumulation triggers stomatal development and a Ca²⁺ shortage in stomatal
145 lineage cells, and the latter resulted in the repression of *GTL1*-controlled *SDD1*
146 expression. Stomatal density and cluster on the leaf epidermis are thereby
147 increased, leading to increased seedling wilting and even death under intensified
148 drought.

149 **Materials and methods**

150 **Plant materials and growth conditions**

151 *Arabidopsis thaliana* (Columbia-0 ecotype) was used as WT. The different ACS2
152 expression lines, including mutant *acs2-1* (CS16564) with a T-DNA insertion,
153 ACS2-complementation (*ACS2/acs2-1*), ACS2-overexpression (*ACS2-OE*), and

154 *pACS2::ACS2-GUS* lines, have been described previously (Han *et al.*, 2019). The
 155 T-DNA insertion mutant *acs6-1* (CS16569) was obtained from the Arabidopsis
 156 Biological Resource Center (USA). The double mutant *acs2-lacs6-1* was created
 157 by crossing *acs2-1* with *acs6-1*. Seeds of *spch-3* mutant with a T-DNA insertion
 158 were a friendly gift from Professor Sui-wen Hou (MOE Key Laboratory of Cell
 159 Activities and Stress Adaptations, Lanzhou, China). These homozygotes with
 160 T-DNA insertion were screened according to the method provided by the Salk
 161 Institute (<http://signal.salk.edu>). Seeds of the point mutant *spch-1* was a friendly
 162 gift from Professor Xiao-lan Chen (School of Life Sciences, Yunnan University,
 163 China), and was identified by PCR amplification and sequencing of the fragment
 164 containing the mutation site. All primers used in this study are listed in the
 165 [Supplementary Table](#).

166 Seeds of the transgenic *pSPCH::SPCH-GFP* line were a friendly gift from
 167 Professor Xiao-lan Chen (School of Life Sciences, Yunnan University, China),
 168 and GFP expression was detected by hygromycin screening and measurement of
 169 fluorescence in leaves. The seeds of Ca^{2+} sensor NES-YC3.6-expressing line were
 170 kindly gifted by Professor Jörg Kudla (Molecular Genetics and Cell Biology of
 171 Plants, University of Munich, Germany). NES-YC3.6-expressing *acs2-lacs6-1*
 172 line was created by crossing *acs2-lacs6-1* with NES-YC3.6-expressing WT plants.
 173 Progeny were selected on kanamycin-containing medium and by measuring
 174 fluorescence in leaves. All F_3 progeny meeting the requirements were used in
 175 subsequent experiments.

176 All seeds were collected and stored under the same conditions. Prior to
 177 experiments, seeds were surface-sterilized and sown on Murashige-Skoog
 178 medium. After 3 days at 4°C in darkness, plates were transferred to a greenhouse
 179 ($21 \pm 2^\circ\text{C}$, 70% humidity, $100 \mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity, and a 16-h light/8-h
 180 dark photoperiod). After germination and growth for 7 days, young seedlings were
 181 transplanted into water-saturated soil. Watering was halted according to the
 182 requirements of each specific drought treatment described in this paper.

183 **Creation of transgenic plants**

184 To generate *ACS6*- and *SPCH*-overexpression lines, the full-length coding
 185 sequence (CDS) of *ACS6* or *SPCH* was amplified and cloned into the pSUPER
 186 1300 vector. Each construct was then introduced into *Agrobacterium* strain

GV3101 and transformed into the target plants by floral infiltration. The same method was used to generate the transformants described below. To generate ACS6-complementation (*ACS6/acs6-1*) lines, the promoter and CDS of *ACS6* were cloned into a pCAMBIA1300 vector, which was transformed into *acs6-1* plants. To generate *pACS6::ACS6-GUS* lines, the *ACS6* promoter fragment and full-length CDS were cloned into the promoter-less GUS expression vector pCAMBIA1391, which was then transformed into WT. To generate *pSDD1::SDD1-GFP* lines, the promoter fragment and CDS of *SDD1* were cloned into a pCAMBIA1300 vector, which was then introduced into *acs2-lacs6-1* and WT. The T₁ transgenic plants were selected on hygromycin-containing medium, and the T₃ progeny were used for subsequent experiments.

Water loss assay

True leaves were collected from 28-day-old plants following previously described methods (Xie *et al.*, 2019). The fresh weight of leaves was determined immediately. Leaves of five plants per line were weighed hourly on an electronic balance (Sartorius, Germany) at room temperature (23°C). Water loss was calculated using the following formula: $((W1-W2)/W1) \times 100\%$, where W1 is the initial leaf fresh weight, and W2 is the leaf weight at a given time point.

Evaluation of stomatal density and rate of stomatal clustering

Stomatal density and clustering ratio were determined according to previously described methods (de Marcos *et al.*, 2017; Qi *et al.*, 2019). The 6th fully expanded rosette leaves (count up from cotyledons) were used for analyzing the stomatal phenotype of 28-day-old seedlings. Strips were peeled from leaf abaxial epidermis, fixed on a slide, and photographed under a differential contrast interference microscope (LSM710, Zeiss, Germany). Images were acquired under the 20× objective (0.18 mm²). Randomly selected images are shown in figures.

For analyses of stomatal density and clustering rate, 25 plants per line per plant were examined. In all counts, a stoma was considered to have a pair of complete guard cells. Stomatal density was calculated as follows: stomatal density = stomatal number/area (mm²). The rate of clustered stomata was calculated as follows: number of clusters/(number of stomata + number of clusters).

RNA extraction and quantitative real-time polymerase chain reaction analyses

220 Total RNA was extracted using a plant RNA MIDI kit (Life-Feng, Shanghai,
221 China). First-strand complementary DNA (cDNA) was synthesized with a
222 Reverse Transcription system (Toyobo, Osaka, Japan) and was used as the
223 template for quantitative real-time polymerase chain reaction (qRT-PCR) analyses
224 along with 2× SYBR Green I master mix (Vazyme, Nanjing, China). The
225 qRT-PCR analyses were performed on a Roche 480 real-time PCR system (Roche,
226 Mannheim, Germany). The RNA levels were calculated as described by Livak
227 and Schmittgen (2001). The reference gene was *ACTIN8* (AT1G49240).

228 **GUS staining**

229 Leaves excised from 21-day-old plants or drought-treated plants were incubated
230 overnight in darkness at 37°C in GUS staining solution (0.1 M sodium phosphate
231 buffer, pH 7.0; 0.05 mM K₃[Fe(CN)₆]; 0.05 mM K₄[Fe(CN)₆]; 1 mg ml⁻¹ X-Gluc
232 (Sigma, USA); and 0.1% Triton X-100). After staining, leaves were de-stained
233 with 75% (v/v) ethanol until the chlorophyll was completely removed. The stained
234 leaves were photographed using a Nikon Coolpix or Canon 760D digital camera.
235 Representative photographs are shown in figures.

236 **Measurement of ACC content**

237 Leaves from the same line of 21-day-old plants were collected and ground into a
238 powder. A 0.1-mg aliquot of powdered sample was transferred into an Eppendorf
239 tube along with 1 ml ultrapure water. To completely extract ACC from leaf tissue,
240 the sample was further fragmented using an ultrasonic crusher (Branson, Danbury,
241 CT, USA). The supernatant was collected, the pH was adjusted to <4, and
242 impurities were removed using 1 ml chloroform. The supernatant was then passed
243 through a column containing C18 adsorbent (Oasis MCX, 30 µm, 3 cc/60 mg,
244 Waters, Milford, MA, USA). The column was eluted with 1 M ammonia in water,
245 with chromatographic methanol as the solvent. The eluent was evaporated to
246 dryness in a Concentrator Plus evaporator (Eppendorf, Hamburg, Germany) under
247 vacuum at 30°C and then re-suspended in solution (chromatographic methanol:
248 0.1% (v/v) acetic acid, 1:9). Samples were analyzed using an Applied Biosystems
249 MDS SCIEX 4000 QTRAP liquid chromatography-tandem mass spectrometry
250 system (AB Sciex, Foster City, CA, USA). Standard ACC (Sigma-Aldrich,
251 Steinheim, Germany) was used for the quantitative analysis.

252 **Protein extraction and western blotting**

253 Leaves were collected according to the experimental requirements and ground
254 into a powder. Powdered samples were transferred to RIPA lysis buffer (Boster
255 Biotechnology, Wuhan, China) and micro-centrifuged at 16,000 *g* for 15 min at
256 4°C. The concentration of crude protein in the supernatant was determined using a
257 NanoDrop 2000 (Thermo Scientific, Wilmington, DE, USA). The crude protein
258 was separated by 12% SDS-PAGE and then transferred to a nitrocellulose filter
259 membrane (Millipore, Billerica, MA, USA) using a Trans-Blot Semi-Dry transfer
260 cell (Bio-Rad, Hercules, CA, USA). The membrane was then incubated at room
261 temperature for 1-2 h in blocking solution before incubation with anti-GFP mouse
262 monoclonal antibodies (1:10,000; Proteintech, Chicago, IL, USA) for 2 h at room
263 temperature. The membrane was subjected to three 10-min washes with TBST and
264 then incubated overnight at 4°C with horseradish peroxidase-conjugated
265 secondary antibody (Proteintech). Protein bands were detected using a BeyoECL
266 Plus kit (Beyotime, Shanghai, China) and then visualized using a Fusion FX7
267 Spectra system (Vilber Lourmat, Marne-la-Vallée, France). An anti-GAPDH
268 antibody (1:5000; Proteintech) was used as the loading control.

269 **Chromatin immunoprecipitation analyses**

270 Immature leaves collected from *pSPCH::SPCH-GFP* of 21-day-old plants were
271 cross-linked using 1% formaldehyde under vacuum for 10 min according to the
272 EZ-ChIP chromatin IP kit protocol (Thermo Scientific). After washing with
273 phosphate-buffered saline solution, leaves were ground in liquid nitrogen and then
274 suspended in SDS lysis buffer containing protease inhibitor cocktail. The DNA
275 was sheared into small fragments (300–500 bp). The sheared chromatin was
276 immune-precipitated with GFP antibodies (Proteintech) overnight at 4°C. The
277 ChIP DNA products were analyzed by RT-qPCR using three pairs of primers
278 synthesized to amplify approximately 200-bp DNA fragments of the promoter
279 region of *ACS2* or *ACS6*, which were used in the ChIP analysis. Primers annealing
280 to promoter regions of two *Arabidopsis* genes lacking an SPCH binding site were
281 used as negative controls. An unrelated DNA sequence from the *ACTIN8* gene
282 was used as an internal control.

283 **Transient transcription dual-luciferase assays**

284 Detection was performed according to previously described methods (Bao *et al.*,
285 2014). The 2400-bp promoter sequence of *ACS2* was divided into 3 fragments (-1

286 to -1000, -900 to -1600, and -1500 to -2400 bp). The 2600-bp promoter sequence
287 of *ACS6* was also divided into 3 fragments (-1 to -1000, -900 to -2000, and -1900
288 to -2600 bp), and the -1 to -780 bp promoter sequence of *GTL1* was selected.
289 Each fragment was cloned into pGreen II 0800-Luc to construct the corresponding
290 reporter plasmid. The coding sequence of *Arabidopsis SPCH* was cloned into
291 pGreenII 62-SK to construct the 35S-SPCH effector plasmid. The *Agrobacterium*
292 strain GV3101 (pSoup-p19) was incubated in yeast mannitol medium and finally
293 re-suspended in buffer to a final concentration of $OD_{600} = 1.0$. Equal amounts of
294 different combined bacterial suspensions were infiltrated into young leaves of
295 tobacco plants using a needleless syringe. After 3 days, the infected leaves were
296 sprayed with D-luciferin (sodium salt) (Yeasen, Shanghai, China) and placed in
297 darkness for 5 min. Firefly luciferase (LUC) signals were then detected using the
298 NightSHADE system (LB 985, Berthold Technologies, Bad Wildbad, Germany).
299 The ratio of LUC activity to Renilla luciferase (REN) activity was measured using
300 a Dual-Luciferase Reporter Gene Assay kit (Solarbio, Beijing, China). Briefly, the
301 tobacco leaves were ground in liquid nitrogen, and the extract was incubated in a
302 low-temperature buffer. The LUC/REN ratio was measured using an enzyme
303 standard instrument (Tecan, Männedorf, Switzerland).

304 **Monitoring of Ca^{2+} levels in stomatal lineage cells**

305 The Ca^{2+} levels in stomatal lineage cells were monitored according to [Krebs et al.](#)
306 [\(2012\)](#). Immature leaves of *Arabidopsis* seedlings expressing the fluorescence
307 resonance energy transfer (FRET)-based Ca^{2+} sensor NES-YC3.6 ([Nagai et al.,](#)
308 [2004; Krebs et al., 2012](#)) were collected from the same position. During confocal
309 laser scanning, strips were peeled from leaf abaxial epidermis and then fixed on a
310 slide on the loading platform. The relative fluorescence intensity of YC3.6 protein
311 was recorded under a Nikon A1 Plus laser scanning confocal microscope (Nikon,
312 Tokyo, Japan) with the following scanning parameters: image dimension = $1024 \times$
313 1024 , pinhole radius = $38.31 \mu m$, scanning speed = 0.25 , zoom = $3\times$, objective =
314 $60\times$ (water), numerical aperture = 1.27 , plan apochromat objective, power = 6%
315 (445 nm solid laser). Images were acquired every $5s$. Emissions from cyan
316 fluorescent protein (CFP; $465\text{-}499 \text{ nm}$) and FRET-dependent cpVenus ($525\text{-}555$
317 nm) in stomatal lineage were detected simultaneously. The cpVenus/CFP
318 emission ratio was analyzed using NIS-Elements AR software.

319 **Statistical analysis**

320 All experiments were independently repeated using three biological replicates and
321 three technical replicates at least. Differences among treatments were compared
322 using Student's *t*-test. *P*-values < 0.05 (*) and < 0.01 (**) were considered to
323 correspond to significant and extremely significant differences, respectively.

324 **Results**

325 **ACS2/6 activation and ACC accumulation facilitated water evaporation from** 326 **leaves in response to drought**

327 Studies have shown that the activity of ACS2 in rice (Zhang *et al.*, 2013) and
328 ACS6 in maize (Young *et al.*, 2004) regulates seedling sensitivity to drought, and
329 that drought induces ACS2/6 activation and ACC accumulation in *Arabidopsis*
330 (Catalá *et al.*, 2014; Dubois *et al.*, 2018). Therefore, we examined the possible
331 roles of ACS2 and ACS6 in the drought response of *Arabidopsis* seedlings.

332 The expressions of ACS2 and ACS6 were modified in several genetic
333 materials. For example, compared with WT, the loss-of-function mutant lines
334 *acs2-1*, *acs6-1*, and *acs2-lacs6-1* showed significantly reduced ACS2 or ACS6
335 mRNA levels; the transgenic ACS2-OE(#1) and ACS6-OE(#1) over-expression
336 lines had significantly elevated ACS2 and ACS6 mRNA levels, respectively; the
337 transgenic complemented lines ACS2/*acs2-1*(#1) and ACS6/*acs6-1*(#1) exhibited
338 no changes in ACS2 or ACS6 expression, respectively (Fig. 1A). Interestingly, the
339 expressions of ACS6 and ACS2 were relatively unchanged in the single mutants
340 *acs2-1* and *acs6-1*, respectively (Fig. 1B). We first checked the growth
341 phenotypes of the various ACS2/6 expression lines in response to drought. Under
342 normal watering (control) conditions, no significant water-losing phenotypes were
343 apparent among these diverse expression lines. Under gradually intensifying
344 drought caused owing to stopping watering, however, the phenotypes were
345 obviously different. In particular, WT, ACS2/*acs2-1*(#1), ACS6/*acs6-1*(#1),
346 ACS2-OE(#1), and ACS6-OE(#1) seedlings withered and some even died after
347 water was withheld for 12 days and the soil water content dropped to ~39%,
348 whereas wilting and drying symptoms were clearly alleviated in the mutants
349 *acs2-1*, *acs6-1*, and *acs2-lacs6-1* seedlings (Supplementary Fig. S1).
350 Preliminary data showed that ACS2/6 activation promoted dehydration and

351 wilting of seedlings under drought conditions.

352 The rate of water evaporation from leaves of these lines was monitored under
353 drought conditions. The water evaporation rate was decreased in *acs2-1*, *acs6-1*,
354 and *acs2-lacs6-1* leaves, compared with that in WT. This decrease was less
355 pronounced in the double mutant *acs2-lacs6-1* than in the single mutants *acs2-1*
356 and *acs6-1* (Fig. 1C). Conversely, the water evaporation rate was significantly
357 increased in ACS2-OE(#1) and ACS6-OE(#1) compared with that of WT, whereas
358 no significant change was observed in ACS2/*acs2-1*(#1) or ACS6/*acs6-1*(#1) (Fig.
359 1C). Data suggest that ACS2/6 activation was positively correlated with the rate
360 of water evaporation from leaves under drought conditions.

361 The characteristics of ACS2/6 expression in leaves were examined in
362 response to drought treatment. Histochemical staining revealed that the higher
363 GUS-marked ACS2/6 expression was in immature leaves, followed by senescent
364 leaves, and then mature leaves in WT plants; After withholding water for 6 days,
365 ACS2 and ACS6 expressions in WT were significantly increased in immature
366 leaves, slightly increased in mature leaves, and unchanged in senescent leaves
367 (Fig. 2A). Meantime, quantitative PCR showed the same results (Fig. 2B). This is,
368 ACS2 and ACS6 expressions were always higher in senescent leaves regardless of
369 drought, whereas both expressions increased in response to drought in
370 non-senescent leaves. Next, ACS2/6-dependent ACC accumulation was analyzed
371 in leaves. Under normal conditions, ACC mainly accumulated in immature and
372 senescent leaves of WT seedlings, whereas ACC accumulated primarily in
373 immature leaves in response to a 6-day halt in watering. More specifically, ACC
374 levels were, respectively, 1.94- and 1.33-times higher in immature and mature
375 leaves of WT after withholding water (Fig. 2C). In contrast to WT, the double
376 mutant *acs2-lacs6-1* did not significantly accumulate ACC in the leaves in
377 response to drought (Fig. 2C). These data indicate that drought induced ACS2/6
378 activation and ACC accumulation in non-senescent leaves of *Arabidopsis*
379 seedlings.

380 ACS2/6 activation affected stomatal density and pattern on leaf abaxial 381 epidermis

382 Evidences suggest that the ACS activity was required for stomatal development
383 (Serna and Fenoll, 1996; Saibo *et al.*, 2003; Young *et al.*, 2004; Zhang *et al.*, 2013;

384 [Lau et al., 2014; Yin et al., 2019](#)), we thus analyzed the effects of ACS2/6 activity
385 on stomatal density and pattern on the leaf abaxial epidermis.

386 Images of stomata on the abaxial epidermis of the 6th (count up from
387 cotyledon) mature leaves of the various ACS2/6 expression lines under normal
388 and drought conditions are shown in [Fig. 3A](#). Under normal watering conditions,
389 stomatal density and the percentage of clustering were slightly decreased in the
390 mutants *acs2-1*, *acs6-1*, and *acs2-lacs6-1*, but slightly increased in ACS2-OE(#1)
391 and ACS6-OE(#1), compared with that in WT ([Fig. 3B](#)). After halting watering for
392 6 days, however, stomatal density was significantly reduced in *acs2-1* (177.8 ± 8.2
393 mm^{-2}), *acs6-1* ($183.3 \pm 6.2 \text{ mm}^{-2}$), and *acs2-lacs6-1* ($161.1 \pm 8.2 \text{ mm}^{-2}$), but
394 significantly increased in ACS2-OE(#1) ($255.6 \pm 6.9 \text{ mm}^{-2}$) and ACS6-OE(#1)
395 ($261.1 \pm 4.9 \text{ mm}^{-2}$), compared with that in WT ($205.6 \pm 5.4 \text{ mm}^{-2}$) ([Fig. 3B](#)). The
396 percentage of the pairs of directly contacting stomata was significantly higher in
397 ACS2-OE(#1) ($\sim 3.1\%$) and ACS6-OE(#1) ($\sim 2.7\%$) than in WT ($\sim 0.2\%$). In
398 contrast, the stomatal clustering rates were lower in the mutants *acs2-1* ($\sim 0.12\%$),
399 *acs6-1* ($\sim 0.15\%$) and *acs2-lacs6-1* ($\sim 0.1\%$) than in WT ([Fig. 3C](#)). Evidently,
400 ACS2/6 activation increased stomatal density and cluster on the abaxial epidermis.
401 In addition, application of 5–10 μM ACC also significantly increased the stomatal
402 density and clustered stomata on the leaf abaxial epidermis of WT seedlings
403 ([Supplementary Fig. S2](#)). This validation experiments indicate that appropriate
404 concentrations of ACC increased stomatal density and cluster on leaf epidermis.

405 **SPCH could bind to promoters of ACS2/6 and regulate their expression**

406 The above data suggest that the effect of ACS2/6-dependnet ACC accumulation is
407 similar to that of SPCH ([Tripathi et al., 2016; Zoulas et al., 2018](#)) in promoting
408 stomatal density on the leaf epidermis, we speculated that SPCH may mediate
409 ACS2/6 expression activity. Although a profile list generated by genome-wide
410 ChIP-based sequencing of the targets of SPCH included both ACS2 and ACS6
411 ([Lau et al., 2014](#)), direct experimental evidence was still lacking.

412 To explore whether SPCH affect ACS2 and ACS6 expression, we checked
413 mRNA levels of ACS2 and ACS6 in the loss-of-function *spch-1* and *spch-3* mutant
414 seedlings, respectively. In order to explore the stomatal development on the
415 epidermis of true leaves, the heterozygote of *spch-1* and *spch-3* were used,
416 because the two homozygotes cannot grow true leaf ([MacAlister et al., 2007](#);

417 Pillitteri *et al.*, 2007; Han and Torii, 2016). Observations indicated that both
418 *spch-1* and *spch-3* had significantly reduced *ACS2* and *ACS6* mRNA levels,
419 respectively, whereas *SPCH*-OE lines had significantly increased mRNA levels,
420 compared with the WT control (Fig. 4A). Interestingly, drought similarly induced
421 the expressions of *SPCH*, *ACS2*, and *ACS6* genes (Fig. 4B). Data implies that
422 *SPCH* activity was positively correlated with the expression of *ACS2* and *ACS6*.

423 To confirm this experimentally, we used ChIP assays to detect the interaction
424 between the transcription factor *SPCH* and the promoters of *ACS2* and *ACS6*. The
425 *in silico* analyses revealed three E-box motifs in the 3.0-kb promoter region of the
426 *ACS2* gene: CGCGTG and CACGTG (at -1079 and -1090), collectively named
427 *ACS2*-P1 because of their close proximity, and CACGTG (at -2326), designated
428 as *ACS2*-P2. Only one E-box motif was present in the 3.0-kb promoter region of
429 the *ACS6* gene: CACGTG (at -2537), named *ACS6*-P1 (Fig. 4C). After randomly
430 selecting DNA fragments from their promoter regions with the same length as the
431 E-boxes (named *ACS2*-P and *ACS6*-P) as the reference, ChIP assays were
432 performed to measure levels of immune-precipitated DNA fragments by *SPCH*
433 protein *in vivo*. In these assays, the abundance of DNA fragments from *ACS2*
434 promoters *ACS2*-P1 and *ACS2*-P2 was, respectively, 6.13- and 1.18-fold higher
435 than that of the control *ACS2*-P (Fig. 4D). Similarly, the abundance of *ACS6*-P1
436 immune-precipitated by *SPCH* protein was 4.57-fold higher than that of the
437 control *ACS6*-P (Fig. 4D). Next, we conducted transient transcription activity
438 assays to verify the binding of *SPCH* to the promoters of *ACS2* and *ACS6*.
439 According to the results, the fluorescence intensity of LUC linked to the specific
440 promoter fragment of *ACS2* (-900 to -1600 bp, containing *ACS2*-P1) was
441 increased in the presence of *SPCH*, with LUC activity 4.2-times higher than that
442 of the blank LUC control (Fig. 4E, F). Similarly, the activity of LUC linked to the
443 promoter fragment of *ACS6* (-1900 to -2600 bp, containing *ACS6*-P1) in the
444 presence of *SPCH* was 3.7-times higher than that of the blank LUC control (Fig.
445 4E, F). This stimulatory effect was specific, as *SPCH* did not induce LUC activity
446 alone or when linked to E-box-free promoter fragments of *ACS2* or *ACS6*
447 (Supplementary Fig. S3). In other words, *SPCH* directed the transcription of *ACS2*
448 or *ACS6* by docking to each of their promoter regions.

449 To verify that *SPCH* promoted *ACS2/6* expression, we monitored the effects

of SPCH activity on ACC levels. The results showed that the SPCH-overexpressing lines *SPCH*-OE(#1), *SPCH*-OE(#2), and *SPCH*-OE(#3) had significantly increased ACC levels (Fig. 5A), but the mutants *spch-1* or *spch-3* had reduced ACC levels in immature leaves, as compared with the ACC levels in leaves of WT (Fig. 5B). Evidently, SPCH-directed ACS2/6 expression was directly related to ACC accumulation in immature leaves. Further observations helped to explain how SPCH mediated stomatal development *via* ACS2/6-dependent ACC production. The single mutant *spch-1*, the double mutant *acs2-lacs6-1*, and the triple mutant *spch-lacs2-lacs6-1* had significantly reduced stomatal densities under normal or drought conditions, compared with WT (Fig. 5C, D). In addition, the stomatal density on leaves was lower in the triple mutant *spch-lacs2-lacs6-1* than in its parents *spch-1* and *acs2-lacs6-1* (Fig. 5C, D). Notably, ACS2- or ACS6-overexpression in *spch-1* reversed the reduction in stomatal density (Fig. 5D). These observations suggest that SPCH activity induced ACS2/6 activation and ACC accumulation.

ACC accumulation decreased SDD1 expression levels in leaves

Evidences have shown that SDD1 expression reduces stomatal density and cluster (Yoo *et al.*, 2010; Yoo *et al.*, 2019), but exogenously applied ACC increases stomatal density and cluster (Serna and Fenoll, 1996; Saibo *et al.*, 2003; Acharya and Assmann, 2009). We therefore verified whether ACC cooperates with SDD1 to establish stomatal density and cluster.

The effect of ACS2/6-dependent ACC accumulation on SDD1 expression was surveyed in immature leaves. The *SDD1* mRNA levels in *acs2-1*, *acs6-1*, *acs2-lacs6-1*, *ACS2*-OE(#1), and *ACS6*-OE(#1) were, respectively, 1.88-, 1.89-, 2.18-, 0.64-, and 0.67-fold that in WT (Fig. 6A). This result suggests that *SDD1* expression was negatively correlated with ACS2/6 activation in immature leaves. Further monitoring of SDD1 protein levels by western blotting indicated that GFP-marked SDD1 protein levels in immature leaves were higher in *acs2-lacs6-1* than in WT under drought conditions (Fig. 6B). Likewise, ACC treatment reduced protein levels of SDD1 in WT leaves compared with the blank control (Fig. 6B). This result is consistent with the expectation that ACC treatment would reduce *SDD1* mRNA transcript levels in immature leaves of WT (Supplementary Fig. S4). These observations suggest that ACS2/6-generated ACC impeded *SDD1* gene

expression and SDD1 protein levels, thereby increasing stomatal density and cluster on the leaf epidermis.

SPCH activity positively regulated GTL1 expression

The trihelix transcription factor GTL1 is known to be the direct controller of SDD1 activity (Yoo *et al.*, 2010; Weng *et al.*, 2012; Viridi *et al.*, 2015), and ChIP-sequencing data suggest that GTL1 is a target of SPCH (Lau *et al.*, 2014). However, experimental evidence that SPCH affects GTL1 activity was lacking.

We investigated the effects of SPCH activity on *GTL1* expression. The *GTL1* mRNA levels in *spch-1*, *spch-3*, and *SPCH-OE*(#3) were, respectively, 0.43-, 0.36-, or 4.47-fold higher than that in WT (Fig. 7A). These data suggest that SPCH promoted *GTL1* expression. We verified this promoting effect by carrying out a transient transcription activity assay in tobacco leaves. Imaging analyses indicated that the presence of SPCH protein increased LUC fluorescence intensity linked to the specific promoter fragment of *GTL1* compared with that of the blank control, LUC alone (Fig. 7B). Interestingly, SPCH-stimulated LUC activity was 4.1-times higher than that of LUC alone (Fig. 7C), indicating that SPCH activated *GTL1* expression by binding to its promoter.

ACC buffered Ca^{2+} activity in stomatal lineage cells

Studies have shown that Ca^{2+} -loaded CaM can relieve the inhibition of *SDD1* expression by GTL1, while the susceptibility of GTL1 to Ca^{2+} levels mainly occurs in stomatal lineage (Weng *et al.*, 2012; Viridi *et al.*, 2015; Yoo *et al.*, 2019). Thus, we monitored how ACC mediates Ca^{2+} levels in stomatal lineage cells on the leaf epidermis. Using the Ca^{2+} fluorescence probe Fluo-4/AM, we preliminarily evaluated the Ca^{2+} levels in stomatal lineage cells on the immature leaf epidermis. Under normal conditions, the Ca^{2+} levels in stomatal lineage cells were slightly higher in *acs2-lacs6-1* than in WT. However, after halting watering for 6 days, the Ca^{2+} levels in stomatal lineage cells were higher in *acs2-lacs6-1* than in WT (Supplementary Fig. S5). This hints that the decreased ACC accumulation increased Ca^{2+} levels in stomatal lineage cells.

The Ca^{2+} -sensitive yellowameleon protein YC3.6 has been developed as a Ca^{2+} biosensor (Krebs *et al.*, 2012; Behera *et al.*, 2017). Therefore, we created NES-YC3.6 expressing *acs2-lacs6-1* lines to obtain *in vivo* data on Ca^{2+} accumulation in the stomatal lineage cells on the leaf epidermis. Both

fluorescence-symbolized and cpVenus/CFP ratio-labelled Ca^{2+} levels were analyzed. Under normal watering conditions, the fluorescence intensity of YC3.6 protein was slightly higher in *acs2-lacs6-1* than in WT (Fig. 8A). Moreover, the cpVenus/CFP ratio was slightly higher in *acs2-lacs6-1* than in WT (Fig. 8B). After halting watering for 6 days, the fluorescence intensity of YC3.6 protein was significantly higher in *acs2-lacs6-1* than in WT (Fig. 8A). Specifically, the Ca^{2+} levels in the stomatal lineage cells was 3.38-times higher in *acs2-lacs6-1* than in WT (Fig. 8B). This result shows that ACC accumulation in leaves significantly reduced Ca^{2+} levels or activity in stomatal lineage cells.

Discussion

These findings reveal the specific role of ACS2/6 activity in the establishment of stomatal density and pattern under drought. A schematic overview of the inter-relationships among these processes is provided in Fig. 9.

The activation of ACS2/6 lays the foundation for ACC-induced stomatal generation and pattern under drought. According to our data, stomatal density and clustering on the leaf epidermis were reduced in loss-of-function mutants *acs2-1*, *acs6-1*, and *acs2-lacs6-1*, but were increased in ACS2- and ACS6-overexpression lines (Fig. 3); to put it in another way, drought-activated ACS2/6 increased stomatal density and cluster, and these facilitated stomata-based water evaporation, in turn, seedlings withered and some even died with drought escalating. This finding provides a genetic explanation for the decrease in stomatal density and clustering caused by inhibitors of ACS activity, such as AVG or PAC, in *Arabidopsis* (Serna and Fenoll, 1996; Saibo *et al.*, 2003; Yin *et al.*, 2019), and also provide theoretical explanations why ACS2- or ACS6-deficient rice (Zhang *et al.*, 2013) or maize (Young *et al.*, 2004) are less sensitive to water deficit than are WT controls. Considering the specificity of ACS activity (Tsuchisaka and Theologis, 2004; Tsuchisaka *et al.*, 2009; Han *et al.*, 2019; Lv *et al.*, 2019), we presume that ACS2/6 activation is specific to stomatal development and spacing on the leaf epidermis when *Arabidopsis* seedlings are under drought. Because drought can induce ACS2/6 activation and ACC accumulation (Catalá *et al.*, 2014; Dubois *et al.*, 2018), the observed increase in stomatal density and cluster under drought conditions (Fig. 2, 3) is easily understandable. Simply, ACS2/6-dependent ACC accumulation increases the susceptibility of seedlings to

drought. Furthermore, activated ACS2 and ACS6 may function in parallel, as growth phenotypes (Supplementary Fig. S1), stomatal densities (Fig. 3B), and clustering (Fig. 3C) were similar among *acs2-1*, *acs6-1*, and *acs2-1acs6-1* mutants, and the expressions of ACS6 and ACS2 were relatively unaffected in the two single mutants *acs2-1* and *acs6-1*, respectively (Fig. 1B). Importantly, our results show that SPCH separately regulates the expression of ACS2 and ACS6 by binding to their promoters (Fig. 4). We speculate that ACS2 and ACS6 jointly ensure plants to fully respond to frequent drought stimuli.

ACS2/6-dependent ACC production is an important node of SPCH-based regulation of stomatal development and pattern. In line with a previous prediction (Lau *et al.*, 2014), our results provide evidence that SPCH acts as a transcription factor to control the expression of ACS2 and ACS6 (Fig. 4). The ability of SPCH to promote ACS2 and ACS6 expression was evidenced by the fact, for example, that *spch-1* and *spch-3* mutants showed reduced expressions of these genes (Fig. 4) and ACC content, whereas SPCH overexpression led to increased ACC levels (Fig. 5). This finding explains why plant tolerance to osmotic stress requires reduced SPCH activity (Han and Torii, 2016; Tripathi *et al.*, 2016; Zoulas *et al.*, 2018).

ACS2/6-generated ACC accumulation acts as a bridge between SPCH-initiated stomatal individual development and SDD1-directed stomatal spacing between stomata. Evidence for this conclusion is as follows: First, ACC mimics SPCH to reduce SDD1 activity, thereby increasing stomatal density and cluster. The mutants *acs2-1*, *acs6-1*, and *acs2-1acs6-1* seedlings (Fig. 2) mimicked transgenic *SDD1*-overexpressing plants by showing reduced stomatal density and cluster on leaves. Consistent with this observation, both the *sdd1-1* mutant (Von *et al.*, 2002) and ACS2- and ACS6-overexpressing plants (Fig. 6) exhibited increased stomatal density and cluster on the leaves. Second, ACC-associated Ca^{2+} insufficiency reduced SDD1 activity, or, alternatively, Ca^{2+} activity, in stomatal lineage cells, so that ACC levels were linked to SDD1 expression. Our findings indicated that Ca^{2+} levels in stomatal lineage cells on the leaf epidermis were higher in *acs2-1acs6-1* plants than in WT (Fig. 8; Supplementary Fig. S5). This suggests that ACC accumulation inhibits SDD1 activity by controlling Ca^{2+} activity in stomatal lineage cells. This result is reasonable because a Ca^{2+} shortage can stabilize the binding of GTL1 to the *SDD1*

promoter to prevent its expression in stomatal lineage cells (Yoo *et al.*, 2019). These findings explicate the mechanisms in the recent discovery that Ca^{2+} activity intensifies stomata-based water evaporation from leaves of *Arabidopsis* seedlings under drought conditions (Teardo *et al.*, 2019).

In brief, these findings first indicated that the ACS2/6 activity may specifically integrated SPCH-initiated stomatal individual development with SDD1-directed space setting between stomata under drought. This integration increased stomatal density and cluster on the leaf epidermis under moderate drought, which laid foundation for seedling wilting and death with drought escalating. The promotion of moderate drought to stomatal density and cluster provided a hint that the evolutionary memory of plants from aquatic to terrestrial may be evoked (Croxdale, 2000); while this evolutionary memory appears to override routine terrestrial regulation determining stomatal development (Croxdale, 2000; van Veen and Sasidharan, 2021).

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Authors' contributions

JJ and CPS formulated the experimental strategy. MZJ, JJ, LYL and CG performed experiments. JJ and MZJ wrote the paper.

Data availability

All data relevant to this study are presented in figures and supplementary data.

Supplementary data

Table. Primer list.

Fig. S1. ACS2- or ACS6-based growth phenotype of *Arabidopsis* seedling or with

613 drought treatment.

614 *Fig. S2.* ACC-increased stomatal density and percentage of clustering stomata on

615 leaf epidermis.

616 *Fig. S3.* The control experiment of SPCH-activated ACS2 or ACS6 expression,

617 respectively, in the transient transcription dual-luciferase assay system.

618 *Fig. S4.* RT-qPCR analysis on the inhibitory effect of ACC treatment on the

619 expression activity of *SDD1* gene in immature leaves.

620 *Fig. S5.* Ca^{2+} -stimulated Fluo-4/AM fluorescence intensity in stomatal lineage

621 cells on leaf epidermis.

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735

736 **Figure legends**

737 **Fig. 1.** Effect of ACS2/6 expression on water loss from leaves of *Arabidopsis*
738 seedlings.

739 (A) qRT-PCR analysis of ACS2 and ACS6 mRNA levels in various lines,
740 including the WT; loss-of-function mutants *acs2-1*, *acs6-1*, and *acs2-1acs6-1*;
741 overexpression lines ACS2-OE(#1) and ACS6-OE(#1); and complementation lines
742 ACS2/*acs2-1*(#1) and ACS6/*acs6-1*(#1). *ACTIN8* was used as a reference gene.
743 Experiments were repeated three times. Values are means $\pm$ SD (Student's *t*-test; *,
744 $P < 0.05$; **, $P < 0.01$).

745 (B) qRT-PCR analysis of ACS6 mRNA levels in *acs2-1* and ACS2 mRNA levels in
746 *acs6-1*. Experiments were repeated three times with similar results.

747 (C) Relative rate of water loss over time from detached rosette leaves of
748 28-day-old plants. All true leaves of five plants of the same line grown under
749 identical conditions were collectively weighed every hour. The data represent the
750 water loss percentage at a given time point, calculated as follows: ((initial weight
751 – weight at each time point) / initial weight) $\times$ 100. Experiments were repeated at
752 least three times with similar results. Values are means $\pm$ SD (Student's *t*-test; *, P
753 < 0.05).

754

755 **Fig. 2.** Effects of drought on ACS2 and ACS6 expressions and ACC accumulation
 756 in leaves of different ages.

757 (A) ACS2 or ACS6 expression was monitored in immature, mature and
 758 senescence leaves of recombinant *pACS2::ACS2-GUS* or *pACS6::ACS6-GUS* lines,
 759 respectively, with or without drought treatment. Representative images are shown.
 760 Experiments were repeated three times with similar results.

761 (B) ACS2 or ACS6 expression was monitored by qPCR in immature, mature and
 762 senescence leaves of WT, respectively, with or without drought treatment.
 763 Representative images are shown. Experiments were repeated three times with
 764 similar results. Values are means $\pm$ SD (Student's *t*-test; *, $P < 0.05$).

765 (C) HPLC analysis of ACC accumulation in leaves of 21-day-old seedlings with
 766 or without drought treatment. Experiments were repeated three times with similar
 767 results. Values are means $\pm$ SD (Student's *t*-test; *, $P < 0.05$).
 768

769 **Fig. 3.** Correlation between ACS2/6 activation and stomatal density and rate of
770 stomatal clustering.

771 (A) Images of stomata distributed on strips of leaf abaxial epidermis under
772 differential interference contrast (DIC) microscopy. Stomata and stomatal clusters
773 are colored in green, and stomatal clusters are indicated by red arrows. The
774 number of stomata is given in parentheses in each image. 6th rosette were
775 collected under drought or normal watering conditions from 28-day-old seedlings
776 of the WT control; mutants *acs2-1*, *acs6-1*, and *acs2-1acs6-1*, overexpression
777 lines *ACS2*-OE(#1) and *ACS6*-OE(#1), and complementation lines
778 *ACS2/acs2-1*(#1) and *ACS6/acs6-1*(#1). Experiments were performed three times
779 with similar results. The black scale bar represents 100 μ m.

780 (B) and (C) Statistical analysis of stomatal density (B) and percentage of stomatal
781 clusters (C). Stomata and stomatal clusters on 25 leaves of 25 seedlings were
782 counted. Values are means $\pm$ SD (Student's *t*-test; *, $P < 0.05$; **, $P < 0.01$).

783

784 **Fig. 4** Evidence for the function of SPCH as a transcription factor of *ACS2* and
785 *ACS6* genes.

786 (A) Levels of *ACS2* and *ACS6* mRNA in immature leaves of WT control,
787 loss-of-function *spch-1* and *spch-3* mutant, and *SPCH*-overexpressing
788 *SPCH*-OE(#3) plants based on qRT-PCR. Experiments were repeated three times
789 with similar results. Values are means $\pm$ SD (Student's *t*-test; *, $P < 0.05$).

790 (B) Relative levels of *ACS2*, *ACS6*, and *SPCH* mRNA transcripts in leaves of
791 21-day-old WT seedlings under normal watering conditions or after 6 days
792 without watering. Experiments were repeated three times with consistent results.
793 Values are means $\pm$ SD (Student's *t*-test; *, $P < 0.05$).

794 (C) Diagram of the relative position of E-boxes (CACGTG or CGCGTG) and a
795 reference DNA region in *ACS2* and *ACS6* gene promoters. Black rectangles
796 indicate E-boxes in *ACS2* (*ACS2*-P1 and *ACS2*-P2) and *ACS6* (*ACS6*-P1)
797 promoter regions, while white rectangles represent reference regions, namely
798 randomly selected DNA fragments from *ACS2* (*ACS2*-P) and *ACS6* (*ACS6*-P)
799 promoter regions.

800 (D) Relative abundance of SPCH-immunoprecipitated DNA fragments as
801 determined by qRT-PCR. All experiments, which included three biological
802 replicates, gave similar results. Values are means $\pm$ SD (Student's *t*-test; **, $P <$
803 0.01).

804 (E) and (F) Binding of SPCH protein to *ACS2* and *ACS6* genes in tobacco leaves
805 in a transient transcription dual-luciferase assay. The size and intensity of LUC
806 fluorescence signals recorded by IndiGO software are proportional to binding
807 ability (E). Relative binding ability was evaluated quantitatively by calculating the
808 ratio of the fluorescence intensity of firefly luciferase (LUC) to that of an internal
809 control, Renilla luciferase (REN) (F). Values are means $\pm$ SD ($n = 3$). Asterisks
810 indicate significant differences (*, $P < 0.05$) compared with leaf regions injected
811 with *Agrobacterium* harboring an empty vector.

812

813 **Fig. 5.** Correlation between SPCH activity and ACS2/6-dependent ACC
814 accumulation and stomatal density and pattern.
815 (A) and (B) qRT-PCR-based relative levels of *SPCH* mRNA transcripts and ACC
816 content in immature leaves of WT control, loss-of-function mutant *spch-1* and
817 *spch-3*, and *SPCH*-overexpressing *SPCH*-OE(#1), *SPCH*-OE(#2), and
818 *SPCH*-OE(#3) plants. The *ACTIN8* gene was used as an internal control. The
819 experiment was repeated three times with consistent results. Values are means $\pm$
820 SD (Student's *t*-test; *, $P < 0.05$; **, $P < 0.01$).
821 (C) and (D) DIC images and statistical summary of stomatal density and
822 patterning on the abaxial epidermis of *spch-1*, *acs2-lacs6-1*, and
823 *spch-lacs2-lacs6-1* plants. Numbers of stomata are indicated in parentheses in
824 each image. Stomata are false colored in green. The black scale bar represents 100
825 μm (C). Stomata on 25 leaves of 25 seedlings were counted (D). Values are means
826 $\pm$ SD. Significant differences are indicated by asterisks (Student's *t*-test; *, $P <$
827 0.05; **, $P < 0.01$).
828

829 **Fig. 6.** Inhibitory effects of ACS2/6 activation and ACC treatment on SDD1
830 expression and protein accumulation.

831 (A) qRT-PCR-based relative levels of *SDD1* mRNA transcripts in immature leaves
832 of WT, *acs2-1*, *acs6-1*, *acs2-lacs6-1*, ACS2-OE(#1), and ACS6-OE(#1) seedlings.
833 The experiment was repeated three times with consistent results. Values are
834 means $\pm$ SD (Student's *t*-test; *, $P < 0.05$; **, $P < 0.01$).

835 (B) Evaluation of SDD1 protein levels by western blotting. The fusion protein
836 GFP-SDD1 was collected from immature leaves of
837 *pSDD1::SDD1-GFP*-expressing WT or *acs2-lacs6-1* lines with or without
838 drought and ACC (0 or 10 μ M) treatment. Levels of GFP-SDD1 fusion protein
839 were determined using GFP antibody. GAPDH protein was used as a loading
840 control. The experiment was repeated three times with consistent results.
841

842 **Fig. 7. SPCH promotion of GTL1 expression.**

843 (A) qRT-PCR-based relative levels of *GTL1* mRNA transcripts in immature leaves
844 of WT, *spch-1*, *spch-3*, and *SPCH*-OE(#3) plants. *ACTIN8* was used as an internal
845 control. The experiment was repeated three times with consistent results. Values
846 are means $\pm$ SD (Student's *t*-test; *, $P < 0.05$).

847 (B) and (C) Binding of SPCH protein to the *GTL1* promoter in tobacco leaves in a
848 transient transcription dual-luciferase assay. The size and intensity of LUC
849 fluorescence signals recorded by IndiGO software are proportional to binding
850 ability (B). Relative binding ability was evaluated quantitatively by calculating
851 the ratio of the fluorescence intensity of firefly luciferase (LUC) to that of an
852 internal control, Renilla luciferase (REN) (C). Values are means $\pm$ SD ($n = 3$).
853 Asterisks indicate significant differences (*, $P < 0.05$) compared with leaf regions
854 injected with *Agrobacterium* harboring an empty vector.

855

856 **Fig. 8.** ACS2/6-generated ACC buffering of Ca^{2+} activity in stomatal lineage cells.
 857 (A) The relative fluorescence intensity of YC3.6 expression in stomatal lineage
 858 cells of immature leaves of NES-YC3.6-expressing WT or *acs2-lacs6-1* plants or
 859 by drought treatment. Representative images from at least 25 leaves in each line
 860 are shown. Pseudocolors in images correspond to relative Ca^{2+} levels according to
 861 the color scale on the right. Scale bar, 10 μm .
 862 (B) Evaluation of Ca^{2+} levels in stomatal lineage cells (labeled with red circle) of
 863 immature by calculating the ratio of the FRET acceptor cpVenus (at 525-555 nm)
 864 to the FRET donor CFP (at 465-499 nm). The experiment was repeated three
 865 times with consistent results. Values are means $\pm$ SD (Student's *t*-test; **, $P <$
 866 0.01).
 867

868 **Fig. 9.** Diagram illustrating the role of ACS2/6 expression and ACC accumulation
869 in the integration of SPCH-initiated stomatal development with SDD1-dependent
870 stomatal spacing.

871 In the top row depicting stomatal development, non-stomatal lineage cells, such as
872 leaf epidermal cells, protodermal cells, and meristemoid mother cells (MMCs),
873 are shown in blue, while stomatal lineage cells, including meristemoid cells, guard
874 mother cells (GMCs), and guard cell (GCs), are indicated in yellow. Under water
875 deficit conditions, SPCH increases ACS2, ACS6, and GTL1 expressions. Next,
876 ACS2/6-generated ACC accumulation may be involved in two processes: 1)
877 promoting the transformation of GMCs into GCs, and 2) inducing a shortage of
878 Ca^{2+} in stomatal lineage cells and increasing SDD1 activity. As a consequence of
879 these two processes, ACS2/6-generated ACC accumulation increases stomatal
880 density and the rate of clustering, which ultimately leads to plant wilting and
881 death under conditions of escalating drought. Relationships among these events
882 are indicated by arrows: blue for conclusions drawn from the literature, and black
883 for findings of the present study.

884

Figures

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8

Figure 9