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ORIGINAL RESEARCH

A Pleiotropic Phenotype is Associated with Altered Endogenous Hormone Balance in the Developmentally Stunted Mutant (*dsm1*)

Hui-Fang Zhao · Kai Qiu · Guo-Dong Ren · Yong Zhu · Ben-Ke Kuai

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Abstract A developmentally stunted mutant (*dsm1*) of *Arabidopsis*, isolated from an EMS mutant screen, had a pleiotropic phenotype, including repressed germination, retarded growth, delayed flowering, and impaired fertility. Additionally, *dsm1* had a lifespan of approximately 160 days, which was more than twice the lifespan of the wild type (Col-0). Fine morphological and anatomical characters, such as the shoot apical meristem, root apical meristem, seed shape, and seed surface, were obviously altered in *dsm1*. We found that both abscisic acid and zeatin riboside levels were significantly greater in *dsm1* than in Col-0 at all stages of development, while the levels of indole-3-acetic acid and gibberellins varied by age. The expressions of some abscisic acid-related genes were higher in *dsm1* than in Col-0. These data indicate that DSM1 may play a general role in plant growth and development.

Keywords ABA · Development · *dsm1* · Hormone balance · Pleiotropic phenotype

Phytohormones are chemical messengers produced in one part of an organism and transported to other regions, where they can exert a substantial effect at very low concentrations. Abscisic acid (ABA) regulates many aspects of plant development, including the synthesis of seed storage proteins and lipids, the promotion of seed desiccation tolerance and dormancy, and the inhibition of stage transitions from embryonic to germinative growth and from vegetative to reproductive growth (Leung and Giraudat 1998; Rock 2000; Rohde et al. 2000; Brocard-Gifford et al. 2004; Frey et al. 2006). In addition, ABA mediates some aspects of the physiological responses to environmental stresses, such as drought, cold, and the pathogen (Leung and Giraudat 1998; Rock 2000; Shinozaki and Yamaguchi-Shinozaki 2000; Millar et al. 2006). ABA is involved in the gradual conversion of embryonic leaves to abnormal leaves and thereby regulates heteroblastic leaf shape changes. Changes in ABA concentration influence the intensity of shoot apical meristem (SAM) organogenic activity (Le Hir et al. 2006). In *Arabidopsis*, ABA biosynthesis is controlled by a small family of 9-cis-epoxycarotenoid dioxygenase (NCED) genes. Five *AtNCED*s (2, 3, 5, 6, and 9) have been cloned and studied (Bowman et al. 1989; Lefebvre et al. 2006).

ABA seems to antagonize the effects of gibberellins (GAs) in many cases (Piskurewicz et al. 2008). In addition, recent studies have demonstrated an interaction between ABA-induced signaling and light (Rock 2000; Finkelstein and Rock 2002). Developing seedlings devote their nutritional reserves, including storage proteins and lipids, almost exclusively to hypocotyl extension initially and then develop the machinery for photosynthesis upon exposure to light (McNellis and Deng 1995; Fujiwara et al. 2002; Penfield et al. 2006). However, the molecular basis of many cross-talks among different plant hormones and signals remain poorly understood (Peng et al. 2009).

We previously reported that *dsm1* (former *drm1*) has an average lifespan of 160 days, which is more than twice that

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H.-F. Zhao · K. Qiu · G.-D. Ren · Y. Zhu · B.-K. Kuai (✉)
State Key Laboratory of Genetic Engineering,
Institute of Plant Biology, School of Life Sciences,
Fudan University,
Shanghai 200433, China
e-mail: bkkuai@fudan.edu.cn

H.-F. Zhao
Department of Biology, School of Life and Environment Science,
Shanghai Normal University,
Shanghai 200234, China

of Col-0, as well as a pleiotropic phenotype, including lower germination rate, slow growth, an increased number of rosette leaves, and significantly retarded bolting. DSM1 may define a novel autonomous pathway controlling several floral repressors such as FLC, EMF1/EMF2, and TFL1 (Zhu et al. 2005). Given that *dsm1* had a pleiotropic phenotype, we tried to uncover the physiological mechanism underlying it. In this study, we further examined several endogenous hormones level and found that hormone balance of *dsml* was altered. Furthermore, ABA level was elevated due to increased expression of major ABA synthetases. Higher endogenous ABA level thus upregulated expression level of *ICK1*, which inhibited cell cycle progression and plant growth. These findings deepened our understanding of function of DSM1 in plant growth and development.

Materials and Methods

Plant Materials and Growth Conditions

Plants were grown under standard greenhouse conditions as described in the previous report (Zhu et al. 2005). To examine the root morphology, seeds of Col-0 and *dsm1* were germinated and grown in Petri dishes filled with 1× MS medium and 1% agar. Dishes were kept under 16-h-light/8-h-dark photoperiod for 7 days.

Light Microscopy

Plant samples at different developmental stages were fixed overnight in FAA (50% ethanol (*v*/*v*), 5.0% acetic acid (*v*/*v*), and 3.7% formaldehyde (*v*/*v*)), dehydrated in a graded ethanol series (50%×2, 60%, 70%, 80%, 90%, 95%, and 100%×2), transferred to xylene, and ultimately embedded in resin. Longitudinal and transverse sections were then stained in toluidine blue at 42°C for 30 min and observed under an OLYMPUS BX51 microscope (Olympus, Tokyo, Japan). Images were taken with an OLYMPUS DX51 digital camera.

For visualizing leaf epidermal cells, leaves were placed in methanol overnight to remove chlorophyll and cleared in lactic acid (De Veylder et al. 2001). Epidermal cells were examined under microscope.

Electron Microscopy

Samples were fixed, dried, dissected, and coated, and specimens were then examined with scanning electron microscopy as described (Bowman et al. 1989). Fresh tissues were fixed in 2.5% glutaraldehyde in 0.05 M phosphate buffer, pH 7.2, overnight at 4°C, and then post-fixed in 2% osmium in 0.05 M phosphate buffer, pH 7.2 for 2 h. Following dehydration in a graded ethanol series, samples were sputter-coated with 8 nm gold and examined using a JSM-840 scanning electron microscope (Jeol Pty Ltd, Tokyo, Japan).

Measurement of Anthocyanin and Chlorophyll Content

Leaves from three plants during the vegetative stage and three during the flowering stage were collected (about 0.2 g fresh weight). Anthocyanin and chlorophyll contents were measured as described previously (Rabino and Mancinelli 1986; Ren et al. 2007).

Drought Treatment and Water Loss Assay

Plants were irrigated regularly. Wild type and *dsm1* at the transitional stage were then completely withheld from water for 21 days and re-watered at day 22. Experiments were repeated at least three times, with similar results.

Leaves at the same developmental stage from wild type and *dsm1* were detached and placed in room condition. Fresh weight was measured at each time point after detachment (0–6 h). Water loss of each point was calculated as a relative to the initial fresh weight.

Determination of Hormone Levels

Samples (30 plants for each sample) were collected and weighed respectively in the vegetative, transitional, and flowering stage. ABA, Indole-3-acetic acid (IAA), zeatin riboside (ZR), and GA4 contents were quantified by ELISA conducted by Phytohormones Research Institute at China Agricultural University (He 1993).

Gene Expression Analysis

Total RNA was extracted from seedlings at the stage of approximately 1.05 (five rosette leaves, with the youngest

Table 1 Primer sequences used for real-time PCR

Primer names	Primer sequences
NCED2-F	5′-TGCAGATCGACGTAACGGAATT-3′
NCED2-R	5′-GAAGATGTTTAGCCGGAGAGGAT-3′
NCED3-F	5′-AGGTCGCAAGATTCGGGATT-3′
NCED3-R	5′-GCGGATTTCAGACAGGACACTC-3′
CYP707A2-F	5′-GGCGGCTCGAATGGTGTTAGT-3′
CYP707A2-R	5′-AATGGTATGGACCTTGGTGGAA-3′
ICK1-F	5′-TCTCCGTCGTCGGTGATAATG-3′
ICK1-R	5′-ATCGTTTTCCTCCCGCTACAA-3′
ACT2-F	5′-GGCTCCTCTTAACCCAAAGGC-3′
ACT2-R	5′-CACACCATCACCAGAATCCAGC-3′

>1 mm in length; Boyes et al. 2001), from plants at the transitional stage and from flower buds, using TRIzol (Invitrogen, Carlsbad, CA, USA) as described (Kinoshita et al. 1999). Real-time quantitative PCR was performed in an iCycler Thermal Cycler (Bio-Rad, Hercules, CA, USA) using the SYBR Green I real-time PCR Master Mix (TOYOBO Co., Osaka, Japan). The primer sequences used are listed in Table 1. Relative expression was analyzed using the Genex Microsoft Excel macro (Bio-Rad). The observed threshold cycle (CT) value of the RNA sample for the *dsm1* mutant was compared with that of an equivalent Col-0 RNA sample. *ACT2* was used as a reference gene for normalization.

Results

Morphological and Anatomical Characters were Obviously Altered in *dsm1*

Apart from developmental retardation (Fig. 1a, b), the mutant *dsm1* had a variety of abnormalities, including twisted leaves, increased number of branches (Fig. 1a, b), thick leaves (Fig. 1c, d), and anomalous siliques (Fig. 1b). Additionally, it had increased anthocyanin and chlorophyll (Fig. 1e, f). The leaf primordium of *dsm1* emerges more slowly. Longitudinal sections through the SAM confirmed that the SAM of *dsm1* was more anisomerous and the organization was defective (Fig. 2A1, A2). Hypocotyl elongation of *dsm1* was clearly reduced (Fig. 2B, right). Transverse sections through the hypocotyls showed an irregular size and arrangement of cortex cells in *dsm1* (Fig. 2C1, C2). The reduced root growth in *dsm1* may be related to defects in the root apical meristem (RAM). This was illustrated in longitudinal sections of 5-day-old primary roots (Fig. 2D1, D2).

We found more than harf of anthers was maldeveloped in *dsm1* (Fig. 2E1, E2). Our results indicate that the low ratio of self-fertility was due not only to a deficiency in stamen filament elongation (Zhu et al. 2005) but also to anther defects. Scanning electron microscopy showed that most *dsm1* seeds were anomalous and wizened (Fig. 2F, right) and that there were crevices on *dsm1* seed surface (Fig. 2G1, G2).

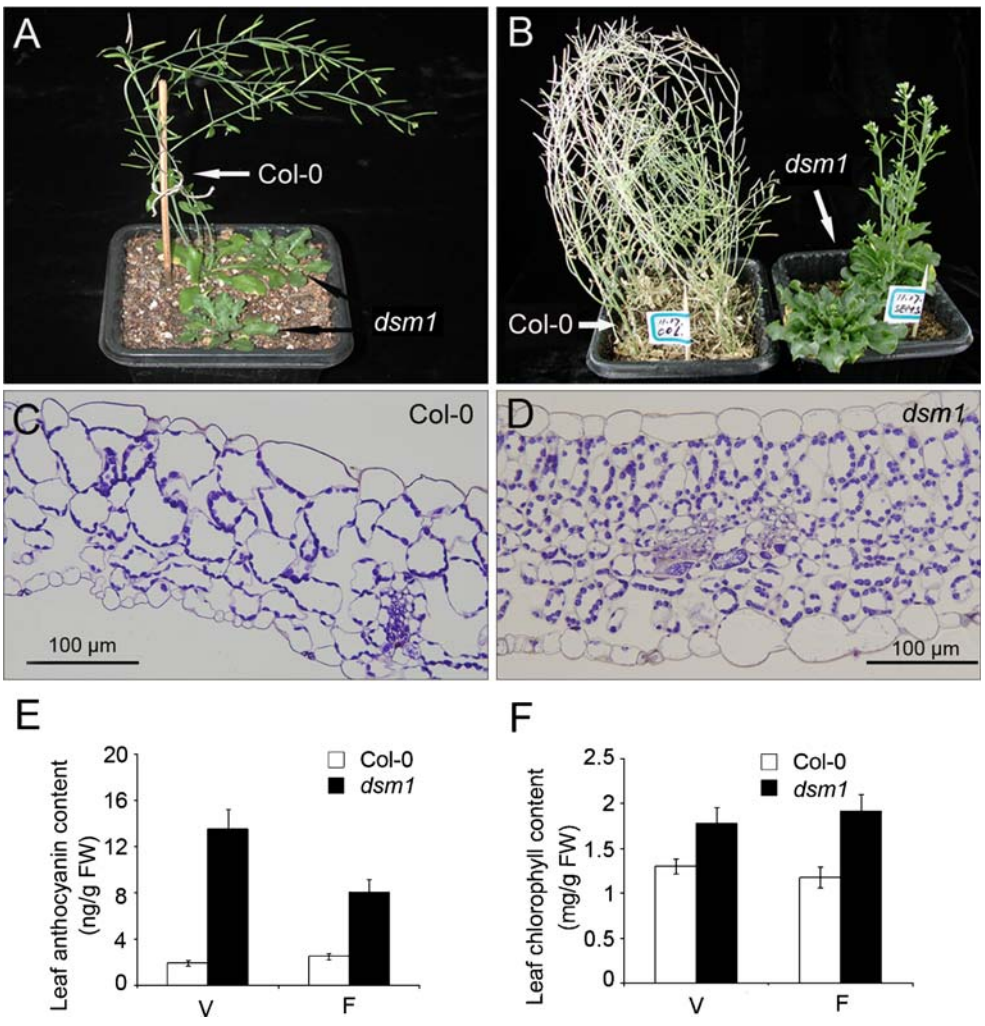

Fig. 1 General pleiotropic phenotype and leaves in *dsm1*. **a** Pleiotropic phenotype of 46-day-old plants. **b** Eighty-eight-day-old plants showed the extremely different growth rate of Col-0 and *dsm1*. **c**, **d** More chloroplasts in *dsm1* showed by transverse sections at the expanded lamina of the first leaves. **e**, **f** Anthocyanin and chlorophyll levels were higher in *dsm1*. *V* vegetative stage, *F* flowering stage

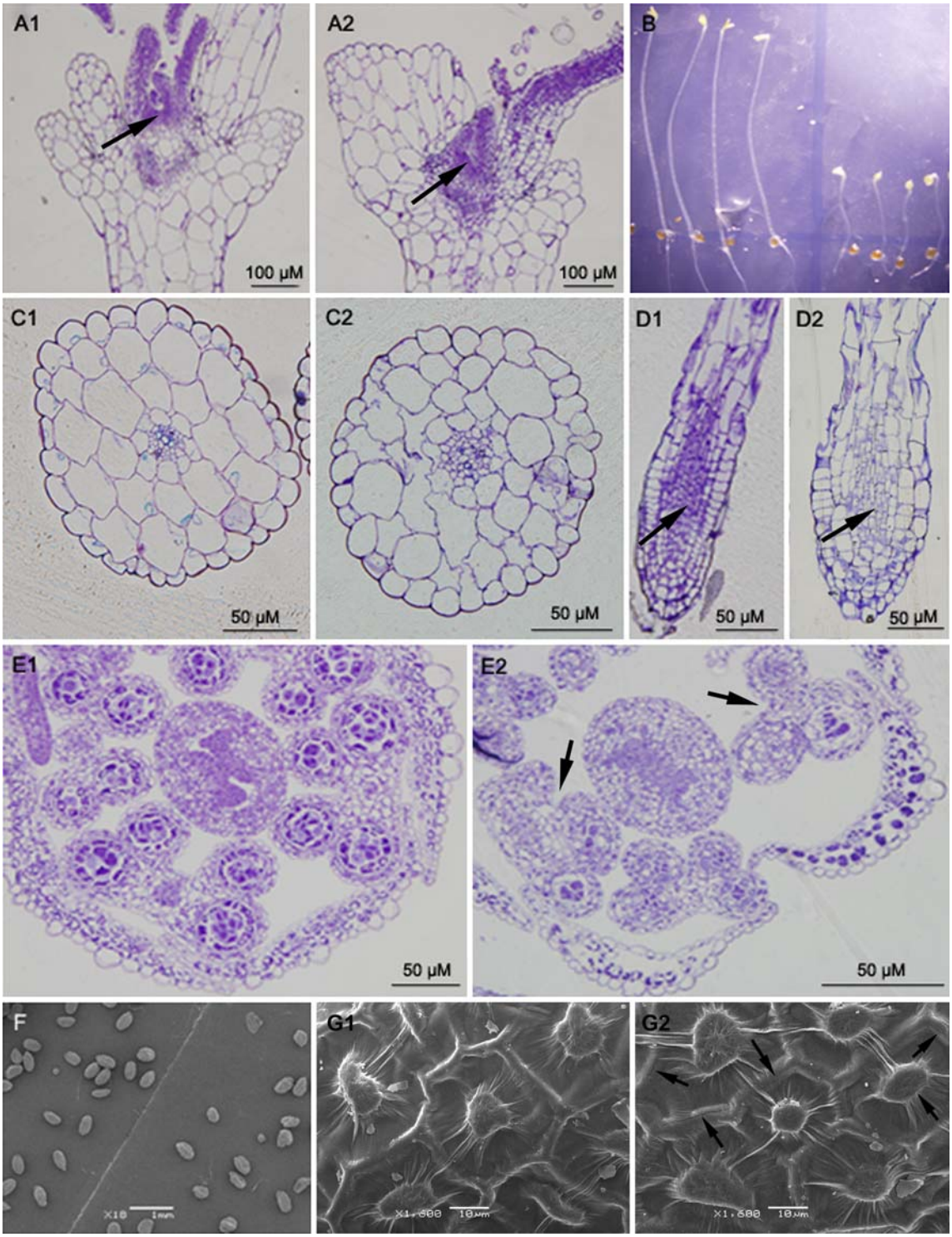

Fig. 2 Anatomical comparisons of Col-0 and *dsm1*. *A1*, *A2*: Longitudinal sections through Col-0 and *dsm1* SAM (indicated by *arrowheads*) of 10-day-old plants showed that the SAM was more anisomerous in *dsm1* (*A2*) than in Col-0 (*A1*). *B*: The elongation of the hypocotyl in the dark was obviously reduced in *dsm1* (*right*) than in Col-0 (*left*). *C1*, *C2*: Transverse sections through the hypocotyls showed that the cortex cells were more anomalous in size and arrangement in *dsm1* (*C2*) than in Col-0 (*C1*). *D1*, *D2*: Longitudinal sections through the RAM (indicated by *arrowheads*) of 5-day-old plants showed that RAM was more anisomerous in *dsm1* (*D2*) than in Col-0 (*D1*) and that the epidermis and cortex cells in the meristem zone and in the elongation zone were more expanded in *dsm1* than in Col-0. *E1*, *E2*: Comparison of transverse sections of developing flowers between Col-0 (*E1*) and *dsm1* (*E2*) showed some defective anthers (indicated by *arrowheads*) in *dsm1*. *F*: Scanning electron microscopy showed that most *dsm1* seeds were anomalous, shriveled, and wizened (*right*). *G1*, *G2*: There were crevices in the surfaces of *dsm1* seeds (*G2*, denoted by *arrowheads*). *A1*, *C1*, *D1*, *E1*, and *G1*: Col-0; *A2*, *C2*, *D2*, *E2*, and *G2*: *dsm1*

Those defects may account for the severely impaired fertility of *dsm1*. The germination rate of *dsm1* under light was only about 60% (Fig. 5f).

Hormone Balance was Altered in *dsm1*

The endogenous ABA level was 24.1%, 405.5%, and 62.8% higher in *dsm1* than in Col-0 during the vegetative stage, the transitional stage, and the flowering stage, respectively (Fig. 3a). The difference during the transitional stage was particularly notable. The data showed that both abscisic acid and zeatin riboside levels were significantly greater in *dsm1* than in Col-0 at all stages of development, while the levels of indole-3-acetic acid and gibberellins varied by age.

The endogenous IAA content in *dsm1* was 44.5% and 71% of that in Col-0 during the vegetative stage and the transitional stage, respectively (Fig. 3b). In the flowering stage, the endogenous IAA level in *dsm1* was 103.5% higher than that in Col-0 (Fig. 3b). The ZR levels were 61.2%, 31.5%, and 132.1% higher in *dsm1* than in Col-0 at the vegetative, transitional, and flowering stages, respectively (Fig. 3c). When endogenous GA4 contents were compared, no obvious difference was observed between *dsm1* and Col-0 during the vegetative and transitional stages. However, in the flowering stage, the GA4 content in *dsm1* was only 58.9% (Fig. 3d) of that in Col-0.

Based on elevated level of endogenous ABA in *dsm1*, we further examined whether seeds of *dsm1* was sensitive to exogenous ABA. The result showed that seeds of *dsm1*

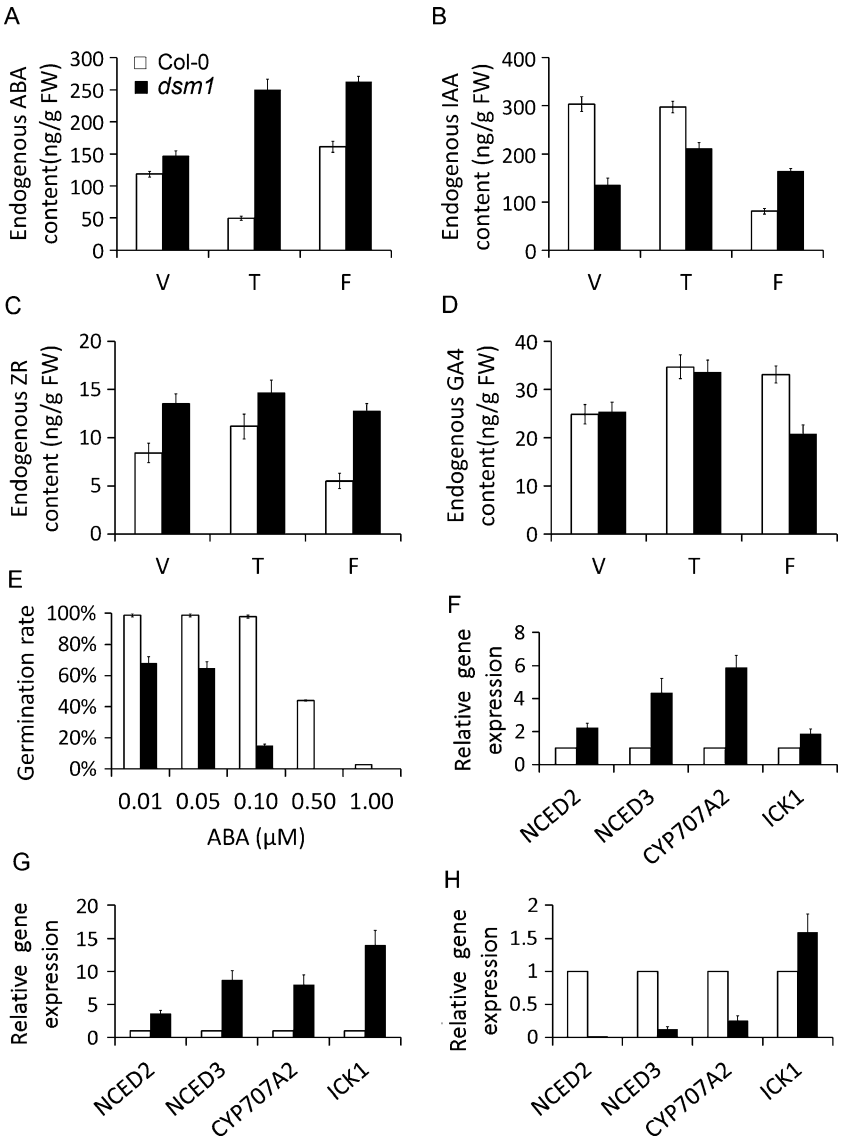

Fig. 3 Measurement of endogenous hormones, germination rate, and gene expression levels in *dsm1*. **a**–**d** The endogenous ABA, IAA, ZR, and GA4 levels in *dsm1* and Col-0 at different development stages. *V* vegetative stage, *T* transitional stage, *F* flowering stage. **e** The seed germination rate was more drastically reduced upon ABA treatment in *dsm1*, especially with 0.1 μM ABA. **f**–**h** Quantitative analysis of ABA metabolism-related genes and *ICK1* by real-time PCR. **f** Vegetative stage. **g** Transitional stage. **h** Flowering stage

were extremely sensitive to 0.1 μM ABA in agar medium (Fig. 3e).

ABA-Related Gene Expression Analysis

We analyzed the gene expression of key enzymes involved in ABA metabolism by real-time RT-PCR. Expression levels of *NCED2* and *NCED3*, which encode two key enzymes that are involved in ABA biosynthesis, and a key ABA catabolism enzyme *CYP707A2* were significantly elevated in *dsm1* as compared to Col-0 during the vegetative stage and the transitional stage (Fig. 3f, g), but much lower in flowering stage (Fig. 3h). This indicated that the increased contents of endogenous ABA was due to an alteration of the ABA metabolic genes expression.

We also examined the transcription level of *ICK1* (*Inhibitor 1 of Cdc2 Kinase*) which is induced by ABA and inhibits cell cycle (Wang et al. 1998, 2000) and found the *ICK1* transcript level was higher in *dsm1* than Col-0 during three stages of plant development we had examined. Remarkably, it was more than ten times higher in transitional stage (Fig. 3g).

Responses of *dsm1* to Stresses

Based on the fact that endogenous ABA content was much higher than in wild type, we further examined whether *dsm1* was more tolerant to drought stress. After 21 days drought treatment and recovered for 1 day, *dsm1* was able to survive, while Col-0 was not (Fig. 4a–c). Water loss assay also showed that detached leaves of *dsm1* lost less water than that of wild type (Fig. 4d). Consistent with that *dsm1* was more tolerant to drought stress, the root of *dsm1* was found to be more branched even at early stage of seedling (Fig. 4e). The root of *dsm1* seedling was also shorter than wild type due to slow growth rate but can be compensated by long-term growth. Additionally, *dsm1* showed less and smaller stomata on leaf surface compared with wild type (Fig. 4f, g).

The germination rate and growth rate of *dsm1* were lower compared to wild type under light (Fig. 5a, c). Interestingly, the difference of germination rate and growth rate between *dsm1* and wild type could be partially restored under dark treatment (Fig. 5b, d). A closer look at seedlings of *dsm1* and wild type under light and dark were showed (Fig. 5e).

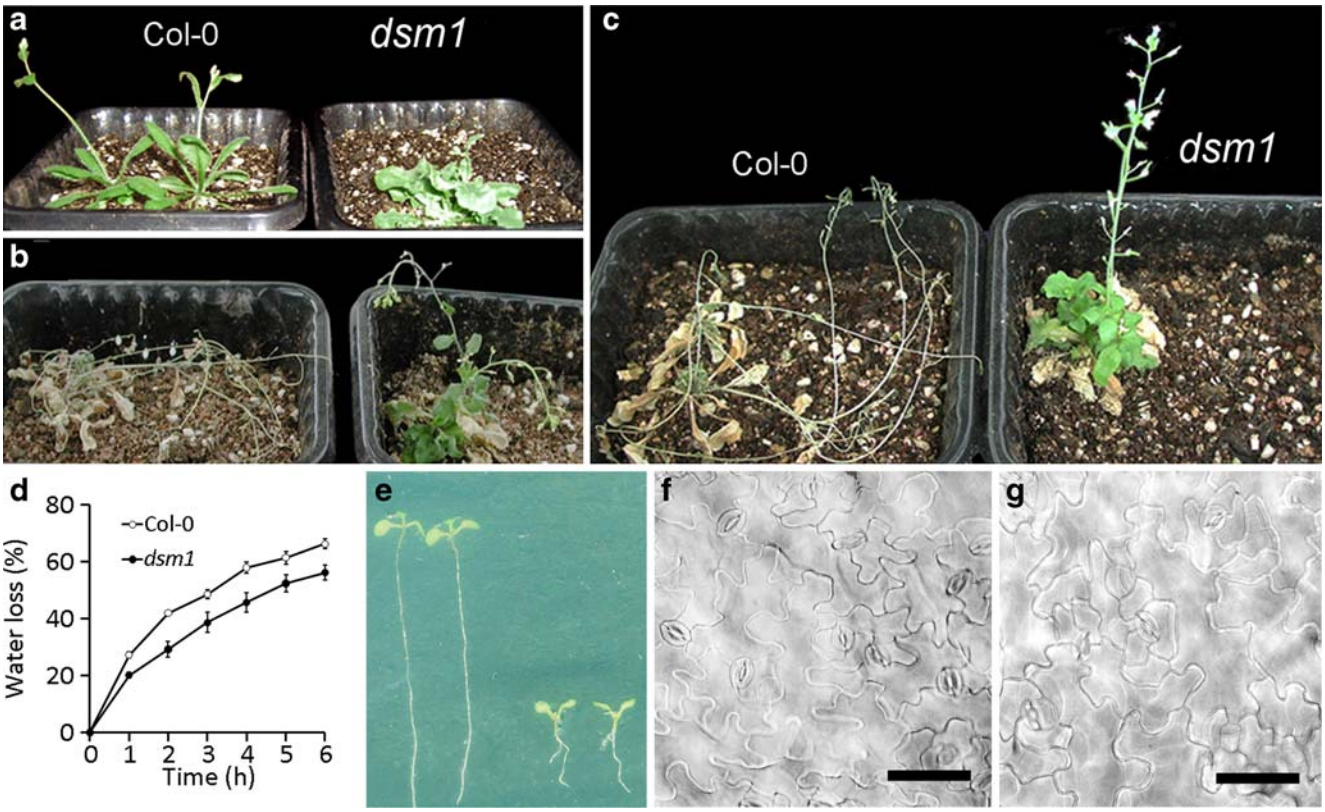

Fig. 4 *dsm1* was more tolerant to drought stress. **a** No drought treatment. **b** After 21 days drought treatment. **c** After one day of recovery. **d** Detached leaves of *dsm1* were more tolerant to water loss. **e** More branch roots in *dsm1* seedlings (*right*). **f**, **g** *dsm1* (**g**) had fewer and smaller stomata than Col-0 (**f**). Bar=50 μM

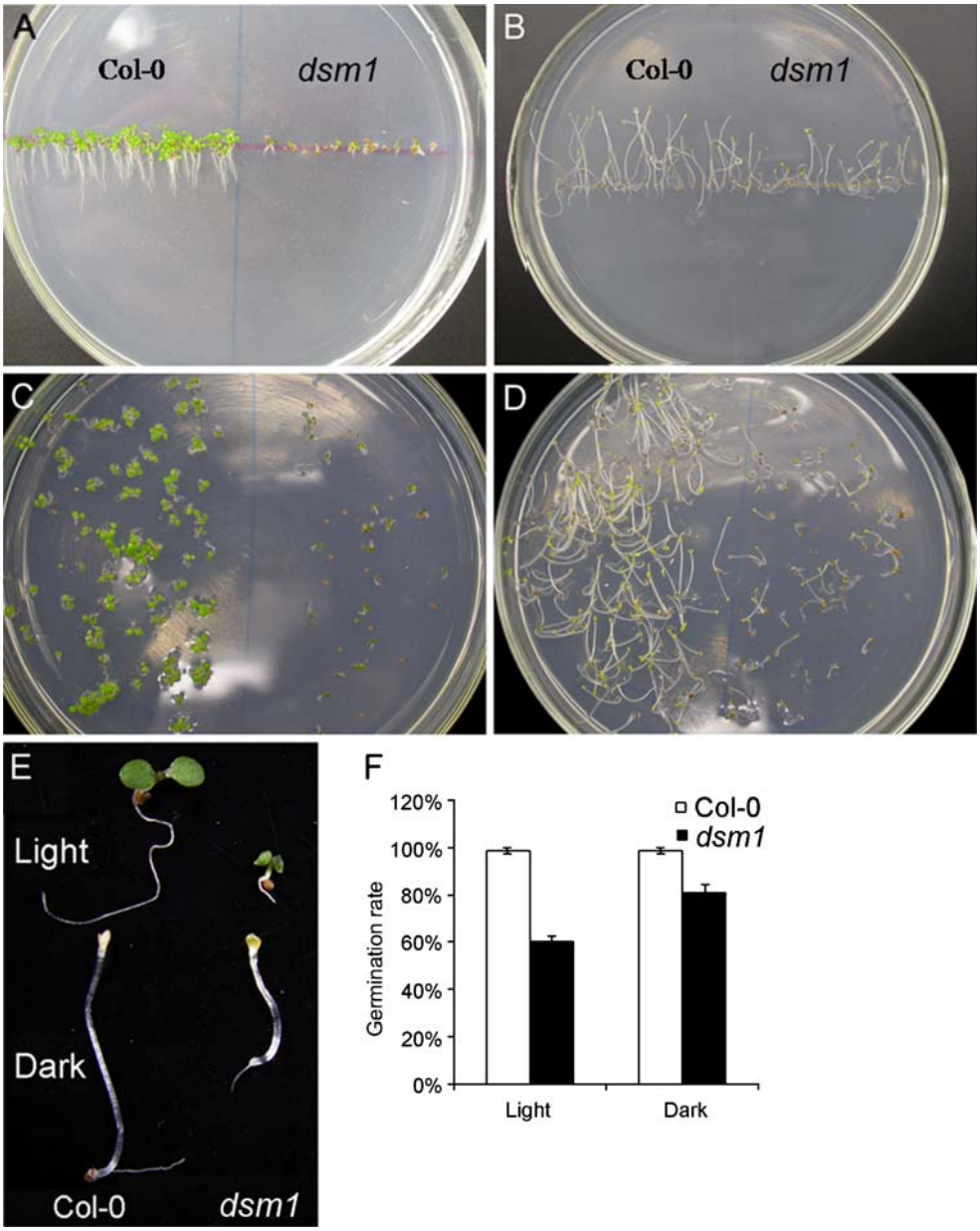

Fig. 5 Higher germination rate and increased growth in darkness. **a**, **c** In the light. **b**, **d** In the dark. **e** Growth rate of Col-0 and *dsm1* under light and dark condition. **f** Germination rate of Col-0 and *dsm1* under light and dark condition

Discussion

ABA regulates many aspects of plant growth and development including seed germination, seedling growth, and flowering and plays a critical role in plant adaptation to environmental stresses such as drought. ABA shows inhibitory effects on bud growth, seed, and bud dormancy. Our results showed that the germination of *dsm1* seeds was severely repressed. The structures of SAM and RAM in *dsm1* were anomalous in size and arrangement, and the flowering time of *dsm1* was more than 1 month later than wild type. Those growth defects were probably caused by the dramatically elevated content of ABA in *dsm1* throughout the plant lifecycle. We also examined the transcription level of *ICK1* which can be induced by ABA and inhibit cell cycle and found that the *ICK1* transcription level was higher in *dsm1*, especially in transitional stage. The increased *ICK1* expression could inhibit cell division in SAM and RAM in *dsm1* thus led to less dividing cell number in meristem and even abnormal structure.

To find out the reason why ABA content in *dsm1* was elevated, we investigated the expression of some ABA metabolic genes in transcript level and found that the expressions of two key ABA biosynthetic genes *NCED2* and *NCED3* were greatly upregulated in *dsm1* during vegetative stage and transitional stage. Interestingly, a major ABA catabolic gene *CYP707A2* expression was also elevated in those two stages, which is probably due to ABA metabolic feedback mechanism. During flowering stage, the expressions of all the three ABA metabolic genes

examined in *dsm1* were much lower than in wild type, which could explain that ABA content of wild type in flowering stage increased several times compare to transitional stage, while ABA content in *dsm1* almost maintained at the same level.

Many biological processes are coordinately regulated by multiple hormones. Recent physiological and genetic studies indicated that there exist a wide range of cross-talks among different types of plant hormones. The combination of these signals controls plant growth, development, and response to numberless biotic and abiotic stresses in a complex manner. Different hormones regulate a common process likely by sharing an interconnected regulatory network, but not by the same genes (Peng et al. 2009). Balancing hormone metabolisms play critical roles in proper regulation of plant growth and development. In *dsm1*, the endogenous IAA content was found to be lower than in Col-0 during the vegetative stage and the transitional stage, respectively. In the flowering stage, the endogenous IAA level in *dsm1* was much higher than that in Col-0. This could partially explain slow growth during vegetative stage and prolonged flowering stage of *dsm1*. The ZR levels were higher than Col-0 at the three stages. The increased anthocyanin levels, increased number of inflorescent branches, and delayed leaf senescence were probably due to the increased level of endogenous cytokinin. In the flowering stage, the GA4 content in *dsm1* was much lower than wild type. This explained the abnormal petal numbers and flower structure in *dsm1*, and was consistent with that ABA antagonizes the effects of GAs (Piskurewicz et al. 2008).

In summary, first, *dsm1* showed many growth and development defects. Second, several endogenous hormones of *dsm1* were dramatically altered, and the hormone balance was broken. We propose that the function of DSM1 may play a general role in plant lifecycle. It may target diverse aspects of plant growth depending on the different developmental context. Further study is needed to confirm this finding.

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