Journal of Experimental Botany, Vol. 67, No. 5 pp. 1461–1475, 2016
doi:10.1093/jxb/erv538 Advance Access publication 17 December 2015

RESEARCH PAPER

The Hippo/STE20 homolog SIK1 interacts with MOB1 to regulate cell proliferation and cell expansion in Arabidopsis

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Received 3 July 2015; Accepted 26 November 2015

Editor: James Murray, Cardiff University

Abstract

Multicellular organisms co-ordinate cell proliferation and cell expansion to maintain organ growth. In animals, the Hippo tumor suppressor pathway is a master regulator of organ size. Central to this pathway is a kinase cascade composed of Hippo and Warts, and their activating partners Salvador and Mob1/Mats. In plants, the Mob1/Mats homolog MOB1A has been characterized as a regulator of cell proliferation and sporogenesis. Nonetheless, no Hippo homologs have been identified. Here we show that the Arabidopsis serine/threonine kinase 1 (SIK1) is a Hippo homolog, and that it interacts with MOB1A to control organ size. SIK1 complements the function of yeast Ste20 in bud site selection and mitotic exit. The *sik1* null mutant is dwarf with reduced cell numbers, endoreduplication, and cell expansion. A yeast two-hybrid screen identified Mob1/Mats homologs MOB1A and MOB1B as SIK1-interacting partners. The interaction between SIK1 and MOB1 was found to be mediated by an N-terminal domain of SIK1 and was further confirmed by bimolecular fluorescence complementation. Interestingly, *sik1 mob1a* is arrested at the seedling stage, and overexpression of neither *SIK1* in *mob1a* nor *MOB1A* in *sik1* can rescue the dwarf phenotypes, suggesting that SIK1 and MOB1 may be components of a larger protein complex. Our results pave the way for constructing a complete Hippo pathway that controls organ growth in higher plants.

Key words: *Arabidopsis thaliana*, cell division, cell expansion, Hippo, MOB1, organ growth, SIK1, STE20.

Introduction

I _ _t _ , _t _ _ _ y _ _ t _t . A t _ _t_ _ t_ _ t - _ t _ _ t _t_ - _t _ _ _ _ _ t t t t _ _ _ (K , 2009; G _ e a ., 2012; _ L _ , 2012; H _t _ L _ , 2014). _y y _y _t _t- _ _ _ t . _ _ t _ t _ G - *Reg a g Fac* (*GRF*) (_ _ e a ., 2015), *TEOSINTEBRANCHED1/CYCLOIDEA/PCF* (*TCP*) (_ - C _ , 2010), _ *AINTEGUMENTA* (*ANT*) (K , 1999; _ _ H , 2000), t _ *ARGOS* _ _ *ARGOS-LIKE* (*ARL*) _ *ORGAN SIZE RELATED1* (*OSR1*) (H e a ., 2003, 2006; F e a ., 2011), t *AUXIN RESPONSE FACTOR ARF2* (e a ., 2006), t _ - t *DA1* (e a ., 2008), t E3 _ *DA2* (e a ., 2013) _ *ENHANCER OF DA1(EOD1)/BIG BROTHER*

[illegible]

Materials and methods

[illegible] *JXB* [illegible].

Accession numbers

[illegible] IK1, At1g69220; [illegible] 1A, At5g45550; [illegible] 1B, At4g19045.

Plant materials and growth conditions

[illegible] *1-1* (*SALK_051369*), [illegible] *1-2* (*SALK_010630*), [illegible] *1-3* (*SALK_046158*), [illegible] *1-4* (*SAIL_636_C05, CS875528*), [illegible] *b1a-1* (*GABI_719G04, CS469004*), [illegible] *b1b-1* (*SALK_062070*) [illegible] *SAT1-cEYFP-N1* [illegible] *cB1;1:GUS* [illegible] 75% [illegible] 100% [illegible] (1/2 [illegible] 0.8% [illegible] 1% [illegible] 5.7 [illegible] 16 (22 [illegible] C)/8 (18 C) [illegible] 90 μE [illegible] 165FC [illegible] CCD [illegible] 33) [illegible] A800).

Constructs and transgenic plants

SIK1 [illegible] 81 [illegible] 1) [illegible] *1g69220.1* (2511 [illegible] *SIK1* [illegible] 1) [illegible] *1g69220.1*. [illegible] *UBQ10:GFP-SIK1* [illegible] *SIK1* [illegible] *CAMBIA1302* (35 [illegible] *UBQ10*) [illegible]

35S:YFP-M [illegible] *CAMBIA1302* [illegible] *SIK1:GUS* [illegible] *SIK1:SIK1-GUS* [illegible] *1g69230* [illegible] *1g69220* (*SIK1*), [illegible] 417 [illegible] *SIK1* [illegible] *CAMBIA1301*. [illegible] *SIK1* [illegible] β [illegible]

[illegible] *SIK1* [illegible] *MOB1A* [illegible] *MOB1B* [illegible] *GADT7* (AD) [illegible] *GBKT7* (BD) [illegible] *SAT1-cEYFP-N1* [illegible] *Ag bac e* [illegible] (G 3101) [illegible] (C [illegible] B [illegible], 1998) [illegible] *cB1;1:GUS* [illegible] *1-4* [illegible]

Gene expression analysis

[illegible] (*et al.*, 2012) [illegible] 8-, 11-, [illegible] 14- [illegible] (L [illegible] 165FC, G [illegible]) [illegible] 200 [illegible] *SIK1:GUS* [illegible] *SIK1:SIK1-GUS* [illegible] *1-4* [illegible] *cB1;1:GUS* [illegible] (*et al.*, 2010).

Yeast complementation, yeast two-hybrid screen, and verification

[illegible] 124 (*MAT* [illegible] *2-3,112* [illegible] *3::LEU2*) [illegible] *e20Δ* [illegible] *STE20* [illegible] *URA3*), [illegible] *RS414-ScS e20* [illegible] *-SIKI* [illegible] *e20Δ* [illegible] (*et al.*, 2012) [illegible] 30 [illegible] 2500 [illegible] AH109 [illegible] 2H [illegible] IK1 [illegible] (6−10⁶ [illegible] >300 [illegible] 10 [illegible] BLA [illegible] AGI [illegible] 2. [illegible] IK1 [illegible] B1 [illegible] BD [illegible] 30 [illegible] 220 [illegible] A 5 [illegible] AD [illegible] (DD [illegible] DD [illegible] 3 [illegible]

Quantification of cell size and numbers

[illegible] 7- [illegible] (L [illegible] 165FC, G [illegible]) [illegible] 6- [illegible] (3:1 [illegible] (8 [illegible] CCD [illegible] (DIC) [illegible] D 72 [illegible] 10 [illegible] 10 [illegible] 28 [illegible] J [illegible] (F-t [illegible]) [illegible] 2010.

Measurement of nuclear DNA content of leaf cells by flow cytometry

[illegible] 28- [illegible] (*et al.*, 1983) [illegible] 50 [illegible] FAC [illegible] II [illegible] (BD [illegible] A).

Transient transformation of tobacco leaves

(... *e a*., 2005) ... 4- ... *N. be a a* ... A ... 2 ... -

... y.

Laser scanning confocal microscopy (LSCM)

(...) ... 5% ... 5 (L ... G ... y).

Results

The Arabidopsis SIK1 *encodes a STE20-like kinase*

... E20/H ... A ... BLA (B ... L ... A ...) ... AI 10) ... 20 ... *S. ce e ae* (HL007C, AAA35039.1), H ... *D. e a ga e* (AAF57543.2), ... t1 ... t2 ... *H a e* (AAA83254.1 ... AAC50386.1). A ... A ... /t ... IK1 (At1G69220) ... =39%, ... =58% ... 20; 51% ... 68% ... H ... ; 50% ... 66% ... t1; ... 44% ... 60% ... t2).

A ... -, ... t, y, ... IK1 ... /t ... t ... y, ... -

... (... *t e a*., 2011). ... t ... t ... (... 249 ... 503) (... 1A) ... , ... t, ... t2 (... 1B). H ... IK1 ... t

... y t ... t ... t, 20, H ... t (... 1A; ... t ... y ... 2). ... t ... IK1 ... (CBI ... t, t CL, 2689098, ... 1C). ... t ... t ... IK1 ... t. 20. A ... t (... *e a*., 2000; H ... , 2002), *e20Δ* ... y ... t, ... (... 1D). ... t ... 131 ... , 45.8% ... tt ... I ... t, *e20Δ* ... t ... *S e20:SIK1* ... t ... 124 (... 1D), ... y 5.0% ... tt ... =140), ... t ... 124 (4.3%, =138). ... ty ... y ... t, t ... IK1, ... t, t 20 ... .

... t ... t, *D a* ... t ... IK1 ... (... t ...), ... t ... y ... t ... .

SIK1 is expressed in mature tissues and is post-transcriptionally regulated

... t ... t, y ... IK1, ... - ... *SIK1* ... t ... . *SIK1* ... A ... t, t ... y ... C (... 2A). ... *SIK1:GUS* ... t ... 418, (417 t +1) ... t t ... - ... t ... t ... *SIK1* t ... . I ... y, *SIK1* ... t ... y ... y ... t ... , ... y ... ty ... t ... t ...

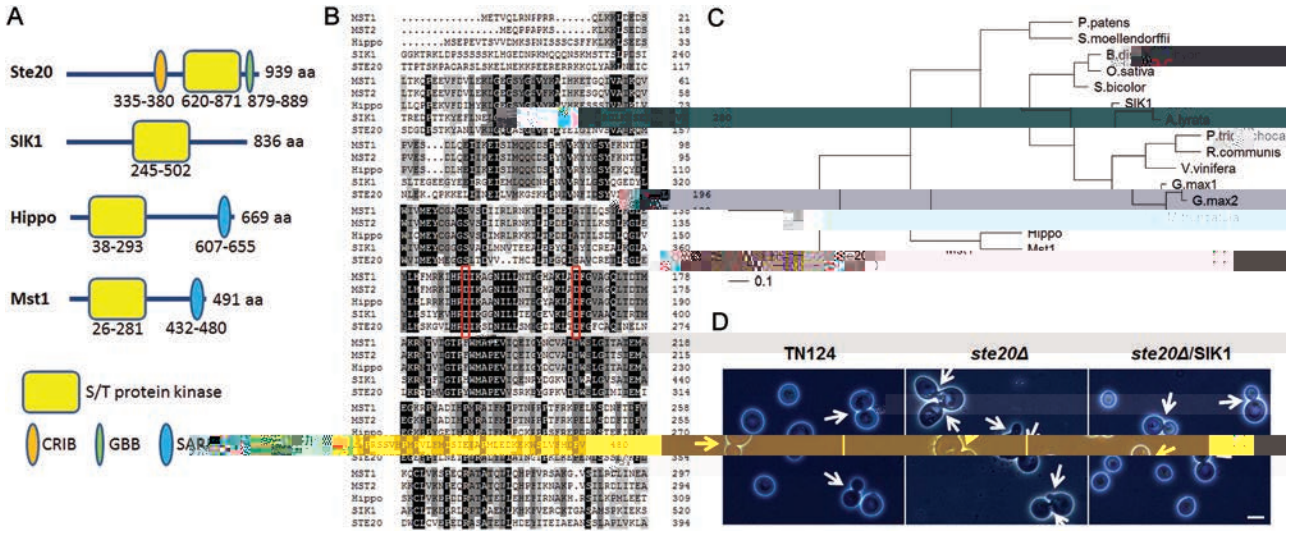

Fig. 1. Arabidopsis SIK1 is a Ste20 and Hippo homolog. (A) Schematic representation of the domain structure of Ste20 family proteins. The serine/threonine protein kinase domain and the domains that mediate protein–protein interaction (PPI), including the Cdc42/Rac interactive binding (CRIB) and G beta-binding (GBB) motif of Ste20, and the Sav/Rassf/Hpo (SARAH) domain of Hippo and Mst1, are shown. No PPI domain can be identified in SIK1. (B) Alignment of the Ste20 family proteins. The kinase domains are highly conserved. D371 and D389 of SIK1 are predicted active sites (boxes). (C) Phylogenetic tree of SIK1 homologs from selected land plants, with metazoan and yeast Ste20 homologs as outliers. The tree is constructed with Clustal W2-generated multiple sequence alignment of SIK1 homologs (NCBI protein cluster CLSN2689098, protein kinase domain-containing protein) using the Neighbor–Joining method and plotted in Treeview. (D) SIK1 restores abnormal budding phenotypes in *ste20*Δ. Yeast cells from the background strain TN124 and *ste20*Δ complemented with SIK1 (*ste20*Δ/SIK1) are normal in cell shape and budding site selection. *ste20*Δ cells are irregular in shape with an abnormal budding pattern. Arrows indicate budding sites. Scale bar=5 μm in (D). (This figure is available in colour at *JXB* online.)

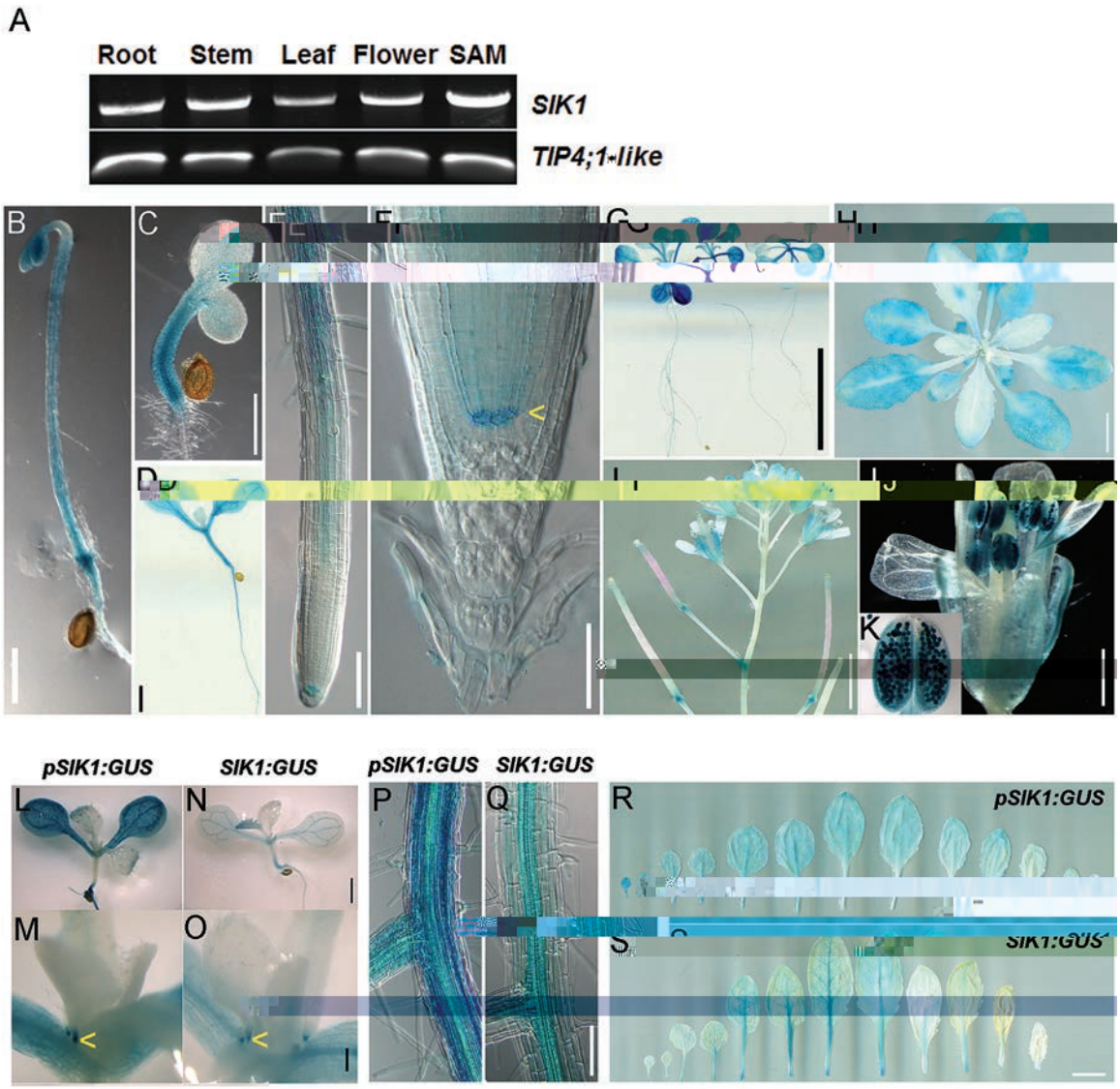

Fig. 2. Developmental expression and post-translational regulation of SIK1. (A) Semi-quantitative RT-PCR analysis of *SIK1* transcript in different organs. *TIP4;1-like* used as internal control. (B–K) Histochemical staining of *pSIK1:GUS* T3 homozygous plants. SIK1 promoter activity is detected in (B) 3-day-old etiolated seedlings, (C) 3-day-old light-grown seedlings, (D) 7-day-old seedlings, (E) primary root of 7-day-old seedlings, (F) quiescent center of the root apical meristem, (G) 14-day-old seedlings, (H) 4-week-old plants, (I) inflorescence, (J) opened flowers, and (K) pollen grains. (L–S) Comparison between expression of *SIK1* transcriptional and translational fusions. GUS activity in *SIK1:GUS* transgenic lines is further restricted compared with *pSIK1:GUS* lines in (L–Q) 10-day-old seedlings and (R, S) rosette leaves of 4-week-old plants. The arrows indicate the quiescent center in (F), and stipules of leaves one and two in (M) and (O). Scale bars=500 μm in (B), (C), (J); 1 mm in (D), (N); 100 μm in (E), (O), (Q); 50 μm in (F); 1 cm in (G), (H), (S); 5 mm in (I). (This figure is available in colour at *JXB* online.)

Growth of sik1 *mutants is retarded*

[illegible]

Cell number, cell size, and the ploidy level are reduced in sik1-4

[illegible]

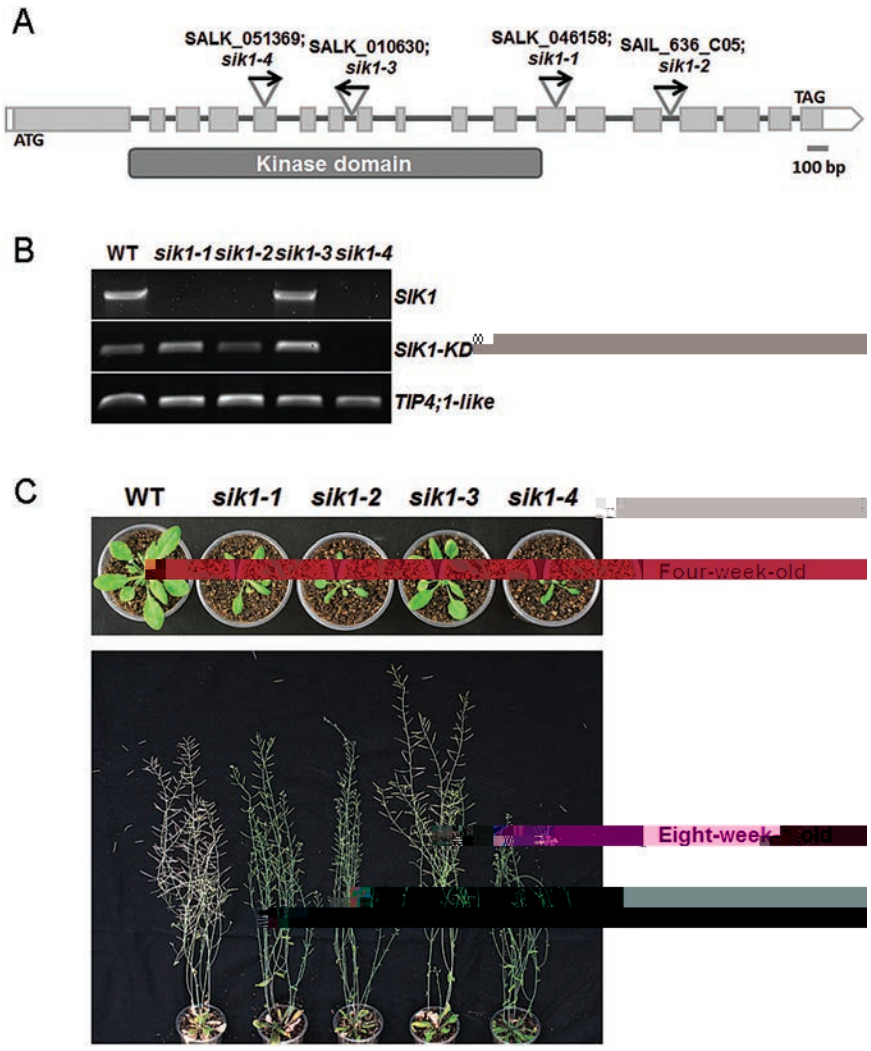

Fig. 3. The *sik1* null mutant has a dwarf phenotype. (A) *SIK1* gene structure and the T-DNA insertions. Exons, introns, and untranslated regions are represented by gray boxes, gray lines, and white boxes, respectively. Insertion positions of the T-DNA in the four alleles are shown. (B) *sik1-4* is a null allele. An mRNA fragment representing the kinase domain (KD; exons 2–12) can be detected in *sik1-1*, *sik1-2*, and *sik1-3*, but not in *sik1-4*. *sik1-3* is a knock-down allele, with the T-DNA inserted into the seventh intron. (C) Phenotypes of four alleles. The three null alleles are slow in growth compared with the wild type, with *sik1-4* having the most severe phenotypes. Diameter of pot=6.5 cm in (C). (This figure is available in colour at *JXB* online.)

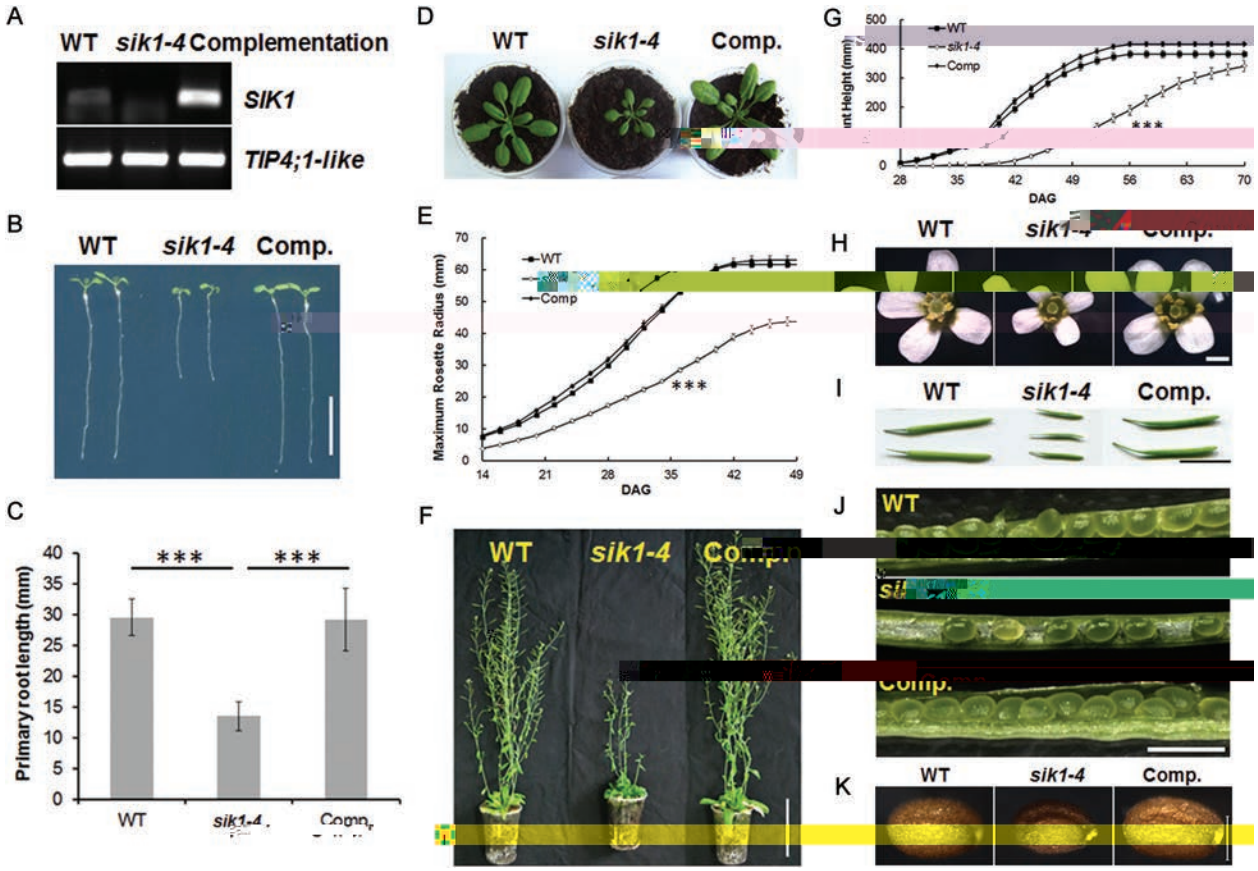

Fig. 4. Complementation of *sik1-4* with *pUBQ10:GFP-SIK1*. (A) Semi-quantitative RT-PCR of *SIK1* in the wild type, *sik1-4*, and the complementation (Comp.) line. *TIP4;1-like* is used as internal control. (B) Seven-day-old, vertically grown seedlings. (C) Quantification of primary root length of seedlings at 7 days post-germination (dpg). (D) Four-week-old plants. (E) Maximum rosette radii of the wild type, *sik1-1*, and the complementation line, measured over a period of 5 weeks. (F) Eight-week-old plants. (G) Plant height measured over a period of 6 weeks. (H) Fully opened flowers. (I) Mature siliques. (J) Halves of siliques. (K) Dry seeds. Bars=SE in (C), (E), and (G). *** indicates $P<0.001$ (Student's *t*-test) in (C), (E), and (G). Scale bars=1 cm in (B), (I), 10 cm in (F), 1 mm in (H), (J), 200 μm in (K). (This figure is available in colour at *JXB* online.)

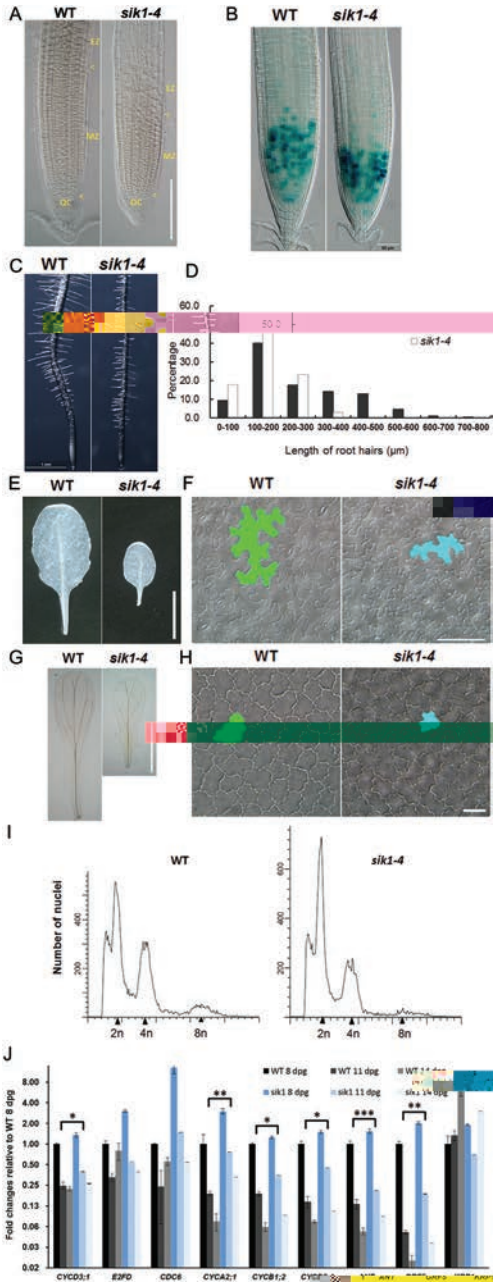

Fig. 5. *sik1-4* has reduced cell numbers, cell sizes, and ploidy levels compared with the wild type (WT). (A) Root tips of 6-day-old vertically grown WT and *sik1-4* seedlings. QC, quiescent center; MZ, meristematic zone; EZ, elongation zone. Arrows indicate the beginning of the MZ and EZ. (B) GUS staining showing *pCYCB1;1* promoter activity in WT and *sik1-4* root tips 7 days post-germination (dpg). (C) Root hairs (n >1000) of WT and *sik1-4* seedlings at 7 dpg. (D) Distribution of root hair lengths in the WT and *sik1-4*. (E) The fifth rosette leaves of the WT and *sik1-4* at 28 dpg. (F) Lower epidermis of (E) with representative cells highlighted. (G) Petals from fully opened flowers. (H) Lower epidermis of (G) with representative cells highlighted. (I) Flow cytometric analysis of the fifth rosette leaf of 28-day-old WT and *sik1-4*. A total of 20 000 nuclei are sorted for each sample. (J) Quantitative RT-PCR of core cell cycle marker genes and regulators from the first and second true leaves of 8-, 11-, and 14-day-old seedlings. *CYCD3;1*, G_1 phase-specific marker; *E2FD/DEL2*, G_1/S specific; *CDC6*, S-phase specific; *CYCA2;1*, S/G_2 specific; *CYCB1;2* and *CYCB2;1*, G_2/M-phase specific markers. *ANT* and *GRF5*, transcription factors that regulate cell proliferation; *KRP1*, cell cycle inhibitor. Bars=SD. *GAPC2* used as internal control. ***P<0.001, ** P<0.01, and *P<0.05 (Student's t-test) in (J). Scale bars=100 μm in (A), 50 μm in (B), (F); 1 mm in (C), (G); 1 cm in (E), 20 μm in (H). (This figure is available in colour at *JXB* online.)

e a., 2006; A[illegible] *e a*., 2012). I[illegible] 8 [illegible] 11 [illegible] 14 (Fig. 5J). A[illegible] *1-4*. H[illegible] *1-4* [illegible] C[illegible] *KRP1*, [illegible] (*e a*., 2003; [illegible] *e a*., 2005), [illegible] *1-4* [illegible] IK1 [illegible].

MOB1A and MOB1B were identified as SIK1-interacting proteins

[illegible] IK1 [illegible] 2H [illegible] IK1 [illegible]. I[illegible] 46 [illegible] 266 [illegible] 2). A[illegible] 1A (At5[illegible]45550) [illegible] 1B (At4[illegible]19045) [illegible] 212 [illegible] 1A [illegible] 1B [illegible] B (O[illegible] *e a*., 2005, 2006). [illegible] *b1* -D A[illegible] A[illegible] *1* (G[illegible] *e a*., 2011; [illegible] *e a*., 2013), [illegible] IK1 [illegible] 1[illegible].

SIK1 interacts with MOB1 at its N-terminal domain

[illegible] IK1 [illegible] IK1 [illegible] (Fig. 6A), [illegible] B1 [illegible] 2H. [illegible] BD [illegible] (Fig. 6B), [illegible] E [illegible] ([illegible] L), [illegible] (Fig. 6C). [illegible] L H[illegible] 1- IK1 ([illegible] 1 235), - IK1 ([illegible] 1 504), [illegible] - [illegible] IK1 [illegible] B1A [illegible] B1B (Fig. 6C). [illegible] L H[illegible] A[illegible] - IK1 [illegible] - [illegible] IK1 [illegible] B1 (Fig. 6C). [illegible] AD [illegible] BD [illegible] IK1 [illegible] B1 [illegible] (Fig. 6D). [illegible] IK1, [illegible] (K278 [illegible] D371A), [illegible] *E c e c a c* ([illegible] 5), [illegible] [illegible] IK1 [illegible] B1A [illegible] [illegible] FC[illegible] [illegible] IK1 E F [illegible] - [illegible] B1A [illegible] E F , F [illegible] [illegible] (Fig. 6D). I[illegible] 1- IK1 E F [illegible] B1A [illegible] E F [illegible] - [illegible] F [illegible] [illegible] (Fig. 6D).

[illegible] IK1 [illegible] B1A [illegible] (F) IK1 [illegible] - [illegible] *a* -G [illegible] /EE)

Table 1. *sik1 has a lower cell number and reduced cell sizes compared with the wild type.*

Parameter	Wild type (average ±SE)	*sik1-4* (average ±SE)	Student's *t*-test
Length of RAM (μm), 7 dpg	296.6±4.2 (*n*=86)	182.8±2.5 (*n*=84)	*P*<1E-50
No. of protoderm cells in RAM, 7 dpg	38.2±0.4 (*n*=86)	24.0±0.3 (*n*=84)	*P*<1E-61
Length of RAM region with *pCYCB1;1:GUS* activity (μm)	186.8±2.5 (*n*=108)	116.1±2.1 (*n*=73)	*P*<1E-51
Area of petal (mm^2)	1.85±0.03 (*n*=123)	1.00±0.02 (*n*=109)	*P*<1E-61
Area of petal epidermal cell (μm^2)	220.8±2.7 (*n*=100)	191.0±2.8 (*n*=86)	*P*<1E-12
Area of the fifth rosette leaf, 28 dpg (mm^2)	165.9±7.2 (*n*=28)	47.8±3.4 (*n*=28)	*P*<1E-17
Area of lower epidermal cell (μm^2)	8955.4±121.9 (*n*=420)	3271.5±37.3 (*n*=440)	*P*<1E-99
No. of palisade cells per 250 000 μm^2	93.5±3.0 (*n*=43)	210.2±6.7 (*n*=44)	*P*<1E-23

dpg, days post-germination.

[illegible]

Genetic analysis of SIK1 *and* MOB1A

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Discussion

The molecular function of Hippo is probably conserved among eukaryotes

[illegible]

A possible structural basis for the kinase–scaffold interaction

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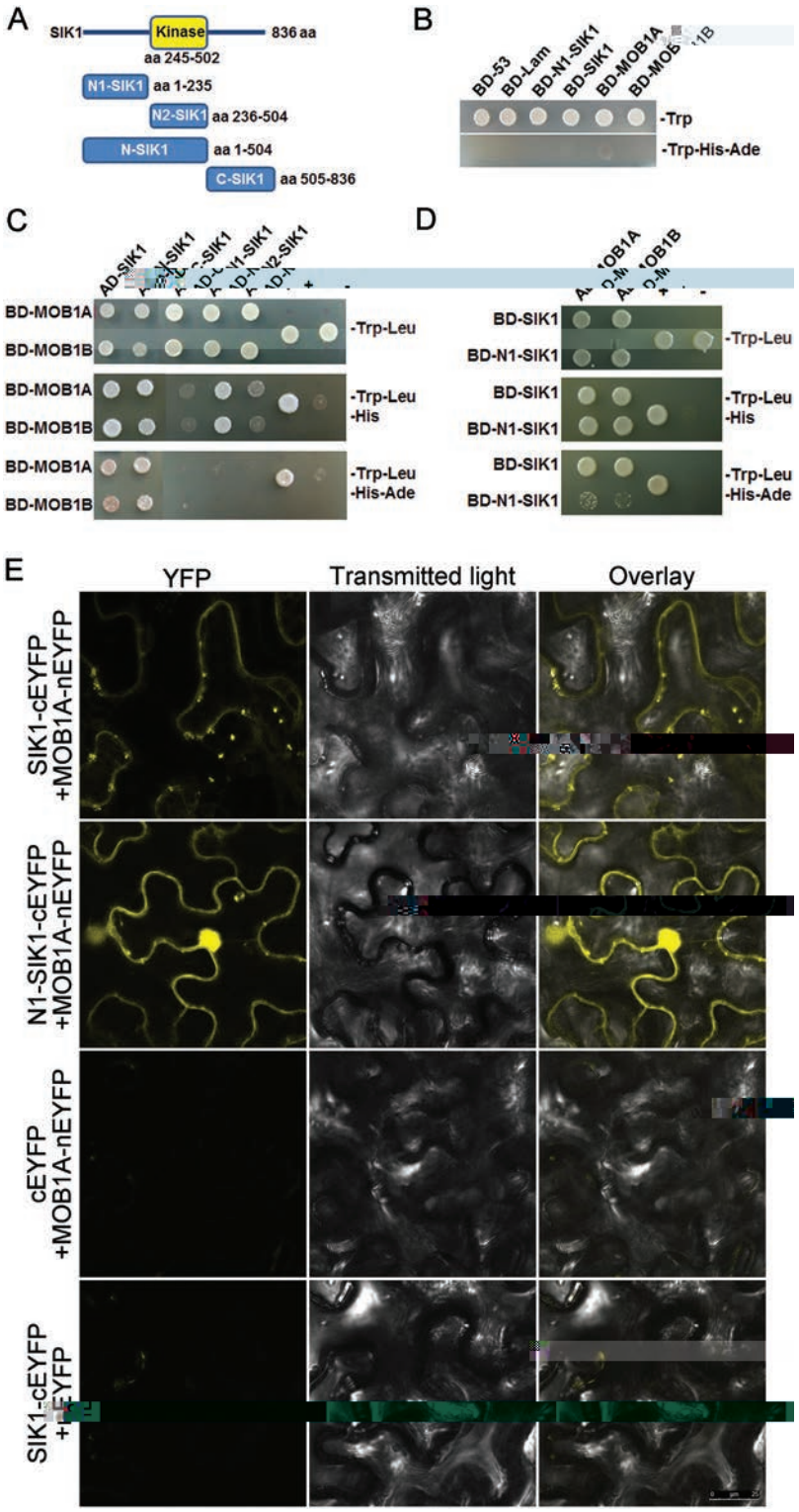

Fig. 6. The N-terminal domain of SIK1 (N1-SIK1) is responsible for its interaction with MOB1s. (A) SIK1 is subdivided into four fragments for yeast two-hybrid (Y2H) analysis. (B) SIK1 and MOB1s do not have self-activation activity. (C) Interaction between the full length and fragments of SIK1 (plus the activation domain, AD) and MOB1s (plus the binding domain, BD) verified on triple- and quadruple-dropout plates. + and –, positive and negative controls. (D) Interactions between AD-MOB1s and BD-SIK1. (E) Interaction between SIK1 and MOB1A confirmed in tobacco leaf epidermal cells with bimolecular fluorescence complementation (BiFC). Scale bar=50 μm in (E). (This figure is available in colour at *JXB* online.)

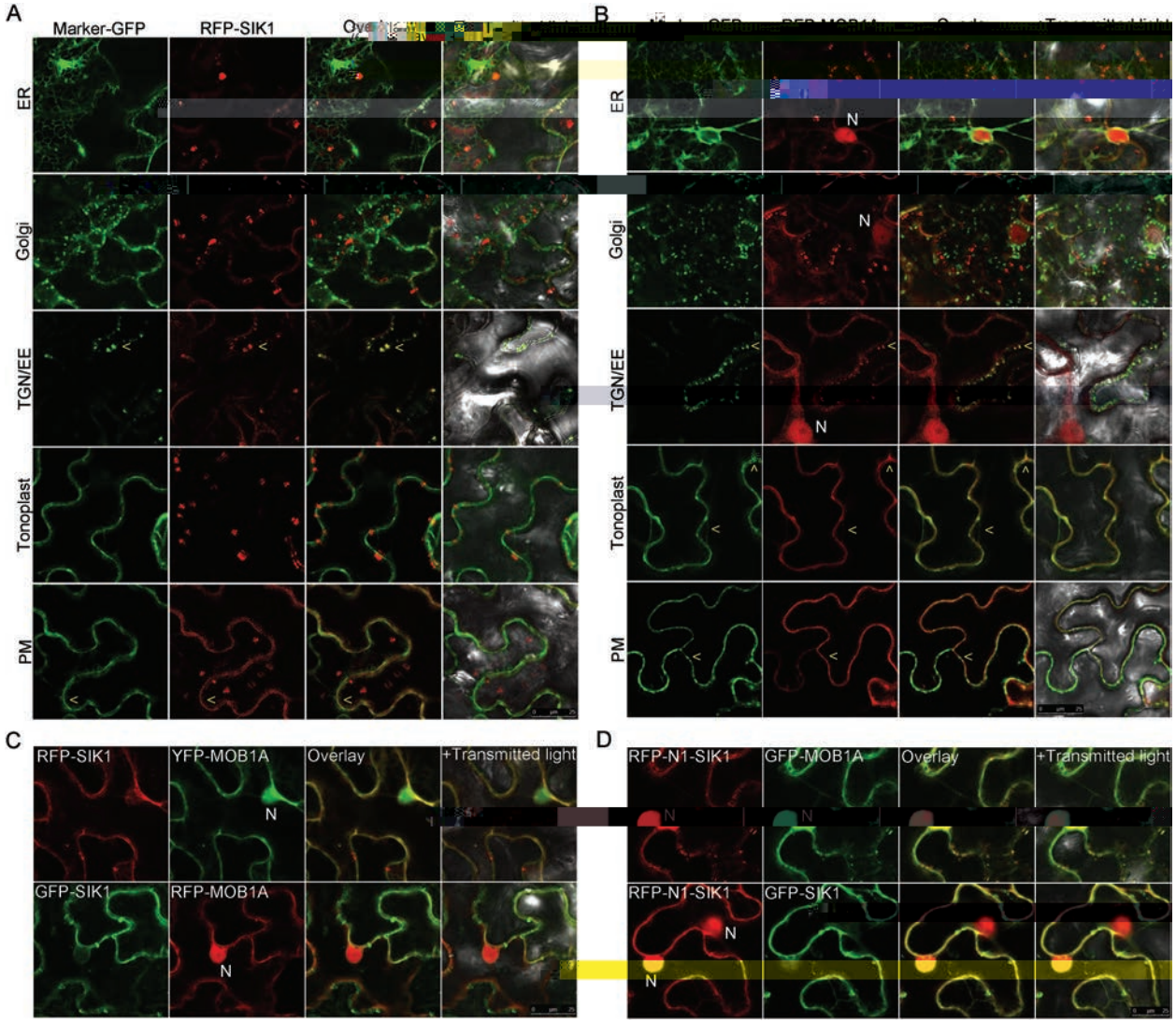

Fig. 7. Subcellular localization patterns of SIK1 and MOB1A. (A) RFP–SIK1 is co-expressed with GFP-tagged organelle markers. SIK1 is co-localized with *trans*-Golgi-network (TGN)/early endosome (EE) and plasma membrane (PM) markers. (B) RFP–MOB1A localizes to the nucleus (N), and co-localizes with TGN/EE, tonoplast, and PM markers. (C) When co-expressed with MOB1A, SIK1 can be detected in the nucleus (N). (D) N1-SIK1 localizes to the nucleus, and co-expression with N1-SIK1 brings SIK1 into the nucleus. Scale bars=25 μm in (A–D).

How SIK1 may participate in organ size control

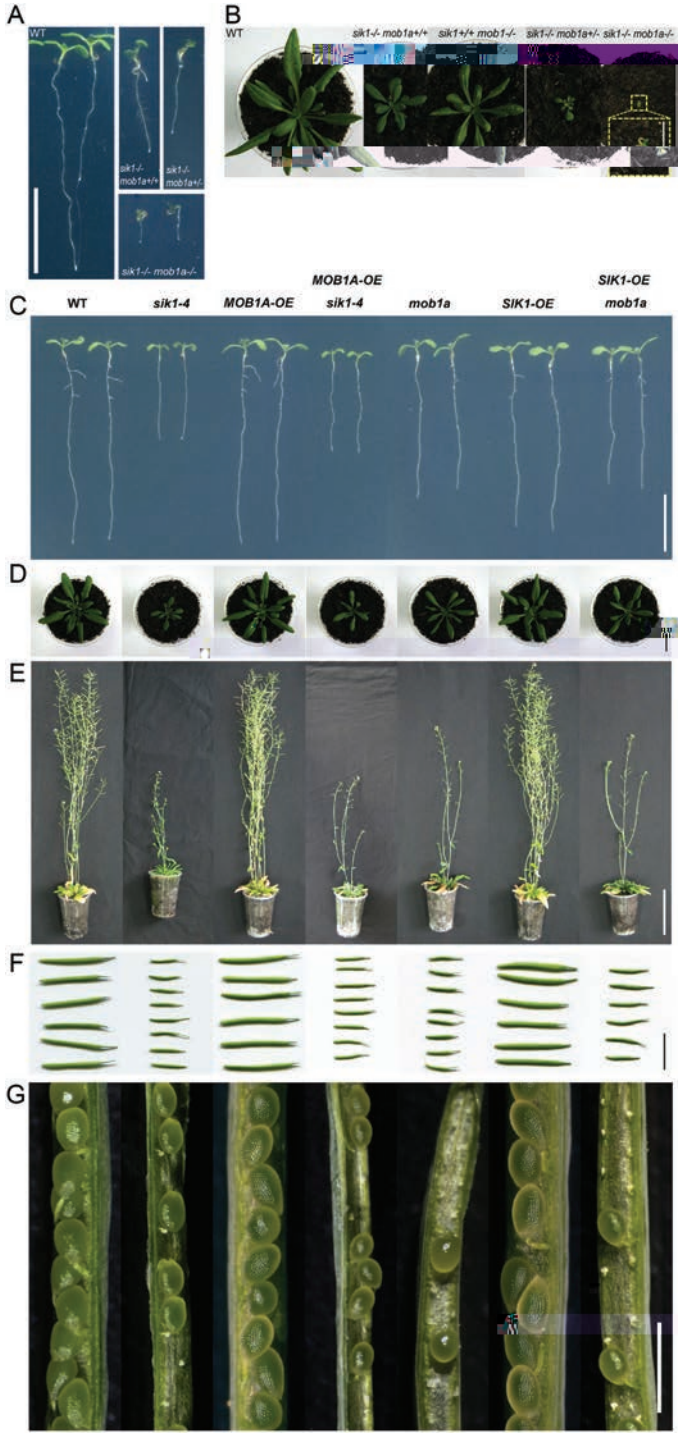

Fig. 8. Genetic interactions between *SIK1* and *MOB1A*. (A) Ten-day-old *sik1–/– mob1a+/–* has more severe phenotypes than *sik1* alone, and *sik1–/–mob1a–/–* is growth arrested. (B) The same phenotypes are observed on 4-week-old, soil-grown plants. (C) The 10-day-old transgenic line carrying *35S:MOB1A* in the *sik1-4* background has the same phenotypes as *sik1-4*. *35S:SIK1* can also not rescue *mob1a*. (D–G) Phenotypes consistent with (C) are observed in (D) 4-week-old, soil-grown plants; (E) 8-week-old plants; (F) mature siliques; (G) halves of siliques. Scale bars=1 cm in (A), (B) (by the double mutant), (C), (D), (F); 10 cm in (E); 1 mm in (G). (This figure is available in colour at *JXB* online.)

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