Two independent cis-acting elements are required for the guard cell-specific expression of SCAP1, which is essential for functionalization of stomata

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Two independent *cis*-acting elements are required for the guard cell-specific expression of *SCAP1*, which is essential for functionalization of stomata

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ABSTRACT

Regulating the stomatal aperture to adapt to environmental changes is critical for plants as stomatal guard cells are responsible for gas exchange between plants and the atmosphere. Previous study showed that a plant-specific DNA-binding with one finger (Dof)-type transcription factor, SCAP1, functions as a key regulator in the final stages of guard cell differentiation. In the present study, I performed deletion and gain-of-function analyses with the 5′ flanking region of *SCAP1* to identify the regulatory region controlling the guard cell-specific expression of *SCAP1*. The results revealed that two *cis*-acting elements, 5′-CACGAGA-3′ and 5′-CACATGTTTCCC-3′, are crucial for the guard cell-specific expression of *SCAP1*. Consistently, when an 80-bp promoter region including these two *cis*-elements was fused to a gene promoter that is not active in guard cells, it functioned as a promoter that directed gene expression in guard cells. Furthermore, the promoter region of *HT1* encoding the central regulator of stomatal CO_2 signaling was also found to contain a 5′-CACGAGA-3′ sequence, which was confirmed to function as a *cis*-element necessary for guard cell-specific expression of *HT1*. These findings suggest the existence of a novel transcriptional regulatory mechanism that synchronously promotes the expression of multiple genes required for the stomatal maturation and function.

INTRODUCTION

The molecular mechanisms of stomatal development are well studied and are suggested to be similar to those that control the development of nerves and muscles in animals (Pillitteri and Torii, 2007). Stomatal development is mediated by three related basic helix-loop-helix (bHLH) transcription factors, SPEECHLESS (SPCH) (MacAlister *et al.,* 2007), MUTE (Pillitteri *et al.,* 2007), and FAMA (Ohashi-Ito and Bergmann, 2006). These bHLH transcription factors form a heterodimer with bHLH proteins, SCREAM (SCRM) and SCRM2 (Kanaoka *et al.,* 2008) to mediate developmental steps of stomata. The initial transition of a protodermal cell to a meristemoid mother cell requires the activity of SPCH, whereas MUTE is required for the termination of meristemoid asymmetric division and promotion of guard mother cell transition. FAMA controls the final transition from the guard mother cell to the guard cell by restricting further symmetric division (Pillitteri and Torii, 2012). Although the early developmental stages of stomata have been elucidated, the details of the late stages of development when stomata become functional have not been unraveled yet.

Previous study revealed that one of the DNA-binding with one finger (Dof) transcription factors, STOMATAL CARPENTER 1 (SCAP1), is involved in the development of functional stomata (Negi *et al.,* 2013). The loss-of-function mutant, *scap1*, develops irregularly shaped guard cells and completely lacks the ability to control the stomatal aperture. The *scap1* mutation also causes decreased expression of several genes, including those for several factors involved in stomatal movement and construction of cell wall architecture. SCAP1 directly promotes the expression of *MYB60* encoding a guard cell-specific transcription factor involved in stomatal opening

(Cominelli *et al.,* 2005) and *GORK* encoding an outward potassium ion channel, which has an important role in stomatal closing (Ache *et al.,* 2000; Hosy *et al.,* 2003). SCAP1 is a key factor that promotes the development of functional stomata by regulating the expression of genes involved in stomatal movement. Although *SCAP1* is expressed in the maturation steps of guard cell differentiation, the regulatory mechanisms of *SCAP1* expression remain to be elucidated.

Previously, a [T/A]AAAG Dof-binding site was identified as a *cis*-element for guard cell-specific gene expression (Plesch *et al.,* 2001). Furthermore, disruption of the Dof-binding sites found in two guard cell-specific gene promoters, p*GC1* and p*MYB60*, impeded the ability of these promoters to direct guard cell-specific expression (Cominelli *et al.,* 2011; Yang *et al.,* 2008). The DNA sequences of some guard cell-specific promoters identified by screening using gene trap lines have also been reported to contain several Dof-binding sites (Galbiati *et al.,* 2008; Gardner *et al.,* 2009). Considering that SCAP1 directly binds to the Dof-binding site-containing promoters of guard cell-specific genes such as *MYB60* and *GORK* (Negi *et al.,* 2013), it is likely that Dof-binding sites interacting with SCAP1 function as *cis*-elements critical for guard cell-specific gene expression. However, no guard cell-specific *cis*-element other than Dof-binding sites has been reported so far.

In this study, I analyzed the *SCAP1* promoter to identify the promoter regions important for the expression of *SCAP1* and found new *cis*-elements for guard cell-specific expression other than Dof-binding sites. Furthermore, the same DNA sequence in another guard cell-specific promoter was also found to function as a *cis*-element for guard cell-specific expression. These findings suggest that a transcriptional cascade involving SCAP1 plays an essential role in stomatal functionalization.

RESULTS

Identification of the region required for guard cell-specific gene expression in the *SCAP1* promoter

To identify the *cis*-regulatory elements required for guard cell-specific gene expression of *SCAP1*, I produced transgenic *Arabidopsis* plants harboring a GUS reporter gene under the control of the *SCAP1* promoter truncated at positions 1898, 1668, 1658, 1648, 1638, 1618, 1608, 1588 or 1306 bp upstream of the transcription start site and the 5' untranslated region (5'-UTR) of *SCAP1* (Figure 1a). Guard cell-specific expression of the reporter gene can be assessed using leaves of T1 generation of transgenic lines. Therefore, in order to speed up the analysis, I decided to perform GUS staining of T1 generation seedlings of multiple independent transgenic lines as a bioassay. Quantitative GUS activity assay was also performed using leaves of T2 generation of transgenic lines (Figure 1b). The *SCAP1* promoter truncated at 1898 or 1668 bp upstream directed guard cell-specific *GUS* expression in all analyzed independent lines, while other truncated *SCAP1* promoters did not (Figure 1a,b). These results indicated the validity of my assay system, suggesting that the 10-bp sequence from -1668 to -1659 in the *SCAP1* promoter is an important region for the expression of *SCAP1*.

To identify the core element in the 10-bp sequence, I performed base substitution analysis using four mutant *SCAP1* promoters (M1, M2, M3, and M4) harboring different 2-bp substitutions in the 10-bp sequence (Figure 1c). GUS staining assay and quantitative GUS activity assay revealed that the wild-type *SCAP1* promoter (truncated at -1668) and the M1 and M4 mutant promoters similarly directed guard cell-specific *GUS* expression, while the M2 and M3 mutant promoters failed to direct expression of

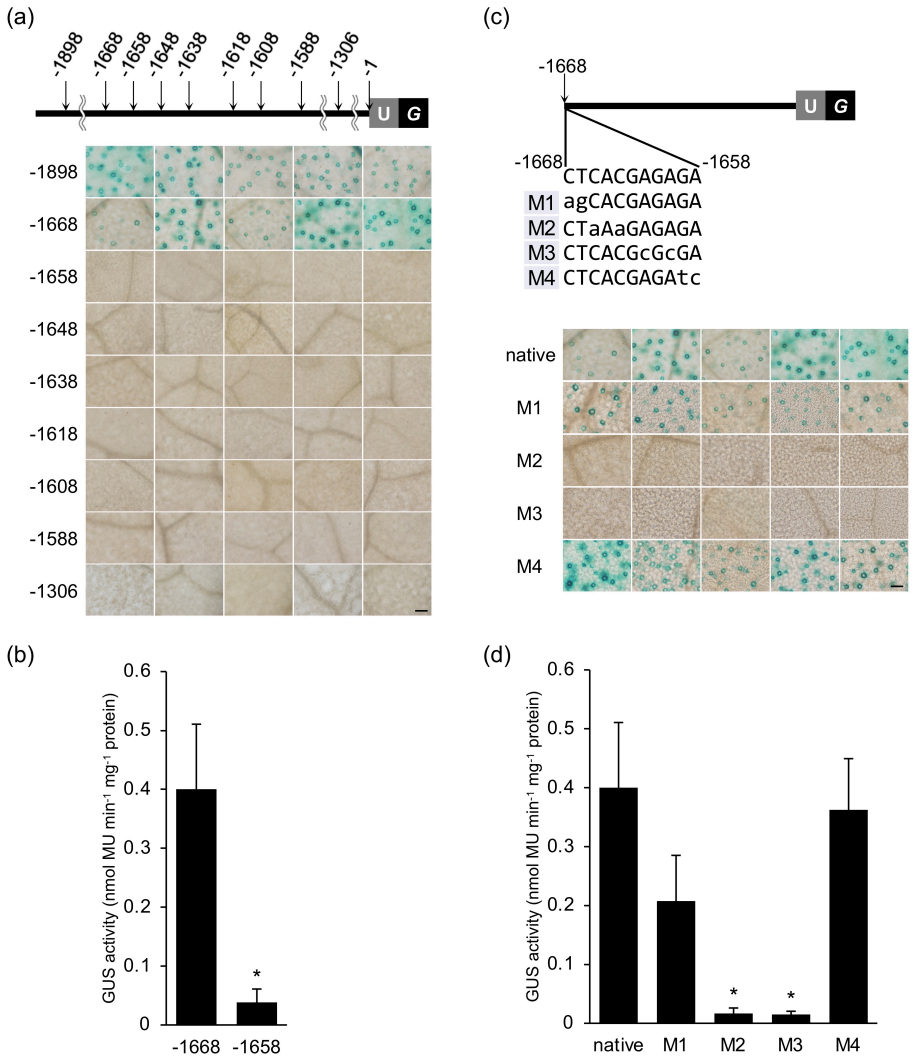


Figure 1. 5′-CACGAGA-3′ is a *cis*-element required for *SCAP1* expression.

(a) 5′ Deletion analysis between -1898 and -1306 of the *SCAP1* promoter. The histochemical GUS staining assay used five independent lines of T1 transformants from each construct that were grown for 16 days on MS medium. U, untranslated region; *G*, *GUS* (*uidA*) reporter gene. Scale bar represents 100 μm.

(b) Effects of deletion on GUS activity in leaves of the *SCAP1* promoter-*GUS* lines. Values shown are means ± SE ($n \geq 5$ independent lines). Asterisk indicates significant difference ($P < 0.05$, Student's *t* test).

(c) Base substitution analysis of the 1668-bp *SCAP1* promoter. The 1668-bp promoter was used as control. Substituted sequences from -1668 to -1658 of the 1668-bp *SCAP1* promoter are indicated. Constructs M1–M4 contained base substitutions A to C, C to A, G to T, and T to G in a sequential 2-bp substitution. U, untranslated region; *G*, *GUS* (*uidA*) reporter gene. Scale bar represents 100 μm.

(d) Effects of base substitution on GUS activity in leaves of the *SCAP1* promoter-*GUS* lines. Values shown are means ± SE ($n \geq 4$ independent lines). Ordinary one-way ANOVA was used and multiple comparisons were achieved with Dunnett's test. Asterisks indicate significant differences ($P < 0.01$).

the GUS reporter gene in guard cells (Figure 1c,d). To determine the accurate sequence, single base pair substitution analysis was also performed (Figure 2) and the results revealed that the two base pairs at both ends of 5′-CACGAGA-3′ were needed for *SCAP1* expression. Thus, the 5′-CACGAGA-3′ sequence (-1666 to -1660) is a *cis*-element necessary for the expression of *SCAP1* in guard cells.

Another promoter region of *SCAP1* is also involved in guard cell-specific expression

Although the 5′-CACGAGA-3′ sequence from -1666 to -1660 was identified as a *cis*-element necessary for the expression of *SCAP1*, it was possible that this sequence was only an enhancer for transcription and not involved in determining the cell type in which *SCAP1* is expressed. To eliminate this possibility, I proceeded to identify the region that is sufficient for the guard cell-specific expression in the *SCAP1* promoter using a gain-of-function analysis. First, I generated transgenic *Arabidopsis* plants harboring GUS gene under the control of a synthetic promoter in which a 362-bp sequence from -1668 to -1307 was fused to the *35S* minimal promoter (Figure 3, region I). GUS staining and GUS quantification of leaves of the generated plants revealed that the 362-bp sequence is sufficient for guard cell-specific expression (Figure 3, region I). Further analysis was performed using three additional synthetic promoters in which four tandem repeats of the 30-bp sequence from -1668 to -1639, the 40-bp sequence from -1648 to -1609, or the 40-bp sequence from -1618 to -1579 were fused to the *35S* minimal promoter (Figure 3, regions IV-VI). Not only the 30-bp sequence containing the 5'-CACGAGA-3' element, but also the 40-bp sequence from -1648 to -1609 was found to display the ability to induce guard cell-specific expression, revealing that the

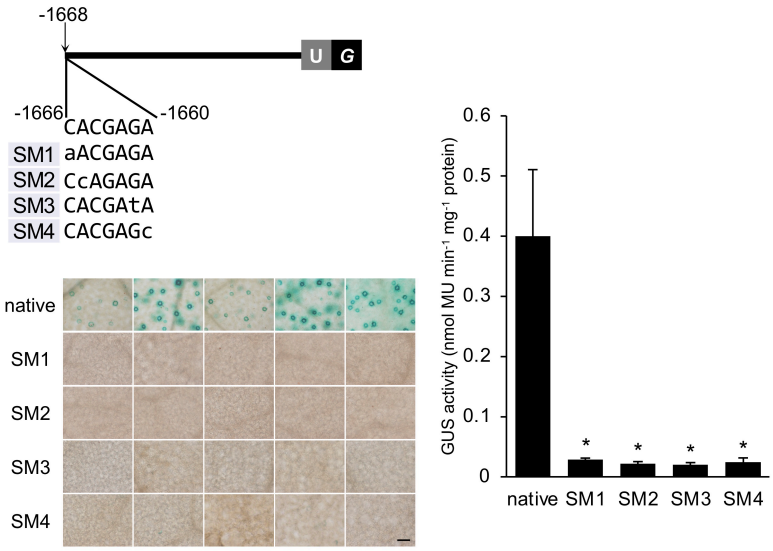


Figure 2. Single base pair substitution analysis. Both GUS expression and GUS activity are shown. Base substitution analysis of the 1668-bp *SCAP1* promoter. The 1668-bp promoter was used as control. Substituted sequences from -1666 to -1660 of the 1668-bp *SCAP1* promoter are indicated. Constructs SM1–SM4 contained base substitutions A to C, C to A, G to T, and T to G in both ends of 5′-CACGAGA-3′. U, untranslated region; *G*, *GUS* (*uidA*) reporter gene. Scale bar represents 100 μm. Values shown are means ± SE ($n \geq 5$ independent lines). Ordinary one-way ANOVA was used and multiple comparisons were achieved with Dunnett's test. Asterisks indicate significant differences ($P < 0.001$).

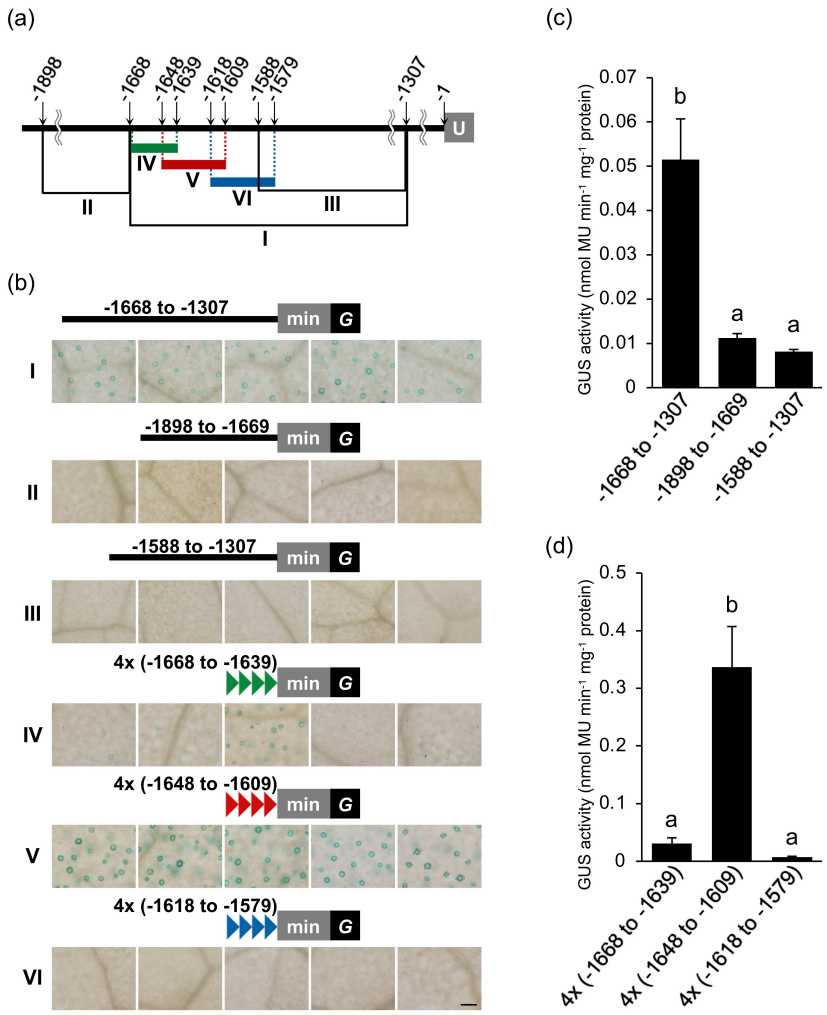


Figure 3. Sequences from -1648 to -1609 of *SCAP1* promoter are also important for the expression of *SCAP1*.

(a-b) Schematic representation of a reporter construct with a synthetic promoter, (I) (-1668 to -1307)::min::*GUS*, (II) (-1898 to -1669)::min::*GUS*, (III) (-1588 to -1307)::min::*GUS*, (IV) 4x(-1668 to -1639)::min::*GUS*, (V) 4x(-1648 to -1609)::min::*GUS*, (VI) 4x(-1618 to -1579)::min::*GUS*. U, untranslated region; min, *35S* minimal promoter truncated at -72; *G*, *GUS* (*uidA*) reporter gene. Scale bar represents 100 µm.

(c-d) GUS activity in leaves of the gain of function promoter-*GUS* lines. Values shown are means ± SE ($n \geq 4$ independent lines). The statistical significance was determined by a one-way ANOVA with Tukey–Kramer multiple comparison tests. Different letters (a and b) indicate statistically significant differences ($P < 0.05$).

30-bp sequence is sufficient to elicit guard cell-specific gene expression, while another *cis*-element is also involved in guard cell-specific expression of *SCAP1*. On the other hand, the regions from -1898 to -1669 and from -1588 to -1307 were suggested to be unrelated to guard cell-specific expression because synthetic promoters containing these regions did not direct GUS expression in guard cells (Figure 3, regions II,III).

Identification of another *cis*-element responsible for the guard cell-specific expression of *SCAP1*

To identify *cis*-element(s) involved in guard cell-specific expression in a sequence from -1648 to -1609, I performed a bioassay of four mutant *SCAP1* promoters with an internal deletion from -1648 to -1639, from -1638 to -1629, from -1628 to -1619, or from -1618 to -1609. GUS staining and GUS quantification revealed that a 20-bp sequence from -1638 to -1619 is needed for the guard cell-specific expression but other regions are not (Figure 4a,c). Next, I performed base substitution analysis with mutant *SCAP1* promoters (M5-M9 promoters) that contained a 2-bp substitution of nucleotides in the region from -1638 to -1620 (Figure 4b,d). GUS staining and GUS quantification assay revealed that M8 and M9 promoters and the wild-type *SCAP1* promoter showed similar expression patterns, while 2-bp substitutions in the M5, M6 and M7 promoters brought the promoter activity below the detection limit. To determine the accurate sequence, single base pair substitution analysis was also performed (Figure 5) and the results revealed that the two base pairs at the 5′ end and three base pairs at the 3′ end of 5′-CACATGTTTCCC-3′ were needed for *SCAP1* expression. These results indicated that a 5′-CACATGTTTCCC-3′ sequence (-1638 to -1627) is another *cis*-element required for the expression of *SCAP1*.

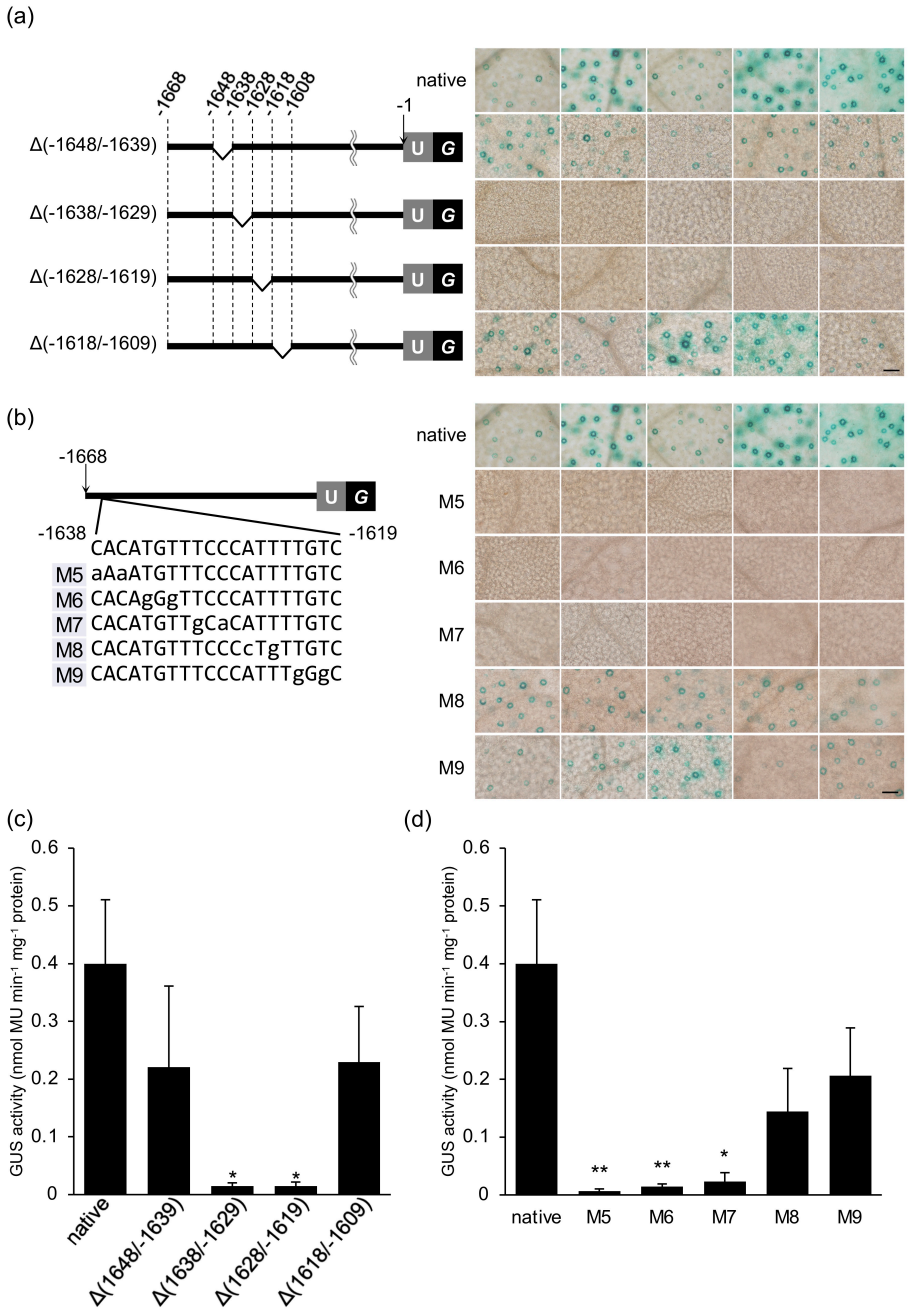


Figure 4. The other *cis*-element required for the expression of *SCAP1* is 5′-CACATGTTTCCC -3′.
(a) Internal deletion analysis of the *SCAP1* promoter. 10-bp sequences are internally deleted from -1648 to -1639, from -1638 to -1629, from -1628 to -1619, and from -1618 to -1609 of the 1668-bp *SCAP1* promoter. U, untranslated region; *G*, *GUS* (*uidA*) reporter gene. Scale bar represents 100 μm.
(b) Base substitution analysis of the 1668-bp *SCAP1* promoter. Substituted sequences from -1638 to -1620 of the *SCAP1* promoter are indicated. Constructs M5–M9 contained base substitutions A to C, C to A, G to T, and T to G in a sequential 2-bp substitution. U, untranslated region; *G*, *GUS* (*uidA*) reporter gene. Scale bar represents 100 μm.

(c) Effects of internal deletion on GUS activity in leaves of the *SCAP1* promoter-*GUS* lines. Values shown are means ± SE ($n \geq 3$ independent lines). Ordinary one-way ANOVA was used and multiple comparisons were achieved with Dunnett's test. Asterisks indicate significant differences ($P < 0.01$).
(d) Effects of base substitution on GUS activity in leaves of the *SCAP1* promoter-*GUS* lines. Values shown are means ± SE ($n \geq 3$ independent lines). Ordinary one-way ANOVA was used and multiple comparisons were achieved with Dunnett's test. One asterisk ($P < 0.05$) and two asterisks ($P < 0.01$) indicate significant differences.

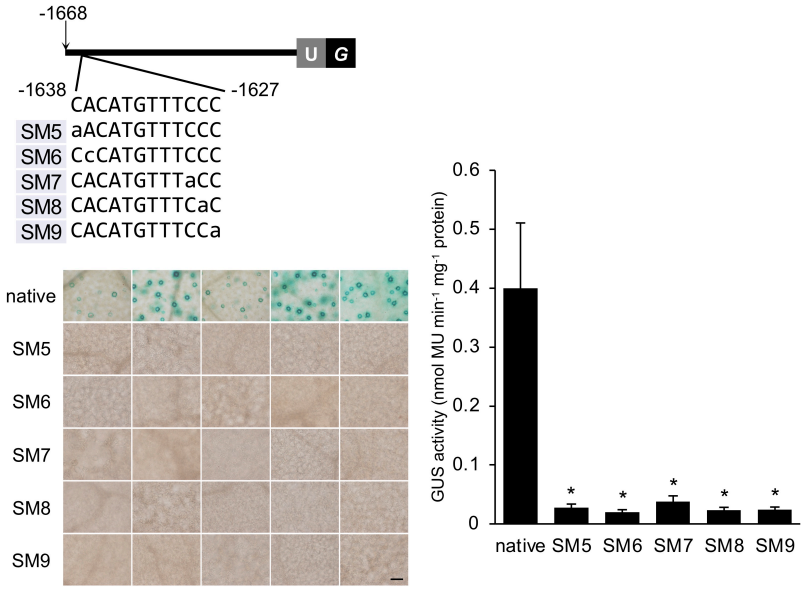


Figure 5. Single base pair substitution analysis. Both GUS expression and GUS activity are shown. Base substitution analysis of the 1668-bp *SCAP1* promoter. The 1668-bp promoter was used as control. Substituted sequences from -1638 to -1627 of the 1668-bp *SCAP1* promoter are indicated. Constructs SM5–SM9 contained base substitutions A to C, C to A, G to T, and T to G in both ends of 5′-CACATGTTTCCC-3′. U, untranslated region; *G*, *GUS* (*uidA*) reporter gene. Scale bar represents 100 μm. Values shown are means ± SE ($n \geq 5$ independent lines). Ordinary one-way ANOVA was used and multiple comparisons were achieved with Dunnett's test. Asterisks indicate significant differences ($P < 0.001$).

Addition of the *SCAP1* promoter region containing the two *cis*-elements confers ectopic activity in guard cells on a root-specific gene promoter

To investigate whether the *SCAP1* promoter region of *SCAP1* including the two *cis*-elements is sufficient to confer ectopic activity in guard cells on unrelated promoters, I conducted gain-of-function analysis using the promoter of *SLAC1 HOMOLOGUE 1* (*SLAH1*), which is functional in roots but not in stomatal guard cells (Figure 6a,b : Negi *et al.,* 2008). Thus, a synthetic promoter in which the 80-bp sequence from -1668 to -1589 of the *SCAP1* promoter was fused to upstream of the 1505-bp *SLAH1* promoter (Figure 6c). GUS staining and GUS quantification revealed that this synthetic promoter is active in guard cells as well as the root (Figure 6d,e).

The CACGAGA element is needed for the expression of *SCAP1* and *HT1*

The 5′-CACGAGA-3′ sequence was found in the 2000-bp region of 5′ upstream regions (relative to the transcription start site) of 3893 genes in *Arabidopsis* (Figure 7a,c). Using previous microarray data (Negi *et al.,* 2018), I found that about a quarter of these genes show more than two-fold higher expression levels in guard cells than in mesophyll cells (Figure 7b). The Gene Set Enrichment Analysis (GSEA) performed using previous microarray data (Negi *et al.,* 2018) revealed that the genes that have 5′-CACGAGA-3′ in their promoters tend to be expressed higher in guard cells than in mesophyll cells (p-value < 0.001, FDR q-value = 0.091). To further assess the stages of stomatal development in cells that express genes with 5′-CACGAGA-3′, I conducted statistical analysis using RNA-seq data (Adrian *et al*., 2015), which revealed that the 5′-CACGAGA-3′ element was enriched in the promoters of genes that were expressed dominantly in mature guard cells compared to the promoters of genes that

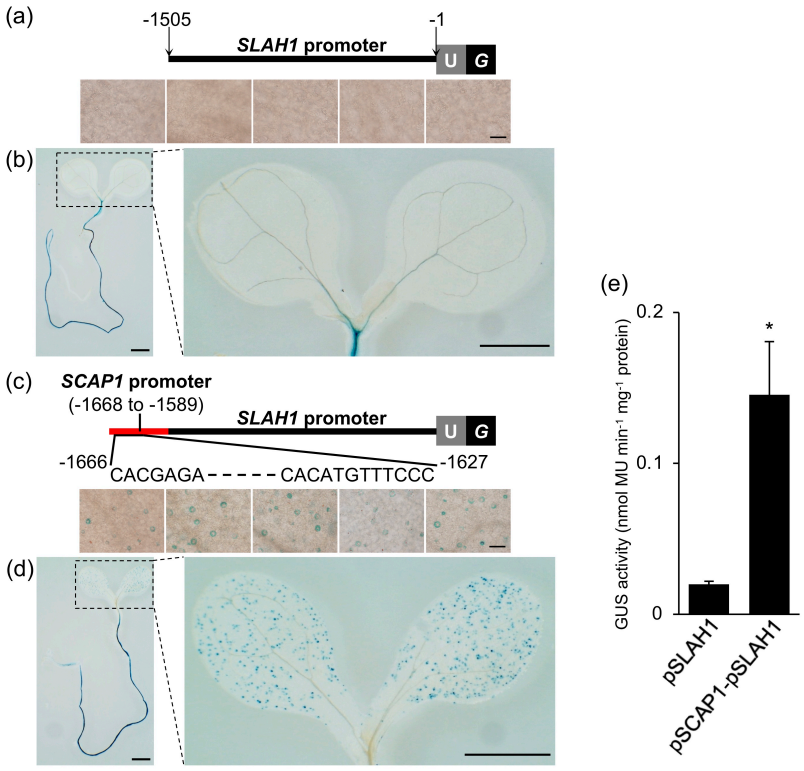
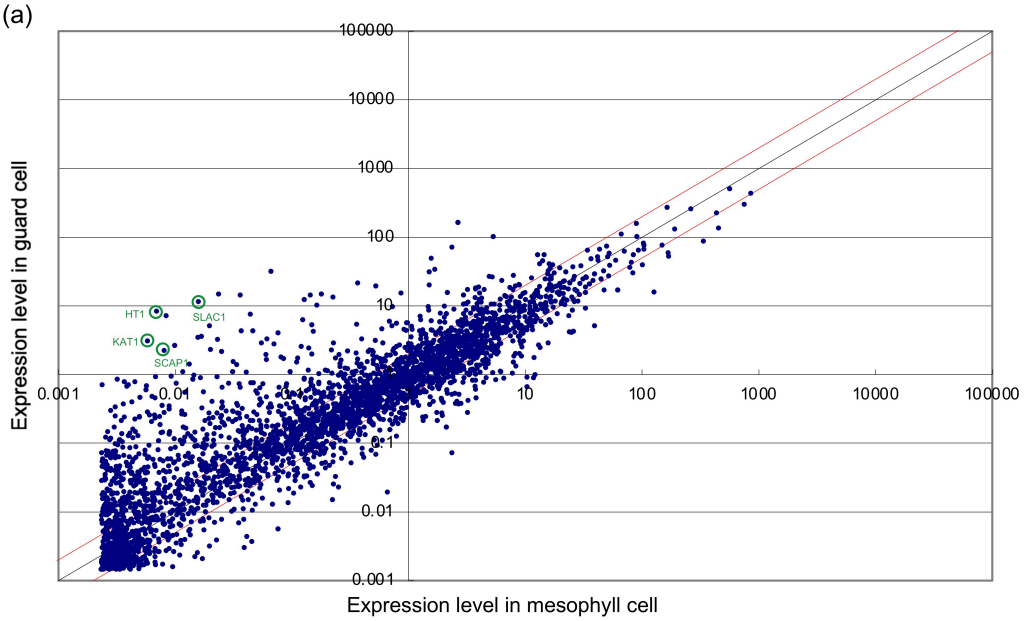


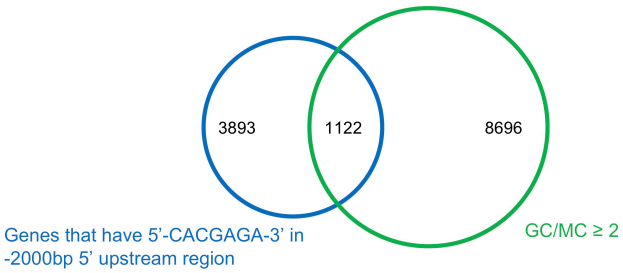
Figure 6. *SCAP1* promoter including the two *cis*-elements confer ectopic guard cell-expression on the promoter of the *SLAH1*.

Schematic representation of reporter constructs with synthetic promoters, (a) p*SLAH1*::*GUS* and (c) p*SCAP1*(-1668 to -1589)::p*SLAH1*::*GUS*. Promoter region of *SCAP1* used in (c) has the two *cis*-elements, 5′-CACGAGA-3′ and 5′-CACATGTTTCCC-3′. Histochemical GUS staining assay used five independent lines of T1 transformants from each construct that were grown for 16 days on MS medium. U, untranslated region; *G*, *GUS* (*uidA*) reporter gene. Scale bars represent 100 μm. Histochemical GUS staining assay used a plant harboring (b) p*SLAH1*::*GUS* construct and (d) p*SCAP1*(-1668 to -1589)::p*SLAH1*::*GUS* construct were grown for 7 days on MS medium. Scale bars represent 1 mm. (e) GUS activity in leaves of the gain of function *SLAH1* promoter-*GUS* lines. Values shown are means ± SE ($n \geq 3$ independent lines). Asterisk indicates significant difference ($P < 0.05$, Student's *t* test).

(a)


100000
10000
1000
100
10
1
0.1
0.01
0.001
0.0001
0.001
0.01
0.1
1
10
100
1000
10000
100000
HT1
SLAC1
KAT1
SCAP1
Expression level in guard cell
Expression level in mesophyll cell

(b)


3893
1122
8696
Genes that have 5'-CACGAGA-3' in -2000bp 5' upstream region
GC/MC ≥ 2

(c)

	GeneSymbol	GeneName	GC/MC
AT1G62400	HT1	protein high leaf temperature 1	1212
AT1G33811	AT1G33811	GDSL esterase/lipase	862
AT2G40260	AT2G40260	myb family transcription factor	829
AT3G51760	AT3G51760	hypothetical protein	745
AT1G12480	SLAC1	guard cell S-type anion channel SLAC1	729
AT1G16960	AT1G16960	ubiquitin domain-containing protein	632
AT5G46240	KAT1	potassium channel KAT1	536
AT1G64370	AT1G64370	hypothetical protein	403
AT2G28870	AT2G28870	hypothetical protein	303
AT5G65590	SCAP1	Dof-type zinc finger DNA-binding family protein	282
AT3G55110	AT3G55110	ABC transporter G family member 18	269
AT2G47880	AT2G47880	glutaredoxin-C13	265
AT5G18600	AT5G18600	monothiol glutaredoxin-S2	221
AT1G11340	AT1G11340	G-type lectin S-receptor-like serine/threonine-protein kinase	215
AT1G45201	TLL1	triacylglycerol lipase-like 1	215
AT3G55500	EXPA16	expansin A16	206
AT1G17200	AT1G17200	hypothetical protein	188
AT2G34850	MEE25	putative UDP-arabinose 4-epimerase 2	181
AT4G04890	PDF2	homeobox-leucine zipper protein PROTODERMAL FACTOR 2	175
AT3G10280	KCS14	3-ketoacyl-CoA synthase 14	175
AT4G14010	RALFL32	protein ralf-like 32	173
AT5G09220	AAP2	amino acid permease 2	173
AT2G28260	CNGC15	cyclic nucleotide-gated channel 15	140
AT1G76190	AT1G76190	SAUR-like auxin-responsive protein	137
AT4G38770	PRP4	proline-rich protein 4	122
AT5G24800	BZIP9	basic leucine zipper 9	117
AT3G51400	AT3G51400	hypothetical protein	115
AT2G46630	AT2G46630	hypothetical protein	114
AT1G52910	AT1G52910	hypothetical protein	112
AT3G19710	BCAT4	branched-chain aminotransferase4	109
AT4G16008	AT4G16008	hypothetical protein	108
AT3G01960	AT3G01960	hypothetical protein	105
AT3G05730	AT3G05730	defensin-like protein 205	100
AT4G17970	ALMT12	aluminum-activated, malate transporter 12	98
AT1G56580	SVB	protein SMALLER WITH VARIABLE BRANCHES	97
AT5G18970	AT5G18970	AWPM-19-like protein	95
AT5G42146	AT5G42146	hypothetical protein	95
AT4G24480	AT4G24480	protein kinase family protein	93
AT5G19580	AT5G19580	glyoxal oxidase-related protein	93
AT2G16740	UBC29	ubiquitin-conjugating enzyme E2 29	87
AT1G22885	AT1G22885	hypothetical protein	83
AT5G04230	PAL3	phenylalanine ammonia-lyase 3	81
AT2G16630	FOCL1	pollen Ole e 1 allergen and extensin family protein	80
AT5G05160	AT5G05160	putative leucine-rich repeat receptor-like protein kinase	78
AT1G12490	AT1G12490	F-box associated ubiquitination effector family protein	76
AT1G77145	AT1G77145	hypothetical protein	74
AT2G19110	HMA4	putative cadmium/zinc-transporting ATPase HMA4	68
AT3G27400	AT3G27400	putative pectate lyase 11	67
AT3G61380	AT3G61380	phosphatidylinositol N-acetyglucosaminlytransferase subunit P-like protein	66
AT5G02380	MT2B	metallothionein 2B	64
AT2G24520	HA5	H(+)-ATPase 5	62
AT1G15580	IAA5	auxin-responsive protein IAA5	62
AT3G54000	AT3G54000	hypothetical protein	60
AT1G50590	AT1G50590	Pirin-like protein	60
AT1G17180	GSTU25	glutathione S-transferase TAU 25	59
AT2G19980	AT2G19980	CAP (Cysteine-rich secretory proteins, Antigen 5, and Pathogenesis-related 1 protein) superfamily protein	57
AT1G12890	AT1G12890	ethylene-responsive transcription factor ERF088	56
AT3G47600	MYB94	putative transcription factor MYB94	55
AT2G22320	AT2G22320	hypothetical protein	54
AT1G12030	AT1G12030	hypothetical protein	54
AT5G43150	AT5G43150	hypothetical protein	53
AT3G51600	LTP5	pathogenesis-related protein LTP5	53
AT4G33150	AT4G33150	lysine-ketoglutarate reductase/saccharopine dehydrogenase bifunctional enzyme	51
AT1G64195	AT1G64195	defensin-like protein 35	50
AT5G01040	LAC8	laccase 8	50

Figure 7. Comparison of guard cell–expressed genes versus mesophyll cell–expressed genes that have 5′-CACGAGA-3′ in the -2000bp 5′ upstream region.

(a) Scatter plot of the normalized expression level of genes that have 5′-CACGAGA-3′ in the -2000bp 5′ upstream region in guard cell versus mesophyll cell.

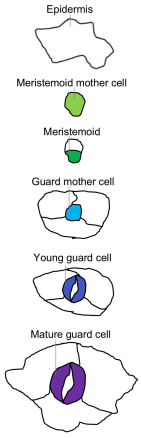
(b) Venn diagram of genes. Blue circle represents the number of genes that have 5′-CACGAGA-3′ in the -2000bp 5′ upstream region, and green circle represents the number of genes whose expression level in guard cells are twice or higher than in mesophyll cells.
(c) List of genes that have 5′-CACGAGA-3′ in the -2000bp 5′ upstream region whose expression level in guard cells are 50 times or higher than in mesophyll cells.

were preferentially expressed at earlier stages in the stomatal lineage (Table 1, Fisher's exact test, p-value = 0.001). These results suggest that 5′-CACGAGA-3′ is a *cis*-element that contributes to guard cell-specific expression, especially in matured stages of stomatal development.

The genes whose expression levels in guard cells was more than 100 times higher than those in mesophyll cells and whose functions in stomata were known were *HIGH LEAF TEMPERATURE 1 (HT1)* (Hashimoto *et al.,* 2006), *SLOW ANION CHANNEL1 (SLAC1)* (Negi *et al.,* 2008), *POTASSIUM CHANNEL IN ARABIDOPSIS THALIANA 1 (KAT1)* (Anderson *et al.,* 1992) and *SCAP1* (Figure 7c). A 5′-CACGAGA-3′ sequence exists at -219 in the *HT1* promoter, at -162 in the *SLAC1* promoter, and at -1323 in the *KAT1* promoter (Figure 8a). On the other hand, although nine genes including *SCAP1* were also found to have another *cis*-element, 5′-CACATGTTTCCC-3′, in their 5′ upstream regions (the 2 kb region from the transcription start site), the functions of these genes, except for *SCAP1*, have not been clarified in stomata (Figure 9). Therefore, I investigated the contribution of the 5′-CACGAGA-3′ sequence to the expression of guard cell-specific genes by base substitution analysis using the *HT1*, *SLAC1*, and *KAT1* promoters. The same mutation as in the M2 promoter in Figure 1c was introduced into the 5′-CACGAGA-3′ sequences of these promoters (Figure 8a). GUS staining and GUS quantification of the plants harboring GUS gene under the control of the wild-type or mutant promoter revealed that the 2-bp mutation strongly repressed the activity of the *HT1* promoter, whereas the mutation exerted no effect on the activity of the *SLAC1* and *KAT1* promoter (Figure 8b).

To examine the physiological significance of these 5′-CACGAGA-3′ sequences, I carried out the complementation analysis (Figure 8c). SCAP1 and HT1 were expressed

Table 1. Rate of genes that have CACGAGA in their promoters.



The stage that genes are dominantly expressed in		Number of genes	Number of genes that have CACGAGA in their promoters	%	p-value
Epidermis	Cluster I	1012	132	13.04	0.6718
Meristemoid mother cells	Cluster II	920	118	12.83	0.7342
Meristemoids	Cluster III	563	83	14.74	0.2021
Epidermis and Guard mother cells	Cluster IV	248	34	13.71	0.4844
Guard mother cells and Young guard cells	Cluster V	335	53	15.82	0.1191
Mature guard cells	Cluster VI	588	106	18.03	0.0010
all		28897	3893	13.47	

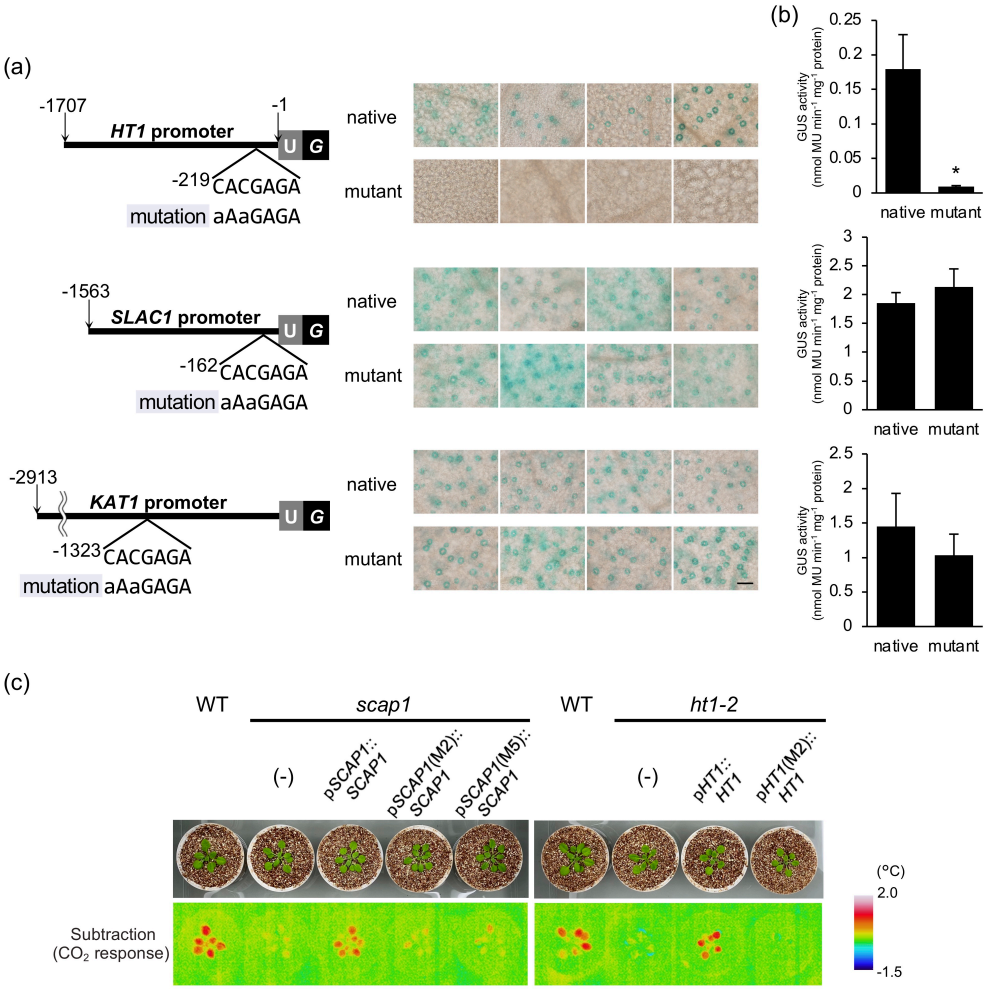


Figure 8. 5′-CACGAGA-3′ is a crucial *cis*-element for the expression of *SCAP1* and *HT1*.

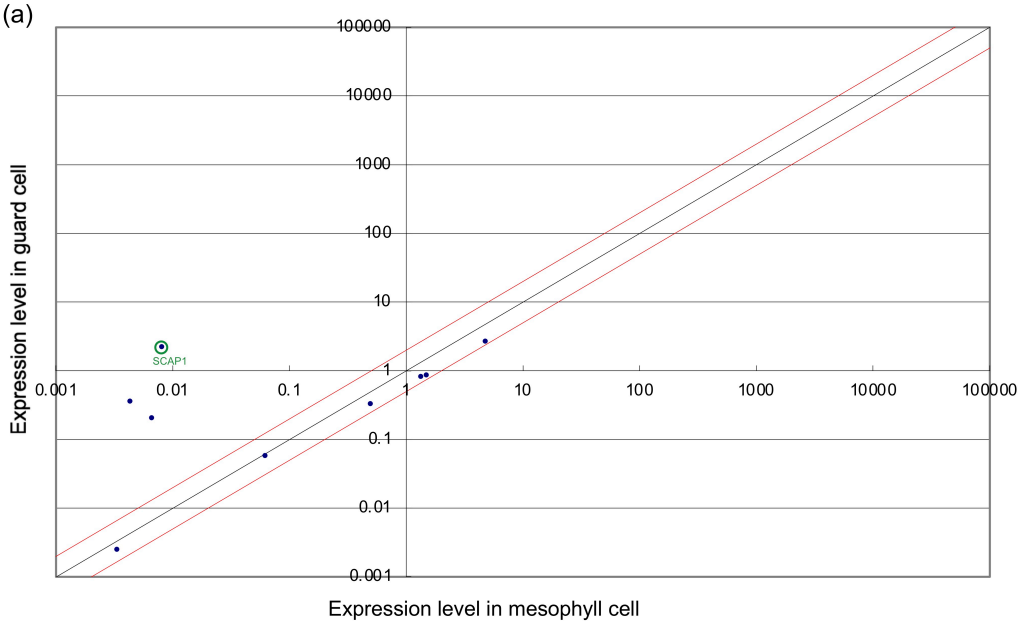
(a) Base substitution analysis of guard cell-specific promoters. Substituted sequences 5′-CACGAGA-3′ of the *HT1* promoter, *SLAC1* promoter, and *KAT1* promoter are shown. 5′-CACGAGA-3′ exists at -219 in the 1707-bp *HT1* promoter, at -162 in the 1563-bp *SLAC1* promoter, and at -1323 in the 2913-bp *KAT1* promoter. Substitutions were the same as M2 described in Figure 1c, containing base substitutions C to A in a sequential 2-bp substitution. U, untranslated region; *G*, *GUS* (*uidA*) reporter gene. Scale bar represents 100 μm.

(b) Effects of base substitution on GUS activity in leaves of the *HT1*, *SLAC1*, *KAT1* promoter-*GUS* lines. Values shown are means ± SE ($n \geq 5$ independent lines). Asterisk indicates significant difference ($P < 0.01$, Student's *t* test).

(c) Thermal subtractive images of the response to [CO_2] in wild-type (WT), *scap1* mutant, *ht1-2* mutant and transgenic plants. p*SCAP1*::*SCAP1*, p*SCAP1*(M2 substitution, described in Figure 1)::*SCAP1*, p*SCAP1*(M5 substitution, described in Figure 4)::*SCAP1* were introduced into the *scap1* mutant. p*HT1*::*HT1*, p*HT1*[M2 substitution, described in (a)]::*HT1* were introduced into the *ht1* mutant. Plants were incubated at high [CO_2] (1000 ppm) for 60 min after incubation at low [CO_2] (0 ppm) for 90 min.

The subtractive images show changes in leaf temperature in response to the transfer from low (0 ppm) to high (1000 ppm) [CO_2].

(a)



(b)

	GeneSymbol	GeneName	GC/MC
AT5G65590	SCAP1	Dof-type zinc finger DNA-binding family protein	282
AT2G25770	AT2G25770	polyketide cyclase/dehydrase and lipid transport superfamily protein	85
AT3G09730	AT3G09730	hypothetical protein	32
AT5G11780	AT5G11780	hypothetical protein	0.95
AT5G66150	AT5G66150	Glycosyl hydrolase family protein	0.77
AT5G66170	STR18	thiosulfate sulfurtransferase 18	0.68
AT2G46505	SDH4	succinate dehydrogenase subunit 4	0.62
AT3G09720	AT3G09720	DEAD-box ATP-dependent RNA helicase 57	0.59
AT3G58170	BS14A	BET1P/SFT1P-like protein 14A	0.57

Figure 9. Comparison of guard cell–expressed genes versus mesophyll cell–expressed genes that have 5′-CACATGTTTCCC-3′ in the -2000bp 5′ upstream region.

(a) Scatter plot of the normalized expression levels of genes that have 5′-CACATGTTTCCC-3′ in the -2000bp 5′ upstream region in guard cells versus mesophyll cells.

(b) List of genes that have 5′-CACATGTTTCCC-3′ in the -2000bp 5′ upstream region.

in the *scap1* mutant (Negi *et al.,* 2013) and *ht1-2* mutant (Hashimoto *et al.,* 2006), respectively, using a native promoter (*SCAP1* promoter and *HT1* promoter) or a base substitution promoter [*SCAP1* promoter (M2), *SCAP1* promoter (M5), and *HT1* promoter (M2)]. Phenotypic recovery was detected by measuring the leaf temperature. Leaf temperature provides a convenient indicator of transpiration and can detect mutant phenotypes with altered stomatal control (Hashimoto *et al.,* 2006; Merlot *et al.,* 2002; Xie *et al.,* 2006). Thermographic measurements revealed that *SCAP1* expressed under a native promoter could complement the CO_2 insensitive phenotype of *scap1* mutant. In contrast, the complementation rate of *scap1* mutant by *SCAP1* expressed under a base substitution promoter was low (Table 2). Similarly, *HT1* expressed under a base substitution promoter could not complement the CO_2 insensitive phenotype of *ht1-2* mutant (Table 2). To quantify the stomatal CO_2 response, I also measured stomatal conductance in these transgenic lines and the results revealed that *SCAP1* or *HT1* expressed under a base substitution promoter could not recover the CO_2-insensitive *scap1* or *ht1-2* phenotypes, respectively (Figure 10). These results indicate that 5′-CACGAGA-3′ is crucial to SCAP1 and HT1 activity in stomatal guard cells.

Table 2. Phenotypic complementation of *scap1* mutant and *ht1-2* mutant.

Name of a construction	Number of inserted lines	Number of lines that restore wild-type phenotype	Phenotypic recovery rate of complementation plants (%)
p*SCAP1*::*SCAP1*	24	22	91.7
p*SCAP1*(M2)::*SCAP1*	24	0	0
p*SCAP1*(M5)::*SCAP1*	26	0	0
p*HT1*::*HT1*	22	21	95.5
p*HT1*(M2)::*HT1*	22	0	0

Transgenic lines exhibited normal CO_2 response same as WT were counted as 'Number of lines that restore wild-type phenotype'.

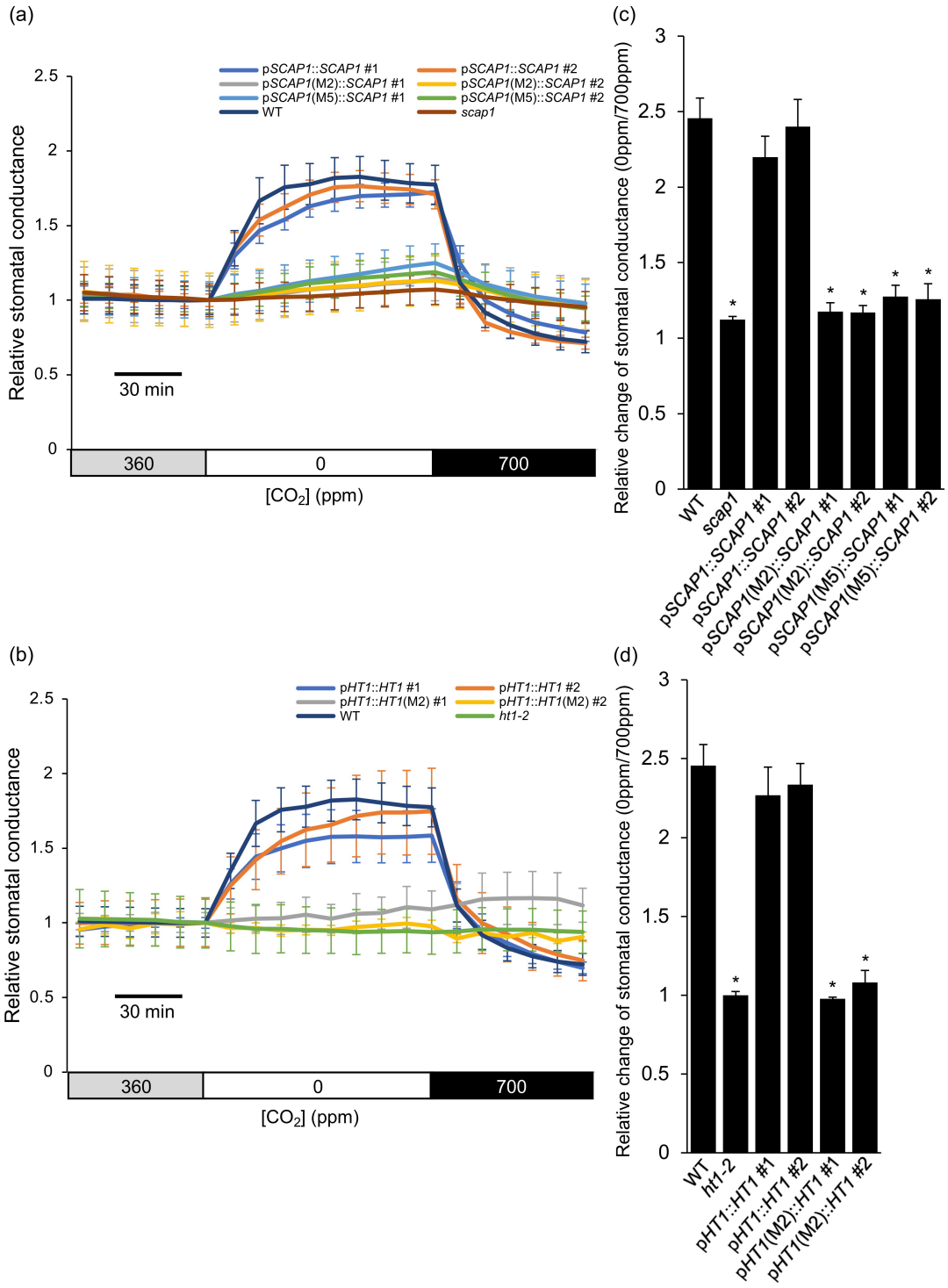


Figure 10. Measurement of stomatal conductance.

(a-b) Relative stomatal conductance of *scap1* and *ht1-2* complementation lines response to [CO_2]. Time course of stomatal conductance in wild-type (WT), *scap1* mutant, *ht1-2* mutant and transgenic plants. p*SCAP1*::*SCAP1*, p*SCAP1*(M2)::*SCAP1*, p*SCAP1*(M5)::*SCAP1* were introduced into the *scap1* mutant. p*HT1*::*HT1*, p*HT1*(M2)::*HT1* were introduced into the *ht1-2* mutant. Stomatal conductance was

normalized to the average conductance at the last 360 ppm CO_2 data point. Values shown are means ± SE (n = 5).

(c-d) The ratio of stomatal conductance after switching from 360 ppm to 0 ppm CO_2 for 60 min to that after switching from 0 ppm to 700 ppm CO_2 for 90 min. Values shown are means ± SE ($n \geq 3$ independent plants). Ordinary one-way ANOVA was used and multiple comparisons were achieved with Dunnett's test. Asterisks indicate significant differences ($P < 0.001$).

DISCUSSION

The regulation of stomatal aperture is critical for plants to adapt to environmental changes. Since SCAP1 is an essential regulator for the development of functional stomata (Negi *et al.,* 2013), identifying the *cis*-elements required for the expression of *SCAP1* is necessary to understand the initiation of stomatal functionalization. In this report, I analyzed the *SCAP1* promoter and identified a 7-bp sequence, 5′-CACGAGA-3′, and a 12-bp sequence, 5′-CACATGTTTCCC-3′, as *cis*-elements necessary for the expression of *SCAP1* (Figure 1, 4 and 8c). Furthermore, I demonstrated that the *SCAP1* promoter region, which has the two *cis*-elements, is sufficient to induce the expression in guard cells (Figure 6). My findings indicate the existence of guard cell-specific transcriptional regulatory mechanisms that promote stomatal functionalization.

The two *cis*-elements independently regulate *SCAP1* expression

Gain of function analysis using synthetic promoters in which four tandem repeats of a part of the *SCAP1* promoter were fused to the *35S* minimal promoter revealed that each of the two *cis*-elements has the ability to independently induce the expression of a reporter gene in stomata (Figure 3b,d). On the other hand, mutation of only one of the two *cis*-elements drastically reduced the reporter gene expression (Figure 1c,d and 4b,d), and the *SCAP1* expression levels obtained using the mutant promoters with only a single *cis*-element did not reach the threshold of *SCAP1* expression level for phenotypic recovery of the *scap1* mutant (Figure 8c, 10a,c and Table 2). These results indicate that although each *cis*-element could work for the expression of *SCAP1* independently, the contribution of a single *cis*-element is not enough for the *SCAP1*

expression level required for stomatal functionalization. Hence, identification of the transcription factors that bind to these two *cis*-elements would be necessary to fully understand the mechanism underlying guard cell-specific transcription involved in stomatal functionalization.

It had been reported that the genomic DNA fragment adjacent to the T-DNA RB in E1728, a guard cell specific-expressing enhancer trap line, includes a part of *SCAP1* promoter region (Gardner *et al.,* 2009). The truncated DNA fragment (-2448 to -1485 of the *SCAP1* promoter), which has the two *cis*-elements, directed GUS expression in guard cells, while any of the other DNA fragments that do not have the two *cis*-elements had no detectable GUS activity (Gardner *et al.,* 2009). It is possible that the guard cell-specific expression in E1728 line depends on the presence of the two *cis*-elements.

Furthermore, because *SCAP1*, which is expressed in the early stages of stomatal development, is also involved in the stomatal lineage specification (Castorina *et al.,* 2016), it might be possible to elucidate the mechanism of stomatal lineage specification by analyzing *SCAP1* expression and the involvement of each of the two *cis*-elements in *SCAP1* expression at that stage.

The CACGAGA-mediated guard cell-specific transcriptional regulation

Promoter activity is generally determined by multiple *cis*-elements. So far, only the Dof-binding site has been identified as a *cis*-element for guard cell-specific gene expression (Cominelli *et al.,* 2011; Galbiati *et al.,* 2008; Gardner *et al.,* 2009; Plesch *et al.,* 2001; Yang *et al.,* 2008). Although there are some Dof-binding sites in the promoter region of *SCAP1*, this study revealed that the 80-bp region without Dof-binding sites is

an important region for the expression of *SCAP1* and that 5′-CACGAGA-3′ and 5′-CACATGTTTCCC-3′ sequences in this region are *cis*-element sequences for guard cell-specific gene expression. These two *cis*-elements differ from the known *cis*-elements, suggesting the existence of novel mechanisms for guard cell-specific gene expression. Furthermore, the 5′-CACGAGA-3′ sequence found in the *HT1* promoter was confirmed to be essential for the guard cell-specific expression of *HT1*, which encodes a kinase required for stomatal movement in response to CO_2. These suggest that 5′-CACGAGA-3′ sequence is not a *cis*-element unique to *SCAP1* expression, but it functions as a ubiquitous *cis*-element for the expression of guard cell-specific genes. If this is true, then in addition to the known Dof-mediated transcriptional regulation of guard cell-specific genes, there may be other mechanisms that allow guard cell-specific expression. However, although 5′-CACGAGA-3′ sequences are also present in the *SLAC1* promoter and *KAT1* promoter, these were not essential for the expression of these genes. It is possible that other *cis*-elements important for the expression of *SLAC1* and *KAT1* exist in their promoters and their expression are regulated under the transcriptional mechanisms different from those of *SCAP1*. Alternatively, SCAP1 may either directly or indirectly regulate *SLAC1* and *KAT1* expression because the expression levels of these genes in *scap1* mutant were reduced compared with that of wild-type (Negi *et al.,* 2013). Therefore, multiple mechanisms likely regulate guard cell-specific gene expression. But my results indicate that the two independent *cis*-acting elements control the guard cell-specific expression of *SCAP1* that is essential for the functionalization of stomata.

Another *cis*-element, 5′-CACATGTTTCCC-3′ is present in the promoters of only nine genes (Figure 9) in *Arabidopsis*, and the function of these genes other than *SCAP1*

have not been known in stomatal guard cells. Thus, it is possible that 5′-CACATGTTTCCC-3′ functions as a *SCAP1*-specific enhancer sequence rather than a *cis*-element common to guard cell-specific genes. Since this sequence is longer than standard *cis*-elements, some nucleotide substitutions within this sequence may not affect DNA-protein interaction. Therefore, it is impossible to completely rule out the possibility at this stage that modified versions of this sequence function as *cis*-elements in other guard cell-specific promoters.

On the other hand, the 5′-CACGAGA-3′ sequence exists in the promoter regions of many genes essential for stomatal function (Figure 7). In this study, I analyzed the functionality of 5′-CACGAGA-3′ sequences in the promoter regions of the genes whose expression level in guard cells is 100 times or more than that in mesophyll cells. However, the 5′-CACGAGA-3′ sequence was also found in the promoters of genes whose expression level in guard cells is 50 times or more than that in mesophyll cells. Such genes include *ALMT12* (*QUAC1*) encoding R-type anion channel that evoke rapid stomatal closure (Meyer *et al*., 2010; Sasaki *et al*., 2010) and *FUSED OUTER CUTICULAR LEDGE1* (*FOCL1*) encoding a putative cell wall glycoprotein that is required for the formation of the stomatal outer cuticular ledge (Hunt *et al*., 2017). Because the functions of *HT1*, *QUAC1* and *FOCL1* are intimately associated with the late stage of stomatal development, it is possible to speculate that the CACGAGA-mediated mechanism may have a role at the late stage of stomatal development to regulate a group of genes specifically expressed at this time.

So far, it remains unclear as to which transcription factor directly regulates the expression of *SCAP1.* Using published DAP-seq data (O'Malley *et al*., 2016), I have identified BIM2 (BES1-INTERACTING MYC-LIKE 2) as a transcription factor that

has the potential to bind to 5′-CACGAGA-3′ in *SCAP1* promoter, but the stomatal CO_2 response of the *bim2* mutant was normal and promoter activity of *SCAP1* was not activated by BIM2 (Figure 11). I also examined the possibility that FAMA is an upstream factor of *SCAP1*. 5′-CACGAGA-3′ resembles N-box, a *cis*-element recognized by the bHLH transcription factor. FAMA is a bHLH transcription factor involved in late stomatal development (Ohashi-Ito and Bergmann, 2006). Moreover, *SCAP1* expression was upregulated by *FAMA* overexpression (Hachez *et al*., 2011, Shirakawa *et al*., 2014). Therefore, I performed a transient assay to clarify whether FAMA activates the *SCAP1* promoter. The results showed that *SCAP1* promoter was activated by *FAMA* overexpression, but mutation in 5′-CACGAGA-3′ did not affect this activation (Figure 12). FAMA is possibly involved in the expression of *SCAP1*, but such FAMA-mediated regulation is independent of the 5′-CACGAGA-3′ *cis*-element. Finding the transcription factor that can bind to 5′-CACGAGA-3′ and regulate *SCAP1* expression will help to further understand gene expression mechanisms that are specifically associated with stomatal functionalization.

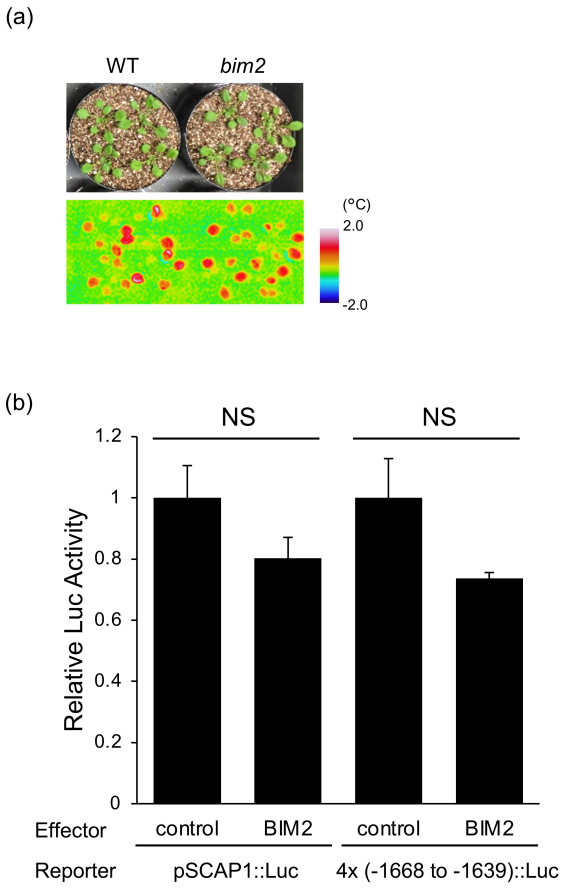


Figure 11. BIM2 is not involved in *SCAP1* expression

(a) Thermal subtractive images of the response to [CO_2] in wild-type (WT), *bim2* mutant. Plants were incubated at high [CO_2] (1000 ppm) for 60 min after incubation at low [CO_2] (0 ppm) for 90 min. The subtractive images show changes in leaf temperature in response to the transfer from low (0 ppm) to high (1000 ppm) [CO_2].

(b) Transactivation of *SCAP1* expression by BIM2 in protoplasts. The p*SCAP1*::*LUC* or 4x (-1668 to -1639)::*Luc* reporter plasmid and the 35S::BIM2 effector plasmid were co-transfected into protoplasts. 4x (-1668 to -1639) represents tandem repeats of the 30-bp sequence from -1668 to -1639 of *SCAP1* promoter described in Figure 3. An empty vector (pBI221) was introduced as a control. Firefly luciferase (Luc) activity was normalized by the activity of *Renilla* luciferase. Values shown are means ± S.E. (n = 3). NS represents no significance (Student's t test).

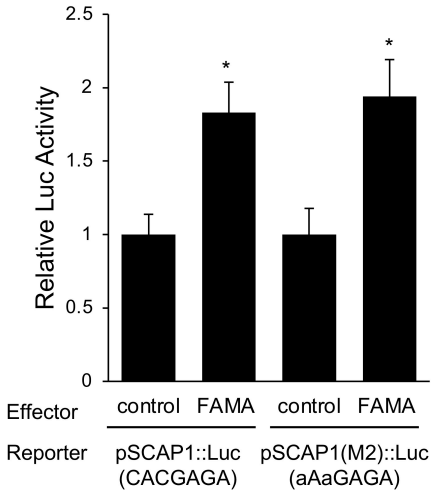


Figure 12. Transient luciferase assay

Transactivation of *SCAP1* expression by FAMA in GCPs. The p*SCAP1*::*LUC* or p*SCAP1*(M2)::*LUC* reporter plasmid and the 35S::FAMA effector plasmid were co-transfected into GCPs. p*SCAP1*(M2) represents mutated *SCAP1* promoter that has a M2 mutation (aAaGAGA, Figure 1). An empty vector (pBI221) was introduced as a control. Firefly luciferase (Luc) activity was normalized by the activity of *Renilla* luciferase. Values shown are means ± S.E. ($n = 4$). Asterisks indicate significant differences compared to the control ($P < 0.05$, Student's t test).

METHODS

Plasmid construction

All primers used for plasmid construction are listed in Table 3. For the construction of p*SCAP1*::*GUS* transgenes, fragments including the 5′ upstream region and 5′ UTR of *SCAP1* gene (1898-bp, 1668-bp, 1658-bp, 1648-bp, 1638-bp, 1618-bp, 1608-bp, 1588-bp, 1306-bp) were generated by PCR amplification from genomic DNA. For the construction of p*HT1*::*GUS*, p*SLAC1*::*GUS*, p*KAT1*::*GUS* transgenes, fragments including the 5′ upstream region and 5' UTR of *HT1* gene (1707 bp), *SLAC1* gene (1563 bp), or *KAT1* gene (2913 bp) were generated by PCR amplification from genomic DNA. The PCR fragments were inserted in front of the *NOS* terminator of pBI101, together with a fragment of the *GUS* (*uidA*) gene.

For M1-M4 base substitution constructs, forward primers incorporating corresponding base substitution were used. For M5-M9 base substitution constructs and internal deletion constructs, fragments including base substitution or internal deletion were prepared by recombinant PCR and inserted into the corresponding sites.

For the construction of synthetic promoters for gain-of-function analysis, fragments of part of the 5′ upstream region of *SCAP1* (-1668/-1307, -1898/-1669, -1588/-1307) were generated by PCR amplification from genomic DNA. Four tandem repeats of the part of 5’ upstream region of *SCAP1* (-1668/-1639, -1648/-1609, -1618/-1579) were synthesized by GenScript. A fragment of *35S* minimal promoter was produced by amplifying the sequence of the *35S* minimal promoter from -72 to -1 in a 4xNRE construct (Konishi and Yanagisawa, 2010). The fragments of 5′ upstream region of

SCAP1 and the *35S* minimal promoter were inserted in front of the *NOS* terminator of pBI101, together with a fragment of the *GUS* (*uidA*) gene.

For gain-of-function analysis using the *SLAH1* promoter, synthetic promoters of *SCAP1* (-1668/-1589) was generated by PCR amplification from genomic DNA. A fragment of *SLAH1* promoter (-1505/-1) was produced by amplifying the sequence of the *SLAH1* promoter from the pBI101-p*SLAH1*::*GUS* construct (Negi *et al.,* 2008). These PCR products were inserted in front of the *NOS* terminator of pBI101, together with a fragment of the *GUS* (*uidA*) gene.

For the construction of a binary vector for functional complementation of the *scap1* mutant or *ht1-2* mutant, a fragment of the sequence including the promoter, UTR and coding region of *SCAP1* or *HT1* was generated by PCR amplification from genomic DNA. The termination codon at the 3′ end of the fragments was deleted. The PCR fragments were inserted into pBI101, together with a fragment of *GFP*::*NOS* terminator from the plasmid CaMV35S-s*GFP*(S65T)::*NOS* terminator (Niwa *et al.,* 1999).

To construct reporter plasmids for the transient assay, native *SCAP1* promoter (1668-bp, Figure 1), mutated *SCAP1* promoter (M2, Figure 1) and tandem repeats of the 30-bp sequence from -1668 to -1639 of *SCAP1* promoter [4x (-1668 to -1639), Figure 3] were inserted into pUC18, together with a DNA fragment harboring the luciferase gene (LUC) and NOS terminator. Effector constructs, 35S::BIM2 and 35S::FAMA, were generated by inserting the full-length *BIM2* or *FAMA* cDNA into downstream of the cauliflower mosaic virus *35S* promoter in pBI221 (Clontech, Palo Alto, CA). A plasmid containing *Renilla reniformis* luciferase gene (Rluc) under the control of the *Arabidopsis* UBQ10 promoter (-1329 to +1) was also generated as an internal control plasmid.

Table 3. Oligonucleotide Primers Used in This Study.

Oligo Name	DNA sequence (5'-to-3')
SCAP1 promoter cloning	
pSCAP1_2k-F_Sal	CGCGTCGACATGTTGTAATATGCTACTTA
pSCAP1_1668-F-SalI	CGCGTCGACCTCACGAGAGATCCATTCCT
pSCAP1_1658-F_Sal	CGCGTCGACATCCATTCCTCGAGGGCTCT
pSCAP1_1648-F_Sal	CGCGTCGACGAGGGCTCTCACATGTTTC
pSCAP1_1638-F_Sal	CGCGTCGACACATGTTTCCCATTTTGTC
pSCAP1_1628-F_Sal	CGCGTCGACCATTTTGTCTTCTCCGTTC
pSCAP1_1618-F_Sal	CGCGTCGACTTCTCCGTTCTCTCGTGTTT
pSCAP1_1608-F_Sal	CGCGTCGACTCTCGTGTTTCTTCACAGAT
pSCAP1_1598-F_Sal	CGCGTCGACTTCACAGATGGAGACGTTT
pSCAP1-R-NcoI	CGCCATGGTGATGATGATGGGAGGAG
pSCAP1_1669-R_XbaI	GCTCTAGAACTAAACCGATCATCCACAA
pSCAP1_1589-R_XbaI	GCTCTAGAATCTGTGAAGAAACACGAGA
SCAP1 base substitution and internal deletion cloning	
pSCAP1_F_M1	CGCGTCGACAGCACGAGAGATCCATTCCT
pSCAP1_F_M2	CGCGTCGACCTAAAGAGAGATCCATTCCT
pSCAP1_F_M3	CGCGTCGACCTCACGCGCGATCCATTCCT
pSCAP1_F_M4	CGCGTCGACCTCACGAGATCTCCATTCCT
pSCAP1_F-Sal_internal(1648-1639)	CGCGTCGACCTCACGAGAGATCCATTCCTCACATGTTTC
pSCAP1_F_internal(1638-1629)	ATCCATTCCTCGAGGGCTCTCCATTTTGTC
pSCAP1_R_internal(1638-1629)	GAACGGAGAAGACAAAATGGAGAGCCCTCG
pSCAP1_F_internal(1628-1619)	CGAGGGCTCTCACATGTTTCTTCTCCGTTC
pSCAP1_R_internal(1628-1619)	AAACACGAGAGAACGGAGAAGAAACATGTG
pSCAP1_F_internal(1618-1609)	CACATGTTTCCCATTTTGTCTCTCGTGTTT
pSCAP1_R_internal(1618-1609)	ATCTGTGAAGAAACACGAGAGACAAAATGG
pSCAP1_F_M5	GGGCTCTAAAATGTTTCCCA
pSCAP1_R_M5	TGGGAAACATTTTAGAGCCC
pSCAP1_F_M6	TCTCACAGGGTTCCCATTTT
pSCAP1_R_M6	AAAATGGGAACCCTGTGAGA
pSCAP1_F_M7	CACATGTTGCACATTTTGTC
pSCAP1_R_M7	GACAAAATGTGCAACATGTG
pSCAP1_F_M8	TGTTTCCCCTGTTGTCTTCT
pSCAP1_R_M8	AGAAGACAACAGGGGAAACA
pSCAP1_F_M9	TCCCATTTGGGCTTCTCCGT
pSCAP1_R_M9	ACGGAGAAGCCCAAATGGGA
pSCAP1_F_SM1	CGCGTCGACCTAACGAGAGATCCATTCCT
pSCAP1_F_SM2	CGCGTCGACCTCCCGAGAGATCCATTCCT
pSCAP1_F_SM3	CGCGTCGACCTCACGAGCGATCCATTCCT
pSCAP1_F_SM4	CGCGTCGACCTCACGATAGATCCATTCCT
pSCAP1_F_SM5	CGAGGGCTCTAACATGTTTC
pSCAP1_R_SM5	GAAACATGTTAGAGCCCTCG
pSCAP1_F_SM6	GAGGGCTCTCCCATGTTTCC
pSCAP1_R_SM6	GGAAACATGGGAGAGCCCTC
pSCAP1_F_SM7	CACATGTTTACCATTTTGTC
pSCAP1_R_SM7	GACAAAATGGTAAACATGTG
pSCAP1_F_SM8	CACATGTTTCACATTTTGTC
pSCAP1_R_SM8	GACAAAATGTGAAACATGTG
pSCAP1_F_SM9	CACATGTTTCCAATTTTGTC
pSCAP1_R_SM9	GACAAAATTGGAAACATGTG
Gain of function promoter cloning	
pSCAP1_1307-R_XbaI	GCTCTAGAAAGTAACGTTTGCTTAACT
pSCAP1_1898-F_SalI	CGCGTCGAATGTTGTAATATGCTACTTA
pSCAP1_1669-R_XbaI	GCTCTAGAACTAAACCGATCATCCACAA
pSCAP1_1589-R_XbaI	GCTCTAGAATCTGTGAAGAAACACGAGA
min-F-XbaI	GCTCTAGATGACGCACAATCCCACTA
min-R_NcoI	CGCCATGGATCCGTCGACCTGCAG
pSLAH1-F-Sal	CGCGTCGACGCCGTGTGAAAAATGTAAC
pSLAH1-R-Nco	CGCCATGGCTTGGTTTATCGATGAAAAA
pSLAH1-F-Xba	GCTCTAGAGCCGTGTGAAAAATGTAAC
HT1 promoter cloning	
pHT1_F_Sal	CGCGTCGACTCGATGCAAACCAAACTCTC
pHT1_R_Nco	CGCCATGGTGAAACATAAACCAGACATA
pHT1_F_M2	AGCAATAAAGAGAAACCTT
pHT1_R_M2	AAGGTTTCTCTTTATTGCT
SLAC1 promoter cloning	
pSLAC1_F_Sal	CGCGTCGACACAGCCGTGAATCTCAGCAG
pSLAC1_R_Nco	CGCCATGGTGATCAGAGCAATTTGATAA
pSLAC1_F_M2	AGCTTTAAAGAGAAACCTA
pSLAC1_R_M2	TAGGTTTCTCTTTAAAGCT
KAT1 promoter cloning	
pKAT1_F_Sal	CGCGTCGACTCCTTACGATTTTGACCCTA
pKAT1_R_Nco	CGCCATGGCTTTTTGATGATCTCTA
pKAT1_F_M2	ACTAATTAATAAAGAGAAAAT
pKAT1_R_M2	ATTTTCTCTTTATTAATTAGT
Complementation analysis sequence cloning	
pHT1-F-Sal	CGCGTCGACTCGATGCAAACCAAACTCTC
HT1-R-Nco_Glycine linker	CGCCATGGCTCCACCTCCACCGGCATTTACAGGAACAGAGG
BIM2 cloning	
BIM2-F-BamHI	CGGATCCATGAGAACCGGAAAAGGAAA
BIM2-R	TCACAGTGTTTTCATCCGCT
FAMA cloning	
FAMA-F-Xba	GCTCTAGAATGGATAAAGATTACTCGGC
FAMA-R	TCAAGTAAACACAATATTTC

Plant Materials and Growth Conditions

The *Arabidopsis* ecotype Columbia (Col) (as wild-type), the *scap1* mutant (Negi *et al.,* 2013), and *ht1-2* mutant (Hashimoto *et al.,* 2006) were used in this study. The mutant line *bim2* (SALK_074689C) was obtained from the Arabidopsis Biological Resource Center. Transgenic plants were generated by Agrobacterium-mediated transformation as previously described (Clough and Bent, 1998). Plants were grown on solid Murashige-Skoog (MS) medium for 19 days in a growth chamber (constant white light of 35 μmol m^{-2} s^{-1} at 22°C, 60% RH), and then transplanted into vermiculite pots supplemented with mineral nutrients.

GUS staining assay

Two-week-old promoter-GUS transformants grown on MS medium were incubated in a staining buffer that contained 100 mM sodium phosphate (pH 7.0), 10 mM EDTA, 0.5 mM K4Fe(CN)6, 0.5 mM K3Fe(CN)6, 0.1% Triton X-100, and 1 mM 5-bromo-4-chloro-3-indolyl β-D-glucuronic acid.

Quantitative GUS activity assay

A mature leaf of two-week-old promoter-GUS transformants was lysed in 100 μl extraction buffer that contained 50 mM sodium phosphate (pH7.4), 10 mM EDTA, 0.1% Triton X-100, 0.1% N-lauroylsarcosine sodium salt, and 10 mM 2-mercaptoethanol. Following centrifugation of the crude extract, the supernatant (80 μl) was incubated in 160 μl of assay buffer that contained 0.5 mM 4-methylumbelliferyl-β-D-glucuronide. After incubation, 5 μl of reaction liquid was removed and the reaction was stopped with 200 μl of 0.2 M Na_2CO_3. Fluorescence of the 4-methylumbelliferone

product was quantified with a plate reader (STRATAGENE Mx3000P, Agilent Technologies, Santa Clara, CA, USA). Protein concentrations were measured with the protein-dye-binding assay (Bradford, 1976) using bovine serum albumin as standard.

Thermal Imaging

Plants grown in a growth chamber were transferred to a growth cabinet (constant white light of 80 μmol m^{-2} s^{-1} at 22°C, 60% RH) equipped with an automatic CO_2 control unit (FR-SP, Koito Co. Ltd., Tokyo, Japan). After 90 min of adaptation to low [CO_2] (0 ppm), thermal images were captured under high [CO_2] (1000 ppm) using a thermography apparatus (TVS-8500, NEC/Avio Infrared Technologies, Tokyo, Japan).

Measurement of stomatal conductance

The stomatal conductance of whole plants response to CO_2 was measured using a portable gas-exchange fluorescence system (GFS-3000, Heinz Walz, Effeltrich, Germany) equipped with a 3010-A *Arabidopsis* chamber. CO_2 concentration was increased from 0 to 700 ppm at a constant light intensity (150 μmol m^{-2} s^{-1}) and 60% RH. The flow rate in the system was kept constant (750 μmol s^{-1}) throughout the gas exchange experiments. Measurements were recorded every minute.

Gene Set Enrichment Analysis (GSEA)

We selected 3893 genes with 5′-CACGAGA-3′as the gene set and carried out the Gene Set Enrichment Analysis (GSEA) (www.broadinstitute.org/gsea; Subramanian *et al*., 2005) for the guard cell protoplasts and mesophyll cell protoplasts expression data

(Negi *et al*., 2013) by setting the significant P value at less than 0.05 and false discovery rate at less than 0.25.

Statistical analysis using RNA-seq data

The ratio of genes with 5′-CACGAGA-3′ in the genes that were expressed dominantly in different stages of stomatal development was investigated using epidermal and stomatal lineage cell-specific expression profiles, in which genes were clustered based on RNA-seq data (Supplementary Material 2 from Adrian *et al*., 2015). The statistical analysis for tissue-specific enrichment of the genes that have the *cis*-element was carried out with Fisher's exact test.

Isolation of guard cell protoplasts (GCPs)

Guard cell protoplasts were isolated from 4- to 5-week-old *Arabidopsis* plants as described previously (Negi *et al*. 2013; Yamamoto *et al*. 2016).

Transient Luciferase assay

Effector and reporter plasmids and an internal control plasmid were co-transfected into GCPs according to the polyethylene glycol (PEG)-calcium transfection protocol (Yoo *et al*., 2007), and transfected protoplasts were incubated at 22 °C in the dark for 18 h. Then, Luc and Rluc activity in each protoplast sample was measured using the Dual-luciferase reporter assay system (Promega, Madison, USA) and Lumino (Stratec, Berlin, Germany). Luc activity was normalized using Rluc activity, and the relative Luc activity was shown as the activity obtained with the empty vector (pBI221) set to 1.

Four independent transfection assays were performed to determine the mean and S.E. for luciferase expression.

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