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Opening a New Era of ABA Research

Mg-chelatase H subunit affects ABA signaling in stomatal guard cells, but is not an ABA receptor in *Arabidopsis thaliana*

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Abstract Mg-chelatase H subunit (CHLH) is a multifunctional protein involved in chlorophyll synthesis, plastid-to-nucleus retrograde signaling, and ABA perception. However, whether CHLH acts as an actual ABA receptor remains controversial. Here we present evidence that CHLH affects ABA signaling in stomatal guard cells but is not itself an ABA receptor. We screened ethyl methanesulfonate-treated *Arabidopsis thaliana* plants with a focus on stomatal aperture-dependent water loss in detached leaves and isolated a *rapid transpiration in detached leaves 1* (*rtl1*) mutant that we identified as a novel missense mutant of CHLH. The *rtl1* and *CHLH* RNAi plants showed phenotypes in which stomatal movements were insensitive to ABA, while the *rtl1* phenotype showed normal sensitivity to ABA with respect to seed germination and root growth. ABA-binding analyses using ^{3}H-labeled ABA revealed that recombinant CHLH did not bind ABA, but recombinant pyrabactin resistance 1, a reliable ABA receptor used as a control, showed specific binding. Moreover, we found that the *rtl1* mutant showed ABA-induced stomatal closure when a high concentration of extracellular Ca^{2+} was present and that a knockout mutant of Mg-chelatase I subunit (*chli1*) showed the same ABA-insensitive phenotype as *rtl1*. These results suggest that the Mg-chelatase complex as a whole affects the ABA-signaling pathway for stomatal movements.

Keywords ABA · Ca^{2+} · Mg-chelatase H subunit · Receptor · Signal transduction · Stomatal guard cell

Introduction

In higher plants, the stomata, surrounded by pairs of guard cells, are the pores in the plant epidermis that regulate gas exchange between the leaves and the atmosphere. Opening of the stomata allows both transpiration and CO_2 entry for photosynthesis. Under drought stress, the phytohormone abscisic acid (ABA) induces stomatal closure to prevent water loss (Schroeder et al. 2001; Shimazaki et al. 2007). ABA-induced stomatal closure is driven by an efflux of K^+ from the guard cells through voltage-dependent outward-rectifying K^+ channels in the plasma membranes. Activation of the K^+ channels requires depolarization of the plasma membrane, and this depolarization is mainly achieved by the activation of anion channels within the plasma membrane (Schroeder et al. 1987; Kim et al. 2010).

Recently, the family of proteins containing pyrabactin resistance (PYR), pyrabactin resistance 1-like (PYL), and regulatory component of ABA receptor (RCAR) has been identified as a reliable family of ABA receptors, and ABA recognition by the PYR/PYL/RCAR family of proteins activates the SnRK2 family of protein kinases through

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inactivation of their central negative regulators, the type 2C protein phosphatases (PP2Cs) (Ma et al. 2009; Park et al. 2009; Santiago et al. 2009; Cutler et al. 2010). The *pyr1 pyl1 pyl2 pyl4* quadruple mutant exhibited a phenotype with strong ABA-insensitive seed germination, root growth, gene expression (Park et al. 2009), and stomatal opening and closing responses (Nishimura et al. 2010), indicating a functional redundancy within the PYR/PYL/RCAR family proteins. More recently, SLAC1, which is thought to be a slow-type anion channel (Negi et al. 2008; Vahisalu et al. 2008), was shown to undergo phosphorylation via an SnRK2 family protein kinase and induce depolarization of the plasma membrane (Geiger et al. 2009; Lee et al. 2009). In addition to PYR/PYL/RCAR family proteins, several candidate ABA receptors have been reported, including the Mg-chelatase H subunit (CHLH) (Shen et al. 2006; Wu et al. 2009), G-protein coupled receptor 2 (GCR2) (Liu et al. 2007), and G-protein coupled receptor-type G proteins (GTG1 and GTG2) (Pandey et al. 2009). It should be noted that CHLH and GCR2, the ABA receptor candidates, have been controversially debated (McCourt and Creelman 2008; Cutler et al. 2010).

CHLH is one of the three subunits of Mg-chelatase (D, H, and I subunits). Mg-chelatase complex is involved in the biosynthetic pathway of chlorophyll, catalyzing the insertion of Mg^{2+} into protoporphyrin IX to form Mg-protoporphyrin IX (Gibson et al. 1995; Willows et al. 1996; Huang and Li 2009). CHLH has also been reported as genomes uncoupled 5 (GUN5), a regulator of plastid-to-nucleus retrograde signaling (Mochizuki et al. 2001). Furthermore, CHLH was identified as an ABA-specific binding protein in *Vicia faba* (Zhang et al. 2002). Subsequent extensive genetic and biochemical analyses using Arabidopsis suggested that CHLH specifically binds ABA and mediates ABA-signaling pathways involved in seed germination, root elongation, gene expression, and stomatal closure (Shen et al. 2006; Wu et al. 2009). More recently, knockout of a group of WRKY transcription factors (*WRKY40*, *WRKY18*, and *WRKY60*) in *cch* mutant has shown to rescue ABA-insensitive phenotypes of *cch*, including stomatal movements, seed germination and post-germination growth, suggesting that these WRKY transcription factors function as negative regulators of ABA signaling (Shang et al. 2010). The expression of *CHLH* is also suppressed by a key component of the circadian clock, TOC1, which interacts with the *CHLH* promoter; overexpression of TOC1 was shown to give rise to a phenotype with stomatal guard cells that were insensitive to ABA, as did also RNAi-mediated knockdown of *CHLH* (Legnaioli et al. 2009). In contrast, Müller and Hansson (2009) reported that recombinant Xan-F, an ortholog of CHLH in barley, did not bind ABA, and that *xan-f* loss-of-function mutants showed normal ABA responsiveness. Thus, whether CHLH functions as an ABA receptor remains controversial. Further investigations are required to elucidate the role of CHLH in ABA signaling.

Several secondary messengers regulate ABA signaling in stomatal guard cells, including Ca^{2+}, reactive oxygen species, nitric oxide, phosphatidic acid, inositol derivatives, and sphingolipids (Kim et al. 2010). Of these, involvement of Ca^{2+} in ABA signaling has been well established. Cytosolic Ca^{2+} elevation and/or oscillation play an important role in ABA-induced stomatal closure (Allen et al. 1999, 2000, 2001; Islam et al. 2010). Treatment of the epidermis with Ca^{2+}-chelating agents such as EGTA suppresses ABA-induced stomatal closure (Hwang and Lee 2001). These results suggest that Ca^{2+} acts as a signal mediator in ABA-induced stomatal closure. Moreover, the sensitivity of stomatal closing in response to elevations in the cytosolic Ca^{2+} concentration has been suggested to be enhanced (primed) by ABA (Young et al. 2006). A Ca^{2+}-independent pathway in the ABA signaling of stomatal guard cells has also been reported (Allan et al. 1994; Marten et al. 2007; Siegel et al. 2009).

In the present study, we performed a screen focused on stomatal aperture-dependent transpiration in detached leaves from *Arabidopsis thaliana* that had been treated with ethyl methanesulfonate (EMS) to induce mutations. Consequently, we isolated a *rapid transpiration in detached leaves 1* (*rtl1*) mutant in which the stomatal movements were insensitive to ABA, and which we identified as a novel missense mutant of the Mg-chelatase H subunit (CHLH). Phenotypic and ABA-binding analyses suggested that CHLH affects ABA signaling in stomatal movements but is not itself an ABA receptor. We propose a novel hypothesis regarding the role of CHLH in ABA signaling of stomatal guard cells.

Materials and methods

Plant materials and growth conditions

Arabidopsis thaliana gl1 [Columbia (Col), carrying homozygous recessive *gl1*], used here as the wild type (WT), is the background ecotype of an *rtl1* mutant. *rtl1* was backcrossed with *gl1* three times. Col is the background ecotype of a *cch* mutant (Mochizuki et al. 2001) and a T-DNA insertion mutant of *CHLI1* (*chli1*; SAIL_230_D11). The transgenic line pOCA107-2 is the genetic background of a *gun5-1* mutant (Mochizuki et al. 2001). The *chli1* mutant was obtained from the Arabidopsis Biological Resource Center (Ohio State University, Columbus, OH, USA). Plants were grown in soil under 16-h fluorescent light (50 μmol m^{-2} s^{-1})/8-h dark cycle at 24°C in 55–70% humidity in a growth room.

To obtain the homozygous mutant line of *chli1*, the plant was identified by PCR using T-DNA left-border primer LB1 (5′-GCCTTTTCAGAAATGGATAAATAGCCTTG CTTCC-3′) and *CHLI1* gene-specific primer (5′-GGAAT CCAAATAAGGCCAAAG-3′).

Isolation of the *rtl1* mutant and identification of the *RTL1* locus

EMS-treated *gl1* M_2 seeds, purchased from Lehle Seeds (Round Rock, TX, USA), were germinated and grown under the same conditions as above. For the screening of leaf transpiration mutants, the fresh weight of a detached rosette leaf was measured at 0 and 90 min after detachment from each 4-week-old M_2 plant. Individuals that showed a rapid or slow weight change compared to WT plants were selected as candidates for rapid-transpiration or slow-transpiration mutants, respectively. To determine stomatal phenotypes, we measured stomatal apertures in the isolated epidermis of 4-week-old candidate plants under several conditions using a microscope. With this screening strategy, we successfully isolated three *rapid transpiration in detached leaves* (*rtl*) mutants and two *slow transpiration in detached leaves* (*stl*) mutants.

To generate mapping populations, the *rtl1* mutant was crossed with the Landsberg erecta (L*er*) accession of *A. thaliana*. The *rtl1* DNA was isolated from 143 F_2 plants that showed a pale-green phenotype. DNA was isolated from individual mutant plants and mapping was performed using simple-sequence length polymorphism (SSLP) markers.

Vector construction for plant transformation

A genomic DNA fragment containing the *CHLH* gene, including its promoter region (from −2,814 to 5,748 bp of the *CHLH* locus; g*CHLH*), from WT plants was amplified by PCR using the specific primer set 5′-CAGCAGCCAC GAGTCCTGATACAGCTCG-3′ and 5′-GTCTCGTGTC ACGGCTACTGCAGATGAAGATG-3′. The amplified 8,562-bp DNA was cloned into the Gateway entry vector pCR8/GW/TOPO (Invitrogen, Carlsbad, CA, USA) and recombined by the LR reaction into the binary vector pGWB1 (Nakagawa et al. 2007). The resulting pGWB1-g*CHLH* vector was used to transform *rtl1* plants for the complementation test. RNA interference (RNAi) lines with downregulated *CHLH* were generated as previously reported (Shen et al. 2006), with some modifications. A gene-specific 653-bp fragment, which was located 2,363–3,015 bp downstream from the start codon, was amplified by PCR using the primer pair 5′-ACAGA GATTCTGTGGTTGGGAAAG-3′ and 5′-GGCACTTGC CATTGCTGCTG-3′. The PCR product was introduced into pCR8/GW/TOPO and transferred into the binary vector pYU501 (Ueno et al. 2007). The resulting pYU501-*CHLH* RNAi was then used for transformation of WT plants. Transformation was performed using the GV3101 strain of *Agrobacterium tumefaciens* and the floral dip method (Clough and Bent 1998).

Measurement of stomatal aperture

Stomatal apertures were measured according to Inoue et al. (2008) with some modifications. The epidermal tissues isolated from dark-adapted 4- to 6-week-old plants were incubated in basal buffer (5 mM MES-BTP, pH 6.5, 50 mM KCl, and 0.1 mM $CaCl_2$). For inhibition of light-induced stomatal opening by ABA, the epidermal tissues were incubated under blue/red light [blue light (Stick-B-32; EYELA, Tokyo, Japan) at 10 μmol m^{-2} s^{-1} superimposed on background red light (LED-R; EYELA) at 50 μmol m^{-2} s^{-1}] for 2.5 h at 24°C in the presence or absence of 20 μM ABA. For the ABA-induced stomatal closure, the pre-illuminated epidermal tissues were incubated under blue/red light for 2.5 h with or without 20 μM ABA. Stomatal apertures in the abaxial epidermis were measured microscopically. Stomatal apertures are presented as the mean of 25 stomata with standard deviation (SD). Results were confirmed by blind reassessment by another researcher.

RT-PCR

The total RNA was extracted from seedlings or leaves of 4- to 6-week-old plants using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. First-strand cDNA was synthesized using the Takara PrimeScript II 1st Strand cDNA Synthesis Kit (Takara, Tokyo, Japan) and used as a template. A 741-bp fragment of *CHLH* cDNA was amplified using the primer pair 5′-GTGTGAGACCAATTGCTGATAC-3′ and 5′-ACTCCATCCCACAGTGTTGG-3′. A 952-bp fragment of *CHLI1* cDNA was amplified using the primer pair 5′-GGAATCCAAATAAGGCCAAAG-3′ and 5′-ACC CATCAACATTGAGCTCTG-3′. *TUB2* (At5g62690), used as a control, was amplified using the primer pair 5′-CAT TGTTGATCTCTAAGATCCGTG-3′ and 5′-TACTGCTG AGAACCTCTTGAG-3′.

Immunoblot

Immunoblot analysis was performed according to Hayashi et al. (2010) with modification. Seedlings or leaves from 4- to 6-week-old plants were ground in extraction buffer (50 mM MOPS–KOH, pH 7.5, 2.5 mM EDTA, 100 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 20 μM

leupeptin, and 2 mM DTT) using a mortar and pestle. Fifty micrograms of protein was loaded and separated by SDS-polyacrylamide gel electrophoresis. To detect CHLH, the polyclonal antibody raised in rabbits against recombinant CHLH protein was used for immunoblot analysis. The 14-3-3 proteins were detected with the anti-14-3-3 protein (GF14ø) antibody (Kinoshita and Shimazaki 1999) as a control. The anti-CHLH and anti-14-3-3 protein antibodies were used at a 3,000-fold dilution.

Measurement of chlorophyll contents

The chlorophyll content of rosette leaves from 4-week-old plants was determined as previously described (Moran 1982).

Preparation of recombinant proteins

The coding sequences of *CHLH* (from 145 to 4,146 bp; At5g13630) and full-length *PYR1* (At4g17870) and *ABI1* (At4g26080) were amplified by PCR using cDNA prepared from WT plants. The primers used were the following: *CHLH* (5′-TCTGCTGTATCTGGAAACGGC-3′ and 5′-TTATCGATCGATCCCTTCGATCTTG-3′), *PYR1* (5′-ATGCCTTCGGAGTTAACACC-3′ and 5′-TCACGTCACCTGAGAACCAC-3′), *ABI1* (5′-ATGGAGGAAGTATCTCCGGC-3′ and 5′-TCAGTTCAAGGGTTTGCTCTTGAG-3′). The PCR products were initially cloned into pCR8/GW/TOPO and transferred into a pDEST17 destination vector containing a 6× His epitope-tag (Invitrogen) or into pDEST15 destination vector containing GST-tag (Invitrogen) by the LR reaction. Each construct was transformed into *E. coli* BL21 strains, and protein expression was induced by the addition of 0.1 mM isopropyl thiogalactoside, with overnight incubation at 30°C. Purification of recombinant His-tagged CHLH and PYR1 proteins was carried out using the His Bind Kit (Novagen, Madison, WI, USA) and Ni–NTA agarose (Qiagen). Purification of recombinant His-tagged ABI1 protein was performed by the same method as CHLH or PYR1, except that all buffers contained 5 mM $MgCl_2$ (Melcher et al. 2009). The purified CHLH protein was used for the ABA-binding assay and as an antigen for preparing the anti-CHLH antibody. The recombinant GST-CHLH and GST-PYR1 proteins were purified with glutathione Sepharose 4B beads (GE Healthcare, Uppsala, Sweden). Protein concentrations were determined with the Bio-Rad protein assay kit using bovine serum albumin as a standard.

ABA-binding assay

ABA binding to purified *E. coli*-expressed recombinant CHLH protein was assayed using ^{3}H-labeled (±)-ABA (370 MBq μmol^{-1}; American Radiolabeled Chemicals, Inc., St. Louis, MO, USA) by both the filter and pull-down methods.

For the filter method (Melcher et al. 2009; Wu et al. 2009), 2 μM purified His-tagged CHLH, PYR1, and ABI1 proteins and 50 nM ^{3}H-labeled ABA were incubated in 0.2 mL binding buffer (10 mM Tris-Mes, pH 7.0, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM DTT, and 250 mM mannitol) with or without 1,000-fold unlabeled ABA (no. A1049; Sigma, St. Louis, MO, USA) for 1 h at 25°C. The ^{3}H-labeled ABA-bound protein was separated from free ^{3}H-labeled ABA by filtering using GF/F glass fiber filter (Whatman, Little Chalfont, Buckinghamshire, UK) and washed with 5 mL ice-cold binding buffer three times. Then, the ^{3}H-labeled ABA remaining on the filter was quantified using a liquid scintillation counter (LSC-5100; Aloka, Tokyo, Japan). PYR1 with ABI1 was used as a control of ABA binding.

For the pull-down method (Melcher et al. 2009), GST-CHLH (30 μg) or GST-PYR1 (4.3 μg) protein-bound glutathione Sepharose 4B beads and 50 nM ^{3}H-labeled ABA were incubated in 0.2 mL binding buffer with or without 1,000-fold unlabeled ABA for 1 h at 25°C. For the binding assay of GST-PYR1, the reaction mixture were supplemented with the purified His-ABI1 (9.5 μg). Then, the beads were washed three times with the binding buffer and the radioactivity of the bound ^{3}H-labeled ABA was measured using a liquid scintillation counter.

Seed germination and root growth assays

Seed germination and root growth assays were performed as previously reported (Shen et al. 2006). For the seed germination test, approximately 100 sterilized seeds were planted on a plate containing Murashige and Skoog inorganic salts (MS medium, pH 5.9), 3% sucrose, and 0.8% agar in the presence of 0 and 3 μM ABA. The plate was kept at 4°C for 3 days, then incubated at 24°C under fluorescent light (50 μmol m^{-2} s^{-1}). The number of germinated seeds was determined at the indicated times after start of incubation. For the root growth assay, 4-day-old seedlings grown on a MS plate under fluorescent light were transferred to MS medium containing 0, 5, 10, or 20 μM ABA. After 7 days, the root length was measured.

Results

An *rtl1* mutant phenotype had stomatal guard cells that were insensitive to ABA

To elucidate the signaling mechanisms of stomatal opening and closing, we performed a screen focused on stomatal

aperture-dependent water loss via transpiration in detached leaves using a microbalance to weigh the leaves. We selected mutants showing rapid or slow weight changes compared to the WT in detached leaves from 12,338 M_2 plants treated with EMS and successfully isolated three rapid-transpiration mutants, which we designated as *rtl*, and two slow-transpiration mutants, which we designated as *stl*. Of these, *rtl1* showed rapid weight changes in detached leaves under the growth conditions. The average weight of the detached rosette leaves from WT plants decreased to 65% of their initial weight over the course of 90 min, whereas the weight of the *rtl1* leaves decreased to 35% of their initial weight (Fig. 1a). In addition, the *rtl1* mutant was recessive and showed semi-dwarf and pale green phenotypes (Fig. 1b). The chlorophyll content of the *rtl1* mutant was approximately 35% that of the WT (Fig. 2d).

We then analyzed the stomatal responses of *rtl1* in more detail. The WT stomata closed in darkness and opened when exposed to light, and this was inhibited by 20 μM ABA. Notably, the stomata of *rtl1* plants opened moderately in darkness, and light-induced stomatal opening was not inhibited by ABA (Fig. 1c). Moreover, the *rtl1* plants did not show ABA-induced stomatal closure (Fig. 1d). Thus, we suspected that rapid transpiration phenotype of *rtl1* is likely due to insensitivity to ABA after detachment of rosette leaves since stomatal aperture under light condition in *rtl1* is almost same with that in wild type (Fig. 1c, d). In addition, seed germination and root growth showed normal ABA sensitivities in the *rtl1* plants (Fig. S1). These results indicate that the ABA-insensitive phenotype of *rtl1* is specific to stomatal movements.

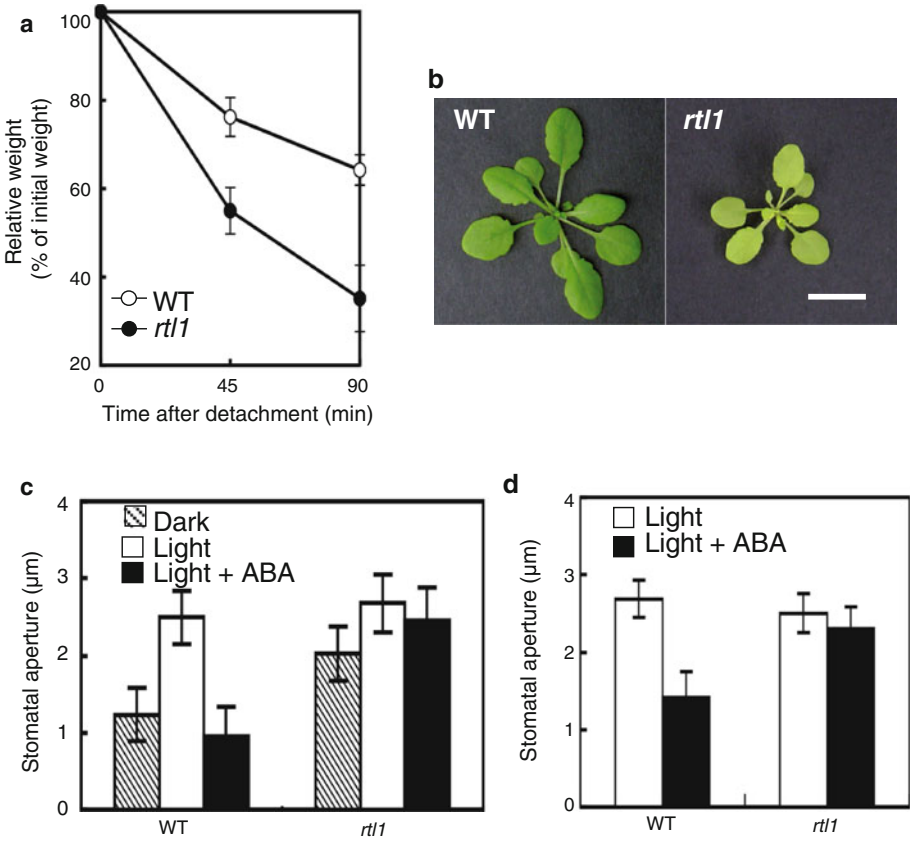

Fig. 1 Characterization of the *rtl1* mutant. **a** Kinetics of the fresh weight change in the detached rosette leaves from 4-week-old wild-type (WT) (*open circles*) and *rtl1* (*closed circles*) plants. The relative weights of leaves are presented as a percentage of the initial weight, which was the weight of each rosette leaf immediately after detachment from the plants, with the standard deviation (SD). **b** Four-week-old WT and *rtl1* plants. *Bar* 10 mm. **c** Effect of ABA on light-induced stomatal opening in WT and *rtl1* plants. Epidermal tissues from dark-adapted plants (*shaded*) were incubated under light (blue light at 10 μmol m^{-2} s^{-1} superimposed on background red light at 50 μmol m^{-2} s^{-1}) for 2.5 h with (*black*) or without (*white*) 20 μM ABA. Data represent the mean with SD. **d** ABA-induced stomatal closing in WT and *rtl1* plants. Pre-illuminated epidermal tissues were incubated under light (same as above) for 2.5 h with (*black*) or without (*white*) 20 μM ABA. Data represent the mean with SD. All experiments were repeated three times on different occasions with similar results

A missense mutation of CHLH was responsible for the ABA-insensitive *rtl1* phenotype

To identify the *RTL1* locus, we performed a map-based analysis, which revealed that *rtl1* showed a strong linkage to the SSLP marker nga151 on chromosome 5 (Fig. 2a). Our search of The Arabidopsis Information Resource (TAIR) database revealed Mg-chelatase H subunit (*CHLH*, At5g13630) as a candidate gene because the *CHLH* locus is very close to nga151, and a known CHLH missense mutant, *cch* (P642 to L), is pale green and ABA-insensitive (Mochizuki et al. 2001; Shen et al. 2006). We then sequenced both genomic *CHLH* DNA and *CHLH* cDNA from *rtl1* and found a single nucleotide substitution from C2068 to T, which induced a novel missense mutation from L690 to F (Fig. 2b). Transformation of the WT genomic *CHLH* gene with its own promoter into *rtl1* (*gCHLH/rtl1*) complemented all phenotypes that had been lost in *rtl1*. That is, *gCHLH/rtl1* plants showed normal plant growth and chlorophyll content compared to the WT (Fig. 2c, d). Furthermore, ABA sensitivity of the stomata was restored in the *gCHLH/rtl1* plants (Fig. 2e). These results indicate that the missense mutation of CHLH (L690 to F) was responsible for the *rtl1* phenotype with ABA-insensitive stomatal movements. Also, the level of *CHLH* transcript in rosette

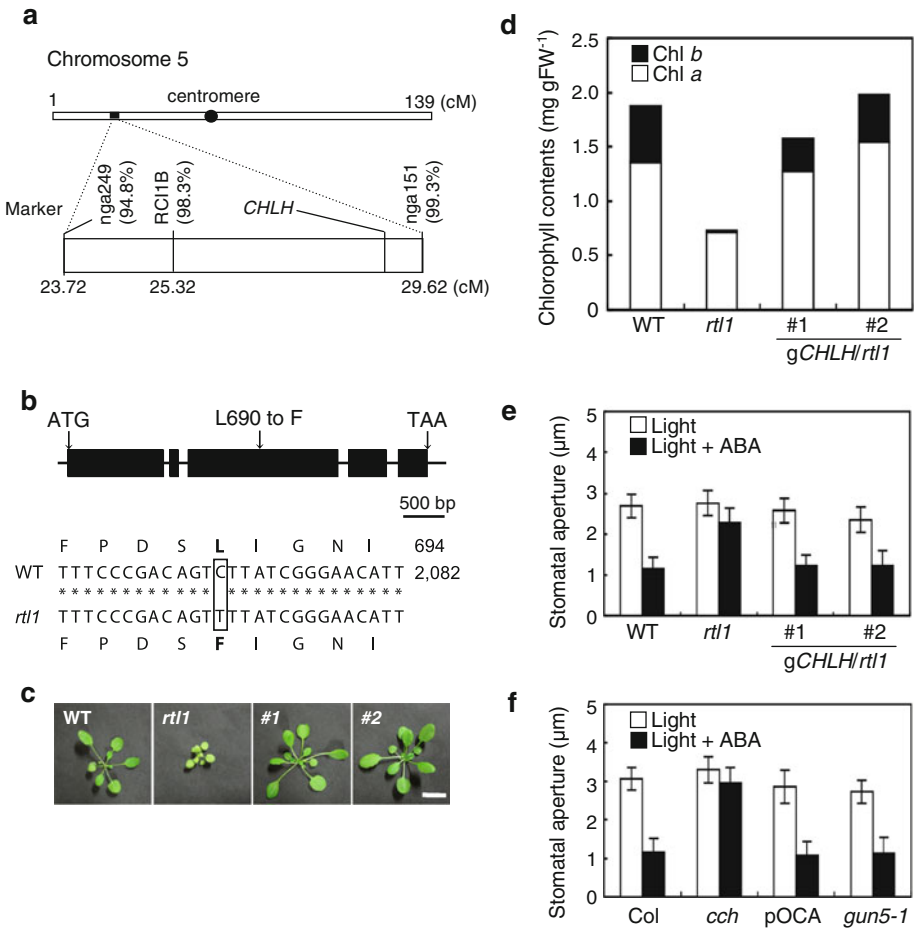

Fig. 2 CHLH missense mutation is responsible for the *rtl1* phenotype. **a** Mapping analysis of the *RTL1* locus. The *RTL1* locus was close to SSLP marker nga151 and *Mg-chelatase H subunit* (*CHLH*). **b** Schematic representation of the structure of the *CHLH* gene (*upper*). *Black boxes* and *lines* represent exons and introns, respectively. The position of the amino acid substitution (L690 to F) in *rtl1* is indicated. The partial sequences of *CHLH* cDNA and the deduced amino acid in wild-type (WT) and *rtl1* are shown (*lower*). A single nucleotide substitution (C2068 to T) is shown by a *box*. The amino acid and nucleotide numbers are indicated on the right. **c** Typical phenotypes in the WT, *rtl1*, and two independent *gCHLH/rtl1* complementation lines (#1 and #2). Plants were grown on soil for 4 weeks. *Bar* 10 mm. **d** Chlorophyll contents in rosette leaves of 4-week-old WT, *rtl1*, and *gCHLH/rtl1* lines (#1 and #2). The *bars* show the contents of chl *a* (*white*) and chl *b* (*black*). **e** ABA-induced stomatal closing in WT, *rtl1*, and *gCHLH/rtl1* lines (#1 and #2). Conditions were the same as shown in Fig. 1d. Data represent the mean with SD. **f** ABA-induced stomatal closing in the known *chlh* mutants, *cch* and *gun5*. Col and pOCA are the background plants of the *cch* and *gun5-1* mutation, respectively. Conditions were the same as shown in Fig. 1d. Data represent the mean with SD. All experiments were repeated three times on different occasions with similar results

leaves of *rtl1* plants was almost identical to that of the WT, whereas the amount of CHLH protein in rosette leaves of *rtl1* plants was approximately three times higher than that of the WT (Fig. 3a, b).

To confirm these results, we prepared *CHLH* knock-down plants. Because T-DNA insertion mutants in the *CHLH* gene are lethal (Shen et al. 2006), we instead prepared *CHLH* RNAi plants, which exhibited lower amounts of the *CHLH* transcript and CHLH protein (Fig. 3a, b). The *CHLH* RNAi plants exhibited a semi-dwarf and pale-green phenotype and did not show ABA-induced stomatal closure (Fig. 3c–e). The CHLH missense mutant, *cch* (P642 to L), also demonstrated ABA insensitivity, but another CHLH missense mutant, *gun5-1* (A990 to V), showed normal ABA-induced stomatal closure (Fig. 2f), consistent with a previous report (Shen et al. 2006). The ABA sensitivities of seed germination and root growth were normal in both *cch* and *rtl1* plants under our growth conditions (Fig. S1). Taken together, these results suggest that CHLH plays a role in the ABA-signaling pathway involved in stomatal movements.

Recombinant CHLH did not bind ABA

Assays of ABA binding to recombinant CHLH have implicated CHLH as an ABA receptor in Arabidopsis (Shen et al. 2006; Wu et al. 2009). On the other hand, Müller and Hansson (2009) showed that recombinant XanF, an ortholog of CHLH in barley, did not bind ABA. To clarify whether CHLH acts as an ABA receptor, we examined the ABA-binding ability of recombinant CHLH protein using ^{3}H-labeled ABA, according to the previous conditions (Melcher et al. 2009; Wu et al. 2009). Figure 4a shows the purified recombinant CHLH (49–1,381 a.a.) truncated *N*-terminal plastid-transit peptide used for the ABA-binding assay. Figure 4b shows the purified reliable ABA receptor PYR1 with ABI1 as a positive control. Using the filter method, no specific ABA binding to the recombinant CHLH was detected (Fig. 4c). In contrast, PYR1 in the presence of ABI1 did bind ^{3}H-labeled ABA, and this binding was competitively inhibited by unlabeled ABA. To confirm these results, we performed the ABA-binding assay using the pull-down method. GST-CHLH did

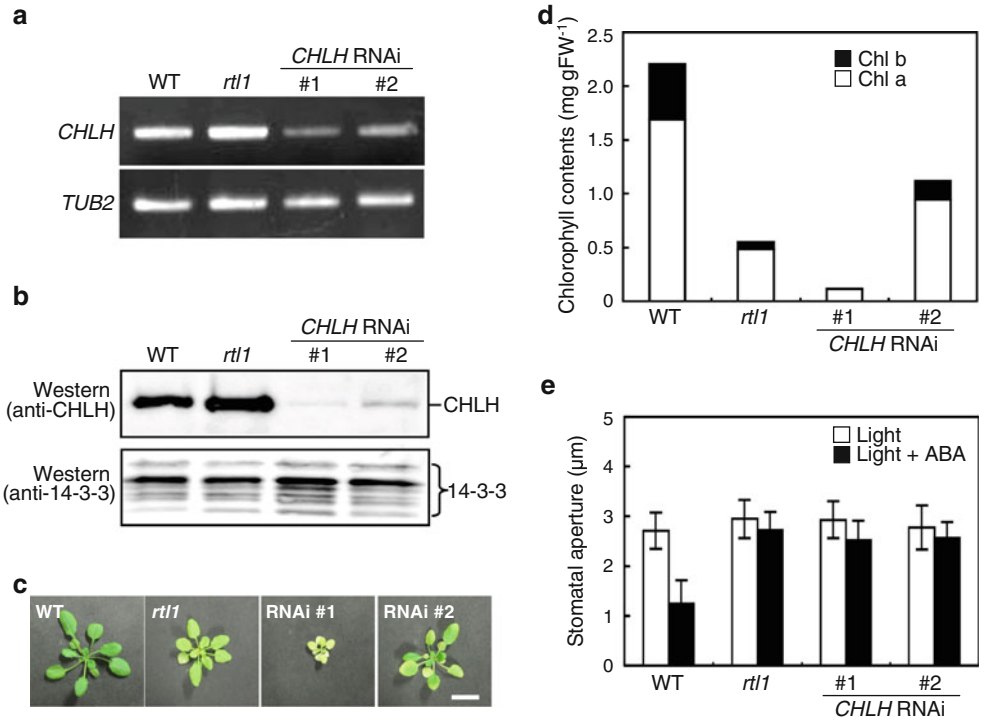

Fig. 3 Phenotypes in wild-type (WT), *rtl1*, and *CHLH* RNAi lines. **a** RT-PCR analysis of CHLH in WT, *rtl1*, and two *CHLH* RNAi lines (#1 and #2). Total RNA was isolated from rosette leaves from 4-week-old plants. PCRs were performed with 26 cycles. *TUB2* was amplified as a control. **b** Immunoblot analysis of CHLH protein in WT, *rtl1*, and *CHLH* RNAi lines. Fifty micrograms of protein extracted from rosette leaves was loaded on each *lane*. The 14-3-3 proteins were detected using anti-14-3-3 antibody as a control. **c** Four-week-old WT, *rtl1*, and *CHLH* RNAi plants. *Bar* 10 mm. **d** Chlorophyll content of rosette leaves from 4-week-old WT, *rtl1*, and *CHLH* RNAi plants. The *bars* show the content of chl *a* (*white*) and chl *b* (*black*). **e** Effect of ABA on stomatal closing in WT, *rtl1*, and *CHLH* RNAi plants. Conditions were the same as in Fig. 1d. Data represent the mean with SD. All experiments repeated three times on different occasions gave similar results

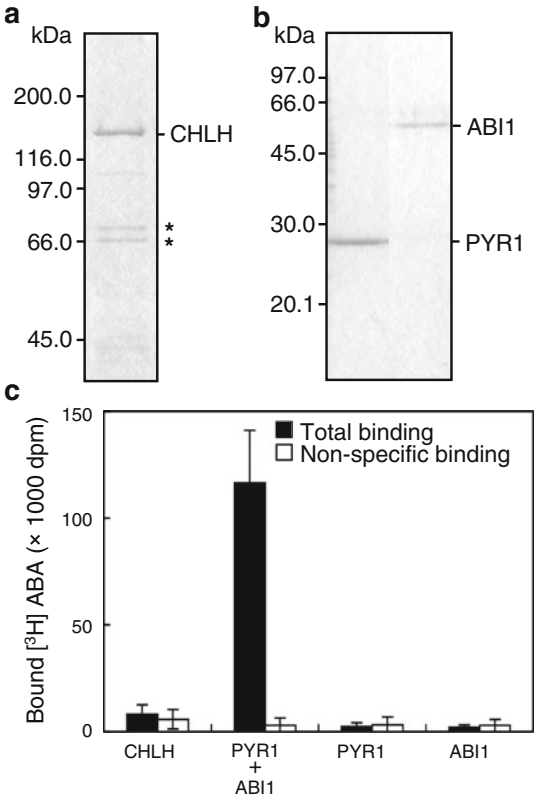

Fig. 4 ABA-binding assay of the recombinant CHLH protein. **a**, **b** Coomassie Brilliant Blue staining of the recombinant His-tagged CHLH, PYR1, and ABI1 proteins used for the ABA-binding assay. *Asterisks* indicate degradation products of CHLH. The sizes of the molecular weight markers are shown on the *left*. **c** Determination of ABA-binding activity of the recombinant proteins by the filter method. Two micromolar recombinant protein and 50 nM ^{3}H-labeled ABA were incubated in the absence (*black*) or presence of 1,000-fold unlabeled ABA (*white*) for 1 h at 25°C. Data represent the mean of three independent experiments with SD

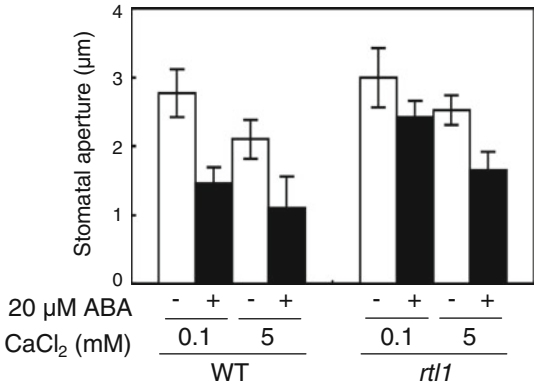

Fig. 5 Effect of a high concentration of Ca^{2+} on the ABA-induced stomatal closing in *rtl1* mutant. Stomatal apertures of WT and *rtl1* were measured after incubation for 2.5 h in the presence of 0.1 and 5 mM Ca^{2+} with or without 20 μM ABA. Other conditions were the same as in Fig. 1d. Data represent the mean with SD. Experiments repeated three times on different occasions gave similar results

not bind ABA, but GST-PYR1 and ABI1 specifically bound ABA (Fig. S2). These results indicate that CHLH does not bind ABA under our experimental conditions.

Note that in our assays, we could not detect binding of ABA to PYR1 alone, and ABA binding by PYR1 required the presence of ABI1 (Fig. 4c). In accord with this finding, the ABA-binding affinity of PYL5 and RCAR1/PYL9 was reported to increase more than tenfold in the presence of PP2Cs (e.g., HAB1 and ABI2) (Ma et al. 2009; Santiago et al. 2009).

High concentration of extracellular Ca^{2+} restored ABA responsiveness of *rtl1* stomata

Although the results of the previous section suggest that CHLH is not an ABA receptor (Fig. 4), the CHLH missense mutants, *rtl1* and *cch*, as well as *CHLH* RNAi plants, all exhibited phenotypes in which stomatal movements were insensitive to ABA (Figs. 1, 2, 3). To explain these results, CHLH may have affected downstream signaling after ABA perception in the guard cells. Studies have demonstrated that Ca^{2+} acts as a signal mediator in ABA-induced stomatal closure (Schroeder et al. 2001, Kim et al. 2010). Thus, we examined the effect of a high extracellular concentration of Ca^{2+} on ABA-induced stomatal closure in the *rtl1* mutant (Fig. 5). Application of 5 mM Ca^{2+} to the epidermis slightly reduced stomatal apertures in WT and *rtl1* plants. Unexpectedly, however, ABA also induced stomatal closure in *rtl1* in the presence of 5 mM Ca^{2+}. These results suggest that a high concentration of extracellular Ca^{2+} restored ABA responsiveness to the *rtl1* stomata.

A *chli1* knockout mutant also had an ABA-insensitive phenotype

In addition to the H subunit, two other subunits of Mg-chelatase, the D and I subunits, are required for enzymatic activity in chlorophyll biosynthesis (Gibson et al. 1995; Willows et al. 1996; Huang and Li 2009). To investigate whether other subunits of Mg-chelatase affect ABA sensitivity in stomatal guard cells, we attempted to obtain knockout mutants by T-DNA insertion. However, *CHLD* (At1g08520) is a single gene in Arabidopsis, as is *CHLH*, and a knockout mutation of *CHLD* was shown to be albino or lethal (Shen et al. 2006; Huang and Li 2009). Indeed, we could not obtain the homozygous *chld* mutants (SALK_150219 and SALK_026379). *CHLI*, however, has two isogenes, designated *CHLI1* (At4g18480) and *CHLI2* (At5g45930), and *CHLI1* is thought to be the major isoform. Consequently, a *CHLI1* knockout mutant was able to grow due

to the redundant role of CHLI2 (Rissler et al. 2002; Kobayashi et al. 2008; Huang and Li 2009). Indeed, a knockout mutant of *CHLI1* (SAIL_230_D11) exhibited a severe dwarf and pale-green phenotype, but we could determine stomatal responses using the isolated epidermis (Fig. 6). In addition, we found that ABA-induced stomatal closure was impaired in this mutant, suggesting that the Mg-chelatase complex as a whole most likely affects the ABA-signaling pathway in guard cells.

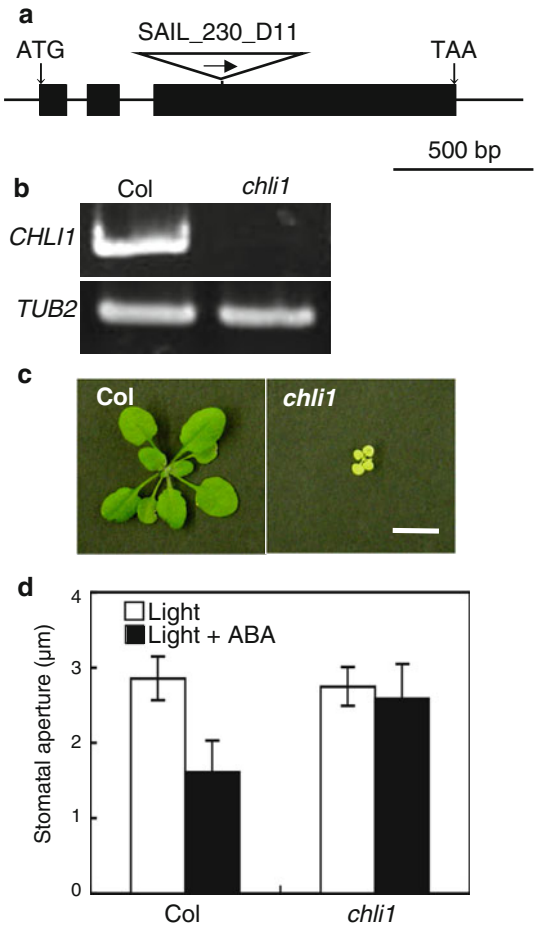

Fig. 6 Characterization of the T-DNA insertion mutant for *Mg-chelatase I subunit 1* (*chli1*). **a** Schematic representation of the structure of the *CHLI* gene and the location of the T-DNA insertion site. *Black boxes* and *lines* represent exons and introns, respectively. The T-DNA was inserted into the third exon of the *CHLI1* genomic DNA. **b** *CHLI1* expression verification by RT-PCR in the *chli1* mutant (*upper panel*). *TUB2* was used as a control (*lower panel*). **c** Four-week-old plants, Col and *chli1*, grown under a 16-h photoperiod. *Bar* 10 mm. **d** Effect of ABA on stomatal closing in Col and *chli1* plants. Stomatal apertures were measured after incubation for 2.5 h in the basal buffer with (*black*) or without (*white*) 20 μM ABA. Other conditions were the same as in Fig. 1d. Data represent the mean with SD. Experiments repeated three times on different occasions gave similar results

Discussion

A screen focused on stomata aperture-dependent water loss via transpiration

One powerful tool for identifying signaling components of stomatal opening and closing is the generation of mutants that show impaired stomatal movements. However, direct microscopic measurement of stomatal apertures is difficult with a large number of plants. Mustilli et al. (2002) reported that the open-stomata mutants, *ost1-1* and *ost1-2*, which were isolated by thermal imaging, exhibit a high rate of water loss via transpiration in their detached leaves. Therefore, measurement of weight changes in detached leaves is an effective method to identify stomatal-aperture mutants. Here, we performed a screen focused on stomatal aperture-dependent water loss in EMS-treated Arabidopsis by weighing detached leaves with a microbalance and successfully isolated three *rtl* and two *stl* mutants. To our knowledge, this is the first report of stomatal aperture mutants isolated by this method.

CHLH affects the ABA-signaling pathway in guard cells but is not itself an ABA receptor

Previous reports have suggested that CHLH localized in chloroplasts is an ABA receptor, and that the known missense mutant, *cch*, has a phenotype that is ABA-insensitive in seed germination, root growth, gene expression, and stomatal movements (Shen et al. 2006; Wu et al. 2009). It should be noted that at the beginning CHLH was identified as an ABA-binding protein using ABA-immobilized at its carboxylate on an affinity resin (Zhang et al. 2002). Given that carboxylate in ABA is needed for bioactivity, this approach potentially possessed a problem (Cutler et al. 2010). In the present study, we could not detect ABA binding to recombinant CHLH protein using ^{3}H-labeled ABA (Fig. 4), and we found no evidence of ABA resistance in either seed germination or root growth in the *cch* and *rtl1* mutants (Fig. S1), even though we performed these experiments in accordance with reported methods (Shen et al. 2006; Melcher et al. 2009; Wu et al. 2009). In contrast, however, the CHLH missense mutants, *cch* and *rtl1* (Figs. 1, 2), as well as *CHLH* RNAi plants (Fig. 3), showed ABA-insensitive stomatal movements, in agreement with previous reports (Shen et al. 2006; Wu et al. 2009). From these results, we conclude that CHLH specifically affects ABA signaling in guard cells but is not itself an ABA receptor.

The *rtl1* mutation is a single nucleotide substitution (C2068 to T), leading to a missense mutation in the protein (L690 to F) (Fig. 2). The missense mutation of *cch* (P642 to L) is proximally close to that of *rtl1*, and indeed, both mutants have nearly identical phenotypes. In contrast to

these, the missense mutation of *gun5-1* (A990 to V), whose phenotype is ABA-sensitive (Shen et al. 2006), is relatively distant from the *rtl1* and *cch* mutations. These results suggest that the region around the *rtl1* and *cch* mutations is important for ABA responsiveness in stomatal guard cells, and that the region around *gun5-1* mutation has no effect on the ABA responsiveness. In addition, the loss-of-function mutant, *xan-f10*, a mutant of Xan-F10 that is an ortholog of CHLH in barley, showed normal ABA responsiveness (Müller and Hansson 2009). However, the mutation of *xan-f10* is a 3-bp deletion that removes the conserved amino acid residue E424, suggesting that the region around the *xan-f10* mutation has no effect on the ABA responsiveness of stomatal guard cells.

We observed significant stomatal closure in the CHLH missense mutant, *rtl1*, when ABA was applied simultaneously with a high extracellular concentration of Ca^{2+} (Fig. 5). Therefore, the CHLH missense mutations of *cch* and *rtl1* may depress Ca^{2+} mobilization from intracellular Ca^{2+} stores in response to ABA, thereby damping the cytosolic Ca^{2+} elevation and/or oscillation in stomatal guard cells. Moreover, these results suggest that chloroplasts may have a crucial role for Ca^{2+} mobilization since CHLH is a chloroplast-localized protein. It is worthy of note that an important role of chloroplasts for Ca^{2+} signaling in guard cells has been reported (Nomura et al. 2008; Weinl et al. 2008). Further investigations will be needed to examine intracellular Ca^{2+} changes in response to ABA in guard cells of *rtl1* in the presence and absence of a high concentration of extracellular Ca^{2+}.

Mg-chelatase complex plays an indirect role in ABA signaling in guard cells

Mg-chelatase is a complex enzyme of three subunits, H, D, and I, and all subunits are required for Mg-chelatase activity in chlorophyll biosynthesis (Gibson et al. 1995; Willows et al. 1996; Huang and Li 2009). Our results indicate that not only CHLH missense mutants, but also a *CHLI1* knockout mutant, showed ABA insensitivity of stomatal movements (Fig. 6). Therefore, the Mg-chelatase complex as a whole probably affects the ABA-signaling pathway in stomatal guard cells. In addition, Mg-protoporphyrin IX may be involved in the regulation of the ABA signaling in guard cells, since Mg-chelatase complex is Mg-protoporphyrin IX-producing enzyme (Matsuda 2008). Further investigation will be needed to clarify this.

Note that GUN4 is shown to stimulate the activity of Mg-chelatase (Larkin et al. 2003). It is worthy to examine whether GUN4 is involved in ABA signaling in stomatal guard cells, although *gun4-1*, a missense mutant (Larkin et al. 2003), did not show ABA-insensitive phenotype (Shen et al. 2006).

A possible physiological role of CHLH in the ABA-signaling pathway in guard cells

In the *CHLH* RNAi experiments, the expression level of *CHLH* affected the ABA sensitivity of the stomatal guard cells (Fig. 3). This observation is consistent with a report stating that overexpression of a key circadian clock component, TOC1, which interacts with the *CHLH* promoter and suppresses expression of *CHLH*, gave rise to a phenotype in which stomatal guard cells were ABA-insensitive (Legnaioli et al. 2009). Taken together, these observations suggest that expression level of CHLH affects the ABA sensitivity of stomatal guard cells. Further investigations of diurnal changes of *CHLH* expression in guard cells and effects of drought stress on *CHLH* expression will provide important information on the physiological role of CHLH in the ABA-signaling pathway of stomatal guard cells.

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