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The genetic and physiological analysis of late-flowering phenotype of T-DNA insertion mutants of *AtCAL*1 and *AtCAL*2 in *Arabidopsis*

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Abstract The homozygous T-DNA mutants of *AtCAL*1 (*Rat1*) and *AtCAL*2 (*Rat2*) were obtained. The double mutant of *Rat2/Rat1RNAi* was constructed which showed obvious late-flowering phenotype from others. The expression of various flowering-related genes was studied among mutants and wild-type plants by quantitative RT–PCR. The double mutant plants showed the shortest root length compared with T-DNA insertion mutants and wild type plants under red light, blue light, and white light. The double mutants showed hypersensitivity to NaCl and ABA. However, these mutants had no effect on stomatal closure by ABA.

Keywords Late-flowering phenotype · Quantitative RT–PCR · Stomatal closure · Seed germination

Introduction

The phytohormone abscisic acid (ABA) plays important regulatory roles in many plant stress and developmental responses throughout the plant life cycle, particularly in the ability to sense and respond to various unfavorable environmental conditions, including drought, salt, and cold stresses during vegetative growth [1, 2]. Abscisic acid (ABA) modulates a wide variety of plant processes ranging from seed dormancy to leaf-water relations (reviewed in [3]). Recently, however, careful phenotypic analysis has determined that several of the hormone response mutants have altered sensitivities to more than one hormone. Many of the biochemical and physiological changes under stress result from ABA induced changes in gene expression patterns [4, 5].

In seeds, ABA is involved in the acquisition of nutritive reserves, desiccation tolerance, maturation, development, and maintenance of dormancy and germination [6]. Most of the physiological responses regulated by ABA include changes in gene expression, and many genes and proteins have been identified as involved in ABA signaling, although the signal transduction cascades are not yet clearly established [2, 7]. However, substantial progress has been made in the characterization of several ABA signaling molecules, including second messengers such as cADPR and Ca^{2+} [8, 9]. In particular, ABA signaling appears to involve RNA-binding proteins HYL1, ABH1, or SAD1 [10, 11, 12], and a complex network of positive and negative regulators, including kinases, phosphatases, and transcriptional regulators (for review, see [13, 14]).

Salinity is one of the most severe abiotic stresses that reduces the productivity of plants. Most importantly, salinity is increasing worldwide because of particular land use practices, such as over-clearing, urban development, river regulation and the cultivation of crops. The injurious effects of salt (NaCl) on plants can be divided into osmotic and ionic effects [15]. Earlier studies have shown that gene expression is greatly altered by salinity in rice and other plants [16, 17, 18, 19]. Flowering Locus C (FLC), which encodes a MADS box transcription factor, functions as a repressor of flowering [20, 21, 22].

For flowering-time analysis, flowering time was scored as the rosette leaf number at bolting and the number of days

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from the treatments to bolting; bolting was noted as the time the florescence appeared in the center of the rosettes [23]. Genetic analyses have revealed that the difference in flowering time between early- and late-flowering ecotypes is largely dependent on allelic variation in FLC [20, 21, 24]. Many genes that control flowering time have been identified by analyzing *Arabidopsis* mutants [25]. These studies have shown that multiple pathways are involved in controlling flowering time in *Arabidopsis*, including the photoperiod, vernalization, and GA pathways [26, 27].

In this report, we described the phenotypes of *Arabidopsis AtCAL*1 and *AtCAL*2 loss-of-function lines. The results from functional analyses in protoplasts revealed that *AtCAL*1 and *AtCAL*2 contributed not only to ABA signalling but also to salt stress.

Materials and methods

Plant material and growth conditions

The T-DNA insertion mutant lines of *Rat2* (At5g63510) and *Rat1* (At3g48680) coming from U.S. Salk Library were in *Col-4* and in *WS* background respectively. To identify individuals homozygous for the T-DNA insertion, genomic DNA was obtained from seedlings and subjected to PCR-based genotyping using the following primers. The sequences for *Rat1* of the forward and reverse primers of their detection were 5′-ATGGCGACTTCGTTAGCA-3′ and 5′-GAGGGAAGTAGCCAGAAA-3′, respectively. The sequences for *Rat2* of the forward and reverse primers of their detection were 5′-ATGGCGACTTCGATAGCT-3′ and 5′-AAGCTTCTAAACGGCGAT-3′, respectively. *Arabidopsis* seeds were sown on soil, cold treated for four days in the dark, and exposed to white light for 4 h to enhance germination. Following white light treatment, plants were moved to the respective conditions employed for each experiment in temperature-controlled growth chambers or dark rooms and grown at approximately 22–25°C. Light for experiments involving continuous blue illumination was provided by Bili Blue fluorescent bulbs (F48T12/B-450/HO; Interlectric Corp., Warren, PA) filtered through a blue plexiglass filter (2424 Blue; Polycast Technology Corp., Stamford, CT). Continuous red light was provided by red fluorescent bulbs (F48T12/R-660/HO; Interlectric) filtered through a red fluorescent bulbs (F48T12/R-660/HO; Interlectric Corp., Interlectric) filtered through a red plexiglass filter (2423 Red; Polycast). The wavelengths of the emission peak for the blue light and red light are 436 and 658 nm, respectively, with a half bandwidth of less than 25 nm for both the blue light and red light (Interlectric Corp., Warren, PA) [14].

Double mutant analysis

Seed were collected from individual F_2 plants exhibiting the *Rat1* and *Rat2* mutant phenotype. F_3 seeds were planted to identify seed stocks segregating the double mutant. Phenotypic analysis was carried out with these F_3 plants. Whenever possible, crosses were carried out to verify double mutant genotype. We compared only flowers 1–10 in all single and double mutants [28].

Stomatal aperture assays

To measure sensitivity to ABA-mediated inhibition of germination, approximately 200 seeds were plated on solid medium composed of Murashige and Skoog basal salts, 1% Suc. After 7 day, these seedings were planted in culture bowl. After one month, the assays of ABA-induced stomatal closing were performed [29]. Data were the average of three experiments where 30–40 stomata were measured for each one.

Leaf water-loss assays

Water loss assays were performed in detached leaves at the same developmental stage and size from 20-day-old plants. Five leaves per individual were excised and fresh weight was determined after submitting the leaves to the drying atmosphere, kept in the growth chamber (Controlled Environments Limited). Kinetics analysis of water loss was performed and represented as the percentage of initial fresh weight at each time point in three independent experiments [30].

Gene expression analysis by quantitative real time-PCR

Total RNA was isolated using Purprep RNA kit (Invitrogen, USA) from at least three plants per sample. cDNA was prepared from 2 μg total RNA using Moloney murine leukemia virus reverse transcriptase according to the manufacturer's instruction (Promega).

Real-time RT–PCR reactions were prepared according to the following protocol: 12.5 μl 2 × SYBR™ Green PCR Mix (Applied Biosystems), 500 nM forward primer, 500 nM reverse primer, cDNA template from plants and 25 μl H_2O. A master mix of sufficient cDNA and 2 SYBR® Green reagent was prepared prior and was dispensed into individual wells reducing pipetting errors and to ensure that each reaction contained an equal amount of cDNA. Stratagene M × 3000P QPCR System was used for the quantification of mRNA transcripts using RT–PCR. The PCR protocol starts with a denaturing step for 10 min at 95°C followed by 40 cycles of 15 s at 95°C and a primer extension reaction at 60°C for 1 min. After the run, the

entire mix was denatured for 5 min at 95°C before it was slowly cooled down to 60°C while the kinetics of the re-association of the complementary DNA strands of the PCR products were monitored. Reassociation kinetics provided information about additional unspecific product accumulation in a dissociation curve. All PCR reactions and the negative controls were run in duplicates with three biological replicates each. Data were analyzed with MxPro software (Stratagene) [31]. Act2 was used as the internal control. The sequence of all other primers is given in Table 1.

Results

Characterization of the phenotype of defect mutants of *AtCAL*1 and *AtCAL2* plants

Knockout of the gene encoding *AtCAL*1 and *AtCAL2* results in absence of the corresponding transcript (Fig. 1). To achieve this aim, obtained cDNA identify the differences between mutant of *Rat1*,*Rat2*, *Rat2/Rat1 RNAi* and *Col-4 WS* five genotypes by PCR and gel electrophoresis. In electrophoresis map by *Rat1* gene primers, cDNA template of *Col-4 WS* and *Rat2* showed more obvious bands (Fig. 1a). In electrophoresis map by *Rat2* gene primers, cDNA template of *Col-4* and *WS* showed more obvious bands, cDNA template *Rat1* showed weaker bands (Fig. 1a). The results in lines with expectations, indicated the source material accurately.

However, comparison of the phenotypic properties and developmental stages between *AtCAL*1, *AtCAL2* and wild-type plants did reveal an obvious difference. Both lines exhibited a very different fertility. Furthermore, mutants and wild-type plants were compared upon cultivation on solid agarose for one week under different light quality (Fig. 2). The double mutant of *Rat2/Rat1RNAi* had obvious late flowering phenotype which was different from others. It had short hypocotyl and root length (Fig. 2). Under normal growth conditions, mutant plants were distinguishable from wild-type plants concerning morphology of roots, leaves, stems and flowers. *Rat2/Rat1 RNAi* mutant showed a clear late-flowering phenotype (Fig. 3). *Rat1* mutant had a relatively weak late-flowering phenotype. *Rat2* mutant and wild-type had similar flowering phenotype (Fig. 3). The comparison of rosette leaves number showed *Rat2/Rat1 RNAi* mutant had the more leaves, which were consistent with the flowering phenotype (Fig. 4).

Table 1 Primers used for PCR and qPCR analysis

Genes	Forward (5′–3′)	Reverse (5′–3′)
Actin2	CACTGTGCCAATCTACGAGGGT	CACAAACGAGGGCTGGAAGAGC
qCO-F	CATAGGTAGTGGAGAGAACAAC	GCAGAATCTGCATGGCAATACA
qCLV-1	GGGTGAGGAACACGGAAGAG	TCATTGCGATCTTGAACACATG
qFT	CAACCCTCACCTCCGAGAATAT	TGCCAAAGGTTGTTCCAGTTGT
qLAS	CGACGGAGACAAACGATAACG	CGGTTATGCGGAGAGATGGA
qLFY	TTAAAGAACGCGGTGAGAACG	AGCGATGTTCACAAGTGGCTT
qSOC-1	CAACAGATTGAGCAACAGCTTGAG	AGCTTCTCGTTTTCTGCAGCTAG

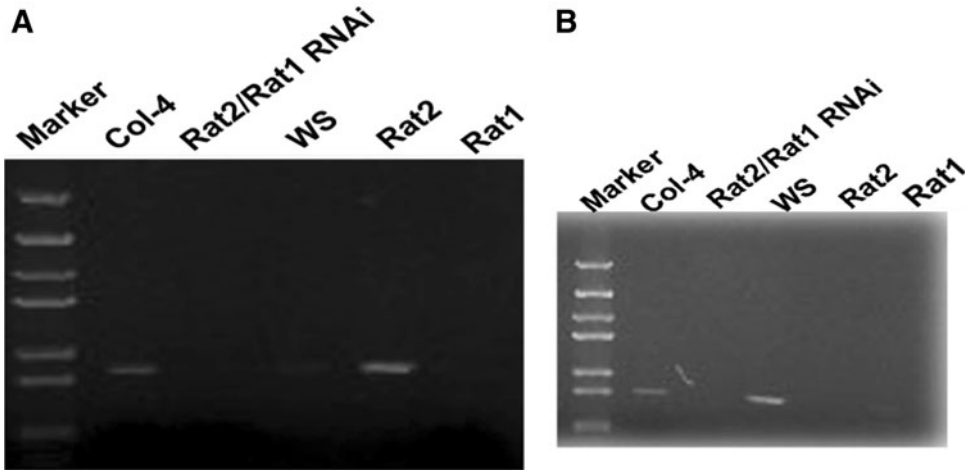

Fig. 1 Seven days seedling MS dish gel electrophoresis map under white light.**a** *Rat1* gene primers to detect the mutant and wild-type cDNA template by electrophoresis. **b** *Rat2* gene primers to detect the mutant and wild-type cDNA template by gel electrophoresis

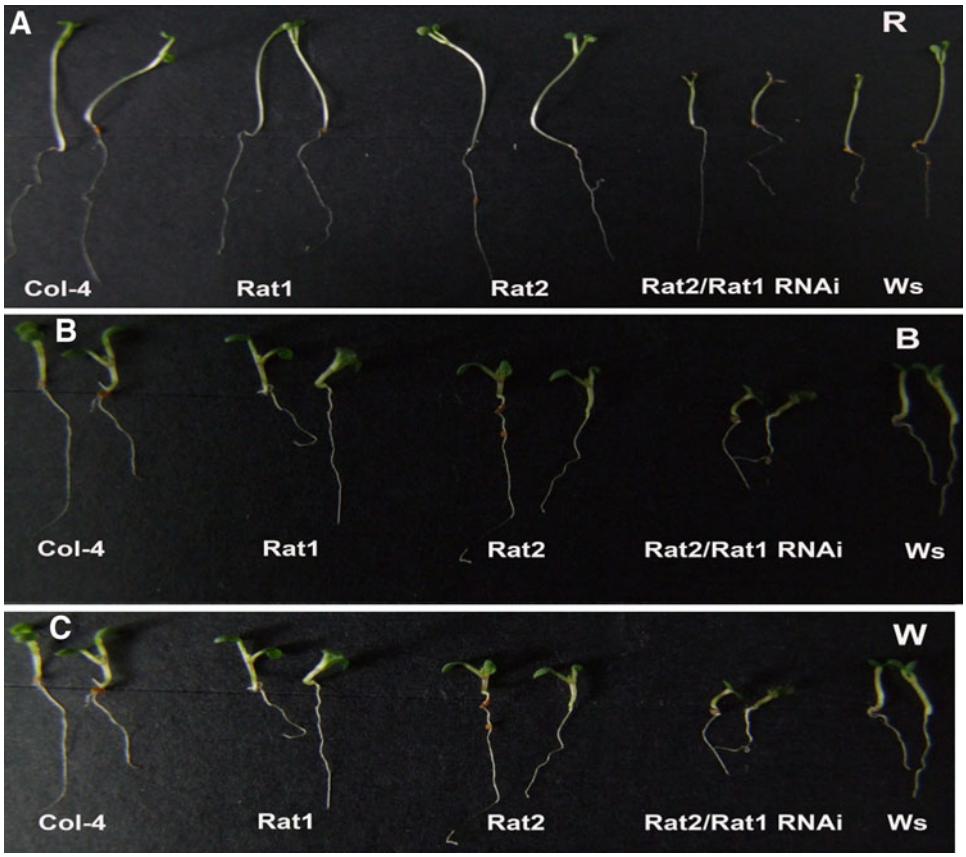

Fig. 2 The comparison of different quality light regulated root and hypocotyls in *Arabidopsis thaliana*. **a** The hypocotyl and root elongation comparison of *Rat1 Rat2* mutant and *Col-4* under red light in *Arabidopsis*. **b** The hypocotyl and root elongation comparison of *Rat1 Rat2* mutant and *Col-4* under blue light in *Arabidopsis*. c The hypocotyl and root elongation comparison of *Rat1 Rat2* mutant and *Col-4* under white light in *Arabidopsis*

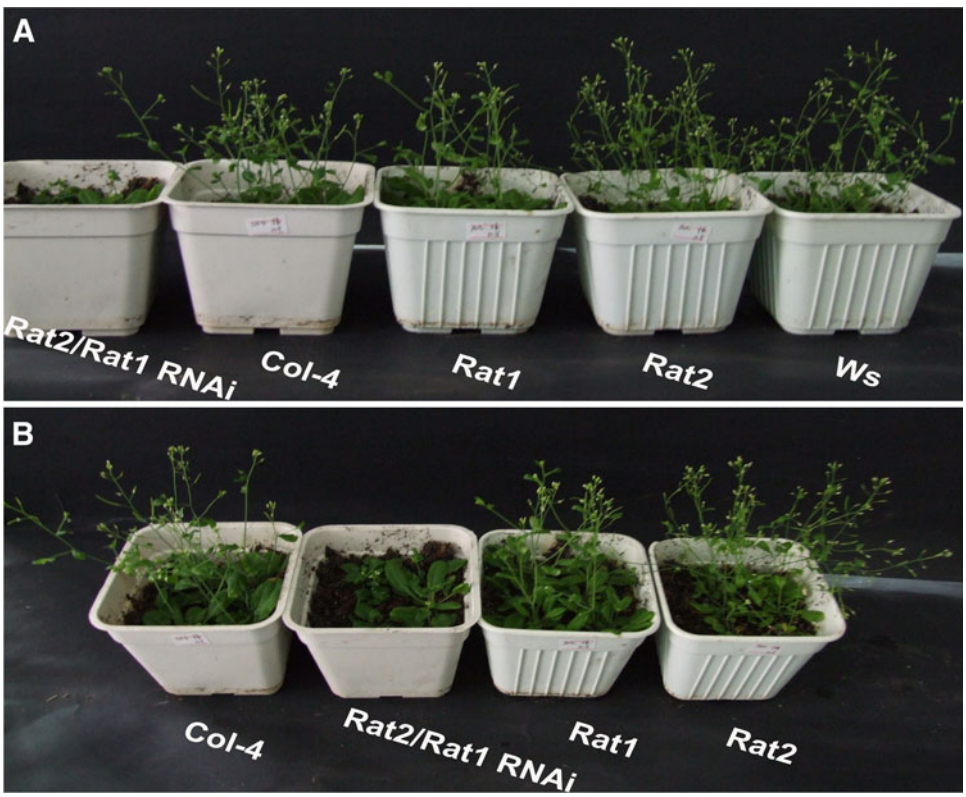

Fig. 3 The pictures of vegetative growth stage of wild-type plant and Rat1 mutant etc. **a** *Rat1* and other four plants during the vegetative growth of five samples of positive picture. **b** *Rat1* and other three samples of plant vegetative growth stage side image

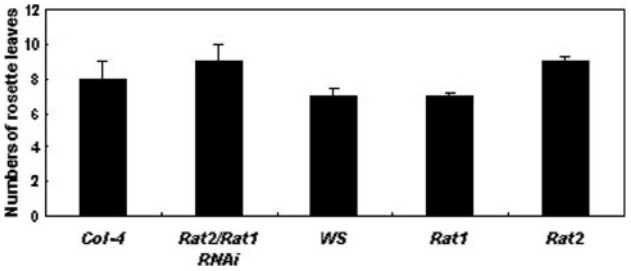

Fig. 4 The comparison of rosette leaves numbers of *Rat1* and other mutants. 25 plants of each sample were selected, repeated for twice, data represent the means (±SD)

Rat1 and *Rat2* gene modifies transcription expression of a series of flower homeotic genes

In order to explore the molecular mechanisms of mutants on floral development, the expression of flower homeotic genes were monitored. 7-day seedlings of *Rat1*, *Rat2* mutants and wild-type cDNA as a template were carried on quantitative PCR analysis. The mutants of *Rat1*, *Rat2* showed lower levels of *FT*, *CO*, *SOC-1* and *LAS*, and showed high levels of *LFY*, especially upregulated in *Rat2*. While the expression levels of *LAS* was not measurable in double mutant of *Rat2/Rat1 RNAi*. The expression levels of *FT*, *LAS*, *LFY* and *SOC-1* genes showed minimum expression in *Rat2/Rat1 RNAi* (Fig. 5). The above results were consistent with the phenotype (Fig. 6).

Stress tolerance of mutant of *AtCAL1* and *AtCAL2* plants

Seeds had more and more low germination rate with ABA and NaCl concentration increasing [32]. Among the five mutants, the germination of *Rat2/Rat1 RNAi* was the lowest. Almost none of them germinated under 0.8μM ABA and 150mM NaCl (Fig. 7a).

NaCl inhibition of root length experiments showed that *Col-4* root length reduced significantly with the NaCl concentration increasing. *Rat2/Rat1 RNAi* mutant root length was the shortest among all mutants and wild-type plants, and which was the minimal reductions (Fig. 7b).

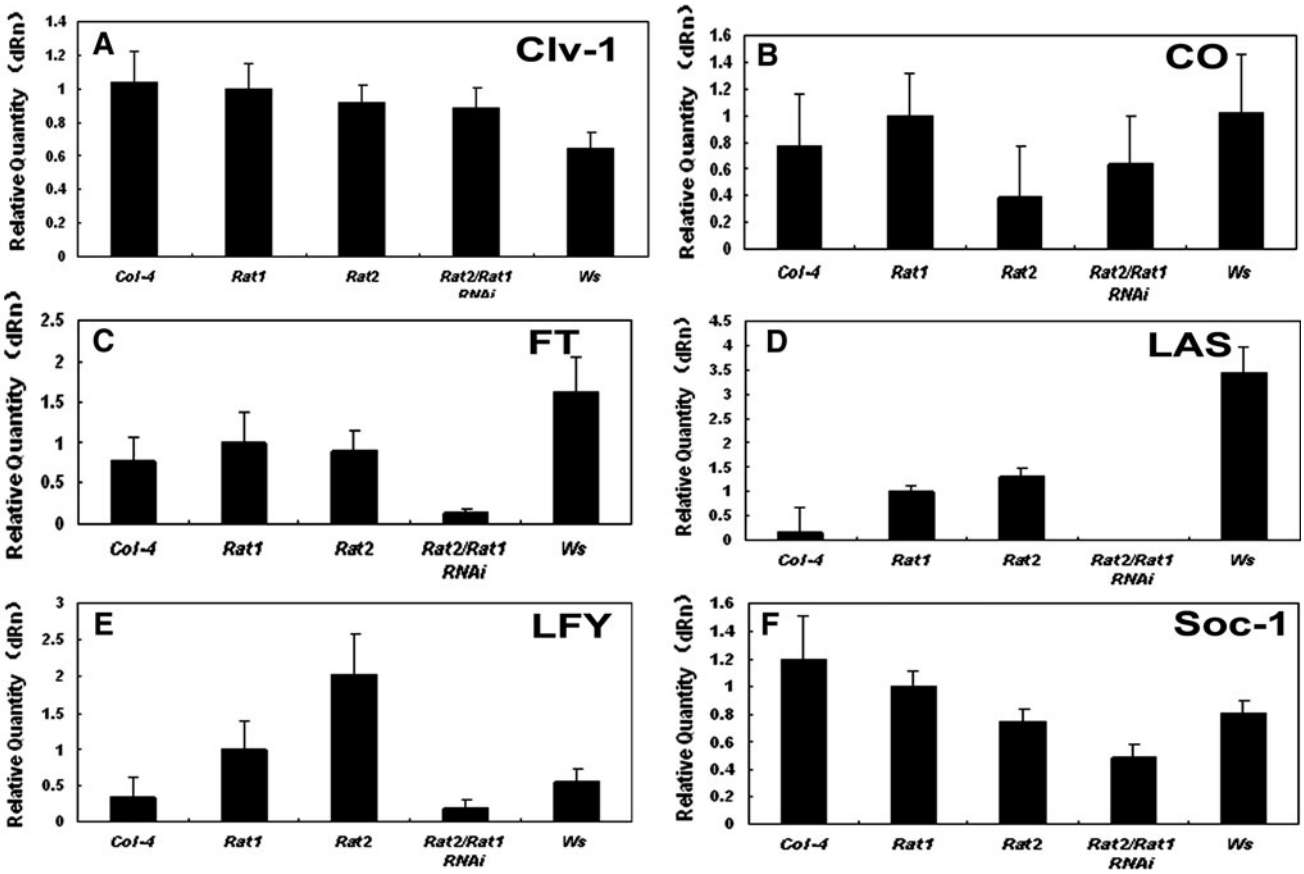

Fig. 5 Comparison of various flowering-related genes transcriptive levels in a set of mutants and wild-type plants by quantitative RT–PCR. Quantitative of RT–PCR analyses of transcript abundance of genes of *Clv-1*, *CO*, *FT*, *LAS*, *LFY*, *Soc-1* in Rat1 mutant and *Col-4*, etc. (**a**–**f**). Each sample was tested in triplicate, and data represent the means (± SD)

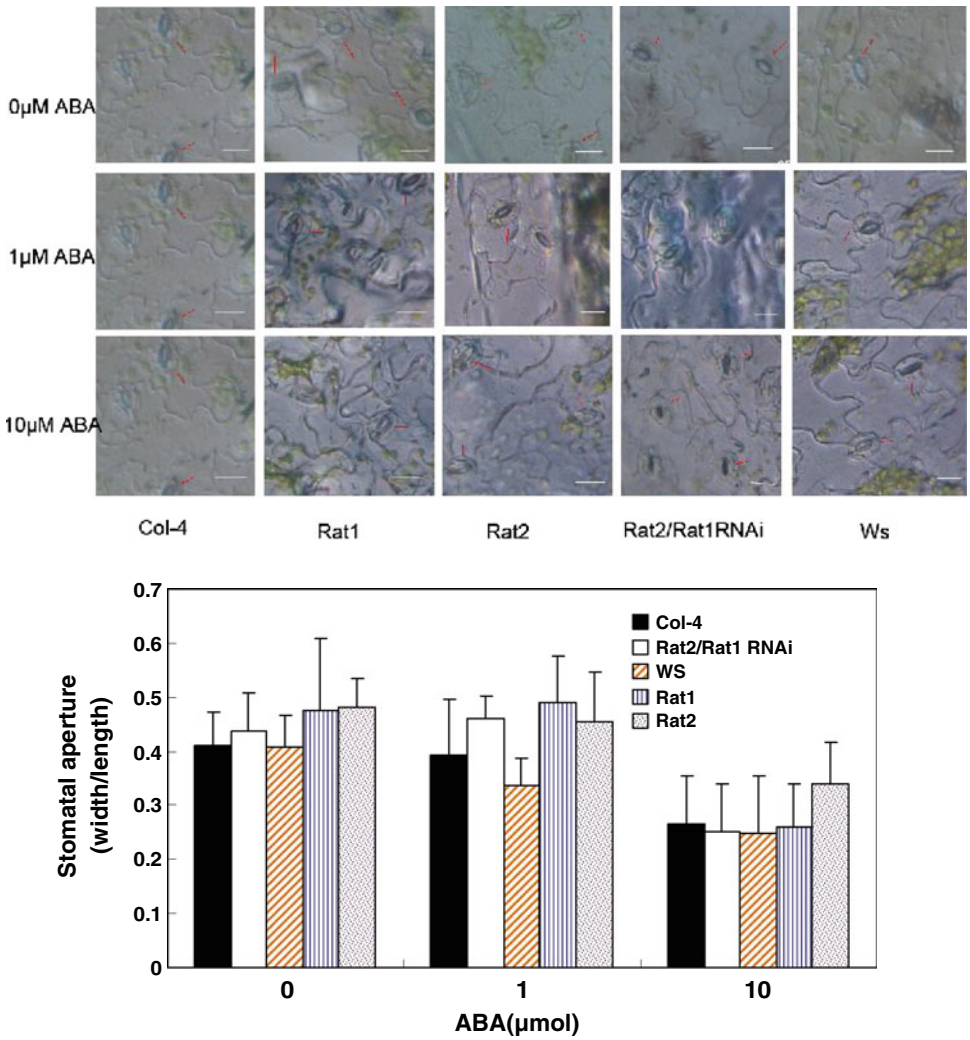

Fig. 6 ABA-hypersensitive stomatal closing in double *Rat1Rat2* mutant. Stomatal apertures were measured 2 h and 30 min after addition of 1 or 10 mM ABA. Data represent average aperture ratio (width/length) of three independent experiments ($n = 30$–40 stomata per experiment). The scale *bars* represent 50 μm

The rate of water loss from *Rat2/Rat1 RNAi* mutant was higher than that from wild-type plants, as measured by the fresh-weight loss of detached shoots (Fig. 7c). After dehydration for 4 h, the fresh weight of *Rat2/Rat1 RNAi* mutant plants was reduced to approximately 20%, whereas wild-type and *Ws* plants retained 30% of their initial weight.

Conclusion and discussion

Carbonic anhydrases $(CA)^2$ are zinc-containing enzymes that catalyze the reversible interconversion of CO_2 and HCO_3^-. In higher plants, carbonic anhydrases have been localized to the chloroplast stroma and to the cytoplasm; in algae additionally to the mitochondrion (reviewed in [33]). Chloroplast carbonic anhydrases are important for efficient delivery of CO_2 to ribulose-bisphosphate carboxylase/oxygenase (RubisCO). In contrast, the physiological role of carbonic anhydrases of other subcellular compartments is still a matter of debate. They are called carbonic anhydrase 1 (CA1) (At1g19580), CA2 (At1g47260), and CA3 (At5g66510) in *Arabidopsis*. Two further related complex I subunits of *Arabidopsis* have a less conserved primary structure and are termed carbonic anhydrase-like protein 1 (CAL1) (At5g63510) and CAL2 (At3g48680) [34].

Arabidopsis complex I is composed of three different subcomplexes: the main matrix-exposed domain constituting the NADH oxidation activity, the membrane arm involved in proton translocation across the inner mitochondrial membrane, and the spherical extra matrix domain most likely constituting aγ-type carbonic anhydrase domain [35].

Plants have developed multiple physiological and biochemical systems which enable them to tolerate abiotic environmental stresses [36, 37]. The change in osmotic potential in cells caused by water loss and high salinity triggers various molecular responses in plants [38, 39]. Defect mutants of *AtCAL*1 and *AtCAL*2 reduced root length inhibition under the salt tolerance than wild-type plants.

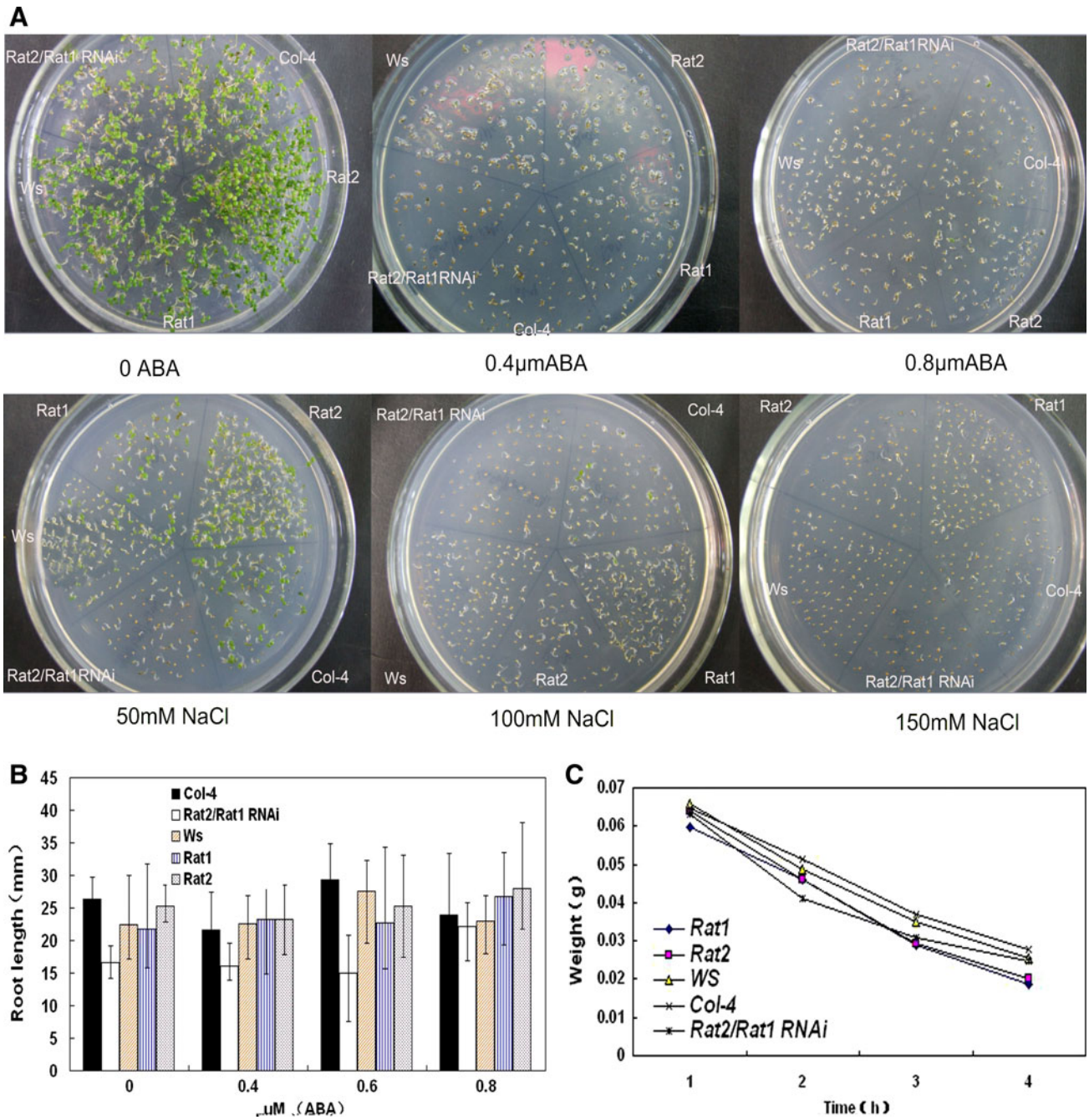

Fig. 7 The mutants treated under different stress. **a** Germination of mutant plants compared to wild type, under ABA and NaCl treatment. 0, 0.4, 0.8 μM ABA; 50, 100, 150 mM NaCl processing materials, seeds vernalized 3 days and growed 7 days. **b** Root length in the presence of the indicate concentration of NaCl. Approximately 200 seeds of *Rat1*,*Rat2*,*Rat2/Rat1 RNAi*,*WS* and *Col-4* were vernalized 3 days and growed 7 days transferred to either 50,100 or 150 mM NaCl, respectively. Scored 4 days later or 6 days later, data represent the means (±SD). **c** Enhanced transpiration rate of mutant plants compared to wild type. Loss of FW was measured in whole plants of *Rat1*, *Rat2*, *Rat2/Rat1 RNAi*, *WS* or *Col-4*. About 0.1 g mutant materials were measured, data represent the means (±SD)

However, loss of function mutant of *AtCAL*1 and *AtCAL*2 specifically *Rat2/Rat1 RNAi* mutant increased the rate of water loss than that from wild-type plants, as measured by the fresh-weight loss of detached shoots (Fig. 7c). In this paper, the mutants was successfully detected and confirmed by semi-quantitative PCR. All of our data indicated that defect mutants were more tolerant than WT to salt stress. *Rat2/Rat1 RNAi* mutant root length was the shortest among all mutants and wild-type plants, however, which was the minimal reduction. Mechanism retained unclear, may be related to the lack of the electronics and proton transfer.

The transition to flowering is controlled by diverse environmental and developmental signals, and many genes that control flowering in Arabidopsis have been identified (for review, see [25, 40, 41]). The pathway integrators *Flowering Locus T* (*FT*) and the MADS-box gene Suppressor of Overexpression of Constans 1 (*SOC1*) combine signals from several pathways to induce flowering (for review, [42, 43]). Signal convergence for floral induction pathways is the regulation of *SOC1* and *FT* expression. *SOC1*, a MADS-box transcription factor, and *FT*, a RAF-kinase inhibitor-like protein, are positive regulators of the expression of floral meristem identity genes and floral initiation [4, 5, 13, 14, 44]. The expression of *SOC1* and *FT* is positively regulated by *CO*, which encodes a B-box zinc-finger transcription factor [5]. *Leafy* (*LFY*) and *Apetala1* (*AP1*) have been demonstrated that play central roles in the initiation and development of flowers [45, 46]. In *Arabidopsis thaliana*, constitutive expression of either *LFY* or *AP1* is sufficient to confer floral meristem identity to the shoot meristems [47]. Therefore, both genes are involved in the transition from vegetative to reproductive development. It appears that *LFY* and *AP1* do not only have overlapping functions, but they also reinforce each other's activities [48, 49]. The mutants of *Rat1*, *Rat2 and Rat1/Rat2 RNAi* showed lower levels of *FT*, *CO*, *SOC-1* and *LAS* (Fig. 5), these mutants obviously appeared late flower (Figs. 3, 4). However, the results showed high levels of *LFY*, especially upregulated in *Rat2*, so offseted with the former function, occurred with the earlier flower phenotype. The expression levels of *LAS* was detected in double mutant of *Rat2/Rat1 RNAi*, therefore it showed the most obvious late flowering phenotype (Figs. 3, 5).

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