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ORIGINAL ARTICLE

Identification and molecular characterization of a *low acid phosphatase 3* (*lap3*) mutant based on the screening of an *Arabidopsis* activation-tagged population

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Abstract A *low acid phosphatase 3* (*lap3*) mutant was identified and characterized from an *Arabidopsis* activation-tagged (Weigel) population. The roots of the *lap3* plants showed lower acid phosphatase (APase) activity compared to wild-type ones under low-Pi conditions (10 μM Pi). Plasmid rescue experiments revealed that the activation-tagging vector was inserted into the intergenic region between *At4g31540* and *At4g31550* in the *Arabidopsis* genome. The genotypic segregation of the *lap3* mutation was tightly linked with the phenotypic segregation of root APase activity in the prgeny of *lap3*. The transcript level of the *At4g31520* (*SDA1*: *SEVERE DEPOLYMERIZATION OF ACTIN 1*), located 7.4 kb from the CaMV 35S enhancers in the *lap3* mutant, was significantly reduced compared to that in the wild type. It was speculated that cellular actin polymerization may be involved in Pi acquisition in higher plants.

Keywords *Arabidopsis thaliana* · Pi deficiency · Acid phosphatase activity · Activation-tagged population screening

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Introduction

Phosphorus (P), an essential macronutrient for all living organisms, is a component of many important biological compounds involved in the regulation of biochemical and physiological processes (Miura et al. 2005; Lin et al. 2009). Plants can acquire phosphorus as phosphate (Pi) anion through Pi transporters in the root. However, Pi is one of the most limiting nutrients for plant growth and crop productivity due to its immobilization in the soil (Holford 1997; Sánchez-Calderón et al. 2005). The major adaptive processes that respond to Pi starvation in plant roots include root system development, Pi mobilization, and changes in Pi metabolism, such as its transport (Fang et al. 2009). Many plants, including rice, wheat, tomato, and *Arabidopsis*, are known to respond to deficiency in exogenous Pi by increasing the activities of the acid phosphatases (APases) secreted from their roots, thereby enhancing the capacity for Pi acquisition (Tadano et al. 1993; Duff et al. 1994; Coello 2002). The molecular mechanisms that monitor Pi availability and transfer the nutritional signal in plants are still not clear, and even the organs or tissues that are responsible for Pi sensing is still a controversial topic (Fang et al. 2009). Hence, efforts to identify the signaling networks that coordinate the response of plants to Pi limitation are needed.

T-DNA activation-tagging mutagenesis is a gain-of-function genetic method that can supplement the limitation of loss-of-function mutagenesis (Weigel et al. 2000). T-DNA activation vector containing tetrameric CaMV 35S enhancers mediates the transcriptional activation of the nearby genes after its integration into the plant genome. However, the frequency of the mutations obtained by transformation using this vector was known to be only around 0.1–1%, which is much lower than expected

(Weigel et al. 2000; Marsch-Martinez et al. 2002). The methylation of the enhancer elements and subsequent transcriptional silencing is considered to be a plausible explanation for the low frequency of the gain-of-function mutation (Chalfun-Junior et al. 2003).

In this study, we isolated and characterized a *low acid phosphatase 3* (*lap3*) mutant that showed reduced root APase activity in response to P starvation, by screening an *Arabidopsis* activation-tagged (Weigel) population.

Materials and methods

Mutant screening

Activation-tagged T-DNA mutant lines (Weigel et al. 2000) were obtained from the Nottingham Arabidopsis Stock Centre (NASC, UK). The sterilized seeds were germinated on MS medium (Murashige and Skoog 1962) for 7 days and transferred to MS medium containing a reduced Pi concentration (10 μM) where they were maintained under continuous light at 23°C for an additional 10 days. Then, 0.008% 5-bromo-4-chloro-3-indolyl phosphate (BCIP) was added to perform a colorimetric assay of the acid phosphatase activities of *Arabidopsis* roots.

Quantification of acid phosphatase activity

Arabidopsis seeds of the wild type and mutants were germinated on MS media for 7 days, transferred to Pi-deficient (10 μM) MS media, and then grown for an additional 10 days. Total proteins were then extracted from *Arabidopsis* roots according to Yen's method (Yen and Green 1991), with minor modifications. The protein concentration was determined according to the Bradford method (Bradford 1976). Acid phosphatase (APase) activity was measured using ρ-nitrophenyl phosphate as the substrate, as described previously (Chen et al. 2000). The total volume of the reaction was 0.06 mL, comprising 100 mM acetic acid–NaOH (pH 5.5), 5 mM ρ-nitrophenyl phosphate as substrate, and appropriate amounts of plant protein (5–10 μg). The reaction was terminated by adding 1 M Na_2CO_3 after incubating at 37°C, and assayed spectrophotometrically. APase activities were calculated using a molar extinction coefficient of 18.3 cm^2 μmol^{-1} for ρ-nitrophenyl at 405 nm.

Plasmid rescue and Southern hybridization analyses

In order to locate the position of the activation-tagging vector within the mutant genome, plasmid rescue was performed as described previously (Weigel et al. 2000), with minor modifications. One microgram of genomic DNA was digested overnight with *BamH*I, and then enzyme was inactivated at 65°C for 15 min. Restricted DNA was self-ligated and transformed into *Escherichia coli* DH10B. The plasmid containing activation-tagging vector with pUC19 sequences was derived based on ampicillin selection of the bacterial transformants and then sequenced. For the Southern hybridization analyses, approximately 5 μg of genomic DNA were digested with *Bam*HI and separated on 0.8% agarose gel. After electrophoresis, the digested DNA was transferred to Hybond N^+ Nylon membrane (Amersham, UK) and hybridized with ^{32}P-dCTP-labeled DNA corresponding to the tetrameric CaMV 35S enhancers as a probe (see Fig. 2a). The primer sequences used for probe amplification were as follows: 35S_F: 5′-TAA TAC GAC TCA CTA TAG GG-3′; 35S_R, 5′-ACC CGC CAA TAT ATC CTG-3′.

Co-segregation analyses of the *lap3* mutant

Co-segregation analyses were performed using the progeny of self-pollinated *lap3* heterozygous plants: phenotyping was carried out using a BCIP-staining-based colorimetric assay of *Arabidopsis* roots (as mentioned above) under Pi-deficient conditions, while genotyping was performed by PCR using two gene-specific primers (P1 and P2; see Fig. 3a) flanking the T-DNA insertion site and T-DNA left border primer (SKC12; see Fig. 3a). The sequences of the P1, P2, and SKC12 primers were as follows: P1, 5′-AAG TCT TCT TCC TCT TGC CT-3′; P2, 5′-TAA TGA GTT GGT TGG GTT TT-3′; SKC12, 5′-TTG ACA GTG ACG ACA AAT CG-3′.

Semiquantitative RT-PCR

Total RNA was extracted from 2-week old *Arabidopsis* seedlings using an RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Two step RT-PCR was used to detect the levels of the transcripts. One microgram of total RNA was used for cDNA synthesis via a high-capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, CA, USA). Two microliters of tenfold-diluted cDNA were used for PCR amplification. The gene-specific primers used in semiquantitative RT-PCR are listed in Table 2.

Results and discussion

Isolation of *Arabidopsis* mutants showing low acid phosphatase activities from an activation-tagged population

Plant roots are known to induce acid phosphatases (APases) in response to Pi starvation (Goldstein et al.

1989). In this study, colorimetric assays were performed to screen for *Arabidopsis* mutants that show low APase activities of the roots under low-Pi conditions (10 μM Pi). APase activities were visually detected around the roots of Arabidopsis seedlings: in other words, wild-type plants grown on low-Pi medium containing 5-bromo-4-chloro-3-indolyl phosphate (BCIP, Fig. 1a) showed a strong blue color change on the medium due to the breakdown of the BCIP by the APases secreted from the roots (Fig. 1b).

From the screening of 33,597 seedlings of an *Arabidopsis* activation-tagged population that represents 11,199 independent tagging lines, three independent mutant lines whose roots showed lower APase activities than those of wild-type plants under Pi-deficient conditions were obtained and named *lap* (*low acid phosphatase*) mutants (*lap1*, *lap2*, and *lap3*). Among the three mutants, *lap3* showed the smallest plant size and least developed root system, as well as the lowest root APase activity compared to wild-type plants under Pi-deficient conditions (Fig. 1b). The APase activity of *lap3* was further tested based on a quantitative enzyme assay using total proteins extracted from the roots and *ρ*-nitrophenyl phosphate as the substrate. As shown in Fig. 1c, the roots of the *lap3* mutant showed 19% lower APase activity than those of wild-type plants under Pi-deficient conditions.

The abnormally low APase activity of the *lap3* mutant roots, revealed by both BCIP staining and a quantitative enzyme assay using *ρ*-nitrophenyl phosphate, provided clear evidence that the *lap3* mutant has the decreased APase activity of roots under Pi-deficient conditions.

Identification of the activation-tagging vector insertion on the *lap3* genome

Plasmid rescue experiments were performed to determine the location of the activation tag within the *lap3* genome. Among the various restriction enzymes tested, only the digestion of the *lap3* genome with *Bam*HI successfully rescued the plasmid, yielding 8 independent positive clones. After digesting the rescued plasmids with *Bam*HI and *Kpn*I, it was possible to obtain a 2.9 kb DNA fragment that corresponded to a pUC19 fragment present in the activation-tagging vector (Fig. 2a, b): all of the 8 rescued plasmids exhibited the same band patterns after being digested with *Bam*HI and *Kpn*I, indicating that *lap3* mutant was likely to contain a single copy of T-DNA inserted in its genome (data not shown; van der Fits et al. 2001). The single-copy T-DNA insertion was confirmed by Southern hybridization analyses using the *Bam*HI-digested *lap3* genome and T-DNA-specific CaMV 35S enhancers as the DNA probe (Fig. 2c).

The T-DNA flanking region in the rescued plasmid was sequenced and the sequence was compared to the *Arabidopsis* genomic DNA sequences deposited in GenBank. The activation-tagging vector PSKI015 was found to be inserted into the intergenic regions of the *At4g31540* and

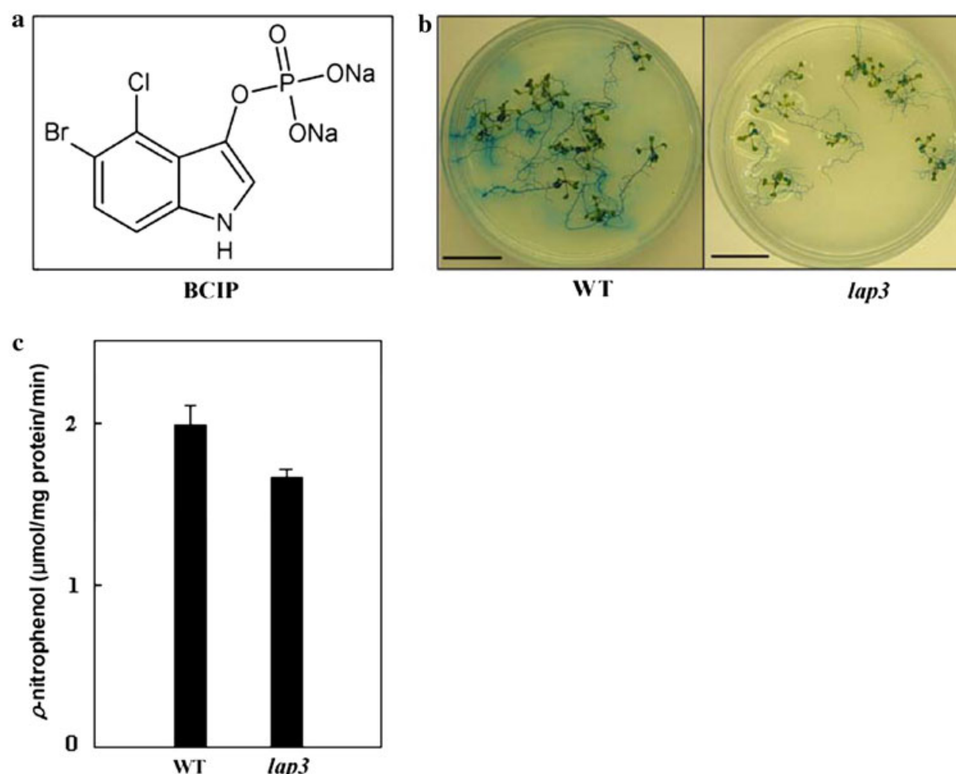

Fig. 1 Chemical structure of BCIP as well as seedling phenotype and APase activity of the *lap3* mutant. **a** Chemical structure of BCIP as a substrate for the colorimetric assay of acid phosphatase activities from *Arabidopsis* roots. **b** Phenotype of the *lap3* mutant. Wild-type and *lap3* mutant plants were germinated and then transferred to MS media containing reduced Pi. After 10 days, histochemical staining for APase activity of roots was performed using BCIP as the substrate. *Scale bars* represent 2 cm. **c** Quantitative measurement of APase activity. Seedlings were grown on Pi-deficient MS media. Total proteins were prepared from the roots of the seedlings and APase activity was measured spectrophotometrically using 5 mM *ρ*-nitrophenyl phosphate as a substrate. The *lines on the bars* indicate standard errors for three replicates

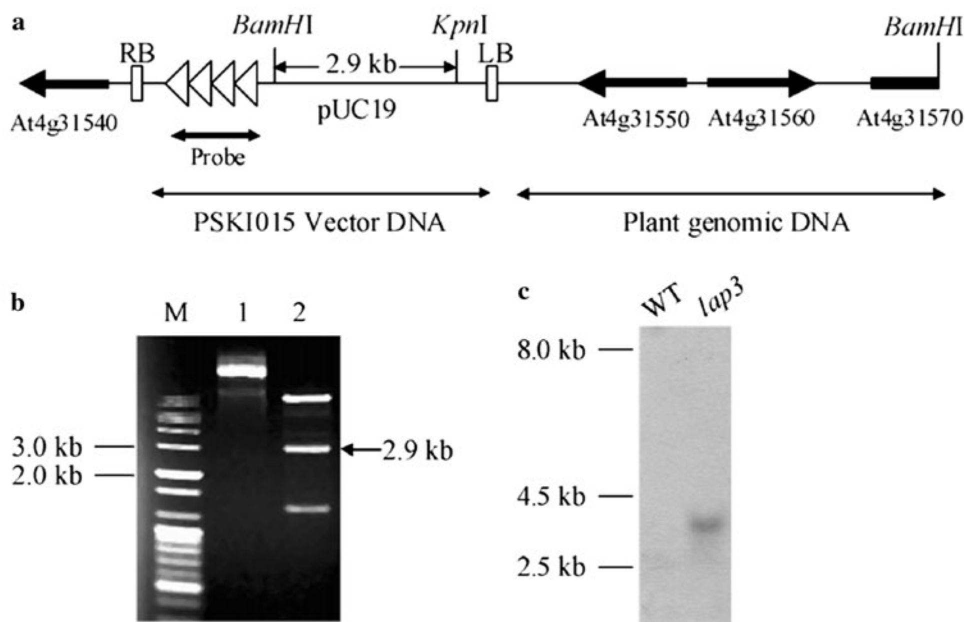

Fig. 2 Plasmid rescued from *lap3* genomic DNA. **a** Map of activation-tagging plasmid rescued by the restriction digestion of *lap3* genomic DNA with *Bam*HI. *White triangles* indicate tetrameric CaMV 35S enhancer elements, and *black arrows* indicate rescued plant genomic DNA. **b** Electrophoresis image of the *Bam*HI-rescued plasmid in an agarose gel. *M* 1 kb plus DNA ladder, *lane 1* uncut plasmid, *lane 2* plasmid cut with *Bam*HI and *Kpn*I. The fragment of 2.9 kb corresponds to the pUC19 segment in the activation-tagging vector. **c** Genomic Southern hybridization analyses. *Bam*HI-digested genomic DNA separated on agarose gel was hybridized with labeled tetrameric 35S enhancer elements as a probe

At4g31550 genes, which were located approximately 2.3 kb upstream from the start codon of the *A4g31540* and 7.4 kb downstream from the *At4g31550* (Fig. 2a, b).

Genetic characterization of *lap3* mutant based on co-segregation analyses

PCR-based genotyping using gene-specific and T-DNA-bordered primers revealed that the *lap3* obtained from the aforementioned root APase-based screening was a heterozygous mutant (data not shown). Then, when 100 progeny of the self-pollinated parental *lap3* mutant were subjected to phenotyping of their root APase activities under Pi-deficient conditions, 64 plants exhibited the mutant phenotype (pale blue BCIP staining), while 36 plants exhibited the wild-type one (strong blue BCIP staining), as shown in Table 1 and Fig. 3. The segregation between the mutant and wild-type phenotypes fitted well to the ratio of 2:1 ($\chi^2 = 0.3$, $P = 0.572$), not to the ratio of 3:1 ($\chi^2 = 6.5$, $P = 0.011$). In addition, when the same progeny were subjected to PCR-based genotyping, the aforementioned 64 and 36 plants were found to be heterozygous mutants and wild-type plants, respectively (Fig. 3). Meanwhile, none of the homozygous mutant plants were found among the progeny, probably due to their lethality. Considering the closely linked co-segregation of the *lap3* phenotype for root APase activity with its genotypic segregation, the loss-of-function phenotype in *lap3* probably occurred due to the dominant mutation effect of the T-DNA inserted in the *lap3* genome.

Phenotypic and genotypic segregation with a ratio of 2:1, which was observed in the self-pollinated *lap3* progeny, is well known to occur for homozygous lethal mutations (Kandasamy et al. 2005; Xu et al. 2010). The lethality could be due to a failure or defects at any stage of reproductive development, such as reduced fertility of gametes, failure of fertilization, or abortion of embryo development (Xu et al. 2010). The mechanism underlying the homozygous lethality of the *lap3* progeny needs to be elucidated in the future.

Identification of the candidate gene responsible for the low APase activity of *lap3* mutant

To monitor the perturbation of gene expression caused by the activation tag in *lap3* plants, we investigated the levels of the transcripts expressed from the eight genes that are located in the region about 10 kb from either side of the T-DNA (left or right border). The genes whose transcript levels were compared between wild-type and *lap3* plants included *At4g31500* (*CYP83B1*, cytochrome P450 monooxygenase 83B1), *At4g31510* (unknown function), *At4g31520* (*SDA1*, *SEVERE DEPOLYMERIZATION OF ACTIN* 1), *At4g31530* (nucleoside-diphosphate-sugar epimerase), *At4g31540*

Table 1 Phenotypic segregation analyses with the progeny of a self-pollinated *lap3* heterozygous mutant

Number of progeny			Observed ratio (mutant/WT)	χ^2-test	
Mutant[a] (pale blue)	WT[b] (strong blue)	Total		3:1	2:1
64	36	100	1.8:1	6.5*	0.3 NS

* $P < 0.05$, *NS* not significant at the 0.05 level

[a,b] Phenotyping was performed by applying a BCIP-staining-based colorimetric assay to *Arabidopsis* roots grown under Pi-deficient conditions

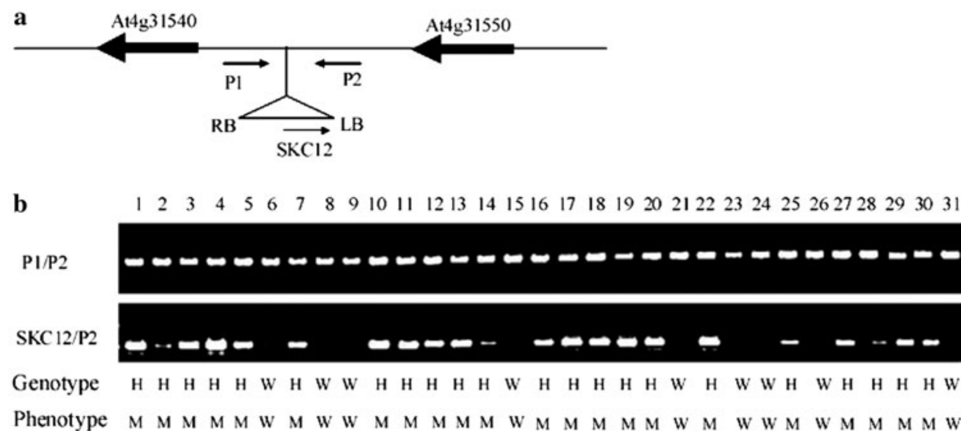

Fig. 3 Co-segregation of the *lap3* mutation with the phenotypic segregation of root APase activity. **a** A diagram showing the insertion site of the activation-tagging vector in the *lap3* genome and the locations of the primers (P1, P2, and SKC12) used for genotyping. **b** Co-segregation analyses of genotype and phenotype in the progeny of the self-pollinated heterozygous *lap3* mutant. *Arabic numbers* represent the tested plants; *P1/P2* PCR product amplified using primers P1 and P2, *SKC12/P2* PCR product amplified using SKC12 and P2 primers, *H* heterozygote, *W* wild type, *M* mutant. Phenotyping was done based on a BCIP-staining-based colorimetric assay of *Arabidopsis* roots under Pi-deficient conditions

(exocyst subunit EXO70 family protein G1), *At4g31550* (*WRKY11*), *At4g31560* (unknown function), and *At4g31570* (unknown function) (Fig. 4a). Semiquantitative RT-PCR analyses were performed using sets of gene-specific primers (Table 2), with total RNA extracted from the wild-type and heterozygous *lap3* plants in order to compare the transcript levels. The levels of most of the transcripts, including those from *β-tubulin* (*At5g62690*), as well as the *At4g31540* and *At4g31550* genes that are immediately adjacent to the T-DNA insertion site, were little changed compared to the wild-type ones (Fig. 4b). Interestingly, however, *lap3* plants showed significantly reduced accumulation of the transcript for the *At4g31520* gene that is located at a distance of approximately 7.4 kb from the 35S enhancer elements. It is thought that the reduced level of the *At4g31520* transcript in *lap3* plants was due to the gene silencing mediated through the 35S enhancer tag. Activation-tagging vector containing four copies of the CaMV 35S enhancer element most often activates the transcription of neighboring genes after random integration into the plant genome, thereby resulting in a gain-of-function phenotype (Weigel et al. 2000; Tani et al. 2004). However, in some cases, like the *lap3* mutant in this study, dominant or semidominant loss-of-function phenotypes of the activation-tagging lines are caused by a reduced transcript level of the gene that is located adjacent to the enhancers. Similar cases have been reported for Z26013L and ACC oxidase genes, as they are located 12 and 11.4 kb from their enhancers, respectively, in activation-tagging lines of *Arabidopsis* and legume plants (Ichikawa et al. 2003; Imaizumi et al. 2005).

The *At4g31520* encodes the SDA1 (SEVERE DEPOLYMERIZATION OF ACTIN 1) family protein in *Arabidopsis*, the function of which is still unknown. Considering the facts that yeast SDA1 is required for actin cytoskeleton organization and actin filaments are involved in the delivery of the Golgi-derived secretory vesicles to the bud region in yeast (Govindan et al. 1995; Buscemi et al. 2000), it is tempting to speculate that SDA1 may be involved in the Golgi-mediated secretion of APases from roots through the control of actin polymerization in *Arabidopsis*. However, the biological function of SDA1 in the regulation of APase activities in *Arabidopsis* roots needs to be elucidated in the future.

So far, only two *phosphatase under-producer (pup)* mutants (*pup1* and *pup3*), which show reduced APase activities at the root surface or in root exudates, have been isolated and characterized physiologically (Trull and Deikman 1998; Tomscha et al. 2004). The genes

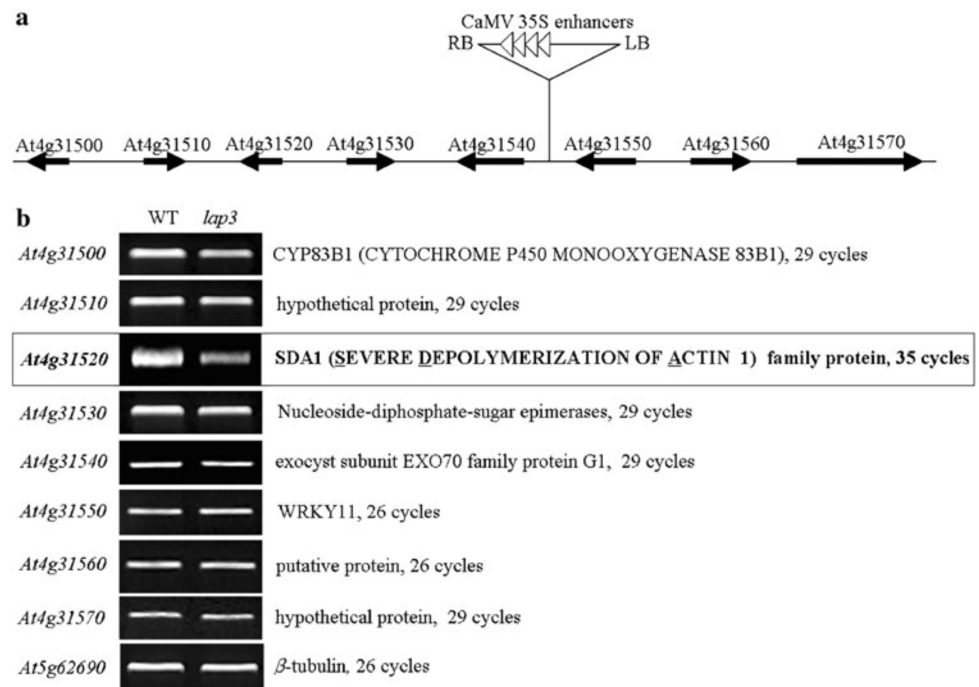

Fig. 4 Levels of the transcripts expressed from the genes adjacent to 35S enhancers. **a** Map of the genes adjacent to 35S enhancers located in the region 10 kb from either side of the enhancers. **b** Semiquantitative RT-PCR analyses of the transcripts, including At4g*31500*, *At4g31510*, *At4g31520*, *At4g31530*, *At4g31540*, *At4g31550*, *At4g31560*, and *At4g31570*, in the wild-type and *lap3* plants. Gene-specific primers were used to measure the transcript levels. *β-tubulin* (*At5g62690*) gene was used as mRNA quantity control. The number of cycles for RT-PCR analysis of each transcript was optimized, and are shown in the figure

Table 2 Primers used for semiquantitative RT-PCR analyses

Locus	Forward primer (5′–3′)	Reverse primer (5′–3′)
At4g31500	TCAACGGATGATGGACAAGA	CCGGCACAACAATATCCAAT
At4g31510	TTTAGGGATCGTAGCAGCGT	GCAACGGCATAGAATTAGGG
At4g31520	AAAAAGCAAGCAAAACTGCAA	ACAGGAATAGCCTCCGGACT
At4g31530	TATCTTCGTCAATCACGCCA	AAGGTTCATGATGCTCCAGG
At4g31540	GAAAGCGTATCCGGATCAAA	ACTTGCTTGAACTCGCGTCT
At4g31550	CTAAACCAAGCATCTTCGGC	TAGTAACCACGTGGGTGTGG
At4g31560	TGGCGAGACTTTTCGTCTCT	TTTAGGCCGGTTACGTGTTC
At4g31570	TGTGAATGTTGCTGAGGAGG	TCCTGCATTTCCCCTACATC
At5g62690	CTCAAGAGGTTCTCAGCAGT	TCACCTTCTTCATCCGCAGT

responsible for these mutations still await identification. Molecular characterization of the SDA1 in the context of plant Pi acquisition would help to expand our limited knowledge about the signaling network that coordinates plant response to Pi availability, which could then be applied to the development of high-efficiency crops in terms of acquiring P nutrients.

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