A Study of Natural Adaptation to Water Loss in *Arabidopsis thaliana* Accessions

Imai Hiroe

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Abbreviations

A. thaliana	*Arabidopsis thaliana* (L.) Heynh.
ABA	abscisic acid
ABRE	ABA-responsive element
CA	carbonic anhydrase
Col	Columbia
DRE	dehydration-responsive element
EDTA	ethylenediaminetetraacetic acid
GUS	*β-glucuronidase*
LOD	logarithm of odds
NRT	nitrate transporter
PPFD	photosynthetic photon flux density
QTL	Quantitative Trait Locus
RI	recombinant inbred
RILs	recombinant inbred lines
SLAC1	SLow Anion Channel-associated 1
SUMP	Suzuki's Universal Micro Printing
Ws	Wassilewskija

1. Abstract

Dehydration tolerance is a major issue in adaptation and geographic distribution for terrestrial plants. Despite the importance, little is known about the genes and molecular mechanisms that could explain its naturally occurring variations in the environment. The variation of intraspecific water loss tolerance between two accessions of *Arabidopsis thaliana*, Columbia (Col)-0 and Wassilewskija (Ws)-2, was analysed by measuring weight loss in detached seedlings when it showed a clear difference between water loss-tolerant Col-0 and water loss-sensitive Ws-2. They also differed in their stomatal response under the water loss condition. Since it was presumably due to genetic differences, a QTL analysis was performed to identify the applicable trait loci. The result indicated a locus on chromosome 1. Being surveyed in the locus, it was extrapolated that the *SLAC1* gene, which is associated with stomatal closure, was likely responsible for the difference in water loss. Comparison of their nucleotide and amino acid sequences revealed that there was no distinct difference in regions encoding SLAC1 protein but was a distinctive alteration in *SLAC1* promoter region. Histochemical GUS staining showed that the *SLAC1* expressed dominantly in guard cells of Col-0, but did less in guard cells of Ws-2. Quantitative PCR analysis also showed that transcript level of *SLAC1* in guard cells was higher in Col-0 than in Ws-2. These *SLAC1* transcription analyses indicated low accumulation of SLAC1 in guard cells of Ws-2. When taken together, the results suggested that

the *SLAC1* promoter was associated to the natural variation between Col-0 and Ws-2 in stomatal control for water loss.

2. Introduction

Plants cope with severe climatic conditions through physiological acclimatisation and evolutionary adaptation. Water availability and temperature strongly limit the natural distribution of terrestrial plant species (Stebbins, 1952; Whittaker, 1975). Since water deficiency can inhibit plant growth (Alward, 1999; Morillon and Lassalles, 2002; Andrioli and Sentelhas, 2009; Balducci et al., 2013) and limit the propagation of wild plants and crop yields (Weaver and Albertson, 1943; Boyer, 1982), understanding plant responses to water deficiency is of great importance to know their adaptation to the environments.

To understand how plants response to water-deficient stresses, detailed genetic analyses of plant physiological responses to the stresses have been carried out in a few crop and model species (Bray, 1997; Bartels and Sunkar, 2005; Bressan et al., 2009). In *Arabidopsis thaliana* (L.) Heynh. (*A. thaliana*), genes associated with the response to water deficiency have been identified largely through the use of knockout mutants and transgenic plants (reviewed by Golldack et al., 2014). Analyses using these tools are useful although the function of only a single gene is able to be examined at a time. When many genes work at the same time under such water deficient condition (Bhatnagar-Mathur et al., 2008), we need to examine not only each gene function but also their harmonious function for studying plant adaptation to their local environmental conditions.

*A. thaliana*s are distributed widely in the Northern Hemisphere and have experienced a wide range of climatic conditions and selective pressures for thousands of generations (Alonso-Blanco *et al*., 2009). Their large genetic variation occurring in nature provides a unique resource for studying its effects on stress tolerance. The variances in traits affecting the tolerance to water deficiency, such as stomatal closure, reduction in leaf growth, and water use efficiency for biomass production, have been documented among different natural accessions of *A. thaliana* (Hausmann *et al*., 2005; McKay *et al*., 2008; Juenger *et al*., 2010; Monda *et al*., 2011). A locus or gene that affects natural adaptation can be identified with quantitative trait locus (QTL) mapping. In *A. thaliana*, several recombinant inbred lines (RILs) have been constructed from genetically and phenotypically distinct parents and have been used for QTL mapping for a variety of traits involved in adaptation to local climatic conditions, including seed dormancy, flowering time and disease resistance (Alonso-Blanco *et al*., 2009). Although several studies have reported differences in the responses to water deficiency among *A. thaliana* accessions (reviewed by Verslues and Juenger, 2011), the genes responsible for the differences between the accessions in adaptation to those water deficient conditions have not been identified.

In this study, I used natural variation between two *A. thaliana* accessions, Columbia (Col)-0 and Wassilewskija (Ws)-2, and carried out QTL mapping of water loss tolerance. An

alteration in *SLAC1* promoter was sufficient to change the transcript level of *SLAC1* in the

guard cells. I conclude that this alteration may affect the level of the SLAC1 protein in the

guard cells and cause the difference in stomatal closing response under water loss conditions

between the two accessions.

3. Materials and methods

3-1. Plant materials and growth conditions

Seeds of *A. thaliana* accessions used in this study were obtained from the Arabidopsis

Biological Resource Center (ABRC, Columbus, OH, USA; Supporting information) (Table 1).

Seeds of homozygous T-DNA knockout lines for *At1g12490* (SALK_038601, SALK_133974

and SALK_151792) were also obtained from the ABRC. The location of each T-DNA insertion

is figured at Fig. 8A. All plants were watered with Hyponex liquid fertilizer (NPK = 6-10-5;

Hyponex Japan, Osaka, Japan) diluted 1:2000.

3-1-1. Growth condition for water loss test

Seeds were sown on blocks of rockwool (Rockwool Grodan, Roermond, The

Netherlands) and vernalized for 1 week at 4°C. Seedlings were grown in a growth chamber at

25°C, a relative humidity of 50 % to 60 % and a photosynthetic photon flux density (PPFD) of

100 μmol photons m^{-2} s^{-1} under a 14-hr light/10-hr dark cycle.

3-1-2. Growth condition for dehydration test

Seeds were sown on pots (15 cm in diameter, 10 cm in depth) filled with perlite

(Nenisanso 2, MITSUI MINING & SMELTING CO., LTD. Japan) and vermiculite (VERMI,

Fukushima, Japan) (1:1, v/v). Seedlings were grown in a growth chamber at 25°C, a relative humidity of 50 % to 60 % and a photosynthetic photon flux density (PPFD) of 100 μmol photons m^{-2} s^{-1} under a 14-hr light/10-hr dark cycle.

3-1-3. Growth condition for quantitative PCR test

Seeds were sown on blocks of rockwool (Rockwool Grodan, Roermond, The Netherlands) and were vernalized for 1 week at 4°C. Seedlings were grown in a growth chamber at 22°C, a relative humidity of 50 % to 60 % and PPFD of 100 μmol photons m^{-2} s^{-1} under an 8-hr light/16-hr dark cycle. Two weeks after germination, plants were transplanted individually into pots (5 cm in diameter, 5 cm in depth) filled with vermiculite (VERMI, Fukushima, Japan) and PROMIX (PREMIER HORTICULTURE INC., PA, USA) (1:1, v/v) and were grown in the same growth chamber.

3-2. Water loss tolerance test

All experiments in water loss were carried out with 2-weeks-old plants. For examining water loss, aerial parts of 10 plants were detached and placed on a tray abaxial side up. They were weighed immediately (0 min) and every 10 min to 60 min. Weight loss was expressed as a percentage of the initial fresh weight.

3-3. Dehydration tolerance test

Dehydration tolerance test was carried out according to Yoshida *et al*. (2010) with a few modifications. In brief, 10 or 11 seeds were sown per pot (13 pots and 143 seeds in total for Col-0, 10 pots and 99 seeds in total for Ws-2). After seedlings were grown with watering for 20 days, the watering was withheld for another 29 days, the plants were then rewatered for 5 days and the number of plants surviving at the 5th day was counted.

3-4. Chronological observation of leaf surface temperature under water loss condition

The aerial parts of each plant (Col-0, Ws-2) were detached and arranged on filter paper, and the leaf surfaces were photographed with an infrared camera (CPA-E60; FLIR Systems, Inc., OR, USA) immediately (0 min) and at 10 and 20 min. The images were analysed with FLIR Tools ver. 4 software (FLIR Systems).

3-5. Determination of stomatal density on leaf surface

The number of stomata on leaf was counted with Suzuki's Universal Micro Printing (SUMP) Method. The first or second leaves of two weeks old plants of Col-0 (n=11) and Ws-2 (n=12) were detached and each leaf of abaxial side was immediately put on a plastic plate on which SUMP solution (KENIS, Osaka, Japan) was spread. The leaf was removed off in 3

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minutes and three areas on the print were observed (352.5 µm x 251.3 µm in area) under the

Olympus BX53 microscope. The number of stomata in each area was counted and stomatal

density was calculated.

3-6. RIL population and QTL analysis of water loss

QTLs for *A. thaliana* water loss tolerance were analysed by using a recombinant inbred

(RI) population of 49 lines derived from six independent crosses between Col-0 (as the male

plant) and Ws-2 (as the female plant). The F_1 seeds from each cross were sown individually,

and each plant was allowed to self-pollinate. Six or seven additional cycles of single-seed

descent were performed to obtain F_7 or F_8 seeds. Sixty DNA markers, distributed along all

chromosomes at 15-cM intervals, were used to determine genotypes in the RI population. All

RI population plants were analysed with the data of water loss at 30 min. Linkage analysis was

performed by interval mapping (Lander and Botstein, 1989) as implemented in the program

R/qtl (Broman *et al*., 2003) using the expectation-maximization algorithm (Haley and Knott,

1992). The recombination fractions were converted to cM by using the Haldane mapping

function (Haldane, 1919). Putative QTLs were also detected by R/qtl.

3-7. Comparison of promoter regions

Genomic DNAs were extracted from leaves with Plant Genomic DNA Extraction Miniprep System (VIOGENE, USA) and the intended regions were amplified by PCR (TAKARA Ex Taq®). The primers used are At1g12500 and SLAC1F (sequences at Table 2). The procedures were followed by the manufacturer's instructions. The DNAs were then analysed electropholetically. 10 μL of each amplified DNA was blended with 2 μL of loading dye (6x), loaded to 0.7 % of agar gel (Agar-powder, WAKO 013-11875), and separated in a bath of TRIS (NACALAI TESQUE) buffer solution with ethidium bromide (0.5 μg mL^{-1}) at 100 V for 30 min. Lambda HindIII Ladder (TOYOBO, Japan) was also loaded as a scale.

3-8. Construct preparation and plant transformation for histochemical GUS experiments

The *SLAC1* promoter regions were amplified from Col-0 and Ws-2 genomic DNA by PCR with primers shown in the Table 2. The fragments were cloned into *p*Drive vector (Qiagen), excised by *Bam*HI and *Xba*I restriction enzymes (at the flanking sites present in the vector) and re-cloned into the same sites of the binary vector pBI101-Hm3 (Sato *et al*., 1999). The resulting constructs (*pSLAC1-Col::GUS* and *pSLAC1-Ws::GUS*) carrying *β-glucuronidase* (*GUS*) under the control of the Col-0 or Ws-2 *SLAC1* promoter were transformed into Col-0 or Ws-2 plants (Fig. 9) by the *Agrobacterium*-mediated floral-dip method. T3 homozygous lines

were used for all experiments.

3-9. Determination of histochemical GUS activity

Aerial parts of two-weeks-old transgenic plants were immersed in adequate amounts of staining solution (50 mM sodium hydrogen phosphate, pH7.0, made of sodium dihydrogen phosphate dihydrate and di-sodium hydrogen phosphate-12 water, 0.1 % (v/v) Triton, 10 mM EDTA, 0.5 mM potassium hexacianoferrate (II) trihydrate, 0.5 mM potassium hexacyanoferrate (III) and 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide) and the solution was vacuum-infiltrated 3 times (5 min each) in a vacuum desiccator. The samples were then immediately incubated in the staining solution at 37°C for 3 to 5 hr. The staining solution was removed and the samples were washed with several changes of ethanol : water : glycerol (7 : 2 : 1, v / v / v) to remove chlorophyll. GUS staining was detected under an Olympus BX53 microscope (Olympus, Tokyo, Japan).

Sodium dihydrogen phosphate dihydrate, di-sodium hydrogen phosphate-12 water, ethylenediaminetetraacetic acid (EDTA), potassium hexacianoferrate (II) trihydrate, potassium hexacyanoferrate (III), glycerol and x-glucose were purchased from NACALAI TESQUE. Water was purified by Academic A10 water purification system (MILLIPORE).

3-10. Sample and standard preparation for quantitative-PCR

For analysing gene expressions in leaf, RNAs from two weeks old leaves were extracted with RNeasy Plant Mini Kit (QIAGEN). For analyzing gene expression in guard cells and in mesophyll cells, guard cell and mesophyll protoplasts were first isolated individually with a method developed by Pandey *et al*. (2002). The plants used were nine weeks old. For guard cell protoplast isolation, the over-night large-scale isolation method was used. RNAs from the protoplasts were extracted with an RNeasy Plant Mini Kit (QIAGEN). The RNAs obtained were then reverse-transcribed into single cDNAs using a ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO, Japan). Genomic DNAs were extracted from leaves with a Plant Genomic DNA Extraction Miniprep System (VIOGENE, USA). The cDNA samples and genomic DNA samples were then mixed with KOD SYBR qPCR Mix (TOYOBO, JAPAN) for quantitative-PCR analysis. The procedures described above were all followed by the manufacturer's instructions. Serial dilutions of genomic DNAs were used for the calibration curves in the analyses. Quantitative-PCR analyses were performed with LightCycler$^{®}$480 II (Roche Diagnostics, Basel, Switzerland). 20 μL of each sample was subjected and the condition of the procedure was as follows: 98°C for 2 min, followed by 55 cycles of 98°C for 10 sec – 60°C for 10 sec – 68°C (1 min 30 sec for *SLAC1* (*At1g12480*) and *Actin* (*At3g18780*), and 1 min for *Carbonic anhydrase* (*CA*) (*At3g01500*)). Each test was run in triplicate. The expression

ratios were calculated as the expression of each gene relative to that of *Actin*. Primer pairs are

shown in Table 2. *CA* expressions was analysed together with *SLAC1* expression to check if the

expression of *SLAC1* had derived from guard cells or from mesophyll. *CA* expresses

significantly higher in mesophyll than in guard cells (Pandey *et al*., 2002).

4. Results and Discussion

4-1. Water loss tolerance of *Arabidopsis thaliana* accessions

The goal of this work is to understand how plant natural variation in water loss tolerance is associated to their living environment. When plants lose water supply and water transpiration only occurs, they face the danger of drying so that they are in need to take actions to tolerate the water loss for keeping their internal water. I preliminarily compared the natural variation in water loss tolerance with 41 *A. thaliana* accessions. Fig. 1 showed that the 41 *A. thaliana* accessions differed in the amount of water lost for 30 min, indicating that each of them had its own water loss tolerance and none of which was the same. Hodja-obi-Garm, which lost approximately 15.4 % of its internal water 30 min after detachment, showed the highest water loss tolerance and Ws-2, which lost approximately 30.8 % of that, showed the lowest in them. Col-0, which was often used in studies, lost approximately 23.5 % of its internal water and showed intermediate water loss ratio in roughly between the Hodja-obi-Garm and Ws-2.

This result gave me a question why they differed in water loss tolerance even as they were the same *A. thaliana*s and whether the difference contributed to each of their adaptation to water deficient environment. To simplify the way to find what was behind, I selected Col-0 and Ws-2 as the test samples to be determined for this study. I first chose Col-0 since the entire sequence had been determined (The Arabidopsis Genome Initiative, 2000) and it was widely

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used in many studies, and then did Ws-2 as the counterpart of Col-0 since it showed the weakest to water loss tolerance in the experiment (Fig. 1).

Detailed further measurement of weight loss in two *A. thaliana* accessions (Col-0 and Ws-2) at 10-min intervals revealed that Col-0 kept water better than Ws-2 throughout 60 min of estimation although both of their weights decreased over time (Fig. 2). The weight of Ws-2 was 20 % less than that of Col-0 in 60 min. Significant differences were determined by a student's *t*-test analysis (P < 0.01). This result indicates that Col-0 can keep water better than Ws-2 and that Col-0 is more tolerant to water loss stress than Ws-2 (Fig. 2).

4-2. Dehydration tolerance of Col-0 and Ws-2

Measurement of survival rate of two *A. thaliana* accessions (Col-0 and Ws-2) under water deficient condition was carried out to determine the difference of water loss tolerance. The survival rate of Col-0 in 5 days after re-watering followed by 29 days of water deficiency was 100 % (143/143), while that of Ws-2 was 71.7 % (71/99). It was also noticeable that Ws-2 showed its weakness to dehydration with partially withering yellowish leaves at the end of the experiment (Fig. 3, lower right). Significant difference from Col-0 was determined by a student's *t*-test analysis (P < 0.01). This result indicates that Ws-2 is less tolerate to dehydration and can hardly survive longer than Col-0 under such water deficient condition (Fig. 3). When

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taking together with the result of the water loss experiment described earlier, it is indicated that

there certainly exists a difference in water loss tolerance between Col-0 and Ws-2.

4-3. Chronological observation of leaf surface temperature under water loss condition

Increase of leaf surface temperature under water loss condition occurs with stomatal

closure, which does not let out heat of evaporation. To characterize the difference in water loss

tolerance between Col-0 and Ws-2, I used thermal imaging of detached seedlings. In both

accessions, leaf surface temperature was almost stable within 10 min after detachment but rose

gradually the subsequent 10 min (Fig. 4A). In Col-0, it rose more than that of Ws-2 in 20 min

(Fig. 4A, right). Actual measured values obtained from the thermal image also showed that the

leaf surface temperature in Col-0 was significantly higher than that in Ws-2 20 min after

detachment of seedlings (Fig. 4B). There were two possibilities that could explain why they

differed in leaf surface temperature of detached seedlings. One was that they differed in

stomatal density and the other was that they differed in stomatal response. The determination of

stomatal density resulted that the stomatal density of Col-0 (119 ± 29 / mm^2) was not

significantly different from that of Ws-2 (121 ± 37 / mm^2). This result denied the former

possibility but implicated the latter one, which indicated that the stomata of Col-0 closed faster

than those of Ws-2 did in response to water loss.

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4-4. Searching the locus for water loss tolerance with QTL analysis

Considering that the difference in stomatal conductance was derived from genetically

based variation between Col-0 and Ws-2, I made a search with a QTL analysis for any

appropriate genes responsible for the difference. A significant QTL was obtained with the LOD

(logarithm of odds) score of over 3 on chromosome 1 (jv26/27 marker locates at 12.6 cM) (Fig.

5A), suggesting this QTL was considered to be a candidate for the loci associating to the

difference in water loss tolerance between Col-0 and Ws-2. To narrow down the location of this

QTL, I searched the vicinity of the detected peak for genes known to be involved in stomatal

movement (Daszkowska-Golec and Szarejko, 2013), and found that the jv26/27 marker lied

close to two genes that were associated to stomatal conductance (Fig. 5B). One of them was

SLOW ANION CHANNEL–ASSOCIATED 1 (*SLAC1*; *At1g12480*) gene, and the other was

NITRATE TRANSPORTER 1.1 (*NRT1.1; At1g12110*). Although the latter gene, *NRT1.1,* is

associated to stomatal response, the protein of the gene acts in stomatal opening (Guo *et al*.,

2003). It is therefore hard to be considered that the gene associates with keeping water under

water loss condition. The former gene, *SLAC1*, encodes a central guard cell S-type anion

channel. This gene is essential for stomatal closure in response to abscisic acid (ABA), CO_2,

ozone, humidity changes and light/dark cycles (Negi *et al*., 2008; Saji *et al*., 2008; Vahisalu,

2008). In guard cells, ABA triggers a cascade of reactions that leads to stomatal closure, with an

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increase in intracellular Ca^{2+} concentration (Schroeder and Hagiwara, 1989; Hedrich *et al*., 1990; McAinsh *et al*., 1990), which activates slow-sustained (S-type, e.g. SLAC1) and rapid-transient anion channels. The release of anions from the guard cells through these channels and the following release of potassium ions through potassium channels reduce turgor pressure in the guard cells and the stoma closes (Siegel *et al*., 2009). Stomatal closure is a natural response to dryness as a means of conserving water, so that I guessed that the *SLAC1* was a good candidate for the gene that contributes to the difference in water loss tolerance between Col-0 and Ws-2 by controlling stomatal response.

4-5. Comparison of Col-0 and Ws-2 in *SLAC1* and their promoter regions

I examined the nucleotide sequence of *SLAC1* and found only one amino acid substitution (alanine to methionine at position 8) in Ws-2 SLAC1 (Fig. 6). SLAC1 has 10 transmembrane α-helices, which compose a functional gating pore (Chen *et al*., 2010). Since the detected substitution was located outside the pore and was almost at the end of its N-terminal tail, I presumed that the substitution could hardly influence its gating role so that the difference in water loss tolerance between Col-0 and Ws-2 was not associated with the amino acid substitution.

The result of PCR amplifications of *SLAC1* promoter region in Col-0 and Ws-2 is figured

in Fig. 7B. It shows that the regions amplified differ in length between Col-0 and Ws-2. Sequencing of both PCR products revealed a ~2.8 kbp deletion in Ws-2. The deletion starts 541 bp upstream of the start codon of *SLAC1* and ends 949 bp downstream of the stop codon of *At1g12500*, including a gene *At1g12490*. Col-0 and Ws-2 share the proximal part of the *SLAC1* promoter spanning 541 bp, of which the 377-bp region proximal to the *SLAC1* start codon is identical in both accessions. The remaining 164 bp represents a repeated region; the other repeated region (with forward direction) is found between *At1g12490* and *At1g12500* only in the Col-0 genome (Fig. 7A). I noticed that the upstream region of *SLAC1* varied with the difference that Col-0 had a region with a gene (*At1g12490*) that Ws-2 did not have next door to *SLAC1* (Fig. 7). The gene *At1g12490* encodes an F-box-associated ubiquitination effector protein although its function has not been completely understood.

4-6. *At1g12490* showed no association to water loss tolerance

There were two possibilities that could explain the functional difference of *SLAC1* between Col-0 and Ws-2. One was that the *At1g12490* was the gene that made the difference in water loss tolerance between them, and the other was that the difference in *SLAC1* promoter made the difference in water loss tolerance between these accessions. Water loss test with *At1g12490*-knock-out mutants (Fig. 8), however, denied the possibility that the *At1g12490* was

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the water loss tolerance associated gene. In three T-DNA insertion lines (SALK_038601,

SALK_133974 and SALK_151792 in the Col-0 background) in which this gene was disrupted

by T-DNA insertions (Fig. 8A), each of their expression of *At1g12490* gene was completely

eliminated (Fig. 8B). In these lines, the weights of the detached plants decreased much as the

same as that of Col-0 and did not of Ws-2 (Fig. 8C). Significant differences between Col-0 and

the three lines and between Ws-2 and the three lines were determined by student's *t*-test

analyses ($P < 0.05$).

4-7. Determination of histochemical GUS activities

Following the result that the *At1g12490* had no association to water loss tolerance, I next

paid attention to the involvement of promoter region of *SLAC1* (Fig. 9A). To perform

histochemical GUS analyses, transgenic plants, which carried GUS under the control of *SLAC1*

promoter derived from each of Col-0 and Ws-2, were generated (Fig. 9B). Histochemical GUS

activity, which signifies the location of gene expression, clarified that both of the *SLAC1*

promoters expressed *SLAC1*s at guard cells (Fig. 10). In plants carrying the *pSLAC1-Col::GUS*

construct, GUS activity was predominantly localized in the guard cells (Fig. 10A and 10B).

Only trace levels of GUS activity were detected in vascular strands, and no GUS activity was

observed in other organs (data not shown). In plants carrying the *pSLAC1-Ws::GUS* construct,

GUS activity was predominantly observed in the vascular strands (Fig. 10C and 10D), with

trace levels detected in the guard cells, although prolonged incubation resulted in clear GUS

activity at the guard cells. The GUS result of this study was in line with a previous study

(Vahisalu *et al*., 2008) in location although the results of GUS staining indicated that the Col-0

SLAC1 promoter had higher activity in the guard cells whereas the Ws-2 *SLAC1* promoter had

lower activity in the guard cells but had higher activity in the vascular strands when compared

with the Col-0 (Fig. 10B and 10D). These suggest that the expression level of *SLAC1* varies in

location. In addition, the subsequent GUS experiments of host exchange denied that the

expressions of *SLAC1* in veins or mesophyll of Ws-2 were the manners of the promoters (data

not shown).

4-8. Determination of *SLAC1* transcript levels

To confirm the difference in the *SLAC1* transcript levels between Col-0 and Ws-2, I

determined each *SLAC1* transcript level of whole leaves, guard cell protoplasts and mesophyll

cell protoplasts by quantitative-PCR analyses. In whole leaves, the *SLAC1* transcript level had

no significant difference between Col-0 and Ws-2 (Fig. 11A). In guard cell protoplasts, it varied

between Col-0 and Ws-2 and was 2.5 times higher in Col-0 than in Ws-2 (Fig. 11B). In

mesophyll protoplasts, both transcript levels were very low in comparison with those in the

guard cells without any significant difference between Col-0 and Ws-2 (Fig. 11C). Taken

together, these data suggest that alteration of the *SLAC1* promoter influences the *SLAC1*

transcript level in the guard cells.

The difference in the *SLAC1* transcript level between Col-0 and Ws-2 may depend on the

structure of *SLAC1* promoter. In this region, a number of (T/A)AAAG elements are found

(Plesch *et al.*, 2001) in both Col-0 (5 on the sense strand and 7 on the antisense strand) and

Ws-2 (7 on the sense strand and 11 on the antisense strand) (Fig. 12). Galbiati *et al.* (2008)

suggested that a cluster of at least three (T/A)AAAG elements located on the same strand

within a 100-bp region was of great importance for guard cell–specific gene expression. Both

Col-0 and Ws-2 have several such clusters (Fig. 12). While on the other hand, two *cis*-elements,

ABA-responsive element (ABRE; ACGTGT) and the core motif of the dehydration-responsive

element (DRE; ACCGAT), were found only in the *SLAC1* promoter of Col-0 (Fig. 12). These

elements are present in many ABA-inducible genes and play important roles in drought stress

responses downstream of ABA (Yamaguchi-Shinozaki and Shinozaki, 2005; Hirayama and

Shinozaki, 2010). These elements in the *SLAC1* promoter of Col-0 may contribute to the high

level of *SLAC1* transcription in the guard cells and to the efficient response to water loss stress

in this accession. The contribution of these elements to *SLAC1* transcription in the guard cells

of Col-0 under non-stress conditions should be clarified in further experiments.

5. Conclusion

Deducing from the results described above, I had a conclusion that the difference of the water loss tolerance between Col-0 and Ws-2 was due to the difference in *SLAC1* promoter region, since the *SLAC1* promoter of Col-0 had a stronger driving force to express *SLAC1* than Ws-2 had, and more SLAC1 proteins are to be produced in Col-0 than in Ws-2 consequently. As a result, the stomata in Col-0 were able to close more robust than those in Ws-2 under water loss condition. Natural variations between Col-0 and Ws-2 have been studied for such traits as photosystem II function, freezing tolerance, ozone sensitivity and induction of volatile terpenes by herbivores (Tamaoki *et al*., 2003; McKhann *et al*., 2008; Brosché *et al*., 2010; Huang *et al*., 2010; Yin *et al*., 2012). I am now satisfied to present a new insight that a natural genetic variation contributes to plant adaptation to water loss. Plants, however, have various mechanisms for tolerating water loss stress (Ingram and Bartels, 1996; Shinozaki and Yamaguchi-Shinozaki, 2007), so that further research is necessary to understand the correspondence of plants to water loss for their adaptation to the environment.

6. Acknowledgements

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8. Tables and Figures

Table 1. Names, Arabidopsis Biological Resource Center identification codes (ABRC ID) and origin of 41 *A. thaliana* accessions used in the study

Accession	ABRC ID	Origin	Accession	ABRC ID	Origin
Bay-0	CS954	Germany	Kn-0	CS1286	Lithuania
Be-0	CS28062	Germany	Kondara	CS916	Tajikistan
Berkeley	CS8068	USA	Ler-1	CS1642	Germany
Bur-0	CS1028	Ireland	Limeport	CS8070	USA
C24	CS28126	-	Lip-0	CS1336	Poland
Chi-0	CS1072	Russia	Mh-1	CS1368	Poland
Cl-0	CS28159	-	Mt-0	CS1380	Libya
Col-0	CS1092	USA	Nd-0	CS28528	Germany
Ct-1	CS1094	Italy	NFA-8	CS22687	UK
Cvi-0	CS902	Cape Verdi	Oy-0	CS1436	Norway
Db-1	CS1102	Germany	Petergof	CS926	Russia
Di-G	CS910	France	Rak-2	CS1484	Czech Republic
Dra-0	CS28212	Czech Republic	RLD-2	CS1641	Russia
En-T	CS920	Tajikistan	Santa-Clara	CS8069	USA
Est-0	CS1148	Russia	Sav-0	CS1514	Czech Republic
Gr-3	CS1202	Austria	Sha	CS929	Tajikistan
Hodja-Obi-Garm	CS922	Tajikistan	St-0	CS1534	Sweden
Jl-3	CS1252	Czech Republic	Stw-0	CS28751	Russia
Jm-0	CS1258	Czech Republic	Tsu-0	CS1564	Japan
Ka-0	CS1266	Austria	Wil-1	CS1594	Russia
			Ws-2	CS28827	Russia

Table 2.
Primers used in this study

| Name of primer	Amplified *locus*	Sequence 5' to 3'
Carbonic anhydrase−newF	cDNA for *Carbonic anhydrase*	GTTGGAGCAGCCATTGAATA
Carbonic anhydrase−R	(*At3g01500*)	ATTCAAGTCCCCAAAGCTCA
actin−F	cDNA for *Actin*	GTTGGGATGAACCAGAAGGA
actin−R	(*At3g18780*)	GAACCACCGATCCAGACACT
At1g12480−lead3	cDNA for *SLAC1*	GAGATGGTCGGAAAACAGATTTTAG
At1g12480−lag3	(*At1g12480*)	CTCCTCTTTTGACAGCTTCAAAGTAG
At1g12500	Genomic region from first exon of	GACGATTATGGGTGTGGTTCTCTATAG
SLAC1F	*SLAC1* to final exon of *At1g12500*	CATTGATGTCGGCGAAAGTGGAATGA
ColSLACPro−F	*SLAC1* promoter	CCTCAAGCAGCGTTGCTTTTTTGAC
SLAC1Pro−R	from Col−0	TGATCAGAGCAATTTGATAATTTG
WsSLACPro−F	*SLAC1* promoter	AAAGTGTGAAGAGAAAGATTATTGG
SLAC1Pro−R	from Ws−2	TGATCAGAGCAATTTGATAATTTG
At1g12490−F	cDNA for *At1g12490*	TAAAGACAAAATCACGGGCACATAC
At1g12490−R		TTCGACTTCGCACACGACAA
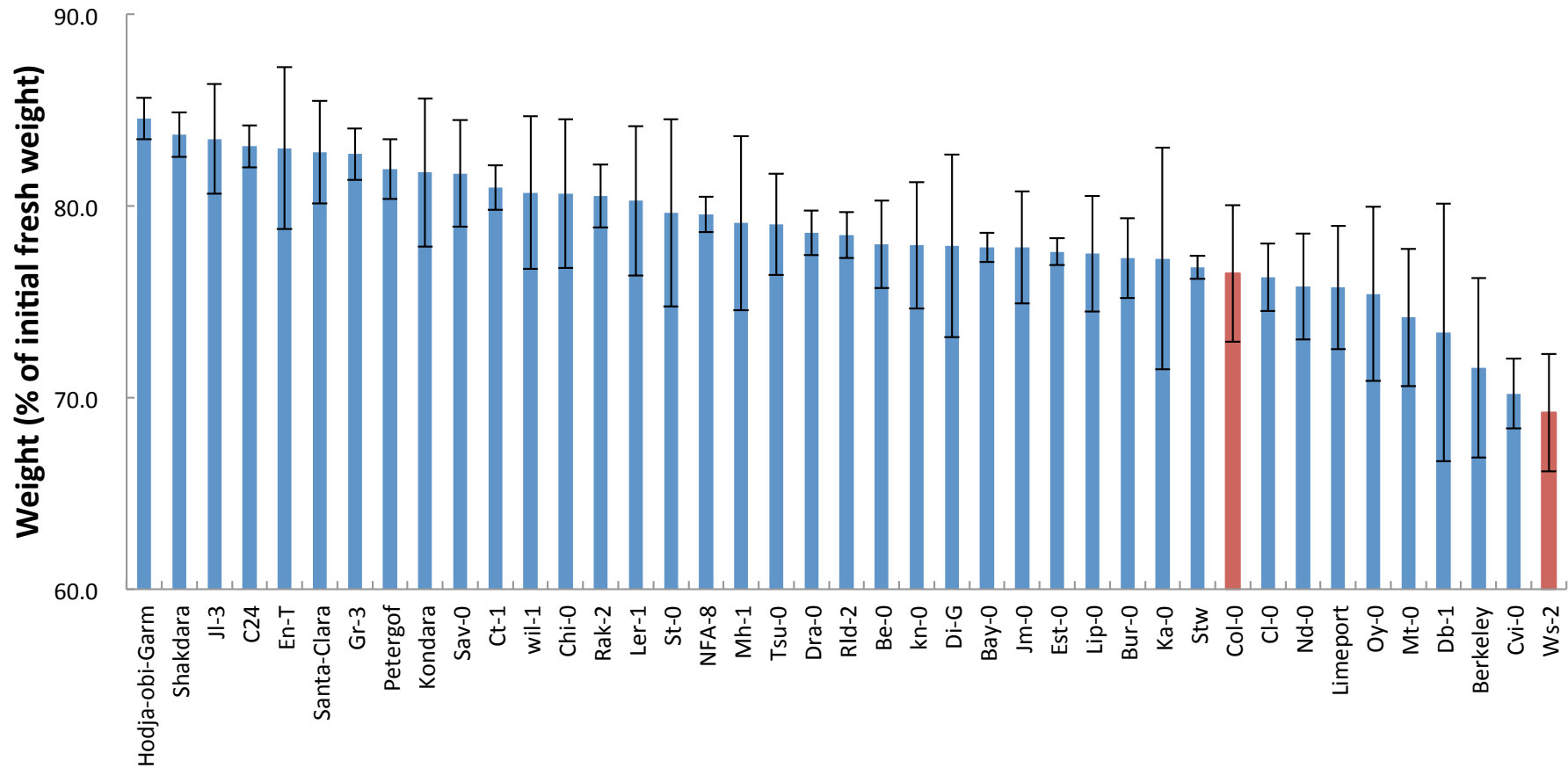
Figure 1. Water loss examination in 41 _A. thaliana_ accessions
The seeds were sown and vernalized for one week. Aerial parts of 10 plants of two-weeks-old were collected in a tray. The weight of the plants in the tray was measured at 0 min and 30 min. Weight at 30 min was expressed as % of the initial weight. Values are mean ± SD; _n = 3_ except for Col-0 (_n_ = 11) and Ws-2 (_n_ = 10).
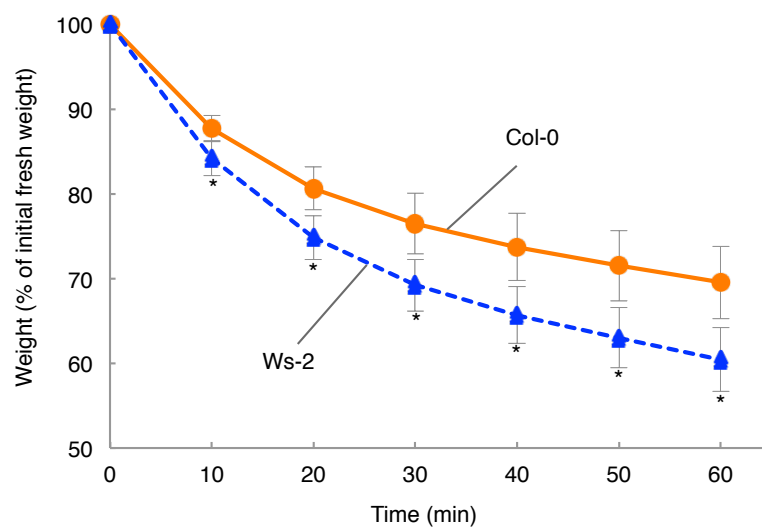
Figure 2. Comparison of water loss in detached seedlings of Col-0 and Ws-2
   Data are expressed as the relative decrease in fresh weight (the value at 0 min was set to 100%). Values are means ± SD (n=11 for Col-0; n=10 for Ws-2). Asterisks indicate significant differences from Col-0 ($p < 0.01$).
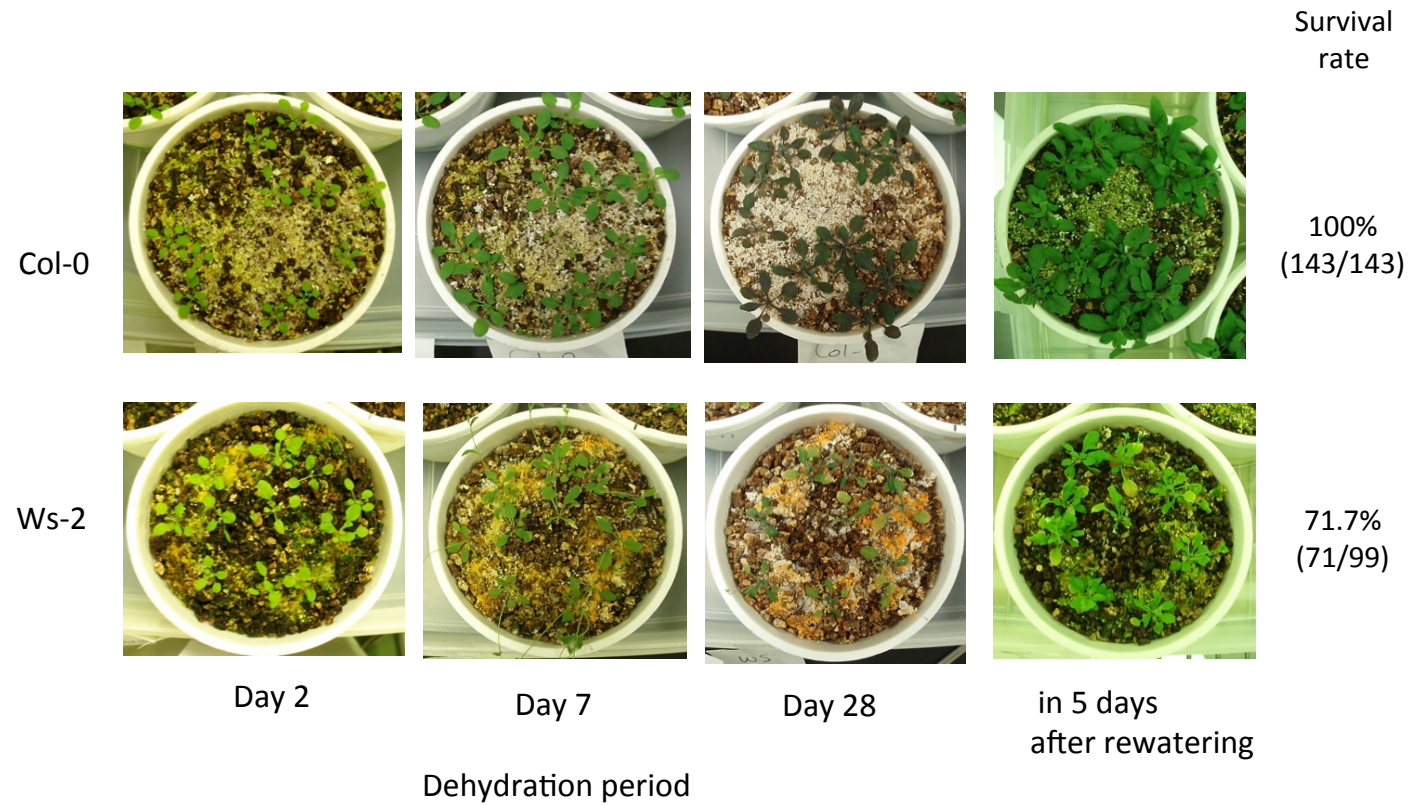
Figure 3. Dehydration tolerance of Col-0 and Ws-2
Photographs show Col-0 (upper) and Ws-2 (lower) in 2, 7, 28 days after watering
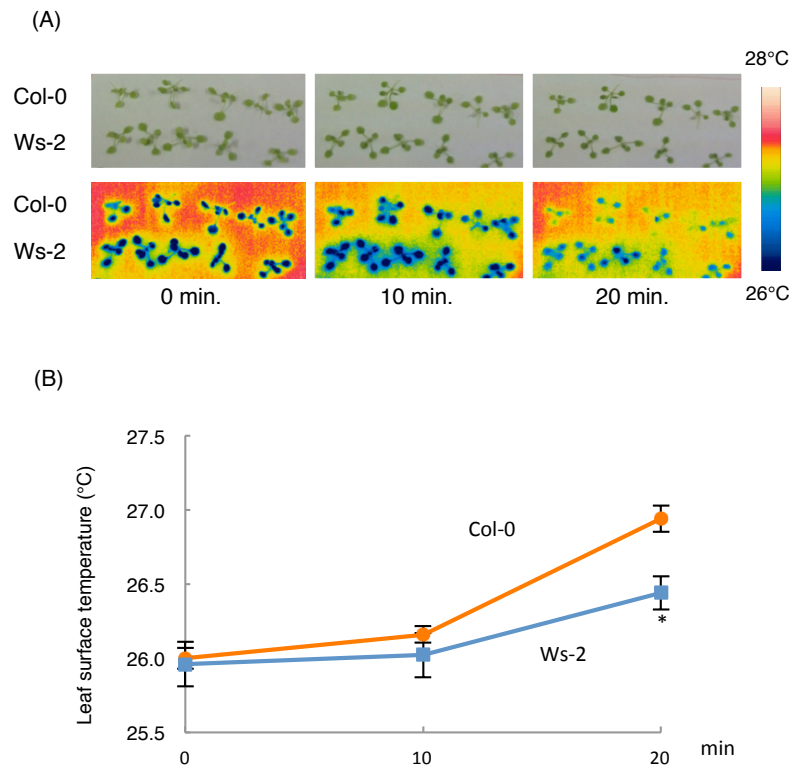was withheld, and the plants rewatered for 5 days.

(A)

(B)

Figure 4. Changes in leaf surface temperature under water loss condition in detached seedlings

(A) Upper panels: visible light images of Col-0 and Ws-2 at 10-min intervals after detachment.

Lower panels: false-colour thermal images of the leaf surface of the same seedlings. Colour coding is explained on the right side.

(B) Average leaf surface temperature of Col-0 and Ws-2. Data represent means ± SD. Asterisk indicates a significant difference from Col-0 ($p < 0.01$).
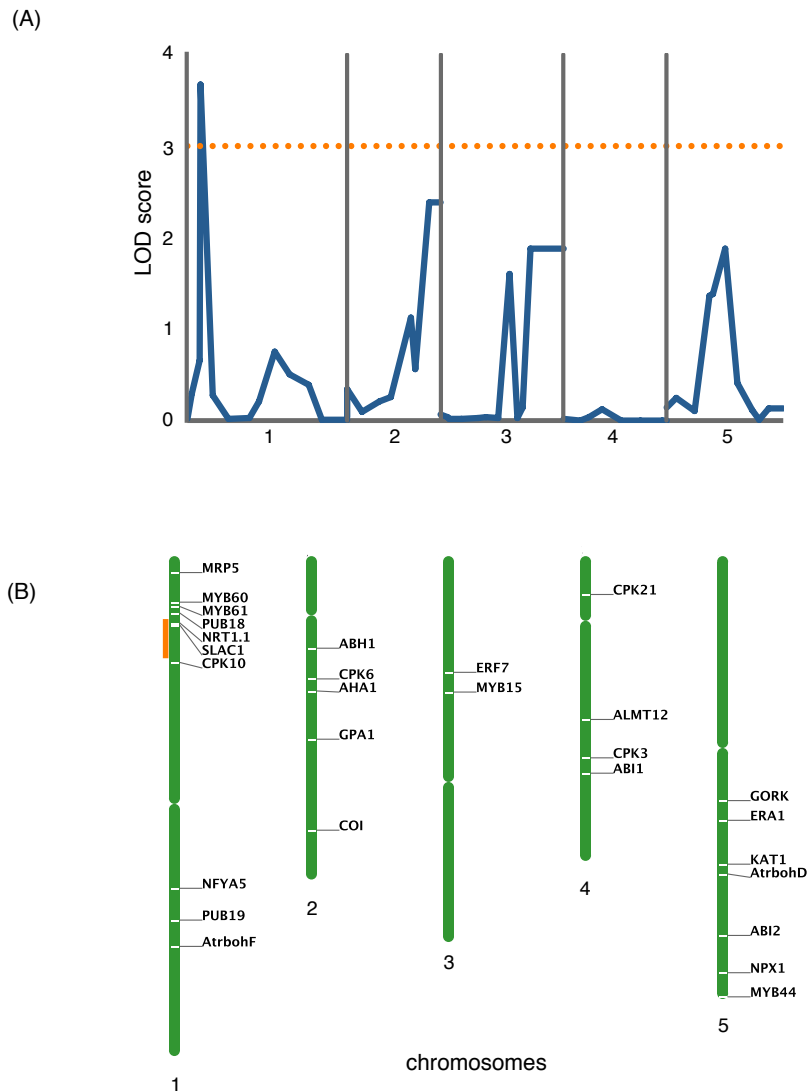
Figure 5. Genome scans for water loss tolerance between Col-0 and Ws-2

(A) QTL likelihood maps produced by composite interval mapping using the water loss tolerance experiment data at 30 min. The dotted orange line shows the LOD score of 3.

(B) Location of the genes known to be involved in the regulation of stomatal movement in *A.thaliana* (Daszkowska-Golec and Szarejko, 2013) is shown at the right of each chromosome. The region marked in orange corresponds to the highest LOD peak found in (A).
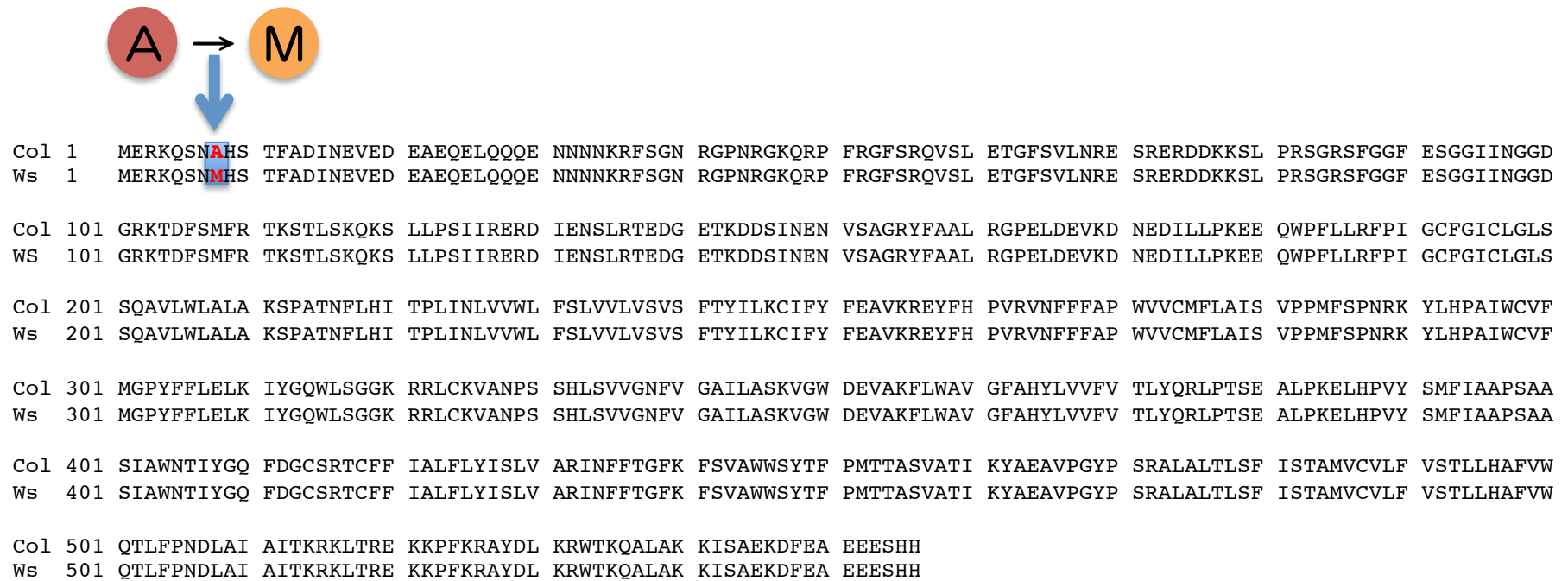
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Col 1    MERKQSNAHS TFADINEVED EAEQELQQQE NNNNKRFSGN RGPNRGKQRP FRGFSRQVSL ETGFSVLNRE SRERDDKKSL PRSGRSFGGF ESGGIINGGD
Ws  1    MERKQSNMHS TFADINEVED EAEQELQQQE NNNNKRFSGN RGPNRGKQRP FRGFSRQVSL ETGFSVLNRE SRERDDKKSL PRSGRSFGGF ESGGIINGGD

Col 101  GRKTDFSMFR TKSTLSKQKS LLPSIIRERD IENSLRTEDG ETKDDSINEN VSAGRYFAAL RGPELDEVKD NEDILLPKEE QWPFLLRFPI GCFGICLGLS
WS  101  GRKTDFSMFR TKSTLSKQKS LLPSIIRERD IENSLRTEDG ETKDDSINEN VSAGRYFAAL RGPELDEVKD NEDILLPKEE QWPFLLRFPI GCFGICLGLS

Col 201  SQAVLWLALA KSPATNFLHI TPLINLVVWL FSLVVLVSVS FTYILKCIFY FEAVKREYFH PVRVNFFFAP WVVCMFLAIS VPPMFSPNRK YLHPAIWCVF
Ws  201  SQAVLWLALA KSPATNFLHI TPLINLVVWL FSLVVLVSVS FTYILKCIFY FEAVKREYFH PVRVNFFFAP WVVCMFLAIS VPPMFSPNRK YLHPAIWCVF

Col 301  MGPYFFLELK IYGQWLSGGK RRLCKVANPS SHLSVVGNFV GAILASKVGW DEVAKFLWAV GFAHYLVVFV TLYQRLPTSE ALPKELHPVY SMFIAAPSAA
Ws  301  MGPYFFLELK IYGQWLSGGK RRLCKVANPS SHLSVVGNFV GAILASKVGW DEVAKFLWAV GFAHYLVVFV TLYQRLPTSE ALPKELHPVY SMFIAAPSAA

Col 401  SIAWNTIYGQ FDGCSRTCFF IALFLYISLV ARINFFTGFK FSVAWWSYTF PMTTASVATI KYAEAVPGYP SRALALTLSF ISTAMVCVLF VSTLLHAFVW
Ws  401  SIAWNTIYGQ FDGCSRTCFF IALFLYISLV ARINFFTGFK FSVAWWSYTF PMTTASVATI KYAEAVPGYP SRALALTLSF ISTAMVCVLF VSTLLHAFVW

Col 501  QTLFPNDLAI AITKRKLTRE KKPFKRAYDL KRWTKQALAK KISAEKDFEA EEESHH
Ws  501  QTLFPNDLAI AITKRKLTRE KKPFKRAYDL KRWTKQALAK KISAEKDFEA EEESHH
```

Figure 6. Amino acid sequences of SLAC1 from Col-0 and Ws-2
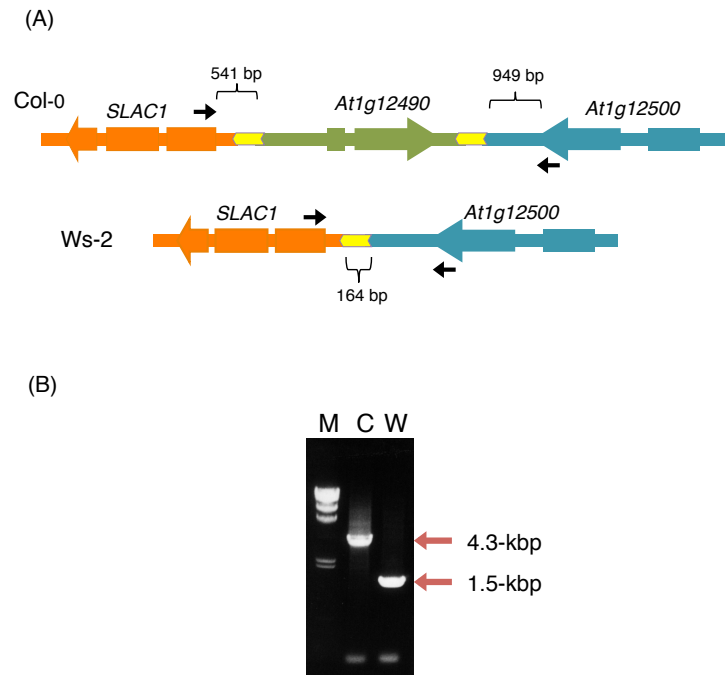The substitution of alanine in Col-0 with methionine (M) in Ws-2 is shown.

Figure 7. Differences in genomic structure upstream of *SLAC1* between Col-0 and Ws-2

(A) Genomic structure upstream of *SLAC1*. Genes are shown as arrows in the direction of transcription. Regions syntenic in Col-0 and Ws-2 are indicated by the same color. Direct repeats in the *SLAC1* promoter are shown as yellow chevron arrows. Locations of the primers used in (B) are shown by black arrows.

(B) PCR amplification of the *SLAC1* promoter region. Positions of PCR products are indicated by red arrows. M, λ/*Hin*d III size marker; C, Col-0; W, Ws-2.
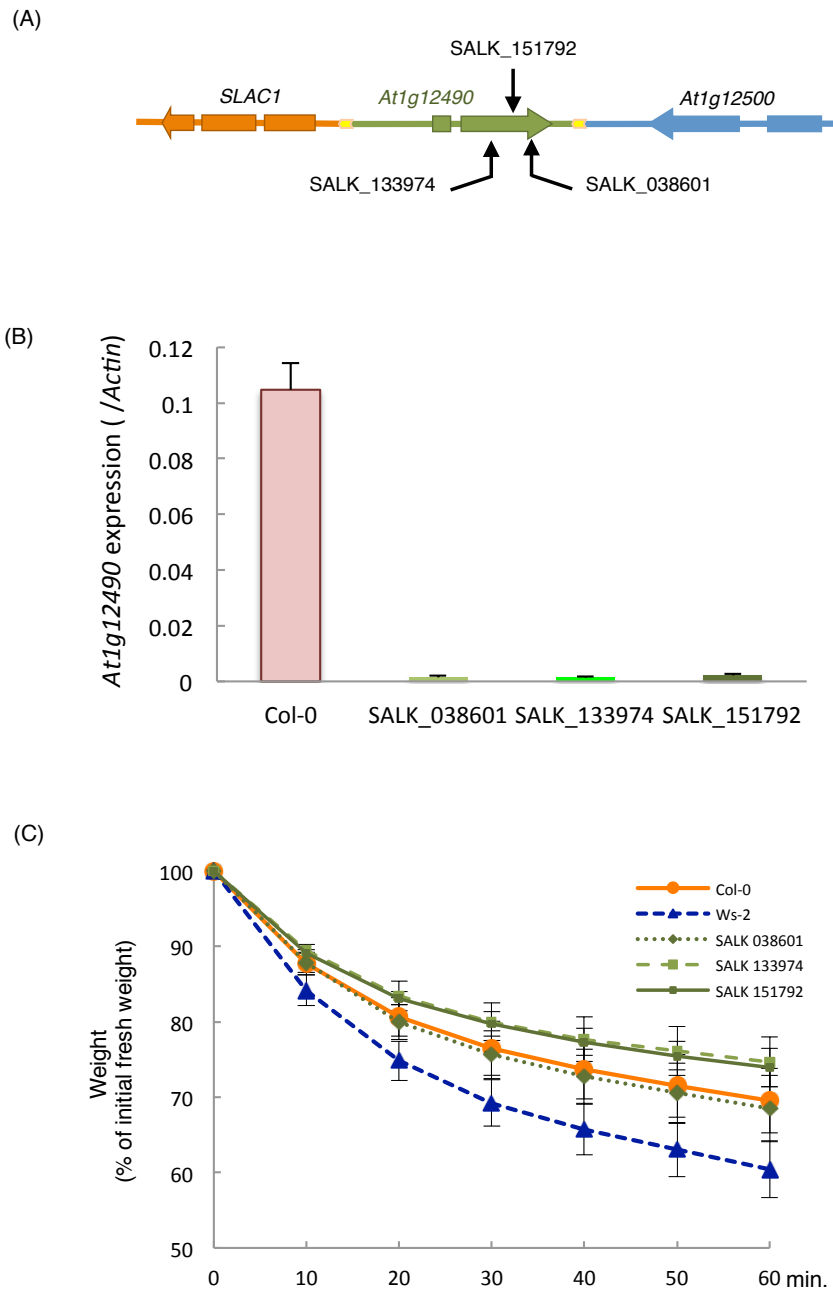
Figure 8. Water loss tolerance in *At1g12490* knockout mutants

 (A) Locations of the T-DNA insertions in *At1g12490* are indicated by arrows.

 (B) Transcript levels of *At1g12490* in Col-0 and three *At1g12490* knockout mutants. Transcript levels are presented relative to *Actin* expression level.

 (C) Water loss tolerance in Col-0, Ws-2 and knock-out plants. Data are expressed as the relative decrease in fresh weight (the value at 0 min was set to 100%). Values are means ± SD. n = 11 for Col-0, n = 11 for Ws-2 and n = 3 for the other three.
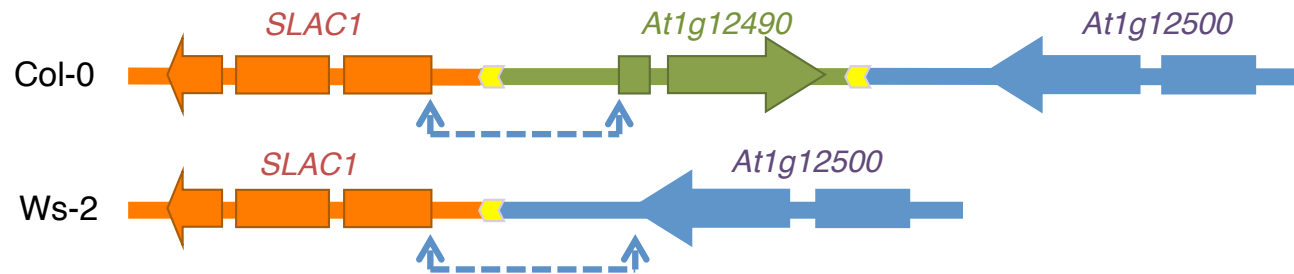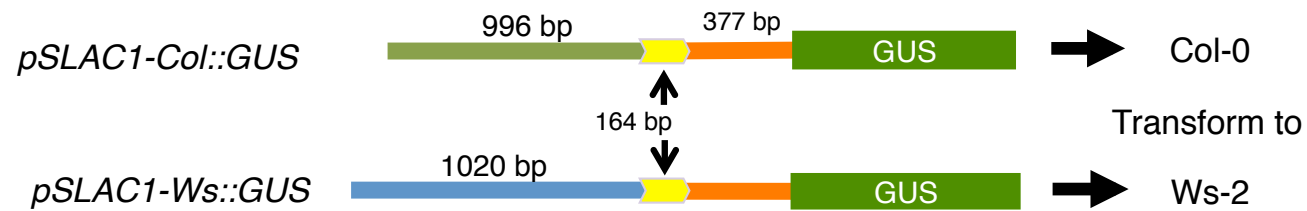
Figure 9. Constructs for histochemical GUS analysis
(A) Genomic structure upstream of *SLAC1* in Col-0 and Ws-2. Blue dotted lines indicate estimated
   SLAC1 promoter regions of Col-0 and Ws-2.
(B) The constructs *pSLAC1-Col::GUS* and *pSLAC1-Ws::GUS* used for plant transformation. Direct
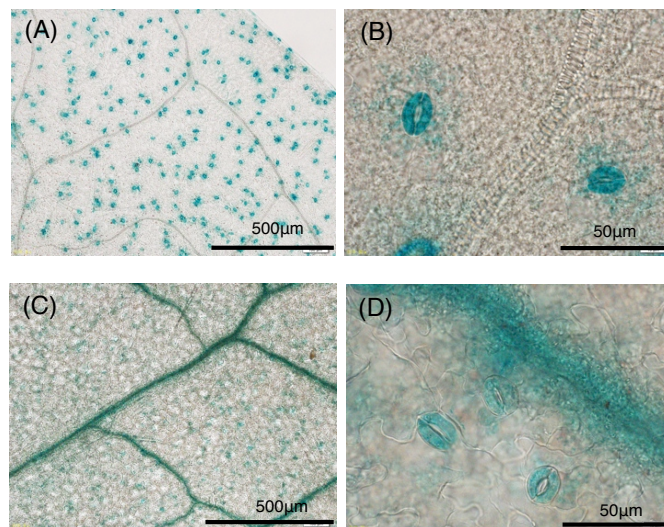   repeats found in the *SLAC1* promoter are shown as yellow chevron arrows.

Figure 10. Histochemical localization of GUS activity in transgenic plants

(A, B) GUS staining in the leaves of Col-0 transformants carrying of
pSLAC1-Col::GUS was specifically observed in guard cells.
(C, D) GUS staining in the leaves of Ws-2 transformants carrying
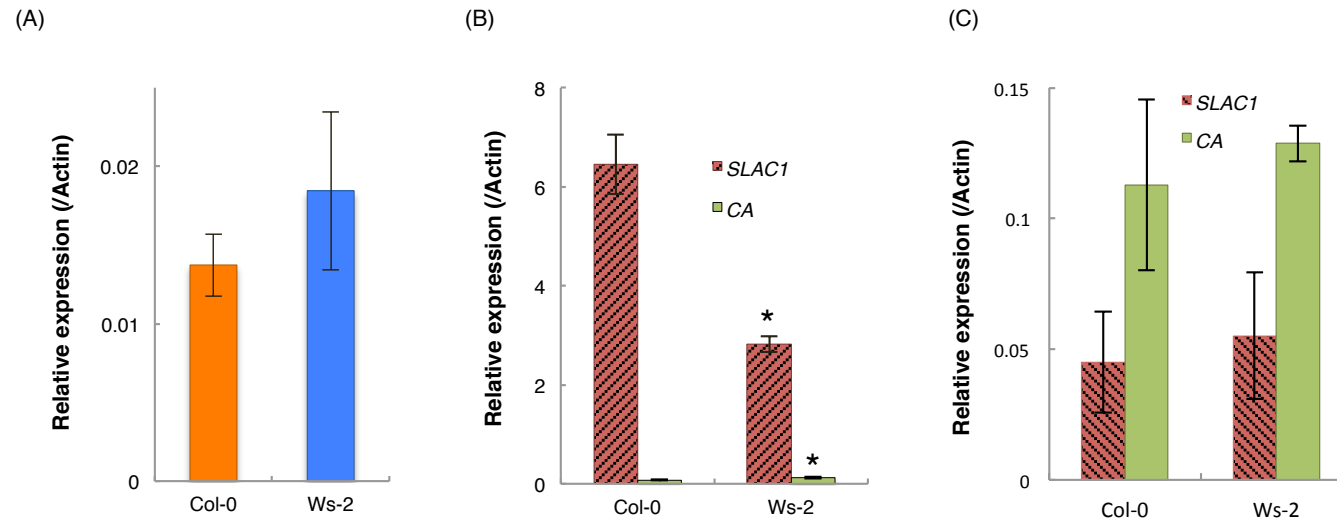*pSLAC1-Ws::GUS* was detected in guard cells and predominantly
in leaf veins.

Figure 11. *SLAC1* expression in leaves

(A) Transcript levels of *SLAC1* in whole leaves.

(B) Transcript levels of *SLAC1* and *Carbonic anhydrase* (*CA*) in guard cell protoplasts. *CA* expression was assessed to check the purity of guard cell protoplasts. Low level of *CA* expression shows that the guard cells were highly purified.

(C) Transcript levels of *SLAC1* and *CA* in mesophyll protoplasts.

Transcript level of each gene is presented relative to the *Actin* expression level. Values are means ± SD ($n = 3$). Asterisks indicate significant differences from Col-0 ($p < 0.01$).
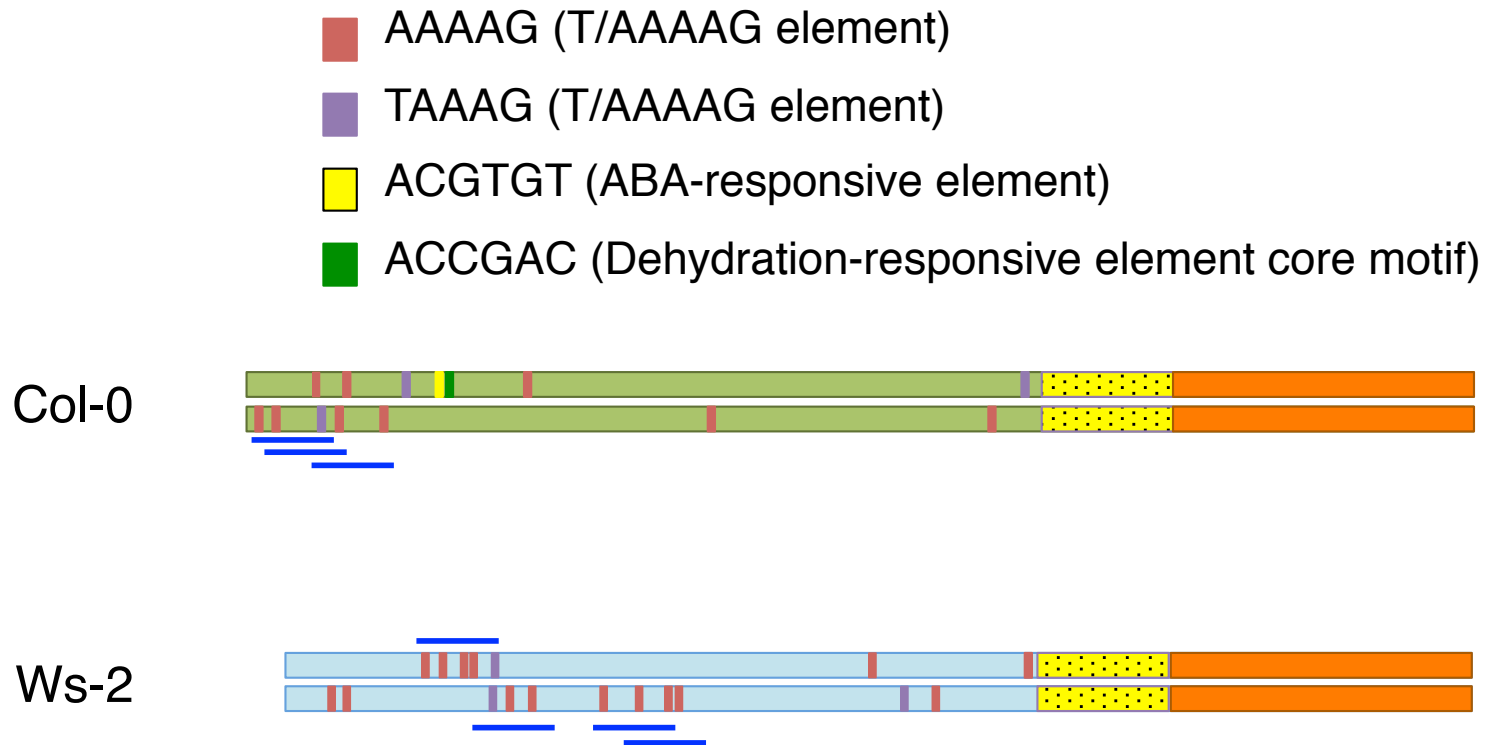
Figure 12. *cis*-elements in the *SLAC1* promoters from Col-0 and Ws-2

Structure of the *SLAC1* promoters in Col-0 and Ws-2 from distal (left) to proximal (right) . Shared and repeated regions are in orange and dotted yellow, respectively. The expected *cis*-elements are shown only in the regions of the *SLAC1* promoters that are different in Col-0 (green) and Ws-2 (pale blue). Thin blue lines indicate clusters of (T/A)AAAG elements.