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Title:

Arabidopsis *ANTHOCYANINLESS1* gene encodes a UDP-glucose:flavonoid-3-*O*-glucosyltransferase

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Abstract We isolated several mutants of *Arabidopsis thaliana* that accumulated less anthocyanin in the plant tissues, but had seeds with a brown color similar to the wild-type. These mutants were allelic with the *anthocyaninless1* mutant that has been mapped at 15.0 cM of chromosome 5. We made fine mapping of *anl1* locus and determined that *ANL1* locates between the nga106 marker and a marker designed on the MKP11 clone. About 70 genes were located between these two markers, including three UDP-glucose:flavonoid-3-*O*-glucosyltransferase like genes and a glutathione transferase gene (*TT19*). A mutant of one of the glucosyltransferase genes (At5g17050) did not compliment the *anl1* phenotype, showing that the *ANL1* gene encodes UDP-glucose:flavonoid-3-*O*-glucosyltransferase. *ANL1* was expressed in all the tissues examined, including rosette leaves, stems, flower buds and roots. *ANL1* was not regulated by *TTG1*.

Key words *anl1* mutant - *ANTHOCYANINLESS1* - *Arabidopsis thaliana* - UDP-glucose:flavonoid-3-*O*-glucosyltransferase

Anthocyanin is a plant pigment responsible for the color of flowers, fruits and autumn leaves. *Arabidopsis thaliana* accumulates much anthocyanin in the leaves and stems especially under stressed conditions. However, most research on flavonoids in *A. thaliana* is focused on tannin accumulation in the seed coat. The seed color of *A. thaliana* is brown because of the accumulation of tannins in the seed coat. More than 20 mutants that produce yellow or pale-brown seeds referred to as *transparent testa* (*tt*) mutants have been isolated (Winkel-Shirley 2001). Some *tt* mutants lack not only tannin but also anthocyanin because tannin and anthocyanin have a common pathway until leucoanthocyanidin. In *tt3*, *tt4*, *tt5*, *tt6* and *tt7*, that are defective in the genes encoding dihydroflavonol reductase (DFR), chalcone synthase (CHS), chalcone flavanone isomerase (CHI), flavanone-3-hydroxylase (F3H) and flavanone-3'-hydroxylase (F3'H), respectively, both seed color and anthocyanin accumulation were affected. On the other hand, in some mutants such as *tt1*, *tt2* and *tt12* only seed color was affected and anthocyanin level in vegetative tissues was similar to that in the wild-type plants (Koornneef 1990; Debeaujon et al. 2001). These mutants are defective in a tissue-specific regulatory gene or a gene required for the later step of tannin accumulation. Another Arabidopsis mutant, *anthocyaninless* (*anl*), has a reduced anthocyanin content but a normal seed color. In this mutant, a gene in the later step of anthocyanin biosynthesis or a regulatory gene specific for anthocyanin accumulation is considered to be affected. Two *anl* loci, *ANL1* and *ANL2*, have been reported. *ANL2*, which is located at the top of chromosome 4, has been isolated by transposon tagging. *ANL2* encodes a homeobox gene and is considered to regulate tissue specificity of subepidermis of shoot and epidermis of root (Kubo et al. 1999). *ANL1*, which is located 15 cM from the top of chromosome 5, has not been identified. Here, we isolated several *anl1* alleles and determined that it encodes an UDP-glucose:flavonoid-3-*O*-glucosyltransferase gene.

For isolation of *anl1* mutants, the M_2 population of fast neutron-irradiated Columbia seeds (Lehle Seeds, Tucson AZ) was sown on agar medium containing half- strength nutrient solution (Somerville and Ogren 1982) and 1% (w/v) sucrose. They were grown for 1 week at 22 C, then grown at 4 C under strong light (20 W/m^2). After 1-2 weeks, plants that accumulated less anthocyanin were selected. The *anl1-1* mutant was obtained from Dr. Maarten Koornneef (Wageningen University, Wageningen , The Netherlands). For fine mapping of *anl1* gene, mutant plants were selected from the F_2 population of the *anl1-2* mutant backcrossed twice, then crossed with L*er*. DNA was isolated by the method described by Liu et al. (1995). ASA1, nga249, nga151, nga106 and r89998 were used to map the *anl1* near nga106 marker. For fine mapping, CAPS markers were designed by the Monsant SNP database between Col and L*er* (Jander et al. 2002). Table 1 shows the nucleotide sequences of these markers (MSH12, MUA22, MQK4, MPI7, F5E19, F2K13 and MKP11). For the detection of polymorphism between Col and L*er*, PCR products were digested with a restriction enzyme shown in Table 1.

These genes were amplified using *Ex Taq* (Takara, Japan), and the PCR products were used for

direct sequencing of mutant alleles. Table 1 shows the nucleotide sequences of the primers used for the sequencing (AL1, AL2 and AL3). For RT-PCR, total RNA was isolated by the standard phenol method. First strand cDNA was synthesized by M-MuLV reverse transcriptase (Takara) with an oligo-d(T)$_{15}$ primer. PCR was performed for 24, 27 and 30 cycles and the product was loaded on an agarose gel, electrophoresed and stained with ethidium bromide. Table 1 shows the nucleotide sequences of the primers used for RT-PCR (AtCHS, AtDFR, AtTUB and AL3).

Seedlings were extracted with 1 ml of 0.5% HCl in methanol for 1 day at 4 C to determine the amount of anthocyanin. The absorbance was measured at 530 nm and 657 nm. A_{530}-0.25A_{657} was used to compensate for the Chl absorption at 530 nm (Mancinelli 1990).

Several plants with reduced anthocyanin content were isolated under a stress condition (4 C; 20 W/m^2). Besides several *tt* mutants, we found several plants that produced brown seeds in spite of the reduced anthocyanin content. Some had a long hypocotyl and were considered to be phytochrome mutants. We searched for *anl* mutants with only a defective anthocyanin phenotype and isolated three *anl* mutants. They were allelic with the *anl1* that had been isolated from EMS-mutagenized Landsberg. We named the *anl1* with a L*er* background *anl1-1* and those with a Col background *anl1-2*, *anl1-3* and *anl1-4*. All the mutants had a normal phenotype except for the anthocyanin content.

In WT, a 4-d-old seedling accumulated anthocyanin in the cotyledons and the upper part of the hypocotyl (a and d in Fig. 1). However, the *anl1* mutants did not accumulate a visible level of anthocyanin (b, c and e in Fig. 1). A slight amount of anthocyanin accumulated at the bottom of the flower stalk at room temperature (data not shown). When mutants were grown at 4 C under a strong light, they accumulated a small amount of anthocyanin but the amount was much less than that in WT (Fig. 2). On the other hand, the seed color of the *anl1* mutants was brown (k and n in Fig. 1) while that of *tt* mutants was yellowish (l and o in Fig. 1).

Mutants with an *anl* phenotype are considered to be defective in a regulatory gene of the anthocyanin-specific pathway or a synthetic gene in the late step of anthocyanin biosynthesis. To examine whether *ANL1* is a regulatory gene for anthocyanin biosynthesis, we examined the expression of the genes encoding phenylalanine ammonia lyase, CHS and DFR by semiquantitative RT-PCR. The expression of these genes was almost the same between WT and *anl1* (data not shown), suggesting that *ANL1* does not regulate these genes.

We used the F_2 population of *anl1-2* x L*er* for mapping. F_2 progeny of *anl1-2* x L*er* showed an approximately 3:1 (558:177) segregation of wild type: mutant phenotype, indicating that the mutation is recessive and occurred in a single nuclear gene. We mapped *anl1-2* with CAPS and SSLP markers and determined that *ANL1* located near the nga106 marker. We designed additional CAPS markers near the nga106 marker and determined that *ANL1* is located between the nga106 marker (5.397 Mb) and a marker that was designed near the top of MKP11 clone (5.679 Mb) (Fig.

3a). We tried to find a plant in which recombination occurred between these two markers but could not find such a plant among more than 400 F_2 plants.

About 70 genes were located between these two markers, including three tandem flavonoid 3-*O*-glucosyltransferase-like genes and a glutathione transferase gene (*TT19*), which were the most probable candidates. Plants mutated in the flavonoid-3-*O*-glucosyltransferase-like genes (At5g17040 and At5g17050) and the glutathione transferase gene (At5g17220) were obtained from SALK T-DNA tag line (SALK_021216, SALK_049338 and SALK_105779, respectively). SALK line in which T-DNA is inserted in At5g17040 accumulated normal amount of anthocyanin (data not shown), indicating that this gene is not involved in glucosylation of anthocyanin. Both SALK_105779 and SALK_049338 accumulated less anthocyanin (f and g in Fig. 1) as reported previously (Kitamura et al. 2004; Tohge et al. 2005). When these mutants were crossed with the *anl1* mutant, SALK_105779 complimented the mutant phenotype but SALK_049338 did not (h and i in Fig. 1; Table 2), showing that SALK_049338 is allelic to *anl1*.

To determine the mutation in the *anl1-1*, *-2*, *-3* and *-4* alleles, we sequenced these genes (Fig. 3b). In *anl1-1*, the 810th nucleotide G was changed to A. This nucleotide change resulted in the change of the 270th codon from tryptophan to stop codon. In *anl1-2*, *anl1-3* and *anl1-4*, the 958th nucleotide C is changed to T. This nucleotide change resulted in the change of the 320th codon from glutamine to stop codon.

From these results, we conclude that the *anl1* phenotype is caused by the mutation in the At5g17050 gene that encodes a anthocyanidin-3-*O*-glucosyltransferase. Our result corresponds with the result reported by Tohge et al. (2005). They showed that At5g17050 encodes an UDP-glucose:flavonoid-3-O-glucosyltransferase and mutation in the At5g17050 gene decreased the amount of anthocyanin.

Expression of *ANL1* in different organs of *A. thaliana* was examined by semiquantitative RT-PCR. *ANL1* was expressed in all the organs examined, including leaves, stems, flower buds and roots (Fig. 4a). *TRANSPARENT TESTA GLABROUS1* (*TTG1*) is known to regulate the genes such as *DFR* and *ANTHOCYANIDIN SYNTHASE* (*ANS*), which are involved in the later step of flavonoid synthesis (Winkel-Shirley et al. 1995; Pelletier et al. 1997). To determine whether *ANL1* is regulated by *TTG1*, we examined the expression of *ANL1* on a *ttg1* background. The expression of *DFR* was reduced, but the expression of *ANL1* was similar to that in L*er* (Fig. 4b).

UDP glycosyltransferase have a consensus sequence in the carboxy terminal that is thought to be involved in binding to the UDP moiety (Mackenzie et al. 1997). *ANL1* also has the consensus sequence in its carboxy terminal (amino acid residues 338-381). All the *anl1* alleles lack this conserved sequence because of the aberrant stop codon or the T-DNA insertion. Therefore, these mutants are considered to be null mutants. However, all the *anl1* mutants accumulated a trace amount of anthocyanin under strong light at a low temperature. Probably there is another gene that

has very low anthocyanidin 3-*O*-glucosyltransferase activity. *A. thaliana* contains more than 100 UDP glycosyltransferases (Ross et al. 2001). There are three genes (At1g30530, At5g17030, At5g17040) that are quite similar to *ANL1*. At1g30530 is shown to encode an UDP-rhamnose:flavonol-3-*O*-rhamnosyltransferase (Jones et al. 2003). At5g17030 and At5g17040 are located just upstream of *ANL1* and obviously triplicated from an original gene but the function of these two genes is unknown. Some of them may have a small effect especially under stressed conditions.

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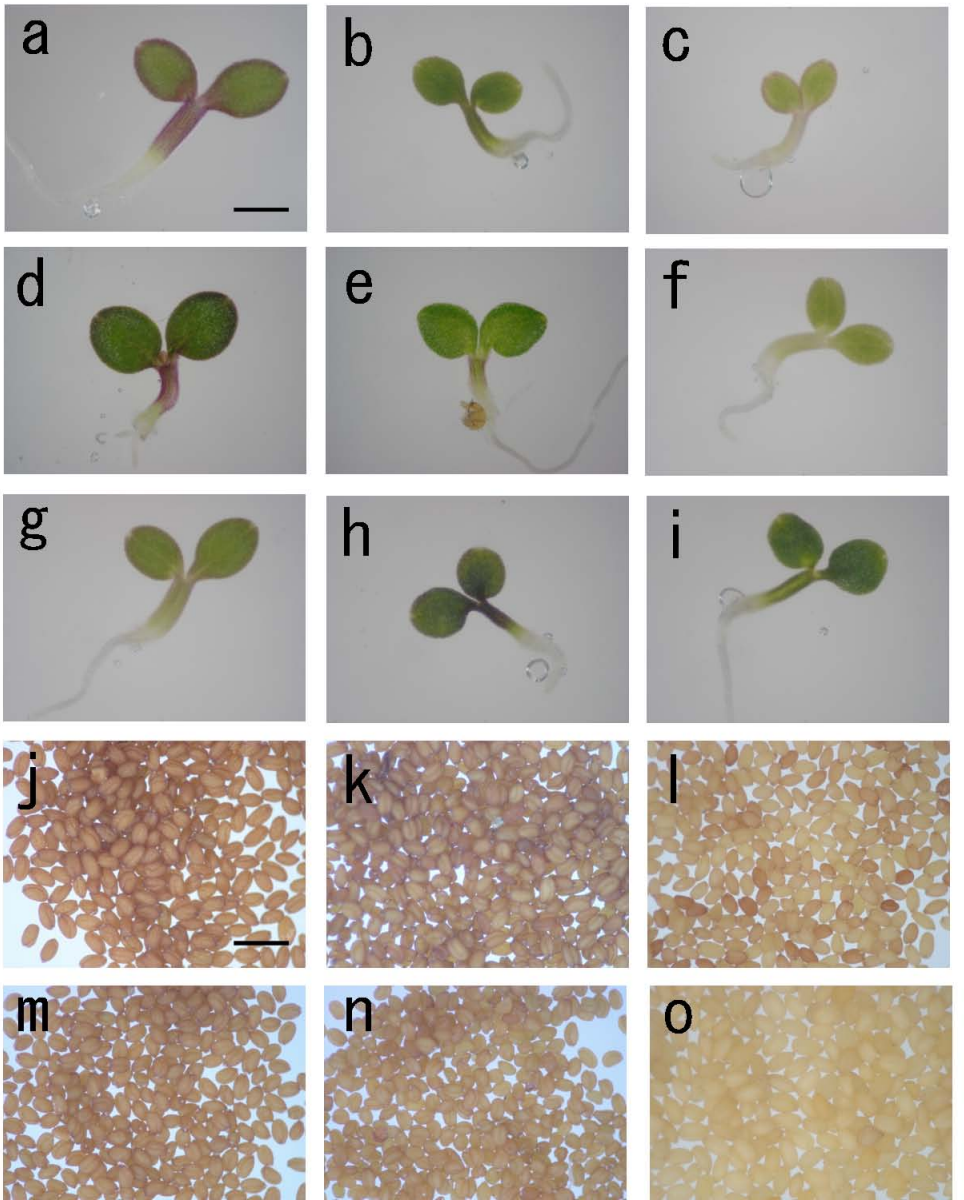
Legends for figure

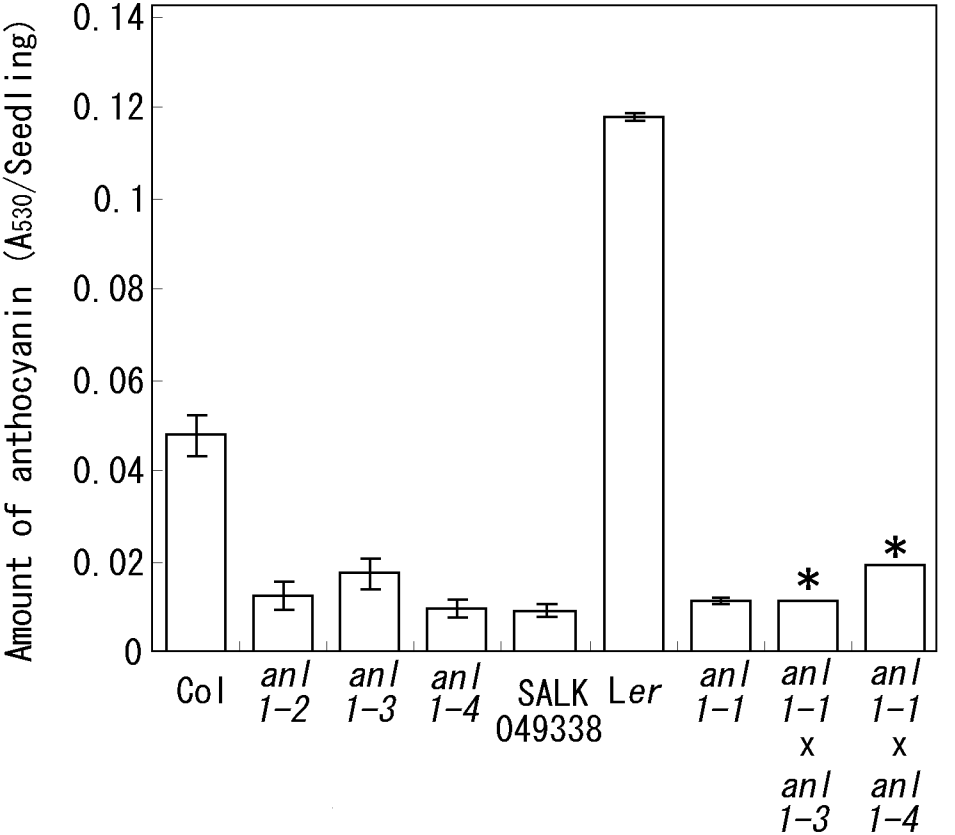
Fig. 1. Phenotype of *anl1* mutants.
Seedlings of Col (a), *anl1-2* (b), *anl1-4* (c), L*er* (d), *anl1-1*(e), SALK_105779 (*tt19*) (f), SALK_049338 (g), F_1 of SALK_105779 (*tt19*) x anl*1-2* (h), F_1 of SALK_049338 x anl*1-2* (i). Seeds of Col (j), *anl1-2* (k), SALK_105779 (*tt19*) (l), L*er* (m), *anl1-1*(n), *ttg1* (o). Bars=1mm.

Fig. 2. Amount of anthocyanin in *anl1* mutants.
Plants were grown for 1 week at room temperature, then grown at 4 C under strong light (20 W/m^2). After 4 days, anthocyanin was extracted with 0.5% HCl-MetOH and the absorbance was measured. Bars=S.D. *:Data from a single experiment.

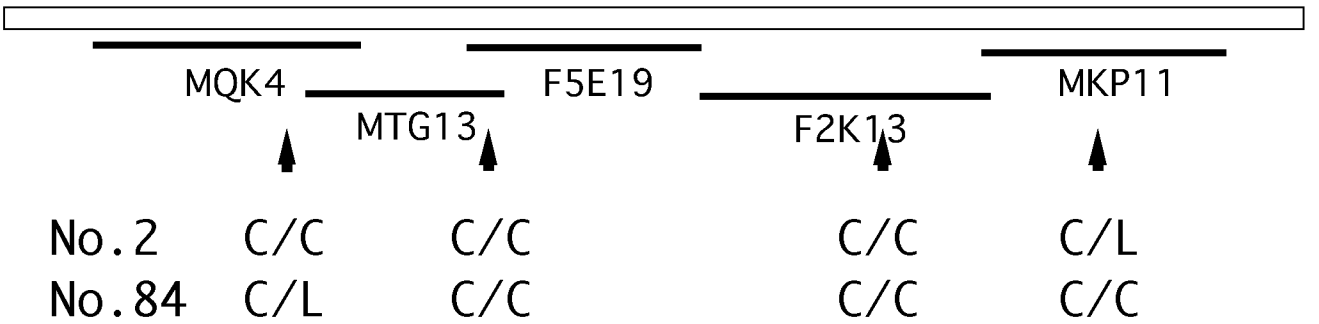
Fig. 3. a: Fine map of *ANL1* region. Arrows show the sites in which primers were designed. The genotypes of No. 2 and No. 84 that are the closest recombinant to *anl1-2* are shown. C: Columbia. L: Landsberg.
b: Schematic representation of *ANL1* gene. The sites of point mutation and T-DNA insertion are shown. Shaded box shows a consensus sequence conserved among UDP glycosyltransferase.

Fig. 4. Expression of *ANL1* gene.
a: Expression of *ANL1* gene in leaves (L), stems (S), buds (B), and roots (R). Expression was examined by semiquantitative RT-PCR. The number of PCR cycles was 30 for *ANL1* and 27 for tubulin.
b: Expression of *ANL1* gene in seedlings of L*er* and *ttg1* mutant. Expression was examined by semiquantitative RT-PCR. The number of PCR cycles was 24 (n), 27 (+3), 30 (+6) for *CHS* and tubulin, and 27 (n), 30 (+3), 33 (+6) for *DFR* and *ANL1*.

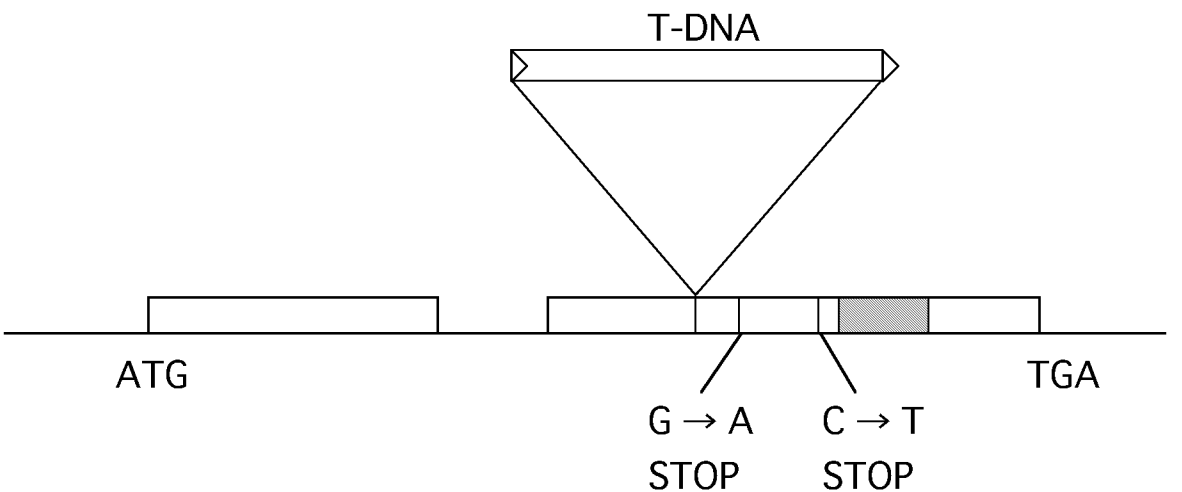

a
b
c
d
e
f
g
h
i
j
k
l
m
n
o


Amount of anthocyanin (A530/Seedling)
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
*
*
Col
anl 1-2
anl 1-3
anl 1-4
SALK 049338
Ler
anl 1-1
anl 1-1 x anl 1-3
anl 1-1 x anl 1-4

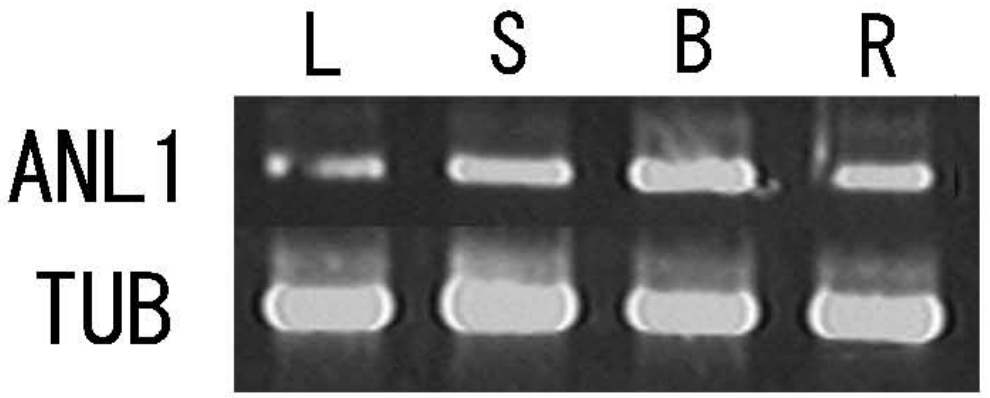
a


MQK4
MTG13
F5E19
F2K13
MKP11
No.2 C/C C/C C/C C/L
No.84 C/L C/C C/C C/C

b


T-DNA
ATG
TGA
G → A
STOP
C → T
STOP

a



b

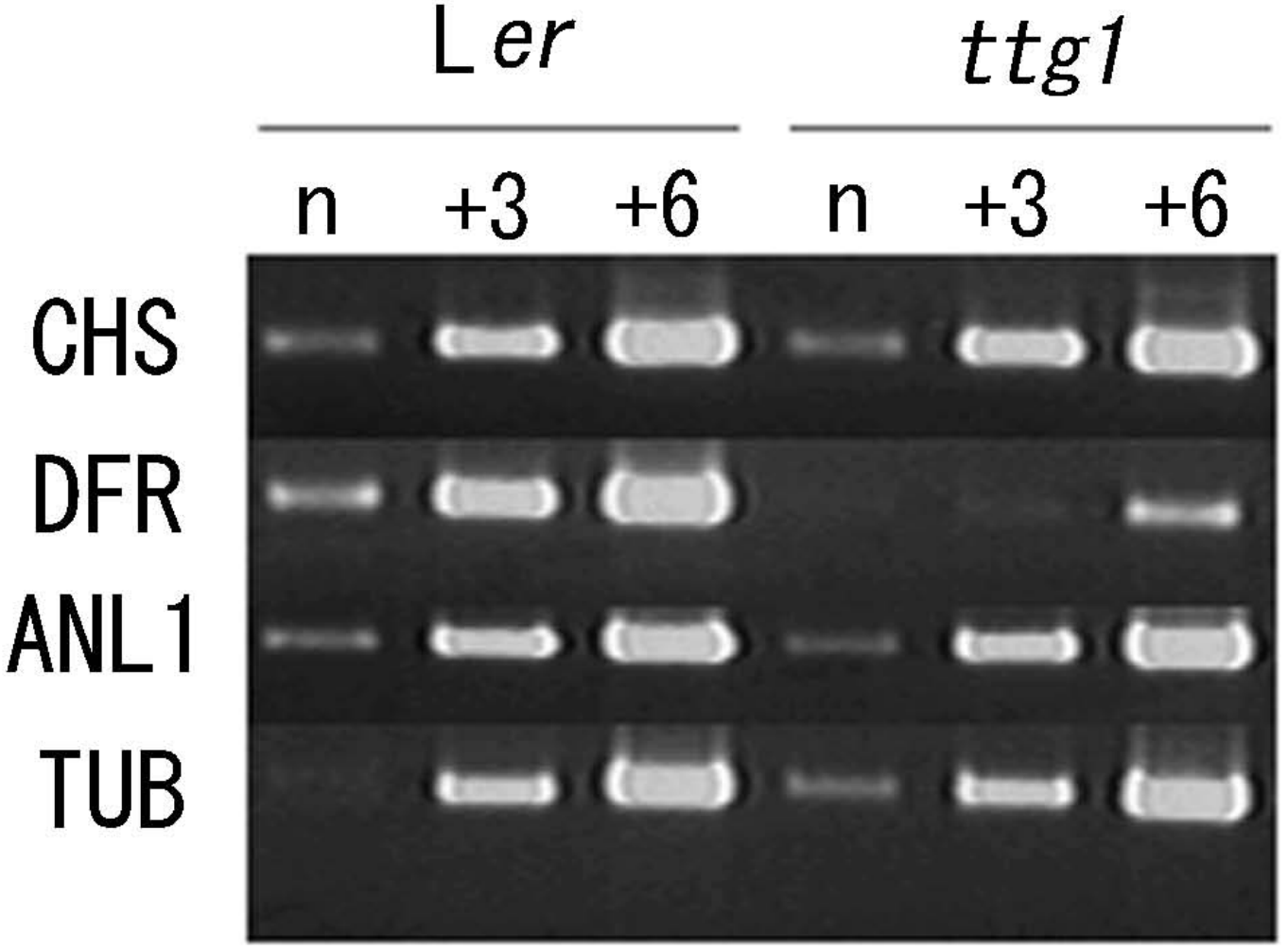


Table 1. Primer sequences used for mapping sequencing and RT-PCR

MSH12 Fwd	5'-TACCTTCTGTATCACAACAA-3',	*Mbo*I
Rev	5'-CAATGGATACCAATTGTCAT-3',	
MUA22 Fwd	5'-TGCAAACACGAACTTTTCTT-3',	*Mbo*I, *Taq*I
Rev	5'-TACTTATCTCTTCTGGTTAG-3',	
MQK4 Fwd	5'-TGTTTCTTTGCATTTGCGTT-3',	*Cla*I, *Mbo*I
Rev	5'-AAGCTTGTTCCACATACTAT-3',	
MPI7 Fwd	5'-CCGTATCGTCTGATTACAAA-3',	*Taq*I
Rev	5'-TTGGGCACTAGCATTTACAG-3',	
F5E19 Fwd	5'-CATTCGGTATCCTTTCTTCT-3',	*Mbo*I
Rev	5'-AGACGTTTAATAAGCGGTAC-3',	
F2K13 Fwd	5'-ATGGTGCGAGTAATGAAGAA-3',	*Mbo*I
Rev	5'-CACCCTTTTTGCTAGTGTTA-3',	
MKP11 Fwd	5'-CTTGAAGGGTATAGGGTTAT-3',	*Taq*I
Rev	5'-GAGATGATGGCATATTGATC-3',	
AL1-F	5'-GTTGTTTACTTGTTATATAA-3'	
AL1-R	5'-AGACAAAATCACAAGTCGGA-3'	
AL2-F	5'-CTTCTGGTTCTGTGGCGTAC-3'	
AL2-R	5'-TCGATTCCAACCCTTCTGCT-3'	
AL3 Fwd	5'-CTGCTCATCTCTACACAGAT-3'	
Rev	5'-CATTTCACATTCAAATAATG-3'	
AtCHS Fwd	5'-ATGGTGATGGCTGGTGCTTC-3'	
Rev	5'-AGCAGACAACGAGGACACGT-3'	
AtDFR Fwd	5'-GTGTGTGTAACCGGCGCTTC-3'	
Rev	5'-TCCCTTGGCGGCTGCTTGTT-3'	
AtTUB Fwd	5'-ATCTCGAGCCTGGTACTATG-3	
Rev	5'-CATTCCTTCTCCTGTGTACC-3'	

Table 2. Number of plants with or without anthocyanin

Strain	anthocyanin	no visible anthocyanin
Col	19	0
anl1-2	0	23
anl1-3	0	25
anl1-4	0	37
anl1-1 x *anl 1-3*	0	20
SALK049338	0	32
SALK105779 (*tt19*)	0	28
anl1-2 x SALK049338	0	18
anl1-2 x SALK105779 (*tt19*)	21	0
anl1-3 x SALK049338	0	13
anl1-3 x SALK105779 (*tt19*)	29	0
anl1-4 x SALK049338	0	17
anl1-4 x SALK105779 (*tt19*)	22	0