Paramutation-Like Interaction of T-DNA Loci in Arabidopsis

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Abstract

In paramutation, epigenetic information is transferred from one allele to another to create a gene expression state which is stably inherited over generations. Typically, paramutation describes a phenomenon where one allele of a gene downregulates the expression of another allele. Paramutation has been described in several eukaryotes and is best understood in plants. Here we describe an unexpected paramutation-like trans SALK T-DNA interaction in Arabidopsis. Unlike most of the previously described paramutations, which led to gene silencing, the trans SALK T-DNA interaction caused an increase in the transcript levels of the endogenous gene (COBRA) where the T-DNA was inserted. This increased COBRA expression state was stably inherited for several generations and led to the partial suppression of the cobra phenotype. DNA methylation was implicated in this trans SALK T-DNA interaction since mutation of the DNA methyltransferase 1 in the suppressed cobra caused a reversal of the suppression. In addition, null mutants of the DNA demethylase ROS1 caused a similar COBRA transcript increase in the cobra SALK T-DNA mutant as the trans T-DNA interaction. Our results provide a new example of a paramutation-like trans T-DNA interaction in Arabidopsis, and establish a convenient hypocotyl elongation assay to study this phenomenon. The results also alert to the possibility of unexpected endogenous transcript increase when two T-DNAs are combined in the same genetic background.

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Introduction

Epigenetic modifications can be defined as heritable information that is not encoded in the nucleotide sequence of DNA. An important epigenetic mark is cytosine methylation of DNA, as severe defects in DNA methylation in mammals are embryonic lethal and in plants lead to pleiotropic morphological defects [1]. To avoid these deleterious effects, DNA methylation patterns are carefully maintained and stably inherited.

DNA methylation has also been implicated in paramutation [2–4] where specific DNA sequences interact in trans to establish mitotically heritable gene expression states [5]. The maize b1 locus encoding a transcription factor regulating anthocyanin biosynthesis provides a classic example of paramutation. Two alleles of the b1 locus, the b’ and B-I, are involved in paramutation. The B-I allele has a high and the b’ low level of expression and when B-I and b’ are combined in the same nucleus the B-I gets converted to b’ [6,7,8]. A hepta-repeat DNA sequence required for the B-I to b’ paramutation is located approximately 100 kb upstream of the transcription start site of b1 [9]. Several other paramutation loci have been documented in maize and other plants [reviewed in [5]]. Paramutation has also been described at the tyrosine kinase receptor encoding Kit locus in mice [10] indicating that the phenomena occurs across eukaryotes. The exact mechanism of paramutation is not clear but has been shown to involve RNA mediated transfer of information between paramutable and paramutable alleles in both plants and animals [10,11,12,13]. This has led to models where RNA directed DNA methylation (RdDM) is responsible for the differential DNA methylation observed in paramutation [6,14].

In plants, cytosine DNA methylation is found in all sequence contexts (CG, CHG and CHH) and several enzymes involved in DNA methylation have been identified. Existing DNA methylation is maintained by three different pathways: DNA METHYLTRANSFERASE 1 (MET1) maintains CG methylation, CHROMOMETHYLASE 3 (CMT3) maintains CHG methylation [15] and CHH methylation is maintained by DOMAINS REARRANGED METHYLTRANSFERASE 1 and 2 (DRM1 and DRM2) [16,17]. De novo methylation of previously unmethylated sequences is also carried out by DRM2 [16]. Plants also have a mechanism to remove DNA methylation, for example through the REPRESSOR OF SILENCING1 (ROS1) DNA demethylase activity [18]. The final DNA methylation pattern of a genome is established by the combined activity of DNA methyltransferases and demethylases [19].
We discovered that non-allelic SALK T-DNA insertions in Arabidopsis genome can interact in trans and cause epigenetic changes creating a DNA methylation dependent paramutagenic allele in the process. DNA methylation mediated trans T-DNA interactions, where one T-DNA induces an epigenetic silencing effect on a second T-DNA, have previously been documented in tobacco [20]. A similar trans silencing T-DNA effect was also observed in Arabidopsis and attributed to the presence of the cauliflower mosaic virus 35S promoter in the SALK T-DNA inserts [21]. In our case the SALK T-DNA triggered epigenetic changes led to increased expression of the endogenous locus where the T-DNA was residing, and in the process this locus became paramutagenic. Characterisation of this SALK T-DNA interaction indicated the involvement of DNA methylation, which was modulated by MET1 and possibly ROS1. The results alert to an unexpected phenomenon associated with T-DNA insertions and describe a new paramutagenic interaction in Arabidopsis.

Results

Suppression of the Primary Cell Wall cobra T-DNA Insertion Mutant

The concept that co-expressed genes tend to be functionally related [22] led us to investigate the genetic interactions in the primary cell wall co-expressed gene network of Arabidopsis (Figure S1) [23]. We discovered that SALK T-DNA mutants of the receptor-like kinase SRF6 (srf6-1 and srf6-5) were able to partially suppress the growth defect of the cellulose deficient mutant cobra (cob-6) [24], but did not suppress mutants of CELLULOSE SYNTHASE 6 (pc1) [25] or CELLULOSE SYNTHASE 3 (cl1) [26] (Figure 1A and Figure S2). cob-6 carries a SALK T-DNA insertion in the first intron of the COBRA gene [24] whereas pc1 and cl1 contain a single nucleotide change in the corresponding gene [25,26]. The locus of the different srf6 and cob alleles used in this study are illustrated in Figure S3. COBRA is an extracellular glycosylphosphatidylinositol anchored protein, which is essential for cellulose synthesis and anisotropic growth [27]. The cob phenotype is consequently most obvious in young roots and dark grown hypocotyls (Figure 1B) [28]. Etiolated epicob-6 hypocotyls were slightly shorter than the srf6cob-6 double mutant suggesting that the srf6 allele had a small additional effect on the phenotype. The epicob-6 plants were grown for four generations but no reversion back to cob-6 phenotype was observed (Figure 1C and Table S2). Hence, epicob-6 can be inherited to progeny independent of the srf6 mutation, and this inheritance is stable for at least four generations.

Increased COBRA Transcript Level Explained cob-6 Suppression

A cob null mutant is seedling lethal [28] but homozygous cob-6 plants are viable, and produce viable seeds [24]. The cob-6 phenotype was fully complemented by a genomic fragment of the COBRA gene (Figure S6), confirming that the cob-6 phenotype is due to the T-DNA insert in the first intron of the COBRA gene. We tested for the possibility that the T-DNA-containing intron could

Figure 1. srf6 SALK T-DNA triggered suppression of cobra phenotype and inheritance of epicob-6. (A) Quantification of hypocotyl length in four-day-old dark grown seedlings. Genotypes, mean and SE are indicated, n = 30–40. (B) Four-day-old dark grown seedlings of Col-0, srf6-1, srf6-cob-6, epicob-6 and cob-6. (C) Phenotype comparison of etiolated epicob-6 for four generations. doi:10.1371/journal.pone.0051651.g001

Epigenetic Inheritance of cob-6 Suppression

The segregation ratio of the F2 progeny from the cross between srf6-1 and cob-6 deviated substantially from the expected for recessive mutations, which would be one suppressed cob-6 seedling per 16 seedlings. Instead we observed one suppressed cob-6 seedling per ca. four seedlings (N = 228 of which 60 were suppressed homozygous cob-6) in the F2 progeny. The same result was also obtained with a second SRF6 knock-out allele srf6-3. To clarify the mechanism of this unusual phenotypic segregation ratio, and the genetic interaction between SRF6 and COBRA, we genotyped the F2 plants. We discovered that the cob-6 suppressor phenotypes were always homozygous for cob-6 but either wild-type, hetero- or homozygous for srf6. Hence, cob-6 single and srf6cob-6 double mutants showed a very similar suppressed phenotype in the F2 progeny of the srf6×cob-6 cross. To further investigate the suppressor mechanism we backcrossed the srf6-1cob-6 double mutant with the parental cob-6 and surprisingly found that the F1 plants still showed the suppressed cob-6 phenotype (Figure S5). Thus, once the cob-6 suppression was established even a wild-type copy of SRF6 was unable to completely reverse the suppression. These results, together with the deviant F2 segregation from the srf6×cob-6 cross, suggested that srf6 acts dominantly and suppresses the cob-6 phenotype through an epigenetic mechanism.

To distinguish between the original cob-6 line and the suppressed cob-6 lines with wild-type SRF6 locus derived from the F2 of the srf6×cob-6 cross, the suppressed cob-6 lines were named epicob-6 (Figure 1B and Table S1). The etiolated epicob-6 hypocotyls were slightly shorter than the srf6cob-6 double mutant suggesting that the srf6 allele had a small additional effect on the phenotype. The epicob-6 plants were grown for four generations but no reversion back to cob-6 phenotype was observed (Figure 1C and Table S2). Hence, epicob-6 can be inherited to progeny independent of the srf6 mutation, and this inheritance is stable for at least four generations.

Figure 1. srf6 SALK T-DNA triggered suppression of cobra phenotype and inheritance of epicob-6. (A) Quantification of hypocotyl length in four-day-old dark grown seedlings. Genotypes, mean and SE are indicated, n = 30–40. (B) Four-day-old dark grown seedlings of Col-0, srf6-1, srf6-cob-6, epicob-6 and cob-6. (C) Phenotype comparison of etiolated epicob-6 for four generations. doi:10.1371/journal.pone.0051651.g001
be correctly spliced out in cob-6. Indeed, we were able to amplify the full-length cDNA from cob-6 plants (Figure S7). Sequencing of this COBRA cDNA showed that it encodes for a wild-type COBRA protein (data not shown). Quantitative real-time PCR (qPCR) experiments using primers amplifying across the intron containing the cob-6 T-DNA showed that COBRA mRNA levels in cob-6 mutant are about 10% of wild type (Figure 2A). Since the cob-6 mutant only resulted in reduced COBRA mRNA, it is plausible that the suppressed cob-6 phenotype could be due to a change in COBRA mRNA levels, especially since most of the described epigenetic phenomena affect gene expression [29,30]. We, therefore, compared COBRA mRNA levels in Col-0, srf6-1, cob-6, srf6-1cob-6, and epicob-6 (Figure 2A). srf6-1cob-6 and epicob-6 displayed a significant increase of COBRA mRNA compared to cob-6, approx. 20% and 17% of wild-type COBRA mRNA levels, respectively. To confirm that the suppressed cob-6 phenotype was due to increased levels of COBRA transcript we crossed srf6-1 with a complete knock-out of COBRA (cob-6) [29]. The cob-6 mutant was not suppressed by srf6-1 (Figure 2B). These results established that srf6 SALK T-DNA mutations suppress the cob-6 SALK T-DNA knock-down mutant through a transcript increase mechanism.

Epigenetic cob-6 Suppression is Caused by trans SALK T-DNA Interaction

We suspected that the cob-6 suppression was linked to the SALK T-DNA insertion in the srf6 lines rather than the SRF6 defect. To test this we crossed cob-6 with three randomly selected SALK T-DNA insertion lines and a SAIL T-DNA insertion in the SRF6 homologue SRF4. All three SALK T-DNA lines suppressed cob-6 to varying degrees, but the srf4-1 SAIL line had no effect on the cob-6 phenotype (Table 1). Hence it appeared that the SALK T-DNAs could somehow interact with each other to promote COBRA expression. To further test this hypothesis we obtained a premature stop codon allele of SRF6 (srf6-4) from a Landsberg TILLING population (Figure S3) and crossed this line to a cob-6, which had been backcrossed to Landsberg live times. No suppressed cob-6 plants were observed in the F2 progeny of the cross between the cob-6 in Landsberg and srf6-4 (Figure S8). Thus it could be concluded that the cob-6 suppression is caused by a dominant trans interaction of SALK T-DNA insertions.

To further elucidate the SALK T-DNA mediated epigenetic effects, we crossed epicob-6 to cob-6 and Col-0. In the F1 and F2 population derived from the cross between epicob-6 and cob-6, all the plants showed suppression of the cob-6 phenotype (Figure 3A and Table S3). This result showed that the epicob-6 established by the srf6 SALK T-DNA was able to convert cob-6 into the suppressed epicob-6 state. Therefore the epicob-6 suppressor state behaved similarly to a paramutagenic allele in that it could convert a cob-6 paramutable allele to a higher expression state. Interestingly, in the F2 population of epicob-6×Col-0 the suppression was lost and only Col-0 and cob-6 were observed, suggesting that two allelic copies of the cob-6 T-DNA were required for the maintenance of the suppression state (Table S3).

Increased COBRA Expression in cob-6 was Associated with Increased DNA Methylation

To assess the epigenetic nature of the SALK T-DNA mediated increase in COBRA transcript levels in cob-6, we analysed whether DNA methylation or histone acetylation could be involved in this process. We grew etiolated seedlings on solid growth media containing the DNA methylation inhibitors 5-azacytidine and zebularine, and the histone deacetylase inhibitor trichostatin A (TSA). 5-azacytidine and zebularine reduce DNA methylation levels through deactivating DNA methyltransferases [31,32], and TSA leads to increased acetylation of histones [33]. We discovered that the srf6-1cob-6 and epicob-6 mutant reversed to cob-6 phenotype when grown on either 5-azacytidine (Figure 4A), or zebularine (Figure 4B), but not on TSA (Figure 4C). The 30 μM of 5-azacytidine or zebularine had no visible effects on the wild-type phenotype (Figure 4A and 4B). We also measured the COBRA mRNA levels in the srf6-1cob-6 seedlings from the 5-azacytidine experiment and established that 5-azacytidine can repress the COBRA expression in srf6-1cob-6 and epicob-6 seedlings (Figure 4D).

Several proteins have been identified that affect DNA methylation in Arabidopsis. The DNA methyltransferases DRM1, DRM2 and CMT3 are involved in de novo DNA methylation and in CHG methylation, respectively [15,16]. MET1 is a methyl transferase thought to be primarily responsible for maintenance of CG methylation [34]. We crossed the srf6-1cob-6 with met1-3 and drm1 drm2 cmt3-11 mutants. Interestingly, we found cob-6 mutant phenotypes in seedlings from the F2 progeny of the cross between

Figure 2. COBRA transcript level increase in the suppressed cob-6. (A) qPCR determined COBRA transcript level in etiolated seedlings grown on 1/5 MS medium. Genotypes, mean and SE are indicated, n = 3 pools of seedlings. Asterisks indicate P values for comparison with cob-6: * P<0.05; ** P<0.001 (Student’s t-test). (B) Phenotype comparison of etiolated srf6-1cob-6 and srf6-1cob-4 seedlings. Pictures are representative of multiple plants for each genotype.

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Table 1. Hypocotyl length of the randomly selected SALK T-DNA r1, r2 and r3cob-6 and the srf4-1 SAIL T-DNA cob-6 double mutants.

| Genotype       | Genomic locus of the additional T-DNA | Number of plants | Three-day-old etiolated hypocotyl length (mm)* |
|----------------|---------------------------------------|------------------|-----------------------------------------------|
| cob-6          | –                                     | 29               | 1.5±0.1Aa                                     |
| srf4-1cob-6    | AT3G13065                             | 30               | 1.6±0.1Aa                                     |
| r1cob-6        | AT3G30980                             | 25               | 3.4±0.2Bb                                     |
| r2cob-6        | AT1G56340                             | 21               | 2.4±0.1Cc                                     |
| r3cob-6        | AT2G35050                             | 29               | 3.3±0.1Bb                                     |

*A, B and C indicate ranking by Duncan test at P≤0.01; a, b and c indicate ranking by Duncan test at P≤0.05.
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**srf6-1cob-6 and met1-3.** The genotypes of the plants displaying the cob-6 phenotypes were either homo- or heterozygous for met1-3 and srf6-1 and homozygous for cob-6 (Figure 4E and Table 2). It is not unexpected that the heterozygous met1-3 can reverse the cob-6 suppression since the heterozygous met1-3 has been shown to cause DNA methylation changes [35]. Also the srf6-1cob-6dm1-1dm2-2emd1-11 mutant seedlings showed a small but significant reversal in the suppression of the cob-6 phenotype (Figure 4E and Table 2). These data indicated that an increase in DNA methylation was involved in the SALK T-DNA interaction triggered suppression of the cob-6 phenotype, and that this methylation mark was removed in the met1-3 background and in seedlings treated with methylation inhibitors and reduced in the dm1-1dm2-2emd1-11 background.

**Mutation in the DNA Demethylase ROS1 also Suppressed cob-6**

We tested whether the increased DNA methylation responsible for cob-6 suppression could be established through a decreased DNA demethylation activity. ROS1 is a DNA demethylase that prevents DNA hypermethylation of both endogenous genes and transgenes [18,36,37]. We crossed cob-6 with a SAIL T-DNA insertion line in ROS1 (ros1-4) and discovered that the phenotype of rosl-4cob-6 was also suppressed, similar to srf6cob-6, and that the phenotype also responded to 5-azacytidine (Figure 4E). Furthermore, the transcript level of COB1 was elevated in rosl-1cob-6 (Figure S9). However, unlike in the progeny of srf6 x cob-6, the rosl-4 mutation suppressed cob-6 only in the homozygous rosl-4cob-6 lines and did not create the epicob-6 phenotype. A possible reason for the lack of epicob-6 in the rosl-4 cross is that the SALK T-DNAs act dominantly in establishing cob-6 suppression, whereas the rosl-4 SAIL T-DNA and other rosl mutations are recessive [18]. Hence the heterozygous rosl-4 is not able to create the paramutagenic epicob-6 allele. We observed no significant additive cob-6 suppression effect in a srf6-1ros1-3cob-6 triple mutant compared to srf6-1cob-6 suggesting that the SALK T-DNA suppressor effect acts on the same locus as ROS1 (Table S4).

**Discussion**

We discovered a trans interaction of SALK T-DNA insertions in Arabidopsis, which led to increased transcript levels of the endogenous gene in the SALK T-DNA insertion site. The affected SALK T-DNA allele (cob-6) was an intron insertion in COBRA gene, which is required for cellulose biosynthesis [28]. While preparing this manuscript Gao and Zhao published a very similar observation but with a different set of SALK T-DNA insertion mutants indicating that such T-DNA interactions are not unusual and may represent a common phenomenon [38]. Furthermore Gao and Zhao also observed suppression of the cob-6 root phenotype by a SALK T-DNA insertion in the auxin biosynthesis gene *YUCCA1* [38]. In both our study and Gao and Zhao (2012) the transcript level increase of the endogenous gene occurred in lines where the SALK T-DNA was inserted in an intron. In both cases this intron insertion caused a reduction in the transcript levels of the endogenous gene, which was partially rescued by the trans SALK T-DNA interaction. A SAIL T-DNA insertion in SRF4 did not induce cob-6 suppression (Table 1) suggesting that the trans T-DNA interaction may require the sequence similarity between two SALK T-DNAs.

Inhibitors of DNA methylation were able to reverse the trans SALK T-DNA interaction induced transcript increase suggesting that DNA methylation was responsible for the increased expression of the endogenous locus (Figure 4). The involvement of DNA methylation was confirmed by introducing a mutation in the main maintenance DNA methyl transferase MET1 into the srf6cob-6. The srf6-1cob-6met1 mutant seedlings were phenotypically identical to the original cob-6 and srf6cob-6, as were the epicob-6 treated with DNA methylation inhibitors. MET1 is therefore involved in the maintenance of the trans SALK T-DNA interaction induced cob-6 suppression. A SAIL T-DNA mutation of the ROS1 DNA
demethylase had a similar effect on cob-6 phenotype and COB expression as the trans SALK T-DNA interaction (Figure 4E, Figure S9 and Table S4). The COB expression was not changed in ros1-4, and the ros1-4 mutant had no additive effect on the suppression of cob-6 implying that both ROS1 and the trans SALK T-DNA effect acted on the same locus. Based on the observation that several SALK T-DNAs were able to trigger the suppression of the cob-6 while an EMS induced srf6 null mutant was not (Table 1 and Figure S8), we hypothesise that the cob-6 T-DNA is most likely the target of the suppressor modifications. Consequently our data also suggested that already the cob-6 T-DNA alone has a tendency to become methylated but that this is counteracted by ROS1 activity.

Once established the cob-6 suppressor locus became paramutagenic in that the epicob-6 was able to convert cob-6 to epicob-6 (Figure 3 and Table S3). The COB transcript level was significantly increased in epicob-6 compared to cob-6 (Figure 2A). We suspected that the increase in COB transcript levels is due to a secondary effect of the trans SALK T-DNA interaction. The 35S promoter in cob-6 T-DNA may result in the expression of a COB antisense transcript, which is reduced in response to the paramutation causing an increase in the wild-type COB transcript. The fact that the SAIL T-DNA lines, srf4-1 or heterozygous ros1-4, which do not contain a 35S promoter were unable to suppress cob-6 suggested the 35S promoter homology or activity may be causing the suppression and the paramutation effect.

**Table 2. Effect of the different DNA methylation mutants on the trans cob-6 SALK T-DNA interaction.**

| Genotype                | Number of plants | Four-day-old etiolated hypocotyl length (mm)* |
|-------------------------|------------------|-----------------------------------------------|
| Col-0                   | 35               | 11.0±0.2Aa                                    |
| ros1-4                  | 47               | 11.0±0.2Aa                                    |
| drm1drm2cmt3            | 41               | 11.0±0.2Aa                                    |
| srf6-1cob-6             | 46               | 6.2±0.2Bb                                     |
| drm1drm2cmt3-11srf6-1cob-6 | 32              | 4.1±0.1Cc                                     |
| ros1-4cob-6             | 46               | 4.6±0.1Dd                                     |
| met1-srf6-1cob-6        | 9                | 1.7±0.1Ee                                     |
| cob-6                   | 50               | 1.8±0.1Ee                                     |

*A, B,C,D,E indicate ranking by Duncan test at P=0.01; a,b,c,d,e indicate ranking by Duncan test at P=0.05.

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hypothesis the cauliflower mosaic virus 35S promoter in the SALK T-DNA inserts has also previously been linked to trans T-DNA effects in Arabidopsis [21]. Another possible factor influencing the degree of the suppression might be the locus of the T-DNA insertion. It is important to note that the randomly selected additional SALK T-DNA loci displayed a relatively minor suppression of the cob-6 phenotype compared to the two srf6 SALK-alleles (Table 1 and Table 2). Thus, several components might affect the proposed trans SALK T-DNA interaction mechanism.

Interestingly, when epicob-6 was crossed to Col-0 the phenotype of the epicob-6 reverted back to the original cob-6 phenotype indicating that homozygosity of the cob-6 T-DNA allele was important for the maintenance of the paramutagenic epicob-6 allele (Table S3). This suggested that the maintenance of the paramutagenic epicob-6 may require the presence of a second paramutagenic (epicob-6) or paramutable (cob-6) allele to be introduced during fertilisation. The fact that mutation of the ROS1 demethylase could also suppress the cob-6 phenotype (Figure 4E and Table S4) implied that the cob-6 T-DNA is actively demethylated. It is therefore possible that ROS1 is involved in reverting the epicob-6 back to cob-6 after the cross of epicob-6 to Col-0. The mechanism of this allele effect and involvement of ROS1 in the cob-6 SALK T-DNA suppression deserve further study. Our results establish a new Arabidopsis system where this question and the trans SALK T-DNA paramutation phenomena can be studied with a convenient hypocotyl elongation assay as a reporter.

Materials and Methods

All Arabidopsis lines used in this study are in accession Columbia with the exception of srf6-4, which is in accession Landsberg glabra. For the cross with srf6-4 the cob-6 mutation was introduced to the Landsberg background by backcrossing the cob-6 to Landsberg erecta five times. All Arabidopsis lines are listed in Table S5. Plants were grown in a growth chamber with 16 hours light (150 μmol m⁻² s⁻¹) and 8 hours dark, temperature 22°C (day) and 18°C (night), relative humidity 60–70%. Etiolated seedlings were first stratified for 2 days at 4°C and then grown on ½ MS medium for 3–5 days in the dark. For inhibitor experiments the respective inhibitor was added directly to the MS medium using the indicated final concentrations. All the primers used in this study are listed in Table S5.

Complementation of cob-6

A fragment containing 1.3 kb upstream the transcription start site of COBRA and the whole COBRA gene was isolated by PCR and cloned into the binary vector pGWB1 [39]. The construct was introduced into the Agrobacterium strain GV3101 and transformed into cob-6 plants. Several T2 homozygote lines were grown for phenotyping.

Analysis of COBRA Expression

RNA was isolated from three to nine 20 mg (fresh weight) pools of etiolated seedlings using the QIAGEN RNeasy mini kit (QIAGEN, www.qiagen.com). All expression experiments were repeated a minimum of three times with similar results. About 500 ng total RNA was reverse-transcribed in 20 μl reaction volume using the Bio-Rad iScript cDNA Synthesis Kit (Bio-Rad, www.bio-rad.com). The quantitative RT-PCR was performed with 0.1 μl cDNA 0.25 μM gene specific primers and 10 μl SYBR Green Master Mix (Bio-Rad iQ SYBR Green Supermix) in 20 μl reaction volume on Roche Lightcycler 480. The quantification was done according to the advanced relative quantification method [40], and the HELICASE reference gene (AT1G58050) chosen after an evaluation of reference genes according to [41] and [42]. The primers used for COBRA expression spanned the cob-6 T-DNA inserted in the first intron.

Cellulose Measurement

To determine the crystalline cellulose content, etiolated seedlings were transferred to 2 ml reaction tubes and treated with Updegraff-reagent [43]. On the resulting pellet Seaman hydrolysis [43] was performed and the hexose content was determined with the anthrone assay described in [44].

Supporting Information

Figure S1 Truncated co-expression network from Cluster 86 in [23]. Brief annotations of genes are indicated in black text. Different coloured edges indicate strength of transcriptional coordination. Green; mutual rank ≤10, Orange; mutual rank ≤20, Red; mutual rank ≤30. Low mutual rank indicates stronger co-expression relationships. Coloured nodes indicate embryo lethality (red), other described phenotypes (green), and no reported phenotype (grey) of mutants corresponding to the respective gene. (TIF)

Figure S2 Phenotype of six-week-old Col-0, srf6-1, cob-6 and srf6-1cob-6 plants grown in 16-h light, 8-h dark. Scale bar 5 cm. (TIF)

Figure S3 Location of the premature stop codon in srf6-4 and the T-DNA insertions in COBRA and SRF6. (TIF)

Figure S4 Cellulose content in four-day-old dark grown seedlings. Genotypes, mean and SE are indicated. A, B, and C indicate significant difference of the genotypes ranked by Duncan’s test at P<0.01, a, b, c indicate ranking by Duncan test at P≤0.05. (TIF)

Figure S5 The phenotype of etiolated F1 seedlings derived from the cross between srf6-1cob-6 and cob-6. Picture is representative of multiple seedlings. (TIF)

Figure S6 Complementation of cob-6 with a genomic COBRA construct. (TIF)

Figure S7 Amplification of full length COBRA cDNA from cob-6 and Col-0. (TIF)

Figure S8 Phenotype comparison of the srf6-4cob-6 and cob-6 etiolated seedlings. Picture is representative of multiple seedlings. (TIF)

Figure S9 The effect of DNA demethylase ros1-1 mutation on COBRA transcript levels. Also shown is the effect of DNA methylation inhibitor 5-azacytidine (5-Aza) on COBRA expression in ros1-1 and ros1-1cob-6. Genotypes, mean and SE are indicated. RNA was extracted from etiolated seedlings, n = 3 pools of seedlings. (TIF)

Table S1 Hypocotyl length of Col-0, srf6-1, srf6-1cob-6, epicob-6 and cob-6. (PPTX)
Table S2 Hypocotyl length of the different epicob-6 generations.

Table S3 Hypocotyl length of the epicob-6 and the F2 plants derived from epicob-6 crossed with cob-6. And the cob-6 and F2 cob-6 plants derived from epicob-6 crossed with Col-0.

Table S4 Hypocotyl length of three-day-old etiolated rosl-1 mutant combinations.

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Author Contributions

Conceived and designed the experiments: WX CR SP WBF TN. Performed the experiments: WX CR SP TN KH NS CC. Analyzed the data: WX CR SP WBF TN KH NS CC. Contributed reagents/materials/analysis tools: WBF TN SP. Wrote the paper: WX CR SP WBF TN.

Table S5 List of Arabidopsis mutants and primers used in the study.

(DOC)
