**RESEARCH PAPER**

**jaw-1D: a gain-of-function mutation responsive to paramutation-like induction of epigenetic silencing**

Wen Jiang1, Zhongfei Li1, Xiaozhen Yao1,2, Binglian Zheng3, Wen-Hui Shen1,4 and Aiwu Dong1,*

1 State Key Laboratory of Genetic Engineering, Collaborative Innovation Center for Genetics and Development, International Associated Laboratory of CNRS-Fudan-HUNAU on Plant Epigenome Research, Institute of Plant Biology, School of Life Sciences, Fudan University, Shanghai 200438, China
2 College of Life and Environment Sciences, Shanghai Normal University, Shanghai 200234, PR China
3 State Key Laboratory of Genetic Engineering, Collaborative Innovation Center of Genetics and Development, Institute of Plant Biology, School of Life Sciences, Fudan University, Shanghai 200438, PR China
4 Université de Strasbourg, CNRS UPR2357, F-67000 Strasbourg, France

* Correspondence: aiwudong@fudan.edu.cn

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**Abstract**

The *Arabidopsis thaliana* gain-of-function T-DNA insertion mutant jaw-1D produces miR319A, a microRNA that represses genes encoding CIN-like TEOSINTE BRANCHED1/CYCLOIDEA/PROLIFERATING CELL FACTORS (TCPs), a family of transcription factors that play key roles in leaf morphogenesis. In this study, we show that jaw-1D is responsive to paramutation-like epigenetic silencing. A genetic cross of jaw-1D with the polycomb gene mutant curly leaf-29 (clf-29) leads to attenuation of the jaw-1D mutant plant phenotype. This induced mutation, jaw-1D*, was associated with down-regulation of miR319A, was heritable independently from clf-29, and displayed paramutation-like non-Mendelian inheritance. Down-regulation of miR319A in jaw-1D* was linked to elevated levels of histone H3 lysine 9 dimethylation and DNA methylation at the CaMV35S enhancer located within the activation-tagging T-DNA of the jaw-1D locus. Examination of 21 independent T-DNA insertion mutant lines revealed that 11 could attenuate the jaw-1D mutant phenotype in a similar way to the paramutation induced by clf-29. These paramutagenic mutant lines shared the common feature that their T-DNA insertion was present as multi-copy tandem repeats and contained high levels of CG and CHG methylation. Our results provide important insights into paramutation-like epigenetic silencing, and caution against the use of jaw-1D in genetic interaction studies.

**Keywords:** *Arabidopsis thaliana*, DNA methylation, epigenetic silencing, histone methylation, jaw-1D, paramutation.

**Introduction**

MicroRNAs (miRNAs) are non-coding RNAs about 21–24 nucleotides in length that are important regulators of gene expression in both animals and plants. In *Arabidopsis thaliana*, miRNAs are involved in a wide range of biological processes, including meristem identity, leaf polarity, flowering patterning, signaling pathways, and responses to environmental stress...
For miRNA synthesis, an miR gene is first transcribed into primary miRNA (pri-miRNA) by RNA polymerase II. Pri-miRNA is then processed into pre-miRNA by DICER-LIKE 1 (DCL1) assisted by HYPOASTONIC LEAVES and SERRATE proteins. The pre-miRNA is further processed into a miRNA/miRNA* duplex consisting of the guide-strand miRNA and the passenger-strand miRNA*. Lastly, one strand of the duplex is incorporated into an ARGONAUTE (AGO) protein to carry out downstream functions, such as slicing/degradation and/or translational repression of target RNA molecules (Xie et al., 2015; Yu et al., 2017).

Arabidopsis miR319A was initially identified through the analysis of jagged and wavy Dominant (jaw-D) mutant alleles in a large-scale T-DNA insertion activation tagging gain-of-function mutagenesis (Weigel et al., 2000; Palatnik et al., 2003). jaw-D mutants exhibit a curly, uneven shape and serrated leaves because of miR319A overexpression. A subset of Teosinte branched1/Cycloidea/Proliferating cell factor (TCP) family transcription factors has been characterized as the targets of miR319A (Palatnik et al., 2003). TCP proteins are plant-specific transcription factors that share a conserved basic helix–loop–helix DNA-binding domain, termed the TCP domain (Cubas et al., 1999). In Arabidopsis, the TCP family consists of 24 members with 13 in the class-I group and 11 in the class-II group, based on the conservation and organization of the TCP domain. Class-II TCPs are further classified into the subgroups CIN-like (eight members) and CYC/TFB1-like (three members) (Martin-Trillo and Cubas, 2010). Five CIN-like TCP genes (TCP2, TCP3, TCP4, TCP10, and TCP24) have been shown to be miR319A targets, and their transcript levels are dramatically reduced in jaw-D because of the ectopic expression of miR319A (Palatnik et al., 2003). Because single tcp loss-of-function mutants only have mild phenotypes through functional redundancy of different TCPs, jaw-D has been widely used to study the functions of CIN-like TCPs and their genetic interactions with other factors in the regulation of plant development (Palatnik et al., 2003; Schommer et al., 2008; Nag et al., 2009; Liu et al., 2011; Danisman et al., 2012; Tao et al., 2013; Zhang et al., 2017). Our own previous work has demonstrated that miR319A-regulated TCPs physically interact with ASYMMETRIC LEAVES2 (AS2), and showed that the as2-1 jaw-1D double-mutant has enhanced leaf developmental defects (Li et al., 2012).

In contrast to these examples that successfully demonstrate the use of jaw-D as a TCP-knockdown mutant, we show here that jaw-1D is responsive to paramutation-like induction of epigenetic silencing, which prevents its use as a bona fide TCP-knockdown tool. Paramutation is an epigenetic phenomenon first described in depth in maize (Brink, 1956) and later found to be present in many multicellular organisms (Hövel et al., 2015; Hollick, 2017; Piu, 2015). It describes the heritable trans-interactions that occur between two homologous alleles that exhibit different transcriptional activities. Usually, the weakly expressed allele (the paramutagenic allele) can transform the highly expressed allele (the paramutable allele) into a new paramutagenic allele. Importantly, the newly transformed/paramutated allele is mitotically and meiotically stable and can induce paramutable-to-paramutagenic allele transformation.

The emerging molecular mechanisms of paramutation implicate self-reinforcing feedback loops carried out by small RNA biogenesis and chromatin modifications (Hollick, 2017). While animals deploy the PIWI-interacting RNA pathway together with repressive histone modifications, plants have evolved the RNA-dependent DNA methylation (RdDM) pathway. In this RdDM model, siRNA (24 nt in length) produced by a paramutagenic allele reinforces the silenced state of the paramutagenic allele and initiates silencing of the corresponding paramutable allele via cytosine (C) methylation in all different DNA sequence contexts (CG, CHG, and CHH; H=A,T, or C) together with methylation of histone-3 lysine-9 (H3K9) and H3K27 (Hövel et al., 2015; Hollick, 2017). Using a multi-copy pRD29A-LUC transgene as a paramutable allele in Arabidopsis, it has been demonstrated that numerous factors involved in RdDM, CG/CHG methylation, or histone modifications are required for paramutation-like silencing of the pRD29A-LUC transgene (Zheng et al., 2015). Nevertheless, the paramutation phenomena identified thus far are limited to a relatively small number of examples, and detailed case-by-case examination has unraveled differences regarding the frequency of paramutation, the heritability and stability of the paramutated state, as well as the occurrence of spontaneous and secondary paramutations (Hövel et al., 2015). The identification of additional paramutation examples is likely to help in understanding the conservation and diversity of the molecular mechanisms underlying paramutation.

In the present study, we report the identification of jaw-1D as a novel paramutable allele. We provide evidence that the heterochromatin mark di-methylation of histone-3 lysine-9 (H3K9me2) and DNA methylations are associated with jaw-1D paramutation. Furthermore, we show that numerous T-DNA insertion mutants of various genes are paramutagenic to jaw-1D, which not only provides useful information about cis-elements involved in paramutation but also cautions against the use of jaw-1D in genetic interaction studies where combined mutants are generated.

**Materials and methods**

**Plant material and growth conditions**

All Arabidopsis alleles used in this work were derived from the Columbia ecotype (Col-0). The following SALK line mutants were obtained from the Arabidopsis Biological Resource Center (www.arabidopsis.org): jaw-1D (CS6948), dfl-29 (SALK_010003), dcl-2 (SALK_064627), sdtg-8 (SALK_065480), dkg-4.3 (SALK_128428), mapkkb21 (SALK_018714C), atg19340-1 (SALK_070222), atg19340-2 (SALK_133379), atg19340-3 (SALK_086845), chr24-1 (SALK_152488), chr24-1 (SALK_149003), chr19 (SALK_069014), nap1-2-1 (SALK_131746), agos2-2 (SALK_035533C), hy11-2 (SALK_068463), smc6b-1 (SALK_101968C), arid1-1 (SALK_047099), nrp1-1-2 (SALK_033832), mpk71 (SALK_020801C), nrp2-2a-1 (SALK_046208), hyd3 (SALK_039784), and atg70810 (SALK_127508). For seed production, genetic interaction, and phenotypic and genotyping analyses, plants were grown in soil in greenhouses under a 16/8 h light/dark photoperiod (light intensity 100~120 μmol photons m⁻² s⁻¹ at leaf level). For all other experiments, plants were cultured in vitro on agar-solidified Murashige and Skoog medium M0255 (Duchefa Biochemie) with 0.9% sucrose at 22 °C under a 16/8 h light/
dark photoperiod (light intensity ~100 μmol photons m⁻² s⁻¹ at leaf level). For the kanamycin (Km) resistance test, the medium was supplemented with 50 ng ml⁻¹ Km.

Gene expression analysis
Total RNA was prepared from 2-week-old seedlings using TRI Reagent (Invitrogen) according to the manufacturer’s instructions. Reverse transcription was performed using standard procedures with iScript reverse transcriptase (Promega). PCR amplifications from the cDNA template were performed using gene-specific primers (Supplementary Table S1 at JXB online). UBQ10 was used as the reference gene in normalization.

Chromatin immunoprecipitation assays
Chromatin immunoprecipitation (ChIP) assays were performed on 2-week-old seedlings according to a previously described method (Saleh et al., 2008) using the following antibodies: anti-trimethyl-H3K27 (07-449; Millipore), anti-trimethyl-H3K4 (07-473; Millipore), and anti-dimethyl-H3K9 (ab1220; Abcam). The gene-specific primers used in PCR are listed in Supplementary Table S1.

Bisulfite genomic sequencing
Genomic DNA was extracted from 2-week-old seedlings using the standard CTAB protocol (Clarke, 2009). A total of 2 μg of R.Nase-treated genomic DNA was subjected to bisulfite conversion using an EpiTect Bisulfite Kit (Qiagen) according to the manufacturer’s instructions. Converted DNA then underwent PCR using primers listed in Supplementary Table S1. The PCR products were cloned into the pGEM-T Easy vector (Promega). Over 10 independent clones were sequenced and analysed for DNA methylation.

T-DNA copy number analysis
T-DNA copy numbers were estimated by real-time quantitative (q)PCR as previously described (Ingham et al., 2001; Bubner and Baldwin, 2004). Genomic DNA was extracted from 2-week-old seedlings using the standard CTAB protocol (Clarke, 2009). T-DNA copy number analysis

Results
Silencing of jaw-1D by clf-29
Our previous studies showed that both CIN-like TCPs and polycomb group (PcG) proteins are involved in AS1/AS2-mediated repression of Class-I KNOX genes (Li et al., 2012, 2016). To further analyse the functional relationship between CIN-like TCPs and PcG proteins, we crossed jaw-1D with clf-29, a T-DNA insertion mutant with the CURLY LEAF gene coding for H3K27-methyltransferase (Xu and Shen, 2008). Because jaw-1D represents a gain-of-function mutation whereas clf-29 is a recessive loss-of-function mutant, plants from the F1 generation of this cross (jaw-1D+/− clf-29+/−) were expected to exhibit a phenotype similar to heterozygous jaw-1D (jaw-1D+/−). Surprisingly, however, while jaw-1D+/− plants displayed a phenotype largely similar to jaw-1D with jagged and serrated leaves, jaw-1D+/− clf-29+/− plants showed a wild-type phenotype (Fig. 1A).

Quadruple repeats of the CaMV35S enhancer (4xCaMV35S), which ectopically activate miR319A expression (Palatnik et al., 2003), were inserted downstream of the miR319A locus in jaw-1D (Fig. 1B). To identify the molecular mechanisms underlying the suppressed phenotype of jaw-1D+/− clf-29+/−, we analysed transcript levels of miR319A in rosette leaves by reverse-transcription qPCR. miR319A transcript levels were increased about 90-fold in jaw-1D and by over 20-fold in jaw-1D+/− but were almost unchanged in jaw-1D+/− clf-29+/− compared with the Col-0 wild-type control (Fig. 1C). These results indicated that the overexpression of miR319A in jaw-1D+/− was sufficient to induce the mutant plant phenotype, and that the wild-type phenotype of jaw-1D+/− clf-29+/− was associated with the attenuation of miR319A overexpression.

The silencing of jaw-1D is inheritable and paramutagenic
Because PcG proteins are generally known as repressors of transcription, the miR319A suppression observed in jaw-1D+/− clf-29+/− was unlikely to be linked with reduced CLF activity. Nevertheless, we directly examined this by crossing jaw-1D with clf-2, a transposon insertion mutant of CLF (Goodrich et al., 1997). In contrast to jaw-1D+/− clf-29+/−, all jaw-1D+/− clf-2+/− plants displayed a phenotype similar to jaw-1D (Fig. 2A), indicating that clf-2 did not induce silencing of jaw-1D. Next, we analysed the progeny of jaw-1D+/− clf-29+/− to investigate
whether the silencing of jaw-1D was released in the next generation. In contrast to the expectation from classical genetic segregation, none of the individual plants from a selfing population of jaw-1D+/− df-29+/− (over 80 individual plants examined) displayed a jaw-1D-like phenotype. The progeny plants with a jaw-1D genotype displayed a phenotype similar to wild-type Col-0, and we refer to them hereafter as jaw-1D* plants. We also tested the interaction between jaw-1D* and jaw-1D using reciprocal crosses. All F1 plants behaved like jaw-1D* and showed a wild-type phenotype (Fig. 2B). To check the genetic inheritance of jaw-1D*, we examined plant phenotypes of the next four successive selfing-generations of jaw-1D* (G1 to G4). As shown in Fig. 2C, plants from all the generations examined showed a wild-type phenotype, indicating stable inheritance of jaw-1D*. Taken together, our data indicate that jaw-1D is responsive to paramutation-like silencing, such that the silencing of jaw-1D (jaw-1D*) induced by df-29 is inheritable independently from df-29, and that jaw-1D* is paramutagenic to jaw-1D.

Analysis of epigenetic modifications associated with jaw-1D silencing

To gain information about the chromatin basis of jaw-1D silencing, we first analysed levels of different histone modifications. In Arabidopsis, H3K4me3 and H3K27me3 are known to be associated with transcription activation and transcription repression in euchromatin, respectively, whereas H3K9me2 marks stably silenced heterochromatin (Liu et al., 2010). Consistently, our ChIP analysis revealed a high level of H3K4me3 at the actively transcribed gene UBQ10, a high level of H3K27me3 at the repressed gene FUS3, and a high level of H3K9me2 at the heterochromatic silenced transposon TA3 (Fig. 3). It also showed no significant difference in jaw-1D and jaw-1D* heterochromatin compared with Col-0. We further analysed different regions covering miR319A to downstream of the T-DNA insertion site of jaw-1D (Fig. 3A).

In Col-0, high levels of H3K27me3 were detected at various regions ranging from the promoter to at least 2000 bp downstream of the miR319A open reading frame (Fig. 3B), whereas H3K4me3 and H3K9me2 were barely detectable (Fig. 3C, D). In contrast, in jaw-1D, H3K27me3 levels were drastically reduced at all the regions examined, and high levels of H3K4me3 were detected at regions close to the miR319A transcription start site (Fig. 3B, C). These data were in agreement with the location of miR319A in euchromatin and indicated that active miR319A transcription was associated with removal of the repressive mark H3K27me3 and the deposition of H3K4me3 in jaw-1D. Most strikingly, in jaw-1D* the heterochromatin mark H3K9me2 was detected at 4xCaMV35S regions, and high levels of H3K27me3 were observed at both miR319A and 4xCaMV35S regions (Fig. 3). Thus, elevations of both H3K9me2 and H3K27me3 appeared to be associated with the stable silencing of miR319A in jaw-1D*. Region-9 downstream of the T-DNA insertion site did not show elevated H3K27me3 in jaw-1D* as in Col-0 (Fig. 3), suggesting that the H3K9me2-associated heterochromatinization may have caused H3K27me3 elevation at specific regions.

In Arabidopsis, H3K9me2 acts closely together with DNA methylation in heterochromatic silencing. The two types of modifications appear to be part of a reinforcing loop, in that H3K9 methylation recruits RdDM components and DNA methyltransferases, and H3K9-methyltransferases bind methylated DNA to favor H3K9 methylation (Law et al., 2013; Johnson et al., 2014; Liu et al., 2014). We carried out bisulfite sequencing analysis to examine DNA cytosine methylation levels at the 4xCaMV35S enhancer in jaw-1D and jaw-1D*. About 92% of CG (n=120), 85% of CHG (n=80), and 25% of CHH (n=600) were methylated in jaw-1D* but were at almost undetectable levels in jaw-1D (Fig. 3E, Supplementary Table S2). The DNA methylation status was stably inherited because the plants of the next generation (jaw-1D* G1) showed similar levels of CG, CHG, and CHH methylation to jaw-1D*.
the parental jaw-1D* plants (Fig. 3E, Supplementary Table S2). Taken together, we conclude that the silencing of jaw-1D is associated with both H3K9me2 and DNA methylation occurring at the 4xCaMV35S enhancer.

**Analysis of epigenetic modifications associated with clf-29**

 jaw-1D paramutation-like silencing was induced by the T-DNA insertion mutant clf-29 but not by the transposon insertion mutant clf-2. To better understand paramutagenic cis-determinants, we analysed the conformation of the T-DNA structure in clf-29. We found that besides NEOMYCIN PHOSPHOTRANSFERASE II (NPTII) driven by the NOS promoter, which served as a selection marker of plants resistant to Km, T-DNA also carried a copy of CaMV35S (Fig. 4A). Considering that paramutation is induced by homolog trans-interactions (Hövel et al., 2015), the CaMV35S sequence may be responsible for jaw-1D paramutation, and the paramutagenic clf-29 T-DNA could also be silenced. Indeed, the clf-29 mutant

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Fig. 3. Analysis of epigenetic modifications at the jaw-1D locus. (A) Diagram of the regions analysed. The structure of the locus is the same as described in Fig. 1B. The numbers p1–p9 indicate the regions analysed. (B–D) ChiP–PCR analysis of H3K27me3, H3K4me3, and H3K9me2 levels in wild-type Col-0 and the mutants jaw-1D and jaw-1D*. The different regions of the jaw-1D locus and the controls UBQ10, TA3, and FUS3 were analysed by real-time qPCR. Percentage-of-input values are shown as means (±SD) from three independent biological replicates. (E) Comparison of DNA methylation levels of CaMV35S in jaw-1D, jaw-1D*, and jaw-1D* G1 as examined by bisulfite sequencing. The CaMV35S sequence contained 12 CGs, eight CHGs, and 60 CHHs. Percentage-of-methylation values are shown based on a total of 11, 10, and 11 CaMV35S sequences cloned from jaw-1D, jaw-1D*, and jaw-1D* G1 samples, respectively. Detailed information can be found in Supplementary Table S2. (This figure is available in colour at JXB online.)
failed to show Km resistance, although the control T-DNA insertion mutant \textit{Atnap1;2-1} \cite{Liu2009} did (Fig. 4B). We further analysed H3K9me2 and H3K4me3 levels at several regions on T-DNA. High levels of H3K4me3 were specifically detected at \textit{NPTII} and closely downstream of it in \textit{Atnap1;2-1}, whereas H3K4me3 was barely detectable in any of the regions examined in \textit{clf-29} (Fig. 4C). In contrast, H3K9me2 was detected at various levels in all regions examined in \textit{clf-29} but was barely detectable in \textit{Atnap1;2-1} (Fig. 4D). These data are in agreement with \textit{NPTII} being active in \textit{Atnap1;2-1} but silenced in \textit{clf-29}. We then analysed DNA methylation levels within the CaMV35S region. CG and CHG methylation were at much higher levels in \textit{clf-29} than in \textit{Atnap1;2-1}, whereas CHH methylation was at a similar level in both mutants (Fig. 4E, \textit{Supplementary Table S2}). Taken together, our data indicate that \textit{clf-29} carries T-DNA in a heterochromatinized state containing...
high levels of H3K9me2 and DNA CG/CHG-methylation of CaMV35S, which is presumably the cis-determinant causing paramutation of jaw-1D.

jaw-1D is responsive to silencing induced by numerous T-DNA insertion mutant lines

The Atnap1;2-1 mutant did not show T-DNA heterochromatinization and is thus anticipated not to be paramutagenic. Indeed, our cross-test between Atnap1;2-1 and jaw-1D revealed that all F1 plants had a jaw-1D mutant phenotype, which was in contrast to the wild-type phenotype of jaw-1D* (Table 1). The visual phenotype screen for the paramutable jaw-1D together with the Km-resistant screen for NPTII activity in T-DNA provided a powerful tool for the analysis of paramutagenic lines. We tested 19 additional T-DNA insertion mutant lines (Table 1), and first determined their Km resistance. Nine behaved like clf-29 and displayed no resistance, one showed weak resistance, and the remaining nine showed good resistance like Atnap1;2-1 (Table 1). We then crossed these different mutant lines with jaw-1D and observed the phenotype of F1 plants. Interestingly, all mutant lines displaying no or weak Km resistance could induce jaw-1D silencing, leading to wild-type phenotype F1 plants. In contrast, none of the Km-resistant mutant lines could induce jaw-1D silencing because their F1 plants showed the jaw-1D mutant phenotype. Hereafter, we class the first group of mutant lines as S-group (Silencing group) and the second as N-group (Non-silencing group) (Table 1).

Tandem repeats and heterochromatinization of T-DNA probably cause jaw-1D silencing

Because our tested T-DNA insertion mutant lines were all from the SALK collection that originated from transformation using the same vector (pROK2, http://signal.salk.edu/tdna_protocols.html), the T-DNA sequence alone was insufficient to explain why a line belonged to the S-group or to the N-group. During transformation, T-DNA can be inserted with a varied copy number into a site of the plant genome (Ingham et al., 2001; Bubner and Baldwin, 2004). Therefore, we next examined the copy number of T-DNA in our studied mutants using qPCR. The endogenous Arabidopsis retrotransposon TA3 was used as a normalization control. As expected,

| Stock number   | Mutant name      | Gene function                        | Kanamycin resistance | Silencing jaw-1D | Classification | CaMV35S copy number |
|----------------|------------------|--------------------------------------|----------------------|-----------------|-----------------|---------------------|
| SALK_021003    | clf-29           | CURLY LEAF, encoding a H3K27-methyltransferase | no                   | yes             | S-group         | 16.13 ± 1.20        |
| SALK_064627    | dcl2-1           | DICER-LIKE 2                         | no                   | yes             | S-group         | 31.92 ± 1.87        |
| SALK_065480    | sdb8-1           | SET DOMAIN GROUP 8, encoding a H3K27-methyltransferase | no                   | yes             | S-group         | 17.62 ± 1.65        |
| SALK_128428    | sde4-3           | SDE4                                 | no                   | yes             | S-group         | 27.82 ± 2.64        |
| SALK_018714C   | mapkklk21        | MAPKKK21                             | no                   | yes             | S-group         | 10.72 ± 0.84        |
| SALK_070222    | at1g19340-1      | Methyltransferase MT-A70 family protein | no                   | yes             | S-group         | 8.1 ± 0.62          |
| SALK_133379    | at1g19340-2      | CHROMATIN REMODELING 24              | no                   | yes             | S-group         | 22.76 ± 1.52        |
| SALK_008485    | at1g19340-3      | CHROMATIN REMODELING 19              | no                   | yes             | S-group         | 9.79 ± 0.94         |
| SALK_152488    | chr24-1          | ARABIDOPSIS TRITHORAX1                | no                   | yes             | S-group         | 6.82 ± 0.67         |
| SALK_149002    | atx1-2           | CHROMATIN REMODELING 19              | weak                 | yes             | S-group         | 7.59 ± 0.74         |
| SALK_069014    | chr19            | AT-RICH INTERACTING DOMAIN 1         | yes                  | no              | N-group         | 12.18 ± 0.79        |
| SALK_131746    | nap1,2-1         | NAP1                                 | yes                  | no              | N-group         | 2.56 ± 0.33         |
| SALK_031553C   | agb6-2           | ARGONAUTE 6                          | yes                  | no              | N-group         | 3.38 ± 0.37         |
| SALK_064863    | hyl1-2           | HYponastic LEAVES 1                  | yes                  | no              | N-group         | 1.57 ± 0.16         |
| SALK_101968C   | smc6b-1          | STRUCTURAL MAINTENANCE OF CHROMOSOMES 6B | yes                  | no              | N-group         | 1.74 ± 0.18         |
| SALK_047099    | arid1-1          | AT-RICH INTERACTING DOMAIN 1         | yes                  | no              | N-group         | 3.05 ± 0.33         |
| SALK_033852    | nrpe1-12         | NUCLEAR RNA POLYMERASE D1B           | yes                  | no              | N-group         | 1 ± 0.00            |
| SALK_020801C   | mpk17            | MAP KINASE 17                        | yes                  | no              | N-group         | 1.03 ± 0.12         |
| SALK_046208    | nrpd2a-2         | NUCLEAR RNA POLYMERASE D2A           | yes                  | no              | N-group         | 3.12 ± 0.42         |
| SALK_039784    | hdt3             | HISTONE DEACETYLASE 3                | yes                  | no              | N-group         | 1.14 ± 0.16         |
| SALK_127508    | at5g07810        | SNF2 domain-containing protein       | yes                  | no              | N-group         | 3.43 ± 0.36         |
the FUS3 endogenous gene showed no copy number difference between the S-group and N-group lines (Fig. 5A). In contrast, the CaMV35S (T-DNA) copy number varied among the mutant lines (Table 1), with S-group mutant lines showing considerably higher numbers than N-group mutant lines (Fig. 5A).

Next, we checked whether multiple copies of T-DNA were inserted as tandem repeats by using a pair of primers that would give a PCR product only if two or more T-DNA copies were present as tandem repeats (Fig. 5B). In all 11 T-DNA insertion mutant lines belonging to the S-group, we detected the PCR product indicating the presence of tandem T-DNA repeats (Fig. 5C). In contrast, among the 10 N-group T-DNA insertion mutant lines, seven showed an absence and three showed a weak signal of the PCR product for the presence of tandem T-DNA repeats (Fig. 5D). Together, these data indicated that tandem repeat multi-copies of T-DNA (CaMV35S) were associated with the induction of jaw-1D silencing.

Finally, we analysed DNA methylation levels at CaMV35S sequences in the S-group and N-group mutant lines. Both CG and CHG methylation were at much higher levels in S-group than in N-group mutant lines, whereas CHH methylation was at similar levels (Fig. 5E, Supplementary Table S2). These data clearly establish the crucial functions of the symmetric cytosine methylations (CG and CHG) but not the asymmetric cytosine methylation (CHH) in paramutation-like silencing.

Discussion

In this study, we identified jaw-1D as a novel paramutation allele. The jaw-1D allele carrying the 4xCaMV35S enhancer overexpressed miR319A in an active chromatin mark-indexed manner (Fig. 3). It was also responsive to epigenetic silencing induced by clf-29 as well as by many other SALK T-DNA insertion mutant lines (Table 1). Once generated, the induced silent state of jaw-1D, namely jaw-1D*, was not only mitotically and meiotically stable but also able to transform de novo the active jaw-1D allele into the silent jaw-1D* allele (Fig. 2). Our results further demonstrate that CaMV35S tandem repeats together with heterochromatin mark enrichments are key factors associated with jaw-1D paramutation (Figs 4, 5).

The 4xCaMV35S activation tagging lines are commonly used for studying gene function in Arabidopsis. When inserted upstream of a gene, the 4xCaMV35S enhancer can promote/activate its expression, leading to a gain-of-function of the gene in the mutant line. Remarkably, even when inserted downstream of a gene, such as in the case of jaw-1D, the 4xCaMV35S enhancer could also activate expression of the target gene. Our ChIP data revealed that it transformed the miR319A locus from an H3K27me3-enriched repressive chromatin state in Col-0 to an H3K4me3-enriched active chromatin state in jaw-1D (Fig. 3). These results are in agreement with a previous report by Chen et al. (2013), and together support the general notion that enhancers generate a chromatin environment that is favorable for neighboring gene transcription (Calo and Wysocka, 2013).
of CaMV35S–driven reporter genes by SALK T–DNA insertion lines has been previously observed (Daxinger et al., 2008). Paramutation–like phenomena have also been described in two examples where T–DNA was inserted in the intron of an actively transcribed Arabidopsis gene. In the first example of cob–6 where a SALK T–DNA was in the first intron of COBRA, the cob–6 mutant phenotype was suppressed by crossing with the T–DNA mutant sfd–1 or other randomly selected SALK T–DNA insertion lines (Xue et al., 2012). In the second example of ag–TD where T–DNA was in the second intron of AGAMOUS, the ag–TD mutant phenotype was suppressed by yuc1–1 containing the same T–DNA sequence (Gao and Zhao, 2013).

Our results extend on, and differ from, these previous studies by showing that: first, the responsive 4xCaMV35S is at the 3’-end (Fig. 1) of miR319A in jaw–1D, so the previous finding of T–DNA location within an intron is not absolutely required; and second, that inducible lines (S–group) contain tandem repeats of CaMV35S sequences interrupted by other T–DNA sequences (Fig. 5), so paramutagenic and paramutable alleles do not need to contain the same full-length T–DNA. In maize, a hepta-repeat DNA sequence of 853 bp required for the B–I to B’ paramutation is located approximately 100 kb upstream of the transcription start site of b1 (Stamp et al., 2002). Collectively, it appears that cis–elements involved in paramutation can be in various positions or configurations in different examples of the studied genes.

Our data showing that S–group but not N–group SALK lines contain high copy numbers and tandem repeats of CaMV35S (T–DNA) are consistent with the general knowledge that paramutation is associated with DNA repeats (Hövel et al., 2015). Not only was jaw–1D silenced in response to numerous S–group SALK T–DNA insertion lines (Table 1), but also the paramutagenic T–DNA line yuc1–1 can induce the silencing of other different T–DNA insertion mutants such as ag–TD and cob–TD (Gao and Zhao, 2013). Nevertheless, the presence of ‘DNA repeats’ alone is insufficient to define a line as paramutagenic, as exemplified in our study by jaw–1D and the three N–group lines containing tandem DNA repeats that could not induce jaw–1D silencing. Our chromatin analyses also showed that the paramutagenic jaw–1D* and clf–29 alleles were marked with high levels of H3K27me3, H3K9me2, and DNA CG/CHG/CHH-methylation at CaMV35S (Figs 3, 4), suggesting that repressive heterochromatinization represents a key feature for paramutagenic alleles. In support of an essential role for DNA methylation in paramutation establishment, the multicopy pRD29A-LUC allele has previously been shown to be actively expressed in wild-type plants but to be converted to a silent and paramutagenic allele in nos1, a mutant lacking DNA glycosylase and thus accumulating a high level of DNA methylation (Zheng et al., 2015).

In general, tandem DNA repeats are associated with siRNA production and are targets of DNA methylation via the RdDM pathway (Matzke and Mosher, 2014). It is highly likely that multi-copy tandem repeats of T–DNA, which occurred during Agrobacterium–mediated plant transformation, caused T–DNA heterochromatinization in the S–group SALK lines, and that the trans–interaction via homologous CaMV35S sequences between an S–group SALK line and jaw–1D led to jaw–1D* formation. Our study unraveled variable levels of DNA methylation of CaMV35S in different SALK T–DNA insertion mutant lines. Importantly, S–group SALK lines showed higher levels of DNA methylation than N–group SALK lines (Fig. 5E). The RdDM pathway is involved in both symmetric (CG and CHG) and asymmetric (CHH) DNA methylation (Matzke and Mosher, 2014), and components of RdDM are required in paramutation (Hövel et al., 2015; Zheng et al., 2015; Hollick, 2017). Symmetric CG and CHG but not asymmetric CHH within CaMV35S were found to be methylated at higher levels in S–group than N–group SALK lines, suggesting a primary role for symmetric DNA methylation in paramutation. The two methylation types are maintained by different mechanisms. CG and CHG methylations can be maintained independently of RdDM by the methyltransferases MET1 and CMT3, respectively, both of which act on hemimethylated DNA to copy the methylation from the parental strand to the daughter strand during DNA replication. In contrast, CHH methylation cannot be maintained in the absence of siRNAs, and requires re-establishment following each DNA replication cycle by the methyltransferase DRM2 (Matzke and Mosher, 2014). It is possible that both symmetric and asymmetric DNA methylations are involved in the establishment of silencing but that subsequently CG and CHG methylation is advantageous over CHH methylation in the stable maintenance of silencing, which is crucial in paramutation.

In conclusion, our study identified jaw–1D as a paramutable allele and highlighted the crucial functions of tandem DNA repeats and epigenetic marks in paramutation. The observed paramutation phenomena also caution against the use of jaw–1D in genetic interaction studies. Our results obtained from numerous T–DNA insertion lines provide a valuable and important source of information for the future investigation of gene silencing and epigenetic regulation.

Supplementary data

Supplementary data are available at JXB online.

Table S1. List of primers used in this study.

Table S2. Analysis of DNA methylation of CaMV35S in different mutants.

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