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REGULAR PAPER

The CaMV 35S enhancer has a function to change the histone modification state at insertion loci in *Arabidopsis thaliana*

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Abstract Chromatin regions with different states usually harbor distinct epigenetic information, through which gene expression is regulated. Recent studies using mammalian cells showed that a chromatin state signature is associated with active developmental enhancers, defined by high levels of histone H3 lysine 27 acetylation (H3K27ac) and strong depletion of H3K27 trimethylation (H3K27me3). These findings also imply that active enhancers may play a role in creating a chromatin state by changing histone modification markers, which in turn affects gene expression. To explore whether an active enhancer in plants affect histone modifications, we investigated the cauliflower mosaic virus 35S enhancer ($35S_{enh}$) for understanding its action model in Arabidopsis. We report that the $35S_{enh}$ has a function to change the histone modification pattern at its presenting loci, by characterization of the $35S_{enh}$ activated *BREVIPEDICELLUS* (*BP*) silencing lines and the randomly selected $35S_{enh}$ activation tagging lines. By analyzing histone modification markers reflecting the plant chromatin state, we show that the $35S_{enh}$ is generally correlated with the reduced level of H3K27me3 and the increased level of H3K4me3 at the insertion loci. Our data are consistent with those in mammals and suggest that the enhancer sequence correlating with the active chromatin state signature may be generally present in the eukaryotic kingdom.

Keywords Arabidopsis · Histone modification · 35S enhancer · Epigenetics

Introduction

Eukaryotic genomes are packaged into chromatin, and diverse histone modifications can define the active or inactive chromatin domains based on whether they facilitate or block gene expression. A recent study using mouse and human cells showed that chromatin regions close to known endogenous enhancer elements are usually correlated with high levels of histone H3 lysine 27 acetylation (H3K27ac) but low levels of H3K27 trimethylation (H3K27me3) (Cotney et al. 2012; Ernst and Kellis 2010; Ernst et al. 2011), suggesting that the states of epigenetic markers may rely on the enhancer sequences. These findings reveal a fundamental aspect of genome-wide gene regulation, but also raise further questions. It is known that different types of histone modifications affect gene expression by changing the chromatin structure. However, based on the reported data in animals (Cotney et al. 2012; Ernst and Kellis 2010; Ernst et al. 2011), it is still unclear whether the endogenous animal enhancers could recruit epigenetic factors that change local chromatin structures through the histone modification. Furthermore, it is not yet known whether the relationship between enhancer sequences and the corresponding histone modification in mammals is also present in plants. In this study, we extend this research to plants, by testing the effect of the 35S enhancer ($35S_{enh}$), which was originally identified from the cauliflower mosaic virus (CaMV)

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promoter (Benfey et al. 1989; Franck et al. 1980; Guilley et al. 1982; Haas et al. 2002; Odell et al. 1985; Olszewski et al. 1982), on the levels of two types of histone methylation markers, H3K27me3 and H3K4me3, in Arabidopsis.

Different types of histone modifications have been well characterized in the model plant *Arabidopsis thaliana*, among which H3K27me3 and H3K4me3 are the two important types of histone modifications corresponding to the states of the euchromatin. In particular, the H3K27me3 chromatin signature is coordinated with the inactive chromatin state (Roudier et al. 2011; Schubert et al. 2006; Zhang et al. 2007), similar to that in animals. However, whether H3K27ac in Arabidopsis genome links to the active chromatin state is currently unknown. H3K4me3 in plants is the chromatin signature marking the active chromatin state (Alvarez-Venegas et al. 2003; Roudier et al. 2011; Zhang et al. 2009). Therefore, H3K27me3 and H3K4me3 could be the suitable markers in plants for determining chromatin states in the regions where enhancers locate.

Compared to that in mammals, the genome-wide enhancer information in plants is fairly limited. For example, more than twenty eight thousands of putative enhancer sequences have been identified from a single mouse embryonic limb database (Cotney et al. 2012), with the number much greater than that known in Arabidopsis. We choose to use the $35S_{enh}$ in this study mainly for the following two reasons. Firstly, the $35S_{enh}$ is widely used in plant researches and after recruiting transcription factors the enhancer is able to constitutively drive gene expression in plants (Haas et al. 2002; Jupin and Chua 1996; Lam et al. 1989; Lam and Chua 1989; Ruth et al. 1994). Secondly, there is a large collection of the $35S_{enh}$ activation tagging lines available for use (Hayashi et al. 1992; Nakazawa et al. 2003; Weigel et al. 2000), so that the histone modification changes in many different tagged loci can be analyzed. In this work, we show that the $35S_{enh}$ insertion loci generally associate with the reduced H3K27me3 and the increased H3K4me3 levels, suggesting that the $35S_{enh}$ promotes gene expression in an epigenetically regulating manner.

Materials and methods

Plant materials and growth conditions

jaw-1D, *jaw-2D*, *jaw-3D*, *jaw-4D*, *jba-1D*, *stip-D*, and *yuc-1D* are mutants of *Arabidopsis thaliana* L. in the Columbia-0 (Col-0) background (Palatnik et al. 2003; Weigel et al. 2000; Williams et al. 2005; Wu et al. 2005; Zhao et al. 2001), and *sef* and *tcp1-1D* are mutants of *A. thaliana* in the Ws background (Guo et al. 2010; Xu et al. 2005). All seeds were obtained from Dr. J. C. Fletcher, Dr. Y. Y. Xu, Dr. K. Chong, Dr. D. Weigel, Dr. J. Li, Dr. Y. Zhao and Arabidopsis Biological Resource Center (ABRC). Plants were grown according to our previous methods (Xu et al. 2003).

Construction of transgenic plants

A 3.5-kb *BP* promoter (BP_{pro}) was amplified by PCR using the wild-type Col-0 genomic DNA as template, and the sequence was verified and inserted before the *β-glucuronidase* (*GUS*) gene of the plasmid pBI101 to create a BP_{pro}*:GUS* construct. The $35S_{enh}$*:*BP_{pro}*:GUS* construct was constructed by fusing a $35S_{enh}$ DNA fragment to the 5′ end of the BP_{pro}*:GUS* construct. All constructions were introduced into the wild-type Col-0 plants by Agrobacterium-mediated transformation (Xu et al. 2003), and transgenic plants at the T2 generation were used in analysis. Primers for plasmid construction are listed in Table S1.

Chromatin immunoprecipitation (ChIP) and GUS staining

ChIP experiments were performed as previously described (Xu et al. 2008). Seedlings of 20-day-old wild-type, mutant, and transgenic plants were subjected to chromatin extraction. Immunoprecipitation was performed using a rabbit anti-trimethyl-histone H3 (lys27) antibody (Cat. 07-449, Millipore, USA) and a rabbit polyclonal antibody to histone H3 (tri methyl K4) (Cat. ab8580, Abcam, UK). H3K27me3 and H3K4me3 levels of each target gene were semi-quantified by real-time PCR, using the results for *PISTILLATA* and *ACTIN* as normalization control, respectively. Two independently prepared samples were analyzed by ChIP-qPCR, and the results were consistent. Thus, the results are shown for one of the two biological repeats. Standard errors (s.e.) were calculated using the data from three technical repeats. Primers used in qPCR are listed in Table S1. GUS staining was performed as previously described (He et al. 2012; Xu and Shen 2008).

Results and discussion

To test the possible function of $35S_{enh}$ in regulating histone methylation patterns, we first made a BP_{pro}*:GUS* construct as described above (Fig. 1a, upper panel). *BP* is known as a gene that is controlled by H3K27me3 (Katz et al. 2004; Lodha et al. 2013; Zhang et al. 2007); it is expressed in the shoot apical meristem but is strictly silenced in the leaf (Lincoln et al. 1994; Ori et al. 2000). We found that the BP_{pro} is sufficient to cause a high level of H3K27me3 modification on the *GUS* gene (Fig. 1d, upper panel), as

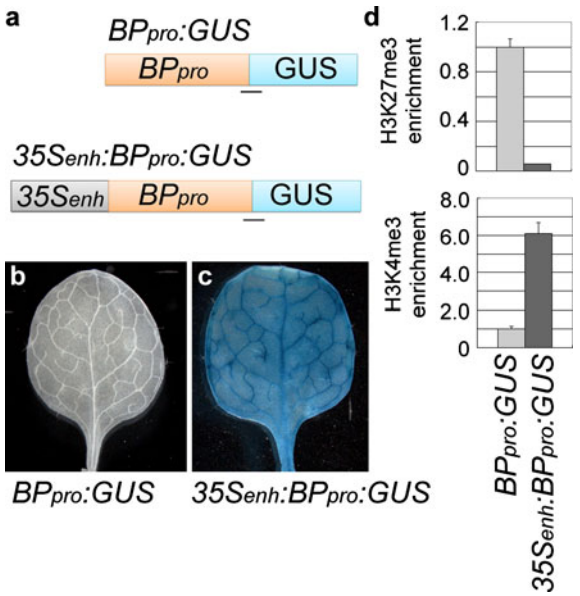


Fig. 1 Analysis of the BP_{pro}:*GUS* and 35_{enh}:BP_{pro}:*GUS* transgenic lines. **a** Diagram of the BP_{pro}:*GUS* and 35_{enh}:BP_{pro}:*GUS* constructs. The *black horizontal lines* spanning the *GUS* transcription initiation region indicate the PCR fragments in analyses of histone modifications. GUS staining of leaves from the BP_{pro}:*GUS* (**b**) and the 35_{enh}:BP_{pro}:*GUS* (**c**) transgenic lines. We obtained 7 and 8 independent transgenic lines for the BP_{pro}:*GUS* and 35_{enh}:BP_{pro}:*GUS* plants, respectively, and GUS staining among each group was consistent. **d** ChIP analysis of H3K27me3 and H3K4me3 levels on the *GUS* reporter gene. Values of BP_{pro}:*GUS* were arbitrarily fixed at 1.0. *Bars* show s.e

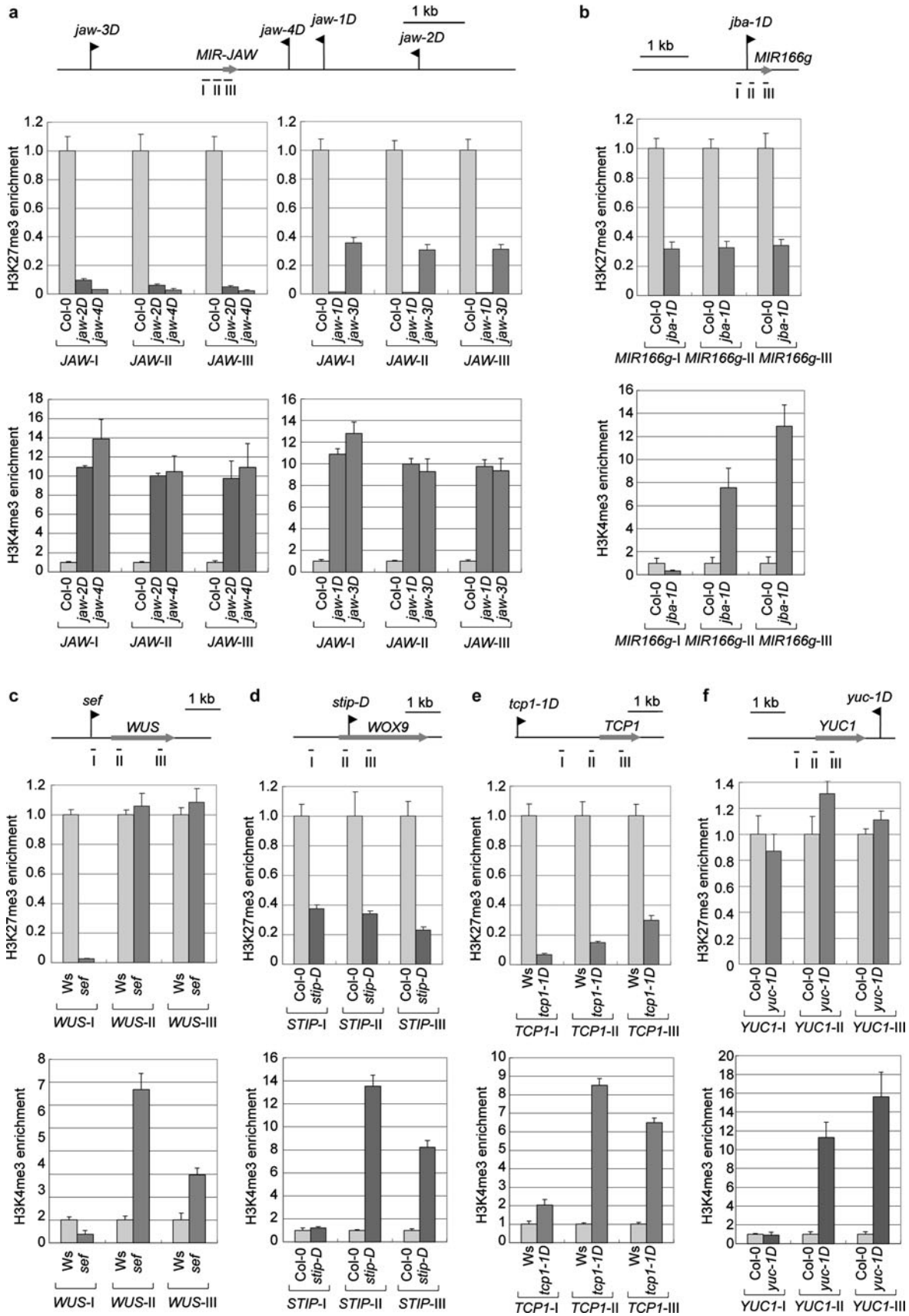
demonstrated by undetectable GUS staining in the leaves of BP_{pro}:*GUS* transgenic plants (Fig. 1b). However, Col-0 transgenic plants carrying a $35S_{enh}$:BP_{pro}:*GUS* construct (Fig. 1a, lower panel) showed a dramatically reduced level of H3K27me3 (Fig. 1d, upper panel) and an increased level of H3K4me3 level (Fig. 1d, lower panel) on *GUS*. These histone modification changes were accompanied by the ectopic *BP* expression, as demonstrated by prominent GUS staining in the entire leaf of $35S_{enh}$:BP_{pro}:*GUS* transgenic plants (Fig. 1c). These results suggested that the $35S_{enh}$ has an ability to change histone modification states at the insertion region and activate the corresponding gene.

To provide more evidence that the $35S_{enh}$ affects histone methylation levels, we randomly selected nine gain-of-function mutants, i.e., *jaw-1D*, *jaw-2D*, *jaw-3D*, *jaw-4D* (Fig. 2a) (Palatnik et al. 2003; Weigel et al. 2000), *jba-1D* (Fig. 2b) (Williams et al. 2005), *sef* (Fig. 2c) (Xu et al. 2005), *stip-D* (Fig. 2d) (Wu et al. 2005), *tcp1-1D* (Fig. 2e) (Guo et al. 2010), and *yuc-1D* (Fig. 2f) (Weigel et al. 2000; Zhao et al. 2001), all of which have 4 × $35S_{enh}$ T-DNA insertion (activation tagging) (Hayashi et al. 1992; Nakazawa et al. 2003; Weigel et al. 2000) upstream, downstream, or in the middle of the target genes (see Fig. 2). *jaw-D* and *jba-1D* alleles correspond to two microRNA genes that are important for leaf development (Palatnik et al. 2003; Williams et al. 2005); *stip-D*, *tcp1-1D* and *sef* loci activate *WOX9*, *TCP1* and *WUS* transcription factor genes, respectively, which are important in plant developmental regulations (Guo et al. 2010; Wu et al. 2005; Xu et al. 2005); and *yuc-1D* allele corresponds to the *YUC1* gene that is involved in auxin biosynthesis (Zhao et al. 2001). All the upregulated genes in these lines are known as a target of H3K27me3 (Zhang et al. 2007). Our ChIP analyses demonstrated that H3K27me3 levels decreased dramatically in eight of the nine lines and H3K4me3 levels increased in all these lines (Fig. 2).

Our results suggest that the $35S_{enh}$ has a general role in creating an active chromatin state via changes of histone modifications at the insertion loci. In mammalian systems, it has been reported that the reduced H3K27me3 and increased H3K27ac levels mark the active chromatin state, and these histone signatures associate with the enhancer sequences (Cotney et al. 2012; Ernst and Kellis 2010; Ernst et al. 2011). Instead of H3K27ac, we examined changes of the H3K27me3 and H3K4me3 levels, because in plants the low H3K27me3 and high H3K4me3 levels mark the active chromatin state (Roudier et al. 2011; Zhang et al. 2009; Zhang et al. 2007). We found that, being consistent with those found in mammals, the $35S_{enh}$ has an ability to change nearby histone modifications, such that the inserted $35S_{enh}$ associates with the plant active chromatin state signatures.

Notably, in *jaw-1D*, *jaw-2D*, *jaw-4D* and *yuc-1D* lines, the insertion of $35S_{enh}$ occurred downstream of the affected genes, and their $35S_{enh}$ sequences are located in the opposite DNA strand of the affected genes, being oriented towards the opposite direction of expression of the target genes (Fig. 2a, f; arrowheads). Therefore, it seems likely that the enhancers in these lines promote gene expression by changing histone modifications. It is known that the $35S_{enh}$ has an ability to recruit various kinds of transcription factors (Haas et al. 2002; Jupin and Chua 1996; Lam et al. 1989; Lam and Chua 1989; Ruth et al. 1994). We propose that when bound with the $35S_{enh}$ each transcription factor may change chromatin conformations by blocking or facilitating the action of protein complexes required for histone modification or chromatin remodeling. Because the strong gene expression caused by the $35S_{enh}$ may cause changes of histone modification patterns, it is also possible that the enhancer activates the chromatin state in a positive feedback manner, thereby promoting gene expression.

We could not rule out the possibility that the changes of chromatin signatures are the consequences of strong gene expression. Thus, the altered histone methylation patterns may be due largely to an indirect effect of the $35S_{enh}$. To address the correlation between histone modifications and


a
jaw-3D
jaw-4D
jaw-1D
1 kb
jaw-2D
MIR-JAW
I II III
H3K27me3 enrichment
H3K4me3 enrichment
Col-0
jaw-2D
jaw-4D
jaw-1D
jaw-3D
JAW-I
JAW-II
JAW-III
b
jba-1D
1 kb
MIR166g
jba-1D
MIR166g-I
MIR166g-II
MIR166g-III
c
sef
WUS
Ws
WUS-I
WUS-II
WUS-III
d
stip-D
WOX9
STIP-I
STIP-II
STIP-III
e
tcp1-1D
TCP1
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TCP1-I
TCP1-II
TCP1-III
f
yuc-1D
YUC1
YUC1-I
YUC1-II
YUC1-III

◀**Fig. 2** Altered histone methylation patterns in several selected activation tagging lines. ChIP analyses of H3K27me3 and H3K4me3 levels in seedlings of *jaw-1D*, *jaw-2D*, *jaw-3D* and *jaw-4D* (**a**), *jba-1D* (**b**), *sef* (**c**), *stip-D* (**d**), *tcp1-1D* (**e**) and *yuc-1D* (**f**). Gene structures and insertion positions of the activation tagging T-DNAs were shown above each illustration of ChIP data. *Black arrowheads* indicate the orientation of the cauliflower mosaic virus $35S_{enh}$. *Grey arrows* show the orientation of the activated genes. *Black lines* indicate the PCR fragments. Values of the wild-type were arbitrarily fixed at 1.0. *Bars* show s.e

expression, genome-wide profiling of H3K27me3 and H3K4me3 (Roudier et al. 2011; Zhang et al. 2009; Zhang et al. 2007) combined with systematically analyzing the known endogenous functional elements would be important.

Among our randomly selected activation tagging lines, *yuc-1D* did not change the H3K27me3 levels at the targeting locus, though the H3K4me3 level was increased. Recent results revealed that PRC2-mediated H3K27me3 is inhibited by active chromatin markers including H3K4me3, leading to activation of gene expression (Schmitges et al. 2011). In addition, it is known that the conserved transcriptional elongation regulator Paf1c affects histone modifications on its targets in Arabidopsis (Oh et al. 2008). The flowering regulator *FLC* is the target of both Paf1c and H3K27me3 pathways, and it was reported that depletion of Paf1c results in loss of H3K4me3 methylation, accompanied by accumulation of H3K27me3. Based on these findings, it is possible that the reduced H3K27me3 level may be a secondary effect controlled by H3K4me3. Therefore, H3K4me3 may play the critical role in upregulation of *YUC1* expression in the *yuc-1D* mutant, albeit without obvious changes of the H3K27me3 level. However, we could not rule out the possibility that we have not yet identified a specific region of the *YUC1* gene where H3K27me3 levels were significantly reduced, because of the limited use of PCR primers. New mechanism regarding the complex epigenetic regulations needs to be further explored.

Conclusions

Although well-defined chromatin signatures offer an elegant framework to interpretation of different chromatin states, very little is known about the action model between the functional elements and the chromatin signatures. The present study using Arabidopsis shows that the $35S_{enh}$ is closely correlated with high levels of H3K4me3 and low levels of H3K27me3 at the loci where the enhancer is inserted. This finding is consistent with those observed in mammal cells, suggesting a general role of the enhancer sequences in contribution to the changes of epigenetic information.

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