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BRIEF COMMUNICATION

# Involvement of histone modification in regulating *CUP-SHAPED COTYLEDON* genes during shoot regeneration in *Arabidopsis*

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## Abstract

Histone modification is a ubiquitous regulator of gene transcription. *Arabidopsis CUP-SHAPED COTYLEDON* (*CUC*) genes serve as a marker for shoot apical meristem initiation, but how they are regulated during shoot regeneration from *in vitro* culture, it is not yet understood. Here, the histone modification status of *CUC1*, *CUC2*, and *CUC3* was analysed using a combination of chromatin immunoprecipitation (ChIP) and real time quantitative PCR. The activation of *CUC1* and *CUC2* was associated with an increased level of histone H3K4 trimethylation and/or H3K9 acetylation, as well as a reduced level of H3K9 demethylation in various parts of their promoter and coding sequences. Histone modification is suggested to play an important role in regulating *CUC1* and *CUC2* expression during shoot regeneration.

*Additional key words*: *Arabidopsis thaliana*, chromatin immunoprecipitation, *in vitro* culture.

Many higher plants are able to regenerate shoots and/or roots from an explant cultured *in vitro* (Cary *et al*. 2002). Regeneration can occur either *via* somatic embryogenesis or *via* organogenesis (Tang 2000), both of which require the establishment of stable patterns of gene expression during cell division and growth. *Arabidopsis thaliana CUP-SHAPED COTYLEDON* (*CUC*) genes encode NAC domain-containing proteins and are expressed in cells forming a boundary domain around the cotyledon primordium (Aida *et al*. 1997, Daimon *et al*. 2003). Because of their functional redundancy, single mutants do not express a detectable phenotype. However, in a *cuc1*/*cuc2* mutant, the cotyledons are fused together, reflecting a compromised boundary specification (Vroemen *et al*. 2003), whereas a*cuc2*/*cuc3* mutant is defective with respect to ovule separation (Kamiuchi *et al*. 2014, Gonçalves *et al*. 2015). The expression of the *CUCs* is restricted to a small number of apical cells required for meristem initiation; they are thought to regulate expression of *SHOOT MERISTEMLESS* (*STM*), which encodes a central determinant of meristem identity (Hibara *et al*. 2003, Spinelli *et al*. 2011). In *in vitro* cultures, both *CUC1* and *CUC2* affect the capacity of a callus to regenerate shoots since regenerability is clearly reduced in the *cuc1*/*cuc2* mutant (Gordon *et al*. 2007).

Temporal and spatial expression of any genes during the process of shoot regeneration from *in vitro* cultures has consequences for the developmental fate of cell types. In some cases, their transcription has been shown to be dependent on epigenetic modification: for example, histone modification to *WUSCHEL* (*WUS*) is known to regulate shoot regeneration in *A. thaliana* (Li *et al*.

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*Abbreviations*: ChIP - chromatin immunoprecipitation; CIM - callus-inducing medium; *CUC* - *CUP-SHAPED COTYLEDON*; H3K9me2 - H3K9 demethylation; H3K9ace - H3K9 acetylation; H3K4me3 - H3K4 trimethylation; MS - Murashige and Skoog; qPCR - quantitative PCR; SIM - shoot-inducing medium.
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2011). Here, the aim was to explore the pattern of histone modification to the members of the *CUC* gene family, with a view to determine what (if any) relationship is between their modification status and their transcription during regeneration from *in vitro* culture.

Seeds of the *Arabidopsis thaliana* L. (ecotype Col-0), were grown on Murashige and Skoog (1962; MS) medium under a 16-h photoperiod, an irradiance of 350 μmol $m^{-2}$ $s^{-1}$, day/night temperatures of 25/20 °C, and a relative humidity of 55 % for 7 d. Root explants were harvested and then transferred to a callus-inducing medium (CIM), comprising Gamborg B5 medium (Gamborg *et al*. 1968) supplemented with 0.5 g $dm^{-3}$ 2-(N-morpho-line)-ethanesulphonic acid, 2.2 μM 2,4-di-chlorophenoxy-acetic acid, 0.2 μM kinetin, and 0.8 % (m/v) agarose. After incubation on the CIM for 4 d, they were transferred to a shoot-inducing medium (SIM) comprising an MS medium with 5.0 μM isopentenyladenine and 0.9 μM indole-3-acetic acid (Qiao *et al*. 2012).

To analyse the transcriptional level, RNA was extracted using a *TRIzol* reagent (*Invitrogen*, Carlsbad, USA). The first cDNA strand was generated by the *SuperScript* first-strand synthesis system (*Invitrogen*) according to the manufactuter's instructions. The expression of *CUC1/2/3* was analysed by real time quantitative PCR (qPCR) as reported previously (Song *et al*. 2015). Primer sequences are listed in Table 1 and the *UBIQUITIN5* gene (*At3g62250*) was chosen as a reference. A relative *CUC1/2/3* transcript abundance was determined using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). All real time qPCRs were repeated three times using templates prepared from three independent materials.

Table 1. Primers for real time quantitative PCR and histone modification analysis.

| Primer | Sense sequence | Antisense sequence |
|---|---|---|
| qRT-*CUC1* | CCTTGACGGCAAATTCTCTT | GCCGCTTTTCAGACAAACTT |
| qRT-*CUC2* | CAACTGTGAGCGTAAGCAG | GGAGTGAGACGGAGGAAGG |
| qRT-*CUC3* | ACTTCCCATCACTACTCTACG | AAGGAAAGATGATACCGTTG |
| qRT-*Tubulin2* | TTTGTGCTCATCTTGCCACGGAAC | CTCAAGAGGTTCTCAGCAGTACC |
| *CUC1*-Region I | GAGGTCGGCGGAGATGAT | GGTCATAAATGACGCTGAGGAT |
| *CUC1*-Region II | TTATCTTTCAACATTTGGGAACT | AACCCAGGTGGCATAAGG |
| *CUC1*-Region III | ACCCAACGGGACTGAGAACGA | AGCGGAGGAGGAAATGTAATG |
| *CUC1*-Region IV | CATACATTCCTTCCCGCTCCA | TCTGTCCCGATGATCCCAAAT |
| *CUC2*- Region I | ACGCACGCATACTCAGATAG | AGAGCAAAGCCAAAGCAGAT |
| *CUC2*-Region II | ACCTTCTCCGCAAAGTCCTCG | GCTCTGTTAGTTCTCAGTCCC |
| CUC2-Region III | TTTCATCTCAAGAAGCTCCAA | ATCAACGTCACCGACTATGTC |
| *CUC2*-Region IV | CTCATTCAGCCGTCTTCCAC | GGTAGCCAGTAATTCATCCC |
| *CUC3*-Region I | TCACAACTTAACATCACCCAG | TGTTTGAGGTTAGAGGAGCAT |
| *CUC3*-Region II | ACCTGGCTTCCGGTTTCAC | AGCTCCCAAGGTTCACAGC |
| *CUC3*-Region III | TAGTATGGGGAGGACAGGGGAGA | CGGTGAACAGAAAACGGCAAG |
| *CUC3*-Region IV | CTCAACGGTATCATCTTTCCT | AACCCAACAGACCATAACTCG |

To analyse *CUC1/2/3*'s histone modification, chromatin immunoprecipitation (ChIP) assay was undertaken exactly as described in Song *et al*. (2012). A 2 $mm^3$ aliquot of a final solution that immunoprecipitated by H3K9 acetylation (H3K9 ace), H3K4 trimethylation (H3K4me3), and H3K9 demethylation (H3K9me2) antibodies (*Millipore*, Boston, USA) was used as a template for real time qPCR analysis. A 10 000 × diluted input DNA obtained from 50 $cm^3$ of the extract was purified in parallel with the immunoprecipitated samples as a control, and ChIP reactions were also performed in the absence of antibody to detect the occurrence of any non-specific binding. Relative levels of H3K9 acetylation, H3K9 demethylation, and H3K4 trimethylation were normalised to an internal control (GenBank accession No. AY139810). The sequences of all PCR primers used are given in Table 1.

The transcription of *CUC1*, *CUC2*, and *CUC3* was monitored using real time qPCR in mock treated roots (taken from seedlings cultured on the MS medium for 7 d), in root explants cultured on the CIM for 4 d (CIM4), and in root explants cultured on the CIM for 4 d and then transferred to the SIM for 4 d (SIM4), 7 d (SIM7), or 10 d (SIM10). As a result, the transcription profiles of *CUC1* and *CUC2* were very similar to one another. Once the root explants were transferred to the CIM and then to the SIM, the abundance of *CUC1* and *CUC2* transcripts rose gradually reaching their highest level (about 10-fold compared with the mock) in the SIM4 treatment (Fig. 1*A*); the implication was that *CUC1* and *CUC2* activities were required for callus formation on the CIM and for shoot regeneration on the SIM. In contrast, *CUC3* transcription did not change significantly over time (Fig. 1*A*).

The histone content (H3K4me3, H3K9ace, and inactive H3K9me2) of the *CUC* genes was characterised

using a combination of ChIP and real time qPCR. Four regions that localised in their promoter (region I), translation start site (region II), exon and/or intron (regions III and IV) were analysed (Fig. 1*B*). The content of H3K9me2 was high and that of H3K9ace low within region I of *CUC1* in both the mock treatment and the CIM4 treatment; once the cultures were transferred to the SIM, there was a marked decrease in the content of H3K9me2 and an increase in that of H3K9ace (Fig. 2*A*,*C*). The H3K4me3 sites remained unmodified throughout experiment (Fig. 2*B*). Within *CUC2* (regions II and IV), a high content of H3K9me2 and a low content of H3K4me3 and H3K9ac was present in the mock; when the explants were cultured on the CIM and SIM, a significant decrease in H3K9me2 and increases in H3K4me3 and H3K9ace were observed (Fig. 2*D,E,F*). In regions III and IV of *CUC3*, the H3K9me2 content was high in the mock treatment, and substantially reduced in the CIM4 and SIM4/7/10 (Fig. 2*G*). The content of H3K4me3 and H3K9ace was comparable across all four regions of *CUC3* in all of the treatments (Fig. 2*H.I*).

A relationship between *CUC1*/*2/3* histone modify-cation status and the genes transcription was also investigated. The transcript abundance of *CUC1* was low in both the mock treatment and the CIM4 treatment (Fig. 1*A*), whereas its region I was highly enriched for

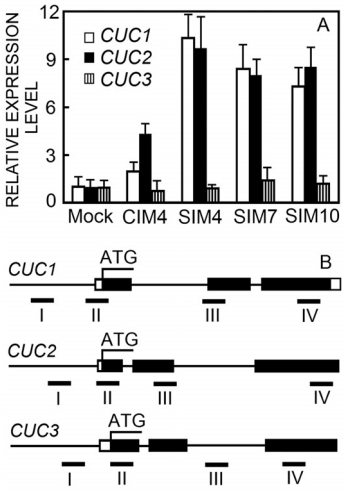


Fig. 1. Transcription profiling of *CUC* genes and sequences subjected to chromatin immunoprecipitation (ChIP) analysis. *A* - Real time quantitative PCR based assessment of transcription of *CUC1*, *CUC2*, and *CUC3* in mock treatment and after culture on a callus-inducing medium (CIM) for 4 d and a shoot-inucing medium (SIM) for 4, 7, and 10 d. The *Tubulin2* gene was used as a reference sequence. Means ± SEs of three biological replicates. *B* - The *black* and *white boxes* indicate exons and untranslated regions, respectively. The *short bars* labelled I, II, III, and IV indicate the sequences subjected to ChIP analysis.

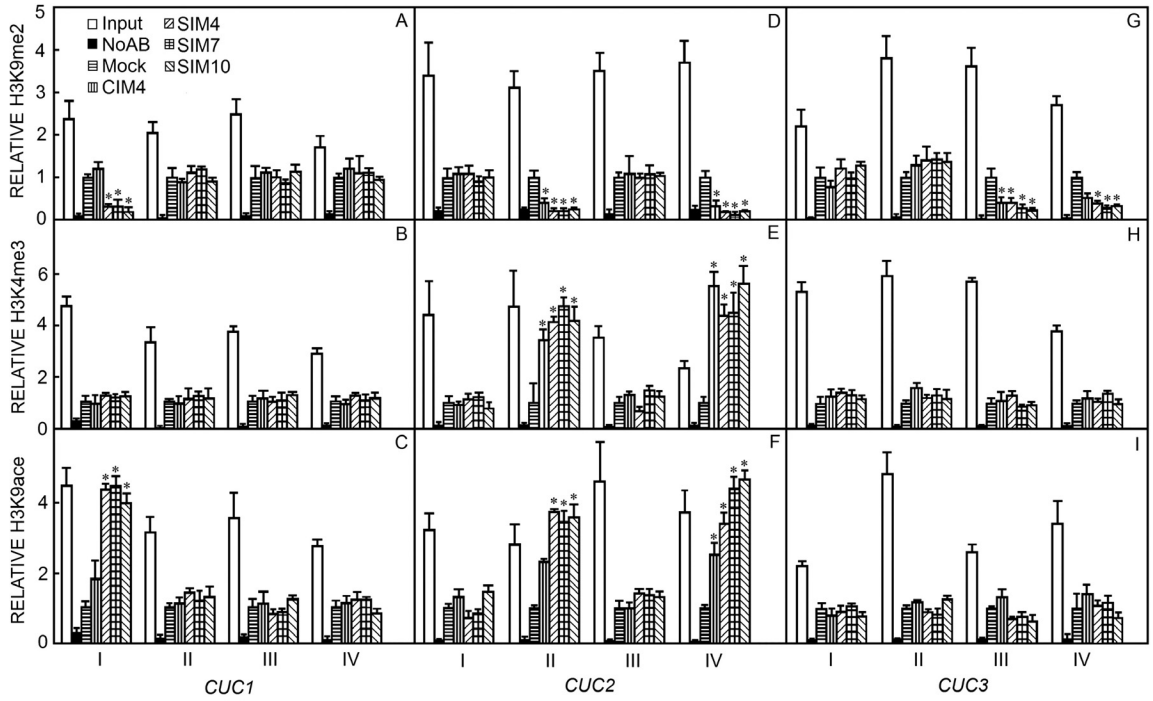


Fig. 2. Histone modification status of *CUC* genes. The content of H3K9 demethylation (H3K9me2; *A*,D,*G*), H3K4 trimethylation (H3K4me3; *B*,*E*,*H*), and H3K9 acetylation (H3K9ace; *C*,*F*,*I*) in *CUC1, CUC2*, and *CUC3*, respectively, in mock treatment and in explants exposed to a callus-inducing medium (CIM) for 4 d and to a shoot-inucing medium (SIM) for 4, 7, or 10 d. Input - input DNA used in PCR in a 1:10 000 dilution; No AB - chromatin immunoprecipitation (ChIP) reactions carried out in the absence of an antibody. Relative H3K9ace, H3K9me2, and H3K4me3 levels were determined by real time quantitative PCR and normalised against an internal control (GenBank accession AY139810). Means ± SEs of three biological replicates. *Asterisks* indicate means differing significantly from the mock ($P \leq 0.05$).

H3K9me2 and depleted for H3K9ace (Fig. 2*A*,*C*). In the SIM4, SIM7, and SIM10, the abundance of the *ZCUC1* transcript rose rapidly (Fig. 1*A*), the content of H3K9me2 in the promoter gradually fell and that of H3K9ace increased (Fig. 2*A*,*C*). The *CUC2* behaved similarly, in the mock, the expression of *CUC2* was low (Fig. 1*A*), whereas its regions II and IV were highly enriched for H3K9me2 and depleted for H3K9ace and H3K4me3 (Fig. 2*D*,*E*,*F*). When the explants were transferred to the CIM and SIM, *CUC2* expression rose rapidly (Fig. 1*A*), whereas the content of H3K9me2 gradually fell and that of H3K9ace and H3K4me3 increased (Fig. 2*D*.*E*.*F*). A suggestion is that transcription of *CUC1* and *CUC2* may be controlled by different histone modifications. Unlike *CUC1* and *CUC2*, the expression of *CUC3* did not change significantly during the whole culture process (Fig. 1*A*), whereas a decrease of H3K9me2 was observed in the CIM4 and SIM4/7/10 in the coding regions (regions III and IV) compared with the mock (Fig. 2*G*). Thus, it seems probable that for *CUC3*, transcription is independent on histone modification.

In conclusion, the up-regulation of *CUC1* during the period of the explant culture on the CIM and SIM may have been achieved by the depletion of H3K9me2 and the enrichment of H3K9ace in region I. The expression of *CUC2* may have been driven by a depletion of H3K9me2 and the enrichment of H3K4me3 and H3K9ace in regions II and IV. These behaviours provide an example of region-specific regulation of histone modification, which is consistent with the histone modify pattern of *WUS* during shoot regeneration (Li *et al*. 2011). The involvement of histone modification during *CUC1/2/3* expression appears to be heterogeneous.

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