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Original Paper

Expression of the auxin biosynthetic genes *YUCCA1* and *YUCCA4* is dependent on the boundary regulators *CUP-SHAPED COTYLEDON* genes in the *Arabidopsis thaliana* embryo

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Abstract During embryogenesis of eudicots, the apical region of the embryo develops two cotyledon primordia and the shoot meristem. In *Arabidopsis thaliana*, this process is dependent on the functionally redundant activities of the CUP-SHAPED COTYLEDON (CUC) transcription factors, namely CUC1, CUC2, and CUC3, as well as the phytohormone auxin. However, the relationship between the CUC proteins and auxin has yet to be fully elucidated. In the present study, we examined whether the expression of auxin biosynthetic genes is dependent on *CUC* gene activities. Comprehensive quantitative RT-PCR analysis of the main auxin biosynthetic gene families of *TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS1*/*TRYPTOPHAN AMINOTRANSFERASE RELATED* and *YUCCA* (*YUC*) showed that *YUC1* and *YUC4* expression levels were lower in *cuc* double mutant embryos than the expression levels of these genes in wild type embryos. Reporter analysis also revealed that the expression of *YUC1* and *YUC4* in the cotyledon boundary region was reduced in *cuc* double mutant embryos. In contrast, the loss of function mutation in the *SHOOT MERISTEMLESS* gene, a shoot stem cell regulator that acts downstream of the *CUC* genes, did not markedly affect *YUC1* expression levels. These results demonstrate that *CUC* genes play an important role in the regulation of auxin biosynthetic gene expression during embryogenesis; furthermore, they raise the possibility that the auxin produced by this regulation contributes to cotyledon boundary development.

Key words: auxin, *CUC*, embryogenesis, shoot meristem, *YUC*.

Introduction

Plant development proceeds with a series of patterning and differentiation events in which regulatory transcription factors and signaling molecules, including phytohormones, play important roles. In eudicots, the apical region of the globular-shaped embryo is subdivided into two cotyledon primordia; the shoot meristem is established at the center of the boundary region of these two primordia (Barton 2010; Barton and Poethig 1993). In *Arabidopsis thaliana*, this developmental process is regulated by the CUP-SHAPED COTYLEDON (CUC) transcription factors (i.e., CUC1, CUC2, and CUC3), which are expressed in the presumptive cotyledon boundary region from the globular stage on (Aida et al. 1999, 1997; Hibara et al. 2006; Takada et al. 2001; Vroemen et al. 2003). By coordinating the expression of regulatory genes including *SHOOT MERISTEMLESS* (*STM*), which is involved in stem cell maintenance in the shoot, these transcription factors ensure proper shoot meristem activity in postembryonic development (Aida et al. 1999, 2020; Belles-Boix et al. 2006; Hibara et al. 2006; Scofield et al. 2018; Takada et al. 2001). Because of their functional redundancy, multiple mutations in the *CUC* genes result in major developmental defects in the apical region of the embryo, whereas *CUC* genes with single mutations show no defects or those that are mild (Aida et al. 1997; Hibara

Abbreviations: Col, Columbia; *CUC*, *CUP-SHAPED COTYLEDON*; qRT-PCR, quantitative RT-PCR; *STM*, *SHOOT MERISTEMLESS*; *TAA1*, *TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS1*; *TAR*, *TRYPTOPHAN AMINOTRANSFERASE RELATED*; WT, wild type; *YUC*, *YUCCA*.

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et al. 2006). Typically, *cuc* multiple mutants develop a single cup-shaped structure consisting of the two fused cotyledons and they fail to establish the embryonic shoot meristem.

The phytohormone auxin plays important roles in various developmental processes including embryogenesis (Teale et al. 2006). The main pathway of auxin biosynthesis is catalyzed by sequential actions of two kinds of enzymes, each of which is encoded by the 5 *TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS1*/*TRYPTOPHAN AMINOTRANSFERASE-RELATED* (*TAA1*/*TAR*) and 11 *YUCCA* (*YUC*) family genes in *A. thaliana* (Cheng et al. 2006; Mashiguchi et al. 2011; Stepanova et al. 2008). Several multiple mutant combinations of auxin biosynthetic genes, such as *yuc1 yuc4 yuc10 yuc11*, show the abnormal embryonic phenotype with fused cotyledons and the lack of a root (Cheng et al. 2007; Robert et al. 2013; Stepanova et al. 2008). Moreover, various mutations in other classes of auxin-related genes (e.g., transport, perception, and response genes) cause defects in embryogenesis that include abnormal cotyledon morphology (Möller and Weijers 2009). The phenotype of these mutants suggests that a close relationship exists between the *CUC* genes and auxin signaling. However, studies on the relationship between *CUC* genes and auxin signaling in embryogenesis have only rarely been reported (Aida et al. 2002; Furutani et al. 2004). Thus, in the present research, we examined whether the *CUC* genes regulate the expression of auxin biosynthetic genes.

Materials and methods

The *A. thaliana* accession Columbia (Col) was used as the wild type (WT). The allelic combination of the *cuc1 cuc2* double mutant was *cuc1-1 cuc2-1*, which had originally been isolated from the Landsberg *erecta* background (Aida et al. 1997) and was backcrossed five times to Col prior to the analyses. The *cuc2 cuc3* and *stm* mutants, which were described previously (*cuc2-3 cuc3-105*: Hibara et al. 2006; *stm-1C*: Takano et al. 2010), were in the Col background. The *GFP-TAA1*, *YUC1p-n3xGFP*, and *YUC4p-n3xGFP* reporter lines were also previously reported (Robert et al. 2013; Stepanova et al. 2008). Plants were grown using previously described methods (Takeda et al. 2011).

To conduct quantitative RT-PCR (qRT-PCR) experiments, 20 heart-stage embryos were collected for each sample. The sampled embryos of the *cuc1 cuc2* double mutant were cup-shaped progeny of *cuc1 cuc2*/+ plants. For each genotype, four replicates were obtained from the independent parental plants. Embryos were dissected from seeds in 7% glucose solution as previously described (Imoto et al. 2021). An equal volume of the solution containing embryos and Monarch DNA/RNA Protection Reagent (New England Biolabs, Ipswich, USA) was mixed and then thoroughly homogenized mechanically at room temperature. Total RNA was purified from this lysate using a Monarch Total RNA Miniprep Kit (New England Biolabs). The concentration of total RNA in each sample was measured with a Qubit Fluorometer (Thermo Fisher Scientific, Waltham, USA). Reverse transcription reactions were performed with a LunaScript RT SuperMix Kit (New England Biolabs), and then real-time PCR was performed with a LightCycler system (Roche Diagnostics, Basel, Switzerland) and Luna Universal qPCR Master Mix (New England Biolabs). All RNA samples were adjusted to a final concentration of 25 pg $10\,\mu l^{-1}$ for PCR reactions. *ACTIN8* (*ACT8*; AT1G49240) was employed as the internal control gene. The Ct values of each gene relative to that of *ACT8* were measured; these were then used to calculate relative expression values. With the exception of *YUC11* primers (Song et al. 2019), all primers were designed using QuantPrime (Arvidsson et al. 2008) and are listed in Supplementary Table S1.

In preparation for confocal imaging, embryos were prepared as previously described (Imoto et al. 2021). Images were captured using LSM 5 Live (Carl Zeiss, Oberkochen, Germany) and FV3000 (Olympus, Tokyo, Japan) confocal laser scanning microscopes.

Results

First, we performed comprehensive expression analyses of auxin biosynthetic genes in WT, *cuc1 cuc2*, and *cuc2 cuc3* double mutant embryos using qRT-PCR. Two *cuc* double mutant combinations were employed because each shows strong apical defects (i.e., the failure of shoot meristem establishment and cotyledon separation) with high penetrance (Hibara et al. 2006). Five *TAA1*/*TAR* family genes and 11 *YUC* family genes were analyzed; according to their relative expression values (R), these genes were classified into the three groups (Figure 1): Group 1 with high expression levels ($R>0.1$), Group 2 with medium levels ($0.1\geq R>0.01$), and Group 3 with low levels ($R\leq 0.01$). In Group 1, the expression level of *TAA1* was significantly higher in the *cuc1 cuc2* mutant than that in the WT, whereas *YUC3* expression was significantly lower in the *cuc2 cuc3* mutant than that in the WT (Figure 1, left panel). On the other hand, *YUC1* and *YUC4* were expressed at lower levels in both the *cuc1 cuc2* and *cuc2 cuc3* mutants relative to their expression in the WT, and the differences were significant except for the expression of *YUC4* in *cuc1 cuc2*. In Group 2, *TAR4* in *cuc1 cuc2* and *YUC8* in *cuc2 cuc3* mutants showed significantly lower expression levels than those detected in the WT (Figure 1, middle panel). The expression of *TAR2*, which has redundant functions with *TAA1*, was not significantly different between the WT and *cuc* double mutant embryos. Among the eight genes in Group 3, the expression of *YUC5* and *YUC9* was significantly increased in *cuc1 cuc2* relative to their expression in the WT (Figure 1, right panel).

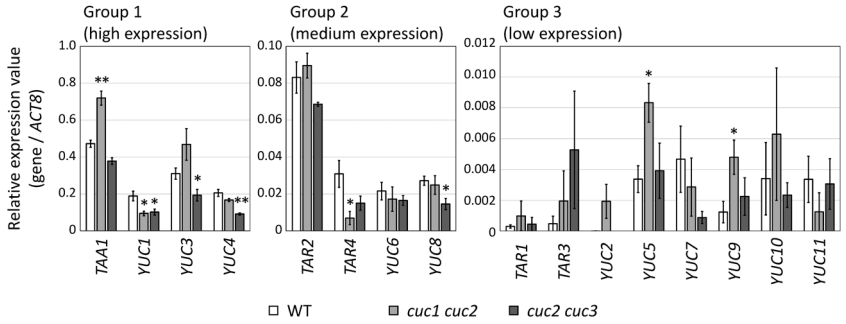

Figure 1. Expression level of auxin biosynthetic genes in the heart-stage embryos of WT, *cuc1 cuc2*, and *cuc2 cuc3*. Each gene was classified according to its relative expression value (see main text). Means±SE from four biological replicates are shown. Single and double asterisks indicate $p<0.05$ and $p<0.01$, respectively, compared with the WT and *cuc* double mutants (Welch's *t*-test).

Table 1. Summary of qRT-PCR results.

Group	Gene	*cuc1 cuc2*	*cuc2 cuc3*	Previously reported expression pattern*
1	*TAA1*	Up	—	AP[1], CB[2]
	YUC1	Down	Down	AP[1], CB[3]
	YUC3	—	Down	S[1]
	YUC4	—	Down	AP[1], CB[3], CT[3], R[1], S[1]
2	*TAR2*	—	—	n.a.
	TAR4	Down	—	n.a.
	YUC6	—	—	n.a.
	YUC8	—	Down	P[1]
3	*TAR1*	—	—	n.a.
	TAR3	—	—	n.a.
	YUC2	—	—	n.a.
	YUC5	Up	—	n.a.
	YUC7	—	—	n.a.
	YUC9	Up	—	S[3]
	YUC10	—	—	overlaps *YUC1* and *YUC4*[3]
	YUC11	—	—	overlaps *YUC1* and *YUC4*[3]

[1]Robert et al. 2013; [2]Stepanova et al. 2008; [3]Chen et al. 2007; *Domains of expression are abbreviated as follows: AP, apical protoderm; CB, cotyledon boundary; CT, cotyledon tip; P, provascular; R, root pole; S, suspensor. n. a., not applicable.

Our qRT-PCR results together with the reported spatial expression pattern of each gene are summarized in Table 1. Among the *YUC* family genes, only *YUC1* was consistently downregulated in both the *cuc1 cuc2* and *cuc2 cuc3* mutants, whereas several other genes, including *YUC3*, *YUC4*, and *YUC8*, were specifically downregulated in the *cuc2 cuc3* mutant. Two other *YUC* genes of Group 3, namely *YUC5* and *YUC9*, were upregulated in the *cuc1 cuc2* mutant; however, because their expression levels were very low, the biological significance of this expression is unclear. Two *TAA1*/*TAR* family genes, namely *TAA1* and *TAR4*, were significantly upregulated in the *cuc1 cuc2* mutant, whereas the expression of the remaining genes in this family did not differ significantly between the WT and the mutants. Notably, mutant combinations that reportedly show the cotyledon fusion phenotype similar to that of the *cuc* mutants are the *taa1 tar1 tar2* triple, *yuc1 yuc4 yuc10 yuc11* quadruple, and *yuc3 yuc9* double mutants (Cheng et al. 2007; Robert et al. 2013; Stepanova et al. 2008); all of these mutants include at least one gene for which the expression was altered in our qRT-PCR experiments. This raises the possibility that changes in these auxin biosynthetic genes are at least partly responsible for the *cuc* mutant phenotype.

We also performed reporter analysis of *TAA1*, *YUC1*, and *YUC4*, the expression of which spatially overlaps that of the *CUC* genes in the cotyledon boundary region (Table 1; Cheng et al. 2007; Robert et al. 2013; Stepanova et al. 2008), and we compared their expression patterns in the WT and *cuc2 cuc3* double mutant embryos at the heart stage. The expression of *TAA1* was detected in the protodermal cells that lay along the cotyledon boundary in the WT (Figure 2A). Within the boundary region, *TAA1* expression was localized at the center, as revealed by transverse and sagittal longitudinal sections reconstructed from serial, frontal longitudinal sections (Figure 2A, left and bottom panels). In the *cuc2 cuc3* mutant, *TAA1* expression was detected in the protodermal cells of the embryo apex corresponding to the cotyledon boundary (Figure 2B). Consistent with the results of the qRT-PCR experiments, the GFP signals of the *TAA1* reporter were essentially the same in the WT and *cuc2 cuc3* embryos, both in terms of their distribution and intensity. In contrast to *TAA1*, both *YUC1* and *YUC4* showed significantly decreased expression in *cuc2 cuc3* mutants compared with their expression in the WT (Figure 2C, D, E, F). A belt-shaped expression pattern was observed in the WT for the *YUC1* reporter in the protodermal layer along the cotyledon boundary; *YUC1* was also expressed in the inner cells that lay just above the provascular cells (Figure 2C). In WT embryos, *YUC4* expression was observed in the peripheral region of the cotyledon boundary; additionally, weak *YUC4* expression was detected in the cotyledon tips, root primordium, and suspensor (Figure 2E). Contrastingly, *YUC1* and *YUC4* expression in the *cuc2 cuc3* mutant was substantially weakened and often difficult to detect (Figure 2D, F); when expression was observed, it was detected in the apical and central

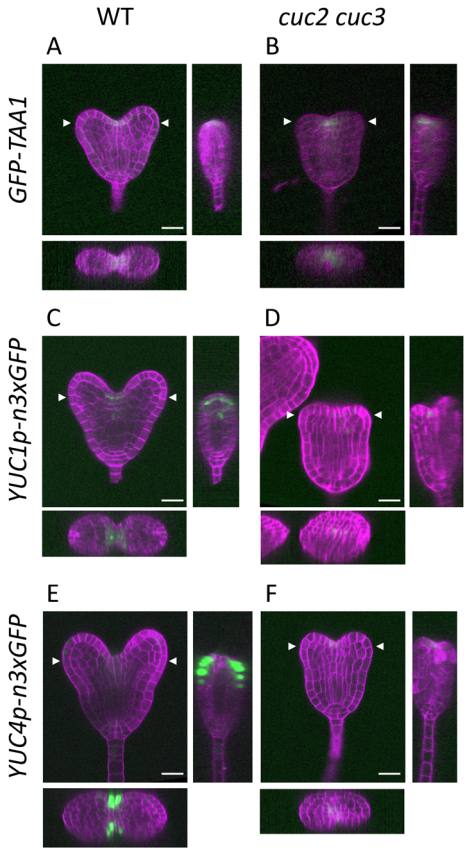

Figure 2. Spatial expression patterns of auxin biosynthetic genes in heart-stage embryos. Confocal images of the embryos carrying the GFP reporters of *TAA1* (A and B), *YUC1* (C and D), and *YUC4* (E and F) in the WT (A, C, F) and *cuc2 cuc3* (B, D, F) backgrounds. Images of the reconstructed cross and sagittal sections are shown in the bottom and right panels, respectively. Arrowheads indicate the y-axis position of the cross sections. The sagittal sections were reconstructed from the medial lines of each embryo. The signals of Calcofluor White staining of the cell wall and GFP are represented in magenta and green, respectively. Scale bars: 20 μm.

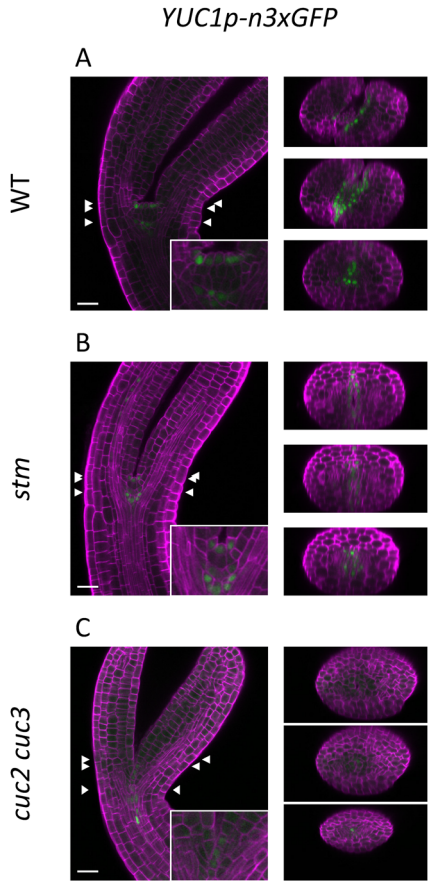

Figure 3. Spatial expression patterns of the auxin biosynthetic gene *YUC1* in bending-cotyledon-stage embryos. Confocal images of the embryos carrying the GFP reporter of *YUC1* in the WT (A), *stm* (B), and *cuc2 cuc3* (C) backgrounds. The three pairs of arrowheads indicate the y-axis positions of the reconstructed cross sections shown in the right panels. The signals of Calcofluor White staining of the cell wall and GFP are represented in magenta and green, respectively. Scale bars: 20 μm.

region corresponding to the cotyledon boundary. The reduction of *YUC1* and *YUC4* expression in the *cuc2 cuc3* mutant was not restricted to the boundary region; their expression was reduced in other regions such as in the inner cells for *YUC1* and in the cotyledon tips, root primordium, and suspensor for *YUC4* (Figure 2D, F). These results indicate that *CUC2* and *CUC3* are required to promote expression of *YUC1* and *YUC4* but not the expression of *TAA1*.

CUC genes are reportedly required for the expression of several downstream genes, among which *STM* plays a major role in promoting cotyledon separation and shoot meristem establishment (Aida et al. 1999, 2020; Scofield et al. 2018). Thus, it is possible that *STM* is required to promote *YUC1* and *YUC4* expression, and the observed reduction in expression in *cuc2 cuc3* embryos may be due to the reduced expression of *STM* (Hibara et al. 2006). To test this possibility, we performed reporter analysis of *YUC1* in the *stm* mutant background. Because the homozygous *stm* mutant is sterile, we selected homozygous progeny from heterozygous parental plants based on their morphology; the homozygous *stm* embryos are initially recognizable at the bending-cotyledon stage as those lacking a dome-shaped shoot meristem between the cotyledon primordia (Barton and Poethig 1993; compare Figure 3A, B, insets). In the WT, *YUC1* expression was detected in the protoderm cells of the shoot meristem, inner cells underneath the meristem, and the boundary region between the cotyledon primordia and the meristem (Figure 3A). In *stm*, despite the lack of a shoot meristem, *YUC1* expression was detected in the boundary region of the cotyledon primordia and inner cells positioned a few cells away from the protoderm; a similar intensity to that of the signals in the WT was shown (Figure 3B). The *cuc2 cuc3* double mutant was also examined at the same stage; only a weak signal at the base of the cotyledon primordia was detected (Figure 3C). Taken together, these results indicate that the expression of *YUC1* is dependent on

CUC2 and *CUC3* but not markedly dependent on *STM*.

Discussion

Our results indicate that the expression of the auxin biosynthetic genes *YUC1* and *YUC4* are positively regulated by *CUC2* and *CUC3*; at least for *YUC1*, this regulation does not require *STM*, which is a major downstream gene of the *CUC* genes. Expression of these two *YUC* family genes overlaps that of the *CUC* genes in the cotyledon boundary region; additionally, their expression was found to be reduced in *cuc2 cuc3* double mutant embryos. Our qRT-PCR experiments indicate that *CUC1* also positively regulates *YUC1* expression. Conversely, among the *TAA1*/*TAR* family genes, only *TAA1* is reportedly expressed in the cotyledon boundary region and its expression did not require any of the *CUC* genes. Because the YUC family proteins catalyze a rate-limiting step of indole-3-acetic acid biosynthesis (Mashiguchi et al. 2011), it is possible that the regulation of *YUC1* and *YUC4* expression by the *CUC* genes plays a role in auxin biosynthesis in the apical embryo.

The biological relevance of *CUC* gene-regulated expression of *YUC1* and *YUC4* is currently unclear. The *yuc1 yuc4* double mutant does not show any prominent phenotype of embryogenesis (Cheng et al. 2006); thus, the cotyledon fusion phenotype observed in the *cuc2 cuc3* mutant cannot be explained by the mere reduction of *YUC1* and *YUC4* expression levels. Studies of the cellular localization and activity of the auxin efflux protein PIN1 indicate that auxin molecules that are synthesized in the apical region of the embryo are transported to the tips of the cotyledon primordia (Petrášek and Friml 2009). Previous researchers have suggested that the auxin that accumulates in the primordium tips contributes to restricting the expression domain of the *CUC* genes to the cotyledon boundary region (Aida et al. 2002; Furutani et al. 2004). One possibility is that *CUC* genes may fine-tune their own expression domain via this negative feedback regulation involving (*YUC* gene-dependent) auxin biosynthesis.

Our results also indicate the cell-nonautonomous effect of the *CUC* genes on *YUC1* and *YUC4*, as well as on other *YUC* genes that are expressed outside the cotyledon boundary region. The expression of *YUC1* and *YUC4* is not only restricted to the boundary region but also occurs in the inner cells above the provascular cells (*YUC1*) and in the tips of the cotyledon primordia and the root (*YUC4*), where a reduction in expression was observed in the *cuc2 cuc3* mutant embryos (Figure 2E, F). Moreover, the *cuc2 cuc3* double mutation also significantly reduced the expression levels of *YUC3* and *YUC8*, which are reportedly expressed in the suspensor and provascular cells, respectively (Figure 1; Table 1; Robert et al. 2013); this further supports the cell-nonautonomous actions of the *CUC* genes on auxin biosynthetic genes. The expression of several *YUC* family genes, including *YUC1*, *YUC4*, and *YUC8*, is negatively regulated by active auxin in seedlings or in roots (Suzuki et al. 2015; Takato et al. 2017). Because a reduction in local auxin biosynthesis can systemically affect auxin distribution in the embryo (Robert et al. 2013), it is possible that the reduced expression of the *YUC1* and *YUC4* in the cotyledon boundary region of the *cuc2 cuc3* mutant would affect overall distribution of auxin, which would in turn alter *YUC* gene expression in other regions of the embryo. Therefore, the regulation of *YUC1* and *YUC4* expression by *CUC* genes may contribute to the fine-tuning of systemic auxin biosynthesis in the whole embryo rather than specifically affecting the differentiation of the apical region of the embryo.

It is also possible that interactions occur between auxin signaling and other *CUC* downstream factors such as *STM*. Auxin is known to negatively regulate expression of *STM*, which in turn positively regulates cytokinin biosynthesis (Heisler et al. 2005; Jasinski et al. 2005; Yanai et al. 2005). The antagonistic crosstalk of auxin and cytokinin signaling plays an important role in establishing and regulating the meristems (Kurepa et al. 2019; Su et al. 2011). Moving forward, further research should aim to clarify the role of *CUC* genes in balancing auxin and cytokinin signaling through the regulation of *YUC1*, *YUC4*, and *STM* expression.

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