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Direct shoot regeneration from *Arabidopsis thaliana* shoot apical meristems

W. XIN[1], Z. LIU, Y. SONG, T. HOU and F. XIANG*

The Key Laboratory of Plant Cell Engineering and Germplasm Innovation, School of Life Sciences, Shandong University, Jinan 250100, Shandong, China

Abstract

In *Arabidopsis thaliana in vitro* culture, shoots were induced from the shoot apical meristem (SAM) of germinating seeds in the presence of 2,4-dichlorophenoxyacetic acid. Primary shoot primordia developed leaf-like structures, from which secondary shoot primordia were produced. Regenerated shoots were recovered when the material was transferred to a medium lacking auxin. Adventitious roots formed from a callusing basal region of the secondary shoots. The *CUC1* transcription factor was expressed at the apex of the primary shoot primordium and at the boundary between the regenerated SAM and the developing leaf primordia. The *DR5::GUS* transgene was used to localize sites of maximum auxin occurrence. Auxin was firstly detected in the dividing cells beneath the SAM epidermis, which coincided with sites where primary shoot primordia were initiated. In the regenerated shoots, auxin response was not detected in the basal region of the stem, suggesting that the regenerating structures were shoots rather than somatic embryos. Direct shoot regeneration from the *A. thaliana* SAM requires a localized accumulation of auxin.

Additional key words: auxin accumulation, 2,4-dichlorophenoxyacetic acid, shoot apical meristem, transcription factors.

Introduction

Shoots can be regenerated from *in vitro* cultures of root, cotyledon, leaf or hypocotyl explants, provided the appropriate hormonal balance is applied (Che *et al*. 2007). Detailed analysis of the process of shoot regeneration in the model plant *Arabidopsis thaliana*, along with its underlying molecular basis, has shown that both indirect and direct organogenesis are involved, depending on whether or not the explant has initially been allowed to form undifferentiated callus. The composition of the culture medium is the critical factor in determining how the process plays out. An effective protocol has been developed in which the explant was initially incubated on an auxin-rich callus-induction medium, and then it was transferred to a cytokinin-rich shoot-induction medium (Valvekens *et al*. 1988, Cary *et al*. 2002, Che *et al*. 2007).

Somatic embryogenesis could be induced from immature explants through direct or indirect ways. For direct somatic embryogenesis (DSE) in *A. thaliana*, immature embryos were used as primary explants to induce somatic embryos directly on the surface of the cotyledons (Gaj 2001, Mordhorst *et al*. 2002, Bassuner *et al*. 2007, Kurczynska *et al*. 2007, Salaj *et al*. 2008). In the indirect somatic embryogenesis system, embryogenic calli were formed from primary somatic embryos induced from immature zygotic embryos, and secondary somatic embryos were formed from embryonic calli (Ikeda-Iwai

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Abbreviations: 2,4-D - 2,4-dichlorophenoxyacetic acid; DSE - direct somatic embryogenesis; *CUC* - *CUP-SHAPED COTYLEDON*; DAG - day after germination; MES - 2-(morpholin-4-yl)ethanesulfonic acid; QC - quiescent center; SAM - shoot apical meristem.

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[1] Present address: Center for Signal Transduction and Metabolomics, Institute of Botany, Chinese Academy of Sciences, Beijing 100093, China.

* Corresponding author; fax: (+86) 531 88565610, e-mail: xfn0990@sdu.edu.cn

et al. 2002, Su *et al*. 2009). Although direct shoot regeneration can be induced in *Arabidopsis*, the focus has been mostly directed to somatic embryogenesis. As a result, the direct shoot regeneration process remains rather poorly described, and the origin of adventitious shoots not well understood.

CUC1 and *CUC2* are important transcription factors in the *A. thaliana* shoot formation process; they regulate both the formation of the stem apical meristem (SAM) and organ separation (Takada *et al*. 2001). The over-expression of *CUC1* induces the formation of adventitious shoots on the cotyledon adaxial surface (Takada *et al*. 2001).

Auxin induced organogenesis in both the vegetative tomato meristem and the *A. thaliana* inflorescence meristem (Reinhardt *et al*. 2000, 2003). Auxin accumulated around the site of organ initiation, and the subsequent direction of its transport, mediated by PIN proteins (Benková *et al*. 2003), determined the direction of growth of the new organ. The fusion of the highly active synthetic auxin response element DR5 with the *β-glucuronidase* (*GUS*) gene (Ulmasov *et al*. 1997) has been widely used to visualize localization of auxin maximum *in planta* (Sabatini *et al*. 1999, Benkova *et al*. 2003). This allowed the recognition of importance of an auxin gradient for indirect shoot regeneration (Gordon *et al*. 2007, Su *et al*. 2009). Here, we established a regeneration system using mature seeds in *A. thaliana* and studied the direct shoot regeneration in detail.

Materials and methods

Seeds of wild type *Arabidopsis thaliana* L. ecotype Col-0 and of transgenic lines *DR5::GUS* (kindly provided by Prof. E.C. Yeung), *CUC1::GUS* (by Prof. D. Wagner) and *QC25::GUS* (by Prof. C.M. Liu) were surface-sterilized for 10 min in 5 % sodium hypochlorite, rinsed three times in sterile distilled water and put in 4 °C for 48 h. Then they were placed (~40 seeds per 40 cm^3 medium) in a liquid medium containing Murashige and Skoog (1962; MS) salts, vitamins of B-5 medium (100 mg dm^{-3} inositol, 10 mg dm^{-3} vitamin B1, 1 mg dm^{-3} vitamin B6, 1 mg dm^{-3} nicotinic acid; Gamborg *et al*. 1968), 1 mg dm^{-3} 2,4-dichlorophenoxyacetic acid (2,4-D, *Sigma*, St. Louis, USA), 20 g dm^{-3} sucrose, 0.5 mg dm^{-3} acid hydrolyzed casein (*Amresco*, USA) and 0.5 mg dm^{-3} 2-(morpholin-4-yl)ethanesulfonic acid (MES, pH 5.8) and cultured on a rotary shaker at 100 rpm in the dark at 25 °C. This medium induced the formation of leaf-like structures from the SAM of the germinating seeds. The cultures were maintained by sub-culturing on a weekly basis into a liquid medium containing MS salts and B-5 vitamins, 20 g dm^{-3} sucrose, and 1 mg dm^{-3} 2,4-D, pH 5.8. The material was maintained on a rotary shaker at 100 rpm in the dark at 25 °C. After three weeks, the materials were transferred onto a solid medium containing MS salts and B-5 vitamins, 10 g dm^{-3} sucrose, and 8 g dm^{-3} agar, pH 5.8. This medium induced the formation of shoot-like structures which finally produced fertile flowers.

At the end of the induction period (21 d after germination, DAG), leaf-like structures from one germinating seed usually connected with each other at the base. So they were taken as a unit. The induction frequency of leaf-like structures was taken as the ratio of the number of leaf-like structure units to the number of germinating seeds. The number of regenerated shoots per unit of leaf-like structure was counted after 14 d on solid medium without 2,4-D. The regeneration frequency of shoots was evaluated by the ratio of the number of regenerated shoots to the number of germinating seeds.

The explant samples were fixed in a solution containing 50 % alcohol, 10 % acetate acid, 5 % formaldehyde overnight, dehydrated by passing through an ethanol series and embedded in paraffin wax. Sections of 8 μm thickness were cut with a rotary microtome (*Leica RM2235*, Nussloch, Germany) and stained either in a solution of 0.05 % (m/v) toluidine blue O in sodium phosphate buffer or used for the detection of GUS activity. The latter was carried out according to Salaj *et al*. (2008). Briefly, samples were placed in GUS solution for 4 - 12 h at 37 °C and X-Gluc stained preparations were washed briefly in sodium phosphate buffer and cleared in 70 % ethanol before fixation. Scanning electron microscopy was carried out as described by Inoué and Osatake (1988). Processed sections were viewed with *Olympus BX51* microscope (Tokyo, Japan), and micrographs captured using an *Olympus DP72 CCD*. The morphology as well as the GUS staining pattern was recorded using a *Nikon* (Tokyo, Japan) *SM21500* stereomicroscope equipped with a digital camera *DXM1200F CCD*.

Results

After 3 d of germination in the presence of 2,4-D, protuberances with a smooth surface became visible at the SAM, which increased in size over time (Fig. 1*a*,*j*). The cells in these structures tended to be small, and their cytoplasm was denser than that of cells from the cotyledons (Fig. 1*j*). After 7 d (7 DAG), primary shoot primordia appeared on these protuberances and developed into curled leaf-like structures (Fig. 1*b*,*k*), which increased in size over time (Fig. 1*c*). By the end of the induction period (21 DAG), a mass of large leaf-like structures had been generated from the SAM, connected with one another at the base (Fig. 1*d*). Cells in the upper layers of the emerging leaf-like structures were well-ordered and mitotically active (Fig. 1*l*). It appeared that these dividing

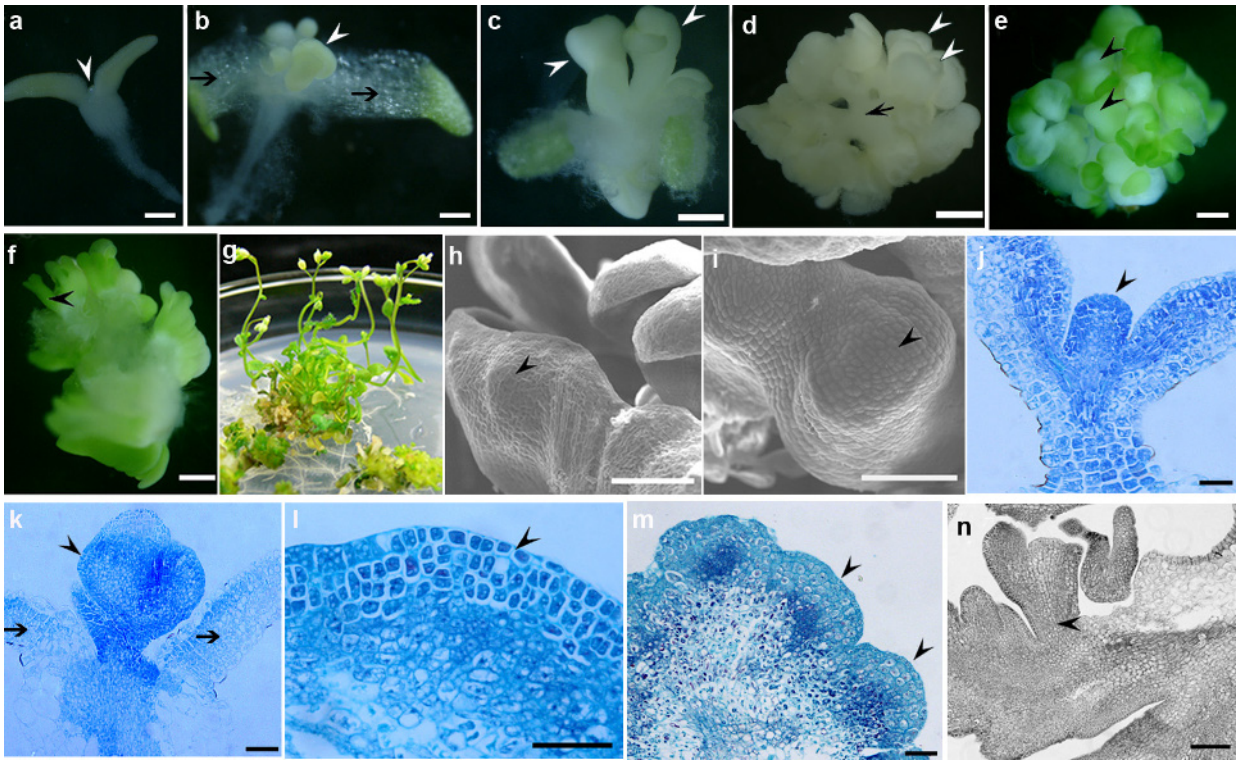

Fig. 1. Morphogenesis of *Arabidopsis* direct shoot regeneration: *a,j* - seedling explants showing a protuberance at the SAM at 3 DAG; *b,k* - a curled leaf-like structure (*arrowheads*) emerging from a protuberance at 7 DAG, *arrows* show the cotyledons; *c* - leaf-like structures continuing to grow; *d,m* - large leaf-like cultures formed at 21 DAG, upon which secondary protuberances (*arrowheads*) were formed; *e* - protuberances developing after 2 d on a solid medium in the absence of 2,4-D (*arrowheads*); *f* - regenerated shoots (*arrowhead*) were observed after 4 d; *g* - fertile regenerated plants; *h* - secondary protuberances (*arrowhead*) developing on the surface of a leaf-like structure observed by scanning electron microscope; *i* - the secondary protuberances continued to grow upon transfer to solid medium; *l* - well-ordered cells (*arrowhead*) in the upper layers of the emerging leaf-like structures; *n* - the central vascular tissues of a set of fused shoots directly connected to the vasculature of the explant (*arrowhead*). *Scale bars* in *a* - *f*, *l* - *n*: 100 μm, in *h* - *i*: 200 μm, in *j* - *k*: 50 μm.

cells retained the competence to form new organs (Lo *et al*. 1997). The surface of some of these leaf-like structures was uneven (Fig. 1*d*), due the emergence of secondary hemispherical protuberances (Fig. 1*h*,*m*). After transfer to a medium lacking 2,4-D, the secondary protuberances continued to increase in size (Fig. 1*i*), and greening began at their periphery (Fig. 1*e*), before the emergence of shoot-like structures, whose basal regions were constricted at their point of attachment to the explant (Fig. 1*f*). Finally, they produced fertile flowers (Fig. 1*g*). Root meristems, however, were not formed from these structures. Procambium-like cells occupied the central core of the stem axes, and the central vascular tissues of the adventitious shoots connected directly to the explant's vasculature (Fig. 1*n*). Even at a later developmental stage, no identifiable root meristem was formed. The base of some of the shoots was made up of a set of regularly arranged, but small cells, which appeared to be the site of the later development of adventitious roots. The induction frequency of leaf-like structures was 19.3 ± 0.5 % at the end of the induction period (21 DAG). The number of regenerated shoots is 13.6 ± 1.3 per unit of leaf-like structure after 14 d on solid medium without 2,4-D. So the regeneration frequency was 262 % in our system. In the absence of 2,4-D in liquid medium, the hypocotyls elongated but the cotyledons did not expand at 3 DAG, while by 7 DAG, the first pair of true leaves had emerged. Neither protuberances nor subsequent leaf-like structures developed.

A transgenic line carrying the construct *CUC1::GUS* was used to track the process of shoot formation and regeneration. A GUS signal was detectable at the SAMs of seedlings growing on ½ MS medium at both 3 and 7 DAG. So *CUC1::GUS* reporter line was used as a molecular marker for SAM formation. This signal was detected at 1 - 3 DAG in seedlings grown in the presence of 2,4-D (Fig. 2*a*). The surface of the enlarged SAM at 5 DAG was free of any GUS activity (Fig. 2*b*). Subsequently, GUS activity was detected at the apex of the primary shoot primordium, and its zone gradually expanded over time (Fig. 2*c*,*d*). After removing 2,4-D from the medium, GUS activity became visible in the SAM region of the secondary shoots (Fig. 2*e*). The expression pattern of *CUC1* in plants grown in liquid medium in the absence of 2,4-D was similar to that in plants grown on ½ MS.

To show that the regenerating structures were shoots rather than somatic embryos, a simple germination test was carried out, by detaching a large number of

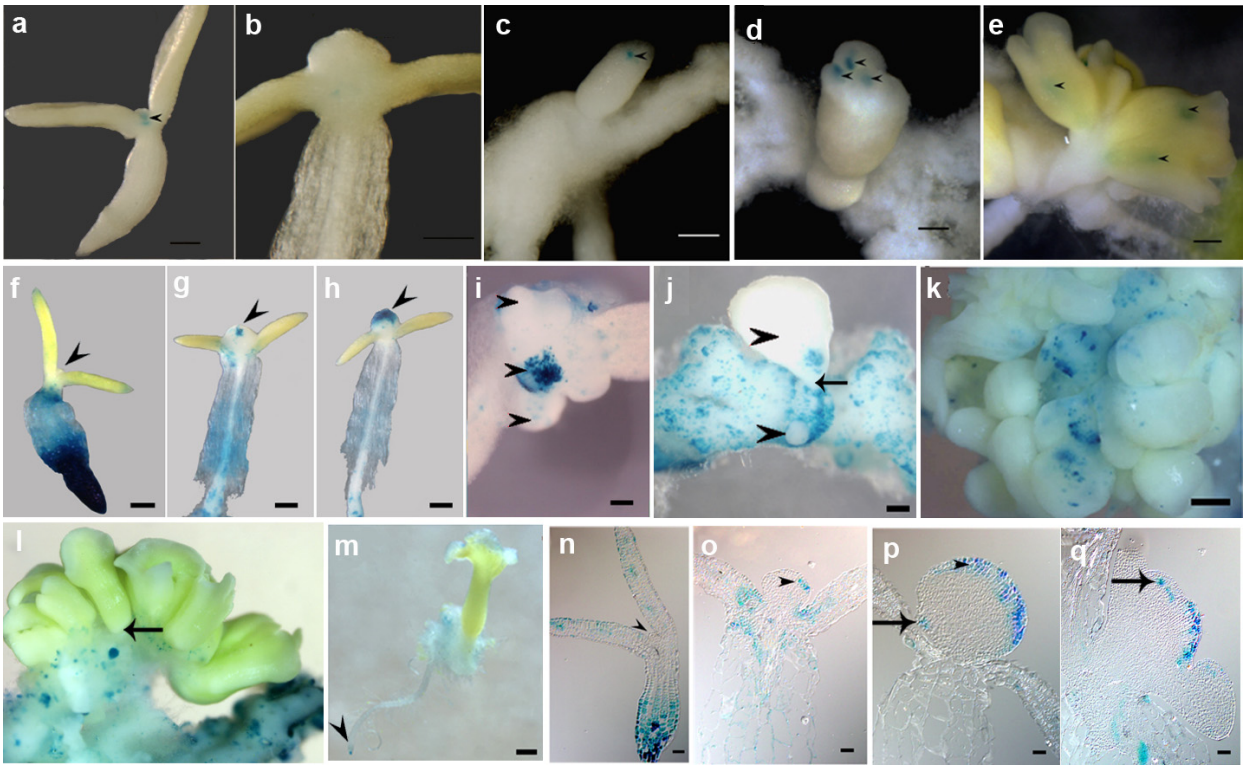

Fig. 2. Expression patterns of *CUC1::GUS* and *DR5::GUS* during direct shoot regeneration: *a* - *CUC1::GUS* activity was restricted to the apical area (*arrowhead*) from 1 to 3 DAG; *b* - No *CUC1::GUS* activity at the surface of the enlarged SAM at 5 DAG; *c*,*d* - between 6 and 10 DAG, *CUC1::GUS* activity first appeared at the apex of the primary shoot primordium and then gradually spread (*arrowheads*); *e* - pattern of expression *CUC1::GUS* after transfer to medium lacking 2,4-D; *f*,*n* - the SAM had enlarged and begun to form protuberances (*arrowheads*) at 3 DAG, but no *DR5::GUS* activity was detected; *g*,*o* - *DR5::GUS* activity was initiated on the SAM surface at 4 DAG (*arrowheads*); *h*,*p* - *DR5::GUS* activity expanded over the whole surface of the primary protuberances at 5 DAG (*arrowheads*); *i* - between 6 and 10 DAG, the distribution of *DR5::GUS* activity had become rather concentrated and *q* - primary shoot primordia were ready to form at the places where *DR5::GUS* staining was visible in the enlarged SAM (*arrow*); *j* - one or several primary shoot primordia from the enlarged SAM developed into leaf-like structures (*arrowheads*), with weak GUS activity in their basal region (*arrow*); *k* - upon transfer to a medium lacking 2,4-D, *DR5::GUS* activity receded quickly, and *l* - it was not detected in the basal region of the regenerating shoots (*arrow*); *m* - *DR5::GUS* activity rose at the root tip as adventitious roots were formed. *Scale bars* in *a* - *m*: 200 μm, in *n* - *q*: 50 μm.

adventitious shoots from the explant and placing them on MS medium. Most of them did not form roots directly, but rather produced adventitious roots from a callus at their base. *QC25* is a marker gene for quiescent center (QC) of roots in seedlings (Blilou *et al*. 2005). Thus, a transgenic line carrying the construct *QC25::GUS* was used to clarify regenerated roots. The GUS signals mainly appeared at QC area of regenerated roots, which was similar to roots in seedlings grown on ½ MS, suggesting that regenerated structure from the callus is adventitious root (data not shown).

The *DR5::GUS* transgene was exploited to visualize auxin maximum perception during shoot induction and regeneration. GUS activity was not detected in the SAM in the period 1 - 3 DAG (Fig. 2*f*,*n*), but as the SAM enlarged, GUS activity began visible at its surface (Fig. 2*g*,*o*), and later expanded over the surface of the protuberances (Fig. 2*h*,*p*). By 6 - 7 DAG, the distribution of GUS activity had become rather concentrated (Fig. 2*i*), and one or several primary shoot primordia developed from the enlarged SAM (Fig. 2*j*), growing into leaf-like structures, with weak GUS activity in their basal region. Primary shoot primordia were formed at the same places where GUS staining was visible in the enlarged SAM (Fig. 2*p*,*q*). Since GUS expression reflected auxin response, the indications were that the initiation of the primary shoot primordia was triggered by a localized build up of auxin response. Upon transfer to a solid medium where exogenous auxin was not supplied, GUS activity in the explants disappeared quickly (Fig. 2*k*). When the shoots formed, GUS activity was not detected in the basal region of the leaflets (Fig. 2*l*). Finally, GUS activity rose at the root tip as adventitious roots were formed (Fig. 2*m*). Thus, it appeared that auxin is needed for shoot primordium induction.

In the absence of 2,4-D in the medium, GUS activity was detected at the tips of the cotyledons by 3 DAG, and its extent was larger than in equivalent seedlings grown on ½ MS. By 7 DAG in liquid medium, GUS activity was visible at the leaf apex and the cotyledon surface, while it was restricted to cotyledon tips in seedlings grown on ½ MS.

Discussion

Here, we explored the detailed process of the direct shoot regeneration in *A. thaliana*. In the presence of 2,4-D, primary shoot primordia emerged from enlarged SAMs, and developed into leaf-like structures (Fig. 1*b*,*k*). Secondary shoot primordia were later generated on the surface of the leaf-like structures (Fig. 1*d*), and these differentiated into shoots when the 2,4-D was removed (Fig. 1*f*,*g*). Histological analysis revealed that their axis was directly connected to the vascular tissue of the explants (Fig. 1n). Most of regenerating structures did not form roots directly, but rather produced adventitious roots from a callus at their base. These results suggest that the regenerating structures were shoots rather than somatic embryos.

Shoots can also be regenerated from *A. thaliana* root explants *via* the *de novo* formation of a SAM (Valvekens *et al*. 1988, Cary *et al*. 2002, Che *et al*. 2007, Gordon *et al*. 2007). In this *in vitro* system, the process is an indirect regeneration pathway, with the shoots regenerated from callus. In the direct somatic embryogenesis (DSE) system, the somatic embryos generated from immature seeds emerged from cotyledon abaxial cells, and SAM was not involved in somatic embryo formation (Bassuner *et al*. 2007, Kurczynska *et al*. 2007). Then they went on to develop into seedlings in a fashion similar to the progress of a zygotic embryo germination. In the indirect somatic embryogenesis system, green primary somatic embryos were formed from immature zygotic embryos after 10 d on solid medium. Then embryonic calli were formed from primary somatic embryos, and secondary somatic embryos were formed from embryogenic calli (Ikeda-Iwai *et al*. 2002, Su *et al*. 2009).

Direct shoot regeneration from immature seed explants has been described several times in the DSE system (Gaj 2001, Mordhorst *et al*. 2002, Bassuner *et al*. 2007, Kurczynska et al. 2007), but somatic embryogenesis was relatively common in this system. In our experiment, adventitious shoots were observed more often, though somatic embryogenesis was also observed (data not shown). The major difference in these regeneration pathways probably reflects the different physiological maturity of the two explant types. The seedling SAM is primed to produce leaf primordia, and these are prompted by the presence of 2,4-D to form shoots.

The expression of the *DR5::GUS* construct confirmed the importance of 2,4-D in shoot initiation. After the SAM became enlarged, auxin was accumulated in the dividing cells beneath the SAM epidermis (Fig. 2*o*). The sites of auxin accumulation and primary shoot primordia formation coincided (Fig. 2*p*,*q*), which implies that auxin response is important for the formation of the primary shoot primordia. Auxin accumulation was also seen on surface of hypocotyl or cotyledon, sites which could have been associated with callus initiation. Once the 2,4-D was removed, auxin content decreased quickly (Fig. 2*k*). When the shoots formed, GUS activity was not detected in the basal region (Fig. 2*l*), further demonstrating that the regenerating structures were shoots rather than somatic embryos. In the absence of 2,4-D, neither protuberances nor subsequent leaf-like structures developed.

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