DE GRUYTER OPEN

Nova Biotechnol Chim (2017) 16(1): 20-25
DOI: 10.1515/nbec-2017-0003

Nova Biotechnologica *et* Chimica

Transient expression of green fluorescent protein in parasitic dodder as a tool for studying of cytoskeleton

Peter Kaštier[a], Michaela Martinčová[a,✉], Roderik Fiala[b] and Alžbeta Blehová[a]

[a] *Department of Plant Physiology, Faculty of Natural Sciences, Comenius University in Bratislava, Ilkovičova 3278/6, Bratislava, SK-841 04, Slovak Republic*

[b] *Institute of Botany, Plant Science and Biodiversity Centre SAS, Dúbravská cesta 9, Bratislava, SK-845 23, Slovak Republic*

Article info

Article history:
Received: 22nd March 2017
Accepted: 16th May 2017

Keywords:
Actin
Agrobacterium tumefaciens
Cuscuta europaea
gfp
Parasitic plants
Transient transformation

Abstract

Dodder (*Cuscuta*) species cause severe agricultural damage in many countries throughout the world. To establish strategies for control of its growth and spreading it is important to study its life cycle and survival strategies. For these efforts genetic modification would represent a powerful tool. Here we report on *Agrobacterium*-mediated transformation of dodder using green fluorescent protein (GFP) fused to actin-binding protein as a vital marker. Since the shoot of germinating *C. europaea* contains a functional apical meristem and grows quickly comparing to the root-like structure, the shoot apex was used here as explant. The transgene expression was only transient, nevertheless it enabled to detect allocation of actin filaments and studying the cytoskeleton organization in dodder shoot apex. Transient expression of GFP appears to be a suitable method for studying *Cuscuta* development through cytoskeleton organisation that is presently largely unexplored.

© University of SS. Cyril and Methodius in Trnava

Introduction

Parasitic *Cuscuta* (dodder) genus (*Convolvulaceae*) contains 194 species divided into four subgenera, namely *Monogynella*, *Cuscuta*, *Grammica* and *Pachystigma* (Costea *et al.* 2015). They are spread nearly worldwide (García *et al.* 2014). Dodders are twining herbs with reduced vegetative organs (leaves are minute scales). Dodder species cause severe agricultural damage in many countries throughout the world resulting in yield decrease of important crops such as tomatoes and potatoes (Heide-Jørgensen 2011). In spite of their agricultural importance, many questions remain unanswered and an efficient transformation system would greatly accelerate the progress in understanding host-parasite relationships.

Research on mechanisms of haustorium formation as of key organ necessary for dodder survival is a prerequisite for developing efficient strategies to control its development and spreading. Understanding the interaction between host plants and parasitic dodders at the ultrastructural and molecular levels may generate knowledge applicable to control plant-parasitic interactions and/or engineer dodder-resistant plants. Though efficient transformation protocols have been described for dodder calli (Borsics *et al.* 2002; Švubová and Blehová 2013), these have never resulted in regeneration of whole plants (Ichihashi *et al.* 2015). Here we report on transformation of excised dodder shoot explants *via Agrobacterium* that resulted in transient expression of the vital marker *gfp* gene.

✉ *Corresponding author:* martincova21@uniba.sk

Bereitgestellt von Slovenská poľnohospodárska knižnica | Heruntergeladen 27.02.20 14:36 UTC

To study the transgenic nature and development of transformed tissue we used a GFP protein fused in frame to a protein that binds to actin. Actin as a component of the cytoskeleton coordinates numerous cellular processes required for plant development, including cell division and cell expansion (Wasteneys and Ambrose 2009), targeted-vesicle transport to establish hormone gradients (Muday 2000), polarity determination to direct localized wall biosynthesis (Baluška *et al.* 2003), and organelle positioning in response to environmental cues (Šamaj *et al.* 2006). In *Cuscuta* species, the presence of α-tubulin, though in quantities lower than in roots, has been associated with the degradation processes of the root-like structures (Sherman *et al.* 2008). Further, indirect immunolocalization of actin as well as tubulin has indicated important role in prehaustorial cells in *C. europaea* (Kaštier *et al.* 2015). The techniques used in those studies, however, do not allow to study the dynamic changes in cytoskeleton organization in real-time in living cells, therefore would largely benefit from a use of a vital marker like GFP (Švubová and Blehová 2013; Blehová *et al.* 2015).

Experimental

Plant material

Parasitic dodder seeds (*C. europaea)* were collected in Ivanka pri Dunaji (2014, Slovakia, latitude 48°19´, longitude 17°22´) and their host plant was *Urtica dioica*. The seeds were scarified at 4 °C and then treated with concentrated H_2SO_4 for 60 min at room temperature in order to disrupt the seed coat integration. Acid was washed-off by sterile distilled water. The seeds were then sterilized with 5 % Savo (0.24 % NaClO) solution for 15 min and thoroughly washed with sterile distilled water. Dodders were cultivated *in vitro* on MS medium (Murashige and Skoog 1962) containing agar (7 g/L), pH 5.8, in the growth chamber (16/8 h long-day photoperiod) at 23±2 ºC, 100 µmol/m^2s PAR.

Bacterial strains and cultivation conditions

Agrobacterium tumefaciens LBA4404 carrying the plasmid pCB302 (Voigt *et al.* 2005) was used for dodder transformation. The T-DNA of the pCB302 contained a single GFP fused with N-terminus of *Arabidopsis thaliana* fimbrin actin-binding domain that is driven by constitutive CaMV 35S promoter. As a plant selection marker, the neomycin phosphotransferase II gene (nptII) was used under control of the nos promoter. *A. tumefaciens*/pCB302 was cultivated on LB (Luria-Bertani) medium, pH 6.9. Bacterial suspension (300 µL) and 100 mg/L of kanamycin were added into a liquid LB medium and cultivated for 12 hours on a shaker (SHAKER DOS-20L; 120 rpm) in the dark at 23 °C.

Bacterial transformation

Segments of *C. europaea* stems (7–8 mm shoot apices) were infected by A. tumefaciens/pCB302 in the liquid LB medium for 10 min and subsequently co-cultivated on MS medium without antibiotics overnight in the dark at 23±2 °C. Infected segments were washed by a liquid MS medium containing cefotaxime (200 mg/L). After drying on a sterile paper, explants were grown on a selective MS medium containing agar and antibiotics – kanamycin (100 mg/L) and cefotaxime (200 mg/L). They were inoculated on fresh selective media in one week sub-cultivation intervals. To monitor plant growth, shoot lengths were measured every week for six weeks of in vitro cultivation.

Microscopy of dodders

Light microscopy of *C. europaea* seedlings was carried out using a stereomicroscope (Leica M165 FC). GFP positive dodder stem segments were examined by a confocal microscope(Olympus FV1000) at an excitation wavelength 488 nm. GFP fluorescence and plastid autofluorescence were detected using 505 – 550 nm and 655 – 755 nm barrier emission filters, respectively.

Bereitgestellt von Slovenská poľnohospodárska knižnica | Heruntergeladen 27.02.20 14:36 UTC

Results and Discussion

Technical advances in transformation techniques have facilitated research in parasitic plants, too. Transgenic dodder calli or roots derived from *A. rhizogenes*-mediated transformations still were able to form lateral haustoria that penetrated the host (Fernández-Aparicio *et al.* 2011; Ishida *et al.* 2011). However, stable and heritable transformation of *Cuscuta* plants, enabling molecular genetics studies of these species, have not been established yet, in part due to challenges in developing transformation protocols, but also because regeneration of a parasitic organism *in vitro* is difficult (Borsics *et al.* 2002; Švubová and Blehová 2013). Since the *C. europaea* seedlings germinate as cotyledonless and leafless shoots with ephemeral root-like structure, transformation of shoot apex can be used as an alternative for *Agrobacterium*-mediated transformation of monocotyledonous crop species (Gould *et al.* 1991; this study).

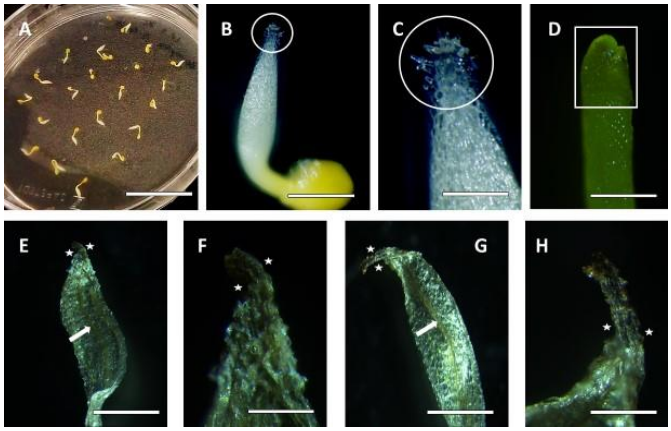

Fig. 1. Growth and development of *C. europaea* seedlings *in vitro*. **A.** Germinating dodder seeds on MS medium on 1st day after germination. **B.** Germinating seed with tuberous radicle and root tip (circle). **C.** Detail of the root tip (circle) with hairs. **D.** Dodder leafless stem with shoot apex (rectangle) on 7th day after germination. **E.** Early degradation (7th day after germination; asterisks) of root-like structure with visible xylem (arrow). **F.** Detail of the degrading root-like structure (asterisks) on 7th day after germination. **G.** Progressing degradation (asterisks) of the root-like structure on 14th day after germination, xylem (arrow). **H.** Detailed view on root-like structure in the late stage of degradation (asterisks) on 14th day after germination. Scale bars: A = 2 cm; B, D, E, G = 2 mm; C, F, H = 0.5 mm.

Dodder seeds were transferred to *in vitro* conditions. According to some authors, stratification by cold and scarification are essential for their successful germination (Švubová and Blehová 2013; Furuhashi *et al.* 2016). Variability of seed coat structure among different dodder species determines their germination characteristics impacting adaptation to natural environment (Behdarvandi *et al.* 2015).

The seeds started to germinate 2–3 days after sowing (Fig. 1A). A visibly swollen radicle of *C. europaea* emerged from the seeds as the first structure (Fig. 1A and B) and this was followed by the emergence of a stem (Fig. 1D). Since the first day after germination, differentiation of root hairs at the radicle tip was observed (Fig. 1C). In contrast, in the case of *C. campestris*, the bulges resembling root hairs are absent and the surface of the radicle-like structure is smooth (Lyshede 1985). According to Sherman *et al.* (2008), these root hairs do not elongate and have a different ultrastructure compared to the root hairs of typical dicots. In addition, the root-like structure of dodder is considered to be a remnant of the true shoot that was significantly modified during evolution (Sherman *et al.* 2008). For example, in *C. europaea* it lacks both root cap and root apical meristem (Fig. 1B and C) as observed by other authors (Lyshede 1986; Lee *et al.* 2000; Behdarvandi *et al.* 2015). Several days after germination this root-like organ degrades through a senescence-like process from apex towards the stem base (Fig. 1E – H), possibly due to low abundance of microtubules in its cells and lack of mitotic division (Sherman *et al.* 2008). Nevertheless, this structure is still capable of interacting with microorganisms, therefore Behdarvandi *et al.* (2015) consider this organ to be a modification of true root. Moreover, xylem strands with non-lignified cell walls were clearly visible even under a light microscope (Fig. 1E and G) indicating to functional translocation of some nutrients into the rapidly growing shoot (Sherman *et al.* 2008).

The development of *C. europaea* plants shows a remarkable degree of plasticity to a large extent attributed to shoot apical meristem. In contrast to the pale root-like structure *C. europaea* stem tips are green (Fig. 1B and D). Several photosynthesis-related proteins have been described in dodder stems (e.g. RbcL, THF1, D1) indicating that

Bereitgestellt von Slovenská poľnohospodárska knižnica | Heruntergeladen 27.02.20 14:36 UTC

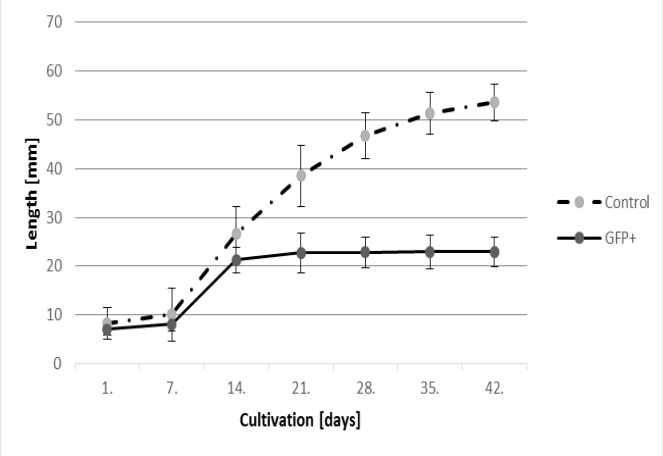

Fig.2. Lengths of dodder shoot explants during 35 days of *in vitro* cultivation. GFP positive (full line) shoot explants transformed by *A. tumefaciens*/pCB302. Dot dotted line represents the length of control shoot explants.

dodders in this non-parasitic developmental stage are photosynthetically active (Švubová *et al.* 2013). In addition, they contain much more actin and microtubule elements compared to roots (Sherman *et al.* 2008) enabling rapid growth of dodder shoot explants on hormone free MS media (Fig. 2). Dynamic reorganization of the cytoskeleton in combination with photosynthetic activity of young dodder seedlings is likely responsible for ability to survive without host plants for up to 3 weeks (Heide-Jørgensen 2008). Transformation of excised shoot explants was performed using the construct pCB302 which has successfully been used for actin visualization in transgenic *Arabidopsis* plants (Voigt *et al.* 2005; Wang *et al.* 2007). After transformation, dodder tissue was cultivated and its growth was monitored. We observed strong GFP fluorescence, especially in the shoot tips (Fig. 3A). Since the marker protein (GFP) was fused to actin-binding protein, higher magnification images of cells in the transformed shoot's apical zone, revealed a network of fine F-actin surrounding the nuclei (Fig. 3B) as described previously for other plant species (Chiu *et al.* 1996; Wang *et al.* 2007). However,

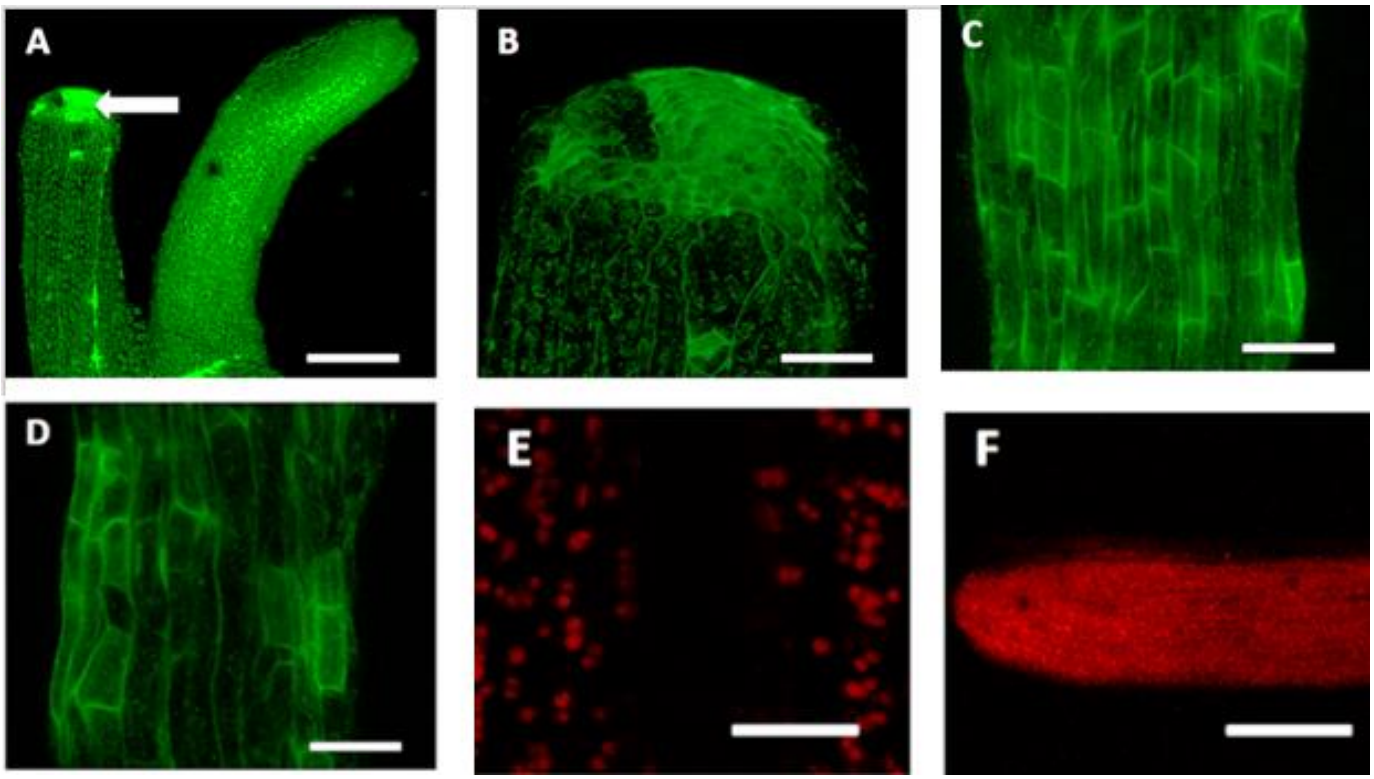

Fig. 3. GFP fluorescence of *C. europaea* shoot explants analysed by confocal microscopy. A) GFP positive dodder shoots on 7th day after transformation, arrow indicates stronger GFP positive signal in the meristematic region of the shoot apex. B) GFP fluorescence of the stem tip epidermal cells. C) The positive signal in the cells of elongation zone on 7th day after transformation. D) The elongating cells with lowered GFP fluorescence on 14th day after transformation. E) Autofluorescence of GFP negative dodder stem on 35th day after transformation. F) Autofluorescence of control stem of non-transformed *C. europaea*. Scale bars: A, F = 200 µm; B, C, D = 50 µm; E = 25 µm.

Bereitgestellt von Slovenská poľnohospodárska knižnica | Heruntergeladen 27.02.20 14:36 UTC

in the distal elongation zone the GFP fluorescence turned considerably weaker (Fig. 3C–E). This observation is consistent with significantly reduced growth of the transformed dodder stems in comparison with the control explants (Fig. 2). Nevertheless, the exact reason for this phenomenon is unclear and might also include problems with the transformation procedure (e.g. gradual degradation of un-incorporated T-DNA, gradual silencing of the transgene expression, recalcitrant dodder genotype etc.) or failing of the binding of the fusion protein in the tissue, or both. Nevertheless, fluorescence of the transgene product in dodder tissue (cultivated in presence of selection agent for 7–14 days), reflects to transient transgene expression that allows for non-invasive observation of cytoskeleton organisation in developing shoot tissue of the parasite. The presence and activity of the transgene in transformed dodder tissue needs to be verified in more detail at molecular level. Further, since dodder shoots appeared as GFP positive only for a relatively short period of time, further optimization of the transformation procedure using e.g. sonication followed by vacuum treatment (Fernández-Aparicio *et al.* 2011). Enhancement of assays for transient gene expression will probably involve optimization of factors promoting T-DNA transfer e.g. over-expression of bacterial virulent *virE* and *virG* genes can enhance transient expression (Wroblewski *et al.* 2005). Also *virD* that probably plays a major role in targeting T-strands to the nucleus through α importins localized in nuclear envelope (Gelvin 2012). In addition, it is also beneficial to apply *Agrobacterium* strains that carry specific P19 helper plasmids as silencing suppressors (Voinnet *et al.* 2003; Canto *et al.* 2006), what will be a challenge for future.

Conclusions

The shoot of germinating *C. europaea* contains a functional apical meristem and grows quickly comparing to the root-like structure, therefore shoot apex was used here for genetic transformation *via A. tumefaciens.* The fluorescence pattern of the marker gene product suggests only a transient transformation. Fusion of GFP with actin-binding element appeared as feasible for not only confirming the presence of transgene product in host tissue but also for studying the organization and of cytoskeleton in the vital dodder tissue. This is the first report on (transient) transgene expression in vegetative tissue of parasitic dodder.

Acknowledgements

This work was supported by the Slovak Research and Development Agency under the contract No. APVV-16-0051.

References

Baluška F, Šamaj J, Wojtaszek P, Volkmann D, Menzel D (2003) Cytoskeleton plasmamembrane-cell wall continuum in plants. Emerging links revisited. Plant Physiol 133: 482-491.

Behdarvandi B, Guinel FC, Costea M (2015) Differential effects of ephemeral colonization by arbuscular mycorrhizal fungi in two *Cuscuta* species with different ecology. Mycorrhiza 25: 573-585.

Blehová A, Švubová R, Lukáčová Z, Moravčíková J, Matušíková I (2015) Transformation of sundew: pitfalls and promises. Plant Cell Tiss. Org. Cult. 120: 681-687.

Borsics T, Mihálka V, Oreifig AS, Bárány I, Lados M, Nagy I, Jenes B, Told O (2002) Methods for genetic transformation of the parasitic weed dodder (*Cuscuta trifolii* Bab. et Gibs) and for PCR-based detection of early transformation events. Plant Sci. 162: 193-199.

Canto T, Uhrig JF, Swanson M, Wright KM, Macfarlane SA (2006) Translocation of tomato bushy stunt virus P19 protein into the nucleus by ALY proteins compromises its silencing suppressor activity. J. Virol. 80: 9064-9072.

Chiu W, Niwa Y, Zeng W, Hirano T, Kobayashi H, Sheen J (1996) Engineered GFP as a vital reporter in plants. Curr. Biol. 6: 325-330.

Costea M, García MA, Stefanović S (2015) A phylogenetically based infrageneric classification of the parasitic plant genus *Cuscuta* (Dodders, *Convolvulaceae*). Syst. Bot. 40: 269-285.

Fernández-Aparicio M, Rubiales D, Bandaranayake PCG, Yoder JI, Westwood JH (2011) Transformation and regeneration of the holoparasitic plant *Phelipanche aegyptica*. Plant Methods 7: 36-46.

Furuhashi T, Nakamura T, Iwase K (2016) Analysis of metabolites in stem parasitic plant interactions: interaction of *Cuscuta – Momordica* versus *Cassytha – Ipomoea*. Plants 5: 43-57.

García MA, Costea M, Kuzmina M, Stefanović S (2014) Phylogeny, character evolution, and biogeography of *Cuscuta* (dodders; *Convolvulaceae*) inferred from coding plastid and nuclear sequences. Am. J. Bot. 101: 670-690.

Bereitgestellt von Slovenská poľnohospodárska knižnica | Heruntergeladen 27.02.20 14:36 UTC

Gelvin SB (2012) Traversing the cell: *Agrobacterium* T-DNA´s journey to the host genome. Front Plant Sci. 3: 52-62.

Gould J, Devey M, Hasegawa O, Ulian EC, Peterson G, Smith RH (1991) Transformation of *Zea mays* L. using *Agrobacterium tumefaciens* and the shoot apex. Plant Physiol. 95: 426-434.

Heide-Jørgensen HS (2011) Parasitic plants. *In*: Simberloff D, Rejmánek M (Eds.) Encyclopedia of biological invasions. University of California Press, Berkeley and Los Angeles, 504-510.

Heide-Jørgensen H (2008) Parasitic flowering plants. 1st ed. Danvers, MA 01923, USA, 438 p.

Ichihashi Y, Mutuku JM, Yoshida S, Shirasu K (2015) Transcriptomics exposes the uniqueness of parasitic plants. Brief. Funct. Genomics 14: 275-282.

Ishida JK, Yoshida S, Ito M, Namba S, Shirasu K (2011) *Agrobacterium rhizogenes*-mediated transformation of the parasitic plant *Phtheirospermum japonicum*. PLoS ONE 6: e25802.

Kaštier P, Švubová R, Fiala R, Blehová A (2015) Organization of the cytoskeleton in various stages of *Cuscuta europaea* haustorium development. *In:* Proceedings of 3rd International Conference: Green for Good. Olomouc: Palacky University of Plant Biotechnology, 15.6.-18.6.2015, p. 64.

Lee KB, Park JB, Lee S (2000) Morphology and anatomy of mature embryos and seedlings in parasitic angiosperm *Cuscuta japonica*. J. Plant Biol. 43: 22-27.

Lyshede OB (1985) Morphological and anatomical features of *Cuscuta pedicellata* and *C. campestris*. Nord. J. Bot. 5: 65-77.

Lyshede OB (1986) Fine-structural features of the tuberous radicular end of the seedling of *Cuscuta pedicellata*. Ber. Deutsch. Bot. Ges. Bd. 99: 105-109.

Muday G (2000) Interactions between the actin cytoskeleton and an auxin transport protein. *In:* Staiger CJ, Baluska F, Volkmann D, Barlow P (eds.) Actin: a dynamic framework for multiple plant cell functions. Kluwer Academic Press, Dordrecht, Netherlands, 541-556.

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol. Plant. 15: 473-497.

Sherman TD, Bowling AJ, Barger TW, Vaughn KC (2008) The vestigial root of dodder (*Cuscuta pentagona*) seedlings. Int. J. Plant Sci. 169: 998-1012.

Šamaj J, Müller J, Beck M, Bohm N, Menzel D (2006) Vesicular trafficking, cytoskeleton and signalling in root hairs and pollen tubes. Trends Plant Sci. 11: 594-600.

Švubová R, Blehová A (2013) Stable transformation and actin visualization in callus cultures of dodder (*Cuscuta europaea*). Biologia 68: 633-640.

Švubová R, Ovečka M, Pavlovič A, Slováková Ľ, Blehová A (2013) *Cuscuta europaea* plastid apparatus in various developmental stages: Localization of THF1 protein. Plant Signal. Behav. 8: e24037.

Voigt B, Timmers ACJ, Šamaj J, Müller J, Baluška F, Menzel D (2005) GFP-FABD2 fusion construct allows *in vivo* visualization of the dynamic actin cytoskeleton in all cells of *Arabidopsis* seedlings. Eur. J. Cell Biol. 84: 595-608.

Voinnet O, Rivas S, Mestre P, Baulcombe D (2013) An enhancement transient expression system in plants based on suppression of gene silencing by the p19 protein of tomato bushy stunt virus. Plant J. 33: 949-956.

Wang Y-S, Yoo C-M, Blancaflor EB (2007) Improved imaging of actin filaments in transgenic *Arabidopsis* plants expressing a green fluorescent protein fusion to the C- and N-termini of the fibrin actin-binding domain 2. New Phytol. 177: 525-536.

Wasteneys GO, Ambrose JCH (2009) Spatial organization of plant cortical microtubules: close encounters of the 2D kind. Trends Cell Biol. 19: 62-71.

Wroblewski T, Tomczak A, Michelmore R (2005) Optimization of *Agrobacterium*-mediated transient assays of gene expression in lettuce, tomato and *Arabidopsis*. Plant Biotechnol. J. 3: 259-273.

Bereitgestellt von Slovenská poľnohospodárska knižnica | Heruntergeladen 27.02.20 14:36 UTC