Threshold Change in Expression of GFP-FABD2 Fusion Protein During Development of Arabidopsis thaliana Leaves

Justyna Labuz, Weronika Krzeszowiec, and Halina Gabrys

Department of Plant Biotechnology, Jagiellonian University,
Gronostajowa 7, 30-387 Cracow, Poland

Received October 20, 2010; revision accepted December 23, 2010

One of the most important technical challenges in cell biology is visualization of the actin cytoskeleton. The widely used GFP-FABD2 fusion protein is a helpful tool for investigating actin architecture in living plants. Here we report our attempt to visualize F-actin in mature leaves of transgenic Arabidopsis thaliana. With a confocal microscope we observed loss of GFP fluorescence in mature Arabidopsis leaves between 19 and 21 days of development. As this pattern was characteristic of all investigated plants and dependent on the age of the plants, we performed precise expression studies at the mRNA (semiquantitative PCR) and protein (Western blot) levels. Our results clearly show a sudden decrease of GFP-FABD2 expression in Arabidopsis leaves after the third week of growth. This means that transgenic Arabidopsis bearing the GFP-FABD2 construct is not a good model system for visualization of the actin cytoskeleton in leaves of mature plants.

Key words: Actin, Arabidopsis thaliana, gene silencing, GFP-FABD2, mature leaves.

INTRODUCTION

Visualization of actin cytoskeleton architecture in plant cells presents a challenge. Actin filaments may be labeled with phalloidin conjugated to a fluorescent marker (Krzeszowiec et al., 2007), or immunocytochemically with antibodies against actin and fluorescence-labeled secondary antibodies (van Gestel et al., 2001). The main drawback of these methods is that they require cell fixation. The unique features of green fluorescent protein (GFP) enable imaging of cells in vivo. Since efforts to visualize actin conjugated to GFP have remained unsuccessful, an indirect method using fusion constructs bearing GFP and actin-binding proteins has been developed. Among them, the GFP-FABD2 fusion protein derived from the C-terminal part of Arabidopsis FIMBRIN1 (Sheenan et al., 2004; Wang et al., 2004; Voigt et al., 2005) is considered one of the best systems. It enables visualization of very detailed F-actin structures in practically all cell types of Arabidopsis seedlings (Voigt et al., 2005). Stably transformed Arabidopsis does not show phenotypic changes, nor any alteration of phototropic and gravitropic responses (Voigt et al., 2005), unlike GFP mouse talin construct GFP-mTn, which perturbs the diffuse growth of cells (Sheenan et al., 2004). The GFP-mTn system also shows diffuse labeling of the nucleoplasm, a phenomenon not observed in plants expressing GFP-FABD2 (Sheenan et al., 2004). Along with some positive features, the GFP-FABD2 system also has some drawbacks. Actin networks observed in the pollen tubes of Nicotiana tabacum transformed with GFP-FABD2 are differently visualized in fixed cells (Wilsen et al., 2006). Moreover, the presence of this fusion protein reduces cytoplasmic streaming (Holweg, 2006). Finally, we can report here that Arabidopsis stably transformed with GFP-FABD2 shows rapid loss of fluorescence during the growth and maturation of leaves.

MATERIALS AND METHODS

PLANT GROWTH CONDITIONS

Arabidopsis thaliana wild-type Columbia was acquired from Lehle seeds (Round Rock, TX, U.S.A.). The seeds of Arabidopsis transformed with a GFP-FABD2 construct were given us by Dr. Boris Voigt (Bonn, Germany). Arabidopsis thaliana plants were vernalized for 2 days after sowing and grown in commercial soil in an environmental chamber (Sanyo MLR 350H, Japan) at 23°C and constant 85% humidity, with a 10 h photoperiod. Illumination was
provided by fluorescent lamps (Sanyo, FL40SS.W/37; light intensity 70–100 μmol m⁻² s⁻¹).

**SEMIQUANTITATIVE PCR**

Total RNA was isolated from leaves using Trizol reagent (Invitrogen, U.S.A.) according to the manufacturer’s protocol. First-strand cDNA synthesis was performed using a Revertaid™ First Strand cDNA Synthesis Kit (Fermentas UAB, Lithuania) with random hexamer primers. A QuantumRNA™ 18S RNA Kit (Ambion Europe Ltd, UK) was used as an internal standard (18S primer : competimer ratio 3:7) in simultaneous PCR amplification with 25 cycles. For amplification of the **GFP-FABD2** construct the primers used were forward 5'CCCGGGATGC
GAAGGGAGAAGAACTTTTCACTGGAGTT3' and reverse 5'GCTTGCAGTTCATTCTCTCTGC3'. For amplification of **Arabidopsis FIMBRIN1** the primers were forward 5'ATGTCAGGTACGTGGGTG3' and reverse 5'ATCCCTTGCACTCAGTGAGCC3'.

**WESTERN BLOT ANALYSIS**

Leaves were lyophilized overnight, homogenized in liquid nitrogen, suspended in denaturing Laemmli buffer and incubated 10 min at 100°C. Samples were separated by SDS-PAGE on 10% gel and transferred to a PVDF membrane. Detection was performed using an anti-GFP primary antibody (Living Colors GFP Monoclonal Antibody, Clontech, no. 632375, Canada), followed by secondary antimouse antibody conjugated with alkaline phosphatase (Sigma, No. A3562, U.S.A.).

**CONFOCAL MICROSCOPY**

GFP fluorescence was tested every 3 days starting from the seventh day of growth up to the end of the third week. Seedlings were gently infiltrated with distilled water in a plastic syringe at room temperature. Images, taken with a BioRad MRC 1024 confocal microscope (BioRad, Hercules, CA, U.S.A.), were collected using a 60x (NA 1.4) PlanApo oil-immersion objective mounted on a Nikon microscope. The excitation wavelength was 488 nm (blue light) emitted by a 100 mW argon-ion air-cooled laser (ITL, U.S.A.) used at 10–30% maximum power for imaging. GFP fluorescence was collected in the green channel with a 540 DF30 filter, and chloroplast autofluorescence in the red channel with a 585LP filter.

**RESULTS**

GFP fluorescence was monitored starting from the first week of plant growth up to the end of the third week, that is, until complete decay of fluorescence. Distinct bundles of actin were seen in the epidermis and mesophyll cells of 11-day-old **Arabidopsis** leaves (Fig. 1a,b). Particularly strong fluorescence was observed in guard cells (Fig. 1a). Fluorescence diminished during plant growth and maturation. Only the cytoskeleton of guard cells remained labeled in 20-day-old leaves (Fig. 1c), but fluorescence in these cells dimmed. We emphasize that in our growth conditions and photoperiod these plants were still very immature, at a very early stage of development. At the end of the fourth week no actin network could be observed; only the inner part of the guard cells showed weak and diffuse fluorescence unrelated to GFP (Fig. 1d).

Semiquantitative PCR with a low cycle number was performed to determine the expression profile of the **GFP-FABD2** construct in transgenic **Arabidopsis thaliana** plants. The primers were designed to anneal to the 5' end of GFP and the 5' end of the FABD2 domain. The results (Fig. 2) clearly show that the amount of **GFP-FABD2** transcript rapidly decreased to a very low level after the third week of growth, as maturation of the **Arabidopsis** leaves proceeded. This expression pattern was matched at the protein level (Fig. 3). On Western blots, sudden loss of the GFP-FABD2 fusion protein (the band corresponding to ~72 kDa of the protein marker) was observed after the third week. Interestingly, the decrease of the **GFP-FABD2** transcript coincided with a decline in the mRNA level of natural **FIMBRIN1** in transgenic **Arabidopsis** leaves (Fig. 4), but this effect was not observed in wild-type plants (Fig. 5). To amplify the natural **FIMBRIN1** from **Arabidopsis**, the primers had been designed to anneal to the 5' end of the gene in order to avoid any amplification of the **GFP-FABD2** construct.

**DISCUSSION**

Stable expression of the **GFP-FABD2** construct in **Arabidopsis** has been widely described, but all studies have focused on root tissues and seedlings at very early stages of development. Images of trichomes, leaf epidermis and stomata come from young plants 4–7 days old (Sheanan et al., 2004; Voigt et al., 2005), and no data from mature leaves are available. Our results make it plain that **GFP-FABD2** expression rapidly decreases in **Arabidopsis thaliana** after the third week of growth. The loss of GFP fluorescence observed with a confocal microscope and the decrease in the amount of fusion protein were due mainly to reduction of the mRNA level.

It is unlikely that **GFP-FABD2** transcription can be regulated by modulating the activity of the 35S promoter, as the latter is a constitutive promoter (Odell et al., 1985). Reports show that the 35S promoter is more active in young leaves of tobacco (Williamson et
al., 1989) than in old ones, as its expression is S-phase-specific (Nagata et al., 1987). Three-week-old Arabidopsis leaves are still developing and actively growing. A more recent study on transgenic cotton transformed with GFP under the 35S promoter demonstrates good GFP fluorescence in leaf mesophyll (Sunilkumar et al., 2002). All these data point to RNA silencing as a probable explanation of the effect observed in Arabidopsis thaliana.

Cosuppression of an endogenous gene by a transgene has been reported in many plant species (Napoli et al., 1990; de Carvalho et al., 1992; de Borne et al., 1994). Truncated transgenes also trigger this phenomenon (Smith et al., 1990). Two findings of ours support transgene-induced gene silencing in GFP-FABD2-expressing Arabidopsis. First, loss of natural FIMBRIN1 transcript was observed along with the decrease in GFP-FABD2 mRNA. Second, the mRNA level of FIMBRIN1 remained unchanged in wild-type plants. Rapid loss of the transgene mRNA at some stage of development, with subsequent reset after meiosis, has also been reported in transgenic tobacco (de Borne et al., 1994; Balandin and Castresana, 1997) and Arabidopsis (Scheid et al., 1991; de Carvalho et al., 1992; Dehio and Schell, 1994; our observations). A similar pattern of gene silencing, with guard cells unaffected, was shown for tobacco leaves transformed with GFP (Voinnet et al., 1998), and was attributed to the lack of symplastic connections of these cells via plasmodesmata (Wille and Lucas, 1984). Very efficient silencing has been attributed to 35S promotor with a double enhancer (Elmayan and Vaucheret, 1996; Que et al., 1997).
same as that used in the GFP-FABD2 construct (Voigt et al., 2005). The resulting rise in expression exceeds the putative threshold level beyond which RNA is degraded (Que et al., 1997). This threshold level depends on the nature of the coding region (Schubert et al., 2004). The postulated mechanism by which postranscriptional gene silencing triggered by a sense transgene occurs consists of three steps: conversion of single-stranded RNA to double-stranded RNA by RNA-DEPENDENT RNA POLYMERASE6, cleavage of long dsRNA into siRNA by DICER-LIKE 4, and subsequent sequence-specific ARGONAUTE1-guided mRNA cleavage (reviewed by Vaucheret, 2006). Although this is the most probable scenario, it is worth mentioning that transgene-induced gene silencing of GFP under the 35S promoter also has been reported (Schubert et al., 2004), and different classes of siRNA have been detected (Hamilton et al., 2002).

A less likely hypothesis is that the FABD2 part of the construct is regulated via an endogenous miRNA pathway. Using software developed by Adai et al., 2005, (http://sundarlab.ucdavis.edu/mirna/) we found out that the FABD domain of Arabidopsis FIMBRIN1 has three putative miRNA target sites, but none of the miRNA candidate molecules have been discovered so far.

Interestingly, the loss of fluorescence during maturation of FABD2-based GFP fusion proteins appears to be a general characteristic. It was reported to a lesser extent in Arabidopsis plants stably transformed with GFP-ABD2-GFP and ABD2-GFP constructs (Wang et al., 2008).

Our work shows that the GFP-FABD2 system cannot serve as a model for investigation of the actin cytoskeleton network in mature leaves of Arabidopsis. Finding a universal system for F-actin visualization remains a challenge.

ACKNOWLEDGEMENTS
We thank Dr. Boris Voigt (Institute of Cellular and Molecular Botany IZMB, University of Bonn, Germany) for kindly providing seeds of Arabidopsis transformed with the GFP-FABD2 construct, and Dr. Jerzy Dobrucki (Division of Cell Biophysics, Jagiellonian University, Cracow) for the use of the confocal microscope. This study was supported by the Polish Ministry of Science and Higher Education (PB 1395/B/P01/2007/33). The Faculty of Biochemistry, Biophysics and Biotechnology of the Jagiellonian University is a beneficiary of European Union structural funds (grant no. POIG.02.01.00-12-064/08, "Molecular biotechnology for health").

REFERENCES
Adai A, Johnson C, Mlotshwa S, Archer-Evans S, Manocha V, Vance V, and Sundaresan V. 2005. Computational prediction of miRNAs in Arabidopsis thaliana. Genome Research 15: 78–91; http://sundarlab.ucdavis.edu/mirna/.
Balandin T, and Castresana C. 1997. Silencing of α-1.3-glucanase transgene is overcome during seed formation. Plant Molecular Biology 34: 125–137.
De Borne F D, Vincentz M, Chipeau Y, and Vaucheret H. 1994. Co-suppression of nitrate reductase host genes and transgenes in transgenic tobacco plants. Molecular Genetics and Genomics 243: 613–621.
de Carvalho F, Gheysen G, Kushnir S, van Montagu M, Inze D, and Castresana C. 1992. Suppression of 3-1,3-glucanase transgene expression in homozygous plants. The EMBO Journal 11: 2595–2602.

Dehio C, and Schell J. 1994. Identification of plant genetic loci involved in a posttranscriptional mechanism for mitotically reversible transgene silencing. Proceedings of the National Academy of Sciences 91: 5538–5542.

Elmayan T, and Vaucheret H. 1996. Expression of single copies of a strong expressed 35S transgene can be silenced post-transcriptionally. The Plant Journal 9: 787–797.

Hamilton A, Voinnet O, Chappell L, and Baulcombe D. 2002. Two classes of short interfering RNA in RNA silencing. The EMBO Journal 21: 4671–4679.

Holweg CL. 2006. Living markers for actin block myosin-dependent motility of plant organelles and auxin. Cell Motility and the Cytoskeleton 64: 69–81.

Kreziszowiec W, Rajwa R, Dobrucki J, and Gablys H. 2007. Actin cytoskeleton in Arabidopsis thaliana under blue and red light. Biology of the Cell 99: 251–260.

Nagata T, Okada K, Kawai-T, and Takebe I. 1987. Cauliflower mosaic virus 35 S promoter directs S phase specific expression in plant cells. Molecular Genetics and Genomics 207: 242–244.

Napoli C, Lemeux C, and Jorgensen R. 1990. Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous genes in trans. The Plant Cell 2: 279–289.

Odei JT., Nag F, and Chua N-H. 1985. Identification of DNA sequences required for activity of the cauliflower mosaic virus 35S promoter. Nature 313: 810–812.

Odegard OM, Paszkowski J, and Potrykus I. 1991. Reversible inactivation of transgene in Arabidopsis thaliana. Molecular Genetics and Genomics 228: 104–112.

Sheahan M, Stagger C J, Rose R J, and McCurdy DW. 2004. A green fluorescent protein fusion to actin-binding domain 2 of Arabidopsis fimbrin highlights new features of a dynamic actin cytoskeleton in live plant cells. Plant Physiology 136: 3968–3978.

Schubert D, Lechtenberg B, Forssbach A, Gils M, Bahadir S, and Schmidt R. 2004. Silencing in Arabidopsis T-DNA transformants: the predominant role of a gene-specific RNA sensing mechanism versus position effects. The Plant Cell 16: 2561–2572.

Smith C J S, Watson CF, Bld CR, Ray J, Schuch W, and Grierson D. 1990. Expression of a truncated tomato polygalacturonase gene inhibits expression of the endogenous gene in transgenic plants. Molecular Genetics and Genomics 224: 477–481.

Sunilkumar G, Mohr LA, Lopata-Finch E, Emani C, and Rathore KS. 2002. Developmental and tissue-specific expression of CaMV 35S promoter in cotton as revealed by GFP. Plant Molecular Biology 50: 463–474.

Que Q, Wang H-Y, English JJ, and Jorgensen RA. 1997. The frequency and degree of cosuppression by sense chalcone synthase transgenes are dependent on transgene promoter strength and are reduced by premature nonsense codons in the transgene coding sequence. The Plant Cell 9: 1357–1368.

van Gestel K, Le J, and Verbeelen JP. 2001. A comparison of F-actin labeling methods for light microscopy in different plant specimens: multiple techniques supplement each other. Micron 32: 571–578.

Vaucheret H. 2006. Post-transcriptional small RNA pathways in plants: mechanisms and regulations. Genes and Development 20: 759–771.

Voigt B, Timmers CJ, Samaj J, Muller J, Baluska F, and Menzel D. 2005. GFP-FABD2 fusion construct allows in vivo visualization of the dynamic actin cytoskeleton in all cells of Arabidopsis seedlings. European Journal of Cell Biology 84: 595–608.

Voinnet O, Vain P, Angelis S, and Baulcombe DC. 1998. Systemic spread of sequence-specific transgene RNA degradation in plants is initiated by localized introduction of ectopic promoterless DNA. The Cell 95: 177–187.

Wang YS, Motes CM., Mohamalawri DR, and Blancaflor EB. 2004. Green fluorescent protein fusions to Arabidopsis fimbrin I for spatio-temporal imaging of F-actin dynamics in roots. Cell Motility and the Cytoskeleton 59: 79–93.

Wang YS, Yoo CM, and 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 fimbrin actin-binding domain 2. New Phytologist 177: 525–536.

Wille AW, and Lucas WJ. 1984 Ultrastructural and histochemical studies on guard cells. Planta 160: 129–142.

Williamson JD, Hirsh-Wyncott ME, Larkins BA, and Gelvin SB. 1989. Differential accumulation of a transcript driven by the CaMV 35S promoter in transgenic tobacco. Plant Physiology 100: 1570–1576.

Wilsen KL, Lovel Wheeler A, Voigt B, Menzel D, Kunkel JG, and Hepler PK. 2006. Imaging the actin cytoskeleton in growing pollen tubes. Sexual Plant Reproduction 19: 51–62.