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FEATURES OF FLUORESCENT PROTEIN APPLICATION TO STUDY THE ROOT SYSTEM DEVELOPMENT OF CUCURBITS (*Cucurbitaceae*)

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Abstract

Modern studies of detailed processes in plant development would not be possible without using a wide range of fluorescent proteins (R. Day and M. Davidson, 2009; D. Chudakov et al., 2010). However, applications of fluorescent proteins are restricted due to problems with their visualization in plant tissues. Plants are difficult objects for microscopic studies. Indeed, even the most advanced methods have significant limitations regarding the depth of light penetration due to the scattering and absorption of light by cell walls. Therefore, to study the distribution of reporter fluorescent proteins in large organs typical for most plants, it is necessary to fix the plant material and prepare thick histological sections with a vibrating-blade microtome. Chemicals traditionally used for fixing, dehydrating, and embedding of plant tissues samples lead to changes in the structure of fluorescent proteins and, as a result, often to the loss of their fluorescence. Therefore, it is important to optimize the protocols for fixing plant tissues, preparing sections, and studying the distribution of fluorescent proteins by laser scanning confocal microscopy. In this work, we propose a novel, integrated, and potentially universal approach to fixation of tissues of transgenic plants and preparation of sections in the course of studying the patterns of cellular response to auxin and expression of transcription factors using laser scanning confocal microscopy. Our aim was to sum up modern approaches to the application of this technique for visualization of tissue and cellular patterns of fluorescent reporter proteins distribution on sections of large non-model plants. The first step for using fluorescent proteins in plants is the generation of genetic constructs that carry the promoter of the gene of interest fused to a reporter gene encoding a fluorescent protein. For this, a transformation protocol has to be available for the selected plant species. We have described the use of Gateway® cloning technology for the construction of vectors for plant transformation that meet modern experimental requirements. To study auxin localization *in vivo* we developed a series of vectors with genes encoding various fluorescent proteins (eGFP, tdTomato, mRuby3) under the control of the auxin-sensitive *DR5* promoter (E. Ilina et al., 2012). We now demonstrate the advantage of nuclear-targeted fluorescent proteins (mNeonGreen-H2B, tdTomato-H2B, mRuby3-H2B), as well as the possibility of their application for additional visualization of cell nuclei in combination with highly specific cell wall staining using SCR1 Renaissance2200. An effective method is presented for constructing vectors to study cell-specific expression patterns of developmental regulators using the transcription factor genes *GATA24* (A. Kiryushkin et al., 2019) and *LBD16* in some *Cucurbitaceae* species as examples. We also applied an expression cassette, *pAtUBQ10::DsRED1* (E. Limpens et al., 2004), carrying a gene encoding red fluorescent protein under the control of a constitutive promoter, to demonstrate the advantages of the use of fluorescent proteins in screening for transgenic vs. wild type roots. A new method of fixation and clearing of plant tissues containing reporter fluorescent proteins and preparation of sections is presented, using transgenic roots of *Cucurbitaceae* as an example. The advantage of using melted agarose compared to embedding media for

orienting plant parts during the preparation of samples is revealed. The increased photostability of fluorescent proteins in sections due to the use of clearing reagent ClearSee (D. Kurihara et al., 2015) as a mounting medium is demonstrated. In sum, we apply a complex of several modern methodological approaches of sample preparation and laser scanning confocal microscopy that offers significant advantages for studying the developmental biology of large non-model plants.

Keywords: agrobacterial transformation, fluorescent proteins, confocal microscopy, plant development, transcription factors

In recent decades, significant advances in knowledge concerning the structure and functions of plant parts have allowed us to better understand specific events of plant life. This has occurred through use of model plants *Arabidopsis thaliana*, rice, tobacco, corn, *Medicago truncatula*, and *Lotus japonicus*. In recent years, omix technologies and the next top models in plant sciences [1] have contributed to a significant breakthrough in understanding of molecular mechanisms of plant development and functioning. The range of model plant species and sequenced genomes in the plabiPD (https://www.plabipd.de/plant_genomes_pa.ep) and Phytozome (<http://phytozome-next.jgi.doe.gov>) databases has increased, and the transcriptomes and proteomes of these plants are receiving much attention. This has led to renewed interest in in-depth studies of more complex non-model plants, including members of the *Cucurbitaceae* family [2-4].

Nowadays, various reporter fluorescent proteins (FP) are used to study molecular and physiological mechanisms of plant development [5]. The areas of FP application are diverse, including tissue patterns of gene expression; visualization of protein distribution, transport, and interaction; labelling of organelles, cells, and tissues; search for new drugs; breeding and selection. [6-8]. However, reporter fluorescent proteins have significant limitations when considering the tissues of many plants.

As a convenient model organism, *Arabidopsis* was applied in most microscopic studies employing fluorescent proteins. *Arabidopsis* has thin roots of ~ 100 μm in diameter, which allows examination of fluorescent protein location in root tissues without sectioning of plant samples, e.g., by noninvasive *in vivo* confocal microscopy. The leaves of *Arabidopsis* are also thin but optically opaque due to chlorophyll, making only the surface layers available to study the distribution of fluorescent proteins. For *Arabidopsis*, the ClearSee tissue optical clearing technique has been developed. ClearSee reagent allows for clearing while maintaining fluorescence of some reporter proteins to permit the visualization of fluorescent protein distributions in optically non-transparent organs [9]. Combining visualization of fluorescent proteins and cell walls stained with nonspecific Direct Red 23 or Direct Yellow 96 dyes [10], the ClearSee method can be leveraged to explore patterns and localization of gene expression in a specific cell or tissue. ePro-ClearSee, an immunohistochemical method that also does not require sectioning of plant organs, was adapted for epigenetic investigations to detect modifications of histones and DNA [11]. In addition, Schiff staining of organs followed by clearing has been modified to study the distribution of indigo (a product of GUS staining) over the entire thickness of any *Arabidopsis* organ [12]. However, this staining is incompatible with the use of fluorescent proteins.

The vast majority of plants, including important crops, have a more complex anatomical structure compared to *Arabidopsis*, their organs consist of a greater number of cell layers in tissues and, accordingly, are larger in size. The maximum penetration depth of the confocal microscope laser into plant tissue is relatively shallow due to light scattering and absorption by cell walls and reaches a maximum value of 100 μm . Multiphoton laser microscopy is rather difficult to use, although

it allows deeper penetration into plant tissues using a near-infrared (900-1000 nm) femtosecond laser [13, 14]. The latest technology of light-sheet fluorescence microscopy based on wide-field fluorescence microscopy allows penetration to a depth of 500 μm [15-17] but is only applicable to transparent or cleared animal and plant objects. Even for *Arabidopsis*, this technology, successfully combined with image deconvolution and reconstruction using a Fiji Multiview-Reconstruction plug-in (<https://imagej.net/Multiview-Reconstruction>), has significant limitations in terms of light penetration depth and resolution like traditional microscopy methods [18].

Despite the undoubted recent progress in microscopic technologies, investigations into the distribution of reporter fluorescent proteins or fusion of their genes with the genes of target proteins (fusion proteins as reporters) in the thick organs of plants requires an optimized protocol for fixing and sectioning to produce thick histological sections of plant material. Chemicals traditionally used for fixing, dehydrating, and embedding plant tissue samples alter the structure of fluorescent proteins and, as a result, lead to a loss of fluorescence. Some researchers use cryomicrotomy to obtain thick (25-50 μm) sections of plant tissues where the fluorescence of such proteins is preserved [19]. However, such approaches have not become widespread.

In plants, the brightest and most photostable fluorescent proteins should be used (e.g., mNeonGreen, mRuby3, or mScarlet) [20, 21] which provide sample scanning to a greater depth. Plant cell autofluorescence in different spectral regions should also be considered. Therefore, vectors for plant transformation must correspond to the objectives of the experiment and the object. The existing sets of vectors for marking of plant cell organelles [22] or commercial genetic constructs are not always best suited to a specific scientific problem, and there is a need for a flexible and efficient methodology to design an optimal vector. Cloning technologies Gateway® [23] and Golden Gate [24] are efficient, provide targeted cloning, and allow the assembly of almost any combination of DNA fragments (e.g., a promoter, a coding part, and a terminator) in a vector suitable for the plant transformation.

This paper reports a novel methodology which leverages fluorescent reporter proteins for investigating the molecular genetic mechanisms of development of large organs of non-model plants, using cucurbits as an example. More specifically, an integrated approach to fixation of transgenic plant tissue and sample sectioning for laser scanning confocal microscopy is put forward to study patterns of cellular auxin response and expression of transcription factors. The paper also describes an application of Gateway® cloning technology to produce new genetic constructs (vectors) for plant transformation that overcomes experimental challenges.

Our aim was to sum up modern approaches to the application of an effective universal technique for visualization of tissue and cellular patterns of fluorescent reporter proteins distribution on sections of large organs of non-model plants (on the example of cucurbits).

Methods. Design of DNA constructs using Gateway® recombination cloning technology. *Gateway® cloning guidelines.* To deliver the genes encoding reporter fluorescent proteins into the plant cells, DNA constructs (vectors) are required. In constructing vectors suitable for plant transformation, we used Gateway® cloning technology (Thermo Fisher Scientific, USA) [25]. For the LR clonase plus reaction, three different entry vectors containing either the promoter, the coding part of the target gene, or the transcription terminator were mixed with the destination vector in a tube containing LR Clonase™

II Plus enzyme mix (Thermo Fisher Scientific, USA; hereinafter LR clonase plus) that catalyzes the *in vitro* recombination between att-sites of entry clones and a destination vector (Fig., A).

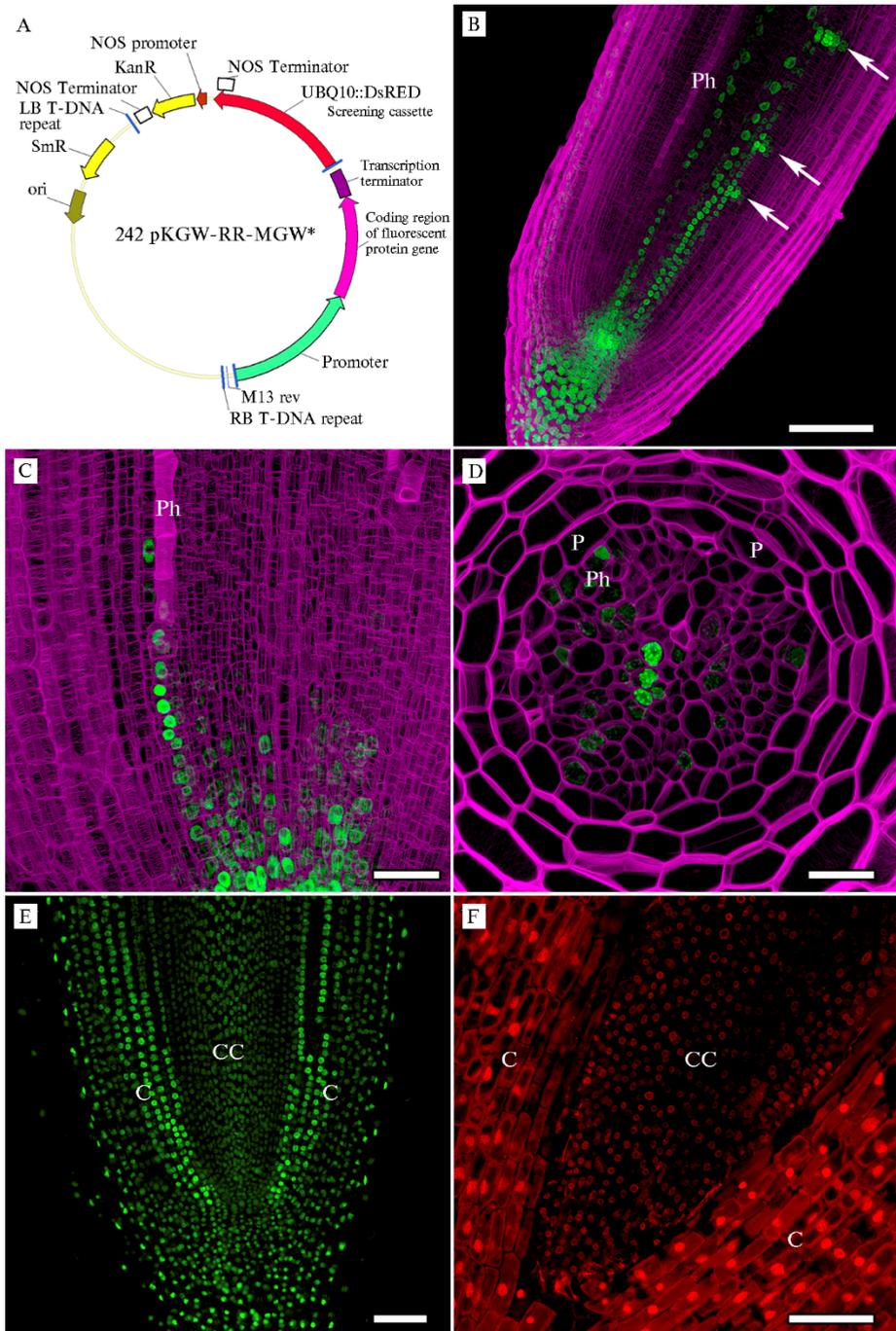
To construct vectors for plant transformation, we also used LR Clonase™ II enzyme mix which transfers one DNA fragment from the entry vector to the destination vector via *attL-attR* recombination. This technology is based on the bacteriophage lambda recombination system [26]. The entry and destination vectors contain heteronomous homologous regions of the bacteriophage lambda DNA, the *attL* (left), and *attR* (right) sites capable of site-specific recombination. The recombination is mediated by LR plus clonase which is a mixture of integrase and excisionase enzymes of the bacteriophage lambda and the Integration Host Factor protein of *Escherichia coli* [26]. Divergent *attL* regions flank the edges of DNA fragments cloned into the entry vector, which allows them to be transferred into the destination vector in a specific order. To generate entry vectors, a BP Clonase™ enzyme mix was used, which catalyzes recombination between the attB sites flanking the target DNA fragment and the *attP* sites of the donor vector. Also the target DNA fragment was conventionally cloned into the entry vector by restriction and ligation performed sequentially.

The entry vectors used for the experiments were 369_pEN-TRattL4attR1_BSAI (Thermo Fisher Scientific, USA) to clone the promoter region, pUC18-entry 8 [27] to clone the reporter DNA fragment, and the pDONR P2-P3 donor vector (Thermo Fisher Scientific, USA) to clone the transcription terminator fragment. pKGW-GG-RR, the destination vector for the LR clonase reaction [28], contained the fused reporter gene encoding for green fluorescent protein eGFP and β -glucuronidase (GUS) *eGFP-GUS* [25] adjacent to *att*-sites [28] and the *pAtUBQ10::DsRED1* cassette [29] to screen transgenic roots by fluorescence of the DsRED1 protein. The destination vectors for the LR plus clonase reaction were 242 pKGW-RR-MGW containing the same cassette *pAtUBQ10::DsRED1* (see Fig., A), and 236 pKGW-RR-MGW without such a cassette for selection of transgenic material.

LR plus, LR, and BP clonase reactions were undertaken according to the manufacturer's recommendations (Thermo Fisher Scientific, USA). The presence and correct assembly of the cassette in the destination vector was verified by PCR and sequencing. The destination vector was transformed into *Rhizobium rhizogenes* R1000 and then used to transform squash and cucumber seedlings according to a previously developed method [3] to generate composite plants with wild-type shoot and transgenic root system.

Construction of a vector plasmid family to study the in vivo localization of the cellular response to auxin. Auxin is one of the most important factors affecting root development in higher plants [30]. To study the role of auxin in lateral root initiation and development in cucurbits, a family of vectors was developed containing the synthetic auxin-sensitive *DR5* promoter [31] and a reporter gene for detecting the cellular response to auxin. The pKGW-RR-MGW-*DR5::eGFP-GUS* vector was produced by an LR clonase-mediated reaction between the entry vector pUC18-entry8-*DR5* carrying the *DR5* promoter and the destination vector pKGW243-GG-RR [3]. The cellular response to auxin was studied on whole transgenic squash roots (*DR5::eGFP-GUS*) stained for β -glucuronidase activity (GUS assay) and on longitudinal and cross sections of the transgenic squash roots. A limitation of GUS staining is caused by diffusion of indigo, a product of the β -glucuronidase reaction, which can lead to erroneous results on tissue localization of the response to the plant hormone. Therefore, fluorescent proteins with nuclear localization are most suitable for a more detailed study of

the role of auxin in the lateral root initiation in squash.



The use of reporter fluorescent proteins to study patterns of cellular response to auxin and expression of transcription factors in *Cucurbitaceae* plants (confocal laser scanning microscopy, LSM 780 microscope, Zeiss, Germany; longitudinal sections of the tips of fixed roots, the section thickness is 65 μm ; maximum intensity projections of optical sections are shown).

A. Chart of vector 242 pKGW-RR-MGW including promoter, coding region of fluorescent protein gene, transcription terminator, and *pUBQ10::DsRED* cassette for screening transgenic material.

B. Visualization of auxin response maxima (nuclear localization of mRuby3 fluorescence, green channel) in transgenic roots of cucumber seedlings carrying the *DR5::mRuby3-H2B* insert. Cell walls are counter stained with SCR1 Renaissance2200 (purple channel), white arrows indicate lateral root primordia. Ph — protophloem. Scale bar, 100 μm .

C, D. Visualization of the *CpLBD16b* gene expression pattern in the root tip of squash carrying the *CpLBD16b::mNeonGreen-H2B* insert, a longitudinal (C) and cross (D) sections. *CpLBD16b* is detected by mNeonGreen fluorescence (nuclear localization, green channel), cell walls are counter stained with SCRI Renaissance2200 (purple channel). Ph — protophloem, P — pericycle. Scale bar, 50 μm .

E. Visualization of cell nuclei (Venus fluorescence, green channel) in transgenic roots of squash seedlings carrying the *UBQ10::H2B-Venus* insert. C — cortex, CC is central cylinder. Scale bar, 100 μm .

F. Visualization of cell nuclei (tdTomato fluorescence, red channel) in transgenic roots of squash seedlings carrying the *pAct::tdTomato-H2B* insert. C — cortex, CC — central cylinder. Scale bar, 100 μm .

We also developed constructs 242 pKGW-RR-MGW-*DR5::mRuby3-H2B* and 242 pKGW-RR-MGW-*DR5::tdTomato-H2B* which provide nuclear localization of the reporter protein. The cellular response to auxin was analyzed on longitudinal and cross 60 μm sections of tips of transgenic squash and cucumber roots bearing constructs *DR5::NLS-eGFP-GUS*, *DR5::mRuby3-H2B* and *DR5::tdTomato-H2B*. The use of reporter proteins tdTomato and mRuby3 fused to human histone H2B [32] allows for the most accurate data on the spatial patterns of the cellular response to auxin in dividing cells during lateral root initiation in the meristem of the parental root in cucumber (see Fig., B) compared to the *DR5::NLS-eGFP-GUS* reporter construct [3] encoding the NLS signal of the nuclear localization. Importantly, the brightness of the tdTomato and mRuby3 proteins is three times higher than that of the eGFP protein. In addition, mRuby3 exhibits increased photostability [33, 34]. The auxin response maximum associated with the initiation site of the lateral root primordium in cucumber occurs at a distance of 200-300 μm from the initial cell prior to the first anticlinal division of the pericycle and endoderm. In addition, auxin is involved in further development of the lateral root primordium.

Therefore, the auxin-sensitive promoters combined with reporter constructs are appropriate tools for investigating the involvement of the hormone in a particular morphogenetic process. The proposed approach requires the use of the brightest photostable fluorescent proteins with nuclear localization to increase the brightness of fluorescence probes by tens of times compared to proteins with cytoplasmic localization, since when studying the early stages of lateral root initiation or similar processes, it is necessary to detect a weak signal from several cells of the pericycle in the thickness of the root. Fluorescent tags of nuclear localization should also correspond to the objectives of the experiment. Thus, the NLS nuclear localization signal did not allow reliable detection of the presence or absence of a marker in a dividing cell, since the nuclear envelope is disassembled during cell division, and the fluorescent protein diffuses into the cytoplasm. Nevertheless, NLS visualizes nuclei in a shape close to that of native nuclei. The combination of reporter proteins with H2B histone visualizes the figures of mitosis, since histone remains bound to nuclear DNA during mitosis. However, in cucurbit plants, uneven distribution of the fluorescent tags in aldehyde-fixed chromatic nuclei may be a disadvantage of fusion of the reporter with the histone protein (see Fig., D).

Construction of vectors for studying the tissue expression pattern of developmental regulator genes. To study a fine coordination of the developmental processes in a plant organ, for example, the initiation of a lateral root, it is necessary to identify the tissues and cells in which the gene of interest is expressed. Asymmetric cell division often mediates cell differentiation in multicellular organisms and plays an important role in the development of new organs. In most flowering plants, lateral roots are formed from pericycle cells located at the xylem pole. These cells are asymmetrically divided into small central and larger flanking cells with different fates. In *Arabidopsis*, LATERAL ORGAN BOUNDARIES DOMAIN 16 (LBD16) and other members of the LBD family play an

important role in establishing the asymmetry of the lateral root founder cells. *LBD16* is specifically expressed in a pair of pericycle cells at the xylem pole prior to the first anticlinal division during lateral root initiation. The pericycle cells at the xylem pole expressing *LBD16* are presumably the lateral root founder cells [35], the specification of which occurs in the basal part of the root meristem and depends on the oscillatory expression of genes, including the *GATA23* gene encoding for transcription factor [36].

Using the NCBI (<https://www.ncbi.nlm.nih.gov/>), Phytozome (<https://phytozome.jgi.doe.gov>), Cucurbit Genomics Database (<http://cucurbitgenomics.org>), and PlantTFDB (<http://planttfdb.gao-lab.org>) databases, we identified the *CpGATA24* [37] and *CpLBD16b* genes in the *Cucurbita pepo* genome, which are the orthologs of *Arabidopsis* auxin-inducible genes *GATA23* and *LBD16* which are involved in specification of pericycle cells for lateral root initiation.

Two vectors, 242 pKGW-RR-MGW-*pCpLBD16b::mNeonGreen-H2B* and 242 pKGW-RR-MGW-*pCpGATA24::mNeonGreen-H2B*, were developed by an LR plus clonase reaction to localize the expression pattern of *CpLBD16b* and *CpGATA24* orthologous genes. The promoter sequences of the *CpLBD16b* and *CpGATA24* genes were amplified using a squash genomic DNA template and cloned into the 369_pENTRattL4attR1_BSAI vector. mNeonGreen, one of the brightest and most photostable proteins with a short maturation time [38, 39] fused to histone H2B, was used as a reporter for the localization of the promoter activity of these genes. The *mNeonGreen-H2B* nucleotide sequence was amplified on a plasmid template (Allele Biotechnology and Pharmaceuticals, Inc., USA, plasmid # H2B-213) [20] and cloned into the pUC18-entry8 vector. The 373_pENTRattR2attL3-TermAct vector containing the *Actin2* gene terminator sequences from *Arabidopsis* was used as a transcription terminator [24]. The resulting binary vectors were used for plant transformation to localize activity of the *CpGATA24* [37] and *CpLBD16b* promoters in the squash plant tissues by confocal laser scanning microscopy (see Fig. C, D). It was revealed for the first time that the *CpGATA24* gene is involved in lateral root primordium initiation during the first anticlinal divisions in the pericycle. *CpLBD16b* is not expressed in the pericycle cells at the xylem pole of the root but it occurs in the initial cells and in the developing protophloem cell file (see Fig., C, D).

To sum up, the expression of reporter genes encoding fluorescent proteins (especially those with nuclear localization) under the control of species-specific promoters of the genes of interest provides reliable data on the expression pattern of these genes in plant tissues and on their involvement in morphogenesis processes, even in large plant organs.

Constitutive promoters as a visualization tool. Selection of transgenic plant organs is essential during plant transformation. During transformation of squash plants with *R. rhizogenes*, a callus formed at the site of inoculation, consisting of cells carrying the T-DNA insert of the binary vector, of non-transformed cells, and, possibly, of cells in which the insert has undergone silencing. The roots developing from such a callus are either transgenic, or wild-type, or chimeric, i.e., formed by both transformed and non-transformed cells. This causes difficulties in the analysis of transgenic roots. A cassette consisting of a gene encoding a fluorescent protein under the control of a constitutive promoter, located within the T-DNA in a binary vector, provides effective screening of transgenic roots. There should be differences in the emission spectra of the screening and reporter proteins. Traditionally, these are pairs with fluorescence in red and green spectral regions. In vector 242 pKGW-RR-MGW, the *pAtUBQ10::DsRED1* cassette containing gene for the red fluorescent protein DsRED1 under the control of constitutive promoter of the ubiquitin gene *AtUBQ10* from *Arabidopsis* provided

the selection of transgenic roots (see Fig., A). In this case, the reporter genes encoded fluorescent proteins from the non-red part of the spectrum (see Fig. B-D).

In most microscopic applications, it is necessary to visualize cell nuclei. Usually, cell nuclei are stained with dyes for DNA (for example, DAPI or propidium iodide), however, such dyes penetrate unevenly into the sample and stain all nucleic acids, including mitochondrial and chloroplast DNA, and sometimes RNA. An advanced approach to nuclear imaging is the use of constitutive promoters which control a gene encoding a fluorescent protein with nuclear localization. The presence of such a cassette in the T-DNA of the vector allows simultaneous screening of transgenic roots and visualization of nuclei in cells on sections.

We have developed new vectors for nuclei visualization in the root tissues of cucurbits, both containing genes for screening proteins under the constitutive promoters. In 242 pKGW-RR-MGW-*pUBQ10::H2B-Venus*, the yellow fluorescent protein reporter gene *Venus* is fused to the human histone gene *H2B* under control of ubiquitin gene *pUBQ10* promoter from *Arabidopsis*. The Venus reporter protein is fast-maturing, highly photostable, and is brighter compared to eGFP [33, 40]. Vector 236 pKGW-RR-MGW-*pACT2::tdTomato-H2B* contains the reporter gene *tdTomato* fused to the histone gene *H2B* under the constitutive promoter of the actin gene *pACT2* from *Arabidopsis*.

It was found that promoters of the ubiquitin and actin genes provide high expression of genes encoding fluorescent proteins but exhibit different activities depending on localization in root tissues (see Fig. E, F). Both promoters were more active in cortex and rhizodermis cells than in the central cylinder, which creates difficulties in studying lateral root initiation and other processes occurring in the pericycle and other tissues of the central cylinder.

Thus, constitutive promoters in combination with a gene encoding a fluorescent protein are a valuable tool both for screening transgenic material and for visualizing cell nuclei by confocal laser microscopy. Nevertheless, the constitutive promoter should be chosen carefully. In the screening cassette, many vectors for plant transformation contain the strong constitutive promoter *35S* of the cauliflower mosaic virus CaMV. We have previously shown that, upon transformation of squash plants with the “empty” vector pMDC162-GFP [23], the *35S* promoter located in the T-DNA in *p35S::GFP* cassette drives ectopic expression of the *GUS* gene which lacks a promoter and, therefore, should not be expressed in root tissues [3]. Cases when the *35S* promoter affects the expression of target cassettes, possibly leading to erroneous results, have hitherto been described [41, 42]. Native promoter *pAtUBQ10* in the *pAtUBQ10::DsRED1* cassette of the vector 242 pKGW-RR-MGW provides robust expression of the target cassette [3]. Uneven activity of constitutive gene promoters in different tissues and plant organs is also known to occur. The *35S* promoter is more active in the central cylinder, leading to brighter fluorescence of the cell nuclei, compared to the cortex [43]. The promoters of constitutively expressed ubiquitin and actin genes of *Arabidopsis* are most popular [18, 29, 44, 45]. However, other constitutive gene promoters are also known. For example, the *PtMCP* has been cloned from poplar *Populus tomentosa* [46]. The promoter of the *RIBOSOMAL PROTEIN S5A (RPS5A)* gene, which is selectively expressed in proliferating cells [47], can be used as a marker of proliferative activity.

Sample preparation technique for localization of fluorescent proteins on sections of fixed organs. In experiments using reporter proteins, preserving their fluorescence ability for as long as possible represents an important task. Accordingly, we modified the method of fixing plant tissues and preparing sections, using transgenic roots of squash and cucumber plants as examples.

Root cells are known to undergo plasmolysis upon fixation in a 100 mM

phosphate buffer. For this reason, we changed the composition of the fixative [48] proposed by Brian Lin (Tufts University, Boston, MA, USA). A 5-fold decrease in the molarity of the phosphate buffer (up to 20 mM) addresses the problem of plasmolysis. A similar approach was also used for immunolocalization of the cytoskeleton on sections of fixed symbiotic nodules [49, 50]. For most fluorescent proteins (GFP, Venus, tdTomato, mRuby3), the optimum pH is 7.2. However, to prevent rapid decay of mNeonGreen in the roots of transgenic plants, we used a buffer with a higher pH, 8.0. The final fixative composition was as follows: 1 % paraformaldehyde, 5 % dimethyl sulfoxide (DMSO), 75 mM L-lysine, 10 mM sodium m-periodate in 20 mM phosphate buffer, pH 7.2-8.0. The tips of the main root (6-8 mm long) were fixed. To remove air from tissues, containers with plant material and the fixative were left to stand in an exicator under vacuum (-1 atm) for 3-5 min, then the vacuum was removed. The procedure was repeated 3-4 times. The roots were kept in the fixative for 1 h at room temperature. After fixation, the material was washed three times for 15-20 min with 20 mM phosphate buffer containing 75 mM L-lysine.

Fixed roots were placed in rubber molds and mounted in molten 2 % LE agarose (Lonza Group, Switzerland) at 40-50 °C. The agarose blocks were glued onto flat metal holders using Super Moment cyanoacrylate glue (Henkel, Russia). An automatic precision microtome with a vibrating blade HM650V (Thermo Fisher Scientific, Microm International GmbH, Germany) was used to prepare 65- μ m-thick sections of roots. The sapphire knife (Delaware Diamond Knives, Inc., USA) provided significantly improved section quality. Distilled water was the optimal cutting medium because different buffers, e.g., phosphate, Tris buffered saline (TBS), and microtubule stabilizing buffer (MTSB) significantly deformed root sections. The cell walls were counter stained for 1 h with fresh 0.1 % solution of an SCRI Renaissance 2200 dye (SR2200, Renaissance Chemicals, Ltd, UK) [51] in dH₂O (pH 8.0) without subsequent washing. If necessary, cell nuclei were alternatively stained for 30-50 min with 0.3 μ g/ml DAPI.

Sections were placed into embedded medium under coverslips. It was revealed that the majority of fluorescent proteins (GFP, Venus, tdTomato), after the proposed fixation, retain fluorescence in the CFMR2 liquid final medium (Citifluor, Division of Electron Microscopy Sciences, USA). However, clearing reagent ClearSee [9] in our modification (13.7 g/l L-lysine monochloride, 100 g/l xylitol, 150 g/l sodium deoxycholate, 240 g/l urea in 20 mM phosphate buffer, pH 8.0) turned out to be optimal for embedding sections, including those of transgenic roots carrying mNeonGreen.

Thus, many methodological problems associated with reporter fluorescent proteins must be considered to preserve their fluorescence. Plant material should be embedded into 2-3 % agarose convenient to hold small plant parts during cutting. It has hitherto been shown that neither dehydration in a series of alcohols nor embedding media should be applied for immunolocalization of cytoskeleton in nitrogen-fixing root nodules of legumes [49, 50]. When the transgenic material carrying the reporter fluorescent proteins is embedded in paraffin or Steedman's wax (a medium with a more gentle sample preparation suitable for the immunolocalization of sensitive proteins) [52-54], dehydration in alcohols is required which leads to rapid decay of the fluorescence of such proteins. When mounted in molten agarose, short-term exposure to 40 °C does not lead to denaturation of the fluorescent protein. The agarose concentration also requires optimization. Specifically, the softer the object, the lower the agarose concentration should be. If the agarose is too dense it can cause strong compression and dehydration of the soft object, which appears as a compression of the stretch zone of the root tip. Instead of the traditionally used propidium iodide (PI) or FM4-64 (plasmalemma

staining), we propose a simplified staining protocol with SCRI Renaissance 2200 cell wall specific dye, which allows a clear visualization of the anatomical structure on tissue sections (see Fig., C, D). Our findings also testify to the importance of optimal embedding medium for the sections, as this parameter affects the stability and duration of fluorescence.

Laser scanning confocal microscopy for visualization of fluorescent proteins in fixed tissues. Analysis of sections and imaging were performed using a confocal laser scanning microscope LSM 780 (Zeiss, Germany; objectives 10×/0.45 and 20×/0.8 of the Plan-Apochromat series with a high numerical aperture, and 40×/1.3 with oil immersion). Reporter fluorescent proteins eGFP and mNeonGreen were detected at the laser wavelength of 488 nm, yellow Venus protein at 514 nm, red proteins mRuby3 and tdTomato at 561 nm. Cell walls (SCRI Renaissance 2200) or nuclei (DAPI) were detected by excitation at 405 nm. To visualize unstained anatomical structures, differential interference contrast (DIC) in an additional transmitted light channel was applied. For imaging and image processing, we used the ZEN v. 2.3 software package (Zeiss, Germany).

Currently, fluorescent proteins cannot be visualized in thick sections of plant tissues in any other way than using confocal microscopy. The possibilities offered by a laser scanning microscope include a significant increase in the spatial resolution of the signal, which makes it possible to separate structures that merge when using wide-field microscopy due to out-of-focus light. The spatial structure of a tissue or organ site is difficult to determine using relatively thin sections (7-16 μm), however, only thin sections can be examined by traditional light microscopy. Thick sections (50-70 μm) in combination with confocal microscopy makes it possible to obtain a series of optical sections (z-stack). Further, depending on the purpose of the study, it is possible to represent the z-stack in the form of a two-dimensional image using the maximum intensity projection algorithm or to obtain a 3D reconstruction. This approach has been successfully used to study the development of various plant organs, in particular, the apical meristems of shoots and roots and the development of lateral roots [55, 56].

An essential problem in studying the development of plant organs is the overlap of the spectra of several fluorescent proteins and autofluorescence of cell and tissue elements [57-59]. The main sources of autofluorescence in plant tissues are chlorophyll (red region of the spectrum) and lignin (green and yellow regions). Autofluorescence masks and complicates the registration of the fluorescence of reporter proteins. Autofluorescence can be strong enough to be mistaken for reporters' fluorescence, leading to erroneous data. The use of confocal microscopy allows the separation of autofluorescence and reporter signal. A linear spectral unmixing algorithm can be used to eliminate autofluorescence, as well as to separate the overlapping spectra of several fluorescent reporter proteins [58, 60]. It is convenient to assess the nature of autofluorescence using emission spectra on unstained sections [61]. Usually, the samples contain several dyes/fluorescent proteins, each of which marks the cell structure or individual cells. Upon spectral imaging, fluorophores are detected either individually or as a mixture, depending on their spatial distribution within the object. The linear spectral unmixing algorithm allows the relative contribution of the intensity of each fluorophore to be determined for each pixel [60]. If the spectra of all fluorophores in the sample are known, then their signals within a pixel can be calculated from the spectral curve of each pixel, which is determined and recorded during the lambda scan. After the spectral contribution of each fluorophore has been determined, the lambda stack can be divided into separate channels for each fluorophore. In particular, the autofluorescence spectrum can be represented as a separate channel and eliminated

from the final image of the sample. Thus, using a complex of modern approaches in confocal laser microscopy addresses the problems of large and dense samples by high resolution three-dimensional imaging for cell or tissue distribution of fluorescent proteins.

Altogether, we have successfully developed and applied a new complex of techniques that allows us to study the localization of reporter fluorescent proteins on fixed sections of large organs of non-model plants. It is shown that the genetic constructs containing screening and reporter cassettes should be carefully chosen. It is also important to consider particularities of the plants, primarily their autofluorescence. The optimal solution is to construct vectors in accordance with the tasks of a specific experiment. Therefore, a necessary condition for the application of the proposed approach is the availability of a transformation technique for the species of interest, which allows the T-DNA of the vector to be delivered to the plant genome. The critical factors are the composition and pH of the fixative, which must be adapted with consideration for both the plant species and the applied fluorescent reporter protein. The method of section preparation is also important to preserve the fluorescence of the fluorescent proteins. Embedding plant material into agarose is optimal. For visualization of cell walls and tissue morphology, we have proposed a simplified protocol for staining with SCR1 Renaissance 2200. Correct selection of the medium for embedding sections is also important, since its properties affect the photostability of the protein during the study of preparations. Finally, the accuracy of the final result will depend on the resolution of the selected microscopy method. Applications of fluorescent reporter proteins for studying plant development are not limited to localization of the cellular response to phytohormones, tissue localization of promoter activity of the gene encoding a developmental regulator, or to detection of the protein itself. Expression of fluorescent protein genes under the control of promoters of genes of interest is an extremely specific fluorescent label produced directly by the plant. Therefore, not only cell structures, but also physiological processes are marked, which cannot be achieved by simple staining a sample with fluorescent dyes. However, all studies using fluorescent proteins require careful experimental design and sample preparation to ensure sample integrity.

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