Istraživanje interakcija proteina BPM1, DMS3 i RDM1 bimolekulskom fluorescencijskom komplementacijom u stanicama duhana

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University of Zagreb

Faculty of Science

Department of Biology

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Ovaj rad je izrađen u Laboratoriju za molekularnu biologiju biljaka na Zavodu za molekularnu biologiju Prirodoslovno-matematičkog fakulteta Sveučilišta u Zagrebu, pod vodstvom izv. prof. dr. sc. Nataše Bauer. Rad je dio projekta PHYTOMETHDEV financiranog od Hrvatske zaklade za znanost. Rad je predan na ocjenu Biološkom odsjeku Prirodoslovno-matematičkog fakulteta Sveučilišta u Zagrebu radi stjecanja zvanja magistra molekularne biologije.

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DOKUMENTACIJSKA KARTICA

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Toni Rendulić

Rooseveltov trg 6, 10 000 Zagreb, Hrvatska

Proteini BPM sudjeluju u regulaciji nekoliko transkripcijskih faktora kroz ubikvitinsko-proteasomski put. Proteini DMS3 i RDM1 su važne komponente sustava za RNA-posredovanu metilaciju DNA (RdDM). U preliminarnom istraživanju pokazalo se da su DMS3 i RDM1 potencijalni interakcijski partneri proteina BPM1. U ovom radu su istražene interakcije proteina BPM1, DMS3 i RDM1 pomoću bimolekulske fluorescencijske komplementacije (BiFC). Radi provedbe analize BiFC, generirani su prikladni plazmidni konstrukti tehnikom kloniranja In-Fusion. Stanice epiderme duhana *Nicotiana benthamiana*, epiderme luka *Allium cepa* i stanice duhana BY-2 transformirane su generiranim plazmidnim konstruktima pomoću agroinfiltracije ili bombardiranjem mikročesticama zlata. Detekcija proteinskih interakcija provedena je fluorescencijskom mikroskopijom. DMS3 i RDM1 su imali interakciju u svim istraživanim stanicama, dok je formacija dimera DMS3 detektirana u epidermalnim stanicama duhana *N. benthamiana* i luka *A. cepa*. Interakcija proteina BPM1 i DMS3 je potvrđena u duhanu *N. benthamiana* i u stanicama BY-2. Interakcija između BPM1 i RDM1 nije detektirana u ovom radu. Potrebna su dodatna istraživanja kako bi se bolje razumjele pojedinosti interakcije između BPM1 i DMS3 te potencijalni utjecaj BPM1 na RdDM.

(39 stranica, 13 slika, 7 tablica, 26 literaturnih navoda, jezik izvornika: engleski)

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Ključne riječi: BTB, MATH, RdDM, BiFC, transformacija

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dr. sc. Martina Šeruga Musić. izv. prof.

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BASIC DOCUMENTATION CARD

University of Zagreb

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Department of Biology

Graduation Thesis

Protein interaction analysis of BPM1, DMS3 and RDM1 in tobacco cells using bimolecular fluorescent complementation

Toni Rendulić

Rooseveltov trg 6, 10 000 Zagreb, Croatia

BPM proteins are known to participate in the regulation of several transcription factors via the ubiquitin proteasome pathway. DMS3 and RDM1 proteins are important components of the RNA-directed DNA methylation (RdDM) machinery. In preliminary research, DMS3 and RDM1 were shown to be potential interactors of BPM1. Here, interactions of BPM1, DMS3 and RDM1 proteins were analyzed via bimolecular fluorescence complementation (BiFC). To perform BiFC analysis, suitable plasmid constructs were generated using In-Fusion cloning technique. *Nicotiana benthamiana*, *Allium cepa* and BY-2 cells were transiently transformed with the generated constructs via agroinfiltration or microparticle bombardment. Finally, fluorescence microscopy was used to detect and analyze protein interactions. DMS3 and RDM1 interacted in all examined cells, while DMS3 dimer formation was detected in *N. benthamiana* and *A. cepa* epidermal cells. Interaction between BPM1 and DMS3 was proved in *N. benthamiana* leaf epiderm and in tobacco BY-2 cells. By using BiFC, no interaction was detected between BPM1 and RDM1 in this study. Further research is required to better understand the details of interaction between BPM1 and DMS3 proteins and the potential effect of BPM1 on RdDM.

(39 pages, 13 figures, 7 tables, 26 references, original in: English)

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Key words: BTB, MATH, RdDM, BiFC, transformation

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Thesis accepted: 5 April 2018

Table of Contents

List of Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
ATP	Adenosine triphosphate
ABA	Abscisic acid
AGO4	Argonaute 4
APC2	Anaphase Promoting Complex 2
BiFC	Bimolecular fluorescence complementation
BPM	BTB/POZ-MATH
BTB	Bric a brac, Tramtrack and Broad Complex
CLSY1	Classy 1
CUL	Cullin
DCL3	Dicer-like 3
DMS3	Defective in Meristem Silencing 3
DREB2A	Dehydration-Responsive Element-Binding 2A
DRM2	Domains Rearranged Methyltransferase 2
DRD1	Defective in RNA-Directed DNA Methylation 1
ERF/AP2	Ethylene Response Factor/Apetala 2
GFP	Green Fluorescent Protein
HECT	Homology to E6-AP C-Terminus
HEN1	Hua Enhancer 1
KTF1	Kow Domain-Containing Transcription Factor 1
Lys	Lysin
MATH	Meprin and TRAF-Homology
MYB56	Myb-related protein 56
MORC6	Microrchidia 6
PCR	Polymerase chain reaction
Pol IV	RNA polymerase IV
Pol V	RNA polymerase V
POZ	Pox virus and Zinc finger
RAP2.4	Related to Apetala 2.4
RBX1	RING-Box protein 1
RdDM	RNA-directed DNA Methylation
RDM1	RNA-directed DNA Methylation 1
RDR2	RNA dependent RNA polymerase 2
RING	Really Interesting New Gene
SHH1	Sawadee Homeodomain Homologue 1
siRNA	Small interfering RNA
SUVH2	Su(var) homologue 2
SUVH9	Su(var) homologue 9
T-DNA	Transfer DNA
WRI1	Wrinkled 1
YFP	Yellow Fluorescent Protein

1 INTRODUCTION

Living organisms possess cellular mechanisms which regulate physiological and developmental processes in response to internal and external cues. Such mechanisms are particularly important in plants due to their sessile lifestyle and include control of gene expression, mRNA processing and stability, as well as protein posttranslational modifications and stability.

1.1 Proteasomal degradation of proteins

Majority of the intracellular proteins are degraded by the ubiquitin proteasome pathway (Fig. 1), and this pathway has emerged as a central player in the regulation of diverse cellular processes. Ubiquitin is a highly conserved protein composed of 76 amino acids and serves as a reusable tag for selective protein degradation in this pathway. Specific protein targets are designated for degradation by covalent attachment of polyubiquitin chain to their lysine residues (Smalle and Vierstra, 2004). This ATP-dependent cascade reaction is accomplished by sequential action of three enzyme families, ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2) and ubiquitin ligases (E3). Initially, E1 activates ubiquitin by coupling ATP hydrolysis to the formation of thioester bond between C-terminal ubiquitin glycine and E1 cysteine residue. Activated ubiquitin is then transferred to an E2 cysteine via transesterification. Finally, E3 binds the E2 and a substrate protein to facilitate transfer of ubiquitin moiety. As a result, isopeptide bond is formed between C-terminal ubiquitin glycine and free lysine ε-amino group in the target. Reiterative transfer of additional ubiquitin molecules to lysine residues (typically lysine 48) on each previously attached ubiquitin generates a polyubiquitin chain (Smalle and Vierstra, 2004). Protein targets conjugated with polyubiquitin chains are degraded in an ATP-dependent process by the 26S proteasome, a 2.5-MDa proteolytic complex. The 26S proteasome is comprised of the 20S core particle and one or two 19S regulatory particles. Core particle is a cylindrical stack created by the assembly of four heptameric rings and its central chamber houses protease-active sites. Regulatory particle binds to one or both ends of the core particle. It serves to recognize ubiquitylated protein targets and participates in their unfolding and direction into the lumen of the core particle for breakdown. After breakdown of protein target, bound ubiquitin moieties are released and thus recycled by the activity of deubiquitylating enzymes (Smalle and Vierstra, 2004). The

importance of this pathway in plants is clearly demonstrated by the number of genes encoding its components, which represent over 5% of the total proteome in *Arabidopsis* (Hua and Vierstra, 2011). The specificity of this pathway is provided by E3 ligases which are also its most numerous and diverse component, with over 1500 different E3 complexes being predicted by comprehensive genetical analyses in *Arabidopsis* (Hua and Vierstra, 2011).

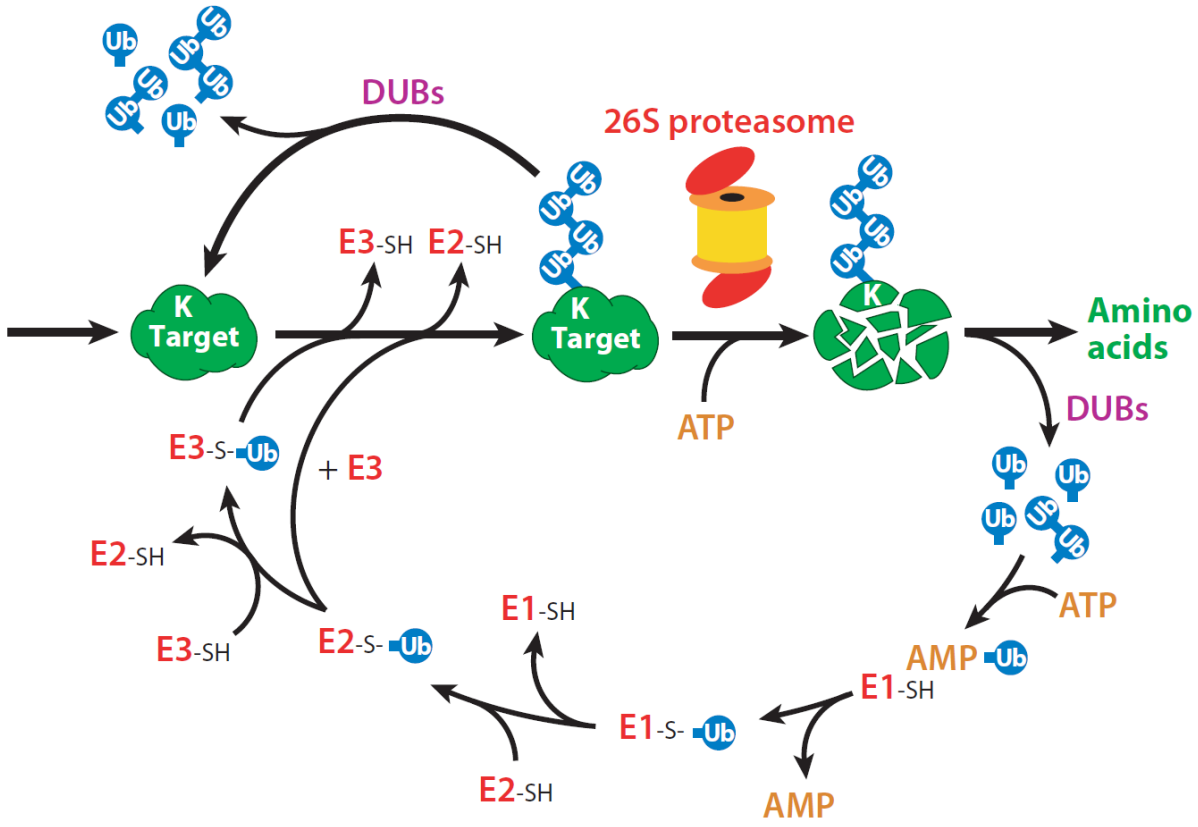


Figure 1. The Ubiquitin proteasome pathway. Ubiquitin is bound to an E1 in an ATP-dependent manner and subsequently transferred to an E2. The E2-ubiquitin conjugate assembles with an E3 which facilitates ubiquitylation of substrate proteins. Substrates with a covalently attached polyubiquitin chain become targets of the 26S proteasome. Protein targets are cleaved into peptides, while the ubiquitin moieties are recycled by deubiquitylating enzymes (DUB). Image acquired from Hua and Vierstra, 2011.

1.2 CUL3 E3 ligases

There are two major classes of E3 ligases: HECT E3 ligases which form a thioester intermediate with ubiquitin prior to transfer of ubiquitin moiety to the substrate protein, and RING-Finger E3 ligases (also called cullin-RING ligases) which facilitate ubiquitylation by positioning

activated E2s near the substrate protein. Multimeric RING-Finger ligases are comprised of a cullin protein as a central and scaffolding subunit which recruits RING-finger protein at its C-terminal region while its N-terminal region binds a variety of substrate adapters (Chen and Hellman, 2013). In plants, there are four known major E3 ligase families that contain either a cullin (CUL1, CUL3, CUL4) or a cullin-like protein (APC2) (Hua and Vierstra, 2011). The CUL3 based E3 ligases (Fig. 2) are comprised of a CUL3 protein which functions as a molecular scaffold by assembling with RBX1 and employing BTB-domain protein as a substrate adapter. The RBX1 protein binds the E2-ubiquitin conjugate and allosterically promotes the direct transfer of ubiquitin moiety from the E2 to the substrate. Proteins with a BTB domain can bind the N-terminal region of CUL3, while their secondary domain such as MATH functions as an actual substrate adapter, thus forming a bridge between CUL3 and the target substrate (Chen and Hellman, 2013).

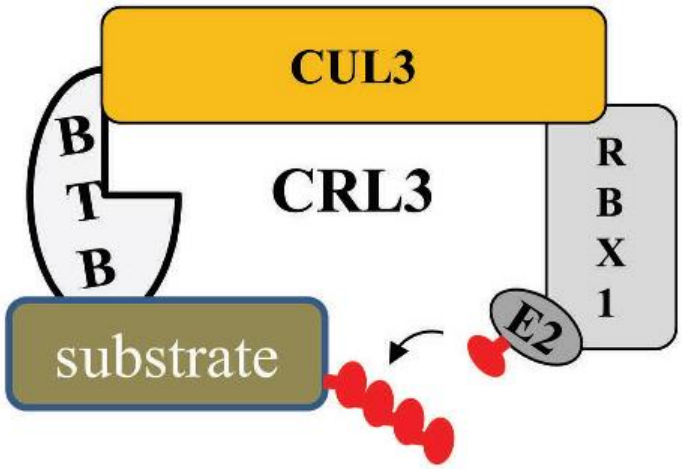


Figure 2. Structure and organization of CUL3 E3 ligase. CUL3 recruits RBX1 at its C-terminal region, while its N-terminal region binds substrate adapters via their BTB domain. The RBX1 protein binds the E2-ubiquitin conjugate and promotes transfer of ubiquitin moiety from the E2 to the substrate. Image acquired and adapted from Chen and Hellman, 2013.

1.3 MATH BTB proteins

The BTB domain, also known as POZ domain, is a protein-protein interaction motif which enables dimerization, oligomerization, as well as interactions with non-BTB proteins, such as previously mentioned CUL3. It is a sequence of approximately 95 amino acids, forming 5 α-helices and 3 β-sheets. Despite widely divergent sequences, the BTB fold is structurally well conserved (Stogios et al., 2005). MATH domain is about 180 amino acids long fold of 7-8

antiparallel β-sheets that participates in protein-protein interactions. All different MATH encompassing proteins subfamilies seem to have a role in the regulation of protein processing (Zapata et al., 2007). Among 80 BTB proteins found in *Arabidopsis*, 6 of them contain MATH domain (BPM1-6; Chen and Hellman, 2013). BPM1 and BPM2 are primarily localized inside the nucleus, while BPM3, BPM4, BPM5 and BPM6 can be found both inside and outside of the nucleus (Lechner et al., 2009; Morimoto et al., 2017). It is demonstrated that all *Arabidopsis* BPM proteins are involved in regulation of abscisic acid (ABA) response. ABA regulates certain aspects of plant growth and development such as seed dormancy and germination while also being a key component in plant response to biotic and abiotic stress. The class I homeodomain leucine zipper (HD-ZIP) transcription factor ATHB6 is a negative regulator of ABA responses such as sensitivity towards ABA during seed germination and stomatal closure. Via their MATH domain, BPM proteins interact with the leucine zipper domain of ATHB6 and consequently target it for proteasomal degradation (Lechner et al., 2011). Members of the ERF/AP2 transcription factor family are also shown to interact with BPM proteins. Weber and Hellman (2009) demonstrate that RAP2.4 interacts with BPM proteins via their MATH domain and propose a working model in which BPM proteins bind with ERF/AP2s, potentially interfering with their DNA-binding ability, but which ultimately results in the degradation of ERF/AP2 proteins. This is furthermore demonstrated by Chen et al. (2013) who show that BPM proteins assemble with WRI1 at the DNA level, causing its destabilization and subsequent degradation. WRI1 is also a member of ERF/AP2 transcription factor family and an important factor in fatty acid and carbohydrate metabolism. A recent study reports that BPM proteins interact with DREB2A, a key transcription factor in drought and heat stress tolerance and a member of ERF/AP2 family. BPM proteins negatively regulate DREB2A stability and therefore modulate the heat stress response and prevent adverse effects of excess DREB2A on plant growth (Morimoto et al., 2017). A role of BPM proteins in regulation of flowering is reported. Transcription factor MYB56 is established as a negative regulator of flowering locus T (FT), a central regulator of flowering time in *Arabidopsis*. Interaction of BPM proteins and MYB56 results in instability and subsequent proteasomal degradation of MYB56. Therefore, BPM proteins positively affect flowering via their antagonistic functions with MYB56 on FT expression (Chen et al., 2015). While previously mentioned studies establish and broaden the role of BPM proteins as regulators in transcriptional processes in *Arabidopsis* via CUL3-based E3 ligase activities, Leljak Levanić et al. (2012) report primarily nucleolar localization of BPM1, a compartment which is to a large extent devoid of CUL3, thus proposing a cullin-independent role of BPM proteins. Further studies (unpublished) found proteins DMS3 and

RDM1, members of the RNA-directed DNA methylation (RdDM) pathway, to be potential interaction partners of BPM1.

1.4 RNA-directed DNA methylation

RdDM is an epigenetic pathway which facilitates *de novo* DNA methylation in plants. It is guided by 24-nucleotide small interfering RNAs (siRNAs) and involves two unique plant RNA polymerases Pol IV and Pol V. Cytosine residues within all sequence contexts can be methylated in this pathway (CG, CHG and CHH, H being A, T or C). The RdDM pathway is implicated in transposon silencing, pathogen defense, stress responses, reproduction, and interallelic and intercellular communication (Matzke and Mosher, 2014). RdDM (Fig. 3) is initiated by Pol IV which transcribes single-stranded RNAs (ssRNA) at its target loci, which are primarily transposons and other repeats. Generated ssRNA is then copied into double-stranded RNA (dsRNA) by RNA-dependent RNA polymerase 2 (RDR2). Dicer endonuclease DCL3 processes dsRNA into 24-nucleotide siRNA which are then exported to cytoplasm and incorporated into AGO4. Formed siRNA-AGO4 complexes are then re-imported to the nucleus. The ssRNA-AGO4 complex is guided by the ssRNA towards complementary scaffold transcripts from Pol V, ultimately leading to recruitment of methyltransferase DRM2 which catalyzes *de novo* DNA methylation (Matzke and Mosher, 2014). Subjects of this research, the DMS3 and RDM1 proteins, are components of the RdDM machinery that appear to be only present in flowering plants. The DMS3 protein possesses a hinge domain of the structural maintenance of chromosomes (SMC) proteins. The SMC proteins are involved in modulating higher-order chromosome organization and dynamics and form heterodimers via their hinge domains. DMS3 is thought to stabilize the unwound state of the DNA duplex, which may ease the Pol V transcription (Matzke et al., 2015). RDM1 is a small protein that has a unique fold and a domain of unknown function. A nearly complete loss of DNA methylation in *rdm1* mutants indicates that RDM1 has a crucial role in RdDM pathway. RDM1 is thought to act as a bridging protein between AGO4 and DRM2 and facilitate Pol V transcription as a part of a complex with DRD1 and DMS3 (Matzke et al., 2015). DMS3 and RDM1 proteins interact with each other and are also capable of forming homodimers (Sasaki et al., 2014).

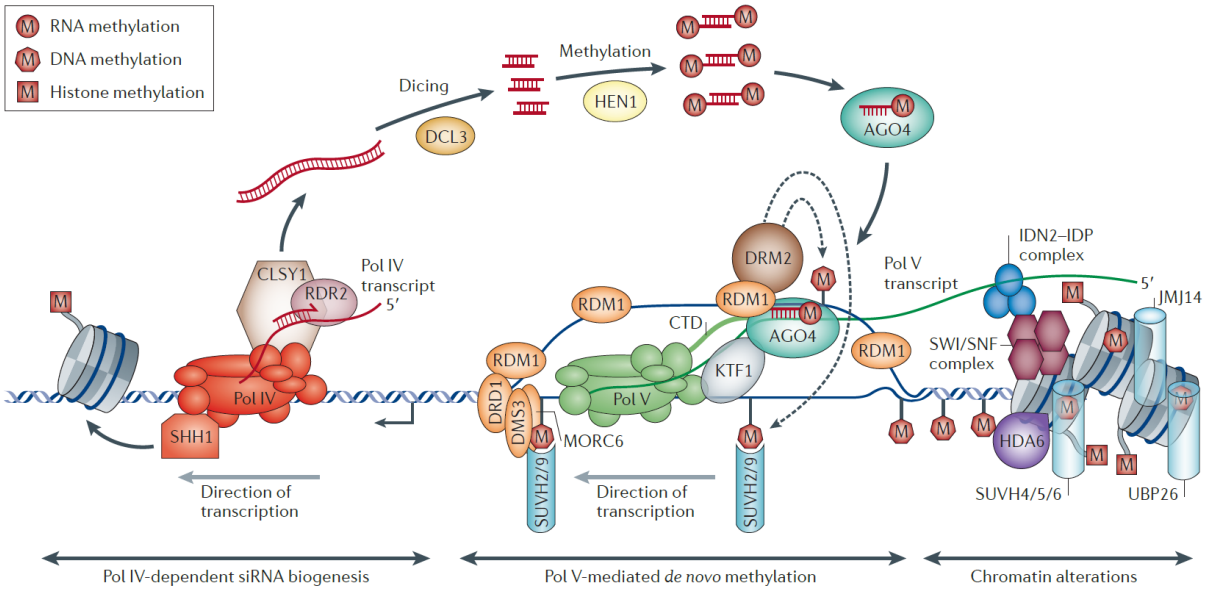


Figure 3. RdDM pathway model. Pol IV transcribes ssRNA which is then copied into dsRNA by the RDR2 with the assistance of CLSY1. The dsRNA is processed into 24-nucleotide siRNAs by DCL3. The siRNAs are methylated at their 3' ends by HEN1 and incorporated into AGO4. The SHH1 protein, which binds to histone H3 methylated at lysine 9, interacts with Pol IV and recruits it to target loci. Pol V transcribes scaffold RNA that base-pairs with AGO4-bound siRNAs. AGO4 interacts with C-terminal region of the largest Pol V subunit and with KTF1. RDM1 protein links AGO4 and DRM2, which catalyzes *de novo* methylation of DNA. Pol V transcription may be enabled by the activity of chromatin remodeler DRD1, which unwinds the DNA duplex, while RDM1, DMS3 and MORC6 may help at generating and stabilizing the unwound state. SUVH2 and SUVH9 bind methylated DNA and may assist in recruitment of Pol V to some loci. Image acquired from Matzke and Mosher, 2014.

1.5 Bimolecular fluorescence complementation (BiFC)

Protein interaction studies in living cells are of particular significance in acknowledging the mechanisms of cellular processes. The interactions that occur in a particular cell depend on the full complement of proteins present in the cell, their posttranslational modifications and the external stimuli that influence the cell. Most protein-protein interactions are studied by *in vitro* methods (pull down) or in yeast (by yeast two hybrid system). BiFC analysis (Fig. 4) enables direct visualization of protein interactions in living cells and can be performed in plant cells. It is based on the formation of a fluorescent complex by two non-fluorescent fragments of the yellow fluorescent protein (YFP) brought together by association of interacting proteins fused to these fragments (Hu et al., 2002).

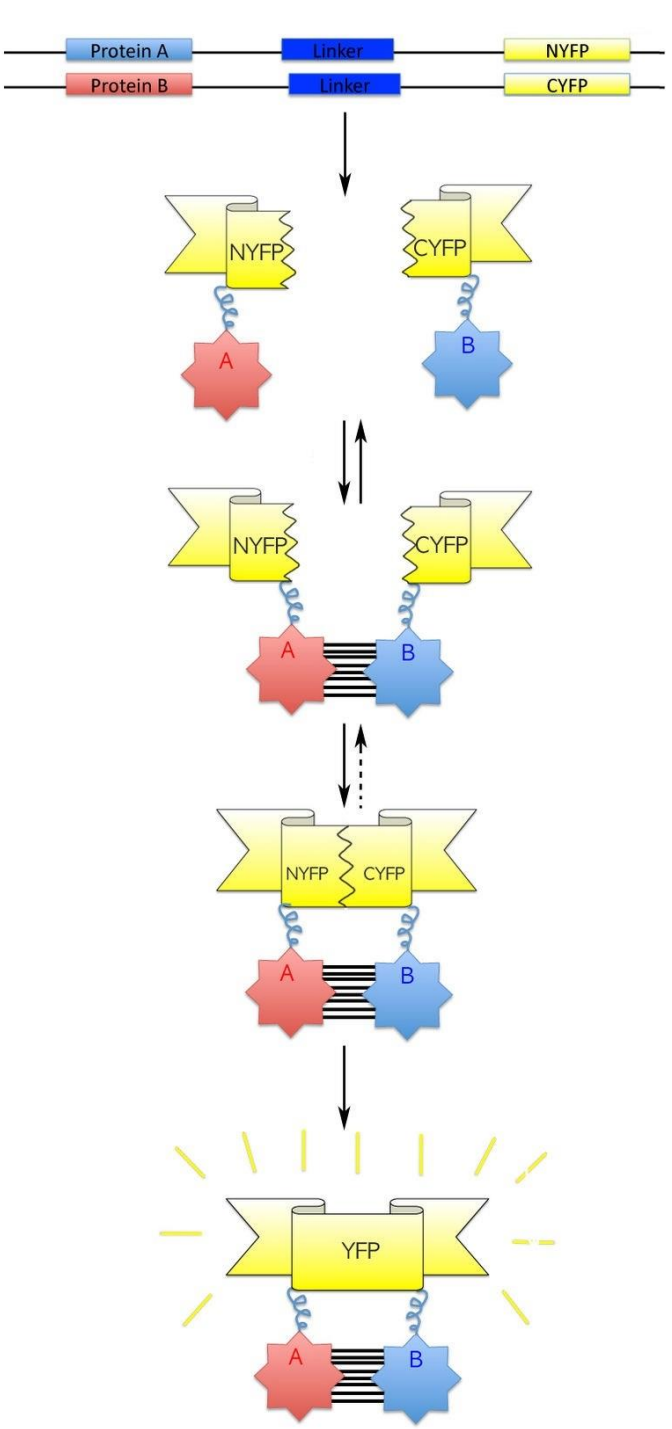


Figure 4. Formation of the bimolecular fluorescent complex. BiFC method is based on complementation between two non-fluorescent YFP fragments when they are brought together by interactions between proteins fused to each fragment. Initial steps of complex formation are mediated by contacts between proteins fused to the YFP fragments. The complex is stabilized by association of the YFP fragments. Reconstitution of the YFP complex is irreversible. Image acquired and adapted from https://commons.wikimedia.org/w/index.php?curid=9646287

1.6 Thesis objective

The main objective of this study is to establish the BiFC method in Laboratory of molecular plant biology, Faculty of Science. Based on a recent study, a cullin-independent function of BPM proteins was proposed (Leljak Levanić et al., 2012) and preliminary research found DMS3, and RDM1, the components of the RdDM machinery, to be potential interactors of BPM proteins (unpublished). The second aim of this work was to analyze protein-protein interactions of BPM1, DMS3 and RDM1 in transiently transformed tobacco cells via BiFC. A positive result would strengthen the recent findings that substantially expand the role of BPM proteins in plants.

2 MATERIALS AND METHODS

2.1 Materials

2.1.1 Bacteria strains and growth conditions

2.1.1.1 *Escherichia coli*

E. coli HST08 strain (Stellar™ Competent Cells) (http://www.clontech.com/US/Products/Cloning_and_Competent_Cells/Competent_Cells/Chemically_Competent_Cells) supplied with In-Fusion HD Cloning Kit was used for vector cloning. Bacteria were cultured in liquid LB medium or on LB agar plates at 37 °C.

2.1.1.2 *Agrobacterium tumefaciens*

A. tumefaciens GV3101-pMP90 strain (Koncz and Schell, 1986) was used for agroinfiltration of *Nicotiana benthamiana* leaves. This strain contains rifampicin resistance gene in its genome, while pMP90 contains gentamicin resistance gene. Bacteria were cultured in liquid LB medium or on LB agar plates at 28 °C.

2.1.2 Plant material and growth conditions

2.1.2.1 *Nicotiana benthamiana*

N. benthamiana plants were grown in a climate chamber at 24 °C under a 16 h/8 h light/dark cycle and light intensity of 80 $\mu E/s^2$, in a relative air humidity of 40 to 60%.

2.1.2.2 *Allium cepa*

Onion bulbs were bought in the market and were stored in the dark.

2.1.2.3 *Nicotiana tabacum* BY-2 cell suspension.

Tobacco BY-2 cells (Brandizzi et al., 2003) are non-green, fast growing plant cells widely used for examination of cell cycle. Cell suspensions were cultivated *in vitro* in BY-2 medium in the dark with shaking at 120 rpm.

2.1.3 Plasmids

2.1.3.1 pSPYNE and pSPYCE

To observe the protein-protein interactions in living plant cells via BiFC, pSPYNE and pSPYCE (shorter for *split YFP N-terminal/C-terminal fragment expression*) were used (Fig. 5) (Walter et al., 2004). Both plasmids contain kanamycin resistance gene as selectable marker in bacteria.

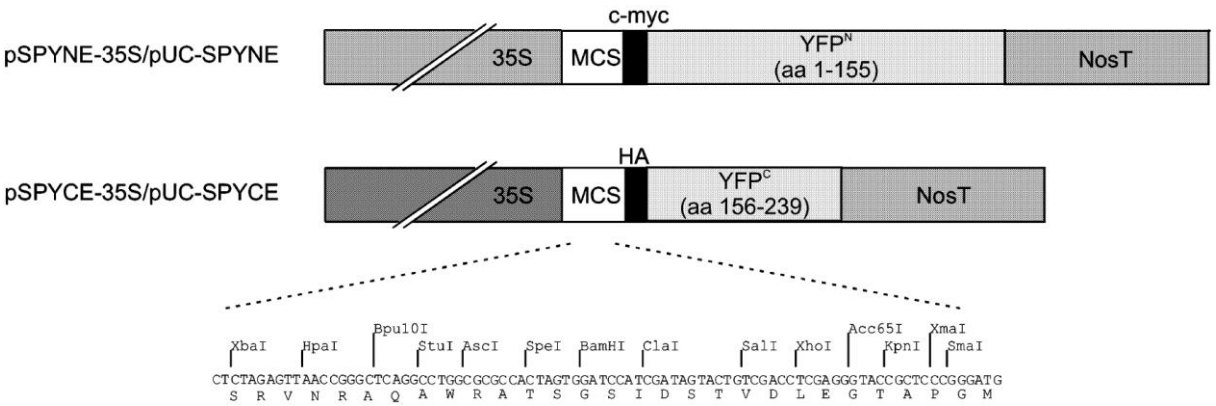


Figure 5. Plant-compatible BiFC vectors used in this research. c-myc, c-myc affinity tag; HA, hemagglutinin affinity tag; MCS, multi-cloning site; 35S, 35S promoter of the cauliflower mosaic virus; NosT, terminator of the *Nos* gene; YFP^N, N-terminal fragment of YFP reaching from amino acid (aa) 1 to 155; YFP^C, C-terminal fragment of YFP reaching from aa 156 to 239. Image acquired from Walter et al., 2004.

2.1.3.2 pB7WGF-GFP

To confirm the efficacy of all used plant transformation techniques, pB7WGF-GFP (Fig. 6) containing *EGFP* gene under control of strong constitutive 35S promoter was used. This plasmid contains spectinomycin resistance gene as selectable marker in bacteria.

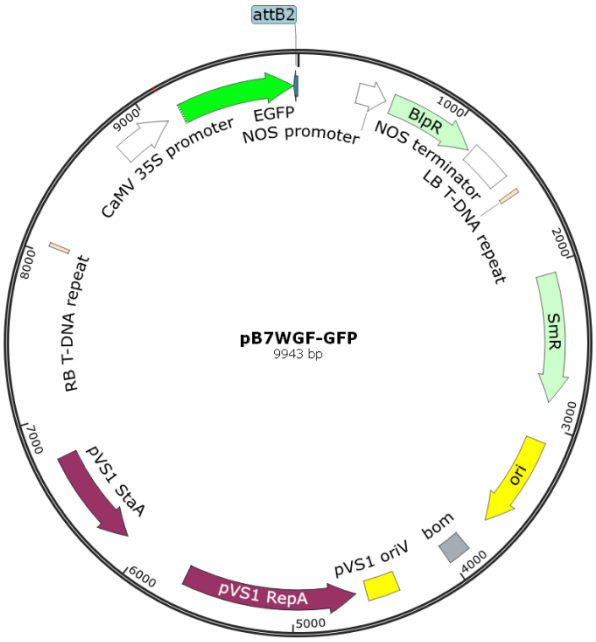


Figure 6. Map of pB7WGF-GFP. This plasmid contains *EGFP* gene under control of strong constitutive 35S promoter. Its expression cassette is bordered by two 25-bp T-DNA repeats, which make it suitable for plant transformation by *A. tumefaciens*.

2.1.3.3 pCB301-p19

To enhance the over-expression of transgenes, pCB301-p19 was used (Win and Kamoun, 2004). The p19 protein functions as a suppressor of post-transcriptional gene silencing in certain plants.

2.1.4 Primers

All primers used in this study are listed in Table 1. Primer sequences were designed using Snapgene® software and ordered from Macrogen. Primers were prepared as 100 µM stock solutions and 10 µM working solutions. Stock and working solutions were stored at -20 °C.

Table 1. Primers used in this study. (1-6) Primer used for generation of gene inserts. Each primer contained 15 bp extensions (5') homologous to the ends of linearized vectors. Primer ends (3') homologous to gene inserts are underlined. Additional nucleotides were added between the 5' overlap region and 3' gene-specific region to restore the pre-existing BamHI restriction site (marked in blue). (7-9) Plasmid-specific primers used for amplification of gene expression cassettes.

	Primer name	Nucleotide sequence 5'-3'
1	BPM1_BIFC_IFf	CGCCACTAGTGGATCCATGGGCACAACTAGGGTCTGC
2	BPM1_BIFC_IFrev	TACTATCGATGGATCCGTGCAACCGGGGCTTCACTC
3	DMS3_BIFC_IFf	CGCCACTAGTGGATCCATGTATCCGACTGGTCAACAGA
4	DMS3_BIFC_IFrev	TACTATCGATGGATCCTCTGGGTGTGTTCATTGGCTG
5	RDM1_BIFC_IFf	CGCCACTAGTGGATCCATGCAAAGCTCAATGACAATG
6	RDM1_BIFC_IFrev	TACTATCGATGGATCCTTTCTCAGGAAAGATTGGGTC
7	BiFC_BY2fw	CCCACTGAATCAAAGGCCATG
8	BiFC_BY2rev	GAATTCCCGATCTAGTAACATAGATGACAC
9	35S-3	CACTGACGTAAGGGATGACGCAC

2.1.5 Media and buffers

2.1.5.1 LB medium

LB medium (10 g/L tryptone, 5 g/L yeast extract, NaCl 10 g/L, pH 7.0) was used for growth of bacteria. When needed, the medium was solidified with agar (15 g/L) and supplemented with antibiotics.

2.1.5.2 SOC medium

SOC medium (5 g/L yeast extract, 20 g/L tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, $MgSO_4$, 20 mM glucose) was used for recovery of competent bacteria during the final stage of bacterial transformation.

2.1.5.3 BY-2 medium

The composition of modified MS (Murashige and Skoog, 1962) growth medium used for cultivation of BY-2 suspension culture is indicated in Table 2. Liquid medium was used for BY-2 culture maintenance. For transformation via Biolistic, BY-2 cells were subcultivated on the same medium solidified with agar.

Table 2. BY-2 medium used for cultivation of BY-2 cells.

Sucrose	30 g/L
Myo-inositol	100 mg/L
Macroelements	1x MS
KH_2PO_4	0.21 g/L
Microelements	1x MS
Thiamine	1 mg/L
2,4- D	0.2 mg/L
Agar (for growth on plates)	8 g/L
pH	5.6

2.1.5.4 Seed germination medium

The composition of growth medium used for germination of *N. benthamiana* seeds is indicated in Table 3. Onion epidermis was placed on the same medium prior to transformation via Biolistic.

Table 3. Seed germination medium used for germination of *N. benthamiana* seeds.

Sucrose	20 g/L
Myo-inositol	100 mg/L
Macroelements	1x MS
Microelements	1x MS
Thiamine	0.1 mg/L
Niacin	0.5 mg/L
Pyridoxine	0.5 mg/L
Glycine	2 mg/L
Agar	8.5 g/L
pH	5.8

2.1.5.5 Buffers

For agarose electrophoresis, 1x TAE buffer (40 mM Tris, 20 mM acetate, 1 mM EDTA) was used.

For preparation of washing and infiltration solution in agroinfiltration procedure, 2-morpholinoethanesulfonic acid (MES) buffer (10 mM, pH 6.0) was used.

2.2 Methods

2.2.1 Generation of plasmid constructs via In-Fusion cloning technology

2.2.1.1 Generation of gene inserts

In-Fusion Enzyme fuses DNA fragments (e.g., PCR-generated inserts and linearized vectors) by recognizing 15-bp overlaps at their ends. Therefore, gene-specific primers with 15 bp extensions (5') homologous to the ends of linearized pSPYNE and pSPYCE vectors were designed (Table 1). pSPYNE and pSPYCE share the same multiple cloning site, thus pairs of primers could be used interchangeably for gene cloning in both vectors. Each gene was amplified using the 2x CloneAmp HiFi PCR Premix (12.5 µL) along with 5 pmol of each gene-specific primer and 10 ng of template DNA (Table 4). PCR was performed in a 2720 Thermal Cycler (Applied Biosystems). Initial denaturation step was performed at 98 °C for 1 min, followed by 35 cycles of denaturation at 98 °C for 10 s, annealing at 58 °C for 10 s, elongation at 72 °C for 5 s/kb and a final elongation step at 72 °C for 1 min. Total reaction volume was 25 µL. Gene inserts (5 µL) were analyzed on 1% agarose gel in 1x TAE buffer and the rest was purified using the NucleoSpin Gel and PCR Clean-Up kit according to manufacturer's protocol (http://www.mn-net.com/tabid/1452/default.aspx). Concentrations of purified inserts were measured using NanoVue^{TM} spectrophotometer (GE Healthcare). Purified inserts were stored at -20 °C.

Table 4. Gene inserts generated via PCR for In-Fusion cloning into pSPYNE and pSPYCE vectors. Gene name, gene ID from the TAIR database, template DNA, primer name and product size are indicated. Compared to their respective original coding sequences, gene inserts lack stop codons and contain extensions homologous to linearized vectors at each end.

Gene name	Gene ID	Template DNA	Primer name	Product size (bp)
BPM1	AT5G19000.1	pB7WGF2-BPM1	BPM1_BIFC_IFf	1253
			BPM1_BIFC-IFrev	
DMS3	AT3G49250.1	pB7WGF2-DMS3	DMS3_BIFC-IFf	1292
			DMS3_BIFC_IFrev	
RDM1	AT3G22680.1	pGEX5.1-RDM1	RDM1_BIFC_IFf	521
			RDM1_BIFC_IFrev	

2.2.1.2 Cloning procedure

Vectors (pSPYNE and pSPYCE) were linearized using FastDigest BamHI (Thermo Scientific) restriction enzyme. Reaction mixtures with a total volume of 50 µL, containing 10x FastDigest Buffer (5 µL), FastDigest BamHI (5 µL) and plasmid DNA (5 µg), were prepared and incubated for 30 min at 37 °C. Linearized vectors were purified with NucleoSpin Gel and PCR Clean-Up kit. Concentrations of purified linearized vectors were measured using NanoVue^{TM} spectrophotometer (GE Healthcare). Previously generated gene-inserts were cloned into linearized vectors using the In-Fusion kit as follows. Each In-Fusion reaction mixture, with a total volume of 10 µL, was prepared in a 0.2-mL reaction tube by adding PCR generated gene insert (50 ng), linearized plasmid (100 ng), 5x In-Fusion HD Enzyme Premix (2 µL) and deionized water. The reaction mixtures were incubated for 15 min at 50 °C and then placed on ice.

2.2.2 Transformation of chemically competent cells

Chemically competent Stellar cells were thawed in an ice bath before use. Aliquots (50 µL) of Stellar cells were transferred into 14-mL round-bottom tubes. In-Fusion reaction mixtures (2.5 µL) containing the plasmid constructs were added to each aliquot. Aliquots were incubated on ice for 30 min. Transformation was performed by heat-shocking the Stellar cells for 45 s at 42 °C and placing them on ice for 2 min afterwards. SOC medium was warmed up to 37 °C before use and 500 µL were added to each tube with the heat-shocked cells. Stellar cells were incubated at 37 °C for 1 hour with shaking at 250 rpm. Afterwards, cells were spread on LB agar plates supplemented with kanamycin (50 ng/µL) and grown overnight at 37 °C.

2.2.3 Colony screening via PCR

Single *E. coli* colonies were picked and cultured overnight at 37 °C in 3 mL of LB liquid medium supplemented with appropriate antibiotic. Portions of cultured bacteria (200 µL) were transferred into 1.5-mL microcentrifuge tubes and pelleted by centrifugation for 10 min at 14 000 rpm. Supernatant was discarded and pellets were resuspended in 200 µL of sterile water. The resuspended bacteria were denatured at 95 °C for 5 min. The denatured bacterial suspensions were used as templates for PCR. Reaction mixtures were prepared by adding 12.5 µL of EmeraldAmp MAX PCR Master Mix (Takara), 2 µL of denatured bacterial

suspension, 5 pmol of each gene specific primer and 9.5 µL of deionized water into each 0.2-mL reaction tube. Initial denaturation step was performed at 98 °C for 3 min, followed by 40 cycles of denaturation at 98 °C for 10 s, annealing at 58 °C for 30 s, elongation at 72 °C for 1 min/kb and a final elongation step at 72 °C for 7 min. Total reaction volume was 25 µL. Finally, PCR mixtures were analyzed on agarose gel.

2.2.4 Plasmid DNA isolation

Remaining portions of liquid cultures positive for DNA-inserts were pelleted by centrifugation for 10 min at 14 000 rpm. Plasmid DNA was isolated using the Wizard® Plus SV Minipreps DNA Purification System (Promega) according to manufacturer's protocol (https://worldwide.promega.com/resources/protocols/technical-bulletins/0/wizard-plus-sv-minipreps-dna-purification-system-protocol/). Isolated plasmid DNA was stored at -20° C.

2.2.5 Restriction analysis

Isolated plasmid DNA (500 ng) was digested using the FastDigest BamHI restriction enzyme (0.5 µL) and FastDigest Green reaction buffer. Digestion mixtures were incubated at 37 °C for 30 min. After digestion, plasmid DNA was analyzed on 1% agarose gel in 1x TAE buffer. Plasmid DNA samples that contained appropriate inserts were submitted to Macrogen for sequencing. After sequence analysis via SnapGene®, approved plasmids were used for subsequent transformation of *A.tumefaciens* and plants.

2.2.6 Glycerol stock preparation

Glycerol (100 µL, 100%) was added into a 1.5 mL tube. Bacterial culture (400 µL) was transferred into the same tube and was gently mixed with glycerol. Stocks were frozen with liquid nitrogen and stored at -80 °C.

2.2.7 Cultivation of *N. benthamiana*

Seeds were rinsed for 1 min in 70% ethanol and then washed in an aqueous solution containing 1% Izosan® G (Pliva) and 0.1% Mucasol (Merz Consumer Care GmbH) for 10 min. After

removing the solution, seeds were rinsed in 1 mL of sterile water and vortexed. Seeds were rinsed and vortexed 4 more times. Sterilized seeds were plated on seed germination medium solidified with 0.8% agar. Plated seeds were stratified overnight at 4 °C in the dark and further cultivated in climate chamber as stated in (2.1.2.1). After approximately 2 weeks, seedlings were transferred into soil and were grown in the same conditions.

2.2.8 Preparation of *A. tumefaciens clones* for agroinfiltration

Premade electrocompetent *A. tumefaciens* bacteria (50 μL) were thawed in an ice bath. Into each 1.5-mL tube containing the bacteria, a different plasmid construct (50 ng) was added. Mixtures were transferred to pre-chilled electroporation cuvettes. Outsides of the cuvettes were wiped down to remove moisture and cuvettes were inserted into the MicroPulser™ electroporator (BIO-RAD). Strength and duration of the pulse were 2200 V and 5 ms, respectively. SOC medium (500 μL) was added into each cuvette. Mixtures were transferred back into the tubes and incubated at 28 °C for 2 hours with shaking (200 rpm). Bacteria were spread on LB agar plates supplemented with respective selective antibiotics (Table 5).

Single *A. tumefaciens* colony was picked and cultured overnight at 28 °C in 3 mL of LB liquid medium supplemented with respective selective antibiotics (Table 5). Bacterial cultures were pelleted, resuspended, denatured and subjected to further PCR analysis as described in 2.2.3

Table 5. Antibiotics added to LB medium for selection of transformed *A. tumefaciens*.

A. tumefaciens clones	Supplemented antibiotics
pSPYNE-BPM1 pSPYCE-BPM1 pSPYNE-DMS3 pSPYCE-DMS3 pSPYNE-RDM1 pSPYCE-RDM1 pSPYNE pSPYCE	Kanamycin (50 ng/µL) Rifampicin (50 ng/µL) Gentamicin (50 ng/µL)
pCB301-p19	Kanamycin (50 ng/µL) Rifampicin (50 ng/µL)
pB7WGF-GFP	Spectinomycin (200 ng/µL) Rifampicin (50 ng/µL) Gentamicin (50 ng/µL)

2.2.9 Agroinfiltration procedure

Each *A. tumefaciens* colony (Table 5.) was grown separately overnight at 28 °C with shaking at 200 rpm in 5 mL of LB liquid medium supplemented with respective selective antibiotics. Bacteria were pelleted by centrifugation at 4000 rpm for 20 min at room temperature. Supernatant was discarded and pellets were resuspended in 2.5 mL of washing solution (10 mM $MgCl_2$, 10 mM MES). Bacteria were pelleted and resuspended again, as described in the previous step. Acetosyringone (100 mM stock, 150 µM final) was added to bacterial suspensions, which were then incubated for 3-4 hours at room temperature. Dilutions (1:10) were made in a total volume of 1 mL to measure the optical density of each suspension. Agroinfiltration mixtures with desired combinations of clones were prepared by calculating required volumes of bacterial suspensions so that each clone in the mixture had an optical density of 0.5 (Table 6). The total volume of each agroinfiltration mixture was 3 mL. Small holes were created on *N. benthamiana* leaves using a needle. Mixtures were injected into leaves by a syringe (without a needle). Syringe was pressed on the hole from the underside of the leaf while applying counter-pressure from the opposite side with a finger. Each plant was infiltrated with a different mixture (2-3 leaves per plant). Plants were watered and placed into the climate chamber.

Table 6. Agroinfiltration mixtures used for analysis of protein-protein interactions. Each clone had an optical density of 0.5 in 3 mL of infiltration mixture.

Protein-protein interaction analyzed	Clones in infiltration mixture		
BPM1 – DMS3	pSPYNE-BPM1	pSPYCE-DMS3	pCB301-p19
	pSPYCE-BPM1	pSPYNE-DMS3	pCB301-p19
BPM1 – RDM1	pSPYNE-BPM1	pSPYCE-RDM1	pCB301-p19
	pSPYCE-BPM1	pSPYCE-RDM1	pCB301-p19
DMS3 – RDM1	pSPYNE-DMS3	pSPYCE-RDM1	pCB301-p19
	pSPYCE-DMS3	pSPYNE-RDM1	pCB301-p19
DMS3 – DMS3	pSPYNE-DMS3	pSPYCE-DMS3	
Positive control	pB7WGF-GFP	pCB301-p19	
Negative control	pSPYNE	pSPYCE	pCB301-p19

2.2.10 Microparticle bombardment

2.2.10.1 Preparation of gold particles

Forty milligrams of Gold Microcarriers (0.6 μm diameter) (BIO-RAD) were added into a 2-mL microcentrifuge tube and thoroughly resuspended in 1 mL of 96% ethanol. The tube was centrifuged at 14 000 rpm for 30 s and the supernatant was discarded. This washing step was repeated twice. The pelleted gold particles were resuspended in 1 mL of sterile water and divided into 50-μL aliquots (2 μg of gold per aliquot) in 1.5-mL microcentrifuge tubes. Tubes with gold particles were sonicated for 2 min. Desired combinations of plasmid constructs were added to each tube (Table 7). According to established protocol, up to 1 μg of DNA could be loaded on gold particles. Spermidine (20 μL, 0.1 M) and calcium chloride (50 μL, 2.5 M) were placed on the inner side of the tube lid. In a quick manner, the lid was closed and the tube was vortexed for 2 min. DNA-coated particles were pelleted by centrifugation. Supernatant was discarded while particles were resuspended in 250 μL of 96%-ethanol. This step was repeated one more time. Finally, gold particles were pelleted again and resuspended in 85 μL of 96% ethanol.

Table 7. Plasmid combinations used for transformation of onion epidermal cells and BY-2 cells via Biolistic.

Protein-protein interaction analyzed	Plasmid combination	
BPM1 – DMS3	pSPYNE-BPM1	pSPYCE-DMS3
	pSPYCE-BPM1	pSPYNE-DMS3
BPM1 – RDM1	pSPYNE-BPM1	pSPYCE-RDM1
	pSPYCE-BPM1	pSPYNE-RDM1
DMS3 – RDM1	pSPYNE-DMS3	pSPYCE-RDM1
	pSPYCE-DMS3	pSPYNE-RDM1
DMS3 – DMS3	pSPYNE-DMS3	pSPYCE-DMS3
Positive control	pB7WGF-GFP	
Negative control	pSPYNE	pSPYCE

2.2.10.2 Plant material preparation

A thin layer of onion epidermis was peeled from the inner side of the onion bulb and was laid flat on the seed germination agar plate and subsequently transformed by Biolistic.

BY-2 cells were transferred from 3 days old suspension culture to solidified BY-2 medium (agar plates) by spreading 200 μL of cell suspension in a circular form (2 cm in diameter). Transferred cells were grown overnight at 24 °C, in the dark and then transformed by Biolistic.

2.2.10.3 Bombardment procedure

To transform the plant cells, PDS-1000/He biolistic system (BIO-RAD) was used. DNA-coated particles (7.5 μL) were spread on the central part of the carrier disk by a micropipette. The disks were left to dry for 10 min and were installed into the microcarrier launch assembly. The assembly was inserted on the highest slot within the chamber. The 1100-psi rupture disks were used to achieve the desired burst strength. Plant samples were placed on the target plate shelf, 6 cm beneath the launch assembly. To increase the efficiency of the process, the chamber was evacuated to sub-atmospheric pressure (27 mmHg on the vacuum gauge) before each bombardment procedure. Each plant sample was bombarded 2 times.

2.2.11 Microscopy

2.2.12.1 Plant sample preparation

Infiltrated area of *N. benthamiana* leaf was excised around the needle mark. Leaf samples were gently evacuated using a syringe filled with water. Epidermis was peeled from the underside of the leaf samples and placed into a drop of 0.1 M MES buffer on a slide and was covered with a cover slip.

Onion epidermis was removed from the agar plate and placed into a drop of 0.1 M MES buffer on a slide and was covered with a cover slip.

Transfected BY-2 cells were rinsed off the agar plates with 2 mL of liquid BY-2 medium by a pipette and were cultured as cell suspensions in 35 mm cell culture dishes. Transfected BY-2 cell suspensions were stored in the dark with shaking at 120 rpm. To observe the transfected BY-2 cells on a microscope, 150 µL of cell suspension was transferred onto a slide and covered with a cover slip.

2.2.11.2 Plant sample microscopy

Microscopy of plant samples was performed using Axiovert 200M (Zeiss) inverted fluorescence microscope. The microscope was equipped with AxioCam MRc microscope camera which was, coupled with the AxioVision imaging software (version 4.5), used for image acquisition. The fluorescence of reconstituted YFP in plant cells was observed using Filter Set 13 (excitation BP 470/20, emission BP 505-530). Plant cells were also observed using Filter Set 14 (excitation BP 510-560, emission LP 590) to distinguish true YFP signal from false positives caused by plant cell autofluorescence. Acquired images were further processed using ImageJ software.

3 RESULTS

3.1 Generation of plasmid constructs for BiFC

To analyze interactions between BPM1, DMS3 and RDM1 in living plant cells, 6 plasmids were constructed. Each gene (*BPM1, DMS3,* and *RDM1*) was cloned into pSPYNE and pSPYCE. *BPM1*, *DMS3* and *RDM1* gene inserts were amplified via PCR and pSPYNE and pSPYCE were linearized by BamHI restriction enzyme. After analysis in 1% agarose gel, it was determined that the obtained gene inserts and linearized plasmids matched their expected size (Fig. 7). Bends that correspond to linearized plasmids were excised from the gel and purified by using Nucleospin Gel and PCR Clean-up Kit. The same kit was used to purify amplified genes.

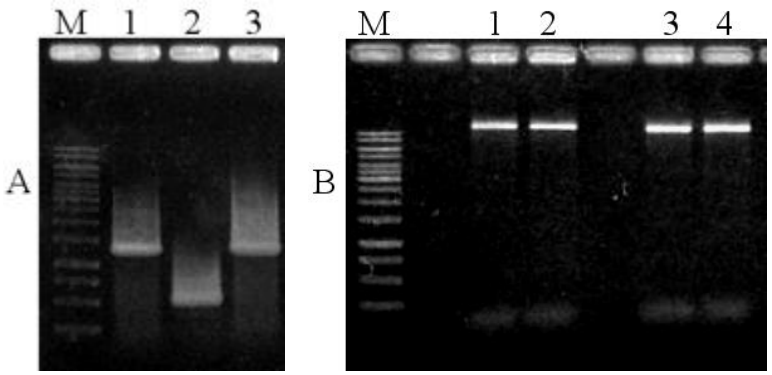


Figure 7. Amplified gene inserts and linearized plasmids. (A) Gene inserts generated via PCR. PCR mixtures (5 µL each) were loaded on 1% agarose gel. 1 – BPM1 (1253 bp); 2 – RDM1 (521 bp); 3 - DMS3 (1292 bp). (B) Visualization of vectors linearized by BamHI restriction enzyme. M – GeneRuler™ 1kb DNA ladder (ThermoScientific); 1,2 - linearized pSPYNE duplicates; 3,4 - linearized pSPYCE duplicates.

Appropriate amount of purified gene insert (50 ng) was mixed with appropriate linearized plasmid (100 ng) and treated for 15 minutes with In-Fusion enzyme. Chemically competent *E. coli* were transformed with 2.5 µL of In-Fusion reaction and plated on selective LB agar plates supplemented with kanamycin. Plates were incubated at 37 °C overnight, and dozens of bacterial colonies developed. Single colonies were picked and cultured overnight in liquid LB medium at 37 °C supplemented with appropriate antibiotic. A portion of each bacteria suspension was denatured at 95 °C and used as a template in PCR to screen the bacteria for

presence of gene inserts (Fig. 8). A combination of plasmid-specific (35S-3) and gene-specific primers was used (Table 1). In analysis on 1% agarose gel, all gene inserts were successfully detected. Plasmid DNA was isolated from bacterial colonies that were PCR-positive for gene inserts. Additional proof of successful cloning was digestion of plasmid DNA with the BamHI restriction enzyme. The analysis of restriction mixture on 1% agarose gel showed expected bands of desired gene inserts and linearized plasmids.

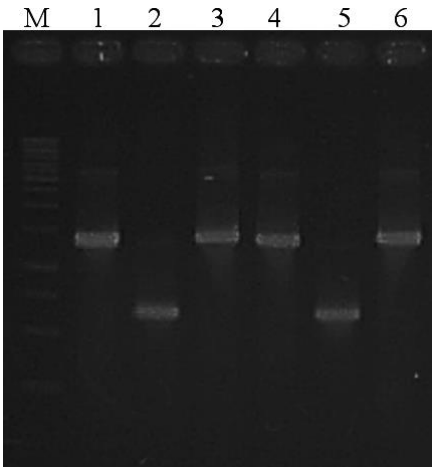
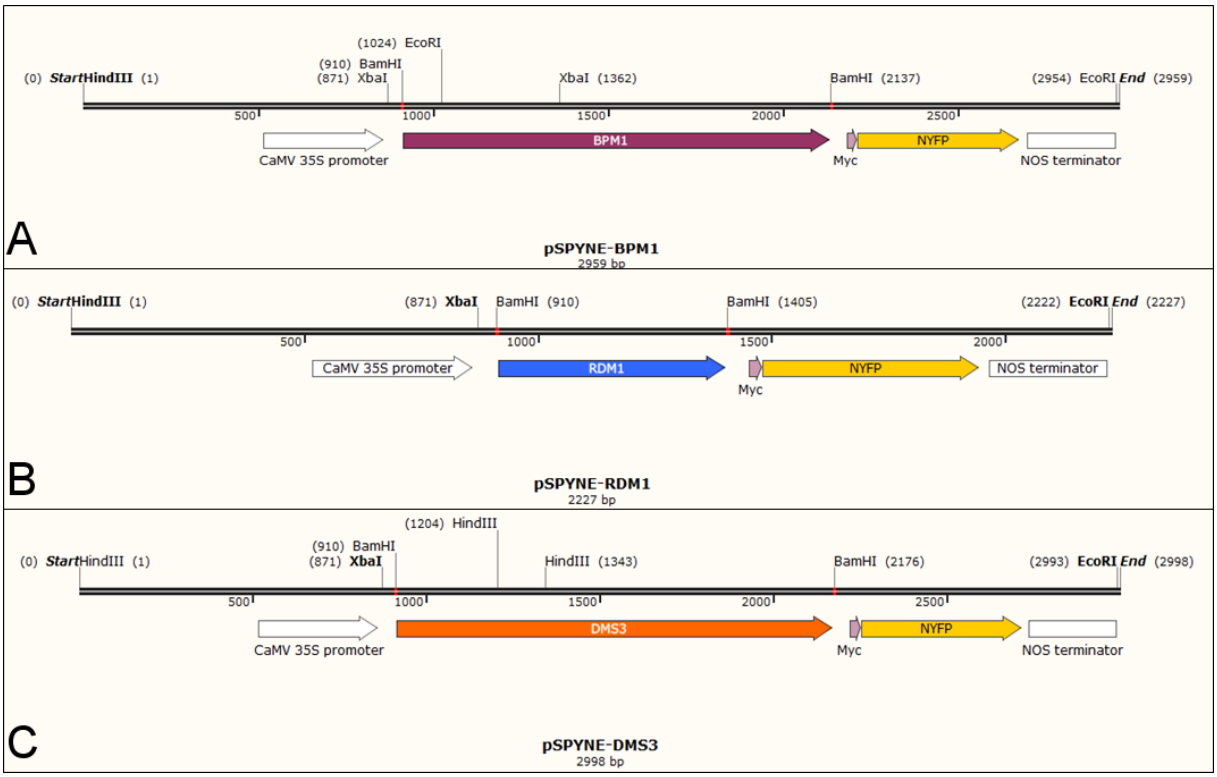


Figure 8. PCR analysis of plasmid constructs using a combination of gene-specific reverse and plasmid-specific (35S-3) primers (Table 1). PCR mixtures (5 µL each) were loaded on 1% agarose gel. M – GeneRuler™ 1kb DNA ladder (ThermoScientific); 1 – pSPYNE-BPM1 (1378 bp); 2 – pSPYNE-RDM1 (646 bp); 3 – pSPYNE-DMS3 (1417 bp); 4 – pSPYCE-BPM1 (1378 bp); 5 – pSPYCE-RDM1 (646 bp); 6 – pSPYCE-DMS3 (1417 bp).

As a final mean of verification, plasmid constructs were submitted for sequencing. After analysis of sequences via SnapGene software, it was determined that all plasmid constructs contained the appropriate gene sequence, as well as fusion tags, within the appropriate reading frame. Thus, 6 plasmid constructs (pSPYNE-BPM1, pSPYCE-BPM1, pSPYNE-DMS3, pSPYCE-DMS3, pSPYNE-RDM1 and pSPYCE-RDM1) were successfully generated (Fig. 9).



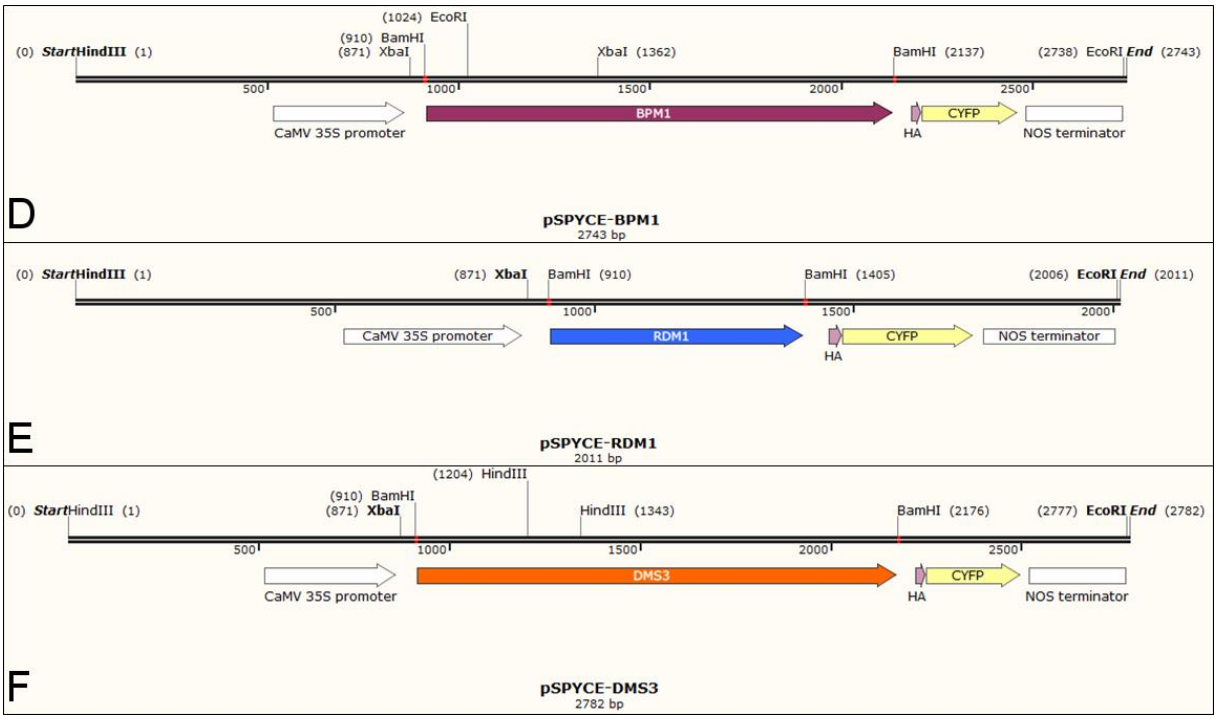


Figure 9. Expression cassette maps of plant-compatible BiFC vectors (pSPYNE and pSPYCE) carrying *BPM1*, *DMS3* and *RDM1* gene inserts. A – pSPYNE-BPM1; B – pSPYNE-RDM1; C – pSPYNE-DMS3; D – pSPYCE-BPM1; E – pSPYCE-RDM1; F – pSPYCE-DMS3. Gene inserts are under control of strong constitutive 35S promoter and contain C-terminal fusion tags. In pSPYNE constructs, c-myc affinity tag and N-terminal fragment of YFP are fused to gene inserts, while HA affinity tag and C-terminal fragment of YFP are fused to gene inserts in pSPYCE constructs. Gene inserts are bordered by BamHI restriction sites.

3.2 Regeneration of *A. tumefaciens* for agroinfiltration

To transiently transform *N. benthamiana* plants via agroinfiltration, 10 different *A. tumeaficens* clones were first established. Besides 6 clones which carried plasmid constructs generated in this study (pSPYNE-BPM1, pSPYCE-BPM1, pSPYNE-DMS3, pSPYCE-DMS3, pSPYNE-RDM1 and pSPYCE-RDM1), clones carrying pB7WGF-GFP, pCB301-p19, and empty pSPYNE and pSPYCE vectors were also established. Electrocompetent *A. tumefaciens* were transformed with 50 ng of appropriate plasmid via electroporation and selected on LB agar plates supplemented with respective antibiotics (Table 5). After 3 days of incubation at 28 °C, dozens of bacterial colonies developed. Single colony was picked and cultured overnight in liquid LB medium at 28 °C supplemented with appropriate antibiotics. A portion of each bacteria suspension was denatured at 95 °C and used as a template in PCR. Plasmid specific primers BiFC_BY2fw and BiFC_BY2rev were used (Table 1). As expected, plasmid constructs were detected in transformed agrobacteria (Fig. 10).

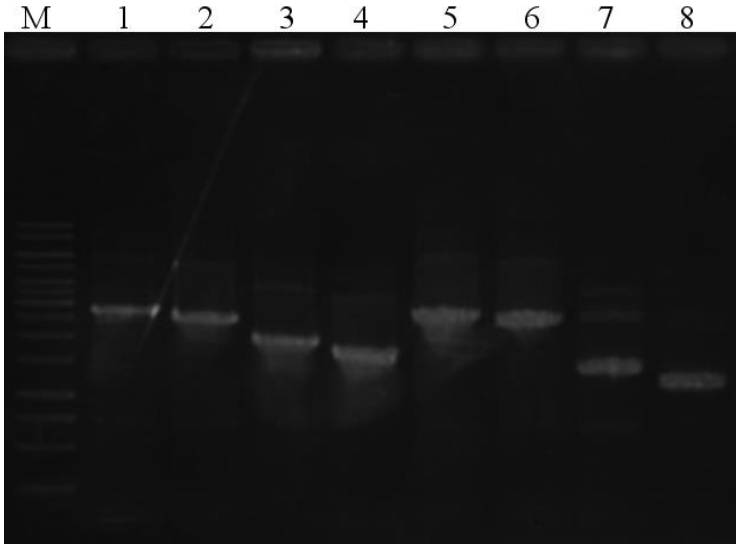


Figure 10. Screening of agrobacteria suspensions via PCR. Presence of plasmid constructs was detected by PCR and 1% agarose gel electrophoresis. Agrobacteria suspensions were used as templates for amplification of transgenes with plasmid-specific primers (BiFC_BY2fw and BiFC_BY2rev) (Table 1). M – GeneRuler™ 1kb DNA ladder (ThermoScientific); 1 – pSPYNE-BPM1 (2643 bp); 2 – pSPYCE-BPM1 (2427 bp); 3 – pSPYNE-RDM1 (1911 bp); 4 – pSPYCE-RDM1 (1695 bp); 5 – pSPYNE-DMS3 (2682 bp); 6 – pSPYCE-DMS3 (2466 bp); 7 – pSPYNE (1416 bp); 8 – pSPYCE (1200 bp).

3.3 Agroinfiltration of *N. benthamiana*

Each *A. tumefaciens* colony was cultured overnight at 28° C in liquid LB medium supplemented with appropriate antibiotics. Appropriate agroinfiltration mixtures with desired combinations

of *A. tumefaciens* clones were prepared (Table 6). Mixtures were injected into *N. benthamiana* leaves. Each plant was infiltrated with an exact mixture (2-3 leaves per plant). Leaf epidermis of agroinfiltrated plants was subjected to fluorescence microscopy during the following days. Overall, strongest fluorescence signals were observed 2-3 days after the agroinfiltration.

To determine the efficacy of transformation via agroinfiltration, *N. benthamiana* leaves were infiltrated with *A. tumefaciens* which carried pB7WGF-GFP (Fig. 11A). Strong fluorescence signal was observed with a high occurrence in cells, indicating a satisfactory transformation rate.

As a negative control for BiFC analysis, *N. benthamiana* co-transformed with empty pSPYNE and pSPYCE was used. As expected, no fluorescence signal was observed in such plants.

As a positive control for BiFC analysis, *N. benthamiana* was co-transformed with pSPYNE-DMS3 and pSPYCE-RDM1 (Fig. 11B) as well as with pSPYNE-DMS3 and pSPYCE-DMS3 (Fig. 11C) constructs. As expected, fluorescence signal was detected in both cases, proving *in vivo* DMS3-RDM1 interaction and DMS3 dimerization in *N. benthamiana* leaf epidermal cells. In analysis of DMS3-RDM1 interaction, fluorescence signal was mostly concentrated in the nuclei, although it was also present in the cytoplasm. In analysis of DMS3 dimerization, intense fluorescence was concentrated in certain areas of cytoplasm, while pale fluorescence was also present in the nuclei.

In explants agroinfiltrated with pSPYNE-DMS3 and pSPYCE-BPM1constructs, fluorescence signal was detected, indicating the existence of interaction between BPM1 and DMS3 (Fig. 11D). Cells which exhibited fluorescence were scarce. Signal was equally distributed in the nuclei and cytoplasm.

In any of the three performed agroinfiltration experiments, interaction between BPM1 and RDM1 was not detected.

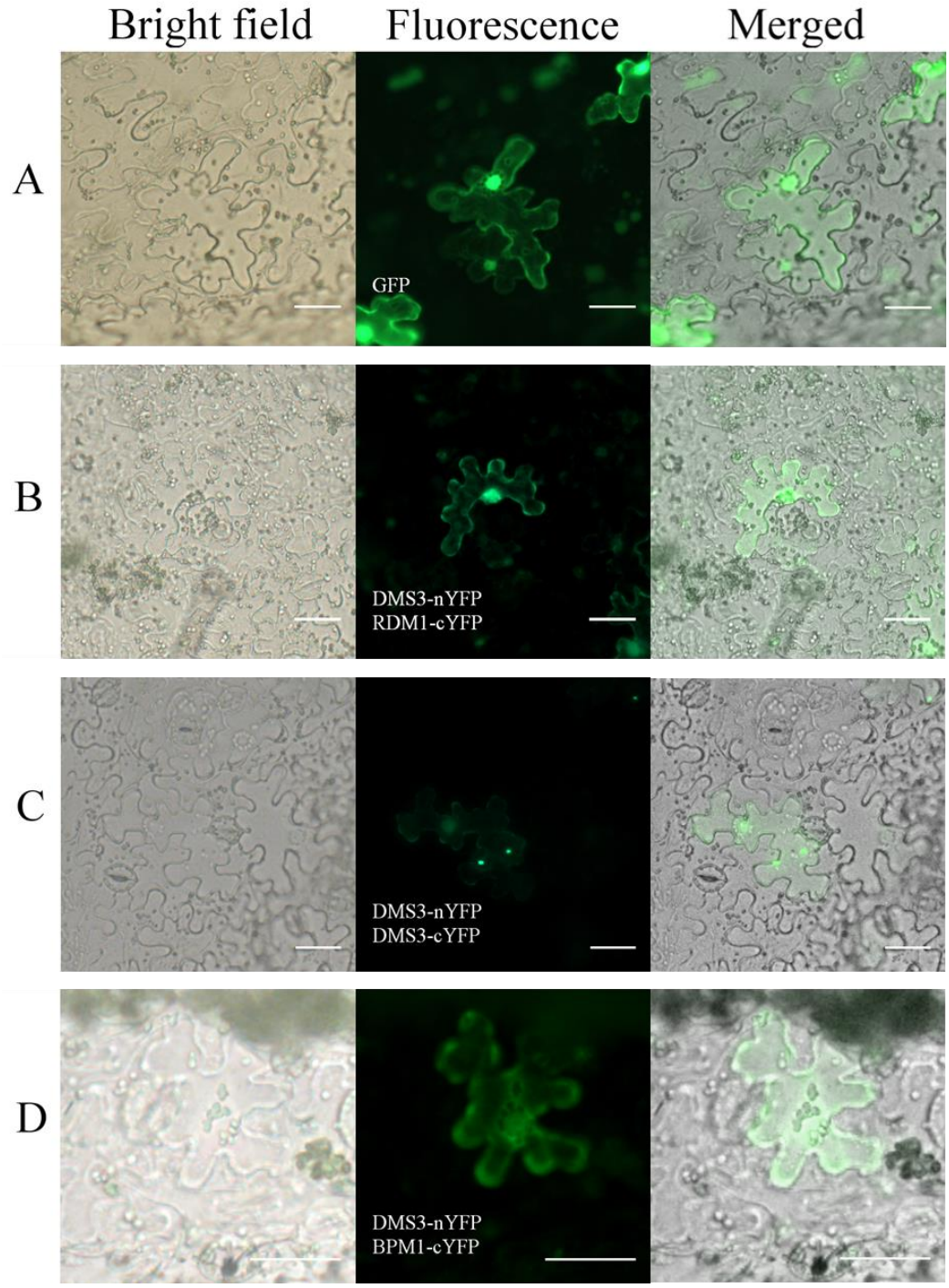


Figure 11. BiFC fluorescence of transiently transformed *N. benthamiana* leaf epidermal cells. Leaves were agroinfiltrated with: A – pB7WGF-GFP; B – pSPYNE-DMS3 and pSPYCE-RDM1; C – pSPYNE-DMS3 and pSPYCE-DMS3; D – pSPYNE-DMS3 and pSPYCE-BPM1. Scale bars = 50 μm.

3.4 Microparticle bombardment of *A. cepa*

Gold particles coated with desired combinations of plasmids (Table 7) were prepared. *A. cepa* epidermis was bombarded with the prepared DNA-coated particles. The epidermis samples

were subjected to fluorescence microscopy during the following 2 days. Strongest fluorescence was observed 2 days after the bombardment.

To check the efficacy of *A. cepa* transformation via microparticle bombardment, pB7WGF-GFP (Fig. 12A) was used.

As a negative control for BiFC analysis, *A. cepa* epidermis was bombarded with particles coated with empty pSPYNE and pSPYCE plasmids. Fluorescence signal was not observed.

To determine the efficacy of BiFC analysis in *A. cepa*, epidermis samples were bombarded with particles coated with pSPYNE-DMS3 and pSPYCE-RDM1 (Fig. 12B) as well as with pSPYNE-DMS3 and pSPYCE-DMS3 (Fig. 12C). Fluorescence signal was detected in both types of positive control, proving *in vivo* DMS3-RDM1 interaction and DMS3 dimerization in *A. cepa* epidermal cells. Fluorescence signal was most intense in the nuclei, while also being present in the cytoplasm. Cells which exhibited fluorescence signal were scarce.

Interaction between BPM1 and DMS3 as well as interaction between BPM1 and RDM1 were not detected in *A. cepa* epidermal in any of the 4 performed experiments.

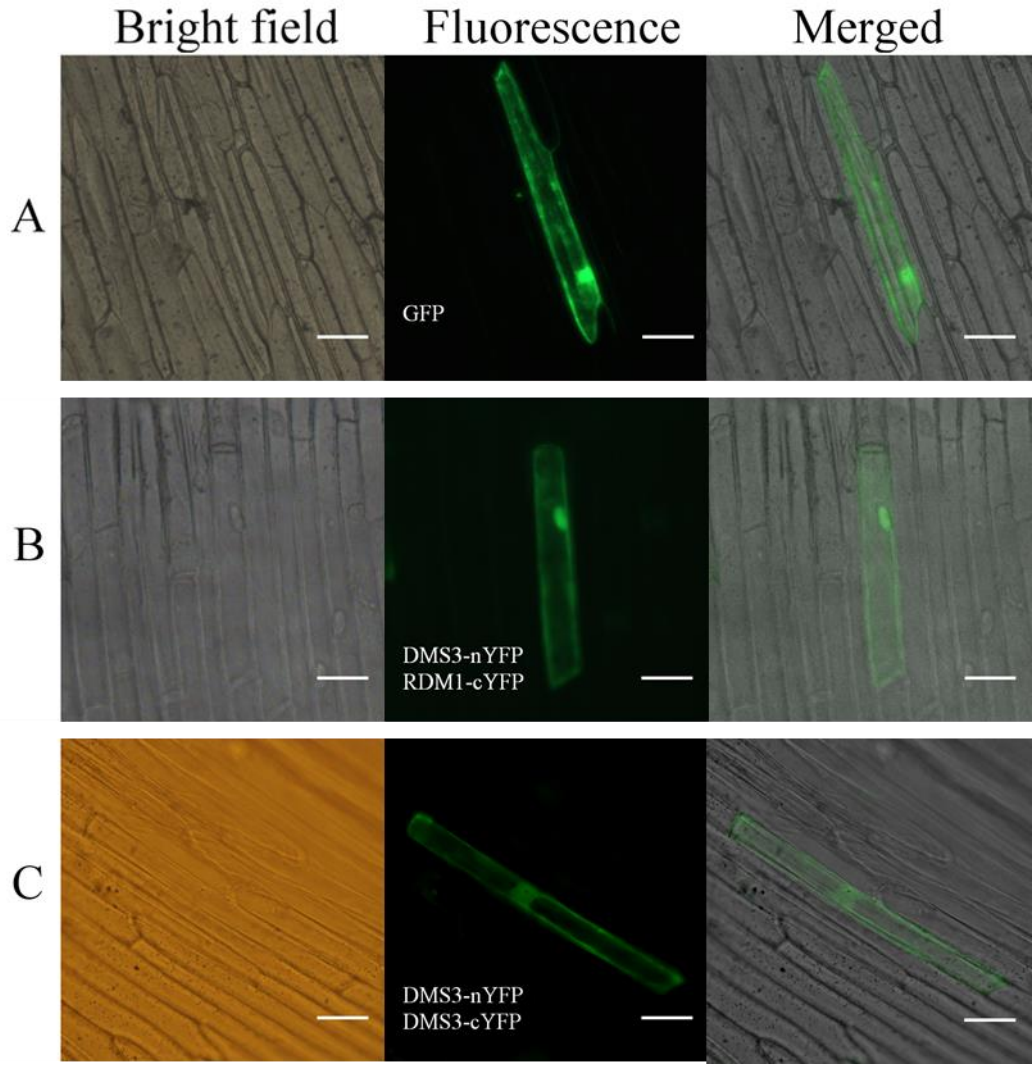


Figure 12. BiFC fluorescence of transiently transformed *A. cepa* epidermal cells. Cells were bombarded with gold particles carrying: A – pB7WGF-GFP; B – pSPYNE-DMS3 and pSPYCE-RDM1; C – pSPYNE-DMS3 and pSPYCE-DMS3. Scale bars = 50 μm.

3.5 Microparticle bombardment of BY-2 cells

Same gold particles prepared for microparticle bombardment of *A. cepa* were used for the bombardment of BY-2 cells. After biolistic transformation, BY-2 cells were observed via fluorescence microscopy during the following 2 days. Stronger fluorescence signal was observed one day after the bombardment procedure.

To determine the efficacy of BY-2 cell transformation via microparticle bombardment, BY-2 cells were bombarded with gold particles coated with pB7WGF-GFP (Fig. 13A). Strong GFP signal was observed in the cytoplasm and in the nuclei.

BY-2 co-transformed with empty pSPYNE and pSPYCE plasmids were used as a negative control for BiFC analysis and as expected, no fluorescence signal was detected.

As a positive control, BY-2 cells were co-transformed with pSPYNE-DMS3 and pSPYCE-RDM1 as well as with pSPYNE-DMS3 and pSPYCE-DMS3. DMS3 – RDM1 interaction was detected (Fig. 13B), although cells which exhibited fluorescence were scarce. Fluorescence signal was present throughout the cytoplasm and in the nuclei. DMS3 dimerization was not detected in BY-2 cells.

Interaction between BPM1 and DMS3 (Fig. 13C) was detected in BY-2 cells via BiFC analysis. Fluorescent cells were scarce. Signal was most intense in the nuclei, while a weak signal was also observable in the cytoplasm.

After performing the experiment 3 times, BPM1-RDM1 interaction was not detected in BY-2 cells.

Additionally, with the aim to improve transformation efficiency, BY-2 cells were also bombarded with gold particles coated with PCR-products that contained expression cassettes (amplified with BIFC_BY2_fw and BIFC_BY2_rev primers) of appropriate recombinant gene, but no fluorescent cell was detected.

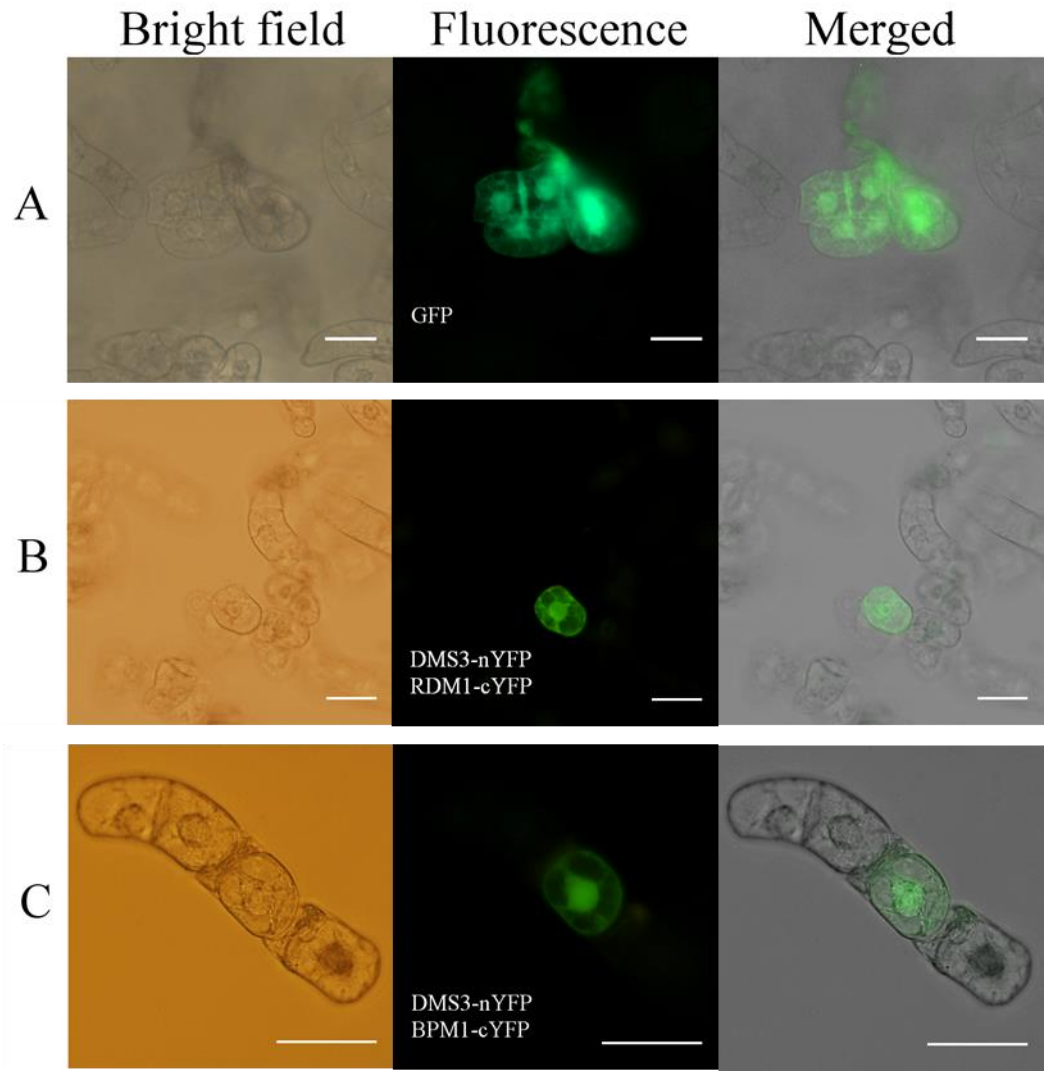


Figure 13. BiFC fluorescence of transiently transformed BY-2 cells. Cells were bombarded with gold particles carrying: A – pB7WGF-GFP; B – pSPYNE-DMS3 and pSPYCE-RDM1; C – pSPYNE-DMS3 and pSPYCE-BPM1. Scale bars = 50 μm.

4 DISCUSSION

4.1 BiFC establishment

BiFC method was first established by Hu et al. (2002) for determination of the locations of protein interactions in living mammalian cells. To enable BiFC analyses in plants, Walter et al. (2004) generated complementary sets of expression vectors which enable protein interaction studies in transiently or stably transformed plant cells. One of the aims of this work was to establish BiFC method in Laboratory of molecular plant biology, Faculty of Science. To check the efficacy of transformation methods performed in this study, pB7WGF-GFP was used. Transformation rate was higher when agroinfiltration was performed compared to microparticle bombardment, and GFP fluorescence was observable during longer time period after transformation. Based on the successful detection of interaction among DMS3 and RDM1, the efficacy of BiFC method in all three expression systems was confirmed. Both DMS3 and RDM1 proteins are important components of the RdDM pathway (Matzke et al., 2014) known to interact with each other and to form homodimers (Sasaki et al., 2014). Fluorescence signal produced by DMS3 and RDM1 interaction was most intense in the plant cell nucleus, a compartment where RdDM occurs. These results additionally confirm that BiFC analysis can indeed be a useful tool in studying subcellular localization of protein interactions. The pale fluorescence signal in the cytoplasm was probably caused by overexpression of studied proteins, considering that they were under control of 35S promoter. Interestingly, the occurrence of detection of DMS3 dimerization was significantly lower compared to detection of interaction between DMS3 and RDM1. This suggests that DMS3 dimers are less stable or their formation occurs less frequently. DMS3 dimerization was not observed in BY-2 cells, either due to unsatisfactory co-transformation rates or such complexes do not form in this cell type or in dividing cells. Since no fluorescence signal was detected in any of the three plant cell types co-transformed with empty pSPYNE and pSPYCE vectors the reliability of BiFC analysis was shown and thereby I can conclude that this method was successfully established. The rate of observable fluorescent cells in BiFC positive controls was much lower in plant samples transformed via Biolistic compared to plants transformed via agroinfiltration. This might be caused by the usage of suboptimal concentrations of plasmid constructs when preparing gold particles for bombardment, as I had problems in achieving highly concentrated samples of plasmid DNA that are required for this transformation method. Usage of different bacteria

strains for vector cloning or different plasmid DNA isolation methods might be helpful in future experiments at achieving desirable plasmid concentrations. Transformation via microparticle bombardment is more efficient when linear DNA is used compared to circular DNA (Glick et al., 2009). Using PCR-products that contain expression cassettes of appropriate recombinant genes might improve efficiency of BiFC analysis. However, when this approach was used here, no fluorescence signal was detected in bombarded cells and was abandoned after fluorescence signal was successfully observed in cells transformed with circular DNA.

Two key steps that affect the efficiency of BiFC analysis in plants transformed via agroinfiltration are proper induction of *A. tumefaciens* by acetosyringone and inclusion of *A. tumefaciens* that harbor the pCB301-p19 plasmid in the infiltration mixtures. Acetosyringone is a phenolic compound that induces transcription of virulence (*vir*) genes in *A. tumefaciens*. *Vir* genes are necessary for T-DNA transfer and subsequent transformation of plant cells. For successful detection of protein-protein interaction via BiFC, at least two different T-DNAs must be transferred into the same plant cell. One from agrobacterium carrying recombinant pSPYNE plasmid, and another T-DNA from agrobacterium carrying recombinant pSPYCE plasmid. This double T-DNA transformation has a significantly lower rate of occurrence compared to single T-DNA transformation (as in control transformation with pB7WGF-GFP). For additional signal enhancement, p19 protein that functions as a suppressor of post-transcriptional gene silencing in plants and significantly enhances transient expression of transgenes in *N. benthamiana* (Win and Kamoun, 2004) was used. Therefore, 3 different T-DNAs have to be transferred into the plant cell to achieve optimal fluorescence in BiFC analysis.

4.2 BPM1 interacts with DMS3

Recent studies found DMS3 and RDM1 to be potential interaction partners of BPM1 (unpublished). In accordance with these findings, interaction between BPM1 and DMS3 was detected via BiFC in this work, albeit in very low number of cells. Here I must mention that fluorescence signal was only observed in plant cells which were transformed with pSPYNE-DMS3 and pSPYCE-BPM1 constructs. In plant cells transformed with the opposite combination (pSPYNE-BPM1 and pSPYCE-DMS3), no fluorescence signal was detected. A fused protein might interfere with the ability of N-YFP and C-YFP fragments to assemble, and this might be the case in BPM1-DMS3 interaction. This shows that usage of both combinations of constructs is crucial when studying protein interactions via BiFC analysis. Here, the

interaction between BPM1 and RDM1 was not detected in any of the three expression systems, indicating that BPM1 protein either does not interact with RDM1 in plant cells used in this research, or complexes formed by their interaction are not stable enough to produce observable fluorescence signal. Since low transformation rate was achieved in my experiments after biolistic transformation, additional BiFC experiments should be performed in future studies before ruling out the existence of RDM1-BPM1 interaction. Recently, BPM proteins are described to interact with DREB2A transcription factor and are involved in DREB2A ubiquitylation by CUL3 E3 ligase (Morimoto et al., 2017). There, interaction between BPM proteins and DREB2A is confirmed via co-purification and yeast two hybrid assays, but fluorescence signal is not observed when BPM proteins and DREB2A are subjected to BiFC. Morimoto et al. (2017) suggest that upon formation of BPM-DREB2A complex, both proteins may degrade, hindering the observation of the BiFC interaction signal. To overcome this problem, they generate BPM proteins which lack the BTB domain. Such truncated BPM proteins are unable to interact with CUL3 and as such cannot facilitate ubiquitylation of DREB2A. After BTB domain deletion, interaction between truncated BPM2 and DREB2A is clearly observed via BiFC (Morimoto et al., 2017). Similar approach could be conducted in the follow-up of this work. First it will be necessary to find out which BPM1 domain is responsible for interaction with DMS3 or RDM1. This could be discovered by performing yeast two hybrid assays with truncated forms of BPM1. Alternatively, to overcome the problem of rapid degradation of BPM complexes, *cul3* mutants could be used as expression systems for BiFC, or plant samples could be treated with 26S proteasome inhibitors prior to microscopy. These approaches may prove effective regardless which domain (MATH or BTB) of BPM1 interacts with DMS3.

4.3 BPM1 activity may affect *de novo* DNA methylation

It is not clear how does BPM1 affect RdDM pathway through interaction with DMS3. Both DMS3 and RDM1 are key components of RdDM considering that plants defective in their genes experience significant loss of *de novo* methylation (Matzke et al., 2015). This implies that BPM1 may have a considerable role in regulation of the RdDM pathway. BPM proteins are known to interact with transcription factors by interfering with their activity and ultimately targeting them for degradation via 26S proteasome (Lechner et al., 2011; Chen et al., 2013; 2015; Morimoto et al., 2017). It is possible that BPM1 affects DMS3 in the same manner. In this scenario, BPM1 would be a negative regulator of RdDM and its activity would probably

lead to the reduction of cytosine methylation in loci targeted by *de novo* methylation in plants. The other possibility is that BPM1 guides the RdDM complex towards the loci occupied by transcription factors, thus functioning as a mediator between Pol II-based transcription and Pol V-based *de novo* methylation in plant cells. Such activity might lead to an increase in cytosine methylation in regions that surround the sites occupied by BPM-related transcription factors. BPM proteins and RdDM pathway are both linked to abiotic stress response in plants. BPM proteins control transcription factors that regulate stress-inducible genes, such as DREB2A (Morimoto et al., 2017). While response to abiotic stress increases the chance of plant survival, it hinders the long-term plant growth. Upon destabilization of transcription factors that induce transcription of stress-related genes, BPM proteins may additionally guide the RdDM machinery towards the stress-related genes, further silencing their transcriptional activity. Methylation and transcription studies of regions that surround stress-related genes that are regulated by activity of BPM proteins might shed some new light on the connection between BPM proteins and RdDM.

5 CONCLUSION

BiFC analysis method in epidermal *N.benthamiana* cells, onion epidermal cells and tobacco BY-2 cells was established in the Laboratory of molecular plant biology, Faculty of Science.

In vivo interaction between DMS3 and RDM1 was confirmed in epidermal *N.benthamiana* cells after agroinfiltration, and in onion epidermal cells and tobacco BY-2 cells after biolistic transformation.

In vivo dimerization of DMS3 was confirmed in epidermal *N.benthamiana* cells after agroinfiltration, and in onion epidermal cells after biolistic transformation.

Interaction between BPM1 and DMS3 was detected via BiFC analysis in epidermal *N.benthamiana* cells after agroinfiltration, and in tobacco BY-2 cells after transformation via biolistic.

Interaction between BPM1 and RDM1 was not detected via BiFC analysis in this study.

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