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The Diverse Salt-Stress Response of Arabidopsis *ctr1-1* and *ein2-1* Ethylene Signaling Mutants is Linked to Altered Root Auxin Homeostasis

Irina I. Vaseva [1,*], Kiril Mishev [1], Thomas Depaepe [2], Valya Vassileva [1] and Dominique Van Der Straeten [2]

[1] Department of Molecular Biology and Genetics, Institute of Plant Physiology and Genetics, Bulgarian Academy of Sciences, Acad. Georgi Bonchev Str., Bldg. 21, 1113 Sofia, Bulgaria; mishev@bio21.bas.bg (K.M.); valyavassileva@bio21.bas.bg (V.V.)

[2] Laboratory of Functional Plant Biology, Department of Biology, Ghent University, K.L. Ledeganckststraat 35, B-9000 Ghent, Belgium; thdpaepe.Depaepe@UGent.be (T.D.); Dominique.VanDerStraeten@UGent.be (D.V.D.S.)

* Correspondence: vaseva@bio21.bas.bg or irina.vaseva@abv.bg

Abstract: We explored the interplay between ethylene signals and the auxin pool in roots exposed to high salinity using *Arabidopsis thaliana* wild-type plants (Col-0), and the ethylene-signaling mutants *ctr1-1* (constitutive) and *ein2-1* (insensitive). The negative effect of salt stress was less pronounced in *ctr1-1* individuals, which was concomitant with augmented auxin signaling both in the *ctr1-1* controls and after 100 mM NaCl treatment. The R2D2 auxin sensorallowed mapping this active auxin increase to the root epidermal cells in the late Cell Division (CDZ) and Transition Zone (TZ). In contrast, the ethylene-insensitive *ein2-1* plants appeared depleted in active auxins. The involvement of ethylene/auxin crosstalk in the salt stress response was evaluated by introducing auxin reporters for local biosynthesis (*pTAR2::GUS*) and polar transport (*pLAX3::GUS*, *pAUX1::AUX1-YFP*, *pPIN1::PIN1-GFP*, *pPIN2::PIN2-GFP*, *pPIN3::GUS*) in the mutants. The constantly operating ethylene-signaling pathway in *ctr1-1* was linked to increased auxin biosynthesis. This was accompanied by a steady expression of the auxin transporters evaluated by qRT-PCR and crosses with the auxin transport reporters. The results imply that the ability of *ctr1-1* mutant to tolerate high salinity could be related to the altered ethylene/auxin regulatory loop manifested by a stabilized local auxin biosynthesis and transport.

Keywords: Arabidopsis *ctr1-1* and *ein2-1* mutants; auxin homeostasis; ethylene signals; crosstalk; salt stress

Citation: Vaseva, I.I.; Mishev, K.; Depaepe, T.; Vassileva, V.; Van Der Straeten, D. The Diverse Salt-Stress Response of Arabidopsis *ctr1-1* and *ein2-1* Ethylene Signaling Mutants is Linked to Altered Root Auxin Homeostasis. *Plants* **2021**, *10*, 452. https://doi.org/10.3390/plants10030452

Academic Editor: Yuichi Tada

Received: 30 January 2021
Accepted: 24 February 2021
Published: 27 February 2021

1. Introduction

Saline soils present a serious agricultural constraint especially in coastal areas and in regions with industrial pollution or intensive plant breeding. Moderate salt stress could remain undetected since it causes no apparent injuries other than restricted growth. This means that high salinity has a negative effect on the signaling cascades involved in the regulation of plant growth and development. The growth restriction caused by high salt concentrations is further complicated by impaired photosynthesis which ultimately leads to accelerated aging and death [1,2].

Plants adapt to environmental challenges through anatomical, metabolic, and morphological changes and the gaseous plant hormone ethylene modulates many of these growth-related processes. As a major stress hormone, ethylene causes growth reduction primarily due to the inhibition of cell expansion which is an adaptive response to the adverse environment [3,4]. Several studies have demonstrated that plants exposed to salt stress show induced ethylene biosynthesis and have enhanced ethylene signaling maintaining both shoot and primary root growth [5–10]. Some ethylene mutations, which

of 60–95 °C in 0.2 °C increment for 60 s. The relative expression of the target genes was calculated using the $2^{-\Delta\Delta Cq}$ method [71] with two reference genes (*At3g18780*, *At5g60390*) for normalization of the relative quantification. Primers used in the qRT-PCRs are provided in Table S3 (Suppl. Materials).

4.6. Statistical Analyses

The data in Figures 1 and 4 were obtained from at least three independent experiments. Plants from three independent crosses with the respective reporters were analyzed to form three independent datasets. Each dataset contain measurements of at least 10 (up to 20) control or salt-treated individuals from the tested lines.

The assumed differences among the tested genotypes at control and salt stress conditions were analyzed by Student's *t*-test (Figure 1) and one-way ANOVA (Figures 2–6) using Excel software. Error bars on the graphs indicate standard deviation (SD) and the values were considered statistically significant at $p \leq 0.05$.

5. Conclusions

Plant susceptibility/tolerance to salt stress is defined by multiple stress-responsive genes controlled by various signal transduction pathways. Our results demonstrate that ethylene signaling could also be engaged in salt stress response through the regulation of local auxin availability. This is sustained by the relative salt tolerance of *ctr1-1* mutation which is characterized by an altered ethylene/auxin regulatory loop leading to stabilized local auxin biosynthesis and polar transport. The salt hypersensitivity of the ethylene-insensitive mutant *ein2-1* could be due to the chronic root auxin deficiency.

Supplementary Materials: The following are available online at https://www.mdpi.com/2223-7747/10/3/452/s1, Table S1: TF DeCON in silico screen genes from the auxin Trp-dependent biosynthesis: *TAA1 (At1g70560), TAR1 (At1g23320), TAR2 (At4g24670)* and *YUC1-11* (resp. *At4g32540*, *At4g13260*, *At1g04610*, *At5g11320*, *At5g43890*, *At5g25620*, *At2g33230*, *At4g28720*, *At1g04180*, *At1g48910*, *At1g21430*), and Table S2: TF DeCON in silico screen of the auxin transport genes *PIN1, PIN2, PIN3, PIN4, PIN5, PIN6, PIN7, PIN8 (At1g73590, At5g57090, At1g70940, At2g01420, At5g16530, At1g77110, At1g23080, At5g15100), AUX1 (At2g38120), LAX1 (At5g01240), LAX2 (At2g21050), LAX3 (At1g77690)* and *ABCB1 (At2g36910), ABCB4 (At2g47000), ABCB19 (At3g28860)* using DAP-Seq data from the Ecker lab (Salk Institute for Biological Studies); Table S3: Primers used for qRT-PCR analyses; Figure S1: AthaMap analyses of promoter regions of the genes coding for *TAA1, TAR1, TAR2, and YUC (1-11)* genes.

Author Contributions: Conceptualization, I.I.V. and D.V.D.S.; methodology, I.I.V., K.M. and T.D.; investigation, I.I.V., K.M., T.D.; resources, V.V., D.V.D.S.; writing—original draft preparation, I.I.V.; writing—review and editing, K.M., T.D., V.V. and D.V.D.S.; visualization, I.I.V., K.M. and T.D.; funding acquisition, V.V., I.I.V. and D.V.D.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by a joint research project "Cell type specificity of ethylene signaling in plant responses to abiotic stress" between the Bulgarian Academy of Sciences (BAS) and the Research Foundation – Flanders (FWO), Belgium, grant number VS.078.18N.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Acknowledgments: The support by the Euro-BioImaging Node in Sofia, Bulgaria (https://www.eurobioimaging.eu, PID: 336) which provided the confocal microscope facility is highly appreciated. DVDS gratefully acknowledges the Research Foundation Flanders (Projects G.0298.09 and G.0656.13N) and Ghent University (BOF-BAS 01B02112) for financial support.

Conflicts of Interest: The authors declare no conflict of interest.

Oleg Georgiev, Kiril Mishev, Maria Krasnikova[a], Meglena Kitanova, Anna Dimitrova* and Luchezar Karagyozov

The *Hordeum bulbosum* 25S-18S rDNA region: comparison with *Hordeum vulgare* and other Triticeae

https://doi.org/10.1515/znc-2018-0109
Received July 19, 2018; revised July 4, 2019; accepted July 18, 2019

Abstract: *Hordeum vulgare* and *Hordeum bulbosum* are two closely related barley species, which share a common H genome. *H. vulgare* has two nucleolar organizer regions (NORs), while the NOR of *H. bulbosum* is only one. We sequenced the 2.5 kb 25S-18S region in the rDNA of *H. bulbosum* and compared it to the same region in *H. vulgare* as well as to the other Triticeae. The region includes an intergenic spacer (IGS) with a number of subrepeats, a promoter, and an external transcribed spacer (5′ETS). The IGS of *H. bulbosum* downstream of 25S rRNA contains two 143-bp repeats and six 128-bp repeats. In contrast, the IGS in *H. vulgare* contains an array of seven 79-bp repeats and a varying number of 135-bp repeats. The 135-bp repeats in *H. vulgare* and the 128-bp repeats in *H. bulbosum* show similarity. Compared to *H. vulgare*, the 5′ETS of *H. bulbosum* is shorter. Additionally, the 5′ETS regions in *H. bulbosum* and *H. vulgare* diverged faster than in other Triticeae genera. Alignment of the Triticeae promoter sequences suggests that in *Hordeum*, as in diploid *Triticum*, transcription starts with guanine and not with adenine as it is in many other plants.

Keywords: external transcribed spacer; *Hordeum*; intergenic spacer; promoter; rDNA.

[a]**Permanent address:** A.N. Belozersky Institute of Physico-Chemical Biology, Lomonosov Moscow State University, Moscow 119992, Russia
***Corresponding author: Anna Dimitrova,** Institute of Plant Physiology and Genetics, Bulgarian Academy of Sciences, Acad. G. Bonchev Str., Bl. 21, 1113 Sofia, Bulgaria, Phone: +359 2 9792677, Fax: +359 2 9785516, E-mail: annadimitrova0@gmail.com, annad@bas.bg
Oleg Georgiev: Institute of Molecular Life Sciences, University Zurich-Irchel, Winterthurer Str. 190, CH-8057 Zurich, Switzerland
Kiril Mishev: Institute of Plant Physiology and Genetics, Bulgarian Academy of Sciences, Acad. G. Bonchev Str., Bl. 21, 1113 Sofia, Bulgaria
Maria Krasnikova, Meglena Kitanova and Luchezar Karagyozov: Department of Genetics, Faculty of Biology, St. Kl. Ohridsky University of Sofia, 8 Dragan Tsankov bld., 1164 Sofia, Bulgaria

1 Introduction

In eukaryotes, the 18S, 5.8S, and 25S-28S ribosomal RNAs (rRNAs) are generated by the processing of a larger 35–45S rRNA precursor (pre-rRNA), synthesized from a single transcription unit. The processing of the pre-rRNA involves excision of the external (5′external transcribed spacer (ETS) and 3′ETS) and the internal transcribed spacers (ITS1 and ITS2) [1]. In plants, there are several hundreds to several thousands of nuclear rRNA genes, repeated head-to-tail in one or more chromosomes [2]. The pre-rRNA transcription units are separated by noncoding intergenic spacers (IGS), which in plants, as a rule, are shorter than in animals [3]. Sequences in the IGS may have defined functions. Transcription initiation and termination are characterized in many plants (e.g. [4, 5]). The rRNA gene promoter is part of the IGS. It is studied in the model plant *Arabidopsis* both by transient expression and in vitro [6, 7]. Typically, the IGS regions contain a variety of repetitive elements. Some repeated IGS sequences were shown to be involved in transcription enhancement [8]. The origin of replication [9] and replication fork barrier [10] were also demonstrated to reside in the IGS. Unavoidably, the search for a function within the IGS region, either by comparison with other sequences or directly, begins with rDNA sequencing.

The tribe Triticae of the grass family Poaceae is composed of about 360 species classified into 20–30 genera. Rye and the oldest domesticated plants, wheat and barley, as well as their wild relatives and some important forage grasses, are among the species that belong to that tribe [11, 12]. The genus *Hordeum* is composed of 33 species, subdivided into two subgenera (listed in Ref. [13]). *Hordeum* ITS sequences, chloroplast sequences, and single nuclear loci were used to construct comprehensive phylogenetic trees [13–15]. The most studied *Hordeum* species is the cultivated barley *Hordeum vulgare* L. *H. vulgare* has two nucleolar organizer regions (NORs), one on chromosome 5H and the other on chromosome 6H [16]. As a rule, each *H. vulgare* plant has two rDNA repeat length variants: a short variant on NOR 5H, and

nature chemical biology

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ARTICLES

https://doi.org/10.1038/s41589-019-0262-1

Disruption of endocytosis through chemical inhibition of clathrin heavy chain function

Wim Dejonghe[1,2,16,17], Isha Sharma[1,2,17], Bram Denoo[3], Steven De Munck[4,5], Qing Lu[1,2], Kiril Mishev[1,2,6], Haydar Bulut[7], Evelien Mylle[1,2], Riet De Rycke[1,2,8], Mina Vasileva[9], Daniel V. Savatin[1,2], Wim Nerinckx[10,11], An Staes[10,12], Andrzej Drozdzecki[13,14], Dominique Audenaert[13,14], Klaas Yperman[1,2], Annemieke Madder[3], Jiří Friml[9], Daniël Van Damme[1,2], Kris Gevaert[10,12], Volker Haucke[15], Savvas N. Savvides[4,5], Johan Winne[3] and Eugenia Russinova[1,2*]

Clathrin-mediated endocytosis (CME) is a highly conserved and essential cellular process in eukaryotic cells, but its dynamic and vital nature makes it challenging to study using classical genetics tools. In contrast, although small molecules can acutely and reversibly perturb CME, the few chemical CME inhibitors that have been applied to plants are either ineffective or show undesirable side effects. Here, we identify the previously described endosidin9 (ES9) as an inhibitor of clathrin heavy chain (CHC) function in both *Arabidopsis* and human cells through affinity-based target isolation, in vitro binding studies and X-ray crystallography. Moreover, we present a chemically improved ES9 analog, ES9-17, which lacks the undesirable side effects of ES9 while retaining the ability to target CHC. ES9 and ES9-17 have expanded the chemical toolbox used to probe CHC function, and present chemical scaffolds for further design of more specific and potent CHC inhibitors across different systems.

Clathrin-mediated endocytosis (CME) is a major route for internalization of plasma membrane proteins and molecules from the extracellular environment[1,2], but its dynamic and essential nature makes it difficult to dissect using classical genetics approaches. Chemical inhibitors of endocytosis are an attractive alternative to the current methods available for disrupting protein functions. However, despite the extensive structural and biochemical knowledge about CME in eukaryotic cells[3], the development of chemicals that interfere with this process is still at a relatively early stage. To date, a few small molecules have been shown to target the CME machinery in mammalian, yeast or plant systems[4]. Among the most commonly used small-molecule CME inhibitors in mammalian systems are Pitstop2 (ref. [5]), targeting the N-terminal domain (nTD) of the CHC, Dynasore[6] and the Dynasore-based series of small molecules called Dyngo[7], the latter pair affecting the dynamin function. A natural product, ikarugamycin, has recently been used to inhibit CME in different systems, but neither its potency nor specificity toward CME has been extensively examined[8]. As none of the above-mentioned molecules displayed consistent effects in plant cells, the plant cell biology has taken advantage of tyrphostin A23, a CME-inhibiting small molecule[9]. However, tyrphostin A23 has recently been described as a protonophore in *Arabidopsis thaliana*, and its inhibition of endocytosis was shown to occur through non-specific cytoplasmic acidification[9]. Therefore, CME research in plants would benefit from novel, potent small-molecule inhibitors that dissect endocytosis to improve our understanding of the many physiological processes that rely on it.

Previously, ES9 (**1**) was characterized as an endocytosis inhibitor in different model systems[9]. Although ES9 is a protonophore, its interference with CME did not originate solely from cytoplasmic acidification. In *Drosophila melanogaster*, ES9 blocked synaptic vesicle recycling, mimicking the phenotypes in mutants defective in clathrin or dynamin functions whereas in *Arabidopsis*, ES9 was found to retain its ability to inhibit endocytosis at an increased apoplastic pH[9]. These results suggested that, despite its protonophore activity, ES9 may directly interfere with proteins involved in CME. Here, we demonstrated that ES9 binds the nTD of the *Arabidopsis* clathrin heavy chain (CHC). Further structure activity relation (SAR) analysis identified a non-protonophoric ES9 analog with a similar mode of action, as confirmed by cellular thermal shift assay (CETSA)[10] and drug affinity-responsive target stability (DARTS)[11]. Altogether, we expand the current chemical toolbox for CME inhibition and present promising scaffolds for further development of chemical probes targeting CHC across different systems. For further information on ES9 and other agents featured in this article, see the sections at the end of the article titled Synthetic functions. In the main text, these agents are flagged as numerals within parentheses and are cross-linked to the appropriate section(s).

[1]Department of Plant Biotechnology and Bioinformatics, Ghent University, Ghent, Belgium. [2]Center for Plant Systems Biology, VIB, Ghent, Belgium. [3]Department of Organic and Macromolecular Chemistry, Ghent University, Ghent, Belgium. [4]Unit for Structural Biology, Department of Biochemistry and Microbiology, Ghent University, Ghent, Belgium. [5]Unit for Structural Biology, Center for Inflammation Research, VIB, Ghent, Belgium. [6]Institute of Plant Physiology and Genetics, Bulgarian Academy of Sciences, Sofia, Bulgaria. [7]Department of Biology, Chemistry and Pharmacy, Freie Universität Berlin, Berlin, Germany. [8]Ghent University Expertise Centre for Transmission Electron Microscopy and VIB BioImaging Core, Ghent, Belgium. [9]Institute of Science and Technology, Klosterneuburg, Austria. [10]Center for Medical Biotechnology, VIB, Ghent, Belgium. [11]Department of Biochemistry and Microbiology, Ghent University, Ghent, Belgium. [12]Department of Biomolecular Medicine, Ghent University, Ghent, Belgium. [13]VIB Screening Core, Ghent, Belgium. [14]Expertise Centre for Bioassay Development and Screening, Ghent University, Ghent, Belgium. [15]Department of Molecular Pharmacology and Cell Biology, Leibniz Research Institute for Molecular Pharmacology, Berlin, Germany. [16]Present address: Department of Botany and Plant Sciences, Institute of Integrative Genome Biology, University of California, Riverside, CA, USA. [17]These authors contributed equally: Wim Dejonghe, Isha Sharma. *e-mail: eurus@psb.vib-ugent.be

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Acknowledgements

We thank S. Vanneste for fruitful discussions, R. Kumar for providing the pDONR211-AP2S plasmid, D. Martinez Molina for help with the CETSA protocol and M. De Cock for help in preparing the manuscript. This work was supported by the Research Foundation-Flanders (project No. G022516N to E.R., project No. G009415N to D.V.D and project No. G0E5718N to E.R. and J.F.); the European Research Council (ERC Co T-Rex, grant No. 682436 to D.V.D); the Deutsche Forschungsgemeinschaft (No. TRR186/A08 to V.H.); the Agency for Innovation by Science and Technology for postdoctoral (K.M.) and predoctoral (W.D. and S.D.M) fellowships; the China Science Council for a predoctoral fellowship (Q.L.); the joint research projects (Nos. VS.025.13N and VS.095.16N) within the framework of cooperation between the Research Foundation-Flanders and the Bulgarian Academy of Sciences (K.M.); and the Belgian Science Policy Office for a postdoctoral fellowship to non-EU researchers (I.S.).

Author contributions

W.D. and E.R. initiated the work. W.D., I.S. and E.R. designed the experiments. W.D., I.S., B.D., A.M. and J.W. performed SAR. W.D., K.M., A.S. and K.G. performed affinity purification and MS analysis. H.B. and V.H. performed the X-ray crystallography. I.S., S.D.M. and S.N.S. performed the in vitro binding assay. W.N. did the molecular docking. W.D., I.S. and Q.L. performed CETSA. W.D., I.S. and Q.L. performed DARTS. W.D., I.S., E.M., D.V.S., and D.V.D. carried out the imaging and data analysis. I.S. performed the cloning and generated transgenic *Arabidopsis* cell cultures. W.D., I.S., A.D. and D.A. performed ATP measurements. M.V. and J.F. contributed to the HeLa cell assays. K.Y. generated the TPLATE antibody. Q.L. and R.D.R performed TEM. W.D., I.S. and E.R. wrote the manuscript. All authors commented on the results and the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information is available for this paper at https://doi.org/10.1038/s41589-019-0262-1.

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Correspondence and requests for materials should be addressed to E.R.

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The Plant Cell, Vol. 30: 2553–2572, October 2018, www.plantcell.org © 2018 ASPB.

The Inhibitor Endosidin 4 Targets SEC7 Domain-Type ARF GTPase Exchange Factors and Interferes with Subcellular Trafficking in Eukaryotes[OPEN]

Urszula Kania,[a,b,1] Tomasz Nodzyński,[c] Qing Lu,[b] Glenn R. Hicks,[d] Wim Nerinckx,[e,f] Kiril Mishev,[b,g] François Peurois,[h] Jacqueline Cherfils,[h] Riet De Rycke,[b,i] Peter Grones,[a,j] Stéphanie Robert,[j] Eugenia Russinova,[b] and Jiří Friml[a,2]

[a]Institute of Science and Technology Austria, 3400 Klosterneuburg, Austria
[b]Department of Plant Biotechnology and Bioinformatics, Ghent University and Center for Plant Systems Biology, VIB, 9052 Ghent, Belgium
[c]Mendel Centre for Plant Genomics and Proteomics, Central European Institute of Technology, Masaryk University, CZ-625 00 Brno, Czech Republic
[d]Center for Plant Cell Biology and Department of Botany and Plant Sciences, University of California, Riverside, California 92521
[e]VIB-UGent Center for Medical Biotechnology, 9052 Ghent-Zwijnaarde, Belgium
[f]Laboratory for Protein Biochemistry and Biomolecular Engineering, Department of Biochemistry and Microbiology, Ghent University, 9000 Ghent, Belgium
[g]Institute of Plant Physiology and Genetics, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria
[h]Laboratoire de Biologie et Pharmacologie Appliquée CNRS, Ecole Normale Supérieure Paris-Saclay, 94235 Cachan, France
[i]VIB BioImaging Core, 9052Ghent, Belgium
[j]Umeå Plant Science Centre, Department of Forest Genetics and Plant Physiology, Swedish University of Agricultural Sciences, 90183 Umeå, Sweden

ORCID IDs: 0000-0001-5416-4287 (U.K.); 0000-0002-1422-4924 (T.N.); 0000-0002-2502-7782 (G.R.H.); 0000-0002-6563-8342 (W.N.); 0000-0001-5849-8786 (K.M.); 0000-0003-1216-1162 (F.P.); 0000-0002-8966-3067 (J.C.); 0000-0001-8270-7015 (R.D.R.); 0000-0003-4132-4151 (P.G.); 0000-0002-0013-3239 (S.R.); 0000-0002-0569-1977 (E.R.); 0000-0002-8302-7596 (J.F.)

The trafficking of subcellular cargos in eukaryotic cells crucially depends on vesicle budding, a process mediated by ARF-GEFs (ADP-ribosylation factor guanine nucleotide exchange factors). In plants, ARF-GEFs play essential roles in endocytosis, vacuolar trafficking, recycling, secretion, and polar trafficking. Moreover, they are important for plant development, mainly through controlling the polar subcellular localization of PIN-FORMED transporters of the plant hormone auxin. Here, using a chemical genetics screen in *Arabidopsis thaliana*, we identified Endosidin 4 (ES4), an inhibitor of eukaryotic ARF-GEFs. ES4 acts similarly to and synergistically with the established ARF-GEF inhibitor Brefeldin A and has broad effects on intracellular trafficking, including endocytosis, exocytosis, and vacuolar targeting. Additionally, Arabidopsis and yeast (*Saccharomyces cerevisiae*) mutants defective in ARF-GEF show altered sensitivity to ES4. ES4 interferes with the activation-based membrane association of the ARF1 GTPases, but not of their mutant variants that are activated independently of ARF-GEF activity. Biochemical approaches and docking simulations confirmed that ES4 specifically targets the SEC7 domain-containing ARF-GEFs. These observations collectively identify ES4 as a chemical tool enabling the study of ARF-GEF-mediated processes, including ARF-GEF-mediated plant development.

INTRODUCTION

Structural integrity, chemical homeostasis, and, thus, the functionality of eukaryotic cells, including plant cells, depends on a complex network of intracellular membrane trafficking routes that act in concert with each other. A large number of critical components of endomembrane trafficking have been identified using various approaches; some of them are evolutionarily conserved, and some are more specific for particular model systems. Among the most prominent regulators of trafficking are ARF (ADP-ribosylation factor) GTPases that, together with their activators ARF-GEFs (ARF guanine nucleotide exchange factors), regulate the budding of trafficking vesicles (Yorimitsu et al., 2014). ARF proteins constantly switch between active (GTP-bound) and inactive (GDP-bound) states (Yorimitsu et al., 2014). The inactive ARF-GDP form localizes to the cytosol or associate loosely with membranes and become activated by the catalytic SEC7 domain of ARF-GEFs by exchanging GDP for GTP (Nielsen et al., 2008). Following activation to the GTP-bound state, ARFs bind to membranes and recruit cytosolic coat proteins Coat Protein Complex I (COPI), COPII, and clathrin to specific sites of vesicle budding at the Golgi apparatus

[1]Current address: Sainsbury Laboratory University of Cambridge, CB21LR Cambridge, UK.
[2]Address correspondence to jiri.friml@ist.ac.at.
The author responsible for distribution of materials integral to the findings presented in this article in accordance to the policy described in the Instructions for Authors (www.plantcell.org) is: Jiří Friml (jiri.friml@ist.ac.at).
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www.plantcell.org/cgi/doi/10.1105/tpc.18.00127

(At2g47170), GNOM (At1g13980), BRI1 (At4g39400), PID (At2g34650), CHC1 (At3g11130), CHC2 (At3g08530), SNX1 (At5g06140), VTI12 (At1g26670), RCN1 (At1g25490), AXR2 (At3g23050), BIG3 (At1g01960), FABD (AT4G26700), and MAP4 (M72414).

Supplemental Data

Supplemental Figure 1. Chemical screen of a set of 11 small molecules implicated in polarity changes.

Supplemental Figure 2. Characterization of the effects of ES4.

Supplemental Figure 3. Effect of ES4 on intracellular trafficking.

Supplemental Figure 4. Effect of ES4 on vacuolar trafficking.

Supplemental Figure 5. Effect of ES4 on intracellular compartments.

Supplemental Figure 6. Effect of ES4 on ARF1^{T31N}-CFP marker.

Supplemental Figure 7. ES4 sensitivity of yeast deletion strains.

Supplemental Figure 8. Representative nucleotide exchange kinetic curves.

Supplemental Figure 9. SDS-PAGE and fluorescence intensity and absorbance of ES4.

Supplemental Table 1. Yeast deletion strains used in the growth assay.

Supplemental Table 2. Docking-calculated affinities.

Supplemental Table 3. ANOVA tables.

ACKNOWLEDGMENTS

We thank Gerd Jürgens, Sandra Richter, and Sheng Yang He for providing antibodies; Maciek Adamowski, Fernando Aniento, Sebastian Bednarek, Nico Callewaert, Matyás Fendrych, Elena Feraru, and Mugurel I. Feraru for helpful suggestions; Siamsa Doyle for critical reading of the manuscript and helpful comments and suggestions; and Stephanie Smith and Martine De Cock for help in editing and language corrections. We acknowledge the core facility Cellular Imaging of CEITEC supported by the Czech-BioImaging large RI project (LM2015062 funded by MEYS CR) for their support with obtaining scientific data presented in this article. Plant Sciences Core Facility of CEITEC Masaryk University is gratefully acknowledged for obtaining part of the scientific data presented in this article. We acknowledge support from the Fondation pour la Recherche Médicale and from the Institut National du Cancer (J.C.). The research leading to these results was funded by the European Research Council under the European Union's 7th Framework Program (FP7/2007-2013)/ERC grant agreement numbers 282300 and 742985 and the Czech Science Foundation GAČR (GA18-26981S; J.F.); Ministry of Education, Youth, and Sports/MEYS of the Czech Republic under the Project CEITEC 2020 (LQ1601; T.N.); the China Science Council for a predoctoral fellowship (Q.L.); a joint research project within the framework of cooperation between the Research Foundation-Flanders and the Bulgarian Academy of Sciences (VS.025.13N; K.M. and E.R.); Vetenskapsrådet and Vinnova (Verket för Innovationssystem; S.R.), Knut och Alice Wallenbergs Stiftelse via "Shapesystem" Grant 2012.0050 (S.R.), Kempe stiftelserna (P.G.), Tryggers CTS410 (P.G.).

AUTHOR CONTRIBUTIONS

U.K., S.R., G.R.H., and J.F. conceived the project. U.K., S.R., T.N., and J.F. designed the research strategies. R.D.R. conducted transmission electron microscopy experiments. U.K. and P.G. performed root growth assays. W.N. performed the docking simulations. Q.L., K.M., and E.R. performed the DARTS assays. T.N. performed Imaris software analysis and imaging of cytoskeleton marker lines. F.P. and J.C. performed nucleotide exchange assay and protein expression and purification. U.K. performed the majority of the remaining experiments and analyzed the data. U.K., T.N., G.R.H., and J.F. edited the manuscript. U.K., T.N., and J.F. wrote the article. All authors revised the article.

Received February 12, 2018; revised June 29, 2018; accepted July 17, 2018; published July 17, 2018.

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The Plant Cell, Vol. 30: 2573–2593, October 2018, www.plantcell.org © 2018 ASPB.

Nonselective Chemical Inhibition of Sec7 Domain-Containing ARF GTPase Exchange Factors

Kiril Mishev,[a,b,c,1] Qing Lu,[a,b,1] Bram Denoo,[d] François Peurois,[e] Wim Dejonghe,[a,b,2] Jan Hullaert,[d] Riet De Rycke,[a,b,f] Sjef Boeren,[g] Marine Bretou,[h] Steven De Munck,[i,j] Isha Sharma,[a,b] Kaija Goodman,[k] Kamila Kalinowska,[l,3] Veronique Storme,[a,b] Le Son Long Nguyen,[m,n] Andrzej Drozdzecki,[m,n] Sara Martins,[o] Wim Nerinckx,[i,p] Dominique Audenaert,[m,n] Grégory Vert,[o] Annemieke Madder,[d] Marisa S. Otegui,[k] Erika Isono,[l,q] Savvas N. Savvides,[i,j] Wim Annaert,[h] Sacco De Vries,[g] Jacqueline Cherfils,[e] Johan Winne,[d] and Eugenia Russinova[a,b,4]

[a] Department of Plant Biotechnology and Bioinformatics, Ghent University, 9052 Ghent, Belgium
[b] Center for Plant Systems Biology, VIB, 9052 Ghent, Belgium
[c] Institute of Plant Physiology and Genetics, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria
[d] Department of Organic and Macromolecular Chemistry, Ghent University, 9000 Ghent, Belgium
[e] Laboratoire de Biologie et Pharmacologie Appliquée, Centre National de la Recherche Scientifique, Ecole Normale Supérieure Paris-Saclay, 94235 Cachan, France
[f] VIB BioImaging Core, 9052 Ghent, Belgium
[g] Laboratory of Biochemistry, Wageningen University, 6708 Wageningen, The Netherlands
[h] Laboratory for Membrane Trafficking, VIB Center for Brain and Disease Research, KU Leuven, Department of Neurosciences, 3000 Leuven, Belgium
[i] Laboratory for Protein Biochemistry and Biomolecular Engineering, Department of Biochemistry and Microbiology, Ghent University, 9000 Ghent, Belgium
[j] Center for Inflammation Research, VIB, 9052 Ghent, Belgium
[k] Laboratory of Cell and Molecular Biology and Departments of Botany and Genetics, University of Wisconsin-Madison, Wisconsin 53706
[l] School of Life Sciences Weihenstephan, Technical University of Munich, 85354 Freising, Germany
[m] VIB Screening Core, 9052 Ghent, Belgium
[n] Expertise Centre for Bioassay Development and Screening (C-BIOS), Ghent University, 9052 Ghent, Belgium
[o] Institute for Integrative Biology of the Cell (I2BC), CNRS/CEA/Université Paris Sud, Université Paris-Saclay, Gif-sur-Yvette 91198, France
[p] Center for Medical Biotechnology, VIB, 9052 Ghent, Belgium
[q] Department of Biology, University of Konstanz, 78457 Konstanz, Germany

ORCID IDs: 0000-0001-5849-8786 (K.M.); 0000-0002-4703-3286 (Q.L.); 0000-0003-1216-1162 (F.P.); 0000-0003-3869-2228 (W.D.); 0000-0002-7860-378X (J.H.); 0000-0001-8270-7015 (R.D.R.); 0000-0002-0847-8821 (S.B.); 0000-0002-4461-4077 (K.G.); 0000-0003-0080-5557 (K.K.); 0000-0003-4762-6580 (V.S.); 0000-0001-9979-4346 (A.D.); 0000-0003-3910-4168 (S.M.); 0000-0002-6563-8342 (W.N.); 0000-0002-0844-9991 (G.V.); 0000-0003-0179-7608 (A.M.); 0000-0003-4699-6950 (M.S.O.); 0000-0003-3420-5947 (S.N.S.); 0000-0001-9454-775X (S.D.V.); 0000-0002-9015-4497 (J.W.); 0000-0002-0569-1977 (E.R.)

Small GTP-binding proteins from the ADP-ribosylation factor (ARF) family are important regulators of vesicle formation and cellular trafficking in all eukaryotes. ARF activation is accomplished by a protein family of guanine nucleotide exchange factors (GEFs) that contain a conserved catalytic Sec7 domain. Here, we identified and characterized Secdin, a small-molecule inhibitor of *Arabidopsis thaliana* ARF-GEFs. Secdin application caused aberrant retention of plasma membrane (PM) proteins in late endosomal compartments, enhanced vacuolar degradation, impaired protein recycling, and delayed secretion and endocytosis. Combined treatments with Secdin and the known ARF-GEF inhibitor Brefeldin A (BFA) prevented the BFA-induced PM stabilization of the ARF-GEF GNOM, impaired its translocation from the Golgi to the *trans*-Golgi network/early endosomes, and led to the formation of hybrid endomembrane compartments reminiscent of those in ARF-GEF-deficient mutants. Drug affinity-responsive target stability assays revealed that Secdin, unlike BFA, targeted all examined Arabidopsis ARF-GEFs, but that the interaction was probably not mediated by the Sec7 domain because Secdin did not interfere with the Sec7 domain-mediated ARF activation. These results show that Secdin and BFA affect their protein targets through distinct mechanisms, in turn showing the usefulness of Secdin in studies in which ARF-GEF-dependent endomembrane transport cannot be manipulated with BFA.

INTRODUCTION

The small GTP-binding proteins from the ADP-ribosylation factor (ARF) family are major regulators of vesicle biogenesis and intracellular trafficking in all eukaryotes, including plants (Donaldson and Jackson, 2011; Yorimitsu et al., 2014). Like other small GTPases, the ARF proteins are controlled by a GTP-binding and GTP hydrolysis cycle that activates and inactivates them, respectively. ARF activation is facilitated by the ARF guanine-nucleotide exchange factors (ARF-GEFs), whereas ARF

absorbs light at 290 nm, which determines the differences in the plateau of the kinetics traces with and without the compound. This decrease was taken into account by fitting the entire kinetics curve. The fluorescence emission spectra of Secdin with and without ARF (excitation wavelength 290 nm) were also controlled. The emission spectra of ARF in the presence of Secdin matched well the addition of the separate spectra, revealing no obvious direct interference of Secdin with ARF. All experiments were done in triplicate.

Statistical Analysis

Unless otherwise specified, P values were calculated with a two-tailed Student's *t* test with Excel software.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: GNOM (At1g13980), GNL1 (At5g39500), GNL2 (At5g19610), BIG1 (At4g38200), BIG2 (At3g60860), BIG3 (At1g01960), BIG4 (At4g35380), BIG5/BEN1 (At3g43300), ARF1 (At2g47170), BRI1 (At4g39400), PIN2 (At5g57090), BOR1 (At2g47160), VHAa1 (At2g28520), RABF2a (At5g45130), RABF2b (At4g19640), PIP2A (At3g53420), BSK1 (At2g17090), SYP61 (At1g28490), SYP22 (At5g46860), and AMSH3 (At4g16144).

Supplemental Data

Supplemental Figure 1. Subcellular localization of different plasma membrane proteins and endomembrane markers in Arabidopsis root epidermal cells treated with Secdin.

Supplemental Figure 2. Lack of cytotoxic effects of Secdin.

Supplemental Figure 3. Effects of Secdin on the vacuolar degradation pathway.

Supplemental Figure 4. The effect of Secdin on plasma membrane protein degradation is dependent on protein ubiquitination.

Supplemental Figure 5. Structure-activity relationship analysis of Secdin analogs.

Supplemental Figure 6. DARTS analysis for validation of the putative Secdin protein targets in Arabidopsis and human cells.

Supplemental Figure 7. Representative nucleotide exchange kinetics curves used to estimate the exchange rates (k_{obs}) of spontaneous (EDTA) and GEF-stimulated ARF1 activation after small-molecule treatment.

Supplemental Figure 8. Golgi apparatus disruption in Secdin-treated human cell cultures.

Supplemental Data Set 1. NMR spectra of the synthesized Secdin-related compounds.

Supplemental Data Set 2. Affinity purification of protein interactors of biotin-tagged Secdin derivative (Secdin24) in Arabidopsis PSB-D cell cultures.

ACKNOWLEDGMENTS

We thank V. Vassileva, D. Van Damme, S. Vanneste, N. Callewaert, F. Coppens, A. Staes, and K. Gevaert for useful discussions; G. Jürgens, S. Richter, S. Y. He, S. Naramoto, J. Friml, M. Hothorn, K. Kasai, T. Fujiwara, C. Luschnig, Z. Wang, and H. Tanaka for sharing published materials; I. Vanhoutte, E. Mylle, T. De Saedeleer, B. Vanderstraeten, and B. Canher for technical assistance; and M. De Cock for help in preparing the manuscript. This work was supported by the Agency for Innovation by Science and Technology for postdoctoral (K.M.) and predoctoral (W.D.) fellowships; the China Scholarship Council for a predoctoral fellowship (Q.L.); a joint research project within the framework of cooperation between the Research Foundation-Flanders and the Bulgarian Academy of Sciences (VS.025.13N; K.M. and E.R.); the Belgian Science Policy Office for a postdoctoral fellowship (I.S.); the German Science Foundation for Grant IS 221/4-1 (E.I.); a grant from the Fondation pour la Recherche Médicale (J.C.); a predoctoral grant from the Ecole Normale Supérieure Paris-Saclay (F.P.); the Research Foundation-Flanders for an SBO project S006617N (W.A. and M.B.); and the Hercules Foundation for Grant AKUL/09/037, 13/39 (W.A.).

AUTHOR CONTRIBUTIONS

K.M, J.W., and E.R. conceived the study and designed the experiments. B.D., J.H., A.M., and J.W. synthesized compounds and consulted with chemistry. K.M., Q.L., W.D., I.S., W.N., R.D.R., K.G., K.K., L.S.L.N., S.D.M., and A.D. performed the experiments. K.M., Q.L., V.S., D.A., M.S.O., E.I., S.N.S., and E.R. analyzed the data. S.M. and G.V. contributed materials. S.B. and S.D.V. did the mass spectrometry. M.B. and W.A. carried out the imaging in human cells. F.P. and J.C. contributed ARF-GEF activity assays. K.M. and E.R. wrote the article. All authors commented on the results and the manuscript.

Received February 14, 2018; revised June 25, 2018; accepted July 17, 2018; published July 17, 2018.

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Regulation of *Arabidopsis* brassinosteroid receptor BRI1 endocytosis and degradation by plant U-box PUB12/PUB13-mediated ubiquitination

Jinggeng Zhou[a,b,c,1], Derui Liu[d,e,1], Ping Wang[b,f], Xiyu Ma[a,b], Wenwei Lin[b,f,2], Sixue Chen[g,h,i], Kiril Mishev[d,e,3], Dongping Lu[a,b,4], Rahul Kumar[d,e,5], Isabelle Vanhoutte[d,e], Xiangzong Meng[c], Ping He[a,b], Eugenia Russinova[d,e,6], and Libo Shan[b,f,6]

[a]Department of Biochemistry and Biophysics, Texas A&M University, College Station, TX 77843; [b]Institute for Plant Genomics and Biotechnology, Texas A&M University, College Station, TX 77843; [c]Shanghai Key Laboratory of Bio-Energy Crops, School of Life Sciences, Shanghai University, Shanghai 200444, China; [d]Department of Plant Biotechnology and Bioinformatics, Ghent University, 9052 Ghent, Belgium; [e]Center for Plant Systems Biology, VIB, 9052 Ghent, Belgium; [f]Department of Plant Pathology and Microbiology, Texas A&M University, College Station, TX 77843; [g]Department of Biology, University of Florida, Gainesville, FL 32610; [h]Genetics Institute, University of Florida, Gainesville, FL 32610; and [i]Plant Molecular and Cellular Biology Program, University of Florida, Gainesville, FL 32610

Edited by Cyril Zipfel, The Sainsbury Laboratory, Norwich, United Kingdom, and accepted by Editorial Board Member Caroline Dean January 6, 2018 (received for review July 11, 2017)

Plants largely rely on plasma membrane (PM)-resident receptor-like kinases (RLKs) to sense extracellular and intracellular stimuli and coordinate cell differentiation, growth, and immunity. Several RLKs have been shown to undergo internalization through the endocytic pathway with a poorly understood mechanism. Here, we show that endocytosis and protein abundance of the *Arabidopsis* brassinosteroid (BR) receptor, BR INSENSITIVE1 (BRI1), are regulated by plant U-box (PUB) E3 ubiquitin ligase PUB12- and PUB13-mediated ubiquitination. BR perception promotes BRI1 ubiquitination and association with PUB12 and PUB13 through phosphorylation at serine 344 residue. Loss of PUB12 and PUB13 results in reduced BRI1 ubiquitination and internalization accompanied with a prolonged BRI1 PM-residence time, indicating that ubiquitination of BRI1 by PUB12 and PUB13 is a key step in BRI1 endocytosis. Our studies provide a molecular link between BRI1 ubiquitination and internalization and reveal a unique mechanism of E3 ligase–substrate association regulated by phosphorylation.

Arabidopsis | BRI1 | ubiquitination | E3 ligase | endocytosis

Being sessile and autotrophic organisms, plants live in a relatively constrained niche with constant challenges from environmental stresses while coordinating growth and developmental processes. Plants have evolved a largely expanded collection of plasma membrane (PM)-resident receptor-like kinases (RLKs), many of which have been implicated in sensing external or internal signals and relaying the signaling cascades to various downstream outputs that are central to plant growth, development, and immunity (1, 2). For instance, BRASSINOSTEROID INSENSITIVE1 (BRI1) perceives the polyhydroxylated steroid hormone brassinosteroids (BRs) in regulating growth and development (3), and FLAGELLIN-SENSING2 (FLS2) perceives bacterial flagellin or its conserved 22-aa peptide (flg22) in regulating plant pattern-triggered immunity (PTI) (4). Both BRI1 and FLS2 belong to the leucine-rich repeat (LRR) domain-containing RLK family with more than 200 members in *Arabidopsis* (1). Despite distinct signaling outputs in growth and immunity, both BRI1 and FLS2 heterodimerize with another LRR RLK, BRI1-ASSOCIATED RECEPTOR KINASE1 (BAK1), also known as SOMATIC EMBRYOGENESIS RECEPTOR KINASE3 (SERK3), and other SERK members upon the cognate ligand perception (5–9). BAK1/SERKs are the shared coreceptors of FLS2, BRI1, and several other LRR RLK receptors, including ELONGATION FACTOR-TU (EF-Tu) RECEPTOR (EFR) regulating immunity and ERECTA regulating stomatal patterning (10). FLS2 and BRI1 reside in PM nanoclusters, the majority of which are spatiotemporally separated at the steady state (11).

The activity of LRR RLK receptor complexes is under multilayered positive and negative regulations to fine-tune signaling outputs (1, 12). Protein posttranslational modifications, such as phosphorylation and ubiquitination, play key roles in the activation and attenuation of LRR RLK complexes. BAK1-mediated transphosphorylation with its associated LRR RLK receptors is essential to activate or amplify intracellular signaling (13). FLS2 is ubiquitinated by two closely related plant U-box (PUB) E3 ubiquitin ligases PUB12 and PUB13. Upon flg22 perception, FLS2 associates with PUB12 and PUB13, resulting in ligand-induced FLS2 ubiquitination

Significance

The brassinosteroid (BR) receptor BRI1 provides a paradigm for understanding receptor-mediated signaling in plants. Different posttranslational modifications have been implicated in the regulation of BRI1 activity. Here, we show that BR perception promotes BRI1 association with plant U-box E3 ubiquitin ligases PUB12 and PUB13, which in turn directly ubiquitinate BRI1. Importantly, the BRI1 protein abundance and plasma membrane-residence time are increased while the endosomal pool of BRI1 is reduced in the *pub12pub13* mutant, indicating that PUB12/PUB13-mediated ubiquitination regulates BRI1 endocytosis and degradation. BRI1 phosphorylates PUB13 on a specific residue to enhance its association with BRI1, suggesting a unique regulatory circuit of phosphorylation-regulated E3 ligase–substrate association. Our study elucidates a mechanism of BRI1 internalization through E3 ubiquitin ligase-mediated ubiquitination.

Author contributions: J.Z., D. Liu, D. Lu, P.H., E.R., and L.S. designed research; J.Z., D. Liu, P.W., X. Ma, W.L., S.C., K.M., D. Lu, R.K., I.V., and X. Meng performed research; J.Z., D. Liu, P.W., X. Ma, W.L., S.C., K.M., D. Lu, R.K., I.V., and X. Meng analyzed data; and J.Z., D. Liu, P.H., E.R., and L.S. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission. C.Z. is a guest editor invited by the Editorial Board.

[1]J.Z. and D. Liu contributed equally to this work.

[2]Present address: Department of Botany and Plant Sciences, Center for Plant Cell Biology, University of California, Riverside, CA 92521.

[3]Present address: Institute of Plant Physiology and Genetics, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria.

[4]Present address: State Key Laboratory of Plant Genomics, Center for Agricultural Research Resources, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Shijiazhuang, Hebei 050021, China.

[5]Present address: Department of Plant Sciences, School of Life Sciences, University of Hyderabad, Hyderabad, 500046 Telangana, India.

[6]To whom correspondence may be addressed. Email: eurus@psb.vib-ugent.be or lshan@tamu.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1712251115/-/DCSupplemental.

Journal of Plant Physiology 205 (2016) 97–104

Contents lists available at ScienceDirect

Journal of Plant Physiology

journal homepage: www.elsevier.com/locate/jplph

Mapping of unmethylated sites in rDNA repeats in barley NOR deletion line

Anna D. Dimitrova[a,*], Oleg Georgiev[b], Kiril Mishev[a], Stefan Tzvetkov[a], Evgueni D. Ananiev[c], Luchezar Karagyozov[c]

[a] Institute of Plant Physiology and Genetics, Bulgarian Academy of Sciences, Acad. G. Bonchev Str., Bl. 21, 1113 Sofia, Bulgaria
[b] Institute of Molecular Life Sciences, University Zurich-Irchel, Winterthurer Str. 190, CH-8057 Zurich, Switzerland
[c] Department of Plant Physiology, Faculty of Biology, St. Kl. Ohridsky University of Sofia, 8 Dragan Tsankov bld., 1164 Sofia, Bulgaria

ARTICLE INFO

Article history:
Received 15 May 2016
Received in revised form 20 July 2016
Accepted 21 July 2016

Keywords:
rDNA hypomethylation
Ribosomal RNA genes
Intergenic spacer
Barley NOR 6H deletion line

ABSTRACT

Extensive cytosine methylation is characteristic of plant rDNA. Evidence exists, however, that the active rRNA genes are less methylated. In this work we report on the mapping of unmethylated CCGG sites in *Hordeum vulgare* rDNA repeats by digestion with methylation sensitive restriction enzyme HpaII and indirect end-labeling of the generated fragments. For mapping we used genomic DNA from barley deletion line with a single NOR on chromosome 5H. This NOR is more active in order to compensate for the missing NOR 6H. The enhanced NOR 5H activity in the deletion mutant is not due to higher multiplicity of the rRNA genes or, as sequencing showed, to changes in the subunit structure of the intergenic spacer. The HpaII sites in barley rDNA are heavily methylated. Nevertheless, a fraction of the rDNA repeats is hypomethylated with unmethylated CCGG sites at various positions. One unmethylated CCGG sequence is close to the transcription start site, downstream of the 135 bp subrepeats. Unmethylated sites are also present in the external transcribed spacer and in the genes coding mature rRNAs. The patterns of unmethylated sites in the barley deletion line and in lines with two NORs were compared. It is hypothesized that the occurrence of unmethylated sites on a fixed subset of rDNA repeats correlates with their transcriptional activity.

1. Introduction

It is well established that cytosine in plant nuclear DNA is extensively methylated into 5-methylcytosine (^{m5}C). The ^{m5}C in higher plants as well as in vertebrates occurs primarily in the sequence CpG. Members of conserved DNA methyltransferases DNMT1 (mammalian) and MET1 (plants) are mainly responsible for the CpG methylation. These enzymes are specific for regions of newly synthesized hemimethylated DNA, with only the old strand being methylated (maintenance methylation). However, in contrast to vertebrate DNA, in plants, cytosine in non-CpG contexts may also be methylated. Sequences CpHpG and CpHpH (H=C, A or T) are methylated by different DNA methyltransferases, such as chromomethylases (CMT) and domains rearranged methyltransferases (DRM) (Goll and Bestor, 2005; Feng et al., 2010; Zhang et al., 2010; Stroud et al., 2014).

The distribution of ^{m5}C in the plant genome is not uniform. In a large fraction of the protein-coding genes, the gene body is more methylated than the flanking 5′ and 3′ regions. A majority, but not all, of the RNA polymerase II promoters are hypomethylated (Zhang et al., 2006; To et al., 2015; Xu et al., 2016). The most methylated parts of the *Arabidopsis* genome are the transposable elements, the telomers, the centromers and other repeated elements (Zhang et al., 2006).

It has long been established that the tandem rRNA genes in plants are hypermethylated (Delseny et al., 1984; Watson et al., 1987; Flavell et al., 1988; Torres-Ruiz and Hemleben, 1994). However, these, as well as other studies have shown that some rDNA repeats are hypomethylated. The number of rRNA genes in plants is large, in the hundreds and quite often in the thousands (Rogers and Bendich, 1987). It has been suggested that the hypomethylated repeats represent the transcriptionally active fraction of all genes. This view is supported by several observations. Examination of the

Abbreviations: rRNA, ribosomal RNA; rDNA, DNA coding pre-rRNA; NOR, nucleolus organizer region; IGS, intergenic spacer; ETS, external transcribed spacer; TSS, transcription start site; 5H and 6H, *Hordeum* chromosomes 5 and 6.

* Corresponding author.
E-mail address: annad@bas.bg (A.D. Dimitrova).

http://dx.doi.org/10.1016/j.jplph.2016.07.019
0176-1617/

nature COMMUNICATIONS

B4_4

ARTICLE

Received 17 Jul 2015 | Accepted 21 Apr 2016 | Published 8 Jun 2016

DOI: 10.1038/ncomms11710 OPEN

Mitochondrial uncouplers inhibit clathrin-mediated endocytosis largely through cytoplasmic acidification

Wim Dejonghe[1,2], Sabine Kuenen[3,4], Evelien Mylle[1,2], Mina Vasileva[5], Olivier Keech[6], Corrado Viotti[7], Jef Swerts[3,4], Matyáš Fendrych[5], Fausto Andres Ortiz-Morea[1,2], Kiril Mishev[1,2], Simon Delang[8], Stefan Scholl[8], Xavier Zarza[9], Mareike Heilmann[10], Jiorgos Kourelis[9,†], Jaroslaw Kasprowicz[3,4], Le Son Long Nguyen[11], Andrzej Drozdzecki[11], Isabelle Van Houtte[1,2], Anna-Mária Szatmári[1,2], Mateusz Majda[12], Gary Baisa[13], Sebastian York Bednarek[13], Stéphanie Robert[12], Dominique Audenaert[11], Christa Testerink[9], Teun Munnik[9], Daniël Van Damme[1,2], Ingo Heilmann[10], Karin Schumacher[8], Johan Winne[14], Jiří Friml[5], Patrik Verstreken[3,4] & Eugenia Russinova[1,2]

ATP production requires the establishment of an electrochemical proton gradient across the inner mitochondrial membrane. Mitochondrial uncouplers dissipate this proton gradient and disrupt numerous cellular processes, including vesicular trafficking, mainly through energy depletion. Here we show that Endosidin9 (ES9), a novel mitochondrial uncoupler, is a potent inhibitor of clathrin-mediated endocytosis (CME) in different systems and that ES9 induces inhibition of CME not because of its effect on cellular ATP, but rather due to its protonophore activity that leads to cytoplasm acidification. We show that the known tyrosine kinase inhibitor tyrphostinA23, which is routinely used to block CME, displays similar properties, thus questioning its use as a specific inhibitor of cargo recognition by the AP-2 adaptor complex via tyrosine motif-based endocytosis signals. Furthermore, we show that cytoplasm acidification dramatically affects the dynamics and recruitment of clathrin and associated adaptors, and leads to reduction of phosphatidylinositol 4,5-biphosphate from the plasma membrane.

[1] Department of Plant Systems Biology, VIB, 9052 Gent, Belgium. [2] Department of Plant Biotechnology and Bioinformatics, Ghent University, 9052 Gent, Belgium. [3] VIB Center for the Biology of Disease, Laboratory of Neuronal Communication, 3000 Leuven, Belgium. [4] Department for Human Genetics, and Leuven Institute for Neurodegenerative Diseases, KU Leuven, 3000 Leuven, Belgium. [5] Institute of Science and Technology Austria, 3400 Klosterneuburg, Austria. [6] Department of Plant Physiology, Umeå Plant Science Centre, Umeå University, 90187 Umeå, Sweden. [7] Department of Plant Physiology, Institute of Biochemistry and Biology, University of Potsdam, 14476 Potsdam, Germany. [8] Developmental Biology of Plants, Centre for Organismal Studies, Heidelberg University, 69120 Heidelberg, Germany. [9] Department of Plant Cell Biology, Swammerdam Institute for Life Sciences, University of Amsterdam, 1090 GE Amsterdam, The Netherlands. [10] Department of Cellular Biochemistry, Institute for Biochemistry and Biotechnology, Martin-Luther-University, 06120 Halle, Germany. [11] Compound Screening Facility, VIB, 9052 Ghent, Belgium. [12] Department of Forest Genetics and Plant Physiology, Umeå Plant Science Centre, Swedish University of Agricultural Sciences, 90183 Umeå, Sweden. [13] Department of Biochemistry, University of Wisconsin-Madison, Madison, Wisconsin 53706, USA. [14] Laboratory for Organic Synthesis, Department of Organic and Macromolecular Chemistry, Ghent University, 9000 Gent, Belgium. † Present address: The Plant Chemetics Laboratory, Department of Plant Sciences, University of Oxford, Oxford OX1 3RB, UK. Correspondence and requests for materials should be addressed to E.R. (email: eurus@psb.vib-ugent.be).

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Acknowledgements

We thank Yvon Jaillais, Ikuko Hara-Nishimura, Akihiko Nakano, Takashi Ueda and Jinxing Lin for providing materials, Natasha Raikhel, Glenn Hicks, Steffen Vanneste, and Ricardo Tejos for useful suggestions, Patrick Callaerts for providing S2 *Drosophila* cell cultures, Michael Sixt for providing HeLa cells, Annick Bleys for literature searches, VIB Bio Imaging Core for help with imaging conditions and Martine De Cock for help in preparing the article. This work was supported by the Agency for Innovation by Science and Technology for a pre-doctoral fellowship to W.D.; the Research fund KU Leuven (GOA), a Methusalem grant of the Flemish government and VIB to S.K., J.K. and P.V.; by the Netherlands Organisation for Scientific Research (NWO) for ALW grants 846.11.002 (C.T.) and 867.15.020 (T.M.); the European Research Council (project ERC-2011-StG-20101109 PSDP) (to J.F.); a European Research Council (ERC) Starting Grant (grant 260678) (to P.V.), the Research Foundation-Flanders (grants G.0747.09, G094011 and G095511) (to P.V.), the Hercules Foundation, an Interuniversity Attraction Poles Poles Program, initiated by the Belgian State, Science Policy Office (to P.V.), the Swedish VetenskapsRådet grant to O.K., the Ghent University 'Bijzonder Onderzoek Fonds' (BOF) for a predoctoral fellowship to F.A.O.-M., the Research Foundation-Flanders (FWO) to K.M. and E.R.

Author contributions

W.D., A.-M.S., S.R. and E.R. initiated the work and designed the experiments; W.D. carried out all experiments in *Arabidopsis*. S.K., J.K. J.S. and P.V. contributed *Drosophila* experiments; M.V. and J.F. contributed the HeLa cells assays; W.D., F.A.O.-M., K.M., E.M., and D.V.D. did imaging and data analysis. M.M. produced preliminary data; O.K. performed respiratory measurements; C.V. did the TEM work in *Arabidopsis*; M.F., J.F., S.D., S.S. and K.S. contributed to the pH measurements; X.Z., J.K. C.T. and T.M. measured phospholipids; G.B., I.V.H., and S.B. did cloning and generated *Arabidopsis* transgenic lines; L.S.L.N., A.D., W.D. and D.A. did ATP measurements; J.W. contributed chemistry expertise; M.H. and I.H contributed the PIP5K1 and PIP5K2 data; W.D. and E.R. wrote the manuscript; All authors revised the manuscript.

Additional information

Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

Competing financial interests: The authors declare no competing financial interests.

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How to cite this article: Dejonghe, W. *et al.* Mitochondrial uncouplers inhibit clathrin-mediated endocytosis largely through cytoplasmic acidification. *Nat. Commun.* 7:11710 doi: 10.1038/ncomms11710 (2016).

Γ7_5

Sequence-Specific Protein Aggregation Generates Defined Protein Knockdowns in Plants[1][OPEN]

Camilla Betti, Isabelle Vanhoutte, Silvie Coutuer[2], Riet De Rycke, Kiril Mishev[3], Marnik Vuylsteke, Stijn Aesaert, Debbie Rombaut, Rodrigo Gallardo, Frederik De Smet[4], Jie Xu[5], Mieke Van Lijsebettens, Frank Van Breusegem, Dirk Inzé, Frederic Rousseau*, Joost Schymkowitz*, and Eugenia Russinova*

Department of Plant Systems Biology, VIB, 9052 Gent, Belgium (C.B., I.V., S.C., R.D.R., K.M., S.A., D.R., M.V.L., F.V.B., D.I., E.R.); Department of Plant Biotechnology and Bioinformatics, Ghent University, 9052 Gent, Belgium (C.B., I.V., S.C., R.D.R., K.M., S.A., D.R., M.V.L., F.V.B., D.I., E.R.); Switch Laboratory, VIB, 3000 Leuven, Belgium (R.G., F.D.S., J.X., F.R., J.S.); Switch Laboratory, Department of Cellular and Molecular Medicine, University of Leuven, 3000 Leuven, Belgium (R.G., F.D.S., J.X., F.R., J.S); and Gnomixx, 9000 Gent, Belgium (M.V.)

ORCID IDs: 0000-0003-3522-6440 (C.B.); 0000-0003-2878-134X (I.V.); 0000-0002-7539-1005 (S.C.); 0000-0001-8270-7015 (R.D.R.); 0000-0001-5849-8786 (K.M.); 0000-0003-1584-3564 (R.G.); 0000-0002-3147-0860 (F.V.B.); 0000-0002-3217-8407 (D.I.); 0000-0003-2020-0168 (J.S.); 0000-0002-0569-1977 (E.R.).

Protein aggregation is determined by short (5–15 amino acids) aggregation-prone regions (APRs) of the polypeptide sequence that self-associate in a specific manner to form β-structured inclusions. Here, we demonstrate that the sequence specificity of APRs can be exploited to selectively knock down proteins with different localization and function in plants. Synthetic aggregation-prone peptides derived from the APRs of either the negative regulators of the brassinosteroid (BR) signaling, the glycogen synthase kinase 3/Arabidopsis SHAGGY-like kinases (GSK3/ASKs), or the starch-degrading enzyme α-glucan water dikinase were designed. Stable expression of the APRs in Arabidopsis (*Arabidopsis thaliana*) and maize (*Zea mays*) induced aggregation of the target proteins, giving rise to plants displaying constitutive BR responses and increased starch content, respectively. Overall, we show that the sequence specificity of APRs can be harnessed to generate aggregation-associated phenotypes in a targeted manner in different subcellular compartments. This study points toward the potential application of induced targeted aggregation as a useful tool to knock down protein functions in plants and, especially, to generate beneficial traits in crops.

In order to function properly, proteins must fold into their native structure, but protein folding is often challenged by protein misfolding and aggregation (Tyedmers et al., 2010). Although protein aggregation has long been considered as a disordered process mediated by nonspecific hydrophobic interactions, it is now understood to be a sequence-specific self-association process (Mitraki, 2010; Tyedmers et al., 2010). Indeed, both in bacterial (Sabaté et al., 2010) and mammalian systems (Rajan et al., 2001), aggregation of nonhomologous proteins has been shown to occur preferentially in distinct inclusion bodies. In vitro aggregation of protein solutions can be accelerated by seeding with preformed aggregates, and this process efficiency depends critically on the sequence homology between seed and target protein (Krebs et al., 2004; O'Nuallain et al., 2004). Self-seeding is generally several orders of magnitude more efficient than cross-seeding (Ganesan et al., 2015; Surmacz-Chwedoruk et al., 2014). Aggregation-associated human diseases, such as Alzheimer's or Parkinson's disease, are in line with this notion because the processes underlying these diseases are highly specific and characterized by the aggregation of one or a few proteins in particular tissues and cell types (Jucker and Walker, 2013).

The elucidation of the structure of amyloid-forming peptides and protein fragments has shed light on the molecular origin of the sequence specificity of protein aggregation. The amyloid structure consists of the formation of a so-called cross-β conformation, whereby the peptide backbone of the aggregate creates hydrogen bond-mediated β-strand interactions, whereas the side chains contribute to the stability of these β-strands by aligning with, and closely packing to, the identical sequence of the neighboring strand (Sawaya et al., 2007; Makin et al., 2005). The registered stacking of side chains explains the aggregation sequence specificity. Indeed, backbone interactions contribute comparatively more to the amyloid structure than to the globular protein structure (Fitzpatrick et al., 2011).

The portions of a protein sequence that are susceptible to associate into aggregates by β-strand-mediated interactions are limited to short segments, defined as aggregation-prone regions (APRs). The APRs consist of 5 to 15 amino acids in length (Rousseau et al., 2006; Goldschmidt et al., 2010) and can be identified by prediction algorithms (Fernandez-Escamilla et al., 2004). The determining role of APRs has been demonstrated by "aggregation-grafting" experiments, in which insertion of an APR of an aggregating protein into the

sequence of a nonaggregating protein results in a protein with aggregation propensity and morphology similar to those of the original protein (Ventura et al., 2004).

Application of the prediction algorithm TANGO (Fernandez-Escamilla et al., 2004) to the Arabidopsis (*Arabidopsis thaliana*) proteome revealed that 80% of the proteins contain APRs, implying that, similar to other eukaryotes, plant proteomes are also susceptible to protein aggregation (Rousseau et al., 2006). As most of the Arabidopsis proteins harbor aggregation-prone sequence segments within their primary structure and as aggregation is sequence specific, it should, in principle, be possible to induce aggregation and, subsequently, functional depletion of a protein by exposing it to a short target-specific aggregating peptide in plants. First, we tested this hypothesis by targeting proteins with kinase activity in Arabidopsis plants. We selected the cytosolic glycogen synthase kinase 3/Arabidopsis SHAGGY-like kinases (GSK3/ASKs) and the chloroplast-localized α-glucan water dikinase (GWD). Arabidopsis possesses 10 ASKs grouped into four clades (Youn and Kim, 2015) that share a 50% overall sequence identity across the whole protein. Among the ASKs, BRASSINOSTEROID INSENSITIVE2 (BIN2) was characterized as a negative regulator of BR signaling (Li and Nam, 2002; Vert and Chory, 2006; Yan et al., 2009). In addition to BIN2 and its two close homologs, BIN2-LIKE1 (BIL1) and BIL2 (clade II), at least four other ASKs redundantly convey BR signals via a mechanism similar to that of BIN2 (De Rybel et al., 2009; Kim et al., 2009; Rozhon et al., 2010).

The GWD enzyme catalyzes the phosphorylation of starch in the chloroplasts by transferring β-ATP phosphate to either the C6 or the C3 position of the glycosyl residue of amylopectin and, thus, plays an essential role in starch metabolism (Mitsui et al., 2010). The phosphate groups influence the susceptibility of the starch granules to degrading enzymes, such as β-amylases. As a result, the starch breakdown is impaired in GWD-deficient plants. In GWD-antisense potato (*Solanum tuberosum*) plants (Lorberth et al., 1998), as well as in the GWD-deficient *starch excess1* (*sex1*) mutants of Arabidopsis (Yu et al., 2001), the foliar starch content is significantly higher than that of the respective wild-type plants. In addition to the model plant Arabidopsis, we applied the APR-mediated aggregation by targeting the GWD enzyme in maize (*Zea mays*).

Our work demonstrates that overexpression of different APRs, derived from a single protein or protein family, fused to a fluorescent carrier, results in specific knockdowns similar to previously described genetic mutants. We show that direct interactions between the APRs and the target proteins caused the loss of function of the proteins. Moreover, specific subcellular targeting of the synthetic APRs can be achieved in both model and crop plant species. Hence, the APR expression approach presented here can be used as an innovative knockdown method to inactivate proteins by specific in vivo pull-down in defined subcellular compartments of plants. In addition, the results also underline that, at least in plants, protein aggregation is not cytotoxic per se, but rather that the functional effect of the aggregates observed here appear to be dominated by sequence-specific cross-seeding of the aggregation of cellular APR-sharing proteins.

RESULTS

Design of the Aggregation Constructs

To simultaneously knock out the function of all 10 ASKs in Arabidopsis by inducing specifically their misfolding and inactivation, we applied the aggregation prediction algorithm TANGO (Fernandez-Escamilla et al., 2004) to BIN2 in order to identify overlapping aggregation-prone peptides in the 10 target proteins. One APR of nine amino acids with a TANGO aggregation score greater than 50 (out of a maximum of 100) and coding for the sequence ^{249}QLVEIIKVL^{257} in BIN2 was detected (hereafter referred to as BIN2$^{249\text{-}257}$; Fig. 1A; Supplemental Table S1). The BIN2$^{249\text{-}257}$ APR was situated in the kinase domain preceding the highly

[1] This work was supported by grants from the Agency for Innovation by Science and Technology ("Strategisch Basisonderzoek" project no. 60839), Ghent University ("Industrieel Onderzoeksfonds" F2014/IOF-StarrTT261 and Multidisciplinary Research Partnership "Biotechnology for a Sustainable Economy" no. 01MRB510W), the Research Foundation-Flanders (Joint Project Bulgarian Academy of Sciences VS.025.13N), the Interuniversity Attraction Poles Program (IUAP VII/29), initiated by the Belgian State, Science Policy Office, University of Leuven, and the European Research Council under the European Union's Horizon 2020 Framework Programme (ERC Grant agreement 647458).

[2] Present address: Bekintex at Bekaert NV, 8000 Bruges, Belgium.

[3] Present address: Institute of Plant Physiology and Genetics, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria.

[4] Present address: Dana Farber Cancer Institute and the Broad Institute/MIT, Cambridge, MA, 02141.

[5] Present address: State Key Laboratory for Oncogenes and Related Genes, Division of Gastroenterology and Hepatology, Renji Hospital, Shanghai Institute for Digestive Diseases, Shanghai Jiao-Tong University School of Medicine, Shanghai 200001, China.

* Address correspondence to frederic.rousseau@switch.vib-kuleuven.be, joost.schymkowitz@switch.vib-kuleuven.be, or eugenia.russinova@psb.vib-ugent.be.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is Eugenia Russinova (eugenia.russinova@psb.vib-ugent.be).

D.I., F.R., J.S., and E.R. conceived the research and designed and supervised the experiments; S.C. initiated the work; C.B. designed and performed most of the experiments; I.V. provided technical assistance to C.B.; R.D.R. performed the TEM work; K.M. and M.V. did the expression studies and data analysis; S.A. and M.V.L. made transgenic maize lines; S.A., D.R., M.V.L., F.V.B., and D.I. contributed to the maize analyses; R.G., F.D.S., and J.X. performed the FTIR and expression analysis; C.B., F.R., J.S., and E.R. wrote the article with contributions of all the authors.

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www.plantphysiol.org/cgi/doi/10.1104/pp.16.00335

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Current Opinion in Plant Biology

The brassinosteroid chemical toolbox

Wim Dejonghe[1,2], Kiril Mishev[1,2,3] and Eugenia Russinova[1,2]

Chemical biology approaches have been instrumental in understanding the mode of action of brassinosteroids, a group of plant steroid hormones essential for plant development and growth. The small molecules used for such approaches include inhibitors of biosynthetic enzymes and signaling components. Additionally, recent structural data on the brassinosteroid receptor complex together with its ligand brassinolide, the most active brassinosteroid, and knowledge on its different analogs have given us a better view on the recognition of the hormone and signaling initiation. Moreover, a fluorescently labeled brassinosteroid enabled the visualization of the receptor–ligand pair in the cell. Given the insights obtained, small molecules will continue to provide new opportunities for probing brassinosteroid biosynthesis and for unraveling the dynamic and highly interconnected signaling.

Addresses
[1] Department of Plant Systems Biology, VIB, 9052 Ghent, Belgium
[2] Department of Plant Biotechnology and Bioinformatics, Ghent University, 9052 Ghent, Belgium
[3] Institute of Plant Physiology and Genetics, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria

Corresponding author: Russinova, Eugenia (jenny.russinova@psb.vib-ugent.be)

Current Opinion in Plant Biology 2014, **22**:48–55

This review comes from a themed issue on **Cell biology**

Edited by **Shaul Yalovsky** and **Viktor Žárský**

For a complete overview see the Issue and the Editorial

Available online 1st October 2014

http://dx.doi.org/10.1016/j.pbi.2014.09.002

Introduction

Brassinosteroids (BRs) are plant steroid hormones that play an essential role in plant growth and development and their biosynthesis, signaling, and transcriptional responses are among the best studied and understood processes in plant biology [1,2]. Once perceived by the plasma membrane-localized leucine-rich repeat (LRR) receptor kinase BRASSINOSTEROID INSENSITIVE1 (BRI1), BRs trigger a signaling cascade that will regulate transcriptional responses (Figure 1). Small molecules have proven to be a valuable tool in elucidating BR biosynthesis and signaling. Examples include the well-known BR biosynthesis inhibitor brassinazole [3] and the BR signaling effector bikinin [4].

The major advantages of chemical tools are their simplicity for translational research, capability to account for gene redundancy and lethality, and the conditionality of treatment. The latter implies that time, duration, and concentration of treatments can be adapted, allowing the study of any process of interest in a dynamic manner opposed to a steady-state situation at the genomic level.

Over recent years, plant research has embraced chemical biology more and more as an alternative to classical methods, encouraging the development of new chemical tools. Here, we provide an overview of the latest reports on small molecules affecting BR processes, improving the chemical toolbox at our disposal to venture farther into the complex cellular and molecular mechanisms upon BR action.

Small molecules affecting BR biosynthesis

Specific inhibitors of BR biosynthesis offer a powerful tool to manipulate BR levels in different plant tissues and have been crucial for identifying novel components involved in the BR signal transduction pathway [5]. All known inhibitors of BR biosynthesis (Figure 2) share an azole moiety that is thought to bind the iron-containing heme prosthetic group of P450 cytochrome enzymes. The best known examples with a specific impact on BR biosynthesis are brassinazole (brz) [3] and brz2001 [6]. Both small molecules are similar to uniconazole, a known gibberellin biosynthesis inhibitor that also affects BR biosynthesis [7], and result from uniconazole derivatization to obtain specific BR biosynthesis inhibitors. A similar strategy has been adopted with another BR biosynthesis inhibitor propiconazole [8] used to develop a more potent inhibitor, namely brz220 [9]. Brz, brz2001, and brz220 target DWARF4 (DWF4), a P450 monooxygenase hydroxylating the C-22 position during BR biosynthesis (Figure 2) [10,11]. Brz2001, recently used in a quartz-crystal microbalance-based T7 phage display target identification strategy, confirmed DWF4 as its target, mapping the binding site as a potential disordered region of the enzyme [12•].

Several other BR biosynthesis inhibitors have been found. Voriconazole inhibits the BR-dependent sterol biosynthesis at the level of the P450 cytochrome CYP51 (Figure 2) resulting in accumulation of the sterol biosynthesis intermediate obtusifoliol [13••]. In addition, voriconazole appears to be specific to BR biosynthesis, because gibberellin application did not rescue the voriconazole-caused growth phenotypes. A group of BR biosynthesis inhibitors, designated the YCZ series, was developed from a ketoconazole scaffold [14] (Figure 2).

Chemistry & Biology
Review

Small Molecules for Dissecting Endomembrane Trafficking: A Cross-Systems View

Kiril Mishev,[1,2,3] Wim Dejonghe,[1,2] and Eugenia Russinova[1,2,*]
[1]Department of Plant Systems Biology, VIB, 9052 Ghent, Belgium
[2]Department of Plant Biotechnology and Bioinformatics, Ghent University, 9052 Ghent, Belgium
[3]Institute of Plant Physiology and Genetics, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria
*Correspondence: eurus@psb.vib-ugent.be
http://dx.doi.org/10.1016/j.chembiol.2013.03.009

Endomembrane trafficking has a key role for ensuring homeostasis, growth and development, hormonal signaling, and adaptation of eukaryotes to the constantly changing environmental conditions. The complex organization of the endomembrane system implies the need for searching novel tools to specifically probe the regulatory components and dissect the tightly interconnected vesicle transport pathways. Here, we review the large-scale chemical genetic screens, which led to the identification of small molecules with an impact on various parts of the vesicle trafficking network. We discuss the similarities and differences in the organization of the endomembrane systems in yeasts, mammals, and plants based on studies of small molecules and their effects on trafficking hubs, routes, and conserved protein targets.

Eukaryotic cells are characterized by functional compartmentalization of macromolecule trafficking, which is performed by an elaborate and fine-tuned endomembrane system. The endomembrane network comprises dynamic organelles with discrete morphology, localization, and functions and ensures both the secretion of biomolecules and the uptake of extracellular material that is delivered to intracellular locations in a highly coordinated manner. The exchange of cargo, membrane components, and solutes between the cellular compartments is a continuously ongoing process that involves alternating steps of membrane deformation, budding, fission, tethering, and fusion. The secretory and endocytic pathways are largely interconnected at the level of common trafficking hubs and protein regulators, which substantially increases the complexity of the endomembrane system. When key protein components of the endomembrane system are compromised, pleiotropic phenotypes may occur, making it difficult to elucidate the underlying molecular mechanism with classical genetic approaches. Significant limitations of forward and reverse genetics are the lack of phenotypic changes if functionally redundant components of vesicular trafficking are analyzed and, in the opposite case, the lethality of loss-of-function mutants due to the essential role of the intracellular transport for cell viability (Hicks and Raikhel, 2012). An alternative approach that circumvents these limitations deals with the use of small organic molecules as specific effectors of endomembrane trafficking. An important advantage of chemical genetics in dissecting complex biological processes is the enormous diversity of chemical structures that could be used for probing protein functions in a dose-dependent and reversible manner (Kaschani and van der Hoorn, 2007). Hence, it should be possible to perturb almost any protein in a specific way, allowing the targeting of essential genes. In addition, entire gene families can be targeted by bioactive chemicals through perturbation of a common feature of the corresponding proteins, thus addressing redundancy among genes (Hicks and Raikhel, 2012; Tóth and van der Hoorn, 2010).

Recent advances in systems biology, bioinformatics, and modern cell imaging opened novel opportunities for identifying small molecules that can be used as tools to dissect endomembrane trafficking pathways. Here, we outline the progress in current chemical genetics related to intracellular vesicle trafficking, with an emphasis on the use of small molecules in plants, mammals, and yeasts identified through high-throughput screens (Figure 1). The central routes and hubs of the endomembrane trafficking network in eukaryotic cells will be discussed for their sensitivity to the compounds (Figure 2; Table 1), which is determined by the extent of conservation of the protein targets.

Endomembrane Trafficking Routes in Yeasts, Plants, and Mammals: A Comparative View

The eukaryotic endomembrane system is composed of membrane-delimited organelles, small vesicular compartments that shuttle between those organelles, and a spectrum of transiently associated, peripheral membrane components from the cytosol that support, regulate, and define endomembrane trafficking (Foresti and Denecke, 2008). Proteins that are cotranslationally translocated into the endoplasmic reticulum (ER) are subsequently sorted for trafficking through the Golgi apparatus and the *trans*-Golgi network (TGN). Anterograde transport from the ER is mediated by COPII-coated vesicles, which fuse with pre-Golgi intermediates to release their cargo, while Golgi-to-ER protein recycling requires COPI vesicle formation. Unlike mammals, the plant endomembrane system lacks an ER-Golgi intermediate compartment (Foresti and Denecke, 2008). After reaching the TGN, secretory proteins are packaged into secretory vesicles and targeted to the plasma membrane (PM) (Vázquez-Martínez et al., 2012). So far, the secretory route from the TGN to the PM in plants is poorly understood. The TGN compartment also produces clathrin-coated vesicles directed to the lytic compartment (the lysosome in mammalian cells or the vacuole in plants and yeasts). The latter route involves late endosomes (LEs) in mammals, also called prevacuolar compartments/multivesicular bodies (PVCs/MVBs) in plant cells. Unlike mammals,

nature chemical biology

Γ7_8

ARTICLE

PUBLISHED ONLINE: 6 MAY 2012 | DOI: 10.1038/NCHEMBIO.958

Fluorescent castasterone reveals BRI1 signaling from the plasma membrane

Niloufer G Irani[1,2,7], Simone Di Rubbo[1,2,7], Evelien Mylle[1,2], Jos Van den Begin[3], Joanna Schneider-Pizoń[1,2], Jaroslava Hniliková[4], Miroslav Šíša[5], Dieter Buyst[3], Josep Vilarrasa-Blasi[6], Anna-Mária Szatmári[1,2], Daniël Van Damme[1,2], Kiril Mishev[1,2], Mirela-Corina Codreanu[1,2], Ladislav Kohout[4], Miroslav Strnad[5], Ana I Caño-Delgado[6], Jiří Friml[1,2], Annemieke Madder[3] & Eugenia Russinova[1,2]*

Receptor-mediated endocytosis is an integral part of signal transduction as it mediates signal attenuation and provides spatial and temporal dimensions to signaling events. One of the best-studied leucine-rich repeat receptor–like kinases in plants, BRASSINOSTEROID INSENSITIVE 1 (BRI1), perceives its ligand, the brassinosteroid (BR) hormone, at the cell surface and is constitutively endocytosed. However, the importance of endocytosis for BR signaling remains unclear. Here we developed a bioactive, fluorescent BR analog, Alexa Fluor 647–castasterone (AFCS), and visualized the endocytosis of BRI1–AFCS complexes in living *Arabidopsis thaliana* cells. Impairment of endocytosis dependent on clathrin and the guanine nucleotide exchange factor for ARF GTPases (ARF-GEF) GNOM enhanced BR signaling by retaining active BRI1-ligand complexes at the plasma membrane. Increasing the *trans*-Golgi network/early endosome pool of BRI1–BR complexes did not affect BR signaling. Our findings provide what is to our knowledge the first visualization of receptor-ligand complexes in plants and reveal clathrin- and ARF-GEF–dependent endocytic regulation of BR signaling from the plasma membrane.

Numerous reports in animals support the concept that integrated networks of endocytosis and plasma membrane–regulated signaling outputs control cellular processes. Endocytosis either physically removes activated receptor-ligand complexes from the cell surface, thus attenuating signaling, or moves them to endosomes to perpetuate their signaling activity before recycling or degradation[1,2].

Plants have a large number of cell-surface receptor–like kinases (RLKs) that control development and respond to an ever-changing extracellular environment[3]. Although several plant RLKs accumulate in endosomes and endocytosis controls their recycling and degradation[4], the mechanism and function of receptor-mediated endocytosis (RME) in signal transduction in plants are largely unexplored. The growth-promoting plant steroid hormones known as BRs and their plasma membrane–localized receptor, the leucine-rich repeat RLK BRI1, are the best-known ligand-receptor pair in plants[5,6]. Binding of BRs to the extracellular domain of BRI1 (refs. 7–9) activates its intracellular kinase activity and triggers the dissociation of the inhibitory BRI1 KINASE INHIBITOR 1 (BKI1)[10]. This dissociation allows the interaction and transphosphorylation between BRI1 and its co-receptor BRI1-ASSOCIATED KINASE 1 (BAK1)[11,12]. The BR signals are then conveyed from the cell surface to the nucleus through sequential signaling modules including the GSK3/SHAGGY-like kinase BR-INSENSITIVE 2 (BIN2)[13]. The BR-induced inactivation of BIN2 leads to dephosphorylation of its targets, the transcription factors BRASSINAZOLE-RESISTANT 1 (refs. 14,15) and BRI1-EMS-SUPPRESSOR 1 (BES1, also known as BZR2)[16], resulting in their relocation to the nucleus for transcriptional regulation of plant development processes[15].

Although much is known about the regulation of the kinase activity of BRI1 and BRI1's role in initiating the BR response, the interplay between receptor-mediated signaling and BRI1 trafficking is less understood. The functional green fluorescent protein (GFP)-tagged BRI1 has been used to describe the subcellular dynamics of the receptor in *Arabidopsis* roots[6,12,17–19]. BRI1 constitutively cycles between the plasma membrane and the *trans*-Golgi network/early endosome (TGN/EE), and it is targeted to the vacuole for degradation[17] via the late endosomes/multivesicular bodies (MVBs)[19] independently of its ligand[12,17]. It has been proposed that an increase in the endosomal pool of BRI1-GFP, induced by the ARF-GEF inhibitor brefeldin A (BFA), promotes BR signaling[17]. However, the localization of BRI1-GFP alone is not sufficient to distinguish between the endocytosed ligand-bound or free receptors and those traversing the secretory pathway, a serious caveat in identifying the intracellular sites for signaling. This limitation, together with the lack of knowledge of the endocytic machinery[20] involved in BRI1 endocytosis, represents a limiting step in studying the role of endocytosis in the regulation of BRI1 signaling.

Here we developed a bioactive fluorescently labeled BR, AFCS (**1**), and followed the endocytic route of receptor-ligand complexes in living cells for what is to our knowledge the first time in plants. We used chemical and genetic approaches to interfere with the trafficking of the BRI1–BR complexes and examined their effect on BR signaling. Our data identified BRI1–BR endocytosis to be dependent on clathrin, ARF-GEFs and the Rab5 GTPase pathway. Interference with clathrin- or ARF-GEF–mediated endocytosis of BRI1 enhanced BR signaling, whereas retaining BRI1–BR complexes at the TGN/EE did not affect signaling, indicating that the

[1]Department of Plant Systems Biology, VIB, Ghent, Belgium. [2]Department of Plant Biotechnology and Bioinformatics, Ghent University, Ghent, Belgium. [3]Laboratory for Organic and Biomimetic Chemistry, Department of Organic Chemistry, Ghent University, Ghent, Belgium. [4]Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Prague, Czech Republic. [5]Laboratory of Growth Regulators, Palacký University and Institute of Experimental Botany, Academy of Sciences of the Czech Republic, Olomouc, Czech Republic. [6]Department of Molecular Genetics, Centre for Research in Agricultural Genomics, Barcelona, Spain. [7]These authors contributed equally to this work. *email: jenny.russinova@psb.vib-ugent.be

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Acknowledgments

We thank N. Geldner, Y. Yin, S. Clouse, Xiaofeng Wang, S. Huber and Xuelu Wang for sharing materials and protocols; S. Figaroli for technical assistance; M. De Cock for the help with manuscript preparation; and J. Martins for NMR and structure analysis. This work is supported by the Odysseus program of the Research Foundation-Flanders (to J.F.), the BRAVISSIMO Marie-Curie Initial Training Network (predoctoral fellowships to S.D.R. and J.S.-P.), the Research Foundation-Flanders (postdoctoral fellowship to D.V.D.), the Agency for Innovation by Science and Technology (postdoctoral fellowship to K.M.), the Research Foundation-Flanders (research grant to E.R.) and projects 1M06030 and Z4 055 0506 (to J.H., M.S. and L.K.). The 700-MHz equipment of the Interuniversitary NMR Facility used in this work was financed by Ghent University, the Free University of Brussels and the University of Antwerp via the 'Zware Apparatuur' Incentive of the Flemish Government.

Author contributions

N.G.I., S.D.R., J.F. and E.R. conceived the study and designed the experiments. N.G.I., S.D.R., E.M., J.S.-P., A.-M.S., J.V.-B., K.M., M.-C.C. and D.V.D. performed experiments and analyzed the results. N.G.I., J.V.d.B., J.H., M.S., M.Š., L.K., D.B. and A.M. performed the chemical synthesis and analyzed the results. N.G.I., S.D.R., A.I.C.-D., D.V.D., A.M., J.F. and E.R. planned experiments and analyses. N.G.I., S.D.R., J.F. and E.R. wrote the manuscript. All authors commented on the results and the manuscript.

Competing financial interests

The authors declare no competing financial interests.

Additional information

Supplementary information and chemical compound information is available in the online version of the paper. Reprints and permissions information is available online at http://www.nature.com/reprints/index.html. Correspondence and requests for materials should be addressed to E.R.

Plant Growth Regul (2011) 65:459–471
DOI 10.1007/s10725-011-9616-8

Г7_9

ORIGINAL PAPER

Local induction of senescence by darkness in *Cucurbita pepo* (zucchini) cotyledons or the primary leaf induces opposite effects in the adjacent illuminated organ

Kalina Ananieva · Evguéni D. Ananiev · Snejana Doncheva · Detelin Stefanov · Kiril Mishev · Miroslav Kamínek · Vaclav Motyka · Petre I. Dobrev · Jiři Malbeck

Received: 29 April 2011 / Accepted: 15 July 2011 / Published online: 4 September 2011

Abstract Local darkening of zucchini cotyledons or the primary leaf affected in an organ-specific manner the adjacent ones which remained under the initial light regime. Individual darkening of either the pair of cotyledons or the primary leaf led to acceleration of senescence expressed by lowering of chlorophyll content and net photosynthetic rate. Darkening of the pair of cotyledons induced a reduction in total cytokinin (CK) levels and increased CK oxidase/dehydrogenase (CKX) activity in the adjacent illuminated primary leaf. In addition, abscisic acid (ABA) content was increased which correlated with reduced stomatal aperture leading to decreased stomatal conductance and transpiration rate. In contrast, darkening of the adjacent primary leaf led to increased metabolic activity in the illuminated cotyledons including increased total CK levels in parallel with decreased CKX activity, decreased ABA content in correlation with increased stomatal aperture, stomatal conductance and transpiration rate. On the other hand, the functional activity of the photosynthetic apparatus as well as the transcript levels of the three photosynthesis-related genes *psbA*, *psaB* and *rbcL* remained almost unaffected in both illuminated organs. Thus, compared with the primary leaves, cotyledons appeared to be much more resistant to the dark stress applied either directly or to the adjacent primary leaf. Our results indicated the involvement of CKs and ABA signalling in the control of the communication mechanisms between cotyledons and the primary leaf that could operate in response to changing environmental factors like shading during earlier stages of plant development.

Keywords Abscisic acid · Cotyledons · Cytokinins · Cytokinin oxidase/dehydrogenase · Dark treatment · Primary leaves

Abbreviations

ABA	Abscisic acid
CK	Cytokinin
*cis*Z	*cis*-Zeatin
*cis*ZR	*cis*-Zeatin riboside
CKX	Cytokinin oxidase/dehydrogenase
DHZ	Dihydrozeatin
DHZR	Dihydrozeatin 9-riboside
DHZ7G	Dihydrozeatin 7-glucoside
DHZ9G	Dihydrozeatin 9-glucoside
DHZROG	Dihydrozeatin 9-riboside *O*-glucoside
DHZRMP	Dihydrozeatin 9-riboside-5′-monophosphate
IAA	Indole-3-acetic acid
iP	N^6-(2-isopentenyl)adenine
iPR	N^6-(2-isopentenyl)adenine 9-riboside
iP7G	N^6-(2-isopentenyl)adenine 7-glucoside
iP9G	N^6-(2-isopentenyl)adenine 9-glucoside
iPRMP	N^6-(2-isopentenyl)adenine 9-riboside-5′-monophosphate
PSII	Photosystem II

K. Ananieva (✉) · S. Doncheva · D. Stefanov · K. Mishev
Institute of Plant Physiology and Genetics, Bulgarian Academy of Sciences, Acad. G. Bonchev Str., Bl. 21, 1113 Sofia, Bulgaria
e-mail: kalina@bio21.bas.bg

E. D. Ananiev (✉)
Department of Plant Physiology, Faculty of Biology, St. Kliment Ohridsky University of Sofia, 8 Dragan Tsankov blvd., 1164 Sofia, Bulgaria
e-mail: ananiev@biofac.uni-sofia.bg

M. Kamínek · V. Motyka · P. I. Dobrev · J. Malbeck
Institute of Experimental Botany, Academy of Sciences of the Czech Republic, Rozvojová 263, 165 02 Prague 6, Czech Republic

Г7_10

Darkness Affects Differentially the Expression of Plastid-Encoded Genes and Delays the Senescence-Induced Down-Regulation of Chloroplast Transcription in Cotyledons of *Cucurbita pepo* L. (Zucchini)

Kiril Mishev[a], Anna Dimitrova[b], and Evguéni D. Ananiev[c,*]

[a] Acad. M. Popov Institute of Plant Physiology, Bulgarian Academy of Sciences, Acad. G. Bonchev Str., Bl. 21, 1113 Sofia, Bulgaria
[b] Acad. D. Kostoff Institute of Genetics, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria
[c] Department of Plant Physiology, Faculty of Biology, St. Kl. Ohridski University of Sofia, 8 Dragan Tsankov Blvd., 1164 Sofia, Bulgaria. Fax: +359-2-856-56-41. E-mail: ananiev@biofac.uni-sofia.bg

* Author for correspondence and reprint requests

Z. Naturforsch. **66c**, 159–166 (2011); received June 21/October 22, 2010

In contrast to differentiated leaves, the regulatory mechanisms of chloroplast gene expression in darkened cotyledons have not been elucidated. Although some results have been reported indicating accelerated senescence in *Arabidopsis* upon reillumination, the capacity of cotyledons to recover after dark stress remains unclear. We analysed the effect of two-days dark stress, applied locally or at the whole-plant level, on plastid gene expression in zucchini cotyledons. Our results showed that in the dark the overall chloroplast transcription rate was much more inhibited than the nuclear run-on transcription. While the activities of the plastid-encoded RNA polymerase (PEP) and nuclear RNA polymerase II were strongly reduced, the activities of the nuclear-encoded plastid RNA polymerase (NEP) and nuclear RNA polymerase I were less affected. During recovery upon reillumination, chloroplast transcription in the cotyledons was strongly stimulated (3-fold) compared with the naturally senescing controls, suggesting delayed senescence. Northern blot and dot blot analyses of the expression of key chloroplast-encoded photosynthetic genes showed that in contrast to *psbA*, which remained almost unaffected, both the transcription rate and mRNA content of *psaB* and *rbcL* were substantially decreased.

Key words: Cotyledon Senescence, Dark Stress, NEP, PEP

Introduction

Darkness can affect the senescence progression in plants by modulating the photosynthetic efficiency, the generation of reactive oxygen species, as well as by activation of different signalling cascades (Lers, 2007). One of the earliest targets of dark-induced senescence is the chloroplast where a number of ultrastructural and functional alterations cause a rapid drop in the photosynthetic activity (Nooden *et al.*, 1997; Krupinska and Humbeck, 2004). Chloroplast senescence includes intensive degradation of pigments, membrane lipids, nucleic acids, and stroma-localized proteins such as ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco). The degradation of thylakoid proteins from photosystem II (PSII), photosystem I (PSI), and the light-harvesting complexes (LHCs) of both photosystems is accelerated at later stages of leaf senescence compared to the stromal proteins (Humbeck and Krupinska, 2003).

It has been shown that darkness can induce two opposite senescence-related responses depending on the level at which it is perceived (whole plant or individual leaf organ) as revealed by experiments with *Arabidopsis thaliana* rosette leaves (Weaver and Amasino, 2001). One response represents the locally induced promotion of senescence when dark treatment is applied to individual leaves. In these leaves, a rapid decline in photosynthetic activity has been found while high mitochondrial respiration is maintained associated with the

Abbreviations: DP, whole darkened plants; IDC, individually darkened cotyledons; NEP, nuclear-encoded plastid RNA polymerase; PEP, plastid-encoded RNA polymerase; PSI, PSII, photosystem I, photosystem II.

0939–5075/2011/0300–0159 $ 06.00

Г7_11

BIOLOGIA PLANTARUM 55 (2): 269-278, 2011

Organ-specific effects of dark treatment on photosynthesis and the expression of photosynthesis-related genes

K. MISHEV[1], E.D. ANANIEV[2] and K. HUMBECK[3]*

Institute of Plant Physiology, Bulgarian Academy of Sciences, Acad. G. Bonchev Str. 21, 1113 Sofia, Bulgaria[1]
Faculty of Biology, St. Kl. Ohridsky University of Sofia, 8 Dragan Tsankov Bld., 1640 Sofia, Bulgaria[2]
Institute of Biology/Plant Physiology, Martin-Luther University Halle-Wittenberg, Weinbergweg 10, 06120 Halle, Germany[3]

Abstract

The effects of two-day dark treatment, applied to whole plants or to individual organs, on the photosynthetic apparatus in cotyledons and first rosette leaves of young *Arabidopsis thaliana* plants were studied. Darkness affected the individually darkened pair of cotyledons as well as the cotyledons of whole darkened plants (DP) in a similar manner as revealed by the significant decrease in the actual yield of photosystem 2 electron transport and the down-regulation of the *psaB* and *rbcL* transcript levels. However, cotyledons and rosette leaves responded differently to darkness with respect to the non-photochemical quenching (NPQ) and the non-regulated energy dissipation (Φ_{NO}), indicating different capacity for photoprotection depending on the type of the applied dark treatment. Besides, the expression of the genes for the two plastid proteases FtsH5 and Deg1 involved in D_1 protein degradation was inhibited in both leaf organs, suggesting that these proteases function mainly under irradiance. Upon re-irradiation, dark-treated cotyledons recovered from the applied stress and during further senescence the changes in the photosynthetic parameters and the mRNA levels of *psaB*, *rbcL* and *SAG12* were similar as in the control plants. However, in the course of recovery typical chloroplast senescence symptoms were observed only in individually darkened leaves while re-irradiated DP leaves maintained high photosynthetic capacity.

Additional key words: cotyledons, photosystem 2, plastid protease, rosette leaves, senescence.

Introduction

The photosynthetic apparatus is highly dynamic and able to respond to environmental stresses, including changes in the quality and quantity of incident radiation (Szabo *et al.* 2005, Chowdhury *et al.* 2009), mineral starvation (Dannehl *et al.* 1996), drought and extreme temperatures (Smart 1994, Humbeck *et al.* 2007, Zhang *et al.* 2009). The transfer of individual intact or detached leaves, as well as whole plants, to darkness is a widely used experimental approach to study the molecular basis of the stress response and the adaptive mechanisms allowing plants to survive the adverse conditions (Biswal and Biswal 1984, Oh *et al.* 1996, Weaver *et al.* 1998). Darkness has been implicated in provoking a decline in total chlorophyll and protein contents, photosystem 2 (PS 2) activity and transcript levels of photosynthesis-related genes (Kleber-Janke and Krupinska 1997, Lu and Zhang 1998, Lin and Wu 2004). Moreover, transcripts of senescence-associated genes (*SAG*s), which normally appear during natural (age-mediated) senescence, have also been detected in dark-treated leaves (Azumi and Watanabe 1991, Weaver *et al.* 1998). On the other hand, genes induced during dark treatment are not necessarily expressed during natural senescence, suggesting that these two senescence processes are not identical (Becker and Apel 1993). As revealed by microarray analysis, the number of genes which are up-regulated during natural senescence is higher compared with those which are down-regulated, whereas in individually darkened intact or detached leaves the fractions are similar or opposite (Van der Graaff *et al.* 2006). In addition, only half of the genes up-regulated during developmental senescence are also strongly enhanced in leaves of darkened plants

Received 15 May 2009, *accepted* 24 April 2010.
Abbreviations: DP - whole plants transferred to darkness; IDC - individually darkened cotyledons; IDL - individually darkened first rosette leaves; NPQ - non-photochemical quenching; PS - photosystem; Φ_{NO} - quantum yield of the non-regulated energy dissipation; Φ_{PS2} - actual quantum yield of the PS 2 electron transport in the light-adapted state.
Acknowledgements: We acknowledge Nicole Ay for providing the primers for *SAG12* and Olaf Barth for his useful suggestions. The Land Sachsen-Anhalt is also thanked for financial support.
* Corresponding author; fax: (+49) 345 552 7245, e-mail: klaus.humbeck@pflanzenphys.uni-halle.de

Γ7_12

Biophysical Chemistry 109 (204) 387–397

Biophysical Chemistry

www.elsevier.com/locate/bpc

Melittin-induced changes in thylakoid membranes: particle electrophoresis and light scattering study

Virjinia Doltchinkova[a,*], Katya Georgieva[b], Nelly Traytcheva[b], Chavdar Slavov[a], Kiril Mishev[a]

[a]*Faculty of Biology, Department of Biophysics and Radiobiology, Sofia University, 8 Dragan Tzankov Boulevard, 1164 Sofia, Bulgaria*

[b]*Institute of Plant Physiology, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria*

Received 18 September 2003; received in revised form 18 September 2003; accepted 23 October 2003

Abstract

Thylakoids were used as a model system to evaluate the effect of bee venom peptide melittin (Mt) on membrane surface charge. At neutral pH, thylakoid membrane surfaces carry excess negative electrical charge. Mt strongly altered the electrophoretic mobility (EPM) of 'low-salt' thylakoids and did not significantly change the EPM of 'high-salt' thylakoids. Mt increased the primary ionic-exchange processes across the 'low-salt' thylakoid membranes, while it did not affect those of 'high-salt' thylakoids. Mt decreased the proton gradient generation on the membranes at both ionic strengths, but it affected more strongly the 'high-salt' than that of 'low-salt' thylakoids. The primary photochemical activity of photosystem II, estimated by the ratio F_v/F_m, was not influenced by the low Mt concentrations. It decreased only when chloroplasts had been incubated with higher Mt concentrations and this effect was better expressed in 'low-salt' than in 'high-salt' thylakoid membranes.

Keywords: Melittin; Thylakoid membranes; Electrophoretic mobility; Surface charge; Light scattering; Chlorophyll fluorescence

1. Introduction

Melittin (Mt), the main component of the European honeybee *Apis mellifera*, is a cationic amphiphilic peptide, which binds to membranes. Mt is a surface-active, amphipathic peptide and serves as a useful model for a variety of membrane interactions [1]. Mt exhibits voltage-dependent channel formation in lipid bilayers [2]. Mt carries highly hydrophilic residues (2 Lys, 2 Arg, 2 Gln at the C-terminus) [3]. Its binding to negatively charged membranes was distinctly enhanced compared to neutral membranes. Because of its amphiphilic properties, it can disrupt lipid bilayers like detergents [4,5]. It was also suggested the possibility of action of Mt on membrane proteins

Abbreviations: EPM, electrophoretic mobility; LS, light scattering; Mt, melittin; PMS, phenazine methosulfate; Chl, chlorophyll; F_0, initial chlorophyll fluorescence; F_v, variable chlorophyll fluorescence; F_m, maximum chlorophyll fluorescence; PS I, photosystem I; PS II, photosystem II.

*Corresponding author. Tel.: +359-2-63-30-281; fax: +359-2-656-641.

E-mail address: virjird@biofac.uni-sofia.bg (V. Doltchinkova).

0301-4622/4/$ - see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/j.bpc.2003.10.030