ISSN 1022-7954, Russian Journal of Genetics, 2018, Vol. 54, No. 11, pp. 1378–1382. © Pleiades Publishing, Inc., 2018.
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SHORT COMMUNICATIONS

Development of Methods for the Target-Specific Protein Elimination in Plants

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Received December 18, 2017; in final form, May 30, 2018

Abstract—Here we present the method for the target-specific elimination of certain intracellular proteins in plants using the ubiquitin-proteasome system. We modified the E3 ubiquitin ligase Chip of *A. thaliana* to obtain two variants carrying the deletions at the N-terminus and the GFP recognition domain. The interaction of the GFP protein and the chimeric ubiquitin ligase was confirmed via yeast two-hybrid assay. Fluorescence microscopy and fluorimetry showed that, when an infiltration of the *gfp*-expressing *N. benthamiana* plants was performed with agrobacteria carrying the hybrid E3 gene of the ubiquitin ligase *Chip* with the GFP recognition domain, a significant decrease in the fluorescence was observed for both variants: carrying the N-terminal deletion of 100 amino acids or the deletion of 140 amino acids.

Keywords: ubiquitin, proteasome, ubiquitin ligase E3, plant, cell, *Chip*, *gfp*

DOI: 10.1134/S1022795418110091

The discovery of the RNA interference phenomenon gave researchers a powerful tool for studying the functions of genes by suppressing their expression. It is still very actively used in studying the functions of plant genes [1–6]. However, the use of RNA interference is impossible in systems where there is no mRNA of the target protein, such as when the cells are infected with a pathogen, and the phytopathogen introduces virulence factors into the host cell using its transport system. For their inactivation, protein interference can be used, i.e., directed degradation of target proteins with the help of intrinsic cell systems.

One such system is the ubiquitin-proteasome degradation system. The selection of substrates for proteolysis is ensured by the fact that the entrance to the proteasome is usually closed, and only proteins bearing a special label can enter it. A chain of polyubiquitin (polyUb) acts as a label, and those proteins that are conjugated to polyUb consisting of at least four ubiquitin monomers (Ub) are degraded.

In eukaryotes, most substrates of the proteasome are polyubiquitinated, and the ubiquitylation process is performed in a cascade of reactions catalyzed by three enzymes: E1, E2, and E3. At the first stage, the ubiquitin-activating enzyme E1, using ATP, activates ubiquitin by the formation of a high-energy thioester intermediate (E1-S~Ub). Then, one of the ubiquitin-transferring enzymes, E2 (ubiquitin-carrier protein, UBC), through the formation of one more intermediate (E2-S~Ub), transfers the activated ubiquitin to E3 ligase specifically bound to the substrate. In the case of RING-domain-containing E3 ligases, ubiquitin is transferred by ligase directly to the substrate. In the case of NEST-domain-containing E3 ligases, the transfer of ubiquitin to the substrate occurs through the formation of one more intermediate—E3-S~Ub. After the attachment of the first ubiquitin to the substrate, the E3 ligase sequentially adds several more Ub molecules to the lysine residue on the first ubiquitin molecule. The minimum degradation signal for a proteasome is a chain of four ubiquitin molecules sequentially connected by an isopeptide bond between the C-terminus of one molecule and the lysine of another molecule [7].

In *A. thaliana* plants, about 5% of all genes are involved in ubiquitylation processes. At the same time, more than 1000 genes are E3 ubiquitin ligases, which perform specific degradation of target proteins [8]. The use of one of these genes and its modification to recognize a specific target make it possible to create a method for regulating the expression of genes in plants at the post-translational level—directed elimination of the protein product by the ubiquitin-proteasome system of the cell. The potential possibility of using the proposed approach was demonstrated in [9], where on human cell lines the authors succeeded in "training" E3 ubiquitin ligase to recognize some proteins and carry out their degradation with the help of the ubiquitin-proteasome system.

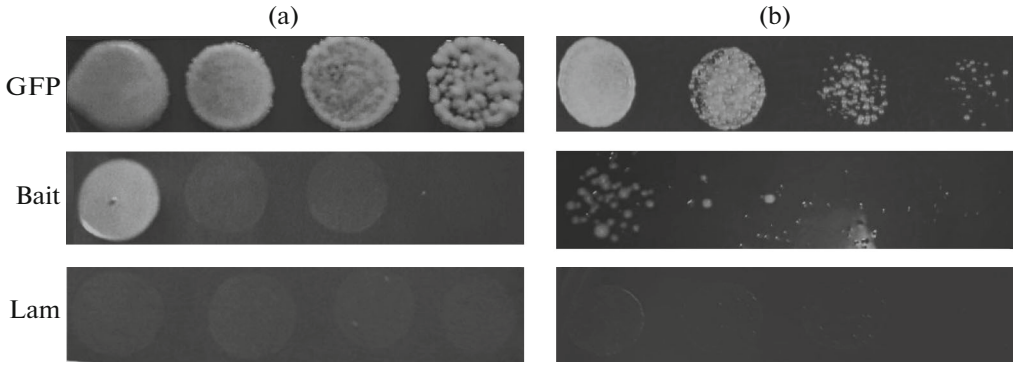

Fig. 1. Joint cotransformation of *Chip100mbody* with GFP (a) and *Chip140mbody* (b) with the evaluation of the interaction by the yeast two-hybrid system. Negative controls were empty "bait"—pGBKT7 and pGBKT7 vectors, carrying the Lamin-C human protein gene, the product of which does not interact with either *Chip100mbody* or *Chip140mbody*. A culture of cells with a density of 0.2 at A600 and 10-fold dilution was used.

To create a convenient model for the selective elimination of proteins by the ubiquitin-proteasome pathway, it is necessary to select a pair of interacting proteins that could be easily detected. A green fluorescent protein (GFP) and a monobody were selected for this purpose [10]. A monobody is an artificially created protein that functions on the same principle as antibodies (although it is not an antibody itself) and is created using combinatorial libraries and phage and mRNA display methods based on the FN3 domain of fibronectin type III.

The gene coding for the monobody sequence was chemically synthesized on the basis of the amino acid sequence of a monobody (VSSVPTKLEVVAATPTSLLISWDAPAVTVDHYYITYGETGHYWYYQAFAVPGSKSTATISGLSPGVDYTITVYAPFSVPVMSPISINYRT), taking into account the codon frequency in plants and yeast and the absence of splicing sites. The interaction of the GFP protein and monobody was confirmed using yeast two-hybrid analysis. The analysis was performed by cotransformation of AH109 yeast cells by two plasmids: pGADT7, carrying the *gfp* gene, paired with pGBKT7, carrying the monobody gene.

The gene *Chip* (AT3G07370) of *A. thaliana* was chosen as the basis for the construction of E3 ubiquitin ligase with a modified domain responsible for the recognition of the target GFP protein. *A. thaliana* ubiquitin ligase Chip refers to ubiquitin ligases of E3-U-box type, which performs specific degradation of target proteins.

It is known that the N-terminal TPR domain of ubiquitin ligase Chip of *Arabidopsis thaliana* is responsible for recognition of the substrate (Fig. S1, see Appendix in the electronic version of the journal). Using molecular cloning techniques, we obtained two variants of the *Chip* gene, devoid of 100 and 140 amino acids at the N-terminus of the protein molecule, and fused in the reading frame with the monobody gene (*Chip100mbody* and *Chip140mbody*, respectively). Verification of the protein-protein interactions of the resulting chimeric proteins with the target—GFP protein—was performed using a yeast two-hybrid system. As a result, interaction of the ubiquitin ligase chimeric proteins bearing the target recognition domain (monobody) and the GFP protein (Fig. 1) in the absence of this interaction with the Lamin-C human protein (negative control) was shown.

For the analysis and effective visualization of the work of created selective protein elimination system in plant cells, transgenic *N. benthamiana* plants expressing the *gfp* gene under the control of the 35S promoter of the cauliflower mosaic virus were obtained. *N. benthamiana* plants, in contrast to *N. tabaccum*, are easily and effectively subjected to transient expression by agroinfiltration of a suspension of agrobacteria into a plant leaf.

N. benthamiana plants were introduced into the culture in vitro using standard protocols for *N. tabaccum*. For the transformation of *N. benthamiana* plants, a vector where a *gfp* gene was placed under the control of the 35S promoter of the cauliflower mosaic virus was created. The *nptII* gene was used as a selective marker, which determines the resistance of plants to kanamycin. Transformation was carried out by the method of leaf disks, resulting in three explants with a resistance to a selective antibiotic. A full-grown plant was obtained from each explant and seeds were collected. Plants of the next generation were analyzed for localization of insertion and GFP expression. In all three lines of the obtained plants, the insert was localized in the intergenic regions. The presence of the GFP protein was checked by fluorescence microscopy (Fig. S2, Appendix).

Plant vectors based on pCXSN plasmid in which the gene encoding the chimeric enzyme was placed under the control of the 35S strong constitutive promoter of the cauliflower mosaic virus were created for transient expression of the hybrid E3 ubiquitin-ligase Chip genes fused to the monobody to the GFP protein

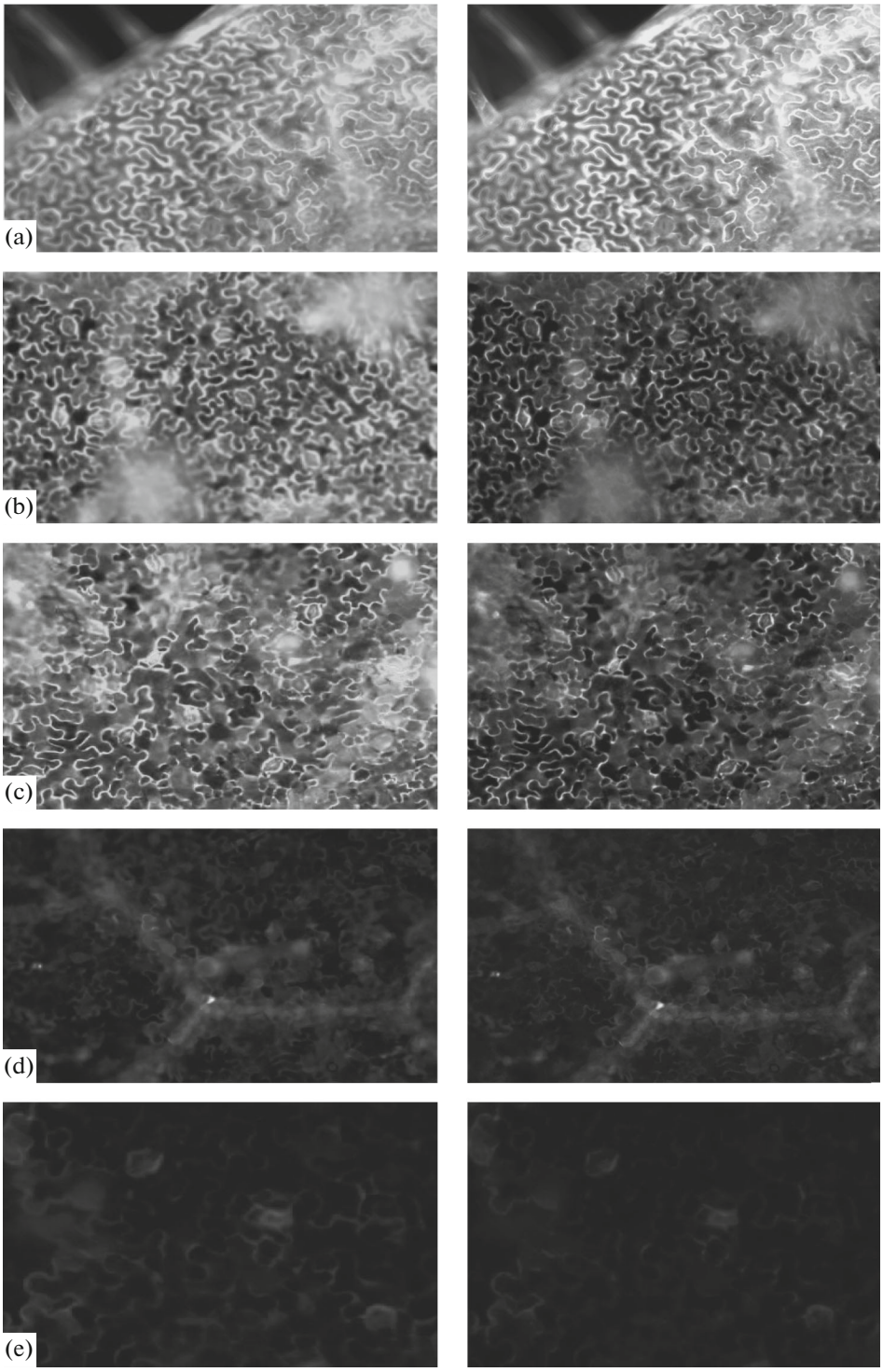

Fig. 2. Fluorescence of GFP protein in the leaves of the transgenic *N. bentamiana* plants. (a) GFP in a zone without infiltration (exposure 500 ms). (b) Control. Fluorescence in the infiltration zone of agrobacteria carrying the empty pCXSN vector 7 days after infiltration (exposure 500 ms). (c) Control. GFP fluorescence in the infiltration zone of agrobacteria bearing the pCXSN-Chip vector 7 days after infiltration (exposure 500 ms). (d) Fluorescence in the infiltration zone of agrobacteria carrying the pCXSN-Chip100mbody vector 7 days after infiltration (exposure 500 ms). (e) Fluorescence in the infiltration zone of agrobacteria carrying the pCXSN-Chip140mbody vector 7 days after the infiltration (exposure 500 ms).

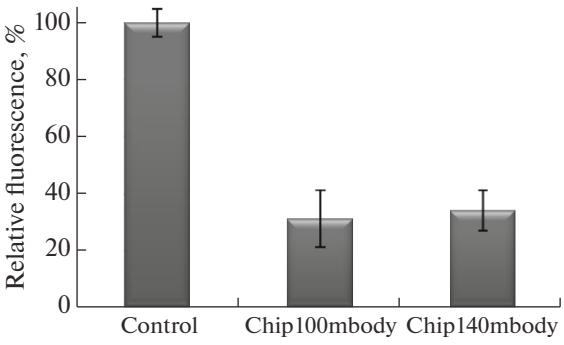

Fig. 3. Change in the fluorescence level of GFP protein in the infiltration zones, %. Chip140mbody and Chip100mbody—the ratio of the average specific fluorescence value of GFP in the infiltration zone of agrobacteria carrying the pCXSN-Chip140mbody and pCXSN-Chip100mbody vectors, respectively, to the fluorescence level of GFP in the control samples. The fluorescence level of GFP in the control samples is assumed to be 100%. The average values obtained from three independent measurements with triplicates, ±standard deviation, are given. The fluorescence was measured using a Spark 10M reader (Tecan, Switzerland).

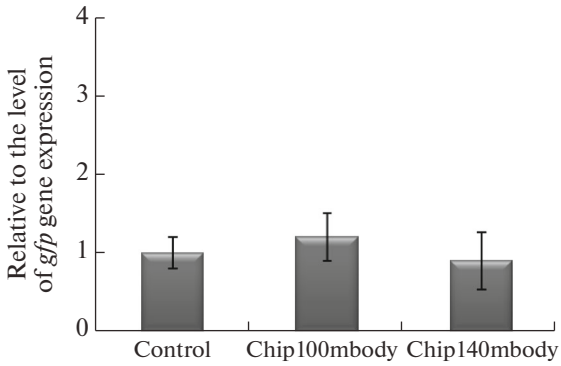

Fig. 4. The level of mRNA of *gfp* gene in the leaves of the transgenic *N. benthamiana* plant upon the infiltration with agrobacterium suspension carrying plasmids for the expression of the hybrid ubiquitin ligase genes: pCXSN-Chip100mbody, pCXSN-Chip140mbody. The level of *gfp* expression in the leaves of the transgenic *N. benthamiana* plant infiltrated with the empty control pCXSN vector was used as a reference. The average values obtained from three independent measurements with triplicates, ±standard deviation, are given. Evaluation of the expression was performed using the Eco Real-Time PCR System (Illumina, United States).

in the plant cells. The resulting vectors were used to transform the cells of C58 *Agrobacterium tumefaciens* bacteria. Infiltration of the leaves of *N. benthamiana* plants was carried out using a standard protocol [11].

The GFP protein was detected using the fluorescence microscopy method (Fig. 2), and the fluorescence intensity values in the control and test samples were determined by fluorimetry (Fig. 3).

It was shown that, when infiltrating *N. benthamiana* plants expressing the *gfp* gene with agrobacteria bearing the hybrid gene of E3 ubiquitin ligase *Chip* with the GFP domain recognition protein, a significant decrease in the fluorescence signal was observed for both variants, in the deletion of the 100 and 140 amino acid N-terminal domain. Thus, when infiltrating *N. benthamiana* plants expressing the *gfp* gene with agrobacteria bearing the *Chip100mbody* gene, the fluorescence level of the GFP protein with respect to control was reduced by 60–75%, and when infiltrating with agrobacteria bearing the *Chip140mbod* gene, the fluorescence level of the GFP protein relative to control was reduced by 60–80%. In this case, all control variants—in the zone without infiltration, in the infiltration zone of agrobacteria bearing the empty pCXSN vector, and in the infiltration zone of agrobacteria carrying the pCXSN-Chip vector (without the monobody to GFP)—do not change the fluorescence of the GFP protein.

To verify the obtained results, RNA was isolated for each analyzed sample, cDNA was synthesized, and quantitative real-time PCR was performed. The actin (AY594294) and β-tubulin (At5g12250) genes were used as housekeeping genes. The mRNA level of the *gfp* gene in the leaves of the transgenic *N. benthamiana* plant after infiltration by a suspension of agrobacterium carrying plasmids for the expression of hybrid ubiquitin ligase genes, pCXSN-Chip100mbody and pCXSN-Chip140mbody, is shown in Fig. 4.

The level of expression of the *gfp* gene in the leaves of the transgenic *N. benthamiana* plant in infiltration with a pCXSN empty control vector was taken as reference. The values presented are the mean ± standard deviation of three independent experiments with triplicates. It was shown that the level of the *gfp* gene expression in the infiltration zones in the control and in the experiment did not change, which indicates the regulation of the amount of accumulated GFP protein in plant cells, not at the expression level, but at the protein level.

Thus, we created a system of selective protein elimination in plant cells making it possible to effectively degrade plant proteins along the ubiquitin-proteasome pathway.

ACKNOWLEDGMENTS

This work was performed with the support from the Russian Foundation for Basic Research, project no. 15-04-09365 A.

REFERENCES

1. de Ronde, D., Butterbach, P., and Kormelink, R., Dominant resistance against plant viruses, *Front. Plant Sci.*, 2014, vol. 5, p. 307. doi 10.3389/fpls.2014.00307
2. Szittya, G. and Burgyán, J., RNA interference-mediated intrinsic antiviral immunity in plants, *Curr. Top.*

Microbiol. Immunol., 2013, vol. 371, pp. 153—181. doi 10.1007/978-3-642-37765-5_6

3. Castel, S.E. and Martienssen, R.A., RNA interference in the nucleus: roles for small RNAs in transcription, epigenetics and beyond, *Nat. Rev. Genet.*, 2013, vol. 14, no. 2, pp. 100—112. doi 10.1038/nrg3355
4. Li, J., Todd, T.C., Lee, J., and Trick, H.N., Biotechnological application of functional genomics towards plant-parasitic nematode control, *Plant Biotechnol. J.*, 2011, vol. 9, no. 9, pp. 936—944. doi 10.1111/j.1467-7652.2011.00601.x
5. McGinnis, K.M., RNAi for functional genomics in plants, *Brief Funct. Genomics*, 2010, vol. 9, no. 2, pp. 111—117. doi 10.1093/bfgp/elp052
6. Tiwari, M., Sharma, D., and Trivedi, P.K., Artificial microRNA mediated gene silencing in plants: progress and perspectives, *Plant Mol. Biol.*, 2014, vol. 86, nos. 1—2, pp. 1—18. doi 10.1007/s11103-014-0224-7
7. Thrower, J.S., Hoffman, L., Rechsteiner, M., and Pickart, C.M., Recognition of the polyubiquitin proteolytic signal, *EMBO J.*, 2000, vol. 19, pp. 94—102. doi 10.1093/emboj/19.1.94
8. Mazzucotelli, E., Belloni, S., Marone, D., et al., The E3 ubiquitin ligase gene family in plants: regulation by degradation, *Curr. Genomics*, 2006, vol. 7, no. 8, pp. 509—522. PMCID: PMC2269001
9. Portnoff, A.D., Stephens, E.A., Varner, J.D., and DeLisa, M.P., Ubiquibodies, synthetic E3 ubiquitin ligases endowed with unnatural substrate specificity for targeted protein silencing, *J. Biol. Chem.*, 2014, vol. 289, pp. 7844—7855. doi 10.1074/jbc.M113.544825
10. Koide, A., Wojcik, J., Gilbreth, R.N., et al., Teaching an old scaffold new tricks: monobodies constructed using alternative surfaces of the FN3 scaffold, *J. Mol. Biol.*, 2012, vol. 415, no. 2, pp. 393—405. doi 10.1016/j.jmb.2011.12.019
11. Leuzinger, K., Dent, M., Hurtado, J., et al., Efficient agroinfiltration of plants for high-level transient expression of recombinant proteins, *J. Visualized Exp.*, 2013, vol. 77, p. 50521. doi 10.3791/50521

Translated by P. Kuchina