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https://orcid.org/0000-0002-2553-8961

VILNIUS UNIVERSITY

Erna
DENKOVSKIENĖ

Construction of inducible expression systems for controlled transfer of *Agrobacterium tumefaciens* T-DNA in plant transient expression systems

DOCTORAL DISSERTATION

Natural Sciences,
Biochemistry (N 004)

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The study presented in this doctoral dissertation has been carried out during doctoral studies (2010–2014) at the Institute of Biotechnology of Vilnius University in a collaboration with Nomads UAB, during 2010–2021. The dissertation is defended on an external basis.

Academic consultant:

Dr. Aušra Ražanskienė (Vilnius University, Natural Sciences, Biochemistry – N 004).

This doctoral dissertation will be defended in a public meeting of the Dissertation Defence Panel:

Chairman – **Prof. Dr. Saulius Serva** (Vilnius University, Natural Sciences, Biochemistry – N 004).
Members:
Dr. Julija Armalytė (Vilnius University, Natural Sciences, Biochemistry – N 004).
Dr. Dukas Jurėnas (French National Centre for scientific research, Natural Sciences, Biochemistry – N 004).
Prof. Dr. Rolandas Meškys (Vilnius University, Natural Sciences, Biochemistry – N 004).
Dr. Eugenijus Šimoliūnas (Vilnius University, Natural Sciences, Biochemistry – N 004).

The dissertation shall be defended at a public meeting of the Dissertation Defence Panel at 2 p.m. on 25th November 2021 in auditorium R401 of the Life Sciences Centre of Vilnius University. Address: Saulėtekio Ave. 7, R401, Vilnius, Lithuania. Tel. +370 620 39 370; e-mail: erna.denkovskiene@gmail.com
The text of this dissertation can be accessed at the library of Vilnius University, as well as on the website of Vilnius University: *www.vu.lt/lt/naujienos/ivykiu-kalendorius*

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VILNIAUS UNIVERSITETAS

Erna
DENKOVSKIENĖ

Indukuojamų raiškos sistemų, skirtų griežtam *Agrobacterium tumefaciens* T-DNR pernašos reguliavimui augalų laikinos raiškos sistemose, konstravimas

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Mokslinė konsultantė

Dr. Aušra Ražanskienė (Vilniaus universitetas, gamtos mokslai, biochemija – N 004).

Gynimo taryba:
Pirmininkas – prof. dr. Saulius Serva (Vilniaus universitetas, gamtos mokslai, biochemija – N 004).
Nariai:
dr. Julija Armalytė (Vilniaus universitetas, gamtos mokslai, biochemija – N 004).
dr. Dukas Jurėnas (Prancūzijos nacionalinis mokslinių tyrimų centras, gamtos mokslai, biochemija – N 004).
prof. dr. Rolandas Meškys (Vilniaus universitetas, gamtos mokslai, biochemija – N 004).
dr. Eugenijus Šimoliūnas (Vilniaus universitetas, gamtos mokslai, biochemija – N 004).

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CONTENTS

LIST OF ABBREVIATIONS

AHL	Acyl homoserine lactone
AMCV	Artichoke mottle crinkle virus
AMP	Adenosine monophosphate
AS	Acetosyringone
ATc	Anhydrotetracycline
ATP	Adenosine triphosphate
bp	Base pair
CAF-1	Chromatin assembly factor one
CFU	Colony forming units
CP	Coat protein
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CumA	Cumic acid
CuO	Cumate operator
DIMBOA	2,4-dihydroxy-7-methoxy-2H-1,4-benzixazin-3-(4H)-one
DMSO	Dimethyl sulfoxide
dpi	Days post infiltration
dps	Days post spray
DRM	DNA recognition module
ESM	Environmental sensing modules
GAN	General adversarial networks
GFP	Green fluorescent protein
GMM	Genetically modified microorganism
GMO	Genetically modified organism
GOI	Gene of interest
GUS	β-glucuronidase
HR	Hypersensitive response
IAA	Indole-3-acetic acid
IAM	Indole-3-acetamide
IPTG	Isopropyl β-D-1-thiogalactopyranoside
Km	Kanamicin
lacO	Lac operator
LB	Left border repeat
LB (medium)	Luria-Bertani
MDIBOA	2-hydroxy-4,7-dimethoxybenzoxazin-3-one
MP	Movement protein
mRNA	Messenger RNA
MU	Miller units

NAD(P)H	Nicotinamide adenine dinucleotide phosphate reduced form
ncAA	Non-coading amino acids
NLS	Nuclear localisation signal
NPC	Nuclear pore complex
OD	Optical density
PCR	Polymerase chain reaction
PVX	Potato virus X
qPCR	Quantitative polymerase chain reaction
RASTA	Rapid and automated scan for toxins and antitoxins
RB	Right border repeat
rpm	Rotations per minute
SA	Salicylic acid
SAR	Systemic acquired resistance
SD	Standard deviation
siRNA	Small interfering RNA
SOB	Super optimal broth
sRNA	Small RNA
TA	Toxin-antitoxin
TBSV	Tomato bushy stunt virus
T-DNA	Transferred DNA
TF	Transcription factor
TMV	Tobacco mosaic virus
UV	Ultraviolet light
WT	Wild type

INTRODUCTION

Plants were discovered as new potential hosts for recombinant protein expression in the early eighties. Promising initial experiments raised to the understanding that various desired proteins can be successfully produced in plants and promoted the rapid development of plant genetic engineering methods, accumulation of valuable experimental data and ideas for the application of plants as bio-factories. Two main strategies used for plant modification are stable nuclear plant transformation and transient plant transfection. During the stable nuclear transformation, foreign DNA sequences are inserted into a plant cell nuclear genome, transgenic plants are created, and transgenes can be inherited and expressed in successive generations of plants. Differently, when plants are transfected transiently, grown up plants are transfected, the transgene is expressed from non-integrated DNA, plants are used only as expression hosts and are harvested after recombinant protein was accumulated in their tissues. Some stable insertions into the plant genome can occur during the transient plant transfection, but transgenic plants are not sought to be created. The principal tool used for both stable and transient transfer of transgenes into plants is *Agrobacterium tumefaciens*, a soil plant pathogenic *bacterium*, which has a natural and unique ability of horizontal gene transfer between organisms of different phylogenetic kingdoms. *A. tumefaciens* causes a crown gall disease on a wide variety of dicotyledonous plants. With the help of its virulence proteins, induced by plant factors, *Agrobacterium* transfers a DNA fragment from its Ti plasmid, called T (transferred)-DNA, harbouring opine synthase genes, producing opines, an important source of nitrogen and carbon for *A. tumefaciens*, and oncogenes responsible for tumour growth, ensuring further production of nutrients needed for the survival of *Agrobacterium*. For use in a laboratory, *Agrobacterium* was genetically modified so that it lost its pathogenicity but retained its ability to transfer DNA into the host plant.

Stable *Agrobacterium*-mediated transformation is irreplaceable for many aspects of molecular plant biology and agriculture is applied and reported in many plant species. However, in many cases, *Agrobacterium*-mediated transient plant transfection is a more convenient approach as it is time-saving and allow induction of transferred gene expression rapidly (from hours to several days) and produce large amounts of recombinant proteins in days. In comparison, several months or years are needed to create a transgenic plant. Transient expression systems have been created, improved, and applied to express different recombinant proteins (antibodies, vaccines, enzymes) in plants in many research laboratories and companies[1]. Our laboratory took part

in the research of transient plant transfection systems with a particular goal – to enhance the biosafety of transient transfection for open environment applications in agrobiotechnology. Outdoor cultivation is superior to cultivation in contained facilities because of lower capital investments required for plant biomass production, no need for substrate or nutrient solution management and other factors. However, the release of genetically modified *Agrobacterium* strains used for plant transfection into the open environment causes potential biosafety issues. Several strategies are created for biocontainment of genetically modified microorganisms (GMM), multiple auxotrophy and genetic circuits based on the insertion of expression systems controlling the survival of GMM, among them. However, only the combination of several strategies can minimize the risks for the environment as systems may be inactivated because of horizontal gene transfer or mutagenesis. Besides the biocontainment, the unintended spread of the transgene can be controlled by regulating the T-DNA transfer process so that GM *Agrobacterium* would only be able to transfer T-DNA in specific conditions, for example, in the presence of the specific inducer molecule. Two different approaches for the construction of regulated T-DNA transfer were used in a dissertation study.

For the first approach, vectors with inducible *virE2* were constructed. The VirE2 participates in protecting *Agrobacterium* T-DNA in a plant cell and is one of the essential virulence proteins that allow for T-DNA transfer. *VirE2* mutants of *A. tumefaciens* almost lose their ability to transfer T-DNA [2]. In our search for the strongest and most stringently regulated promoters in *Agrobacterium*, for the regulated expression of *virE2*, the efficiency of conventional IPTG inducible promoters P_{lac}, P_{tac}, $P_{T5/lacOlacO}$, $P_{T7/lacO}$, and the cumic acid inducible hybrid promoters $P_{T5/CuO}$, $P_{tac/CuO}$, $P_{lacUV5/CuO}$ and $P_{virE/CuO}$ in *A. tumefaciens* was evaluated. Cumic acid (or cumate)-inducible expression system, referred to as a cumate gene switch, is based on the elements of *Pseudomonas putida* F1 *cym* and *cmt* operons. It is composed of repressor gene *CymR*, wich expression is driven by a weak constitutive kanamycin promoter (Km), and operator *CuO* which is inserted behind the promoter of interest. The addition of inducer removes the repressor CymR from the operator and allows gene transcription from a promoter. Cumic acid inducible systems showed very low background expression of *virE2* and were highly inducible in *A. tumefaciens*, which allowed an efficient control of transfection of *Nicotiana benthamiana* plants, model plants often used for evaluation of transient expression systems and agrobiotechnology.

For the second approach, a transient expression system was constructed for preventing unintended T-DNA transfer by using the potential of natural

bacterial toxin-antitoxin (TA) modules. TA modules are abundant genetic elements in bacteria and archaea and function in a cellular stress tolerance, phage infection inhibition or maintenance of genetic elements. TA systems are composed of two or more genes that encode a toxic molecule, the toxin, and toxin neutralizing molecule, the antitoxin. Several putative toxins and antitoxins from TA modules were identified in the *Agrobacterium* genome, toxins' ability to cause bacteria death when overexpressed ectopically was demonstrated in dissertation work. Next, the *pemIK* toxin-antitoxin pair was used to construct a transient expression system, the action of toxin and antitoxin modulated for strictly regulated T-DNA transfer. Model *Nicotiana benthamiana* plants were used for transient transfection experiments.

The goal of the study

- To construct and evaluate inducible expression systems for a tightly controlled T-DNA transfer from *Agrobacterium tumefaciens* bacteria to *Nicotiana benthamiana* plants.

Objectives of the study

- To identify, construct and evaluate inducible promoters suitable for tightly regulated expression of genes of interest in *A. tumefaciens*.

- To construct expression vectors for inducible expression of *Agrobacterium* virulence gene *virE2*.

- To evaluate the level of T-DNA transfer using the *ΔvirE2 A. tumefaciens* strain and selected best performing vectors for *virE2* expression.

- To identify active toxins from *A. tumefaciens* toxin-antitoxin systems and evaluate their activity against agrobacteria.

- To evaluate the efficacy of a regulated T-DNA transfer system in *A. tumefaciens* based on different regulations of toxin and antitoxin expression.

Scientific novelty

At the beginning of our research, the information about the over-expression and expression control of foreign genes in *Agrobacterium* was scarce. Our study, for the first time, provided detailed information about relative activity and regulation of several inducible promoters (P_{lac}, P_{tac}, $P_{T5/lacOlacO}$, $P_{T5/CuO,}$ $P_{T7/lacO}$) in *A. tumefaciens.* By being not satisfied by obtained results, we constructed and evaluated several new strong and tightly regulated promoters $P_{tac/CuO}$, $P_{lacUV5/CuO}$ and $P_{virE/CuO}$ for expression in *Agrobacterium*. These new promoters were used for controlled expression of essential agrobacterial virulence protein coding gene *virE2*, which resulted into stringent regulation of T-DNA transfer from *Agrobacterium* to the host plant *N. benthamiana*.

For further perfection of the biosafe transient expression system in plants, several *A. tumefaciens* toxins were identified from putative TA systems, and their activity evaluated. Toxins Dead, PIN, VapC, IetS and PemK showed high efficiency against *A. tumefaciens*. Further, an efficient regulated T-DNA transfer system, based on inducible expression of toxin *pemK* and antitoxin *pemI*, was constructed and evaluated, thus providing a supplementary approach for higher biosafety of *Agrobacterium*-mediated transient transfection of plants.

Major findings presented for defence in the thesis

- Cumic acid inducible promoters $P_{T5/CuO}$, $P_{tac/CuO}$, $P_{lacUV5/CuO}$ and $P_{virE/CuO}$ are active and tightly regulated in *A. tumefaciens.*

- Controlled expression of *Agrobacterium* virulence factor *virE2* in *virE2* deficient *A. tumefaciens* strains results in an efficient and tightly regulated T-DNA transfer from *Agrobacterium* to plants.

- Agrobacterial toxins PemK, PIN, VapC, IetS and Dead from bacterial toxin-antitoxin modules are capable of causing *A. tumefaciens* cells death *in vitro.*

- Controlled expression of toxin *pemK* and antitoxin *pemI* from agrobacterial toxin-antitoxin module *pemIK* results in an efficient regulation of T-DNA transfer from *Agrobacterium* to plants.

1. REVIEW OF LITERATURE

1.1. Plants as recombinant protein expression hosts

Plants were discovered as new potential biofactories almost four decades ago. The first recombinant plant-derived pharmaceutical protein, a chimera of agrobacterial nopaline synthase and human growth hormone, was expressed in tobacco and sunflower callus tissue in 1986 [3]. Only ten years later, transgenic plants were already used as commercial expression hosts for the production of hen egg protein avidin [4]. These and many later achievements proved plants to be suitable for the large-scale recombinant protein production, including very complex functional mammalian proteins such as antibodies, vaccine antigens, hormones and enzymes [5]. Similarly to other eukaryotic systems, plants showed their ability to modify proteins post-translationally and maintain their structure and functionality for therapies [6].

While bacterial, yeast, insect and mammalian cells remain the most commonly used hosts for the commercial expression of recombinant biopharmaceuticals, plant expression platforms are rising as next generation alternatives. As an advantage over other expression systems, plant systems have a very high scalability. The bioreactors can be replaced with greenhouses or even open fields, which significantly reduces energy and material consumption in upstream applications. An advantage is even more significant if we think about edible fruits, tubers and leafy crops expressing various antigens and simply used as oral vaccines. Recent studies concerning plant-derived oral vaccines have covered a broad range of diseases, including respiratory infections, gastroenteritis, hepatitis, HIV/AIDS, cervical cancer, malaria, botulism and toxoplasmosis [7]. An oral vaccine approach reduces costs of downstream processing as protein extraction and purification steps are unnecessary. Moreover it reduces cold storage and transportation costs. Plant-produced proteins already include protein biopolymers, such as plant-expressed human collagen, used for wound healing and skin tissue engineering [8, 9], industrial proteins and enzymes such as cellulases, produced for conversion of cellulosic biomass into ethanol as a fuel extender [10] and also, therapeutic proteins, such as monoclonal antibodies (mAbs), cytokines, enzymes, growth factors, growth hormones and vaccine antigens [11]. Plant production systems include microalgae, cells, hairy roots, moss, and whole plants with either stable (transgenic plants) or transient transgene expression. For entire plants, both field and automated greenhouse cultivation methods are used with products expressed and produced either in leaves or seeds. Many

successful expression systems currently exist for a variety of different products including already successfully commercialised ones and ones still in clinical development [1,12].

1.2. Plant transformation strategies

A stable nuclear transformation is the most common gene engineering approach used for genetic modification of plants. During the stable nuclear transformation, foreign DNA sequences of interest are inserted into a plant cell nuclear genome. The two most popular and efficient methods for a nuclear transformation are biolistics (or microprojectile bombardment) and *A. tumefaciens* mediated transfer.

1.2.1. Biolistics

In biolistics approach, gold or tungsten particles are covered with DNA vectors containing a gene of interest and accelerated to a high speed with a particle gun, targeting plant cell culture or parts of a grown-up plant, such that they penetrate plant tissues [13]. Subsequently, plant cells culture undergo selective regeneration process when plant cells, transformed and resistant to introduced antibiotics, proliferate and differentiate to a grown-up plant. Thus, a transgenic plant is obtained from one transformed cell, and all cells of the plant have a foreign gene of interest inserted. However, the method is not as simple as it could seem, as more than one copy of transgene can be inserted into each genome and thus result in significant variations of foreign gene expression levels between transgenic individuals. Besides that, a foreign gene can be inserted into silenced regions of a plant genome and thus not be expressed [14, 15]. The biolistics is also used for stable transformation of plant cell plastids (most commonly – chloroplasts), as an alternative to a nuclear transformation, and has some advantages over the nuclear transformation. The plastid genome of higher plants is depicted as a circular molecule of 120–160 kb, containing approximately 130 genes. The main advantage of this transformation method is an opportunity to insert a transgene to the exact location of the plastid genome, as homologous recombination, mediated by a bacterial-like RecA-based system, occurs in plant plastids – whereas nuclear transformation is always random. Such integration into a preselected genome region enables an insertion of a single copy of a foreign gene. This prevents uncontrollable variation in the expression of transgenes which often occurs after nuclear transformation. In addition, some proteins are more efficiently

expressed in plastids than in plant cell cytoplasm because of a different set of proteases and different post-translational modifications [16]. However, a stable and reliable insertion of transgene is usually obtained only after several generations of the transformed plant. Therefore, this process requires careful selection and time – from several months to several years depending on species of plants.

1.2.2. *Agrobacterium* mediated plant transformation

1.2.2.1. Mechanisms of *Agrobacterium* mediated transformation of plants

A soil *bacterium Agrobacterium tumefaciens* is the best-known member of the genus *Agrobacterium*, which has a natural and unique ability of horizontal gene transfer between organisms of different phylogenetic kingdoms. *A. tumefaciens* causes a crown gall disease in a wide variety of dicotyledonous plants. Other pathogenic *Agrobacterium* species include *A. rhizogenes,* which causes hairy root disease, *A. vitis,* which causes tumours on grape and few other plant species, and *A. rubi*, which causes cane gall disease [17]. The pathogenesis mechanism of all these species is similar as a large single-stranded DNA fragment from Ti-plasmid (tumour inducing) or Ri-plasmid (root inducing) is being transferred, integrated into the host genome and expressed in a host cell. The region which is recognised by *Agrobacterium* and transferred to a plant cell is called T (transferred)-DNA region and the transferred DNA is termed T-DNA. T-DNA in a Ti-plasmid is delineated by 23 base pair (bp) border repeats, left border repeat (LB) and right border repeat (RB). LB and RB repeats are primary and essential sights to be recognised by *Agrobacterium* molecular machinery for the transfer of any T-DNA occurring between them – of natural origin or a DNA sequences, inserted by genetic engineering techniques. Mainly two groups of genes are transferred by wild type *Agrobacterium tumefaciens* within their T-DNA. The first group is opine synthase genes, producing opines, an important source of nitrogen and carbon for *A. tumefaciens*. The second group includes oncogenes, responsible for tumour growth. Main tumour growth-controlling genes, coded by *Agrobacterium* T-DNA, are *ipt, iaaM* and *iaaH,* participating in the biosynthesis of important plant phytohormones – cytokinins and auxins. *Agrobacterium* gene *iaaM* encodes tryptophan monooxygenase, which converts tryptophan to indole-3-acetamide (IAM). Another enzyme, indoleacetamide hydrolase, encoded by *iaaH,* converts IAM to indole-3-acetic acid (IAA) – the most abundant and basic auxin natively occurring and

functioning in plants. *Ipt* encodes an isopentenyl transferase. This enzyme catalyses the condensation of adenosine monophosphate (AMP) and isopentenyl pyrophosphate to form isopentenyl AMP, one of the adenine-type cytokinins. *A. tumefaciens* secretes auxin and cytokinin from its cells to initiate crown gall development. The action of transferred enzymes promotes hyper-proliferation of plant cells leading to the formation of a tumour at the agrobacterial infection site, which increases the concentration of opines and helps for *Agrobacterium* to survive [18].

1.2.2.2. Activation of *Agrobacterium* virulence

Genes responsible for the virulence of *Agrobacterium* are called *vir* genes and are mainly located in an *Agrobacterium* Ti plasmid, outside the T-DNA. *Vir* genes, except transcription factors *virA* and *virG*, are virtually silent unless bacteria are co-cultured with plant cells. Still, *vir* induction is related not to direct bacterial attachment to a plant cell but rather dependent on molecules released by plant cells. However, other studies showed that attachment is also necessary for transformation, but the dependence on the attachment is regulated not by *vir* genes but by chromosome encoded genes [19]. When *Agrobacterium* is interacting with a plant cell, two proteins VirA and VirG are activated to induce expression of other *vir* genes (Fig. 1). VirA and VirG are proteins of a two-component regulatory system in which a sensor kinase VirA is activated by an external signal and then changes the phosphorylation status of response regulator VirG. The membrane-bound sensor kinase, VirA, autophosphorylates at a conserved histidine residue and transfers phosphate to a conserved aspartate residue on the cytoplasmic response regulator, VirG. Phosphorylated VirG (VirG-PO_4) binds to specific 12bp DNA sequences (*vir* boxes) of *vir* promoters and activates transcription [20]. External signals, sensed by VirA while being in contact with a plant cell, are phenols, aldose monosaccharides, low pH and low PO_4 [20]. Phenols are absolutely required for the induction of *vir* gene expression, whereas other signals, such as monosaccharides, low pH and PO_4, sensitise VirA to phenol perception. For example, sugars allow induction of the VirA and VirG system at much lower phenol concentrations and significantly increase the response [21] (Fig. 2). Acetosyringone (AS) is one of the identified phenols routinely used for enhancing the efficiency of *Agrobacterium*-mediated plant transformation. Naturally, *Agrobacterium* gets into contact with plant cells trough the wounded tissues. Wounds are not only entry sites for the *bacterium* but also facilitate the transformation process because wound-secreted phenols and sugars promote *vir* gene expression. Phenylpropanoid pathway, low pH and

sugars associated with cell wall synthesis for wound repair serve as invasion and transformation factors for *Agrobacterium* [22].

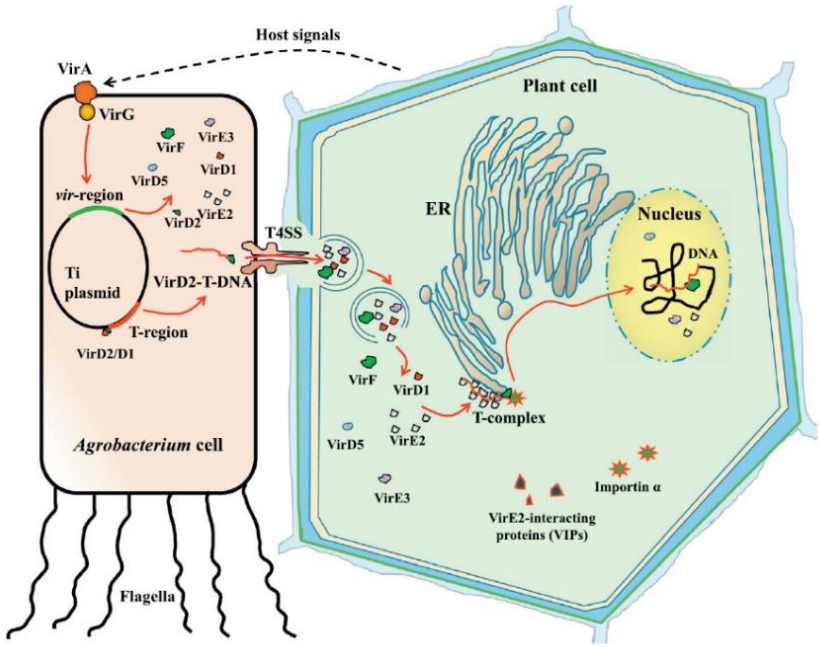

Figure 1. A general model of *Agrobacterium*-mediated transformation of a plant cell [23].

Chemicals, emitted by some plant species, can also act as inhibitors of the *Agrobacterium* virulence. This may help to explain an unequal resistance to *Agrobacterium* infection between different plant species. Two chemical compounds found in corn seedling homogenates were shown to have such inhibitory effect: DIMBOA and MDIBOA (Fig. 2). DIMBOA (2,4-dihydroxy-7-methoxy-2H-1,4-benzixazin-3-(4H)-one) is an inhibitor of both *Agrobacterium* growth and AS-dependent virulence activation [24]. Another compound MDIBOA (2-hydroxy-4,7-dimethoxybenzoxazin-3-one) is a potent inhibitor of *Agrobacterium* virulence with a limited effect on bacterial growth [25]. DIMBOA and MDIBOA derive from the tryptophan biosynthetic pathway [26]. Moreover, auxin indole acetic acid (IAA) acts as an inhibitor of *vir* gene induction, likely by competing with the inducing phenolic compounds, such as AS, for binding to VirA. As mentioned before, IAA is produced at high concentrations by *Agrobacterium* T-DNA encoded factors during the development of crown gall tumours. The likely predicted function of IAA is inhibition of a secondary transformation of the same plant cell by

the same or another competing strain of *Agrobacterium*. Other two signal molecules involved in a plant response stress are salicylic acid (SA) and ethylene [27]. *Arabidopsis* or tobacco mutants deficient in SA accumulation are more sensitive to an *Agrobacterium* infection, but plant mutants overproducing SA or plants treated with exogenous SA are relatively resistant to the transformation [28,29].

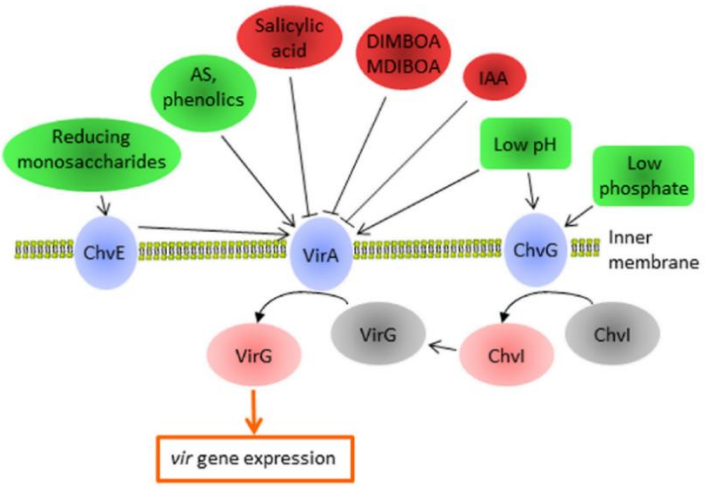

Figure 2. Plant factors affecting *vir* gene expression. The *Agrobacterium* VirA/VirG two-component regulatory system integrates numerous plant and environmental signals to regulate transcription of *vir* genes. Phenolic compounds bind directly to VirA to promote VirG activation, while salicylic acid, DIMBOA, MDIBOA, IAA inhibit VirG activation and enhancement of its expression. Reducing monosaccharides bind to ChvE, which in turn interacts with VirA to enhance AS-induced *vir* activation. Low pH and low phosphate concentration have a positive effect on *vir* activation by affecting either directly VirA or the ChvG-ChvI two-component system that in turn activates the VirG expression [30].

1.2.2.3. T-DNA transfer mechanism

When *vir* genes are activated and *Agrobacterium* is attached to a plant cell, a formation of agrobacterial secretion system starts. A secretion system is consisted of VirB and VirD4 proteins and belongs to the class of type IV secretion systems T4SS. T4SS are found in many gram-negative bacteria and serve for a conjugative transfer of plasmids between bacteria and for a horizontal gene transfer from *Agrobacterium* to plant [31]. VirB complex is

composed of at least 12 proteins, VirB1-11 and VirD4 which form a multisubunit envelope-spanning structure [32] (Fig. 3). The complex crosses the inner membrane, the peptidoglycan layer and outer membrane of *Agrobacterium*, also the cell wall and membrane of the plant. Bacterial factors which are transferred through the complex are VirD2-T-DNA, VirE2, VirE3, VirF, VirD5, MobA and Atu6154 in *A. tumefaciens* and the GALLS protein in *A. rhizogenes* [33].

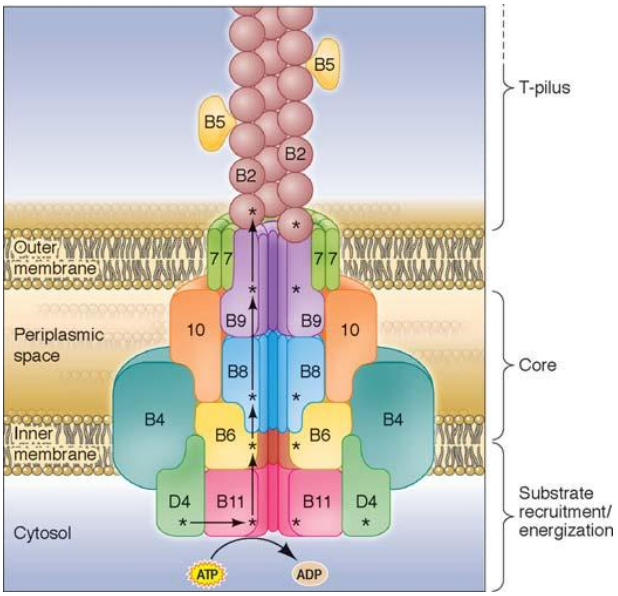

Figure 3. Generalised scheme representing the VirB and VirD4 complex. Asterisks indicate proteins shown to interact with the transported DNA substrate, and arrows indicate the order of DNA transfer through the complex [276].

1.2.2.4. Functions of main virulence proteins

VirD2 is a nuclease which nicks DNA at RB and LB sequences at both ends of T-DNA and then covalently binds to excised single-stranded T-DNA and forms the T-complex. VirD2 seems to lead T-strands through the T4SS complex into the plant cell. Protein probably serves in the targeting of T-strands to the nucleus in a host cell, because the C-terminal region of VirD2 contains a nuclear localisation signal (NLS) sequence which targets affixed reporter protein to the nucleus of a plant cell [34]. Importin α proteins also seem to be participating in nuclear targeting of VirD2/T-strand complex as they interact both with conservative NLS motifs containing proteins and with

nuclear shuttle protein importin β. The complex of α/β importins and cargo protein traverse through nuclear pores into the nucleoplasm and then dissociates [35]. Probably, a complex of α/β importins and VirD2/T-strand is similarly transported into a plant cell nucleus.

VirE2 is a DNA binding protein that forms complexes with any single-strand DNA sequence *in vitro* [36]. Since T-DNA is transferred as an unstable single-strand molecule from *Agrobacterium* to a host plant, a proposed function of VirE2 is the protection of T-DNA during the molecule's journey from bacterium to a plant cell [37]. Early research indicated that VirE2 interacts with T-DNA in *Agrobacterium* [38], but more recent publications claim that VirE2 is rather transported to a host cell, where it coats and protects T-strands from nucleolytic degradation [30]. Recent studies which have taken advantage of split-GFP (Green fluorescent protein) technology to visualise VirE2 movement in *N. benthamiana* leaf cells, showed that VirE2 is transported into a plant cell via clathrin-mediated endocytosis [39] and trafficked through an endoplasmic reticulum/F-actin network. VirE2 movement is powered by host myosin XI-K [23,40]. VirD2-T-strand and VirE2 proteins form mature T-strand complex after their independent transfer to a plant cell [2,41].

A typical mature T-complex from a nopaline-specific *Agrobacterium* strain is composed of a 22-kb T-DNA strand, 1,176 molecules of VirE2 and one molecule of VirD2, with a total molecular mass of 90 MDa. The cytoplasmic movement of such a large complex to the nucleus by passive diffusion would be minimal, and the entry to the nucleus just impossible [42]. Therefore T-complex is most likely being transported by active mechanisms. Many DNA viruses are dependent on cellular molecular motors, such as dynein and microtubule network, in their trans-cytoplasmic transport. Probably, T-complex uses the same model for movement through the plant cell cytoplasm, as VirE2 interacts with host molecular motors (myosin XI-K) and the surface of T-complex is covered by hundreds of VirE2 molecules [40]. It was also shown that synthetic T-complexes are actively transported along the microtubule network in a cell-free system, suggesting the involvement of cytoskeletal elements in the infection process [43]. *Agrobacterium virE2* mutant strains are not completely avirulent, however, their virulence is severely decreased [37]. These strains can completely recover their virulence in transgenic *virE2* expressing plants because of function complementation [2,44]. VirE2 interacts with its chaperone VirE1, which likely protects VirE2 from self-aggregation and association with T-strands in *Agrobacterium* [45,46].

The role of VirE2 in nuclear targeting of T-strands remains controversial and seem to be more complex than VirD2, which interacts with importin α for nuclear targeting. Unlike VirD2, VirE2 does not interact efficiently with

importin α [47]. A long-standing assumption that VirE2 nuclear targeting relies on the presence of host protein VIP1 (VirE2 interacting protein 1) and also is dependent from interaction with virulence protein VirE3 [47,48,49] turned out to be inaccurate. Recent studies indicated that VIP1 is not significant for *Agrobacterium* transformation [50,51]. Also VirE3 was indicated as an anchorage protein targeting VirE2 to the host plasma membrane, not the nucleus, and facilitating VirE2 protection for T-DNA [52]. A new hypothetical model for the nuclear import of the T-complex to the plant cell nucleus was proposed in a very recent study [53]. As VirE2 was proven to be interacting directly with the host nuclear pore complex (NPC) component nucleoporin CG1 *in vitro*, it was suggested that the T-complex is transported into the host nucleus with a cooperative participation of both VirD2 and VirE2. It was indicated that VirE2 can not enter the host nucleus solely, in the absence of VirD2 or T-DNA, probably because of internal localization of CG1 in a nucleopore. Thus, in a suggested model, VirD2 provides a leading guidance for the T- complex to the nucleus through the NLS-based interaction with plant importin α, whereas VirE2 facilitates T-DNA transport on the lateral side of the complex via interaction with CG1 (Fig.4).

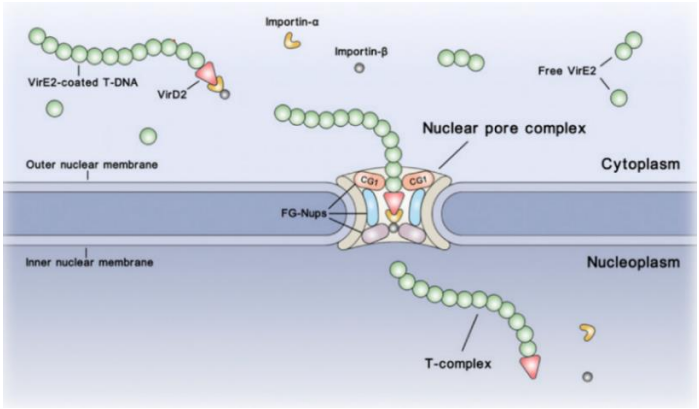

Figure 4. A hypothetical model for the nuclear import of the T-complex. VirE2 coats the VirD2 associated T-DNA in a host cytoplasm. Then VirD2 interacts with host importins α/β and T-complex is guided to the host nuclear pore. Soon interactions between importin β and NPC components translocate the nucleoprotein complex into the channel where interactions between VirE2 and nucleoporins CG1 are enabled. When T-complex enters the nucleus, importins are removed and nucleoprotein can be further targeted for the integration into host chromosomes [53].

1.2.2.5. Chromatin targeting of T-complex

T-DNA needs to be targeted to chromatin and then inserted into a plant genome after T-complex entered the nucleus. An answer has been sought for many years for the question if T-DNA is being inserted to a plant genome randomly or if there are any preferable regions of a plant genome to be inserted into. Several early investigations indicated that T-DNA is mostly inserted into transcriptionally active regions of chromatin, however, later studies proved insertions to be truly random [54,55]. These findings raised several interesting questions. If T-DNA insertions are random and can access both euchromatin and heterochromatin, then there must be special mechanisms modifying heterochromatin so that T-DNA could reach and integrate into condensed chromatin during *Agrobacterium* infection. It has been also proposed that T-DNA integration may occur at the host cell chromatin decondensation step during the S-phase of the cell cycle [56]. Also, there is a hypothesis that *Agrobacterium* may use a plant cell dedifferentiation process occurring during the response to stress when chromatin is also decondensed [57].

1.2.2.6. Disassembly of T-complex

Upon the arrival of T-complex in the host nucleus or its anchorage to chromatin, its protein components have to be removed so that the T-strand would become accessible for T-DNA second strand synthesis and integration to chromatin. It is thought that the disassembly of the complex is mainly mediated by the host ubiquitin-proteosome system [58]. The transferred *Agrobacterium* virulence factor VirF is proposed to participate in the disassembly of T-complex. It is a typical F-box protein which mediates protein degradation by the ubiquitin-proteosome system and is a homolog of eukaryotic F-box proteins [59,60]. VirF likely participates in the destabilisation of all the T-complex. VirF itself is a target of proteosomes and is unstable in a plant cell but is protected by transferred virulence factor VirD5 [61,62]. However, VirF is not necessarily required for *Agrobacterium* infectivity in all recipient plant species. For example, VirF enhances *Agrobacterium* infectivity in tomato and *Nicotiana glauca* but is not necessary for infection of *Nicotiana tabacum* or *Arabidopsis* plants [63]. Potentially, plant species that do not require agrobacterial VirF to infect them, possess endogenous F-box protein which compensates functions of VirF [56].

1.2.2.7. Integration of T-DNA

Studies of T-DNA integration in plants, yeast and *in vitro* experimental systems have shown that the integration of T-DNA is absolutely dependent on the host DNA repair machinery and the roles of agrobacterial T-DNA-associated proteins could be described as more minor than significant. Bacterial proteins likely serve as molecular links between T-DNA and host factors. Even if early research works proposed models of single-stranded T-DNA integration into plant cell genome [64], more recent works challenged them. Thousands of sequences of T-DNA/plant DNA junctions were analysed, and micro homologies, deletions, fillers, target-site duplications and various duplications were often found at that junctions. As similar changes are also characteristic to repair sites of DNA double-strand break repair sites, the model of double-strand T-DNA integration was proposed [65]. Besides DNA repair machinery, host proteins that are mainly involved in chromatin structure or remodelling are also vital for T-DNA integration. Nucleosomal histones have been suggested mediating T-DNA interactions with a host chromosomes before integration. Histone H2A and others were shown to be important for an efficient plant cell transformation [66, 67]. Also *Arabidopsis* mutants deficient in chromatin assembly factor 1 (CAF-1) were more sensitive to stable transformation by *Agrobacterium* than the wild-type plants [68].

1.2.2.8. Regulation of T-DNA expression

Natural agrobacterial T-DNA expression is classified into two modes: transient and stable. Transient expression is usually defined as a peak in T-DNA expression that occurs within 2–4 days after transformation [69–71]. After 2–4 days post-transformation, both the number of transgene-expressing cells and the level of expression are lowered. Differently, late gene expression, which occurs 10–14 days after agrobacterial transformation [69] is stable and inheritable in the case of germline transformation, resulting from the integrated T-DNA. Transient expression from T-DNA likely occurs from T-DNA molecules that have not yet integrated into the host genome; however, the duration of such expression is limited because of the instability of such non-integrated DNA. Several studies showed that transient and stable expression could be uncoupled. It has been demonstrated that several plant mutants with reduced tumour formation still display normal levels of transient T-DNA expression [72,73]. Expression of non-integrated T-DNA has been demonstrated by co-transformation of two T-DNAs: the C-T-DNA with *Cre* recombinase expression cassette and the K-T-DNA with the *nptII* and β-

glucuronidase (GUS) expression cassettes flanked by two *lox* sites which are recognition sites of recombinase. Transgenic plants were generated that contained K-T-DNA integrated into the host genome, indicating *Cre* expression from C-T-DNA, however, no integrated C-T-DNA was detected [74].

1.2.2.9. Defence mechanisms of the host

Expression levels of integrated T-DNA are affected by different host-related factors, mostly participating in defence mechanisms of the host. The expression is mainly regulated by RNA silencing, DNA methylation and hypersensitive response as a protective reaction. RNA silencing is one of the defence mechanisms used by plants mostly for protection from viruses; however, this mechanism is also used against *Agrobacterium* infection. Small interfering RNAs (siRNAs) specific for the T-DNA sequence are generated by the host plant during *Agrobacterium* infection [75,76]. Plants deficient in siRNA pathways are very susceptible to *Agrobacterium* transformation. Also, ectopic expression of RNA silencing suppressors encoded by diverse plant viruses, such as P19 of *Tomato bushy stunt virus*, HcPro of *Potato Virus Y* (PVY) or V2 of *Tomato yellow leaf curl virus*, during *Agrobacterium* transformation, significantly increased the transformation efficiency and duration of transient T-DNA expression [77,78]. However, while most plant viruses evolutionary gained RNA silencing suppressors, no agrobacterial silencing suppressor was identified to date.

Another mechanism regulating T-DNA expression may be DNA methylation, which is often observed in integrated transgenes, especially when several T-DNA copies are integrated into the host genome [79,80]. A study described genome-wide changes in DNA methylation in crown gall tumours [81]. However changes may not be directly related to infection or genetic transformation but connected to the expression of crown gall regulators and changes in the regulation of a plant cell division and development instead [82].

The hypersensitive response is another mechanism used by plants for defence against various plant pathogens which are capable of suppressing basal defence of the plant. Hypersensitive response (HR) is characterised by deliberate plant cell suicide resulting in tissue necrosis at the infection site. HR may limit pathogen access to water and nutrients by sacrificing a few cells to save the rest of the plant. Once the HR has been triggered, plant tissues may become highly resistant to a broad range of pathogens for an extended time. This phenomenon is called systemic acquired resistance (SAR) and represents a heightened state of readiness in which plant resources are mobilised in case of further attack [83]. Although *Agrobacterium* infection usually does not result

in visible symptoms, such as tissue necrosis, studies of changes in the host transcriptional activity induced by *Agrobacterium* infection have shown that related defence mechanisms are activated. In *Arabidopsis* cells, many genes known to be involved in pathogen defence are activated 48 h after inoculation with *Agrobacterium* [84]. However, later decrease in gene expression has been observed, probably due to the expression of yet unknown *Agrobacterium* factors, participating in resistance to the host response [85].

1.2.2.10. *Agrobacterium* as a tool for transfer of foreign DNA into plant

As agrobacterial T-DNA expression can be stable or transient by its nature (described earlier), two *Agrobacterium*-mediated transformation methods were adapted for transgene expression in plants: stable expression in transgenic plants and transient expression. For transient expression, mature plants are transfected, T-DNA integration into the host genome is not required, and T-DNA expression usually lasts for a few days. For stable transformation, the T-DNA must integrate into the host cell genome, and transgenic plant regeneration process must be undertaken, so that it is subsequently passed on to the next generation.

To utilise pathogenic *A. tumefaciens* as a DNA vector for plant transformation, several obstacles needed to be overcome. In the early 1980s, soon after the reason of crown gall disease was understood, researchers predicted that *Agrobacterium* and its Ti plasmid would become a unique vehicle for the transfer of genetic material into plants. However, wild-type *Agrobacterium* strains needed genetic modification so that they could be easily used under laboratory conditions. First of all, T-DNA region, which carries genes responsible for tumorigenesis and opine synthesis in a host, was deleted entirely in order to make *Agrobacterium* non-pathogenic. However, this did not affect its ability to transfer DNA as only flanking sequences of T-DNA called left border (LB) and right border (RB) of T-DNA are needed for T-DNA recognition and transfer machinery of *Agrobacterium*. Hence, any desired transgenes can be inserted between them and transferred to a plant cell. However, Ti plasmids are usually very large (often over 200 kbp) and carry more than 200 genes. Therefore, they are low copy number, typically unstable and contain few unique sites for restriction endonucleases. The finding that *vir* genes can act *in trans* (expressed from another replicon than T-DNA) and two border sequences are the only *cis*-elements required for T-DNA production led to the establishment of a binary vector system [86,87]. In a binary vector system, *vir* genes and T-DNA are located on two separate

replicons. The vector carrying *vir* genes is called *vir* helper (or helper Ti plasmid). T-DNA border sequences LB and RB are located on another plasmid, called a binary vector. The plasmid can replicate both in *Agrobacterium* and in *Escherichia coli*. After genes of interest are inserted into the T-DNA region of a binary vector and cloned into *E. coli*, this binary vector can be easily purified from *E. coli* and introduced into an *Agrobacterium* strain that carries a helper Ti plasmid [23]. Transformed agrobacteria can then be used for plant transformation. The binary vector must have two types of selectable markers for the selection in bacteria and plant, respectively. As transformation efficiency is dependent from characteristics of the binary vector (size, copy number, stability), versatile binary vectors were constructed for different purposes [88–90].

When *Agrobacterium*-mediated transformation is used for stable transformation, plant cells cultures, explants of plant tissues (leaves, roots, shoots) or plant blossoms (when floral dipping method is used) are infected with *Agrobacterium* carrying genes of interest. Then plant cells culture undergoes a selective regeneration process, during which transformed and resistant to some antibiotic plant cells proliferate and differentiate into a grown-up plant. After floral dipping transformation, seeds are placed on selective medium and only transformed seedlings germinate [91].

1.2.3. Transient expression

1.2.3.1. *Agrobacterium* infiltration

Although transgenic crops generated by *Agrobacterium*-mediated stable transformation have many prospects in agriculture and are irreplaceable for many aspects of molecular plant biology [92], in many cases, *Agrobacterium*-mediated transient plant transformation allows faster timelines and higher expression levels than stable transgenic plants [56].

One of the approaches for transient expression in plant tissues is *Agrobacterium* suspension infiltration. During the process, recombinant *A. tumefaciens* bacteria harbouring a binary expression vector are introduced directly into grown up plant leaves using syringe injection or more scalable methods like vacuum infiltration or *Agrobacterium* spray (agrospray). When the syringe agroinfiltration is applied, suspension of agrobacteria are injected into plant leaves abaxial side through the stomata with a syringe without a needle. During vacuum agroinfiltration, a whole plant leaf system is submerged into the suspension of agrobacteria and leaves are infiltrated by

applying and releasing vacuum [93]. When agrospray is used, then *Agrobacterium* suspension is simply sprayed on plant leaves [10,94]. Agroinfiltration is a convenient and time-saving technique which lets to obtain a desirable product in plants in 4–6 days. Thus, in terms of application for molecular plant biology, the transient expression can be used to evaluate the activity of expression constructs rapidly and to produce a sufficient quantity of recombinant protein for a functional analysis including protein-protein interaction or subcellular protein localisation [95]. The transient expression system is also flexible, as it allows for the expression of multiple genes simultaneously, and provides a reliable and reproducible indicator of expression construct performance since it avoids the effects of T-DNA integration position in a plant chromosome generally associated with stable transgenic plants [96].

Traditional binary expression systems are convenient for application of transient expression in a laboratory scale; however, further improvements of the approach were needed to meet commercial needs for recombinant production in *planta.* Construction of plant virus-based vectors for transient expression, described in the next section, is one of the solutions to enhance the yield of recombinant proteins.

1.2.3.2. Plant virus-based vectors for transient expression in plants

Transient expression systems allow the production of ectopic proteins in days. However, the use of traditional binary expression systems faced limitations in expression levels of intended proteins in plants. Product yields remained relatively limited, and one of the reasons of the indicated problems was the RNA silencing initiated by plant cells in response to the introduction of foreign nucleic acids [97]. One solution to increase the level of transient expression was the exploitation of plant viral proteins that function as RNA silencing suppressors, a function gained during coevolution with plants [98]. The inhibitory effect of the P19 suppressor from *Tomato bushy stunt virus* (TBSV) and *Artichoke mottle crinkle virus* (AMCV) was successfully used for enhancement of transient and constitutive expression levels of recombinant proteins in plants [99,100]. Later even cocktails of suppressors targeting different distinct steps of the RNA silencing were used for expression enhancement [101]. The next significant step forward in the development of transient expression systems was the coupling of *Agrobacterium*-mediated transformation with the delivery of cDNA encoding viral RNA and acting as an "amplifier" for the production of a protein of interest in plant cells. Introduction of plant viruses engineered to contain a gene of protein of interest and able to replicate many

times in a plant cell, allowed to obtain a much higher level of foreign protein expression than in a non-viral context [102,103]. Since the first plant virus, *Tobacco mosaic virus* (TMV), is exploited for the production of diverse recombinant proteins in plants, scientists and industry continue to improve viral vectors to develop convenient and highly efficient expression systems for high scale production of recombinant proteins. Plant virus expression vectors have been designed from genomes of both positive-sense RNA viruses and single-stranded DNA viruses [104] (Tab. 1).

Table 1. Description of plant viruses commonly used for construction of viral expression vectors for heterologous expression in plants (according to [105]).

Virus	Family	Type of genetic material
PVX (potato virus X)	*Potexviridae*	(+) ssRNA
PapMV (Papaya mosaic potexvirus)	*Potexviridae*	(+) ssRNA
TMV (Tabaco mosaic virus)	*Tobamoviridae*	(+) ssRNA
SHMV (Sun hemp mosaic virus)	*Tobamoviridae*	(+) ssRNA
CPMV (Cowpea mosaic virus)	*Comoviridae*	(+) ssRNA
BeYDV (Bean yellow dwarf virus)	*Geminiviridae*	(+) ssDNA
BCTV (Beet Curly top virus)	*Geminiviridae*	(+) ssDNA
TYDV (Tobacco yellow dwarf virus)	*Geminiviridae*	(+) ssDNA

Plant viral vectors used for plant transfection can be divided into two categories based on the manner they were constructed. At the early stage of viral vector application for plant transformation, a full-virus strategy was used. A foreign gene of interest was inserted and expressed as part of a fusion protein with a viral protein from a viral promoter, or separately from an additional strong sub-genomic promoter which was inserted into the viral genome. Fusions with viral coat proteins were reported to be successfully expressed [106,107]. However, the full-virus strategy has some drawbacks as the size of the transgene is limited because of the instability of oversized viral genome [108]. Also, the usage of a full virus faces safety concerns as the amplicon is autonomous and can potentially spread in the environment. Therefore, researchers have moved to the development of another more

versatile and efficient strategy of viral vector construction called deconstructed virus strategy. Deconstructed viral vectors are composed only of the genomic regions required for viral replication in plant cells and sometimes for movement from cell to cell or systemic spread throughout the plant to obtain higher efficiency of transformation (Fig. 5).

Figure 5. Schematic representation of plant virus-based binary vector used for transient transfection of plants. A typical vector is composed of a binary vector backbone harbouring replication *ori* for replication in *E. coli* and *A. tumefaciens* and antibiotic-resistance genes for selection of positive clones. A viral amplicon, containing viral polymerase, movement or coat protein (MP/CP), and the target gene is inserted between T-DNA borders (LB and RB) for recognition and transfer into a plant cell by *Agrobacterium* T-DNA transfer machinery.

Additionally, even more developed approaches are used, for example, a deconstructed viral vector approach based on *in planta* assembly of functional viral vectors from separate pro-vector modules. In this approach, different agrobacteria strains are used to deliver different modules of the viral vector and various genes of interest instead of inserting the full vector as a linear DNA molecule. Separate components are then delivered to a plant cell and assembled inside the cell with the help of site-specific recombinase which coding sequence is also delivered as a separate component of the expression system. The resulting DNA is transcribed and spliced removing undesired elements such as recombination sites, and perfect gene/vector sequence is being created within a fully functional infective replicon [109] (Fig. 6).

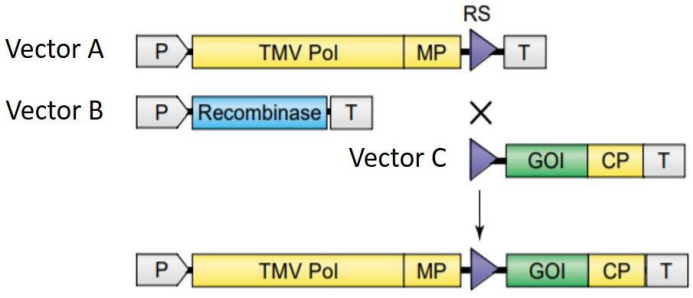

Figure 6. Schematic representation of in planta assembly of functional viral vectors from separate pro-vector modules. The system consists of the vector A carrying T-DNA with viral replication components – viral polymerase (TMV Pol) and movement protein (MP) coding genes, vector C harbouring a gene of interest (GOI) and a gene of viral coat protein (CP). Also, separate vector B with the coding sequence of recombinase. Purple triangles indicate recombination sites, P – promoter, T – terminator [110].

To summarise, significant advances have been made in the field of *Agrobacterium*-mediated transient expression in plants in the last ten years, which include improvement of expression vectors [111,112], modification of a host cell, for example, glycoengineering plants for proper glycosylation of proteins [113], and even development of commercial manufacturing platforms for large-scale production of high-value therapeutic proteins in plants such as antibodies for the treatment of Ebola virus disease [114], *N. benthamiana*-based influenza vaccine [115] and others [116]. *A. tumefaciens* has become a very powerful DNA vector used for transient expression of pharmaceutical and non-pharmaceutical proteins in such plant species as *Nicotiana benthamiana*, tobacco, lettuce, tomato [60,117–119] (Fig. 7).

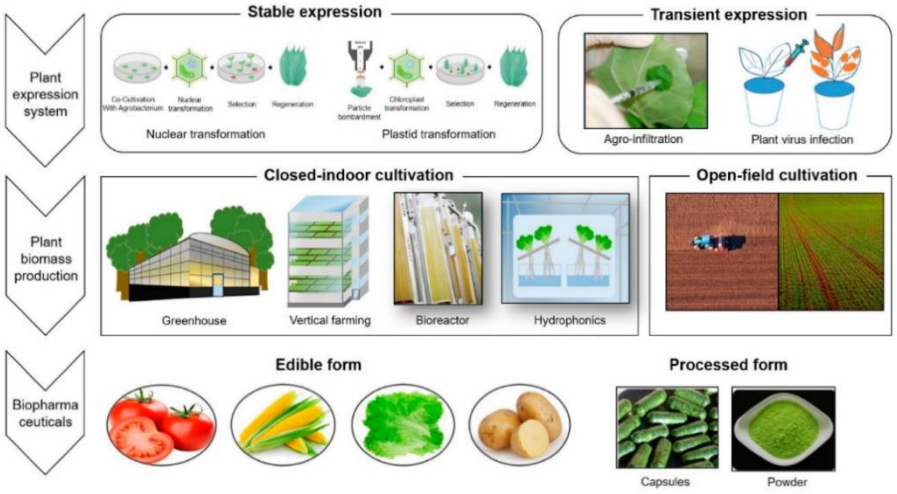

Figure 7. Overview of plant-based biopharmaceuticals expression strategies, biomass production platforms and forms of the biopharmaceuticals consumption**.** Stable nuclear or plastid transformation by particle bombardment or *Agrobacterium*-mediated transformation; also transient transformation by agroinfiltration or plant virus infection are used as plant transformation strategies. Different platforms for plant biomass growth are

used: closed indoor cultivation which include cultivation in greenhouses, vertical farming, bioreactors and hydrophonics and also open-field cultivation. Finally plant produced biopharmaceuticals can be used in edible form if expressed in fruits or leaves of edible plants or in a processed form as capsules, powder or as purified pharmaceuticals [120].

1.2.4. Biosafety concerns related to *Agrobacterium*-mediated transfection

The wide application of *Agrobacterium*-mediated plant transformation leads to the concern about biosafety and risk of use of recombinant *Agrobacterium*, especially, for potential open environment applications. Main risks related to the use of *Agrobacterium* are horizontal gene transfer from *Agrobacterium* to non-target plant species or other microorganisms including avirulent *Agrobacterium* [121,122] and transfer of selectable markers to the host [123,124].

Since the 1970s, when genetically engineered organisms were put into use for various purposes, scientists and the public have voiced concern that the accidental or intentional release of genetically modified organisms (GMOs) might impact the environment [125]. Despite there is no reliable research data confirming GMOs being biological hazards till date, there are many publications indicating invasion of transgenes into the natural environment [126–128]. One warning example shows that the spread of antibiotic resistance genes, often incorporated into genetically modified organisms as selectable markers, can directly impact human health. It was reported that resistance genes, found in water polluted by farmland faecal waste, could be transferred to natural bacterial species by bacteriophages [129]. Such transgene spread can increase antibiotic resistance of natural microorganisms, invaded by resistance genes harbouring bacteriophages, and subsequently burden a cure of bacterial diseases in human. Because of a rapid rise in development and use of GMOs in recent years, impact of engineered organisms for the environment and human health is proposed to increase [130,131]. Therefore, special safeguard measures to prevent GMO escape was designed and is updated periodically [132,133]. The design of bacterial biocontainment strategies focus on the synthetic auxotrophism, a genetic circuit-actuated killing and blocking of horizontal gene transfer, involving a wide range of cellular mechanisms. Effective biocontainment approach must meet essential biosafety requirements to prevent the release and proliferation of genetically engineered organisms in the natural environment. As GMOs are living entities that can grow and self-replicate, any biocontainment system has to be extremely robust to prevent the release of even a small fraction of organisms that could grow to dominate an ecosystem [134]. Despite that some biosafety

guidelines considered GMO escapee rate below 1 in 10^8 cells acceptably safe [135] and the number of designed biocontainment approaches met this criterion, continuous improvements in systems robustness and efficiency are needed to ensure biosafety as scale of GMO deployment increases. In addition to robustness, the stability of the biocontainment system is another important criterion for the effective protection of the environment. Random DNA recombination and mutagenesis may silence the biocontainment system in the long term. Moreover, biocontainment systems themselves are often toxic for the host cells or trigger their metabolism, which may limit the desired cellular performance and further increase the selective pressure to disrupt the biocontainment apparatus. Therefore, biocontainment systems must be tightly regulated under permissive conditions [134]. Several biocontainment approaches designed to meet these biosafety criteria are going to be described below.

1.2.4.1. Biocontainment through auxotrophy

One of the earliest and still widely used methods for biocontainment is auxotrophy. Auxotrophic microorganism is obtained by random mutagenesis or targeted engineering (Curtiss et al. 1977). Regarding biosafety, auxotrophic microorganisms are unable to synthesise compounds essential for their survival or replication, such as nucleotides or amino acids, so the missing components have to be provided externally [130] (Fig. 8a). Therefore, microorganisms are unable to survive outside of bioreactor where missing compounds are provided for their growth. Many auxotrophic organisms have been engineered for various purposes. As an example, where auxotrophism is used instead of antibiotic selection markers, is the auxotrophic *E. coli* strain M15, which carries an inactive *glyA* gene, needed for the production of intracellular glycine. Here, auxotrophy empowers transformation of only plasmid harbouring functional *glyA* gene into the strain and this prevents from loss of the plasmid [137]. Another example representing a more sophisticated approach of natural containment systems, is a strategy to treat inflammatory bowel disease in humans by a genetically modified *Lactococcus lactis* strain. In this strain, gene encoding thymidylate synthase, which is essential for the growth of the bacterium, was replaced with a human interleukin-10 gene. The resulting strain expresses interleukin-10, which is used as a therapeutic protein for the cure of intestine disease [138]. Moreover, this strain is biologically contained in humans as it is dependent on thymidine or thymine for survival. The strain was successfully tested in a clinical trial [139]. The possibility to use auxotrophic *Agrobacterium* strains in a plant transformation was also considered early. First, tryptophan auxotrophs were intended to be used,

however just for control of tumorigenesis, not for T-DNA transfer [140]. Also cysteine auxotroph was selected for T-DNA transfer, but showed high expression of reporter gene without addition of cysteine and appeared to be a poor candidate for biocontainment [141]. Obviouslly, the classic auxotrophic strategy may not always be efficient as auxotrophic microorganism may still survive in natural environments by obtaining essential compounds provided by other organisms.

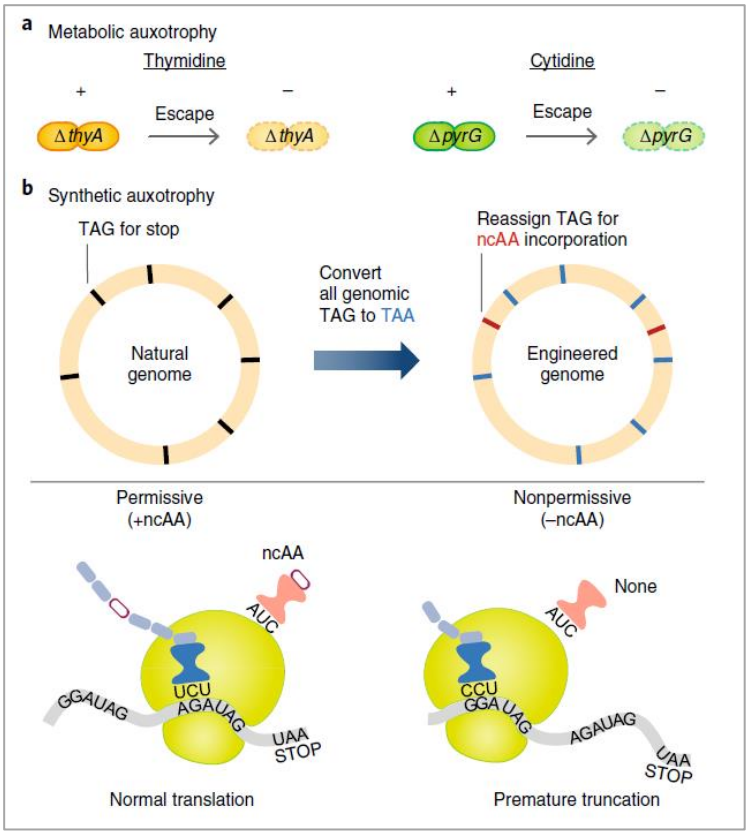

Figure 8. Biocontainment through auxotrophy. a. Metabolic auxotrophy: deletion of the *thyA* gene or *pyrG* gene in *L. lactis* led the strain to the critical dependence from external thiamine or cytidine, respectively. b. A synthetic auxotrophic strain was developed by replacing all amber stop codons (TAG) with ochre stop codons (TAA) and using the amber codon sequence to incorporate a ncAA into essential enzymes in the permissive environment [134].

Besides that, deleted functions can be regained by horizontal gene transfer, and thus biosafety will be compromised. Therefore, auxotrophy is usually

combined with other containment systems to achieve lower escapee frequencies.

One of the strategies designed to increase the biosafety of auxotrophism is the recent development of synthetic auxotrophic GMOs that depend on noncoding amino acids (ncAA) to survive. In these strains, all natural amber stop codons UAG have been replaced with an ochre stop codon UAA. These engineered strains express a modified tRNA and aminoacyl-tRNA synthase pair which incorporates ncAA into amber codons that have been intentionally inserted into the sequences of essential genes (Fig. 8b). Therefore ncAA are critically needed for cellular survival and are only supplied in the designed environment [142,143]. To protect the system from accidental incorporation of naturally existing amino acids, researchers redesigned structures of essential enzymes so, that their enzymatic activity became dependent on ncAAs' residues [144]. However, the application of such an approach is limited because of the need for extensive genome editing in the microbial strain, and also because this strategy does not completely safeguard from horizontal gene transfer.

1.2.4.2. Biocontainment using synthetic gene circuits

Another frequently used strategy for biological containment is biocontainment via synthetic gene circuits. This strategy couples environmental sensing with synthetic circuit-based control of cell survival. For example, an *E. coli* strain which survival is critically dependent on the quorum-sensing molecule at a concentration which is only achievable in high cell density cultures was engineered [145]. *Bacterium* proliferation is prevented in non-contained environments because of the low concentration of acyl homoserine lactone (AHL) (Fig. 9a).

Some biocontainment systems are designed in a two-layered gene expression regulation architecture in which first inducible regulator represses the expression of a second regulatory element which in turn represses expression of some toxin which, if de-repressed, causes cell death. The regulated and constant presence of the inducer of the first regulator is required for the suppression of toxin expression and consequent cell survival. Two recent examples of two-layered biocontainment systems in *E. coli* are "Deadman" and "Passcode" kill switches. The "Deadman" circuit is based on reciprocal repression by the LacI and tetR transcription factors which form transcription states that are maintained by the circuit's linked feedback loops. The presence of the TetR inhibitor anhydrotetracycline (ATc) is required to maintain the circuit in the survival state. Bacterial toxin gene (*ecoRI*, *ccdB* or *mazF*) was inserted into the circuit to kill the cell in the case ATc is not present

in a non-permissive environment. Additional palindromic LacI operator sites were inserted into the promoter of the toxin to minimise leaky expression and terminating sequence was introduced upstream to the promoter to insulate gene from spurious transcription. To increase the dynamics of the kill switch, degradation site was tagged to C-terminus of LacI that is recognised specifically by mf-Lon protease expressed from LacI-dependent promoter. After inducer ATc had been removed, toxins reduced bacterial colony forming units (CFU) counts by 3–5 orders of magnitude within 6 hours. The robustness of the system was further increased by using mf-Lon protease for degradation not only of LacI but also of some proteins essential for the survival of *bacterium* such as murC, participating in the biosynthesis of peptidoglycan [146].

For the design of the "Passcode" kill switch, hybrid transcription factors were constructed combining environmental sensing modules (ESM) of cellobiose-responsive transcription factor (TF) CelR from *Thermobifida fusca* and the galactose-responsive TF GalR with DNA recognition module (DRM) of TF LacI, both from *E.coli*. Hybrid TFs CelR-LacI and GalR-LacI were obtained. Another hybrid transcription factor, LacI-Scr was constructed by a combination of ESM of LacI and DRM of transcription factor ScrR from *Klebsiella pneumoniae.* Transcription factors CelR-LacI and GalR-LacI are used to control the expression of TF LacI-ScrR, which in turn represses toxin expression. In this circuit, loss of galactose or cellobiose allows CelR-LacI and GalR-LacI to bind the *lacO* operator, blocking LacI-ScrR expression, thus enabling toxin expression and causing cell death. Any exposure to inducer isopropyl β-d-1-thiogalactopyranoside (IPTG) releases repression of LacI-ScrR and allows expression of toxin, which also results in cell death (Fig. 9b). It is important that rearranging of EMSs in hybrid TFs allows modulating connections between the environmental sensing and transcriptional regulation [146].

A kill-switch was also introduced into *Agrobacterium.* A pair of genes, a *sacB/R,* commonly applied for creation of targeted knockouts in Gram-negative bacteria, was integrated into *Agrobacterium* genome. *SacB/R* encode levansucrase, an enzyme involved in hydrolysis of sucrose and biosynthesis of a toxic sugar – levan. The generated mutant *Agrobacterium* strain GV2260-*SacB/R*, under treatment with 5% or 3% of sucrose, was used for reduction of *Agrobacterium* overgrowth after stable leaf-disk transformation. Killing was significant, however not complete. The hypothesis was raised that some agrobacteria gained mutations in *SacB-SacR* genes and became resistant [147].

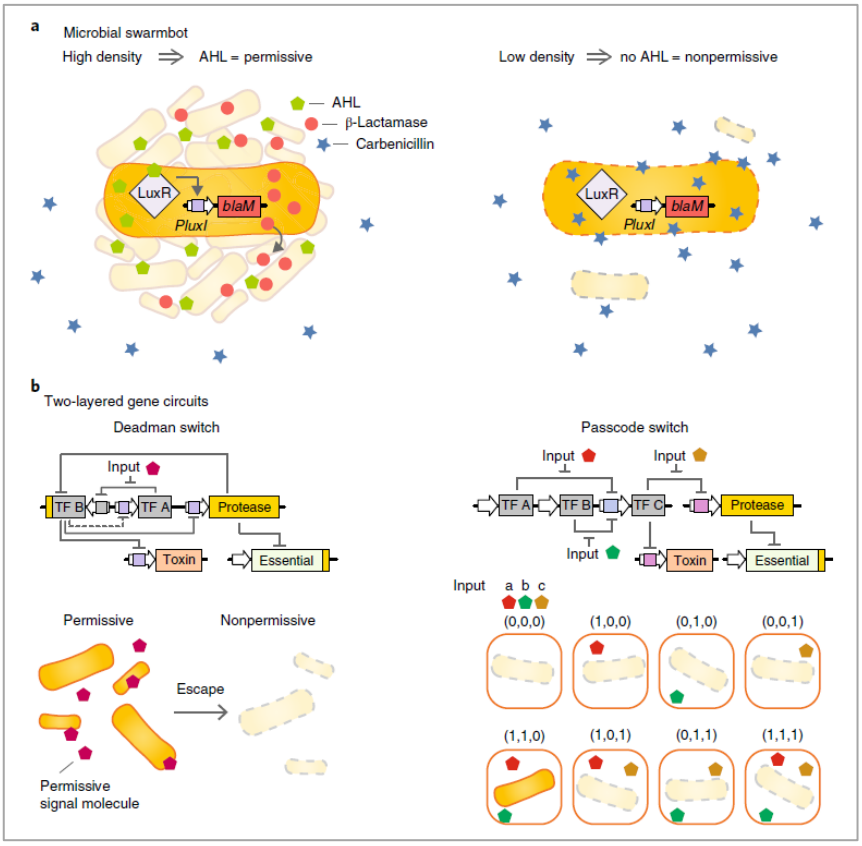

Figure 9. Biocontainment using synthetic gene circuits**.** a. Biocontainment through quorum sensing. High cell density leads to accumulation of quorum-sensing molecules AHL in the permitted conditions, inducing the expression of β-lactamase, which confers resistance to carbenicillin. At low cell density, β-lactamase is not expressed, leading to cell death by the antibiotic. b. Schematic representation of two-layered kill switches. In the "Deadman" system, the absence of a permissive signal allows the repression of transcription factor LacI (TF B) by TetR (TF A), thereby allowing toxin expression, as well the expression of a protease Lon-mf that targets an essential enzyme and any remaining LacI, causing cell death. In the "Passcode" switch, hybrid TFs are applied to rewire input-output connections in the genetic circuit. In the presented case, cell survival occurs if two signals, galactose (a) and cellobiose (b), are present and a third, signal IPTG (c), is absent [134].

1.2.4.3. Prevention of horizontal gene transfer

Ability to transfer genes to other organisms by horizontal transfer is a characteristic of many prokaryotes. Therefore, prevention of horizontal gene transfer from genetically engineered prokaryotes to naturally existing organisms is very critical and essential for biocontainment. One of the implemented approaches for the prevention is systems, pairing bacterial toxins and antitoxins from natural bacterial toxin-antitoxin (TA) modules [148–150]. In such systems, the toxin gene is inserted into the plasmid, and the organism, where this plasmid is intended to be transformed, must express an appropriate antitoxin gene for the avoidance of toxic action of toxin and the survival. Another strategy uses a conditional origin of replication, that allows plasmid replication only in bacteria with a specific combination of replication activating factors [151,152]. However, these strategies can be used to reduce gene transfer from plasmids or other mobile DNA elements but not within chromosomal DNA [134].

1.2.4.4. Future directions for biocontainment

As described above, various strategies are being designed for biocontainment of genetically modified microorganisms to prevent their unintended spread into the environment. However, none of them is perfect as all of them have specific or universal disadvantages and cannot ensure the complete containment. Only a combination of existing strategies, further improvements of already constructed systems or search for entirely new ideas for biocontainment can lead to the desired biosafety level. Some of the future directions for decreasing escapee rates involves specific degradation of transgenes in escapee microorganisms using target-specific CRISPR technologies, mutation recovery systems protecting from inactivation of synthetic gene circuits by Cas9 protein, appropriate modifications of cellular DNA-repair and mutagenesis pathways. Finally, use of multispecies consortia as a biocontainment strategy, where auxotrophic microorganisms are dependent from required elements supplied by co-existing other members of the community in a designed environment would be a powerful strategy [134].

1.3. Toxin-antitoxin systems

1.3.1. General description of bacterial TA systems

Toxin-antitoxin sytems are abundant genomic elements found in most bacteria and archea strains [153]. TA systems are typically composed of two elements: a toxic molecule toxin and its' antidote – antitoxin, which neutralizes the toxin activity. Toxins disrupt some key cellular functions: interfere with membrane integrity preventing proton movement [154,155], act as endoribonucleases or disturb DNA replication and translation by inhibiting DNA gyrase [156]. A small portion of known toxins stops cell division by interfering with the synthesis of bacterial peptidoglycan [157] or inhibiting polymerization of bacterial cytoskeletal proteins [158].

Usually, toxin and antitoxin are organized into operons where antitoxin gene aither precedes the toxin gene or is located downstream of the toxin. Essentialy TA systems are expressed from autoregulated promoters, antitoxin acts as a transcription repressor while toxin is a corepressor [159]. TA systems have been categorized into eight types, according to the mechanism by which antitoxin interacts with a toxin. In type I systems RNA antitoxins inhibits the translation of the toxin mRNA, while in type III systems RNA antitoxins bind directly to protein toxin. In type II and IV systems, antitoxins are proteins. In type II, antitoxin protein directly binds and inhibits the toxin protein, in type IV – indirectly counteract the toxin. In type V systems antitoxin is an RNase which degrades toxin-coding mRNA [154]. In type VI TA systems, antitoxin interacts with specific protease which degrades the toxin [160]. Type VII toxin-antitoxin system is similar to type V systems in such way, that antitoxin is an enzyme as well, but differently to V type systems, type VII antitoxin inactivates toxin by oxidizing a cysteine residue [161]. In recently discovered type VIII systems antitoxin RNA inactivates the toxin, which is a small RNA, by anti-sense binding [162].

Conventionally, TA systems have been claimed to function in stabilization of mobile genetic elements, bacterial stress tolerance, virulence, phage defence and also persister cells and biofilm formation [163] (Fig. 10). For several decades TA systems were considered associated with a programmed cell death in prokaryotes, which allow them to survive environmental stresses [164]. However, some notions of TA systems have been challenged by recent reports [159, 165, 166, 167]. Claims on bacteria determination to death by toxins were based on effects observed when toxic molecules were overexpressed ectopicaly, but there is no reliable evidence that bacteria are killed by toxins under physiological conditions [165]. It is clearly agreed that TA systems function in

stabilization of non-chromosomal mobile genetic elements like plasmids or prophages. However, physiological functions of chromosomal TA systems are disputed. Although TA systems appear to control bacterial growth rates during stress response but they are probably not utilized for persister cells formation [166]. A very recent review article states, that a phage inhibition is the primary physiological role of TA systems, as different types of TA systems inhibit phage and because phages evolved to inactivate or prevent activation of host TA systems [166]. More interestingly, it is presumed that TA systems evolved into components of CRISPR-Cas systems, specified for phage defence [168] , moreover, bacteria may use both CRISPR-Cas and TA systems for phage inhibition [169] . In addition, it was recently suggested to consider TA systems as members of interconnected gene networks rather than individual entities as cross-regulations (interactions) between TA systems of similar or distinct types, homologous and nonhomologous, chromosomic or encoded from plasmids were described in different microorganisms [170].

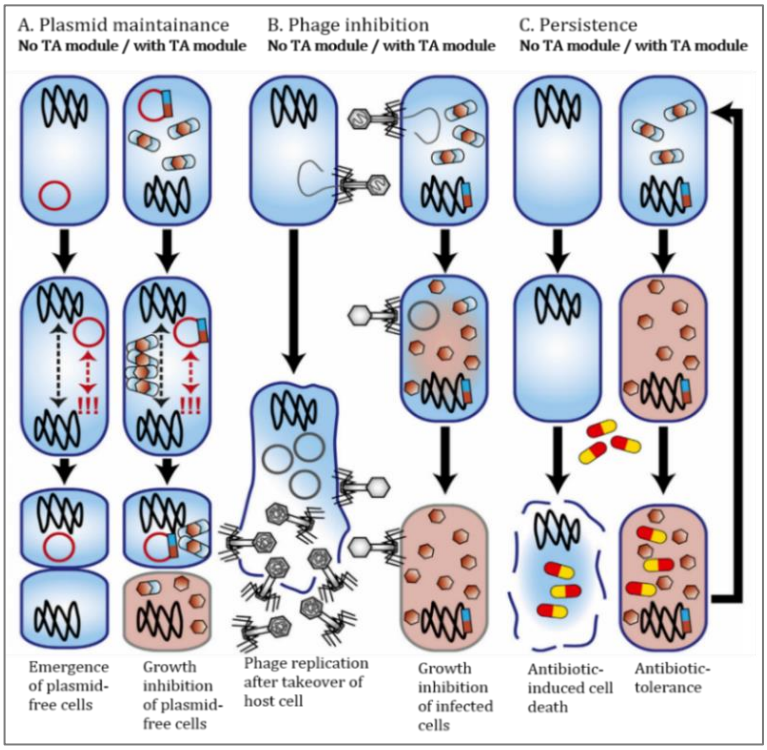

Figure 10. Representation of the main biological functions of TA modules ([171], corrected according to [167]).

1.3.1.1. Types of bacterial toxin-antitoxin systems

1.3.1.1.1. Type I TA systems

In type I systems, antitoxin is a small noncoding antisense RNA (sRNA) which binds toxins' mRNA and inhibits translation of the protein [172]. Under normal growth conditions, such antitoxin-toxin mRNA duplexes are quickly degraded by RNAse III. However, when bacterial cells experience stress, the pool of antitoxin sRNA is reduced and the translation of toxin is not triggered anymore resulting in the accumulation of the toxin. Toxins in type I systems are short hydrophobic peptides that penetrate membranes leading to a loss of membrane potential and resulting in growth inhibition. The hok/sok TA system (*parB* locus), initially discovered on plasmid R1 in *E. coli* [173] and later in many other microorganisms, is a well-known example of type I systems which was discovered the first. Locus encodes three genes – a stable highly toxic *hok* (host killing), *mok* (modulation of killing) which overlaps *hok* site and is responsible for *hok* expression and transcription regulation, and *sok* (suppressor of killing) – small antisense RNA, which is expressed in *cis* from a strong promoter, but is extremely unstable. Only mature *hok* transcripts are available for ribosome translation and also Sok-RNA binding and related to translation inhibition. Duplex of *hok* mRNA and *Sok*-RNA is also accessible for RNAse III degradation. In cases when the plasmid is lost, the amount of *sok*-RNA quickly decreases (half-life of the *sok* is about 30s) in a pool and mature and stable *hok* mRNA (half-life of the *hok* is about 30min) is freed for translation and subsequent growth arrest or even death of the plasmid-free cell progeny in such way maintaining beneficial plasmid in a culture of bacteria [174].

1.3.1.1.2. Type II TA systems

Type II TA modules are the largest group of TA systems with thousands of TA loci identified in most living bacteria. Differently from type I TA modules, toxin and antitoxin are proteins in type II TA systems. Type II antitoxins are usually composed of two domains – DNA binding N-terminal domain for autoregulation of transcription and C-terminal domain for direct binding and inactivation of the toxin. Inactivation of the toxin is performed in two modes of action: interference of catalysis at the active site of toxin (examples are TA modules MazEF [175], RelBE [176], VapBC [177] of *E.coli*) or steric obstruction of target binding for example, CcdBA [178].

One of the main paradigms about type II TA systems states that type II TA complexes are activated by proteolysis of antitoxin. Antitoxin is determined for proteolysis because of intrinsic instability and unfolded regions. In a model microorganism *E. coli* K-12 strain most antitoxins are degraded by Lon protease, although some are targeted by ClpP with the help of its adapters ClpA or ClpX [179]. The significant difference between half-lives of toxin and antitoxin (ten times on *E. coli* RnIAB system case [180]) is very important for biological functions of TA modules when living conditions change quickly as bacteria experience phage infection or lose necessary plasmid during segregation. Quick degradation of antitoxin leads to the growth arrest of the cell due to cellular effects of the free toxins – inhibition of replication by inhibiting DNA gyrase [181] or translation by cleaving mRNA [182,183], inactivating ribosome elongation factors [184] and other processes.

Interestingly, a very recent report insist to revise the paradigm about toxin activation because of antitoxin degradation. The paradigm is challenged by observation that not all antitoxins are unstructured so can not be targeted by Lon protease since Lon preferentially degrades unstructured proteins. The protease can participate in antitoxins degradation during cellular stress however there is little evidence that they target antitoxins when bound to toxins. It was proposed that toxins are more likely activated by their *de novo* synthesis laking sufficient amount of antitoxins. As toxin and antitoxin are co-transcribed from their operon in type II systems, probably there are some still unknown factors which determine a antitoxin mRNA portion for inactivation while leave the toxin portion for translation during strees [165].

The majority of type II toxins are endoribonucleases (RNases). They are ribosome-dependent or ribosome-independent RNase toxins. Ribosome-dependent toxins bind directly to A site of ribosome where they cleave ribosome-associated mRNA, for example toxins HigB [185,186], RelE [187,188] or YafQ [189] in *E. coli*. Ribosome association is not required for the activity of other type II RNase toxins like MqsR [153] or dimer of toxin MazF [175,190] (Fig. 11).

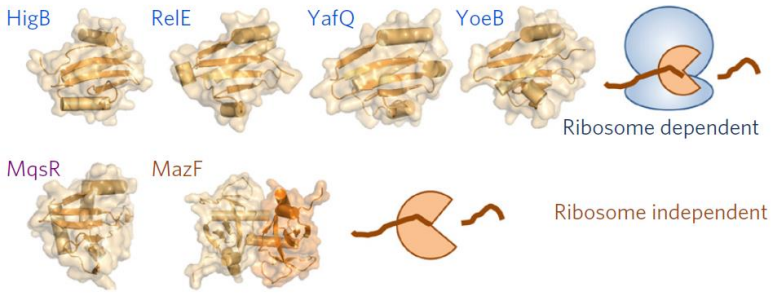

Figure 11. Ribosome-dependent and ribosome-independent RNase toxins**.** All ribosome-dependent RNase toxins (HigB, RelE, YafQ, YoeB) adopt microbial RNase fold. Although MqsR adopts RNase fold, it does not require ribosome for activity as well as dimer of MazF [191].

1.3.1.1.3. Type III TA systems

In type III TA systems antitoxin is a small RNA like in type I TA systems, however, differently from type I systems, antitoxin does not interact with toxin mRNA but forms secondary structure and binds directly to the toxin. Best studied type III TA system is *toxIN* [192,193]. ToxN is RNAse and cleaves *toxIN* transcript into active antitoxin sRNA fragments and degrades other mRNAs. The activity of toxin is inhibited when toxI sRNA blocks its active site.

1.3.1.1.4. Type IV-VIII TA systems

In type IV TA systems as in type II systems, both toxin and antitoxin are proteins. However, in type IV systems toxin and antitoxin do not interact with each other directly. For example, toxin inhibits growth by binding and inhibiting the polymerization of the bacterial cytoskeletal proteins MreB and FtsZ, thus blocking cell division. Antitoxin in such TA system does have a contrary effect: it stabilizes proteins and promotes cytoskeletal formation [194].

There is only one known type V TA system named GhoST. Antitoxin GhoS is an RNase which cleaves toxins GhoT mRNA under normal growth conditions. However, when bacteria experience stress, antitoxin GhoS is degraded by type II toxin MqsR and the toxin is freed for action as a small hydrophobic peptide that damages the cell membrane like toxins in type I TA systems [154]. *GhoST* TA system is an example of how one TA system can be regulated by another.

An example of type VI TA systems is *SocBA* module. The toxin SocB blocks replication elongation by binding directly to the β sliding clamp DnaN and outcompeting other clamp-binding proteins. Antitoxin SocA is a proteolytic adaptor which promotes toxins SocB degradation by protease ClpXP [160]. In a type VII system, Hha/TomB an antitoxin is an enzyme which does not cleave mRNA of a toxin as in type V systems but inactivates the toxin by oxidizing a cysteine residue [161]. In type VIII TA system SdsR/RyeA both toxin and antitoxin are sRNAs. The research showed that RyeA, as a *cis*-encoded sRNA, inhibited the observed SdsR-driven cell death by facilitating the degradation of SdsR [162].

1.3.1.2. Strategies for identification of TA systems and confirmation of their activity

First described TA systems were identified according to their plasmid stabilizing effect [195]. Also some later TA modules were identified by this approach. A genome or plasmid, where TA systems were searched for, was randomly fragmented, fragments were inserted into vector pALA136, containing P1 replicon and ColE1 origin from which replication results into a moderate copy number of the vector with a fragment, inserted for creation of a library, in a wild-type *E. coli*. The library is then transformed into the strain, where ColE1 origin is not functional, and low copy number of the vector is obtained by control of P1 replicon. The transformants are then plated several times on non-selective medium and, finally, plasmid maintenance is investigated by plating on antibiotic-containing selective medium. *StbD/stbE* TA systems of *relE/parE* superfamily, inhibiting translation, were also isolated by this approach [196].

Some of new type II TA systems were discovered by shotgun cloning [197]. The principle of this strategy is to randomly fragment the investigated genome, ligate fragments into a vector and transform into *E.coli*. Only transformants containing both toxin and antitoxin or antitoxin gene alone grow up because the growth of transformants containing solely toxin without its cognate antitoxin is arrested and clones are not obtained. Eight previously unidentified families of TA systems were identified using an application of additional selection criteria by shotgun strategy and six of them were later evaluated experimentally. However, TA systems with tiny antitoxin genes are difficult to identify because of a low possibility of such system fragmentation and almost only TA systems which are active in *E.coli* can be identified using shotgun approach [198].

Currently, new TA systems are usually discovered by bioinformatic analysis using sequences of already known TA systems by created algorithms [199,200]. Important criteria for the selection of TA modules are physical proximity and size of genes of toxin and antitoxin. Also type I TA systems were identified by searching for small ORFs, transmembrane motifs and aromatic or polar amino acids at C-terminus of predicted proteins [200]. Furthermore, the tendency of TA modules to be located in tandem duplicates were incorporated into the algorithms and enhanced the efficiency of TA module detection. To date, several bioinformatic tools are available for the detection of TA modules in bacterial genomes: RASTA-Bacteria [201], TASmania [202] or TADB 2.0 database tool TA-finder [203].

After preliminary identification, the functionality of TA systems must be confirmed experimentally using several criteria. The overexpressed toxin should inhibit the growth of its host and co-expression of antitoxin should revert the action of the toxin. For such evaluation, toxin and its antitoxin are usually inserted under the control of different inducible promoters, often an IPTG-inducible promoter (*lac*) and an arabinose inducible promoter (*ara*) [204,205,206]. The first evidence of the toxic activity of a predicted toxin can be morphological changes noticeable after the induction of toxin expression. Inhibition of DNA replication may result in cell filamentation, impaired cell wall synthesis may cause bulge formation: toxins targeting the cytoskeleton may change the shape of the cell from spherical to lemon. However, morphological changes must be confirmed additionally by other methods. A pulse-chase method using radiolabelled nucleotides or amino acids is used to determine if the action of toxin impairs DNA, RNA or protein biosynthesis [205,154,207]. If toxin targets cell membrane, staining techniques can be used together with fluorescence microscopy [208]. Membrane staining techniques and evaluation of adenosine triphosphate (ATP) level and activity of nicotinamide adenine dinucleotide phosphate (NAD(P)H)-dependent oxidoreductases is useful to distinguish living and dead cells [205]. Application of such methods can also be helpful for investigation if TA systems are bacteriostatic or bactericidal as this is still a matter for debate [197].

1.4. Inducible promoters for heterologous gene expression in bacteria

Among many factors impacting prokaryotic gene expression, careful selection of a suitable promoter for expression of genes of interest is still a challenging task in many biological experiments. While strong inducible promoters with minimal basal expression are a frequent choice of bioprocess specialists, many

other promoters with varying strength and regulation profiles are desired by metabolic engineers and synthetic biologists. As a result, a significant increase in a variety of newly identified and newly engineered promoters from and for various microorganisms can be recognized in recent years.

Gene regulation of bacteria has been studied for many years and even if many aspects of regulatory mechanisms are still missing and not all participating factors are known, undeniable progress has been made in a field over a relatively short period. Mechanism of DNA transcription, promoter architecture, three-dimensional structures of transcription factors are only a small part of all the knowledge the scientific community has gathered and which enabled the development of novel tools for prokaryotic microorganism engineering. Early studies of transcription regulation of model microorganism *E. coli* pointed in directions for further deeper and broader research in a field. At the early stages of transcription studies, promoters have been described as one of the most fundamental regulatory elements present in bacterial operons. Structure and consensus sequence of *E. coli* promoters were determined by numerous sequencing experiments (Fig. 12). However, only a small part of prokaryotic promoters identified up to now fit the typical structure, and many more of them vary significantly in length and content of nucleotides in their motifs and their regulation undoubtedly. Therefore, despite numerous tools and approaches created for the prediction of prokaryotic promoters, identification and engineering of them remains a very challenging task (*according to* Jajesniak and Seng Wong 2015).

1.4.1. Design of promoters

Because of considerable variation and complexity of naturally existing promoters, biotechnologically significant promoters were generally not constructed *de novo* but were engineered using three main strategies: saturation mutagenesis of spacer regions, error-prone PCR and hybrid promoter engineering. Saturation mutagenesis of spacer regions changes nucleotides surrounding –35 and –10 motifs and relies on the fact that changes in consensus regions of promoters decrease RNA polymerase binding to the promoter. This strategy allows the creation of a library of promoters of different strengths [210,211]. A similar strategy to obtain promoters with variant strength is error-prone PCR which also generates point mutations, however, in the whole promoter, but the library of mutants contains a much smaller fraction of functional members compared to saturation mutagenesis of spacer regions method [209].

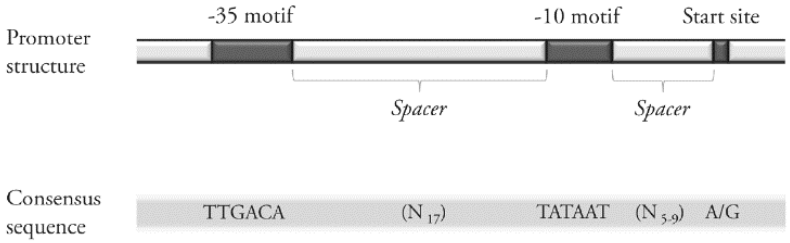

Figure 12. Representation of the typical structure of prokaryotic promoter – the simplified structure and consensus sequence of *E. coli* σ^{70} promoter [209].

The third strategy of obtaining new synthetic promoters is hybrid promoter engineering which combines parts of promoters of different sources. A widely used *tac* promoter is an excellent example of successful hybrid promoter engineering. *Tac* promoter was constructed by composing fragment downstream to –20 position of *lacUV5* promoter and fragment upstream to -20 position of stronger *trp* (tryptophan) promoter. In other words, –36 region of *lavUV5* promoter (mutant *lac* promoter containing two mutated nucleotides at –10 hexamer region which makes the promoter no longer sensitive to catabolite repression and promoter recruits RNA polymerase more effectively than *lac*) was replaced with –35 region of *trp* promoter (Fig. 13). It was shown that *trp* promoter in trpR- host was three times stronger than *lacUV5* promoter. Interestingly, constructed *tac* promoter was even measured to be about 11 times stronger than *lacUV5*. Thus, hybrid promoter is far more efficient than parent promoters. Authors of the promoter predict that increased efficiency can be related to the optimized distance between –10 and –35 hexamers in the hybrid promoter (17 bp) compared to the parental *lacUV5* (18 bp) [212]. Other examples of hybrid promoters are *Bacillus subtilis* P_{SPAC} promoter [213] and *E. coli rha*P_{BAD} expression system [214].

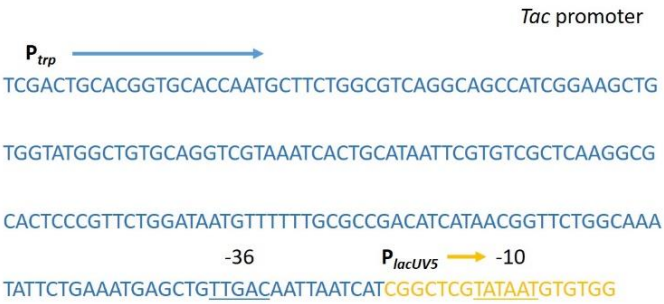

Figure 13. Composition of the *tac* promoter.

Novel methods of synthetic biology are also being recruited for generation of new active promoters. For example the use of General adversarial networks (GANs) model for creation of new promoters. Evaluated in other fields, such as natural image generation, image-to-image translation and super resolution image creation, GAN models were used for successful generation of novel synthetic promoters. A GAN model was trained to extract features from natural promoters using dataset of experimentally identified promoters. Generated millions of brand new artificial sequences were further filtered by activity predictive model and then their activity evaluated experimentaly. Up to 70.8% of the designed promoters were functional, some of them showed activities similar to some most active natural promoters or even performed better [215]. Application of such methods facilititates promoter search and creation and, definitely, starts a new era for fundamental research and biotechnology.

1.4.2. Selection of promoters

Selection of an appropriate promoter is one of the most crucial and impactful factors. Most commonly, the desired properties of a suitable promoter are robust and low-cost induction, low basal expression level and high yield of functional protein. However, the decision is complicated in most cases as expression systems do not meet the ideal criteria. For example, phage T7 expression system which is characterized by high expression of the heterologous protein, is inducible by a very expensive inducer IPTG and is associated with considerable basal expression [216]. Furthermore, T7 promoter requires the co-expression of T7 RNA polymerase from a separate vector or bacterial chromosome (*E. coli* strains BL21, C41, C43 contains T7 RNA polymerase expression cassette stably inserted into their chromosome), as this promoter is specifically recognized only by T7 polymerase. Moreover, high-level expression from the promoter does not always result in a high yield of the functional heterologous protein as part of the protein may be insoluble and aggregated into intracellular inclusion bodies [217 218]. However, a high level of expression is not always a purpose if toxic biomolecules or membrane proteins are wanted to be expressed. In such case, low expression and minimal basal expression would be desirable characteristics for the promoter. As many promoters are present for the choice of the researchers and all of them do have advantages and disadvantages in individual cases Jajesniak and Wong summarized main properties of each of the most popular promoters to facilitate the choice [209] (Tab. 2).

Table 2. Most commonly used inducible *E. coli* expression systems and their essential properties [209].

Name	Expression level	Basal expression	Catabolite repression	Inducer	Inducer cost	References
lac	Low-medium	High	Yes	IPTG	High	[219]
lacUV5	Low-medium	High	Reduced	IPTG	High	[220] [221]
trp	High	High	No	IAA	High	[222] [223]
tac	High	High	Reduced	IPTG	High	[212]
trc	High	High	Reduced	IPTG	High	[224]
phoA	High	Low	No	Phosphate starvation	-	[225]
P_L	High	Low	No	Temperature shift	-	[226] [227]
tetA	Medium-high	Low	No	Anhydrotetracycline	Low	[228]
araBAD	Low-high	Low	Yes	L-arabinose	Low	[229] [230]
$rhaP_{BAD}$	Low-high	Low	Yes	L-rhamnose	High	[214]
T5/lac	Very high	High	Reduced	IPTG	High	[231]
T7	Very high	High	Reduced	IPTG	High	[232]
T7/proU	Very high	Low	No	NaCl	Very low	[233]

As most of known and most frequently used expression systems exhibit undesirable properties, alternative strategies for promoter engineering and even novel expression systems were developed with a purpose to broaden the area of choices. Good examples are propionate-inducible [234] and cumate-inducible expression systems [235]. However, even if the addition of chemical inducer into bacterial culture is comfortable in small scale studies, it can be problematic because of potential contamination or too high costs in large scale protein production. Therefore, expression systems with other modes of induction were constructed. One example of such a system consists of temperature-inducible bacteriophage P_L promoter controlled by temperature-sensitive repressor cI857 [226,227]. Another example is auto-induction by glucose depletion in a growth medium of the T7 expression system. A low amount of glucose inhibits protein expression at the early stages of bacterial growth, but expression starts when cells run out of glucose [236].

A great variety of expression systems developed for *E. coli*, *B. subtilis*, *Pseudomonas* spp. and others used till now demonstrate a rapid and broad development of promoter engineering strategies and high-throughput screening technologies. Even if definition and comparison of promoter

strength remain complicated task till date, expansion of existing dababases and development of bioinformatics tools let us assume that promoter selection is going to be a more defined and standardized procedure in the near future.

1.4.2.1. IPTG and cumate inducible expression systems

IPTG inducible expression systems. IPTG (isopropyl β- d-1-thiogalactopyranoside) is a synthetic chemical reagent, mimicking a natural chemical – allolactose, an inducer of *lac* operon present in *E. coli* and many other bacterial species. *Lac* operon encodes several bacterial proteins responsible for lactose catabolism at the scarcity of glucose, a primary carbon source. Gene regulation of the *lac* operon was the first understood clearly and became the foremost example of regulation mechanisms in prokaryotic organisms. Allolactose is an isomer of lactose formed after lactose enters a bacterial cell. Many regulated expression systems were constructed on the base of the lac operon. The main components of these expression systems are lacI gene, encoding lac repressor, a promoter of choice and lac operator lacO sequence inserted right downstream the promoter. Expression from the promoter is blocked by the bind repressor protein when the IPTG inducer is not present. Under induction, IPTG removes the repressor from the operator, and the expression of the desired protein from the promoter starts. At low concentrations, IPTG can be actively transported to bacterial cell by lac permease LacY, also encoded by lac operon. However, IPTG also enters the bacterial cell by simple diffusion at higher concentrations, used for routine induction of expression systems [237].

Several different promoters were used for the construction of different IPTG inducible expression systems (Tab. 2). In expression systems containing promoters *tac*, *lacUV5* or bacteriophage T5 promoter are inserted between *lacI* and *lacO* and are induced by lac repressor removal by IPTG (Fig), while, for example, bacteriophage T7 expression system (already mentioned in a previous section) is regulated by more sophisticated mechanism. The original T7 expression system is a two-part system composed of two IPTG inducible expression cassettes. GOI is expressed from T7/lacO promoter recognized specifically by T7 polymerase, inserted into the vector. At the same time, T7 polymerase is expressed from the cassette with lacUV5 promoter inserted into *E.coli* genome. Under induction with IPTG, T7 polymerase specifically recognizes promoter T7/lacO and transcription of GOI starts [233]. Different variants with various combinations of elements of T7 expression system were created since the original system was constructed.

Cumate-inducible expression system. Cumate-inducible expression system, referred to as a cumate gene switch, is based on the elements of *Pseudomonas putida* F1 *cym* and *cmt* operons. Both operons participate in catabolism of a terpene *p*-cymene (*p*-isopropyltoluene), which is found in essential oils from over 100 plant species [238]. *Cym* operon encodes the conversion of *p*-cymene to *p*-cumate, while *cmt* operon located downstream to *cym* operon encodes further catabolism of p-cumate. Cumate gene switch was firstly constructed and used for regulation of heterologous gene expression from promoter PmxaF in *Methylobacterium extorquens* [239]. An operator of the *cmt* operon was inserted downstream of the cloned PmxaF promoter in the broad-host-range expression vector (pCHOI3). The repressor gene (*cymR*) located upstream of the *cym* operon in *P. putida* F1 was amplified by PCR. As overexpression of *CymR* was toxic for *M. extorquens* and caused growth inhibition, single and/or double copies of *cymR* were integrated into the chromosome of *M. extorquens* [239]. Differently from this strategy, *cymR* was inserted under the control of a weak and constitutive kanamycin promoter P_{km} whereas the cumate gene switch was adapted for *E.coli* and used for regulation of a strong partial T5 phage promoter in an expression vector pNEW in the work of another research group [235]. Mechanistically, the addition of inducer cumate removes the repressor CymR (which is constitutively expressed from P_{km} promoter in the same vector) from the operator and allows gene transcription from T5 promoter by bacterial RNA polymerase (Fig. 14). Initially, the expression system was used for the expression of several genes in *E.coli* and green fluorescent protein GFP was among them. GFP expression reached its maximum after 4h induction with only 30 μM cumate [235]. Later, cumate induction system was successfully adopted for transgene expression in other microorganisms, such as industrial production hosts like *Bacillus subtilis* and *Bacillus megaterium* [240] and for expression in mammal cells [241]. Cumate-inducible expression systems are already available commercially as a SparQ lentivectors from System Biosciences company for expression in mammal cells.

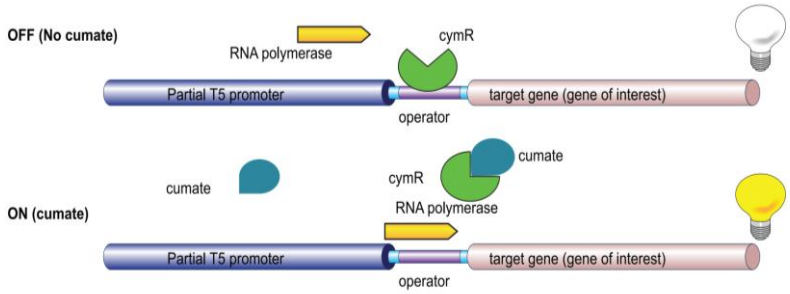

Figure 14. A schematic representation of the mechanism of action of cumate gene switch in *E. coli* [235].

1.5. Summary

An *Agrobacterium*-mediated plant transformation is a conventional approach for creating transgenic plants and transient expression of GOI in a small laboratory or production scale. However, the use of GM *Agrobacterium* for transfection faces biosafety issues for open environments and contained facilities as a risk of agrobacteria escape can not be ruled out. Despite several attempts to address agrobacteria containment issues, such as constructing auxotrophic *Agrobacterium* strains [140, 141] or introducing a kill switch into *Agrobacterium* [147], biocontainment issues of GM agrobacteria or biosafety of agro-transformation are rarely mentioned in a published data. Still, the importance of biosafety of contained or open environment transformation is increasing as plants become recognized expression hosts for the recombinant proteins. In a dissertation work, we addressed biosafety issues of *Agrobacterium*-mediated transient transfection with two novel strategies – control of a horizontal gene transfer, T-DNA transfer, by inducible expression of an essential *Agrobacterium* virulence gene *virE2* and induced expression of the toxin-antitoxin system from *A. tumefaciens* genome.

Although much is known about the role and importance of *virE2* in an *Agrobacterium*-mediated plant transformation, little data is published about the inducible expression of heterologous genes in agrobacteria. An early study demonstrated the activity of *araC* and *lacI* based induction systems in *A. tumefaciens* [242]. Also a *tetR* system was used to express Cre-recombinase in *Agrobacterium* [243], other groups demonstrated an activity of P_{lac} promoter [244, 245]. In addition, lacI-regulated T7 expression system showed functionality in *Agrobacterium* [246]. However, a more comprehensive analysis and development of heterologous gene expression control in *A. tumefaciens* were undoubtedly needed for the tight inducible regulation of horizontal gene

transfer between agrobacteria and plants. Thus, a search for efficient inducible promoters in *Agrobacterium* became a primary task of a dissertation work.

Scarce information was also apparent in the field of TA systems in *Agrobacterium.* The only agrobacterial TA system, *ietA/ietS*, was confirmed to be functional in agrobacteria at the begining of our research [247]. Another one, *yoeB/yefM*, has been described in a very recent publication [248]. A broad interest in TA systems and accumulating data about them in other microorganisms became our reasons to search for active toxins from TA modules in a *A. tumefaciens* genome and evaluate a TA system's suitability for control of *Agrobacterium*-mediated transient transfection of plants.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Chemicals

Table 3. Chemicals used in this work.

Chemicals	**Manufacturer**
DMSO, TRIS, EDTA, Hepes, $CaCl_2$, KCl, spectinomycin, acetic acid, cumic acid (4-isopropylbenzoic acid), acetosyringone (3',5'-dimethoxy-4'-hydroxyacetophenone), KOH.	Sigma
Pipes, $MnCl_2$, $MgSO_4$, methanol	Fluka
Glycerol, agarose, nystatin	Fisher Scientific
Ampicillin, ethidium bromide	Roth
Kanamycin	Applichem
Silwet L-77	Kurt Obermeier GmbH And Co
IPTG	Thermo Fisher Scientific Baltics
Ethanol	Vilniaus Degtinė

2.1.2. Buffers and solutions

TAE buffer: 40 mM Tris-acetate, 1 mM EDTA, pH 7.9 at 25 °C.
TB buffer: 10 mM Pipes, 15 mM $CaCl_2$, 250 mM KCl, 55 mM $MnCl_2$, pH 6.7 at 25 °C. 1 M $CaCl_2$, 2 M KCl and 1 M $MnCl_2$ stock solutions were prepared in advance then particular amount stock solutions were added to the final mixture. 10 mM Pipes, 15 mM $CaCl_2$, 250 mM KCl were mixed all together in this order, pH 6.7 was set up with KOH (pH increases very slowly, therefore starting volume of the solution should not be high). Afterwords 55 mM $MnCl_2$ was added from 1 M stock solution, the mixture was filter sterilized.
1mM Hepes buffer: 1mM Hepes solution, pH=7, sterilized at 121°C for 20 min, stored in the dark.
1mM Hepes/10% glycerol buffer: 1 mM Hepes solution, 10% of glycerol added, pH=7. Solution sterilized at 121°C for 20 min, stored in the dark.

Cumic acid solution: because of slight solubility in water, the stock solution of 150 mM cumic acid was prepared in 96% ethanol, further dilutions were made in distilled or tap water as needed.
IPTG solution: stock solution of 100 mM was prepared in distilled water, filter sterilized.
Antibiotics stock solutions: Rifampicin stock solution of 25 mg/mL was prepared in methanol. Ampicillin stock solution of 100 mg/mL, kanamycin, spectinomycin and nystatin stock solutions of 50 mg/mL were prepared in distilled water, filter sterilized. All stock solutions of antibiotics stored at –20 °C.

2.1.3. Enzymes

DNA restriction and modification enzymes (T4 DNA ligase, polymerases *Pfu*, *Phusion* and *Taq*, Klenow fragment and restriction endonucleases), reagents and buffers for the reactions from Thermo Fisher Scientific Baltics were used for all cloning steps according to the manufacturer's recommendations.

2.1.4. DNA vectors

Table 4. DNA vectors used in the study.

Vector	Description	Source
pTZ57R	Amp^r, PCR cloning vector	Thermo Fisher Scientific Baltics
pJET1.2	Amp^r, PCR cloning vector	Thermo Fisher Scientific Baltics
pNMD803	Km^r ,binary viral vector, TMV virus-based, ΔMP	Nomad Bioscience
pICH18711	Km^r, binary viral vector, TMV virus-based, MP	Icon Genetics [249]
pICH27566	Km^r, binary viral vector, PVX virus-based, CP	Icon Genetics
pGEX5x-1	Amp^r, expression vector	GE Healthcare
pET16	Amp^r, expression vector	Novagen
pQE30	Amp^r, expression vector	Qiagen
pNDC	$Spec^r$, in house binary vector	Department of Eukaryote gene engineering, Life Sciences Center of Vilnius University.

2.1.5. Kits used for molecular cloning, nucleic acid purification and qRT-PCR

A. GeneJET Genomic DNA extraction Kit (K0721, Thermo Fisher Scientific Baltics).
B. CloneJET™ PCR Cloning Kit (K1231, Thermo Fisher Scientific Baltics).
C. InsTAclone™ PCR Cloning Kit (K1213, Thermo Fisher Scientific Baltics).
D. GeneJET™ Plasmid Miniprep Kit (K0502, Thermo Fisher Scientific Baltics).
E. GeneJET™ Gel Extraction Kit (K0691, Thermo Fisher Scientific Baltics).
F. GeneJET™ RNA Purification Kit (K0732, Thermo Fisher Scientific Baltics).
G. Verso 1-Step qRT-PCR Kit SYBR Green, with separate ROX vial (AB4104A, Thermo Fisher Scientific Baltics).

2.1.6. Bacterial strains

E. coli strains used in the study are listed in Table 5. *A. tumefaciens* strains used and newly transformed during the study are listed in Table 6.

Table 5. *E.coli* strains used in the study.

Strains	Description	References
DH5α	F– *gyrA96* (NaI^r) *recA1 relA1 endA1 thi-1 hsdR17* (r_k^- m_k^+) *glnV44 deoR* Δ (*lacZYA-argF*) *U169* [φ80dΔ(*lacZ*) *M15*]	[250]
DH1	F– *gyrA96* (NaI^r) *recA1 relA1 endA1 thi-1 hsdR17*(r_k^- m_k^+) *glnV44*	[251]
BL21(DE3) pLysS	F– *ompT gal dcm lon hsdS$_B$* (r_B^- m_B^-) λ(DE3[lacI lacUV5-T7 gene 1 ind1 sam7 nin5]) pLysS(cm^R)	[252]

Table 6. *A. tumefaciens* strains used in the study.

Strains	Description	References
GV3101	(pMP90RK), nopaline, Rif^r	[253]
ICH011	GV3101*ΔvirE2* (pMP90RK), nopaline, Rif^r	[254]
KYRT-1	Disarmed Chry 5, Rif^r	[255]
GV3101(pICH18711)	contains TMV virus-based vector with GFP	This study
GV3101(pICH27566)	contains PVX virus-based vector with GFP	This study
GV3101(pNMDV36)	contains pNMD803-*lacIq*, P_{lac}::*lacZ*	This study
ICH011(pNMDV148)	contains pICH18711-*lacIq*, P_{tac}::*virE2*	This study
ICH011(pNMDV150)	contains pICH18711-*lacIq*, P_{lac}::*virE2*	This study
GV3101(pNMDV163, pNMDV223)	contains pNDC-*lacIq*, P_{lacUV5}::*T7pol*, pICH18711- *lacI*, $P_{T7/lacO}$::*lacZ*	This study
ICH011(pNMDV163, pNMDV164)	contains pNDC-*lacIq*, P_{lacUV5}::*T7pol*, pICH18711-*lacI*,$P_{T7/lacO}$::*virE2*	This study
ICH011(pNMDV281)	contains pICH18711-*cymR*, $P_{T5/CuO}$::*virE2*	This study
GV3101(pNMDV299)	contains pNDC-*cymR*, $P_{T5/CuO}$::*lacZ*	[254]
ICH011(pNMDV355)	contains pICH18711-*cymR*, $P_{tac/CuO}$::*virE2*	This study
ICH011(pNMDV356)	contains pICH27566-*cymR*, $P_{tac/CuO}$::*virE2*	This study
GV3101(pNMDV376)	contains pNDC-*cymR*, $P_{lacUV5/CuO}$::*lacZ*	[254]
ICH011(pNMDV381)	contains pICH27566-*cymR*, $P_{T5/CuO}$::*virE*, *virE2*	This study
ICH011(pNMDV386)	contains pICH27566-*cymR*, $P_{T5/CuO}$::*virE2*	This study
GV3101(pNMDV395)	contains pNDC-*cymR*, $P_{tac/CuO}$::*lacZ*	[254]
GV3101(pNMDV417)	contains pNDC-*lacIq*, $P_{T5/lacOlacO}$::*lacZ*	This study
ICH011(pNMDV437)	contains pICH27566-*cymR*, $P_{virE/CuO}$::*virE2*	This study
GV3101(pNMDV442)	contains pICH27566- *cymR*, $P_{virE/CuO}$::*lacZ*	This study
ICH011(pNMDV457)	contains pICH27566-*cymR*, $P_{lacUV5/CuO}$::*virE2*	This study

Strains	Description	References
ICH011(pNMDV519)	contains pICH18711-*lacIq*, $P_{T5/lacOlacO}$::*virE2*	This study
GV3101Δ*pemIK*	(pMP90RK), nopaline, Rif ^{r}, *pemIK* region deleted	[256]
GV3101 (pNMDV621)	contains pNDC-cymR-$P_{tac/CuO}$::*dead*	[256]
GV3101 (pNMDV622)	contains pNDC- cymR-$P_{tac/CuO}$:: *PIN*	[256]
GV3101 (pNMDV623)	contains pNDC- cymR-$P_{tac/CuO}$:: *vapC*	[256]
GV3101 (pNMDV624)	contains pNDC- cymR-$P_{tac/CuO}$:: *nuclease_like*	[256]
GV3101 (pNMDV371)	contains pNDC- cymR-$P_{T5/CuO}$:: *pemK-*P_{tetR}	[256]
GV3101 (pNMDV375)	contains pNDC- cymR-$P_{T5/CuO}$:: *pemIK*	[256]
GV3101 (pNMDV536)	contains pNDC- cymR-$P_{tac/CuO}$:: *ietS*	[256]
GV3101 (pNMDV371+pICH27566)	contains pNDC- cymR-$P_{T5/CuO}$:: *pemK-*P_{tetR} + pICH27566	[256]
GV3101Δ*pemIK* (pNMDV515+pNMDV535)	contains pNDC- P_{virE}::*pemK* + pICH27566-cymR-$P_{tac/CuO}$::*pemI*	[256]
GV3101Δ*pemIK* (pNMDV515+pNMDV507)	contains pNDC- P_{virE}::*pemK* + pICH27566-cymR-$P_{lacUV5/CuO}$::*pemI*	Constructed by Šarūnas Paškevičius
GV3101 (pNMDV535)	contains pICH27566-cymR-$P_{tac/CuO}$::*pemI*	[256]
GV3101 (pNMDV507)	contains pICH27566-cymR-$P_{lacUV5/CuO}$::*pemI*	Constructed by Šarūnas Paškevičius

2.1.7. DNA primers

Table 7. List of DNA primers used in the study.

	Gene or promoter	Description	Primer name	Sequence	Restriction site inserted
A	*virE2*	Primers used for *virE2* coding sequence amplification from genomic DNA of *Agrobacterium* strain GV3101.	virE2 Fwd virE2 Rev	ACCATGGATCCGAAGGCCGAAGGC TACTAGTCTACAGACTGTTTACGGTTGGG	*Nco*I *Bcu*I
B	*virE1-virE2*	Primers used for the entire *virE1-virE2* coding sequence amplification from genomic DNA of *Agrobacterium* strain GV3101.	VirE1 (NcoI) Fwd virE2 Rev	ACCATGGTGATCATCAAGCTAAATGCG TACTAGTCTACAGACTGTTTACGGTTGGG	*Nco*I *Bcu*I
C	*lacIq*,P_{lac}	Repressor gene lacIq together with P_{lac} promoter sequence was amplified from *E. coli* strain DH1 genomic DNA for the construction of IPTG inducible constructs.	LacIq Fwd LacI Rev	AATTTAAATGTATACGACACCATCGAATGGT GCAAAAC TACTAGTTTTATACCATGGCTGTTTCCTGTGT GAAATTGTTATC	*Smi*I, *Bst1107*I *Bcu*I, *Nco*I
D	P_{tac}	The tac promoter was PCR - amplified from vector pGEX5x-1.	tac1 EcoRI Fwd tac1 NcoI Rev	AGAATTCGACTGCACGGTGCACCAAT TCCATGGAATACTGTTTCCTGTGTGAAATTG	*EcoR*I *Nco*I
E	*lacI truncated*	Primers used for the construction of vector pNMDV148 with *lacIq*, P_{tac}*::virE2*.	lacI EheI Fwd lacI mRNA EcoRI Rev	CTGGCGCCCAATACGCAAACC AGAATTCAGCCGGAAGCATAAAGTGTAAAG	*lacI* internal *Ehe*I *EcoR*I
F	P_{lacUV5}*::T7pol*	The T7 polymerase expression cassette P_{lacUV5}**::**T7pol was PCR amplified from E. coli strain BL21	BL21 (749727) EheI Fwd BL21(753066) BcuI Rev	AGGCGCCCAATACGCAAACCGC TACTAGTTACGCGAACGCGAAGTCCGAC	*lacI* internal *Ehe*I *Bcu*I

	Gene or promoter	Description	Primer name	Sequence	Restriction site inserted
		genomic DNA for IPTG inducible constructs.			
G	$P_{T5/lacOlacO}$	The $P_{T5/lacOlacO}$ promoter was PCR-amplified from vector pQE30 for the construction of IPTG inducible constructs.	T5 (Eco32I) Fwd T5 (NcoI) Rev	AGATATCAAATCATAAAAAATTTATTTGCTTTGT ACCATGGTTAATTTCTCCTCTTTAATGAATTCT	*Eco32*I *Nco*I
H	P_{virE}	The P_{virE} promoter region was PCR - amplified from *Agrobacterium* strain KYRT1 genomic DNA for the construction of plant factors inducible constructs.	PvirE/CumO Fwd PvirE/CumO Rev	TATTTAAATGCATGCCCAGGCCGCGTGCGTCCTGT AGAATTCATAATACAAACAGACCAGATTGTCTGTTT GTTATGTTCTCTCCTGCAAAATTGCGGTTTTCTA	*Smi*I, *Pae*I *EcoR*I,CuO inserted
I	*LacZ*	The *lacZ* sequence was PCR - amplified from *E.coli* strain DH1 genomic DNA for construction of inducible expression vectors used in β-galactosidase activity assays.	LacZ Fwd LacZ Rev	ACCATGGCCATGATTACGGATTCACTGGC TACTAGTTTATTTTTGACACCAGACCAACTG	*Nco*I *Bcu*I
J	*virE2*	Primers used for qRT-PCR evaluation of induced *virE2* expression.	VirE2-qPCR Fwd 2 VirE2-qPCR Rev 2	CGGCAAAGTCATGCGAATA GGACAAATCTGGAACCTCAC	
K	*rpoB*	Primers used for qRT-PCR evaluation of reference gene *rpoB* expression.	rpoB-qPCR Fwd rpoB-qPCR Rev	CGACATCTACCGCGTCATGC GCCAACAGCCGAGCGATCGT	
L	*16S rRNA*	Primers used for qRT-PCR evaluation of reference gene 16S rRNA expression.	16S rRNA-qPCR Fwd 16S rRNA-qPCR Rev	CGTGAGATGTTGGGTTAAGT ACCTTCCTCTCGGCTTAT	

2.1.8. Synthetic and constructed promoters

Table 8. List of synthetic and constructed promoters.

Promoter	Description		Source
$P_{tac/CuO}$	**Description**	Red characters: P_{tac} promoter, blue characters: CuO. Restriction sites of *Pae*I and *Nco*I are underlined.	Eurofins
	Sequence	GCATGCTCGACTGCACGGTGCACCAAT GCTTCTGGCGTCAGGCAGCCATCGGAA GCTGTGGTATGGCTGTGCAGGTCGTAA ATCACTGCATAATTCGTGTCGCTCAAGG CGCACTCCCGTTCTGGATAATGTTTTTT GCGCCGACATCATAACGGTTCTGGCAA ATATTCTGAAATGAGCTGTTGACAATTA ATCATCGGCTCGTATAATGTGTGGAAC AAACAGACAATCTGGTCTGTTTGTATTA TGAATTCCAGCACACTGGCGGCCGTTA CTAGAAATAATTTTGTTTAACTTTAAGA AGGAGATATACCATGG	
$P_{lacUV5/CuO}$	**Description**	Red characters: P_{lacUV5} promoter, blue characters: CuO. Restriction sites of *Pae*I and *Nco*I are underlined.	Eurofins
	Sequence	GCATGCGCGCAACGCAATTAATGTAAG TTAGCTCACTCATTAGGCACCCCAGGCT TTACACTTTATGCTTCCGGCTCGTATAA TGTGTGGAACAAACAGACAATCTGGTC TGTTTGTATTATGAATTCCAGCACACTG GCGGCCGTTACTAGAAATAATTTTGTTT AACTTTAAGAAGGAGATATACCATGG	
$P_{virE/CuO}$	**Description**	Red characters: P_{virE} promoter region, blue characters: CuO. Restriction sites of *Smi*I, *Pae*I and *Eco*RI are underlined.	This study (the P_{virE} promoter region was PCR-amplified from *Agrobacterium* strain KYRT1 genomic DNA, sequence of *CuO* operator was added to the promoter).
	Sequence	ATTTAAATGCATGCCCAGGCCGCGTGC GTCCTGTCTTTCCAGTTCCTCCCTTTCAG CTCGATTGTGGCATCATTTATTGCCTGC TCATTGCAGTTGAAACGCGATATCCGTT TCAAGACCCGGGTATGGATGGTACTTT GGAGATATGAGCACTTGCCAGACTTCC CGGTCGGAGACTTAAAAATTCCGATAA GAGGTTACGGACGACGGACCTTATAAG TGGATCGTCTAGTGGTGGCGCCGATCA AAACAGTTCCGCCCCCGGCTTTGCTCAA AGTAGCAAAGCAGCTTTAACTCGGGTT GCGGAGGATTTTCTAGAAAACCGCAAT TTTGCAGGAGAGAACATAACAAACAGA CAATCTGGTCTGTTTGTATTATGAATTC	

2.2. METHODS

2.2.1. Bacterial growth conditions

Both *E. coli* and *A. tumefaciens* cells were grown in LB (Luria-Bertani) medium at 37 °C or 28 °C, respectively. All media were supplemented, when necessary, with antibiotics: 100 μg/ml ampicillin, 50 μg/ml spectinomycin, 50 μg/ml kanamycin, 50 μg/ml rifampicin or 50 μg/ml nystatin.

2.2.2. Plant growth conditions

Nicotiana benthamiana plants were grown under controlled conditions (25 °C and 50 % relative humidity) at long-day photoperiod (16 h light and 8 h dark with illumination of approx. 320 lx). 5–6 weeks-old *N. benthamiana* plants were used for agroinfiltration experiments.

2.2.3. Construction of expression cassettes

Polymerase chain reaction (PCR) was used for amplification of DNA fragments from plasmid DNA or genomic DNA of *E.coli* or *A. tumefaciens*. Genomic DNA was extracted from cells using a commercial kit (see 2.1.5 A) according to the manufacturer's recommendations. Primers used in PCR reactions are listed in Table 7. PCR amplified genes were cloned into pJET1.2/blunt or pTZ57R/T – a commercial linearized positive selection cloning vectors (listed in Table 4) from commercial kits (see 2.1.5 B and C).

Gene sequences were confirmed by sequencing analysis and inserted into binary expression vectors under the control of inducible promoters for induced expression in *Agrobacterium*. *E. coli* strain DH5α (Table 5) was used as a recipient for all sub-cloning steps. Plasmid DNA was extracted from *E. coli* cells using a commercial plasmid DNA extraction kit according to the manufacturer's recommendations (see 2.1.5 D). DNA electrophoresis was performed in 0.8–2 % agarose gels using TAE buffer at a voltage of 10 V/cm. 0.1–0.2 μg/mL of Ethidium bromide was added to gels for visualization of DNA fragments. Gels were analysed in a transilluminator under UV light. DNA fragments were extracted from agarose gels using a commercial kit according to the manufacturer's recommendations (see 2.1.5 E). DNA concentration was determined by a spectrophotometer.

Constructed vectors were transferred into *A. tumefaciens* by electroporation (see 2.2.7). Detailed descriptions and schemes of plasmid construction are provided in Supplementary Figures.

2.2.4. Preparation of competent *E. coli* cells

Competent *E. coli* cells were prepared and transformed according to described methodology [257] with some modifications. *E.coli* cells were inoculated and grown overnight in LB medium. The next day OD_{600} of o/n culture was measured, and bacteria inoculated in SOB medium (100 mL of medium to 1L flask) to OD_{600} 0.2–0.3. 20 mL/L of 1 M $MgSO_4$ stock solution was added to the medium. *E. coli* culture was grown at 30 °C with rotation of 200 rpm till OD_{600} reached 0.6. The culture was cooled on ice for 10 min and transferred to two sterile tubes of 50 mL. Cells were spun at 2465 g 10 min at 4 °C, then resuspended in 0.5 mL ice-cold TB buffer (see 2.1.2). An additional 1.5 mL of TB buffer was added, cells resuspended and combined to one tube. Finally, 20 mL of ice-cold TB was added (total volume ~30 mL). Suspension kept on ice for 20 min. Cells were spun 10 min again at 4 °C at 2465 g and resuspended in 0.5 mL of ice-cold TB buffer, then 3.5 mL of the same buffer was added (total volume of suspension ~ 5 mL). 7 % of DMSO (0.35 mL of DMSO to 5 mL of bacterial suspension) was added to *E.coli* suspension by gentle swirling the tube and kept on ice for 10 min. Finally, bacterial suspension was divided into aliquots of 150 µL and frozen in liquid nitrogen immediately. Competent cells were stored at -80 °C.

2.2.5. Transformation of competent *E. coli* cells

A frozen aliquot (150 µL) of competent *E.coli* cells was thawed on ice. 15 µL (10 % of cells' volume) of ligation mixture or 1–2 µL plasmid DNA (~200–400 ng) was added gently. DNA-cells mixture was incubated on ice for 30 min. Cells were heat-shocked in a 42 °C thermostat for 90 s and returned to the ice for 2 min. Then 1 mL of sterile LB medium was added, tube with DNA-cells mixture incubated at 37 °C with shaking at 220 rpm for 45–60 min. The tube was spun 1 min at 20 000 g, the supernatant discarded, leaving ~50 µL of supernatant for cells resuspension. Cells were plated on LB medium with appropriate antibiotic selection.

2.2.6. Preparation of electro-competent *A. tumefaciens* cells

A. tumefaciens strain was inoculated from frozen stock in 4 mL LB medium with appropriate antibiotics and grown overnight. O/n culture was diluted to OD_{600} = 0.1 in 100 mL of LB medium (100 mL of LB medium went to 0.5 L or 1 L flask). The diluted culture was grown to OD_{600} = 0.5 (normally about 4 h of growth). Bacterial culture was centrifuged 10 min at 3220 g at 4 °C,

resuspended in 40 mL of sterile 1 mM Hepes buffer (pH=7) and centrifuged 10 min again at 3220 g at 4 °C. Bacteria pellet was resuspended in 40 mL of sterile 1 mM Hepes/10 % Glycerol buffer (pH=7) and centrifuged 10 min at 3220 g at 4 °C, then resuspended in 2 mL of 1 mM Hepes/10 % Glycerol buffer (pH=7). The obtained volume was divided into 2 microcentrifuge tubes and centrifuged 2 min at 3381 g at 4 °C. Pellet resuspended in 200 µL of 1 mM of Hepes/10 % Glycerol buffer pH=7. The suspension was aliquoted into 40 µL samples and stored at -80 °C.

2.2.7. Transformation of electro-competent agrobacteria by electroporation

Electrocompetent *Agrobacterium* cells were thawed on ice. 40 µL of cells were mixed with 1–2 µL of plasmid DNA (~500 ng). DNA and cells mixture was transferred to a chilled on ice cuvette avoiding air bubbles. The capacitance of the electroporator was set to 25 µF, voltage to 2.5 kV, and resistance to 400 Ω. Outside of the cuvette was dried with tissue paper, and the cuvette was inserted into the cuvette chamber of electroporator. Immediately after electrical pulse, cuvette was removed and 1 mL of cold LB medium (without antibiotics) was added. The solution was mixed by gentle pipetting up and down. Cells were transferred to microcentrifugation tube and incubated at 28 °C for 2–4 h in the shaker for recovery. Then cells were centrifuged 2 min at 6000 rpm and resuspended in 100 µL of LB medium. 1/10 part of the volume of transformed cells was plated on first LB plate with antibiotics and the rest on a second LB plate with antibiotics. Plates were incubated at 28 °C for 2–3 days growth while colonies appeared.

2.2.8. β-galactosidase activity assay

The β-galactosidase activity of agrobacterial strains was determined with a colourimetric assay described earlier [258] with an extra step added: cells were pelleted and resuspended in assay buffer after incubation step to avoid errors in the evaluation of β-galactosidase activity related to different carbon sources in the growth medium. *Agrobacterium* strains were grown overnight from frozen stocks, diluted with fresh LB medium to an OD_{600}~0.4 and then incubated with shaking for 4 hours at 28 °C in LB medium with inducer 150 µM cumic acid or 2 mM IPTG (solutions preparation see 2.1.2) or without inducer (agrobacteria usually reached OD_{600}~0.8) to compare the levels of β-galactosidase expression.

2.2.9. Syringe agro-infiltration of *N. benthamiana* plants

A. tumefaciens cultures carrying different constructs were grown in LB medium overnight, diluted with fresh medium to an OD_{600}=1 and then further diluted 10^3 fold in tap water containing IPTG or cumic acid. Syringe agroinfiltrations were performed on the abaxial side of the plant leaves. 200–300 μL of diluted *Agrobacterium* suspension were used for one syringe infiltration.

2.2.10. Evaluation of the T-DNA transfer efficiency and leakiness by agrospray

Agrobacterium strains carrying different constructs were grown overnight and diluted with fresh medium to an OD_{600}=1. For testing transfer efficiency, suspensions were further diluted $1x10^4$ fold in tap water containing 0.05 % Silwet L-77 and 150 μM cumic acid and incubated for 1h. For evaluation of leakiness, suspensions were diluted 10 fold in tap water + 0.05 % Silwet L-77. 7–10 mL of suspension were used for one plant spraying. Sprayings were performed on the abaxial side of the plant leaves. Five to seven independent induction experiments were conducted for each *Agrobacterium* strain. Each induction experiment utilized 3–5 plants with 4–5 leaves per plant being treated with *Agrobacterium* suspension. At 5–6 days post spraying (dps) plant leaves were detached and photographed under UV illumination. The number of GFP expression foci on plant leaves was counted to evaluate the frequency of the T-DNA transfer events. Average foci number and standard deviation were calculated and compared with wild type T-DNA transfer.

2.2.11. Evaluation of toxins' ability to induce death of *A. tumefaciens* ectopically

The overnight cultures of *A. tumefaciens* containing an inducible toxin were inoculated into 4 ml LB medium from frozen stock and cultivated at 28 °C shaker at 220 rpm. The optical density of o/n cultures was measured, and cultures were diluted to OD_{600} ~0.1–0.2. Each diluted culture was divided into two parts – the medium with an inducer (150 μM cumic acid) and the inducer-free medium. Induced and non-induced cultures were grown in the shaking incubator (28 °C, 220 rpm) for 4 h. Serial dilutions of induced and non-induced cultures were made and plated on the selective LB-agar medium without inducer. After two nights of growth, colony forming units of *Agrobacterium* were counted. The effectiveness of each toxin was evaluated

by the following formula: $\Delta\log_{10} = \log_{10}$ (CFU/ml non-induced culture)-$\log_{10}$ (CFU/ml induced culture).

2.2.12. Evaluation of GFP expression foci number and amount of agrobacteria on plant leaves after vacuum agroinfiltration

Agrobacterium strains were inoculated from frozen stocks in 4 ml LB medium and cultivated at 28 °C with shaking at 220 rpm. Overnight cultures were diluted $1x10^5$ fold starting from $OD_{595} = 1$ in tap water and supplemented with 0.005 % Silwet L-77 and 50 μM or 150 μM cumic acid if needed. *Agrobacterium* suspension was loaded into a desiccator vessel connected to a vacuum pump. The entire leaf system of a 5–6 week old plant was then submerged into the suspension. Agroinfiltration was achieved by applying (till the pressure of 200 mbar) and releasing vacuum through the pump. The air is extracted, and the suspension is drawn into the leaf intercellular space during the procedure. 6 days post agroinfiltration, 4 infiltrated leaves from every plant were detached, and GFP loci were counted under UV light illumination. Then leaves were weighed and frozen in liquid nitrogen for further evaluation of *A. tumefaciens* CFU number on plant leaves. Plant leaves were ground in liquid nitrogen, 2 mL distilled water per 1 g of fresh plant tissues were added, and tissue suspension was prepared. The suspension was incubated for 30–40 min at room temperature, filtrated, serial dilutions of suspension then plated on the LB medium plates with antibiotics – rifampicin (50 μg/mL), kanamycin (50 μg/mL), spectinomycin (50 μg/mL) and nystatin (50 μg/mL) to ensure *Agrobacterium* growth and prevent the growth of other microorganisms. The number of bacteria per 1 g of fresh leaves was calculated on plates after two nights of incubation in a 28 °C incubator. Two plants were used for each experimental point. Three individual experiment repeats were made.

2.2.13 *VirE2* expression evaluation by qRT-PCR

Agrobacterium strains with cumic acid inducible promoters ICH011 (pNMDV386) and ICH011 (pNMDV356) and strain GV3101 (pICH27566) (Table 2) for wild type *virE2* expression evaluation were syringe-infiltrated into *N. benthamiana* plants as described in Methods section 2.2.7. 150 μM cumic acid was added for the induced expression of *virE2*. Infiltrated plant leaves were harvested 4, 24, 48 and 72 h after agroinfiltration, leaves were frozen in liquid nitrogen and stored at -80 °C till RNA extraction. Plant tissue samples were ground in liquid nitrogen, total RNA was extracted using a

commercial kit according to the manufacturer's recommendations (see 2.1.5 F). A commercial kit for SYBR Green chemistry was used for qRT-PCR reactions (see 2.1.5 G). Reactions were performed in a Corbett Rotor gene 6000 thermocycler according to the given recommendations. The expression ratio of *virE2* from induced and non-induced promoters $P_{tac/CuO}$, $P_{T5/CuO}$ and a native promoter of *virE* region were evaluated. RT-qPCR data were normalized to the expression of two reference genes *rpoB* and *16sRNA*, as they showed a high and stable expression in *A. tumefaciens*. Primers used for reactions are listed in Table 7 J, K, L. Reactions were repeated in triplicate.

2.2.14. Statistical analysis

Program MaxStat Pro 3.6 was used for statistical analysis of results. A two-tailed unpaired t-test was used to determine significant differences between results. Results were considered different when encountered significance level $p < 0.05$.

3. RESULTS

Two approaches were used to achieve higher environmental biosafety of transient transfection of plants by *A. tumefaciens*. Inducible expression of *virE2* in *ΔvirE2 Agrobacterium* strain and inducible expression of *pemK* and *pemI* from agrobacterial toxin-antitoxin system *pemIK* in *ΔpemIK Agrobacterium* strain were used for T-DNA transfer control. Also toxins from other agrobacterial toxin-antitoxins systems were identified and their activities evaluated.

3.1. Construction and evaluation of expression systems based on inducible expression of *A. tumefaciens* virulence gene *virE2* for regulation of T-DNA transfer from *Agrobacterium* to plant.

3.1.1. Introduction to the experiments performed

T-DNA transfer assays were carried out in *N. benthamiana* plants. Two types of plant virus-based binary vectors were used for T-DNA transfer assays. Both tobacco mosaic virus TMV-based pICH18711 and potato virus X PVX-based pICH27566 vectors contain viral polymerase and GFP coding sequences in their T-DNA region. Additionally, they both also contain coding sequences of proteins responsible for viral replication and movement within plant tissues. The pICH18711 has a TMV movement protein MP which is responsible for the intercellular spread of viral replicons through plasmodesmata, and pICH27566 contains a set of three proteins (triple gene block proteins) and a coat protein CP which together are responsible for intercellular and systemic replicon spread throughout all of the plant vasculature (Fig. 15). Transient expression assays in *N. benthamiana* following agroinfiltration with *A. tumefaciens* strain GV3101 transformed with either pICH18711 or pICH27566 show strong GFP fluorescence in plant leaves 4–5 days post-infiltration. In these assays the number of T-DNA transfer events can be easily quantified as it corresponds to the number of GFP expression foci. An *A. tumefaciens* strain, ICH011 (*ΔvirE2*), lacking the entire *virE2* ORF was constructed and kindly provided by Stefan Werner from Nomad Bioscience GmbH for inducible *virE2* cassettes evaluation [259]. The transient expression assay in *N. benthamiana* plants infiltrated either with ICH011 (pICH18711) or ICH011 (pICH27566) showed no detectable GFP expression thus confirming ICH011 inability to accomplish the T-DNA transfer (Fig. 16).

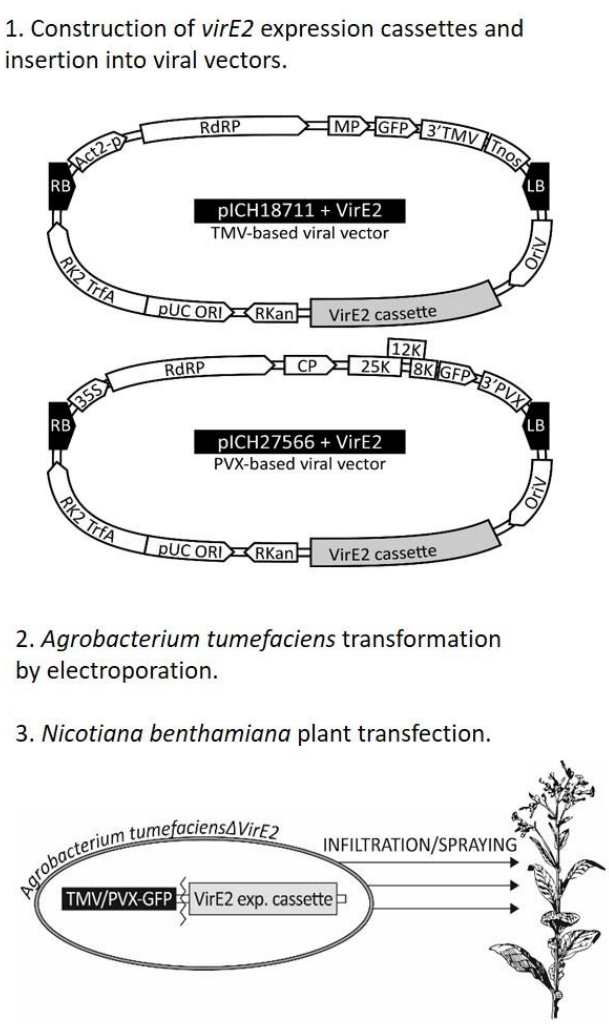

Figure 15. The experimental set-up. Inducible *virE2* expression cassettes were inserted into the unique *Eco47*III site in the backbone of either TMV-based vector pICH18711 or PVX-based vector pICH27566 and transformed into *A. tumefaciens* strain ICH011 (*ΔvirE2*) lacking the entire *virE2* ORF. Transformed agrobacteria were used in agroinfiltration or agrospray assays**.** Description of TMV- and PVX-based vectors, containing *virE2* expression cassette: LB and RB, binary T-DNA left and right borders, respectively; *Act2*, *Arabidopsis thaliana* actin 2 promoter; *35S*, Cauliflower mosaic virus promoter; *Tnos*, *nos* terminator; *RdRp*, RNA-dependent RNA polymerase; *MP*, movement protein; *CP*, coat protein; *25K*, *12K*, *8K*, PVX triple gene block; *GFP*, green fluorescent protein; *3'TMV*, 3´untranslated region of TMV; *3'PVX*, 3´untranslated region of PVX.

When transgenic *N. benthamiana* (P_{35S}*::virE2*) plants constitutively expressing *virE2* (provided by Stefan Werner from Nomad Bioscience GmbH [259]) were used for agroinfiltration, both ICH011 (pICH18711) and ICH011 (pICH27566) were able to accomplish the T-DNA transfer as confirmed by strong GFP fluorescence (Fig. 16). These data are in complete agreement with the data that describes the ability of VirE2 protein to complement a *virE2* mutant when provided in *trans* [260].

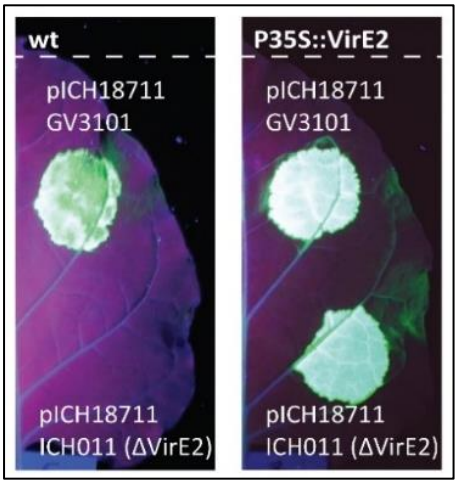

Figure 16. T-DNA transfer by *A. tumefaciens* strain ICH011. The WT *N. benthamiana* agroinfiltration with *virE2* deficient *A. tumefaciens* strain ICH011 (pICH18711) shows no detectable GFP expression thus confirming its inability to accomplish the T-DNA transfer. In comparison, strong GFP expression is observed with control strain GV3101 (pICH18711). When transgenic *N. benthamiana* (P_{35S}::*virE2*) plants are used for agroinfiltration, strain ICH011 (pICH18711) can accomplish the T-DNA transfer as confirmed by strong GFP fluorescence.

We constructed inducible *virE2* cassettes and inserted them into the backbone of both pICH18711 and pICH27566, which were then transfected into strain ICH011 (*ΔvirE2*). The suitability of various inducible promoters to create an *Agrobacterium* mediated transient plant transfection system with greater environmental biosafety was assessed by comparing their ability to restore the T-DNA transfer in their induced state while exhibiting minimal background expression when not induced. Regulation of all inducible promoters was evaluated in β-galactosidase assays. *VirE2* constructs were first tested in plant syringe-infiltration assays with the number of GFP fluorescence foci evaluated visually. The best-performing constructs were then evaluated in large scale quantitative plant spraying assays on *N. benthamiana* plants (agrospray).

3.1.2. Evaluation of IPTG inducible promoters in *A. tumefaciens* and their use for inducible T-DNA transfer

3.1.2.1. P_{lac} promoter

The functionality of P_{lac} promoter in *A. tumefaciens* has already been described [244,245]. The successful use of this promoter in *Agrobacterium* and the theoretical possibility to use an inexpensive and non-toxic product – lactose as an inducer in large-scale plant transfection [261] were our reasons for examining the P_{lac} promoter.

β-galactosidase assays with *Agrobacterium* strain GV3101 (pNMDV36) harbouring P_{lac}::*lacZ* construct (Suppl. Fig. 1) confirmed P_{lac} promoter activity in *Agrobacterium*. The detected activity amounted to 100 MU after 5 hours of induction (Fig. 17), which is in good agreement with previously described values [244]. P_{lac} promoter activity was also tested in the construct without the *lacI* repressor and was found that the measured activity in this strain was slightly higher and amounted to ~400 MU (not shown). Also native P_{lac} promoter conformation with conserved positions of O_1 and O_3 operators, and lacking O_2 operator in the *virE2* construct, but present in *lacZ* construct (O_2 operator is located in *lacZ*) was used in a study. Next the P_{lac}::*virE2* construct (Suppl. Fig. 2) was examined in plant agroinfiltration assays in order to evaluate the T-DNA transfer efficiency provided by P_{lac} controlled *virE2* expression. However, GFP expression foci were not obtained in syringe infiltrated *N. benthamiana* plant leaves, indicating that no T-DNA transfer events occurred (Fig. 17).

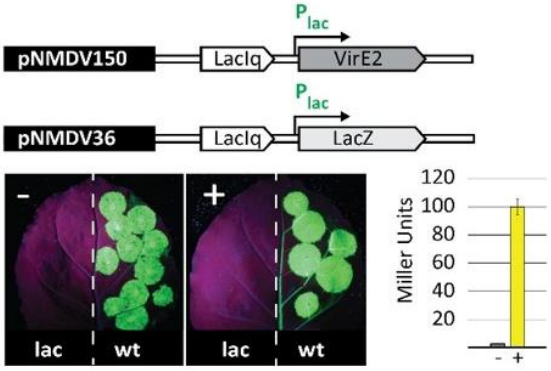

Figure 17. Evaluation of P_{lac} promoter activity in *A. tumefaciens* ICH011. *VirE2* expression from IPTG-induced promoter P_{lac} did not result in detectable T-DNA transfer; β-galactosidase activity assay showed activity of ~100 MU in *Agrobacterium*. *N. benthamiana* leaf pictures were taken under UV light 5 d.p.i. + : induction with 1mM IPTG. - : no inducer added. wt : control syringe

infiltrations of *Agrobacterium* strain GV3101 carrying viral vector with GFP. β-galactosidase assay values correspond to the average of three experiments. Bars represent standard deviation. Photos of leaves demonstrate representative result after 2-3 agroinfiltration repeats.

Then several slightly different constructs (expression cassette in PVX- and TMV-based vectors, with or without *lacY* permease) were evaluated, but none of these constructs were able to induce detectable T-DNA transfer (not shown). We thus presumed that P_{lac} promoter activity (~100 MU) is not sufficiently strong to provide the amounts of VirE2 necessary for efficient T-DNA transfer.

Several engineered IPTG inducible promoters (P_{tac}, $P_{T5/lacOlacO}$ and $P_{T7/lacO}$) are stronger than the P_{lac} promoter and are used for recombinant protein production in *E. coli* and other organisms. We decided to continue with the construction of *lacZ* and *virE2* expression cassettes utilizing these promoters.

3.1.2.2. P_{tac} promoter

The *virE2* coding sequence was inserted under control of hybrid P_{tac} promoter [212] in a TMV-based viral vector (pNMDV148) (Suppl. Fig. 3). This construct was tested in a agroinfiltration assay. In contrast to P_{lac}*::virE2*, the P_{tac}*::virE2* expression cassette provided sufficient amount of VirE2 for highly efficient T-DNA transfer to occur. Plant infiltration with ICH011 (pNMDV148) resulted in intense GFP fluorescence. However, although very strong, P_{tac} was poorly regulated by IPTG and gave similar GFP fluorescence in the non-induced state as when induced by IPTG (Fig. 18). We tried to combat this leaky expression by providing an additional copy of *lacIq* in *trans* in a separate vector, but no significant improvements were obtained.

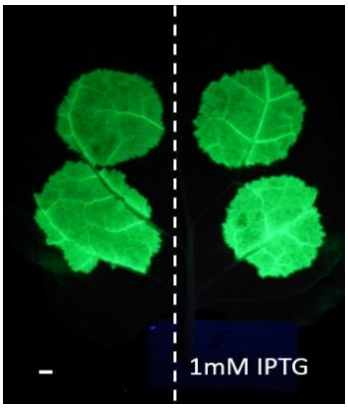

Figure 18. Evaluation of P_{tac} promoter activity in *A. tumefaciens* ICH011. *VirE2* expression from promoter P_{tac} resulted into intense GFP fluorescence when infiltrated with 1mM IPTG and without inducer. Photo of a leaf demonstrate representative result after 2–3 agroinfiltration repeats.

3.1.2.3. $P_{T5/lacOlacO}$ promoter

Bacteriophage T5 early promoter P_{N25} (later in the text P_{T5}) has been described as very strong promoter recognized by bacterial RNA polymerase [262]. The P_{T5} hybrid promoter is used in Qiagen expression vectors and was tested in several microorganisms including *Agrobacterium* [263]. A hybrid P_{T5} promoter with two *lac* operator sequences, as in Qiagen pQE30 vector ($P_{T5/lacOlacO}$), was used for *virE2* and *lacZ* expression in *Agrobacterium*. A strong positive correlation between induction strength and concentration of IPTG was observed and no toxic effect of IPTG was noticed with concentrations as high as 10 mM (Fig. 19).

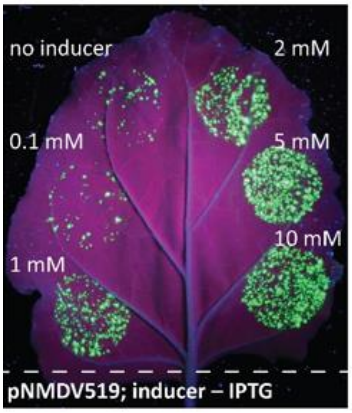

Figure 19. IPTG - induced T-DNA transfer is regulated in a dose-responsive manner in *A. tumefaciens*. Strain ICH011 was transformed with pNMDV519 ($P_{T5/lacOlacO}$::*virE2*). No inducer was added to the first sample as a negative control, 0.1, 1, 2, 5 or 10 mM IPTG was added to experimental samples just before infiltration. Photo of leaf demonstrate representative result after 2–3 agroinfiltration repeats.

A. tumefaciens strain GV3101 (pNMDV417) containing the $P_{T5/lacOlacO}$*::lacZ* expression cassette showed high β-galactosidase activity (~1500 MU) when induced with 2 mM IPTG for 5 hours and low activity (~ 27 MU) in an un-induced state (Fig. 20). The plant agroinfiltration assay with *A. tumefaciens* strain ICH011 (pNMDV519) containing $P_{T5/lacOlacO}$*::virE2* expression cassette (Suppl. Fig. 4) showed almost undetectable GFP fluorescence in the absence and strong GFP fluorescence in the presence of the inducer (Fig. 20).

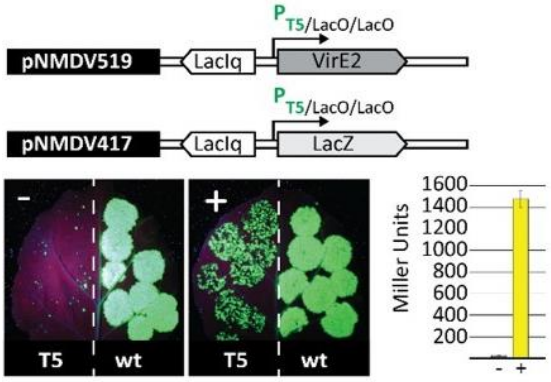

Figure 20. Evaluation of $P_{T5/lacOlacO}$ promoter activity in *A. tumefaciens* ICH011. *VirE2* expression from promoter $P_{T5/lacOlacO}$ resulted in efficient and tightly regulated T-DNA transfer, *lacZ* expression resulted in medium β-galactosidase activity (~ 1400 MU) in induced state and very low activity (~ 27 MU) without induction. *N. benthamiana* plant leaves pictures were taken under UV light 5 d.p.i. + : induction with 1mM IPTG. - : no inducer added. wt : control syringe infiltrations of *Agrobacterium* strain GV3101 carrying viral vector with GFP. β-galactosidase assay values correspond to the average of three experiments. Bars represent standard deviation. Photos of leaves demonstrate representative result after 2-3 agroinfiltration repeats.

3.1.2.4. $P_{T7/lacO}$ promoter

The bacteriophage T7 RNA polymerase driven hybrid $P_{T7/lacO}$ promoter is one of the strongest and most stringent promoters used for heterologous protein expression in *E. coli* [232]. It has also been tested in other microorganisms including *A. tumefaciens* [246]. *T7* expression system was also tested for inducible *virE2* expression in our study. In contrast to previously described *T7* expression systems, our P_{lacUV5}*::T7* polymerase expression cassette was not integrated into the bacterial genome but was cloned in a separate plasmid

pNMDV163. The $P_{T7/lacO}$*::virE2* expression cassette was cloned into pICH18711 giving construct pNMDV164 (Suppl. Fig. 5). *A. tumefaciens* strain ICH011 (pNMDV163, pNMDV164) was tested in agroinfiltration assay and demonstrated the restored ability of T-DNA transfer (Fig. 21). The efficiency of T-DNA transfer was significantly lower than transfer obtained with $P_{T5/lacOlacO}$*::virE2*, and some leakiness of the promoter was noticed in the absence of the inducer. Further, the activity of *T7*-polymerase regulated $P_{T7/lacO}$ promoter was tested in a β-galactosidase assay using the *A. tumefaciens* strain GV3101 (pNMDV163, pNMDV223). Surprisingly, almost no activity was detected in *Agrobacterium* even in the presence of IPTG, contradictory to the results with *virE2*. Both plasmids, pNMDV163 and pNMDV223, were retransformed into *E. coli* and then β-galactosidase activity measured. The results showed high promoter activity, confirming the functionality of the system in *E. coli* (Fig. 21). We cannot explain the observed discrepancy between lack of β-galactosidase activity in *Agrobacterium* and successful inducible T-DNA transfer. It can be speculated that the observed difference is due to different induction times and conditions between the β-galactosidase activity test in liquid culture and infiltration experiment *in planta*. In general *virE2* expression from $P_{T7/lacO}$*::virE2* resulted in markedly weaker GFP fluorescence in plant leaves in comparison with wild type strain therefor we continued our search for inducible promoters of higher efficacy.

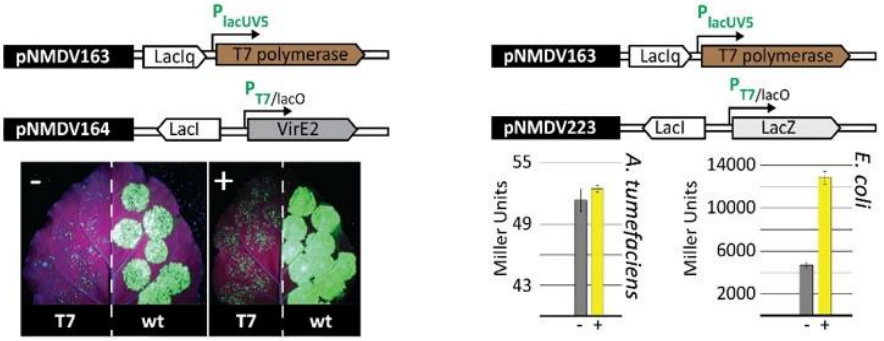

Figure 21. Evaluation of $P_{T7/lacO}$ promoter activity in *A. tumefaciens* ICH011. *VirE2* expression from promoter $P_{T7/lacO}$ resulted in moderate T-DNA transfer in induced state and detectable T-DNA transfer in the absence of inducer; very low β-galactosidase activity was measured in *Agrobacterium* when *lacZ* was expressed from $P_{T7/lacO}$ promoter, while activity was high in *E.coli*. *N. benthamiana* leaf pictures were taken under UV light 5 d.p.i. + : induction with 1mM IPTG. - : no inducer added. wt : control syringe infiltrations of *Agrobacterium* strain GV3101 carrying viral vector with GFP. β-galactosidase

assay values correspond to the average of three experiments. Bars represent standard deviation. Photos of leaves demonstrate representative result after 2–3 agroinfiltration repeats.

3.1.3. Evaluation of cumic acid inducible promoters in *A. tumefaciens* and their use for inducible T-DNA transfer

A novel tightly regulated gene expression system was developed for *E. coli* by applying the regulatory elements of the *Pseudomonas putida* F1 *cym* and *cmt* operons to control target gene expression at the transcriptional level by using *p*-isopropylbenzoate (cumate) as an inducer. This system is referred to as the cumate gene switch [235]. We evaluated the possibility of using cumate-inducible promoters in *A. tumefaciens*.

3.1.3.1. $P_{T5/CuO}$ promoter

$P_{T5/CuO}$*::virE2* cassette was inserted into viral vectors pICH18711 and pICH27566 resulting in constructs pNMDV281 and pNMDV386, respectively (Suppl. Fig. 6A). Unlike Choi et al. we used cumic acid (4-isopropylbenzoic acid) as an inducer instead of cumate because cumic acid gave better results in *Agrobacterium* (data not shown). Cumic acid induced relatively strong *virE2* expression in *A. tumefaciens* resulting in strong GFP fluorescence in plant leaves (Fig. 22). In contrast to IPTG inducible $P_{T5/lacOlacO}$, cumic acid inducible $P_{T5/CuO}$ did not provide any detectable GFP fluorescence in the absence of the inducer. β-galactosidase activity of GV3101 (pNMDV299) strain harboring $P_{T5/CuO}$*::lacZ* expression cassette was evaluated and compared with β-galactosidase activity of GV3101 (pNMDV417) harboring $P_{T5/lacOlacO}$*::lacZ* expression cassette (Fig. 20 and Fig. 22). β-galactosidase activity was much higher when the $P_{T5/CuO}$ promoter was used for inducible expression of *lacZ* and reached ~5200 MU when induced, while expression from $P_{T5/lacOlacO}$ promoter resulted only in ~1500 MU. However, the basal activity of non-induced $P_{T5/CuO}$ promoter was also higher (~240 MU) than the basal activity of $P_{T5/lacOlacO}$ (~27 MU). This higher leakiness of $P_{T5/CuO}$ however did not result in detectable T-DNA transfer in the plant infiltration assays with $P_{T5/CuO}$*::virE2* construct (Fig. 22), while lower leakiness of $P_{T5/lacOlacO}$ did (Fig. 20). In order to understand why the $P_{T5/CuO}$ promoter was less leaky in agroinfiltration experiments than $P_{T5/lacO\text{lacO}}$, the activity of the *lacIq* repressor promoter and the activity of the P_{Km} promoter used for *CymR* expression were measured in *Agrobacterium*. The β-galactosidase activity of P_{lacIq}*::lacZ* was ~100 MU, while P_{Km}*::lacZ* activity

was 4 times greater and amounted to ~400 MU. We suggest that *lacI* expression from its native promoter in *Agrobacterium* may be insufficient for stringent regulation of the $P_{T5/lacOlacO}$. We sought to replace *lacIq* with P_{Km}*::lacI* cassettes in our constructs, but failed to obtain *Agrobacterium* transformants. We believe that the higher permeability of IPTG-induced promoters in plant tissues was due to unknown, possibly plant-related, factors affecting repressor stability or operator binding affinity.

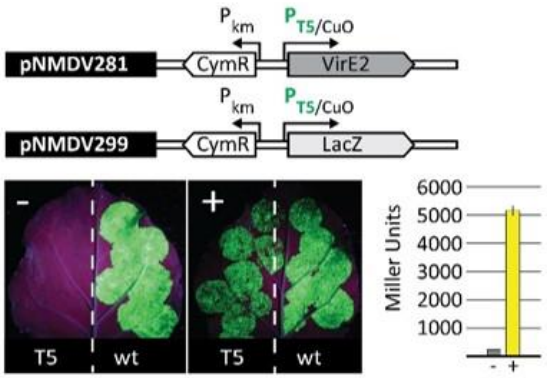

Figure 22. Evaluation of $P_{T5/CuO}$ promoter activity in *A. tumefaciens* ICH011. *VirE2* expression from promoter $P_{T5/CuO}$ resulted in highly inducible and very stringent T-DNA transfer, *lacZ* expression resulted in high β-galactosidase activity while induced (~5200 MU) and low leaky activity (~ 240 MU). *N. benthamiana* leaf pictures were taken under UV light 5 d.p.i. + : induction with 150μM cumic acid. - : no inducer added. wt : control syringe infiltrations of *Agrobacterium* strain GV3101 carrying viral vector with GFP. β-galactosidase assay values correspond to the average of three experiments. Bars represent standard deviation. Photos of leaves demonstrate representative result after 2–3 agroinfiltration repeats.

Next the correlation between cumic acid inducible *virE2* expression, resulting in T-DNA transfer and GFP fluorescence in plant, and inducer concentration was evaluated. Cumic acid concentrations of 50–250 μM were tested. As little as 50 μM of cumic acid was able to induce GFP fluorescence in *N. benthamiana* leaves. This fluorescence seemed to reach its maximum induction between 100 μM and 250 μM of cumic acid (Fig. 23). Therefore the concentration of 150 μM of cumic acid was chosen for further induction experiments.

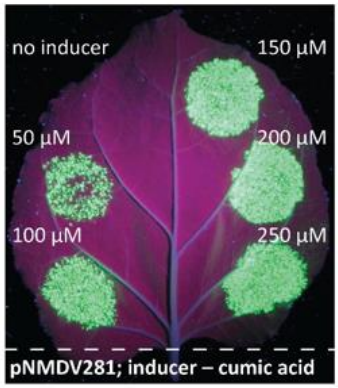

Figure 23. Cumic acid induced T-DNA transfer is regulated in a dose-responsive manner in *A. tumefaciens*. ICH011 transformed with pNMDV281 ($P_{T5/CuO}$::*virE2*): no inducer was added to the first sample, 50, 100, 150, 200 or 250 µM cumic acid was added to experimental samples just before infiltration. The photo of a leaf demonstrate representative result after 2–3 agroinfiltration repeats.

As a following step, the correlation between strength of expression from $P_{T5/CuO}$ and length of incubation with cumic acid was analyzed. Suspensions of ICH011 (pNMDV281) were incubated with 50 µM cumic acid for 0.5, 1, 2, 3, 4 and 5 h and then syringe-infiltrated into plants. It appeared that <0.5 h incubation was sufficient for maximal induction of *virE2* expression. Increased incubation time did not increase *virE2* expression or transformation efficacy (Fig. 24).

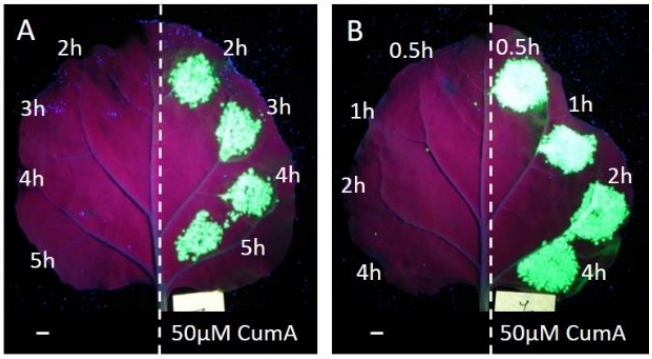

Figure 24. Evaluation of correlation between strength of cumic acid induced expression and length of indubation with an inducer. Two agroinfiltration experiments with strain ICH011 (pNMDV281) were made. **A.** At the first experiment agrobacteria were infiltrated after 0.5, 1, 2, 3 and 4 h indubation with 50 µM cumic acid. **B.** At the second experiment agrobacteria were infiltrated after 2, 3, 4 and 5h indubation with 50 µM cumic acid. Photos of leaves demonstrate representative result after 2–3 agroinfiltration repeats.

Next we examined if it would be possible to induce bacteria with cumic acid, and then remove the inducer prior to plant infiltration. Such a procedure would have both environmental and economic benefits for large-scale plant transfection applications, because no cumic acid would be applied to the field and smaller quantities of cumic acid would be required. *Agrobacterium* suspensions were supplemented with cumic acid, bacteria incubated with inducer for 1, 2, 3, 4, 5, 6 or 24 h, then inducer removed just before agroinfiltration. Unfortunately, GFP fluorescence was not detected under such conditions, proving that an inducer needs to be present in the infiltration suspension to allow for efficient T-DNA transfer induction. Addition of acetosyringone, a natural plant origin phenolic product used for induction of *Agrobacterium* virulence, to the induction medium or co-expression of VirE1, which was described as a stabilizing chaperone of VirE2 [46], did not influence the results (not shown). It therefore appears that the timing of VirE2 expression is crucial. Apparently, functional VirE2 cannot be pre-accumulated in bacterial cytoplasm before the induction of other constituents involved in T-DNA transfer.

In summary, the first results obtained with cumic acid regulated $P_{T5/CuO}$ promoter were promising and prompted us to construct and analyse new cumic acid regulated promoters.

3.1.3.2. $P_{tac/CuO}$ promoter

P_{tac} promoter was very strong in *Agrobacterium* as P_{tac}*::virE2* expression resulted in a very efficient T-DNA transfer. However, P_{tac} promoter was very poorly repressed in the absence of IPTG induction (see 3.1.2.2). Because we observed the tight regulation of $P_{T5/CuO}$*::virE2* expression in *A. tumefaciens*, we expected that the P_{tac} promoter could also be regulated more tightly by cumic acid. A synthetic $P_{tac/CuO}$ promoter was designed by replacing the *lac* operator sequence in the P_{tac} promoter with the cumate operator sequence (Table 7). As an additional means to achieve higher stringency, the repressor gene expression cassette was cloned in opposite orientation to the $P_{tac/CuO}$ promoter, the same orientation as was utilized in the $P_{T5/CuO}$ construct. The $P_{tac/CuO}$*::virE2* expression cassette was inserted into viral vectors pICH18711 and pICH27566, giving constructs pNMDV355 and pNMDV356, respectively (Suppl. Fig. 7). Indeed, as anticipated, plant infiltration with cumic acid induced ICH011 (pNMDV355) strain resulted in strong GFP fluorescence. No GFP fluorescence was observed in plant leaves infiltrated with the non-induced ICH011 (pNMDV355) strain (Fig. 25). We also tested the $P_{tac/CuO}$ promoter in β-galactosidase assays using the GV3101

(pNMDV395) strain harbouring the $P_{tac/CuO}$*::lacZ* cassette. The β-galactosidase activity was ~5400 MU in the induced state and only ~110 MU in the non-induced state. These results define the hybrid $P_{tac/CuO}$ promoter as a very strong and tightly regulated promoter in *Agrobacterium* (Fig. 25).

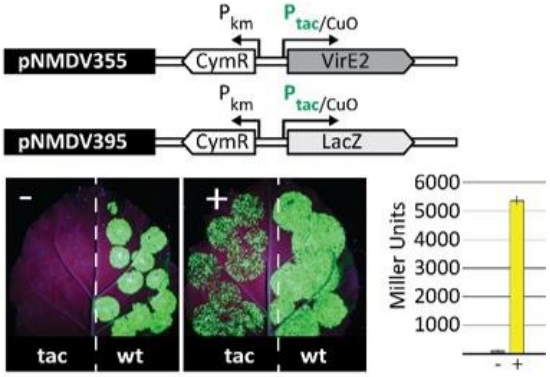

Figure 25. Evaluation of $P_{tac/CuO}$ promoter activity in *A. tumefaciens* ICH011. *VirE2* expression from promoter $P_{tac/CuO}$ resulted in highly inducible and very stringent T-DNA transfer, *lacZ* expression resulted in high β-galactosidase activity while induced (~5400 MU) and low leaky activity (~110 MU). *N. benthamiana* leaf pictures were taken under UV light 5 d.p.i. + : induction with 150 μM cumic acid. - : no inducer added. wt : control syringe infiltrations of *Agrobacterium* strain GV3101 carrying viral vector with GFP. β-galactosidase assay values correspond to the average of three experiments. Bars represent standard deviation. Photos of leaves demonstrate representative result after 2–3 agroinfiltration repeats.

3.1.3.3. $P_{lacUV5/CuO}$ promoter

The tight regulation of cumic acid inducible promoters in *Agrobacterium* suggests that this characteristic could be exploited in order to evaluate the minimal strength of the *virE2* promoter necessary for the T-DNA transfer to occur. We have previously determined that the leakiness of the $P_{T5/CuO}$ promoter (~240 MU) is insufficient to provide enough VirE2 molecules to allow efficient T-DNA transfer to occur and that the promoter strength of 1500 MU of $P_{T5/lacOlacO}$ promoter is largely sufficient for that aim. We then searched for a promoter weaker than $P_{T5/lacOlacO}$. The P_{lac} promoter in its native conformation is weak in *Agrobacterium* and reaches only ~100 MU under our induction conditions. However, we expected that by changing both the regulation of this promoter (cumic acid instead of IPTG) and the conformation

of the construct (*cymR* gene cloned in an inverted position in comparison to *lacIq* in pNMDV150) greater activity of P_{lac} promoter could be achieved. A synthetic $P_{lacUV5/CuO}$ promoter was constructed by replacing the *lac* operator sequence with a cumate operator sequence (Table 7). The $P_{lacUV5/CuO}$*::virE2* and $P_{lacUV5/CuO}$*::lacZ* expression cassettes were used to construct plasmids pNMDV457 (Suppl. Fig. 8) and pNMDV376, respectively. The GV3101 (pNMDV376) strain was tested in β-galactosidase assays and had activity of ~1100 MU when induced with cumic acid, which is considerably higher than activity of native P_{lac} promoter (~100 MU). The transient expression assays with cumic acid induced ICH011 (pNMDV457) strain showed efficient T-DNA transfer in infiltrated *N. benthamiana* leaves (Fig. 26). Only several GFP expression foci were detected when non-induced ICH011 (pNMDV457) strain was infiltrated into plant leaves (Fig. 26). The result suggests that promoter activity greater than ~240 MU and less than or equal to ~1100 MU is necessary to provide minimal quantity of VirE2 protein molecules to allow efficient T-DNA transfer to occur.

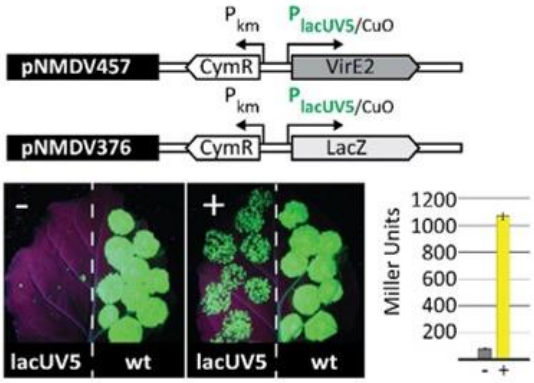

Figure 26. Evaluation of $P_{lacUV5/CuO}$ promoter activity in *A. tumefaciens* ICH011. *VirE2* expression from promoter $P_{lacUV5/CuO}$ resulted in moderately efficient and relatively stringent T-DNA transfer; β-galactosidase activity assay showed activity of ~1100 MU when induced and ~80 MU in the absence of the inducer. *N. benthamiana* leaf pictures were taken under UV light 5 d.p.i. + : induction with 150μM cumic acid. - : no inducer added. wt : control syringe infiltrations of *Agrobacterium* strain GV3101 carrying viral vector with GFP. β-galactosidase assay values correspond to the average of three experiments. Bars represent standard deviation. Photos of leaves demonstrate representative result after 2–3 agroinfiltration repeats.

3.1.3.4. $P_{virE/CuO}$ promoter

Although $P_{T5/CuO}$ and $P_{tac/CuO}$ promoters were highly inducible in *Agrobacterium* neither of them proved itself as strong as the native *virE* region promoter P_{virE}. Neither the $P_{T5/CuO}$*::virE2* nor the $P_{tac/CuO}$*::virE2* constructs could provide GFP fluorescence intensity comparable to that obtained with GV3101 strain carrying the intact native *virE* operon. We decided then to construct a cumic acid regulated $P_{virE/CuO}$ promoter. For this two steps of construction were needed. First, the putative P_{virE} promoter region of *Agrobacterium* strain KYRT-1 [255], homologous to the described P_{virE} promoter in *A. tumefaciens* strain A6 [264] and 99% identical to the same region of pTiBo542 [265] was PCR amplified. The cloned putative P_{virE} promoter was confirmed to be able to drive *virE2* expression resulting in efficient T-DNA transfer (not shown). Next, the cumate operator sequence was added to the isolated P_{virE} promoter (Table 7.H). The $P_{virE/CuO}$*::virE2* cassette was inserted into a pICH27566 viral vector resulting in construct pNMDV437 (Suppl. Fig. 9). Plant infiltration assays with cumic acid induced ICH011 (pNMDV437) demonstrated very strong GFP fluorescence (Fig. 27).

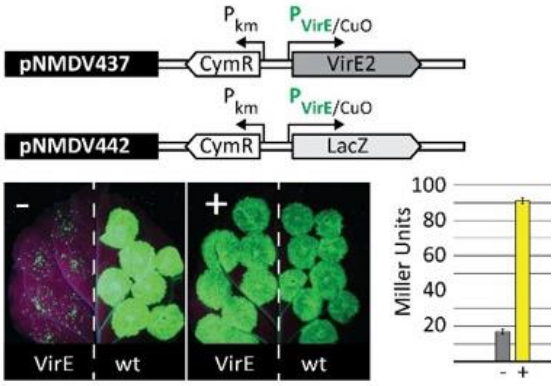

Figure 27. Evaluation of $P_{virE/CuO}$ promoter activity in *A. tumefaciens* ICH011. *VirE2* expression from induced promoter $P_{VirE/CuO}$ resulted in highly efficient T-DNA transfer, however several GFP expression foci were detected in the absence of the inducer; *lacZ* expression from this promoter resulted in low β-galactosidase activity (~ 90 MU) only when 100μM acetosyringone was added to the *Agrobacterium* suspension. *N. benthamiana* leaf pictures were taken under UV light 5 d.p.i. + : induction with 150μM cumic acid. - : no inducer added. wt : control syringe infiltrations of *Agrobacterium* strain GV3101 carrying viral vector with GFP. β-galactosidase assay values correspond to the average of three experiments. Bars represent standard

deviation. Photos of leaves demonstrate representative result after 2–3 agroinfiltration repeats.

However, infiltration of the non-induced ICH011 (pNMDV437) strain also showed detectable GFP fluorescence, indicating greater leakiness than that of the $P_{tac/CuO}$ or $P_{T5/CuO}$ promoters. Also a $P_{virE/CuO}$*::lacZ* expression vector harboring *Agrobacterium* strain GV3101(pNMDV442) was constructed and tested it in β-galactosidase activity assays. As expected, promoter activity could not be evaluated appropriately *ex planta*, with or without inducer, confirming that the $P_{virE/CuO}$ promoter needs additional factors for induction, which consist of VirG activation by plant phenolic compounds. Only when 100 μM AS was used as the inducer of VirG together with cumic acid, weak β-galactosidase activity could be detected (Fig. 27). This activity was poor and did not correspond to high T-DNA transfer efficiency observed in the plant infiltration assay. We presume that full induction of $P_{virE/CuO}$ cannot be reached *ex planta* in the conditions we used.

3.1.4. Quantitative evaluation of transfection efficiency with *Agrobacterium* strains carrying cumic acid inducible *virE2* constructs.

After preliminary syringe agroinfiltrations we performed plant sprayings with the most efficient constructs in order to evaluate their efficiency in a large scale transfection. The expression cassettes with $P_{T5/CuO}$, $P_{tac/CuO}$ and $P_{virE/CuO}$ promoters in PVX-based vectors in ICH011 strain were used for these experiments. The level of wild type T-DNA transfer obtained by spraying plants with GV3101 (pICH27566) positive control was defined as 100% efficient.

The $P_{T5/CuO}$*::virE2* expression cassette containing ICH011 (pNMDV386) provided 47% of wild type transfection efficiency. $P_{tac/CuO}$ and $P_{virE/CuO}$ promoters containing strains ICH011 (pNMDV356) and ICH011 (pNMDV437) performed better, with T-DNA transfer efficiency reaching 71-72% of the wild type expression (Fig. 28). In order to further improve T-DNA transfer efficiency *virE1* was co-expressed together with *virE2*. VirE1 protein is described as a chaperone preventing VirE2 from aggregation, important for stability of VirE2, or as being helpful for VirE2 translation [46,266,267]. *VirE1* and *virE2* coding sequences as in the native *virE* operon were inserted under control of the $P_{T5/CuO}$ promoter in viral vector giving construct pNMDV381 (Suppl. Fig. 6B). However, co-expression of *virE1* and *virE2* did not significantly improve T-DNA transfer efficiency in ICH011 (pNMDV381) in comparison to *virE2* expressing strain ICH011 (pNMDV386) (Fig. 28).

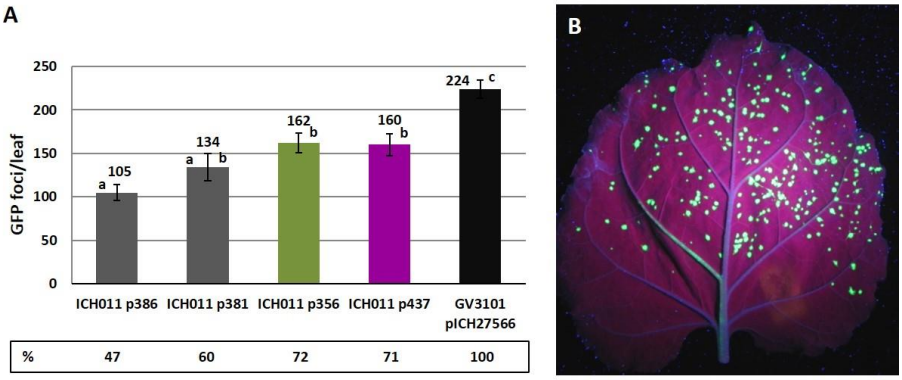

Figure 28. Evaluation of T-DNA transfer efficiency by agrospray in cumic acid induced constructs (*Agrobacterium* suspension OD_{600}=1x10^{-4}). A. *N. benthamiana* plants were sprayed with *Agrobacterium* strains carrying different constructs : p386 corresponds to pNMDV386 ($P_{T5/CuO}$::virE2), p381 to pNMDV381 ($P_{T5/CuO}$::virE1,virE2), p356 to pNMDV356 ($P_{tac/CuO}$::virE2), and p437 to pNMDV437 ($P_{virE/CuO}$::virE2). The different colors indicate different promoters: $P_{T5/CuO}$ and $P_{T5/CuO}$ – grey, $P_{tac/CuO}$ – green, $P_{virE/CuO}$ – purple, black columns indicate control strains. The efficiency of T–DNA transfer in each separate experiment was evaluated as the percentage of this number in comparison with a wild type T–DNA transfer (GFP expression foci number of GV3101 sprayed plants). Wild type T–DNA transfer was considered as 100%. Bars represent standard deviation (SD). Marks a, b and c indicate significance of difference between values. Values are not significantly different within group a**,** b or c but are significantly different between groups. B – representative picture of *N. benthamiana* leaf with GFP expression foci.

3.1.5. Quantitative evaluation of transfection leakiness with *Agrobacterium* strains carrying cumic acid inducible *virE2* constructs

The leakiness from the inducible promoter cannot be avoided completely with any known inducible expression system. Even with our best performing constructs, several GFP expression foci were observed on syringe infiltrated plant leaves when cumic acid was not added to the *Agrobacterium* suspension. It was important to quantify the extent of this leakiness more precisely in *N. benthamiana* agrospray experiments.

Plant sprayings were performed with all constructs previously evaluated for T-DNA transfer efficiency. In order to evaluate the significance of the

background expression, all tested *Agrobacterium* strains were diluted 10, 10^2 and 10^3 fold and sprayed on *N. benthamiana* plants without the inducer. No GFP expression foci were detected after spraying with 10^2 and 10^3 fold diluted agrobacteria. When agrobacteria were diluted only 10 fold, several GFP expression foci were detected with all *virE2* expression constructs and also with the negative control strain ICH011 (pICH27566). The incomplete loss of T-DNA transfer ability in *virE2* deficient strain has been already described [2]. However the frequency of non-induced T-DNA transfer events was extremely low and reached only 0.002-0.03 percent of wild type T-DNA transfer (Fig. 29). Cumic acid inducible *virE* promoter showed the greatest leakiness, which however was 3000 times lower than the wild type T-DNA transfer. The leakiness of the other strong promoter $P_{tac/CuO}$ was 50000 times lower in comparison with the wild type T-DNA transfer and approached the leakiness obtained with the negative control.

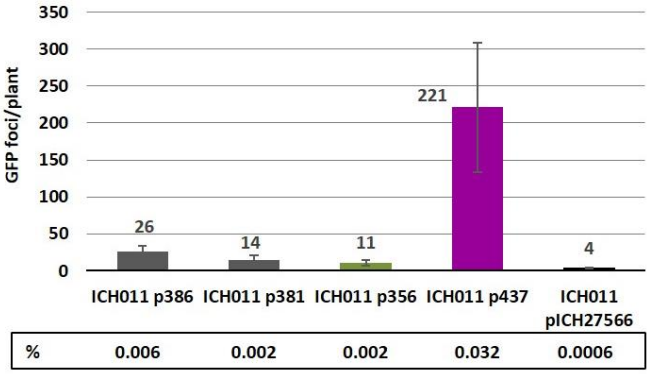

Figure 29. Evaluation of T-DNA transfer efficiency by agrospray in non-induced constructs *(Agrobacterium* suspension $OD_{600}=1x10^{-1}$). *N. benthamiana* plants were sprayed with *Agrobacterium* strains carrying different constructs: p386 corresponds to pNMDV386 ($P_{T5/CuO}$::*virE2*), p381 to pNMDV381 ($P_{T5/CuO}$::*virE1*,*virE2*), p356 to pNMDV356 ($P_{tac/CuO}$::*virE2*), and p437 to pNMDV437 ($P_{virE/CuO}$::*virE2*). The different colors indicate different promoters: $P_{T5/CuO}$ – grey, $P_{tac/CuO}$ – green, $P_{virE/CuO}$ – purple, black columns indicate control strains. The leakiness of T-DNA transfer in each separate experiment was evaluated as the percentage of this number in comparison with a wild type T-DNA transfer (GFP expression foci number of GV3101 sprayed plants). Wild type T-DNA transfer was considered as 100%. Bars represent standard deviation (SD).

3.2. Control of T-DNA transfer from *A. tumefaciens* to plants based on inducible bacterial toxin-antitoxin system

3.2.1. Identification of *A. tumefaciens* toxin-antitoxin modules

The preliminary screening of putative TA loci in *A. tumefaciens* strain C58 genome was performed with a web-based tool – RASTA-Bacteria by Šarūnas Paškevičius during Master thesis work. RASTA-Bacteria cut-off score >47% was manually settled and TA systems were selected – three from the circular, one from the linear chromosome and one from the Ti plasmid. Additionally, the *IetAS* TA module located at the Ti plasmid was selected from a published data [247] (Table 9). *IetAS* module was also included among potential TA systems, identified by previous RASTA bacteria analysis (as Atu6082-Atu6083), however, scored only 27%, thus was not recognized as a candidate system at the bigining of our research.

Table 9. Toxins and antitoxins from *A. tumefaciens* (C58) genome TA systems. The tool http://genoweb1.irisa.fr/duals/RASTA-Bacteria/ was used to search for TA systems in the *Agrobacterium* genome.

Putative toxin	Putative antitoxin	Locus tags of the TA module	Location in the genome	Predicted function of the toxin and related references
pemK	*pemI*	*Atu0939-Atu0940*	Circular chromosome AE007869.2	Originally genes of *pem* (plasmid emergency maintenance) were found in *E. coli* plasmid R100 [268]. Has endoribonuclease activity [269].
vapC	*vapB*	*Atu1004-Atu1005*	Circular chromosome AE007869.2	VapC family toxin from type II TA systems. VapC family members are thought to be site-specific endoribonucleases that cleave tRNA (fMet) in the anticodon stem-loop between nucleotides +38 and +39 *in vivo* and *in vitro* [270271].
PIN	*Atu2141*	*Atu2140-Atu2141*	Circular chromosome AE007869.2	VapC family toxin from type II TA system. Functions as ribonuclease, contains the PIN domain [272].
dead	*Atu3014*	*Atu3013-Atu3014*	Linear chromosome AE007870.2	VapC family toxin. Predicted nucleic acid binding protein, contains the PIN domain.
Nuclease -like	*Atu6123*	*Atu6122-Atu6123*	Ti plasmid AE007871.2	Thermonuclease family protein, part of traCDG operon regulated by quorum sensing, gene named *yci* [273].
ietS	*ietA*	*Atu6082-Atu6083*	Ti plasmid AE007871.2	Serine protease function, plasmid stabilization [247].

3.2.2. Evaluation if ectopically expressed putative toxins are capable of causing *Agrobacterium* death

All cloned toxin genes were placed under the control of strong and tightly regulated cumic acid inducible promoters and constructed binary vectors were transformed into the *A. tumefaciens* GV3101 strain (Table 6). The cloning of toxin genes was done by Šarūnas Paškevičius, binary vectors were constructed by Justina Stankevičiūtė during her Master thesis work. Resultant strains were used to investigate the toxic effect of cloned putative toxins. The expression of *Agrobacterium* toxins PemK, Dead, VapC, PIN and IetS reduced bacterial CFU counts by a very similar range, 2.7 to 3.8 orders of magnitude, after 4 h induction with 150 µM cumic acid in liquid medium. By contrast, the Nuclease-like toxin demonstrated a much weaker activity against *A. tumefaciens* reducing CFU count by only 0.5 order of magnitude (Fig. 29). Thus, five of cloned toxins are capable of killing agrobacteria efficiently while expressed ectopically.

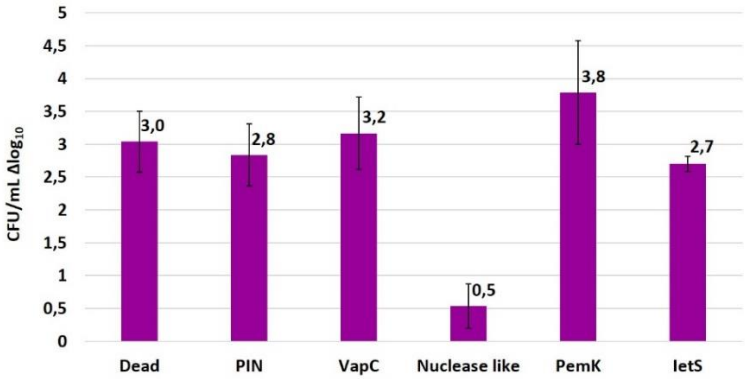

Figure 29. The ability of ectopically expressed toxins to kill *A. tumefaciens*. Overnight cultures of *A. tumefaciens* GV3101 with plasmids carrying toxin expression cassettes were diluted to OD_{595} ~ 0.1–0.2. Each diluted culture was split in two – the medium with 150 µM cumic acid and the inducer-free medium. The cultures were further incubated at 28 °C, 220 rpm for 4 hours, then serial dilutions were made and plated on selective LB-agar medium without inducer (25 µL of each culture). CFU were counted after 48h incubation at 28 °C. The effectiveness of each toxin was evaluated by using the formula: $\Delta\log_{10} = \log_{10}$ (CFU/mL non-induced culture) - $\log_{10}$ (CFU/mL induced culture). Data are the mean ± SD of three independent experiments.

3.2.3. PemK effectively supresses agrobacteria-based plant transient expression

Previous results obtained by Justina Stankevičiūtė during her master thesis work have demonstrated that *pemK* inserted under cumic acid regulated promoters can suppress the agrobacteria-based transient expression, when bacteria are sprayed onto *N. benthamiana* plants. Briefly, two *Agrobacterium* strains, control strain GV3101 (pICH27566) and GV3101 (pICH27566, pNMDV371), containing $P_{T5/CuO}$::*pemK* construct in addition to the GFP-expression vector, were used for agro-spray experiments. CFU counts of viable agrobacteria seven days post-spraying demonstrated significant differences. 4.1 $\log_{10}$ of bacteria were retrieved from a gram of fresh plant leaves sprayed with the control strain (pICH27566), but no bacteria were retrieved from the cumic acid induced strain, containing $P_{T5/CuO}$::*pemK* cassette (pNMDV371, pICH27566). Even without induction by cumic acid, toxin expression cassette-harbouring strain demonstrated CFU counts almost a hundredfold lower than the control strain and reached 2.2 $\log_{10}$ per 1 g of fresh plant leaf (Fig. 30A).

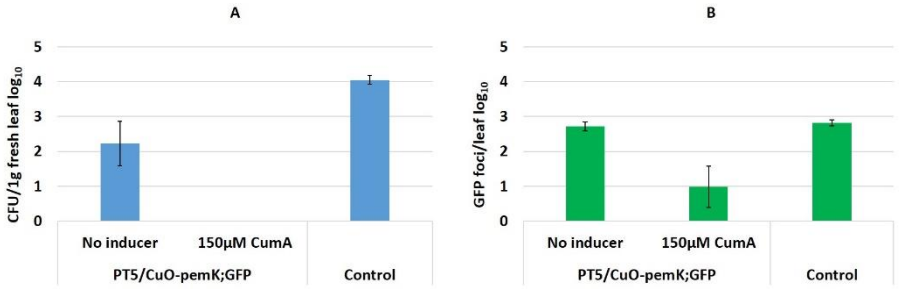

Figure 30. Evaluation of toxin PemK activity against *Agrobacterium* and GFP expression foci number in agro-spray experiments. *Agrobacterium* CFU count per 1g of fresh leaf $\log_{10}$ in a chart **A**, and GFP foci number per plant leaf $\log_{10}$ in a chart **B.** *N. benthamiana* plants were sprayed with cumic acid-induced or non-induced $P_{T5/CuO}$::*pemK* construct-containing strain GV3101 (pICH27566, pNMDV371) and with a control strain GV3101 (pICH27566). GFP loci were counted on sprayed leaves 7 dps**.** Next, same leaves were ground in liquid nitrogen for agrobacteria isolation and CFU count evaluation. The amount of bacteria per 1 g of fresh leaves was calculated**.** Data are the mean ± SD of three independent experiments. Justina Stankevičiūtė master thesis.

We presume that this reduction in the number of bacteria resulted from the basal activity of the $P_{T5/CuO}$ promoter. Count of GFP expression foci was similar between the control strain and the un-induced $P_{T5/CuO}$::*pemK* cassette-containing strain, with the numbers of GFP foci per leaf of 2.8 $\log_{10}$ and 2.7 $\log_{10}$, respectively. The cumic acid induced $P_{T5/CuO}$::*pemK* cassette containing strain demonstrated strongly reduced T-DNA transfer (1 $\log_{10}$) (Fig. 30B). Thus, the induced expression of toxin *pemK* efficiently killed recombinant agrobacteria and most killing seemed to occur before the T-DNA transfer was initiated, as demonstrated by reduced GFP foci numbers.

3.2.4. Evaluation of induction kinetics of P_{virE} and cumic acid-regulated promoters

In an ideal regulated T-DNA transfer system, the bacteria should be able to transfer the T-DNA to plants in a programmed time manner, for example, just one or two days after plant transformation, and then lose this ability. In our expression system, the cumic acid-induced *pemK* expression kills bacteria too rapidly, before the T-DNA transfer fully takes place. Thus, to give time for agrobacteria to transfer the T-DNA, either the *pemK* expression or its action should be delayed.

The *pemIK* toxin-antitoxin module belongs to type II TA systems. In type II systems the antitoxin, which is a labile protein, blocks the protein toxin action by tight binding [274,275]. Thus, including *pemI* into our transient expression system seemed appropriate for delaying the toxic effect of PemK activity. In the initial stages of testing the expression system, the entire toxin-antitoxin module was loaded under a single cumic acid inducible promoter, and it was expected from the literature that the unstable antitoxin PemI would protect agrobacteria from death for some time and later degrade to release the more stable PemK toxin. Co-expression of the toxin and antitoxin simultaneously blocked the action of PemK, but later death of agrobacteria could not be achieved (the results are presented in the master's thesis of Šarūnas Paškevičius). In order to obtain a tightly regulated system, we aimed to express both the toxin and the antitoxin from the separate promoters with different regulation profiles. We sought an early expression of *pemI* antitoxin and a delayed but strong expression of the *pemK* toxin.

In the quest for an appropriate promoter pair, the expression kinetics of a native P_{virE} promoter of *Agrobacterium* strain GV3101 and two cumic acid inducible promoters $P_{T5/CuO}$ and $P_{tac/CuO}$ were compared. As described earlier, P_{virE} is induced by plant phenolic compounds released during pathogen-plant

interaction. P_{virE} induction begins the transcription of genes from *virE* operon, and the T-DNA transfer chain of events is thus initiated [276].

Three *Agrobacterium* strains, the first harbouring $P_{T5/CuO}$::*virE2* (ICH011 (pNMDV386)), the second harbouring $P_{tac/CuO}$::*virE2* (ICH011 (pNMDV356)), and GV3101 strain containing vector pICH27566 were used for plant infiltration. The infiltrated leaves were collected at 4, 24, 48 and 72 h post-infiltration, frozen in liquid nitrogen and subjected to RNA extraction. The *virE2* expression analysis by real-time qPCR revealed different expression kinetics from synthetic cumic acid-regulated promoters and a natural *virE* promoter. At 4 h post-infiltration, the first time point investigated, *virE2* expression from cumic acid induced $P_{T5/CuO}$ and $P_{tac/CuO}$ was already almost five times higher than the basal expression from un-induced promoters. By this time, the expression from the native *virE* promoter was still very low and comparable to the expression from un-induced $P_{T5/CuO}$ and $P_{tac/CuO}$. However, at 24 h post-infiltration, P_{virE}-driven *virE2* expression is multiplied sevenfold and is similar to the expression from $P_{T5/CuO}$ and $P_{tac/CuO.}$ At 48 and 72 h post-infiltration the relative expression from P_{virE} is equal or higher than the expression from cumic acid-inducible promoters. *VirE2* expression from all three promoters reached a maximal level after 48 h and then started to decrease, demonstrating a two-fold lower relative expression level after 72 h (Fig. 31).

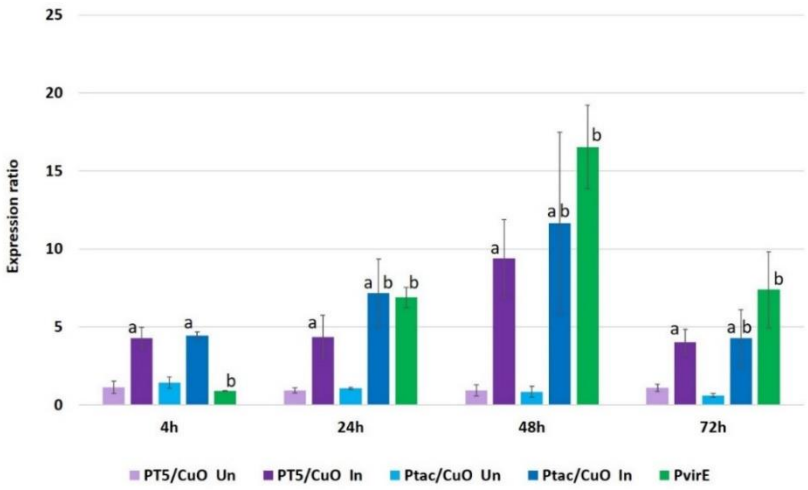

Figure 31. RT-qPCR analysis of *virE2* expression from cumic acid inducible promoters $P_{T5/CuO}$ and $P_{tac/CuO}$ and from the native promoter P_{virE}. *A. tumefaciens* ICH011 (*ΔvirE2*) strains containing pNMDV386 ($P_{T5/CuO}$::*virE2*) or pNMDV356 ($P_{tac/CuO}$::*virE2*) expression constructs, and GV3101 (pICH27566) strain were syringe-infiltrated into *N. benthamiana* plants.

Infiltrated plant leaves were harvested 4, 24, 48, 72 h after agroinfiltration. The expression ratio of *virE2* was evaluated by RT-qPCR. Un – *virE2* expression from uninduced $P_{T5/CuO}$ and $P_{tac/CuO}$ promoters, In- *virE2* expression from 150 μM cumic acid induced $P_{T5/CuO}$ and $P_{tac/CuO}$ promoters, P_{virE} – *virE2* expression from wild type P_{virE} promoter in GV3101. Data are the mean ±SD of three independent experiments. Marks a and b indicate significance of difference between values. Values are not significantly different within group a and b but are significantly different between groups.

3.2.5. Evaluation of transfection efficiency of *Agrobacterium* strains carrying $P_{lacUV5/CuO}$ :: *pemI* or $P_{tac/CuO}$ :: *pemI* and P_{virE}:: *pemK* constructs.

We presumed that if the antitoxin is expressed before the toxin and accumulated in the cell in a certain amount before the expression of toxin starts, it will be able to inactivate the toxin and to allow bacteria to survive for the time lapse sufficient for the T-DNA transfer. As the natural induction of P_{virE} is delayed in comparison with the chemical induction, the antitoxin *pemI* was inserted under cumic acid inducible weaker $P_{lacUV5/CuO}$ and stronger $P_{tac/CuO}$ promoters and the toxin *pemK* under control of P_{virE}.

The $P_{lacUV5/CuO}$::*pemI* and $P_{tac/CuO}$::*pemI* was inserted into the backbone of the PVX-based GFP expression vector pICH27566, and P_{virE}::*pemK* was cloned in a separate plasmid pNDC of a different incompatibility group. Vectors were transformed into GV3101*ΔpemIK* strain in order to avoid any interference with the native *pemIK* module expressed from *Agrobacterium* genome. Strains GV3101*ΔpemIK* (P_{virE}::*pemK;* $P_{lacUV5/CuO}$::*pemI*) and GV3101*ΔpemIK* (P_{virE}::*pemK;* $P_{tac/CuO}$::*pemI*) were used for agroinfiltration experiments and T-DNA transfer evaluation by counting the GFP foci. Essentially no T-DNA transfer was observed with *Agrobacterium* strains harbouring both *pemK* and *pemI* constructs when no cumic acid was added (1 GFP foci per leaf with $P_{lacUV5/CuO}$ and 4 GFP foci per leaf with $P_{tac/CuO}$ in average). When *Agrobacterium* suspension contained cumic acid for the induction of *pemI*, the T-DNA transfer was reconstituted and 2.4–2.5 times more efficient with stronger $P_{tac/CuO}$ than with $P_{lacUV5/CuO}$ promoter: T-DNA transfer was 45.3 % efficient after induction with 50 μM cumic acid and even 115.3% efficient after induction with 150 μM cumic acid in comparison with T-DNA transfer of a control strain, containing only $P_{tac/CuO}$::*pemI* construct (Fig. 32A). The induced expression of *pemI* from $P_{tac/CuO}$ promoter was strong enough to protect agrobacteria from the action of PemK and induced T-DNA transfer was similar or even more efficient than the wild type T-DNA transfer. Only 24% T-DNA transfer efficiency was obtained after induction with 50

μM cumic acid and 48 % after induction with 150 μM cumic acid with a strain harboring $P_{lacUV5/CuO}$::*pemI* compared to control strain containing only vector $P_{lacUV5/CuO}$::*pemI* (Fig. 32B).

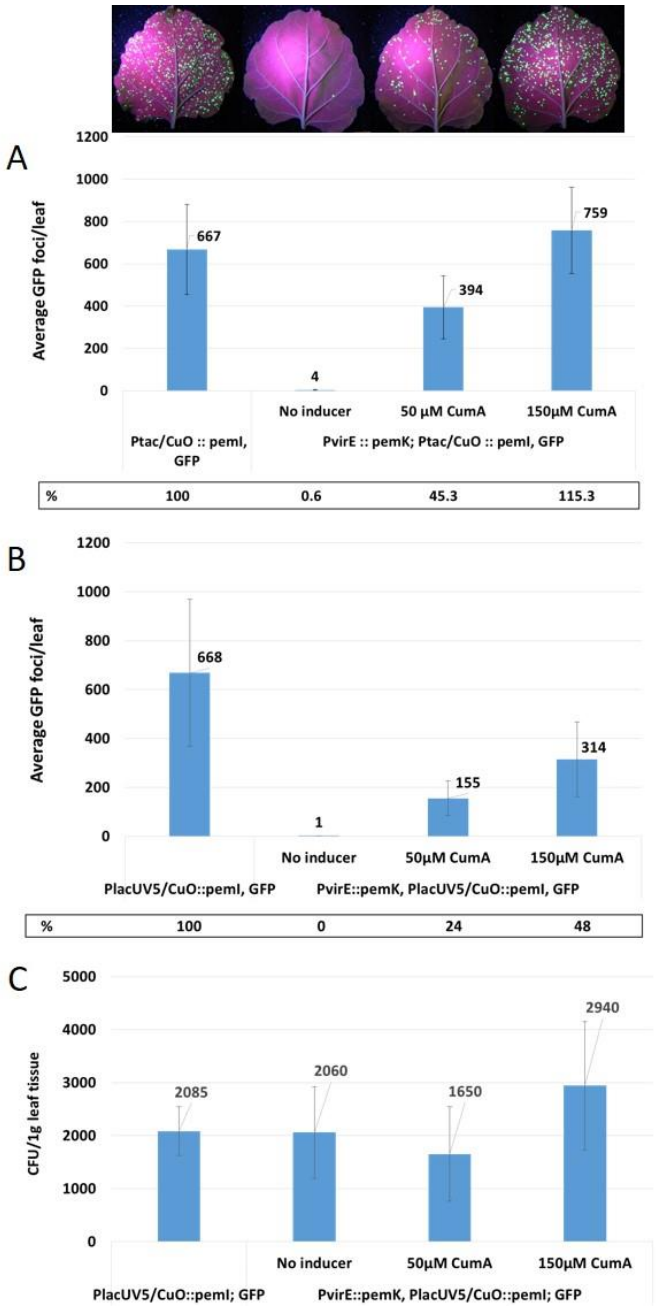

Figure 32. Evaluation of GFP expression foci number and CFU count of live agrobacteria after agroinfiltration with *Agrobacterium* strains harbouring P_{virE}*::pemK* and $P_{tac/CuO}$*::pemI* or $P_{lacUV5/CuO}$*::pemI* constructs. A. P_{virE}*::pemK* and $P_{tac/CuO}$*::pemI* constructs. GV3101*ΔpemIK* (pNMDV515, pNMDV535), containing $P_{tac/CuO}$*::pemI* and P_{virE}*::pemK*, was vacuum infiltrated into *N. benthamiana* plants after addition of 50 μM, 150 μM cumic acid or without inducer. GV3101 (pNMDV535), containing only $P_{tac/CuO}$*::pemI* was used as a positive T-DNA transfer control. **B.** P_{virE}*::pemK* and $P_{lacUV5/CuO}$*::pemI* constructs. GV3101*ΔpemIK* (pNMDV515, pNMDV507), containing $P_{lacUV5/CuO}$*::pemI* and P_{virE}*::pemK*, was vacuum infiltrated into *N. benthamiana* plants after addition of 50 and 150 μM cumic acid or without inducer. GV3101 (pNMDV507), containing only $P_{lacUV5/CuO}$*::pemI* was used as a positive T-DNA transfer control. GFP loci were counted on agroinfiltrated leaves 6 dpi**.** C. Evaluation of CFU count of live agrobacteria on plant leaves after agroinfiltration with *Agrobacterium* strain harbouring P_{virE}*::pemK* and $P_{lacUV5/CuO}$*::pemI* constructs. After plant leaves were photographed for GFP foci number evaluation 4 infiltrated leaves were detached from each plant and grinded in a liquid nitrogen for agrobacteria isolation and CFU enumeration. For every gram of fresh leaf 2 ml of distilled water was added, incubated for 30-40 min at room temperature, filtrated, and then plated on LB medium with antibiotics – rifampicin, kanamycin, spectinomicin and nystatin. The amount of bacteria per 1 g of fresh leave was calculated on plates**.** Bars represent standard deviation. Data are the mean of three independent experiments. Representative pictures of *N. benthamiana* leaves with GFP expression foci are seen on the top of the Figure.

Toxin expression from the P_{virE} promoter does not significantly reduce the number of bacteria on plant leaves when no inducer is added for antitoxin expression, CFU counts does not corelate to GFP foci counts and are similar between different experimental groups in agroinfiltration experiments with strain harbouring P_{virE}*::pemK* and $P_{lacUV5/CuO}$*::pemI* constructs. 2060 CFU/g of leaf tissue of *Agrobacterium* were counted when no inducer was added, 1650 CFU/g of leaf tissue and 2940 CFU/1g of leaf tissue counted when 50 or 150 μM Cumic acid was added respectively, 2085 CFU/g leaf tissue were counted on plant leaves infiltrated with $P_{lacUV5/CuO}$*::pemI, GFP* strain (Fig. 32C). We presume that only a minor fraction of bacteria in contact with the plant leaf started the T-DNA transfer events cascade and underwent *virE* promoter induction. The significant part of bacteria left on the leaf surface after infiltration was not induced for the T-DNA transfer event cascade and thus was not affected by the toxic effect of PemK. This is in contrast to P_{lacUV5}, $P_{tac/CuO}$ or $P_{T5/CuO}$ which are induced by cumic acid despite virulence genes induction state.

DISCUSSION

With the evolution of the technology surrounding the field of transient plant transformation, there is an increasing need for innovations to address environmental biosafety issues, particularly those related to the use of GM *Agrobacterium* in open environments. There were individual attempts to address agro-transformation biosafety issues by creating auxotrophic agrobacteria or inserting a kill switch into them [141, 147], however, approaches were not effective enough to gain a broader practical application. Ultimately several different biocontainment strategies are needed to be used to assure biosafety. To address some of the environmental biosafety issues, we constructed expression systems for controlled T-DNA transfer from *A. tumefaciens* to the host plant. Two distinct approaches for regulation of transient plant transformation were used in the study – an inducible expression of essential *Agrobacterium* virulence gene *virE2* and regulated expression of toxin and antitoxin from *Agrobacterium* TA system *pemIK*.

Starting with inducible expression of *virE2* for T-DNA transfer regulation, this essential *Agrobacterium* virulence gene was inserted under the control of both IPTG or cumic acid inducible promoters and then expression cassettes transformed into *virE2*-deficient *A. tumefaciens* strain. Due to the paucity of information available regarding the over-expression and expression control of foreign genes in *A. tumefaciens*, we tested the regulation of several inducible promoters in *Agrobacterium*. Though IPTG inducible P_{lac} promoter, P_{T5} promoter and lacI-regulated T7 expression system were confirmed functional in *Agrobacterium* earlier [244, 263, 246], our investigation showed that activity of these promoters and expression system was not sufficient for expression of *virE2* and subsequent efficient control of T-DNA transfer from *Agrobacterium* to plants. Several IPTG inducible promoters were investigated, and several new hybrid cumic acid regulated promoters were constructed. We observed a better correlation between β-galactosidase activity assays and inducible T-DNA transfer with cumic acid regulated promoters than with IPTG regulated promoters. Different cumic acid activated constructs were both tightly regulated and highly inducible in *A. tumefaciens*, both in assays *in vitro* and in plant transfection experiments.

Other than promoter strength, several additional factors may influence the efficiency of regulated T-DNA transfer from *ΔvirE2 Agrobacterium* strains harbouring expression cassettes for inducible expression of *virE2*. As the inducer has to be co-infiltrated with *Agrobacterium* to obtain T-DNA transfer, the observed difference between the wild type T-DNA transfer and induced T-DNA transfer efficiency might be caused by non-equal distribution and

penetration of the inducer into the intercellular space of plant leaves, especially in agrospray experiments. This factor may be less impactful when vacuum agroinfiltration is used, however, we did not compare these methods directly to base this presumption. Also, the timing of induced *virE2* expression, as shown by qRT-PCR analysis, do not correspond precisely to the timing of the wild-type *virE2* expression and probably is also shifted temporally compared to the expression of other *vir* genes (i.e. chaperone VirE1 and VirB/D4 transport system, necessary for VirE2 translocation into the plant cell) and may impact the difference between induced and wild type T-DNA transfer efficiencies in agrospray experiments. For further perfection of the system, future insertion of *virE2* expression cassettes into the *Agrobacterium* genome could stabilize the system and simplify the use of inducible *Agrobacterium.* In such a case, a viral vector with any gene of interest could be transformed without further modifications into the engineered chemically regulated *Agrobacterium* and used for transient transfection of plants.

After examining promoter strength, we were interested in evaluating the leakiness of different *virE2* expression systems. $P_{virE/CuO}$, the leakiest promoter, contains a *vir* box and is naturally inducible by the agrobacterial transcription factor VirG [277]. The expression of VirG is increased during the interaction of *Agrobacterium* and the plant cell [278]. High concentration of VirG may impact the leakiness of this promoter. Low leakiness from the promoters $P_{tac/CuO}$ and $P_{T5/CuO}$ as determined by β-galactosidase assays, seemed to be insufficient to provide the necessary amount of VirE2 for T-DNA transfer in syringe infiltration experiments. No leaky T-DNA transfer were observed from these promoters when agrospray assays were performed with diluted *Agrobacterium* suspensions (OD_{600} = 0.001 and 0.01). Only when an agrobacterial density of OD_{600} = 0.1 (corresponding to ~10^8 cfu/ml) was used in agrospray assays, several T-DNA transfer events caused by leaky expression from $P_{tac/CuO}$ and $P_{T5/CuO}$ promoters were detected. However, the efficiency of this observed unwanted T-DNA transfer was very low and would not be relevant under field conditions. The working bacterial concentration for agrospray is typically 10^7-10^6 cfu/ml, which is 1-2 orders of magnitude less than the concentration used in our experiments to evaluate promoter leakiness.

Additionally, a complementary mechanism for the regulation of the T-DNA transfer, based on bacterial type II toxin-antitoxin system *pemIK* was constructed. Bacterial TA systems seemed *a priori* attractive, however challenging tools for our purposes because of limited understanding of the upstream and downstream regulation of TA systems and the scarcity of specific knowledge about *Agrobacterium* TA systems. Although several

TA systems were identified in *A. tumefaciens* bioinformatically, the functionality of a single agrobacterial TA system *ietA/ietS* was confirmed at the very beginning of our research [247]. Another one, *yoeB/yefM*, has been described recently [248].

Toxins from bacterial TA modules have already been successfully used for the construction of bio-containment systems. In particular, bacterial TA systems were shown to work in yeast containment systems. *E. coli* RelBE and Kis-Kid (homolog of *pemI-pemK*) TA modules have been introduced into genetically modified *Saccharomyces cerevisiae* [279, 280]. Kis-Kid controlled expression system was engineered using a strategy similar to ours, with the toxin and antitoxin being inserted under the control of two different inducible promoters *Met25* and *CUP1*. Yeast was transformed with the plasmid in which *kis* (antitoxin) expression is repressed in the presence of methionine, and *kid* (toxin) expression is activated in the presence of Cu^{2+}. CFU count of yeast was lowered by five orders of magnitude in the presence of methionine and Cu^{2+}, with no growth inhibition detected in the absence of both inducers or the presence of Cu^{2+} only. Co-expression of the antitoxin together with the toxin protected yeast from growth inhibition and confirmed the functionality of the bacterial toxin-antitoxin system in eukaryotic cells [280]. Later, alternative containment systems for *E. coli* "Deadman" and "Passcode" based on toxins *mazF* and *ccdB*, respectively, were constructed [146]. These works demonstrated high potential of bacterial TA systems for bio-containment of prokaryotes, lower eukaryotes or even for cell ablation-based strategies for the control of spread of genetically modified plants (expression of *yoeB* toxin from *Streptococcus pneumoniae* TA module *yoeB*/*yefM* shown to be lethal in *Arabidopsis*) [281].

Examples of successful application of TA systems in other microorganisms encouraged us to test whether TA systems could be suitable as a means of control of T-DNA transfer from genetically modified agrobacteria. Initially, we intended to co-express the toxin *pemK* and antitoxin *pemI* from the same inducible promoter expecting a rapid antitoxin degradation and subsequent release of the toxic molecule as such mode of toxin activation was described. We obtained a complete neutralization of a toxin at the co-expression with an antitoxin but were not able to achieve later toxicity (Šarūnas Paškevičius master thesis). Probably our efforts were destined to be ineffective in the light of a very recent publication, where authors raised the hypothesis of a toxin activation by *de novo* synthesis at the reduced pull of the antitoxin rather than by the degradation of a bound antitoxin because not all antitoxins are unstructured and susceptible to Lon protease during cell stress [165]. Interestingly, functional characterization of the same agrobacterial TA module

pemIK (named *mazEF*) has been provided in a very recent publication [282], where antitoxin PemI was described as having a stable secondary helix structure, thus probably not susceptible for proteolytic degradation. The investigation showed that toxin PemK has a ribosome-independent endoribonuclease activity and is neutralized by direct binding of antitoxin PemI. Also four new active toxins from TA modules were identified in *A. tumefaciens* C58 genome by authors.

We changed our strategy to a separate expression of toxin and antitoxin from different inducible promoters. Cumic acid-inducible promoters $P_{tac/CuO}$, $P_{T5/CuO,}$ $P_{lacUV5/CuO}$ and $P_{virE/CuO}$ demonstrated different expression levels and strict regulation of *virE2* expression by the inducer in *Agrobacterium*. These data were definitely facilitating the choice of promoter pair for toxin *pemK* and antitoxin *pemI* expression. Pathogen-plant interaction induced expression of the toxin *pemK* from a natural *Agrobacterium* promoter P_{virE} and the expression of the antitoxin *pemI* from a cumic acid inducible promoter $P_{tac/CuO}$ resulted in very efficient transient transfection of *N. benthamiana* plants after 6 days post agroinfiltration. T-DNA transfer was possible only for a short time after agroinfiltration, while cumic acid, the inductor of expression of the antitoxin, was present. When the inductor is depleted, the T-DNA transfer becomes impossible as bacteria with induced virulence genes are equally induced for transcription of the *pemK* toxin. Any subsequent T-DNA transfer from the bacteria remaining in the soil (in the absence of the chemical inducer) should lead to the death of bacteria. We used plasmid-expressed *pemI* and *pemK* in our experimental system, but for further improvement of the control system, both expression cassettes should be integrated into *Agrobacterium* genome. We also identified and evaluated the activity of several other *A. tumefaciens* toxins from TA systems for the first time. Toxins Dead, PIN, VapC and IetS were highly efficient in reducing *Agrobacterium* CFU counts and may be further used for the construction of biosafety systems.

We assume that our constructed expression systems for controlled T-DNA transfer, based on the expression of *virE2* and expression of *pemI* and *pemK*, could be used with other efficient TA systems besides the *pemIK*, as well as with other approaches for biocontainment of GM microorganisms: with multiple metabolic or synthetic auxotrophy, genetic circuits constructed till date. Only the combination of different biocontainment traits can minimize the risk of unintended T-DNA spread, which may happen because of the natural inactivation of the biosafety strategy applied, caused by horizontal gene transfer between microorganisms, random or targeted mutagenesis. We can never expect a zero risk as with any technological endeavour, but diligently working towards reducing environmental and human health risks to

the lowest levels possible is a worthwhile and necessary goal for adopting new agronomic and biomanufacturing approaches.

CONCLUSIONS

- Cumic acid inducible hybrid promoters $P_{tac/CuO}$, $P_{T5/CuO}$, $P_{lacUV5/CuO}$ are highly active and tightly regulated in *Agrobacterium tumefaciens*, as demonstrated both by agroinfiltration and β-galactosidase assays. Hybrid promoter $P_{virE/CuO}$ showed high activity and tight regulation in agroinfiltration assays.

- Controlled expression of *Agrobacterium* virulence factor *virE2* from cumic acid inducible promoters in *virE2*-deficient *Agrobacterium tumefaciens* strain results in a tightly regulated T-DNA transfer from *Agrobacterium* to plants: up to 72% efficient T-DNA transfer when induced by cumic acid and only 0.002–0.03% in non-induced state.

- Agrobacterial toxins PIN, VapC, PemK, IetS and Dead are functional and capable of reducing *Agrobacterium* CFU counts by 2.7–3.8 orders of magnitude in liquid culture while expressed from cumic acid inducible promoters.

- T-DNA transfer from *Agrobacterium* strain containing toxin and antitoxin expression cassettes P_{virE}::*pemK* and $P_{tac/CuO}$::*pemI* is tightly controlled by cumic acid and as efficient as a wild type transfer.

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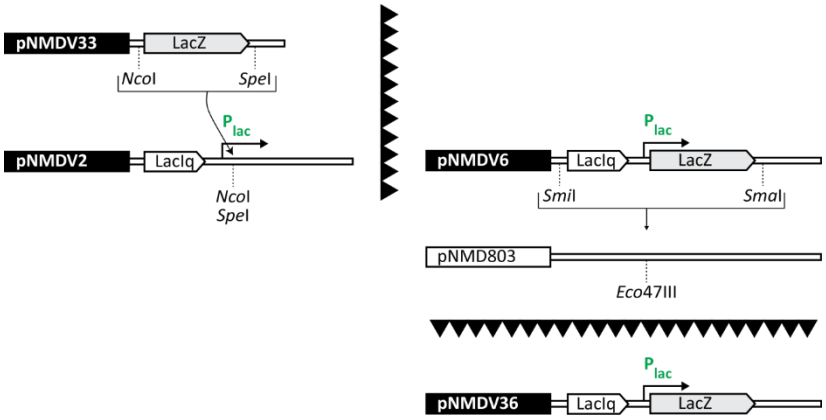
1. Cloning of lacZ coding sequence.

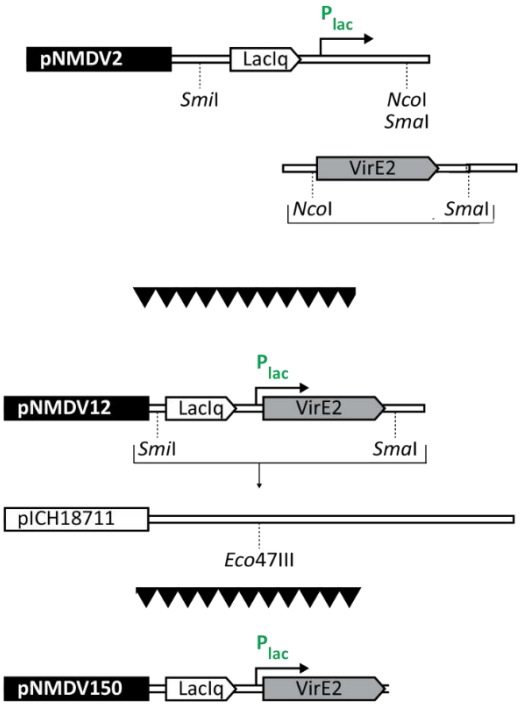


Suppl. Figure 1. Construction of vector with P_{lac}*::lacZ*. The *lacZ* sequence was PCR - amplified from *E. coli* strain DH1 genomic DNA (primers listed in Table 7. I) and inserted into pNMDV2 through NcoI-SpeI, resulting in pNMDV6. The expression cassette *lacIq*,P_{lac}*::lacZ* was then excised from pNMDV6 as a *Smi*I-*Sma*I fragment and inserted into viral vector pNMD803 through a unique restriction site *Eco47*III resulting in construct pNMDV36.

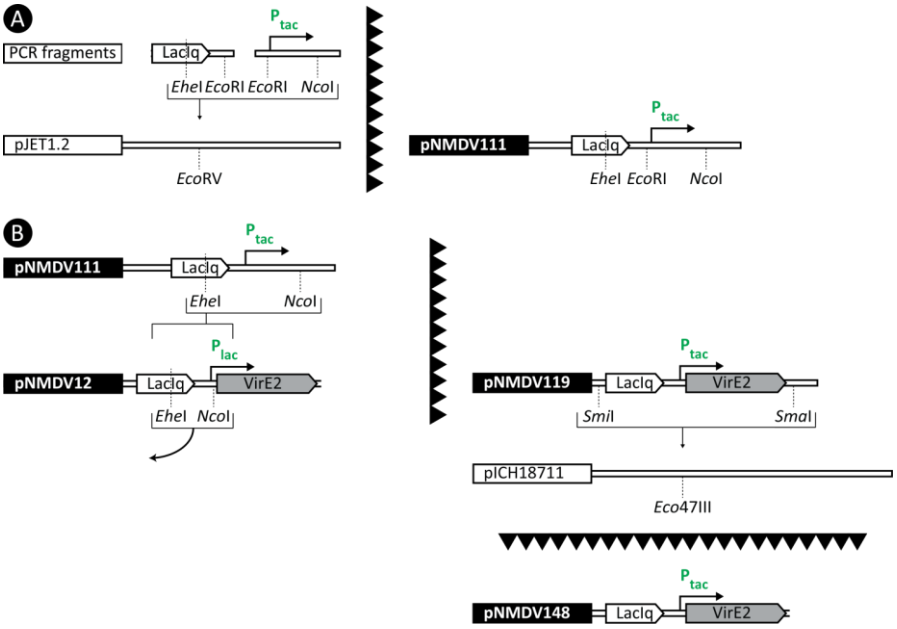
2. Cloning of virE2 and virE1-virE2 coding sequences.

The *virE2* coding sequence was PCR-amplified from *A. tumefaciens* strain GV3101 genomic DNA (primers listed in Table 7. A) and inserted into vector pTZ57R resulting in the construct pNMDV32. The entire *virE1virE2* coding region was amplified from *A. tumefaciens* strain GV3101 genomic DNA (primers listed in Table 7. B) and inserted into vector pJET1.2 resulting in construct pNMDV95.

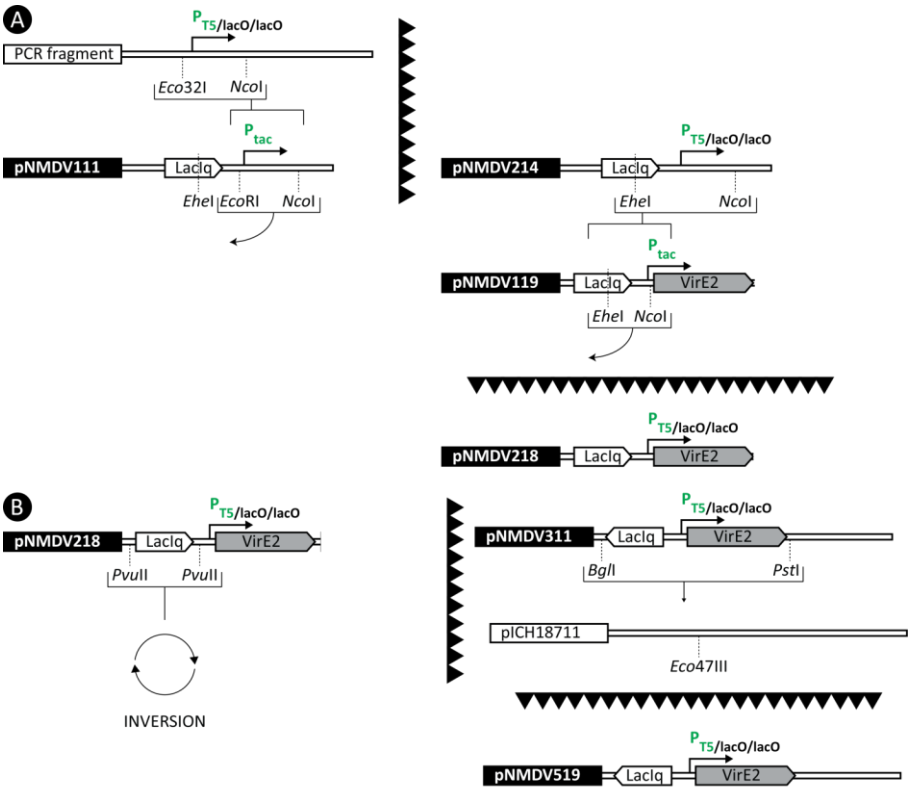
2.1. IPTG inducible virE2 constructs



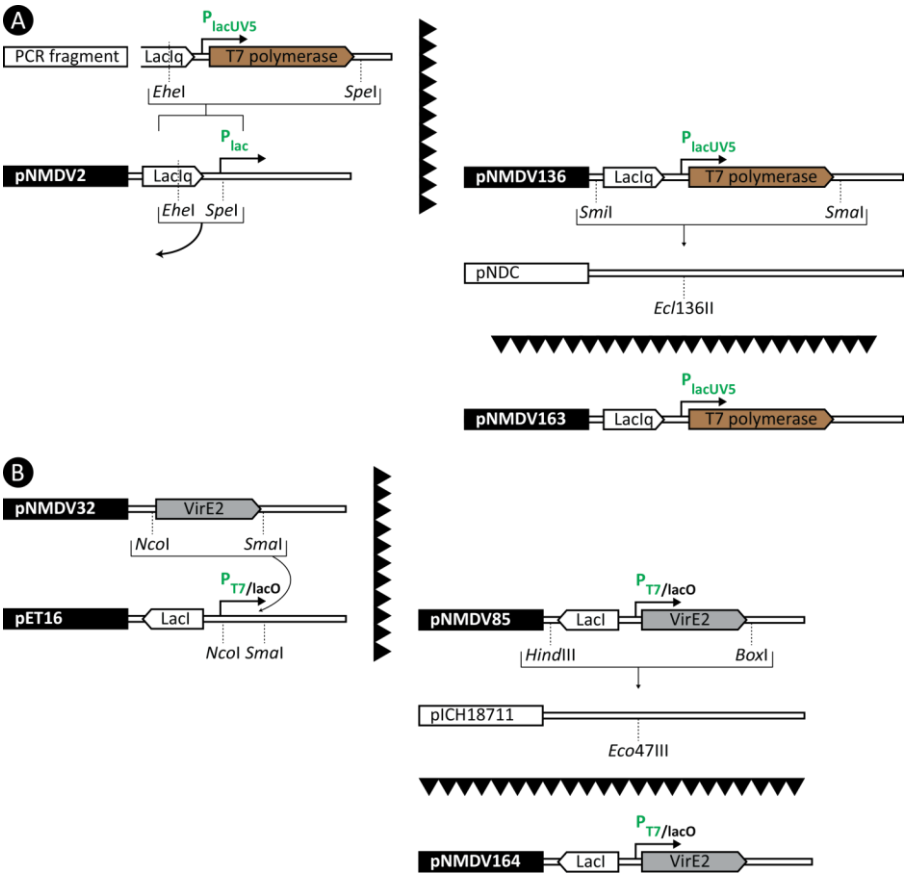
Suppl. Figure 2. Construction of vector with P_{lac}*::virE2***.** Repressor gene *lacIq* together with P_{lac} promoter sequence was amplified from *E. coli* strain DH1 genomic DNA (primers listed in Table 7. C). The amplicon *lacIq*, P_{lac} was cloned into pTZ57R resulting in vector pNMDV2. *VirE2* was inserted downstream to P_{lac} using *Nco*I-*Sma*I, resulting in vector pNMDV12. Expression cassette *lacIq*, P_{lac}*::virE2* was inserted as a *Smi*I-*Sma*I fragment into *Eco47*III digested viral vector pICH18711 giving construct pNMDV150.



Suppl. Figure 3. Construction or vector with P_{tac}*::virE2*. The *tac* promoter was PCR - amplified from pGEX5x-1 (primers listed in Table 7. D). A short *lacI* gene fragment was amplified from previously constructed pNMDV2 (primers listed in Table 7.E). PCR fragments of truncated *lacI* and P_{tac} were joined together in vector pJET1.2 at an *Eco*RI site resulting in construct pNMDV111. The *lacI*, P_{tac} was inserted as a *Ehe*I-*Nco*I fragment into pNMDV12 digested with EheI-NcoI and replaced the *lac* promoter resulting in vector pNMDV119. The *lacIq*, P_{tac}*::virE2*, cassette was then inserted as a *Smi*I-*Sma*I fragment into viral vector pICH18711 digested with *Eco47*III resulting in vector pNMDV148.

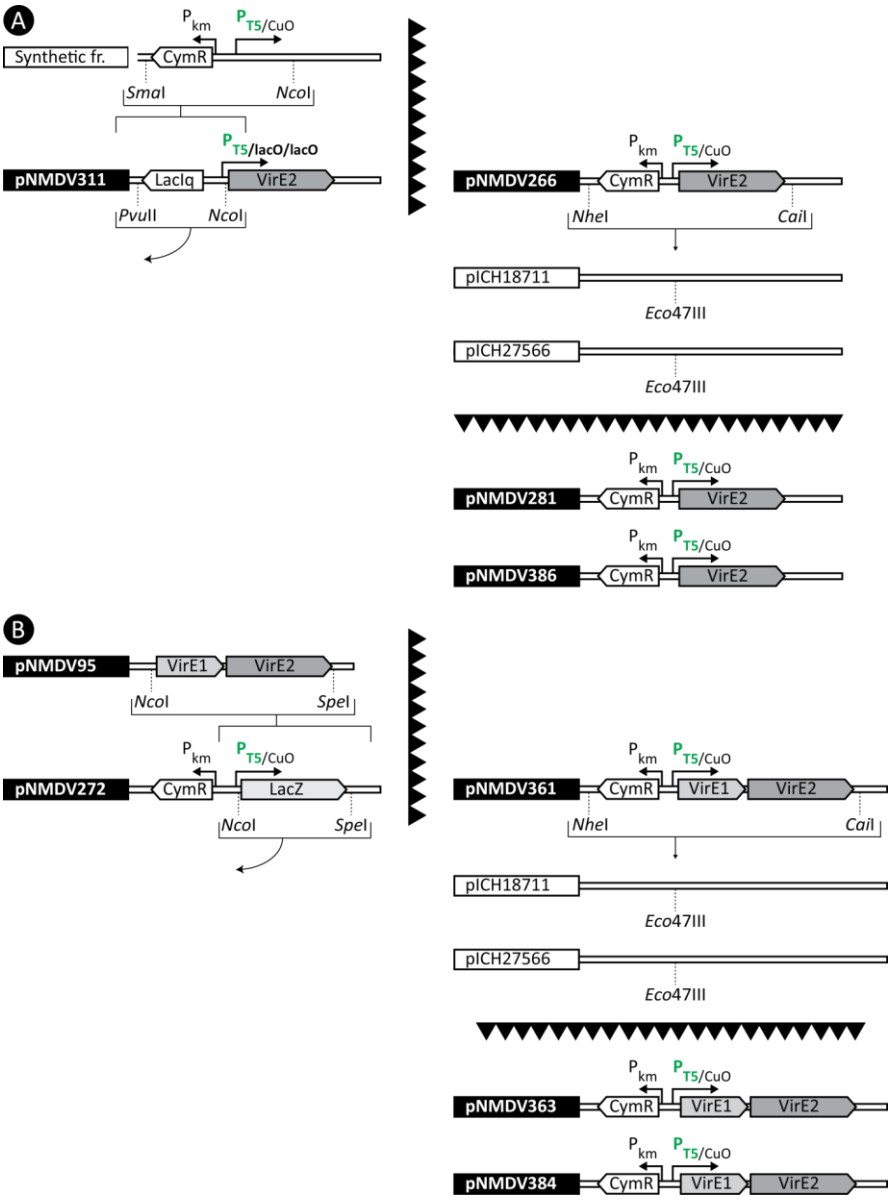


Suppl. Figure 4. Construction of vector with $P_{T5/lacOlacO}$*::virE2***.** The $P_{T5/lacOlacO}$ promoter was PCR-amplified from vector pQE30 (primers listed in Table 7. G) and was inserted as *Eco32*I-*Nco*I fragment (ends blunted with Klenow fragment) into pNMDV111 which also resulted in the removal of the P_{tac} promoter resulting in pNMDV214. An *Ehe*I-*Nco*I fragment with the $P_{T5/lacOlacO}$ promoter was excised from pNMDV214 and inserted into pNMDV119 upstream to *virE2* replacing the P_{tac} promoter and yielding construct pNMDV218. The *lacIq* was inverted in a vector at *Pvu*II in such a way that expression from the *lacI* promoter and from $P_{T5/lacOlacO}$ would go in opposite directions to reduce leaky expression from $P_{T5/lacOlacO}$ (construct pNMDV311). Expression cassette *lacIq*,$P_{T5/lacOlacO}$*::virE2* was inserted as a *Bgl*I-*Pst*I fragment (ends blunted with Klenow fragment) into a viral vector pICH18711 digested with *Eco47*III giving construct pNMDV519.



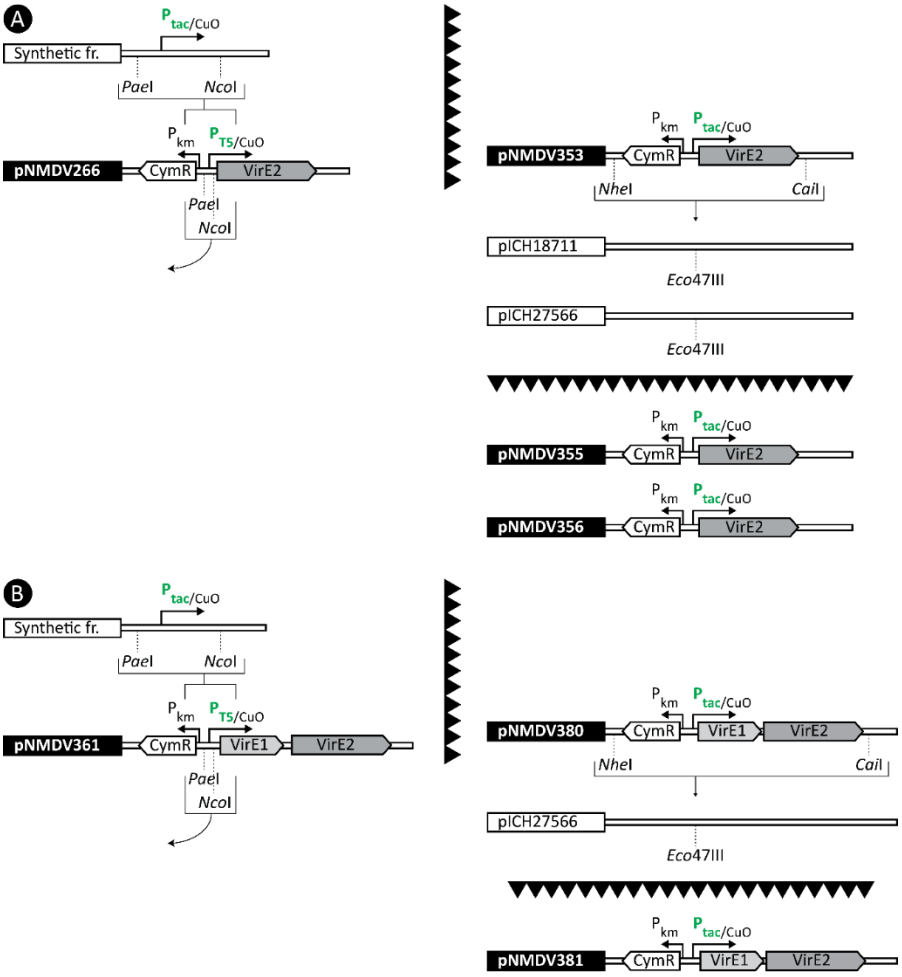
Suppl. Figure 5. Construction of vectors with P_{lacUV5}*::T7polymerase* and $P_{T7/lacO}$*::virE2*. The *T7* polymerase expression cassette P_{lacUV5}*::T7pol* was PCR amplified from *E. coli* strain BL21 genomic DNA (primers listed in Table 7. F). The P_{lacUV5}*::T7pol* fragment was inserted as *Ehe*I-*Spe*I fragment into pNMDV2 digested by *Ehe*I-*Spe*I giving construct pNMDV136. We obtained a *lacIq*,P_{lac}***::T7pol*** cassette from pNMDV136 by digestion with *Smi*I-*Sma*I and inserted this into plant binary vector pNDC, digested with *Ecl136*II resulting in the construct pNMDV163. For construction of $P_{T7/lacO}$*::virE2* expression cassette *virE2* was excised as a *Nco*I-*Sma*I fragment from pNMDV32 and inserted into NcoI-SmaI digested in-house modified pET16 vector downstream to $P_{T7/lacO}$ promoter giving vector pNMDV85. Expression cassette *lacI*,$P_{T7/lacO}$*::virE2* was inserted as *Hind*III-*Box*I (Klenow) fragment into pICH18711 *Eco47*III site, resulting in pNMDV164.

2.2. Cumate inducible virE2 and virE1-virE2 constructs.



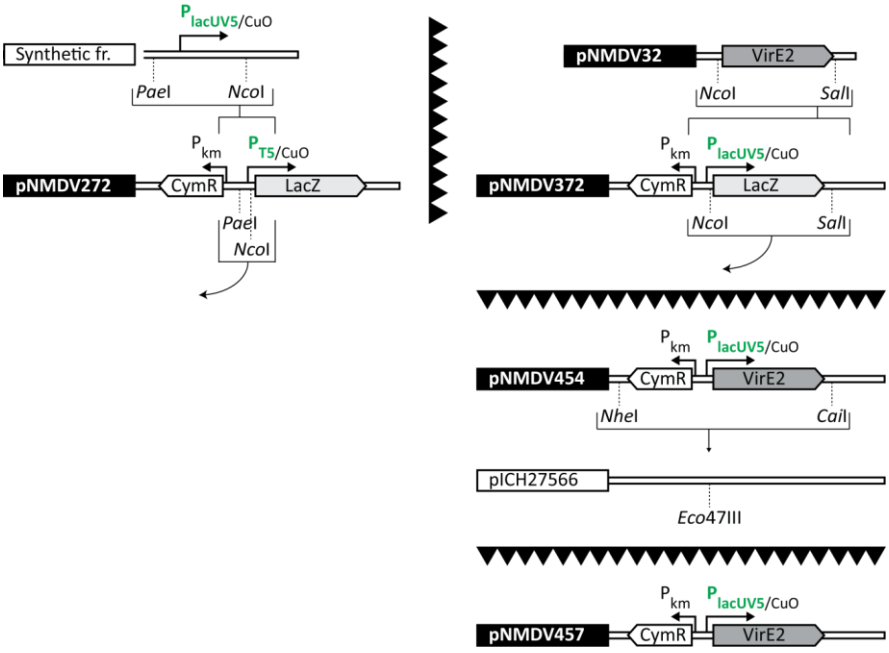
Suppl. Figure 6. Construction of vectors with $P_{T5/CuO}$*::virE2* and $P_{T5/CuO}$*::virE1-virE2***.** A. Promoter $P_{T5/CuO}$ together with repressor gene *CymR* as in pNew [235] was synthesized by GeneScript (USA). The *CymR*,$P_{T5/CuO}$ fragment was inserted as a *Sma*I-*Nco*I fragment into pNMDV311 replacing *lacIq*,$P_{T5/lacOlacO}$ and resulting in pNMDV266. The expression cassette *CymR*, $P_{T5/CuO}$::*virE2* was then inserted as a *Nhe*I-*Cai*I (ends blunted with Klenow

fragment) fragment into viral vectors pICH18711 and pICH27566 at a unique *Eco47*III restriction site resulting in pNMDV281 and pNMDV386, respectively. B. The *virE1-virE2* coding region was inserted into the $P_{T5/CuO}$ regulated expression cassette in pNMDV272 as a NcoI-SpeI fragment giving intermediate construct pNMDV361. The *CymR*, $P_{T5/CuO}$::*virE1-virE2* cassette was then inserted as *Nhe*I-*Cai*I (ends blunted with Klenow fragment) fragment into viral vectors pICH18711 and pICH27566, yielding constructs pNMDV363 and pNMDV384.

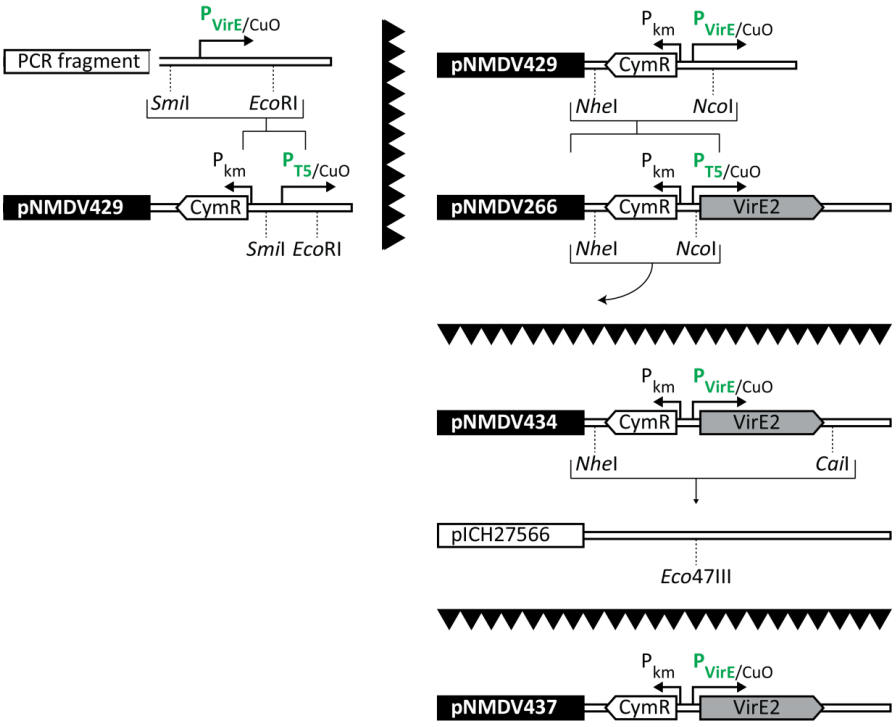


Suppl. Figure 7. Construction of vectors with $P_{tac/CuO}$*::virE2* and $P_{tac/CuO}$*::virE1-virE2*. Hybrid promoter $P_{tac/CuO}$ was designed by adding the *CuO* operator sequence to the P_{tac} promoter and synthesized by Eurofins

(Germany). The $P_{tac/CuO}$ was inserted as a *Pae*I-*Nco*I fragment into the *Pae*I-*Nco*I digested-vector pNMDV266, thereby replacing the $P_{T5/CuO}$ promoter and providing the construct pNMDV353. The obtained *CymR*, $P_{tac/CuO}$::*virE2* cassette was excised with *Nhe*I-*Cai*I (ends blunted with Klenow fragment) and inserted into viral vectors pICH18711 and pICH27655 resulting in constructs pNMDV355 and pNMDV356. The $P_{tac/CuO}$ was inserted as a *Pae*I-*Nco*I fragment into *Pae*I-*Nco*I digested vector pNMDV36, which replaced the $P_{T5/CuO}$ promoter and resulted in pNMDV380. The obtained *CymR*, $P_{tac/CuO}$::*virE1-virE2* cassette was then inserted as a *Nhe*I-*Cai*I (ends blunted with Klenow fragment) fragment into viral vector pICH27566, yielding pNMDV381.



Suppl. Figure 8. Construction of vector with $P_{lacUV5/CuO}$::*virE2*. The hybrid $P_{lacUV5/CuO}$ promoter was designed by adding a *CuO* operator sequence to the P_{lacUV5} promoter and synthesized by Eurofins (Germany). The $P_{lacUV5/CuO}$ sequence was inserted as a *Pae*I-*Nco*I fragment into pNMDV272, replacing $P_{T5/CuO}$ and resulting in pNMDV372. The *virE2* was inserted into pNMDV372 as a *Nco*I-*Sal*I fragment thereby creating pNMDV454. Expression cassette *CymR*, $P_{lacUV5/CuO}$::*virE2* was inserted as *Nhe*I-*Cai*I (Klenow) fragment into vector pICH27566 at the unique *Eco47*III restriction site and resulted in construct pNMDV457.



Suppl. Figure 9. Construction of vector with $P_{virE/CuO}$*::virE2*. The P_{virE} promoter region was PCR - amplified from *Agrobacterium* strain KYRT1 genomic DNA (primers listed in Table 7. H). The P_{T5} in pNMDV429 was replaced with $P_{virE/CuO}$ as an *Smi*I-*Eco*RI fragment. The obtained *CymR*, $P_{virE/CuO}$ fragment was then inserted as a *Nhe*I-*Nco*I fragment into vector pNMDV266 upstream to *virE2* resulting in pNMDV434. The *CymR*, $P_{virE/CuO}$::*virE2* cassette was then inserted as *Nhe*I-*Cai*I (ends blunted with Klenow fragment) fragment into pICH27566 producing pNMDV437.

SANTRAUKA

SUTRUMPINIMAI IR SANTRUMPOS

apm – apsisukimai per minutę,
angl. – anglų kalba,
bp – bazių pora,
BVX – bulvių virusas X (angl. *PVX – potato virus X*),
dpi – dienos po infiltracijos,
dpp – dienos po purškimo,
GM – genetiškai modifikuotas,
IPTG – izopropyl-β-D-tiogalaktopiranozidas,
KFV – kolonijas formuojantys vienetai,
LB – Luria-Bertani terpė,
MV – Milerio vienetai,
OT – optinis tankis,
PGR – polimerazinė grandininė reakcija (angl. *PCR – polymerase chain reaction*),
SN – standartinis nuokrypis,
TA – toksino-antitoksino,
T-DNR – transportuojama DNR (angl. *transfered DNA*),
TL-PGR – tikro laiko polimerazinė grandininė reakcija,
TMV – tabako mozaikos virusas (angl. *TMV – tobacco mosaic virus*),
UV – ultravioletinė šviesa,
ŽFB – žaliai fluorescuojantis baltymas (angl. *GFP – green fluorescent protein*).

ĮVADAS

Augalai, kaip nauji potencialūs šeimininkai rekombinantinių baltymų gamybai, buvo atrasti aštuntojo dešimtmečio pradžioje, kai į augalų ląsteles pirmą kartą įkeltas atsparumo antibiotikui G418 genas[1]. Atradimas, kad įvairūs tiksliniai baltymai gali būti sėkmingai ekspresuojami ne tik mikroorganizmuose ar gyvūnų ląstelių kultūrose, paskatino spartų augalų genų inžinerijos metodų tobulinimą, vertingų eksperimentinių duomenų kaupimą ir naujų idėjų atsiradimą, norint augalų teikiamas galimybes panaudoti vis efektyviau.

Dvi pagrindinės augalų modifikavimo strategijos rekombinantinių baltymų raiškai augaluose yra stabili transformacija ir laikina augalų transfekcija. Atliekant stabilią augalo transformaciją, tikslinės DNR sekos įterpiamos į augalo ląstelių branduolio arba plastidžių genomą, vykdoma transgeninių augalų regeneracija bei atranka, transgenai paveldimi ir kitose transformuoto augalo kartose. Laikinos raiškos atveju perkeltų genų raiška vyksta transfekuotų suaugusių augalų audiniuose nuo neintegruotos svetimos DNR, augalai netrukus yra nuskinami ir iš jų išskiriami susintetinti tiksliniai rekombinantiniai baltymai. Tiek stabiliai, tiek laikinai augalų transformacijai dažniausiai naudojama patogeninė dirvožemio bakterija *Agrobacterium tumefaciens*, turinti natūralų ir unikalų gebėjimą perduoti genus tarp skirtingų filogenetinių karalysčių organizmų, konkrečiai, tarp bakterijų ir augalų. *A. tumefaciens* sukelia auglius įvairiems dviskilčiams augalams. Pasitelkdamos augalų veiksnių indukuotus virulentiškumo veiksnius, daugiausia koduojamus bakterijų *vir* operono, agrobakterijos perkelia iš savo Ti plazmidės DNR fragmentą, vadinamą T-DNR (angl. *transferred DNA*), į augalo ląstelės branduolį, kur fragmentas integruojamas. Agrobakterijų T-DNR fragmenteyra genai opinų sintezei, kurie yra svarbus azoto ir anglies šaltinis agrobakterijoms, taip pat onkogenus, atsakingus už naviko augimą augalo audiniuose. Šis navikas užtikrina tolesnę maisto medžiagų, reikalingų agrobakterijų išlikimui, gamybą. Kai T-DNR fragmentas integruojamas į augalo ląstelės genomą, dalyvaujant augalo šeimininko fermentams ir veiksniams, pradedama opinų sintezės genų ir onkogenų raiška[2]. Agrobakterijos, kad galėtų būti naudojamos tikslingai augalų transformacijai laboratorijose, buvo „nuginkluotos“, t. y. genetiškai modifikuotos taip, kad prarastų savo patogeniškumą, tačiau išlaikytų gebėjimą pernešti svetimą DNR į augalų ląsteles. Pernašai būtinos tik T-DNR fragmentą iš abiejų pusių ribojančios sekos LB (angl. *left border*) ir RB (angl. *right border*), kurias specifiškai atpažįsta agrobakterijų T-DNR pernašoje dalyvaujantys

veiksniai[3, 4]. Buvo sugalvota atskirti *vir* genus nuo likusios Ti-plazmidės dalies ir juos perkelti į atskirą vektorių, pavadintą pagalbiniu *vir* vektoriumi. Taip buvo gauta dvinarė T-DNR plazmidė. Jei abu vektoriai yra toje pačioje bakterijos ląstelėje, *vir* veiksniai puikiai atlieka savo T-DNR sekos perkėlimo funkcijas[5]. Įvairiems tikslams buvo sukonstruoti skirtingi dvinariai vektoriai augalų transformacijai[6–8].

Nors transgeninių augalų kūrimas kai kuriais atvejais yra nepamainomas molekulinės augalų biologijos tyrimuose ar agrobiotechnologijoje, laikina augalų transfekcija dažnai yra patogesnė naudoti. Laikinos raiškos sistemose tiksliniai rekombinantiniai baltymai gali būti susintetinami per kur kas trumpesnį laiką, nei kuriant transgeninius augalus. Laikinos raiškos galimybės būtų dar didesnės, jei transfekciją būtų galima atlikti lauko sąlygomis, pavyzdžiui, purškiant agrobakterijų suspensiją ant tikslinių augalų. Augalus auginant ir transfekuojant lauko sąlygomis, biomasės gamybai būtų reikalingos mažesnės finansinės investicijos nei auginant uždarose patalpose. Tačiau genetiškai modifikuotų agrobakterijų naudojimas laukuose būtų rizikingas dėl galimo jų paplitimo natūralioje aplinkoje ir atsirandančios rizikos transformuoti netikslinius augalus. Šiuo metu yra sukurtos kelios biosaugos strategijos, skirtos sumažinti įvairiais tikslais naudojamų genetiškai modifikuotų mikroorganizmų (GMM) paplitimo riziką natūralioje aplinkoje. Tarp šių strategijų yra daugybinis auksotrofizmas ir genetinių grandinių naudojimas (angl. *genetic circuits*). Vis dėlto, vertinant šių ir kitų sukurtų strategijų biosaugumą, tapo akivaizdu, kad tik derinant kelias GMM paplitimo užkardymo strategijas galima sumažinti riziką aplinkai, nes taikomos biosaugos sistemos gali būti išaktyvuojamos dėl natūralaus horizontalaus genų perkėlimo ar mutagenezės[9].

Laikinos augalų transfekcijos biosaugumas gali būti padidintas kontroliuojant *A. tumefaciens* T-DNR pernašą. Reguliuojant T-DNR pernašą taip, kad GM agrobakterijos turėtų galimybę perduoti T-DNR augalams tik esant induktoriui ir prarastų šią galimybę induktoriui pasišalinus iš bakterijų aplinkos, sumažėtų rizika, kad GM agrobakterijos transformuos netikslinius natūralioje aplinkoje augančius augalus. Turėdami tikslą sukurti reguliuojamos T-DNR pernašos sistemą *A. tumefaciens*, sukonstravome indukuojamos agrobakterijų virulentiškumo veiksnio *virE2* raiškos sistemą. VirE2 baltymai yra būtini T-DNR pernašai iš agrobakterijų į augalo ląsteles. Tai buvo patvirtinta analizuojant *virE2* iškritą turinčio agrobakterijų kamieno gebėjimą pernešti T-DNR į augalo ląsteles. Agrobakterijos beveik visiškai prarado gebėjimą transformuoti augalus[10]. Indukuojamos raiškos sistemai konstruoti ieškojome stiprių, efektyviai induktoriumi kontroliuojamų promotorių, kurie būtų aktyvūs agrobakterijose. Šiuo tikslu įvertinome kituose

mikroorganizmuose naudojamų IPTG induktoriumi indukuojamų promotorių P_{lac}, P_{tac}, $P_{T5/lacOlacO}$, $P_{T7/lacO}$ ir kumine rūgštimi indukuojamo promotoriaus $P_{T5/CuO}$ aktyvumą bei kontrolę agrobakterijose, taip pat sukonstravome kelis hibridinius kumine rūgštimi indukuojamus promotorius $P_{tac/CuO}$, $P_{lacUV5/CuO}$ ir $P_{virE/CuO}$ ir įvertinome jų tinkamumą kontroliuojamai *virE2* raiškai *A. tumefaciens*. Kumine rūgštimi indukuojami promotoriai pasižymėjo stipria raiškos indukcija ir itin mažu pralaidumu agrobakterijose.

Taip pat T-DNR pernašai reguliuoti sukonstravome kitą, *virE2* raiškos sistemą papildančią, indukuojamos raiškos sistemą, paremtą natūraliomis bakterijų toksino-antitoksino (TA) modulių savybėmis. TA moduliai plačiai paplitę bakterijų bei archėjų genomuose, dalyvauja jų atsake į stresą, apsaugoje nuo bakteriofagų infekcijos ir palaiko genetinių elementų stabilumą. TA modulius (sistemas) sudaro du ar daugiau genų, koduojančių toksišką molekulę – toksiną ir jį neutralizuojančią molekulę – antitoksiną. Remdamiesi homologijomis su kitų mikroorganizmų toksinais, *A. tumefaciens* genome identifikavome kelis naujus TA moduliams priklausančius toksinus ir įvertinome jų aktyvumą prieš agrobakterijas. Vieno iš modulių, *pemIK*, pagrindu, kuriame *pemK* yra toksinas, o *pemI* – antitoksinas, sukonstravome efektyvią kumine rūgštimi reguliuojamos T-DNR pernašos sistemą agrobakterijose. *VirE2* bei toksino ir antitoksino raiškos sistemų efektyvumą įvertinome infiltruodami agrobakterijas į modelinius augalus *Nicotiana benthamiana*.

Tyrimų tikslas. Sukonstruoti ir įvertinti indukuojamos raiškos sistemas, skirtas griežtai T-DNR pernašos iš *A. tumefaciens* bakterijų į *N. benthamiana* augalus kontrolei.

Uždaviniai:

- Nustatyti tinkamus promotorius indukuojamai genų raiškai *A. tumefaciens* bakterijose.
- Sukonstruoti efektyvią indukuojamą virulentiškumo veiksnio *virE2* raiškos sistemą *A. tumefaciens* bakterijose.
- Įvertinti reguliuojamos *virE2* raiškos sistemos efektyvumą T-DNR pernašoje iš agrobakterijų į *N. benthamiana* augalus.
- Identifikuoti aktyvius toksinus, priklausančius agrobakteriniams TA moduliams.
- Įvertinti TA moduliams priklausančių toksinų aktyvumą prieš agrobakterijas.
- Sukonstruoti efektyvią indukuojamos toksino ir antitoksino raiškos sistemą augalų transfekcijos kontrolei.

- Įvertinti toksino ir antitoksino raiškos sistemos efektyvumą T-DNR pernašoje iš agrobakterijų į *N. benthamiana* augalus.

Mokslinis naujumas

Trūkstant informacijos apie transgenų raiškos kontrolę agrobakterijose, šiame moksliniame darbe įvertinome kituose mikroorganizmuose tyrinėtų ir plačiai naudojamų chemiškai indukuojamų promotorių aktyvumą *A. tumefaciens*. Įvertinome IPTG indukuojamų promotorių P_{lac}, P_{tac}, $P_{T5/lacOlacO}$, $P_{T7/lacO}$ bei kumine rūgštimi indukuojamo promotoriaus $P_{T5/CuO}$ aktyvumą ir kontrolę *A. tumefaciens*. Promotorius $P_{T5/CuO}$ buvo itin aktyvus ir griežtai kontroliuojamas agrobakterijose, todėl papildomai sukonstravome visiškai naujus hibridinius kumine rūgštimi indukuojamus promotorius $P_{tac/CuO}$, $P_{lacUV5/CuO}$ ir $P_{virE/CuO}$. Šiuos promotorius sėkmingai panaudojome konstruodami reguliuojamas agrobakterijų virulentiškumo veiksnio *virE2* raiškos sistemas, kurios mums leido kontroliuoti T-DNR pernašą iš *A. tumefaciens* bakterijų į *N. benthamiana* augalus. Taip pat *A. tumefaciens* genome identifikavome toksinų Dead, PIN, VapC ir PemK, priklausančių agrobakteriniams TA moduliams, genų sekas ir patvirtinome jų aktyvumą prieš *A. tumefaciens*. Sukonstravome efektyvią T-DNR pernašos reguliavimo sistemą, paremtą kontroliuojama agrobakterijų toksino *pemK* ir antitoksino *pemI* raiška. Tokiu būdu buvo sukurta *virE2* raiškos sistemas papildanti raiškos sistema, skirta padidinti agrobakterijų atliekamos laikinos augalų transfekcijos biosaugumą.

Ginamieji teiginiai

- Kumine rūgštimi indukuojami promotoriai $P_{T5/CuO}$, $P_{tac/CuO}$, $P_{lacUV5/CuO}$ ir $P_{virE/CuO}$ yra aktyvūs ir griežtai reguliuojami *A. tumefaciens*.
- Kontroliuojama agrobakterijų virulentiškumo veiksnio *virE2* raiška *virE2* iškritą turinčiame *A. tumefaciens* kamiene lemia efektyviai reguliuojamą T-DNR pernašą iš *A. tumefaciens* į *N. benthamiana* augalus.
- *A. tumefaciens* toksinai PemK, PIN, VapC, IetS ir Dead, priklausantys bakteriniams toksino-antitoksino moduliams, sukelia agrobakterijų žūtį *in vitro*.
- Kontroliuojama toksino *pemK* ir antitoksino *pemI* iš toksino-antitoksino modulio *pemIK* raiška lemia efektyvų T-DNR pernašos reguliavimą iš *A. tumefaciens* į *N. benthamiana* augalus.

MEDŽIAGOS IR METODAI

Medžiagos

Medžiagos, fermentai bei rinkiniai

DNR ir RNR išskyrimui buvo naudojami UAB „Thermofisher Scientific Baltics“ rinkiniai, fermentinėms reakcijoms – UAB „Thermofisher Scientific Baltics“ fermentai, jiems skirti buferiniai tirpalai ir kiti reakcijoms reikalingi reagentai, juos naudojant buvo vadovaujamasi gamintojo pateiktomis rekomendacijomis.

Vektoriai

Komerciniai klonavimo vektoriai pTZ57R ir pJET1.2 (*Thermofisher Scientific Baltics*) buvo naudojami pradiniuose raiškos sistemų konstravimo etapuose, dvinariai vektoriai pNMD803 (*Nomad Bioscience*), pICH18711, pICH27566 (*Icon Genetics*) ir pNDC (Gyvybės mokslų centras, Vilniaus universitetas) buvo naudojami galutinių vektorių konstravimui, kuriais vėliau buvo transformuojamos agrobakterijos, jomis transfekuojami *N. benthamiana* augalai.

Pradmenys ir sintetinių promotorių sekos

PGR reakcijoms naudoti pradmenys buvo susintetinti įmonėje „Metabion“, promotorių $P_{tac/CuO}$ ir $P_{lacUV5/CuO}$ sekos buvo susintetintos įmonėje „Eurofins“, promotoriaus $P_{T5/CuO}$ seka kartu su jo represoriumi *CymR* susintetinta „Genescript“.

E. coli kamienai

E. coli kamienas DH5α buvo naudojamas visuose tarpiniuose vektorių konstravimo etapuose. *E. coli* kamienų DH1 ir BL21 bakterijų genominė DNR buvo naudojama tikslinių genų sekų padauginimui PGR metodu.

1 lentelė. Šiame darbe naudoti *A. tumefaciens* kamienai.

Kamienas	Kamieno apibūdinimas	Šaltiniai
GV3101	(pMP90RK), nopalino, Rif^r	[11]
ICH011	GV3101*ΔvirE2* (pMP90RK), nopalino, Rif^r	[12]
KYRT-1	Nuginkluotas Chry 5, Rif^r	[13]
GV3101(pICH18711)	Turi TMV virus-based vector with GFP	Šis darbas
GV3101(pICH27566)	Turi PVX virus-based vector with GFP	Šis darbas
GV3101(pNMDV36)	Turi pNMD803-*lacIq*, P_{lac}::*lacZ*	Šis darbas
ICH011(pNMDV148)	Turi pICH18711-*lacIq*, P_{tac}::*virE2*	Šis darbas
ICH011(pNMDV150)	Turi pICH18711-*lacIq*, P_{lac}::*virE2*	Šis darbas
GV3101(pNMDV163, pNMDV223)	Turi pNDC-*lacIq*, P_{lacUV5}::*T7pol*, pICH18711- *lacI*, $P_{T7/lacO}$::*lacZ*	[12]
ICH011(pNMDV163, pNMDV164)	Turi pNDC-*lacIq*, P_{lacUV5}::*T7pol*, pICH18711-*lacI*,$P_{T7/lacO}$::*virE2*	Šis darbas
ICH011(pNMDV281)	Turi pICH18711-*cymR*, $P_{T5/CuO}$::*virE2*	Šis darbas
GV3101(pNMDV299)	Turi pNDC-*cymR*, $P_{T5/CuO}$::*lacZ*	[12]
ICH011(pNMDV355)	Turi pICH18711-*cymR*, $P_{tac/CuO}$::*virE2*	Šis darbas
ICH011(pNMDV356)	Turi pICH27566-*cymR*, $P_{tac/CuO}$::*virE2*	Šis darbas
GV3101(pNMDV376)	Turi pNDC-*cymR*, $P_{lacUV5/CuO}$::*lacZ*	[12]
ICH011(pNMDV381)	Turi pICH27566-*cymR*, $P_{T5/CuO}$::*virE*, *virE2*	Šis darbas
ICH011(pNMDV386)	Turi pICH27566-*cymR*, $P_{T5/CuO}$::*virE2*	Šis darbas
GV3101(pNMDV395)	Turi pNDC-*cymR*, $P_{tac/CuO}$::*lacZ*	[12]
GV3101(pNMDV417)	Turi pNDC-*lacIq*, $P_{T5/lacOlacO}$::*lacZ*	[12]
ICH011(pNMDV437)	Turi pICH27566-*cymR*, $P_{virE/CuO}$::*virE2*	Šis darbas
GV3101(pNMDV442)	Turi pICH27566- *cymR*, $P_{virE/CuO}$::*lacZ*	[12]
ICH011(pNMDV457)	Turi pICH27566-*cymR*, $P_{lacUV5/CuO}$::*virE2*	Šis darbas
ICH011(pNMDV519)	Turi pICH18711-*lacIq*, $P_{T5/lacOlacO}$::*virE2*	Šis darbas
GV3101Δ*pemIK*	(pMP90RK), nopalino, Rif ^{r}, *pemIK* regiono iškrita	[14]
GV3101 (pNMDV621)	Turi pNDC-cymR-$P_{tac/CuO}$::*dead*	[14]
GV3101 (pNMDV622)	Turi pNDC- cymR-$P_{tac/CuO}$:: *PIN*	[14]
GV3101 (pNMDV623)	Turi pNDC- cymR-$P_{tac/CuO}$:: *vapC*	[14]
GV3101 (pNMDV624)	Turi pNDC- cymR-$P_{tac/CuO}$:: *nuclease_like*	[14]
GV3101 (pNMDV371)	Turi pNDC- cymR-$P_{T5/CuO}$:: *pemK-*P_{tetR}	[14]
GV3101 (pNMDV375)	Turi pNDC- cymR-$P_{T5/CuO}$:: *pemIK*	[14]
GV3101 (pNMDV536)	Turi pNDC- cymR-$P_{tac/CuO}$:: *ietS*	[14]
GV3101 (pNMDV371+pICH27566)	Turi pNDC- cymR-$P_{T5/CuO}$:: *pemK-*P_{tetR} + pICH27566	[14]
GV3101Δ*pemIK* (pNMDV515+pNMDV535)	Turi pNDC- P_{virE}::*pemK* + pICH27566-cymR-$P_{tac/CuO}$::*pemI*	[14]
GV3101Δ*pemIK* (pNMDV515+pNMDV507)	Turi pNDC- P_{virE}::*pemK* + pICH27566-cymR-$P_{lacUV5/CuO}$::*pemI*	Sukonstruota Šarūno Paškevičiaus
GV3101 (pNMDV535)	Turi pICH27566-cymR-$P_{tac/CuO}$::*pemI*	[14]
GV3101 (pNMDV507)	Turi pICH27566-cymR-$P_{lacUV5/CuO}$::*pemI*	Sukonstruota Šarūno Paškevičiaus

Metodai

Bakterijų auginimo sąlygos

E. coli ir *A. tumefaciens* bakterijos auginamos LB terpėje, atitinkamai 37 °C ir 28 °C temperatūroje. Į terpes pagal poreikį pridedama antibiotikų: 100 μg/ml ampicilino, 50 μg/ml spektinomicino, 50 μg/ml kanamicino, 50 μg/ml rifampicino ar 50 μg/ml nistatino.

Augalų auginimo sąlygos

Nicotiana benthamiana augalai auginami kontroliuojamomis sąlygomis (25 °C temperatūroje ir 50 % santykinėje drėgmėje) esant ilgos dienos fotoperiodui (16 val. šviesus periodas, 8 val. tamsus periodas, esant ~ 320 lx apšviestumui). 5–6 savaičių amžiaus augalai buvo naudojami agroinfiltracijos eksperimentuose.

Raiškos kasečių konstravimas ir vektorių transformavimas į imliąsias *E.coli* bei *A. tumefaciens* ląsteles

Genų sekos padauginamos PGR metodu ir įterpiamos į komercinius klonavimo vektorius pJET1.2 ir pTZ57R/T. *E. coli* kamienas DH5α buvo naudojamas visuose tarpiniuose vektorių konstravimo etapuose. Imliosios *E. coli* ląstelės ruošiamos ir transformuojamos pagal anksčiau aprašytą metodologiją su nedideliais pakeitimais[15]. Nustačius įterptų fragmentų sekas, naudojant restrikcijos endonukleazes, jie buvo perkelti į dvinarius raiškos vektorius, skirtus indukuojamai genų raiškai agrobakterijose. Detali informacija apie tai, kaip vektoriai buvo konstruojami, ir vektorių konstravimo schemos yra pateiktos disertacijos priede. Sukonstruoti vektoriai transformuojami į elektroimliąsias *A. tumefaciens* ląsteles elektroporacijos metodu.

β galaktozidazės aktyvumo tyrimas

β galaktozidazės aktyvumas agrobakterijose buvo nustatomas naudojant anksčiau aprašytą kalorimetrinį metodą[16], pridėjus vieną papildomą etapą – agrobakterijų ląstelės po inkubacijos etapo buvo centrifuguojamos ir resuspenduojamos tyrimo buferiniame tirpale. Iš šaldytų kultūrų inokuliuotos agrobakterijos auginamos per naktį, skiedžiamos šviežia LB terpe ir 4 val. inkubuojamos 28 °C purtyklėje su induktoriumi (150 μM kuminės rūgšties arba 2 mM IPTG) ar be induktoriaus β galaktozidazės aktyvumo kontrolei.

N. benthamiana augalų agroinfiltracija švirkštu

A. tumefaciens kultūros auginamos per naktį LB terpėje su antibiotikais, tada skiedžiamos šviežia terpe iki OT_{600} = 1 ir toliau skiedžiamos tūkstantį kartų vandeniu iš čiaupo. Į bakterijų suspensiją pridedama induktoriaus IPTG arba kuminės rūgšties. Agroinfiltracija atliekama švirkštu be adatos bakterijų suspensiją sušvirkščiant į apatinę augalo lapo pusę. Suspensija į augalo audinius patenka per lapo žioteles.

T-DNR pernašos ir pralaidumo įvertinimas purškiant augalus *A. tumefaciens* bakterijomis

A. tumefaciens kultūros auginamos per naktį LB terpėje su antibiotikais, tada skiedžiamos šviežia terpe iki OT_{600} = 1 ir toliau skiedžiamos vandeniu iš čiaupo santykiu 1:10 000. Į bakterijų suspensiją buvo pridedama 0,05 % Silwet L-77 ir 150 μM kuminės rūgšties, inkubuojama 1 val. Raiškos sistemos pralaidumui įvertinti kultūros buvo skiedžiamos vandeniu iš čiaupo santykiu 1:10, tada pridedama 0,05 % Silwet L-77. Bakterijų suspensija purškiama ant apatinės augalų lapų pusės. 5–6 dieną po purškimo augalų lapai nuskinami ir fotografuojami UV šviesoje. T-DNR pernašos efektyvumas vertinamas skaičiuojant šviečiančius žaliai fluorescuojančio baltymo (ŽFB) taškus apatinėje lapo pusėje. Apskaičiuojamas vidutinis taškų skaičius ir palyginamas su laukinio tipo T-DNR pernaša.

Toksinų aktyvumo prieš agrobakterijas įvertinimas

A. tumefaciens kultūros inokuliuojamos iš šaldytų kultūrų į LB terpę ir auginamos inkubatoriuje per naktį purtant 220 apm greičiu. Tada kultūros skiedžiamos ir padalijamos į dvi dalis – į agrobakterijų suspensiją su induktoriumi (150 μM kuminės rūgšties) ir suspensiją be induktoriaus. Indukuota ir neindukuota kultūros buvo auginamos 4 val. 28 °C inkubatoriuje purtant 220 apm greičiu. Vėliau kultūros išsėjamos ant agarizuotos LB terpės be induktoriaus. Po dviejų parų augimo 28 °C inkubatoriuje bakterijų kolonijos suskaičiuojamos, toksinų aktyvumas įvertinamas pagal formulę: $\Delta\log_{10}$ = $\log_{10}$ (KFV/mL neindukuotoje kultūroje) – $\log_{10}$ (KFV/mL indukuotoje kultūroje).

T-DNR pernašos efektyvumo bei agrobakterijų kiekio ant lapų įvertinimas agrobakterijas infiltruojant vakuumu

A. tumefaciens kultūros buvo auginamos per naktį LB terpėje su antibiotikais, tada skiedžiamos šviežia terpe iki $OT_{600} = 1$ ir toliau skiedžiamos vandeniu iš čiaupo santykiu 1:100 000. Į bakterijų suspensiją pridedama 0,05 % Silwet L-77 ir, kai reikia, 50 μM ar 150 μM kuminės rūgšties. Agrobakterijų suspensija supilama į vakuuminį indą, prijungtą prie vakuumo pompos. Augalo lapų sistema pamerkiama į bakterijų suspensiją vakuuminiame inde, sukuriamas (iki 200 mbar slėgio) ir vėl panaikinamas vakuumas. Sukuriant vakuumą, iš augalo lapo tarpuląsčių pašalinamas oras, o slėgį vėl atkuriant, tarpuląsčiai prisipildo bakterijų suspensijos. 6-ą dieną po vakuuminės transformacijos augalų lapai buvo nuskinami ir fotografuojami UV šviesoje. Suskaičiuojami šviečiantys ŽFB taškai ant lapų. Apskaičiuojamas vidutinis taškų skaičius ir įvertinamas T-DNR pernašos efektyvumas, palyginant su laukinio tipo T-DNR pernaša. Toliau tie patys nuskinti lapai buvo pasveriami ir sutrinami skystame azote, kad iš jų audinių būtų galima išskirti bakterijas. Į sutrintus audinius pridedama sterilaus distiliuoto vandens (1 g lapų audinių pridedama 2 ml sterilaus vandens), paruošiama lapų audinių suspensija. Suspensija buvo filtruojama plieniniu sieteliu, atliekami serijiniai skiedimai ir išsėjama ant LB terpės su antibiotikais rifampicinu, kanamicinu, spektinomicinu bei nistatinu. Kolonijos suskaičiuojamos ant lėkštelių po dviejų parų auginimo 28 °C inkubatoriuje, tada apskaičiuojamas agrobakterijų kiekis viename grame lapų masės.

VirE2 raiškos įvertinimas TL-PGR metodu

Kumine rūgštimi indukuojama *virE2* raiška pasižymintys agrobakterijų kamienai ir kamienas GV3101 laukinio tipo *virE2* raiškos įvertinimui buvo švirkštu infiltruojami į *N. benthamiana* augalų lapus su induktoriumi kumine rūgštimi arba be jos. Augalų lapai buvo nuskinami praėjus 4, 24, 48 ir 72 val. po infiltracijos. Lapai sutrinami skystame azote, iš jų išskiriama bendroji RNR. TL-PGR atliekamos naudojant komercinį rinkinį, kuriame reakcijoms pasitelkiama interkaliuojančio dažo *SYBR Green* chemija. Reakcijos atliktos rotoriniame termocikleryje „Corbett Rotor gene 6000“ vadovaujantis gamintojo rekomendacijomis. Reakcijų duomenys buvo normalizuojami pagal referentinių genų *rpoB* ir *16sRNA* raišką agrobakterijose.

Statistinė analizė

Statistinė duomenų analizė atlikta naudojantis programine įranga „MaxStat Pro 3.6“. Rezultatų skirtumai laikyti statistiškai reikšmingais, kai reikšmingumo lygmuo $p < 0,05$, pagal dvipusį neporinį t skirstinį.

REZULTATAI IR JŲ APTARIMAS

Turėdami tikslą padidinti GM agrobakterijų tarpininkaujamos laikinos augalų transfekcijos biosaugumą, šiame darbe taikėme dvi skirtingas agrobakterijų T-DNR pernašos kontrolės strategijas. T-DNR perdavimo kontrolės buvo siekiama induktoriumi reguliuojant agrobakterijų virulentiškumo veiksnio *virE2* raišką *ΔvirE2 A. tumefaciens* bakterijose, taip pat derinant agrobakterijų toksino *pemK* ir antitoksino *pemI* raišką *ΔpemIpemK A. tumefaciens* bakterijose.

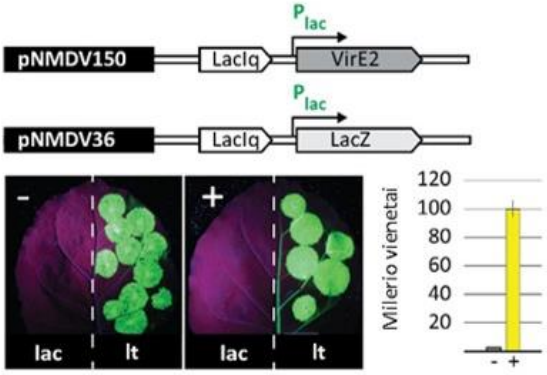
Indukuojamos agrobakterijų virulentiškumo veiksnio *virE2* raiškos sistemos konstravimas, sistemos tinkamumo reguliuojamai T-DNR pernašai iš *A. tumefaciens* bakterijų į *N. benthamiana* augalus įvertinimas

Indukuojamos *virE2* geno raiškos sistemai konstruoti buvo naudojami du skirtingi dvinariai raiškos vektoriai – tabako mozaikos viruso (TMV) pagrindu sukonstruotas vektorius pICH18711 ir bulvių X viruso (BVX) pagrindu sukonstruotas vektorius pICH27566. Abu vektoriai savo T-DNR regione turi virusinę polimerazę ir ŽFB koduojančius genus, taip pat genus, koduojančius virusinių dalelių judėjimo baltymus. Šie baltymai užtikrina virusinių replikonų plitimą iš vienos augalo ląstelės į kitą (pICH18711) ar sisteminį plitimą visame augale (pICH27566). Šiuos vektorius naudojant laikinai augalų transfekcijai agrobakterijomis, praėjus 4–5 dienoms po agroinfiltracijos, augalo audiniuose matoma stipri ŽFB raiškos taškų fluorescencija, o taškų kiekis koreliuoja su T-DNR pernašos efektyvumu. *VirE2* raiškos kasetės buvo įkeliamos į pICH18711 ir pICH27566 vektorius, kurie transformuojami į agrobakterijų kamieną ICH011 (*ΔvirE2*). *VirE2* raiškos kasetėms konstruoti naudoti IPTG arba kumine rūgštimi indukuojami promotoriai. Indukuojamų promotorių efektyvumas ir tinkamumas raiškos sistemai buvo įvertinamas infiltruojant gautus agrobakterijų kamienus į *N. benthamiana* augalų lapus su induktoriumi ar neindukuojant ir vėliau lyginant ŽFB raiškos taškų skaičių augalo audiniuose. Raiškos kasetės, pasižymėjusios didžiausiu aktyvumu pridėjus induktoriaus ir mažiausiu

pralaidumu neindukuojant, vėliau buvo analizuojamos agropurškimo eksperimentuose.

IPTG indukuojamų promotorių įvertinimas

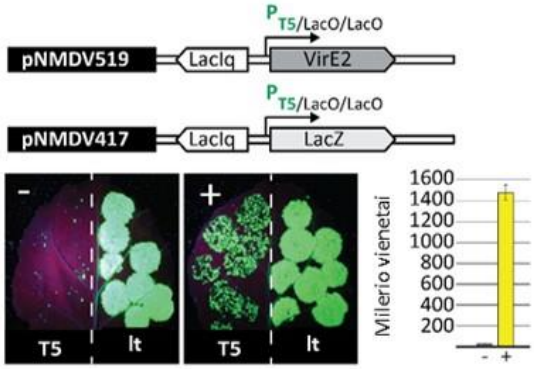
Lac promotorius. P_{lac} promotoriaus aktyvumas agrobakterijose pirmiausia buvo įvertintas β galaktozidazės aktyvumo tyrime, atliktame su agrobakterijų kamienu GV3101 (pNMDV36 (P_{lac}::*lacZ*)) (1 lent.). Nustatytas promotoriaus aktyvumas po 5 val. indukcijos IPTG siekė 100 MV (1 pav.). Tada promotoriaus aktyvumas buvo įvertintas agroinfiltracijos eksperimentuose, kai agrobakterijos ICH011 (pNMDV150 (P_{lac}::*virE2*)) buvo infiltruojamos į *N. benthamiana* augalus. Deja, ŽFB raiškos taškų augalo audiniuose užfiksuoti nepavyko (1 pav.). Promotorius P_{lac} nebuvo pakankamai stiprus produkuoti reikiamą VirE2 baltymų kiekį efektyviai T-DNR pernašai vykti. Tada nusprendėme įvertinti kelių stipresnių IPTG indukuojamų promotorių aktyvumą *A. tumefaciens*. Promotorių P_{tac}, $P_{T5/lacO/lacO}$ ir $P_{T7/lacO}$ aktyvumas anksčiau buvo patvirtintas *E. coli* bei kituose mikrooorganizmuose, todėl juos pasirinkome ištyrimui *A. tumefaciens*.



1 pav. P_{lac} promotoriaus aktyvumo įvertinimas *A. tumefaciens* kamiene ICH011**.** *VirE2* raiška nuo IPTG indukuoto P_{lac} promotoriaus nelėmė ŽFB raiškos augalo lapuose; β galaktozidazės aktyvumo tyrimas agrobakterijose parodė ~ 100 MV indukuoto promotoriaus aktyvumą. *N. benthamiana* augalų lapai fotografuoti UV šviesoje 5 d.p.i. „+“ – indukcija su 1 mM IPTG. „–“ – neindukuota. „lt“ – kontrolinės agroinfiltracijos su *A. tumefaciens* kamienu GV3101, turinčiu virusinį vektorių su ŽFB raiškos kasete. β galaktozidazės aktyvumo tyrimų rezultatų reikšmės atitinka trijų nepriklausomų eksperimentų rezultatų vidurkį. Stulpelių viršuje nurodyti standartiniai nuokrypiai.

***P_{tac}* promotorius.** P_{tac} promotoriaus aktyvumas buvo įvertintas agroinfiltracijos eksperimente. Eksperimentui naudotas *A. tumefaciens* kamienas ICH011 (pNMDV148 (P_{tac}::*virE2*)) (1 lent.). Skirtingai nei P_{lac}*::virE2, virE2* raiška nuo P_{tac} promotoriaus lėmė intensyvią ŽFB fluorescenciją augalo audiniuose. Vis dėlto promotorius taip pat pasižymėjo dideliu raiškos pralaidumu, lėmusiu beveik lygiavertišką ŽFB fluorescencijos intensyvumą, kai induktoriaus nebuvo pridėta (duomenys neparodyti). Siekėme sumažinti promotoriaus pralaidumą, įkeldami į raiškos sistemą papildomą represoriaus *lacIq* geno seką atskirame vektoriuje, tačiau papildoma represoriaus raiška *in trans* sistemos pralaidumo nesumažino.

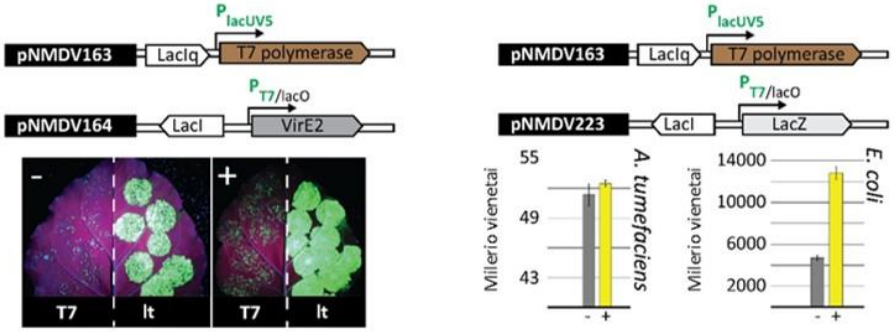
***$P_{T5/lacO/lacO}$* promotorius.** $P_{T5/lacO/lacO}$ promotoriaus, tokio, koks yra naudojamas komerciniame Qiagen vektoriuje pQE30 (hibridinis P_{T5} promotorius, turintis dvi *lacO* operatoriaus sekas), aktyvumą įvertinome β galaktozidazės aktyvumo bei agroinfiltracijos eksperimentuose. *A. tumefaciens* kamienas GV3101 (pNMDV417 ($P_{T5/lacO/lacO}$ *::lacZ*)) (1 lent.) po 5 val. indukcijos su 1 mM IPTG pasižymėjo dideliu β galaktozidazės aktyvumu (~ 1500 MV) ir mažu raiškos pralaidumu neindukavus (~ 27 MV). Į augalų lapus infiltravus 1 mM IPTG indukuotą kamieną ICH011 (pNMDV519 ($P_{T5/lacO/lacO}$ *::virE2*)) (1 lent.), buvo matoma ryški ŽFB taškų fluorescencija, o induktoriaus nepridėjus buvo matomi vos keli ŽFB raiškos taškai (2 pav.).



2 pav. $P_{T5/lacO/lacO}$ promotoriaus aktyvumo įvertinimas *A. tumefaciens* kamiene ICH011. *VirE2* raiška nuo $P_{T5/lacO/lacO}$ promotoriaus lėmė efektyvią ir griežtai reguliuojamą T-DNR pernašą, stiprią ŽFB fluorescenciją lapuose. *LacZ* raiška nuo promotoriaus sąlygojo vidutinį β galaktozidazės aktyvumą (~ 1400 MV) esant induktoriui ir labai mažą pralaidumą neindukavus (~ 27 MV). *N. benthamiana* augalų lapai fotografuoti UV šviesoje 5 d.p.i. „+“ – indukcija su 1 mM IPTG. „–“ – neindukuota. „lt“ – kontrolinės agroinfiltracijos su

A. tumefaciens kamienu GV3101, turinčiu virusinį vektorių su ŽFB raiškos kasete. β galaktozidazės aktyvumo tyrimų rezultatų reikšmės atitinka trijų nepriklausomų eksperimentų rezultatų vidurkį. Stulpelių viršuje nurodyti standartiniai nuokrypiai.

$P_{T7/lacO}$ promotorius. Hibridinio plačiai kituose mikroorganizmuose naudojamo $P_{T7/lacO}$ promotoriaus, tokio, koks yra naudojamas komerciniame vektoriuje pET16 „Novagen“ raiškos sistemoje, aktyvumas agrobakterijose buvo įvertintas β galaktozidazės aktyvumo ir agroinfiltracijos tyrimuose. Sukonstravome dvigubą raiškos sistemą, sudarytą iš vektoriaus, turinčio bakteriofago T7 polimerazės raiškos kasetę, ir dvinario vektoriaus, turinčio T7 polimerazės specifiškai atpažįstamas ir IPTG indukuojamas raiškos kasetes $P_{T7/lacO}$::*lacZ* arba $P_{T7/lacO}$*::virE2*. Agrobakterijų kamieno ICH011 (pNMDV163 (P_{lacUV5}::*T7 polymerase*), pNMDV164 ($P_{T7/lacO}$::*virE2*)) (1 lent.) efektyvumas T-DNR pernašoje buvo įvertintas agroinfiltracijos eksperimentuose. 1 mM IPTG indukuota raiškos sistema agrobakterijose pasižymėjo didesniu raiškos pralaidumu ir silpnesne ŽFB fluorescencija nei sistema, turinti $P_{T5/lacO/lacO}$:: *virE2* (3 pav.). Mus nustebino tai, kad raiškos sistema, turinti kasetę $P_{T7/lacO}$::*lacZ* (kamienas ICH011 (pNMDV163, pNMDV223)), parodė itin mažą β galaktozidazės aktyvumą agrobakterijose, tačiau buvo labai aktyvi ją transformavus į *E. coli* ląsteles (> 12 000 MV) (3 pav.). Taigi ši dviguba raiškos sistema buvo funkcionali, tačiau dėl nežinomų priežasčių neaktyvi *A. tumefaciens* β galaktozidazės aktyvumo vertinimo eksperimente.



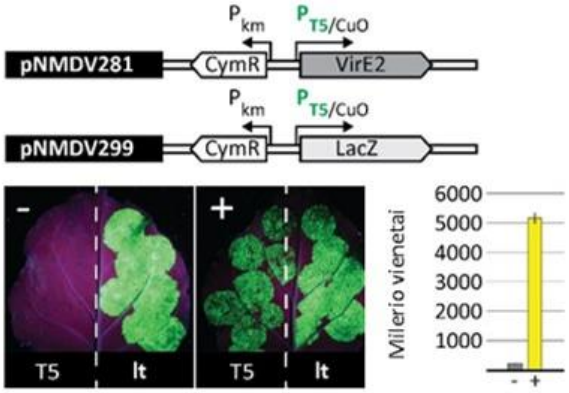
3 pav. $P_{T7/lacO}$ promotoriaus aktyvumo įvertinimas *A. tumefaciens* kamiene ICH011. *VirE2* raiška nuo $P_{T7/lacO}$ promotoriaus lėmė vidutinišką ŽFB taškų fluorescenciją augalo lapuose pridėjus induktoriaus ir nedidelę, tačiau matomą fluorescenciją neindukavus. *LacZ* raiška sąlygojo itin mažą β galaktozidazės aktyvumą *A. tumefaciens* tiek esant induktoriui, tiek neindukuojant (~ 52 MV), tačiau sistemos aktyvumas buvo didelis *E. coli* (> 12 000 MV).

N. benthamiana augalų lapai fotografuoti UV šviesoje 5 d.p.i. „+“ – indukcija su 1 mM IPTG. „–“– neindukuota. „lt“ – kontrolinės agroinfiltracijos su *A. tumefaciens* kamienu GV3101, turinčiu virusinį vektorių su ŽFB raiškos kasete. β galaktozidazės aktyvumo tyrimų rezultatų reikšmės atitinka trijų nepriklausomų eksperimentų rezultatų vidurkį. Stulpelių viršuje nurodyti standartiniai nuokrypiai.

Kumine rūgštimi indukuojamų promotorių įvertinimas

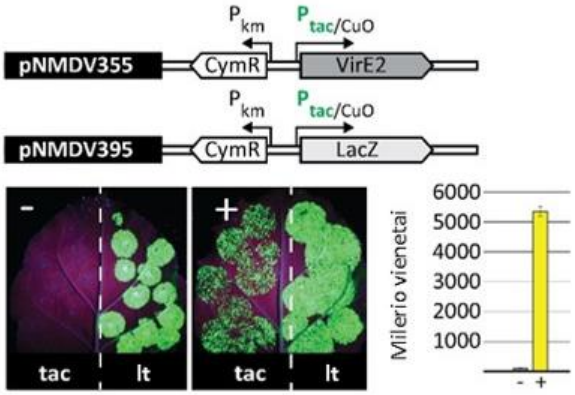
Ištyrę IPTG kontroliuojamų promotorių aktyvumą *A. tumefaciens*, indukuojamų *virE2* raiškos sistemų konstravimui nusprendėme panaudoti anksčiau apibūdintą efektyvią kumine rūgštimi indukuojamą genų raiškos sistemą (angl. *cumate gene switch*), kurios pirminis šaltinis yra *Pseudomonas putida* bakterijų kamieno F1 operonai *cym* ir *cmt*[17].

$P_{T5/CuO}$ promotorius. $P_{T5/CuO}$ promotoriaus seka, kartu su represoriaus seka *cymR*, tokia, kokia ji yra pateikta originalią raiškos sistemą turinčiame komerciniame vektoriuje pNew[17], buvo susintetinta (*Genescript*) ir įterpta į dvinarius vektorius *virE2* ar *lacZ* raiškos kontrolei. Kuminė rūgštis indukavo stiprią *virE2* raišką agrobakterijų kamiene ICH011 (pNMDV281 ($P_{T5/CuO}$::*virE2*)), kuri lėmė T-DNR pernašą ir matomą jos rezultatą – stiprią ŽFB fluorescenciją *N. benthamiana* augalų lapuose (4 pav.). $P_{T5/CuO}$ promotorius pasižymėjo stipresne indukcija (~ 5200 MV) nei $P_{T5/lacO/lacO}$ promotorius (~1500) β galaktozidazės aktyvumo tyrime. Nors kumine rūgštimi indukuojamo promotoriaus pralaidumas taip pat buvo didesnis (~ 240 MV) nei IPTG indukuojamo promotoriaus (~ 27 MV), tačiau nepakankamas pradėti T-DNR pernašai, kuri lemtų matomą ŽFB fluorescenciją augalų audiniuose (4 pav.), o santykinai nedidelis raiškos kasetės $P_{T5/lacO/lacO}$ *::virE2* pralaidumas agroinfiltracijos eksperimentuose buvo matomas kaip ŽFB fluorescencija augalų lapuose. Manome, kad IPTG indukuojamų promotorių didesnį pralaidumą augalo audiniuose lėmė nežinomi galbūt su augalu susiję veiksniai, turintys įtakos represoriaus stabilumui ar prisijungimo prie operatoriaus afiniškumui.



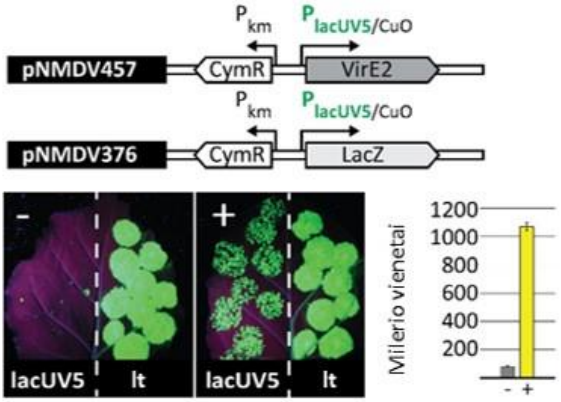
4 pav. 4. $P_{T5/CuO}$ promotoriaus aktyvumo įvertinimas *A. tumefaciens* kamiene ICH011. *VirE2* raiška nuo $P_{T5/CuO}$ promotoriaus lėmė labai stiprią ir labai griežtai kontroliuojamą T-DNR pernašą; *lacZ* raiška lėmė didelį β galaktozidazės aktyvumą (~ 5200 MV) ir mažą pralaidumą (~ 240 MV). *N. benthamiana* augalų lapai fotografuoti UV šviesoje 5 d.p.i. „+“ – indukcija su 150 µM kuminės rūgšties. „–“ – neindukuota. „lt“ – kontrolinės agroinfiltracijos su *A. tumefaciens* kamienu GV3101, turinčiu virusinį vektorių su ŽFB raiškos kasete. β galaktozidazės aktyvumo tyrimų rezultatų reikšmės atitinka trijų nepriklausomų eksperimentų rezultatų vidurkį. Stulpelių viršuje nurodyti standartiniai nuokrypiai.

$P_{tac/CuO}$ promotorius. Kaip minėta anksčiau, IPTG indukuojamas P_{tac} promotorius *A. tumefaciens* pasižymėjo stipria indukcija, tačiau ir dideliu pralaidumu (duomenys neparodyti). Kadangi mūsų ekperimentuose kumine rūgštimi indukuojamas $P_{T5/CuO}$ buvo mažiau pralaidus nei IPTG indukuojamas $P_{T5/lacOlacO}$ promotorius, tikėjomės, kad pridėdami kumato operatorių už P_{tac} promotoriaus sekos, sumažinsime šio promotoriaus pralaidumą. Įmonėje „Eurofins“ buvo susintetintas $P_{tac/CuO}$ promotorius, kuriame *lacO* operatoriaus seka buvo pakeista kumato operatoriumi *CuO*. Agrobakterijų kamiene ICH011 (pNMDV355 ($P_{tac/CuO}$*::virE2*)) (1 lent.) pridėjus 150 µM kuminės rūgšties, buvo indukuota stipri *virE2* raiška, kuri lėmė efektyvią T-DNR pernašą ir matomą jos rezultatą – stiprią ŽFB fluorescenciją *N. benthamiana* augalų lapuose. Į agrobakterijų suspensiją nepridėjus induktoriaus, ŽFB fluorescencija nevyko, taigi raiškos sistema pasižymėjo itin mažu pralaidumu (5 pav.). β galaktozidazės aktyvumo tyrime $P_{tac/CuO}$ promotorius taip pat pasižymėjo stipresne indukcija (~ 5400 MV) ir mažesniu pralaidumu (~ 110 MV) nei $P_{T5/CuO}$ promotorius. Šie rezultatai leidžia teigti, kad hibridinis $P_{tac/CuO}$ promotorius yra labai stiprus ir griežtai reguliuojamas *A. tumefaciens*.



5 pav. $P_{tac/CuO}$ promotoriaus aktyvumo įvertinimas *A. tumefaciens* kamiene ICH011. *VirE2* raiška nuo $P_{tac/CuO}$ promotoriaus lėmė labai efektyvią T-DNR pernašą; *lacZ* raiška lėmė didelį β galaktozidazės aktyvumą pridėjus induktoriaus (~ 5400 MU) ir mažą aktyvumą be induktoriaus (~ 110 MU). *N. benthamiana* augalų lapai fotografuoti UV šviesoje 5 d.p.i. „+“ – indukcija su 150 µM kuminės rūgšties. „–“– neindukuota. „lt“ – kontrolinės agroinfiltracijos su *A. tumefaciens* kamienu GV3101, turinčiu virusinį vektorių su ŽFB raiškos kasete. β galaktozidazės aktyvumo tyrimų rezultatų reikšmės atitinka trijų nepriklausomų eksperimentų rezultatų vidurkį. Stulpelių viršuje nurodyti standartiniai nuokrypiai.

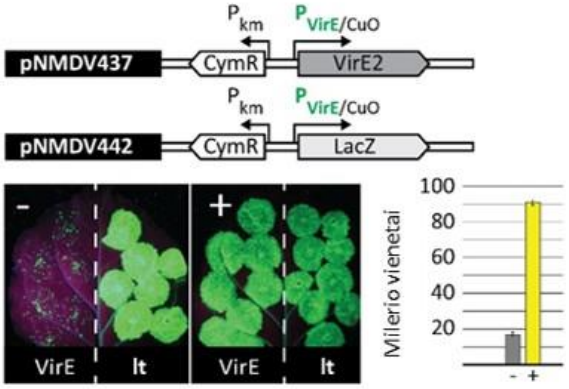
$P_{lacUV5/CuO}$ promotorius. Toliau mums buvo įdomu nustatyti minimalų reikiamą promotoriaus stiprumą *virE2* raiškai ir efektyviai T-DNR pernašai vykti. Kaip minėta anksčiau, $P_{T5/CuO}$ promotoriaus pralaidumas (~ 240 MV) buvo nepakankamas T-DNR pernašai įvykti, tačiau *virE2* raiška nuo $P_{T5/lacOlacO}$ promotoriaus (~ 1500 MV) jau lėmė efektyvią T-DNR pernašą ir ryškią ŽFB fluorescenciją *N. benthamiana* augalų lapuose. Todėl ieškojome silpniau indukuojamo promotoriaus nei $P_{T5/lacOlacO}$. Šiuo tikslu įmonėje „Eurofins“ buvo susintetintas kumine rūgštimi indukuojamas $P_{lacUV5/CuO}$ promotorius. Raiškos kasečių $P_{lacUV5/CuO}$::*virE2* ir $P_{lacUV5/CuO}$::*lacZ* indukcijos stiprumas buvo įvertintas β galaktozidazės aktyvumo bei agroinfiltracijos eksperimentuose. 1100 MV siekęs promotoriaus aktyvumas buvo pakankamas vykti T-DNR pernašai iš agrobakterijų į augalus (6 pav.). Taigi gauti rezultatai parodė, kad, norint susintetinti reikiamą VirE2 baltymo kiekį ir pasiekti minimalų T-DNR pernašos efektyvumą, promotoriaus aktyvumas turėtų būti tarp ~ 240 MV ir 1100 MV β galaktozidazės aktyvumo tyrimuose.



6 pav. $P_{lacUV5/CuO}$ promotoriaus aktyvumo įvertinimas *A. tumefaciens* kamiene ICH011. *VirE2* išraiška nuo $P_{lacUV5/CuO}$ promotoriaus lėmė vidutiniškai efektyvią ir santykinai griežtą T-DNR pernašos kontrolę; β galaktozidazės aktyvumo tyrimas parodė ~ 1100 MV aktyvumą pridėjus induktoriaus ir ~ 80 MV nesant induktoriaus. *N. benthamiana* augalų lapai fotografuoti UV šviesoje 5 d.p.i. „+“ – indukcija su 150 µM kuminės rūgšties. „–“– neindukuota. „lt“ – kontrolinės agroinfiltracijos su *A. tumefaciens* kamienu GV3101, turinčiu virusinį vektorių su ŽFB raiškos kasete. β galaktozidazės aktyvumo tyrimų rezultatų reikšmės atitinka trijų nepriklausomų eksperimentų rezultatų vidurkį. Stulpelių viršuje nurodyti standartiniai nuokrypiai.

$P_{virE/CuO}$ promotorius. Kumine rūgštimi indukuojami $P_{T5/CuO}$ ir $P_{tac/CuO}$ promotoriai buvo stiprūs agrobakterijose, tačiau savo stiprumu neprilygo natūraliam agrobakterijų *virE* operono P_{virE} promotoriui, kurio stiprumą galėjome įvertinti infiltruodami kontrolinį kamieną GV3101 (pICH18711). Todėl nusprendėme sukonstruoti kumine rūgštimi reguliuojamą $P_{virE/CuO}$ promotorių. P_{virE} promotoriaus seka PGR metodu buvo padauginta nuo *A. tumefaciens* genominės DNR, už promotoriaus sekos buvo įterpta kumato operatoriaus *CuO* seka. Raiškos kasetė $P_{virE/CuO}$*::virE2,* pasitelkiant restrikcijos endonukleazes, buvo įterpta į virusinį vektorių pICH27566. Augalų agroinfiltracijos eksperimente, kai į augalų lapus buvo infiltruotas 150 µM kumine rūgštimi indukuotas agrobakterijų kamienas ICH011 (pNMDV437 ($P_{virE/CuO}$*::virE2*)) (1 lent.), augalų lapuose buvo matoma itin stipri ŽFB fluorescencija (7 pav.). Vis dėlto, infiltravus agrobakterijų suspensiją be induktoriaus, ant augalų lapų buvo matomas gana didelis ŽFB fluorescencijos taškų kiekis, taigi promotorius pasižymi didesniu pralaidumu nei promotoriai $P_{T5/CuO}$ ar $P_{tac/CuO}$. Promotoriaus aktyvumas negalėjo būti

tinkamai įvertintas β galaktozidazės aktyvumo tyrime, nes visiška promotoriaus indukcija galėjo būti pasiekta tik augalo audiniuose – natūralaus *virE* regiono indukcijai reikalingi papildomi su augalu susiję veiksniai.

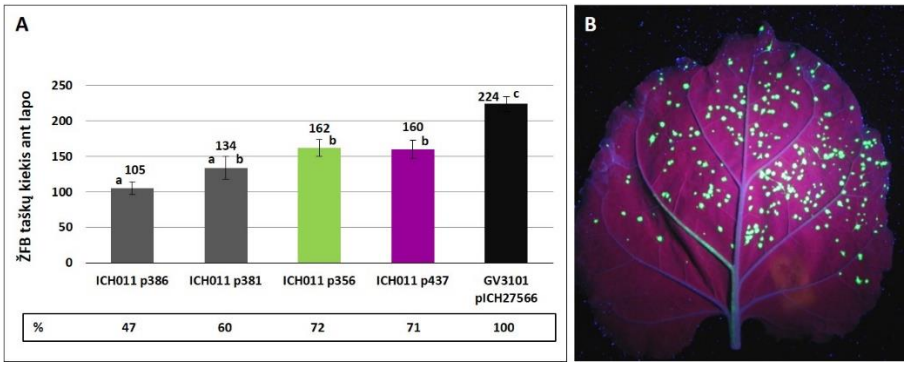


7 pav. $P_{virE/CuO}$ promotoriaus aktyvumo įvertinimas *A. tumefaciens* kamiene ICH011. Indukuoto promotoriaus raiška lėmė labai efektyvų T-DNR pernešimą, tačiau nepridėjus induktoriaus augalų lapuose taip pat buvo matomi ŽFB raiškos taškai; *lacZ* raiška nuo šio promotoriaus lėmė mažą β galaktozidazės aktyvumą (~ 90 MV) ir tik tada, kai į bakterijų suspensiją buvo pridėta 100 µM acetosiringono – *vir* regiono induktoriaus. *N. benthamiana* augalų lapai fotografuoti UV šviesoje 5 d.p.i. „+“ – indukcija su 150 µM kuminės rūgšties. „–“– neindukuota. „lt“ – kontrolinės agroinfiltracijos su *A. tumefaciens* kamienu GV3101, turinčiu virusinį vektorių su ŽFB raiškos kasete. β galaktozidazės aktyvumo tyrimų rezultatų reikšmės atitinka trijų nepriklausomų eksperimentų rezultatų vidurkį. Stulpelių viršuje nurodyti standartiniai nuokrypiai.

Kiekybinis augalų transfekcijos efektyvumo įvertinimas naudojant agrobakterijų kamienus su kumine rūgštimi indukuojamomis *virE2* raiškos sistemomis

Po preliminaraus efektyviausių *virE2* raiškos kasečių įvertinimo agroinfiltracijos ekperimentuose, toliau jų efektyvumas buvo analizuojamas didesnės apimties augalų transfekcijoje juos purškiant agrobakterijomis. Agropurškimo eksperimentuose buvo įvertintos *virE2* raiškos kasetės, turinčios promotorius $P_{T5/CuO}$, $P_{tac/CuO}$ ir $P_{virE/CuO}$. *VirE2* raiškos kasetes turinčių agrobakterijų atliekama T-DNR pernaša buvo lyginama su laukinio tipo T-DNR pernaša, kuri šiuose eksperimentuose buvo laikoma 100 % efektyvia. Agrobakterijų kamieno ICH011 (pNMDV386 ($P_{T5/CuO}$::*virE2*)) (1 lent.)

T-DNR pernaša siekė 47 % laukinio tipo pernašos. Tačiau raiškos sistemos, kuriose *virE2* raiška vyksta nuo promotorių $P_{tac/CuO}$ ir $P_{virE/CuO}$, pasižymėjo didesniu efektyvumu, kuris siekė net 71–72 % (8 pav.). Siekdami dar labiau padidinti T-DNR pernašos efektyvumą, kartu su *virE2* ekspresavome agrobakterijų virulentiškumo veiksnį *virE1*, kuris yra apibūdintas kaip šaperonas, apsaugantis VirE2 baltymus nuo agregacijos, taigi padidinantis baltymo VirE2 stabilumą, ar dalyvaujantis VirE2 transliacijoje[18]. VirE1 ir VirE2 koduojančios sekos buvo įterptos po $P_{T5/CuO}$ promotoriumi tokia pačia tvarka, kuria jos yra aptinkamos natūraliame *virE* operone, gautas agrobakterijų kamienas ICH011 (pNMDV381 ($P_{T5/CuO}$*::virE1, virE2*)) (1 lent.). Vis dėlto papildomai ekspresuojant VirE1 reikšmingo T-DNR pernašos efektyvumo padidėjimo užfiksuoti nepavyko (8 pav.).



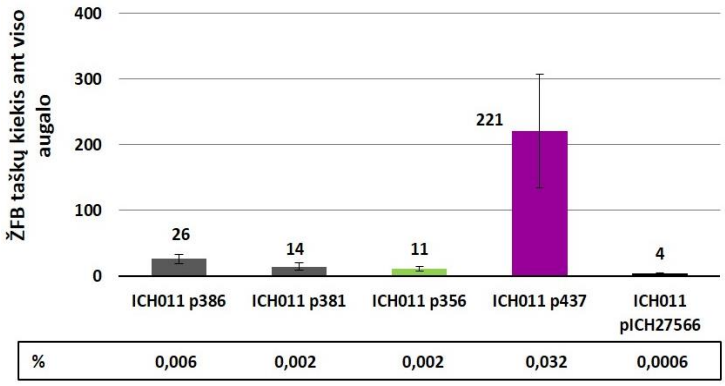
8 pav. ŽFB raiškos taškų skaičiaus įvertinimas po agropurškimo, pridėjus kuminės rūgšties. A. *N. benthamiana* augalai buvo purškiami agrobakterijų kamienais, turinčiais skirtingus vektorius: p386 atitinka pNMDV386 ($P_{T5/CuO}$*::virE2*), p381 – pNMDV381 ($P_{T5/CuO}$*::virE1, virE2*), p356 – pNMDV356 ($P_{tac/CuO}$*::virE2*), p437 – pNMDV437 ($P_{virE/CuO}$*::virE2*). Skirtingos spalvos nurodo skirtingus promotorius: $P_{T5/CuO}$ – pilka, $P_{tac/CuO}$ – žalia, $P_{virE/CuO}$ – violetinė, juoda spalva nurodo kontrolinį kamieną. ŽFB raiškos taškų skaičius kiekviename atskirame eksperimente buvo įvertinamas kaip procentinė ŽFB raiškos taškų skaičiaus, gauto augalus nupurškus kontroliniu kamienu GV3101, dalis (laikoma 100 %). Stulpelių viršuje nurodyti standartiniai nuokrypiai. **B.** Reprezentatyvi *N. benthamiana* augalo lapo, ant kurio matomi ŽFB raiškos taškeliai, nuotrauka.

Be promotorių aktyvumo, indukuojamai T-DNR pernašai gali turėti įtakos keli papildomi veiksniai. Kad vyktų T-DNR pernaša, induktorius turi būti infiltruojamas kartu su agrobakterijomis, todėl nustatytas skirtumas tarp

laukinio tipo ir indukuotos T-DNA pernašos efektyvumų gali būti nulemtas netolygaus induktoriaus pasiskirstymo ir patekimo į augalų lapų tarpuląsčius. Taip pat, kaip parodė vėliau atlikta *virE2* raiškos analizė TL-PGR metodu (12 pav.), chemiškai indukuota *virE2* raiška yra ankstyvesnė nei laukinio tipo *virE2* raiškos indukcija ir, greičiausiai, taip pat yra ankstyvesnė nei kitų virulentiškumo veiksnių raiškos pradžia (pavyzdžiui, šaperono VirE1 ir VirB/D4 transportavimo sistemos raiška, būtina VirE2 transportui į augalo ląstelę[19]), tai gali lemti mažesnį indukuotos pernašos efektyvumą, palyginti su laukinio tipo pernaša.

Kiekybinis augalų transfekcijos pralaidumo įvertinimas naudojant agrobakterijų kamienus su kumine rūgštimi indukuojamomis *virE2* raiškos sistemomis

Atlikus kiekybinį *virE2* raiškos sistemų efektyvumo įvertinimą T-DNR pernašoje iš *A. tumefaciens* į augalus, taip pat buvo svarbu nustatyti kiekybinį raiškos sistemų pralaidumą. Raiškos sistemų pralaidumui nustatyti agrobakterijų, turinčių *virE2* raiškos sistemas, suspensijos buvo skiedžiamos 10 kartų ir purškiamos ant *N. benthamiana* augalų nepridėjus induktoriaus (T-DNR pernašos efektyvumui įvertinti, taikant agropurškimą, agrobakterijos buvo skiedžiamos 1000 kartų). Po agropurškimo ant lapų buvo suskaičiuoti tik keli ŽFB fluorescencijos taškai. Neindukuotos T-DNR pernašos dažnis buvo itin mažas ir tesiekė 0,002–0,032 % laukinio tipo T-DNR pernašos (9 pav.). Rutiniškai naudojama bakterijų koncentracija agropurškimo eksperimentuose paprastai yra 10^7–10^6 kfv/ml, t. y. 1–2 eilėmis mažesnė už koncentraciją, naudotą pralaidumo vertinimo eksperimentuose. Kadangi agrobakterijų koncentracija gamtoje yra nedidelė, antrinių transformacijų, galimų dėl promotoriaus pralaidumo, tikimybė būtų labai maža. Tam tikrą T-DNR pernašos pralaidumą (0,0006 %) parodė ir kontrolinis agrobakterijų kamienas ICH011 (pICH27566) (9 pav.). Kamieno pralaidumas patvirtino anksčiau aprašytą nevisišką T-DNR pernašos funkcijos praradimą *virE2* deleciją turinčiuose agrobakterijų kamienuose[10].



9 pav. ŽFB raiškos taškų skaičiaus įvertinimas po agropurškimo nepridėjus kuminės rūgšties. *N. benthamiana* augalai buvo purškiami agrobakterijomis, turinčiomis skirtingus vektorius: p386 atitinka pNMDV386 ($P_{T5/CuO}$*::virE2*), p381 – pNMDV381 ($P_{T5/CuO}$*::virE1, virE2*), p356 – pNMDV356 ($P_{tac/CuO}$*::virE2*), p437 – pNMDV437 ($P_{virE/CuO}$*::virE2*). Skirtingos spalvos nurodo skirtingus promotorius kamienuose: $P_{T5/CuO}$ – pilka, $P_{tac/CuO}$ – žalia, $P_{virE/CuO}$ – violetinė, juoda spalva nurodo kontrolinį kamieną. ŽFB raiškos taškų skaičius kiekviename atskirame eksperimente buvo įvertinamas kaip procentinė ŽFB raiškos taškų skaičiaus, gauto augalus nupurškus kontroliniu kamienu GV3101, dalis (laikoma 100 %). Stulpelių viršuje nurodyti standartiniai nuokrypiai.

T-DNR pernašos iš agrobakterijų į augalus kontrolės sistema, paremta indukuojamu bakteriniu toksino-antitoksino moduliu

Tik skirtingų biosaugumo strategijų derinys gali sumažinti nenorimą T-DNR paplitimą gamtoje, taikant laikiną augalų transfekciją. Biosaugumo strategijos išaktyvavimas gali įvykti dėl horizontalaus genų perdavimo tarp mikroorganizmų, atsitiktinės arba kryptingos mutagenezės. Todėl sukūrėme papildomą reguliuojamos T-DNR pernašos mechanizmą, pagrįstą bakteriniu II tipo toksino-antitoksino (TA) moduliu *pemIK*. Vis dėlto, planuojant eksperimentus, mums labai trūko detalesnės informacijos apie TA sistemų reguliavimą ir ypač informacijos apie agrobakterijų TA sistemas. Bakterijų TA modulių toksinai yra sėkmingai naudojami biosaugumo sistemoms kurti kituose mikroorganizmuose. Pavyzdžiui, buvo parodyta, kad bakterijų TA sistemos veikia GM mielių bioizoliavimo sistemose. *E. coli RelBE* ir *Kis-Kid* (*pemI-pemK* homologai) TA moduliai buvo įterpti į genetiškai modifikuotas *Saccharomyces cerevisiae* mieles[20, 21]. Vėliau buvo sukurtos alternatyvios *E. coli* izoliavimo sistemos („Deadman“ ir „Passcode“), pagrįstos atitinkamai

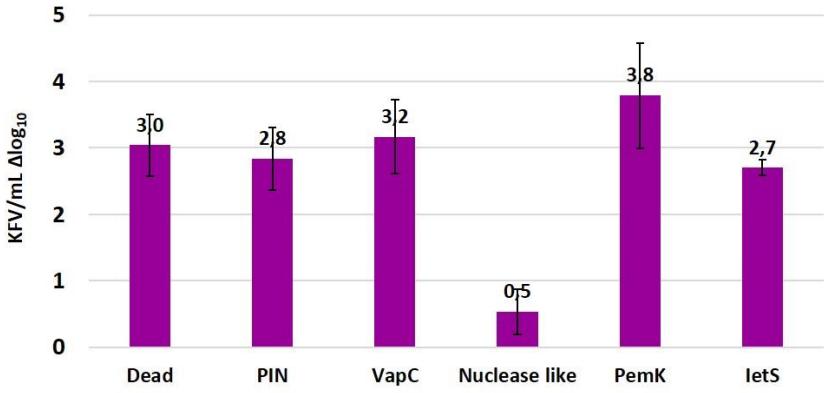
toksinų *mazF* ir *ccdB* veikimu[22]. Šie darbai parodė didelį bakterinių TA sistemų potencialą prokariotų, žemesniųjų eukariotų ar net genetiškai modifikuotų augalų plitimo kontrolės strategijose (buvo parodyta, kad YoeB toksino, priklausančio *Streptococcus pneumoniae* TA moduliui yefM-yoeB, raiška yra toksiška *Arabidopsis thaliana* augalų ląstelėms[23]).

A. tumefaciens toksino-antitoksino modulių paieška

Sėkmingo TA sistemų pritaikymo kitų mikroorganizmų bioizoliacijai pavyzdžiai paskatino mus patikrinti, ar TA sistemos galėtų būti naudojamos kontroliuoti T-DNR pernašą iš genetiškai modifikuotų *A. tumefaciens* į augalus. Pirminę potencialių agrobakterijų TA modulių paiešką bei atranką atliko Šarūnas Paškevičius, naudodamasis internetiniu įrankiu „RASTA-bacteria“. Įrankis atrenka galimus TA modulius pasirinkto organizmo genome pagal įrankio autorių nustatytus kriterijus[24]. Iš įrankyje pateikto sąrašo pagal didžiausios tikimybės principą buvo pasirinkti penki potencialūs *A. tumefaciens* genome esantys TA moduliai – trys esantys žiedinėje agrobakterijų chromosomoje (jų toksinų genai *pemK, vapC, PIN*), vienas esantis linijinėje chromosomoje (toksino genas *dead*) ir vienas esantis Ti plazmidėje (toksino genas *Nuclease-like*). Papildomai toksinas *ietS*, priklausantis Ti plazmidėje esančiam moduliui, buvo pasirinktas remiantis publikuotais duomenimis[25].

Toksinų antibakterinio aktyvumo įvertinimas

TA moduliams priklausančių toksinų aktyvumui įvertinti toksinų genai buvo padauginti nuo genominės *A. tumefaciens* bakterijų DNR PGR metodu ir įterpti po stipriais kumine rūgštimi indukuojamais promotoriais virusiniuose dvinariuose vektoriuose, sukonstruoti vektoriai transformuoti į *A. tumefaciens* kamieną GV3101. Vektorių konstravimas buvo atliktas Justinos Stankevičiūtės jos magistro darbo metu. 150 µM kumine rūgštimi indukuota agrobakterijų toksinų PemK, Dead, VapC, PIN ir IetS raiška sumažino bakterijų KFV skaičių suspensijose 2,7–3,8 eilėmis. Nuclease-like toksino antibakterinis aktyvumas buvo daug mažesnis – indukuota toksino raiška sumažino bakterijų KFV skaičių tik 0,5 eilės (10 pav.). Taigi penki iš šešių įvertintų toksinų buvo aktyvūs prieš *A. tumefaciens* bakterijas.



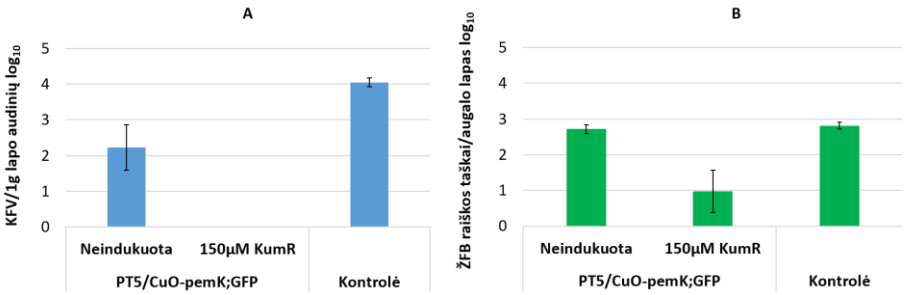
10 pav. Toksinų gebėjimo nužudyti *A. tumefaciens* įvertinimas. *A. tumefaciens* GV3101 vienos nakties kultūros su plazmidėmis, turinčiomis toksinų raiškos kasetes, buvo skiedžiamos iki OT_{600} ~ 0,1–0,2. Kiekviena kultūra buvo padalyta į dvi suspensijas – į suspensiją, į kurią pridėta 150 µM kuminės rūgšties, ir suspensiją be induktoriaus. Suspensijos buvo inkubuojamos 28 °C temperatūroje jas purtant 220 apm greičiu 4 val., atliekami serijiniai suspensijų skiedimai ir bakterijos išsėjamos ant selektyvios agarizuotos LB terpės lėkštelių be induktoriaus (kiekvienos suspensijos po 25 µL). KFV buvo skaičiuojami ant lėkštelių po 48 val. inkubacijos 28 °C temperatūroje. Kiekvieno toksino veiksmingumas buvo vertinamas naudojant formulę: $\Delta\log_{10} = \log_{10}$ (KFV/mL neindukuotos kultūros) – $\log_{10}$ (KFV/mL indukuotos kultūros). Pateikti duomenys yra trijų nepriklausomų eksperimentų vidurkis. Stulpelių viršuje nurodyti standartiniai nuokrypiai.

Toksino PemK efektyvumo įvertinimas agropurškimo eksperimentuose

Patvirtinus toksinų aktyvumą prieš agrobakterijas, toliau buvo įvertinta galimybė panaudoti toksinus reguliuoti laikinos raiškos sistemoms, kai agrobakterijos yra purškiamos ant *N. benthamiana* augalų. Šie eksperimentai buvo atlikti Justinos Stankevičiūtės jos magistro darbo metu. Agrobakterijų kamienas GV3101 (pNMDV371 ($P_{T5/CuO}$::*pemK*), pICH27566) ir kontrolinis kamienas GV3101 (pICH27566) buvo naudojami agropurškimo eksperimentuose (1 lent.). *PemK* raiškos indukcijai į agrobakterijų suspensiją buvo pridedama 150 µM kuminės rūgšties. Praėjus septynioms dienoms po agropurškimo, ant augalų lapų buvo suskaičiuojami ŽFB fluorescencijos taškai, iš tų pačių lapų išskiriamos agrobakterijos, įvertinamas agrobakterijų KFV skaičius. Iš vieno gramo šviežių *N. benthamiana* augalų lapų, purkštų

kontroliniu kamienu, buvo išskirtos 4,1 eilės bakterijų. O iš augalų lapų, kurie buvo purkšti su toksino raiškos kasetę turinčiu agrobakterijų kamienu pridėjus induktoriaus, gyvybingų agrobakterijų išskirta nebuvo. Net ir nepridėjus induktoriaus, *pemK* toksiną turinčių gyvybingų bakterijų skaičius buvo šimtą kartų mažesnis nei kontrolinio kamieno (11 pav.). Tokį rezultatą galėjo lemti pralaidi *pemK* toksino raiška nuo $P_{T5/CuO}$ promotoriaus.

ŽFB raiškos taškų skaičius ant *N. benthamiana* augalo lapų buvo panašus tiek nupurškus kontroliniu kamienu (2,8 eilės), tiek neindukuotu *pemK* raiškos kasetę turinčiu kamienu (2,7 eilės). Indukavus *pemK* raišką, ŽFB fluorescencijos taškų skaičius labai sumažėjo ir tesiekė 1 eilę (11 pav.). Taigi indukuota toksino *pemK* raiška efektyviai nužudė didžiąją dalį rekombinantinių agrobakterijų dar prieš joms pradedant T-DNR pernašos procesą.

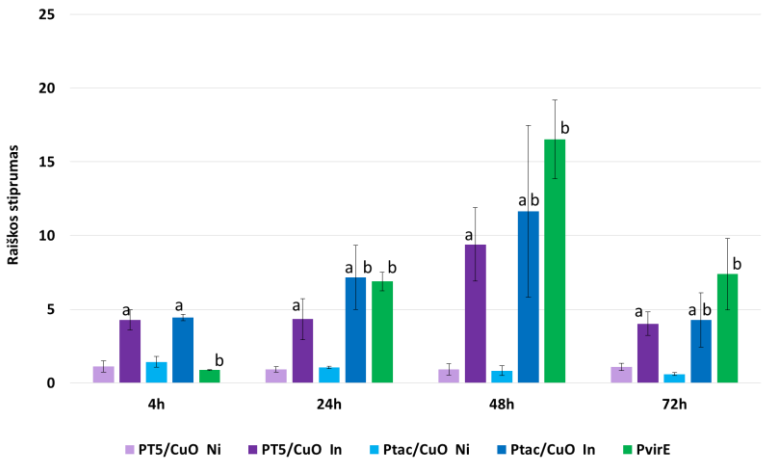


11 pav. Toksino PemK aktyvumo prieš agrobakterijas (A) ir ŽFB raiškos taškų skaičiaus įvertinimas (B) agropurškimo eksperimentuose. *N. benthamiana* augalai buvo purškiami į agrobakterijų suspensiją pridėjus arba nepridėjus kuminės rūgšties. Purškimui naudoti $P_{T5/CuO}$*::pemK* raiškos kasetę turintis GV3101 (pICH27566, pNMDV371) kamienas ir kontrolinis kamienas GV3101 (pICH27566). ŽFB raiškos taškai buvo skaičiuojami ant nupurkštų lapų 7-ą dieną po purškimo (dpp). Tada tie patys lapai buvo sutrinti skystame azote, iš lapų audinių išskirtos agrobakterijos, jos išsėtos ant agarizuotos terpės. Po dviejų parų augimo apskaičiuotas bakterijų KFV skaičius 1-am gramui šviežių lapo audinių. Duomenys yra trijų nepriklausomų eksperimentų vidurkis. Stulpelių viršuje nurodyti standartiniai nuokrypiai.

Reguliuojamų agrobakterijų toksino-antitoksino moduliu paremtų raiškos sistemų konstravimas bei efektyvumo įvertinimas agropurškimo eksperimentuose

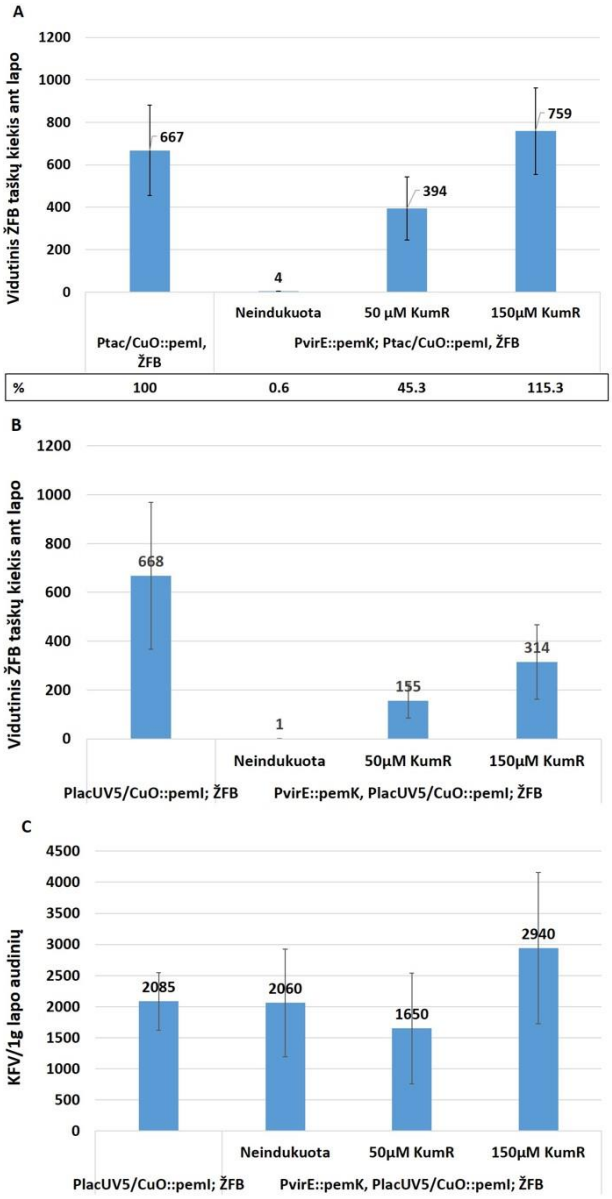
Pirmiau aprašyto eksperimento rezultatai parodė, kad kumine rūgštimi indukuota toksino *pemK* raiška nužudo daugelį agrobakterijų anksčiau, nei jos gali atlikti augalo ląstelių transfekciją. Taigi, norint agrobakterijoms suteikti

daugiau laiko T-DNR pernašai, toksino raiška arba jo veikimas turėtų būti atidėtas. Turėdami tikslą pavėlinti toksino *pemK* veikimą laike ir sukonstruoti efektyvią reguliuojamos raiškos sistemą, nusprendėme į ją įterpti modulio *pemIK* antitoksiną *pemI*. Antitoksinas blokuoja toksino veikimą prie jo stipriai prisijungdamas[26, 27]. Toksiną ir antitoksiną koduojančias sekas siekėme įterpti po indukuojamais, tačiau skirtingai reguliuojamais promotoriais, kurie aktyvintų ankstesnę antitoksino *pemI* raišką ir vėlesnę, tačiau stiprią *pemK* raišką. Ieškodami tinkamos promotorių poros mūsų raiškos sistemai, atlikome kumine rūgštimi indukuojamų promotorių $P_{T5/CuO,}$ $P_{tac/CuO}$ ir natūralaus agrobakterijų *virE* regiono promotoriaus P_{virE} indukcijos kinetikos analizę TL-PGR metodu. Siekiant nustatyti *virE2* raiškos kitimą laike, *N. benthamiana* augalai buvo infiltruojami su agrobakterijų kamienais ICH011 (pNMDV386 ($P_{T5/CuO}$::*virE2*)), ICH011 (pNMDV356 ($P_{tac/CuO}$::*virE2*)) ir GV3101 (pICH27566) pridėjus ar nepridėjus induktoriaus. Praėjus 4, 24, 48 ir 72 val. po agroinfiltracijos, augalų lapai buvo surenkami, iš jų išskiriama bendroji RNR, atliekamos TL-PGR reakcijos naudojant *virE2* genui specifiškus pradmenis. Praėjus 4 val. po agroinfiltracijos, *virE2* raiška nuo kumine rūgštimi indukuotų promotorių buvo penkis kartus stipresnė nei bazinis raiškos lygis nuo neindukuotų promotorių. *VirE2* raiška nuo natūralaus *virE* regiono promotoriaus šiame laiko taške vis dar buvo labai silpna. Vis dėlto, praėjus 24 val. po agroinfiltracijos, *virE2* raiška nuo promotoriaus P_{virE} išaugo septynis kartus ir pasiekė panašų stiprumą kaip ir nuo kumine rūgštimi indukuotų promotorių. Po 48 val. ir 72 val. raiška nuo natūralaus promotoriaus išliko lygiavertiška ar šiek tiek stipresnė nei nuo kumine rūgštimi indukuojamų promotorių. Raiška nuo visų trijų promotorių pasiekė piką po 48 val. ir susilpnėjo praėjus 72 val. po agroinfiltracijos (12 pav.).



12 pav. *VirE2* raiškos nuo kumine rūgštimi indukuojamų promotorių $P_{T5/CuO}$ ir $P_{tac/CuO}$ bei natūralaus *virE* operono promotoriaus P_{virE} analizė TL-PGR metodu. *A. tumefaciens* ICH011 (*ΔvirE2*) kamienai, turintys vektorių pNMDV386 ($P_{T5/CuO}$*::virE2*) arba pNMDV356 ($P_{tac/CuO}$*::virE2*), ir kamienas GV3101 (pICH27566) buvo agroinfiltruojami į *N. benthamiana* augalus. Infiltruoti augalų lapai buvo nuskinami praėjus 4, 24, 48, 72 val. po agroinfiltracijos. *VirE2* raiškos stiprumas įvertintas TL-PGR metodu. Ni – *virE2* išraiška nuo neindukuotų $P_{T5/CuO}$ ir $P_{tac/CuO}$ promotorių, In – *virE2* išraiška nuo 150 μM kumine rūgštimi indukuotų $P_{T5/CuO}$ ir $P_{tac/CuO}$ promotorių, P_{virE} – *virE2* raiška nuo laukinio tipo P_{virE} promotoriaus agrobakterijų kamiene GV3101. Duomenys yra trijų nepriklausomų eksperimentų vidurkis. Stulpelių viršuje nurodyti standartiniai nuokrypiai. Žymėjimas a ir b nurodo verčių skirtumo reikšmingumą. a ir b grupėse esančios vertės nesiskiria tarpusavyje reikšmingai, tačiau tarp grupių verčių skirtumai yra reikšmingi.

Kadangi natūralaus P_{virE} promotoriaus indukcija buvo vėlesnė nei kumine rūgštimi indukuojamų promotorių, toksiną *pemK* koduojančią seką įterpėme už promotoriaus P_{virE}, o antitoksiną *pemI* koduojančią seką už kumine rūgštimi indukuojamų promotorių, stipresne raiškos indukcija pasižyminčio $P_{tac/CuO}$ ir silpnesne indukcija pasižyminčio $P_{lacUV5/CuO}$. Raiškos kasetės buvo įterptos į vektorius pICH27655 ir pNDC, priklausančius skirtingoms nesuderinamumo grupėms (angl. *incompatibility groups*). Vektoriai buvo kotransformuoti į *pemIK* regiono iškritą turintį agrobakterijų kamieną GV3101Δ*pemIK*. Gauti kamienai GV3101Δ*pemIK* (P_{virE}::*pemK*, $P_{tac/CuO}$::*pemI)* ir GV3101Δ*pemIK* (P_{virE}::*pemK*, $P_{lacUV5/CuO}$::*pemI*) vakuumu infiltruoti į *N. benthamiana* augalus, T-DNR pernašos reguliacija įvertinta skaičiuojant ŽFB raiškos taškus augalų lapuose. T-DNR pernaša faktiškai visiškai nevyko abiejuose agrobakterijų kamienuose, kai kamienai buvo infiltruojami be induktoriaus (vidutiniškai buvo registruojami tik 4 ŽFB raiškos taškai viename augalo lape su $P_{tac/CuO}$ ir tik 1 raiškos taškas su $P_{lacUV5/CuO}$). Tačiau ŽFB raiškos taškų skaičius padidėjo į agrobakterijų suspensiją pridėjus induktoriaus ir buvo 2,4–2,5 karto didesnis infiltruojant kamieną, turintį raiškos kasetę $P_{tac/CuO}$::*pemI,* nei kamieną su $P_{lacUV5/CuO}$::*pemI.* Infiltravus kamieną GV3101Δ*pemIK* (P_{virE}::*pemK*, $P_{tac/CuO}$::*pemI*) su 150 μM induktoriaus, vidutiniškai buvo gauti 759 ŽFB raiškos taškai ant augalo lapo. O infiltravus kontrolinį kamieną, turintį tik raiškos kasetę $P_{tac/CuO}$::*pemI,* vidutiniškai buvo suskaičiuoti 667 ŽFB raiškos taškai (13 pav.). Taigi kumine rūgštimi indukuota T-DNR pernaša buvo ne mažiau efektyvi ar efektyvesnė nei laukinio tipo pernaša.



13 pav. ŽFB raiškos taškų ir agrobakterijų KFV skaičiaus įvertinimas po agroinfiltracijos naudojant agrobakterijų kamienus GV3101*ΔpemIK* (P_{virE}*::pemK*, $P_{tac/CuO}$*::pemI, ŽFB*) ir GV3101*ΔpemIK* (P_{virE}*::pemK*, $P_{lacUV5/CuO}$*::pemI, ŽFB*). A**.** Kamienas GV3101*ΔpemIK* (pNMDV515, pNMDV535), turintis raiškos kasetes $P_{tac/CuO}$*::pemI* ir P_{virE}*::pemK*, buvo

vakuumu infiltruotas į *N. benthamiana* augalų lapus, pridėjus 50 μM, 150 μM kuminės rūgšties ar be induktoriaus. Kamienas GV3101 (pNMDV535), turintis tik raiškos kasetę $P_{tac/CuO}$*::pemI, GFP* buvo infiltruotas teigiamai T-DNR pernašos kontrolei. **B.** Kamienas GV3101*ΔpemIK* (pNMDV515, pNMDV507), turintis raiškos kasetes $P_{lacUV5/CuO}$*::pemI* ir P_{virE}*::pemK*, vakuumu infiltruotas į *N. benthamiana* augalų lapus, pridėjus 50 μM, 150 μM kuminės rūgšties ar be induktoriaus. Kamienas GV3101 (pNMDV507), turintis tik raiškos kasetę $P_{lacUV5/CuO}$*::pemI, GFP* buvo infiltruotas teigiamai T-DNR pernašos kontrolei. ŽFB raiškos taškai suskaičiuoti ant lapų praėjus 6-ioms dienoms po agroinfiltracijos. **C.** Agrobakterijų KFV skaičiaus įvertinimas po agroinfiltracijos. Lapai buvo pasveriami ir sutrinami skystame azote. Į sutrintus audinius pridedama sterilaus distiliuoto vandens, paruošiama lapų audinių suspensija. Suspensija filtruojama, atliekami serijiniai skiedimai ir išsėjama ant lėkštelių su LB terpe su antibiotikais. Agrobakterijų kiekis vienam gramui lapų masės buvo suskaičiuojamas ant lėkštelių po dviejų naktų auginimo 28 °C inkubatoriuje. Duomenys yra trijų nepriklausomų eksperimentų vidurkis. Stulpelių viršuje nurodyti standartiniai nuokrypiai.

Toksino PemK raiška nuo promotoriaus P_{virE}, kai antitoksino raiška nebuvo indukuota, bakterijų skaičiaus ant augalų lapų reikšmingai nesumažino. KFV skaičius nekoreliavo su ŽFB taškelių skaičiumi ir skirtingų eksperimentinių grupių buvo panašus (13C pav.). Manome, kad tokį rezultatą lėmė tai, kad tik maža dalis ant augalo lapo patekusių agrobakterijų yra indukuojamos T-DNR pernašai, taigi dauguma agrobakterijų išvengia P_{virE} promotoriaus indukcijos ir toksiško PemK poveikio. Tačiau agrobakterijų ir augalų sąveikos indukuota toksino *pemK* raiška nuo P_{virE} ir antitoksino *pemI* raiška nuo kumine rūgštimi indukuojamo promotoriaus $P_{tac/CuO}$ lemia efektyvią agrobakterijų tarpininkaujamos augalų transfekcijos kontrolę. T-DNR perdavimas šioje raiškos sistemoje galimas tik trumpą laiką po agroinfiltracijos, kol agrobakterijoms yra prieinama suspensijoje esanti kuminė rūgštis – antitoksino raiškos induktorius. Nesant induktoriaus, augalo ląstelių transfekcija bus blokuojama kiekvieną kartą, agrobakterijoms aktyvuojant T-DNR pernašos mechanizmą, nes tuo pat metu bus indukuojama ir toksiškojo *pemK* raiška nuo P_{virE} promotoriaus. Taigi vėlesni dirvožemyje likusių bakterijų bandymai pernešti T-DNR augalams turėtų lemti bakterijų žūtį.

Sukurtos raiškos sistemos, paremtos indukuojama *virE2* geno ar toksino *pemK* ir antitoksino *pemI* genų raiška, skirtos kontroliuoti T-DNR pernašai iš agrobakterijų į augalus, galėtų būti naudojamos ir su kitais efektyviais TA moduliais, taip pat su kitomis sukurtomis biosaugumo strategijomis, tokiomis kaip daugybinė metabolinė ar sintetinė auksotrofija. Be abejonės, kaip ir bet

kurių kitų technologijų atveju, niekada negalime tikėtis absoliutaus saugumo, tačiau kruopštus darbas, siekiant sumažinti įtaką natūraliai aplinkai ir žmonių sveikatai iki mažiausios įmanomos, yra būtinas ir vertingas, siekiant pritaikyti naujus metodus rekombinantinių baltymų produkcijoje ir agrobiotechnologijoje.

IŠVADOS

- Kumine rūgštimi indukuojami promotoriai $P_{T5/CuO}$, $P_{tac/CuO}$, $P_{lacUV5/CuO}$ buvo efektyviai reguliuojami ir stipriai indukuojami *A. tumefaciens* bakterijose tiek β galaktozidazės aktyvumo matavimo, tiek agroinfiltracijos eksperimentuose, hibridinis promotorius $P_{virE/CuO}$ buvo labai aktyvus ir griežtai reguliuojamas agroinfiltracijos eksperimentuose.

- Reguliuojama agrobakterijų virulentiškumo veiksnio *virE2* raiška nuo kumine rūgštimi indukuojamų promotorių *ΔvirE2 A. tumefaciens* bakterijose lemia griežtą T-DNR pernašos iš agrobakterijų į augalus kontrolę: 72 % laukinio tipo efektyvumo siekiančią indukuotą T-DNR pernašą ir tik 0,002–0,03 % siekiantį pralaidumą be induktoriaus.

- *A. tumefaciens* toksinai PIN, VapC, PemK, IetS ir Dead, kurių raiška vyksta nuo kumine rūgštimi indukuojamų promotorių, yra funkcionalūs ir geba sumažinti *A. tumefaciens* KFV skaičių *in vitro* kultūroje 2,7–2,8 eilėmis.

- Agrobakterijų kamieno, turinčio toksino ir antitoksino raiškos kasetes P_{virE}::*pemK* ir $P_{tac/CuO}$::*pemI*, T-DNR pernaša yra griežtai kontroliuojama kumine rūgštimi, jos efektyvumas panašus į laukinio tipo pernašos efektyvumą.

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CURRICULUM VITAE (*lietuviškai*)

Vardas, pavardė	Erna Denkovskienė
Gimimo data:	1985 11 07
Darbovietės adresas:	UAB „Nomads“, Geležinio Vilko g. 29A, Vilnius, Lietuva
Mobilusis tel.	+ 370 620 39 370
El. paštas:	erna.denkovskiene@gmail.com

Išsilavinimas

2010 10–2014 10	Biochemijos doktorantūros studijos. Eukariotų genų inžinerijos skyrius, Biotechnologijos institutas, Vilniaus universitetas.
2008 09–2010 06	Biochemijos magistro laipsnis. Chemijos fakultetas, Vilniaus universitetas. Magistro darbo tema: *Brassica napus* transkripcijos veiksnių *BnICE, BnCBF3* ir *BnMYB15* genų raiškos analizė.
2004 09–2008 06	Biochemijos bakalauro laipsnis. Chemijos fakultetas, Vilniaus universitetas. Bakalauro darbo tema: *Brassica napus* šalčio atsako genų *BnICE, BnCBF3* ir *BnMYB15* raiškos analizė.

Mokslinio darbo patirtis:

Nuo 2010 09	Mokslo darbuotoja įmonėje UAB „Nomads“. Dalyvavimas moksliniuose projektuose: 1. „Intelektas LT“ priemonės finansuojamas projektas „Antimikrobiniai baltymai maisto saugai“ (2013–2015 m.). 2. „Intelektas LT“ priemonės finansuojamas projektas „Antimikrobinis preparatas *P. aeruginosa* infekcijų gydymui“ (2016–2018 m.). 3. „Intelektas LT“ priemonės finansuojamas projektas „Antimikrobinis preparatas *Klebsiella* infekcijų gydymui“ (2018–2020 m.). Rezultatai paskelbti publikacijoje:

Denkovskienė, Erna, Šarūnas Paškevičius, Audrius Misiūnas, Benita Stočkūnaitė, Urtė Starkevič, Astra Vitkauskienė, Simone Hahn-Löbmann, Steve Schulz, Anatoli Giritch, Yuri Gleba, and Aušra Ražanskienė. 2019. „Broad and Efficient Control of *Klebsiella* Pathogens by Peptidoglycan-Degrading and Pore-Forming Bacteriocins Klebicins“. *Scientific Reports* 9 (1):15422.

Paskelbta patento paraiška:

WO2020245376: **Erna Denkovskienė**, Audrius Misiūnas, Aušra Ražanskienė. Klebicins for the control of *Klebsiella.* 2020.

4. „Eksperimentas LT“ priemonės projektas „Naujos kartos antimikrobinio preparato sukūrimas *Klebsiella pneumoniae* ir *Pseudomonas aeruginosa* infekcijoms gydyti“ (2020–2022 m.).

2012 09–2015 07 Jaunesnioji mokslo darbuotoja. Eukariotų genų inžinerijos skyrius, Biotechnologijos institutas, Vilniaus universitetas. Dirbau Lietuvos mokslo tarybos finansuotame projekte „Antocianinų biosintezės kelio genų raiška sodo augaluose“ (2012–2015 m.). Rezultatai paskelbti publikacijose:

Starkevič, Pavel, Jurgita Paukštytė, Vaiva Kazanavičiūtė, **Erna Denkovskienė**, Vidmantas Stanys, Vidmantas Bendokas, Tadeušas Šikšnianas, Aušra Ražanskienė, and Raimundas Ražanskas. 2015. „Expression and Anthocyanin Biosynthesis-Modulating Potential of Sweet Cherry (Prunus Avium L.) MYB10 and BHLH Genes“ edited by C. Xu. PLOS ONE 10 (5):e0126991.

Starkevič, Pavel, Aušra Ražanskienė, Urtė Starkevič, Vaiva Kazanavičiūtė, **Erna Denkovskienė**, Vidmantas Bendokas, Tadeušas Šikšnianas, Rytis Rugienius, Vidmantas Stanys, and Raimundas Ražanskas. 2020. „Isolation and Analysis of Anthocyanin Pathway Genes from *Ribes* Genus Reveals *MYB* Gene with Potent Anthocyanin-Inducing Capabilities“. *Plants* 9 (9):1078.

2008 01–2010 06	Laborantė. Eukariotų genų inžinerijos skyrius, Biotechnologijos institutas, Vilniaus universitetas.
2007 03–2008 01	Studentė praktikantė. Eukariotų genų inžinerijos skyrius, Biotechnologijos institutas, Vilniaus universitetas.

CURRICULUM VITAE (*In English*)

First name/Surname:	Erna Denkovskienė
Date of birth:	07/11/1985
Work address:	Nomads UAB, Geležinio Vilko g. 29A, Vilnius, Lithuania
Mobile telephone:	+370 620 39 370
E-mail:	erna.denkovskiene@gmail.com

Education

2010 10–2014 10	PhD studies in Biochemistry. Department of Eukaryote Gene Engineering, Institute of Biotechnology, Vilnius University.
2008 09–2010 06	Master degree in Biochemistry. Faculty of Chemistry, Vilnius University. Master degree thesis: Expression analysis of *Brassica napus* transcription factors *BnICE*, *BnCBF3* and *BnMYB15*.
2004 09–2008 06	Bachelor degree in Biochemistry**.** Faculty of Chemistry, Vilnius University. Bachelor degree thesis: Expression Analysis of *Brassica napus* Cold response Genes *BnCBF3*, *BnICE* and *BnMYB15*.

Work experience:

2010 09 (present)	Researcher at Nomads UAB. Participation in research projects: 1. The project "Antimicrobial proteins for food safety" financed by the "Intellect LT" measure (2013–2015). 2. The project "Antimicrobial substance for treatment of *P. aeruginosa* infections" financed by the "Intellect LT" measure (2016–2018). 3. The project "Antimicrobial substance for treatment of *Klebsiella* infections" financed by the "Intellect LT" measure (2018–2020). The results are published in the publication:

Denkovskienė, Erna, Šarūnas Paškevičius, Audrius Misiūnas, Benita Stočkūnaitė, Urtė Starkevič, Astra Vitkauskienė, Simone Hahn-

Löbmann, Steve Schulz, Anatoli Giritch, Yuri Gleba, and Aušra Ražanskienė. 2019. "Broad and Efficient Control of *Klebsiella* Pathogens by Peptidoglycan-Degrading and Pore-Forming Bacteriocins Klebicins". *Scientific Reports* 9(1):15422.

Published patent application:

WO2020245376: **Erna Denkovskiene**, Audrius Misiunas, Aušra Ražanskiene. Klebicins for the control of *Klebsiella*. 2020.

4. The project "Development of a new generation antimicrobial substance for treatment of *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* infections" financed by "Experiment LT" measure (2020–2022).

2012 09–2015 07 Junior researcher at Department of Eukaryote Gene Engineering, Institute of Biotechnology, Vilnius University. Worked in a project "Expression of anthocyanin biosynthesis genes in garden plants" financed by Research council of Lithuania (2012–2015). The results are published in two publications:

Starkevič, Pavel, Jurgita Paukštytė, Vaiva Kazanavičiūtė, **Erna Denkovskienė**, Vidmantas Stanys, Vidmantas Bendokas, Tadeušas Šikšnianas, Aušra Ražanskienė, and Raimundas Ražanskas. 2015. "Expression and Anthocyanin Biosynthesis-Modulating Potential of Sweet Cherry (Prunus Avium L.) MYB10 and BHLH Genes" edited by C. Xu. PLOS ONE 10(5):e0126991.

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2008 01–2010 06	Laboratory assistant at Department of Eukaryote Gene Engineering, Institute of Biotechnology, Vilnius University**.**
2007 03–2008 01	Student trainee at Department of Eukaryote Gene Engineering, Institute of Biotechnology, Vilnius University**.**

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1. Denkovskienė, Erna, Šarūnas Paškevičius, Stefan Werner, Yuri Gleba, and Aušra Ražanskienė. 2015. "Inducible Expression of *Agrobacterium* Virulence Gene *VirE2* for Stringent Regulation of T-DNA Transfer in Plant Transient Expression Systems". *Molecular Plant-Microbe Interactions*® 28(11):1247–55.

2. Denkovskienė, Erna, Šarūnas Paškevičius, Justina Stankevičiūtė, Yuri Gleba, and Aušra Ražanskienė. 2020. "Control of T-DNA Transfer from *Agrobacterium tumefaciens* to Plants Based on an Inducible Bacterial Toxin-Antitoxin System". *Molecular Plant-Microbe Interactions*® 33(9):1142–49.

CONFERENCE PRESENTATIONS

1. Erna Denkovskienė, Šarūnas Paškevičius, Stefan Werner, Anatoli Giritch, Aušra Ražanskienė, "Inducible Expression of *Agrobacterium* Virulence Gene *VirE2* for Stringent Regulation of T-DNA Transfer in Biosafe Plant Transient Expression Systems". 2014.07.13-16. "16th European Congress on Biotechnology", Edinburgh, Scotland.

2. Erna Denkovskienė, Šarūnas Paškevičius, Stefan Werner, Anatoli Giritch, Aušra Ražanskienė "Chemically regulated *Agrobacterium* T-DNA transfer for biosafe transient plant transfection". 2014.09.12. "Life Sciences Baltics 2014", Vilnius, Lithuania.

NOTES

Vilnius University Press
9 Saulėtekio Ave., Building III, LT-10222 Vilnius
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