Control of T-DNA Transfer from *Agrobacterium tumefaciens* to Plants Based on an Inducible Bacterial Toxin-Antitoxin System

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High-value pharmaceutical products are already successfully produced in contained facilities using *Agrobacterium*-mediated transient transformation of plants. However, transfection methods suitable for open field applications are still desirable as a cheaper alternative. Biosafety concerns related to the use of recombinant *agrobacteria* in an industrial transfection process include possible transformation or transfection of unintended hosts or spread of the genetically modified *agrobacteria* in the environment. In this paper, we explored a novel biocontrol approach resulting in greater biosafety of the transient expression process in plants. Our proposed solution involves inducible expression of *Agrobacterium tumefaciens* toxin PemK and antitoxin PemI that provides for strictly regulated T-DNA transfer from *agrobacteria* to plants. We also identified several other toxins from putative *Agrobacterium* toxin-antitoxin modules and demonstrate their potential usefulness in the control of *Agrobacterium tumefaciens* as a DNA vector.

Keywords: biocontrol, toxin-antitoxin system, transient expression

Agrobacterium tumefaciens, a soil plant-pathogenic bacterium, has become a very powerful DNA vector widely used in development of genetically modified plants. It is also used for transient expression of pharmaceutical and nonpharmaceutical proteins in *Nicotiana benthamiana*, tobacco, lettuce, tomato, and other plant species (Krensek et al. 2015; Schulz et al. 2015; Tschöfen et al. 2016; Yao et al. 2015). Transient production of high-value therapeutic proteins in plants in contained facilities is increasingly applicable also in large-scale production such as a *Nicotiana benthamiana*-based influenza vaccine manufacturing by the Medicago company (Hodgins et al. 2019). However, transfection methods suitable for field applications are still desirable for the industrial-scale production of cost-sensitive products, such as the low-caloric sweetener thaumatin, for the partial substitution of the sugar in food, or cellulases produced

for the conversion of cellulosic biomass into ethanol as a fuel extender (Hahn et al. 2015; Szwacka et al. 2012; Tusé et al. 2014). Outdoor cultivation is superior to that in contained facilities because of lower upstream process 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 for plant transfection into the open environment causes potential biosafety issues. There are several approaches being investigated for the biocontainment of genetically modified microorganisms, including multiple metabolic or synthetic auxotrophy or synthetic gene circuits. However, none of them can ensure complete biosafety because of specific or universal disadvantages, such as function regain because of horizontal gene transfer or biocontainment system disruption because of mutagenesis (Lee et al. 2018). Thus, only a combination of different strategies can help to reach the goal of the minimum risk. Earlier, we have demonstrated that efficient control of T-DNA transfer from recombinant *Agrobacterium* spp. to plants can be achieved by engineering inducible expression of *agrobacterial* virulence gene *virE2* (Denzkovskienė et al. 2015). The goal of our recent research was to explore the possibility of constructing a more biosafe transient expression system for preventing unintended T-DNA transfer by using the potential of natural bacterial toxin-antitoxin (TA) modules.

In eukaryotes, the classical form of programmed cell death is apoptosis (Fadell and Orrenius 2005). Many recent studies have shown that particular mechanisms of programmed cell death also exist in prokaryotes, allowing them to withstand environmental stresses, such as nutrient deprivation, antibiotics exposure, and more (Hu et al. 2010). For most eubacteria and archaea, the best-known mechanisms involved in bacterial programmed cell death are TA systems. TA systems function in bacterial stress tolerance, virulence, phage defense, and also biofilm formation (Wang and Wood 2011). All TA systems are normally composed of two elements, a toxin and an antitoxin. Basically, the toxin suppresses bacterial growth and exists as a stable protein, while the antitoxin is a labile protein or RNA that neutralizes the toxin (Yamaguchi and Inouye 2016). TA systems have been categorized into seven types according to the mechanism by which the antitoxin interacts with the toxin. In most cases, toxin and antitoxin genes are organized into operons. The expression of a TA operon is autoregulated by the antitoxin at the transcription level, while the toxin acts as a corepressor (Christensen et al. 2004). In normal conditions in type II and type III systems, the antitoxin blocks toxin action

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by tight binding (Goeders and Van Melder 2013; Sala et al. 2014). However, when a microorganism experiences nutrient deficiency, antibiotic treatment, environmental stress, immune system response, bacteriophage infection, or oxidative stress, the concentration of antitoxin may decrease and the free toxin suppresses bacterial growth or causes cell death. Toxins interfere with membrane integrity preventing proton movement (Wang et al. 2012; Weel-Sneve et al. 2013), act as endoribonucleases, or disturb DNA replication and translation by inhibiting DNA gyrase (Yamaguchi and Inouye 2016). A small portion of known toxins stop cell division by interfering with the synthesis of bacterial peptidoglycan (Mutschler and Meinhardt 2011) or inhibiting the polymerization of bacterial cytoskeletal proteins (Tan et al. 2010).

We identified several putative toxins, homologs of toxins from TA modules in the *Agrobacterium* genome and demonstrated the ability of these toxins to cause bacterial death when expressed ectopically. We used one TA pair, *pemIK*, for the construction of a transient expression system with strictly regulated T-DNA transfer.

RESULTS

Identification of *A. tumefaciens* TA modules.

The preliminary screening of putative TA loci in the *A. tumefaciens* C58 genome was performed with the web-based tool RASTA-Bacteria. All open reading frames found by the RASTA-Bacteria webtool in a provided sequence, whether annotated or not, undergo a scoring process to determine their likelihood to belong to the TA family of genes, according to several criteria settled by the authors (Sevin and Barloy-Hubler 2007), and are ranked accordingly in a given list of candidate TA systems. Scores above 70% indicate high probability of an active TA module, while lower scored candidates require further manual verification. A RASTA-Bacteria cut-off score >47% was manually settled and TA systems were selected—three from the circular chromosome, one from the linear chromosome, and one from the Ti plasmid. Additionally, the *ietS* toxin from the Ti plasmid was selected based on data by Yamamoto et al. (2009) (Table 1). The *Iets* gene was also included among potential TA systems provided by RASTA-Bacteria (as Atu6083), however, it scored only 27% and was not recognized as a candidate toxin initially.

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 (constructed strains are listed in Table 2). The 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 colony-forming unit (CFU) counts by a very similar range, 2.7 to 3.8 orders of magnitude, after 4 h of induction with 150 μ M 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. 1). Thus, we demonstrated that five of cloned toxins are capable of killing agrobacteria efficiently while expressed ectopically.

PemK effectively suppresses agrobacteria-based plant transient expression.

As the next step, we investigated whether toxins inserted under cumic acid-regulated promoters could be used to suppress the agrobacteria-based transient expression system when bacteria are sprayed onto *Nicotiana benthamiana* plants. As *pemK* was the first toxin cloned, it was used for the construction of the model system.

Vector pNMDV371 ($P_{TS/CuO}::pemK$) was cotransformed into *Agrobacterium tumefaciens* with pICH27566, a PVX (potato virus X)-based binary expression vector for the expression of green fluorescent protein (GFP) in plants (Supplementary Fig. S1). As we demonstrated previously, transient expression assays in *N. benthamiana* following agroinfiltration with *A. tumefaciens* GV3101 (pICH27566) show strong GFP fluorescence in plant leaves 4 to 5 days postinfiltration. In these assays, GFP expression foci, as they are a phenotypic result of the T-DNA transfer, were calculated on plant leaves (Denkovskienė et al. 2015). Two *Agrobacterium* strains, control strain GV3101 (pICH27566) and GV3101 (pICH27566, pNMDV371), containing the $P_{TS/CuO}::pemK$ construct in addition to the GFP-expression vector, were used for agrospray experiments. To induce *pemK* expression in the agrobacterium, 150 μ M of cumic acid was added into the spraying solution. Seven days after the spraying, both GFP foci counts on plant leaves and *Agrobacterium* CFU counts in the same leaves were evaluated. CFU counts of viable agrobacteria 7 days after spraying demonstrated significant differences. A total of 4.1 log₁₀ of bacteria were retrieved from a gram of fresh plant leaves sprayed with the control strain (pICH27566). By sharp contrast, no bacteria were retrieved from the cumic acid-induced strain containing the $P_{TS/CuO}::pemK$ cassette (pNMDV371, pICH27566). Even without induction by cumic acid, the toxin expression cassette-harboring strain demonstrated CFU counts almost 100-fold lower than the control

Table 1. Toxins from *Agrobacterium tumefaciens* (C58) genome toxin-antitoxin (TA) systems^a

Putative TA module or toxin alone	Locus tags	Location in the genome	Predicted function of the toxin and related references
<i>pemIK</i>	Atu0939-Atu0940	Circular chromosome AE007869.2	Originally genes of <i>pem</i> (plasmid emergency maintenance) were found in <i>E. coli</i> plasmid R100 (Tsuchimoto et al. 1992). Has endoribonuclease activity (Lee et al. 2012)
<i>vapC</i>	Atu1004	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 (Winther and Gerdes 2011; Winther et al. 2016)
<i>PIN</i>	Atu2140	Circular chromosome AE007869.2	VapC family toxin from type II TA system. Functions as ribonuclease, contains the PIN domain (Arcus et al. 2011)
<i>dead</i>	Atu3013	Linear chromosome AE007870.2	VapC family toxin. Predicted nucleic acid binding protein, contains the PIN domain
<i>Nuclease-like</i>	Atu6122	Ti plasmid AE007871.2	Thermonuclease family protein, part of traCDG operon regulated by quorum sensing, gene named <i>yci</i> (Cho and Winans 2007)
<i>ietS</i>	Atu6083	Ti plasmid AE007871.2	Serine protease function, plasmid stabilization (Yamamoto et al. 2009)

^a The RASTA-Bacteria tool was used to search for TA systems in the *Agrobacterium* genome.

strain and reached $2.2 \log_{10}$ per gram of fresh plant leaf (Fig. 2A). We presume that this reduction in the number of bacteria resulted from the basal activity of the $P_{T5/CuO}$ promoter. A growth curve of uninduced $P_{T5/CuO}::pemK$ strain showed similar growth compared with control strain harboring $P_{T5/CuO}::lacZ$ in a period of 24 h (Supplementary Fig. S4); however, the effect of a basal expression of *pemK* may accumulate in a longer period of time, as CFU counts of *Agrobacterium* colonies was evaluated after 7 days post-agro-spray in our experiment. Count of GFP expression foci was similar between the control strain and the uninduced $P_{T5/CuO}::pemK$ cassette-containing strain, with the numbers of GFP foci per leaf of $2.8 \log_{10}$ and $2.7 \log_{10}$, respectively. The cunic acid-induced

$P_{T5/CuO}::pemK$ cassette-containing strain demonstrated strongly reduced T-DNA transfer ($1 \log_{10}$) (Fig. 2B). Thus, the induced expression of toxin *pemK* efficiently killed recombinant agrobacteria and most of the killing seemed to occur before the T-DNA transfer took place, as demonstrated by reduced GFP foci numbers.

Construction of a regulated T-DNA transfer system and evaluation of induction kinetics of P_{virE} and cunic 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 timely manner, for example, just one or two days after plant

Table 2. List of *Agrobacterium tumefaciens* strains used in this study

Strain	Description	References
GV3101	(pMP90RK), nopaline, Rif ^r	Koncz and Schell 1986
GV3101 $\Delta pemK$	(pMP90RK), nopaline, Rif ^r , <i>pemK</i> region deleted	This study
ICH011	GV3101 $\Delta virE2$ (pMP90RK), nopaline, Rif ^r	Denkovskienė et al. 2015
ICH011 (pNMDV386)	Contains pICH27566-cymR, $P_{T5/CuO}::virE2$	Denkovskienė et al. 2015
ICH011 (pNMDV356)	Contains pICH27566-cymR, $P_{lac/CuO}::virE2$	Denkovskienė et al. 2015
GV3101 (pICH27566)	Contains potato virus X-based vector with green fluorescent protein	Icon Genetics
GV3101 (pNMDV621)	Contains pNDC-cymR- $P_{lac/CuO}::dead$	This study
GV3101 (pNMDV622)	Contains pNDC-cymR- $P_{lac/CuO}::PIN$	This study
GV3101 (pNMDV623)	Contains pNDC-cymR- $P_{lac/CuO}::vapC$	This study
GV3101 (pNMDV624)	Contains pNDC-cymR- $P_{lac/CuO}::nuclease_like$	This study
GV3101 (pNMDV371)	Contains pNDC-cymR- $P_{T5/CuO}::pemK-P_{tetR}$	This study
GV3101 (pNMDV350)	Contains pNDC-cymR- $P_{T5/CuO}::lacZ-P_{tetR}$	This study
GV3101 (pNMDV375)	Contains pNDC-cymR- $P_{T5/CuO}::pemK$	This study
GV3101 (pNMDV536)	Contains pNDC-cymR- $P_{lac/CuO}::letS$	This study
GV3101 (pNMDV371+pICH27566)	Contains pNDC-cymR- $P_{T5/CuO}::pemK-P_{tetR}$ + pICH27566	This study
GV3101 $\Delta pemK$ (pNMDV515+pNMDV535)	Contains pNDC- $P_{virE}::pemK$ + pICH27566-cymR- $P_{lac/CuO}::pemI$	This study
GV3101 (pNMDV535)	Contains pICH27566-cymR- $P_{lac/CuO}::pemI$	This study

Fig. 1. The ability of ectopically expressed toxins to kill *Agrobacterium tumefaciens*. The overnight cultures of *A. tumefaciens* GV3101 with plasmids carrying toxin expression cassettes were diluted to an optical density at 595 nm approximately 0.1 to 0.2. Each diluted culture was split in two. The medium with 150 μM cunic acid and the inducer-free medium. The cultures were further incubated at 28°C and 220 rpm for 4 h, then, serial dilutions were made and were plated on selective Luria Bertani-agar medium without inducer (25 μl of each culture). Colony-forming units (CFU) were counted after 48 h of incubation at 28°C. The effectiveness of each toxin was evaluated by using the formula $\Delta \log_{10} = \log_{10}(\text{CFU per milliliter of noninduced culture}) - \log_{10}(\text{CFU per milliliter of induced culture})$. Data are the mean $\pm$ standard deviation of three independent experiments.

transformation, and then lose this ability. We have demonstrated that 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 *PemK* TA 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 (Goeders and Van Melder 2013; Sala et al. 2014). Thus, including *pemI* into our transient expression system seems appropriate for delaying the toxic effect of PemK activity. 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, we compared the expression kinetics of a native P_{virE} promoter of *Agrobacterium* sp. strain GV3101 and two cumic acid–inducible promoters $P_{T5/CuO}$ and $P_{tac/CuO}$ (Denkovskienė et al. 2015). 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 (McCullen and Binns 2006).

Three *Agrobacterium* strains, the first harboring $P_{T5/CuO}::virE2$ [ICH011 (pNMDV386)], the second harboring $P_{tac/CuO}::virE2$ [ICH011 (pNMDV356)], and the GV3101 strain containing vector pICH27566 were used for plant infiltration. The infiltrated leaves were collected at 4, 24, 48, and 72 h post-infiltration, were frozen in liquid nitrogen, and were subjected to RNA extraction. The *virE2* expression analysis by real-time PCR revealed different expression kinetics from synthetic cumic acid–regulated promoters and a natural *virE* promoter. At 4 h postinfiltration, the first timepoint 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 uninduced promoters. By this time, the expression from the native *virE* promoter was still very low and comparable to the expression from uninduced $P_{T5/CuO}$ and $P_{tac/CuO}$. However, at 24 h postinfiltration, 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 postinfiltration, the relative expression from P_{virE} is equal to or higher than the expression from cumic acid–inducible promoters. *VirE2* expression from all three promoters reached its peak after 48 h and then started to decrease, demonstrating a two times lower relative expression level after 72 h (Fig. 3).

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 of $P_{tac/CuO}$, we inserted the antitoxin *pemI* under the $P_{tac/CuO}$ promoter and the toxin *pemK* under the control of P_{virE} of *Agrobacterium* sp. strain KYRT-1, described in our previous publication (Denkovskienė et al. 2015). The $P_{tac/CuO}::pemI$ was inserted into the backbone of the PVX-based GFP expression vector pICH27566, and $P_{virE}::pemK$ was cloned in a separate plasmid of a different incompatibility group (Supplementary Fig. S2). Both vectors were transformed into GV3101Δ*pemK* strain, to avoid any interference with the native *pemK* module expressed from the *Agrobacterium* genome. The strain obtained ($P_{virE}::pemK$; $P_{tac/CuO}::pemI$) was used for agroinfiltration experiments and T-DNA transfer evaluation by counting the GFP foci. Practically no T-DNA transfer was observed with the *Agrobacterium* strain harboring both *pemK* and *pemI* constructs when no cumic acid was added (four GFP foci per leaf, on average). When *Agrobacterium* suspension contained cumic acid for the induction of *pemI*, the T-DNA transfer was reconstituted; 394 GFP foci per leaf were obtained after induction with 50 μM cumic acid, and 759 GFP foci per leaf after induction with 150 μM cumic acid (Fig. 4). For comparison, infiltration with the strain containing only the $P_{tac/CuO}::pemI$ construct resulted in 667 GFP foci per leaf, meaning that the induced T-DNA transfer was similar or even more efficient than the wild-type T-DNA transfer (115.3%).

Toxin expression from the P_{virE} promoter does not significantly reduce the number of bacteria (not shown). We presume that only a minor fraction of bacteria in contact with the plant leaf will start the T-DNA transfer events cascade and undergo *virE* promoter induction. Most bacteria left on the leaf surface after infiltration will not be induced for the T-DNA transfer event cascade and, thus, will not be affected by the toxic effect of PemK. This contrasts with $P_{tac/CuO}$ or $P_{T5/CuO}$, which are induced by cumic acid despite the induction state of their virulence genes.

In summary, we achieved tight regulation in a plant transient expression system, based on the expression of the toxin *pemK* under control of the native plant *virE* promoter and expression of the *pemI* antitoxin from a chemically inducible $P_{tac/CuO}$ promoter. In the proposed system, the T-DNA transfer is possible only for a short time after agroinfiltration, while

Fig. 2. Evaluation of toxin PemK activity against *Agrobacterium tumefaciens* and green fluorescent protein (GFP) expression foci number in agrospray experiments. *Nicotiana benthamiana* plants were sprayed with cumic acid–induced or noninduced $P_{T5/CuO}::pemK$ construct-containing strain GV3101 (pICH27566, pNMDV371) and with control strain GV3101 (pICH27566). GFP loci were counted on sprayed leaves 7 days after spraying. Next, the same leaves were ground in liquid nitrogen for agrobacteria isolation and counting colony-forming units (CFU). The amount of bacteria per gram of fresh leaves was calculated. Data are the mean $\pm$ standard deviation of three independent experiments.

pemI expression is induced by cumic acid, present in the agroinfiltration solution. When the inductor is depleted, *pemI* expression stops and the T-DNA transfer becomes impossible as bacteria with induced virulence genes are simultaneously induced for transcription of the *pemK* toxin. Every subsequent T-DNA transfer attempt from the bacteria remaining in the soil should lead to the death of bacteria upon induction of the *virE* promoter.

DISCUSSION

The main goal of this work was to create a tightly controlled T-DNA transfer system for large-scale applications of genetically modified agrobacterium in agrobiotechnology, such as those requiring the release of genetically modified agrobacteria into an open environment. In our previous study, we described the construction of a regulated transient expression system by deleting the *A. tumefaciens virE2* gene essential for T-DNA transfer and placing it under different cumic acid-inducible promoters. In that system, agrobacteria were able to transfer T-DNA to plants only when cumic acid was present in the spraying solution. After depletion of the inductor, no T-DNA transfer was possible, except for very rare events, probably explained by the residual activity of promoters (Denkovskienė et al. 2015). Our previously constructed T-DNA transfer regulation system may be further supplemented with a TA-based expression system, thus minimizing unintended T-DNA spread. Alternatively, this proposed mechanism is useful in cases when an appropriate *virE2*-deficient *Agrobacterium* strain is not available.

Bacterial TA systems seemed to be *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. Toxins from bacterial TA modules have already been successfully used for the construction of biocontainment systems. In particular, bacterial TA systems were shown to work in yeast containment systems. *Escherichia coli RelBE* and *Kis-Kid* (homolog of *pemI-pemK*) TA modules have been introduced into genetically modified *Saccharomyces cerevisiae* (de la Cueva-Méndez et al. 2003; Kristoffersen et al. 2000). The *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 in the presence of Cu^{2+} only. Coexpression of the antitoxin together with the toxin protected yeast from growth inhibition (de la Cueva-Méndez et al. 2003). Later, an alternative containment system for *E. coli* ('Deadman') based on toxins *mazF* and *ccdB* was constructed (Chan et al. 2016). These works demonstrated the high potential of bacterial TA systems for biocontainment of prokaryotes, lower eukaryotes, or even for cell ablation-based strategies for controlling the spread of genetically modified plants (expression of *yoeB* toxin from *Streptococcus*

Fig. 3. Reverse transcription-quantitative PCR (RT-qPCR) analysis of *virE2* expression from cumic acid inducible promoters $P_{TS/CuO}$ and $P_{tac/CuO}$ and from the native promoter P_{virE} . *Agrobacterium tumefaciens* ICH011 ($\Delta virE2$) strains containing pNMDV386 ($P_{TS/CuO}::virE2$) or pNMDV356 ($P_{tac/CuO}::virE2$) expression constructs, and GV3101 (pICH27566) strain were syringe-infiltrated into *Nicotiana benthamiana* plants. Infiltrated plant leaves were harvested 4, 24, 48, and 72 h after agroinfiltration. The expression ratio of *virE2* was evaluated by RT-qPCR. Un = *virE2* expression from uninduced $P_{TS/CuO}$ and $P_{tac/CuO}$ promoters, In = *virE2* expression from 150 μM cumic acid-induced $P_{TS/CuO}$ and $P_{tac/CuO}$ promoters, P_{virE} = *virE2* expression from wild-type P_{virE} promoter in GV3101. Data are the mean $\pm$ standard deviation of three independent experiments. Unpaired *t* test was used for statistical analysis. Lower case a and b indicate significance of difference between values. Values are not significantly different within group a or b but are significantly different between groups.

pneumoniae TA module *yefM-yoeB*, shown to be lethal in *Arabidopsis* [Bakar Yeo and Harikrishna 2015]).

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. We provide, here, an efficient inducible expression system based on the action of the bacterial TA system utilizing a type II TA system, *pemIK*. In a previous study, we constructed and analyzed several inducible promoters to be used in *A. tumefaciens* (Denkovskienė et al. 2015). Cumic acid-inducible promoters $P_{lac/CuO}$, $P_{T5/CuO}$, $P_{lacUV5/CuO}$, and $P_{virE/CuO}$ demonstrated different expression levels and strict regulation by the inducer in *Agrobacterium tumefaciens*. We now used these data for the choice of a 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, $P_{lac/CuO}$, resulted in 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 inducer of expression of the antitoxin, is present. When the inducer is depleted, the T-DNA transfer becomes impossible as bacteria with induced virulence genes were equally induced for transcription of the *pemK* toxin. Any subsequent T-DNA transfer from bacteria remaining in the soil after agroapplication (in the absence of the chemical inducer) should lead to the death of bacteria. Controlled T-DNA transfer would be beneficial in combination with other safeguard approaches for biocontainment, for example, multiple auxotrophy or genome recoding (Lajoie et al. 2013; Mandell et al. 2015). We used plasmid-expressed *pemI* and *pemK* in our experimental system, but, for further improvement, both expression cassettes should be integrated into the *Agrobacterium* genome. We also identified and evaluated, for the first time, activity of several other *A. tumefaciens* toxins from TA systems. Toxins Dead, PIN, VapC, and IetS were all highly efficient in reducing *Agrobacterium* CFU count by 2.7 to 3.8 orders of magnitude after a 4-h incubation with an inducer.

As with any biosafety or biocontainment technology applied for microorganisms, we can never expect a zero risk of biosafety system disruption because of horizontal gene transfer or

Fig. 4. Evaluation of green fluorescent protein (GFP) expression foci number after agroinfiltration with an *Agrobacterium* strain harboring $P_{virE}::pemK$ and $P_{lac/CuO}::pemI$ constructs. GV3101 $\Delta pemIK$ (pNMDV515, pNMDV535), containing $P_{lac/CuO}::pemI$ and $P_{virE}::pemK$ was vacuum infiltrated into *Nicotiana benthamiana* plants after addition of 50 or 150 μM cumic acid or without inducer. GV3101 (pNMDV535), containing only $P_{lac/CuO}::pemI$, was used as a positive T-DNA transfer control. GFP loci were counted on agroinfiltrated leaves 6 days postinfiltration. Data are the mean $\pm$ standard deviation of three independent experiments. Representative pictures of *N. benthamiana* leaves with GFP expression foci are seen on the top of the chart.

mutagenesis of inserted genes or promoters. Only a combination of different strategies and further improvements of various systems can lead to the desired biosafety level.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

Agrobacterium tumefaciens strains used in the study are listed in Table 2. *E. coli* strain DH5 α was used for all sub-clonings. Both *E. coli* and *A. tumefaciens* cells were grown in Luria Bertani (LB) medium at 37 and 28°C, respectively. All media were supplemented, when necessary, with the following rates of antibiotics: 100 μ g/ml ampicillin, 50 μ g/ml spectinomycin, 50 μ g/ml kanamycin, 50 μ g/ml rifampicin, or 50 μ g/ml nystatin. Restriction and modification enzymes from Thermo Fisher Scientific were used for all cloning steps.

Construction of expression cassettes.

PCR-amplified genes were cloned into pJET1.2/blunt or pTZ57R/T—commercial linearized positive-selection cloning vectors. Gene sequences were confirmed by sequencing and were inserted into binary expression vectors under the control of inducible promoters for induced expression in *Agrobacterium* strains. Constructed vectors were transferred into *A. tumefaciens* by electroporation.

A detailed description and schemes of plasmid construction are provided in Supplementary Figure S3, sequences of primers used in the study are listed in Supplementary Table S1, and relevant characteristics of plasmids are summarized in Supplementary Table S2.

Plant growth conditions.

Nicotiana benthamiana plants were grown under controlled conditions (25°C and 50% relative humidity) with a long-day photoperiod (16 h of light and 8 h of dark, with illumination at approximately 320 lux). Five- to six-week-old *N. benthamiana* plants were used for agroinfiltration experiments.

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

The overnight cultures of *A. tumefaciens* containing an inducible toxin were inoculated into 4 ml of LB medium from frozen stock and were cultivated at 28°C in a shaker at 220 rpm. The optical density of o/n cultures were measured and cultures were diluted to an optical density at 595 nm (OD₅₉₅) of approximately 0.1 to 0.2. Each diluted culture was divided into two parts—the medium with an inducer [150 μ M cumic acid (4-isopropylbenzoic acid)] and the inducer-free medium. Induced and noninduced 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, CFU of *Agrobacterium* were counted. The effectiveness of each toxin was evaluated by the following formula: $\Delta\log_{10} = \log_{10}(\text{CFU/ml noninduced culture}) - \log_{10}(\text{CFU/ml induced culture})$. Raw data of the experiment are provided in Supplementary Table S3.

Evaluation of GFP expression foci number and residual amount of agrobacterium after agro-spray transformation.

Agrobacterium strains were inoculated from frozen stocks in 4 ml of LB medium and were cultivated at 28°C with shaking at 220 rpm. Overnight cultures were diluted 1:1,000, starting from OD₅₉₅ = 1 in tap water, and were supplemented with 0.005% Silwet L-77 and 150 μ M cumic acid if needed. Plants were sprayed five “sprays” to both sides of four upper leaves. GFP loci were counted on sprayed leaves 7 days after spraying,

under UV light illumination. Next, the same four sprayed leaves were weighed and ground in liquid nitrogen for agrobacteria isolation and CFU enumeration. For every gram of fresh leaf, 3 ml of sterile water was added, the plant tissue suspension was incubated for 30 to 40 min at room temperature, were filtrated through Miracloth, and then, 1 ml was plated on the LB medium with the antibiotics rifampicin (50 μ g/ml), kanamycin (50 μ g/ml), spectinomycin (50 μ g/ml), and nystatin (50 μ g/ml), to ensure *Agrobacterium* growth and prevent the growth of other organisms. The amount of bacteria per gram 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. Raw data of the experiment are provided in a Supplementary Table S4.

Evaluation of GFP expression foci number after vacuum agroinfiltration.

Agrobacterium strains were inoculated from frozen stocks in 4 ml of LB medium and were cultivated at 28°C with shaking at 220 rpm. Overnight cultures were diluted 1:100,000, starting from OD₅₉₅ = 1 in tap water, and were supplemented with 0.005% Silwet L-77 and 50 or 150 μ M cumic acid, if needed. *Agrobacterium* suspension was loaded into a desiccator vessel connected to a vacuum pump. The entire leaf system of a 5- to 6-week-old plant was then submerged into the suspension. Agroinfiltration was achieved by applying (till pressure of 200 mbar) and releasing vacuum through the pump. At 6 days after agroinfiltration, four infiltrated leaves from every plant were detached and GFP loci were counted under UV light illumination. Two plants were used for each experimental point. Three individual experiment repeats were made. Raw data of the experiment are provided in a Supplementary Table S5.

VirE2 expression evaluation by reverse transcription-quantitative PCR (RT-qPCR).

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 by Denkovskienė et al. (2015). Cumic acid (150 μ M) was added for induced expression of *virE2*. Infiltrated plant leaves were harvested 4, 24, 48, and 72 h after agroinfiltration, and leaves were frozen in liquid nitrogen and were stored at –70°C till RNA extraction. Plant tissue samples were ground in liquid nitrogen and total RNA was extracted, using Thermo Scientific GeneJET RNA purification kit. Thermo Scientific Verso 1-Step qRT-PCR kit SYBR green was used for qRT-PCR reactions. Reactions were performed according to given recommendations in a Corbett Rotor gene 6000 thermocycler. 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 Supplementary Table S1. Reactions were repeated in triplicate.

Construction of GV3101 Δ pemIK strain.

The region of *pemIK* was PCR-amplified together with upstream and downstream sequences from *A. tumefaciens* GV3101 genomic DNA (primers used can be found in Supplementary Table S1) and were inserted into a pJET1.2/blunt vector (Thermo Fisher scientific), resulting in a pNMDV90 construct. pNMDV90 was digested with *Bgl*III-*Xba*I, and a fragment was ligated into vector pDNR1 (Clontech) digested with *Bgl*III-*Nhe*I. The resulting plasmid pNMDV421 was digested with *Mss*I-*Nde*I

(removing the coding sequence for *pemIK*) and re-circularized. The final plasmid pNMDV426 contains the *sacB* gene for counterselection on sucrose-containing medium. The construct pNMDV426 was transformed into *A. tumefaciens* GV3101 (list of constructed vectors can be found in Supplementary Tables S2 and S3). The *pemIK* genes were deleted by two homologous recombination events as described earlier (Berger and Christie 1993). The deletion of the *pemIK* region was confirmed by PCR and Southern blot analyses. The resulting *Agrobacterium* strain was named GV3101 Δ *pemIK*.

AUTHOR-RECOMMENDED INTERNET RESOURCE

The RASTA-Bacteria tool: <http://genoweb1.irisa.fr/duals/RASTA-Bacteria>

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