Inducible Expression of *Agrobacterium* Virulence Gene *VirE2* for Stringent Regulation of T-DNA Transfer in Plant Transient Expression Systems

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Agrotransfection with viral vectors is an effective solution for the transient production of valuable proteins in plants grown in contained facilities. Transfection methods suitable for field applications are desirable for the production of high-volume products and for the transient molecular reprogramming of plants. The use of genetically modified (GM) *Agrobacterium* strains for plant transfections faces substantial biosafety issues. The environmental biosafety of GM *Agrobacterium* strains could be improved by regulating their T-DNA transfer via chemically inducible expression of *virE2*, one of the essential *Agrobacterium* virulence genes. In order to identify strong and stringently regulated promoters in *Agrobacterium* strains, we evaluated isopropyl- β -D-thiogalactoside-inducible promoters P_{lac} , P_{lac} , $P_{T7/lacO}$, and $P_{T5/lacOlacO}$ and cumin acid-inducible promoters $P_{lacUV5/CuO}$, $P_{lac/CuO}$, $P_{T5/CuO}$, and $P_{virE/CuO}$. *Nicotiana benthamiana* plants were transfected with a *virE2*-deficient *A. tumefaciens* strain containing transient expression vectors harboring inducible *virE2* expression cassettes and containing a marker green fluorescent protein (GFP) gene in their T-DNA region. Evaluation of T-DNA transfer was achieved by counting GFP expression foci on plant leaves. The *virE2* expression from cumin acid-induced promoters resulted in 47 to 72% of wild-type T-DNA transfer. Here, we present efficient and tightly regulated promoters for gene expression in *A. tumefaciens* and a novel approach to address environmental biosafety concerns in agrobiotechnology.

Transient expression systems are widely used for small-scale protein expression in plants (Xu et al. 2012). Among several strategies used for transient transformation, including *Agrobacterium* infiltration and virus vector infection methods, the combination of these two—agrobacterial delivery of plant virus-based expression vectors—holds particular promise. This combinatorial approach seems to be the most universal method to obtain high-level transient expression of polypeptides or hetero-oligomeric proteins in just days after the initial molecular cloning event (Rybicki 2010). Several very efficient approaches of agro- or viral transformation are currently being used, from Icon Genetics' 'deconstructed' viral vectors (Gleba et al. 2005) to

launch vectors (Musiyshuk et al. 2007) and combinations of transient and stable transformations that allow for in-plant activation of viral amplification based on the transgenic expression from inducible promoters (Werner et al. 2011).

Transient plant transformation is not only a very convenient method for efficient production of biomolecules in plants, it also reduces biosafety concerns related to unintended transgene spread, because the transformation of plant tissue occurs via cytoplasmic viral RNA amplification (Marillonnet et al. 2004). Despite being nonpathogenic to humans and animals, the un- welcome spread of GM *Agrobacterium* strains in soil or the transformation of unintended hosts must be considered with agro-mediated plant transfections. While the production of high value proteins in plants in contained facilities is economically viable and minimizes the environmental biosafety concerns of agrotransfections, field applications would be necessary to achieve the production of lower-value and high-volume products such as amylases or cellulases in industrially relevant scale (Hahn et al. 2015; Tusé et al. 2014). Additionally, field application would be essential for transient molecular reprogramming of plants to modulate agronomic traits, such as resisting stresses or altering the quantity and quality of useful plant secondary metabolites (Glenn et al. 2013; Sainsbury and Lomonosoff 2014). While some strains of GM *Agrobacterium* strains are already approved for open field use in the United States, such as *Agrobacterium radiobacter* KI026 (Gleba et al. 2014), the release of GM agrobacteria into an open field environment still requires an assessment of potential environmental biosafety risks compared with the potential benefit from the biotechnological product. Among several possible ways to reduce the environmental risks associated with the release of a GM *Agrobacterium* sp., a promising one is the introduction of multiple auxotrophy and genetic manipulations to prevent conjugative plasmid transfer (Gleba et al. 2014). The environmental safety of GM *Agrobacterium* strains would be further increased by controlling its T-DNA transfer. If T-DNA transfer could be controlled so that GM *Agrobacterium* strains could only transfer a target T-DNA with the application of an inducer and lose that ability with the depletion of the inducer, it would provide additional biosecurity and dramatically reduce the environmental risks associated with field use of GM *Agrobacterium* strains. In order for such a system to be biosafe, industrially useful, and economically relevant, it must prevent background expression in the absence of the inducer and provide high transformation efficiency upon induction. With that goal in mind, we chose the *Agrobacterium* virulence gene *virE2* for T-DNA transfer regulation. The *VirE2* protein participates in a protection of *Agrobacterium* T-DNA in a plant cell and is one of the essential virulence proteins that allows for T-DNA

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transfer. *VirE2* mutants of *A. tumefaciens* lose almost all their ability to transfer T-DNA (Citovsky et al. 1992). In our search for the strongest and most stringently regulated promoters for expression of *virE2* in *Agrobacterium* strains, we evaluated efficiency of isopropyl- β -D-thiogalactoside (IPTG)-inducible promoters P_{lac} , P_{tac} , $P_{T5/lacOlacO}$, $P_{T7/lacO}$, and the cumic acid-inducible hybrid $P_{T5/CuO}$, $P_{lac/CuO}$, $P_{lacUV5/CuO}$, and $P_{virE/CuO}$ promoters in *A. tumefaciens*. Cumic acid-inducible systems showed extremely low background expression of *virE2* and were highly inducible in *A. tumefaciens*.

RESULTS

The experimental setup.

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 (Hahn et al. 2015) and *Potato virus X* (PVX)-based pICH27566 vectors contain viral polymerase and green fluorescent protein (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. pICH18711 has a TMV movement protein, 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, which, together, are responsible for intercellular and systemic replicon spread throughout all of the plant vasculature (Fig. 1). Transient expression assays in *N. benthamiana* following agroinfiltration with *A. tumefaciens* GV3101 transformed with either pICH18711 or pICH27566 show strong GFP fluorescence in plant leaves 4 to 5 days postinfiltration. In these assays, the number of T-DNA transfer events can be easily quantified, as it corresponds to the number of GFP expression foci.

Fig. 1. *Tobacco mosaic virus* (TMV)- and *Potato virus X* (PVX)-based vectors containing the *virE2* expression cassette. Inducible *virE2* expression cassettes were inserted into the unique *Eco47III* site in the backbone of either TMV-based pICH18711 or PVX-based pICH27566 and were transformed into *Agrobacterium tumefaciens* ICH011 ($\Delta virE2$), lacking the entire *virE2* open reading frame. Transformed *agrobacteria* were used in agroinfiltration or agrospray assays. 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, and 8K = PVX triple gene block, GFP = green fluorescent protein, 3'TMV = 3'untranslated region of TMV, and 3'PVX = 3'untranslated region of PVX.

An *A. tumefaciens* strain, ICH011 ($\Delta virE2$), lacking the entire *virE2* open reading frame was constructed for inducible *virE2* cassette evaluation. 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. 2A). When transgenic *N. benthamiana* ($P_{35S}::virE2$) plants constitutively expressing *virE2* 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. 2A). These data are in complete agreement with the data describing the ability of *VirE2* protein to complement a *virE2* mutant when provided in *trans* (Gelvin 1998; Otten et al. 1984).

We constructed inducible *virE2* cassettes and inserted them into the backbone of both pICH18711 and pICH27566, which were then transfected into strain ICH011 ($\Delta virE2$). The suitability of various inducible promoters to create an *Agrobacterium*-mediated transient plant transformation 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. All 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).

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

Lac promoter. The functionality of P_{lac} promoter in *A. tumefaciens* has already been described (Chen and Winans 1991; Khan et al. 2008). The successful use of this promoter in *Agrobacterium* strains and the theoretical possibility to use an inexpensive and nontoxic product as an inducer in large-scale plant transfection (Viitanen et al. 2003) were our reasons for examining the P_{lac} promoter.

β -galactosidase assays with *Agrobacterium* sp. strain GV3101 (pNMDV36) confirmed P_{lac} promoter activity in *Agrobacterium* strains. The detected activity amounted to 100 Miller units (MU) after 5 h of induction (Fig. 3A), which is in agreement with previously described values (Khan et al. 2008). We also tested P_{lac} promoter activity in the construct without the *lacI* repressor and found that the measured activity in this strain was slightly higher and amounted to approximately 400 MU (not shown). We used native P_{lac} promoter conformation with conserved positions of the O_1 and O_3 operators and with an O_2 operator lacking in the *virE2* construct but present in *lacZ* construct (the O_2 operator is located in *lacZ*).

We examined the $P_{lac}::virE2$ construct in plant agroinfiltration assays in order to evaluate the T-DNA transfer efficiency provided by P_{lac} controlled *virE2* expression. However, we did not obtain GFP expression foci in syringe-infiltrated *N. benthamiana* plant leaves, indicating that no T-DNA transfer events occurred (Fig. 3A). We then tried several slightly different constructs (expression cassette in PVX- and TMV-based vectors, with or without *lacY* permease), but none of these constructs were able to induce detectable T-DNA transfer (not shown). We thus presumed that P_{lac} promoter activity (approximately 100 MU) is not sufficiently strong to provide the amounts of *VirE2* necessary for efficient T-DNA transfer.

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

***P_{lac}* promoter.** The *virE2* coding sequence was inserted under control of hybrid *P_{lac}* promoter (de Boer et al. 1983) in a TMV-based viral vector (pNMDV148). This construct was tested in our agroinfiltration assay. In contrast to *P_{lac}::virE2*, the *P_{lac}::virE2* expression cassette provided a 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_{lac}* was poorly regulated by IPTG and gave similar GFP fluorescence in the noninduced state as when induced by IPTG. 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 (not shown).

***P_{T5/lacOlacO}* promoter.** Bacteriophage T5 early promoter *P_{N25}* (hereafter called *P_{T5}*) has been described as a very strong promoter recognized by bacterial RNA polymerase (Rommens et al. 1983). The *P_{T5}* hybrid promoter is used in Qiagen expression vectors and was tested in several microorganisms, including *Agrobacterium* species (Wang et al. 2000). 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* strains. 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. 2B). *A. tumefaciens* GV3101 (pNMDV417), containing the *P_{T5/lacOlacO}::lacZ* expression cassette, showed high β -galactosidase activity (approximately 1,500 MU) when induced with 2 mM IPTG for 5 h and low activity (approximately 27 MU) in an uninduced state (Fig. 3B). The plant agroinfiltration assay with *A. tumefaciens* ICH011 (pNMDV519) containing the *P_{T5/lacOlacO}::virE2* expression cassette showed almost undetectable GFP fluorescence in the absence of the inducer and strong GFP fluorescence in its presence (Fig. 3B).

***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* (Studier et al. 1990). It has also been tested in other microorganisms, including *A. tumefaciens* (Kang et al. 2007). We tested the T7 expression system for inducible *virE2* expression. 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. *A. tumefaciens* ICH011(pNMDV163, pNMDV164) was tested in our agroinfiltration assay and demonstrated the restored ability of T-DNA transfer (Fig. 3C). 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. We next tested the activity of T7 polymerase-regulated *P_{T7/lacO}* promoter in a β -galactosidase assay using the *A. tumefaciens* strain GV3101(pNMDV163, pNMDV223). Surprisingly, almost no activity was detected in *Agrobacterium* strains even in the presence of IPTG, contradictory to our results with *virE2*. We retransformed both pNMDV163 and pNMDV223 plasmids into *E. coli* and, then measured β -galactosidase activity. The results showed high promoter activity, confirming the functionality of the system in *E. coli* (Fig. 3C). We cannot explain the observed discrepancy between lack of β -galactosidase activity in *Agrobacterium* strains and successful inducible T-DNA transfer. We can speculate that the observed difference is due to different induction times and conditions between the β -galactosidase activity test in liquid culture and the 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 the wild-type strain, and as such, the obtained T-DNA transfer efficiency did not meet our goals and expectations.

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

A novel tightly regulated gene-expression system was recently 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 (Choi et al. 2010). We evaluated the possibility of using cumate-inducible promoters in *Agrobacterium tumefaciens*.

***P_{T5/CuO}* promoter.** *P_{T5/CuO}::virE2* cassette was inserted into viral vectors pICH18711 and pICH27566, resulting in constructs pNMDV281 and pNMDV386, respectively. Unlike Choi et al. (2010), cuminic acid (4-isopropylbenzoic acid) was used as an inducer instead of cumate (p-isopropylbenzoate) because cuminic

Fig. 2. T-DNA transfer by the *Agrobacterium tumefaciens* ICH011 strain. **A**, The wild-type *Nicotiana benthamiana* agroinfiltration with *virE2* deficient *A. tumefaciens* ICH011 (pICH18711) shows no detectable green fluorescent protein (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) is able to accomplish the T-DNA transfer, as confirmed by strong GFP fluorescence. **B**, Isopropyl- β -thiogalactoside (IPTG) and cuminic acid-induced T-DNA transfer is regulated in a dose-responsive manner in *A. tumefaciens* ICH011 transformed with inducible *virE2* constructs. Left panel, 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. Right panel, pNMDV281 (*P_{T5/CuO}::virE2*). No inducer was added to the first sample, 50, 100, 150, 200, or 250 μ M cuminic acid was added to experimental samples just before infiltration.

acid gave better results in *Agrobacterium* strains. Cumic acid induced relatively strong *virE2* expression in *A. tumefaciens*, resulting in strong GFP fluorescence in plant leaves (Fig. 3D). 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. We evaluated β -galactosidase activity of GV3101(pNMDV299) strain, harboring the $P_{T5/CuO}::lacZ$

expression cassette, and compared it with β -galactosidase activity of GV3101(pNMDV417) harboring the $P_{T5/lacOlacO}::lacZ$ expression cassette (Fig. 3B). β -galactosidase activity was much higher when the $P_{T5/CuO}$ promoter was used for inducible expression of *lacZ* and reached approximately 5,200 MU when induced, while expression from $P_{T5/lacOlacO}$ promoter resulted only in approximately 1,500 MU. However, the basal activity of

noninduced $P_{T5/CuO}$ promoter was also higher (approximately 240 MU) than the basal activity of $P_{T5/lacOlacO}$ (approximately 27 MU). This higher leakiness of $P_{T5/CuO}$, however, did not result in detectable T-DNA transfer in the plant infiltration assays (Fig. 3D) while lower leakiness of $P_{T5/lacOlacO}$ did (Fig. 3B). Here again, we hypothesize that expression from IPTG-inducible promoters while *Agrobacterium* strains are infiltrated in plant tissue may be influenced by additional factors, resulting in the observed differences with the promoter activity measured in *Agrobacterium* strains while in nutrient medium.

We next evaluated the correlation between cumic acid-inducible *virE2* expression, resulting in T-DNA transfer and GFP fluorescence in plants, and inducer concentration. Cumic acid concentrations of 50 to 250 μ M were tested. As little as 50 μ M of cumic acid was able to induce GFP fluorescence in *N. benthamiana* leaves. This fluorescence seemed to reach its maximum induction between 100 and 250 μ M of cumic acid (Fig. 2B). Therefore, we chose the concentration of 150 μ M of cumic acid for further induction experiments.

As the next step, we analyzed the correlation between strength of expression from $P_{T5/CuO}$ and length of incubation with cumic acid. Suspensions of ICH011(pNMDV281) were incubated with 150 μ M cumic acid for 0.5, 1, 2, 3, 4, and 5 h and, then, were syringe-infiltrated into plants. It appeared that <0.5 h of incubation was sufficient for maximal induction of *virE2* expression. Increased incubation time did not increase *virE2* expression or transformation efficacy. Next, we determined 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. We induced *VirE2* synthesis by cumic acid, incubated the treated bacteria for 1, 2, 3, 4, 5, 6, or 24 h and removed the inducer just before infiltrating bacteria into plants. Unfortunately, we did not detect any GFP fluorescence 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 to the induction medium or coexpression of *VirE1*, which was described as a stabilizing chaperone of *VirE2* (Zhao et al. 2001), did not change the results (not shown). Therefore, it appears that the timing of *VirE2* expression is crucial and that functional *VirE2* cannot be preaccumulated 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 analyze new cumic acid-regulated promoters.

$P_{lac/CuO}$ promoter. We demonstrated that the P_{lac} promoter was very strong in *Agrobacterium* strains, as $P_{lac}::virE2$ expression resulted in very efficient T-DNA transfer. However, the P_{lac} promoter was very poorly repressed in the absence of IPTG induction. Because we observed the tight regulation of $P_{T5/CuO}::virE2$ expression in *A. tumefaciens*, we expected that the P_{lac} promoter could also be regulated more tightly by cumic acid. A synthetic $P_{lac/CuO}$ promoter was designed by replacing the *lac* operator sequence in the P_{lac} promoter with the cumate operator sequence. As an additional means to achieve higher stringency, the repressor gene expression cassette was cloned in opposite orientation to the $P_{lac/CuO}$ promoter, the same orientation as was utilized in the $P_{T5/CuO}$ construct. The $P_{lac/CuO}::virE2$ expression cassette was inserted into viral vectors pICH18711 and pICH27566, giving constructs pNMDV355 and pNMDV356, respectively. Indeed, as anticipated, plant infiltration with the 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. 3E). We also tested the $P_{lac/CuO}$ promoter in β -galactosidase assays, using the GV3101(pNMDV395) strain harboring the $P_{lac/CuO}::lacZ$ cassette. The β -galactosidase activity was approximately 5,400 MU in the induced state and only approximately 110 MU in the noninduced state. These results define the hybrid $P_{lac/CuO}$ promoter as a very strong and tightly regulated promoter in *Agrobacterium* strains (Fig. 3E).

$P_{lacUV5/CuO}$ promoter. The tight regulation of cumic acid-inducible promoters in *Agrobacterium* strains 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 (approximately 240 MU) is insufficient to provide enough *VirE2* molecules to allow efficient T-DNA transfer to occur and that the promoter strength of 1,500 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* strains and reaches only approximately 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 with *lacIq* in pNMDV150) we could achieve greater activity of the P_{lac} promoter. We constructed a synthetic $P_{lacUV5/CuO}$ promoter by replacing the *lac* operator sequence with a cumate operator sequence. The $P_{lacUV5/CuO}::virE2$ and $P_{lacUV5/CuO}::lacZ$ expression cassettes were used to construct plasmids pNMDV457 and pNMDV376, respectively. The GV3101(pNMDV376) strain was

Fig. 3. Evaluation of inducible promoters in *Agrobacterium tumefaciens* ICH011. Isopropyl- β -D-thiogalactoside (IPTG) or cumic acid-inducible promoters were evaluated in β -galactosidase assays (*lacZ*-carrying constructs) and in plant agroinfiltration assays (*virE2*-carrying constructs) for their ability to restore the T-DNA transfer. **A**, $P_{lac}::virE2$ expression from IPTG-induced promoter did not result in detectable T-DNA transfer; the β -galactosidase activity assay showed activity of approximately 100 Miller units (MU) in *Agrobacterium* strains. **B**, $P_{T5/lacOlacO}::virE2$ expression from this promoter resulted in efficient and tightly regulated T-DNA transfer; *lacZ* expression resulted in medium β -galactosidase activity (approximately 1,400 MU) in the induced state and very low activity (approximately 27 MU) without induction. **C**, $P_{T7/lacO}::virE2$ expression from this promoter resulted in moderate T-DNA transfer in the 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 the $P_{T7/lacO}$ promoter, while activity was high in *Escherichia coli*. **D**, $P_{T5/CuO}::virE2$ expression resulted in highly inducible and very stringent T-DNA transfer; *lacZ* expression resulted in high β -galactosidase activity while induced (approximately 5,200 MU) and low leaky activity (approximately 240 MU). **E**, $P_{lac/CuO}::virE2$ expression resulted in highly inducible and very stringent T-DNA transfer; *lacZ* expression resulted in high β -galactosidase activity while induced (approximately 5,400 MU) and low leaky activity (approximately 110 MU). **F**, $P_{lacUV5/CuO}::virE2$ expression resulted in moderately efficient and relatively stringent T-DNA transfer; β -galactosidase activity assay showed activity of approximately 1,100 MU when induced and approximately 80 MU in the absence of the inducer. **G**, $P_{VirE/CuO}::virE2$ expression from the induced promoter resulted in highly efficient T-DNA transfer, however, several green fluorescent protein (GFP) expression foci were detected in the absence of the inducer; *lacZ* expression from this promoter resulted in low β -galactosidase activity (approximately 90 MU) only when 100 μ M acetosyringone was added to the *Agrobacterium* suspension. *Nicotiana benthamiana* leaf pictures were taken under UV light 5 days postinoculation. + indicates induction with 1 mM IPTG or with 150 μ M cumic acid, – indicates no inducer added, wt = control syringe-infiltrations of *Agrobacterium* sp. strain GV3101 carrying viral vector with GFP. β -galactosidase assay values correspond to the average of three experiments. Bars represent standard deviation.

Fig. 4. Evaluation of T-DNA transfer efficiency by agrospray in **A**, cumic acid-induced and **B**, noninduced constructs. *Nicotiana 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 ($P_{T5/CuO}::virE2$), and p437 to pNMDV437 ($P_{VirE/CuO}::virE2$). The different colors indicate different promoters: gray = $P_{T5/CuO}$, green = $P_{T5/CuO}$, purple = $P_{VirE/CuO}$. Black columns indicate control strains. The efficiency and 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 (green fluorescent protein [GFP] expression foci number of GV3101-sprayed plants). Wild-type T-DNA transfer was considered as 100%. Bars represent standard error. **C**, Representative picture of *N. benthamiana* leaf with GFP expression foci.

tested in β -galactosidase assays and had activity of approximately 1,100 MU when induced with cumic acid, which is considerably higher than activity of native P_{lac} promoter (approximately 100 MU). The transient expression assays with the cumic acid-induced ICH011(pNMDV457) strain showed efficient T-DNA transfer in infiltrated *N. benthamiana* leaves (Fig. 3F). Only several GFP expression foci were detected when the noninduced ICH011(pNMDV457) strain was infiltrated into plant leaves (Fig. 3F). The result suggests that promoter activity greater than approximately 240 MU and less than or equal to approximately 1,100 MU is necessary to provide minimal quantity of VirE2 protein molecules to allow efficient T-DNA transfer to occur.

$P_{VirE/CuO}$ promoter. Although $P_{T5/CuO}$ and $P_{T5/CuO}$ promoters were highly inducible in *Agrobacterium* strains, neither of them proved itself as strong as the native $virE$ region promoter P_{virE} . Neither the $P_{T5/CuO}::virE2$ nor the $P_{T5/CuO}::virE2$ 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, we proceeded in two steps. First, the putative P_{virE} promoter region of *Agrobacterium* sp. strain KYRT-1 (Torisky et al. 1997), homologous to the described P_{virE} promoter in *A. tumefaciens* A6 (Das et al. 1986) and 99% identical to the same region of pTiBo542 (Komari et al. 1986), was polymerase chain reaction (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, we added the cumate operator sequence to the isolated P_{virE} promoter. The $P_{VirE/CuO}::virE2$ cassette was inserted into a pICH27566 viral vector, resulting in construct pNMDV437. Plant infiltration assays with cumic acid-induced ICH011 (pNMDV437) demonstrated very strong GFP fluorescence (Fig. 3G). However, infiltration of the noninduced ICH011 (pNMDV437) strain also showed detectable GFP fluorescence, indicating greater leakiness than that of the $P_{T5/CuO}$ or $P_{T5/CuO}$ promoters. We also constructed a $P_{VirE/CuO}::lacZ$ expression vector harboring *Agrobacterium* sp. strain GV3101(pNMDV442) and tested it in β -galactosidase activity assays. As expected, in the absence of acetosyringone, we could not detect any promoter activity in the presence or absence of cumic acid ex planta, confirming that the $P_{VirE/CuO}$ promoter needs additional factors for induction, which consist of VirG activation by plant phenolic compounds. Only when 100 μ M acetosyringone was used as the inducer of VirG together with cumic acid could β -galactosidase activity be detected (Fig. 3G). This activity was weak and did not correspond to the 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.

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 $P_{T5/CuO}$, $P_{T5/CuO}$, and $P_{VirE/CuO}$ promoters in PVX-based vectors in the ICH011 strain were used for these experiments. The level of wild-type T-DNA transfer obtained by spraying plants with the 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. $P_{T5/CuO}$ and $P_{VirE/CuO}$ promoters containing strains ICH011(pNMDV356) and ICH011(pNMDV437) performed better, with T-DNA transfer efficiency reaching 71 to 72% of the wild-type expression (Fig. 4A). In order to further improve T-DNA transfer efficiency, we tried to coexpress $virE1$ 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 (Deng et al. 1999; Sundberg and Ream 1999; Zhao et al. 2001). *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. We found that *virE1* and *virE2* coexpression did not significantly improve T-DNA transfer efficiency in ICH011 (pNMDV381) in comparison with *virE2*-expressing strain ICH011 (pNMDV386) (Fig. 4A) (analysis of variance test, $P > 0.05$).

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, we observed several GFP expression foci 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.

We performed plant sprayings with all constructs previously evaluated for T-DNA transfer efficiency. In order to evaluate the significance of background expression, all tested *Agrobacterium* strains were diluted 10^{-1} , 10^{-2} , and 10^{-3} -fold and were 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 the *virE2*-deficient strain has been already described (Citovsky et al. 1992). However, the frequency of noninduced T-DNA transfer events was extremely low and reached only 0.002 to 0.03% of wild-type T-DNA transfer (Fig. 4B). Cumic acid-inducible *virE* promoter showed the greatest leakiness, which, however, was 3,000 times lower than the wild-type T-DNA transfer. The leakiness of the other strong promoter $P_{lac/CuO}$ was 50,000 times lower in comparison with the wild-type T-DNA transfer and approached the leakiness obtained with the negative control.

DISCUSSION

With the evolution of technology surrounding the field of transient plant transformation, there is increasing need for innovations to address environmental biosafety issues, particularly those related to the use of GM *Agrobacterium* strains in open field environments. To address some of the environmental biosafety issues, we constructed expression vectors for controlled T-DNA transfer from *A. tumefaciens* to the host plant. We inserted an essential *Agrobacterium* virulence gene, *virE2*, under control of either IPTG- or cumic acid-inducible promoters and, then, transformed expression cassettes into a *virE2*-deficient *A. tumefaciens* strain. Due to the paucity of information available regarding the overexpression and expression control of foreign genes in *A. tumefaciens*, we tested several inducible promoters. We investigated several IPTG-inducible promoters and constructed several new hybrid cumic acid-regulated promoters for expression of *virE2* in *Agrobacterium* strains. We observed better correlation between β -galactosidase activity assays and inducible T-DNA transfer with cumic acid-regulated promoters. The most efficient of the IPTG-regulated promoters was the $P_{T5/lacOlacO}$ promoter. The $P_{T5/lacOlacO}$ promoter was strong and showed low leakiness in the absence of the inducer in β -galactosidase measurements. However, although this promoter seemed to be tightly regulated in the β -galactosidase assay, it demonstrated

considerable leakiness in planta. In order to understand why the $P_{T5/CuO}$ promoter is less leaky in agroinfiltration experiments than $P_{T5/lacOlacO}$, we measured, in *Agrobacterium* strains, the activity of the *lacIq* repressor promoter and the activity of the P_{Km} promoter used for *CymR* expression. The β -galactosidase activity of $P_{lacIq}::lacZ$ was approximately 100 MU, while $P_{Km}::lacZ$ activity was four times greater and amounted to approximately 400 MU. We suggest that *lacI* expression from its native promoter in *Agrobacterium* strains may be insufficient for stringent regulation of the $P_{T5/lacOlacO}$. We tried to replace *lacIq* with $P_{Km}::lacI$ cassettes in our constructs but failed to obtain *Agrobacterium* transformants. Moreover, we cannot rule out other possibilities for the higher leakiness of the $P_{T5/lacOlacO}$ promoter, i.e., different stabilities of repressors, different operator binding affinities, etc.

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. *VirE2* expression from $P_{T5/CuO}$ and our newly constructed hybrid promoters $P_{lac/CuO}$ and $P_{virE/CuO}$ resulted in 47 to 72% efficient T-DNA transfer.

Other than promoter strength, several additional factors may influence the efficiency of inducible T-DNA transfer. As the inducer has to be cosprayed with *Agrobacterium* strains in order 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 nonequal distribution and penetration of the inducer into the intercellular space of plant leaves. Also, the timing of induced *virE2* expression may not correspond exactly to the timing of the wild-type *virE2* expression and may be shifted temporally compared with the expression of other *vir* genes (i.e., chaperone VirE1 and VirB/D4 transport system, necessary for VirE2 translocation into the plant cell) (Vergunst et al. 2000). In the future, insertion of *virE2* expression cassettes into the *Agrobacterium* genome could stabilize the system and simplify the use of inducible *Agrobacterium* strains. In such a case, a viral vector with any gene of interest could be transformed without further modifications into the engineered chemically regulated *Agrobacterium* strains and could be 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 transcription factor VirG. VirG is highly expressed during the interaction of *Agrobacterium* strains and the plant cell. It is possible that high concentration of VirG may impact the leakiness of this promoter.

Low leakiness from promoters $P_{lac/CuO}$ 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. We observed no leaky T-DNA transfer from these promoters when agrospray assays were performed with diluted *Agrobacterium* suspensions (optical density at 600 nm [OD_{600}] = 0.001 and 0.01). Only when an *agrobacterial* density of $OD_{600} = 0.1$ (corresponding to approximately 10^8 CFU/ml) was used in agrospray assays were several T-DNA transfer events caused by leaky expression from $P_{lac/CuO}$ and $P_{T5/CuO}$ 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 to 10^6 CFU/ml, which is one to two orders of magnitude less than the concentration used in our experiments to evaluate promoter leakiness. Since *agrobacteria* persist only at low concentrations in the field, the risk of secondary transfections resulting from promoter leakiness would be extremely low. As with any technological endeavor, we can never expect zero risk; but diligently working toward reducing environmental and human health risks to the lowest levels possible is a worthwhile and necessary goal for the adoption of new

agronomic and biomanufacturing approaches. We believe that inducible T-DNA transfer in combination with other biosafety traits (multiple auxotrophy, programmable death of agrobacteria) could help meet the main expectations for environmental biosafety and increase trust in transient plant transformation technology for large-scale applications in the near future.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

All *Agrobacterium tumefaciens* strains and plasmids used in this study are listed in Table 1. *E. coli* DH5 α was used as a recipient for all cloning procedures. Both *E. coli* and *A. tumefaciens* cells were grown in Luria Bertani (LB) medium at 37 or 30°C, respectively. When necessary, all media were supplemented with ampicillin (100 μ g/ml), spectinomycin (50 μ g/ml), kanamycin, (50 μ g/ml), or rifampicin (25 μ g/ml). Restriction and modification enzymes from Thermo Scientific were used for all cloning steps.

Construction of ICH011 (Δ VirE2) strain.

Sequences upstream and downstream of the *virE2* coding sequence were PCR amplified from *A. tumefaciens* GV3101 genomic DNA (Supplementary Table 1) and were inserted into a pGEM-T vector (Life Technologies), resulting in constructs pICH19247 and pICH19252. pICH19247 and pICH19252 were digested with *Nde*I-*Eco*RI and *Eco*RI-*Xho*I, respectively, and were ligated into vector pDNR1 (Clontech) digested with *Nde*I-*Xho*I. The resulting plasmid, pICH19500, contains a *sacB* gene for counterselection on sucrose-containing medium. Construct pICH19500 was transformed into *A. tumefaciens* GV3101. The *virE2* gene was deleted by two homologous recombination events as described earlier (Berger and Christie 1993). Deletion of *virE2* was confirmed by PCR analysis. The resulting strain was named ICH011 (Table 1).

Construction of P_{35S}::VirE2 *N. benthamiana* plants.

The *virE2* coding sequence was PCR-amplified from *A. tumefaciens* GV3101 genomic DNA and was inserted as a *Nco*I-*Xba*I fragment into vector pICH5290 (Icon Genetics), resulting in construct pICH19160. Transcription of the *virE2*

gene is under the control of the *Cauliflower mosaic virus* 35S promoter and the agrobacterial nos terminator. This vector also contains a BAR resistance cassette for selection of transgenic plants. Transgenic *N. benthamiana* plants expressing VirE2 were generated by *Agrobacterium*-mediated leaf disk transformation using a standard procedure. Insertion of the *virE2* expression cassette was confirmed by PCR.

Construction of expression cassettes.

Description of plasmid constructs and construction schemes and sequences of synthetic cumic acid inducible promoters can be found in Supplementary Figures 1 and 2.

β -galactosidase activity assay.

The β -galactosidase activity of agrobacterial strains was determined with a colorimetric assay (Miller 1972). Agrobacterial strains were grown overnight from frozen stocks, were diluted with fresh medium to an OD₆₀₀ of approximately 0.4, and then, were incubated with shaking for 4 h at 30°C in LB medium with inducer (150 μ M cumic acid [Sigma Aldrich] or 2 mM IPTG [UAB Fermentas]) and without inducer (agrobacteria usually reached OD₆₀₀ of approximately 0.8) to compare the levels of β -galactosidase expression.

Plant growth conditions and syringe-infiltration.

Wild-type *N. benthamiana* plants were grown in soil in an environment-controlled room at 25°C under long-day conditions of 16 h of white light and 8 h of dark. Five- to six-week-old plants were used for syringe-infiltration or spraying. *Agrobacterium tumefaciens* cultures were grown in LB medium overnight, were diluted with fresh medium to an OD₆₀₀ = 1, and then, were further diluted 10³-fold in tap water containing IPTG or cumic acid. Syringe agroinfiltrations were performed on the abaxial side of the plant leaves. One syringe-infiltration used 200 to 300 μ l of diluted *Agrobacterium* suspension.

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

Agrobacterium strains carrying different constructs were grown overnight and were diluted with fresh medium to an OD₆₀₀ = 1.

Table 1. *Agrobacterium tumefaciens* strains used in the study

<i>A. tumefaciens</i> strains	Description	References
GV3101	(pMP90RK), nopaline, Rif ^r	(Koncz and Shell 1986)
ICH011	GV3101 Δ virE2 (pMP90RK), nopaline, Rif ^r	This study
KYRT-1	Disarmed Chry 5, Rif ^r	(Torisky et al. 1997)
GV3101(pICH18711)	Contains TMV virus-based vector with GFP	Icon Genetics
GV3101(pICH27566)	Contains PVX virus-based vector with GFP	Icon Genetics
GV3101(pNMDV36)	Contains pNMD803- <i>lacIq</i> , P _{lac} :: <i>lacZ</i>	This study
ICH011(pNMDV148)	Contains pICH18711- <i>lacIq</i> , P _{lac} :: <i>virE2</i>	This study
ICH011(pNMDV150)	Contains pICH18711- <i>lacIq</i> , P _{lac} :: <i>virE2</i>	This study
GV3101(pNMDV163, pNMDV223)	Contains pNDC- <i>lacIq</i> , P _{lacUV5} :: <i>T7pol</i> , pICH18711- <i>lacI</i> , P _{T7/lacO} :: <i>lacZ</i>	This study
ICH011(pNMDV163, pNMDV164)	Contains pNDC- <i>lacIq</i> , P _{lacUV5} :: <i>T7pol</i> , pICH18711- <i>lacI</i> , P _{T7/lacO} :: <i>virE2</i>	This study
ICH011(pNMDV281)	Contains pICH18711- <i>cymR</i> , P _{T5/CuO} :: <i>virE2</i>	This study
GV3101(pNMDV299)	Contains pNDC- <i>cymR</i> , P _{T5/CuO} :: <i>lacZ</i>	This study
ICH011(pNMDV355)	Contains pICH18711- <i>cymR</i> , P _{lac/CuO} :: <i>virE2</i>	This study
ICH011(pNMDV356)	Contains pICH27566- <i>cymR</i> , P _{lac/CuO} :: <i>virE2</i>	This study
GV3101(pNMDV376)	Contains pNDC- <i>cymR</i> , P _{lacUV5/CuO} :: <i>lacZ</i>	This study
ICH011(pNMDV381)	Contains pICH27566- <i>cymR</i> , P _{T5/CuO} :: <i>virE</i> , <i>virE2</i>	This study
ICH011(pNMDV386)	Contains pICH27566- <i>cymR</i> , P _{T5/CuO} :: <i>virE2</i>	This study
GV3101(pNMDV395)	Contains pNDC- <i>cymR</i> , P _{lac/CuO} :: <i>lacZ</i>	This study
GV3101(pNMDV417)	Contains pNDC- <i>lacIq</i> , P _{T5/lacO} :: <i>lacZ</i>	This study
ICH011(pNMDV437)	Contains pICH27566- <i>cymR</i> , P _{virE/CuO} :: <i>virE2</i>	This study
GV3101(pNMDV442)	Contains pICH27566- <i>cymR</i> , P _{virE/CuO} :: <i>lacZ</i>	This study
ICH011(pNMDV457)	Contains pICH27566- <i>cymR</i> , P _{lacUV5/CuO} :: <i>virE2</i>	This study
ICH011(pNMDV519)	Contains pICH18711- <i>lacIq</i> , P _{T5/lacO} :: <i>virE2</i>	This study

For testing transfer efficiency, suspensions were further diluted 1×10^4 -fold in tap water containing 0.05% Silwet L-77 (Kurt Obermeier GmbH and Co) and 150 μ M cumic acid and were incubated for 1 h. For evaluation of leakiness, suspensions were diluted 1×10^1 -fold in tap water plus 0.05% Silwet L-77. A 7- to 10-ml suspension was 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 three to five plants with four to five leaves per plant being treated with *Agrobacterium* suspension. At 5 to 6 days after spraying, plant leaves were detached and were 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 error were calculated and compared with wild-type T-DNA transfer.

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