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The food additive vanillic acid controls transgene expression in mammalian cells and mice

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ABSTRACT

Trigger-inducible transcription-control devices that reversibly fine-tune transgene expression in response to molecular cues have significantly advanced the rational reprogramming of mammalian cells. When designed for use in future gene- and cell-based therapies the trigger molecules have to be carefully chosen in order to provide maximum specificity, minimal side-effects and optimal pharmacokinetics in a mammalian organism. Capitalizing on control components that enable *Caulobacter crescentus* to metabolize vanillic acid originating from lignin degradation that occurs in its oligotrophic freshwater habitat, we have designed synthetic devices that specifically adjust transgene expression in mammalian cells when exposed to vanillic acid. Even in mice transgene expression was robust, precise and tunable in response to vanillic acid. As a licensed food additive that is regularly consumed by humans via flavoured convenience food and specific fresh vegetable and fruits, vanillic acid can be considered as a safe trigger molecule that could be used for diet-controlled transgene expression in future gene- and cell-based therapies.

INTRODUCTION

Synthetic control devices providing precise expression fine-tuning of mammalian transgenes in response to molecular cues have been fundamental for functional genomic research (1), biopharmaceutical manufacturing of difficult-to-express protein therapeutics (2–4), drug discovery (5,6), tissue engineering (7–9), the design of complex synthetic gene networks (10–13), prototypic gene therapy applications (9,14,15) and the design of functional materials (16). Most of the currently available mammalian transgene-control devices share a common design principle, which ensures that the basic transcription controllers are compatible and could be assembled to higher order control networks (10–13). The basic control switches consist of synthetic transcription factors, prokaryotic response regulators fused to either a transactivation or a transsilencing domain, which bind and induce or repress chimeric promoters containing specific operator sites (17–19). Transcription can be fine-tuned by a specific trigger compound that modulates the promoter affinity of the chimeric transcription factors in a dose-dependent manner (20–24).

Besides optimal regulation performance the characteristics of the trigger molecule is of key importance when control circuits are designed for future gene- and cell-based therapies. Therefore, the latest generation of transgene control systems considers inducer molecules with minimal potential side effects such as those derived from endogenous metabolites (15,25–27) or physiologically inert molecules, such as licensed cosmetic compounds (28).

Vanillic acid is the oxidized form of vanillin and found at high concentrations in vanilla beans (29,30) and in *Angelica sinensis*, a plant used in traditional Chinese medicine (31). Vanillic acid has been associated with a variety of pharmacologic activities such as inhibiting snake venom activity (32,33), carcinogenesis (34), apoptosis (35,36) and inflammation (37) but it has become most popular for its pleasant creamy odour that is widely used in fragrances and licensed as a food additive (FAO/WHO Expert Committee on Food Additives, JECFA no. 959). Vanillic acid is also one of the main metabolites found in humans after consumption of green tee infusions (38). Overall, vanillic acid has all it takes to

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be a safe and physiologically inert inducer for gene switches designed for future therapeutic applications.

Caulobacter crescentus is a Gram-negative, oligotrophic freshwater bacterium that plays an important role in the carbon cycle by disposing of the soluble phenolic intermediates such as vanillic acid that result from fungal oxidative cleavage of lignin originating from decaying vascular plant material (39–41). *Caulobacter crescentus* can utilize vanillic acid as its sole carbon source by metabolizing it to protocatechuate and then to succinyl-CoA as well as acetyl-CoA, which is converted to metabolic energy in the citric acid cycle (42,43). Unless *C. crescentus* comes across vanillic acid the corresponding monooxygenase encoded by the VanAB gene cluster remains shut down as the transcriptional repressor VanR binds a distinct perfect inverted repeat operator (VanO; ATTGGATCCAAT) upstream of the *vanAB* promoter region (43). However, vanillic acids binds VanR and derepresses the metabolic pathway (43–47). Capitalizing on the *C. crescentus* vanillic acid-responsive transcriptional repressor VanR, we engineered different variants of a synthetic vanillic acid-responsive mammalian expression system (VAC) that show excellent regulation performance in mammalian cells as well as in mice receiving vanillic acid.

MATERIALS AND METHODS

Plasmid construction

Table 1 lists all the plasmids used in this study and provides detailed information about their construction. Using the basic local alignment search tool (BLAST) all of our promoter and operator sequences were shown to be free of binding sites specific from mammalian proteins.

Cell culture

Wild-type Chinese hamster ovary cells (CHO-K1, ATCC: CCL-61) and their derivatives were cultivated in standard medium: ChoMaster® HTS (Cell Culture Technologies, Gravesano, Switzerland) supplemented with 5% (v/v) foetal calf serum (FCS, PAN Biotech GmbH, Aidenbach, Germany, Cat. No. 3302, Lot No. P251110) and 1% (v/v) penicillin/streptomycin solution (Biowest, Nuaillé, France, Cat. No. L0022-100, Lot No. S07497L0022). Human embryonic kidney cells (HEK293-T, ATCC: CRL-11268), human cervical carcinoma cells (HeLa, ATCC: CCL-2), African green monkey kidney cells (COS-7, ATCC: CRL-1651), baby hamster kidney cells (BHK-21, ATCC: CCL10), human fibrosarcoma cells (HT-1080, ATCC: CCL-121) and mouse fibroblasts (NIH/3T3, ATCC CRL-1658) were cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS and 1% penicillin/streptomycin solution. All cell lines were cultivated at 37°C in a humidified atmosphere containing 5% CO_2. Viable cell numbers were determined using a Casy® Cell Counter and Analyser Model TT (Roche Diagnostics GmbH, Basel, Switzerland).

Transfection

The CHO-K1 cells were transiently transfected using 0.5 µg of total plasmid DNA (equimolar plasmid ratios were used for co-transfections) according to an optimized calcium phosphate-based protocol (48). As for other cell lines the transfection efficiency was tuned to reach 35% ± 5% as determined by parallel transfections using pd2EYFP (Clontech, Mountain View, CA, USA) (15). In brief, 50 000 CHO-K1 cells were seeded into each well of a 24-well plate and cultured overnight. The plasmid DNA was then diluted to a total volume of 25 µl with 0.5 M $CaCl_2$ solution, which was mixed with 25 µl 2 × HBS solution (50 mM HEPES/280 mM NaCl/1.5 mM Na_2HPO_4, pH 7.1). This mixture was incubated for 15 min at room temperature before the precipitates were directly added into the well and centrifuged onto the cells (5 min at 1200 *g*). After 3 h, the cells were treated with 0.5 ml glycerol solution (ChoMaster® HTS medium containing 15% glycerol) for 60 s. After washing once with phosphate-buffered saline (PBS, Dulbecco's phosphate-buffered saline; Invitrogen, Basel, Switzerland, Cat. No. 21600-0069), the cells were cultivated in 0.5 ml standard ChoMaster® HTS medium in the presence or absence of different concentrations of vanillic acid or its derivatives. For the transient transfection of BHK-21, COS-7 and HEK-293 cells, a plasmid DNA-Ca_2PO_4 precipitate was prepared and applied to the 50 000 cells cultivated per well of a 24-well plate, as described above. The HEK-293 and COS-7 cells were washed once with PBS after 3 h incubation with the DNA-Ca_2PO_4 precipitate and subsequently cultivated in standard DMEM, while BHK-21 and HeLa cells were incubated overnight with the precipitates and then cultivated in DMEM after being washed once with PBS. The HT-1080 and NIH/3T3 cell lines were transfected with Fugene™ 6 (Roche Diagnostics AG, Basel, Switzerland, Cat. No. 11814443001) according to the manufacturer's protocol and cultivated in the cell culture medium specified above. After transfection, all cells were cultivated in DMEM supplemented with different concentrations of vanillic acid and reporter protein levels were profiled 48 h after transfection, unless indicated otherwise.

Construction and characterization of the stable cell line CHO-VAC

The CHO-VAC_{12} cell line, transgenic for vanillic acid-controlled SEAP expression, was constructed by sequential co-transfection and clonal selection of (i) pMG250 (P_{SV40}-$VanA_1$-pA) and pSV2*neo* (Clontech, Cat. No. 6172-1) at a ratio of 20:1 to result in the cell line CHO-VanA, (ii) CHO VanA was subsequently co-transfected with pMG252 (P_{1VanO2}-SEAP-pA) and pPur (Clontech, Cat. No. 6156-1) (ratio of 20:1), and the resulting double-transgenic cell line CHO-VAC_{12}, showing vanillic acid-responsive SEAP production, was chosen after clonal selection. To assess the vanillic acid-mediated transgene regulation characteristics, 100 000 cells/ml of CHO-VAC_{12} were cultivated for 48 h in standard ChoMaster® HTS medium supplemented with increasing concentrations of vanillic acid, ranging from 0 to 1000 µM. Reversibility of the vanillic acid-mediated

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Table 1. Expression vectors and oligonucleotides designed and used in this study

Plasmid	Description	Reference
pBP99	Vector encoding a tetracycline-responsive SAMY expression unit ($P_{hCMV*-1}$-SAMY-pA). pCF59 was restricted with BprPI/EcoRV and religated.	unpublished
pBP100	Vector encoding an erythromycin-responsive SAMY expression unit (P_{ETR3}-SAMY-pA).	18
pcDNA3.1	Commercial cloning vector containing a constitutive promoter (P_{hCMV}).	Invitrogen
pCF59	Vector encoding a P_{PIR}-driven SAMY expression unit ($P_{hCMV*-1}$-pA-IRES-P_{PIR}-SAMY-pA). SAMY was excised from pSS158 using SpeI/BglII and ligated into pMF187 (SpeI/BglII).	unpublished
pCK9	Vector encoding a P_{UREX8}-driven SEAP expression unit (P_{UREX8}-SEAP-pA).	15
pCK73	Vector encoding a uric acid-responsive SEAP expression unit, driven by a size-reduced P_{hCMV} (P_{hCMV}-8xhucO-SEAP-pA). Size reduced P_{hCMV} was amplified using OCK72: 5′- ccg*ctcgag*g*aagatct*CGCGTTGACATTGATTATTGAC-3′ and OCK74: 5′- gc*gacgtcc*a*agctt*GCTTATATAGACCTC CCACC-3′ (lower case italics, restriction sites; upper case italics, annealing), digested with XhoI/HindIII and ligated into pCK9 (XhoI/HindIII).	15
pCK188	Constitutive $VanA_4$ expression vector (P_{SV40}- $VanA_4$-pA; $VanA_4$, VanR-KRAB). VanR was PCR-amplified from pMG250 using OCK190: 5′- g*gaattc*cacc*ATGGACATGCCGCGCATAAAG*-3’ and OCK191: 5′ ggcg*acgcgta*GTCGGCGCGAATGCTCCAC-3′ (lower case italics, restriction sites; upper case italics, annealing), digested with EcoRI/XbaI and ligated into pWW43 (EcoRI/BssHII).	This work
pCK189	Constitutive $VanA_4$ expression vector (P_{hCMV}-$VanA_4$-pA). VanR was PCR-amplified from pCK188 using OCK190: 5′- g*gaattc*cacc*ATGGACATGCCGCGCATAAAG*-3′ and OCK192: 5′- g*ctctaga*CTTACCAGAGATCATTCCTTGC-3′ (lower case italics, restriction sites; upper case italics, annealing), digested with EcoRI/XbaI and ligated into pcDNA3.1 (EcoRI/XbaI).	This work
pCK191	Vector encoding a P_{vanON8}-driven SEAP expression unit (P_{VanON8}-SEAP-pA; P_{vanON8}, P_{hCMV}-$VanO_8$). Oligonucleotides OCK193: 5′- *agctt*ATTGGATCCAATGCATTGG ATCCAATGGATTGGATCCAATCGATTGGATCCAAT*gatatc*ATTGGATCCAATGCATTGGATCCAATGGATTGGATCCAATCGATTG-GATCCAAT*g*-3′ and OCK194: 5′- *aattc*ATTGGATCCAATCGATTGGATCC AATCCATTGGATCCAATGCATTGGATCCAAT*gatatc*ATTGGATCCAATCGATTGGATCCAATCCATTGGATCCAATGCATTGGATCC-AAT*a*-3′ (lower case italics, restriction sites; upper case, 8xVanO) were annealed and ligated directly into pCK73 using HindIII/EcoRI.	This work
pd2EYFP	Mammalian d2EYFP expression vector	Clontech
pMF187	Dual-regulated expression vector ($P_{hCMV*-1}$-MCSI-IRES-MCSII-pA_I-P_{PIR}-MCSIII-pA_{II}).	68
pMG10	Vector encoding a P_{TtgR1}-driven SEAP expression unit (P_{TtgR1}-SEAP-pA; P_{TtgR1}, O_{TtgR}-0bp-$P_{hCMVmin}$).	28
pMG18	Constitutive $TtgA_2$ expression vector (P_{SV40}-$TtgA_2$-pA).	28
pMG19	Constitutive $TtgA_3$ expression vector (P_{SV40}-$TtgA_3$-pA).	28
pMG20	Vector encoding a P_{TtgR2}-driven SEAP expression unit (P_{TtgR2}-SEAP-pA; P_{TtgR2}, O_{TtgR}-2bp-$P_{hCMVmin}$).	28
pMG21	Vector encoding a P_{TtgR3}-driven SEAP expression unit (P_{TtgR3}-SEAP-pA; P_{TtgR3}, O_{TtgR}-4bp-$P_{hCMVmin}$).	28
pMG22	Vector encoding a P_{TtgR4}-driven SEAP expression unit (P_{TtgR4}-SEAP-pA; P_{TtgR4}, O_{TtgR}-6bp-$P_{hCMVmin}$).	28
pMG23	Vector encoding a P_{TtgR5}-driven SEAP expression unit (P_{TtgR5}-SEAP-pA; P_{TtgR5}, O_{TtgR}-8bp-$P_{hCMVmin}$).	28
pMG24	Vector encoding a P_{TtgR6}-driven expression unit (P_{TtgR6}-SEAP-pA; P_{TtgR6}, O_{TtgR}-10bp-$P_{hCMVmin}$).	28
pMG250	Constitutive $VanA_1$ expression vector (P_{SV40}-$VanA_1$-pA; $VanA_1$, VanR-VP16). VanR was PCR-amplified from *C. crescentus* genomic DNA using ODF150: 5′-cg*gaattc*cacc*ATGGACATGCCGCGCATAAAGCCGGGC*-3′ and ODF151: 5′- gtacg*acgcgt*ggctgtacgcgga*GTCGGCGCGAATGCT CCACGCCGCGC*-3′ (lower case italics, restriction sites; upper case italics, annealing), digested with EcoRI/MluI and ligated into pWW35 (EcoRI/BssHII).	This work
pMG252	Vector encoding a P_{1VanO2}-driven SEAP expression unit (P_{1VanO2}-SEAP-pA; P_{1VanO2}, $VanO_2$-0bp-$P_{hCMVmin}$). $VanO_2$ was created by annealing Oligos OMG65 (5′-phosphate-*tcgac*gtcaat*tcgcgaATTGGATCCAATagc gctATTGGATCCAATgatatc*gga*cctgca*-3′; lower case italics, restriction sites; upper case italics, VanO) and OMG66 (5′-phosphate-gcagtta*agc gctTAACCTAGGTTAtcgcgaTAACCTAGGTTActatag*cctgg-3′; lower case italics, restriction sites; upper case italics, VanO), and was directly ligated into SalI/SbfI-digested pWW1088 thereby resulting in P_{1VanO2}.	This work
pMG256	Constitutive $VanA_2$ expression vector (P_{SV40}-$VanA_2$-pA; $VanA_2$, VanR-p65). VanR was PCR-amplified from *C. crescentus* genomic DNA using ODF150: 5′-cg*gaattc*cacc*ATGGACATGCCGCGCATAAAGCCGGGC*-3′ and ODF151: 5′- gtacg*acgcgt*ggctgtacgcgga*GTCGGCGCGAATGCT*	This work

(continued)

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Table 1. Continued

Plasmid	Description	Reference
	CCACGCCGCGC-3′ (lower case italics, restriction sites; upper case italics, annealing), digested with EcoRI/MluI and ligated into pMG18 (EcoRI/BssHII).	
pMG257	Constitutive $VanA_3$ expression vector (P_{SV40}-$VanA_3$-pA; $VanA_3$, VanR-E2F4). VanR was PCR-amplified from *C. crescentus* genomic DNA using ODF150: 5′-cgg*aattc*cacc*ATGGACATGCCGCGCATAAAGCCGGGC*-3′ and ODF151: 5′- gtacg*acgcgt*ggctgtacgcgga*GTCGGCGCGAATGCT CCACGCCGCGC*-3′ (lower case italics, restriction sites; upper case italics, annealing), digested with EcoRI/MluI and ligated into pMG19 (EcoRI/BssHII).	This work
pMG262	Vector encoding a P_{1VanO1}-driven SEAP expression unit (P_{1VanO1}-SEAP-pA; P_{1VanO1}, $VanO_1$-0bp-$P_{hCMVmin}$). pMG252 was digested using either NruI/HindIII or Eco47III/HindIII. The resulting fragments were ligated to result in pMG262 harboring one VanO-operator element.	This work
pMG263	Vector encoding a P_{1VanO3}-driven SEAP expression unit (P_{1VanO3}-SEAP-pA; P_{1VanO3}, $VanO_3$-0bp-$P_{hCMVmin}$). pMG252 was digested using either EcoRV/HindIII or Eco47III/HindIII. The resulting fragments were ligated to result in pMG263 harboring three VanO-operator elements.	This work
pMG264	Vector encoding a P_{1VanO4}-driven SEAP expression unit (P_{1VanO4}-SEAP-pA; P_{1VanO4}, $VanO_4$-0bp-$P_{hCMVmin}$). pMG252 was digested using either EcoRV/HindIII or NruI/HindIII. The resulting fragments were ligated to result in pMG264 harboring four VanO-operator elements.	This work
pMG265	Vector encoding a P_{2VanO2}-driven SEAP expression unit (P_{2VanO2}-SEAP-pA; P_{2VanO2}, $VanO_2$-2bp-$P_{hCMVmin}$). 2bp-$P_{hCMVmin}$-SEAP was excised from pMG20 (SbfI/XhoI) and ligated into pMG252 (SbfI/XhoI).	This work
pMG266	Vector encoding a P_{3VanO2}-driven SEAP expression unit (P_{3VanO2}-SEAP-pA; P_{3VanO2}, $VanO_2$-4bp-$P_{hCMVmin}$). 4bp-$P_{hCMVmin}$-SEAP was excised from pMG21 (SbfI/XhoI) and ligated into pMG252 (SbfI/XhoI).	This work
pMG267	Vector encoding a P_{4VanO2}-driven SEAP expression unit (P_{4VanO2}-SEAP-pA; P_{4VanO2}, $VanO_2$-6bp-$P_{hCMVmin}$). 6bp-$P_{hCMVmin}$-SEAP was excised from pMG22 (SbfI/XhoI) and ligated into pMG252 (SbfI/XhoI).	This work
pMG268	Vector encoding a P_{5VanO2}-driven SEAP expression unit (P_{5VanO2}-SEAP-pA; P_{5VanO2}, $VanO_2$-8bp-$P_{hCMVmin}$). 8bp-$P_{hCMVmin}$-SEAP was excised from pMG23 (SbfI/XhoI) and ligated into pMG252 (SbfI/XhoI).	This work
pMG269	Vector encoding a P_{6VanO2}-driven SEAP expression unit (P_{6VanO2}-SEAP-pA; P_{6VanO2}, $VanO_2$-10bp-$P_{hCMVmin}$). 10bp-$P_{hCMVmin}$-SEAP was excised from pMG24 (SbfI/XhoI) and ligated into pMG252 (SbfI/XhoI).	This work
pMG270	Autoregulated vanillic acid-controlled SEAP expression vector (P_{1VanO2}-SEAP-$IRES_{PV}$-$VanA_1$-pA). $VanA_1$ was excised from pMG250 (SspI/NotI) and ligated into pMG252 (SspI/NotI).	This work
pPur	Selection vector conferring puromycin resistance.	Clontech
pSAM200	Constitutive tTA expression vector (P_{SV40}-tTA-pA).	69
pSEAP2-Control	Constitutive SEAP expression vector (P_{SV40}-SEAP-pA).	Clontech
pSS158	P_{hCMV}-driven SAMY expression vector (P_{hCMV}-SAMY-pA).	50
pSV2neo	Selection vector conferring neomycin resistance.	Clontech
pWW35	Constitutive ET1 expression vector (P_{SV40}-ET1-pA; ET1, E-VP16).	18
pWW43	P_{SV40}-driven expression vector encoding the macrolide-dependent transrepressor ET4: P_{SV40}-ET4-pA; ET4, E-KRAB.	18

d2EYFP, destabilized variant of the yellow fluorescent protein; E2F4, transactivation domain of the human E2F transcription factor 4; ET1, macrolide-dependent transactivator (E-VP16); ET4, macrolide dependent transrepressor (E-KRAB); ETR, operator specific for macrolide-dependent transactivators; $IRES_{PV}$, polioviral internal ribosome entry site; KRAB, Human Kruppel-associated box protein; NF-κB, human transcription factor; O_{TtgR}, TtgR-specific operator; p65, transactivation domain of NF-κB; pA, polyadenylation site; P_{ETR3}, macrolide-responsive promoter; P_{hCMV}, human cytomegalovirus immediate early promoter; $P_{hCMVmin}$, minimal P_{hCMV}; $P_{hCMV*-1}$, tetracycline-responsive promoter; P_{SV40}, simian virus 40 promoter; $P_{TtgR1-6}$, phloretin-responsive promoters containing different spacers between O_{TtgR} and $P_{hCMVmin}$; P_{UREX8}, uric acid- responsive promoter containing 8 hucO-operator sites in 3′ of a P_{CMV} promoter; $P_{1-6VanO2}$, vanillic acid-responsive promoters containing different spacers between VanO and $P_{hCMVmin}$; $P_{1VanO1-4}$, vanillic acid-responsive promoters harboring 1, 2, 3 or 4 VanO-operator repeats in front of $P_{hCMVmin}$; SEAP, human placental secreted alkaline phosphatase; SAMY, *Bacillus stearothermophilus*-derived secreted α-amylase; TtgR, repressor of the *Pseudomonas putida* DOT-T1E ABC multi-drug efflux pump; $TtgA_2$, phloretin-dependent transactivator (TtgR-p65); $TtgA_3$, phloretin-dependent transactivator (TtgR-E2F4); VanAB, gene cluster within *C. crescentus* that plays a role within the vanillic acid metabolism; VanO, VanR specific operator; VanR, repressor of the *C. crescentus* VanAB gene cluster; VP16, *Herpes simplex* virus-derived transactivation domain.

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SEAP production was assessed by culturing CHO-VAC_{12} (100 000 cells/ml) for 144 h while alternating vanillic acid concentrations from 0 to 500 μM every 48 h.

Quantification of reporter gene expression

Production of the human placental secreted alkaline phosphatase (SEAP) and the heat-stable *Bacillus stearothermophilus*-derived secreted α-amylase was quantified as described previously (49,50).

Inducer compounds: vanillic acid and its derivatives

The following were prepared as 50 mM stock solutions in 70% (v/v) EtOH and adjusted to pH 7 using 2.5 M NaOH when required: 2-vanillic acid (2-hydroxy-3-methoxy benzoic acid, ABCR, Karlsruhe, Germany, Cat. No. AB177480), 2-vanillin (2-hydroxy-3-methoxybenzal dehyde, ABCR, Cat. No. AB117268), acetovanillone (4′-hydroxy-3′-methoxyacetophenone, ABCR, Cat. No. AB125832), benzaldehyde (Acros, Geel, Belgium, Cat. No. 378361000), benzoic acid (ABCR, Cat. No. AB113879), benzyl acetate (ABCR, Cat. No. AB131641), benzyl alcohol (ABCR, Cat. No. AB171491), ethyl-vanillate (ABCR, Cat. No. AB178082), ethyl-vanillin (3-ethoxy-4-hydroxybenzaldehyde, ABCR, Cat. No. AB126381), eugenol (ABCR, Cat. No. AB111881), homovanillic acid (Sigma, St Louis, MO, USA, Cat. No. H1252-1G), isovanillic acid (3-hydroxy-4-methoxybenzoic acid, ABCR, Cat. No. AB117271), isovanillin (3-hydroxy-4-methoxybenzaldehyde, ABCR, Cat. No. AB117270), methyl-vanillate (ABCR, Cat. No. AB132603), protocatechualdehyde (3,4-dihydroxy benzaldehyde, ABCR, Cat. No. AB110948) and vanillin (ABCR, Cat. No. AB117415). Vanillic acid (Fluka, Buchs, Switzerland, Cat. No. 94770-10G) was prepared as a 50 mM stock solution in water and adjusted to pH 7 with 2.5 M NaOH. All solutions were used at a final concentration of 250 μM unless otherwise indicated. Tetracycline (Sigma, Cat. No. T7660) was prepared as a 1 mg/ml stock solution in H_2O, and erythromycin (Fluka, Cat. No. 45673) as a stock solution of 1 mg/ml in ethanol. Both antibiotics were used at a final concentration of 2 μg/ml.

In vivo methods

The CHO-K1 cells, engineered for vanillic acid-controlled SEAP expression (CHO-VAC_{12}) and for constitutive SEAP expression [CHO-$SEAP_{18}$ (51)], were encapsulated in 400 μm alginate-poly-(L-lysine)-alginate beads (400 cells/capsule) using an Inotech Encapsulator Research IE-50R (EncapBioSystems Inc., Greifensee, Switzerland) according to the manufacturer's instructions and the following parameters: 0.2 mm nozzle, 20 ml syringe at a flow rate of 405 U, nozzle vibration frequency of 1116 Hz and 950 V for bead dispersion. Female OF1 mice (oncins France souche 1, Charles River Laboratories, France; $n = 8$ for all experiments) were implanted intraperitoneally with 700 μl of FCS-free ChoMaster® HTS containing 4×10^6 encapsulated CHO-VAC_{12} cells. Control mice were implanted with microencapsulated CHO-K1 or CHO-$SEAP_{18}$. One hour after implantation, vanillic acid (50 mg/ml in PBS, pH7) was administered twice daily by injection for the next three days at doses ranging from 0 to 500 mg/kg. After 72 h, the mice were sacrificed, blood samples were collected retroorbitally and SEAP levels were quantified in the serum, which was isolated using a microtainer SST tube according to the manufacturer's instructions (Beckton Dickinson, Plymouth, UK, Cat. No. 365968). All the experiments involving mice were performed according to the directives of the European Community Council (86/609/EEC), approved by the French Republic (No. 69266310) and performed by Marie Daoud El-Baba at the Université de Lyon, F-69622 Villeurbanne, France.

Preparation of mouse organ extracts

Organs (kidney, liver, lung, muscle) of female OF1 mice were homogenized in PBS (1:2 (v/v); organ:PBS) using an IKA T18 basic ULTRA-TURRAX® disperser (IKA GmbH, Staufen, Germany), mixed with 10% (v/v) hydrochloric acid (Fluka, Cat. No. 35327) for 5 s using a vortex and incubated with 6 ml ethyl acetate (Sigma Cat. No. 270989) at 4°C while shaking overnight to extract vanillic acid from the tissue samples. As positive control a liver and a kidney sample were spiked with 100 μl of a 50 mM vanillic acid stock solution prior to tissue homogenization. The tissue homogenates were then centrifuged for 10 min at 6000 *g* and 4°C and the supernatants were dried using a rotary evaporator (Rotavapor R-215, Büchi Labortechnik AG, Flawil, Switzerland). Dried samples were re-suspended in 300 μl methanol (Sigma, Cat. No. 322415), dried again using an Eppendorf concentrator (Eppendorf concentrator plus, Vaudaux-Eppendorf, Schönenbuch, Switzerland, Cat. No. 5305 000.304) and were finally resuspended in 300 μl dimethyl sulfoxide (DMSO, Sigma, Cat. No. D4540). 5 μl of organ extracts were mixed with 500 μl ChoMaster® HTS and added to CHO-K1 cultures containing the VAC_{OFF} or the VAC_{ON} expression systems.

RESULTS

Design of vanillic acid-responsive mammalian transcription-control devices

Using the basic parts that control vanillic acid metabolism in *C. crescentus*, the transcriptional repressor VanR and its target operator VanO, we have designed two different vanillic acid-responsive transcription-control devices that either induce (VAC_{ON}) or repress (VAC_{OFF}) target gene transcription in the presence of the food additive vanillic acid.

The VAC_{ON} system was assembled by fusing VanR C-terminally to the human Krueppel-associated box [KRAB; (52)] domain to generate a mammalian transsilencer ($VanA_4$) that binds and represses a chimeric promoter (P_{VanON8}) consisting of a constitutive human cytomegalovirus immediate early promoter (P_{hCMV}) containing an octameric VanO operator module ($VanO_8$) immediately downstream. Vanillic acid triggers the release of $VanA_4$, which derepresses P_{VanON8} and results in P_{hCMV}-driven transgene expression (Figure 1A). When

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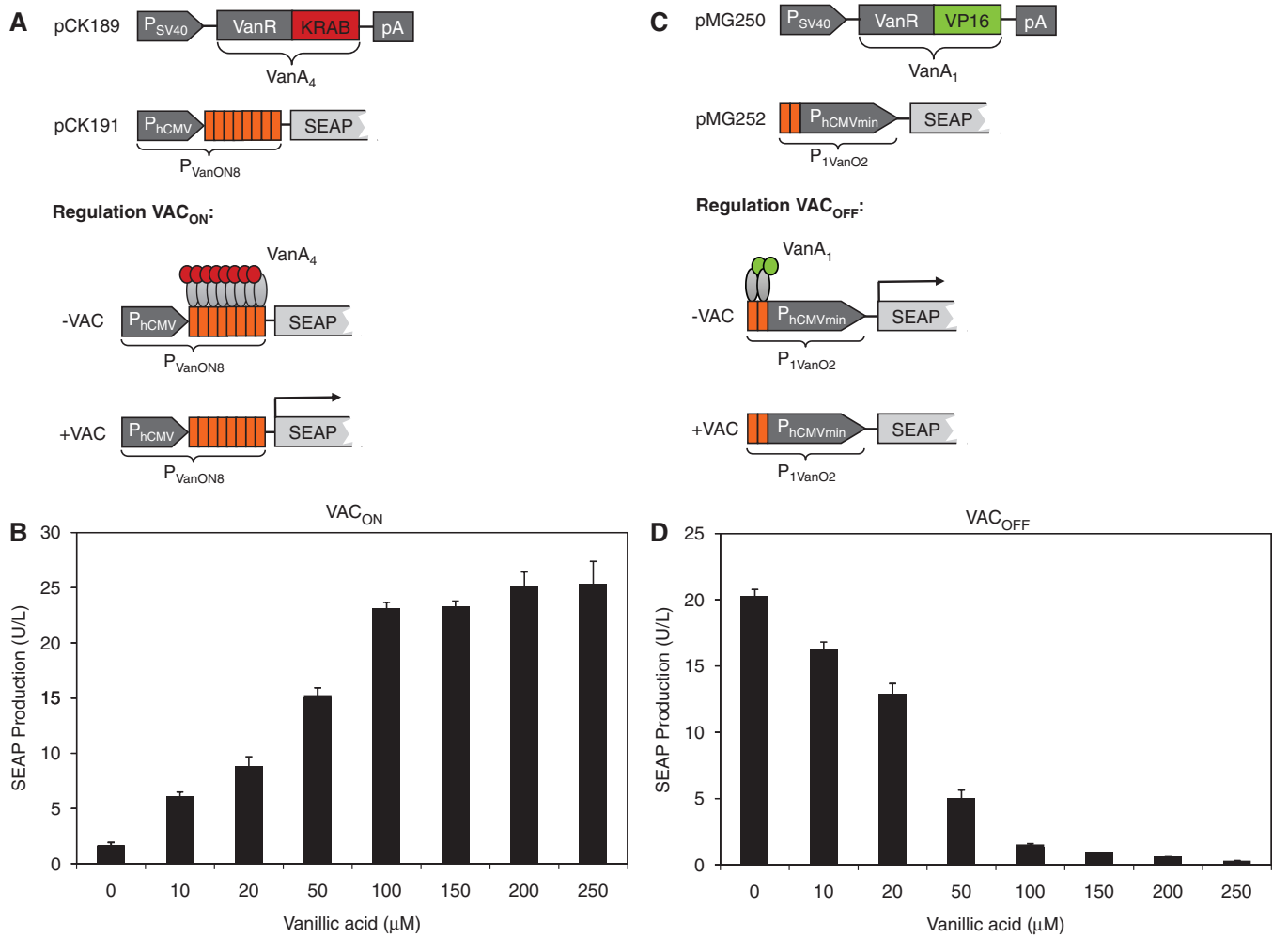

Figure 1. Design and validation of the VAC_{ON} and VAC_{OFF} systems. (**A** and **B**) Diagram and functionality of the VAC_{ON} system. (A) VanR was fused to the KRAB transrepressor domain, a human Krueppel-associated box protein, resulting in $VanA_4$ (VanR-KRAB), which was expressed by the constitutive human cytomegalovirus immediate early promoter (P_{hCMV}) (pCK189). The vanillic acid-inducible promoter P_{VanON8} harbors eight VanO operator sites immediately 3′ of a constitutive Simian virus 40 promoter (P_{SV40}) that was set to drive the human placental secreted alkaline phosphatase (SEAP) (pCK191). OFF status: $VanA_4$ is constitutively expressed and, in the absence of vanillic acid (–VAC), binds to P_{VanON8} and represses SEAP expression. ON status: in the presence of vanillic acid (+VAC), $VanA_4$ is released from P_{VanON8} which fully induces SEAP expression. (B) CHO-K1 cells were transiently transfected with pCK189 (P_{SV40}-$VanA_4$-pA) and pCK191 (P_{VanON8}-SEAP-pA) and SEAP-expression profiles were assessed 48 h after cultivation of the cells in medium containing different concentrations of vanillic acid (0–250 µM). (**C** and **D**) Diagram and functionality of the VAC_{OFF} system. (C) VanR was fused to the VP16 transactivation domain of the *Herpes simplex* virus, resulting in $VanA_1$ (VanR-VP16), which was expressed by the constitutive Simian virus 40 promoter (P_{SV40}) (pMG250). The vanillic acid-responsive promoter P_{1VanO2} contains two VanO operator sites (*ATTGGATCCAAT*AGCGCT*ATTGGATCCAAT*; VanR binding sites in italics) immediately 5′ of a minimal human cytomegalovirus immediate-early promoter ($P_{hCMVmin}$), which was set to drive the human placental secreted alkaline phosphatase (SEAP) (pMG252). ON status: $VanA_1$ is constitutively expressed and, in the absence of vanillic acid (–VAC), binds to P_{1VanO2} and activates SEAP expression. OFF status: in the presence of vanillic acid (+VAC), $VanA_1$ is released from P_{1VanO2} which shuts down SEAP expression. (D) CHO-K1 cells were transiently transfected with pMG250 (P_{SV40}-$VanA_1$-pA) and pMG252 (P_{1VanO2}-SEAP-pA) and SEAP-expression profiles were assessed 48 h after cultivation of the cells in medium containing different concentrations of vanillic acid (0–250 µM).

co-transfecting pCK189 (P_{hCMV}-$VanA_4$-pA) and pCK191 (P_{VanON8}-SEAP-pA) into CHO-K1 expression of SEAP was insignificant (1.70 ± 0.23 U/L). However, when adding increasing concentrations of vanillic acid (0–250 µM) SEAP expression was dose-dependently induced up to a level of 25.3 ± 2.08 U/l (Figure 1B).

The VAC_{OFF} system was designed by fusing VanR C-terminally to the *Herpes simplex* virus transactivation domain VP16 to generate a chimeric transcription factor ($VanA_1$) that binds to two VanO-operator sequences separated only by an Eco47III restriction site ($VanO_2$, 5′-ATTGGATCCAATagcgctATTGGATCCAAT-3′, VanO operator upper case) and activates a 3′-placed minimal version of P_{hCMV} ($P_{hCMVmin}$) (P_{1VanO2}). Vanillic acid triggers the release of $VanA_1$, which inactivates P_{1VanO2} and shuts transgene expression down (Figure 1C). Co-transfection of pMG250 (P_{SV40}-$VanA_1$-pA), and pMG252 (P_{1VanO2}-SEAP-pA) into CHO-K1 resulted in high SEAP expression levels (20.3 ± 0.5 U/l) comparable to an isogenic constitutive SEAP control vector (pSEAP2-Control; P_{SV40}-SEAP-pA) (33.9 ± 0.2 U/l). However, when adding increasing concentrations of

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vanillic acid (0–250 μM) SEAP was dose-dependently shut down to almost complete repression (0.31 ± 0.01 U/l) (Figure 1D).

In order to assess the impact of vanillic acid on mammalian cell cultures, we exposed CHO-K1 cells, transiently transfected with pSEAP2-control (P_{SV40}-SEAP-pA), to increasing concentrations of vanillic acid (0–1000 μM) and profiled SEAP production as well as viable cell numbers for 48 h. The observation that SEAP levels and cell numbers remained equally high at all vanillic acid concentrations (0 μM: 33.9 ± 0.17 U/l, 9.08 ± 0.84 (10^6 cells/ml); 1000 μM: 35.4 ± 1.74 U/l, 9.22 ± 0.9 (10^6 cells/ml)) indicates that the licensed food additive has no obvious impact on cell physiology below a concentration of 1 mM. This observation was confirmed by scoring the viability of CHO-K1 and HEK-293 exposed to regulation-effective vanillate concentrations (Supplementary Figure S1). Since the basic VAC_{OFF} system shows tighter regulation performance, no epigenetic imprinting compared to the KRAB-containing VAC_{ON} design (53,54) and is the configuration of choice for the assembly of advanced synthetic multi-control gene networks (13), we chose to use VAC_{OFF} in all follow-up studies.

VAC_{OFF}-controlled transgene expression in various mammalian cell lines

Versatility of the VAC_{OFF}-system was assessed by co-transfection of pMG250 (P_{SV40}-$VanA_1$-pA) and pMG252 (P_{1VanO2}-SEAP-pA) into different rodent, monkey and human cell lines followed by cultivation for 48 h in the presence (+) and absence (–) of 250 μM vanillic acid (BHK-21: –, 0.52 ± 0.02 U/l; +, 0.05 ± 0.01 U/l; COS-7: –, 17.95 ± 0.55 U/l; +, 0.45 ± 0.02 U/l; HEK-293: –, 484.96 ± 54.24 U/l; +, 20.85 ± 1.24 U/l; HeLa: –, 7.89 ± 0.99 U/l; +, 1.38 ± 0.11 U/l; HT-1080: –, 0.65 ± 0.11 U/l; +, 0.07 ± 0.01 U/l; and NIH/3T3: –, 7.41 ± 0.16 U/l; +, 0.28 ± 0.12 U/l). SEAP production levels indicated that VAC_{OFF}-controlled transgene regulation was functional in all tested cell lines, suggesting a broad applicability of this control technology.

Optimizing the VAC_{OFF} system I—assessment of promoter variants containing varying numbers of VanO operator modules

Altering the number of operator modules in front of an inducible minimal promoter impacts the regulation performance regarding (i) maximal expression levels, as an increasing number of operator sites can recruit more transactivators and (ii) basal expression of the system's repressed state, as more transactivators have to be released from an increasing number of operator sites (27,55). To evaluate the optimal number of VanO-operator sites for VAC_{OFF}-controlled gene regulation, we constructed $VanA_1$-responsive promoter variants harbouring either one (pMG262, P_{1VanO1}-SEAP-pA; P_{1VanO1}, *Nru*I-VanO-0bp-$P_{hCMVmin}$), two (pMG252, P_{1VanO2}-SEAP-pA; P_{1VanO2}, *Nru*I-VanO-*Eco*47III-VanO-0bp-$P_{hCMVmin}$), three (pMG263, P_{1VanO3}-SEAP-pA; P_{1VanO3}, *Nru*I-VanO-*Eco*47III-VanO-6bp-VanO-0bp-$P_{hCMVmin}$) or four (pMG264, P_{1VanO4}-SEAP-pA; P_{1VanO4}, *Nru*I-VanO-*Eco*47III-VanO-6bp-VanO-*Eco* 47III-VanO-0bp-$P_{hCMVmin}$) operators immediately 5′ of the minimal promoter. Co-transfection of corresponding SEAP expression vectors harbouring the different promoter variants with pMG250 (P_{SV40}-$VanA_1$-pA) into CHO-K1, HEK-293 and BHK-21 cells and scoring of SEAP levels 48 h after cultivation in the presence and absence of 250 μM vanillic acid revealed their transcriptional performances. In all cell lines, it was observed that an increasing number of operator modules resulted in higher maximum expression levels but also in higher basal expression of the repressed status. The dimeric promoter configuration (pMG252, P_{1VanO2}-SEAP-pA) resulted in the best regulation performance with a 72-fold induction factor in CHO-KI cells and 23-fold in HEK-293 cells, while the trimeric promoter set-up showed the best performance in BHK-21 cells with a ON/OFF control factor of 11 (Figure 2A–C).

Optimizing the VAC_{OFF} system II—engineering of different vanillic acid-dependent transactivator variants

Basal and maximum expression of synthetic mammalian gene regulation systems can be influenced by the kind of mammalian transactivation domain, which is fused to the bacterial DNA-binding protein (19,56). We have therefore evaluated different transactivation domains by designing vectors containing VanR fused to the *Herpes simplex*-derived VP16 domain (pMG250, P_{SV40}-$VanA_1$-pA; $VanA_1$,VanR-VP16), the human nuclear factor kappa B (NF-κB) -derived transactivation domain p65 (pMG256, P_{SV40}-$VanA_2$-pA; $VanA_2$, VanR-p65) or the transactivation domain of the human E2F transcription factor 4 (E2F4) (pMG257, P_{SV40}-$VanA_3$-pA; $VanA_3$, VanR-E2F4). Co-transfection of corresponding transactivator-encoding expression vectors with pMG252 (P_{1VanO2}-SEAP-pA) into CHO-K1, HEK-293, BHK-21, HT-1080 and HeLa cells and scoring of SEAP levels 48 h after cultivation in the presence and absence of 250 μM vanillic acid revealed the performances of the individual transactivators. The basal as well as maximum expression levels varied considerably amongst the different transactivators. In general, E2F4 showed the weakest maximum expression levels in all tested cell lines and could therefore not compete with the best-in-class regulation performance of VP16 and p65. In CHO-K1, VP16 exhibited the highest maximum expression paired with the lowest leakiness, making it the transactivator of choice for this cell line (Table 2). Peak expression in HEK-293 cells was achieved by p65, although the high basal expression again rendered VP16 the best choice in terms of the regulation factor. In HeLa cells, only VP16 provided significant transactivation, while in BHK-21 and HT-1080 cells p65 offered the best performance (Table 2). Due to their cell-type specificity, their graded maximum transcription initiation capacities and their basal expressions in the repressed status, the three transactivators offered a selection of different expression windows and

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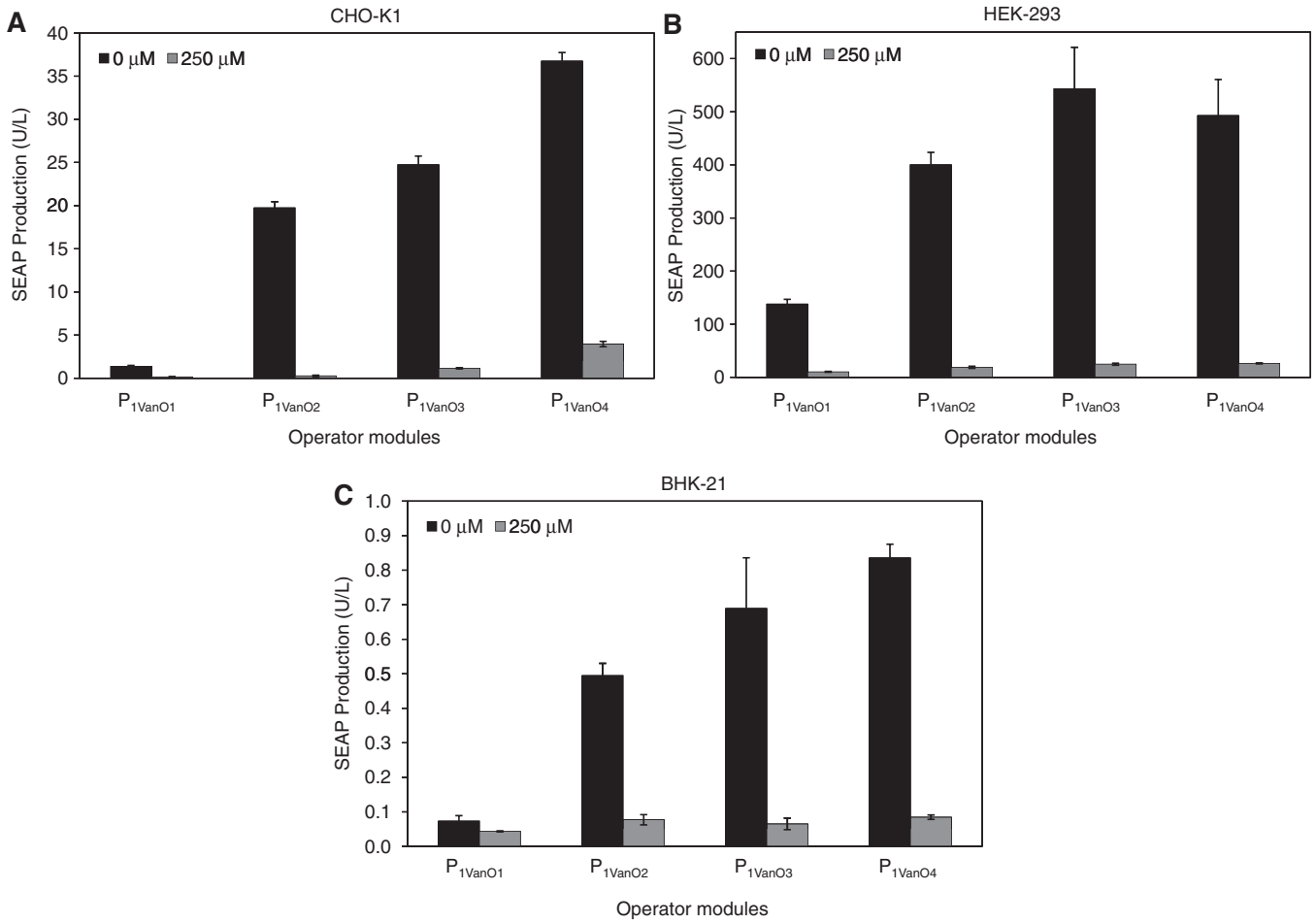

Figure 2. Validation of vanillic acid-responsive promoter variants containing different numbers of VanO operator modules. Vectors encoding SEAP expression driven by a vanillic acid-responsive promoter harbouring monomeric (pMG262), dimeric (pMG252), trimeric (pMG263) or tetrameric (pMG264) operator modules were co-transfected with pMG250 (P_{SV40}-$VanA_1$-pA) into (**A**) CHO-K1, (**B**) HEK-293 and (**C**) BHK-21 cells and SEAP production was scored after cultivation for 48 h in the presence and absence 250 μM vanillic acid.

Table 2. Combinatorial profiling of different VAC_{OFF} transactivators and promoters in various cell types

	SEAP Production (U/l)					
	pMG252/pMG250 (VP16)		pMG252/pMG256 (p65)		pMG252/pMG257 (E2F4)	
Vanillic acid (250 μM)	–	+	–	–	–	+
BHK-21	0.52 ± 0.02	0.05 ± 0.01	1.22 ± 0.05	0.08 ± 0.01	0.24 ± 0.05	0.04 ± 0.01
CHO-K1	27.06 ± 0.16	0.59 ± 0.01	26.31 ± 1.38	2.13 ± 0.17	15.02 ± 0.44	1.35 ± 0.13
HEK-293	484.96 ± 54.24	20.85 ± 1.24	1032.46 ± 63.34	54.2 ± 4.00	203.79 ± 36.96	12.79 ± 2.12
HeLa	7.89 ± 0.99	1.38 ± 0.11	1.37 ± 0.06	1.49 ± 0.08	1.49 ± 0.12	1.44 ± 0.07
HT-1080	0.65 ± 0.11	0.07 ± 0.01	1.28 ± 0.15	0.13 ± 0.02	0.13 ± 0.03	0.02 ± 0.00

SEAP production was quantified 48 h after transient co-transfection of pMG252 (P_{1VanO2}-SEAP-pA) and either pMG250 (P_{SV40}-$VanA_1$-pA; VanR-VP16), pMG256 (P_{SV40}-$VanA_2$-pA; VanR-p65) or pMG257 (P_{SV40}-$VanA_3$-pA; VanR-E2F4).

regulation factors depending on the cell line and application chosen.

Optimizing the VAC_{OFF} system III—promoter variants that differ in the distance between VanO operator modules and the minimal promoter

Maximum transcription levels and minimum leakiness are not only influenced by the number of operator modules recruiting the transactivators, but also by the relative spacing of the operator modules to the minimal promoter and the resulting torsion angle of the operator-bound transactivator (57,58). To further assess the optimal design of P_{VanO} configurations, we engineered spacers of 2 bp increments between the two VanO modules ($VanO_2$) and $P_{hCMVmin}$, resulting in SEAP expression vectors, isogenic to pMG252 (P_{1VanO2}-SEAP-pA) which harbours the default 18 bp between $VanO_2$ and

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$P_{hCMVmin}$, while pMG265 (P_{2VanO2}, $VanO_2$-2bp-$P_{hCMVmin}$), pMG266 (P_{3VanO2}, $VanO_2$-4bp-$P_{hCMVmin}$), pMG267 (P_{4VanO2}, $VanO_2$-6bp-$P_{hCMVmin}$), pMG268 (P_{5VanO2}, $VanO_2$-8bp-$P_{hCMVmin}$) and pMG269 (P_{6VanO2}, $VanO_2$-10bp-$P_{hCMVmin}$) contain an additional 2, 4, 6, 8 or 10 bp between $VanO_2$ and $P_{hCMVmin}$. Any of the vectors comprising the vanillic acid-responsive promoter variants were co-transfected with pMG250 (P_{SV40}-$VanA_1$-pA), pMG256 (P_{SV40}-$VanA_2$-pA) or pMG257 (P_{SV40}-$VanA_3$-pA) into CHO-K1 cells to evaluate the optimal promoter configuration independently of the transactivator variant. The expression of SEAP was scored 48 h after cultivation of the transfected cells in media containing 0, 50 or 250 μM of vanillic acid (Figure 3A–C). Generally, P_{1VanO2} exhibited the best regulation performance in terms of maximal expression and minimal leakiness. The promoter variants with 2, 4 and 10 bp spacers (pMG265, pMG266 and pMG269) showed expression performances, which were comparable to the best-in-class configuration harbouring no spacer (pMG252). However, pMG267 and pMG268 (6 and 8 bp increments) resulted in a much lower maximal expression. All promoter variants displayed similar expression profiles for all VanA transactivator variants, implying that chimeric promoters and transactivators can be independently optimized.

Design of an autoregulated version of the vanillic acid-responsive VAC_{OFF} expression system

Besides the conventional two-vector design, consisting of a plasmid for constitutive expression of a transactivator and a second vector encoding the responsive promoter that drives transcription of the gene of interest, we also designed an autoregulated single-vector set-up for vanillic acid-controlled transgene expression. The autoregulated design consists of P_{1VanO2} producing a dicistronic transcript sequentially encoding SEAP and $VanA_1$ preceded by a polioviral internal ribosome entry site ($IRES_{PV}$) (P_{1VanO2}-SEAP-$IRES_{PV}$-$VanA_1$-pA). Whereas SEAP is translated in a classical cap-dependent manner, translation initiation of $VanA_1$ is mediated by $IRES_{PV}$. Such an autoregulated configuration represents the most compact transgene-control design, is convenient for overcoming undesired expression variations in transient transfections and is useful for the design of noise-resistant gene networks (59). Transfection of pMG270 (P_{1VanO2}-SEAP-$IRES_{PV}$-$VanA_1$-pA) into CHO-K1 cells started leaky expression of $VanA_1$, which then in an autoregulated feedback initiated full activation of the VanA-responsive promoter P_{1VanO2} to reach maximum levels of SEAP production and co-cistronic expression of $VanA_1$. When 250 μM vanillic acid was added to the culture, the autoregulated induction was interrupted and SEAP expression remained in the fully repressed state (0 μM vanillic acid: 9.58 ± 0.29 U/l; 250 μM vanillic acid: 0.30 ± 0.04 U/l). The SEAP levels for this experiment were profiled after 48 h.

Expression kinetics, adjustability and reversibility of VAC_{OFF}-controlled transgene expression in a stably transgenic CHO-K1 cell line

Detailed characterization of long-term expression, adjustability and reversibility of the VAC_{OFF} system requires the creation of a stable cell line. We therefore established stable CHO-K1-derived VAC_{OFF}-containing cell lines (CHO-VAC) by sequential transfection of pMG250 (P_{SV40}-$VanA_1$-pA) and pMG252 (P_{1VanO2}-SEAP-pA) and subsequent clonal selection. The expression of SEAP was scored for five randomly chosen single clones after cultivation for 48 h in the presence and absence of 500 μM vanillic acid. All clones showed similar basal expression, but varied substantially in their maximum SEAP expression levels (Figure 4A). With a regulation factor of 92-fold repression, CHO-VAC_{12} showed the best regulation performance out of the five single clones. Furthermore, CHO-VAC_{12} revealed precise adjustability according to the level of vanillic acid administered to the medium (Figure 4B) and displayed unchanged maximal expression and repression levels in long-term cultures of up to 90 days (Day 0, ON: 109.13 ± 3.80 U/l, OFF: 1.19 ± 0.04 U/l; Day 90, ON: 104.36 ± 5.75 U/l, OFF: 1.45 ± 0.09 U/l). Besides excellent adjustability, rapid response kinetics and reversibility are essential for high-performance mammalian gene regulation systems. When cultivating CHO-VAC_{12} for 72 h, the system showed exponential SEAP expression kinetics without vanillic acid in the medium, whereas upon addition of 500 μM of the trigger compound, SEAP expression levels did not significantly exceed the background levels (Figure 4C). Full reversibility of the VAC_{OFF}-system was monitored when cultivating CHO-VAC_{12} for 144 h while alternating the vanillic acid concentration every 48 h between 0 and 500 μM (Figure 4D).

Compatibility of the VAC_{OFF}-system with other transgene regulation systems

The broad applicability of mammalian transgene regulation systems within complex synthetic gene networks is also determined by their ability to function interference-free alongside other regulation systems that capitalize on different inducers (11,12,60,61). To assess this important requirement, we transiently transfected CHO-VAC_{12} with the established components of the tetracycline- (TET_{OFF}) (21) or the erythromycin- (E_{OFF}) (18) responsive expression systems. Both, the TET_{OFF} (pSAM200, P_{SV40}-tTA-pA; pBP99, $P_{hCMV*-1}$-SAMY-pA) and the E_{OFF} (pWW35, P_{SV40}-ET1-pA; pBP100, P_{ETR3}-SAMY-pA) systems drove the expression of the heat-stable *Bacillus stearothermophilus*-derived secreted α-amylase [SAMY, (50)] under a tetracycline- or an erythromycin-responsive promoter, respectively. The levels of SEAP and SAMY were scored 48 h after transfection and cultivation of the CHO-VAC_{12} cell line in the presence or absence of the different inducers (500 μM vanillic acid/2 μg/ml tetracycline/2 μg/ml erythromycin) and a completely compatible, interference free and fully functional regulation performance in the same

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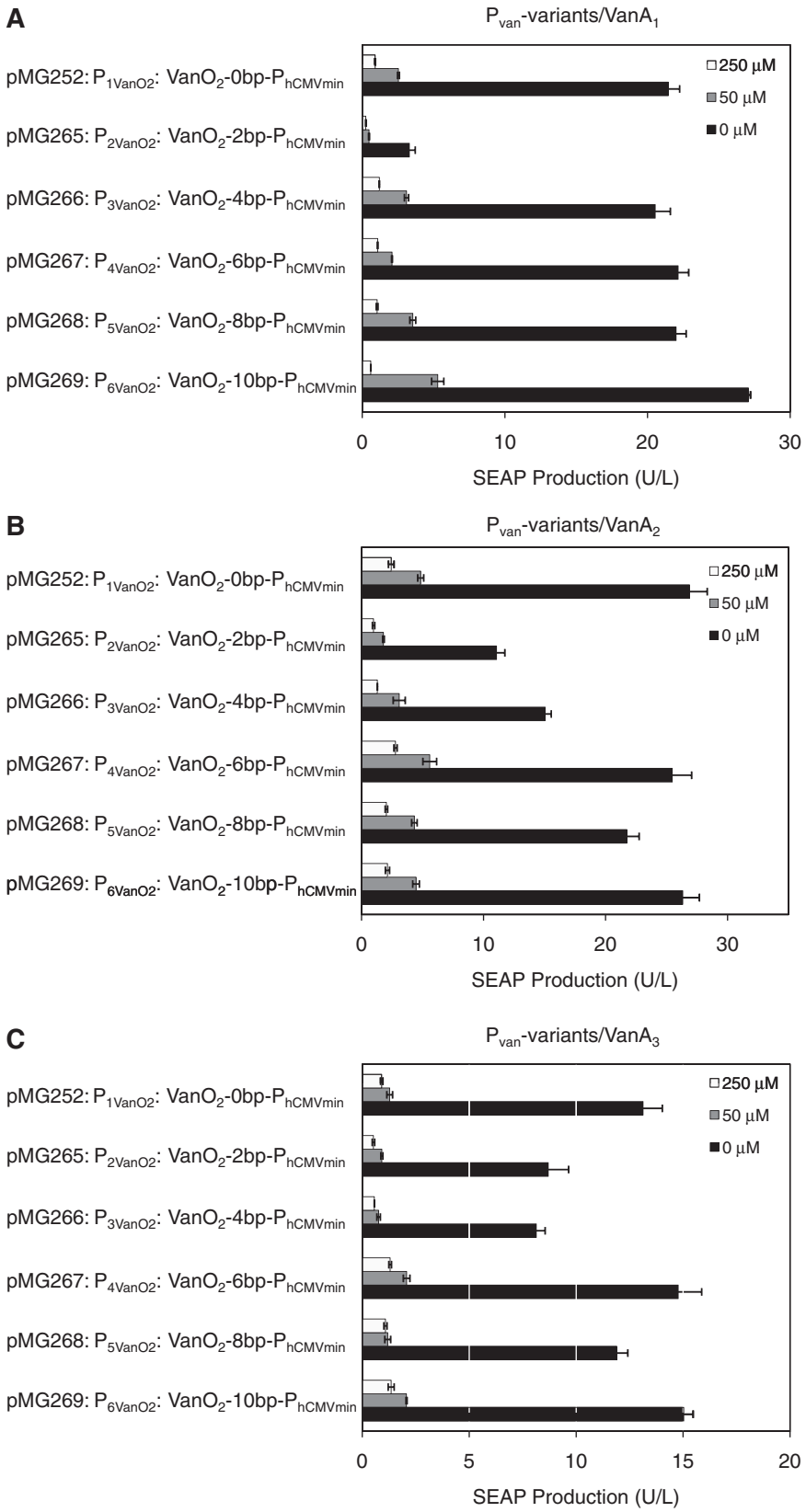

Figure 3. Combinatorial validation of the VAC_{OFF} system in different transactivator and promoter configurations. VAC_{OFF} transactivators employing different transactivation domains (**A**: $VanA_1$, VanR-VP16; pMG250) (**B**: $VanA_2$, VanR-p65; pMG256) (**C**: $VanA_3$, VanR-E2F4; pMG257) were co-transfected with different vanillic acid-responsive promoter variants containing 0 (P_{1VanO2}; pMG252), 2 (P_{2VanO2}; pMG265), 4 (P_{3VanO2}; pMG266), 6 (P_{4VanO2}; pMG267), 8 (P_{5VanO2}; pMG268) and 10 (P_{6VanO2}; pMG269) base-pair linkers between VanO and the minimal promoter into CHO-K1 cells. All promoter variants drove SEAP expression and the production was profiled 48 h after cultivation of the cells in media containing different concentrations of vanillic acid (0, 50 and 250 μM).

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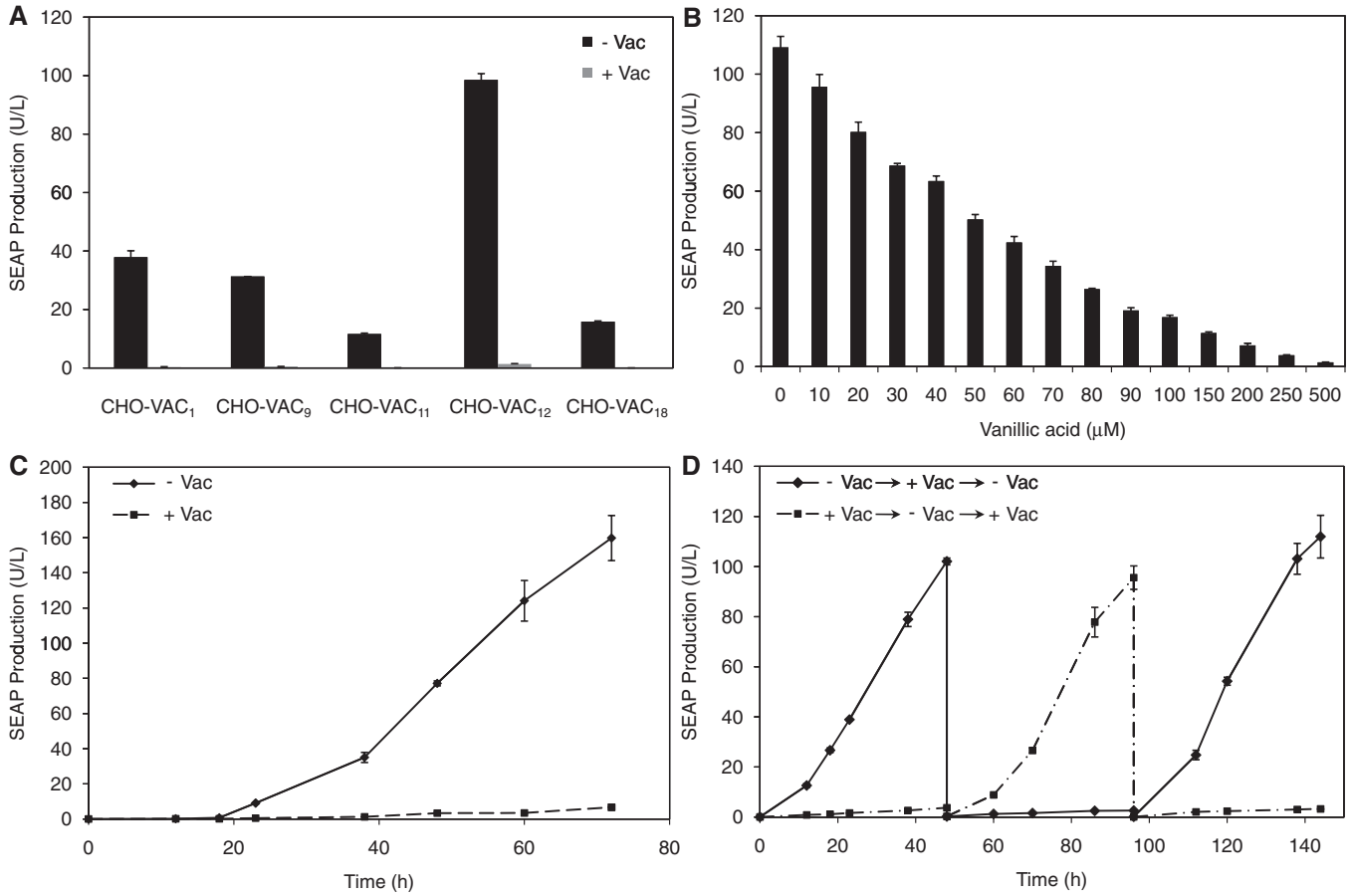

Figure 4. Characterization of stably transgenic vanillic acid-responsive CHO-K1 cell lines. CHO-K1 was stably co-transfected with pMG250 (P_{SV40}-$VanA_1$-pA) and pMG252 (P_{1VanO2}-SEAP-pA) and vanillic acid-responsive SEAP expression of the resulting CHO-VAC cell lines was analysed. (**A**) After clonal expansion, individual clones were assessed for their vanillic acid-responsive regulation performance. SEAP levels were profiled after cultivation for 48 h in the presence and absence of vanillic acid (± VAC). (**B**) The dose–response profile of CHO-VAC_{12} was profiled after cultivation for 48 h in medium containing increasing concentrations of vanillic acid (0–500 μM). (**C**) SEAP expression kinetics of CHO-VAC_{12} cultivated for 72 h in the presence and absence of 250 μM vanillic acid (± VAC). (**D**) Reversibility of vanillic acid-responsive transgene expression following periodic addition and removal of the inducer. CHO-VAC_{12} (80 000 cells/ml) were cultivated for 144 h in the presence and absence of 250 μM vanillic acid (± VAC). Every 48 h, the cell density was re-adjusted to 80 000 cells/ml and the cells were cultivated in fresh medium with reversed vanillic acid concentrations.

mammalian cells was demonstrated for the VAC_{OFF}, TET_{OFF} and E_{OFF} systems (Table 3).

Specificity of the VAC_{OFF} system

VanR plays a key role in controlling lignin biodegradation of *C. crescentus*. One of the commonly produced compounds in this pathway is vanillic acid, but closely related compounds were also suggested as being able to directly interact with VanR (43). To assess the specificity of the VAC system and the capability of isomeric compounds of vanillic acid to interact with the synthetic mammalian-adapted $VanA_1$ transactivator, we cultivated CHO-VAC_{12} for 48 h in media containing 0, 250 and 500 μM of a comprehensive set of compounds closely related to vanillic acid (2-vanillic acid, 2-vanillin, acetovanillone, benzaldehyde, benzoic acid, benzyl acetate, benzyl alcohol, ethyl-vanillate, ethyl-vanillin, eugenol, homovanillic acid, isovanillic acid, isovanillin, methyl-vanillate, protocatechualdehyde and vanillin). In parallel, we assessed the toxicity of these compounds on a stable CHO-K1-derived cell line constitutively expressing SEAP [CHO-$SEAP_{18}$; (51)]. Some of the compounds were toxic when administered to CHO-$SEAP_{18}$ cells at concentrations of 500 μM (2-vanillin, eugenol, isovanillin, methyl-vanillate and protocatechualdehyde), but none of the 16 tested structures was able to regulate the VAC_{OFF} system. This implies an extraordinary specificity of the VAC_{OFF} system for vanillic acid (Supplementary Table S1).

Vanillic acid—transgene expression in mice is mediated by a food additive

For subsequent applications in functional genomics research or future gene- and cell-based therapies, it is essential that state-of-the-art gene regulation systems are functional within entire organisms. To validate the vanillic acid-controlled gene regulation system *in vivo*, we implanted microencapsulated CHO-VAC_{12} intraperitoneally into mice. The treated mice were given a dose of vanillic acid within the range of 0–500 mg/kg

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Table 3. Compatibility of vanillic acid-, erythromycin- and tetracycline-responsive transgene control systems

Inducer	−Tet /−Vac	−Tet /+Vac	+Tet/−Vac	+Tet/+Vac
CHO-VAC_{12} transfected with the tetracycline-responsive regulation system				
Relative SEAP production (%)	100 ± 5.62	2.18 ± 0.31	101.04 ± 6.21	2.07 ± 0.29
Relative SAMY production (%)	100 ± 5.03	99.06 ± 4.53	4.53 ± 0.52	5.01 ± 1.61
Inducer	−EM/−Vac	−EM/+Vac	+ EM/−Vac	+EM/+Vac
CHO-VAC_{12} transfected with macrolide-responsive regulation system				
Relative SEAP production (%)	100 ± 6.31	2.56 ± 0.09	102.19 ± 7.08	1.95 ± 0.59
Relative SAMY production (%)	100 ± 5.67	98.97 ± 7.73	5.20 ± 0.68	4.83 ± 1.22

CHO-VAC_{12} were co-transfected with pSAM200 (P_{SV40}-tTA-pA) and pBP99 ($P_{hCMV*-1}$-SAMY-pA) (A) or pWW35 (P_{SV40}-ET1-pA) and pBP100 (P_{ETR3}-SAMY-pA) and grown for 48 h in the presence and absence of vanillic acid (Vac, 250 µM), erythromycin (EM, 2 µg/ml) or tetracycline (Tet, 2 µg/ml) before SEAP and SAMY production was assessed.

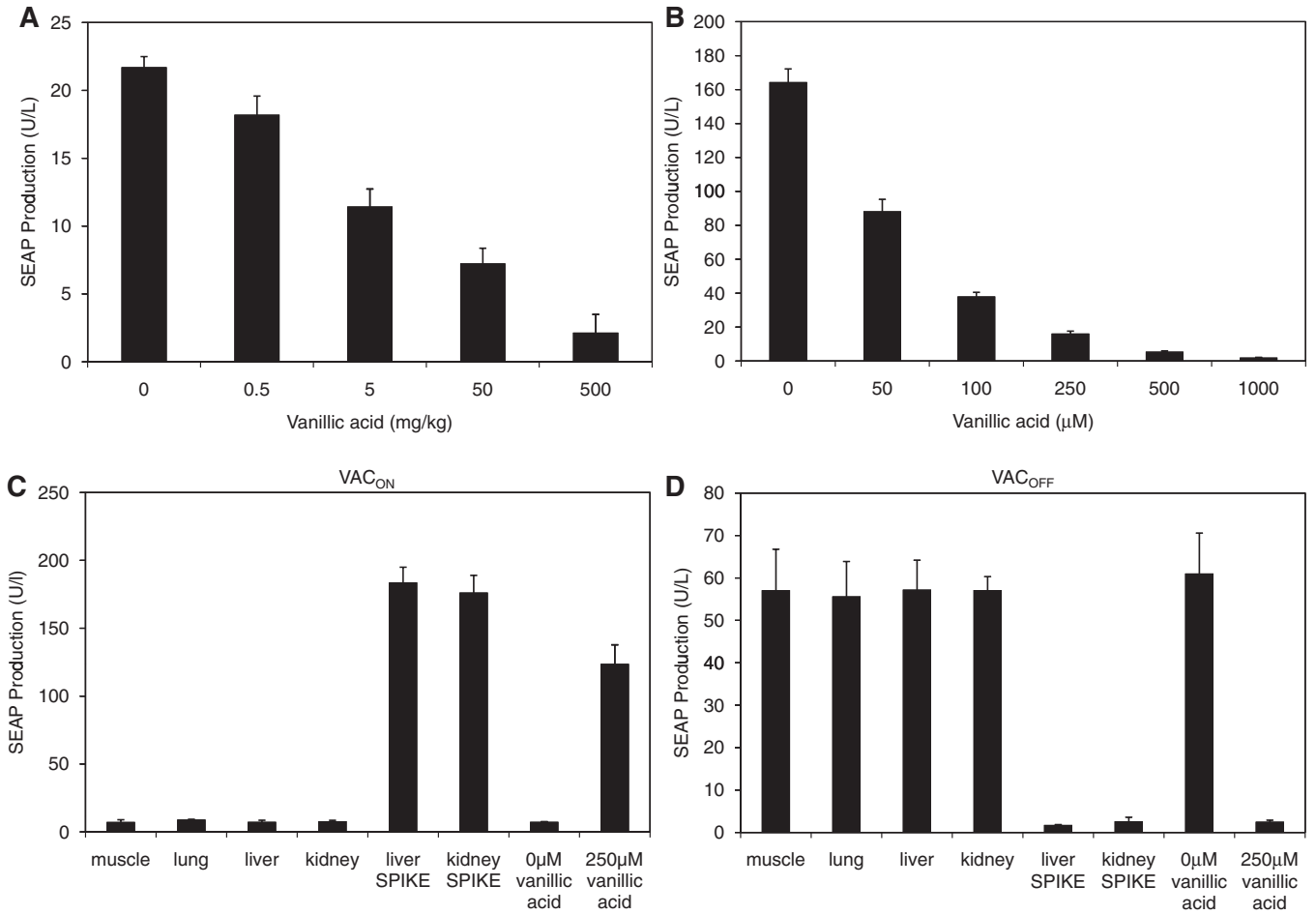

Figure 5. Vanillic acid-controlled SEAP expression in mice. (**A**) CHO-VAC_{12} cells were microencapsulated in alginate-poly-(L-lysine)-alginate beads and implanted intraperitoneally into female OF1 mice (4×10^6 cells per mouse). The implanted mice received different concentrations of vanillic acid twice daily. Seventy-two hours after implantation, the level of SEAP in the serum of the mice was determined. Data represent mean ± SEM of 8 mice per treatment group. (**B**) SEAP expression profiles of the microencapsulated CHO-VAC_{12} implant batch were cultivated *in vitro* for 72 h at different vanillic acid concentrations. (**C** and **D**) Extracts of wild-type mouse organs were assessed for their vanillic acid content based on their ability to induce the (C) VAC_{OFF} or (D) VAC_{ON} systems. Vanillic acid-spiked organs were used as positive control. All samples were compared to the effect of 250 µM vanillic acid to show the fully induced state of the systems. All extracts were added to CHO-K1 cells transiently transfected with either the VAC_{ON} or the VAC_{OFF} systems and SEAP expression was assessed after a cultivation period of 48 h.

twice daily. SEAP levels quantified in the blood of treated animals 72 h after implantation showed vanillic acid-dependent dose–response characteristics comparable to the control experiment using the same batch of microencapsulated CHO-VAC_{12} cells exposed to vanillic acid in an *in vitro* setting (Figure 5A and B). The serum SEAP levels of control mice, encapsulated with constitutively expressing CHO-$SEAP_{18}$, were unresponsive to

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vanillic acid treatment of a twice-daily dose of 500 mg/kg and thus showed similar expression levels as untreated mice containing CHO-VAC_{12} implants (0 mg/kg vanillic acid: 15.37 ± 1.57 U/l; 500 mg/kg vanillic acid: 16.61 ± 1.33 U/l). Since vanillic acid is a standard food additive it may in principle be present in the animal and interfere with VAC_{OFF}-based fine-tuning of transgenes. We have therefore analysed whether liver, kidney, muscle and lung tissue extracts of mice kept on a standard diet could interfere with CHO-K1 cultures engineered for VAC_{OFF}- and VAC_{ON}-controlled SEAP expression. Unlike positive controls consisting of organ extracts spiked with vanillic acid, none of the organ extracts produced from wild-type mice kept on a standard diet showed any interference with the VAC_{ON} or VAC_{OFF} systems (Figure 5C and D).

DISCUSSION

Heterologous transgene expression control by non-toxic small molecule inducers remains one of the major challenges for future gene- and cell-based therapies as well as for biopharmaceutical manufacturing of difficult-to-produce protein therapeutics. The employed inducers must meet high medical standards and also need to be physiologically inert for long-term applications in humans. Antibiotics, steroid hormones, immunosuppressive drugs and a multitude of other regulating molecules fail to meet these requirements due to their high levels of side effects, particularly when given over a long period of time (62–64).

Phenolic acids are a class of compounds that are naturally produced by plants, and are therefore present in vegetables and fruits that are widely distributed throughout human dietary products, like coffee, wine, beer and vanilla (65). In general, phenolic acids are said to possess many physiological and pharmacological functions (66) and vanillic acid, in particular, was successfully evaluated as a suppressor of a potent snake venom (33), cell apoptosis in Neuro-2A cells (35,36), immune-mediated liver inflammation in mice (37) and carcinogenesis (34). Being a licensed food additive with a very agreeable smell (which also enables vanillic acid to be used in fragrances), this specific phenolic acid combines the ideal properties for functioning as a physiologically inert inducer molecule in future gene- and cell-based therapies. This judgment is supported by the reported LD50 value of 5 g/kg, which was tested intraperitoneally in rats (67).

Combining the elements of the *C. crescentus* VanR-regulated VanAB gene cluster and mammalian transactivation and transsilencing domains, we designed the novel mammalian heterologous transgene regulation systems VAC_{ON} and VAC_{OFF}, which respond exclusively to the licensed food additive vanillic acid. Even closely related compounds with very similar structure are unable to regulate the vanillic acid control circuits. The generic design of the VAC_{OFF} system allows for several configurations using (i) diverse numbers of operators, (ii) different mammalian transactivation domains and (iii) variable distances between the specific operator site and the minimal promoter, to provide a toolbox for a wide variety of applications. Due to the high modularity of the VAC_{OFF} system, we were able to provide a specific configuration for all of the tested cell types, which exhibited an optimal regulation performance with high maximal expression and full reversibility. Furthermore, the VAC_{OFF} system demonstrated interference-free regulation characteristics when employed in parallel settings with the TET_{OFF} (21) and the E_{OFF} systems (18). Owing to its unprecedented specificity the presented regulation unit is likely to be an ideal building block for complex synthetic networks operating with various inducer inputs. The fact that vanillic acid is a natural plant component, which is licensed as food additive may facilitate its approval by governmental agencies for applications in future biopharmaceutical manufacturing scenarios as well as in gene- and cell-based therapies.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Table 1 and Supplementary Figure 1.

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