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TALE-induced cell death executers – an origin outside immunity?

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Abstract

Phytopathogenic bacteria inject effector proteins into plant host cells to promote disease. Plant resistance (*R*) genes encoding nucleotide-binding leucine-rich repeat (NLR) proteins mediate recognition of functionally and structurally diverse microbial effectors, including transcription-activator like effectors (TALEs) from the bacterial genus *Xanthomonas*. TALEs bind to plant promoters and transcriptionally activate either disease-promoting host susceptibility genes or cell death-inducing executor-type *R* genes. It is perplexing that plants contain TALE-perceiving executor type *R* genes in addition to NLRs that also mediate recognition of TALE-containing xanthomonads. We will review recent findings on the evolvability of TALEs, which suggest that the native function of executors is not in plant immunity, but possibly in regulation of developmentally-controlled programmed cell death (PCD) processes.

Keywords

Xanthomonas ; executor; programmed cell death (PCD); transcription activator-like effector (TALE); evolution; nucleotide-binding leucine-rich repeat (NLR) protein

How did the TALE DNA binding domain evolve and what are its implications for plant breeding?

Transcription-activator like effectors (TALEs) from the plant-pathogenic bacterial genus *Xanthomonas* have attracted considerable attention in the scientific community due to their programmable DNA binding domain that can be engineered to bind DNA motifs of desired lengths, nucleotide composition, and epigenetic makeup [1–3]. Recent studies uncovered that native TALE DNA binding domains are highly evolvable and capable of rapidly adjusting their target specificity to either induce expression of disease-promoting plant susceptibility (*S*) genes or to avoid transcriptional activation of matching plant resistance (*R*) genes [4,5]. Herein, we will discuss the implications of these new findings in the context of

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recently developed molecular breeding strategies, whereby TALE-targeted *S* and *R* gene promoters have been engineered to generate crops with broad spectrum *Xanthomonas* resistance [6–10]. Moreover, we will discuss *executors*, a functionally and structurally unique plant *R* gene class that trap TALEs via their 5' upstream DNA motifs causing transcriptional activation of downstream encoded cell death-inducing executor proteins [11]. We will review evidence suggesting that executor *R* genes have been mistakenly interpreted solely as components of the plant immune system, while their native function possibly lies in the execution of programmed cell death (PCD) as an integral part of developmental processes.

What are the functional domains that TALEs have evolved to activate transcription in eukaryotic host cells?

Bacteria inject TALE proteins into eukaryotic host cells where TALEs activate host gene transcription to increase susceptibility of the host. The functional domains of bacterial TALE proteins include *i*) nuclear localization signals (NLSs) that by interaction with eukaryotic importin α proteins facilitate the transfer into the host's nucleus, *ii*) a DNA binding domain that mediates interaction with host promoters, *iii*) a transcription factor binding (TFB) motif domain that by interaction with the general transcription factor TFIIAγ, recruits the host's RNA polymerase II complex, and *iv*) an activation domain (AD) that by yet unknown means stimulates eukaryotic transcription (Figure 1).

The DNA binding domain is the largest functional unit, located in the centre of the TALE polypeptide and is composed of a variable number (typically 15-20 modules) of almost identical, tandem-arranged 33-35 amino-acid modules, referred to as repeats. Each TALE repeat binds to one base in a given DNA target sequence (referred to as effector binding element; *EBE*) and the TALE repeats form collectively a superhelical structure that winds around target sequences in the DNA helix (Figure 2). TALE repeats differ from one another predominantly in residues 12 and 13, the repeat variable diresidue (RVD). Repeat residue 13, the base-specifying residue (BSR), faces towards the DNA bases in the *EBE* sense strand and defines the base preference of a given repeat. Notably, native *Xanthomonas* TALEs contain RVDs with pronounced base preference ('specific RVDs'; e.g. "NI" pairs with adenine and "HD" pairs with cytosine) as well as RVDs that have either relaxed specificity (e.g. "NN" pairs with guanine or adenine) or no base preference (e.g. "NS"). RVDs with relaxed or no base preference are a typical feature of native TALE repeat arrays and probably enable TALEs to activate plant *S* genes across different accessions of a given host species even if these show single nucleotide polymorphisms (SNPs) within TALE *EBEs*. Mismatch tolerance of native TALE repeat arrays is presumably the cause of why TALEs typically not only activate the disease-promoting *S* gene, but also other plant genes that do not increase host susceptibility and that are likely off-targets. By contrast, *in vitro* assembled designer TALEs (dTALEs), fabricated for specific activation of user-defined target genes [12], typically only contain RVDs with pronounced base preference and therefore have generally few, if any, off targets.

Base-preferences of all 400 possible two-amino acid combinations in the RVD position have been decoded experimentally [13,14] and accordingly, the preferred *EBE* can be predicted for any TALE based on its RVD composition. While the preferred *EBE* can be easily predicted for any given TALE, it remains challenging to predict whether or not DNA sequences related to the preferred *EBE* will indeed mediate TALE-dependent gene activation.

In summary, predictability of TALE DNA binding specificity provides indications on host target genes and the linked biological processes that a given TALE possibly manipulates. However, *in silico* prediction of TALE targets typically produces many false-positives, and experimental validation of these predicted targets is imperative to uncover TALE-manipulated plant processes.

How do TALEs manipulate plant processes to promote bacterial disease?

Xanthomonads populate plant intercellular spaces (the apoplast) and increase their virulence via injection of TALEs into plant cells. Upon injection, TALEs translocate to the nucleus, scan the host genome for compatible *EBEs*, then bind to and transcriptionally activate typically a handful of plant genes. Generally, TALEs target only one disease-promoting host *S* gene while all other TALE-target genes are likely off-targets. As such, TALE-activated host *S* genes that promote bacterial virulence have been identified for several *Xanthomonas*-host interactions [2,15]. The mechanistic basis of how *S* gene expression increases host susceptibility is most well understood for plant *SWEET* genes, which are transcriptionally activated by TALEs from xanthomonads infecting rice (*Oryza sativa*) [16,17], cotton (*Gossypium* spp.) [18], and cassava (*Manihot esculenta*) [19]. In non-infected plant tissue, expression of *SWEETs* is exclusive to phloem parenchyma cells whereby SWEETs mediate sugar efflux from the cytoplasm into the apoplast followed by sugar uptake into sieve element companion cells [20]. TALE-induced, ectopic expression of *SWEETs* results in a release of sugar from host cells into the apoplastic lumen, where increased sugar concentrations promote bacterial proliferation, most likely by serving as a carbon source for xanthomonads.

In *Xanthomonas*-host interactions where TALE-dependent *S* gene activation is crucial for bacterial pathogenicity, plant accessions where the *S* gene is not preceded by a TALE-compatible *EBE* will not support bacterial growth and will accordingly be resistant to *Xanthomonas*. The recessively inherited rice resistance genes *xa13*, *xa25*, and *xa41* are such examples where naturally occurring DNA polymorphisms upstream of rice *SWEET* genes inhibit *S* gene activation by matching TALE proteins [16,21,22]. Recently, gene editing approaches have been used to mutate functional *EBEs* upstream of host *S* genes in rice and citrus plants to engineer pest resistant plants [23].

Continuous research on rice-infecting *Xanthomonas oryzae pv. oryzae* (*Xoo*) strains has uncovered an arsenal of TALEs with distinct repeat arrays that bind to *EBEs* located within the 5’ upstream regions of *OsSWEET11, OsSWEET13,* and *OsSWEET14* genes, all encoding functionally-equivalent sugar export channels, suggesting convergent evolution of *Xoo* TALEs. Knowledge of existing TALE repertoires in *Xanthomonas* species and their

S gene targets facilitates mutagenesis of several *EBEs* in parallel, consequently generating *EBE*-depleted crops with broad-spectrum resistance to numerous *Xanthomonas* strains [7,24]. Ultimately, having the insight on TALE-targeted *S* genes and their upstream *EBEs* provides a basis to engineer pathogen-resistant crops.

Are TALE DNA binding domains evolving to promote disease or to avoid detection by the plant immune system?

TALE repeat encoding DNA segments (*repeats*) are almost identical in their DNA sequences and this sequence similarity is expected to favour evolvability of *repeat arrays* [25]. Therefore, are TALE repeat arrays with modified DNA target specificity positively selected for by evolutionary constraints? Indeed, such evolvability has been recently demonstrated for derivatives of PthA4, a TALE from the citrus (*citrus* spp.) pathogen *Xanthomonas citri* subsp. *citri* (*Xcc*) that transcriptionally activates the host *S* gene *CsLOB1* [4]. Notably, transcriptional activation of *CsLOB1* is crucial to pathogenicity of *Xcc* since *in planta* growth of bacterial mutants lacking PthA4 is severely attenuated [4]. To study if *Xcc* would evolve *in planta* TALE repeat arrays that activate *CsLOB1*, *in vitro* mutagenesis was used to generate PthA4 derivatives, that due to modification of up to five PthA4 RVDs were no longer capable of transcriptional activation of *CsLOB1*. Repeated *in planta* incubation cycles of *Xcc* strains containing these PthA4 mutant derivatives resulted in recovery of evolved TALEs (eTALEs) that regained the ability to activate *CsLOB1*. Notably each of the *CsLOB1*-activating eTALEs had an RVD composition that was distinct from PthA4, and likely these eTALEs evolved by *repeat* rearrangements of the *in vitro*-generated PthA4 mutants [4].

Complementary experimental evolution studies were carried out on PthAW1, a TALE protein identified in *Xcc* that triggers cell death in sweet orange (*Citrus sinensis*) but not in other tested citrus varieties [4]. Repeated *in planta* incubation cycles resulted in PthAW1 derivatives that had lost individual repeats, presumably to escape detection by a yet unknown executor *R* gene in sweet orange. These observations demonstrate that the DNA binding specificity of TALE repeat arrays is shaped by evolutionary constraints.

Do EBE-depleted plant cultivars provide durable resistance to TALE-containing xanthomonads?

What are the implications of the observed evolvability of TALEs on recently engineered plant lines in which *EBEs* matching to known TALEs have been depleted? The use of such plants in agriculture would cause a strong selection pressure on the pathogen population towards eTALEs that regain the capability of activating *S* genes. It is also possible that *Xanthomonas* populations already comprise strains containing a large variety of eTALEs matching to different *S* gene upstream regions as observed for *OsSWEET14* (Figure 3) and that the use of *EBE*-depleted cultivars will simply induce a shift in the population towards strains with TALEs that promote disease in the given host genotypes. Moreover, even if the CRISPR-engineered *EBE*-depleted plant varieties would offer no targets for any of the TALEs present in a pathogen population, a single *S* gene-activating eTALE in a

given *Xanthomonas* strain will still be sufficient to regain susceptibility in such engineered crops. Given the evolvability of the TALE DNA binding domain it is conceivable that the occurrence of such resistance-breaking eTALEs is just a matter of time.

How do host plants perceive the presence of TALEs?

Molecular analysis of dominantly-inherited TALE-triggered plant-immune reactions uncovered two functionally distinct *R* gene classes: *i)* constitutively-expressed *R* genes encoding nucleotide-binding-leucine rich repeat proteins (NLRs) and *ii)* transcriptionally-controlled executor *R* genes (Figure 4). NLR proteins are defined by their unifying domain composition that enables members of this immune receptor class to either directly or indirectly sense structurally and functionally diverse microbial effectors and to trigger a defence reaction that typically correlates with HR cell death to inhibit proliferation of the strictly biotrophic pathogen *Xanthomonas*. Tomato (*Lycopersicum esculentum*) Bs4, rice Xa1, and rice Xo1 are three examples of TALE-sensing NLR proteins [26–30]. Notably, all three NLRs mediate detection of multiple TALEs with distinct RVD composition [26–28,31,32]. This suggests that these TALE-sensing NLRs recognize conserved non-RVD repeat residues that collectively constitute the outer shell of the superhelical structure formed by tandem arranged TALE repeats (Figure 2). Accordingly, rearrangements in TALE repeat arrays that change the RVD composition are not expected to affect these NLR-dependent defence reactions.

While the NLR proteins Bs4, Xa1, and Xo1 all perceive non-RVD TALE repeat residues, the tomato and rice NLR proteins differ substantially at the structural and functional level. Tomato Bs4 is a predicted cytoplasmatic protein while the highly similar rice Xa1 and Xo1, that are likely encoded by allelic genes [30], are nuclear-targeted NLRs [28,33]. Moreover, recognition of full-length TALEs by rice Xa1/Xo1 is suppressed by atypical TALEs lacking the C-terminal transcription activation domains, that are known as iTALEs (interfering TALEs) [32] or truncTALEs (truncated TALEs) [34]. iTALEs/truncTALEs have been observed only in rice-infecting xanthomonads and it is currently unclear how these atypical TALEs supress Xa1/Xo1-mediated recognition of full-length TALEs.

In TALE-perceiving executor *R* genes, an upstream *EBE* sequence motif serves as a DNA-based receptor that binds consecutive TALE RVDs and forces trapped TALEs to induce transcription of the downstream encoded executor gene. The translated executor R protein triggers cell death and stops proliferation of the strictly biotrophic pathogen *Xanthomonas*. Given that executor R proteins only execute cell death but are not involved in the perception of TALEs, this has been the defining characteristic for this peculiar *R* gene class. With the exception of rice Xa10 and Xa23 which are likely paralogues of one another, the six executor R proteins that are known to date share little to no sequence homology to each other, which is in marked contrast to the structural uniformity of NLR proteins [11]. However, aside from the pepper (*Capsicum annuum)* executor Bs3, which is the only executor with homology to a protein of known function, a flavin monooxygenase [35], executors share some commonalities including their small size (about 100 amino acids), predicted transmembrane domains, and localization in the endoplasmic reticulum (Figure 5), suggesting that executors might trigger cell death by similar means.

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All in all, TALE-sensing NLRs mediate recognition of conserved non-RVD repeat residues in TALE proteins, while TALE-sensing *EBEs* upstream of executor *R* genes mediate recognition of the highly evolvable RVDs of TALE-repeats. These functional features suggest that NLRs will provide more durable and substantially broader-spectrum resistance towards TALE- but not iTALE/truncTALE containing *Xanthomonas* strains, when compared to executor *R* genes.

How can the durability of native executor R genes be improved?

To overcome the problem of the predicted lack of durability in native executor *R* genes which are only equipped with single *EBEs*, scientists engineered executor *R* genes that are preceded by several tandem-arranged *EBEs* matching to a variety of TALEs present in corresponding *Xanthomonas* pathovars, providing broad-spectrum resistance [8–10]. The concept to equip a single executor coding sequence with numerous tandem-arranged *EBE*s seems reminiscent of the aforementioned CRISPR-based depletion of multiple *EBE*s to provide broad-spectrum resistance. However, there is a marked difference since *Xanthomonas* strains that contain several TALEs each being recognized by one of many tandem-arranged *EBE*s, the given strain must mutate several TALEs simultaneously to escape detection by such engineered executor alleles. This is in contrast to the aforementioned *EBE*-depleted pathogen-resistant plants, where a single eTALE is sufficient to regain host compatibility. In essence, promoter traps with tandem-arranged *EBEs* are likely superior in terms of durability as compared to *EBE*-depleted host plants. However, this approach relies on the presence of a transgene which still lacks acceptance in the public view.

TALE-trapping EBEs upstream of executors; evolved due to selection pressure?

As stated before, TALEs are highly evolvable and can escape detection by matching executor *R* genes via repeat rearrangements resulting in TALEs with modified DNA target specificity. This raises the following question: why would the plant immune system evolve TALE-specific immune receptors that rely on the most evolvable TALE domain? Circumstantial evidence suggests that executors with TALE-compatible *EBEs* did not evolve as TALE-specific immune receptors, but that their identification is simply a consequence of their specific *in planta* activity (see below). We postulate that the discovery of TALE-recognizing executor alleles is simply the result of extensive germplasm screens in which TALE-containing xanthomonads were used to uncover plant accessions that show TALE-dependent HRs. In such phenotypic screens TALEs effectively act as molecular probes that scan the genome of a given plant accession for the presence of TALE-compatible *EBE*s upstream of cell-death inducing open reading frames. Similar to an activation-tagging screen that uses random promoter integration resulting in the expression of nearby genes, the *EBE* mediates TALE-dependent ectopic expression of downstream plant genes, with the potential to induce cell death. Genetic diversity present across the studied germplasm is the fuel that boosts discovery of executor alleles preceded by a TALE-compatible *EBE*. Our considerations suggest that TALEs could be also used as probes to uncover phenotypes

other than HR if these are induced by transcriptional activation of a given host gene. In summary, it seems plausible that executors are transcriptionally-controlled activators of cell death programs for which the biological relevance remains to be discovered. In this context, executor alleles that contain a TALE-compatible *EBE* in their 5' upstream sequence have been generally interpreted by the scientific community as TALE-specific immune receptors that evolved due to evolutionary pressure to mediate resistance to TALE-containing xanthomonads. However, the presence of TALE-compatible EBEs in some executor alleles might occur simply by chance followed by human selection, an alternative hypothesis which is generally neglected. Given that there is no direct evidence that executor *R* alleles with TALE-compatible *EBEs* evolved due to evolutionary pressure, we should consider alternative models that explain the presence of TALE-activated executor alleles in the host germplasm.

What if TALE-perceiving executor alleles did not evolve due to evolutionary pressure?

If plant executor *R* genes would have evolved primarily to mediate protection against *Xanthomonas* infections, executor *R* genes should be present in plant species adapted to warm and humid environments since these conditions favour *in planta* growth of *Xanthomonas* [36]. Indeed TALE-sensing executor *R* genes have been isolated from pepper (*Capsicum* spp.) and rice, two plant species that are generally adapted to warm and humid climate conditions, supporting the idea that executors evolved to provide resistance against *Xanthomonas*. Upon careful inspection, at least two published cases reveal executor *R* genes that have been identified in plants in the absence of obvious evolutionary pressure.

For example, the executor *R* gene *Bs4C* that contains an *EBE* compatible to the *X. euvesicatoria* TALE protein AvrBs4 was identified in *C. pubescens* accession PI 235047 [37,38]. *C. pubescens* is the most cold-tolerant of the cultivated peppers that is grown in highland climates and does not tolerate the heat of lowland tropics [39]. This ultimately suggests that *Bs4C* resistance is not an evolutionary relevant trait in natural habitats of *C. pubescens*. *Bs4C* alleles are also present in *C. annuum* [38], the most extensively cultivated of the five domesticated *Capsicum* species that is grown throughout the world in warm temperate to tropical climates [40]. Interestingly, *Bs4C* alleles that are preceded by an AvrBs4-trapping *EBE* have not been identified in *C. annuum* genotypes, although evolutionary constraints should have favoured occurrence of such *EBE*-armed *Bs4C* alleles. It should also be noted that *Bs4C*-like genes are not restricted to *Capsicum* species but are present across all members of the nightshade family (*Solanacea*) including numerous plant species that are not *Xanthomonas* host plants [38], further supporting the notion that the native function of *Bs4C* might not reside in *Xanthomonas* resistance. In summary, the presence of *EBE*-armed *Bs4C* alleles in plants of the nightshade family does not correlate with presence of TALE-equipped xanthomonads in given habitats, suggesting that *EBE*s in identified executor *R* genes did not evolve due to pathogen pressure.

A second line of evidence in support of our hypothesis that executors did not evolve as TALE-specific immune sensors arises from studies of PthA4^{AT}, a recently identified 7.5

repeat TALE protein that presumably evolved from PthA4, a 17.5 repeat TALE from *X. citri* that activates *CsLOB1* [41]. PthA4^{AT}, but not its NLS-mutant derivatives, triggers HR in lemon (*Citrus limon*) and *Nicotiana benthamiana*. This observation suggests that both *N. benthamiana* and lemon contain executor genes that trigger HR upon transcriptional activation of PthA4^{AT}. Notably, the commonly used *N. benthamiana* laboratory (LAB) genotype is endemic to arid regions in Australia [42] which are unfavourable conditions to *Xanthomonas* that requires high humidity to proliferate *in planta* [36,43,44]. It therefore seems unlikely that the PthA4^{AT}-induced *N. benthamiana* executor gene is a product of evolutionary constraints. One might also wonder why PthA4^{AT} would uncover executors in both lemon and *N. benthamiana*. In this context it is worth noting that the 7.5 repeat TALE protein PthA4^{AT} is predicted to target a sequence of merely 9 base pairs, which will occur by chance >1000 times in both the lemon and *N. benthamiana* genomes. Considering these numbers it seems less of a surprise that within the PthA4^{AT}-activated plant genes at least one triggers a cell death reaction when being expressed ectopically.

In summation, the presence of *EBE*-armed executor genes in plants that are unlikely infected by *Xanthomonas* in their native habitats suggests that these executors did not evolve as TALE-sensing immune receptors, but that TALE-trapping *EBE*s occur by chance and were subsequently selected by breeders as monogenically-inherited plant *R* genes.

What is the native function of executor genes?

If executors did not evolve as TALE-trapping immune sensors, then what is their native function? In this context it is worth highlighting that distinct executor alleles present in accessions of a given plant species generally encode full length executor proteins, irrespective if they are equipped with TALE-trapping *EBE*s [45–47]. Maintaining cell-death inducing genes poses a risk to plant health since leaky expression would be detrimental. Indeed, executor transcripts have so far only been identified in plant tissue containing TALEs that transcriptionally activate the given executors. This tight expressional control resembles known features of developmental PCD (dPCD) programs that are generally confined to a few select cells within a specific organ at a defined developmental stage [48]. It is conceivable that executor genes could therefore possibly be transcriptionally-regulated activators of dPCD repurposed to provide resistance to the biotrophic pathogen *Xanthomonas* if the given executor coding sequence contains a TALE-compatible upstream *EBE* coincidentally (Figure 6, Key Figure).

Transcriptomic profiling of different cell death types revealed that transcriptional signatures of dPCD are distinct from the ones associated with pathogen-induced HR or cell death elicited by abiotic stresses [49]. If executor-triggered cell death would indeed be related to dPCD rather than an immunity-linked HR, executor- and NLR-triggered cell death reactions should be characterized by largely distinct transcriptional changes. Indeed, transcriptomic profiling of cell death executed by the TALE-sensing rice NLR Xo1 and the rice executor Xa23 revealed that the Xo1-mediated response is more similar to two other NLR type resistance proteins than it is to the response induced by the executor Xa23 [28]. While detailed transcriptomic studies of executors are still missing, the currently available data

would be in line with the hypothesis that executor *R* genes are indeed part of dPCD programmes.

While we propose here a generalised model for executors in dPCD, it is possible that our model might only be applicable for some, but not all executors. Moreover, it is possible that executors trigger cell death only if expression is driven by TALEs, but not if expression is driven by plant transcription factors. In this context, it is worth noting that native- and TALE-induced transcripts of the same plant host gene typically differ in the length of their 5' UTRs. 5' UTRs often contain *cis* elements regulating translation of the downstream coding sequence. This could explain why native- and TALE-induced mRNAs can be derived from the same host gene, but have distinct transcriptional start sites, and can differ in their translational activity [50].

Concluding remarks and future perspectives

Whether or not executor *R* genes are transcriptionally-controlled regulators of dPCD remains an outstanding question that should be studied experimentally in the future. To this end, executor knock out mutants could uncover informative phenotypes that might provide insight on their native function as well as spatial and temporal executor expression. Promoter-reporter fusions would provide a complementary experimental approach to uncover sites of native expression of executors.

Another outstanding question in the context of executor research is why the involvement of executors in dPCD processes has not been noted thus far. The few known executor genes from pepper and rice encode rather small proteins (Figure 5) that had not been annotated before TALE-dependent expression uncovered their function as cell death inducers. If indeed most executor genes are not yet annotated in plant genomes, then corresponding reads would be discarded as unmapped reads in RNA-Seq approaches even if executor reads are present in the raw data. Similarly, the lack of annotation possibly also prevented identification of executors in genetic forward screens where typically only mutations in annotated genes are considered as potentially causal mutations. Therefore, future studies of plant dPCD should consider that even in well-established model species like *Arabidopsis thaliana*, executor genes might have escaped annotation.

We consider it likely that executors are present in all plant species including the model plant arabidopsis. As explained above, TALEs with a small number of repeats (short TALEs; sTALEs) have the potential to transcriptionally activate numerous genes in parallel and could be used as an executor gene discovery tool in arabidopsis and other plant species. Due to sequence diversity across arabidopsis ecotypes, some sTALEs could trigger HR in an ecotype-specific fashion. Such differential, ecotype-specific phenotypes would provide the prerequisites for positional cloning of yet unknown executor genes. Given that dPCD has been studied most intensively in arabidopsis, it seems likely that newly identified executor genes can be integrated into known dPCD pathways. Newly identified arabidopsis executor genes could provide a tool to compare executor- and the numerous known NLR-induced cell death reactions and to clarify whether or not these proteins use the same or distinct genetic elements to execute cell death. Due to their tightly controlled expression, executor

promoter-reporter fusions would be a valuable addition to the existing tools that visualize activation of dPCD at a cellular resolution. Moreover, it will be interesting to identify and study the upstream regulators that activate executors outside an immunity context. All in all, we postulate that the discovery and analysis of executor genes will provide a valuable tool to gain insight into the regulatory mechanisms of cell death control.

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Glossary

Designer TALE (dTALE)
In vitro assembled TALE that binds to user-defined DNA target sequence. dTALE repeat arrays typically contain only RVDs with high base specificity to avoid off-targets.

Effectors
pathogen-derived proteins that increase pathogen virulence. Bacterial effectors are injected into host cells via a syringe-like injection device, the bacterial type III secretion system.

Effector binding element (*EBE*)
DNA sequence element to which a TALE binds. The presence of an *EBE* in the 5' upstream sequence of a gene makes this gene TALE-inducible. The transcriptional start site (TSS) of a TALE-induced transcript is dictated by the *EBE*, typically 50-100 basepairs downstream of the *EBE* and often differs from the TSS of the native mRNA.

Evolved TALE (eTALE)
TALE that evolved new DNA target specificity due to selection pressure.

Executor
transcriptionally-controlled, cell death-inducing protein. Executor transcripts have been detected only upon transcriptional activation by matching TALEs, suggesting stringent transcriptional control. Executor proteins are structurally diverse, but often encode transmembrane proteins.

Executor-type *R* genes
consist of two functional elements, a TALE binding site (*EBE*) and a downstream encoded executor protein, which induces cell death when being expressed. Note that only executor *R* genes combine the capability to recognize an effector and to trigger defence, while the translated executor has no recognition function at the protein level.

Nucleotide binding leucine rich repeat proteins (NLRs)
NLRs are the predominant structural class of intracellular plant immune receptors and combine pathogen recognition and immune signalling activities at the protein level.

Programmed cell death (PCD)
cellular suicide as a result of events inside of a cell; a process that is usually beneficial to the organism as a whole.

***Resistance* (*R*) gene**
mediates resistance against a microbial pathogen if present in an otherwise susceptible plant genotype. The vast majority of plant *R* genes encode nucleotide binding leucine rich repeat (NLR) proteins.

***Susceptibility* (*S*) gene**
encode host proteins that promote microbial disease. TALEs increase host susceptibility by transcriptional activation of host *S* genes. *S* alleles that are not TALE inducible can provide resistance to TALE-containing xanthomonads and coins these *S* alleles as recessively-inherited resistance (*r*) genes.

Transcription activator-like effectors (TALEs)
effector class present in strains of the bacterial genus *Xanthomonas* with a structurally unique DNA binding domain, comprised of tandemarranged, almost identical 33-35 amino acid modules, a structural feature that promotes evolvability of DNA target specificity in TALEs. TALE-like proteins with tandem arranged DNA binding repeats have been found in the bacterial root pathogen *Ralstonia solanacearum* [51], the endofungal bacterium *Mycetohabitans rhizoxinica* [52,53] and unknown microbial marine organisms [54].

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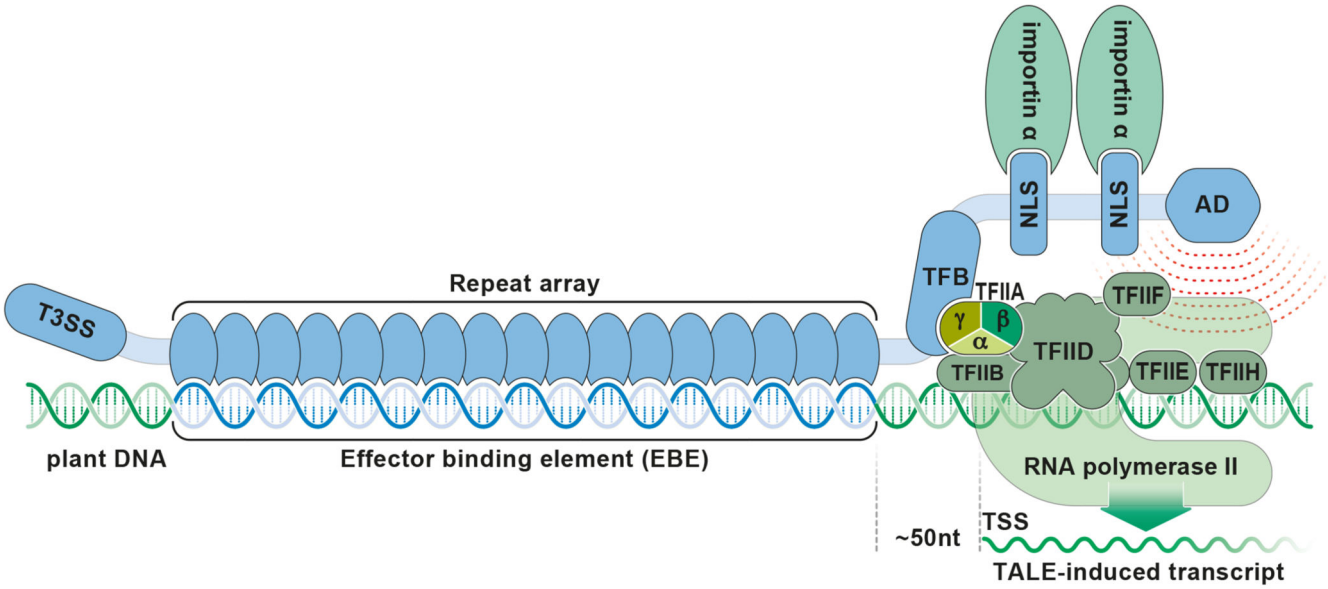

Figure 1. Transcriptional activation of plant gene by a TALE protein.

An N-terminal type III secretion signal (T3SS) marks effector proteins for translocation from the bacterial pathogen into the plant cytoplasm. Tandem-arranged repeat modules form a repeat array that binds in a sequence-specific fashion to a DNA target sequence (effector binding element; *EBE*). The transcription factor binding (TFB) domain mediates interaction with the gamma (γ) subunit of the general transcription factor TFIIA that is part of the host's RNA polymerase II preinitiation complex. The C-terminal activation domain (AD) stimulates transcriptional activation. The transcriptional start site (TSS) of the TALE-induced transcript (green waved line) starts about 50-100 nucleotides downstream of the *EBE*. Notably, the TALE-induced TSS is typically distinct from the TSS of the corresponding native transcript.

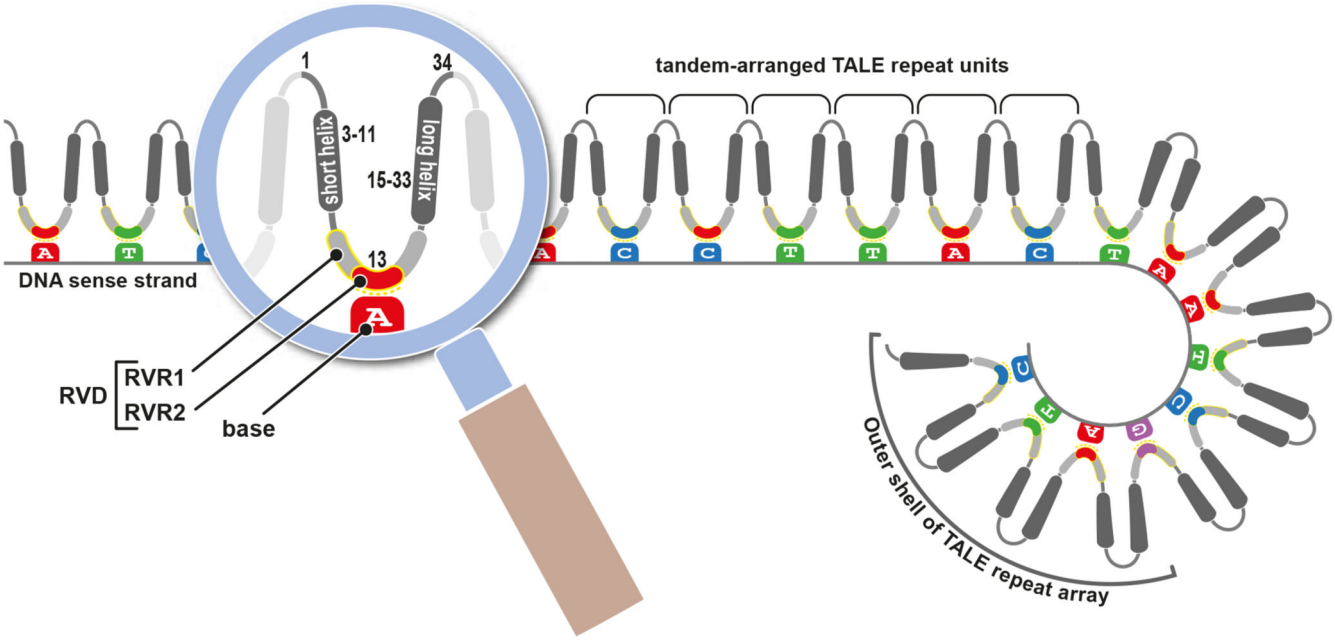

Figure 2. Structural basis of TALE-DNA interaction.

TALE proteins bind to DNA by a variable number of tandem-arranged repeats, with each repeat pairing with one DNA base in the sense strand of matching *EBEs*. The TALE-DNA interaction is depicted in a simplified linear display (left) and the helical arrangement (right) where the TALE repeat array wraps around the helical structure of the DNA. Repeats are generally highly sequence related to each other with residues 12 and 13 being most variable and accordingly designated as repeat variable diresidue (RVD; highlighted by yellow frame). The repeat variable residue 1 (RVR1; residue 12) and RVR2 (residue 13) define DNA base preference of a given repeat. RVR2, also known as the base-specifying residue (BSR), makes the major contribution to the base preference of a given repeat. Residues 12-14 collectively form the DNA binding loop that is flanked by short and long helical regions. Tandem-arranged repeats wind around the DNA and form a superhelical structure. Given that the outer shell of the repeat array is formed by conserved repeat residues, its shape is likely not affected by variation in the RVD composition and thus will be mostly highly similar for each TALE protein. Please note the simplified display of the TALE protein where only the TALE-repeat array is depicted and not the N- and C-terminal parts of the TALE protein, which are displayed in Figure 1. Note that TALEs bind to double stranded DNA but that in this simplified depiction only the DNA sense strand is shown.

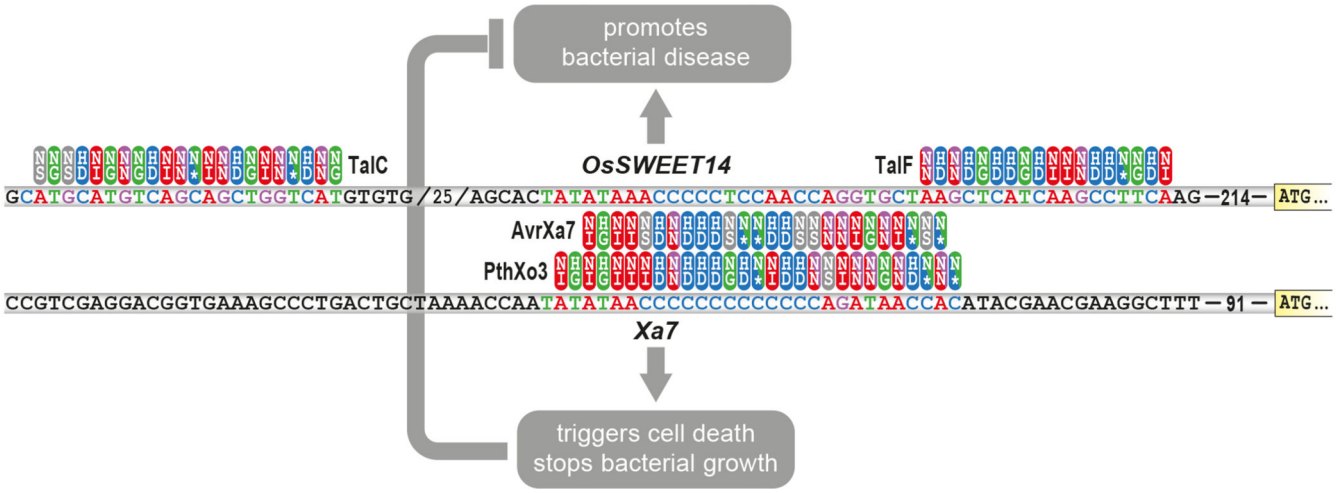

Figure 3. TALEs with a dual function that either promote disease or trigger plant resistance.

Strains of the rice pathogen *Xanthomonas oryzae* evolved distinct TALEs to transcriptionally activate the rice *OsSWEET14* gene as depicted above. This includes the TALE proteins TalC, TalF, AvrXa7, and PthXo3. The rice executor *R* gene *Xa7* contains in its 5' upstream sequence an AvrXa7- and PthXo3-compatible *EBE* but not TalC- and TalF-compatible *EBEs*. TALEs are represented by repeats (ovals) with their RVDs (one-letter amino acid code). Colour coding indicates base preference of a given RVD. 5' upstream nucleotide sequences of the rice *S* gene *OsSWEET14* and the executor *R* gene *Xa7* are shown in the upper and lower line, respectively.

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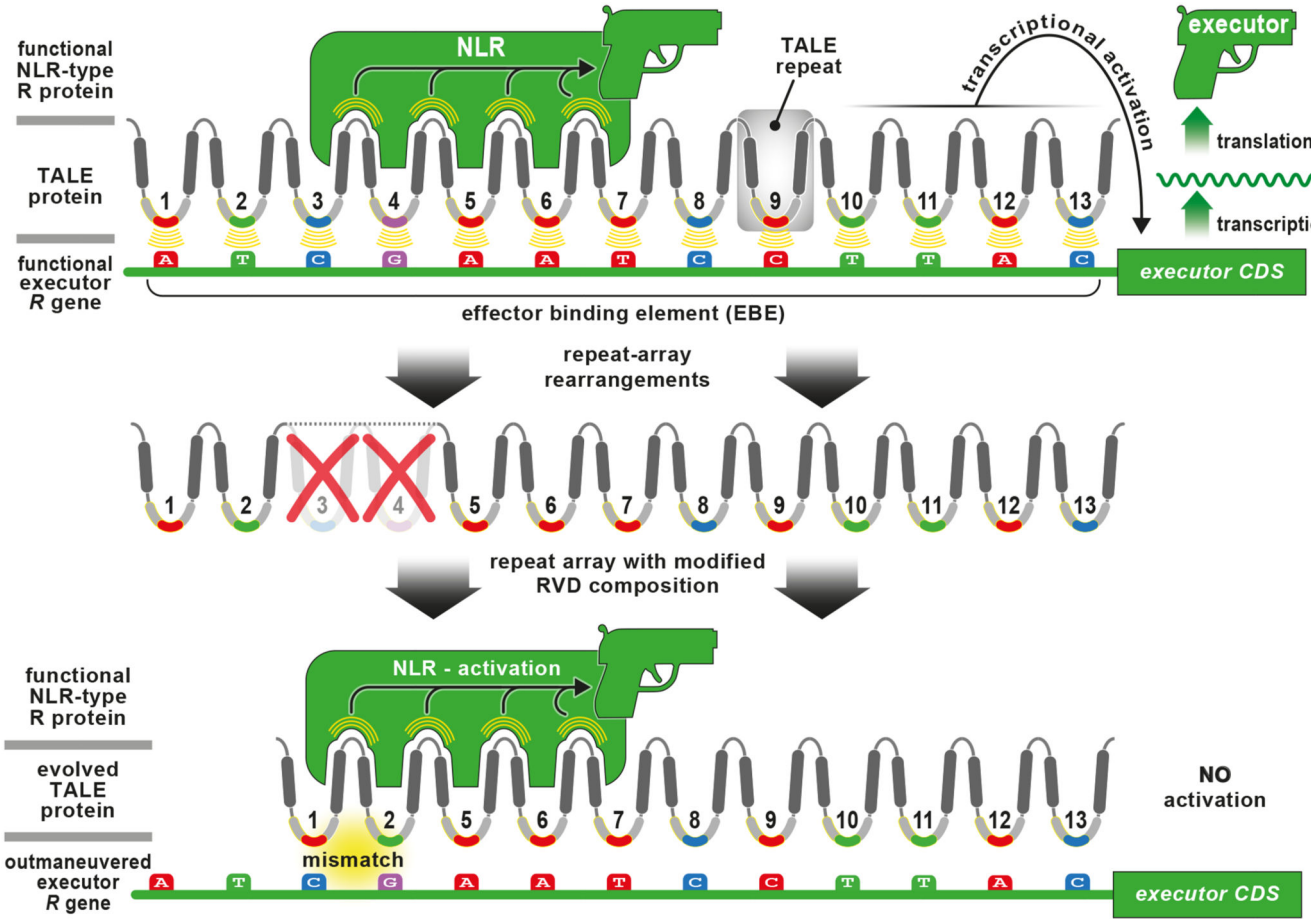

Figure 4. The impact of TALE repeat rearrangements on NLR- and executor-mediated recognition.

The displayed TALE protein is recognized either by a matching NLR protein or an executor R gene. The NLR perceives non-RVD residues of the repeat array and in turn executes an immune reaction (depicted by a pistol) that typically culminates in cell death. The TALE can also be perceived by a DNA-based receptor (*EBE*) of an executor *R* gene. Interaction of tandem-arranged RVDs with the *EBE* results in transcription of the downstream executor R gene that upon translation triggers cell death. TALEs can rapidly change the composition of their repeat array by inserting or deleting repeats (e.g.: deletion of repeats 3 and 4). The evolved TALE is still recognized by the NLR but no longer by the executor R gene. Note the simplified depiction of the TALE protein where only the TALE repeat array is shown. Note, that suppression of NLR-mediated recognition of TALEs by iTALEs/truncTALEs is not shown here since these atypical TALEs have been observed only in Asian rice-infecting xanthomonads.

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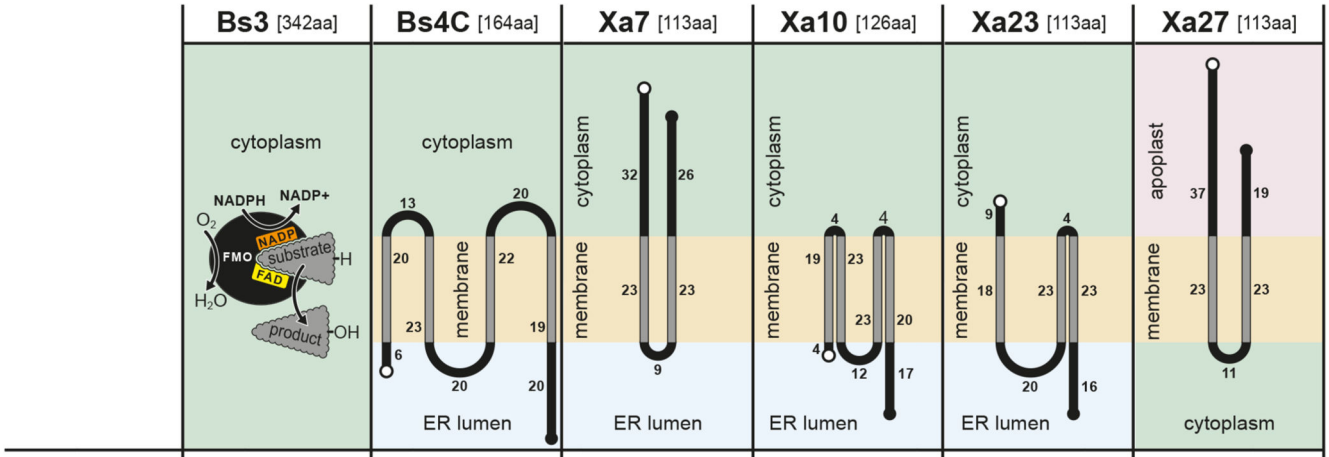

	Bs3 [342aa]	Bs4C [164aa]	Xa7 [113aa]	Xa10 [126aa]	Xa23 [113aa]	Xa27 [113aa]
Executor *R* gene donor species	*Capsicum annuum* PI271322	*Capsicum pubescens* PI235047	*Oryza sativa* DV85	*Oryza sativa* Cas 209	*Oryzae rufipogon*	*Oryza minuta* Acc. 101141
Distribution	*Solanaceae*	*Capsicum*	*~10% of Oryza species*	*Oryza*	*Oryza*	*Oryza*
Matching TALEs	AvrBs3 (*Xeu* 71-21) AvrHah1 (Xg XV444)	AvrBs4 (*Xeu* 82-8)	AvrXa7 (*Xoo* PXO86) PthXo3 (*Xoo* PXO61)	AvrXa10 (*Xoo* PXO86)	AvrXa23 (*Xoo* PXO99A)	AvrXa27 (*Xoo* PXO99A)
References	[35, 55]	[38, 56]	[57-59]	[60]	[61]	[62]

Figure 5. Structural and functional of executor R genes and encoded executor proteins.

Designation of executors is given on top with the size of the executor protein in square brackets. Depictions display the predicted topology and subcellular localization of executor proteins with terminal white and black circles indicating N- and C-termini, respectively. Transmembrane stretches were predicted with TMHMM (http://www.cbs.dtu.dk/services/TMHMM-2.0/) and are indicated with grey-coloured lines. Digits indicate the number of amino acids domains that a given domain is composed of.

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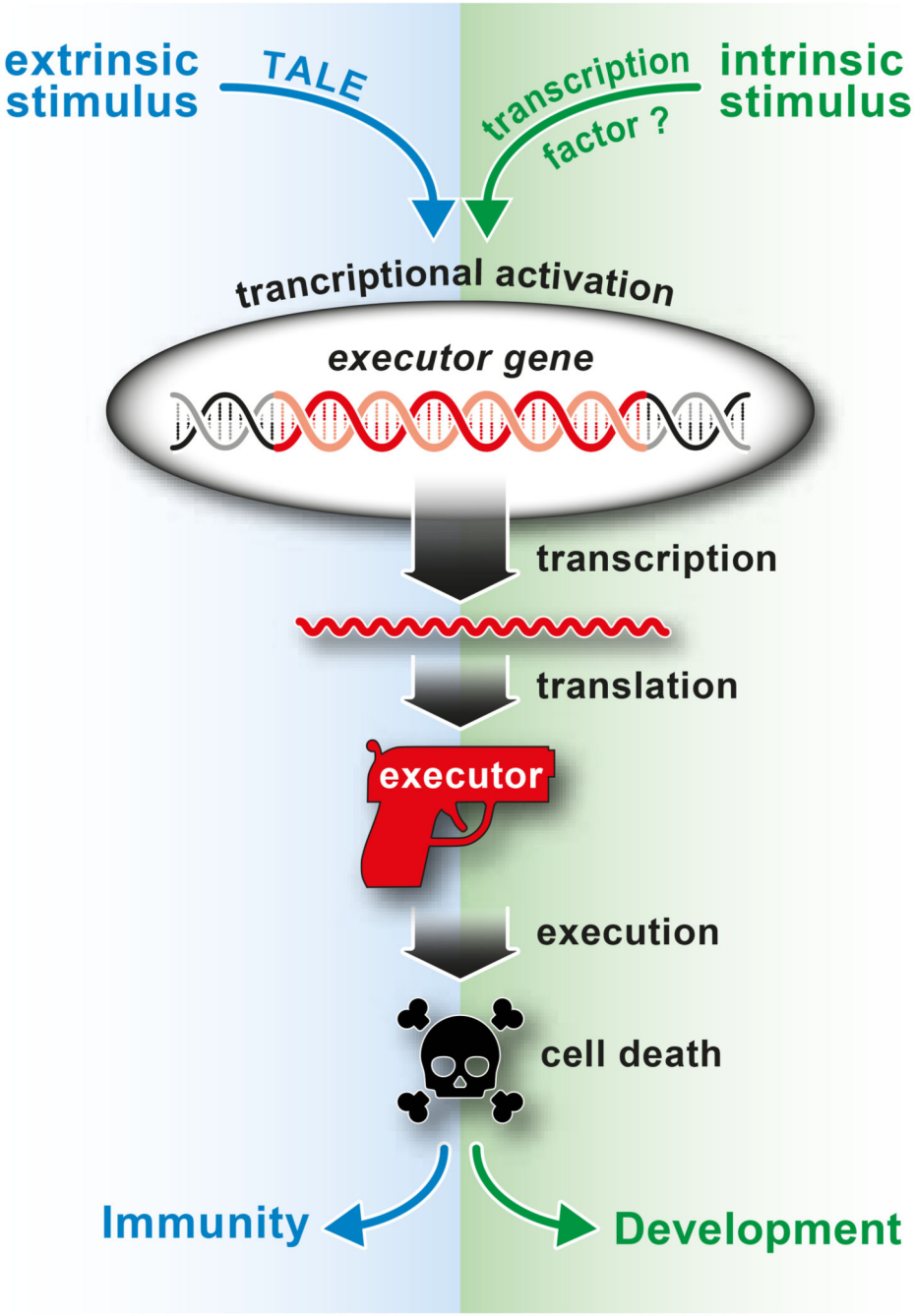

Figure 6. Transcriptional activation of executor genes.

Proposed working model where executor genes are transcriptionally activated either by TALEs, resulting in plant immunity, or by the to-be-identified plant transcription factors that transcriptionally activate executors upon intrinsic stimuli. Expression of executors triggered by an intrinsic stimulus is likely restricted to a specific tissue or developmental stage and is likely involved in formation of specific cells, tissues, or plant organs.