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Plant synthetic biology for molecular engineering of signalling and development

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Abstract

Molecular genetic studies of model plants in the past few decades have identified many key genes and pathways controlling development, metabolism and environmental responses. Recent technological and informatics advances have led to unprecedented volumes of data that may uncover underlying principles of plants as biological systems. The newly emerged discipline of synthetic biology and related molecular engineering approaches is built on this strong foundation. Today, plant regulatory pathways can be reconstituted in heterologous organisms to identify and manipulate parameters influencing signalling outputs. Moreover, regulatory circuits that include receptors, ligands, signal transduction components, epigenetic machinery and molecular motors can be engineered and introduced into plants to create novel traits in a predictive manner. Here, we provide a brief history of plant synthetic biology and significant recent examples of this approach, focusing on how knowledge generated by the reference plant *Arabidopsis thaliana* has contributed to the rapid rise of this new discipline, and discuss potential future directions.

In the 15 years since the first plant genome was fully sequenced[1], plant biology has been at the forefront of developing tools to connect genotype to phenotype. It is a significant technical challenge to make the leap from a sequenced genome to cellular signalling architecture to forecasting systems-level outputs. Among the greatest challenges is the fact that several pervasive features of biological networks, such as redundancy, convergence on shared signalling components and feedback, are not easily resolved by molecular genetics and systems approaches. Synthetic biology, as a complementary bottom-up approach, offers an opportunity to significantly accelerate our understanding of normal plant growth and development.

In this Review, we define `synthetic biology' as an engineering approach to design, build and analyse dynamic molecular devices and/or pathways from biological components to produce cells and organisms with customized functionality (Fig. 1). This broad definition builds

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bridges to approaches with natural affinity to synthetic biology (for example, chemical biology) and spans work anywhere along the spectrum, from solving explicit engineering problems to answering fundamental biological questions. A hallmark of synthetic biology is the incorporation of precise models and computational predictions of the properties of the engineered systems[2]. Although quantitative predictability is already crucial for rewiring or designing pathways and networks, precise prediction of outcomes is quite daunting, given the complex nature of higher plants as multicellular organisms and multiscale levels of regulation. Such approaches have been successful for producing simple genetic switches in bacteria[3] and are now being applied to re-engineering photosynthesis in cyanobacteria[4,5]. Recent major advances in plant synthetic biology incorporate engineered variants of receptors, ligands, signal transduction components, epigenetic regulatory machinery and cytoskeletal motors with a diverse array of potential applications (Fig. 1).

Identification and remediation of toxins

Long before the term synthetic biology was introduced and recognized in the plant biology community, the first generation of these approaches was already being implemented. Although here we focus primarily on the engineering of plant parts drawn from signalling and cell biology, there is significant research activity in synthetic metabolic engineering[6,7], with many recent headlines stemming from successful porting of opiate[8] and cannabinoid[9] production into yeast.

Phytoremediation was the intended application of some of the earliest examples of successful metabolic engineering. For instance, engineered transgenic tobacco plants expressing human cytochrome P450 2E1 showed substantially enhanced ability to oxidize the toxic halogenic environmental pollutants trichloroethylene (TCE) and ethylene dibromide (EDB)[10]. Transgenic hybrid poplar expressing P450 2E1 effectively removes TCE, chloroform and even the gaseous form of benzene[11].

The classic concept of directed protein evolution has also been incorporated into synthetic biology to boost the performance of transgenic organisms. Traditionally, mutagenesis and iterative cycles of selection have been used to produce proteins or pathways with enhanced activity[12]. Together with structure-guided rational design and high-throughput cloning/screening methods, the directed evolution approach could accelerate future protein engineering[13–16]. By applying such techniques to enzymes such as the P450 proteins involved in phytoremediation approaches, it may be possible in the future to design and optimize new functionalities.

The ground-up design of a complete, artificial signalling pathway to detect environmental pollutants was reported recently using an engineered bacterial two-component system[17]. This system consists of membrane-bound sensor histidine kinases and response regulators, which trigger conformational changes following phosphorylation, resulting in target gene expression[18]. One study[17] introduced into *Arabidopsis* and tobacco a complete set of signalling components, from upstream ligand binding to downstream gene expression: (i) modified bacterial periplasmic binding protein (PBP) in the plant apoplast that could bind trinitrotoluene (TNT) as a ligand; (ii) a chimaeric bacterial two-component receptor histidine

kinase (Trg:PhoR) that would bind TNT–PBP; (iii) adapted response regulator with synthetic transcriptional activation domain (PhoB–VP64); and (iv) transcriptional signalling readout using the modified PhoB promoter driving a reporter enzyme (β-glucuronidase, or GUS) or a suite of `degreening' genes that interfere with chlorophyll biosynthesis[17,19].

The two-component system, although lost in animals, is retained in yeasts and plants, where the conserved circuits were adopted into hormone signalling pathways[20–24]. For example, the cytokinin-sensing pathway retains the basic framework of two-component circuitry. Using yeast, a synthetic signal transduction pathway was created, replacing the yeast osmosensor SLN1 with the *Arabidopsis* cytokinin receptor CRE1. This synthetic pathway rescued the lethal phenotype of the *sln1D* mutant only when cytokinin was exogenously applied[23]. The result implicated that the two-component systems can be introduced or exchanged between the kingdoms of life, providing a compelling rationale for repurposing this module for diverse applications. Future work can probably address some limitations of the engineered two-component systems, including leaky (background) activation, crosstalk with the endogenous two-component signalling pathways, and efficacy of synthetic circuit in the contexts of feedback regulation (tissue-specific and plant phase/age-related) and diurnal fluctuations of transgene expression and responsiveness.

Receptors

Engineered plant receptors could be powerful tools to hijack or bolster endogenous signal transduction pathways to manipulate plant behaviours. Such an approach does not require introduction of a complete set of synthetic signalling components. Rather, structural information on receptors at an atomic resolution, as well as computational structural modelling and simulation, are essential. Crystal structures of several key plant receptors have been solved recently, including receptors for auxin[25], gibberellins[26] (GA), abscisic acid (ABA)[27–29], jasmonates (JA)[30], brassinosteroids (BR)[31,32] and peptides[33]. This deep structural knowledge has been instrumental in the engineering of new hormone biosensors, including DII–VENUS[34] and its ratiometric variant R2D2[35] for auxin, Jas9–VENUS for JA[36], and ABACUS[37] and ABAleons[38] for ABA.

Molecular structural information on plant hormone–receptor interaction and signal activation can be used directly to engineer receptors to confer novel recognition specificity. Among the ongoing efforts, engineering of the ABA receptor PYRABACTIN RESISTANCE 1 (PYR1) has been particularly noteworthy. PYR1 was identified through chemical genetic screen using pyrabactin, a selective ABA agonist that preferentially interacts with PYR1[39]. Structure-guided receptor engineering has been used to increase pyrabactin selectivity between PYR1 and PYR2, illustrating a path to manipulating signalling outputs via receptor modification[40]. One study[41] further engineered PYR1 to perceive mandipropamid, an agrochemical compound used to fight blight pathogens (Fig. 2). A wide mutational search followed by directed mutagenesis yielded a receptor variant called $PYR1^{MANDI}$ that essentially piggybacks on the ABA response in transgenic *Arabidopsis* or tomato plants (Fig. 2b,c). The synthetic pyrabactin–$PYR1^{MANDI}$ ligand–receptor system has important implications in both basic and translational research for plant biology. For instance, cell-type-specific expression of $PYR1^{MANDI}$ probably triggers ABA responses in the specific cell

type of interest following mandipropamid spraying. The cell-type-specific roles of ABA signalling could be investigated without being hampered by the complexity owing to redundancies among PYR/PYR-like (PYL) proteins or systemic ABA transport. In the crop fields, mandipropamid spraying could enhance the drought tolerance of transgenic plants expressing $PYR1^{MANDI}$, but not of non-transgenic weeds, thereby selectively protecting the crops from abiotic stress (Fig. 2d,e).

Receptors can also be expressed in heterologous settings, making it possible to test the function of potential orthologues across a vast range of species. These head-to-head comparative studies in a common, evolutionarily distant background could lead to a more sophisticated assessment of how gene function evolves. Porting orthologous pathways from divergent lineages into a `blank slate' background could be combined with other synthetic approaches, such as ancestral sequence reconstruction (ASR)[42]. ASR aligns extant protein sequences within phylogenies and then uses statistical models of amino acid substitution rates to determine the maximum-likelihood sequence at an ancestral node. Such studies have helped resolve apparent paradoxes where co-evolution of complex components leave a `chicken or egg' dilemma, such as in the diversification of animal hormone receptors[43]. ASR and related approaches could aid in the transfer of knowledge from model plants to other plants of interest. For example, evolutionary and molecular phylogenetic studies coupled with developmental and physiological analysis may shed light on the specialization and diversification of plant receptors for strigolactones (SL) (which are butenolide hormones for branching and also used as a cue for parasitic plants and arbuscular mycorrhizal fungi) and karrikins (chemical compounds found in smoke)[44–46]. Engineering receptors using an ASR approach could be applied for manipulating plant growth and symbiotic relationships while limiting parasitism.

Signals

Small chemical analogues represent a powerful tool to discover, visualize and manipulate plant signalling pathways[47,48]. Here again, structural resolutions of small chemical hormone binding sites to corresponding receptors enable the rational design of analogues that interfere with endogenous hormone signalling. For instance, a series of auxin agonists and antagonists were created by extending alkyl chains to the α-position of indole-3-acetic acid (IAA)[49]. The crystallographic and molecular-docking analyses revealed that introduction of butyl or longer alkyl chains at this position blocks access of the Aux/IAA degron to the TRANSPORT INHIBITOR RESPONSE 1 (TIR1) auxin-binding pocket, thereby acting as an antiauxin[49]. Similarly, ABA analogues have been engineered with a long alkyl chain that interferes with PYL–protein phosphatase 2C (PP2C) interactions, thus acting as ABA antagonists[50].

Fluorescent analogues of hormones and other signalling molecules are a major advance for probing *in vivo* events in real time and across different spatial scales. Perhaps the greatest advantage of these compounds is that they can be used in any plant species, even those without established transformation protocols. This overcomes a significant limitation of the fluorescence resonance energy transfer (FRET)-based biosensors, which require the introduction and expression of recombinant, synthetic protein fusions in the whole plant or

in cell/tissue types of interest. Ideally, both methods can be used to validate each other, as data from chemical analogues may not always accurately reflect the activities or dynamics of endogenous chemical signals.

Some recent examples include fluorescently labelled bioactive GA (GA–FI) and BR analogues (Alexa Fluor 647-castasterone; AFCS)[51,52]. Treatment of *Arabidopsis* roots with GA–FI revealed the selective accumulation of signals in elongating endodermal cells, suggesting the presence of active GA transport and specific tissues as GA sinks[51]. AFCS enabled visualization of the internalization of BRI1–AFCS ligand–receptor complexes at the plasma membrane[52]. In these cases, conjugation of a large fluorescent dye with the introduction of a long alkyl chain reduces the binding affinity to the receptor. This inevitably makes such analogues less competitive against the endogenous, non-tagged counterparts. Similarly, the fluorescent auxin analogues 7-nitro-2,1,3-benzoxadiazole (NBD)–α-naphthalene acetic acid (NAA) and NBD–IAA allow for visualization of auxin transport, but are unable to mediate signalling through TIR1–Aux/IAA[53,54]. To improve the design, each plant hormone needs to be carefully investigated to elegantly replace the non-essential backbone with a fluorescent moiety. Recently, a fluorescent SL analogue, CISA-1 (cyano-isoindole strigolactone analogue-1), was created on the basis of such a design principle[55].

The creative and effective design of synthetic analogues could enable the visualization of the actual receptor perception and signal transduction with fluorescence. One study recently reported the synthesis and application of such an innovative synthetic chemical, Yoshimulactone Green (YLG), which can visualize the SL receptor activity in real time with high spatial resolution[56]. The SL receptor's α/β-hydrolase activity cleaves SLs into two pieces: the ABC-ring, which is structurally free, and the D-ring, which is required for bioactivity (Fig. 3a). In YLG, the ABC-ring is replaced by a fluorescein derivative, which emits fluorescence only if the D-ring is hydrolysed (Fig. 3a,b). Hence, YLG retains SL bioactivity. YLG and its variant with a higher on/off ratio, YLG-double (YLGW), trigger the germination of *Striga* seeds (Fig. 3b)[56]. Strikingly, the long-term time-lapse imaging of *Striga* germination by YLGW revealed the biphasic response of SL perception and signal activation: the first `wake up wave' sharply illuminates the root tip of *Striga* embryos within 20 minutes of YLGW application, diffuses towards cotyledons and then disappears after six hours[56]. This initial strong reaction is specific to *Striga* embryos and is not observed in *Arabidopsis* embryos, whose germination is not SL-specific. The second `elongation tide' of fluorescent wave coincided with germination and root elongation[56].

As molecular structures of ligand–receptor associations are increasingly being resolved at atomic resolutions[25,30,31], it will be possible to design diverse ligand analogues with specific properties to visualize and manipulate signalling. Although the new chemical approach offers great promise, there are many potential pitfalls. For instance, conjugating fluorescent dyes to hormones inevitably affects receptor binding affinity and diffusion rates, as well as degradation and transport kinetics. As a result of these differential effects, synthetic analogues may not accurately reflect the full activity of their endogenous counterparts. In addition, synthetic hormone agonists, antagonists and fluorescent probes are added exogenously to plants, rather than synthesized endogenously. Exogenous application may lead to a highly artificial spatial distribution of pathway activation, perturb the effect of

endogenous hormones and provoke feedback regulation of endogenous hormone biosynthesis, signalling and transport pathways. Careful, multifaceted approaches that combine genetic tools, biosensors and mathematical modelling[57–59] could moderate the impact of these limitations.

Combining engineering approaches with metabolic and signalling pathways is the obvious next step in synthetic signalling, as this would allow implementation of entire synthetic networks. The network motifs needed for complex dynamic output functions would be greatly facilitated by plants that could synthesize multiple synthetic signalling molecules that are, in turn, selectively perceived by engineered receptors and downstream components (Fig. 1). New approaches for rapid prototyping of synthetic parts in plants[57] are key to developing the library of components needed to scale-up synthetic pathway engineering. Implementation of engineered networks in basal plant lineages may also be a means to accelerate the design–build–test engineering cycle, as these organisms often have smaller families of competing signalling components and more streamlined genome editing[58,60].

Epigenetics

A synthetic approach has also been effective at manipulating plant gene expression. One powerful example makes use of the C-function homeotic transcription factor AGAMOUS (AG), which acts during floral development to turn on the transcription factor KNUCKLE (KNU; Fig. 4)[61]. KNU represses the expression of the stem cell gene *WUSCHEL* (*WUS*) to terminate stem cells in flower primordia. Interestingly, *KNU* induction by AG requires two days, and this involves displacement of Polycomb group (PcG) proteins by AG at the *KNU* locus (Fig. 4a)[61]. To unravel the mechanism underlying the time lag, one study[62] generated an artificial DNA-binding protein, LacI–GR, which binds to the bacterial lactose repressor, coexpressed with a *KNU* reporter whose AG-binding domains within the promoter region were replaced by a *lac* operator sequence. Furthermore, this promoter region contains a Polycomb response element (PRE)-like domain from other plant genes, so that the *KNU* reporter is silenced unless the PcG proteins are removed from the system. Notably, following nuclear localization of LacI–GR, this synthetic system recapitulated the *KNU* expression with a two-day time lag (Fig. 4b).

This work was extended with a second synthetic epigenetic timer, consisting of a synthetic promoter-driven YFP construct and an artificial transcription activator-like (TAL)–GR DNA-binding protein, which was designed to bind the DNA sequence around the AG-binding site[62]. The culture cells expressing these constructs beautifully recapitulated the two-day lag of gene expression (Fig. 4c). These results indicate that any DNA-binding protein at the *KNU* promoter region that physically and competitively interferes with the association of the PcG protein complex would be sufficient to induce *KNU* gene expression. Moreover, the work shows the power and promise of a synthetic approach to controlling timing of gene expression using artificially designed epigenetic timers. Such an approach could be expanded and coupled with feedback/feedforward circuits and genetic switches to control artificial gene expression programs in plants.

A synthetic transcription factor can also be used to uncouple chromatin architecture and chemical-stimulus-induced gene expression. Very recently, a study reported that an auxin-regulated chromatin switch, mediated by MONOPTEROS (MP)/ARF5 (AUXIN RESPONSE FACTOR 5), triggers epigenetic reprogramming for floral primordial initiation[63]. Auxin perception normally frees MP/ARF5 from repression by degradation of Aux/IAA co-repressors. In this new work, the authors propose that degradation of Aux/IAAs allows MP/ARF5 to recruit a chromatin-remodelling complex containing SPLAYED (SYD) or BRAHMA (BRM) to open up nearby chromatin. In support of this model, an artificial transcription factor that fuses the MP/ARF5 DNA-binding domain and BUSHY, a protein that recruits SYD/BRM complexes, was able to mimic MP function and rescue the *mp* `pin' inflorescence phenotype[63]. These findings provide a blueprint for engineering artificial DNA-binding proteins with the capacity to directly recruit the SYD/BRM complex to any known *cis*-regulatory elements and precisely manipulate the chromatin landscape.

Molecular motors

Plant cytoskeleton and motor proteins are intimately coupled with plant cell division, cell elongation, cell shape and polarity specification[64–66]. For example, cortical microtubules position the cellulose synthase complex, and thereby determine the site of cellulose microfibril deposition[67,68]. As such, understanding and manipulating their dynamics in a controlled manner could have a huge impact on engineering cell wall biosynthesis and plant biomass.

Myosin is a motor protein that uses energy from ATP hydrolysis to `walk' along actin filaments. Researchers have engineered myosin VI and performed single-molecule imaging to investigate its behaviour *in vitro*, and compared this with predictions from mathematical models. Three- and four-headed myosins with various arm lengths, instead of natural myosins with two heads, have been created and their processivity and behaviours were tested *in vitro*[15]. These researchers tactically combined four-head myosin with *Chara* myosin XI, the fastest myosin known[69], and created the fastest synthetic myosin known to date[15]. The next and most important question is how such engineered motor proteins perform in living cells. The plant myosin (myosin XI) was engineered to manipulate its velocity and processivity. Faster myosin, and thus faster cytoplasmic streaming, led to bigger *Arabidopsis* plants, whereas slower myosin resulted in smaller plants[70]. This work revealed a surprising connection between myosin velocity and plant size control. If such an approach can be expanded to also engineer actins and tubulins, it would allow precise control of the cytoskeleton, as well as cortical microtubule density and dynamics. This would allow researchers to manipulate cell growth and shape in a predictable manner.

Engineering other organisms with plant-derived pathways

Plant proteins and small molecules are increasingly being used to engineer heterologous systems. For example, the field of optogenetics has harvested a number of plant proteins involved in light perception that work effectively to trigger activation of neurons in living animals with exquisite spatial resolution[71]. A similar logic and related plant components were exploited to develop tools for light-induced gene expression[72–74], protein splicing[75,76]

and nuclear localization[77]. Plant hormone pathways have been engineered to allow small-molecule regulation of target proteins, including triggering their turnover[78–80] or relocalization[81]. Recently, an enzyme for auxin biosynthesis has been combined with components from the auxin response pathway to produce a robust sender–receiver system in yeast[82]. A sender strain expresses an enzyme from bacteria that converts a precursor called indole-3-acetamide into auxin. A library of auxin-degradable CRISPR transcription factors coexpressed with an auxin receptor translates the auxin signal in receiver cells.

In addition to producing new tools for heterologous systems, synthetic recapitulation of entire pathways or networks can generate new hypotheses about plant function. A suite of auxin response circuits encompassing signal perception to gene expression has been analysed in yeast[83]. Sensitivity analysis of the isolated pathway guided studies in plants by generating the hypothesis that auxin sets an Aux/IAA-regulated timer, coordinating progression through development[84]. In this way, parallel analysis of synthetic and natural systems can be synergistic. In a recent review outlining plans for re-engineering photosynthesis to meet the urgent need for increased crop yields, the authors highlighted the critical role of synthetic biology in realizing the most ambitious, and probably most effective, of these ideas[85].

Perspective

Synthetic biology is rapidly becoming as essential to molecular biology as crystallography, *in vitro* biochemistry, genetics and the many `omics'. When combined with high-fidelity genomic engineering[86,87], higher efficiency transformation[88], high-throughput phenotyping platforms[89] and improved *in silico* tools for pathway design and testing[90–94], the scale of reasonable plant engineering projects expands dramatically. Detailed knowledge of a genome, specifically the genotype-to-phenotype map, is essential for targeting and rapidly prototyping the optimal candidates for engineering. Synthetic biology, as a multiscale and cross-disciplinary approach, offers a deeper understanding of plant development and signalling. This new perspective will make it possible to unleash the full potential of plants for the benefit of our health and environment.

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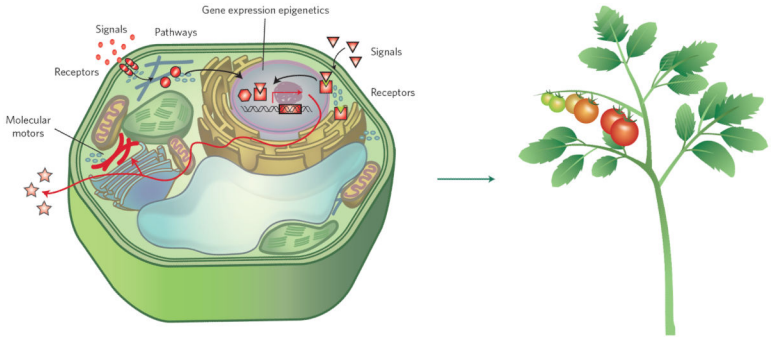

Figure 1. A schematic diagram of an idealized plant cell with synthetic engineered pathways to produce a plant with ideal traits and functionality

Each synthetic engineered component is coloured in red. Figure courtesy of Haruko Hirukawa (ITbM, Japan).

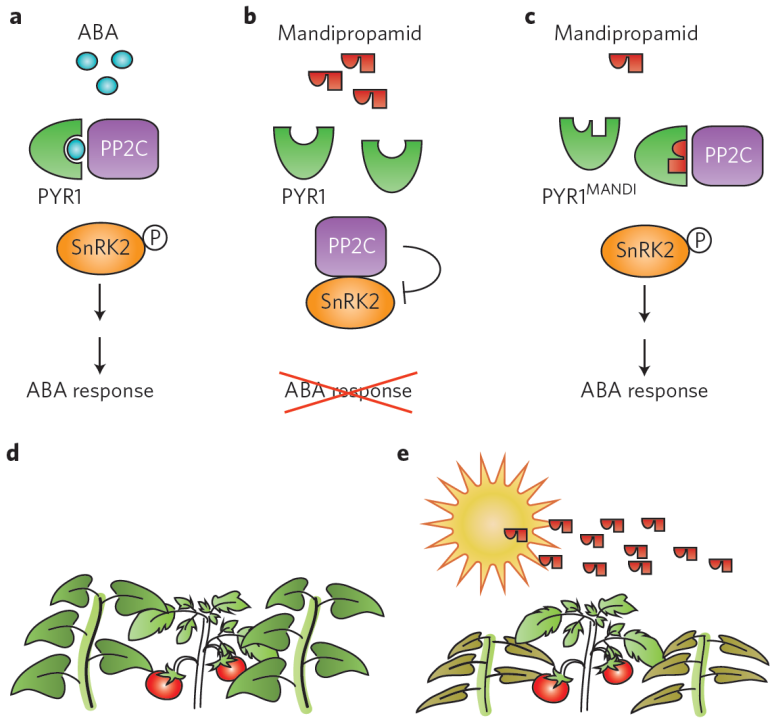

Figure 2. An engineered ABA receptor can perceive a fungicide and trigger an ABA response

a, Endogenous ABA signalling. Binding of ABA to the PYR1 receptor promotes PYR1–PP2C association, which in turn activates the downstream protein kinase SnRK2. Active SnRK2 triggers ABA responses, including stomatal closure and drought tolerance. **b**, Mandipropamid treatment does not elicit an ABA response in wild-type plants. **c**, In a plant expressing the engineered PYR1^{MANDI} receptor, mandipropamid treatment triggers ABA response and, consequently, drought tolerance. **d**,**e**, Potential and idealized translational application of a synthetic ligand–receptor system in a crop field. Here, a transgenic tomato crop plant expressing PYR1^{MANDI} is outcompeted by nearby weeds (**d**). During drought season, mandipropamid application triggers ABA response to the transgenic tomato plant, thereby boosting its drought tolerance. Surrounding non-transgenic weeds do not respond to the chemical spray. Mandipropamid has already been approved by the Environmental Protection Agency as a fungicide (reg. no. 100-1281) for field application.

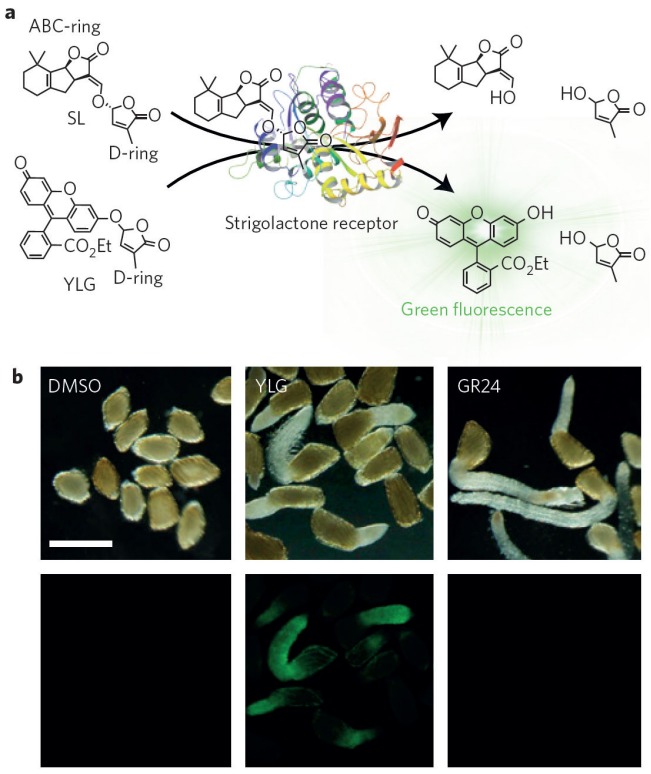

Figure 3. Mode of action of YLG

a, Perception of SL (top, left) by the SL receptor results in hydrolysis of SL, releasing the D-ring (top, right). YLG (bottom, left) is also recognized by the SL receptor with high affinity. The receptor perception cleaves off the D-ring of YLG, releasing a fluorescein derivative, which emits green fluorescence. **b**, Visualization of YLG perception in germinating *Striga* seeds. Although both YLG and the synthetic SL analogue GR24 trigger germination, strong green fluorescence is visible in the roots of only YLG-treated seedlings. Scale bar, 0.5 mm; DMSO, dimethylsulfoxide. Figure courtesy of Shinya Hagihara, Kenichiro Itami and Masahiko Yoshimura (ITbM, Japan).

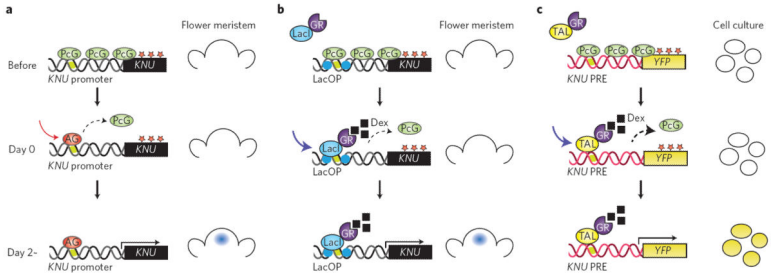

Figure 4. A synthetic epigenetic timer for gene expression

a, The endogenous timer. During flower development, *KNU* is covered with H3K27me2 repressive marks (red stars). The PcG complex maintains such marks (top). Binding of AG to the *KNU* promoter elements triggers eviction of PcG (middle). After two days, cell-division-dependent loss of repressive histone marks facilitates *KNU* gene expression in the flower meristem (bottom; right). **b**, A partially synthetic timer. On dexamethasone (Dex) treatment, LacI–GR, a synthetic DNA-binding protein consisting of the LacI DNA-binding domain fused to the glucocorticoid receptor (GR), can trigger the eviction of the PcG complex from the *KNU* promoter region that contains Lac operator (LacOp) sequences (middle). This leads to *KNU* gene expression after two days, thus mimicking the AG action (bottom, right). **c**, A completely synthetic timer. YFP driven by an unrelated promoter can be silenced with repressive histone marks (asterisks) if a Polycomb response element from *KNU* (*KNU* PRE) is inserted (top). A synthetic DNA-binding protein consisting of GR fused with TAL that is designed to bind near the *AG*-binding site, TAL–GR, can trigger the eviction of PcG (middle). This leads to *YFP* reporter gene expression in cultured cells (bottom; right).