Tunable control of insect pheromone biosynthesis in *Nicotiana benthamiana*

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Summary

Synthetic regulatory elements that provide control over the timing and levels of gene expression are useful for maximizing yields from heterologous biosynthetic pathways. Previous work has demonstrated that plants and microbes can be engineered to produce insect sex pheromones, providing a route for low-cost production of these compounds, which are valued for species-specific control of agricultural pests. Strong constitutive expression of pathway genes can lead to toxicity and metabolic loads, preventing normal growth and development and thus limiting biomass and affecting overall yields. In this study we demonstrate the ability to inducibly control the accumulation of Lepidopteran sex pheromones in leaves of *Nicotiana benthamiana*. Further, we show how construct architecture influences expression and product yields in multigene constructs, applying this to control the relative expression of genes within the pathway, thereby tuning the accumulation of pheromone components. The approaches demonstrated here provide new insights into the heterologous reconstruction of metabolic pathways in plants.

Introduction

The reconstruction of biosynthetic pathways in heterologous organisms has become an established route for the production of important biomolecules for agriculture, industry and medicine. Although recombinant DNA technologies have been in use for decades, recent advances in metabolic engineering and synthetic biology have expanded the breadth and complexity of molecules produced by heterologous biosynthesis (Keating and Young, 2019; Romero-Suarez et al., 2022). Indeed, a major advantage of biological manufacturing is the ability to produce complex molecules, including those for which chemical synthesis has proven difficult or commercially non-viable due to the requirement for multiple stereoselective steps (Cravens et al., 2019). For example, there is growing interest in the biological production of insect sex pheromones (many of which are stereochemically complex) for species-specific control of agricultural pests (Ding et al., 2014; Holkenbrink et al., 2020; Mateos Fernández et al., 2022; Mateos-Fernández et al., 2021; Xia et al., 2021).

Most progress in biomanufacturing natural products has been achieved by the engineering of industrially established microbes. However, there is increasing interest in plant and algal production systems (Brodie et al., 2017; Burnett and Burnett, 2020; Stephenson et al., 2020). The use of photosynthetic hosts grown in glasshouses and photosynthetic bioreactors negates the requirement for feedstocks such as sugars, which, depending on the sources from which they are derived, can raise new issues of sustainability (Dammer et al., 2019; Matthews et al., 2019). Due to the presence of the necessary metabolic precursors and cofactors and the ability to express, fold and post-translationally modify eukaryotic proteins, plants can often successfully express eukaryotic metabolic pathways without the need for engineering of the host

(Patron, 2020; Stephenson et al., 2020). Tobacco (*Nicotiana tabacum*) and other species in the *Nicotiana* genus are highly amenable to *Agrobacterium*-mediated transformation and, consequently, have become widely used in the plant sciences both as model plants for studying gene function and, more recently, in plant biotechnology owing to their ease of genetic manipulation (Bally et al., 2018; Lein et al., 2008; Molina-Hidalgo et al., 2021). Tobacco accumulates considerable biomass and it has been estimated that field-grown transgenic tobacco are more cost-effective than cell culture methods for the production of recombinant proteins (Conley et al., 2011; Schmidt et al., 2019). *N. benthamiana*, a non-cultivated species native to Australia, has a comparatively shorter life cycle and does not accumulate large biomass in field conditions. However, it is particularly amenable to *Agrobacterium*-mediated transient expression (agroinfiltration), which has been exploited for the large-scale production of recombinant proteins including the production of coronavirus-like particles, approved as a COVID-19 vaccine in Canada (Chen et al., 2013; Hager et al., 2022; Stephenson et al., 2020). In recent years, this method has also been shown to be amenable for the reconstruction of many metabolic pathways, including the production of preparative quantities (van Herpen et al., 2010; Molina-Hidalgo et al., 2021; Reed et al., 2017; Stephenson et al., 2020). Thus, together with tools for manipulating their endogenous metabolism, the availability of characterized regulatory elements and design rules for the reconstruction of heterologous pathways in these species are highly desirable.

Many metabolic engineering experiments have aimed solely at maximizing the expression levels of all genes of interest by strong constitutive expression of pathway genes. However, particularly when pathways are introduced into the genome to produce transgenic lines, the use of such promoters can be detrimental, leading to toxicity and metabolic loads preventing normal growth and development and thus limiting biomass and affecting overall yields (Bernabé-Orts et al., 2019). We recently encountered this when engineering the production of volatile moth pheromones in *Nicotiana benthamiana* (Mateos-Fernández et al., 2021). In that study, we were able to obtain plants producing moderate levels of (Z)-11-hexadecenyl acetate (Z11-16OAc) and high levels of (Z)-11-hexadecenol (Z11-16OH), the two main volatile components in many Lepidoptera sex pheromone blends. However, higher levels of production were accompanied by a reduction in plant growth (Mateos-Fernández et al., 2021). The development of genetic circuits that enable control over both the timing and levels of gene expression might, therefore, be useful for increasing the yield of such compounds. Further, better regulation of gene expression might also allow control over the relative yields of the major pheromone components, the ratio of which is known to differ between moth species (Zavada et al., 2011).

There are numerous ways to fine-tune expression, including post-transcriptional methods (Krishnamurthy et al., 2015) and protein stability (Trauth et al., 2019). However, information flow from synthetic circuits inserted into engineered cells begins at transcription. It is therefore desirable to control transcriptional activity from synthetic genetic circuits. Significant efforts have focused on engineering synthetic regulatory sequences and switches for plants (Andres et al., 2019; Belcher et al., 2020; Bernabé-Orts et al., 2020; Cai et al., 2021; Garcia-Perez et al., 2022; McCarthy and Medford, 2020). Many inducible systems for controlling gene expression in plants use expensive molecules such as estradiol or dexamethasone, or require the application of stresses such as heat or wounding, which cannot easily be applied in field conditions and may affect plant fitness (Corrado and Karali, 2009). However, expression systems inducible by copper, a relatively low-cost molecule that is readily taken up by plants and registered for field use, have also been demonstrated (Kumar et al., 2021; Mett et al., 1993; Saijo and Nagasawa, 2014). In recent work, we optimized this system, achieving tight, high-level activation of reporter and endogenous genes in *N. benthamiana* (Garcia-Perez et al., 2022). In this study, we combine copper inducibility with synthetic programmable transcription factors to regulate the production of Lepidopteran pheromones, gaining the ability to control inducible control of expression as well as tuning the relative levels of the major pheromone components.

Results

Comparison of diacylglycerol acetyltransferases

Heterologous production of moth sex pheromones from endogenous 16C fatty acyl CoA is achieved by constitutive expression of Δ11 desaturase, fatty acid reductase and diacylglycerol acetyltransferase (Figure 1A). The genes encoding moth acetyltransferases involved in pheromone biosynthesis have not yet been identified and previous attempts at heterologous biosynthesis have employed either an enzyme from the plant, *Euonymus alatus* (EaDAct) (Ding et al., 2014; Mateos-Fernández et al., 2021), or from the yeast, *Saccharomyces cerevisiae* (ScATF1) (Ding et al., 2016; Xia et al., 2022), with the latter producing more acetate. We first compared EaDAct and ScATF1 with two further diacylglycerol acetyltransferases from

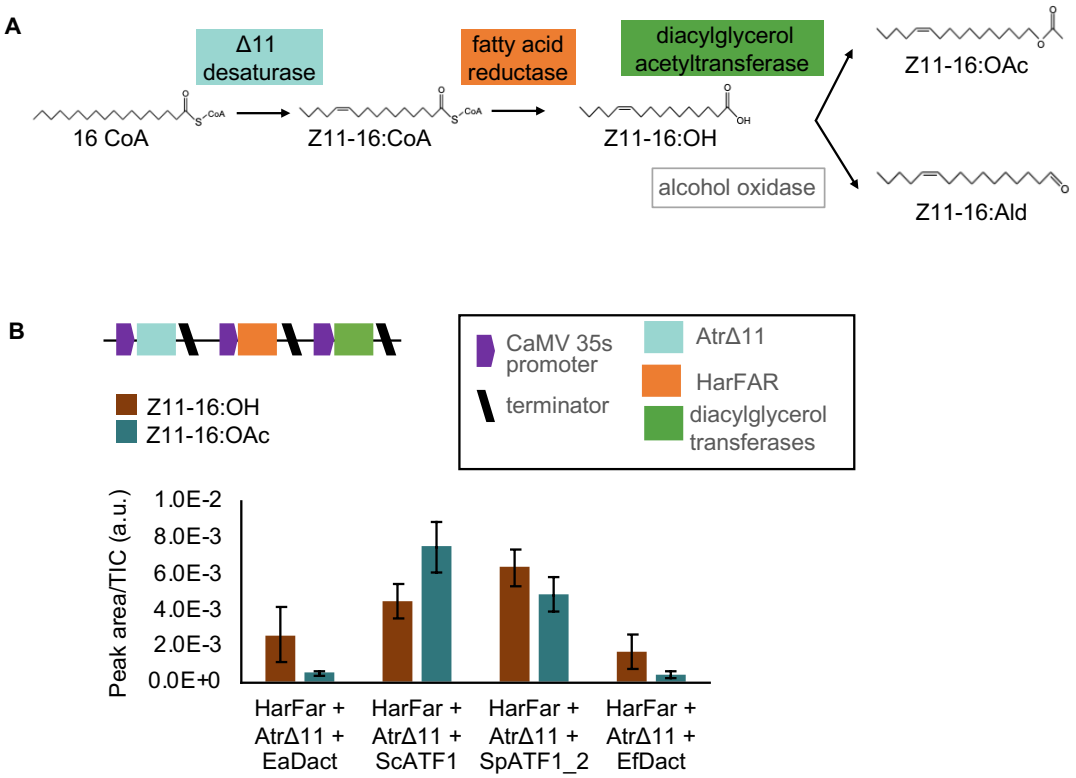

Figure 1. Heterologous production of Lepidopteran sex pheromones. (**A**) Plant production of the two main volatile components in many Lepidopteran sex pheromones *(Z)*- 11-hexadecenol (Z11-16:OH) and *(Z)*-11-hexadecenyl acetate (Z11-16:OAc) from endogenous 16C fatty acyl CoA (Z11-16:CoA) was previously achieved by heterologous expression of a Δ11 desaturase, a fatty acid reductase and a diacylglycerol acetyltransferase. The accumulation of *(Z)*-11-hexadecenal (Z11-16:Ald) was also observed, presumably catalyzed by an endogenous alcohol oxidase (Mateos-Fernández *et al.*, 2021). (**B**) Differences in the quantities and ratios of Z11-16:OAc and Z11-16:OH obtained by co-expression of diacylglycerol transferases from *Euonymus alatus* (*EaDAct), E. fortunii (EfDAct), Saccharomyces cerevisiae* (ScATF1) and *S. pastorianus* (SpATF1-2) with a fatty acid reductase from *Helicoverpa armigera* (HarFAR) and a Δ11 desaturase from *Amyelois transitella* (AtrΔ11).

Euonymus fortunii (EfDAct) (Tran et al., 2017) and *Saccharomyces pastorianus* (SpATF1-2) (Yoshimoto et al., 1999). To do this, each gene was co-expressed with the coding sequences of enzymes encoding a Δ11 desaturase from *Amyelois transitella* (*AtrΔ11*) and a fatty acid reductase from *Helicoverpa armigera* (*HarFAR*) (Figure 1B). We found that both yeast enzymes produced more Z11-16OAc than those from plants (Figure 1B). Although the sequences of ScATF1 and SpATF1-2 only differ at K183R, we also observed some differences in their activity in *N. benthamiana*, resulting in different ratios of Z11-16OH : Z11-16OAc.

Copper inducible expression of Lepidopteran pheromones

In previous work, we found that fusions of copper-responsive factor CUP2 with the Gal4 domain resulted in strong activation of reporter genes (Garcia-Perez et al., 2022). To test copper-inducible accumulation of pheromone components, we assembled the coding sequences of *AtrΔ11*, *HarFAR* and *SpATF1-2* with a minimal 35s promoter preceded by four copies of the CBS (Figure 2A). These three synthetic genes were then co-assembled with a synthetic gene in which the *A. tumefaciens* nopaline synthase promoter (*AtuNos*) was fused to CUP2:GAL4 for moderate constitutive expression (Figure 2A). The construct was agroinfiltrated into *N. benthamiana* leaves in a 1:1 ratio with an Agrobacterium strain carrying a plasmid encoding the P19 suppressor of silencing (Garabagi et al., 2012). Three days post-infiltration, leaves were sprayed with either water or 2.5 mM copper sulfate ($CuSO_4$), previously identified as the optimal concentration (Garcia-Perez et al., 2022). The total volatile organic compound (VOC) composition of all samples was analyzed five days post-infiltration by gas chromatography/mass spectrometry (GC/MS). GC peaks corresponding to the pheromone compounds Z11-16OH and Z11-16OAc were detected in samples treated with $CuSO_4$, but not in untreated samples or in control samples infiltrated with P19 alone (Figure 2B).

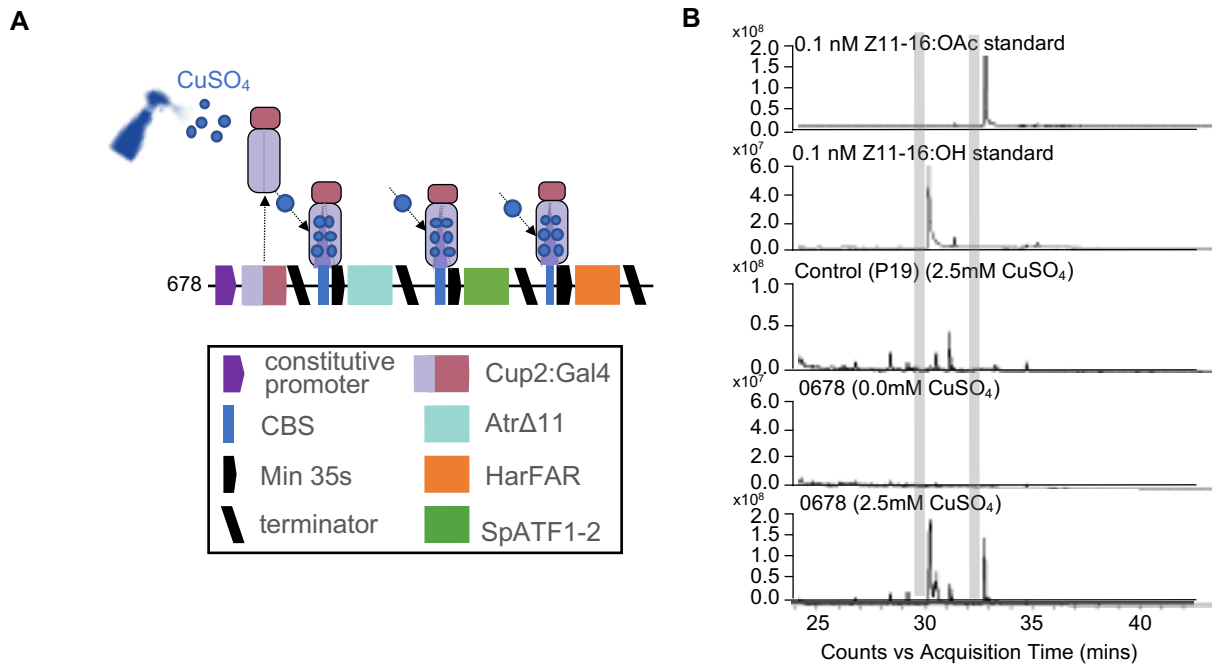

Figure 2. Copper inducible expression of Lepidopteran pheromones. (A) Schematic of a plant expression construct containing synthetic genes encoding the copper-responsive transcription factor CUP2 in translational fusion with the Gal4 activation domain and the coding sequences of AtrΔ11, HarFAR and SpATF1-2 under control of a minimal 35s promoter preceded by four copies of the CUP2 binding site (CBS). (B) Total ion chromatogram showing the accumulation of Z11-16:OH and Z11-16:OAc in leaves of *N. benthamiana* co-infiltrated with *Agrobacterium* strains containing the expression construct (678) and a construct expressing the P19 suppressor of silencing only after application of 2.5mM copper sulfate ($CuSO_4$).

Construct architecture influences expression and product yield

It has long been known that the repetition of some genetic elements within constructs as well as the insertion of T-DNA as tandem repeats can trigger gene silencing (Stam et al., 1997; Vaucheret et al., 1998). This presents a challenge for designing synthetic circuits in which coordinated expression of multiple genes in response to a single signal is desirable. For transient expression, it is possible to avoid co-assembly onto a single T-DNA by the co-delivery of multiple strains of A. tumefaciens. However, it is unknown what proportion of cells receive all strains and whether this might constrain maximum yields. For the production of transgenic lines, it is highly desirable that all synthetic genes are co-assembled to enable integration into a single genomic locus, preventing segregation of pathway genes in the progeny.

To determine if and how the expression levels of copper-inducible synthetic genes are affected by co-assembly, we first compared the relative expression levels of two luciferase reporters driven by identical copper-inducible promoters in single and multi-gene configurations (Figure 3A). We found that the amount that the relative expression of each gene in the multi-gene constructs was dependent on its position in the assembly (Figure 3B). In the absence of the P19 silencing suppressor, we observed that expression from the second gene in the assembly, regardless of identity, was significantly reduced (Figure 3B). Although co-expression of P19 improved the expression balance, it did not fully restore it (Figure 3B). Constructs in which the two genes were assembled on opposing strands were unstable. Similar results were also obtained using constitutive promoters (Supplementary Figure S1).

From these results, we reasoned that altering the relative position of each gene in the pheromone biosynthesis pathway would likely affect relative expression and thus alter the accumulation of total and relative quantities of Z11-16OH and Z11-16OAc. To investigate this, we constructed and compared three T-DNAs in which we varied the relative positions of each gene (Figure 2C). As expected, we observed variations in both the overall yields and the relative ratios of Z11-16OH and Z11-16OAc components (Figure 2D).

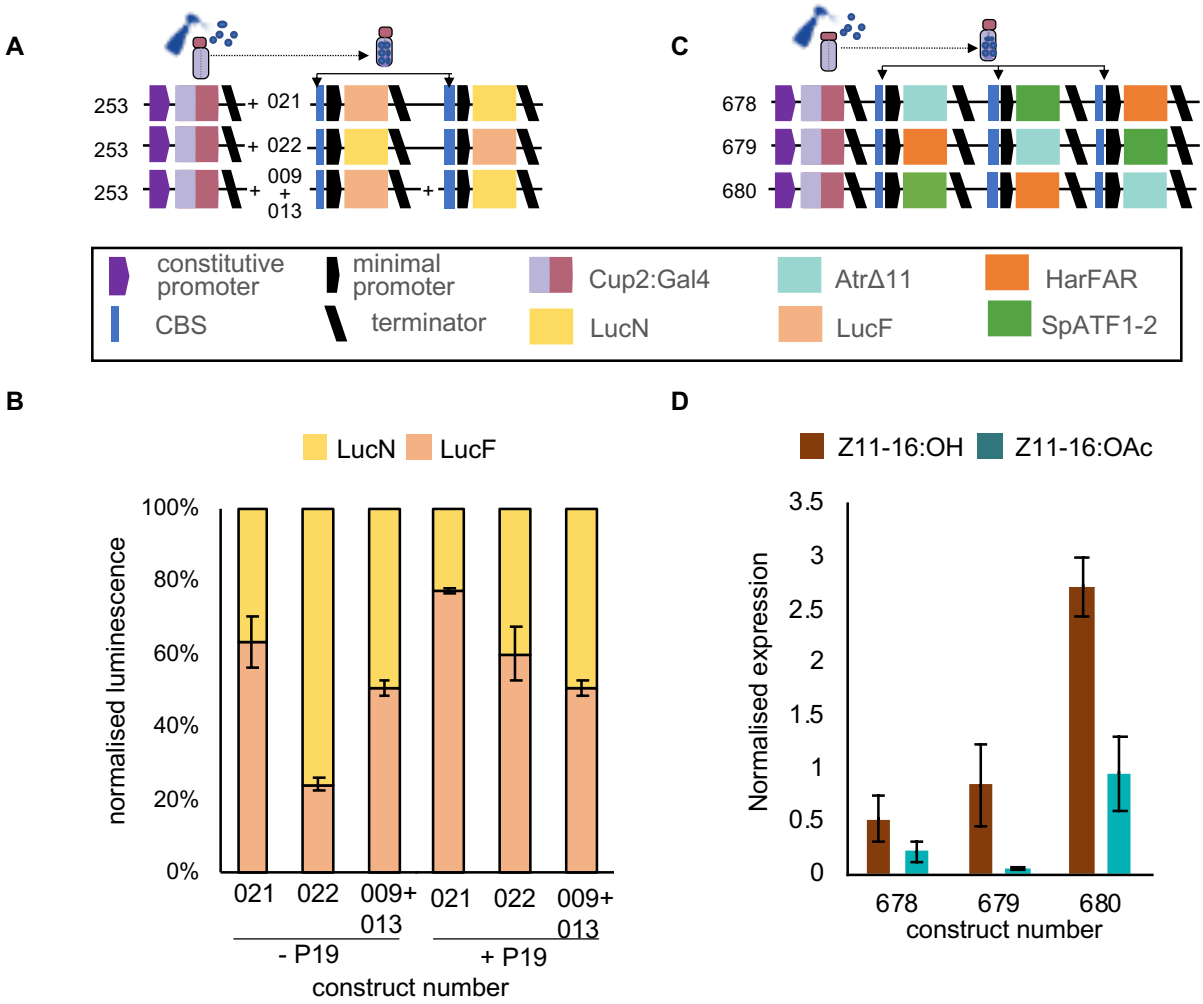

Figure 3. Construct architecture influences expression and product yield. (**A**) Schematics of plant expression constructs containing synthetic genes for copper-inducible expression of firefly luciferase (LucF) and nanoluciferase (LucN). (**B**) The level of expression from each gene in a multigene construct is dependent on the position in which the gene is assembled. (**C**) Schematics of plant expression constructs containing synthetic genes for copper-inducible expression of lepidopteran sex pheromones. (**D**) The relative positions of pathway genes influenced the overall yield and the relative ratios of pheromone products

The configuration that produced the most pheromone production was having AtrΔ11 in the last position.

Copper inducible transcriptional cascades

Another method of controlling expression levels is to alter the strength of the promoters. In previous work, we designed minimal synthetic promoters from which expression levels correlated with the number of binding sites for cognate synthetic transcriptional activators (Cai et al., 2021). To investigate if this might provide an alternative method for controlling the relative expression of co-assembled pathway genes we first compared the activation and background expression levels obtained from three types of previously reported synthetic promoters activated by (i) a transcription activator-like effector (TALE) (Cai et al., 2021), (ii) a Gal4:ΦC3 fusion protein (Bernabé-Orts et al., 2020; Cai et al., 2021), or (iii) dCasEV2.1, which consists of three components: dCas9 fused to the EDLL transcriptional activation domain, a fusion of the MS2 phage coat protein and a VPR activation domain, and a guide RNA (gRNA) that guides the complex to the promoter (Selma et al., 2019). We found that the dCasEV2.1 system achieved the highest levels of activation with the lowest levels of background (Figure 4A). We then compared how each of these systems performed in a transcriptional cascade in which expression of the synthetic transcription

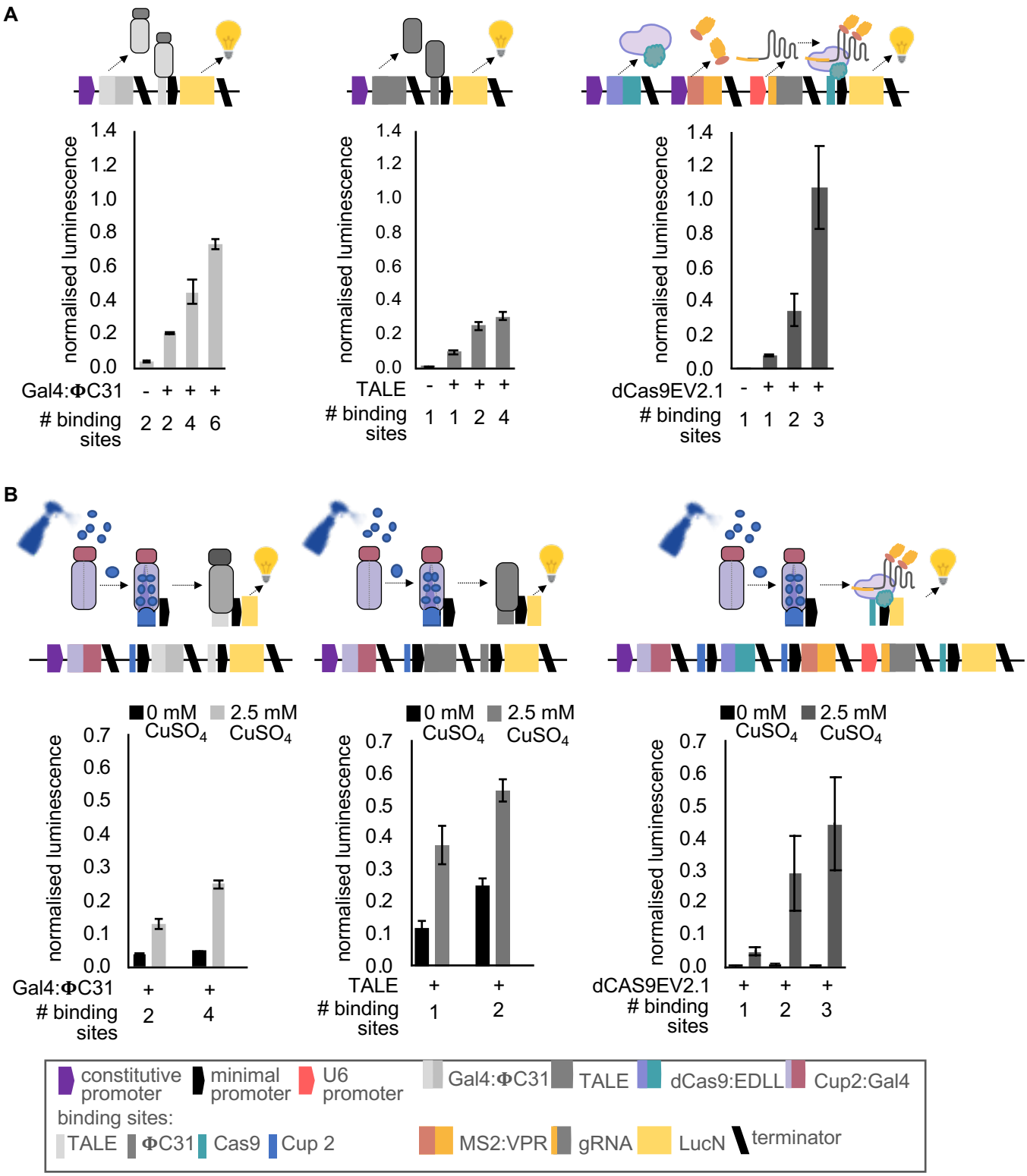

Figure 4. Comparison of constitutive and copper-inducible expression by synthetic transcriptional activators. (A) Expression levels of nanoluc luciferase (LucN) correspond to the number of binding sites in the promoter for constitutively expressed synthetic transcriptional activators, GAL4: ΦC31, transcription activator-like effector (TALE) and dCasEV2.1. **(B)** Copper inducible expression of dCasEV2.1 maintains tight control (low-background) of gene expression

factor (TALE, Gal4:ΦC3 or dCasEV2.1) was controlled by copper-inducible elements. In these experiments, we observed that the relative strength of promoters with different numbers of binding elements was conserved across all three systems, but the total level of expression decreased (Figure 4B). In addition, background expression in the absence of copper also increased for TALE and Gal4:ΦC3 transcription factors. However, the background expression for dCasEV2.1 mediated expression remained low (Figure 4B).

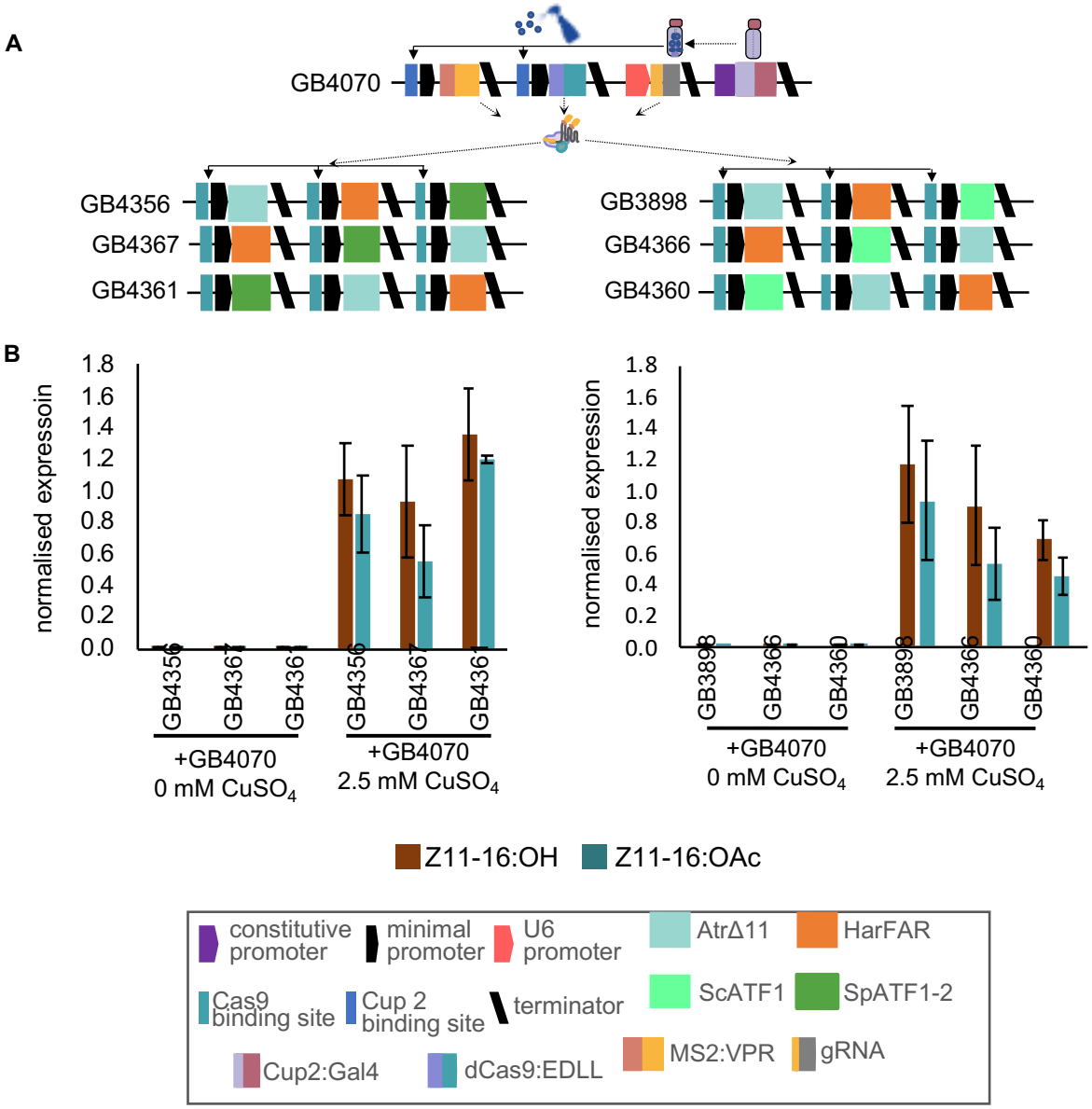

Figure 5. Copper inducible, CRISPR/Cas9-mediated control of pheromone biosynthesis. **(A)** Schematic of plant expression constructs containing elements for copper inducible expression of the dCasEV2.1 transcriptional activator (above) and six multigene constructs containing coding sequences for AtrΔ11, HarFAR and SpATF1-2 or ScATF1, each assembled with a minimal DFR promoter with cognate target sequences for the gRNA (below) (**B**) Application of $CuSO_4$ results in dCasEV2.1 mediated production of *(Z)*-11-hexadecenol (Z11-16:OH) and *(Z)*-11-hexadecenyl acetate (Z11-16:OAc).

Copper inducible, CRISPR/Cas9-mediated control of pheromone biosynthesis

To test if the copper-inducible dCasEV2.1 system would enable control over pheromone biosynthesis, we assembled the coding sequence of each pathway enzyme with a synthetic promoter activated by this complex. As our experiments with reporters indicated that expression from the transcriptional cascade was reduced at least two-fold in comparison to constitutively expressed dCas9 (Figure 4B), all pathway genes were assembled with a promoter consisting of a minimal DFR together with a unique proximal region containing three dCasEV2.1 recognition

sites, thus maximizing strength and minimizing sequence repetition within the multigene assembly.

We first compared the yields of pheromone components obtained using constitutively expressed and copper-inducible control of dCasEV2.1. In similarity with the reporter constructs, pheromone yields were reduced with the copper-inducible transcriptional cascade (Supplementary Figure S2). We also compared two construct configurations in which the gRNA element was co-assembled with either the dCasEV2.1 elements, or with the pathway genes (Supplementary Figure S3). These two configurations provide different advantages for reuse: co-assembly with dCasEV2.1 enables the control module to be reused by simple co-delivery of the dCas9 module with modules containing new pathways. However, separating the gRNA from the dCas9 module allows new gRNA/promoter pairs to be deployed without the need to reassemble the dCas9 module. While both configurations were functional, greater yields were obtained by co-assembly of the gRNA with the dCasEV2.1 components (Supplementary Figure S3).

Finally, to investigate if these regulatory components would enable copper-inducible control of yields and product ratios, we altered both the relative position of each pathway gene and the identity of the diacylglycerol transferase (Figure 5A). In contrast to the direct copper activation, in which greater yields were obtained (Figure 2), all constructs produced similar ratios of pheromone components, with slightly more Z11-16OH than Z11-16OAc (Figure 5B). Although differences in yields of Z11-16OH were not significant, the construct containing SpATF1-2 in the first position, followed by AtrΔ11 and HarFAR produced the most Z11-16OAc (Figure 5B).

Discussion

The use of chemical formulations to control insect pests in food crops has a long history. However, the increasing use of synthetic pesticides in the twentieth century led to concerns about the deterioration of biodiversity in agricultural landscapes, as well as risks to farmworkers and consumers (Köhler and Triebskorn, 2013). The advent of molecular biotechnology in the 1980s provided alternatives to foliar applications of broad-spectrum pesticides, most successfully, transgenic insect-resistant crops (ISAAA, 2018). Advances in synthetic biology and metabolic engineering are now providing alternatives to both synthetic chemicals and to these first-generation GM plants (Mateos Fernández et al., 2022). One such alternative is expanding the use of insect sex pheromones. Insect sex pheromones are typically volatile molecules produced by females to attract a mate and the presence of even minute quantities from alternative sources can disrupt breeding and behavior (Mateos Fernández et al., 2022). Unlike other methods of biocontrol, the evolution of resistance to sex pheromones is considered unlikely due to strong selection against losing the ability to find a mate (Rizvi et al., 2021). Consequently, the global insect pheromone market is growing (Agricultural Pheromone Market Report, 2021). However, while the pheromones of some species can be cheaply manufactured by synthetic chemistry, the pheromones of many insect species have complex structures requiring stereoselective steps making them difficult and expensive to produce (Petkevicius et al., 2020). To provide proof-of-concepts for the biological synthesis of pheromone components, several studies have successfully produced Lepidopteran sex pheromones in heterologous systems including yeast (Hagström et al., 2013; Holkenbrink et al., 2020; Konrad et al., 2017; Petkevicius et al., 2020) and *N. benthamiana* (Ding et al., 2014; Mateos-Fernández et al., 2021; Xia et al., 2022, 2020). In the latter, both transient agroinfiltration and stable transgenic approaches have been trialed, though retardation of growth was observed in the best-producing transgenic lines (Mateos-Fernández et al., 2021).

In this study, we tested and compared a number of gene regulatory components to gain control of the levels and timing of expression. We deployed inducible components (Figure 2) that, in transgenic lines, would enable expression to be delayed until biomass has accumulated. We also demonstrated methods to control the relative expression of pathway genes, altering the relative yields of the major pheromone components (Figure 3), the ratio of which is known to differ between moth species (Zavada et al., 2011).

The genes encoding moth acetyltransferases involved in pheromone biosynthesis have not yet been identified and previous studies have employed either a plant enzyme, EaDAcT (Ding et al., 2014; Mateos-Fernández et al., 2021) or the yeast enzyme ScATF1 (Ding et al., 2016; Xia et al., 2022), with the latter producing more acetate. When comparing the activities of these enzymes we observed differences in the levels of product obtained from two yeast enzymes, ScATF1 and SpATf1-2, which differ at only one amino acid (Figure 1)

Inducible gene expression systems are essential tools in metabolic engineering. They are commonly employed to switch metabolism from growth to production, enabling biomass to accumulate before energy is redirected into the biosynthesis of desired products. While several inducible systems have been developed for plants, many of these rely on stimuli that are either expensive or challenging to

deploy in large-scale or field conditions (e.g., environmental stress, hormones etc.). In previous work, we optimized a copper-inducible system demonstrating that this enabled high levels of expression of reporter and endogenous genes in *N. benthamiana* and, in the absence of copper, very low background expression (Garcia-Perez et al., 2022). Here, we demonstrate that this system is suitable for controlling the expression of biosynthetic pathways (Figure 2). As copper sulfate is approved for agricultural use, it provides a possible tool for large-scale bioproduction systems. The range of concentrations at which copper is active as a signaling molecule is much lower than those employed for antifungal applications in field conditions, therefore the employment of copper sulfate as a trigger for recombinant gene expression could be compatible with the current reduction trend in copper-based antifungal formulations.

The development of modular cloning systems such as Golden Braid (2013; Vazquez-Vilar et al., 2017), used in this study, has eased the assembly of constructs. It is now relatively easy to build variants of genetic constructs that enable the properties of individual components such as promoters and untranslated regions to be compared (Bernabé-Orts et al., 2020; Cai et al., 2021). These cloning systems have also facilitated the design and assembly of multigene constructs (Pollak et al., 2019; Sarrion-Perdigones et al., 2011; Weber et al., 2011). However, few studies have investigated how co-assembly affects the performance of synthetic genes. While it has long been known that gene-silencing can reduce expression from transgenes and that some regulatory elements and construct architectures (e.g. the inclusion of inverted repeats) are more susceptible (Stam et al., 1997; Vaucheret et al., 1998), the effects of co-assembly on individual genes within multigene plant constructs have not been quantified. Using ratiometric reporter assays, we found that the position in which genes are located within multigene constructs differentially affects their expression (Figure 3). In this simple experiment, we investigated constructs with only a small number of synthetic elements. Larger combinatorial studies are required to investigate more complex constructs and also to determine the best strategy to co-assemble synthetic genes with different transcriptional activities and with more diverse components. This is especially important as more information emerges about the impact of different regulatory elements, including the effects of untranslated sequences and terminators on expression and post-transcriptional silencing (F de Felippes et al., 2020; Wang et al., 2020). It may be possible to achieve equal levels of expression from multiple co-assembled genes by conducting a combinatorial study to select appropriate regulatory elements for each gene and/or by testing the efficacy of insulator sequences, shown to reduce the effects of genomic locations on transgenes, between transcriptional units (Pérez-González and Caro, 2019). In this study, however, rather than attempting to avoid the unequal expression observed by co-assembling inducible and constitutively expressed genes (Figure 3B, Supplementary figure S1), we investigated whether the different levels of expression could be used to alter the product profile obtained from our biosynthetic pathway. We found that changing the relative position of each pathway gene altered both overall yield and the relative quantities of the major pheromone components (Figure 3B).

We also investigated whether we could achieve inducible control over the relative expression of genes by combining the copper-inducible system with a second synthetic transcriptional activator (Figure 4). We first compared the activity and background levels of three previously described synthetic activators; TALEs, Gal4:ΦC3 and dCasEV2.1 with their cognate minimal synthetic promoters (Figure 4A). In all cases we observed that the expression levels were reduced when coupled to copper inducibility (Figure 4B). We also observed reduced pheromone yields with the pathway genes (Supplementary Figure S2). The reduced expression may be due to increased competition for transcriptional complexes. However, we noted that the transcription, translation and maturation of TALEs, Gal4:ΦC3 or dCasEV2.1 will commence only after the application of $CuSO_4$, and additional time may be required for optimal activity. As gene expression following agroinfiltration is known to peak and then decline (Yamamoto et al., 2018), this complicates the quantitative comparison of pathways with different numbers of transcriptional tiers. We also noted that combining copper-inducible control with TALE and Gal4:ΦC3 activators led to an increase of background expression in the absence of copper but that dCasEV2.1 maintained low background levels (Figure 4B). The combination of copper-inducibility with dCasEV2.1-activated promoters using different numbers of gRNA binding sites, therefore, provides a basis for inducible-control of relative expression. The reduction in expression in the transient system deterred us from testing the weaker promoters with pathway genes, however, we were able to demonstrate copper-inducible, dCasEV2.1-controlled production of pheromone biosynthesis (Figure 5).

Conclusions

In this study we have applied synthetic biology approaches to control the expression of pheromone biosynthesis in *N. benthamiana*, using synthetic transcriptional elements and construct architectures to modulate the time of expression and the quantities and composition of pheromone components. These provide novel ways to control yields via either transient expression or stable transgenesis and can be combined with further control mechanisms. For example, trichome-specific promoters have recently been shown to increase the release of pheromones from leaves (Xia et al., 2022), which might enable the utility of the transgenic plant itself as live dispensers, proposed as an alternative to extraction from harvested biomass for use in traditional dispensers (Mateos Fernández et al., 2022). Finally, the regulatory systems demonstrated here and developed as modular genetic elements for facile reuse, are not limited to controlling pheromone biosynthesis, but are broadly useful to the design of constructs for plant metabolic engineering.

Methods

Assembly of expression constructs

All constructs were assembled using the GoldenBraid (GB) cloning system (Sarrion-Perdigones et al., 2013; Vazquez-Vilar et al., 2017). Standardized DNA parts (promoters, coding sequences, and terminators) were cloned as Level 0 parts using the GoldenBraid (GB) domestication strategy described by Sarrion-Perdigones et al. (2013). Transcriptional units (Level 1) were then assembled in parallel, one-step restriction-ligation reactions and transformed into bacteria as previously described (Cai et al., 2020). Hierarchical stepwise assembly of transcriptional units into multigene constructs was achieved using binary assembly via BsaI or BsmBI-mediated restriction ligation as defined by the GB system. GB constructs employed in this study are provided in Supplementary Table S1, and sequences have been deposited at Addgene. Expression constructs were transformed into electrocompetent *Agrobacterium tumefaciens* GV3101.

Transient expression in *N. benthamiana*

N. benthamiana plants were grown in a controlled environment room with 16 hr light, 8 hr hours dark, 22℃, 80% humidity, ~200 µmol/m^2/s light intensity. Strains of A. tumefaciens GV3101 harboring the expression constructs were grown in LB medium supplemented with 50 µg/mL kanamycin or spectinomycin and 50 µg/mL rifampicin for 16 hours at 28℃/250 rpm. Overnight saturated cultures were centrifuged at 3,400 x g for 30 min at room temperature and cells were resuspended in infiltration medium (10 mM 2-(N-morpholino)ethanesulfonic acid (MES) pH 5.7, 10 mM $MgCl_2$, 200 µM 3′,5′-Dimethoxy-4′-hydroxyacetophenone (acetosyringone)) and incubated at room temperature for 2-3 hours with slow shaking. Healthy plants (29-37 days old) with 3-4 fully expanded true leaves were infiltrated on the abaxial side of the leaf using a 1 mL needleless syringe and grown for five days in a growth chamber with 16 hr light, 8 hr hours dark at 22℃ and 120-180 µmol/m^2/s light intensity. Infiltrated leaves were treated with 2.5 mM copper sulfate by spray at three days post infiltration. The spray was applied to both the adaxial and abaxial surfaces of the leaf. All chemical compounds were purchased from Sigma-Aldrich (St. Louis, MO).

Quantification of reporter gene expression

Luciferase expression was detected using the Nano-Glo® Dual-Luciferase® reporter assay system (Promega, Madison, WI, USA). Two 8 mm-diameter discs per infiltrated leaf were homogenized in180 µL passive lysis buffer (Promega) containing protease inhibitor (P9599, Sigma-Aldrich, Dorset, UK). Following incubation on ice for 15 min and centrifugation (100 × g, 2 min, 4°C), the supernatant was diluted to a 1:5 dilution. 10 µL of the dilution was mixed with 20 µL of passive buffer which was then mixed with 30 µL ONE-Glo™ EX Luciferase Assay Reagent (Promega) and incubated at room temperature for 10 min. LucF luminescence was detected using either a GloMax 96 Microplate Luminometer (Promega) or a Clariostar microplate reader (BMG Labtech, Aylesbury, UK) with a 10 s read time and 1 s settling time. LucN luminescence was detected from the same sample by adding 30 µL NanoDLR™ Stop & Glo® Reagent (Promega). After incubation for 10 min at room temperature, luminescence was detected as above. To calculate the proportion of expression from each reporter, luminescence from firefly luciferase (LucF) was scaled to the nanoluciferase (LucN) signal by an experimentally determined factor obtained from expression from single gene LucN and LucF constructs. Normalized (relative) expression levels of synthetic promoters were obtained as previously described (Cai et al., 2021) and are reported as the ratio of luminescence from the test promoter (LucN) to the calibrator promoter (LucF), normalized to the luminescence of an experiment control LucN/LucF expressed from calibrator promoters.

Metabolite extraction and quantification

Standards, extraction methods and analysis of pheromone compounds were as previously described (Mateos-Fernández et al., 2021). Briefly, synthetic samples of Z11-16OH were obtained as described by Zarbin et al. (2007)

and purified by column chromatography using silica gel and a mixture of hexane : Et2O (9:1 to 8:2) as an eluent. Acetylation of Z11-16OH was carried out using acetic anhydride (1.2 eq) and trimethylamine (1.3 eq) as a base in dichloromethane (DCM). For biological samples, 8 mm leaf disks were snap frozen in liquid nitrogen and ground to a fine powder. 50 mg of frozen powder was transferred to 10 mL headspace vials and stabilized with 1 mL 5M $CaCl_2$ and 150 µL 500 mM EDTA (pH=7.5). Tridecane was added to a final concentration of 10 ppb for use as an internal standard and vials were bath-sonicated for 5 minutes. For volatile extraction, vials were incubated at 80°C for 3 minutes with 500 rpm agitation, after which the volatile compounds were captured by exposing a 65 µm polydimethylsiloxane/divinylbenzene (PDMS/DVB) SPME fiber (Supelco, Bellefonte, PA) to the headspace of the vial for 20 minutes. Volatile compounds were analyzed using a 6890N gas chromatograph (Agilent Technologies, Santa Clara, CA) with a DB5ms (60 m, 0.25 mm, 1 µm) J&W GC capillary column (Agilent Technologies) with helium at a constant flow of 1.2 mL min^{-1}. Fiber was desorbed for 1 minute in the injection port at 250°C and chromatography was performed with an initial temperature of 160°C for 2 min, 7°C min^{-1} ramp until 280°C, and a final hold at 280°C for 6 minutes. All pheromone values were divided by the tridecane value of each sample for normalization. Alternatively, if tridecane was not added, pheromone values were normalized using the total ion count (TIC) of the corresponding sample (Wu and Li, 2016).

Data Availability

Plasmids and their complete sequences have been submitted to Addgene (See Supplementary Table S1).

Conflict of interest statement

None declared

Author contributions

KK, EMG, DO and NP conceptualized the study. KK, EMG, RMF, SG and CT were responsible for the design and assembly of constructs. KK, EMG and CT conducted molecular and expression analyses of constructs. KK, EMG, RMF and SG conducted biochemical analyses of leaf extracts. All authors contributed to the analysis and visualization of data. DO and NP were responsible for supervision and funding acquisition. EMG, CT, and NP drafted the text. All authors contributed to revising and editing the text.

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Supplementary Data

Supplementary Table S1. Constructs used in this study
Supplementary Figure S1. Construct architecture influences expression from constitutive promoters.
Supplementary Figure S2. Comparison of pheromone yields obtained by constitutively expressed and copper-inducible expression of dCasEV2.1.
Supplementary Figure S3. Comparison of pheromone yields obtained by altering the location of the sgRNA expression cassette

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