Production of Volatile Moth Sex Pheromones in Transgenic *Nicotiana benthamiana* Plants

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The use of plant systems for the bio-production of insect sex pheromones represents an innovative strategy to make pest control more efficient and sustainable. Insect pheromones are an appealing alternative to broad-spectrum pesticides, and different pheromone-based approaches can be employed to contain herbivore populations and reduce the damage to crops and stored goods. Contrary to conventional pesticides, pheromones are highly species-specific, non-toxic and prevent the insurgence of genetic resistance in target populations. The global insect pheromone market was worth 1.9 billion USD in 2017, and it is predicted to reach over 6 billion USD by 2025. Currently, the use of insect sex pheromones in agriculture is limited by their cost, which makes them affordable only for certain high value crops, and their sustainability is hampered by the complex and polluting processes involved in their chemical synthesis. Engineered biological systems, both microbial and plant-based, thus represent an excellent alternative to improve sustainability coupled with metabolic flexibility. Plant biofactories can be devised both for the extraction of the molecules of interest to formulate conventional pheromone traps, or for the active release of pheromones into the environment as live biodispensers in sexual confusion strategies. The engineering of such systems is not, however, free from complications and biological bottlenecks, which derive chiefly from the toll that the sustained production of insect sex pheromones can impose on plant metabolism.

We engineered *Nicotiana benthamiana* plants to produce (*Z*)-11-hexadecenol (Z11-16OH) and (*Z*)-11-hexadecenyl acetate (Z11-16OAc), two main volatile components in many Lepidoptera sex pheromone blends. To do this, we assembled a biosynthetic pathway comprising three genes encoding key enzymes for the biosynthesis of fatty-acid derived pheromones: the *Amyelois transitella* desaturase *AtrΔ11*, the *Helicoverpa armigera* fatty acyl reductase *HarFAR*, and the *Euonymus alatus* diacylglycerol acetyltransferase *EaDAct*. The ability of *N. benthamiana* to produce these pheromone components was first assayed transiently, and then stable transformants were obtained and their phenotype assessed throughout various generations. These pheromone-producing plants were dubbed 'Sexy Plants' (SxP). Different versions of the SxP (v1.0, 1.1 and 1.2) were generated. Production of insect sex pheromone components resulted in plants with dwarf phenotypes, whose severity increased with production levels. All but one of the recovered transgenic lines produced high levels of Z11-16OH, but only one transgenic line (SxPv1.2) produced appreciable levels of Z11-16OAc (11.8 µg g^{-1}FW), next to high levels of Z11-16OH (111.4 µg g^{-1}FW). SxPv1.2 was thus used to measure the rates of volatile pheromone release, which resulted in 8.48 ng g^{-1}FW per day for Z11-16OH and 9.44 ng g^{-1}FW per day for Z11-16OAc. Changes in the general volatile profile of the SxP with respect to WT *N. benthamiana* were investigated, and the ability of the plant-produced pheromone components to elicit a response in *Sesamia nonagrioides* antennae was confirmed.

Our results establish a roadmap for biotechnological improvements of pheromone-producing plants. Based on the biomass and release constraints identified in this study, different strategies can be envisioned to regulate the production of insect sex pheromones in plants. The temporal regulation of the biosynthetic pathway through induction with eco-friendly agrochemicals would allow the plant to develop a normal biomass before starting to accumulate pheromone compounds. On the other hand, the spatial regulation of their biosynthesis by targeting gene expression to the trichomes, coupled with the expression of transporters, would allow cellular detoxification and increased volatilization.