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The GRAPE selection escapes from slow *in planta* directed evolution

Directed evolution is a powerful engineering technique that mimics natural selection in the laboratory to create biomolecules with enhanced or novel properties. A typical directed evolution system involves the generation of a large library of diversified variants through mutagenesis of an initial sequence, screening or selection of variants with improved or desirable properties, and repeat of the cycle with the best hits to accumulate beneficial mutations (Zeymer and Hilvert, 2018). While established systems, such as phage-assisted continuous evolution in bacteria and OrthoRep in yeast, have enabled the continuous evolution of genes of interest (GOIs), they are less suited for plant-specific traits due to their unique cellular environment and complex regulatory networks (Esvelt et al., 2011; Tian et al., 2023). Many valuable targets, such as plant resistance genes, do not fit easily in these systems. Prior plant-based directed evolution approaches required screening thousands of GOI variants individually following *in vitro* mutagenesis. No true *in planta* selection was achieved, largely due to the low proliferation rate of plant cells. In a recent breakthrough study, Zhu et al. (2025) developed an innovative geminivirus-replicon-assisted *in planta* directed evolution (GRAPE) platform that enables high-throughput selection of GOI variants *in planta*, accelerating the selection of a defined trait within a single leaf of *Nicotiana benthamiana* in just 4 days. These technological advances will comprehensively reshape the landscape of *in planta* directed evolution.

The GRAPE selection: Linking gene function to geminivirus replicon replication

A key innovation of GRAPE is that, unlike previous labor-intensive screening, it links the function of GOIs to the replication efficiency of geminivirus replicons by equipping the replicon module with genetic circuits (Figure 1).

Geminiviruses are a group of single-stranded DNA viruses that replicate in the nucleus of host cells via rolling circle replication (RCR) and recombination-dependent replication (Hanley-Bowdoin et al., 2013). Due to their rapid and high-yield replication and wide flexibility for cargo insertion, geminivirus replicons have been utilized for a plethora of functions, such as enhancing the yield of biomanufactured products and increasing template concentration for gene editing (Lozano-Durán, 2016; Garcia-Perez et al., 2025). To overcome the obstacles of fast directed evolution in plants, Zhu et al. (2025) harnessed the RCR machinery of geminiviruses and engineered a geminivirus-replicon-assisted platform for high-throughput *in planta* directed evolution.

The rate of viral replication is a central consideration in designing virus-based directed evolution. To identify the optimal geminivirus, the authors selected 19 geminiviruses from three well-identified genera of the family *Geminiviridae* (*Begomovirus*, *Mastrevirus*, and *Curtovirus*) and evaluated the replication efficacy of the artificial replicons of each geminivirus. They demonstrated that 14 geminivirus replicons could self-replicate in *N. benthamiana*. After several rounds of assessment, they found that the controllable replicon derived from bean yellow dwarf virus achieved the highest copy number, reaching up to ~80 000 copies per cell in the presence of *trans*-supplied Rep/RepA proteins. This replicon comprises two components: a replicative vector where the GOI is flanked by the geminiviral intergenic region and Rep/RepA provided in *trans*. Upon expression, Rep/RepA recognizes the iteron in the intergenic region, produces a site-specific nick, and mediates the circularization of the sequences. The released episomal DNA molecules will then be replicated at a faster rate than the reproduction of plant cells. To meet the diverse demands of selection, the bean yellow dwarf virus replicon was equipped with genetic circuits, including inteins, proteases, and recombinases, to positively link GOI functions to RCR or with homologous recombination factors, sequence-specific nucleases, and plant immunity elicitors to negatively link GOI functions to RCR. If the biological function of GOIs is positively linked to RCR, functional variants drive replicon amplification, while non-functional ones are depleted. Conversely, functional variants inhibit replicon amplification and are thus depleted from the replicon library in a negative selection. Evolved variants could be subsequently identified after deep sequencing of the replicon library.

To support the construction of a large library of given GOIs, they further developed pPhi, a 2-kb *Agrobacterium tumefaciens* vector that maintains a high copy number in *Escherichia coli* and *A. tumefaciens*. It also performs well via *Agrobacterium*-mediated infiltration in *N. benthamiana*. Finally, using diversification barcodes and deep sequencing, they optimized agroinfiltration conditions. At a low OD_{600} (0.01–0.03), most *N. benthamiana* cells are typically transformed by only a single *Agrobacterium* strain, which ensures unbiased library representation and is relevant for interpreting combinatorial delivery outcomes. Accordingly, a throughput of up to 10^5 variants can be screened per leaf within 4 days.

Proof of concept: Evolving plant immune receptors with expanding functions

Intracellular nucleotide-binding leucine-rich repeat (NLR) receptors confer plant resistance against pathogens by detecting pathogen effectors and initiating immune responses. While the high specificity of NLRs enables precise recognition of their cognate effectors, it also creates narrow spectrum that can be sometimes circumvented by pathogens. Harnessing stepwise evolution or engineering of NLRs represents a promising strategy for developing crops with elevated disease resistance (Wang et al., 2025a, 2025b). As a proof of concept, Zhu et al. (2025) demonstrated

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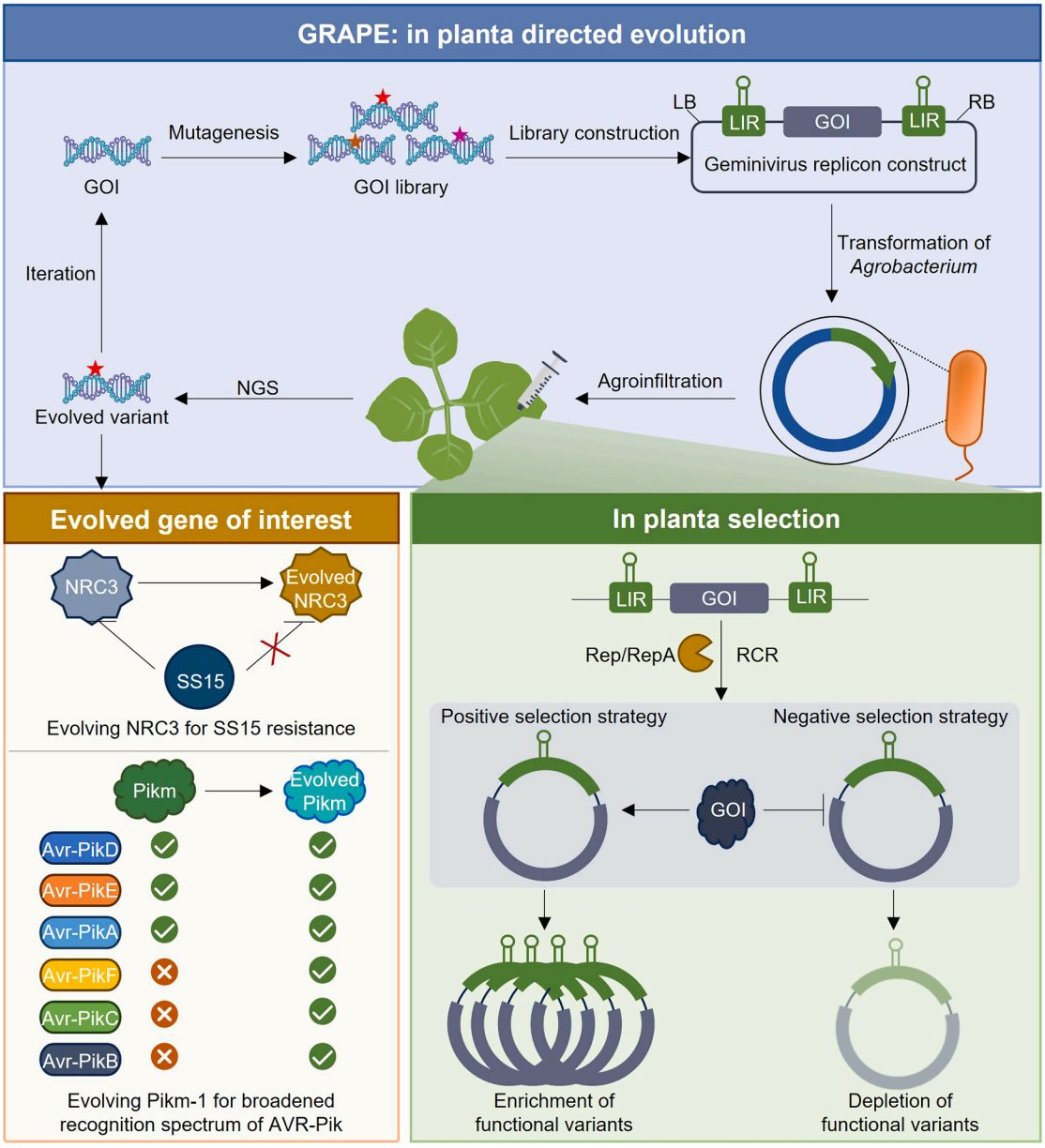

Figure 1. Schematic of geminivirus replicon-assisted *in planta* directed evolution.

A gene of interest (GOI) is mutagenized *in vitro*, and the resulting library of mutagenized GOIs is cloned into a controllable geminivirus replicon. A genetic circuit links the desired GOI function to rolling circle replication (RCR) of the geminivirus replicon. After transformation of the construct into *Agrobacterium tumefaciens*, the cultures were suspended and agroinfiltrated into *Nicotiana benthamiana* leaves. In *N. benthamiana*, when GOI function is positively linked with RCR, the functional GOI variant triggers RCR, leading to their amplification and enrichment, while non-functional variants are depleted. When GOI function is negatively linked to RCR, functional variants inhibit replication and are thus depleted. Afterward, next-generation sequencing (NGS) is used to identify evolved variants. If no desired variant is obtained, the selection cycle is iterated. As a proof of concept, the negative selection strategy was used to evolve NRC3 variants resistant to the nematode effector SS15-mediated suppression and Pikm-1 variants with expanded recognition of Avr-Pik alleles—from three to all six. LIR, long intergenic region; LB, left border; RB, right border.

the power of GRAPE through the directed evolution of two plant disease resistance genes, *NRC3* and *Pikm-1*. NRC3 is a helper NLR receptor in *Solanaceae* plants that triggers hypersensitive response cell death upon detection of pathogens by NLR. However, the nematode effector SPRYSEC15 (SS15) suppresses NRC3 activity by directly interacting with NRC3. Using a negative selection strategy, they used GRAPE to obtain NRC3 variants that fail to interact with SS15 and thus evade the suppression of SS15 while retaining hypersensitive response-inducing capability and plant immunity. This overcomes a dual constraint that would be difficult to enforce in non-plant systems. They next engineered the rice receptor Pikm-1 and obtained evolved Pikm-1 variants with expanded recognition of the *Magnaporthe oryzae* effector Avr-Pik alleles—from three to all six. These results highlight the great potential of GRAPE in the optimization of complex traits in a plant-native context.

Concluding remarks and perspectives

GRAPE provides key advances to the field of *in planta* directed evolution. GRAPE offers high-throughput selection but the shortest experimental timescales and, in principle, enables the fastest and broadly applicable evolution of diverse biomolecules *in planta*. By tethering replicon replication to the function of GOIs, it allows simultaneous selection of desired variants on rapid timescales. It also confines the selection of desired variants to a relevant, tunable plant context, ensuring that evolved variants can be seamlessly translated into practical applications without further optimization in plants. Based on the demonstrated success in evolving plant immune receptors with broader resistance spectra, proof-of-principle experiments are required to extend this platform to rapidly test additional biomolecules that have been inaccessible through other methods. Although the GRAPE platform does not yet support *in planta* mutagenesis, a feature available in virus-based continuous directed evolution in mammalian cells (Esvelt et al., 2011; Hendel and Shoulders, 2021), it nevertheless represents a quantum leap over prior *in planta* methods, laying the groundwork for new discoveries and innovative applications in plant cells.

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