Commentary

Dude, where is my mutant? *Nicotiana benthamiana* meets forward genetics

Nicotiana benthamiana, a solanaceous species native to Australia, is one of the most commonly used model plant organisms. It was initially used by the virology community because of its hypersusceptibility to plant viruses. Later, this feature was exploited with the development of viral vectors that can express foreign genes and the establishment of virus-induced gene silencing (VIGS), which enabled knocking down endogenous plant genes. Benth (or Benthi), as it is colloquially known, was further popularized by the development of agroinfiltration, a method that enabled transient protein expression in plants. Agroinfiltration has been extensively used in cell biology, biochemistry, protein–protein interaction analyses and other *in planta* studies (Goodin *et al.*, 2008). However, genetic analyses of *N. benthamiana* remained limited due to its allotetraploid nature and incomplete sequence of the 3.1 Gb genome. In this issue of *New Phytologist*, Schultink *et al.* (pp. 1001–1009) used a forward genetic screen in *N. benthamiana* to determine that NbZAR1, an orthologue of the *Arabidopsis thaliana* NLR (nucleotide-binding domain and leucine-rich repeat domain-containing) ZAR1, is responsible for the perception of XopJ4 from the tomato bacterial pathogen *Xanthomonas perforans*. Although forward genetic screens using gene silencing have been performed in *N. benthamiana*, Schultink *et al.* are among the first to use a chemical mutagenesis-based screen to dissect plant biological processes. Together with CRISPR genome editing and improved genomics resources, this study ushers in a new era of forward and reverse genetic analyses for this much-cherished model plant system (Fig. 1).

How conserved are immune pathways across plants?

All plant species have immune systems that help them fend off invading pathogens (Kourelis & van der Hoorn, 2018). The plant immune system is activated upon perception of pathogen molecules by host immune receptors, which are often NLR proteins or pattern recognition receptors (PRRs). Some of these immune receptors directly associate with pathogen molecules, while others indirectly perceive pathogen molecules through associated proteins (Kourelis & van der Hoorn, 2018). The degree to which immune pathways are conserved across different plant botanical families is unclear. Thus, identification of shared immune pathways, using comparative genomics, across different plant lineages should be of interest to plant pathologists and plant breeders.

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Schultink *et al.* used an ethyl methanesulfonate (EMS)-based forward genetics screen in *N. benthamiana* to identify the immune pathway responsible for perception of the type III effector XopJ4 from *X. perforans*. Two EMS mutants unable to perceive XopJ4 were identified. These mutants were back-crossed to wild-type plants, and the mutant phenotype was genetically mapped in one of the resulting F_2 populations using Illumina whole genome sequencing to identify single nucleotide polymorphisms (SNPs) that associate with the loss-of-function phenotype. Alterations in both lines turned out to be due to mutations in the NLR protein named NbZAR1. NbZAR1 is orthologous to *A. thaliana* ZAR1, which recognizes several bacterial effectors, including HopZ1a from *Pseudomonas syringae* (Lewis *et al.*, 2010; Kourelis & van der Hoorn, 2018). Since *A. thaliana* receptor-like cytoplasmic kinases (RLCK) XII family members are required by ZAR1 for recognition of different pathogen effectors, Schultink *et al.* hypothesized that this may also be the case for NbZAR1. To test this, they used VIGS to knock-down members of the RLCK XII family in *N. benthamiana* and found that silencing one of these family members, namely XopJ4 Immunity 2 (JIM2), compromised XopJ4 recognition in *N. benthamiana*. This indicates that, in *N. benthamiana*, NbZAR1 adopts an effector response pathway similar to that of ZAR1 in *A. thaliana*, indicating some level of immune pathway conservation between the Solanaceae and Brassicaceae plant lineages. Interestingly, Schultink *et al.* observed that the two ZAR1 orthologues were not functionally interchangeable across the examined species. This implies that ZAR1 orthologues display a degree of functional diversification among different plant lineages. By combining mutant screens with VIGS and genetic complementation assays, Schultink *et al.* provide a platform for comparative functional studies in *N. benthamiana* to further dissect the evolutionary diversification of the ZAR1 pathway.

The approach used by Schultink *et al.* could be generalized to study pathogen effectors that activate immunity in *N. benthamiana*. This may eventually lead to the identification of resistance genes and other components of the *N. benthamiana* immune pathway that are

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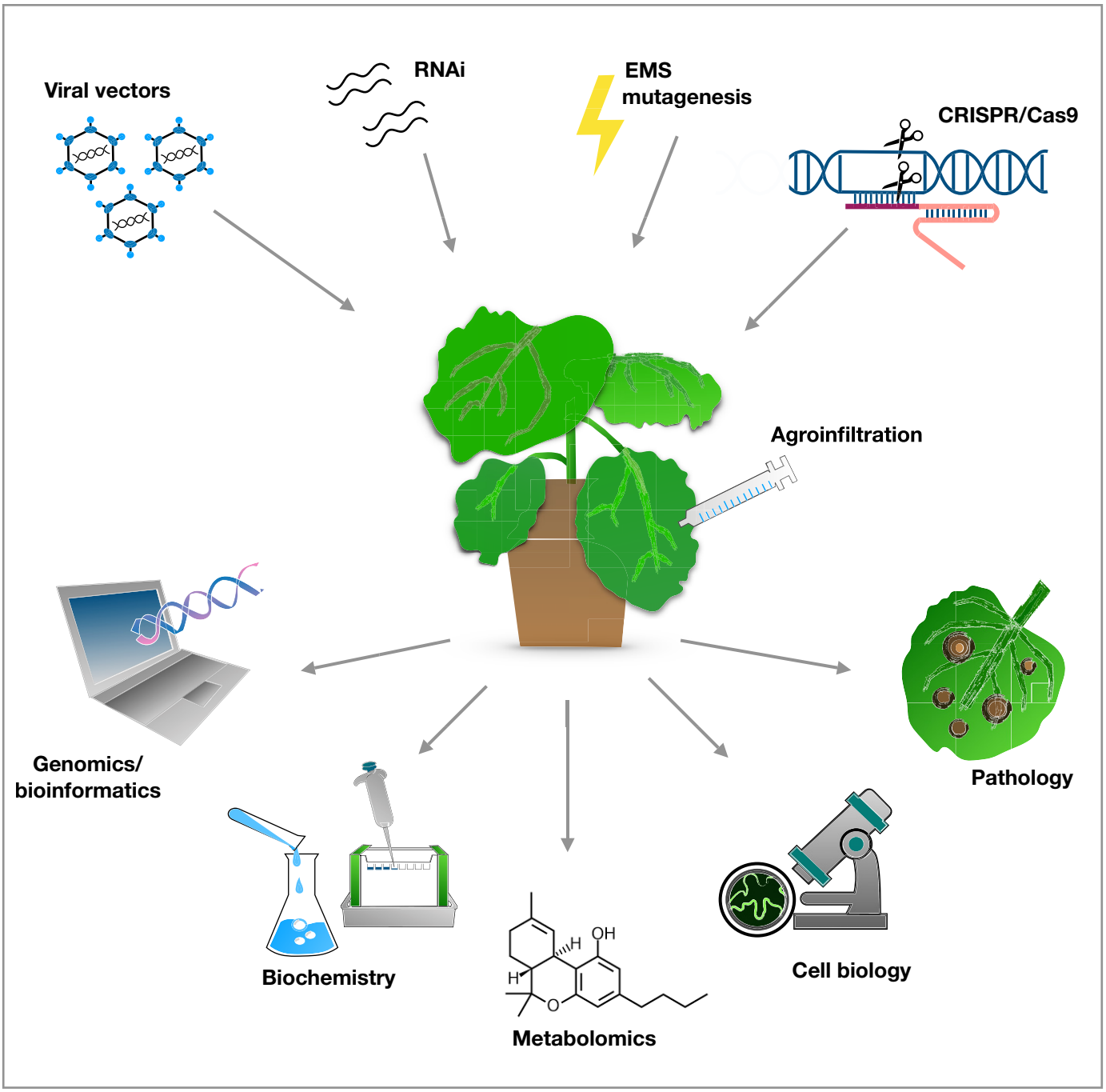

Fig. 1 *Nicotiana benthamiana* is an amenable model organism for plant biology study. Several functional genomics tools, including viral vectors, RNAi, ethyl methanesulfonate (EMS) mutagenesis, CRISPR-mediated genome editing, and agroinfiltration, are available in the *N. benthamiana* experimental system. These tools can be applied to research in genomics, biochemistry, metabolomics, cell biology and pathology as well as other topics in plant biology.

valuable in agriculture. These genes would be useful resources in breeding disease resistance in different crop plants.

How diverse are the *Nicotiana benthamiana* laboratory lines?

Schultink *et al.* noted differences in the response to XopJ4 between different laboratory lines of *N. benthamiana*. This raises questions about the degree of genetic variation among *N. benthamiana* laboratory accessions. Goodin *et al.* (2008) investigated the genetic diversity of different sources of *N. benthamiana* and concluded that the laboratory accessions probably originated from a single source that carries a mutation in the RNA-dependent RNA polymerase RDR1, which is uncommon in wild populations (Bally *et al.*, 2015; Wylie *et al.*, 2015). However, agroinfiltration of XopJ4 induced cell death in the *N. benthamiana* accession used by Schultink *et al.* but not in the accession used by Schwartz *et al.* (2015). Although the reasons leading to this differential response have not yet been

identified, these results suggest, perhaps unsurprisingly, that not all laboratory accessions have an identical genetic background. A comprehensive study of the history and genetic variation of laboratory accessions is essential to further improve *N. benthamiana* as a model system for plant biology. Cell death mediated by effector recognition, such as that of XopJ4, can act as a quick read out, and therefore, could potentially be used as a phenotypic marker to characterize and identify differences between different laboratory *N. benthamiana* accessions. These observations also call for a centralized repository of *N. benthamiana* genetic resources, and for a naming system of the different *N. benthamiana* accessions.

Genomics resources for *Nicotiana benthamiana*

Next-generation sequencing technologies have revolutionized biology, for example, by providing genomics resources for mechanistic studies in many plant species. Several *N. benthamiana* draft genome assemblies and *de novo* transcriptome sequences have been released since 2012 (Bombarely *et al.*, 2012; Naim *et al.*, 2012; Nakasugi *et al.*, 2014). However, genomic resources of this species remains incomplete. Although *N. benthamiana* lacks a complete reference genome sequence, the existing information was sufficient for the completion of the Schultink *et al.* study. More recently, Kourelis *et al.* (2018) re-analysed the four available draft genomes (Niben 1.0.1; Niben 0.4.4; Nbv0.5; and Nbv0.3), and improved the quality of the current gene annotations by taking advantage of genomes of other *Nicotiana* species. These datasets have greatly facilitated *N. benthamiana* research, allowing for more precise genetic studies, such as VIGS, proteomics and genome editing. The current gaps within the draft genome assemblies should be closed by several ongoing projects that aim to improve the *N. benthamiana* genome sequence using single molecule sequencing technologies.

Looking forward: mutants galore for *Nicotiana benthamiana*?

Functional genomics studies have been somewhat limited in *N. benthamiana* due to the incomplete genome information and genetic complexity attributed to the allotetraploid genome. Nonetheless, several gene silencing-based strategies have been used to perform informative genetic screens (Senthil-Kumar & Mysore, 2014; Brendolise *et al.*, 2017). These screens have led to important discoveries in plant biology, notably in the field of plant–microbe interactions. The work of Schultink *et al.* opens up additional opportunities for forward genetics studies in *N. benthamiana*. Combined with tools, such as transient gene expression, gene silencing, and genetic complementation, cell biology, biochemistry and metabolomics, the link between gene and phenotype-of-interest can quickly be validated (Fig. 1). Furthermore, *N. benthamiana* is highly amenable to CRISPR-mediated genome editing and targeted mutagenesis is now routine in several laboratories. CRISPR also enables directed mutagenesis of multiple homologues and paralogues to overcome the challenges posed by genetic redundancy. With further improvements of the *N. benthamiana* genome assembly and annotation, as well as the development of associated functional genomics tools, we anticipate that the popularity of this model system will continue to rise in plant biology research.

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