# *Nicotiana benthamiana*, A Popular Model for Genome Evolution and Plant–Pathogen Interactions

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**Abstract**

*Nicotiana benthamiana* originates from northern Australia and belongs to the Suaveolentes section. It is used extensively as a model organism for many types of research, including plant–pathogen interactions, RNA interference, and functional genomics. Recent publications that used *N. benthamiana* as a model for plant–pathogen interactions focused mainly on bacteria, viruses, oomycete, and fungi. Two different *N. benthamiana* whole genome assemblies were published in 2012. These assemblies have been improved and structurally annotated in later versions but are still incomplete. The lineage most widely used in research originates from a population that has retained a loss-of-function mutation in *Rdr1* (RNA-dependent RNA polymerase 1) that makes it highly susceptible to viruses. In this chapter, we review some of the techniques used in *N. benthamiana* to study plant–pathogen interactions, including virus-induced gene silencing, transient protein expression by agroinfiltration, stable genetic manipulation, and transcriptomics analysis, and discuss some of the results. Descriptions and links to some of the most relevant online resources for *N. benthamiana* are also provided.

## 14.1 Evolutionary History of *Nicotiana benthamiana*

*N. benthamiana* Domin is among the most popular plants used for plant pathology studies. Despite this, specific details about the origin of this species are still unknown. *N. benthamiana* is a herbaceous plant with white flowers that is native to Australia where it can be found along the north coast, the Northern Territory, and Queensland (Fig. 14.1) (Global Biodiversity Information Facility (GBIF) 2018).

It can be identified following the dichotomous key described by Burbidge. *N. benthamiana* is distinguished from *N. umbratica* specimens by: "Upper cauline leaves sessile and forming leafy

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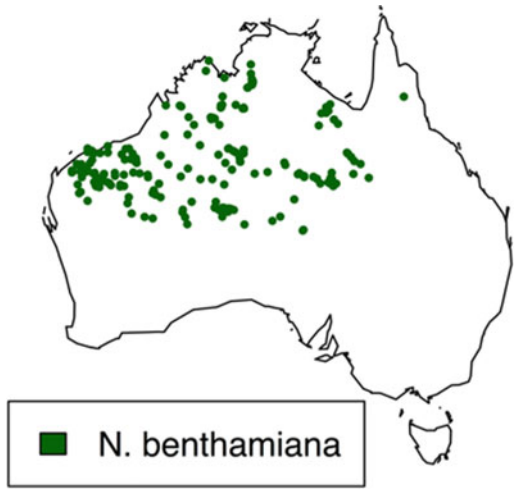


**Fig. 14.1** Natural distribution of *N. benthamiana* in Australia

bracts. Laminae ovate to broad lanceolate, obtuse. Corolla lobes obtuse compared with the corolla lobes acute of the last one" (Burbidge 1960). The first available record of *N. benthamiana* can be found in the Herbarium Hookerianum at Kew Royal Botanical Gardens (http://specimens.kew.org/herbarium/K000196107), with a specimen collected by Benjamin Bynoe during the voyage of the Beagle (1837–1843) (Orchard 1999). Like other Australian *Nicotiana* species, *N. benthamiana* belongs to the Suaveolentes section, which contains 30 species and represents an important radiation of the *Nicotiana* genus in Australia (26 species), South Pacific (3 species), and Africa (1 species) (Marks et al. 2011). They are adapted to a wide range of conditions, from the high humidity coastal regions of Cairns (e.g., *N. debneyi*) to the extremely dry Great Sandy and Tanami deserts (e.g., *N. benthamiana*). All the Suaveolentes species have been described as allotetraploids with a variable number of chromosomes ranging from $2n = 4 \times = 30$ (*N. suaveolens*) to $2n = 4 \times = 48$ (*N. debneyi*).

Elucidation of the origin of the Suaveolentes section is still ongoing. An early publication by DeWolf and Goodspeed (1957) proposed that it could have formed after the hybridization of members of the Petunioides, Alatae/Sylvestres, and/or Noctiflorae sections. Later phylogenetic studies using plastid gene sequences such as *matK* indicated a Noctiflorae species as maternal donor (Aoki and Ito 2000; Clarkson et al. 2004). The use of interspacers ITS1 and ITS2, and 5.8S ribosomal genes indicated that an ancestral member of Alatae could be the possible paternal progenitor (Chase et al. 2003). A study using the *GS* (glutamine synthetase) gene, with both copies usually retained in polyploid *Nicotiana* species, indicated Sylvestres as the maternal donor and Trigonophyllae as the paternal donor (Clarkson et al. 2010). Nevertheless, the most accepted hypothesis based on several genes, such as *ADH* (alcohol dehydrogenase), *LFY/FLO* (LEAFY/FLORICAULA), *GS*, and nrITS (nuclear ribosomal interspacer), suggests a complex history where the most probable donors are Sylvestres (paternal) and Noctiflorae/Petunioides (maternal) (Kelly et al. 2013). The divergency ages between the Suaveolentes subgenomes and the corresponding proposed progenitors were estimated as 6.4 million years ago for the maternal contribution from the Noctiflorae section and 5.5 million years ago for the paternal contribution from the Sylvestres section (Clarkson et al. 2017).

## 14.2 *Nicotiana benthamiana* Genome Assemblies and Genetic Data

*N. benthamiana* was one of the first plant models positively affected by next-generation sequencing (NGS) technologies. Although several plant whole genome assemblies were built using NGS before 2012, most of them were for crops such as cucumber (Huang et al. 2009), apple (Velasco et al. 2010), and soybean (Schmutz et al. 2010). Two different *N. benthamiana* whole genome assemblies were published in 2012 (Bombarely et al. 2012; Naim et al. 2012). *N. benthamiana* is an allotetraploid plant ($2n = 4 \times = 38$) with a large genome of 3.1 Gb, which made it difficult to assemble using the short reads obtained by NGS. Although both genomes were incomplete,

**Table 14.1** Details of the two *N. benthamiana* 2012 genome assemblies

| Assembly | Niben0.4.2[a] | | Nbv0.3[b] | |
|---|---|---|---|---|
| Assembly statistics | Contigs | Scaffolds | Contigs | Scaffolds |
| Total assembly size (Gb) | 2.46 | 2.63 | 2.44 | 2.44 |
| Total assembled sequences | 461,463 | 141,339 | 300,384 | 275,036 |
| Longest sequence length (Kb) | 208.21 | 615.59 | 307.11 | 447.13 |
| Average sequence length (Kb) | 5.37 | 18.61 | 8.12 | 8.88 |
| N50 index (sequences) | 42,984 | 8,897 | 22,438 | 22,068 |
| N50 length (Kb) | 16.48 | 89.78 | 31.25 | 31.83 |

[a]Bombarely et al. 2012; [b]Naim et al. 2012

they have been used extensively for plant–pathogen research. Statistics of the two *N. benthamiana* genome assemblies are summarized in Table 14.1.

Although a chromosome level assembly has still not been achieved (as October 2018), these assemblies have been improved. Statistics of the latest versions are summarized in Table 14.2. Evaluation of these genome assemblies using BUSCO (Simao et al. 2015) indicated they had similar completeness (95.4% for Niben1.0.1 and 94.4% for Nbv0.5), but Niben1.0.1 had a higher proportion of duplicated genes (46.0%) than Nbv0.5 (43.4%). These assemblies were also structurally annotated revealing 59,814 and 49,818 primary transcripts for Niben1.0.1 and Nbv0.5, respectively. Because of the ongoing diploidization process in *N. benthamiana,* its gene number is higher than the gene numbers for diploid Solanaceae species such as *Solanum lycopersicum* (Tomato Genome Consortium 2012), *Solanum tuberosum* (Potato Genome Sequencing Consortium 2011), *Capsicum annuum* (Kim et al. 2014), *N. sylvestris* and *N. tomentosiformis* (Sierro 2013), and *Petunia axillaris* (Bombarely et al. 2016), but lower than the gene number for allotetraploid *N. tabacum* (Sierro et al. 2014; Edwards et al. 2017). As expected, the BUSCO evaluation of the completeness of the annotations produced a lower value (88.1% for Niben1.0.1 and 75.2% for the transcriptome Nbv5.1 from the assembly Nbv0.5) than the evaluation of the genome assembly. Kourelis et al. (2018) used the genomes of other *Nicotiana* species to reanalyze the four genomes (Niben1.0.1, Niben0.4.2, Nbv0.5, and Nbv0.3) improving the quality of the *N. benthamiana* gene annotations.

To date, dozens of experiments using NGS technologies to investigate Solanaceae species have been published by the National Center for Biotechnology Information (NCBI). Indeed, the NCBI's Taxonomy Browser has links to 342 SRA datasets, 442 BioSamples, and 74 BioProjects (up

**Table 14.2** Details of the latest publicly available *N. benthamiana* genome assemblies (up to October 2018)

| Assembly | Niben1.0.1[a] | | Nbv0.5[b] | |
|---|---|---|---|---|
| Assembly statistics | Contigs | Scaffolds | Contigs | Scaffolds |
| Total assembly size (Gb) | 2.49 | 2.97 | 2.51 | 2.55 |
| Total assembled sequences | 288,736 | 56,094 | 180,357 | 147,949 |
| Longest sequence length (Kb) | 187.66 | 2,838.18 | 965.20 | 2,628.40 |
| Average sequence length (Kb) | 8.62 | 52.94 | 13.92 | 17.23 |
| N50 index (sequences) | 37,001 | 1,738 | 7,104 | 1,936 |
| N50 length (Kb) | 20.13 | 520.10 | 105.31 | 396.15 |

[a]Niben1.0.1 is available on the Sol Genomics Network (https://solgenomics.net/organism/Nicotiana_benthamiana/genome); [b]Nbv0.5 is available from Queensland University of Technology (http://benthgenome.qut.edu.au/)

to December 2018) for *N. benthamiana*. These BioProjects not only study plant–pathogen interaction using resistance gene enrichment sequencing (RenSeq) (e.g., BioProject accession PRJNA496490) and RNA sequencing (RNA-Seq) (e.g., PRJNA360110), but also study expression regulatory mechanisms involving small RNAs (e.g., PRJNA481240, PRJNA309389) and circular RNAs (e.g., PRJNA422356), transcriptomic landscapes in several organs such as nectaries (e.g., PRJNA448133), transition and floral meristems (e.g., PRJNA343677), and grafting experiments (e.g., PRJDB3306).

## 14.3 *Nicotiana benthamiana* as a Model for the Study of Plant–Pathogen Interactions

The use of *N. benthamiana* as a model for plant–pathogen interactions has been thoroughly reviewed by Goodin et al. 2008; therefore, we will focus mainly on the information that has been generated since then. In their review, Goodin et al. performed a PubMed search using the term "Nicotiana benthamiana" and found 1,743 publications until 2006. We conducted a similar search, which yielded 3,606 hits (as of October 2018). By curating the publications since 2008, we identified 314 papers in which *N. benthamiana* was used as a tool to study different pathosystems, not taking into account studies in which *N. benthamiana* was used merely for transient protein expression and was unrelated to plant immunity. Among the 314 publications, the focus of study included plant interactions with bacterium (Senthil-Kumar and Mysore 2010; Kim et al. 2016), virus (Pavli et al. 2011; Zhu et al. 2014), oomycete (Adachi et al. 2015; King et al. 2014), fungus (De Jonge et al. 2012; Li et al. 2015), nematode (Mantelin et al. 2011; Ali et al. 2015), aphid (Peng et al. 2016; Atamian et al. 2013), insect (Chen et al. 2014), and viroid (Adkar-Purushothama et al. 2015). Among these pathogen types, the first four were the most widely studied using *N. benthamiana* (Fig. 14.2).

*N. benthamiana* was adopted as a model in the plant–virus research field because of its remarkable susceptibility (Goodin et al. 2008). In particular, the lineage employed at that time, which continues to be widely used in research, has a disruptive insertion in the gene coding RNA-dependent RNA polymerase I (*Rdr1*), which enhances plant fitness but simultaneously leads to its high susceptibility to viruses (Balli et al. 2015). Other reasons why *N. benthamiana* is so extensively used include high efficiency of


**Fig. 14.2** Pathogen types studied in publications involving *N. benthamiana* (2009 to October 2018). The input for WordSift (https://wordsift.org) was derived from the information from 314 publications in which *N. benthamiana* was employed in studies of plant–pathogen interaction published after 2008. Word font size indicates frequency of use

gene silencing, ease of protein transient expression using *Agrobacterium tumefaciens*, the possibility of stable genetic manipulation (Todesco and De Felippes 2016), and the availability of a draft genome (Bombarely et al. 2012; Naim et al. 2012). In addition, the ongoing development of NGS techniques may further enhance the use of *N. benthamiana* to study plant–pathogen interactions. In the following sections, we describe and discuss each of the uses and techniques mentioned in this section.

## 14.4 Virus-Induced Gene Silencing (VIGS)

This technique relies on the silencing machinery of plants in order to be able to target the transcripts of genes of interest (Burch-Smith et al. 2004). Tobacco rattle virus (TRV)-based vectors are commonly used in numerous plant species, particularly those belonging to the Solanaceae family (Senthil-Kumar and Mysore 2014). VIGS technology offers several advantages such as rapid reverse genetic screens, mainly because the time-consuming process of plant transformation is avoided (Velasquez et al. 2009). VIGS also allows the simultaneous knockdown of multiple genes by selecting a single fragment with enough homology to the target genes or by arranging several fragments in a single construct (Miki et al. 2005; Zhou and Zeng 2017). An important general aspect of VIGS is the choice of a control against which the target gene's silencing performance can be compared. An empty vector (TRV2::00) is not recommended as a control, rather TRV2 carrying an insert is preferred (Hartl et al. 2008; Wu et al. 2011). Inserts derived from green fluorescent protein (*GFP*) (Ryu et al. 2004), β-glucuronidase (Gonorazky et al. 2014), or an *Escherichia coli* gene (*Ec1*) (Rosli et al. 2013) have been used previously. Another key aspect of VIGS is the selection of an insert that will effectively target the gene(s) of interest while avoiding off-targets. The Sol Genomics Network (SGN) VIGS Tool allows the interactive identification of most probable targets and off-targets, thereby assisting in construct design (Fernandez-Pozo et al. 2015a, b) (see the section "Online resources for *Nicotiana benthamiana*" in this chapter for more details). In *N. benthamiana*, VIGS has been used to silence some target genes (Kang et al. 2010; Choi et al. 2011; Liebrand et al. 2012 Rosli et al. 2013; Pombo et al. 2014), as well as large sets of candidate genes in a high-throughput fashion (Chakravarthy et al. 2010; Zhu et al. 2010; Mantelin et al. 2011; Rojas et al. 2012; Senthil-Kumar and Mysore 2012; Xu et al. 2012; Du et al. 2013; Lee et al. 2013; Nakano et al. 2013). The silencing step is followed by a readout experiment that depends on the particular process under study and may involve transient protein expression and elicitation of programmed cell death (PCD) (see below), pathogen challenge (Asai et al. 2008; Tanaka et al. 2009; Senthil-Kumar and Mysore 2010; Chaparro-Garcia et al. 2011; Kiba et al. 2012; Du et al. 2013; Ohtsu et al. 2014; Adachi et al. 2015; Bruckner et al. 2017; Turnbull et al. 2017), reactive oxygen species (ROS) production (Shibata et al. 2010; Segonzac et al. 2011; Deng et al. 2016; Pfeilmeier et al. 2016; Saur et al. 2016), nitric oxide production (Zhang et al. 2010), and stomatal aperture measurement (Zhang et al. 2012, 2016). Although *N. benthamiana* is susceptible to many pathogens, it may not be a host to the pathogen under study. This has been overcome by engineering the pathogen (Wei et al. 2007) or using a related species that causes disease in *N. benthamiana* (Yu et al. 2012; Yin et al. 2013; Wang et al. 2016). Chakravarthy et al. (2010) developed an assay that can be used to test the effect of silencing a candidate gene on the pattern-triggered immunity (PTI) response. This requires the infiltration of a PTI inducer (*Pseudomonas fluorescens*) and, a few hours later, a second infiltration performed in an overlapping manner with a challenger (*Pseudomonas syringae* pv. *tomato*, Pst). The speed of PCD progression in the overlapping area is related to the functionality of PTI. The authors coupled this assay with VIGS high-throughput screening and identified genes involved in the PTI response.

Recently, Zhou and Zeng 2017 developed a novel VIGS strategy to specifically and

efficiently knockdown members of a highly homologous gene family using fragments of approximately 70 base pairs. The authors combined the SGN VIGS Tool (Fernandez-Pozo et al. 2015a, b) with a manual optimization step to select the fragments in order to analyze functional redundancy among members of a gene family.

## 14.5 Transient Protein Expression by Agroinfiltration

This technique uses *Agrobacterium tumefaciens* carrying an expression vector system. Usually a suspension is infiltrated into a leaf and within 1–3 days the tissue is ready for downstream analysis or treatment. The most commonly used vector cloning methods rely mainly on Gateway (Life Technologies, Carlsbad, CA, USA) (Karimi et al. 2002; Nakagawa et al. 2007) and type IIS assembly-based technologies (Golden Gate and GreenGate) (Engler et al. 2008; Lampropoulos et al. 2013). Expression vectors allow the targeted proteins to be expressed under different promoters, depending on the final purpose of the experiment. In plant–pathogen studies, transient expression in *N. benthamiana* has been driven mainly by the constitutive cauliflower mosaic virus 35S (CaMV 35S) promoter (Kang et al. 2010; Anderson et al. 2012; Stirnweis et al. 2014; Song et al. 2015; Saur et al. 2016) and in some cases under the native promoter (Sato et al. 2014; El Kasmi et al. 2017; Ramachandran et al. 2017). Alternatively, when controlled timing of protein expression was required or when prolonged protein expression could lead to detrimental effects, gene expression was modulated by inducible promoters (Stork et al. 2015; Hwang et al. 2017). To study plant immunity, transient expression in *N. benthamiana* has been followed mainly by subcellular and tissue localization (Thiel et al. 2012; Rodriguez et al. 2014; Su et al. 2015; Zhuang et al. 2016), protein–protein interactions through co-immunoprecipitation (Co-IP) (Zhao et al. 2013; Hurni et al. 2014; Kim and Hwang 2015; El Kasmi et al. 2017), bimolecular fluorescence complementation (Sun et al. 2014; Du et al. 2015; Liu et al. 2016) or luciferase complementation imaging assay (Du et al. 2013), effect on pathogen performance (Bae et al. 2011; Medina-Hernandez et al. 2013; Song et al. 2015), expression of immune-related or marker genes (Nguyen et al. 2010; Li et al. 2014; Rodriguez et al. 2014; Su et al. 2014; Chaparro-Garcia et al. 2015), electrolyte leakage (Yu et al. 2012; Teper et al. 2014; Gupta et al. 2015), and ROS production (Stork et al. 2015). Because *N. benthamiana* does not respond with a ROS burst when challenged with the microbe-associated molecular pattern (MAMP) flgII-28 from flagellin, transient overexpression of the tomato receptor-like kinase flagellin-sensing FLS3 was used to confer responsiveness to *N. benthamiana* (Hind et al. 2016). The transient expression of a mutated version of FLS3 showed that its kinase activity was required for downstream signaling associated with the flgII-28 ROS burst.

Transient protein expression in *N. benthamiana* also was employed to identify the receptor of another MAMP from the bacterial cold shock protein, the csp22 peptide (Saur et al. 2016). Under the hypothesis that this receptor should interact with the coreceptor BAK1 (that is part of several activated receptor complexes) upon csp22 challenge, BAK1 was expressed fused to a GFP tag that was used for immunoprecipitation, followed by liquid chromatography–mass spectrometry (LC-MS/MS). This strategy allowed the identification of the *N. benthamiana* receptor-like protein required for csp22 responsiveness, NbCSPR.

Attachment of fatty acids as a post-translational modification is important for the regulation of protein location and is of particular interest in the study of plant–pathogen interactions (Boyle and Martin 2015). In *N. benthamiana*, transient protein expression coupled with click chemistry has been exploited for the detection of modifications such as N-myristoylation and S-acylation of both pathogen and host proteins (Boyle et al. 2016).

Transient overexpression of the transcription factor CabZIP63 from *Capsicum annuum* (pepper) in *N. benthamiana* leaves, followed by chromatin immunoprecipitation combined with

PCR, was used to study the transcriptional regulation that CabZIP63 exerted on CaWRKY40, a transcription factor involved in the response to the bacterial pathogen *Ralstonia solanacearum* (Shen et al. 2016).

One of the most frequently used outcomes following transient protein expression is the observation of, usually macroscopic, PCD symptoms (Kang et al. 2010; Cunnac et al. 2011; Chronis et al. 2013; Mafurah et al. 2015). The large *N. benthamiana* leaves allow testing several elicitors using different concentrations or combinations. Coupled with VIGS, this approach has been used to test if the targeted candidate gene participates in PCD associated with highly divergent types of pathogens, such as bacterium, virus, nematode, and oomycete (Del Pozo et al. 2004; Oh and Martin 2011; Pombo et al. 2014). This approach also has been used to test PCD-suppressing activity of pathogen effectors by co-infiltration with inducers of PCD (Teper et al. 2014; Stork et al. 2015). The importance of using appropriate controls in these types of experiments has been highlighted by Adlung and Bonas 2017 who found that some effectors affected *Agrobacterium tumefaciens* performance in leaf tissues, which could lead to overall lower amounts of the PCD-eliciting protein than expected.

Protein expression also has been used in high-throughput approaches. A simple toothpick method (Takken et al. 2000) coupled with the observation of PCD development allowed the identification of plant proteins involved in resistance (Nasir et al. 2005; Coemans et al. 2008; Takahashi et al. 2009). High-throughput transient *in planta* expression assays were performed to study the biological activities of pathogen effector proteins (Caillaud et al. 2012; Stam et al. 2013; Petre et al. 2015). The availability of genome sequences from a variety of pathogens allows the computational prediction of candidate effector genes based on conserved host translocation motifs and their presence in well-defined genome regions (Pais et al. 2013). Cloning and transient *Agrobacterium*-mediated expression of candidate effectors can give valuable insights into the virulence activities of effector proteins, particularly regarding the suppression of host plant immunity (Pais et al. 2013). Using this approach named "effectoromics," Petre et al. 2015 selected, cloned, and expressed 20 candidate effectors in *N. benthamiana* leaf cells to determine their subcellular localizations and to identify the plant proteins they interacted with, through downstream experiments such as Co-IP and mass spectrometry. A similar approach was used for the phenotypic characterization of 84 members of a subclass of *Phytophthora capsici* effectors, which allowed the identification of one member that, when expressed *in planta*, enhanced *P. capsici* virulence in *N. benthamiana* (Stam et al. 2013). By transiently expressing 49 RxLR effector candidates (HaRxLs) of the filamentous phytopathogen *Hyaloperonospora arabidopsidis* fused to fluorescent tags in *N. benthamiana*, two major classes of HaRxLs were defined as those that accumulated in the plant cell nucleus and those that accumulated in the plant membranes. Functional analysis revealed that, in particular, a membrane-localized effector, HaRxL17, enhanced the susceptibility of *N. benthamiana* to this pathogen (Caillaud et al. 2012).

## 14.6 Stable Genetic Manipulation

The technique has been used frequently in studies of plant–virus interactions for stably overexpressing virus-derived transcripts by taking advantage of post-transcriptional gene silencing (PTGS) to generate *N. benthamiana* plants more resistant to a pathogen (Ling et al. 2008; Reyes et al. 2009). Plant PTGS machinery has been exploited to improve resistance by overexpressing transcripts derived from viral DNA fragments (Lin et al. 2012), double-strand RNA from viral replicase (Pavli et al. 2012), artificial microRNA (Ali et al. 2013; Wagaba et al. 2016), and interfering satellite RNA and RNA interference (RNAi) (Montes et al. 2014). Expression of a whitefly GroEL chaperonin, a protein that can bind to several viruses, produced *N. benthamiana* plants more tolerant to tomato leaf curl virus and cucumber mosaic virus (Edelbaum et al. 2009).

Broad-spectrum resistance was explored using stable expression of artificial transcript activator-like effectors, assembled based on highly conserved regions within begomovirus genomes, that conferred partial resistance to three begomoviruses tested (Cheng et al. 2015). The CRISPR/Cas9 system (clustered regulatory interspaced short palindromic repeat/CRISPR-associated DNA endonuclease 9), which revolutionized plant and animal genome editing (Samanta et al. 2016), has been used successfully in *N. benthamiana* (Nekrasov et al. 2013; Li et al. 2013). CRISPR/Cas9 *N. benthamiana* plants with an inactivated Argonaute 2 gene were used to investigate broad range resistance, which showed that the Argonaute 2 protein had antiviral activity against at least three viruses in a virus-specific manner (Ludman et al. 2017). Stable plastid protein expression (transplastomics) in *N. benthamiana* using a plastid-transformation vector and biolistic was employed to express multiple defense genes (Chen et al. 2014). The results showed that a combination of sweet potato sporamin, taro cystatin, and chitinase from *Paecilomyces javanicus* conferred broad-spectrum resistance against insects, pathogens, and abiotic stresses.

As mentioned in the "Transient protein expression by agroinfiltration" section, *N. benthamiana* has been used to study the molecular mechanisms of pathogen effectors. *Plasmopara viticola* effectors with immune-suppressing activities have been identified by combining transient and stable protein expression. In particular, the overexpression of the effector PvRxLR28 in *N. benthamiana* and grapevine produced plants with enhanced susceptibility to this oomycete (Xiang et al. 2016). Interestingly, the stable overexpression of two *Phytophthora sojae* effectors enhanced disease resistance and tolerance to salt and drought stresses in *N. benthamiana* plants (Rajput et al. 2015; Zhang et al. 2015). These results suggested the possible use of these effectors in crop breeding strategies.

A good degree of conservation of certain molecular pathways, which allows interfamily gene transfer, has been key to the use of *N. benthamiana* in plant–pathogen studies. For example, stably expressed proteins from *Arabidopsis thaliana* (Lacombe et al. 2010; Narusaka et al. 2013; Huang et al. 2014; Wang et al. 2016), tomato (Rommens et al. 1995), and cotton (Lu et al. 2013; Li et al. 2014; Xu et al. 2014) were shown to have functional roles in *N. benthamiana* immunity. A pathogen-induced nucleotide-binding (NB)-leucine-rich repeat (LRR) candidate gene from *Vitis amurensis* was stably overexpressed in *N. benthamiana* (Li et al. 2017) and the transgenic plants were more resistant not only to the oomycete *Plasmopara viticola*, but also to drought and salt stresses, suggesting that the NB-LRR protein may have immune and non-immune roles.

Some stably modified *N. benthamiana* lines have been employed as tools to study the plant immune response. Line SLJR15 expresses the reporter protein Aequorin (Knight et al. 1993), which allows cytoplasmatic $Ca^{2+}$ dynamics to be studied through luminescence imaging (Segonzac et al. 2011; Saur et al. 2016). Line 16c, which expresses *Aequorea victoria* GFP targeted to the endoplasmic reticulum (Ruiz et al. 1998), is the most frequently used *N. benthamiana* line, in particular, to study small RNAs (Philips et al. 2017). Using NGS, the T-DNA insertion region was identified in line 16c and, surprisingly, a portion of a bacterial transposon was found to have co-integrated with this insertion, raising the concern that such events may occur in lines designed for commercial use (Philips et al. 2017). A Cas9-overexpressing (Cas9-OE) *N. benthamiana* line was developed as part of a virus-mediated genome editing system (Ali et al. 2015). In these plants, the DNA endonuclease Cas9 is stably overexpressed under the 35S promoter, and the single guide RNA (sgRNA), which determines the target sequence, is systemically delivered via tobacco rattle virus (Ali et al. 2015). This approach was used to rapidly test different sgRNAs to confer better immunity more efficiently against the DNA virus, tomato yellow leaf curl virus.

## 14.7 Transcriptomics Analysis of Plant–Pathogen Interactions Using *N. benthamiana*

Before a microarray derived entirely from *N. benthamiana* expressed sequence tags (EST) was developed, potato cDNA arrays were used to determine changes in gene expression in response to virus infection (Senthil et al. 2005; Dardick 2007). Subsequently, a *N. benthamiana* microarray was developed and used to comparatively analyze gene expression changes in response to the necrotrophic *Pectobacterium carotovorum* and hemibiotrophic Pst DC3000 bacteria (Kim et al. 2011). Analysis of the data showed that the transcriptomic expression profiles of *N. benthamiana* in response to *P. carotovorum* were similar to those in response to a mutated Pst DC3000 without a type III secretion system.

The development of NGS techniques and the availability of a draft genome (Bombarely et al. 2012; Naim et al. 2012) may further enhance the use of *N. benthamiana* as a model plant. So far, the Illumina RNA-Seq approach has been used to analyze changes in messenger RNA (mRNA) or small RNA levels. High-throughput small RNA sequencing was used to study the effectiveness of different RNA silencing approaches in the control of virus infections based on the expression of large virus-derived sequences (Montes et al. 2014; Zhao et al. 2015). NGS also was employed to identify and characterize microRNAs involved in the N protein-mediated immune response to tobacco mosaic virus (Yin et al. 2015). RNA-Seq was recently used for the identification of a set of stably expressed genes in *N. benthamiana* which were validated as reference genes for reverse transcription-quantitative PCR (qPCR) in plant–bacteria interaction experiments (Pombo et al. 2019).

The use of integrated omics, which included RNA-Seq analysis of healthy and Odontoglossum ringspot virus (ORSV)-infected *N. benthamiana* leaves combined with proteomics, allowed the identification of putative host proteins that interacted with ORSV capsid protein, which is important for viral long-distance movement in *N. benthamiana* (Lin et al. 2015). Recently, transcriptomic differences detected between mock-treated and *Phytophthora parasitica*-inoculated *N. benthamiana* leaves provided broad insights into *N. benthamiana* defense mechanisms against this oomycete pathogen (Shen et al. 2016).

## 14.8 Online Resources for *Nicotiana benthamiana*

Many online resources are available for *N. benthamiana* ranging from bioinformatics tools and sequence databases to germplasm collections. Some of the most relevant of these resources are summarized in Table 14.3.

The three main genomics and transcriptomics resources for *N. benthamiana* sequences and annotations are the Queensland University of Technology (QUT) database, the SGN, and NCBI's GenBank. The data in the QUT database are based mostly on the Nbv0.5 genome assembly and transcriptomes v5.1 and v6.1 from Naim et al. 2012. The main features and tools in this database are BLAST and keyword searches, data downloading, genome browsing, expression visualization, and a transcript lookup tool to find corresponding transcripts among the four genome assemblies (Niben1.0.1, Niben0.4.4, Nbv0.5, and Nbv0.3) of Bombarely et al. (2012) and Naim et al. (2012). The SGN contains resources and tools for Niben0.4.4 and Niben1.0.1 (Bombarely et al. 2012). The sequences, annotations, and proteomics resources from these genome versions are available for downloading and the data also can be queried in BLAST and JBrowse (genome browser) tools. The SGN also hosts the SGN VIGS Tool (see below) and SolCyc, a bioinformatics tool to visualize metabolic pathways based on genes from Solanaceae species. GenBank is a large database that contains sequences, annotations, scientific publications, and much more information for all species. Links to the most useful NCBI resources for *N. benthamiana* can be found on the *N. benthamiana* page in NCBI's Taxonomy Browser (https://www.ncbi.nlm.nih.

**Table 14.3** Online resources for *N. benthamiana*

| Resource | Type | URL |
|---|---|---|
| The Plant List | Taxonomic/Systematics | http://www.theplantlist.org/tpl1.1/record/kew-2382877 |
| TimeTree[a] | Taxonomic/Systematic | http://timetree.org/ |
| GBIF | Populations/Natural occurrences | https://www.gbif.org/species/3800423 |
| Atlas of Living Australia | Populations/Natural occurrences | http://bie.ala.org.au/species |
| GRIN | Germplasm collections | https://npgsweb.ars-grin.gov/gringlobal/taxonomydetail.aspx?25258 |
| IPK Gatersleben | Germplasm collections | https://gbis.ipk-gatersleben.de/ |
| NCBI/GenBank[b] | Molecular/Genomics/Literature | https://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?id=4100 |
| QUT *N. benthamiana*[c] | Molecular/Genomics/Transcriptomics | http://benthgenome.qut.edu.au/ |
| Sol Genomic Network[d] | Molecular/Genomics/Transcriptomics | https://solgenomics.net/organism/Nicotiana_benthamiana/genome |
| miRNEST[e] | MicroRNAs and predicted targets | http://rhesus.amu.edu.pl/mirnest/copy/browse.php |
| SolCyc[f] | Metabolic pathways | https://solgenomics.net/tools/solcyc/index.pl |
| CRISPR-P[g] | Biotechnology (CRISPR) | http://crispr.hzau.edu.cn/CRISPR/ |
| CCTop[h] | Biotechnology (CRISPR) | https://crispr.cos.uni-heidelberg.de/ |
| VIGS database[i] | Biotechnology (VIGS) | https://vigs.noble.org/ |
| SGN VIGS Tool[j] | Biotechnology (VIGS) | http://vigs.solgenomics.net/ |
| JOVE VIGS videos[k] | Biotechnology (VIGS) | https://www.jove.com/video/1292/virus-induced-gene-silencing-vigs-in-nicotiana-benthamiana-and-tomato |
| BTI *N. benthamiana* | Biotechnology | https://btiscience.org/our-research/research-facilities/research-resources/nicotiana-benthamiana/ |
| iGEM Foundation | Biotechnology (Synthetic biology) | http://parts.igem.org/Collections/Plants#Nicotiana_benthamiana |

[a]Kumar et al. (2017); [b]Benson et al. (2004); [c]Nakasugi et al. (2013); [d]Fernandez-Pozo et al. (2015a, b); [e]Szczesniak and Makalowska (2014); [f]Foerster et al. (2018); [g]Lei et al. (2014); [h]Stemmer et al. (2015); [i]Senthil-Kumar and Mysore (2014); [j]Fernandez-Pozo et al. (2015a, b); [k]Velasquez et al. 2009)

gov/Taxonomy/Browser/wwwtax.cgi?id=4100). In addition to these three web portals, the Boyce Thompson Institute (BTI) *N. benthamiana* website (https://btiscience.org/our-research/research-facilities/research-resources/nicotiana-benthamiana/) has a collection of links to bioinformatics tools and experimental protocols and resources for *N. benthamiana*.

Two bioinformatics tools, CRISPR-P and CCTop, provide support to design targets for genome editing using CRISPR with Niben0.4.4 and Niben1.0.1, respectively, as the reference genomes (see Table 14.3).

VIGS is an important and efficient tool for functional genomics in *N. benthamiana* (see the "Virus induced gene silencing (VIGS)" section). Several resources for designing and performing VIGS analysis in *N. benthamiana* are available, including, for example, the SGN VIGS Tool (Fernandez-Pozo et al. 2015), the VIGS database (Senthil-Kumar and Mysore 2014), and a video titled "Virus-induced gene silencing (VIGS) in *Nicotiana benthamiana* and tomato" (Velasquez et al. 2009). The SGN VIGS Tool assists in the design of VIGS constructs based on Niben1.0.1 or Niben0.4.4 using an interactive and intuitive

interface. This tool predicts the best target of a gene of interest, thereby allowing the design of constructs to silence multiple genes and minimizing the silencing of off-target genes. The VIGS database contains phenotypic information for a large number of genes silenced in *N. benthamiana*. Currently, the database contains about 1,300 descriptions and/or photographs of gene-silenced plants as well as sequence information of about 4,500 ESTs used for VIGS. This database also includes keyword and BLAST searches to explore all the resources.

Another resource of interest for biotechnology is the International Genetically Engineered Machine (iGem) Foundation's Registry of Standard Biological Parts for *N. benthamiana* (http://parts.igem.org/Collections/Plants#Nicotiana_benthamiana), which provides a collection of expression constructs, reporters, promoters, and other elements tested or that could be used in *N. benthamiana*.

More information about *N. benthamiana* taxonomic resources, populations and natural occurrence, and germplasm collections can be found in the links provided in Table 14.3.

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