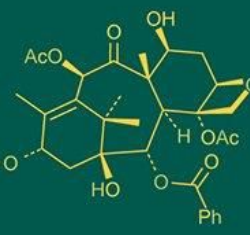
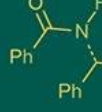


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Gateway® cloning: A versatile platform for high-throughput functional genomics and synthetic biology

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Abstract

By substituting a reliable, high-fidelity recombination technology based on bacteriophage λ for enzyme-dependent restriction-ligation techniques, gateway cloning has revolutionised molecular cloning. DNA transfer between entrance and destination vectors is accurate, directed, and modular because to its att-site architecture and BP/LR recombination processes. Due to its reproducibility, scalability, and compatibility with many vector platforms, this technology quickly became essential for big ORFeome endeavours, high-throughput functional genomics, and plant transformation operations. Its ability to assemble multigene complexes, transcriptional units, synthetic circuits, and pathway modules has been increased by later innovations like MultiSite Gateway and hybrid ligation-free techniques. Promoter-reporter systems, CRISPR expression backbones, and Gateway-compatible binary vectors continue to simplify pathway building, genome editing, and gene function investigations in plant biology. In comparison to Golden Gate, MoClo, and Gibson assembly, this review summarises the mechanistic principles, methodological developments, and real-world applications of Gateway cloning, assesses its advantages and disadvantages, and describes how hybrid cloning workflows combine the modularity of Gateway with the flawless precision of new assembly technologies. Together, these advancements establish Gateway as a key element of contemporary molecular engineering pipelines as well as a reliable cloning platform.

Keywords: Gateway Cloning, Recombinant DNA Technology, BP/LR Recombination, Functional Genomics, Synthetic Biology, Multigene Assembly

1. Introduction

By substituting a high-fidelity, site-specific recombination mechanism derived from bacteriophage λ for restriction-ligation, recombinational cloning utilising the Gateway® technology revolutionised molecular cloning. DNA fragments are moved directionally between entry and destination vectors by the system using designated recombination sites (attB/attP and the resulting attL/attR) and specialised BP and LR recombinase reactions. This allows for quick, orientation-specific transfer without the need for compatible restriction sites or ligases (Karimi *et al.*, 2007) [11]. Gateway cloning has become a key tool for creating massive ORFeome collections and for high-throughput functional genomics because it is simple to automate and maintains the precise sequence of inserted open reading frames (ORFs). The approach expedited proteome-scale cloning, protein localisation, interaction mapping, and systematic perturbation research across model organisms, as demonstrated by large community ORFeome initiatives that cloned thousands of protein-coding sequences into Gateway entry vectors (Lamesch *et al.*, 2007) [13].

Single-fragment transfers gave way to MultiSite/Multisite Pro versions over time, which allow the assembly of several functional modules (such as promoter-ORF-tag or multigene cassettes) in predetermined order and orientation. These multisite recombinational techniques increased Gateway's usefulness for pathway creation, combinatorial testing, and complicated structures, particularly in plant biology where binary T-vector compatibility and modularity are essential (Gillespie *et al.*, 2024) [10]. Many Gateway-compatible binary vectors and toolboxes have been created to support Agrobacterium-mediated transformation, transient expression

assays, subcellular localisation studies, and tissue-specific expression analyses, demonstrating Gateway's unique advantages for plant research. For many plant molecular laboratories looking for repeatable, modular cloning that easily interacts with downstream functional tests, Gateway is a practical option because of these community resources (Mejias *et al.*, 2025) ^[17]. By providing scarless assemblies and frequently reduced per-reaction costs for multiplexed, seamless structures, more recent "seamless" and Type IIS-based assembly techniques (Golden Gate/MoClo and variations) and homology-based approaches (Gibson, Nimble, etc.) have challenged Gateway's hegemony. As a result, researchers are now using hybrid tactics and method comparisons to determine which cloning technique best suits their needs, taking into account factors like cost, convenience of use, fidelity, and whether or not the intended application permits the retention of small att-site scars (Bird *et al.*, 2022) ^[12].

In order to place Gateway cloning in the current molecular cloning toolkit, this review will first summarise its molecular logic and workflows. Next, it will examine the use of Multisite and high-throughput Gateway applications in plant functional genomics. Lastly, it will compare Gateway with emerging assembly platforms and highlight hybrid or complementary strategies that capitalise on the advantages of each method.

2. Principles and Workflow of gateway cloning

2.1 Conceptual framework: recombination as design language

Site-specific recombination from bacteriophage λ is recast into a predictable engineering grammar for DNA using gateway cloning. Gateway integrates the logic of construct assembly into specific recombination loci (att sites), whose

identity dictates the direction and result of each transfer, as opposed to depending on random restriction sites or end-to-end ligation chemistry. Every cloned fragment becomes a reusable module when sequence capture (creating an entry clone) and functional deployment (moving that fragment into expression or binary backbones) are separated. Once an ORF or regulatory element is present in an entry plasmid, it can be transferred into any compatible destination vector without the need for re-amplification or re-subcloning. The long-term usefulness of Gateway in ORFeome projects and in labs that need quick iteration across expression settings is supported by this conceptual modularity (Qin *et al.*, 2022) ^[22].

2.2 The BP and LR reactions molecular mechanics with engineered specificity

Two complimentary recombination reactions are used to execute Gateway operationally. An entrance clone with attL sites is created in the BP reaction when an insert flanked by attB sequences recombines with attP sites on a donor (pDONR) vector. To produce the final expression construct and regenerate attB sites, those attL sites recombine with attR sites on a destination backbone during the LR reaction. In order to enforce orientation and order, the att site repertoire has been designed so that certain couples (like attB1/attP1) recombine with high fidelity whereas incompatible subtypes do not (Fig 1). To make the right recombinants the dominant survivors after transformation, the system's genomic design frequently incorporates differential antibiotic markers and negative selection cassettes (ccdB). This helps explain the high practical cloning efficiency observed across laboratories (Kramme *et al.*, 2021) ^[12].

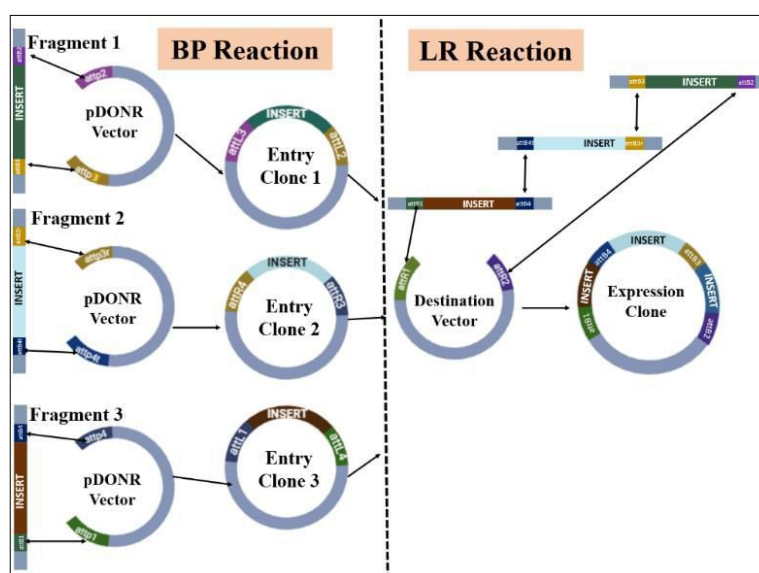


Fig 1: Workflow of Multisite Gateway Cloning Using BP and LR Recombination Reactions

The schematic illustrates the assembly of multiple DNA fragments into a final expression construct using the Gateway recombination system. In the BP reaction (left panel), each PCR-amplified fragment flanked by appropriate attB sites recombines with the corresponding pDONR vector containing matching attP sites to generate individual Entry Clones flanked by attL sites (Entry Clone 1, 2, and 3). Each Entry Clone retains the insert in a defined orientation

based on the att site configuration. In the LR reaction (right panel), the set of Entry Clones recombines with a Destination Vector carrying attR sites to produce the final Expression Clone, in which all fragments are assembled directionally according to the att site logic. The process ensures high-fidelity, modular, and ordered multi-fragment assembly suitable for constructing complex expression cassettes.

2.3 Multisite gateway and the move to modular, multi-fragment assembly

MultiSite Gateway, which increased the att-site repertoire and allowed for the orderly assembly of several modules (such as promoter–ORF–tag or multigene cassettes) in a single LR reaction, is a significant advancement of the platform. With this innovation, Gateway was transformed from a single-insert shuttle system into a hierarchical assembly platform appropriate for multigene engineering and synthetic biology. MultiSite workflows have been utilised in plant biology to effectively construct inducible, cell-type specific, and multigene expression constructs. More recent hybrid approaches, such as Gibson, combine Gateway with overlap-based techniques to get around size or sequence limitations while maintaining Gateway's plug-and-play deployment advantage. Robust multigene stacking in crops and experimental model systems has been proven using these mixed pipelines (Gibson + Gateway) (Omeng *et al.*, 2021) ^[19].

2.4 Selection logic, throughput, and practical considerations

Large ORFeome collections have traditionally been created in Gateway entry vectors to enable subsequent re-deployment into numerous functional backbones because of Gateway's built-in selection (ccdB negative selection plus antibiotic markers) and uniform reaction chemistry, which make it especially amenable to parallelisation and automation. However, practical users must strike a balance between primer design (attB tails must be added without adding frame or regulatory artefacts), insert size (efficiency may drop with very big fragments), and reagent cost (commercial Clonase mixes remain the standard). To achieve consistent high yields in both the BP and LR phases, protocol tuning molar ratios, reaction time/temperature, and competent strain selection remains crucial (Omelina *et al.*, 2022) ^[18].

2.5 Constraints, artefacts, and hybrid strategies

The current application of Gateway is shaped by two frequently cited restrictions. First, recombination leaves short att-site "scars" at junctions; these remnant sequences can obstruct nuanced regulatory tests or precise translational fusions, even though they are usually harmless. Second, infrastructure requirements and per-sample costs are impacted by the use of proprietary enzyme mixes and negative-selection cassettes. Many groups respond by using hybrid strategies, such as using MultiSite Gateway for ordered assembly while using scarless methods when junction sequence is crucial, or first assembling modules using sequence-overlap methods (Gibson) followed by a single Gateway LR to consolidate modules into a destination vector. These practical combinations take advantage of the scarless benefits of more recent assembly approaches while maintaining Gateway's conveniences (reusability, compatibility with community vector collections) (Zeng *et al.*, 2023).

3. Evolution of Gateway cloning: From Single-fragment capture to modular synthetic biology

3.1 Expanding the recombination architecture

The Gateway system has been greatly improved over time, especially in terms of its att-site architecture. Several att variations (such as attB1/B2, attB3/B4, etc.) that allow for

more intricate recombination patterns are included in more recent versions, which were initially intended for single-fragment transfers. The construction of multi-part constructs in a single recombination reaction (LR) is made possible by this diversification, which enables researchers to recombine several entrance modules in predetermined sequence and orientation. Because it allows for precise control over assembly logic without depending on ligase or restriction sites, this structural flexibility is essential to modular build design. Even in the presence of many elements, this designed specificity minimises cross-reactivity while maintaining excellent recombination fidelity (Ferguson *et al.*, 2022) ^[6].

3.2 Multisite gateway for multigene and modular circuit construction

The development of MultiSite Gateway, which enables the simultaneous recombination of three (or more) fragments such as promoter, gene, and tag into a single destination vector, is a significant turning point in the history of Gateway cloning. This makes it possible to build synthetic circuits or transcriptional units without having to clone each component one after the other. Researchers have greatly accelerated the speed of synthetic biology in plants by using MultiSite Gateway to create stacked multigene vectors, inducible expression systems, and cell-type-specific reporter lines. For example, a study created Gateway-compatible binary vectors that enable high-throughput investigation in a variety of tissues by integrating root-specific promoters, fluorescent protein tags, and other functional modules using three-part LR recombination (Gillespie *et al.*, 2024) ^[10].

Another study reported a hybrid multigene stacking technique known as Pyramiding Stacking of Multigenes (PSM), which is another significant breakthrough. In their method, they combine these modules in a single Gateway LR reaction after initially assembling several expression cassettes into entry vectors using Gibson assembly. They successfully produced binary vectors with up to nine genes in a single T-DNA using this technique, showing stable expression in Arabidopsis (Zeng *et al.*, 2023).

3.3 Hybrid strategies: Merging gateway with other assembly techniques

Despite the strength of Gateway's recombinational logic, its drawbacks such as the cost of enzyme mixes and the existence of leftover att sites, or "scars" have prompted researchers to switch to hybrid cloning procedures. These procedures usually construct complicated modules or multi-kilobase chunks using overlap-based assembly techniques (like Gibson Assembly), which are subsequently loaded into Gateway entry vectors for modular deployment.

The GuanNan Stacking (GNS) system, is a stunning illustration of this concept. GNS combines Cre-lox systems for marker removal, Golden Gate (Type IIS) for expression cassette construction, and Gateway LR recombination for multigene stacking. Using this technique, they created transgenic rice lines with all features by assembling a binary vector with five expression cassettes (>100 kb T-DNA). The smooth building of intricate modules using Golden Gate or Gibson and the modular exportability and reusability of Gateway-based vectors are both captured in this hybrid technique. For synthetic biology initiatives that call for combinatorial testing and iterative design, this kind of convergence is very helpful (Bitebo, 2024) ^[3].

3.5 Community vector repositories, orfeome resources, and their influence on gateway cloning

The concurrent emergence of huge, well selected vector libraries and ORFeome-scale resources has been one of the key elements in the endurance and widespread use of Gateway cloning. Reproducible genetic engineering is now possible on a worldwide scale thanks to the ecosystem these community-driven platforms have developed, which allows Gateway-compatible constructions to be easily shared, re-deployed, and standardised among research organisations.

3.4.1 ORFeome projects: Establishing standardized entry clone libraries

Although the idea of an ORFeome complete collections of open reading frames cloned into a single vector system became popular in the early 2000s, its influence is still felt today because Gateway entry vectors were used in the construction of the majority of ORFeome libraries. ORFs became inherently compatible with hundreds of destination vectors for functional testing across species and experimental settings once they were captured into attL-flanked entry clones (Wierbowski *et al.*, 2020) ^[28].

The Human ORFeome v8.1 project, which offers over 16,000 completely sequence-validated ORFs in Gateway entry clones, is a notable example. Because Gateway allows each ORF to be quickly moved into different tagging, inducible, or viral-expression backbones, this resource has been widely used for interactome mapping, subcellular localisation studies, and overexpression screening. There are comparable ORFeome libraries for *Saccharomyces cerevisiae*, zebrafish, mice, *Arabidopsis thaliana*, and numerous crop species. With the help of these collections, Gateway evolved from a cloning technique into a functional genomics platform where a single entry clone can support numerous downstream uses (Steffens *et al.*, 2014) ^[26].

3.4.2 Model plant vector kits and standardized expression backbones

The impact of Gateway is especially noticeable in plant biology. Gateway recombination is the foundation of several popular binary vector suites, such as pGWB, pK7WG series, pMDC vectors, and pEarleyGate vectors. These toolkits have grown during the last ten years to include

- Promoters particular to a certain tissue
- Modules of hormone-responsive regulation
- Luminescent and fluorescent tags
- Fusions of epitopes
- Variants of CRISPR-Cas9 and Cas12a

reporter cassettes for stress and developmental pathways

These libraries have recently been expanded to include synthetic regulatory switches, advanced localisation tags (APEX2, TurboID), and brilliant fluorophores (mNeonGreen, mScarlet) in Gateway format. Researchers can swap ORFs, tags, and promoters without changing the DNA sequence or repeating PCR amplifications by keeping compatible att sites throughout the whole backbone library (McLellan *et al.*, 2022) ^[16].

3.4.3 Repository-driven standardization and reproducibility

Thousands of Gateway plasmids that are dispersed over the world are kept in large community repositories like Addgene, DNASU, ABRC, and EU-ORFEOME. This

environment is significant for two reasons:

- **Standardisation:** For multi-site research, interactome mapping, and multi-lab cooperative efforts, using universal att site architecture guarantees consistent recombination results across labs.
- **Reproducibility:** Gateway lowers the possibility of sequence drift, mutation, or lab-specific cloning discrepancies by allowing the same entry clone to produce expression constructs in various research settings. Gateway-compatible plasmids continue to be one of the most popular plasmid groups globally, particularly for plant systems, viral expression systems, and protein interaction research, according to a recent study of synthetic biology archives (Li *et al.*, 2024) ^[14].

3.4.4 Gateway as the backbone of interactome and high-throughput screening pipelines

Researchers frequently need dozens to thousands of expression constructs that reflect the same gene fused to various tags or promoters in high-throughput functional genomics. For these pipelines, Gateway's recombination-based mobility is perfect. For instance, Gateway entry clones are crucial for systematic protein tagging and expression in the BioPlex project, which maps the human proteome interactome. Similar to this, large-scale yeast two-hybrid (Y2H), split-YFP, and transient expression tests are driven by Gateway ORFeomes in Arabidopsis interactome investigations. Thousands of constructs can be produced with little overhead because each ORF is kept in a stable entry clone, enabling high-throughput tests that would not be feasible using restriction-ligation techniques (Paul *et al.*, 2025) ^[21].

4. Advances in Gateway Cloning for Plant Molecular Biology

4.1 Gene Overexpression and Knockdown Applications:

Because the recombinational transfer of ORFs into various binary vectors removes the need for repeated PCR amplification and restriction site modification, gateway cloning has emerged as a fundamental technique for producing overexpression and knockdown constructs in plant systems. Rapid functional studies of signalling components, transcription factors, and metabolic enzymes in Arabidopsis, rice, and tomato were made possible by the explicit design of high-efficiency expression vectors like the pEarleyGate and pGWB series, which are both widely used across model and crop species. Gateway-compatible overexpression libraries are still used in recent functional genomics projects (Samtani *et al.*, 2023) ^[23]. For instance, a study on transcriptional regulators of heat tolerance in tomatoes used Gateway-based ORF cloning to systematically test candidate regulators by stable and transient expression assays. Similar to this, Gateway multisite cloning modules have helped RNA interference (RNAi) and artificial microRNA (amiRNA) techniques by enabling the assembly of hairpin structures without creating restriction-site scars. The use of Gateway-compatible hairpin constructs to silence immunity-related kinases in *Nicotiana benthamiana* was shown in a recent plant-pathogen interaction study, demonstrating the ongoing importance of Gateway-based RNA interference in quick functional characterisation pipelines (Sohi *et al.*, 2025) ^[25].

4.2 Gene Overexpression and Knockdown applications

Promoter-reporter experiments, which are essential for comprehending transcriptional control, stress-responsive gene expression, and epigenetic modulation, have also been greatly aided by gateway cloning. Promoter-swap experiments that examine the cis-regulatory architecture of plant genes are made possible by Multisite Gateway cloning, which enables the smooth exchange of promoters, reporters (GFP, LUC, GUS), and terminators. For instance, a study examining the drought-responsive chromatin landscape in sorghum validated stress-inducible regulatory motifs found using ATAC-seq and ChIP-seq datasets using Gateway-based promoter-LUC constructs (Seth *et al.*, 2025) ^[24]. Crucially, Gateway is widely utilised to clone long non-coding RNA (lncRNA)-associated regulators, histone variations, and chromatin-modifying enzymes into overexpression or CRISPR activation backbones. In order to map nucleosome-depleted areas necessary for transcriptional activation, recent research on the dynamics of histone deacetylase (HDAC) and H3K27me3 in rice stress memory used Gateway-compatible promoter deletion constructs. In the larger framework of plant epigenetics, these results demonstrate how Gateway cloning makes combinatorial testing of promoter elements and gene regulators easier (Omolehin *et al.*, 2021) ^[20].

4.3 Gateway-Based tools for plant transformation and genome editing

Because of their dependability and adaptability, gateway-compatible binary vectors are still widely utilised in Agrobacterium-mediated transformation systems. In Arabidopsis and other horticultural crops, the pK7WG/pK7GWI and pHGW series facilitate the quick exchange of gene cassettes for stable transformation by integrating attR sites into T-DNA regions. Furthermore, Gateway-based expression backbones including pEAQ-, pBIN19-, and pGWB-derivatives dominate transient tests in *Nicotiana benthamiana*, a key platform for plant bioengineering. For example, Gateway-compatible binary vectors were used in a work on immune receptor engineering to quickly infiltrate Agrobacterium and check for receptor variations (Baskar *et al.*, 2019) ^[11].

Additionally, CRISPR-Cas delivery modules incorporate gateway cloning. Multisite Gateway assembly of promoters, Cas nucleases, and polycistronic guide RNA arrays is a key component of many multiplexed CRISPR systems. Using Gateway-based modular vectors to effectively exchange tissue-specific promoters and crRNA modules, a recent approach for CRISPR-Cas12a genome editing in Arabidopsis and Setaria has increased editing precision and expression control. Gateway's function as a modular framework for assembling CRISPR components continues to be crucial as genome editing platforms diversify, particularly in labs that need to generate high-throughput constructs (Ma *et al.*, 2016) ^[15].

5. Integration of Gateway Cloning With Emerging Technologies

5.1 Gateway Cloning and CRISPR/Cas Genome Editing

Because of its modular vector design, particularly for assembling promoters, Cas nucleases, and reporter or selection cassettes, Gateway cloning continues to be deeply ingrained in genome-editing processes, despite Golden Gate cloning dominating CRISPR guide RNA assembly because

to its scarless Type IIS logic. The Cas9/Cas12a expression cassette is fixed in many CRISPR platforms' Gateway-compatible destination vectors, however ORFs and regulatory modules can be switched via LR recombination. Multisite Gateway cloning was used in recent CRISPR-Cas12a systems in Arabidopsis and *Setaria viridis* to interchange polycistronic CRISPR arrays with little gRNA module redesign and insert tissue-specific promoters upstream of Cas12a. Better promoter flexibility and quick testing of crRNA configurations were made possible by this method, which eliminated the need for repetitive PCR procedures. Similarly, deactivated Cas9 (dCas9) coupled to transcriptional regulators was expressed in rice using Gateway vectors in a study, allowing CRISPRa/CRISPRi-based transcriptional reprogramming (Dias *et al.*, 2023) ^[15]. Gateway-compatible binary vectors offer a reliable and repeatable foundation in cereals and other resistant crops where transformation processes take a long period. Using Gateway-based donor constructs for homology-directed repair (HDR), a wheat genome-editing work made it simple to replace reporter or selectable marker cassettes via LR recombination. These examples show that Cas expression units and regulatory modules still mostly rely on Gateway's modular recombination, even if gRNA modules are frequently constructed utilising Type IIS techniques (Chen *et al.*, 2006) ^[4].

5.2 Gateway in Synthetic Biology and Modular DNA Design

Although hierarchical assembly techniques are being used more and more in synthetic biology, Gateway cloning is still crucial for promoter engineering, circuit prototyping, and quick ORF swapping. The method is frequently used to create synthetic promoters by screening various cis-regulatory modules or minimal promoters through recombination into reporter backbones (Gao *et al.*, 2019) ^[9]. Using Gateway-mediated assembly of promoter variants into LUC/GFP vectors for transient experiments, a recent study in *Nicotiana benthamiana* produced artificial salicylic acid-responsive promoters. Biosensor engineering also uses gateway cloning, particularly in plants where investigating input-output correlations requires high-throughput promoter swapping. In order to precisely tune auxin responsiveness, a constructed auxin biosensor tested degron-tag combinations and fluorescent reporters using Gateway-compatible modules. Gateway-based Multisite assembly facilitates the simultaneous integration of route enzymes into multi-cassette complexes in metabolic pathway reformation (Varghese, 2008) ^[27]. For instance, Gateway multisite recombination was utilised to transfer many enzyme-coding sequences into binary vectors for stable transformation in an Arabidopsis flavonoid pathway reconstruction. This greatly reduced the cloning burden involved with redesigning each enzyme cassette. Gateway is still crucial for quick prototyping, entry-vector storage of pathway enzymes, and vector diversification, particularly when combinatorial testing of regulatory variations is needed, even if Golden Gate and MoClo dominate multi-gene assembly (Zhang *et al.*, 2021).

5.3 Coupling Gateway Cloning with Omics-Driven Target Discovery

The need for cloning systems that can rapidly validate candidate genes found through high-throughput data has

increased due to the growth of genomics, transcriptomics, proteomics, and metabolomics. Because entry-vector cloning enables ORFs to be permanently preserved and reused across numerous destination vectors without the need for further PCR steps, gateway cloning is especially well suited for this. Gateway-based overexpression and promoter-reporter vectors continue to be the gold standard for verifying stress-responsive transcription factors in transcriptomics-guided functional studies (Fowler *et al.*, 2016). For instance, transcription factors found in regulatory network models were tested using Gateway vectors in an RNA-seq investigation on drought-responsive signalling modules in sorghum (Zhen *et al.*, 2022).

Gateway multisite assembly pathway reconstruction enables systematic candidate enzyme verification in metabolomics-guided investigations. Candidate enzymes were transferred

into expression vectors for heterologous testing via Gateway cloning in a recent reconstruction of the benzoxazinoid biosynthesis modules in maize. Gateway-based bait and prey vectors are widely used in shotgun proteomics and yeast two-hybrid screens in plant-microbe interaction studies. Several thousand potential interactors were screened in a standardised protein–protein interaction pipeline using Gateway ORFeome libraries in a study investigating effector–host interactions in *Phytophthora infestans*. These illustrations demonstrate how Gateway's stability and reusability make it essential for systematic experimental validation in the high-throughput omics age, particularly when dozens or hundreds of gene candidates need to be functionally tested across various vector backbones (Lamesch *et al.*, 2007) ^[13].

Table 1: Applications of Gateway Cloning in Plant Molecular Biology and Biotechnology

Application	Gateway Component Used	Purpose / Outcome	Examples
Overexpression vectors	LR recombination into pGWB / pEarleyGate	Functional gene analysis	Stress-responsive transcription factors
Promoter–reporter assays	Multisite Gateway (Promoter–LUC/GFP)	Promoter validation, cis-element mapping	Drought- and hormone-responsive promoter studies
RNAi / amiRNA constructs	Hairpin modules in Entry clones	Gene silencing	Reverse genetics, pathogen-defense genes
CRISPR/Cas expression	Modular Cas9/Cas12a Gateway cassettes	Genome editing and promoter swapping	Tissue-specific Cas delivery in rice / Arabidopsis
Binary plant transformation	Gateway-compatible T-DNA vectors	Stable transformation via <i>Agrobacterium</i>	Arabidopsis, tomato, rice, maize
Synthetic promoter libraries	Promoter modules recombined into reporter vectors	Engineering expression systems	Hormone biosensors, synthetic switches
Pathway engineering	Multisite Gateway assembly	Multi-enzyme pathways in metabolic engineering	Flavonoid/terpenoid pathway reconstruction

Conclusion and future prospects

Because of its dependability, modular design, and suitability for large-scale and high-throughput applications, gateway cloning continues to be one of the most adaptable and durable platforms in molecular biology. Expression vectors, promoter–reporter systems, CRISPR modules, and multigene assemblies can be quickly constructed using its recombination-based logic without the need for restriction-site availability or repetitive PCR. Functional genomics, plant transformation, interactome mapping, and synthetic biology processes across model organisms and agriculturally significant crops are thus still supported by Gateway. Despite its wide range of applications, some drawbacks, such as att-site scarring, the expense of specialised recombinase mixes, and sporadic recombination inefficiencies with large inserts, have prompted the integration of Gateway with new cloning techniques. While maintaining Gateway's benefits of modularity and reusability, hybrid pipelines that integrate Gateway with Type IIS assembly, Gibson assembly, Cre-lox recombination, or other ligation-free techniques have proven particularly effective for building vast and complex DNA constructions. These convergent approaches show how adaptable, system-level design frameworks are replacing method-specific clones.

As research advances towards automation, genome-scale analysis, and the creation of synthetic traits, gateway cloning is anticipated to continue to play a major role in molecular engineering. Its usefulness for creating multi-layered regulatory circuits, stacked characteristics, and combinatorial variant libraries will be improved by the

ongoing development of Gateway-compatible vector libraries, community ORFeome resources, and CRISPR toolkits. Functional validation workflows will be accelerated by integration with single-cell omics, cell-free systems, and next-generation sequencing. In the end, Gateway cloning will continue to develop as a fundamental component of increasingly interconnected cloning ecosystems rather than as a stand-alone technique, enabling quicker, more accurate, and more scalable genetic editing across biology.

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