

R-Gene and Resistance-Gene-Analog Marker Development for Biotic-Stress Resistance and Tolerance in Grapevine Integrating candidate-gene cloning, sequence polymorphism, and defense-signaling methyltransferases for breeding and germplasm evaluation

Davis W. Cheng

chengdw1980@gmail.com

Independent Researcher

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Abstract

Grapevine improvement for durable resistance to pathogens and pests is constrained by the high heterozygosity of *Vitis*, the perennial breeding cycle, and the complex architecture of many resistance phenotypes. Here we integrate experimentally generated grapevine molecular-marker datasets to identify, characterize and map resistance (R) genes and resistance-gene analogs (RGAs) associated with biotic-stress resistance and tolerance. The archived program combined candidate-gene discovery, PCR amplification, cloning, sequencing, polymorphism screening and segregation analysis across diverse *Vitis* germplasm and multiple mapping populations. The resource comprises 341 NBS-LRR R genes from public grape genomic data, 26 cloned NBS-LRR R genes and 101 NBS-LRR RGAs, and more than 50 non-NBS-LRR defense-related genes, of which at least 64 NBS-LRR and 15 non-NBS-LRR candidates were pathogen-inducible. Eighty-three primer pairs were designed or retrieved, and 46 R-gene plus 30 RGA pairs were tested across nine parental pairs and 24 wild or breeding lines. Polymorphism arose from both SNPs and indels, including a diagnostically useful class of indels in upstream/UTR and promoter regions. Seven candidate genes were polymorphic; R9, R18 and R19 showed clear parental differentiation, yet R9 and R19 segregation did not track the resistance phenotype—an important negative control. The program also cloned defense-network genes—a cytochrome P450 (CYP736B), a dioxygenase, a zinc-finger protein, a brassinosteroid-6-oxidase and ethylene-response-factor-4—and a set of defense-signaling methyltransferases and a methylesterase. These observations anticipated later resistance-locus discoveries and are readily upgraded by phased genomes, graph pangenomes and haplotype-aware marker systems, positioning candidate-gene polymorphisms as a bridge between defense biology and marker-assisted breeding.

1. Introduction

Grapevine (*Vitis* spp.) is a highly heterozygous perennial crop in which conventional selection for resistance is slowed by long generation intervals, complex pedigrees, and the frequent contribution of multiple loci to disease and pest phenotypes. Many of the most valuable resistance sources reside in wild *Vitis* and *Muscadinia* germplasm and must be introgressed into elite *V. vinifera* backgrounds, where linkage drag and the perennial cycle make phenotype-based selection slow and expensive. Molecular markers that are physically close to, within, or functionally connected with resistance loci can shorten the breeding cycle and permit early seedling selection before phenotypes are expressed (Douceff et al. 2004; Riaz et al. 2009).

The original research program was explicitly designed around this principle. Its central hypothesis was that sequence diversity within candidate R genes and RGAs provides a rich source of polymorphic markers that are more biologically informative than anonymous markers, because they occur in or near defense-related genes (Di Gaspero and Cipriani 2002; Di Gaspero and Cipriani 2003; Donald et al. 2002). The program combined three linked objectives: (i) PCR-mediated identification of R-gene polymorphisms in resistant and susceptible parents and diverse wild germplasm; (ii) genetic and physical mapping of polymorphic R-gene markers in segregating populations; and (iii) application of validated markers to

marker-assisted selection (MAS) and germplasm evaluation. The archived proposal describes candidate R genes as including NBS-LRR proteins together with other defense-related gene classes, and emphasizes that useful marker polymorphisms can occur in coding, untranslated, upstream and downstream regions.

This conceptual framework remains highly relevant. Modern grapevine studies have shown that resistance loci are often genetically complex and may contain clusters of NLR genes, receptor-like kinases, or other defense-related genes (Michelmore and Meyers 1998; Hulbert et al. 2001; Meyers et al. 2003). The powdery-mildew locus Run1 was genetically and physically mapped to a resistance-gene cluster (Pauquet et al. 2001; Barker et al. 2005), the Pierce's-disease resistance region PdR1b contains candidate leucine-rich-repeat receptor-kinase genes (Agüero et al. 2022), and XiR1, a major locus for dagger-nematode resistance, was physically delimited to approximately 115 kb containing NB-LRR candidates (Hwang et al. 2010). More recently, phased grape genomes and graph-based pangenomes have revealed extensive structural variation, presence/absence variation, and lineage-specific resistance-gene repertoires (Liu et al. 2024; Guo et al. 2025). The original RGA-marker strategy can therefore be reframed as an early form of haplotype-aware candidate-gene marker development.

The purpose of this manuscript is not to present the 2007–2009 program as if it had generated a modern genome-wide association dataset. Instead, it reconstructs the experimental evidence actually preserved in the project files—including the original gel images and cloned sequences—distinguishes what was demonstrated from what was proposed, integrates a previously separate class of defense-signaling methyltransferase and methylesterase genes cloned in the same program, and develops a publication-ready framework that connects those observations to subsequent peer-reviewed discoveries and current genomic breeding technology.

2. Research objectives and hypotheses

The research can be organized around four testable hypotheses:

H1. Candidate R genes and RGAs are sufficiently polymorphic among *Vitis* parents and wild germplasm to generate reproducible DNA markers.

H2. Sequence polymorphisms located within candidate genes or their regulatory neighborhoods are enriched for biologically informative variants and can be converted into co-dominant or allele-specific assays.

H3. A subset of R-gene/RGA and defense-signaling markers will cosegregate or show close linkage with resistance phenotypes in mapping populations, thereby identifying candidate regions for genetic and physical mapping.

H4. Validated markers can be transferred into breeding and germplasm-evaluation pipelines and used in combination with existing SSR markers (Adam-Blondon et al. 2004; Riaz et al. 2004) to increase marker

density and support resistance pyramiding.

The archived program was broader than a single pathogen. Its stated biotic-stress scope included fungal, bacterial, viral and pest interactions; the principal experimentally documented examples are powdery mildew, Pierce's disease, dagger-nematode/root-associated resistance, fanleaf-associated breeding objectives, and R-gene/RGA variation across diverse *Vitis* germplasm.

3. Materials and methods

The experimental program followed a seven-stage candidate-gene marker-development workflow: candidate discovery, primer design, PCR/RT-PCR screening, cloning and sequencing, sequence-polymorphism characterization, marker-to-segregation assays, and application in a resistance-locus and breeding context. This workflow is summarized in Fig. 1, and each stage is detailed in the subsections below.

3.1 Study design and germplasm

The archived material comprised cultivated *V. vinifera*, wild *Vitis* accessions, interspecific hybrids, parental breeding lines, and segregating mapping populations. The germplasm inventory (Table 6) includes *V. aestivalis* 1394; *V. arizonica* b43-17; *V. candicans* T56; *V. champinii* 3-48; *V. rufotomentosa* 1416; *V. shuttleworthii*; *V. simpsonii* c56-94; *V. smalliana*; *V. vinifera* Chardonnay, Mission, Red Globe, Alvarinho, Sauv Gris and Vioziniio; *Muscadinia rotundifolia* Cowart; *V. rupestris* Metallique 1421; *V. girdiana* 1379; *V. monticola*; *V. nesbitiana* 2235.4; and *V. arizonica* × *V. rupestris* hybrids including 8909-15, 8909-19, 8909-67, 8909-94 and 8909-17. Twenty-four wild or breeding lines and nine pairs of parental lines were screened in the 2008–2009 phase (Tables 2 and 6).

3.2 Candidate-gene discovery and primer development

Candidate sequences were assembled from public grape genomic resources, ESTs, expression resources and prior functional-genomics work (Lin et al. 2007; Velasco et al. 2007). The program reported 341 NBS-LRR R genes from public grape genomic resources, 26 cloned NBS-LRR R genes, 101 cloned NBS-LRR RGAs, and more than 50 non-NBS-LRR defense-related genes; at least 64 NBS-LRR and 15 non-NBS-LRR sequences were described as pathogen-inducible (Fig. 2). Eighty-three R-gene primer pairs were designed and/or retrieved, and 46 R-gene plus 30 RGA primer pairs were tested during the 2008–2009 phase. An earlier screen used 20 candidate-gene primer pairs, seven of which showed detectable polymorphism; R9, R18 and R19 displayed clear parental differences in three populations.

3.3 PCR, restriction analysis, cloning and sequencing

Genomic DNA and total RNA were isolated from grape tissues; the archived protocol used TRIzol for RNA isolation (Chomczynski and Sacchi 1987), DNase treatment, oligo(dT)₂₀-primed cDNA synthesis, and Taq-mediated PCR/RT-PCR (Saiki et al. 1988). A representative RT-PCR program used 98 °C denaturation, 55 °C annealing and 72 °C extension for 30 cycles. PCR products were separated on agarose gels. Where direct size polymorphism was not evident, restriction digestion was used: the R19 assay in population 04191 (MspI) and the R19 assay in population 04373 (MseI) illustrate this PCR-RFLP approach. Candidate products were cloned, sequenced using dye-terminator (Sanger) chemistry (Sanger et al. 1977), and analyzed with sequence- and fragment-analysis software.

3.4 Sequence-polymorphism characterization

Cloned candidate-gene amplicons were sequenced and aligned across parental and wild lines to characterize sequence polymorphism. Deduced RGA coding regions were screened for single-nucleotide polymorphisms and insertions/deletions, with each substitution classified as synonymous or non-synonymous and mapped relative to the conserved NBS-LRR motifs (P-loop, Kinase-2, Kinase-3a and the hydrophobic domain (Meyers et al. 2003; Di Gaspero and Cipriani 2003)); upstream/UTR and promoter regions were examined in the same way for length (indel) variants. Polymorphisms that distinguished parental lines were selected as candidate markers, and approximately 30 primer pairs were designed to target informative UTR/promoter regions for the segregation and germplasm assays described below. Variants identified in this way are treated as candidate marker polymorphisms rather than as validated causal variants.

3.5 Mapping populations and phenotyping

The archived research plan used population 03-3004 for powdery mildew and the V. arizonica b43-17/8909-derived populations 04190, 04191 and 04373 and 9621 for Pierce's-disease- and nematode/fanleaf-related resistance (Table 2). The 9621 population was derived from D8909-15 × F8909-17, with D8909-15 carrying V. arizonica-derived resistance and F8909-17 containing V. arizonica/candicans ancestry (Douceff et al. 2004; Riaz et al. 2008). The proposed linkage-analysis workflow called for allele scoring, a LOD threshold of 4.0, Kosambi mapping and linkage-map construction. Greenhouse and field evaluations used three biological repeats; Ramming's group performed powdery-mildew evaluation and Walker's group performed Pierce's-disease, nematode and fanleaf-related evaluation. Because the archived files available for this reconstruction do not contain complete individual-level phenotype tables, formal QTL-effect estimates and marker-by-marker recombination fractions are not reconstructed here.

3.6 Contemporary genetic reinterpretation

For a contemporary publication, archived candidate sequences should be remapped to a current phased *Vitis* reference (e.g., PN40024 v4) or graph pangenome, and all historical marker loci converted to stable coordinates and haplotypes. This is important because modern pangenomes reveal extensive structural variation and reference bias in grapevine, and resistance loci frequently occur in highly variable gene clusters (Michelmore and Meyers 1998; Liu et al. 2024; Guo et al. 2025).

3.7 Phylogenetic analysis

To position the archived RGA clones within grapevine R-gene diversity, all *Vitis* resistance-gene-analog and NBS-LRR/NB-ARC nucleotide records were retrieved from NCBI GenBank (queries: *Vitis*[Organism] AND (“resistance gene analog” OR RGA), and *Vitis*[Organism] AND (“NBS-LRR” OR “NB-ARC”)). Each record and each of the six RGA clones from this study were translated in all six reading frames, and the longest open peptide containing the NBS P-loop signature was retained. An *Arabidopsis thaliana* RPS2 NB-ARC sequence was added as the outgroup. Records were obtained through NCBI E-utilities (Sayers et al. 2022). Peptides were aligned with FAMSA (Deorowicz et al. 2016) and columns with more than 50–55% gaps were trimmed. Two trees were built. For a comprehensive tree (supplementary), near-identical sequences ($\geq 88\%$ aligned identity) were collapsed, leaving 129 sequences, and a neighbor-joining tree (Saitou and Nei 1987) was inferred from amino-acid p-distances with 100 bootstrap replicates (Felsenstein 1985). For the class-level tree shown in Fig. 5A, sequences were clustered and one representative was retained per major cluster, giving 25 representative *Vitis* RGAs (including the characterized *V. amurensis* RGA1) plus the six clones from this study and the outgroup; this reduced set was re-aligned and analyzed by neighbor-joining with 1,000 bootstrap replicates. Node support was mapped onto each reference tree. The alignments (FASTA), the Newick trees, the accession list and a high-resolution image of the comprehensive tree are provided as supplementary files.

4. Results

4.1 A large candidate defense-gene resource was assembled

The program established a substantial candidate-gene resource before contemporary grapevine pangenomics existed. The archived records report 341 NBS-LRR genes from public grape genomic resources, 26 cloned NBS-LRR genes, 101 NBS-LRR RGAs, and more than 50 non-NBS-LRR defense-related genes—at least 518 candidate sequences in total (Fig. 2, Table 1). At least 64 NBS-LRR and 15 non-NBS-LRR candidates were identified as pathogen-inducible. Bioinformatic analysis placed most NBS-LRR candidates on 18 of the 19 grape chromosomes and described approximately 70 common R genes/RGAs concentrated on five chromosomes, consistent with the well-established tendency of plant resistance genes to occur in polymorphic clusters (Michelmore and Meyers 1998; Hulbert et al. 2001; Meyers et al. 2003). These chromosome assignments should now be re-evaluated against phased and graph-based references (Liu et al. 2024; Guo et al. 2025).

4.2 Candidate-gene PCR screening revealed extensive parental polymorphism

The program progressed from 20 candidate R-gene primer pairs in the early experiment to 83 R-gene primer pairs designed or retrieved in the subsequent phase; during 2008–2009, 46 R-gene and 30 RGA primer pairs were tested across nine parental pairs and 24 wild or breeding lines. The preserved gels document reproducible parental differentiation for numerous candidate genes (Fig. 3). Group-1 and Group-2 panels show clear presence/absence and size polymorphism across parental pairs 1–5 for NB-ARC-LRR primers (RF2, RF3–RF11, RF10), the ethylene-response-factor-4 marker (Er4), and—screened in the same panels—the SABATH/JMT/SMT methyltransferase markers (Section 4.7). A further panel resolves R3, R5, R6, R9, R18 and R19 across parental pairs 7–10, with R9, R18 and R19 among the most clearly differentiated. Seven candidate genes were polymorphic in the early screen, establishing the key premise of the study: sequence-informed candidate genes generate markers that distinguish resistance-source parents and can therefore be tested in segregating progeny.

4.3 Non-coding regions yielded diagnostic indel markers

A distinctive and durable finding was that regulatory regions, not only coding sequence, are a productive source of markers. For a powdery-mildew candidate gene (designated PM1-R), primers spanning the promoter (P), coding (C) and terminator (T) regions were screened between parental lines (Fig. 4). Promoter primers PM1-P1/P2 produced clear presence/absence and size differences across five parental pairs, and a broader promoter/coding/terminator panel showed that several non-coding amplicons were more polymorphic than the corresponding coding amplicons. Because coding regions are often constrained by protein function, extending marker design into flanking promoter and UTR sequence recovered stable indels suitable for genotyping—a design principle that modern long-read and pangenome assemblies now make substantially more powerful by resolving structural and presence/absence variation directly (Liu et al. 2024; Guo et al. 2025).

4.4 The cloned RGAs represent at least two distinct grapevine RGA subfamilies

To determine which grapevine R-gene classes the archived clones belong to, all *Vitis* resistance-gene-analog and NBS-LRR/NB-ARC records available in NCBI GenBank were retrieved and translated to their conserved NB-ARC peptide region, and one representative was selected from each major sequence cluster to give a compact, class-level reference set. These 25 representative *Vitis* RGAs — including the characterized *V. amurensis* RGA1 — were combined with the six RGA clones sequenced here from 9621-67 resistant-associated (67CS: A7-1/2/3) and susceptible-associated (67TS: B4-1/2/3) material and rooted with an *Arabidopsis* RPS2 outgroup. A neighbor-joining tree was inferred with 1,000 bootstrap replicates, and the ingroup resolved into four bracketed clades, I–IV (Fig. 5A). The six clones do not form

a single novel clade; instead they fall into two of these established grapevine RGA lineages. Clones A7-2 and A7-3 group together (70% bootstrap) within clade I, which also contains published RGAs AY427078/AY427154 and DQ656096 (clade I support 93%), whereas A7-1 and the three B4 clones fall in clade III, forming a strongly supported subclade (100% bootstrap; B4-1/B4-2 identical) sister to a group of published AY427 RGAs. Clades II and IV contain only published *Vitis* RGAs and no clones from this study. Two conclusions follow. First, the clones are authentic members of established grapevine RGA subfamilies rather than a new structural class—so the historical work sampled genuine, and diverse, portions of the grape NBS-LRR repertoire. Second, the resistant-associated (A7) and susceptible-associated (B4) materials contributed to different subfamilies, and one subfamily contains both a resistant- and susceptible-associated clone, underscoring that subfamily membership alone does not predict phenotype. Definitive assignment to the TIR-NBS-LRR versus CC-NBS-LRR superclasses, and any claim of a truly novel class, would require full-length NB-ARC/LRR sequences and the remaining cloned RGAs rather than the short amplicons available here. Complementing the tree, alignment of 16 representative RGAs from grape parental lines resolves both SNPs and indels across the P-loop, Kinase-2, Kinase-3a and hydrophobic-domain motifs, with many non-synonymous substitutions outside the conserved domains (Fig. 5B); these variants define haplotypes that can track candidate loci and prioritize functional assays.

4.5 R19 and R9 provided representative segregation assays

Two candidate-gene markers were carried into segregating progeny (Fig. 6). In population 04373 (F2-35 × b43-17; b43-17 is the *V. arizonica* Pierce's-disease resistance source), R19 was assayed by PCR followed by *Mse*I digestion and R9 by PCR with an actin internal control; the parents (b43-17 resistant, F2-35 susceptible) and ten numbered progeny are documented directly on the gels. In population 04191, R19 was resolved as an undigested amplicon and as *Msp*I-digested fragments across the two parents and ten F₂ progeny. Both markers were clearly polymorphic and segregated among progeny. Importantly, the archived written summary explicitly states that R9 and R19 segregated in 04373 but did not segregate in accordance with the resistance phenotype. This negative result is scientifically valuable: it demonstrates that a polymorphic candidate-gene marker is not automatically a validated resistance marker, and that segregation analysis—not band-counting—must decide the point. R9, R18 and R19 are therefore reported here as validated polymorphic candidate-gene markers requiring further linkage validation, not as causal resistance markers.

4.6 Non-NBS-LRR defense genes broadened the candidate-gene concept

The cloning records contain several full-length defense-associated genes beyond canonical NBS-LRRs (Table 3). A cytochrome P450 monooxygenase cloned from grape selection 9621-67 was a 1,512-bp

gene (1,507-bp CDS; 495-aa protein) with strong similarity to elicitor-inducible plant P450s; a later peer-reviewed study characterized this defense context as grape CYP736B and demonstrated differential expression associated with Pierce's-disease resistance and susceptibility (Cheng et al. 2010). The records also include a dioxygenase (1,834-bp complete sequence; 610-aa protein), a zinc-finger protein (2,075 bp; 689 aa), a brassinosteroid-6-oxidase (1,451-bp clone; functional CDS reported as 750 bp/250 aa with four amino-acid substitutions relative to the reference), and ethylene-response-factor-4 (864-bp clone; 843-bp CDS; ~280 aa, differing from the grape reference at multiple positions). These genes are best classified as defense-network or signaling candidates rather than canonical R genes, and they extend the marker resource into metabolism, redox chemistry, transcriptional regulation and hormone signaling.

4.7 A defense-signaling and secondary-metabolism methyltransferase resource

A coherent additional class of defense-related genes was cloned in the same program and is integrated here for the first time: SABATH-family and related methyltransferases together with a methylesterase (Fig. 7, Table 4). These enzymes control the reversible methylation of the two central defense hormones and of antimicrobial secondary metabolites. Salicylic-acid methyltransferase/benzoic-acid–salicylic-acid methyltransferase activity (represented here by SABATH MT3 and SMT1 clones) converts salicylic acid to methyl salicylate, the mobile signal for systemic acquired resistance (Ross et al. 1999; Chen et al. 2003; Park et al. 2007), while a methylesterase (V29-3) hydrolyzes methyl salicylate back to active salicylic acid—the two halves of the SAR methylation cycle (Park et al. 2007). A jasmonic-acid carboxyl methyltransferase (JMT; clones T3-3 and T4-2, 1,128 bp/375 aa) generates methyl jasmonate (Seo et al. 2001), and a set of O-methyltransferases (OMT2.1, OMT2.2 and OMT3.1) act in the phenylpropanoid/stilbene branch that produces methylated grape phytoalexins such as pterostilbene (Schmidlin et al. 2008); an L-isoaspartate-O-methyltransferase (MT5-1) contributes stress-induced protein repair.

Two lines of archived evidence justify treating these genes as part of the same marker resource rather than a separate story. First, the methyltransferase markers were screened directly alongside the R genes in the 2008–2009 parental panels: JMT (CA061119), SMT (CA022803), SMT1 and SABATH MT3 primer pairs appear in the same gels as the NB-ARC-LRR and ERF4 markers (Fig. 3A,B). Second, the methyltransferase genes were themselves polymorphic across the wild-germplasm panel, showing clear genomic-DNA size differences among 24 wild *Vitis* accessions (Fig. 7A). Together with the cloned coding sequences from Pierce's-disease-resistant and -susceptible 9621-67/9621-94 materials and Chardonnay, these data provide both a functional-gene resource and an additional, independent set of candidate markers that broaden the manuscript from an R-gene study to a recognition-plus-signaling defense-gene marker platform.

4.8 RGA sequence diversity was preserved from resistant and susceptible 9621-67 materials

PCR-derived RGA sequences from 9621-67 material designated 67CS and 67TS were archived, including clones A7-1, A7-2, A7-3, B4-1, B4-2 and B4-3. These records provide a molecular archive for allelic comparison between resistant- and susceptible-associated materials. The most defensible contemporary use of these sequences is as a source of historical haplotypes for re-alignment to current grapevine assemblies and for retrospective identification of SNP/indel markers within or near mapped resistance loci.

4.9 The mapping-population design suited resistance-marker development

The program used multiple populations with different resistance sources and genetic backgrounds (Table 2), an arrangement well suited to distinguishing source-specific polymorphism from broadly conserved candidate-gene variation, because a marker that is polymorphic in one cross can be monomorphic in another. The 04190, 04191 and 04373 populations share *V. arizonica* b43-17/8909-derived ancestry, whereas 9621 derives from D8909-15 × F8909-17 and 03-3004 targets powdery mildew. Using related populations that segregate for arizonica-derived resistance provides internal replication for candidate-marker behavior.

4.10 Subsequent published studies validate the biological logic of the program

Several later studies provide independent evidence that the archived strategy was directionally correct (Table 5 lists the loci). b43-17-derived Pierce's-disease resistance (PdR1a/PdR1b) was mapped to chromosome 14, with the PdR1a interval refined to 0.6 cM in the 9621 population and PdR1b to 0.4 cM in 04190 (Riaz et al. 2008); molecular and functional characterization subsequently identified RGA-type genes at PdR1b, including LRR receptor-kinase candidates (Agüero et al. 2022). XiR1, derived from *V. arizonica*, was fine-mapped to a ~115-kb interval containing NB-LRR candidates using populations from the same pedigree (Hwang et al. 2010). For powdery mildew, the Run1 locus was mapped to a resistance-gene cluster containing full-length NBS-LRR genes, and additional Ren and Run loci and fungal-resistance QTL were resolved (Pauquet et al. 2001; Barker et al. 2005; Fischer et al. 2004; Massonnet et al. 2022), and REN12 from *V. amurensis* was mapped to a ~780-kb interval containing candidate NLR genes with markers suitable for MAS (Sapkota et al. 2023). These studies do not retroactively prove that any archived R9/R18/R19 marker is causal; rather, they validate the program's broader strategy of interrogating candidate resistance genes around mapped loci.

4.11 The historical strategy fits naturally within modern pangenomic breeding

Current grapevine genomic resources provide a major opportunity to reanalyze the archived material (Fig. 8). A 2024 graph-based grapevine pangenome integrated 18 phased haplotypes and 11 published assemblies and detected more than 236,000 structural variants across a 466-accession variation map (Liu et al. 2024), while a 2025 *Vitis* super-pangenome analyzed 72 haplotype-resolved accessions and showed that European cultivars susceptible to downy mildew carry a smaller repertoire of TIR-NBARC-LRR NLR genes, linking more than 63 structural variants and associated genes to downy-mildew resistance (Guo et al. 2025). The historical SNP/indel, RGA and methyltransferase sequences can therefore be upgraded into haplotype-aware markers that are less vulnerable to reference bias and better suited to structural-variant genotyping and resistance pyramiding (Possamai et al. 2024; Höschele et al. 2024).

5. Discussion

The central contribution of this work is methodological and biological: it establishes an experimentally grounded route from defense-gene discovery to breeding-marker development, and it does so with primary data—original gels and cloned sequences—rather than assertion. The archived dataset demonstrates that grapevine R genes, RGAs and defense-signaling genes are not merely conserved sequences; they contain sufficient interspecific and parental variation to produce useful PCR and PCR-RFLP markers (Di Gaspero and Cipriani 2002; Di Gaspero and Cipriani 2003; Donald et al. 2002). At the same time, the data show why candidate-gene polymorphism must not be equated automatically with resistance. R9 and R19 were polymorphic and segregating in 04373, yet the archived summary records that their segregation did not correspond to the resistance phenotype. This negative result is a strength of the dataset because it demonstrates, from primary evidence, that candidate-gene markers require genetic validation.

The distinction between marker and gene is especially important for NLR-rich loci. Resistance-gene clusters evolve through duplication, recombination, divergence and selection, producing paralogs with related motifs but potentially different functions (Michelmore and Meyers 1998; Hulbert et al. 2001; Richly et al. 2002), so a primer designed from a conserved NBS motif may amplify multiple loci (Di Gaspero and Cipriani 2002; Di Gaspero and Cipriani 2003). The 16-RGA comparison illustrates this directly: conserved P-loop and kinase motifs coexist with lineage-specific substitutions and indels (Fig. 5). Consequently, the best contemporary strategy is to use the archived sequences to define locus-specific haplotypes rather than simply to score bands.

The finding that non-coding regions contain useful indels is equally consequential. Coding regions are often constrained by protein function and may be relatively conserved, whereas promoter and UTR regions accumulate indels and regulatory SNPs (Bakker et al. 2006); the PM1-R promoter/terminator panels (Fig. 4) show this empirically. The program therefore anticipated a marker-design principle that

remains useful today: when coding variation is insufficient for robust genotyping, extend sequencing into flanking genomic regions and identify stable indels or diagnostic SNPs. Modern long-read and pangenome assemblies make this approach far more powerful because structural and presence/absence variation can now be resolved directly (Liu et al. 2024; Guo et al. 2025).

Integrating the methyltransferase and methylesterase genes strengthens the manuscript in a way that is biologically—not merely bibliographically—justified. Methyl salicylate and methyl jasmonate are central mobile and volatile signals of induced and systemic resistance (Seo et al. 2001; Park et al. 2007), and the enzymes that make and hydrolyze them (SABATH SAMT/BSMT, JMT and SABP2-like methylesterases (Ross et al. 1999; Seo et al. 2001; Chen et al. 2003)), together with the O-methyltransferases that decorate stilbene phytoalexins such as pterostilbene (Schmidlin et al. 2008), sit at the interface of pathogen recognition and defense output. That these genes were both polymorphic across wild *Vitis* (Fig. 7A) and screened as markers in the same parental panels as the NLRs (Fig. 3) means the program had already assembled a recognition-plus-signaling marker set. Reframing the dataset around this axis—rather than around NLRs alone—better reflects the biology of durable resistance and enlarges the pool of candidate markers available for pyramiding.

The 9621 materials are especially valuable because they connect the archived molecular work to a well-documented resistance pedigree. Published genetic mapping subsequently showed that b43-17-derived PdR1a mapped in the 9621 population to a 0.6-cM interval on chromosome 14, while a sibling population carried PdR1b in a 0.4-cM interval (Riaz et al. 2008), and RGA-type genes were later characterized at PdR1b (Agüero et al. 2022). This provides a strong independent framework for revisiting the RGA sequences generated from 9621-67: aligning the A7/B4 sequences to the PdR1 region and determining whether they represent candidate receptor-like kinases or linked NLRs is a concrete, testable next step. The connection to dagger-nematode resistance is similarly compelling, because D8909-15—part of the 9621 pedigree—was later used to delimit XiR1 to ~115 kb containing NB-LRR candidates (Hwang et al. 2010). The original program proposed essentially the same conceptual bridge, although the archived files do not contain a complete fine-scale physical map for the RGA markers.

The modern genomic context further elevates the significance of the dataset. Grapevine pangenomics now shows that structural variation contributes substantially to phenotype and that a single reference genome does not capture the full complement of grape genes (Liu et al. 2024; Guo et al. 2025). This matters directly for R-gene marker development because resistance loci are frequently structurally dynamic and enriched in duplicated gene families. The historical marker pipeline should therefore be upgraded from a single-reference PCR strategy to a graph/haplotype strategy: identify the original sequence, place it on multiple phased assemblies, define allele-specific SNP/indel/SV signatures, test transferability across resistant germplasm, and then validate linkage in segregating populations (Fig. 8).

A practical publication strategy follows from these observations. The dataset is best presented as an integrative original-research/resource paper with three evidence layers: primary archived experimental observations from the program; later peer-reviewed studies that independently validate resistance loci or

functional genes related to the same germplasm and populations; and a contemporary reanalysis framework that converts historical sequences into modern markers. This preserves the originality and value of the experimental work without overclaiming unpublished historical data.

Limitations. The available archived material does not include complete individual-level genotype matrices, full phenotype datasets, marker coordinates, recombination fractions, LOD scores, or a final integrated linkage map. The present manuscript therefore does not claim newly calculated QTL positions or marker effects. It reports marker-discovery outcomes, sequence resources, representative segregation assays and the mapping-population design, and it identifies the datasets required for a full quantitative linkage analysis. If the original laboratory notebooks, raw gels, genotype sheets, phenotype scores or sequencing chromatograms are recovered, the paper can be upgraded with formal linkage statistics without altering its central framework.

6. Conclusions

This integrated analysis supports a clear conclusion: grapevine R genes, RGAs and connected defense-network genes—including the defense-signaling methyltransferases integrated here—provide a biologically rational and experimentally productive substrate for molecular-marker development. The archived program generated a large candidate-gene resource, demonstrated parental and interspecific polymorphism from primary gels, identified SNP and indel variation including diagnostic non-coding indels, developed PCR and PCR-RFLP marker assays, and established multiple mapping populations for resistance-related phenotypes. The data also correctly revealed that not every R-gene polymorphism is resistance-associated, emphasizing the need for segregation analysis and validation.

The research concept is even more powerful today. Modern phased genomes, graph pangenomes, structural-variant maps, NLR repertoires, transcriptomics and haplotype-aware marker platforms can transform the archived sequences into transferable markers with higher specificity and lower reference bias (Liu et al. 2024; Guo et al. 2025; Possamai et al. 2024). The strongest publication frame is therefore an integrative one: historical candidate-gene and defense-signaling marker development, plus independent later validation of related resistance loci, plus modern pangenomic reanalysis and marker conversion. Such a paper preserves the originality of the experimental work while placing it firmly within the current state of grapevine resistance genetics and precision breeding.

Declarations

Data availability

The primary experimental evidence for this manuscript was supplied as archived project files: (i) the 2009 grant-renewal document; (ii) the 2008–2009 research progress report and the identification of functional PD-resistant gene analogs (2008); (iii) the 2007 grape R-gene cloning results; (iv) the 04373 R19/R9 polymorphism presentation and the 2009 parental-line polymorphism presentation (source of Figs. 3–6); (v) the brief R-gene polymorphism/segregation summary (2007); (vi) the 9621-67 RGA

sequence file and RGA accession list; (vii) the cloned methyltransferase/methylesterase sequence files and the wild-Vitis methyltransferase polymorphism image (source of Fig. 7); and (viii) the archived wild-species/germplasm tables. These files are cited as supplementary experimental records or can be deposited as a project dataset where the journal permits. Supporting the Fig. 5A phylogeny, the following are provided as supplementary files: the FAMSA protein alignment (FASTA), the Newick tree with bootstrap support, the list of GenBank accessions used, and a high-resolution tree image in which every accession and support value is legible. All GenBank sequences are public and identified by accession. The manuscript intentionally distinguishes archived observations from later published validation and from proposed contemporary reanalysis.

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Competing interests

The author declares no competing financial or non-financial interests.

Author contributions

D.W.C. is the sole author. D.W.C. conceived and designed the research program, generated and analyzed the data, interpreted the results, and wrote the manuscript.

Data Archiving Statement

Newly reported sequences generated in this study (the cloned RGA clones A7-1/2/3 and B4-1/2/3 and the cloned defense-network and methyltransferase genes) will be deposited in GenBank and their accession numbers added prior to publication. All third-party sequences analyzed are publicly available GenBank records and are identified by accession number in the text and supplementary files.

Use of generative AI and AI-assisted technologies

During preparation of this manuscript the author used a generative AI assistant (Claude, Anthropic) to help reformat the manuscript into the journal's style, to copy-edit the text for readability, and to verify the reference list against primary sources. The author designed the study, generated and interpreted all data, drafted the manuscript, reviewed and edited all AI-assisted output, and takes full responsibility for the content.

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support of that original work. The analyses, interpretations, and conclusions presented here are solely those of the author and do not necessarily reflect the views of USDA-ARS or the American Vineyard Foundation.

Recommended contemporary validation experiments

- Recover and digitize the original genotype and phenotype spreadsheets for 03-3004, 04190, 04191, 04373 and 9621.
- Map all historical R-gene, RGA and methyltransferase sequences to PN40024 v4 and to phased wild/cultivated *Vitis* assemblies, retaining multi-mapping information for duplicated NLR loci.
- Redesign locus-specific assays from historical SNPs/indels using KASP, rhAmpSeq, amplicon sequencing, or another validated platform.
- Re-genotype informative recombinants and calculate recombination fractions, LOD scores, confidence intervals and conditional QTL effects.
- Test whether archived 9621-67 RGA sequences fall within or near PdR1a/PdR1b, XiR1, or other resistance-associated regions.
- Validate candidate-gene and methyltransferase expression or allelic function using RT-qPCR, RNA-seq, transient assays, or transformation where biologically justified.
- Evaluate marker transferability across the archived wild germplasm and modern breeding populations and quantify false-positive/false-negative rates for MAS.
- Combine candidate-gene and defense-signaling markers with genome-wide haplotype markers in resistance-pyramiding experiments to minimize linkage drag.

References

1. Adam-Blondon AF, Roux C, Claux D, Butterlin G, Merdinoglu D, This P (2004) Mapping 245 SSR markers on the *Vitis vinifera* genome: a tool for grape genetics. *Theor Appl Genet* 109:1017–1027. <https://doi.org/10.1007/s00122-004-1704-y>
2. Agüero CB, Riaz S, Tenschler AC, Bistué C, Walker MA (2022) Molecular and functional characterization of two RGA-type genes in the PdR1b locus for Pierce's disease resistance in *Vitis arizonica/candicans*. *Plant Cell Tissue Organ Cult* 151:497–510. <https://doi.org/10.1007/s11240-022-02366-6>
3. Bakker EG, Toomajian C, Kreitman M, Bergelson J (2006) A genome-wide survey of R gene polymorphism in *Arabidopsis*. *Plant Cell* 18:1803–1818. <https://doi.org/10.1105/tpc.106.042614>
4. Barker CL, Donald T, Pauquet J, Ratnaparkhe MB, Bouquet A, Adam-Blondon AF, Thomas MR, Dry IB (2005) Genetic and physical mapping of the grapevine powdery mildew resistance gene, Run1, using a bacterial artificial chromosome library. *Theor Appl Genet* 111:370–377. <https://doi.org/10.1007/s00122-005-2030-8>

5. Chen F, D'Auria JC, Tholl D, Ross JR, Gershenzon J, Noel JP, Pichersky E (2003) An *Arabidopsis thaliana* gene for methyl salicylate biosynthesis, identified by a biochemical genomics approach, has a role in defense. *Plant J* 36:577–588. <https://doi.org/10.1046/j.1365-313X.2003.01902.x>
6. Cheng DW, Lin H, Takahashi Y, Walker MA, Civerolo EL, Stenger DC (2010) Transcriptional regulation of the grape cytochrome P450 monooxygenase gene CYP736B in response to *Xylella fastidiosa* infection. *BMC Plant Biol* 10:135. <https://doi.org/10.1186/1471-2229-10-135>
7. Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162:156–159. [https://doi.org/10.1016/0003-2697\(87\)90021-2](https://doi.org/10.1016/0003-2697(87)90021-2)
8. Deorowicz S, Debudaj-Grabysz A, Gudyś A (2016) FAMSA: fast and accurate multiple sequence alignment of huge protein families. *Sci Rep* 6:33964. <https://doi.org/10.1038/srep33964>
9. Di Gaspero G, Cipriani G (2002) Resistance gene analogs are candidate markers for disease-resistance genes in grape (*Vitis* spp). *Theor Appl Genet* 106:163–172. <https://doi.org/10.1007/s00122-002-1062-6>
10. Di Gaspero G, Cipriani G (2003) Nucleotide binding site/leucine-rich repeats, Pto-like and receptor-like kinases related to disease resistance in grapevine. *Mol Genet Genomics* 269:612–623. <https://doi.org/10.1007/s00438-003-0884-5>
11. Donald TM, Pellerone F, Adam-Blondon AF, Bouquet A, Thomas MR, Dry IB (2002) Identification of resistance gene analogs linked to a powdery mildew resistance locus in grapevine. *Theor Appl Genet* 104:610–618. <https://doi.org/10.1007/s00122-001-0768-1>
12. Doucleff M, Jin Y, Gao F, Riaz S, Krivanek AF, Walker MA (2004) A genetic linkage map of grape, utilizing *Vitis rupestris* and *Vitis arizonica*. *Theor Appl Genet* 109:1178–1187. <https://doi.org/10.1007/s00122-004-1728-3>
13. Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39:783–791. <https://doi.org/10.1111/j.1558-5646.1985.tb00420.x>
14. Fischer BM, Salakhutdinov I, Akkurt M, Eibach R, Edwards KJ, Töpfer R, Zyprian EM (2004) Quantitative trait locus analysis of fungal disease resistance factors on a molecular map of grapevine. *Theor Appl Genet* 108:501–515. <https://doi.org/10.1007/s00122-003-1445-3>
15. Guo L, Wang X, Ayhan DH et al (2025) Super pangenome of *Vitis* empowers identification of downy mildew resistance genes for grapevine improvement. *Nat Genet* 57:741–753. <https://doi.org/10.1038/s41588-025-02111-7>
16. Höschele T, Malagol N, Bori SO, Müllner S, Töpfer R, Sturm J, Zyprian E, Trapp O (2024) Rpv10.2: a haplotype variant of locus Rpv10 enables new combinations for pyramiding downy mildew resistance traits in grapevine. *Plants (Basel)* 13:2624. <https://doi.org/10.3390/plants13182624>
17. Hulbert SH, Webb CA, Smith SM, Sun Q (2001) Resistance gene complexes: evolution and utilization. *Annu Rev Phytopathol* 39:285–312. <https://doi.org/10.1146/annurev.phyto.39.1.285>
18. Hwang CF, Xu K, Hu R, Zhou R, Riaz S, Walker MA (2010) Cloning and characterization of XiR1, a locus responsible for dagger nematode resistance in grape. *Theor Appl Genet* 121:789–799.

<https://doi.org/10.1007/s00122-010-1349-y>

19. Lin H, Doddapaneni H, Takahashi Y, Walker MA (2007) Comparative analysis of ESTs involved in grape responses to *Xylella fastidiosa* infection. *BMC Plant Biol* 7:8. <https://doi.org/10.1186/1471-2229-7-8>
20. Liu Z, Wang N, Su Y et al (2024) Grapevine pangenome facilitates trait genetics and genomic breeding. *Nat Genet* 56:2804–2814. <https://doi.org/10.1038/s41588-024-01967-5>
21. Massonnet M, Riaz S, Pap D, Figueroa-Balderas R, Walker MA, Cantu D (2022) The grape powdery mildew resistance loci Ren2, Ren3, Ren4D, Ren4U, Run1, Run1.2b, Run2.1 and Run2.2 activate different transcriptional responses to *Erysiphe necator*. *Front Plant Sci* 13:1096862. <https://doi.org/10.3389/fpls.2022.1096862>
22. Meyers BC, Kozik A, Griego A, Kuang H, Michelmore RW (2003) Genome-wide analysis of NBS-LRR-encoding genes in *Arabidopsis*. *Plant Cell* 15:809–834. <https://doi.org/10.1105/tpc.009308>
23. Michelmore RW, Meyers BC (1998) Clusters of resistance genes in plants evolve by divergent selection and a birth-and-death process. *Genome Res* 8:1113–1130. <https://doi.org/10.1101/gr.8.11.1113>
24. Park SW, Kaimoyo E, Kumar D, Mosher S, Klessig DF (2007) Methyl salicylate is a critical mobile signal for plant systemic acquired resistance. *Science* 318:113–116. <https://doi.org/10.1126/science.1147113>
25. Pauquet J, Bouquet A, This P, Adam-Blondon AF (2001) Establishment of a local map of AFLP markers around the powdery mildew resistance gene Run1 in grapevine and assessment of their usefulness for marker assisted selection. *Theor Appl Genet* 103:1201–1210. <https://doi.org/10.1007/s001220100664>
26. Possamai T, Scota L, Velasco R, Migliaro D (2024) A sustainable strategy for marker-assisted selection (MAS) applied in grapevine (*Vitis* spp.) breeding for resistance to downy (*Plasmopara viticola*) and powdery (*Erysiphe necator*) mildews. *Plants (Basel)* 132001. <https://doi.org/10.3390/plants13142001>
27. Richly E, Kurth J, Leister D (2002) Mode of amplification and reorganization of resistance genes during recent *Arabidopsis thaliana* evolution. *Mol Biol Evol* 19:76–84. <https://doi.org/10.1093/oxfordjournals.molbev.a003984>
28. Riaz S, Dangl GS, Edwards KJ, Meredith CP (2004) A microsatellite marker based framework linkage map of *Vitis vinifera* L. *Theor Appl Genet* 108:864–872. <https://doi.org/10.1007/s00122-003-1488-5>
29. Riaz S, Tenschler AC, Rubin J, Graziani R, Pao SS, Walker MA (2008) Fine-scale genetic mapping of two Pierce's disease resistance loci and a major segregation distortion region on chromosome 14 of grape. *Theor Appl Genet* 117:671–681. <https://doi.org/10.1007/s00122-008-0802-7>
30. Riaz S, Tenschler AC, Graziani R, Krivanek AF, Ramming DW, Walker MA (2009) Using marker-assisted selection to breed Pierce's disease-resistant grapes. *Am J Enol Vitic* 60:199–207. <https://doi.org/10.5344/ajev.2009.60.2.199>

31. Ross JR, Nam KH, D'Auria JC, Pichersky E (1999) S-adenosyl-L-methionine:salicylic acid carboxyl methyltransferase, an enzyme involved in floral scent production and plant defense, represents a new class of plant methyltransferases. *Arch Biochem Biophys* 367:9–16.
<https://doi.org/10.1006/abbi.1999.1255>
32. Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB, Erlich HA (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* 239:487–491. <https://doi.org/10.1126/science.2448875>
33. Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 4:406–425. <https://doi.org/10.1093/oxfordjournals.molbev.a040454>
34. Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* 74:5463–5467. <https://doi.org/10.1073/pnas.74.12.5463>
35. Sapkota S, Zou C, Ledbetter C, Underhill A, Sun Q, Gadoury D, Cadle-Davidson L (2023) Discovery and genome-guided mapping of REN12 from *Vitis amurensis*, conferring strong, rapid resistance to grapevine powdery mildew. *Hortic Res* 10:uhad052. <https://doi.org/10.1093/hr/uhad052>
36. Sayers EW, Bolton EE, Brister JR et al (2022) Database resources of the National Center for Biotechnology Information. *Nucleic Acids Res* 50:D20–D26. <https://doi.org/10.1093/nar/gkab1112>
37. Schmidlin L, Poutaraud A, Claudel P et al (2008) A stress-inducible resveratrol O-methyltransferase involved in the biosynthesis of pterostilbene in grapevine. *Plant Physiol* 148:1630–1639.
<https://doi.org/10.1104/pp.108.126003>
38. Seo HS, Song JT, Cheong JJ, Lee YH, Lee YW, Hwang I, Lee JS, Choi YD (2001) Jasmonic acid carboxyl methyltransferase: a key enzyme for jasmonate-regulated plant responses. *Proc Natl Acad Sci USA* 98(8):4788–4793. <https://doi.org/10.1073/pnas.081557298>
39. Velasco R, Zharkikh A, Troggio M et al (2007) A high quality draft consensus sequence of the genome of a heterozygous grapevine variety. *PLoS ONE* 2(12):e1326.
<https://doi.org/10.1371/journal.pone.0001326>

Tables

Table 1 Summary of archived marker-development evidence.

Dataset component	Archived evidence	Publication-level interpretation
R-gene / RGA inventory	341 public NBS-LRR + 26 cloned NBS-LRR + 101 cloned RGAs + >50 non-NBS genes	≥ 518 candidate sequences; substantial defense-gene resource
Primer development	83 R-gene primer pairs designed / retrieved	Candidate-gene marker platform
2008–2009 screening	46 R-gene + 30 RGA primer pairs	Broad parental / wild-germplasm polymorphism screen
Early candidate screen	20 primer pairs; 7 polymorphic; R9/R18/R19 clearly differentiate parents	Evidence of marker polymorphism, not proof of causal resistance
Sequence variation	SNPs and indels; V1–V7 variable regions	Candidate haplotypes and marker-conversion sites
Non-coding variation	~30 UTR/promoter primer pairs; PM1-R promoter/terminator gels	Useful source of diagnostic indels
Segregation assays	R19 + MseI (04373); R19 + MspI (04191); R9 PCR + actin	Representative segregation evidence; not phenotype-concordant
Methyltransferase set	SABATH MT3, SMT1, JMT, OMTs, methylesterase; polymorphic in 24 wild Vitis	Defense-signaling marker & functional-gene resource
9621-67 sequence resource	A7/B4 RGA sequences from 67CS/67TS	Historical haplotype archive for retrospective mapping

All evidence in Table 1 was generated by the archived experimental program of this study; no external references apply. Published context for the resistance loci and phenotype associations is cited in Tables 2 and 5.

Table 2 Mapping populations and resistance-oriented applications. The crosses are from this study; the published locus/phenotype associations are cited in the Reference column. b43-17 is the *V. arizonica* PdR1 (Pierce's-disease) donor; R9/R18/R19 were scored against PD resistance/susceptibility in the archived segregation summary.

Population	Cross / ancestry (archived)	Target phenotype / purpose	Reference
03-3004	{V. vinifera Raisin de Palestine × (V. romanetii C166-026 × V. vinifera)} × V. vinifera B70-57, BC2	Powdery-mildew resistance	This study
04191	F2-7 × F8909-07 (V. arizonica b43-17 pedigree)	PD-associated R-gene segregation (R9/R18/R19)	This study
04190	F2-7 × F8909-08 (V. arizonica b43-17 pedigree)	PD-associated resistance; later linked to PdR1b	This study; PdR1b (Riaz et al. 2008)
04373	F2-35 × b43-17	PD resistance/susceptibility; archived R9/R19 assays (Fig. 6A,B)	This study
9621	D8909-15 × F8909-17	Pierce's-disease pedigree (PdR1); nematode/fanleaf context; RGA sequence source	This study; PdR1 (Riaz et al. 2008; Riaz et al. 2009)

Table 3 Representative cloned candidate R and defense-network genes.

Candidate / gene class	Archived observation	Functional / modern interpretation
R9	Clear parental polymorphism; segregated in 04373	Candidate marker; not phenotype-concordant in archived summary
R18	Clear parental polymorphism (04190/04191/04373)	Candidate marker requiring validation
R19	PCR / restriction polymorphism; 04373 & 04191 panels	Candidate marker; not phenotype-concordant in archived summary
NBS-LRR / RGA clusters	~70 common genes/RGAs on five chromosomes	Clustered resistance-gene architecture; re-map with pangenome
CYP736B / P450	Cloned from 9621-67; 1,512-bp gene, 1,507-bp CDS, 495 aa	Later work showed Xylella-associated expression
ERF4	864-bp clone; 843-bp CDS; ~280 aa	Transcriptional defense/signaling candidate
Dioxygenase	Complete sequence 1,834 bp; 610-aa protein	Defense/metabolic-network candidate
Zinc-finger protein	Complete sequence 2,075 bp; 689 aa	Regulatory candidate
Brassinosteroid-6-oxidase	1,451-bp clone; functional CDS 750 bp/250 aa	Hormone/defense-network candidate

Table 4 Defense-signaling and secondary-metabolism methyltransferase / esterase genes cloned in the program.

Gene / clone	Size	Source material	Function / defense role
SABATH MT3 (Y3-22)	1,218 bp / 405 aa	9621-67 leaf	SA/BA carboxyl methyltransferase; MeSA – SAR signal
SMT1 (X4/X5)	1,164 bp / 387 aa	9621-94 leaf; Chardonnay stem	SABATH methyltransferase; SA/BA methylation
JMT (T3-3, T4-2)	1,128 bp / 375 aa	9621-67; 9621-94	Jasmonate carboxyl methyltransferase; MeJA defense signal
Methyltransferase V28-2	898 bp / 298 aa	Wild Vitis panel	SABATH-type methyltransferase (VsamtF3/R3)
Methylesterase V29-3	786 bp / 261 aa	Wild Vitis panel	Methylesterase (SABP2-like); MeSA → SA hydrolysis
OMT2.1 (M3-8)	986 bp	9621-67	O-methyltransferase; phenylpropanoid/stilbene branch
OMT2.2 (M2-2)	1,077 bp	9621-67	O-methyltransferase; methylated phytoalexins
OMT3.1 (M1-4, MT1-2/3)	~564 bp	9621-67; 9621-94	O-methyltransferase; secondary-metabolite methylation
L-isoaspartate-OMT (MT5-1)	729 bp	9621-94	Stress-induced protein repair

Table 5 Independently mapped grapevine resistance loci that provide biological context for the archived candidate-gene strategy. All loci in this table are from the published work cited in the Reference column, not from this study.

Locus	Pathogen / pest	Source & interval	Relevance to archived program	Reference
PdR1a / PdR1b	Pierce's disease (Xylella)	V. arizonica b43-17; chr 14; 0.6 cM (9621) / 0.4 cM (04190)	Same germplasm/populations; RGA-type genes later found at PdR1b	(Riaz et al. 2008; Agüero et al. 2022)
XiR1	Dagger nematode (Xiphinema)	V. arizonica; ~115 kb; NB-LRR candidates	Shares 9621/D8909 pedigree; candidate-gene physical mapping	(Hwang et al. 2010)
Run1 / Ren loci	Powdery mildew (Erysiphe)	AFLP & BAC mapping; NBS-LRR (Run1) candidates	Clustered NLR architecture consistent with RGA screen	(Donald et al. 2002; Pauquet et al. 2001; Fischer et al. 2004; Massonnet et al. 2022)
REN12	Powdery mildew (Erysiphe)	V. amurensis; ~780 kb; candidate NLRs; MAS markers	Validates candidate-gene marker approach for MAS	(Sapkota et al. 2023)
Rpv loci	Downy mildew (Plasmopara)	Multiple loci; pangenome-linked NLRs & SVs	Modern targets for haplotype-aware pyramiding	(Liu et al. 2024; Possamai et al. 2024; Höschele et al. 2024)

Table 6 Archived *Vitis* germplasm represented in the program.

Germplasm group	Representative archived materials
Wild Vitis	V. aestivalis 1394; V. arizonica b43-17; V. candicans T56; V. champinii 3-48; V. rufotomentosa 1416; V. shuttleworthii; V. simpsonii c56-94; V. smalliana; V. girdiana 1379; V. monticola; V. nesbitiana 2235.4; V. rupestris Metallique 1421
Muscadinia	M. rotundifolia Cowart
Interspecific hybrids	8909-15, 8909-19, 8909-67, 8909-94, 8909-17; V. arizonica × V. rupestris ancestry
V. vinifera	Chardonnay; Mission; Red Globe; Alvarinho; Sauv Gris; Vioziniio

Figures

Candidate-gene R/RGA marker development pipeline

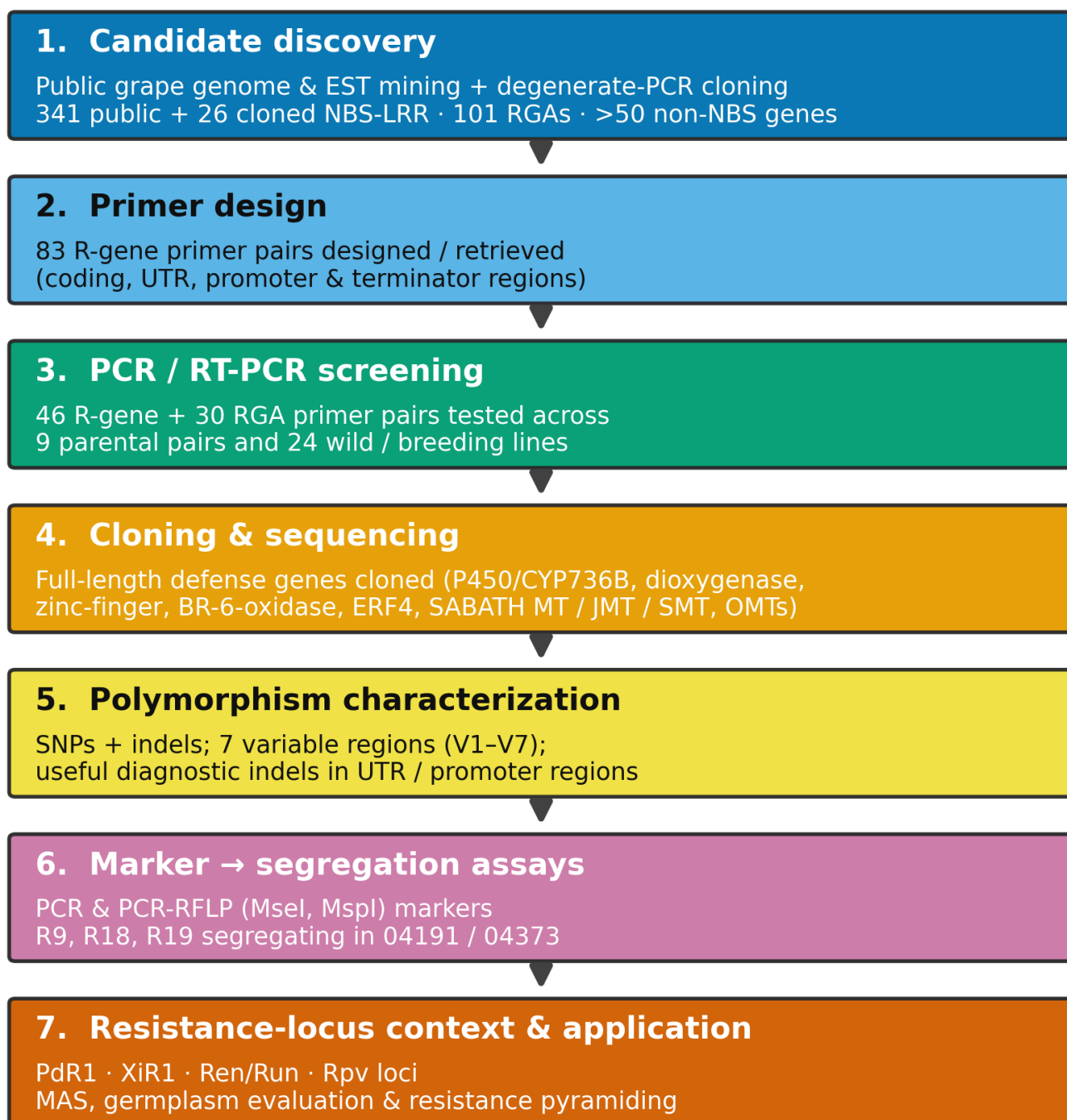
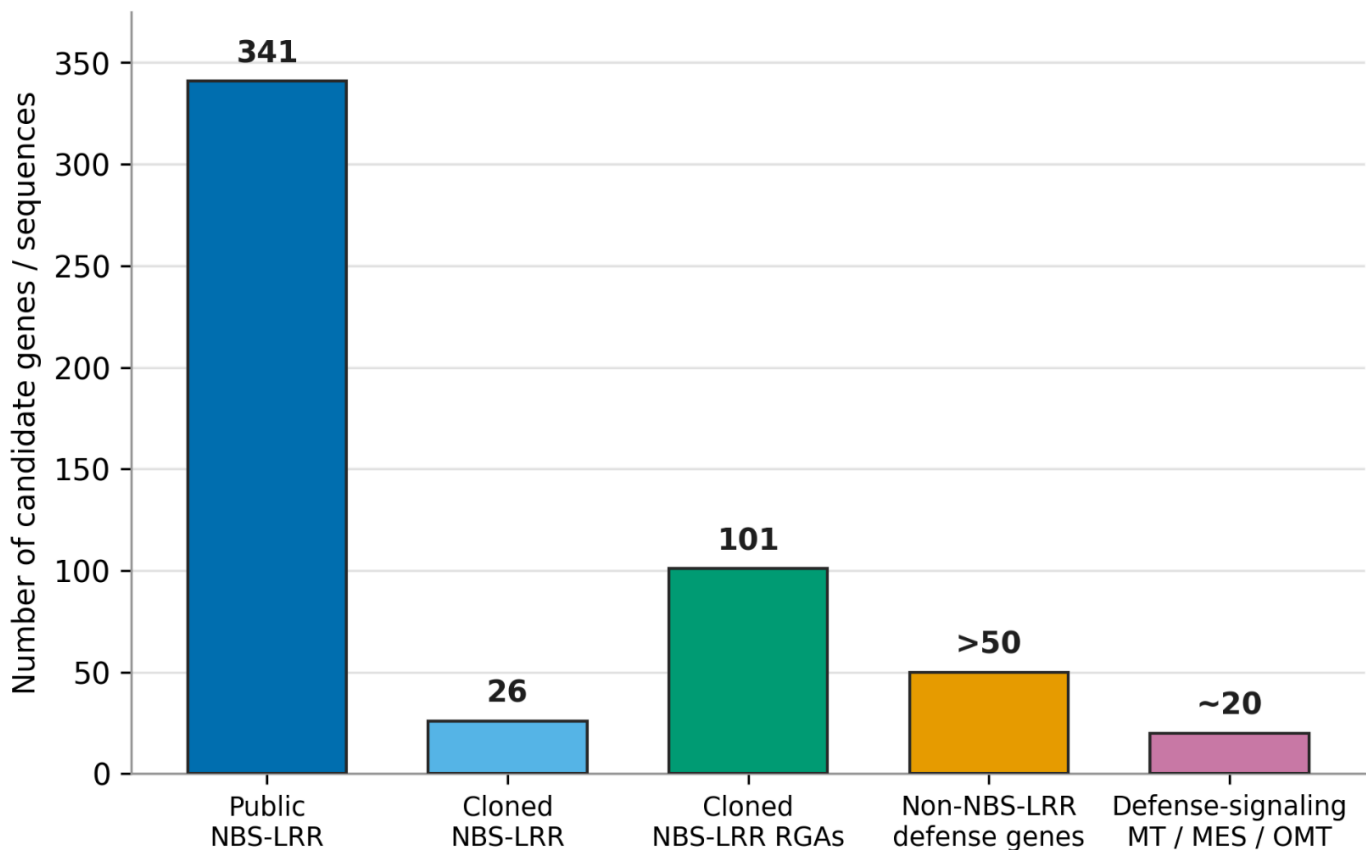


Figure 1

Candidate-gene R/RGA marker-development pipeline used in this study. The workflow proceeds from candidate discovery through primer design, PCR/RT-PCR screening, cloning and sequencing, polymorphism characterization, marker-to-segregation assays, and finally to the resistance-locus and precision-breeding context in which the markers are deployed. Numbered stages correspond to the Materials and methods subsections (3.2–3.7) and the Results.

Archived grapevine defense-gene candidate resource (≥ 518 sequences)



≥ 64 NBS-LRR and ≥ 15 non-NBS-LRR candidates were reported as pathogen-inducible.

Figure 2

Archived grapevine defense-gene candidate resource (≥ 518 sequences). Counts of public and cloned NBS-LRR genes, cloned NBS-LRR RGAs, non-NBS-LRR defense genes, and the defense-signaling / secondary-metabolism methyltransferase–esterase set. At least 64 NBS-LRR and 15 non-NBS-LRR candidates were reported as pathogen-inducible.

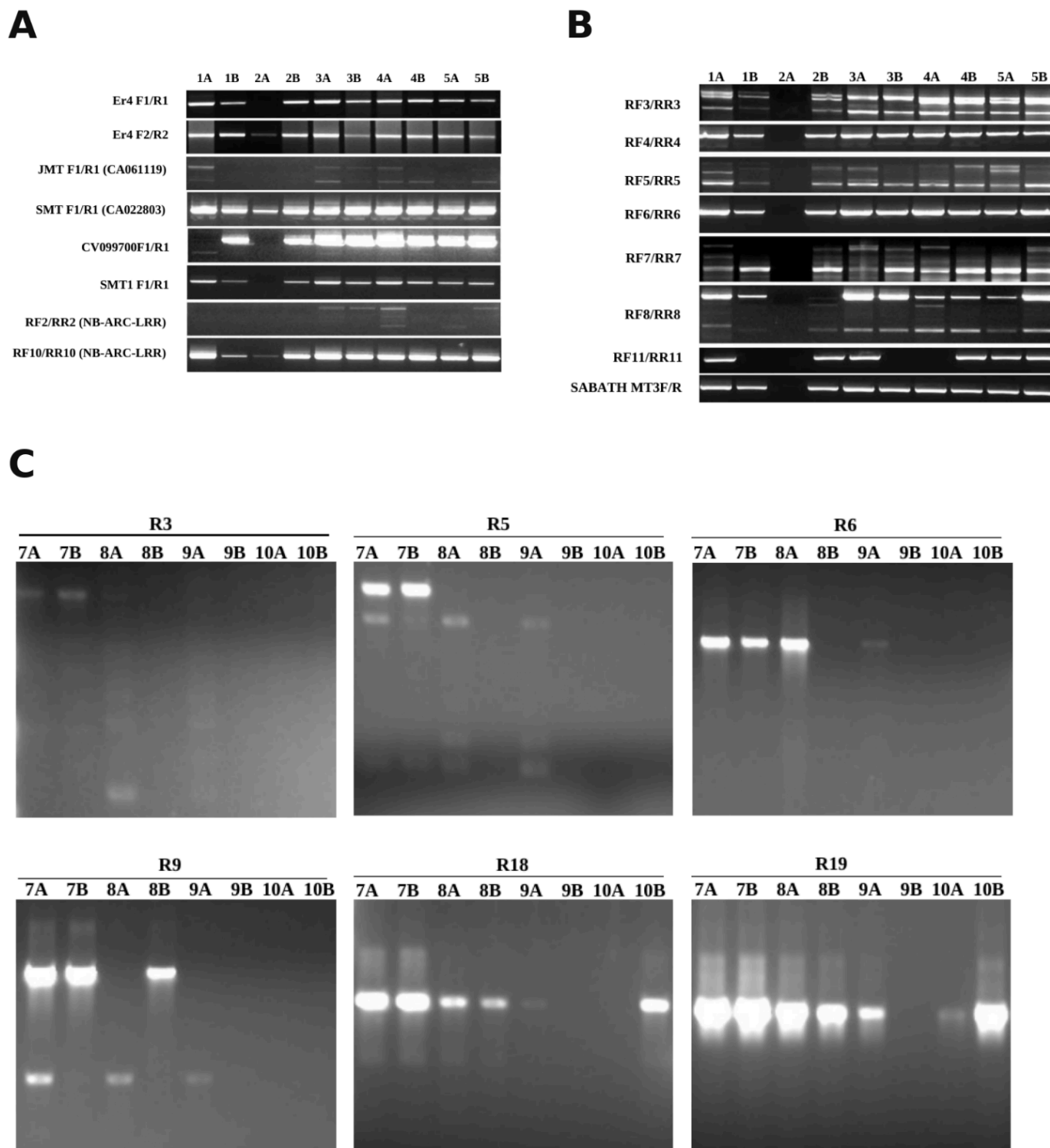
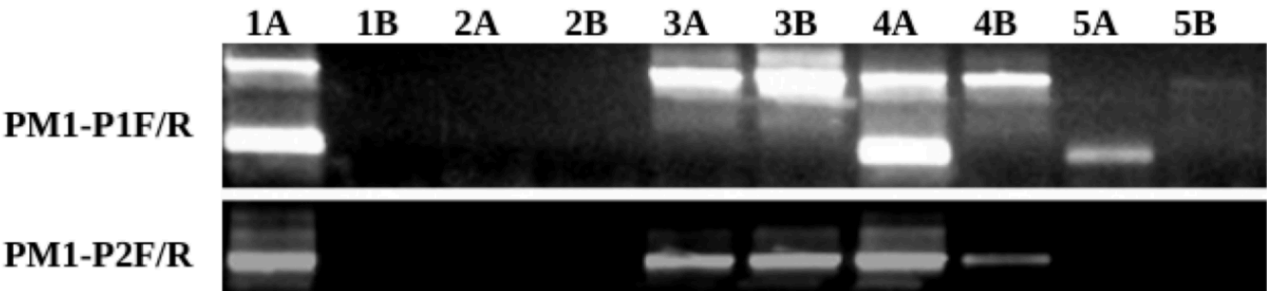


Figure 3

Candidate-gene polymorphism among grape parental lines (2008–2009 screen; original gels). Lanes are grouped by parental pair. Each pair is one candidate breeding/mapping cross, and within a pair the two lanes labeled “A” and “B” are its two parent lines (one a resistant/wild source, the other a susceptible or complementary parent); a marker is informative for that cross when the A and B lanes differ. Nine parental pairs were screened; pairs 1–5 (lanes 1A–5B) appear in panels A and B and pairs 7–10 (lanes

7A–10B) in panel C. (A) Group 1: Er4 (F1/R1 and F2/R2), JMT (CA061119), SMT (CA022803), CV099700, SMT1 and NB-ARC-LRR primers RF2 and RF10. (B) Group 2: NB-ARC-LRR primers RF3–RF11 and the SABATH MT3 marker. (C) R3, R5, R6, R9, R18 and R19 across parental pairs 7–10. Reproducible A-vs-B band differences identify candidate co-dominant and presence/absence markers. The specific line identity of each numbered pair is held in the archived breeding records and can be tabulated once that key is supplied.

A



B

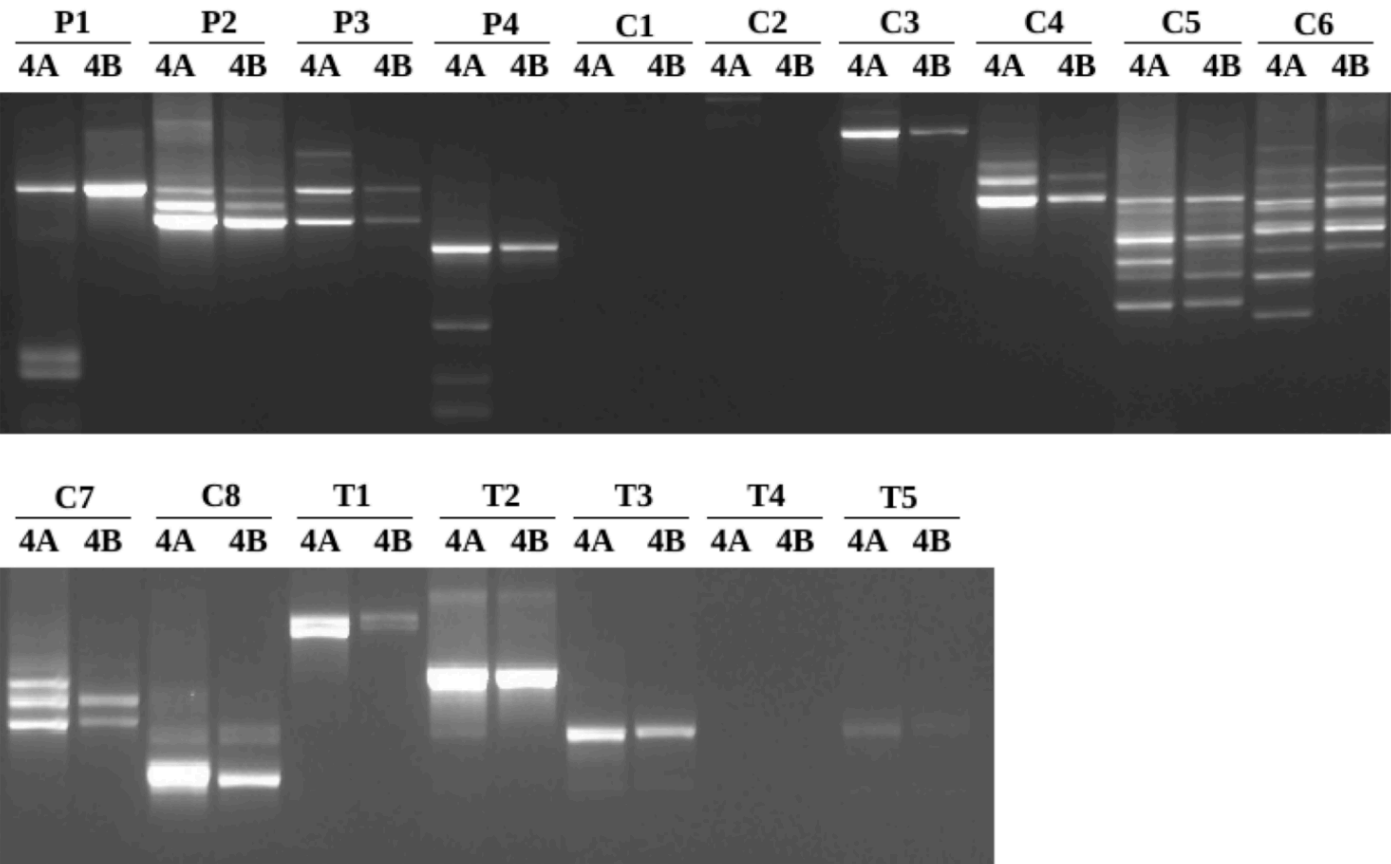


Figure 4

Polymorphism in non-coding regions of a powdery-mildew candidate gene (PM1-R; original gels). (A) Promoter markers PM1-P1F/R and PM1-P2F/R across five parental pairs (lanes 1A–5B). (B) Promoter (P1–P4), coding (C1–C8) and terminator (T1–T5) region markers between parental line 4 (4A/4B), showing that several non-coding regions are more polymorphic than the coding regions.

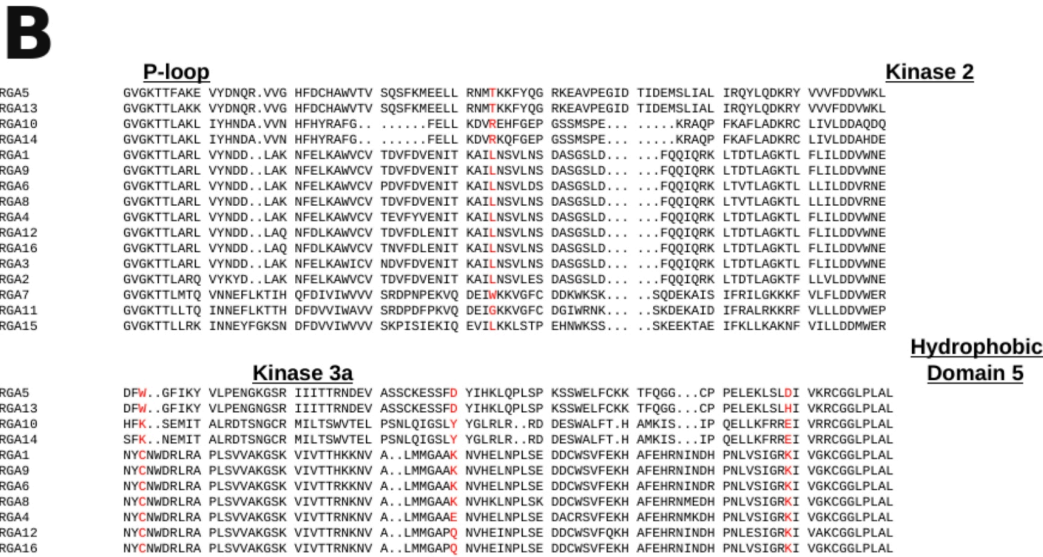
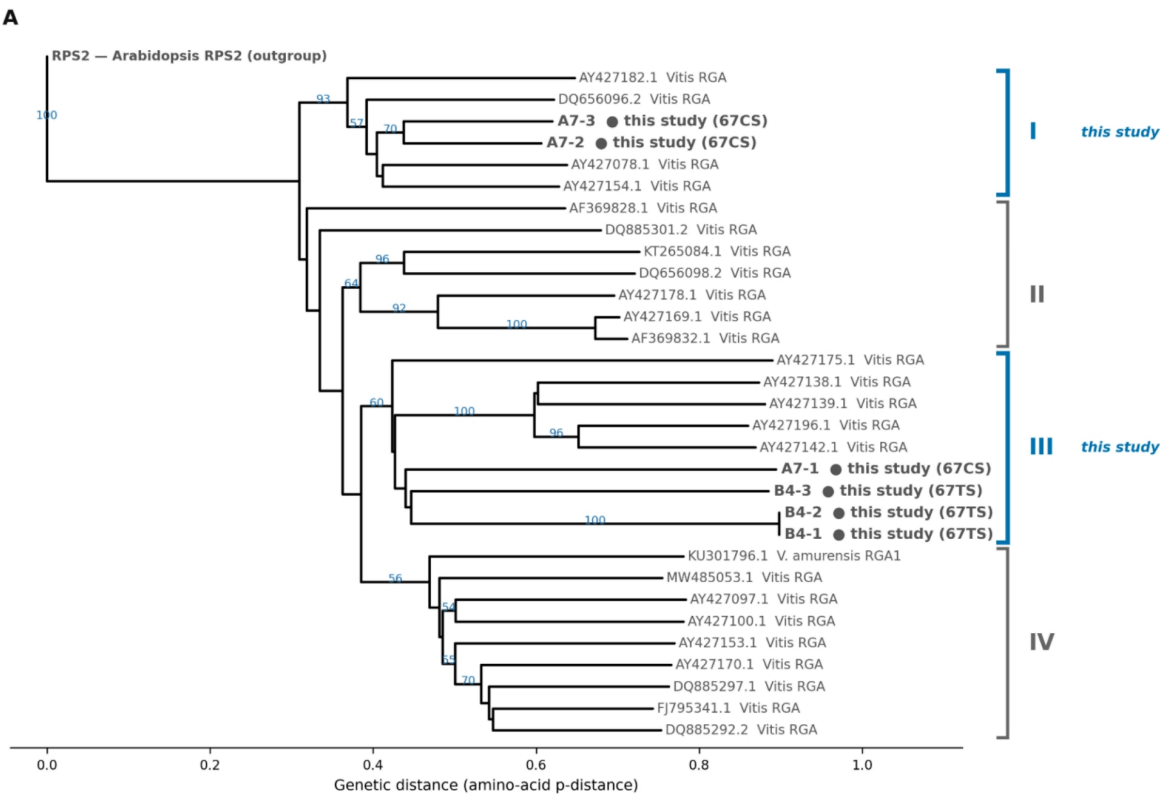
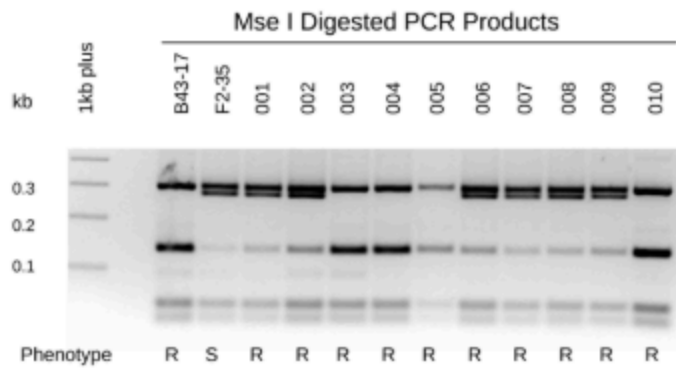


Figure 5

Grapevine RGA phylogeny and RGA sequence polymorphism. (A) Neighbor-joining phylogeny of 31 NB-ARC peptides: 25 representative *Vitis* RGAs selected one-per-cluster from all *Vitis* resistance-gene-analog and NBS-LRR/NB-ARC records in GenBank (grey accession labels; KU301796 = characterized *V. amurensis* RGA1), the six RGA clones from this study (bold; blue = 67CS resistant-associated, orange = 67TS susceptible-associated, marked ●), rooted with an *Arabidopsis* RPS2 outgroup. Blue numbers are bootstrap support (%; 1,000 replicates; shown for nodes $\geq 50\%$); the scale bar is amino-acid p-distance. Brackets I–IV mark the four major ingroup clades; clades I and III (blue, “◀ this study”) each contain clones from this study—A7-2/A7-3 in clade I and A7-1 with B4-1/2/3 in clade III—while clades II and IV contain only published *Vitis* RGAs. The clones thus belong to at least two established grapevine RGA lineages rather than a single new class. A comprehensive 129-taxon tree of all retrieved sequences, the alignment (FASTA), the Newick tree and the accession list are provided as supplementary files. (B) SNP/indel pattern across 16 RGAs from grape parental lines: non-synonymous SNPs (red) and indels fall outside the four conserved domains (P-loop, Kinase-2, Kinase-3a, hydrophobic domain 5), contributing to R-gene diversity.

A R19 · MseI-digested · population 04373



B R9 · PCR with actin control · population 04373



C R19 · MspI-digested · population 04191

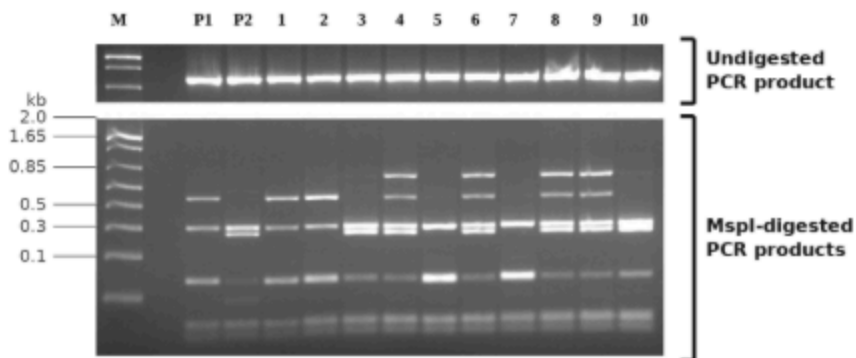
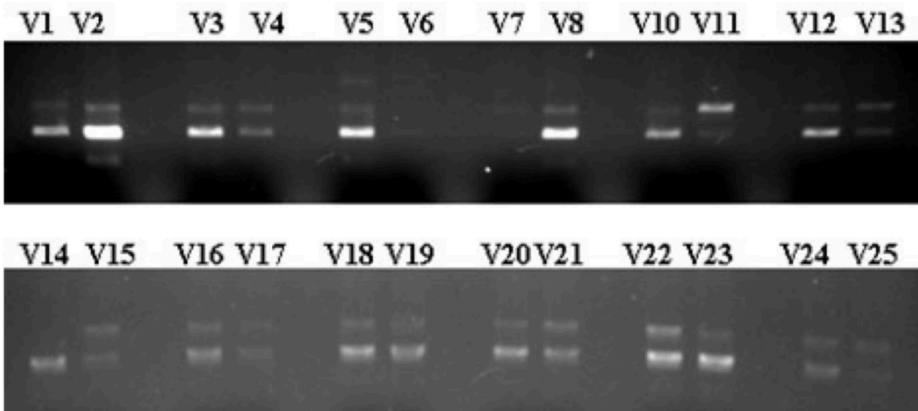


Figure 6

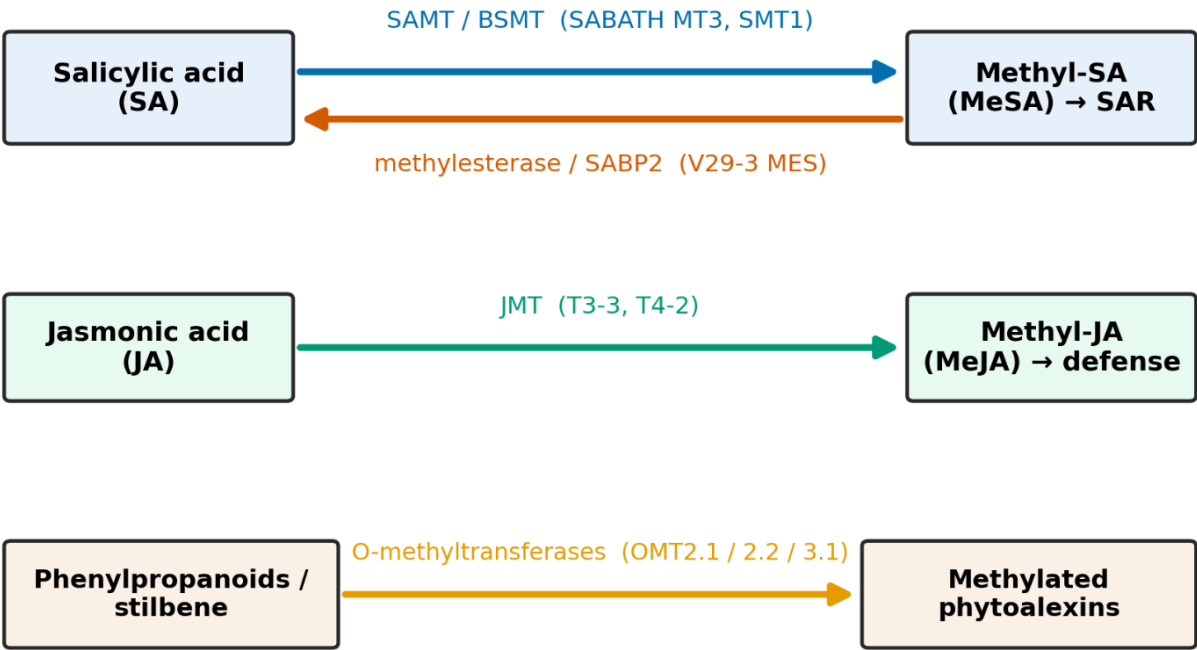
Representative candidate-gene segregation assays (original gels). (A) R19 PCR products digested with MseI in population 04373 (B43-17 = R, F2-35 = S, progeny 001–010). (B) R9 PCR with an actin internal control in the same 04373 materials. (C) R19 in population 04191: undigested PCR product (top) and MspI-digested fragments (bottom); P1, P2 and ten F₂ progeny. Markers are polymorphic and segregate, but the archived summary records that R9/R19 segregation did not track the resistance phenotype in 04373. Molecular sizes (kb) at left of panels A and B are from the 1 kb Plus DNA ladder; the sizes shown

for panel C are inferred from the same ladder, as panel C was not size-annotated in the original record, and should be treated as approximate.

A



B



L-isoaspartate-O-methyltransferase (MT5-1): stress-induced protein repair

Cloned from PD-resistant/susceptible 9621-67 & 9621-94, Chardonnay and 24 wild *Vitis* accessions

Figure 7

Defense-signaling and secondary-metabolism methyltransferase / esterase resource. (A) Genomic-DNA polymorphism of methyltransferase genes across 24 wild *Vitis* accessions (V1–V25; original gel). (B)

The defense methylation cycle showing where the cloned grapevine genes act: SABATH SAMT/BSMT (SABATH MT3, SMT1) and a methylesterase/SABP2 (V29-3) interconvert salicylic acid and methyl salicylate (systemic acquired resistance); JMT (T3-3, T4-2) forms methyl jasmonate; O-methyltransferases (OMT2.1/2.2/3.1) methylate stilbene/phenylpropanoid phytoalexins; and an L-isoaspartate-O-methyltransferase (MT5-1) performs stress-induced protein repair.

From candidate-gene RGA markers (2007-2009) to haplotype-aware pangenomic breeding

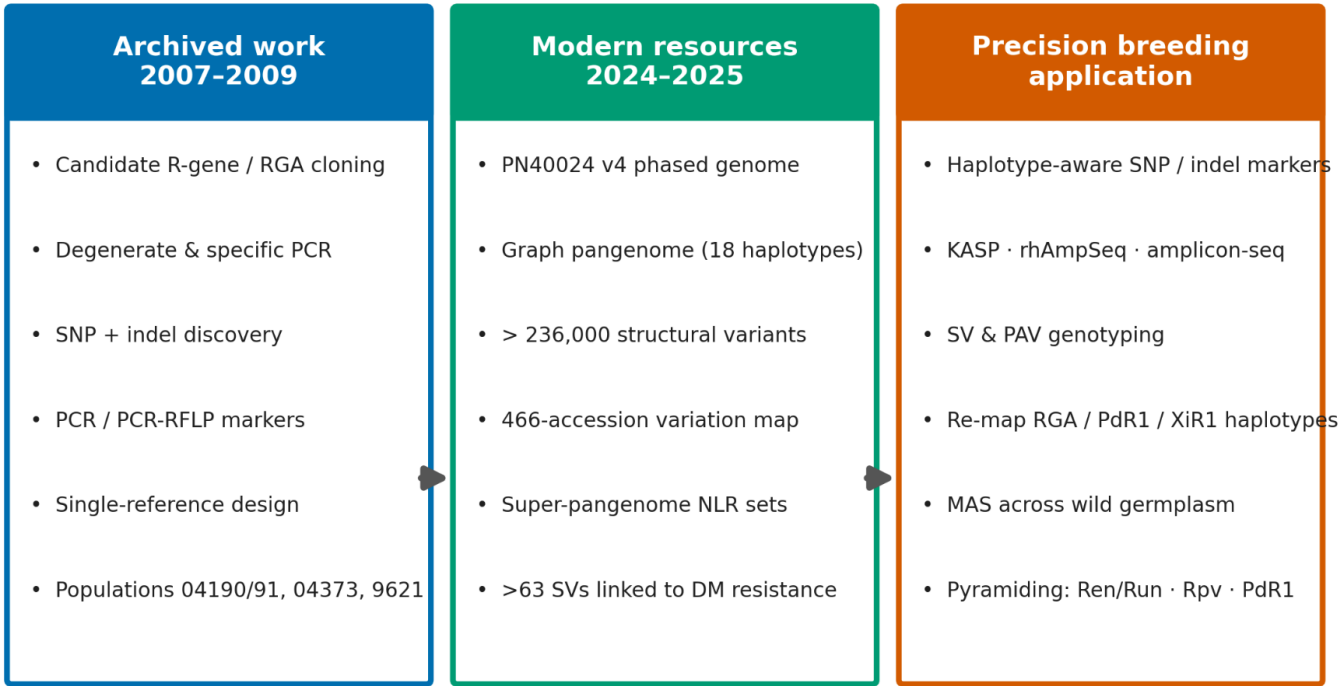


Figure 8

From candidate-gene RGA markers to haplotype-aware pangenomic breeding. The archived single-reference PCR/PCR-RFLP marker pipeline (left) is upgraded using modern phased genomes, graph pangenomes and structural-variant maps (center) into haplotype-aware KASP/rhAmpSeq/amplicon-sequencing assays, structural-variant and presence/absence genotyping, and marker-assisted pyramiding of Ren/Run, Rpv and PdR1 loci (right).