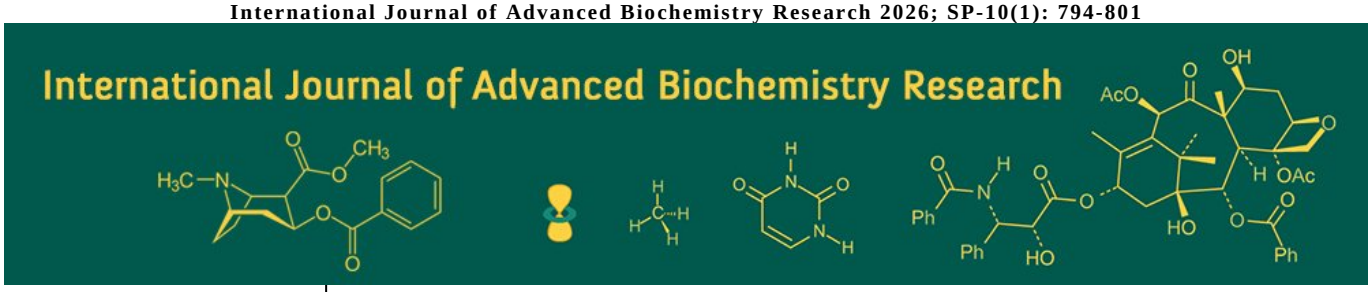

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Building resilient crops: A comprehensive review of gene pyramiding for durable resistance and multi-stress tolerance

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Abstract
Gene pyramiding represents a fundamental paradigm shift in modern crop improvement, empowering breeders to transcend the limitations of single trait selection by synchronizing the simultaneous integration of multiple elite alleles into superior, multi stress tolerant cultivars. This strategy is no longer a luxury but a mandate; as global yield trajectories falter against the projected necessity to double food production by 2050, the stability of our food systems is increasingly besieged by a synergistic onslaught of virulent biotic pathogens and volatile abiotic stressors. Leveraging the precision of Marker-Assisted Selection (MAS), contemporary breeding programs can now stack numerous genes or complex Quantitative Trait Loci (QTLs) with accuracy, ensuring the rapid recovery of the elite recurrent parent genome while minimizing the deleterious effects of linkage drag. This strategic consolidation of genetic assets not only reinforce immediate phenotypic performance but also provides a critical biological buffer against the evolution of pathogen virulence, thereby enhancing the durability of resistance in the field. This review synthesizes the current landscape of gene pyramiding, examining the biological mechanisms that underpin multi genic durability. Furthermore, it explores the integration of transformative molecular technologies most notably CRISPR-Cas9 mediated genome editing and Genomic Selection (GS) to bypass the logistical bottlenecks of traditional breeding.

Keywords: Gene pyramiding, marker assisted selection, quantitative trait loci, genomic selection.

Introduction
Gene pyramiding is defined as the simultaneous selection for and introduction of multiple genes during plant breeding to assemble them into a single genotype (Joshi and Nayak, 2010) [20]. This technique addresses a critical limitation of single gene resistance: the rapid evolution of pathogen populations that can overcome resistance through spontaneous mutations, resulting in epidemic outbreaks and substantial yield losses (Mundt, 2018) [30]. By combining two or more resistance genes or quantitative trait loci (QTLs) with different modes of action, plant breeders can significantly delay or prevent the emergence of virulent pathogen races, thereby ensuring the sustainability and longevity of genetic resistance in commercial varieties (Mundt, 2018) [30].

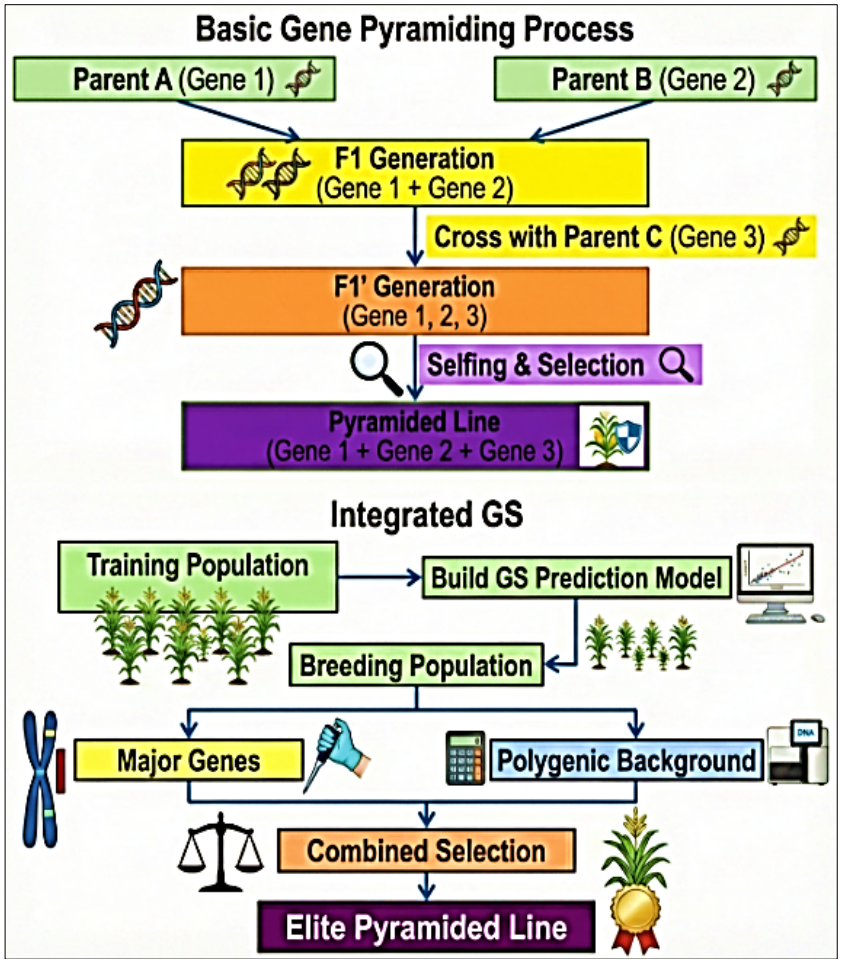

Fig 1: Steps in gene pyramiding to develop an elite line with desirable genes

Theoretical Foundations and Core Breeding Objectives of Gene Stacking

Gene pyramiding involves the deliberate combination of two or more genes conferring favourable phenotypes into a single plant genotype through hybridisation and subsequent selection strategies (Ashkani *et al*., 2016; Das and Rao, 2015) [5, 10]. The primary objectives of these programs include the enhancement of trait performance by combining complementary genes that exert additive or synergistic effects on the phenotype (Das and Rao, 2015; Akos *et al*., 2019) [10, 3]. A second objective involves the remediation of genetic deficits by retrogression genes from diverse germplasm sources, including wild relatives or exotic cultivars, into elite genetic backgrounds (Dormatey *et al*., 2020) [11]. Furthermore, gene pyramiding increases the durability of resistance by deploying multiple resistance genes simultaneously, thereby raising the genetic barrier against pathogen population evolution (Mundt, 2018) [30]. Finally, the strategy aims to widen the genetic basis of released cultivars to enhance their adaptability and stability across diverse agro ecological environments (Hoisington *et al*., 1994) [17]. The consolidation of desirable alleles from multiple parents into a single genotype represents a fundamental departure from traditional breeding approaches that select for one trait sequentially across numerous generations (Akos *et al*., 2019; Pradhan *et al*., 2015) [3, 35].

Biological Drivers and Genetic Models for Sustained Pathogen Resistance

The theoretical advantage of gene pyramiding for durable resistance rests on the "gene dosage hypothesis," which assumes that simultaneous overcoming of multiple resistance genes by pathogens requires independent mutations at multiple loci (Vanderplank, 1975) [48]. This hypothesis posits that such a simultaneous event has a significantly lower probability than single mutations conferring virulence to monogenic resistance (Mundt, 2018) [30]. In cereal rust systems, pyramided cultivars combining major resistance genes (R-genes) with adult plant resistance (APR) genes have maintained high levels of disease control for extended periods despite intense selection pressure (Singh *et al*., 2011) [43]. The synergistic interaction between major R-genes and APR genes is particularly noteworthy, as APR exerts incomplete resistance that imposes continuous selection pressure on pathogen populations without creating a strong selective advantage for specific virulent races (Roelfs, 1989; Singh *et al*., 2011) [39, 43]. Several scientific hypotheses explain this observed durability, including the lineage exclusion hypothesis, which suggests that by stacking genes so that avirulence mutations must occur at low frequency, breeders can exclude all pathogenic lineages (McDonald and Linde, 2002) [27]. The negative fitness cost hypothesis posits that mutations allowing a pathogen to overcome specific resistance genes often carry fitness penalties, reducing its competitive advantage or reproductive capacity (Vera Cruz *et al*., 2000) [49].

Technological Evolution: Transitioning from Phenotypic Selection to Molecular Precision

Prior to the advent of molecular markers, gene pyramiding relied entirely on conventional phenotypic selection, where breeders manually crossed parents carrying desired genes

and evaluated segregating generations through labour intensive field screening (Jena and Mackill, 2008) [19]. The conventional process typically involved designing crossing schemes, phenotypic evaluation under disease pressure, and identifying plants carrying combined genes through selfing (Ye and Smith, 2008) [52]. The fundamental limitation of conventional approaches is the inability to directly select for plants carrying multiple target genes in early generations when heterozygosity predominates (Lande and Thompson, 1990) [43]. For traits controlled by recessive alleles or those manifesting late in development, conventional screening is inefficient because the phenotype may not accurately reflect the genotype (Joshi and Nayak, 2010) [20]. Environmental variation also impacts the reliability of conventional selection, as disease severity and stress expression fluctuate across seasons (Frisch and Melchinger, 2005) [14]. The combinatorial challenge becomes intractable as the number of target genes increases; for five independent genes, the probability of finding a homozygous individual in an F_2 population is approximately 1 in 1,048,576 plants, necessitating population sizes exceeding practical feasibility (Lynch and Walsh, 1998; Joshi and Nayak, 2010) [24, 20].

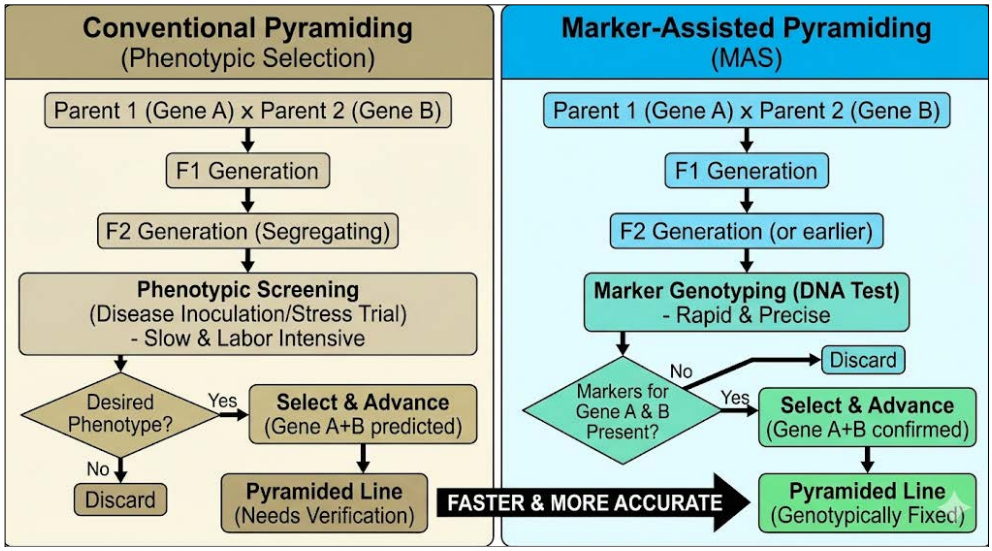

Fig 2: Comparision between traditional and Marker assisted gene pyramiding process.

Molecular Toolkits: Diverse DNA Markers and High-Throughput Genotyping Platforms; The development of molecular markers has transformed gene pyramiding into a precise and accelerated process compatible with modern breeding timelines (Collard and Mackill, 2008) [8]. Marker-assisted pyramiding (MAP) leverages DNA markers tightly linked to target genes to track inheritance without requiring phenotypic evaluation (Ashkani *et al.*, 2016) [5]. Key advantages include non-destructive selection, which enables the testing of seed tissues or seedling leaves for early identification of desired genotypes (Jena and Mackill, 2008) [19]. Simple Sequence Repeats (SSRs), or microsatellites, are highly informative, co-dominant markers that are cost effective and compatible with most laboratories (Miah *et al.*, 2013) [29]. Single Nucleotide Polymorphisms (SNPs) represent high frequency base variations that enable extremely high-density genotyping through SNP chips or sequencing (Rafalski, 2002) [36]. KASP (Kompetitive Allele-Specific PCR) assays represent a cost effective SNP genotyping approach valuable for marker assisted breeding, requiring minimal laboratory infrastructure compared to SNP arrays (Semagn *et al.*, 2014) [11]. Insertion-Deletion (indel) polymorphisms offer high information content comparable to SNPs and can be genotyped using PCR-based assays. High density platforms now allow for comprehensive genome wide background selection, accelerating the breeding cycle by identifying plants with optimized distributions of the recurrent parent genome (Karunarathna and Mason, 2021) [21].

Operational Frameworks of Marker Assisted Backcrossing and Introgression

Marker assisted backcrossing (MABC) represents the most widely implemented application of markers in gene pyramiding, encompassing three distinct, sequential steps (Ye and Smith, 2008) [52]. Foreground selection involves using markers tightly linked to target genes to identify and select plants that have inherited desired alleles at all target loci (Jena and Mackill, 2008) [19]. The efficiency is enhanced when multiple independent markers are used for each locus to avoid false positives resulting from marker gene recombination (Frisch and Melchinger, 2005) [14]. Background selection employs distributed markers to identify individual plants that possess the highest proportion of recurrent parent genome outside the target regions (Frisch *et al.*, 2000) [15]. By using large numbers of distributed markers, breeders can identify BC_1 or BC_2 plants possessing genome recovery equivalent to BC_3 or BC_4 generations, saving substantial time (Jena and Mackill, 2008) [19]. Localized background selection specifically targets markers immediately flanking target genes to break the linkage between target genes and undesired donor alleles, thereby minimising linkage drag (Young and Tanksley, 1989) [53]. This comprehensive strategy allows for the recovery of up to 97% of the recurrent parent genome in early generations (Miah *et al.*, 2016; Das and Rao, 2015) [55, 10].

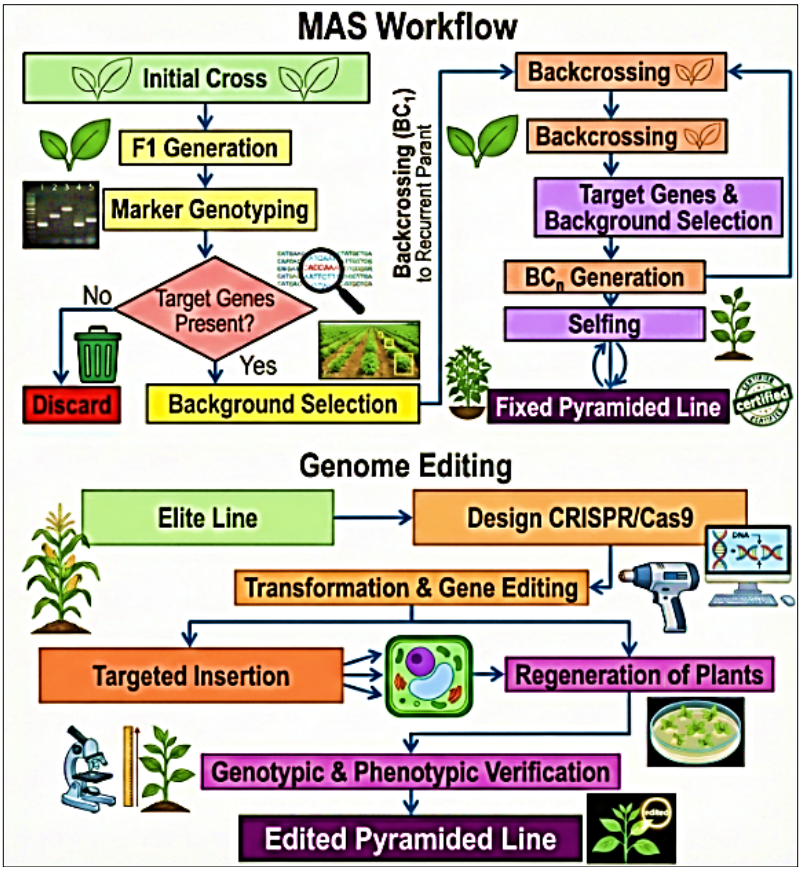

Fig 3: Marker assisted selection and Genome editing technologies in Gene pyramiding

Empirical Evidence of Durable Resistance against Devastating Biotic Pathogens

Rice production is severely constrained by multiple fungal, bacterial, and viral diseases that can cause yield losses exceeding 50% (Savary *et al.*, 2019) [40]. Bacterial leaf blight (BLB), caused by *Xanthomonas oryzae pv. oryzae*, reduces yield drastically by destroying the photosynthetic area (Pradhan *et al.*, 2015) [35]. Successful pyramiding of genes like *Xa21, Xa4, xa5,* and *xa13* has resulted in highly durable resistance to diverse bacterial isolates (Singh *et al*., 2001; Pradhan *et al.*, 2015) [44, 35]. For rice blast resistance, more than 146 *Pi* genes have been identified, and successful programs have combined three to five *Pi* genes within single cultivars (Pesaresi *et al*., 2023) [56]. A prominent example involved pyramiding *Piz, Pib, Pita,* and *Pik* into a susceptible variety, achieving 95.65% recovery of the recurrent parent genome (Pesaresi *et al.*, 2023) [56]. The Asian rice gall midge is another serious pest causing significant yield loss; pyramiding the *Gm1* and *Gm4* genes is considered ideal for conferring robust resistance against multiple biotypes (Biradar *et al.*, 2004; Das and Rao, 2015) [7, 10]. These multi gene barriers ensure that if one gene is overcome by a new pathogen race, the other genes continue to provide protection.

Enhancing Environmental Resilience through Abiotic Stress Tolerance Pyramiding

Abiotic stresses, including drought, salinity, and submergence, reduce major crop yields by more than 50% globally (Wang *et al.*, 2003; Akos *et al.*, 2019) [50, 3]. Submergence is a vital issue in flash flood prone areas; the *Sub1* gene allows rice to survive up to two weeks of complete flooding by regulating ethylene response factors (Xu *et al.*, 2006; Iftekharuddaula *et al.*, 2015) [51, 18]. Salinity affects over 150 million hectares of potential rice land; the *Saltol* QTL on chromosome 1 is the primary target for improving tolerance at the seedling stage (Thomson *et al.*, 2010) [47]. Drought stress imposes a massive threat, affecting over two million hectares in Asia and leading to increased spikelet sterility (Swamy and Kumar, 2013; Akos *et al.*, 2019) [46, 3]. Breeders target yield related QTLs such as *qDTY2.2, qDTY3.1,* and *qDTY12.1* to improve adaptability in rain fed regions (Shamsudin *et al.*, 2016) [42]. Soil related stresses like iron toxicity cause an average yield loss of 30% in poor drainage lowland soils (Becker and Asch, 2005; Akos *et al.*, 2019) [6, 3]. Pyramiding these tolerance traits into high yielding backgrounds is essential for developing stable varieties capable of withstanding the increasingly volatile environments caused by climate change (Singh *et al.*, 2014a) [57].

Case Study: Integrated Multi Stress Stacking in Elite Recurrent Parent Genotypes

A landmark study successfully demonstrated the potential of MABC by pyramiding 10 genes/QTLs into the elite rice cultivar "Improved Lalat". This variety already contained four BB resistance genes (*Xa4, xa5, xa13, Xa21*) and was further fortified with blast resistance (*Pi2, Pi9*), gall midge resistance (*Gm1, Gm4*), submergence tolerance (*Sub1*), and salinity tolerance (*Saltol*) (Das and Rao, 2015) [10]. Molecular analysis revealed clear polymorphism between the donor and recipient parents for all markers tagged to the target traits. Conventional backcrossing was followed till the BC_3F_1 generation, with MAS employed at each step to monitor the transfer of alleles. Out of the gene pyramids tested, two lines (ILGP5, ILGP19) contained all 10 resistance/tolerance genes and showed adequate levels of resistance against all five target stresses. Most of these

pyramided lines showed a high degree of similarity to the recurrent parent for morphological and grain quality traits, proving that multi stress stacking can be achieved without disturbing the elite background (Das and Rao, 2015) [10].

Quantitative Trait Loci (QTL) Integration and Complex Phenotypic Architecture

Many agronomically important traits including grain yield, quality, and abiotic stress tolerance are controlled by multiple QTLs exhibiting quantitative inheritance (Lynch and Walsh, 1998; Mao *et al.*, 2022) [24, 25]. The identification and mapping of these QTLs often utilise Chromosomal Segment Substitution Lines (CSSLs) to enable systematic detection across the genome. By evaluating CSSL collections, researchers can identify specific regions harbouring QTLs for diverse traits and quantify their allelic effects (Mao *et al.*, 2022) [25]. In rice, researchers detected thirteen QTLs across five chromosomes controlling grain length, width, and plant height (Mao *et al.*, 2022) [25]. Significantly, pyramiding three target QTLs (*qGL-3*, *qGL-6*, and *qGL-7*) resulted in plants with the longest grain length, exceeding expectations based on simple additive effects (Mao *et al.*, 2022) [25]. This demonstrates the power of QTL pyramiding for improving complex, multigenic traits that cannot be easily managed through single-gene approaches (Ashikari *et al.*, 2006) [4].

Deciphering Epistatic Networks and Non-Additive Allelic Interactions

The pyramiding of multiple loci often reveals epistatic interactions, where the combined phenotypic effect of alleles at two loci differs substantially from additive expectations (Cordell, 2002) [9]. These interactions can be synergistic (positive) or antagonistic (negative) (Lynch and Walsh, 1998) [24]. For instance, while pyramiding *qGL-6* in rice increased grain length, its interaction with *qGL-3* was negative, actually reducing grain length compared to expectations (Mao *et al.*, 2022) [25]. Conversely, the three-locus combination of *qGL-3*, *qGL-6*, and *qGL-7* revealed a complex network that ultimately resulted in a superior phenotype (Mao *et al.*, 2022) [25]. These findings underscore that the sequence of gene pyramiding which loci are combined first versus later can significantly impact the phenotypic outcome. Characterising these interaction networks allows for a more rational design of breeding strategies to ensure that stacked genes do not inhibit each other's expression (Mao *et al.*, 2022) [25].

Inherent Technical Hurdles and Logistical Constraints in Gene Stacking Programs

Despite its successes, gene pyramiding faces significant challenges, primarily linkage drag, where deleterious alleles from wild parents are introgressed alongside target genes (Jena and Mackill, 2008) [19]. Overcoming this requires strategic recombinant selection using high-density marker panels to identify rare recombination events (Frisch and Melchinger, 2005) [14]. Population size requirements also increase geometrically as the number of target genes rises; pyramiding five independent genes requires evaluating at least 2000-5000 plants to reliably identify a few desired genotypes (Lynch and Walsh, 1998; Joshi and Nayak, 2010) [24, 20]. Furthermore, pyramiding in polyploid crops like hexaploid wheat or cotton is more complex due to multiple chromosome sets, dosage uncertainty, and ambiguous allele patterns (Mason *et al.*, 2014) [26]. Polyploids also exhibit reduced recombination frequencies and pairing irregularities, necessitating larger populations and denser marker panels (Dufresne and Mason *et al.*, 2015) [58]. Negative epistasis can also limit the achievement of superior phenotypes, forcing breeders to select only subsets of available alleles (Mao *et al.*, 2022) [25].

Pioneering Frontiers: CRISPR-Cas9, Genomic Selection, and Multiplex Gene Editing

Emerging genomic technologies offer the potential to further enhance gene pyramiding efficiency (Ahmad *et al.*, 2021) [2]. CRISPR-Cas9 genome editing enables the simultaneous targeting and modification of multiple genes within a single plant individual, potentially circumventing the need for extended backcrossing (Endo *et al.*, 2020). Rice lines have been generated through multiplex editing of genes controlling grain size (*GS3*, *GW2*, *Gn1a*), photosynthesis (*rbcS*), and drought tolerance (*OsMYB30*) (Ahmad *et al.*, 2021) [2]. Genomic Selection (GS) predicts breeding values using genome wide marker data, allowing for the simultaneous consideration of numerous loci affecting complex traits (Sinha *et al.*, 2023) [45]. GS accelerates genetic gain by predicting phenotypes more accurately than single locus approaches and has been explored in cassava to maintain yield while introgressing resistance (Sinha *et al.*, 2023) [45]. The integration of these tools with traditional pyramiding offers a path toward even higher precision in trait assembly (Ndudzo and Kamara, 2024) [33].

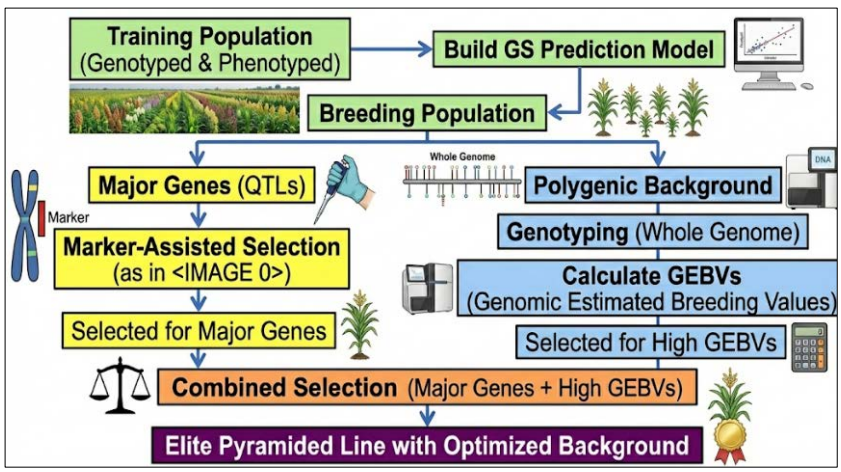

Fig 4: Genomic Selection and Gene pyramiding

Strategic Recommendations for Precision Breeding and Haplotype Based Selection

Future advancement in gene pyramiding depends on the development of low-cost SNP genotyping and portable sequencing technologies to enhance accessibility in resource limited regions (Ribaut *et al.*, 2010) [38]. Systematic characterisation of epistatic interactions and synergistic effects through transcriptomic and proteomic approaches will provide a stronger scientific foundation for pyramiding decisions (Sinha *et al.*, 2023) [45]. For polyploid species, the integration of long read sequencing to determine haplotype phases will reduce ambiguity in genotype assignment (Mason *et al.*, 2015) [59]. Furthermore, multi objective optimisation using machine learning algorithms can help identify superior allele combinations across diverse traits, identifying strategies that deliver maximal improvements across yield, quality, and resistance targets simultaneously (Sinha *et al*., 2023) [45].

Synthesis and Future Trajectory of Pyramided Crop Varieties

Gene pyramiding has evolved into a standard, indispensable tool for modern crop improvement, fundamentally enabled by DNA marker technology. It effectively compresses breeding timelines and enables the development of "multiline" varieties with broad spectrum resistance to biotic stresses and enhanced tolerance to abiotic stressors. While challenges such as linkage drag and polyploid complexity persist, the integration of advanced tools like CRISPR-Cas9 and Genomic Selection offers a promising path forward. As global food security challenges intensify due to climate change and expanding human populations, the continued refinement and deployment of gene pyramiding strategies will be essential for agricultural stability. Continued refinement of these strategies will be required to develop high yielding, resilient crops capable of withstanding the increasingly volatile environments of the twenty first century.

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