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Identification, Mapping and Pyramiding of Genes/Quantitative Trait Loci (QTLs) for Durable Resistance of Crops to Biotic Stresses

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Abstract

Biotic stresses significantly limit global crop production. Identification and use of resistant cultivars is currently seen as the best strategy, cheapest, durable and environmentally friendly method to manage biotic stresses. However, resistance gained through single gene/quantitative trait loci (QTLs) transfer leads to resistance breakdown within a short period. Hence, current breeding programs targeted at developing durable and/ broad spectrum resistant cultivars by pyramiding multiple resistant genes/QTLs. Despite its significant contributions to crop improvement, gene pyramiding through conventional breeding suffers from being laborious, time consuming, costly and less efficient. Recently, the use of modern molecular tools like molecular markers and genetic engineering has dramatically enhanced the gene pyramiding strategy for biotic stress resistance. Molecular markers are very helpful for precise identification, mapping and introgression of multiple desirable genes/QTLs underlying trait of interest. Moreover, Genetic engineering has enabled scientists to transfer novel genes from any source into plants in a single generation to develop cultivars with the desired agronomic traits. Therefore, the current paper targeted to review the different types of biotic stress resistance in plants and the methodologies for identification, mapping and pyramiding of resistance genes/QTLs to develop durable and/or broad spectrum biotic stress resistant cultivars. So far, numerous crops with durable/broad spectrum resistance to pathogens, insect pests and herbicides have been developed by pyramiding multiple resistant genes/QTLs using marker assisted selection and genetic engineering techniques to contribute to increased crop production and productivity to maintain food security globally.

Keywords: Biotic stress; Durable resistance; Linkage mapping; Gene pyramiding; Marker assisted selection; Genetic engineering

Introduction

Biotic stresses remain the greatest constraint to crop production [1] accounting for 52% of the global yield loss [2]. Bacteria, viruses, fungi, nematodes, insect pests and weeds are considered to be biotic factors that limit crop production [2-4]. For years, chemicals have been used to control biotic damage of crop plants. Nowadays, interest in the use of chemicals against biotic stress is decreasing because of its various limitations such as the requirement for more than one chemical application, an investment that is not affordable by most small-scale farmers [5]. Besides, using chemical spray may have adverse effects on human health and the environment, including beneficial organisms and may lead to the development of chemical-resistant pathogen races, insects, and weeds [4,6]. On the other hand, the use of resistant cultivars is currently seen as the best strategy, durable, economical, and environmentally friendly means of biotic stress control [7-9].

Usually, breeding efforts made to incorporate single resistant gene leads to resistance breakdown within a short period [10]. Hence, recent breeding programs have targeted at developing cultivars that can withstand multiple stresses by assembling series of genes from different parents into a single genotype in a phenomenon called gene pyramiding or stacking [1,2]. Malav et al. [11] stated that gene pyramiding is a breeding method that aimed at assembling multiple desirable genes from multiple parents into a single genotype. The technique is very helpful for developing crops that confer broad spectrum resistance against different races of pathogens or pests or combination of stresses [12]. For several years, traditional breeding has been used to identify and incorporate multiple resistant genes/QTLs into cultivars of interest to develop durable resistance to biotic stresses [7]. However, conventional method of crop improvement has been complained to be slow, less precise, less flexible, labor-intensive and expensive [13,14]. With traditional breeding, breeder's capability to track the presence or absence of the target genes is very slow and limited. This limits the number of genes to be stacked into elite cultivars at any times [11].

Hence, a technological interventions that can reduce the time and costs necessary to develop and release new cultivars with durable resistance are always welcome. Recently, biotechnological tools like molecular markers and genetic engineering are widely used in crop improvement program for rapid and efficient accumulation of desirable genes from various sources into a single background to produce broad spectrum/durable resistance [2,7,11,15]. The advent and application of molecular marker technology made it easier to identify, map and efficiently pyramid resistant genes/QTLs into crop plants [16]. DNA markers tightly linked (<5 cM) to the desired gene serve as chromosomal landmark, 'signs' or 'flags' to track the introgression of the desired gene in progenies in a cross [17]. Hence, identification of resistant genes/QTLs with closely linked DNA-markers is useful for successful transfer of the gene/QTLs into improved cultivars via marker-assisted selection (MAS) [18].

So far, various resistance genes/QTLs of crop plants have been identified and mapped using marker assisted selection. For instance, Yadav et al. [19] identified and mapped nine QTLs associated with sheath blight resistance in rice using MAS. Similarly, Perchepied et al. [20] identified and mapped two new pear resistance loci against the fungal pathogen *Venturia pirina* using MAS. Moreover, molecular markers are widely used for successful pyramiding of several resistance genes

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into crops including powdery mildew resistance genes (*Pm2+Pm4a, Pm2+Pm21, Pm4a+Pm21*) into wheat line [21], bacterial blight resistance genes (xa5, xa13, and Xa21) into rice [22], rust resistance genes (*Lr41*, *Lr42* and *Lr43*) into wheat [23], late blight resistance genes (*Rpi-mcd1* and *Rpi-ber*) into potato [24] etc. Therefore, it is important to deduce that molecular markers have remarkable applications in resistance gene/QTLs identification, mapping and pyramiding into crop plants to develop durable/ broad spectrum resistance to biotic stresses.

Moreover, the advent of genetic engineering (GE) has enabled scientists to transfer novel genes from any source to crop plants in a single generation [12]. Unlike conventional and MAS breeding methods which allow the transfer of desired genes between related species [25], Genetic engineering allows the specific transfer of gene of interest from any source (from animals, viruses, bacteria, or even from totally man-made sequences) into crop plants [26]. It has been reported that single gene transformation results in insufficient or narrow spectrum disease resistance [27], and hence a genetic transformation of crop plants with a combination of resistance genes would be more logical [1]. So far, a number of transgenic crops with durable resistance to bacterial diseases [28,29], viral diseases [30-32], fungal diseases [33], insect pests [34,35] and herbicides [36,37] have been developed. Thus, it is possible to deduce that genetic engineering is also another useful tool to pyramid novel resistance genes into crop plants to develop durable resistance to biotic stresses [2]. Therefore, the present review paper is aimed at reviewing the methodologies involved in identification, mapping and pyramiding of resistance genes/QTLs into to crop plants to develop durable and/or broad spectrum biotic stresses resistant cultivars.

Biotic stress resistance in crop plants

Being sessile organisms, plants are often exploited as a source of food and shelter by a wide range of parasites including viruses, bacteria, fungi, nematodes, insects, and even other plants [38]. Hence, biotic stress resistance in plants refers to the collective heritable characteristics of plant species to reduce the possibility of successful utilization of that plant as a host by these parasites [2]. As successful establishment of these biotic factors can cause severe damage on crop production, identification of the resistance genes and their utilization in breeding program makes the crop production system sustainable, economical, and environmentally friendly strategy [9]. Resistance in plants can be classified into two major categories and various terms have been used to describe the two categories of resistance, such as vertical versus horizontal resistance [39], qualitative versus quantitative resistance [40], and complete versus partial resistance [41].

Vertical resistance

Vertical resistance also called major-gene or single-gene resistance is a type of resistance where the plant possesses one or a few specific, well-defined genes that confer a high level of resistance to a specific pathogen. In this type of resistance, a particular gene gives the plant resistance to only one race of a pathogen and if other race comes, the plant needs different major genes for resistance to each race. It is sometimes called qualitative resistance because plants are either resistant or susceptible, without intermediate levels.

The simple model for how the host- pathogen recognition operates is that there is a dominant resistance (R) gene in the plant encoding a product that recognizes a pathogenicity factor (produced by a dominant *Avirulent or Avr* gene) in the pathogen to confer resistance [38,42,43]. There is mutual signaling between hosts and pathogens. Briefly, up on landing to the plant surface, the pathogen avirulence gene leads to

	Resistance or susceptibility genes in the plant	
Virulence or avirulence genes in the pathogen	**R (resistant) dominant**	**r (susceptible) recessive**
A (avirulent) dominant	AR (-)	Ar (+)
A (virulent) recessive	aR (+)	ar (+)

[a]Minus signs indicate incompatible (resistant) reactions and therefore no infection. Plus, signs indicate compatible (susceptible) reactions and therefore infection develops.

Table 1: Summary of host–pathogen reaction types based on the gene-for-gene concept.

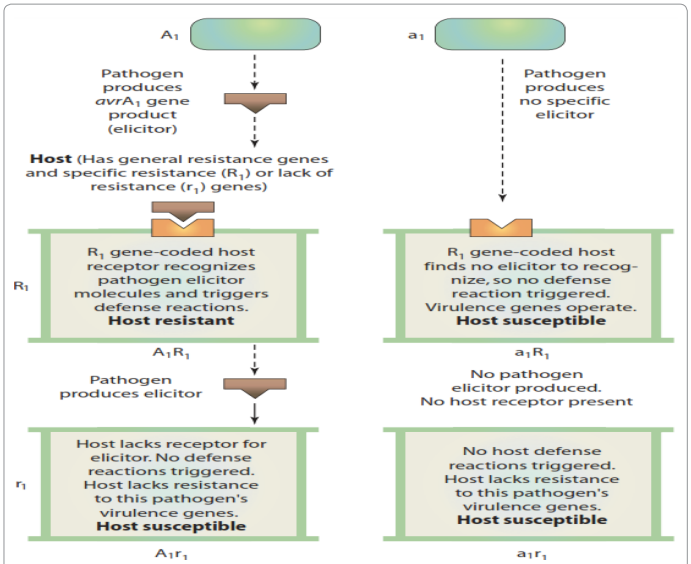

Figure 1: Basic host-pathogen interaction: Based on gene-for gene concept. Basic interaction of pathogen avirulence (A)/virulence (a) gene with host resistance (R)/ susceptibility (r) genes in a gene for gene relationship, and the final outcomes of the interactions. Source: Agrios (2005).

the production of some "signal" molecule called elicitors (pathogen-associated molecular patterns (PAMPs)). The elicitors bind specifically to the plant Pattern Recognition Receptors (PRRs). This activates the PRRs and triggers a signal-transduction pathway leading to expression of the plant R gene to be expressed [44]. This ultimately results in recognition of the pathogen by the plant to be destroyed. Such type of resistance that depends on a precise match-up between a genetic allele in the plant and an allele in the pathogen is called gene- for- gene resistance [45]. On the other hand, absence of R gene in the plant and/ or absence of the avirulence gene in the pathogen make the pathogen to be unrecognized by the plant. This results in the pathogen virulent gene to operate and makes the plant susceptible (diseased) [45]. Table 1 and Figure 1 summarize the gene-for gene concept when two cultivars, one with resistant gene (R) and the other with susceptible gene (r) are inoculated with two pathogen races; one carrying an avirulence (A) gene and the other with virulent (a) gene against the resistance gene R. According to the gene-for-gene concept, when the plant is resistant, the pathogen is called avirulent and the interaction is incomplete. While when the plant is susceptible, the pathogen is virulent and the interaction is complete [38].

As the effect of major gene is easy to recognize and select, most of the resistance exploited by plant breeders is of the major gene type [43]. However, major gene resistances are easier for a pathogen or an insect pest to break down in short period [10,46-51]. Because of this, recent plant breeding programs targeted at the identification and pyramiding of several major genes against a number of pathogenic races [11,15].

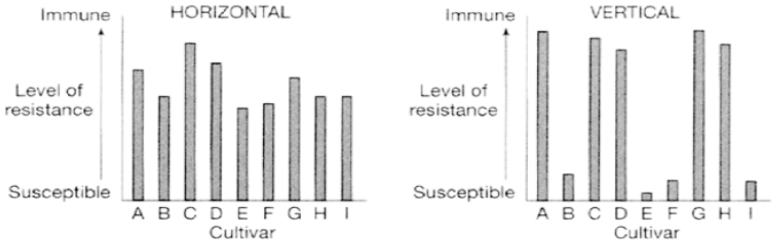

Figure 2: Horizontal versus vertical (gene-for-gene) resistance. In horizontal resistance, numerous genes have small additive effects so that the resistance varies by small amounts between cultivars. In vertical resistance, controlled by single genes, resistance is either close to complete immunity if the gene is present, or complete susceptibility if it is absent. Source: Dickinson (2005).

Horizontal resistance

Horizontal or quantitative resistance is defined as a race non- pacific or general resistance to a range of pathogens or pests [41] as a result of many genes expression with minor additive effects. As it is controlled by the collective effects of numerous genes known as quantitative trait loci (QTLs), horizontal resistance is important to control a broad range of pathogen races. Hence, horizontal resistance is durable and never breaks down. In a crop containing both major- and minor genes derived resistance, the minor gene resistance becomes visible after the "breaks down" of major gene resistance [43,52]. However, unlike vertical resistance that can protect the crop completely from the parasite, horizontal resistance does not protect plants from becoming infected. Rather it reduces the rate of disease development and spread. There is little difference in the level of horizontal resistance among crops (Figure 2).

Most reports indicate that horizontal resistance is polygenically inherited: does not obey the simple Mendelian inheritance. Mundt [53] reported that the resistance to the leaf rust pathogen *P. hordei* in barely is inherited polygenically and controlled by five or six minor genes. As a rule, a combination of major (R) genes and minor genes or QTLs for resistance against a pathogen is the most desirable makeup for any plant variety. Therefore, to successfully transfer desired resistance genes through modern breeding techniques, their precise location in the genome shall be known through genome mapping. The following section of this review presents the methodology involved in mapping genes/QTLs controlling important agronomic traits. Nowadays, molecular markers are becoming very helpful tools for precise detection and mapping of genes/QTLs controlling trait of interest.

Identification and Linkage Mapping of Resistance Genes/QTLs in Crop Plants

Gene mapping describes the methods used to identify the locus of a gene and the distances between genes. There are two distinctive types of "maps" used in the field of genome mapping: genetic maps and physical maps. They differ in techniques used to construct them and in the degree of resolution. Genetic map distances are constructed based on the genetic linkage information while physical maps use actual physical distances (has high resolution) usually measured in number of base pairs [54]. QTL map is a type of genetic map, which indicates the approximate location of a quantitative trait locus (QTL) within an interval delineated by two or more markers on a genetic map.

Genetic mapping/linkage mapping of genes/QTLs

Genetic mapping can be defined as the process of determining the linear order of molecular markers or genes (generally, loci) along a stretch of DNA or chromosome [55]. Linkage map indicate the relative position of markers on chromosome or linkage groups (LGs) based on the frequencies of recombination that occur between markers on homologous chromosomes during meiosis. Recombination frequency between two markers is proportional to the distance separating the markers. The greater the frequency of recombination, the greater the distance between two genetic markers; conversely, the smaller the recombination frequency, the closer the markers are to one another. The distance between markers on a genetic map is given as Morgan (M) or centimorgan (cM), where one cM is the distance that separates two markers (or genes), between which a 1% chance of recombination exists (corresponding to one recombination event in 100 meioses). That means 99% of the times these two markers (genes) co-segregate, and hence MAS can be applied to select progenies with desired traits during crossing. The following steps are prerequisites for a successful linkage or genetic mapping of a target genome [55].

Selection of parent plants: The first step in linkage mapping is the selection of genetically divergent parents that exhibit sufficient polymorphisms for the trait of interest, but are not so distant as to cause sterility of the progeny [55]. Accordingly, in determining the chromosomal position of resistant genes/QTLs toward a particular pathogen, parental lines with sufficient polymorphism (pure resistant and pure susceptible parental liens) should be selected phenotypically in the field and/or using marker system [55].

Developing mapping population: Following the selection of polymorphic parental lines, the next key step is developing a mapping population [55]. Several types of mapping populations may be suitable for a particular project [56] including:

1. Double haploid lines (DHLs): Regenerated plants from pollen (which is haploid) of the F_1 plants and treated to restore diploid condition in which every locus is homozygous.

2. Backcross (BC) population: The F_1 plants are backcrossed to one of the parents.

3. F_2 population: F_1 plants are selfed.

4. Recombinant inbred lines (RILs): Inbred generation derived by selfing individual F_2 plants and further single seed descent. A population of RILs represents an 'immortal' or permanent mapping population.

Each of the above mapping populations has both advantages and disadvantages, and the choice of the type of mapping population depends on many factors such as the plant species, type of marker system used, and the trait to be mapped [56]. Accordingly, F_2 populations and BC populations are simple and can be developed in short period for self-pollinating species. While RIL population takes six to eight generations. Although development of a DH population takes much less time than RIL; it is only possible in species that are amenable to tissue culture. RIL and DH populations are good in that they produce homozygous or 'true-breeding' lines that can be multiplied and reproduced without genetic change occurring. This allows undertaking

replicated trials across different locations and years. With regard to the marker choice, co-dominant markers are best informative in F_2 population, while information obtained by dominant marker systems can be maximized by using RILs or DHLs. Double haploids, F_2 families, or RILs are advantageous if the trait to be mapped cannot be accurately measured on a single-plant basis but must be assessed in replicated field experiments [56].

Determining mapping population size: In linkage mapping, the resolution of a map and the ability to determine marker order largely depend on population size [55]. A vague lower threshold that can localize quantitative trait loci (QTL) is a size of 100 individuals. However, high-resolution maps for map-based cloning of target genes ideally require population sizes of more than 500 or even 1000 individuals. Yadav et al. [19] used 210 F_2 and 150 BC_1F_2 mapping population to map QTLs governing the sheath blight resistance in rice. Similarly, Klarquist et al. [8] used 151 $F_{2:5}$ RIL populations to identify and map QTLs involved in stripe rust resistance in wheat. Moreover, Perchepied et al. [20] mapped two new pear resistance loci using three F_1 segregating populations (182, 144 and 81). Hence, it is important to decide the appropriate mapping population size required in locating chromosomal position of trait of interest, and generally the larger (>100) the mapping population, the better the map resolution would be [55].

Phenotype evaluation: Once a population segregating traits of interest is obtained, mapping the trait typically involves measuring the phenotype. Phenotypic evaluation can be undertaken in the field under natural condition (where high disease pressure can be expected) or in greenhouse/growth room in which the plants are inoculated with specific pathogen strains. Compared to the field evaluation, a greenhouse seedling inoculation can assess disease reactions quickly, reduce some sources of environmental variation by use of characterized pathogen strains and defined inoculum concentrations, and avoid confounding effects from other pests or diseases [9].

Genotype profiling: Generation of genotypic data for the mapping population involve two steps. First, DNA samples from the parental lines are screened for polymorphisms, using markers that span the chromosome(s) of interest. To scan the whole genome, polymorphic markers spaced approximately every 25 cM to 30 cM are needed. The second step is genotyping the mapping population with the selected polymorphic markers [55]. It is important to include many markers as much as possible [56].

Construction of linkage maps: The marker data collected through genotyping of the mapping population are used to construct the linkage map. Linkage analysis is based on the fact that two marker loci that are close to each other on the same chromosome tend to co-segregate; i.e., will be inherited together [55]. The frequency of recombinant (non-parental) genotypes is used to calculate recombination frequency, which is then used to infer the genetic distance between markers. By analyzing the segregation of markers, the relative order and distances between markers can be determined. The lower the frequency of recombination between two markers, the closer they are situated on a chromosome; conversely, the higher the frequency of recombination between two markers, the further away they are situated on a chromosome [56].

Simple statistical tests such as a χ^2 analysis will test the independent assortment of two loci and hence linkage. For two loci, a recombination frequency <50% indicates linkage. Usually, Kosambi's mapping function is used to derive genetic distances (cM) between linked loci from their recombination frequency. Linkage between two loci is usually calculated with an odds ratio (i.e., the ratio of linkage versus no linkage). This ratio is more conveniently expressed as the logarithm of the ratio and is called a logarithm of odds (LOD) value or LOD score. A LOD score of 3 is normally accepted as a lower significance threshold to assert linkage [55], and the QTLs of interest are thought to exist at positions where an LOD score exceeded the corresponding significant threshold. Linked markers are grouped together into linkage groups (LG). In QTL analysis, the proportion of phenotypic variation explained by each QTL is calculated as R^2 value, and the degree of dominance of a QTL is estimated as the ratio of dominance effect to additive effect. A number of mapping computer programs are available for mapping traits controlled by single genes as well as quantitative traits like MAPMAKER/EXP [57] and JoinMap v.4.0 [58].

Applications

Identification and mapping of resistant genes/QTLs in two selected crops

A) Identification of QTLs and possible candidate genes conferring sheath blight resistance in rice (*Oryza sativa* L.)

Sheath blight is one of the most devastating diseases of rice caused by the fungus *Rhizoctonia solani Kühn*. Wang et al. [16] stated that pyramiding of diverse Sheath blight resistant (ShBR) QTLs could help to achieve higher levels of resistance to ShB. In line with this, Yadav et al. [19] aimed at identifying and mapping QTLs and candidate genes associated with sheath blight resistance in rice. As a procedure, two mapping populations namely 210 F_2 (derived from the cross between the susceptible BPT-5204 and moderately resistant ARC10531) and 151 BC_1F_2 populations (derived from the same cross) were developed. After greenhouse phenotypic evaluation in the presence of the pathogen *R. solani,* the F_2 population was genotyped using 70 polymorphic SSR markers. A linkage map was constructed using MAPMAKER 3.0 and significance threshold of >3 was considered for linkage grouping. Finally, 9 ShBR QTLs have been identified and mapped to five chromosomes (1, 6, 7, 8 and 9) with phenotypic variance ranging from 8.40% to 21.76% (Table 2). They identified new markers linked to the ShB resistances QTLs on chromosome 1, 6 and 8 (Figure 3). The study also identified two major ShBR-QTLs: *qshb7.3* (explained 21.76% of the total phenotypic variance) and *qshb9.2* (explained 19.81% of the phenotypic variance) that can be transferred using MAS into elite cultivars.

Validation of linked microsatellite markers associated with sheath blight resistance in rice

Another crucial step in linkage mapping is validation of the co-segregation of the identified marker and the trait. Usually, Bulk Segregant Analysis (BSA) has been employed to identify the DNA markers linked to the sheath blight resistance gene. Accordingly, in their validation analysis Yadav et al. [19] pooled the DNA from 10 extremes resistant and 10 extreme susceptible plants of the BC_1F_2 separately. And then, amplified along with both parents using the same SSR markers: RM336 and RM205 (Figures 4A and 4B). Finally, it was found that the resistance alleles show co-segregation among the parents ARC10531and BPT-5204 i.e., presence of the markers confirm presence of the resistant genes (Figure 4). Moreover, an *in-silico* analysis using rice data base RAP-DB for search of defense responsive gene identified 32 genes within QTL region near to the marker RM205 on chromosome 9. Functional annotation of predicted genes by blastp revealed one defense responsive gene ß 1-3 glucanase like protein present in a single copy within the cluster and it may be responsible for sheath blight resistance in the rice line ARC-10531. This shows that the identified markers are very efficient and helpful to select progenies carrying the desired genes/QTLs in crop breeding program. Hence, genetic mapping is helpful to

S.no.	QTLs	Chr.	Marker	Marker interval	LOD	%R²
1	qshb1.1	1	RM151	RM151-RM12253	10.7	10.99
2	qshb6.1	6	RM400	RM400-RM253	4.43	13.25
3	qshb7.1	7	RM81	RM81-RM6152	8.8	10.52
4	qshb7.2	7	RM10	RM10-RM21693	6.7	9.72
5	qshb7.3	7	RM336	RM336-RM427	4.12	21.76
6	qshb8.1	8	RM21792	RM21792-RM310	4.2	10.52
7	qshb9.1	9	RM257	RM257-RM242	5.9	8.4
8	qshb9.2	9	RM205	RM205-RM105	7	19.81
9	qshb9.3	9	RM24260	RM24260-RM3744	3.5	12.58

Source: Yadav et al. [20].

Table 2: QTLs identified for Sheath Blight resistance by Composite Interval Mapping (CIM).

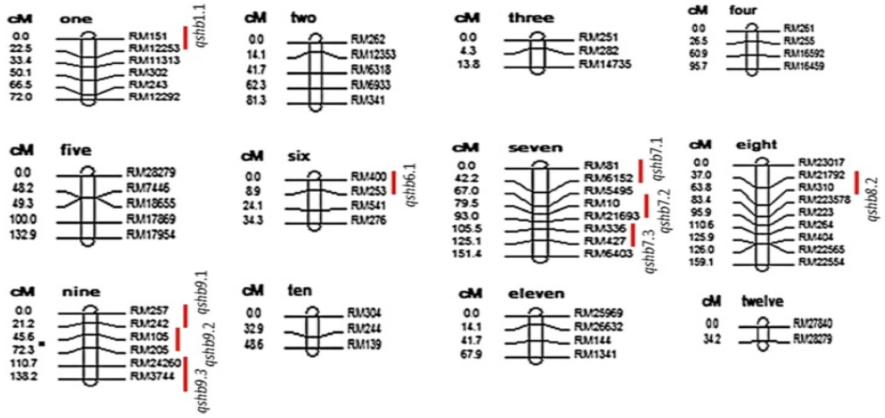

Figure 3: Molecular genetics map of rice along with positions of QTLs for sheath blight resistance. Source: Yadav et al. [20].

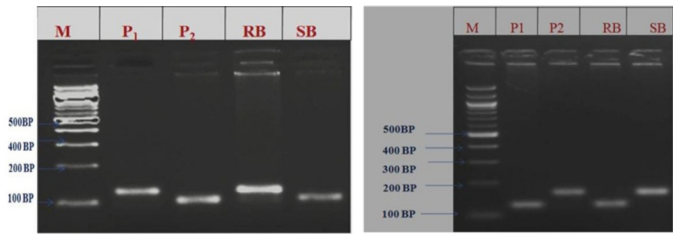

Figure 4: Bulk segregate analysis to validate SSR markers linkage to sheath blight resistance genes in rice.

(A)RM 205; M-100 bp ladder; P1 ARC10531; P2 -BPT-5204; RB-Resistance bulk; SB–susceptible bulk. (B) SSR marker RM 336; M-100 bp ladder; P1 ARC10531; P2 -BPT-5204; RB-Resistance bulk; SB–susceptible bulk.

identify and map markers linked to desired agronomic traits to be used in genome-assisted crop improvement.

B) Identification and mapping of new pear resistance loci against the fungal pathogen *Venturia pirina*

Scab is one of the major fungal diseases infecting pear trees. Perchepied et al. [20] targeted to identify and map new pear resistance loci against the fungal pathogen *Venturia*. As a procedure, they developed three F_1 segregating populations derived from the cross of: (1) Ange´lys (scab susceptible) × P3480 (scab-resistant), (2) Euras (resistant) × P2896 (susceptible) and (3) Euras × P3480. After phenotypic evaluation through artificial inoculation, the mapping populations including the parental lines were genotyped using 153 SSR markers. Linkage map was constricted using JoinMap v.4.0 software [58] at LOD significance threshold of 3. Kosambi's function was used to calculate genetic distances (cM). The position of marker-trait association was identified using CIM by the software MapQTL 5.0 at a significance threshold of LOD>3. A QTL with the largest LOD value is the major QTL controlling the trait. A position on the LG where the LOD plot reaches its peak is the position of the major QTL (Figures 5A and 5B). The proportion of phenotypic variation explained (PVE) by each significant QTL indicated by the R^2. Hence, the study identified two new major QTLs namely, qrvp-LG01 on LG01 with a LOD score of 36.5 at the QTL peak located close to the SAmsCO865608 marker, and a second significant QTL qrvp-LG04 on LG04 with a LOD score of 19.2 (Table 3).

The percentage of phenotypic variation explained by qrvp-LG01 is 67.0 (Table 3). It was reported that the qrvp-LG04 QTL is located between the TsuGNH244 and TsuGNH076 markers and it is responsible for 52.8% of the phenotypic variation (Table 3).

Pyramiding of genes /QTLs for biotic stress resistance

Concept of gene pyramiding: Gene pyramiding is defined as a method of transferring multiple desirable genes/QTLs from multiple parents into a single genotype [2]. It is a breeding technique amid at assembling several genes with known effect on target trait [59]. The

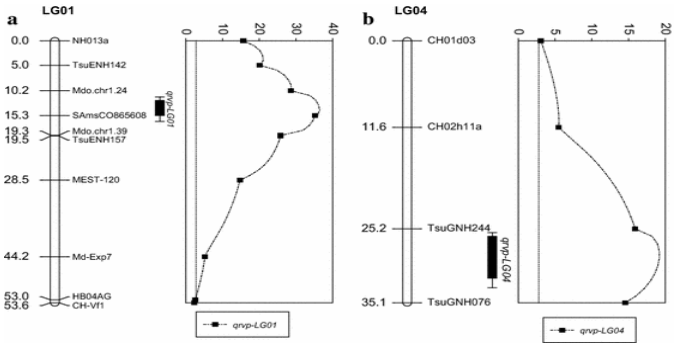

Figure 5: Genetic maps of the linkage groups 01 and 04, and LOD plot for the quantitative trait locus (QTL) detected for scab resistance (sporulation severity). The one- and two-LOD support intervals of the QTL are shown. a) Linkage group 01 and QTL, named *qrvp-LG01*, detected for the pear scab-resistant hybrid P3480 in the F1 segregating population deriving from the cross 'Angélys' × P3480. b) Linkage group 04 and QTL, named *qrvp-LG04*, detected for the resistant cultivar 'Euras' in the F1 segregating population deriving from the cross 'Euras' × P2.

The percentage of phenotypic variation explained by qrvp-LG01 is 67.0 (Table 3). It was reported that the qrvp-LG04 QTL is located between the TsuGNH244 and TsuGNH076 markers and it is responsible for 52.8% of the phenotypic variation (Table 3).

Progeny	Parental map	LG[a]	Position (cM)[b]	Marker closest to the QTL peak	LOD	R^2
A × P3480	P3480	1	13.33	SAmsCO865608	36.5	67.0
E × P2896	Euras	4	28.18	TsuGNH244	19.2	52.8
E × P3480	P3480	1	10.95	SAmsCO865608	4.3	22.6
	Euras	4	14	TsuGNH244	7.2	35.5

[a]Linkage group
[b]Position of the QTL peak on the LG

Table 3: Parameters associated with the quantitative trait loci (QTL) detected on the linkage groups 1 and 4 of the F1 segregating populations deriving from the crosses 'Angélys' × P3480 (A × P3480), 'Euras' × P2896 (E × P2896), and 'Euras' × P3480 (E × P3480).

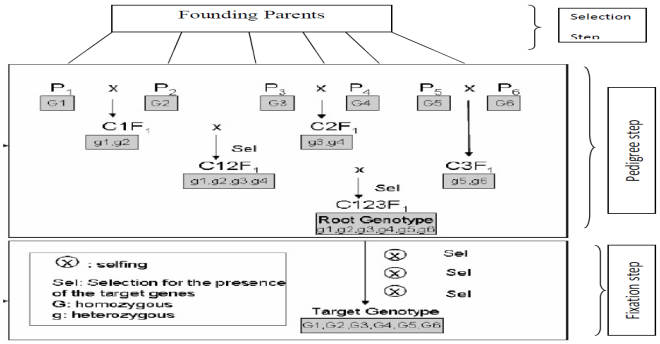

Figure 6: Diagrammatic representation of a gene-pyramiding scheme cumulating six target genes from six parental lines. Source: Suresh and Malathi [2].

technique is helpful in conferring broad spectrum resistance against different races of pathogens or pests or combination of stresses. Similarly, Ye and Smith [59] stated that genes are pyramided for one or combination of the following objectives: 1) enhancing trait performance by combining two or more complementary genes, 2) remedying deficits by introgression of genes from other sources, 3) increasing the durability of disease resistance, and 4) broadening the genetic basis of released cultivars.

Rationale behind gene/QTL pyramiding: The rationale behind gene pyramiding originates from the age-old philosophy of the use of insecticide mixtures to broaden the spectrum of insects controlled in one spray event [60]. In similar fashion, if two or more genes are stacked into a single variety, it is less probable for the plant to lose both resistant genes at the same time or a pathogen race with resistance to two genes to evolve. Nowadays, gene pyramiding is becoming an important breeding approach for developing durable or broad-spectrum resistance in crop plants against biotic stresses. It is a cost effective and environmentally friendly strategy to manage crop production loss due to biotic factors. Recently, molecular techniques like molecular markers and genetic engineering are widely used for rapid and efficient accumulation of novel resistant genes from various sources into a single background to produce broad spectrum/ durable resistance in crop plants [1,8,10].

Designing a gene pyramiding strategy: The ultimate objective of a gene pyramiding program is to generate an ideal genotype having all desirable genes brought from various sources [12]. Successful gene pyramiding involves three steps [12,61] (Figure 6). The first step is identification/selection of parents containing the desirable genes (founding parents). This will be followed by a second step also called the pedigree step, which involves assembling single copy of (heterozygous) of the targeted genes (g1, g2, g3, g4, g5 and g6 in the example) through successive crossings to produce root genotype. The final step is called the fixation step which aims at fixing the target genes into a homozygous state to avoid their segregation in successive generations. Frequently, double haploid (DH) production and recombinant inbred line (RIL) techniques are used for homozygous line production. In this regard, the DH production technique that involves *in vitro* culturing of

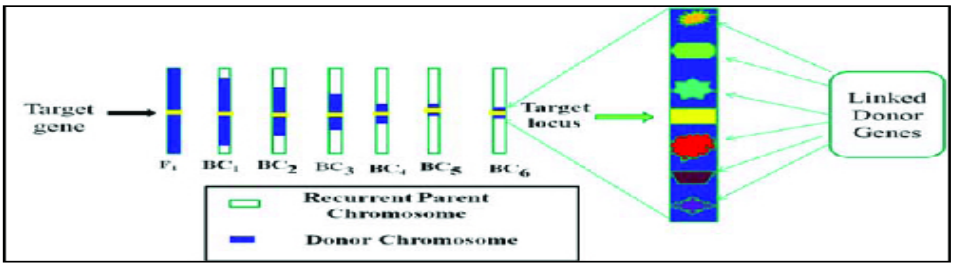

Figure 7: Schematic representation of transferring undesirable genes with target gene.

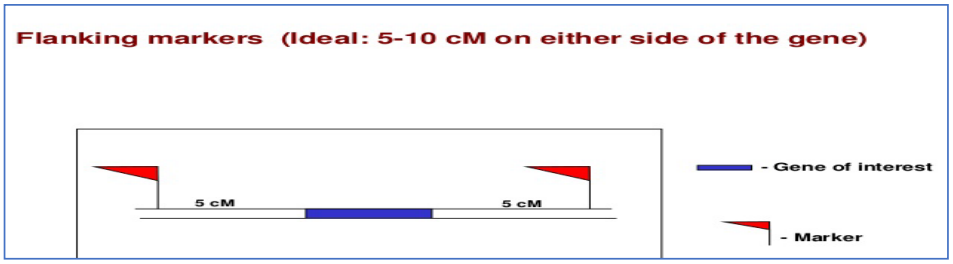

Figure 8: DNA markers tightly linked to the target gene.

gametes (anther, microspores or ovules) of the root genotype produces a population of fully homozygous individuals in single generation, among which the target genotype/ideotype can be found [62,63]. Application of co-dominant markers makes the ideotype selection process fast, efficient and cost effective [64]. However, producing large population of doubled haploid is difficult and cumbersome in certain plant species.

Alternatively, RIL development method could be used to fix the pyramided genes in root genotype. This involves selfing of the root genotype followed by intensive selection of progenies carrying the desired genes (Figure 6). It may take several generation selfing to develop fully homozygous lines of the pyramid genes [12]. Traditionally, breeders differentiate progenies carrying the desired genes based on phonotype which makes the screening process difficult. However, application of marker technology simplifies the pyramiding process by assisting the identification and maintenance of plants that carries the desired allele combination and discarding those that don't have [64].

Gene Pyramiding Methods

Gene pyramiding through traditional backcrossing

Recurrent backcrossing is a breeding method used to incorporate one or a few desirable traits into an elite variety containing large number of desirable traits but deficient in only a few traits [65,66]. Thus, the target of backcrossing is to transfer one or more genes of interest from donor parent into the genetic background of the improved variety and recover the recurrent parent genome (RPG). During backcrossing, together with the target gene, some unwanted genomic regions (gene drag) of the donor parent can transfer into the backcross progenies. Removing the linkage drag and recovering the recurrent parent genome requires six to eight backcrossing [67]. At each backcross generation, the proportion of recurrent parent genome recovered could be estimated using the formula $1-(1/2)^{n+1}$, where n is the number of backcross generations. At backcross six (BC6) up to 99.2% of the RPG would be recovered. Then, the resulting F_7 populations will be selected and selfed to generate three genotypes: homozygous resistant, heterozygous resistant and the susceptible ones [7]. After one generation field screening, progenies homozygous for the resistant gene will be identified and maintained as improved line for resistance [64]. Surprisingly, the linkage drags (Figure 7) may remain even after six generations of backcrossing [1]. That is why conventional method of gene pyramiding for crop improvement is complained to be slow, tedious and inefficient. Hence a technology that can circumvent these limitations of conventional backcrossing and promotes the crop improvement program is always welcome [2].

Gene pyramiding using marker assisted selection

Molecular markers are identifiable DNA sequences found at specific location of the genome and inherited by the standard laws of inheritance [2,14]. DNA markers tightly linked (<5 cM) to the desired gene (Figure 8) serve as chromosomal landmark, 'signs' or 'flags' [17] to track the introgression of the desired gene in progenies in a cross i.e., identification of the marker indicates presence of the desired gene. The use of molecular marker technology in breeding to select progenies with the desired genes is called marker assisted selection (MAS), marker-assisted breeding or 'smart breeding' [20].

Application of MAS in plant breeding program has multiple advantages over the conventional phenotypic selection [64]. First, DNA based selection allows breeders to identify and select desirable plants at seedling stage, savings resources like greenhouse and/or field space, water, and fertilizer. Secondly, when assembling multiple genes for resistance to the same disease, using phenotypic selection alone, it can be difficult to distinguish those plants that carry all desired alleles from those that only have some of them. However, molecular markers are very powerful to precisely identify genotypes carrying the stacked desired genes. Thirdly, unlike phenotypic selection, genotypic selection is not affected by environmental factors. In addition to this, molecular markers are very important in backcross breeding to pyramid two or more genes associated with biotic stress resistance [1]. And marker assisted backcrossing (MABC) involves three levels of selection (Figure 9) [7]. The firs level of selection is called foreground selection where progenies carrying the desired gene would be selected using markers linked to the target gene. The second level of selection is called

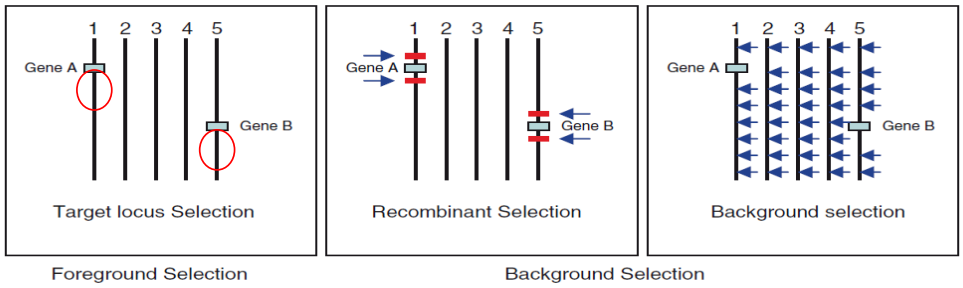

Figure 9: Schematic diagram showing whole genome selection process: Foreground selection, recombinant selection and background selection respectively. Source: Jain and Brar [1].

Features	Some DNA Markers				
	RFLPs	RAPDs	AFLPs	SSRs	SNPs
DNA quality	High	High	Moderate	Moderate	High
PCR-based	No	Yes	Yes	Yes	Yes
Ease of use	Not easy	Easy	Easy	Easy	Easy
Amenable to automation	Low	Moderate	Moderate	High	High
Reproducibility	High	Unreliable	High	High	High
Development cost	Low	Low	Moderate	High	High
Cost per analysis	High	Low	Moderate	Low	Low

Table 4: Comparison of most commonly used marker systems. Source: Korzun [59].

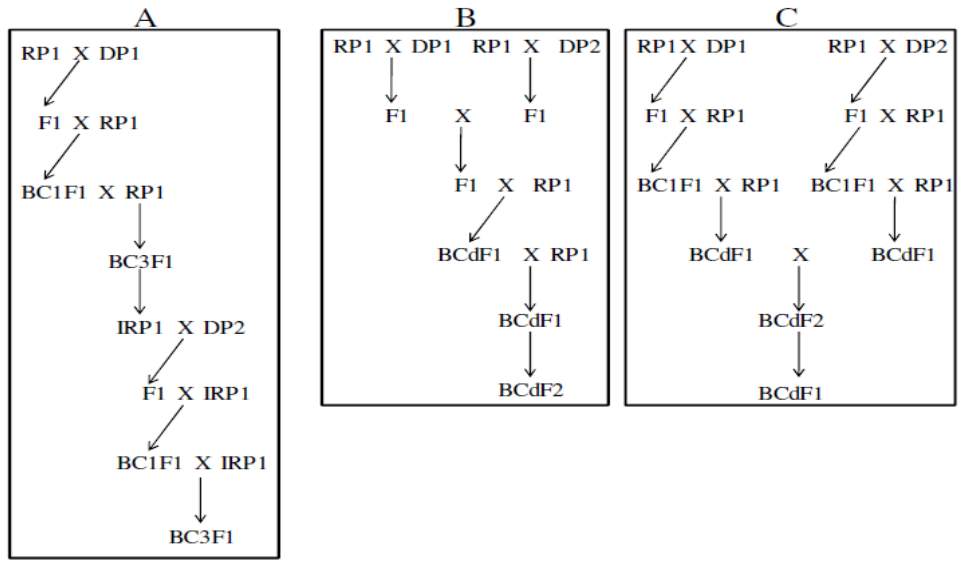

Figure 10: Different schemes of backcrossing for gene pyramiding. RP- Recurrent parent; DP- Donor parent; BC- Backcross; IRP- Improved recurrent parent. A. Stepwise transfer; B. Simultaneous transfer; C. Simultaneous and stepwise transfer. Source: Joshi and Nayak [1].

recombination selection where homozygous alleles of the recurrent parent will be selected using tightly liked markers flanking the target alleles. This step is important to reduce linkage drag [67]. The third level of selection is called the background selection which involves the selection of individuals carrying homozygous alleles of the recurrent parent at a number of unlinked marker loci covering the entire genome (chromosome 2, 3 and 4 in Figure 9 in background selection) [59]. Background selection accelerates the recovery of the recurrent parent genome and hence the use of MAS in backcross breeding reduce the time required to recover the recurrent genome by two to four backcrosses.

Commonly used marker systems in crops: So far, several marker systems have been developed and are applied to a range of crop species. These include Restriction Fragment Length Polymorphisms (RFLPs), Random Amplification of Polymorphic DNAs (RAPDs), Amplified Fragment Length Polymorphisms (AFLPs), Simple Sequence Repeats (SSRs) or microsatellites, inter- SSRs (ISSRs), and most recently single nucleotide polymorphism (SNP) [14,68]. It is beyond the scope of this review to discuss the technical methods of how each of these DNA markers is functioning. However, each marker system has its own pros and cons, and the various factors to be considered in selecting one or more of these marker systems have been described in Table 4.

MAS gene pyramiding strategies: Generally, there are three possible MAS gene pyramiding strategies: Stepwise transfer, simultaneous transfer, and simultaneous and stepwise transfer (Figure 10) [2,12]. In stepwise transfer method, the recurrent parent (RP_1) is

crossed with donor parent (DP_1) to produce the F_1 hybrid which then backcrossed up to third backcross generation (BC_3) to produce the improved recurrent parent (IRP_1). This improved recurrent parent is then crossed with other second donor parent (DP_2) to pyramid multiple genes (Figure 10A). Although this pyramiding strategy is very precise, the method is less accepted as it is time taking. In simultaneous gene pyramiding strategy, the recurrent parent (RP_1) is crossed with series of donor parents (DP1, DP2, etc.) to get the F1 hybrids which are then intercrossed to produce improved F1 (IF1). This improved F1 is then backcrossed with the recurrent parent to get the improved recurrent parent (IRP) (Figure 10B). The third strategy is a combination of the first two strategies which involve simultaneous crossing of recurrent parent (RP1) with many donor parents and then backcrossing them up to the BC_3 generation. The backcross populations with the individual gene are then intercrossed with each other to get the pyramided lines (Figure 10C) [12]. This is the most acceptable way as in this method not only time is reduced but fixation of genes is fully assured.

Efficiency of MAS gene pyramiding: Compared to conventional method, marker assisted backcrossing have been found to be rapid and efficient method of transferring multiple desirable genes into an elite cultivar. In line with this, Tanksley et al. [69] stated that using traditional backcross breeding, it takes six backcross generation to recover 99.2% of the recurrent parent genome and the same proportion of recurrent genome reconstruction can be achieved in less than two to four backcrossing generation using marker assisted backcross breeding [66]. Similarly, Jain and Brar [1] stated that MAS enable to monitor the introgression of many traits (up to six traits) at a time which often needs to conduct separate trials to screen for individual traits with conventional method. Moreover, MAS make the gene pyramiding process cost effective by allowing breeders to identify and select desirable plants very early (at seedling stage), saving resources like greenhouse and/or field space, water, and fertilizer. Furthermore, when multiple genes for resistance to the same disease are assembled, molecular markers are so efficient and powerful to discriminate those plants carrying all desired alleles from those that only have some of them. Therefore, it is possible to deduce that MAS gene pyramiding is a rapid, efficient, cost effective and a straight forward strategy in plant breeding for pyramiding genes/QTLs to crop plants to increase their durable resistance to biotic stresses.

Major achievements of marker aided gene pyramiding: Marker-assisted gene pyramiding has been used extensively for pyramiding major or qualitative disease resistance genes in plants [70]. So far, a number of promising achievements have been reported in developing durable disease resistance in crop plants through marker assisted gene pyramiding. Table 5 summarize some of the achievements made in controlling biotic stresses by pyramiding multiple resistant genes into crop plants through marker assisted breeding. Therefore, it is possible to deduce that molecular marker technology is a very helpful tool in the identification and introgression of multiple desirable genes into cultivars of interest to develop durable and broad spectrum biotic stress resistant cultivars to boost crop reduction and productivity.

Crop	Trait	Pyramided genes	References
Rice	Bacterial blight resistance	*xa5, xa13, Xa21*	Pradhan et al. [13]
	Bacterial blight resistance	*Xa4, xa5, xa13* and *Xa21*	Shanti et al. [98]
	Blast resistance	Pi1, Pi2 and Pi33	Usatov et al. [99]
Wheat	Leaf rust resistance	*Lr41*, *Lr42* and *Lr43*	Cox et al. [24]
	Powdery mildew resistance	*Pm2+Pm4a, Pm2+Pm21, Pm4a+Pm21*)	Liu et al. [22]
	Stripe rust resistance	*Yr5*and *Yr15*	Santra et al. [100]
Barley	Barley Yellow Mosaic Virus resistance	rym4, rym5, rym9 and rym11	Werner et al. [101]
Potato	Late blight resistance	*Rpi-mcd1* and *Rpi-ber*	Tan et al. [25]
Soybean	Soybean mosaic virus (SMV)-resistance	*Rsv*1, *Rsv*3, and *Rsv*4	Zhu et al. [102]

Source: Joshi and Nayak [12] and Suresh and Malathi [2].

Table 5: Summary of the success history of pyramiding genes using MAS to develop biotic stress tolerant cultivars.

Gene pyramiding through genetic engineering

Concepts of genetic engineering and its applications in crop improvement program: Genetic engineering or recombinant DNA technology (rDNA) is defined to be the technology that is used to cut a known DNA sequence from one organism and introduce it into another organism thereby altering the genotype (hence the phenotype) of the recipient [26,71,72]. The organism whose genes have been artificially altered for a desired trait is often called genetically modified organism (GMO). The techniques use highly sophisticated laboratory tools and specific enzymes to cut out, insert, and alter pieces of DNA that contain one or more genes of interest [72].

Unlike conventional and MAS breeding methods which allow the transfer of desired genes between related species [25], genetic engineering allows the specific transfer of gene of interest from any source (from animals, viruses, bacteria, or even from totally man-made sequences) into crop plants [26], to generate crops with the desired agronomic trait/s. Jain and Brar [1] stated that genetic engineering is the only option to transfer genes of interest originates from cross barrier species, distant relatives, or from non-plant sources in a very fast way than through conventional or molecular breeding. Although, genetic engineering is a universal, precise, and fast method to transfer desired gene/s into crop plants [72], it will not replace conventional breeding but it will add to the efficiency of crop improvement.

Major steps in plant genetic engineering: The process of genetic engineering requires the successful completion of the following series of steps [73].

1) Identifying the target gene and isolating the DNA from the desired organism: To identify a desirable new trait or gene it is important to look to nature. It means in searching for a trait that would allow a crop to survive in a specific environment, it is important to look for organisms that naturally are able to survive in that specific environment. For instance, Monsanto created "Roundup Ready" plants after finding bacteria growing near a Roundup factory that contained a gene that allowed them to survive in the presence of the herbicide [74]. The other desired gene identification techniques are comparative genome analysis of organisms showing the trait and lacking the trait as well as mutational analysis i.e., purposeful deletion, or "knock out," of parts of the genome of interest until the desired trait is lost [75].

2) Gene Cloning: After the target gene has been identified and isolated, it will be multiplied by inserting it into bacterial plasmid (cloning vector). Plasmids are small circular DNA capable of replicating independently [73]. During gene cloning, both the DNA with target gene and the vector are cut open with the same restriction endonuclease. After the insertion of the target gene, the cut ends will be sealed by molecular glue called DNA ligase. Then, the construct (vector plus target gene) will be reintroduced into bacterial cells to allow it to replicate together with the replication of the host cell. There are also other types of coning vectors used to transport target gene into

host cells including: phages, cosmids, bacterial artificial chromosomes (BACs), and yeast artificial chromosomes (YACs).

3) Selection of the transformed host cells: For easy identification of the bacteria carrying the target gene, selectable markers like antibiotic resistance gene is included in the construct. Hence, bacteria cells that have incorporated the plasmids with their antibiotic resistance gene will grow in the medium containing the antibiotics and those that do not take the plasmid are killed by the antibiotics. In addition to this some reporter genes such as ß-galactosidase gene are incorporated in the construct to aid easy selection of host cells carrying the target genes. After selection of the transformed colonies, the multiplied target gene will be again isolated to be transferred into the target organism. In plants, callus (masses of undifferentiated cells produced through tissue culture of immature embryos, leaf disks, and apical meristems) is the appropriate stage for incorporation of desired transgenes [73].

4) Insertion of target gene and regeneration of the transgenic plant: There are various techniques used to transfer desired genes into plant cells. Some of the more commonly used methods include: gene gun (particle bombardment), agrobacterium mediated, microfibers, electroporation, chemical method (calcium phosphate), microinjection, use of liposomes, and Polyethylene glycol (PEG). After gene transfer, successfully transformed plant cells are selected and then regenerated into full transgenic plants. The presence of the target gene in the regenerated plants can be checked through Southern, Northern, or Western blotting techniques. Then, transgenic plants are acclimatized in greenhouses and the transgenic seeds will be collected [73].

5) Backcross breeding: Usually, the transformed plants are poor in their agronomic or quality traits. Therefore, the transgenic plants are repeatedly backcrossed with an improved variety using traditional plant breeding methods. This will ultimately result in a crop with a high yield potential expressing the trait encoded by the new transgene in this case biotic stress resistance.

Genetic engineering methods for gene pyramiding: The commonly used transformation methods for pyramiding desirable genes are hybrid stacking, co-transformation, linked genes or multigene cassette transformation and re-transformation [76]. A biotech crop variety that bears stacked traits is called a biotech stack or simply stacks. The easiest and quickest way to stack up genes into a plant is to make crosses between parental plants that have different biotech traits, an approach known as hybrid stacking. Most of the commercially available biotech stacks, like triple stack, and quadruple stack, are products of serial hybrid stacking which is widely adapted and accepted [76]. Another method of gene stacking, known as molecular stacking involves the introduction of gene constructs simultaneously or sequentially into the target plant by standard delivery systems such as *Agrobacterium*-mediated and biolistic methods [77,78]. In some stacks, molecular stacking has been done with conventional breeding approaches to put together the desirable traits [76]. In co-transformation method, a plant is transformed with two or more independent transgenes. The transgenes of interest are in separate gene constructs and delivered to the plant simultaneously. Re-transformation method involves the transformation of a plant harboring a transgene with additional transgenes. In linked genes or multigene cassette transformation a plant is transformed with a single gene construct that harbors two or more linked transgenes [76].

Applications: Genetic engineering for pyramiding Bt genes for insect resistance: Genetic engineering offers an expanding array of strategies for enhancing insect pest resistance of crop plants [4]. The deployment of Cry proteins for insect control serves as an excellent example of how a transformation approach can contribute to increased and stable crop production by reducing input cost and environmental contamination due to pesticides application. So far, a number of crops such as rice, corn, cotton, and potato [79,80] have been successfully transformed to express Cry proteins that kill certain insects when they feed on the plants. The proteins are from the gram-positive soil bacterium *Bacillus thuringiensis* (Bt) coded by its Cry gene. In nature, there are many variants of Bt-Cry toxins like Cry1Ab, Cry1Ac, Cry2A, Cry2Ab etc coded by Cry gene family [1,60].

To increase pest control efficacy and delay resistance evolution, a gene pyramiding strategy has been employed in transgenic plants to produce two or more Bt toxins of dissimilar mode of action effective against the same target pest species [81-83]. Thus, crops pyramided with Bt genes express the insecticidal toxin in all of their tissues. When the target insect pests eat any parts of these crops, the crystal inclusion (Cry proteins) in the food will solubilize by the alkaline pH of the mid-gut to release an inactive toxin called protoxin. The protoxin is then converted in to an active toxin after processed by the host proteases present in the midgut [84]. The activated toxin binds to insect-specific receptors found on the surface of the plasma membrane of midgut epithelial cells and then inserts into the membrane to create transmembrane pores that cause cell lysis and eventually death of the insect [84]. Interestingly, Bt-toxins have been found to be safe to human consumption because the intestinal walls of mammals do not have the endotoxin receptor necessary for the toxic effect, and the proteins are degraded quickly in the stomach. Table 6 below presents some examples of the transgenic crops pyramided with the different versions of Cry genes to improve their resistance against selected insect pests.

Therefore, it is important to deduce that resistance gene pyramiding is a good strategy to increase insect control efficiency thereby contributing to increased agricultural production and productivity with low inputs and in environmentally friendly manner, and genetic engineering is an efficient technique to pyramid multiple desirable genes from any source (e.g., Bt*)* into crop plants to develop durable resistance against insect pests.

Genetic engineering for pathogen resistance: In contrast to herbicide- or insect-resistant transgenic plants, which have been grown extensively worldwide, the development of transgenic plants with enhanced resistance to fungal and bacterial pathogens has received only limited success [85,86]. There are various reasons for this limited

Crop	Trait	Engineered Genes	Reference
Corn	Asian corn borer resistance	cry1Ie and cry1Ac	Jiang et al. [84]
Rice	Corn borer resistance	*Cry1Ab* and *Cry1Ac*	Ahmad et al. [103]
	Striped stem borers and yellow stem borers resistance	*cry1Ab* and *cry1Ac*	Cheng et al. [104]; Datta et al. [105].
	Leaf folder, yellow stem borer and brown plant hopper resistance	*Cry1Ac, Cry2A* and *gna*	Maqbool et al. [35]
Cotton	*Spodoptera litura and Heliothis armigera resistance*	Cry9C gene and Cry 2A or Cry 1Ac	Li et al. [106]
	Bollworm resistance	*Cry1Ac* and *Cry2Ab*	Jackson et al. [107]
	Insect pest resistance	*Cry 1Ac, Cry 2Ac*	Gahan et al. [108]
Brassica	Diamondback moth larvae and lepidopteran insect pest's resistance	*cry1Ac* and *cry1C*	Cao et al. [109]
Chickpea	Lepidopteran resistance	*cry1Ac* and *cry1Ab*	Meenakshi et al. [110]

Table 6: Summary of selected transgenic crops pyramided with Bt genes for durable resistance to insect pests.

success rate. First, these pathogens are taxonomically highly diverse and physiologically very different from each other, and therefore no single gene transformation product can be expected to have a direct toxic effect on all types of pathogens. Secondly, most pathogens use two major life strategies, namely biotrophy and necrotrophy. Biotrophic pathogens essentially act as a sink for the host's anabolic assimilates, and therefore keep it alive. Meanwhile, necrotrophic pathogens consume the host tissues as invaded. Consequently, plants have developed quite different ways for dealing with these two strategies [86], which is of course difficult to achieve through genetic engineering. Thirdly, the pathogen avirulence (Avr) gene undergoes strong diversifying selection pressure or mutation to avoid recognition by host (R) genes [81]. The low levels of pathogenic resistance by some transgenic crops coupled with the negative perception of genetically modified plants have resulted in a relatively small number of transgenic lines being brought to late stage field testing and even fewer that have been successfully brought to market. With the exception of virus-resistant plants, currently there are no commercially available transgenic plant species with increased resistance towards fungal and bacterial pathogens [87].

So far, many transformation strategies have been used to increase resistance of crop plants against bacterial, fungal and viral pathogens including: introgressing R genes, introducing genes coding for antimicrobial compounds (chitinase or glucanase enzymes that break down fungal cell walls-chitin or glucan respectively), up regulating defense pathways (through promoter transfer), disarming host susceptibility genes, detoxifying pathogen virulence factors (toxins), increasing structural barriers and silencing essential pathogen genes (RNA silencing, RNA interference, or RNAi) [4,85,87]. For instance, Zhou et al. (2009) introgressed two R genes (*Xa23* and *Rxo1*) to develop rice cultivars resistant to bacterial blight and bacterial streak diseases. Table 7 presents published examples of genetic engineering based disease resistance development in major crops.

Crop	Disease resistance	Mechanism	Reference
Tomato	Bacterial spot	R gene from pepper	Horvath et al., [29]
Apple	Apple scab fungus	Thionin gene from barley	_Krens et al. [34]
	Fire blight	Antibacterial protein from moth	Borejsza-Wysocka et al. [111]
Banana	Xanthomonas wilt	Novel gene from pepper	Tripathi et al [112]
Barley	Stem rust	Resting lymphocyte kinase (RLK) gene from resistant barley cultivar	Horvath et al. [29]
Papaya	Ring spot virus	Pathogen-derived resistance	Ferreira et al [31]
Plum	Plum pox virus	Pathogen-derived resistance	Malinowski et al [32]
Potato	Potato virus Y	Pathogen-derived resistance	Bravo-Almonacid [33]
	Late blight	R genes from wild relatives	Foster et al [113]
	Late blight	R gene from wild relative	Bradeen et al [114]
	Late blight	R gene from wild relative	Halterman et al. [115]
	Potato virus X	Mammalian interferon-induced enzyme	Truve et al. [116]
Rice	Bacterial blight and bacterial streak	Engineered E gene	Hummel et al. [30]
	Bacterial streak	R gene from maize	Zhao et al. [117]
Squash	Three mosaic viruses	Pathogen-derived resistance	Lius et al. [118]
Tomato	Multibacterial resistance	PRR from Arabidopsis	Lacombe et al. [119]
Wheat	Powdery mildew	Over expressed R gene from wheat	Brunner et al. [120]

Table 7: Example of genetic engineering based disease resistant development in crop plants.

Genetic engineering for pyramiding herbicide tolerance genes: The long-lasted problem in weed control was selectively killing of the weeds through herbicide application leaving the crop plants unaffected. For instance, glyphosate or roundup is a broad-spectrum herbicide: kills all green plants. It inhibits the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) in the shikimate pathway, causing insufficient production of essential aromatic amino acids like tryptophan, tyrosine, and phenylalanine [88,89]. To circumvent this problem, glyphosate-tolerant crops have been developed by engineering with glyphosate resistance gene CP4 *epsps identified from Agrobacterium.* Nowadays, numerous crops such as corn, cotton, canola, tomato, soybean, sugar beets and, most recently, wheat, have been genetically transformed for glyphosate tolerance [90-94].

However, repeated use of glyphosate has resulted in the evolution of glyphosate-resistant weeds [95-97]. It has been reported that in 2016, 34 weed species had evolved resistance to glyphosate in 26 countries [37]. Thus, glyphosate should no longer be applied alone anytime on any weed anywhere [36]. This shows that single gene transformation for herbicide tolerance is not a guarantee. Hence, to use mixtures of herbicides, stacking multiple herbicide tolerant gens through genetic engineering is important. So far, the glyphosate resistance gene CP4 *epsps* has been stacked with the *pat* gene from *Streptomyces viridochromogenes* conferring resistance to herbicide glufosinate and/or with the *dmo* gene conferring resistance to herbicide dicamba [36].

Hence, it is appropriate to deduce that genetic engineering is a very helpful tool for pyramiding herbicide resistant genes from diverse sources into crop plants to develop broad spectrum herbicide tolerant crops to enable the use of herbicides combination for effective weed control which ultimately contributes to increased and stable crop production and productivity to maintain food security.

Conclusion

Biotic factors such as viruses, bacteria, fungi, nematodes, insect pests cause significant yield loss across the world. The use of resistant cultivars is seen as the best strategy, economical, durable and environmentally friendly to control these biotic stresses. As single gene based resistance breakdown within a short period, current breeding programs targeted at stacking multiple resistance genes/QTLs into a single genotype to develop durable biotic stress resistant cultivars. The present seminar paper reviewed the types of genetic resistance (major and minor genes) in plants and the methodologies involved in identification, mapping and then pyramiding of genes/QTLs into crop plants to develop durable/broad-spectrum resistance to biotic stresses. Usually gene mapping is the starting point of many important downstream studies. Herein, linkage map construction procedures are reviewed in detail and supported with practical examples of mapping QTLs conferring resistance to different diseases.

It has been reviewed that successful gene pyramiding involves three successive steps namely selection of parental lines containing the desirable genes, crossing the selected lines to transfer single copy of all the target genes into a single genotype and finally fixing the stacked genes to avoid their segregation. Moreover, the three gene pyramiding methods: conventional breeding, marker assisted selection and genetic engineering are discussed in detail. It was found that gene pyramiding through traditional breeding is slow, costly and inefficient. Thus, recently molecular markers and genetic engineering are widely used in crop improvement program to stack series of resistance genes into elite cultivar to develop durable and/or broad-spectrum resistance to biotic stress. Therefore, to minimize yield loss due to biotic stress, developing countries including Ethiopia shall include gene pyramiding in their

resistance breeding and also start the use of modern molecular tools to speed up the crop improvement program.

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Conflicts of Interest

We the authors have not declared any conflict of interests.

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