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A REVIEW

Gene stacking: Approach of genetic engineering

Omprakash, Aparna*, Bapsila Loitongbam[1], S. K. Bairwa **and** Kailash Chandra
Sri Karan Narendra Agriculture University, Jobner, Jaipur (Rajasthan) India
(Email: omprakash.pbg@sknau.ac.in)

Abstract : Gene stacking is the process of addition of two or more gene of interest into a single plant. The combination or stacking of different traits or genes in plants is rapidly gaining popularity in biotech crop production. The new evolved trait is known as stacked trait and the crop is known as biotech stacked or simply stacked. This can be accomplished in many ways, one of which is gene pyramiding. Biotech stacks give crops a larger genetic and agronomic boost, allowing them to perform better in challenging farming situations. Biotech stacks are designed to increase productivity by overcoming biotic and abiotic challenges like as insect pests, diseases, weeds, and environmental stress. This review will explain about the gene stacking principle, the need for biotech stacking, and the many gene stacking methods.

Key Words : Gene stacking, Biotech, Trait, Productivity, Biotic, Abiotic challenges

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INTRODUCTION

Gene stacking is a method of gene cloning that involves introduction of two or more transgenes into a single plant. In a nutshell, it's anamalgamation of two or more genes controlling disease resistance, yield and others traits of economical importance and quality. Gene stacking can be achieved in many ways *viz*; gene pyramiding, molecular and biotechnological approaches etc. Stacked traits are the resulting commingled traits. A plant that has been transformed with two or more genes coding for *Bacillus Thuringiensis* (Bt) proteins with different modes of action is an example of a stack crop. Dual hybrid cotton stackwas first biotech stack approved by FDA in 1995, which was developed by crossing bollgard TM cotton (which expresses the Bt toxin cry1ab) with roundup readyTM cotton, which generates the epsps enzyme providing resistance to glyphosate (Singh *et al*., 2018). In 2018, stack plants were planted on an area of about 80.5 million hectares which accounts for more than 42% of the total world's area under biotech crops (ISAAA, 2018). As per USDA data, stacked seeds cover 89 per cent of cotton acreage and 80 per cent of corn acreage in 2019 (USDA-ERS, 2019). During the last one or two decades, the number of biotech stacks available in the market has been increased. Herbicide tolerance and insect resistance were the most common combination features in crops like soybean and maize. However, only a few varieties have been introduced that possesses various combinations of drought resistance gene and other stresses. In a nutshell, biotech stacks can improve food safety and sustainability. Golden Rice,

***Author for correspondence:** Department of Agriculture, Jagannath University, Jaipur, Rajasthan
[1]College of Agriculture, (CAU, Imphal), Pasighat (Arunachal Pradsh) India

Blue Rose, and SmartStax are example of promising stacks.

Need for gene stacking:

Biotech stacks provide a genetic and agronomic boost to crops, helping them to perform better in difficult farming settings. Insect pests, diseases, weeds, and environmental stress are all examples of biotic and abiotic stresses that biotech stacks are meant to overcome. Furthermore, if the target insect pest continues to mutate, the resistance conferred by a single Bt gene may be lost and here comes the role of biotech stack.Biotech stacks illustrate varied insect resistance using Bt gene technology. To delay the breakdown of Bt resistance, a refuge or non-Bt variety should be planted alongside the Bt crop. In case of mono-Bt. crop, refuge contributes only about 20% of the total crop area. Refuge crop approach minimizes the risk of resistance breaking down.The gene stacking strategy was utilized to develop the next generation of Bt crops with multiple mechanisms of action for pest control by distinct classes of Bt genes. This new generation of Bt crops has a lesser probability of resistance breakdown since it is difficult for insects pest to adapt against numerous insecticidal proteins. As Bt stacks are more resilient, they may have a slight production disadvantage due to reduced refuge crop area. (Storer *et al*., 2012).Weeds exhibiting resistance to commercial herbicides with numerous herbicidal modes of action has been discovered. Recently cotton plants have been developed by stacking epsps gene conferring resistance to glyphosate, pat gene conferring resistance to glufosinate and dmo gene imparting resistance to herbicide dicamba. These stacked palntspossess metabolic processeswith large number of genes interacting with one another.Plant metabolic engineering relies heavily on gene stacking (Naqvi *et al*., 2009). The biosynthetic route for provitamin A (beta carotene) in Golden Ricewas constructed in the endosperm by stacking three carotenoid genes (Ye *et al*., 2000). Another successful biotech stack is the rose (Biotech rose) with modified flower colour, which was created by stacking two genes in the anthocyanin biosynthetic pathway. Thsesgenes were engineered for flower color to impartunique blue colour to biotech rose (Tanaka *et al*., 2009).

Approaches of gene stacking:

The easiest and quickest method for developing biotech stacks is hybrid stacking, which involves establishing crosses between parental plants with discrete biotech traits. Another option is molecular stacking, which involves delivering gene constructs to the target plant simultaneously or sequentially using common delivery mechanisms such Agrobacterium-mediated and biolistic ways (Haplin *et al*., 2000). In general, the optimum strategy is determined by the species of interest, as well as the availability of genetic constructs and pre-existing transgenic events.

Hybrid stacking:

It's a type of sexual hybridization in which a plant having one or more transgenes hybridize with another plant that has different transgenes. Stacks containing numerous transgenes can be created by crossing parents with different transgenes until all of the required genes are accumulated in a single progeny. Stacks such as triple stack and quadruple stack are common and successful, and they are the outcome of segregation.Stacks like triple stack and quadruple stack are common and successful, and they're the outcome of serial hybrid stacking, which has been widely adopted and accepted (ISAAA, 2017). For Example: Cross-breeding of tobacco to combine four genes expressing various immunoglobulin polypeptides to produce secretory IgA antibodies (Ma *et al.,* 1995). Other examples are: Maize: Agrisure™ Viptera™ 3220 (Bt11 x MIR162 x TC1507 x GA21), Cotton: Roundup Ready™ Flex Bollgard™ II (MON88913x MON15985).

The inserted transgene will integrate at random loci in the plant, which is a major limitation in sexual hybridization. It's laborious and time-consuming also.

Molecular stacking:

The most prevalent approaches for developing genetically modifying plants with gene stacking comprise the simultaneous introduction of genes using a co-transformation processes or the sequential introduction of genes using re-transformation processes. Stacking would be caused by intermolecular or intramolecular hydrophobic interactions between aromatic nuclei such as anthocyanidins, flavones, and aromatic acids (Taverniers, 2008).

Re-Transformation:

A transgene-carrying plant can be transformed with other transgenes. It has the ability to silence transgenes and requires a variety of selectable marker genes so that each successive transformation can employ a different one. Bollgard TM II (ex-cotton).

Advantages:

The multiple "effect" gene can be introduced in a single process. Transgenes frequently co-integrate in the same chromosome. Multiple transgene integration, fewer transformation events, and reduced time advantages this method.

Limitations:

Incorporation of a complicated T-DNA molecule from several sources is undesirable.

Co- Transformation:

Two or more separate transgenes are used to transform a plant. The transgenes of interest with different gene constructs are introduced into the plant at the same time. NaturGard™ Knockout™ (Bt176), BtXtra™ (DBT418), Yield Gard™ (MON810, MON809, MON802) are few examples in case of maize.

Single Plasmid Co-Transformation of linked transgenes:

Genes to be introduced linked as single piece of DNA with each gene having its own promoter.

Multiple Plasmid Co-transformations of linked transgenes:

Consist of several plamid or discrete fragments each carrying a transgene (including promoter) that are transferred together in plant via agrobacterium mediated transformation or Biolistic method.

Linked genes or multigene cassette transformation:

A plant is transformed with a single gene construct that harbors two or more linked transgenes. Maize: Herculex™ I (TC1507), Herculex™ RW (59122), Agrisure™ CB/LL (Bt11), Soybean: Vistive™ Gold (MON87705)

Regulation of stacked product:

Regulation of stacked product: genetically modified and stacked products are being developed by various countries and for which, regulation and approval procedures to commercialize that product are also varying in different countries. If hybrid stack is the product of crossing in which already approved lines are used, then there is no necessity of additional regulatory approval for commercializing of that product (in USA and Canada). In contrary to these countries, Japan and some countries from European Union, stacked product is always considered new and has to pass the regulatory approval system even the individual component lines of that product had already gone through and passed the regulatory approval system. The first stack that gained regulatory approval in 1995 was a dual hybrid cotton stack produced by crossing Bollgard™ cotton that expresses the *Bt* toxin *cry1Ab* and Roundup Ready™ cotton that produces the *epsps* enzyme conferring resistance to herbicide glyphosate (Singh *et al*., 2018). Following commercial success of this hybrid stack, developers sought to stack up more biotech traits into their crop portfolio to create multi-stack hybrids. The cotton triple stack which combines two *Bt* genes with the glyphosate resistance gene occupied more than 54 percent of the US cotton area in 2008. The recently released eight-gene maize stack known by its trade name Smart Stax™ is the result of crossing four different biotech maize lines to combine two herbicide tolerance genes with six *Bt* genes. The resulting stack features dual modes of control for weeds, lepidopteran insects and coleopteran insects and allowed for the refuge requirement to be reduced from 20 per cent to 5 per cent in the US Corn Belt (USDA-EPS, 2015).

The trend for the future generation of biotech crops has been set by the increasing number of biotech traits in recent stacks. Future stacks are anticipated to include not just numerous pest and disease resistances, but also the combination of the traits with engineered metabolic pathways and the simultaneous introduction of various routes (for example, pathways for beta carotene, ascorbate, and folate and vitamin E synthesis) through metabolic engineering.

Few examples of the stacked crops developed by different giant companies through incorporation of number of genes for more than one trait are given in the table (Source: Helpin *et al.,* 2005):

Technological Challenges: The developer's decision between a huge molecular stack and a sophisticated hybrid stack will depend on the cost and time to construct and register a stack. The one-shot molecular stacking might be more cost effective than the lengthy hybrid stacking in a nations where a stack must pass a separate regulatory review. However, there are technological difficulties in molecular stacking, such as the design of massive multi-gene complexes, delivery methods into plant cells, and the stability of multiple gene expression.

Table 1 : Examples of the stacked products

Trait	Crop	Genes	Method
Herbicidetolerance+ fertilityrestoration	Canola	*Bar;barnaseor barstar;neo*	*Agrobacterium*linkedgene,1t-DNA
Herbicidetolerance+ fertilityrestoration	Chicory	bar;barnase; neo	Agrobactarium/linked gene, 1 T- DNA
Multiplevirus resistance	Squash	Neo*; CP- ZYMV;;CP-WMV2	Agrobactarium/linked gene, 1 T- DNA
Modifidecolour + herbicidetolerant†	Carnation	surB†;dfr;bp40	Agrobactarium/linked gene, 1 T- DNA
Insect+viruseresistant	Potato	cer3A;PLRVrep;ne oor7	Agrobactarium/linked gene, 1 T- DNA
Insectresistance+herbicidetolerance	Maize	Bar;cry1Ac;pin//‡	Biolisics/3plasmidco-transformation
Insect resistance + herbicidetolerance	Maize	cry1Ab;EPSPS;gox	Biolisics/2plasmidco-transformation
Potato late blight	Potato	Rpi-sto1, Rpi-vnt1.1 and Rpi-blb3	Transformation using single binary vector pBINPLUS

To overcome these challenges, molecular biologists are developing new genetic engineering technologies. Site-specific gene recombination systems in combination with designed DNA cutting enzymes, as well as artificial gene assembly known as mini chromosome assembly, are among the promising technologies (Haplin *et al.,* 2005). The numbers of transgenes that can affect the plant's general physiology, as well as the number of genes and combinations of genes that can be stacked into a plant, are of primary concerned to the developers. The yield is expected to decline if repeated transgenic insertions have aadverse impact on the plant's protein synthesis and metabolic activities. While yield drag isn't always a biosafety issue, the yield loss must be offset by lower production costs and allow farmers to harvest the benefits of improved value of the biotech trait in order to make stack a viable and lucrative biotech product (s).

Conclusion:

For gene stacking, there are a variety of traditional and more unique ways available; nevertheless, no single method has yet proven to be perfect. When compared to re-transformation, co-transformation is a more successful approach for gene stacking. Chimeric transgenes with fused sequences of multiple "effect genes" controlled by a single promoter provide a number of advantages. Insect and disease resistance, multiple resistance, abiotic stress tolerance, quality enrichment, and modulation of metabolic pathways in crop plants are all possible with gene stacking technology.

Future thrust:

Our knowledge on metabolic pathways and the identification of genes involved must continue to expand. Refinement of the present approach will be necessary for coordinated multigene modification in plants, resulting in more durable and cleaner transgene technologies that will ease the regulatory clearance process and reassure consumers about GM product safety and stability. A better vector technology should be developed that can transfer several genes in a single transfer. Due to the emergence of stacking transgenes methods, a tiny but growing percentage of genetically engineered crops have two or more unique features. Stacking can be accomplished using a variety of approaches, each with its own set of constraints. Transgene stacking has the potential to expand the scope of present plant genetic manipulation, allowing for the introduction of entirely new biochemical pathways or the elimination of a variety of problems.

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