Training on

Use of Biotechnological Tools for Varietal Development

Date: 12-16 January, 2025

Compiled and edited by:

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Crops Division

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Bangladesh Agricultural Research Council (BARC)

New Airport Road, Farmgate, Dhala-1215

Training on Use of Biotechnological Tools for Varietal Development

Organized by : Crops Division, Bangladesh Agricultural Research Council (BARC)
Date and time : 12-16 January, 2025, Time: 9.30 AM - 5.00 PM
Venue : Conference room-2, BARC, Farmgate, Dhaka.
Participants : 25 Scientist from NARS and Agricultural University.
Course Director : Dr. Md. Abdus Salam, Member Director (Crops), BARC.
Course Coordinator : Dr. Md. Mahfuz Alam, Principal Scientific Officer (Crops), BARC.

Day 1 (12.01.2025)		
Time	**Topics**	**Facilitators**
09.00-09.30	**Registration**	**Dr. Md. Mahfuz Alam**, Principal Scientific Officer(Crops), BARC
09.30-10.00	**Inaugural program**	**Welcome address: Dr. Md. Mahfuz Alam,** PSO (Crops), BARC **Special Guest: Dr. Md. Ashraful Alam**, APD, PARTNER, BARC **Chief Guest: Dr. Nazmun Nahar Karim,** Executive Chairman, BARC **Chair: Dr. Md. Abdus Salam,** Member Director (Crops), BARC
10.00-10.30	**Tea break**	
10.30-11.30	Role of Biotechnology in Crop Improvement	**Dr. Md. Salimullah**, Chief Scientific Officer, NIB
11.30-12.30	Genetic Engineering for Crop Improvement: Concepts, Tools and Examples in Varietal Development	**Dr. Md. Salimullah**, Chief Scientific Officer, NIB
12.30-13.15	Status for Biotechnological Research in Bangladesh	**Dr. Md. Abdus Salam** Member Director (Crops), BARC
13.15-14.15	**Lunch and prayer break**	
14.15-15.15	DNA Extraction, Visualization and Analysis	**Dr. Md. Wali Ullah** SSO, Genome Research Center, BJRI
15.15-16.30	Primer Designing for Varietal Development	**Dr. Md. Abu Sadat** SSO, Genome Research Center, BJRI
Day 2 (13.01.2025)		
Time	**Topics**	**Facilitators**
09.30-10.30	Molecular Cloning for Varietal Development	**Dr. Md. Wali Ullah** SSO, Genome Research Center, BJRI
10.30-11.00	**Tea break**	
11.00-12.00	Plasmid DNA Isolation, Validation and Analysis	**Dr. Md. Wali Ullah** SSO, Genome Research Center, BJRI
12.00-13.00	Agrobacterium Mediated Transformation of Desired Gene(s) in Plants for Varietal Development	**Dr. Md. Wali Ullah** SSO, Genome Research Center, BJRI
13.00-14.00	**Lunch and prayer break**	
14.00-15.30	Salinity Tolerance: Exploring Potential Genes to Enhance Salt Stress Adaptation	**Dr. Borhan Ahmed** SSO, Genome Research Center, BJRI
15.30-16.30	Identification of Candidate Genes and Cross-Talk Mechanisms using Pathway and Network Analysis for Varietal Development	**Dr. Borhan Ahmed** SSO, Genome Research Center, BJRI

Day 3 (14.01.2025)		
Time	**Topics**	**Facilitators**
09.30-10.30	Genome editing, CRISPR workflow and its potential in agriculture	**Dr. Tahmina Islam,** Assoc. Professor, Department of Botany, Dhaka University
10.30-11.00	**Tea Break**	
11.00-12.00	Case studies on genome-edited crops	**Dr. Tahmina Islam,** Assoc. Professor, Department of Botany, Dhaka University
12.00-13.00	Biosafety Guidelines for Transgenic Research in Bangladesh.	**Dr. Md. Aziz Zillani Chowdhury** Ex. Member Director (Crops), BARC
13.00-14.00	**Lunch and prayer break**	
14.00-15.00	Development of Abiotic and Biotic Stress Tolerance/Resistant rice lines through Over expression of *OsHAP2E* gene	**Dr. Md. Mahfuz Alam** Principal Scientific Officer Crops Division, BARC
15.00-16.30	Study on Population Genetic Structures of *Puccinia striiformis* f. sp. *tritici*	**Dr. Md. Ashraful Alam** Agency Program Director, APCU-BARC, Partner & Principal Scientific Officer
Day 4 (15.01.2025)		
Time	**Topics**	**Facilitators**
09.30-10.30	Functional Genomics in Crop Improvement	**Prof. Dr. Jamilur Rahamn** GPB, SAU
10.30-11.00	**Tea Break**	
11.00-12.15	Epigenetics, DNA methylation, histone modifications, and their impact on trait inheritance	**Prof. Dr. Jamilur Rahamn** GPB,SAU
12.15-13.30	Transcriptomics, proteomics, and metabolomics for trait discovery	**Prof. Dr. Jamilur Rahamn** GPB,SAU
13.30-14.30	**Lunch and prayer break**	
14.30-16.00	Development of abiotic stress tolerant crop variety through Molecular Approach	Dr. Kamrun Nahar, SSO, BTD, BARI
Day 5 (16.01.2025)		
09.30-10.30	Varietal Development of Eggplant using *Bt* gene (BSFB resistance)	Dr. Mohammad Kamrul Hasan, PSO, BTD, BARI
10.30-11.00	**Tea Break**	
11.00-12.00	Varietal Development of Potato using *3R* gene (late blight resistance)	Dr. Mahmuda Khatun, CSO& Head BTD, BARI
12.00-13.00	Group discussion on challenges and way forward	**Dr. Md. Mahfuz Alam**, Principal Scientific Officer, BARC
13.00-14.00	**Lunch and prayer break**	
14.00-14.30	**Post Evaluation**	**Dr. Md. Mahfuz Alam**, Principal Scientific Officer, BARC
14.30-16.00	**Closing & Certificate giving ceremony**	**Chief Guest: Dr. Nazmun Nahar Karim,** Executive Chairman, BARC **Chair: Dr. Md. Abdus Salam,** Member Director (Crops Division), BARC

Contents

Basics of Biotechnology in Crop Improvement

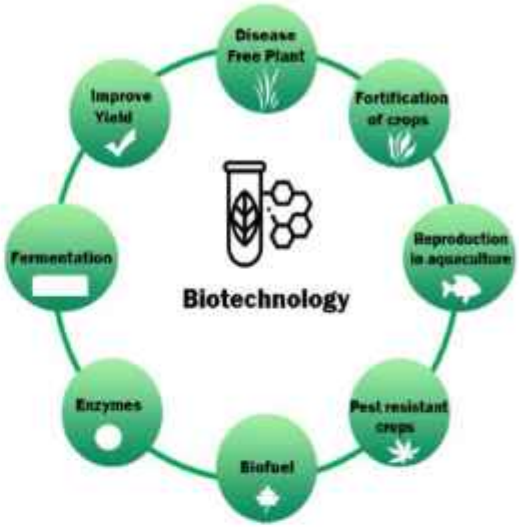
12th January, 2025

Dr. Md. Salimullah
Chief Scientific Officer
Molecular Biotechnology Division
National Institute of Biotechnology
Bangladesh

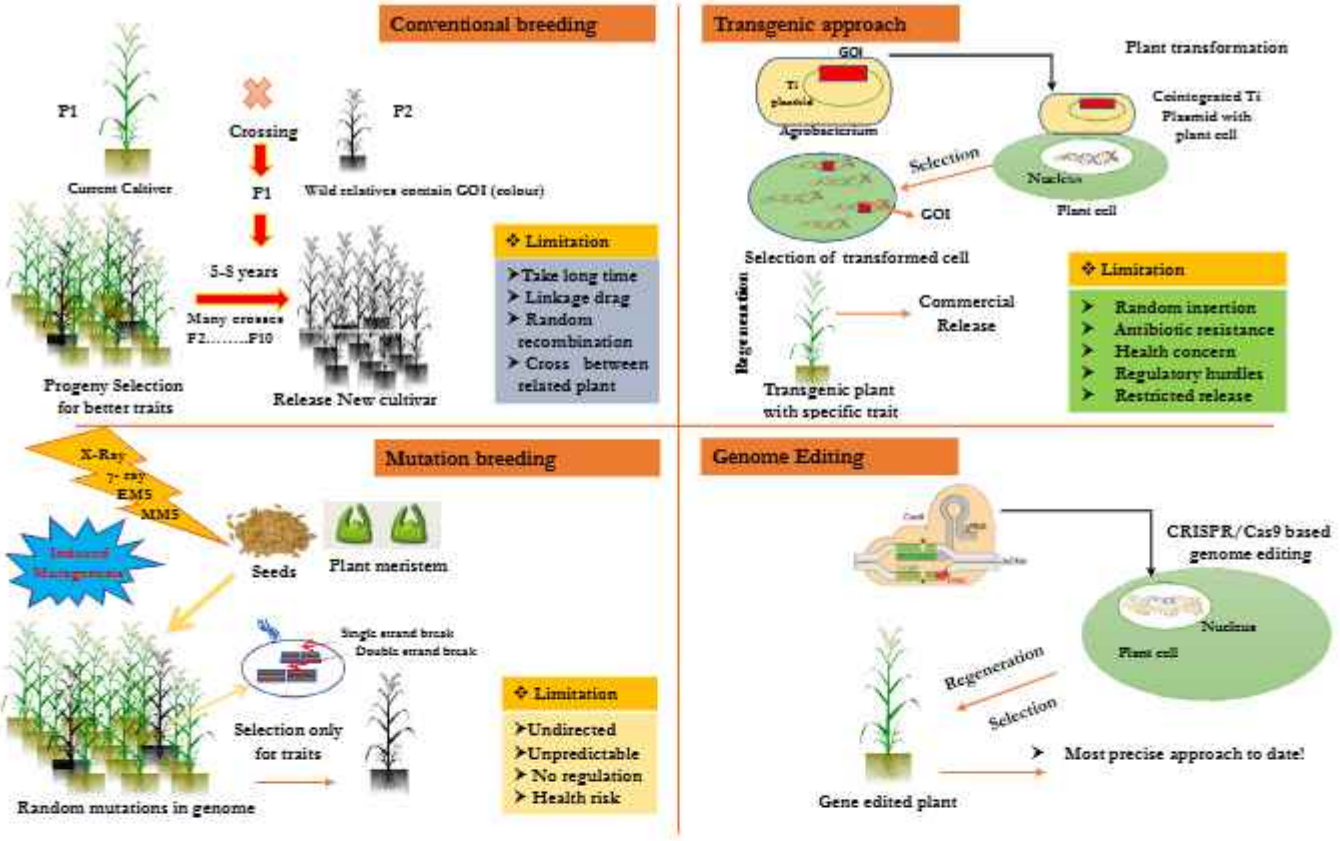
Life is twisted!!!

Biotechnology is a multidisciplinary field that involves using living organisms, biological systems, or derivatives to develop or modify products and processes for specific uses.

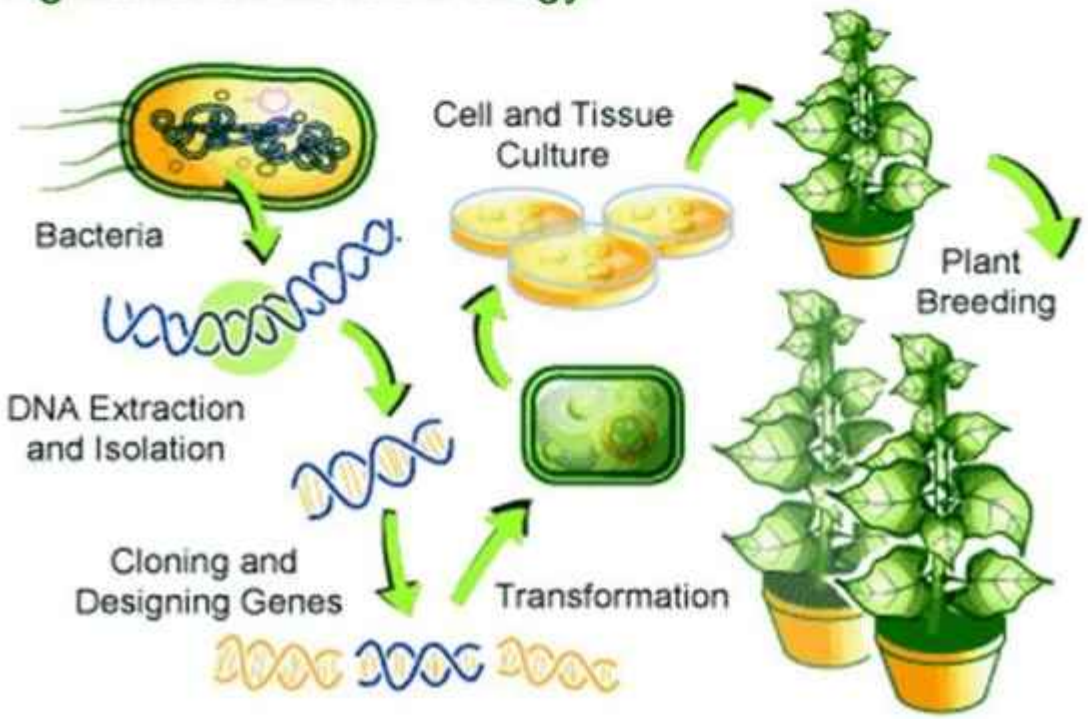
Agricultural biotechnology is a scientific field that uses tools and techniques to modify living organisms for agricultural purposes. Agricultural biotechnology can help address climate change, improve global food security, and reduce environmental impacts from agriculture. These techniques include genetic engineering, molecular markers, and tissue culture.



Key Techniques for Crop Improvement???



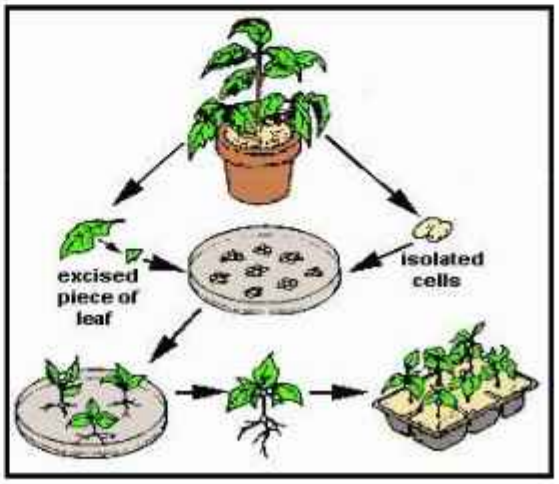
Agricultural Biotechnology: include genetic engineering and tissue culture technique



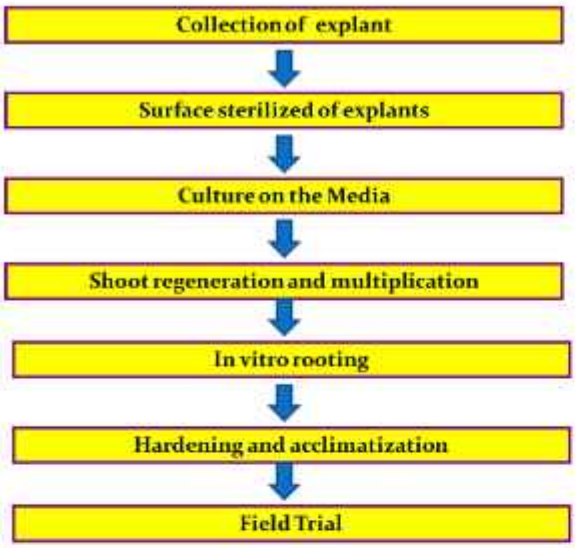
Method	Description
Tissue culture	Used to regenerate transformed plant cells into whole plant
***Agrobacterium*-mediated transformation**	Uses the bacterial pathogen Agrobacterium tumefaciens to deliver genes into a plant. The transferred DNA can integrate into the plant genome or remain transiently in the nucleus.
Gene gun	Uses tungsten or gold particles coated with plasmid DNA to deliver genes into plant tissue. The particles are accelerated with air pressure and shot into the plant.
Molecular marker	Molecular markers are DNA fragments that used to identify a specific gene or character in a plant, and to select plants based on their genotype.

What is plant tissue culture?

✓ It is the process of producing plants from tissue of the desired plant in an artificial nutrient medium under controlled environment



Plant Tissue Culture is the process of creating more plants from one plant



Application of Tissue Culture

The Success Story of Plant Tissue Culture Technology for Crop Improvement in Bangladesh

Banana

Strawberry

Potato

Aloe vera

Eggplant | *Aloe vera* | Rice

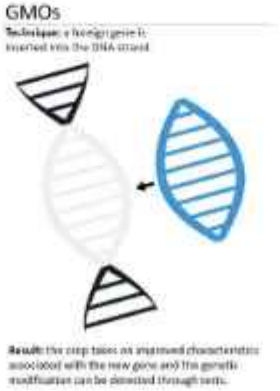
What is plant genetic engineering?

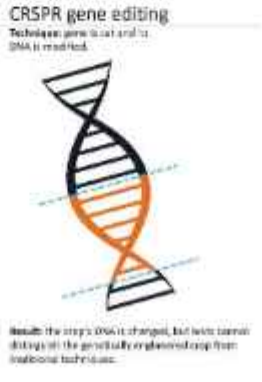
Plant genetic engineering, also known as plant genetic modification or manipulation, is the key that opens up the doors for introducing crops with valuable traits

Although the global crop yield has been improved over the recent years, the growth of crops in many areas are still stressed from plant diseases, pests and multiple abiotic stress such as salt, drought, coldness and heavy metal pollution.

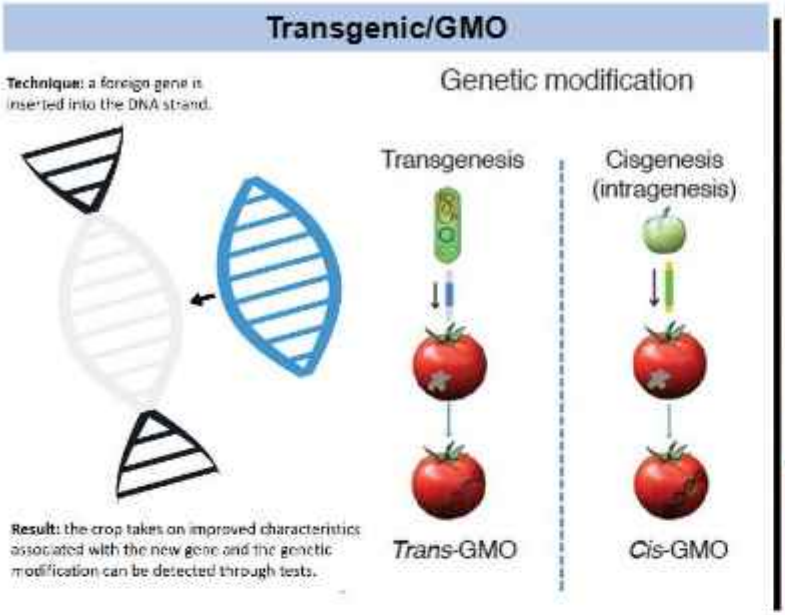
What is genetic modification (GM) of crops

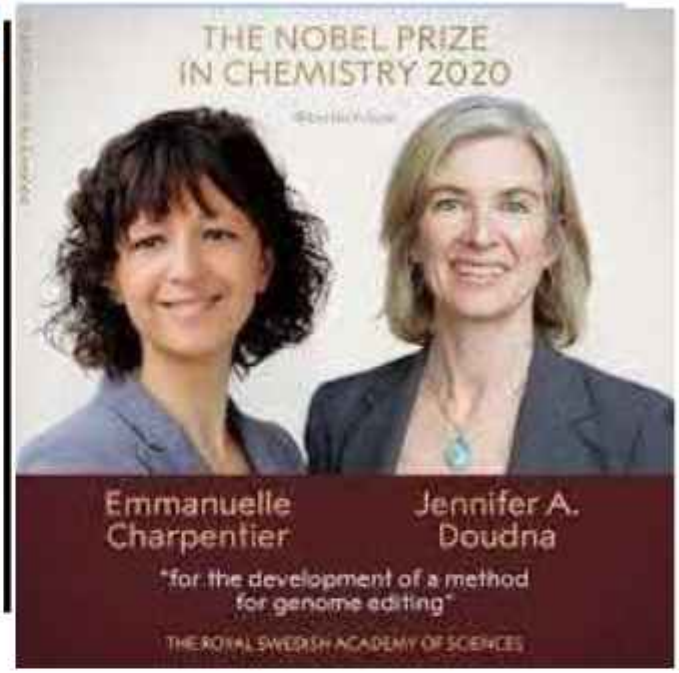
GM is a technology that involves inserting DNA into the genome of an organism. To produce a GM plant, new DNA is transferred into plant cells. Usually, the cells are then grown in tissue culture where they develop into plants. The seeds produced by these plants will inherit the new DNA.



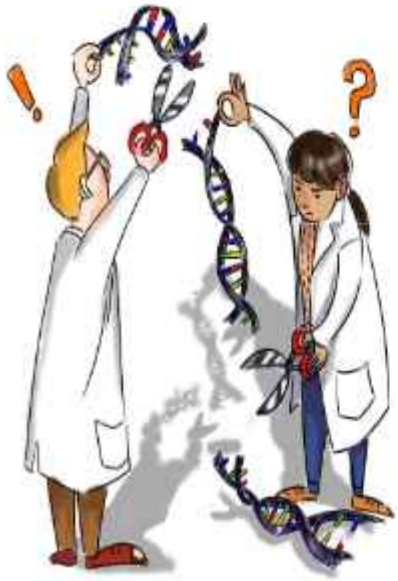


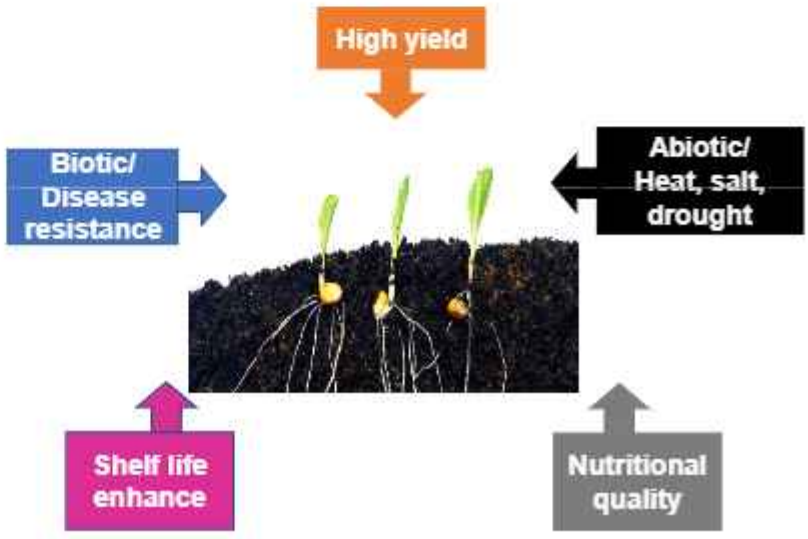
Biotechnological Approaches (GMO VS. Genome Editing)



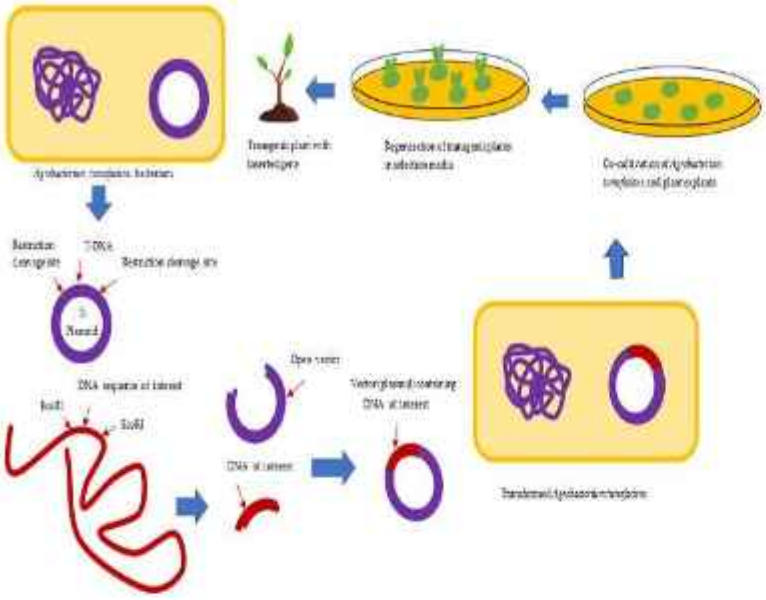


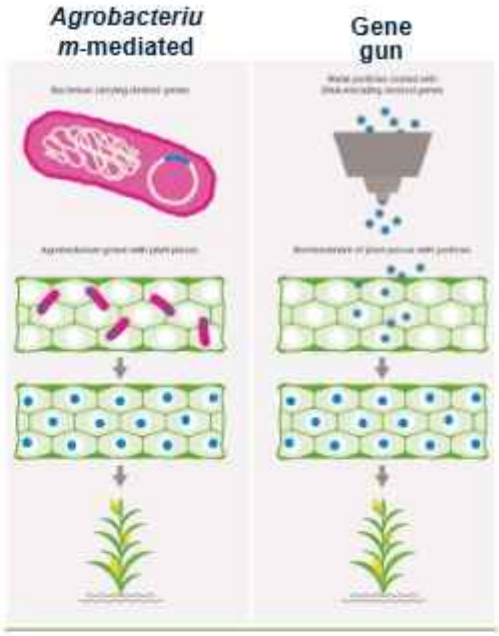
Biotechnological Approaches (GMO/Genome Editing) in Crop Plants: Potential benefits and real





Agrobacterium-mediated gene transfer steps





Bt-Brinjal: The first GM crop in Bangladesh

First genetically engineered transgenic crop, commercially released in Bangladesh on 30th October 2013

Bt -Brinjal resistant to the fruit and shoot borer pest

Plant Genetic Engineering Technology for Crop Improvement in Bangladesh

Bangladesh has started growing **Bt cotton**, a genetically modified (GM) cotton variety. The National Committee on Biosafety (NCB) of the Ministry of Environment & Forest officially released Bt cotton on May 31, 2023

Bangladesh is waiting for regulatory approval to cultivate vitamin A-enriched rice known as **Golden Rice**

Late blight (*Phytophthora infestans*) is a common and destructive fungal disease that threatens potato production in Bangladesh also waiting for regulatory approval

Indo-Bangla collaborative project under the India-Bangladesh Joint Committee on Science and Technology (JSTC) between NIB and ICGEB (2017 – 2020)

- **Molecular cloning and characterization of mitochondrial small heat-shock protein genes in eggplant for abiotic stress tolerance**

ICGEB-এর PMB গ্রুপের দলীয় প্রধান এবং গবেষক Dr. M. K. Reddy

Goverment Of India

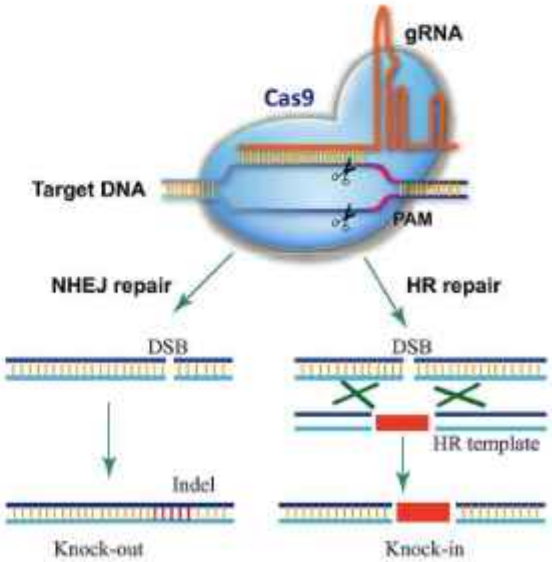
✓ Development of abiotic stress tolerant transgenic eggplant and rice lines

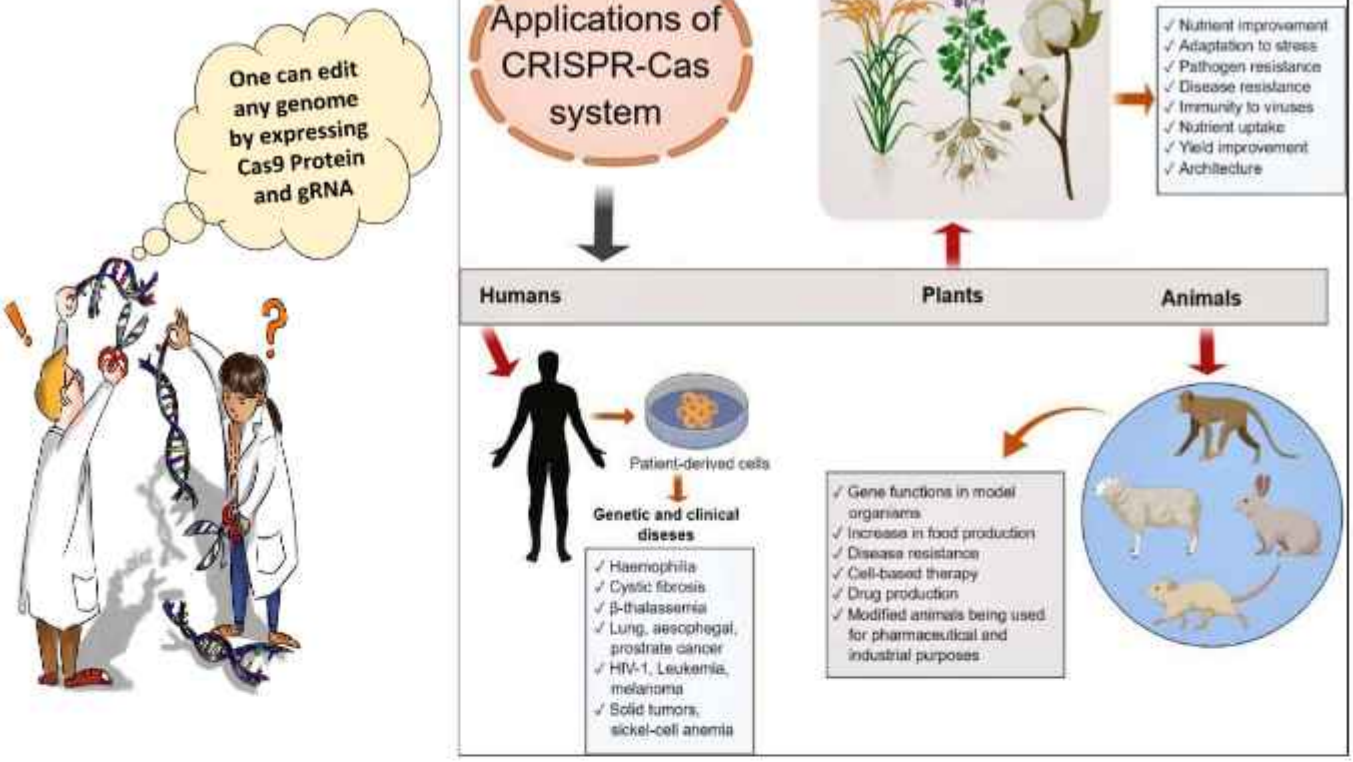
NIB হতে উদ্ভাবিত প্রথম প্রতিকূল পরিবেশ-সহিষ্ণু ট্রান্সজেনিক বেগুনের জাত তৈরি এবং বিজ্ঞান ও প্রযুক্তি মন্ত্রণালয়ের মাননীয় সিনিয়র সচিব জনাব মোঃ আনোয়ার হোসেন-এর উদ্ভাবিত ট্রান্সজেনিক বেগুনের জাত পরিদর্শন।

National Technical Committee on Crop Biotechnology (NTCCB) visited our Proteogenomics facility at National Institute of Biotechnology (NIB)

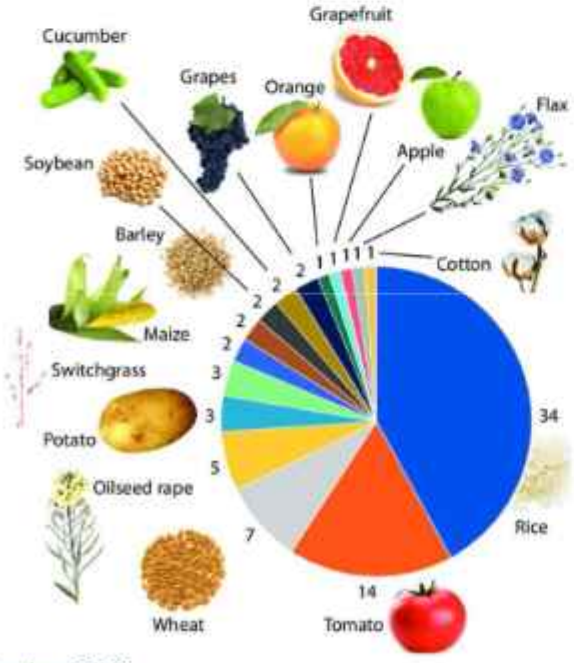
Plant Genetic Modification by CRISPR/Cas9?

CRISPR/Cas9 gene editing is a technique that rapidly edits genomic DNA at very high efficiency and specificity. Cas9 is an RNA -guided DNA endonuclease enzyme associated with the CRISPR. sgRNA (single-guide RNA), which packages with multiple crRNAs and the tracrRNA, can combine Cas9 and guide this complex to a complementary sequence in the target DNA. Then the HNH and RuvC domains of Cas9 cut DNA on 3bp upstream of the PAM (protospacer adjacent motifs) site (typically NGG), making DSBs (DNA double strand breaks). The DSBs are repaired by either NHEJ (non-homologous end joining) or HDR (homology directed repair) allowing for nucleotide insertions, deletions, substitutions or site-specific mutations in the broken regions. The simple design of sgRNA strictly follows Watson-Crick base pairing and has made CRISPR/Cas9 a very effective and promising gene editing tool.

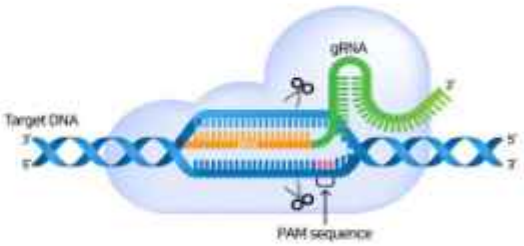




Number of genes and plants modified using CRISPR/Cas system for crop improvement



A. M. Korotkova 2020



❖ The success story of CRISPR

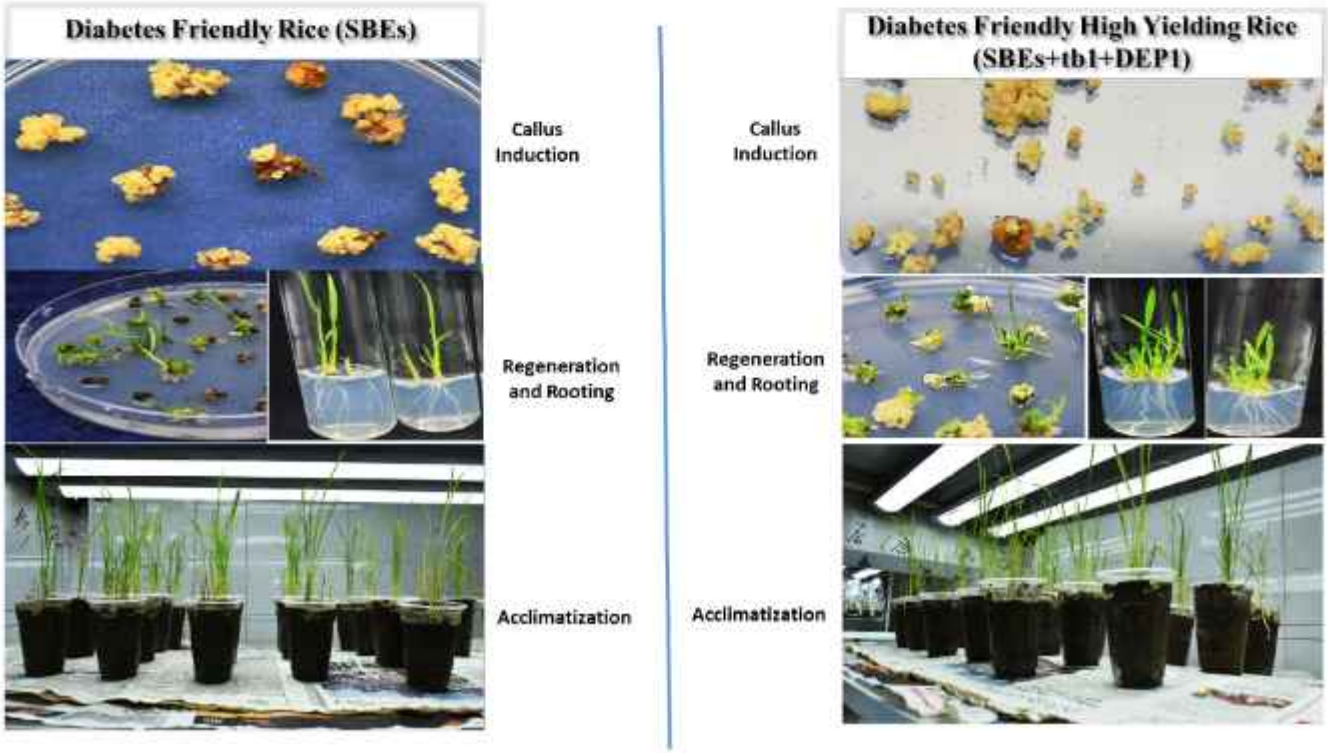
- ❖ CRISPR tomatoes: wild and groundcherry, lower blood pressure and promote relaxation.
- ❖ CRISPR mushrooms: stop them from browning.
- ❖ CRISPR rice: improving the yield, and stress tolerance.
- ❖ CRISPR citrus fruits: Disease resistance.
- ❖ CRISPR chocolate: boost immune system.

❑ Our Ongoing Projects

❖ Engineering Multiple Isoforms of Starch Branching Enzyme Through Multiplex CRISPR/Cas9 to Generate Diabetes Patient Friendly High-Amylose Containing Rice

❖ Down Regulation of Gn1a (Grain Number 1a) Gene by CRISPR/Cas9 to Increase Yield in Bangladeshi Aromatic Rice Variety

CRISPR/Cas9 Cassette Transfer by *A. tumefaciens* mediated transformation in BRRI-92



Achievements of The Project

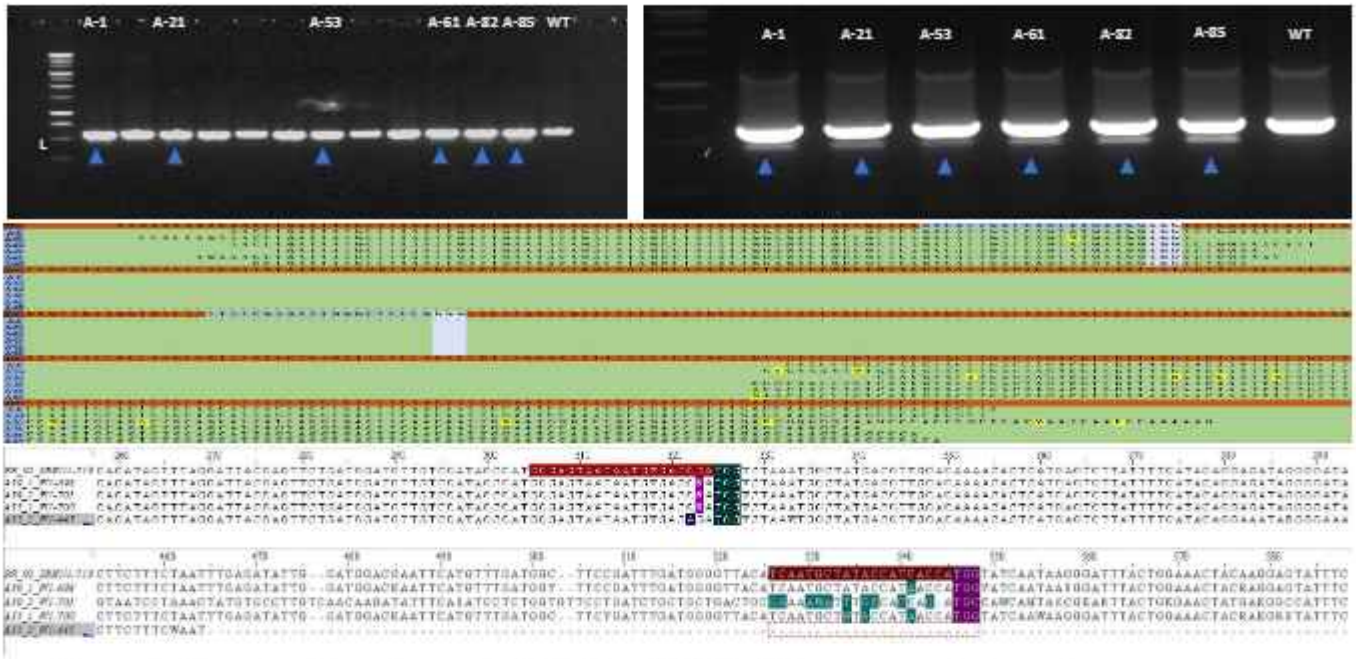


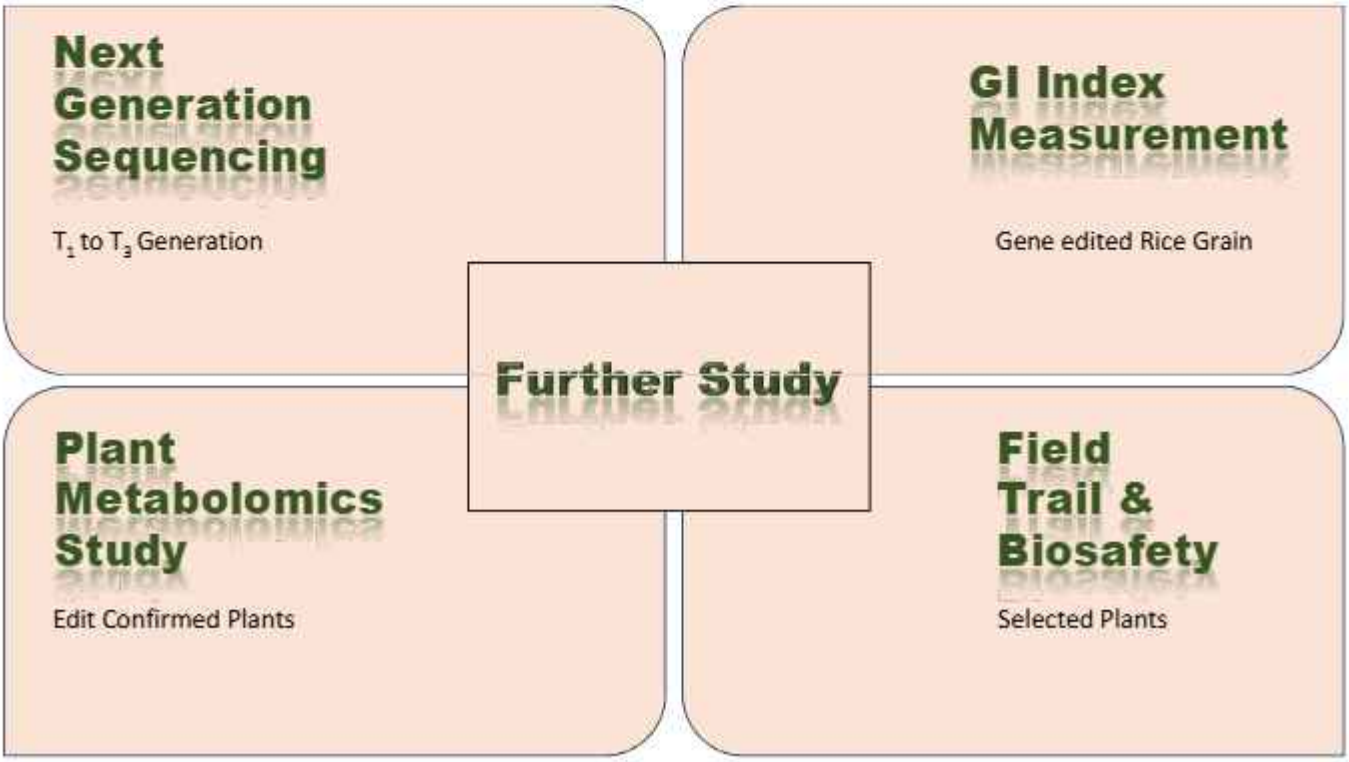
Figure: Confirmation of genetic mutation in SBE1 and SBE3 in different transgenic rice variety

Cultivation of T1 generation and mutation inheritance analysis

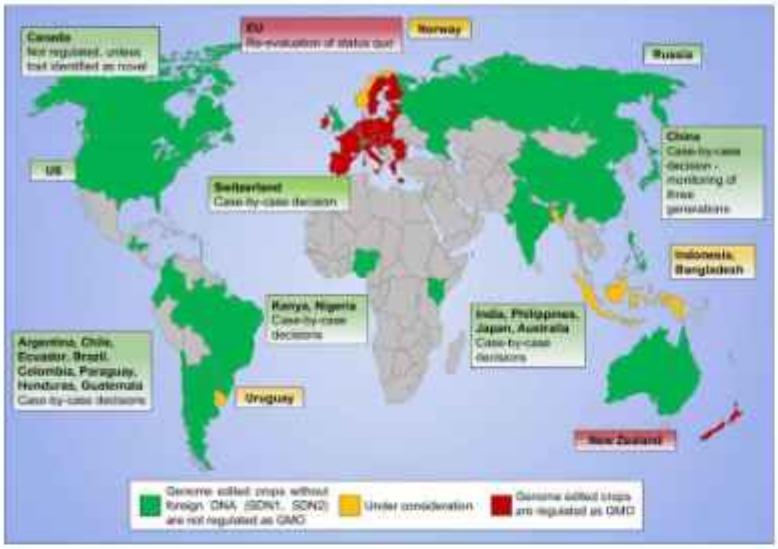
Achievements of The Project

Rice Variety	Absorbance 1	Absorbance 2	Absorbance 3	Calculated Value 1	Calculated Value 2	Calculated Value 3	Weighted Cal 1	Weighted Cal 2	Weighted Cal 3	Mean Value
A-9	0.386	0.387	0.385	1.3663	1.36996337	1.3626374	29.3286	29.328628	29.32863	29.3286276
A-33	0.357	0.355	0.357	1.260073	1.25274725	1.2600733	29.4609	29.460933	29.46093	29.4609331
A-39	0.378	0.375	0.365	1.336996	1.32600733	1.2893773	30.8625	30.862473	30.86247	30.8624726
A-44	0.36	0.362	0.373	1.271062	1.27838828	1.3186813	30.2379	30.237899	30.2379	30.2378993
BR-92	0.343	0.336	0.336	1.208791	1.18315018	1.1831502	26.0023	26.00229	26.00229	26.0022904

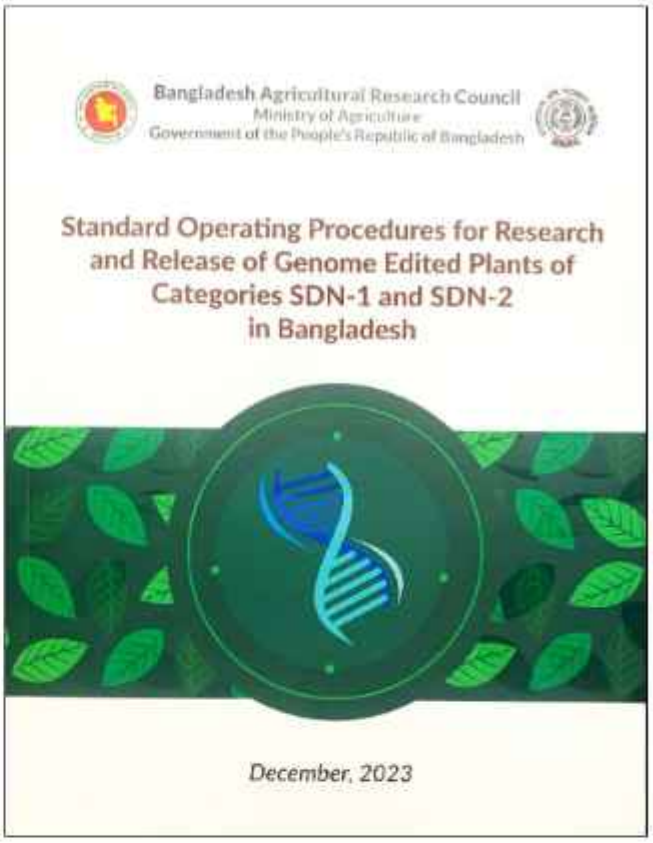
Figure: Estimated Amylose Content (%) in different edited rice variety.

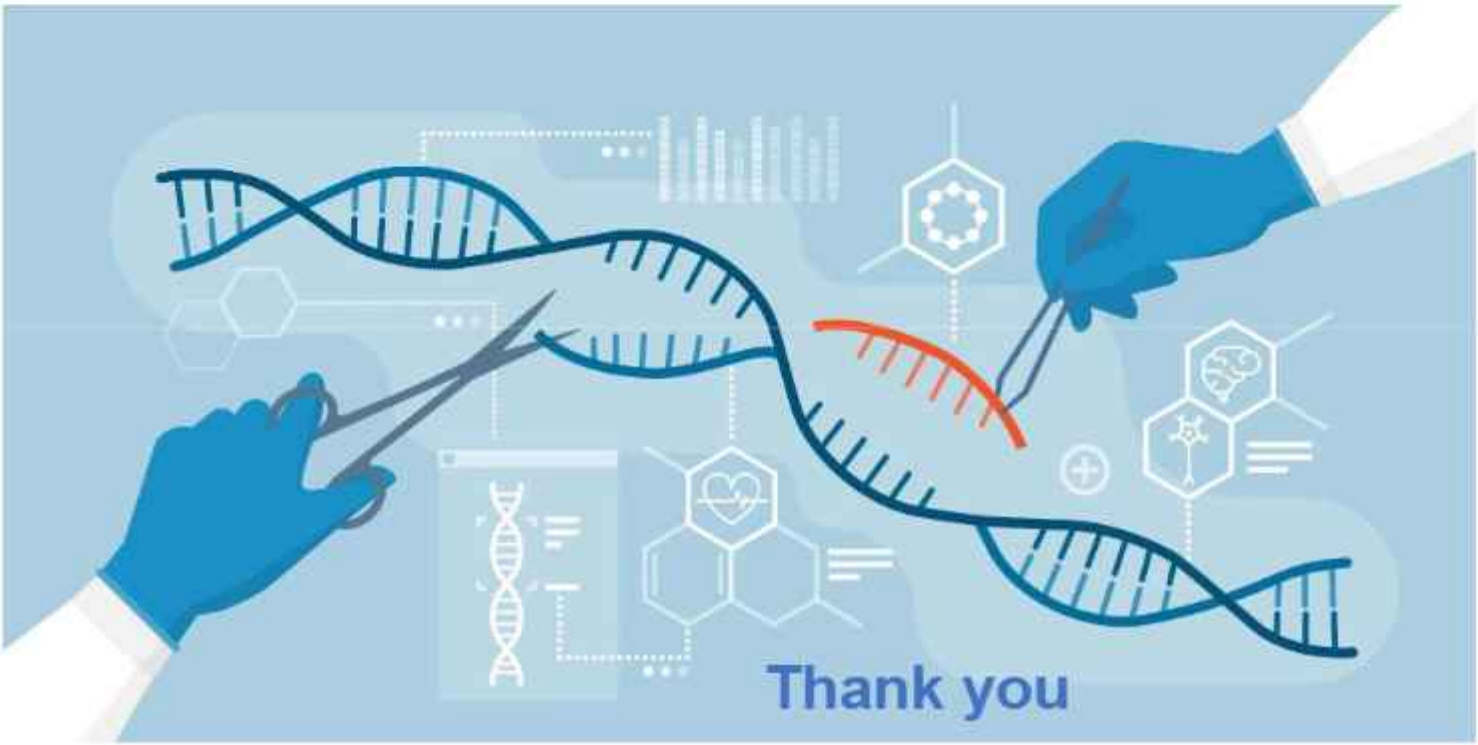


Current state of genome editing legislation



- USA and several other countries classified transgene-free, genome-edited lines as equivalent to conventionally bred lines.





Agricultural Biotech Policy and Research in Bangladesh

Training on Use of Biotechnological Tools for Varietal Development
12-16 January 2025

Md. Abdus Salam, *PhD*
Member Director (Crops)
Bangladesh Agricultural Research Council

Outline of the presentation

- Agriculture in Bangladesh: General Features
- Major Challenges of Agriculture in Bangladesh
- Transformation of Bangladesh Agriculture
- Biotech Research Priority Areas
- Biotech Pertinent Policies/Documents
- Achievements of Biotechnology/Genome Research
- Biotech Progress: BARI, BRRU, BJRI, BINA, BSRI, MWMRI, BFRI (Forest), NIB, BRAC, ACI
- Biotech Research Area: Based on the Biotechnology Policy 2012.
- Output based on Biotechnology Policy 2012: BARI, BRRI, BJRI, BINA, BSRI, MWMRI, BFRI (Forest), NIB, BRAC, ACI, BAU, BSMRAU, SAU.
- International Collaboration for GM/Biotech Research
- Way forward

Towards Building Advanced Intelligent System for Agriculture

Agriculture in Bangladesh: General Features

Major Physiographic Units (Lands)

- Alluvial Plain 78%
- Mangrove Forest 2%
- Terrace 8%
- Hill 12%

Climate: Humid Sub-tropical

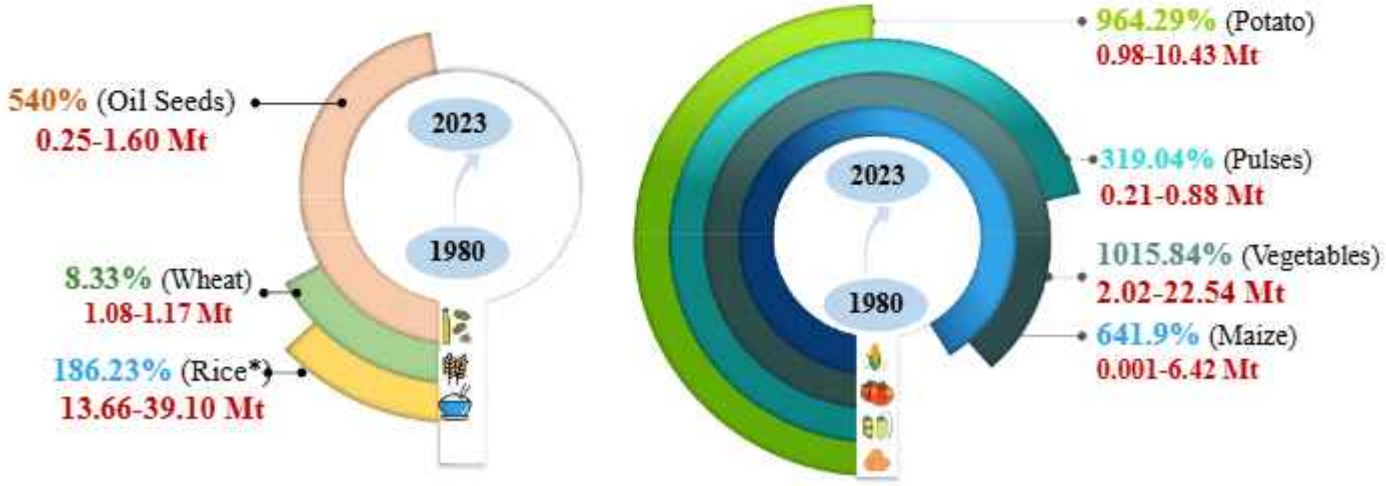
Temperature: Summer 25-30^{0}C
Winter 15-20^{0}C

Annual Rainfall: 2200 mm

7th most climate vulnerable country

- Crops are intense in floodplains
- Contribution to GDP 11.40%
- Total area 14.76 million ha
- Arable land 8.81 million ha
- Forest land 1.44 million ha (≈10%)
- AEZ 30
- **Cropping intensity 198%**
- **Rice based cropping system >50%**
- **Farm households** 16.88 million
- Population 170 million
- Population density 1,305/km^2
- Per capita income $ 2,784 (Tk 3,06,144)

Production Increased (%) of Major Crops in last 43 Years



(**Source:** MoA Annual Report, 2022-23; BBS, 2023 *Milled rice)

Towards Building Advanced Intelligent System for Agriculture

Population vs Food Production with Requirement (up to 2100)

Total food production needs to be **increased** by-

29% in **2030**

43% in **2050**

24% in **2100**

(if not otherwise constrained by severe impact of climate change)

Source: FAO & WHO (2014); BIRDEM (2013); UNEP (2021)
https://www.populationpyramid.net/bangladesh/2100/

Major Challenges of Bangladesh Agriculture

Protect arable land, land and soil degradation
(*Loosing agril. land annually 0.24% from 9.8 million ha in 1976 to 8.33 ha in 2021*)

Climate vulnerability

Production risk factors
(*Climate, biological,* and *market*)

Socio-economic factors for technology adoption
(*Education, training, income, simplicity of the technology, size of holding, farmers' awareness, access to input, access to credit, etc.*)

Food security
(Increasing population)

Productivity of ecologically constrained areas
(*Fragile ecosystems*)

Towards Building Advanced Intelligent System for Agriculture

Transformation of Bangladesh Agriculture

Green Revolution to *Gene Revolution*

Gene Revolution Era - **rapid innovations** in **biotechnology field** provide **alternative strategies** to **further improve crop yield, quality,** and **resilience towards biotic** and **abiotic stresses.**

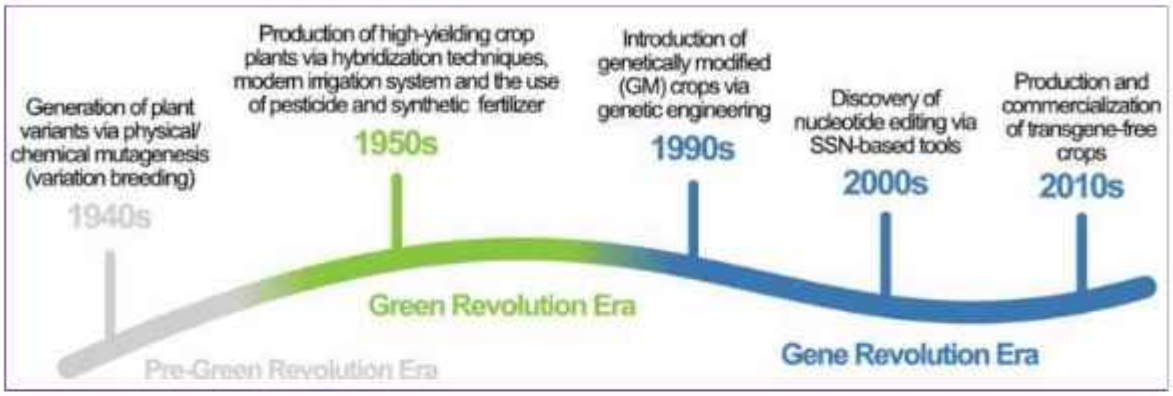


Figure : A roadmap showing Shift from Green Revolution to Gene Revolution Era.

(**Source:** Hamdan *et al.* 2022; *Plants.* https://doi.org/10.3390/plants11101297)

Application of Biotechnology

Used when all other techniques have been exhausted-

1) the trait to be introduced is not present in the GP/crop;
2) the trait is very difficult to improve by conventional breeding
3) take a very long time to introduce and/or improve such trait in the crop by conventional breeding.

Biotech Research Priority Areas

- Stress Tolerant variety (salinity, drought, heat, cold, submergence etc)
- Yield maximization (gene editing and other biotech approach)
- Crop diversification and intensification
- Agro processing

Towards Building Advanced Intelligent System for Agriculture

Potential Benefits of Agricultural Biotechnology

- Assist crop-breeders to improve the yields and quality of crops
- Through genetic modification, desirable genes can be transferred to crops irrespective of species barriers
- Plants can be made more tolerant (drought, heat, cold)
- More resistant to diseases & insect pests, reducing the input of agrochemicals and other agro inputs
- Development of crops with improved storage properties and nutritional characteristics (e.g. proteins and vitamins)

Biotech Pertinent Policies/Documents

1. Biosafety Framework 2006
2. Biosafety Guidelines of Bangladesh 2008
3. National Biotechnology Policy 2012
4. Biosafety Rules 2012
5. Guidelines for The Environmental Risk Assessment (ERA) of GEP (2016)
6. National Agriculture Policy (NAP) 2018
7. SOP for Genome Edited Plants in Bangladesh (2023)
8. National Biosafety Policy, 2024/বাংলাদেশ জীব নিরাপত্তা নীতি, ২০২৪

Towards Building Advanced Intelligent System for Agriculture

Achievements of BT/ Genome Research

Status of GM Crops in Bangladesh

BARI: 4 Bt Brinjal (2013) and CDB: 2 Bt Cotton (2023)

BARI
BFSF

			
BARI Bt Begun 1	BARI Bt Begun 2	BARI Bt Begun 3	BARI Bt Begun 4

CDB
Cotton bollworms

	
JKCH 1947 Bt cotton	JKCH 1050 Bt cotton

Towards Building Advanced Intelligent System for Agriculture

BARI: Biotech Research Progress

Protocol developed

- Microprogation of Strawberry and Banana.
- *In vitro* propagation
  - potato, sweet potato, mukhikachu.
  - gerbera, lilium, gladiolus, phaius orchid, dendrobium hybrid, orchid.
  - cassava (*Manihot esculenta* crantz).
- *In vitro* regeneration-
  - papaya, sweet gourd, okra, brinjal, tomato, chickpea, potato, soybean.
  - mungbean, field pea, lentil, country bean (BARI varieties).

- MAS of disease resistance R genes in tetraploid potato (LBR).
- Chloroplast genome sequencing and QTL analysis of heat tolerant and LBR potato varieties
- Fingerprinting of BARI released cloned potato varieties using SSR marker
- Fingerprinting of Indigenous Potato Varieties of Bangladesh using SNP Marker.
- Molecular fingerprinting eggplant, potato, onion, guava, mango and banana.
- Molecular characterization of BARI sweet potato varieties using SSR marker.
- Molecular diagnosis of sweet potato infecting viruses in Bangladesh

- *In vitro* conservation of mycelia of Oyster mushroom (*Pleurotus sp.*).

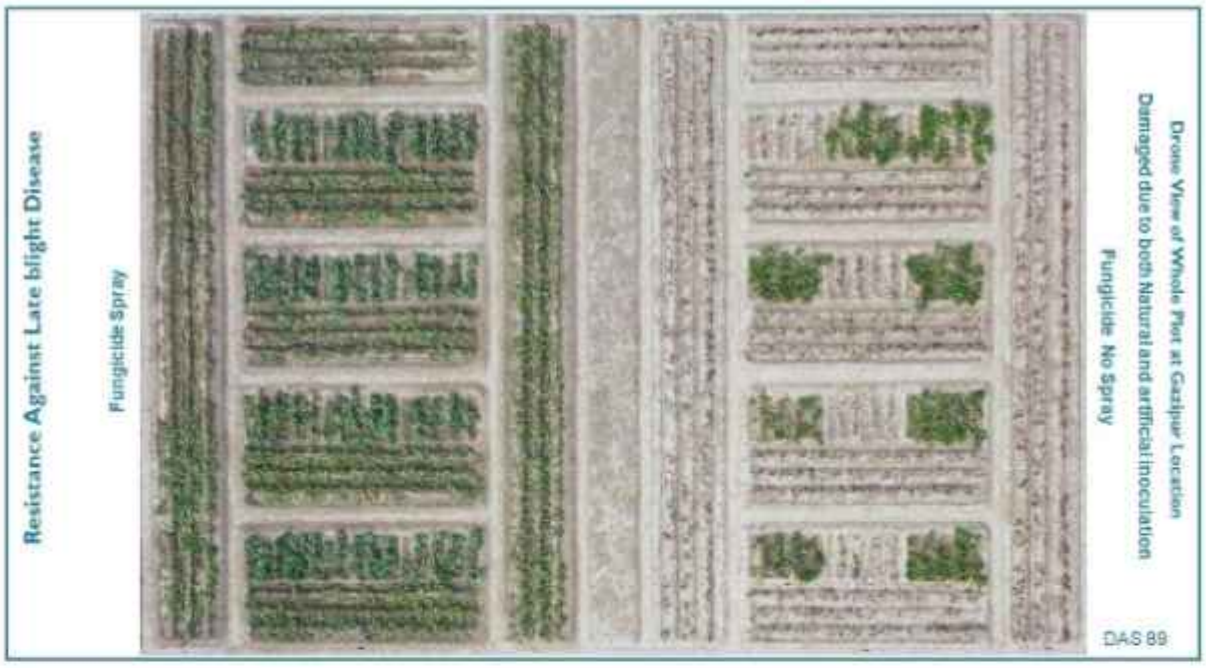
BARI: Biotech Research Progress

- Papaya ring spot virus has been detected and the coat protein sequences determined.
- MAS of wheat for bread making quality and heat stress tolerance (BARI wheat varieties).
- Salt tolerant wheat lines developed using marker-assisted transfer (*Nax* genes).
- Genome sequencing: 32 tomato leaf curl virus isolates and their molecular characterization.
- Waterlogged tolerant 2 sesame genotypes confirmed by *SiWRKY* gene expression.
- Molecular characterization of sunflower mutants for dwarfism and high oleic acid have been done.
- Transformation of tomato for resistance against tomato Leaf Curl Virus.
- Genome editing for increasing the shelf life of tomato.
- Cloning, characterization and transformation of biotic and abiotic stress tolerant gene (Na^+, K^+, H^+ transporters) of *Arabidopsis*

Towards Building Advanced Intelligent System for Agriculture

Confined field trial of 3R-gene LBR potato

BARI



BRRI: Biotech Research Progress

Developed Rice Variety

MAS: BRRI dhan51,52, 78, 79, 101, 109, 110 (7 variety)
Anther culture: BRRI dhan86, 92, 103 (2 variety)
Embryo culture: BRRI dhan58, 88 (2 variety)

Development of vitamin-A rich golden rice

- Vitamin-A rich BRRI dhan29 golden rice developed and applied for commercial release.
- Pro-vitamin-A synthesizing gene is introgressed in the background of BRRI dhna48, 67, 71, 84, 87 and 89 (on six varieties).

Golden rice event (GR2-E BRRI dhan29)

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BRRI: Biotech Research Progress

QTL identified (Stress and quality)

- *qSL1* - Shoot length for salinity tolerance from Akundi
- *qK8* - K^+ Concentration for salinity tolerance from Akundi
- *qPN6.1* - Panicle Number from *O. rufipogon*
- *qF1.1* - Percent fertility from *O. rufipogon*
- *qGZN13.1* - Grain Zn content from Kalobokri
- *qCTS1.1* - Cold tolerance at seedling stage
- *qRTVR10.4* - Tungro virus resistance from Kumragoir
- *qAsTRLT8* - Root length for As phyto-toxicity tolerance from BRRI dhan47
- *qSES1.3 and qST12.2* - Salinity tolerance and Sterility percentage
- *qB5* - Blast resistance from Basmati380
- *HATPase* - Isolated from *O. coarctata* and construct was made to overexpress

BRRI: Biotech Progress

Gene introgressed (for stress and quality)

- Salinity tolerant (QTL *SALTOL) into* BRRI dhan28 and 49;
- Flash flooding tolerant (QTL *SUB1)* into BR22, BRRI dhan33, 44, 49, 79;
- Seedling stage cold tolerance (QTLs HbjBVI) into BRRI dhan28;
- Three salinity tolerance QTLs from Horkush into BRRI dhan63, 74, 67 and SNP markers designed;
- Heat tolerant QTL *qHTSF4.1* into BRRI dhan28 and 29;
- Provitamin A (2E) into BRRI dhan28, 48, 67, 71, 84, 87 and 89;
- Three events (IRS1030-039,-031, -059) of high Iron and Zinc traits into BRRI dhan71, 81, 87, 89, 98;

- MAS-for fragrance gene, aromatic and submergence tolerance, developing short stature Biroi rice;
- Cloning of drought tolerant genes from wheat;
- Development of GI rice, antioxidant enriched black rice, Protein enriched rice, high-yielding colored and low-amylose content rice for hill ecosystem;

Genome Editing for Targeted Gene in Rice

Genes of interest

- *OsRR22* gene for improving salinity tolerance (BRRI dhan89, 92)
- *OsERF922* gene for improving blast resistance (BRRI dhan28, 81)
- *TMS5* gene for developing male sterile rice line for two-line hybrid system
- *CYP71A1* gene for the suppression of serotonin synthesis for insect control (BRRI dhan89, 92)

Status of Genome Editing

- Plants were regenerated, and Cas9 +ve plants selected using PCR.
- The Cas9 +ve plants sequenced to detect mutation in the target DNA of the selected genes.

Genome edited plants in BRRI (CRISPR Cas9)

For IR : BPH BRRI dhan 92)


জিন বদলে এলো প্রথম সাফল্য

BJRI : Achievements of Genome Research Center

- BJRI Tossa Pat 8 (Robi-1) variety has been developed that has about 20% yield advantage than conventional varieties (genome sequencing).
- Three advanced lines (One Tossa and two Deshi jute) developed which are now in field evaluation stage.
- Using Genome information, BT Jute, Fungal Resistant, Low Lignin content, Salt Resistant, Drought Resistant Variety Development on-going.
- Bacterial Consortia has been developed for rapid retting of jute.

Whole Genome Sequence
- *Corchorus olitorius – 2020*
- *Corchorus capsularis- 2013*
- *Macromophina phaseolina – 2012*
- *Sesbania sesban - 2018*

Transgenic research in jute
- Short duration - Gen 5 (evaluation stage)
- Low lignin content- Gen 3 (evaluation stage)
- Stem rot resistant - Gen 4 (evaluation stage)
- Wilt resistant - Gen 1 (evaluation stage)

BINA: Biotech Research Progress

- Regeneration Protocol developed : rice, tomato, mustard, mungbean and wheat.
- One salinity and drought tolerant gene *OsNHX2* transferred to BINA tomato 12 and BARI tomato 18.
- Four salinity and drought tolerant gene construct (*OsARP, OsNHX2, OsDREB* and *OsHKT8*) prepared from rice.
- Five deep water and two boro rice potential lines developed through callus irradiations.
- Three submerged and tidal tolerant rice line developed through MAS.
- Three new bacterial sps discovered from lentil (*Rhizobium lentis, R. binae* and *R. bangladeshense*).

Biotech Research Progress

BSRI

Micropropagation
- Around 10,000 plantlets and somaclones from eight sugarcane varieties.
- Plantlets have been produced from BSRI Stevia 1.

- Protocol of Arabian date palm and Palmyra palm (Micropropagation and callus culture).
- Drought and salinity resistant gene(PsCBL and PsCIPK) transferred in Isd 37 and 39.
- Five red rot resistant mutants selected at molecular level by different markers analysis.
- Quality parent selection of sugarcane, sugar beet, stevia, Arabian date palm, and palmyra palm by using SSR, SNP, and ISSR marker.
- Selection of disease resistant somaclones of different sugar crops.
- Molecular characterization of 15 sugarcane varieties.

BWMRI

- Developed BARI gom 33, BWMRI gom 1 & 5, using molecular markers.
- Seven heat-resistant lines identified using molecular marker.
- Protocol for double haploid technology developed (for heat and disease resistant).
- Detection of wheat blast pathogen in 250 isolates (out of 380) using molecular markers (MoT3/ITS).
- Genome sequencing of 87 isolates completed, and the analysis of these sequences is ongoing.
- DNA fingerprinting of 28 wheat varieties using 13 SSR markers.

BFRI (Forest): Biotech Research Progress

BFRI (Forest)

- DNA sequence of ITS2 and rpoC2 genes of Shada garjan, Teli garjan and Bailam has been achieved under barcoding research.
- Shoots produced on culture of Taxodium and Boilam.
- TC protocol developed for Asper and Moso bamboo.
- Root induction and optimization of ten village bamboos.
- Induced tap roots in *in vitro* grown Rubber shoots and transferred to soil.

NIB

- Developed Leaf-spot-resistant variety 'NIB Aloe Vera-1'
- About 200 *in vitro* large cardamom plantlets produced through TC.
- A gene construct necessary for beta-carotene synthesis in eggplant has been developed. Five transgenic lines evaluating in a greenhouse.
- Transferred pRGEB32_sgRNA_SBE1_SBE3 construct in BRRI dhan92 using Agrobacterium-mediated transformation. Ten lines showed positive phenotype and found significant changes in amylose (rice) concentration.

Biotech Research Progress

BRAC

Plantlets produced (numbers)

- Potato (12 variety): 22,74,583
- Banana (47 variety): 36,000
- Strawberry (2 variety) : 5,000
- Stevia : 256,000
- Ornamental plants (Gerbera, orchid, cactus) : 5,000

Using SSR marker 12 Blast resistant rice genotypes identified.

ACI

- Developed and Released 29 Potato varieties of different types (Short duration, Industrial type, long shelf life, LBR, Anti-oxidant rich) through MAS.
- 2.3 Lakh plantlets produced through Meristem culture from 78 germplasm in 2024.
- Diversity Analysis of 20 Newly Developed Wheat Cross Lines Using Molecular Techniques.
- High-yielding & Disease Resistant Wheat lines developed through molecular markers.
- LBR potato variety development through hybridization followed by MAS.

Biotech Research Area: Based on the Biotechnology Policy 2012

Short term: 2 Yr (2017-18; 18-19);
Medium term: 5 Yrs (2017-18 to 21-22);
Long term: 10 Yrs (2017-18 to 26-27)

1.	Improvement of Tissue culture/Micro-propagation System for quality, disease free seedling/saplings in important crops
2.	Marker Assisted Selection for trait specific improvement
3.	Transgenic plant development: nutrient enrich, insect-pest and disease resistant, stress tolerant, climate resilient crops
4.	Crop improvement through gene transfer (gene identification, isolation, and characterization)
5.	Molecular characterization of PGRs and agriculturally important micro- organisms
6.	Genome sequencing for specific use of important crops and medicinal plants
7.	Introduction, evaluation and monitoring of transgenic crop
8.	Identification of plant diseases at molecular level

Output based on Biotechnology Policy 2012

(Short term- 2 yr; Medium term- 5 yr): 2017-2022

BARI

- Protocol development: Micropropagation of BARI strawberry-2 & 3 and BARI banana-3 & 4.
- Improvement of plantlet production method of potato, sweet potato and cassava.
- Biofortified and anthocyanin rich potato plantlet production.
- Fingerprinting of 21 indigenous potato varieties.
- Preparation of molecular plasmid vector for development of virus resistant tomato varieties.

BRRI

- Developed 175 doubled haploid and 1130 somaclonal inbred rice lines.
- QTL fingerprinting of 835 rice genotypes.
- DNA fingerprinting of 96 local rice varieties.
- CFT of Golden Rice conducted in 8 locations across the country
- Using molecular markers-
  - Developed 21 advance inbred lines resistant to rice leaf blight disease.
  - Developed submergence inbred lines by transferring Sub1 QTL in BRRI dhan62 and 22.
- Developed 300 advance inbred lines resistant to rice tungro disease.

Output based on Biotechnology Policy 2012

(Short- 2 yr; Medium- 5 yr): 2017-2022

BJRI

- Identification of genes related to flower production and flower retention using jute genome data and bioinformatics analysis.
- Molecular characterization of 69 jute germplasm including 13 jute varieties using SSR primers.

BINA

- Developed two somaclonal variants from BINA tomato-12 and one somaclonal variant from BINA tomato-11.
- Selection of 02 tidal flood resistant lines by applying gamma-ray on embryogenic callus of Sadamota rice.
- Transfer of salinity and drought tolerant gene to tomato varieties
- DNA fingerprinting of 21 BINA developed rice varieties.
- Isolation of 15 Rhizobium strains of Faba bean.

BSRI

- Plantlet production of six sugarcane varieties (Isd 16, 37, 39, 40 and BSRI Sugarcane 41, 42) through micropropagation.
- DNA fingerprinting of seven sugarcane varieties (Isd 34 to Isd 40)
- Identified 34 sugarcane red rot tolerant and 30 susceptible somaclones.

Towards Building Advanced Intelligent System for Agriculture

Output based on Biotechnology Policy 2012

(Short- 2 yr; Medium- 5 yr): 2017-2022

BWMRI	• Developed 42 wheat lines through double haploid technology for blast resistant. • Five wheat lines and BARI gom 33 identified as blast resistant using molecular markers. • DNA fingerprinting of 28 wheat varieties using SSR markers.
CDB	Released cotton bollworm resistant varieties (JKCH 1947 Bt and JKCH 1050 Bt).
BRAC	Plantlet production of potato, banana, strawberry, gerbera, chrysanthemum and stevia.
ACI	• Potato plantlet production. • Detection of Late Blight pathogen (*Phytopthora infestans*) of potato, YVMV of pumpkin and MV of cucumber using molecular markers.

Output based on Biotechnology Policy 2012

(Short- 2 yr; Medium- 5 yr): 2017-2022

BAU	• Molecular characterization of rice blast resistance genes. • Selection of bean and tomato mosaic virus resistant lines. • Developed induced hexaploid wheat.
BSMRAU	• Identified gene responsible for aroma from fragrant rice. • Developed four S-gene mutant wheat lines resistant to wheat blast. • Genome sequence of jackfruit.
SAU	• Development of protocols for *in vitro* regeneration of potato, sweet potato, ginger, aloe vera, pineapple, turmeric and orchids. • Molecular diversity of brinjal, potato, turmeric, ginger, cotton, capsicum and rice germplasm (7 crop)

Towards Building Advanced Intelligent System for Agriculture

International Collaboration for GM/Biotech Research

- Cornell University, USA (*Bt brinjal*)
- Michigan State University, USA (*3R gene potato*)
- Global Institute for Food Security (GIFS), University of Saskatchewan, Canada
- Consultative Group of International Agricultural Research (CGIAR): IRRI, CIMMYT
- South Asia Biosafety Programme (SABP)

Way Forward

- Strengthen national capability and core research infrastructures for Biotechnology innovations
- Skill development in frontier biotechnologies and research capabilities
- Strengthen collaboration with int. and regional res. org. and development partners
- Effective integration of knowledge, information, and resources on the current status and trends of commercial agriculture
- Strengthen inter-ministerial coordination for market intelligence and marketing
- Establishment of legal and conducive environment to promote PPP in R&D
- Promote collaboration and linkage among local organizations
- Ensuring fund for implementing demand driven biotech research specially for time bound research.

Thanks to all

Topic: DNA Extraction, Visualization and Analysis

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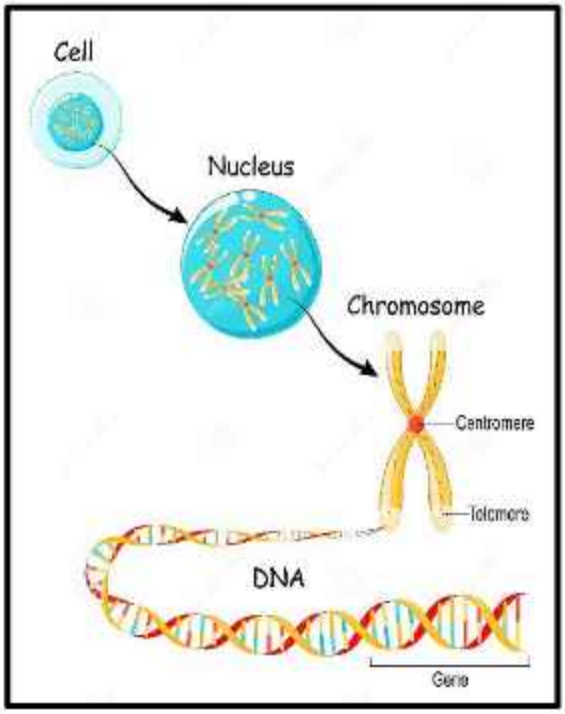
What is DNA?

DNA stands for Deoxyribonucleic Acid. It is a molecule that carries the genetic instructions necessary for the growth, development, functioning, and reproduction of all known living organisms and many viruses. DNA is often referred to as the "blueprint of life" because it contains the instructions that make each organism unique.

Key Features of DNA:

Structure:

DNA has a double helix structure, resembling a twisted ladder. It is composed of two long strands of nucleotides running in opposite directions.



Each nucleotide consists of:

- A phosphate group.
- A sugar molecule (deoxyribose).
- A nitrogenous base [Adenine (A); Thymine (T); Cytosine (C) and Guanine (G)]

These bases pair specifically:

Adenine pairs with Thymine (A-T).

Cytosine pairs with Guanine (C-G).

Function:

DNA stores genetic information used to produce proteins, which perform most biological functions. It undergoes replication to pass genetic information from one generation to the next.

Mutations (changes in DNA sequences) can lead to variations, some of which are beneficial, harmful, or neutral.

What is DNA Extraction?

DNA extraction is a process that used to purify DNA by using chemical or physical methods from a biological sample separating DNA from protein, cell membranes, and other cellular components. It is the first critical step in many molecular biology techniques, including polymerase chain reaction (PCR), sequencing, cloning, and forensic analysis. Extracting DNA in a pure form is essential because it allows scientists to study the genetic material without interference from other cellular substances.

What is DNA Extraction Process/Principle?

DNA extraction is a fundamental technique in molecular biology, enabling scientists to isolate and study genomic DNA. The process typically involves several key steps: cell lysis, removal of proteins and other cellular components, and finally, the isolation of purified DNA.

- **Cell Lysis:** The first step, cell lysis, involves breaking open the cell membranes to release the cellular contents. This is often achieved using physical methods (like vortexing) and chemical reagents (such as SDS, or sodium dodecyl sulfate). The goal is to disrupt the cell sufficiently to release the nucleic acid (DNA and RNA) contained within.
- **Removal of Proteins & Other Contaminants:** After lysis, proteins, lipids, and other cellular debris must be removed to extract DNA in its pure form. Protease enzymes are commonly used to digest proteins, while organic compounds like phenol and chloroform help separate DNA from other cellular components. Centrifugation is a critical step here, aiding in separating different layers, with DNA remaining in the aqueous phase.
- **DNA Isolation & Purification:** The final step involves isolating and purifying the genomic DNA from the mixture. This usually involves the addition of ethanol or isopropanol, which precipitates DNA. In some methods, the RNase enzyme is added to remove RNA. The DNA precipitate is then washed with a cold alcohol solution, such as ethanol, to remove any remaining contaminants. After washing, the DNA pellet is dried and dissolved in a suitable buffer like TE buffer (Tris-EDTA).

Various DNA extraction methods can be employed throughout this process, ranging from traditional organic extraction to modern DNA extraction kits that utilize spin columns and silica membranes. The choice of method often depends on factors such as the type of starting material, desired yield of DNA, and the specific requirements of downstream applications like PCR, gel electrophoresis, DNA sequencing, or cloning.

The Key Components and Reagents for DNA Extraction

The effectiveness of DNA extraction largely depends on the quality and specificity of the components and reagents used. Understanding these elements is crucial for optimizing the extraction of DNA, ensuring its purity and suitability for further analysis, such as PCR, sequencing, or cloning.

- **Detergents & Surfactants (SDS):** Detergents like Sodium Dodecyl Sulfate (SDS) play a critical role in cell lysis, helping to break down cell membranes and nuclear envelopes. This disruption releases DNA into the solution by dissolving lipids and proteins that make up cellular and nuclear membranes.
- **Enzymes (Protease, Proteinase K, RNase):** Enzymatic treatment is essential for degrading proteins and RNAs that could contaminate the DNA sample. Protease and Proteinase K are commonly used to digest protein contaminants. RNase is specifically used to remove RNA, ensuring the isolation of pure genomic DNA.
- **Solvents (Phenol, Chloroform, Isopropanol, Ethanol):** Organic solvents like phenol and chloroform are employed to extract DNA in the phase separation step. They help in removing proteins and other organic contaminants. Alcohols like isopropanol and ethanol are crucial in the DNA precipitation step, where they facilitate the aggregation and settling of DNA, forming a DNA pellet.
- **Salts (Sodium Acetate, EDTA):** Salts are used to stabilize and protect the DNA. Sodium acetate aids in DNA precipitation, while EDTA (Ethylenediaminetetraacetic acid) is used to chelate divalent cations, inhibiting the activity of nucleases that could degrade the DNA.
- **Buffers (TE Buffer, Phosphate Buffers):** Buffers like TE buffer (a mixture of Tris base and EDTA) are used to resuspend and store the purified DNA. They help maintain the pH and stability of the DNA sample, ensuring its integrity for downstream applications.

Each reagent in DNA extraction has a specific and essential role, from breaking down cells to purifying and protecting the DNA. The choice and quality of these components directly affect the yield, purity, and success of downstream processes like amplification, sequencing, and analysis. Using high-quality reagents and well-optimized protocols ensures reliable DNA extraction and enhances experimental outcomes.

Common Methods of Plant DNA Extraction

1. CTAB Method (Cetyltrimethylammonium Bromide): This is a widely used method for plant DNA extraction, particularly for samples with high polysaccharide and phenolic compound content. This method is a specially prepared liquid-liquid and solution-based extraction method for plant DNA extraction. The CTAB buffer removes polysaccharides and polyphenols effectively and gives excellent yield for plant DNA. This method required extensive chemical preparation and additional techniques like tissue homogenization and the use of liquid nitrogen. It also takes a long time.

2. Silica Column-Based Extraction: A commercial method utilizing silica membranes to bind DNA, offering high purity and speed. Spin column extraction involves passing the lysed sample through a silica gel membrane within a centrifuge tube. DNA binds to the silica membrane under high salt conditions and is eluted after washing steps. This method is prized for its simplicity, speed, and efficiency, making it a popular choice in research and clinical laboratories.

3. Magnetic Bead-Based Extraction: A modern approach that uses magnetic beads to bind DNA, ensuring minimal contamination. This method employs tiny magnetic beads coated with silica. DNA binds to the silica surface in the presence of certain salts. When a magnetic field is applied, the beads (with the bound DNA) are pulled to the side of the tube, allowing for easy removal of contaminants. This technique is known for its high purity yield and is suitable for automated high-throughput DNA extraction.

4. Phenol-Chloroform Extraction: A traditional method employing organic solvents for DNA purification, effective but labor-intensive.

5. Alkaline Lysis Method: A simple and rapid method for DNA extraction from young plant tissues with low secondary metabolites.

6. Automated DNA Extraction Systems: Automation has significantly improved DNA extraction, especially regarding reproducibility and throughput. Automated systems use robotic mechanisms to perform cell lysis, DNA binding, washing, and elution in a closed system, minimizing the risk of contamination and human error. These systems are ideal for laboratories handling large sample volumes or requiring consistent results.

Key steps for DNA extraction

1. Tissue Preparation: Collect 100 mg of fresh or frozen plant tissue and grind it into a fine powder using liquid nitrogen.

2. Lysis: Add 500 µL of preheated CTAB buffer (65°C) and incubate at 65°C for 30 minutes with occasional mixing.

3. Phase Separation: Add an equal volume of chloroform:isoamyl alcohol (24:1), mix thoroughly, and centrifuge at 12,000 rpm for 10 minutes.

4. DNA Precipitation: Transfer the aqueous phase to a new tube and add an equal volume of isopropanol. Incubate at -20°C for 30 minutes.

5. Pellet Washing: Centrifuge at 12,000 rpm for 10 minutes, discard the supernatant, and wash the DNA pellet with 70% ethanol.

6. Resuspension: Air-dry the pellet and dissolve it in TE buffer.

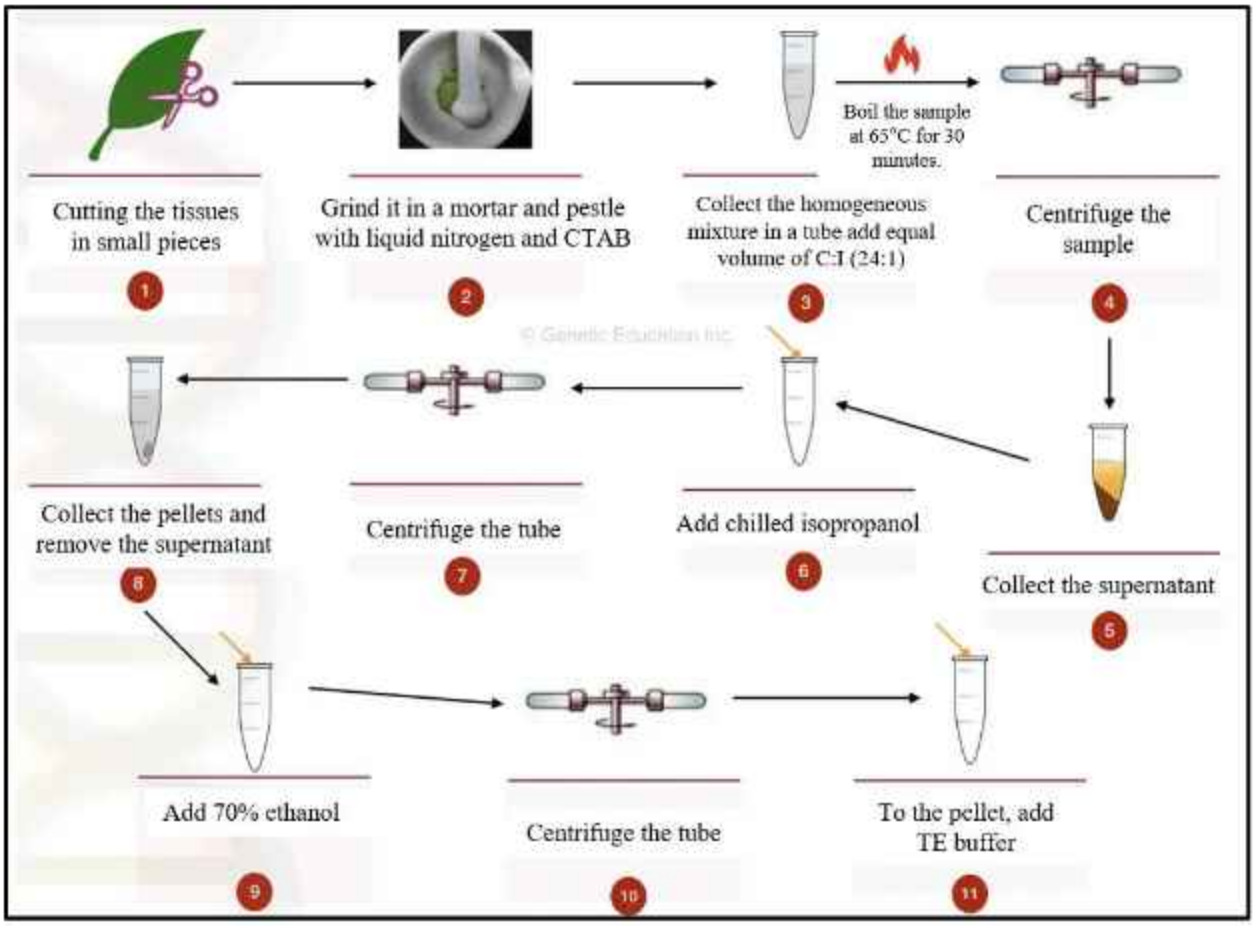


Illustration of the process of plant DNA extraction

DNA Visualization

Visualization confirms the successful extraction of DNA and evaluates its integrity.

Techniques for Visualization

1. Agarose Gel Electrophoresis

- DNA is separated by size in an agarose gel matrix under an electric field.
- Ethidium bromide (EtBr) or safer alternatives like SYBR Safe are used for staining DNA.
- A UV transilluminator is used to visualize bands, indicating the presence and size of DNA.

2. Qubit, Nanodrop or Spectrophotometric Analysis

- Measures DNA concentration and purity based on absorbance at 260 nm and 280 nm.

3. Fluorescent Dye-based Methods

- Uses fluorescent intercalating dyes for highly sensitive detection of DNA.

DNA Analysis

DNA analysis provides insights into the genetic information encoded in DNA and enables applications such as gene identification, phylogenetics, and breeding programs.

Common Techniques

1. Polymerase Chain Reaction (PCR)

- Amplifies specific DNA sequences for further study.
- Used for marker-assisted selection and genetic diversity studies.

2. DNA Sequencing

- Determines the nucleotide order of DNA, providing comprehensive genetic information.
- Enables gene identification and functional annotation.

3. Restriction Fragment Length Polymorphism (RFLP)

- Analyzes DNA polymorphism by digesting DNA with restriction enzymes and separating fragments via electrophoresis.

4. Microarray Analysis

- Allows parallel analysis of thousands of DNA sequences, identifying genes associated with specific traits.

5. Next-Generation Sequencing (NGS)

- Offers high-throughput analysis for genomic and transcriptomic studies, accelerating varietal development.

Common issues found in plant DNA extraction

Generating sufficient yield and quality of DNA during plant DNA extraction is more difficult than in animals because of the plant's rigid cell wall. Furthermore, plants also contain varying levels of

carbohydrates or polyphenols which combine with nucleic acids during DNA isolation and further affect the quality of the extracted DNA.

These plant components have a similar nucleic acid structure that allows secondary metabolites and polysaccharides to interfere with total DNA isolation. Due to their chemical properties, polysaccharides co-precipitate with genomic DNA, giving viscous solutions.

Phenolics are chemicals which, once released from plant tissue, irreversibly bind to the phosphate backbone of DNA and generate the typical browning observed in plant tissues. Both contaminants hamper the use of DNA for molecular biology purposes, such as restriction digest, PCR, or sequencing (like Next Generation Sequencing), by inhibiting the action of polymerases or endonucleases.

Some plant taxa are also more likely to contain a high level of specific metabolites, making DNA extraction harder. For instance, cereals are rich in carbohydrates, whereas stressed plants are rich in polyphenols. A way to overcome these issues is to look for protocols that specialize in eliminating these contaminants.

Factors to consider while choosing the suitable method for your DNA extraction

You can choose the best DNA extraction method for your project by checking the following factors:

- **The type of Sample:** each type of sample (Bacteria, animal tissue, plants, water, blood, soil, saliva) requires a specific method to extract the DNA. Your type of sample is in fact the most crucial factor to consider when selecting a DNA extraction method.
- **Plasmid DNA vs Genomic DNA:** The protocol for plasmid DNA isolation is different from the one used to isolate the genomic DNA. The difference is mainly in the lysis process, and some buffer formulations.
- **Downstream applications:** The quality and purity of extracted DNA must fulfill the requirements of the downstream applications.
- **Sample quantity:** Some methods are designed specifically for a certain amount of sample.
- **Yield:** The DNA extraction method you choose should meet the yield expectations for your purpose.

Conclusion

Selecting an appropriate DNA extraction method depends on the plant species, tissue type, and downstream applications. While traditional methods like CTAB and phenol-chloroform offer high yields, modern methods like silica column and magnetic bead-based extraction provide higher purity and ease of use. Each method has its unique advantages and limitations, making it essential to choose based on experimental needs.

Primer designing for varietal development

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A primer is a short single-stranded DNA fragment used in many molecular techniques from polymerase chain reaction (PCR) to DNA sequencing. There can be a set of primers (forward and reverse) with a sequence complementary to the template DNA -a point of initiation synthesis.

How primers work:

DNA polymerases can only add new nucleotides to an existing DNA fragment, so primers are required for DNA replication.

Types of primer:

There are different types of primers used in molecular biology, including:

Gene-specific primers: These are oligonucleotide primers that guide polymerases to amplify gene-specific sequences.

Non-specific primers: These include random hexamers (oligodeoxyribonucleotides of random sequence [d(N)6]) and oligo-dT primers, which are used to synthesize cDNA and DNA hybridization probes.

***DNA oligos*:** These are used as primers for DNA sequencing. DNA oligos are engineered to anneal specific target sequences.

Importance of primer:

Primers are used in several molecular biology techniques, including:

Polymerase chain reaction (PCR): In PCR, a pair of primers hybridizes with a sample DNA to define the region to be amplified.

DNA sequencing: Primers are used in DNA sequencing.

Recombinant DNA technology: Primers are used in recombinant DNA technology methods such as restriction cloning and DNA assembly methods.

Molecular cloning: Primers are used to isolate the target gene to be cloned.

***Genome editing*:** Making Single-guide RNA (sgRNA) for the CRISPR-Cas9 genome editing system, which is used to cut double-stranded DNA.

Degenerated primer:

A degenerate primer is a mixture of similar oligonucleotides used in a PCR to amplify unknown DNA sequences. The primers are designed based on sequence data from related genes that have already been sequenced.

The process of designing degenerate primers is called degenerate primer design (DPD). DPD involves designing a pair of primers that match and amplify as many input sequences as possible. The degeneracy of the primer must be bounded to reduce the chance of amplifying non-related sequences.

- Degenerate primers are useful when the exact nucleotide sequence of the target DNA is unknown but can be inferred from an amino acid sequence.
- Degenerate primers are also useful when a population of organisms is evolving rapidly or is highly diverse.

Some challenges of using degenerate primers include:

- Only a limited number of primer molecules complement the template within a degenerate primer mixture.
- The melting temperature (Tm) of primer sequences may vary significantly.
- The sequences of some primers can be complementary to those of others.
- The likelihood of amplifying unwanted alternative products increases as the number of degenerate bases specified in a pair of primers rises.

Allele specific primer

Allele-specific primers are oligonucleotide primers that are used to detect specific alleles in DNA. They are used in a variety of techniques, including:

***Allele-specific polymerase chain reaction (ASPCR)*:** A PCR technique that uses allele-specific primers to detect point mutations in DNA. ASPCR is used to diagnose genetic and infectious diseases.

***Allele-specific primer extension (ASPE)*:** A technique that uses an enzymatic reaction to determine a target genotype, and then captures it on a solid microsphere surface for detection.

***Allele-specific probe and primer amplification assay (ASPPAA PCR)*:** A real-time PCR method that uses allele-specific primers and probes to quantify SNPs in DNA.

Allele-specific sequencing primers

A method that uses allele-specific primers to directly sequence separate alleles.

Here are some tips for designing allele-specific primers:

- Place the SNP site at a penultimate base in each primer to increase reaction specificity.
- Design the 3′ end of each primer to be unique and specific to its target.

- Use short primers, around 15-13 nucleotides, to hinder the amplification of non-target alleles.
- Optimize the primer sequences for similar thermodynamic parameters.

Primer designing criteria

***Primer uniqueness*:** Only one target site in the template DNA where the primer will be attached, which means the primer sequence will be unique in the template DNA

Primer length: Oligonucleotides between 18-24 are said to be quiet enough and advantageous so that short primers would bind easily to the template at the annealing temperature.

GC content: The G-C content should be in the range of 30% to 80%, with 50% to 55% being ideal. If the primers G-C content is less than 50%, the length of the primer may need to be increased to maintain the proper Tm.

Melting temperature (52°C-56°C): The GC results of the sequence gives a fair indication of the primer Tm. However, the difference of the primer should not be less than 2°C.

Primer Annealing (Ta): The high Ta results in low PCR product with insufficient primer-template hybridization, while too low Ta will lead to non-specific PCR products caused as a result of a high number of base pairs mismatches.

Ta= 0.3*Tm (primer) +0.7 (product) – 14.9, Tm (primer)

Melting Temperature of the Primer:

Tm (primer)- It measures the least stable primer-template pair.

Tm (product)- It measures the melting temperature of the PCR product.

The modified step annealing can be performed using gradient PCR where temperature can be set to bind primers.

3'-clamp properties: GC bp with 3 H bonds that are stronger than AT bonds with 2 bonds with the high stability of the primer along with the improvement and specificity of the primer binding. Presence of a G or C base in the last 5 bases (the 3′ end) of a PCR primer. This can help to improve the specificity of primer binding to the complementary sequence

Care need to be taken during primer designing

The primers can be formed as following types:

***Hairpins*:** The loop structure formed by the intramolecular interactions within the primer which optimally 3' end with -2kcal/m and internal hairpin with -3kcal/m can be tolerated.

Dimers: A structure forming ds DNA by intermolecular interactions between 2 primers. Likewise, if the interaction formed between 2 homologous or the same sense of primer, – called as self-dimers while the opposite primers are called as cross dimers.

Repeats & Run: The consecutive occurrence of dinucleotide runs in the continuous stretch of a single nucleotide is considered the most important property. The maximum no. of repeats and runs was of 4 dinucleotides and 4 base pairs.

Primer- Template Cross Homology: Primers should be designed in such a way that no homology within the template is been noticed other than the target site which resulted in non-specific binding and amplification. This can be categorized into 2 types:

a) **Intra-primer homology:** The complementary bases within the same pair in the region of more than 3 bases can cause intramolecular bonding

b) **Inter-primer homology:** Forward and reverse primers with complementary sequences are responsible for intermolecular bonding.

Analyzing primer dimer formation is the primary important caution to be taken care of. However, it involves the determination of Gibbs free energy which aids to be the one. Although 5' end was found to be more reliable than 3' end.

Important links for primer designing

1. Primer designing tool (nih.gov)
2. Primer3 Input
3. Primer3Plus (bioinformatics. NL)
4. PrimerQuest – design qPCR assays | IDT (idtdna.com)
5. PerlPrimer (sourceforge.net)
6. Primer Design with Oligo Primer Analysis Software v. 7
7. Real-Time PCR Primer Design – Real-Time PCR Probe Design – GenScript
8. www.autoprime.de
9. https://www.genscript.com/
10. https://sg.idtdna.com/

Topic: Molecular Cloning for Varietal Development

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What is Molecular Cloning?

Molecular cloning is a primary procedure in contemporary biosciences. This involves introducing a specific piece of DNA (most often a gene) into a cell where it does not generally belong. Furthermore, this process ensures that this foreign DNA fragment replicates (copies itself) and expresses itself (through transcription) in the new host.

Basic materials needed to clone a fragment of target DNA:

- Target DNA fragment
- Host cells and vectors for gene cloning
- Cloning enzymes
- Host cells and vectors for expressing the cloned DNA
- Growth media for host cell culture

Beyond the *basic requirements* listed, PCR, electrophoresis, and cell culture resources are also necessary.

Types of Molecular Cloning Techniques

1. Traditional Cloning:

- Relies on restriction enzyme digestion and DNA ligation.
- Restriction enzymes cut the DNA at specific sequences, which are then joined to the vector.
- Though effective, this method is time-consuming and less precise.

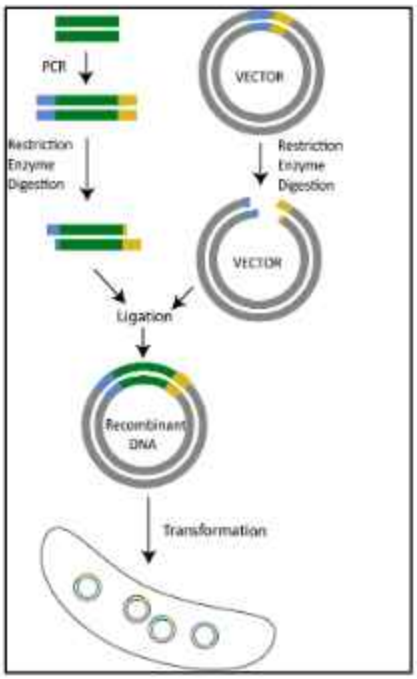


Fig: Restriction Enzyme Based Cloning. 1. Short sequences containing restriction sites are added into the 5' ends of primers for DNA amplification by PCR. 2. Both the vector and DNA fragment are digested with restriction enzymes to create cohesive ends. 3. The vector and DNA fragment are ligated. 4. The recombinant DNA enters the host cell during transformation.

2. TA/PCR Cloning:

- Utilizes the complementary overhangs of Taq polymerase-amplified PCR products.
- DNA fragments with "A" overhangs are inserted into vectors with complementary "T" overhangs.
- This method is faster and does not require restriction enzyme digestion.

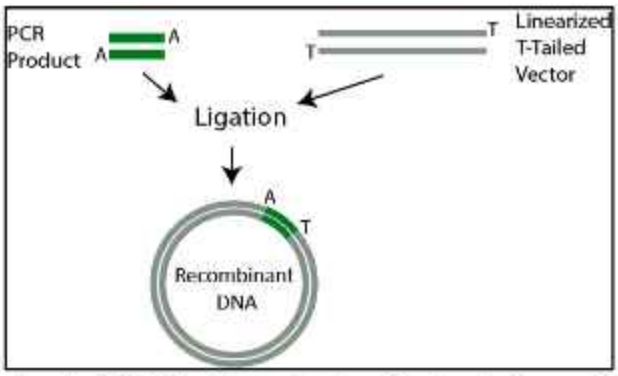


Fig: PCR Cloning. 1. PCR Product with A-tailed ends is combined with T-tailed vector. 2. During ligation, PCR product is inserted into the vector.

3. Gateway Cloning:

- A highly efficient, recombination-based cloning system that does not require restriction enzymes.
- Uses site-specific recombination to insert DNA fragments into vectors.
- Allows easy transfer of DNA between multiple vectors, making it suitable for complex experiments.

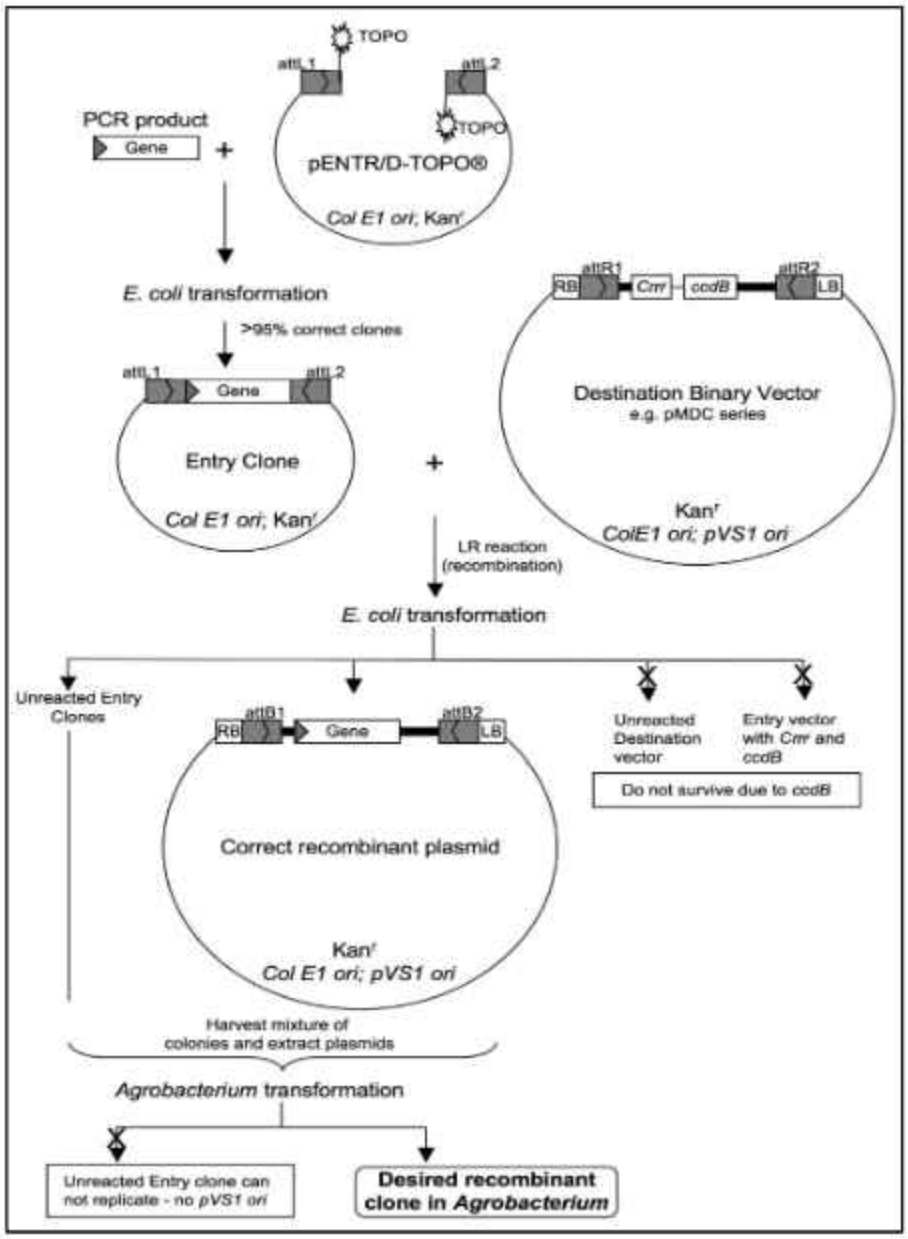


Fig: Procedure for construction of entry clone and destination vector (Gateway system)

4. Gibson Assembly:

- An enzyme-based method that joins overlapping DNA fragments in a single reaction.
- Ideal for assembling large DNA fragments or complex constructs.
- Highly precise and efficient, with no need for restriction enzymes or specific sequence requirements.

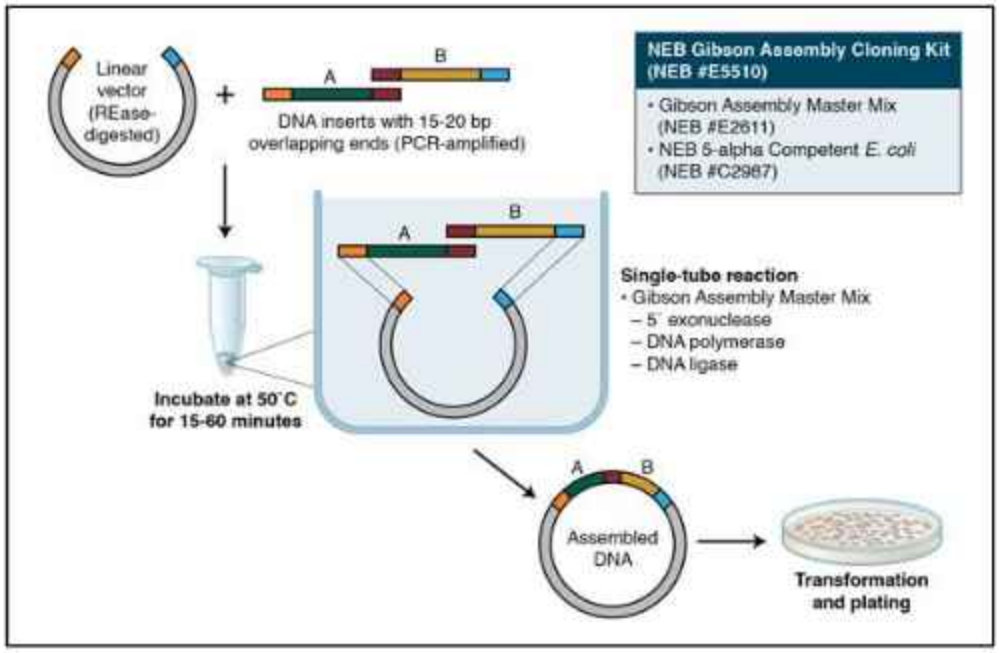


Fig: Gibson Assembly employs three enzymatic activities in a single-tube reaction: 5′ exonuclease, the 3′ extension activity of a DNA polymerase and DNA ligase activity. The 5′ exonuclease activity chews back the 5′ end sequences and exposes the complementary sequence for annealing. The polymerase activity then fills in the gaps on the annealed regions. A DNA ligase then seals the nick and covalently links the DNA fragments together. The overlapping sequence of adjoining fragments is much longer than those used in Golden Gate Assembly, and therefore results in a higher percentage of correct assemblies.

5. CRISPR-Based Cloning:

- Combines molecular cloning with genome-editing technology.
- Utilizes the CRISPR-Cas9 system to introduce specific genetic modifications.
- Allows precise editing and insertion of DNA sequences into target locations in the genome.

6. Seamless Cloning:

The seamless cloning (SC) technique (similar to LIC) depends on matching short sequences at the ends of a DNA fragment to the short sequences on a plasmid vector. SC method requires an enzyme with 5' to 3' exonuclease activity to create 3' overhangs, a DNA polymerase to fill in gaps, and a DNA ligase to seal the nicks.

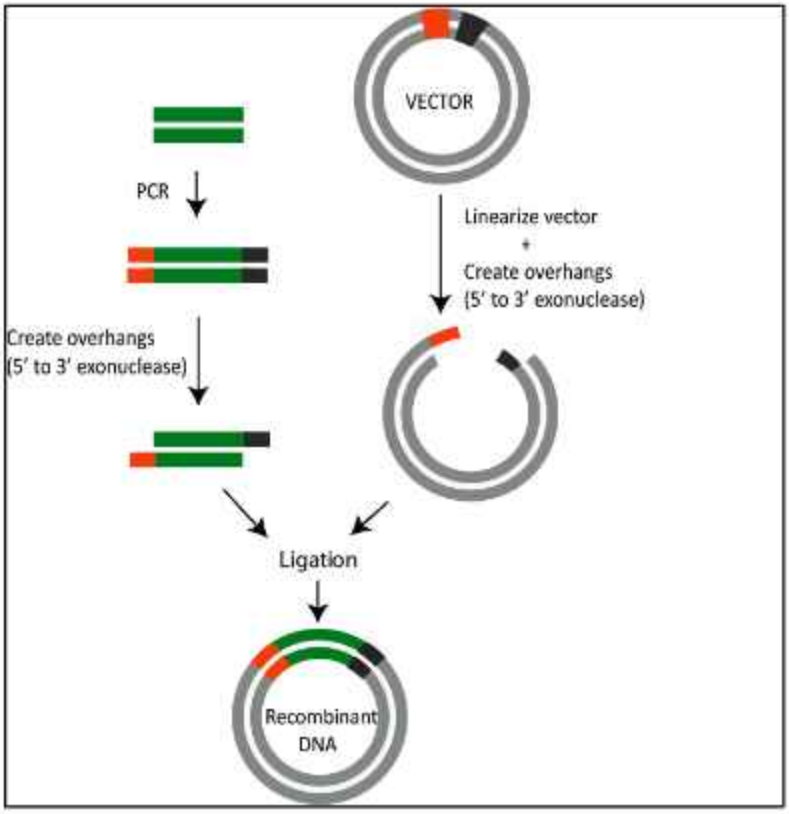


Fig: Seamless Cloning. 1. Short sequences are added into the 5' ends of primers for DNA amplification by PCR. 2. Vector is digested by a restriction enzyme. 3. Both DNA fragment and vector are treated with an enzyme with 5' to 3' exonuclease activity to create cohesive overhangs. 4. During ligation, the DNA fragment is inserted into the vector.

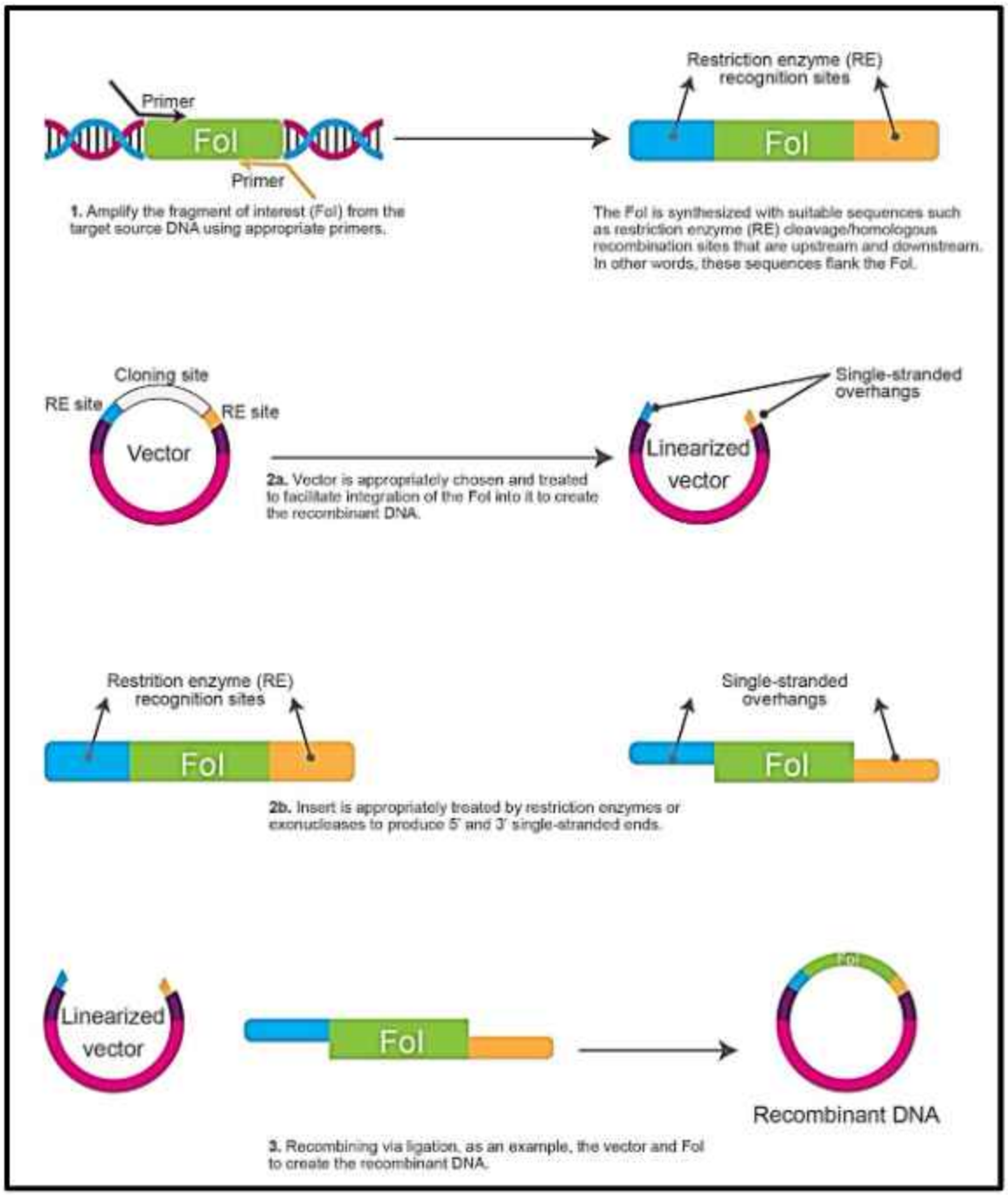
General gene cloning steps:

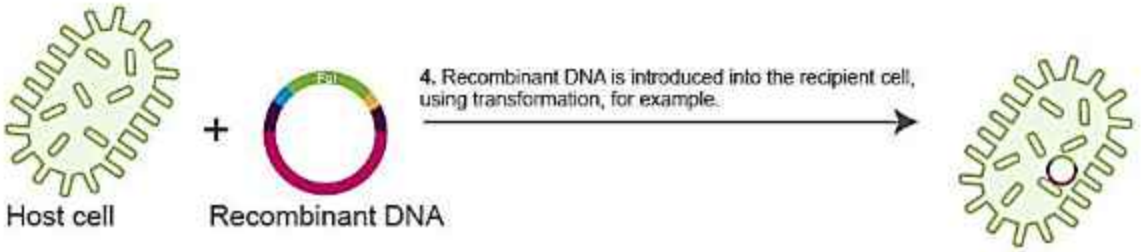
The following steps give you a general idea of the scope of the molecular gene cloning process. Each protocol you encounter will have specific steps and key techniques to optimize the process, but this part lets you point out the typical workflow for molecular gene cloning.

1. Isolation and preparation of the source DNA that you want to clone.
2. Preparation of the cloning vector.
3. Combining the vector and DNA fragments suitably so they form the recombinant DNA molecule.

4. Introducing this recombinant DNA (vector + insert) into the host recipient.
5. Selecting the host cells that have the correct recombinant DNA introduced into them.
6. Ensuring the insert is expressing itself to serve the purposes it was cloned (mass-production of a foreign protein etc.). Often, transgene expression is carried out using a strain or cell line different from the one that was used to clone it.

A basic schematic representation of the molecular cloning process is depicted below:





Host cell **did not** take up the plasmid: _____

Host cell **did** take up the plasmid: _____

5. Host cells that survive under the appropriate selection pressure are further tested by colony PCR, for example, to ensure the clone has the FoI in the right orientation.

6. Suitable clones identified are further tested for expression of the FoI through diagnostic RNA or protein techniques, such as Norther and Western blot respectively.

► Target DNA fragment isolation and Preparation

The first step of gene cloning involves identifying and preparing the desired DNA fragment referred to as the fragment of interest (FoI).

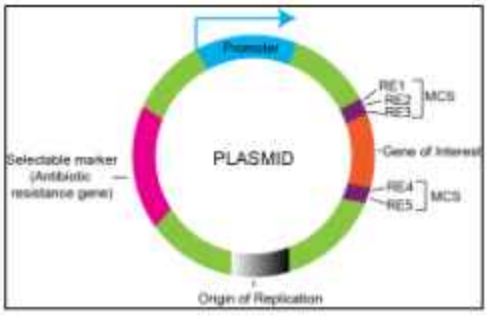
The source DNA from which your target DNA fragment is cloned can be either genomic DNA or cDNA. So, to begin, you will first need to isolate the cDNA or genomic DNA. Both are considered to be your source DNA. Once isolated, the target DNA sequence in your source cDNA or genomic DNA needs to be amplified through PCR before it can be inserted into a vector. As with any PCR, you need to confirm your results using DNA gel electrophoresis to be certain you have the fragment you intend. The quality and integrity of the isolated and prepared target DNA are essential for successful gene cloning experiments.

► Preparing your vector for gene cloning

A cloning vector carries the cloned DNA fragment into the desired host organism. But it not only functions as a vehicle delivering your target DNA fragment, it also ensures efficient replication, expression, and maintenance of the cloned DNA fragment within the host organism.

There are four crucial elements that cloning vectors must have for successful gene cloning:

- Origin of replication (ori)
- Selectable marker
- Multiple cloning site (MCS)
- Promoter



Something very important to note is that within gene cloning, there are two types of vectors needed, the cloning vector and the expression vector.

The cloning vector is responsible for cloning your target gene of interest.

The expression vector enables the cloned gene to be expressed. Expression vectors are DNA molecules used in gene cloning to facilitate the expression of a specific gene or genes in a host organism. By cloning a gene of interest into an expression vector, you can introduce the vector into a suitable host organism, such as bacteria or mammalian cells. The host cells then replicate the vector and transcribe and translate the cloned gene, leading to the production of the desired

protein. Your cloning and expression vectors may be different, or they might be the same depending on your experimental needs.

► Cloning enzymes

Within any gene cloning setup, there are certain enzymes that are necessary to carry out the procedure. Most importantly are your restriction enzymes that cut DNA at specific points, and DNA ligase that join DNA fragments together.

► Restriction enzymes

To clone your target DNA fragment into the vector, both the fragment and the vector might need to be cut and then stitched back together. This is where an enzyme class called restriction endonucleases, also known as restriction enzymes, are useful. Restriction enzymes cut DNA at very specific cut sites. Within a vector's multiple cloning site (MCS) are multiple restriction sites where restriction endonucleases cut. To ensure compatibility between the digested vector and the insert, choosing the most appropriate restriction enzymes is crucial in a cloning reaction. Restriction enzymes are specifically selected to generate compatible sticky ends between the digested vector and insert.

► DNA ligase

Once you have your digested DNA fragment and the vector with their sticky ends, you will have to join the DNA fragment with the plasmid backbone through covalent bonding. This is facilitated by an enzyme called DNA ligase.

► Host cells and organisms for cloning

The desired host organism is a critical component in gene cloning experiments because it serves as the recipient for the introduction and propagation of the cloned DNA and its expression. Most of the time, people doing gene cloning will use *Escherichia coli* (*E. coli*) strains. Other bacteria, yeast, mammalian or plant cells also may be used. Horizontal gene transfer methods are used to introduce the recombinant vector construct into the recipient host. There are several methods for doing this:

- Transformation
- Conjugation
- Transfection

Bacterial host organisms like *E. coli* are commonly used due to their ease of manipulation and rapid growth. Yeast and mammalian cells offer advantages in studying eukaryotic gene expression and protein function. Plant cells are utilized for cloning plant genes and investigating plant molecular biology.

► **Growth media for host cell culture**

Growth media, also known as culture media, are solid or liquid mixtures that provide the necessary nutrients, vitamins, and minerals for cell growth and proliferation during gene cloning experiments. They serve as an environment for the host organisms to propagate, to replicate, maintain and express the recombinant DNA.

The culture media composition can vary depending on the host organisms and gene cloning experimental requirements. Typically, culture media contains a carbon source such as glucose, a nitrogen source such as amino acids or ammonium salts, salts and other essential nutrients. Growth media are also extensively used when you want to culture the host cells for expressing the recombinant DNA you've cloned in those cells. Choosing your growth media for your host cell culture depends on the specific host organism being used. Different organisms have different nutritional requirements. For example, if the expression cell line is mammalian, you would need to use the appropriate cell culture methods, which are different from bacterial cell culture.

► **Commonly used growth media for host cell culture:**

- LB (lysogeny broth/ Lauria broth): commonly used for bacterial cell culture.
- TB (Terrific broth): used for bacterial cell culture.
- YPD (Yeast extract Peptone Dextrose): used for growth of yeast cells.
- Minimal media: used for selective bacterial growth or for yeast strains with specific nutrient requirements.
- DMEM (Dulbecco's Modified Eagle's Medium): commonly used for mammalian cell culture.
- RPMI 1640: another widely used medium for mammalian cell culture.

In addition, antibiotics or selective agents may be added to media to select cells carrying the recombinant DNA. This type of media is known as selective media. Selective media enables host organisms that contain the desired recombinant target DNA to grow and be identified. Components within selective media allow transformed or transfected cells to grow while not being inhibited by non-transformed cell growth. This is achieved by incorporating antibiotics, nutritional markers, or other selectable markers into the media. Using selective media enhances the efficiency and accuracy of gene cloning experiments because it enables researchers to select cells that carry the desired genetic material.

Applications of Molecular Cloning in Varietal Development

Molecular cloning has far-reaching applications in developing improved crop varieties. Some of the major applications include:

1. Development of Genetically Modified Crops:

- Crops like *Bt* cotton and *Bt* maize, developed using molecular cloning, exhibit resistance to pests such as bollworms and corn borers.
- This reduces the need for chemical pesticides, promoting environmentally friendly farming practices.

2. Improved Stress Tolerance:

- Introduction of genes conferring tolerance to abiotic stresses such as drought, salinity, and extreme temperatures.
- Enhances crop resilience to changing climatic conditions.

3. Enhanced Nutritional Quality:

- Molecular cloning is used to develop biofortified crops such as Golden Rice, enriched with vitamin A to combat malnutrition.
- Other examples include protein-enriched maize and iron-fortified wheat.

4. Disease Resistance:

- Cloning and insertion of resistance genes (R-genes) provide immunity to crops against fungal, bacterial, and viral pathogens.
- Example: Papaya plants resistant to papaya ringspot virus (PRSV).

5. Herbicide Tolerance:

- Development of crops that can tolerate herbicides, allowing effective weed control without harming the crop.
- Example: Glyphosate-resistant soybean.

6. Hybrid Seed Production:

- Cloning systems aid in developing hybrid seeds by introducing male sterility or fertility restorer genes.
- Ensures higher yield and better-quality crops.

7. Metabolic Engineering:

- Cloning of genes involved in metabolic pathways to produce high-value compounds such as vitamins, antioxidants, and secondary metabolites in plants.

Advantages of Molecular Cloning in Varietal Development

1. Precision:

- Targeted introduction of desirable traits ensures precision in crop improvement.

2. Efficiency:

- Molecular cloning is faster compared to traditional breeding methods, accelerating the development of new varieties.

3. Overcoming Genetic Barriers:

- Enables the transfer of genes across species barriers, which is not possible through conventional breeding.

4. Sustainability:

- Reduces dependency on chemical inputs, promoting sustainable and eco-friendly agricultural practices.

5. Uniformity in Crops:

- Produces genetically identical plants, ensuring uniformity in yield, quality, and performance.

Challenges and Limitations

1. Cost and Infrastructure:

- Molecular cloning requires advanced laboratory facilities and skilled personnel, making it expensive and inaccessible for small-scale farmers.

2. Regulatory Hurdles:

- Genetically modified crops often face stringent regulatory approval processes and public resistance.

3. Unintended Consequences:

- Introduction of foreign genes may have unforeseen effects on the environment or non-target organisms.

4. Ethical Concerns:

- Genetic manipulation raises ethical questions, particularly with respect to biodiversity and ecosystem balance.

Conclusion

Molecular cloning has emerged as a powerful biotechnological tool for advancing varietal development. By enabling precise genetic modifications, it has revolutionized agriculture, addressing global challenges such as food security, climate change, and malnutrition. While challenges remain, ongoing research and innovation in molecular cloning promise a future of sustainable and resilient agricultural systems. Its integration with other biotechnological advancements will continue to shape the future of crop improvement and global agriculture.

Topic: Plasmid DNA Isolation, Validation and Analysis

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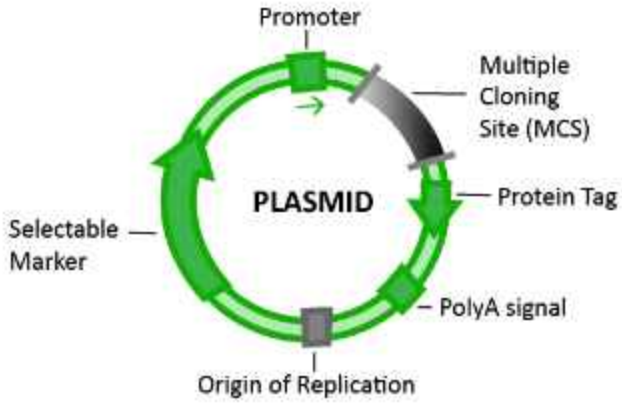
What is Plasmid DNA?

Plasmids are small, circular, double-stranded DNA molecules found in bacteria and some eukaryotes. They are distinct from chromosomal DNA and can replicate independently. Plasmids often carry genes that confer advantageous traits such as antibiotic resistance, making them valuable tools in molecular biology, biotechnology, and genetic engineering. Their application in varietal development is crucial for introducing and propagating desirable traits in plants and microorganisms.

Key Features of Plasmids:

- **Origin of Replication (ORI):** a required sequence or element on the plasmid for its replication inside the host.
- **Selectable Marker:** a required element for cloning to select a host, which carries the vector. Only those host cells that have the vector inside them will grow in the media that has the selection pressure, most commonly an antibiotic; while those host cells that do not have the vector, will die out because they cannot tolerate the antibiotic. In this way, the experimenter will be able to select host cells that have the vector, over those that do not have the vector.
- **Multiple Cloning Site (MCS):** an element on the plasmid fragment which contains restriction enzyme sites to allow DNA insertion. Compatible restriction enzymes cut on the MCS of plasmid and a DNA insert during preparation step of cloning.
- **Promoter Region:** a region which drives the protein expression of the cloned DNA.
- **Protein Tag:** a particular sequence which produces a protein with specific function, and it is usually attached to the recombinant protein. An example of a protein tag is luciferase or GFP, to monitor or quantify the protein.
- **Poly-adenylation signal:** an element containing poly-A which is important to produce a protein.



Principles of Plasmid DNA Isolation

Plasmid DNA isolation involves separating plasmid DNA from chromosomal DNA, RNA, and proteins. The process typically uses the differences in size, structure, and chemical properties between plasmid DNA and other cellular components.

General Steps:

1. **Cell Lysis:** Breaking open the bacterial cells to release their contents.
2. **Separation:** Using differential solubility to separate plasmid DNA from chromosomal DNA and other cellular debris.
3. **Purification:** Removing contaminants like RNA, proteins, and salts to obtain pure plasmid DNA.

Methods of Plasmid DNA Isolation

A. Alkaline Lysis Method

This is the most commonly used method for plasmid isolation. It leverages the different denaturation and renaturation properties of chromosomal and plasmid DNA.

Procedure:

1. **Growth of Bacterial Culture:** Grow bacteria containing the plasmid in a suitable medium with selective antibiotics.

2. **Harvesting Cells:** Centrifuge the culture to pellet the cells.

3. Cell Lysis:

- Resuspend the pellet in a lysis buffer containing Tris (pH buffer), EDTA (to chelate divalent cations), and glucose.
- Add a solution of sodium hydroxide and SDS (alkaline lysis solution) to lyse the cells.

4. Neutralization: Add potassium acetate or acetic acid to neutralize the solution. Plasmid DNA renatures, while chromosomal DNA and proteins precipitate.

5. Centrifugation: Remove the precipitate by centrifugation.

6. Plasmid Precipitation: Add isopropanol or ethanol to the supernatant to precipitate plasmid DNA.

7. Washing and Resuspension: Wash the DNA pellet with ethanol and resuspend it in TE buffer (Tris-EDTA).

B. Commercial Kits

Commercial kits simplify plasmid isolation by providing pre-formulated reagents and spin columns. These kits often use silica membrane technology for DNA binding and elution. The process includes:

- Cell lysis and neutralization.
- DNA binding to the silica membrane.
- Washing to remove impurities.
- Elution of pure plasmid DNA.

C. Other Methods

- **Cesium Chloride Density Gradient Centrifugation:** A high-purity method used for large-scale applications.
- **Magnetic Beads:** Uses beads with affinity for DNA to isolate plasmids efficiently.

Validation of Plasmid DNA

After isolation, the quality and integrity of plasmid DNA must be validated. Key parameters include concentration, purity, and integrity.

1. Quantification

- **UV Spectrophotometry:**
- Measure absorbance at 260 nm (A260) to determine DNA concentration.
- Measure A260/A280 ratio to assess purity (ideal range: 1.8–2.0).
- **Fluorometry:**
- Use intercalating dyes like SYBR Green or PicoGreen for sensitive quantification.

2. Purity Assessment

- Check for protein contamination (A260/A280 ratio) and RNA contamination (presence of extra bands in gel electrophoresis).

3. Gel Electrophoresis

- Run the plasmid DNA on an agarose gel to assess integrity and confirm size.
- Supercoiled, nicked, and linear forms of plasmid DNA may be visible.

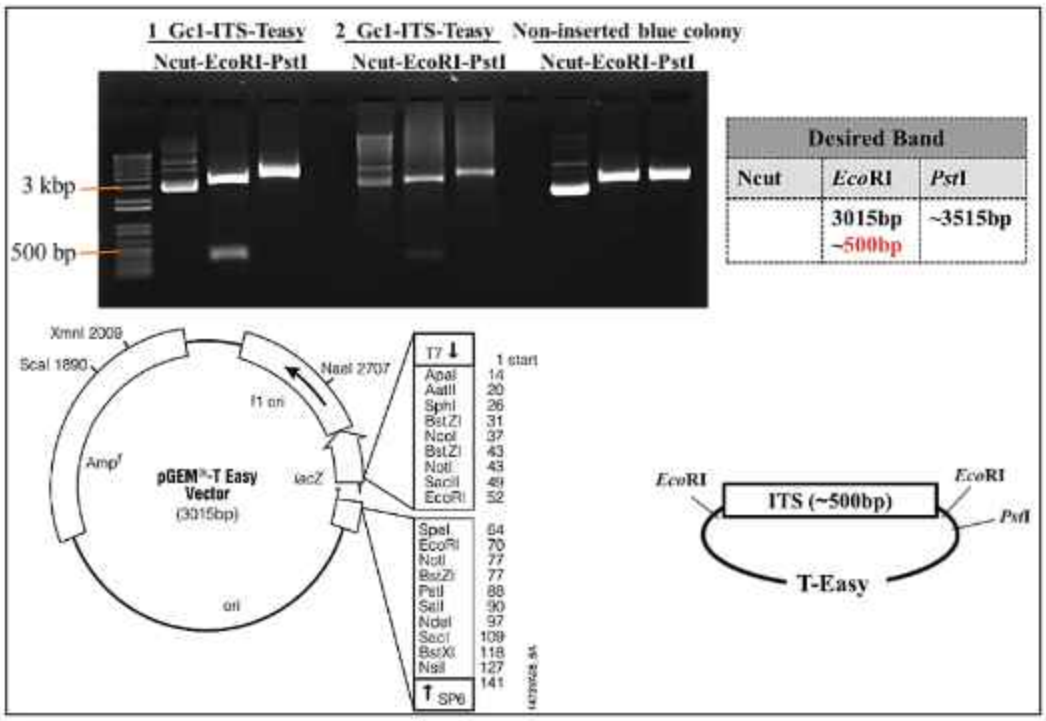


Figure: After plasmid isolation digestion with restriction enzyme and validation of desired clones.

4. Functional Validation

- Transform the plasmid into competent cells and check for the expression of the gene of interest or selectable marker.

Analysis of Plasmid DNA

1. Restriction Digestion

- Use restriction enzymes to cut the plasmid at specific sites.
- Analyze the digested DNA on an agarose gel to verify the plasmid map.

2. Polymerase Chain Reaction (PCR)

- Amplify specific regions of the plasmid to confirm the presence of inserted genes.

3. Sequencing

- Perform Sanger or next-generation sequencing to verify the sequence of the plasmid, including the insert.

4. Southern Blotting

- Hybridize a labeled probe to the plasmid DNA to confirm the presence of specific sequences.

Application in Varietal Development

1. Introduction of Desirable Traits

- Plasmids can be used as vectors to introduce genes for desirable traits, such as:
- Pest
- Herbicide tolerance: Genes for tolerance to specific herbicides.
- Nutritional enhancement: Genes for increasing vitamin or mineral content.

2. Development of Transgenic Plants

- Transform plants with plasmids carrying the gene of interest using techniques like Agrobacterium-mediated transformation or gene gun methods.

3. Microbial Strain Improvement

- Use plasmids to enhance microbial strains for:
- Biocontrol agents: Improving efficiency against pests and diseases.
- Biofertilizers: Enhancing nitrogen fixation or nutrient solubilization capabilities.

4. Marker-Assisted Selection

- Plasmids carrying molecular markers aid in identifying and selecting varieties with desirable traits.

Common issues found in plasmid DNA and solutions

Issue	Cause	Solution
Low yield	Incomplete lysis or poor growth	Optimize lysis conditions and culture time
RNA contamination	Ineffective RNase treatment	Add RNase to the lysis buffer
Protein contamination	Inefficient precipitation	Ensure thorough neutralization and washing
Smearing on gel electrophoresis	Degraded DNA or improper handling	Use fresh reagents and minimize shearing

Topic: Agrobacterium-Mediated Transformation of Desired Gene(s) in Plants for Varietal Development

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Introduction

Agrobacterium-mediated transformation is a widely used and highly efficient technique for introducing desired gene(s) into plants. This method leverages the natural genetic engineering capabilities of Agrobacterium tumefaciens, a soil bacterium capable of transferring part of its DNA into plant cells. By harnessing this mechanism, scientists can introduce specific genes responsible for desired traits, such as improved yield, pest resistance, stress tolerance, or enhanced nutritional quality, into target plant species.

This approach has become a cornerstone of modern plant biotechnology and plays a crucial role in varietal development, addressing global challenges like food security and sustainable agriculture.

What is Agrobacterium-Mediated Transformation?

Agrobacterium tumefaciens is a naturally occurring bacterium that infects plants and causes crown gall disease. The pathogenicity of this bacterium is due to its unique ability to transfer a segment of DNA (called T-DNA) from its plasmid (the Ti plasmid) into the plant genome. Scientists have modified this process to introduce beneficial genes into plants instead of disease-causing genes, making it a powerful tool for plant genetic engineering.

Agrobacterium used as a vector in plant biotechnology

After the discovery of Agrobacterium, researchers explored and understood the mechanism of how wild and pathogenic Agrobacterium strains transferred DNA into the plants.

The Agrobacterium tumor-inducing plasmid, or Ti-plasmid, does the DNA transfer. Later, to use Agrobacterium as a "tool" for plant genetic engineering, scientists modified the T-DNA region by removing the oncogenes to avoid the tumorigenicity to avoid getting sick plants while introducing

a gene of interest. So, researchers created a disarmed (nonpathogenic) Agrobacterium strains and used it as a vector.

In cloning, a vector is a DNA molecule used as a vehicle to artificially carry foreign genetic material into another cell, where it can be replicated and expressed. Thus, a vector allows you to create transgenic organisms (not only plants) with improved traits (e.g., disease resistance), or understand a gene or protein function.

Today, many agriculturally and horticulturally important species are routinely transformed using this bacterium, and the list of Agrobacterium-mediated transformation susceptible species grows at an accelerated rate.

How does *Agrobacterium* infect plants?

Agrobacterium strains, such as Agrobacterium tumefaciens and Agrobacterium rhizogenes, infect plants and cause the development of large tumor or root hairs in plants.

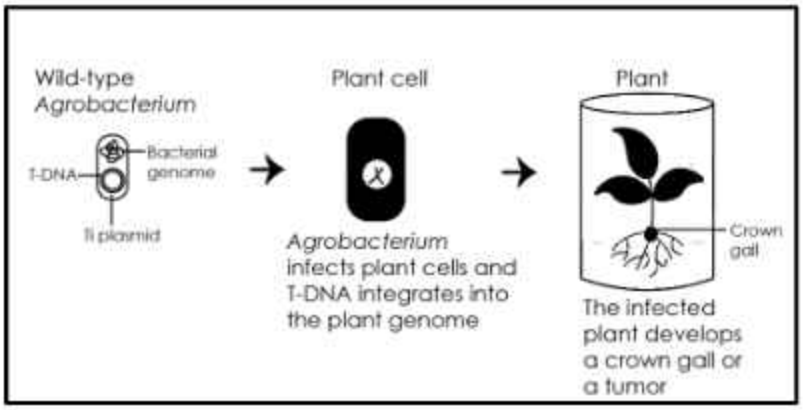

Fig 1. *Agrobacterium* infection in the host plants. The *Agrobacterium* infects a plant cell. The T-DNA within *Agrobacterium* integrates with the plant's genome causing the plant to develop crown gall disease (in the case of *Agrobacterium tumefaciens* or another type of tumor).

Tumor inducing (Ti) or root inducing (Ri) plasmids carried by Agrobacterium play a significant role in this process. A plasmid is a small and circular DNA. Agrobacterium transfers a part of Ti or Ri plasmids, called T-DNA, to plant cells. T-DNA carries a number of genes, important for the survival of Agrobacterium and the bacterial infection in plants.

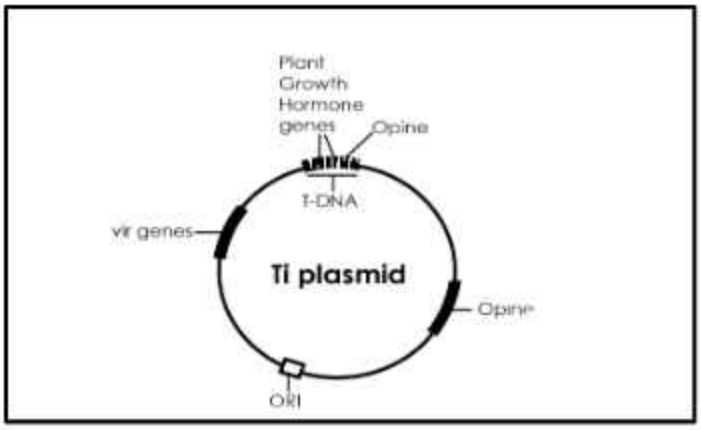

Fig 2. The Ti plasmid carries a T-DNA region, *vir* genes and genes encoding opine catabolism. The T-DNA region contains tumor-associated genes, causing abnormal growth in plants by encoding plant growth hormone. It also has opine synthase genes, encoding for enzymes involved in the synthesis of opine.

Among the genes located on the Ti or Ri plasmids, some virulence genes, called vir genes, encode Vir proteins in Agrobacterium. The activity of Vir proteins promotes the T-DNA integration into the plant genome. Not only Vir proteins, some other proteins encoded by genes in the bacterial chromosomes are also important for this integration. After detecting plant molecules, Agrobacterium activates its chromosomal genes (chv genes) and vir genes. Several Chv proteins participate in Agrobacterium attachment to the plant cells, whereas Vir proteins assist in the cleavage and the transfer of T-DNA from the bacterial cell into the plant cell.

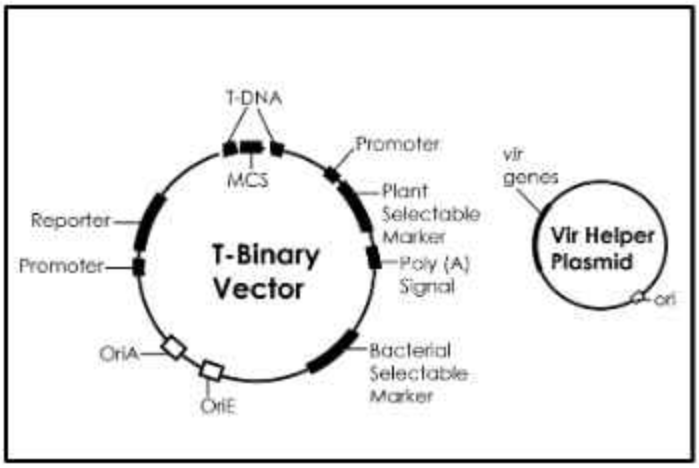

Fig 3: Illustration of the T-binary system showing both vectors. The first vector is the T-binary vector on the left. The second vector, on the right, is the Vir Helper Plasmid.

T-DNA also contains genes encoding plant growth hormones. When expressed in the plant genome, the overproduction of plant growth hormones in plants stimulates the development of tumor or root hairs. Some genes, located on the T-DNA, encode for enzymes important for the synthesis of unique amino acids, called opines. After T-DNA integration, the large crown galls in the plants provide Agrobacterium with this essential nutrient.

Why is Agrobacterium used to make transgenic plants?

Agrobacterium is a useful tool for plant transformation because it can carry, transfer, and integrate a gene of interest into the plant genome.

In the development of transgenic plants, this system allows plants to stably harbor and pass a particular gene of interest to the next generations relatively quicker than by using the more traditional plant breeding method. This method is relatively inexpensive and easy to perform. In addition, it provides convenient way to screen and select the transformed plant tissues.

Agrobacterium-mediated transformation and CRISPR

A recent advantage of *Agrobacterium*-mediated gene transfer comes with the advent of gene-editing tools like CRISPR/Cas.

Genome editing technologies provide powerful tools for precise manipulation of targeted genome sequences, setting up unprecedented opportunities for crop breeding and functional genomics research. However, the lack of appropriate methods to deliver genome-editing reagents (for example, constructs encoding the nuclease and target-specific RNAs) is the primary barrier to CRISPR/Cas-mediated gene editing in a variety of plants.

Currently, Agrobacterium-mediated transformation is the preferred method of CRISPR/Cas reagent delivery. Studies have already reported this success in maize, wheat and tobacco. Also, compared to the biolistics approach, the Agro-infiltration systems have significantly improved, progressing from the single Ti plasmid to the binary vector. Even today, more advanced Agrobacterium vectors have been developed like the superbinary and ternary vectors systems.

In a binary vector, T-DNA could be separated from the Ti plasmid and placed on shuttle vectors. In the superbinary vector, the binary vector carries additional virulence genes and enhances Agrobacterium-mediated gene transfer of recalcitrant plants.

With a ternary vector, a third plasmid—called the accessory plasmid or the virulence helper plasmid is used to carry the virulence gene cluster. These improvements have increased transformation efficiency throughout Agrobacterium and expanded the transformability of a wide range of plant genotypes!

Process of Agrobacterium-Mediated Transformation

The Agrobacterium-mediated transformation process involves several key steps, which can be broadly categorized as follows:

1. Construction of the Recombinant Ti Plasmid

a) Gene Isolation and Cloning:

- Identify and isolate the desired gene(s) responsible for traits such as pest resistance, drought tolerance, or higher yield.
- Clone the gene into a T-DNA region of a modified Ti plasmid, ensuring that it is flanked by the left and right border sequences for transfer into the plant genome.

b) Incorporation of Selectable Markers:

- Include marker genes (e.g., antibiotic resistance or herbicide tolerance) to facilitate the selection of transformed plant cells.

2. Preparation of Agrobacterium Culture

a) Transform *Agrobacterium tumefaciens* with the recombinant Ti plasmid using electroporation or chemical methods.

b) Culture the transformed Agrobacterium in a suitable growth medium.

3. Co-Cultivation with Plant Tissue

a) Selection of Explants:

- Choose appropriate plant tissue, such as leaf disks, cotyledons, or stem segments, as explants for transformation.

b) Co-Culture:

- Incubate the explants with the transformed Agrobacterium under controlled conditions to facilitate T-DNA transfer into plant cells.

4. Selection of Transformed Cells

a) Transfer the co-cultivated explants onto a selection medium containing the appropriate antibiotic or herbicide to eliminate non-transformed cells.

b) Include plant growth regulators in the medium to stimulate callus formation or shoot regeneration.

5. Regeneration of Transgenic Plants

a) Transfer the selected callus or regenerated shoots to a rooting medium to develop complete plants.

b) Harden the regenerated plants in a greenhouse before transferring them to soil for further growth and evaluation.

6. Validation of Transformation

a) Use molecular techniques like PCR, Southern blotting, or RT-PCR to confirm the integration and expression of the desired gene in the transgenic plants.

b) Perform phenotypic and agronomic evaluations to assess the expression of the introduced trait.

Advantages of Agrobacterium-Mediated Transformation

1. High Efficiency:

- Achieves stable integration of the desired gene into the plant genome with high success rates.

2. Wide Host Range:

- Can be used for a variety of dicotyledonous plants and, with modifications, for monocots as well.

3. Precision:

- Targets specific genes, allowing precise modification of plant traits.

4. Cost-Effectiveness:

- Relatively simple and cost-effective compared to other transformation methods like particle bombardment.

5. Environmental Friendliness:

- Reduces the need for chemical inputs like pesticides by introducing pest resistance traits directly into crops.

Applications in Varietal Development

1. Development of Pest-Resistant Crops:

- Example: Bt cotton and Bt brinjal, engineered with Bacillus thuringiensis toxin genes to combat insect pests.

2. Improved Stress Tolerance:

- Introduction of genes conferring drought, salinity, and temperature tolerance to enhance crop resilience.

3. Enhanced Nutritional Quality:

- Biofortification of crops such as Golden Rice, enriched with provitamin A to address malnutrition.

4. Herbicide Tolerance:

- Crops engineered for herbicide tolerance allow effective weed control without harming the crop. Example: Glyphosate-resistant soybean.

5. Disease Resistance:

- Insertion of R-genes provides resistance against viral, bacterial, and fungal pathogens. Example: Papaya resistant to papaya ringspot virus.

6. Production of Hybrid Crops:

- Engineering male sterility or fertility restorer genes facilitates hybrid seed production, enhancing crop yield and uniformity.

Conclusion

Agrobacterium-mediated transformation has revolutionized plant biotechnology, providing a reliable and efficient method for introducing desired gene(s) into plants. This technique has

significantly contributed to varietal development, enabling the creation of crops with improved yield, resilience, and nutritional value. While challenges remain, continued advancements in this field hold the promise of transforming agriculture to meet the needs of a growing global population sustainably. As research progresses, Agrobacterium-mediated transformation will remain an indispensable tool in the quest for food security and sustainable agriculture.

Salinity Tolerance: Exploring Potential Genes to Enhance Salt Stress Adaptation

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Salinity stress poses a significant challenge to global agriculture by reducing crop yields and rendering fertile farmland unsuitable for cultivation. The escalating global population and declining agricultural land due to urbanization and industrialization further emphasize the need to adapt saline soils for food production. Traditional breeding for salt-tolerant crops has shown limited success, primarily due to reproductive barriers and limited genetic diversity. In contrast, genetic engineering offers precise tools for modifying genes or introducing new traits to enhance salinity tolerance. Advances in this field have demonstrated success in improving the salt stress adaptation of crops, but more field-applicable research is essential.

This module delves into the molecular mechanisms underlying salinity tolerance, emphasizing the identification and utilization of key stress-responsive genes. It explores genetic engineering techniques, such as CRISPR-Cas9 and transgenic approaches, to develop resilient crop varieties. Additionally, it examines the integration of modern biotechnological tools with traditional breeding for sustainable agricultural practices in saline environments.

Mechanisms of Salinity Tolerance

Plants naturally respond to salinity stress through a variety of metabolic mechanisms, including ion homeostasis, production of osmolytes, activation of antioxidative defenses, and signaling responses. These adaptive responses are controlled by specific genes and signaling pathways, which can be exploited for the development of salt-tolerant crop varieties. Genetic engineering strategies are focused on manipulating or introducing genes that regulate essential processes such as ion transport, osmoprotection, transcriptional control, and stress signaling. With advancements in biotechnology, scientists have been able to transfer stress-responsive genes from naturally

salt-tolerant species or modify the expression of native genes in crops, resulting in transgenic plants that exhibit enhanced tolerance to salinity. This progress has opened new pathways for developing crops that can thrive in saline-prone areas, contributing to more sustainable agriculture. Numerous key genes involved in salinity tolerance have been successfully identified and utilized in genetic engineering efforts, furthering the potential to create robust, salt-tolerant crop varieties (Figure 1).

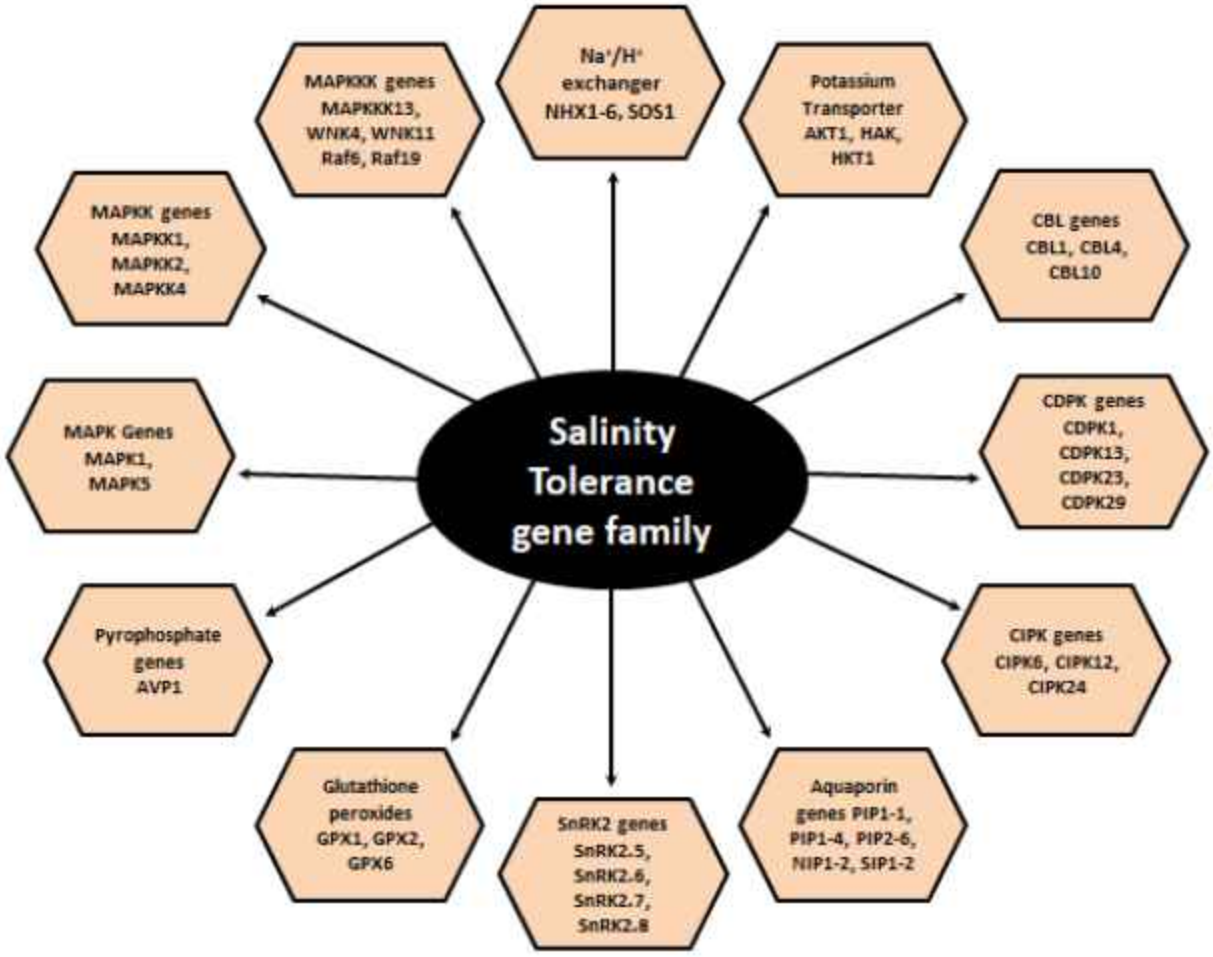

Figure 1: Genes Enhancing Salinity Tolerance Across Plant Species through Overexpression

Key Mechanisms of Salinity Tolerance

1. Sodium (Na^+) Exclusion Mechanisms

Sodium (Na^+) exclusion mechanisms are critical for plants to maintain ionic homeostasis and ensure survival under salt stress conditions. High sodium levels can disrupt cellular processes by causing ion toxicity and osmotic stress. To combat this, plants employ specialized transport systems to limit sodium accumulation in sensitive tissues. The SOS (Salt Overly Sensitive) pathway plays a pivotal role in sodium exclusion, where SOS1, a plasma membrane Na^+/H^+ antiporter, actively pumps excess sodium out of the cytosol into the apoplast or back into the soil. Additionally, tonoplast-localized transporters such as NHX1 help sequester Na^+ into vacuoles, thereby reducing its toxic effects in the cytoplasm. Other mechanisms include the selective uptake of potassium (K^+) over sodium through high-affinity K^+ transporters, such as HKT1, which prevent sodium accumulation in the shoot while maintaining essential cellular functions. These exclusion strategies, combined with the regulation of osmoprotectants and antioxidant enzymes, enable plants to withstand saline environments. Understanding and enhancing these mechanisms through genetic engineering can improve salt tolerance in crop species, contributing to sustainable agriculture in saline-prone regions.

2. Potassium (K^+) Homeostasis

Potassium (K^+) homeostasis is essential for plant growth, development, and stress tolerance, particularly under salt stress conditions. As a vital macronutrient, K^+ is involved in numerous physiological processes, including enzyme activation, osmotic regulation, and maintaining electrical potential across membranes. In saline environments, excessive sodium (Na^+) levels can disrupt K^+ uptake and distribution, leading to ionic imbalance and impaired metabolic functions. Plants maintain K^+ homeostasis through selective ion transport systems, such as high-affinity potassium transporters (HKT), potassium channels (AKT), and H^+-ATPase-driven pumps. These mechanisms prioritize K^+ retention in cells while minimizing Na^+ interference. Additionally, K^+ plays a critical role in osmotic adjustment, enabling plants to sustain water uptake and cell turgor under salt stress. The activation of stress-responsive signaling pathways, including those mediated by the SOS (Salt Overly Sensitive) system, further aids in maintaining K^+/Na^+ balance. Enhancing K^+ homeostasis through genetic engineering or nutrient management strategies can significantly improve plant salt tolerance, ensuring better growth and productivity in saline-prone agricultural regions.

3. Calcium (Ca^{2+}) Signaling

Calcium (Ca^{2+}) signaling plays a central role in plant salt tolerance by acting as a second messenger in stress perception and response. Under salt stress, elevated sodium (Na^{+}) levels trigger rapid changes in cytosolic Ca^{2+} concentrations, which are sensed by calcium-binding proteins such as calmodulins (CaMs), calcineurin B-like proteins (CBLs), and Ca^{2+}-dependent protein kinases (CDPKs). These proteins decode the Ca^{2+} signal and activate downstream pathways to mitigate the detrimental effects of salt stress. For instance, the CBL-CIPK (calcineurin B-like protein-CBL-interacting protein kinase) complex activates the SOS1 (Salt Overly Sensitive 1) Na^{+}/H^{+} antiporter, facilitating sodium efflux and ionic balance. Ca^{2+} signaling also modulates the expression of stress-responsive genes, osmoprotectant production, and antioxidant defenses, which collectively enhance cellular resilience. Additionally, localized Ca^{2+} spikes in specific tissues help fine-tune responses to salt stress. Manipulating Ca^{2+} signaling components through genetic or biotechnological approaches holds potential for improving salt tolerance in crops, enabling sustainable agriculture in saline environments.

4. MAPK cascade

Mitogen-activated protein kinase (MAPK) signaling pathways play a pivotal role in salinity tolerance by mediating stress perception, signal transduction, and downstream gene expression in plants. These pathways are activated in response to salt stress through receptors that sense external stimuli, leading to the phosphorylation cascade involving MAPKKK, MAPKK, and MAPK proteins. Once activated, MAPKs regulate the expression of stress-responsive genes, including those involved in ion transport, osmoprotection, and antioxidant defense. Key ion transporters, such as SOS1, HKT1, and NHX1, are modulated through MAPK signaling to maintain ion homeostasis. Additionally, MAPK pathways influence the production of osmoprotectants like proline and glycine betaine and enhance the activity of antioxidant enzymes such as SOD and CAT to mitigate oxidative stress. Transcription factors like DREB and WRKY, regulated by MAPK signaling, orchestrate complex gene networks that reinforce salinity tolerance. These mechanisms make MAPK pathways integral to improving plant resilience against salinity through targeted breeding and genetic engineering approaches.

4. Biotechnological Approaches to Enhance Salt Stress Adaptation

Biotechnological approaches offer promising solutions to enhance salt stress adaptation in plants, addressing a major challenge in agriculture. These methods involve genetic engineering, genome editing, and molecular breeding to develop salt-tolerant crop varieties. Key strategies include the

introduction or overexpression of genes involved in ion homeostasis, such as SOS1 (Salt Overly Sensitive 1), NHX1 (vacuolar Na^+/H^+ antiporter), and high-affinity potassium transporters, to regulate sodium exclusion and potassium retention. Genes encoding osmoprotectants like proline and glycine betaine, as well as those enhancing antioxidant enzyme activities, such as superoxide dismutase (SOD) and catalase (CAT), are targeted to mitigate oxidative damage under salt stress. Advanced genome-editing tools like CRISPR-Cas9 enable precise modifications of stress-related genes to improve salt tolerance. Additionally, transcription factors such as DREB and NAC families are engineered to regulate stress-responsive gene networks. Integrating these approaches with marker-assisted breeding and omics technologies ensures efficient identification and manipulation of salt-tolerance traits, paving the way for the development of resilient crops suited for saline-prone agricultural regions.

5. Emerging Insights from Omics Studies

Emerging insights from omics studies have significantly advanced our understanding of salt tolerance mechanisms in plants, offering new avenues for crop improvement. Genomics, transcriptomics, proteomics, metabolomics, and ionomics collectively provide a comprehensive view of the molecular and physiological changes plants undergo under salt stress. Genomic studies have identified key salt-tolerance genes, such as those involved in ion transport, osmoprotection, and antioxidant defense. Transcriptomic analyses reveal the dynamic regulation of stress-responsive pathways, including transcription factors like DREB, NAC, and bZIP, that orchestrate downstream gene networks. Proteomic studies highlight post-translational modifications and stress-induced protein complexes, while metabolomics identifies crucial osmolytes, secondary metabolites, and signaling molecules involved in stress adaptation. Ionomics sheds light on ion homeostasis and distribution patterns, essential for maintaining cellular function under salinity. Integrating data from these omics platforms with bioinformatics and systems biology enables the identification of key regulatory hubs and cross-talk mechanisms, facilitating the development of salt-tolerant crops through targeted genetic engineering and breeding strategies. These insights are vital for addressing the challenges of salinity in agriculture and ensuring food security.

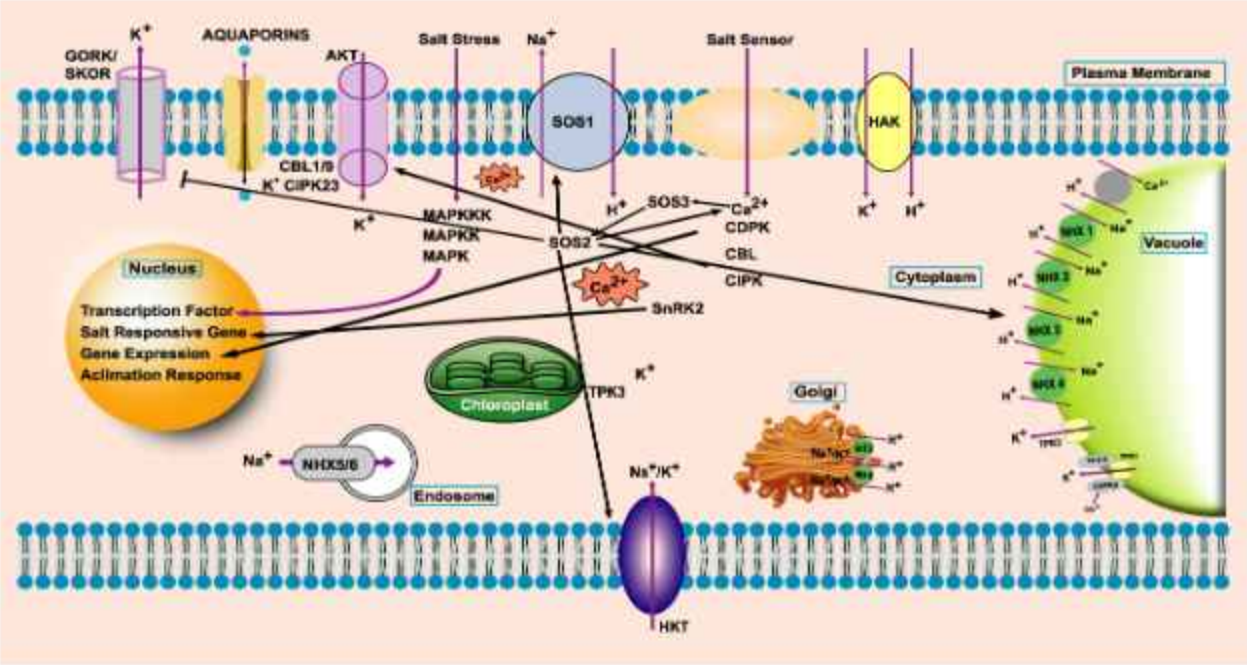

Figure : Schematic Representation of Salt Tolerance Mechanisms and Key Components of the Salt Stress Response Network in Jute

Future Perspectives

The integration of multi-omics data, machine learning, and systems biology is revolutionizing the discovery of novel genes and pathways for salinity tolerance. These advanced tools accelerate the identification of genetic targets and enhance our understanding of complex stress-response mechanisms. Additionally, harnessing natural genetic variation through genome editing and speed breeding offers rapid solutions for developing resilient crop varieties tailored to specific saline environments. Genome editing, in particular, represents a transformative approach to improving salinity tolerance by precisely modifying key genes involved in salt stress adaptation. This technology, combined with advancements in genomics, bioinformatics, and systems biology, holds immense potential to produce high-yielding, salt-tolerant crops, addressing the challenges of soil salinization.

In conclusion, leveraging salinity tolerance genes through cutting-edge biotechnological approaches will be instrumental in achieving global food security and fostering sustainable agriculture amidst escalating soil salinization and the impacts of climate change.

Identification of Candidate Genes and Cross-Talk Mechanisms Using Pathway and Network Analysis for Varietal Development

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The development of improved plant varieties is a cornerstone of agricultural advancement, driven by the need to enhance productivity, resilience, and adaptability to environmental challenges. The identification of candidate genes and the elucidation of cross-talk mechanisms between signaling pathways are pivotal in understanding and engineering traits for varietal development. Modern bioinformatics tools and systems biology approaches, such as pathway and network analysis, offer powerful methods to achieve these goals.

In plants, various types of pathways are involved in regulating growth, development, response to environmental cues, and stress management. Below are key types of pathways in plants, including metabolic, regulatory, signaling, and other essential pathways:

1. Metabolic Pathways

A metabolic pathway is a series of interconnected biochemical reactions that occur within a cell to convert substrates into end products, which are essential for maintaining cellular functions and homeostasis. These pathways are often catalyzed by enzymes, which facilitate the transformation of molecules by reducing activation energy. Metabolic pathways can be classified into two broad categories: catabolic pathways, which break down molecules to release energy (e.g., glycolysis and the citric acid cycle), and anabolic pathways, which use energy to build complex molecules (e.g., protein and nucleic acid synthesis). Pathways often involve intermediates, which can be used in multiple processes or regulated to respond to environmental or internal signals. The coordination of various metabolic pathways ensures the efficient use of resources and the balance of energy, nutrients, and waste products necessary for the growth, development, and survival of organisms. Detailed analysis of metabolic pathways helps us understand diseases, metabolic disorders, and the regulation of biological systems, and provides insights for drug design, agriculture, and biotechnology.

2. Regulatory Pathways

Regulatory pathways are intricate networks of signaling mechanisms that control various cellular processes, ensuring that the organism's development, growth, and response to environmental stimuli occur in an orderly and balanced manner. These pathways involve the activation or inhibition of specific genes, proteins, or metabolites through signal transduction cascades, which often begin with receptors detecting external signals such as hormones, light, temperature, or nutrients. The receptors trigger intracellular signaling molecules like kinases, phosphatases, and transcription factors, which then regulate the expression of target genes or modify the activity of enzymes, leading to cellular responses such as growth, differentiation, stress adaptation, or metabolic regulation. Examples of key regulatory pathways include the MAPK pathway, which controls cell division and stress responses, and the TOR (Target of Rapamycin) pathway, which regulates cell growth in response to nutrient availability. These regulatory networks ensure the dynamic and efficient functioning of biological systems, adapting to both internal and external changes, and are crucial in maintaining homeostasis and enabling the organism to thrive in diverse conditions.

3. Signaling Pathways

Signaling pathways are complex systems of molecular interactions that allow cells to communicate with each other and respond to various internal and external stimuli. These pathways typically begin when a signaling molecule, such as a hormone, nutrient, or environmental factor, binds to a receptor on the cell membrane or inside the cell. This binding activates intracellular signaling proteins, often through processes like phosphorylation, which transmit the signal through a cascade of molecular events. These events can lead to changes in gene expression, protein activity, or cellular behavior, ultimately influencing processes like growth, differentiation, immune responses, or stress adaptation. Key examples of signaling pathways include the MAPK/ERK pathway, which regulates cell growth and division, the Wnt/β-catenin pathway, which is crucial for cell fate determination and development, and the JAK-STAT pathway, which mediates immune responses. Signaling pathways are tightly regulated by feedback mechanisms to ensure appropriate responses, and dysregulation of these pathways can lead to diseases such as cancer, autoimmune disorders, and metabolic diseases. Understanding signaling pathways is vital for advancing therapeutic strategies in medicine and biotechnology.

4. Developmental Pathways

Developmental pathways are crucial networks of molecular and cellular processes that guide the growth, differentiation, and morphogenesis of organisms throughout their life cycle. These pathways orchestrate the formation of tissues, organs, and structures by regulating gene expression, cell division, migration, and apoptosis in response to both intrinsic genetic instructions and external signals. Key developmental pathways include the Notch signaling pathway, which influences cell fate decisions and tissue patterning, the Hedgehog pathway, which governs the development of limbs, the nervous system, and other tissues, and the TGF-β (Transforming Growth Factor Beta) pathway, which regulates cell differentiation and immune responses. These pathways are tightly regulated at multiple levels, ensuring precise control of developmental timing and processes. Disruptions in developmental pathways can lead to developmental disorders, congenital diseases, and cancers. Understanding these pathways is essential for advancing regenerative medicine, tissue engineering, and developmental biology, providing insights into how organisms grow and develop from a single fertilized cell into complex multicellular structures.

5. Defense Pathways

Defense pathways are essential biological mechanisms that enable organisms to detect and respond to harmful stimuli such as pathogens, toxins, and environmental stressors. In plants, these pathways involve a complex network of signaling systems that activate immune responses, including the recognition of pathogen-associated molecular patterns (PAMPs) through pattern recognition receptors (PRRs), which trigger the PAMP-triggered immunity (PTI) response. Following pathogen invasion, plants can activate effector-triggered immunity (ETI), where intracellular receptors recognize pathogen effectors and initiate stronger defense responses, such as the production of reactive oxygen species (ROS), antimicrobial compounds, and cell wall fortifications. In animals, defense pathways involve the innate immune system, where receptors like Toll-like receptors (TLRs) detect foreign pathogens and activate signaling cascades, including the NF-κB pathway, leading to inflammation and the recruitment of immune cells. Additionally, the adaptive immune response, involving T and B cells, is activated to target specific pathogens. These defense pathways are tightly regulated to balance effective protection while minimizing collateral damage to the host's own tissues. Disruptions in defense pathways can result in autoimmune diseases, chronic inflammation, or increased susceptibility to infections, highlighting the importance of these systems in maintaining organismal health.

6. Symbiotic Pathways

Symbiotic pathways are complex molecular networks that govern the interactions between organisms living in close association, where both parties typically benefit from the relationship. In plants, one of the most well-known examples is the mycorrhizal symbiosis, where fungi colonize plant roots, enhancing the plant's nutrient uptake, especially phosphorous, in exchange for carbon

compounds. Similarly, rhizobial symbiosis involves nitrogen-fixing bacteria that live in the roots of legumes, converting atmospheric nitrogen into a form the plant can use, while the plant provides carbohydrates to the bacteria. These interactions are regulated through signaling pathways that allow plants to recognize and respond to their symbiotic partners. In the case of mycorrhizal and rhizobial symbioses, plant receptors on root cells detect signaling molecules from the fungi or bacteria, triggering a cascade of responses that lead to the formation of symbiotic structures, such as root nodules or fungal hyphae networks.

Cross-Talk Mechanisms

Pathway and network analysis of cross-talk mechanisms refers to the study of how different signaling pathways and biological networks interact and influence each other within a cell or organism. These cross-talks are crucial for integrating diverse signals from environmental, physiological, and developmental stimuli, enabling the cell to produce a coordinated response. For instance, hormonal pathways, such as those regulated by auxin, gibberellins, and cytokinin, can interact with stress response pathways, including those triggered by drought, salinity, or pathogens, through shared signaling components or downstream transcription factors. This integration allows plants to adapt to changing conditions while maintaining overall homeostasis. Similarly, metabolic pathways involved in energy production can cross-talk with pathways controlling growth and cell division, ensuring that energy is efficiently used for developmental processes. Understanding these complex interactions through pathway and network analysis helps in identifying key regulatory nodes and biomarkers that can be targeted for improving stress tolerance, disease resistance, or growth characteristics in crops. Additionally, it opens opportunities for designing new biotechnological strategies, such as gene editing or synthetic biology, to optimize plant performance in various agricultural contexts.

Some practical use of pathway and network as Use of Biotechnological Tools for Varietal Development

Understanding pathways and networks in biological systems has significant practical applications, particularly in agriculture, where biotechnological tools are employed for varietal development. By analyzing metabolic, signaling, and regulatory pathways, scientists can pinpoint crucial genes responsible for important traits like disease resistance, drought tolerance, yield, and nutritional content. With tools like genetic engineering, CRISPR-Cas9, and marker-assisted selection, researchers can modify specific pathways to enhance desirable crop traits. In the context of Bangladesh, pathway and network analysis has been applied in various biotechnological research projects to improve crop quality and resilience are listed below. These advancements not only help in developing high-yielding and robust crop varieties but also play a key role in promoting sustainable agriculture by reducing dependence on chemical inputs.

Fiber formation pathway in Jute

The fiber formation pathway in jute, a major natural bast fiber crop, involves a complex process of cellular differentiation and biochemical pathways that lead to the development of its characteristic strong and durable fibers. Jute fibers are primarily composed of cellulose, hemicellulose, and lignin, with cellulose being the major structural component. The fiber formation begins with the differentiation of vascular tissues in the stem, where specialized cells known as fiber cells are formed. These cells undergo a process of secondary wall thickening, primarily through the deposition of cellulose and hemicellulose. Lignin, which is involved in strengthening plant tissues, is also deposited but in lower amounts compared to other fiber crops like flax or hemp, making jute fibers more flexible. The biosynthesis of cellulose in jute is regulated by key enzymes, including cellulose synthase, and the formation of the lignin structure is controlled by genes involved in the phenylpropanoid pathway, such as PAL (phenylalanine ammonia-lyase), C4H (cinnamate-4-hydroxylase), and 4CL (4-coumarate-CoA ligase). These genes play a crucial role in lignin biosynthesis, contributing to the structural integrity of the fiber. The process of fiber development also involves hormonal regulation, with auxins and cytokinins influencing cell elongation and differentiation. Understanding the fiber formation pathway in jute can help improve the quality and yield of fibers through genetic and biotechnological interventions.

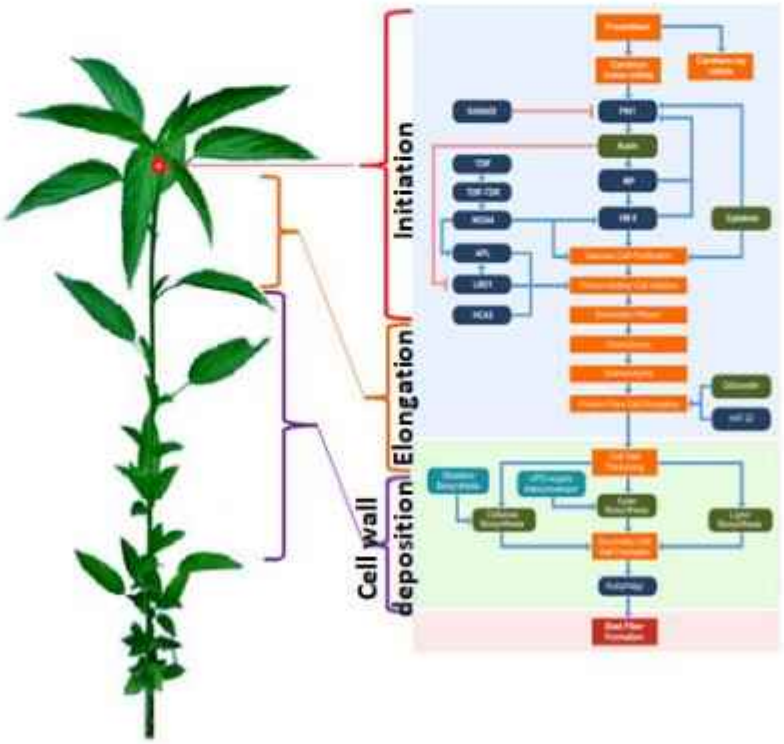


Figure 1. Schematic representation of the fiber formation pathway in jute, depicting the key stages of fiber development, including fiber initiation, differentiation of vascular tissues, cell elongation, and the biosynthesis of cellulose, hemicellulose, and lignin.

Regulation of transcription in secondary cell wall formation.

Transcriptional regulation of secondary cell wall formation is a highly orchestrated process that controls the expression of genes involved in the synthesis of cellulose, hemicellulose, and lignin, which are the major components of the secondary cell wall. This regulation is essential for the structural integrity of plant cells, particularly in cells like fibers, xylem vessels, and sclerenchyma that require strong, rigid walls for support and water transport. Several key transcription factors, such as MYB, NAC, WRKY, and bHLH families, play critical roles in activating or repressing genes related to secondary wall biosynthesis. For example, the MYB46 and MYB83 transcription factors are central regulators of secondary cell wall formation, activating the expression of genes involved in cellulose, lignin, and xylan biosynthesis. Additionally, NAC transcription factors like NST1, SND1, and VND6 are involved in promoting secondary wall biosynthesis in specific cell types, particularly in xylem and fiber cells. These transcription factors often work in a cascade, with early regulators activating downstream genes that further fine-tune the deposition of cell wall components. Understanding the transcriptional regulation of secondary cell wall formation is crucial for improving plant biomass, fiber quality, and resistance to environmental stresses in agricultural and industrial applications.

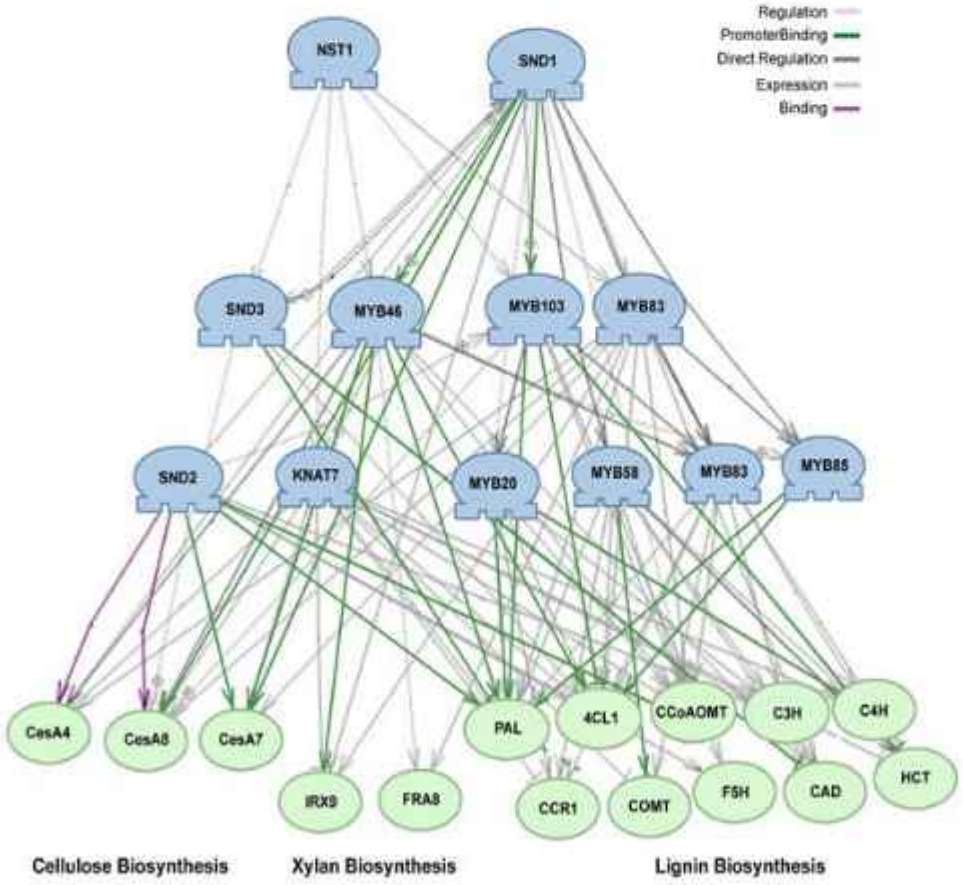


Figure 2: Transcriptional regulation of secondary cell wall formation

Lignin Biosynthesis Pathway

Reducing lignin content in plants by targeting the lignin biosynthesis pathway involves manipulating specific genes or enzymes responsible for lignin production. This pathway is a part of phenylpropanoid metabolism, which leads to the synthesis of monolignols, including p-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol. These monolignols polymerize to form lignin, a key structural component of plant cell walls. By analyzing the genes involved in this pathway, such as Phenylalanine Ammonia-Lyase (PAL), Cinnamate-4-Hydroxylase (C4H), 4-Coumarate-CoA Ligase (4CL), Caffeic Acid O-Methyltransferase (COMT), and Caffeoyl-CoA O-Methyltransferase (CcAoMT) family genes, it becomes possible to identify suitable targets for genetic manipulation. Modifying these genes can reduce lignin synthesis while preserving other metabolic processes, enabling applications in agriculture and industry where reduced lignin content is advantageous, such as improved forage digestibility or enhanced biofuel production efficiency.

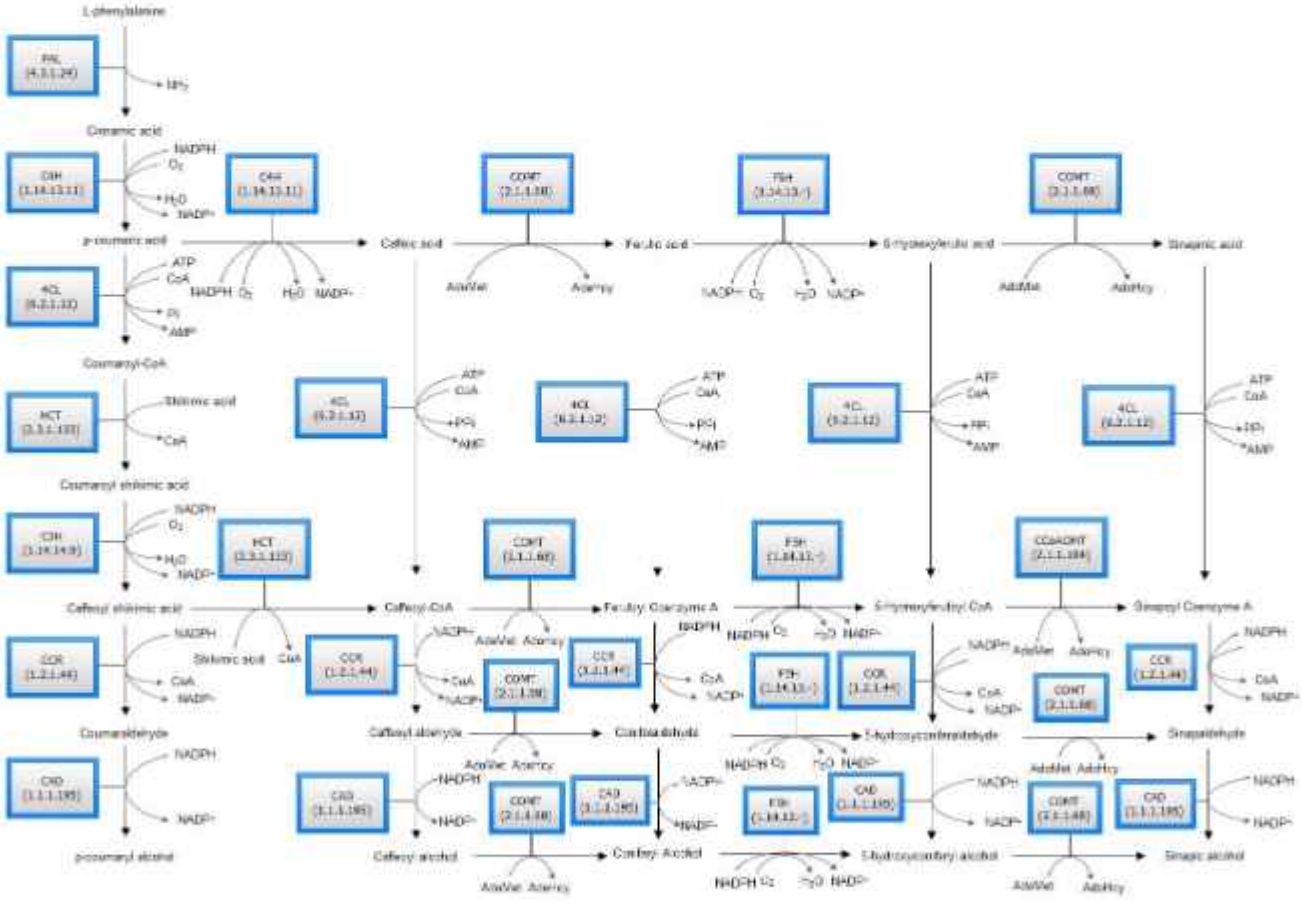


Figure 3. Schematic representation of the lignin biosynthesis pathway, illustrating the key enzymatic steps involved in the synthesis of lignin monomers from phenylalanine

Flower induction Pathway

The flower induction pathway governs the transition from vegetative to reproductive growth in plants and integrates environmental cues (like light and temperature) with internal signals (such as developmental stages and hormones). Key pathways include the photoperiod pathway, where light-regulated genes like CONSTANS (CO) activate FLOWERING LOCUS T (FT) under specific day lengths; the vernalization pathway, where prolonged cold exposure suppresses FLOWERING LOCUS C (FLC) to permit flowering; and the gibberellin (GA) pathway, which promotes flowering through hormonal signaling. The autonomous and age pathways regulate flowering independently, with the latter involving miR156-SPL interactions to time flowering based on plant maturity. Integrative genes like SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1 (SOC1) act as hubs, coordinating inputs from these pathways. Analyzing this process involves studying gene expression, protein interactions, and chromatin modifications, providing insights into optimizing flowering time for agriculture and adapting plants to changing environments.

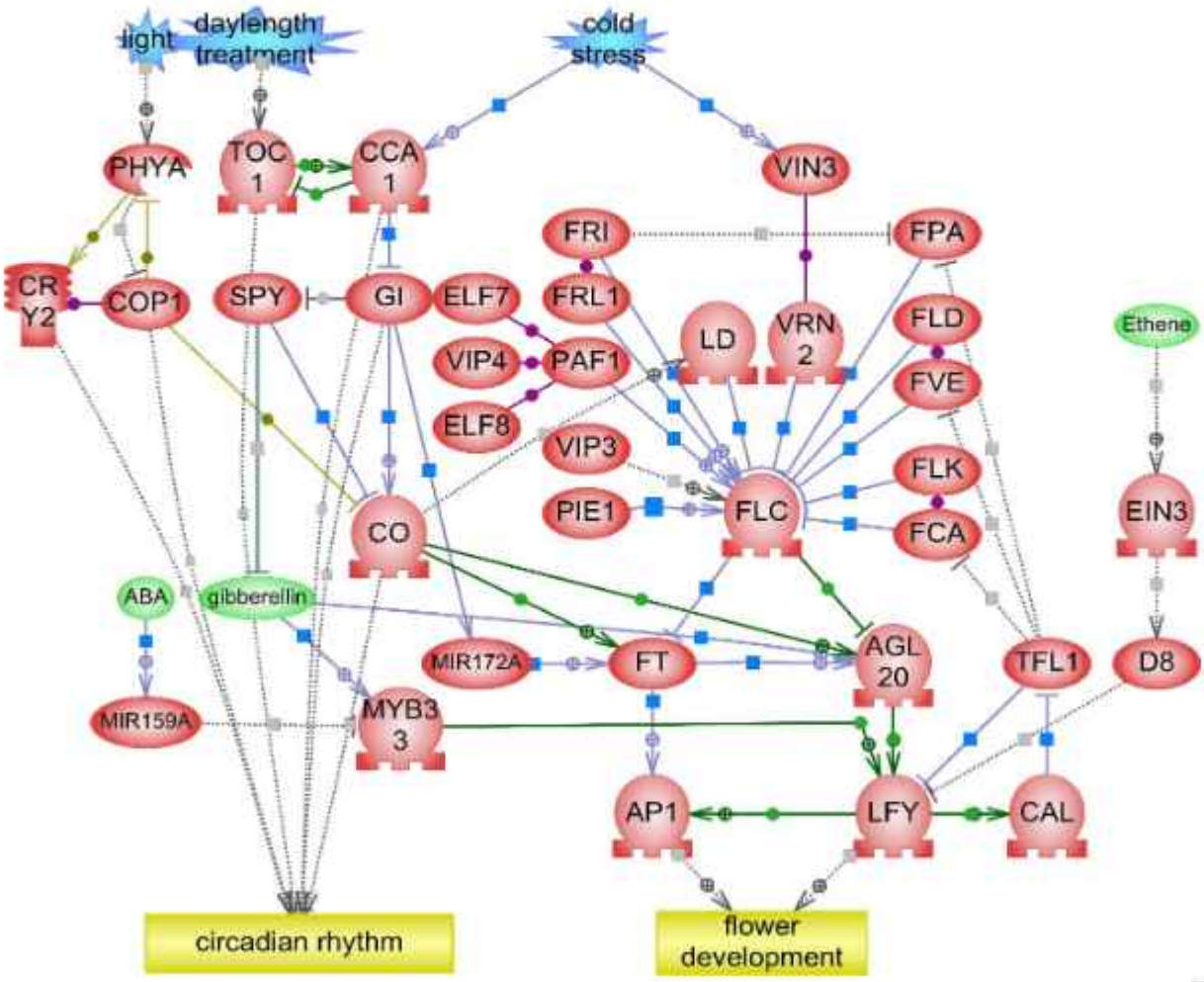


Figure 4. Possible flowering induction pathway during the flower development along with circadian rhythm.

Heavy metal sequestration pathway

The heavy metal sequestration pathway in plants enables tolerance and detoxification of toxic metals by regulating their uptake, transport, and compartmentalization. Key mechanisms include metal chelation by molecules such as phytochelatins (PCs) and metallothioneins (MTs), which bind heavy metals to form stable complexes. Transporters like Heavy Metal ATPases (HMAs), Natural Resistance-Associated Macrophage Proteins (NRAMPs), and Zinc/Iron-Regulated Transporter (ZIP) family proteins facilitate metal uptake, translocation, and sequestration in vacuoles or cell walls. Additionally, secondary metabolites like organic acids (e.g., citric acid) chelate metals in the cytosol, reducing toxicity. Phytoremediation processes also involve root exudates altering metal bioavailability and the role of antioxidant enzymes mitigating oxidative stress caused by heavy metals. Understanding these pathways provides insights into engineering plants for enhanced metal tolerance and remediation of contaminated environments.

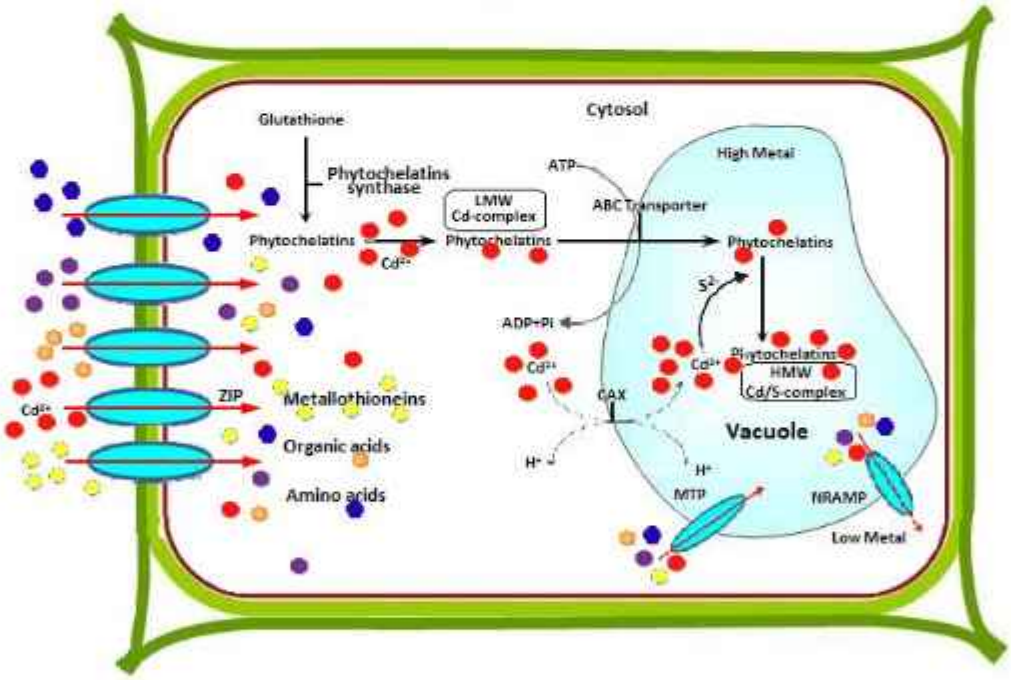


Figure 5. Schematic representation of the vacuolar sequestration pathway for heavy metal detoxification, illustrating the process by which plants uptake, transport, and sequester toxic heavy metals such as cadmium, lead, and arsenic into vacuoles.

Gibberellic acid biosynthesis pathway

The gibberellic acid (GA) biosynthesis pathway plays a pivotal role in regulating plant growth and development, including seed germination, stem elongation, and flowering. This pathway involves the conversion of geranylgeranyl diphosphate (GGDP) to active GAs through a series of enzymatic steps catalyzed by key enzymes such as ent-copalyl diphosphate synthase (CPS), ent-kaurene synthase (KS), ent-kaurene oxidase (KO), and GA 20-oxidase (GA20ox). The balance between active and inactive GAs is further controlled by GA 3-oxidase (GA3ox) and GA 2-oxidase (GA2ox). Genes encoding these enzymes are central to GA biosynthesis and are potential targets for enhancing growth rates. Identifying "quick-growing" genes involves analyzing expression profiles of GA-related genes and their regulatory elements under various conditions to determine their contribution to rapid growth phenotypes. CRISPR-Cas9 or overexpression techniques can be used to manipulate these genes, enhancing plant growth for agricultural productivity while maintaining optimal developmental and stress response balance.

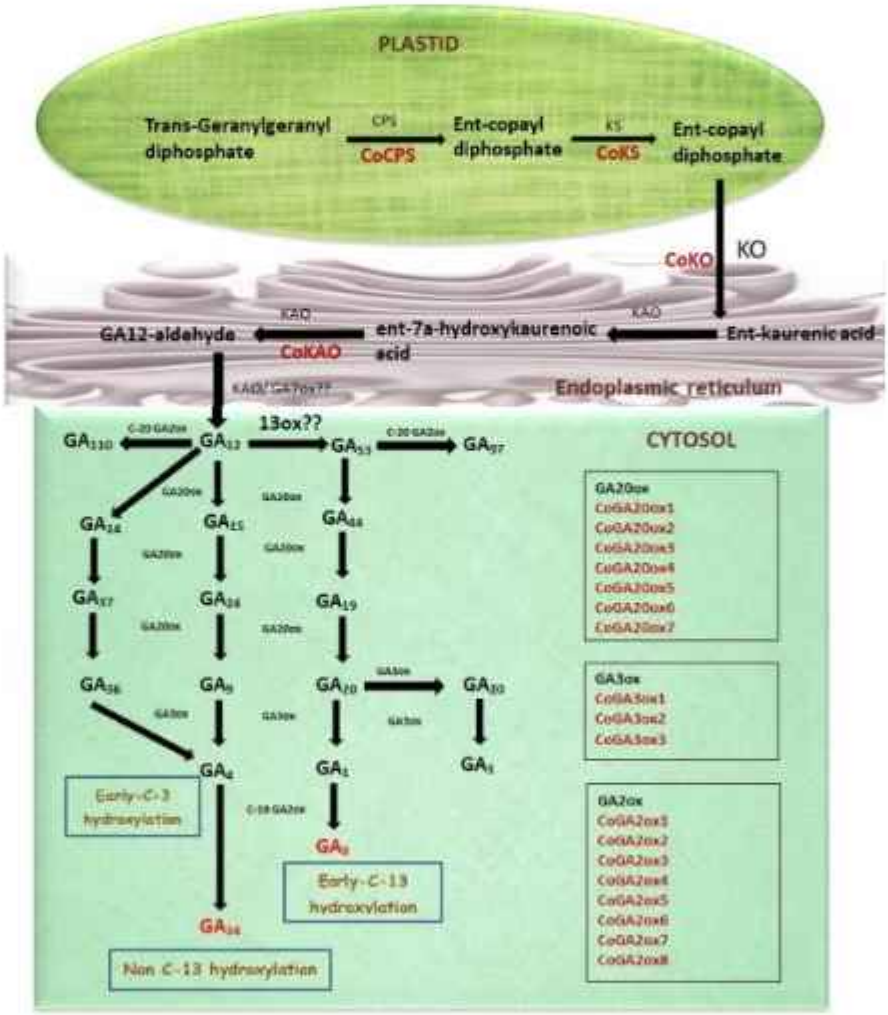


Figure 6. Schematic representation of the gibberellic acid (GA) biosynthesis pathway in jute, detailing the enzymatic steps involved in the production of gibberellic acid from geranylgeranyl pyrophosphate (GGPP).

Aroma in rice, mung bean

Knocking out the BADH2 (Betaine Aldehyde Dehydrogenase 2) gene is a key strategy to produce fragrant rice by disrupting the conversion of gamma-aminobutyraldehyde (GABald) to gamma-aminobutyric acid (GABA) in the GABA shunt pathway. In non-fragrant rice, BADH2 activity prevents the accumulation of 2-acetyl-1-pyrroline (2-AP), the compound responsible for the characteristic aroma in fragrant rice varieties. A loss-of-function mutation in BADH2 causes GABald to accumulate, enhancing 2-AP synthesis and developing the desired fragrance. Techniques such as CRISPR-Cas9 genome editing are used to specifically disrupt the BADH2 gene, and the success of this intervention is validated through gene expression analysis, metabolite profiling (quantifying 2-AP levels), and sensory evaluation of grain aroma. While this approach effectively enhances fragrance, potential impacts on stress tolerance due to disruption of the GABA shunt must be mitigated by monitoring plant metabolic balance and agronomic performance to ensure high-quality fragrant rice production.

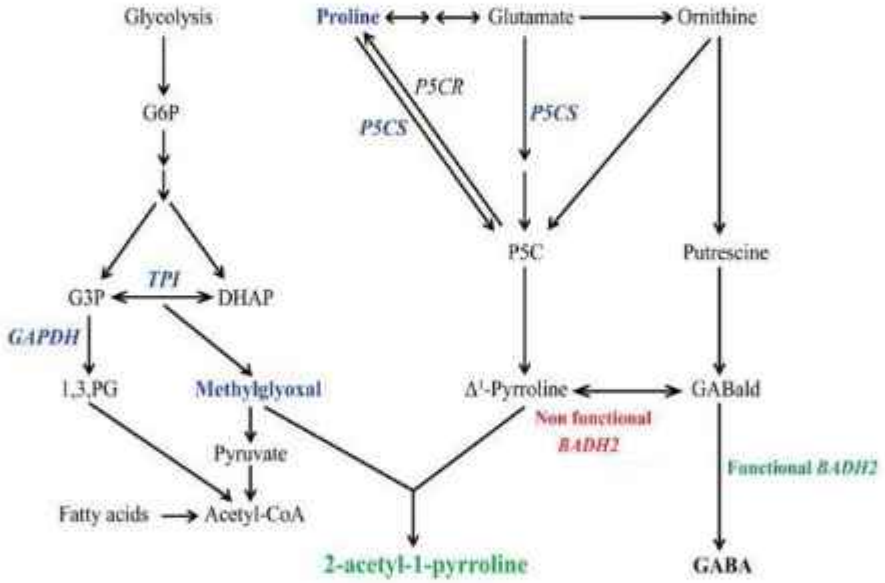


Figure 7. Schematic representation of the biosynthetic mechanism of 2-AP for fragrance production

Concluding remarks

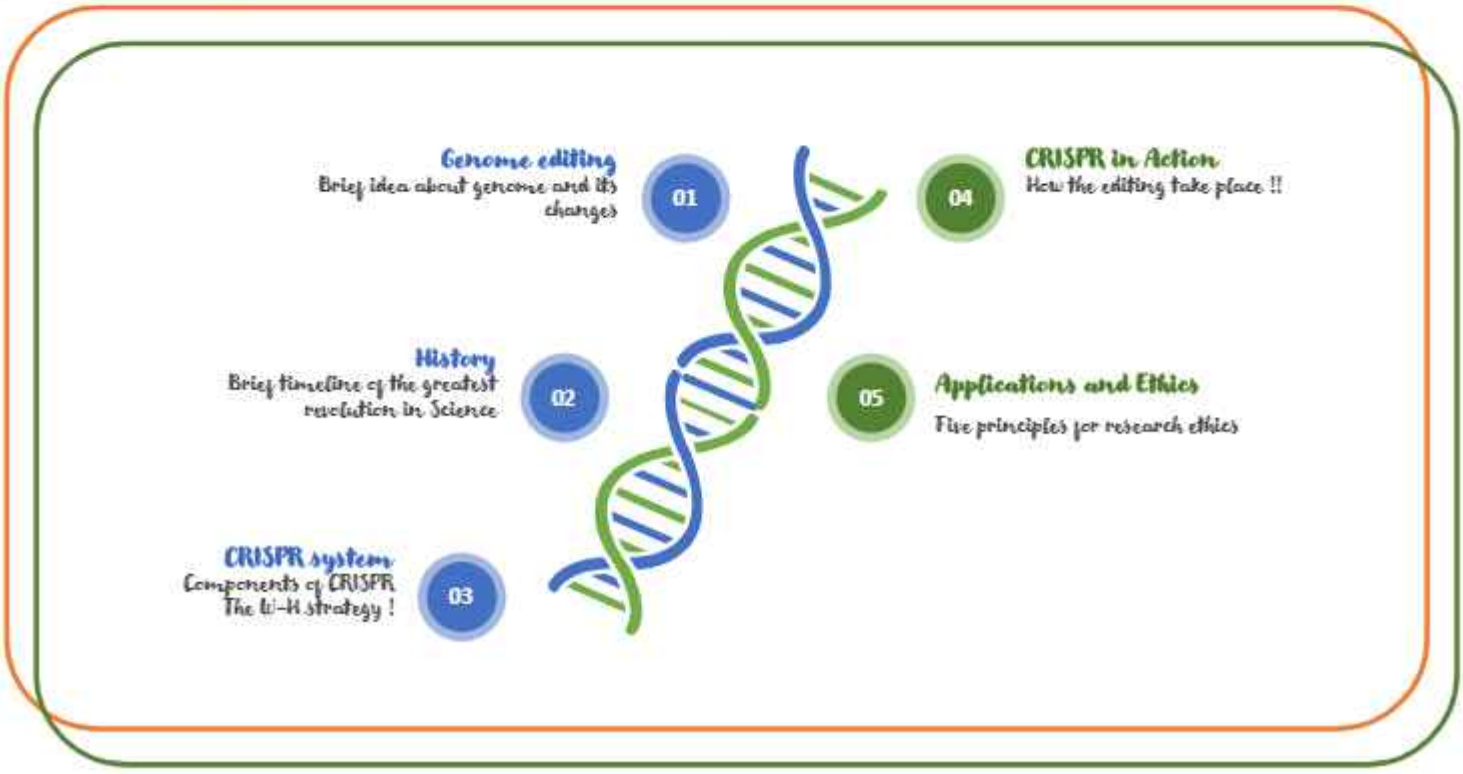
The integration of multi-omics data (genomics, transcriptomics, proteomics, and metabolomics) will further refine the identification of candidate genes and their roles in cross-talk mechanisms. Additionally, advancements in machine learning and artificial intelligence promise to revolutionize network analysis, making predictions more accurate and actionable for varietal development.

In summary, pathway and network analysis serve as indispensable tools in the identification of candidate genes and cross-talk mechanisms, paving the way for the development of improved plant varieties tailored to the demands of modern agriculture.

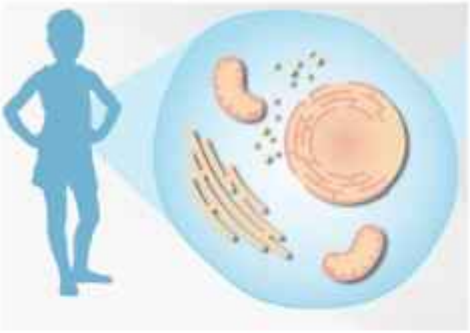
Genome editing, CRISPR workflow and it's potential in Agriculture

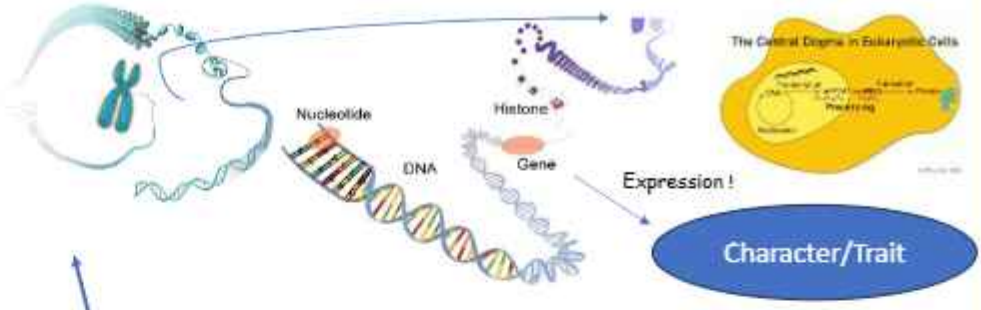
Tahmina Islam, PhD
Associate Professor
Plant Breeding and Biotechnology Laboratory
Department of Botany
University of Dhaka

Contents

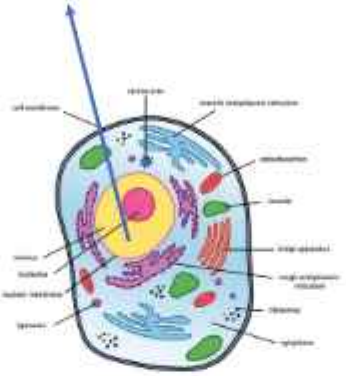


DNA and Gene





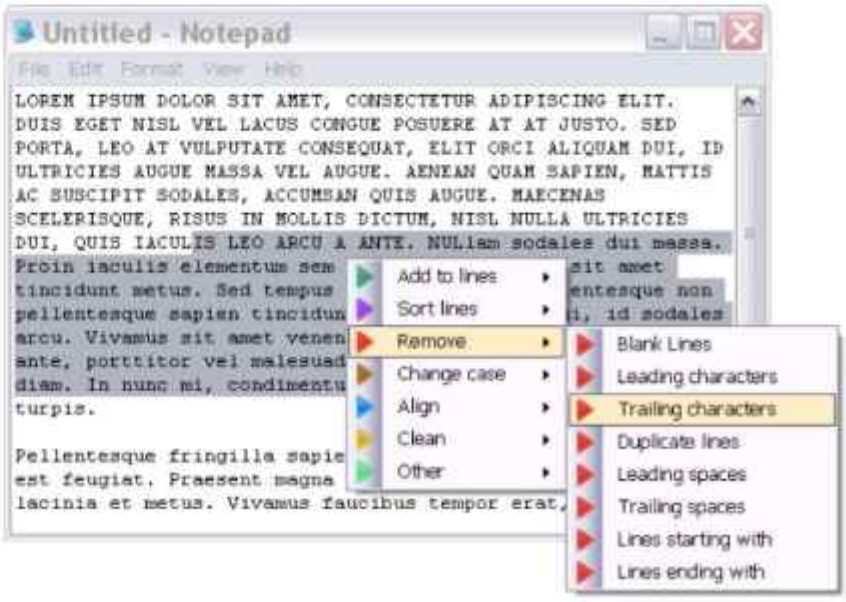
APPROX. 37.2 TRILLION CELLS



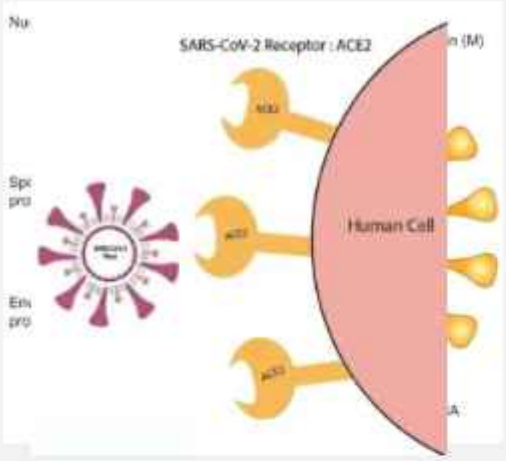
Gene is the functional Unit of DNA !

Background

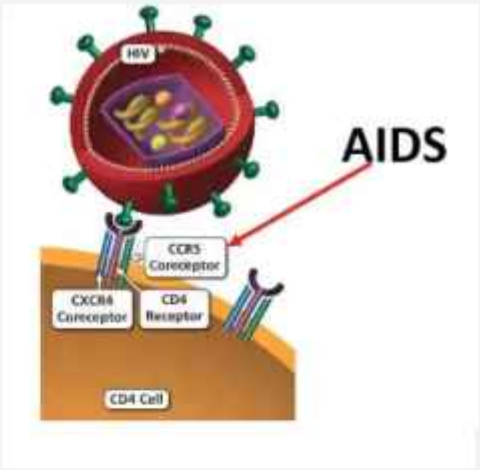
What if a cell's DNA could be edited just like the text of a document...



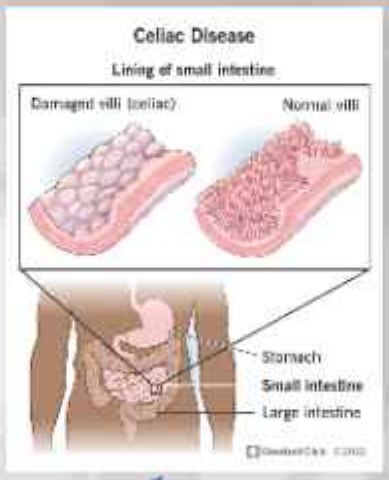
BRAIN Storming !!!



SARS-CoV2/Corona Virus



Is there a way to disrupt specifically the receptors??



Intestinal villi are tiny, finger-like projections that absorb nutrients from the food and then shuttle them into your bloodstream so they can travel where they're needed.

Immune response

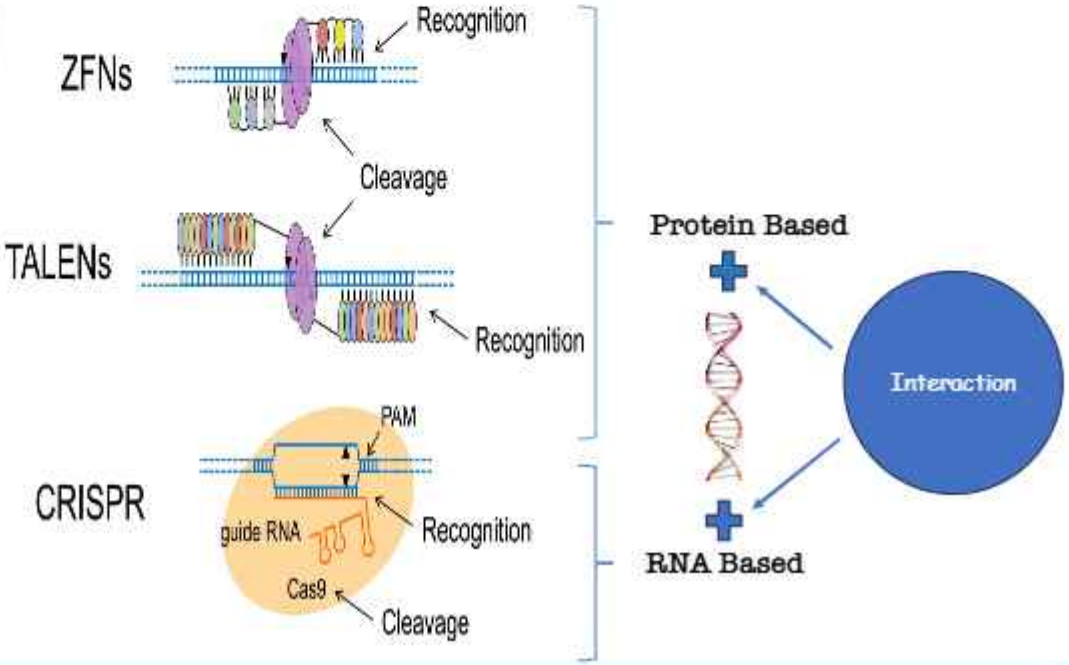
Antigenic

14 common foods with potential health risks

Can we specifically deactivate the gliadin forming gene in wheat ??

The Way: Genome editing

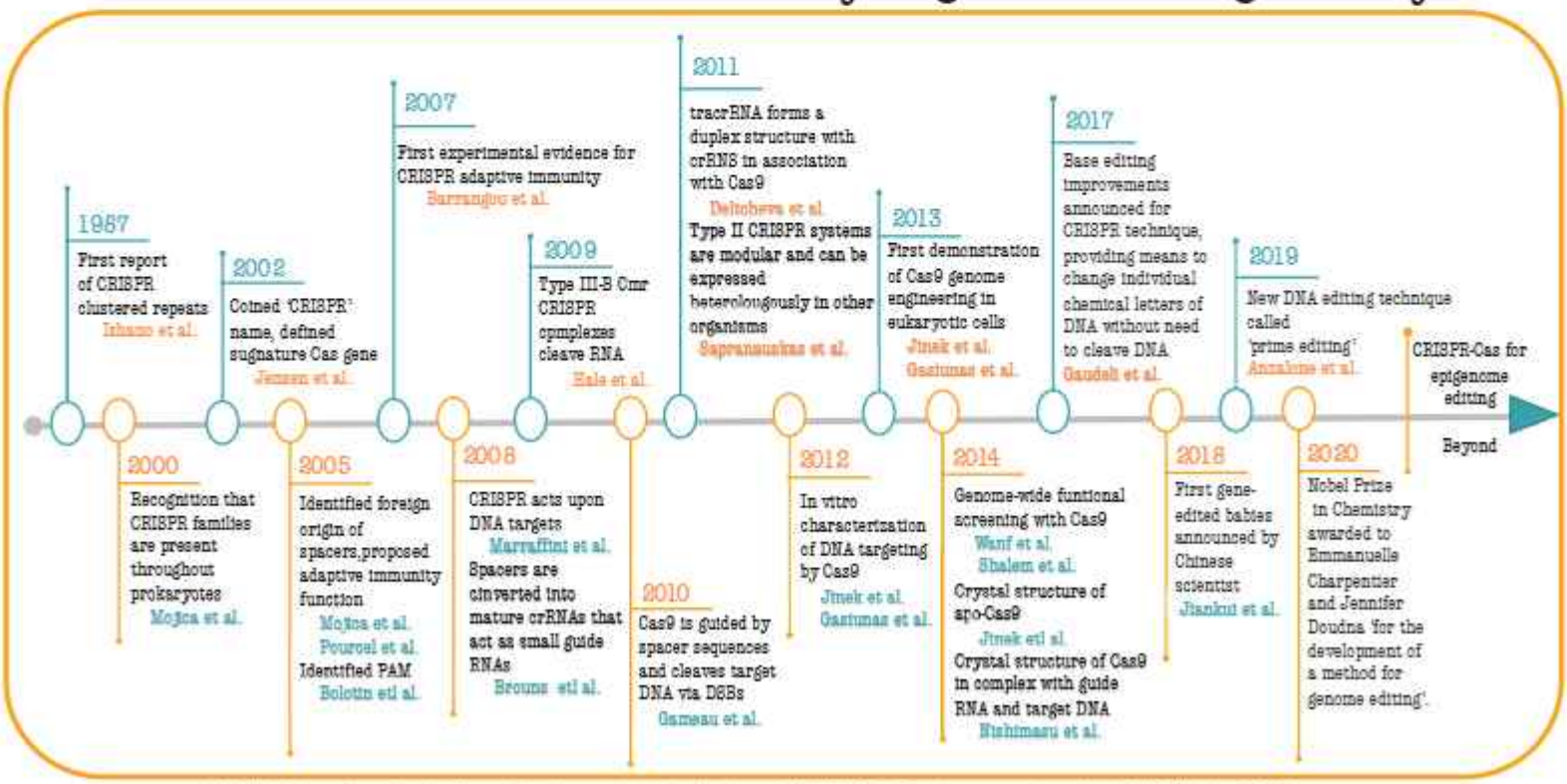
TOOLS TO DEACTIVATING THE GENES



History

Brief timeline of the greatest revolution in Science

The CRISPR Timeline: From discovery to genome editing and beyond



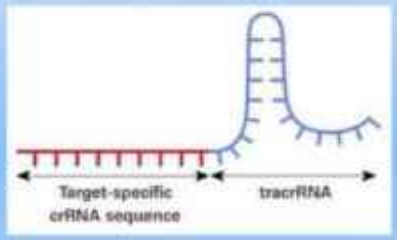
*Modified from Hsu, Lander,Zhang: Development and application of CRISPR-Cas9 for genome engineering: Cell 157, June6,2014

Main actors in CRISPR-Cas9*

Read: Antonio Regalado@Technologyreview.com:Who owns the biggest Biotech Discovery of the century ?
(*) Ingnores many other researchers and inventors such George Church (Harvard), Philippe Horvath, Podolphe Barrangou (Danisco?Dupont)

CRISPR loci and Cas nuclease nomenclature

CRISPR COMPONENTS



✓**crRNA/ CRISPR RNA :** Guide RNA present in Host DNA that bind with tracrRNA and form hairpin complex.

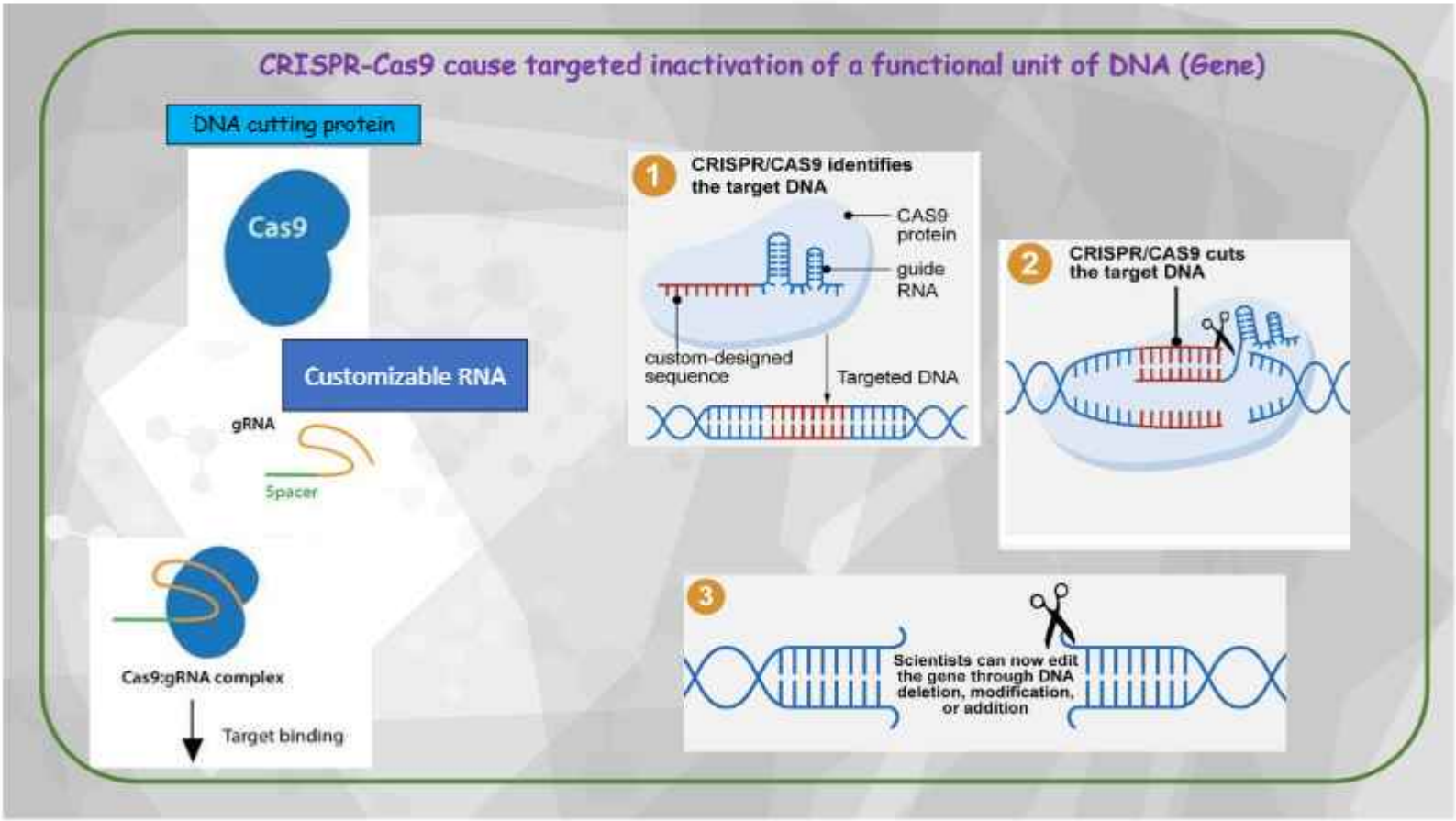
✓**tracrRNA :** Trans active RNA that bind with crRNA form active complex.

✓**sgRNA :** Single guide RNA (crRNA + tracrRNA)

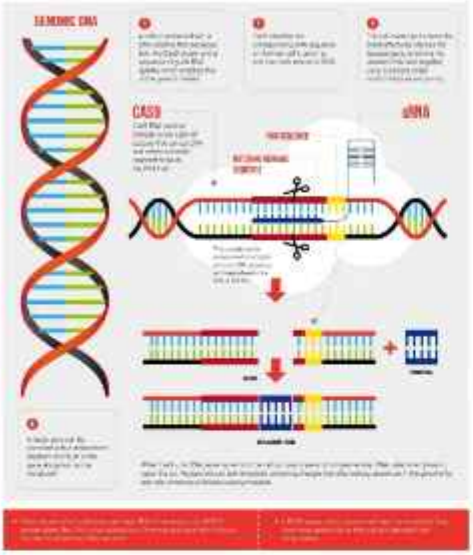
✓**Cas9 :** Protein / nuclease that can modify DNA

CRISPR in Action

How the editing takes place



HOW THE GENOME EDITOR WORKS

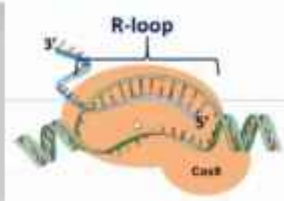


1 **Binding with NGG PAM (SpCas9)**

ATGGCAGGAGGTTTCTTGTCCATGGCTAACCCGGcggTCACCCTCTCCGGTGTTGCAG

20 bp genomic target PAM

2 • R-loop= double stranded to single stranded DNA

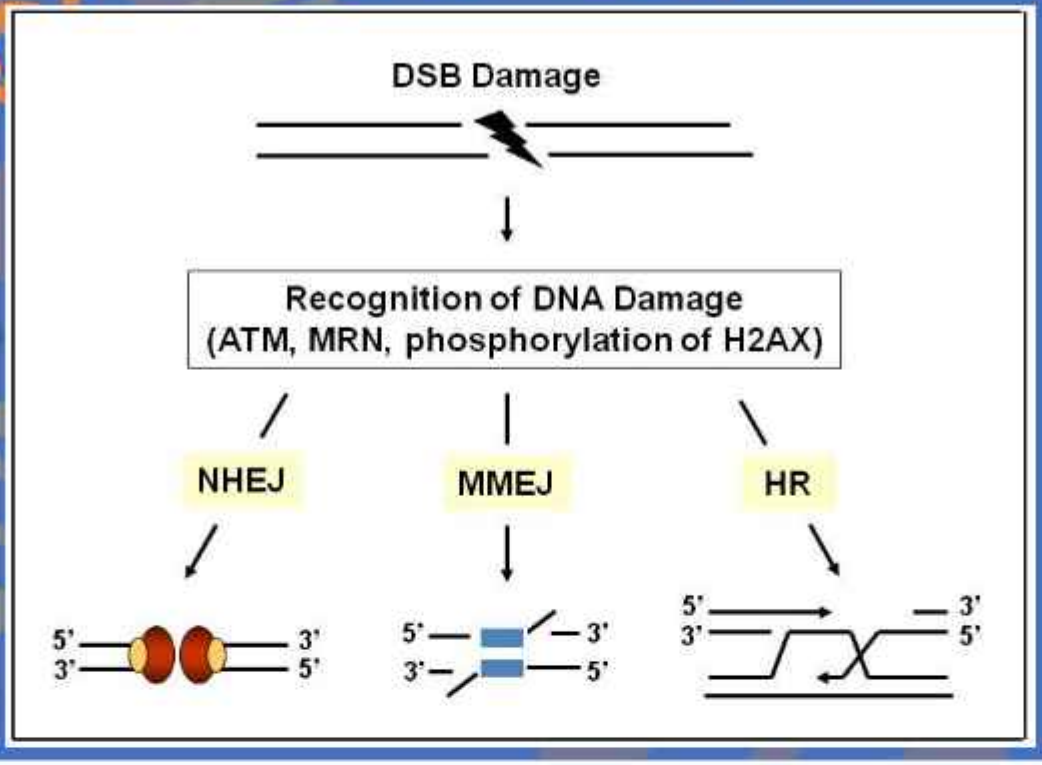


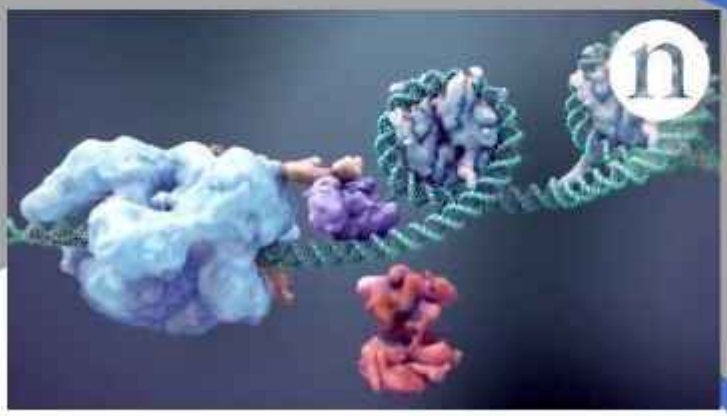
3 sgRNA binding with homologous sequence

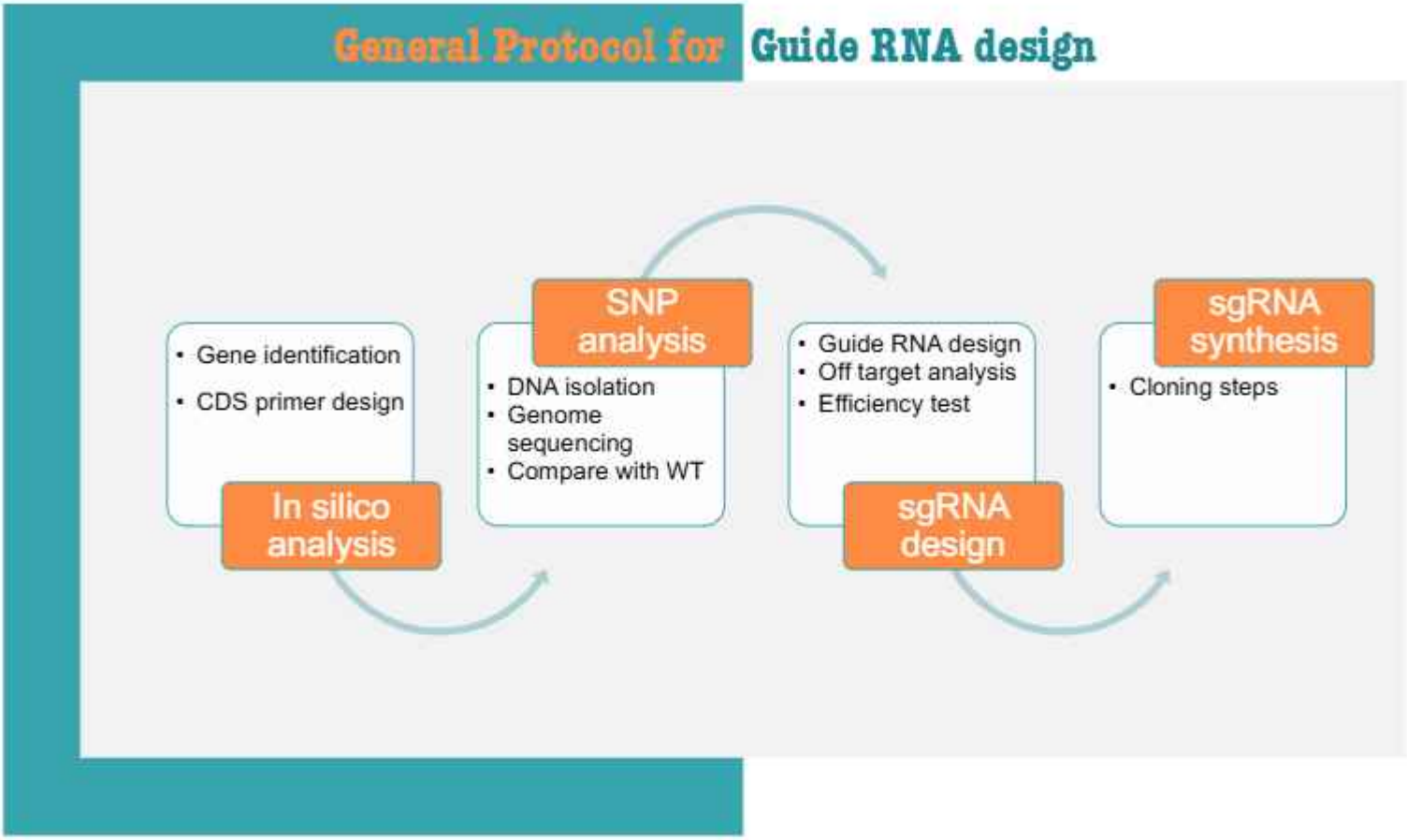
4 Cas9 makes a cut

What if you don't have a NGG PAM at your target locus

Two major repair pathways of DSBs






General Protocol for Guide RNA design
In silico analysis
• Gene identification
• CDS primer design
SNP analysis
• DNA isolation
• Genome sequencing
• Compare with WT
sgRNA design
• Guide RNA design
• Off target analysis
• Efficiency test
sgRNA synthesis
• Cloning steps


Selection of a target gene

CRISPR-based experiments follow two basic approaches into genome editing

1. Knock-in experiments(HDR) **2.** Knock-out experiments(NHEJ)

Selecting a trait gene as a target depends heavily on the kind of experiment that will be conducted

Usually, the first thing to decide is whether to work on a positive regulator or negative regulator of a significant physiological function in a species.

Functions that are usually looked for:

Abiotic stress tolerance

Biotic stress tolerance

Defense against antagonists

Induced Production of certain biochemicals etc.

(This headings cover a huge area of functions in general)

Negative regulators

- Crispr directed mutations in the target gene results in loss of it's function, hence the term knock-out

Positive regulators

- Any genetic element that is of hindrance to the positive regulator can be targeted through CRISPR
- Homology directed knock-in experiments can be conducted to induce functions of some positive regulators

Trait genes

- Conducting various expression experiments to identify physiological responder genes
- Or by searching through existing scientific literature

Case study:

A CRISPR genome editing experiment of the *OsbZIP52* Basic leucine zipper (bZIP) transcription factor, Negative regulator of cold and drought stress response in Oryza sativa (Rice)

Planta (2012) 235:1157–1169
DOI 10.1007/s00425-011-1564-z

ORIGINAL ARTICLE

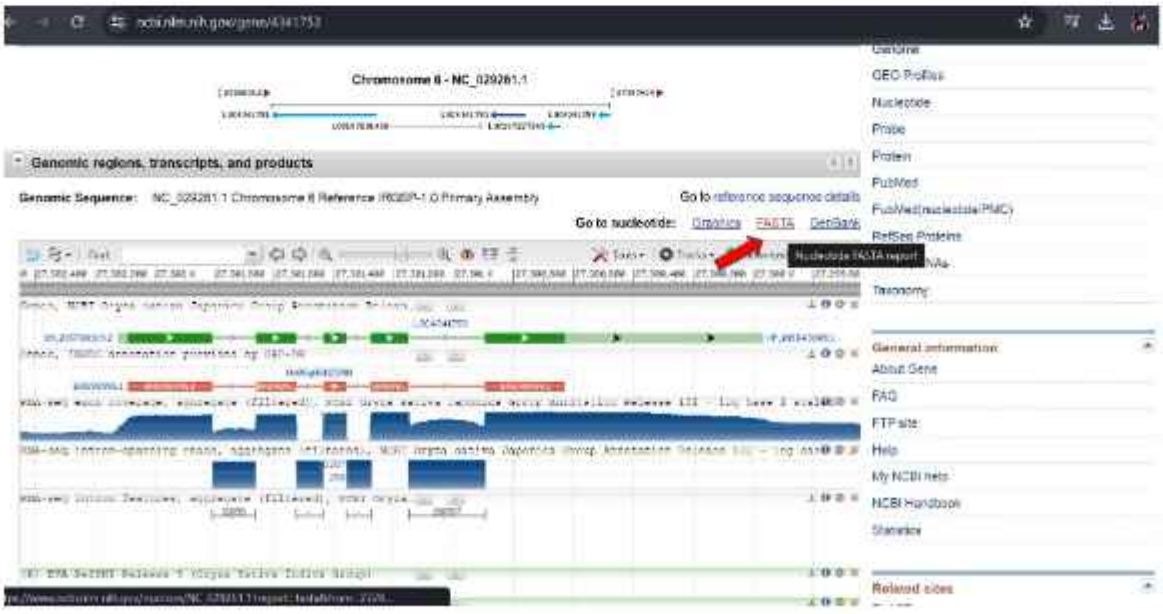
bZIP transcription factor *OsbZIP52/RISBZ5*: a potential negative regulator of cold and drought stress response in rice

Citao Liu · Yanbin Wu · Xiping Wang

Gene description & Primer design

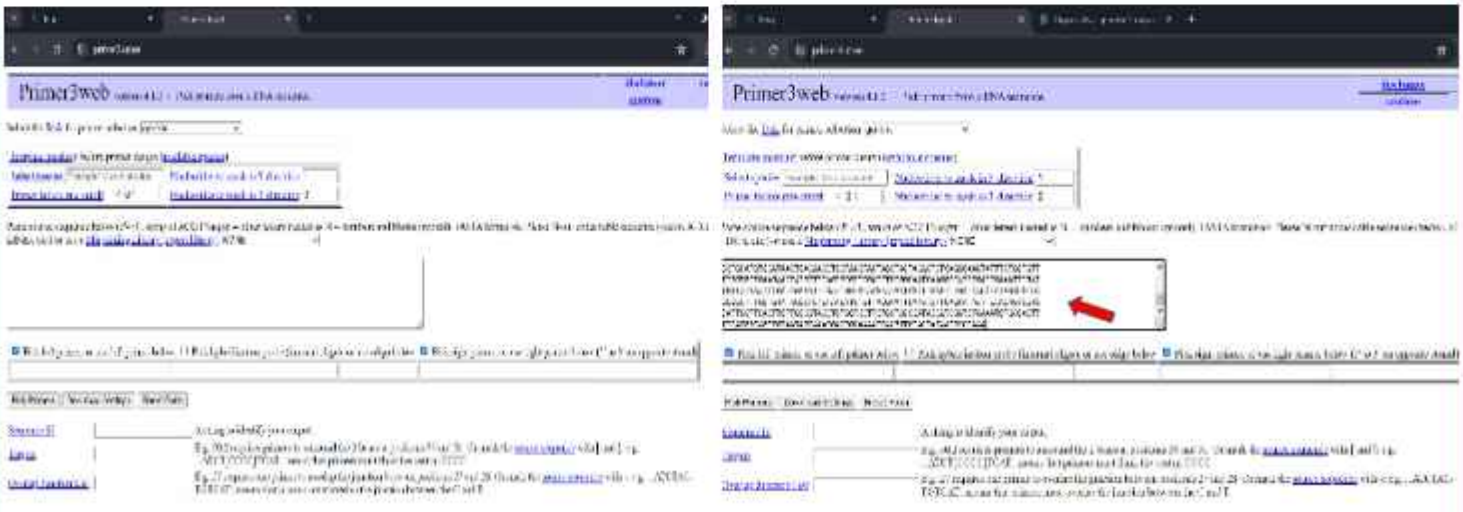
- After selection of the target gene the gene description must be downloaded from an authentic database.
- The NCBI- The National Center for Biotechnology database is a universal website for a wide range of genomic information download
- From this database, gene descriptions like the nucleotide sequence of the gene, accession ID, chromosomal location etc. can be downloaded.
- The downloaded nucleotide sequence of the gene will then be used to design target specific primers for the experiment

Use any proper or well-known website for genomic information of the gene.



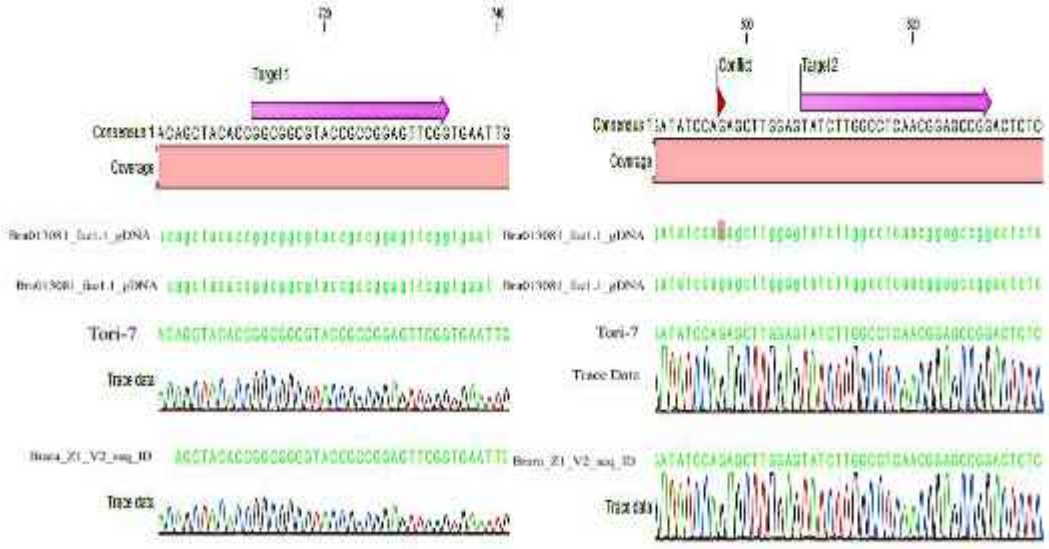
The nucleotide sequence is key to designing a proper target, save it for further analysis

Make sure to design primers needed in the experiment beforehand



3. Genome sequencing & SNP analysis

A standard SNP analysis is a molecular experimental procedure. Upon obtaining sequencing results, the results are aligned against genomic database and target region. Usually, The plant variety which shows lowest occurrence of SNPs in the target region compared to the genomic sequence available in the database (which will be used to construct sgRNAs) is selected for the project

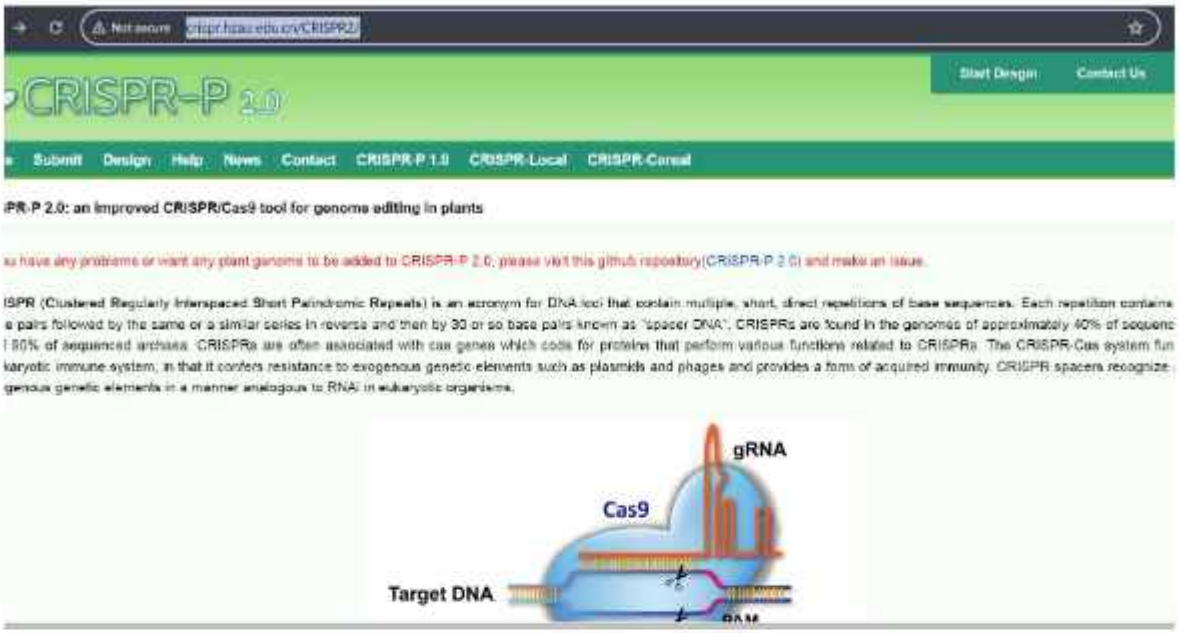


After sequencing and SNP analysis, plant material selection would be done. Upon completion, the sgRNAs for the target gene will be generated using an online tool.

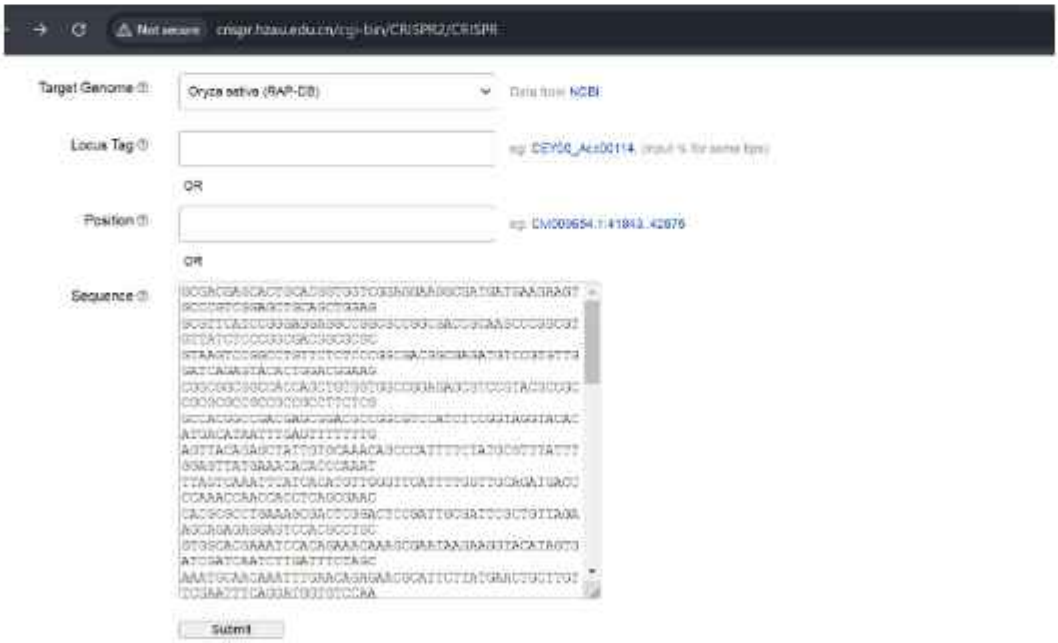
While designing sgRNAs via online tool, we are to take note that,

1. **The CRISPR-Cas system must be the standards sp-cas9 (Streptococcus pyogenes cas9 system) system**
2. **Guides should be no more than 20 nucleotides (excluding PAM)**
3. **Minimum 3 mismatches can be preferred while considering off-targets**

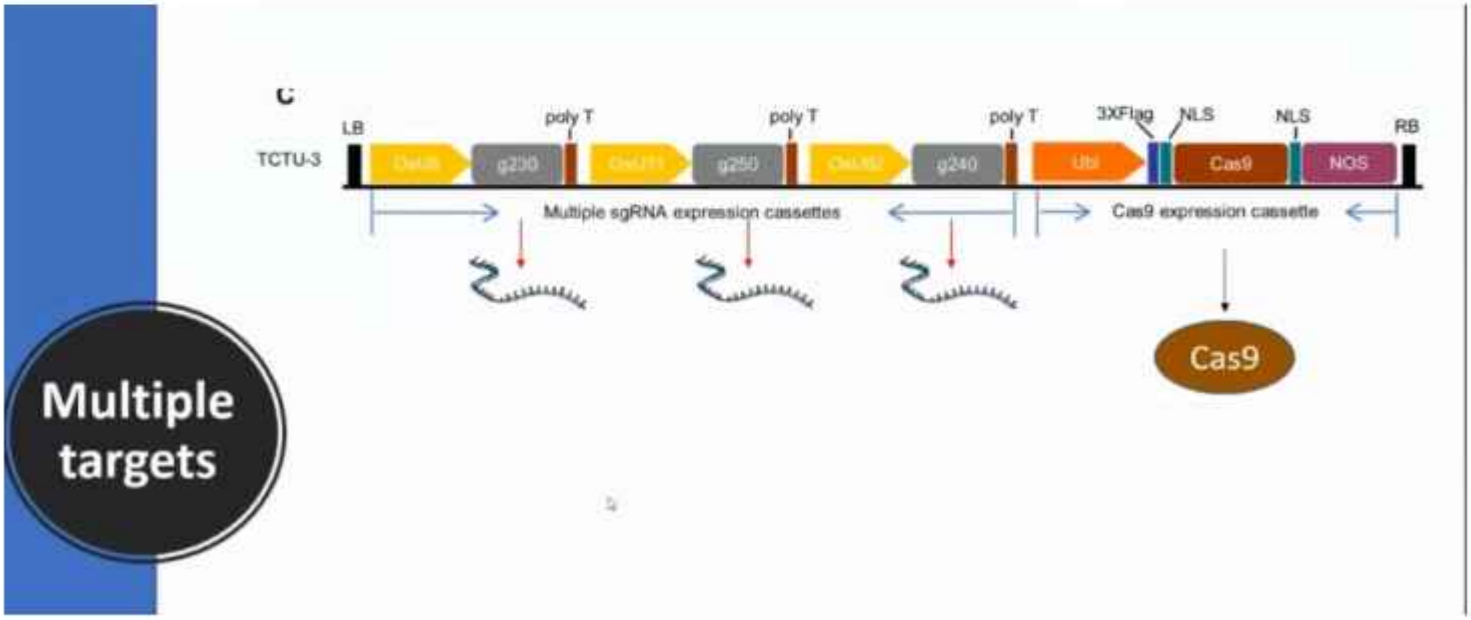
Use CRISPR sgRNA designing websites like CRISPR-P, CHOPCHOP, E-CRISPR to design your target

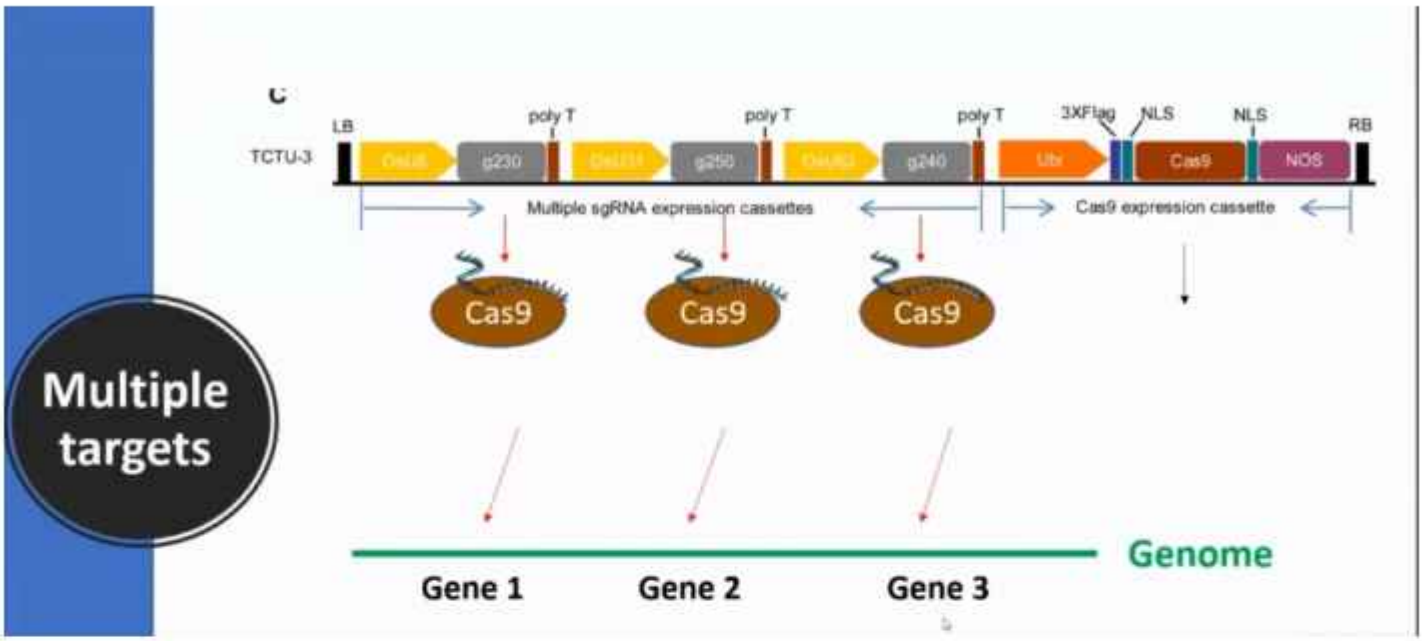


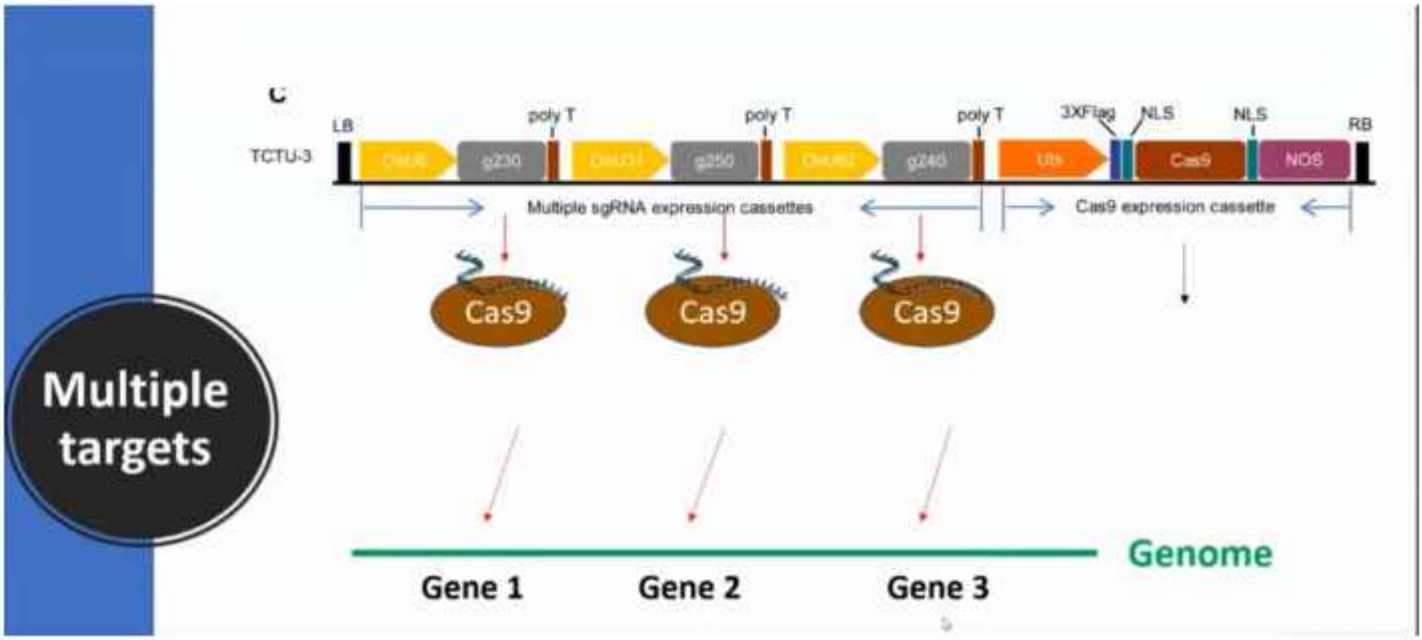
Pay attention to species, database, pam site and the sequence pasted

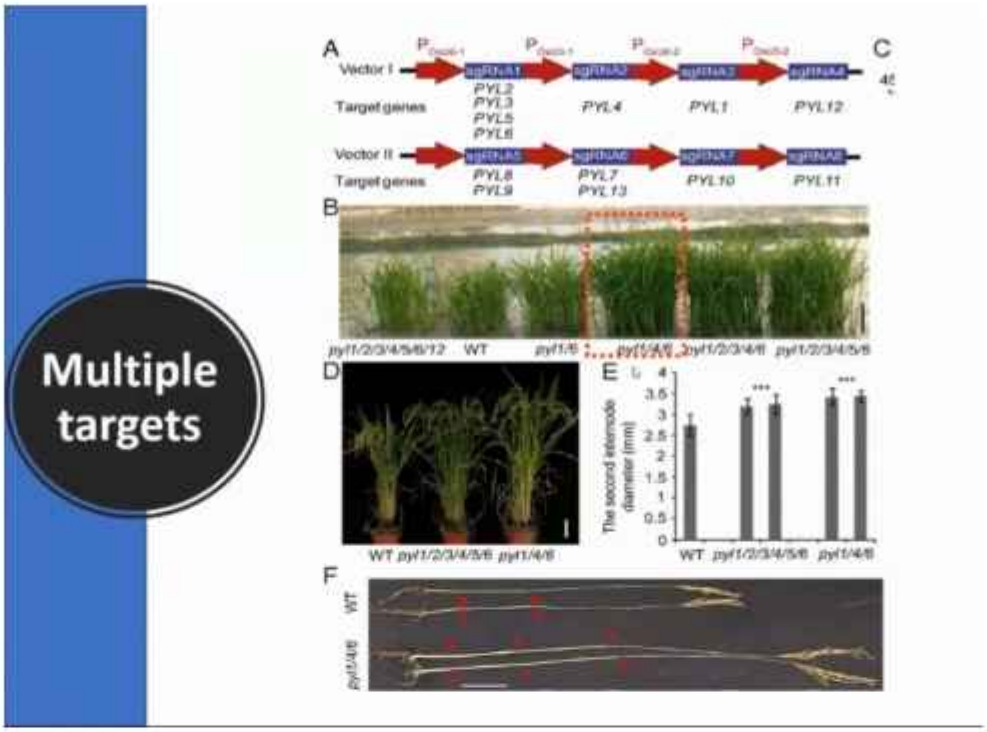


Use Select suitable guides preferably from the top choices with high On-score. Exonic region sgRNAs are the most suitable for CRISPR genome editing. Avoid UTR regions and introns (except for in special cases). GC content and temperature can be within usual range.

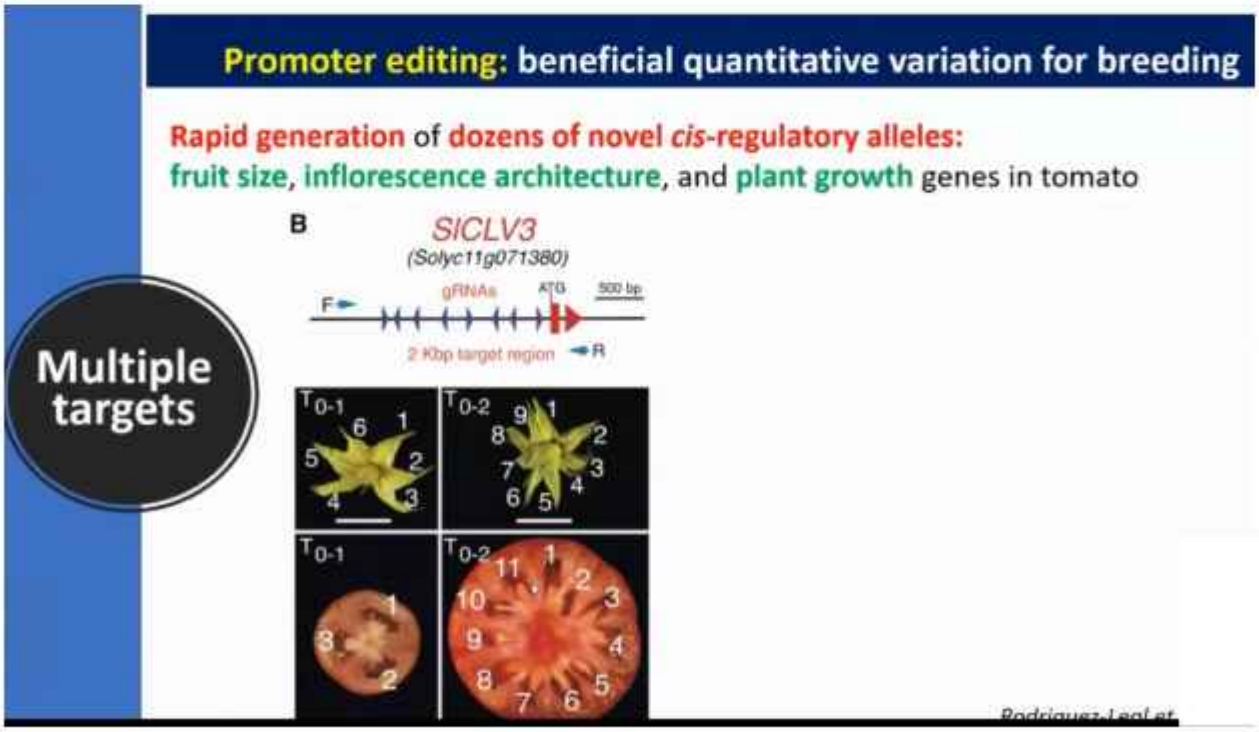



Multiple targets
LB
TCTU-3
poly T
poly T
poly T
3XFlag
NLS
NLS
RB
g230
g250
g240
Ubi
Cas9
NOS
Multiple sgRNA expression cassettes
Cas9 expression cassette
Cas9
Cas9
Cas9
Genome
Gene 1
Gene 2
Gene 3


Multiple targets
LB
TCTU-3
poly T
poly T
poly T
3XFlag
NLS
NLS
RB
g230
g250
g240
Ubi
Cas9
NOS
Multiple sgRNA expression cassettes
Cas9 expression cassette
Cas9
Cas9
Cas9
Genome
Gene 1
Gene 2
Gene 3



Miao et al, 2018,PNAS

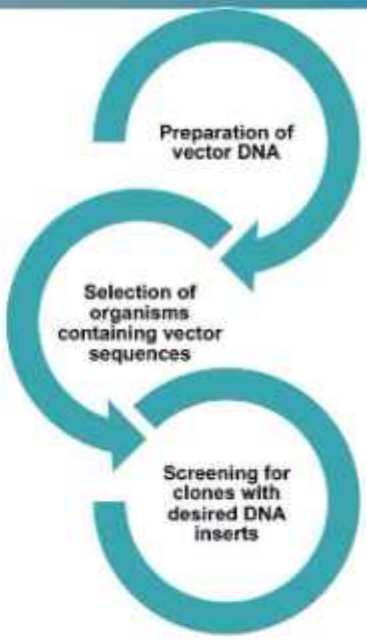


Steps involved in molecular cloning

Preparation of DNA to be cloned

Creation of recombinant DNA with DNA ligase

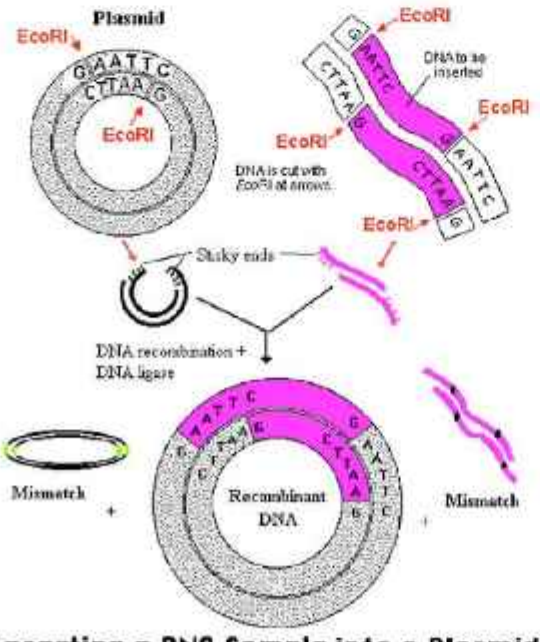
Introduction of recombinant DNA into host organism



Creation of recombinant DNA with DNA ligase

DNA prepared from the vector and foreign source are simply mixed together with DNA ligase that covalently links the ends together. This is called ligation.

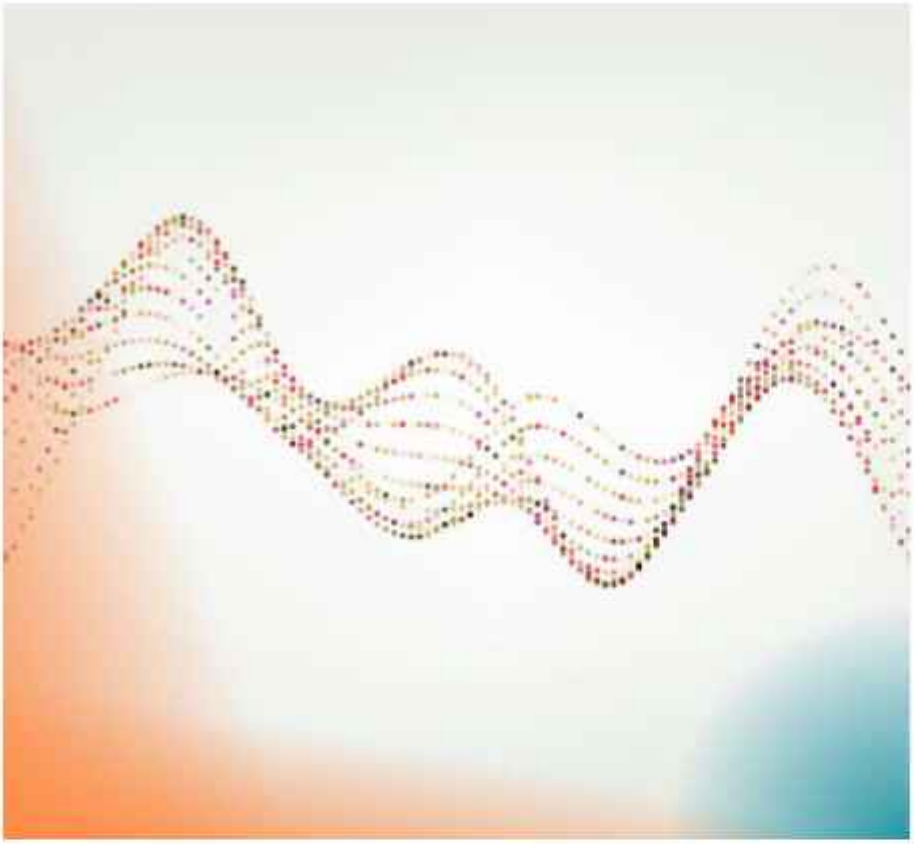
Alkaline phosphatases



Inserting a DNA Sample into a Plasmid

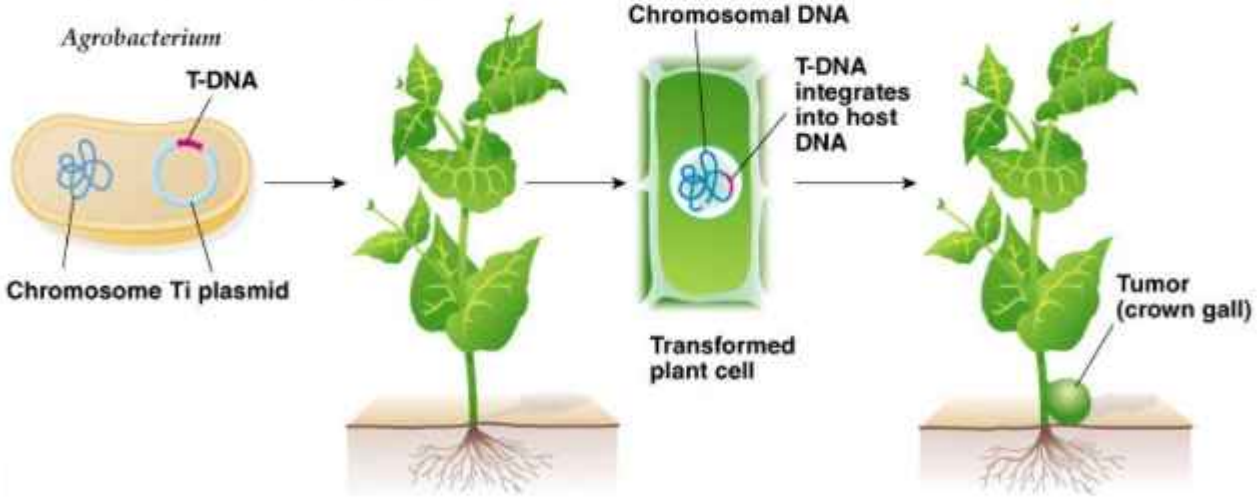
Recombinant DNA

The plasmid and the foreign DNA are cut by a restriction endonuclease (*EcoRI* in this example) producing intermediates with sticky and complementary ends. Those two intermediates recombine by base-pairing and are linked by the action of DNA ligase. A new plasmid containing the foreign DNA as an insert is obtained. A few mismatches occur, producing an undesirable recombinant.

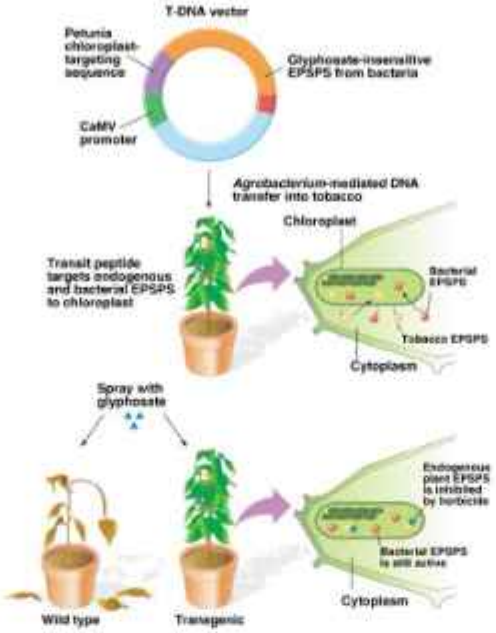


Transforming Bacteria

- After you create your new plasmid construct that contains your insert of interest , you will need to insert it into a bacterial host cell so that it can be replicated.
- The process of introducing the foreign DNA into the bacterial cell is called transformation.

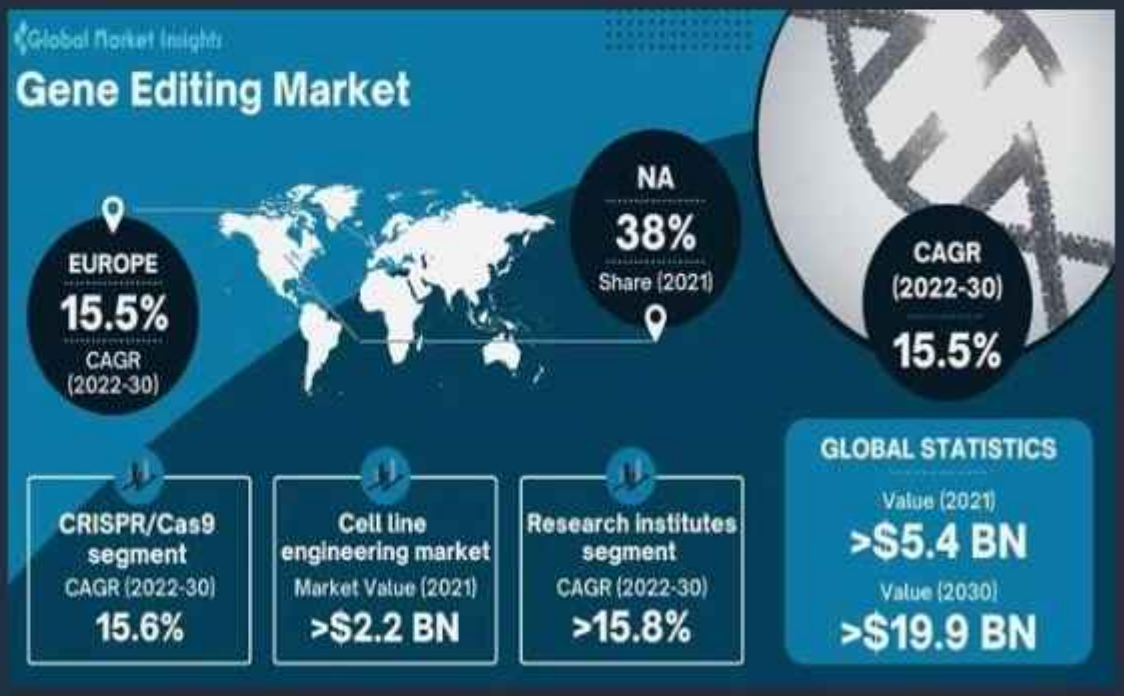


Making a transgenic, Roundup™-tolerant tobacco plant by introducing a modified form of the bacterial gene for the enzyme EPSPS that is resistant to the herbicide

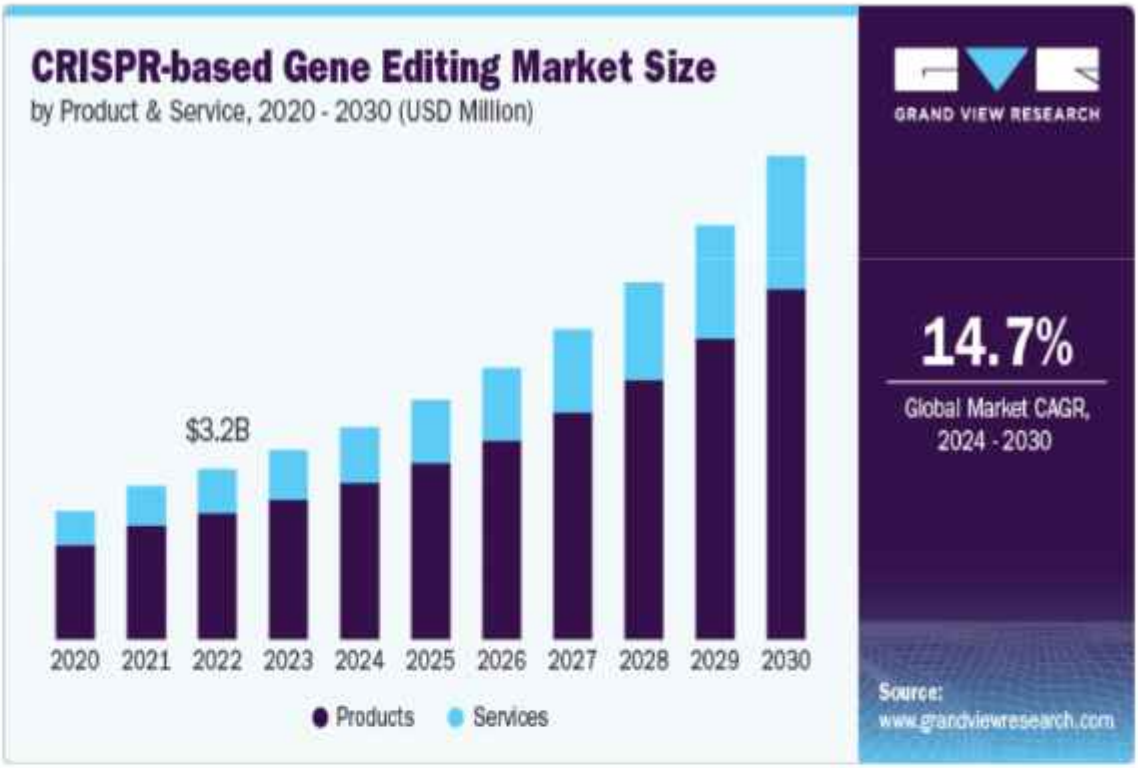


Why Bangladesh musn't fall behind

As the global market for CRISPR grows, the application requires huge leap as well. Each and every nation is now trying to catch upto the CRISPR market and CRISPR based agronomy



Exponential growth is expected to continue for next 7-10 years at the rate of 14.7%

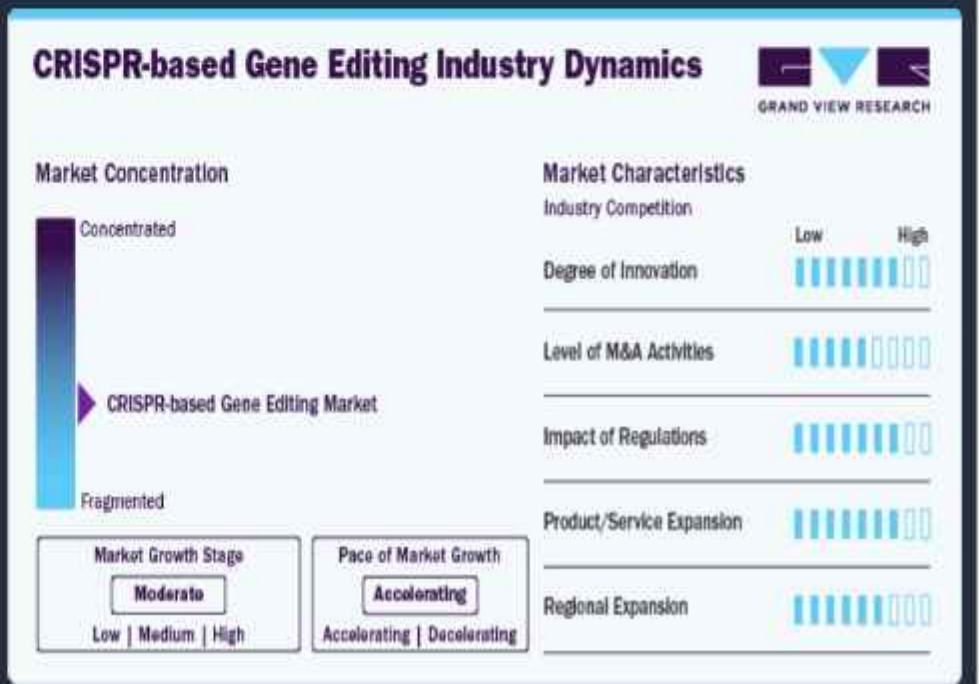


Leading countries

1.United States: Leads with over 8,000 CRISPR-related publications. Major focus areas include gene therapies and agricultural CRISPR crops. The U.S. invests heavily in biotech, with billions in venture and federal funding for CRISPR.

2.China: China has rapidly grown in CRISPR research, now publishing thousands of papers yearly. Focuses include genetic modification in agriculture and disease research, with substantial government funding.

3.European Union: Germany, France, and the UK lead in CRISPR research, publishing around 4,000 papers annually. Investment focuses on healthcare and agriculture.



Bangladesh has a formidable SOP developed for genome edited plants; bypassing restrictions of GM crops

foreign investments have supported Bangladesh's agricultural sector, particularly in areas such as climate-smart agriculture, agro-processing, and sustainable resource management.

The Food and Agriculture Organization (FAO) has estimated an investment of USD 456 million for cold storage and USD 165 million for agro-processing projects by 2030.

However, investements can increase 10 folds if climate smart and sustainable agriculture through genome editing is possible in bangladesh


Bangladesh Agricultural Research Council
Ministry of Agriculture
Government of the People's Republic of Bangladesh

Standard Operating Procedures for Research and Release of Genome Edited Plants of Categories SDN-1 and SDN-2 in Bangladesh

December, 2023


Translated in English from Bangla

Government of the People's Republic of Bangladesh
Ministry of Agriculture
Research-1 Section
www.moa.gov.bd

Memo no. 12.00.0000.062.99.005.23.646 — Dated: 26 Agrahan 1430 / 11 December 2023

Subject: Approval of 'Standard Operating Procedures for Research and Release of Genome Edited Plants of Categories SDN-1 and SDN-2 in Bangladesh'.

Reference: Bangladesh Agricultural Research Council's Memo no 12.20.0000.004.99.30.2023-2776, Date: 18-11-2023

With the above reference the approved SOP entitled 'Standard Operating Procedures for Research and Release of Genome Edited Plants of Categories SDN-1 and SDN-2 in Bangladesh' related to develop desired crop varieties after deletion/addition of specific genetic traits through advance 'Genome Editing' technology is hereby forwarded as per direction of competent authority.

Muhammad Zahirul Islam
Deputy Secretary
Phone: 223351198
Email: dsresearch@moa.gov.bd

Executive Chairman
Bangladesh Agricultural Research Council
Farmgate, Dhaka

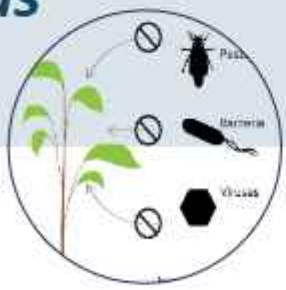
Memo no. 12.00.0000.062.99.005.23.646 — Dated: 26 Agrahan 1430 / 11 December 2023

Copy forwarded for kind information (Not According to the Seniority)

1. PS to Honorable Minister, Ministry of Agriculture, Bangladesh Secretariat, Dhaka
2. PS to Secretary, Ministry of Agriculture, Bangladesh Secretariat, Dhaka
3. PO to Joint Secretary, Research Wing, Ministry of Agriculture, Bangladesh Secretariat, Dhaka
4. PO to Joint Secretary, Research Branch, Ministry of Agriculture, Bangladesh Secretariat, Dhaka

Muhammad Zahirul Islam
Deputy Secretary

CRISPR now is the major concern of three fields



Agriculture

For global food security

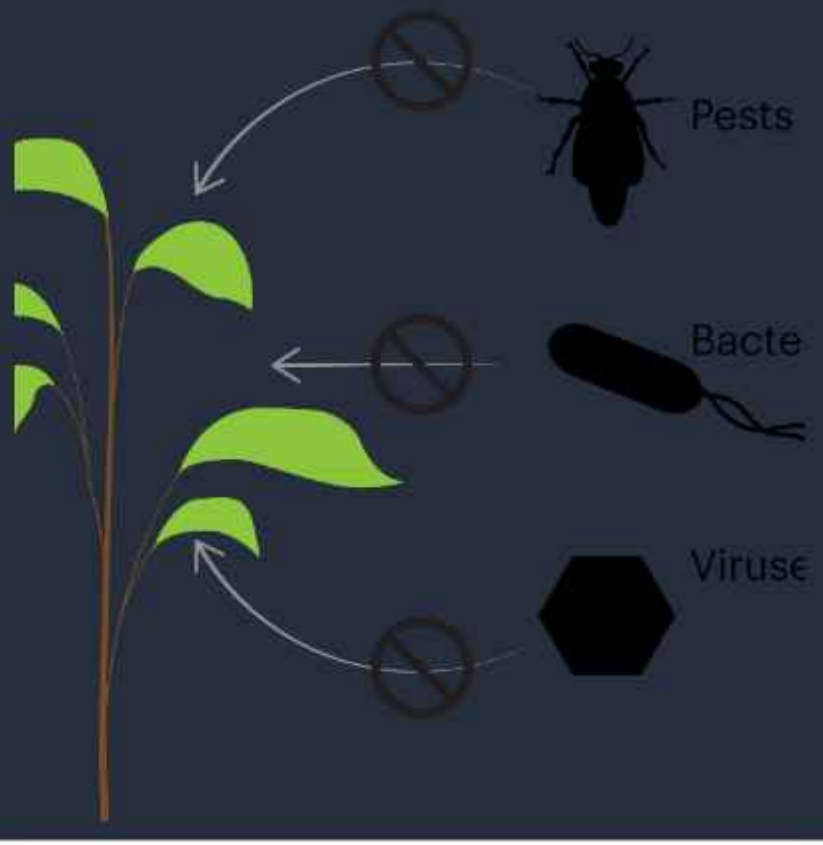
Therapeutics

Treatment of diseases

Biodiversity

Saving endangered species

Agriculture

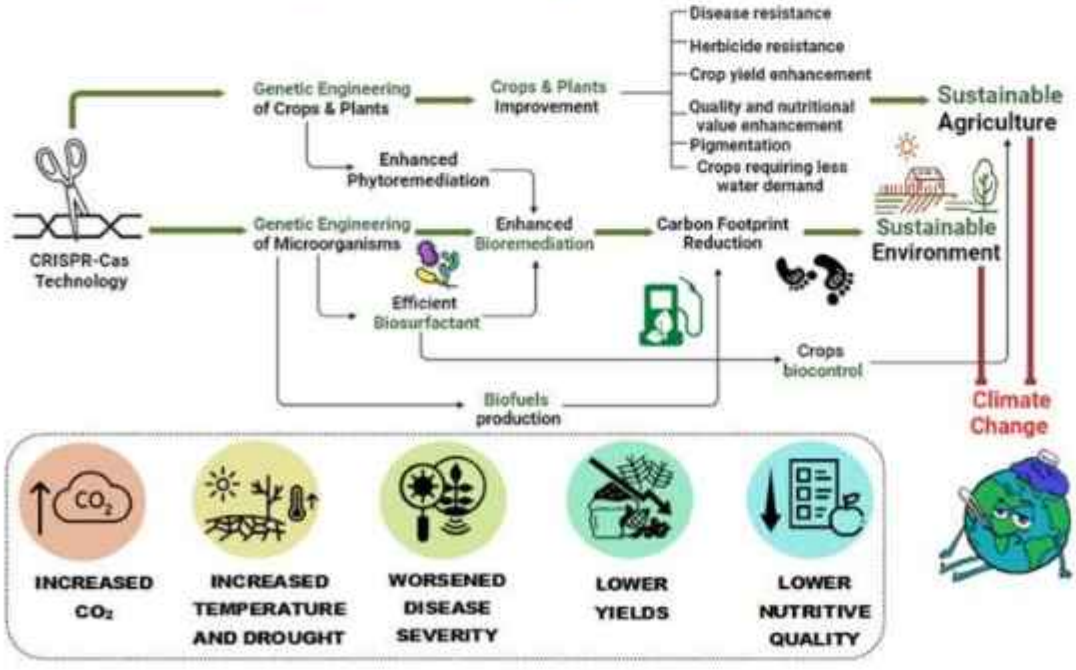


CRISPR can change the current status and approach to food yield and cultivation

CRISPR/Cas9 mediated crop improvement

Improved Yield	Improved quality & herbicide resist.	Improved Pathogen resistance	Improved Abiotic tolerance
OsIPA1-number of tillers	*SlIAA9*-seedless tomato fruit, eating quality	*CsLOB1*-citrus canker resistance	*AtOST2*-stomata closure, abiotic stress tolerance
OsGS3-grain size, higher yield	*CsFAD2*-oil quality, enhanced oleic acid	*OsERF922*-rice blast resistance	*TaMIR169, MIR827a*-drought tolerance
OsDEP1-dense and erect panicles	*CsFAD2*-oil composition, ↓linolenic & linoleic acids	*SlMlo*-powdery mildew resistance	*ZmARGOS8*-higher yield under drought stress
OsGn1a-grain number	*GmFAD2-1A & 1B* -oil quality, oil composition	*NbCPs-CLCuKoV, TYLCV* resistance	*AtCBF1, 2, and 3*-cold and salinity tolerance
SlSPSG-early flowering and yield	*OsSBE1 & OsSBEIIb*-higher amylose & resist. starch	*NbCPs-TYLCSV, BCTV* resistance	*UGT79B2 & B3-ROS, salt, cold, drought tolerance*
OsGW2, 5 and 6-grain weight, higher yield	*ZmRPL & PPR*-reduced kernel zeins proteins	*AteIF(iso)4E-TMV* resistance	*OsSAPK2*-drought & salt tolerance, ABA signaling
OsHD2, 4 and 5-early heading and maturity	*OsEPSPS*-Glyphosate herbicide resistance	*eIF4E*-cucumber *PRSMV, ZYMV, CuVYV* resistance	*SSADH, GABA-TP1,2 & 3; CAT3*-abiotic signaling
OsSWEET11-sugar transport, grain filling	*OsALS*-Bispyribac sodium herbicide resistance	*ToMLO*-powdery mildew resistance	*AtAVP1*-enhanced drought tolerance
SU2-jointless fruit stem, less fruit dropping	*ZmALS2*-Chlorsulfuron herbicide resistance	*OsMPK1 and 2*-biotic & abiotic signaling	*OsMPK1, 2, 5 and 6*-biotic & abiotic signaling
SlEJ2-larger fruit, higher yield	*ALS, FTIP1e*-Imazamox herbicide resistance	*OsMPK5 and 6*-biotic & abiotic signaling	*PpAP2/ERF*-ABA in/dependent signaling

CRISPR-Cas Impacts on the Agriculture and Environment



Climate Change Impacts on the Agriculture and Environment

CRISPR FOODS

1. CRISPR tomatoes: wild and groundcherry

- To create compact plants with less sprawling bushes,
- larger fruits that can ripen at the same time,
- Higher vitamin C levels
- Resistance to bacterial spot disease
- Fruits that stay attached to their stem better
- Resistance to salt, and more.
  Timeframe: 3 years
- Compared to 10 with previous approaches

CRISPR FOODS

2. CRISPR mushrooms: stop them from browning

- To alter the white button mushroom, preventing it from browning quickly and elongating its shelf life
- Timeframe: Instant modification compared to decades of work with traditional methods

CRISPR FOODS

3. CRISPR rice: improving the yield

- To improve crop yields in rice, a staple food for a significant number of the world's population, yet one that is overly susceptible to negative environmental factors.
- Timeframe: Modification of multiple genes at one time compared to decades of work with traditional methods

CRISPR FOODS

4. CRISPR citrus fruits: saving the oranges from greening

- Oranges and other citrus foods are at risk from decimation due to the "citrus greening" disease. CRISPR could create a resistance to this disease, and save the industry which is at a complete risk of collapse
- Timeframe: Currently undetermined

CRISPR FOODS

5. CRISPR chocolate: saving the cacao trees

- To modify the cacao plant to equip it with enhanced resistance to diseases, and ultimately to prevention eventual extinction
- Timeframe: Currently undetermined

CRISPR FOODS

6. CRISPR wheat: removing the gluten

- To create strands of wheat that do not contain gluten, allowing those with Celiac Disease to consume wheat varieties without immunoreactivity
- Timeframe: Success using CRISPR with simultaneous gene manipulation while no current success using breeding/GMOs

New York Times:

SCIENCE

Scientists Seek Ban on Method of Editing the Human Genome

By NICHOLAS WADE MARCH 19, 2015

MIT Technology Review:

Engineering the Perfect Baby

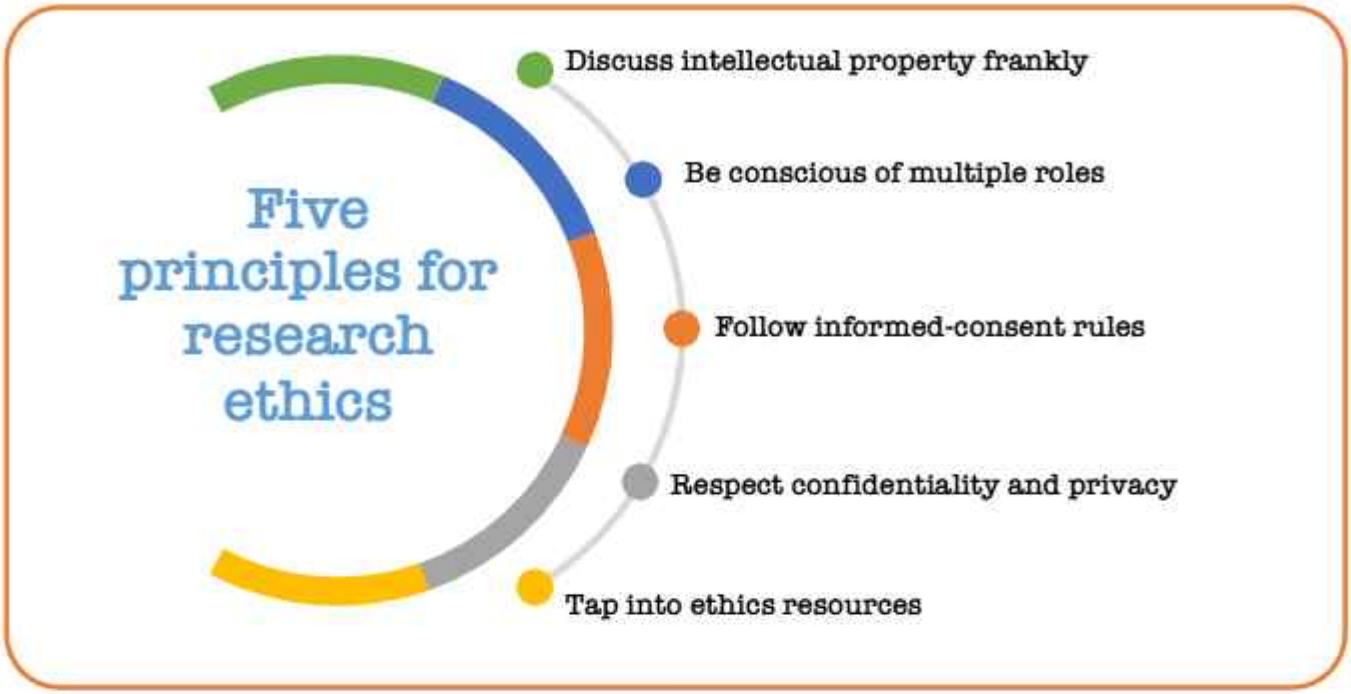
Scientists are developing ways to edit the DNA of tomorrow's children. Should they stop before it's too late?

By Antonio Regalado on March 5, 2015

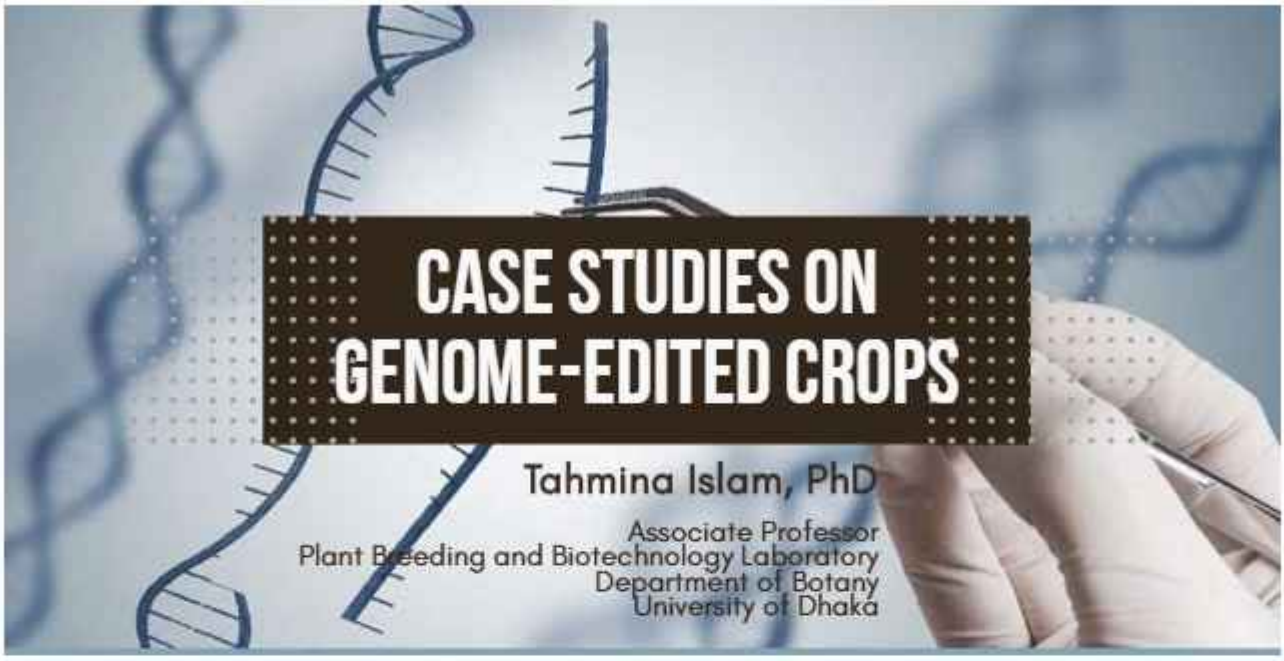
Forbes:

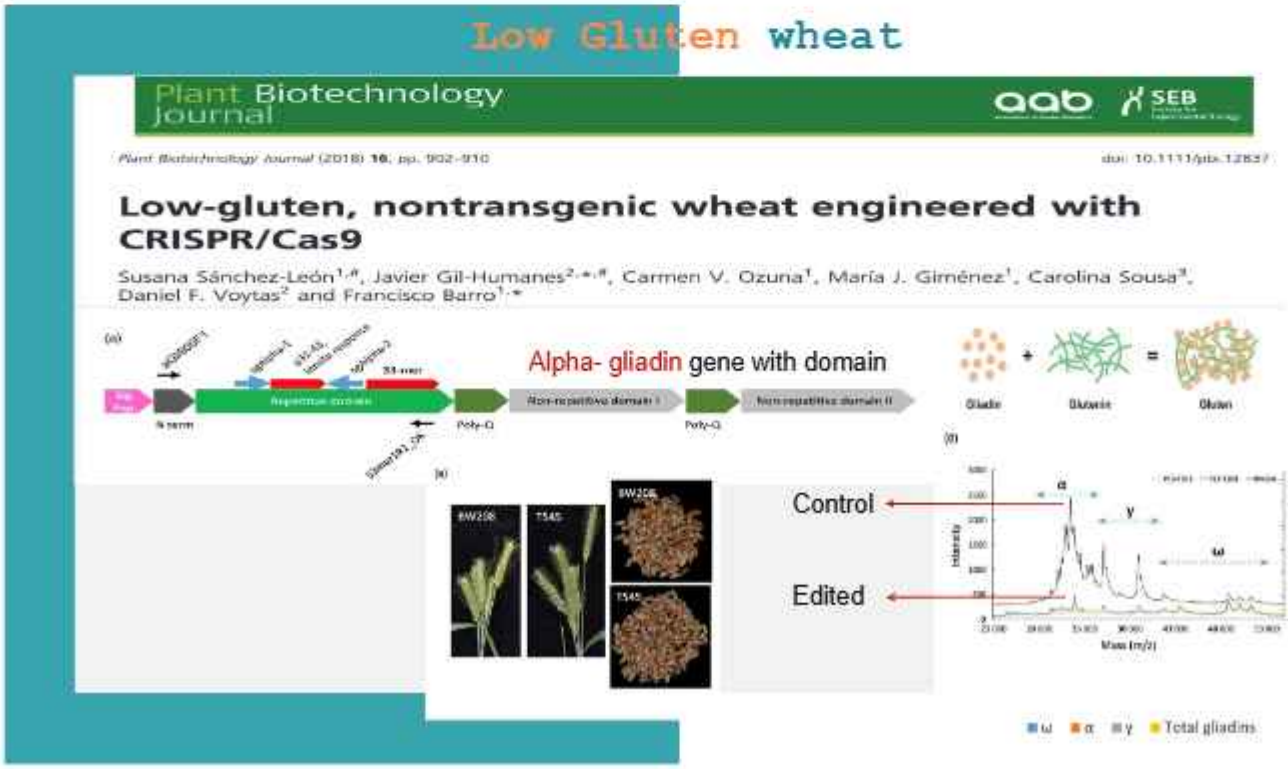
TECH 3/23/2015 @ 12:13PM | 2,339 views

'Ban DNA Editing Of Sperm And Eggs'


Five principles for research ethics
Discuss intellectual property frankly
Be conscious of multiple roles
Follow informed-consent rules
Respect confidentiality and privacy
Tap into ethics resources

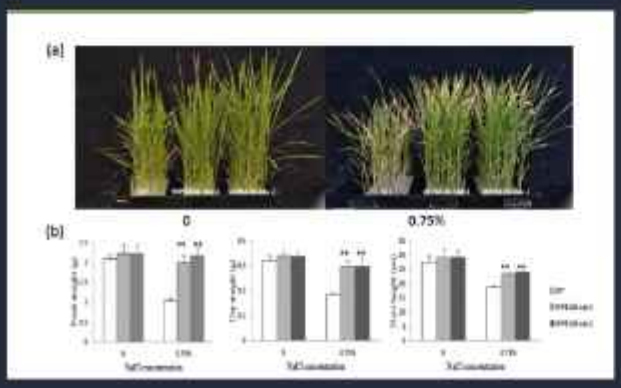

Thank You
RE-ENGINEER YOURSELF.....
DO CRISPR !

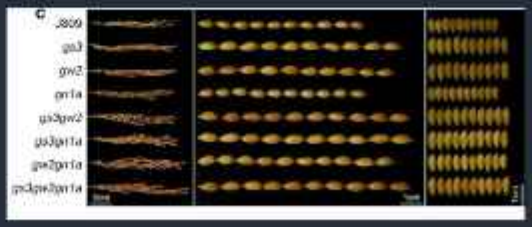

CASE STUDIES ON
GENOME-EDITED CROPS
Tahmina Islam, PhD
Associate Professor
Plant Breeding and Biotechnology Laboratory
Department of Botany
University of Dhaka


Low Gluten wheat
Plant Biotechnology Journal
aab
SEB
Plant Biotechnology Journal (2018) 16, pp. 902–910
doi: 10.1111/pbi.12837
Low-gluten, nontransgenic wheat engineered with CRISPR/Cas9
Susana Sánchez-León[1,#], Javier Gil-Humanes[2,*,#], Carmen V. Ozuna[1], María J. Giménez[1], Carolina Sousa[3], Daniel F. Voytas[2] and Francisco Barro[1,*]
Alpha- gliadin gene with domain
Gliadin
Glutenin
Gluten
Control
Edited
ω α γ Total gliadins

Penn State develop gene-edited mushroom

OsRR22 gene encodes a 696–amino acid B-type response regulator transcription factor that is involved in both cytokinin signal transduction and metabolism; its loss of function has been reported to significantly increase salt tolerance





Crispr-induced mutations in the multiples genes, OsGS3, OsGW2 and OsGn1a,
which negatively regulate the grain size, width and weight, and number, respectively resulted in up to 68% increase in yield per panicle.

The OsCKX2 dilemma

The gene OsCKX2, associated with cytokinin degradation, was knocked out using CRISPR-Cas9 technology to increase grain number per panicle.
The knockout led to increased panicle size and plant height, resulting in a higher yield. However, the unintended trade-off was a reduction in the number of grains per panicle, highlighting a classic "seed size vs. seed number" dilemma often observed in grain crops.

SPECIAL SECTION: GENOME EDITING AND CHROMOSOME ENGINEERING IN PLANTS

Loss-function mutants of *OsCKX* gene family based on CRISPR-Cas systems revealed their diversified roles in rice

Xuelian Zheng | Shuling Zhang | Yanling Liang | Rui Zhang | Li Liu | Pengchen Qin | Zhe Zhang | Yan Wang | Jianping Zhou | Xu Tang | Yong Zhang

Abstract

Cytokinin (CTK) is an important plant hormone that promotes cell division, controls cell differentiation, and regulates a variety of plant growth and development processes. Cytokinin oxidase/dehydrogenase (CKX) is an irreversible cytokinin-degrading enzyme that affects plant growth and development by regulating the dynamic balance of CTKs synthesis and degradation. There are presumed 11 members of the CKX gene family in rice (*Oryza sativa* L.), but limited members have been reported. In this study, based on CRISPR-Cas9 and CRISPR-Cas12a genome-editing technology, we established a complete set of *OsCKX1*-*OsCKX11* single gene mutants, as well as double-gene and triple-gene mutants of different *OsCKXs* gene combinations with high similarity. The results revealed that CRISPR-Cas12a outperformed Cas9 in generate biallelic mutations, multi-gene mutants, and more diverse genotypes. And then, we found, except the reported *OsCKX2*, *OsCKX4*, *OsCKX9* and *OsCKX11*, *OsCKX5*, *OsCKX6*, *OsCKX7*, and *OsCKX8* also had significant effects on

IN OTHER DISCIPLINES

CRISPR IS EXTENSIVELY USED IN ALL BRANCHES OF AGRICULTURE EVEN ASIDE FARMING

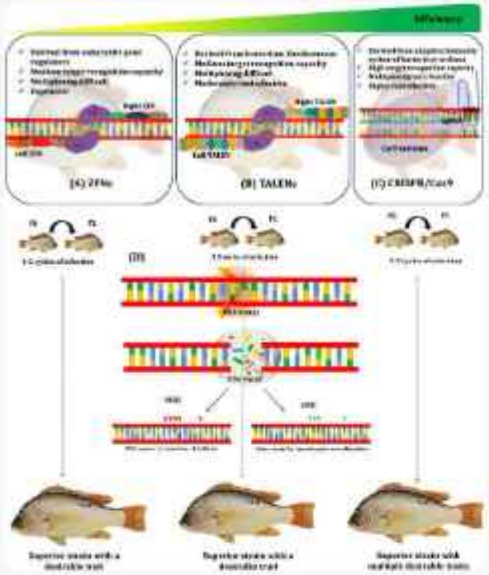
Disease Resistance in Tilapia (Orexin Gene :Knockout):

- Objective: Improve disease resistance by reducing susceptibility to common infections.
- Gene Targeted: Orexin (also known as hypocretin), a neuropeptide that regulates feeding behavior and is linked to immune response in fish.
- Experiment Details: Researchers used CRISPR to knock out the orexin gene in tilapia embryos. By targeting the orexin gene, they observed increased resistance to the *Streptococcus iniae* bacteria, a pathogen that affects farmed tilapia.
- The CRISPR technique introduced specific guide RNAs into the fertilized eggs, effectively disrupting the orexin gene sequence. Surviving edited fish showed lower mortality rates upon exposure to the bacteria, demonstrating increased disease resistance.

Growth Acceleration in Catfish (Myostatin Gene Knockout):

- Objective: Enhance growth rates to improve production efficiency.
- Gene Targeted: Myostatin (MSTN), a negative regulator of muscle growth, which when knocked out, can lead to increased muscle mass.
- Experiment Details: CRISPR was used to disable the MSTN gene in catfish, leading to faster growth and higher muscle mass. Researchers microinjected CRISPR/Cas9 constructs, including guide RNAs specific to MSTN, directly into fertilized catfish embryos. This gene disruption led to a significant increase in muscle development, producing fish that grew faster and had more flesh, benefiting aquaculture.

Aquaculture



Cold Tolerance in Salmon (Leptin Receptor Gene):

- Cold Tolerance in Salmon (Leptin Receptor Gene):Objective: Improve survival rates in colder environments to expand the habitat range.
- Gene Targeted: Leptin receptor gene, which influences energy homeostasis and can play a role in stress responses to temperature.
- Experiment Details: CRISPR was used to alter the leptin receptor gene in Atlantic salmon to improve cold tolerance. Researchers introduced guide RNAs to target specific exons of the leptin receptor gene, creating mutations that affected its function. Edited fish exhibited better cold tolerance, allowing aquaculture to expand to colder waters with reduced risk of fish loss during winter.

Effects of CRISPR/Cas9 on Biological and Environmental Aspects of Fish Species

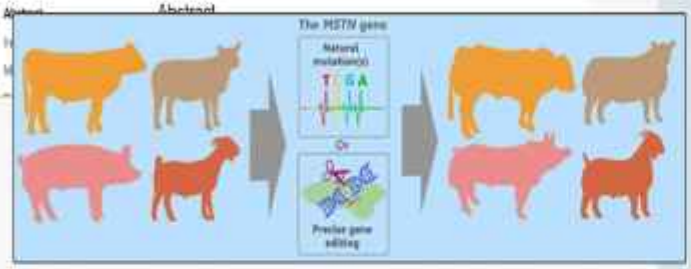
Applicable Fields	Impacts
Disease resistance	It is used to reduce the viral hemorrhagic septicemia virus (VHSV) infection of olive flounder hirame natural embryo (HINAE) cells. It enables gene editing in fish species such as salmon, tilapia, and shrimp to increase their resistance to diseases. It helps in the deletion of the *JAM-A* gene in grass carp cells, which significantly enhances resistance to grass carp reovirus (GCRV) infection. It helps enhance fish cell lines for host response and genetic resistance against infectious diseases, using Atlantic salmon and rainbow trout as model systems in aquaculture.
Environmental adaptation	It helps to edit genes in fish species, such as farmed salmon, to adapt to changing environments.
Improved growth rates and muscles	It increases muscle growth by knocking out melanocortin (*mc4r*) receptor genes and has been experimentally tried on channel catfish and medaka fish. It improved the growth rates and increased muscle mass of the channel catfish by modifying the myostatin gene in channel catfish embryos. It helps increase the muscle mass of blunt snout bream due to the disruption of the *mstna* and *mstnb* genes.
Bone development	It helps in *myostatin* gene disruption of genes, such as transcription factor *sp7*, causing bone defects in common carp, and increases muscular cells, resulting in a more robust muscular phenotype.
Colour defects	It can be used to edit genes involved in pigmentation pathways, potentially leading to loss of skin pigmentation, e.g., edited mutant of large-scale loach, causing skin pigmentation loss and black patch dispersion in the Oujiang color common carp. It helps identify and introduce mutations in genes responsible for pigmentation, such as *tyrosinase* or *mitf*, which can lead to pigmentation defects in fish species like salmon. It helps to reveal a recessive inheritance pattern for the white-albino phenotype, lacking pigment-containing chromatophores, in rainbow trout.
Sex determination	It can be used to disrupt or modify the gonadal soma-derived factor (*gsdf*) gene, which is a crucial gene in teleost fish. Disruption of genes such as dmrt1 and cyp19a1a can lead to sex reversal phenotypes in zebrafish.
DNA integration	It facilitates the integration of exogenous DNA into the zebrafish genome, but it may also cause additional genetic mutations or disruptions, depending on the editing conditions and precision of the technique.
kidney and gonads development	It helps disrupt the *Wilms tumor 1* (*wt1a*) gene, which may lead to abnormal gonad and kidney development in Nile tilapia.
Immune genes improvement	It has been used to knock out or edit genes in salmon fish. However, overexpressing interferon (*IFN*) or inducing stimulated genes (*ISGs*) does not guarantee broad disease resistance.

Livestock

PLOS ONE

Knockdown of Myostatin Expression by RNAi Enhances Muscle Growth in Transgenic Sheep

Article | Authors | Metrics | Comments | Media Coverage



Enhanced Muscle Growth in Cattle (Myostatin Gene Knockout):

- Objective: Increase muscle mass in cattle, similar to the "double-muscling" trait seen in Belgian Blue cattle.
- Gene Targeted: Myostatin (MSTN), a protein that inhibits muscle growth.
- Experiment Details: Scientists used CRISPR to knock out the MSTN gene in cattle embryos. Guide RNAs were designed to target specific regions of the MSTN gene to cause a frameshift mutation, which disrupted its function. The resulting cattle displayed increased muscle growth due to reduced inhibition from the MSTN protein, producing leaner and more muscular cattle that may provide higher meat yield per animal.

PLOS PATHOGENS

Precision engineering for PRRSV resistance in pigs: Macrophages from genome edited pigs lacking CD163 SRCR5 domain are fully resistant to both PRRSV genotypes while maintaining biological function

Antiviral Research

CD163 knockout pigs are fully resistant to highly pathogenic porcine reproductive and respiratory syndrome virus

Livestock

Disease Resistance in Pigs (CD163 Gene for PRRS Resistance):

- Objective: Develop pigs resistant to porcine reproductive and respiratory syndrome (PRRS), a costly viral disease in swine farming.
- Gene Targeted: CD163, a receptor required by the PRRS virus to enter pig cells.
- Experiment Details: Researchers used CRISPR to remove the specific exon responsible for PRRS susceptibility in the CD163 gene, without affecting other immune functions. They introduced CRISPR/Cas9 into pig embryos to target and delete this portion of the gene. The edited pigs showed strong resistance to PRRS infection, significantly reducing the impact of the disease. Importantly, the pigs remained healthy and developed normally, as CD163 was altered specifically to resist PRRS without compromising other cellular functions.

Livestock

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Gene Editing Cattle for Enhancing Heat Tolerance: A Welfare Review of the "PRLR-SLICK Cattle" Case

Original Research Paper | Open access | Published: 22 June 2024

Volume 18, article number 6, (2024) Cite this article

Download PDF You have full access to this open access article

Heat Tolerance in Cattle (SLICK Gene):

- Objective: Increase heat tolerance in cattle to reduce heat stress impacts in warm climates.
- Gene Targeted: SLICK locus, associated with a trait for short, sleek hair which helps in heat dissipation.
- Experiment Details: CRISPR/Cas9 was used to insert the SLICK allele into cattle embryos of breeds not naturally carrying the gene. By editing the embryos, researchers introduced this allele, resulting in cattle with shorter, sleek hair that better tolerated heat. This approach helps maintain productivity and animal welfare in tropical regions by reducing the effects of heat stress..

DETAILED CASE STUDY-

CRISPR/CAS9-MEDIATED MULTIPLEX GENOME EDITING OF THE *BNWRKY11* AND *BNWRKY70* GENES IN *BRASSICA NAPUS L.*

Qinfu Sun, Li Lin, Dongqiao Liu, Dewei Wu, Yujie Fang, Jian Wu* and Youping Wang*

International Journal of Molecular Sciences

MuPI

Article

CRISPR/Cas9-Mediated Multiplex Genome Editing of the *BnWRKY11* and *BnWRKY70* Genes in *Brassica napus* L.

Qinfu Sun, Li Lin, Dongqiao Liu, Dewei Wu, Yujie Fang, Jian Wu * and Youping Wang *

Jiangsu Provincial Key Laboratory of Crop Genetics and Physiology, Yangzhou University, Yangzhou 225009, China; dx120159@yzu.edu.cn (Q.S.); MX6790@yzu.edu.cn (L.L.); DX120131159@yzu.edu.cn (D.L.); dwwu@yzu.edu.cn (D.W.); yjfang@yzu.edu.cn (Y.F.)
* Correspondence: wu_jian@yzu.edu.cn (J.W.); wangyp@yzu.edu.cn (Y.W.); Tel.: +86-514-879-97303 (Y.W.); Fax: +86-514-879-97747 (Y.W.)

Received: 19 July 2016; Accepted: 7 September 2016; Published: 13 September 2016

Abstract: Targeted genome editing is a desirable means of basic science and crop improvement. The clustered, regularly interspaced, palindromic repeat (CRISPR)/Cas9 (CRISPR-associated 9) system is currently the simplest and most commonly used system in targeted genomic editing in plants. Single and multiplex genome editing in plants can be achieved under this system. In *Arabidopsis*, *AtWRKY11* and *AtWRKY70* genes were involved in JA- and SA-induced resistance to pathogens, in rapeseed (*Brassica napus* L.), *BnWRKY11* and *BnWRKY70* genes were found to be differently expressed after inoculated with the pathogenic fungus, *Sclerotinia sclerotiorum* (Lib.) de Bary. In this study, two Cas9/sgRNA constructs targeting two copies of *BnWRKY11* and four copies of *BnWRKY70* were designed to generate *BnWRKY11* and *BnWRKY70* mutants respectively. As a result, twenty-two *BnWRKY11* and eight *BnWRKY70* independent transformants (T_0) were obtained, with the mutation rates of 54.5% (12/22) and 50% (4/8) in *BnWRKY11* and *BnWRKY70* transformants respectively. Eight and two plants with two copies of mutated *BnWRKY11* and *BnWRKY70* were obtained respectively. In T_1 generation of each plant examined, new mutations on target genes were detected with high efficiency. The vast majority of *BnWRKY70* mutants showed editing in three copies of *BnWRKY70* in examined T_1 plants. *BnWRKY70* mutants exhibited enhanced resistance to *Sclerotinia*, while *BnWRKY11* mutants showed no significant difference in *Sclerotinia* resistance when compared to non-transgenic plants. In addition, plants that overexpressed *BnWRKY20* showed

WRKY transcription factors (TFs), defined by their DNA-binding domain, namely, the WRKY domain, have been identified in different plants and are widely involved in defense to diverse plant stress conditions, especially in plant immune responses.

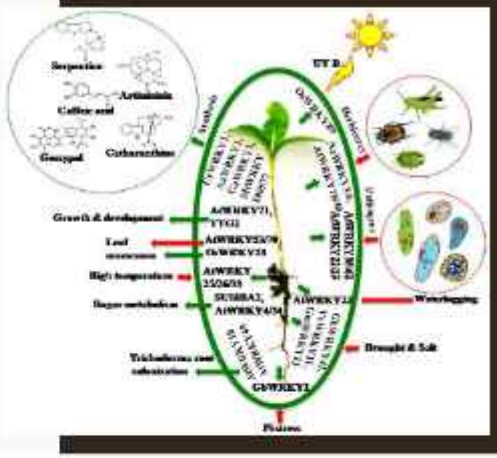
In *Arabidopsis*, many WRKY transcription factors have been reported to be associated with disease resistance, including WRKY8, WRKY11, WRKY33, WRKY38 and WRKY62, WRKY46, WRKY53 and WRKY70

Studies have shown that overexpression or loss function of *WRKY11* or *WRKY70* affects SA (Salicylic acid) and JA (Jasmonic Acid) induced disease resistance response to pathogens in Arabidopsis.

Previous reports suggest that some *BnWRKY* genes might be involved in the response to pathogens in B. napus as well.

Here, we explored the patterns of targeted mutagenesis of the B. napus genome mediated by the CRISPR/Cas9 system. CRISPR/Cas9 vectors with multiple sgRNA expression cassettes were constructed to target the BnWRKY11 and BnWRKY70 genes of *B. napus* to establish multiplex genome editing possibility.

TWO WRKY TF'S WERE IDENTIFIED AS NEGATIVE REGULATOR FOR PATHOGEN DEFENCE



OBJECTIVES

In-silico analysis of the *Arabidopsis* defense genes and detection of the homologs in *Brassica napus*

Expression profiling and determining target copies to create sgRNAs and construct the binary vector

Conducting *Agrobacterium*-mediated genetic transformation to obtain transgenic plants

Mutation analysis at target sites

Infection assay of the transgenic mutated plants to analyze resistance to pathogens

METHODS

1. Target Design and Vector Construction:

- Creating multiple sgRNAs targeting selected copies of *BnWRKY11* and *BnWRKY70*
- Constructing the binary vector **pLYCRISPR/Cas935S-N** by golden gate clone

2. Genetic Transformation of *B. napus*:

- Conducting *Agrobacterium*-mediated genetic transformation on *B.napus* line "J9712"
- Obtaining putative transgenics and confirmation via PCR using primers in the flanking region of Cas9/sgRNA targets

3. Mutation Analysis

- Amplification of the Cas9/sgRNA target sequences and sequencing of the PCR products to analyze nature of mutagenesis

4. *S. sclerotiorum* Infection Assay:

- Inoculation of selected mutated plants with *S. sclerotiorum* (Lib.) de Bary isolate SS-1
- Measurement of lesion size 48h post-inoculation

RESULTS

1

1. Sequence Identification and Expression Analysis of *BnWRKY11* and *BnWRKY70* Genes in *B. napus:*

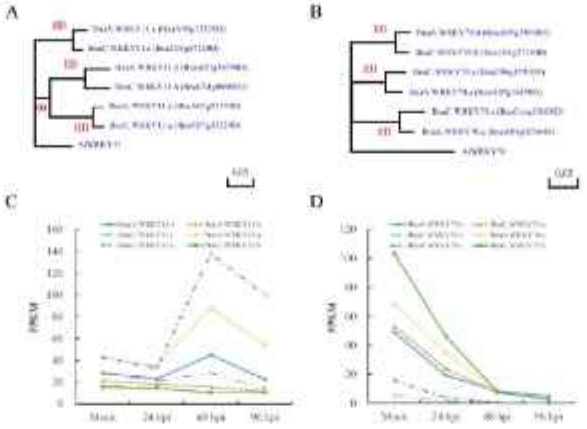


Fig 2. Phylogenetic tree of *WRKY11* and *WRKY70* and the expression level of *BnWRKY11* and *BnWRKY70* in response to *S. sclerotiorum* inoculation. (A) Phylogenetic tree of BnWRKY11 and the homologs from *Arabidopsis*; (B) Phylogenetic tree of *BnWRKY70* and the homologs from *Arabidopsis*; (C,D) The expression level of *BnWRKY11* and *BnWRKY70* in response to *S. sclerotiorum* inoculation.

2

2. CRISPR/Cas9 Binary Vector Construction, Rapeseed Transformation and Screening of Positive Transformants:

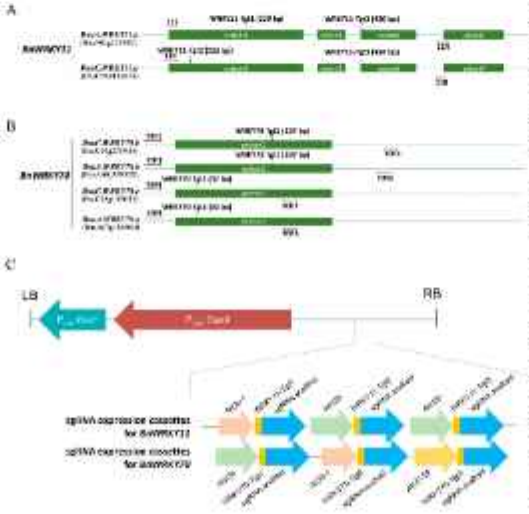


Fig 3. Position of target sites and primers on *BnWRKY11* and *BnWRKY70* and physical maps of the T-DNA regions of Cas9/sgRNA constructs. (A,B) the target sites for *BnWRKY11* and *BnWRKY70* respectively and the primers for the amplification were shown as well. Tgt1 Tgt3 means the chosen target sites, the locations of target sites are marked with black arrows; primers are shown in red arrows. (C) Physical maps of the T-DNA regions of Cas9/sgRNA constructs. LB/RB, left/right border of T-DNA; P35S:Cas9, Cas9 gene which driven by CMV35S promoter; P35S:KanR, NPT gene which driven by CMV35S promoter. AtU3/AtU6, Arabidopsis U3/U6 promoter.

3

3. Confirmation of Cas9-Induced Mutagenesis in Transgenic Plants of *B. napus:*

Table 1. The targets and primers designed for *BnWRKY11* and *BnWRKY70* and mutation rates in T0 plants

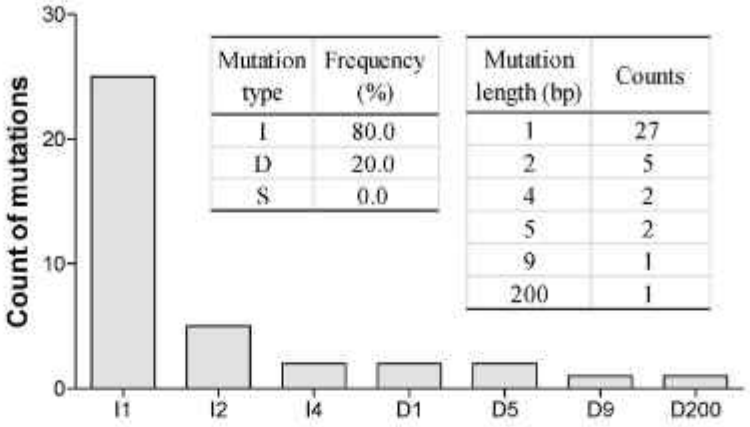
Target Gene (Number of Transformants)	Copies	Target	Amplification Primer	No. of Plants with Mutations	Mutation Frequency (%)
BnWRKY1122	BnaA.WRKY11.a	WRKY11-Tgt1, WRKY11-Tgt3	11wdyF/11wdyR	0, 7	0, 31.8%
	BnaC.WRKY11.a	WRKY11-Tgt2, WRKY11-Tgt3	11F/11R	12, 9	54.5%, 40.9%
BnWRKY708	BnaC.WRKY70.b	WRKY70-Tgt1	70F3/70R3	0	0
	BnaA.WRKY70.a	WRKY70-Tgt1	70F3/70R3	8	37.5%
	BnaC.WRKY70.a	WRKY70-Tgt2	70F1/70R2	0	0
	BnaA.WRKY70.a	WRKY70-Tgt3	70F1/70R1	8	37.5%

4

Fig 4. Multiplex mutagenesis of *B. napus* genome in T0 generation. The protospacer adjacent motif (PAM) is shown in bold blue letters; red dashes mark the deletions; the inserted nucleotide is marked by a green letter. The numbers on the right show the type of mutation and how many nucleotides are involved, with "–" and "+" indicating deletion or insertion of the given number of nucleotides, respectively. Tgt1-Tgt3 means the target sequence used to generate sgRNA expression cassette.

5

4. Variety and Frequency of Mutations



Mutation type	Frequency (%)
I	80.0
D	20.0
S	0.0

Mutation length (bp)	Counts
1	27
2	5
4	2
5	2
9	1
200	1

Fig 5. Mutation types and frequency in transgenic plants. Mutation types and frequency from combined data of four different targets at T0 generation. Left insert, occurrence of insertion (I), deletion (D) and substitution (S) mutation types. Right insert, counts of different mutation length. In x-axis: I#, # of bp inserted at target site; D#, # of bp deleted from target site

6

5. Mutagenesis in T1 Plants

Table 2. Sum of the edited T1 plants of CRI-W11 and CRI-W70.

Line	Number of Examined Plants	Cas9:sgRNA Constructs [a]	Number of Edited Plants [b]			
			BnWRKY11			
			BnaA.WRKY11.a (WRKY11-Tg1)	BnaA.WRKY11.a (WRKY11-Tg2)	BnaC.WRKY11.a (WRKY11-Tg2)	BnaC.WRKY11.a (WRKY11-Tg3)
CRI-W11-6	10	10	10	10	0	10
CRI-W11-10	10	10	10	10	0	10
			BnWRKY70			
			BnaA.WRKY70.b	BnaA.WRKY70.a	BnaC.WRKY70.a	BnaC.WRKY70.b
CRI-W70-6	10	8	8	8	0	8
CRI-W70-7	10	8	8	8	0	8
CRI-W70-9	10	10	10	10	0	10
CRI-W70-10	10	8	10	10	0	10

Tgt means the target sequence used to generate sgRNA expression cassette. a Cas9:sgRNA construct in the plants was identified by PCR, with the primer pair: Cas9-F/Cas9-R. b Detailed mutation types for every plant were listed on Table S3.

7

6. *BnWRKY70* Mutants Enhance Resistance to *S. sclerotiorum*.

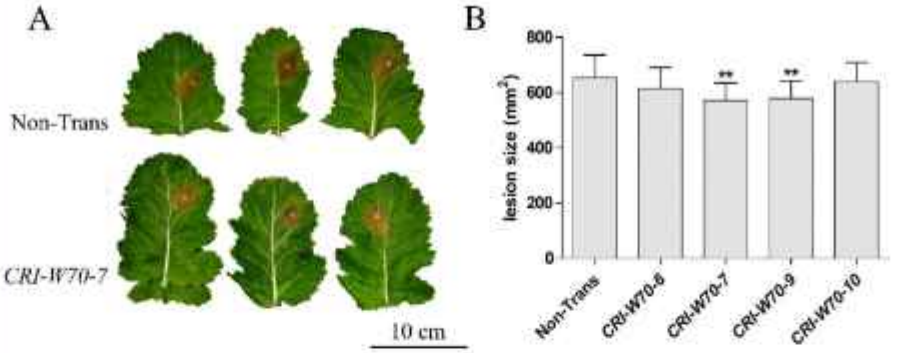


Fig 6. Lesion area on leaves of *BnWRKY70* knockout *B. napus* lines inoculated with *S. sclerotiorum*. (A) Representatives of disease symptom on the Non-Transgenic (Non-Trans), *BnWRKY70* knockout lines. Leaves of 6-week-old plants were inoculated with *S. sclerotiorum*. Photos were taken 48 h post-inoculation. (B) Lesion area on leaves of *BnWRKY70* overexpression lines. ** indicate that the means are statistically different ($p < 0.01$).

8

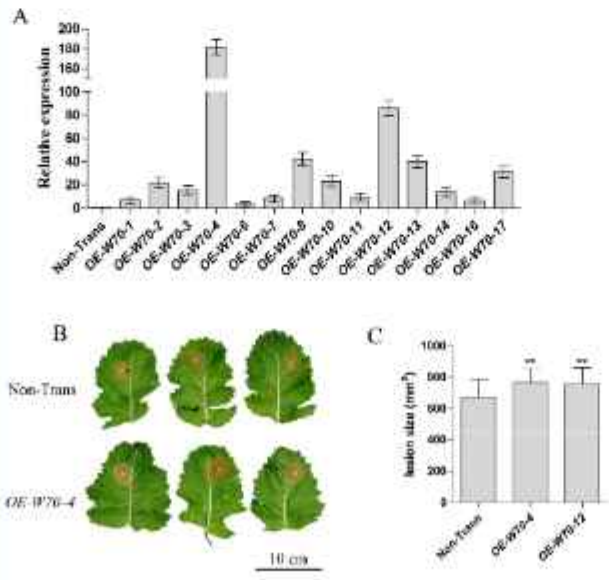


Fig 7. Expression analysis and lesion area on leaves of *BnWRKY70* overexpression *B. napus* lines inoculated with *S. sclerotiorum*. (A) RT-qPCR analysis of *BnWRKY70* expression in overexpression plants. *BnActin7* was used as reference gene. The data shown are the mean of three independent experiments ± standard error (SE). (B) Representatives of disease symptom on the Non-Transgenic (Non-Trans), *BnWRKY70* knockout lines. Leaves of 6-week-old plants were inoculated with *S. sclerotiorum*. Photos were taken 48 h post-inoculation. (C) Lesion area on leaves of *BnWRKY70* overexpression lines. ** indicate that the means are statistical.

SUMMARY

In this study it was demonstrated that . CRISPR/Cas9 system can be an effective tool for multiplex genome editing in *B. napus*.

Out of the 12 homologues of *Arabidopsis WRKY11* and *WRKY70* genes found in *B.napus* (6 homologues each), 2 from *BnWRKY11* and 4 from *BnWRKY70* were chosen as candidate gene.

Multiple sgRNAs targeting exons of the candidate genes were constructed each with different target sites, driven by different promoters. Two sgRNAs rendered non-functional probably due to AtU6-1 promoter.

Total 30 transgenic plants were obtained in T0 generation, 22 with mutation in the *BnWRKY11* gene and 8 in the *BnWRKY70* gene

Targeted mutagenesis revealed various category of mutations in different target sites, heterozygous mutations were seen, heritable mutations were seen in T1 generation. And Chimerism was also seen in some plants in T1 generation

14 Overexpression lines were obtained, expression profiling of these lines helped reveal *BnWRKY70* as a major negative regulator of SA mediated defense pathway in *B.napus*

The knockout mutants of *BnWRKY11* and *BnWRKY70* both were analyzed but only the latter showed significant increase in resistance during infection.

THANK YOU FOR YOUR ATTENTION

ANY QUESTIONS AS A FOLLOW UP?

Development of Abiotic and Biotic Stress Tolerance/Resistant rice lines through Over expression of *OsHAP2E* gene

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Summary

Heme activator protein (HAP), also known as nuclear factor Y or CCAAT binding factor (HAP/NF-Y/CBF), has important functions in regulating plant growth, development, and stress responses. The expression of rice *HAP* gene (*OsHAP2E*) was induced by probenazole (PBZ), a chemical inducer of disease resistance. To characterize the gene, the chimeric gene (*OsHAP2E::GUS*) engineered to carry the structural gene encoding β-glucuronidase (GUS) driven by the promoter from *OsHAP2E* was introduced into rice. The transgenic lines of *OsHAP2Ein::GUS* with the intron showed high GUS activity in the wounds and surrounding tissues. When treated by salicylic acid (SA), isonicotinic acid (INA), abscisic acid (ABA) and hydrogen peroxide (H_2O_2), the lines showed GUS activity exclusively in vascular tissues and mesophyll cells. This activity was enhanced after inoculation with *Magnaporthe oryzae* or *Xanthomonas oryzae* pv. *oryzae*. The *OsHAP2E* expression level was also induced after inoculation of rice with *M. oryzae* and *X. oryzae* pv. *oryza* and after treatment with SA, INA, ABA and H_2O_2, respectively. We further produced transgenic rice overexpressing *OsHAP2E*. These lines conferred resistance to *M. oryzae* or *X. oryzae* pv. *oryzae* and to salinity and drought. Furthermore, they showed a higher photosynthetic rate and an increased number of tillers. Microarray analysis showed up-regulation of defense related genes. These results suggest that this gene could contribute to conferring biotic and abiotic resistances and increasing photosynthesis and tiller numbers.

Introduction

We previously reported microarray analysis in rice treated with probenazole (PBZ), a chemical inducer of disease resistance (Shimono *et al.*, 2003; Nishiguchi *et al.*, 2007). Through this study, the expression of rice heme activator protein gene (*OsHAP2E*, accession number AK071595, Os03g29760) was induced by PBZ (Nishiguchi, unpublished). Heme activator protein (HAP) is a transcription factor, also known as nuclear factor Y or CCAAT binding factor (HAP/NF-Y/CBF), has important functions in regulating plant growth, development, and stress responses (Ballif *et al.*, 2011; Laloum *et al.*, 2012; Petroni *et al.*, 2012). HAP is a transcription factor which is highly conserved in all living organisms and has a high affinity and sequence specificity for the CCAAT box, a cis-element present in 25% of

eukaryotic gene promoters. HAP is a heterotrimeric complex composed of HAP2 (also known as NF-YA or CBF-B), HAP3 (NF-YB or CBF-A), and HAP5 (NF-YC or CBF-C) (Li *et al.,* 2008). Rice has 10 HAP2s, 11 HAP3s and 7 HAP5s (Thirumurugan *et al.,* 2008) and *Arabidopsis* genome encodes 10 HAP2s, 13 HAP3s and 13 HAP5s (Gusmaroli *et al.,* 2002). These family member genes showed different expression patterns and various combinations of overlapped expression of the *HAP2*, *HAP3* and *HAP5* were observed in rice (Thirumurugan *et al.,* 2008). *HAP2* is expressed in sperm, involved in pollen tube guidance, and essential for seed formation (von Besser *et al.,* 2006). *HAP2/NF-YA* functions against some abiotic stresses like drought, flowering, cold and heat (Leyva-Gonzalea *et al.,* 2012), flowering time control (Wenkel *et al.,* 2006), ER stress (Liu and Howell, 2010), plant fertility (Levesque-Lemay *et al.,* 2003), nodule development (Combier *et al.,* 2008) and nitrogen nutrition (Zhao *et al.,* 2011). However, any function of HAP related to disease resistance has never been reported so far. It has been demonstrated that NFYB9 (LEC1) (a member of the HAP3 family) plays a pivotal role in embryo development (Lee *et al.,* 2003). Recently, the overexpression of *Arabidopsis NF-YB1/HAP3* in *Arabidopsis* and maize (*Zea mays*) was shown to significantly improve drought resistance and yield under drought stress conditions (Nelson *et al.,* 2007). *NF-YB5 and NF-YB7* in *Arabidopsis* can improve drought tolerance (Li *et al*. 2008; Han *et al.,* 2013). *NF-YB2 and NF-YB3* play additive roles in the promotion of flowering (Kumimoto *et al.,* 2008) Recently, *NFYA1* involves in regulation of post germination growth restriction under salt stress in *Arabidopsis* (Li *et al.,* 2013). The *OsHAP3E*-overexpressing plants were dwarf with erected leaves while silencing of this gene resulted in lethality (Ito *et al.,* 2011). However, the biological roles of most of the HAP family members in plants are not understood yet.

Since *OsHAP2E* was induced by PBZ, it would be interesting to know whether *OsHAP2E* is involved in defense reactions against pathogen infection. We investigated the roles of *OsHAP2E* using transgenic plants carrying *β-glucuronidase* (*GUS*) with the upstream region of *OsHAP2E* or overexpressing *OsHAP2E* and microarray analysis of the *OsHAP2E* overexpressed lines. We report the expression patterns of *GUS* and *OsHAP2E* after treatment with SA, INA, H_2O_2 and ABA, and infection with blast fungus (*Magnaporthe oryzae*) and leaf blight bacterium (*Xanthomonas oryzae* pv. *oryzae*). We also report that the overexpression of *OsHAP2E* not only conferred resistance to fungal and bacterial pathogens and to salinity and drought, but also increased photosynthesis and tiller number. We propose that *OsHAP2E* plays an important role in these functions.

Materials and methods

Plasmid constructs and rice transformation

The *OsHAP2E* promoter region was isolated using a combination of gene-specific primers designated OsHAP2E pro-5′, OsHAP2E in pro-3′ and OsHAP2E pro-3′ (Table 3.1) that carry extra sequences for the *Sbf*I, *Nco*I and *Kpn*I recognition sites, respectively. The OsHAP2E promoter and first intron fragment (3752 bp) was amplified by PCR with a DNA polymerase (KOD -Plus-; Toyobo, Osaka), and genomic DNA as a template. The amplified fragment was digested with *Sbf*I, *Nco*I and *Kpn*I and inserted into the binary vector pSMAHdN627-M2GUS (Hakata *et al.*, 2010) treated with the same restriction enzymes followed by dephosphorylation (Figure 3.2a, b). OsHAP2Ein::GUS plasmid contained 1,993 bp of the promoter and first intron of the OsHAP2E gene while OsHAP2E::GUS contained only the promoter (1,993 bp). Rice was transformed by *Agrobacterium* as described by Toki *et al.* (2006).

Plant materials

Oryza sativa L. cv. Nipponbare and the transgenic lines were used. Rice seeds on the MS agar medium with hyg (30 µg/ml) and without hyg were incubated in a Petri dish at 27°C under a daily cycle of 16 h continuous light and 8 h dark. One week later the seedlings were transferred to small plastic boxes containing commercial soil for rice cultivation (Iseki, Matsuyama, Japan) and placed in growth chambers (temperature ranged between 23 and 26°C under 16 h daylight and 8 h dark). Approximately three weeks later the plants were transferred to buckets containing commercial soil (Rice Ikubyo Baido; Iseki, Matsuyama, Japan) and left in the growth chambers. The fourth leaves of plants at the 4-leaf stage were used as the experimental material.

Histological GUS analysis

Histochemical GUS staining was performed at 37°C essentially as described by Jefferson (1987). The reaction was stopped by adding ethanol. The samples were treated several times with fresh 70% ethanol several times, if necessary, until the plant tissues were mostly discolored. Some of the samples were cut into 30-µm thick cross-sections using a microtome (Retoratome REM-710; Yamato Kohki Industrial, Asaka, Saitama, Japan) and observed under a microscope (Labphoto-2; Nikon, Tokyo, Japan).

DNA extraction and PCR amplification

Rice genomic DNA was isolated using the cetyltrimethyl ammonium bromide (CTAB) method (Doyle and Doyle, 1987). Integration of the expression cassette in the transgenic genome was confirmed by genomic PCR using DNA polymerase (Taq polymerase; Takara,

Japan) and a pair of primers directed against the *OsHAP2E* promoter (OsHAP2E pro-5′, OsHAP2E in pro-3′ and OsHAP2E pro-3′) and *GUS*-coding region (ΔGUS-5′ and ΔGUS-3′) (Table 3.1). Genomic DNA (100 ng) from each transgenic line was used as a template. The pBI221 vector (Jefferson, 1987) was used as a template DNA for positive control with the *GUS*-specific primers (Table 3.1). The PCR reaction was 2 min of 94°C preheating, followed by a 30 cycle amplification program (1 min at 94°C for denaturation, 1 min at 58°C for annealing, and 1 min at 72°C for extension) and a final extension at 72°C for 5 min. The PCR products were analyzed by electrophoresis on a 1.0% agarose gel.

Fungal and bacterial inoculations

Magnaporthe oryzae virulent strain 001 (MAFF #238988) and avirulent strain 102.0 (MAFF #238991) were grown on oat-meal medium (Difco) for 2 weeks and conidia were induced under BLB light (FL20S BLB; Toshiba) for 2 days at 25°C. The rice seedlings of Nipponbare and transgenic plants at the 4-leaf stage were needle-inoculated with a conidia suspension of 2×10^5 conidia/ml (Shimono *et al.,* 2003) containing 0.05% Tween 20, and a droplet was put on the same position. The inoculated plants were incubated under high humidity in the dark for 48 h, and then moved to the greenhouse. For bacterial blight infection, Nipponbare plants, which are compatible with *Xanthomonas oryzae* pv. *oryzae* (Xoo) strain T7174 (race I, MAFF 311018), were inoculated using a needle that had been dipped in a suspension containing 0.3 OD at 600 nm of Xoo and a droplet was put on the same position. The plants were then incubated in the greenhouse.

Treatment of defense related signaling molecules

Twelve-day-old rice seedlings were submerged in 1.0 g/l Oryzemate (24% granules of PBZ) (Meiji Seika Pharma, Tokyo, Japan). After 7 days of treatment with Oryzemate, the youngest leaf was used for GUS staining (Shimono *et al.,* 2003). Rice seedlings at the 4-leaf stage were dipped in solutions containing 10 mM SA, 5 mM INA, 10 mM MeJA, 20 mM H_2O_2, or 10 mM ABA and incubated for 72 h in the growth chamber following the procedure of Mitsuhara *et al.*. (2008) with a slight modification. For JA treatment, rice seedlings at the 4-leaf stage were put in an air-tight clear plastic box, and a cotton pad with volatile MeJA dissolved in ethanol was put in the corner of the box to give a final concentration of 100 µM, and incubated for 72 h.

Extraction of total RNA and gene expression analysis by RT-PCR

Total RNA was extracted from the young leaves of rice plants using a TRI reagents kit (Molecular Research Centre, Ohio, USA) according to the supplier's protocol with some modifications. RT-PCR was carried out using the RT primer OsHAP2E-ORF-R-3′. The cDNA products from RT-PCR were then amplified using the pair of primers

OsHAP2E-ORF-F-5′ and OsHAP2E-ORF-R-3′. Amplifications were performed at 94°C for 5 min, followed by 30 cycles of amplification (94°C for 1 min, 58°C for 1 min, 72°C for 1 min). RT-PCR reaction for the house-keeping gene, the small subunit of *actin* gene, was performed using the primers Actin-5′ and Actin-3′ under the same condition as described above to estimate equal amounts of RNA among samples. PCR was been done at least three times for each gene, Rice *Actin* gene was used as an internal standard. Quantitative RT-PCR analysis followed the procedure described by Chen *et al.* (2009). SYBR Green was used to monitor the kinetics of PCR product formation in qPCR. The actin transcript, as an internal control, was used to quantify the relative expression levels of genes in samples. The primer (OsHAP2E-ORF-F1-5′and OsHAP2E-ORF-F-3′) sequences are shown in Table 3.1.

Plasmid construct for *OsHAP2E* overexpressed transgenic plants

A 954-bp cDNA fragment (accession number AK071595) encoding the full-length *OsHAP2E* was PCR amplified from the rice mRNA using a pair of primers OsHAP2E-ORF-F-5′ and OsHAP2E-ORF-R-3′ (Table 3.1) that carry extra sequences for the *Eco*RI and *Spe*I recognition sites, respectively. The amplified DNA fragments were digested with *Eco*RI and *Spe*I and used for ligation with pCAMBIA1390-35SThe binary plasmid pCAMBIA-*OsHAP2E* was constructed by inserting a 954-bp *Eco*RI/*Spe*I fragment into the *Eco*RI/*Spe*I site of the pCAMBIA1390-35S. *Oryza sativa* L. cv. Nipponbare was transformed by *Agrobacterium tumefaciens* (EHA101) as previously described by Toki *et al.* (2006).

Analysis of salinity and drought stresses

For germination assay, the seeds were surface sterilized with 70% ethanol for 3 min, with 20% hypochlorite for 10 min, and then rinsed with sterile deionized water. Germination assays were carried out with three replicates of 12 seeds. Rice seedlings were grown in ½ MS media in growth chamber a 16 h light/8 h dark cycle at 26C. Up to 7 days seedling were grown in ½ MS media containing final concentration of 200 mM mannitol and 200 mM NaCl, respectively. For direct comparison of germination rates (%), growth rate (shoot and root length in mm), fresh weight (g) and dry weight (g) (drying at 80 ^{0}C for overnight) each plate was subdivided, and seeds of all genotypes were put on the same plate.

Statistical Analysis

Data were subjected to software package used for statistical analysis (SPSS version 16, 2007) and significant differences between individual means established using a Student's t test. Differences at the 1% level were considered significant and denoted by the lowercase letters among different groups.

Analysis of chlorophyll content, photosynthesis rate and tiller number

The leaf chlorophyll content was measured using a Chlorophyll Meter SPAD-502Plus (Konica Minolta Optics, Inc., Osaka, Japan). The photosynthetic rate was measured using an Li-6400 Portable Photosynthesis System (Li-Cor, Lincoln, Nebraska, USA). The young, fully expanded leaves of 8-week-old plants were measured at an ambient CO_2 concentration of 400 μmol mol^{-1}, photosynthetic photon flux density of 1000 μmol m^{-2} s^{-1}, and a chamber temperature of 22°C. Rice seedlings (3–4) were grown in a bucket and the number of tillers was measured at the 8 week-old stage.

Microarray analysis

Total RNA was extracted from rice leaves following the manufacturer's protocol with a slight modification (Total RNA purification kit; Jena Bioscience, Jena, Germany, http://www.jenabioscience.com). The concentration of total RNA was checked by Nanodrop (Thermo Fisher Scientific Inc, USA) and gel electrophoresis and stored it at -80 °C. The concentration of total RNA was checked by nano drop and gel electrophoresis and stored at −80°C. Microarray analysis was performed according to Agilent Oligo DNA Microarray Hybridization protocols using an Agilent 44 K Rice Oligo DNA Microarray RAP-DB (Agilent Technologies, USA). The hybridized slides were scanned using the Agilent G2505C DNA microarray scanner. Signal intensities were extracted by Feature Extraction software version 10.5.1.1 (Agilent Technologies, USA). For statistical analysis, we excluded genes with low signal intensities (less than 500) in all treatments of the wild-type and the transgenic lines. We extracted genes up-regulated by more than three-folds in the transgenic overexpressed lines #4 and #18. The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE54865 (http://www.ncbi.nlm.nih.gov/ geo/query/acc.cgi?acc=GSE54865).

Results

Upstream region of *OsHAP2E*

The genomic DNA of *OsHAP2E* is 5587 nucleotides including six introns and seven exons (http://rice.plantbiology.msu.edu/cgi-bin/ORF_infopage.cgi?orf=LOC_Os03g297 60). A 1,993-bp fragment of a 5′-flanking region of *OsHAP2E* was isolated by combining the primers OsHAP2E pro-5′ and OsHAP2E in pro-3′, OsHAP2E pro-5′ and OsHAP2E pro-3′ (Table 3.1). Generally, stress-responsive cis-acting elements are found in the promoter regions of stress-inducible genes (Hwang *et al.,* 2010). In this region, several stress-related transcription factor-binding sites including ABA-response elements (ABREs), CCAAT boxes, stress response elements (STREs), the putative cis-acting elements responsible for vascular tissue expression (VTRE) and W-boxes were predicted and their locations are presented in

Figure 3.1 and Table 3.2. TATA and CAAT boxes were identified, which is consistent with the core promoter consensus sequences (Yang *et al.*, 2011; Hwang *et al.*, 2010). In addition, other motifs or elements are also presented in Table 3.2. Since the promoter region contains many cis-elements related to stress and wounding, the expression of *OsHAP2E* may be controlled by complex regulatory mechanisms that respond to external factors.

Table 3.1 PCR primers used in this study

Primer	Nucleotide sequence[a]
OsHAP2E pro-5′	5′-CCTGCAGGATCCTACATTTAACCGTCTG -3′
OsHAP2E in pro-3′	5′-GAGAATCATCCTGTCCAATCCATGG -3′
OsHAP2E pro-3′	5′-GCATATTTGGCAGGAAGAGCCGGTACC -3′
ΔGUS-5′	5′-AGCTCTAGACTATCCCGCCGGGAATGGTGA-3′
ΔGUS-3′	5′-ACGAATTCGGTAGCAATTCCCGA-3′
OsHAP2E-5′	5′-CACCATGATAATGCTGTTGCAAGA-3′
OsHAP2E-3′	5′-ACAGTTCGTTCAGTCATAGC-3′
Actin-5′	5′-GAGTATGATGAGTCGGGTCCAG-3′
Actin-3′	5′-ACACCAACAATCCCAAACAGA-3′
OsHAP2E-ORF-F-5′	5′-CGGAATTCATGATAATGCTGTTGCAAG-3′
OsHAP2E-ORF-F1-5′	5′-GCCGTTGTCCTTGGCAAGAACCT-3′
OsHAP2E-ORF-R-3′	5′-CCACTAGTTCACCTCATGACGGGGAC-3′
HPT-ORF-5′	5′-ATGAAAAAGCCTGAACTCACC -3′
HPT-ORF-3′	5′-CTATTCCTTTGCCCTCGGAC-3′
OsPR1a-F-5′	5′-GTATGCTATGCTACGTGTTTATGC-3′
OsPR1a-R-3′	5′-GCAAATACGGCTGACAGTACAG-3′
OsPR1b-F-5′	5′-ACGCCTTCACGGTCCATAC-3′
OsPR1b-R-3′	5′-CAGAAAGAAACAGAGGGAGTAC-3′
OsPAL-F-5′	5′-AGGTCAACTCCGTGAACGAC-3′
OsPAL-R-3′	5′-AGGTCAGCCCGTTGTTGTAG-3′

[a] Underlined nucleotides in OsHAP2E pro-5′, OsHAP2E in pro-3′, OsHAP2E pro-3′, OsHAP2E-cDNA-F-5′ and OsHAP2E-cDNA-R-3′ indicate *Sbf*I, *Nco*I, *Kpn*I, *Eco*RI and *Spe*I sites, respectively.

-1993	ATCCTACATTTAACCGTCTGATAATTATCTTTGTAGTGGTGTCATAAATCGAATCGCTTG
-1933	TCACTAGAAAAGTAGTCTAGTACATGTACTTCATTTCTTAGAAAACAAAGAAATAATTAT
-1873	GAGGCAGTTGCTGCTTAAAACCTTTTATTAGTGTCCCGCAATAAATTTCTTCATAGAAAA
-1813	TTAAAGAGAAAAACCCTTCATCTGTCATGAAGATTTGAAGTAGCTGTGAATCAGCACCCA
-1753	CAAGCTTCATTCCCTTTATCAACTTCATTCAACAACTTCCAAAAATAAGTTGAAAACAAA
-1693	TGCTTTTCTAAAAGTGCATTGCAACCCTACATAAAATGCACGACATACATGAACATCTTC
-1633	TACATGCTCCCTCCGTCCCCTAATATAAAGGATTTTGATATTTTATTTGCATTATTTGAC
-1573	CATTCATCTTATTTAAAATTTTTTTGGAATTATTATTTATTTTTTGTGACTTACTTTATT
-1513	ATCCAAAGTACTTTAAGCACAACTATTCGTTTTTTATATCTGCAAAAAAAATTTGAATAA
-1453	GACGAGTGGTCAAACAGTGCAAACAAAATATTAAAATCCCTTATATTATGAGGCGGATGG
-1393	AGTACTTAATAGTATAGCCAACTATCAGCTCTAAATCATCAGCCAATCTAATAGCCTATT
-1333	TATACAATAGTTAACTACAAATATACTCCCTCTGTACTTGTAAAGGAAGTCGTTTATGAC
-1273	AATATTTAAGTCAAACCTTGGGAATATAAATCATGAATAACTCTCAAGTTATTGAGTTTG
-1213	AAAATTAAAAATTATATGAATAGATTTGTCTTGAAAAATACTTTCATAAAAGTATACATA
-1153	TATCAATTTTCAATAAATATTTTTATAGAAACAAGAAGTCAAAGTTTTTTTTAGACTGTG
-1093	TCGCTGTCCAAAACGACTTTTTTTAGGCATCCACAATGTAGTCCAAAAGTGGTCCATAAG
-1033	TAATACAATATTTCATTCAACTACCTAGCAACATTGTGGAACTAGTTAATAGGAAAGAAA
-973	TAACAAAAGCTACCTGTAAGCATATGTGCTGCCCATAGAGAATATAATATATTATTTGTC
-913	TCTACTTCTTCTCTCCTCCTAATACTATTTGCTATCTATATACATTGTGAAGATTACAAT
-853	AGTTAAAGTCCATATATGTGAGTCCCATCTATATGTACTACTTTTACTATATATATTGTT
-793	GTTGCCCTTACGAGTACGGAGGGAGTATACCACATCATTAATAATTGATCTCACATGTCA
-733	TACACACAACGTTTTATAGTCCGTGCTGCAGCTGATCTACACTTCTCTTCTCTCTCATCT
-673	CTTATCTTTTTCACATGTAGTTATAGCTGATTTATACTTCCTCAATTTACTTTTGATAGT
-613	CATATTTCATCTTAACACACAGACTAAGGATAAGTAATTCTACTTATAATCTATTTAAAC
-553	ATACTACTAGTCATTCCTCGTAAACAAGTAATTCGTTAATATTTATATTTCTCGATGCCC
-493	ATGTAGCCAATCTTGTATGGAAGAATAGAGTCACGTACTAAATCTGAGAAAGTCATTAAG
-433	ATGATAGGTTAAATCTAAGAGTCATTAAGATGCTAGGTTGTTGGATTGAAATATTCCGTA
-373	TACCTATAAAAATAAATTTTTCAGATTTGAAAATATATCTATGAAAAGTATATGAAGTAT
-313	ATGGAGGGAGTACTGCTAAAGATGCCGTCATAGAATTGCCGTCATTTTTAAAGATGGAGT
-253	CAAGTCATCATCTGTTCGTAGATCAACTTCCAACGCGTTCATGCAAACACACTTTTCATA
-193	CATTCGGCGAGAACTTGCGCACTGGGTTCTCCATGGACCAATGTATGGTGATGATGGTGT
-133	GTGTGTGTGGCTGTGTGCACCTAAGAATTTCTCCGGTGGATGATGTATGATGGTGGTGCC
-73	GTAGTGGCGAGAGCGTGACATAATCCTTTTTTTCCCCCTAAATACACATAGGAGAGAGAA
-13	AGACTCAATAGTA^{+1}gtacaaaggagagagagagagactctagtgcatatttggcaggaagagccgaagaggggag
+64	gtgaggatcagaggaggcagcctcatcgtc**atg**

Figure 3.1 Sequences and structural features of the *OsHAP2E* flanking sequence. The nucleotide sequences of the 5′-flanking region of*OsHAP2E*. The numbering of nucleotides relative to the putative transcriptional initiation site (+1) is shown on the left of the sequences. The translation start site, ATG, is underlined. The putative TATA box is identified by grey back ground and the putative core promoter (CCAAT) consensus sequences are highlighted in pink background. The CPBCSPOR motifs are highlighted in violet, W-boxes in yellow, ABA-responsive & MYCCONSENSUAT in light blue, GT1 boxes in dark blue and the putative cis-acting elements responsible for vascular tissue expression is indicated by green. A stress responsive, SA-reponsive and pollen specific elements are indicated in double-underlined, dashed-lined and dotted-lined, respectively. The locations of cis-elements of interest were identified by using PLACE and Plant CARE databases. The functions and consensus sequences of the corresponding elements are shown in Table 3.2 (#AC092262, http://www.ncbi. nlm.nih.gov/). Here current/sense strand is indicated only.

Table 3.2 Putative *cis*-acting elements and their sequences, positions and possible functions in the 5′-regulatory region of the *OsHAP2E* gene

Cis-element	Sequence and position	Function
ABRE (Hwang *et al.*, 2010)	ACGT: -725 to 722,-459 to -456(2)	The abscisic acid responsiveness
EBOXBNNAPA	CANNTG: -1869 to -1864, -1775 to -1770, -1697 to -1992, -953 to -948, -742 to -737, -705 to -701, -657 to -652, -245 to -240 (8)	The abscisic acid responsiveness
CAAT-box (Yang *et al.*, 2011; Hwang *et al.*, 2010)	CCAAT:-1351 to -1347, -487 to 483, -156 to -152, CAAT: -1835 to -1832, -1350 to -1347, -1329 to -1326, -1274 to -1271, -1150 to -1147, -1143 to -1140, -1060 to -1057, -1028 to -1025, -857 to -854, -631 to -628, -486 to -483, -155 to -152, -8 to -5 (16)	Common *cis*-acting element in promoter and enhancer regions
CPBCSPOR	TATTAG: -1848 to -1843 (1)	NADPH-protochlorophyllide reductase gene expression
GT1CONSENSUS	GRWAAW: -1974 to -1969, -1818 to -1813, -1806 to -1801, -1214 to -1209, -1181 to -1176, -345 to -340 (6)	SA inducible gene expression

Cis-element	Sequence and position	Function
GT1GMSCAM4	GAAAAA: -1806 to -1801, -1181 to -1176 (2)	Pathogen, salt and SA regulated gene expression
GTGANTG10	GTGA: -1768 to -1765, 1528 to -1525, -867 to -864, -836 to -833, -146 to -143, -59 to -56 (6)	*Cis*-acting regulatory element required for pollen specific expression
MYCCONSENSUAT	CANNTG: -1869 to -1864, -1775 to -1770, -1697 to -1992, -953 to -948, -742 to -737, -705 to -701, -657 to -652, -245 to -240 (8)	Drought and cold stress regulate gene expression
STRE (Hwang *et al.*, 2010)	CCCCT:-1617 to -1613, -39 to -35(2)	Stress responsive elements
TATA-box (Yang *et al.*, 2011; Hwang *et al.*, 2010)	TATAAAT: -1249 to -1243, TTATTT: -1591 to -1586, -1582 to -1577, -1565 to -1560, -1541 to -1536, -1537 to -1532, -922 to -917 (7)	Core promoter element around −30 of transcription start
VTRE (Yin *et al.*, 1997;Hatton *et al.*, 1995)	CCCCT: 1617 to-1613, -39 to -35, GATA: -1974 to -1971,-1597 to 1594, -619 to -616, -585 to -582, -431 to -428 (7)	*Cis*-acting regulatory element required for vascular tissue expression
W-box (Hwang *et al.*, 2010)	TGAC: -1577 to 1574, -1527 to -1524, -1277 to -1274, -58 to -55 (4)	Wonding and pathogen responsive elements

Figure in parenthesis represents the total number of sites. Here current/sense strand indicated only.

PCR analysis and *GUS* staining of transgenic lines

The transgene in hygromycin-resistant (hyg-resistant) rice plants was tested by PCR amplification (Figure 3.2 c and Table 3.3). In this study, *OsHAP2Ein::GUS* including the first intron showed GUS activity, but *OsHAP2E::GUS* lacking the first intron showed no GUS acitivity (Figure 3.2 a, b and Table 3.3). Finally, line #772 + 10B was selected for GUS assay under various conditions.

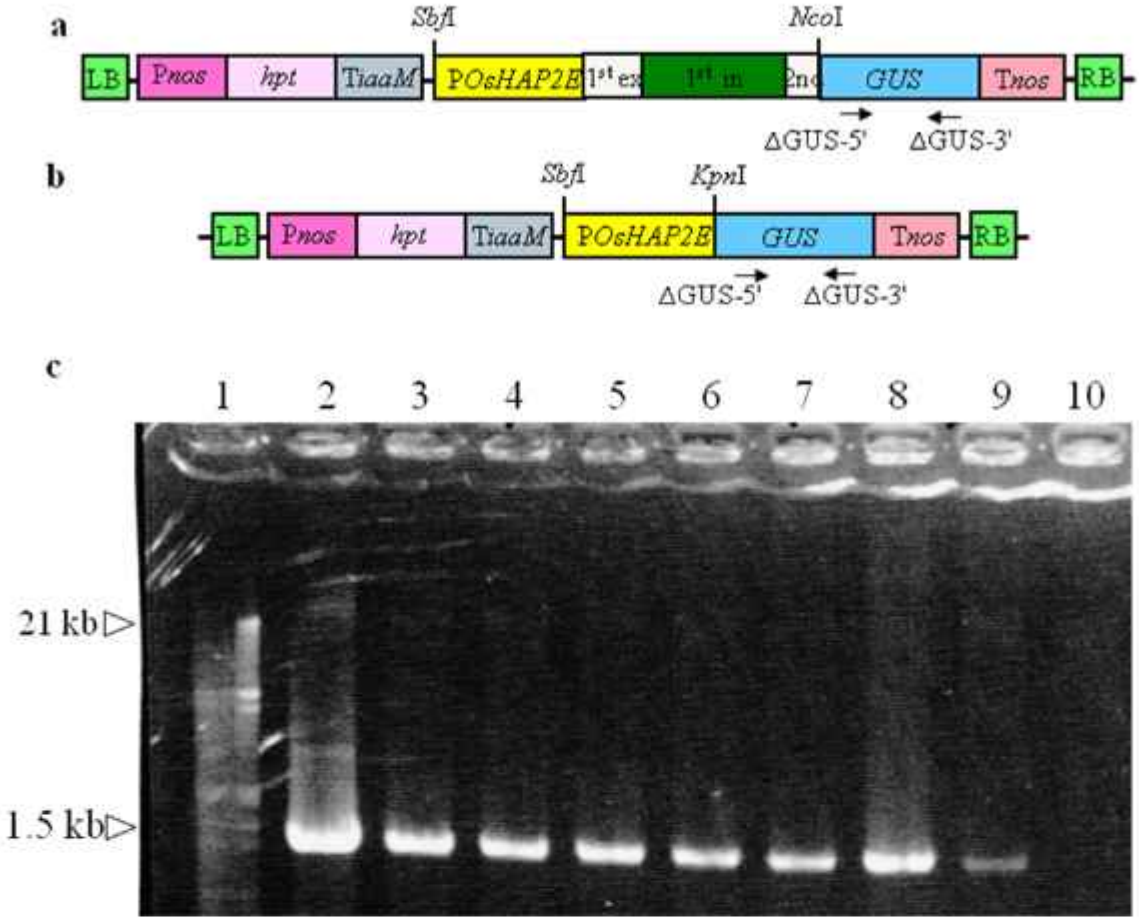

Figure 3.2 Plasmid constructs of *OsHAP2E*. (a) *OsHAP2Ein::GUS* with the first intron. (b) *OsHAP2E::GUS* without the intron. P*nos* and T*nos* are the promoter and terminator of the *nopaline synthase* gene, respectively. *hpt*, hygromycin (hyg) phosphotransferase; T*iaaM*, terminator of *iaa monooxygenase* gene. Arrows indicate the positions of *GUS* gene-specific primers (Table 3.1). (c) Schematic genomic structure of *OsHAP2E*. (d) Gel electrophoresis of PCR-amplified *GUS* fragment. Lane 1, marker (λ/*Hin*dIII.*Eco*RI); lane 2, positive control (pBI221); lane 3, 772+1A; lane 4, 772+1B; lane 5, 772+2A; lane 6, 772+2B; lane 7, 772+10A; lane 8, 772+10B; lane 9, 772-3A; lane 10, 772-6B.

Table 3.3 Analysis of *OsHAP2E*(+)*::GUS* and *OsHAP2E::GUS* -transgenic rice plants by genomic PCR and GUS staining

Plasmid	Transgenic line	Plants tested (T2 generation)	Plants stained blue	PCR-positive plants
OsHAP2E(+)*::GUS*	772+1A	5	4	5
	772+1B	5	4	4
	772+2A	5	4	4
	772+2B	5	3	4
	772+10A	5	4	5
	772+10B	5	4	4
OsHAP2E(-)*::GUS*	772-3A	5	0	4
	772-6B	5	0	4
No	Control	6	0	0

GUS activities induced by probenazole and pathogen infections

Since PBZ is a well-known chemical inducer of plant disease resistance (Watanabe *et al.*, 1977), PBZ inducible genes may play a role in plant disease resistance. To confirm that the *OsHAP2E* promoter is induced by PBZ, we treated 12-day-old rice plants with PBZ and examined the GUS activity. We found that GUS activity increased from 1 to 5 days and degreased at 7 days after PBZ treatment (Figure 3.3). There has never been any information reported about the function of HAP family genes on pathogen infection. We studied the GUS activity 2 days after inoculation (dpi) with *M. oryzae* which could cause severe symptoms in young plants. The result showed that increased GUS activity was detected around the symptom site as well as the wound site after inoculation with a virulent strain of *M. oryzae* (Figure 3.4 a). Weakly and strongly induced activities of GUS were observed by wounding (mock) and inoculating with the fungus, respectively (Figure 3.4 a). In the *OsHAP2E* promoter regions, there are several W-boxes which act as a wounding and pathogen responsive element (Figure 3.1 and Table 3.2) (Hwang *et al.*, 2010). When inoculated with an avirulent strain of *M. oryzae*, the plants showed an increased level of GUS activity in most leaf tissues (Figure 3.4 b). These data indicate that expression of *OsHAP2E* responds to *M. oryzae* and wounding.

To study the response of *OsHAP2E* to a bacterial pathogen, the fourth leaves of the plants were inoculated with *X. oryzae* pv. *oryzae* for leaf blight disease. The results showed that GUS activity appeared to increase at 2 dpi in response to *X. oryzae* pv. *oryzae* (Figure 3.4 c), while mock inoculation caused weak GUS activity in the tissues surrounding the wound site, suggesting that the expression of *OsHAP2E* plays a role in defense response to *X. oryzae* pv. *oryzae*.

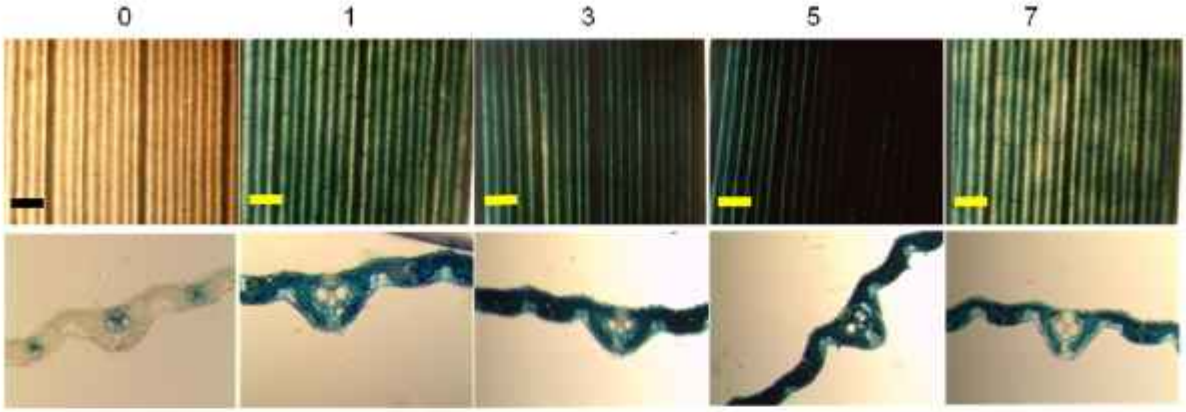

Figure 3.3 *OsHAP2Ein::GUS* expression induced by probenazole (PBZ). *GUS* activity was analyzed in the leaves treated with PBZ. Rice seedlings at 12 days were dipped in PBZ solution at 28°C. Up to 7 days after, from the youngest leaves 1 cm cuttings were used for GUS staining. Scale bar indicates 400 μm long.

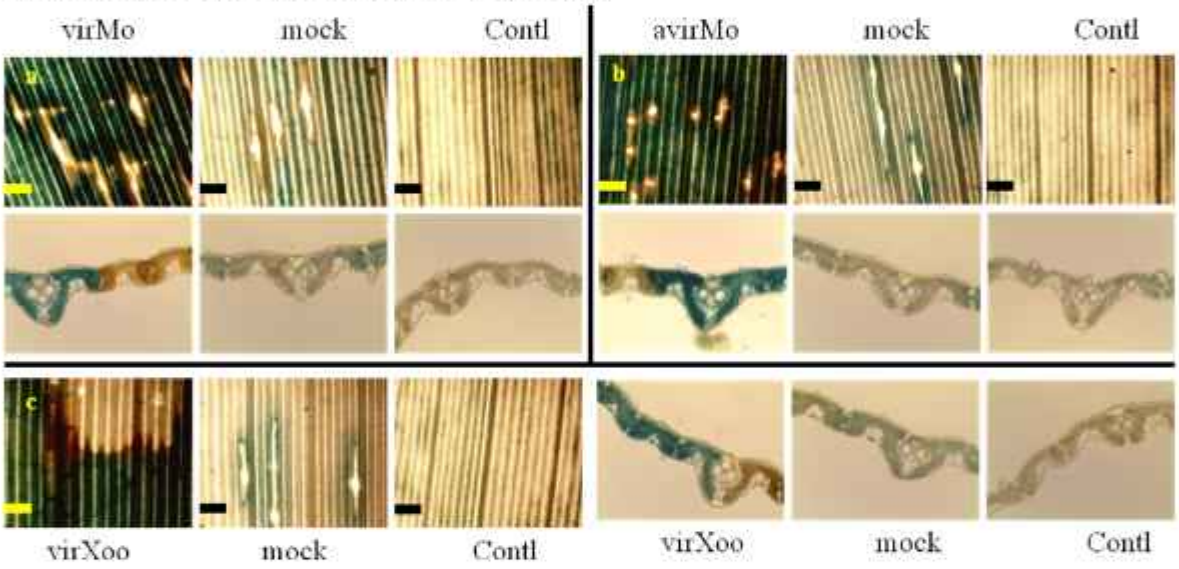

Figure 3.4 Expression patterns of *OsHAP2Ein::GUS* after infection with fungal and bacterial pathogens. (a) *Magnaporthe oryzae* (virulent strain 001); (b) *M. oryzae* (avirulent strain 102.0); (c) *Xanthomonas oryzae* pv. *oryzae*. Removed leaves were wounded by needle inoculation with and without droplets of conidia suspension ($2x10^5$ conidia ml^{-1}) of *M. oryzae* and suspension of *X. oryzae* pv. *oryzae* at OD_{600} of 0.3, respectively. Two days later the leaves were then subjected to GUS staining. Transverse sections of leaves are shown just below the leaf image. Brown portion is symptom of infection by blast fungi and leaf blight bacteria. The area surrounding the symptoms was stained blue, which is clearly shown in the leaf images as well as in transverse sections. Mo/Xoo, inoculation with *M. oryzae*/*X. oryzae* pv. *oryzae*; mock, needle inoculation with water (wounded); Contl, no treatment. Scale bar indicates 400 μm long.

GUS activity induced by defense related signaling molecules

To determine whether the expression of *GUS* is inducible by defense related signaling molecules, GUS activity was analyzed in transgenic plants after treatment with SA, INA, ABA, H_2O_2 or MeJA. It was observed that SA induced GUS activity at 1 day post treatment (dpt), reached a peak at 2 dpt and declined at 3 dpt (Figure 3.5a). INA, a biologically active analogue of SA, shared the same mechanism with SA (Chen *et al.,* 1995). After INA treatment, the activity level of GUS increased dramatically at 1-2 dpt and declined thereafter (Figure 3.5b). To determine whether the expression of *OsHAP2E* is also inducible by other chemical signal molecules, we analyzed the levels of GUS in rice plants after treatment with H_2O_2, ABA and MeJA, respectively. As shown in Figure 3.5c, GUS activity increased gradually with H_2O_2, reached a maximum level at 1-2 dpt and then declined slightly at 3 dpt. The result showed that, like NF-YA/HAP2 family genes from *Arabidopsis thaliana* (Leyva-Gonzalez *et al.,* 2012), *OsHAP2E* was also positively regulated at 2 dpi by ABA (Figure 3.6d). However, the GUS activity showed a basal level with MeJA (Figure 3.5e). These results indicate that *OsHAP2Ein::GUS* expression is up-regulated in response to SA, INA, ABA and H_2O_2, but not to MeJA.

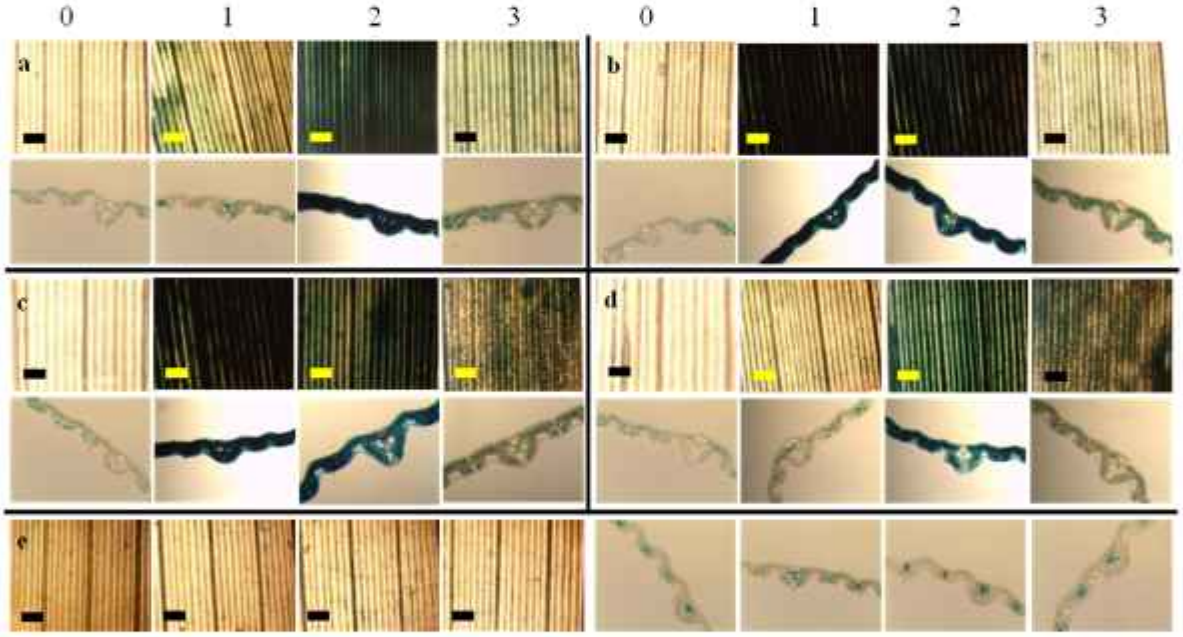

Figure 3.5 Effect of signaling molecules on *OsHAP2Ein::GUS* expression. (a) 10 mM salicylic acid; (b) 5 mM isonicotinic acid; (c) 20 mM H_2O_2; (d) 10 mM abscisic acid; (e) 10 mM methy jasmonate. GUS activity was analyzed in the leaves treated for the indicated duration (days) after each treatment. Rice seedlings at the 4-leaf stage were dipped in 3 ml GUS staining solution and kept at 28°C. The 4th young leaves were cut into 1 cm pieces and used for GUS staining. Scale bar indicates 400 μm long.

RT-PCR analysis of *OsHAP2E* expression in response to pathogen infections and signaling molecules

To check the expression pattern of *OsHAP2E* in response to abiotic and biotic stress, rice plants were inoculated with *M. oryzae* and *X. oryzae* pv. *oryzae*, treated with wounding, PBZ, SA, INA, H_2O_2, ABA and MeJA, respectively. The results showed that *OsHAP2E* expression was induced from 1 dpt to 7 dpt (Figure 3.6a) with PBZ. *OsHAP2E* expression reached a plateau at 3 and 5 days post inoculation (dpi) with a virulent strain of *M. oryzae*, but it was induced earlier at 12-48 hrs after inoculation by an avirulent strain (Figure 3.6b and 4c). Moreover, *OsHAP2E* expression reached a plateau at 3-5 dpi with *X. oryzae* pv. *oryzae* (Figure 3.7d). We observed that *OsHAP2E* expression was induced by PBZ, fungus, bacterium, wounding and signaling molecules like SA, INA, H_2O_2 and ABA, but not by MeJA (Figure 3.6e–j). All of these induction patterns coincided with those of GUS activities (Figures 3.3, 3.4 and 3.5). The results showed that endogenous *OsHAP2E* expression patterns under various conditions coincided with those using transgenic rice expressing *OsHAP2Ein::GUS*.

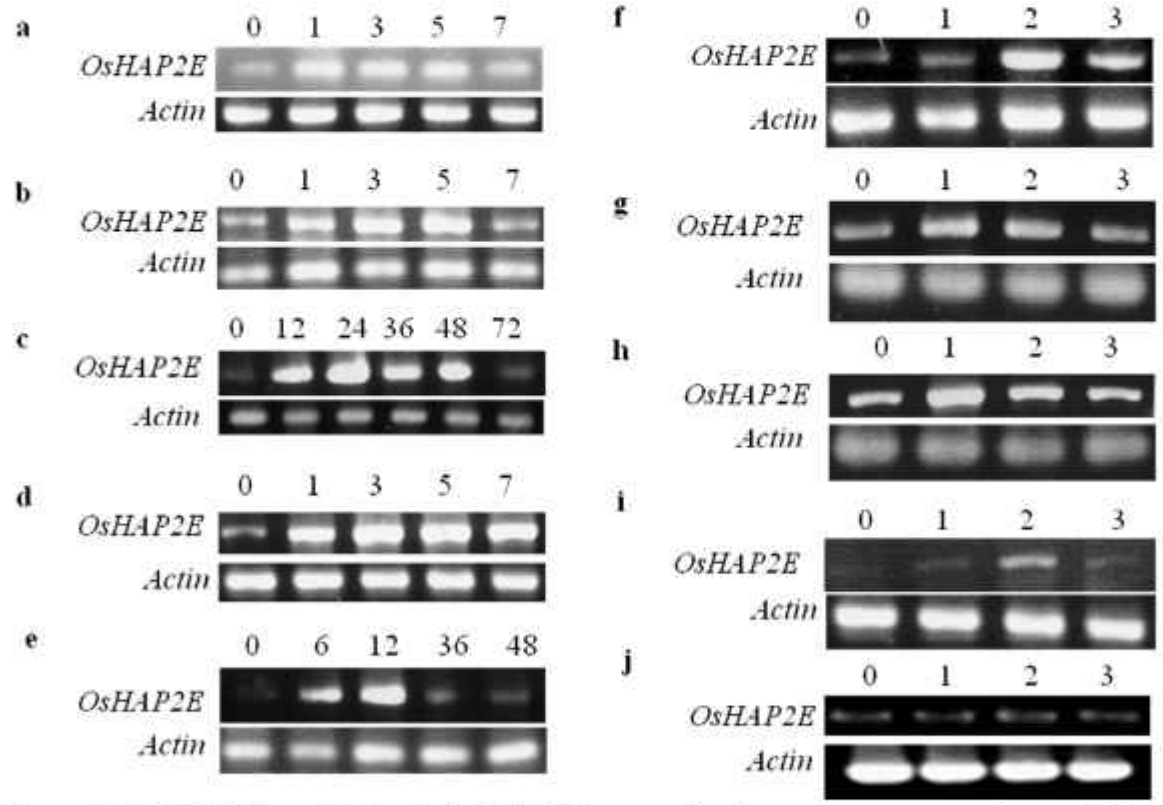

Figure 3.6 RT-PCR analysis of *OsHAP2E* expression in response to pathogen infection and signaling molecules. (a) probenazole (PBZ); (b) *Magnaporthe oryzae* (virulent strain 001); (c) *M. oryzae* (avirulent strain 102.0); (d) *Xanthomonas oryzae* pv. *oryzae*; (e) wounding; (f) salicylic acid; (g) isonicotinic acid; (h) H_2O_2; (i) abscisic acid; (j) methy jasmonat. Numbers represent days after treatment, but for the avirulent strain of *M. oryzae* and wounding they represent hours after treatment. Total RNA was extracted from leaves at

the indicted time. The *actin* gene was used as the standard control to show normalization of the amount of templates in PCR reactions.

Generating *OsHAP2E* overexpressed transgenic plants

Oryzae sativa L. cv. Nipponbare was transformed with the plasmid carrying *OsHAP2E* ORF by *Agrobacterium* (Figure 3.7). Twenty transgenic lines with pCAMBIA-OsHAP2E were regenerated in the presence of hyg. All transgenic lines appeared to have normal development. The genomic DNA samples from T1 plants (Figure 3.7b) and pCAMBIA1390–OsHAP2E (Figure 3.7b) produced the amplified DNA fragment of hyg, indicating the integration into the rice genome. In RT-PCR, a 954-bp amplified fragment confirmed the expression of *OsHAP2E* in T1 transgenic plants (Figure 3.7c). A higher level of *OsHAP2E* transcript expression was recorded in #4, 5, 6 and 18 than in #2, 3, 8, 9, 10 and 14 (Figure 3.7c). Two highly expressed transgenic lines (#4 and #18) were selected for further analyses. These overexpressed plants exhibits normal growth (Figure 3.8a). RT-PCR and quantitative RT-PCR analysis revealed that their transcript levels were higher than the control plants (Figure 3.8b and c).

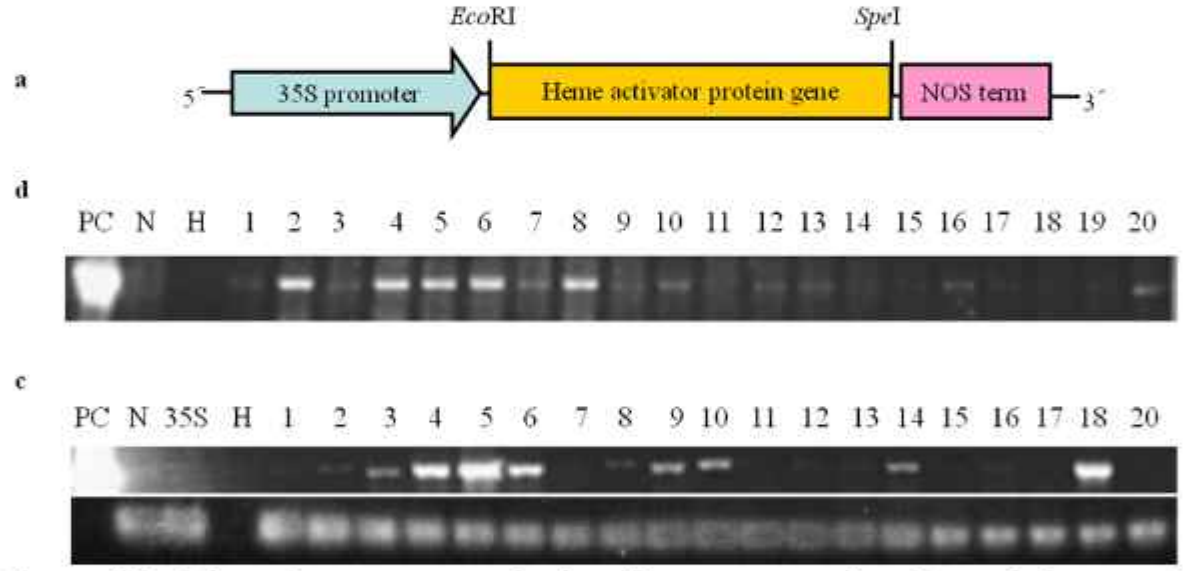

Figure 3.7 Schematic structure of plasmid construct, detection of hygromycin phosphotransferase gene and expression of *OsHAP2E*. (a) Plasmid construct for transformation. (b) PCR analysis of transformed rice plants (T1) for the presence of hygromycin (hyg). PC, pCAMBIA1390-OsHAP2E; N, non-transformed rice; H, H_2O; lanes 1–20, transformed rice. Using hyg specific primers (Table 3.1), hyg fragment was PCR-amplified. PCR conditions were 94°C for 2 min, followed by 30 cycles of amplification (94°C for 30 s, 57°C for 30 s, 72°C for 1 min). (c) RT-PCR analysis of the expression level of *OsHAP2E* in transformed rice. PC, pCAMBIA1390–OsHAP2E; N, non-transformed rice; 35S, transformed rice with pBI121; H, H_2O; lanes 1–20, transformed rice. RT-PCR was carried out as described in materials and method.

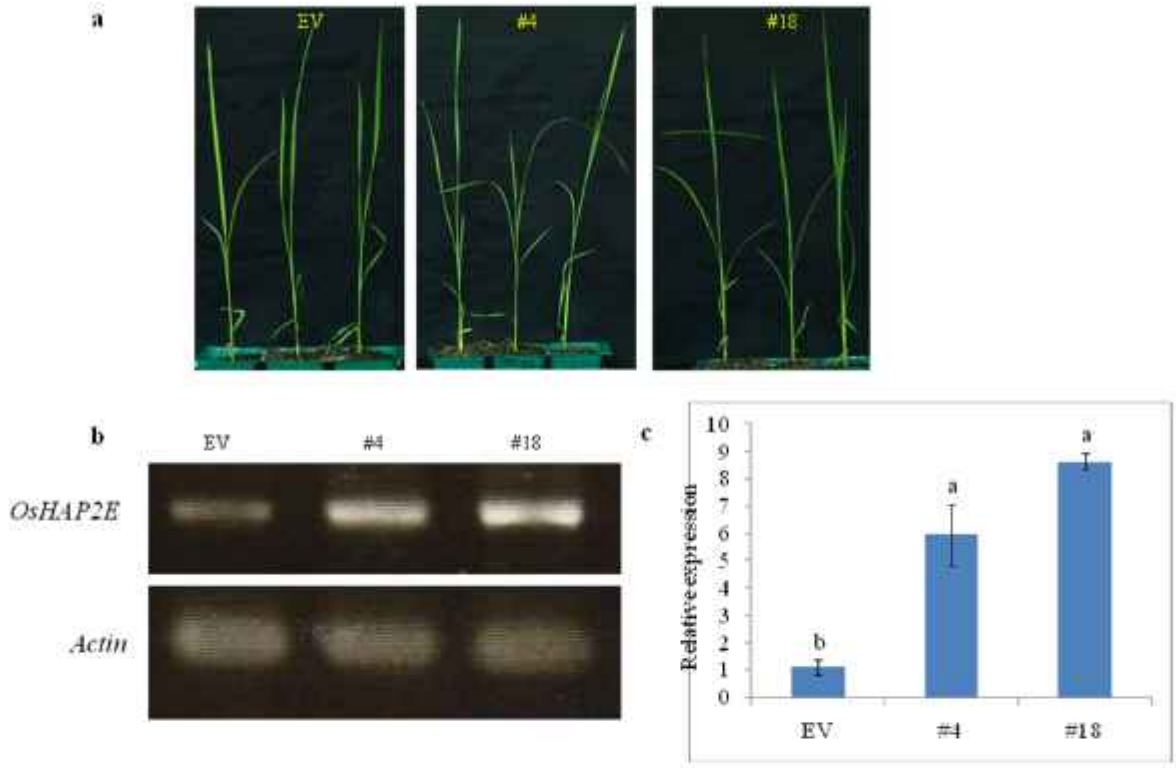

Figure 3.8 Phenotypes of overexpressing *OsHAP2E* lines under normal conditions. (a) Four-week-old seedlings. (b) Expression patterns of *OsHAP2E* in control and *OsHAP2E* overexpressed plants. Upper panel shows *OsHAP2E* and lower panel shows *actin* as a control. (c) Quantitative RT-PCR for *OsHAP2E* transcript. The expression level of rice ubiquitin gene was used as the internal control. Data are mean ± SE (n = 3). Values with different letters are significantly different: $p<0.01$. EV represents *Agrobacterium* mediated transformants without the *OsHAP2E* transgene.

Resistance to fungal infection in overexpressed lines

To confirm the positive role of *OsHAP2E* in resistance against *M. oryzae*, *OsHAP2E* overexpressed lines, together with wild-type rice; Nipponbare, Asominori (a variety resistant to *X. oryzae* pv. *oryzae*), Sensyou (a variety resistant to *M. oryzae*) and transgenic plant containing pBI121 vector were inoculated by *M. oryzae*. The *OsHAP2E* overexpressed lines showed smaller lesions than wild-type rice; Nipponbare, Asominori and transgenic plant containing pBI121 (Figure 3.9a). Statistical analysis by the t test revealed that the disease indices of *OsHAP2E* overexpressed lines were significantly lower than those of wild-type rice plants; Nipponbare, Asominoi and the transgenic plants containing pBI121 vector (Figure 3.9b). The resistance extent of *OsHAP2E* overexpressed lines is similar to that of a resistant variety of Sensyou (Figure 3.9b). These results show that overexpression of *OsHAP2E* led to conferring of resistance to *M. oryzae*.

Resistance to bacterial infection in overexpressed lines

To assess the effect of *OsHAP2E* on bacterial pathogens, the fourth leaves of T1 plants were inoculated with *X. oryzae* pv. *oryzae* and 7 days later the lesions were measured. The transgenic lines exhibited much smaller lesions compared with those in the non-transgenic or GUS transgenic control plants at 7 dpi (Figure 3.9c, d). Even the lesion length and width were smaller in the transgenic lines than those in a resistant variety, Asominori (Figure 3.9d). These results suggest that *OsHAP2E* is involved in the defense reaction against *X. oryzae* pv. *oryzae*.

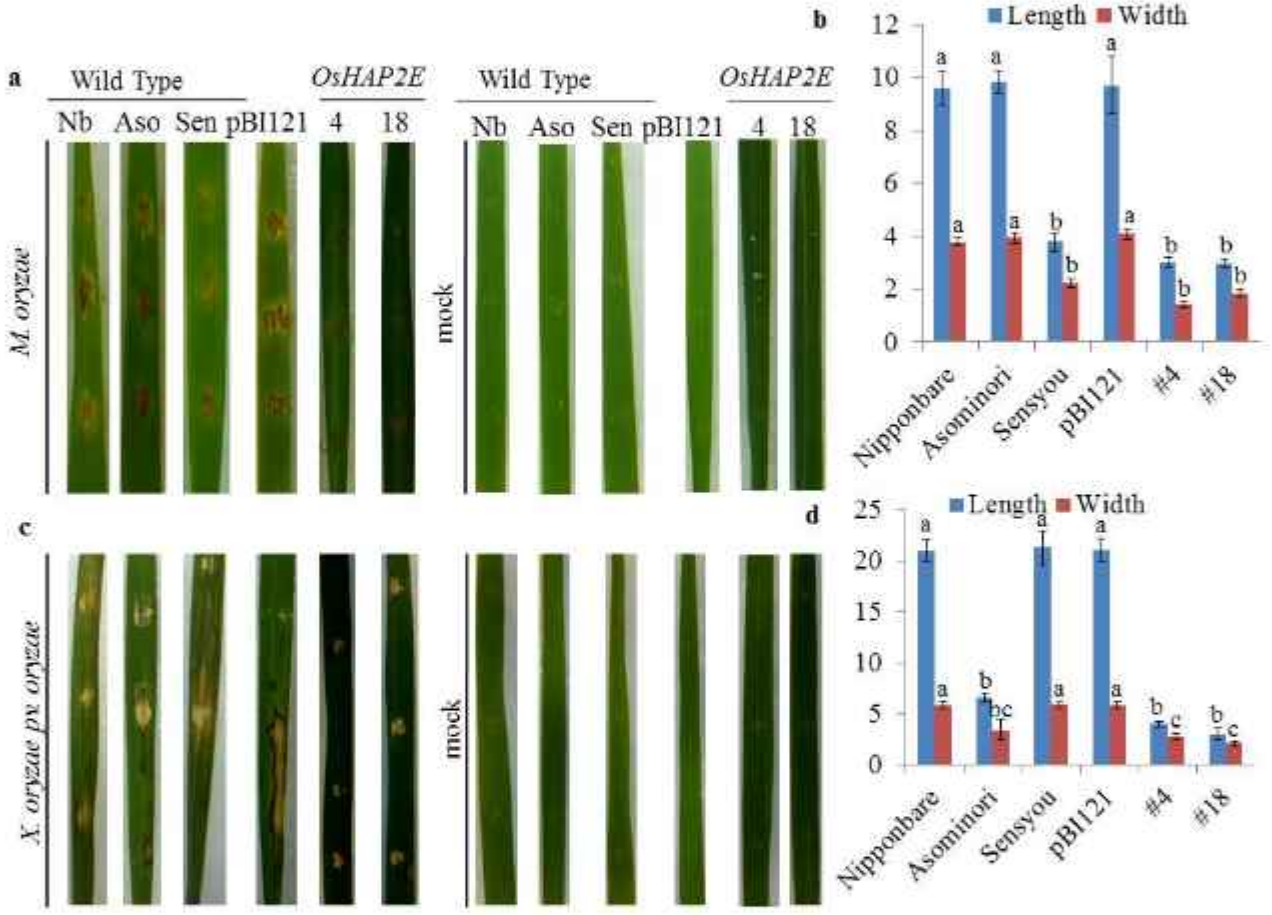

Figure 3.9 Effect of *OsHAP2E* overexpression on *Magnaporthe oryzae* or *Xanthomonas oryzae* pv. *oryzae* infection in transgenic rice. (a) *M. oryzae* at 7 days after inoculation; (b) Lesion size (n = 20). (c) *X. oryzae* pv. *oryzae* at 7 days after inoculation; (d) Lesion size (n = 20) measured at 7 days after inoculation. Vertical axis of a graph represents mm. Upper leaves were removed from at least 5 individual plants inoculated by dropping spores of *M. oryza* or bacterial solution of *X. oryzae* pv. *oryzae* and 7 days later used for measurement. Experiments were repeated three times and data presented are the mean ± SE of three independent experiments. Samples with different letters within each parameter are significantly different: $p<0.01$. Nb, Nipponbare; Aso, Asominori; Sen, Sensyou. Asominori and Sensyou are varieties resistant to *X. oryzae* pv. *oryzae* and *M. oryzae*, respectively.

Resistance to salinity and drought in overexpressed lines

To test whether the overexpression of *OsHAP2E* affects resistance to salinity and drought, rice seeds were sown and grown on the ½ MS media containing 200 mM NaCl (salinity) or 200 mM mannitol (drought), the growth (shoot length, root length, fresh weight and dry weight) and the germination rate ware measured. As a result, each of them was higher in overexpressing *OsHAP2E* lines than that in control plants under salinity and drought conditions, respectively while there are no significant difference between overexpressed lines and control plants under normal conditions (Figure 3.10). Furthermore, these plants were continuously grown for three weeks under salinity and osmotic stress conditions, respectively. It was found that the growth of the control plants were dramatically restricted while the overexpressed plants showed normal growth performance under the same conditions (Figure 3.11). These results show that higher level of resistance to both drought and salinity were conferred in the overexpessed lines.

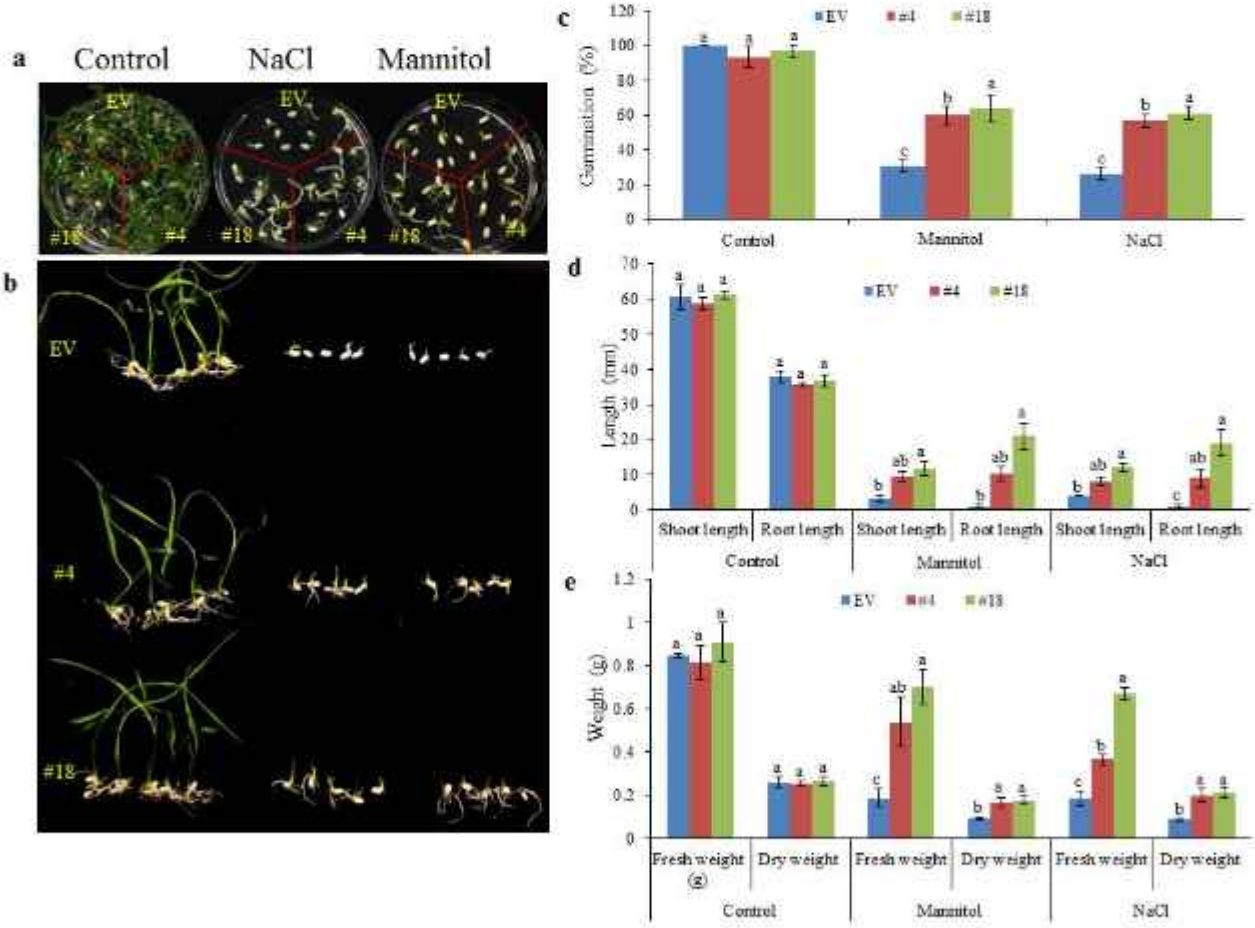

Figure 3.10 Effect of *OsHAP2E* overexpression on drought and salinity. (a), (b) Growth on ½ MS medium containing 200 mM mannitol (drought) or 200 mM NaCl. (c) Germination (%), (d) Length, (e) Weight. After 7 days of sowing the seeds shoot length and root length (mm) were measured. Fresh weight and dry weight (g) of 12 seedlings under each condition

was measured. For these treatments, three independent biological repeats were done. Samples with different letters within each parameter are significantly different: $p<0.01$.

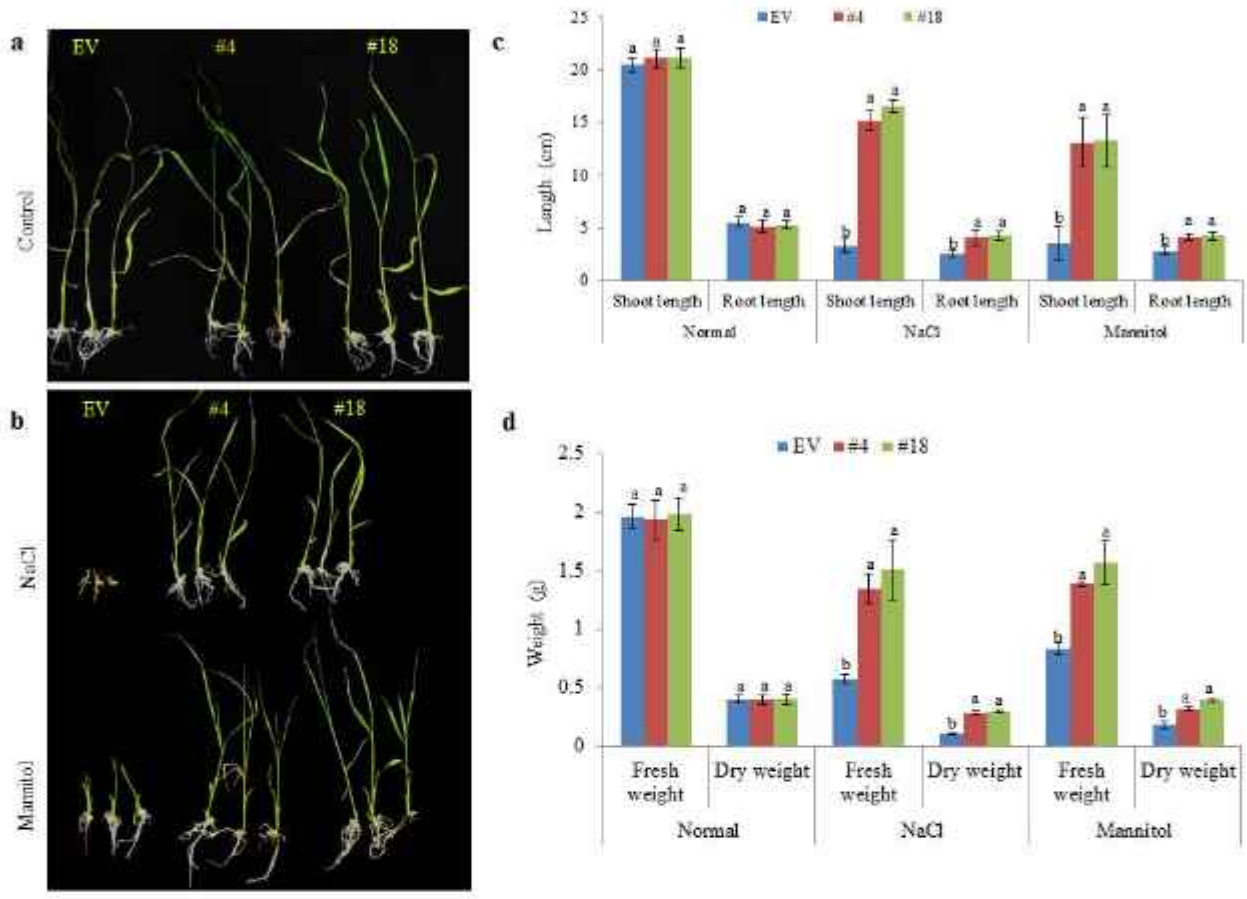

Figure 3.11 Salinity and osmotic stress sensitivity of the overexpressed lines of *OsHAP2E*. (a) Seedlings grown in 1/2 MS media. (b) Seedlings grown in 1/2 MS media containing 200 mM NaCl or 200mM mannitol. (c) Shoot and root length of plants. (d) Fresh weight and dry weight (g) of plants. Total 10 rice seedlings were measured three weeks after sowing. For these treatments, three independent biological repeats were done. Different letters shown above the bar represent results significantly different from control: $p<0.01$. EV represents *Agrobacterium* mediated transformants without the *OsHAP2E* transgene.

Photosynthesis and tiller number in overexpressed lines

Since the leaf color of transgenic overexpressed lines showed green darker than that of control plants, we examined chlorophyll content and photosynthesis rate. The transgenic overexpressed lines showed higher chlorophyll content and photosynthetic rates (Figure 3.12). Tillering is an important factor in increasing rice gain yield because it is a specific grain-bearing branch formed on the unelongated basal internodes and grows separately from the mother stem by way of its adventitious roots (Li *et al.*, 2003). The transgenic

overexpressed lines produced more tillers than control plants did (Figure 3.13a, d). Furthermore, 4-week-old seedlings were grown in 200 mM NaCl or without watering (drought) for 15 days and then allowed to recover for 15 days. Under these stress conditions the transgenic lines as well as control plants showed fewer tillers than under normal conditions (Figure 3.13b, c, e and f).

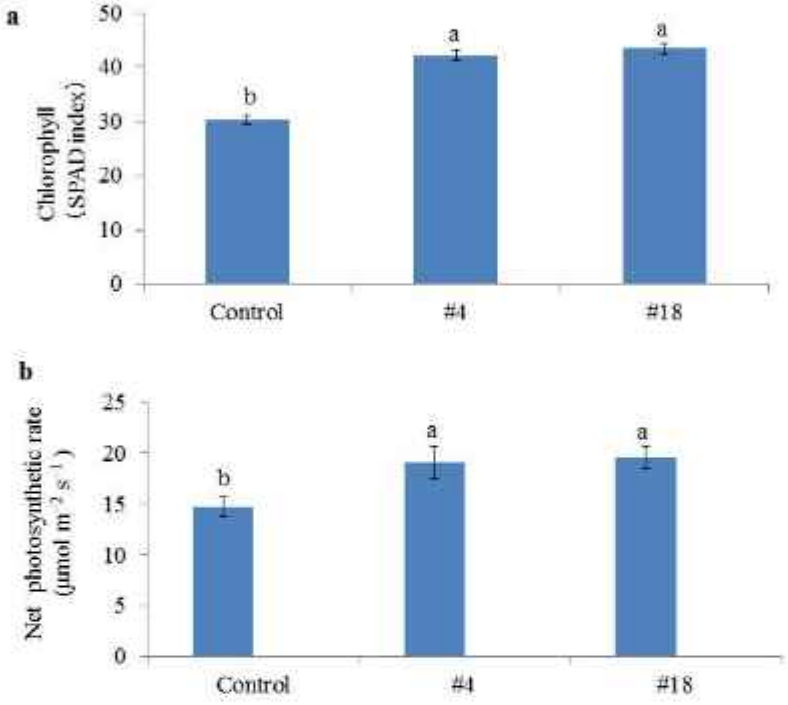

Figure 3.12 Chlorophyll content and photosynthesis in the overexpressed lines of *OsHAP2E*. (a) chlorophyll content of leaf (SPAD Index); (b) net photosynthetic rate at PPFD 1000 μmol m^{-2} s^{-1}. Samples with different letters within each parameter are significantly different: $p<0.01$. EV represents a *Agrobacterium* mediated transformants without the *OsHAP2E* transgene.

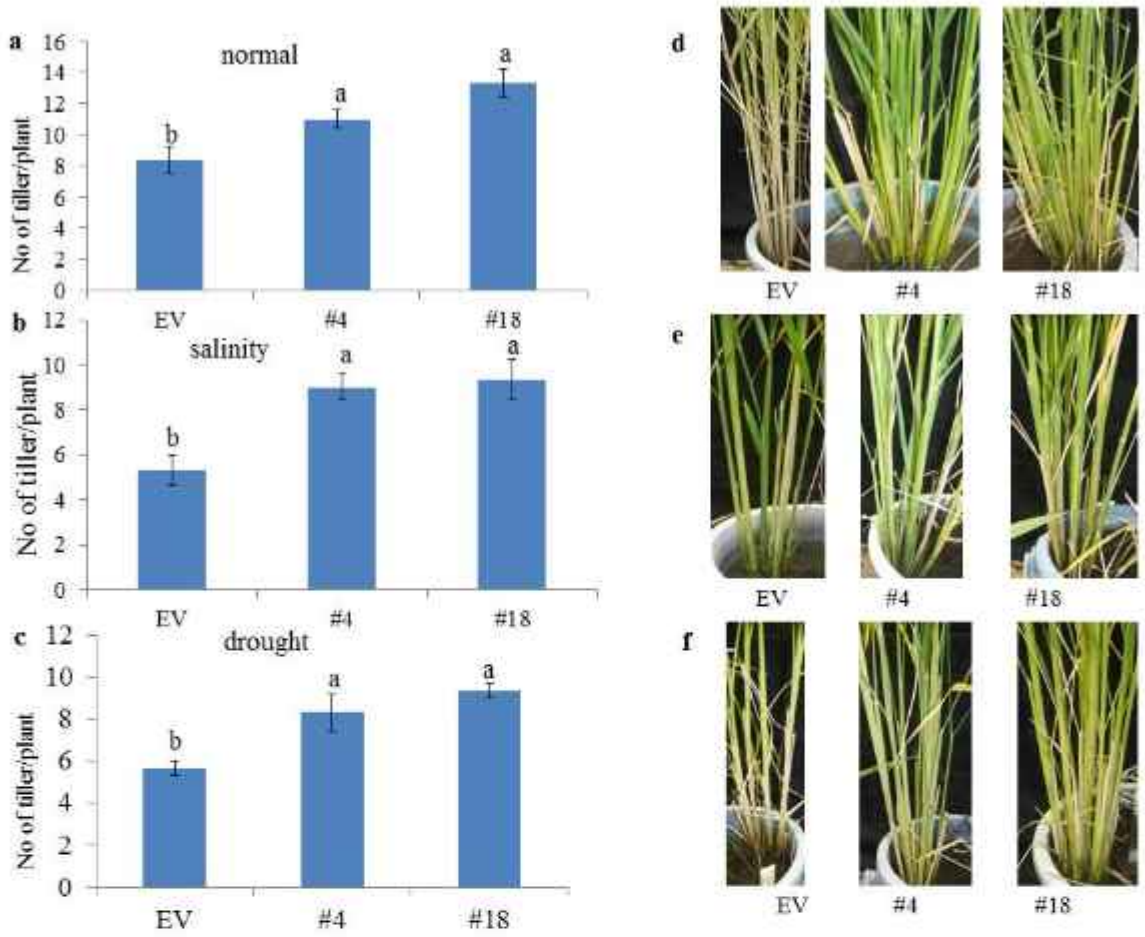

Figure 3.13 *OsHAP2E* overexpression increases tiller numbers under normal, salt and drought conditions. (a, d) Under normal conditions; (b, e) Under salinity conditions for 15 days and then under normal growth condition for 15 days; (c, f) Under drought conditions (no watering) were transferred to grow under normal conditions for 15 days. Tiller number was counted at the heading stage when tillering was completed at 12 week-old stage. EV represents *Agrobacterium* mediated transformants without the *OsHAP2E* transgene. Samples with different letters within each parameter are significantly different: $p<0.01$.

Up-regulation of defense and signaling-related gene expression in overexpressed lines

We examined the microarray analysis of 44K rice genes in the transgenic lines #4 and #18, using a 60-mer oligo DNA microarray (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE54865). Statistical analysis using analysis of variance false discovery rate (p value) < 0.01 identified 95 and 65 up-regulating genes among by 3 folds higher ones in lines #4 and #18, respectively. Among these, 38 genes were shared in the those lines, including defense related genes, chitinase, PBZ1, beta-1,3-glucanase, and thaumatin-like protein, some of which are also induced in *Arabidopsis* by salinity and drought stress (Seki *et al.*, 2002). Some highly induced genes are shown in Table 1. We also used RT-PCR analysis to compare the expression of defense-related genes between the transgenic and non-transgenic control

lines. *OsPR1a*, *OsPR1b* and *OsPAL1* (accession numbers AU163470, AK107926 and X16099, respectively) were selected. An increased level of expression was observed for *OsPR1a*, *OsPR1b* and *OsPAL1* in *OsHAP2E* overexpressed lines (Figure S6), suggesting a relationship with the activation of the SA signaling pathway (Chern *et al.*, 2005; Mitsuhara *et al.*, 2008; Shimono *et al.*, 2007; Yuan *et al.*, 2007). This finding suggests that *OsHAP2E* may play an important role in regulating the expression of *OsPR1a*, *OsPR1b* and *OsPAL1*.

Table 3.4 Up-regulated genes in the overexpressed plants compared with non-transgenic controls

		Line #4		Line #18	
Accession	Description	Foldchange	P-value	Foldchange	P-value
Os10g0542900	Chitinase.	53.28	1.01E-22	11.04	1.34E-19
Os01g0940700	Beta-1,3-glucanase.	18.45	3.59E-21	9.68	4.68E-19
Os10g0491000	Plant Basic Secretory Protein family protein.	10.00	3.39E-19	8.10	3.32E-18
Os02g0758000	Low molecular weight heat shock protein precursor.	3.54	5.07E-12	7.40	1.03E-17
Os12g0555500	Probenazole-inducible protein PBZ1.	26.88	6.50E-22	7.03	2.05E-17
Os10g0569400	RIR1a protein precursor.	12.51	4.68E-20	6.53	5.77E-17
Os12g0628600	Thaumatin-like protein precursor.	10.24	2.69E-19	6.36	8.53E-17
Os10g0570200	RIR1b protein precursor.	7.68	6.40E-18	6.35	8.71E-17
Os06g0591400	Conserved hypothetical protein.	12.02	6.47E-20	5.81	3.42E-16
Os03g0663400	Thaumatin-like protein.	13.34	2.85E-20	5.78	3.74E-16
Os09g0537700	Ribonuclease T2 family protein.	5.26	1.80E-15	5.72	4.38E-16
Os12g0268000	Cytochrome P450.	12.93	3.63E-20	5.11	3.01E-15
Os10g0527800	Tau class GST protein 3.	19.61	2.60E-21	4.98	4.82E-15
Os03g0319000	Hypothetical protein.	3.99	3.72E-13	4.73	1.20E-14
Os01g0734800	UDP-glucuronosyl/UDP-glucosyltransferase family protein.	3.93	4.93E-13	4.73	1.23E-14
Os10g0416500	Chitinase 1 precursor.	6.11	1.53E-16	4.29	8.24E-14
Os03g0580200	Hypothetical protein.	3.23	4.25E-11	4.26	9.43E-14
Os01g0720400	HAD-superfamily subfamily IB hydrolase.	3.33	2.05E-10	4.20	1.25E-13
Os12g0247700	Beta-glucosidase aggregating factor.	63.56	7.46E-23	4.18	1.40E-13
Os12g0555000	Bet v I allergen family protein.	14.36	1.67E-20	4.15	1.62E-13

The genes are listed from highly upregulated genes in line #18. These microarray data can be found at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE54865.

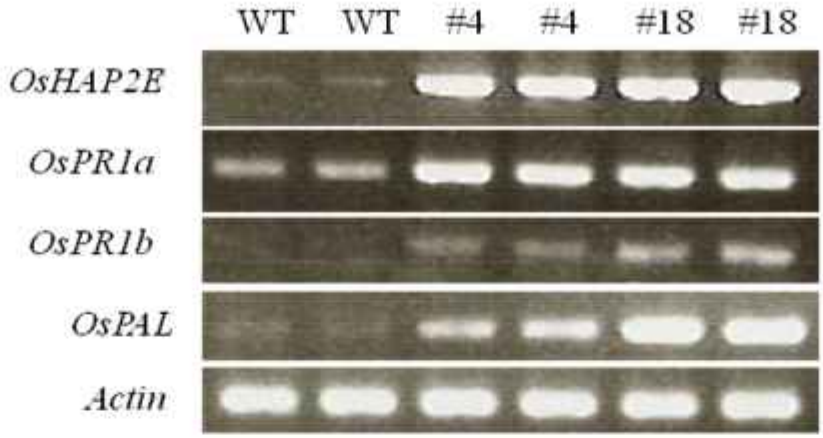

Figure 3.14 RT-PCR analysis of *OsPR1a*, *OsPR1b* and *OsPAL* gene expression. Transcript levels of *OsPR1a*, *OsPR1b* and *OsPAL* were determined for each primer set using non-saturating cycling parameters, and PCR products were electrophoresed on 1.5% agarose gels. WT, wild type; #4 and #18, *OsHAP2E* overexpressed lines. Total RNA was extracted from 4 week-old rice plants. The expression levels of a rice actin were used as an internal control. Amplifications were performed at 94°C for 5 min, followed by 30 cycles of amplification (94°C for 1 min, 58°C for 1 min, 72°C for 1 min).

Discussion

For efficient gene expression in transgenic plants with a desired phenotype, the choice of promoter is an important factor that affects not only the transgene transcription level, but also the stage, tissue and cell specificity of its expression (Koyama *et al.,* 2005). Control of the expression of any gene is regulated by its promoter sequence through interaction with specific transcription factors and making the gene responsive to various biotic and abiotic stimuli (Xu *et al.,* 2010). Therefore, to know the regulatory mechanisms controlling *OsHAP2E* gene expression, the construct consisting of the *GUS* reporter gene under the control of the *OsHAP2E* promoters was introduced into rice via *Agrobacterium* mediated transformation (Figure 3.2 and Table 3.3) (Toki *et al.,* 2006).

We hypothesized that the selective induction of these genes may be due to the presence of specific promoter elements. Thus, the upstream promoter sequences (1,993 bp) of the *OsHAP2E* were analyzed to search for any stress-related cis-elements (Figure 3.1 and Table 3.2). This analysis revealed that *OsHAP2E* contains several putative vascular tissue and wounding stress-related cis-elements, including ABREs, CCAAT-boxes, VTREs, STREs, and W-boxes. The ABRE and W-box motifs have been identified as binding sites for ABRE-binding factors (ABFs) (Choi *et al.,* 2000), and WRKY transcription factors (Eulgem and Somssich, 2007), respectively. ABREs control ABA stress-responsive gene expression, as mentioned by Larkindale and Knight (2002). These motifs were found in the *OsHAP2E*

promoter region and may modulate expression of corresponding genes in response to biotic and abiotic stresses.

In this study, *OsHAP2E* promoter containing the first intron was positive, but the same promoter lacking the first intron was negative for GUS expression (Figure 3.2 and Table 3.3). It has been proposed that an intron's ability to enhance transgene expression depends on the sequence of the intron (Bourdon *et al.*, 2001; Rose, 2002; Rose *et al.*, 2008) and the position of the intron within the transgene (Rose, 2004). In most studies, an intron mediated effect has been attributed to an increased accumulation of mRNA (Rose, 2002); however, some reports provide evidence of enhanced translation (Bourdon *et al.*, 2001).

The expression of *OsHAP2Ein::GUS* as well as endogenous *OsHAP2E* was induced by *M. oryzae* and *X. oryzae* pv. *oryzae* (Figures 3.3 and 3.6). *OsHAP2Ein::GUS* expression was induced in most leaf tissues, with a somewhat weaker expression by wounding (Figure 3.4). *OsHAP2Ein::GUS* expression was induced in the epidermal cells, mesophyll cells and vasculature around the wounding sites (Figure 3.4).

Transcriptional levels of *OsHAP2E* were significantly increased by SA, INA, H_2O_2, but not by MeJA (Figure 3.5). Previous studies have shown that SA plays a critical signaling role in the activation of plant defense responses after pathogen attack (Klessig *et al.*, 2000), while jasmonic acid (JA) is important in inducing non-specific disease resistance through signaling pathways that are distinct from the classical systemic acquired resistance (SAR) response pathway regulated by salicylic acid (Dong, 1998). So it is reasonable to speculate that the expression of *OsHAP2E* could be regulated through the salicylic acid-dependent pathways.

ABA controls the SAR pathway both upstream and downstream of SA induction in *Arabidopsis* and tobacco, as well as inhibits the accumulation of crucial defense compounds (Kusajima *et al.*, 2010). We found that *OsHAP2Ein::GUS* and the endogenous *OsHAP2E* was up-regulated by ABA. The cis-acting regulatory elements responding to ABA were also found in the 5′-flanking regions of *OsHAP2E*. These might be why *OsHAP2E* is up-regulated by ABA. Furthermore, ROS production is required for ABA-driven stomatal closure. In our study, H_2O_2 was found to induce *OsHAP2E* expression. Because ABA is an essential signal for plant resistance to pathogens (Adie *et al.*, 2007) and *OsHAP2E* can be up-regulated by ABA, it is reasonable to predict that *OsHAP2E* may play an important role in resistance to pathogens. This prediction could be supported by the up-regulated expression of *OsHAP2Ein::GUS* and endogenous gene by inoculation with *M. oryzae* and X. *oryzae* pv. *oryzae*.

The rice HAP family consists of more than 28 members (Thirumurugan *et al.*, 2008). To date, none has been demonstrated to regulate defense responses against any pathogens. In this study, we examined the role of *OsHAP2E* in response to *M. oryzae* and *X. oryzae* infection. These findings extend our understanding of the biological functions of rice *OsHAP2E*. Moreover, the overexpressing of *OsHAP2E* resulted in enhanced resistant to *M. oryzae* and *X.*

oryzae and up-regulation of expressions of defense and signaling-related genes. Previous reports indicated that different mechanisms may be involved in defense responses against different rice pathogens (Wen *et al.,* 2003; Ahn *et al.,* 2005). The SA and ethylene/JA mediated signaling pathways might operate in concert in rice to regulate defense response against *M. oryzae* (Qiu *et al.,* 2007). Because the expression of *OsHAP2E* can be induced by exogenous SA (Figure 3.6f), it is reasonable to postulate that overexpression of *OsHAP2E* could up-regulate the expression of *OsPR1a,* which is activated by the SA signaling pathway (Mitsuhara *et al.,* 2008). We propose that *OsHAP2E* may also participate in the SA signaling pathways. However, the role of *OsHAP2E* in the SA and JA signaling pathways requires further investigation. Enhanced resistance to *M. oryzae* and *X. oryzae* (Figures 5) and up-regulation of the defense and signaling-related genes were found in the *OsHAP2E* overexpressed plants (Table 1). We consider that this method fulfills the demand for breeding useful crops with high resistance to fungal and bacterial pathogens. Moreover, it may be worthwhile testing resistance against viruses.

Salinity and drought harmfully affect plant growth and crop yields. The physiological reaction to stress brings about changes in gene expression. The expression of numerous genes is induced in plant cells in response to stress, and these genes are thought to function in stress resistance through signal transduction (Yamaguchi-Shinozaki and Shinozaki, 2006). Transcription factors likely control expression of the stress-responsive genes by binding specifically to the motif of the promoters to alter resistance to stress (Yamaguchi-Shinozaki and Shinozaki 2006). HAP/NF-Y/CBF is an essential transcription factor family involved in salinity and drought resistance in plants (Nelson *et al.,* 2007; Stephenson *et al.,* 2007; Li *et al.,* 2008; Leyva-Gonzalez *et al.,* 2012; Li *et al.,* 2013). Overexpression of *OsHAP2E* increased tiller numbers, chlorophyll content and photosynthetic rate, which are important factors in increasing yields. Different HAP/NF-Y/CBF proteins have been identified as regulators of salinity and drought resistance in different plant species such as *AtNF-YA*, *ZmCBF3*, *ZmNF-YB2*, and *TaNF-YB2* in *Arabidopsis*, maize, and wheat, respectively (Nelson *et al.,* 2007; Stephenson *et al.,* 2007; Li *et al.,* 2008; Xu *et al.,* 2011; Li *et al.,* 2013). A number of studies have focused on HAP/NF-Y/CBF in controlling photosynthesis by regulating the chloroplast ATP synthase (Kusnetsov *et al.,* 1999) and some nuclear-encoded photosynthesis genes such as *RBCS* and *CAB* (Miyoshi *et al.,* 2003). Transgenic wheat with *TaNF-YB3* has significantly enhanced leaf chlorophyll content and photosynthesis rate (Stephenson *et al.,* 2011). Leyva-Gonzalez *et al.* (2012) reported that the overexpression of *AtNF-YA2*, *7* and *10* resulted in dwarf late-senescent plants with enhanced tolerance to several types of abiotic stress. The *OsHAP2E* (*OsNF-YA2*) overexpressed lines did not show dwarf phenotype. This may be reflected by the functional difference between the two plant species, monocot and dicot plants. *OsHAP2E* is classified into a different clade from that of *AtHAP2E* (*AtNF-YA2*) (Laloum *et al.,* 2012; Petroni *et al.,* 2012).

Additionally, stress causes photo-oxidative reactions and degrades the membranes of the cell organelles, especially thylakoids of the chloroplasts which eventually leads to chlorophyll degradation (Husaini and Abdin, 2008). Chlorophyll changes are important in understanding plant response to stress. In this study, the rice plants overexpressing *OsHAP2E* maintained a higher content of chlorophyll than the control plants possibly because the expression of *OsHAP2E* activated the expression of downstream genes that prevented chlorophyll decomposition and maintained a higher chlorophyll concentration (Zhang *et al.,* 2004; Gao *et al.,* 2009). The results of the present study agree with *GhDREB* expression in transgenic wheat (Gao *et al.,* 2009), which showed a higher chlorophyll content than that in non-transformed plants. The transgenic overexpressed lines maintained a higher net photosynthetic rate than the control plants (Figure 3.12). The increased photosynthesis in our results agrees with previous reports in which maize expressing ZmNFYB2 (Nelson *et al.,* 2007), cotton expressing the *AVP1* (Pasapula *et al.,* 2011) and *Populus tomentosa* expressing *mtlD* genes (Hu *et al.,* 2005).

In conclusion, the overexpression of OsHAP2E contributed to high resistance to biotic and abiotic stress and to increased photosynthesis and tiller number, which would lead to developing useful crops of these traits.

plants

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Genetic Diversity of Wheat Stripe Rust Fungus *Puccinia striiformis* f. sp. *tritici* in Yunnan, China

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Abstract: The stripe rust of wheat is one of the devastating diseases in China, which is caused by fungus *Puccinia striiformis* f. sp. *tritici* (*Pst*). The Yunnan Province of China is located in the south-western part, and holds distinctive geographical and climate features, while wheat growth and epidemics of stripe rust fungus are fully dissimilar to the major wheat-growing regions of China. It is important to discover its origin and migration to control the disease. In this study, 352 isolates were sampled from 11 spots of the Yunnan Province during the wheat growing season from 2004 to 2015 and analyzed with SNPs markers of housekeeping genes. Results revealed that 220 haplotypes were inferred from the concatenating sequences; among them, 5 haplotypes (*viz.*, 'H86', 'H18', 'H8', 'H15' and 'H23') comprised over 24.5% of the population. The haplotype diversity, nucleotide diversity, mutation rate and recombination events were 0.992, 6.04×10^{-3}, 4.46×10^{-3} and 18.0 respectively, which revealed the genetic diversity of *Pst* populations among all locations. Four grouping methods, such as UPGMA-tree, PCA, PLS-DA and STRUCTURE, were employed for the categorization of the *Pst* populations conferring to their races and topographical localities. All methods were found significant and mostly had co-linear relations with each other. The analysis of molecular variance (AMOVA) conferred total variation was 9.09%, and 86.20% of variation was within the populations. The current study also exposed a comparatively high genetic multiplicity within the population, while low genetic inconsistency among the populations. Furthermore, the molecular records on the gene pole (Nm = 18.45) established that the migration of the stripe rust pathogen occurred among all locations in Yunnan province. The ancestral haplotype was detected in Yuxi. Based on the trajectories of upper airflow and genetic diversity of *Pst* populations in different locations, it is suggested that the locations Dehong, Dali, Lincang and Baoshan are probably a major source of *Pst* in Yunnan.

Keywords: genetic diversity; population structure; wheat; *Puccinia striiformis* f. sp. *tritici*; Yunnan Province; China

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1. Introduction

Around the globe, the stripe rust disease of wheat is considered the most devastating disease, and it is caused by the fungus *Puccinia striiformis* f. sp. *tritici (Pst)* [1,2]. In terms of the area that can be affected by the disease, China is the largest epidemic region for stripe rust disease of wheat in the world [3]. During 1950, 1964, 1990 and 2002, yield losses of wheat due to the disease were > 6.0, 3.0, 1.8 and 1.3 million metric tons, respectively [3,4]. Generally, stripe rust disease of wheat in China is the most devastating, due to distinctive inter-regional features that help to migrate the disease to a long distance over similar geographic regions [5]. It is noted that most of these races of *Pst* were first detected in the Gansu Province of China [6]. The genetic diversity of stripe rust pathogens was high in Gansu [6–8], where the *Pst* population can easily complete their lifecycle due to different

elevations of mountains on wheat [9–11] and/or alternative hosts (*Berberis* spp.) for sexual reproduction [12,13].

The Yunnan Province is one of the wheat-producing Provinces in China, which is situated in the south-western part of the country [14]. From west to east, the Yunnan–Guizhou Plateau of China crosses this Province. This area shows an enormous dissimilarity of agro-climate, vegetation, agricultural systems as well as cultivation of wheat compared to other provinces in China. Wheat is generally cultivated in Yunnan province from the valleys' lowland to highland, with overlapping growth stages of wheat along with the elevation, which delivers a year-round host for stripe rust pathogens [5,12]. A survey estimated that the incidence of numerous races of stripe rust fungus in Yunnan Province was dissimilar to that in original epidemic regions such as Gansu Province [5–7]. In addition, the earliest epidemic of stripe rust was recorded in Yunnan province of China in the 1930s [15]. Furthermore, Yunnan possesses this disease year-round, like Gansu [5].

Recently, in Europe, Australia and New Zealand, population structures of various pathogens have been recognized by using various molecular markers [15–18]. However, a recombination signature in *Pst* has been documented in Gansu Province [9,10]. In Europe, the genetic diversity of *Pst* fungus was exceptionally studied by Justesen et al. [19] by using the amplified fragment length polymorphism (AFLP) markers; similarly, in North America, Markell and Milus [20] also used the same markers for the identification of the genetic diversity of *Pst* populations. Single nucleotide polymorphisms (SNPs) are relatively different types of molecular markers and AFLP, RAPD (Random Amplification of Polymorphic DNA) and SSR (Simple Sequence Repeats) are replaced by SNPs with the development of sequencing technology. Polymorphisms can be detected in coding and non-coding regions by the SNP marker of an organism that can cover a large part of the genome [21]. A SNP is the mutation of a single base pair at a specific locus position, and SNPs can conserve during evolution [22]. In recent years, SNP markers have been used to study population structure in plant pathogens. Li et al. [5] exposed three SNP primers of housekeeping genes to study the origin, evolution and movement of *Pst* in China. Similarly, in the USA, Parks et al. [23] used SNP markers and found 25 haplotypes during the investigation of the population structure of *Blumeria graminis* f. sp. *tritici*. The current study aimed to investigate the genomic assortment and population structure of *Pst* in Yunnan Province from the year 2004 to 2015 by using SNPs markers of housekeeping genes.

2. Materials and Methods

2.1. Sampling and Multiplication

Wheat stripe rust samples were collected in the year of 2004 to 2015 (main wheat growing season from February to May) from eleven locations of Yunnan province in China. Naturally infected green leaves were taken randomly from different wheat nurseries and farmers' fields (Table S1). The sampling distance between the two places was more than 1000 m. The sampling sites covered altitudes from 906 to 2480 m and the main profitable wheat cultivars to increase the multiplicity of the *Pst* pathogens. During sampling, a leaf of the wheat plant was collected as a sample and wrapped in a piece of clean paper. A total of 352 isolates were used in this study (Table 1).

Then, the dried sample leaf was placed on a wet filter paper in a Petri-dish, Φ100 mm, for 6 to 12 h in a dark place at a temperature of around 20 °C. The pustules were scraped with a dissecting needle and urediniospores spread to seedlings of 'Mingxian 169', grown in pots, Φ100 mm, when the first leaf was fully expanded. The inoculated seedlings were sprayed with water and kept in humid condition in a dark place for 24 h at a temperature of 10 ± 1 °C. The pots were then moved to a greenhouse covered with glass shade with an open-top to insulate each isolate. The temperature was kept at 14 ± 3 °C in the greenhouse with a lighting time of 10 to 14 h each day. Then, the fresh urediospores were collected with a test tube by tapping the tube when the symptoms were fully appeared and each pot was harvested 3 to 4 times to obtain about 10 mg of urediniospores. For DNA extraction,

the spores of stripe rust pathogens were then shifted to a centrifugal tube, then desiccated and deposited/stored at 4 °C.

Table 1. The numbers of *Puccinia striiformis* f. sp. *tritici* isolates were obtained from 11 different locations of Yunnan from 2004 to 2015 (main wheat growing season: February to May).

Location	Year						Total
	2004	2008	2011	2012	2014	2015	
Lijiang (LJ)	16	-	12	4	-	-	32
Dehong (DH)	-	3	19	-	2	5	29
Baoshan (BS)	-	16	7	-	-	-	23
Dali (DL)	-	13	13	-	10	-	36
Qujing (QJ)	-	22	-	-	5	5	32
Zhaotong (ZT)	5	13	4	-	-	12	34
Yuxi (YX)	-	-	14	-	21	4	39
Lincang (LC)	-	-	12	-	9	7	28
Wenshan (WS)	-	-	9	-	2	14	25
Chuxiong (CX)	6	-	4	-	6	10	26
Kunming (KM)	-	-	20	-	28	-	48
Total	27	67	114	4	83	57	352

2.2. Primer Design

The sequences of *Pst* housekeeping genes were searched in Gen-Bank. Three protein-coding housekeeping genes were identified for developing SNP primers, namely heat shock protein 90 kDa (HSP), ubiquitin-activating enzyme E1 (UBA) and ubiquitin-conjugating enzyme E2 (UBC). The SNP primers were designed using Premier 5.0 software [https://en.freedownloadmanager.org/users-choice/Primer_Premier_5_64.html] (Accessed on 12 August 2021). Designed primer pairs were synthesized by Tsingke Biological Technology Co. Kunming, China. The other three primers were designed by Li et al. [22], namely, Elongation factor (EF-1), Map kinase 1 (MAPK) and Cyclin-dependent kinase 2 (CDC2). Details of all primers' information are available in Table 2.

Table 2. Primers and corresponding sequences used in this study.

Gene	Gene Name	Organism	Gene Bank acc. No.	Primer Sequence (5′–3′)	Product Size (bp)	Temperature
Ef-1	Elongation factor	*Pgt*	X73529.1	Ef137S: AAGCCGCATCCTTCGTTG Ef531A: TTGCCATCCGTCTTCTCG	395	51
Mapk1	Map kinase 1	*Pst*	HM535614.1	Map1351S: GTCGGTCGGGTGTATCCT Map1683A: GGTTCATCTTCGGGGTCA	332	53
Cdc2	Cyclin dependent kinase 2	*Pst*	GQ911579.1	Cdc28S: AAATCATCCACATCTGCTCCAC Cdc352A: TCCTACAAACCCCTCCAAAGGA	325	55
HSP	heat sock protein 90 kDa (hsp90)	*Pst*	AJIL01000023.1	Hsp2396S: TGCTCGTCACTGGTCAGTTC Hsp2680A: CGAAGAGGAGGACACTCAGG	285	52
UBA	ubiquitin-activating enzyme E1 (UBA)	*Pst*	AJIL01000094.1	Uba1715S: ACCCAAACCACGGAACCC Uba2088A: TCGCTCCAGCACCAACTA	374	59
UBC	ubiquitin-conjugating enzyme E2	*Pst*	AJIL01000007.1	Ubc279S: TTTGCGAATGGAGTATGG Ubc581A: GAGGGACTGACCTTTGAC	303	52

Puccinia graminis f. sp. *tritici* (*Pgt*), *Puccinia striiformis* f. sp. *tritici* (*Pst*), Elongation factor (EF-1), Map kinase 1 (MAPK), Cyclin-dependent kinase 2 (CDC2), heat shock protein 90 kDa (HSP), ubiquitin-activating enzyme E1 (UBA) and ubiquitin-conjugating enzyme E2 (UBC).

2.3. Procedures of DNA Extraction

The DNA was extracted directly from urediniospores by using a reformed cetyltrimethylammonium bromide (CTAB) technique, which was previously characterized by Chen et al. [24] with some modifications. For each segregate, 10 mg of urediniospores were taken into a 2.0 mL Eppendorf tube with 5–7 sterile glass balls (3–4 mm). Then, 500 µL of preheating abstraction buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl and 100 mM EDTA) and 5 mL of protease (10 mg·mL^{-1}) were added and shaken by vortex for 2 min, and the tube was then hatched at 65 °C for 60 min. The tube was cooled at room temperature, and 500 µL of chloroform was added, mixed gently and then centrifuged at 12,000 rpm for 10 min. Then, the supernatant was transferred into a 1.5 mL tube and 500 mL of pre-cooling isopropyl alcohol was added, mixed gently and kept for 30 min at −20 °C. After centrifuging for 10 min at 12,000 rpm, the discarded supernatant pellet was washed two times, then separately cold-washed with 70% ethanol and 100% ethanol, and then desiccated and melted in 40 µL of TE buffer. The DNA-solution was treated with RNase (final concentration 20 µg mL^{-1}) and reserved for 60 min at 37 °C to entirely digest RNA. The DNA was re-hastened, rinsed with ethanol, dried and dissolved in 40 µL of TE buffer. The DNA concentrations were diluted to 20 ng µL^{-1} with TE buffer before storing at −20 °C in small aliquots.

2.4. PCR (Polymerase Chain Reaction) and Sequencing

The PCR was performed in a 20 µL volume, and all primers were amplified under similar warm air cycling circumstances and chemical reagent concentrations, except the annealing temperatures. Every reaction occupied 10 µL of TIANGEN 2 × Taq PCR MasterMix (0.1 units of Taq Polymerase µL^{-1}, 500 µM dNTPs each, 20 mM Tris-HCl (pH8.3), 100 mM KCl, 3 mM $MgCl_2$ and other steadying and strengthening agents) 1 µL of each 10 µM primer, 1 µL of 20 ng of genomic DNA and 7 µL of ddH_2O. The cycling situations were one cycle of 94 °C for 5 min, then 34 cycles of 94 °C for 25 s, strengthening (51–59 °C) for 25 s and 72 °C for 45 s, monitored by an ultimate extension phase of 72 °C for 5 min. Before sequencing, agarose gel electrophoresis used an output of 1 × TAE buffer for 40 min at 110 volts to perceive if the band is the distinctive directed band. Sequencing was carried out at Tsingke Biological Technology Co., Kunming, China. The sequencing instrument was a 3730 × l DNA Analyzer. The sequencing substance was Big-Dye Terminator v3.1.

2.5. Analysis of the Recorded Data

Recorded data were analyzed by the multi-evolutionary analysis software. The arrangements were aligned and split for a single gene and all of the samples by using MEGA 4.0 [25]. The sequences were concatenated conferring to the instruction of *Cdc2*-(*Ef-1α*)-*Hsp-Mapk1-Uba-Ubc*. The haplotypes, counting the records of SNP loci and the category, collapse sequence, ancestral and isolate number of haplotypes, were examined by Map tariff options for collapsing sequences and removing indels into haplotypes and eliminating infinite-sites' desecrations, using SNAP Workbench 2.0 [26], and execution of numerous evolutionary analysis under a distinct interface [27]. It was assumed that all data were in accordance with the unrestricted model, and there was only a sole modification at an individually mutated locus. The diversity of haplotype (*Hd*) and nucleotide (*Pi*), neutrality tests (Tajima's *D* and Fu's *Fs* values), re-amalgamation incident (*Rm*) and coefficient of genetic differentiation (*Gst*) based on haplotypes and gene flow (*Nm*) was calculated in DnaSP v.5.10 [28]. Negative and significant *D* and *Fs* values were taken as one source of the indications of population expansion. The mutation rate of the populations (Θ) was computed in MEGA 4.0. Analysis of molecular variance (AMOVA) was carried out using Arlequin 3.1 [29]. AMOVA is a technique of segregating genetic assortment into within-population and between populations for distinguishing population dissimilarities [30]. To assess the degree of isolates' concentration changes (heat maps), PCA and PLS-DA clustering were performed using the Metabo-Analyst 2.0 software [31]. Heatmaps were created based on the Pearson distance. The UPGMA-tree was constructed using MEGA 5.0 software [25] and illustrated by FigTree v1.4.2. The STRUCTURE 2.3.4 was used for

inferring population structure [32]. For the Evanno plot, the Structure Harvester was followed for imagining the structure outputs [33].

Bayesian-based clustering was performed using STRUCTURE v.2.3.4 [32], testing three independent runs with K from 1 to 14, with each run having a burn-in period of 50,000 iterations and 500,000 Monte Carlo Markov iterations, assuming an admixture model. The most likely K value was processed with STRUCTURE HARVESTER v.0.9.94 [33] and was detected using the Evanno transformation method [34]. To assign samples to clusters, a membership coefficient of $q > 0.8$ was used, while coefficients ≤ 0.8 were considered "genetically admixed".

3. Results

3.1. Genetic Diversity in the Yunnan Pst Isolates

From 11 counties of Yunnan province in the years of 2004 to 2015, 220 haplotypes were detected from 352 samples using 6 SNP primers collected. There were 42 SNP loci samples collected from all locations, where 33 were phylogenetically informative (Table S2). No supplements or removals were identified, and all recorded data were constant with an infinite-sites model, where each variable locus has only a distinct metamorphosis. By using 6 primers, a total of 1354 polymorphic alleles were found across all populations (Table S2). Among them, 161 polymorphic alleles were detected across SNP primer CDC2, 88 were detected across EF-1, 379 were detected across HSP, 218 were detected across MAPK-1, 147 were detected across UBA and 359 were detected across UBS (Supplementary Table S2). There were 25, 24, 19, 30, 23, 30, 31, 22, 22, 22 and 37 haplotypes found in Lijiang (LJ), Dehong (DH), Baoshan (BS), Dali (DL), Qujing (QJ), Zhaotong (ZT), Yuxi (YX), Lincang (LC), Wenshan (WS), Chuxiong (CX) and Kunming (KM), respectively (Table 3).

Table 3. SNP information of different locations in Yunnan.

Location	LJ	DH	BS	DL	QJ	ZT	YX	LC	WS	CX	KM	Total
SNP locus	26	34	18	27	24	26	26	23	33	26	25	42
Haplotype	25	24	19	30	23	30	31	22	22	22	37	220
Private. hap	13	18	10	20	16	17	21	12	13	15	26	181
Total	32	29	23	36	32	34	39	28	25	26	48	352

The private haplotypes were 13 in Lijiang (LJ), 18 in Dehong (DH), 10 in Baoshan (BS), 20 in Dali (DL), 16 in Qujing (QJ), 17 in Zhaotong (ZT), 21 in Yuxi (YX), 12 in Lincang (LC), 13 in Wenshan (WS), 15 in Chuxiong (CX) and 26 in Kunming (KM), respectively. Private haplotypes are those haplotypes that are found in one particular population sample but are absent in the samples from other populations. Haplotypes H86, H18, H8, H15 and H23 had the maximum incidence among the haplotypes, which added up to 24.5%, and were shared in the populations of Dehong, Yuxi, Lincang, Qujing and other counties (Figure 1; details in Table S3). Among them, H18 and H86 were comparatively widespread haplotypes and shared in six counties. The haplotypes were distributed at altitudes of 906 to 2480 m and were composed of local and introduced varieties and near-isogenic lines (Table S1). We constructed a dendrogram using Metabo-Analyst analysis to infer phylogenetic relationships among the *Pst* populations within the locations. The locations were assembled into six groups. Group 1: Qujing and Wenshan, Group 2: Zhaotong, Group 3: Yuxi and Lincang, Group 4: Chuxion, Lijiang and Baoshan, Group 5: Dali and Group 6: Dehong and Kunming (Figure 1 and Figure S1).

The outcomes of the diversity of haplotypes designated that the maximum *Hd* value was in Zhaotong, 0.993, and the lowest was in Qujing, 0.946 (Table 4). The diversity of nucleotides (*Pi*) fluctuated from 3.91×10^{-3} to 5.98×10^{-3} in the diversified populations. The maximum was in Wenshan and the lowest in Lijiang. The mutation rate was the highest in the Wenshan population (5.98×10^{-3}) and lowest in the Baoshan (3.34×10^{-3}) popu-

lation. The recombination tests revealed that Zhaotong and Kunming had the maximum recombination, with $Rm = 11$, and it was lowest in Lijiang, $Rm = 6$ (Table 4).

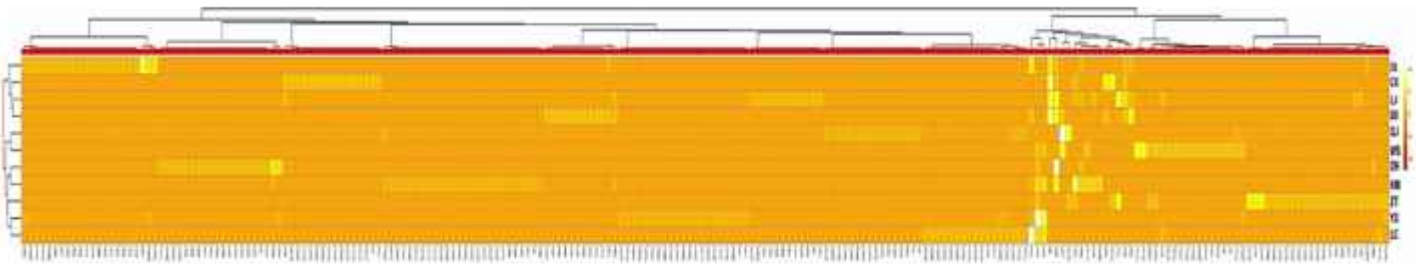

Figure 1. Heat-map visualization and hierarchical clustering analysis with Metabo-Analyst's data annotation tools were constructed based on the different haplotypes for 11 locations. Rows: locations; Columns: haplotypes. Color key indicates haplotypes value, red: lowest, white: highest.

Table 4. Indices of molecular diversity in *Pst* population.

Regions	Haplotype Diversity (*Hd*)	Nucleotide Diversity (*Pi*)	Population Mutation Rate (θ)	Recombination Event (*Rm*)	Tajima's *D*/*p*-Value	Fu's *Fs*/*p*-Value
Lijiang (LJ)	0.98	3.91×10^{-3}	4.42×10^{-3}	6	−0.40263/0.4410	−16.332/0.000 **
Dehong (DH)	0.98	4.22×10^{-3}	4.18×10^{-3}	8	−0.15861/0.4530	−12.02127/0.000 **
Baoshan (BS)	0.980	3.92×10^{-3}	3.34×10^{-3}	7	0.63788/0.7890	−10.793/0.000 **
Dali (DL)	0.989	5.47×10^{-3}	4.45×10^{-3}	7	0.79275/0.8100	−19.035/0.000 **
Qujing (QJ)	0.946	5.95×10^{-3}	4.08×10^{-3}	8	1.61410/0.1095	−7.865/0.000 **
Zhaotong (ZT)	0.993	5.94×10^{-3}	4.35×10^{-3}	11	1.280431/0.9120	−19.836/0.000 **
Yuxi (YX)	0.972	5.05×10^{-3}	4.21×10^{-3}	9	0.680891/0.7940	−19.998/0.000 **
Lincang (LC)	0.968	5.49×10^{-3}	4.04×10^{-3}	7	1.278558/0.9210	−9.420/0.000 **
Wenshan (WS)	0.990	6.48×10^{-3}	5.98×10^{-3}	9	0.31318/0.6420	−10.373/0.000 **
Chuxiong (CX)	0.985	5.93×10^{-3}	4.66×10^{-3}	7	1.002346/0.8780	−10.233/0.000 **
Kunming (KM)	0.987	5.40×10^{-3}	3.85×10^{-3}	11	1.323307/0.9230	−24.891/0.000 **
Total	0.992	6.04×10^{-3}	4.46×10^{-3}	18	0.8238/0.77364	−369.901/0.000 **

** indicates statistically highly significant.

The overall Tajima's D was positive and not significant ($D = 0.8238$, $p = 0.77364$), indicating low levels with low-frequency polymorphisms within locations. The Fu's Fs was highly significant and negative ($Fs = -369.901$, $p = 0.0000$), indicating an excess number of alleles, as would be expected from a recent population expansion or genetic hitchhiking. The individual Tajima's D values for populations of different counties in Yunnan were positive and not significant, except Lijiang and Dehong. Lijiang and Dehong were negative and not significant (Table 4). Fu's Fs, which was considered extra subtle to population demographic expansion [35], displayed different results. Fu's Fs was undesirable and extremely substantial for all counties' populations. The ancestral haplotype (H148) was detected in Yuxi, but all other results indicated frequent pathogen exchange within the locations (Table 4). The sequence of H148 is listed in Supplementary Table S2. The phylogeny tree (UPGMA) of haplotypes indicated that some haplotypes collected from the different locations were grouped, such as H4 from Wenshan, H6 from Lijiang and H161 from Kunming. Some haplotypes were from the same locations but grouped to different clusters, such as H1 and H2 from Qujing and H6 and H8 from Lijang. This indicates that the clustering of haplotypes was not related to geographical sources.

The coefficient of genetic differentiation (Gst) among all populations of Yunnan was 0.01337, while it was 0.0123, 0.00875, 0.01199, 0.00819, 0.02104, 0.00634, 0.00495, 0.00307, 0.0046 and 0.01126 between Yuxi and Lijiang (LJ), Dehong (DH), Baoshan (BS), Dali (DL), Qujing (QJ), Zhaotong (ZT), Kunming (KM), Lincang (LC), Wenshan (WS) and Chuxiong

(CX), indicating a low differentiation among the ten counties, except Qujing (Table 5). The *Gst* was low among all populations, indicating lower heterogeneity.

Table 5. *Gst* and *Nm* between Yuxi and other populations.

Parameter	Lijiang (LJ)	Dehong (DH)	Baoshan (BS)	Dali (DL)	Qujing (QJ)	Zhaotong (ZT)	Kunming (KM)	Lincang (LC)	Wenshan (WS)	Chuxiong (CX)	Among All Populations
Gst	0.0123	0.00875	0.01199	0.00819	0.02104	0.00634	0.00495	0.00307	0.0046	0.01126	0.01337
Nm	20.08	28.33	20.61	30.28	11.63	39.19	50.30	81.17	54.09	21.96	18.45

Among all populations in Yunnan, the gene flow strength, *Nm*, was 18.45, indicating a recurrent pathogen interchange among the provinces (Table 5). While computing the tradeoff of *Pst* between Yuxi (YX) and Lijiang (LJ), Dehong (DH), Baoshan (BS), Dali (DL), Qujing (QJ), Zhaotong (ZT), Kunming, Lincang (LC), Wenshan (WS) and Chuxiong (CX) Provinces, the *Nm* was 20.08, 28.33, 20.61, 30.28, 11.63, 39.19, 50.30, 81.17, 54.09 and 21.96, respectively. *Gst* was the lowest, 0.00307, and *Nm* the highest, 81.17, between Yuxi and Lincang. These results indicated that *Pst* was extremely consistent in Yuxi and Lincang as compared to other provinces, and there was a huge scale of pathogen substitution between the two provinces. The results of AMOVA signposted that modification largely originated from within populations, accounting for 86.20% (Table 6), while it accounted for 9.09% among populations within assemblies and accounted for 4.71% among clusters.

Table 6. AMOVA of *Pst* pathogens during the years from 2004 to 2015.

Source of Variation	df	Sum of Square	Variance Components	Percentage of Variation (%)	*p*-Value
Among groups	5	151.381	0.21516	4.71	0.11926
Among populations within groups	5	82.068	0.41509	9.09	0.00
Within populations	341	1342.014	3.93552	86.20	0.00
Total	351	1575.463	4.56578		

The dendrogram (Figure 2) was prepared from the genetic variation matrix derivatives from 42 SNP loci for 220 haplotypes. In the UPGMA (unweighted pair-group method using arithmetic averages) dendrogram, the haplotypes were assembled into seven groups; however, three of them contained less than five haplotypes. An additional 4 groups were characterized as key groups comprising more than 15 haplotypes. Group 1 contained 79 haplotypes with 92 isolates, where 85.7% of isolates were from Yuxi (17), Kunming (14), Lincang (14), Wenshan (13), Chuxiong (12) and Qujing (8). Group 2 contained 18 haplotypes with 38 isolates, where 89% of isolates were from Qujing (16), Wenshan (6), Zhaotong (6) and Dali (4). Group 4 contained 35 haplotypes with 81 isolates, where 68% of isolates were from Dehong (22), Kunming (20) and Lijiang (12). Group 6 contained 82 haplotypes with 135 haplotypes, where 79% of isolates were from Dali (24), Baoshan (17), Yuxi (17), Zhaotong (13), Kunming (12) and Qujing (8).

Principal component analysis (PCA) was used as a way to deliver a three-dimensional graphical image of the proportional genetic detachments between the populations. It also measures the strength of the diversity between the groups categorized by a dendrogram. The haplotypes grouped by PCA and PLS-DA were carefully arranged with a UPGMA-based tree. In PCA scatterplots, the first two principal components explained 20.8% and 20.2% (Figure 3A), and in PLS-DA, the first three principal components explained 15.7%, 11.7% and 5.8% (Figure 3B) of the entire dissimilarity, respectively. In agreement with the UPGMA-tree, haplotypes were obviously detached by PC1 and 4 distinct groups were found; however, group 1 and group 4 were very close. In PLS-DA, the haplotypes were also found in 4 distinct groups, and group 2 and group 3 were close.

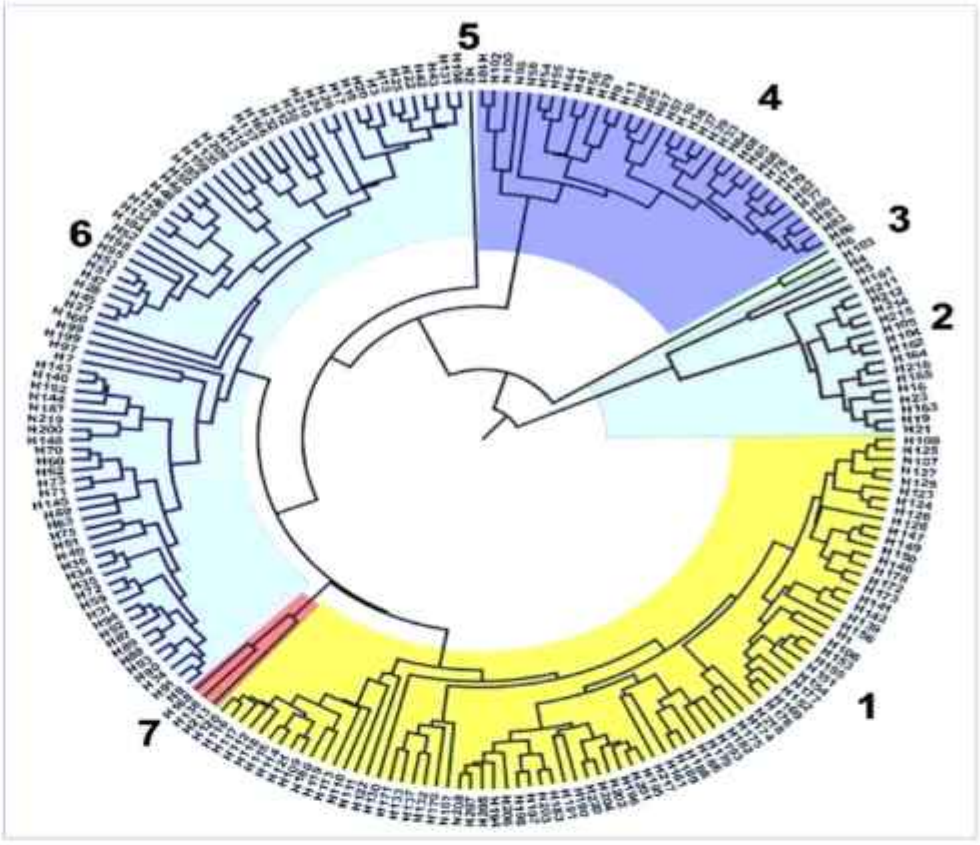

Figure 2. UPGMA dendrogram based on dissimilarity index of 42 SNP loci for 220 haplotypes.

3.2. Population Structure of the Yunnan Pst Isolates

For population genetic structure analysis, Bayesian clustering modeling was performed in the STRUCTURE software using 220 haplotypes, where data were generated by SNP markers. As the clustering model assumes the fundamental reality of K clusters, an Evano test was carried out and generated K = 3 as the maximum log-likelihood (Figure S2; Figure 4).

This means that three was the optimal number of sub-populations, representing that all populations characterize three dissimilar clusters. The analysis of structure according to the geographical origin was performed by setting the range of a possible number of sub-populations (K) from 2 to 10. In STRUCTURE software analysis, concurrences were further characterized as unadulterated or admixture, where concurrences with a score > 0.80 were measured as pure and < 0.80 as an admixture. The population I comprised 17.7% of haplotypes (39 haplotypes), where 24 haplotypes were pure and 15 were admixed. There was a total of 90 isolates in population I, with 20 isolates from Qujing, 10 from Zhaotong, 9 from Lincang, 9 from Kunming, 8 from Dali and 9 from Wenshan, which covered 72% of the population I isolates. Population II comprised of a total of 93 haplotypes with 115 isolates, of which, 12 haplotypes were found admixed. Out of 113 isolates, 25 isolates from Yuxi, 20 isolates from Kunming, 13 isolates from Chuxiong, 12 isolates from Wenshan, 15 isolates from Lincang, 10 isolates from Zhaotong and 9 isolates from Qujing covered 92% of population II isolates. In population III, there was a total of 88 haplotypes with 149 isolates, where 19 were admixed. Out of 149 isolates, 5 locations, Dehong (25), Dali (25), Lijiang (19), Baoshan (19) and Kunming (19), covered 71.8% of the population III isolates (Figure 4). The incidental descent particulars with SNP markers for the strength of character of population structure of the 220 haplotypes are specified in Table S2.

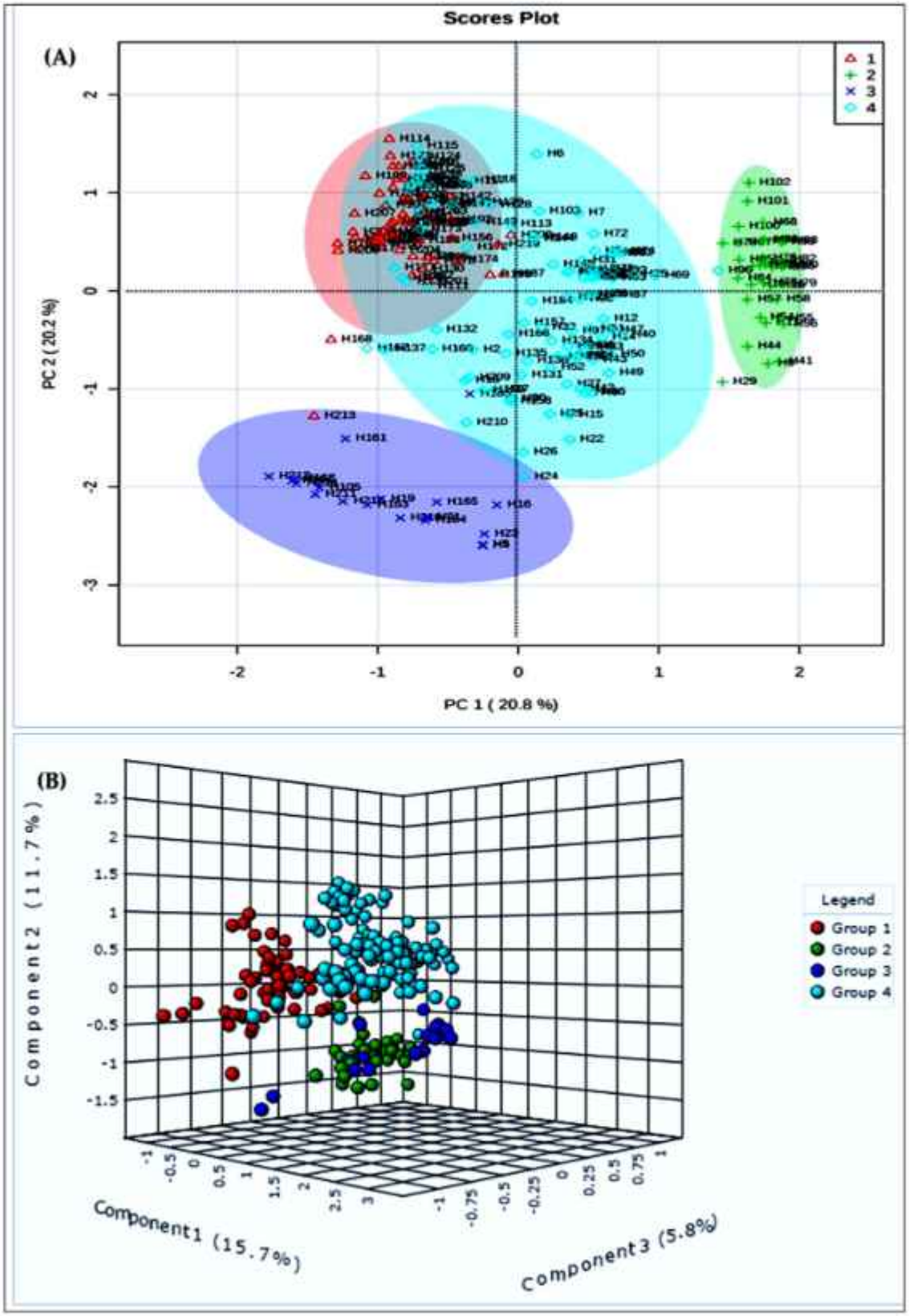

Figure 3. (**A**). Principal component analysis of 220 haplotypes based on SNP markers' data; (**B**) Partial least squares-discriminant analysis of 220 haplotypes based on SNP markers' data.

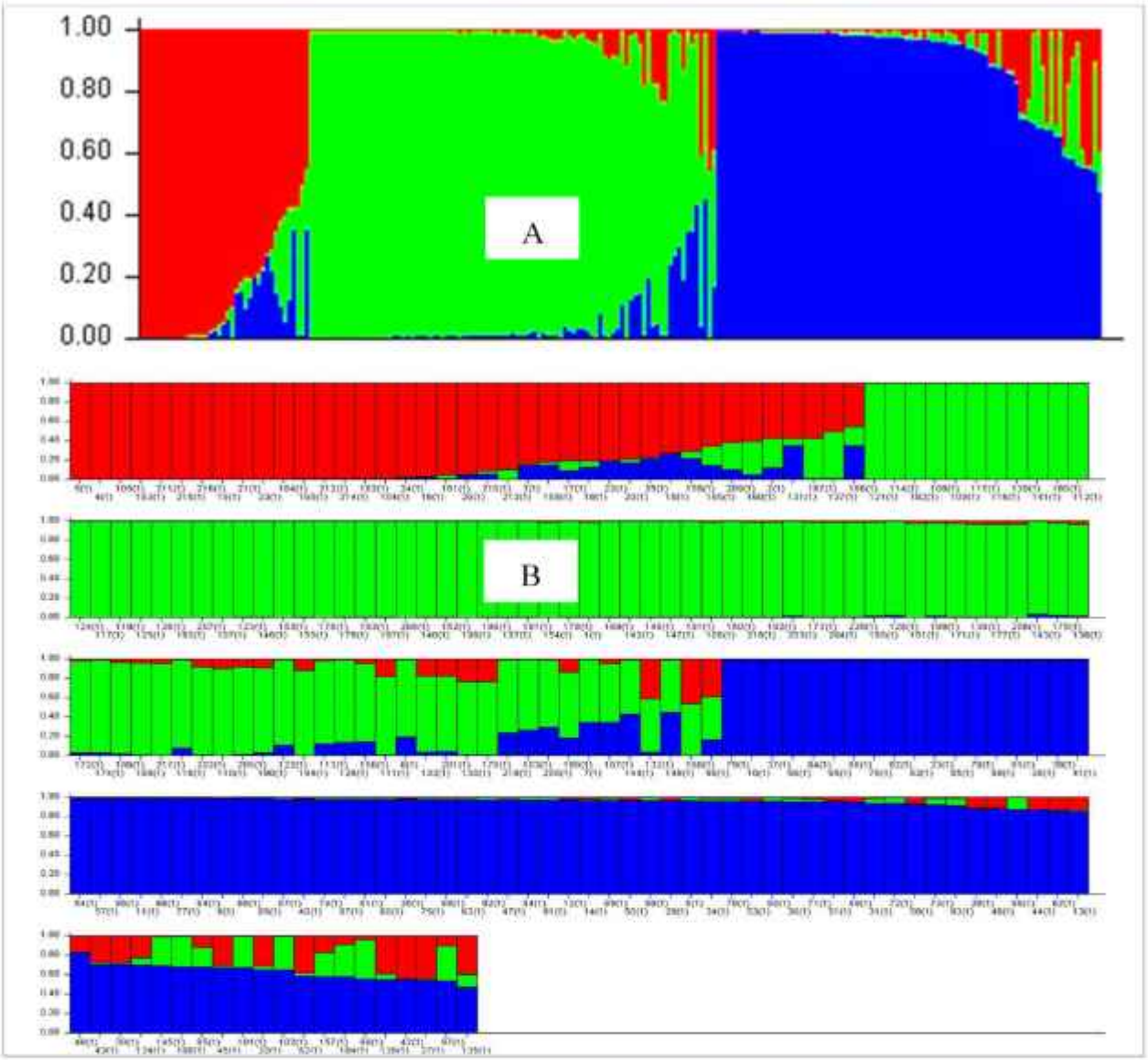

Figure 4. Model-based population structure plot for each isolate with K = 3, using structure with SNP markers' data. Note: Color codes are as follows: Population I: red, Population II: green, Population III: blue. (**A**) The color code of each haplotype corresponds to the description in (**B**).

4. Discussion

4.1. Genetic Diversity and Population Structure

The results from our study demonstrated that the genetic diversity, as well as the mutation rate of the Pst population, is very high in different locations of Yunnan. The strong ultraviolet rays due to the high altitude may be the drivers of mutation. Mutation is the important process for virulence variation [5]. The climate and the agricultural practices provide the year-round growth of wheat or volunteer wheat plants to ensure the emergence of new mutants in Yunnan. Previously, several population structure studies of *Pst* were conducted in China. Zeng and Luo [7] classified 15 stripe rust epidemiological regions in China. Wan et al. [3] and Chen et al. [35] recognized that the southern part of Gansu Province was the main source of *Pst* in China. Hu et al. [36] revealed 13 natural populations in Gansu, Shaanxi, Sichuan and Tibet, and the genetic diversity was highest in Gansu and Sichuan populations. Recently, Ali et al. [37] mentioned that the Himalayas region, such as China, seems more likely the center of origin for stripe rust pathogens worldwide, recognized based on the presence of the maximum levels of

multiplicity, secluded alleles, the structure of the recombinant population, the capability to produce sex-related structures and the self-governing preservation of *Pst* pathogens' center. Li et al. [5] conducted a study in Yunnan, Guizhou, Sichuan and Shaanxi, analyzing their data combined with the trajectory analysis of upper airflow, and presented to the overall story of the pathogen migration and revised that Yunnan is the main source of *Pst* in China. Wang et al. [38] documented that the genetic diversity was reliably high in Gansu and Shaanxi, but low in Sichuan, and there was a closer relationship between Gansu and Sichuan. A total of 1454 multi-locus genotypes (MLGs) were detected in the USA from 2010 to 2017 and observed that populations in the western part were more MLGs and higher divergence than in the eastern part of country [39].

Recombination of *Pst* was first reported in Yunnan by Li et al. [5]. Our study has intensively investigated different counties of Yunnan province. To explore the molecular genetic variation of the wheat stripe rust population, we adopted SNP neutral markers to carry out multi-locus sequence typing analysis on the *Pst* population. The phylogenetic investigation of *Pst* exhibited a structure in which long-distance dispersion and self-regulating progression harmonize. The haplotype diversity of the pathogen population was high ($Hd = 0.992$), and the pathogen diversity was rich in different counties in Yunnan. This is probably due to the diverse geographic environment and complex climate, and the natural conditions differ extremely between regions. It is, therefore, likely that the gene-flow is close to an island model between Yuxi and Lincang, and it is also more like a neighborhood model between Yuxi and Lincang (Table 5 and Figure 1).

Of these 220 detected haplotypes, H18 and H86 had a high frequency, occurred in the 6 areas and represented a stable genotype that was the best adapted to the current environment in the history of pathogen evolution. Other haplotypes that had lower frequencies probably occurred more recently in history or had lower adaptation. The high gene-flow of the pathogen population ($Nm = 18.45$) suggested a frequent exchange between the sub-populations. Considering all locations, the gene-flow was positive and significant, suggesting a higher exchange of *Pst* isolates within locations. The evolution analysis ($D = 0.8238$, $p = 0.77364$ and $Fs = -369.901$, $p = 0.0000$) of population structures suggested that there was a leftover digit of alleles, as would be anticipated from a current population extension or from genomic hitching in the Yunnan population. This is in accordance with the relatively low divergence among populations (9.09% variation). This provides the DNA proof for the long-distance dispersal of stripe rust pathogens [5,32,34] and the possible explanation as to why wheat stripe rust affects Yunnan Province year-round. The high level of genetic diversity also indicated a rapid population growth after a bottleneck [38,40]. This agrees with the results of surveys carried out in the past years [5]. In previous studies, the ancestral haplotype was detected in Yunnan, indicating that the pathogen of *Pst* in Yunnan was older [5]. It was also suggested that Yunnan is the center of origin of *Pst* in China. Using AFLP, the population of *P. striiformis* in Yunnan Province has been detected as a clonal population [41].

There was no significant difference in the diversity of haplotypes and nucleotides within all populations of Yunnan (Table 4). All regions likely play a significant role in supplying new emergences. The mutation rate of the pathogen was the highest ($\theta = 4.46 \times 10^{-3}$) considering all tested populations (locations) in Yunnan. In accordance with the mutation rate, the recombination events were high ($Rm = 18$), suggesting that the new pathotype emerged in Yunnan due to mutation. The virulence metamorphosis is a significant process of virulence variation [38]. Li et al. [5] also observed the high mutation rate ($\theta = 3.81 \times 10^{-3}$) and recombination event ($Rm = 5$) in Yunnan in 2008 and 2011, indicating that the new pathotype emerged earlier in Yunnan. As mentioned above, the strong ultraviolet rays due to the high altitude may be the drivers of mutation. The topography, the elevation between 77 and 6749 m, the climate and the farming practices provide the year-round growth of wheat or volunteer wheat plants and ensure the emergence of new mutations and variations in Yunnan [5]. The element factors related to the formation of such dynamic genetic structure may include mutation, recombination, host selection, the size and composition

of an incurred population, the distance the wind can reach, the hitch-hiking, if there are alternate hosts or not, etc.

The expansion of the *Pst* population was measured by Tajima's *D* tests in different locations in Yunnan, suggesting that the *Pst* population acts as a source of dispersal. Among all populations in Yunnan, the gene-flow strength, *Nm*, was 18.45, indicating a recurrent pathogen interchange among the provinces (Table 5). Li et al. [5] documented that the *Nm* was extensively higher in different locations of Yunnan: 142.60 and 9.47 for the two years studied (2008 and 2011). Chen [40] studied 20 natural populations of *Pst* in the main epidemiological region and found that the *Nm* values of Shaanxi and Gansu ranged from 1.1 to 9.0, and the highest *Nm* values between Gansu and Sichuan varied from 1.1 to 2.5. Hu et al. [36] stated that the populations of Gansu, Shaanxi and Sichuan of China had extensive gene exchange ($Nm > 4$) compared to Tibet. *Gst* was the lowest, 0.00307, and *Nm* the highest, 81.17, between Yuxi and Lincang. These results indicated that *Pst* was extremely consistent in Yuxi and Lincang as compared to other provinces and there was a huge scale of pathogen substitution between the two provinces.

The UPGMA-trees formed by SNP markers were similar to the outcomes of structure. Most of the isolates from groups 2 and 5 were allocated to population I of the structure. Groups 1, 3 and 7 were allocated to population II, and most of the isolates from group 4 were allocated to population III. The isolates from group 6 were distributed among three population groups. Population structure analysis showed that 8% of haplotypes were highly admixed. Using the SNP dataset, most of the genomic diversity has been clarified by the first axis of the PCA investigation. Nevertheless, SNP indicators were established as insignificant for the group of the existing set of segregates to some magnitude, rendering to their topographical positions. All four methods (UPGMA-tree, PCA, PLS-DA and STRUCTURE) were applied in the present study to categorize the *Pst* populations conferring to their races and physical sites, which were recognized as significant, and the furthermost of clutches were co-linear in all methods [6].

The present study also aimed to estimate the genetic relationship among populations of stripe rust pathogens in different locations of Yunnan province. AMOVA based on multilocus sequences revealed a lower genetic differentiation among populations (9.09%), and most of the diversity was due to individuals within the populations (86.20%), indicating that the genetic divergence of the pathogen mainly came from inside the population. All these results indicate that the *Pst* population changes quickly. A lower level of genomic assortment between populations and a higher level within the population in Yunnan Province [5,38]. It is reported that there was geographic divergence for both wheat and stripe rust [5]. Bai et al. [39] also stated that the genetic variation was higher among years in the USA using AMOVA.

4.2. Route of Pst Dispersal in Yunnan

China is constantly under the westerly winds, the Himalayas are located at the border between China and countries west of China, and the wind that may carry urediniospores blows, along the south face of the Himalayas, into southwestern China, e.g., Yunnan, from the countries such as Pakistan, Nepal, etc. [37]. Then, *Pst* evolves locally and independently and disperses further to the northeast and the northern part of China. Yunnan, having all the characteristics of being a center of origin, provides new incursions and new emergences to the northern regions of Yunnan, including Gansu. Li et al. [5] suggested that Yunnan is the primary source of *Pst* in china. Our results also suggested that the high genetic diversity of *Pst* isolates is present in different counties of Yunnan. The exchange rate was also high within the populations. The trajectory of upper airflow is the main indicator for the detection of *Pst* urediniospores' dispersal. The earlier studies performed by Li et al. [5], looking at the trajectories of upper airflow between Yunnan and Gansu during wheat growing seasons from 2005 to 2012, showed that the direction of airflow was from southwest Yunnan to north and northeast Yunnan. In our study, we intensively analyzed the population structure in different locations of Yunnan. Based on the trajectories of

upper airflow and genetic diversity (Figure 1) of *Pst* populations in different locations, we suggested that Dehong, Dali, Lincang and Baoshan are probably the sources of *Pst* in Yunnan (Figure S3; Figure 5).

Figure 5. Route of stripe rust pathogen dispersal in Yunnan.

5. Conclusions

In this study, results from SNPs of 352 segregates showed that 6 housekeeping genes were established to comprise a total of 42 SNP positions. From the concatenated sequences, 220 haplotypes were found, with 5 haplotypes (*viz.*, 'H86′, 'H18′, 'H8′, 'H15′ and 'H23′) comprising over 24.5% of the population. The haplotype diversity, nucleotide diversity, mutation rate and recombination events were 0.992, 6.04×10^{-3}, 4.46×10^{-3} and 18.0 respectively, which revealed the genetic diversity of *Pst* populations among all locations. Four grouping methods, UPGMA-tree, PCA, PLS-DA and STRUCTURE, were applied in the present study to categorize the *Pst* populations, conferring to their races and physical localities, and the majority of the groups were co-linked in all methods for grouping. By using AMOVA, the study recognized about 9.09% of total dissimilarity, and 86.20% within populations. The findings of the study also showed that comparatively, the maximum hereditary assortment resulted from inside the population, but lower genetic discrepancy was found among populations. Furthermore, the genomic data on gene-flow (Nm = 18.45) established that the movement of pathogens occurred among all locations in Yunnan Province. Based on the trajectories of upper airflow and genetic diversity of *Pst* populations in different locations, it is suggested that Dehong, Dali, Lincang and Baoshan are probably the sources of *Pst* in Yunnan.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/plants10081735/s1, Figure S1: The best number of groups among locations estimated by Evano test methods; Figure S2: The determination of the best number of clusters among 220 haplotypes by Evano test methods, Figure S3: Distinct groups among 11 locations; Table S1: Isolates collected from different counties of Yunnan and Sichuan Provinces; Table S2: Haplotypes and their SNP loci of *Pst* population; Table S3: Number of haplotypes and their distribution among the different locations.

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Abbreviations

AFLP	Amplified fragment length polymorphism
AMOVA	Analysis of molecular variance
CDC2	Cyclin-dependent kinase 2
CTAB	cetyltrimethylammonium bromide
EDTA	Ethylenediamine tetra-acetic acid
EF-1	Elongation factor
HSP	heat shock protein 90 kDa
MAPK	Map kinase 1
MCMC	Markov chain Monte Carlo (MCMC) methods comprise a class of algorithms for sampling from a probability distribution
MEGA	Molecular Evolutionary Genetics Analysis
PCA	Principal component analysis
PLS-DA	Partial Least-Squares Discriminant Analysis
Pst	*Puccinia striiformis* f. sp. *tritici*
RAPD	Random Amplification of Polymorphic DNA
SNPs	Single nucleotide polymorphisms
SSR	Simple Sequence Repeats
UBA	ubiquitin-activating enzyme E1
UBC	ubiquitin-conjugating enzyme E2
UPGMA	Unweighted Pair Group Method with Arithmetic Mean

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Functional Genomics in Crop Improvement

Jamilur Rahman

1. Introduction

Functional genomics focuses on understanding the functions and interactions of genes and their roles in biological processes. It provides insights into how genes contribute to the development of specific traits and how they respond to environmental factors. In the context of crop breeding, functional genomics aids in identifying gene functions and pathways that contribute to desirable traits, such as yield improvement, stress resistance, and nutrient content. With advancements in molecular biology and computational technologies, functional genomics has become a cornerstone of modern crop improvement strategies.

Crop breeders face increasing challenges such as climate change, growing population demands, and the need for sustainable agricultural practices. Traditional breeding methods, while effective, often require lengthy cycles and extensive field trials. Functional genomics addresses these challenges by offering precise tools to identify and manipulate genes of interest. By integrating data from various "omics" technologies, functional genomics accelerates the development of improved crop varieties tailored to specific environmental and consumer needs. This article explores the applications, tools, and future prospects of functional genomics in crop breeding, highlighting its transformative potential in addressing global food security and agricultural sustainability.

2. Key concept

Functional genomics is a specialized branch of genomics that seeks to understand the dynamic roles of genes and their regulatory networks within an organism. Unlike structural genomics, which focuses on the static architecture of genomes, functional genomics explores how genetic information is translated into biological functions and phenotypes. This approach involves studying the expression, interaction, and regulation of genes in various contexts, such as development, stress responses, and environmental interactions. One of the primary goals of functional genomics is to bridge the gap between genotype and phenotype. By identifying how specific genes influence observable traits, researchers can develop predictive models that link DNA sequences to functional outcomes. Functional genomics also aims to elucidate gene networks and pathways, providing insights into complex biological systems. Ultimately, the field strives to enable precision breeding by targeting genes that confer desirable traits, such as enhanced yield, pest resistance, or stress tolerance, thus revolutionizing agricultural practices.

Functional genomics also plays a pivotal role in understanding epigenetic modifications, transcriptional regulation, and post-translational changes that affect gene function. These insights enable researchers to tackle intricate biological questions, such as how genes adapt to environmental changes or how multiple genes collectively contribute to a single phenotype. By leveraging advanced tools and technologies, functional genomics lays the foundation for transformative breakthroughs in crop improvement.

3. Importance in Crop Breeding

Functional genomics offers transformative potential in modern crop breeding by addressing limitations associated with traditional methods. Its contributions can be categorized as follows:

- **Identification of Target Genes**: Functional genomics empowers breeders to identify specific genes responsible for agronomically important traits. By linking gene function to phenotypic outcomes, researchers can target key genetic factors that drive improvements in yield, quality, and resilience. For instance, genes involved in drought tolerance, such as DREB transcription factors, have been identified through functional genomics studies.
- **Acceleration of Breeding Programs**: Traditional breeding relies on phenotypic selection, which is often time-consuming and resource-intensive. Functional genomics reduces the dependency on trial-and-error approaches by providing molecular markers and genomic data that facilitate faster selection of desirable traits.
- **Development of Climate-Resilient Crops:** With climate change posing significant challenges to agriculture, functional genomics helps in understanding how genes contribute to stress tolerance. By identifying genes associated with heat, salinity, and water-deficit tolerance, breeders can develop crops capable of thriving under adverse conditions.
- **Reduction of Agricultural Inputs:** Functional genomics enables the creation of crops that require fewer resources, such as water, fertilizers, and pesticides. For example, nitrogen-use efficient crops can significantly reduce the environmental impact of agriculture while maintaining productivity.

By integrating functional genomics into breeding pipelines, researchers can achieve more sustainable, efficient, and precise crop improvement strategies. This integration not only addresses current agricultural challenges but also lays the groundwork for future innovations in global food security and environmental sustainability.

4. Techniques and Tools in Functional Genomics

4.1 Transcriptomics

Transcriptomics involves studying RNA molecules to understand gene expression patterns. It provides insights into how genes are activated or repressed under various conditions.

Key Tools: RNA sequencing (RNA-seq), microarrays.

Applications:

- Identifying genes and pathways involved in stress responses (e.g., drought or heat).
- Understanding temporal gene expression during crop development.

4.2 Proteomics

Proteomics examines the full set of proteins produced by a crop. Proteins are the functional molecules in cells, and their abundance and interactions are closely linked to phenotypes.
Key Tools: Mass spectrometry, 2D gel electrophoresis.

Applications:

- Identifying stress-responsive proteins and their roles in tolerance mechanisms.
- Linking protein expression patterns to agronomically important traits.

4.3 Metabolomics

Metabolomics is the study of small molecules (metabolites) produced by plants. Metabolites play vital roles in growth, stress responses, and nutrient composition.

Key Tools: Gas chromatography-mass spectrometry (GC-MS), Liquid chromatography-mass spectrometry (LC-MS), Nuclear Magnetic Resonance (NMR).

Applications:

- Enhancing flavor and aroma through manipulation of metabolic pathways.
- Engineering crops with improved nutritional content, such as increased vitamins and minerals.

4.4 Genome Editing

Genome editing allows precise modifications of the DNA sequence to achieve specific traits.

Key Tools: CRISPR-Cas9, TALENs (Transcription Activator-Like Effector Nucleases), ZFNs (Zinc Finger Nucleases).

Applications:

- Developing disease-resistant crops by knocking out susceptibility genes.
- Improving yield and abiotic stress tolerance through targeted gene insertion or deletion.

4.5 Functional Annotation of Genes

Functional annotation assigns roles to genes based on experimental data and comparative analyses with well-characterized organisms.

Methods: Gene knockout studies, overexpression experiments, comparative genomics.

Applications:

- Elucidating the role of unknown genes in critical traits.
- Identifying candidate genes for marker-assisted breeding programs.

5. Applications in Crop Breeding

5.1 Improving Stress Tolerance

Crop plants face various abiotic stresses, including drought, heat, and salinity. Functional genomics helps breeders:

- Discover and manipulate genes associated with stress resilience.

- The DREB (Dehydration-Responsive Element-Binding) transcription factors that enhance drought tolerance in multiple crops. In India, the incorporation of DREB genes into rice varieties has led to improved drought tolerance and better yields in arid regions, significantly benefiting smallholder farmers.

5.2 Enhancing Disease Resistance

Plant diseases caused by pathogens like fungi, bacteria, and viruses lead to significant yield losses. Functional genomics:

- Identifies resistance (R) genes and their associated pathways.
- Blight-resistant rice developed using Xa21 has been deployed in Southeast Asia, reducing crop losses by over 50% in heavily affected regions.

5.3 Boosting Nutritional Quality

Functional genomics supports biofortification the enhancement of crops with essential nutrients:

- Golden Rice with increased Vitamin A content through the introduction of carotenoid biosynthetic pathway genes. Golden Rice has been a pivotal innovation in addressing Vitamin A deficiency in populations across Asia and Africa, reducing malnutrition-related blindness and diseases in children.
- Iron-enriched wheat varieties by manipulating iron transporter genes.

5.4 Accelerating Breeding Cycles

The integration of genomic data with traditional breeding practices:

- Improves marker-assisted selection (MAS) and genomic selection (GS) efficiency.
- Shortens breeding cycles by predicting phenotypes based on genotype data. In maize breeding programs across the United States, genomic selection has reduced the development time for new hybrid varieties by nearly 30%, increasing adaptability to changing climates.

6. Challenges and Limitations

6.1 Complexity of Gene Networks

Gene interactions and regulatory mechanisms are intricate and context-dependent. Genes rarely act independently but interact with one another in complex networks. This complexity makes it difficult to predict the outcomes of genetic modifications accurately. Variations in gene expression and the presence of epistatic effects add another layer of challenge in functional genomics studies.

6.2 High Costs and Technical Expertise

Advanced tools and technologies, such as CRISPR, RNA sequencing, and metabolomics platforms, require substantial financial investments. Many research institutions, especially in

developing countries, may lack the infrastructure and skilled personnel needed to implement these technologies effectively. This cost barrier limits the widespread application of functional genomics in crop breeding.

6.3 Ethical and Regulatory Issues

Public concerns about genetically modified organisms (GMOs) and genome editing techniques often lead to stringent regulations and slow the adoption of improved crop varieties. Ethical debates surrounding the use of gene editing technologies also create uncertainty, potentially delaying research progress and practical applications.

6.4 Environmental Variability

Gene function and expression can vary significantly across different environmental conditions. Traits that show promise in controlled laboratory environments may not perform consistently under field conditions, making the translation of research findings to practical crop breeding challenging. Extensive field trials are often required to validate the effectiveness of genomic interventions.

7. Future Directions

7.1 Integration of Multi-Omics Data

The future of functional genomics lies in integrating genomics, transcriptomics, proteomics, and metabolomics data to gain holistic insights into crop systems. This multi-omics approach will enable a more comprehensive understanding of complex traits and provide breeders with detailed molecular blueprints to guide their strategies.

7.2 Advances in Genome Editing

Ongoing advancements in genome editing technologies, such as CRISPR-Cas systems, are expected to improve their precision and reduce off-target effects. These next-generation tools will make it easier to develop crops with specific traits, such as disease resistance, stress tolerance, and higher nutritional content, with minimal unintended consequences.

7.3 AI and Machine Learning in Genomics

Artificial intelligence (AI) and machine learning (ML) have immense potential in functional genomics. These technologies can analyze vast datasets, identify gene functions, predict interactions, and simulate breeding outcomes. The adoption of AI and ML will streamline the discovery of candidate genes and optimize breeding programs for maximum efficiency.

7.4 Sustainable Agriculture

Functional genomics will play a crucial role in developing crops that support sustainable agricultural practices. By breeding nitrogen-use-efficient crops, for instance, farmers can reduce their reliance on synthetic fertilizers, minimizing environmental impact. Similarly, drought-tolerant and pest-resistant crops will contribute to resource conservation and lower agricultural inputs.

8. Conclusion

Functional genomics has revolutionized the field of crop breeding by unraveling the genetic basis of traits and enabling precise genetic interventions. By integrating functional genomics with traditional breeding methods, researchers and breeders can address the challenges of food security, climate change, and sustainable agriculture. As tools and techniques continue to evolve, functional genomics will play an increasingly vital role in shaping the future of crop improvement.

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Epigenetics: DNA Methylation, Histone Modifications, and Their Impact on Trait Inheritance

Jamilur Rahman

1. Introduction

Epigenetics refers to heritable changes in gene expression that do not involve changes to the underlying DNA sequence. These changes are crucial in regulating gene activity and have profound implications for development, health, and disease. While genetic sequences serve as the blueprint for life, epigenetics provides the dynamic regulation necessary for fine-tuning gene activity in response to developmental cues and environmental signals. Through processes like DNA methylation, histone modifications, and the actions of non-coding RNAs, epigenetics orchestrates when and where genes are turned on or off, ensuring proper growth, development, and adaptability.

In both plants and animals, epigenetic mechanisms influence not only individual development but also trait inheritance across generations. They enable organisms to rapidly respond to environmental changes without altering the genetic code. This phenomenon plays a significant role in evolution, disease susceptibility, and agricultural productivity. For instance, stress-induced epigenetic modifications in plants can enhance resilience and be passed on to subsequent generations, shaping crop performance. Thus, understanding epigenetics is essential for advancing fields like medicine, agriculture, and evolutionary biology. This article explores the principles of epigenetics, focusing on the roles of DNA methylation and histone modifications, and examines their impact on trait inheritance and their potential applications in science and agriculture.

2. Key Concepts

Epigenetics encompasses a range of molecular processes that regulate gene activity without altering the underlying DNA sequence. The primary mechanisms include DNA methylation, histone modifications, and the involvement of non-coding RNAs. These processes work in concert to modulate chromatin structure and gene accessibility, influencing whether a gene is actively transcribed or remains silent. For instance, DNA methylation often represses gene expression by preventing transcription factors from accessing DNA, while histone acetylation generally promotes gene activation by loosening chromatin structure. Together, these epigenetic marks establish and maintain patterns of gene expression that are essential for cellular differentiation, development, and adaptation to environmental stimuli.

A key goal of epigenetics is to bridge the gap between genotype and phenotype by elucidating how external factors can induce heritable changes in gene expression. Epigenetic modifications act as a molecular memory, capturing environmental influences such as stress, temperature changes, and nutrient availability, and encoding them into stable marks that may persist through cell divisions or even across generations. This capability offers a framework for understanding

phenomena like transgenerational inheritance, hybrid vigor, and adaptive evolution. Another critical goal is to explore the implications of epigenetic regulation for health, agriculture, and evolution. In agriculture, for example, leveraging epigenetic mechanisms can improve crop resilience, enhance productivity, and reduce the environmental impact of farming practices. In health sciences, understanding epigenetic mis regulation provides insights into diseases like cancer and opens avenues for targeted therapies. By studying epigenetic mechanisms, scientists aim to unlock strategies for sustainable development, personalized medicine, and climate-resilient agriculture, ultimately contributing to a better understanding of biological complexity and trait inheritance.

3. Mechanisms of Epigenetic Regulation

3.1 DNA Methylation

DNA methylation involves the addition of a methyl group to the 5th carbon of cytosine residues, primarily in CpG dinucleotides in animals and more diverse contexts in plants. This process is a key regulatory mechanism that influences chromatin structure and gene expression.

- **Key Features:**
  - DNA methylation is often associated with gene silencing and transcriptional repression.
  - It plays a crucial role in regulating transposable elements (TEs) and maintaining genome stability.
  - Essential developmental processes, such as genomic imprinting, X-chromosome inactivation, and stress responses, depend on DNA methylation.
- **Key Enzymes:**
  - DNA methyltransferases (DNMTs), including DNMT1 for maintenance methylation and DNMT3 for de novo methylation, catalyze the addition of methyl groups to DNA.
  - Demethylases, such as the TET family proteins, remove methyl groups to reverse methylation.
- **Applications in Trait Inheritance:**
  - In plants, methylation patterns regulate important traits such as flowering time (*FWA* gene in Arabidopsis) and pathogen resistance.
  - Aberrant methylation in mammals is linked to developmental disorders and diseases like cancer.

3.2 Histone Modifications

Histones are proteins that package DNA into chromatin, and their post-translational modifications (PTMs) significantly influence chromatin structure and gene activity. PTMs regulate accessibility of transcriptional machinery to DNA, thus controlling gene expression.

- **Common Modifications:**
  - **Acetylation:** Associated with gene activation by loosening chromatin and increasing transcription factor accessibility.
  - **Methylation:** Can lead to activation or repression, depending on the specific histone residue and methylation state (e.g., H3K4me3 promotes activation, while H3K9me3 is linked to repression).
  - Other modifications include phosphorylation, ubiquitination, and sumoylation, each contributing to chromatin dynamics.
- **Key Enzymes:**
  - Histone acetyltransferases (HATs) and histone deacetylases (HDACs) regulate acetylation levels.
  - Histone methyltransferases (HMTs) and demethylases modulate methylation patterns.
- **Applications in Trait Inheritance:**
  - In plants, histone modifications regulate genes associated with stress responses, such as heat-shock proteins during temperature stress.
  - Specific histone marks are critical for developmental plasticity and hybrid vigor in crops.

3.3 Non coding RNAs (ncRNAs)

Non-coding RNAs play a central role in directing epigenetic modifications by guiding chromatin-modifying complexes to target genomic regions.

- **Examples:**
  - **Small interfering RNAs (siRNAs):** Involved in RNA-directed DNA methylation (RdDM) in plants, silencing transposable elements and repetitive sequences.
  - **Long non-coding RNAs (lncRNAs):** Act as scaffolds or decoys, modulating chromatin accessibility and gene expression.

4. Applications of Epigenetics in Trait Inheritance

4.1 Adaptation to Environmental Stress

Epigenetic modifications allow organisms to rapidly adapt to environmental challenges by altering gene expression without changes to DNA sequence.

- **Examples:**
  - In plants, stress-induced DNA methylation leads to increased drought tolerance.
  - Histone modifications activate protective genes during heat or cold stress.
- **Realtime implementation:** In maize, drought-induced DNA methylation patterns correlate with improved yield stability under water-deficient conditions, providing a template for breeding resilient crops.

4.2 Crop Improvement

Harnessing epigenetic mechanisms can enhance crop traits, including yield, resilience, and nutritional quality.

- **Examples:**
  - Altering methylation patterns can regulate flowering time, optimize fruit ripening, and improve resistance to diseases.
  - Epigenome editing tools, such as CRISPR-dCas9, enable precise modification of epigenetic marks to achieve desired traits.
- **Realtime implementation:** In tomatoes, targeted epigenetic reprogramming extended fruit shelf life by modulating methylation patterns of ripening-related genes.

4.3 Transgenerational Inheritance

Epigenetic changes induced by environmental factors can be stably inherited across generations, impacting trait inheritance.

- **Examples:**
  - Stress-induced DNA methylation in Arabidopsis persists for multiple generations.
  - Nutritional stress in mammals affects metabolic traits in subsequent offspring.
- **Realtime implementation:** In rice, heat stress-induced epigenetic modifications improved tolerance in the next generation, demonstrating the potential for breeding climate-resilient crops.

4.4 Epigenetics in Disease Resistance

Epigenetic regulation influences plant immune responses and pathogen resistance by modulating the expression of defense-related genes.

- **Examples:**
  - DNA methylation represses transposable elements that can activate during pathogen attack.
  - Histone acetylation enhances the expression of immune-responsive genes.

- **Realtime implementation:** In barley, histone acetylation promoted resistance to powdery mildew by upregulating defense-related pathways, highlighting the role of epigenetics in crop protection.

5. Challenges and Limitations

5.1 Epigenetic Plasticity

Epigenetic changes are reversible and highly dynamic, influenced by environmental and developmental contexts. This plasticity complicates efforts to stabilize desirable traits over generations.

5.2 Technical Challenges

Advanced techniques like bisulfite sequencing, ChIP-seq, and epigenome editing require sophisticated technology and expertise. Distinguishing causative epigenetic changes from correlative marks remains a significant challenge.

5.3 Inheritance Complexity

Not all epigenetic marks are stably inherited, as environmental reprogramming can override epigenetic patterns. Long-term studies are required to confirm the heritability of specific epigenetic traits.

6. Future Directions

6.1 Epigenome Editing

Future research will focus on refining tools like CRISPR-dCas9 for precise and stable modification of epigenetic marks, enabling the development of crops with enhanced traits such as yield and stress tolerance.

6.2 Integration with Multi-Omics

Combining epigenomics with genomics, transcriptomics, proteomics, and metabolomics will provide a holistic understanding of complex traits, aiding in more targeted breeding strategies.

6.3 Epigenetics in Personalized Agriculture

Customizing crops based on their epigenetic profiles to suit specific environmental conditions and farming practices will become a key focus, enabling sustainable agriculture.

6.4 Transgenerational Studies

Long-term studies will investigate the stability and evolutionary impact of epigenetic modifications, deepening our understanding of phenotypic diversity and adaptation.

7. Conclusion

Epigenetics adds a crucial layer of complexity to our understanding of gene regulation and trait inheritance. DNA methylation, histone modifications, and ncRNAs play pivotal roles in

controlling gene expression, shaping phenotypic diversity, and enabling organisms to adapt to environmental changes. With advances in epigenetic technologies, researchers can harness these mechanisms to improve crops, enhance stress resilience, and explore new frontiers in evolutionary biology and agriculture.

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Transcriptomics, Proteomics, and Metabolomics for Trait Discovery

Jamilur Rahman

1. Introduction

Modern biological research integrates multiple "omics" technologies to unravel the molecular mechanisms underlying traits. Transcriptomics, proteomics, and metabolomics are pivotal fields that systematically study RNA transcripts, proteins, and metabolites, respectively. These approaches enable researchers to bridge the gap between genotype and phenotype, offering comprehensive insights into the biological pathways influencing traits in plants, animals, and microbes.

The integration of these "omics" technologies has transformed trait discovery, enabling the identification of genes, proteins, and metabolites linked to stress tolerance, disease resistance, yield improvement, and nutritional quality. By understanding how these molecular components interact, scientists can uncover novel targets for crop improvement and precision agriculture. This lecture delves into the principles, methods, and applications of transcriptomics, proteomics, and metabolomics in trait discovery.

2. Key Concepts

2.1 Transcriptomics

Transcriptomics focuses on the study of RNA transcripts (mRNA, rRNA, tRNA, and non-coding RNA) to understand gene expression dynamics under specific conditions. This field provides critical information about how genes are activated or repressed, revealing molecular responses to environmental stimuli or developmental cues.

- **Key Tools and Methods:**
  - RNA sequencing (RNA-seq): Provides high-throughput analysis of the transcriptome, offering quantitative and qualitative data on gene expression.
  - Microarrays: Analyze the expression of predefined sets of genes using hybridization-based techniques.
- **Applications in Trait Discovery:**
  - Identifying stress-responsive genes (e.g., drought, salinity, or heat).
  - Discovering regulatory networks and transcription factors controlling key traits.
  - Example: Transcriptomic analysis in maize identified *ZmDREB2A*, a gene involved in drought tolerance.

2.2 Proteomics

Proteomics is the large-scale study of proteins, including their structure, function, and interactions. Proteins are the functional molecules of cells and directly influence phenotypic traits. Studying proteomes helps link gene expression to functional outcomes.

- **Key Tools and Methods:**
  - Mass spectrometry (MS): Identifies and quantifies proteins with high precision.
  - 2D gel electrophoresis: Separates proteins based on their isoelectric point and molecular weight.
  - Protein interaction assays: Identify physical or functional interactions between proteins.
- **Applications in Trait Discovery:**
  - Identifying proteins involved in stress tolerance or disease resistance.
  - Mapping post-translational modifications (e.g., phosphorylation) critical for protein activity.
  - Example: Proteomic studies in rice uncovered proteins linked to blast disease resistance.

2.3 Metabolomics

Metabolomics investigates the complete set of small molecules (metabolites) within a biological system. Metabolites are the end products of cellular processes and provide a snapshot of an organism's metabolic state.

- **Key Tools and Methods:**
  - Gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS): Detect and quantify metabolites with high sensitivity.
  - Nuclear Magnetic Resonance (NMR) spectroscopy: Provides structural information about metabolites.
- **Applications in Trait Discovery:**
  - Identifying metabolic pathways linked to traits such as flavor, aroma, and nutritional quality.
  - Uncovering biomarkers for stress responses or disease susceptibility.
  - Example: Metabolomic profiling in tomato identified metabolites associated with fruit ripening and shelf life.

3. Applications of Multi-Omics Integration

The integration of transcriptomics, proteomics, and metabolomics offers a holistic view of biological processes, enabling the discovery of complex traits. Combining these approaches helps identify molecular mechanisms, validate targets, and bridge the gap between genotype and phenotype.

3.1 Stress Tolerance

Omics technologies have been instrumental in identifying key components involved in stress tolerance. Transcriptomics identifies genes activated under stress, such as those encoding heat-shock proteins or enzymes involved in osmolyte biosynthesis. Proteomics complements this by uncovering post-translational modifications in proteins that regulate stress responses. Metabolomics reveals metabolites such as proline and trehalose, which act as osmoprotectants under drought or salinity stress.

Realtime implementation: In rice, multi-omics analysis revealed the involvement of proline metabolism and its regulation at the transcript, protein, and metabolite levels, contributing to enhanced drought tolerance.

3.2 Nutritional Quality

Nutritional enhancement through omics involves identifying and modifying pathways responsible for the synthesis of essential nutrients. Transcriptomics highlights genes in nutrient biosynthesis pathways, while proteomics maps the enzymes catalyzing these processes. Metabolomics quantifies nutrient levels and metabolites that influence bioavailability and flavor.

Realtime implementation: In wheat, multi-omics integration identified genes and pathways responsible for higher iron and zinc content, aiding in biofortification efforts.

3.3 Disease Resistance

Omics approaches dissect the molecular mechanisms underlying disease resistance. Transcriptomics identifies upregulated defense-related genes during pathogen attacks, proteomics maps changes in defense proteins, and metabolomics reveals the production of phytoalexins and other antimicrobial compounds.

Real implementation: In soybean, multi-omics revealed the role of specific secondary metabolites and defense proteins in resistance to root rot, paving the way for developing resistant varieties.

3.4 Crop Improvement

Omics-guided breeding integrates molecular insights to develop high-yielding, stress-resistant, and disease-tolerant varieties. The combined analysis of transcripts, proteins, and metabolites enables the identification of key molecular hubs that drive yield and resilience.

Real implementation: In maize, omics analysis under drought stress conditions identified molecular pathways contributing to increased kernel weight and overall yield stability.

4. Challenges and Limitations

4.1 Complexity of Data Analysis

The sheer volume and complexity of data generated by transcriptomics, proteomics, and metabolomics require advanced computational tools for integration and analysis. Data integration across omics layers is challenging due to differences in data types, scales, and biological contexts. Moreover, distinguishing causative molecular changes from correlative ones remains a significant hurdle.

4.2 Cost and Technical Expertise

High-throughput omics technologies, including RNA-seq, mass spectrometry, and metabolomic profiling, are expensive to implement. They require sophisticated instrumentation, skilled personnel, and computational resources, making them less accessible to resource-constrained laboratories.

4.3 Environmental Variability

Omics data are highly influenced by environmental factors, which can introduce variability and complicate reproducibility. Field-level validation of traits discovered in controlled environments is essential but challenging, as environmental conditions in the field are dynamic and unpredictable.

5. Future Directions

5.1 Advances in Multi-Omics Platforms

Future research will focus on developing integrated platforms capable of simultaneously analyzing transcripts, proteins, and metabolites. These platforms will facilitate seamless data acquisition and integration, reducing complexity and enhancing biological insights. Improved algorithms for multi-omics data analysis will allow for more accurate modeling of complex traits.

5.2 AI and Machine Learning

Artificial intelligence (AI) and machine learning (ML) technologies are set to revolutionize omics data analysis. These tools can efficiently handle large datasets, identify patterns, and predict trait-linked molecular targets. Machine learning algorithms will also improve the identification of key regulators and interactions across omics layers.

5.3 Precision Agriculture

Omics-guided precision agriculture involves tailoring crop varieties to specific environmental conditions or agricultural practices. By combining omics data with precision phenotyping, researchers can design crops optimized for resource efficiency, resilience, and productivity.

5.4 Expansion to Non-Model Organisms

Expanding omics research to underutilized crops, wild relatives, and non-model organisms offers untapped potential for discovering novel traits and pathways. These studies will provide valuable genetic and metabolic diversity to support crop improvement and food security.

6.Conclusion

Transcriptomics, proteomics, and metabolomics provide complementary insights into the molecular basis of traits, enabling a comprehensive understanding of biological systems. Their integration has revolutionized trait discovery, offering novel targets for crop improvement, stress tolerance, and disease resistance. As technologies advance, multi-omics approaches will continue to drive innovations in agriculture, enhancing productivity, sustainability, and resilience to global challenges.

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DEVELOPMENT OF ABIOTIC STRESS TOLERANT CROP VARIETY THROUGH STRESS BREEDING

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Abiotic stresses, including drought, salinity, extreme temperatures, and nutrient deficiencies, are major constraints to crop production and productivity worldwide. These stresses adversely affect plant growth, development, and yield, leading to significant economic losses and threatening global food security. With the increasing world population and the impact of climate change, the development of crop cultivars with enhanced tolerance to abiotic stresses has become a pressing need. Breeding for abiotic stress tolerance is a complex process that requires a comprehensive understanding of the physiological, biochemical, and molecular mechanisms underlying stress responses in plants.

Abiotic stress

Abiotic stress is the negative impact of non-living factors on plant growth and productivity in a specific environment. Abiotic stresses are:

- Drought
- Salinity
- Temperature
- Heavy metal
- Nutrient deficiency

Major Abiotic Stress: Drought

- Drought stress involves timing, duration and intensity of the water deficit.
- Impact on yield depends on duration, crop growth stage, crop species or variety, soil type and management practices.

Drought Tolerance

Drought tolerance is a plant's ability to survive in water deficit condition. It's a complex trait that involves a combination of morphological, physiological, and biochemical adaptations.

Basis of Drought Tolerance

- Earliness
- Reduced tillering
- Leaf characters: leaf rolling, leaf folding, leaf shading
- Reduced leaf area: Narrow leaf, change in leaf angle
- Hairiness: Presence of hairs on leaf and other parts
- Wax content
- Awns
- Root system (Rooting depth and intensity)

Major abiotic stress: Salinity

- There are 954 million hectares of salt affected land
  - 69% is in Asia and Australia
  -half of cultivated land in India is threatened by salinity problems
  - about 1.056 million hectares of land are affected by salinity in Bangladesh
- Salinization of arable land will result in 30% land loss in the next 25 years and 50% by 2050
- Salinization is due to increased irrigation

Salinity Tolerance

The ability of plants to prevent, reduce or overcome injurious effects of soluble salts present in their root zone.

Basis of Salinity Tolerance

It's a complex process that involves multiple physiological, biochemical, and molecular mechanisms. Here are some of the ways plants tolerate salt stress:

- Ion homeostasis
- Osmolytes
- Antioxidant defense system
- Gene expression
- Microbes
- Salt exclusion
- Tocopherols
- Superoxide dismutase (SOD)
- Ascorbate peroxidase (APX)

Major abiotic stress: Heat

- Every 1ºC increase in temperature between 30ºC and 40ºC during flowering reduces grain of different crops by approximately 10%
- As temperature increases above 30ºC, crop yield decreases because
  - plants use more energy for respiration
  - crops mature earlier and have less time for producing yield
  - high temperature causes thermal damage to vital plant tissues

Heat stress

- The typical response to heat stress is a decrease in the synthesis of normal proteins, accompanied by an accelerated transcription and translation of new proteins known as heat shock proteins (HSPs)
- May arise in leaves
  - when transpiration is insufficient
  - when stomata are partially or fully closed

- May arise in germinating seedlings
  - when the soil is warmed by the sun
- May arise in organs with reduced capacity for transpiration e.g. fruits

Effects of Abiotic Stresses

Abiotic stresses affect the plant's morphological, biochemical, and physiological mechanisms, resulting in

- reduced germination
- reduced growth
- reduced photosynthesis rates
- modifications in gene expression
- disruptions of hormone and enzyme activities
- increased oxidative stress and
- declines in yields

Sources of Abiotic Stress Resistance

There are several sources of abiotic stress tolerance in crop plants-

- Tolerance may exist in land races, wild relatives, high yielding varieties.
- Initial breeding materials and advance breeding materials. Landraces from dry habitats have been used successfully in breeding toward developing open pollinated varieties or hybrids for water limited environments.
- Wild species and progenitors of cultivated crops were always on the agenda as possible donors for abiotic stress tolerance.
- Large genetic variation for drought and salinity resistance attributes exists in the breeding materials and some of the improved cultivars of different crop species.

Genetics of abiotic stress tolerance

Necessary information for developing abiotic stress tolerant variety-

- Mode of inheritance

- Association studies

- Mode of gene action

- Heritability

- Combining ability

- Heterosis

Methods for developing abiotic stress tolerant crop variety

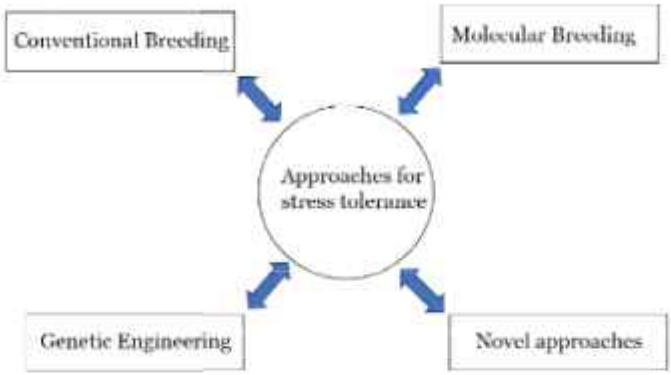


Fig. Breeding approaches for developing abiotic stress tolerant crop varieties

The choice of suitable breeding program for the development of tolerant crop variety to a defined abiotic stress depends upon a number of factors:

- Screening techniques
- Sources and mechanism of tolerance
- Mode of gene action
- Heritability
- And their relationship to agronomic traits.

Conventional Breeding

Conventional breeding approaches have been widely used to develop abiotic stress tolerant crop cultivars. These approaches rely on the exploitation of natural genetic variation present in germplasm collections, wild relatives, and landraces. The selection of superior genotypes is based on their performance under abiotic stress conditions in field trials or controlled environments. The general phases of conventional breeding are:

- ✓ variability creation
- ✓ selection
- ✓ evaluation and
- ✓ cultivar release

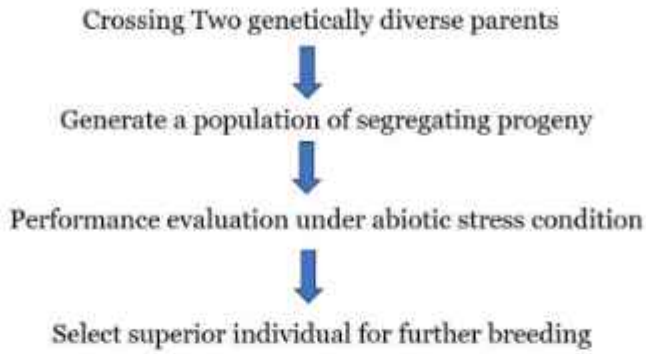
Conventional breeding techniques for abiotic stress tolerance in plants include:

- Hybridization and selection
- Mutation breeding

Selection methods

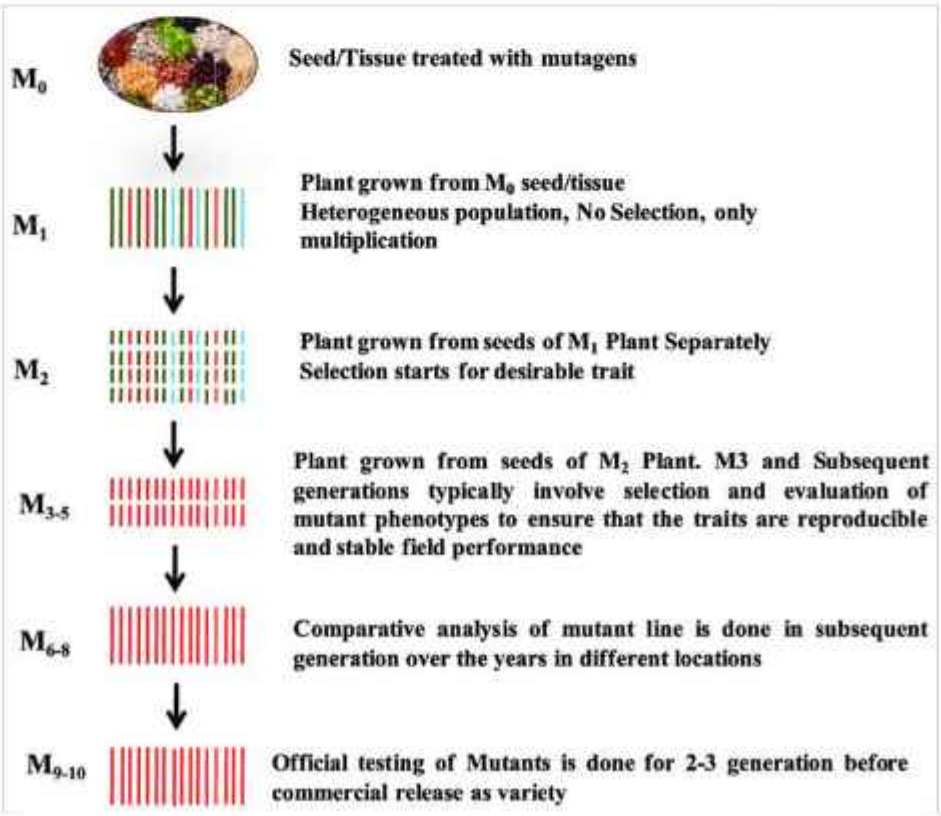
- Pedigree selection
- Bulk selection
- Single seed descent method

Hybridization and Selection:



Mutation Breeding

Mutation breeding involves the use of physical or chemical mutagens to induce random mutations in the genome of a crop species. The mutants are then screened for desirable traits, such as enhanced abiotic stress tolerance, and the selected individuals are used as parents in breeding programs. Mutation breeding has been successfully used to develop abiotic stress tolerant cultivars in various crop species, such as rice, wheat, and barley. The advantage of mutation breeding is that it can generate novel alleles and traits that are not present in the existing germplasm. However, the
mutation process is random, and the majority of the induced mutations are deleterious or have no effect on the trait of interest. Therefore, large mutant populations and efficient screening methods are required to identify the rare beneficial mutations.



Molecular Breeding

- Marker-assisted selection
- Quantitative trait loci mapping
- Genome-wide association studies

Marker-Assisted Selection (MAS)

Marker-assisted selection (MAS) is a breeding approach that uses molecular markers to select for desirable traits in segregating populations. Molecular markers are DNA sequences that are closely linked to genes or quantitative trait loci (QTLs) controlling the trait of interest. The identification of such markers requires the construction of genetic linkage maps and the mapping of QTLs for abiotic stress tolerance traits. Once the markers are identified, they can be used to screen breeding populations and select individuals carrying the desirable alleles. MAS has several advantages over conventional phenotypic selection, including increased selection accuracy, reduced breeding time, and the ability to select for traits that are difficult or expensive to phenotype.

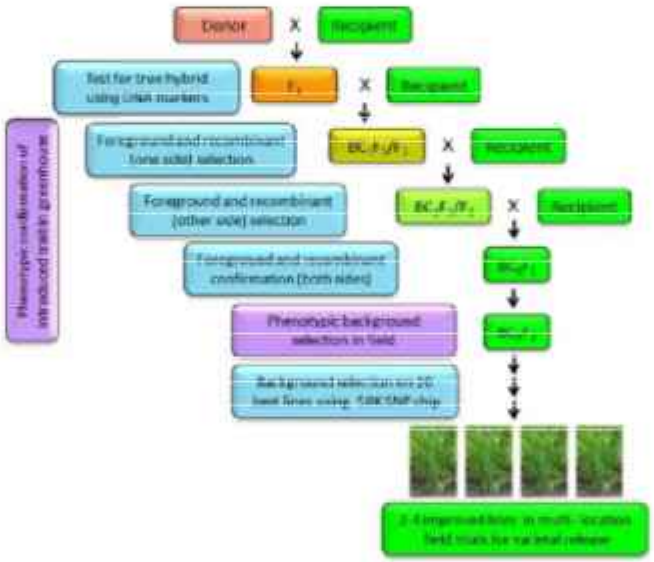


Fig. Schematic representation of marker assisted selection for stress tolerant crop variety development

QTL mapping

Steps involved in QTL mapping:

- Selection of parental lines
  - Sufficient polymorphism
  - Parental lines are highly contrasting phenotypically
  - Genetically divergent
- Selection of molecular markers (dominant/codominant)

- Making crosses
- Creation of progeny (mapping population)
- Phenotyping of the progenies
- Genotyping of the progenies
- Construction of linkage map
    - Link trait data with marker data

Genome-Wide Association Studies

Genome-wide association studies (GWAS) are a powerful genomics approach for dissecting the genetic architecture of complex traits, such as abiotic stress tolerance. GWAS involve the genotyping of a large number of individuals from diverse germplasm collections using high-density molecular markers, such as single nucleotide polymorphisms (SNPs). The marker data is then associated with phenotypic data collected under abiotic stress conditions to identify genomic regions and candidate genes underlying stress tolerance. GWAS have been successfully applied to identify genetic loci associated with drought tolerance in maize, salt tolerance in rice, and heat tolerance in wheat. The identified loci and candidate genes provide targets for marker assisted selection and genetic engineering approaches to improve abiotic stress tolerance in crop plants.

Other Omics technologies for abiotic stress tolerant variety development

- Transcriptomics and gene expression profiling
- Proteomics and Metabolomics

Transcriptomics and gene expression profiling

Transcriptomics involves the study of the complete set of RNA transcripts (transcriptome) in a cell or tissue under specific conditions. Gene expression profiling using microarrays or RNA sequencing (RNA-seq) technologies allows for the identification of differentially expressed genes (DEGs) under abiotic stress conditions. Comparative transcriptome analysis of contrasting genotypes, such as stress-tolerant and stress-sensitive varieties, can reveal key genes and pathways associated with abiotic stress tolerance. For example, transcriptome analysis of drought-tolerant and drought sensitive rice genotypes identified several stress-responsive transcription factors, such as DREB, NAC, and bZIP, that were differentially expressed under drought stress.

Proteomics and Metabolomics

Proteomics and metabolomics are complementary approaches to transcriptomics that provide information on the functional components of the cell under abiotic stress conditions. Proteomics involves the study of the complete set of proteins (proteome) in a cell or tissue, while metabolomics focuses on the identification and quantification of small molecules (metabolites) involved in cellular processes. Comparative proteomics and metabolomics of stress-tolerant and stress-sensitive genotypes can identify proteins and metabolites that are differentially

accumulated under abiotic stress conditions. These molecules can serve as biomarkers for stress tolerance and provide
targets for genetic improvement. For example, proteomic analysis of salt-tolerant and salt-sensitive rice genotypes identified several proteins, such as osmotin, salt-stress-induced protein, and glyceraldehyde-3-phosphate dehydrogenase, that were differentially accumulated under salt stress.

Genetic Engineering and Genome Editing

Transgenic approach:
Transgenic approach involved the introduction of foreign genes or the over expression of endogenous genes in crop plants to enhance abiotic stress tolerance. These genes encode proteins with diverse functions, such as ion transporters, osmoprotectants, antioxidants, and regulatory proteins. One of the most successful examples of transgenic approaches for abiotic stress tolerance is the development of Bt eggplant, which carries a gene from the bacterium Bacillus thuringiensis that confers resistance to brinjal shoot and fruit borer.
There are several methods:

- Agrobacterium mediated gene transfer
- Particle bombardment
- Electroporation
- Protoplast transformation

While transgenic approaches have been successful in improving abiotic stress tolerance in several crop species, they also face challenges such as public acceptance, regulatory hurdles, and potential ecological risks. Therefore, alternative approaches, such as marker-assisted breeding and genome editing, are gaining prominence for developing stress tolerant crop varieties.

CRISPR/Cas9 mediated Genome Editing

CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9) is a revolutionary genome editing technology that allows for precise and targeted modification of genes in crop plants. It is based on the bacterial immune system and consists of a guide RNA (gRNA) that directs the Cas9 endonuclease to a specific genomic location, where it creates a double-strand break (DSB). The DSB is then repaired by the cell's endogenous repair mechanisms, leading to either gene knockout or precise gene editing.

CRISPR/Cas9 has been successfully applied to improve abiotic stress tolerance in various crop species. For example, knockout of the OsDST gene in rice using CRISPR/Cas9 has been shown to enhance drought and salt tolerance. Similarly, targeted mutagenesis of the OsPYL9 gene in rice using CRISPR/Cas9 has been reported to improve drought tolerance. CRISPR/Cas9 has also been used to edit the ZmDREB2A gene in maize for improved drought tolerance. The advantages of CRISPR/Cas9-mediated genome editing over transgenic approaches include the ability to introduce precise modifications without the integration of foreign DNA, reduced off-target effects, and the potential for multiplexing (editing multiple genes simultaneously).

Brinjal Shoot and Fruit Borer resistant Eggplant varietal development using *Bt* gene

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Brinjal (*Solanum melongena*), also known as eggplant aubergine, is one of the leading vegetable crops grown extensively across the country. It holds significant importance in Bangladesh due to its role in the country's agriculture, economy, and daily diet. It is grown on nearly 55 thousand hectares. Brinjal is prone to attack from insect pests and diseases, the most serious and destructive of which is the the Brinjal Shoot and Fruit Borer (BSFB) leading to substantial yield losses, increased production costs, and reduced quality of produce. Farmers are often caught in a cycle of pesticide use that only partially controls the pest while harming the environment and public health. Bt brinjal, a genetically modified variety that incorporates a gene from the *Bacillus thuringiensis* bacterium, offers a breakthrough solution by providing resistance to the BSFB. This innovative crop reduces the need for harmful chemical pesticides, leading to higher yields, improved crop quality, and reduced environmental impact. The introduction of Bt brinjal in Bangladesh is a landmark achievement in the country's agricultural biotechnology efforts.

Mode of Action of Bt gene: The *Bacillus thuringiensis* bacterium produces these Cry proteins as part of its normal life cycle. When insects ingest the Cry proteins (typically via ingestion of plant material or soil), the proteins are activated by the alkaline conditions in the insect's gut. The activated Cry proteins then bind to specific receptors in the gut cells, causing the cells to break down, leading to the insect's death by gut paralysis and disruption of digestion.

Source of nucleic acid (s): The Cry1Ac gene encodes for an insecticidal protein, derived from the crystalliferous spore-forming gram-positive soil bacterium *Bacillus thuringiensis subsp. kurstaki (B.t.k)*. The nptII gene encodes the selectable marker enzyme neomycin phosphotransferase II (NPTII) was used to identify transformed cells containing the Cry1Ac protein. It has no pesticide properties. The nptII gene is derived from the prokaryotic transposon Tn5. The aad gene, which encodes for the bacterial selectable marker enzyme 3"(9)-Oaminnoglycoside adenyl transferase (AAD).

Transformation of Bt gene : The *Agrobacterium tumefaciens* strain LBA4404 carrying the vector pMON10518 (carrying genes *cry1Ac, nptII* and *aad*) was used in the transformation process. The *cry1Ac* gene is under the transcriptional control of the enhanced *CaMV35S* promoter (P-E35S). The aforesaid genes have been introduced by *Agrobacterium* mediated transformation and transferred into the genome of brinjal cells and thereby allowing selection on kanamycin medium. The plant expression vector was assembled and then transformed into *Escherichia coli* and mated into *Agrobacterium* strain LBA4404 by the triparental conjugation system. The T-DNA was transferred to the plant cells via the functions of the vir genes located in the disarmed Ti plasmid present in the *Agrobacterium*. The Ti plasmid does not transfer to the plant cells but remains in the *Agrobacterium*. The brinjal tissues were then transferred on shoot induction medium for the regeneration of shoots and ultimately plantlets were obtained on tissue culture medium containing kanamycin. The presence of Cry1Ac protein and insect resistance was analysed in the plantlets and the plants carrying the protein were tested for several generations to identify the lines in which the transgene segregated. A single line (Event EE-1) was introduced into the breeding program.

Development of Bt Brinjal in Bangladesh: Under the public-private partnership, Agricultural Biotechnology Support Project II (ABSP II) project, BARI, India-based Maharashtra Hybrid Seed Company (Mahyco) , Sathguru management consultants of India, and Cornell University started the work of Bt eggplant research in Bangladesh in 2005. The event was donated to BARI in Mahyco and backcrossed into nine popular eggplant varieties of Bangladesh selected based on the regional consumer preference. Efficacy trials were conducted for a period from 2007 to 2012.

Approval Process: To launch the best 4 varieties, BARI applied to the National Technical Committee on Crop Biotechnology (NTCCB) to release Bt eggplant. Following the recommendation from NTCCB, the application for release was forwarded to the NTCCB Core Committee followed by the Biosafety Core Committee (BCC) and National Committee on BioSafety (NCB). The Bangladesh government granted approval for release of four varieties (BARI Bt Begun-1, BARI Bt Begun- 2, BARI Bt Begun-3, and BARI Bt Begun-4) for limited cultivation in the field on 30 October 2013. The approval process was rigorous, with assessments of both the environmental and health safety of the modified crop.

Sustaining of the technology: As with any new technology, stewardship is vitally important, and this is true of Bt brinjal. Planting borders of non-Bt brinjal as a refuge is critical for the sustainability of the Bt brinjal technology. BARI plays a critical role in the sustainable adoption of Bt technology in the country. BARI has been actively involved in conducting field trials, capacity-building through training programs for farmers, DAE personnel, BADC seed marketing officers, and seed dealers; continuous monitoring; maintaining biosafety compliance; production of good quality seed; partnership with international, government, and non-government organizations; using electronic and printed media, etc.

The development and adoption of Bt brinjal in Bangladesh marked a key moment in the country's agricultural innovation. After successfully releasing four Bt brinjal varieties, BARI Begun-10 and BARI Begun-11, BARI is now developing varieties with resistance to both BSFB and bacterial wilt. With the fast adoption rate of Bt brinjal in Bangladesh, the researchers are hopeful that the new varieties will have the potential to help the farmers increase their profits even more.

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Late Blight Resistant Potato Variety Development Using 3R-gene

Dr. Mahmuda Khatun

CSO and Head

Biotechnology Division

Bangladesh Agricultural Research Institute

Potato

Potato is an important crop in Bangladesh, grown in about 0.5 million hectares of land. It ranks third after rice and wheat but in terms of per unit area and time, production of potato is higher compared to other crops. Nutrient-density in potato is also higher than that of rice and wheat. For example, energy obtained from potato is about 60200 KJ per hectare of land whereas rice and wheat can produce 38075 and 36053 KJ energy per hectare, respectively. In addition, 100 gm potato contains 13 mg Vit C (FAO, 2008) but rice and wheat do not have any Vit C. Potato is also an important source of vitamin B_6, potassium, iron, protein and dietary fiber.

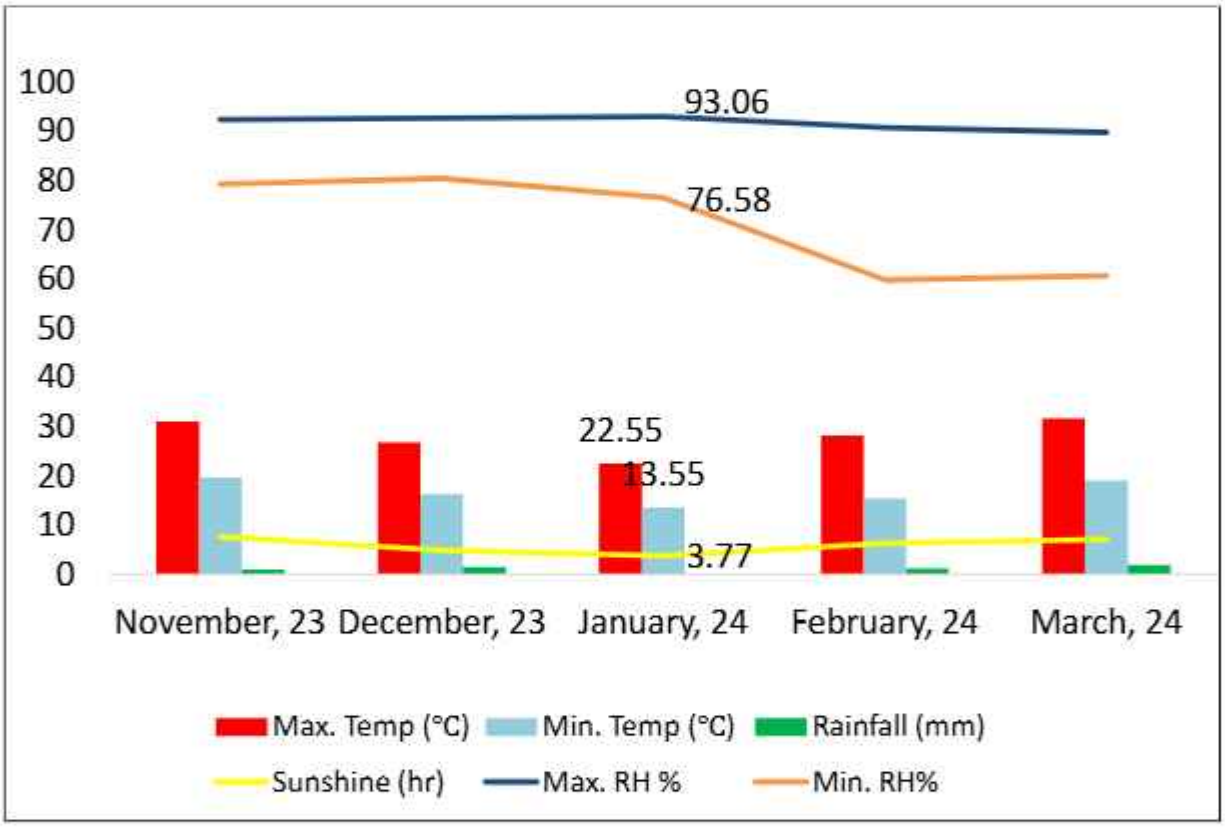
Bangladesh is among the **top ten** potato producers in the world, grown chiefly for the domestic market. Potato production in Bangladesh has increased substantially over the last decade due to the availability of high yielding varieties, better seed and improved cultivation practices. Despite significant increase in potato production, its yield is often affected due to frequent infestation by the late blight disease- the most devastating disease of potato in the world.

Serial No.	**Top 10 Countries**	**Potato Production in Year 2022 (Kilotonns)**
1	China	95,570
2	India	56,176
3	Ukraine	20,899
4	Russia	18,888
5	United States	17,792
6	Germany	10,683
7	**Bangladesh**	10,145
8	France	8,067
9	Pakistan	7,937
10	Netherlands	6,916

Late blight disease of Potato

- Caused by fungal-like oomycete *Phytophthora infestans*
- Favored by cool, moist weather
- Can infect potato foliage and tubers at any stage of crop development
- Produce water-soaked lesions
- White, fluffy fungal growth is found on the bottoms of leaves
- Can kill plants within two weeks

Optimum weather for Late blight disease occurrence in Bangladesh



Optimum night temperatures: 9-15 °C

Optimum day temperatures of 15-19 °C

Optimum RH: 90%

Consequences of Late blight disease of Potato

- A major cause in the 19th century Irish and European famines.
- Annual potato yield losses due to late blight have been estimated at 25-57% (Geopotato, 2019)
- Up to six billion dollars of potato loss annually.
- About Tk. 100 crore is spent on fungicides.
- Late blight can be controlled by frequent and costly applications of fungicides.
- The degree of control heavily depends on the timing of the fungicide application in relation to local weather conditions, crop development and disease pressure.
- The disease is of common occurrence in Bangladesh for over 30 years and causes considerable yield loss
- Farmers spend up to a fourth of their investments on fungicide sprays to fight late blight, with over 7-8 sprays per season in 3-5 days interval
- Damages 20 percent of the potato yield in Bangladesh every year
- Severe infestation of late blight occurrence was recorded in 2006-07 crop season where the mean disease incidence in the country ranged 50.0-78.4% (Dey et. al, 2010)
- In 2023-24 season severe late blight outbreak occurred in the whole country with most devastation in Northern region of Bangladesh

Why late blight is so difficult to control in Bangladesh?

- Up to the years 2001-02 simple races of *P. infestans* with a limited genetic diversity and fewer virulence factors were predominant
- In recent years, several genetically diversed complex races carrying multiple virulence genes have been detected
- Over 20 races of the fungus have been identified in the country (Dey et. al, 2010)
- Several fungicide resistant strains of *P. infestans* were detected across the country
- Due to farmer's limited knowledge, indiscriminate application of fungicides over several decades has caused this fungus to develop resistance.

Number of registered fungicides under different hazard category reported from the potato growing farmers of Bangladesh (Hossain, 2024)

Hazard category	Fungicide
Ib	-
II	1
III	10
III+II	4
U+III	18
U+II	1
U	46
Not classified	1
Total	**81**

*Ia, Extremely hazardous; Ib, Highly hazardous; II, Moderately hazardous; III, slightly hazardous; U, Unlikely to present acute hazard in normal use (WHO)

Use of 321 pesticides were reported by 460 potato farmers in a potato growing area of Bangladesh, of which only 161 was found registered pesticides. Out of 161 registered pesticides, 81 were fungicides (50.31%) which are used by Potato farmers with a frequency of more than 5 times application (Hossain,2024).

Limitations of existent conventionally bred resistant varieties

- Many potato varieties are bred with race-specific R-genes can be easily broken if the pathogen evolves a corresponding virulence factor. The breakdown of vertical resistance can happen quickly, sometimes within just a few growing seasons.
- The pathogen is highly variable due to recombination in genetic patterns due to both sexual and asexual reproduction and adapt to the newly bred varieties and fungicides quickly
- High level of fluctuating environmental conditions, such as humidity and temperature, can influence the aggressiveness of *P. infestans*

Why 3R-gene LBR potato is a best choice to fight against late blight in Bangladesh?

- Single gene gives some resistance, but potatoes still require some fungicides while double resistance can still be overcome by the late blight pathogen.
- Multiple genes insertion in one potato variety provides stronger, longer lasting resistance
- The 3R-gene LBR potato lines have been developed by inserting three blight resistant genes derived from **three wild potato species** –

  Mexican origin *Solanum bulbocastanum*,

  Argentine origin *Solanum venturii* and

  Peruvian origin *Solanum mochiquense*

In Bangladesh, some varieties showed high to moderate resistance, but none of those were found to be immune to *P. infestans* (Masud et al., 2024), however 3R-gene LBR potato showed complete and durable resistance, even after inoculating with aggressive pathogen strains with a very high concentration (@ $1x10^6$ sporangia/ml)

What happens to a potato variety after the genetic transformation?

- The transgenic materials are morphologically same as the original potato released potato variety to [e.g. 3R-gene LBR events of Diamant variety are same as traditional BARI Alu-7/ Diamant in Bangladesh]
- Completely unaffected to Late Blight Disease
- No change in morphology of potato plants due to expression of 3R- gene.

Benefits of 3R-gene late blight resistant potato

- ❖ Reduce the use of excess pesticides
- ❖ Reduce cost of production through less chemical costs
- ❖ Reduce chemical residue in food, fodder, land, and wastewater enhancing human, livestock, fish and environmental health
- ❖ Reduce risk to farmer and consumer health with less exposure to toxic chemicals
- ❖ Effectively improve yield compared with diseased potatoes

Feed the Future Global Biotech Potato Partnership

- Developing a **durable late blight resistant potato** for smallholder farmers in Bangladesh
- Adding late blight resistance to the Bangladesh **farmer-preferred varieties (e.g. Diamant)**
- Using **3 resistance genes from wild potato species** to provide stronger and more **durable resistance**
- Genes can be isolated from wild species and inserted into domestic potato using genetic engineering **much more efficiently** than through traditional breeding
- Bangladesh is assessing two 3R-gene transformed events (DIA-MSU-015 and DIA-MSU-255) for its suitability

Sources of late blight resistance 3R-gene

3 wild potato species

1. ***Rpi-blb2-*** gene from *Solanum bulbocastanum*, discovered in Mexico (Van der Vossen *et al*., 2005)
2. ***Rpi-vnt1-*** gene from *Solanum venturii*, identified in Argentina (Foster *et al*., 2009)
3. ***Rpi-mcq1-*** gene from *Solanum mochiquense*, found in Peru (Smilde *et al*., 2005).

Steps in 3R-gene genetic transformation in Potato

There are 3 late blight resistance genes that include their native promoters and native terminators, cloned from wild species of potato

- The *Rpi-vnt1*GenBank accession no. FJ423044
- The *Rpi-mcq1*GenBank accession no. GN043561 and
- The *Rpi-blb2*GenBank accession no. DQ122125.1

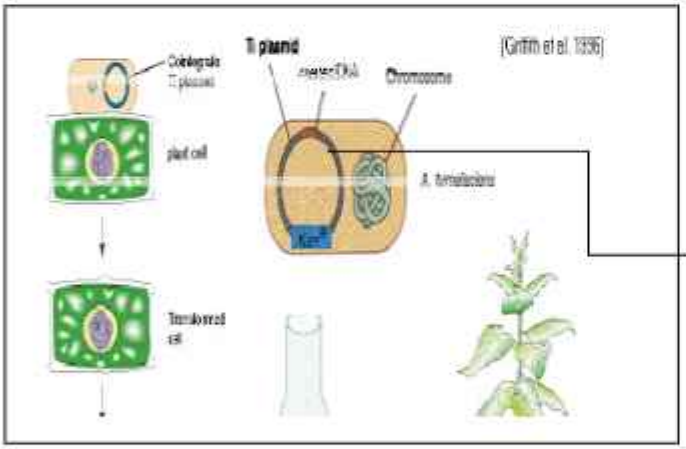


Photo Courtesy of Simplot Plant Sciences

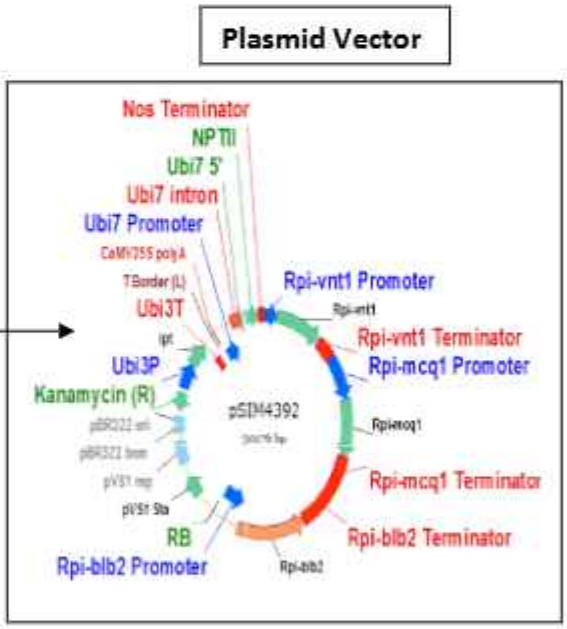


Fig. Schematic diagram of plasmid pSIM4392 including T-DNA region

Steps in *Agrobacterium* – mediated genetic transformation in 3R-gene Potato

Plasmid construction with 3R-genes and *Agrobacterium* vector preparation

⇩

Tissue cultured plantlets obtained from potato sprout culture with well developed internodes

⇩

Co-cultivation of internode/ leaf segments with freshly grown *Agrobacterium* containing pSIM4392 plasmid

⇩

Transformed explants cultured in specific selection media for shoot and subsequent root development

⇩

Propagation of established genetically transformed plantlets

⇩

PCR screening for presence of desired genes in the plantlets

⇩

PCR positive lines were again screened for copy number or vector backbones

⇩

Lab based late blight resistance testing and screening

⇩

PCR and Lab based confirmed 3R-gene lines are identified as individual events

⇩

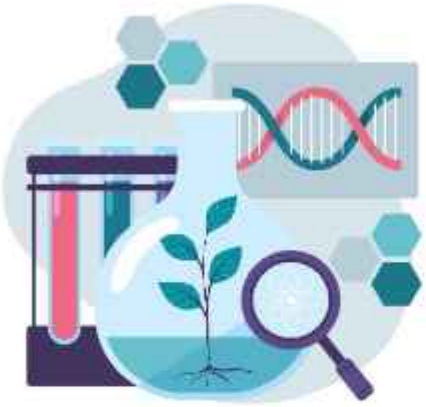
3R-gene containing events are tested in contained trials

⇩

3R-gene containing events are tested in confined field trials

Biosafety Guidelines for Transgenic Research in Bangladesh

14 January 2025



Md. Aziz Zilani Chowdhury, *PhD*
Former Member Director (Crops)
Bangladesh Agricultural Research Council

Biosafety ?

Policies and procedure adopted to ensure the environmentally safe application of modern Biotechnology in medicine, agriculture, fisheries and livestock and in the environmental management, without endangering public health or environmental safety.

Towards Building Advanced Intelligent System for Agriculture

Biotechnology

The UNs convention on biological diversity (CBD) defines as :

"Any technological application that uses biological systems, living organisms, or derivatives thereof, to make or modify products or processes for specific use."

The economic potential of this new biotechnology in agriculture, health, energy and environment is well recognized.

Several branches of Biotechnology (BT)

1. **Green BT:** Applied to **Agricultural** processes (eg. **IR *Bt* maize)**
2. **Blue BT:** **Marine** and **aquatic** applications of BT
3. **Red BT:** Applied to **Medical** processes
4. **White BT:** Applied to **Industrial** processes

Transgene: A DNA fragment or gene from a non-cross compatible species.

Transgenesis: The process of introducing transgene into the genome of a given cell and the propagation of such a fragment thereafter.

Transgenic/GM plants are plants that have been genetically modified to have new traits that are not naturally present.

GMO and LMO are both terms for living organisms that have had their genetic material altered using biotechnology.

Application GE technology?

Used when all other techniques have been exhausted & w/n:

1) the trait to be introduced is not present in the GP/crop;

2) the trait is very difficult to improve by conventional breeding

3) take a very long time to introduce and/or improve such trait in the crop by conventional breeding methods.

What are the potential risks of GM Crops ?

With every new emerging technology, there are potential risks. These include:

- Unintentionally introducing allergens and other anti-nutrition factors in foods
- Likelihood of transgenes escaping from cultivated crops into wild relatives
- Potential for pests to evolve resistance to the toxins produced by GM crops
- The risk of these toxins affecting non-target organisms
- Mixing of genes from unrelated organisms might create natural imbalance that is not yet clearly understood.

To address the issues of public and environmental safety concerning modern biotechnology, its product or its application and over and above all to discharge the obligations of the CBD and the Cartagena Protocol,

there is an urgent need to develop biosafety guidelines and enact related Rules/Acts to regulate laboratory research, field studies and commercial release of GMOs and products thereof.

The Cartagena Protocol on Biosafety (CPB)

The CPB was adopted by the international community in Montreal on 29 Jan 2000 in order to fulfill one of the imp. obj. of the 1992 CBD:

the conservation and sustainable use of biological diversity.

The Convention takes a comprehensive approach to the conservation of biological diversity.

It addresses the threats that might arise from the ***transfer, handling and use*** of LMOs resulting from modern BT.

- The Cartagena Protocol is an international legally binding treaty w/c sets procedures and mechanisms to be applied in the transboundary movements of LMOs –
- Living organisms that possesses a novel combination of genetic material obtained through the use of modern BT (genetic modification).
- The Protocol does not apply to other products of biotechnology.

Why do we need a Protocol ?

- Rapid development and commercialisation of BT and its products, including LMOs
- Recognition of potential contribution that BT can make to improving human well being
- Uncertainties regarding potential risks of LMOs on biodiversity and human health
- Lack/limited capacities of many countries to make enlightened decisions concerning imports of LMOs

Bangladesh as a party to the Protocol

- According to **Article 34** of the Convention, the Protocol is to be ratified by each state separately in order to be binding on states,.
- Bangladesh signed the Protocol on **24 May 2000** and **ratified it on 5 February 2004**.
- According to **Article 36 (4)** of the Convention, the Protocol came into force for Bangladesh on **5 May 2004**, on the ninetieth day after the date of deposit of the instrument of ratification.

Biosafety related documents in Bangladesh

1. National Biosafety Framework, 2008
2. Biosafety Guidelines of Bangladesh, 2008
3. Bangladesh Biosafety Rules 2012
2. Guidelines for The Environmental Risk Assessment (ERA) of Genetically Engineered Plants (2016)
3. National Biosafety Policy 2024 (জাতীয় জীবনিরাপত্তা নীতি ২০২৪)

What are the biosafety guidelines?

Biosafety guidelines are a set of policies, rules, and procedures that personnel must follow in various facilities handling microbiological agents, such as bacteria, viruses, parasites, fungi, prions, and other related agents and microbiological products.

Biosafety policy of Bangladesh ?

The mission of the National Biosafety Policy is to ensure safe use of biotechnology through providing an enabling environment for the safe development, use and application of modern biotechnology for ensuring maximum benefits to agricultural, medical and industrial development.

Bangladesh Biosafety Rules 2012 ?

Bangladesh Biosafety Rules (BR), 2012, provides regulations on the approval process for biotech products developed domestically or by a third country. According to Biosafety Rules 2012, all GE products need to be approved before they can be imported or sold domestically within Bangladesh.

Biosafety Guidelines of Bangladesh (January 2008)

Govern the research, development, and use of GMOs. These guidelines apply to all activities involving GMOs, including laboratory research, field trials, and the transportation of GMOs. The guidelines are intended to protect human health and the environment.

Contents of the Guidelines

Chapter-I : Scopes and Objectives of Biosafety Guidelines

Chapter-II : Institutional Arrangements

Chapter-III: General Provisions on Biosafety: Risk Assessment and Risk Management

Chapter-IV: Physico-chemical and Biological Containments: Procedures and Facilities

Chapter -I. Scope of Biosafety Guidelines

Biosafety guidelines are applicable to all R & D activities of modern BT conducted in the laboratories of the govt. res. Inst., state enterprises, universities, int. orgns located in Bangladesh, private companies and non-governmental orgns.

It applies to laboratory and field trial, trans-boundary movement, transit, handling and use of all GMOs/LMOs that may have adverse effects on the conservation and sustainable use of biological diversity, taking also into account risks to human health.

Chapter –II. Institutional Arrangements : Committees

- National Committee on Biosafety (NCB)
- Biosafety Core Committee (BCC)
- Institutional Biosafety Committee (IBC)
- Field level Biosafety Committee (FBC)

National committee on biosafety (NCB)

Composition of NCB:

The scientists and or experts will be nominated by the MOEF.
The structure of the committee:

Chairperson:

1. The Secretary, MOEF
2. Full time member Secretary to be nominated by MOEF

Members (19):

1. Secretary, Ministry of Sci., Information and Commun.Technology.
2. Secretary, Ministry of Agriculture
3. Secretary, Ministry of Fisheries an Livestock
4. Secretary, Ministry of Health

5. Executive Chairman, Bangladesh Agricultural Research Council
6. Member (relevant), National Board of Revenue
7. Director General, Directorate of Food
8. Director General, Department of Environment
9. Chairman, Bangladesh Atomic Energy Commission
10. Director General, Fisheries Research Institute
11. Director General, Bangladesh Livestock Research Institute
12. Director General, Bangladesh Agriculture Research Institute
13. Director General, Bangladesh Rice Research Institute
14. Director General, Bangladesh Jute Research Institute
15. Director, Bangladesh Forest Research Institute
16. DG, Bangladesh Institute for Nuclear Agriculture
17. Director, National Institute of Biotechnology
18. One representative from NGO working with environment related issues (to be nominated by NGO affairs bureau)
19. One representative from NGO working with legal aspects related to environment (to be nominated by affairs NGO bureau)

Towards Building Advanced Intelligent System for Agriculture

Powers, Functions and Responsibilities of the NCB

- Regulation and Monitoring GMOs
- Formulation and review policies, guidelines, acts and rules standards and manuals on Biosafety and shall supervise RA, RM and the implementation activities.
- Review all existing bilateral and multilateral projects, all research undertaken by NGO/Private/Public organization.
- Review, Monitor and Recommend measures to minimize potential risks that may result from, import, contained use, field trial and release or introduction of new species, strains or varieties of GMOs.
- Facilitate the institutes/faculties/companies working with GMOs to obtain necessary permission/clearance in favor of their activities.
- NCB to examine the applications on case-by-case basis.
- MOEF will notify the decision of NCB to the applicant.

Powers, Functions and Responsibilities of the NCB

- Instruct the respective authority to ensure implementation of Biosafety measures at on-site (field), laboratory, during trans-boundary movement, use, handling and release in the market.
- Prepare different forms for permission to undertake laboratory work; field trial and for commercial release of GMOs including monitoring format.
- Hold public consultation on proposed national policies, guidelines and on the comparative ecological, economic and social impacts of alternative approaches to attain the purposes/ objectives of the proposed genetic modification products and/or services
- Review the appointment of the members of the IBC
- Assess and Identify priorities in HRD capacity building requirements.
- Coordinate the activities of IBCs and FBCs.

Towards Building Advanced Intelligent System for Agriculture

Biosafety Core Committee (BCC)

The BCC shall be composed of 8 members.

The structure of BCC shall be as follows:

- DG, DoE, Convener.
- Director (technical), DoE, Member Secretary
- 4 bio-scientists to be maintained by MOEF
- 1 medical scientist (human health aspects)
- 1 scientist specialized in biosafety affairs.

Functions of BCC

- Monitor the implementation of Biosafety guidelines, policies, acts and rules as complementary to the NCB
- Examine application for any permit/license of import of GMOs/LMOs for contained use, field trial and field release and forward recommendations to the NCB for its consideration.
- Provide technical comments or recommendations to NCB or the govt on policy, legal and technical issues of Biosafety as and w/n requested for.
- BCC will meet, as and when necessary, preferably on quarterly basis.
- BCC shall arrange annual inspection and evaluation of performance of all the laboratory engaged in research, development and demonstration (R&D) of GMOs/LMOs.
- BCC may co-opt experts related to specific issues regarding Biosafety as and when required

Towards Building Advanced Intelligent System for Agriculture

Institutional Biosafety Committee (IBC)

- All institutions engaged in GE shall have an IBC to evaluate and monitor the Biosafety aspects of their research.
- Whenever an institution intends to become involved in planned field release.
- members of the IBC should collectively have the range of expertise necessary to supervise and assess the program.

Composition of IBC

- A senior knowledgeable person with adequate experience in biosafety shall be appointed as the chairperson.
- The committee will be composed of 4 members from amongst the high caliber and experienced scientist of the institute to be appointed by the chairperson according to their expertise in biosafety of the corresponding modern biotechnological activities, they should be able to evaluate, assess and advise precautionary measures for GE work.
- The IBC represent the most important component in the domain of biosafely supervising all GE work in the institute.

Functions of IBC

- Review works conducted by the institutions and recommends research proposals for considerations by NCB.
- Hold discussions or public consultations on the comparative ecological, economic and social impacts of alternative approaches to attain the purposes/objs of the proposed GE products/or services
- Formulate and adopt emergency plans covering accidental spills and personnel contamination resulting from lab and fieldwork
- Report immediately to the appropriate official in the concerned orgn and to the NCB any significant problems with the implementation of the guidelines and any significant research-related accidents or illness.
- Maintain close liaison with the BCC.

Biological Safety Officers (BSO)

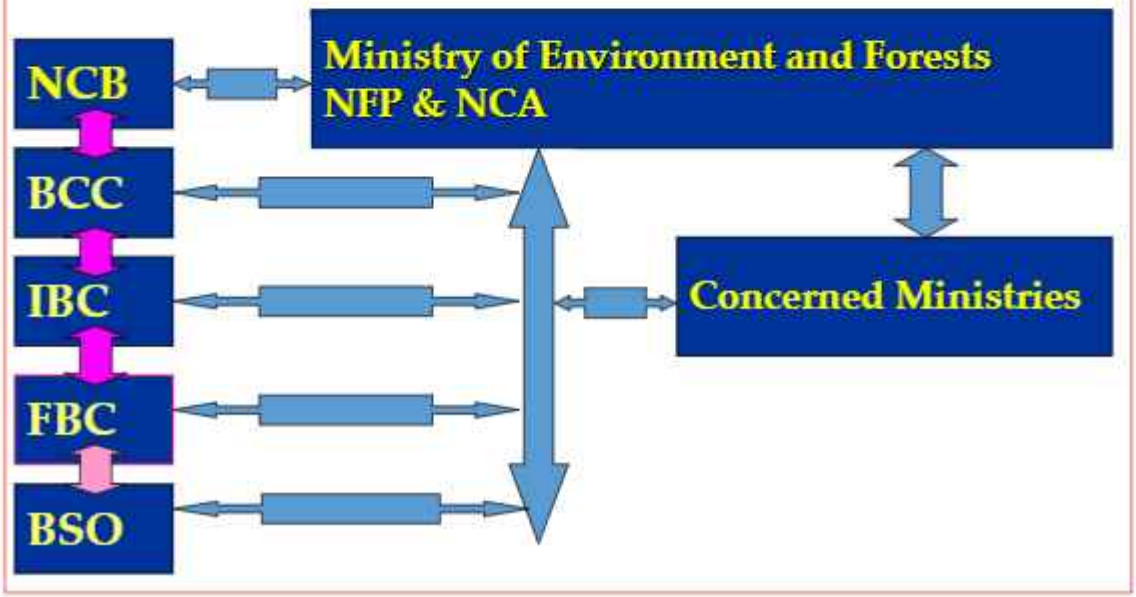
- Each institution shall designate at least one scientist as BSO, should have considerable experience on Biosafety issues and emergency counter measures and is expected to have rigorous training on biosafeguards.
- BSO monitors the compliance of the guidelines at the institution level. BSO has to report regularly to the Chairperson of IBC on any matter regarding Biosafety applications in the institution.

Field level Biosafety Committee (FBC)

There is a provision for constitutions of FBC when permission for field trial of GMOs/LMOs is given.

NCB will appoint 03 scientists from relevant institutions 01 representative from the MoEF to constitute the field level biosafety committee.

Biosafety Administrative structure



Towards Building Advanced Intelligent System for Agriculture

GM Crop Approval Process

Application Approval by the IBC of the Respective Organization (public or private)

↓

Submission of application through the respective ministry (Through the National Technical Committee)

↓

The tech comt evaluates the applications and forwards it to the BCC

↓

Evaluation by the BCC & Respective Experts

↓

BCC forwards recommendation to the NCB

↓

Approval/Rejection by NCB

Contents of the Guidelines

Chapter-III: General Provisions on Biosafety (Risk Assessment and Risk Management)

Guidelines for The Environmental Risk Assessment (ERA) of Genetically Engineered Plants (2016)

1. INTRODUCTION
2. PURPOSE OF THE ERA GUIDELINES IN THE CONTEXT OF THE Biosafety Framework of Bangladesh
3. SCOPE
4. DEFINITIONS
5. GENERAL CONSIDERATIONS IN ERA
   - DESCRIPTION OF GE PLANT
   - DESCRIPTION OF THE NON-TRANSGENIC HOST PLANT OR NON- MODIFIED COUNTERPART OR PARENTAL PLANT
   - DESCRIPTION OF THE DONOR ORGANISMS
   - DESCRIPTION OF THE GENETIC MODIFICATION(S)
   - MOLECULAR CHARACTRIZATION OF TRNSGENE (S)
   - PHENOTYPIC AND AGRONOMIC CHARACTERISTICS OF THE GE PLANT

6. CULTIVATION PRACTICES
7. IMPACT ON NON-TARGET ORGANISMS
8. POST-RELEASE ENVTL MONITORING
9. INSTRUCTIONS ON DATA QUALITY

Contents of the Guidelines

Chapter-IV: Physico-chemical and Biological Containments: Procedures and Facilities

Measures for Containment Since R&D in modern biotechnology may have adverse impact on biodiversity and human health, precautionary measure is a prerequisite for each and every laboratory in the country.

Therefore, measures should be taken to limit the interaction of the regulated organisms with the environment or with human being.

For this the procedures for physico-chemical and biological containments have been given in details in this chapter.

Biosafety Level 1 (BL1) : BL1 is suitable for work involving agents of no known or minimal potential hazard to laboratory personnel and environment.

Biosafety Level 2 (BL2) : BL2 is similar to BL1 and is suitable for work involving agents of moderate potential hazard to personnel and the environment.

Biosafety Level 3 (BL3) : BL3 is applicable to clinical diagnosis, teaching, research or production facilities where work is done with indigenous or exotic agents that may cause serious or potentially lethal diseases as a result of exposure by inhalation.

Biosafety Level 4 (BL4) : BL4 work with dangerous and toxic agents which pose a high individual risk of life, threatening disease, members of the laboratory staff are to receive specific training in handling infectious agents.

Documents/Mechanism Developed to Support Biosafety Regulations

Bangladesh Biosafety Clearing House : http://www.bchbd.org.php

SOPs for Confined Field Trial (CFT)

- Inspectors Manual for CFT
- Data Recording Formats
- Guidelines for the safety Assessment of Food derived from GE Crops
- BSTI adopted this guidelines as BSTI standards for GE food safety assessment

Application format for permission to undertake field release of GMOs in addition to the general information

a) Title of the Project
b) Name address of the Chief Investigator
c) Objectives of the Project i. Overall Objectives ii. Specific Objective
d) Intended date of commencement
e) Intended date of completion
f) Location of release with area to be covered
g) Time of release (Date)
h) Expected date of completion of release
i) Information on similar release elsewhere with adverse effect observed (if any)
j) Experimental details with quantity of materials to be released (number, weight, size etc)
k) Is future field release of the same material expected? If yes,
l) Amount, time location and period of release
m) What is the intended output of the field release?
n) Precautionary measures to be taken as per Biosafety Guidelines in case of adverse situation
o) Additional information, if any

জাতীয় জীবনিরাপত্তা নীতি ২০২৪

বাংলাদেশ গেজেট, আগস্ট ২২, ২০২৪

১. প্রস্তাবনা ও প্রেক্ষিত: (১.১-১.৪)
২. জাতীয় জীবনিরাপত্তা নীতির পরিধি : GMO অথবা LMO-এর আধুনিক জীবপ্রযুক্তির ব্যবহারে Cartagena Protocol on Biosafety অনুসারে জীবনিরাপত্তা নীতির পরিধি: (২.১-২.৫)
৩. জাতীয় জীবনিরাপত্তা নীতির মূলনীতি (Principles) : ৩.১-৩.৮
৪. জাতীয় জীবনিরাপত্তা নীতির শিরোনাম, পলিসি স্টেটমেন্ট, লক্ষ্য এবং উদ্দেশ্য: ৪.১-৪.৬
৫. জীবনিরাপত্তা নীতির বাস্তবায়ন কৌশল (Implementation Strategy) :
৫.১ ক) মানবস্বাস্থ্য, প্রাণিস্বাস্থ্য, জীববৈচিত্র্য, পরিবেশ, কৃষিজ পণ্য, খাদ্যপণ্য পশুখাদ্য, দূষণ নিয়ন্ত্রণ ইত্যাদির ক্ষেত্রে জিএমও/এলএমও-এর গবেষণা ও উন্নয়ন, হস্তান্তর, পরিবহন, আমদানি, রপ্তানি, এবং অন্যান্য ব্যবহারের সময় জিএমও/এলএমও-এর সম্ভাব্য বিরূপ প্রভাব মুক্ত রাখা, হ্রাস করা অথবা উপযুক্ত ব্যবস্থা গ্রহণ করা (৫.১.১-৫.১.৮)

৫.২ (খ) জিএমও শনাক্তকরণ এবং ঝুঁকি নিরূপণের জন্য মানসম্পন্ন অবকাঠামোগত সুবিধা (৫.২.১-৫.২.১৪)

৫.৩ (গ) জিএমও/ এলএমও পণ্যের লেবেলিং নিয়ন্ত্রণ : ৫.৩.১-৫.৩.৪)

৫.৪ (ঘ) Cartagena Protocol on Biosafety-এবং আন্তর্জাতিক বিধিবিধানের সাথে সঙ্গতি রেখে জিএমও/এলএমও-এর কার্যকর নিয়ন্ত্রণ নিশ্চিত করা : (৫.৪.১- ৫.৪.৩)

৫.৫ (ঙ) আধুনিক জীবপ্রযুক্তির ব্যবহারে উদ্ভূত জরুরি পরিস্থিতি মোকাবেলায় উপযুক্ত কলাকৌশল প্রতিষ্ঠা:(৫.৫.১-৫.৫.৪)

৫.৬ Cartagena Protocol on Biosafety-এর ২৬ অনুচ্ছেদের অনুসরণে জিএমও/এলএমও অবসমুক্তির পূর্বে আর্থ-সামাজিক ঝুঁকি নিরূপণ করিতে হইবে। ঝুঁকি মূল্যায়ন করিবার সময় যেমন সংস্কৃতি, আর্থ-সামাজিক এবং নৈতিক বিষয়গুলোকে বিবেচনায় নেওয়া হইবে।

৫.৭ (ছ) জীবনিরাপত্তা সম্পর্কিত কার্যক্রমে তথ্য প্রবাহ, নেটওয়ার্কিং, জবাবদিহিতা এবং স্বচ্ছতা বৃদ্ধিকরণ:(৫.৭.১-৫.৭.৫)

জাতীয় জীবনিরাপত্তা নীতি ২০২৪

৬. বাস্তবায়ন সূচক:

৭. জিএমও/এলএমও সংক্রান্ত তথ্য ভান্ডার তৈরি:

৮. জীবনিরাপত্তা বিষয়ক তহবিল গঠন:

৯. বাংলাদেশ জীবনিরাপত্তা বিধিমালা ২০১২ ও জীবনিরাপত্তা নির্দেশিকা ২০০৮-এর প্রতিপালন:

১০. পরিবীক্ষণ এবং আইন ও বিধিবিধান বাস্তবায়ন প্রক্রিয়া:

১১. জীবনিরাপত্তা ক্ষেত্রে আন্তর্জাতিক সহযোগিতা জোরদারকরণ:

১২. জাতীয় জীবনিরাপত্তা নীতির সংশোধন:

১৩. জাতীয় জীবনিরাপত্তা নীতির ইংরেজিতে অনূদিত পাঠ প্রকাশ:

১৪. শব্দকোষ :

Towards Building Advanced Intelligent System for Agriculture

From Biosafety policy 2024: Annex

Information required concerning living modified organisms intended for direct use as food or feed, or for processing under article 11

a. The name and contact details of the applicant for a decision for domestic use.
b. The name and contact details of the authority responsible for the decision.
c. Name and identity of the living modified organism.
d. Description of the gene modification, the technique used, and the resulting characteristics of the living modified organism.
e. Any unique identification of the living modified organism.
f. Taxonomic status, common name, point of collection or acquisition, and characteristics of recipient organism or parental organisms related to biosafety.
g. Centres of origin and centres of genetic diversity, if known, of the recipient organism and/or the parental organisms and a description of the habitats where the organisms may persist or proliferate.
h. Taxonomic status, common name, point of collection or acquisition, and characteristics of the donor organism or organisms related to biosafety.
i. Approved uses of the living modified organism.
j. A risk assessment report consistent with Annex.
k. Suggested methods for the safe handling, storage, transport and use, including packaging, labelling, documentation, disposal and contingency procedures, where appropriate.

Annex

Risk Assessment

1. Objective

The objective of risk assessment, under this Protocol, is to identify and evaluate the potential adverse effects of living modified organisms on the conservation and sustainable use of biological diversity in the likely potential receiving environment, taking also into account risks to human health.

2. Use of risk assessment

Risk assessment is, inter alia, used by competent authorities to make informed decisions regarding living modified organisms.

General principles

3. Risk assessment should be carried out in a scientifically sound and transparent manner, and can take into account expert advice of, and guidelines developed by, relevant international organizations.
4. Lack of scientific knowledge or scientific consensus should not necessarily be interpreted as indicating a particular level of risk, an absence of risk, or an acceptable risk.
5. Risk associated with living modified organisms or products thereof, namely, processed materials that are of living modified organism origin, containing detectable novel combinations of replicable genetic material obtained through the use of modern biotechnology, should be considered in the context of the risks posed by the non-modified recipients or parental organisms in the likely potential receiving environment
6. Risk assessment should be carried out on a case-by-case basis. The required information may vary in nature and level of detail from case to case, depending on the living modified organism concerned, its intended use and the likely potential receiving environment.

Methodology

7. The process of risk assessment may on the one hand give rise to a need for further information about specific subjects, which may be identified and requested during the assessment process, while on the other hand information on other subjects may not be relevant in some instances.

8. To fulfil its objective, risk assessment entails, as appropriate, the following

(a) An identification of any novel genotypic and phenotypic characteristics associated with the living modified organism that may have adverse effects on biological diversity in the likely potential receiving environment, taking also into account risks to human health;
(b) An evaluation of the likelihood of these adverse effects being realized, taking into account the level and kind of exposure of the likely potential receiving environment to the living modified organism;
(c) An evaluation of the consequences should these adverse effects be realized;
(d) An estimation of the overall risk posed by the living modified organism based on the evaluation of the likelihood and consequences of the identified adverse effects being realized;
(e) A recommendation as to whether or not the risks are acceptable or manageable, including, where necessary, identification of strategies to manage these risks; and
(f) Where there is uncertainty regarding the level of risk, it may be addressed by requesting further information on the specific issues of concern or by implementing appropriate risk management strategies and/or monitoring the living modified organism in the receiving environment.

Points to consider

9. Depending on the case, risk assessment takes into account the relevant technical and scientific details regarding the characteristics of the following subjects:

(a) Recipient organism or parental organisms. The biological characteristics of the recipient organism or parental organisms, including information on taxonomic status, common name, origin, centres of origin and centres of genetic diversity, if known, and a description of the habitat where the organisms may persist or proliferate;
(b) Donor organism or organisms. Taxonomic status and common name, source, and the relevant biological characteristics of the donor organisms;
(c) Characteristics of the vector, including its identity, if any, and its source or origin, and its host range;
(d) Insert or inserts and/or characteristics of modification. Genetic characteristics of the inserted nucleic acid and the function it specifies, and/or characteristics of the modification introduced;
(e) LMO. Identity of the living modified organism, and the differences between the biological characteristics of the living modified organism and those of the recipient organism or parental organisms;
(f) Detection and identification of the LMO. Suggested detection and identification methods and their specificity, sensitivity and reliability;
(g) Information relating to the intended use. Information relating to the intended use of the living modified organism, including new or changed use compared to the recipient organism or parental organisms; and
(h) Receiving environment. Information on the location, geographical, climatic and ecological characteristics, including relevant information on biological diversity and centres of origin of the likely potential receiving environment.

Thanks to all

			
BARI Bt Begun 1	BARI Bt Begun 2	BARI Bt Begun 3	BARI Bt Begun 4