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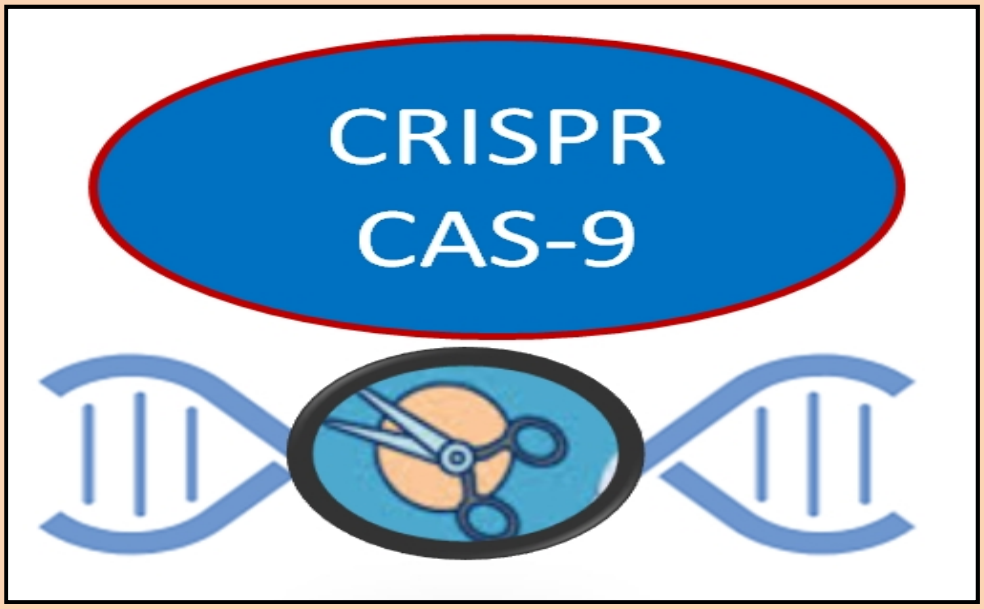
NEWS LETTER

ON

APPLICATION OF CRISPR/CAS9 IN THE ENVIRONMENT



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EDITORIAL

The story of CRISPR (Clustered Regularly Interspaced Short Palindromic Repeat) began in late 1980s, when such sequence was discovered in *E. coli*. Much later, it was realized that this is part of adaptive immune system of bacteria. Using CRISPR associated genes (cas), foreign DNA are cleaved and pasted in DNA known as CRISPR arrays. When a phage DNA invades the bacterial system, CRISPR RNAs (crRNAs) are expressed by some cas proteins, which guide cas nuclease to specific area of invader DNA assisted by protospacer adjacent motif (PAM). The CRISPR-cas complex binds the DNA at a specific location and cleaves it.

Later, it was realized that such DNA cleavage can also be programmed to insert desired sequences in specific sites – using DNA repair. This can happen via non-homologous end joining (NHEJ), or by homology directed repair (HDR). Since the second decade of the new millennium, things have moved quite fast. High fidelity cas systems, precise editing of DNA and RNA sequences, tagging for visualization and other purposes etc. have become routine. These have repercussions in research on epigenetics, drug research etc.

The articles in the present issue are a brief introduction, followed by application to plant systems and in environmental biotechnology.

Asoke Prasun Chattopadhyay
Subhankar Kumar Sarkar

IN THIS ISSUE:

Introduction to CRISPR/Cas9

CRISPR-Cas (Genetic Scissors) are adaptive immune response systems that protect prokaryotes from bacteriophages. It stands for Clustered Regularly Interspaced Short Palindromic Repeats. The discovery of the type II prokaryotic CRISPR "immune system" has allowed for the development for an RNA-guided genome editing tool that is simple, easy and quick to implement. CRISPR genome editing has revolutionized the biological sciences, from medicine to agriculture and industry, making it possible to edit the DNA sequence of any living organism.

Genome editing (also called gene editing) is a group of technologies that give scientists the ability to change an organism's DNA. These technologies allow genetic material to be added, removed, or altered at particular locations in the genome. Several approaches to genome editing have been developed. A recent one is known as CRISPR-Cas9, which is short for clustered regularly interspaced short palindromic repeats and CRISPR-associated protein 9. The CRISPR-Cas9 system has generated a lot of excitement in the scientific community because it is faster, cheaper, more accurate, and more efficient than other existing genome editing methods.

Two scientists who pioneered the revolutionary gene-editing technology are the winners of this year's Nobel Prize in Chemistry, who discovered CRISPR-Cas9. Emmanuelle Charpentier and Jennifer A. Doudna share the 2020 Nobel chemistry prize for their discovery of a game-changing gene-editing technique.

Jennifer Doudna is Professor of the Departments of Chemistry and of Molecular and Cell Biology at University of California, Berkeley and an Investigator of the Howard Hughes Medical Institute. Early in her career, she studied the structure and mechanism of ribozymes (enzymatic RNA molecules) and RNA-protein complexes. Now her research focuses on understanding how RNA molecules control gene expression in bacteria and eukaryotic cells, through CRISPR-Cas9 and RNA-mediated mechanisms, respectively. For her outstanding scientific contributions, she was elected into the American Academy of Arts and Sciences in 2002 and the National Academy of Sciences in 2003, and was awarded the 2015 Breakthrough Prize in the Life Sciences. In 2020, Dr. Doudna received the Nobel Prize in Chemistry for her contribution to the development of CRISPR-Cas technology as a method for genome editing.

Charpentier reported the discovery in 2011 and that year struck up a collaboration with Doudna. In a landmark 2012 paper in Science1, the duo isolated the components of the CRISPR–Cas9 system, adapted them to function in the test tube and showed that the system could be programmed to cut specific sites in isolated DNA. Their programmable gene-editing system has inspired a gold rush of countless application in medicine, agriculture and basic science-and work continues to tweak and improve CRISPR and to identify other gene-editing tools.

The Nobel Committee's selection of Emmanuelle Charpentier, now at the Max Planck Unit for the Science of Pathogens in Berlin, and Jennifer Doudna, at the University of California, Berkeley, puts an end to years of speculation about who would be recognized for their work developing the CRISPR–Cas9 gene-editing tools. The technology allows precise edits to the genome and has swept through laboratories worldwide since its inception in the 2010s. It has countless applications: researchers hope to use it to alter human genes to eliminate diseases; create hardier plants; wipe out pathogens and more.

Jennifer Doudna & Emmanuelle Charpentier
Source:https://www.nature.com/articles/d41586-020-02765-9

CRISPR / CAS9 mediated gene editing in plants for crop improvement and disease resistance

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Abstract

Improvement of agricultural production with the use of modern breeding technologies is needed to enhance the approach for nutritious food around the globe. Among the various new technology developed for enhancement of agricultural production, CRISPR/Cas genome editing enable effective alteration in crops, hence displaying positive outcome. Here, we aim to summarize the advancement in CRISPR/Cas9 and highlight the base-editing tools as an approach for the improved crop production.

Introduction

The modern agricultural techniques that are developed, clearly shows the relation between research and technology to improve crop quality and yield. Although the conventional methods of breeding has been rapid in comparison to what was two decades ago, is likely not able to cope with the increasing food requirement along with the environmental disputes around the globe. Until the breeding of plants remains solely dependent on searching of populace of plants having adequate alternative and on methods of conventional crossing strategies to initiate characteristics into distinct aimed plants, impediment for improvement of crop will still continue. Since the first utilization of CRISPR technology, it has revolutionized research in the field of life sciences. It is thought that CRISPR technologies has the potential to overcome these restrictions and advance the breeding of plants beyond the imagination done earlier (Gao, 2018). It has also been known to improve the shelf life of climacteric fruits which has been reported by Pathak et al.(2018) (**Fig 1**). CRISPR/Cas systems are differentiated chiefly into two classes, one possessing multi-subunit effectors, and the other having single protein effectors, and are further differentiated into six types on the basis of their signature proteins. Among them three are chiefly given importance, namely Type I-III which has signature proteins Cas3, Cas9 and Cas10, respectively. CRISPR/Cas9 is considered one of the most studied and extensively utilized CRISPR systems in plants. CRISPR/Cas9 systems operate by conserving the sequence of DNA from the plasmid or bacteriophage, by which the cell is invaded. Upon re-exposure to some specific plasmid or bacteriophage, the system utilizes the sequences of its transcribed RNA detect and a Cas nuclease is assisted to cleave the DNA. *Streptococcus pyogenes,* bacterium are the main sources of Cas9, and it has been detected as a nuclease having the capability to cleave at two active cleaving sites of the DNA i.e., one of each strand of the DNA. A single Cas9 protein can be remodelled to cleave targeted sites on the bacterium DNA (Esvelt et al., 2013; Jiang et al., 2013). Hence this mechanism of Cas9 has been widely used for the advancement of crop improvement and disease resistance which has been discussed below.

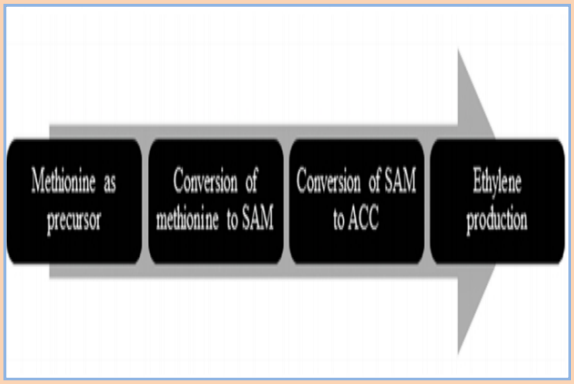

Fig 1: Schematic diagram of the steps involved in ethylene production (Pathak et al., 2018).

CRISPR technology, a promising tool for improved crop production:

The uses of CRISPR technology as promising tool in agriculture has already

been documented in plants. SDN-1 has been documented to be used in wheat, expecting to produce plants with resistance against the destroying powdery mildew. Use of SDN-3 (site-directed nuclease) to the Argos8 gene promoter in maize delivered essential character of the endogenous control gene, showed better results in yield in condition such as drought stress. SDN-1 has also been used in the aim of cause mutations in the modulatory sites of the tomato yield genes, leading to enhancement of genetic variation and yield. As the next generation of CRISPR approach has been initiated for agricultural uses, they can go further DSB-based (double-strand breaks) alteration in increasing the capability of CRISPR approach to particularly aimed sequence of DNA (Gao , 2018) (**Fig 2).** Removal of negative elements is a known technique in the field of genetics enhancement. Moreover, knocking out genes having unwanted characteristics is very common and a commonly used application of CRISPR/Cas9. Improved characteristics using CRISPR/Cas9 include enhancement in crop yield, enhancement in crop quality, and improved resistance of plants from both biotic and abiotic stress.

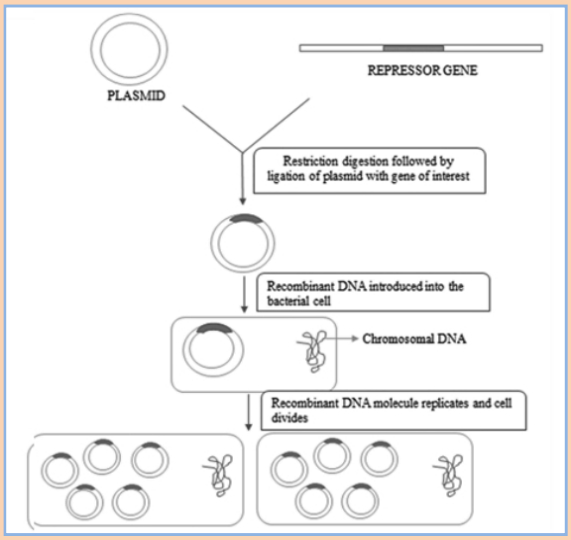

Fig 2: Mechanism of rDNA involved in the production of ethylene (Pathak et al., 2018).

- **Enhancement in crop yield**

The requirement of food security focuses production, the essential aim of gene editing for improving crops quality. Moreover, as production rely on numerous criteria's, knocking out negative modulators proved to have impact in yield-deciding criteria such as grain size (OsGS3), grain count (OsGn1a), panicle size (TaDEP1, OsDEP1), tiller number (OsAAP3) and grain weight (OsGW5, TaGASR7, TaGW2 and OsGLW2), and also generated the aimed phenotypes in crops with deterioration-of-function alterations in these genes, proving the capability of CRISPR/Cas9 in enhancement of crop yield (Chen et al., 2019).

- **Enhancement in crop quality**

The quality characteristics of crops alter depending on the particular breeding necessities. Till the present time the enhancement of crops by genome editing has influenced nutritional value, fragrance, starch content, and storage quality. Deletion of Waxy via CRISPR/Cas9 has enhanced rice cooking and eating quality. Studies has also shown that using CRISPR/Cas9 knockout waxy can generate corn line with higher production for commercial purpose (Ma et al.,2015; Waltz, 2016). Using CRISPR/Cas9 technology SBEIIb, a starch branching enzyme gene was mutated to generate higher amylose and resistant starch rice. Studies has shown that utilizing TALEN-aimed severance of OsBADH2, a fragrant rice line was prepared having the same 2-acetyl-1-pyrroline content of the naturally altered aromatic rice species. CRISPR/Cas9 alterations encourage a unique possibility to change characteristics monitored by families of large gene with superfluous characteristics. Evidence around the globe shows that knocking out of the required genes using CRISPR/Cas9 led a way for the production of crops with the desired characters. (Chen et al.,2019).

- **Resistance of plants from both biotic and abiotic stress**

One of the pivotal criteria involved in crop quality and yield is stress. Plants having enhanced biotic stress resistance, comprises bacterial, fungal, insects, and viral disease resistance is possible using

the CRISPR/Cas9 knockout technology. Studies has showed that utilizing CRISPR/Cas9 and TALEN technology, knocking out the TaMLO alleles in wheat was possible leading to production of plants having very high contention to powdery mildew fungus (Wang et al., 2014), also studies has showed to produce tomatoes having contention to powdery mildew (Nekrasov V et al., 2017). Blast-resistant rice production was also possible by knockout of OsERF922 (Wang et al., 2016). In case of viral diseases CRISPR/Cas9 technology is capable of production of broad potyvirus–resistant eif4e cucumber, eif4g rice with resistant to tungro-disease, and cotton leaf curl disease-contention clcud cotton. In case of abiotic stress, knocking out OsHAK1, OsNramp5, and OsARM1, rancher has produced strains of rice with decreased levels of arsenic, and radioactive cesium (Chen et al., 2019). Recent studies show that pyl1/4/6 triple knockout rice produced by CRISPR/Cas9 editing enhances higher tolerance towards temperature, grain yield, and also reduces pre-harvest of sprouting. (Miao et al., 2018).

Conclusion

The remarkable potential of CRISPR/Cas9 has initiated with rapid advancement in crop breeding and its research. The versatility, robustness, simplicity of this approach have made genome alteration an effective strategy for advancement of crop production. However, challenges have been faced for precisely shifting this approach from the bench site to the field. Hence by the approach of systems and synthetic biology will permit the development of crops with improved features in the fields.

Acknowledgments

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Application of CRISPR/Cas9 in Environmental Biotechnology

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Discoveries of CRISPR/Cas9 technology

The emergence of facile genome editing to manipulate or precisely modify the animal and plant genome using CRISPR/Cas9 technology is an indispensable tool in molecular biology (1). Although various genome engineering systems have been available since the 1970s, CRISPR by far is the most versatile, simple and precise method.

The discovery of the CRISPR editing system was started by a researcher, Francisco Mojica in 1993 to halophilic archaea (haloarchaea). He found an unexpected pattern – multiple copies of partially palindromic 30 bases long repeated sequence at regular interval (2). This regularly spaced repeat sequence had also been shown in bacteria, *Escherichia coli* (3) and *Mycobacterium spp* (4) although having no sequence similarity to haloarchaea (2). These repeat elements were termed as **s**hort **s**equence **r**epeats (SSR) (5), **s**pacer **i**nterspersed **d**irect **r**epeats (SPIDR) (6) or **s**hort **r**egularly **s**paced **r**epeats (SRSRs) (7). Later, the name CRISPR (**c**lustered **r**egularly **i**nterspaced **s**hort **p**alindromic **r**epeats) was accepted because of simplicity and it contemplated the major characteristics. (6).Several studies have shown that CRISPR/Cas is an adaptive bacterial immune system that protects against viral genetic material and contains a target specific protospacer that are expressed as CRISPR RNAs (crRNAs). During the CRISPR/Cas-mediated immunity, this crRNAs form a ribonucleoprotein (RNP) complex with Cas protein and cleaves invading viral DNA (or RNA) via base pairing of crRNA and target nucleic acids (8, 9). There are three distinct types of CRISPR/Cas system - type I, type II and type III. Types I and III are found in both bacteria and archaea and they are distinguished by Cas3 and Cas10 proteins respectively, whereas type II is only found in bacteria and Cas9 protein is the critical component in this system(10).

Mechanism of action of CRISPR/Cas9-mediated Genome engineering

In type II CRISPR system, Cas9 protein forms an RNP complex with dual guide RNA made up of crRNA and a trans-activating crRNA (tracrRNA) which creates double strand break in the DNA (1, 11). The dual tracrRNA:crRNA was fused into a single guide RNA (sgRNA) to generate a simple two-component system in which, by changing sgRNA sequence toCas9, one can edit any DNA sequence of interest (12, 13, 14). The DNA cleavage by Cas9 enzyme creates two endogenous repair pathways, Homology-Directed Repair (HDR) and Non-Homologous End Joining (NHEJ). By exploiting these two DNA repair pathways, we can generate gene knock-outs or knock-in at the site of cleavage (Figure 1).

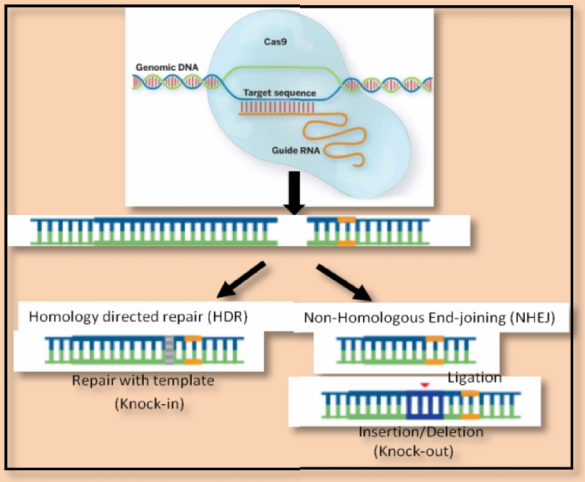

Fig 1. CRISPR/Cas9 gene targeting system

This simple CRISPR/Cas9 editing system has enabled a remarkable advancement in genome editing field to precisely edit genomes in a wide array of cells and organisms in a cost-effective manner. These characteristics make CRISPR/Cas9 technology a tremendously powerful tool to transform the field of basic science, biotechnology, and medicine. Remarkably, this invaluable technology is also helping

scientists to address the complex environmental challenges to protect the environment and human health (Figure 2).

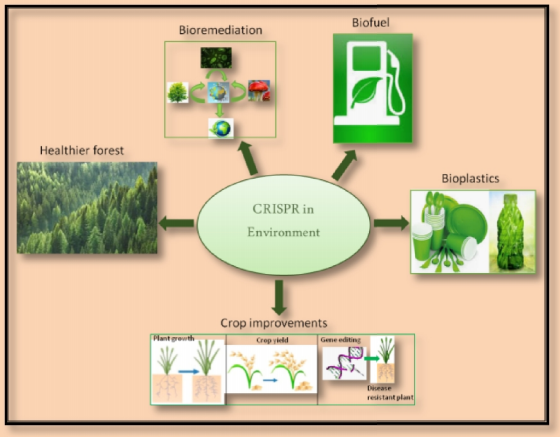

Fig 2. Implementation of CRISPR/Cas9 technology in agriculture and industrial biotechnology.

Applications and advancements of CRISPR/Cas9

Biofuels: To find sustainable solutions for the constant depletion of fossil fuel, biofuels, produced from biomass, are very promising alternative renewable energy resource and are environment-friendly. Plants, algae material or animal waste naturally produce byproducts such as sugars, fats or alcohols which are all potential alternative fuel sources. Usage of CRISPR techniques in biofuel production has remarkable benefits. For example, recently scientists were able to produce double the amount of biodiesel from phototrophic algae by using CRISPR genome editing technique (15). Yeast plays a critical role in the fermentation process and the pre-treatment chemicals in this process are very harmful to yeast. Current research work showed that CRISPR-modified yeasts are resistant to those chemicals during the biofuel production (16). CRISPR-modified acetogenic bacteria has also shown improved efficiency of ethanol production in commercial scale (17).

Bioremediation and Bioplastics: Plastics are non-biodegradable and one of the major sources of environmental pollution. Bioplastics are the alternative source of these toxic pollutants and they are essentially derived from natural biomass, such as cellulose from plants, bacterial cellulose and microalgae. The limitations of these bioplastics are cost and water resistance. However, scientists are using CRISPR/Cas9 technology to modify the carbon chain length to make bioplastics more abundant and cost-effective (18). Further, microorganisms or plants could be engineered using CRISPR for efficient bioremediation and phytoremediation, such as cleaning up oil spills or improving wastewater treatment and taking up heavy metals from soils and water.

Crop improvement: Using CRISPR/Cas9 technologies, we can make our agricultural practices more effective and ethical. Precise genetic manipulation of important agricultural crops generates ideal germplasms, which improve several crops characteristics, including high yield, overall quality, resistance to environmental challenges or microbial infection, which ultimately ameliorate food security while avoiding the introduction of foreign DNA into the food (19). Here are some examples of CRISPR-mediated editing of crops (Table 1).

Healthier forests: Trees are excellent to sequester carbon and tackle the climate crisis. Trees can be vulnerable through viral diseases, like Citrus Tristeza virus (CTV), or bacterial pathogens such as Citrus Greening (also known as Huanglongbing or HLB-disease) and put an entire species at risk. Breeding a tree in a classical method may take decades. On the other hand, CRISPR-based technologies can reduce that time to 1-2 years and hold a great promise to improve quality of trees of interest across the world, which eventually will create more sustainable, healthier, climate- and disease-resistant tree breeders and healthy forests (20). In addition, CRISPR/Cas may edit mitochondria and chloroplasts genome and trace cell lineages to explain the patterns of plant development and detect internal and external signals for other applications in plant synthetic biology (19). Thus CRISPR/Cas technology has undoubtedly reformed and will continue to

refashion both agriculture and plant biotechnology.

Environmental health and toxicology research

Genome editing tools can be used to identify toxic environmental contaminants and their exposure in public health. It advances the researcher's ability to study mechanisms of toxicity in the genomic and epigenomic pathways. For example, using genome-wide CRISPR screening, scientists were able to pinpoint the most important mechanism involved in arsenic trioxide, which is the number one environmental toxic substance according to the 2017 Agency for Toxic Substances and Disease Registry priority list. The genome-wide CRISPR screen identified 100 candidate genes either linked to sensitivity or resistance to arsenic trioxide and its exposure confirmed the increase of reactive oxygen species in cellular content (21).

Limitations and Government regulations

Despite the great potential of CRISPR/Cas technology in the improvement of agriculture and environmental challenges, it has few major concerns of its efficiency and specificity- (i) one possibility of off-target effects because the 20-bp targeting gRNA sequence and 3 bp PAM motif (3 bp) may be present in other parts in the genome (22).(ii) The second challenge is to increase the efficiency of HDR-mediated repair while reducing NHEJ repair path (23).(iii) In addition, a clear understanding of function of Cas9 enzyme is important since different types of Cas9 from different bacteria functions differently, e.g., SpyCas9 from *Streptococcus pyogenes* finds protospacer by search through billions of base pairs and significantly bend the DNA during this process (24). Therefore, it is absolutely imperative to gain the precise control of Cas9 activity in order to have safe and efficient genome editing in animals and plants.

The incredibly easy and cheap CRISPR editing tool has enormous potential for use in various applications such as clinical treatments to agricultural production to climate change. The expeditious progress in this technology has introduced concerns about its safe, secure, and ethical applications. Surely, genomic engineering of crops and livestock has the potential to escalate food production, but it may also affect ecosystems. Unless we have a clear view of the ecological impact of these genetically modified organisms and plants, we should use the technology in a responsible manner. Further, all the genetic modifications in plants and agriculture must be taken care of by government regulatory policy to protect our environment and human health. Additionally, genetically modified crops should assess the impact in long-term for environment and food safety before it is available commercially. It is also wise to label them as GMO so that they would be traceable and publicly acceptable in the benefits to global food security.

Table 1: Applications of CRISPR/Cas9 technology in agricultural crops

Crop species	Target genes	Target traits	References
Rice	GW2, GW5, TGW2	Increased grain size	25
Rice	PYLs	Improved plant growth and production	26
Wheat	Gliadings	Reduced gluten content and exposure	27
Tomato	DMR6, MLO1	Resistance against downy and powderymildew	28, 29
Tomato	Pl, ALC	Long-shelf-life tomato	30, 31
Apple	DIPM1, 2, 4	Resistance against fire blight disease	32
Banana	ORF region of virus	Resistance against banana streak virus	33

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Report of Webinar on 'Promotion of Science'

A webinar on 'Promotion of Science' in the area of Genetic Scissors (CRISPR-Cas9) was organized on 15th October, 2020 by University of Kalyani in collaboration of ENVIS RP on Environmental Biotechnology, DST PURSE -II, NASI and INSA under the banner of "Promotion of Science: Recent developments in interdisciplinary Science such as Physical, Biological including Agriculture, Health and Environmental Science".

The welcome address was given by Prof. Rita Ghosh, Coordinator, DST PURSE II, KU. The webinar was inaugurated by Hon'ble Vice Chancellor, University of Kalyani, Prof. Sankar Kumar Ghosh, in the virtual presence of dignitaries and eminent speakers Prof. Indrajit Lahiri, Dean, PG Faculty of Science, Prof. Rita Ghosh, Coordinator, DST PURSE II; Prof. Asoke Prasun Chattopadhyay, Coordinator, ENVIS RP; Prof. Swapan K Datta, Former VC, Visva-Bharati, Ex-Deputy Director General, (Crop Science), ICAR; Prof. Amit Ghosh, Ex-Director, IMTECH, J. C. Bose Distinguished Chair Professor, National Academy of Sciences, India; Prof. Hemanta K. Majumder, Senior Scientist NASI, IICB-CSIR, Kolkata, Conveners, NASI & INSA, Kolkata Chapter and Dr. Anindya Bandyopadhyay, Vice President, Reliance Industries Limited.

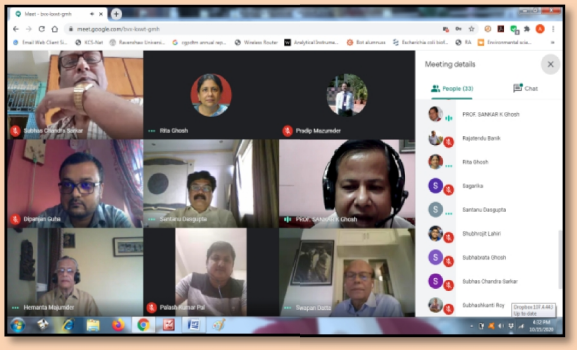
This webinar was organized keeping in the mind on the recent Nobel Prize on CRISPR –Cas9, a method of genome editing. The announcement of this year's Nobel Prize in Chemistry to Prof. Jennifer A Doudna and Prof. Emmanuelle Charpentier for their work on "a method of genome editing" has focused attention to the method called CRISPR. Using this technology, commonly termed as "molecular scissors", plasmid or vector DNA can be suitably modified to produce organisms expressing genetic materials of choice. This opens up enormous possibilities for modifying genetic materials and seeing their effects.

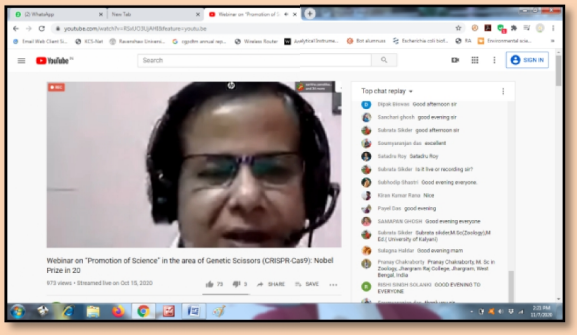
In the webinar students, research scholars and faculties were interacted with the eminent experts.

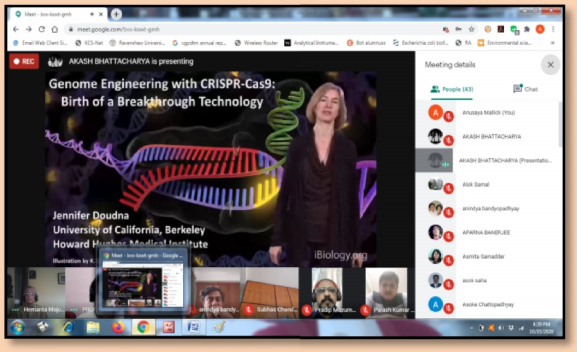
There are 382 participants were participated through google meet and YouTube channel

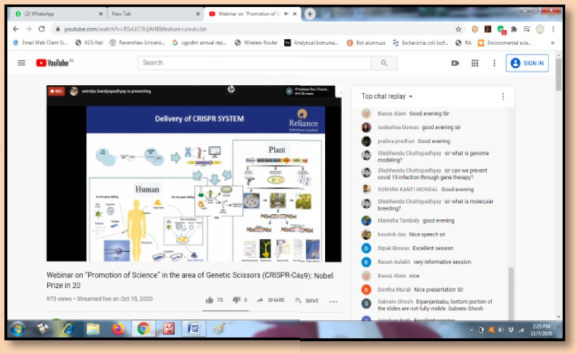
(https://www.youtube.com/watch?v=RSxUO3UjAHI&feature=youtu.be).

Lastly the concluding remarks and vote of thanks was given by Prof. Asoke Prasun Chattopadhyay, ENVIS coordinator.









FORTHCOMING EVENTS		
Events	**Date**	**Place & Correspondence**
3rd International Virtual Conference "The Ecology Of New Economy Post COVID-19"	23rd to 24th April 2021 Online	Chandigarh, India http://cgc.ac.in/international-conference-2021
International Workshop on Technology Innovation of Algae	1st to 3rd May 2021 Online	Taleghani Street, Tehran, Iran https://iranalgae2021.inacc.ir/
02nd International Conference on Marine Science and Technology	24th to 24th July 2021	Aachen, Germany http://sustainableconference.science/aachen/
2nd International Conference on Biomedical Engineering, Bio-Informatics and Design & Software Applications	24th to 25th July 2021	langkawi, Malaysia https://aniceas.com/conferences/bbds-july-2021/
2021 International Agriculture Innovation Conference	3rd to 4th September 2021 Online	TOKYO, JAPAN https://iaic2021.iaas.org.sg/

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