Genome Editing and Engineering
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Module - 05
Zinc Finger Nuclease (ZFN) Technology
Lecture - 12
Applications of Zinc Finger Nucleases - PART A

Welcome to my course on Genome Editing and Engineering. We are discussing Module 5, Zinc Finger Nuclease Technology. In today's lecture, we will discuss about the various Applications of Zinc Finger Nucleases.

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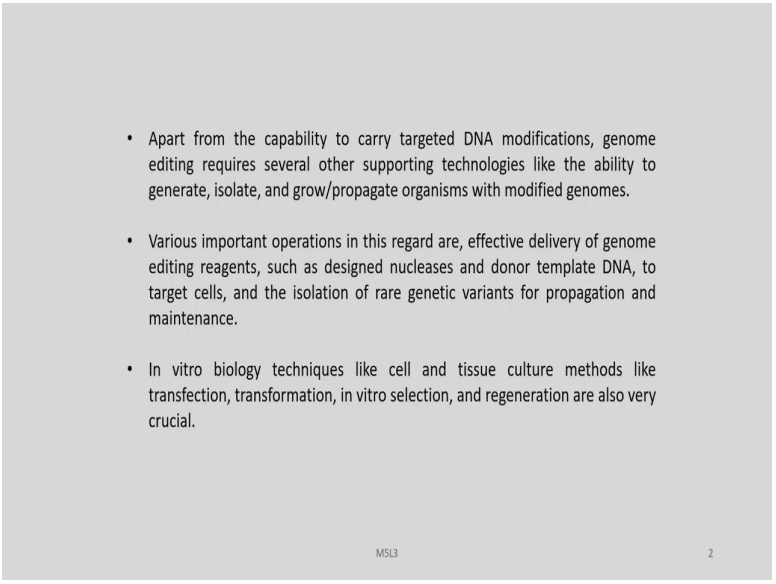

So, till now, you have learnt about the structure of zinc fingers, how they bind to DNA on a sequence specific manner and then we have also learned how the zinc finger nucleases are engineered, and how these engineered zinc finger nucleases are delivered to the cells for genome editing and engineering.

The technology has huge potential. Apart from the ability to carry targeted DNA modifications, genome editing requires several other supporting technologies like the ability to generate, isolate, and grow and propagate organisms with the modified genomes.

There are various important operations which need to be carried out in this regard, like the effective delivery of the genome editing reagents, the design nucleases and the donor template DNA to target cells and the isolation of the rare genetic variants which are generated in the process for propagation and maintenance.

Several in vitro biology techniques; like cell and tissue culture methods like transaction, transformation, in vitro selection, and regeneration are does very very crucial for the entire process of genome editing and engineering.

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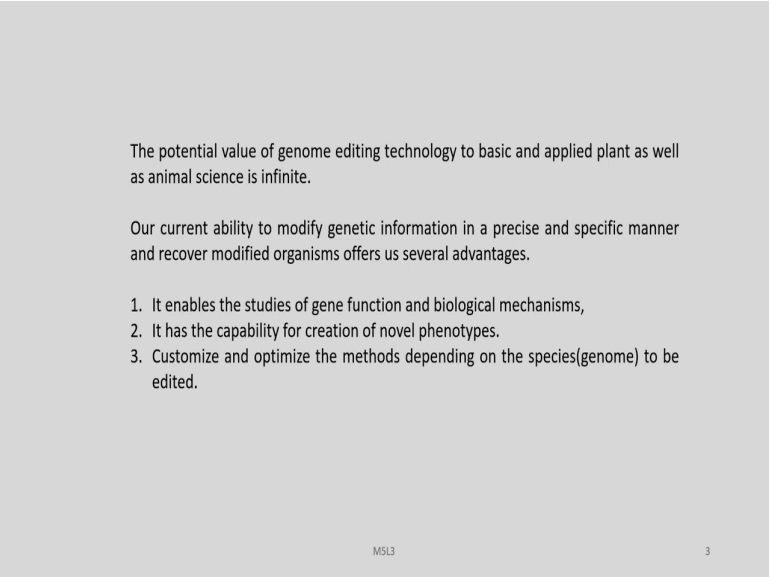

The potential value of this technology to basic and applied plant and animal sciences as well as microbes is infinite. Our current ability to modify genetic information in a precise and specific manner and recover modified organisms does offer us several advantages.

For example, it enables the studies of gene function and biological mechanisms. For example, we can knock out a gene and we can knock in a gene and can understand the exact role of the gene in imparting a particular type of phenotype. Besides that, it has the capability of creating novel phenotypes which are unknown in the wild type organism. Then customize and optimize the methods depending on the species to be edited.

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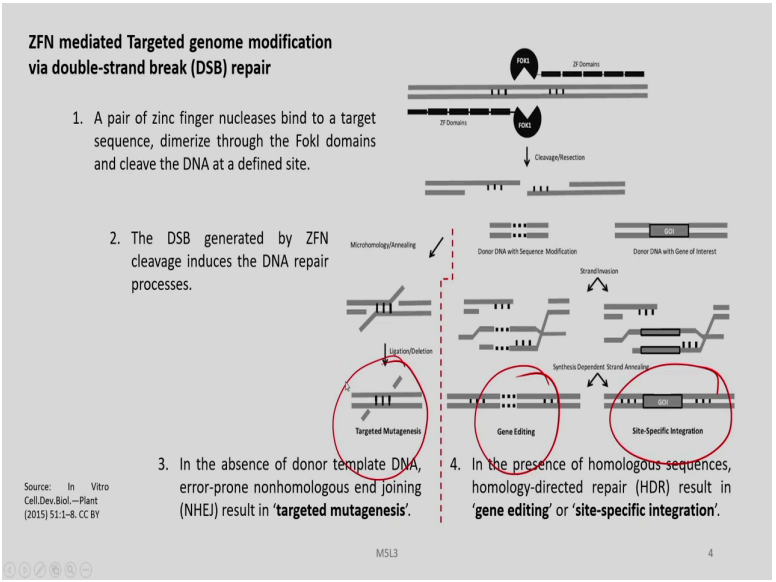

So, you have learnt about the various recombination methods, the homologous recombination and the non-homologous end joining and you have some idea how these repair mechanisms are occurring in nature and also how they help in the genome editing process. So, let us have a brief, you know, revision of these methods with respect to the zinc finger nuclease mediated targeted genome modification and how these are done through the double strain break repair.

So, you can see in step 1 here, there is a pair of engineered ZFNs which bind to a target sequence and the nuclease domains dimerize through the FokI domains and cleave the DNA at a defined site. So, this is the cleavage which is happening as a result of the FokI dimerization. In the last lectures, we discussed how these dimerization occurs.

The double strength break that is generated by these ZFN cleavage will induce the repair process which is known to you and it may follow either of the two parts the non homologous end joining or the homologous repair pathway. So, in the absence of donor template, error prone non-homologous end joining will result in the targeted mutagenesis. So, here the important concept is the microhomology/annealing and then the ligation or the deletion and the final output is the targeted mutagenesis.

In the presence of homologous sequences, so, you have a donor DNA here which is having a sequence modification or we may have altogether a new gene of interest. And, these are having homologous sequences flanking the zone of interest and in the presence of these

homologous sequences, the homology-directed repair or HDR occurs and these leads to either genome gene editing or site specific integration where a new gene is integrated.

So, these are the methods in brief by which ZFN is utilized for genome editing and engineering. So, in this case, this is editing and these are examples of engineering where you have you know incorporated a whole gene or caused some mutation in the wild type gene.

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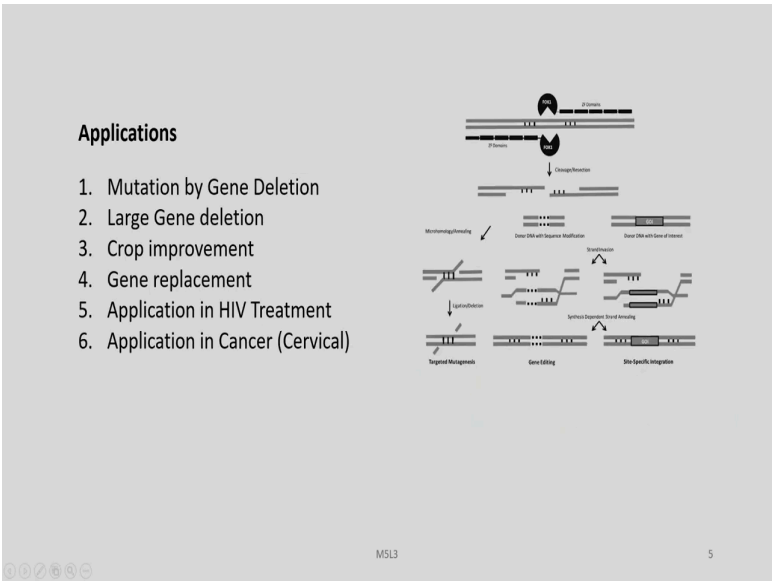

So, what are the various applications? As I have told you in the beginning, the potential of ZFN in engineering and editing plant, microbe, animal genomes are infinite and there are innumerable number of cases, successful cases as well as, you know, kind of trials where ZFN has been utilized. Due to lack of time, we cannot discuss this in every one of such cases; we are taking some representative cases to understand the flexibility as well as the power of these technology platform.

So, we will be discussing about small mutations which happens due to gene deletion, then we will take a case of large gene deletion. And, some cases where they are applied; for example, in crop improvement. And, then also examples where entire genes are being replaced and, then pertaining to human diseases like the application in HIV treatment, as well as the application in cancer.

In cancer, there are so many different types and lot of research has been going on we will be discussing as a case study only one type of cancer, the cervical cancer in which ZFN technology has been used as a therapeutic approach.

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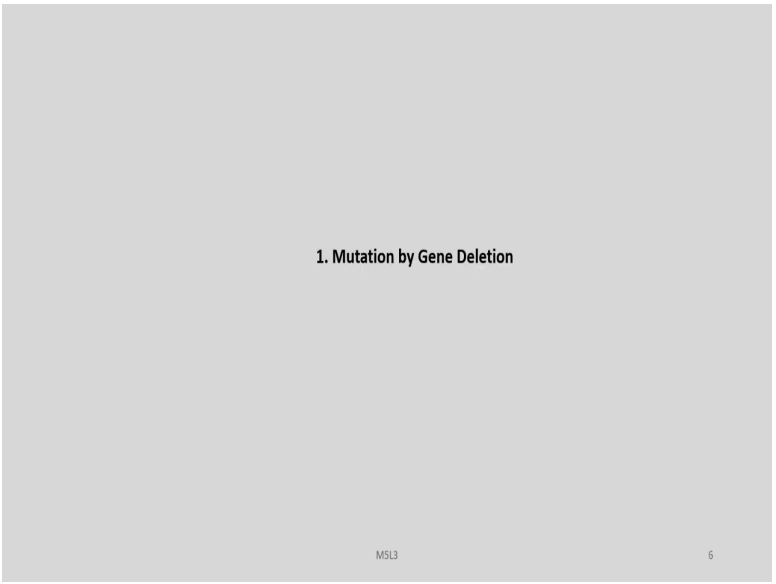

Let us start with mutation by gene deletion.

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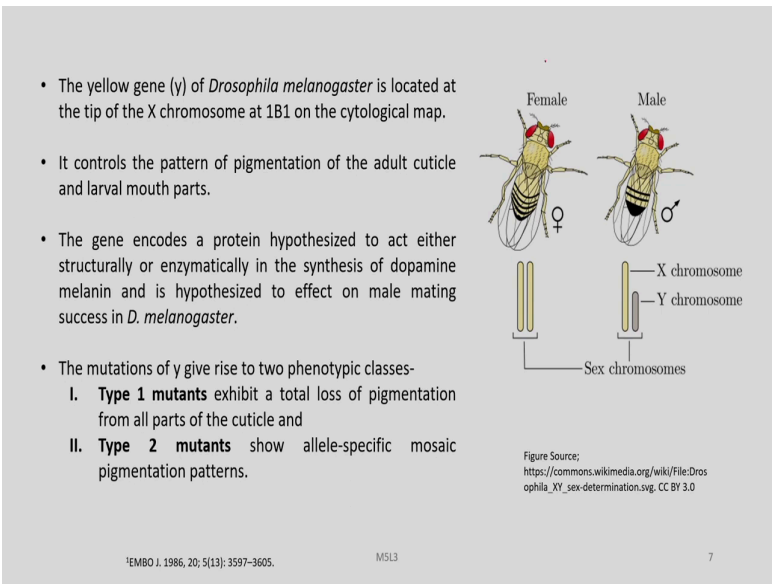

So, this is a model fly drosophila melanogaster and in these drosophila melanogaster, there is a gene called yellow gene which is located at the tip of the X chromosome, ok, and the locus of these particular chromosome is particular gene is 1B1 on the cytological map.

This particular gene controls the pattern of pigmentation of the adult cuticle and also the larval moth parts. And, these gene basically encodes a protein which is hypothesized to act either structurally or enzymatically in the synthesis of dopamine melanin and is hypothesized to effect on male mating success in drosophila melanogaster.

The mutations of these yellow gene gives rise to two type of phenotypic classes. There is a type 1 mutant and a type 2 mutant. The type 1 mutant exhibit a total loss of pigmentation from all parts of the cuticle and the type 2 mutants show allele specific mosaic pigmentation patterns.

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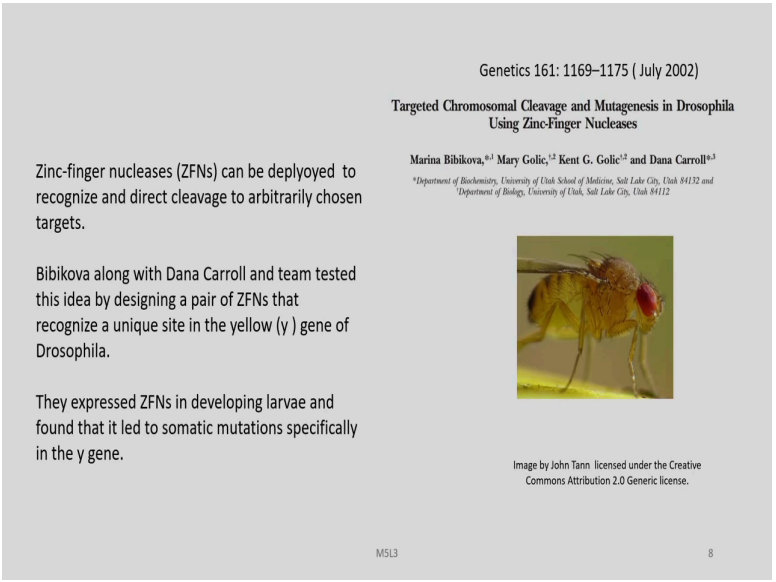

So, this is a very interesting work by Bibikova and associates including Dana Carroll, where they carried out targeted chromosomal cleavage and mutagenesis in drosophila using zinc finger nucleases. So, this work was published in this journal Genetics in the year 2002.

So, these zinc finger nucleases were deployed by Dana Carroll and a team to recognize and direct cleavage to arbitrarily chosen targets. For example, they use these approach by designing a pair of ZFNs that recognize a unique sight in the yellow gene of drosophila which we discussed in the previous slide. So, while targeting these particular yellow gene, they

expressed the ZFNs in developing larvae and found that it lead to somatic mutations specifically in the y gene.

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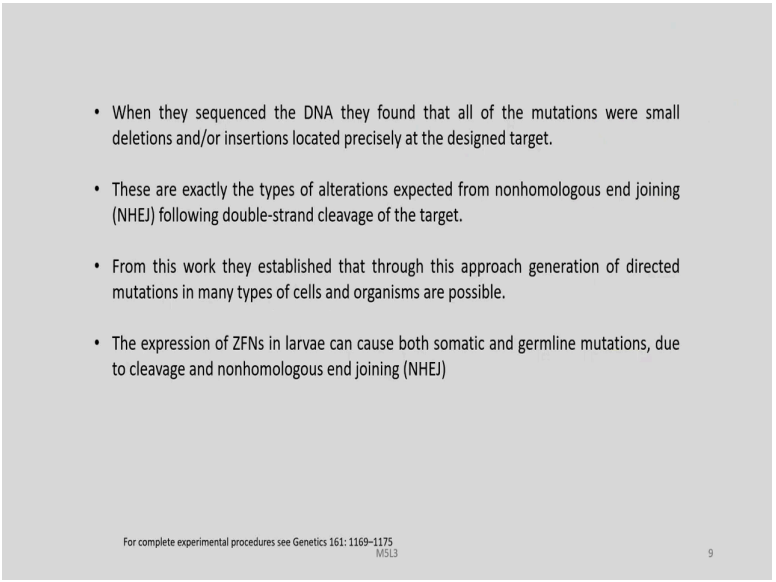

And, when they sequenced the DNA location, they found that all the mutations were small deletions and or insertions located precisely at the design target. So, this is a very successful experiment which build up the confidence that ZFN can be used for targeted deletions.

And, these were exactly the type of alterations which were expected from non homologous end joining following the double strand cleavage of the target. And, this work established that these approach can generate directed mutagenesis in many types of cells and organisms in which these ZFNs can be deployed.

The expression of the ZFNs in larvae can cause both somatic and germline mutations, due to cleavage and nonhomologous end joining.

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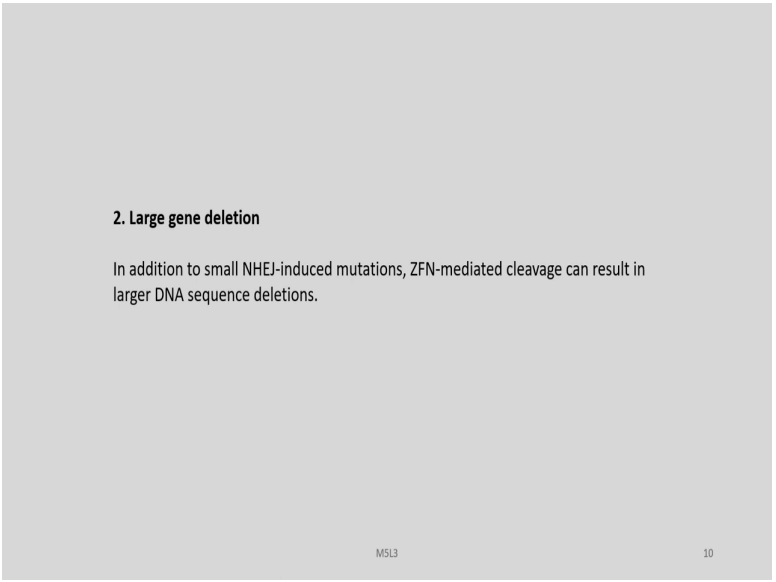

So, with these particular experiment, confidence was gained on the capability of ZFN technology and soon, it was applied for deleting large fragments of DNA or large gene deletions. So, in addition to the small nonhomologous end joining induced mutations, ZFN mediated cleavage can also be used for DNA sequence large DNA sequence deletions.

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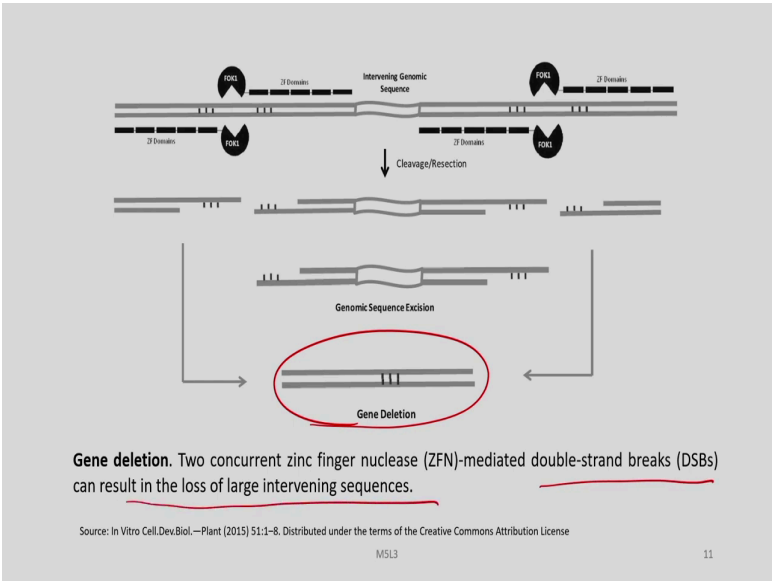

So, this is a schematic for large DNA segment deletion. So, you can see here, there are two pairs of engineered ZFN. So, one pair are here and they bind in a location where they have a common target and where the Folk1 nucleus domains will dimerize and cleave. And, then the

second pair of the engineered ZFNs binding another site which is farther away from the previous site and they also the dimerization of the nucleus takes place and cleavage follows.

So, it is obvious that if we have a cleavage here and a cleavage here, these particular intervening sequence will be lost or deleted. And, that is exactly what is happening over here as you can see due to this cleavage reaction, this large fragment is now lost and excised from the system. And, these sequences are coming together and joined and so, in these final product, we have a large portion which is deleted as a result of these engineered ZFNs which target two different locations spaced quite apart.

So, the two concurrent zinc finger nucleases mediates the double strain breaks and this results in the loss of this large intervening sequence.

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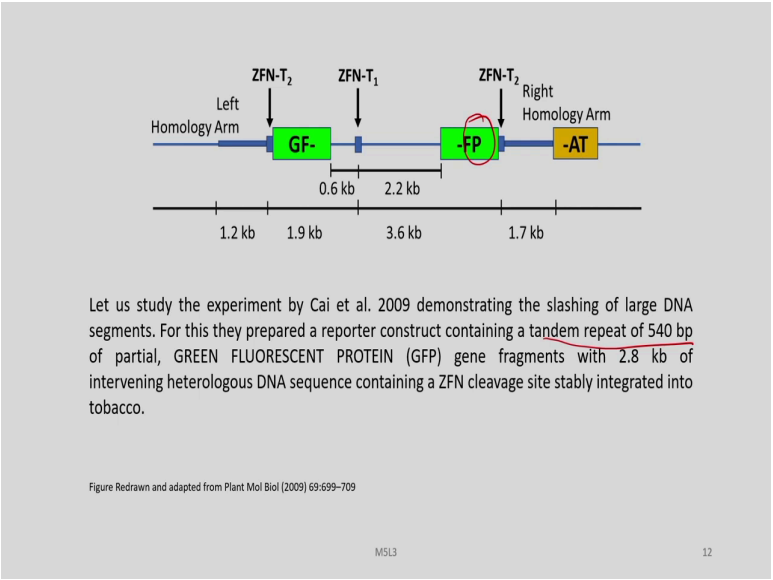

So, this was carried forward to be demonstrated by Cai et al. through certain experiments which we will discuss here in little detail. So, this is kind of slashing and cutting, you know, big chunks of DNA from chromosomes.

So, for their experiment, Cai et al. prepared a reporter construct which contain tandem repeats of 540 base pairs of partial, green fluorescent protein. So, these partial green fluorescent protein, you can see, you have only some portion of the gene GF, the C terminal for example, here a large chunk of information is missing and here you have only FP part, this is just a

schematic representation to show a partial green fluorescent protein and here you can see for example, a large chunk of the N-terminal domain is missing.

So, these partial green fluorescent proteins would not be functional. So, we will not be able to visualize the green fluorescent protein in its active form. Now, these partial GFPs are spaced apart as you can see over here by some kind of intervening sequence.

So, for example, we have these intervening sequence over here and this is the intervening sequence over here and if you remember this. So, our idea is to delete these intervening sequence using ZFN technology. And, for this reason we have certain ZFN target sites including one site in the intervening sequence and you can see here the genomic distances and then this intervening sequence is around 2.2 plus 0.6 which is around 2.8 kilo bases. And then, in these entire construct, you can see some left homology arm and the right homology arm.

So, these tandem repeat of 540 base pairs of partial green fluorescent protein gene fragments have intervening of 2.8 kb of heterologous DNA sequence and this contain a ZFN cleavage site integrated and this is expressed in a crop tobacco.

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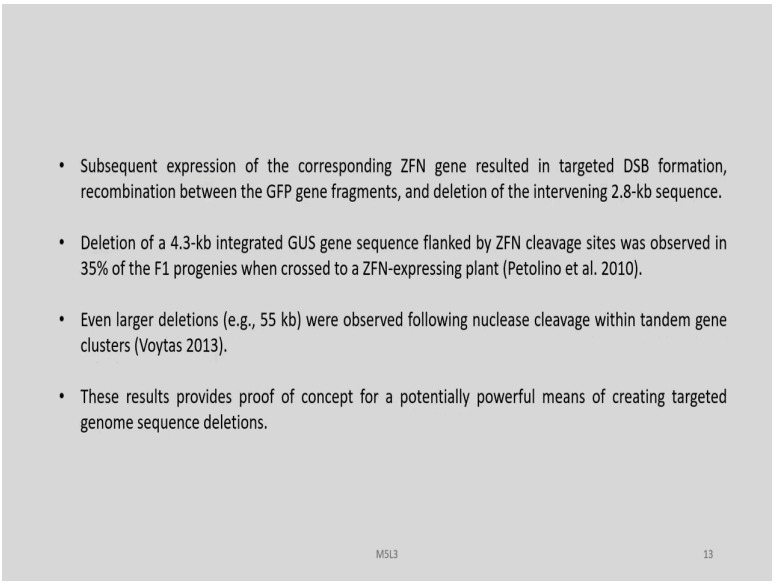

Subsequently, upon expression of the corresponding zinc finger nucleus gene, we have targeted DSB, Double Strand Break formation, and then there will be recombination of the

GFP gene fragments which will show you in the next slide and the deletion of the intervening 2.8 kb sequence.

So, deletion of 4.3 kb integrated GUS gene sequence flying by ZFN cleavage sites was also observed in 35 percent of the F1 progenies when they were crossed to ZFN expressing plants and then even larger deletions were observed following nucleases cleavage within tandem gene clusters.

So, finally, these results gave proof of concept for a potentially powerful means of creating targeted genome sequence deletions of large fragments of DNA.

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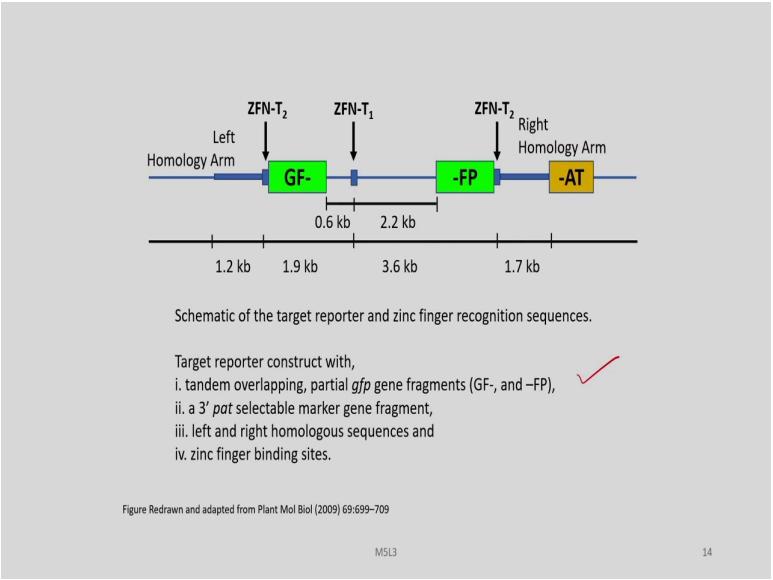

So, here you have the target reporter construct with tandem overlapping partial GF gene fragments, GF minus and dash FP and then you have a 3 prime pat selectable marker gene fragment and left and right homologous sequences and you have many zinc finger binding sites.

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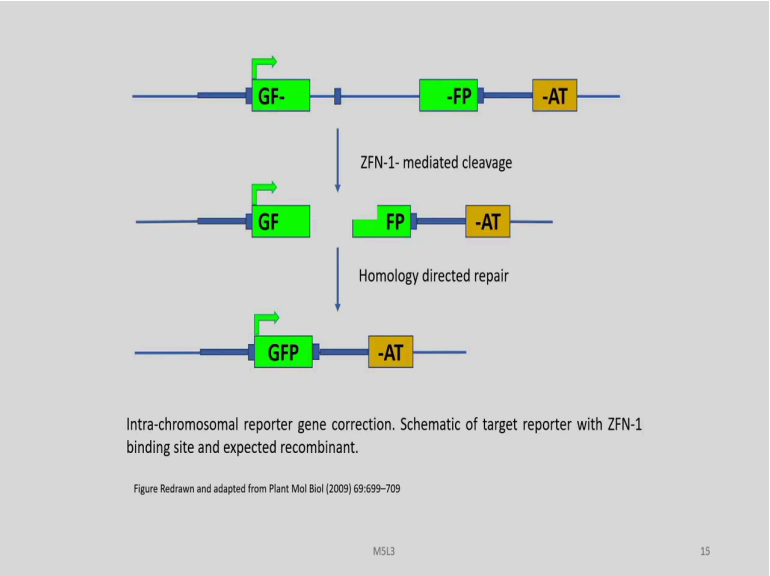

So, upon ZFN mediated cleavage, these fragment will be lost, ok and then the homology directed repair will be taking over due to which these partial genes are joined together to form a complete gene. And, we can now visualize the functionality of this particular protein GFP and we can select the mutants where these large deletions has been carried out.

So, this is the intra-chromosomal reporter gene correction schematic of the target reporter with ZFN – 1 binding side and the expected recombinant. So, these are only some of the technology development part and this has been deployed in various cases to generate different type of large gene deletions, ok.

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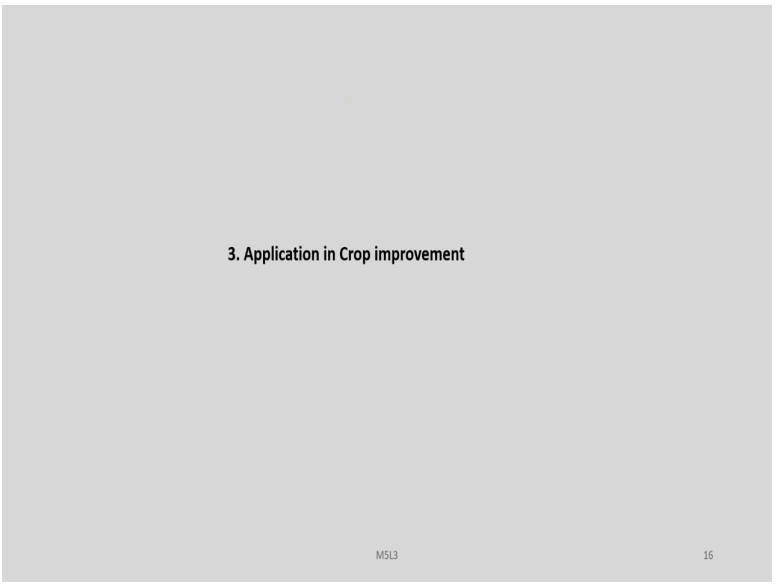

Let us now discuss some of the applications of ZFN in crop improvement.

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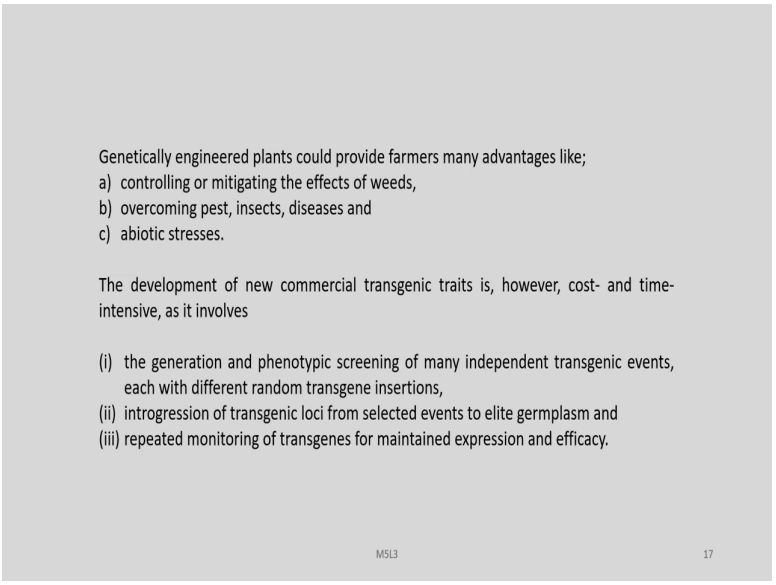

So, genetically engineered plants could provide farmers many advantages. For example, controlling or mitigating the effects of weeds, overcoming pest, insects, diseases and abiotic stresses.

The development of new commercial transgenic traits, however, is cost- and time- intensive, as it follows the generation and phenotypic screening of many independent transgenic events,

each with different random transgenic insertions, introgression of transgenic loci from selected events to elite germplasm and repeated monitoring of transients for maintained expression and efficacy.

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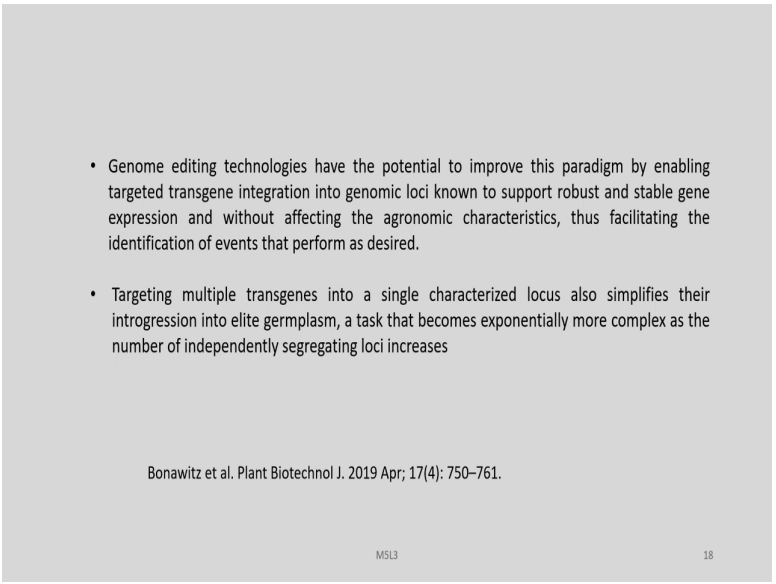

Genome editing technologies have the potential to improve this paradigm by enabling targeted transient integration into genomic loci known to support robust and stable gene expression and without affecting the agronomic characteristics, and facilitate the identification of events that perform as desired.

Targeting multiple transients into a single characterized locus also simplifies their introgration into elite germplasm, which is a task that otherwise becomes exponentially complex as the number of independently segregating loci increases.

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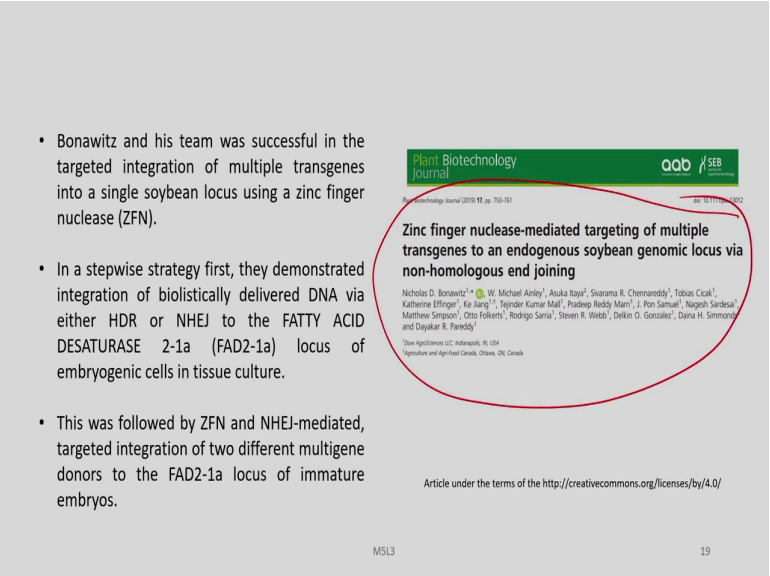

So, in this particular work Bonawitz and his team, I mean very big team, they used zinc finger nucleases for targeting multiple transgenes to an endogenous soybean genomic locus via non homologous end joining.

So, they carried this work in a stepwise manner and as a strategy, first, they demonstrated integration of biolistically derived DNA via either HDR or NHEJ to the fatty acid desaturase 2-1a locus of embryogenic cells in tissue culture. And, this was followed by ZFN and non homologous end joining mediated targeted integration of two different multi gene donors to the FAD 2-1a locus of immature embryos.

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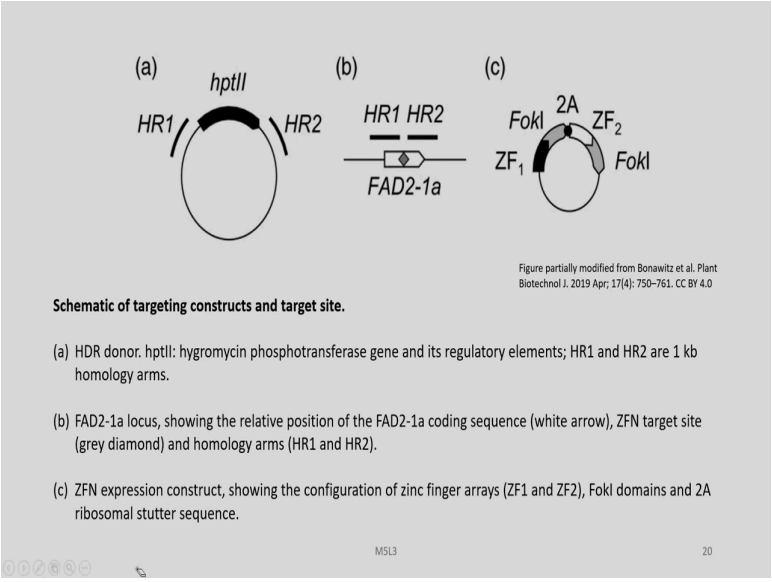

So, these are some of the schematics of the targeting constructs and target side. So, here you have a, the HDR donor and these hpt is the hygromycin phospotransferase gene and its regulatory elements. Then HR1 and HR2 are the 1 kb homology arms flanking the hpt gene.

In figure b, you can see FAD 2-1a locus and this is showing the relative position of the FAD 2-1a coding sequence, this particular white arrow. And, ZFN target site, this particular diamond and these are the homology arms 1 and 2 flanking this particular region.

Then, this is the ZFN expression construct. I hope you remembered about the 2A sequence that we discussed in the last class. So, you have here zinc finger 1 attached to a FokI gene sequence and zinc finger 2 attached to another FokI zinc sequence and these, presence of the 2A gene will ensure that the two constructs ZFN 1 and ZFN 2 are expressed separately.

So, these gene expression/ZFN expression constructs. So, the configuration of the zinc finger arrays folk one domains and the 2A ribosomal stutter sequence or the ribosomal skipping sequence.

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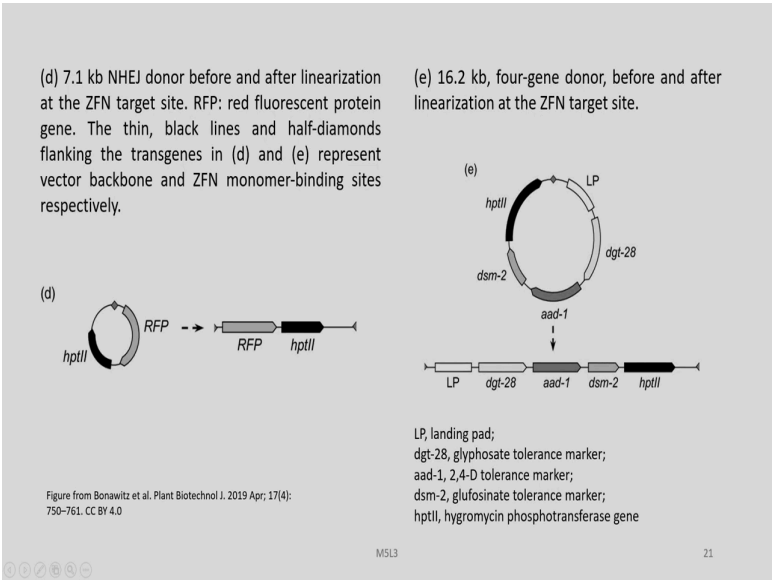

In figure d) you can see the 7.1 kb NHEJ donor before and after linearization. So, this is the same gene when this is linearized here. So, you have the sequence of this gene in this particular order. So, RFP is basically a red fluorescent protein which is something like the green fluorescent protein, but this particular protein will show red fluorescence and these thin black lines and half diamonds flanking the transgenes in d) and e) represent vector backbone and ZFN monomer binding sites respectively.

In figure e), you can see the 16.2 kb four-gene donor. So, we are targeting multiple transients here. So, these are the four-gene donors before and after linearization and the ZFN site. So, you can see this is the landing pad LP, then you have the four-genes dgt-28 glyphosate tolerance marker, aad-1 which is a 2, 4-D tolerance marker and then dsm-2 which is glufosinate tolerance marker and hptII, hygromycin phospotransferase gene.

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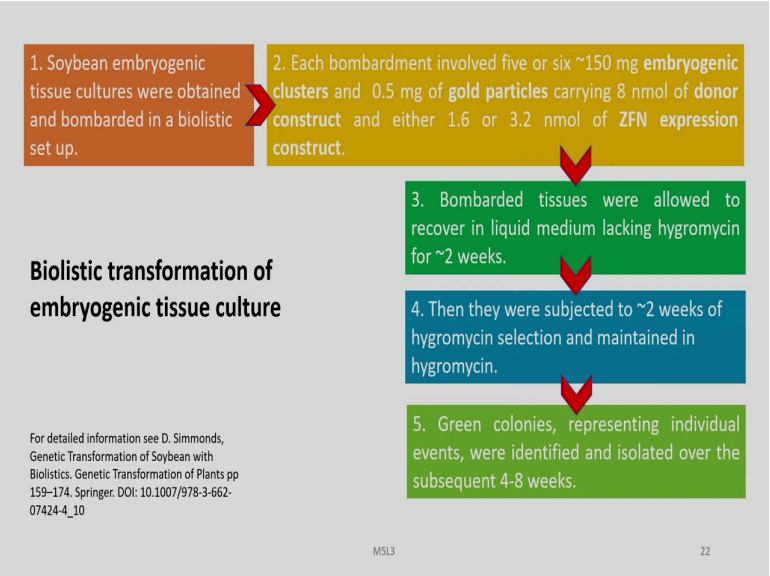

So, how the work is carried out? So, we use biolistic for transformation of the embryogenic tissue culture. Here, first they took soybean embryogenic tissue cultures and bombarded them in a biolistic set up. So, each bombardment involve 5 or 6 roughly around 150 mg embryogenic clusters and 0.5 mg of gold particles carrying 8 nanometer of donor construct which we have discussed in the earlier slide, it is an either 1.6 or 3.2 nano moles of ZFN expression constructs.

The bombarded tissues were allowed to recover in liquid medium lacking hygromyicin for about two weeks and then they were subjected to two weeks of hygromyicin selection and maintained in hygromyicin. Finally, green colonies representing individual events were identified and isolated over the subsequent 4 to 8 weeks.

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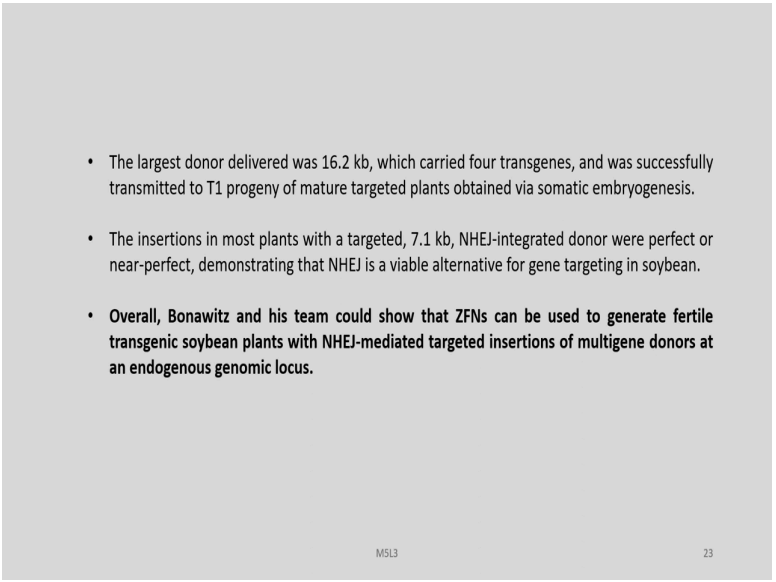

The largest donor delivered was around 16.2 kb which carried four transgenes, and was successfully transmitted to T1 progeny of mature targeted plants obtained via somatic embryogenesis. The insertions in most plants with a targeted 7.1 kb, non homologous end joining integrated donor were perfect or near perfect demonstrating that NHEJ is a viable alternative for gene targeting in soybean.

In conclusion, Bonawitz and his team could show that ZFNs can be used to generate fertile transgenic soybean plants with NHEJ-mediated targeted insertions of multiple donors at endogenous genomic loci.

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Let us now go to the next application, gene replacement where we will be replacing genes.

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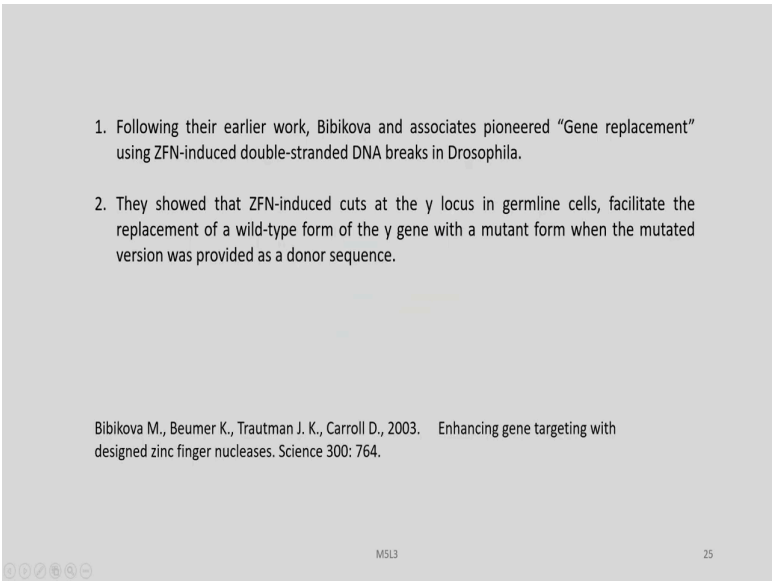

So, following the earlier work, Bibikova and team and associates pioneered gene replacement using ZFN-induced double stranded DNA breaks in Drosophila. They showed that ZFN induced cuts at the y locus in germline cells, and facilitate the replacement of wild type form of the y gene with a mutant form when a mutated version was provided as a donor sequence.

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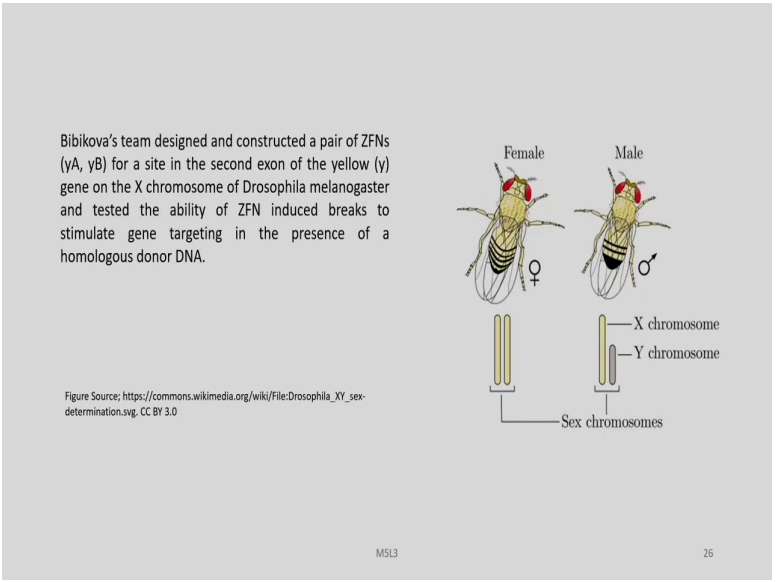

So, their team designed and constructed a pair of ZFN yA and yB for a site in the second exon of the yellow gene on the X chromosome drosophila melanogaster and tested the ability of ZFN induced breaks to stimulate gene targeting in the presence of a homologous donor DNA.

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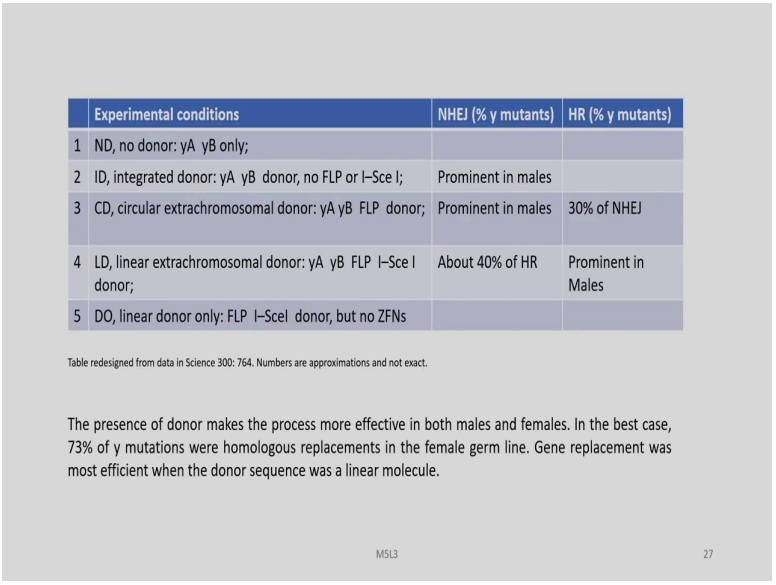

So, this table we have redesigned from the data available in their publication. So, these are the various experimental conditions, you can see here and the mutants which has been obtained by either NHEJ or the homologous recombination.

In the 1st case, there are no donors, only the ZFN constructs and you do not get any kind of mutants here. And, in the 2nd case they use integrated donors and then yA and yB donor, but there were no FLP or I-Sce. So, this kind of mutants were prominent in males under the non homologous end joining pathway.

In 3rd case they took circular extra chromosomal donors and yA yB and FLP donor and they found that again these are prominent in males and also some kind of homologous recombination products are there, but which are about one third of the number found in case of NHEJ.

And, when they used linear extra chromosomal donor and other donors, they found that about 40 percent of these are permanent in males under the homologous recombination pathway and these are less in non-homologous end joining pathway and the numbers achieved here were only 40 percent of what was achieved in the case of homologous recombination.

So, overall the conclusion was that the presence of donor makes the process more effective in both males and females. In the best case, 73 percent of y mutations were homologous replacements in the female germ line. Gene replacement was most efficient where the when the donor sequence was a linear molecule.

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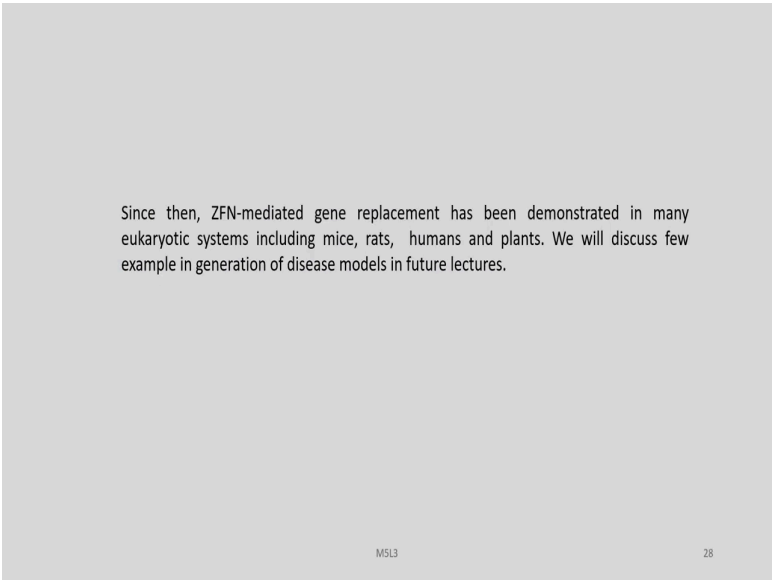

Since these experiment ZFN mediated gene replacement has been demonstrated in many eukaryotic systems including mice, rats, humans particular cell lines and plants. We will be discussing a few of these examples in generation of disease models in our future lectures.

Thank you.