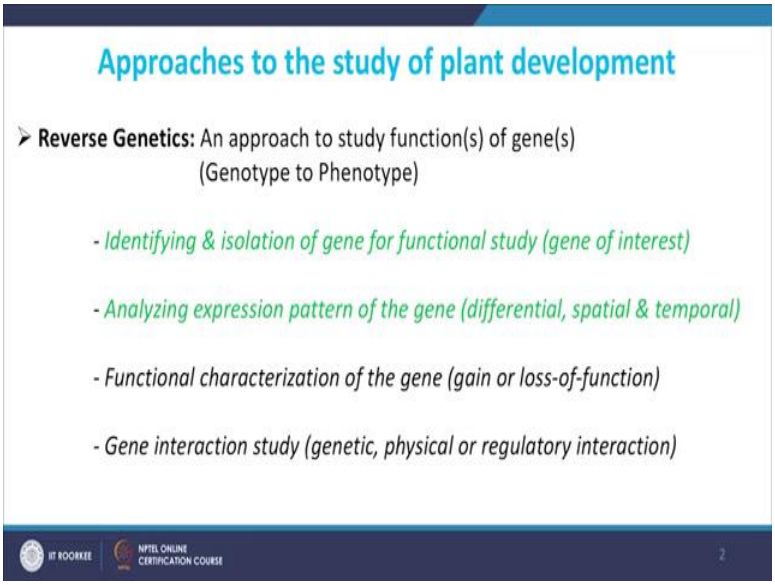
Plant Developmental Biology
Prof. Shri Ram Yadav
Department of Biotechnology
Indian Institute of Technology, Roorkee

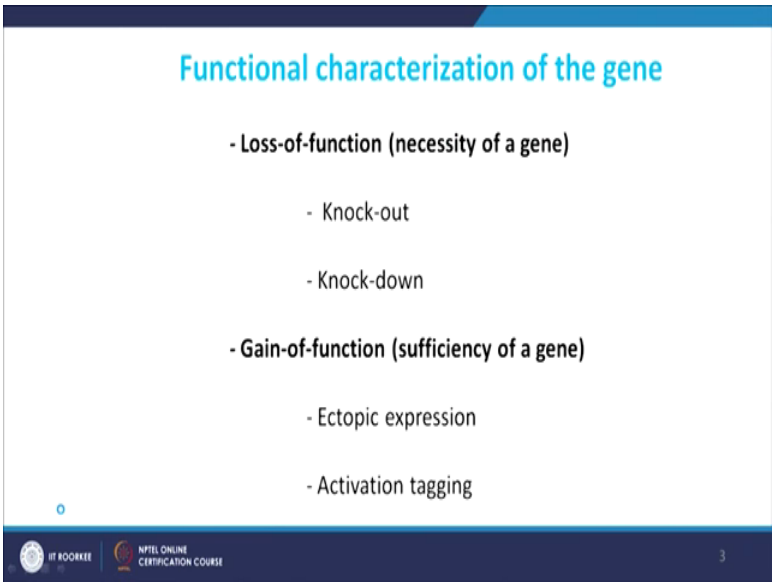
Lecture - 09
Molecular Genetics of Plant Development - Contd…- II

(Refer Slide Time: 00:35)



Welcome to the course of Plant Developmental Biology. So, in previous class, we were discussing the reverse genetics based approaches, and we have already finished how to analyse or how to monitor gene expression pattern. And then now in this class, we are going to discuss about how to functionally characterise a gene. So, to functionally characterise a gene which means that you want to know what is a specific function of a gene in a particular developmental process.

(Refer Slide Time: 00:54)



How will you know that? One approach is loss-of-function. If you somehow disrupt the gene, and then see what kind of developmental defect you get when you make a loss-of-function of a gene. And this will tell that for what function your gene is necessary. And then another complimentary approach to validate it or to understand the function of a gene is the gain-of-function.

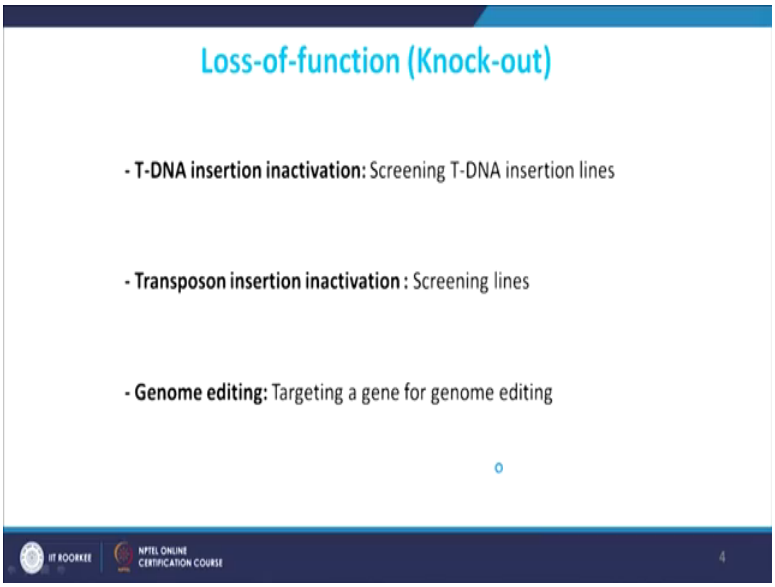
Let us assume a developmental gene is expressed in a particular tissue, but it is not expressed in some other tissue. Then if you take this gene and ectopically express in the tissue where it was not present normally, and then you can look that tissue can it reinitiate some developmental program. And if it is; if this is true, then we can say that a particular gene is sufficient for a particular function. So, these are the two major approach which we take to understand function of a particular gene in development.

First we will take loss-of-function. And here there are two way to do it either you knock-out the gene or you knock-down the gene. In knock-out you totally make a null mutant where the gene is totally disrupted. In knock-down you are reducing the expression of a particular gene, but it is not null mutant.

And in gain-of-function there are two way to do that, one is ectopic over expression or ectopic expression. So, you change the promoter, use some broader promoter to express this gene in a tissue where normally it is not expressed, and second method is activation tagging.

So, first we will take the loss-of-function or how to generate knock-outs. So, if you look the first two approaches, this we have already discussed in the forward genetic based approaches which is to generate insertional inactivation lines for a particular gene.

(Refer Slide Time: 03:17)

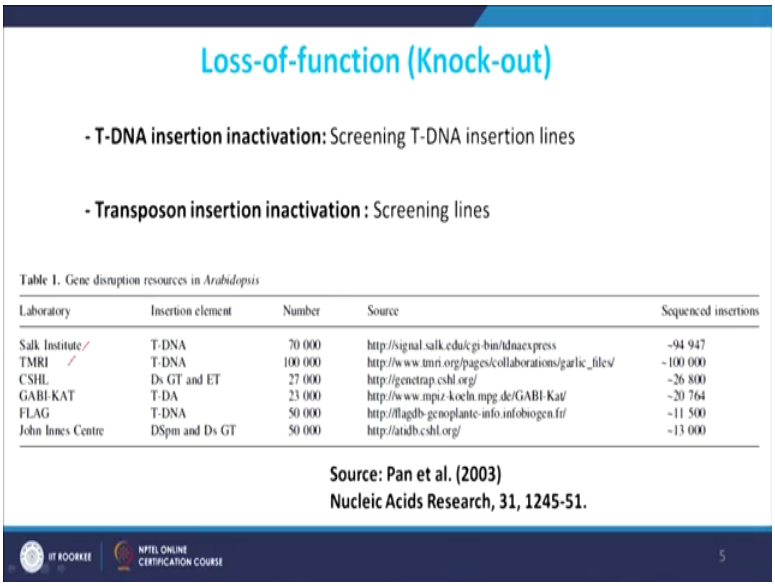


So, one was the T-DNA insertion, second was the transposon mediated insertion. But the difference between forward genetics and reverse genetics is that in forward genetics we were not fixing the gene, we were fixing the function or we were fixing the phenotype or the biological process, and then we are trying to look for T-DNA insertion lines or transposon insertion lines and then try to map that which gene it is.

But in the reverse genetics, we know a specific gene and want to understand what is its biological function, then you can specifically go and look for some T-DNA insertion lines available for that gene or transposon lines available for that gene or not.

Another and now very widely used method is genome editing. So, here you are basically editing at the genome level, and then you are generating knock-outs or loss-of-function mutants. So, first we will take the insertional inactivation lines. As I said that initially people have done at a very global level, they have generated a large number of T-DNA insertion lines or transposon insertion lines, and those lines has been deposited to some databases.

(Refer Slide Time: 04:46)



There are already so many databases or so many laboratory and projects, they have generated a large number of T-DNA insertion lines or transposon insertion lines. And this all mutants has been preserved and protected. And you can choose any of the mutant, you can request, and order, and you will directly get those mutants for your detailed functional analysis. So, there are large number of this kind of collection of mutants available.

(Refer Slide Time: 05:18)

For example, this T-DNA express or Salk Institute they have generated large number of T-DNA insertion line. What simply you have to do, this website is available, you just chose your gene, put the gene ID, search with the gene, chromosome, and then they will show a list of the insertion lines available for that particular gene, and you can chose.

Sometime what happens that you might have more than one insertion lines in your gene. For example, if you look this gene you can have so many insertion you can choose that I want one insertion line in exon 1, I want one insertion line in exon 2. And then you just take these numbers and request from the database, and these seeds the mutant *Arabidopsis* seeds will be available to you, and then you can get this mutant or knock-out lines for your detailed functional characterisation.

(Refer Slide Time: 06:28)

And the first thing what you do, if you get this kind of insertion line, you first establish that actually there is insertion. Second thing is that what is the genetic pattern is it insertion is homozygous, both the alleles are disrupted or it is heterozygous only one alleles are disrupted. How you can do? This is very easy since you know the predicted site of insertion, you know the sequence of T-DNA, you know your gene id and essentially the sequence of your gene. You can design a forward primer here, one reverse primer here and one primer you can design in the T-DNA and do some PCR.

If you do with the genomic DNA forward and reverse primer, and there is insertion, the insertion line will not give it because the fragment size is very big, you will not get the PCR amplification.

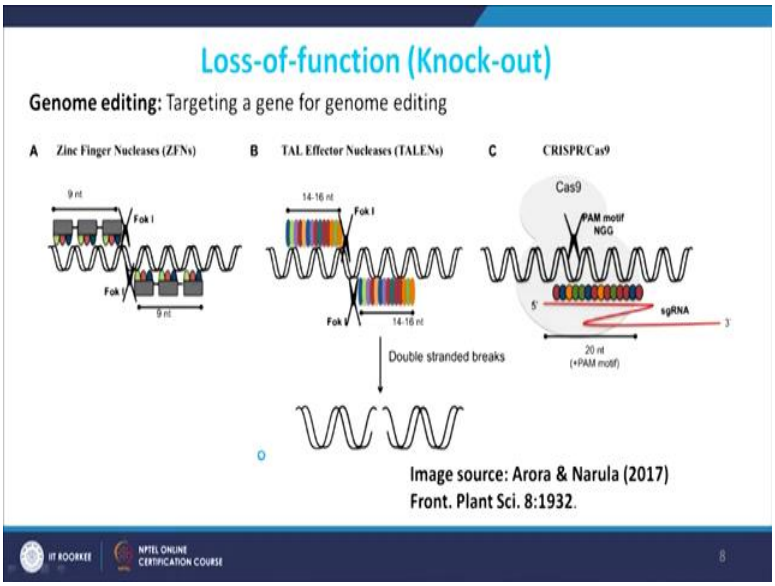
If you have a homozygous line, in homozygous line both the genomic locus has the insertion. Then if you use LP and RP together you will not get a band here. But if it is wild type or heterozygous, in heterozygous one locus has the insertion, another locus does not have insertion. Then from the locus where you do not have insertion you will get this band. So, basically your wild type and heterozygous both will give band with the LP and RP.

Then you choose your LP with this or RP with this depending on in what orientation your T-DNA is, and then if you have a heterozygous you will have a band here. And then if it is wild type, you will not have this T-DNA specific band. So, it is important for you to establish that what is the insertion status of this T-DNA in your gene.

Then another important thing what we do is back cross because since all these has been generated through a random insertion, there is a possibility that your plant there are more than one T-DNA insertion site. So, you might have an insertion in your gene, but there might be some other insertions somewhere else in other genes.

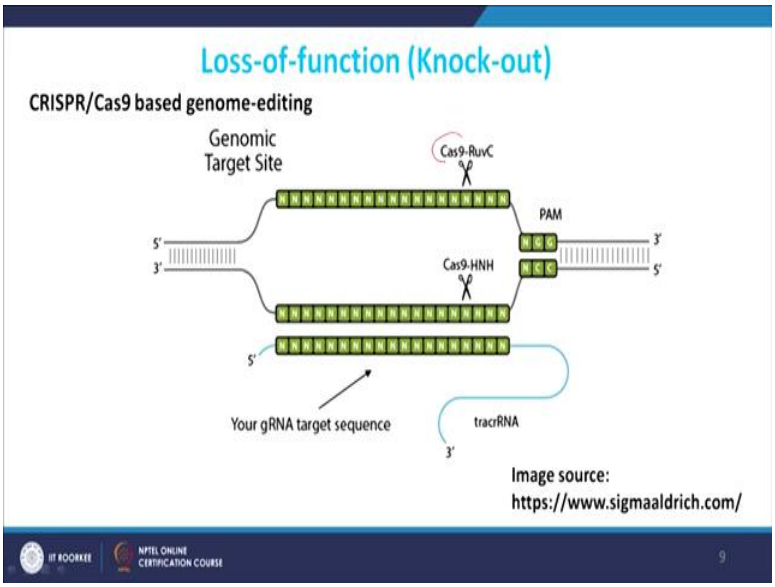
And that you can remove by taking these lines, crossing with the wild type and then going to the F2 generation and again screen for homozygous. And once you have established this line, you can take these lines for detailed functional characterisation or study the developmental defect in this T-DNA or loss-of-functional analysis.

(Refer Slide Time: 09:00)



Another method is genome editing. So, in genome editing, there are many techniques available which you can use to edit the genome ZFN, then TALEN, but recently CRISPR Cas 9 has been used very widely used these days.

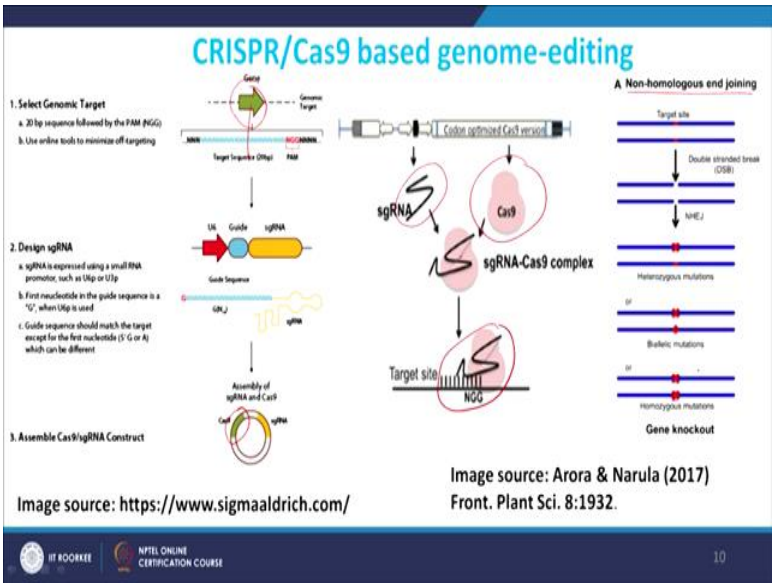
(Refer Slide Time: 09:24)



So, we will see how this is being used to edit the gene. Essentially in CRISPR Cas 9 based genome editing tool we are using bacterial endonucleases which is RNA guided endonucleases, Cas 9.

Then you simply produce a guide RNA and Cas 9 protein in the plant. This guide RNA can target your sequence wherever you want. So, you can design a very targeted guide RNA.

(Refer Slide Time: 10:06)



Let us assume this is your gene and you want to disrupt this gene, what you can do you can choose a target sequence typically here. And there is one requirement which is called PAM sequence. So, you can target your region which is near to the PAM sequence, and you can generate guide RNA which is specific for this sequence.

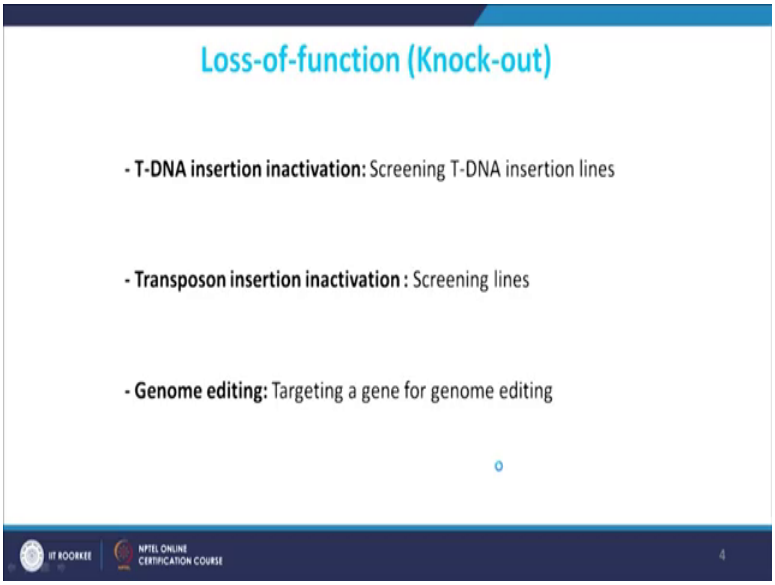
And then you clone this guide RNA in a vector and the same vector you can clone Cas 9 and the endonuclease protein. When you take this and make a transgenic plant both the partners will express there. When Cas 9 protein will bind with the guide RNA, guide RNA will target this endonucleases in the sequence specific manner.

And when this endonucleases is recruited on the genomic copy, it will make a double stranded break in the genomic copy. This break is considered as a damaged DNA. And endogenous mechanism which is called non homologous end joining mechanism will be activated in the plant and it will fill this gap, it will repair here.

But during the process of end joining mechanism there is a possibility that you can generate a deletion, you can generate a insertion, you can disrupt the coding region, and

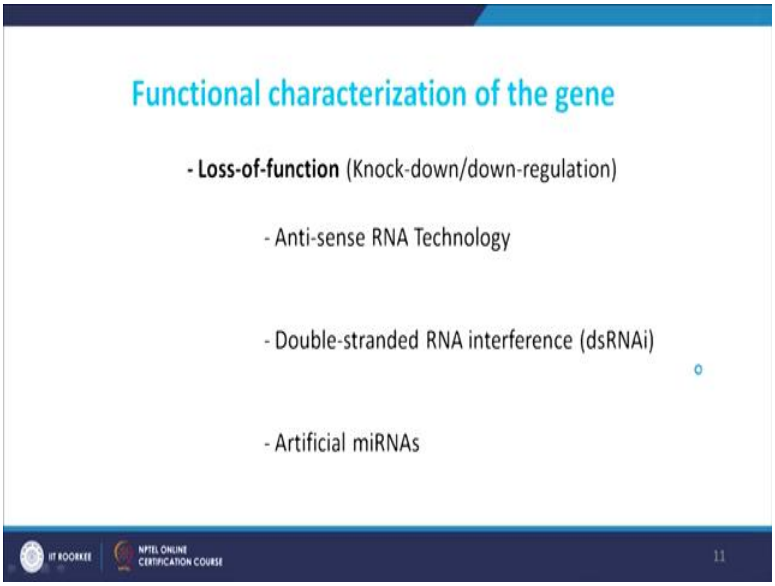
that is how you will have a genomic disruption of a particular gene. So, this is some of the method which is being used for making the knock-out lines.

(Refer Slide Time: 11:42)



Another mechanism what we are using to study the loss-of-function is knock-down or down regulation.

(Refer Slide Time: 11:53)

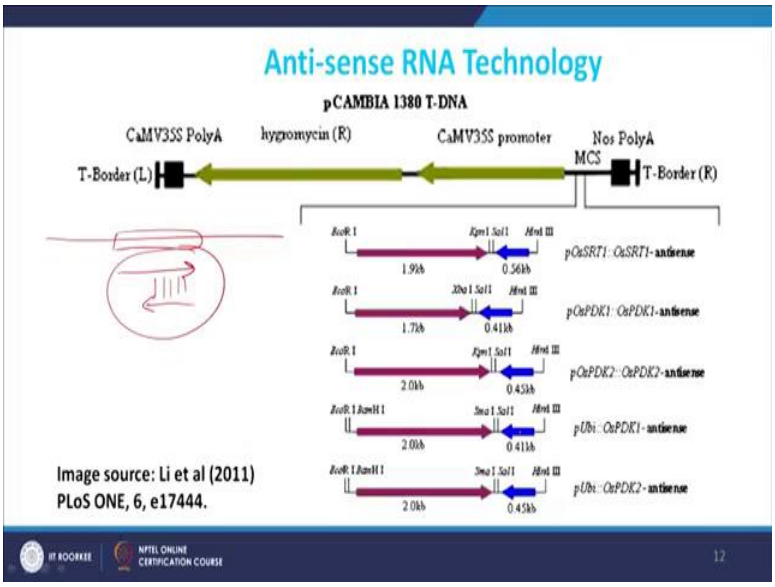


So, here we are not disrupting the genomic copy, but we are regulating after the transcription. So, we are reducing the expression level either at the protein level or at the transcription level. And there are some of the techniques which is being used for this

function; one is the anti-sense RNA technology, this was one of the pioneer technique which was been used, but these days there are many more techniques. The second method is double stranded RNA interference and artificial micro RNA.

In anti-sense RNA what happens that if you have a gene and you know the gene sequence, you just take the same gene or the gene specific fragment of the gene and clone in the anti-sense orientation.

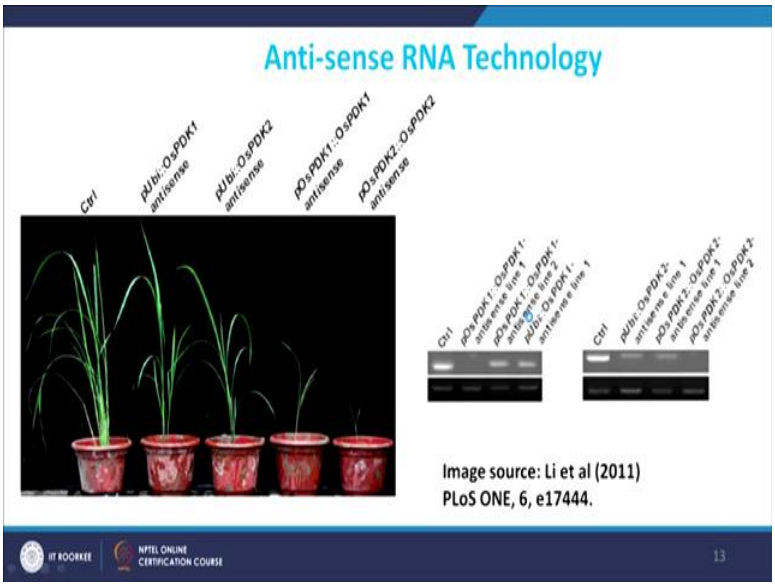
(Refer Slide Time: 12:42)



Then what are you expecting. So, if it is a genomic copy of your gene, it will transcribe in this direction, but now you have generated another transcript which is complimentary to this endogenous transcript because it is anti-sense oriented and then this transcript will go and it will bind with the endogenous transcript, and then this transcript, either it will be degraded or it will be inhibited for protein synthesis and you will have the phenotype.

This is one example, there are few genes targeted through anti-sense RNA, you can see, this is the promoter and the gene specific region has been cloned in the anti-sense orientation.

(Refer Slide Time: 13:23)

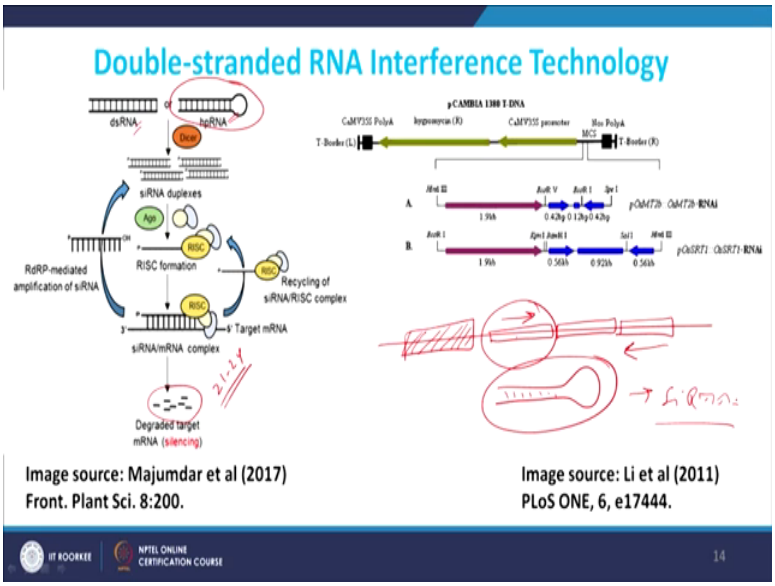


And when you have drive this genes, you can basically suppress the endogenous gene using this method as you can see the phenotype. So, this is control; when the genes has been suppressed, there are lot of developmental defects. And when you check the RNA level or the transcript level, you can clearly see that as compared to controls in this anti-sense lines, there is significant reduction of the transcript level. But there are some something important if you look this line, here almost down regulated, but these lines are partial down regulation.

So, this is not a kind of null mutant, but this is a kind of down regulation. So, you can have here a gradient or you can have some lines where you have near to the null phenotype, but some lines you might have which are weak lines, there the down regulation is not so efficient.

Second method which is being used for down regulation is double stranded RNA interference technology. This technology were developed from the endogenous mechanisms.

(Refer Slide Time: 14:33)



So, in plants siRNA and micro RNAs are well known regulatory mechanism. There is a large number of small RNAs, and they are regulating expression of many important endogenous gene. If you look a general mechanism, there are two way either you have a double stranded RNA or hairpin RNA, this will undergo the process of processing through DICER or AGO mediated processing.

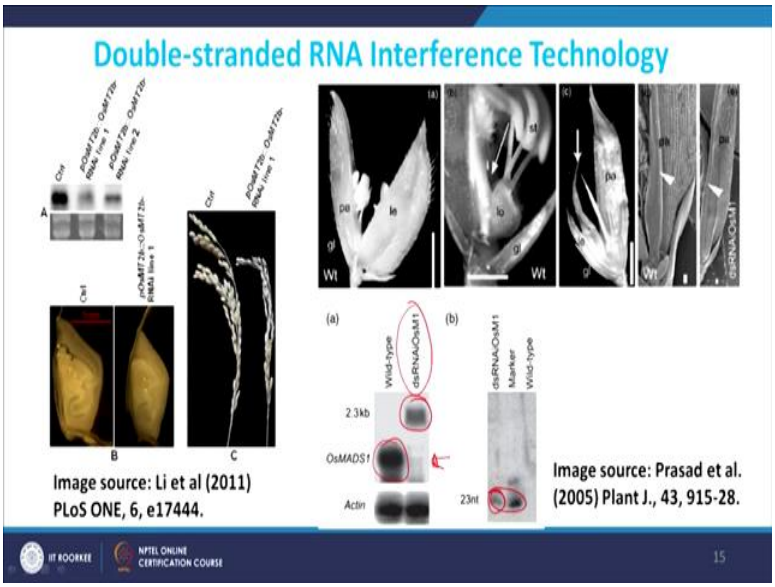
This I will not go in the detail; I am sure you would have already studied. And then it generate a very small 21 to 24 nucleotide single standard antisense RNAs. And these RNA they can go and pair with endogenous messenger RNA, and then they will either target that messenger RNA for the degradation or they can inhibit protein synthesis or they can do epigenetic regulation, any kind of regulation is possible.

To do that, if you want to use this technology, you can generate your own RNA interference construct for a specified genes. What you have to do? You have to simply mimic this hairpin generation. Take a gene specific fragment and clone in sense orientation, and then put some non-specific sequence here or stuffer fragments also called linker fragment, and then you take the same fragment and clone downstream, but in opposite orientation. If this is in the sense orientation, this you put in the antisense orientation and put a promoter here, drive this hair pin loop.

Then what will happen? This messenger RNA will make this kind of structure. So, this region and this region will pair here. And it will generate a hairpin structure, and this will

be endogenously processed and then it will generate siRNA. And this siRNA specifically will target the endogenous RNA or messenger RNA against which it has been designed, and then it will down regulate the expression of those genes.

(Refer Slide Time: 16:45)

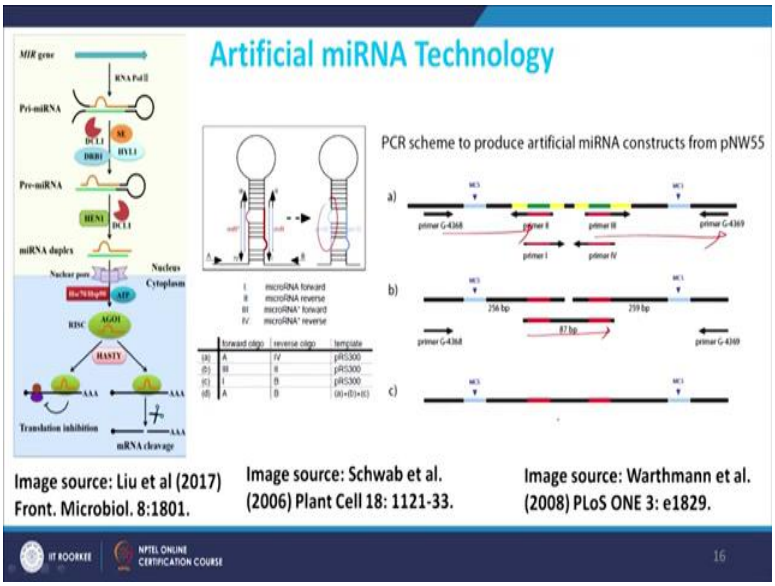


There are plenty of examples were RNA interference has been used to down regulate the gene. Some of them I will show here. If you look here two of the genes has been down regulated by RNA interference based approach. You can look here this is northern blot. In control there is high level of expression, but in RNA interference line the level has been significantly decreased. And you can see the panicle phenotype.

Similarly, this is a very important transcription factor MADS1. And when the RNA interference line was generated for *MADS1* you can see this is northern blot again. In wild type, you have a very high level of expression, but in RNA interference line the endogenous expression is almost null.

Whereas you can detect the hair pin structure which is basically bigger of the size. Not only that if you go and do a very spatial northern blot, you can detect small siRNAs as well. So, these are the small siRNAs which is not present in the wild type, but which is present in the RNA interference line.

(Refer Slide Time: 17:55)



So, this has been very extensively used to down regulate many of the genes.

Then third method which is been used to make a knock-down mutants are artificial micro RNA. So, as I said micro RNA is a natural small RNA present in the genome of the plants, they are being processed through dicer and ago mutated mechanism. And then this micro RNA, they are going and targeting specifically some of the genes, and regulating its expression at different level, transcriptionally, post transcriptionally, translationally, epigenetically. And then this technique was taken and then used to generate some artificial micro RNA.

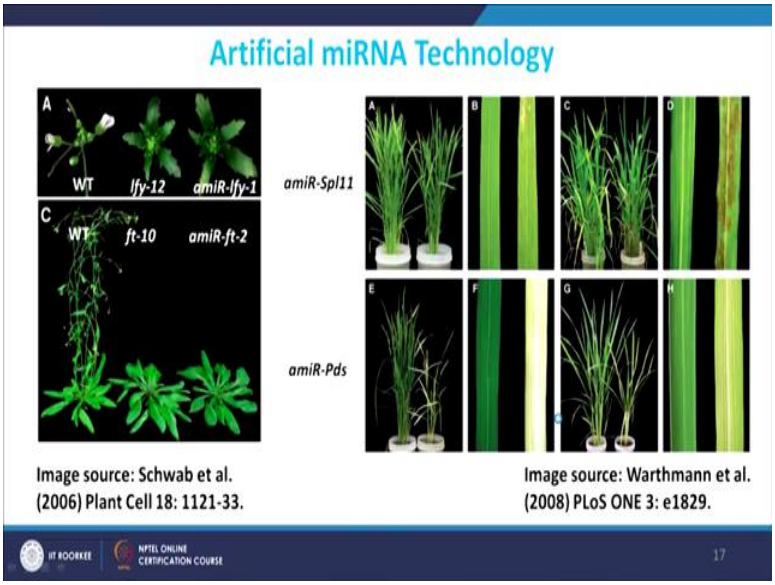
If you take a natural hairpin structure or the loop of the naturally existing micro RNA, and if you can replace this core sequences with the micro RNA core sequences which is basically showing the complementarity with the gene. And if you replace with your own gene of interest, you can down regulate it.

And then this can be easily done by combination of multiple PCR.

For example, if this is your construct here you can use some different set of primers you can amplify this region from here to here, then you can amplify this region from here to here, and you can amplify this region from here to here. So, basically this red is your artificial micro RNA sequence. Then if you use these three as a template together and

use this two primers, so you can combine them. And then this artificial micro RNA, you can clone under promoter and then generate some knockout line.

(Refer Slide Time: 19:43)

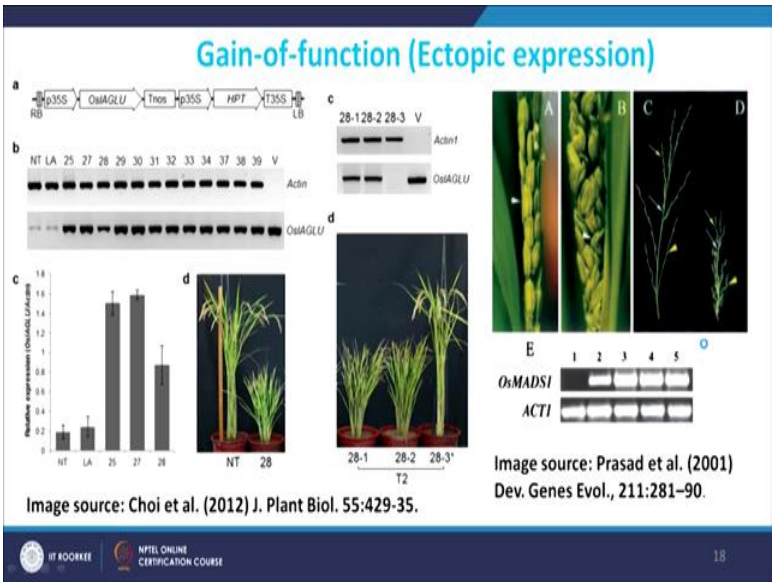


And if you see here this has been quite successfully used in some of the study. For example, in *Arabidopsis*, this is *leafy* mutants, which is an insertional mutant. *leafy12*, this has a significant phenotype as compared to the wild type. But if you target this *LEAFY* through artificial micro RNA against the *LEAFY*, you can mimic the phenotype. So, your artificial micro RNA here is as strong as the other mutants.

Similar is the case if you look *ft* mutant. If you look the wild type, the plant has already flowered it has achieved the reproductive stage whereas, this *ft* mutant has very delayed flowering. But if you generate again artificial micro RNA against the *FT*, you can see that it is showing a severe phenotype as the other mutant. So, this tells that artificial micro RNA is one of the very efficient way to down regulate genes in *Arabidopsis*.

And similar kind of things has been used in other plant species. For example, if you take the case of rice. Here artificial micro RNA has been generated for couple of genes. This is *SPL11*, and this is *PDS* gene. This is the control and this is the RNA or artificial micro RNA line and you can see clear phenotype in artificial micro RNA lines.

(Refer Slide Time: 21:14)



So, for this study either you can use the knock-out based study or you go through the knock-down based study. You can generate a loss-of-functional mutants and you can conclude that this gene is necessary for this particular function.

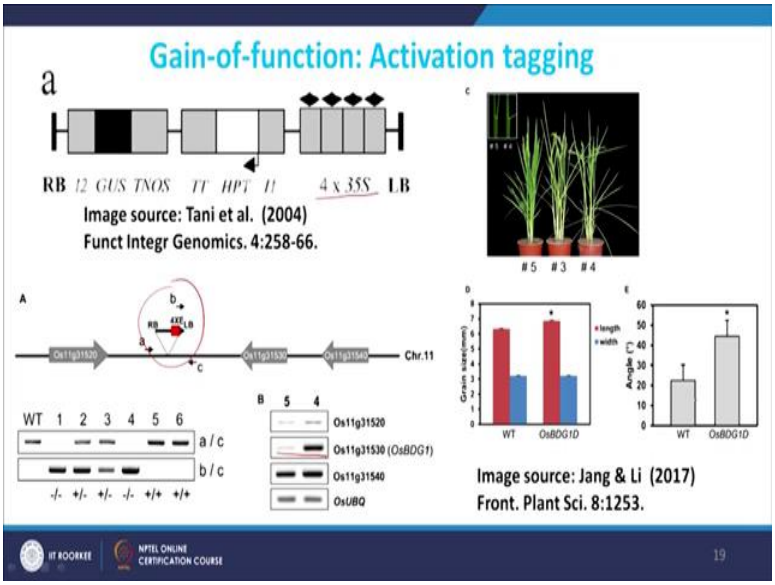
Another complimentary way as I said is gain-of-function. First way to generate ectopic over expression or ectopic expression construct is to use some broader promoter or a promoter which is expressed at the high level. And you can use either tissue specific promoter for the tissue where your gene endogenously does not express or you can use a general 35S promoter or Ubiquitin promoter to drive your gene in the sense orientation.

And then you can have high-level of expression in the tissue where you do not expect your gene endogenously present. In this mutant, you can clearly see that this is your basal level of expression, but when you have over expressed under 35S promoter, you can see that in most of the lines the expression level is very high as compared to the control. And the relative expression is co related with the phenotype.

If you can go to the next generation, may be the lines are segregating. If you look this plant, it is not showing phenotype, but these two plants are showing the phenotype. And if you go and check the over expression level, it clearly co relates. This plant which is not having phenotype does not show the over expression as well. But these two plants which show the over expression, they show the phenotype.

Similar kind of analysis has been done for the transcription factor MADS1. And you can clearly see when *MADS1* was ectopically over expressed in transgenic rice, you can see a very significant phenotype in the panicle architecture and at the spikelet development. And if you see the expression level, you can clearly see that in the control you do not have expression in this tissue, but these lines they have a high level of expression.

(Refer Slide Time: 23:31)

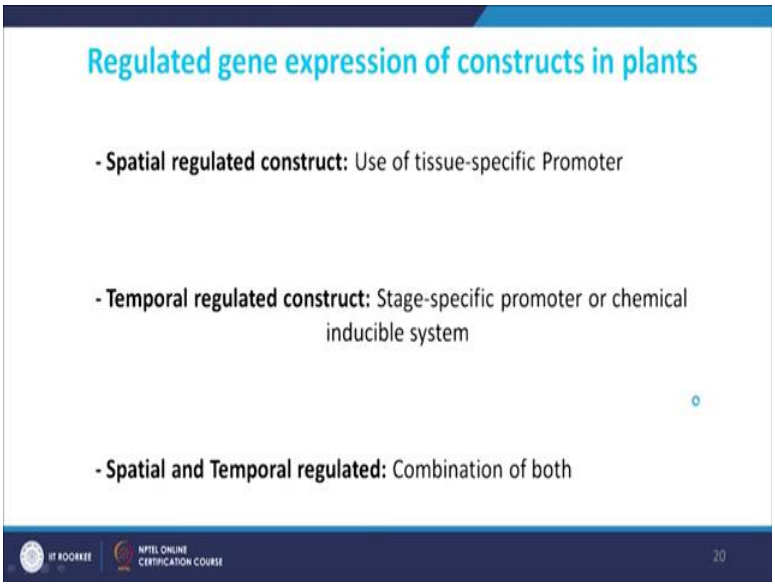


So, another way to generate a gain-of-function mutant is activation tagging. In activation tagging you take T-DNA construct, and put some multiple copy of enhancer elements. Here you can take 4 x copy of the 35S enhancer element. And then randomly throw in the genome and it will go and integrate, but what happens that if it comes in close proximity with some of the basal promoter or some of the promoter which normally is expressed at the very low level or normally it is not expressed.

But now the promoter has got some of the enhancer element in the close proximity and then the promoter will start activity it will start very strong expression. And in that case, you will see a phenotype which could be because of the gain of function phenotype.

These are some of the example. So, if you look here, there is an insertion of enhancer elements here. And if you look the result, this gene is more expressed in the insertion lines than the control. So, these are some of the phenotype of this line. This is called enhancer tagging, you can look for many more genes and identify more activation tagging lines and you can take them for the studying the function .

(Refer Slide Time: 25:03)



This is a typical way to functionally study gene in plant, but there is some problem. Let us assume that if you want to study function of some of the essential genes, and if you make a knock-out or if you make a knock-down, if gene is essential you face a problem of lethality. If it is lethal, if loss-of-function is lethal or gain-of-function is lethal, you will not have transgenic plant or you will not have a plant to study.

In that case, how to solve the issue? Some of the genes are very important at very early stage during embryo development. So, they could be embryonic lethal, some of the gene might not be embryonic lethal, but they could be lethal at the seedling stage or at any stage they complete their vegetative growth, but they cannot enter in the reproductive phase. So, basically they will not make a fertile flowers, they will not make seeds which means that you cannot go to the next generation.

So, if you want to study such kind of genes through the reverse genetics based approach how to do it. In that case, what you have to do, you have to use a regulated gene expression based approaches. So, you cannot take a gene and down regulate all the time or down regulate constitutively or over express constitutively. If you do that, you will not be able to study.

Then what you can do, you can generate a construct or you can make a strategy where you can specifically regulate a gene whenever you want or you can have a kind of more temporal and spatial based regulation of your gene silencing or overexpression. There are

three ways you can regulate gene. One is the spatial regulated construct. So, what you can do that instead of over expressing a gene in all the tissues, you target over expression in some of the tissue where you feel that might not be so harmful that it will have lethality.

For such kind of things you can use some tissue specific promoters or known promoter which is expressed in a particular organ, for example, if you want to check in the leaf then you can use some leaf specific promoter, or express a flowers specific gene in the leaf and look can it start some of the flower specific developmental program in the leaf.

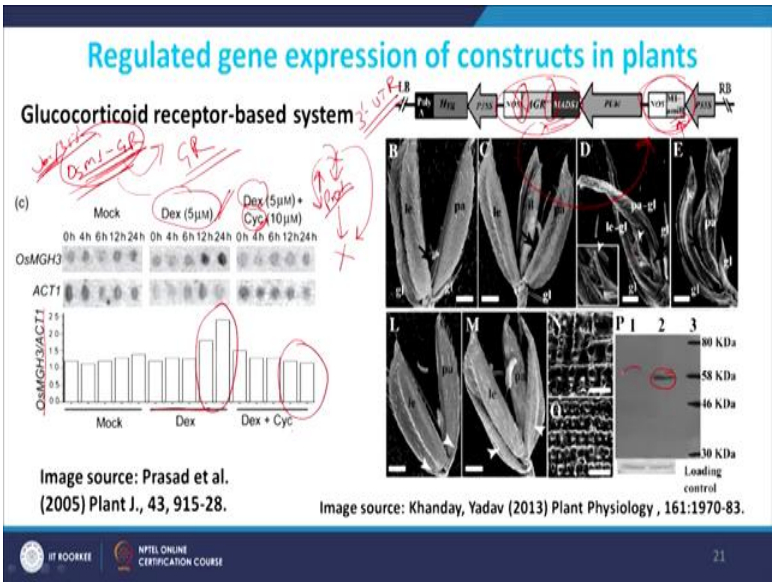
Second way of regulation is the temporal regulation construct. Here you can express or down regulate a gene at a particular stage not before that. For example, let us assume that down regulation of a gene *X* is embryonic lethal, which means that if you down regulate from day one even embryogenesis will not complete, then you will not have plant.

In that case, what you can do, and if you want to study the role of this gene maybe in the vegetative development or maybe in the reproductive development, then you do not need to down regulate or silence or over express this gene during the process of embryogenesis.

In that case you can you some stage specific promoter to drive the over expression construct or RNAi construct. And you can skip the expression of this construct during early stage of embryogenesis. So, embryogenesis will occur normally then later on you can down regulate and see what is the consequence.

Second is that you can even combine both and you can generate a construct where you can do both together. So, you can regulate a gene or a construct over expression, RNAi construct temporally as well as spatially. How you can do it? We will take just one example of both the cases.

(Refer Slide Time: 28:55)



For example, if you are studying a transcription factor, there is a system which is being very widely used is a glucocorticoid receptor - based system. So, we know that transcription factors, they have to enter inside the nucleus for activating the gene expression. And if you somehow retain them in the nucleus and regulate their translocation between cytoplasm and nucleus, you can regulate their activity and that is being done by fusing your transcription factor with a rat or mouse glucocorticoid receptor.

Glucocorticoid is basically a steroid hormone in animals and the glucocorticoid receptors they are normally present in the cytoplasm. But, when they receive signal through the hormones, they then enter inside the nucleus and activate their genes.

I will take an example of *MADS1*; if I take *MADS1* gene and fuse with delta GR and overexpress under any broad promoter such as 35S promoter. Then under normal condition, I have a high level of *MADS1* expressed, protein is already made which is fusion protein, MADS1 delta GR. Fusion protein is already made it is ready, but it is only present in the cytoplasm. Since, MADS1 is a transcription factor until and unless it will enter inside the nucleus, it cannot perform the function.

Then if I take this plant and induce with hormone such as dexamethasone, then this fusion protein will enter in the nucleus and it can activate the gene.

And this is very important technique, which you can use to even distinguish between direct genes which are regulated directly by the *MADS1* or the gene which are consequence of the *MADS1* induction, if you combine this dexamethasone treatment with the protein synthesis inhibitors cycloheximide.

This is an example of *OsMGH3*; *OsMGH3* as I said earlier that this is one of the gene which is regulated by *MADS1*. And what happens, when you treat with the dexamethasone you can see at a particular time point the expression level of *OsMGH3* is going up, which means that *MADS1* is sufficient to activate expression of *OsMGH3*.

But when you treat with the cycloheximide, so basically cycloheximide is a protein synthesis inhibitor. So, it will not allow new protein to be synthesized, in that case whatever MADS1 protein is already made delta GR protein is already made, only that will enter inside the nucleus and it will activate immediate genes, but those genes which are getting activated at the transcription level those RNA will not make protein, because there is cycloheximide.

And if protein is not made, there target will not be activated. So, the *MADS1* indirect target will not be activated, but direct target will be activated and that is how you can see here, when we treat dexamethasone and cycloheximide together you cannot see the activation of *OsMGH3*. This tells that *OsMGH3* is not directly regulated by *MADS1*, but it is indirectly regulated by *MADS*.

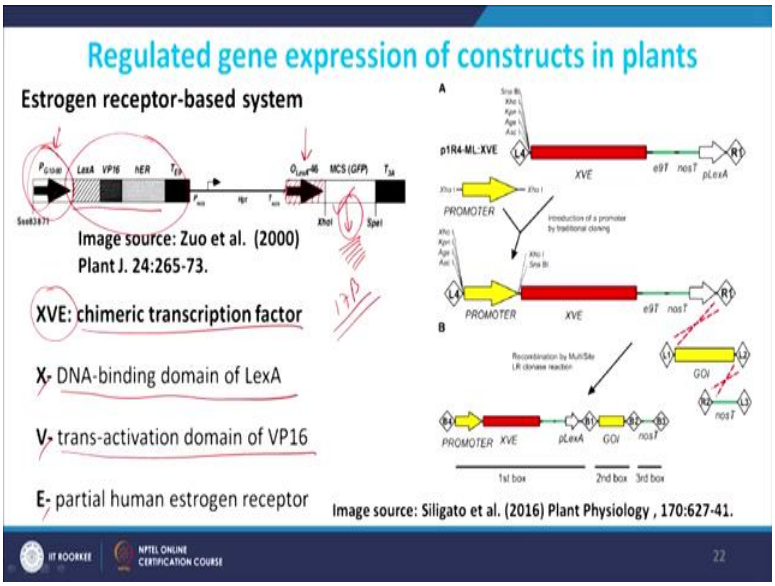
And then if you look this construct, this is another very important construct. So, this is a case when you down regulate *MADS1* gene what happens that it is all the floral organs are defective, you will not make a fertile seeds. You can see here this is the floral organs, they all are getting converted into leaf like structure or lemma palea like structure and you will not have organ formation. So, it is very challenging to go to the next generation. In that case what you can do? You can make a *MADS1* delta GR fusion as well as artificial micro RNA.

And then what you do, normally this artificial micro RNA will suppress the gene and will have the phenotype, but if you grow in presence of dexamethasone this delta GR will go and it will complement the phenotype. So, this construct has over expression as well as artificial micro RNA, but important thing here is that this artificial micro RNA is designed to target 3 prime untranslated region of the *MADS1* and that 3 prime

untranslated region is not present here. Here we have taken *MADS1*, only till the stop codon, untranslated region is not present why? If we take that region, then this artificial micro RNA will go and silence this gene as well.

So, in this construct this micro RNA will specifically silence the endogenous copy of the gene, but it will not silence the transgene copy and this way you can use a complementation based assay and do whatever phenotypic analysis or you can even identify the direct downstream targets. You can see here, when you induce with dexamethasone, protein is getting localised to the nucleus, but when you do not induce, you do not see the protein inside the nucleus.

(Refer Slide Time: 34:08)



Another very important technique which is being used to regulate both temporal as well as spatial expression, simultaneously is XVE bases system or estrogen receptor – based system. Here a chimeric transcription factor had been generated, XVE is a chimeric transcription factor, having three component; X, V and E.

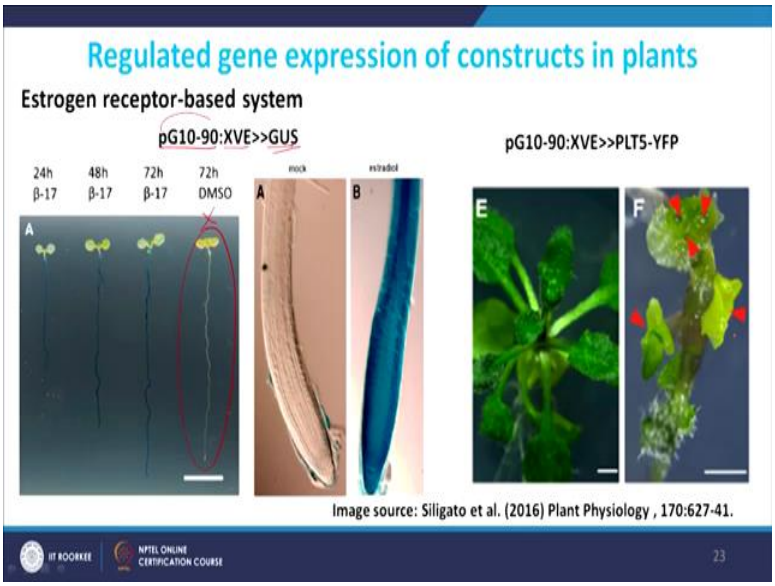
X is a DNA - binding domain of LexA protein, LexA is from the bacteria. And then V is trans-activation domain of VP16 protein which is from virus. And then E is the human estrogen receptor. And when you combine this, so you have a fusion protein which is an artificial transcription factor, which has a DNA binding domain and trans-activation domain, but at the same time it is also fused with the delta GR. So, you can regulate its translocation from cytoplast to the nucleus.

And when you drive its expression under any promoter, if you choose a tissue specific promoter, you can drive this XVE only in a specific tissue. Why this is required? Because the previous delta GR based system you can use only for the protein which are functional in the nucleus. If you want to study a protein which is not functional in the nucleus that technique, might not be very useful.

So, here what you can do, you can basically regulate both ways. You just put a LexA binding site just upstream to a basal promoter and then put your gene of interest, whatever you want to express, you want to express over expression construct, you want to express RNAi construct, artificial micro or whatever construct want to express.

Then what will happen here? Normally this promoter will drive expression of the XVE protein, but this protein will not enter inside the nucleus because of the estrogen receptor, but when you treat this with estradiol, 17 beta estradiol, this artificial protein will go and it has binding site here, it will bind here and the trans-activation domain of this transcription factor will activate the gene which is cloned downstream of it, and this can be used for even non-transcription factor kind of protein. So, this has been recently very detailed discussed and generated lot of gateway based construct, which can be used for making this kind of study.

(Refer Slide Time: 36:41)

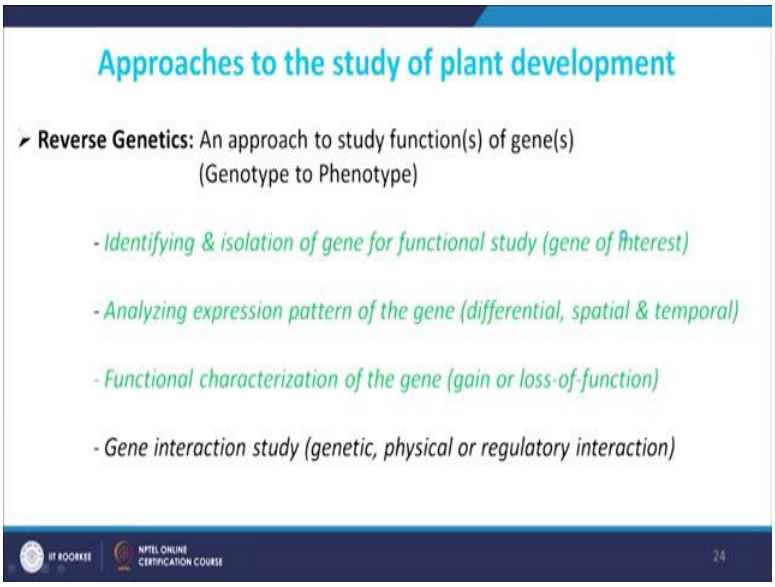


Here the promoter is G1090, which is an artificial very broadly expressed promoter. And XVE is driven by this promoter with downstream cloned gene, GUS.

So, if you see if you do not induce the plant, you cannot see GUS expression at all, but when you induce this with the estradiol at different time point, you can see very strong GUS expression here. This is the enlarged view of the root you can see, under mock treated condition there is no expression, but when you induce with the estradiol, there is a very high expression.

You can see the functional significance here. Here XVE has been cloned under G1090 promoter and then *PLETHORA5*. PLETHORA5 is one of the AP2 domain containing transcription factor in *Arabidopsis* and it has been cloned downstream of the LexA binding site. When you do not induce, you see that your plant is normal, but when you induce with the estradiol, you can see lot of defect, developmental defect which is consequence of over expression of *PLETHORA5*.

(Refer Slide Time: 37:56)



So, to conclude this; once you have generated transgenic, you can go and analyse it's phenotype as per your defined objective. So, now we will finish here and then in next class we will study gene interaction particularly genetic way or physical interaction or regulatory interaction.

Thank you very much.