Plant Developmental Biology
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Lecture - 06
Molecular Genetics of Plant Development - III

Welcome to the course of Plant Developmental Biology again. So, in this class we will continue discussing Molecular Genetics of Plant Developmental biology.

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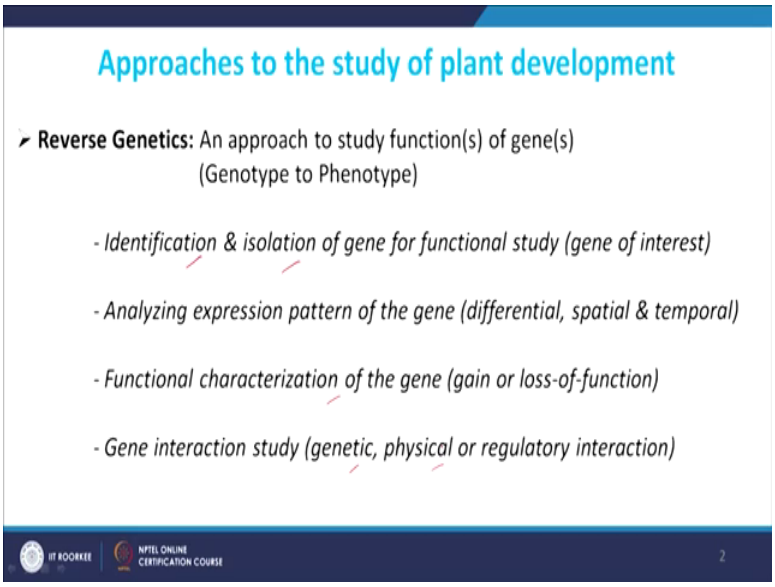

So, in previous class we have discussed the forward genetics based approaches and how forward genetics based approaches are being taken up for analyzing development in case of plants.

Now, we will focus on the reverse genetics based approaches. In reverse genetics based approaches we have first identify the gene and then we try to understand, what is the specific function a particular gene or a group of genes have in particular developmental processes? The process of reverse genetics can be studied in following steps. In first step we aim to identify a gene of interest and isolate that gene of state; gene of interest for studying its function.

Then second step of this is that to analyze the expression pattern of this gene. So, here we are starting from a gene. So, we should know where exactly this gene is expressed

particularly with respect to the developmental stages, developmental tissues or organs. Then once we identify the gene we know the expression, this will allow us to expect some phenotype in the tissue where it is normally expressed.

Then we move ahead for functional characterization of this gene. There are different approaches. Commonly we take the gain of function approach and we express a gene where normally it is not expressed and look what happens. This tells if a gene is sufficient for initiating any developmental program or not.

Second approach is loss of function. In this study what we silence a gene or knock out a gene or knock down a gene and then the look what happens in a development, this allows us to understand if gene is necessary for a particular function. Then try to understand, what is the mechanism behind a particular developmental role? This is being done by studying the interaction of a particular gene, it can be genetic interaction, physical interaction with other regulator or its regulatory interactions.

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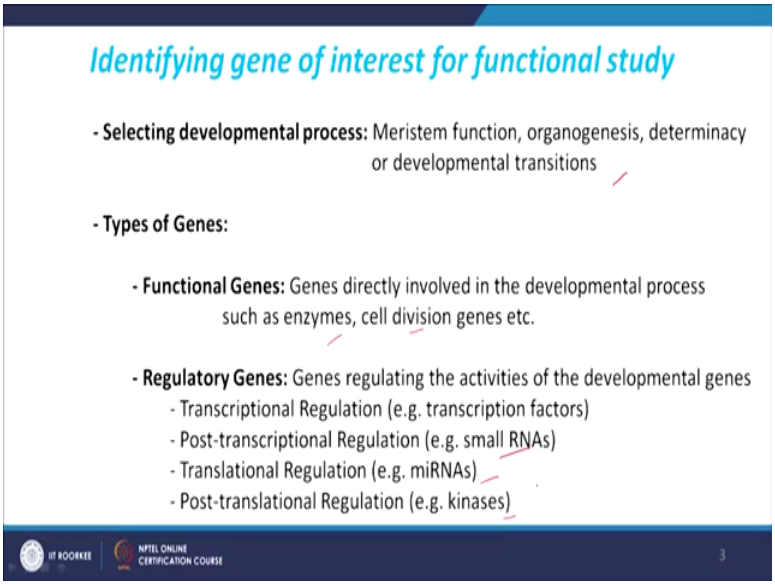

If we want to take a reverse genetics based approach before identifying a gene first we fix the developmental processes we want to study. This is very similar what we have done in the forward genetics as well.

So, in forward genetics to identify a particular mutant or particular phenotype we fix the developmental process, here also to identify a putative genes we will fix the

developmental process. So, are you interested in looking for the meristem function or the genes which is responsible for organogenesis determinacy or developmental transition phase transitions? So, we will fix first the process, then you would like to specify what kind of gene you want to study.

So, there are some genes which are directly involved in some biological process for example, cell division or some enzymes which are basically controlling a fundamental processes and then second class of genes are regulatory genes. So, these genes are mostly regulating the activity of some of the developmental genes.

And when it comes to the regulation there are different step of gene regulation and at least major steps where you would like to look the regulation is transcriptional regulation, here mostly we focus on the specialized transcription factor. Post transcriptional regulation, there are lot of developmental regulator which has been identified and they function after transcription like small RNAs.

Then there are known developmental regulators which regulate protein synthesis or translation of a particular messenger RNA, and then post translational regulation where you control the activity of a particular regulator by regulating or by modifying the protein through post translational modification. So, before starting any process we have to define this step by step we have to fix our biological process, we have to fix type of genes then we move and we try to identify kind of gene.

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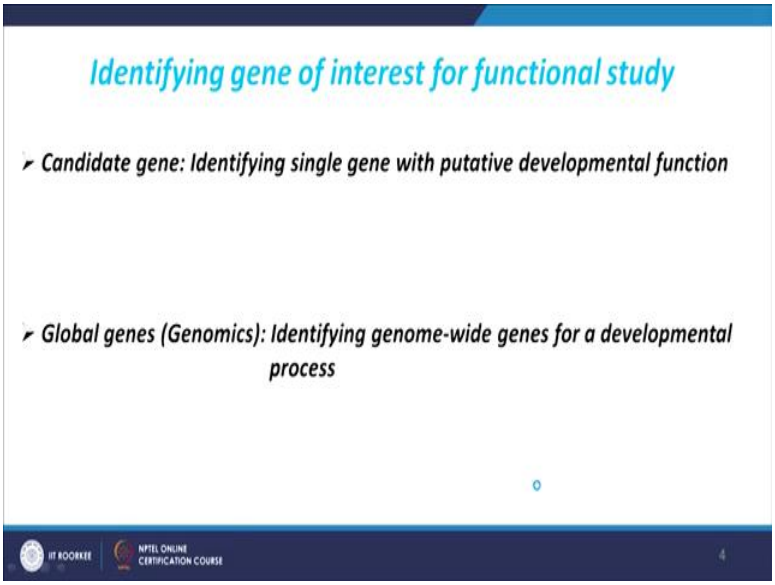

So, we can take two approaches one is called candidate gene approach where you can identify any single genes with a putative developmental functions. In second approach you can take global genes, which is called genomics. Here you do not identify a single gene you identify a group of gene which are specifically present in a particular developmental processes and then you move ahead.

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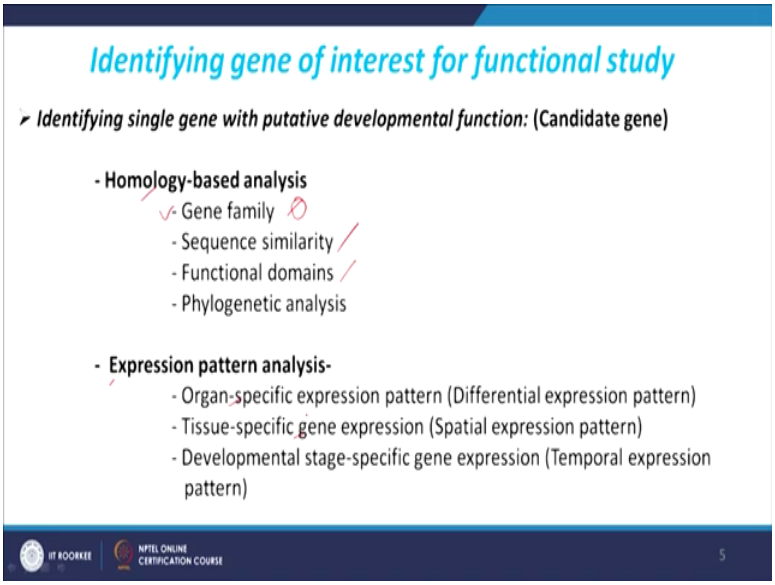

So, what are the criteria which we look for identifying or for selecting a particular genes which might have some function in the developmental process? So, there are two important parameter what we consider, first one is the homology based analysis.

Some of the model organisms, their whole genomes are sequenced. Forward genetics based studies is quite advance and then you can identify some genes which has already been shown for there some of the developmental function. And then you can look in other plant species, related plant species and then you identify if there is any homologous gene for that particular regulators.

For example, some of the regulators they make a gene family. There are multiple genes belonging to that family and sometime what happens the entire family is mostly regulating, some kind of developmental pathways or they are broadly regulating multiple physiological and developmental processes. So, you can look gene family and then you can look the sequence similarity.

Let us assume that you have one particular gene maybe in few slides I will show some of the example how this has been taken, but you can choose one gene which is known to have some developmental function, then you can take the sequences and try to find out what are the homologous gene in another related species. And then if there is a common functional domains you can look for that. You can check the relatedness which is called phylogenetic analysis.

So, typically if a gene is having a high level of homology or if it is related with a particular gene you can expect that it might have a similar function in other species. So, this is *in silico* approaches mostly bioinformatics approaches. This will help you to define some of the genes which you might take for the functional study.

Then second important thing you can check the expression pattern. So, this is very important because if you are interested let us assume you are interested studying some gene which is responsible for floral organ development, then you would like to check the expression pattern and you will prefer a gene which might have a specific expression in the flower or some of the organs of the flower.

So, you can check the organ specific expression and then you can check tissue specific expression if you want to define a very specific function. At the tissue level you can look for the expression pattern. If you are interested in the stage specific gene expression pattern you can look up a different stages and then you can identify some genes which are present in one particular stage absent in another stage.

If you identify a gene which is not expressed in the vegetative stages, but it is expressed in the reproductive stages you may expect that this gene might have some function during the reproductive stages.

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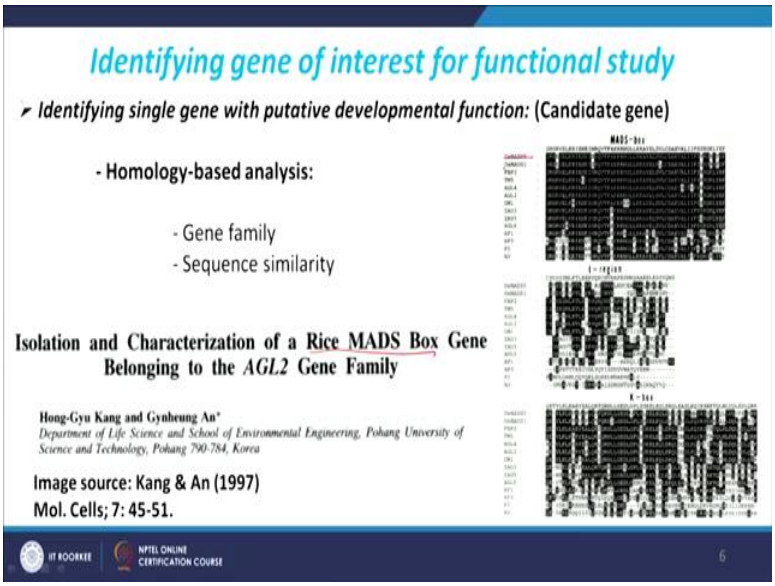

So, these two major approaches we take to define a gene and you can see some of the example, how these approaches has been taken. For example, in *Arabidopsis thaliana* a class of a transcription factor MADS box containing transcription factor has been well studied and it has been identified that they play an important role in the floral organ development and floral organ patterning.

So, this approach was used to identify some of the rice *MADS* box genes. For example, if you look this *MADS 5*, *MADS 1* and they have been taken for reverse genetics based approaches to show their specific function during rice flower development.

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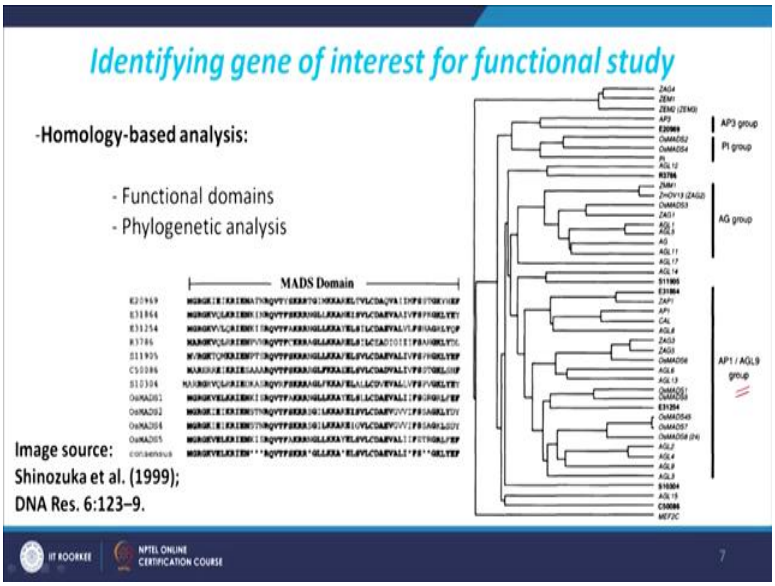

Similarly, flower development is studied in model dicot *Arabidopsis thaliana* and there we have found some classes of genes particularly A class, B class, C class.

So, this is typically A class gene, then you have B class gene and C class gene and A class genes has been shown to regulate sepal and petal identity. B class genes are shown to regulate petal and stamen identity and C class genes are shown to regulate stamen and carpel identity. And then if you look the sequence similarity homology functional domain in other species you may identify the homologous gene. If I take some example for example, if you look this PI; PI is a typical B class gene which interacts with AP3 to specify petal and stamen development in *Arabidopsis*.

Now, there homologues like *MADS2* and *MADS4* has been identified based on the sequence similarity functional domain phylogenetic analysis in rice and when they were studied; it was shown that actually they are involved in regulation of petal equivalent which is lodicule in rice and stamen development. But there is one interesting thing, there might be lot of species specific variations as well.

For example, there is only one PI gene in *Arabidopsis*, but rice the PI is duplicated we have two PI like genes *MADS2* and *MADS4* and when both of they were studied. Functionally it was found that *MADS2* is more important for petal equivalent development whereas, *MADS4* probably is involved more in regulating stamen

development. So, this is one approaches where you can identify a putative genes to study.

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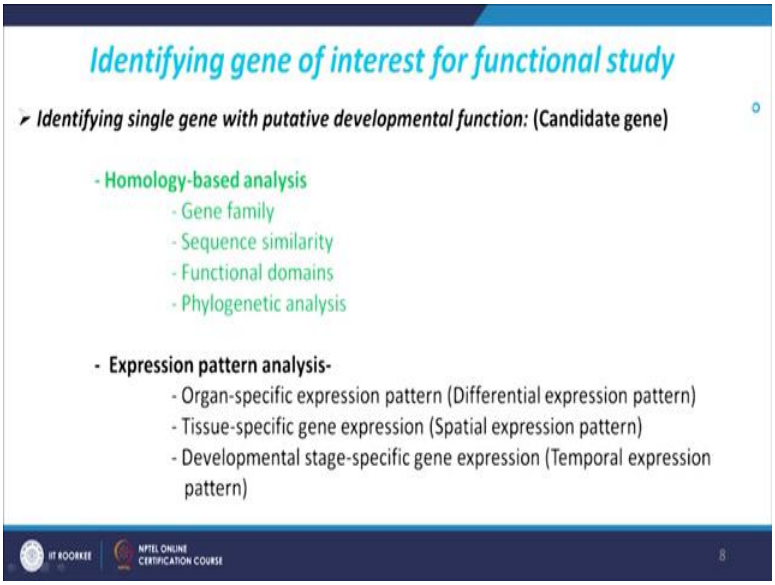

Then coming to the expression pattern analysis. So, expression pattern there are different ways to study expression pattern.

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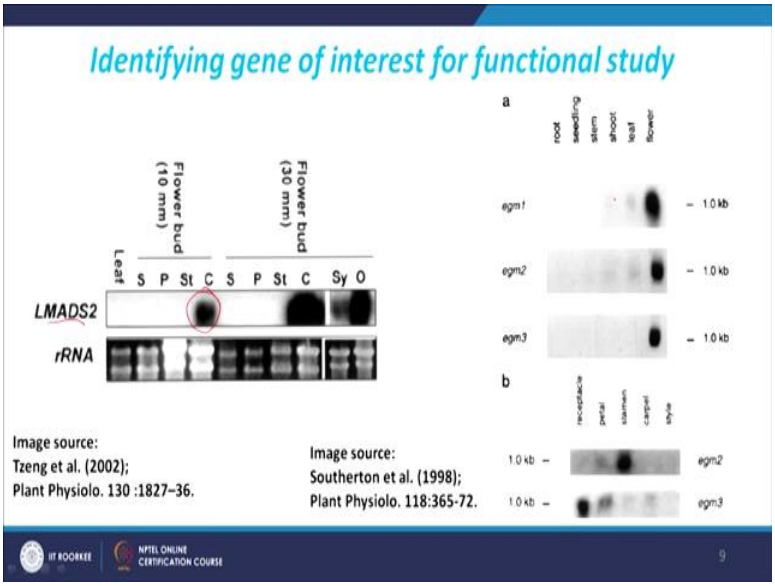

Typically people used to do northern blot analysis in early days when genome or most of the genomes were not sequenced. It is a hybridization based technique maybe we will see some time later. We are extracting total RNA, fractionating through the gel

electrophoresis, transferring on the membrane and then we are using an antisense RNA or specific DNA or RNA probe which is radioactively labeled and then probing with it.

So, it will go and it will hybridize with the messenger RNA which is specific for that probe and then you can detect through the radioactive detection method. For example, if you look some examples, so this is one of the *MADS* gene. This is leaf, sepal, petal, stamen and carpel's you can see that this gene is very specifically expressed in the carpel, but it is not expressed in all other tissues.

Similarly, if you look these are different genes and they are very broad very strongly expressed in the flower, but they do not express or they express very weak in leaf and other tissues. This is a differential expression pattern. Now I can expect that this gene might have some role in the carpel development whereas, this is a kind of general, it has a flower specific expression. So, we can say that it might have some function in the flower.

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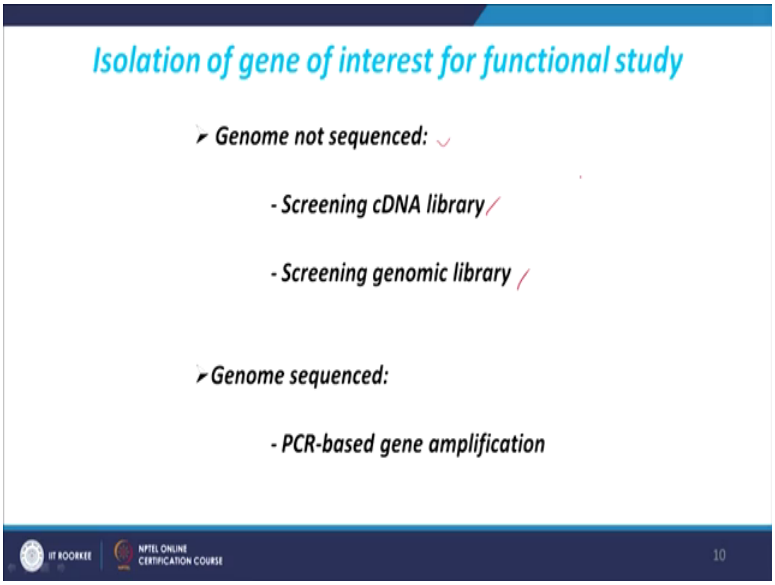

Then once you have defined a gene next question is how to isolate the gene? To isolate a gene if your model organism or if your plant species which you are interested in, it's genome is not sequence then you have to take some approach to identify the gene because you cannot design a primer you cannot do polymerase chain reaction, but if your genome is sequenced then it is relatively easy you can design a specific primer for your gene your all the sequence are known and you can PCR amplify.

But let us take first example if genome is not sequence, then one way of isolating gene is using cDNA library or genomic library. What are this library?

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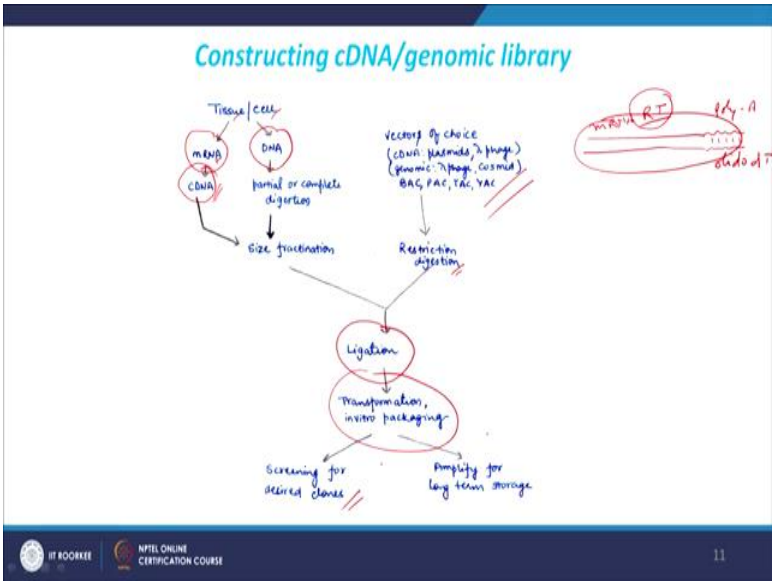

If genome is not sequenced, you can make their library. You can take either tissue specific cell or a particular organ whatever you are interested, extract total messenger RNA or total genomic DNA. If you want to make genomic DNA library, you will extract total genomic DNA, this will provide the information of the entire genome at the DNA level. But if you want to make cDNA library you have to extract total messenger RNA here this will provide the information of the expressed gene.

For genomic library you take the DNA do the partial digestion, you can sheer it, you can digest it and then you can fractionate, you can choose what size or fragment you want to generate library with. Similarly, for messenger RNA you extract total RNA, from total RNA you can isolate or you can specifically fractionate the messenger RNA and then use this messenger RNA to generate the cDNA synthesis.

The typical way of generating cDNA synthesis is, you know that a typical messenger RNA in case of eukaryote have poly A tail and you can design a primer which is oligo dT. This oligo dT is a reverse primer which will bind to the poly A tail of all messenger RNA and then you can do reverse transcription process through which you can make DNA from the RNA. So, now your template is messenger RNA and you are doing RT and then it will make a DNA.

You can generate DNA from the messenger RNA. Take this cDNA, you can fractionate on gel and you can choose what size of library you want to prepare or you can prepare all the library, and then you can use a suitable vector depending on the size of your fragment or size of clones you want to generate, and then digest this vector and do the ligation. Through ligation and transformation you can basically capture all the cDNAs; all the specific cDNAs which are present in a particular tissue or in a particular organ or in a particular developmental stages and then you can clone them and put in form of different libraries.

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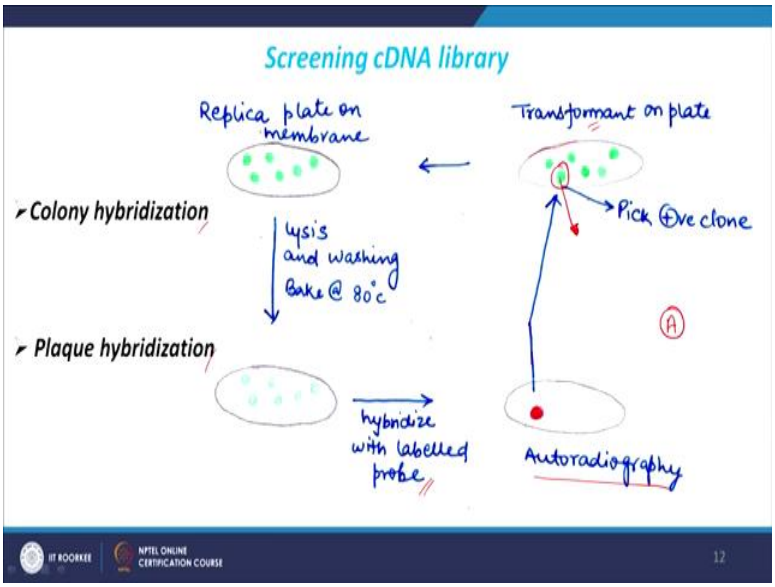

Now, since you have the library. Now you want to identify a specific gene from that library. So, there are two way of doing it depending on what kind of library you have made. So, one way of doing is colony hybridization and other is plaque hybridization. Technology wise both the techniques are very similar. What you do basically let us assume that you have library here and what you can do, you can grow this colony's on a plate and then you can do a replica plating.

So, basically you can take a membrane and you can basically replicate or you can make a replica plate of this particular plate and then you use this membrane, cell lyse and bake it. So, that the DNA which are coming from this bacteria they are getting fixed or they are getting attached with the membrane, then take this membrane and hybridize with your gene specific probe and then you can detect it.

So, let us assume if you do the hybridization and detection if this is the colony which is showing signal with your gene A using a probe against the gene A, then you can assume that the corresponding bacteria or the corresponding colony might have a fragment of A. You isolate this colony isolate DNA, isolate cDNA sequence and then you can identify your gene of interest for your functional study.

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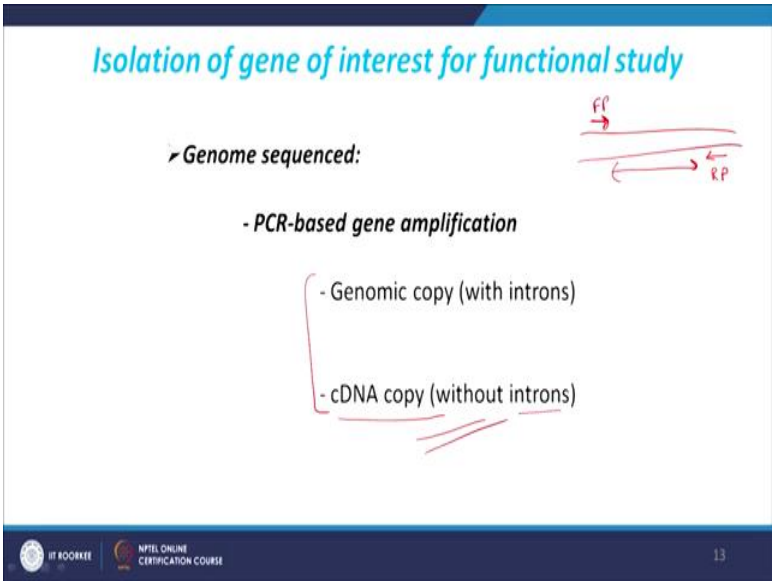

As I said if the genome is sequenced, then probably isolating gene is very easy, you can do a simple PCR based gene amplification where you have to design gene specific primer. So, if this is your gene you can design a short primers, one primer which we call forward primer, one as a reverse primer and then you can do a PCR amplification.

Now, you can clone a gene, this is called gene cloning, you can clone a gene and while you are cloning the gene you can either use the genomic copy or you can make a cDNA. Genomic copy basically, if you know and if you recall your basic molecular biology you know that in case of eukaryotic genomes genes have exons as well as introns.

So, if you use genomic DNA as a template and if you use a primer and amplify the gene then you are going to amplify both exon as well as intron; whereas, if you make cDNA for making cDNA you are using mature messenger RNA and in mature messenger RNA all the introns are already spliced out. So, if you use this method to amplify your gene then here you are going to amplify only the coding region which is spliced already and this will be without the introns.

So, depending on your use depending on the need you might choose what template you want to use and this is the way you can identify or isolate a particular gene of interest. So, now you have defined your gene of interest which you want to study through the reverse genetics based approach.

Now, next step once you have isolated the gene, you can proceed for functional characterization that we will take in some other class.

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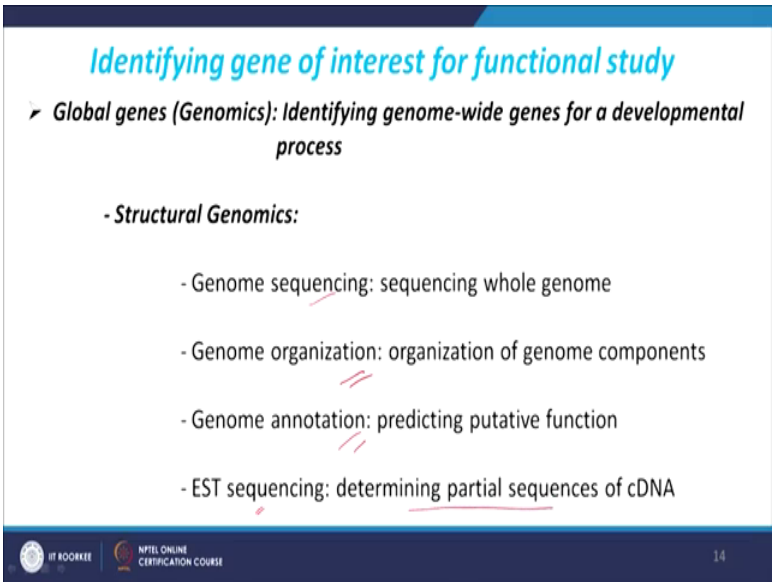

Another approach which we are taking for doing multiple gene identification is the genomics based approach. So, in genomic based approach we fix our biological process our biological organ or question and then identify at the global label what all are the genes which are expressed or which are present there.

There are two classes of genomics; structural genomics and functional genomics. In structural genomics we sequence the whole genome, then we use some of the already sequenced genome as a template and then you can do the genome organization. In genome organization you can basically identify what are the genes, what are the coding region, what are the non coding region.

Then you can do genome annotation where you can predict the gene using bioinformatics tool, doing the homology based analysis, using template of other known sequences or reference genome as a known sequences and then you can predict the putative function

or you can predict a putative domain or class of the protein. Then another important thing what do we do in structural genomics is EST sequencing; EST is basically Expressed Sequence Tag.

Here you can determine the partial sequences of some cDNA and this you can fix in a different tissue, different stages and different conditions. And then clone small fragment of the cDNA clone sequence to identify what are the genes which are expressed in a particular condition.

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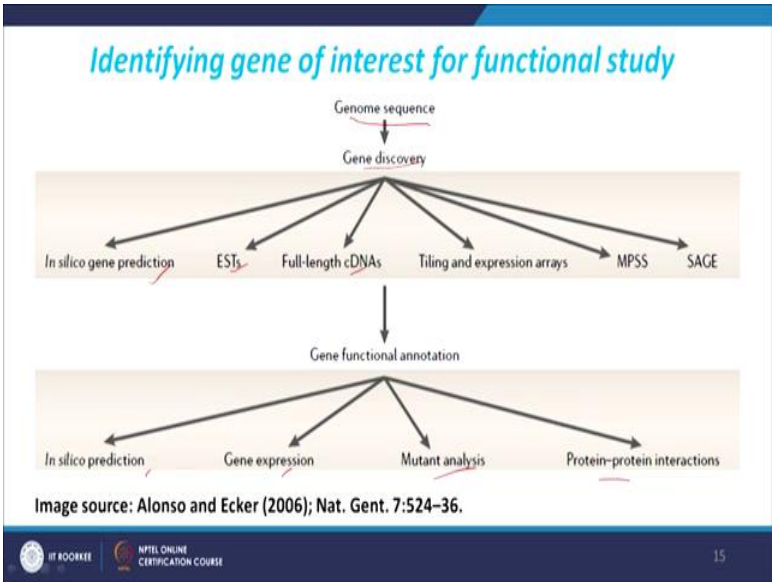

If you look this schematic diagram. So, if you have a genome sequence, you can do gene discovery, how you can do? You can take the *in silico* gene prediction, EST sequencing, full length cDNA by PCR and other methods. Then you can do a gene functional annotation, you can do expression pattern analysis, *in silico* prediction homology based then mutant analysis and protein-protein interaction.

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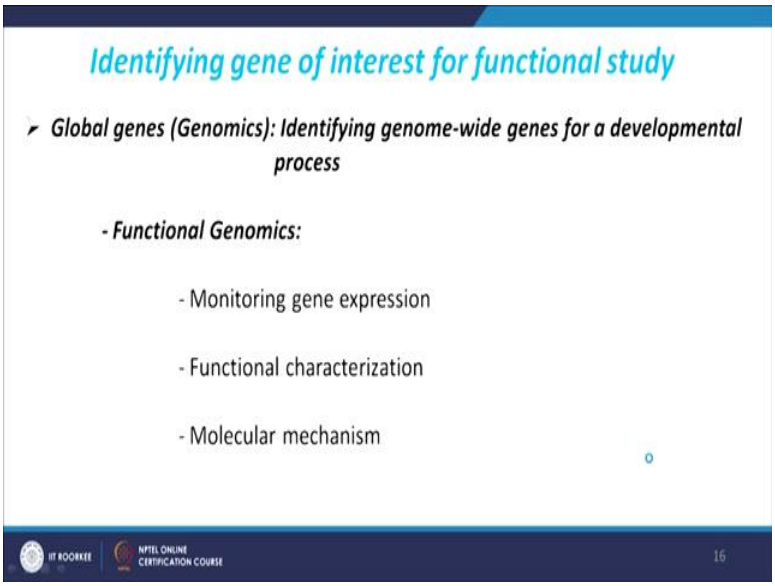

Another genomics approach what we are taking is the functional genomic approach. So, here we first identify or we monitor gene expression and then we go for the functional characterization and then trace out the mechanisms behind a particular process.

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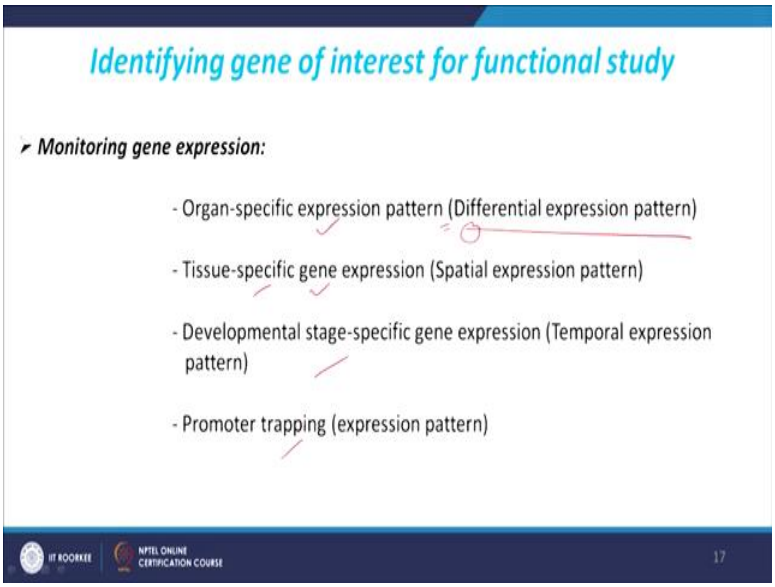

If you have to start monitoring gene expression then first thing what we you want to look that is there any organ specific gene expression or tissue specific gene expression. Organ specific gene expression; you can check in different organs this is called differential expression pattern, which is present in some organ absent in some organ.

It can be organ, it can be stages, it can be tissue whatever you want and then you can identify the differentially expressed genes or you will be more interested if expression of a particular gene is developmentally regulated. If gene is expressed everywhere probably that might not be very regulated and then we would be more interested in a gene which is specifically or present in a some condition, but absent in most of the condition.

Then you can study the tissue specific expression; in tissue specific expression you can even go and look the expression pattern in the organ; if you make anatomy of the organ you can look where exactly the gene is expressed which tissue it is expressed. Then as I said that if you want to study developmental stages, so one development comes after the next and then what you can do here you can identify the onset of a particular gene.

So, let us assume a gene is expressed in a particular development stage, but it is absent in the previous developmental stage. So, you can go and identify what is the first developmental stage where this gene get activated or start expressing; this is called temporal expression pattern. Then we do promoter trapping, how exactly we do we will discuss in another class.

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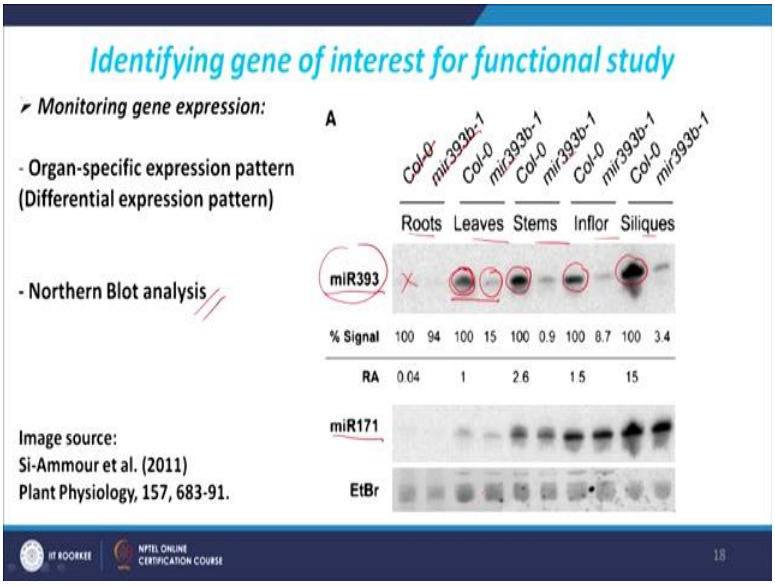

So, what are the different ways in the plant developmental biology to monitor gene expression? First way is Northern blot analysis, this is the old way. Here we extract total RNA from two different organs, two different stages or two different conditions, run them on gel and then we transfer the RNA on membrane and then use a probe which is

very specific for a particular gene. Hybridize the membrane and then check the expression and see how the expressions or amount of RNA varies across the tissues across the organs.

So, these are some of the example if you look this has been done for some micro RNAs; micro RNAs 393 and micro RNA 171 and if you look in different tissues root, leaves, stem, inflorescence and silique. So, the probe was used against this micro RNA and tested in a wild type background as well as some of the mutant background. You can clearly see that the micro RNA is present in some of the tissue, it is present in the leaf, it is present in the stem, it is present in the inflorescence and silique, but it is totally absent from the root.

So, this tells the organ specific expression, in the shoot system it is present, but it is absent in the root system and if you look these mutants, in mutant background the expression is getting reduced. For example, if you take the leaf, so it is very highly present in case of wild type background, but in mutant background the expression is reduced. Similar kind of things you can check here for other micro RNA.

So, northern blot is being used here, but this is like as I said that previously it was being used when advanced techniques technologies were not discovered because it involves a kind of radio activity. Now though we have another method of non radio activity, but this is more kind of laborious method. So, this is being used now in a very special cases, but this is not a kind of routine way of checking expression pattern.

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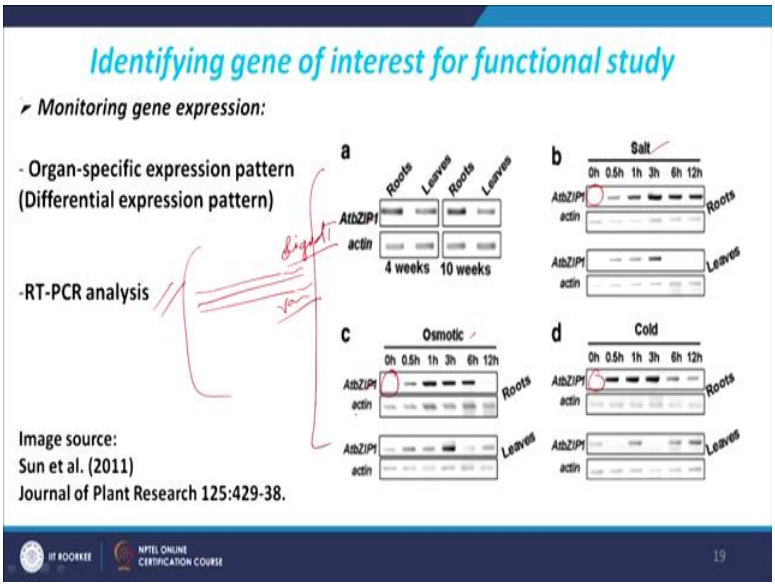

Now, the more quick and more easy to do expression analysis is reverse transcription PCR. So, in reverse transcription PCR you are extracting total RNA, then you use some primers, reverse primer which can be oligo dT or it can be simply random primer and then you are doing reverse transcription to make cDNA and so if you have extracted total RNA from a particular tissue, then you will convert all the messenger RNA into cDNA from that particular tissue. And then you can use some gene specific primer to do PCR amplification from that particular pool and then you can see whether a gene is expressed or not.

For example these are the RT PCR product. So, you can see in a certain condition in different tissues, these genes look quite stable it is expressed in almost all tissues alike, in roots and leaves, but if you look in different conditions. For example, when you are inducing with the salt. When you do not induce the expression level is almost 0. But when you induce at different time point in root, then you can see that expression of these genes are going up. This you can monitor by RT PCR and this RT PCR is called semi quantitative RT PCR.

So, here what we are doing? You are making cDNA, then you are doing some round of PCR cycle and then you are taking the end product of the PCR and running on the gel to check. The drawback here is that you do not know after how many cycles the RT PCR will start getting saturated.

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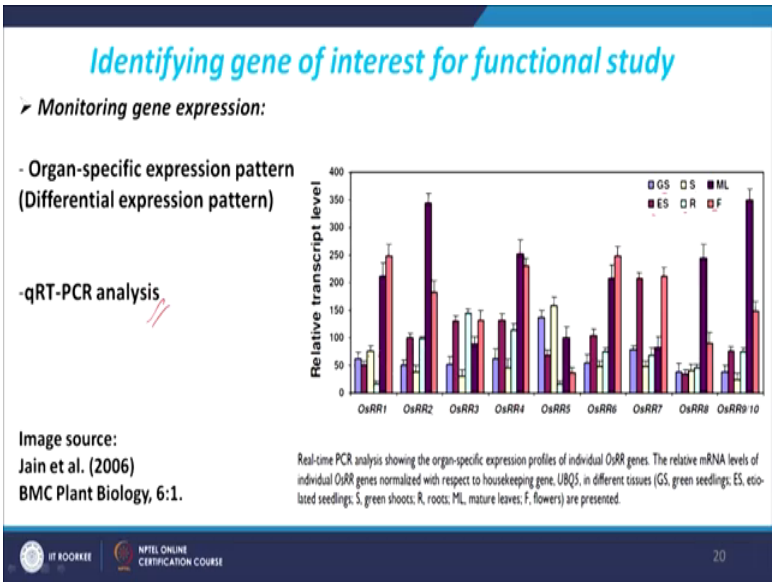

The alternate method which has come, is quantitative RT PCR which is more kind of dye binding based assay. Here Syber green is very routinely being used and you can basically monitor expression pattern more quantitatively. For example, different tissues has been taken, these are different genes and here you can design a gene specific primer and you can monitor expression pattern in the more quantitative manner for different genes in different conditions. This is another very important techniques which is being used for this one.

So, we will stop here for this class and in next class we will take further of the functional genomics based approaches.

Thank you very much.