Plant Development Biology
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Lecture - 07
Molecular Genetics of Plant Development - IV

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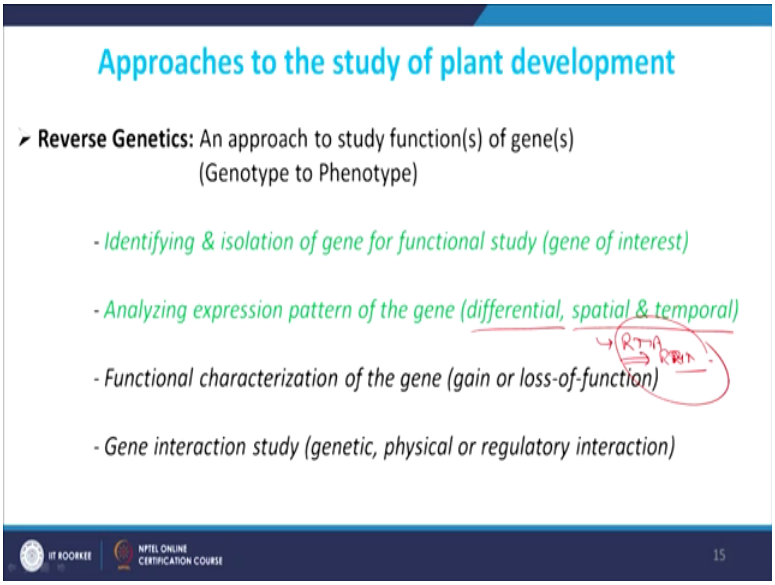

Welcome back to the course of, Plant Developmental Biology. In last class we were discussing, reverse genetics based approaches to study the developmental biology. In that class we have covered identification and isolation of genes for developmental functions. Now, we have started the functional genomics based approaches. In functional genomics based approaches, we discussed the first step; monitoring gene expression.

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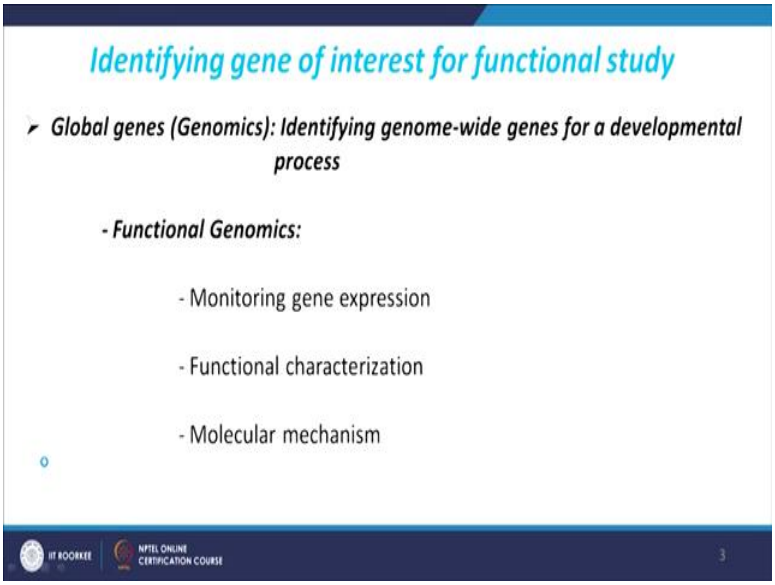

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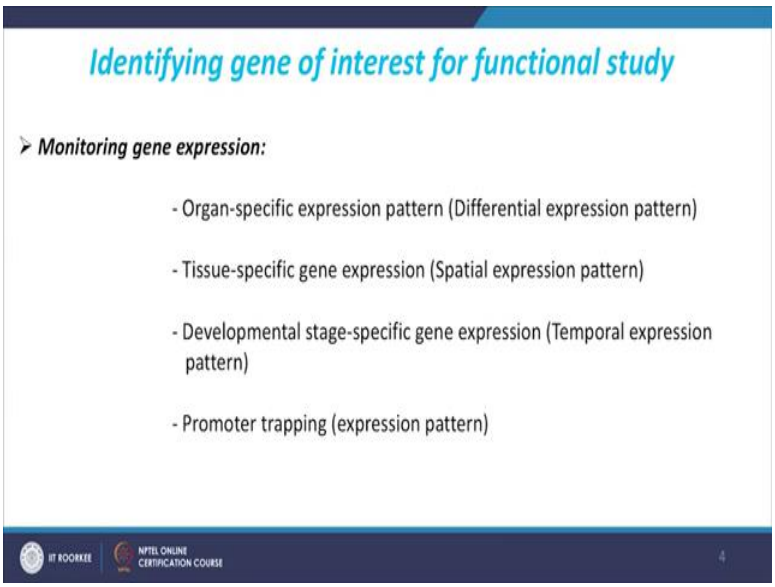

And now, so we have seen how to check the expression pattern of individual genes.

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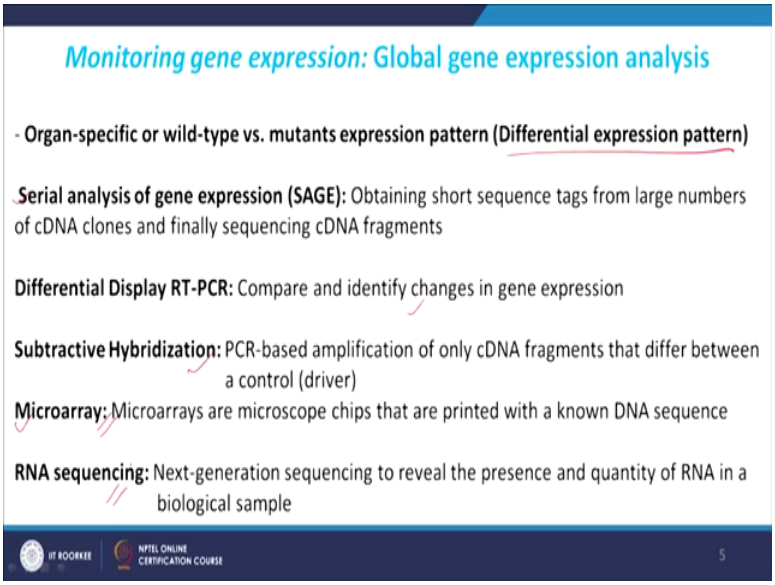

Now, we will mostly focus on the global genes. How to study the expression pattern of the Global genes? And what are the way how to study the expression pattern? You can again fix the problem. So, the main goal of a genomics or Global gene expression analysis is, to identify the differential genes in different conditions.

It can be different organs, it can be different tissues, it can be different stages, it can be different conditions between a wild type and mutants any combination. Whatever you can choose with respect to the development and then you take the genomics based approaches and identify all the genes which are differentially expressed between two conditions two organs or whatever.

And there are many way to study a differential gene expression. So, I will list some of them here, but we will focus on only few.

First was the serial analysis of gene expression, SAGE. Here people started by obtaining some short sequence tags from a large number of cDNA clones. And they, then they sequence it and identify the differential gene expression pattern.

Then another way was differential display RT PCR. Here what people used to do, they used to take two population of total RNA, they used to make cDNA, run a gel and then try to identify which particular band is missing in one condition and present in another

condition or vice versa. And then they used to cut that band, amplify the fragment, clone, sequence and identify what are the genes.

Another method is subtractive hybridization, this was PCR based amplification of only cDNA fragment that differs between two conditions. Here for the two population of the messenger RNA, you make cDNA then do the subtraction. After subtraction, you identify or you isolate only those populations which are differentially expressed and then specifically you can amplify them and then identify them.

The technique which was very widely used in most of the genomics based differential expression pattern, analysis was microarray. Microarray was the technique, where small fragment or small single stranded oligos were spotted on a chip and then the total RNA was, isolated from a particular condition to identify the differential expression.

We will see microarray in detail, how this has been used as this is one of the most used technology in the plant developmental biology.

And now recently next generation sequencing has, actually changed the entire scenario. RNA sequencing has lot of benefits over the microarray. So, these are the different methods which are being used to study the differential expression pattern in different conditions or different developmental processes.

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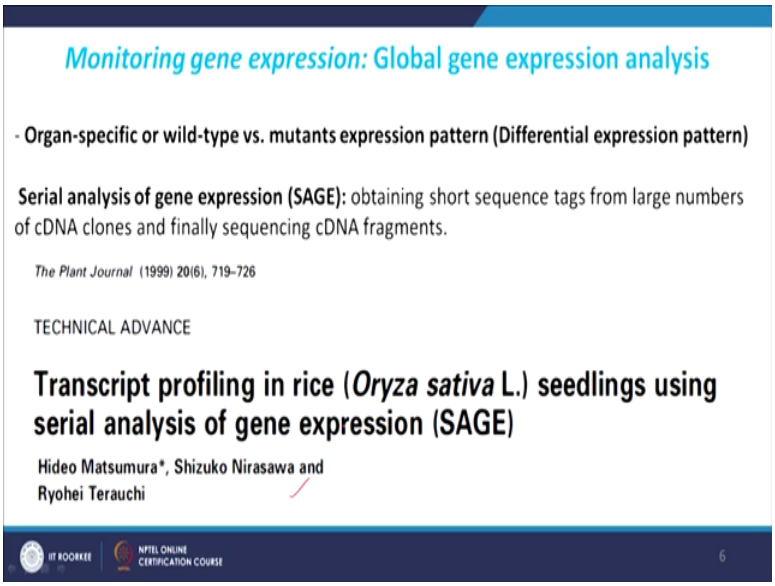

So, before going to the Microarray, I would give some of the typical examples of different technologies, which was used. For example, here the transcript profiling was done in rice seedling using SAGE as a method. So, this was used and people have identified the differential expression here.

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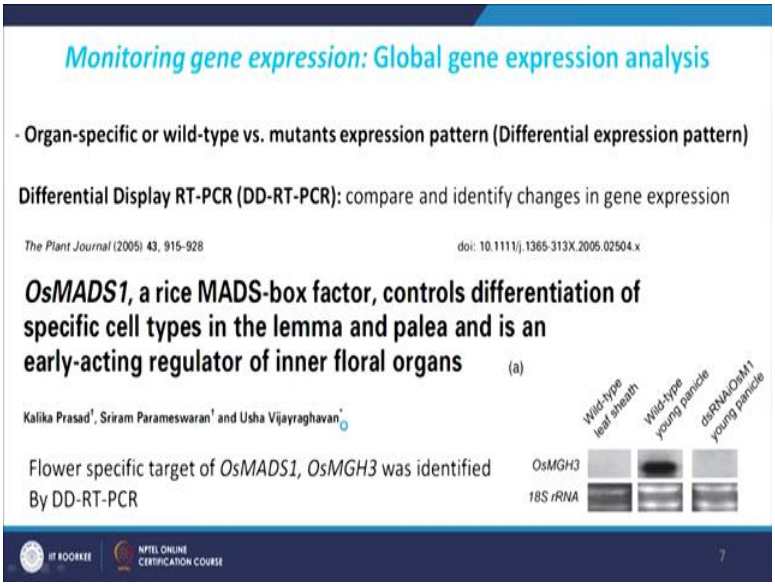

Then this is one example, where differential display RT PCR was done. This is MADS box containing transcription factor in rice. This gene which is *MADS 1*, it was expressed in sepal equivalent which in case of rice, is lemma and palea, but it was not expressed in the inner organ. But when the gene was mutated, when this gene was silenced, the effect was also seen in the inner floral organs for example, lodicules and stamens. Then the question was, if the gene is not present in this tissue how it is regulating?

So, one of the possibility was that, maybe it is activating because at very early stage, it was expressed throughout the floral primordia. It was hypothesized that maybe, it is activating some early regulators at very early stage, whose expression is basically there in inner floral organs and regulating it. To identify such kind of gene, the differential display RT PCR was taken up and then one of such genes was identified which was *OsMGH3*. And it was shown that actually *OsMGH3* was expressed in the inner floral organs.

This is the northern blot and here the expression pattern of *OsMGH3*, you can see that in wild type, leaf sheath it is not expressed whereas, in the young panicle, panicle is inflorescence in case of rice, it has a very high level of expression. But if you look a panicle which is mutant panicle, where *MADS1* is down regulated, the expression of *OsMGH3* also disappears. Which means that there is an organ specific or tissue specific regulation of this gene. The gene is only expressed in the panicle, not in the leaf sheath and it is expression or activation is dependent on the *MADS1*. So, this is one example of differential display RT PCR.

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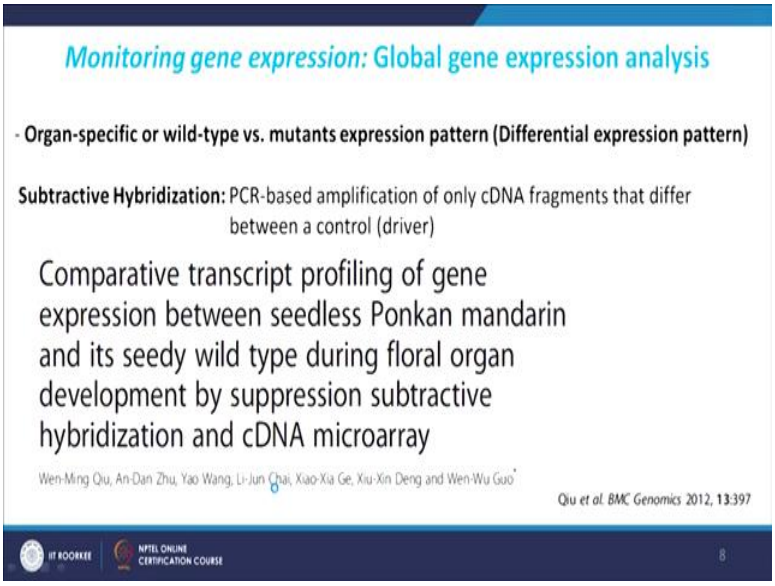

Then subtractive hybridization was used in this study. Where they have done a comparative transcript profiling of gene expression between seedless variety and it's seedy wild type during floral organ and development and I will not go in detail.

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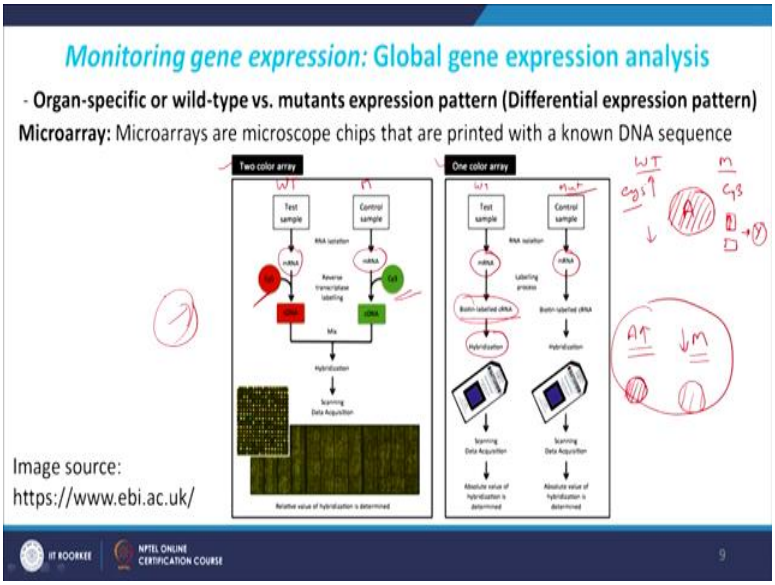

Then the technique which is microarray. So, microarray is a kind of chip generated and widely used in the post genomic era, before RNA sequencing. Once the genome was sequenced and identified the sequence of all the genes. Then the probes for those genes were designed and these probes were spotted in these chips.

So, for every gene we had a probe. And then there was two way to identify the differential expression, one was the two color way and the other is one color way. So, in one color way what you have to do, let us assume that, you want to test a wild type sample which is your test or mutant sample. So, you want to identify the genes which are differentially expressed between this wild type and this mutant. And what you can do, you can extract total RNA from the wild type, total RNA from the mutants then you label this RNA. And one way of labeling was, the biotin labeled cRNA synthesis. Both were basically labeled.

So, entire RNA population in these two conditions, were labeled and then this labeled cRNA were used to do the hybridization on the chips. So now, these chips contains, global genes represented in an equal amount.

Now let us assume that, there is a gene A, which is more in case of wild type, but it is getting down regulated in case of mutant. Then you are expecting more cRNA in wild type, less cRNA in mutant. And when you hybridize on the chip, you are expecting more

signal for that probe in case of wild type whereas, less signal in case of mutant. And then if you compare the signal intensity, you do the data acquisition scanning and if you calculate the absolute value of hybridization, you can identify the difference in the expression pattern of gene A between wild type and mutant.

And then, another way of analysis was that let us assume, a similar case you have wild type RNA, you have mutant RNA and then you take total RNA from both the sample. And then you label them with two different dye for example, this is cy5, this is cy3, cy5 will give red color, cy3 will give green color and then you take equal amount of total cRNA and hybridize on the same chip.

So, basically the difference between these two methods was, here both the cRNAs were labeled with the same label, whereas here they are labeled with the different labels. Here they were probed, they were hybridized on two different independent chips, here both are being hybridized on the same chip.

So, let us assume if there is a chip which is for gene A. Now normally if the expression level of A is same in wild type and mutant, then you are expecting same amount of cy 5 label cRNA and cy 3 level cRNA to go and hybridize with this particular probe. So, you are expecting same amount of red signal, same amount of green signal and the result will be yellow signal here. But if any one of them are differentially present for example, if expression is more in the wild type then you are expecting more red signal than the green and then the color of these spots will change, it will be more towards the red.

If gene is down regulated then it will be more towards the green. So, based on this color and then the quantification you can actually calculate the fold change between wild type and mutant for all these gene.

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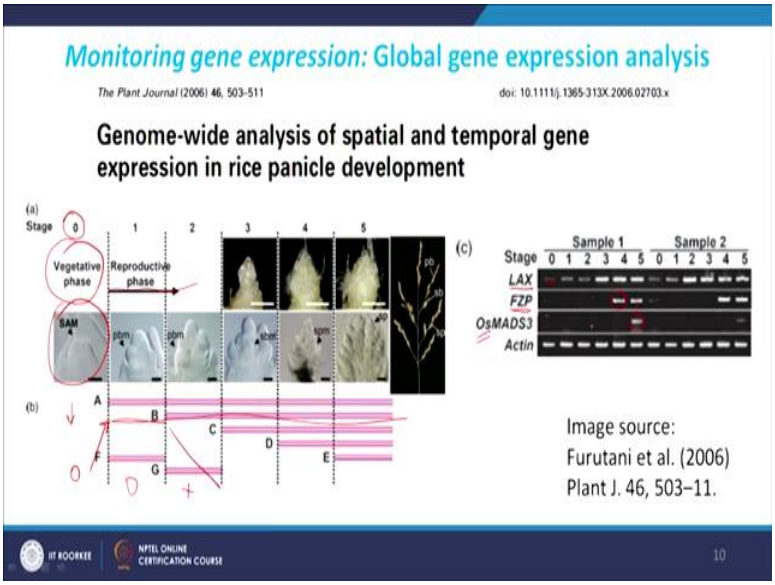

These techniques you can also use for the different stages. For example in this particular case. People have studied the genes which are differentially expressed, at different developmental stage of rice panicle development or flower development. So, for example, if you take these stages, this is stage 0 which is basically vegetative phase and this is your shoot apical meristem.

This is vegetative phase and then there is a transition from vegetative to the reproductive phase. Then after transition, this inflorescence has different stage of development. Here primary branch meristem and then secondary branch meristem, secondary spikelet meristem. So, what people have done, they have taken RNA from all these different stages and they have performed the microarray analysis.

They could identify the gene which is present in a particular developmental stage, but absent in the different developmental stage. Or a particular genes, how it's dynamic expression pattern is across the development. For example, some genes might be 0 here and then they are suddenly getting activated and they are maintained across the development. Or they are activated in this developmental domain and then they are going down.

So, dynamic expression pattern you can analyze, using this technology. And everything, we are doing at the global level and we can identify. For example some of the genes, has been tested here. So, you can check this is RT PCR analysis.

If you see the gene *LAX*, the gene *LAX* is very low at 0 stage but across the stages 1 2 3 4 5 the expression level is increasing, whereas if you take this *FZP* which is *FRIZZY PANICLE*, the expression starts or activates only from the stage 4 onwards and continue in the stage 5. It is not present in the early stage, whereas this *MADS3* it is getting activated in stage 5. So, this is how that microarray was extremely useful, in studying this kind of genome wide or differential expression analysis.

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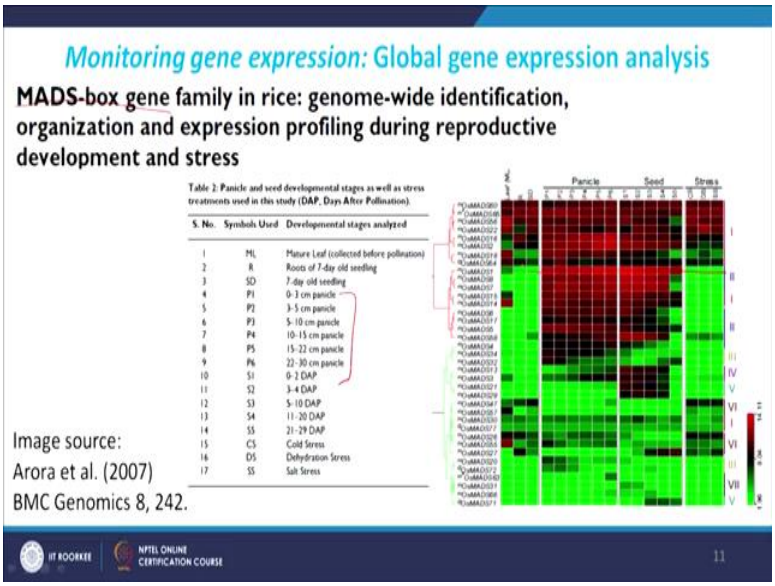

This is another example of a microarray which was used and here they have focused their study on the *MADS* box containing genes. Why I am focusing most on the *MADS* box containing gene? Because this was the class, which is well established to regulate a lot of developmental processes. Here they have taken large number of tissues like mature leaf, roots, 7 day old seedling and then different size of panicle. Different size of panicle or inflorescence signifies different developmental stages in case of this one.

And then they have checked the expression pattern, through microarray analysis. And what they can identify, there are lot of genes. So, if green is less expression, red is high expression. For a particular gene, if you go across the different organs you can see their

expression. How the expression patterns are changing from one developmental stage to another developmental stage or one tissue to another tissue.

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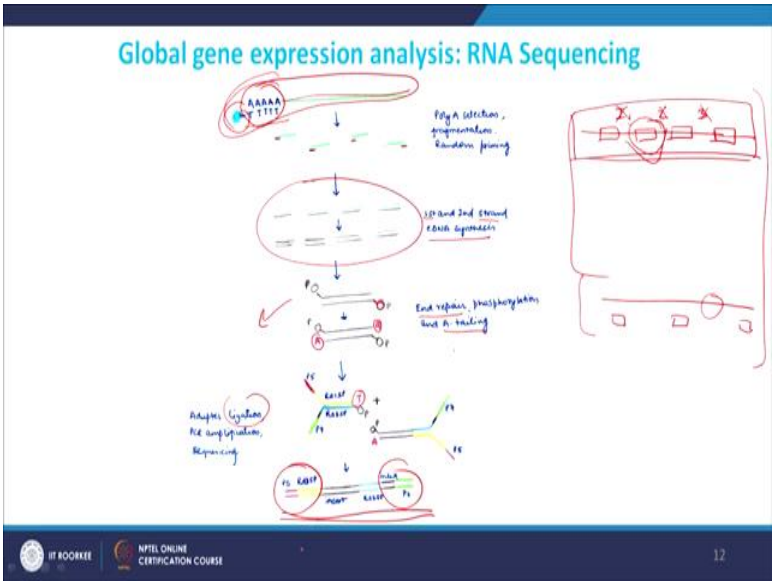

Nowadays it is RNA sequencing, this is next generation sequencing. It is very cheap and very easily available. So, now, people are doing RNA sequencing and has some advantages over the microarray. First advantage is that, for microarray to generate the chips you have to have the sequence information, which means that the genomes of the organism has to be sequenced, then and only then, you can define probes for the chip. But in RNA sequencing even if the genome is not sequenced, it can be studied through RNA sequencing.

Second, an important process in eukaryotic organism is alternative RNA splicing. Alternative RNA splicing occurs if a gene has multiple introns. So, let us assume intron 1, intron 2 and intron 3. So, there are a large number of gene regulation occurring at the level of splicing. Alternative splicing essentially provides, an opportunity to generate more than one type of protein from a single gene, just by alternatively splicing different introns.

For example, one messenger RNA, you can generate from here if you splice all the introns. Then you can generate a messenger RNA, where you do not splice intron 1 or you do not splice intron 2 or you do not splice intron 3 or you splice individual, you can

even skip the introns, you can splice this exon along with the intron. So, this provides a large number of different transcripts from the same gene. And it is very difficult and challenging to keep probes for all splice variant in the chip, in the microarray experiment.

It is challenging because if you do not know, how many splice variant exists for a particular gene, it is very difficult. Because normally when you are designing the chip, you used to keep one or maybe two or maybe three chips for a particular three oligos for a particular gene. But if there is some intron here, you will not have intron or splice variant a specific chips. But in case of RNA sequencing, basically it can capture all the splice variants.

So, if you look at the technique how RNA sequencing functions? Extract total messenger RNA and then you can specifically select messenger RNA from the total RNA. How you can do that? You all know that messenger RNAs, they have poly A tail at the 3' end and then you can use oligo dT tagged. And using this system, you can specifically select messenger RNA from the total RNA population. And then you use this RNA and you can do the fractionation, you can make a small fragment of RNA and then use some random primers.

Random primers are basically small six nucleotide long primer with different sequences. So, it can randomly go and bind different places and then you can make first strand cDNA, synthesis second strand cDNA. So, basically you are generating a different, different small fragments cDNA and it is double standard cDNA. Then you can put the phosphate, normally the primers they do not have phosphate at their end.

So, then you can provide phosphate because if you have to use this for further ligation process, if they do not have phosphate at their end, then they cannot make a phosphodiester bond. So, you can do the end repair by placing the phosphate.

And another thing what you can do, you can add flanking A, this you can be done using poly A polymerase and then if you add this A tail, this will provide you to take this fragment and add/ligate some adapter. This adapter can have a stretch of T. So, T and A, they will anneal to each other and then you can perform a ligation reaction. So, essentially what you are generating, you are generating double stranded DNA fragments

with adapter at both the end. And then you know the sequence of this adapter you can use this adapter sequence and you can take and sequence this entire cDNA population.

So, if you take two different population of RNA, you can generate two different population of fragmented cDNA. And if you sequence them and compare them, you can identify differential expression level of different RNA.

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So, again I will give some of the examples, where RNA sequencing was used to identify differential expression pattern. For example, here different staged fruits were taken and total RNAs were extracted and RNA sequencing was done. And you can clearly see different genes and their expression pattern.

If you look this gene, or any of this gene, this has a very low expression at this stage, but if you come here by the end of this stage the expression level is really going very high. So, this tells, that there are a differential expression of different type of genes. This is heat map of RNA sequencing data which involves the shoot development. So, there they have taken different types of shoots here: secondary shoots, primary shoots, mother variant shoots. And they have extracted total RNA and performed RNA sequencing, across these different types of shoots and they have identified different tissue specific genes which are present here.

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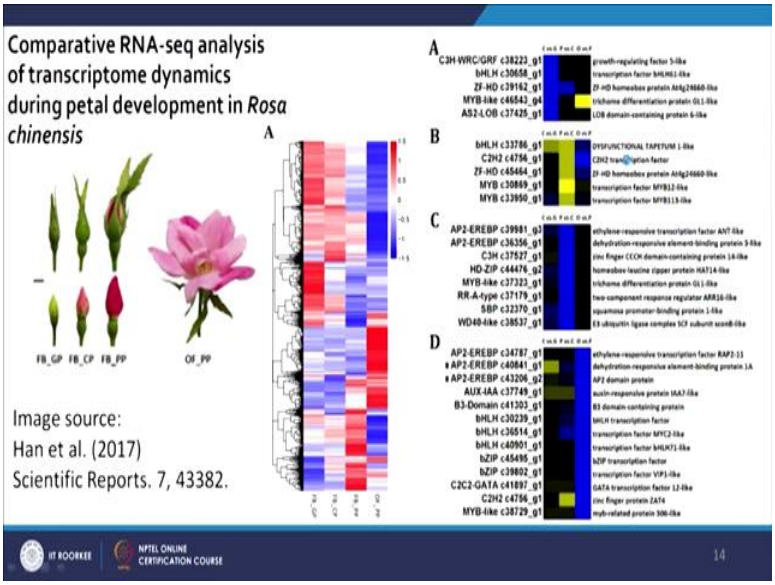

This is another example, where comparative RNA sequencing analysis was done for transcriptome dynamics during petal development in in Rose, one of the Rosaceae member and where you can see that they have taken different stages of the petals from the different developmental stage and they have performed the RNA sequencing analysis, and they could identify different genome expression pattern.

They could identify here all these different types of transcription factor. Depending on your studies, you can focus on transcription factor. So, you can identify all the transcription factors which are differentially expressed.

In RNA sequencing you can combine with a different other technique. So, it is not stand alone technique and then you can perform more specified or more defined way to generate RNA sequencing data. For example, if you have some kind of developmental stages maybe I will draw here.

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So, for example, if you want to study a particular developmental stage, let us assume that you want to study root developmental stage and you know that root development occurs through different stages of the development.

The first stage in the lateral root, branching is that the specification of some cells. So, these are the mature cells, now they are taking stem cell property and then these cells start the cell division. They undergo the process of cell division and they generate primordia which is called root primordia. And then this root primordia undergo the process of cell differentiation. It start root specific differentiation program and then finally, the primordia is emerging out.

Now, if you want to really identify the genes, you can collect this tissue very specifically. There are lot of techniques available, one of the technique which is called laser capture microdissection. You can use and you can collect all these primordia and then extract total RNA from this primordia and then you can identify. Or you can take some wild type sample and the mutant sample, were a particular transcription factor a particular gene is missing.

And you want to identify what are the genes, which are, whose expressions are getting affected, when you mutate a particular gene. Then you can take total RNA, you can do tissue specific total RNA extraction. And then perform RNA sequencing to identify the

differential genes which are present in these two particular condition or two particular mutants.

So, this is about analyzing expression pattern, where we have tried to study today how to identify differential expression pattern. Now for doing spatial and temporal expression pattern analysis, there are several method, which we will discuss more in the next class. But to just briefly introduce them, one way of doing is that to study RNA localization, which we are going to do by RNA-RNA *in situ* hybridization.

Then you can do protein localization, you can use some specific antibody and localize you can study their specific localization in across the tissues. And then you can do promoter trapping, where you can use a promoter less reporter gene or you can do transcriptional fusion, you can do translational fusion. So, maybe we will stop this class here.

And in next class we will discuss in detail, how to study spatial and temporal expression pattern and functional characterization of gene through reverse genetics based approaches.

Thank you very much.