Plant Developmental Biology
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Lecture - 08
Molecular Genetics of Plant Development - Cont.

Welcome to the course of Plant Developmental Biology. We are continuing with the reverse genetics based approaches.

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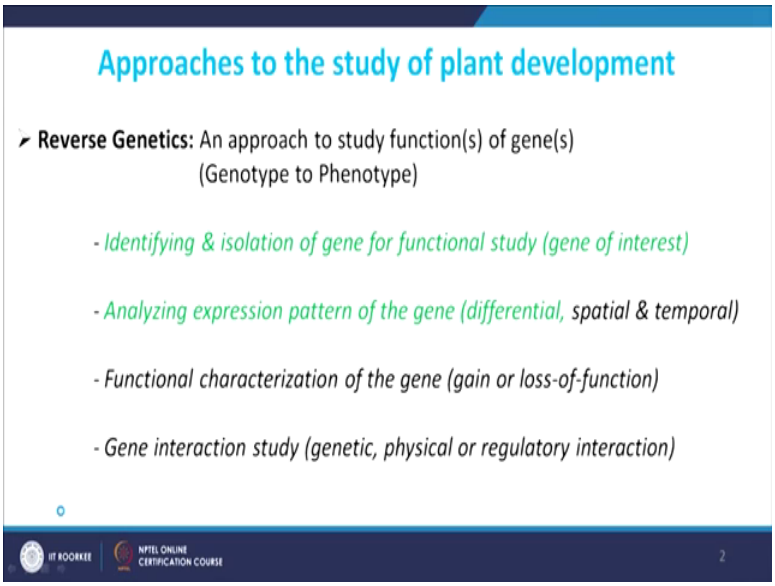

If you recall previous lectures, we have finished identification and isolation of gene for functional study. We have studied analysis of expression pattern particularly global analysis or the expression pattern where differential expressed genes has been already identified.

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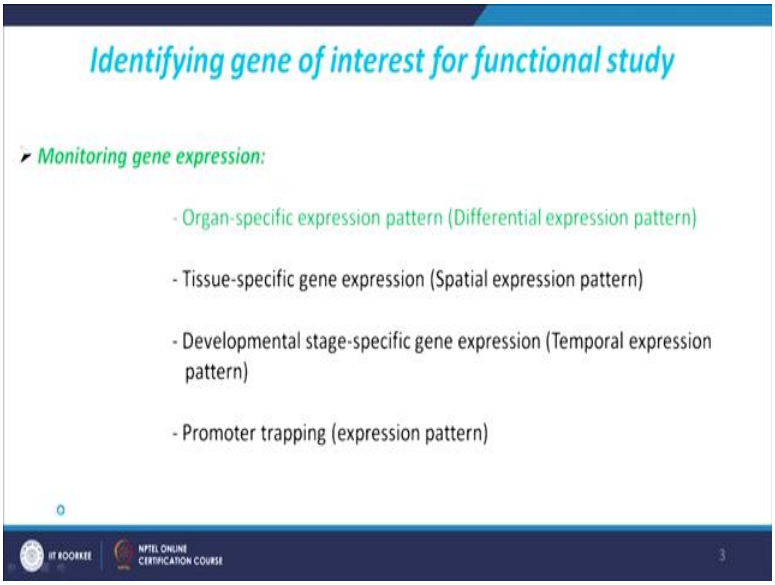

In this class we will focus on the spatial expression pattern analysis.

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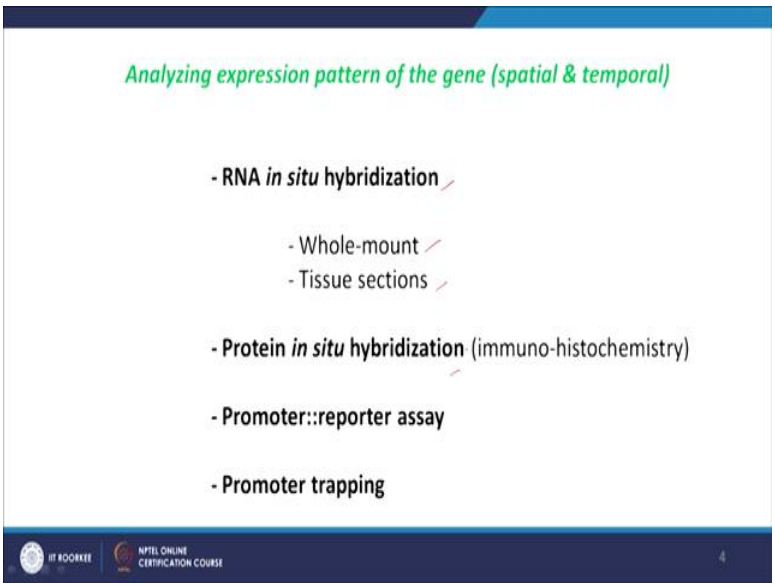

So, this is very important in context with the developmental biology, because development of any organs or any tissue is a context dependent process. We have discussed that, cell-cell communication is one major driving factor in case of the development.

So, if you want to study function of a particular gene through reverse genetic based approach, the first and very important thing what you have to do you have to very precisely study what is the expression pattern particularly it's spatial and temporal

expression pattern. Spatial means which tissue, which cells or which organs it is expressed and temporal means at what stage of the development it has started expressing.

So, there are several ways to analyse expression pattern spatially and temporally, one way is RNA *in situ* hybridization. Here we use whole mount issue or tissue sections. In whole mount issue you can take a full organ or full structure and then you can probe that with a specific gene specific probe and then identify where a particular gene expressed?

In tissue section we first fix the tissue and then make a cross sections of 8 micron or 10 micron thickness and then probe that cross section with the gene specific probe. So, this method you can use to identify messenger RNA which means that you can identify the transcriptional activity of a gene in a tissue specific manner.

Second thing you can study is the protein *in situ* hybridization, where you can look where a particular protein is present. There are other ways also to study temporal and spatial gene expression pattern which is promoter reporter assay and promoter trapping, we will discuss one by one.

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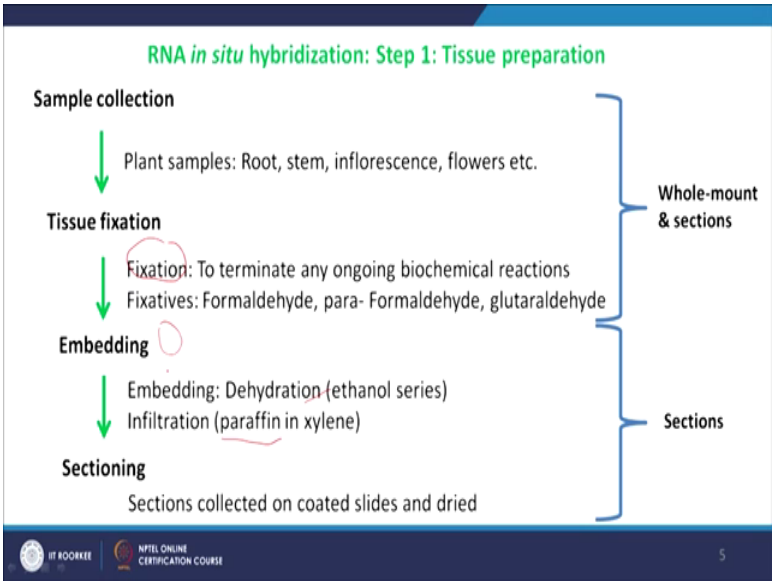

So, first we will take RNA *in situ* hybridization. So either you can do whole mount *in situ* hybridization or you can do on the section. The first major step of RNA *in situ* hybridization is tissue preparation. So, you collect the sample depending on what tissue

or what organs you are going to study, you can collect root, stem, inflorescence, flowers depending on your interest.

Then you have to fix the tissues. This is important to terminate any ongoing biological reactions or bio-chemical reactions. There are various fixatives available to fix the plant tissue and these two processes are common in both whole mount *in situ* as well as *in situ* on the cross sections or longitudinal section, but if you want to do on the sections then another thing what you have to do? You have to take this tissue and embed in embedding media. This is important because you want to make a very thin section, which is 8 micron or 10 micron section. The embedding media preferably what we use is paraffin.

Then there is process of the dehydration through ethanol series to remove the water and then you infiltrate this with the paraffin. And then once your paraffin blocks are ready, you take this samples, make a cross section collect this sections on Poly-L-lysine coated slide, this is important because you want your section to stick on the slide and it should not fall off.

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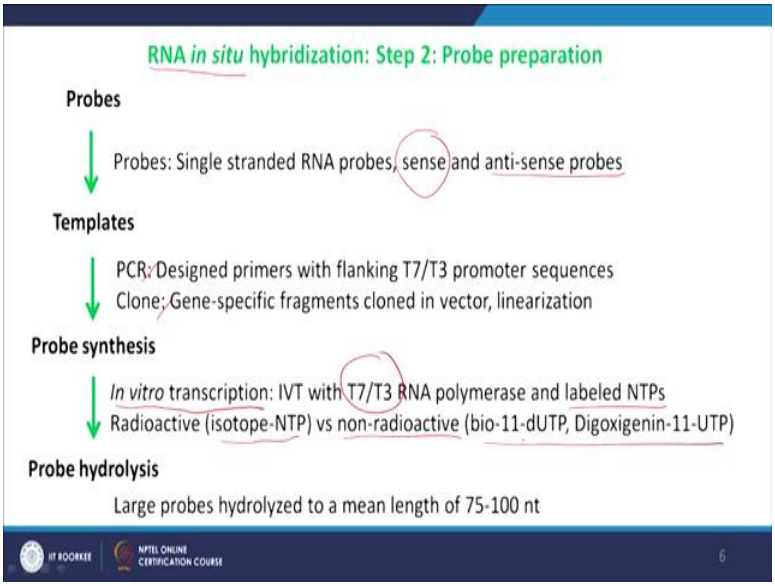

Then you have to generate RNA probe. This is RNA-RNA *in situ* hybridization which means that you are detecting messenger RNA for a particular gene and using RNA as a probe. So, if you have to make probe you will have antisense RNA as a probe because this antisense RNA will go and hybridize with the sense RNA.

But as a control you will also make sense probe to basically check what is the background signal. And how you make this probes I will describe in detail. There are two ways; one is that you can do PCR based probe synthesis or you can clone a gene specific fragment in a vector and then use that as a template to generate the probe. Since we are generating RNA probe the process which involves is *in vitro* transcription and here we are using either T7 RNA polymerase or T3 RNA polymerase depending on which promoter is available and then you can use labelled NTP.

So, there are two way of doing it. Initially people used to make radioactive labelled probe and now because of lot of issues with the radioactivity, we are moving towards the non radioactive labelled probe. And in this probe synthesis either you take one of the NTP as isotope labelled or you can use some of the chemical synthesized NTP. For example, this is biotin labelled UTP, DIG or digoxigenin labelled UTP. And then this probe, depending on what is the size of your gene, you can use them and you can hydrolyse them.

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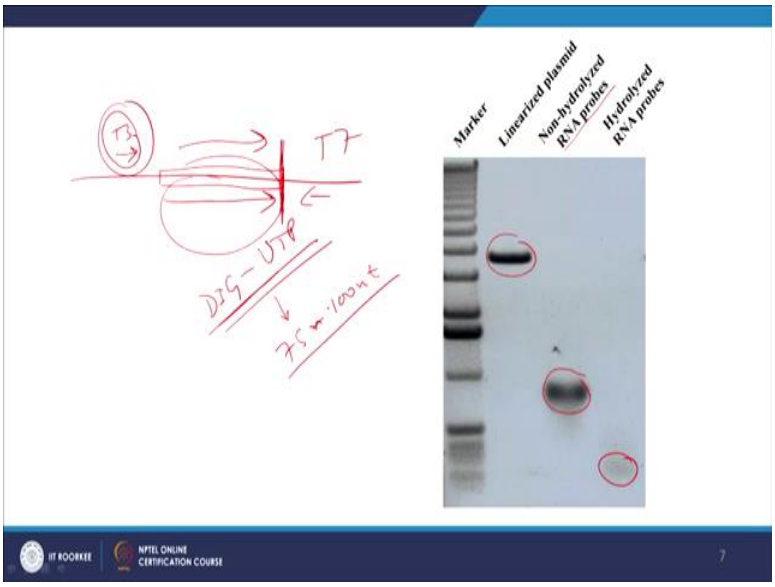

So, basically you take your gene specific fragment and clone in a cloning vector. The important thing is that your cloning vector should have T3 and T7 promoter or you can use SP6 promoter depending on the vector available. And then if your gene specific fragment is in the sense orientation, if you have to generate antisense probe. You will have to use the T 3 promoter and T 3 RNA polymerase. For doing this you linearize this

vector from here and then use T 3 RNA polymerase to transcribe this region as a antisense probe.

But while transcribing this region you are adding UTP which is tagged with digoxigenin which is called DIG-UTP and then if the size of probe is higher than prefer to hydrolyse it and make a probe size of roughly 75 to 100 nucleotide long.

This is important because if size of probe is very high it will not properly penetrate the tissues at the time of hybridization. For example, this is a gel picture which is showing how to generate the probe. This is a linearized plasmid, you have plasmid with your gene specific fragment cloned in it, then you have done *in vitro* transcription and you have generated the probes; this is RNA probe. But this probe size is quite high then we have hydrolysed this probe and we have made a small hydrolysed probe. Now, this hydrolysed probe is ready for hybridization.

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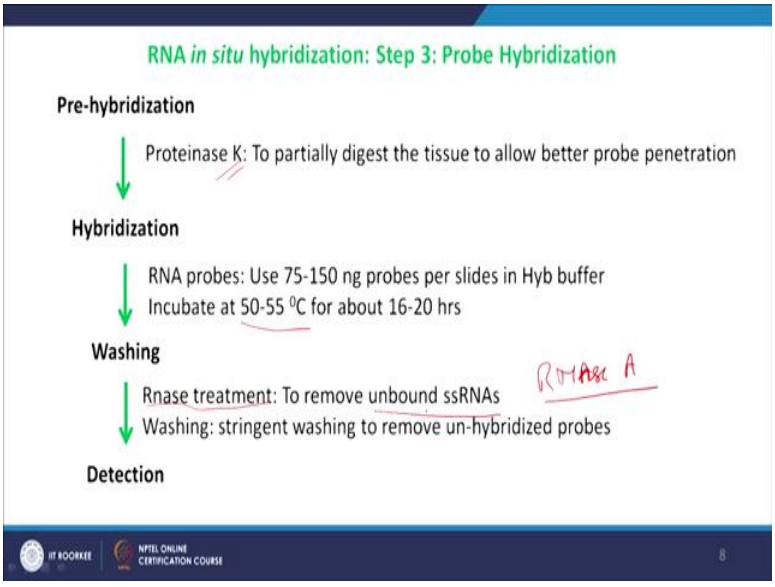

For hybridization what we do? Before going for the hybridization there are some treatment with the tissues, and one very important treatment is proteinase K treatment this is important to partially digest the tissue to allow better probe penetration. And then once you have treated your tissues then use this RNA probe and do hybridization for 16 to 20 hours at 50 to 55 degree centigrade. Then once your hybridization is finished it is

very important for you to remove unbound probes otherwise they will also gives background signal.

To remove them we do RNase treatment typically RNase A. RNase A which specifically degrades single stranded unbound RNA. So, this will remove all unbound RNA from the tissues. Then we do stringent washing to remove even if there are some un-hybridised probes. This is very important before going for the detection of the signal, if you do not do this properly then you are going to have lot of background signals.

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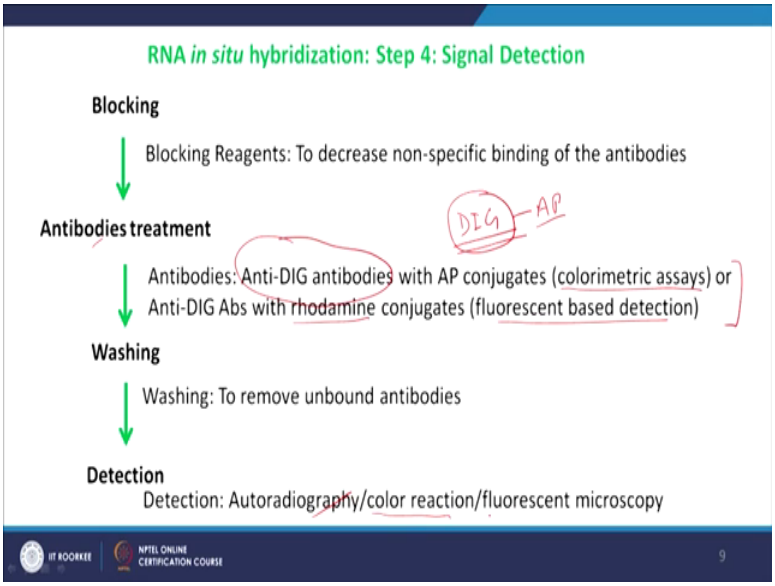

Once probed with the antisense RNA probes, then you use antibody. Typically we use Anti-DIG antibody. So, you know that our antisense RNA probe is tagged with DIG. Now we are using an antibody which recognise digoxigenin. So, this Anti-DIG antibodies we are using. Depending on what method or what way you are going to detect it, if you want to do the colorimetric assay then what we use anti DIG antibodies which has alkaline phosphatase conjugates. But if you want to detect through the fluorescent base detection, then you can use anti DIG antibody with some of the fluorescent tagged protein for example, Rhodamine.

So, basically you have now treated slides with the antibody then you have to wash to remove unbound antibodies, then go for the detection. Again the method of detection will depends on what kind of probes what kind of antibody you have used. So, if you

have used radioactive probe then you can detect the signal by autoradiography, if you have used antiDIG antibody which has alkaline phosphatase or other colour based enzymes then you can do the colour reaction, but if you have fluorescent tagged antibody, then you can do fluorescent microscopy to detect the signal.

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I will give you some of the examples. So, this is a case of whole mount *in situ* and one of the gene which is rice transcription factor MADS2 was detected or analysed for its expression pattern using nonradioactive DIG-UTP labelled antisense RNA probe and this was detected by fluorescence microscope. So this is the organ where we want to see the expression. This organ is called lodicule, it is a small rice flower organ you can see here very clearly, and we want to see that how *MADS2* express across the organ.

We have taken this entire lodicule and we have done the whole mount *in situ* hybridization using antisense probe against the *MADS2* and then detected using anti rhodamine labelled antibody. And you can clearly see here that with this *in situ* method we can tell that that expression of *MADS2* is asymmetrical distributed. You can see that tips and the peripheral region of the lodicule have more *MADS2* signal as compared to the proximal and basal region of this.

So, this analysis not only allow us to check the expression in a particular organ, but it is also allowing us to check if the transcript is asymmetrically distributed.

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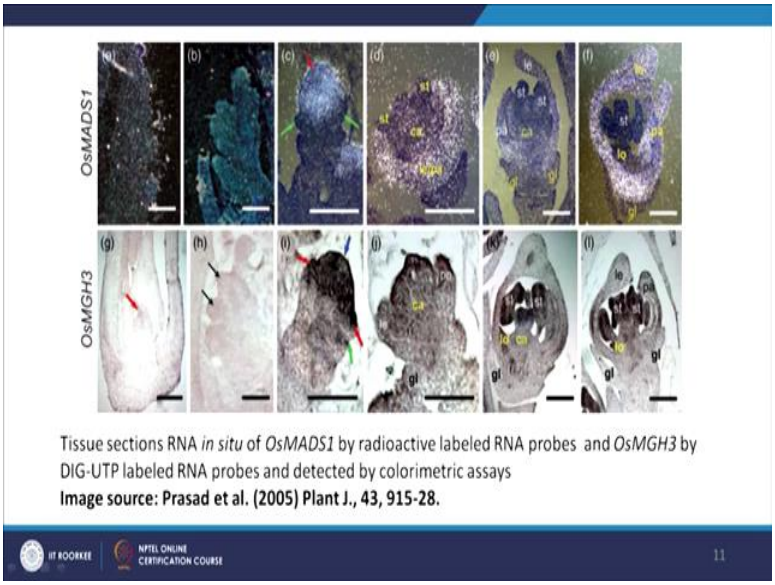

Similarly, if you take this example. Here these were done on the longitudinal sections of the panicle. This is rice panicle, and rice panicle is rice inflorescence it has different florets at different developmental stages and this upper panel if you look they has been labelled with antisense RNA probe for gene which is called *MADS1*, but it is radio labelled probe.

So, you can clearly see the expression is throughout at early stage and then later stage it is more restricted to the lemma and palea and expression in inner whorl organs are disappearing. So, this is a typical example of radioactive label RNA *in situ* hybridization on section. On the other hand if you look this *OsMGH3*. *OsMGH3* is a downstream gene which is regulated by *MADS1*. This is done by non radioactive labelled antisense RNA probe and if you look at this stage you can see that very similar to the *MADS 1* it shows the high level of expression throughout the floral primordia.

But in the later stage when the floral organs are organogenesis are already started you can see the expression in lemma and palea is decreased, but the inner whorl organs are having high level of expression. So, this way; this method is allowing us to study where exactly a particular gene is expressed.

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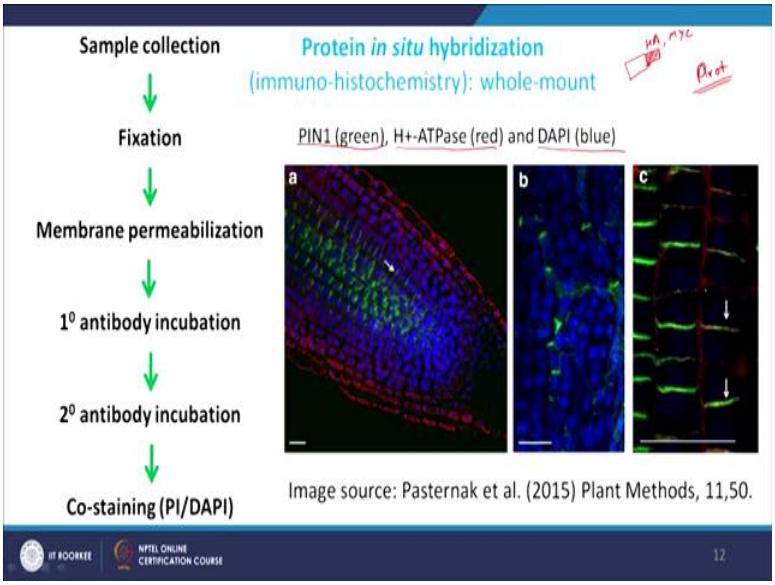

So, this was example of RNA-RNA *in situ* hybridization, but we all know that in most of the cases the functional molecule is protein not the RNA. Of course, there are a lot of RNAs which are functioning without being translated into a protein, but in general cases or typically RNAs are undergoing the process of translation and they are making the protein.

So, if you want to detect the protein in a tissue or if you want to detect the spatial and temporal localization the protein, you can perform protein *in situ* hybridization which is also called immunohistochemistry and what you can do. This is a typical example of a whole mount. Here this is quite similar with the RNA *in situ* hybridization except the detection method is slightly different.

So, here also you collect the tissue then do the fixation, then after fixation membrane permeabilization is done and then you can add the antibody. So, there are two possibility if you want to detect a protein against which you already have antibody then you can use that antibody, if you do not have antibody against a particular protein of interest then you can tag your protein with some tag. For example, you can add HA tag, MYC tag or any kind of tag and then you can use antibody against the tag to detect the protein localization.

And then there are two way, if you have a primary antibody already tagged with the with the conjugates, you can just use primary antibody else you can first detect with the primary antibody. And then you can use secondary antibody which is against the primary

antibody and then you can visualise the signal. Signal visualisation you can do in different way you can in fact, co stain with different dye.

For example, if you take this example here a protein which is PIN1 an auxin transporter protein. It has been studied for its localisation and you can see the green signal is for PIN protein. And another protein which is tagged with the red signal is ATPase, this is going to mark the membrane protein and then DAPI which is blue in colour DAPI basically binds with the DNA. So, it is going to stay in the nucleus.

All three together has been used to check the localisation of PIN protein. If you look here. This is may be enlarged version of it and you can clearly say that this is the cell boundary and in the cell boundary the PIN protein is very specifically localised or asymmetrically localised on one side of the cell membrane, but this side of membrane the protein localisation is very small.

There are plenty of examples available where you can detect protein by protein *in situ* hybridization or immune immune-histochemistry.

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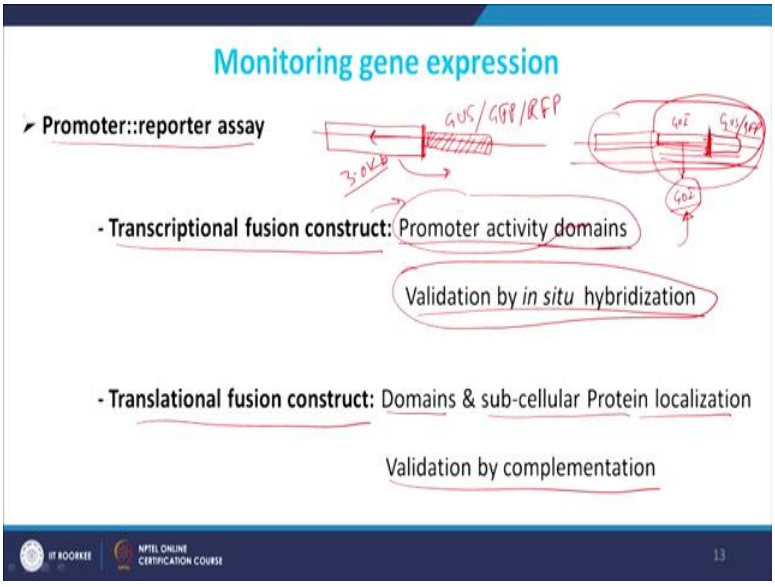

So, next method which is being used to monitor temporal and spatial gene expression is promoter reporter assay. If you recall one of our previous lectures we have discussed that there could be two types of promoter reporter assay.

You can either make a transcriptional fusion construct or make a translational fusion construct, what is difference between them? In a transcriptional fusion construct you only take the promoter or the upstream sequence of your gene. So, if this is your gene you only take the promoter region and this construct will tell you the transcriptional or promoter activity wherever it is available. And then you use a kind of reporter gene here, like GUS, GFP, RFP any kind of reporter gene you can use here.

Then if you generate this kind of construct it is called transcriptional fusion construct, and this construct will tell you where is the promoter activity domain. But this alone cannot be used because in plants most of the time it has been seen that promoters are not very localised, there are some cis regulatory elements which are essential for the promoter activity and they are even found within the gene, sometime in the intron.

So, it is always challenging to choose how much region or how much segment of the DNA has to be taken for the promoter activity. let us assume if you are taking 3 Kb upstream sequence from the transcriptional start side, and clone it and you will see an expression pattern, but if you want to validate it or if you want to be very sure that actually this is the promoter activity you will have to do this analysis together with the *in situ* hybridization.

So, *in situ* hybridization just now we have discussed, there you are just detecting the endogenous expression pattern of a gene or of a RNA. And then you are making the transcriptional fusion construct to compare.

If your promoter activity or if your promoter reporter activity is same as the *in situ* hybridization, then you can say that the promoter element or the promoter region which you have selected, it is complete and it has all the cis regulatory elements which is part of this promoter or which is required by this promoter.

In translational fusion construct it will tell domains of the expression as well as sub cellular protein localisation. So, here what we do? We basically take the desired promoter and then we take the gene as well, but we fuse this gene with some of the reporter.

Here we are mutating the stop codon for the gene of interest and then in frame we are fusing with some of the reporter and if you use this construct this is called translational fusion construct. So, this will tell first thing that, where ever is the promoter activity it will report and second thing is that what is the subcellular localisation of your protein.

Because your protein is now tagged with the GUS and GFP so, wherever your protein will move the reporter will report its presence, but here there is one issue. The issue is that since you are making a fusion protein there is a possibility that this fusion might inactivate your proteins. Your protein might not be in its the proper confirmation and then if you report its localisation it is always questionable that the localisation is because of the artefact or it is the true localisation pattern.

So, this has to be validated by doing the complementation which means that, if you take mutant of this gene of interest, where you see some phenotype and then take the translational fusion construct and put back to the mutant. If the phenotype of mutant is complemented by this construct, which means that your fusion protein is functional and you can rely on its the localisation pattern.

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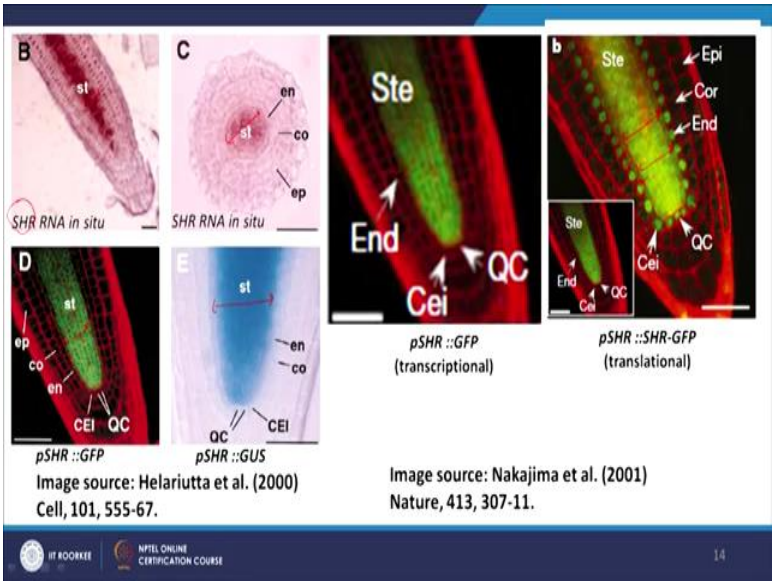

I will give few examples where transcriptional and translational fusion construct has been successfully used to reveal important information. For example, if you look this

SHORTROOT gene. So, *SHORTROOT* gene is important for root development in *Arabidopsis.* So, this is endogenous RNA detection by RNA *in situ* hybridization and here you have taken *SHORTROOT* promoter and fused here with GFP here GUS.

In all this case if you look the expression domain of SHORTROOT protein you see that this gene is expressed in the vascular issue. This is a longitudinal view of the growing *Arabidopsis* root, this is endodermis. So, if you see this in endodermis you do not see any signal, but the tissue after endodermis like pericycle, xylem, phloem, procambium they have the activity this is same you can see here and this is you can see when you do *in situ* hybridization here in the root.

So, this tells that the *SHORTROOT* promoter is active in the vascular tissue, but when you make a translational fusion, where you are using same *SHORTROOT* promoter, but now SHORTROOT protein has been translationally fused with the GFP. What you observe that apart from the expression domain which is in the vascular tissue, you also see that protein is also present in the endodermis and in endodermis it is getting localised to the nucleus, and this has a lot of importance maybe in some of the future classes, we will discuss what is the functional significance of this.

So, how this is possible? If your promoter is active here transcriptions is going on only in the vascular tissue, how come your protein is present in the tissue where transcription is not happening? So, this gives an information that there might be a possibility that your protein is getting synthesised in the vascular tissue, then from vascular tissue it is migrating to the neighbouring cells which is endodermis in this case. And this is only possible if you study both transcriptional as well as translational fusion construct for the gene.

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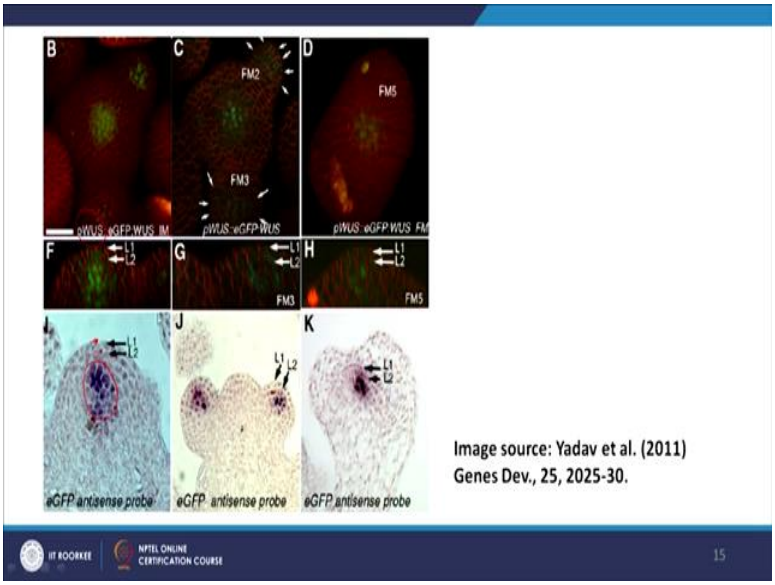

Similar kind of study has been done in shoot apical meristem of *Arabidopsis thaliana*, and here an important protein which is called WUSCHEL is studied. *WUSCHEL* is important to regulate and to maintain the meristematic activity, you will see in the next class. Here if you look the *in situ* hybridization pattern of the *WUSCHEL*. You see that this *WUSCHEL* is expressed somewhere here in this domain. But this domain which is L 1 and L 2 layer, this is inflorescence meristem this is floral meristem, in all the cases you can see that L 1 and L 2 layer they do not have RNA which means that transcription of *WUSCHEL* is not happening in L 1 and L 2 layer.

But when you look for the protein localisation of WUSCHEL. So, this is *WUSCHEL* promoter driving GFP fused with the WUSCHEL protein, you can see that in L 1 and L 2 layer both the layers has the protein. So, transcriptionally it is here, RNA is here and then the protein is also present in the region where transcription the active transcription for *WUSCHEL* is not happening. So, this tells that this protein might be moving from one cell to another cell.

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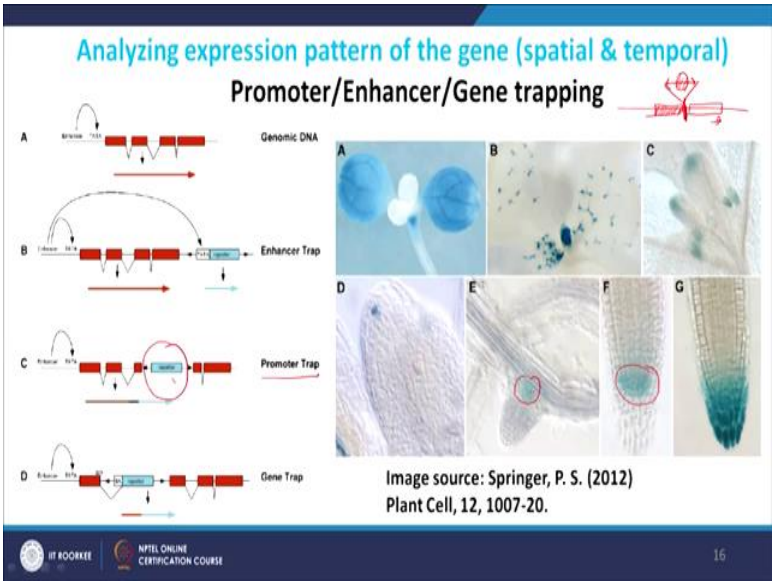

The final method what is being used to study temporal and spatial expression is promoter trapping or enhancer trapping or gene trapping, what is this trapping? There are different types of trapping you can do. This is typical genomic DNA and then for example, you want to identify a gene with a very particular or with a defined expression pattern how you can do?

One way of it doing in enhancer trapping. Here we will focus more on the promoter trapping. If you have to trap a promoter of your interest, if you want to identify a gene which has let us assume expression in leaf or if it is expressed only in the petals and you want to identify that kind of gene for the reverse genetic based study how can you do?

You can generate a construct were you put a reporter gene which is promoter less reporter gene, and make a construct in the T DNA and use this T DNA to raise the transgenic plant. So, this T DNA will randomly get inserted in the genome, and if this T DNA got inserted let us assume in gene of interest which is expressed in the petal. Let us assume that if this is a promoter, this is the gene and here your construct got inserted. Then now it has got a promoter activity from here from the gene which is like petal expression and then you have a reporter gene here.

So, basically you are randomly integrating a reporter in the genome and trying to identify a transgenic line, where your reporter has been integrated in the downstream of a promoter which is active in a particular tissue. And then you can isolate them

Similarly you can do the gene trapping; you can do the enhancer trapping.

In enhancer trapping what basically we do? We take a minimal promoter and then reporters, and then try to trap the enhancer. Here we are trying to trap the promoter. And you can see some of the example that this has been very successfully used to identify a lot of genes whose expressions are in a very restricted manner for example, this gene is expressed in the leaf this is expressed in the trichome then this is expressed in the in the root apical meristem similarly here if you look the lateral primordias are coming.

So, basically then then since you know the T DNA sequence you can use some of the molecular biology based technique to map the gene where is the site of integration, then you can identify the gene for functional study.

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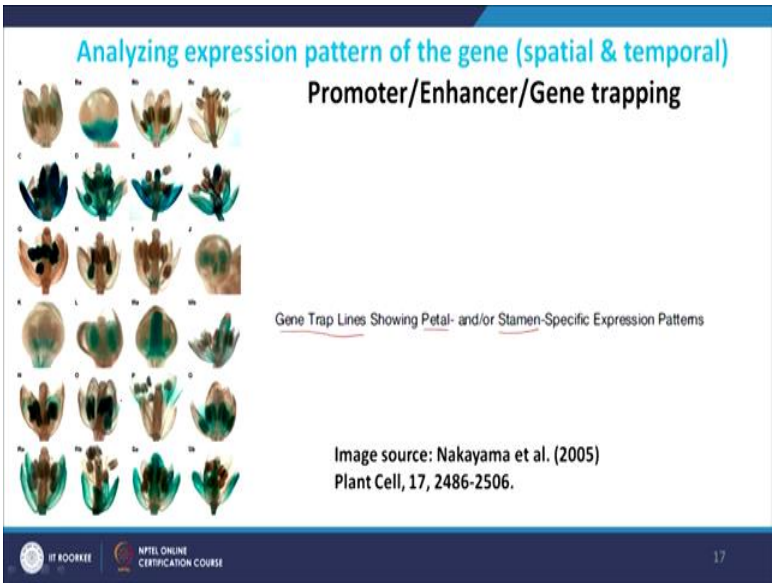

Here is one very good example, where the genes or the lines has been identified which show either petal or stamen and some of them are expressed both in petal and stamen. So, large number of gene trap line has been generated and if you look these lines they are having a different expression pattern.

Some of them are specifically expressed in the petal or specifically expressed in the stamen some of them has been expressed both in petals and stamens. Then you can go, map this gene and then identity and take this gene for the further functional study.

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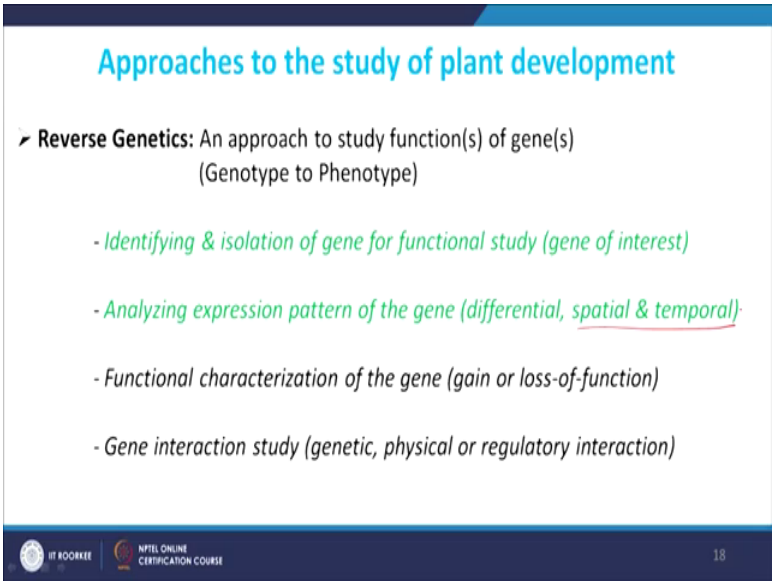

So, if I recap what we have discussed today. Today we have finished the expression pattern analysis particularly we have taken what are the ways and to study the spatial and temporal expression pattern of a gene in plant. And these are very important when you are studying development of a particular organ or if you are identifying some putative regulator of a developmental process, and if you want to study their specific function in the process of development.

So, I will stop here, in next class we will discuss functional characterization of a gene. So, once you have identified a gene, how you will take it for further functional characterization.

Thank you very much.