Plant Developmental Biology
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Lecture - 10
Molecular Genetics of Plant Development - Cont…- III

Welcome back to the course of Plant Developmental Biology. We are still discussing about the Molecular Genetics approach for the studying Plant Development. So, if I recall previous class, we were discussing about the reverse genetics based approaches to study a particular developmental processes in plant.

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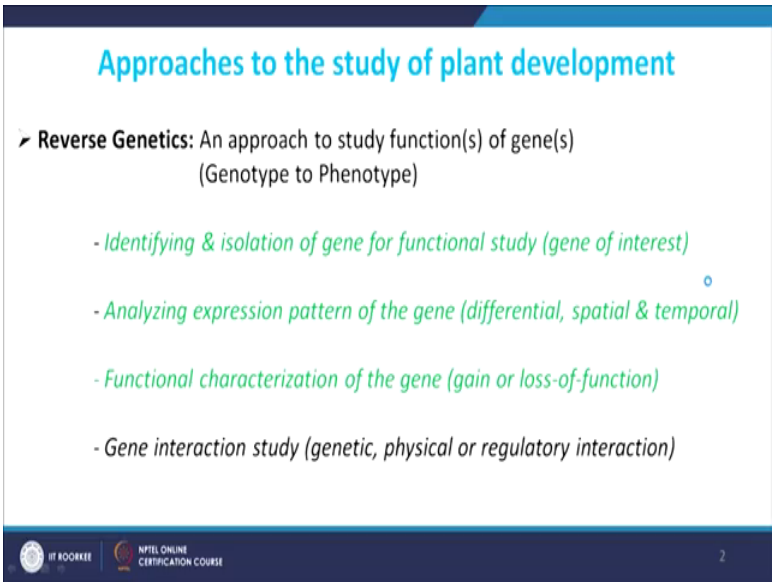

And, we have covered how to identify a particular gene, how to choose a particular gene to study its function, then how to analyse its differential as well as temporal and spatial expression pattern at a particular stage of the development or in a particular organ and tissue, then we have taken this gene for the functional study with two approaches mainly. We have studied the gain of function approach and the loss of function approach.

Now, so we have a gene, we have its function, we know what kind of phenotype this gene has when we down regulate this gene or when we silence this gene. And, second thing we have a phenotype or we know what happens if we ectopically over express this gene. Now, the next part what is remaining is that, if you have a gene what is the

interaction study or the mechanism, what is the mode of action, how a particular gene is regulating a particular process this is important.

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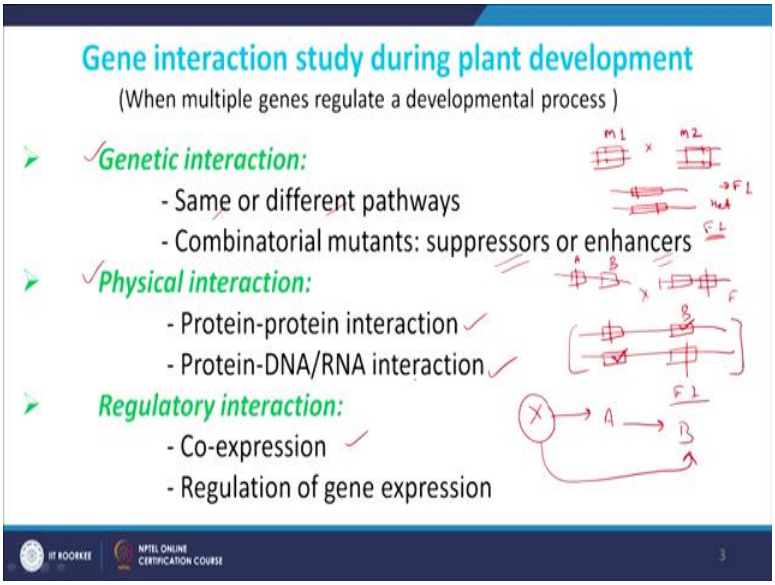

One particular developmental process is not usually regulated by a single gene. So, you might end up identifying multiple genes which might have similar phenotype or same phenotype, then how to study? What kind of interaction? What kind of relationship all those genes have in a particular developmental process.

Let us assume that if you have two genes and if you have loss of function mutant of both the genes with the same phenotype, if you see that in both the mutants phenotype is same then the first question is that, is the both mutation in the same gene or in the different gene. How to rule out this possibility? This you can do by studying the genetic interaction.

So, if one mutant which is m1 and another mutant which is m2 both are having same phenotype both are recessive loss of function mutants and let us assume the phenotype is short root. If I may make a cross and if both the mutations are in the same locus, what is going to happen? You know that for a gene exists two loci and if these mutations are in the same gene then you can expect that somewhere here is the mutation.

Here the site of mutation might be different, but in the same gene. And if you make a cross and go to the F1 generation, what are you going to have? You are going to have

one allele from one mutant and another allele from another mutants, but in this heterozygous F1 plant both the alleles are disrupted. So, you are going to have F1 plants with mutant phenotype. If this is the case, then you can say that these both independent mutants there the mutation site is in the same gene probably in the same gene.

On the other hand, if in this mutant mutation is let us assume in gene A and there is another gene B which is also playing role in the same developmental process. And in second mutant if mutations in gene B, but both are homozygous when you cross them and go to the F1 generation you are going to have gene A disrupted from here, but you will have allele of A as a normal from another plant.

Here you will have gene B allele normal from this plant, but here you are going to have B interrupted, but in this heterozygotes what will happen that the both the wild type copies of the gene will complement the mutant phenotype which means that in F1 you are not going to get any phenotype. This kind of genetic analysis you perform and you can tell that whether both the mutants are in the same gene or in the different gene.

Today we will discuss what are the different types of interactions? The first interaction is the genetic interaction, if genetically two genes are interacting, what does it mean? It means that if there are two mutations for two genes which are regulating same developmental pathway. Let us take example if both are regulating root length then are they working in the same pathway? Are they working in the different pathways? This we can solve by performing genetic analysis, how you can do? You can make combinatorial mutants, you can combine both the mutant and look the phenotype. We will see the example.

Another thing you can identify, suppressors or enhancers of the mutants. So, you take a mutant and then again mutagenize and try to find another double mutants where either the previous mutant phenotype is enhanced or suppressed and then you establish their interaction through the genetic analysis. So, these kinds of interactions are called genetic interactions. Then second thing is that if they are the genetically interacting, are they also interacting physically?

So, there are lot of transcription factor, lot of developmental regulators they are known to interact directly such as protein-protein interaction and they can make higher order complex and that complex is actually regulating the process. So, how to study protein-

protein interaction. Second is protein DNA interactions or RNA interaction. Let us assume that if your developmental regulator is a transcription factor, which means that it will go and bind to some DNA element to activate gene expression or for its function.

There could be two kinds of genes, one genes which your transcription factor is directly regulating, it is physically binding to the promoter or the cis-regulatory elements of your gene and activating or repressing the expression depending on its nature. Or it is indirectly regulating. In indirect case your transcription factor X is regulating gene A and then gene A is regulating gene B.

So, if you take X. With respect to X, B is also regulated by X, but X is not directly regulating B, it is indirectly regulating B. Based on this kind of genetic regulatory physical interaction, you can build and you can understand what is the regulatory network foundation? You can also take help of the co expression analysis. If you check a particular tissue or particular developmental stage and identify what are the genes which are co expressed, you can basically predict that there might be interaction between them, if both are present in the same cell or in the same tissues.

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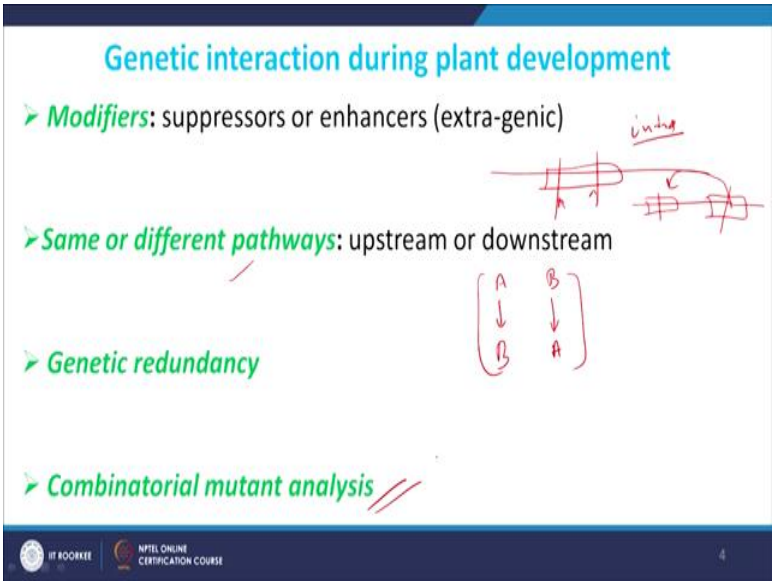

Genetic interactions:- So, as I said that, that you can identify either modifiers which could be either suppressor or enhancers. Let us assume you have a mutant where mutation is in this particular gene, and if you take and try to identify a suppressor mutant, if you mutagenize again there is possibility that another mutation which happens

in the same gene either at the same site or at the different site and the second mutants might restore the function of this particular gene such kind of mutants are called intragenic suppressor.

Another possibility is that you have a mutant, you have a phenotype now second mutation you identify in a totally independent or different gene and if this mutation is suppressing phenotype of this mutants then it is called extragenic. So, you can identify extragenic mutation, you can establish their genetic interaction, you can establish their physical interaction, you can establish their regulatory interaction and try to understand what is the mode of action.

When we are analysing whether they are working in the same pathway or different pathway. If they are working in the different pathway then it means that there are more than one parallel pathways going on and both the parallel pathways, they are regulating the same biological process, but if they are working in the same pathway then you have to establish which gene is upstream which gene is downstream is A regulating B or B regulating A.

So, which gene is upstream and which gene is downstream and how you can establish this? Again through the genetic interaction and the common way of doing is that you make different mutants, higher order mutants, combinatorial mutants, double mutant, triple mutant and then analyze

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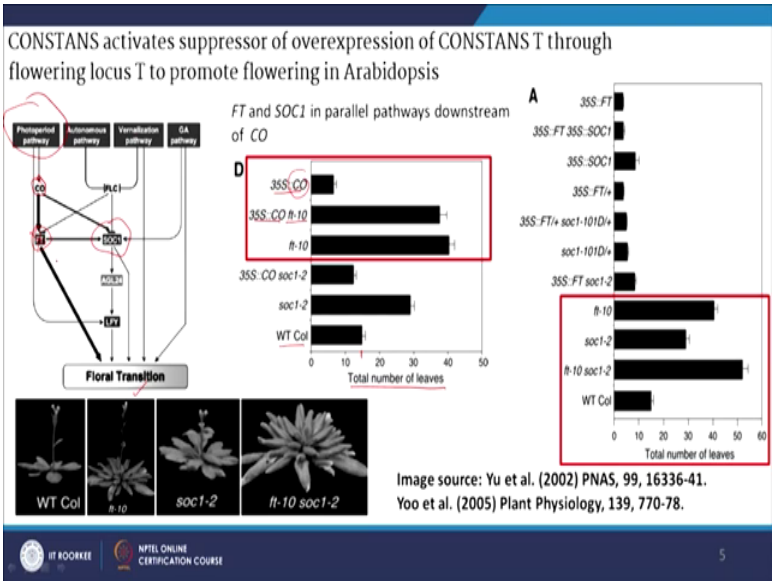

So, I will take some of the example this is a well studied case of floral transition. In plant up to a certain time point, plant undergo the process of vegetative growth then it decide to transit, but the transition from vegetative phase to reproductive phase is a very important process in the plant, to ensure proper sexual reproduction or successful sexual reproduction.

Therefore, it is regulated by multiple regulators, multiple pathways and some of them are working in independent manner and then there are interdependent manner, there is a huge amount of crosstalk.

But how this crosstalk was established for example, if you look here there are four pathways photoperiod, autonomous, vernalization and gibberellic acid pathway and all these pathways, this is a very simple picture, but this pathways are quite complex and eventually regulating the floral transition; so, I will take few of the example.

Photoperiod means the amount of light a plant is getting. Based on that we can define entire plant into three category either they are long day plant, short day plant, or day neutral plant. So, in long day condition, what happens? One gene which is getting activated is *CONSTANST* and then *CONSTANST* activates two pathway, one is *FT* and *SOC1*. And *FT* and *SOC1*, they are having their independent or parallel pathways to regulate the floral transition, how to establish this? If you look this mutant phenotype.

So, here the total number of leaves is counted when there is a transition, if you have more leaves then it is delayed flowering if you have less leaf then it is early flowering. So, if you look a typical wild type plant. So, wild type plants they have usually approximately 15 leaf before they transit from vegetative phase to reproductive phase, but if you look this *ft* mutants, they are making almost 40 leaves before the transition which means that there is a huge delay in the flowering, but if you over express *CONSTANST* if you increase the amount of *CONSTANST*, you can see that there is early flowering.

Even less than 10 leaves are made and plant has already undergone the process of flowering. So, this tells that both are activators of the flowering, but if you take *CONSTANST* over expression in the *ft-10*, *ft* mutant background the *CONSTANST* cannot induce the flowering which means that *CONSTANST* is important, *CONSTANST* is important in activating the flowering, but it is working through *FT*.

So, if you do not have *FT*, *CONSTANST* is not able to activate the flowering. So, it means that there is an interaction, *CONSTANST* is upstream of *FT* and it is regulating through the *FT*.

If you have wild type, you have around 15 leaf at the time of flowering, but *ft* mutants shows delayed flowering. Another gene which is called *SOC1*, *soc1* is also showing delayed flowering, but if you combine *FT* and *SOC1*, the plant is further showing further delayed flowering.This kind of phenotype is called additive phenotype. So, both the mutants are showing additive phenotype. This tells that they are working in two independent pathways.

So, *FT* is working through this pathway, *SOC1* is working through this pathway. If you only mutate *FT*, there is an effect. If you only mutate *SOC1*, there is an effect. But if you mutate both the parallel pathway the effect is enhanced.

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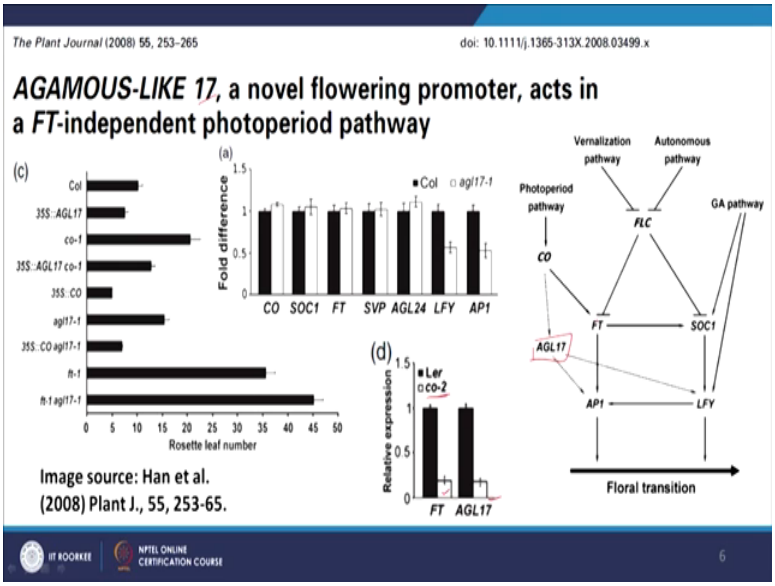

Similar kind of a genetic analysis has been done here to show that *AGAMOUS-LIKE 17*, another gene which is working through a different pathway, but this is also downstream of *CONSTANST*. As you can see in the *constant* mutant background, the expression of both *FT* as well *AGL17* is decreased.

So, *CONSTANST* is activating *AGL17* and *AGL* 17 is also activating finally, *AP1* and *LFY*. So, the final point of regulation is quite conserved, but the upstream pathways are different.

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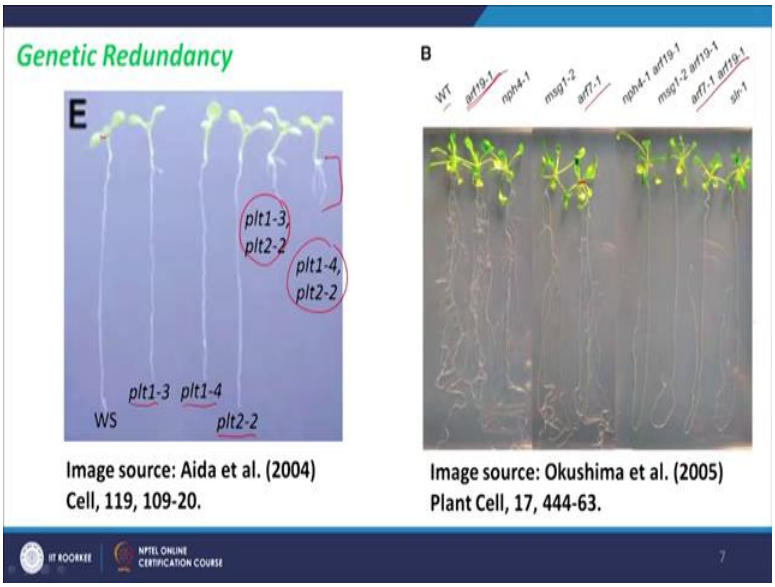

So, this kind of genetic analysis will basically allow you to place all the genes in a particular pathway. Another important thing is the genetic redundancy. Sometime if there is a family of a genes, and there are more than one members, they all or some of them do the same function, which means that if you have mutant of one gene, you might not see a significant effect, but if you mutate more than one genes you start seeing the effect this is called genetic redundancy.

So, for example, if you look this is wild type plant and this is *plethora*. PLETHORA are AP2 domain containing transcription factor, very very important class of transcription factor which regulates meristem function, stem cell maintenance as well as flowering, but here I am showing the effect in the root apical meristem.

If you mutate *plethora1*, these are two different mutant of *plethora1*; you do not see a significant defect in the root growth. Similarly, when you mutate *plethora2* alone, you do not see a significant difference it is quite similar to the wild type, but when you make a double mutant when you mutate both *plethora1* as well as *plethora2*, you can see that root growth is very strongly inhibited.

They form very short root, which means that both *PLETHORA1* and *PLETHORA2*, they are working in a genetically redundant manner to regulate the root growth and single mutant of them, do not have a significant effect. Similar kind of things you can look here, here the effect is on the lateral root. So, primary root development is normal and these are ARF's, ARF's are AUXIN RESPONSE FACTOR they are also a class of transcription factor, but they works in the auxin mediated signalling pathway. Two of auxin response factor, this is *arf19* this is *arf7* and this is wild type. So, if you compare with the wild type, *arf19* and *arf7* alone they do not have much effect on the lateral root development.

You can see that primary roots are developed and these primary roots has significant number of lateral roots, but when you combine both, when you make a double mutant where *arf7* as well as *arf19* both are disrupted, you can see that primary root is very well grown it is equivalent to wild type, but this does not have any lateral roots. So, this also suggests that *AUXIN RESPONSE FACTOR 7* and *19*, they are genetically redundant in regulating lateral root formation in *Arabidopsis*. So, this kind of genetic analysis will allow you to establish a genetic relationship between or among various regulators of a particular developmental pathway.

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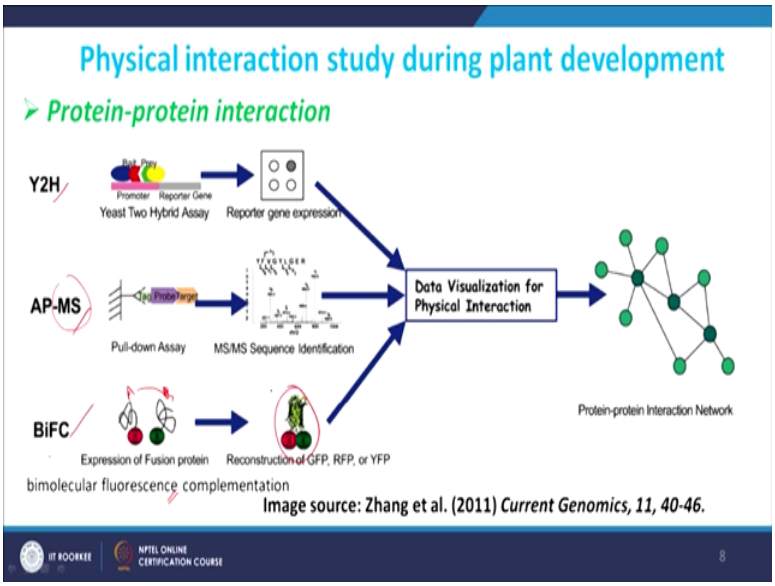

If they are genetically interacting what would be the next question, are they physically interacting as well? If this is so, how to study them? There are many way to study

protein-protein interaction, some of them is highlighted here, this is yeast two hybrid, this is affinity purification based mass spectroscopy and this is bimolecular fluorescence complementation. These are few very commonly used method.

In yeast two hybrid, what we are doing? You have both the gene cloned in a different vector; one will have to be fused with the bait and one with the prey.And if both the genes are interactive in nature, they will interact and then the bait and prey they will come together and they will give of signals.

In affinity purification mass spectroscopy, is based on affinity column or pull down. You pull down some proteins and along with that proteins, there will be lot of other proteins coming and then identify through the mass spectroscopy, establish their identity to know which genes are interacting.

BiFC in principle is similar to the yeast two hybrid, but here the mode of detection is the fluorescence based. So, you have a fluorescent protein which has two domains, if these two domains are together then and only then they will give the fluorescence. So, you clone them separately, use one protein here, another protein here, which you want to see whether they are interacting or not. If these proteins are interacting then they will bring both the domains together. When a and b domains are together they will give fluorescent signal then you can see whether they are interacting or not.

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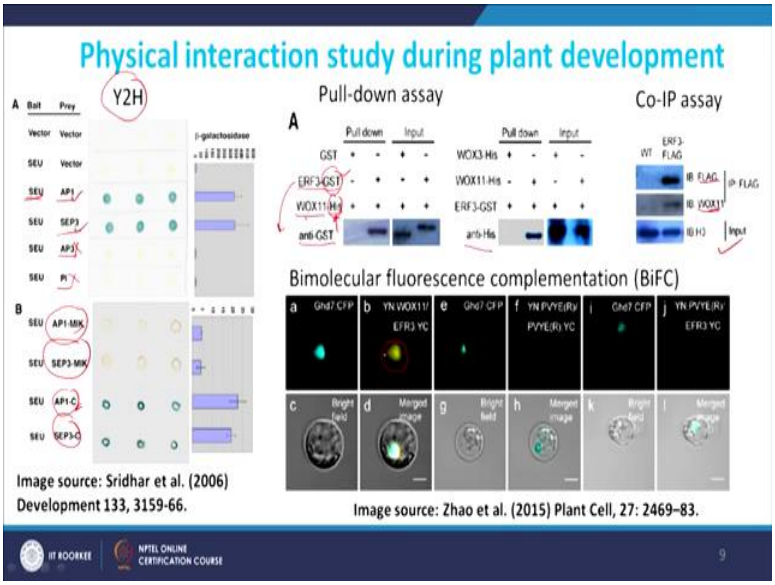

So, here are few examples of this protein-protein interaction. For example, if you look AP1 and SEPALLATA3 , AP3, PI you will study in the next class they are very very important genes which are regulating floral organ development in *Arabidopsis* and *SEU* S E U, this is another gene which is important and here what they have tried to check that whether SEU is interacting with these ABC genes or not. And, this has been done by the yeast two hybrid and what you can see this colour signal will tell if there is an interaction, if there is no colour then there is no interaction.

So, if you look this assay, you can clearly find that SEU is interacting only with AP1 and SEPALLATA3, but it is not interacting with AP3, it is not directly or physically interacting with PI. You have identified AP1 and SEP3 are interacting with C1, you can also establish through yeast two hybrid that what is the domain of interaction. So, this AP1 and SEP3 both are MADS domain containing transcription factor, they have one domain which is called MIK domain and another domain is C terminal domain.

So, here specifically they have taken only MIK domains of both the factors and C terminal domains of both the factor and they have checked the interaction, you can clearly see that only C terminal domain are showing interaction. But, MIK domain is not showing interaction, which means that you can tell that AP1 and SEPALLATA 3 they are interacting with SEU through their C terminal domain.

Another assay this is done for another two very important transcription factor during adventitious root development in rice. ERF3 is an AP2 domain containing transcription factor, WOX11 is a homeobox domain containing transcription factor and both are very very important for adventitious root development. They fused ERF3 with GST tag and WOX11 with His tag and you can pull down using anti GST antibody or anti His antibody. If you are using anti GST for pull down then you can do immune blotting using anti His antibody.

So, if ERF3 and WOX11 are interacting then if you pull down ERF3 you can detect WOX11. If you pull down WOX11 you can detect ERF3 and this is *in vitro* way of doing the detection and here you can see that, either you use anti GST or anti His in both the cases you can detect the band. Another way of doing protein-protein interaction is Co-IP, co immune precipitation, this you can do *in vivo*. You make a transgenic plant where you already have a tagged protein.

So, for example, this is FLAG tag. So, you can tag your protein with any tag and then you can use antibody against the tag and you can do the pull down from the total extract from the plant and then detect or do immune blotting with the another gene which you want to show the interaction. This is your BiFC method and here you can see when both the proteins are interacting you will have yellow signal if they are not interacting there will not be yellow signal.

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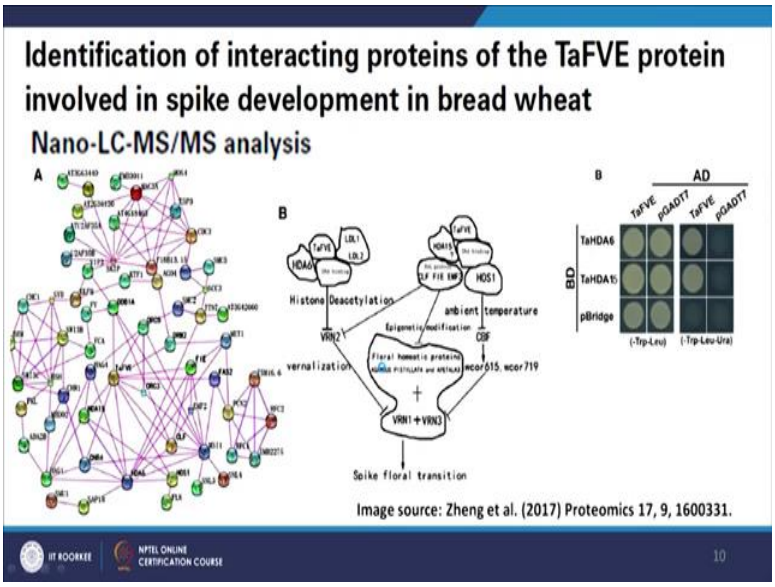

This is example of pull down followed by mass spectroscopy, I will not go in the detail, but why I want to tell here that you can do interaction study at the global level. You can tag a protein, you can use antibody against the tag, you can do the pull down and then you can identify all the protein through mass spectroscopy at the global level to identify all the proteins which are interacting with a particular protein.

Then second study which is important is DNA-protein interaction as you know that most of the master regulators of the developments are transcription factor. So, it is very important to identify what are their binding domain, what are the DNA elements where they bind and how they regulates, what are their direct targets, what are their indirect targets. So, how to do there are again plenty of techniques available, but few of them I am discussing here.

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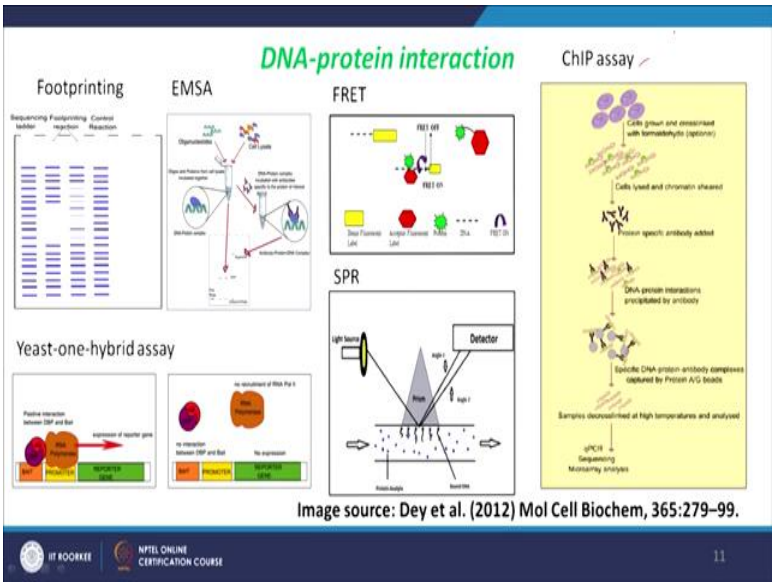

So, there are some *in vitro* way of doing, this is foot printing, electrophoretic mobility shift assay and then yeast one hybrid in yeast, in yeast one hybrid you can use the DNA and then identify what are the proteins which are bound to that DNA elements.

These two, FRET and SPR are physical assay to study the interaction and then ChIP assay chromatin immunoprecipitation which is being very extensively used to study the protein-protein interaction.

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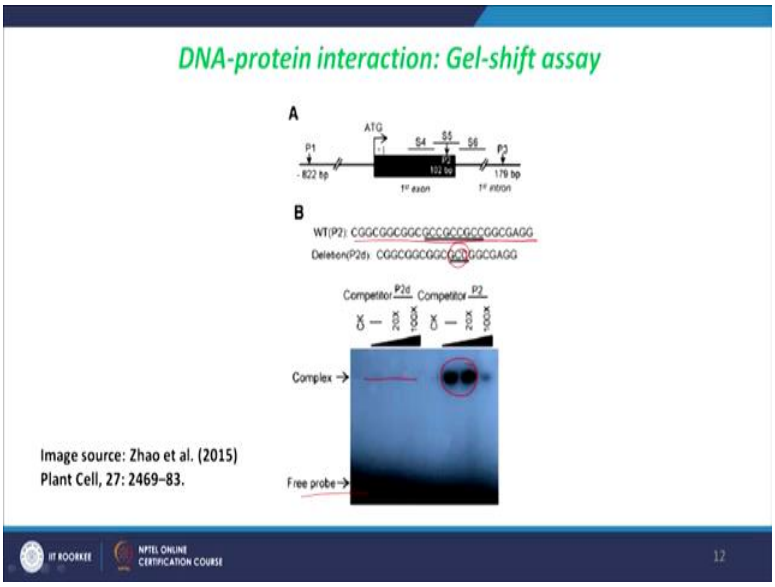

So, this is one example of electrophoretic mobility shift assay or gel shift. In this case what you can do, you can take a putative binding site DNA element you can make a small probe and then this probe can be labelled with radioactive nucleotide and then you mix with your protein and what happens that if your protein is binding with this elements and if you run the gel and detect the binding you will you can see a shift.

So, now this is your free probe. if your free probe is bound with the protein then you will detect a protein at the higher molecular weight size, then you can tell that your protein is basically binding to the cis element. To confirm it, you can mutate your putative binding site and show that when you are mutating this the binding is totally disappearing as you can see here there is no binding, but here there is binding.

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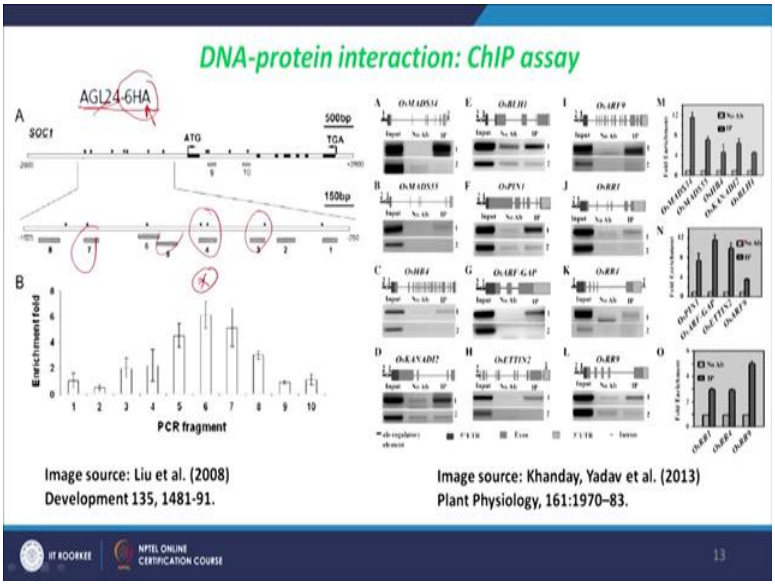

Chromatin immune precipitation can be done at the global level. Here what you want basically you have a putative binding sites. So, what you do, you allow your protein to bind to the chromatin if you have a transcription factor it will go and bind to its target then you fix it you may cross link it.

Your protein will be permanently or covalently bound to the DNA and then you extract total chromatin, make a small fragment and use antibody against either your protein directly or if you have some tag associated with your protein.And pull down those chromatin region or those fragment of chromatins which are bound with your protein and

then you treat with proteinase. You will remove your protein now you have a DNA fragment only.

So, either you take that DNA fragment and if you know what is the putative binding site you can design a specific primer and you can amplify through PCR, qRT PCR or qPCR or if you do not know the sequence you take those fragments clone in some vectors sequence them and identify what are the binding sites. So, these are some examples for example, if you look these are the putative binding sites of AGL24 which is MADS-domain transcription factor and this is tagged with the HA tag; 6HA tag.

So, you can do the chromatin immunoprecipitation using antibody against the HA. You want to see whether AGL24 is binding to *SOC1* promoter or not. These are the putative binding sites for the AGL24 in the *SOC1* promoter. And, then you check by RT PCR or by simple genomic DNA PCR for the different region and you can see that, this region is showing maximum enrichment which means that this region is getting enriched when you are doing chromatin immunoprecipitation for *24* which means that binding is very high at this region. But if you look this region the binding is very very low.

Similar kind of study has been done, where direct targets of MADS1 another transcription factor in rice has been studied. This analysis has been done to show that MADS1 is directly associated with the chromatin of all these direct targets. This is the way to study protein-protein interaction or DNA-protein interaction and this is very important to understand what is the mechanism of a transcription factor.

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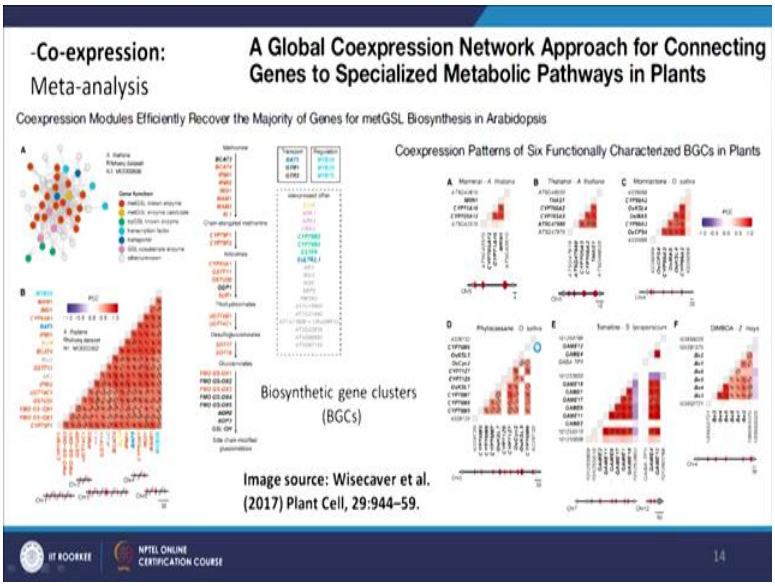

To understand the regulatory interactions among the regulators and the pathways which are being regulated by developmental regulators, meta-analysis can be done or the co-expression analysis. All these data which are already published or submitted to the database all these database can be retrieved and lot of expression analysis can be done. In the database you can you just put id of your gene, you can find what are the genes which are co-expressed with that gene. If they are co expressed with that gene, you may expect that there must be some interaction among themselves. For example, if you look here a global co expression network approach for connecting genes to specialized metabolic pathway has been done.

If you look the co expression then you can identify those genes which might be responsible or which might be functioning in a similar developmental pathway or similar metabolic pathway.

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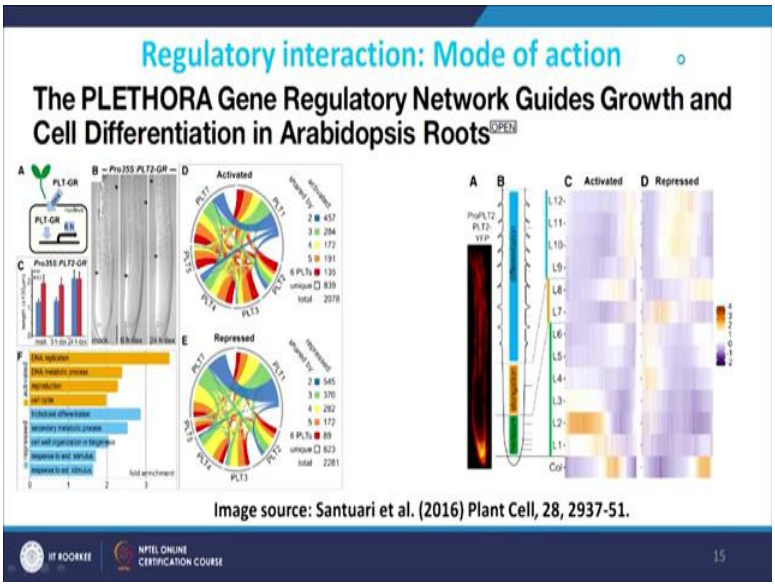

For the transcription factor, if you want to nail down that what is the mechanism how a particular transcription factor is regulating a particular developmental pathway, you have to identify what are the genes which are regulated by this transcription factor.

And this you can do again at gene level or you can do at the global level. For identifying this kind of genes, the global techniques like microarray, RNA sequencing can be used. If you quantify total expression of the genes or global expression of the genes in the wild type and mutant of a particular transcription factor, you can identify what are the genes whose expression labels are altered in the mutants and then you can establish that they are getting regulated.

This kind of study has been done recently to identify global genes regulated by *PLETHORA* genes. So, as I said there are multiple *PLETHORA 1, 2, 3, 4, 5, 6, 7*.

PLETHORA genes were fused with the glucocorticoid receptor. If you recall one of the previous lecture there I have discussed what is glucocorticoid receptor? This way you can control activity of your transcription factor by regulating its translocation from cytoplasmic to nucleus and then after induction, you can perform microarray and identify what are the genes which are getting induced or what are the genes which are getting repressed.

So, by doing this they have basically identified the genes which are shared by two *PLETHORA* three *PLETHORA* four *PLETHORA* five *PLETHORA* and all six *PLETHORA* and they have also identified what are the genes which are specifically or uniquely regulated by either of the *PLETHORA* either activated or repressed. Through this global analysis all the activated genes if you look here, they are expressed in the meristematic region. Which means that in the meristem domains, the genes are mostly getting activated whereas, repressed genes are mostly expressed in the elongation domain. So, by doing this kind of expression or genetic network analysis you can predict genetic regulation.

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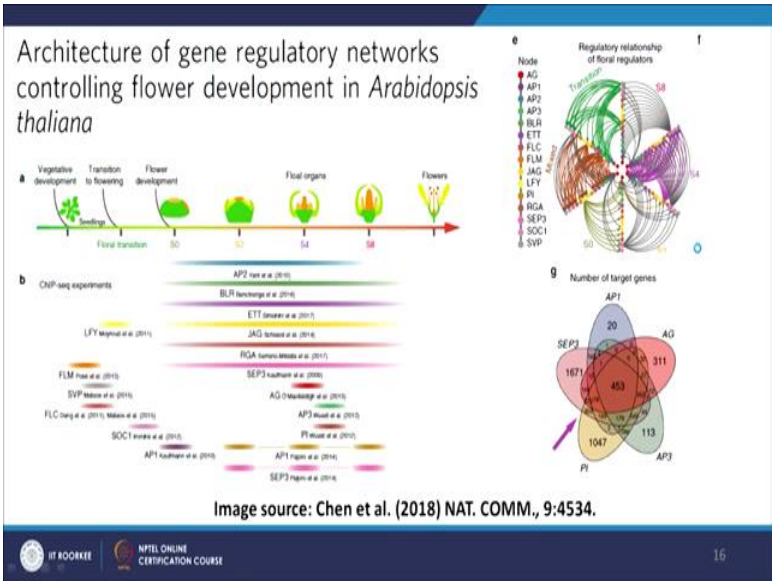

Similar kind of things has been done here for architecture of gene regulatory networks controlling flower development in *Arabidopsis thaliana*. So, as I said that, the flowering starts with the transition from the vegetative phase to reproductive phase and then once this transition has occurred, the floral meristem undergoes the different developmental stage and targets of different developmental stage or transcription factors at different developmental stage has been already identified in different studies.

And, then if you take all these data and do the co-expression analysis, you can establish a regulatory relationship among the regulators and you can identify the genes which are commonly regulated or the genes which are specifically regulated by a particular regulators.

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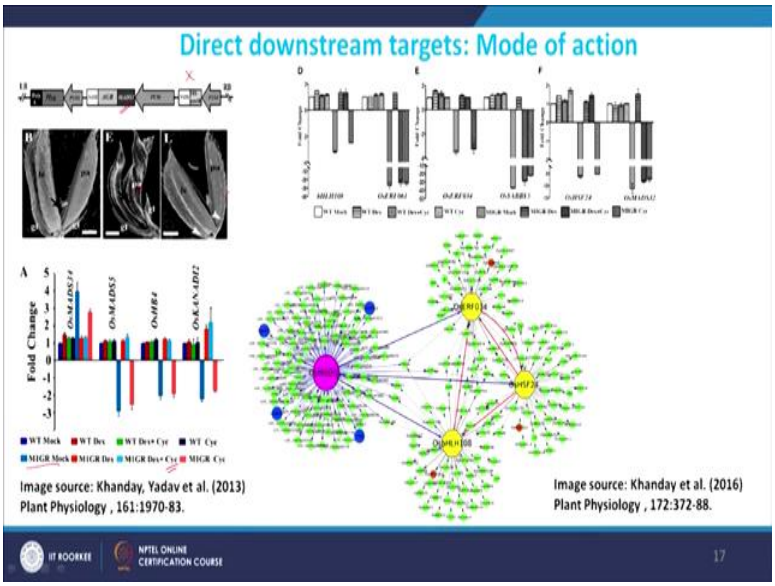

If you want to identify, what is the direct downstream target of a transcription factor, this could be one very good strategy which has been very successfully used with one transcription factor which is MADS1.

I have already told that MADS1 is an important regulator of floral organ development or flower development in rice and if you mutate *MADS*, you will not have a proper flower development and these plants are sterile, it will not make seeds. Then in that case if you want to study this gene, you will have to generate a very special construct.

Here *MADS1* artificial micro RNA against the *MADS1* has been developed and then at the same time, *MADS1* delta GR construct was provided. So, what will happen; this artificial micro RNA will go and silence, and this is designed in a way that, it will not silence the delta GR fusion version of the MADS1. It will only silence the endogenous gene and then this delta GR MADS1, if you induce with dexamethasone it will go and compliment the phenotypes.

So, you will have the plant as you can see this is wild type, when you do not complement with delta GR florets are like this, but when we complement with delta GR after dexamethasone induction, flowers are very normal. If you take these lines and in mock condition; meaning if you do not treat with dexamethasone, what will happen? There is a down regulation because *MADS1* will be down regulated by this gene and you can see

what happens that these genes the expression is going up and this gene expression is going down.

So, when *MADS1* is down regulated *34* is induced, *55* is down. Which means that *MADS1* is negative regulator of *34* and positive regulator of *MADS55*, but when you induce with dexamethasone, the induced expression or repressed expression of the targets are coming back to the normal, but if you do this in presence of cyclohexamide. So, cycloheximide is a protein synthesis inhibitor, if you do that then what will happen? It will activate only the direct target, indirect target will not be activated and if it is direct target then you will see the effect if it is not directed target you will not see the effect.

So, what happens here? When you put in presence of cyclohexamide, this is going down. This is the effect which tells that, these genes are directly regulated by *MADS1* and this approach has been used in combination with the ChIP sequencing. So, basically ChIP sequencing is very similar to the chromatin immune precipitation, identify those genes and use for the sequencing or ChIP sequencing. Through this study, three important genes has been identified which are directly regulated by *MADS1* and then you can use and identify their regulatory networks you can establish their regulatory networks.

We will stop here and in next class, we will start next chapter of plant development.

Thank you very much.