Course Name: Basics of Crop Breeding and Plant Biotechnology

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Lecture-49: Western Blot

Hello everybody, welcome back to SWAYAM NPTEL course on Basics of Crop Breeding and Plant Biotechnology. Today, we will discuss on the selection and screening of transgenic plants and in lecture 2, mostly we will discuss about the analysis of transgenic plants with special emphasis on Western blot analysis, DNA microarray and other different techniques. So, these are the concepts which will be covered mostly under this particular topic. First of all, we will be discussing about the Western blot analysis, how the putative transform plants or transgenic plants are analyzed through Western blot analysis will be discussed. Then DNA microarray will be discussed in detail, what are the principles of DNA microarray, how can we check the gene expression through microarray those things will be discussed. Then quantitative PCR analysis will be discussed under which context we should go for this one and finally, we will be discussing about the transcriptome profiling.

So, just a basic overview will be given to you regarding most of this analysis and if you plan to do such type of experiment, then I think, a lot of troubleshooting a researcher has to face. So, gradually, let us discuss about the basic principles and for which particular condition we have to do this. So, let us start our discussion on Western blot. Earlier we have discussed about Southern blot, in Southern blot we basically detect the DNA. Ok! means whether our target DNA has been integrated within the plant genome or not that could be done through Southern blot analysis.

Whether a particular fragment which is used as a probe, whether it is available within a

genome as single copy or multiple copy. So, this could be done through Southern blot analysis. So basically, copy number determination, ok, that is the most important thing associated with Southern blot analysis. While in case of Northern blot, we have discussed that we can check the expression of a target gene. Ok! So, in case of Northern blot, if some endogenous gene is there and if we are trying to over-express some gene, ok, then in-spite of this endogenous control, if we over express that particular protein we may see stronger band in the transgenic lines.

While, if you do gene silencing compared to the endogenous band, the transcript band which is coming from the endogenous gene in the transgenic lines where the RNAi mediated gene silencing is done, there we can see the reduced transcript expression. Because, the transcriptional silencing, post-transcriptional gene silencing is done through RNAi method. Ok! In our earlier class, we have discussed how basically post-transcriptional gene silencing works initially from the RNAi construct, the small RNA is produced then the small RNA will be single stranded in nature and finally, it can target the endogenous gene based on which we have designed the gene silencing construct. Ok! Now, we will discuss about the Western blot. In Western blot, we will analyze the protein. Ok!

So, this analysis is very important for different transgenic study because until and unless we get the protein product or until and unless our protein product is reduced upon silencing, we cannot tell that our transgenic plant is working fine. Ok! Suppose, someone try to do over expression. Ok! He has or she has taken a rice gene suppose *OsGLP1* and he is trying to over express it in tobacco system. So, he has cloned this particular gene from rice initially and thereafter using promoter, terminator, he is trying to express this gene in tobacco through *Agrobacterium* mediated transformation or non-*Agrobacterium* mediated transformation direct gene transfer method. So, our target is to get a transgenic lines, where this *OsGLP1* will be over-expressed.
So, through Northern blot, we can tell whether our transcript production has been more or not for this particular gene. If we analyze the putative transformed tobacco lines through Northern blot, in control tobacco plant, control means untransformed one, in control

tobacco plants we should not see *OsGLP1* specific band while, if we do over-expression in these different transgenic lines, we can see the transcript band. But, although we are getting the transcript, our job is not done yet, we have to get the protein of this particular gene, the over-expressed protein, then only we can see its phenotype, then only we can see its features. Ok! So, for detection of protein we have to do the Western blot analysis. So first, we need to prepare the samples. Ok!

Suppose, in case of over-expressed lines, we need to isolate the total protein from the putative transformed lines and in addition to that, we have to have the protein from the untransformed control plant. So, let us assume, this is the untransformed plant and these are putative transgenic lines 1, 2 and 3. Suppose, we have over expressed the CDS of a gene, CDS means the coding DNA sequence let us assume its 660 base-pair. Ok! Then how many amino acids will be produced from this particular gene? So, we know that 3 codon means 3 bases in codon they basically code for 1 amino acid. Ok? In this way, we can calculate how many amino acids will be produced from this particular CDS, it will be 220- 1.

If we divide this with 3, if we divide this gene size with 3, because, 3 bases in a codon they code for a particular amino acid it will be 220, but you recall, if you recall the last part of the translation, once the translation has to be done, the last codon will be stop codon that is needed right? So, that will not, in stop codon, no specific amino acid will be produced for that. So here, 219 amino acids will be produced from this particular CDS if we do over expression. Ok! And if we know the sequence then different software are there nowadays in online software are therefrom, you can calculate the expected molecular weight of this particular protein and we should expect this particular protein band in the different plants, while in the untransformed, we should not see any band. So first, we need to isolate the total protein from different transgenic lines and the untransformed plant also, thereafter, we have to run gel electrophoresis.

So, for protein analysis, in DNA we have used the normal agarose gel, in case of RNA we have used the denaturing gel using formaldehyde formamide. In case of protein,

analysis, we have to do gel electrophoresis using polyacrylamide gel electrophoresis means through PAGE. Ok! The polyacrylamide gel electrophoresis is done for separating protein samples. So here, basically, three types of gel could be used. Ok! One will be fully denaturing gel; one will be native gel and one will be semi-native gel.

These three types of gels are used in Western blotting protein analysis. Ok! In normal denaturing gel, in polyacrylamide gel electrophoresis once we have prepared the protein samples using different buffers, mostly Tris buffer is used. Ok! Tris-EDTA buffer is mostly used for plant samples. So, once we have isolated the plants, before loading on the gel, we have to denature the protein using SDS. SDS is sodium dodecyl sulphate or we can use SLS also sodium lauryl sulphate. Ok!

So, using those things we have to denature the protein before loading on the gel. Thereafter, once we have to make the gel for this particular gel, within the gel itself also the SDS is given. Ok! So, once we are adding SDS with the protein or within the gel then all the proteins which are available in the eppendorf tube or in the falcon tube, what we have collected from the plant, all the proteins will be negatively charged in nature. Once we boil it with SDS, or once we load it in the denaturing gel upon boiling with SLS. Ok! So, the protein become fully negatively charged.

So, once all the protein become negatively charged, then on the gel different proteins will be migrated based on their molecular weight. Ok! The proteins will be migrated based on their molecular weight. Suppose, this is from the proteins available from the P_1 plants, P_2 plants, P_3 plants and untransformed plants. So, upon running the gel, for the first time we can do the staining, Coomassie Blue Staining or we can write at CBB, Coomassie brilliant blue staining. Using that staining, we can check whether our protein has been isolated properly or not, different protein bands are visible or not.

If you recall the Southern hybridization process, upon digestion, we have seen a smear like thing. In case of protein, you can see different distinct bands, but some overlapping

bands would be there might be there. Ok! And these are overall proteins available in P_1, P_2, P_3 and untransformed plants. So, suppose, we have done the denaturing gel electrophoresis is done. Then, we have to do the transfer in Southern, Northern, in both the cases once the DNA or RNA sample was electrophoresed on the gel it was transferred into membrane.

If you recall in Southern and Northern, we have used nitrocellulose membrane or nylon membrane, right? While in case of Western, we have to use PVDF membrane, we have to use PVDF membrane for Western blot analysis. So, two types of transfer mechanisms are there- one is the wet transfer method; one is the dry transfer method. If the protein sizes are larger, then generally the scientist used to prefer the wet transfer method ok, because the larger protein size might be denatured or might be degraded during the transfer process through the dry transfer method. So, wet and dry transfer method, two types of transfer methods are there, means through this method we have to transfer the proteins available on this gel into the PVDF membrane.

So, our transfer is done, then we have to do blocking, why blocking is needed? Until and unless we do blocking, during the course of different washing or different incubation, our protein may be washed off. Ok! So, here basically casein is used mostly. So, casein is used as a blocking material on the particular PVDF membrane, then we have to incubate it with antibody. Ok! In Western blot, specific probe is not used, here antibodies are used basically two different types of antibodies are used over here. First one is known as the primary antibody.
In primary antibody, suppose on this gel, just assume in the over-expressed lines let me draw with a different curve. Suppose, over here, our protein is being over-expressed, while in the untransformed one, that protein is not at all available. So, once we will use the primary antibody, the primary antibody will bind with this particular primary antibody become highly specific. Ok! So, it will bind our target protein. So, over here our primary antibody will bind.

So, then we have to use secondary antibody. Then secondary antibody, those antibodies, basically bind with the primary antibody, what we have used earlier, it will bind the primary antibody. Our primary antibody will bind to the targeted region, only to the targeted protein, only then the secondary antibody will bind those primary antibodies and thereafter, we have to do the detection. For detection, different kits are there either we can do the color development kit, Western blot color development kit or we can do chemiluminescence assay, we can do the chemiluminescence technique to detect our targeted protein. Ok! So, in this way basically Western blotting is done.

Now, I have mentioned about 3 different gels, one denaturing, one semi-native and one native gel. So, under denaturing gel, samples are treated with SDS as well as on the gel SDS is available. In case of native gel, basically no SDS is used. In native gel proteins will be migrated based on its molecular weight as well as its charge, ok, both the things will be considered over here. And if a protein is available in number of subunits are there. Ok!

Now, let us think about the RNA polymerase. Ok! RNA polymerase is a big protein, different subunits are there, alpha, beta, beta-prime. So, under denaturing gel, if a protein is multimeric, then it will be denatured into monomer, means all fragments will be separated under denaturing gel. While, in case of native gel, if it is a group of protein if it is multimeric, it will migrate as multimer. Ok! For some analysis, some biochemical analysis, we can go for semi-native gel.

In semi-native gel, from the name itself you can tell that semi-native on the gel, basically the SDS is used during gel preparation, while the samples are not at all treated or boiled with SDS. So that, the specific activity of those proteins will be retained and through semi-native gel, we can analyze a particular enzyme action, means SOD or superoxide dismutase activity you can analyze. And then, the catalase activity also we can analyze through semi-native gel. Ok! So, in this way, different types of gel electrophoresis is done based on our requirement. If we have to see the band whether our targeted protein is properly over expressed or not, we can directly go to denaturing gel.

If we have to check the biochemical activity of the over expressed protein, then we have to go to SDS page, sorry, then we have to go to the semi-native PAGE. Again, that semi-native PAGE could be confirmed through Western blot analysis, means first gel will be loaded under semi-native condition, the proteins will be loaded under semi-native condition and once the migration is done, then we can do this type of steps, the blocking antibody incubation and thereafter, we can tell on the gel if we just take an example of superoxide dismutase gel. Suppose, initially, we have done the assay, SOD assay in the over-expressed protein, we found such type of bands in the over expressed line the P_1, P_2, P_3 while in the untransformed plant, no band was detected. Ok! Then, we can do the Western blotting once again, basically, in plant samples for SOD activity, not a single band will be observed, maybe a number of bands will be there and some bands will be endogenous also, that will be available on the untransformed one, as well as in the transgenic one also. Ok! So, while the band which is probably coming from our transgene it will be available in the P_1, P_2 and P_3 transgenic plants because we are analyzing the over-expression construct right?

Once, this thing is done, then we can confirm, we can do this experiment once again and we can do this Western blot analysis. And therefrom, suppose in this plant, we are getting similar types of bands once we are using *OsGLP11* specific primary antibody, then we may use different types of secondary antibody. Ok! Like a horseradish peroxidase- based antibody or alkaline phosphatase based secondary antibody, while this band will not be available in the untransformed one. So, therefrom we can confirm that our protein is being over expressed in the transgenic lines because these are P_1, P_2, P_3 and our protein is having the SOD activity. Ok! In this way, different types of Western blot analysis is done.

So, now we will just briefly discuss if we have to do the Western blot analysis in RNAi construct. So, suppose in RNAi construct, in RNAi construct basically post transcriptional gene silencing is taken place, post transcriptional gene silencing is taken place. So, after the formation of transcript mRNA, the gene silencing has been done, the

mRNA has been degraded, right? Now through Western blot, we need to confirm it whether the mRNA has been properly degraded or not. So, here, we should expect this type of thing, suppose this is different individual plants which have been generated through transformation and this is the untransformed plant.

In this experiment a rice gene *OSGLP11* has been silenced in rice. So, gene silencing means the gene is endogenous in rice, now we are silencing it. So, in the untransformed plant, we must see the band, while in the putative transformed plants if the silencing is done properly, we may see faint bands because through gene silencing, basically 50 to, not 50, basically 40 to 80% reduction is observed in protein production or in protein synthesis, means we may not see that nothing is there in the putative transformed plants. So, we may see some faint bands, means, some amount of proteins are produced because suppose 1000 mRNA is being produced, out of that, 800 mRNA has been degraded. So, 200 mRNAs are there they will make the protein.

So, in this way 40 to 80% reduction is generally seen in RNAi construct. So, we may see faint expression of the protein once we analyze this RNAi construct. Now another thing, I would like to mention that in most of the cases once we go for Western blot analysis if we use specific antibody, if we use monoclonal antibody as the primary antibody we should expect a single band, we should expect a single protein band which will be based on the molecular weight of the protein. Ok! But in some cases, we may see multiple bands and this type of things are mostly common multiple bands, means more bands in the upper part means wherever the band should be there that band definitely will get, along with that, some other bands might be available. This might be available, means this might occur due to degradation of protein, if our protein is degraded then we may see this type of bands. Ok!

But this type of bands means some specific bands are there which are being highlighted using primary antibody and secondary antibody and those bands are migrating slower than our actual band. This might be due to different protein modification like

sumolyation or the glycosylation of protein, due to this type of protein modification, we may not see a single band of our protein. Ok! So, this is the thing through which Western blot analysis is done. If you do, means if you would like to little bit more about this type of analysis then you can go through some publications on rice germin like protein 1. Ok! You can go to Google scholar you can search about this particular gene rice germin like protein 1 and you can search with my name also, a couple of papers you may get.

So, there such type of Western analysis multiple bands will be detected. Ok! So, one of this was published in BBRC in 2010 another one published in BBRC 2010 and third one has been published in plant cell tissue and organ culture in 2018-19 at that time. So, if you go through this particular publication, you can understand little bit more. Ok!