Course Name: Basics of Crop Breeding and Plant Biotechnology

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Lecture-48: Southern and Northern Blot

Welcome back. So, we will continue again. Now, gradually, we will move into the molecular screening. So, suppose, initially we found at least 20 putative transformants and those putative transformants have been screened through PCR and after PCR screening, out of them, 10 transformants were found to be PCR positive. So, can we tell that those transformants are transgenic? We cannot tell because a plant will be transgenic when the transgene will be integrated within the genome of the plant and it will be available or it will be transmitted to the next generation also, then only we can say as transgenic. So, within a cell, suppose, our plasmid has been somehow inserted or part of the plasmid has been inserted.

If we isolate the total DNA, herefrom we can get the PCR, but until and unless it is integrated within the chromosome, we cannot tell it as transgenic plant. Ok! So, two types of expression study or expression analysis is done in molecular screening. One is transient expression study; another one is stable expression study. So, for transient expression study, basically, PCR screening is not at all needed; within 2 days after infection, we can see that in how many cells or in how many, in how much tissue our transgene has been delivered.

It can be done by using different reporter genes, we can use *GUS* reporter gene, we can use *GFP* reporter genes. So, suppose, a tobacco leaf is there and the leaf has been infiltrated with agro solution, *Agrobacterium* solution, it has been infiltrated means, it is

also known as agro-infiltration. So, once the infiltration is done, the agro solution will go inside of this leaf and the leaf is kept for 48 hours and thereafter, we can screen the leaf. If we use any construct where *GUS* reporter gene is used, then we have to do the histological staining. If we use any construct where *GFP* reporter gene is used, then we have to check this particular leaf under fluorescence microscopy.

In this way, we can tell whether our construct is alright, whether the *Agrobacterium* is working properly or not whether our gene is functional or not, the gene construct, the reporter system is properly working or not. This is the purpose of doing transient expression study, but through transient expression study, we cannot tell that a particular gene will be properly functional in all the tissues. If we have to over-express a particular gene within a specific tissue or throughout the plant, then we need to get stable expression. If we have to reduce the expression of a particular gene through gene silencing construct, then we have to do stable expression. If we have to know that tissue specific expression of a particular promoter, then we have to do stable expression study.

Through stable expression study, basically, we have to make the transgenic plants. So, what is that I have already told the transgene has to be delivered within the chromosome, transgene has to be integrated within the chromosome and it should be transmitted to next generation, then only we can tell it as transgenic. Ok! So, through PCR screening we can do the screening process that out of 20, how much will be selected for further work, but for confirmation, we have to go to Southern hybridization or other different molecular screening methods. Ok! So, gradually, we will come into that. So, first let us discuss about the Southern blot or Southern hybridization.

So, what we have to do? We have to initially digest the DNA with restriction enzymes and then run the digested DNA on an agarose gel, the digested genomic DNA samples will be loaded on agarose gel. Then we have to transfer the denatured DNA to the membrane, nylon membrane or nitrocellulose membranes are used. Then, we have to probe the membrane with labelled single stranded DNA, then washing and visualization will be done. So, these steps are there, now one by one, I will describe the process once

again. So, let us talk about a particular over-expression construct.

A rice gene has been over-expressed in tobacco system. So, we had a promoter, then we had a gene of interest and we had a terminator. So, these things were available in the binary construct where LB and RB; left border and right border was there. So, we are assuming that within the PCR positive, this whole part has been transferred. While, this is available in the putative transformed lines 1 and putative transformed lines 2.

While we have the untransformed plant also, untransformed tobacco plant, here this particular transgene is not at all available. What gene was used? We used *OsGLP11* the *OsGLP11* is not available over here. So, first what we have to do? First, we have to isolate the genomic DNA from untransformed and different putative lines. So, the DNA will be checked on the gel, the DNA quality should be checked on the gel whether any smear is, smear is there or not we need to check. If too much smear is there, we should not use that particular DNA, that DNA should be intact, means if smearing is there then what type of problem will arise, later on we will discuss.

So, thereafter once the proper DNA will be isolated, we are supposed to get a band in all of these cases. So, the band size should be more than 10 kb, the genomic DNA bands and for electrophoresis of genomic DNA, we have to use 0.8% agarose gel and for Southern analysis, basically, we need to transfer, sorry, we need to run the gel or we need to do the electrophoresis process in very less speed for longer duration. Generally, overnight gel run is done for Southern analysis. So, this is the undigested one and once the genomic DNA will be digested, in all these cases, the untransformed P_1 and P_2, suppose, we are digesting with a particular restriction enzyme.

Generally *Eco*RI, *Bam*HI, *Hin*dIII those enzymes are famously used in Southern analysis, but in addition to that, other restriction enzymes are also used. Ok! So, once the digestion will be done, we can see a smear in all the samples. Basically, in all the plants, the untransformed, putative transformed 1 and 2 different chromosomes are available and in each and every chromosome, suppose, we are doing Southern analysis using *Eco*RI

restriction enzyme only. So, in each and every chromosome, different *Eco*RI sites are there. So, for that reason, ultimately, this different bands will be produced and a smear like things will be observed in all the cases.

So, we cannot distinguish them visually. So, after this electrophoresis of the digested DNA samples, we need to transfer it to the membrane. Here, the DNA was in the agarose gel so, we need to transfer in nylon or nitrocellulose membrane. Ok! So, whatever was available over there, that will be transferred. Suppose, this is the membrane where this whole thing has been transferred.

Now, one thing I would like to mention before choosing the restriction enzyme which is being used in Southern analysis, we need to know the information of this gene of interest. Definitely, we have made the over-expression construct. So, we have to have this particular sequence information and suppose, we know *Eco*RI has been used in this particular cloning. Suppose, the gene has been cloned in *Eco*RI and *Sac*I site. So, this information is needed, means where *Eco*RI is available within our construct that is information is needed.

If other than *Eco*RI, any other restriction site is there that could be used in Southern hybridization. Ok! So, now, the digestion has been done, it has been transformed, it has been transferred to the membrane and thereafter, we have to prepare the probe, gene specific probe. In the untransformed plant, this *OsGLP11* is not at all available, right? It is a rice gene and we are expecting that, in the putative transformed plants, it might be available in transformed, untransformed it is not available. Ok! So, for labeling or preparing the probe, from here, we need to PCR amplify this particular gene of interest or we need to amplify the part of this particular gene of interest and that will be labeled, radiolabeled by using different kits. Ok! Different kits are available in the market, using that we can radiolabel the probe.

Nowadays, non-radiolabeled is also done by using other different non-rad methods and

once this type of probe preparation is done, this double-stranded probe should be denatured before using in the hybridizing media. So, initially the probe is prepared in double stranded condition, then it should be denatured and this denatured probe will be used in the hybridization condition. Ok! It should be used for hybridization purpose. So, if you think about these DNA strands, these different DNA fragments which are available in untransformed and putative transformants 1 and 2. Our gene specific probe will bind to the transform plants only, because in the untransformed plants, that specific region is not at all available.

So, it cannot bind. So, in this way, through Southern blot, we can confirm whether our putative transformants are transgenic or not and transgenic we have to confirm in T_2 generation. So, the first generation what we get that is known as T_0 generation, thereafter, once the seeds are formed, we can get to T_1, then gradually we can go to T_2 generation. In T_2 generation, mostly all the transgene become homozygous. Ok! So, this is the thing in through which we have to screen the putative transformants through Southern hybridization. Then later on, we will discuss other different complex problem related to Southern hybridization-based analysis, means how many copies of a transgene has been transferred or how the transgene has been integrated within the genome in single copy or in tandem repeats or in reverse tandem repeats those things will be discussed later on.

So, now, coming to another important aspect of molecular analysis of the transgenic plants, that is done through Northern blot. The Southern blot or Southern hybridization is done for DNA detection, DNA based analysis, while Northern blot is done for RNA analysis. So, over here first we need to isolate the RNA, total RNA is isolated. So, in total RNA, will be having mRNA, rRNA and tRNA all the things are available, but for mRNA you will not see any specific bands, smear like background is available on the gel and for Northern blot, the denaturing agarose gel electrophoresis is done. Ok! Because you know that, RNA is single stranded in nature and it is very unstable, it can form some transient double stranded structure.

Using denaturing gel, using by creating this denaturing condition, we have to linearize

the mRNA. So that, each mRNA can migrate at its proper size. Suppose one mRNA length is 8000 bases while another mRNA is there its length is 800 bases. So, generally RNA is single stranded in nature, but it can form some transient double stranded structure in this way. Under denaturing gel, it will be available in its basic structure.

So, all the mRNA will be migrated based on its size during the electrophoresis process. Ok! So, in RNA gel, basically formaldehyde and formamide this specific combination is used, using these two chemicals, we have to make the denaturing condition and the gel run principle is almost same. So, over here, we need to isolate the untransformed plant DNA, sorry, untransformed plant RNA the putative transformants 1 and putative transformants 2, their total RNA will be loaded on the gel. So, thereafter, electrophoresis will be done under formaldehyde formamide gel and once the electrophoresis is done, then we have to transfer it on the membrane. Here also, nylon or nitrocellulose membranes are used; nitrocellulose membranes are preferred or N^+ membranes are preferred and this Northern blot gel becomes very fragile, become highly fragile and here basically, this gel run is done for shorter period of time because RNAs are not too much longer like DNA samples.

So, basically in a couple of hours or in 3 to 4 hours, this gel run is done thereafter we need to transfer it into the membrane and once we transfer all the mRNA, tRNA, rRNA samples on the membrane then we have to fix it by UV cross linking. Then, we have to prepare the probe what we have discussed earlier. Ok! So, using either radioactive or non rad method by biotin or some other means we can label the probe, we can prepare the probe and thereafter, we have to use the pre-hybridization buffer. In pre-hybridization buffer, basically, this membrane is incubated for approximately 2 hours and after that incubation, we have to use the denatured probe over there to start the hybridization. Ok! So, once the hybridization is done, then we can see what should be the label of RNA of our probe specific target.

So, if you recall our last experiment, we have used *OsGLP11* specific probe, *OsGLP11* specific probe was used. So, in untransformed plant, we should not have this particular

gene in Southern analysis, we did not get any band over there. So, over here also, we should not expect anything or any signal while in plant 1 and plant 2 we can see that particular transcript availability if our transgene that is *OsGLP11* is expressed properly under the particular promoter. So, if we use our particular gene under a promoter and terminator, then only we should see this type of expression, otherwise, just through Southern we can check whether it has been integrated or not. Ok! For expression purpose, gradually we have to think about the promoter, until and unless promoter is there, we should not see any expression. Ok!

So, if you recall the Southern blot, there we mentioned the signal at two different locations. It means in plant 1 and plant 2 our transgene might have been integrated at two different chromosomes or at different positions within a particular chromosome, but over here in plant 1 and plant 2, we are getting the signal at a specific region. Why is it so? This is due to the fact that here, we are talking about the transcript which is produced from this particular gene or specific mRNA, which is produced from this particular gene. If we use a 600 base pair region as *OsGLP11* gene, then this product size will be 600 base pair. It might be little bit more than that because thereafter, some terminator sequence might be there in the pre mRNA ok, but it will be close to that. Ok!

So, here, in both the plants, we should see differential expression, we might see differential expression. Suppose, we got this type of things in untransformed one in plant 1 and plant 2; in plant 1 we are getting strong expression in plant 2 we are getting faint expression. So, this type of scenario might be available if there is variation in the RNA loading, suppose during the loading process in plant 2, we have, we have put less amount of RNA, less amount of total RNA. So, we may get faint signal, for that checking we need to do Northern analysis using a housekeeping gene also. What is housekeeping gene? Those genes which are expressed in all cells, in all types of tissues, in all growth and developmental stages at same rate. Ok!

So, generally actin gene is used as a housekeeping gene in case of plants. So, we can use beta actin gene over here, we can use tubulin genes as housekeeping gene. So, in

housekeeping gene, we have to see the signal in all the plants, because that will be available in untransformed plant also and in putative transformants 1 and 2. So, this will be our loading control, therefrom we have to tell that we have done equal loading, but still, we are getting this differential results. Why it may be? May be in plant 2, our transgene has been integrated close to the centromeric region. Ok!

If a gene is integrated close to centromere region, there a lot of hetero-chromatin are available, it is heterochromatin condensed region. So, their gene expression is highly affected. Ok! If our transgene is integrated in the euchromatin region, then we can see proper expression. So, this type of variation may be available in between plant 1 and plant 2 or within different transformants. Well! Thank you. So, later we will discuss other different process of molecular screening of transgenic plants. Thank you.