Course Name: Basics of Crop Breeding and Plant Biotechnology

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Week: 11

Lecture-51: Molecular Analysis of Transgenic Plants I

Hello everybody. Welcome to SWAYAM NPTEL online course on Basics of Crop Breeding and Plant Biotechnology. Today we will start module 11. Under this particular module we will be discussing on Molecular Analysis of Transgenic Plants and here from we will start analysis of Southern Blot. How the Southern blots are analyzed in different way to understand the copy number, to understand the type of integration in different transgenic plants. So, these are the concepts which will be covered under this particular topic.

First of all we will discuss about the probe selection and restriction enzyme selection in the Southern analysis in the Southern hybridization. Then gradually we will move into the copy number determination, and thereafter we will discuss about different types of double integration or multiple integration of the transgene within the transgenic plant. So, let us start our first discussion on probe and restriction enzyme selection. So, before discussing these things first we need to understand that a particular construct has been generated, and that particular construct has been used for plant transformation.

And few putative transformants have been identified have been isolated, it has been initially screened through PCR, and then we will go through the Southern hybridization. And in the Southern hybridization, what should be our probe type, and what restriction enzymes we should use we will be discussing here. So, let us assume we have used this particular construct where we have a promoter, we have a specific gene of interest and we have a terminator sequence. Let us assume that the promoter has been cloned in *Eco*RI

and *Bam*HI restriction site. While our gene of interest has been cloned in *Bam*HI and *Sac*I restriction sites and the terminator has been cloned in *Sac*I and *Hin*dIII restriction sites.

And this construct is available within the vector which has been used for initially *Agrobacterium* transformation in *Agrobacteria* this vector has been inserted and using that particular *Agrobacterium* strains just assume this is a pCAMBIA vector. So, the *Agrobacterium* cell harboring this particular vector has been used for plant transformation. And suppose this is our left border region and this is our right border region. And this whole chunk available between left border and right border is supposed to be transferred into the plant genome. And within the transgenic plant it will be integrated anywhere within the genome suppose several chromosomes are there 7, 8 different chromosomes are there.

So, it can be integrated in chromosome 1, it can be integrated in chromosome 5, in chromosome 7 wherever. Ok! Similarly, the T-DNA integration is taken place through illegitimate recombination, and it can be integrated anywhere within the genome. Even within a same chromosome this T-DNA part which is available in between the left border and right border it could be integrated multiple times. Ok! So, different scenarios may be aroused. So, first we need to think about the probe selection and restriction enzyme selection.

So, if you recall the Southern process, in Southern hybridization first we need to isolate the genomic DNA, we need to isolate the genomic DNA, then we have to digest the genomic DNA with restriction enzyme. Generally, one restriction enzyme is used sometimes we can use two restriction enzymes also. Ok! Thereafter electrophoresis, then we need to transfer it, transfer the DNA from gel into membrane, then we have to prepare the probe, and we will go for hybridization. So, over here we need to add gene specific probe. So, thereafter we have to develop the autoradiogram.

We are discussing about the Southern techniques or Southern hybridization, where radio

labelled probes are being used. So, we can get the autoradiogram, because those are highly specific and we can properly visualize where our gene has been integrated, whether any variation is there in different transformants, those things could be easily understood. Ok! Now, let us start our restriction enzyme selection part. So, suppose I am considering two different plants over here one is P_1 and another one is P_2 both are putative transformed plants. Ok! Two putative transformed plants have been screened through PCR, those are PCR positive and we are analyzing it through Southern hybridization.

So, what could be the scenarios? So, let us assume that in plant 1, 3 chromosomes are available. Ok! This is the chromosome 1, this is just our assumption, this is the chromosome 2 and this is the chromosome 3. So, in chromosome 1, 2 *Bam*HI sites are available, 2 *Bam*HI sites are available and the size of this fragment, is suppose 5 kb, the size of this fragment is 1 kb and size of this fragment is 1.5 kb. In chromosome 2 no *Bam*HI site is available suppose and its size is approximately 3 kb.

In chromosome 3, again 2 *Bam*HI sites are available ok, its size is 2.5 kb, its size is 3.5 kb and its size is 2 kb. Ok! Suppose this is the scenario. So, if we digest this particular plant, if we digest P_1 plant with *Bam*HI restriction enzyme how many fragments we can get? Here from we will get 1 fragment, 2 fragment, 3 fragments, 4 fragments, 5 fragments, 6 fragments and this fragment will be also available, although no *Bam*HI site is available over there.

So, let me draw these things, that P_1 has been digested with *Bam*HI, what will be the scenario? What will be our smallest fragment? Over here the smallest fragment will be 1 kb. So, we will get a band at around 1 kb here I am writing kb on top this is 1 kb. Then our next fragment will be 1.5 kb, then our third fragment will be 2 kb, then we will be having another fragment of 2.5 kb, then this one done 1.5 done, 2 kb done, 2.5 kb done, then we will be having a 3 kb fragments, this one done then we will be having 3.5 kb fragments and we will be having 5 kb fragments. Ok! This will be the normal scenario. So, this will be the scenario if our transgene has not been integrated, over here and we are

digesting the chromosome available in this putative transform plants with *Bam*HI, we are digesting it with *Bam*HI.

Suppose here our transgene has not been integrated ok, although you are thinking somehow in PCR, we found the band maybe the plasmid was lying somewhere within the cell. So, there from we got some sort of amplification, some artifacts were there and through Southern this type of banding pattern we are getting. In Southern basically those fragments will not be visualized, the digested DNA will not be visualized, for visualization of that we need to use a particular probe. So, this is just our assumption in P_1 putative transform plants no transgene has been integrated. What is the scenario in P_2? In P_2 the situation was almost similar this is chromosome 1, this is chromosome 2 and this is chromosome 3.

In chromosome 1, 2 *Bam*HI sites were available because a particular rice variety or a particular tobacco variety has been used for this type of transformation. So, their basic genetic structure will be same the pattern of *Bam*HI restriction sites available on each chromosome will be same. In chromosome this is the chromosome 1, this is the chromosome 2 and this is the chromosome 3. Ok! In chromosome 3 also 2 *Bam*HI sites were available. So, now let us assume in plant 2 P_2 our T-DNA, that is this part the transfer DNA has been integrated over here, our T-DNA has been integrated over here in chromosome 3. Ok!

So, what will be the new look of its genomic structure? Once the T-DNA is integrated then we will be having chromosome 1 in chromosome 1, 2 restriction sites where available initially sorry, 2 restriction sites were available initially that will be same it was 1.5 kb, it was 5 kb and it was 1 kb. This one will be similar to this one, no integration has been taken place we are assuming it. Ok! In chromosome 2 what will be the scenario? In chromosome 2 nothing has been integrated its size is 3 kb. In chromosome 3, some modification has been taken place due to integration of this T-DNA. Ok!

Now in this T-DNA, once we are making this construct, we have to know the size of

different parts. Ok! Suppose here size of the promoter is 1 kb, the size of the gene of interest is 1.5kb and size of the terminator is 0.3 kb. Suppose this is known to us.

So, this 1 + 1.5 + 0.3 = 2.8 kb. This region will be integrated, in this part on the chromosome 3. So, its size will be changed also.

So, let us try to draw this chromosome part. In chromosome 3 earlier we had 1 *Bam*HI sites at 2.5 kb away from this end, then we had another *Bam*HI site over here, and in between our T-DNA has been integrated right. So, let us draw our T-DNA. Suppose in T-DNA the promoter was there, in T- DNA our gene of interest was there and in T- DNA our terminator was there.

This thing has been integrated over here. We know the size of this part its size is 2 kb. It will be similar to this one, because no integration has been taken place at this position, while in between these two restriction sites our T- DNA has been integrated. Now we know that in our T-DNA the gene of interest has been cloned in *Bam*HI and *Sac*I site. Once we have to do the Southern hybridization we have to design the probe mostly from the gene of interest.

So, that we can effectively tell whether our trans gene has been delivered, has been integrated properly or not. Then in addition to that we can use some promoter specific probe also in Southern hybridization, that is based on our requirement, but the gene of interest specific probes are mostly used. So, here our gene of interest has been cloned in *Bam*HI and *Sac*I site. So, within this construct over here another *Bam*HI site is available that is coming from this particular construct. So, we need to think about this particular band. Ok!

For knowing this particular band, first of all we need to know this distance. This distance here I can tell you, but in nature means once the transformation is done the measuring this distance will be highly difficult. So, based on the autoradiogram we need to calculate. So, just for the sake of some example I am telling that this one was 0.3 and

suppose this part was 3.2 means out of this 3.5 kb from this position after 3.2 kb away our T- DNA has been integrated. So, if it is so, then what should be this fragment size? This fragment size will be 3.2 coming from here plus within the construct another 1 kb right.

So, it will be 4.2 kb this fragment while will be having another 1 fragment, in that fragment 0.3 kb was there from this side and from our transgene this part will be coming because, after the *Hin*dIII site the right border was there, if full T-DNA is transfer is transferred after the right border then this 0.3 kb sequence is available, this 0.3 kb sequence is available right?

So, there from 1.5 + 0.3 = 1.8 + 0.3 = 2.1 kb will be available. Now what will be the banding pattern of this particular plant after digestion with *Bam*HI restriction sites. Ok! So, let us make it in this plant how many fragments will be getting? Fragment 1, fragment 2, fragment 3, fragment 4, fragment 5, then fragment 6, fragment 7 and fragment 8.

Earlier we had 1, 2, 3, 4, 5, 6, 7 fragments now we are getting another 1 extra fragment because within our T-DNA region a *Bam*HI site is available. Ok! So, it is cleaving the chromosome once again. So, let us make these fragments. So, will be having 1.5 kb bands, will be having 5 kb bands, will be having 1kb bands, then will be having 3kb bands let us assume this is 3, this is 5, this is 1.5, this is 1, then will be having 2.5 kb bands, 2.5 kb bands these are all kbs kilo bases. Then will be having 4.2 kb bands a new band is coming over here 4.2 kb bands. Another new bands will be getting that is 2.1 kb bands over here will be getting 2.1 kb bands, then will be having 2 kb bands. So, in this way 1, 2, 3, 4, 5, 6, 7, 8, 8 bands will be coming from this particular genomic DNA after digestion with *Bam*HI restriction enzyme. So, this is P_2 digested with *Bam*HI restriction enzyme.

Now, in Southern blot what happens, I have mentioned the steps first genomic DNA isolation, restriction digestion, this is the restriction digestion and electrophoresis has

been shown over here restriction digestion and electrophoresis. Thereafter, we need to transfer these whole things on the membrane, then we have to use a particular probe. Suppose over here, if we use gene of interest specific probe, I am repeating it once again suppose over here we are using gene of interest specific probe, which was available in our construct. So, this probe will be, this probe will bind the DNA region wherever this particular sequence is available right? So, we are assuming that in P_2 plant our transgene has been delivered.

So, our gene of interest has been integrated while in P_1 plant or gene of interest has not been integrated. So, in P_2 plants our probe can bind within these digested fragments. Now, let us see in which particular fragment our probe will bind. If we think about this particular part, where I have made a chromosomal structure where our transgene T-DNA has been integrated. If we carefully see this part, we are using gene of inter a specific probe, gene of interest, a specific probe is available in this side in the right side of the *Bam*HI available in our construct. Ok!

So, definitely in this side this part our probe will bind. So, basically this 2.1 kb fragment, over here our probe will bind, our probe will bind to this particular fragment. Ok! So, now, once we will do the hybridization and thereafter once we will develop the autoradiogram, the autoradiogram will be like this in P_1 and in P_2. In P_1, will not see any signals while, in P_2 will get a signal at around 2.1 kb region. So, therefore, we can tell that in our P_2 plants the transgene has been integrated, in somewhere in the chromosome while, in P_1 no signals are there, it means our gene specific probe is not at all binding and that particular transgene has not been delivered. Ok! So, let me repeat this part once again using another different type of probe. So, to repeat this part, I am once again drawing the construct, otherwise it will be little bit difficult to understand this construct we have used this one was our promoter, then we had our gene of interest and, then we used the terminator and, these things were available within the vector, and over here the left border and over here the right border region was available. So, if you appear different competitive exams in CSIR or UGC-NET or in GATE you may get such type of questions means gradually we will go into the details means initially the basic part should

be clear 1 kb, 1.5 kb, 3kb. So, its size was 1 kb, its size was 1.5 kb and its size was 0.3 kb, and what restriction sites did we use? *Eco*RI, *Bam*HI, *Sac*I, *Hin*dIII over here we had *Eco*RI, over here we had *Bam*HI, over here we had *Sac*I, and over here we had *Hin*dIII. This restriction size have been used, and this is the plant 1 P_1 and this is the plant 2 P_2 in plant 1, 3 chromosomes were there in plant 2, 3 chromosomes were there its size was 1.5 kb, its size was 5 kb, and its size was 1 kb ,this one was the *Bam*HI sites and over here its size was 2.5 kb, its size was 3.5 kb, and its size was 2 kb and, its size was 3 kb where no restriction site was there means, whatever I had drawn in the previous slide I am repeating it once again to make you understand, if we use different probe how our autoradiogram will look like. Ok! So, in this plant *Bam*HI site is available in chromosome 1, 1.5 kb, it was 5 kb it, was 1 kb, it was 3 kb, over here some sort of changes was there, 2.5 kb then, over here we had our promoter we had our transgene and we had our terminator part. Thereafter another *Bam*HI site was available, this size was 2 kb right, and we already know this part was 0.3 kb, and this part was 3.2 kb, because this total 5 was 3.5, 0.3 was there, and 3.2 was here thereafter our T-DNA has been integrated, right? Now once we have done the autoradiogram what did we get autoradiogram using gene specific probe, means as a probe we have used gene of interest. In plant 1 we did not find anything, in plant 2, we found a band at around at around 2.1 kb. Ok! Because, a new *Bam*HI site is available and this sequence, will be this, *Bam*HI digested sequence will be 1.5 + 0.3 that is coming from this particular construct means 1.8 and 0.3 means 2.1 kb. Ok! Now the same fragments are available this is we are talking about autoradiogram we have discussed in the previous slide.

Now I am again showing the digested DNA in plant 1 how many fragments you are getting, 7 fragments are there means we had 1 kb, we had 1.5kb, we had 2 kb we had 2.5 kb we had 3 kb we had 3.5 kb and we had 5 kb means 5 sorry, 3.5 those are all kbs ,then 3, 2.5, 2, 1.5, and 1, this is in case of P_1 this fragments we are getting 1.5, 5, 1, 3, 2.5, 3.5 and 2 right. Now, in plant 2 what was the scenario we are having 8 bands. Ok! So, 1.5 will be common, 1 kb will be common, 5 kb will be common, those are coming from chromosome number 1, then 3 kb bands will be common, that is coming from chromosome number 2, and will be having 2.5 kb bands, then will be having a 3.2 + 1 =

4.2 specific band will be there. Right? 4.2 specific bands will be there this will be 3, this will be 2.5, this will be 1.5, this will be 1 and will be having another bands of 2.1 kb, 2.1 kb, and will get the band of 2 kb, also right here from 2 kb band will be available. So, in this way 1, 2, 3, 4, 5, 6, 7, 8, 8 bands will be available, this thing we have shown earlier after digestion, but now we are going to use a different probe, earlier during hybridization process we used gene of interest specific probe. Ok! That is the probe was used from this part, this specific DNA was used in hybridization process. Now, we are using promoter specific probe, to see what will be the signal in the autoradiogram. Ok! Now, we are using suppose promoter specific probe, this specific probe we are using and the promoter has been used, suppose we have done rice transformation using CaMV 35S promoter.

So, this promoter will not be available within the rice genome naturally, from the transgene only this promoter could be integrated within the rice genome. So, this part will be unique now let us see if we use CaMV 35S promoter specific probe, and if we digest the plant samples with the same restriction enzyme *Bam*HI what will be the scenario. So, you now focus over here in this part of the chromosome, where our transgene has been integrated. If we use promoter specific probe it will bind this part. So, this particular fragment will be highlighted on the gel what will be the size of this fragment it will be 3.2 + 1 kb means its size will be 4.2 kb, 1kb coming from the construct and 3.2 coming from the genomic DNA. Ok! So, basically our probe will bind over here, in this fragment and our autoradiogram will be like this in P_1 and in P_2, P_1 again will not get any band, but in P_2 at around 4.2 kb region will get the signal. So, in this way if we use different probes here, we used promoter specific probe and over here we have used gene of interest specific probe.

In the same plant if we think about the plant number 2, once we are using gene of interest specific probe we are getting a signal at around 2.1 kb. While once we are getting the promoter specific probe on the same plant, we are getting a signal at around 4.2 kb. So, once we have to analyze this type of Southern related problem, we have to think about the restriction enzyme what is being used in the hybridization in the Southern blot over here *Bam*HI has been used then we have to think about the probe which

particular probe is being used.

If probe we are using from this part, then this region will be bound this region will be bound with the probe. If we use probe from here from the promoter region then our this particular part will bound on the digested DNA. But actually, once we have to do the Southern hybridization this less number of bands, will not be available 7, 8 bands will not be there, thousands of bands will be there and only after hybridization process in the autoradiogram we can see the signal. So, basically this bands what I have told earlier that is just for your understanding to make your concept clear.