Course Name: Basics of Crop Breeding and Plant Biotechnology

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Week: 12

Lecture-57: Characterization of *OsGLP1* Gene from Rice (Part II)

Welcome back. So, we will continue again. Well so, now let us discuss the gene silencing construct preparation. So, for gene silencing construct or RNAi construct preparation, RNAi stands for RNA interference, construct preparation, or this type of construct is also known as hp-RNA construct ok, hairpin RNA construct. In hairpin RNA construct preparation also, we have to put our gene in reverse and forward orientation. You can see over here, that the gene has been placed in reverse orientation, and the gene has been placed in forward orientation flanking a linker region. Ok!

Linker is the non-specific region; non-specific DNA means that DNA will not be available within the rice genome or within that genome, where this RNAi construct will be utilized. Ok! Maybe some bacterial sequence could be put over there. So, in this way this basic part is made then it will be under the control of a particular promoter. If you can recall our expression of the *OsGLP1* gene. Ok! We found that the *OsGLP1* gene was highly expressed in the green tissues, mostly in the leaf tissue, and in the sprout tissue.

So, we have used a high-level expressing promoter, rice polyubiquitin promoter for expressing this gene silencing construct or hp-RNA construct. So, thereafter a nos terminator was used. So, the hairpin RNA will not be read through, which means those RNA will be stopped somewhere. So, eventually, the siRNA could be produced in the plant system. Now within this gene silencing construct, we have used the pCAMBIA 1300 vector.

So, in this vector, the left border, and the right border region, are available, within the multiple cloning sites cloning was done and thereafter we had CaMV 35S promoter, a *hygromycin phosphotransferase* gene, which is a selectable marker gene, and CaMV 35S poly A signal also. Ok! These things are available already in the pCAMBIA 1300 vector, these 3 things are available. So, we have done the construct preparation and the gene silencing construct preparation they are considering these parts. Ok! So, now let me talk a little bit more about the rice polyubiquitin promoter, which has been used over here. So, if you see the rice polyubiquitin promoter, it is a high-level expressing promoter in plant systems, some such types of high-level expressing promoters have been characterized or identified by different research groups.

One is the rice polyubiquitin promoter, and another such type of promoter is the actin gene promoter. Ok! So, in those promoters, along with the initial core promoter region, where the TATA box binding factors are available, some part of the exon of those genes, like this is rice polyubiquitin promoter, the first exon part is part of the second exon of *rice polyubiquitin* gene, is added within this promoter. Ok! And a huge intron region is available. So, once we put some gene-like over here, we have used the gene silencing part under this whole promoter. Ok! Once we put it over here, then in the plant system once the pre mRNA is formed thereafter splicing will take place, and this intron part will be removed and eventually, the mature mRNA will come up from this part gradually.

So, in this way as part of the first exon, the first exon and part of the second exon are available. So, those types of promoters will be highly expressed, which means they will express the gene, under it in an enormous amount, because our target is to make more siRNA. So, whatever the *OsGLP1* transcript is available, in the plant that will be degraded, will be chopped by siRNA and dicer, their mixed action. Ok! So, we expressed it under rice polyubiquitin promoter. Now, let us see what restriction enzymes are available in rice polyubiquitin promoter, because for this construct preparation, for gene silencing construct preparation initially, we had to clone the gene part then the linker was added over there, then the gene was placed in reverse orientation, then we cloned the rice

polyubiquitin promoter, and nos terminator separately and finally, it was taken together within the pCAMBIA 1300 backbone.

Now, let us see the details of this rice polyubiquitin promoter. The accession number, of the rice polyubiquitin promoter, is given over here. So, let us go to the NCBI, here you can see the rice polyubiquitin promoter region, and partial CDS sequence given there, its size is 1773 base pairs, and this is its sequence. So, let us take first the FASTA sequence of this particular gene, of this particular promoter, which was used in this particular construct preparation, and through the NEB cutter we can understand that what are the different restriction enzymes available within this particular promoter. We need to understand it before cloning, because based on that we have to design primers, for this promoter part, primers for this gene part, terminator part separately.

So, this is the 1773 bp size, if you look carefully over here different restriction sites are available, but we might be looking for those enzymes, that are available in the multiple cloning sites region, of different vectors, namely pUC19, pUC18, or pBluescript or pCAMBIA 1300. So, we need to find out those restriction sites whether it is available or not. So, over here if you see an *Eco*RI restriction site is available, a *Pst*I restriction site is also available. So, those things are available approximately, at around 1300 bases means at one site around, 400 to 473 base pairs will be there. Now, let us go to our original discussion once again.

Once, the RNAi construct was prepared or the hairpin RNA construct was prepared, then we did the plant transformation using that particular gene silencing construct, and the T_1 and T_2, the T_1 and T_2 generation plants were eventually screened through initially PCR and thereafter by southern hybridization. In southern hybridization, the genomic DNA of those plants was isolated, and it was digested with a *Pst*I restriction enzyme. If you recall the copy number determination of the *OsGLP1* gene, we found that in *Pst*I digestion we found two signals right. So, using that particular enzyme we planned our analysis of different T_1 and T_2 generation rice plants. So, let us see those experiments.

So, this construct was used. So, first, try to focus on the figure available on the left-hand side. Ok! This is the T_1 generation plant. So, in the T_1 generation plant, we found that in the untransformed plant, once we use the *OsGLP*1 specific probe two signals are available, two signals are available at around 2.2 kb, and at around 0.83 kb. Earlier if you recall in the untransformed plant, after *Pst*I digestion using the *OsGLP1* probe we found similar types of signals. So, in untransformed as well as, in all the transgenic plants this signal will be available. So, these four lines were analyzed, the line 1, line 2, line 3, and line 4, those are independent putative transformed lines. So, in all those independent putative transformed lines, we found this endogenous band over here. Ok! While in the putative transformed lines along with these two bands, other different signals were detected.

It means our *OsGLP1* transcript, which means *OsGLP1* gene silencing construct have been integrated because, over here our *OsGLP1* probe will bind right? And finally, we can see different integrations in the autoradiogram. Now, let me explain these things once again in detail. If you carefully see, line 1, line 2, line 3, and line 4 over there, two common signals are available other than these two endogenous bands. Two common signals are available other than these two endogenous bands, one is over here and another one is over here. So, over here we are getting a signal close to 3.8 kb, 3.9 kb. Ok! While over here we are getting, a signal at around 500 bp to 650 bp region. So, if we think about the construct that was used in transformation within the construct, within the *OsGLP1* gene, the *Pst*I site was available right? So, from here this fragment will be coming from this construct, even if you recall the rice polyubiquitin promoter, which was used over here. There also a *Pst*I site was available, here from another fragment will be coming. So, once we will use the *OsGLP1*-specific probe this part will be highlighted, this part will be highlighted.

So, the size of these parts is close to 500 to 600 bp region, which means this signal is coming in all the transgenic lines it is not available in the endogenous plant right? Because this construct was delivered in the transgenic line. So, they are from this, the lowermost signal is coming at around 500 to 600 bp. While we found another band at

around 3.8 kb, that might be at least due to one copy tandem integration, one copy tandem integration later on, I will explain this part once again. So, other than these two bands, these two common signals, other different signals were observed in lines 1, 2, 3, and 4.

Those are due to the differential integration of this transgene, within the chromosomes of these independent lines. In T_2 generation, means in the progeny of those T_1 generation plants we observed almost similar type of banding pattern which was expected. If you see in line number 1, line number 1 this type of banding pattern was here in the T_1 generation, in the T_2 generation we are getting this type of banding pattern in its progeny. So, the progeny has been almost homozygous for this transgene at least. In line number 2, the progeny of line number 2, you can see such type of banding pattern while in line number 4, we observed such type of banding pattern. Ok!

So, in this way, we can mean, we confirm that in the T_2 generation, for this transgene our different lines became homozygous. Now, let me discuss this particular part where we can tell, how this 3.8 kb band is coming in most of the transgenic lines. Well, so now let us think if this construct is integrated at least in one tandem manner. Ok! We have done the tandem integration, and we have discussed the tandem integration, reverse tandem integration of the construct, again and again.

So, now suppose this one was our construct, this one is the RuBQ promoter, then this is the gene, in reverse and forward orientation, this one is the linker, ok, this one is the terminator of our construct, and then we had the promoter, the *hptII* gene, and the poly-A terminator. Ok! So, its size are written as there its 1773 bp, its size is 642 bp, 377 bp, approximately the terminator was , approximately 275 bp, then the promoter was 787 bp, the *hptII* gene, 1094 bp and a poly A tail of few 100 means, 150 to 200 bp. Suppose, this is the whole thing ok, suppose this is the whole thing and it is present in two copies, which means tandem integration. So, let us draw it over here this is one integration, and this is another integration, the tandem integration has taken place somewhere within the genome, and thereafter, we have done the southern blot analysis using the *Pst*I restriction

enzyme right? So, now we need to think where the *Pst*I sites are available, if we recall within the RuBQ promoter, a *Pst*I site was available ok, while within our gene a *Pst*1 site is available.

So, thereafter from here, no *Pst*I site would be available. Ok? So, let us see, if you think about this part its size is close to 1300 bp. Ok? So, first, let us count the size suppose over here at around 1300 bp, here from first *Pst*I cleavage has been taken place. Ok! Thereafter this size are there, these different *Pst*I sites are there and I am not mentioning the internal sites suppose this one is the site. Ok! Thereafter from here, we may get at least 500 bp, then plus 275 bp, then plus 787 bp, then 1094 bp and let us assume this is as 100 bp or something.

What will be its size? Its size will be around 2,756 bp, this size will be there, Ok? So, now over here if you see again *Pst*I site will be available, within the rice ubiquitin promoter. So, this part will be available as a common site ok, where our part of the gene will be available and part of the other construct will be available if tandem integration takes place. In this way, it has been found that upon at least one tandem integration the size of this will be close to 3.8 kb, it will be close to approximately 3.8 kb. Let me check, it yeah it will be close to, 3.8 kb, 3.9 kb at this position. Ok! So, in this way due to tandem integration, we are getting a particular band and due to cleavage and due to cleavage from this part we are getting a common signal at around 600 bp, 500 bp to 600 bp region. Ok! So, once the transgenic lines were confirmed then finally, we analyzed the different transgenic lines to know, whether our gene silencing, is being taken place or not because we were trying to do RNAi-mediated gene silencing.

So, once the genomic DNA integration of the construct, has been confirmed then we try to find out whether our transgene was working properly or not. So, for that reason again we have to go to another publication. So, first, let us focus on this particular part. Ok! Let us focus over here. So, first when we have isolated the RNA samples from the leaf tissue of the untransformed one, and lines 1, 2, and line 3 in T_2 generation ok.

Their RNA samples were isolated, and an *OsGLP1*-specific probe was used. If you recall the endogenous transcript of *OsGLP1*, was close to 1900 bases in the control northern blot. It was found to be expressed in the green tissue, in the leaf tissue, in the sprout tissue. So, here leaf tissue was taken at 1900 base, and we are getting the band in the untransformed plant. And in line 1, line 2, and line 3 different gene silencing lines, we found that that particular transcript expression has been enormously reduced.

This particular transcript at around 1900 region has been reduced, but additionally, we found some bands in line 1, line 2, and line 4. Some bands at around 2700, and some bands at around 1700. Ok! Those are not available in the untransformed plants. So, where from it is coming? In the transgenic lines, it is coming. So, it might be coming from our transgene because, in the transgene, we have used a high-level expressing promoter, we have used rice ubiquitin promoter.

Now, if you carefully see the rice ubiquitin promoter structure, as I have mentioned earlier, there a huge intron is there, and one exon a part of the second exon is available. So, if this construct is available over here, then different forms will be produced. The pre-mRNA, means unprocessed mRNA will be there, then eventually it will be processed within the system, thereafter dicer will work, and a gene silencing mechanism might come. So, for further confirmation of where from these bands are coming, we have used different probes, and we have used the promoter-specific probe also. In the promoter-specific probe, rice ubiquitin second exon specific probe, this is the second exon we have used this specific probe.

And, we found that the band at around 2700 is being available, at around 2700-2800 bp, is available in untransformed as well as, in different transgenic lines. In untransformed plants, it is coming because if you see the transcript size of rice polyubiquitin, it will come at around this region. Similar to that, in addition to that, band in our transgenic plant, this unprocessed mRNA will be produced. If you see, about its size, it will be also close to 2700. So, we found this particular band that is coming in the transgenic lines

mostly from this unprocessed transcript.

For further confirmation, we have used a linker-specific probe, because a linker will not be available in the plant system, it is the heterologous system, coming from the heterologous system means from bacterial origin or something else. So, in the construct only it will be available. So, we found that in the untransformed plant, no linker-specific signal was obtained, but in the transgenic lines, the 2700 band, the 1700 band, and this mirror at around 400 were detected by a linker. Now, if you see the processing of the, post-transcriptional gene silencing mechanism, how is it taking place? First of all, from our construct, this type of unprocessed transcript will be produced. Within that unprocessed transcript, the big intron of the promoter will be available, then splicing will be taken place, and a processed transcript will be produced, of around 1700 bp. Ok!

In the processed transcript, the gene will be available in reverse and forward orientation ok, with the linker some part of the terminator will be there, and the exon part will be there. Then as the gene is placed in reverse and forward orientation, it will form the hairpin-like structure, over here the double-stranded RNA formation will take place and eventually, it will be cleaved by dicer protein. So, the dicer will cleave it into small siRNA, and the linker part will come out. Hence, we observed a smear at around 400 bases, once we used the linker-specific probe, 400 because, sometimes the cleavage may occur here, and sometimes cleavage may occur over here. So, a smear was available close to 400 bases, while this 1700 base and the 2700 base, in both of the linkers were available.

So, we found our linker-specific hybridization in the northern blot over here. For checking the loading control, we used a beta-actin gene-specific probe to check whether an equal amount of RNA was loaded or not. So, in this way, we confirm that our gene silencing mechanism is working fine. For further confirmation, of whether the small RNA is being produced or not, or whether the protein production has been reduced or not we have done some western blot analysis. Ok!

So, we have done, some western blot analysis. Initially, we found that the siRNA was indeed being produced, in different transgenic lines, and the size of the siRNA was close to the 26 nucleotides, while once we conducted the western blotting using the OsGLP1-specific antibody then we found, that in the untransformed rice strong protein expression was there while in the transgenic lines, the protein expression has been significantly reduced. Ok! As the siRNA was being produced, the gene was silenced properly. So, the protein production was absolutely reduced. Ok! Here also in the western blot we have to use a loading control, here beta-actin antibody was used, to check equal amount of protein samples were loaded over there or not. So, in this way we confirm that our transgenics are fine, and their gene silencing construct has been integrated gene silencing mechanism is properly working. Ok!

Then coming to our original discussion. So, thereafter we started to analyze, the over-expression construct preparation and transgenic development. Ok! Because, for the characterization of a particular gene, two approaches we are taking one is gene silencing, and one is overexpression. So, through here we have made the overexpression construct using the *OsGLP1* gene under 2X CaMV 35S promoter, CaMV 35S promoter with double enhancer was used and nos terminator was used. And, thereafter, the standard *hptII* gene CaMV 35S promoter and CMV 35S poly A were available, and here also pCAMBIA 1300 vector was used to clone this particular thing and here tobacco transformation was done. And once the tobacco plant transformation was done tobacco plants were generated those were eventually screened through southern blotting.

If you try to find out in normal tobacco, the *OsGLP1* gene will not be available right? So, in the transgenic lines if it is integrated then we can see some signals *OsGLP1* specific signals. So, let us see what was observed in the southern blot, the tobacco southern blotting was done in the untransformed plant sample, no signal was detected *OsGLP1* specific. While in lines 1, 2, 3, 4 different tobacco transgenic lines, we found differential integration of *OsGLP1* transgene. It means our transgene, has been somehow integrated within the genome of tobacco. Ok!

So, in this way, we confirmed different tobacco plants thereafter, our subsequent analysis started. So, so far, we have developed the transgenic line gene silencing construct we have developed the over-expression construct. Now we have tried to know the functional significance of this particular gene, which means that this gene does. Ok? So, using those transgenic lines different experiments were planned. First of all, based on the previous study as different *germin-like protein* genes, were found to be associated with disease resistance.

So, first, the *OsGLP1*, down-regulated transgenic lines were grown in well-irrigated fields. And, those transgenic lines were found to be more susceptible to biotic stress as compared to the untransformed plants. This means those plants were screened in the field and we found that the silenced plant where the gene expression was retarded, and protein expression was reduced extremely, those were highly susceptible to different biotic stresses. So, let us find out those publications. Here, you can see over here in this figure you can see, A is the control plant, this one is the control leaf, but in different transgenic leaf, you can see the severe fungal infestation has been occurred. Ok!

In the leaf also, sorry in the panicle also, you can see severe fungal infection. Ok! So, in this way the transgenic rice lines where gene silencing was conducted it was found to be highly susceptible to different fungal diseases. It means; therefore, we get that our gene might be associated with disease resistance. So, for further validation, we need to crosscheck the over-expressed lines. So, we have done cross-checking in the overexpressed lines, and we found that in the untransformed tobacco leaf if you see this type of fungal infection was detected. Ok!

The dose of fungal inoculum was increased, and the leaf was damaged enormously, but in the transgenic lines once, which means although we have increased the dose of the inoculum the infection was very less, it was insignificant means, our gene is indeed responsible for disease resistance. So, in this way we have to characterize a gene, first we have to take the silencing approach, the over-expression approach, then eventually we

have to find out its function and its biological significance. Ok! The transgenic tobacco lines expressing *OsGLP1*, showed tolerance to biotic and abiotic stresses also, later on I will show abiotic stress tolerance also. Then the planting, in planting also we found some significant differences. The *OsGLP1* downregulated transgenic lines, they displayed semi-dwarf phenotype in T_2 generation compared to the untransformed plants.

So, let us see, over here this one is the tobacco plants, in the transgenic leaf you can see more H_2O_2 accumulation in the untransformed plant, you can see less H_2O_2 accumulation. Ok! The more hydrogen peroxide accumulation means, the more it might show more tolerance. So, it is showing more tolerance over here why is it so, why means that H_2O_2 accumulation is associated with the tolerance, later on, I am coming. Ok! So, if you see, this particular analysis we found that different transgenic lines, gene silencing lines of rice where the *OsGLP1* gene, was silenced it was dwarf compared to the untransformed cultivar. Ok! And, once we cross-checked, it by transverse stem section we found that the cell length has been reduced in the transgenic line.

So, somehow due to some means due to its availability in the cell wall region, or due to some type of signal transduction this gene, is making the plant dwarf means once the gene has been silenced then the plant has been dwarf in nature, means the gene might be somehow related to the cell wall cross-linking. Ok! Now different cross-linking study, was also planned. Ok! The transgenic lines, expressing that *OsGLP1* in tobacco background, in tobacco plant they showed enhanced cross-linking of cell wall components. So, in cross-linking, we found evidence in rice, also in tobacco. So, in tobacco such type of evidence we found this one was the untransformed stem. Ok! This is in the unstained condition, in untransformed and transgenic here you cannot see too much difference, but once the staining was done using phloroglucinol! Phloroglucinol, basically stains the secondary cell wall formation. Ok!

So, in the transgenic lines, you can see strong staining, ok, deep red staining while in the untransformed tobacco, we can see relatively lesser staining. It means in the transgenic stem, where our genes have been over-expressed, here more cross-linking has done, we

have done Maule staining to check the lignin deposition and those things, and we found that the wall lignin formation has been taken place in the transgenic stem compared to the untransformed one. Ok! So, due to the formation of different lignins, this type of orange coloration is detected in Maule staining. So, we found that pattern in transgenic lines. So, we thought that the *OsGLP1* is responsible for the disease resistance, as well as, the cell wall cross-linking, but how it can do those things. Ok!

For those confirmations, we have planned some other experiments. We have analyzed the homology modeling of OsGLP1 protein and in its active site, we found that 3 histidine and 1 glutamate residues are available. Through homology modeling, study we have made the protein structure ok, and we found that 3 histidine and 1 glutamate residues are available in its active site, and that might be responsible for metal ion binding, and SOD activity. SOD, is superoxide dismutase activity. If superoxide dismutase activity, is there, then it will convert the superoxide molecule into hydrogen peroxide, by this enzymatic activity, and if this gene is over-expressed. So, H_2O_2 production will be more, if H_2O_2 production is more, cell wall cross-linking will be there. Ok!

So, in this way, we hypothesized the biological significance or biological function of the *OsGLP1* gene. The *OsGLP1* over-expression construct was expressed in tobacco, and it showed a distinct SOD active band along with the endogenous band which was absent in untransformed tobacco plants. So, now I will show you the SOD active band which was found to be available in controlled tobacco plants, and OsGLP1 expressing tobacco plants. Let us see if any additional bands were there or not. This one was the homology modeling, this one was the model, prepared for the *OsGLP1* gene and we found that 3 histidine and 1 glutamate, were available in its active site that might be responsible for metal ion binding and SOD activity.

And, then if you carefully see over here this part of the picture. Ok! Here rice leaf sample has been loaded, and its in-gel SOD activity was analyzed. Then, this one is untransformed tobacco and this one is the transformed tobacco sample. Ok! Now in the

rice, one different SOD active bands were observed. If you carefully see in the tobacco sample, the SOD active band is completely different from the rice one. So, if you see the transgenic tobacco, where our *OsGLP1* gene has been over-expressed along with these endogenous SOD active bands which were available in the controlled tobacco, also some additional signal was available.

So, for confirmation, we have done the immunoblot analysis of the western blotting using an *OsGLP1*-specific antibody. We found that in the semi-native condition, such types of signals were detected one signal is coming to the SOD active band where we observed, and one signal at the lower part. While in the complete denaturing condition we got the band at the lower part. We know that for enzymatic function a protein has to be in its quaternary structure or tertiary structure, but during protein gel electrophoresis, I have mentioned native gel, semi-native gel, and denaturing gel. In complete denaturing condition, we have to put SDS on the gel, and we have to boil the protein with SDS.

For semi-native conditions, we have to put SDS on the gel and we do not have to boil the protein with SDS. OK! So, over here it is the complete denatured condition means on the gel SDS is there the protein has been boiled with SDS. So, the protein has been completely denatured and we can see those monomeric bands, but as GLP are several beta roll-like structures, are available the cupin superfamily-specific structures are available. So, it might show different interactions, it might be available in multimeric form, and in multimeric form, those enzymatic activity was found to be available. Once we have run the semi-native gel, then we found that SOD activity and the particular antibody-based with OsGLP1 specific protein detection, under semi-native conditions.

So, if you go through this publication, if you carefully read the figure legends, I think you guys can understand it better way. So, these are the SOD activity and finally, in this way, a particular gene has been characterized. So, this is a unique case study there from a basic gene that has been characterized, and if you do molecular biology work during your M. Tech or PhD or your M.Sc. So, you may plan such type of experiment you can talk to your supervisors and you may plan such type of experiments. So, these are the references

you can go through two papers were published in BBRC in 2010 and one was published in plant cell tissue and organ culture in 2017. Thank you.