Course Name: Basics of Crop Breeding and Plant Biotechnology

Professor Name: Dr. Joydeep Banerjee

Department Name: Agricultural and Food Engineering

Institute Name: Indian Institute of Technology Kharagpur

Week: 08

Lecture-37: Scorable Marker Gene and Plant Tissue Culture

Welcome back. So, we will continue again. So, so far, we have discussed about different types of negative selectable marker and positive selectable marker. So, using those marker genes, we can easily screen the transformed plants. We can screen, but the confirmation could be done through PCR or other molecular approaches like Southern hybridization, Northern hybridization, later on we will be discussing. Now we will discuss about the scorable marker. Ok!

So, a scorable marker is a genetic marker that provides a visible or measurable phenotype, making it easy to detect or score an organism. In earlier case, we could select within the plate that may be few plants have been positive. It means, in few plants we may think that our transgene has been delivered ok, but through scorable marker, some measurable phenotype should be there. Based on that phenotype we can confirm that our transgene has been delivered successfully over there and here, some detection process is also needed, different types of scorable markers are used.

So, some detection processes are there and these markers are often used in genetic studies to track the inheritance of specific traits or genetic elements. It is another plus point if we have to trace some specific elements. Suppose, we are trying to characterize a particular promoter. Ok! So, we know that under each and every promoter if we put some gene, once the transcription is taken place, suppose it is a promoter and if we make such type of construct after this promoter, if we put any gene of interest and terminator this

gene will be transcribed. Now, suppose we have a new promoter, in spite of using a target gene we can put a scorable marker gene over there ok, later on we will discuss.

So, if a scorable marker gene is used over there, then under this promoter, this gene will be transcribed from this scorable marker gene, protein will be produced and if we can locate those protein, we can tell that our promoter is active in this type of tissues. Ok! Because for different genes, different tissue specific or stress inducible promoters are available in plant system. Suppose a plant is being grown in Kharagpur weather ok, while the same variety is being grown close to the sea water. So, their gene expression will be different, means some genes might be overexpressed under the salt stressed condition. So, if we try to find out the expression of those promoters of some target genes which might be active under salt stress, then we can fuse the promoter of that gene with a scorable marker gene.

Maybe, we can see the expression of those promoters in the root tissue because root is the main part where from the salt enters into the plant system the sodium ions, chlorine ions can be accumulated within the root system. Ok! So, we can see that in which tissue our promoter is acting. Then, we can fuse our gene of interest with the scorable marker also. Suppose, we are trying to characterize a *calmodulin* gene. So, calmodulins are calcium modulated protein, under any types of stress the calcium signature is rapidly developed within the cell and different sets of proteins are activated and calmodulin is one of them, it is modulated in presence of calcium.

So, suppose we have a *calmodulin* gene, let us assume its name is *calmodulin 1*. We do not know in which tissue this particular gene is active, in which tissue the calmodulin protein is active. So, we can fuse the *calmodulin* gene with a scorable marker gene. So, once the expression is will be taken place, along with the calmodulin gene our scorable marker gene will be also expressed and we can detect it that in which tissue those *calmodulin 1* might be expressing. Ok! So, they confer an easily screened phenotype on transformed plant tissues.

Now, let us take some example, first of all genes encoding fluorescence proteins like GFP green fluorescent protein, red fluorescent protein or RFP and other different types of FPs are there like cyan fluorescence protein, CFP, yellow fluorescence protein, YFP those type of scorable markers are mostly used in different plant molecular biology based research to locate different proteins and to characterize various promoters. So, these proteins, means some of this fluorescence proteins they emit light of specific wavelength, means we have to use the fluorescence microscopy to check the fluorescence of that specific wavelength of that specific protein and we can detect in which tissue or in which cell types those type of proteins are being expressed. And with the help of the emitting light under a specific wavelength, the researchers can visualize and track gene expression or protein localization within the cells. So, using fluorescence microscopy or using confocal microscopy we can do such type of experiment. The scorable marker like different fluorescence protein could be utilized using the fluorescence microscopy or confocal microscopy.

Then, the luciferase reporter gene, it is another highly popular scorable marker gene, it was found to be highly beneficial for some of the animal cell line, also some bacterial system, also even in the plant system also. So, luciferase gene produces an enzyme that catalyze a reaction resulting in the emission of light. So, the genes are used as scorable marker in assays where the presence or activity of a particular promoter or gene expression need to be measured. Suppose this is the size of a promoter, thereafter, we have used the luciferase gene, while the promoter has been deleted partially and thereafter, we have used the luciferase gene and both these constructs have been used for transformation maybe in bacterial cell. So, after those transformation, we can check the luciferase activity and from that activity, we can measure whether this promoter is beneficial or the deleted promoter is beneficial. Ok!

In this way, the luciferase reporter gene could be utilized in plant molecular biology for promoter characterization mostly. Then, a very popular gene, that is *GUS* gene it stands for *β-glucuronidase* gene, it is a common reporter gene used in molecular biology and genetic studies since long time and this gene originates from the bacteria *E. coli* and it

encodes an enzyme β glucuronidase. Ok! So, β-glucuronidase is an enzyme that catalyze the hydrolysis of glucuronides. *GUS* gene is often used as reporter gene to study gene expression or promoter activity.

The GUS gene is fused to the gene of interest or placed under the control of a specific promoter sequence, as we were discussing earlier and when the promoter becomes active or when the gene of interest is expressed, the *GUS* gene is also transcribed and translated into that particular enzyme that is the β glucuronidase. The activity of GUS enzyme is performed, how should we test it? By adding a substrate X-gluc, this substrate we should add in the putative transformed tissues. So, its full form is 5-bromo-4-chloro-3-indolyl-β D-glucuronide and then, we can see that in which tissue our GUS gene is being expressed. If GUS enzyme is active, it hydrolyzes X-gluc substrate, resulting in the production of a blue precipitate. A blue precipitate is formed or a color change in the cells or tissues where the *GUS* genes are expressed.. is formed.

Suppose, a plant leaf has been formed from a putative transgenic plant. We have generated a putative transgenic plant and this whole leaf has been treated with the X-gluc solution and in the plant transformation process, suppose, we have used a vector where under a particular promoter, the *GUS* gene was being expressed. So, we may assume that within this leaf tissue may be in some part, this protein will be active or this promoter will be active. If the promoter will be active, from *GUS* gene the transcription will be taken place, translation will be taken place and protein production will be taken place, that is the β glucuronidase enzyme will be produced. So, once that enzyme will be produced, if the whole leaf is immersed in X-gluc solution. So, that enzyme will react with X-gluc and it will show the blue precipitation. So, this type of study is known as histochemical localization. Histochemical localization or histology means in which specific tissues our promoter was active or targeted GUS protein accumulation is being visible. Ok! So, we may see either in the root, in the leaf tip or in the hydathode region, our promoter might be active. Ok! We may see that our promoter might be active in different trichomes available on the leaf.

The promoter may not be activated throughout the leaf tissue, but it may be active in some part of the leaf tissue. So, in this way, we can know that tissue specific localization of that particular promoter which is expressing *GUS* gene. If we recall our previous discussion on binary vectors, we have mentioned about pCAMBIA1300 or pCAMBIA1301 vector. So, in those vectors, some scorable marker genes are also there may be in pCAMBIA1301, the GUS, scorable marker gene is there within the left border and right border region. So, along with our transgene and the selectable marker gene, the disease resistance or hygromycin resistance gene, this scorable marker gene is also proved.

So that, we can confirm that in which plant our transgene has been delivered. Now we will start discussion, we will start our discussion on the plant tissue culture, what are the basic things we need for setting up a plant tissue culture facility and what are the importance of plant tissue culture, what are the things we can do. So, plant tissue culture involves in-vitro cultivation of excised plant tissues under aseptic conditions. So, excised plant tissues one part is mentioned there and aseptic conditions are there. Ok! So, tissue culture media is an enriched media therefore, it is easily contaminated.

Hence, maintaining an aseptic condition is very important. So, what are the things we can do through plant tissue culture? We can do meristem culture, that is the tip part of the shoot, it might be apical meristem or the tip part of the root could be cultured also, but in most of the cases, the shoot apical meristem part is cultured because that part is free from different diseases. And if we culture in-vitro condition under tissue culture condition, then we can develop disease free plantlets, in different crops the meristem culture is being done. Then we can do the protoplast culture, we can do cell suspension culture, then tissue or organ culture and anther or pollen culture. Ok! So, let us talk about the tissue and organ culture. Ok!

For normal crops, normal food crops like rice, wheat, maize those crops if we grow in the field, they can produce hundreds of seeds right? So, for those crops, normal cultivation in tissue culture is not at all needed, but some crops are there those are

maintained through vegetative propagation. Ok! Those are propagated through vegetative means, on those crops, tissue culture is highly successful because if we try to grow by vegetative propagation in conventional way, the process is relatively slower in most of the cases. But through tissue culture, by using in this media, we can fasten the process, the process could be faster. Ok! So, the organ culture or plant tissue culture has been popular for different horticultural, ornamentation plants.

So, the anther culture or pollen culture, those things are important for development of haploids, for development of double haploids by colchicine treatment. So, for those purpose, the anther culture and pollen cultures are famous. Then protoplast culture or cell suspension culture, those are used for some in-vitro study ok, suppose we need to characterize some promoters. Ok! Different deletion constructs have been made using that particular promoter, this is the original promoter. Suppose, different deletion constructs have been made using this promoter.

If we need to find out that which of this particular promoter, which of the deleted fragment will be maximum effective to express a target gene, then through protoplast culture or cell suspension culture we can do easily. Because, if we have to go for the conventional tissue culture process, if we have to go for transgenic plant development, it will take long time. While, through protoplast culture or cell suspension culture, may be within a week we can get the result that which one of these promoters is active. But for knowing the tissue specific role, definitely, we have to go to the full formed plant, the full transgenic plant has to be developed to know the tissue specific role of different promoters. Now, in-vitro propagation of plants is a labor-intensive process.

The regeneration potential from the explants depends on various factors. So, therefore, basic setup of laboratory and standardization of protocols from different explants are important to determine the final success of the process. Ok! So, different pros and cons are involved there in the process. So, we have to be very much cautious, once we have to do the plant tissue culture because a lot of contaminations may come from anywhere. So, these are the basic equipment which are required for setting up a plant tissue culture

facility.

First, this is the water distillation unit. Ok! So, basically the double distillation unit is must to initiate the plant tissue culture because over here, the purified water is produced and that will be contamination free and thereafter, it has to be autoclaved again once we have to prepare different media or stock solutions. Next, coming to other different things, the hot plate or stirrer that is also needed and if heating facility is there, it is good because it will be utilized for different media preparations. We have to mix different salts; I will be mentioning about some basic media compositions also later on. Then, we need at least a refrigerator 4℃ for keeping different media, different stocks, different hormones and a freezer also, -20℃ freezer.

Then, this is the most important thing that is needed in plant tissue culture, that is the laminar air flow unit, a sterilized area for transferring different cultures. So, within this laminar air flow, basically, HEPA filters are available. So, high efficiency particulate air filters are available through which the purified air basically blows within this cabinet and from outside, the contaminated air may not enter. So, whatever we have to do inside of this cabinet that will be free from various microbes. So, it is a very important equipment needed for plant tissue culture.

Then, weighing balance, that is needed for weighing different chemicals for media preparation, a pH meter is needed and at last, but not the least, we must need an autoclave. Autoclave machine is needed for sterilization of each and every media which should be utilized in plant tissue culture. So, some facilities in laboratory that actively performs plant tissue culture are the following- first washing area. The cleaning and washing glass wares and other items used in experiments. So, we need to have a separate washing area that should be away from the tissue culture area or the laminar space. Ok!

Then glass wares and plasticwares used for media preparation, storage of media and setting experiments different petri-plates, conical flasks those are used. Then area for media preparation, some area should be dedicated for preparation of tissue culture media.

They should include the storage area for chemicals, the refrigerator should be there and different chemicals should be kept properly on the racks. Then, we need a greenhouse for acclimatization of in-vitro rich plants through tissue culture, whatever the seedlings were being generated or we are trying to develop within the controlled condition, those plants will be tender. Those will not be too much hardened in nature compared to the plants which are being grown in outside atmosphere.

So, before shifting them from the lab, from the tissue culture lab to the field, a greenhouse is needed for their acclimatization. Ok! The temperature and humidity is maintained in the greenhouse condition also. Within the transfer area, a sterile atmosphere is required to transfer for doing the different plant tissue transfer from one media to another media within the laminar air flow. Ok! So, those transfer area where the transfer process or gene transfer process, gene delivery process is taken place, a designated area is there, basically, two door system is used for tissue culture place basically. So that, the contaminants or dust particles can enter in least amount.

The laminar air flow is used to make transfer of explants in media, maintaining a clean and dry place is important in tissue culture because in the country like India, where the environment is hot and humid, there fungal contamination is very much. So, we have to keep our tissue culture condition clean and dry. AC should be maintained there all the times. So that, the moisture problem could be minimized and fungal contamination could be reduced because fungal problem, fungal contamination is a very tough situation in tissue culture that is mostly faced. Then autoclave, I have mentioned about its utility, it is done for elimination of microorganism and maintaining sterility in plant tissue culture.

So, the microorganism from the media and other equipment like the forceps, scalpels, those things could be sterilized through autoclave also for the first time and the autoclave is done at 15 pounds per square inch (psi) at 121℃ and this pressure and this temperature is maintained for 15 minutes approximately. And the culture rooms and incubators are also needed, means after doing the tissue culture practice in the laminar air flow or laminar hood, we have to keep it under certain light condition, under certain specific

temperature conditions that will vary from plant to plant; for tobacco, little bit cooler temperature is needed compared to rice, in this way, for each and every crop, some specific temperature is needed. Ok! So, in the culture rooms and incubators, the air condition should be there. Ok! So, it should work properly, it is better if at least two ACs are installed. So that, they can work in tandem manner, once one will shut down, will stop, another one will start functioning. Ok!

So, these are the things which are needed, which are the basic facilities needed for plant tissue culture and now coming to the culture media, main components used in plant tissue culture media, these are some inorganic supplements means macronutrients and micronutrients, which are used in macronutrients N, P, K, Ca, Mg, S; means nitrogen, phosphorus, potassium, calcium, magnesium and sulfur, those are used as macronutrients while in micronutrients, iron, manganese, zinc, boron, copper, molybdenum, cobalt. So, those things are mostly used if we think about different media where I will mention later that, these components are available in the form of different salts. The organic supplements which are given in tissue culture media, those are different vitamins like thiamine, nicotinic acid, myo-inositol, those are used different amino acid supplements are given like casein, casein hydrosylate, proline, L-glutamine, L-asparagine, those amino acids are given. Then, several growth regulators or plant hormones, phytohormones are used in tissue culture those are auxins, cytokinins, gibberellins are mostly used; in auxins some natural auxins like IAA, IBA, PAA are used, some synthetic auxins like 2, 4 D, NAA those are used. Then cytokinin, some natural cytokinins are there like zeatin, then isopentenyl adenine, while the synthetic cytokinin, benzyl aminopurine, was found to be very famous in most of the tissue culture.

So, now, coming to different tissue culture medium, some common medium which is used for tissue culture, one is Murashige and Skoog medium commonly known as MS medium, then Linsmaier and Skoog medium; LS medium, Gamborg B5 medium, Nitsch and Nitsch medium, White's medium, those mediums are used by different laboratories and it has been optimized for different crop species by specific laboratories. So, for each and every crop, the media composition might vary. So, either we have to go with some

published literature or we have to plan the experiment in such a way, so that, first we need to optimize the process, then ultimately, we can do the tissue culture in large scale. For certain crops like banana, tissue culture has been very famous in Indian context and these are the references of this particular class. Thank you.