Plant Cell Bioprocessing
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Lecture – 23
Genetic transformations in plant cell – Part 2

Yesterday we spoke about Genetic transformations in plant cells. Now I said genetic transformations in plant cells *in vitro* are generally carried out using vectors.

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Now, in nature genetic transformation happens in plant cells via *Agrobacterium* transformations. So, what happens in *Agrobacterium* transformation? It has a plasmid which is called the tumor inducing plasmid or a root inducing plasmid.

Now, these plasmids have certain genes which can get integrated into the plant chromosome thereby causing transformation in plant cells. Now, this can be exploited *in vitro* conditions by removing some of the genes which are not necessary for the transfer and putting up polylinker sites or multiple cloning sites (MCS) with unique restriction sites so that you can insert your desired gene along with your marker system into the T-DNA.

We were also talking about different types of plasmids - there are conjugative plasmid and there are non transmissible plasmid. For non-transmissible plasmids which are non conjugate the facilitation is done using conjugate plasmid. In that case we use binary vectors.

The Ti and Ri plasmids are quite large in size - 200 kbp, Ri is even larger - it is 250kbp. So, being very big plasmids and low copy numbers for multiplication in *Agrobacterium* the success of transformation might be less. So, generally to remove the bulkiness, the virulence region of the particular Ti plasmid which is inherently present is disarmed. This means the oncogenes which are present which in nature get transmitted as a part of infection into the plant chromosome are removed.

What are oncogenes? Oncogenes are your auxin and cytokinin synthesizing genes which lead to this rapid multiplication and morphogenetic events, change in the biochemical makeup especially with respect to the growth hormones. Now, when these oncogenes are removed this is called as disarmed plasmid but your virulence gene is intact.

This virulence genes region in the disarmed plasmid is kept separately from another artificial plasmid which you prepare which is much smaller in size and is called a binary vector like pCambia which we hear in plant transformation. These are called binary vectors because they have an origin of replication for *E.coli* as well as for *Agrobacterium*.

So, these can multiply and maintain an *E. coli* and in *Agrobacterium* in addition, will have your T-DNA. Now, the inherent Ti plasmid is a disarmed plasmid, but this vector will have the T-DNA with the polylinker site and MCS, with your marker system with your promoter and your 3’ UTR poly A signal.

So, when these two are there they work in trans and this virulence genes which is present in the disarmed plasmid will help in packaging the T-DNA and processing it, helping for its integration into the plant chromosome and thereby the expression of the desired gene which you have inserted in this area.

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So, what happens in nature? Crown gall disease is caused by the infection of *Agrobacterium tumefaciens* and leads to a neoplastic growth. It utilizes opines produced by the host cells - apart from your auxins and your cytokinin genes there are opine genes also present in this T-DNA which once integrated the plant start producing certain compounds which are rich in carbon and nitrogen. This Ti plasmid which is there in the *Agrobacterium* - not everything is getting integrated. In the rest of the Ti plasmid, there are opine catabolism genes.

Now, once the plant produces these opines induce the opine catabolism gene present in the *Agrobacterium* Ti plasmid such that the *Agrobacterium* can use it. So, by nature *Agrobacterium* is a parasite for the plant. Transformation is associated with and accomplished by transfer of a stable replicating portion of Ti plasmid DNA to the plant cell. So, the replicating portion of the Ti plasmid is your T-DNA. We will see how it happens. Precise mechanism is unknown, but molecular analysis has shown that 25 nucleotides which are called as direct repeats; direct repeat means the base pairs which are after a gap continuously found to be present in more than one pair.

So, more than one time they are continuously repeated. Repeated sequencing flanking the boundaries of the integration sites - left and right border. It reads from the right to the left. The genes that are to be introduced in the plant cells must be inserted between the left and the right border.

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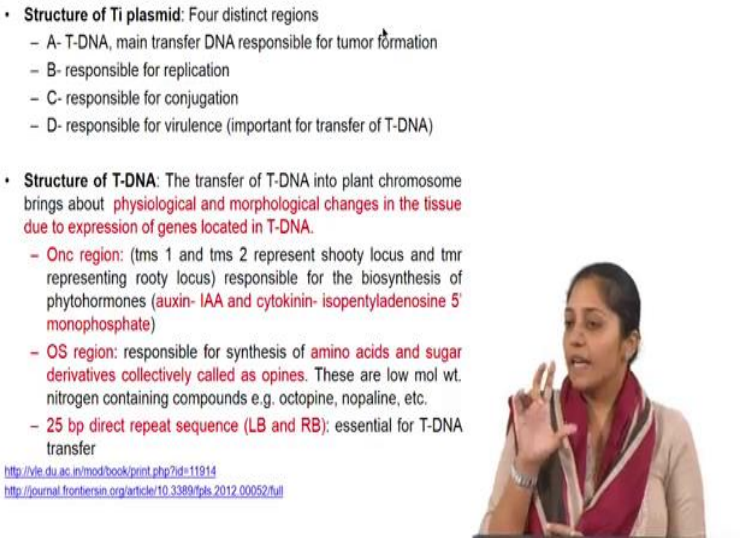

So, what happens? In the structure of the Ti plasmid, there are four regions; one is the T-DNA region which is the main transfer DNA responsible for the tumour induction - it has all those genes. The second region in this Ti plasmid is responsible for replication. The third region is responsible for conjugation - pilus formation such that the transfer can take place. The fourth region is responsible for virulence - where genes are there and it is important for processing and the transfer of T-DNA.

Now, the structure of T-DNA is broken down into three parts. The transfer of T-DNA into the plant chromosome brings about physiological and morphological changes in the tissue due to expression of genes located in the T-DNA. Onc region which is the oncogenic region and Tms 1 - Now, these are different locus or positions of the genes. Tms 1 and Tms 2 represent shooty locus which means they represent or they express to produce cytokinins. What kind of cytokinin? Generally it is isopentyladenosine (IPA).

Then, the tmr region, the rooty locus is responsible for auxin synthesis. Generally what kind of auxin is present? Indole Acetic Acid (IAA). Then the OS region is your Opine Synthesis region which is responsible for synthesis of amino acids and sugar derivatives - amino acids and sugar derivatives are collectively called opines. These are low molecular weight nitrogen containing organic compounds.

There are many different types of opine, some of them are octopine, menopine, nopaline. Sometimes, *Agrobacterium* themselves are classified depending on the type of opine they are producing - some are called monopine types of *Agrobacterium*, some are octopine type.

Now, a 25 base pair repeat sequence which is called as a left border and the right border is essential for the T-DNA transfer. Now, how does it take place? These are some of the links which you can go through to help you to understand (Ref. slide).

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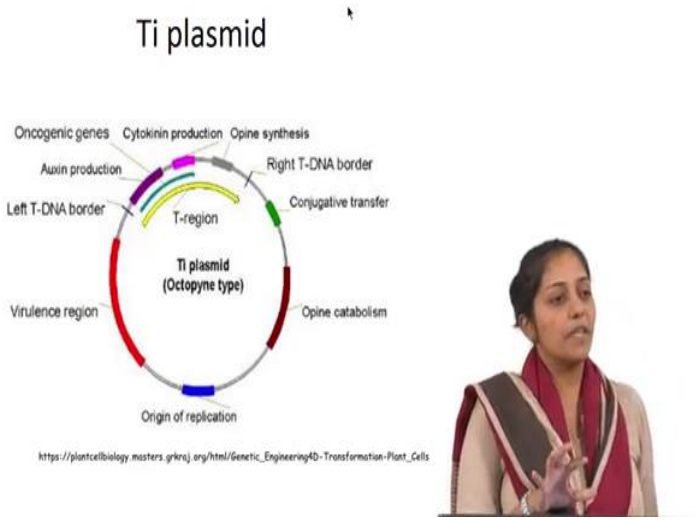

So, this is a pictorial representation of the entire Ti plasmid. Now, you will have oncogenic genes, you will have cytokinin producing genes, you will have opine synthesizing genes, then this entire part is called the oncogenic region.

Then you will have T region - which includes your left border and the right border and all these genes. Then I told you about conjugative transfer - this is the region which is responsible for conjugative transfer (Ref. slide). Then opine catabolism genes, origin of replication, the virulence region are also present.

So, T-DNA does not contain the other genes - neither the catabolism genes nor the origin of replication nor virulence gene. That is I when we say disarmed plasmid it means the T-DNA region has been taken out and replaced.

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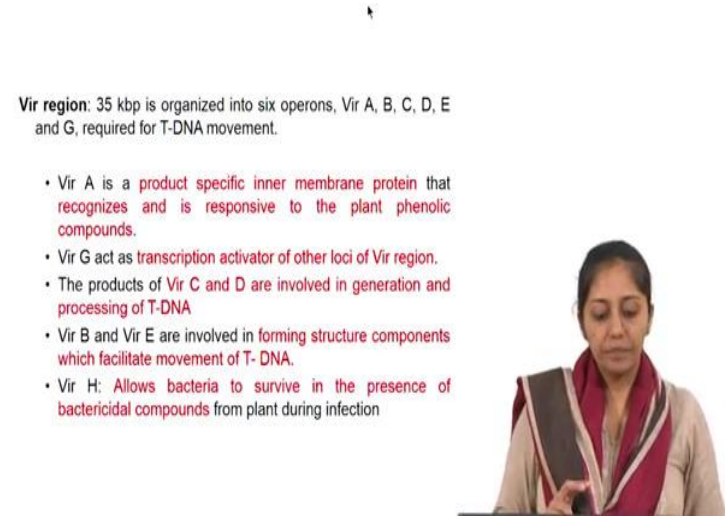

Now, virulence region is generally 35 kbp long, and is organised in six operons virulence A, B, C, D, E and G and each of these regions play a different role during this transfer process. It is not only transfer which is happening - from the time it induces there is the signals like your defence or some molecules like polyphenolic compounds which get released by the injured plant cells, they act as cues - they induce some of these virulence regions in the *Agrobacterium* and these different proteins will be responsible for first induction of the other regions of this virulence gene - some of the proteins will be utilised in packaging, some of the proteins will be utilised in creating a nick, some of the proteins are utilised in protecting the T-DNA as it enters the plant cells, some of the proteins are responsible for conjugation. So, virulence A is a product specific inner membrane protein that recognises and is responsive to the plant phenolic compounds.

So, this is what is responsible to recognise plant phenolic compounds and it acts as a receptor. Virulence G acts as a transcription activator of other loci of the virulence region. The products of Vir C and D are involved in generation and processing of T-DNA - which means creating a nick and then replicating and then packaging it which means the 5’ and is gapped. Virulence B and E are involved in forming structural components which facilitate movement of T-DNA. Virulence H allows bacteria to survive in the presence of bactericidal compounds from plants during the infection.

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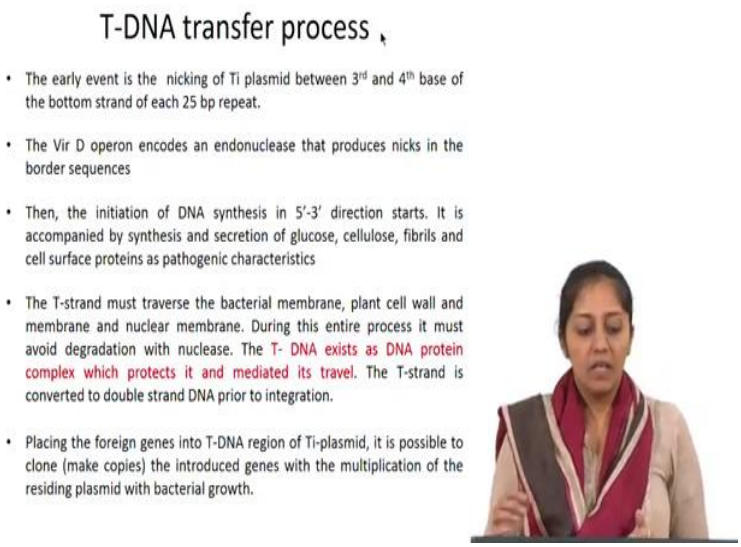

So, how does it happen? The early event is the nicking of the Ti plasmid between the third and the fourth bases of the bottom strand of the 25 base per repeat. The virulence D operon encodes an endonuclease that produces nick in the border sequence. Then the initiation of DNA synthesis begins from 5'to 3' end.

Now, it is accompanied by synthesis and secretion of certain other molecules like glucose, cellulose or surface proteins which have pathogenic characteristics. The T strand which is replicated is a single strand. It has to enter the plant chromosome - through the nuclear membrane into the plant chromosome.

Now, the T strand in order to transverse through the bacterial membrane, plant cell wall, plant cell membrane and then the nuclear membrane - during this entire process it must avoid degradation with nucleases. The T-DNA exists as a DNA protein complex - I talked about capping - which protects it and mediates its travel. The T-DNA is converted to the double strand DNA prior to integration. So, this double strand DNA is happening prior to integration into the plant chromosome.

Placing the foreign genes into the T-DNA region of the Ti plasmid it is possible to clone (clone means making copies) the introduced genes with the multiplication of the residing plasmid with the bacterial growth.

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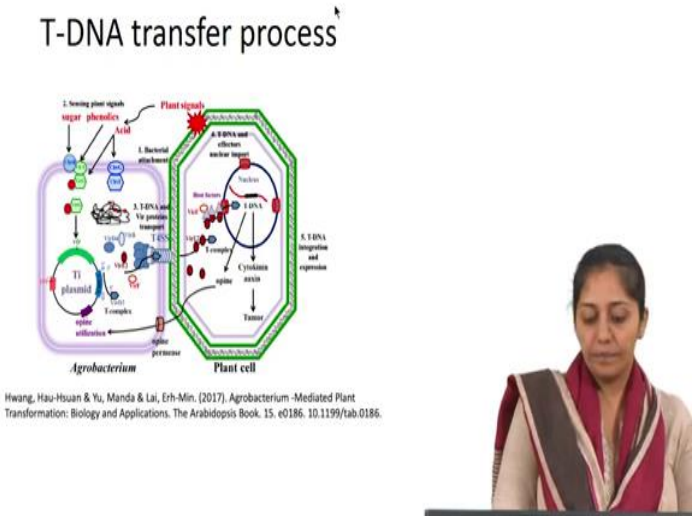

So, this is what we were talking about. This is how it happens - the various events which we were talking about - you can read that and correlate with the figure.

So, they have demonstrated the various virulence proteins. (Refer slide) One is acting as a receptor and it further induces other regions of the virulence gene. After induction - induction involves production of Vir D, Vir E. Vir E is involved in protection - once it enters the plant chromosome to protects it from the nucleases. Then Vir B is involved in conjugations. So, you can correlate and remember how the events take place.

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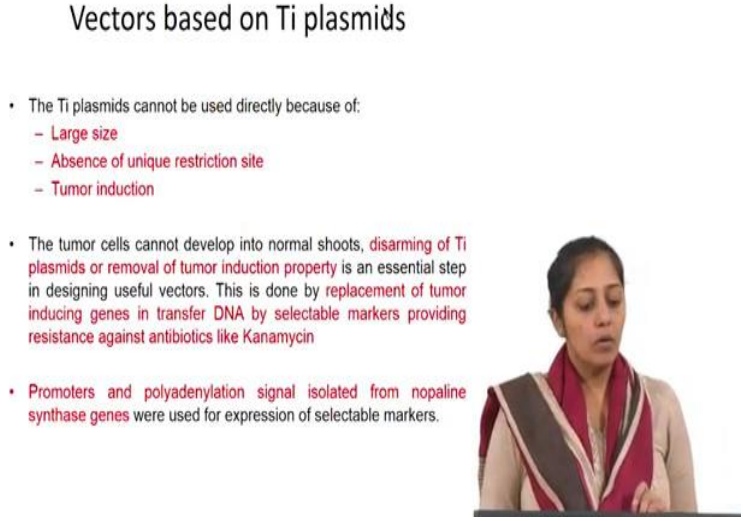

So, now the Ti plasmids cannot be used directly because of - as I was saying, generally we have binary vectors and these vectors are inserted into *Agrobacterium* which are disarmed.

Now, disarmed means the inherent plasmid T-DNA has been replaced and only virulence gene is kept. The reason being they are large in size, absence of unique restriction sites and hence it is difficult to insert your gene of interest. Then tumor induction. Why do you think tumor induction has to be avoided? If you do not disarm there will be oncogenic genes present. So, the morphogenetic event would be causing tumors or causing adventitious roots and that was not the purpose. If that happens what will happen?

It depends on the objective of the study. You are trying to do a targeted transformation into the plant cell. So, the more the number of genetic transformations the more metabolic burden. So, any integration there is leading to removal of certain endogenous genes and replacement. So, more the removal the more there is a chance that some of the crucial endogenous genes get silent or gets replaced and this is a disease. Only if desired, you would like to have it otherwise why would you like to have unnecessary growth or neoplastic growth in the plant.

If you are working on whole plants then obviously, you do not want to have neoplastic growth in the whole plant. But what if the purpose of secondary metabolite production using plant cell cultures? If suppose it is for secondary metabolite production using plant cell cultures, is this useful or not?

Student: Yes useful.

Why?

Student: Because they grow.

They will grow very fast, they will not need hormones in the medium - generally this is what is observed. For oncogenic genes this is how they are exploited for secondary metabolic production because if you take up wild *Agrobacterium* with their natural Ti or Ri plasmid which we call as hairy roots, we are not having any specific MCS or specific gene which we have inserted. This is a natural infection which we exploit in the lab.

So, what would it lead to? It leads to integration of the inherent T-DNA thereby leading to expression of oncogenes. This is what leads to hormone free growth in transformed cultures.

And once you have hormone free growth - we have faster growth, more branching and because it is stress associated the possibility is higher that the secondary metabolite yield increases than the untransformed - is it useful or not? It is advantageous, but still there is a selection of the cell line needed after transformation. Why? You understood what I am trying to ask? I infect the *Agrobacterium* and I will have more than one cell line and I say still there is a selection needed. Even if it is a targeted transformation and I am still saying there is a selection needed in *Agrobacterium* transformations.

Selection is needed because you may end up in many different cell lines isn't it? As you end up in untransformed callus induction. So, why now selection is needed? Once I have confirmed that this particular cell line is transformed generally using PCR (we will talk about those methods) why can't I stick to just that line.

Student: It might not be regenerated in all the generations the plasmids.

It is a stable transformation event because it is not a transient expression but it has got integrated into the plant chromosome. I will give a hint - we were talking about stress. It has been found in literature that *Agrobacterium* infections are stress to the plant cells. So, where will we compromise?

Student: Some cells might got resistant to that stress some might not be affected by that stress.

They act like transposable elements. So, sometimes the copy number and the position is not in your hand. In that case, the cell lines which come out will vary in growth as well as yield. In addition, being a stress, there will be a compromise in growth. So, there is a need for another step of selection even after transformation to reach the highest yield and highest or with better growth in comparison to others lines.

So, among what has been transformed, generally you select based on your secondary metabolite yield and growth index.

The tumor cells cannot develop into normal shoots - disarming of Ti plasmid or removal of tumor inducing property is an essential step. Generally it is observed that once you do *Agrobacterium* infection even if you have ended up in cell lines their regeneration capacity is hampered because of the stress into the whole plant.

So, when you are working for transgenic plants it is all the more important that the tumor regions or the onco regions have to be taken out. This is done by replacement of tumor inducing genes in transfer T-DNA by selectable markers providing resistance against antibiotics like kanamycin. Now, going back to my question, I said it is recommended that the desired gene has to be inserted near the 5' end.

Student: The 5 prime end is capped and protected. So, our inserted DNA is more safe there.

Your marker systems - antibiotic resistance gene is on the other end. So, if it is nicked once the transformation is done, you would not like to have the antibiotic resistance gene in the plant because any extra gene insertion is a metabolic burden. Promoters and polyadenylation signals isolated from nopaline synthase genes were used for expression of selectable markers.

By nature these opines are already getting expressed, so, people have utilised the same expression promoter like CaMV 35S promoter, then here nopaline synthase 3' UTR is used because it has been found to express very well in the plants.

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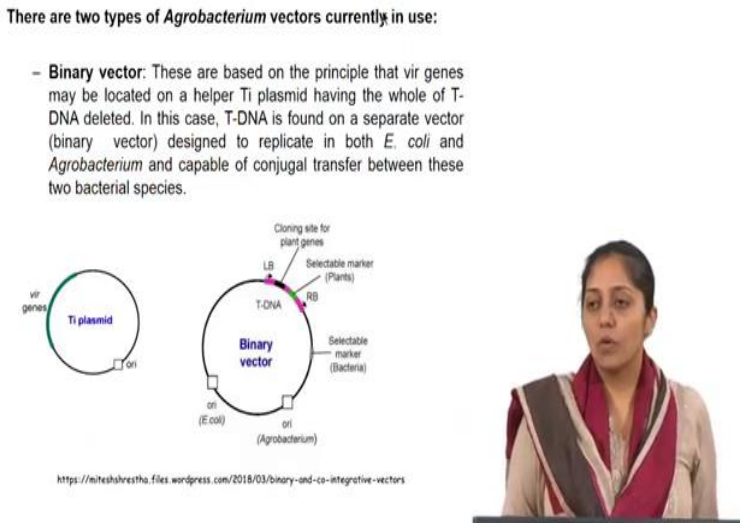

So, what are binary vectors? These are based on the principle that virulence genes may be located on a helper Ti plasmid having the whole of the T-DNA located. So, this disarmed Ti plasmid is called the helper Ti plasmid. In this case T-DNA is found on a separate vector which is a binary vector designed to replicate in both *E. coli* and *Agrobacterium*. So, it has the origin

of replication of both the *E. coli* and *Agrobacterium*. Now, why do you think there is a need for *E. coli* origin of replication, why unnecessarily having *E. coli* step in between?

Student: To transform to it to be able to grow it in *E. coli*because it has multiple copies.

Because this is a conjugate plasmid which is bigger in size. So, it cannot multiply here. So, you need to have more copies and then you transform those into the *Agrobacterium* and then this transformed *Agrobacterium* is used to transform the plant cells. I will stop here. There are other kinds of vectors also present called intermediate and helper vectors, but generally you will find in literature the success rate is very high with binary vectors. So, most of these transformations are done using binary vectors.