Plant Cell Bioprocessing
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Lecture - 24
Genetic transformations in plant cells- Part 3

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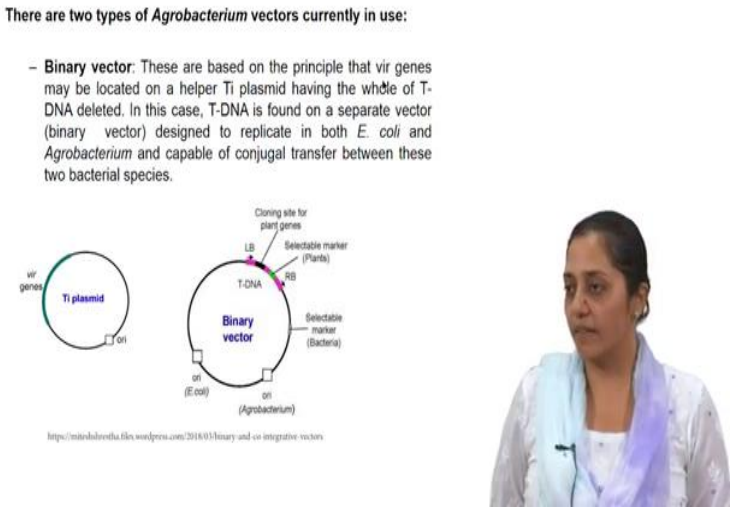

So, last class, we were discussing the various genetic transformations in plant cells.. So, we discussed that generally in literature you will find that *Agrobacterium* mediated transformations are used. *Agrobacterium* themselves are natural engineers to transformation, they have the machinery to successfully transform the plant cells through a section of DNA present in them - plasmid DNA which is called as T-DNA and gets stably integrated.

So, how we have exploited is that the inherent T-DNA present in the *Agrobacterium* is disarmed and another vector which have this segment of T-DNA which *Agrobacterium* has the machinery to integrate - this plasmid vector is exogenously added into the *Agrobacterium* to integrate into the plant cells.

So, now what are the different kinds of vectors which we were discussing last time? The most commonly used vectors are binary vectors. Now, why are they called binary vectors? It is because of the origin of replications - they can replicate in *E. coli* as well as in *Agrobacterium.* And then what else? The conjugal transfer can take place between *E. coli* to *Agrobacterium*, and then from *Agrobacterium* to plant cells.

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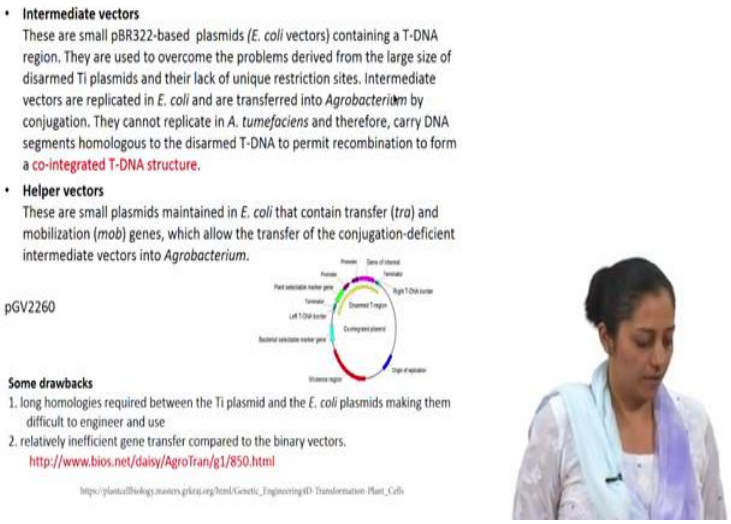

Now, I was also talking about other vectors which can be used for genetic transformation in plant cells. These are intermediate vectors - what are these? For example, pBR322, you will be hearing if you work in this area. These are smaller size vectors in *E. coli*, which contain the T-DNA and they are used to overcome the problems because of the large size of the Ti plasmid.

So, as I was saying there is a helper plasmid present in the *Agrobacterium* which is disarmed. Now, this is called a helper plasmid and will have virulence regions. Now, this small plasmid can multiply in *E. coli* and through conjugal transfers it can be brought into *Agrobacterium*. Now, these plasmids in *Agrobacterium* cannot multiply but they can be used to transform the plant cells.

Now, co-integrative. Sometimes there is homologous recombination. In the *Agrobacterium*, some of the T-DNA regions have been disarmed, but in the other regions if there is a homologous region i.e if there is a similarity between that DNA component and the DNA which is present in these vectors, then transformation would take place, recombination would take place and there will be an exchange of genetic material.

And this T-DNA where your MCS region is there, this gets integrated into the entire one single plasmid which can then act as a complete Ti plasmid for the transformation to happen in the plant cells. This is called a co-integrative plasmid.

Then helper vectors. Either you have your inherent Ti plasmid which has been disarmed, disarmed means the oncogenic regions - T-DNA has been taken out or you have separate plasmids which help in the transformation event - they will have regions which are transfer regions which will help in the transport of this T-DNA into small integrative vectors. These are small plasmids maintained in *E. coli* called helper plasmids and these help in *E. coli* to *Agrobacterium* transfer.

They contain transfer regions and mobilization regions which allow the transfer of the conjugation deficient intermediate vectors into *Agrobacterium*. Suppose if conjugation is absent - not all vectors are conjugal vectors. In that case, you need helper plasmid which can help in the transfer of these vectors into the *Agrobacterium*.

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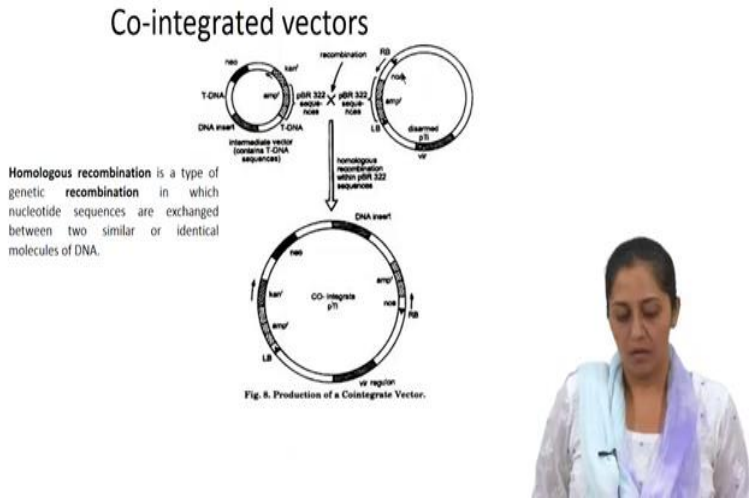

So, this is what I was saying: co-integrative vectors where homologous recombination of the entire one co-integrative plasmid inside the *Agrobacterium* can be obtained.

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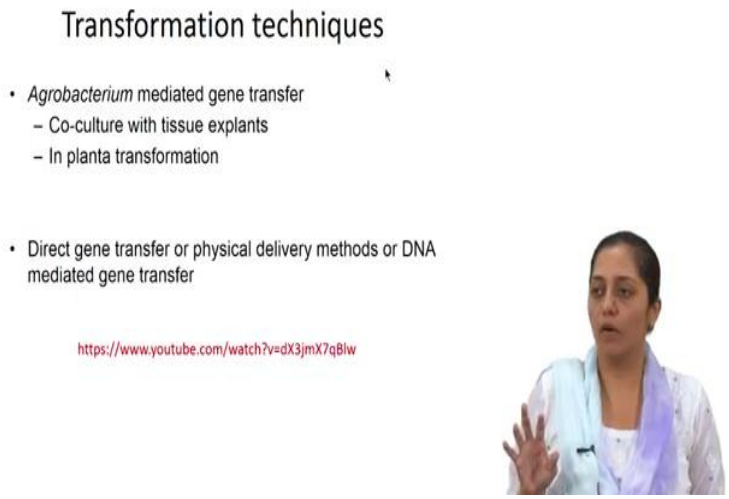

Now, what are the different transformation techniques? One is the well-known co-culture technique which is being used in laboratories and the other is direct techniques in planta transformation - directly the plant is transformed. Now co-culture with tissue explants means, it begins with *in vitro* cultures and from the *in vitro* cultures regeneration is done to get the transformed plant.

Otherwise directly the plant parts can themselves get transformed either the seed itself or the zygote or in case of floral pollination, just before pollination when the flowers come out - there is a flower dip method or floral dip method through which *Agrobacterium* can get into the plant. And after pollination it can transform the zygote, thereby in the progeny you will see transformation happening.

Then direct gene transfer or physical delivery methods. In direct gene transfer either we are using *Agrobacterium* as a tool to transfer. There are also indirect gene transfer methods for example, gene guns which I think you must have heard about. And there are other methods which we will see.

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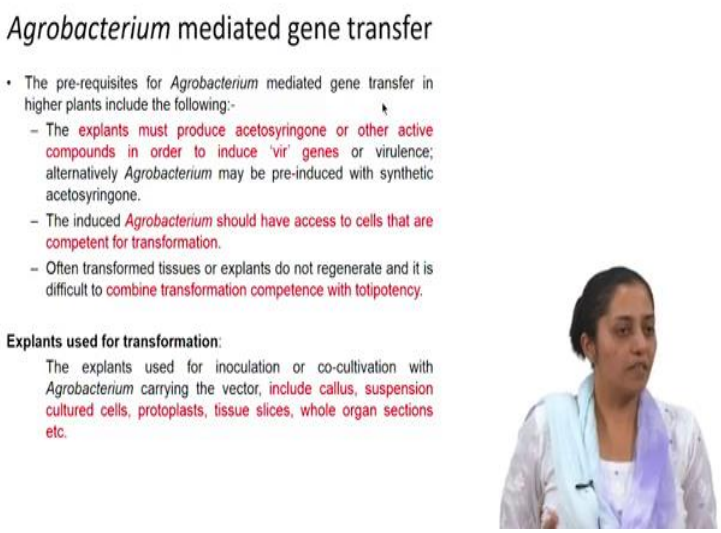

So, *Agrobacterium* mediated methods - the prerequisites for *Agrobacterium* mediated gene transfer is that the explants must produce acetosyringone. What is acetosyringone?

So, this is used to induce the virulence genes present in the *Agrobacterium*. So, acetosyringone, the type of explants, the age of the explant also sometimes matter for successful transformations to happen. So, there has to be production of these signalling molecules which can attract the *Agrobacterium* and increase the proximity between the *Agrobacterium* and the plant cells.

Now, the explant must produce acetosyringone or other active compounds - other phenolic compounds in order to induce the virulence genes. Then, the *Agrobacterium* should have access to cells that are competent for transformations. Sometimes it is observed although transformation is done, but regeneration does not happen. For regeneration to happen, you need competent cells for transformation and totipotency has to be maintained in the transformed cells so as to reach into the regenerant plant, so which is a difficult task.

So, now for successful transformation access to the competent cells is necessary - not all cells will be competent by nature. Sometimes you will see that even if you use a similar method - you will keep on repeating but only some will be successful, the others may not because of all these reasons. Not all cells in the callus will be of the same biochemical nature.

Then explants used for transformation - the type of explant, the age of explants matters. Now, it is generally preferred that they should be actively dividing cells when transformation should be done. Why? Young explants are preferred to old ones, why is it so?

For successful transformation to happen it is preferred that you should work with younger explants with rapidly dividing cells if you are working with cell cultures.

Let us do a comparison between matured cells in suspension and young cells. The secondary cell wall comes when organogenesis and it is in the tissue isn't it.

The probability can be higher - what else? Isn't there one major difference between matured cells and immature cells? If you remember I was telling in the earlier classes that in the younger cells the vacuoles are small while the cytoplasm is more dense. Hence the probability is higher. In mature cells, the vacuoles become large and the cytoplasm is less dense.

So, denser the cytoplasm, higher is the probability for transformation. Moreover vacuoles contain enzymes which can rapidly degrade the extracellular DNA. Hence, it is preferred that you use younger cells in comparison to matured cells. In literature, you will find all kinds of *in vitro* cultures can be transformed - callus, suspension cells, protoplasts, tissue, whole organ, entire plant. So there is a wide range.

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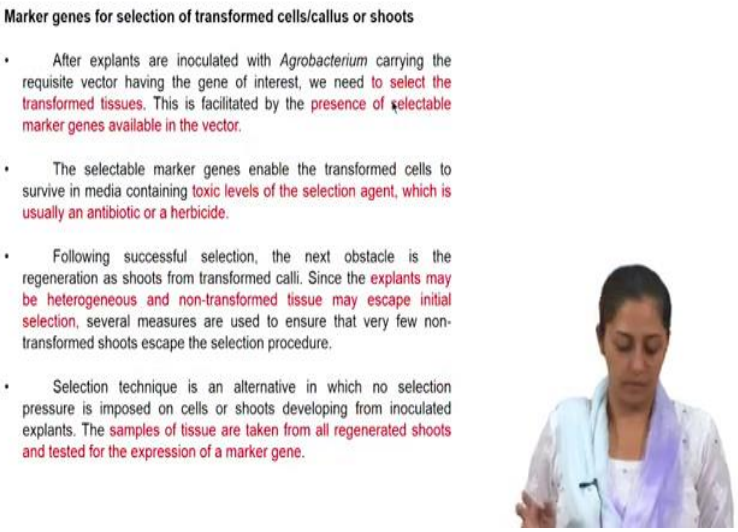

Now, after explants have been co-cultured with *Agrobacterium*, how to detect which cells are successfully transformed? We have already read that it is based on the selection marker or the

marker system present - whether it is histochemical assays that are used or antibiotic selection medium or herbicide selection medium are used to increase the probability that you will end up in successfully transformed cells with subsequent subculturing.

Apart from the marker or the selective agent, *Agrobacterium* growth inhibiting compounds, like carbenicillin to which *Agrobacterium* are sensitive is also used. When is this used or when should it be used? For transformation, what are you doing? You are bringing the *Agrobacterium* in close contact with the cells, but ultimately what you want is transformed plant cells. You cannot keep growing *Agrobacterium* because it will over grow which will subsequently lead to loss in the viability of the cells and the growth.

So, after successful transformation, from successive cultures *Agrobacterium* has to be removed. So, after 48 hours - generally 48 hours is the timeline for exposure with the *Agrobacterium* and then it is kept under high concentration of carbenicillin or any antibiotic to which *Agrobacterium* is sensitive. Subsequently with reduced concentration of the antibiotic you keep subculturing and after subsequent subcultures the antibiotic is removed from the medium. This is because the antibiotic itself can be inhibitory to the plant cell growth which you would not like to have for the transformed cells. In general for transformed cells - because we have genetically manipulated them their growth rates are compromised. So, the selection marker systems - the toxic levels. So the toxic level of the selection agent has to be optimized - it varies with species.

So, it is species specific - the time of exposure is species specific, the antibiotic concentration is species specific, so all this has to be optimized.

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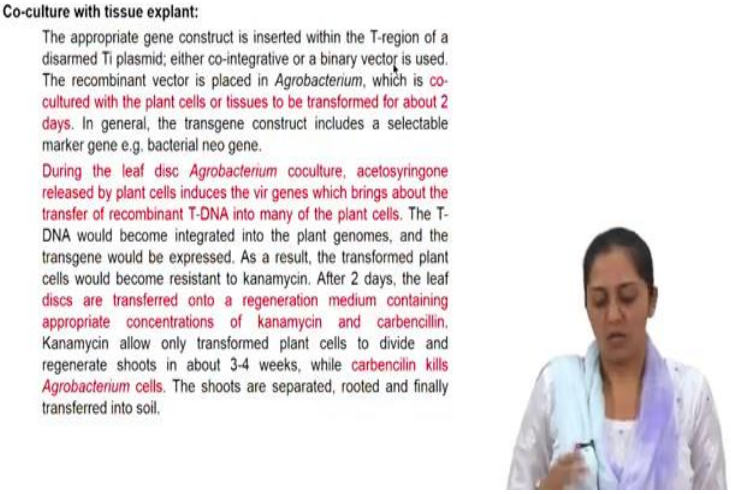

So, this is the entire protocol you can go through. This is for your reference - the theoretical protocol of how it is done. So, what is done is you choose an appropriate explant, then you surface sterilize the explants and you grow the *Agrobacterium* in suspension for 24 hours. You look for the growth phase because then the *Agrobacterium* cultures are active. Generally log phase cultures are preferred.

Once the *Agrobacterium* has grown - there are different strains of *Agrobacterium* which are present and which are virulent for infecting the plant cells, but it is highly dependent on the species. So, if one *Agrobacterium*, for example, *Agrobacterium* LBA 4404 or 9402 - these are different strains which are known to be highly virulent. Note that it is not necessary that because it has worked with one plant species, it will work equally with the other.

So, you need to choose the *Agrobacterium* strain and grow it till log phase. Even for exposing the cells you can use different techniques. You either create a wound in the explant and dip it into the suspension. Now, the time of exposure also varies. You cannot keep it for more than 10 to 15 minutes.

After keeping it exposed for 10 to 15 minutes, you need to wash it so that excess growth of *Agrobacterium* will not happen when you are keeping it in the incubation for 48 hours. So, you need to vary the exposure time, you can vary the *Agrobacterium* concentration - the cell suspension concentration. You can have different ODs when you are using the culture to co-cultivate with the plant cells.

Then you can also vary the type of explant. Then I was talking about the wounding - wounding itself can be created through different ways. So, people have found that using a syringe filled up with the *Agrobacterium* suspension, you make injuries on the midrib of the leaf explants and that has given rise to increased efficiency in transformation.

Then people use a simple dip method, where they wound the explant by a blade or by creating holes in the leaves or explants and then expose it. So, there are different factors which need to be optimized to get a successfully transformed culture.

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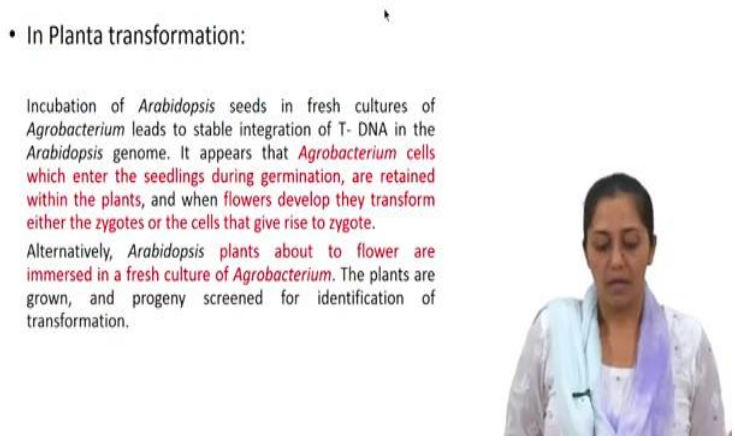

Then in planta transformation. It generally uses the floral dip method in which the flower is dipped in the *Agrobacterium* suspension, and then kept for incubation for some time so that it resides - even the seeds themselves can be surface sterilized and dipped in the suspension so that the *Agrobacterium* can transform the seeds. Once they regenerate or when the seedling comes out, they infect the tissues - the aerial parts or the ground part regions.

Or when the flowers are dipped with *Agrobacterium* or brought in contact with the *Agrobacterium* just before pollination, during pollination or after pollination the *Agrobacterium* present inside may infect the zygote or may infect the germ cells which are going to get involved in the zygote formation, thereby leading to progenies which are transformed.

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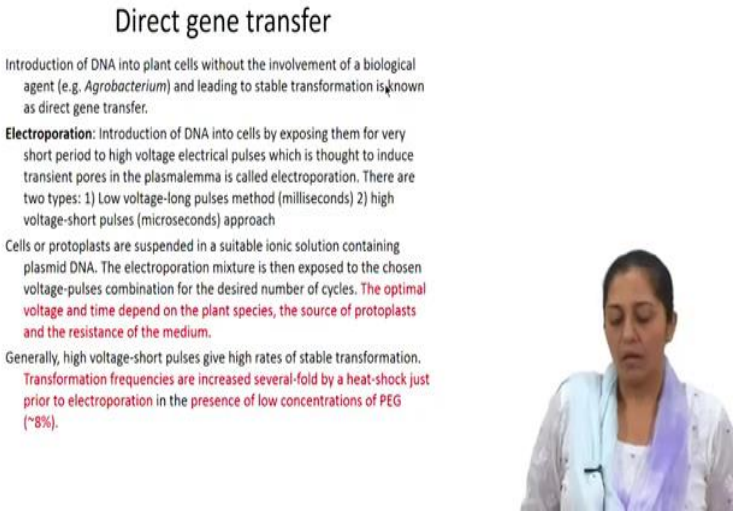

Now, talking about direct gene transfer methods. Electroporation is a well known technique which is used even for bacterial transformations. What are the two different types of electroporation which are frequently used? These are low voltage long pulse methods - time duration is increased, but lower frequencies are used and High voltage short pulse methods. Generally high voltage short pulse methods are found to work better than the low voltage long pulse methods because the damage to the DNA is lesser in that case.

So what can be optimized - the voltage which we are using, the time for which it is being applied. Then transformation frequencies are increased by several folds by using certain techniques like heat shock or PEG. Why do you think it helps? Have you heard about competent cells? How are they made competent? Competent cells means they become more susceptible to uptake of the extracellular DNA. How are they made susceptible?

Any divalent ion, even magnesium, can be used. How is it made susceptible? It is done by increasing the proximity between the extracellular DNA and reducing the repulsion - because both are negatively charged. Now, what about PEG, what does it do?

Student: Facilitates easy uptake

What is it actually doing, everything is done to facilitate easy uptake. Polyethylene glycol is also very frequently used.

Student: Surface tension removal

DMSO, polyethylene glycol.

Student: It reduces the surface tension and makes the membrane more permeable.

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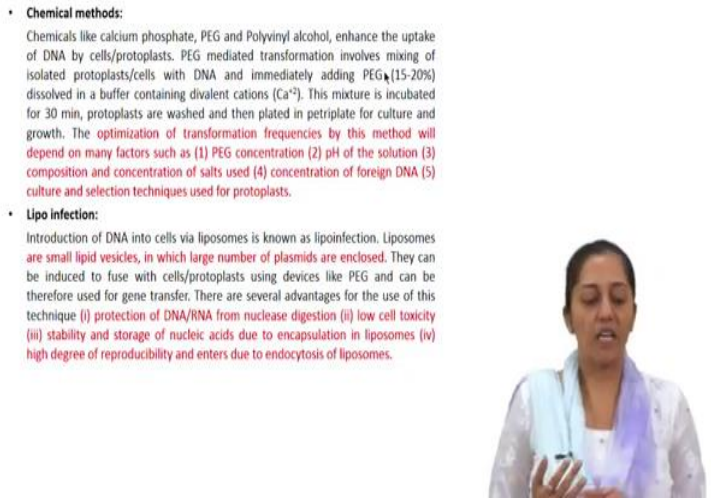

So what is done is in chemical methods? As we were talking about, the cells are first made competent. Divalent ions can be used to reduce the charge repulsion so as to increase the proximity between the two DNA. The other thing is to manipulate pH, add polyethylene glycol in the medium and the concentration of the DNA is also manipulated to enhance the efficiency of the transformation.

Now, some of these methods - you need to go back and check, what they do is, they will try to condense the DNA or concentrate the DNA. Concentrating the DNA and reducing the proximity between the extracellular DNA and the plant cell membrane would increase the probability of success.

Now, lipoinfection. The extracellular uptake of the DNA can be improved by encapsulating them in the form of liposomes. Liposome intake is a well known phenomena in cells. Liposomes not only will increase the uptake of the DNA, but they will also protect the DNA from the nucleases - so that way also it helps. Now, what are liposomes? I hope you know what liposomes are. They will have both hydrophilic hydrophobic ends.

Student: They have a double layer

Like phospholipid bilayer. And they will encapsulate the DNA within. They can be induced to fuse with the cells or the protoplast using PEG and can therefore be used for gene transfer. Then there are several advantages. What are the different advantages with lipoinfection? Low cell toxicity, stability and storage of nucleic acid can be improved by encapsulation, high degree of reproducibility and endocytosis is a phenomena already present in the cell, so that can be easily taken up by the cells.

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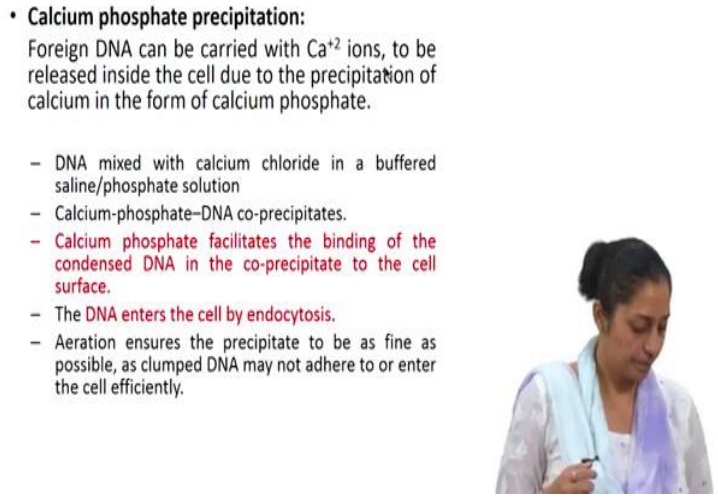

Now, calcium phosphate precipitation is another method. It condenses and precipitates the DNA - because it is condensed, it brings the cell much closer to the cell membrane, thereby increasing the probability of uptake of the extracellular DNA.

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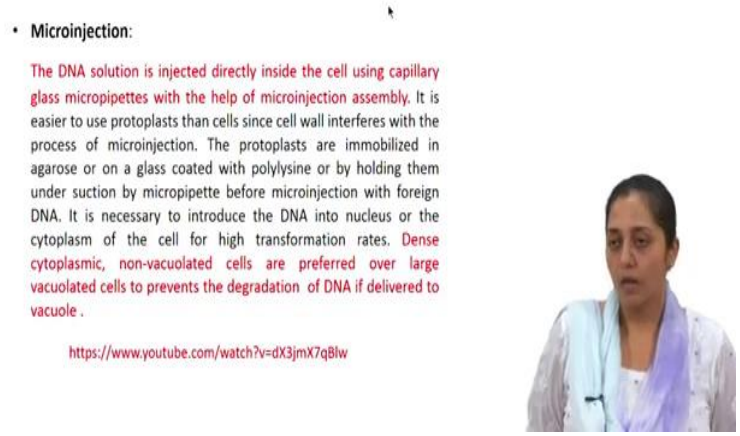

Microinjection - this is direct gene transfer in which a microinjection assembly is used. It is not that easy. Under the microscope you hold the cell by freezing it onto a slide, using polylysine. Polylysine, because of the charges present it will try to stick it at one place and then suction pressure is created such that it does not move. Then through that microinjection assembly, the DNA is tried to either be put in through the cell membrane into the cytoplasm or directly into the nucleus of the plant cell.

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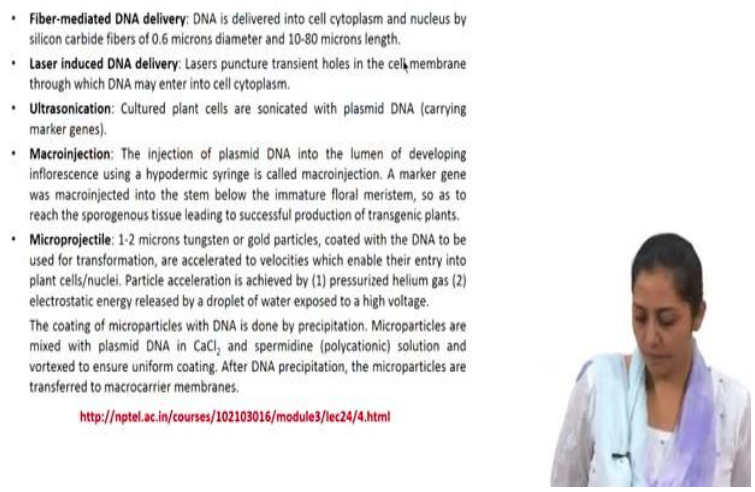

Now, what other direct methods can be used? In literature, you will see that people have used fiber mediated DNA delivery in which very small size nano fibers are used - silicon fibers are used with high speed, so that they can impregnate the plasma membrane and the cell wall and the DNA is delivered into the cytoplasm. Then laser induced DNA delivery is also reported. Ultrasonification is another method. Then macro injection is the hypodermic syringe which we were talking about, which can be directly injected into the cytoplasm or into the nucleus.

Then micro projectile - which is your gene gun method. In this, at a very high speed it can be delivered to the plant cells or the explants kept under an inert atmosphere with helium gas or using water vapor - using the electrical charge under high voltage, high speed is created, and it carries the extracellular DNA coated with the inert material like tungsten or gold nanoparticles. This can be injected under high speed directly into the explants.

I stop here. And in the next class we will begin with the plant cell bioreactors which is the last part of the course.