Course Name: Basics of Crop Breeding and Plant Biotechnology

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Lecture-40: Preparation of Gene Silencing Construct

Hello everybody. Welcome to SWAYAM NPTEL course on Basic of Crop Breeding and Plant Biotechnology. Today we will start preparation of gene constructs and promoter reporter constructs. We will be mostly discussing on gene silencing construct preparation and promoter analysis, as well as the construct preparation for promoter characterization. So, these are the concepts which will be covered under this topic. Firstly, we will discuss the gene silencing or hp-RNA construct preparation. hp-RNA stands for hairpin RNA construct preparation. Then, we will be discussing the unidirectional and bidirectional promoters. Then, the promoter reporter gene fusion construct preparation will be discussed and finally, we will discuss some numerical questions on promoter analysis. So, let us start our discussion on preparation of gene silencing construct. Ok!

To tell about these things, first, we need to think about the eukaryotic gene sequence. In our last class, we have mentioned about two types of gene cloning. One type of gene in which no intron is there, those genes could be cloned from genomic DNA. While if within a gene intron is available, then we need to clone the coding DNA sequence region from the cDNA. The cDNA will be prepared from the mRNA by reverse transcriptase enzyme. Ok!

So, today, we will start the gene silencing construct preparation. So, before discussing the construct part, first, we need to discuss the process through which gene silencing through hairpin RNA mediated construct works. Ok! Suppose, a plant cell is available. So, within that plant cell, the chromosomes are available within the nucleus right?

Different chromosomes are available, chromosome 1, 2, 3, 4, whatever, those are available within the nucleus.

And within the nucleus what is taken place, the transcription is taken place, within the nucleus in eukaryotic cell. And once the transcription is completed, different steps are there in transcription, earlier we have discussed the initiation of transcription, then elongation, then termination. So, after those process, the pre mRNA is formed from the nucleus. So, through transcription, first pre-mRNA is formed, thereafter, different processing is taken place and mature mRNA is formed. In mature mRNA, no introns are there, the intron part has been spliced and once the mature mRNA is available, it will come to the cytosol, the cytoplasm.

So, let us assume, for a particular gene these are mature mRNA of that particular gene. So, the translation is taken place in cytoplasm in eukaryotic cell. So, using those mRNA basically, the translation will be started and through translation the protein production will be taken place. Now, if you have to silence a particular gene using hp-RNA construct; 'hp' stands for hairpin RNA or this mechanism is also known as RNAi, RNA interference. Ok! So, according to this mechanism what happens ok, before describing this mechanism let me tell you two different things.

First of all RNAs, especially mRNAs are mostly single stranded in nature within the system, within a cell it may develop different small secondary structures for a short period of time. Ok! And the RNAs are not too much stable. So, they are produced once the translation is done, then eventually, they are degraded. Ok! So, they are present within a cell for a shorter period of time for a smaller period of time and different lengths are available for mRNA, some of the mRNA might be up to 400, 500 bases long, some mRNA, very less number of mRNA might be more than 5 kb or close to 10 kb. Ok! So, different sized mRNAs are available within the cell those are transcribed from different genes gene A, B, C different genes are there, all of them will produce mRNA and once the mRNA will be processed properly, it will come to the cytosol for subsequent translation.

Now, those mRNA could be silenced, now if you somehow silence the mRNA or if you somehow degrade the mRNA or block the mRNA then what will happen, the translation process will be affected. Suppose in a normal cell suppose 100 mRNA copies are available while in a silenced cell, somehow, we have silenced the mRNA number, we have reduced the mRNA number, suppose there approximately 5 mRNA of a particular gene is available. So, definitely in the normal condition, protein production will be more while over herefrom that particular gene, the protein production will be retarded, will be reduced. So, in this way, once the mRNA level is reduced then the protein production will be reduced and eventually the function of the gene could be observed or could be detected that suppose, a particular gene is responsible for flower colour formation. Somehow, if those genes which are responsible for flower colour formation like some gene associated in anthocyanin pathway, if somehow, we silence those genes through RNA strategy or some other strategy, then initially its mRNA level will be reduced and once the mRNA level will be reduced, then sufficient protein will not be produced. So, we may see some pale color in the flower that may occur.

So, two strategies could be taken place, could be taken for RNA mediated gene silencing. Initially scientist opted for antisense RNA technology, initially, scientist worked on antisense RNA technology thereafter, the hp-RNA technology came. So, according to antisense RNA technology what we have to do, suppose, it is a gene sequence from 5′ to 3′, over here, the sequence was ATG CCC …… finally, TAA, this sequence was available on a particular strand and its complementary strand is as such their sequence is TAC GGG ATT. So, once the transcription will be taken place, first this two strand will be separated from the promoter region, in the promoter region, RNA polymerase will bind, it may interact with other different transcription factors and finally, it will initiate the transcription process.

And if you recall the transcription what we have discussed earlier, then out of this two strands, one strand is known as coding strand or the DNA, another strand is known as template strand. So, using this particular template strand, basically, the mRNA will be formed. So, our mRNA sequence from here will be AUG CCC UAA basically, it will be

complementary to this strand and it will be similar to the coding strand, just one change will be there in-spite of T there will be U residues. So, this is our sense RNA or sense mRNA. So, we know that RNA is available as single stranded condition within a cell, mRNA is mostly available as single stranded condition.

Now, initial strategy came, according to that strategy, scientist tried to synthesize the antisense RNA. Suppose over here, the transcription is supposed to start from here, if we have to use antisense RNA technology, you have to put a promoter at this end and you have to start transcription from here, you have to start transcription from here. So, if you start transcription from here from this particular end, then this strand will be used as a template strand. So, let us try to make the antisense RNA what will be produced from this particular gene sequence if transcription is taken place from this direction? Ok! So, this will be the antisense, antisense mRNA. Ok!

So, let us try to find out what will be the sequence, its sequence will be UUA GGG then CAU. Ok! This is the 5′ to 3′ end and in the sense strand, it was the 5′ to 3′ end because once the transcription will be started from here, you guys can just flip the sequence. Ok! If we flip the sequence what will be this strand? This strand will be TTA then we had GGG and we had CAT right, in this way, one strand of the DNA will be there and the opposite strand will be AAT CCC GTA. Ok! This one the 5′ to 3′ strand, this one will be 3′ to 5′ strand and we started transcription from here, I am just showing this antisense RNA development. Ok! If you have to put the sequence in this way, so that we can prepare the antisense RNA.

So, once the transcription will be started from here, our mRNA sequence will be UUA what I have written here then it will be GGG then it will be CAU. Ok! So, in this way, the antisense RNA could be made by putting the inverted sequence within a particular construct. Already, you have learned about the over expression construct preparation. So, in-spite of over expressing a particular gene we can synthesize the antisense RNA of that gene using this technology just we have to reverse the gene, we have to flip the gene in opposite orientation under a particular promoter. Then we can synthesize this type of

antisense RNA.

Now, within a cell, if sense as well as antisense RNA are available, at the same time then if you see carefully the sense sequence was AUG CCC …. UAA this is the 5′ to 3′ end. While in case of antisense mRNA, if we see we have to start from the 5′ to 3′ because 5′ 5′ pairing will not be there. So, let us start from here, will be having UUA … then GGG and CAU the 3′ end this is the sense RNA, this is the antisense RNA. So, this RNA will pair and once it will pair, suppose within this cell, this particular cell, where the antisense RNA has been formed. So, those mRNA which were initially available it will form pairing with the antisense mRNA and if double stranded mRNA will be there, the translation process will be inhibited because mRNA will do the translation once it is in the single stranded condition.

If double stranded structure formation is taken place, the translation process will be inhibited. So, then, our protein production will be blocked, protein production will be reduced. Ok! In this way, the antisense RNA technology works. Now, we will discuss how the hairpin RNA construct could be prepared. So, for hairpin RNA construct preparation, already we have discussed earlier, a gene sequence should be placed in forward and reverse orientation flanking a linker.

If it is our gene sequence, let us think that our sequence was this one, it was starting with AT like this and it was ending with A. Ok! In the opposite strand, it was TA and it was finishing with T. Let us assume, this one was our gene sequence this is the 5′ to 3′ end and this is the 3′ to 5′ end. So, if we have to make the RNAi construct or RNA interference construct, we need the inverted sequence of some part of the gene. Generally, for RNAi construct preparation, the full-length gene is not needed, but we need a cDNA sequence of at least 300 bp; 300 to 350 bp at least is needed.

Then, we need some linker DNA sequence also. So, what is linker DNA? Linker DNA is some unspecific DNA, non-specific DNA. Suppose, we are planning to do plant transformation in rice or we are planning to do plant transformation in tobacco. So, as a

linker we have to put those DNA sequences where no sequence match will be available in rice or tobacco plants. May be some viral DNA sequence we can put over here, some bacterial DNA sequence we can put in the linker region, why, I will mention later on.

Then what should be the size of the linker DNA? It has been found that 300 to 700 bp was found to be optimum for this linker length. Thereafter, we have to put the gene in reverse orientation. In reverse orientation means this 5′ end will go over here. So, we should start AT from here …. finally, will end at A. While, in this strand will be having TA at the end will be having T.

So, this type of things are generally placed in hp-RNA or RNAi construct. Now, once we have to prepare the RNAi construct, in most of the laboratory, they try to put the construct in this orientation. First, they will put a promoter, then the construct will be placed initially in reverse orientation, that is this part. Initially, the construct will be placed in reverse orientation then, we have to put the linker region and then we have to put the construct in forward orientation i.e., in this orientation. Then thereafter, we have to put a terminator region. Ok!

So, this is gene part in reverse orientation, this is the gene part in forward orientation and in between we have written the linker DNA. Ok! So, what type of promoter will be used in this type of construct preparation. Ok! So, suppose we would like to silence a particular gene through RNA interference and that gene is active in the leaf tissue, in the green tissue. So, we have to use that tissue specific promoter. So, that our hp-RNA construct will be active in those tissues only.

Suppose we have to silence a particular gene which is active in the root tissue. So, we need to use root tissue specific promoter. So, in this way, we need to select a particular promoter. Thereafter, let us talk about the silencing part, the gene will be initially placed in reverse orientation. Why? If the gene is placed in forward orientation as in most of the cases we can put the gene part from the coding DNA sequence.

If the start codon is there, then after end of transcription some mRNA will be formed and from that mRNA, some non-specific protein might be generated if it is available within a frame. Ok! So, to get rid of that particular, those issues, we need to put initially gene in reverse orientation, then linker, then the gene part will be kept in forward orientation. So, once this type of thing is available, then our chances of success will be more through this concept ok, and another thing that is very important in RNAi construct preparation, we need to think about the off-target effect. What is that? I am telling it later on. So, after the antisense part, we have to put the terminator.

So, initially the pre mRNA will be formed, and once this mRNA will be formed, if you try to draw a sequence by yourself using this particular thing what I have drawn earlier then eventually in the system, it will make a hairpin loop like structure. Ok! In this hairpin loop like structure, this is one part of the gene that is in the reverse orientation then, this is another part of the gene that is placed in forward orientation and this one is the linker part. So, hence it has been found that the optimum size of the linker is in between 300 to 700 bp because if 300 to 700 bp linker is available, then proper folding could be formed for this hairpin. Hence, the name is hairpin RNA because hairpin shaped RNA will be formed over here and here, the pairing will be taken place between the reverse orientation and the forward orientation of the gene. So, now in eukaryotic system, once such type of double stranded RNA will be available, then a group of protein known as Dicer or DCL homolog, they will attack this double stranded RNA structure and they will cleave it into small double stranded region of around 18 to 24 nucleotide long. Ok!

In this way, the small double stranded region will be generated from this double stranded RNA throughout the part which has been used in the gene construct. So, now let us assume, a cell is available there the hp-RNA, sorry, hp-RNA construct has been used. So, the native mRNA is there and such type of small double stranded RNA, those are known as siRNA, small interfering RNA those are also available within the gene, within this particular cell if this cell is infected with hp-RNA. So, eventually what this si-RNA duplex will do, they will be single stranded in nature and one strand will bind on the mRNA sequence based on the sequence complementarity. So, if those thing occurs again,

double stranded RNA will be formed within the cell and Dicer will cleave it.

Once the Dicer will cleave it, the endogenous RNA or endogenous mRNA level will be reduced because the endogenous mRNA is being degraded by this gene- silencing construct, siRNA formation and Dicer mediated cleavage. So, eventually we can see the reduction in protein production also. So, in this way, we can prepare gene silencing construct, either initially antisense technology was done in some cases like in case of FLAVR-SAVR, you can see the antisense RNA technology was highly popular and later on, hp-RNA construct was famous in different crops for gene silencing for viral resistance this type of hp-RNA constructs have been used.