



Genetic Engineering and Increasing Resistance of Plants to chilling by Creating Mutants Bacteria Lacking the Ice Nucleation

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Abstract. Ice nucleation active bacteria are important in damages which are caused by chilling and freezing in plants that are sensitive to cold. Every treatment which leads to a reduction in the number of ice-causing nucleation bacteria or activity of bacteria ice-causing nucleation decreases the amount of frost damage in plants. One of these ways is a change in genetic structure of coding inaz protein so that, this protein isn't synthesized in bacteria or is made imperfectly and therefore, ice nucleation formation and freezing is delayed in plant. The Purpose of the present study is recognition of INA isolates of Pseudomonas Putida bacteria and creating INA mutants. For this study, plant samples were gathered from almond trees which showed symptoms of freezing in spring. After separating and recognition of bacteria strains from plant tissue the range of ice nucleation activity was determined by droplet freezing method. Using polymer chain reaction (PCR) the presence or absence of INA gene in bacteria isolates was studied and detected. Production of PCR was done and by Electrophoresis Erwinia herbicola epiphyte strain Vector of colonized gene of inaz was used as positive control. For creating mutation in p.putida strain containing nucleation activity, electroporation method and for creating mutation in ice gene, Tn transposon was used. Mutations got resistant to kanamycin and were chosen with selective medium containing Kanamycin. Studying the effect of mutations on cucumber planting showed that some mutations didn't show freezing symptoms and planting remained normal, whereas some of isolations and control provided positive freezing evidence

Keyword: Genetic Engineering, mutant bacteria INA, freezing, chilling, plant

1. INTRODUCTION

Ice nucleation formed by heterogeneous method in which ice is induced by an external factor such as bacteria, fungus or a special chemical combination can decrease temperature of ice nucleation forming to -2°C in organic and inorganic materials. Freezing is an important noncontagious disease in plants which destroys high percentage of agricultural products across the world every year. In many plant tissues which are sensitive to freezing, water can become very cold to temperature of -12°C without freezing in inter- or intracellular liquid [1]. Ice nucleation active bacteria (INA) have an important role in damage caused by freezing in plants that are sensitive to cold. Maki et al. recognized INA^+ phenotype in types of gram negative bacteria including pseudomonas, Erwinia, and Xanthomonas in 1987 [2]. All INA^+ bacteria are epiphytic and are nearly found on all plants. Frost damage in plants depends directly on logarithm of these bacteria population and logarithm of bacterial ice core number on plants at freezing time. In the presence of INA^+ bacteria, freezing may occur at temperature about -1.2°C [3]. Burke and Lindow believed that each treatment which leads to the decrease in ice nucleation bacteria number or bacteria ice nucleation activity, decreases freezing damage in plants. One of the ways is to change the genetic structure of gene encoding INAZ protein so that this protein is not synthesized in bacterium or it is made defectively. As a result of this change ice nucleation formation and freezing will be postponed, which will subsequently lead to

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increased growth season [4]. The purpose of this study is recognition of INA⁺ isolates of *P. putida* bacteria in almond plant and making INA⁻ mutants.

2. MATERIALS AND METHODS

2.1. Isolation and recognition of bacteria

Plant samples were collected from almond trees which were showing freezing symptoms in Taft city of Yazd in spring. To isolate bacteria from plant tissues, tissues including leaves and the bark of young frosted stems with diameter of 0.5 to 1 centimeter were segmented and dipped in Erlenmeyer containing distilled water. The container of samples was put on the shaker with low round of 200 rpm and after 15 minutes, plant samples were exited from Erlenmeyer after which 1-2 drops of suspension were pervaded on the nutrient agar medium containing sucrose (NAS). After a day, from brown colonies, colored ones suspected to *Pseudomonas* were chosen and some of them were re-cultured for purification on the NAS; biochemical and physiological characteristics were used in order to recognize *P. putida* strains [5]. Determining the amount of ice nucleation activity was done by droplet freezing method and sterile distilled water was considered as a negative control.

2.2. Polymerase chain reaction for detection of INA gene

It was used for checking presence or absence of INA gene in bacterial isolates with sequences of:

5' ATCCAGTCATCGTCCTCGTC 3' 3' CAAGTGTCACGTTACCGGTG 5'

Bacterial strain *Erwinia herbicola* containing inaz colony gene was used as positive control. PCP product electroforz was done on % 1 Agarose gel and with flow intensity of 30, mili Amper.

2.3. Preparation of bacterial cells for Electroporation:

Bacterial isolates were cultured in LB medium (Tipton 10g, yeast extract 3g, NaCl 10g, distilled water 1 liter) for one night. After reaching logarithmic growth phase, bacterial cells were settled with centrifuges and cell sediments were washed with % 10 sucrose for 3 times and after final centrifuges, they were solved in % 10 glycerol.

2.4. Electroporation

For mutagenesis of *P. putida* isolates containing ice nucleation activity electroporation were used in voltage of 1500 with distance of 1mm between 2 electrodes and with 100 ml volume and time pulse of 2.5 milliseconds. For mutagenesis in ice gene Tn₅ transposons was used. Plasmid was used in the selection of mutants. Finally, 22 mutants were obtained. Mutants got resistant to Kanamycin and were chosen on the selective medium containing Kanamycin.

2.5. Investigation and observation of the effects of mutants on cucumber plantings

Proper density was prepared from mutant bacteria suspension in sterile distilled water and was sprayed on cucumber plantings which had 2-5 leaves. Plantings were put in temperature of 4°C for 10 hours. Then plantings were put in vitro for 1 hour in order to dry their surface, after which the effect of treatment was evaluated. Sterile distilled water was used as negative control and mutant bacteria suspension was used as positive control. It was observed that 5 isolates of mutant didn't freeze and plantings got dark and brown completely.

3. DISCUSSION AND CONCLUSION

After investigating the effect of mutants on cucumber plantings, finally, 5 isolates were obtained which showed freezing percentage of 10-15% in droplet freezing test while they didn't lead to freezing in plantings with 2-5 leaves. Hirano et al state that ice nucleation bacteria activity is less in higher temperature and all bacteria don't show ice nucleation activity in ideal situation and even different strains of one type of bacteria are dissimilar in ability to produce ice nucleation [6]. In the present study, in the droplet freezing test, ice nucleation activity was different for *P. putida* various isolates. It could be probably due to difference of strains from each other, ice nucleation inactivity in all cells of a strain on equal terms, difference in the number of ice nucleation active bacteria, and lack of the same conditions for all of the isolates. *Castrieeousedinaz* primer pair for detection of *ina* gene in *P. putida*, *P. fluorescens* and *P. syringae*. The size of propagated pieces by this primer pair was more than 4500bp [7].

In present study, the size of propagated sequence by this primer pair was 4500 for *P. putida* isolates and *E. herbicola* carrying *ina* gene. Wolber studies in 2002 show that ice nucleation genes in different types have a little difference but their overall structure is similar [8].

So, probably, difference in the size of bands between *P. Putida* and *E. herbicola* could be justified with a little difference between *INAZ* and *ICE*. However, it is necessary that more detailed examinations are done on different isolates of two bacteria simultaneously. In addition, for more accurate examination it is necessary to evaluate isolates at the molecular level.

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