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INA GENE INACTIVATION IN ISOLATED STRAINS FROM FROZEN LEAVES AND ITS EFFECTS ON PLANT FREEZING

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ABSTRACT. Freezing is a major environmental stress, which limits plant's distribution, growth and productivity. Ice nucleation active bacteria can catalyze ice formation at temperatures as high as -2°C. A membrane protein confer the ability of ice nucleation, called ice-nucleating proteins (INPs), which is encoded by a single gene. Mutation in this gene will lead to delaying of ice nucleation. In this study, leaf tissues of several plants with freezing symptoms were collected from different locations and 40 bacterial isolates with yellow circular colonies and regular margins were isolated from samples. Finally, total of 12 isolates belong to *Xanthomonas* were selected for ice nucleate activity (INA) by Droplet-freezing test and presence of INA gene was surveyed by PCR. According to the obtained results, isolate 28 was targeted to mutagenesis by using Tn5 transposon. After mutagenesis, isolates with ability to grow on kanamycin, which lack of INAx gene in PCR were considered as mutated isolates and their freezing effects were evaluated on bean seedlings. Results showed that isolates with mutated INA gene cannot induce freezing on bean seedlings, while primary identified isolate (isolate 28) could do it. These results show that if we could replace wild type ice nucleation active bacteria with mutated forms (just different in ice nucleation activity), we could, probably, prevent freezing and subsequent economic losses.

Key words: freezing stress; ice nucleating protein; mutagenesis; transposon; *Xanthomonas*.

INTRODUCTION

Freezing is a major environmental stress which limits plant's distribution, growth and productivity (Kazemi Shahandashti *et al.,* 2013). Annually, chilling and

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freezing cause significant crop losses all over the world (Xin and Browse, 2000). Plants avoid freezing damage by supercooling to temperatures as low as -40°C. Because of absence of nucleating substances, supercooling occurs in some cells and tissues (Burke *et al.,* 1976). However, ice nucleation active (INA) bacteria can catalyze ice formation at temperatures as high as -2°C. The presence of these bacteria on leaf surfaces can alter (i.e., destroy) leaf habitats at subzero temperatures (reviewed by Hirano and Upper, 2000). Therefore, INA have a significant role in freezing derived injuries in cold sensitive plants (Keikhasaber *et al.,* 2007). The bacterial ice nucleation phenomenon was first observed in strains of *Pseudomonas syringae* (Lindow, 1983a). Shortly after, it was proved that some other strains, such as *Erwinia herbicola*, *Pseudomonas fluorescens*, *Pseudomonas viridiflava* and *Xanthomonas campestris* have ability to catalyze ice formation in supercooled water (discussed in Gurian-Sherman and Lindow, 1993).

Subsequent analysis of ice nucleate bacteria revealed that there is a particular membrane protein, called ice-nucleating protein (INP), which has ability to act as a nucleation site. This property is conferred by a single gene encoding for this membrane protein that acts as a template for the arrangement of water molecules in crystals (Jolya *et al.,* 2013). These nucleation sites allow water molecules to become particularly aligned in order to promote freezing. The increase in the number of nucleation sites led to promotion of freezing at higher temperatures (Li *et al.,* 1997).

In the absence of heterogeneous ice nuclei, water associated with leaves will be supercooled. Supercooling in the temperature ranges of 0 to roughly -5°C is primarily limited by the presence of INA bacteria. Therefore, INA bacteria are responsible for ice formation, and hence cause some injuries in plants, mainly in the range from 0 to -5°C (Hirano and Upper, 2000). This line of reasoning led to development of recombinant ice-strains of *P. syringae* and *P. fluorescens* by deleting a roughly 1 to 1.5 kb fragment of the ice gene (Lindow, 1995). These strains of bacteria, which had a mutation in this particular gene, showed lower freezing temperature of water droplets on the surface of plants. Although ice-strains were effective in preventing or minimizing colonization of INA bacteria on plants, no elimination observed in established populations of the target microbes (Hirano and Upper, 2000). Manipulation of INA bacterial population sizes by application of non-INA strains is promising strategy to reduce frost injury of plants. Therefore, if INA protein is not synthesized in bacteria or synthesized incompletely, it would lead to delay in freezing and formation of ice nucleus and as a result, freezing consequences will be decreased (Burke and Lindow, 1990). The objectives of this study were to isolate INA strains from frozen leafs of some plants, expose them to

mutagen and afterward, evaluate their ability to form ice nucleus.

MATERIALS AND METHODS

Isolation and identification of bacteria

Periwinkle (*Vinca minor*), pistachio (*Pistachio nuts*), Alnus (*Alnus serrulata*) and primrose (*Primula vulgaris*) plants samples with frozen symptoms were collected from Sari, Ghaemshahr, Babol, Babolsar and Amol, in Iran. Plant tissues, such as leaves and branch skins, were cut to 0.5-1 cm segments and placed into Petri dishes contain 1ml distilled water and shaked for 15-20 minutes. After that, 1-2 drops of the suspension spread on nutrient agar medium with 3% sugar and incubated at 27°C. After 2 days, single yellow circular colonies with regular margins and glazed were transferred to new medium for subculture. Identification of *Xanthomonas* isolates was done through their biochemical and physiological characteristics (Schaad *et al.,* 1988).

Hypersensitivity test

A suspension at concentration of 108 to 109 cfu/ml was prepared from 24 h cultured isolates and injected on the back side of Pelargonium (*Pelargonium cucullatum*) leaves at several points. Results were evaluated after 24-48 hrs (Schaad *et al.,* 1988).

Determinative tests

A series of determinative tests, including gram, oxidase production, urease production, hydrogen sulfide production, esculin hydrolysis, oxidative/fermentative (O/F), levan production and solubility in ethanol/methanol tests were done to phenotypic characteristic *Xanthomonas* isolates (Schaad *et al.,* 1988).

Droplet-freezing method

Ice nucleation activity levels measured by droplet freezing method as previously described (Lee *et al.,* 1995). Sterile-distilled water was used as negative control and ice nucleation process was assayed at -5 to -15°C.

Polymerase chain reaction

To detect of Ice nucleation active (INA) gene in isolates polymerase chain reaction was carried out using two 20 nucleotides specific primers (forward INAx: 5'- GCCTGGGAAATACTCCGATT-3' and reverse INAx: 5'-CGGTTTCCAGAATTTGCATT-3'). Genomic DNA were isolated by using alkalin method. PCR amplifications were performed in a total volume of 25 µl, containing 1X PCR buffer, 15 ng of genomic DNA, 0.5 mM of each primer, 0.2 mM dNTP, 2.5 mM MgCl 2 and 1 units of Taq DNA polymerase. The thermal cycling conditions are summarized as 5 min at 93°C, followed by 35 cycles of denaturation at 93°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 45 s and then a final extension at 72°C for 6 min. Electrophoresis was performed on 1% agarose gel at 30 watt constant power and stained by ethidium bromide. After amplification of INAx gene, PCR products were sequenced and results checked with NCBI.

Preparation of bacteria for electroporation

Bacterial isolates were cultured in STOLP medium (10 g pepton, 3 g yeast extract, 5 g NaCl prepared in 1 liter distilled water) for overnight. After reaching to the logarithmic growth, bacterial cells were precipitated by centrifugation and sediments were washed

in sucrose 10% for three times. The cells were dissolved in glycerol 10% after final centrifugation.

Electroporation

Mutation in *Xanthomonas* isolates, containing ice nucleating activity, was done by using electroporation method at 1800 v, 1mm distance between electrodes, 100 µl cell volume and 2.5 ms pulse time. Tn5 transposon used for ice gene mutation. At first, plasmid pSUP2021 contain Tn5 transposon were extracted from overnight *E. coli* S17-1 culture by previously described method (Currier and Nester, 1976). Tn5 transposon in (*E. coli* S17-1 pSUP2021) pSUP2021 plasmid had kanamycin resistant gene that used for selection of mutants. Selective medium (NAS+ kanamycin) used for selection of the resistant mutants. Droplet-freezing test was used to evaluate the ice nucleation activity of mutants.

Evaluation of mutant effects on bean seedlings

A concentration of mutant bacteria suspension (about 108 cfu, OD 600: 0.2) in sterile distilled water was sprayed over leaves of bean (*Phaseolus vulgaris*) seedlings. Seedlings were exposed to -10°C for 4 to 7 hrs. The seedlings were placed at laboratory environment for drying, before evaluating the treatments. Sterile distilled water and bacteria with no mutation were used as negative and positive control, respectively (Keikhasaber *et al.,* 2007).

RESULTS AND DISCUSSION

After 48-72 hrs culturing of suspension, 40 bacterial isolates with mentioned colony's charactristics were identified (*Table 1 and Fig. 1*). Finally, after purification and physiological and biochemical tests, 12 isolates belong to *Xanthomonas* were selected for droplet-freezing test. Based on droplet-freezing test results, three isolates had high IN activity in -5 to -10°C (isolates 25, 26 and 28) and four isolates had poor IN activity (isolates 10, 18, 19 and 21, *Fig. 2*). Ice nucleation activity was not found for other isolates. The presence of ice nucleation active gene was evaluated in INA^+ isolates by INAx specific primer pairs. A number of four isolates, including isolates 28, 26, 25 and 10, had shown a 160 bp fragment corresponding to the control *X. translucens* ICMP16317 INA^+. Although isolates 18, 19 and 21 showed poor ice nucleation activity in droplet-freezing test, they had no INA gene (*Fig. 3*). However, non-biological ice nuclei, such as presence of dust particles in suspension droplets, may lead to false results in ice nucleus formation in these isolates. The products of isolates 25 and 28 were sequenced and blasted with NCBI. The sequences showed about 99% similarity to INAx gene from *Xanthomonas translucens*. According to this result, isolate 28 was selected to subsequent mutagenesis. Isolate 28 was exposed to electroporation by using Tn5 transposon. After culture of isolates on solid medium, kanamycin disks were putted on the medium and the cells growing nearby disks were selected as transformed colonies (*Figs. 4a and 4b*). A number of eight isolates were selected on selective medium, and presence of INA gene in

their genome was surveyed by PCR. Total two out of eight isolates did not show any specific product similar to wild type (*Fig. 4c*) and were considered as mutant isolates. These two isolates were sprayed on bean seedlings to evaluate their ability in IN activity. Results of this test showed that seedling sprayed by isolate 28 *Xanthomonas* (as positive control) showed freezing symptoms but in seedling, which sprayed by mutant isolates or sterile water, no freezing signs were observed (*Fig. 5*).

Table 1 - Results of physiological and biochemical tests for 12 selected isolates

Traits	Test result
Oxidase	negative
Gram reaction	negative
Anaerobic growth	negative
Urease production	negative
Levan production	positive
Esculin hydrolysis	positive
Hydrogen sulfide production	positive
Solubility in ethanol/methanol	negative

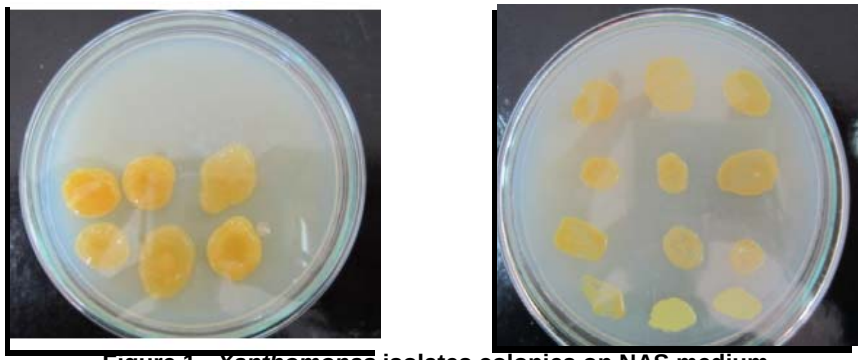

Figure 1 - *Xanthomonas* isolates colonies on NAS medium

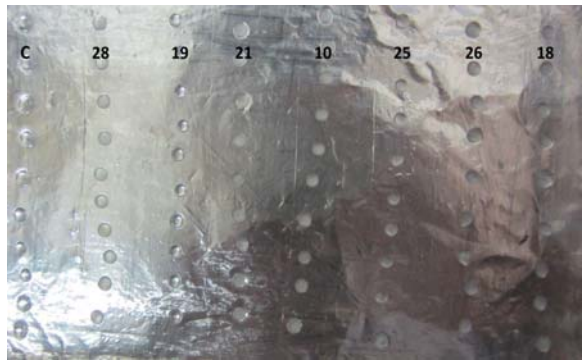

Figure 2 - Droplet-freezing test of Xanthomonas sp. in cold bath: C is sterile distilled water as control, isolates 25, 26 and 28 have shown highest Ice nucleation activity and isolates 10, 18, 19 and 21 were frozen before freezing sterile distilled water. Of 12 isolates, four isolates had not ice nucleation activity, they were not included in the image.

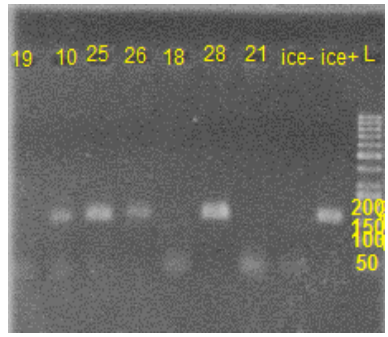

Figure 3 - PCR products on 1% agarose gel: L is size marker SM0373 fermentas, ice+ is *X. translucens* ICMP16317 ice^{+}. 21, 28, 18, 26, 25, 10, 19 are *Xanthomonas isolates*.

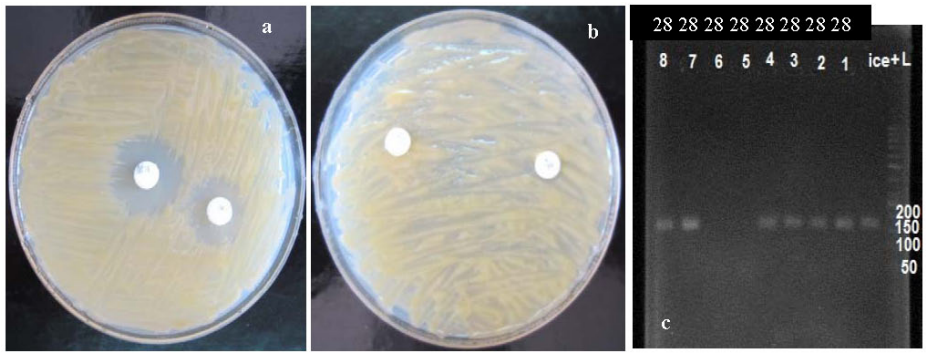

Figure 4 - Kanamycin resistant mutants of isolate 28 *Xanthomonas*, grown on selective NAS medium in front of kanamycin disks: a before mutation and b after mutation. PCR product of INAx amplification in mutated isolates.

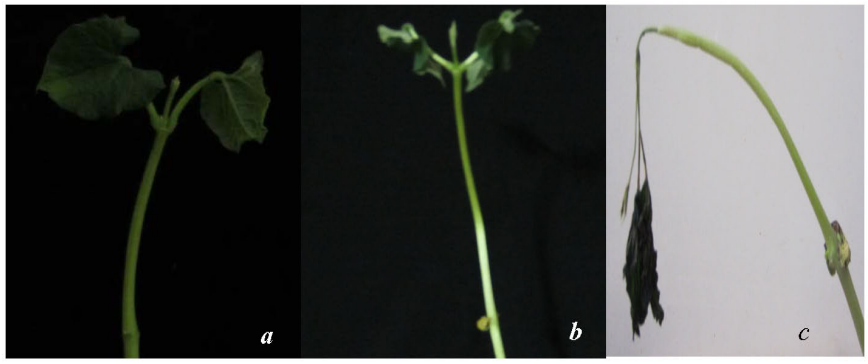

Figure 5 - Bean seedlings after sprayed with mutated and wild type isolates 28 and stored at -10°C: a and b did not freeze, they were sprayed by sterile water and mutated isolate, respectively, and c sprayed by wild type isolate 28 and froze.

In this study, *Xanthomonas* bacteria isolated from plant samples showed different ice nucleation activity in droplet-freezing test. This variation in nucleation was probably because of factors like differences between strains, inactivity of ice nucleus in all cells of a strain for the same conditions and various amounts of bacteria with ice nucleation activity (Keikhasaber *et al.,* 2007).

Bacterial ice nuclei can incite plant frost injury to many plant species. Most plant tissues can supercool extensively, whereas frost damage occurs at temperatures as high as -2°C (Lindow, 1983b). The application of bactericides or antagonistic bacteria to reduce the population size of ice bacteria on plant species in field conditions can reduce the incidence of freezing damage during natural frosts with minimum air temperatures of -5°C or higher (Gurian-Sherman and Lindow, 1993). If genetic structure of iceX protein gene mutate, this protein is not synthesized in bacteria or synthesized incompletely and formation of ice nucleus and freezing are delayed, therefore, growth season increase, the costs of freezing protection reduce leading to reduction in crop prices and (Burke *et al.,* 1990).

We isolated ice nucleation active isolates from some frozen plants in different locations and then targeted one isolate to mutagenesis. Analysis of the effects of mutants on bean seedlings showed that seedling which sprayed by mutated isolates didn't show any freezing signs, while seedling sprayed with a native isolate completely decayed due to freezing. Our results were in accordance with several studies, which have found that ice nucleation bacteria living on leafs lead to freeze plants in low temperatures and disrupting of INA gene, could prevent freezing (Lindow 1983a; Gurian-Sherman and Lindow, 1993; Hirano and Upper, 2000; Keikhasaber *et al.*, 2007).

CONCLUSION

Our results demonstrated that disruption of INP leads to lack of ice formation in temperatures above -10°C on bean leaves. Result showed that if we could replace wild type ice nucleation active bacteria with mutated forms (just different in ice nucleation activity), we could probably prevent freezing and subsequent economic losses.

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