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Root Colonization and Phytostimulation by Phytohormones Producing Entophytic *Nostoc* sp. AH-12

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Abstract *Nostoc*, a nitrogen-fixing cyanobacterium, has great potential to make symbiotic associations with a wide range of plants and benefit its hosts with nitrogen in the form of nitrates. It may also use phytohormones as a tool to promote plant growth. Phytohormones [cytokinin (Ck) and IAA] were determined in the culture of an endophytic *Nostoc* isolated from rice roots. The strain was able to accumulate as well as release phytohormones to the culture media. Optimum growth conditions for the production of zeatin and IAA were a temperature of 25 °C and a pH of 8.0. Time-dependent increase in the accumulation and release of phytohormones was recorded. To evaluate the impact of cytokinins, an ipt knockout mutant in the background of *Nostoc* was generated by homologous recombination method. A sharp decline (up to 80 %) in the zeatin content was observed in the culture of mutant strain *Nostoc* AHM-12. Association of the mutant and wild type strain with rice and wheat roots was studied under axenic conditions. The efficacy of *Nostoc* to colonize plant root was significantly reduced ($P < 0.05$) as a result of ipt inactivation as evident by low chlorophyll *a* concentration in the roots. In contrast to the mutant strain, wild type strain showed good association with the roots and enhanced several growth parameters, such as fresh weight, dry weight, shoot length, and root length of the crop plants. The study clearly demonstrated that Ck is a tool of endophytic *Nostoc* to colonize plant root and promote its growth.

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Introduction

Living organisms interact with each other to live successfully because isolation from other forms of life is unbearable. Cyanobacteria are an ancient (about 3.5 billion years old) and highly diverse, monophyletic group of oxygen releasing photoautotrophic creatures that were the sole source of oxygen to the Earth's atmosphere about 2.5 billion years ago [6]. Their first association with eukaryotic cell through an endosymbiotic event, was responsible for the evolution of chloroplasts in living world, and they evidently entered into symbiotic relationships with primitive land plants, including hornworts and liverworts etc. [23]. Nitrogen-fixing cyanobacteria such as *Nostoc* are famous for their symbiotic associations with diverse taxonomic groups of plants, including algae, bryophytes and angiosperms, and providing nitrogen in the form of nitrates to their host [22]. Naturally, *Nostoc* may occur as epiphytes (growing on the phyllo- or rhizosphere) on aquatic plants or plants growing in high-humidity environments [16], endophytes (growing inside plant's tissues), or symbiontes, making beneficial association [19, 20]. However, natural symbioses between *Nostoc* and crop plants have not been reported [22]. Symbiotic *Nostoc* in addition to fixed-N_2, provides fixed-carbon to their host [2]. Phytohormones such as IAA and cytokinins (Cks) released by symbiotic cyanobacteria are utilized by host plants for their growth and development [9]. However, benefits gain by *Nostoc* and other cyanobacteria from host are less obvious. It is speculated that *Nostoc* may absorb nutrients in the form of organic carbon from their hosts, along with getting shelter from predators and extreme environment, such as high light intensity or desiccation [1]. A great deal of interest

is currently diverted in making simulated associations of cyanobacteria with domesticated plants to improve plant growth and yield, as well as minimize need for chemical fertilizers. Heterocystous cyanobacteria, such as *Nostoc* and *Anabaena*, have been successfully tested under laboratory conditions to form association with rice plant [17, 28].

In current study, we focused on the ability of endophytic *Nostoc* to colonize rice and wheat plants under laboratory conditions. To investigate the impact of Cks on root colonization potential of the cyanobacterium, isopentenyl transferase (ipt) knockout was generated in the background of *Nostoc*. This gene codes for an enzyme known as ipt, which catalyze the first step of Cks biosynthesis. Hence, knocking down ipt gene from *Nostoc* would impair its ability to synthesize Cks.

Methodology

Isolation of Endophytic *Nostoc* Strains

For isolating endophytic strains wheat and rice fields were selected in 15 different locations of district Mardan, Pakistan. Selected plants were uprooted and transported to lab in sterile containers. Fragments of the roots (2 cm) were surface sterilized with 0.1 % solution of $HgCl_2$ and injured by pressed with sterile forceps. The pressed pieces of roots were inoculated on BG 11 medium supplemented with cyclohexamide (400 mg L^{-1}) in petri plates and incubated for 3 weeks at 25 °C in a photoperiod of 8 h light and 12 h dark. Cyanobacterial colonies were picked from the mixed cultures and enriched in liquid BG11 media (20 mL) to sufficient biomass. Biomass was harvested from the cultures by centrifugation (12,000 rpm for 2 min), suspended in BG11 medium and spread on plates of BG11containing cyclohexamide. Purity of the colonies was checked by light microscopy and pure colonies or filaments were transferred to new BG11 plates incubated under the conditions mentioned above.

Screening for Phytohormones

The strains were screened for Cks by cucumber cotyledon bioassay, as described by Hussain et al. [10]. IAA production in the culture of different strains of *Nostoc* was checked by colorimetric method using Salkowski reagent [10].

Identification of the Selected Strain

To identify the selected strain 16S genomic DNA was isolated from the cultures grown for 15 days by UltraCleanSoil DNA isolation Kit TM (Mo Bio Laboratories, Carlsbad,CA, USA) following the manufacturer's instructions. 16S rDNA was amplified with forward primer, PB36: AGRGTTTGATCMTGGCTCAG and PB38 GKTACCTT GTTACGACTT by following the protocol developed by Webb and Maas [29]. Gel purification of the amplified fragment was performed by using aqua pure DNA extraction kit (Bio-Rad, Hercules, CA, USA), which was then cloned in pGEMT vector. After overnight incubation at 4 °C plasmids were transformed into maximum efficiency competent cells (Life Technologies, Rockville, MD, USA), according to themanufacturer's instruction and plated onto LB Amp(60 μg mL^{-1}) agar, incubated overnight at 37 °C. Sequencing was done by using ABI PRISM-3100 GeneticAnalyzer (Applied Biosystems, Foster City, CA, USA), as described earlier [29].

Generation of *ipt* Knockout Mutant in *Nostoc*

Genomic DNA was isolated from the strain by adopting the previously established protocol [10]. Homologous recombination approach was used to generate *ipt* mutants in the background of *Nostoc* by replacing part of the gene sequence with a fragment containing kanamycin resistance gene. To make the construct, genomic DNA of *Nostoc* was used as a template for the amplification of open reading frame (ORF) of *ipt* with forward primer CAGTGCTAGC AAGCAAGGC and reverse primer AGACAGATCGCTC CGTTGTT. After ligating the amplified ORF into the *Xba* I site of the vector pUC19, a 207-bp fragment from the gene was dissected with *Nla* IV and *Xba* I and replaced by a fragment containing kanamycin resistance sequence maintained in vector pKRP11 by digestion with *Xba* I [12]. The construct was used to transform cyanobacterial cells, as mentioned before [21]. The cells/DNA suspension was applied on petri plates containing BG 11 medium (solidified with agarose) for the preliminary selection of transformants. After 24 h of incubation, kanamycin resistance was checked by well diffusion assay by adding 0.45 mg of kanamycin in the agar plate. Successive streaking on BG 11 plates supplemented with kanamycin was used for obtaining homozygous mutants.

Confirmatory PCR reactions were run to assay the accurate incorporation of the knockout cartridge and complete segregation of the wild-type alleles. For *Nostoc,* the complete segregation was evaluated by ACTGTTCGT ACCAGTGCCAG combined with CGTGAAGGTCGAGA ACGAGG. The correct insertion of the kanamycin resistance cassette was confirmed by following Kaczmarzyk [12]. The successful replacement of ipt in the mutant of *Nostoc* was confirmed by PCR method, using a pair of forward primer (5′CTGTTCGTACCAGTGCCAGT3′) and reverse primer (5′ACGTGAAGGTCGAGAACGAG3′).

Optimization of Growth Conditions

The wild-type and ipt knockout strains were grown in BG 11 media incubated under different conditions of temperature

and pH to find optimum growth conditions for the strains. Chlorophyll *a* was extracted from the filaments of *Nostoc* 15 days post inoculation and estimated spectrometrically. Growth was shown as mg of chlorophyll *a* per liter of media.

Cks and IAA Determination in Cyanobacterial Cultures

Phytohormones extraction and determination was performed essentially, as described by Hussain et al. [10]. Selected strains were grown in 250 mL liquid BG11 media supplemented with 500 μg $mL^{-1}$of tryptophan and 10 μM adenine at 25 °C, under a photoperiod of 8 h light (18 L mol photons m^{-2} s^{-1}) and 12 h dark for 3 weeks (inoculum density was adjusted to 0.2 μg mL^{-1} ch-*a*). Phytohormones were determined in the cultures at intervals of 1, 2, and 3 weeks by separating biomass from culture media. After filtering the culture supernatant through filters of 22 μm pore size extraction of phytohormones was carried out with Bieleski buffer (60 % methanol,25 % $CHCl_3$, 10 % HCOOH, and 5 % H_2O) containing 1 pmol [2H5] tZ and 10 pmol [2H5] IAA to monitor recovery and quantify phytohormones. After evaporated to dryness, the pooled extract was reconstituted in 5 mL of acidified water adjusted to pH = 3. The reconstituted extract was purified by solid phase extraction column (CHROMA-BOND®HR-XC, 3 mL, 200 mg) following the manufacturer's instructions. The eluent was dried, re-dissolved in 15 mM ammonium format (pH 4.0), and analyzed via UPLC-ESI–MS/MS, under the conditions described earlier [10].

Association of Wild Type and Knockout *Nostoc* Strains with Plant Roots

Seeds of *Triticum aestivum* var. Uqab 2000 and *Oriza sativa* var. Basmati were surface-sterilized with 0.1 % $HgCl_2$ for 5 min with constant shaking followed by rinsing five times with sterile distilled water. Surface-sterilized seeds were grown in petri plates containing 7 mL of nutrient solution under axenic conditions. After germination 2 mL cyanobacterial suspension (adjusted to inoculum density of 1 μg mL^{-1}ch-*a*) was added to each plate. After incubation for 2 weeks 22 ± 1 °C, 60 % relative humidity, 12 h photoperiod, and light intensity adjusted to 180 μmol $m^{-2}s^{-1}$, seedlings were harvested. Chlorophyll was extracted from the roots with 80 % methanol. Chlorophyll *a* was determined by taking OD of the samples at 660 nm and solving the equation for Ch-*a* by putting the values of OD. Each treatment was replicated three times and the experiment was repeated twice.

Statistical Analysis

A software package SPSS 12 (SPSS Inc., Chicago, IL, USA) was used to analyze the data obtained during current study. Analysis of variance (ANOVA; $P = 0.05$) and Duncan multiple range test ($P = 0.05$) were performed for finding any significant difference between different treatments.

Results

Screening and Identification Growth Characteristics of the Strains

Only one strain (AH-12) out of 17 was selected for its potential to produce Cks and IAA in its culture media. The strain was identified on the basis of 16S rDNA sequence to be *Nostoc*. The DNA sequence was submitted to NCBI, GenBank under accession number KC699844. Optimum growth conditions for both wild type and ipt knockout mutant strains were a temperature of 25 °C and pH 8 (Fig. 1). However, at optimum growth conditions, wild type strain (AH-12) showed significantly greater growth than the ipt knockout mutant (AHM-12).

Generation of *ipt* Knockout Mutants in *Nostoc*

To examine the biological function of Cks in root colonization, ipt knockout was produced in the background of *Nostoc*. The ipt gene in the mutant *Nostoc* strains was inactivated by replacing 207 bp of the gene by a kanamycin resistance cartridge. Since, a single cyanobacterial cell encloses many copies of the chromosome [11], the perfect segregation of the wild-type allele was established by PCR. The presence of ipt was ruled out in the mutant strains by running PCR reaction using primers specific to the replaced part of the gene. However, a fragment of 150 bp was amplified with the same primers from the genome of *Nostoc* AH-12 (wild-type strain). In ipt mutant strains, correct orientation of the fragment containing kanamycin within chromosome was confirmed in the second PCR reaction with kanamycin specific primers. The second reaction verified the effective integration and accurate orientation of kanamycin cassette. The result of these two PCR reactions indicated the removal the functional ipt gene in the mutant *Nostoc* strains. The ipt knockout mutant strain was labelled with AHM-12.

Phytohormones Determination

Seventeen strains of endophytic *Nostoc* were isolated from rice plants. Only one strain identified as *Nostoc* AH-12 was selected for further study on account of its ability to release Cks and IAA in the culture media. Conditions for optimum growth were a temperature of 25 °C, pH of 8.0 and photoperiod of 8 h light and 12 h dark. The wild type strain was shown to synthesize and release both zeatin and IAA in its culture media. Production of both

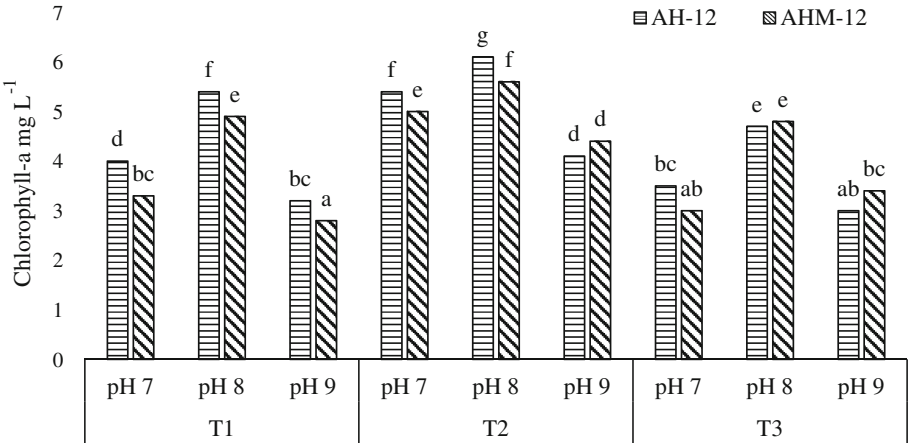

Fig. 1 Effect of pH (7, 8, and 9) and temperature (T1, 20 °C; T2, 25 °C, and T3, 30 °C) on the growth of wild type (AH-12) and ipt knockout mutant (AHM-12) strains of *Nostoc*. Growth was determined as mg of chlorophyll *a* per liter of media 15 days post inoculation. *Bars* labelled with *different letters* are significantly different, tested by Duncan multiple range test ($P < 0.05$)

these phytohormones was time dependent and a steady increase in their production was observed over time (Fig. 1a). Optimum phytohormones production was recorded in the culture incubated at 25 °C with pH of the culture adjusted to 8. Ck to IAA ratio was almost 1 in the culture of *Nostoc* AH-12. A reduction of 80 % in zeatin production was recorded in the culture of mutant strain AHM-12 when compared with the wild strain AH-12 resulting in lowering Ck to IAA ratio drastically (Fig. 1b).

Colonization of Plant Roots with *Nostoc* Strains

Wild type strain was able to associate with roots of both rice and wheat plants equally well. Un-inoculated roots of both plants were devoid of chlorophyll *a* (Fig. 2). Colonization of roots by the strain showed a gradual increase over time. Increase in chlorophyll *a* concentration from 11 to 35 μg g^{-1} FW of roots during 3-week incubation period was observed in rice inoculated with wild-type strain. The efficacy of colonizing wheat root by the same strain was evident from up to 29 μg chlorophyll *a* g^{-1} FW of roots at the end of incubation of 3 weeks. The efficiency of the *Nostoc* to colonize plant root greatly decreased with decreasing zeatin. Knock-out strain AHM-12 with reduced zeatin synthesis could only weakly colonize the roots of both rice and wheat (Fig. 2). After incubation of 1 week the potential of this strain to colonize wheat roots was only 27 %, significantly lower than wild strain of *Nostoc*. At the end of second and third week chlorophyll concentration of wheat roots colonized with the mutant was 41 and 40 % lower than the chlorophyll concentration of roots colonized with wild strain. For rice the colonization efficiency AHM-37 was 46 % lesser than wild strain of *Nostoc* during first week. In the following weeks the root chlorophyll *a* was 53 % lower in root colonized with the mutant as compared with the wild strain.

Effect of Exogenous Application of Ck on Root Colonization

Exogenous application of trans-zeatin (tZ) enhanced colonization of roots by mutant strain AHM-12 (Fig. 3). When 378 pmol of tZ was added to petri plates containing wheat plants inoculated with AHM-12 to reconstitute Ck concentration of the wild type strain, up to 33 % increase in root colonization was observed. Application of exogenous Ck at the time of inoculating plant root with mutant *Nostoc* enhanced its root colonization potential by 33 and 48 % in case of wheat and rice, respectively (Fig. 3). Application of Ck after incubation of 1 week (at the beginning of second week) improved colonization by 20–21 %. In the beginning of third week, the application of exogenous Ck could not significantly improved root colonization by AHM-12. These results suggested critical role for Cks at a very early stage of plant-*Nostoc* association.

Phytostimulation Under Axenic Conditions

The most efficient strain was *Nostoc* sp. AH-12 which caused an increase of 36 % in dry weight of wheat seedlings as compared to control seedlings (un-inoculated). An increase of 45 % was recorded in the fresh weight of wheat seedlings by the end of incubation for 2 weeks. Seedlings primed with wild type strains showed 20 % increase in shoot length and 31 % increase in root length. Fresh weight of rice seedlings was enhanced over control by 63 % in 2 weeks. Increase in dry weight (41 %), shoot length (22 %), and root length (40 %) was evident in rice seedlings inoculated with this strain. Seedlings inoculated with mutant strain AHM-12 showed some increase in these parameters which was not significant statistically (Table 1).

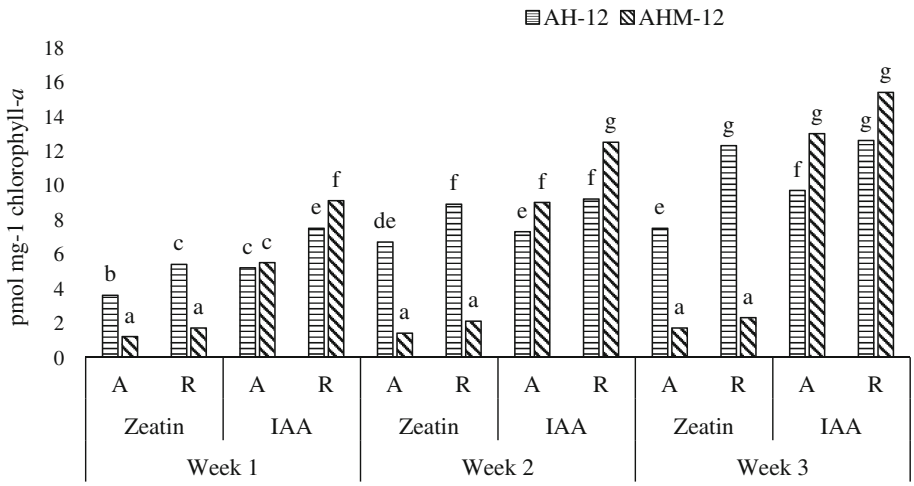

Fig. 2 Phytohormones zeatin (Ck) and IAA in the culture (R) and Biomass (A) of *Nostoc* strain AH-12 and AHM-12 determined by UPLC-ESI–MS/MS after incubation of cultures for one, 2 and 3 weeks. *Bars* labelled with *different letters* are significantly different, tested by Duncan multiple range test ($P < 0.05$)

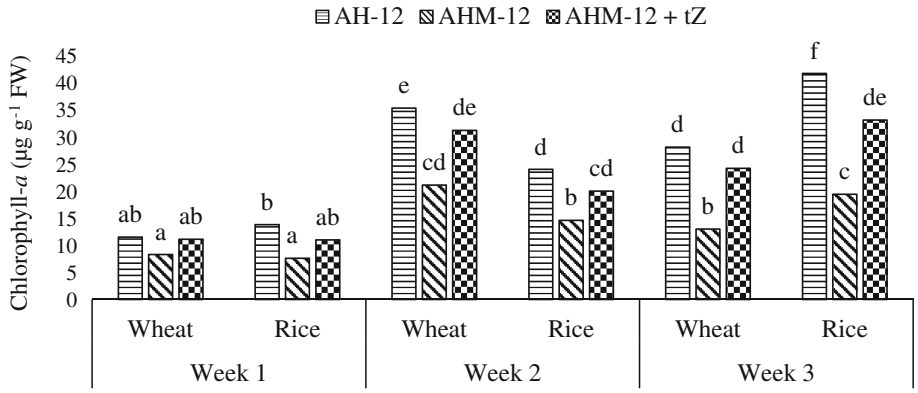

Fig. 3 Colonization of plant roots by wild strain of *Nostoc* (AH-12), ipt mutant (AHM-12) and ipt mutant along with tz (AHM-12+tZ. Colonization was quantified by measuring chlorophyll *a* concentration in the roots of rice and wheat seedlings harvested after one, 2 and 3 weeks of co-cultivation with different strains of *Nostoc*. *Bars* labelled with *different letters* are significantly different, tested by Duncan multiple range test ($P < 0.05$)

Table 1 Impact of wild type and mutant strains of *Nostoc* on growth of wheat and rice seedlings incubated for 2 weeks under axenic conditions

Strain	cm				mg plant-1			
	Shoot length		Root length		Fresh weight		Dry weight	
	Wheat	Rice	Wheat	Rice	Wheat	Rice	Wheat	Rice
Cont.	12.2 (a)	9.3 (a)	3.6 (a)	2.1 (a)	1.6 (a)	1.1 (a)	0.25 (a)	0.16 (a)
AH-12	14.6 (b)	11.3 (b)	3.8 (a)	2.2 (a)	2.3 (b)	1.79 (b	0.34 (b)	0.22 (b)
AHM-12	12.4 (a)	9.8 (a)	4.7 (b)	2.9 (b)	1.7 (a)	1.13 (a)	0.26 (a)	0.17 (a)

Different letters in the same column indicate significant difference between means (Duncan multiple range test; $P < 0.05$)

Discussion

The ability of cyanobacteria to promote plant growth has been attributed to their potential to colonize roots in the rhizosphere and even penetrate them to benefit from the association [3, 14]. Heterocystous cyanobacteria have the ability to fix nitrogen into nitrates and other soluble forms, making it available for plants [2]. *Nostoc* has been widely reported to form symbiotic associations with a wide range of plants, such as angiosperms, bryophytes, as well as with

algae, and provide fixed nitrogen to its host [17, 18, 22]. To study association between cyanobacteria and plant roots, two protocols are normally adopted: (1) chlorophyll *a* measurement to give a quantitative estimation and (2) imaging techniques, such as light microscopy or scanning electron microscopy for qualitative aspect of the association [3, 17]. We followed the former method to study root colonization with *Nostoc*. The strain isolated from rice tissues was proved as a good colonizer of plant roots under axenic conditions. According to previous reports, microbes use Cks as an important tool to interact with plants [8]. To check the involvement of Cks in plant–*Nostoc* interaction, we knocked out ipt gene from our isolated strain. This gene encodes ipt enzyme which catalyze the rate limiting step of Ck biosynthesis [24]. The gene has previously been reported from several cyanobacterial strains including *Nostoc*, *Anabaena*, and *Synechocystis* etc. [4, 13, 27]. Direct evidence of the involvement of Cks in plant–*Nostoc* interaction was provided by a significant decrease of root colonization by *Nostoc* as a result of knocking down ipt gene from it. The role of Cks has been previously implicated in root colonization by Rhizobium and mycorrhizal symbionts [5, 15]. However, direct evidence of the involvement of Cks in plant–cyanobacteria interaction is a novelty. In addition to Cks, bacteria and cyanobacteria also use IAA as a part of their root colonization strategy and a tool of plant growth promotion [17, 26]. In current study, endophytic *Nostoc* strains capable to synthesize equal amount of the phytohormones Ck and IAA were good colonizers of the root in rice as well as wheat system. However, decreasing Ck/IAA ratio in ipt knockout *Nostoc* adversely affected its ability to colonize root. When this gene was knockout by homologous recombination in the background of *Nostoc*, the synthesis of zeatin was drastically reduced followed by significant decrease in the growth of the mutant strain. This behavior of ipt knockout *Nostoc* was a validation of the previous study reporting the important role of Cks in cyanobacterial growth and development [25]. Inactivation of the gene was confirmed by PCR using primers specific to the part of gene replaced by kanamycin construct. Gene knockout has been used as a successful strategy to inactivate genes and impair their function in cyanobacteria [7]. These findings evidenced the importance of the relative amount of Cks and IAA in plant–microbe interaction. The role of Cks in root colonization by *Nostoc* was confirmed by the application of exogenous zeatin to the roots co-cultivated with AHM-12 (low Ck mutant), which restored the colonization potential of the strain lost by mutation. It may be concluded that knocking down ipt from *Nostoc* impairs its ability to synthesize Cks and colonize plant root. The study provides a sound proof that Cks play a very important role in the association of cyanobacteria, particularly *Nostoc* with plant roots.

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