A molecular evidence for the presence of methylobacterial-type Fe siderophore receptor in *Celosia cristata*

A. Gholizadeh[1], B.B. Kohnehrouz[2]

[1]Research Institute for Fundamental Sciences, University of Tabriz, Tabriz, Iran
[2]Department of Plant Breeding and Biotechnology, University of Tabriz, Tabriz, Iran

ABSTRACT

The presence of efficient iron-uptake bacteria was predicted to be localized as endosymbionts within the leaves of *Celosia cristata*, a well known iron-rich plant. On the other hand, the symbiotic methylobacterium having a distinctive pink pigmentation was suggested to be more likely in the leaves of pink-colored plants. These considerations were experimented by priming a cDNA fragment containing methylobacterial-type Fe siderophore receptor domain from *Celosia* leaf cDNA population. Since no detectable homologue was found in plant species sequenced to date, the presence of a Fe-efficient methylobacterium endosymbiosis was reliably predicted in *Celosia* plant. This is the first report that may lead to the way for future studies on molecular interactions between high iron content pink-colored plants and iron-efficient pink-pigmented bacteria. Corresponding cDNA sequence was submitted to EMBL databases under accession number FM955594.

Keywords: *Celosia*; siderophore; iron; methylobacterium; cDNA cloning

Bacteria are able to colonize a wide variety of habitats, including the most extreme environments and even living organisms. This reflects a high degree of adaptability and the presence of specific genetic programs devoted to satisfy the nutritional requirements of the bacteria in their diverse habitats.

Iron had played an essential role in the evolution of life on earth, and is required by most microorganisms, such as bacteria (Posey and Gherardini 2000). This essential trace element is often used as a cofactor in key metabolic processes, including deoxyribonucleotide synthesis, oxidative phosphorylation, and electron transport (Briat 1992). The bacterial ability to use this element implies its abundance in their habitats. Despite the relative abundance of iron in different natures, it is often sparingly available for the living organisms such as bacteria. Iron is considered to play a crucial role in plant-microbe interactions. It is known as one of the factors that limits the bacterial growth in plants (Expert et al. 1996).

Bacteria have commonly developed extensive and efficient siderophore-mediated iron acquisition strategies to colonize habitats and to satisfy their requirements (Braun 1997). They mostly secrete at least one siderophore, a low molecular weight compound (500–1500 Da), having high affinity for ferric iron and often with possession of three bidentate iron chelating groups (Raymond et al. 1989, Neilands 1995).

Siderophores avidly scavenge ferric ions and are bound by specific outer membrane receptors which actively pump them into the periplasm of bacteria against a concentration gradient by utilizing an energy-dependent transport pathway. The gram-negative bacterial outer membrane siderophore transport system is dependent upon the presence of a complex of three cytoplasmic membrane proteins named TonB, ExbB and ExbD. They are generally termed as TonB-dependent receptors (Bradbeer 1993, Kaserer et al. 2008).

The siderophore recognition domain of TonB-dependent receptors is known as Plug domain. Plug domain has been shown to be an independently folding subunit of the TonB-dependent receptors. It acts as the channel gate through conformational

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changes, blocking the pore until the channel is bound by siderophore (Ferguson et al. 2002).

TonB-dependent receptor sequences were characterized from various gram negative bacteria, such as methylobacterium (Loper and Buyer 1991, www.homd.org/download/NCBI_bacteria_genome/Methylobacterium; www.micro-genomes.mpg.de, Braun 1995). This bacterium can be found mostly in soils, on leaves, and in other parts of a wide variety of plants as a natural flora (Lindstrom and Christoserdova 2002). Its mechanisms are not fully understood, but it is a highly studied organism. It is extremely important because it is a methanotroph that utilizes and reduces the methane/methanol emitted by the stomata of plants. From the ecological viewpoints, methanotrophs might be used in facilitating attempts to reduce methane emission in the future (Eller and Frenzel 2001).

The details of the methylobacteium relationship to plants have been unclear so far. They were shown in some cases to stimulate seed germination, plant development, and to contribute to the plant flavor (Corpe and Basile 1982, Holland and Polacco 1992, 1994, Zabetakis 1997). Production of cytokinins observed in different strains of methylobacteium was recently predicted to be sufficient to have an effect on plant growth and developmental processes (Ivanova et al. 2000, 2001). Because of methylobacterial distinctive pink pigmentation, these bacteria are sometimes referred to as PPMFs 'pink-pigmented facultative methylotrophs' (Lindstrom and Christoserdova 2002). The numerous indications exist that these PPMFs interact with plants, but the biochemical and genetic details of these interactions have been remained elusive.

Celosia species are generally pink-colored plants and exceptionally known as high iron content plants (Gupta et al. 2005), therefore, we predicted and investigated the presence of Fe-efficient methylobacterium symbiosis in this plant for the first time.

MATERIAL AND METHODS

Celosia cristata seeds were obtained from the division of Biochemistry, IARI, New Delhi, India and were grown in greenhouse conditions. *E. coli* strain DH5α was used for bacterial transformation. Plasmid vector pGEM-T easy (Cat. No. A1360, Promega) was used for PCR product cloning. Trizol reagent (Cat. No. RN7713C; RNX^{TM}; CinnaGen) was used for total RNA isolation. The mRNA purification kit provided by QIAGEN, USA (Cat. No. 70022). AccessQuick^{TM} RT-PCR System was purchased from Promega (Cat. No. A1701). Fermentas DNA Extraction Kit (Cat. No. K0513) was used for the purification of the PCR product from the agarose gel. All of the other chemicals used in this research work were of molecular biology grades.

Total RNA isolation and mRNA purification. The leaves of *Celosia* plants were collected at vegetative growth stage and rinsed thoroughly with double distilled water three times to wash out any leaf surface bacteria. The leaf samples were finally washed with DEPC-treated water for total cellular RNA isolation using Trizol reagent. About 0.2 g of leaf material was fine-powdered using liquid N_2 and 2 ml of Trizol reagent was added to homogenize it at room temperature (RT). 200 µl of chloroform was added to the mixture, mixed for 15 s, incubated on ice for 5 min and centrifuged at 13000 g for 15 min. The upper phase was transferred to another tube and the RNA was precipitated with equal volume of isopropanol. The pellet was washed in 1 ml of 75% ethanol, dried at RT and dissolved in 30 µl RNase-free water. The integrity of the RNA was tested on 1% non-denaturing agarose gel using TBE running buffer.

Poly (A^+) RNA was purified from total RNA using oligo dT-columns according to the kit protocol. The integrity of the purified mRNA was also analyzed by electrophoresis using 1% non-denaturing agarose gel.

cDNA amplification by RT-PCR. Primers specific for methylobacterium-type Fe siderophore receptor transcript were designed using Primer 3 software http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi based on the siderophore recognition sequence named as Plug domain. The sequences of the primer pairs were as follows: Fw: 5' CAATCGATCACCGTCGTG 3', Rv: 5'CGCTTGCTGATCTGGTTG 3'. Amplification of siderophore receptor gene was carried out by the RT-PCR method. The RT-PCR reactions were performed using one-step AccessQuick^{TM} RT-PCR System. About 0.5 µg of total RNA or mRNA was mixed with 25 µl Master Mix (2×) and 1 µl of each primer. The mixture was adjusted to a final volume of 50 µl using nuclease-free water. The reaction mixture was incubated at 45°C for 45 min and proceeded with PCR cycling. The PCR was carried out after a pre-denaturation stage at 95°C for 3 min in 25 cycles of 1 min denaturation at 95°C, 1 min annealing at 55°C, and 1.5 min of extension at 72°C. The reaction was finally extended at 72°C for 10 min.

Cloning of amplified fragment and sequencing. Amplified RT-PCR end product was extracted from the agarose gel using Fermentas DNA Extraction Kit, cloned in pGEM-T easy cloning vector and transformed to DH5α *E. coli* strain (Ausubel 1991). The plasmid DNA was isolated by alkaline lysis method (Bimboim and Dolly 1979) and proceeded for the sequencing in *Microsynth* DNA sequencing center at Switzerland.

Sequence analysis. The nucleotide and deduced amino acid sequences of the isolated cDNA fragment were analyzed for primary structure similarity by computing at BLAST (Basic Local Alignment Search Tool, http://www. genome.ad.jp) and Multalin servers developed by *Expasy* proteomic tools (http://www.expasy.ch/tools).

RESULTS AND DISCUSSION

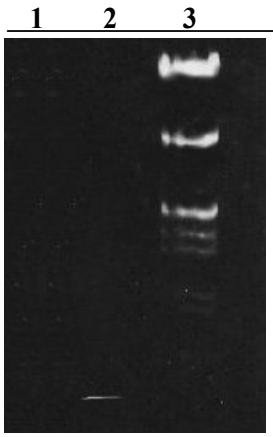
As *Celosia* species belong to high iron content plants and have high pink pigmentation in their leaves and flowers (Gupta et al. 2005), we predicted the presence of efficient iron-uptake bacteria as well as pink pigmented bacteria in the leaves of *C. cristata* plant. These bacteria were more likely thought to be methylobacteria that were known as pink pigmented facultative methylotrophs, commonly found on the surfaces or inside the leaves of a wide varieties of plants (Lidstrom and Chistoserdova 2002). We examined this idea by priming a cDNA fragment containing methylobacterial-type Fe siderophore receptor plug domain from the leaves of *Celosia*. A specific primer set targeted to the methylobacterium plug domain was used in priming reactions by RT-PCR method. As mentioned in the materials and methods section, RT-PCR reactions were performed using leaf total RNA and mRNA as starting materials in two separate samples. The RT-PCR end products of each sample were separated on 1% agarose gel and analyzed for the presence of amplified fragment (Figure 1). This analysis clearly showed a detectable primed product in the reaction sample started with total RNA, while no amplified fragment was observed in the reaction sample started with mRNA. The resulted cDNA was cloned and sequenced for its further characterization. A 322 nucleotide-based sequence data is illustrated in Figure 1.

Comparing the results of two RT-PCR reaction samples is very interesting. It can be deduced that the corresponding amplified fragment is not originated from plant cDNA population, but it more likely comes from coexistent bacteria in the leaf tissues. Analysis of the nucleotide and deduced amino acid sequences of the amplified cDNA using different data analyzing softwares showed the presence of methylobacterial-type Fe siderophore receptor plug domain (Figure 2).

Alignment of *Celosia* cDNA with corresponding gene from methylobacterium showed that they are highly identical in terms of the nucleotide and deduced amino acid levels. Among 107 amino acid residues there are only two substituted positions. These include Gln and Leu in *Celosia* sequence in place of Arg and Val in corresponding sequence in methylobacterium (Figure 2).

Blast search data as well as ClastalW analysis revealed that *Celosia* Fe-siderophore receptor sequence is closer to proteobacterial genes. A dandrogram of genetic distance analysis has been illustrated (Figure 3, sequence alignment data not shown).

Iron is considered to play a crucial role in plant-microbe interactions. Since the micromolar concentrations of iron are necessary to support bacterial growth and manipulation, it is known as



<CAATCGATCACCGTCGTGGGC
CAGCAGCAGCTCGACACCCAGA
ACGCCCAGACCCTCACCCAGGC
GACCCAGTACGTCGCCGGCACC
TACGCGGGCACCTTCGGCGCCG
ACACGCGCCTCGACTTCTTCCA
GCTGCGCGGATTCGTGGTCAGC
GATTACGGCCTCTACCTCAACG
GCCTGCAGCTCCTGAACTACGG
CTTCGCCTACAGCCGCGTGGAC
ACGTTCGGGCTGGAGCGGATCG
AGCTGCTGCGCGGCCCCTCCGC
GGTGCTGTTCGGCGCCGGCAAT
CCCGGCGGCCTGATCAACCAGA
TCAGCAAGCG>

Figure 1. Analysis of RT-PCR amplified cDNA fragment. Two separate RT-PCR reactions were performed using methylobacterial type siderophore receptor specific primers and leaf total RNA/ mRNA populations. The end products were then analyzed on 1% agarose gel. A detectable band was observed in the reaction sample started with total RNA. 1: mRNA-based product; 2: Total RNA-based product and 3: EcoRI and HindIII double digested DNA marker

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Methylobacterium/   ATCGAGACCCCGCAATCGATCACCGTCGTGGGCCGGCAGCAGCTCGACACCCAGAACGCC
Celosia/            ------------CAATCGATCACCGTCGTGGGCCAGCAGCAGCTCGACACCCAGAACGCC

Methylobacterium/   CAGACCCTCACCCAGGCGACCCAGTACGTCGCCGGCACCTACGCGGGCACCTTCGGCGCC
Celosia/            CAGACCCTCACCCAGGCGACCCAGTACGTCGCCGGCACCTACGCGGGCACCTTCGGCGCC

Methylobacterium/   GACACGCGCCTCGACTTCTTCCAGCTGCGCGGATTCGTGGTCAGCGATTACGGCCTCTAC
Celosia/            GACACGCGCCTCGACTTCTTCCAGCTGCGCGGATTCGTGGTCAGCGATTACGGCCTCTAC

Methylobacterium/   CTCAACGGCCTGCAGCTCCTGAACTACGGCTTCGCCTACAGCCGCGTGGACACGTTCGGG
Celosia/            CTCAACGGCCTGCAGCTCCTGAACTACGGCTTCGCCTACAGCCGCGTGGACACGTTCGGG

Methylobacterium/   CTGGAGCGGATCGAGGTGCTGCGCGGCCCCTCCGCGGTGCTGTTCGGCGCCGGCAATCCC
Celosia/            CTGGAGCGGATCGAGCTGCTGCGCGGCCCCTCCGCGGTGCTGTTCGGCGCCGGCAATCCC

Methylobacterium/   GGCGGCCTGATCAACCAGATCAGCAAGCGCCCGACCGAGACCCCGTTCGGGCTCGTCGAG
Celosia/            GGCGGCCTGATCAACCAGATCAGCAAGCG----------------------------

Methylobacterium/   QSITVVGRQQLDTQNAQTLTQATQYVAGTYAGTFGADTRLDFFQLRGFVVSDYGLYLNGL
Celosia/            QSITVVGQQQLDTQNAQTLTQATQYVAGTYAGTFGADTRLDFFQLRGFVVSDYGLYLNGL

Methylobacterium/   QLLNYGFAYSRVDTFGLERIEVLRGPSAVLFGAGNPGGLINQISN
Celosia/            QLLNYGFAYSRVDTFGLERIELLRGPSAVLFGAGNPGGLINQISN
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Figure 2. Nucleotide and deduced amino acid sequence alignment. The amplified Celosia cDNA sequence and its deduced amino acid sequence were aligned with methylobacterium Fe siderophore receptor plug domain gene. The two substituted positions were shown inside the boxes

one of the limiting factors for bacterial growth in planta (Expert et al. 1996). However, bacteria use a common strategy to overcome such problem by utilization of siderophore compounds with high affinity for ferric ions (Braun 1997). In a recent study, it was shown that the bacterial iron-uptake genes are necessary for the induction of hypersensitive response on the non-host plants (Wiggerich and Pühler 2000).

Our present work results indicated that iron-efficient bacteria might exist on plants with high iron content rather than the others. Our study may also provide new ways in understanding the molecular bases of methylobacterium in pink-colored high iron content plants in the future. Methylobacterium are commonly found on the surface of the leaves of the wide varieties of plants. They were also reported to be localized as endo-

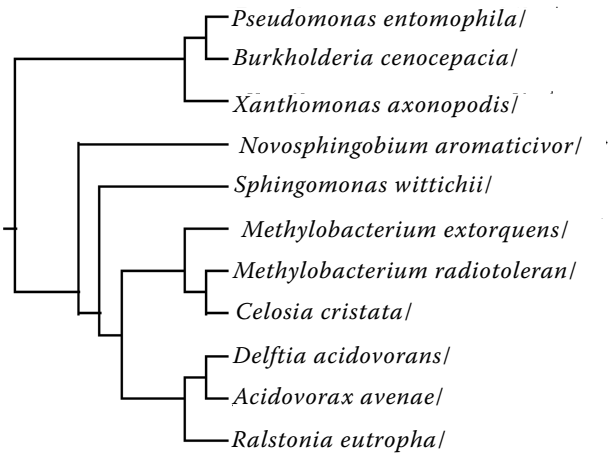

Figure 3. Dendrogram for genetic similarities/distances. Amplified *Celosia* sequence was analyzed for its similarities/distances with bacterial Fe-siderophore receptor plug domains. *Sphingomonas wittichii* (NCBI-GI: 148556463); *Delftia acidovorans* (NCBI-GI: 160898505); *Acidovorax avenae* (NCBI-GI: 120610054); *Ralstonia eutropha* (NCBI-GI:116695045); *Methylobacterium radiotolerans* (NCBI-GI: 170747917); *Celosia cristata* (EMBL Ac. No: FM955594); *Methylobacterium extorquens* (NCBI-GI: 163851022); *Pseudomonas entomophila* (NCBI-GI: 104781151); *Burkholderia cenocepacia* (NCBI-GI: 116689369); *Xanthomonas axonopodis* (NCBI-GI: 21244345); *Novosphingobium aromaticivor* (NCBI-GI: 87201096)

symbionts within the cells of some plants (Pirttila et al. 2000). Earlier evidences showed that these bacteria utilize methanol by the stomata of plants, but the details of their relationship to plants is unclear thus far (Eller and Frenzel 2001). It is not clear whether the methylobacterium strains are commensal bacteria or they do communicate with plants. They were already shown to be involved in some processes such as seed germination, plant growth and development. These may be through the production of phytohormones such as cytokinins or indole acetic acids by coexistent plant bacteria (Ivanova et al. 2000, 2001). Some of the methylobacterium strains were reported to contribute to the flavor of plants or nitrogen fixation process in legumes (Sy et al. 2001). Recently, a partial genome sequence analysis of *Methylobacterium extroquens* revealed a number of genes similar to genes involved in plant association in R*hizobia* and *Agrobacterium* (http://pedant.mips.biochem.mpg.de/).

To date, it is clear that pink-pigmented methylobacterium interact with plants, but the biochemical and genetic details of their interactions are yet elusive. Production of phytohormones by these bacteria provided the new insights into the commensal plant bacterial interactions in general (Lidstrom and Chistoserdova 2002).

Similarly, the present results may lead to the new ways for studies on molecular interactions between high iron content pink-colored plants and iron-efficient pink-pigmented bacteria in the future.

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Corresponding author:

Ashraf Gholizadeh, University of Tabriz, Department of Molecular Biology, Research Institute for Funamental Sciences, Tabriz, Iran
e-mail: aghz_bioch@yahoo.co.in