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Identification and characterization of a bacteria-like sequence in the genome of some *Silene* species

M. TALIANOVÁ*, J. ŽLŮVOVÁ, R. HOBZA, B. VYSKOT and B. JANOUŠEK

Department of Plant Developmental Genetics, Institute of Biophysics, Academy of Sciences of the Czech Republic, Královopolská 135, CZ-61265 Brno, Czech Republic

Abstract

The aim of this work was to characterize a nucleotide sequence MK14 that originated from a plasmid library obtained *via* degenerate oligonucleotide primed polymerase chain reaction (DOP-PCR) amplification of laser microdissected Y-chromosomes of *Silene latifolia*. This sequence showed significant similarity to parts of two adjoining genes from bacterial representatives of the genus *Ralstonia.* MK14 sequence contains a part of a conserved domain, and phylogenetic analysis based on this region confirmed its relationship to *Ralstonia*-derived sequences. Genomic Southern blot analysis proved the presence of this fragment in the genome of *S. latifolia*. We hypothesize that this insertion is of bacterial origin, and was probably gained *via* horizontal gene transfer. Moreover, MK14 insertion is shared by some closely related *Silene* species, suggesting an ancient spontaneous transformation by an ancestor of bacteria from the genus *Ralstonia*.

Additional key words: horizontal gene transfer, microdissection, phylogenetic analysis, *Ralstonia*, sulfate adenylyltransferase, transformation.

Introduction

The genus *Silene* (*Caryophyllaceae*) is an important model for the studies of various evolutionary aspects (*e.g.*, evolution of sexual systems, evolution of sex chromosomes in their early phases), and in ecological studies (Bernasconi *et al*. 2009, Janousek and Mrackova 2010). However, only since recently genomic resources for *Silene* have become available greatly facilitating genetic and molecular studies in this genus. Chromosome microdissection, microcloning and construction of chromosome-specific genomic libraries are widely used tools in studying the structure of genomes and identification of chromosome-specific markers (*e.g.,* Mariotti *et al.* 2006, Hobza and Vyskot 2007). Chromosome microdissection has been also used to study the structure of X and Y sex-chromosomes in *S. latifolia* (Delichère *et al.* 1999, Matsunaga *et al.* 1999, Sugiyama *et al.* 2003, Hobza *et al.* 2004, 2007). Several markers from the Y-chromosome genomic library were isolated and studied in detail (Hobza *et al.* 2006, Kejnovsky *et al.* 2006). The aim of this work was to characterize another sequence named MK14 that originated from the genomic plasmid library derived from the degenerate oligonucleotide primed polymerase chain reaction (DOP-PCR) on the Y-chromosome of *S. latifolia*.

Materials and methods

Plants: In this study, several species of the genus *Silene* were used, including dioecious species *Silene latifolia* (population MAV, inbred line kindly provided by Dr. S. Matsunaga, University of Tokyo, Japan; Bc, interpopulation cross, seed material collection of the Institute of Biophysics, Brno; U9xCH, interpopulation cross, seed material collection of the Institute of Biophysics, Brno; MAVxBystrc, interpopulation cross, of Biophysics, Brno), *S. dioica* and *S. diclinis* (seed material collection of the Institute of Biophysics, Brno) and gynodioecious

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Abbreviations: DOP-PCR - degenerate oligonucleotide primed polymerase chain reaction; HGT - horizontal gene transfer.
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* Corresponding author; e-mail: martina.talianova@biologie.uni-regenburg.de

species *S. vulgaris* (wild type population, Brno). Plants were grown in greenhouse at temperature of 22 °C and 16-h photoperiod with natural irradiance. Axenic plant cultures from surface-sterilized seeds of *S. latifolia* and *S. vulgaris* were grown on the BMS-10 medium (Ye *et al.* 1990) with antibiotic cefotaxime (0.5 mg cm^{-3}) in sterile conditions for 7 weeks in climate chamber at 22 °C and 16-h photoperiod. The presence of bacteria from the genus *Burkholderia* and *Ralstonia* in seeds of *S. latifolia* was tested by inoculation of homogenized seeds on the nonspecific King's B medium (King *et al.* 1954).

Isolation of nucleic acids: DNA samples from individual plants were isolated using commercial kits for plant DNA isolation (*Qiagen*, Germany; *Macherey-Nagel*, Germany). RNA samples were isolated as described by Zluvova *et al.* (2010) from different tissues (leaves, flower buds of various size: 1, 2, 3 and > 3 mm) of both male and female individuals of *S. latifolia* and cDNA was synthesized using the procedure described by Zluvova *et al.* (2006). As a positive control, actin primers were used for the amplification both cDNA and genomic DNA as described by Cegan *et al.* (2010).

PCR amplification: Primers were designed along the MK14 fragment (forward primers: MK14F1n 5'-CGA TATCGGTACGGTCAACA-3', MK14F2n 5'-CGTGCT GGACGTGCATACG-3', MK14Fmt 5'-AACGATATC GGTACGGTCAA-3'; reverse primers: MK14Rn 5'-AAA AGCAAAGGAATCCAGGC-3', MK14Rmt 5'-ATGATC CGTGCATTTTCCTA-3', MK14R1 5'-ATGATCCGT GCATTTTCCTAATGTGGAT-3'). For all primer combinations, PCR amplification was carried out using the PCR mixture according to Michu *et al*. (2010) and following PCR cycles: 94 °C/3 min, 35 × (94 °C/30 s, 55 °C/60 s, 72 °C/45 s), 72 °C/4 min using 20 ng of total genomic DNA of the studied species (*S. latifolia*, *S. diclinis*, *S. dioica*, and *S. vulgaris*). PCR products were cloned into a vector using cloning kits *pGEM-T easy* (*Promega*, USA). Reverse transcription PCR on the cDNA was run with primers MK14F2n and MK14Rmt using the same program as for standard PCR.

Genomic Southern hybridization: For genomic Southern hybridization, 25 - 30 µg of each genomic DNA sample was digested with *HindIII* restriction enzyme (*New England Biolabs*, USA), because this restriction site is absent in the MK14 sequence. Samples were loaded on 0.8 % agarose gel and transferred onto the positively charged nylon membrane (*Amersham*, USA) by capillary transfer. Hybridization was carried out for 16 h at 65 °C with a probe derived from a part of conserved CysN_NoDQ_III domain amplified from *S. latifolia* showing high similarity to the corresponding domain from the representatives of the genus *Ralstonia*. The probe was radiolabelled by [α-^{32}P]-dCTP using the *Prime-It II* random primer labeling kit (*Stratagene*, USA).

Phylogenetic analyses: Comparison of MK14 sequence against the public NCBI sequence database was performed using the *Blast* tool with the default settings. Amino acid sequences of the representatives of sulfate adenylyltransferase large subunit (or large subunit of ATP sulfurylase) were downloaded from the NCBI database creating a dataset of 4990 sequences. Redundant sequences (*i.e.*, sequences that were 100 % identical) were excluded from the dataset in Jalview editor (Waterhouse *et al.* 2009) reducing the dataset to 1923 amino acid sequences. Resulting dataset was aligned together with bacteria-like amino acid sequences from *Silene* species in *Mafft* (Katoh *et al.* 2002). Due to the large number of sequences, phylogenetic analysis was performed using approximately-maximum-likelihood phylogenetic inference in *FastTree* (a program that has been shown to be very efficient in terms of both resolution and computation time and thus it is very suitable for the analyses of large datasets; Price *et al.* 2010). In *FastTree*, statistical support for branches is tested by Shimodaira-Hasegawa (SH) test which has been shown to be a good and very fast alternative to bootstrapping (Guindon *et al.* 2010). When using the SH test, for a given branch, estimated maximum-likelihood branch is compared to the next two most likely nearest-neighbor interchange (NNI) rearrangements of that branch (Guindon *et al.* 2010). Phylogenetic tree was visualized and edited in *Dendroscope* (Huson *et al.* 2007).

Results and discussion

To characterize sex chromosome specific markers we have analysed sequences from the Y-chromosome specific plasmid library of DOP-PCR products (Hobza *et al.* 2006). A DNA sequence of our interest named MK14 (291 bp long) resulted from random selection of clones from this library. Blasting MK14 sequence against *NCBI* database revealed strong similarity (E = $5e^{-152}$) to two adjoining genes in bacterial species from the genus *Ralstonia* (*Burkholderiaceae*), with no significant hits within plant kingdom. One part (85 bp) of this bacteria-like fragment of *S. latifolia* shows similarity to a part of a gene coding for uroporphyrin-III C-methyltransferase (*nirE*), while the second part (203 bp) revealed similarity to the part of a gene encoding the large subunit of sulfate adenylyltransferase (ATP sulfurylase; *cysN*) (Fig. 1). Within the second part, a part of conserved protein domain corresponding to the CysN_NodQ_III protein domain (named according to CysN protein and nodulation protein Q) has been identified. This domain is functionaly related to the domain III of translation elongation factor Tu (EF_Tu), a GTPase which is essential for GTP hydrolysis (Martemyanov and Gudkov 2000). *CysN* and *nodQ* genes have been identified from proteobacteria and few gram-positive bacteria, other

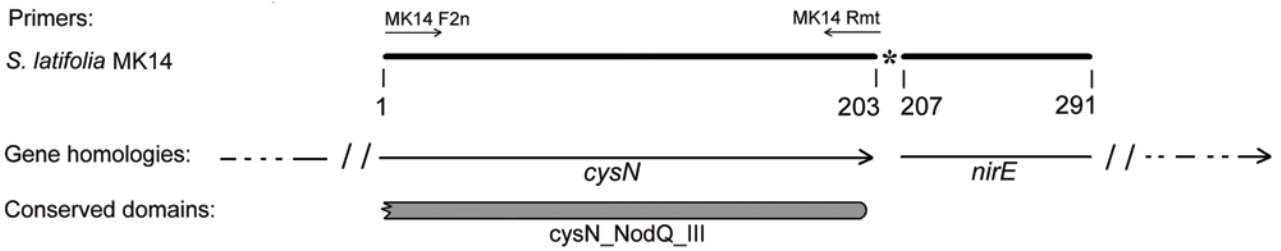

Fig. 1. Graphical representation of *Silene latifolia* MK14 nucleotide sequence along with homologies with corresponding genes *cysN* and *nirE* from *Ralstonia* species, and conservative domain CysN_NodQ_III. Positions of primers MK14F2n and MK14Rmt are denoted. *Asterisk* represents a termination codon.

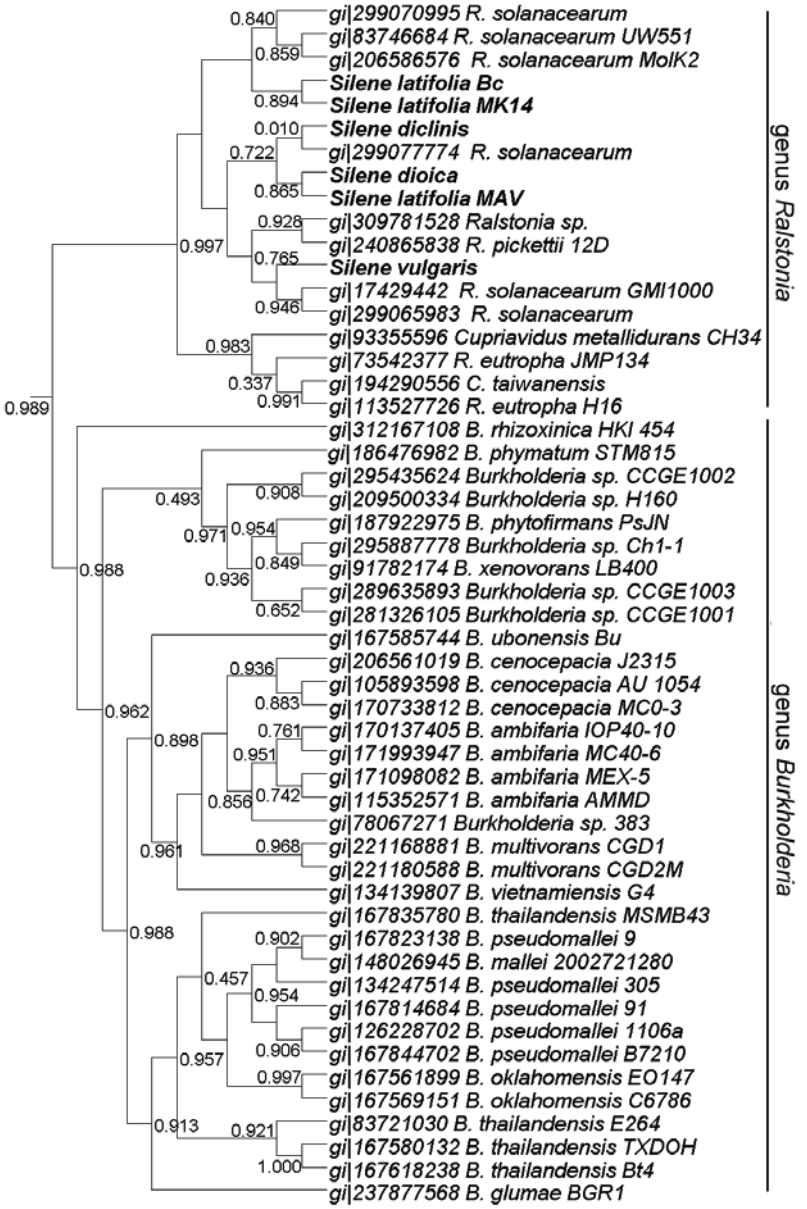

Fig. 2. A subtree of *FastTree* generated approximately-maximum-likelihood phylogeny of sulfate adenylyltransferase large subunit (entire phylogeny available upon request). Sequences from *Silene* species are highlighted *in bold*. Shimodaira-Hasegawa statistical support values are denoted at nodes (significance is measured as 1 - *P*, where *P* is equal to the probability of the null hypothesis - *i.e.*, the reconstructed branch is not significantly more likely than alternative rearrangements).

eubacteria, archaea and eukaryotes use different ATP sulfurylase that shows no amino acid similarity to CysN and NodQ (Inagaki *et al.* 2002).

To check whether this bacteria-like sequence does not result from bacterial contamination, we designed a set of primers covering the sequence MK14 and performed PCR, both on tissues of greenhouse-grown plants of *S. latifolia*, *S. diclinis*, *S. dioica* and on the axenic cultures of plants grown from surface sterilized seeds of *S. latifolia* and *S. vulgaris* on the medium containing antibiotic cefotaxime. Moreover, bacteriological tests ruled out the possibility of presence of bacteria from the genus *Ralstonia* in the seeds of *S. latifolia*.

The best results were obtained with the primer combination MK14F2n and MK14Rmt (ranging over the part of conserved CysN_NodQ_III domain) amplifying fragment of expected size (201 bp). Sequencing and comparison of the sequences with the public *NCBI* databases confirmed that the amplified fragments showed a high similarity to the corresponding DNA sequences of species from the genus *Ralstonia*.

The phylogenetic relationship of the bacteria-like sequences from *Silene* species among all representatives of the sulfate adenylyltransferase (ATP sulfurylase) large subunit retrieved from the *NCBI* database was examined. Since the dataset was rather large (1 928 amino acid sequences), we decided to use approximately-maximum-likelihood phylogenetic inference by *FastTree*. Phylogenetic tree revealed clustering of bacteria-like sequences derived from the *Silene* species with entries of bacteria from the genus *Ralstonia* with high statistical significance (Fig. 2).

To show genomic organization and to further confirm the presence of the bacteria-like sequences in the genome of *S. latifolia*, we performed Southern blot hybridization on genomic DNA isolated from two different populations. By hybridizing with the probe derived from the CysN_NodQ_III part of *S. latifolia,* signals were obtained for both male and female individuals, and in both populations (one strong signal of 5.6 kb common for all individuals plus four minor signals; Fig. 3). This suggests that despite the original *Ralstonia*-like fragment MK14 was isolated from the Y-chromosome of *S. latifolia*, it is not Y-specific and most probably there are more than one copy present in the genome. Length polymorphisms were present both within and between populations. Three out of five hybridization signals are common for females and males in both populations. Within U9xCH population, there is a 8.3 kb long fragment that appears to segregate, however no linkage to sexual phenotype was detected in any fragment. Population MAVxBystrc appears to harbor an extra band that is not present in the U9xCH population.

Given the presence of bacteria-like insertion in several closely related *Silene* species (both dioecious and gynodioecious) it is likely that the insertion was already present in the ancestor of these species. Such insertion might have been acquired from some *Ralstonia*-like bacteria. Present day species of this bacterial genus are associated with various ecological niches, such as water, soil or plant rhizospheres. Some species are phyto-pathogenic, *e.g., R. solanacearum* (previously known as *Pseudomonas solanacearum*), which is a dangerous pathogen with wide host range, a causal agens of bacterial wilt (Strider *et al.* 1981). Other species (*e.g.*, *R. pickettii*) are associated with infections in humans with attenuated immunity system (Stelzmueller *et al.* 2006).

DNA can be asexually transmitted between more or less distantly related species through a process called horizontal gene transfer (HGT; Keeling and Palmer 2008). However, it has been thought that higher eukaryotes only seldom take part in this process (Kurland *et al.* 2003). As the amount of sequences is increasing, new cases of HGT including multicellular eukaryotes are recorded (*e.g.,* Keeling and Palmer 2008, Keeling 2009). In plants, numerous cases of foreign DNA uptake have been described including various donors of sequences (*e.g.*, Richardson and Palmer 2007, Bock 2010, Talianova *et al.* submitted). Examples involve genes from the *Ri* plasmid of *Agrobacterium rhizogenes* (*e.g.,* Furner *et al.* 1986), insertions of gemini- and pararetroviral sequences (*e.g.*, Bejarano *et al.* 1996, Staginnus *et al.* 2007), and transfer of genes between plant species involving mitochondrial and nuclear DNA (*e.g.*, Richardson and

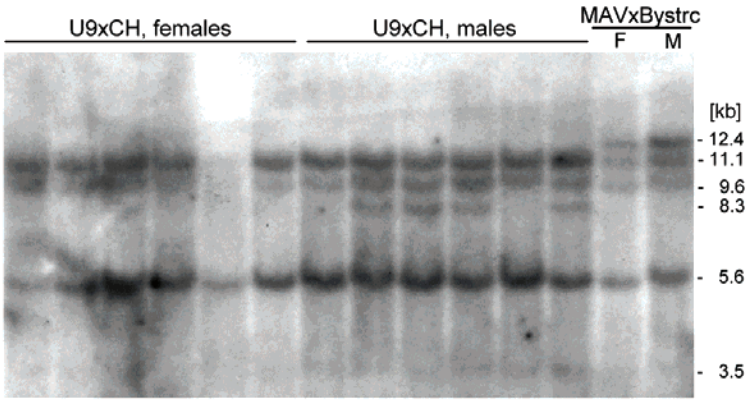

Fig. 3. Southern hybridization on the HindIII digested genomic DNA from individuals of two different *S. latifolia* populations (six female and six male individuals of the U9xCH population; one female and one male individual of the MAVxBystrc population).

Palmer 2007, Roulin *et al.* 2009, Yoshida *et al.* 2010). Moreover, there is also a report of HGT from *Ralstonia solanacearum*-like bacterium into potato (*Solanum tuberosum*) genome accompanied by protein domain reshuffling, which has resulted in a mosaic protein conferring resistance to *R. solanacearum* (Feng *et al.* 2003, Talianova *et al.* submitted). Virtually, plants are good candidates for HGT - given the fact that in contrast to animals, plants lack sequestered germline. Thus transformation of any single meristematic cell giving rise to reproductive tissues, or transformation of a cell with a capability to regenerate a novel individual might be sufficient to pass the foreign DNA to further generations.

Several scenarios could explain how the bacterial DNA entered the genome of *Silene*. A spontaneous transformation might have been promoted during infection (*i.e.*, if the ancestral bacterial donor was pathogenic) or symbiosis. An example of well documented HGT is artificial transformation of plants mediated by bacterial pathogens (*Agrobacterium tumefaciens* and *A. rhizogenes*), and bacterial symbionts (*Sinorhizobium meliloti, Mesorhizobium loti*, and *Rhizobium* sp.) once these were equipped with the tumor-inducing plasmid of *A. tumefaciens* (Broothaerts *et al.* 2005). There are yet several other though less likely possibilities for DNA transfer, such as vector-mediated transfer (*e.g.*, *via* squash-sucking insects as vehicles for bacteria) or root-mediated absorption of the naked DNA from the soil (Richardson and Palmer 2007).

Mechanisms of HGT are difficult to elucidate, since the cases of HGT are rather detected as ancient events. Some clues can be deduced based on studies of the behaviour of putative donors. A good piece of information is, *e.g.*, available from *Agrobacterium rhizogenes* mediated spontaneous transformation of *Nicotiana* species (*e.g.*, Aoki and Syōno 1999). There is a good knowledge of the action of *Agrobacterium* sp. (*A. rhizogenes* and *A. tumefaciens*) leading to plant transformation promoted by the presence of specialized plasmid. Interestingly, many other bacterial species, including species from the genus *Ralstonia,* have been shown to possess specific kind of mobile DNA called biphenyl catabolic transposon Tn4371 (Toussaint *et al.* 2003, Ryan *et al.* 2009). The region of this integrative conjugative element was found to contain several plasmid-related genes (involved in plasmid replication or partition) together with a cluster of genes corresponding to the type IV secretion system (T4SS). T4SS complexes are associated with the pathogenesis of various bacteria and are known to be involved in functions related to the delivery of substrate molecules to target cells, including the horizontal DNA transfer to both other bacteria and eukaryotic cells (Backert and Meyer 2006). Together with the ability of bacteria to easily share their genetic information this implies that there can be also other bacterial species able to deliver their DNA into the cells of eukaryotic hosts.

Another question is what happens with the DNA once incorporated into the host genome. It has been hypothesized that unless such insertions confer some adaptive role to the host organisms, they are often subjected to genetic degeneration (Keeling and Palmer 2008). In some cases expression of foreign DNA is regulated by host silencing mechanisms (Hobbs *et al.* 1990, Staginnus *et al.* 2007). To see whether the bacteria-like fragment is expressed in *S. latifolia*, we performed reverse transcription PCR with primers MK14F2n and MK14Rmt on RNA samples isolated from leaves and flower buds. However, we have not detected any transcripts of the studied bacteria-like fragment (data now shown). This might be due to the reasons mentioned above - either the sequence does not provide any advantage to the host genome and undergoes degeneration, or some regulatory processes operate on it to prevent its expression in the plant.

Our work further contributes to an increasing amount of detected cases of HGT involving plants and other higher eukaryotes. These cases suggest that natural barriers preventing an uptake of foreign DNA are probably surpassed at a higher frequency than previously thought. Our results indicate that also other bacteria than *Agrobacterium* sp. could be able to transfer their genetic material across the borderline of bacterial kingdom.

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