Scientia Horticulturae 125 (2010) 692–699

Contents lists available at ScienceDirect

Scientia Horticulturae

journal homepage: www.elsevier.com/locate/scihorti

Interspecific hybridization in *Lonicera caerulea* and *Lonicera gracilipes*: The occurrence of green/albino plants by reciprocal crossing

Tomomi Miyashita[a], Yoichiro Hoshino[a,b,*]

[a] Division of Biosphere Science, Graduate School of Environmental Science, Hokkaido University, Kita 11, Nishi 10, Kita-Ku, Sapporo 060-0811, Japan
[b] Field Science Center for Northern Biosphere, Hokkaido University, Kita 11, Nishi 10, Kita-Ku, Sapporo 060-0811, Japan

ARTICLE INFO

Article history:
Received 15 February 2010
Received in revised form 26 May 2010
Accepted 28 May 2010

Keywords:
Albino
Interspecific hybridization
Lonicera caerulea var. *emphyllocalyx*
L. gracilipes var. *glabra*
Nuclear–cytoplasmic incompatibility

ABSTRACT

Lonicera caerulea L. var. *emphyllocalyx* (Maxim.) Nakai is a berry crop cultivated in cold regions. So far, commercial cultivars have been mainly introduced from selection of wild plants. Therefore, fruit traits and other agricultural characteristics have been limited. In this study, interspecific crosses between *L. caerulea* var. *emphyllocalyx* and *Lonicera gracilipes* var. *glabra* Miquel were examined to increase genetic variability of *L. caerulea* var. *emphyllocalyx*. Seedlings were obtained from reciprocal crosses between *L. caerulea* var. *emphyllocalyx* and *L. gracilipes* var. *glabra*. The hybrid nature of seedlings was confirmed with random amplified polymorphic DNA analysis. Viable green plants were obtained efficiently from *L. gracilipes* var. *glabra* × *L. caerulea* var. *emphyllocalyx*. In contrast, all plants produced from *L. caerulea* var. *emphyllocalyx* × *L. gracilipes* var. *glabra* were albino. These albino plants were very weak and only survived in culture condition. The chlorophyll deficiency was unilaterally observed, suggesting the occurrence of nuclear–cytoplasmic incompatibility. Viable F_1 hybrids obtained from *L. gracilipes* var. *glabra* × *L. caerulea* var. *emphyllocalyx* are amphidiploid ($2n = 4x = 36$) as showing same to both parents. The hybrid plants are expected to increase the variability of fruit traits, and may have heat tolerance from *L. gracilipes* var. *glabra*.

1. Introduction

The genus *Lonicera* belongs to the family Caprifoliaceae and comprises more than 200 species (Naugžemys et al., 2007). One of them, namely, *Lonicera caerulea* L., belongs to the section *Isika*, subsection Caeruleae (Rehder, 1903). It is a deciduous shrub with edible fruits and is distributed in the northern regions of Eurasia to North America (Thompson and Chaovanalikit, 2003). This plant is commonly known as blue honeysuckles. In Japan, *L. caerulea* L. var. *emphyllocalyx* (Maxim.) Nakai grows in cold regions from alpine in middle mainland to all over the Hokkaido (Sato, 1985), and is known as 'Haskap' in the Ainu language used by the indigenous Ainu people of Hokkaido. Fruits of *L. caerulea* var. *emphyllocalyx* are blue-black in color, sour to sweet taste, and rich in nutrients, such as anthocyanins, minerals and vitamins (Anetai et al., 1996; Tanaka and Tanaka, 1998; Terahara et al., 1993). This plant species has been cultivated as a horticultural crop since 1970s in Hokkaido. Recently, it was introduced to North America as a new berry crop (Thompson, 2006). The commercial cultivars have been mainly introduced from selection of wild plants. Therefore, fruit traits and other agricultural characteristics have been limited. A major problem in the cultivation of *L. caerulea* var. *emphyllocalyx* is that fruits of wild plants are small and thin pericarp. Therefore, harvesting is laborious. In addition, cultivation of *L. caerulea* var. *emphyllocalyx* is restricted to cold region. Fruit yield and other traits also must be improved to increase commercial production. To breed this berry crop, Takada et al. (2003) evaluated the eating qualities and some horticultural characteristics of wild species, and made some elite selections. Breeding program by cross-pollination between elite strains and by producing polyploid plants are in progress (Miyashita et al., 2009; Suzuki et al., 2007). Interspecific hybridization is useful for increasing the genetic variability. However, it has not been reported in *L. caerulea* var. *emphyllocalyx*.

Lonicera gracilipes var. *glabra* Miquel is a deciduous shrub and belongs to the section *Isika*, subsection Purpurascentes (Rehder, 1903) or subsection Monanthae (Hara, 1983). This species is endemic to Japan (Theis et al., 2008). It is found in deciduous forest in altitude 20–600 m of southernmost Hokkaido, mainland, and Shikoku (Hara, 1983), which is a warm region compared to the habitat of *L. caerulea* var. *emphyllocalyx*. Fruits of *L. gracilipes* var. *glabra* are red in color and have sweet taste. This species has been planted

Abbreviations: DAP, days after pollination; DAPI, 4′,6-diamidino-2-phenylindole; MS, Murashige and Skoog (1962); dNTP, deoxyribonucleoside triphosphate; PCR, polymerase chain reaction; PVP, polyvinylpyrrolidone; RAPD, random amplified polymorphic DNA; RFLP, restriction fragment length polymorphism.

* Corresponding author. Tel.: +81 11 706 2857; fax: +81 11 706 2857.
E-mail address: hoshino@fsc.hokudai.ac.jp (Y. Hoshino).

0304-4238/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.scienta.2010.05.032

in gardens for its edible fruits (Hara, 1983). It is expected to be a new breeding material of *L. caerulea* var. *emphyllocalyx*.

In this study, we investigated the characteristics of *L. caerulea* var. *emphyllocalyx* and *L. gracilipes* var. *glabra*. Furthermore, interspecific crosses between *L. caerulea* var. *emphyllocalyx* and *L. gracilipes* var. *glabra* were examined.

2. Materials and methods

2.1. Plant materials

Two strains of *L. caerulea* var. *emphyllocalyx* (Lc-Y47 and Lc-T39) and *L. gracilipes* var. *glabra* (Lg-A and Lg-N) were used in this study. All plants were grown at the Experiment Farms in Hokkaido University.

2.2. Characterizations of plant materials

The characteristics of the plant materials were investigated as flower characters (color and size), fruit characters (color, size, fresh weight, total soluble solids, pH, seed number and seed size), and chromosome number. For measurement of flower size and fruit traits, 10 flowers or fruits were used. The total soluble solids (Brix) and pH of fruits were measured using refractometer (BRX-242, TOKYO GARASUKIKAI Co. Ltd., Tokyo, Japan) and pH meter (B-212, Horiba, Ltd., Kyoto, Japan), respectively. Statistical tests were performed using the SPSS 16.0 J program. The differences were analyzed using one-way analysis of variance (ANOVA) followed by Bonferroni's test, with $P < 0.05$ as the level of statistical significance.

2.3. Interspecific hybridization and seed culture

Interspecies crosses between *L. caerulea* var. *emphyllocalyx* and *L. gracilipes* var. *glabra* were performed. The cross combinations are listed in Table 3. Flowers were emasculated prior to anthesis and then hand-pollination was carried out. All pollinated flowers were covered with paper bags to prevent pollination by other plants. Mature fruits were harvested at more than 40 DAP. The seeds were sterilized with 1% sodium hypochlorite solution containing 1–2 drops of polyoxyethylene (20) sorbitan monolaurate (Wako Pure Chemical Industries, Ltd., Osaka, Japan) for 20 min, and then rinsed three times with sterile-distilled water. These seeds were cultured on a half-strength MS (Murashige and Skoog, 1962) medium containing 30 g l^{-1} sucrose and 2 g l^{-1} gellan gum (Wako Pure Chemical Industries, Ltd.). The pH of the medium was adjusted to 5.8 prior to autoclaving at 121 °C for 20 min. The seeds cultured on the medium were maintained in a controlled growth environment chamber at 20 °C under 24 h photoperiod (35 μmol m^{-2} s^{-1}) provided by 40 W fluorescent tubes.

2.4. Confirmation of hybrid nature with RAPD analysis

Hybrid nature was investigated with RAPD analysis. Total genomic DNA was isolated from fresh leaves using the DNeasy Plant mini kit (Qiagen, Inc., Valencia, CA, USA). PCR was carried out in volume of 25 μl containing 25 ng of genomic DNA, 2.5 μl of 1× buffer for KOD-plus-Ver.2 (Toyobo, Co. Ltd., Osaka, Japan), 0.2 mM of each dNTP, 1.5 mM $MgSO_4$, 0.6 μM primer and 0.5 U KOD-plus-Ver.2 DNA polymerase. A total of 11 RAPD primers reported by Naugžemys et al. (2007) were used in the PCR reactions. Amplification was carried out using a Bio-Rad iCycler Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA) with 1 cycle at 94 °C for 2 min and 40 cycles for 10 s at 98 °C, 30 s at 36 °C, 2 min at 68 °C according to the manufacturer's instructions of KOD-plus-Ver.2 DNA polymerase. PCR products were separated by electrophoresis in 3% agarose gels (Certified Low Range Ultra Agarose, Bio-Rad, USA). Gels were stained with ethidium bromide, visualized on UV light and photographed. Wide-Range DNA ladder (Takara Bio Inc., Shiga, Japan) was used as a size marker.

2.5. PCR-RFLP analysis of plastid DNA inheritance

PCR-RFLP analysis was performed for confirming the inheritance pattern of plastid DNA in hybrids plants. Region of Plastid DNA, *atp*β–*rbc*L, was amplified from total genomic DNA isolated from fresh leaves using primers 377 (F) (5′-GTGGAAACCCCGGGACGAGAAGTAGT-3′) and Z346 (R) (5′-AAATACGTTACCCACAATGGAAGTAAATAT-3′) described in the report of Crayn and Quinn (2000). PCR was carried out in a volumes of 50 μl containing 10 ng of genomic DNA, 2.5 μl of 1× buffer for KOD-plus-Ver.2 (Toyobo, Japan), 0.2 mM of each dNTP, 1.5 mM $MgSO_4$, 0.3 μM each primer and 1 U KOD-plus-Ver.2 DNA polymerase. Amplification was carried out using a Bio-Rad iCycler Thermal Cycler (Bio-Rad, USA) with 1 cycle at 94 °C for 2 min, and 35 cycles for 10 s at 98 °C, 30 s at 53 °C and 1 min at 68 °C according to the manufacturer's instructions of KOD-plus-Ver.2 DNA polymerase. The PCR products were digested with 10 unit of *Hae*III at 37 °C for 3 h. The restriction fragments were separated by electrophoresis in 2% agarose gels (Agarose S, Nippon Gene Co. Ltd., Tokyo, Japan). Gels were stained with ethidium bromide, visualized on UV light and photographed.

2.6. Ploidy analysis using flow cytometry

The relative DNA content of plants of *L. caerulea* var. *emphyllocalyx*, *L. gracilipes* var. *glabra* and their hybrids were determined using flow cytometry (Partec PA; Partec GmbH, Münster, Germany) according to the protocol of Miyashita et al. (in press). Fresh leaves of *Capsicum annuum* (cv. 'Kyonami') were used as the internal standard. Fresh leaves were chopped with a 0.2 ml of nuclei extraction buffer (CyStain UV precise P; Partec, Münster, Germany). After filtration through a 30 μm nylon mesh, crude nuclear samples were stained with 0.8 ml DAPI solution containing 10 mM Tris, 50 mM sodium citrate, 2 mM $MgCl_2$, 1% (w/v) PVP K-30, 0.1% (v/v) Triton X-100, and 2 mg l^{-1} DAPI (pH 7.5) (Mishiba et al., 2000). After incubation for 5 min at room temperature, the relative DNA content was measured with flow cytometry. In this study, description of genome size is expressed as C_x-value, which is designated as DNA content of a monoploid genome with chromosome number x, according to Greilhuber et al. (2005).

2.7. Chromosome analysis

For counting the chromosome number, actively growing root tips were utilized for chromosome observation. According to the procedure of Miyashita et al. (in press), chromosomes were observed as follows. The root tips were pretreated with ice water for 24 h and fixed with acetic acid:ethanol (1:3) at 4 °C overnight. The fixed root tips were treated with an enzyme mixture of 2% (w/v) Cellulase Onozuka RS (Yakult Pharmaceutical Co. Ltd., Tokyo, Japan) and 0.5% (w/v) Pectolyase Y-23 (Seishin Pharmaceutical Co. Ltd., Chiba, Japan) (Shibata and Hizume, 2002) in the citrate buffer (0.01 M citric acid and 0.01 M trisodium citrate dehydrate), pH 4.5, at 37 °C for 20 min. After treatment with the enzyme mixture, the root tips were rinsed with distilled water. Then, the root tips were squashed in a drop of 45% acetic acid on slide glass, covered, and squashed again. Cover glasses were removed by freezing glass slides in liquid nitrogen, and slides were dried at 37 °C. For staining, a drop of DAPI solution [0.233 g 1,4-diazabicyclo(2.2.2)-octane, 1 ml 0.2 M Tris–HCl, pH 8.0, 9 ml glycerol, 0.5 μg ml^{-1} of