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MICROPROPAGATION OF *RHODODENDRON KEISKEI* VAR. *HYPOGLAUCUM* SUTO & SUZUKI AND ASSESSMENT OF CLONAL FIDELITY OF PLANTLETS BY RAPD

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Abstract

An efficient reproducible protocol has been developed for *in vitro* propagation of *Rhododendron keiskei* var. *hypoglaucum*. Nodal explants were cultured on Anderson basal nutrients (AM) medium supplemented with different concentrations and combinations of plant growth regulators for axillary shoot proliferation. The highest percentage of shoot induction (96%) was achieved when nodal explants were cultured on AM medium supplemented with 2.0 mg l^{-1} 2iP, 0.5 mg l^{-1} IAA, and 1.0 mg l^{-1} GA_3 with a mean of 16 shoots per explant. Individual shoots, which were grown to about 2-3 cm long, were transferred onto a full or half strength AM medium with 0, 0.5, 1.0, 2.0 or 4.0 mg l^{-1} IBA or NAA for rooting. The highest percentage of rooting (100%), with maximum number of 7.6 roots per shoot and the greatest root length (4.4 cm) were obtained on the half-strength AM medium supplemented with 0.5 mg l^{-1} IBA. Random amplified polymorphic DNA analysis confirmed that the regenerated plantlets were genetically identical to their donor plant.

Key words: 2iP, Anderson medium, conservation, endangered species, *in vitro* propagation

INTRODUCTION

Rhododendrons are among the most important woody landscape plants (Peng et al. 2008). The genus *Rhododendron* (Ericaceae) consists of around 1200 species distributed throughout Northeast Asia, Eurasia, Western Europe, and North America (Mao et al. 2011). Gibbs et al. (2011) reported that about 25% of all *Rhododendron* taxa are under threat of extinction in the wild. *Rhododendron keiskei* var. *hypoglaucum* Suto and Suzuki is a low compact shrub with pale yellow or lemon-yellow flowers, native to Japan. It is rare and a very small population remains in Kanto District of central Honshu (Gibbs et al. 2011). For the first time we found this important plant species in the rocky cliffs of Daesambudo islet in Yeosu, Korea. Rhododendrons are conventionally propagated through cuttings and seeds. However, the rate of vegetative propagation is very slow and seed germination in nature is also very poor (Mao et al. 2011). The difficulties associated with propagation by conventional techniques may be overcome using tissue culture techniques (Sivanesan et al. 2011). *In vitro* propagation is an important asexual method that can be used for the production of clonal plants; it also forms the basis for the production of genetic variation or improvement of species by genetic transformation or somaclonal variations (Parveen et al. 2012). Although *in vitro* plant regeneration protocols have been developed for *Rhododendron* species (Iapichino et al. 1992, Marks and Simpson 1999, Tomsone and Gertnere 2003, Almeida et al. 2005, Hebert et al. 2010, Mao et al. 2011), there have been no reports on plant regeneration from explants of *R. keiskei* var. *hypoglaucum*.

The genetic homogeneity of *in vitro*-regenerated plants is very important for mass clonal propagation and conservation. Axillary shoot proliferation generally retains clonal fidelity, and variations are rarely observed among the regenerated plants (Sivanesan and Jeong 2012). Somaclonal variation is usually associated with point mutations, chromosomal rearrangements, DNA methylation, altered sequence copy number, and transposable elements (Bairu et al. 2011). Somaclonal variants can be detected using various methods such as phenotypic identification (Kong et al. 2012, Sivanesan and Jeong 2012), cytological analysis (Wang et al. 2012), estimation of nuclear DNA content (Rai et al. 2012), protein analysis (Northmore et al. 2012), and

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secondary metabolites analysis (Verma et al. 2012). Molecular analysis is being widely used for monitoring genetic homogeneity of *in vitro*-regenerated plants (Bairu et al. 2011). Random amplified polymorphic DNA (RAPD) among other molecular marker methods has considerable advantages because it is fast, not expensive, requires only small amount of DNA and in the potential detection of DNA damage and mutation (Jain et al. 2000, Atak et al. 2011). RAPD techniques have been successfully used to assess genetic variations in rhododendrons (Dunemann et al. 1999, Lanying et al. 2008, Liu et al. 2012). Jain et al. (2000) studied genetic variability and relationships of geographically isolated population of *Rhododendron arboretum* by employing RAPD assay. RAPD revealed that higher genetic variability among the individuals of temperate *Rhododendron* populations. Lanying et al. (2008) also studied RAPD analysis to determine the genetic diversity and relationship of *Rhododendron* species. Recently, RAPD has been successfully used to detect *in vitro*-induced *Rhododendron* mutants (Atak et al. 2011).

The objectives of this study were to investigate the effect of plant growth regulators (PGRs) on axillary shoot multiplication and to evaluate the genetic homogeneity of *in vitro*-regenerated plants using RAPD technique.

MATERIALS AND METHODS

Plant materials

Plants were collected from the rocky cliffs of Daesambudo islet in Yeosu, South Korea and maintained in the glasshouse. Actively growing healthy shoots (6 cm in length) were separated from glasshouse-grown plants and were washed thoroughly in running tap water for 30 min and soaked in 0.1% (v/v) detergent solution (Teepol ™ 610 S, Sigma-Aldrich Korea Ltd., Kyunggi-do, South Korea) for 5 min, and then washed three times with distilled water. Thereafter, in aseptic conditions, explants were surface disinfested with a solution of 70% (v/v) ethanol for 60 s, followed by 5.0% (v/v) sodium hypochlorite for 15 mins. Finally, they were rinsed five times with sterile distilled water and cut into nodal segments (0.5-1.0 cm).

Culture medium and conditions

The medium consisted of Anderson (Anderson 1984) basal nutrients (AM) supplemented with 3% (w/v) sucrose, and solidified with 0.8% (w/v) plant agar (P1001.1000, Duchefa Biochemie, The Netherlands). The pH of the medium was adjusted to 5.70 ± 0.02 before autoclaving at 121°C for 15 min. Gibberellic acid (GA_3) was filter-sterilized and added to the autoclaved medium. Other PGRs were added to a basal medium prior to pH adjustment and sterilization. All cultures were maintained at 25 ± 1°C under a 16-h photoperiod with 45 µmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD) provided by cool white fluorescent lamps (Philips Lifemax, TLD 32W/840 RS Cool White).

Effect of PGRs on axillary shoot induction and multiplication

Nodal explants were cultured on AM basal nutrients medium supplemented with 0, 0.5, 1.0, 2.0 or 4.0 mg l^{-1} N^6-(2-Isopentenyl)adenine (2iP) for axillary shoot induction or in combination with 0.1 or 0.5 mg l^{-1} indole-3-acidic acid (IAA), and 1.0 mg l^{-1} gibberellic acid (GA_3) for axillary shoot multiplication. The number of explants initiating shoot buds and average number of shoot buds per explant were recorded after 8 weeks. For each treatment, 15 explants were used and each experiment was repeated three times.

Rooting

Axillary shoots, which were grown to about 2-3 cm long, were transferred onto a full or half strength AM medium with 0, 0.5, 1.0, 2.0 or 4.0 mg l^{-1} indole-3-butiric acid (IBA) or α-naphthaleneacetic acid (NAA) for rooting. After 7 weeks, the percentage of root induction, number of roots per shoot and roots length were recorded. For each treatment, 50 shoots were used and experiment was repeated three times. All data collected were subjected to analysis of variance (ANOVA) by using SAS program (Release 9.1, SAS Institute, NC, USA), and Duncan's multiple range test was used to asses significant differences between mean values.

Molecular analysis of regenerated plantlets by RAPD

To study the clonal fidelity, we carried out RAPD analysis on the genomic DNA of randomly-selected 7 regenerated plants, along with the donor plant. Total genomic DNA of the donor plant and *in vitro* raised plants was extracted from young leaf tissue by using the plant DNA isolation kit (Invitrogen, Seoul, Korea). The concentration and quality of DNA samples were calculated from the Optical Density (OD) values at 260 and 280 nm and they were prepared of standardized DNA (20 ng μl^{-1}). Sixty-four RAPD primers (Enotech, Daejeon, Korea) were used for initial screening. The PCR amplifications were performed in a Thermal Cycler PCR (GeneAmp PCR System 9700). PCR amplification reactions were carried out in a total volume of 20 µl. The mixture contained 0.5 µM of each primer, 40 ng of extracted DNA, 200 µM of each dNTP, 1.5 mM $MgCl_2$, and one unit of DNA polymerase (EnZynomics, Daejeon, Korea). PCR reaction conditions were as follows: an initial denaturizing at 94°C for 4 min followed by 40 cycles of 30 s at 94°C, 30 s at 45°C, 1 min at 72°C and finally extended at 72°C for 10 min. The amplified products were resolved by electrophoresis on 1.8% agarose gel in tris-borate EDTA (TAE) buffer

and stained with ethidium bromide. The fragment sizes were estimated with 100 bp - 1500 bp DNA ladder (Bio Basic Inc, Canada).

RESULTS AND DISCUSSION

The surface disinfection procedure yielded 76% aseptic explants. Nodal explants failed to develop shoots in a PGR-free AM basal nutrients medium even after 8 weeks of culture. Although AM medium with vitamins has been widely used for shoot induction of *Rhododendron* species. The addition of PGRs to the culture medium is required for shoot induction of many *Rhododendron* species (Almeida et al. 2005, Vejsadova 2008). When the AM medium was supplemented with different concentrations of 2iP, axillary shoots were initiated from the nodal explants within 2 weeks of culture. The stimulating effect of 2iP in axillary shoot induction has also been reported in some *Rhododendron* species (Marks and Simpson 1999, Vejsadová 2008, Singh and Gurung 2009, Mao et al. 2011). The percentage of shoot induction and average number of shoots per explant varied among the different concentrations of 2iP studied (Table 1). An increase in the concentration of 2iP from 0.5 to 2.0 mg l^{-1} significantly increased shoot induction percentage, although further increases in the 2iP concentration decreased the percentage of shoot induction. Maximum percentage of shoot induction (63%), with a mean number of 3.6 shoots per explant was obtained on AM medium supplemented with 2.0 mg l^{-1} 2iP. Similar results were also reported in *Erica multiflora* (Iapichino et al. 2012).

The effect of auxin and cytokinin combination on shoot induction showed that a combination of 2iP and IAA maximized shoot induction percentage and the average number of shoots per explant significantly than 2iP alone. This agrees with the results of earlier studies on other *Rhododendron* species (Singh and Gurung 2009, Mao et al. 2011). Of the various combinations studied, AM medium with 2.0 mg l^{-1} 2iP and 0.5 mg l^{-1} IAA was optimum for axillary shoot proliferation (Table 2). On this medium, 92.4% of the explants responded, with a mean number of 12.3 shoots per explant. Similar results were also reported in *Rhododendron* cultivars (Vejsadova 2008) and *R. maddeni* (Singh and Gurung 2009).

Table 1. Effect of 2iP on multiple shoot formation from nodal explants of *Rhododendron keiskei* var. *hypoglaucum*.

2iP (mg l^{-1})	Shoot induction (%)	No. of shoots per explant
0	0.0 ± 0.0	0.0 ± 0.0
0.5	43.6 ± 4.4 d	1.6 ± 0.4 b
1.0	49.4 ± 2.6 c	2.0 ± 0.6 b
2.0	63.0 ± 2.0 a	3.6 ± 0.4 a
4.0	57.0 ± 3.0 b	3.4 ± 1.0 a

Means ± SE within a column followed by the same letters are not significantly different ($p \leq 0.05$).

Addition of GA_3 at 1.0 mg l^{-1} in shoot proliferation medium (2.0 mg l^{-1} 2iP and 0.5 mg l^{-1} IAA) stimulated both shoot induction percentage and number of shoots per explant (Table 2). The positive effect of GA_3 on shoot regeneration has been reported in many plant species (Kaushal et al. 2005, Isogai et al. 2008, Perez-Tornero et al. 2010). The highest percentage of shoot induction (96%) was achieved when nodal explants were cultured on AM medium supplemented with 2.0 mg l^{-1} 2iP, 0.5 mg l^{-1} IAA and 1.0 mg l^{-1} GA_3 with a mean of 16 shoots per explant (Fig. 1A, B). Similar result was also observed in *Rosa rugosa* (Xing et al. 2010).

The shoots failed to develop roots in full-strength AM medium even after 8 weeks of culture. Similar result was observed in *R. maddeni* (Singh and Gurung 2009). In contrast, root induction was observed on AM medium in *R. dalhousiae* var. *rhabdotum*, *R. elliottii*, and *R. johnstoneanum* (Mao et al. 2011). The shoots developed roots on the half-strength AM medium and the percentage of root induction was 63.8%. Reduced strength of basal medium is often used for rooting of adventitious shoots (Sivanesan and Jeong 2007). Thus, half-strength AM medium was used for further rooting experiments. The addition of IBA or NAA at 0.5 mg l^{-1} in the basal medium significantly promoted rooting

Table 2. Effect of plant growth regulators on multiple shoot formation from nodal explants of *Rhododendron keiskei* var. *hypoglaucum*.

PGRs (mg l^{-1})			Shoot induction (%)	No. of shoots per explant
2iP	IAA	GA_3		
1.0	0.1	0	76.4 ± 1.7 d	6.7 ± 1.4 c
2.0	0.1	0	83.0 ± 1.0 c	9.8 ± 1.2 b
1.0	0.5	0	87.4 ± 1.2 b	8.6 ± 1.4 b
2.0	0.5	0	92.4 ± 1.6 ab	12.3 ± 1.8 ab
2.0	0.5	1.0	96.0 ± 1.2 a	16.0 ± 1.0 a

Means ± SE within a column followed by the same letters are not significantly different ($p \leq 0.05$).

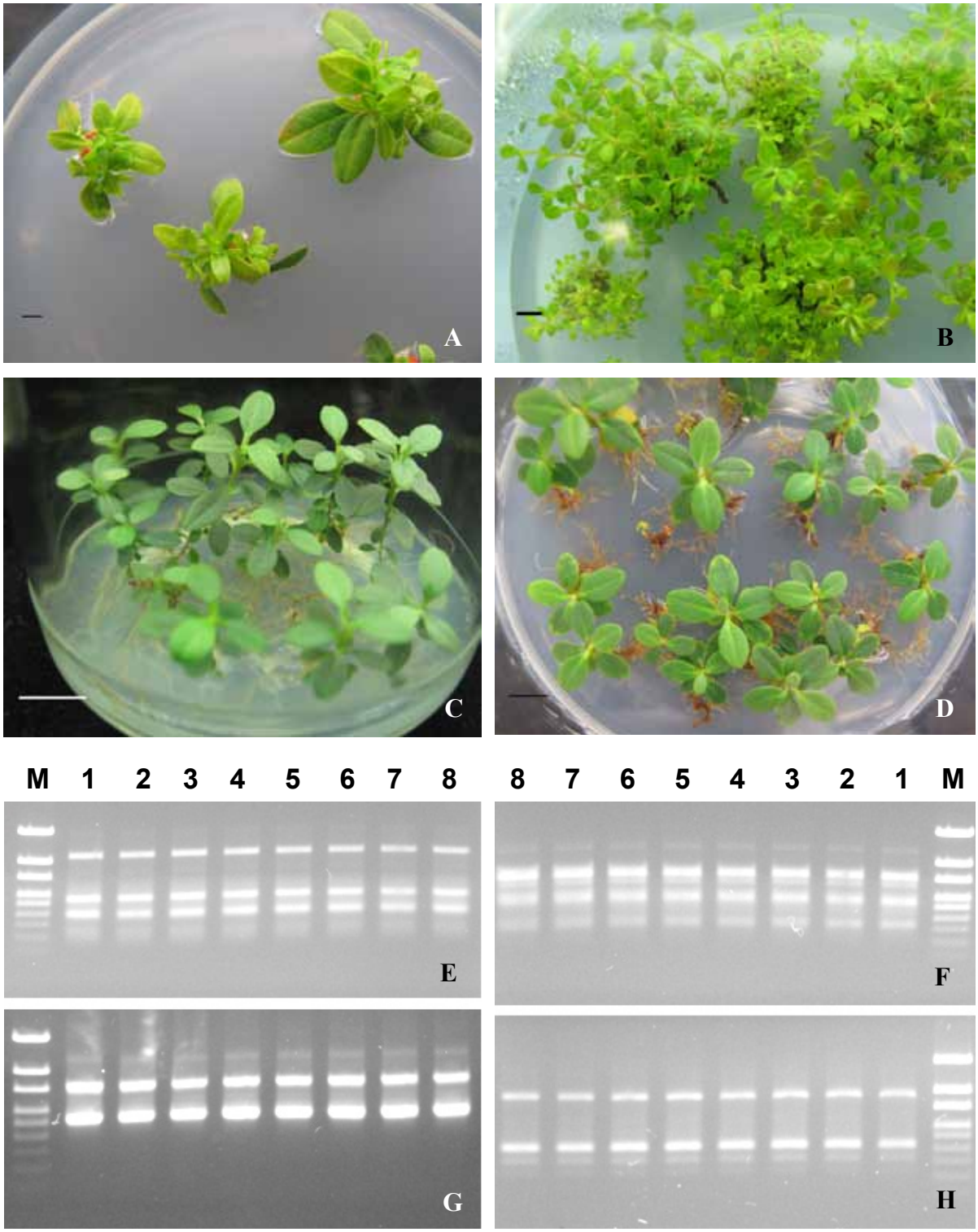

Fig. 1. Axillary shoot multiplication from nodal explants of *Rhododendron keiskei* var. *hypoglaucum* cultured on AM medium supplemented with 2.0 mg l^{-1} 2iP, 0.5 mg l^{-1} IAA and 1.0 mg l^{-1} GA_3. A) after 4 weeks of culture and B) after 8 weeks of culture. Scale bar indicate 1.0 cm. Rooting of *Rhododendron keiskei* var. *hypoglaucum* on half-strength AM medium containing C) 0.5 mg l^{-1} IBA and D) 1.0 mg l^{-1} IBA. Scale bar indicate 2.0 cm. Polymerase chain reaction (PCR) amplification products obtained with RAPD primers E) OPA12, F) OPB5, G) OPC1, and H) OPD6. M represents 100 bp-1500 bp ladder, 1 represents donor plant, Lane 2 to 8 represent in vitro raised clones of *Rhododendron keiskei* var. *hypoglaucum*.

Table 3. Effect of different concentrations of auxins on root induction.

Concentration (mg l^{-1})	Root induction (%)		No. of roots per shoot		Root length (cm)	
	IBA	NAA	IBA	NAA	IBA	NAA
0.0	63.8 ± 3.2 b	63.8 ± 3.2 c	2.3 ± 0.7 c	2.3 ± 0.7 b	1.6 ± 0.4 c	1.6 ± 0.4 b
0.5	100.0 ± 0.0 a	91.2 ± 1.8 b	7.6 ± 1.0 a	5.8 ± 2.2 a	4.4 ± 0.6 a	3.0 ± 0.2 a
1.0	100.0 ± 0.0 a	C + R	5.0 ± 1.0 b	C + R	2.7 ± 0.4 b	C + R
2.0	C + R	C	C + R	C	C + R	C
4.0	C + R	C	C + R	C	C + R	C

C - callus formation, C + R – callus and root formation.
Means ± SE within a column followed by the same letters are not significantly different ($p \leq 0.05$).

(Table 3). The effectiveness of IBA and NAA on rooting of the *in vitro*-regenerated rhododendron shoots has been reported earlier (Singh and Gurung 2009, Mao et al. 2011). The highest percentage of rooting (100%), with maximum number of 7.6 roots per shoot and the greatest root length (4.4 cm) were obtained on the half-strength AM medium supplemented with 0.5 mg l^{-1} IBA (Fig. 1C). The stimulating effect of IBA in root formation has been reported in *Rhododendron* species (Almeida et al. 2005, Singh 2008, Singh and Gurung 2009, Mao et al. 2011). An increase in concentration above 0.5 mg l^{-1} IBA reduced root length and promoted callusing (Fig. 1D).

The genomic variations in phenotypically normal regenerants have been documented in *Amorphophallus albus* (Hu et al. 2008) and *Cliva miniata* (Wang et al. 2012). The genetic homogeneity is required for clonal propagation and germplasm conservation. The RAPD technique has been applied to many plants to evaluate genetic homogeneity or heterogeneity among the micropropagated and the donor plants (Atak et al. 2011, Bairu et al. 2011, Bhatia et al. 2011, Ramesh et al. 2011, Thankappan and Morawala-Patell 2011). Out of 64 different RAPD primers screened, 17 generated clear, reproducible and scorable bands. The number of scorable bands for each primer varied from 2 (OPA-12) to 9 (OPJ-6) (Table 4). The 17 RAPD primers produced 71 distinct and scorable bands in the size range of 200 to >1500 bp, with an average of 4.18 bands per primer. No polymorphic bands were observed between the *in vitro*-regenerated plants and the donor plant (Fig. 1E-H). The absence of genetic variation in the micropropagated plants using RAPD technique has also been reported earlier in *Camellia sinensis* (Mondal and Chand 2002), *Dendranthema grandiflora* (Minano et al. 2009), *Dianthus caryophyllus*, and *D. chinensis* (Kumawat et al. 2013), *Dioscorea prazeri* (Thankappan and Morawala-Patell 2011), and *Swertia chirata* (Chaudhuri et al. 2008). Thus, this micropropagation protocol can be utilized for *in vitro* mass clonal propagation of *R.*

Table 4. The random amplified polymorphic DNA (RAPD) primers utilized to verify *Rhododendron keiskei* var. *hypoglaucum* clones.

Primers	5'-3' motif	No. of scrorable bands	Range of amplification (bp)
OPA-12	TCGGCGATAG	3	350-1000
OPA-13	CAGCACCCAC	3	200-500
OPA-17	GACCGCTTGT	5	100-1500
OPA-18	AGGTGACCGT	3	200-500
OPB-01	GTTTCGCTCC	5	100-700
OPB-03	CATCCCCCTG	4	200-600
OPB-05	TGCGCCCTTC	4	300-1200
OPC-01	TTCGAGCCAG	3	300-1000
OPC-04	CCGCATCTAC	5	100-500
OPD-03	GTCGCCGTCA	5	300-1200
OPD-06	ACCTGAACGG	2	600-1000
OPE-03	CCAGATGCAC	3	500-1000
OPE-07	AGATGCAGCC	7	200->1500
OPG-02	GGCACTGAGG	2	200-700
OPG-03	GAGCCCTCCA	2	500-1100
OPJ-01	CCCGGCATAA	6	200->1500
OPJ-06	TCGTTCCGCA	9	200->1500

keiskei var. *hypoglaucum*.

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