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Morphological, Cytological and Molecular Evidence for Intersubgeneric F_1 Hybrid between *Glycine max* x G. *tomentella*

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This study was carried out to demonstrate morphological, cytological and molecular evidence for intersubgeneric F_1 hybrid between *Glycine tomentella* and *G. max* cv. 'Baemkong'. Morphological features of F_1 plant for pistil and stamen, flower color and growth habit showed intermediate type between *G. tomentella* and *G. max* cv. 'Baemkong'. Chromosome number of F_1 plant was 2n=39, which explained the evidence of F_1 hybrid between *G. tomentella* (2n=38) and *G. max* cv. 'Baemkong' (2n=40). Polyacrylamide isoelectric focusing pattern for esterase and peroxidase also showed that the F_1 plant was true F_1 hybrid between *G. tomentella* and *G. max* cv. 'Baemkong'. From RAPD analysis, we identified that 62 primers showing bands in F_1 hybrid had both bands from *G. tomentella* and *G. max* cv. 'Baemkong', which suggested that this was true F_1 hybrid. Based on our results from morphological, cytological and molecular analyses, we suggest that the F_1 plant was true intersubgeneric hybrid between *G. tomentella* and *G. max* cv. 'Baemkong'. Our results still remain us further study to recover fertility of F_1 hybrids. The occurrence of maternal and/or paternal inheritance in F_1 hybrid from intersubgeneric cross between *G. tomentella* and *G. max* cv. 'Baemkong' need to be explained.

Key words : *G. max*, *G. tomentella*, F_1 hybrid, morphological traits, chromosome number, polyacrylamide isoelectric focusing, RAPD

Introduction

Soybean is one of the most important crops accounting for about 30% of the vegetable oil and 60% of the vegetable protein in world production. In Korea, it has been cultivated for thousands of years, and has been treated as important traditional diets with having many uses such as bean paste, soy source, tofu, bean sprout, etc.. The use of soybean is increasing worldwide. However, the elucidation of soybean genetics has proceeded slowly in comparison with other major crops such as corn and rice due to the large genome size, inherent difficulties in performing crosses, a lack of genetic variation in the germplasm, and a lack of cytogenetic stocks [12]. The soybean genome contains an estimated 1.29×10^9 bp [10] to 1.81×10^9 bp [9]. Delannay et al. [8] reported that 10 plant introductions contributed more than 80% of the northern gene pool, and 7 plant introductions contributed more than 80% of the southern gene pool. Specht and Williams [33] reported that only 16 plant introductions were the maternal or cytoplasmic ancestor or the 136 cultivars released since 1939. And, Cheng and Hadley [5] reported that essentially 5 ancestors served as the ultimate cytoplasm source for the bulk of the 136 cultivars.

The genus *Glycine* Willd. is composed of two subgenera: *Glycine* and *Soja* (Moench) F.J. Herm. The wild perennial soybeans belong to the subgenus *Glycine* and have a wide array of genomes. The cultivated soybean [*Glycine max* (L.) Merr.] and its wild annual progenitor [*G. soja* (Sieb. and Zucc.)] belong to the subgenus *Soja*, contain 2n=40 chromosomes, are cross-compatible, usually produce vigorous fertile F_1 hybrids, and carry similar genomes [22].

Utilization of wild species for genetic improvement of their cultivated counterparts is steadily increasing in various crops [32]. Exploitation of the wild progenitors is a reasonable approach because a cultigen (e.g., the soybean) and its wild progenitor (*G. soja*) are genetically members of the same species, and gene transfer between them is a relatively easy task. Use of other wild species, such as those belonging to the secondary or tertiary gene pools of the cultigen is much more difficult because various types of isolating mechanisms that prevent gene flow between different biological units must be overcome [11]. On the other hand, because of genetic remoteness and unique selection pressures on these wild species possess variation in eco-

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nomically valuable characteristics that may be missing in the cultivated germplasm [22].

Many of the previous studies have reported that wild perennial *Glycine* possesses agronomically useful traits. Lim and Hymowitz [17] reported that several wild perennial *Glycine* accessions carry resistance to brown spot. Resistance to soybean rust [28], yellow mosaic virus [30], and powdery mildew [18] has been reported. Accessions that are salt tolerant [24] and tolerant to herbicides [35] have also been identified.

Wild perennial *Glycine* species have not been exploited in soybean breeding programs because of the extremely low crossability and the need to employ immature seed-rescue techniques to obtain F_1 hybrids [22]. Brown et al. [3] reported the molecular phylogenetic relationships within and among diploid races of *G. tomentella*. Rauscher et al. [25] reported the multiple origins and nrDNA internal transcribed spacer homeologue evolution in the *G. tomentella* allopolyploid complex. And, Zou et al. [36] reported that SSR marker and ITS cleaved amplified polymorphic sequence analysis of soybean x *G. tomentella*. intersubgeneric derived lines.

Several sterile intersubgeneric F_1 hybrids were reported in the previous studies [20,31]. The results suggest that wild perennial *Glycine* species constitute the tertiary gene pool of soybean [11]. Artificially synthesized amphiploids (2n=118) of *G. max* (2n=40) x *G. tomentella* (2n=78) produced a few one- or two-seeded pods [20,29].

Kim and Chang [15] reported the intersubgeneric cross between *G. max* cv. 'Baemkong' and four strains of *G. tomentella* (2n=38, 40, 78 and 80), and reported several morphological features. Results from this study are summarized as follows: (1) Crossing efficiency and pod survival rate were highest from the cross between 'Baemkong' and 2n=38 strain of *G. tomentella* when 'Baemkong' was used as a female parent. (2) In reciprocal cross, they found out that crossing efficiency and pod survival rate were better when 'Baemkong' was used as a female parent. (3) The best time for the intersubgeneric cross between *G. max* cv. 'Baemkong' and *G. tomentella* was Aug. 20 when the temperature and relative humidity were high. However, they suggested the molecular examination to confirm their results.

The objective of this study was to examine morphological, cytological and molecular evidence for intersubgeneric F_1 hybrid between *G. tomentella* (2n=38) and *G. max* cv. 'Baemkong' (2n=40).

Materials and Methods

As Kim and Chang [15] reported previously, 24 F_1 plants were regenerated through ovule culture from intersubgeneric cross between *G. tomentella* (2n=38) as a maternal parent and *G. max* cv. 'Baemkong' (2n=40) as a paternal parent. Morphological traits for pod shape, flower color, and growth habit for both parents and 24 F_1 plants were investigated in this study.

Root tips from both parents; *G. tomentella* and G. max cv. 'Baemkong', and F_1 plants were collected. They were soaked in a few drops of 1 M HCl in a watch glass for 10 min, and transferred to a few drops of acetocarmine stain for 15 min. To prepare a squash, root tips were trimed to 2 mm. One tip/each was transferred to a few drops of water on a slide. Cover slip was lowered onto the tip and pressed down gently to squash. Chromosome number was examined.

Horizontal isoelectric focusing (IEF) was performed in mini-polyacrylamide gels (Amersham Pharmacia Biotech.). After IEF, gels were stained for esterase and peroxidase. Fast Blue RR (16 mg) was dissolved in a mixture of 10 ml 0.1 M KH_2PO_4/NaOH buffer at pH 7.0 (=6.804 g KH_2PO_4+1.164 NaOH in 1,000 ml H_2O), 10 ml H_2O and 800 µl α-naphtylacetate solution (1% w/v α-naphtylacetate in 50% v/v acetone). In order to avoid precipitation of undissolved Fast Blue RR, the solution was filtered before pouring onto the gel. After rinsing with water for 30 min, gels were dried.

Plant leaves from *G. tomentella* and F_1 hybrid were harvested from the plants which have been kept in the glasshouse. Seeds of *G. max* cv. 'Baemkong' were sown in the greenhouse and young leaves from seedlings were harvested. Total genomic DNA was extracted using the CTAB method [26] with modifications for soybeans as outlined by Keim et al [13]. The isolated DNA was quantified on a fluorometer (Hoefer Scientific Instruments, CA) using the Hoechst 33258 dye (Hoefer Scientific, CA). Each DNA was diluted with 1× TE for a final concentration of 60 ng/µl. The 10-base nucleotide primers from Operon Technologies, Inc. were used in Polymerase Chain Reaction (PCR). Approximately 60 ng DNA was used as a template in a 25 µl reaction volume that contained 4.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl, pH 8.0, 200 µM dNTPs

(Pharmacia LKB Biotech.), 0.4 μM primer, and 0.5 units Ampli-Taq Polymerase (Perkin-Elmer). Amplifications (45 cycles of 1 min at 94°C, 1 min at 36°C, and 2 min at 72°C) were performed in a Perkin-Elmer Cetus DNA Thermal Cycler. Amplified products were separated electrophoretically on 1.4 % agarose gel at 65 V for 4 hr and visualized by staining with ethidium bromide. Polymorphisms were scored based on the presence or absence of DNA bands.

Results and Discussion

It is desirable to incorporate agronomically useful traits that wild species possess into the cultivated germplasms in soybean. In this study, we report pistil and stamen, flower color, and growth habit as morphological traits, chromosome number as cytological evidence, and polyacrylamide isoelectric focusing pattern and RAPD analysis as molecular evidence to assure the F_1 hybrid from the intersubgeneric cross between *G. tomentella* and *G. max* cv. 'Baemkong'.

Morphological traits for pistil and stamen, flower color, and growth habit between *G. tomentella*, *G. max* cv. 'Baemkong', and F_1 hybrid are shown in Fig. 1. Flower structure for pistil and stamen in both *G. tomentella* and *G. max* cv. 'Baemkong' were normal, but that of F_1 hybrids was abortive and sterile (Fig. 1). Flower color in *G. max* cv. 'Baemkong' was pink and *G. tomentella* was purple. F_1 hybrids showed purple flower color, which resembles *G. tomentella* (Fig. 1). Growth habit of *G. tomentella* was perennial type and that of *G. max* cv. 'Baemkong' was annual type. F_1 hybrids showed perennial type, but showed intermediate type between both parents (Fig. 1). Based on morphological features, we propose that 24 F_1 plants we tested were true F_1 hybrids between *G. tomentella* and *G. max* cv. 'Baemkong'. Newell and Hymowitz [19] reported that morphological trait of F_1 plants regenerated through ovule culture between *G. max* and *G. tomentella* resembled *G. tomentella* than *G. max*. Singh and Hymowitz [31] also reported that morphological trait of F_1 plants between *G. max* and *G. tomentella* resembled that of *G. tomentella* than that of *G. max*. Chung [7] reported that petiole length and leaf color of F_1 plant resembled those of *G. max* and hypocotyl color and leaf shape resembled those of *G. tomentella*, but flower structure was intermediate type. From these results he proposed that F_1 plant was true hybrid between *G. max* and *G. tomentella*. Our results confirmed these previous results. Especially, F_1 hybrids between *G. max* cv. 'Baemkong' and *G. tomentella* showed perennial growth habit, which explain the possibility for utilization of these hybrids as germplasms for perennial type soybean cultivar. However, the stamen of F_1 hybrids was abortive in flower structure. This remains us further study to recover fertility and the incorporation of useful traits through backcross.

Fig. 2 shows chromosomes from root tips of *G. tomentella* (2n=38), *G. max* cv. Baemkong (2n=40), and F_1 plants. Chromosome number of F_1 plants was 2n=39. Newell and Hymowitz [19] reported that chromosome number of F_1 plants was 2n=59 from the cross between *G.*

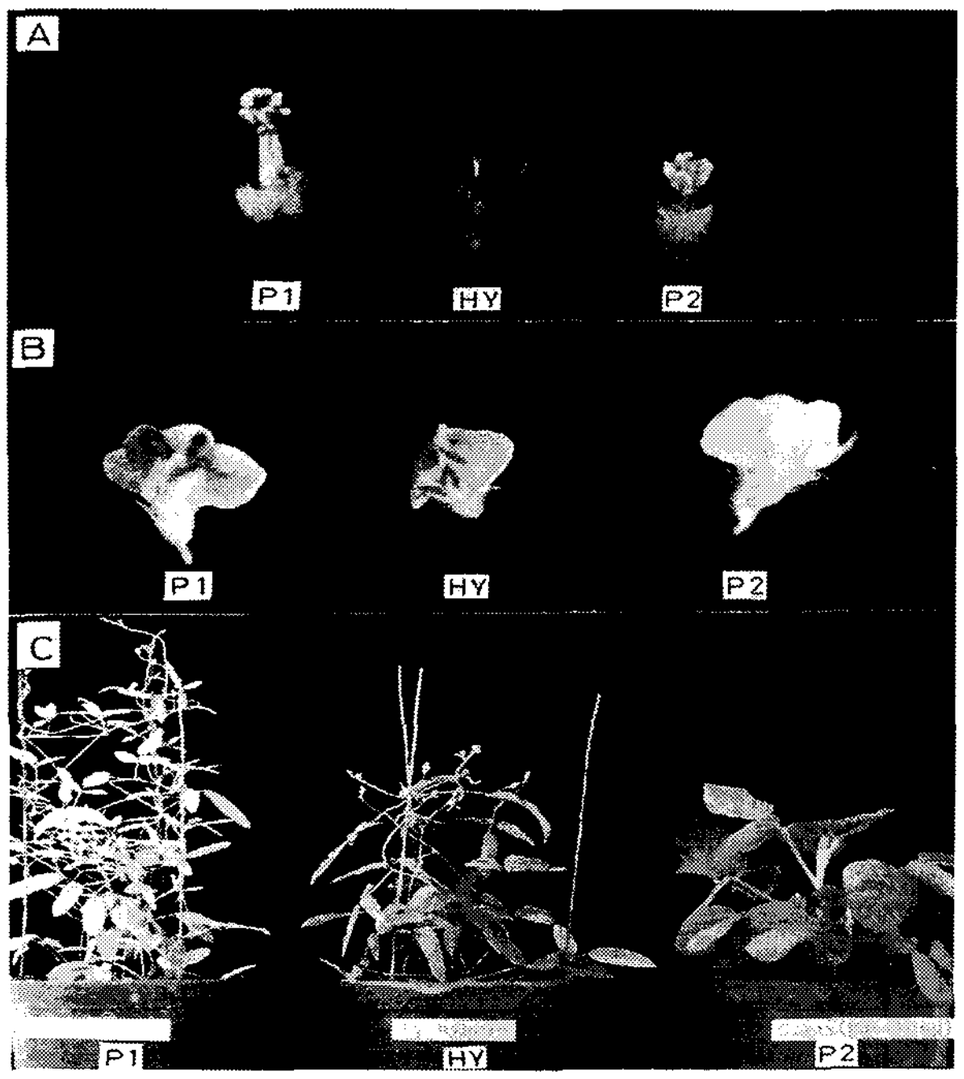

Fig. 1. Morphological features of pistil and stamen, flower color, and growth habit in *Glycine tomentella*, *G. max* cv. 'Baemkong', and F_1 hybrid. A: pistil and stamen, B: flower color, C: growth habit, P_1: *G. tomentella*, P_2: *G. max* cv. 'Baemkong', *HY*: F_1 hybrid.

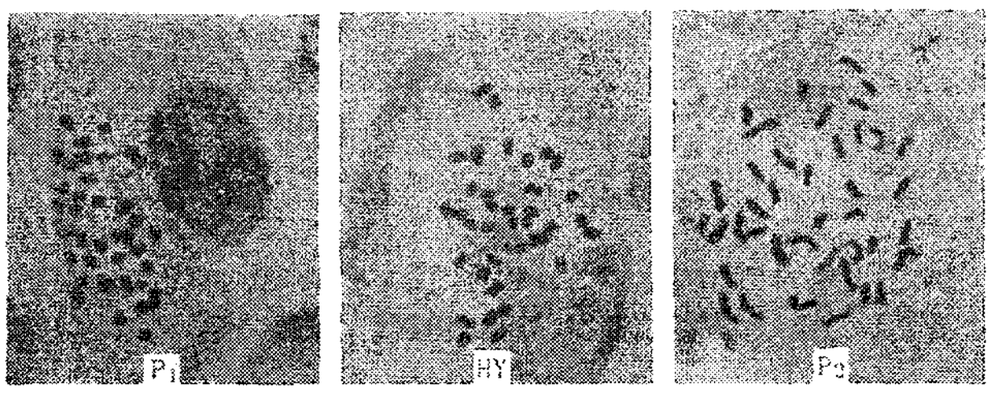

Fig. 2. Chromosome number from root tip of *Glycine tomentella* (2n=38), *G. max* cv. 'Baemkong' (2n=40), and F_1 hybrid (2n=39). P_1: *G. tomentella*, P_2: *G. max* cv. 'Baemkong', *HY*: F_1 hybrid.

tomentella (2n=78) and *G. max* (2n=40). From the result of chromosome number, we suggest that 24 F_1 plants we tested were true F_1 hybrids between *G. max* cv. 'Baemkong' and *G. tomentella*.

Polyacrylamide isoelectric focusing pattern of *G. tomentella, G. max* cv. 'Baemkong', and F_1 plant for esterase and peroxidase is shown in Fig. 3 (A: esterase, B: peroxidase). Band 1 and 3 for esterase isozyme pattern in F_1 plant were inherited from *G. tomentella*. Band 2 turned out to be inherited from *G. max* cv. 'Baemkong'. Peroxidase isozyme band 3 of F_1 plant was inherited from *G. max* cv. 'Baemkong', and band 5 showed identical to that of *G. tomentella*. Band 2 for peroxidase isozyme pattern in F_1 plant was weaker than that of both parents. Band 1 in F_1 plant could not be detected in both parents, and band 4 in both parents could not be detected in F_1 plant. Kiang and Gorman [14] reported that isozyme pattern could be used as an important evidence for confirming F_1 plant from intersubgeneric crosses. Broue et al. [2] reported the identification of synthetic amphiploid and F_1 hybrids between *G. tomentella* and *G. canescens* by indophenol oxidase isozyme pattern. Sakai and Kaizuma [27] reported the confirmation of regeneration of hybrid embryo using peroxidase zymogram. Isozyme patterns of our results agreed with those of previous results by Broue et al. [2], Chung [7], and Sakai and Kaizuma [27]. From polyacrylamide isoelectric focusing pattern, we suggest that 24 F_1 plants we tested were true F_1 hybrids between *G. max* cv. 'Baemkong' and *G. tomentella*.

Results from RAPD analysis are summarized in Table 1. Total number of primer we tested was 100 (OP kit A to E). Sixty-five primers generated well resolved bands, but 35

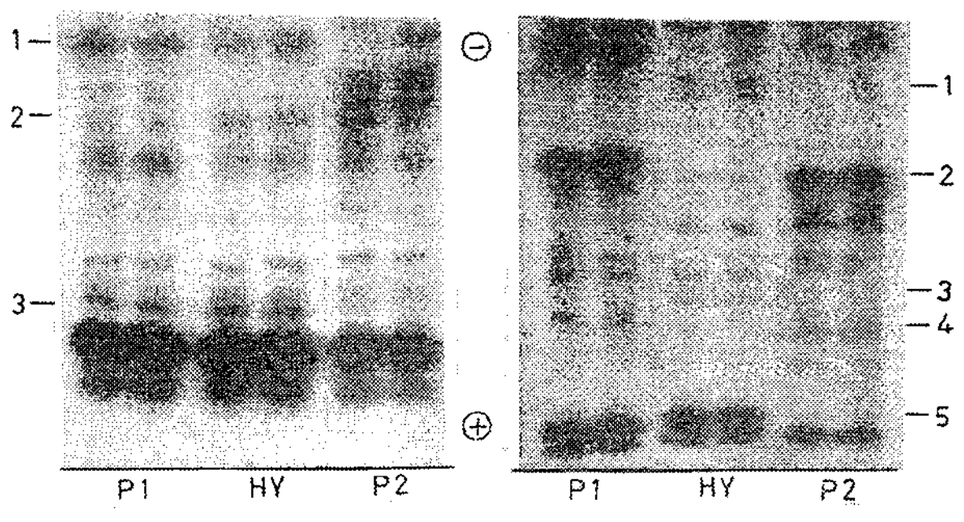

Fig. 3. Polyacrylamide isoelectric focusing pattern of *Glycine tomentella, G. max* cv. 'Baemkong', and F_1 hybrid. A: Esterase, B: Peroxidase, P_1: *G. tomentella*, P_2: *G. max* cv. 'Baemkong', *HY*: F_1 hybrid.

Table 1. RAPD polymorphisms between *Glycine max* cv. 'Baemkong' and *G. tomentella*

Total number of primers tested	100
Number of polymorphic primers	65
Number of monomorphic primers	0
Number of not good working primers	35
Total number of bands generated	431 (100 %)
Number of polymorphic bands	363 (84.2 %)
Number of monomorphic bands	68 (15.8 %)

primers generated not well resolved bands or not worked at all. Thus, we excluded these primers from our analysis. Number of polymorphic primers was 65, which accounted for 100 % of good working primers. In other words, we didn't find out any monomorphic primer (Table 1). The total number of bands we generated from 65 primers which gave us the clear bands was 431. Three hundred sixty-three bands that accounted for 84.2 % of total number of bands were polymorphic, and 68 bands (15.8 %) were monomorphic (Table 1).

Polymorphic frequency from RAPD analysis in this study was extremely high when comparing with previous results from inter- and intraspecific crosses of *G. max* x *G. soja* and *G. max* x *G. max* in soybean and other crops. In RFLP analysis, Apuya et. al. [1] reported the generation of about 20% of the polymorphisms from the probes tested in the interspecific cross of *G. max* x *G. soja*, and about one third of probes mapped in the interspecific cross were useful for mapping in intraspecific cross of *G. max* x *G. max*. Choi [6] has reported the polymorphisms of 35.0% between Essex and PI 437654, 38.3% between Essex and PI 88788, and 40.4% between Essex and Peking from intraspecific cross of *G. max* x *G. max*.

Sixty-two primers from 65 good working primers showed the bands in F_1 hybrid had both bands from both parents, which suggested that this is true F_1 hybrid from *G. max* cv. 'Baemkong' and *G. tomentella*. Examples of these primers are shown in Fig. 4.

Two primers (OPA02 and OPA09) showed the bands in F_1 hybrid had bands from *G. tomentella* (2n=38), which suggested the maternal inheritance. These primers are shown in Fig. 5.

On the other hand, 1 primer (OPD05) showed the bands in F_1 hybrid had bands from *G. max* cv. 'Baemkong', which suggested the paternal inheritance. This primer is shown in Fig. 6.

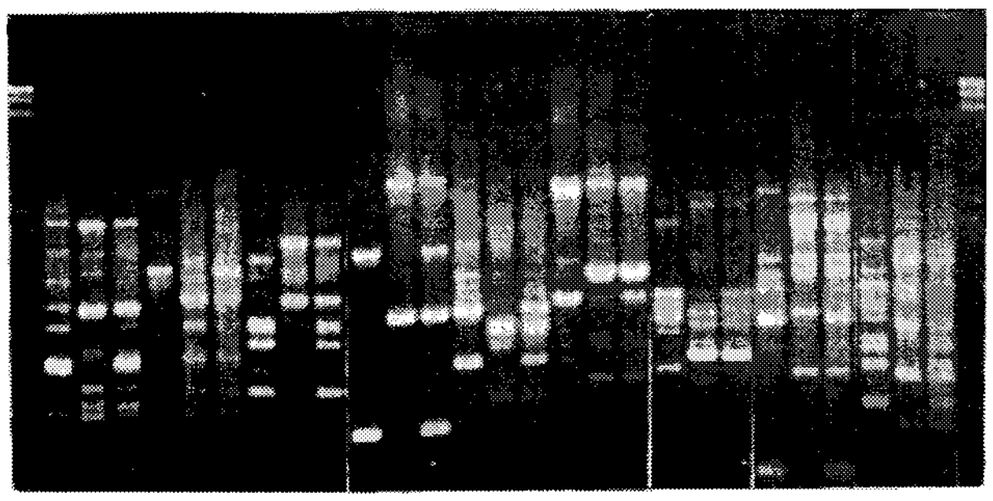

Fig. 4. RAPD polymorphisms between *Glycine max* cv. 'Baemkong' and *G. tomentella*. Lane 1: HindIII digested λDNA, lane 2: *G. max* cv. 'Baemkong', lane 3: *G. tomentella*, lane 4: F_1 hybrid, lane 2, 3, 4: OPA01, lane 5, 6, 7: OPA03, lane 8, 9, 10: OPA08, lane 11, 12, 13: OPA17, lane 14, 15, 16: OPA18, lane 17, 18, 19: OPC09, lane 20, 21, 22: OPC16, lane 23, 24, 25: OPAD08, lane 26, 27, 28: OPD18, lane 29: HindIII digested λDNA.

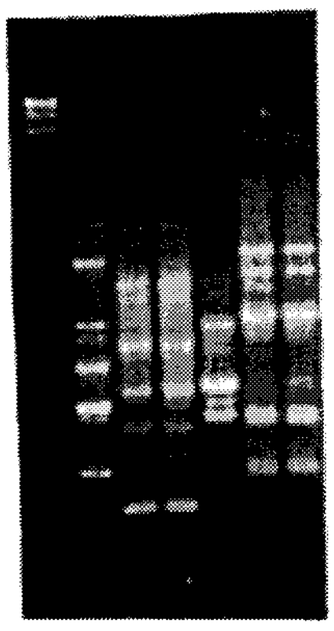

Fig. 5. Primers (OPA02, OPA09) that showed maternal inheritance in F_1 hybrid from the intersubgeneric cross between *Glycine max* cv. 'Baemkong' and *G. tomentella*. Lane 1: HindIII digested λDNA, lane 2: *G. max* cv. 'Baemkong', lane 3: *G. tomentella*, lane 4: F_1 hybrid.

The RAPD banding pattern in F_1 hybrid from *G. max* cv. 'Baemkong' and *G. tomentella* (2n=38) in soybean has not previously been discussed. However many of papers have discussed the segregation distortion in F_2 segregating population. Segregation distortion has been reported in *Arabidopsis* [34], alfalfa [16], common bean [21], and *Drosophila* [23]. The reasons for the segregation distortion were explained as lethal mutations [34], the sterility of the female gametes of the F_1 hybrid caused by a sporogametophytic interaction, and unequal sister chromatid exchange between the tandem repeats in *Drosophila* [23]. Brummer et al. [4] reported that about 50% of the mapped

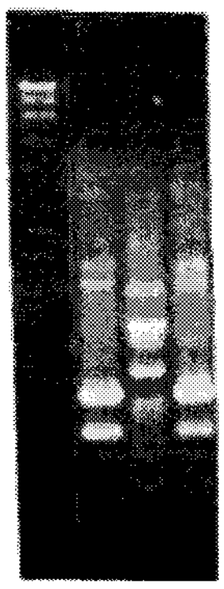

Fig. 6. Primer (OPD05) that showed paternal inheritance in F_1 hybrid from the intersubgeneric cross between *Glycine max* cv. 'Baemkong' and *G. tomentella*. Lane 1: HindIII digested λDNA, lane 2: *G. max* cv. 'Baemkong', lane 3: *G. tomentella*, lane 4: F_1 hybrid.

loci in alfalfa showed segregation distortion, mostly toward excess heterozygotes.

Divergence in the direction of favoring the wild type parent or the heterozygous class in the crosses of tomato has been found, and has ascribed the uneven segregation to gametic selection which favored the wild accession over the cultivated line [12]. In soybean, Keim et al. [12] reported that 20 out of 150 markers deviated ($P<0.05$) from the expected segregation in an interspecific cross between *G. max* and *G. soja*. The deviations occurred in both parental directions.

In this paper, we demonstrated morphological, cytological, and molecular results to confirm intersubgeneric F_1 hybrid between *Glycine max* cv. 'Baemkong' and *G. tomentella*. It is desirable to incorporate agronomically useful traits that wild species possess into the cultivated germplasm in soybean. Our results still remain us further study to recover fertility of F_1 hybrids and the incorporation of useful traits through backcross. And, the occurrence of maternal and/or paternal inheritance in F_1 hybrid from intersubgeneric cross between *G. max* cv. 'Baemkong' and *G. tomentella* should be explained.

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초록 : 콩 *Glycine max*와 *G. tomentella*의 종간교잡으로부터 얻은 F_1식물체 검증을 위한 형태적 · 세포학적 · 분자유전학적 연구

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본 연구는 콩의 *Glycine tomentella*와 *G. max* 밤콩의 종간교잡으로부터 얻은 F_1식물체의 검증을 위하여 형태적, 세포 유전학적, 그리고 분자유전학적 연구를 하였던 바 그 결과를 요약하면 다음과 같다. F_1 식물체의 암술과 수술, 꽃 색깔, 그리고 생육습관 등의 형태적 특징들은 *G. tomentella*의 특징들을 따르거나 중간적 특성을 나타내었다. *G. tomentella* (2n=38) 와 *G. max* 밤콩(2n=40)의 F_1식물체의 염색체수는 2n=39를 가지고 있었다. Esterase와 peroxidase의 동위효소 반응의 결과에서도 F_1 식물체는 *G. tomentella*과 *G. max* 밤콩의 중간적인 밴드유형을 나타내었다. RAPD 분석결과 62 primers들로부터 얻은 F_1 식물체 밴드양상이 모두 *G. tomentella*와 *G. max* 밤콩 양친으로부터 물려받은 것들로 판명되었다. 형태적, 세포학적 그리고 분자유전학적 결과들을 종합하여 볼 때, 본 연구의 *G. max*와 *G. tomentella*의 종간교잡으로부터 얻은 F_1 식물체는 진정 F_1 교배체로 판명되었다. F_1 식물체의 임성회복을 위한 연구와 RAPD 분석에서 나타난 모계유전양상(OPA02, OPA09)과 부계유전양상(OPD05)을 보인 결과에 대한 지속적인 연구를 위한 노력이 요구된다.