TITLE: **Production of Symmetric and Asymmetric Somatic Hybrids via Protoplast Fusion Between Cotton Species**

DISCIPLINE: **Genomics and Biotechnology**

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ABBREVIATIONS: GA3, Gibberellic acid; Asn, Asparagine; Gln, Glutamine; UV, Ultraviolet

Production of Symmetric and Asymmetric Somatic Hybrids via Protoplast Fusion Between Cotton Species

ABSTRACT

An efficient system for protoplast culture and protoplast fusion in cotton has been established in our laboratory. Five cotton species regenerated plants from protoplasts, and efforts have been made to improve the protoplast yield, viability and plating efficiency in protoplast culture. The receptor and the donor in protoplast fusion were treated to produce asymmetric hybrids for special breeding programs. Four interspecific symmetric somatic hybrids and two interspecific asymmetric somatic hybrids were obtained in this program. The hybrids were confirmed by morphological, cytological and molecular analysis, and would be used in further investigation and breeding programs.

KEY WORDS:

protoplast culture, protoplast fusion, cotton

Although conventional breeding programs have made steady improvements in agronomic traits, it is becoming more and more difficult to develop new varieties. For successful crop improvement in Brassica, maize, wheat, Medicago, and rice, protoplast fusion showed potential to bypass sexual-crossing barriers, transfer desirable agronomical relevant traits only expressed in cytoplasmic genome such as cytoplasmic male sterility (CMS), exploit new cytoplasmic combinations and to generate new unexpected alleles (Cardi and Earle, 1997; Liu et al., 1999; Szarka et al., 2002; Tian and Rose, 1999).

The success of protoplast fusion was based on the effective system of protoplast culture. The process of somatic embryogenesis and plant regeneration from protoplast is complex. Protoplasts have to come through cell wall regeneration, cell division, microcalli and calli formation, embryogenesis and plant regeneration. Also, protoplast culture is influenced by many factors, such as explants, culture medium, osmotic pressure, culture density, culture method and the combination of plant growth regulators (Kao and Michayluk, 1975; Kyozuka et al., 1987; Vazquez-Tello et al., 1995; Fellner and Lebeda, 1998). Methods have been developed for plant regeneration from cotton protoplasts (Chen et al., 1989; Finer and Smith, 1982; Peeters et al., 1994), but more details of protoplast culture procedure remains to be elucidated and the low plating efficiency needs improvement. Based on protoplast culture, protoplast fusion in cotton had been occasionally tried, but only the electrofusion conditions were studied (She et al., 1995).

Molecular tools provide convenient ways to confirm the hybrid status of symmetrically fused products, such as randomly amplified polymorphic DNA (RAPD, Zheng et al., 1999; Binsfeld et al,. 2002; Zhou et al., 2005), restriction fragment length

polymorphism (RFLP, Zheng et al., 1999; Cabasson et al., 2001; Zhou et al., 2005), and simple sequence repeat (SSR, Kovtun et al., 1993). However, asymmetric somatic hybrids gained only a few chromosomes, chromosome fragments or no chromosome from the donor genome represent more valuable material. Cleaved amplified polymorphic sequence (CAPS) analysis using mitochondrial or chloroplast universal primer pairs have proved to be efficient and reliable methods for characterizing the cytoplasmic genome of asymmetric hybrids (Zheng et al.,1999; Cheng et al., 2003).

Embryogenesis and organogenesis in several wild cotton species. Embryogenic calli were induced from *G. aridum* S. (D4 genome), *G. davidsonii* K. (D3-d genome), *G. klotzschianum* A. (D3-k genome), *G. raimondii* U. (D5 genome), and *G. stocksii* M. (E1 genome)**,** while others remained nonembryogenic (Sun et al., 2003, 2006a). Plant growth regulator combinations, adding GA3, high inorganic salt stress, and plant growth regulator-free media were used to induce embryogenic calli. Somatic embryogenesis was adjusted by changing sugar sources, regulating combinations of plant growth regulators, and using cell suspension culture. Different sugar sources were attempted to promote embryo maturation and germination. Normal plantlets were regenerated from *G. davidsonii*, *G. klotzschianum*, *G. raimondii*, and *G. stocksii* and abnormal plantlets were obtained in *G. aridum*.

A reproducible plant regeneration system via organogenesis was developed in some wild cotton species. Some factors that influenced organogenesis such as the combination and concentration of plant growth regulators and explants were studied (data omitted).

Establishment of suspension cultures. Suspension cultures of several cotton species (YZ1, Coker201, Coker312, *G. Klotzschianum* and *G. davidsonii*) (Fig. 1) were established, and optimized factors (NH^+/NO_3^-, combination and concentration of plant growth regulators and the concentration of Asn and Gln) of suspensions of Coker 201 were studied for protoplast isolation (data omitted).

Protoplasts isolation, culture and plant regeneration. Protoplasts were isolated from different explants (leaves, hypocotyls, young roots, embryogenic callus, immature somatic embryos and suspension cultures) of Coker 201, Coker 312, YZ1, *G. Klotzschianum* and *G. davidsonii*, and the effect of explants, enzyme combinations, culture density, osmotic potential, combination of plant growth regulators, and culture method on protoplast culture of upland cotton and wild species were studied (Sun et al., 2005a, c; Yang et al., 2007a). As a result, the enzyme combination of 2% cellulose + 1% pectinase + 0.3% hemicellulase or 1.5% cellulose + 1% macerozyme + 2% hemicellulase, the density of $2\text{-}10\times10^5$ /ml, the osmotic potential using 0.1 M glucose plus 0.5 M mannitol and the combination of 0.45 μM 2, 4-dichlorophenoxyacetic acid + 2.68 μM α-naphthalene acetic acid + 0.93 μM kinetin were efficient to protoplast isolation and culture of cotton (Fig. 2).

Treatment of donor and receptor. For the treatment of donor, we used a UV lamp at the dose of 0, 38.7 J/cm^2, 77.4 J/cm^2 and 116.1 J/cm^2 respectively to irradiate the donor protoplast. After UV irradiation, agarose gel electrophoresis in combination with a fast DNA preparation technique was used to determine the degree of DNA damage caused by UV irradiation treatment. Moreover, the viability and division percentage of the irradiated protoplast and control was detected at 20-day culture period and the plating efficiency at the

40th day. We consider irradiation with the dose of 38.7 J/cm^2 as the lethal dose for our asymmetric protoplast fusion experiments (Yang et al. 2007b).

For receptor treatment, we used IOA (Iodoacetamide) dissolved in KM8P medium to the concentrations of 0 mM(CK), 0.5 mM, 1.0 mM, 1.5 mM and 2 mM respectively. After treatment, the viability and division percentage of the irradiated protoplast and control was detected at 20-day culture period and the plating efficiency at the 40th day.

Symmetric protoplast fusion between Upland cotton and wild species. Symmetric protoplast fusions were successfully achieved between upland cotton and wild species (Sun et al., 2004, 2005b and 2006b) (Table 1) using an SSH-2 somatic hybridizer (Shimadzu, Toyota, Japan), and the hybrids were confirmed at morphological, cytological and molecular levels. The morphology of the hybrids was distinct from that of the parents. Flow cytometric analysis revealed that the hybrids had a relative DNA content close to the total DNA contents of the two parents. RAPD analysis revealed 16 of 18 plants were true somatic hybrids. Cytological investigation of the metaphase root-tip cells of seven hybrids revealed there were 72–81 chromosomes in the hybrids, a value close to the expected 78 chromosomes (Table 3, Fig. 3).

Asymmetric protoplast fusion between cotton species. Asymmetric somatic hybrids were obtained between upland cotton and wild species (Table 2), and the hybrids were confirmed at morphological, cytological and molecular levels. Most regenerated plants derived from fused protoplasts displayed a recipient-like morphology, while some showed an intermediate phenotype between Coker 201 and *G. klozschianum*. Chromosome numbers in these somatic hybrids ranged from 54 to 74. RAPD and SSR profiles showed absence or

co-existence of parents' genome DNA fragments. CAPS analysis indicated that recombination and rearrangements might have occurred in some regions of mitochondria (mt) and chloroplast (cp) DNA (yang et al. 2007b) (Table 3, Fig. 4).

DISCUSSION

For protoplast culture of cotton, the successful regeneration from protoplasts was limited within the genotypes which regenerated easily via somatic embryogenesis (Chen et al., 1989; Peeters et al., 1994; Lu et al., 1999). Finer and Smith (1982) cultured protoplasts isolated from nonembryogenic callus cultures of *G. klotzschianum*, only mini-cell group formed. Previously a plating density of 10^5 ml^{-1} has been found to be optimal (Chen et al., 1989; Lu et al., 1999), and 5×10^4 ml^{-1} using feeder layers in Coker 312 (Peeters et al., 1994). Some reports indicated some upland cottons formed callus from protoplasts in K3 medium better than KM8P medium (Saka et al., 1987), but also successful regeneration in KM8P medium (Chen et al., 1989; Lu et al., 1999). In our research, KM8P medium were used, and the enzyme combination of 2% cellulose + 1% pectinase + 0.3% hemicellulase or 1.5% cellulose + 1% macerozyme + 2% hemicellulase, a density of $2\text{-}10\times10^5$ ml^{-1}, the osmotic potential using 0.1 M glucose plus 0.5 M mannitol and the combination of 0.45 μM 2, 4-dichlorophenoxyacetic acid + 2.68 μM α-naphthalene acetic acid + 0.93 μM kinetin were efficient to protoplast isolation and protoplast culture of some explants in some cotton species (Sun et al., 2005a, c; Yang et al., 2007a).

Protoplast fusion between upland cotton and wild species had achieved successfully in our laboratory (Sun et al., 2004, 2005b, 2006b; Yang et al., 2007b), and this progress might be a useful tool for producing variability for breeding programs based on wild species.

However, many abnormal plantlets were produced from fused protoplasts. Along with genetic distance and other factors, the unbalanced high ploidy level may play a major role in the variation of the hybrids. As long as the somatic hybrids were produced, backcross may be required for the establishment of a new cultivar. In addition, these hybrids, especially asymmetric hybrids frequently suffer from chromosome instability and a high degree of sterility (Binsfeld et al., 2000), this may also be useful for the production of monosomic or disomic addition plants by micro-protoplast fusion and recombinant lines after backcrossing (Ramulu et al., 1996). Until now, we have done serials experiments on protoplast fusion, but the advantages or limitations of asymmetric somatic hybrids and symmetric somatic hybrids in breeding programs have not yet been systematically investigated and require more information for further analysis. Asymmetric somatic hybrids as well as symmetric somatic hybrids plants can be used in different breeding programs. Symmetric somatic hybrids plants appeared to produce a wide range of variability in genomic controlled traits, while the aim of asymmetric somatic hybrids was to minimize the disadvantages of donor genome as well as transfer desired traits, chromosomes or chromosome fragments, and produce specific addition lines (Ramulu et al., 1996; Rasmussen et al., 1997; Wardrop et al., 2004; Chen et al., 2004).

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Table 1. The combinations of different species for protoplast fusion.

Species and genome	Origin of protoplast	Fusion ratio	Culture method	Plant regeneration
C $(AD)_1$+K (D_{3-k})	suspensions/embryogenic callus and immature embyro	1:1	Thin layer liquid/ Nurse culture	+
C $(AD)_1$+D (D_{3-d})	suspensions/embryogenic callus	1: 2	Thin layer liquid/ Liquid over agrose	+
C $(AD)_1$+A (E_1)	Suspensions/immature embryo	1:1.5	Thin layer liquid/ Liquid over agrose	+
C (AD)1+B (G)	Suspensions/leaf	1:2	Thin layer liquid/ Liquid over agrose	+

Table 2. The combinations of different species for asymmetric protoplast fusion.

Species and genome	Origin of protoplast	Fusion ratio	Culture method	Cell groups/Callus formation/Plant regeneration
C $(AD)_1$+K (D_{3-k})	Suspensions/suspensions and immature embryo	1:1	Liquid over agrose	+/+/+
C $(AD)_1$+D (D_{3-d})	Suspensions/suspensions	1:2	Liquid over agrose	+/+/+
C $(AD)_1$+B (G)	Suspensions/leaf	1:2	Liquid over agrose	+
C $(AD)_1$+A (E_1)	Suspensions/leaf	1:2	Liquid-over -agrose	+
C$(AD)_1$+Xh$(AD)_2$	Suspensions/leaf	1:1	Thin layer liquid	+
C(AD)1+Zhong(A)	Suspensions/leaf	1:1	Thin layer liquid	+

Table 3. Division percentage and plating efficiency of parental and fused protoplasts.

Cultured protoplants	Density (cells/ml)	Division Efficiency[z](%)	Plating efficiency[y](%)	No. of callus produced
G. klotzschianum	2.0×10^5	45 ± 4.1	11 ± 0.58	211
Coker 201	2.0×10^5	53 ± 3.4	20 ± 2.41	287
K + C[x]	3.5×10^5	39 ± 2.7	13 ± 1.03	176
K × C[w]	2.5×10^5	31 ± 2.4	1.6 ± 1.01	78
K_1[v]	5.0×10^5	10.60±2.69	0	0
C×K_1[u]	5.0×10^5	24.63±4.11	1.05±0.41	47

[z] The ratio of number of protoplasts dividing to the total number of protoplasts at the same visual field of microscope. n = 5

[y] The ratio of number of protoplasts that continued to divide and form cell group related to the total number of protoplasts at the same visual field of microscope. n = 5

[x] The mixture of Coker 201 and *G. klotzschianum* protoplast

[w] Fused protoplasts between Coker 201 and *G. klotzschianum*

[v] Protoplast of G. *klozschianum* irradiated with 38.7 J cm^{-2} UV

[u] Fused protoplasts between Coker 201 and *G. klotzschianum* irradiated with 38.7 J cm^{-2} UV

FIGURE CAPTIONS

Figure 1. Suspension culture of several cotton species. **A:** YZ1. **B:** Coker 201. **C:** Coker 312. **D:** *G. Klotzschianum*. **E:** *G. davidsonii* K.

Figure 2. **A-D:** protoplast isolated from suspension culture, no-embryogenic calli, leaf and immature somatic embryo. **E:** protoplast viability detected by FDA. **F:** cell wall regeneration detected by Fluorescent Brightener 28. **G-J:** cell division after wall regeneration. **K-N:** Calli formation from suspension culture, leaf, immature somatic embryo and hypocotyls. **O:** plant regeneration.

Figure 3. Production and characterization of symmetric hybrids between upland cotton Coker 201 (*Gossypium hirsutum*) and wild cotton (*G. klozschianum* Anderss) (After Sun et al., 2004). **A-D:** protocol of protoplasts electrofusion. **E**: first division. **F:** Cell aggregation. **G**: Calli formation from fusion protoplasts. **H-I:** Grafting. **J:** plant transferred to soil. **K-L:** Chromosome counts in root-tip metaphase cells. **M-O:** Flow cytometry analysis of relative DNA content of parents and somatic hybrid (M: *G. Klotzschianum*. N: Coker 201. O: somatic hybrid). P-Q: RAPD patterns of Parental plants and putative somatic hybrids (P: S1325. Q: S1340).

Figure 4. Production and characterization of asymmetric hybrids between upland cotton Coker 201 (*Gossypium hirsutum*) and wild cotton (*G. klozschianum* Anderss). **A:** Calli formation from protoplast of Coker201. **B-C:** Calli formation from fusion protoplasts. **D:** Single cell derived calli proliferation after transferred to solid medium individually. **E:** Fascicular plants regenerated from abnormal embryo. **F:** Vigorous stem and bigger stipules of hybrid plant. **G:** Grafting. **H:** plant transferred to soil. **I:** Chromosome analysis of

asymmetric hybrids (2n = 54, 58, 60, 64, 62, 66, 68, 74. All bars = 50 μm) **J:** RAPD patterns of asymmetric somatic hybrids and their parents generated by primer S353. **K:** SSR band patterns of the parental species and regenerated plantlet by the primer pair BNL3232. **L:** The cpDNA banding profiles of the regenerated plants and the fusion parents as revealed with polymorphic primer/enzyme combination *trn*K-*trn*K/*Eco*RI. **M:** The mtDNA banding profiles of the regenerated plants and the fusion parents as revealed with polymorphic primer/enzyme combination *nad 4* exon *1-2*/*Eco*RI. (Lane C = Coker 201, lane k = *G. klotzschianum*, Lanes 1–8: ASH hybrids. Lane M: 100bp DNA marker).

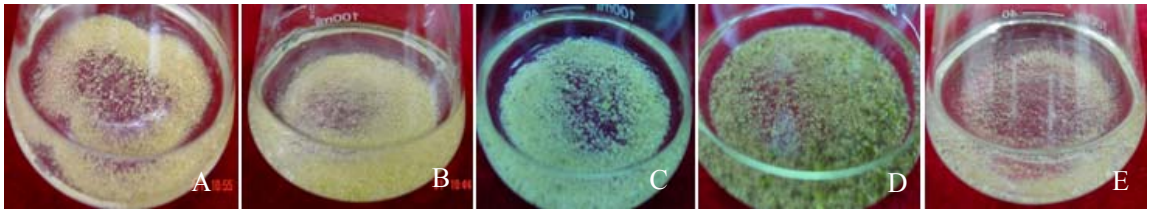


Figure 1.

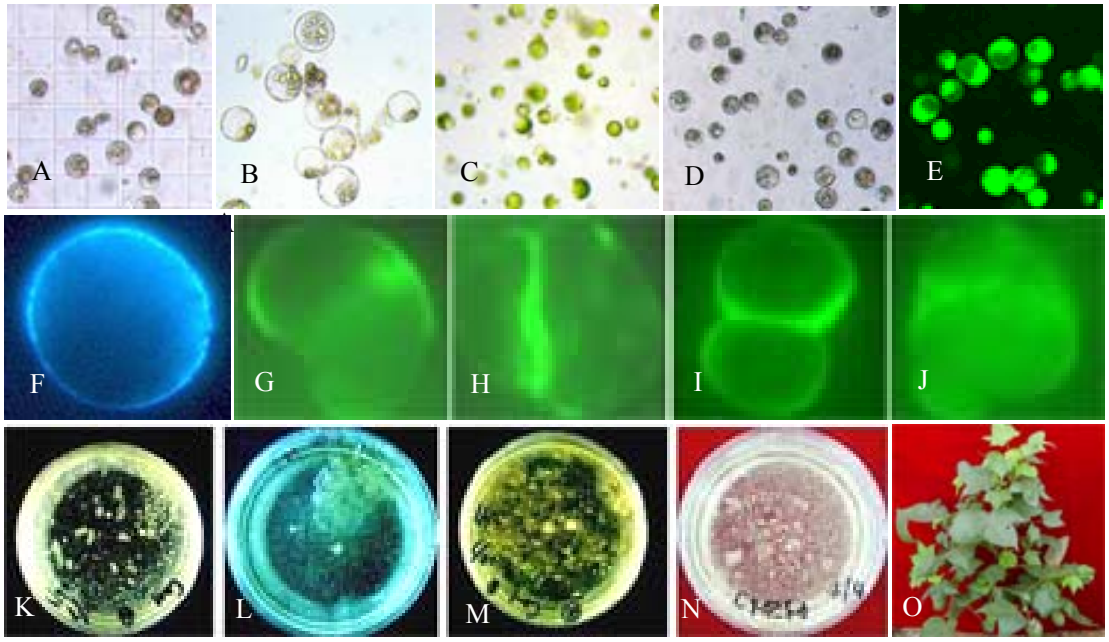
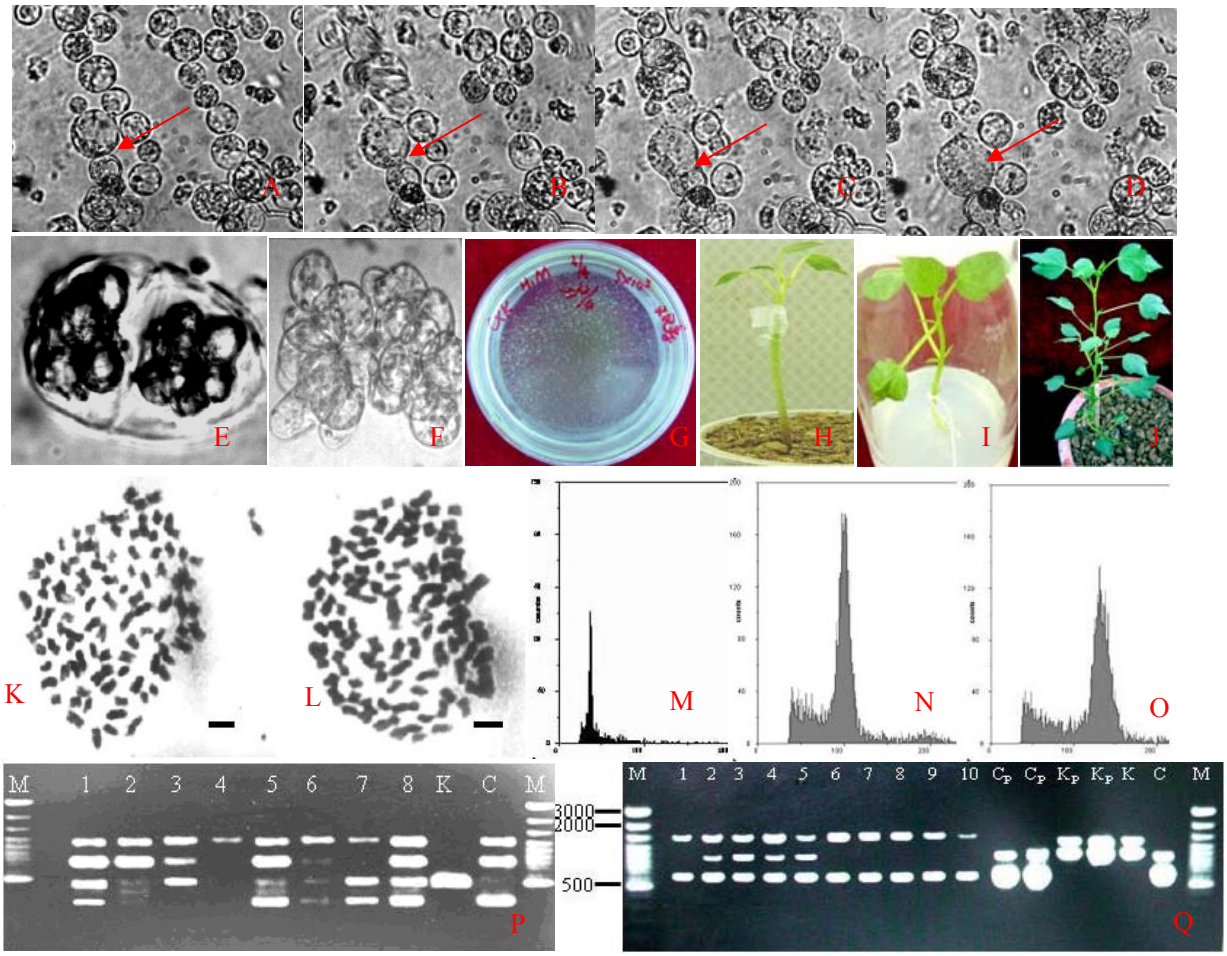


Figure 2.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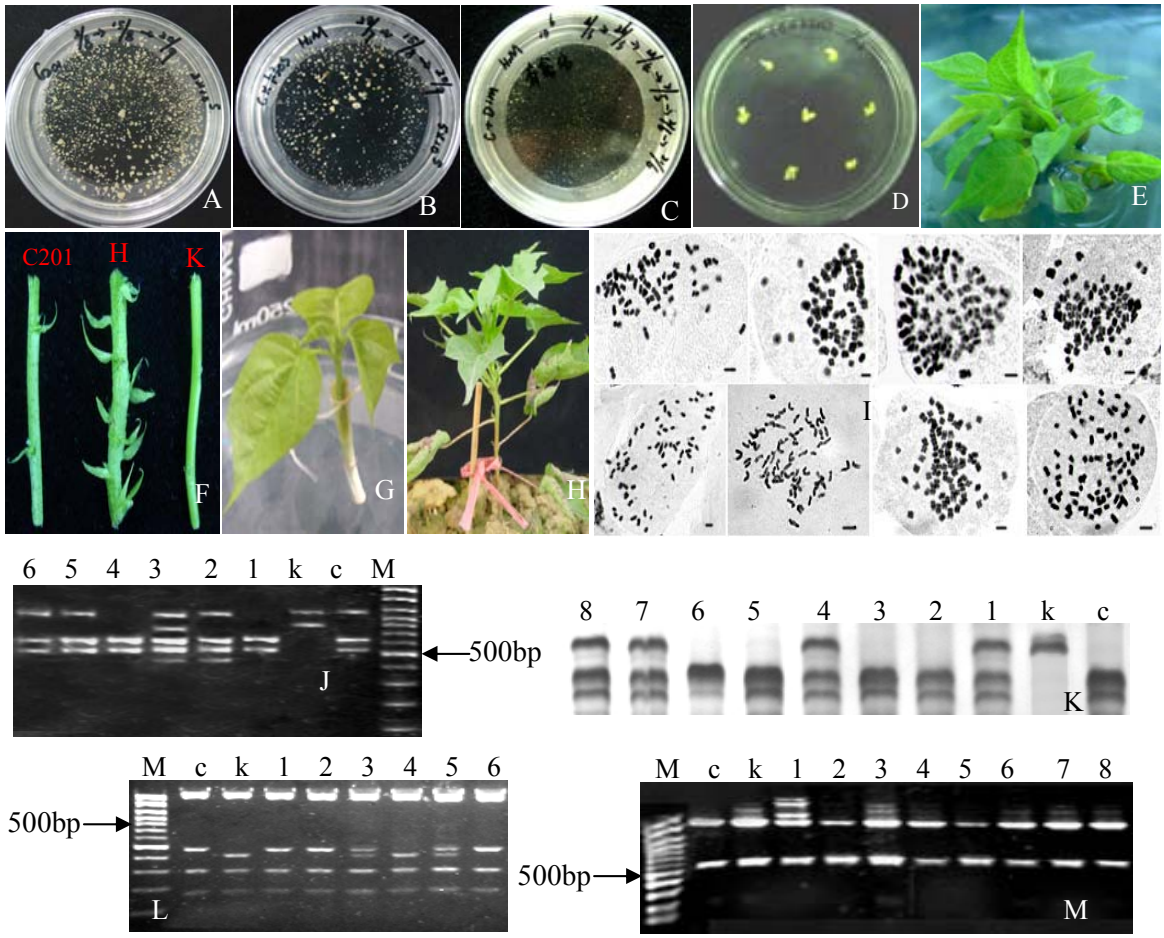
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Figure 4.