Some Results of Cotton Breeding in Vietnam

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1 Introduction

In Vietnam, cotton is an important economic fiber crops with more than 3 million farmers depending on it for their livelihood. At present, cotton growing is mainly concentrated in northern high land, central high land, coastal central and eastern southern regions. In the past, cotton cultivated area of Vietnam had reached more than 100 thousand hectares. However, due to continuous breakout of cotton bollworm in the early 2000s, the country had suffered heavy economic losses. The disaster caused by cotton bollworm became one of the major factors for the sharp drop down of cotton production. Under this severe situation, since 2004, the program of development of transgenic cotton has been launched under participating of RICOTAD. After four years, we have obtained some of the beginning achievements.

More than 95% of cotton area in Vietnam is grown by hybrid cultivars with several good cultivars such as L18, VN20, VN35, VN15, VN01-2, VN02-2, VN04-3 and VN04-4. These cultivars are all intra-specific (*G. hirsutum* x *G. hirsutum)* and widely cultivated now. Hybrid cotton seed production on a commercial scale in Vietnam mainly use hand emasculation and pollination technique and this technique has been applying successfully. The yield of hybrid cottonseed reached 100-150 tons year^{-1} that satisfied the domestic's demand. But now, Vietnam is facing lack and high cost of labor, so that, cost of hybrid cottonseed is increasing. To solve this problem, since 2000, we have studied on hybrid cotton seed production by applying genetic male sterile (GMS) lines and using natural honeybees and insects as pollinators.

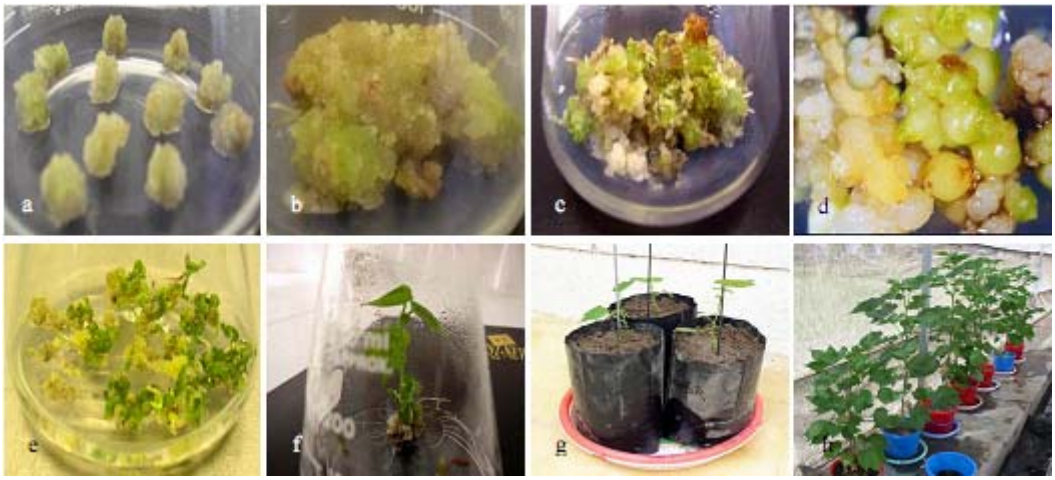
2 Results of cotton biotechnology research in Vietnam

2.1 Establishment of cotton regeneration system

2.1.1 *Regeneration via somatic embryogenesis.*Gene's transfer into cotton through *Agrobacterium nvolves* the development of an efficient regeneration system from transformed tissues. Regeneration through somatic embryogenesis is preferred over organogenesis. However, efficient in vitro techniques for the regeneration of large numbers of plantlets from cotton are limited when compared to other major commercial crops. Price and Smith (1979) were the first to report somatic embryogenesis in cotton (*G. klotzchianum*), although complete plants could not be regenerated. Davidonis and Hamilton (1983) subsequently described plantlet regeneration from two-year-old callus culture of *G. hirsutum var*. Coker 310 through somatic embryogenesis. This procedure involved a lengthy culture period and was difficult to repeat. Since then, several researchers have studied extensively on plant regeneration through somatic embryogenesis from var. Coker 310 (Finer 1988; Firoozabady and DeBoer 1993; Shoemaker et al., 1986; Trolinder and Goodin 1987, 1988a, 1988b; Trolinder and Xhixian 1989), Sicala, Siokara (Cousins et al., 1991; Rangan and Rajasekaran 1996), an Acala (Rangan 1993; Rangan and Rajasekaran 1996). Although regeneration efficiency via somatic embryogenesis has been improved, but genotype dependent response, a prolonged culture period, high frequency of abnormal embryo development, low conversion rate of somatic embryos into plantlets, and a ack of shoot elongation are the problems associated with cotton regeneration.

In RICOTAD, since 2004, an cotton (*G. hirsutum* L.) cultivar SSR60F has been studied for ability of regeneration via somatic embryogenesis. Callus was induced from hypocotyls explants on medium MSB (MS basal salt with B5 vitamins) containing 2,4-D and kinetin. The optimal 2,4-D and kinetin concentrations or callus induction were both 0.1 mg/l. Hormone free MSB medium promoted

the proliferation of embryogenesis callus. The best medium for the differentiation, maturation and germination of somatic embryos was MSB with 1.9 g/l KNO_3, MSB supplemented with 1 g/l glutamine and 0.5 g/l asparagine and ½ MSB, respectively. An efficient protocol for the production of high frequency somatic embryogenesis and plant regeneration of an cotton cultivar has been developed. Completed plant could be regenerated through somatic embryogenesis from hypocotyl explants within 8-9 months (figure 1).



(a-c) induction and proliferation of callus, (d) somatic embryos at various development stages, (e-f) germination of somatic embryos and plant regeneration, (g-h) regenerated plant after transferred to soil

Fig. 1 Somatic embryogenesis and plant regeneration of cotton (*Gossypium hirsutum* L.) cultivar SSR60F.

2.1.2 *Regeneration via multiple shoot from zygotic embryo.*Currently, transgenic are mainly delivered to cultured plant tissues of cotton by co- cultivation with *A. tumefaciens* (Firoozabady et al., 1987; Umbeck et al., 1987) But, this method requires regeneration via somatic embryogenesis which has been successfully applied to only a few regenerable cotton cultivars (e.g., the Coker lines). All cotton cultivars are under cultivation in Vietnam and they are not as amenable to tissue culture techniques as the Coker lines. Therefore, this method involves the following (i) transformation of regenerable cells or callus tissues that were derived from the Coker lines, (ii) regeneration of putative transgenic plants through somatic embryogenesis, (iii) collection of transgenic T1 seeds and advancement of the desired traits into an agronomic background by plant breeding techniques. This strategy requires 10-14 months to obtain mature transgenic plants of the Coker lines and an additional 6-10 years are necessary to backcross the added value traits into more agronomic cultivars. Moreover, plants regenerated from an embryogenesis callus phase are sometimes sterile or show signs of somaclonal variation which affect both the phenotype and genotype of the plant (Stelly et al. 1989; Firoozabady and DeBoer 1993).

Therefore, in RICOTAD, a protocol for multiple shoot regeneration in two cotton (*G. hirsutum* L.) cultivars LRA5166 and C118 has been developed in 2005. Zygotic embryos were isolated from cotton seeds and were passed onto multishoot induction, multishoot formation, shoot elongation and root formation media. The MS medium containing 0.1 mg/l 2,4-D, 2.0 mg/l BAP and 0.1 mg/l NAA seemed to be optimal for induction stage of multishoot formation. The combination of 0.5 mg/L BAP and 0.05 mg/l NAA drove the continuously dividing cells derived from the former event towards the formation of shoots, giving rise to a shoot mass from each original zygotic embryo. The subsequent absence of phytohormons facilitated the elongation and root formation of the shoots. Transferring the in vitro rooted plantlets into soil succeeded to verify the whole plant regeneration procedure. Completed plant could be regenerated via multiple shoot from zygotic

embryos in 4-5 months. An efficient protocol for the production of the relatively high rate of shoot mass formation could be used for genetic transformation in cotton (figure 2).

(a-c) zygotic embryos in multiple-shoot induction, multiple-shoot formation and shoot elongation media, (d) shoots in root formation medium, and (e-f) regenerated plant after transferred to soil.

Fig. 2 Plant regeneration through multiple-shoots from zygotic embryos in three an elite Vietnamese cotton (*Gossypium hirsutum* L.) cultivar LRA5166, VN36P and C118

2.2 Establishment of cotton genetic transformation system

2.2.1 *Agrobacterium-mediated transformation.*Since the first transgenic plant appeared in 1983, studies on plant transformation techniques have achieved a great progress. Apart from the widely used method of *Agmbacterium-mediated* transformation, scientists have developed other techniques, such as particle bombardment, electroporation, microinjection, ultrasonic, carborundum, pollen tube pathway transformation etc. In cotton, *Agrobacterium-mediated* transformation has been widely adopted in the World. Horsch et al., in 1985 developed *Agrobacterium-mediated* transformation of leaf disks of tobacco. In 1987, Agracetus Company firstly used this protocol to introduce nptII and cat genes into upland cotton varieties COKER 312 and COKER 310 and obtained transgenic plants (Umbeck et al., 1987). Perlak et al. (1990) transferred Bt gene and obtained insect resistant cotton plants.

In Vietnam, after developing an efficient protocol for the production of high frequency somatic embryogenesis and plant regeneration of an cotton cultivar SSR60F, since 2006, scientists of RICOTAD have applied this protocol to transfer Cry1Ac gene into this cotton cultivar by *Agrobacterum-mediated* transformation. As a result, a reliable and high efficienc y system of transforming embryogenic callus mediated by *A. tumefaciens* was developed. *Agrobacterium tumefaciens* strain C58 harboring the pC1300-Ubi-Cry1Ac plasmid containing hpt gene as a selection marker, were used for transformation. Hypocotyls were excised from 7 day old seedlings and cut into 5-6 mm segments. Hypocotyl explants were first inoculated with the *A. tumefaciens*. Infected hypocotyl explants were co-cultivated in callus induction medium with 200 mg/l Acetosyringone for two days under 21^0C condition and were then moved on to callus induction medium with 10 mg/l hygromycin for 45 days. After that, the hygromycin resistant calli were subcultured in embryogenic callus proliferation medium with 10 mg/l hygromycin. Transformed embryogenic calli were then transferred to MSB with 1.9 g/l KNO_3, MSB supplemented with 1 g/l glutamine and 0.5 g/l asparagine and ½ MSB media, respectively for differentiation, maturation and germination of somatic embryos. Putative transformants were confirmed by PCR and Southern-blot analysis. Forty five regenerated plants were successfully transferred to soil, of which 12

proved to have the active Cry1Ac gene. Insect resistance will be tested by bioassay in next time (figure 3).

(a) induction of transformed callus, (b) proliferation of transformed embryogenic callus, (c) transformed embryogenic calli on somatic embryo differentiation and maturation media, (e-g) germination of transformed somatic embryos and transgenic plant regeneration, (h-i) Transgenci regeneration plantlet after transferred to soil

Fig. 3 *Agrobacterium*-mediated transformation of cotton (*Gossypium hirsutum* L.) cultivar SSR60F.

2.2.2*Pollen tube pathway (FTP) transformation.*Pollen tube pathway transformation is a method that delivers foreign genes directly into germline cells. It has been proved that pollen tube pathway developed by Chinese scientists, is an efficient and convenient way for gene delivery, particularly for cotton. A great progress has been made in recent years in China, by which numerous transgenic cotton cultivars or lines have been bred, that are now widely used in cotton production. Advantages of method are (i) avoiding somaclonal variation and genotype dependence, (ii) expanding the range of recipient species for plant transformation, (iii) avoiding use of marker gene or reporter gene, (iv) shortening duration of whole transformation process, and (v) the technique is simple and easy for breeders to handle; particularly it is suitable for large scale transformation to get large populations of transformants. In addition, since molecular analysis and agronomic trait selection of transgenic plants can be done at the same time, it is ideally to combine genetic engineering with conventional breeding thus improving the efficiency of cotton improvement (Ni et al., 1996 and Guo et al., 1999).

(a) Smear paint on top of the floral bud on the previous day of flower opening, (b) the flower turn red after 20-24 h of flowering, (c) remove petals and make style flat, (d) vertically insert needle of syringe into ovary at the center of the flat style, (e) gently push plasmid DNA solution into ovary, (f) drop on the end of boll petiole with 40 ppm of gibberellin and tie marker on the branch (g) identify transgenic plants by detecting of expression of the nptII gene (→ kanamycin positive and ← negative plant); (k) identify integration of CryIAc gene in transgenic plants by PCR.

Fig. 4 Some of micro-injection picture and screening transformed plants

In Vietnam, researchers in IBT designed and artificially synthesized Cry1Ac and VIP3 genes in 2004. Since 2005, scientists in RICOTAD, have introduced those genes by PTP into three cotton *G. hirsutum* L.) cultivars C118, LRA5166 and TM1. As a result, a reliable and high efficiency system of transforming was developed. In that, *E.coli* strain DH5α harboring the pIBT(C;P*)-CryIAc and pBI121-VP3 plasmid containing nptII gene as a selection marker were used for extraction of plasmid DNA. About 10 µl plasmid DNA solutions (the concentration of plasmid DNA is 0.01-0.02 µg/µl) were injected into ovary of flowers after 20~24 h of flowering. A total of 161,487 injected seeds were harvested from 15,660 injected cotton flowers. All injected seeds were sown in the field and screened for transformants with kanamycin solution (the concentration of kanamycin is 2,500-5,000 mg/l) spray. Result of 2.27% seedlings (base on the calculation of resistant seedlings out of the total geminated seedlings) showed resistance to the kanamycin (negative plants). Those negative plants were then detected integration of Cry1Ac and VIP3 genes by PCR analysis. Result of 0.96% seedlings (base on the calculation of positive PCR seedlings out of the total geminated seedlings) expressed positive PCR. In the next time, those plants will be analyzed by Southern, Northern, Western blot and bioassay.

3 Researching results of hybrid cotton seed production by applying genetic male sterile (GMS) and using natural honey bees and insects as pollinators

One method frequently proposed for utilizing hybrid vigor involved male sterile plants (Turner, 1948; Christidis and Harrison, 1955; Justus and Leinweber, 1960; Meyer and Roux, 1963; Weaver and Ashley, 1971; etc.). Moreover, investigate natural insect as pollinators to produce hybrid cotton seed has been studied (Allard 1910, 1911; Butler et al, 1960; Kearney, 1923; McGregor, 1959; McMillian, 1959; Stephens and Finkner, 1953; Theis, 1953; etc.), but almost limited on a small scale trial or evaluated the role of insect as pollinators.

In Vietnam, utilizing hybrid vigor involving male sterile cotton plants has been studying since early 1990s. Now, 14 promising genetic male sterile (GMS) lines (BD1, BD2, BD3, BD20, BD21, BD22, BD23, BD24, BD25, K.LRA5166, K.VN36P, K.K11, VN36P.GMS1 and 1247GMS) have been bred. These GMS lines showed 38.8%～55.6% male sterile plants and their agronomic characteristics are suitable for utilizing hybrid vigor. Since 2005, we have carried out a study with three pollinating methods (i) by raising honeybees and natural insects, (ii) by only natural insects and (iii) by hand. Two genetic male sterile (GMS) lines (BD21 and BD24) and one male fertile restoring line D20-24 were grown with 4：1 alternate ratio, respectively. The initial plant density was 70.000 plants·ha^{-1} (or 7.0 plants·m^{-2}). Results showed that percent of male sterile plants about 40.0%~48.8% and density of male sterile plants after removing fertile plants about 3.0~4.8 plants·m^{-2} at harvested period. The boll weighs did not differ from treatments, but the significant difference of boll set (67.2%; 48.3% and 72.6% respectively), therefore the seed cotton yield is varied. Among the treatments, pollination by raised honeybees and natural insects are the highest yield (11.7 quintals/ha). A remarkable event is the second treatment (pollinating by natural insects) attained 48.3% setting boll and that was beyond all our expectations. From these results, a system of hybrid cotton seed production by applying genetic male sterile lines and using natural honeybees and insects as pollinators was developed. Using this system, we are not only saving the large amount of money but also solving deficient situation of labor in our country. In which, cost of hybrid cotton seed reduced 14.1%~41.0% in comparison with hand emasculation and pollination technique.

References:

[1] ALLARD H.A. 1910. Preliminary observations concerning natural crossing in cotton. Amer. Breeders' Mag. (4): 247 - 261.

[2] BUTLER G.D., Todd F.E., McGregor S.E, Werner F G. 1960. Melissodes bees in Arizona cotton fields. Ariz. Agr. Expt. Sta. Tech Bul. 139, 11 pp.

[3] CHRISTIDIS B.G. , Harrison G.J. 1955. Cotton growing problems. 633 pp. McGraw-Hill Book Co., Inc., New York and London.

[4] COUSINS Y L, Lyon B R, Llewellyn D J . 1991. Transformation of an Australian cotton cultivar: Prospects for cotton through genetic engineering. Aust J plant Physiol 18:481-494.

[5] DAVIDONIS G H, Hamilton RH. 1983. Plant regeneration from callus tissue of *Gossypium hirsutum* L. Plant Sci Lett 32:89-93.

[6] FINER J J. 1988. Plant regeneration from somatic embryogenic suspension cultures of cotton. *Gossypium hirsutum* L. Plant Cell Rep 7:399-402.

[7] FIROOZABADY E, DeBoer DL. 1993. Plant regeneration via somatic embryogenesis in many cultivars of cotton (*Gossypium hirsutum* L.). In Vitro Cell Dev Biol 29P:166-173.

[8] FIROOZABADY E, DeBoer DL (1993) Plant regeneration via somatic embryogenesis in many cultivars of cotton (*Gossypium hirsutum* L.). In Vitro Cell Dev Biol 29P:166-173.

[9] FIROOZABADY E, DeBoer DL, Merlo DJ, Halk EL, Amerson LN, Rashka KE, Murray EE. 1987. Transformation of cotton (*Gossypium hirsutum* L.) by *Agrobacterium tumefaciens* and regeneration of transgenic plants. Plant Mol Biol 10:105-116.

[10] GUO S D, Cui H Z, Ni W C, et al. 1999. Development of bivalent insect resistant transgenic cotton plant. Scientia Agricultura Sinica, 32 (2): 1-7 (in Chinese).

[11] HORSCH RB, Fry ZE, Hoffman LN, et al. 1985. A simple and general method for transferring genes in to plant. Science, 217: 1299-1231.

[12] JUSTUS N, Leinweber CL. 1960. A heritable partially male-sterile character in cotton. Jour. Hered. 51: 191-192.

[13] KEARNEY T.H. 1923. Self-fertilization and cross-fertilization in pima cotton. U.S. Dept. Agr. Dept. Bul. 1134, 68.

[14] MCGregor S E. 1959. Cotton-flower visitation and po1len distribution by honey bees. Science 129: 97-98.

[15] MCMILLIAN W W. 1959. The potential pollinators of cotton inmonokova florence and darlington counties, 1955. South Caro1ina. 56 pp. M.S. Thesis, Clemson Agr. Col.

[16] MEYER J.R. and Roux J.B. 1963. A partially male-sterile character in upland cotton. Crop Sci. 3: 428-429.

[17] NI W C, Huang J Q, Guo SD, et al .1996. New germplasm of bollworm resistant cotton derived from Bt gene transfer. Jiangsu J Agri Sci, 12 (1): 1-6 (in Chinese).

[18] PERLAK F J, Deaton RW, Armstrong TA, et al.1990. Insect resistant cotton plants. (Bio/Tech), 8: 939-942.

[19] PRICE H J, Smith RH .1979. Somatic embryogenesis in suspension cultures of Gossypium klotzchianum Anders. Planta 145:305-307.

[20] RANGAN T S .1993. Regeneration of cotton. USA patent no. 5,244,802.

[21] RANGAN T S, Rajasekaran K.1996. Regeneration of cotton plant in suspension culture. USA patent no. 5,583,036.

[22] SHOEMAKER RC, Couche L J, Galbraith DW. 1986 Characterization of somatic embryogenesis and plant regeneration in cotton (*Gossypium hirsutum* L.). Plant Cell Rep 3:178-181.

[23] STELLY D M, Altman DW, Kohel RJ, Rangan TS, Commiskey E. 1989 Cytogenetic abnormalities of cotton somaclones from callus cultures. Genome 32:762-770.

[24] STEPHENS S G., Finkner M D. 1953. Natural crossing in cotton. Econ. Bot. 7(3): 257-269.

[25] THEIS S A. 1953. Agents concerned with natural crossing of cotton in Oklahoma. Amer. Soc. Agron. Jour. 45: 481-484.

[26] TROLINDER N L, Goodin J R.1987. Somatic embryogenesis and plant regeneration in cotton (*Gossypium hirsutum* L.). Plant Cell Rep. 6:231-234.

[27] TROLINDER N L, Goodin JR.1988a. Somatic embryogenesis in cotton (*Gossypium*). I. Effects of source of explant and hormone regime. Plant Cell Tissue Organ Cult 12:178-181.

[28] TROLINDER N L, Goodin J R. 1988b Somatic embryogenesis in cotton (*Gossypium*). II. Requirements for embryo

development and plant regeneration. Plant Cell Tissue Organ Cult 12:43-53.
[29] TROLINDER N L, Xhixian C. 1989. Genotype specificity of the somatic embryogenesis response in cotton. Plant Cell Rep 8:133-136.
[30] TURNER J.H. 1948. Hybrid vigor studies. Ga. Coastal Plain Expt. Sta. Ann. Rpt. Bul. 46, 18-20.
[31] UMBECK P, Johnson G, Barton K, Swain W .1987. Genetically transformed cotton (*Gossypium hirsutum* L.) plants. Biotechnology 5:263-266.
[32] WEAVER J B, Ashley T. 1971. Analysis of a dominant gene for male sterility in upland cotton. Crop Sci. 11: 596-597.