The floral androcarpel organ (*ACO*) mutation permits high alkaloid yields in opium poppy *Papaver somniferum*

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Isolation of a recessive *aco* (androcarpel organ) mutation in opium poppy *Papaver somniferum*, is described. The *aco* (*aco aco*) mutant plants form androcarpels in place of inner whorl stamens in their flowers. The abnormal flowers are self-fertile, as stamens in the outer whorl are normal. In *aco* mutant the calyx, corolla and syncarpous gynoecium are formed like in the wild type *ACO* plants. The androcarpel walls of *aco* plants synthesize and accumulate alkaloids like the main carpel walls. The *aco* mutation provides a means for improving carpel wall husk mass and thereby the yield of alkaloids.

THE opium poppy plant, *Papaver somniferum*, synthesizes more than 40 alkaloids in its root, stem, leaf and/or fruit (capsule), some of which have high biological–pharmacological activities and economic importance. The demand for poppy alkaloids, especially for morphine and related compounds used for suppressing pain and cough, has been growing and is estimated as 75 tonnes; it was about 10 tonnes in 1983 and 55 tonnes in 1997 (ref. 1). The poppy plant is harvested in two ways to produce raw material for alkaloid production. The growing capsule at the advanced stage of development is lanced and the latex that oozes out and forms opium after coagulation on the capsule wall is collected. Alternatively, the dried capsule and part of peduncle are harvested and threshed to collect the husk after separation of seeds. The alkaloids are chemically extracted from the opium or capsule/peduncle husk. Under the 1988 United Nations (UN) Convention, while a few countries have been permitted to grow opium poppy for capsule husk, India has been permitted to cultivate poppy for obtaining opium, to meet the international requirements of opium alkaloids and to control their illicit traffic[2]. Poppy crops are cultivated by identified farmers, of selected districts in Uttar Pradesh, Madhya Pradesh and Rajasthan, who are licenced by the Narcotics Commissioner of the Government of India to grow opium poppy in small fields, for the production of opium all of which is purchased by the Narcotics Commissioner. UN convention requires India to develop new cultivars and processing technique(s) that will increase alkaloid production, reduce the area of poppy cultivation and further check any illicit traffic of opium poppy products[2]. To meet these requirements, one of the objectives of the opium poppy plant breeding programme is to develop cultivars that accumulate higher levels of morphine, codeine, thebaine, narcotine and/or papaverine in capsule husk for industrial extraction[3].

Under dense planting conditions of cultivation, individual *P. somniferum* poppy plants produce 1–3 capsules from equal number of flowers[3]. The poppy flower has four kinds of organs arranged in concentric whorls, two sepals in two outermost whorls, four petals arranged in two whorls, inner to sepals, several whorls of stamens and a gynoecium comprising many fused carpels. The gynoecium has a stigmatic plate, a very short style and an ovary. Each carpel of syncarpous unilocular ovary on the inside bears a placenta, the two sides of which bear the ovules. The ovary (capsule) wall has a vast network of laticiferous vessels which are the principle site of synthesis and accumulation of alkaloids[4,5]. Apparently to breed high alkaloid-yielding opium poppy genotypes, one of the strategies will be to increase the number of carpel organs and thereby capsule wall volume.

In angiosperms, differential activities of homeotic genes in different regions of a developing flower are responsible for the specification of organ identities in flower[6]. Three classes of floral homeotic genes that function in overlapping domains determine the identity of sepals, petals, stamens and carpels in such flowers[7]. In *Arabidopsis*, the A-class genes, *APETALA 1* and *APETALA 2*, act to specify sepal and petal development. The B-class genes, *APETALA 3 (AP3)* and *PISTILLATA (PI)*, act to specify petal and stamen development and the C-class gene(s) *AGAMOUS (AG)* acts to specify stamen and carpel development. The floral homeotic genes have been highly conserved among angiosperm plant species. It has been shown that the orthologous genes are involved in the development of corresponding organs in *Antirrhinum* and *Petunia*[7–9]. Mutations in the homeotic genes are known to change identities of the organs of different floral whorls. For example in *ap2* mutant of *Arabidopsis*, sepals and petals become carpels and stamens, respectively[10]. Here, we describe isolation of a variant (*aco aco*) from a land race (*ACO ACO*) in which inner whorls of stamens have become carpels. The presence of many androcarpels in the poppy mutant is shown to increase the yield of carpel husk and thereby alkaloids.

In the course of evaluation of the Indian landrace genetic resources of *P. somniferum*, plants were raised of accessions using seeds collected from farmers. The accessions were selfed and progeny populations were screened for variation in morphological features and alkaloid profiles. While the seeds of apparently homogenous selfed plants were pooled accession-wise, the

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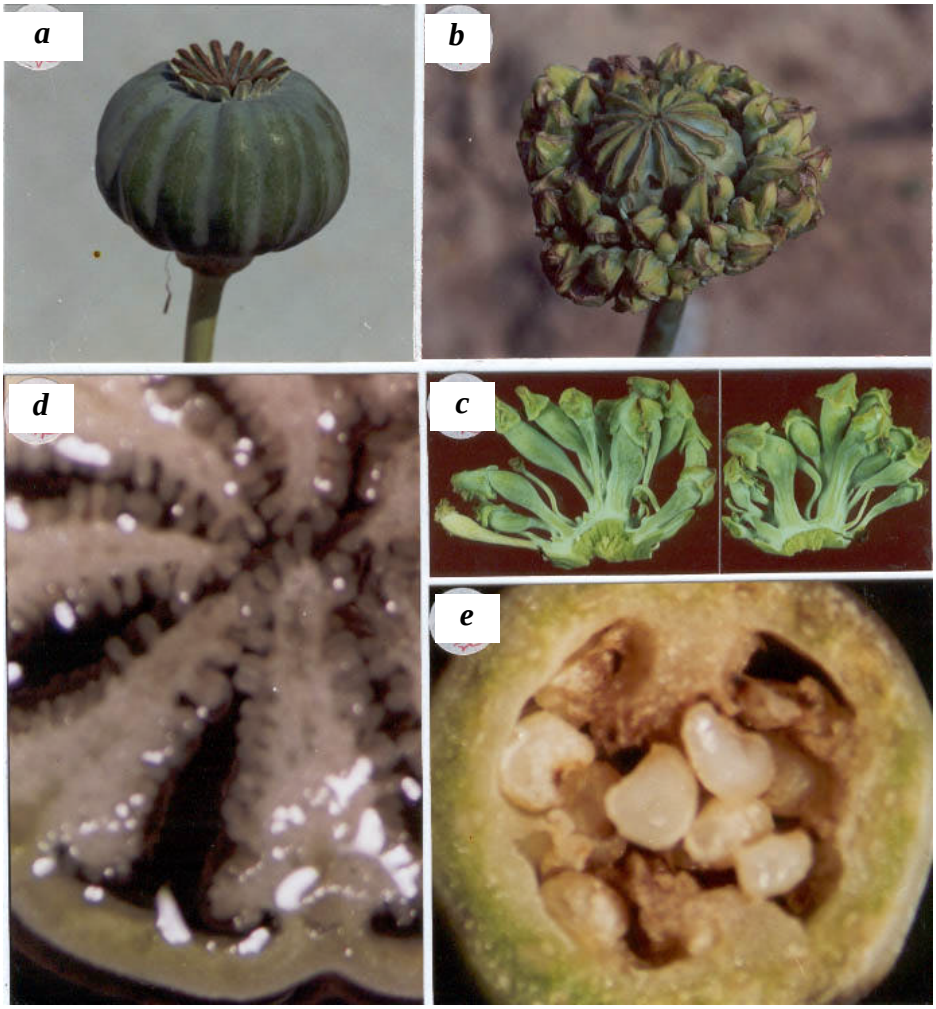

Figure 1. Mature syncarpous capsule of wild type *ACO* and semi-mature main syncarpous capsule and androcarpel capsules of *aco* mutant in opium poppy *Papaver somniferum*. ***a***, *ACO* capsule; ***b***, Central main capsule and many androcarpels in two whorls around main capsule; ***c***, Androcarpels viewed from their adaxial side; ***d***, Parietal placentae bearing many ovules in *Aco* capsule, viewed in transverse section; ***e***, Four parietal placentae bearing ovules in an transversely sectioned androcarpel.

Table 1. Expression of floral organ traits in the *ACO* wild type and *aco* mutant in opium poppy *Papaver somniferum*

Flower organ/whorl type	*ACO* wild type	*aco* mutant
Calyx	Two sepals, arranged in a whorl	Like in *ACO*
Corolla	Four petals arranged in two whorls	Like in *ACO*
Androecium	Stamens numerous (143–189), arranged in three or four whorls, 2.8 ± 0.2 cm in length, pollen fertile	Stamens *ACO* like in outer whorl, those of inner whorls abnormally developed into androcarpels, filamentous at base, cylindrical in body (1.9 ± 0.7 cm *H* and 0.7 ± 0.1 *D*) and capped distally, having ovules, borne on 1–4 parietal placentae, viable seeds produced on maturity
Gynoecium	5–9 carpels, fused to form, unilocular capsule of 4.7 ± 0.7 cm height (*H*) and 14.2 ± 0.4 cm diameter (*D*), flat disc like stigma, very large number of ovules borne on many parietal placentae	Like in *ACO*, except somewhat smaller capsule (4.2 ± 0.2 *H* and 13.2 ± 0.6 *D*)

variant plants were maintained separately. The selfing of the landraces and variants isolated from them was continued for six generations. In the process, a variant was isolated from the accession Sanchita, in which the stamens of the inner two whorls developed into carpels (Figure 1 *b*). The androcarpel organ formation in this spontaneous mutant was found to be inherited as a recessive Mendalian character (S. Bajpai, unpublished observation). The wild type allele of the affected *aco* mutation was given the name *androcarpel organ* (*ACO*). Thus the genotype of wild type plants homozygous at the locus of interest was given the acronym *ACO* (Figure 1 *a*), the mutant plants homozygous for *aco* mutation were called *aco* plants. In the 1999–2000 and 2000–2001 rabi season, the two genotypes were grown under similar conditions in the field and observations were recorded on their characteristic morphological traits and on the content of alkaloids in the carpel wall. The alkaloids were estimated using published procedures[11]. A comparison of the mutant genotype with the parental genotype is presented below (Table 1).

The *aco* androcarpel organ bearing plants and the wild type *ACO* plants were similar in general shoot morphology, height, leaf shape, size and colour, number of flowers formed, size and shape of peduncle, size and colour of sepals, petals, normal stamens, and stigma and ovary of gynoecium. However the inner two whorls of stamens were represented by carpels of different sizes. Each of these androcarpels was borne on filament, had cylindrical ovary and stigma was cap-like (Figure 1 *c*). Many ovules were located on placentae that grew from the androcarpel inner wall into locular space (Figure 1 *e*). The *aco* and *ACO* flowers were self-fertile; the stamen formed in the outer androecium whorl of *aco* flowers produced fertile pollen, like stamen of all the whorls in *ACO* flowers. At maturity, fully formed seeds were present in androcarpels of *aco* plant, like in the capsules of *aco* and *ACO* plants (Figure 1 *d–e*).

Latex oozed out when the androcarpel wall was injured, like from the injured capsules of *aco* and *ACO* plants. Alkaloids were synthesized and accumulated in the androcarpels and capsules (syncarpous organs) of *aco* plants and capsules of *ACO* plants. The profile of morphinane and other alkaloids in androcarpels was similar to that of capsule in *aco* plants. The *aco* androcarpels and capsule together produced about 1.3-fold more carpellary husk and 33% more morphine and 110% more codeine in its andro- and normal-carpels, than that produced by the corresponding wild type *ACO* capsule (Table 2).

Table 2. Effect of *aco* mutation on the yield of morphine and codeine from capsules in *Papaver somniferum*

	Alkaloid yield (mg) in a kilogram of mature carpellary husk produced by flower of	
Alkaloid[a]	*ACO* wild type	*aco* mutant
Morphine (M)	10.2	13.6[b]
Codeine (C)	2.7	5.8

[a]Besides M and C, presence of codeinone, oripavine, reticuline, papaverine and narcotine could also be ascertained in androcarpillary and normal capsules of *aco* and wild type capsule of *ACO* plant; [b]The M and C yields of *ACO* and *aco* were significantly different.

The sepals, petals, stamens in the outer whorl and syncarpel are all wild type *ACO* like in the *aco* mutant. The inner whorl stamens develop into androcarpels. These carpels have filamentous base, cylindrical body and cap-like distal stigmatous end. They possess placenta and form ovules. Upon pollination with pollen formed in anthers of outer whorl stamens or heterologous pollen, androcarpels produce viable seeds. The phenotype of *aco* flowers does not seem to match that of known floral mutations in *Arabidopsis*, *Antirrhinum* and *Petunia*[6]. Since the number of stamenoid whorls has not changed, stamens are formed in the outer stamenoid whorl and the androcarpels are formed from inner whorl stamen initials, the androcarpels maintain certain stamenoid features (capped cylinder borne on a filament) confirming that the organ transformation occurred following organ initiation. The floral genes of B- as well as C-classes must have determined the androcarpel development in inner stamen whorls and that of B-class, the outer stamen whorl. The C-class of genes must have been ectopically expressed in the inner stamenoid whorls, on account of the *aco* mutation. Interaction of over-expressed C-class genes and normally expressed B-class genes is most likely responsible for the androcarpel organ development in the stamenoid inner whorls of *aco* flowers.

The *aco* character which enlarges carpel wall volume where alkaloids are accumulated, also increased the yield of alkaloids. Incorporation of *aco* character together with high alkaloid concentration in carpel walls is expected to generate improved genotypes/cultivars of opium poppy for the production of alkaloid-rich carpel straw raw material for chemical extraction of alkaloids in high yields. In view of the tremendous progress made in the field of molecular genetics of floral organ development in plants, the demonstration that androcarpels of *aco* mutant synthesize and accumulate opium poppy alkaloids opens the way for further genetic mutational and transgenic manipulations of poppy floral organs towards high levels of alkaloid yield per unit area of opium poppy crop.

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Phenotypic variation in cotton (*Gossypium hirsutum* L.) regenerated plants

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Somaclonal variation could be utilized for genetic improvement of cotton (*Gossypium hirsutum* L.). Although significant progress has been made in cotton regeneration, the phenotype of regenerated plants has rarely been investigated. We report here the phenotypic variation of regenerated cotton plants. Extensive variations exist in the F_0 generation and subsequent progenies of regenerated plants. Most of the phenotypic variations in F_0-regenerated plants were physiological or epigenetic and were not inherited by offspring. However, we have obtained sterile plants, and elite lines with characters of bigger boll, higher lint percentage or longer fibre from the progenies of regenerated plants. These variations in the F_1 plants were steadily inherited into the F_2 generation. The results will promote the application of plant tissue culture to cotton improvement.

COTTON is one of the most important fibre crops. Since Davidonis and Hamilton[1] obtained the first plant regeneration via somatic embryogenesis from two-year-old callus of *Gossypium hirsutum* L. cv Coker 310, significant progress has been reported in cotton tissue culture and plant regeneration. *In vitro* cultured cells of cotton have been induced to undergo somatic embryogenesis in numerous laboratories using varied strategies[1–11]; regenerated plants have been obtained from explants such as hypocotyl, cotyledon, root[10] and anther[11] of various cotton species. Regeneration protocols have been used to obtain genetically modified plants (insect-resistant[12,13], herbicide-resistant[13–16], disease-resistant) by *Agrobacterium*-mediated transformation[17,18] or by particle bombardment[19].

Although major progress has been made in cotton regeneration, the phenotype of regenerated plants has rarely been investigated[20,21]. We have regenerated plants from many cultivars of *G. hirsutum* L. via somatic embryogenesis[5,10,11,22–26]. This paper reports extent of phenotypic variation observed in cotton plants regenerated *in vitro*.

Seeds of *G. hirsutum* L. cvs Simian-3, CCRI 12, Sirokral 1-3 and Coker 201 were obtained from the Cotton Research Institute of the Chinese Academy of Agricultural Sciences, Anyang, China. Seed coat was completely removed, and the kernels were surface-sterilized by dipping in 0.1% mercuric chloride ($HgCl_2$) solution for 7 min. After rinsing three times with sterile water, kernels were placed on half-strength Murashige and Skoog (MS) medium[27] for germination. Hypocotyl sections (3–5 mm length) and cotyledon pieces (10 ~ 16 mm^2 surface area) from 5- to 7-day-old seedlings were placed on MS medium supplemented with 0.1 mg/l kinetin (KT), 0.1 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.1 mg/l indoleacetic acid (IAA) for callus induction. After 4 weeks of culture, calli were transferred to embryogenic callus induction medium (MS with 0.1 mg/l IAA and 0.1 mg/l zeatin (ZT)). After 4 to 6 weeks in this medium, embryogenic calli were transferred to the same medium for further proliferation. Every 28 days, embryogenic calli were subcultured on MS medium supplemented with 0.1 mg/l KT, 0.2 mg/l IAA and 0.1 mg/l 2,4-D.

Embryogenic calli, derived from MS medium supplemented with 0.1 mg/l KT, 0.2 mg/l IAA and 0.1 mg/l 2,4-D, were selected and transferred to MS medium supplemented with 0.1 mg/l ZT for the differentiation and maturation of somatic embryos. After 3 to 5 weeks, somatic embryos (Figure 1 *a*) at various developmental stages were observed. Mature somatic embryos, arrested at the late torpedo stage and cotyledonary stage, were selected and placed on MS medium supplemented with 0.1 mg/l ZT and 2 g/l activated charcoal for germination and plant regeneration. All media were supplemented with 30 g/l sucrose, and were solidified with 7 g/l agar (Beijing Biochemistrical Company, China). The pH of the medium was adjusted to 5.8 before autoclaving at

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