HAPLOID *ARABIDOPSIS THALIANA* CALLUS AND PLANTS FROM ANTHER CULTURE

By P. M. GRESSHOFF* and C. H. DOY*

Abstract

Haploid callus and plants were cultured from the anthers of diploid *A. thaliana*. This depends on removing anthers during late prophase of meiosis, selecting a genotype favouring callus formation from dividing sporocytes on a high auxin–low kinetin concentration, fully defined medium, then inducing differentiation by transfer to a low auxin–high kinetin concentration, fully defined medium, with a light–dark cycle. Attempts to produce embryoids directly from anthers were unsuccessful. The view that our approach may have a more general application is discussed in relation to the work of others and our culture of haploid callus and plantlets from tomato (*Lycopersicon esculentum*) and haploid callus from barley (*Hordeum vulgare*).

I. INTRODUCTION

Much of the success of biochemical genetics and molecular biology in providing an understanding of the sequence and regulation of metabolic pathways and the expression of the genome has depended on the ability to obtain a wide range of mutants. This was readily achieved in pro- and eukaryotic microorganisms by making use of largely haploid life cycles in conjunction with the ability to analyse the products of genetic crosses.

In higher green plants the haploid phase of the life cycle is short and not readily available for experimentation and in consequence higher plants are relatively poorly understood at the molecular level. A prolongation of the haploid phase, for example in tissue culture, and the development of systems of genetic analysis of such tissue would constitute a major advance. It has been suggested that the limited spectrum of mutants in higher plants may be because of developmental selection against homozygous auxotrophic mutants during early embryogenesis (Langridge 1958). This would be overcome if mutants were selected in tissue culture.

Methods already published for the production of haploid plants from anthers (Guha and Maheshwari 1964, 1966; Nitsch and Nitsch 1969; Sunderland and Wicks 1969) seem only applicable to the species concerned and depend on the use of poorly defined, highly supplemented media, which are undesirable if auxotrophs are a potential objective.

* Department of Genetics, Research School of Biological Sciences, Australian National University, P.O. Box 475, Canberra City, A.C.T. 2601.

The recent report of six auxotrophs (representing a wide range of nutritional requirements) derived from anther culture of the polyploid *Nicotiana tabacum* (Carlson 1970) encourages the view that a tissue culture approach may be useful. Unfortunately the mutants were leaky, which would be expected of multiple genes coding for the same protein or different genes coding for proteins capable of cross-functioning. To overcome the potential problem of polyploidy we chose the diploid crucifer *Arabidopsis thaliana* for anther culture.

The present paper describes a method for obtaining true haploid callus and plants of *A. thaliana* on completely defined media. This plant is particularly convenient for laboratory use, being small, having a short life cycle, a large number of seeds, and the ability to be cultured aseptically on defined medium (Langridge 1957).

II. MATERIALS AND METHODS

(a) A. thaliana *Races*

Successful anther cultures were obtained with the following races: Wassilewskija (WS); Estland (EST); Martuba (MT).

Attempts to obtain anther cultures with the following 15 races were unsuccessful: Berlin–Dahlem (BD); Bensheim (BE); Blanes (BLA); Bonn (BO); Brunn (BR); Schiedra (CHI); Coimbra (CO); Catania (CT); Darmstadt (DA); Enkheim (EN); Frankfurt-am-Main (FR); Hilversum (HI); Koln (KOL); Bad Vilbel (VI); Wachkiippel (WK).

(b) *Stock Solutions*

The stock solutions contained either mineral salts solution 1 (MS1) (see Gamborg 1968) or mineral salts solution 2 (MS2) (see Blaydes 1966), vitamins–glycine solution [nicotinic acid 10 mg, thiamine 100 mg, pyridoxine 10 mg, myo-inositol 1000 mg, glycine 40 mg, distilled water to 100 ml], trace elements (see Gamborg 1968), and iron chelate [$FeSO_4.7H_2O$ $5 \cdot 57$ g, disodium ethylenediaminetetraacetate $7 \cdot 45$ g, and distilled water to 1 litre].

(c) *Defined Basal Medium*

Defined basal medium 1 (DBM1) was prepared by mixing, before autoclaving, MS1 (200 ml), vitamins–glycine solution (10 ml), trace elements (1 ml), iron chelate (5 ml), sucrose (20 g), agar (8 g), and distilled water to 1 litre, adjusting to pH $6 \cdot 2$–$6 \cdot 5$ with NaOH. Defined basal medium 2 (DBM2) was prepared by substituting MS2 for MS1 in the above. The compositions of growth media 1–4 are listed in Table 1.

TABLE 1

GROWTH MEDIA

Hormones were added before autoclaving

Growth medium	Defined basal medium*	Concn. (mg/l) of hormones added to defined basal medium			
		NAA	IAA	2,4-D	Kinetin
1	DBM1	—	$6 \cdot 0$	$2 \cdot 0$	$1 \cdot 5$
2	DBM2	$20 \cdot 0$	—	—	$0 \cdot 03$
3	DBM2	$0 \cdot 5$	—	—	$10 \cdot 0$
4	DBM2	$2 \cdot 0$	—	—	$0 \cdot 03$

* For composition see Sections II(c) and II(d).

(d) *Excision of Anthers*

The flowers were removed from vigorously growing diploid plants at a bud length of approximately $0 \cdot 5$ mm (which coincided with a stage of development where the pollen mother

cells were in early meiosis) and surface-sterilized by immersion in absolute ethanol for 10 sec. The anthers were removed aseptically, put onto agar slants of sterile growth medium 1 (10 ml) contained in cotton-wool-plugged scintillation vials, then placed at 25°C, 70% relative humidity, and 4000 lux m^{-2} for 24 hr (to ensure continuation of meiosis) after which the light was turned off. Transfer to growth media 2, 3, or 4 for differentiation or accelerated callus growth (see Section III) was accompanied by a 16 hr light–8 hr dark cycle.

(e) Chromosome Counts

Actively growing root tips (or callus) or haploid plants were prepared by the standard Feulgen method (Darlington and La Cour 1947).

III. Results

(a) Preliminary Studies

Preliminary studies with diploid cultures of *A. thaliana* indicated that MS1- and MS2-based media supported callus growth. Other mineral salt solutions (White 1943; Linsmaer and Skoog 1965; medium N of Vogel 1956, 1964), supplemented with vitamins–glycine solution, trace elements, iron chelate, sucrose [see Section II(c) as for DBM1], and 2,4-dichlorophenoxyacetic acid (2 mg/l), failed to initiate and support callus growth from *A. thaliana* stems. Therefore the supplemented MS1 and MS2 (in the form of DBM1 and DBM2) were used as the basis for haploid tissue culture.

(b) Haploid Tissue Culture

Eighteen inbred varieties of *A. thaliana* were used to provide anthers. Anthers of three (race WS, EST, and MT) of these produced solid yellow callus 14–21 days after excision. The yield of callus formation was maximal on growth medium 1 (Table 1), where 80% of the excised anthers of race WS developed as compared to 40–60% of races EST and MT. Other growth media, mainly those used for anther culture of other plant species, failed to produce any growth in the pollen sacs. It is important to take anthers at the correct stage of meiosis [see Section II(d)] since less or more mature pollen was relatively unsuccessful.

After 4 weeks of growth the callus reached a fresh weight of approximately 100 mg. Transfer to growth medium 2 (Table 1) ensured further callus growth at an accelerated rate.

(c) Differentiation of Haploid Plants

Differentiation of the callus to a complete plant was achieved by transfer to growth medium 3 (Table 1) and incubation under controlled conditions including a light–dark cycle [see Section II(d)]. The callus differentiated chloroplasts and shoots when kept in continuous light but root formation was retarded. Conversely, dark incubation prevented differentiation of chloroplasts and shoots but favoured root formation.

Greening of the callus and the development of shoot primordia (dark green depressions on the callus surface) occurred within 21 days after transfer to growth medium 3. A single fasciated shoot developed from one of the shoot primordia in the next 14 days (Fig. 1). This shoot produced a mass of green, leaf-like structures (Fig. 2). Roots formed (Fig. 3) at the base of the shoot, thus producing a plantlet, which
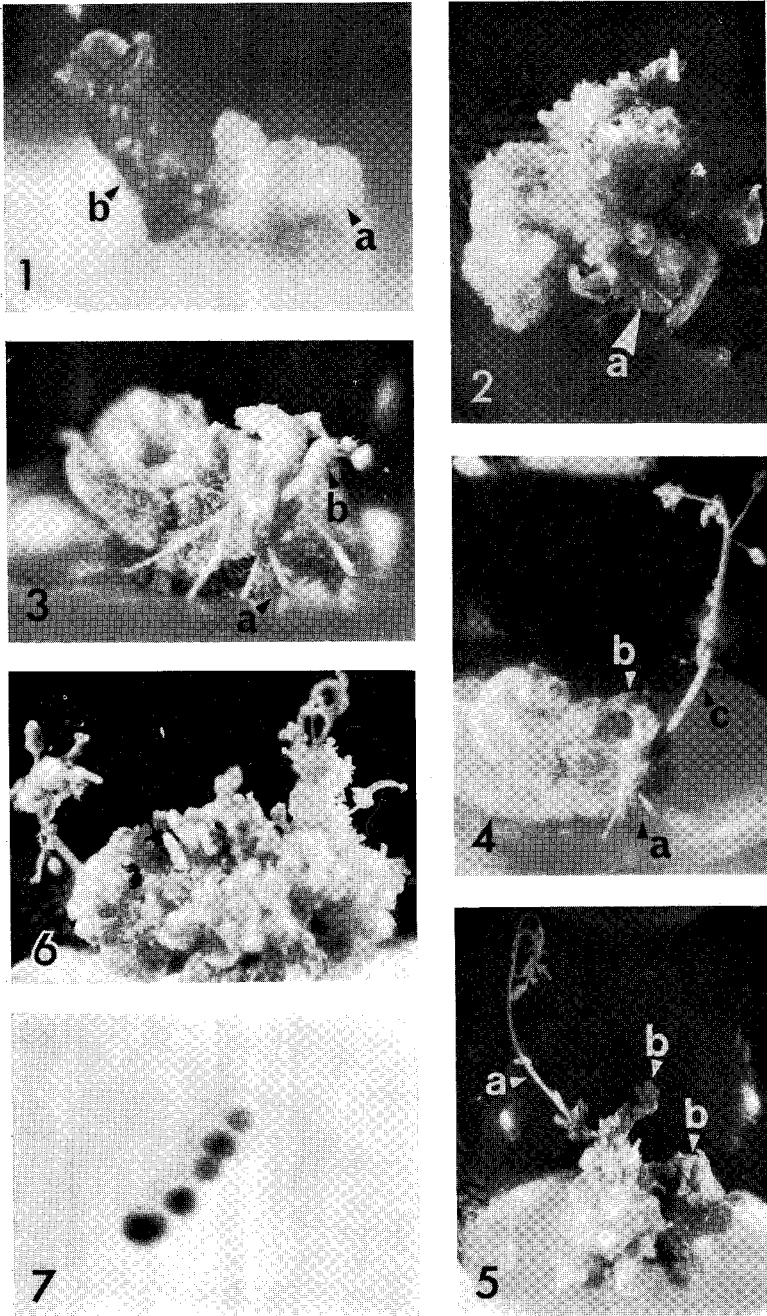
Fig. 1.—Haploid callus (a) with developing shoot (b).

Fig. 2.—Leaf-like structures (a).

Fig. 3.—Advanced root development (a) and small flowering shoot (b).

Fig. 4.—Complete haploid *Arabidopsis thaliana* with roots (a), leaf-like
       structures (b), and mature flowering shoot (c).

Fig. 5.—Dead shoot (a) and regrowth (b) initiated on callus.

Fig. 6.—Regrowth with multiple shoots.

Fig. 7.—Haploid metaphase (n = 5) from root tip squash. × 4000.

could be separated and grown individually on growth medium 4. A flowering shoot developed from the central area of the plantlet (Fig. 4) within 7 days after root formation. This shoot had normal *A. thaliana* flower and stem morphology.

The flowers were sterile as expected for a haploid condition. The shoot died within 15 days after flowering (Fig. 5); at this time other shoot primordia, if present on the callus, developed into new shoots (Fig. 6).

Chromosome counts of root tips and callus material, stained by the Feulgen method (Darlington and La Cour 1947), indicated haploidy ($n = 5$) (Fig. 7).

IV. Discussion

Previous studies with *Oryza sativa* (rice) (Guha, Gupta, and Swaminathan 1970) indicated that successful anther culture may be dependent on genotypic variation. Our results support this conclusion not only for *A. thaliana* but for other important plants. For example, in similar experiments, haploid callus with subsequent plantlet development has occurred from 3 of 43 inbred varieties of *Lycopersicon esculentum* (tomato) and callus was initiated from 1 of 30 varieties of *Hordeum vulgare* (barley). We shall report these experiments in detail elsewhere.

Previous methods for anther culture favoured excision at the late tetrad or uninucleated pollen grain stage. Because of the difficulty of determining this stage we chose to remove anthers during late prophase of meiosis, thereby allowing meiosis to continue on the culture medium.

In our experiments anther culture proceeds in two major steps: (1) haploid callus formation from the dividing sporocytes; and (2) the differentiation of this callus to a haploid plant. This developmental process has been observed in the growth of haploid *O. sativa* (Guha, Gupta, and Swaminathan 1970) and is in contrast with *Nicotiana tobacum* (Carlson 1970) where an embryoid and plantlet are directly induced from the anther. Not surprisingly there are fundamental differences in the hormonal requirements of these processes. The anther–callus–plant sequence requires a high auxin medium initially to induce callus formation from the actively dividing sporocytes, while the anther–plantlet sequence requires a very low auxin concentration so that differentiation can proceed at an early stage, thus producing the embryoid. *A. thaliana, O. sativa, L. esculentum,* and *H. vulgare* anthers do not develop when placed on very low auxin medium suggesting that certain plant species require high auxin activation for successful anther culture. *Datura innoxia* anthers, on the other hand, can undergo development of both kinds as determined by the hormonal activation (Guha, Gupta, and Swaminathan 1970).

Our success with the culture of anthers from a number of plants leads us to conclude that an approach to haploid tissue culture of higher plants which considers genotypic variation, early excision of anthers, and induction of callus prior to plantlet formation may have wide application.

V. Acknowledgments

VI. References

BLAYDES, D. F. (1966).—*Physiologia Pl.* **19**, 748.

CARLSON, P. (1970).—*Science, N.Y.* **168**, 487.

DARLINGTON, C. D., and LA COUR, L. F. (1947).—"Handling of Chromosomes." 2nd Ed. Rev. (George Allen & Unwin: London.)

GAMBORG, O. L. (1968).—*Can. J. Biochem.* **46**, 417.

GUHA, S., and MAHESHWARI, S. C. (1964).—*Nature, Lond.* **204**, 497.

GUHA, S., and MAHESHWARI, S. C. (1966).—*Nature, Lond.* **212**, 97.

GUHA, S., GUPTA, N., and SWAMINATHAN, M. S. (1970).—*Curr. Sci.* **3**, 174.

LANGRIDGE, J. B. (1957).—*Aust. J. biol. Sci.* **10**, 243.

LANGRIDGE, J. B. (1958).—*Aust. J. biol. Sci.* **11**, 58.

LINSMAER, E., and SKOOG, F. (1965).—*Physiologia Pl.* **18**, 100.

NATAKA, K., and TANAKA, M. (1968).—*Jap. J. Genet.* **43**, 65.

NITSCH, J. P., and NITSCH, C. (1969).—*Science, N.Y.* **163**, 85.

SUNDERLAND, N., and WICKS, F. M. (1969).—*Nature, Lond.* **224**, 1227.

VOGEL, H. J. (1956).—*Microb. Genet. Bull.* **13**, 42.

VOGEL, H. J. (1964).—*Am. Nat.* **98**, 435.

WHITE, P. R. (1943).—"A Handbook of Plant Tissue Culture." Vol. 4. (The James Cattrell Press: Lancaster, Pa.)