FLOWER DIFFERENTIATION IN ARABIDOPSIS

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SUMMARY

There is a consensus among developmental geneticists that few generalizations are possible at the present status of the field, and even the boundaries are difficult to define. Yet in few special cases, consistent facts have been accumulated which point to systems of controls of differentiation. - In the facultative long-day plant Arabidopsis, the differentiation of flower primordia is controlled by several gene loci. Recessive mutations may determine in a qualitatively distinct manner the onset of flower development. Continuous illumination in contrast to 8-9 hours daily cycles of light promotes flowering in all genotypes. Mutants at the ld locus are incapable of flowering under short days and entail a critical day-length. Different alleles at the gi locus require several times as long period for flower induction than the wild type under 24 hours light yet under short days they do not differ very conspicuously from the standard type. Mutants at the co locus are late flowering and recessive under long days but they are more precocious than the wild type under short days and they display dominance. In total darkness, the wild type and all mutants flower early. The aseptic feeding of 5-bromodeoxyuridine highly accelerates flower differentiation in all genotypes under long days and also under short days with the exception of the ld mutants. The analog is incorporated into the DNA of all types. Bromodeoxyuridine-grown plants accumulate higher amounts of radioactivity, provided by ^{14}C-amino acids, into a chromatin fraction. The experimental observations support the view that flowering in this plant is under negative control and bromodeoxyuridine is hampering the synthesis of a postulated supressor.

INTRODUCTION

Developmental genetics generated considerable interest in recent years yet when it comes to the definition of the area

proper serious difficulties arise. "The subject, insofar as one can define it, has developed no pervasive set of principles - which is the reason, no doubt, that its borders are vague"(SRB 1972).

"Evolution and phylogeny have endowed organisms, their growing regions and cells, with a multiplicity of feasible functions. The smooth ontogenetic course of normal nutrition, metabolism, growth, and development deceptively hides the baffling complexity of so many discrete steps, genetically imprinted upon cells, being so accurately programmed and coordinated. But when known external variables and stimuli modify this course and produce, predictably, directed and obvious responses, the usual gap in our knowledge concerns the way that stimuli as perceived and transmitted are effectuated. Geneticists do not yet know how genes program development or are "turned on or off"; physiologists still need to understand the varied mechanisms of stimulus and response" (STEWARD and KRIKORIAN 1972).

The understanding of differentiation and development requires a knowledge of selective functioning of genes in a systematic pattern. Even if we consider this problem in Arabidopsis, a perfect higher eukaryotic plant, with the lowest amount of DNA in its cells in the group, the complexity becomes staggering. Ignoring the extranuclear genetic material which has important functions (RÉDEI 1973, RÉDEI and PLURAD 1973) we still have to asses the role of the 4 billion nucleotides estimated in the diploid nuclei (SPARROW, PRICE and UNDERBRINK 1972). Undoubtedly some of this DNA is redundant as guessed from the substantial amount of heterochromatin present in the nuclei (LAIBACH 1907, STEINITZ-SEARS 1963) yet obviously evolution would not have preserved even the reiterated sequences of genes without some purpose or function. Logically one would expect that in the control of differentiation and development the role of a gene is both absolute and relative, relative to pathways which procure substrates or dispose products. Thus even if it would be possible to estimate the number of genes functioning, we would have to face the assesment of the interactions possible among these functions.

WATSON (1970) in his recent textbook concludes (p.513): "*no one* will ever be able to work out *all* the chemical details that accompany embryological development of any higher plant or animal".

In spite of the difficulties of generalizations a few chemical mechanisms became known to participate in the determination of the developmental processes (RUTTER, PICTET and MORRIS 1973, SRB 1972, HESLOP-HARRISON 1972, MEDVEDEV 1970, GROSS 1968).

The mechanisms of differentiation in higher plants is much less well understood in physico-chemical terms than in

lower forms or in higher animals (see Brookhaven Symp. Biol. Vol. 25).

In the followings, the information collected on flower differentiation in mutants of Arabidopsis will be summarized. The review of literature will be restricted only to the most pertinent work since the general area of flowering physiology has been amply covered by several recent works (SCHWABE 1971, EVANS 1971, BRIGGS and RICE 1972, LANG 1965, LOCKHART 1961, CHAILAKHYAN 1968, NOUGARÈDE 1967). Comprehensive treatment would be impossible within the framework of this presentation since the annual number of publications on flowering exceeded 200 in recent years (EVANS 1971).

DESCRIPTION OF THE MUTANTS

Arabidopsis thaliana (L.) Heynh. has a very wide geographical distribution from the near-arctic to the tropical mountain regions (RÉDEI 1970) yet very little genetic variation can be observed in these different habitats with the exception of flowering time. The northern ecotypes generally require vernalization while the rest may or may not respond to cold treatment. Irrespective of their flowering time they respond to continuous illumination with acceleration of flowering. Arabidopsis is generally classified as a facultative long-day plant since the summer annual ecotypes at moderate temperatures can develop flower primordia also in 4-5 hours daily light periods (LAIBACH 1951).

In the summer annual ecotypes studied so far, late flowering was dominant over earliness. The difference was determined generally by one major and several minor genes (HÄRER 1951). These dominant genes do not lend themselves well for a genetic study of flower differentiation since not even the number of loci involved could be determined in the absence of allelism tests.

Table 1. Photoperiodic response of X-ray mutants on mineral agar medium containing 2% glucose.

Genotypes	Number of days required to visible flower primordia under illumination	
	24 hrs	9 hrs
Columbia *wild* type	11.1	36.3
pr	9.5	24.0
co^1	15.7	27.3
gi^1	19.4	41.4
gi^2	32.2	45.9
ld^1	17.0	no flower

REINHOLZ (1947) discovered that in an early flowering line, several late flowering types can be induced by X-rays. Subsequently various early and late flowering mutations were induced by different mutagens (RÉDEI 1962 and unpublished, McKELVIE 1962).

The mutations induced in our laboratory displayed the same wide range of photoperiodic responses as their counterparts in nature but all of them were recessive (Figures 1,2,3). Mutations at the *co* locus behaved unexpectedly, inasmuch as they were recessive under continuous illumination and dominant under short daily light cycles. Several of these photoperiodism mutations have been located to chromosomes. These studies indicated that flowering is under the control of a large number of genes, scattered in the genome.

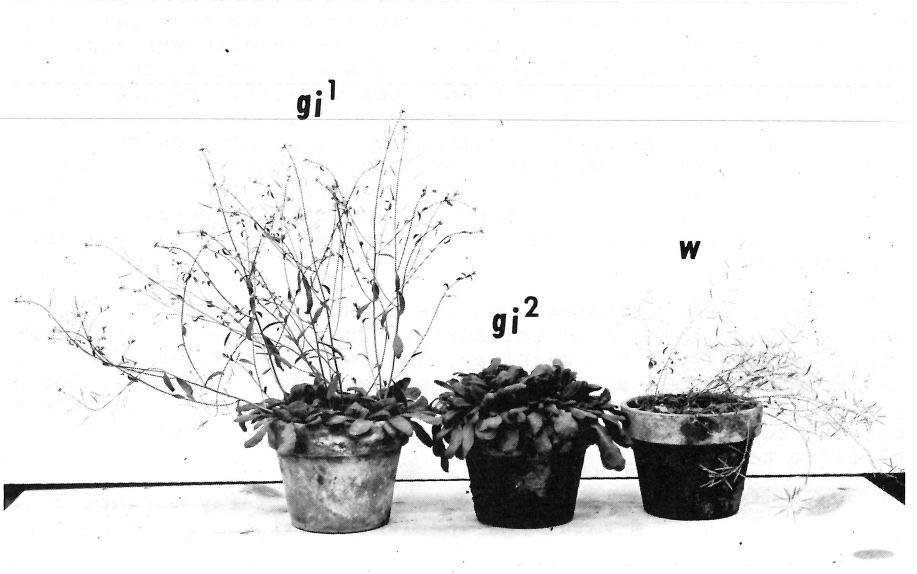

Figure 1. Arabidopsis grown under continuous illumination in a greenhouse. Right: Columbia *wild type*, middle: gi^2, left: gi^1 mutants.

These mutants are certainly unusual inasmuch as they display more vigorous growth than the wild type. The increased vegetative development and growth is accompanied by greater seed production and improved competitive ability resulting in very high selective values in mixed populations (RÉDEI 1962).

Figure 2. Wild type, and mutants grown under 9 hours daily illumination.

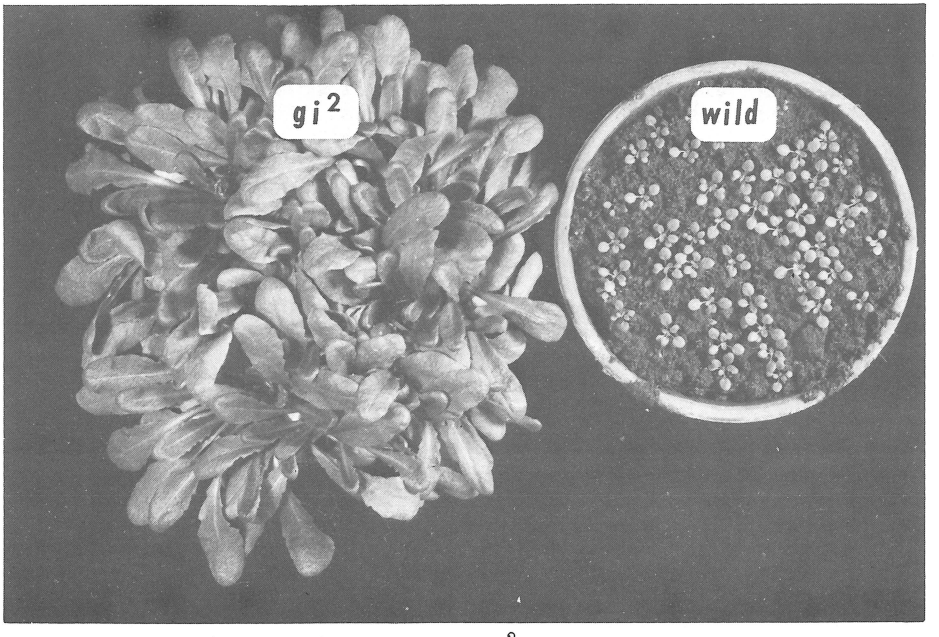

Figure 3. *Wild* type and mutant gi^2 under continuous light at the time when flower buds visibly appear. The age of the plants is not identical.

There are numerous reports in the literature on induced quantitative variation. These mutants, in sharp contrast to that group, represent discrete qualitative changes and perfectly mendelian inheritance.

All these mutations are involved in the control of flower differentiation and thus permit an analysis of the process both by genetic and physiological means.

Because of the small size of the plants, they can be cultured on completely defined media under aseptic conditions and the various environmental factors can be well controlled. A further advantage is, that the genetic background is identical with the exception of the mutations concerned. The morphological, histological aspects of floral differentiation have been thoroughly studied in the past but it was interpreted differently by the investigators.

HISTOLOGICAL EXPRESSIONS OF FLOWER INITIATION

Flower buds arise through some physiological changes affecting the apical meristem and causing visible alterations in the cellular organization there (see for reviews WARDLAW 1965, NOUGAREDE 1967).

The transformation of the vegetative apex to a floral one has been the topic of vigorous debates since the past century. NÄGELI (1845), the famous correspondent of Gregor Mendel, developed the 'apical cell theory' by his suggestion that all the tissues and organs are derived from a single cell through a series of transformations. His concepts were based on the studies of cryptogams yet his errors in this field were relatively minor comparing to those concerned with inheritance. In the mature embryo of Arabidopsis the leaves and other epicotyledonar organs are represented by about 16 cells (RÉDEI and LI 1969) and the germline constitutes of only two cells (LI and RÉDEI 1969).

HANSTEIN (1868) suggested the 'histogen theory', according to what all morphologically distinguishable tissues are supposed to be the descendants of different individual cells or cell groups in a predetermined fashion.

The third group of interpretations are traced to SCHMIDT (1924) who distinguished in the apex an outer layer (tunica) and an inner core (corpus) of cells. His ideas were further developed by PLANTEFOL (1946) and his students. These French botanists were influenced also by the views of GRÉGOIRE (1938) who taught that ..." le receptacle floral...provient... d'un sommet inflorescentiel qui n'a... rien de commun avec un cône végétatif" (p. 331). According to the French school, in the vegetative apex mitotic activity is most intense in a meristematic ring (anneau initial). The critical step in pre-

floral transformation comes when the subapical core, restive in the preceeding phases (méristème d'attente), initiates a greater mitotic activity which is followed by a general rapid cell division over the entire apex resulting in the formation of the well familiar floral parts. This theory of cytohistological zonations has been the target of criticism since a clear distinction between the role of these apical regions is often hard to demonstrate (WETMORE, GIFFORD and GREEN 1959).

Students of differentiation of the apical meristem of Arabidopsis (VAUGHAN 1955, MIKSCHE and BROWN 1965, WIBAUT

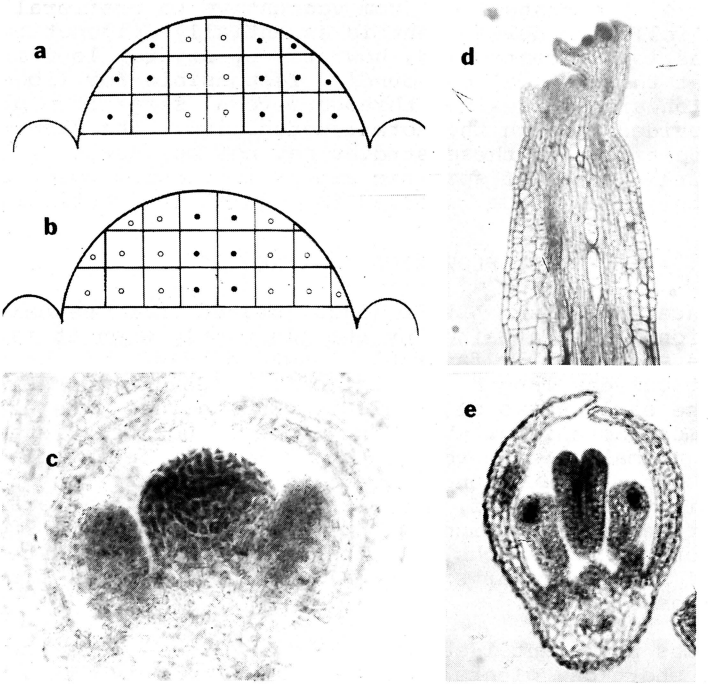

Figure 4. The major histologically visible steps of flower differentiation. The schematic drawings are oversimplified representations of the vegetative (a) and of the prefloral apex (b). The biseriate surface cell layer is omitted since its role in this process is not critical. Solid dots represent the mitotically active and the open circles the quiescent nuclei. In the third major step (c) the apex elongates and mitotic activity expands to the flanks also, then elongation and differentiation of the sex organs follows (d) and in the central region the two carpels and the flanking anthers become easily visible (e).

1966, BESNARD-WIBAUT 1968) agree that in this species floral differentiation proceeds according to the general scheme outlined by the French school.

Thus flower differentiation follows some definable steps in Arabidopsis (Figure 4), yet we were not able to identify any mutant which would be specifically involved in the control of these major steps. Apparently all are affected in the physiological control of the morphologically not distinguishable steps, preparatory to the histological transformations.

It is conceivable that the genes control only the determination of the transition from vegetative to prefloral stages and the following development is essentially epigenetic. This determination is controlled, however, by several loci indicating that the chemical compound(s) responsible for flower initiation are synthesized through several steps from precursors provided through the normal metabolism. The flowering genes identified in these studies may not be, however, the 'structural genes' of specific flower initiating molecules, rather they seem to be involved in regulatory functions.

FLOWERING IN DARKNESS

Since GARNER and ALLARD (1920) stated that "sexual reproduction can be attained by the plant only when it is exposed to a specifically favorable length of day" and the notion of 'critical day length' was introduced (GARNER and ALLARD 1923) the early views of VÖCHTING (1893) gained wide acceptance among botanists. Vöchting wrote: "Um ihre Blüthenbildung in normales Weise vollziehen zu können, bedarf die Pflanze einer Beleuchtung, die unter ein gewisses unteres Maass nicht sinken darf, deren Stärke aber bei den verschiedenen Arten sehr ungleich ist" (p.186). MacDOUGAL (1903) points out that J. Sachs felt that plants with adequate supplies of speciel reserve material might form normal flowers in darkness (p. 10).

Sachs prescient ideas were verified many years later. Several short-day plants like morning glory (TASHIMA and IMAMURA 1953, TAKIMOTO 1960), buckwheat (INOUYE 1965) and long-day plants as peas (BÖRGSTROM 1939, GENTCHEFF and GUSTAFSSON 1940, HAUPT 1952), wheat (SUGINO 1957, INOUYE, TASHIMA and KATAYAMA 1964), barley (INOUYE and ITO 1968), spinach (GENTCHEFF and GUSTAFSSON 1940, TASHIMA and IMAMURA 1953), *Baeria, Silene, Rudbeckia* (TASHIMA and IMAMURA 1953), radish (TASHIMA 1953, TASHIMA and IMAMURA 1954, TASHIMA and KIMURA 1958, KIMURA 1961) have been observed to flower in complete darkness.

According to the majority of these reports flowering in the dark required about the same length of time as under the favorable light regimes. BÖRGSTROM (1939) claims that in the American Wonder variety of pea flowering in the dark took

place in half or less the time than under 15-18 hours daily light cycles.

The majority of these species were cultured in the dark on sucrose media, though radish (a vernalization requiring variety) developed flower primordia in the cold (under 20° C) without this supplement to the agar medium. Thus the natural reserve material in the seed could supply all the organic material needed for floral induction (KIMURA 1961). These experiments proved the correctness of Sachs' predictions a century earlier.

The majority of the plant physiologists being preoccupied with the various conditions of illumination (critical day-length, light intensity, spectral quality, cycling, hormones etc.) did not consider dark flowering of major importance concerning the interpretation of the mechanism of flowering. Comprehensive reviews mentioned the experimental reports but either overlooked its significance (LOCKHART 1965, SCHWABE 1971) or simply dismissed its meaning after some consideration as stated by LANG (1965):"It thus seems premature to use the ability of some photoperiodic plants to form flowers in darkness as a basis for far-reaching reinterpretations of photoperiodism"(p. 1455).

On the basis of some early experiments with the long-day plant *Hyoscyamus niger*, the conclusion was reached that there are two phases of flower induction, one is sensitive, the second is independent from the dark reactions. The dark cycles initiated inhibitory-processes (Hemmungsvorgänge). Consequently dark periods were not believed to be of usefulness in the flowering process of long-day plants (LANG and MELCHERS 1943). These interpretations are generally favored even in recent times (LANG 1965).

Working with Hyoscyamus and rice (both long-day plants) completely different conclusions are expressed by other experimentalists. "No inhibition of floral initiation occurs in continuous darkness, very short photoperiods cause a delay in floral initiation" (BEST 1960). "Inhibition is due to the production of an inhibitor precursor, which becomes active mainly in darkness, following a light period. In dark, the precursor will be gradually transformed into an inhibitor which itself has no measurable persistence so in continuous darkness the plant is no longer inhibited, no more than during light". (DeLINT 1960).

LANG (1965) aware of these conclusions summarized his evaluation as follows:"...these arguments do not possess... a very solid factual foundation. The number of photoperiodic plants in which flower formation in total darkness has been obtained is quite small. Among the long-day plants, on which the argument is principally hinging, there were several (peas, summer wheat, possibly also both radish and spinach) with only a quantitative long-day response, i. e. plants which

are able to form flowers in non-inductive photoperiod conditions and therefore, presumably, also in continuous darkness provided they are able to survive" (p. 1454).

It appeared worthwhile to examine this problem with Arabidopsis. Mutant *ld*[1] is different from the majority of genotypes studied in this species inasmuch as it has a 'critical daylength'. Though the switch point has not been determined yet, it has been firmly established that this mutant does not flower in 9 hours long daily light cycles in periods of time exceeding ten fold that required for continuous illumination. At various temperatures, and in both soil and agar cultures, we failed to obtain flowering plants under short-day conditions. Periods longer than 150 days could not be tested because the plants died by old age after that time. It should be noted that this is an exceptionally vigorous mutant, and under long-day conditions in both vegetative growth and seed output, it surpasses the wild type.

Our standard (Columbia wild type) or any of our mutants tested do not grow in darkness on solid media supplemented with sugars. Similarly this material fails to grow beyond the cotyledonous stage in liquid mineral media free of sugar. If sucrose or glucose is provided, all the genotypes, listed in Table 1, flower under continuous illumination in liquid culture if the temperature does not rise too high due to direct exposure to strong sunlight. Their response to short days is also comparable to that observed on solid media. Slight agitation of the cultures may be somewhat beneficial but not necessary for growth or for flowering. Actually, in shaken culture distorted stem and leaf development can be regularly observed.

Similarly, substantial growth can be obtained in liquid culture - in contrast to solid media - in complete darkness. Generally single seeds are dropped into 5 ml medium in standard size test tubes or 30-80 mls are used in bottles or Erlenmeyer flasks and more seeds planted. After planting, the cultures are placed in a light-tight container for a day and subsequently they are exposed to less than one hour white light to secure germination. The majority of genotypes of Arabidopsis have a light requirement for germination. Then the culture vessels are wrapped with two layers of aluminum foil and placed in the greenhouse or in a constant temperature box for various periods of time. The cultures are never opened before the termination of an experiment. Germination usually takes place in 3-4 days after planting. Thus the plants are not exposed to any light after emergence.

It poses some problems to express flowering in days in in total darkness because flowering within the experimental series does not take place exactly the same day in all plants, and once the cultures have been opened to light for observation, they have to be terminated.

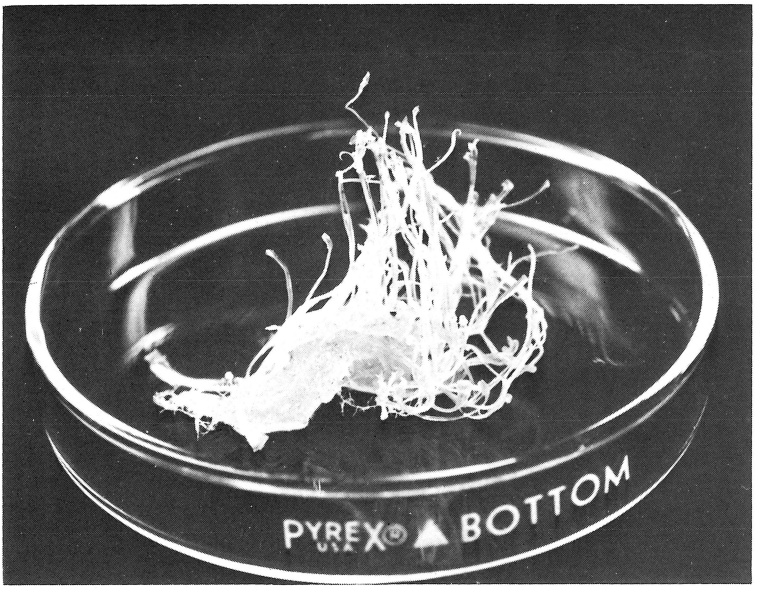

Figure 5. A bundle of plants grown in complete darkness on 80 ml medium in a single Erlenmeyer flask. Age 7 weeks.

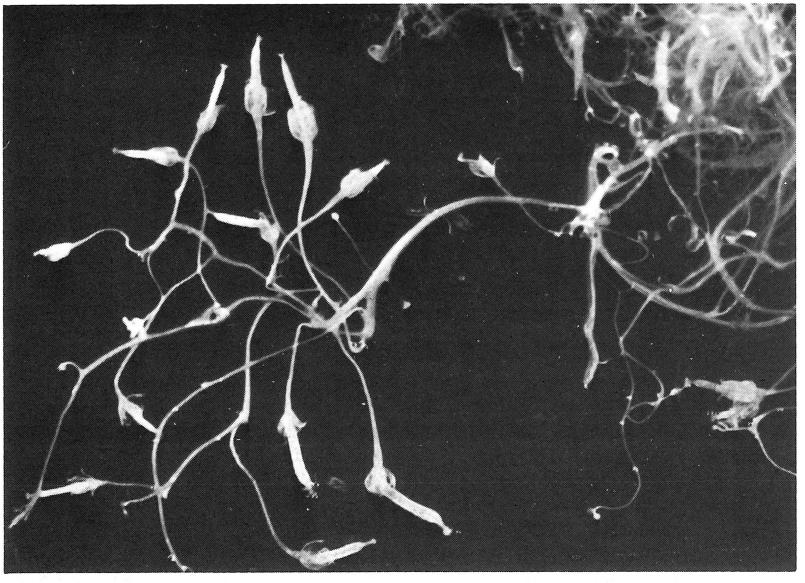

Figure 6. A single plant cultured as indicated in caption of figure 5 at the age of 9 weeks. The number a fruits exceed the usual, formed *in vitro* in long days.

Fortunately, there is a very close correlation between the number of roseette leaves formed before the appearance of flower buds and flowering time (compare Tables 1 and 2). Just as flowering time is determined by the duration of light cycles, by the light intensity and by the temperature so is the number of rosette leaves. During the summer especially in the agar cultures the wild type may develop only four leaves under continuous illumination and under short days we may find only 9-10 leaves. In the dark, year around we were able to observe flowering with a minimal number of leaves. Remarkable was that in total darkness all genotypes, including *ld*, flowered early. In some experiments flower buds were definitely seen in 15 days after germination and in 7 weeks fruits were observed. In some two months old cultures, empty seeds were well visible.

Table 2. Number of rosette leaves developed before the appearance of visible flower primordia in the wild type and mutants under different conditions of culture.

Media →	Soil	Agar	Agar	Liquid
Illumination, hrs →	24	24	9	0
Columbia *wild*	6.9	6.1	26.8	4.0
ld^1	8.4	-	-	3.0
co^1	10.1	-	-	4.4
gi^1	17.9	-	-	-
gi^2	34.9	17.9	32.7	3.0

Observations on dark flowering indicate not only that in this species light is not a prerequisite either for the determination or for the realization of this developmental process. Flower initiation and differentiation is delayed by light, especially when the daily cycles are short. In mutant *ld* under short days flowering was never observed since it was isolated 16 years ago. In continuous dark culture it flowered with about the same facility as the wild type.

The data accumulated during the years seem to indicate that the flowering response is under negative control in this plant. As a minimum, four genes are involved in the process and all respond with delayed flowering to short daily illuminations. In dark the difference among them is practically eliminated, and all appear to be derepressed. The simplest interpretation is that light triggers the synthesis of one or more photosensitive inhibitors which decompose or get inacti-

vated in long light cycles but persist under short days for a long period of time.

Apparently none of these mutants are blocked in the synthesis of an indispensable flowering hormone, rather they control the production or activation of repressor(s) functioning under short light regimes. Present information is insufficient to tell whether these genes mediate sequentially the synthesis of precursors of a single light sensitive repressor or several such repressors exist. At the *gi* locus two very clearly distinguishable alleles exist indicating that the product of that locus has more than one active site or functional groups.

The mutations provide minor clues concerning the nature of flowering hormones. It is conceivable that the production of physiologically active flowering hormones hinges on the activities of these regulator genes. One may assume that flower differentiation *per se* is an epigenetic process, determined by the availability of some common metabolites. Under light regimes unfavorable to flowering the competition for these active compounds prevents their channeling to this type of process of differentiation. The failure of chemical isolation of flower-inducing substances may be considered as a circumstantial support for this assumption as it has been advocated earlier by DENFFER (1950) and BAXTER (1972). Also there is good evidence for the regulatory effect of light on enzymes (see ZUCKER 1972). It is equally plausible that the production of specific flowering hormones is under direct genetic *control* and the failure of isolation is due to some other intrinsic problems.

BROMODEOXYURIDINE AND FLOWERING

Since the experiments of J. SACHS (1865) suggested that the flowering process is initiated in the green leaves, a material basis of induction was postulated (SACHS 1880), and the hypothetical compound was named as 'anthogen'(NĚMEC 1934), 'blastenine', 'anthesin' (CHOLODNY 1939) or 'florigen' (CHAILAKHYAN 1936). Continued efforts either to isolate (HODSON and HAMNER 1970, KOLLI 1969) or to find a known organic compound with florigenic properties met little success (CHAILAKHYAN 1968). A listing of all the compounds which had some apparent or real stimulating effect on flowering cannot be attempted within the frame of this paper.

The majority of the tested organic compounds were ineffective or inhibitory and the relevance of their effect to the process of flowering was implied by 'lucus a non lucendo' syllogisms.

Special consideration is deserved by gibberellic acid which apparently promoted flowering conspicuously in a number of species. CHAILAKHYAN (1972) is tempted to suggest that for long-day plants gibberellic acid or a related compound may serve as 'florigen'. Short-day plants are not satisfied, however, by this kind of substance thus the logical conclusion was that they require at least another hormone, 'anthesin'.

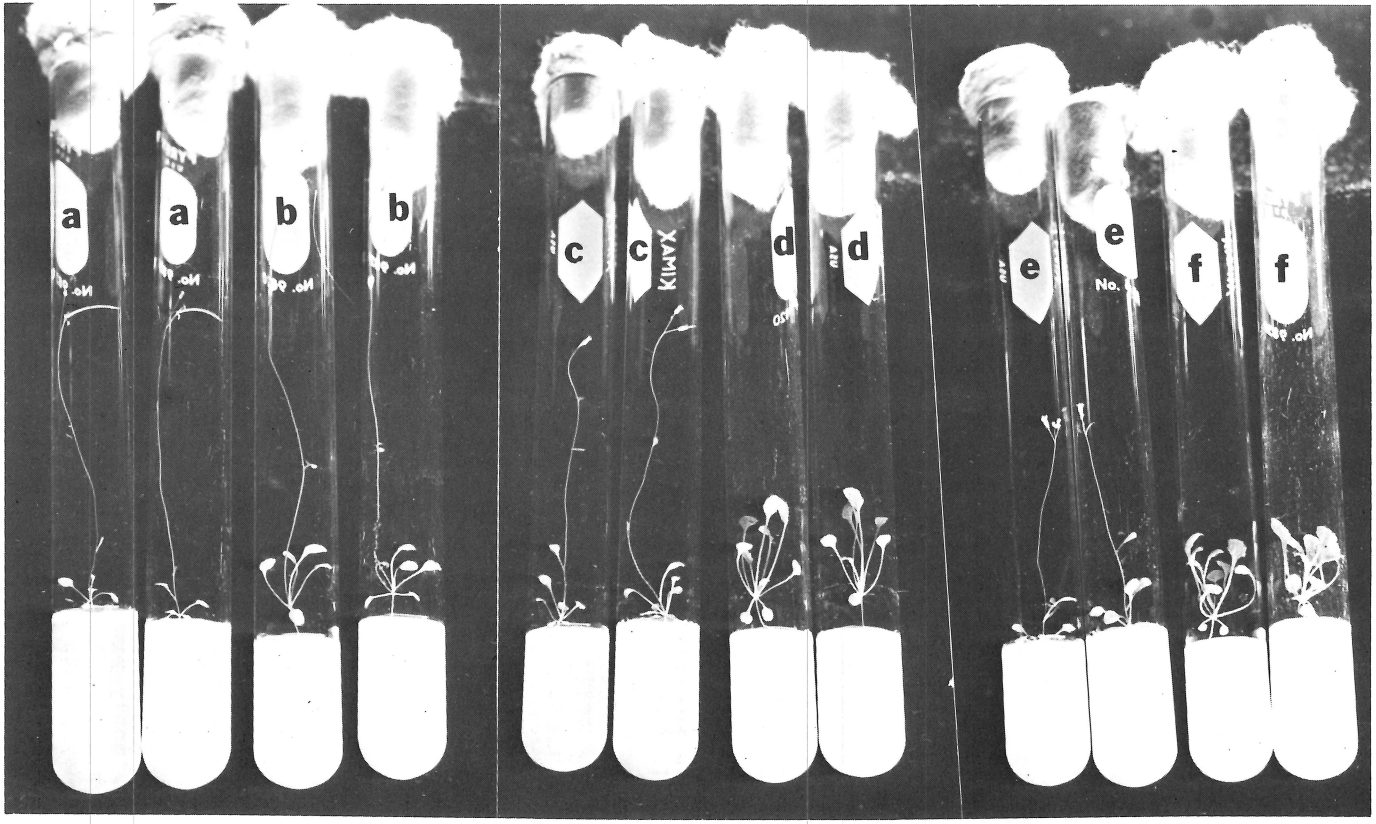

Figure 7. Presence of bromodeoxyuridine in the medium (a: *wild*, c: gi^2, e: ld^2) compared with its absence (b: *wild*, d: gi^2, f: ld^2) under continuous illumination in greenhouse.

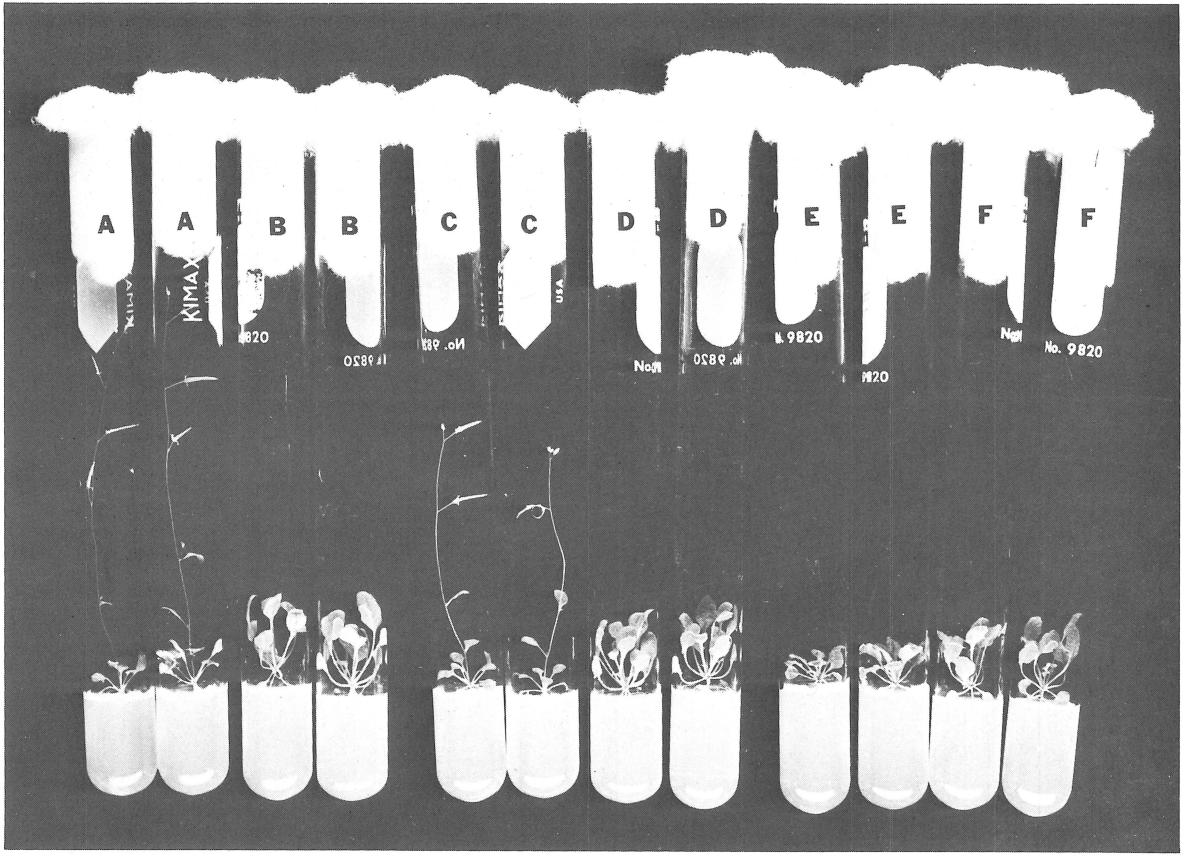

Figure 8. Presence of bromodeoxyuridine in the medium (A: *wild*, C: gi^2, E: ld^2) compared with its absence (B: *wild*, D: gi^2, F: ld^2) under short day conditions in greenhouse.

LANGRIDGE (1957) observed that 2 micrograms of gibberellic acid applied aseptically to 19 days old plants could bring about up to 25% reduction of flowering time in the race Estland of Arabidopsis. Similar beneficial effect of this compound has been confirmed by others (BROWN 1962, JACOBS 1964, MICHNIEWICZ and KAMIENSKA 1965). In our laboratory the flower-promoting effect of gibberellic acid was minimal.

Table 3. Effect of 5-bromodeoxyuridine on flowering time of Arabidopsis, Columbia wild type and mutants

Genotype	5×10^{-6} M BrdU	Days to flowering under illumination	
		24 hrs	9 hrs
wild	present	9.4	19.7
	absent	11.5	40.6
gi^2	present	14.2	22.6
	absent	30.8	41.5
ld^2	present	14.5	>57.7
	absent	22.4	0

In the 1950-es attention was focused on halogenated nucleic acid base analogs and probably the laboratory of Šorm observed first in 1956 some developmental effect of 5-bromouracil on plants (TELTSCHEROVÁ, SEIDLOVÁ and KREKULÉ 1967). Subsequently promotion of flowering by the apical application of 5-iodo-, and 5-bromodeoxyuridine were reported (BROWN 1962), JACOBS 1964) for Arabidopsis. In later experiments, both laboratories noted flowering inhibition by bromodeoxyuridine (BROWN and SMITH 1964, JACOBS 1967). HIRONO and RÉDEI (1966) using low concentrations of 5-bromodeoxycytidine and 5-bromodeoxy uridine by continuous supply through the nutrient medium, observed dramatic acceleration of flowering in various genotypes of the same species.

Actually all genotypes listed in Table 1 responded favorably to bromodeoxyuridine, except the *ld* mutants under short day conditions. Allele *ld*^{1} actually displayed even less response to the analog than *ld*^{2}. 5-Bromodeoxycytidine evoked essentially the same response in this material. Bromouridine, bromouracil and a number of other analogs failed to exhibit any promotory effect on flowering. 8-Azaadenine, however, was also active (HIRONO and RÉDEI 1966a) though to a somewhat lesser extent.

According to our experience, no other chemicals can exert in Arabidopsis a comparable flower-promoting activity as these analogs. The halogenated deoxynucleosides do not have a gener-

al inductive effect within the species; a northern ecotype failed to respond to them under any condition (RÉDEI 1969). Similarly the related crucifer, Sinapis was not sensitive to halogenated nucleosides (BROWN 1968).

Bromodeoxyuridine feeding in total darkness had no stimulatory effect on flowring in any genotype of Arabidopsis, possibly because flowering in darkness proceeded with maximal speed permitted by the metabolism under the conditions.

THE FATE OF BROMODEOXYURIDINE IN THE TISSUES

5-Bromouracil is not incorporated in significant amounts into nucleic acids in plant tissues (SEBESTA, BAUEROVÁ, ŠORM and ŠORMOVÁ 1960). The analog is quickly degraded to CO_2 and only small fractions may enter DNA probably through the degradation products.

BROWN and SMITH (1964) found that ^{125}iododeoxyuridine was incorporated into the apical nuclei of Arabidopsis as seen by autoradiography. This technique does not distinguish among the possible different target molecules, however.

Bromodeoxyuridine-H^3 or-C^{14} were effectively taken up by Arabidopsis plants when provided through the roots in the culture medium but only a few percent of the counts was associated with the DNA fraction isolated by a modified Schmidt - Thannhauser procedure. Approximately the same amount of radioactivity ended up in the RNA fraction and about the same activity remained insoluble. Over 90% of the counts were found in the alcohol and acid soluble fractions. These analytical data did not permit any conclusion concerning the effective target molecules of the analog (RÉDEI, unpublished).

SCHUBERT and JACOB (1970) found that bromodeoxyuridine induced differentiation of mouse neuroblastoma in the absence of DNA replication but protein synthesis was apparently necessary for its biological effect. They suggested that probably membrane components are affected by the analog.

These DNA-nucleoside analogs are very inefficient mutagens in Arabidopsis (HIRONO and SMITH 1969). Thus it appeared necessary to survey other than nucleic acid fractions for possible differences in incorporation between the two mutants which responded differently to bromodeoxyuridine feeding.

It was established that the two genotypes (*gi* and *ld*) take up radioactive bromodeoxyuridine with about equal facility.

Plants grown for about 4-5 weeks under short days on a mineral-glucose-agar medium containing radioactive bromodeo-

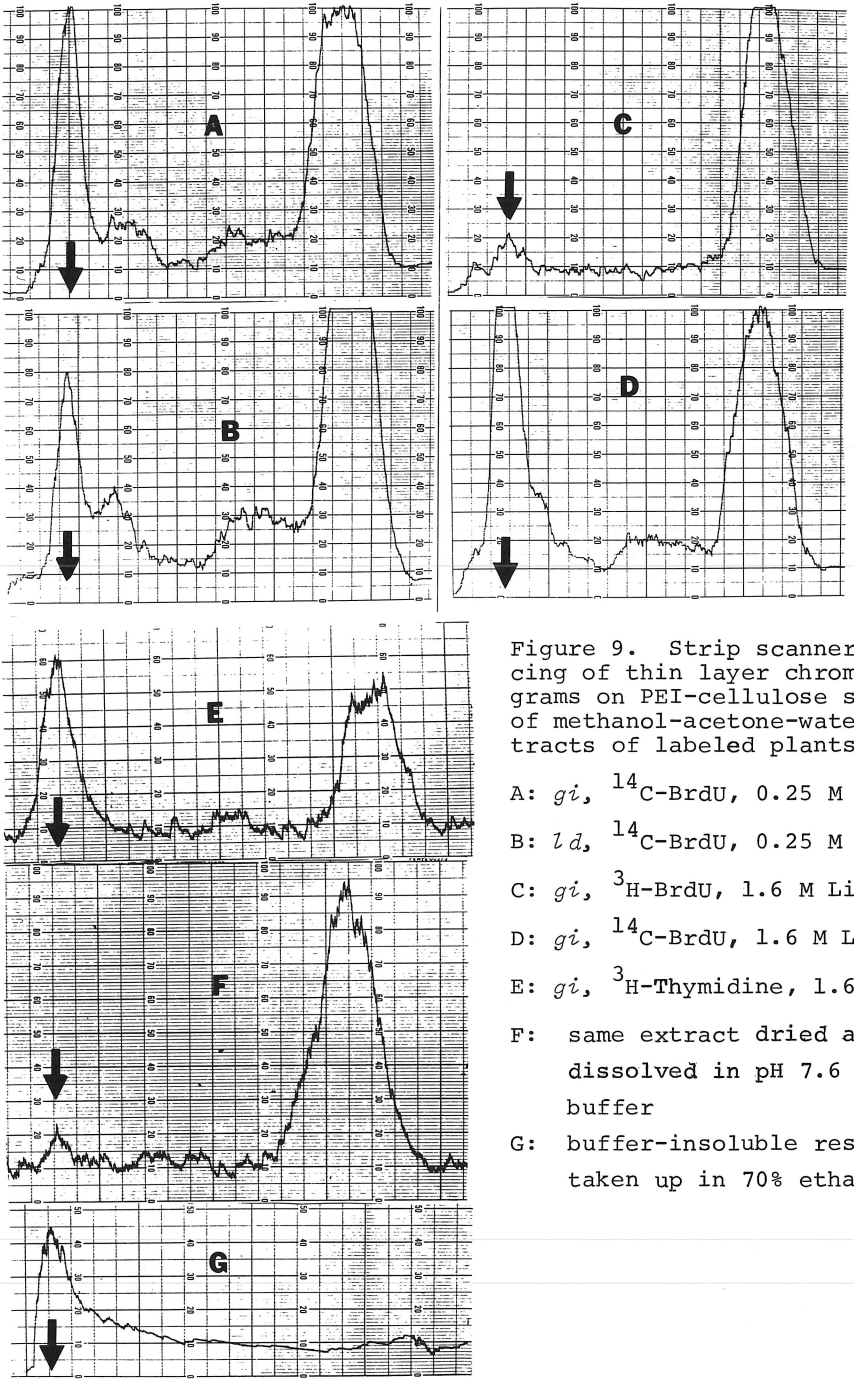

Figure 9. Strip scanner tracing of thin layer chromatograms on PEI-cellulose sheets of methanol-acetone-water extracts of labeled plants.

A: *gi*, ^{14}C-BrdU, 0.25 M LiCl

B: *ld*, ^{14}C-BrdU, 0.25 M LiCl

C: *gi*, ^{3}H-BrdU, 1.6 M LiCl

D: *gi*, ^{14}C-BrdU, 1.6 M LiCl

E: *gi*, ^{3}H-Thymidine, 1.6 LiCl

F: same extract dried and dissolved in pH 7.6 Tris buffer

G: buffer-insoluble residue taken up in 70% ethanol

oxyuridine (3.1 μCi H^3 or 0.33 μCi C^{14} per plant, respectively) were extracted with methanol-acetone-water (45:4:5 v/v) in the cold and the extract was chromatographed to survey radioactive metabolites in this fraction with a Packard gas-flow strip scanner on thin layer plates (Figure 9).

The bulk of the radioactivity - as expected in this fraction - was in bromodeoxyuridine and a significant portion was degraded to bromouracil (distal peaks, Figure 9). When 0.25 M LiCl was used as a solvent (Figure 9 A,B) phosphorylated nucleosides were also detectable though in smaller amounts than in extracts of thymidine-C^{14} fed plants. Nevertheless it was obvious that both the *gi*^{2} and the *ld*^{2} mutants were able to metabolize the analog and the differential response in flowering could not be attributed to a failure in producing DNA building blocks in the latter mutant. In mammalian system bromodeoxyuridine resistance was based on a modified thymidylate kinase (KITT et al. 1963).

The only difference in between this type of extracts of the two mutants was in the fraction which did not move on polyethyleneimine-cellulose thin layer plates with LiCl solvents (Table 4).

Table 4. Radioactivity in the methanol-acetone-water extracts in the chromatographically non-moving first peak (1.6 M LiCl solvent) as shown on figure 9.

Genotype	Label	Percentage of the total organic-soluble counts
gi^2	^{3}H-thymidine	33.3
ld^2	^{3}H-thymidine	35.1
gi^2	^{3}H-BrdU	8.5
*ld*2	^{3}H-BrdU	4.5
gi^2	^{14}C-BrdU	31.1
ld^2	^{14}C-BrdU	17.9

There was no difference between the two genotypes when the label was provided by thymidine. Approximately half as much radioactivity was detectable in this fraction of mutant *ld*^{2} compared to *gi*^{2} irrespective whether the radionuclide of bromodeoxyuridine was ^{3}H or ^{14}C. Generally in both genotypes ^{3}H labeling of the analog gave a smaller percent of radio-

activity in this fraction compared to a ^{14}C label. The radioactivity was measured with scintillation counting in the scraps and recounted with internal standards.

The chemical nature of this fraction could not be identified. We were unable to free it from traces of pigments thus the spectral properties could not be determined. Results of characterization attempts indicate that this chromatographically non-moving fraction is not a single base, nucleoside, nucleotide or polynucleotide. It is not a protein or lipid complex. Most likely it is more than a single compound. A very similar complex occurs in thymidine-fed plants.

Table 5. Characterization of the methanol-acetone-water extracts of bromodeoxyuridine grown plants. The results of the various treatments were followed by chromatography and scintillation counting.

Solubility	in water	poor
	in 70% ethanol	good
	in ethylether	poor
Mobility on PEI-cellulose		
	LiCl solvent	none
	isopropanol:NH_3:H_2O	partial separation
	isobutyric acid:NH_3:H_2O	moves
Digestion with enzymes		
	DN-ase I, pH 7.6	resistant
	phosphodiesterase, pH 7.6	resistant
	acid phosphatase, pH 4.5	resistant
	lipase, pH 7.2	resistant
	pronase, pH 7.0	resistant
	chymotrypsin, pH 7.2	resistant
Stability	0.33 N KOH, 16 hrs, 37° C	resistant
	1 N $HClO_4$, 1 hr, 70° C	slight degradation
	75° C temperature, 2½ hrs	resistant
	fluorescent light	resistant
	ultraviolet, 366 nm, 1 hr	produced more
	X-rays, 150 kV, 270 kR	slight degradation

The various pyrimidines are photosensitive compounds. Bromodeoxyuridine has an absorption maximum at 280 nm, thus at longer wave length than any natural pyrimidines (HUTCHINSON 1973). Though bromouracil does not form UV photoproducts alone, it does so in the presence of other pyrimidines (SMITH 1963, PETER and DREWER 1970). Though our plants were not exposed during the culture to short wave length irradiation, natural day light in the greenhouse or fluorescent light in the growth rooms contain near-UV spectra. Under these

conditions not only pyrimidine-pyrimidine complexes (dimers, hydrates, adducts; RAHN 1972) can form possibly but pyrimidine-amino acid complexes (SMITH 1969, WATAYA, NEGISHI and HAYATSU 1973) may arise during the prolonged time of culture (5-6 weeks). The pyrimidine adducts absorb short wave length rays and upon excitation may become emitters of well visible radiation in the 400-500 nm range (HAUSWIRTH and WANG 1973).

Our data cannot reveal the chemical nature of this substance. It appears that the H atom attached to the 6 C may be involved in the complex formation. The radioactive 5-bromodeoxyuridine used in these experiments were labeled either at the 6 position with ^{3}H or the ring carried the ^{14}C. Conspicuously reduced amount of the total radioactivity taken up by the cells was recovered in the chromatographically non-moving fraction after ^{3}H- compared to ^{14}C-labelling.

This unidentified compound may serve as a light receptor or may be a catabolic product of a flowering suppressor compound destroyed by bromodeoxyuridine. Upon photo decomposition of bromodeoxyuridine, uracilyl radical is formed which can extract hydrogen atoms from several compounds and can cause their decay (HUTCHINSON 1973).

BROMODEOXYURIDINE IN THE DNA

BROWN (1968, 1972) on the basis of histoautoradiography suggested that in the vegetative apical meristem bromo- or iododeoxyuridine are incorporated into the nuclei of the mitotically active flank meristem (see figure 4a) and inhibit transiently cell division in the 'anneau initial'. The cessation of mitotic activity in this area triggers nuclear division in the 'meristème d'attente' which normally signals the onset of the prefloral stage (Figure 4b). Subsequently the halogenated analogs are eliminated also from the flank meristem cells and the whole apex is then converted into a floral meristem.

This theory may appear attractive but is has a number of flaws. According to the generally accepted view, supported by substantial body of evidence (LANG 1965, CHAILAKHYAN 1972) the flower inducing stimulus is transported to the apex from the leaves where it is synthesized as suggested over hundred years ago by J. SACHS (1865). Thus the cyto-histological picture seems to reflect the consequence rather than the cause of the flower inducing changes in the metabolism. Furthermore, no experimental evidence is presented for the rapid degradation of the analog. The autoradiography did not provide evidence even for the incorporation of the analogs into DNA; this was only implied on logical basis.

Arabidopsis has the lowest amount of DNA in its nuclei among the angiosperms. Its diploid cells contain 4×10^{9} nucleotides (SPARROW, PRICE and UNDERBRINK 1972), slightly

over 1% as much as Lilium. This fact makes a little difficult to identify DNA in this plant with crude techniques.

Bromodeoxyuridine increases the buoyant density of the DNA (WAKE and BALDWIN 1962). In CsCl poly deoxyadenylic acid-thymidylic acid has 200 mg/ml less density than poly (dA-dBrU). This property of the analog makes easy the quantitative estimation of the extent of incorporation of the analog into DNA by using simple formulas (DeLEY 1970).

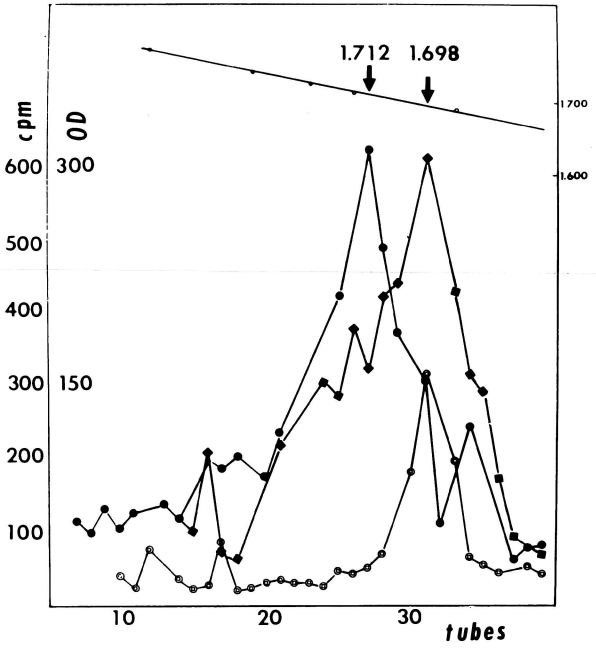

Figure 10. Buoyant density profile of Arabidopsis DNA normal cold ▪—▪, thymidine-C^{14} o-o, bromodeoxyuridine C^{-14} •—•.

After growing the plants on 0.33 µCi ^{14}C-bromodeoxyuridine (47.4 mCi/mmole) for 6 weeks under short days, the leaf pigments were removed with cold methanol-acetone-water and the nucleic acids were extracted by 0.15 M NaCl-0.1 M EDTA containing 1% sodium lauryl sulfate. This was followed by digestions with nuclease free protease and ribonuclease (LEDOUX, HUART and JACOBS 1971) and finally treated with phenol and numerous washings with 70% ethanol. This procedure yielded a white, long fibrous product with an O.D. at 260/280 and 260/230 approaching 2.

In CsCl the cold or thymidine-C^{14} DNA displayed the only major peak at a density of 1.698, the bromodeoxyuridine-C^{14} labeled DNA had a variable density between 1.712- 1.718 g/ml in the different experiments (Figure 10).

In spite of the fact that the DNA was extracted from mature tissues, containing also some senescing leaves, there was only moderate denaturation. The chloroplast and mitochondrial DNAs were not distinguishable clearly in these total cell preparations.

The density data indicate that Arabidopsis DNA contains almost 31% thymine and in the different experiments 18-26% of this was replaced by bromouracil. In spite of the substantial difference in flowering as a response to bromodeoxyuridine feeding of the different genotypes, consistent genotypic differences in the degree of incorporation could not be found.

At a buoyant density of 1.712 the DNA contained 70 million bromouracils, a number much to large to be accountable for by a single or a few loci. Yet some sort of selective effect has to be postulated since at a concentration of 10^{-6} M BrdU flowering is much promoted at a relatively normal growth rate (only root elongation is reduced noticably). It seems that a substitution of a fourth of the normal base has barely if any mutagenic effect and very little general metabolic effect. The obvious flower inducing power is not likely to be due to transient incorporation of the analog per se.

PROTEIN BINDING OF BROMODEOXYURIDINE-DNA

SMITH (1962, 1964) observed that the extractability of DNA from bacteria, substituted with bromouracil, is substantially reduced after ultraviolet irradiation. In density gradients 80% of the DNA irradiated floated on top because of tight association with protein. OPARA-KUBINSKA, KURYLO-BOROWSKA and SZYBALSKI (1963) reported that bromouracil-DNA was almost five times more sensitive to ultraviolet cross-linking than normal DNA, and it was more difficult to elute this material from methylated albumin-kieselguhr columns. LIN and RIGGS (1970,1971,1972) observed that poly dA-dT binds much less efficiently the *lac* repressor of *Escherichia coli* than poly dA-dBrU. Actually the latter competed 40 times as effectively for the repressor. BrU substituted operator sites bound 10 times tighter the *lac* repressor than the normal one. A further increase in binding could be brought about by ultraviolet irradiation (LIN and RIGGS 1974).

Recent years, besides the effect on flowering in Arabidopsis, in a number of mammalian systems morphogenetic-regulatory effect of bromodeoxyuridine has been reported (see RUTTER, PICTET and MORRIS 1973). LIN and RIGGS (1972, 1974) suggested that a regulatory role of the analog may be due to altered binding capacity of regulatory proteins and these proteins

may be preserved for detailed analysis by firmer locking them to specific chromosomal sites by the use of UV irradiation.

Table 6. Binding of ^{14}C-amino acid-labeled non-histone protein(s) to DNA in the presence or absence of bromodeoxyuridine in the culture medium of the plants. Figures are cpm/mg fresh weight of tissue.

Genotypes	arginine		valine	
	BrdU	T	BrdU	T
wild type	765	581	1708	844
gi^{2}	1145	939	1135	993
ld^{1}	1227	726	1345	608

Histones (HUANG and BONNER 1962, GEORGIEV 1969), are capable of regulating transcription in a non-specific way, and recently various kinds of histones in different quantitities have been detected during specific steps of differentiation (RUDERMAN, BAGLIONI and GROSS 1974). Generally the non-histone type of proteins are favored, however, as the most likely regulators of genic activity (STEIN, SPELSBERG and KLEINSMITH 1974). A small fraction of the phosphorylated non-histone proteins were reported to show characteristic binding to DNA and influence the pattern of synthesis of RNA. TURKINGTON and KADOHAMA (1972) observed selective inhibition of the expression of milk protein genes upon bromodeoxyuridine feeding. They concluded that acidic nuclear proteins are the prerequisites for differentiation in mammary cells.

RIGGS and associates (1970) developed a simple and effective technique to isolate repressor bound DNA on a nitrocellulose filter. In Arabidopsis nothing is known on any specific regulatory proteins, yet on the analogy of the Riggs' technique we isolated protein(s) with tight binding to DNA. The crude DNA was extracted with saline-EDTA-SLS as indicated above but no enzymatic digestion or phenol was applied to the extract. After precipitation of the cell-free solution with ethanol the chromatin was trapped on an 'all bias' polyester web (Pellon Corp. New York) and washed with generous amounts of cold 70% ethanol. The filter does not retain any bovine albumin or measurable amounts of cellular proteins but traps chromatin especially when it is filtered along with slightly sheared commercial DNA of high molecular weight.

The filter bound ^{3}H-thymidine-DNA if it was not deproteinized by pronase but on an average of 93.5% of the radioactivity contributed by ^{14}C-valine was eliminated when a nuclease-free protease digestion of 1½ hrs preceeded the filtration. The protein trapped on the filter was substantially free of histones since less than one half percent of the radioactivity could be removed by 30 minutes extraction with 0.4 N H_2SO_4. The absence of histones from the preparation is not unexpected since sodium lauryl sulfate is known to split away proteins from nucleic acids (McCONKEY 1967).

When this procedure was used with extracts derived from plants grown in the presence of ^{14}C-amino acids and amino acids plus bromodeoxyuridine, respectively, significantly more radioactivity was trapped on the filter from the latter types of extracts (Table 6).

The binding of protein to DNA was consistently higher in the BrdU series (in over 30 determinations) yet clear genetic differences could not be established. It appeared the amount of this protein varied substantially during development, an observation not uncommon in non-histone proteins (ELGIN et al. 1974).

CONCLUSIONS

Flower differentiation in Arabidopsis is under the control of several unlinked loci. Three of them studied in detail appear to be suppressed under short daily cycles of illumination and in continuous darkness they behave in a constitutive fashion. Obviously there is no requirement for light for flower determination, inflorescence development or for any phase of the process called flowering.

Mutants at two loci and the wild type can utilize some halogenated deoxypyrimidine nucleosides to eliminate or reduce the deleterious effects of light under all regimes of illumination while mutants at another locus (*1d*) can utilize bromodeoxyuridine only under less inhibitory conditions of light. Also one natural late flowering ecotype tested could not flower earlier on bromodeoxyuridine media under any regime of illumination. These differences clearly reflect the different control mechanisms determined by genetic factors.

Bromodeoxyuridine is well taken up by the cells of this plant and metabolized into different products. The analog is a light sensitizer and may act as a light receptor and/or a wavelength transformer of the absorbed radiation in the cell; alternatively it may disrupt the molecules associated with.

Bromodeoxyuridine - under the experimental conditions- substituted for up to one fourth of the thymidine in the DNA. The altered regions may be incapacitated by single strand breaks and delayed repair processes at the sites involved in the control of flowering. This hypothesis is not supported

by any direct evidence, however, available in this material. The analog containing DNA binds much tighter some non-histone type protein(s). These types of proteins are likely candidates for regulatory type of functions in higher organisms. It is conceivable that in the presence of bromodeoxyuridine in the DNA the protein tightly associated with the flowering suppressor loci hampers the transcription of flowering inhibitors. The amount of this non-histone protein(s) is larger than required for the relatively few loci of the genome to act on them exclusively but their sensitivity to this kind of regulation may be higher than the average genes' performing cell essential functions. HOLTZER and ABBOT (1968) suggested that bromodeoxyuridine can selectively control 'luxury functions', a category applicable to flowering. Flowering is not vital to a cell, it is essential only to the whole organism. The regulation of this particular function in nature is very common both by genetic and/or environmental factors. Thus flowering appears to have evolved as a highly modifiable function in contrast to the synthesis of basic metabolites.

Bromodeoxyuridine regulates several different functions in other unrelated organisms. In certain systems groups of enzymes (STELLWAGEN and TOMKINS 1971, WALTHER et al. 1974) are selectively suppressed. In others certain functions are preferentially stimulated and the same time others are hampered (PRASAD, MANDAL and KUMAR 1973, TOMIDA, KOYAMA and ONO 1974). This different behaviour upon bromodeoxyuridine provision may find its explanation in the specific inhibition of transcription (OSTERTAG et al. 1973, HILL, TSUBOI and BASERGA 1974). Preferential binding of histones and RNA polymerase to BrdU-DNA does occur (WEINTRAUB 1974).

Perhaps the wide variety of functions responding to bromodeoxyuridine by increased or reduced activity have one common property: they are non-constitutive to some degree. A higher or lower activity of enzymes upon bromodeoxyuridine feeding may be determined whether they are inducible or repressible. The basis of these different responses may be simply controlled by differential binding of proteins to BrdU-DNA.

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