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111 Jesse Hall

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Gyorgy Pal Redei - Principal Investigator

Genetic Studies on Somatic Cells of the Higher Plant, Arabidopsis

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Respectfully submitted,

THE CURATORS OF THE
UNIVERSITY OF MISSOURI

R. H. Bezoni, Comptroller

G. P. Redei
Department of Field Crops

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Report of Scientific Progress

A year ago a few problems of the genetics of somatic cells were pointed out and experimental approaches were suggested. In the past few months considerable progress was made though several experiments can not be evaluated yet.

Recently it was confirmed by trisomic analysis that the group of markers used belong to three different chromosomes (Suzanne Lee Chen, 1966). A few mutant trisomes were obtained indicating a definite, though small, frequency of double reduction. This information will make it possible to determine the relative position of the centromere. Knowledge of centromere position is critical for the feasibility of somatic exchange studies.

Somatic recombination studies were initiated in three linkage groups. A large number of somatic sectors, including stem sectors were obtained in all three groups. Progeny tests of the sectors have not been completed yet. It was pleasant to learn that somatic sectoring is several fold more frequent in one of the newly synthesized systems compared to what has been used previously. For the first time very large triple sectors were detected. Thus complementary products and the original genotype are clearly represented by inflorescence bearing sectors.

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Interesting information was obtained -- as a by-product -- on repairability of gene function. All the eight morphological markers, used for the recombination experiments gave evidence of sector formation when irradiated in heterozygous condition. If the direct or indirect products of the genes would be diffusible sectoring would not be expected. The wild type tissues should provide selectively timed the exactly needed nutrients. This hypothesis turned out to be presumptuous. If this information is compared to another observation, obtained in our laboratory, that only 47/2000 of the lethal or viable mutants of *Arabidopsis* respond to exogenous supply of nutrients of a "complete medium" the conclusion to a very high degree of cellular autarky is justified. If 98% of the gene controlled functions are beyond our reach, the experimental approaches to morphogenesis and differentiation of specific structures are seriously limited (Rédei, 1966).

Premeiotic marker exchange has been induced with x-rays. The effect of two chemicals was also investigated with the following genetic set up $\frac{gi^2}{gi^1} + \frac{pa}{ch^1}$. In this material several mechanisms (exchange, deletion, mutation, translocation, etc.) may bring about yellow green sectors if the middle marker is involved in any of the above events. The purpose of the experiment was to see whether the three different agents would have a comparable effect on somatic exchange. Since marker ch¹ fails to

produce chlorophyll b by simple chromatographic procedure the identity of the locus can be easily established.

Though x-ray, ethyl methane sulfonate, and nitrosoimidazolidone produced a very different degree of sectoring (eg 2.0, 11.8 and 5.7% resp.) the frequency of chlorophyll b less sectors was remarkably similar, 1.4%, 1.9%, and 1.1% respectively. It appears that the mutagenic effectiveness can be very well studied by somatic tests. Interpretation of the data requires, however, extreme caution unless the genetic constitution of the material is as favorable as it was in this experiment (Rédei, Röbbelen and Hirono, 1966 manuscript in preparation). As a by product of these studies an improved method of leaf pigment chromatography was also developed.

I believe it is pertinent to report our progress in some other aspects of the genetic studies on somatic cells. This line of investigation was not discussed in the original proposal. The support provided by AEC made it possible however, to study in depth some other related problems. This project discussed below was supported also by National Science Foundation.

Genetic control of differentiation

Recessive mutations at a mapped locus produce a high degree of variegation in homozygous condition (Rédei 1963a, 1963b, 1965). In certain cells chloroplast differentiation can not be completed and consequently leaf pigments are not produced while in others perfectly normal chloroplasts are found. This is an all or none phenomenon. The extent of variegation is extremely influenced by several well defined conditions. High light intensity (6000 Å region) interferes with plastid differentiation. On the other hand low light intensity (4500-5500 Å region) promotes plastid formation. Irradiation with heavy doses of x-rays also favor the appearance of larger and more numerous green sectors. It appears therefore that the variegation is produced not by the loss of a "structural gene" rather a "regulatory element" is malfunctioning. So far our attempts to revert the mutation were not successful. The expression of the variegation can be modified by different chemicals. Cysteine slightly favors normal differentiation while cystine or homocystine have the opposite effect. The photodynamic dye acriflavin if applied to germinating seeds also have an effect similar to cysteine. Dramatic changes in the mutant phenotype can be brought about by azauracil, azauridine or azacytidine. Variegation is strongly suppressed and the number of cells with normal chloroplasts is

increased several fold as measured by the pigment content of the plants. Azathymine or other related pyrimidine or purine analogs do not influence the mutant to any measurable extent. Studies with labelled azauridine indicate that the analog is selectively taken up by the plastids.

The protein content of the white tissues is only slightly less than that of the wild type. The absence of chloroplast differentiation is accompanied by considerable accumulation of inorganic phosphates. There is no difference between mutant and wild type in the activity of acidic or neutral phosphatases (Arabidopsis does not have alkaline phosphatase), 3'-nucleotidase, 5'-nucleotidase, ATP-ase or phosphodiesterase. The white tissues display however an increased ribonuclease activity. So far three ribonucleases were detected in this material. One, a phosphodiesterase occurs in both the soluble and particulate (organelle) fraction of the cell but its amount (compared on protein basis only) is relatively higher in the non-particulate cytoplasmic fraction. The chloroplasts, mitochondria, and microsomes contain an about pH 7.0 optimum ribonuclease, while the soluble fraction of the cytoplasm harbors a pH 5 ribonuclease. The difference between mutant and wild type is limited to the low pH optimum enzyme. The differentiation of the chloroplast is controlled by a nuclear gene through the cytoplasmic, and not by the plastid enzyme. The role of azapyrimidines in the variegation is not quite clear. It was demonstrated the 2', 3'-cyclicphosphate of azauridine is a substrate definitely inferior

to the normal nucleotide for the Arabidopsis ribonuclease. On the other hand mutant plants grown on azapyrimidines synthesize only about normal amount of ribonuclease. Thus it appears that this ribonuclease is an inducible enzyme. The lack of appropriate substrate limits its production. So far qualitative difference was not detected between the mutant and wild type enzyme. This system is one of the best for the study of differentiation through selective, and regulated gene activity (Rédei, 1966).

MOLECULAR ASPECTS OF A GENETIC VARIEGATION AFFECTING DIFFERENTIATION^{1,2}

G. P. RÉDEI

Department of Field Crops, University of Missouri, Columbia

Received

Differentiation and development are controlled by genes (RÉDEI 1962; HIRONO and RÉDEI 1966a, b). These processes require selective synthesis or catabolism of protein and appropriate timing of enzyme activity. Little information is available as to how the genes determine this selectivity and this timing. Yet BONNER and his associates (BONNER 1965) beautifully demonstrated that gene function can be controlled at the chromosomal level. Again little is understood how histones carry specificity for the multitude of loci (BLOCH 1962). Both the operon model (JACOB and MONOD 1961; MONOD and JACOB 1961) and the modulation hypothesis (AMES and HARTMAN 1964) are of heuristic value for the interpretation of development through coordinated gene function. Molecular evidence that differentiation in higher forms is mediated through these models is not yet available. However, there are similarities in the control systems of phylogenetically different forms (McCLINTOCK 1965).

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Differentiation certainly cannot be understood by considering only primary gene action (WAGNER and MITCHELL, 1965). The complexity of an open, dynamic system poses enormous problems. Identification of a single decisive component can rarely be expected. Genetic studies combined with physiological analysis may reveal major factors controlling differentiation of certain structures as exemplified by the investigations on a variegation mutant of *Arabidopsis*.

MATERIAL

Mutant im was obtained by x-ray treatment (10.000 r) of 24 hours presoaked seed of the Columbia wild type of *Arabidopsis thaliana*. In homozygous condition this recessive mutant produces variegation (Fig. 1). Chloroplast differentiation cannot be completed in certain cells; yet in others normal chloroplast development apparently takes place. The cells with imperfect plastids remain white; neither chlorophylls nor carotenoids are synthesized. All plastids within a single cell are either completely normal or abnormal. In the white cells all of the plastids are arrested in the same developmental stage. The cell phenotype is not fixed at the time of cell replication since the same seed sample may germinate with either all green or all white cotyledons depending upon the intensity or quality of light exposed to after imbibition. The expression is irreversible, however, after the completion of differentiation. The two types of cells are genetically identical, since the descendants of both kinds of cells may

become either white or green. This peculiar behavior of the gene results in a rich but non-random pattern of green and white cells with sharply demarcated areas in the various tissues. Some leaves carry large continuous sectors, while others display frequent transitions from green to white cell colonies closely following the pattern of differentiation. Sometimes a larger number of cells undergo an apparently synchronized phase switch. In extreme cases single green cells or cell lineages may be found in a white cell environment (Figures 2, 3, 4, 5, 6).

For most of the biochemical studies, the double mutant im gi² was used. The gi² factor conveys little sensitivity to photo-periodic treatment. Even under continuous illumination, which was desirable for producing extreme mutant phenotype, vigorous vegetative growth was ensured because of the late flowering of the plants (cf. RÉDEI 1962). The im⁺ gi² xv gl² genotype was selected a "wild type" control for most of these experiments. Since homozygous im is very low in pigment content, development is at a considerably slower rate than the im⁺ type. This developmental difference was eliminated by employing the yellow green gene xv in conjunction with im⁺. Marker xv is closely linked to gi (ca. 2 m.u. apart), and both are independent of im. The marker gl², hairless and of no importance in this system, is also independent from all other markers.

METHODS

Plants for biochemical analysis were raised in the greenhouse. The pots were supplied with distilled water from below to prevent contamination of the leaves with mud or water salts and to assure vigorous growth. Occasionally test tube grown material was also used (cf. RÉDEI 1965).

Protein content was determined in the various buffer extracts according to the method of LOWRY, ROSEBROUGH, FARR and RANDALL (1951). Phosphorus was analyzed by the ammonium vanadate-ammonium molybdate technique of GERICKE and KURMIES (1952) from the same type of extracts which were used for the protein assay. Calcium and magnesium were estimated from dry ashed material according to the techniques of SENDROY and SIMON respectively as given by BALLENTINE and BURFORD (1957). The crude enzyme extract was obtained by homogenization of fresh or lyophilized plants in the appropriate buffers followed by centrifugation at 10,000 rpm at room temperature. This crude extract was practically colorless. The amount of inorganic phosphate liberated in the incubation mixture was used as a measure of activity of the different phosphatases. The less sensitive phenolphthalein phosphate technique of HIGGINS and TALALAY (1945) was also employed as given by BERGMEYER (1963). Phosphodiesterase was assayed principally according to RAZZEL and KHORANA (1959). Ribonuclease activity was estimated by a slight modification of the technique of DICKMAN, AROSKAR and KROPP (1956). All enzymes were compared on the basis of equal amounts of protein. The data reported there were obtained from crude extracts. Enzyme extracts purified approximately 15 times were also tested with essentially the same results.

Subcellular fractions were prepared from fresh tissues disintegrated with a high speed Virtis homogenizer containing 0.5 M sucrose in pH 5.2 phosphate buffer (0.05 M). The chopping was stopped when almost all cells were broken, and the great majority of the chloroplasts still appeared intact as ascertained by microscopic examination. The homogenate was filtered through cheesecloth and then separated into fractions with a Spinco Model L analytical centrifuge. The fractions were freeze-dried, defatted by acetone, dried, ground in 0.5 M KCl, and then centrifuged at 10,000 rpm. The supernatants were dialyzed for 24 hours against cold distilled water and were preserved by lyophilization until used. Commercial yeast RNA, purified (FRISCH-NIGGEMEYER, and REDDI 1959) from acid soluble nucleotides, was used as substrate for ribonuclease assay. Other reagents were obtained either from Sigma (St. Louis, Mo.) or from Calbiochem (Los Angeles, California).

RESULTS

The response of the im mutant to temperature, to the quality and intensity of visible light, to x-rays, to the metabolites of the cysteine pathway and to 6-azauracil has already been mentioned (REDEI 1963a, 1965a). The genetic mechanism involved has also been briefly discussed. (REDEI 1963b). The activity of the im locus will be further characterized below.

A surprising observation was that high doses of x-rays (10,000 r) normalized to a small but definite extent the mutant phenotype (REDEI 1967a). Similarly, the antimetabolite 6-azauracil more or less restored normal plastid differentiation when the mutant was grown for at least 3-4 weeks on a medium containing the analog (REDEI 1967b). Feeding cysteine also made the plants greener but its effect was less than that of 6-azauracil. The mutant did not respond much to the nutrients of a complete medium. These observations indicated that the mutant phenotype is not the consequence of the lack of synthesis of an essential nutrient; instead, the overactivity of an enzyme appeared to be responsible for the disturbance.

From the knowledge that 6-azauracil interfered with the decarboxylation of orotidylidic acid (HANDSCHUMACHER, 1960), one might expect that the cells produce free inorganic phosphorus in the process of disposing of the accumulated orotidylidic acid. Indeed, a simple chemical analysis revealed that the readily soluble phosphorus accumulates in the mutant. This difference is especially obvious in the white tissues (Table 1). The two samplings shown in Table 1 were made on slightly different material and with different solvents. This data still proved consistent. In other experiments, the almost completely white im mutants accumulated even larger amounts of phosphorus. The analyses of several different samples gave variable results depending on the extent of variegation of the plants. The mutants displayed, however, more phosphorus than the wild type in every case.

The calcium and magnesium contents of the normal dark green type and a highly variegated im mutant were also determined. Since the mutant sample contained approximately a third of the chlorophyll of that of the wild type, one might expect a comparably lower amount of magnesium in the plants of im genotype. However, the magnesium and calcium contents are practically identical in the mutant to that of the normal green wild type. In another study, 40% of the total magnesium and calcium content of bean and tobacco leaves was found in the chloroplasts (STOCKING and ONGUN 1962). Apparently the variegation, which interfered with plastid differentiation and consequently with leaf pigment synthesis, did not affect the uptake and utilization of these two metals as it did affect the metabolism of phosphorus.

According to GRANICK (1963) 35-55% of the dry weight of the chloroplasts is protein. Most of this is in an insoluble form. More than half of the total nitrogen content of the leaf cells is expected to be in the chloroplasts (STOCKING and ONGUN, 1962). Numerous determinations of the soluble protein content exposed small differences between the plants of im and im⁺ genotypes (Table 3).

Therefore the assumption is that the mutation affects plastid differentiation, a highly complex process, in a very specific way.

Phosphorus may accumulate in the cells if phosphatases, abundant in all tissues, were unrepressed or induced. Neither phosphomonoesterase nor phosphodiesterase activity is different in the two genotypes (Table 4). Though phosphodiesterases do not produce free inorganic phosphates, their products may be acted upon by unspecific enzymes which cleave phosphorus bonds.

In another study, the activity of a few phosphatases was assayed from both genotypes. In order to partially inactivate the heat sensitive phosphomonoesterases, the crude enzyme extract was heated and centrifuged again before incubation. The 3'-nucleotidase is a fairly heat stable enzyme without much difference between the two genotypes in its activity. Actually, the enzymes of the mutant seem to be less active (Table 5). This may be attributed to the inhibitory effect of the increased amount of phosphorus in the mutant tissues. That phosphatases are subject to product inhibition is well-known (MORTON 1955).

The chloroplasts contain a significant amount of RNA (BIGGINS and PARK 1962). WOLLGIEHN and PARTHIER (1964) demonstrated that kinetin prevents RNA breakdown and actually promotes RNA synthesis. When kinetin was incorporated into the nutrient medium of aseptically grown plants, the content of leaf pigments in the mutant was increased. These findings were not surprising. Kinetin was effective in the restoration of chloroplast differentiation only at very toxic concentrations. Apparently its effect was not due to differential inhibition of the growth of the two kinds of tissues, since the total pigment content per plant was approximately doubled at a concentration of 10^{-3} M. A kinetin medium of 5×10^{-3} M killed the plants.

SAHAI SRIVASTAVA and WARE (1965) observed that kinetin treatment of barley leaves reduced the level of DNase and RNase. When kinetin was added to the incubation mixture, the activity of either of these enzymes was not affected.

A ribonuclease assay demonstrated a difference between im mutant and the im⁺ type (Fig. 7). The curve of the ribonuclease activity of the mutant and wild type was not identical or parallel through the pH range of 3 to 8. On the basis of an equal amount of protein at pH 8, the activity of RNase was the same in both genotypes. At pH 7, there was a slightly higher activity in the extract of the mutant. In the acidic range the difference was clear, as the mutant displayed definitely higher activity. Within the range of pH 4 to 6 the mutant always displayed the higher RNase activity. Individual samples from different plants varied considerably. Some samples of the mutant exhibited only 25% excess activity over the wild type; in others five to seven fold increases were observed. This is understandable since a variegated mutant was compared with the stable wild type. If the variegation has any correlation with ribonuclease, such a variegation in enzyme activity is also expected. The observation was that the whiter the tissues were, the more ribonuclease these tissues contained.

Two questions, whether the increased ribonuclease activity was more or less the specific consequence of the mutant gene function or whether albinism per se was responsible for the increase in amount or activity of the enzyme, then arose. To produce enough tissues from albina Arabidopsis plants is difficult. Dr. E. G. ANDERSON supplied maize seedlings, which were almost pigment free, and some green seedlings. Both types of seedlings were germinated in the sand-benches of the greenhouse. The comparison of the

two types of maize did not reveal any difference between the white seedlings and their normal sibs. The variation between the data of the two tests was probably due to sampling error. Only a small amount of material (50 mgs. dry weight) was used for the tests. If there was any variation within the individual plants or in the samplings, such a fluctuation might result.

The lack of parallelism between the two curves (Fig. 7) indicates furthermore, that there is more than one RNA digesting enzyme in the crude extract. There is evidence that the mutant produces an excessive amount of only the lower pH optimum enzyme.

Free ribonuclease is known to occur in the cytoplasm of higher plants, but several organelles also harbor ribonucleases. By differential centrifugation, two cellular fractions were prepared. The chloroplast and nuclei were sedimented at $2,500 \times g$ and processed as indicated in the Methods. The other portion was subjected to $112,000 \times g$ for 90 minutes, and the supernatant was considered as the non-particulate fraction.

The chloroplasts plus the nuclei and the non-particulate fractions were assayed for ribonuclease within the range of pH 4.5 to 8.5. The non-particulate cellular fraction displayed an optimum around pH 6, while the tested organelles contained an enzyme with a definitely higher optimum (Fig. 8). These experiments demonstrate that the mutant is over-producing the enzyme which is not bound to the organelles.

Since most of our assays were carried out with crude extracts, it was important to know whether the RNA digestion in vitro was carried out by

ribonucleases or by phosphodiesterases. Arabidopsis phosphodiesterase is not active at the lower pH values. Since phosphodiesterase is inhibited by PO_4 , the RNase assay was performed in citrate-phosphate buffer. The phosphodiesterase was assayed with p-nitrophenyl-thymidine-5'-P which is not digested by RNase. That Mg^{++} ions are necessary for the activity of venom phosphodiesterase is known (RAZZEL and KHORANA 1959). The diesterase was assayed also in the presence of $1.7 \times 10^{-3} \text{ M Mg SO}_4$.

On the basis of an equal amount of protein, there was about twice as much phosphodiesterase activity in the soluble cellular fraction than in that of the chloroplasts plus nuclei (at pH 7.5). Ribonuclease, on the contrary, was relatively more abundant in the organelle fraction when assayed at pH 7.5. Thus in the non-particulate fraction the RNA was digested at the higher pH values primarily by the phosphodiesterase. In the particulate fraction, apparently RNase has the major role in RNA disposal (Fig. 9).

Attempts to differentiate qualitatively the acid ribonuclease of the mutant from that of the wild type have not been successful.

DISCUSSION

Not much is known about mutants affected in ribonuclease synthesis or activity. The commonly used isolation techniques in microbial systems have failed to identify the mutants even if these types have occurred. In *Escherichia coli*, however, a mutant lacking ribonuclease has been mentioned (WATSON 1964).

Plant ribonucleases appear especially attractive for genetic studies. Most higher plants contain several ribonucleases (WILSON 1963, GOLASZEWSKI and SZARKOWSKI 1964). In addition, plant ribonucleases generally digest the diester bonds of all four nucleotides and most of them hydrolyze the cyclic phosphates of all four bases. Differences between species of higher plants concerning the catabolic functions of RNase have also been detected (REDDI 1958, SHUSTER and KHORNA 1958 etc.). Variations are obvious among ribonucleases of widely different organisms (cf. JOSEFSSON and LAGERSTEDT 1962). In addition to the eight types of catabolic functions, synthetic ability of RNase has also been demonstrated. The sites active in the degradative and synthetic processes are different in pancreatic RNase as has been shown recently (BERNFELD 1965). The wealth of biochemical information available on these enzymes may aid genetic studies if mutants can be produced which are affected in any of these functions. Furthermore, RNases may have an important role in the catabolism of messenger RNA or in controlling other steps of the protein synthesizing machinery (TAL and ELSON 1963, ARTMAN and ENGELBERG 1964, ANRAKU and MIZUNO 1965). However the enzyme discussed in this paper is not associated with the ribosomes.

Ribonuclease activity varies during ontogenesis both in plants (LEDOUX, GALAND and HUART 1962, HANSON, WILSON, CHRISPEELS, KRUEGER and SWANSON 1965) and in animals (BRESNICK, SAGE, LANCLOS 1965). Thus differentiation, organization and growth may be partly controlled by ribonucleases. The genetic control of development may be exercised

through variants of the protein, through the differential activation, through the suppression of one or several enzymes with similar or different function, or through the subcellular localization of the enzymes. Genetic amino acid variants of mammalian ribonucleases have been identified (ANFINSEN 1959). Special natural inhibitors of RNase have been revealed in both plants (BERNHEIMER and STEELE 1955) and in animals (ROTH 1957, SHORTMAN 1961). RNases with different pH optima in the different tissues (WILSON 1963) or different organelles or plants have also been reported (GOLASZEWSKI and SZARKOWSKI 1964).

Thus ribonucleases may play some role in the genetic control of differentiation. In Arabidopsis the variegation in plastid formation is associated with the activity of one of the ribonucleases.

The mutant im has a facultative ability to produce normal chloroplasts; therefore, the mutation must not have affected a "structural gene". Furthermore, the attempts to find a qualitative difference between the enzyme of the mutant and wild type were unsuccessful. Some kind of a controlling factor has been mutated seemingly. Since the im gene is recessive, this controlling factor -- by definition -- cannot be the regulator gene of an operon.

The RNase synthesis, rather than the RNase activation, is more likely involved because of the lack of success in affecting differentially the activity of the wild type and mutant enzymes in vitro. In the green tissues of the mutant, however, apparent normal activity was detected. The effect of chemical treatments (e.g. azapyrimidines), which modified the expression

of the mutant, could be observed after a lag period. Modification of the variegation is easiest by physical agents during germination and by the early stages of development when the synthesis of RNase is rapid.

The activity of the mutant gene is strictly cell limited. Single green cells on a white background or single white cells in a green tissue are frequently observed. This indicates that a macromolecule is involved which does not diffuse through cell membranes. Whether some primary gene product or a corepressor or an inducer is directly responsible for the variegation is not known. Learning that the nuclear gene im controlled plastid differentiation through an enzyme localized outside of the plastid was gratifying. Interesting indeed would be the knowledge as to whether the chloroplast ribonuclease is controlled by the plastom.

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SUMMARY AND CONCLUSIONS

The recessive mutant im in homozygous condition displays variegation. In certain cells plastid differentiation is arrested; in others the chloroplasts are apparently normal. Within a single cell all the plastids develop uniformly, and the distribution of the two types of cells in the

tissues is not random. The prevailing epigenetic factors determine the transition from one state of differentiation to the other. The generative progeny of the two types of sectors is indistinguishable as both repeat the variegation. Heterozygotes never reveal any sectoring; an interpretation of high somatic mutability can not be invoked. Since the mutant cells have a facultative ability to produce normal chloroplasts, the genetic alteration did not affect directly any of the "structural genes". Mutation in a "regulator gene" is ruled out by definition, since im is recessive. Direct evidence for the existence of an "operator site" is not available.-----The white tissues of the mutant accumulate inorganic phosphorus. The calcium and magnesium contents of the two types of cells are the same. Phosphatases in the mutant are apparently not different from those of the wild type plant. When the mutant is grown on 6-azauridine or 6-azacytidine containing media, the variegation is suppressed, and normal green phenotype is more or less restored. Similar is the effect of kinetin. Kinetin is a regulator of RNA anabolism and catabolism. Direct evidence is presented that in the white tissues of the mutant there is an excessive ribonuclease activity. The accumulated phosphorus is apparently a by-product of the increased RNA catabolism. Arabidopsis possesses three ribonucleases. Only an acid ribonuclease is over produced in the mutant. This enzyme occurs in the non-particulate cytoplasm. Apparently the im locus has an important role in the control of the synthesis of this ribonuclease. The possible mechanisms of control are discussed.

LITERATURE CITED

- AMES, B. N., and P.E. HARTMAN, 1964 The histidine operon. Cold Spring Harbor Symp. Quant. Biol. 28: 349-356.
- ANFINSEN, Ch. B., 1959 The Molecular Basis of Evolution. John Wiley and Sons, Inc., N.Y.
- ARTMAN, M., and A. ENGELBERG. 1964 Degradation of rapidly-turned-over ribonucleic acid to acid-soluble compounds by *Escherichia coli* ribosomes. Biochim. Biophys. Acta 80: 517-520.
- BALLENTINE, R., and D. D. BURFORD, 1957 Determination of metals. In Colowick, S. P. and N. O. Kaplan; Methods in Enzymology. Acad. Press. N.Y. Vol. 3 Pp. 981-1049.
- BERGMEYER, H. N., 1963 Methods of Enzymatic Analysis, Acad. Press. N.Y.
- BERNFELD, M. R. 1965 Ribonuclease and oligoribonucleotide synthesis.
I. Synthetic activity of bovine pancreatic ribonuclease derivatives.
J. Biol. Chem 240: 4753-4762.
- BERNHEIMER, A. W. and J. M. STEELE, Jr., 1955 Ribonuclease and ribonuclease inhibitors among higher plants. Proc. Soc. Exp. Biol. Med. 89: 123-126.
- BIGGINS, J., and R. B. PARK, 1962 cited by Granick, 1963.
- BLOCH, D. P., 1962 On the derivation of histone specificity. Proc. Natl. Acad. Sci. (U.S.). 48: 324-326.
- BONNER, J., 1965 Development in J. BONNER and J. E. VARNER. Plant Biochemistry, Acad. Press, N.Y. Pp. 850-866.
- BRESNICK, E., J. SAGE, and K. LANCLOS, 1965 Ribonuclease activity in hepatic nuclei during development. Biochim. Biophys. Acta 114: 631-633.

- DICKMAN, S. R., J. P. AROSKAR, and R. B. KROPP, 1956 Activation and inhibition of beef pancrease ribonuclease. *Biochim. Biophys. Acta* 21: 539-545.
- FRISCH-NIGGEMEYER, W., and K. K. REDDI, 1959 Studies on ribonuclease in tobacco leaves. I. Purification and properties. *Biochim. Biophys. Acta* 26: 40-46.
- FUTAI, M., Y. ANRAKU, and D. MIZUNO, 1965 The roles of three enzymes in messenger RNA degradation in cell free systems from normal or phage-infected *Escherichia coli*. *Biochim. Biophys. Acta* 119: 373-384.
- GERICKE, S., and B. KURMIES, 1952 Colorimetrische Bestimmung der Phosphorsäure mit Vanadat-Molybdat. *Z. Anal. Chem.* 137: 15-22.
- GOLASZEWSKI and SZARKOWSKI, 1964 Carrot ribonucleases. *Acta. Soc. Bot. Poloniae.* 33: 749-758.
- HANSON, J. B., M. J. CHRISPEELS, W. A. KRUEGER, and A. R. SWANSON, 1965 Ribonuclease and other factors involved in respiratory senescence of maize scutellum. *J. Exp. Bot.* 16: 282-293.
- HANDSCHUMACHER, R. E., 1960 Orotidylic acid decarboxylase: inhibition studies with azauridine 5'-phosphate. *J. Biol. Chem.* 235: 2917-2919.
- HIGGINS, Ch., and P. TALALAY, 1945 Sodium phenolphthalein phosphate as a substrate for phosphatase test. *J. Biol. Chem.* 159: 399-410.
- HIRONO, Y., and G. P. RÉDEI, 1966a Acceleration of flowering of the long-day plant, *Arabidopsis* by 8-azaadenine. *Planta* 68: 88-93.
- HIRONO, Y., and G. P. RÉDEI, 1966b Early flowering in *Arabidopsis* induced by DNA base analogs. *Planta* 71: 107-112.
- GRANICK, S., 1963 The plastids: their morphological and chemical differentiation in *M. Locke*, *Cytodifferentiation and Macromolecular Synthesis*. Acad. Press. N.Y. Pp. 144-174.

- JACOB, F., and J. MONOD, 1961 Genetic regulatory mechanisms in the synthesis of proteins. *J. Mol. Biol.* 3: 318-356.
- JOSEFSSON, L., and S. LAGERSTEDT, 1962 Characterization of ribonuclease and determination of its activity. *Methods of Biochem. Anal.* 9: 39-74.
- LEDOUX, L., P. GALAND, and R. HUART, 1962 Nucleic acids and protein metabolism in barley seedlings II. Interrelations of different organs. *Exp. Cell Res.* 27: 132-136.
- LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, 1951 Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193: 265-275.
- McCLINTOCK, B., 1965 The control of gene action in maize. *Brookhaven Symp. Biol. No.* 18: 162-184.
- MONOD, J., and F. JACOB, 1961 General conclusions: telonomic mechanisms in cellular metabolism, growth and differentiation. *Cold Spring Harbor Sym. Quant. Biol.* 26: 389-401.
- MORTON, R. K., 1955 Phosphomonoesterase of milk in Colowick, S. P. and N. O. Kaplan. *Methods in Enzymology*. Acad. Press. N.Y. Vol. 2. Pp. 533-539.
- RAZZEL, W. E., and H. G. KHORANA, 1959 Studies on polynucleotides. *J. Biol. Chem.* 234: 2105-2113.
- RÉDEI, G. P., 1962 Supervital mutants of *Arabidopsis*. *Genetics* 47: 443-460.
- RÉDEI, G. P., 1963a Somatic instability caused by a cysteine-sensitive gene in *Arabidopsis*. *Science* 139: 767-769.
- RÉDEI, G. P., 1963b A genetic oscillatory mechanism in *Arabidopsis*. *Genetics Today. Proc. XI. Int. Congr. Genet.* 1: 41.
- RÉDEI, G. P., 1965a Genetic control of subcellular differentiation. *Arabidopsis Research. Rep. Int. Symp. Arabidopsis Res. Göttingen* 119-127.

- REDEI, G. P., 1965b Genetic blocks of thiamine synthesis in the angiosperm *Arabidopsis*. *Amer. J. Bot.* 52: 834-841.
- REDEI, G. P., 1966 A defective genetic regulation of ribonuclease synthesis in *Arabidopsis*. *Genetics* 54: 356 (Abstr.)
- REDEI, G. P., 1967a Phenotypic reversions induced by x-rays (in manuscript)
- REDEI, G. P., 1967b 6-azapyrimidine induced normal phenocopies of a mutant of *Arabidopsis* (*in manuscript*).
- REDDI, K. K., 1958 Studies on tobacco leaf ribonuclease II. Mechanism of action. *Biochim. Biophys. Acta* 28: 386-391.
- ROTH, J. S., 1956 Ribonuclease V. Studies on the properties and distribution of ribonuclease inhibitor in the rat. *Biochim. Biophys. Acta* 21: 34-43.
- SAHAI SRIVASTAVA, B. I., and G. WARE, 1965 The effect of kinetin on nucleic acids and nucleases of excised barley leaves. *Plant Physiol.* 40: 62-64.
- SHORTMAN, K., 1961 Studies on cellular inhibitors of ribonuclease I. The assay of ribonuclease-inhibitor system and the purification of the inhibitor from rat liver. *Biochim. Biophys. Acta* 51: 37-49.
- SHUSTER, L., H. G. KHORANA, and L. A. HEPPEL, 1959 The mode of action of ryegrass ribonuclease. *Biochim. Biophys. Acta* 33: 452-461.
- SISSAKIAN, N. M., 1958 Enzymology of the plastids. *Adv. Enzymol.* 20: 201-236.
- STOCKING, C. R., and A. ONGUN, 1962 The intracellular distribution of some metallic elements in leaves. *Amer. J. Bot.* 49: 284-289.
- TAL, M., and D. ELSON, 1963 The location of ribonuclease in *Escherichia coli*. *Biochim. Biophys. Acta.* 76: 40-47.

WAGNER, R. P., and H. K. MITCHELL, 1964 Genetics and Metabolism. John Wiley and Sons Inc., N.Y.

WATSON, J. D., 1964 Protein Synthesis Upon Polyribosomes. 6th Int. Congr. Biochem. N.Y. cited by Elson, D. (1965) in Ann. Rev. Biochem. 34: page 475.

WILSON, C. M., 1963 Substrate and product specificity of two plant ribonucleases. Biochim. Biophys. Acta 76: 324-326.

WOLLGIEHN, R., and B. PARTHIER, 1964 Der Einfluss des Kinetins auf den RNS- und Protein Stoffwechsel in abgeschnittenen, mit hemmstoffen behandelten Tabakblättern. Phytochemistry 3: 241-248.

Table 1. Soluble inorganic phosphorus content of the wild type and im plants and green and white sectors of the mutant. Fresh leaves (75 mg tissue) were extracted with pH 7.5 TRIS buffer and pH 6.6 citrate buffer 0.1 M respectively.

Buffers	TRIS		Citrate	
			im leaf sectors	
Genotypes	im ⁺	im	green	white
Protein microgram	292	270	351	301
Phosphorus microgram	5.6	7.3	9.8	16.3
Phosphorus/Protein	0.01918 (100%)	0.02703 (141%)	0.02792 (100%)	0.05411 (194%)

Table 2. Calcium and Magnesium content of two genotypes
micrograms per 100 mgs lyophilized
plant tissue

Calcium		Magnesium	
im ⁺	im	im ⁺	im
329	312	226	227
100.0%	94.8%	100.0%	100.4%

Table 3. Citrate buffer soluble protein
content of the plants of two genotypes
in micrograms per mgs dry weight
(Average of 10 samples)

im ⁺	im
111.3	103.7
100.0%	93.2%

Table 4. Phosphomonoesterase (PME) and phosphodiesterase (PDE) activity of the plants of two genotypes. Enzyme was extracted with pH 7.2 TRIS buffer and after incubation at 37° C (PME for 3 hrs, PDE for 1 hr) the increase of optical density was determined in a Beckman DU spectrophotometer at 4000 Å° and 5400 Å° respectively. The reading was corrected for protein.

Enzyme	Substrate	O.D.	
		im ⁺	im
PME	Phenolphthaleinphosphate pyridine salt	.193 (100%)	201 (105%)
PDE	p-Nitrophenyl thymidine phosphate	.615 (100%)	.632 (103%)

Table 5. Liberation of inorganic PO_4 (microgram/ml) from various substrates. The crude enzyme extract was heated to 60°C for 10 min and assayed at pH 7.5 in TRIS buffer incubated at 37°C for 2 hrs

Enzyme	Substrate	PO_4 liberated		
		im^+	im	im in % of im^+
Phosphatase	RNA	10	8.2	82
Phosphatase	2'-AMP	11.3	10.0	89
5'-nucleotidase	5'-AMP	22.5	23.0	102
3'-nucleotidase	3'-AMP	90.0	75.0	83

Table 6. Effect of kinetin on pigment production of mutant im (rosettes 29 day old test tube culture).

Kinetin	No. of plants	Average fresh weight mg	Pigment/fresh weight gram		Pigment/plant	
			Chlorophyll	Carotenoids	Chlorophyll	Carotenoids
Control	20	1.85	50.4 (100%)	19.7 (100%)	0.0504	0.0036
1×10^{-3}	19	0.43	216.3 (429%)	42.8 (317%)	0.0931	0.0182

Table 7. Ribonuclease assay on albina maize seedlings (0.1 M citrate buffer pH 4.6, yeast RNA substrate)

Genotype	RNase activity	
	Exp. #1	Exp. #2
1351 albina	.094	.121
1351 green sibs	.146	.103
1441 albina	.107	.093
1441 green sibs	.095	.111

Fib. 1a. Wild type of Arabidopsis b im mutant grown at continuous illumination in the greenhouse on soil.

Fig. 2. Stem leaves of the mutant with characteristic non random distribution of sectors. Leaf at left did not contain any green cells at the base, still there is a green spot of considerable size at the tip. In the second and third leaves, several groups of cells simultaneously switched back and forth to the production of green or white mitotic progenies. The greyish areas represent green tissues on the ventral side of the leaf blade which are covered by white cells of one or two layers. The pattern of leaves four and five is the result of a reasonably faithful replication of the cells of the original four cell leaf initial.

Fig. 3. Green cell clusters on white tissue background. Each dark spot consists of several green cells with apparently normal chloroplasts.

Fig. 4. Microscopic photograph of a leaf. Each dark area represents a single cell loaded with green chloroplasts. The green cells are much larger than the white ones. Intact leaves were evacuated in a water : glycerol : formalin (10:2:0.5) mixture and directly mounted on slides for examination.

Fib. 5a. Green sector of the mutant with normal chloroplasts b white cells with small plastids which are apparently devoid of lamellar structure.

Fig. 6. Fruit of the mutant may also carry large sectors, thus the genetic constitution can be easily tested. Progeny of either type repeats the variegation.

Fig. 7. Ribonuclease activity of the wild type and of the mutant in McIlvain buffer. Crude extracts (390 microgram protein per 0.5 ml buffer) were incubated at 37° C with yeast RNA substrate (1.5 mg per 0.5 ml buffer). The reaction was stopped with 3 ml tertiary butanol-acetic acid mixture (2:1, v/v) then chilled for ten minutes and centrifuged at 10,000 rpm for 10 minutes. The supernatant was diluted four fold and the optical density of the soluble material was determined in a Beckman DU spectrophotometer at 2600 Å°. To assure good measurements throughout the whole pH range, the time of incubation was properly adjusted within the linearity range. The heavy solid line indicates enzyme activity of the im mutant in percentage of im⁺.

Fig. 8. Ribonuclease activity of two cellular fractions in McIlvain's buffer. The different pH optima are evident. The data do not give quantitative estimates on total ribonuclease activity in the two fractions but the curves indicate ribonuclease activity per equal amount of protein.

Fig. 9. Comparison of phosphodiesterase (pH 7.5 TRIS buffer) and ribonuclease (pH 7.5 McIlvain's buffer) activity of the two subcellular fractions. Ribonuclease assay performed as indicated at Fig. 7. Phosphodiesterase was assayed with 0.25 ml enzyme, 0.25 ml substrate (1.2 mg p-nitrophenyl thymidine-5'-P per 1 ml buffer) and 0.25 ml H₂O or 5 x 10⁻³ M Mg SO₄. The mixture was incubated for 1 hr diluted with 2.25 ml H₂O and optical density determined at 4,000 Å in a Beckman DU spectrophotometer.