Technical News Feature

Plant Lipid Biotechnology Through The Looking Glass

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The biosynthesis of economically important fatty acids has been resolved in recent years. The individual enzymes have been isolated, purified and characterized; the specific compartmentations of these enzymes in the cell have been determined; the flow of precursors required to supply the substrates for fatty acid synthesis has been defined. With these data at hand, a new thrust has been brought forth, namely the input of the concepts of molecular biology to the solution of a number of problems, which until now have not been resolved. If and when these problems are elucidated, application of these new solutions will have a profound effect on the agroeconomics involved in the annual production and consumption of over 50 million metric tons (m MT) of vegetable oil throughout the world. This discussion focuses on some of the problems facing plant scientists in their attempts to understand and control plant lipid biosynthesis. Some possible solutions will be suggested, and the impact of these solutions on the economy of nations will be examined.

"'The time has come' the Walrus said,
'To talk of many things:
Of shoes-and ships-and sealing wax-
Of cabbages-and kings-
And why the sea is boiling hot-
And whether pigs have wings.'"
Alice's Adventures in Wonderland, Lewis Carroll

INTRODUCTION

Rather then talk of many things, I would like to limit myself to some important implications concering biotechnology and the future direction in which plant lipid research will move during the next decade. I think it is quite safe to say that the next decade will bring remarkable new discoveries in the plant sciences. These discoveries will have a direct bearing not only in giving the research investigator a much better understanding of what transpires in the intact plant but also will make possible a dramatic change in how industry faces its problems and these problems will be resolved effectively and efficiently.

Biotechnology is a new name for fundamental processes that occur continuously in nature. It has been practiced for thousands of years in complete ignorance of any concepts of molecular biology by honey bees, primitive farmers and primitive breeders. The new twist is the explosive growth of knowledge in both the biochemistry and molecular biology of plants, spilling out in ever-increasing volumes. As a result, the application of new knowledge will have a tremendous impact on the efficiency of crop production for food and industrial uses, will force the improvement of the quality of crops (1) and even now is stimulating the dcevelopment of environmentally friendly crop chemicals such as glyphosate.

However, up to now, the vegetable oil industry has benefited largely from the employment of conventional agricultural technologies coupled with an increasingly sophisticated science of plant breeding. As a result in less than 20 years vegetable oil production has been doubled from about 25 million tons (MT) in 1969 to over 50 MT in 1987.

The purpose of this conference, I would assume, is to explore how genetic engineering and associated technologies will interface with the current technologies employed in the production of oil crops and how these will be used to modify and improve the quality of vegetable oils, yet still meet the old as well as the new demands of industry.

THE BASIC CONCEPTS

The ultimate source of carbon in a plant is atmospheric CO_2. We now have a reasonably full understanding of the biochemical events that are involved in the conversion of carbon dioxide to vegetable oil. As depicted in Figure 1, a remarkable number of reactions are required to channel the carbon of CO_2 to the final product, a triglyceride. At least 50 enzymes function in this sequence. First of all, atmospheric carbon dioxide is fixed exclusively in the chloroplast compartment of the leaf canopies of all plants. The key enzyme, ribulose 1,5 bisphosphate carboxylase/oxygenase, converts carbon dioxide to organic carbon, which then enters the Calvin Cycle to be converted eventually to a triose phosphate, a simple sugar phosphate. This intermediate is translocated out of the chloroplast into the cytosolic compartment where enzymes convert this compound to sucrose.

Nature was very wise in selecting sucrose as the carbon carrier; it is the ideal substance. It is very soluble, relatively metabolically inert, neutral and thus readily transported from the leaf canopy to a variety of sinks via the plant vascular systems. In one type of sink, namely the developing pea or corn seed, sucrose is converted to starch, the major storage metabolite. In developing rapeseed, sunflower seed, soya seed, etc., sucrose is converted to acetyl=CoA, a very active metabolite used almost exclusively for fatty acid synthesis. When the fully developed oil seed germinates, the stored fatty acids (as triglycerides) are broken down to acetyl-CoA by β-oxidation and then channeled via a set of enzymically catalyzed reactions to sucrose, which is then translocated to appropriate growing tissues of the new plant. There it is converted into proteins, nucleic acids, etc. Quite differently, in developing fruit mesocarp tissue such as the oil palm fruit and the avocado, fatty acids are synthesized and stored as triglycerides. However, these stored lipids have no physiological function. The ripened fruit drops to the ground where it is degraded by bacteria and fungi to carbon

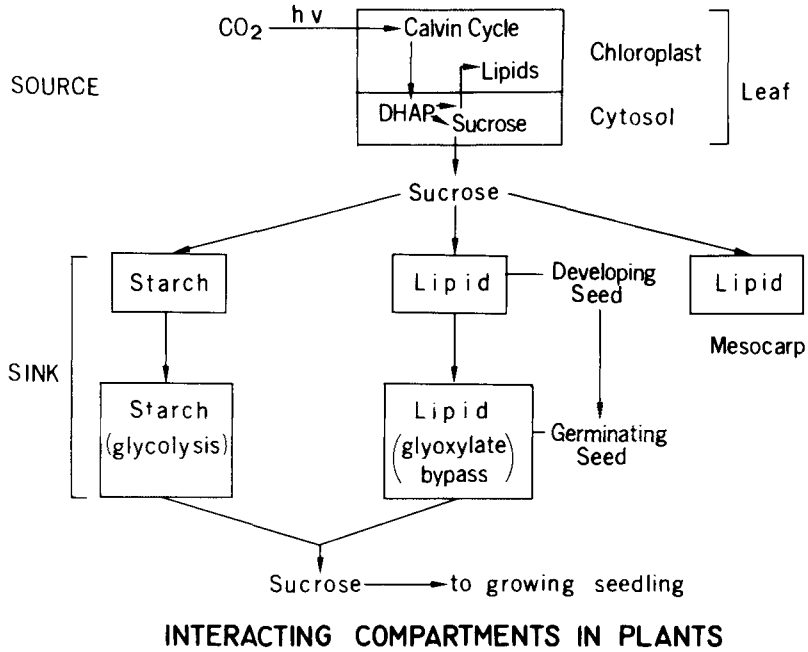


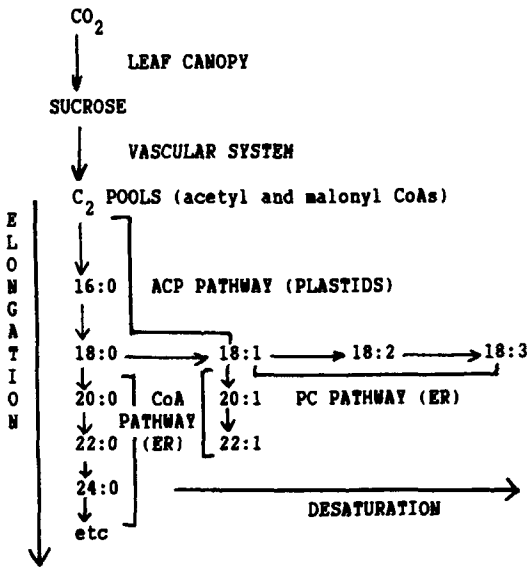
FIG. 1. The interaction of various parts of the plant in the biosynthesis and utilization lipids.

dioxide if it is not sent to the mills for extraction, etc. In summary, the leaf cell traps carbon dioxide and converts it to sucrose. In addition, the leaf cell has the capacity to synthesize those fatty acids and complex lipids that are essential for the construction and maintenance of its membranes (2). The fatty acids found in leaf membrane lipids always consist of palmitic acid and a large amount of linoleic and linolenic acids. It is important to note that these fatty acids, as complex polar lipids, are found in the membrane lipids of all green plants; that is to say, through millions of years of evolution, nature has settled on these acids as essential in the make-up of complex polar lipids that must play key roles in the structure of membranes involved in photosynthesis.

It is in the triglycerides or neutral lipids of the seed and fruit where the fatty acid composition varies according to the genetics of the particular plant. For example, although the leaf lipids of the castor bean plant have the predicted complement of palmitic, oleic, linoleic and linolenic acids, the triglyceride composition of the fully developed seeds contain as much as 90% ricinoleic acid. This acid is associated exclusively with the seed triglycerides but is completely excluded from the lipids of the leaf and the seed membranes. Thus, in developing new varieties the genetic engineer must manipulate a set of genes so that all membrane lipid compositions are conserved. In contrast, the storage lipids can be altered to any desired composition providing that in the germination of the seeds the pathways essential for the conversion of lipid to sucrose remain intact.

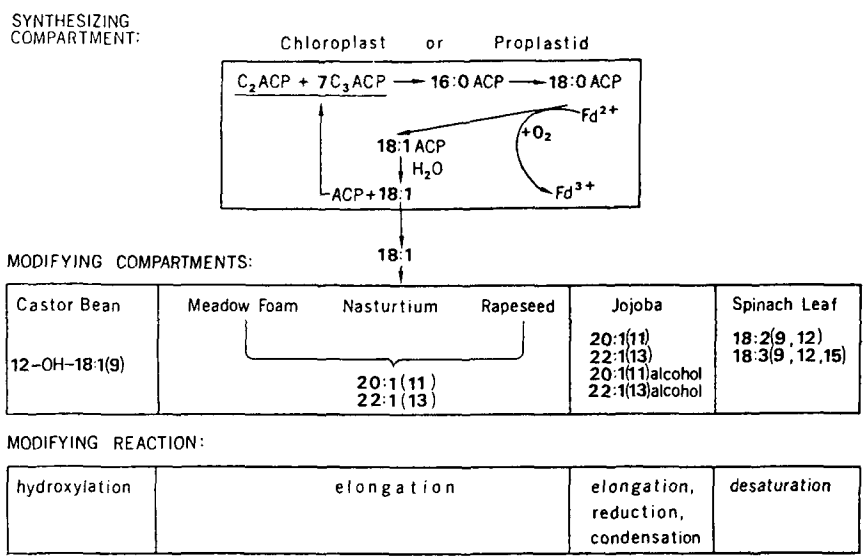
For the actual synthesis of fatty acids, the plant cell must convert sucrose to a very active metabolite called acetyl-CoA, its carboxylation product, malonyl-CoA, and then seven or eight enzymes utilize these substrates for the construction of the typical fatty acid (3). In sharp contrast to the bacterial and animal cells, all the plant enzymes are localized exclusively in organelles. In the leaf cell, the organelle is the chloroplast, a highly efficient multifunctional plastid that also houses all the enzymes required for photosynthesis. In the developing seed or fruit mesocarp cell, the organelle is called the proplastid, and this contains all the enzymes for fatty acid synthesis and the ancilliary enzymes required to convert sucrose to acetyl-CoA. Another interesting facet of the plant fatty acid synthase system (PFAS) is that all the enzymes in this system are individual proteins that make-up what is called the non-associated system, a very similar system also is found in all bacteria. In sharp contrast all animal and yeast FAS systems consist of multifunctional polypeptides with each of the enzyme activities represented by a domain or a stretch of amino acid residues that constitute the active enzymic site scattered up and down the polypeptide chain(s). This difference in molecular architecture is important because it allows the biochemist to separate each of the seven plant enzymes from each other, thus permitting reconstruction experiments in which one enzyme concentration can be varied while all the other six enzyme concentrations are held constant. This type of experiment obviously cannot be done with the animal FAS complex (3). Furthermore, because the nonassociated FAS systems of bacteria and plants are so similar, the genetic engineer might even consider transferring key bacterial FAS enzymes into the plant genome to improve the kinetics of lipid synthesis.

Figure 2 summarizes the present knowledge concerning the synthesis of fatty acids in plants. Suffice it to say that by a series of reactions, acetyl-CoA and malonyl-CoA are condensed in an ordered series of reactions to form palmitic acid, which is elongated to



PATHWAYS, LOCALIZATION AND SUBSTRATE SPECIFICITIES FOR FATTY ACID BIOSYNTHESIS IN OIL SEEDS

FIG. 2. A summary of the pathways involved in the conversion of acetyl-CoA to the final product, the localization of these pathways in the seed and the substrate specificities.



CENTRAL ROLE OF OLEIC ACID

FIG. 3. Central role of oleic acid. A plant cell has a synthesizing compartment and modifying compartments (indicated here as ER, i.e. endoplasmic reticulum, etc.). Modifying reactions are indicated in the figure. Fd, ferredoxin; C_2ACP, C_3ACP, malonyl-ACP.

stearic acid and then desaturated to the mono-, di- and tri- enoic acids (3). Figure 3 emphasizes these reactions as they relate to the various compartments.

TARGET REACTIONS

With these comments as a backdrop, what can be said about the rational application of molecular biology to the improvement of world oil production? As I have indicated, at least 50 enzymes participate in the conversion of carbon dioxide to fats and oils. The source of energy for these reactions is from photosynthetic reactions, which make possible the fixation of carbon dioxide, evolution of oxygen and the generation of

adenosine triphosphate (ATP), the immediate driving force in all biosynthetic reactions. Which of these 50 enzymes can be altered or modified? Obviously, over several billion years plants that make possible the production of the present vast tonnage of vegetable oil have evolved. What is there left for the plant lipid scientist to tackle? As we learn more about the detailed biochemistry of higher plants, possible opportunities in which we can accelerate the very slow process of evolution arise. Thus, genetic engineering can function at several levels: (a) the introduction of a single gene that is expressed at all times throughout the entire plant, (b) the introduction of a single gene that is expressed only in a specific developing seed cell at a given time, (c) the introduction of controlling genes that regulate expression at temporally different stages of development, (d) the insertion of genes that control multiple traits such as drought, temperature or water stress (4) and (e) in the future, a procedure that would allow the deletion of a normally functioning gene and its replacement with a gene specifically designed to alter a specific reaction could be developed.

Before the genetic engineer can carry out these changes, the biochemist must be able to identify target reactions that regulate the synthesis of plant fatty acids. The very difficult problem the biochemist faces is determining which of the 50 or more enzymes fit that bill. I have selected a few reactions that should be examined carefully by the plant biochemists and the plant molecular biologist as possible target reactions.

Carbon dioxide fixation. The key enzyme in all green plants for carbon dioxide fixation is ribulose 1,5 bisphosphate carboxylase/oxygenase (also called rubisco) (5). The most abundant soluble protein in the world, it is located solely in the chloroplast compartment, and in all higher plants it consists of eight small polypeptide subunits (S) and eight large polypeptide subunits (L) held together as the active complex L8S8. The "L" subunit contains the catalytic site and is encoded by chloroplast DNA while the "S" subunit is nuclear-encoded but its function is defined poorly. The enzyme being bifunctional, catalyzes the reactions:

(1) ribulose 1,5 bisphosphate + CO_2 → 2 phosphoglyceric acids
a carboxylation reaction

(2) ribulose 1,5 bisphosphate + O_2 → phosphoglyceric acid + phosphoglycolic acid
an oxygenation reaction

Reaction 2, the oxygenation reaction, deprives the Calvin Cycle of one phosphoglyceric acid because phosphoglycolic acid is formed, becoming a substrate for photorespiration and ultimate conversion to carbon dioxide. One of the features of rubisco is its low rate of fixation. As Andrews and Lorimer (5) stated recently, "Given that for more then 3.5×10^9 years, nature has been conducting a selection experiment with rubisco . . . . is rubisco in higher plants already perfect in the sense that no further increases in these parameters are possible?" This would suggest that if rubisco has not improved over these many years, a barrier too wide to be bridged by natural evolution must exist in the enzyme structure. Perhaps molecular biology, coupled with a detailed knowledge of the structure of rubisco, can be employed to design an enzyme that has suppressed the oxygenase step and increased its affinity for CO_2 and, hence, the rate of carboxylation. If genetic engineering can redesign rubisco, the final product of CO_2 fixation, namely sucrose, would be increased and indirectly lead to great yields of triglycerides. It should be pointed out here that an equally important candidate for the control of sucrose synthesis involves the regulation of the synthesis of another molecule, fructose 2,6 bisphosphate, which recently has been implicated as playing a key role in controlling sucrose synthesis in the leaf cell. Whether these and other reactions can be considered targets of control awaits much more research.

Acetyl-CoA carboxylase. The biotinyl enzyme synthesizes the key substrate necessary for fatty acid synthesis:

acetyl-CoA + CO_2 + ATP → malonyl-CoA + ADP + Pi

The enzyme has been investigated in plant tissue by a number of workers (3). Although the structure appears to be rather complex, its regulation also is poorly understood. Nevertheless, low levels of malonyl-CoA in plant extracts lead to the synthesis of a number of fatty acids ranging from C8 to C18 fatty acids, high levels narrow the range of fatty acid chain lengths. It seems that a much broader understanding of the carboxylase gene(s) presumably encoded for by nuclear DNA would allow a sharper definition of the subunit structure of this important enzyme and its regulation at either the transcriptional or translational level. Perhaps gene transfer of a more active bacterial carboxylase gene into the plant DNA also could be explored.

Fatty acid synthesis enzymes. Before we can discuss what, if any, enzymes could be modified or replaced to improve the fatty acid composition of a commercial vegetable oil, a basic observation must be made. In general, there are at least two types of genes: one that can be labeled as housekeeping genes and is expressed in all cells; their expression is required for the orderly functions common to all cells. The second type is organ- or tissue-specific and is expressed in a temporal or developmental mode. These genes are switched on and off as a function of the development of the cell or tissue, and their gene products or enzymes are involved in synthesizing compounds uniqe to a specific tissue such as pigments, alkaloids or unique fatty acids such as ricinoleic or erucic acids. Thus, although the mechanism of fatty acid synthesis may be identical in the seed or leaf cell, the end product control may be unique in the seed cell. The conclusion one must accept is that in the attempts by the molecular biologist to modify the type and amount of fatty acids in the plant, care must be taken to include in gene transfers suitable regulatory regions that will make possible a normal seed developmental sequence and also the specific assignment of fatty acids to the different sites in the cell.

There are a number of target reactions in fatty acid synthesis that could be considered as potential regulatory reactions:

(1) Developmental control systems are possible regulatory actions. At a critical time after fertilization in the developing seed, a coordinated and intense increase occurs in the activity of at least 40–50

enzymes required for the conversion of glucose phosphate to fatty acids. FAS activity reaches a maximum value and then falls rapidly as the seed approaches dormancy. Evidence strongly suggests that the transcription of specific genes encoded in nuclear DNA takes place when a switch is turned on, and as a result a rapid synthesis of mRNA occurs. Ohlrogge has evidence that this occurs in the formation of acyl carrier protein, which is critical for fatty acid synthesis (6). In addition, all the ancilliary enzymes responsible for the conversion of sucrose to acetyl-CoA and for the formation of the plastid structure also must be synthesized. The fundamental dilemma here is that very little is known about the switching mechanism involved in turning on and off the genes that are responsible for the specific gene products. A complete understanding of this switching mechanism would permit the molecular biologist to manipulate these "on- off" controls with possible amplification of important regulatory enzymes. An even more interesting approach would be the isolation or synthesis of an inducer that could be sprayed onto the whole plant; the inducer then would turn on the capacity of the plant to synthesize triglycerides, for example, in all tissue. The plant would die, of course, but its entirety could be harvested for oil. With no inducer, the plant would go through its normal life cycle to produce the necessary seeds.

(2) Of the seven enzymes involved directly in fatty acid synthesis, at least two or three may have important regulatory effects on the overall formation of fatty acids (3,7,8). These include:

(a) acetyl CoA:ACP transacylase:

$$\text{acetyl CoA} + \text{ACP} \longleftrightarrow \text{acetyl ACP} + \text{CoA}$$

(b) β-ketoacyl ACP Synthase I:

$$\text{acyl ACP} + \text{malonyl ACP} \longrightarrow \beta\text{-ketoacyl ACP} + \text{ACP} + CO_2$$

(c) β-ketostearoyl ACP Synthase II:

$$\text{palmitoyl ACP} + \text{malonyl ACP} \longrightarrow \beta\text{-ketostearoyl ACP} + \text{ACP} + CO_2$$

All these enzyme have low specific activities in a wide variety of seed extracts and thus are candidates for regulatory functions. In reconstitution experiments with highly purified PFAS enzymes, in which one enzyme concentration was varied while the others were kept constant, it was observed that (a) increasing the concentration of the transacylase markedly decreased the chain length of the newly synthesized fatty acids, (b) Synthase I is involved in the synthesis of fatty acids only up to palmitic and (c) Synthase II was responsible for the conversion of palmitic to stearic and was terminated there.

Therefore, one could suggest that a manipulation of the transacylase by recombinant DNA technology may alter greatly the end-product of fatty acid synthesis, favoring the formation of the shorter-chain fatty acids, and that deletion of Synthase II would result in a plant that will synthesize only C16 fatty acids. Deletion of Synthase II would imagine, would prove lethal to the plant.

In summary, a number of possible target reactions may be vulnerable to genetic engineering, providing the precautions I listed earlier are taken into account. Needless to say, to the imaginative plant lipid scientist, a number of other enzymes can be mentioned but those listed above could be of primary importance.

MODIFYING REACTIONS

A number of plants accumulate fatty acids that are quite different from the normal C16 and C18 fatty acids. The production of these have considerable potential in new industrial oleochemical uses. These include the Cuphea, the jojoba and the Brassica, to list just a few. All these plants have a mechanism that terminates chain elongation at a given chain length. With the exception of the Cuphea, we now are able to explain the mechanisms that the plants employ to achieve the synthesis of the fatty acids characteristic of the species. In these cases, we return to the basic concepts outlined earlier, namely the synthesizing compartment in which acetyl-CoA is used to construct the C16 and C18 fatty acids on the ACP track and then the transfer of the end product, be it stearic or oleic acid, to the cytosol for further modifications. Thus, in the developing jojoba seed oleic acid is synthesized in the proplastid compartment, translocated to the cytosol where it then is elongated on the CoA track to C20 and C22 moneoic acids, reduced to the corresponding alcohols and then these are condensed to form the valuable oxygen esters. In Brassica, oleoyl-CoA and stearoyl-CoA are ineffective as primers (9). In leek epidermal tissue, palmitoyl-CoA is the primer for the formation of the very long chain saturated acids and oleoyl-CoA is ineffective as a primer (10). Thus, the specificity of the final product is in large part dictated by the nature of the primer substrate.

leek: *palmitoyl-CoA* + malonyl-CoA → β-ketostearoyl-CoA + CoA etc.

Brassica: *oleoyl-CoA* + malonyl-CoA → β-ketoeicosenoatoyl-CoA + CoA etc.

THE FUTURE TREND—THE LOOKING GLASS

We have discussed a number of target reactions that are being investigated in University and, presumably industrial laboratories. The new techniques of molecular biology have given an enormous impetus to the exploration of the modification of these target reactions. Robbelen, Theimer, Stobart, Harwood, Knowles, Scowcroft, Horsch, Jones and Ohlrogge and others will describe in much greater detail what their researches are beginning to show. In addition, it now is possible to engineer genetically a number of plants that are resistant to virus infections, herbicides and pesticides.

What disadvantages result from these new approaches? For example, if a plant such as rapeseed or soya could be altered genetically to produce any desired fatty acid composition that industry would require—a number of problems would arise. One would be the further drift from genetic diversity to genetic uniformity (11). The next step would be genetic vulnerability, that is vulnerability to pests, disease and weather.

History very dramatically, has documented the problem of genetic uniformity. For example, as recently as 1985, a new strain of a crop pathogen, citrus canker, threatened to wipe out the susceptible citrus varieties grown in Florida.

In addition, a versatile oil crop could affect greatly the economy of an entire nation. The oil palm is the principal agronomic crop in Malaysia, Indonesia and some African countries. If a genetically designed rapeseed or soya seed could produce the same type of triglycerides as economically what now is produced by the oil palm, then the oil palm industry would collapse, and the palm oil producing countries would suffer. Conversely, if the oil palm industry would apply the same techniques to the oil palm that were used to alter rapeseed or soya, then the oil palm would become the prime source of vegetable oils. When one considers the average yield of various oil-producing crops, the oil palm is prodigiously productive with an average yield of 4000 kg oil/ha whereas soya is much lower with only about 400 kg oil/ha. Of course, the soya bean is grown primarily for its protein; the oil is a side product!

Thus, as biotechnology enters the 21st century, the explosive techniques of molecular biology coupled with the equally explosive needs of an ever-growing population will revolutionize the current modes of agriculture and will have a profound effect on the economies of the world. Achieveing these goals will require (a) adequate funding in research from both federal and industrial sources, (b) properly trained biochemists and molecular biologists to develop the basic knowledge needed to solve technical problems, (c) an exchange of ideas not only at the academic level but also at the industrial level. The question arises as to the impact secrecy would have on the development of fruitful research. (d) A solution to the problem of genetic uniformity of plant material. (e) Political considerations to control trade barriers that even now are appearing above the horizon. For example, restrictions placed on oil imports because of nutritional dogma, and (f) competition between temperate zone high technology countries vs third world tropical countries with their evolving agriculture and technology.

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