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Technological Synthesis [and Discussion]

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Phil. Trans. R. Soc. Lond. B 1990 **326**, 687-692
doi: 10.1098/rstb.1990.0039

Phil. Trans. R. Soc. Lond. B **326**, 687–692 (1990)
Printed in Great Britain

Technological synthesis

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In agriculture and horticulture, chilling and freezing injury of plants often results in serious problems of reduced yield and crop loss. The geographical distribution of many field crops is, in many instances, determined by the minimum temperature encountered. If crop responses to reduced temperature could be modified major changes in productivity would result.

In addition to the studies of the effects of environmental temperatures, a separate discipline has arisen in low-temperature biology concerned with the extended storage of viable biological material, generally at the temperature of liquid nitrogen. Until recently, there has been little dialogue between the disciplines of environmental studies and cryopreservation. It is probable that an understanding of the tolerance of organisms to environmental stress would accelerate progress in cryopreservation.

Introduction

It could be argued that a more complete understanding of the cellular mechanisms associated with low temperature and freezing resistance, as evolved by organisms in response to extremes of their natural environment, would bring about practical benefits in two major areas.

The first is in food production from agricultural and horticultural plants, by extending the growing seasons and geographical distribution of crops into seasons and regions where previously unfavorable lower temperatures were experienced. In this context a widely quoted statistic suggests that a 1 °C increment in the freezing resistance of wheat would provide a potential increase in yield of better than 25% of world production. The second area where benefit might be gained is in the improvement of cryopreservative procedures, by modifying perhaps structure and functioning of material to be preserved and making significant alterations to the protocols involved, based on data derived from natural systems.

It is a widely held view that these benefits may be slow to be realized, as a consequence of too little dialogue between those who study low temperatures in the environment and the practitioners of laboratory-based cryobiology. In this paper we examine the role that information from studies in the natural environment might have on the rapid construction of new commercial varieties of crop plants, and on procedures for cryopreservation.

Cold-resistant crop plants

Radical changes in the geographical distribution of a crop plant could occur from relatively small increases in its resistance to chilling or freezing. The creation of plant varieties with such increased resistance to low temperature is therefore becoming one of the goals of current efforts in genetic engineering of plants. The feasibility of achieving such enhanced resistance by gene manipulation in the laboratory, while retaining the other desired features of the crop must however be questioned.

There are three general experimental approaches to the determination of what are in effect symptoms of resistance to all aspects of low temperature exposure. These involve comparison of: (i) closely related stress-sensitive and stress-resistant species; (ii) cold acclimated and non-acclimated stages of an individual species; (iii) resting stages such as seeds with vegetative stages.

The inference from many such studies has been that the observed resistance to low temperature stress is associated simply with the accumulation of a single compound or a step change in the structure of an organelle or membrane. This is unlikely as, for example, in the simple case of chilling injury it is evident that a reduction in temperature may affect the structure and activity of both cellular proteins and lipids, and the resulting effects on cellular physiology are bewildering in complexity. It has proved simple to induce and select mutants of microorganisms with a restricted range of growth temperatures for example, but mutants capable of growth at temperatures significantly lower than that for the wild-type have not been reported. This suggests that many genes determine the natural range for growth, and attempts to identify individual biochemical sites for limiting growth at low temperatures may be naive (Morris & Clarke 1987).

The basis of enhanced low temperature resistance in the natural environment can be seen as a simple sequence of cellular events: (i) the presence of a gene, but more likely a family of genes, that need to be expressed to produce the resistant state; (ii) the production of specific gene products that result in adapted metabolism or morphology or both; (iii) the complete expression of the adapted phenotype that demonstrates low-temperature resistance.

The complete sequence is necessary for the expression of resistance. To use an extreme example, it can be argued that 'resistance genes' are widely distributed among plants and lower animals, but are expressed only in the complex changes that bring about the production of resting stages. For example, a tomato plant has the gene sequences, that result in the changes of function and structure of a seed that confer resistance to extremes of chilling and freezing, whereas the vegetative plant and hydrated seed are susceptible to such stresses.

It is also important to note that there is a trade-off between expression of the resistance genes and the modification of structure and function. Typically a highly freezing-resistant plant diverts little metabolic effort to growth and development, but supports high levels of maintenance metabolism and accumulation of stored solutes. It thus appears that enhanced resistance and active growth are incompatible, at least in plants of agricultural significance. A good example is the winter heading cauliflower (*Brassica oleracea* var. *botrytis*) which has foliage that acquires marked frost resistance, but ceases to grow when so adapted. The flower head of the same crop, for which it is harvested, continues to grow rapidly and acquires little or no frost resistance (Grout 1987).

At an extreme level, therefore, it can be argued that any search for a complete complex of frost resistance genes is of limited value, for the accompanying, apparently necessary, modifications of phenotype would not be welcome in high-performance crop plants.

An alternative, and more likely, approach to the protection of field and horticultural crops may be not by the genetic modification of the plant, but rather, from an understanding of the biochemical basis of injury, to produce a short-lived treatment when low temperatures are predicted.

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Cryopreservation

Cryopreservation of biological material was first demonstrated to be a reality by Polge *et al.* (1949) with mammalian sperm. Since that seminal discovery the range of cell types successfully frozen to, and thawed from, −196 °C has ranged from microorganisms to mammalian embryos. This technology has established several new industries, the largest application being artificial insemination of a variety of domestic animals. In the cattle industry the number of inseminations using cryopreserved sperm now exceeds 130 million per annum (Iritani 1980). Recently, embryo transfer has been established in a range of mammalian species, and the estimate of the size of the market for cattle embryos is £60 million per annum. However, it must be stressed that a significant range of cellular material remains recalcitrant with respect to cryopreservation, and that tissues, and organs, of prime concern for human transplantation cannot yet be stored.

The methods typically employed to develop new cryopreservation techniques are essentially the recipe approach, with the addition of a limited range of potentially protective chemicals and manipulation of rates of cooling. If cells are not amenable to this somewhat limited repertoire then progress is slowed or halted.

The hope is that information from environmental studies may provide novel approaches to cryopreservation. To achieve this, the differing circumstances of stress must be recognized (table 1).

Table 1. Differences between environmental freeze–thaw stress and cryopreservation

	environment	cryopreservation
temperature	lowest recorded −130 °C generally only to −20 °C	industry standard −196 °C
stability	often on a 24 h cycle	no cycling
cooling rates	invariably 'slow', i.e. 1 °C h^{-1}	wide range from < 0.1 °C min to > 100 °C min
cryoprotectants	recognized are sugars glycerol, amino acids	wider range DMSO, propanediol polymers, i.e. PVP
cell injury	dehydration, intracellular ice	dehydration, intracellular ice, devitrification
DNA stability	selection	nuclear DNA stable plasmids? transgenics?

It also must be recognized that in the development of improved cryopreservation methods there may be little to be gleaned from those environmental studies where survival is associated with freeze avoidance rather than freeze resistance. A possible exception could be certain aspects of acclimation of plants and microorganisms and hibernation in mammals, where increased resistance is acquired in a relatively short timespan, as the result of some sort of inductive temperature shock. This acclimation may translate, in some way, to the sort of short-term manipulations that are possible in the laboratory before cryopreservation and result in improved pretreatments.

By contrast, more may be learnt from the cellular modifications of extreme halophiles and desiccation-resistant stages of a range of types. For example, a number of compatible solutes

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have been identified that protect cells against extreme halophilic conditions (> 4.0 M NaCl), many of these being of value in cryopreservation. A clear example is the halophilic alga *Dunaliella* which accumulates intracellular glycerol up to a concentration of 8 M. Organisms that naturally desiccate without extensive injury have been demonstrated to accumulate a limited range of compounds, notable among which is the sugar trehalose, which has been demonstrated to be a useful cryoprotectant in some systems. However, a major factor in their application is the permeability of cells to such compounds.

The natural environment provides further examples of material fully adapted to the extremes of desiccation required for preservation, and seeds of higher plant cells are probably the best example. However, the extremes of adaptation of structure and function in a seed cannot realistically be imposed upon, for example, mammalian cell structure. Yet there is much of value to cryobiology in this situation. At a fundamental level the mechanism by which seeds can tolerate extremes of dehydration is not clear, and compounds generally associated with desiccation tolerance in other types, e.g. nematodes, are not present in many desiccation-resistant seeds. The basis of such an extreme resistance is, however, unquestionably gene based.

To make significant progress in cryopreservation, novel technologies need to be developed and may be helped by clear and open dialogue with many other areas. The possible input from environmental studies has been briefly discussed but if the practical problems associated with cryobiology are examined (table 2) it is perhaps to be physical sciences that more attention should be directed.

TABLE 2. PROBLEMS ASSOCIATED WITH CRYOPRESERVATION

1. With cell types of interest it is often not possible to manipulate the cell biology.
2. Addition or removal of cryoprotectants.
3. Nucleation.
4. Definition of an appropriate rate of cooling.
5. Problems associated with heat transfer, especially for large volumes.
6. Storage and transport.

CONCLUSIONS

Novel cryopreservation methods might be expected to be derived, at least in part from a study of the response of natural systems to environmental stress, the most likely systems being desiccation resistant and halophilic organisms. By contrast, progress in the development of commercially useful cold-resistant crop plants is unlikely to be developed from studies of cold-resistant species followed by genetic manipulation of existing crop plants.

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Discussion

A. Cairns (*Welsh Plant Breeding Station, Plas Gogerddan, U.K.*). Dr Morris introduced his talk with reference to calculations concerning the effect of reducing the lower temperature threshold for growth in plants: a 1 °C lowering of this threshold leading to an estimated 40 % increase in global rice yield was one example presented. In addition, Dr Morris pointed out the potential savings in energy expenditure that could be obtained by engineering cold-tender glasshouse plants, such as tomato, to grow at lower temperatures.

Dr Morris held the view that the previous, and by implication current, attempts to manipulate plant cold hardiness are, in Dr Morris's words, 'naive', because the known adaptive strategies in organisms other than plants, namely membrane lipid changes, accumulation of cryoprotectants, and elimination or production of ice-nucleation foci, are not necessarily those that are involved in the specific cold-tolerance mechanism of any given plant species.

He argued that most plants contain innate genes for cold tolerance, which are not expressed in the growing mature plant. His evidence for the existence of these genes is the known ability of seeds to survive the low temperatures of winter.

Based on this reasoning, his strategy for the engineering of cold tolerance into mature plants was, therefore, to bring about expression of these innate seed genes in the mature plant. This could be accomplished by the application of, in Dr Morris's words, 'some sort of spray'. The specific, innate cryoprotectants, unsaturated lipids, etc., which are characteristic of the seed of that species, would then protect the tissues of the mature plant. He presented no specific examples of biochemical or structural features which could be exploited in this way or means for the regulation of their expression.

It is my opinion that the view presented by Dr Morris is based upon a misunderstanding of the function of the seed in the life cycle of plants and upon a confusion between the concepts of 'survival' at low temperature and 'growth' at low temperature.

Seeds are characteristically dehydrated and hence cannot suffer ice formation and the consequent damage to their tissues. When seeds absorb water in the process of imbibition, resistance to low temperature is lost, indicating dehydration to be of central importance. Enzyme proteins maintained at a low degree of hydration may also be more stable to low temperatures. Seeds are dormant and have a low metabolic rate. They are encased in a resistant seed coat and do not grow during the cold season. In summary, a seed is a device that allows a cold-sensitive species to cease active growth and avoid the ravages of winter, so that it may survive to grow in the next warm season.

These characteristics of the seed may be contrasted with those of the mature plant which may generally contain 80–90 % by mass of water and hence be highly susceptible to low temperatures or ice damage or both. Mature tissues grow, are metabolically active and the mature plant is not protected from the environment by a resistant barrier.

Expression of the genes encoding for the structural and metabolic adaptations of the seed in the mature plant, would result in a plant with very similar properties to a seed, that is, dry, dormant and non-growing. I suspect that such a plant would be of little use in agriculture!

Dr Morris seemed unaware of the great variety of mature plant responses to low temperature which encompass both survival and low temperature growth phenomena. One example is the 'slender' mutant of barley, plants of which continue to grow undamaged at temperatures as

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low as −5 °C. I feel that studies of such characteristics in growing plants, in conjunction with a knowledge of the comparative biochemistry and physiology underlying function of all low temperature adapted organisms is highly likely to yield information of use to plant genetic engineers and are less naive than Dr Morris would suggest.

G. J. Morris. The point being made was not concerned with 'innate seed genes'. What was being aired was the notion that plants producing orthodox seeds have genes that allow cytoplasm to be reduced to minimal water activity without loss of viability. The activity of such genes, were it controlable in the laboratory or field, might be of value in the acquisition of freezing tolerance. Obviously, if the totality of genes encoding for seed morphology and metabolism were expressed a seed would result, but expression of a selection of those genes might produced the required result. The suggestion was that study of such genes might be at least as useful as the searches currently in progress for desiccation resistance genes associated with physiological adaptations of the vegetative state.

The ideas proposed were not intended to refer to adaptations allowing for growth at low temperature in the unfrozen state, but to relate to water activity and stresses in the presence of ice.

U. Heber (*Institute of Biology and Pharmacological Biology, University of Würzburg, F.R.G.*). Can we equate freezing tolerance and dessication evidence?

G. J. Morris. One important aspect of freezing tolerance is the ability to contend with reduced water activity. If the mechanism involved is one of resistance, rather than avoidance, then there are obvious parallels with resistance to dessication.

G. E. Fogg, F.R.S. (*University College of North Wales, U.K.*). Nothing resembling the accumulation of sucrose at low temperatures such as occurs in higher plants seems to have been reported for any group of algae. Smith & Morris (1980), in short term experiments below −1 °C, found that phytoplankton from the Southern Ocean assimilated ^{14}C from bicarbonate mainly into lipid, but it is doubtful whether this bears any relation to cold tolerance.

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