The Environment as an Active Generator of Adaptive Genomic Variation

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I believe there is little reason to question the presence of innate systems that are able to restructure a genome.

We know nothing, however, about how the cell senses danger and instigates responses to it that are often remarkable.

Barbara McClintock 1978 and 1983 in The Dynamic genome: Barbara McClintock's Ideas in the Century of Genetics (1992) (Federoff, N. and Botstein, D., eds), Cold Spring Harbor Press.

I Introduction

All living organisms are affected by their environment. As the environment changes, the organism needs to adapt in order to survive. Clearly, organisms have developed responses to many environmental stresses including thermal stress, wounding, oxidative stress and to control invasive or transposable elements. Many of the responses are physiological and designed to overcome a temporary stress and not designed to generate genetic change which can be transmitted to the following generations. Some epigenetic modifications such as paramutation (Hollick, Dorweiler and Chandler, 1997) and repeat induced point mutation (RIP) (Selker 1997) can be heritable. Rapid modifications of the genome, correlated with changes of gene expression, have also been observed during plant development and under stress conditions. Several mechanisms, such as quantitative modification of repetitive DNA, DNA methylation, excision and insertion of transposable elements, gene amplification or deletion and histone acetylation have been suggested as points of control of these changes in gene expression [Fedoroff, 1989; Cullis 1990; Bassi 1990, 1991; Natali et al. 1993; 1995; Smulders et al 1995; Braunstein et al. 1993; Johnson et al 1996; Richards, 1997]. Thus, in many organisms, the genome may reorganize itself on facing adversity, for which it is unprepared, in order to ensure the organism's survival. It is this response of the genome, that is a modification of the DNA structure, which is the focus here, along with the evidence for such a response, and how such a response may be configured.

Several instances have been documented in which the genome does alter in response to the environment [Evans, Durrant and Rees, 1966; Cullis and Charlton, 1981; McClintock 1984; Walbot & Cullis 1985; Johnson et al 1996]. Examples include adaptive evolution in Escherichia coli, sporulation in Bacillus, mating type switching in yeast, activation of transposable elements, tissue culture induced variation, gene amplification as well as others [Cairns et al. 1988; Cairns & Foster 1991; Foster & Cairns 1992; Foster & Trimarchi 1994; Hall 1988; Stragier et al. 1989; Sprague et al. 1983; McClintock 1984; Roth et al. 1989; Stark et al. 1989]. Each of these systems shares a common feature of DNA variation or mutations arising in an apparent direct response to an environmental cue.

In an evolutionary context, what are the circumstances where such a reorganization would confer a substantial advantage? Firstly, it would need to be an inducible system so that when the organism is surviving well in a given environment there is not a continuous generation of variation. Ideally, the variation would only be generated when the organism "senses" the need.

The way in which such a sensory mechanism may work is certainly not clear at present. Secondly, the regions of the genome which are restructured in response to stress need to be delineated. It appears obvious that any restructuring cannot just be at random. Any random mutagenesis is unlikely to generate useful variation without the concomitant appearance of negative traits. Thus, it is probable that there will regions of the genome, which will be more labile than others. The identification of these regions will also be important in identifying the mechanism(s) by which the genome is restructured. The identification of these regions will also be essential in understanding how the genomic reorganizations result in phenotypic variation. Thirdly, the breeding system of the organism is also likely to be important. In the case of an inbreeding species, there will be little variation left when growing in a favorable environment over an extended period. Thus in such a circumstance, a significant change in the environment is likely to be disastrous, unless a mechanism by which variation can be introduced, or maintained, is present. Therefore, it is likely that evolutionary selection will favor the presence of a controlled plasticity in those inbreeding species which have survived the slings and arrows of outrageous environments.

Therefore, for the genomic response to stress to confer an adaptive advantage, it will need to have the following properties:
1. The mechanism is only activated when the normal physiological responses have been exhausted. Therefore there needs to be a “sensing” system.
2. The genomic variation has to be translated into phenotypic variation.
3. The effects of the changes have to be advantageous to the generation in which they occur as well as capable of being passed onto the next generation.
4. The alterations need to be reversible.
5. The alterations need to be limited in scope

II. Characteristics of stress-induced adaptive genomic reorganizations.

The basic assumption is that the genome is variable and can be reorganized in response to various stresses (Cullis, 1977; Walbot and Cullis, 1985; Roth et al., 1989). However, all of the genome cannot be equally susceptible to change. Thus genomic variations occurring in response to stress must be localized in a subset of the genome. The specific fraction of this variable subset which is modified in response to any given stress will need to depend on the physiological status of the cell (Cullis 1977; 1983; Cullis & Creissen 1987). Thus the regions of the genome which are altered will be dependent on the particular stress being experienced. Some of the labile regions may be more variable than others and, therefore, always alter irrespective of the inducing stress such a stress-sensitive set of regions could act as markers for conditions under which genomic reorganization is taking place.

The response to environmental stress must be under genetic control, that is there must be genes, which control the ability of the organism to respond to environmental stresses. The presence or absence of functional copies of these genes accounts for the ability of the organism to undergo genomic change in response to stress. It is neither known how many different sensing systems for activating the response are present, nor, the range of stresses that can activate the response. However, the presence of independent activating systems would result in lines that would respond to some stresses but not to others. Therefore we should be able to identify lines within a species that will react, and others which are unreactive, to particular stresses, and be able to use these lines to map the genes involved in the response.

The regions of the genome which are altered in response to the environment, as distinct from the regions controlling the ability to respond, can also be identified. The characterization of the alternative states of these regions will indicate the molecular mechanisms by which reorganization occurs (clearly there can be multiple mechanisms involved, and some may be stress specific).

Finally, and most importantly, the genomic restructuring must result in an altered phenotype, ideally one which improves the viability of the organism under the stress conditions. Since all the characteristics of an individual cannot be detailed, the phenotypic variants studied are likely to be those most easily observable, and not necessarily those most important for the adaptation. A second consideration in studying such phenomena is the problem of continuous variation under the influence of environmental pressures. To compare the effects of a set of stresses, progeny need to be grown in a common environment. If the genomic restructuring system is still active, after a small number of generations, all the individuals should be similar, irrespective of their previous experience. Therefore only transient maternal effects are likely to be observed unless the response system is inactivated, possibly as a consequence of the genomic restructuring.

The discussion here will center on the environmentally induced heritable changes in flax. This phenomenon has been extensively described and the genomic alterations characterized. The work was possible as some of the heritable changes occurred in lines which also lost their ability to respond as part of the heritable changes. The stability of the induced lines facilitated an identification of genomic components altered in response to the environment.

III. Examples of Genomic Modification in Plants.

In recent years various lines of research have shown that the genome can be modified both during development and under various types of environmental stresses. One of the results of such modifications is the selective, quantitative variations in specific families of DNA. For example, exposure of cultured Cymbidium protocorms to auxin resulted in amplification of AT-rich satellite DNA, while exposure to gibberellic acid (GA) increased a GC-rich fraction (Nagl & Rucker 1976). These modifications in repetitive DNA were correlated with induction of cytodifferentiation of orchid protocorms suggesting amplification of particular DNA sequences during development. Dedifferentiation of Vicia faba root cells, following root decapitation, induced amplification of GC-rich satellite DNA (Natali et al. 1986). Gense, 1980, demonstrated that wounding or TMV-infection of tobacco leaves induced the synthesis of AT- rich satellite DNA. Low-temperature acclimation induced changes in hybridization with rDNA probe of Southern transfers of DNA isolated from leaves of Brassica napus and of Descurainia sophia

(Laroche et al. 1992). Evidence that the variability of the DNA content in sunflowers may be of importance in buffering the effects of changing environmental conditions (Natali et al., 1993), and be influenced by the light quality and/or quantity (Johnston et al, 1996) has recently been presented. Sorghum bicolor plants have been adapted to salinity [Amzallag et al. 1990; Amzallag et al. 1993] and that adaptation can be passed onto subsequent generations [Lerner and Amzallag, personal communication]. The work of Durrant, Cullis, and coworkers, during the past 30 years, has demonstrated that specific concentration of certain mineral nutrients, or temperature, induced plants to be larger, while other concentrations induced plants to be smaller, in the flax genotype Stormont Cirrus (Durrant 1962, 1971; Cullis, 1981, 1990). These phenotypic changes are transmitted to the offspring that remain stably altered for a large number of generations.

The quantitative DNA variations induced by the environment are generally of transitory character. Under determined conditions, however, they can be of a permanent character and hence, bound to be transmitted to the following generations (Cullis 1981, 1990).

IV. Induced Heritable Chances in Flax

The environmental induction of heritable changes in the inbred flax variety Stormont Cirrus is one well described plant system in which genome alterations occur in response to specific, defined environments. Growth of Stormont Cirrus, termed Plastic (Pl), in different fertilizer combination, or under different temperature regimes, can result in phenotypic and genotypic differences in the first generation progeny. These differences are inherited by the progeny in subsequent generations obtained by self-fertilization [Durrant 1962, Cullis 1977, 1981]. While much remains to be learned about the induction process in flax, there are four established aspects. First, Stormont Cirrus is a predominantly self-fertilizing plant since anther dehiscence and pollination occur during flower opening. Second, nearly all of the seeds planted grow under the inducing conditions and can contribute to the next generation [Durrant 1962]. Thus it is extremely unlikely that any form of selection, in the conventional sense, from a heterogeneous population of plants, is the causative agent for the observed change. Third, all of the self fertilized progeny from all the individuals growing in a specific environment were identical, but different from all the progeny of individuals grown in a different environment. Fourth, the induction of the changes has been repeated with Pl, resulting in the appearance of similar phenotypic, biochemical and molecular changes [Durrant 1962; Cullis 1977, 1981].

IV A. Characterization of the Induction

The induction of heritable changes in flax in response to various nutrient regimes was first described by Durrant [1958, 1962]. Seeds from an inbred line were given different combinations of nitrogen, phosphorus, potassium and calcium for a single generation and allowed to self. These seeds were then grown in a common environment and there were differences in plant weight and height associated with the growth regime in the previous generation. The seed from the treated plants were grown under either the same conditions to which the previous generation was subjected, or a different regime. Again the plants were self fertilized and seed from the various individuals were grown in a common environment. As before, there were differences between the families, but in some cases, the phenotype was correlated with the treatment received two generations earlier, not that of the immediately preceding cycle. These families then remained stably altered for a large number of generations, irrespective of the subsequent growth conditions. However, it was only a minority of the conditions used which resulted in the stable alteration of the lines, and in general, if any phenotypic modifications were observed they were correlated with the conditions of growth in the immediately preceding generation.

The lines in which stable changes were observed were termed genotrophs and had a number of characteristics. All the individuals of a given genotroph, generally all the progeny of a single inbred plant which had undergone an induction cycle, were identical to each other. However, each of the extremes, termed the large and small genotrophs, differed from each other and the line from which they were derived (termed plastic, Pl) in a number of characteristics. The original line was taller than either of the genotrophs, but the large type was much more branched. The small type was shorter than Pl, and even less branched. Another characteristic of the induction of heritable changes was the reproducibility of the phenomenon. The conditions used were not lethal and all, or most (>90%), of the seeds planted and grown survived and produced seed. When, either many seed from a single individual plant, or seed from different plants which have been grown under the same inducing conditions, are grown in a common environment, all are very similar to each other. Thus the outstanding characteristic of the environmental induction of heritable changes in flax is that all the progeny from an individual inbred plant that has been grown in an inducing environment are similar to one another, and to all the progeny from any other plant which has gone through the same regime, but were different from the original line and other genotrophs, the latter having been produced by another set of environmental treatments. The genotrophs differed from each other and the original line from which they were derived in a number of characters. These included plant weight, height [Durrant 1962], total nuclear DNA content as determined by Feulgen staining [Evans et al. 1966], hair number on the false septa of the seed capsules [Durrant a Nicholas 1970], and the isozyme band patterns for peroxidase and acid phosphatase [Fieldes & Tyson 1973].

The alteration of the peroxidase isozyme band pattern was controlled at a single locus, with a dominant and a recessive allele. This gene showed the expected dominance in the F1 and a 3:1 segregation of the dominant:recessive pattern in the F2 in crosses between genotrophs [Cullis 1979].

An important note in the alterations seen in both the peroxidase isozyme pattern and the seed capsule septa hair number is that the change seen in the genotrophs was from a dominant character to a recessive one. However, the parental line gave no indication that it was heterozygous for either of these characters. Since the recessive phenotype shows up in the first generation after the induction treatment, the change must have taken place in both the members of the homologous pair of chromosomes. Whether this change occurs by a conversion event after one of the sites has altered, or by two independent events is not known.

The appearance of homozygosity immediately after the inducing generation has been observed for all the characteristics so far investigated.

Another question is whether or not the altered phenotypes would confer an advantage to the genotrophs? This has not been specifically tested, although the characteristics of the extreme genotrophs (large and small) when grown together under the inducing conditions suggest that the phenotypic alterations may be adaptive. When both the large and small genotrophs are grown under conditions that produced the large genotroph, both lines grow well. However, because of its larger habit, plant weight and number of seed bearing branches, the large genotroph produced about ten times more seed than the small genotroph. When both genotrophs were grown under conditions producing the small type, then a different result was observed. Under these conditions, the small genotroph grows well, forms a short upright plant and produces adequate seed. The large genotroph does not grow as well, fails to form a rigid stem and lodges (falls over). It yields little seed under these conditions (Cullis, unpublished data). Therefore, on a test of the two types grown under the conditions in which they were generated, each would appear to be more successful in their respective inducing environment. This data does not prove that the induced changes are adaptive, but is certainly an indication of such.

One of the characteristics of the genotrophs is that they are stable in subsequent generations. A mechanism, which only allows a single induction event, would not be one that would have a great deal of evolutionary utility. However, an important note to stress again is that only a subset of the inducing conditions generate stable genotrophs, with most of the progeny maintaining their ability to respond repeatedly. Therefore, in general, heritable changes cannot be accompanied by the loss of the ability to subsequently respond, an important property if the mechanism is to be useful in an evolutionary sense. It must also be noted that some of the genotrophs were also still able to alter their genome, only not in response to the original inducing conditions. Thus in the stable genotrophs two separable events must have taken place. One set of events is the changes which result in an altered phenotype, including the DNA variations. The other set is the loss of the ability to respond to the particular environmental stimuli by which the changes were generated. The fact that some of the genotrophs were still able to respond to other environmental stimuli, is consistent with the notion of multiple sensing and/or modification systems.

IV B. Nuclear DNA Variation

The changes in the nuclear DNA associated with the environmentally induced heritable changes have been extensively characterized. The characterization has included renaturation analysis (Cullis 1981; 1983), characterization of the ribosomal RNA genes (Goldsbrough et al. 1981; Cullis & Creissen 1987; Schneeberger et al. 1989; Schneeberger and Cullis 1991), the description of an insertion sequence (Schneeberger 1992), and the use of random amplified polymorphic DNAs (RAPDs) (Song, Swami and Cullis, in prep).

IV B 1. Characterization of a Labile Fraction of the Genome.

Flax has a genome of 1.5 x 10^8 base pairs, distributed among 15 pairs of approximately equal sized chromosomes (Cullis, 1980). Nuclear DNA measurements have shown that the nuclear DNA content of the large and small genotrophs differs by about 15%. However, meiotic analysis of crosses between a large and a small genotroph did not indicate any large chromosomal anomalies (Evans, 1968). Therefore, the DNA differences must be distributed approximately equally over most, or all, of the chromosomes. This widespread distribution of the differences has been confirmed by mapping some of the RAPD polymorphisms between a large and a small genotroph (Song, Swami and Cullis, in prep).

The sequences shown to vary after induced heritable changes span the whole range from highly repetitive to low copy number. The highly repetitive sequence fraction was shown to vary quantitatively between the genotrophs using slot blots with cloned representatives from all the highly repetitive sequence families (Cullis and Cleary, 1986). A particular representative of the highly repetitive sequences, the 5S ribosomal RNA sequences, was shown to be part of the variable component. However, a specific set of restriction fragment length polymorphisms (RFLPs) was characterized in a subset of this gene family (Schneeberger and Cullis, 1991). The interesting part of these results was that identical RFLPs for all restriction enzymes tested were observed in four independent small types produced from independent induction experiments (Schneeberger and Cullis, 1991).

The magnitude of the DNA differences between the genotrophs suggested that a particularly fruitful search could be made using random 10mer primer in a PCR reaction to develop RAPD markers (Williams et al, 1990). A series of eleven genotrophs and the original variety were characterized using random amplified polymorphic DNAs (RAPDs). Over 300 random primers were used, which generated 223 polymorphic fragments (Song, Swami and Cullis, in prep). A subset of the primers which gave polymorphisms between the genotrophs, was also used to compare the genomic organization between the genotrophs and callus tissue to probe any connection between the induced heritable changes and somaclonal variation (Linden, Hehr and Cullis in prep). The result of one of these experiments is shown in Figure 1. It can be seen that there are specific differences between Pl and some of the genotrophs, as well as differences between leaf and callus DNAs. Twenty of the polymorphic RAPD bands have been characterized as to their repetitive frequency in the flax genome. Highly repetitive tandem arrays, dispersed repetitive sequences and low copy number sequences are all represented among the polymorphic bands.

A comparison of the RAPD polymorphisms between Pl and the genotrophs indicated that there is a possible labile set of loci, defined by the inducing stress. A group of three genotrophs, LH, Sh and L6, comprising one large and two small types, were all induced by temperature stress. There were 158 RAPD polymorphisms identified between at least one of this group and Pl. However, these three genotrophs had 126 polymorphisms in common, that is, all three had the same amplification pattern as each other, while Pl had a diffeent patern. All of the other groupings of genotrophs, which included lines generated by different stresses, had fewer than 50% of the polymorphisms in common. These data are again consistent with the notion that the regions of the genome that are susceptible to rearrangement under stress are delineated by the physiological status of the cell.

A group of fourteen RAPDs were followed in a cross between two genotrophs. No close linkages were found amongst this group of RAPD markers (Song, Swami and Cullis, in prep). This observation is again consistent with the earlier evidence that the genomic alterations are spread throughout the genome, and not all localized in a particular small number of chromosomal regions.

IV B 2. An Insertion-like Sequence in Flax (LIS-1)

A single copy RFLP between Pl and seven of the genotrophs was shown to be due to the presence of a 5.8kb insertion sequence named LIS-1 (Schneeberger 1992). The RFLP was identified initially in a screen of single copy sequences adjacent to one of the 5SrDNA arrays. However, this RFLP is not linked to that set of 5SRNA genes.

LIS-1 was shown to be inserted into a 3.7kb EcoRI fragment present in Pl. LIS-1 shows a target site duplication. The sequence TCC is present at the right and left hand borders of the insertion. The sequencing around the insertion site did not reveal any typical terminal inverted repeats. There are several inverted repeats of the sequence TCC (GAA) present in the right most terminus of LIS-1 in addition to the surrounding Pl target sequence. The significance of these sequences is not known but the may be relics of terminal repeats or of earlier insertion and excision events.

All of the data on the DNA differences between the genotrophs and Pl are consistent with the notion that specific alterations are taking place. The same polymorphism, whether it be an RFLP in the 5S ribosomal RNA genes, the presence of an insertion of particular structure or placement, or the occurrence of RAPD polymorphisms, arises again and again. Insufficient data has been generated to conclude whether a common mechanism is responsible for all the RAPD polymorphisms, although at least on of them appears to be caused by an insertion sequence which is distinct from LIS-1. A continued characterization of the molecular differences at these polymorphic loci will help elucidate the molecular mechanisms involved in generating these polymorphisms.

IV C. Mapping the genetic loci controlling the induced variation.

Are all the stress responses controlled by a single mechanism? The data from the environmental induction of heritable changes in flax would suggest that there may be multiple mechanisms. The stable genotrophs were defined by their lack of response to the alternative fertilizer treatments. However, one of them, the large genotroph, was subsequently altered to generate a small type (termed L6). This change was mediated by a low night temperature over the early development for a number of generations (Durrant 1971). Similarly, genomic alterations have been observed in tissue cultured material from all of the genotrophs (Cullis and Cleary, 1986; Linden, Hehr and Cullis, in prep). Therefore, it appears that the ability to respond to different stresses may be separable. However, the original Pl line has been the one in which genomic alterations have been most frequent. Therefore, the stable genotrophs must have an altered ability to respond to stress. Whether this altered response is by a modification of the system by which the changes are generated, or by a variation in the stress sensing system is not yet clear. It will be important to characterize the type of genomic change occurring in response to various stresses to help to understand the relationship between the various steps in the process. However, at first glance, it would appear to be a modification of the sensing system rather than the genomic remodeling that is altered in the stable genotrophs. The evidence for this is that the same types of RAPD polymorphisms are observed for all three stress responses, namely response to nutrients, temperature or tissue culture. Eight of the sixty-four primers, which showed variation among the genotrophs, also gave polymorphisms when used to amplify leaf and callus DNA from nine genotrophs (Linden, Hehr and Cullis, in prep). There were also a large number of common polymorphisms between genotrophs generated by nutrient and temperature treatments (Song, Swami and Cullis, in prep). Therefore, the best fit to the data is of a number of independent stress sensing pathways, each of which can independently activate a single genomic restructuring pathway.

V. Conclusions.

How well does the characterization of environmentally induced heritable changes in flax fit the criteria developed earlier for being an appropriate model to study the influence of the environment on the generation of useful genomic variation?

Flax is basically an inbreeder, so over an extended period the lines generated should be generally homozygous. In keeping with the ideas of survival of the fittest, such a system would tend towards uniformity over an extended time period in a relatively stable environment. Therefore, little variation will be present to provide the raw material to be sekected in the event of perturbations to the environment. Such a system is likely to be an evolutionary dead-end. Therefore, the occurrence of a mechanism by which variation can be generated in response to a perturbation, would in a teleological argument, make sense.

The genomic alterations in flax appear to be restricted to a subset of the genome. Responses to three types of stress all appear to restructure a subset of the genome, thereby raising the possibility of generating variation without causing great lethality. If the system was simply to raise the general mutation rate, then a large number of deleterious mutations are likely to arise which have no chance of being adaptively beneficial. However, this does not imply that a flax plant “knows” what mutations will be advantageous, simply that there is a subset of the genome which can be reorganized and which will generate phenotypic diversity without a high lethality. The resulting variants are then tested in the stress environment, and those that are better adapted are likely to take over the meristem and be propagated into the next generation. In this scheme, there needs to be a selection at the cell level within the meristem among variants generated in response to stress, which then contribute to the next generation. Provided the genomic restructuring does not affect either the restructuring or the sensing mechanisms, the organism can undergo an unlimited number of cycles of response.

At this stage there is no evidence as to how these compartments of the genome are differentiated. However, examples abound of the recognition and selection of particular sections of the genome. In mammals, parts of the genome are imprinted to ensure orderly development of the embryo (Jaenisch, 1997). Gene silencing by transgenes is another example of particular regions being selectively modified. An understanding of the distribution of the labile sequences, their chromosomal environments and their methylation state may shed some light on how they are identified.

Insufficient detail about the nature of the genomic modifications makes any speculation concerning molecular mechanisms premature. An attractive hypothesis involves the activation of transposable elements, a ubiquitous force in genomic restructuring. Transposable elements may play a dual role in these phenomena, both causing “genomic stress” on their movement, as well as being agents of generating diversity on movement around the genome. However, the flax insertion sequence does not fit into any of the typical transposon families previously identified since it has a specific site of insertion and the absence of terminal repeats. In addition there is little previous evidence for transposable elements in flax. However, a characterization of modified loci should shed considerable light on the types of molecular mechanisms that effectively restructure the flax genome.

Finally, the genetic control of the ability to respond to environmental challenges still needs to be characterized. The use of crosses between stable (at least for the environmental challenges to be administered) and responsive lines will allow the inheritance of the ability to respond to be genetically mapped. Although an arduous task, these loci need to be isolated and characterized. Once they have been identified, analogous loci in other species can be sought and the generality of the phenomenon, most spectacularly demonstrated in flax, can be assessed.

Genotypic and phenotypic variability are greatest under conditions of environmental stress. How, or if, the environment plays a role in the generation of this variability is of great importance, and evidence is accumulating for a direct role. The general conclusion to be drawn from these studies is that, in the presence of an inducible variation generator, any significant modification in the environment to which an organism has become adapted should increase the genomic variability. However, since there is also a selection operating on the increased variability, the increased genomic variability may not be reflected in an immediate increase in phenotypic variability among the progeny. The general applicability of this conclusion will be easier to determine when the mechanisms are clearer, and the genetic control characterized. Then it will be possible to detect the presence or absence of specific activities relating to the environmental induction of genetic change in a wide variety of species

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Figure 1.

Polymerase chain reaction (PCR) amplification of DNAs isolated from leaves (a - Pl, c - L1, e - L3, g - L6, i - LH, k - S1, m - S3, o - S6, q - Sh) and callus tissue (b - Pl, d - L1, f - L3, h - L6, j - LH, l - S1, n - S3, p - S6, r - Sh) using a 10- base primer (TCGCAGAACG). The PCR reaction conditions were as described in Aldrich and Cullis (1993). The extreme right and left-hand lanes are molecular weight markers.