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GENETIC ENGINEERING AND MUTATION BREEDING FOR TOLERANCE TO ABIOTIC AND BIOTIC STRESSES: SCIENCE, TECHNOLOGY AND SAFETY

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Summary. Genetic engineering is often presented as a one-step, rapid solution to the improvement of stress tolerance in plants. While it may benefit from but not necessitate, the requirement for backcrossing for gene introgression, it does not reduce the requirement for field trials. The introduction of herbicide and pest resistance into plants has been an applied success and these characteristic singly and combined still dominate the applications for trials permits. However, they do not represent the complexity of the challenge for engineering for durable biotic and abiotic stress resistance. The dissection of stress responses in plants is showing high levels of complexity and redundancy at the perception, signalling and expression levels with cross regulation (cross talk) between stress pathways and over lapping functions between stress metabolites and stress proteins in different stresses. Stress metabolite engineering is complicated by a lack of knowledge of pathways and their regulation and poses the question of how metabolite fluxes between shared pathways can be controlled, indeed redundant homeostatic mechanisms may be discovered. In the case of stress proteins, there are limits on genes of known function that are available but perhaps more importantly is the issue of whether single or multiple gene transformations will confer stable resistance. There are technical limitations in multigene engineering but more important is the global character of stress responses. Some have argued that the solution lies in engineering for constitutive expression of stress pathways but this may confer a yield penalty and plants have evolved to rely on inducible responses. There is also the complication that at least some plant stress pathways are subject to reciprocal regulation i.e. the salicylic acid pathway for pathogen resistance may suppress the jasmonic acid pathway for pest resis-

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tance. Further there is evidence that different pathogens may induce different stress responses in the same host implying a higher level of stress interpretation, customisation of stress responses. Some stress metabolites and stress proteins are anti-nutritional and allergenic respectively. This poses a potential risk to consumers where these are used as the basis of transgenic resistance or where their expression is increased due to the presence of transgenes.

Key words: food safety, pleiotropy, stress cross tolerance, substantial equivalence, transgenic plants

Abbreviations: ACC – aminocyclopropane-1-carboxylic acid; AMPs – antimicrobial proteins; PR – proteins, pathogenesis related proteins; ROS – reactive oxygen species

Introduction

The genetic yield potential of a crop variety is limited by the environment, including abiotic and biotic stresses (Oerke, 1999). The effects of the latter can be ameliorated by the application of fertilizers, herbicides, pesticides and by irrigation etc., as appropriate. However, such treatments have an economic cost which may not be affordable, or in the case of increased pesticide application, meet increasing consumer resistance. Globally, yield potential is being affected in many regions by increasing soil salinity a consequence of intensification of horticultural production under irrigation. There is also the emerging problem of global warming. In Europe, for example, it is predicted that climate change will be, overall, beneficial for the north and disadvantageous for the south (Alexandrov and Hoogenboom, 2000; Fumagalli et al., 2001; Olesen and Bindi, 2001). To exploit the improved climate in northern Europe it will be necessary to develop adapted varieties and/or increase pesticide inputs. Another pressure on crop yields is the need to increase food supply for an expanding global population which in many regions is already malnourished (www.fao.com; Batten, 1999). While world population growth figures have been revised downwards due to a declining birth rate in developed countries and disease pandemics in some less developed regions, food deficits are predicted to increase (www.fao.org). These deficits are compounded in e.g. Africa, by desertification.

Conventional plant breeding has been responsible for the very significant increases in the genetic yield potential of crop plants and to increasing abiotic and biotic stress resistance with support from agronomists, plant stress physiologists and plant pathologists. While crop yields have increased progressively (“the Green Revolution”), this has been dependent on fertilizer application and heavy reliance on herbicides and pesticides. In intensified agriculture, yield is attained at considerable economic and arguably high environmental cost. In spite of the rigorous mandatory toxicological

screening of pesticides, there is increasing consumer resistance to modern intensive agriculture which is viewed subjectively as "non-sustainable". This has been reflected in an increase in "organic crop" (pesticide-independent) production but the latter is subject to consumer price resistance (Rigby et al., 2001).

The principles and methodology of genetic engineering of plants have been validated for traits such as herbicide tolerance (Paoletti and Pimental, 1995). A platform has been established for plant improvement based on rapid advances in the understanding of the plant genome from the *Arabidopsis* model study and of the eukaryote genome in general, from studies on the yeast and the human genomes. This is underpinned by developments in methods for genomic analysis e.g differential arrays. Conventional plant breeding will continue to be important *per se* and in the context of introgressing genetic change achieved by genetic engineering. Genetic engineering, uniquely, has the capability to introduce genes from any origin, singly or sequentially (gene pyramiding) to potentially improve existing elite gene combinations whether in infertile crops like banana or in heterozygous genotypes like potato where the problem of gene segregation has frustrated efforts at improvement due to the requirement for so many characters to be retained in varietal improvement. Genetic engineering has met with strong consumer resistance, particularly in Europe. Arguably due to a failure in science communication where those presenting the arguments for genetic engineering were not sensitive to the general concerns that European consumers have regarding food safety.

In addition to the goal of further increasing genetic crop yield, is that of increasing the attainable yield at existing or at reduced production cost by reducing fertiliser application and by improving stress tolerance, that is by reducing agrochemical usage. There is also the goal of improving plant quality by engineering for improvement in beneficial nutrients and neutraceutical ("functional food") composition and by using plants to deliver vaccines (Daniell et al., 2001; Charglegue et al., 2001; Walmsley and Arntzen, 2000). Currently, we are at the "centre of origin" of plant genetic engineering. Many approaches are being proposed and evaluated. These include metabolome engineering, proteome engineering (Grover et al., 1999), attempts to alter gene expression through engineering of signal transduction pathways and of transcription factors (Cao et al., 1998). These targets are being driven by the genomic model studies referred to above and by the recognition that the chromosome structure, genomic programming and genomic responses to stress are highly conserved in eukaryotes allowing transfer of knowledge from the human and yeast models to higher plants. Here, an overview will be presented of genomic, proteomic and metabolomic responses to stress. This will be followed by a review of strategies reported for the engineering of tolerance to stress in plants. In so far as many of these strategies involve attempts to up- or down-regulate constitutive or induced pathways, comparison will be made with mutation strategies to achieve the same objectives. The goal in genetic engineering of plants is to produce stable improved lines and so the trialling requirements for

transformed lines will be discussed. In the case of food crops, the improved lines must be wholesome, that is free from allergens and anti-nutrients (Conner and Jacobs, 1999; Hollingsworth et al., 2002). The principles for evaluating the wholesomeness of transgenic plants will be considered Charles et al., 2002; Novak and Haslberger, 2000).

Stress responses in plants

Stress is defined as an influence that is outside the normal range of homeostatic control in a given genotype (Lerner, 1999a). Where a stress tolerance is exceeded, response mechanisms are activated (Lerner, 1999b). Where the stress is controlled a new physiological state is established, homeostasis is re-established. When the stress is removed the plant may return to the original state or a new physiological state may be established (Amzallag, 1999). There are well characterised specific responses to abiotic and biotic stresses, however, it appears that commonly if not universally, multiple stress defence pathways are induced (Fig. 1; Inze and Van Montagu, 2002).

In the study of stress, researchers historically have tended to specialise in the study of specific stresses which has resulted in a narrow perspective on this phenomenon (Lerner, 1999a). Current elucidation of stress responses suggest that there is cross induction ("cross talk") in the stress signalling pathways between the specific stress responses and that plants may respond to stress perception by an initial global response ("stress cross signalling") involving initially activation of a global stress response with elements of oxidative, a "heat shock" and a "pathogenesis" stress responses and followed by a more specific or customised stress response specific to the cues abiotic or biotic perceived (Genoud and Metraux, 1999; Netting, 2000; Bartels, 2001; Pieterse et al., 2001).

Both non-specific (activated by reactive oxygen species) and specific e.g osmotic, stress responses depend on perception of the stress, signal transduction, activation of transcription factors and gene expression (Krauss, 2001). The production of the stress response include the production and/or up-regulation of metabolic pathways resulting in changes in the metabolome e.g. the formation of compatible compounds (antioxidants, phytoalexins, protein protectants, cryoprotectants (Bohnert and Shen, 1999) and in the proteome, increased expression of constitutive defence proteins, production of novel defence proteins and protein chaperones (Cushman and Bohnert, 2000; Grover et al., 1999).

Oxidative stress

ROS are generated in the mitochondrion and chloroplast via the electron transport chain and converted by superoxide dismutase to hydrogen peroxide. Hydrogen peroxide is generated in photorespiratrion in the peroxisomes and from fatty acid break-

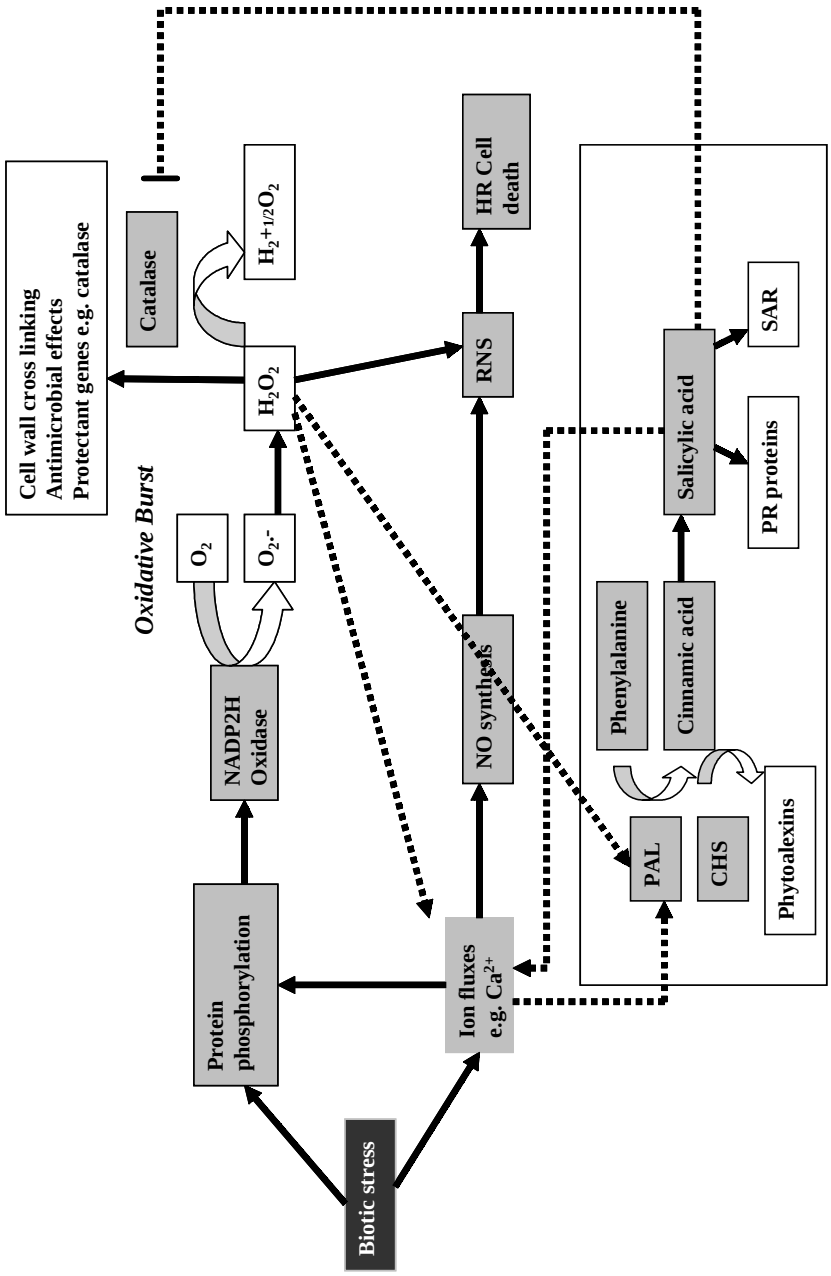

Fig. 1. The global (phase 1) response to pathogen attack initiated by nitrous oxide and the oxidative burst and supported by ion fluxes, phosphorylation and salicylic acid signalling result in increased expression of antimicrobial compounds, cell wall cross linking, increase in oxidative stress protection and activation of synthesis of phytoalexins and pathogenesis-related (PR) proteins and systemic acquired resistance (SAR). A high stress responses may induce the hypersensitive (phase 2) response. PAL, phenylalanine ammonia lyase; CHS chalcone synthase. (Based on Denny, 2002).

down in the glyoxysomes. Each of the cellular compartments has scavenging mechanisms based on e.g. conversion of superoxide radicle to hydrogen peroxide which is passed through the ascorbate glutathione cycle (Van Breusegem et al., 2000)

The oxidative stress response involves up-regulation of antioxidant synthesis including ascorbic acid, glutathione, flavonoids. It also involves up-regulation of the production of antioxidant enzyme production including aldose-aldolase reductase, catalase, superoxide dismutase, ascorbate peroxidase. Cell cycle shut down may also occur depending on the severity of the oxidative stress. The strategy is aimed at minimizing ROS effects on protein inactivation, loss of enzyme and membrane function by breaking down the ROS, by inundating the cytoplasm with antioxidants and by coating the proteins with a shell of protectant molecules ("compatible solutes"). The risk of mutation is reduced by shutting down the cell cycle and by increasing the enzymes of DNA repair. Oxidative stress in plants has recently been reviewed by Inze and Van Montagu (2002).

Hydrogen peroxide is also involved in signalling both locally and to neighbouring cells heat, cold, pathogen and other stresses as the initial stage in the global response strategy. Ozone, for example, activates the ethylene, hydrogen peroxide and salicylic acid signal transduction pathways (Langebartels et al., 2002). ROS stress signalling involves signal transduction by cytosolic Ca^{2+} and downstream participation of the mitogen-activated protein kinase cascade (MAPK) (Nurnberger and Scheel, 2001).

Abiotic stresses

Abiotic stress responses in general involve the up-regulation or de-repression of the synthesis of protective proteins including protein chaperones and enzymes (Lerner, 1999b). There may also be an increase in compatible metabolites (Bohnert and Shen, 1999). Cell division and "house-keeping" functions may be slowed or shut down depending on the severity and type of stress (Guy, 1999; Taiz and Zeigler, 2002).

Heat shock (HS) is well characterised in humans and yeast. Studies on plants confirm that the basic mechanisms are highly conserved (Guy, 1999). It has been hypothesised that changes in membrane fluidity may act as cellular thermometers (Browse and Zhanguo, 2001). There is also evidence from research on cyanobacteria that membrane-bound histidine kinases and other proteins may be involved in temperature sensing. The eukaryote's response to heat stress is to up-regulate the production of heat shock proteins (Fig. 2; Guy, 1999). While some heat shock proteins (Hsps) are known to be produced developmentally e.g. in over-wintering buds, some e.g. Hsp90 are associated with exposure to temperatures of approximately 10 ^{o}C above ambient. These proteins act as molecular chaperones stabilising the confirmation of cellular protein; some act as proteases hydrolysing inactivated proteins. Hsp production is regulated by heat shock transcription factors (HSFs) which are present in uninduced cells (Lam and Meisel, 1999; Fig. 2). Class B HSFs repress transcription to modulate the HS res-

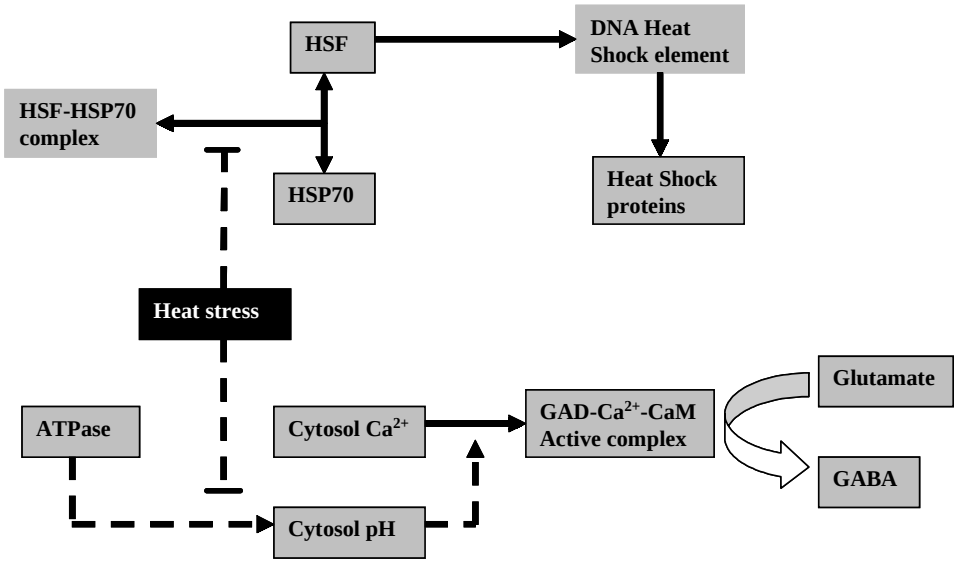

Fig. 2. The activation of the heat shock response. Under non-stressed conditions the heat shock factor (HSF) binds to heat shock protein 70 (HSP70). On heat shock, the HSF-HSP70 complex dissociates and trimers of the HSF bind to the heat shocjk elements in the promoter of the HSP genes and activate HSP mRNA synthesis. Heat shock also causes a reduction in the pH of the cytosol possibly by inhibiting proton-pumping ATPases, with associated changes in calcium influx into or efflux from the cytosol resulting in increased cytosolic calcium. The latter activates calmodulin (CaM) which binds to and activates glutamate decarboxylase (GAD) which converts glutamate to the compatible solute g-amino-butyric acid (Based on Taiz and Zeigler, 2002).

ponse while class A HSFs promote transcription. HS gene expression can increase by 200-fold by temperature stress when there is a concomitant reduction in the expression of housekeeping genes (Czarnecka-Verner et al., 2000).

Aside from Hsp production, there is evidence that heat stress results in an increase in cytoplasmic calcium that combines with calmodulin to activate glutamate decarboxylase (GAD) leading to increased accumulation of 4-aminobutyric acid (GABA) which occurs in a number of stress responses (Fig. 2; Evenas et al., 1998; Snedden and Fromm, 1999). GABA is one of many compatible solutes whose production is increased in parallel with the proteomic changes (Kinnersley, 2000).

Drought, salt and cold stresses are associated with changes in the genome, proteome and metabolome (Fig. 3; Lerner, 1999b; Cherry et al., 2000) While there are some elements of the response that are unique to the specific stress, there are also common responses, arguably related to the common underlying osmotic stress component. The salt overly sensitive (SOS) response involves changes in ion transporters and is an ion homeostasis response to salt stress that has been relatively well characterised. Like the heat stress response, the SOS pathway involves both up and down regulatory controls. There is evidence that calcium signalling and activation of specific mitogen-

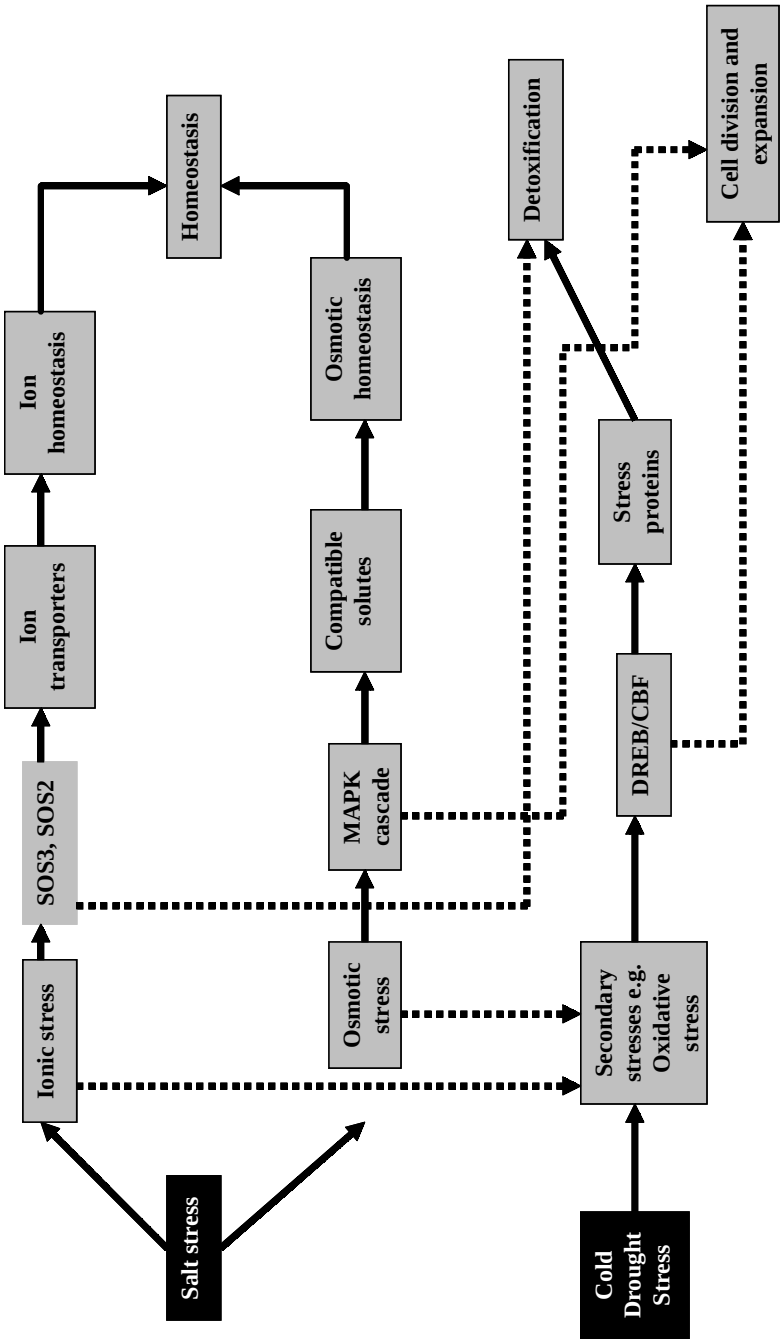

Fig. 3. The salt stress response showing proteomic and metabolomic response components. The figure also shows the common and discrete elements in the salt versus cold and drought stress responses. Redundancy is shown in that both the MAPK cascade and the DREB/CBF genes influence cell division and expansion. (Based on Zhu, 2001).

activated protein (MAP) kinases is involved in osmolyte (compatible solute) accumulation e.g. of glycine betaine. The cold response, unlike heat stress, is very diverse in plants indicating that several responses may have developed independently in plant evolution (Guy, 1999). Among the specific elements of the response is the involvement of the dehydration response element (DRE) factors. Plants expressing these factors accumulate proline and sugars which correlate with increased cold tolerance (Browse and Zhanguo, 2001).

Waterlogging is associated with anoxia in the roots which results in the inhibition of ACC oxidase. ACC is synthesised in waterlogged roots and transported via the xylem to the shoots where it is converted to ethylene which induces epinasty (Voesenek and Blom, 1999).

Light stress occurs when the rate of photon absorption exceeds the rate of photon utilization. Under these circumstances ROS including hydrogen peroxide, superoxide and hydroxyl radicals are formed (Foyer, 2002). The main defences are the alternative oxidase system (Godde, 1999) and the xanthophylls cycle (Demmig-Adams and Adams 1996). Ozone and UV-damage results in the production of ROS which are broken down by the cell redox enzyme system with the involvement of antioxidant molecules (Melis, 1999; Langebartels et al., 2002).

Biotic stress responses

Specific responses to biological stresses involve the induction of antimicrobial proteins and phytoalexins (Figs. 4 and 5; Slusarenko et al., 2000; Boller and Keen, 2000). Following stress perception, stress signal transduction takes place (Bolwell, 1999; Ellis et al., 2000; Heath 2000; Nurnberger and Scheel, 2001). In the case of necrotising pathogens this may lead to a local hypersensitive response and a systemic induction of resistance (SAR) with the production of AMPs (Broekaert et al., 2000) and phytoalexins (Mansfield, 2000). Non-necrotising pathogens, biocontol organisms and insects may induce systemic resistance whose basis is uncertain (Van Loon, 2000). SAR involves ethylene and salicyclic acid as signalling molecules whereas induced systemic resistance (ISR) involves jasmonic acid. There is known to be cross talk between the respective signalling systems with suppression of one by the other leading to cross susceptibility between pathogens and pests in some cases (Fig. 4; Pieterse et al., 2001).

Plant hormones in stress responses

Plant hormones are involved in stress signalling and stress response coordination (Itai, 1999). ABA is involved in signalling heat stress, flooding and drought, ethylene (from ACC export) also signals flooding (Itai, 1999; Taiz and Zeigler, 2002). It has been hypothesised that hormones play the key role in an hierarchical strategy coordinating

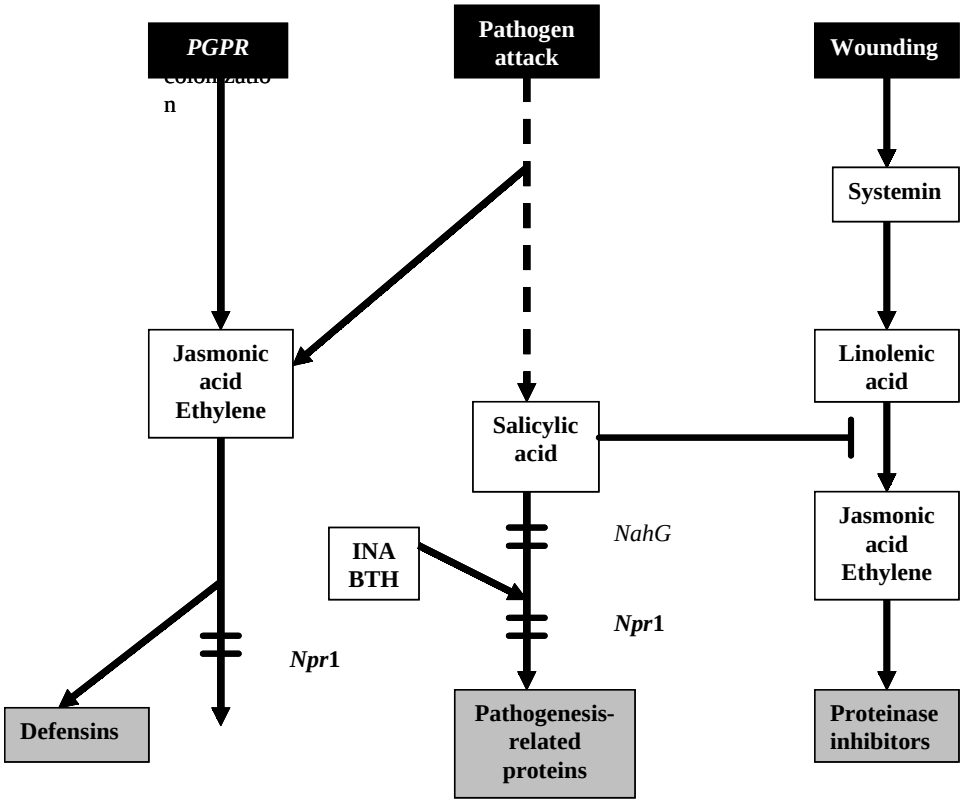

Fig. 4. Systemic stress signalling pathways for induced systemic resistance (ISR) induced by plant growth promoting rhizobacteria (PGPR); systemic acquired resistance (SAR) induced by pathogens and induced pest resistance induced by wounding. Stages blocked by gene mutations are indicated by double crossed lines. Salicylic acid inhibits the wound response pathway and ISR and SAR have common gene and signalling components. Also shown are the points at which the signalling compound analogues INA and BTH activate pathogenesis-related protein synthesis (Based on Van Loon, 1999). INA, 2,6-dichloroisonicotinic acid BTH, thiadiazole-7-carbothioic acid-S-methyl ester.

plant (abiotic) stress responses (see Itai, 1999). In support of this hypothesis is the link between the promotive effects of auxin on ethylene synthesis (via ACC synthase; Fig. 6) which triggers increased ABA synthesis. However, this hypothesis is complicated by the recent findings that carbon homeostasis is tightly coupled to sugar signalling pathways (Gazzarrini and McCourt, 2001) and linkages between carbon and nitrogen sensing and signalling have also been reported (Corruzzi and Zhou, 2001).

Ethylene and jasmonic acid are local and systemic signalling compounds for biotic stresses (Slusarenko et al., 2000) and it is known that there is cross talk between these and the salicyclic acids stress pathway which may result in negative or positive interactions, arguably, to optimise the defences against a perceived pathogen or pest attack (Fig. 4; Pieterse et al., 2001).

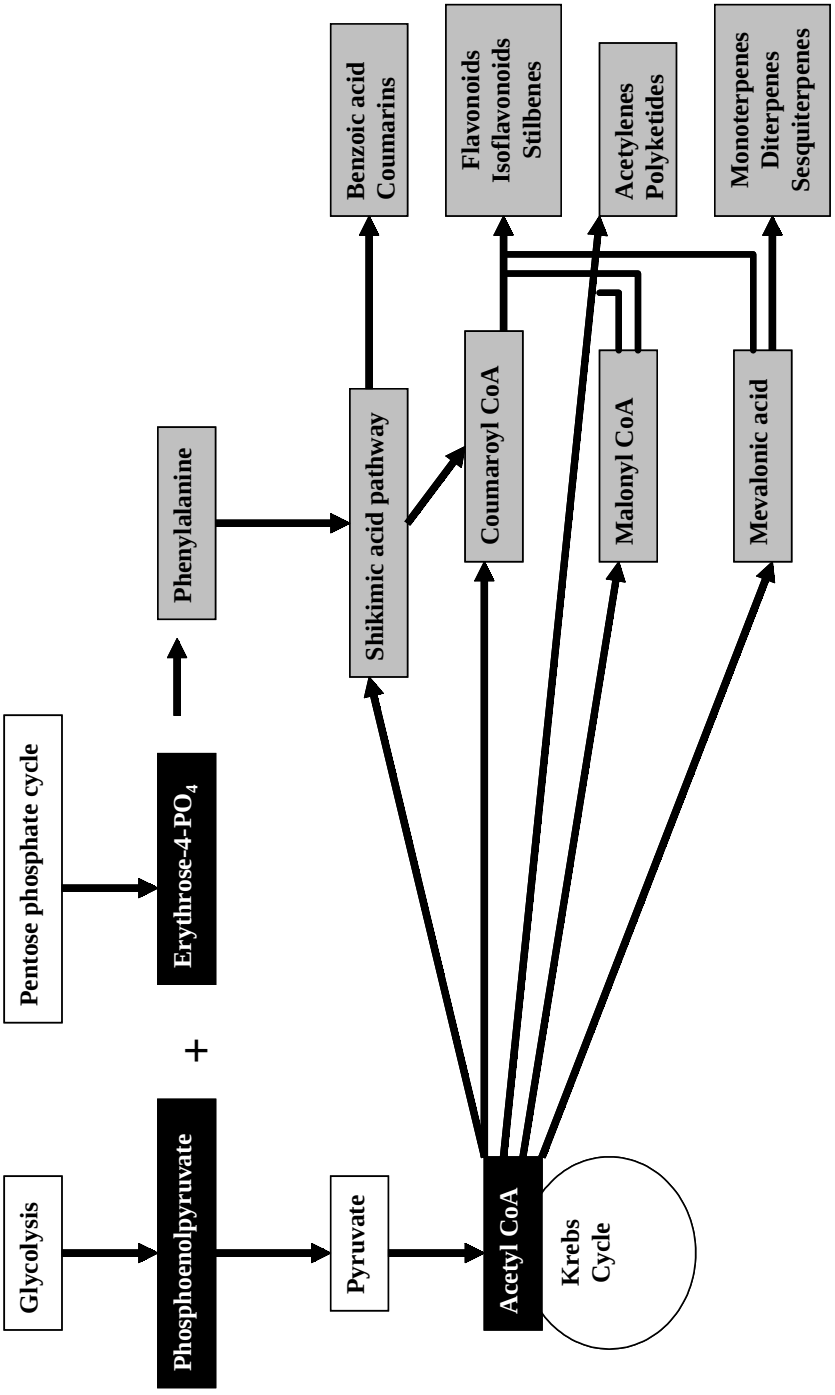

Fig. 5. Showing the complexity of pathways of phytoalexin synthesis. Activation of defense responses leads to a largescale diversion of primary metabolites into the transient up-regulated synthesis of stress metabolites via the shikimic acid, mevalonic acid and mixed pathways of biosynthesis implying complex regulation (Based on Mansfield, 1999).

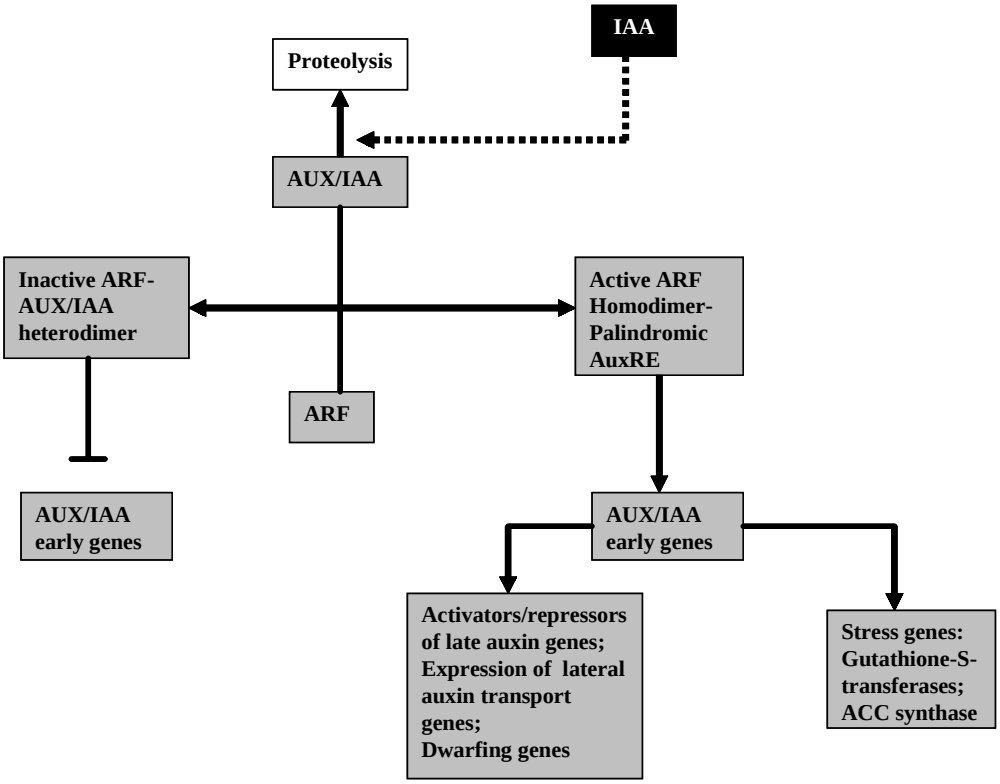

Fig. 6. The regulation of the transcription of the auxin early response genes. In the absence of auxin the transcription factor forms inactive heterodimers with the AUX/IAA protein. In the presence of auxin the AUX/IAA proteins are broken down by ubiquitin ligase and active ARF dimers are formed. These bind to the promoter activating transcription. Early genes activate the AUX/IAA transcription factors, genes affection lateral auxin transport, dwarfism genes and stress genes involved in oxidative stress protection and activation of ethylene synthesis (after Gray *et al*., 2001).

Genetic engineering

Transformation systems

In the years between 1996 and 2000 there was an increase from 4.2 to 104.7 million acres of transgenic plants grown globally (Cockburn, 2002). In terms of the selection of "viable" transgenic plants for commercial production, the foreign gene has to function in the desired way and the chosen elite variety (the transformant) should be free from pleiotropic effects. For most, if not all, of the current transformation protocols a tissue culture stage is a requirement with plants being derived either through somatic embryogenesis or organogenesis. According to Hansen and Wright (1999) there are three methods of transformation that fulfill the criteria for the establishment of trans-

genic plants i.e. protoplast transformation, *Agrobacterium tumefaciens*-mediated transformation and biolistics. These processes have been reviewed in detail in a number of publications (Christou, 1996; Hansen and Wright, 1999). The choice of transformation method will depend on (i) the special requirements (if any) e.g. in tissue culture, of the crop to be transformed; (ii) accessibility to different plant tissue; (iii) financial constraints (especially for academic laboratories that may be working on low budgets); (iv) the availability of specialized laboratory equipment; (v) patent clearance (Hansen and Wright, 1999). The most widely used method for the genetic transformation of plants is *Agrobacterium* transformation (Kumar and Fladung, 2002).

Characteristics of the transformation vector (for nuclear genome transformation)

As a starting point in any transformation system, appropriate constructs need to be made containing, in addition to the gene of interest (GOI), a selectable or screenable marker whereby putative transformants can be selected at an early stage in the regeneration process. Typical examples of selectable markers include genes which confer resistance to either antibiotics or herbicides but these, although useful in selecting for transformants, are known to reduce transformation frequency due to their inhibitory effect on the growth and regeneration of transformed cells (Zuo et al., 2002). The standard constructs for use in transformation experiments will contain (i) the GOI (either side of which is found a promoter (e.g. CaMV 35s promoter for dicotyledons or the ubiquitin promoter for the expression of genes in monocotyledons – both constitutive promoters) and a terminating sequence; (ii) a selectable marker for the selection of plant transformants and (iii) a selectable marker for the selection of bacterial transformants (usually, for example, in either *E.coli* or *Agrobacterium tumefaciens*) and two 25 bp border sequences (left and right T-DNA borders) if an *Agrobacterium*-mediated transformation system is used (Cockburn, 2002). The expression of the gene may be controlled either spatially or temporally or could be induced by a number of abiotic factors (Cockburn, 2002). Various promoters that respond to different spatial and temporal signals are under intense investigation for their potential application in the manipulation of biotic and abiotic plant stress responses. As an alternative to the two constitutive promoters described above there are other tissue specific promoters that may be used in transformation experiments, for example, for seed-specific expression the vicilin promoter from pea, the phytohemaglutinin promoter from bean and the glutenin promoter from wheat have all been used. Additionally, the α-amylase promoter has been used for the tissue specific expression of genes in the aleurone layer of cereals (Christou, 1996). The use of wound inducible promoters or those that respond to a signal from fungal pathogen invasion or ethylene inducible promoters or latex-specific promoters have been described. Other promoters have been described for root-preferential gene expression in both soybean and *Arabidopsis*. Two tissue specific pro-

moters were examined in transgenic cotton, namely, cotton ribulose-1,5-bisphosphate carboxylase small subunit gene (Gh-rbcS) and a seed protein gene (Gh-sp) – patterns of transgenic expression of both of these genes accurately reflected their origin in the native plant i.e. in chlorophyll containing tissue and in the developing seeds, respectively (Song et al., 2000).

Genome Position Effect

According to Gelvin (1998) one of the big challenges facing genetic engineers today is the regulation of transgene expression, with the position of integration of a transgene within a genome influencing its expression. This is known as the genome position effect (Daniell and Dhingra, 2002). The insertion of multiple random copies of a transgene in the genome can effectively abolish its expression and the insertion of a transgene in or close to another gene can result in the production of an undesirable phenotype (Kumar and Fladung, 2002). Therefore, to ensure long term stable expression of a transgene post-transformation, the insertion of a single copy of a gene into a location in the genome where expression of the transgene is not adversely affected by the surrounding genomic sequences is desirable (Kumar and Fladung, 2002). One way of isolating the transgene from the potential deleterious effects of the surrounding plant genomic DNA is to include nuclear matrix attachment regions (MARs) as part of the chimeric binary construct. For a review of some of the possible roles of the (MARs) with respect to transgene expression see Holmes-Davis and Comai, (1998).

Antibiotic marker genes – what are the alteratives?

The recent public concerns over the safety of selectable markers such as antibiotic and herbicide resistant genes for the identification of transgenic plants *in vitro*, particularly in food crops, has fuelled the impetus for the search for alternative selection strategies. Genetically engineered crops containing antibiotic resistance genes have been banned from release in Germany (Daniell et al., 2001b). Any antibiotic resistance genes which are perceived as being potentially detrimental to human health should be prohibited for use by the 31st December 2004 (pertinent for those GMOs that were approved under Part C of Directive, 2001/18/EC) and by 31st December 2008 for those approved under Part B of the same directive according to a notice issued by the EU (Cockburn, 2002). Thus the generation of transgenic plants free of these marker genes is one of the current major challenges facing biotechnologists (Zuo et al., 2002). The removal of these marker genes via a site-specific recombinase post-transformation is becoming more and more important if there is to be any improvement in the public acceptance of transgenic plants generally (Kumar and Fladung, 2002). Another alternative is to deliver two different T-DNAs into the nuclear plant genome (one containing the gene of interest the other the selectable marker gene) and as a consequence

of the genes integrating at two different sites in the genome genetic segregation to separate the gene of interest from the selectable marker should be possible at a later stage.

An alternative to using antibiotic and herbicide selectable markers is for the selection of putatively transformed cells using MPI (mannose-6-phosphate isomerase) as a selectable marker. This gene was originally isolated from *E. coli* (*man*A). Transformants containing the MPI marker gene have the ability to use mannose as a carbon source and its effectiveness has been demonstrated in sugar beet, wheat and maize (Hansen and Wright, 1999). The first report of successful chloroplast genetic engineering without using antibiotic selectable marker genes was made by Daniell et al., (2001b). Here, the betaine aldehyde dehydrogenase gene (from spinach) was used which converts toxic betaine aldehyde to the non-toxic form. Using this selectable marker gene 80 % of the leaf discs cultured were seen to produce shoots within two weeks. The use of antibiotic selectable marker genes in chloroplast transformation could be a real problem as thousands of copies of the gene could in theory be present in one cell.

Multigene engineering

A major advance in the field of plant genetic engineering is a move from the insertion of a single gene to the insertion of multiple genes in a single transformation event (gene stacking) (van Bel et al., 2001). Because the nuclear genome does not process polycistronic mRNA molecules, difficulties can be encountered when trying to introduce multiple genes into the nuclear genome (Daniell and Dhingra, 2002). Therefore, one way of facilitating multigene engineering is to engineer chloroplasts. A distinct advantage of chloroplast transformation over nuclear transformation is that through homologous recombination, foreign DNA can be inserted into the spacer region between functional chloroplast genes thereby determining precisely where the transgene will be located unlike the situation observed in nuclear transformations where the random integration of the transgene (genome position effect) may have a negative effect on its overall expression (Kota et al., 1999). Another advantage of chloroplast transformation over nuclear transformation is that, due to the fact that chloroplasts are maternally inherited in most crops, the risk of gene escape from insect or wind transported pollen is reduced. Cross pollination between transgenic and non-transgenic plants is a serious environmental concern, for example, the risk can be as high as 38 % in sunflower and 50 % in strawberries (Daniell, 1999).

The chloroplast genome has been engineered to express traits such as herbicide insect and disease resistance, drought tolerance and also for the production of biopharmaceuticals (Daniell, 2002a). A comparison of chloroplast versus nuclear transformation is given in Table 1. Transgenic plants with genetically engineered plastids are more productive than plants whose nuclear genome has been altered. Reports to date

Table 1. A comparison of chloroplast and nuclear genetic engineering (Adapted from Daniell *et al*., 2002b).

Transgenic	Chloroplast Genome	Nuclear Genome
Transgene copy number	Up to 10, 000 per cell	Few copies per cell
Gene expression	Foreign gene expression can in some cases account for up to 47% of total soluble protein	Accumulation of foreign protein can be quite low (less than 1 % of total soluble protein)
Gene arrangement and transcription	Polycistronic RNA is often transcribed therefore multiple transgenes could be introduced and expressed in one transformation event	Monocistronic RNA is transcribed
Position effect	No position effect reported as site-specific insertion occurs	Genome position effect often reported due to the random nature of the insertion event
Gene silencing	Not reported	Reports of gene silencing
Gene containment	Due to maternal inheritance in most crops genes can be contained	Risk of gene escape in outbreeders
Toxicity of foreign proteins	Possibility of minimal effects due to the containment of the proteins within the chloroplast organelle	Toxic effects may be due to an accumulation of the toxin in the cytosol
Generation of transgenic lines	Uniform lines generated	Large variability of gene expression seen

indicate that protein production from a transgene inserted into the nuclear genome usually accounts for not more than 1% of overall total soluble protein (TSP) in the transformed plant (Gewold, 2002) compared with reports of greater than 45% of the overall protein production attributed to transgene expression in the chloroplast in some cases.

With respect to the engineering of insect resistance in plants, high expression of the Bt toxin can be achieved via chloroplast engineering as the number of chloroplast genomes per cell is between 5,000 and 10,000 (Kota et al., 1999). The two Bt genes that are found in most of the commercial transgenic crops are either Cry1Ab or Cry1Ac. As a consequence of their amino acid sequence similarity (90% homology) if a resistance allele appears in the insect population to one of these proteins, the chances of

it conferring resistance to the other Bt protein is quite high (Kota et al., 1999). The overexpression of the *Bacillus thuringiensis* Cry2Aa2 protein in chloroplasts demonstrated resistance to plants against both Bt susceptible and resistant insects (Kota et al., 1999). Therefore it may be necessary in some cases to increase the number of Bt proteins in use in the production of transgenic crops in order to pre-empt problems like the development of resistance alleles in the insect population. A recent review of plastid transformation including information on plastid transformation vectors can be found in Maliga (2002).

Genetic engineering for stress tolerance

Much effort in recent years has been devoted to identifying potential target genes for use in genetic engineering for biotic and abiotic stress resistance. The process has been accelerated by reference to the rapidly expanding bioinformatics data bases, by progress in elucidating the human, yeast, *Arabidopsis* and bacterial genomes. The use of mutation techniques in *Arabidopsis* to obtain knock out and up-regulated mutants, and the elucidation of stress defence mechanisms in yeast and humans, where these mechanism are highly conserved in eukaryotes, has also made a major contribution. This background work is extensive with some 24,000 papers on biological calcium alone published in 1995-97 (Evenas et al., 1998).

This is reflected in the applications for trials approval in the period 1987–2001 (www.aphis.usda.gov/ppq/biotech/). Analysis of the latter show that the greatest number of applications in the USA (in total 9204 to date) have been for trials of herbicide tolerant (32.5%), pest resistant (18.5%) and improved product quality (16%) transgenic lines. Genetic engineering for biotic (excluding pests and viruses) and abiotic stress is covered under the category ‘agronomic properties’ which accounts for 7% of the total trials applications. Some genes have not been identified for commercial reasons. In applications for trials for biotic and abiotic stress resistance, aside from the Bt and herbicide resistance genes, the target genes have included those for oxidative and specific stresses; enzymes for antioxidants, compatible solutes and phytoalexins (see: www.nbiap.vt.edu/cfdocs/fieldtests1.cfm). In most cases the applications have been for approval to trial lines with single transgenes but there were a number of applications to trial plants transformed with two genes, namely, herbicide and insect resistance (9%), herbicide resistant and agronomic properties (4.5%) and virus and insect resistance (4.5%); in some cases genes for enzymes of oxidative stress resistance have been combined with those for pathogen resistance in a pyramiding strategy. A few applications have also been made to trial regulatory genes. The latest data (for 2001) shows the insect and herbicide tolerance applications still predominate (total 49%) with pathogen resistance identified at 15%, product quality 14%, agronomic properties 6% and “other” 16%. Some representative examples are discussed below.

Strategies for engineering for virus resistance tend to be specific for viruses and are not discussed here for a review see Beachy (1997); Lorito et al. (2002).

Engineering for changes in the metabolome

Attempts at metabolome engineering for abiotic stress reduction have been based on attempts to increase the constitutive concentration of antioxidants and compatible metabolites in the plant tissues (Bohnert and Shen, 1999; Verpoorte et al., 2000). As discussed above, the oxidative stress response is a component of the global stress response and consequently engineering of gluathione and ascorbate metabolism has been attempted. Enzymes from *E. coli* and higher plants have been introduced and expressed in the cytosol and chloroplasts of tobacco and poplar and the plants exposed to paraquat, ozone salt and other stresses with mixed results (reviewed by Pastori and Foyer, 2000). The conclusions of Pastori and Foyer (2000) were that rather than trying to continue the approach of introducing single enzymes from the gutathione-aspartate pathway, more effort should be placed on attempts to elucidate and manipulate the transcription factors involved. Glycine betaine is a compatible solute associated with tolerance to salt, low temperature and drought. Nuccio et al. (1999) reviewed results for the engineering of a number of compatible solutes including proline, mannitol, sorbitol, trehalose, inositol and glycine betaine. The results showed variability in the improved resistance claimed with in some cases reports of adverse phenotypic effects. They also discuss the merits of attempting regulon engineering rather than the engineering of individual steps and the need for repeated rounds of engineering and detailed analysis of the progeny.

Biotic defence compounds are divided into phytoprecipitins which are constitutive and phytoalexins which are induced on pathogen stress perception. The compounds are products of many metabolic pathways and have been extensively reviewed by Mansfield (1999). The transfer of stilbene synthase from grapevine to tobacco, resulting in resveratrol synthesis was reported to confer resistant against *Botrytis cinerea* but predictable variability in expression of the transgene was reported (Hain et al., 1993).

In summary, experimental results have been published where attempts have been made to engineer plants for the over expression of biotic and abiotic stress compounds. These efforts have at best given only partial alleviation of oxidative or the specific target stress but there is a paucity of field trials data. The issues involved in metabolome engineering are complex varying from lack of understanding of the enzymology of the pathway and of its regulation (Dixon et al., 1996; Nuccio et al., 1999; Verpoorte et al., 2000). The challenges are to overcome rate-limiting steps, the avoidance of flux reductions through competing pathways that would have adverse effects of host fitness, the prevention of breakdown or over expression of the target product(s).

Engineering for changes in the proteome

In comparison with metabolome engineering, there have been many reports of proteome manipulation. Engineering of the proteome for increased oxidative stress tolerance has involved transformation for constitutive high expression of enzymes associated with ROS resistance e.g. Cu/Zn/Fe/MnSOD, APX and GST/GPX activity. The transformed plants have shown variation in stress tolerance in approx 60% of the reports, albeit more recent reports suggest greater success rates (Van Bruesegem et al., 2002). A wide range of target genes have been identified for improvement of plant abiotic stress tolerance (Cushman and Bohnert, 2000). These include specific heat shock proteins, ion transporters, water transporters (aquaporins), as well as signalling components e.g. MAP kinases, Ca^{2+}-dependent protein kinases, transcription factors e.g. DREB, CBF and Myb, and enzymes of plant hormone metabolism (Cushman and Bohnert, 2000; see also Cherry et al., 2000).

Engineering of the proteome for increasing disease resistance primarily focussed on up-regulation of the expression of pathogenesis-related genes e.g. chitanase and glucanase (Broekaert et al., 2000). The results varied with the gene used, the host and the challenge organisms. In some cases e.g. PR-3 (acidic chitinase) in cucumber and carrot, no resistance was detected against a challenge with a range of fungal pathogens of the respective crops. In the case of PR-3 (basic chitinase) resistance was expressed against *Botrytis cinerea, Rhizoctonia solani* and *Sclerotium rolfsii* in carrot but not in cucumber (Punja and Raharjo, 1996). However, field trial data is unavailable for most of these transformed lines. Similar results were obtained with PR-2 (acidic glucanase) in alfalfa where resistance was obtained against some fungal pathogens but not others (Masoud et al., 1996). Similar variability in response has been obtained following transfer with the small antimicrobial proteins, thionins (Epple et al., 1997) and lipid transfer proteins (Molina and Garcia-Olmedo, 1996). Higher resistance, compared with single gene transformations, has been obtained by pyramiding 2 resistance genes (Jach et al., 1995). In a different strategy, Cao and Dong (1998) reported broad spectrum resistance following over expression of the *NPR1* gene a regulator in the SA induced SAR pathway in *Arabidopsis thaliana*. It remains to be confirmed that this approach will work with crop species. Over expression of the *Pto* resistance gene involved in the hypersensitive response has been reported to confer broad resistance to bacterial and fungal pathogens in tomato (Tang et al., 1999). For further examples of target genes see review of Lorito et al. (2002).

Mutation techniques in breeding for stress tolerance

While much of this review relates to plant improvement by genetic engineering it is important to recognise that conventional breeding and mutation breeding also have

contributions to make (Brunner, 1995). The choice of plant breeding method should not be driven by technology solely but with regard to crop (whether sexually-propagated – self or cross pollinated – or clonally propagated; its use and its degree of domestication); the character(s) (whether major and/or polygenic and whether available in sexually compatible germplasm) and infrastructure (including consumer acceptance) (Jones and Cassells, 1995). There are also important lessons to be learned from the attempts of hybridists and mutation breeders to introduce abiotic and biotic stress resistance into plants (Cassells and Jones, 1995).

Mutation techniques, including transposon mutagenesis, have made and will continue to make a valuable contribution to the understanding of the molecular basis of the plant stress response based on information gathered from the *Arabidopsis* and other model studies. Loss and gain of function mutants have identified components of stress reception, signal transduction and transcription factors involved in the stress response Reference to www.nbiap.vt.cfdocs/filedtests1.cfm shows that many trial permits involve the use of both sense and the corresponding antisense gene constructs. This information has been used in identifying targets for genetic engineering for stress tolerance (see above). Furthermore, while often presented as a precise tool for plant improvement, transformation, like mutagenesis, creates random variants. In the case of mutation breeding this is because mutation is a random event, in the case of transformation it is because insertion site and in some cases copy number, are uncontrolled. In both cases, introgression of the mutant gene(s)/trangene(s) by backcrossing can be effective in reducing pleotropic effects (Maluszynski et al., 1995)

Mutation techniques have been used widely in efforts to breed abiotic stress tolerance and disease resistant lines with some success (see www.isea.org for lists of varieties released). The affects of physical and chemical mutagens are well characterised and are very similar to the spontaneous mutation arising *in vitro* (‘somaclonal variation’). Somaclonal variation has contributed to the development of abiotic and biotic stress resistant varieties in major crops (Brar and Jain, 1998). Use of *in vitro* mutagenesis strategies systems, especially for vegetatively propagated crops including the major world crops potato and banana, combined with *in vitro* selection and early *post vitrum* selection for isogenicity with the parental line have significantly improved the efficiency of mutation techniques in breeding (Cassells, 2002).

Safety of genetically engineered lines

Ecological and human health risks associated with the release of transgenic plants

The negative effects of growing transgenic plants from an ecological point of view can be classified as either direct, due to the invasiveness of the plants in a particular habitat or indirect, by influencing changes in agronomic practice. According to Hails

(2000) the ecological risks posed by transgenic plants can be identified under the following headings: (a) the organization of the particular plant genome; (b) the introgression of transgenes into wild relatives and (c) the effect of the transgenes on non-target species and, as a consequence, the broader effect on the ecosystem as a whole. Most of the transgenic crops that have been commercialized to date are a result of a foreign gene being inserted into the nuclear genome and, as a consequence, the possibility of gene escape exists via the movement of pollen. This is different to the situation found with chloroplast engineering where, due to the maternal inheritance of chloroplasts in most crops, the risk of gene escape via pollen is reduced. However, biparental or paternal inheritance of chloroplasts is seen to occur in gymnosperms and also in some of the angiosperms, therefore chloroplast engineering may reduce the risk of gene escape but does not eliminate it (Gray and Raybould, 1998). Up until the beginning of 1998, transgenic herbicide tolerant crops accounted for about 35 % of all genetically modified crops released (Gray and Raybould, 1998). Various problems associated with gene escape have been identified and particularly with outbreeding crops. The literature up to 1998 suggests that gene flow had occurred between the following crops and their wild relatives: sugarbeet, maize, sunflower, carrot, sorghum, strawberries, quinoa and squash (Gray and Raybould, 1998).

Another problem exists with the over-use of glyphosate as a result of the release of these resistant crops i.e., the potential generation of mutant weeds resistant to glyphosate. According to Gray and Raybould (1998), no resistance has occurred to the glyphosate herbicide even though it has been in use for over 20 years and its target is a single enzyme (EPSPS), however, Robert and Baumann (1998) dispute this. They point out that to date there have been at least two cases of resistance evolving in the field to the herbicide glyphosate in *Lolium rigidum*.

In terms of the risks to human health, the possible transfer of antibiotic resistant genes (horizontal gene transfer) from the plant genome to pathogenic microbes present in the soil or in the human intestinal tract has to be addresed (Daniell et al., 2001; Cockburn, 2002) (see above section: “Antibiotic marker genes – what are the alternatives?”). Additional potential identifiable risks to human health could be due to the following; (i) a transgene could be responsible for the production of an allergenic protein, (ii) the introduction of a transgene could effectively result in the inactivation of one or more endogenous genes and (iii) the integrated transgene could result in the switching on of a hitherto silent endogenous gene(s) (Cockburn, 2002).

Food safety

It has been argued that “the potential risks of introducing new food hazards from the application of genetic engineering are no different to the risk that might be anticipated from genetic manipulation of crops via traditional breeding” (Conner and Jacobs, 1999). While in general this a reasonable hypothesis, it should be recognised that some

target genes, e.g. the use of chitinase in engineering for biotic resistance, may be potential allergens (Shewry et al., 2001; Taylor and Hefle, 2001) and some stress metabolites e.g. phytoalexins may be plant toxins or have anti-nutritional properties (Novak and Haslberger, 2000). There is also the possibility that the transgene, possibly depending on its insertion site or other epigenetic interactions, may stress the genome resulting in the up-regulation of the expression of constitutive putative allergens such as members of the antimicrobial proteins.

The Novel Food Regulation of the European Community and the US FDA guidance "Foods Derived from New Plant Varieties" are based on the principle of the "substantial equivalence" of the parent variety and its genetically modified derivative(s), that is, that the concentrations of key toxic, anti-nutritional and allergenic compounds in the GMO are within the range found in the parental variety (Anon., 2002; Novak and Haslberger, 2000; Schauzu, 2000). So, for example, in the case of potatoes transformed with the Bt gene, glycoalkaloid analysis is carried out to confirm that they are within the range of commercial varieties. The transgene is also evaluated for potential allergenicity. A limitation of this approach is that "traditional crops" are immune from the legislation that has been proposed in some countries to which "new or non-traditional crops" should be subjected and is the basis on which anti-GMO campaigners have attacked the principle of substantial equivalence. The anti-GMO lobby, in addition to concerns about transgene escape, are arguing that the safety of plant foods, and by extrapolation feed, should be determined to establish a scientific basis for the principle of substantial equivalence. This proposal has major cost implications for Governments and/or all producers of crops for feed and food, including those not using GMOs.

Conclusions

The human, yeast and *Arabidopsis* genome projects and the high degree of conservation of pathways in eukaryotes, underpin recent rapid advances in dissecting the complexity of stress responses in plants. Jardin's principle states that all problems at first appear simple but as they are investigated are seen to be more complex. That is certainly the case in the emerging elucidation of plant stress responses. Indeed the complexity so far revealed may only be the tip of the iceberg as redundancy is being shown as the way of life for plants (Normanly and Bartel, 1999) There is no doubt that the use of herbicide and pest (Bt) resistance genes, singly and in combination, has been successful in practice, aside from social and environmental concerns. But attempts to confer oxidative and specific stress resistance through single gene transformations appear less successful. In many cases, only an incremental improvement in tolerance was reported and where reported this was in the case of pathogen tolerance, against some pathogens in some hosts. Gene pyramiding or stacking appears to confer

relatively greater benefit as does the reported case of increased expression of a biotic stress regulatory gene. Several authors have argued for engineering of specific stress pathways for constitutive higher expression but this would imply a significant yield penalty. The general conclusion is that neither over expression of phytoalexins (Mansfield, 1999) or of defence proteins (see elicitor fungicides below) (Broekaert et al., 1999) confers broad spectrum resistance (Lorito et al., 2002). The latter is, however, generally expressed in the "global defence response" in non-host resistance (Heath, 2000).

Plant breeders know the absolute requirement for multi-site, multi-annual field trials to evaluate the durability of resistance. There is a paucity, indeed in most cases, a complete lack of field trials data for transformed lines. Pleiotropy is recognised as frequently being associated with the introduction of novel genes by hybridisation. This is likely to apply to the introduction of transgenes. The successful Bt and herbicide resistant genes act peripherally to host pathways thus pleiotropy is minimised (Buiatti and Bogani, 1995). Where host metabolic pathways are altered by the transgene, pleiotropic effects might be predicted or transgenic modification may be restricted by compensation of the host metabolism due to attempts to maintain homeostasis (Buiatti and Bogani, 1995)

Plant breeders have long been aware of the complexity of breeding for stress resistance and in breeding for yield have attenuated such defences. In the case of abiotic stress it is arguably the exception that crops are exposed to single stresses and the stress complexes may be regional as opposed to across the geographic range of the crop (Acevedo and Fereres, 1993). In the case of biotic stress, there is the ability of the pathogens to mutate which has eroded the durability of resistance genes, especially of single genes and, arguably, limits their potential use in transgenic plants (Niks et al., 1993). Also, in the case of pathogens, there is the specificity of the pathogen-host genotype interaction where multiple pathogenicity factors (elicitors, toxins and inhibitors) may induce an array of responses in a given host under given physiological conditions as has been shown in the *Arabidopsis* model (Thomma et al., 2001).

In addition to elucidating the receptors, signal transduction and transcription activation pathways, a key element, namely, the avoidance of the possible growth penalty and lack of flexibility associated with continuous expression of stress defences (Agrawal and Karban, 1999), will be the challenge of modulating the responses such that they are up-regulated rapidly, tissue-specifically, to the level necessary when the stress is perceived and that the ground state is rapidly re-established when the stress abates. In abiotic stress, some stresses may be persistent e.g. salt stress, and a compromise may have to be reached between the growth penalty of expressing stress tolerance and the yield potential; while e.g. in cold stress, the stress may be transient implying lesser yield penalties for transgenic varieties. In the case of biotic stress resistance two broad strategies are being followed; firstly, the search for resistance to specific pathogens based on elucidation of the pathogenicity factors and engineering for specific solutions and, secondly, the search for broad-spectrum resistance based on

engineering for non-host resistance. In engineering for resistance to pathogenicity factors the problem is that faced by conventional breeders of pathogen resistance, namely, mutation of the pathogen to overcome the resistance. In seeking more environmentally acceptable pesticides, the fungicide manufacturers' have developed 'elicitor' fungicides, these analogues of signalling compounds such as salicyclic acid, act by inducing AMPs (Van Loon, 1999). Their effects are transient and consequently do not impose an economic yield penalty but there is the criticism that due to cross-talk they may increase susceptibility to pests (Pieterse et al., 2001). This poses the question as to whether either the hypothesis of a pathogen specific response involving up regulation of pathogenesis-related protein and phytoalexin synthesis, or a global response where a prescribed array of stress defences is activated, represents the plants response to pathogen attack. There is emerging evidence that each pathogen stress-host interaction may be customised by the host (Thomma et al., 2001). It should not be forgotten that the pathogen host interaction is also dynamic in space and time, adding further complexity to attempts at genetic engineering for biotic resistance.

Technical complexity aside, there is the issue of food safety. Many stress metabolites e.g. the potato phytoalexin and stress proteins e.g. the lipid transfer proteins are anti-nutritional and allergenic, respectively (Novak and Haslberger, 2000; www.fao.org). Given that the defence proteins are highly conserved this poses the question of whether transgenic plants expressing higher level of these proteins (and stress metabolites), or their increased expression by putative transgene-induced stress effects on the genome (Matzke and Matzke, 1998), pose consumer health risks. Given consumer concerns about the principle of substantial equivalence and the view of activists that the safety of plant food be evaluated as a baseline for evaluation of the safety of GMOs there is arguably a need for the development of methodologies to analyse plants for unanticipated consequences of genetic transformation (Charles et al., 2002).

In all genetic engineering, while transformation systems are available for most important crops, there remains the inherent unpredictable character of the process, which is based on random insertion and sometimes multiple insertions can result in positional effects, in transgene interactions, gene silencing and result in adverse pleiotropic effects. In fertile crops, some of these problems can be resolved by backcrossing.

Finally there are the critical issues of adequate trialling to confirm the stability and durability of the resistance in the case of pathogen resistance and socio-economic factors. It is unfortunate that journal editors are not more rigorous in requiring that field trials be carried out before papers claiming improved stress tolerance are published. Pleiotropic consequences of transgene incorporation are, as yet, generally unreported. In a recent editorial Radin, (2003) points out that while genetically engineered varieties of canola, flax, papaya, tomato, squash, sugarbeet potato and radicchio have been approved for commercial use, most of these varieties are not grown. He attributes this to the transgenes giving only partial resistance, to unfavourable economics but also to consumer resistance to GM plants.

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