

REVIEW PAPER

Genetic engineering to improve plant performance under drought: physiological evaluation of achievements, limitations, and possibilities

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Abstract

Fully drought-resistant crop plants would be beneficial, but selection breeding has not produced them. Genetic modification of species by introduction of very many genes is claimed, predominantly, to have given drought resistance. This review analyses the physiological responses of genetically modified (GM) plants to water deficits, the mechanisms, and the consequences. The GM literature neglects physiology and is unspecific in definitions, which are considered here, together with methods of assessment and the type of drought resistance resulting. Experiments in soil with cessation of watering demonstrate drought resistance in GM plants as later stress development than in wild-type (WT) plants. This is caused by slower total water loss from the GM plants which have (or may have—morphology is often poorly defined) smaller total leaf area (LA) and/or decreased stomatal conductance (g_s), associated with thicker laminae (denser mesophyll and smaller cells). Non-linear soil water characteristics result in extreme stress symptoms in WT before GM plants. Then, WT and GM plants are rewatered: faster and better recovery of GM plants is taken to show their greater drought resistance. Mechanisms targeted in genetic modification are then, incorrectly, considered responsible for the drought resistance. However, this is not valid as the initial conditions in WT and GM plants are not comparable. GM plants exhibit a form of ‘drought resistance’ for which the term ‘delayed stress onset’ is introduced. Claims that specific alterations to metabolism give drought resistance [for which the term ‘constitutive metabolic dehydration tolerance’ (CMDT) is suggested] are not critically demonstrated, and experimental tests are suggested. Small LA and g_s may not decrease productivity in well-watered plants under laboratory conditions but may in the field. Optimization of GM traits to environment has not been analysed critically and is required in field trials, for example of recently released oilseed rape and maize which show ‘drought resistance’, probably due to delayed stress onset. Current evidence is that GM plants may not be better able to cope with drought than selection-bred cultivars.

Key words: drought, genetic modification, leaf area, soil water; stomata, stress metabolism, transgenic plants, transpiration, water deficits, water stress.

Drought and justification of genetic modification to give drought resistance

For some 30 years (Deckard, 1988; Mullet, 1990; Toenniessen, 1991), the techniques of molecular biology have offered the prospect of directly altering the genomes of higher plants to change their metabolism and improve growth and yield under adverse environmental conditions to better serve human

requirements (Edgerton, 2009). Overcoming abiotic environmental factors which decrease the yield of crops, including those with long generation times (Newton *et al.*, 1991), has been a central aim. Of particular importance is drought—water deficiency—which adversely affects plant and crop

production, often greatly (Kramer and Boyer, 1995; Chaves and Oliveira, 2004). Therefore, the aim has been to genetically modify plants to induce drought resistance (DR; this abbreviation is also used for ‘drought resistant’). The terms genetic engineering or modification (GM; also used for ‘genetically modified’) have been used for the processes of transforming plants. Attempts were made to identify and transfer genes responsible for DR between species such as ‘resurrection plants’ (Iturriaga *et al.*, 1992) or by more conventional hybridization (Jauhar, 1992), cell fusion (Begum *et al.*, 1995), and by transformation of tobacco with a drought-inducible histone gene (Wei and O’Connell, 1996). Of great importance was the exploration of induction of gene expression caused by drought in plants, particularly in the ‘model’ species *Arabidopsis thaliana* (Yamaguchi-Shinozaki and Shinozaki, 1994; Yamaguchi-Shinozaki *et al.*, 1995; Kasuga *et al.*, 1999), which has influenced the direction of drought-related studies since. This has resulted in identification of ‘candidate genes’ (Le *et al.*, 2011), likely to confer DR in crop species. Wide-ranging effects and benefits have been expected from altering or introducing many types of genes and altering their regulation, for example gene promoters and transcription factors (Held and Wilson, 2007; Khan and Liu, 2009). Proponents of genetic engineering have generally assumed that the mechanisms impairing crop production caused by drought are known, and assert that the limitations may be overcome by appropriate alterations to metabolism via changes to the genome (GM). GM lays strong claim to being based on exact knowledge of mechanisms and ability to alter, specifically, key metabolic processes to give a precise outcome—engineering—and to improve both absolute and relative crop production (mass of dry matter and harvestable yield) per area of land surface, and to increase water use efficiency (WUE) when water is limiting and thus ameliorate (or even eliminate) the effects of drought (Nguyen *et al.*, 1997). There is a general, pervasive ethos in the GM literature that natural selection has not given adequately DR plants and that GM is the only way of achieving the desired changes as selective breeding is incapable of doing so in any reasonable time scale, despite scientific (Rebetzke *et al.*, 2002, 2008; Richards *et al.*, 2010) and practical evidence (‘Drysdale wheat’, CSIRO Plant Industry, www.csiro.au) to the contrary. Claims in the GM literature to have produced DR plants require examination.

Social, economic, and scientific context of GM

The social, and thus economic, importance of achieving greater production under deficient water is enormous, and production of truly DR plants would be a major achievement. The need is urgent: the current human population of Earth is 7 billion and is expected to reach 10 or even 12 billion, and will require food, fibre, and energy (Evans, 1998, 1999). Globally, agriculture is practised in many areas where water supply is very frequently deficient compared with the evapotranspiration from crops, which then have insufficient water to achieve their genetic potential yield. Drought over

the long term is a major problem, but in the short term it also decreases crop production even when other conditions are favourable, and may have very serious economic and social consequences (Kramer and Boyer, 1995; Kostandini *et al.*, 2009). There is great variability in water supply occurring at different periods during growth and development of particular crops, and the effects may be very specific. Plant processes from genome to growth and production (total biomass as well as economic yield and quality of crops) depend strongly on water supply (Chaves *et al.*, 2003) and are very susceptible to drought; losses worldwide are difficult to estimate but are certainly many millions of tonnes with large economic value (Wilhite, 2005). During the adoption of agriculture, selection (subconsciously or consciously by people over millennia) of favourable characteristics of plants and development and application of appropriate technology increased yields of basic crops. Despite what must have been a tendency to select for productive genotypes in drought conditions, crops still depend strongly on water. More recent selection breeding, applying scientific principles (Nguyen *et al.*, 1997; Tuberosa *et al.*, 2007), has also not given crops which are unaffected by drought, but has led to smaller improvements; for example, application of carbon isotope discrimination in wheat breeding (Rebetzke *et al.*, 2008; Richards *et al.*, 2010) has improved yields by ~5% with drought which decreased yield by 50%, and by 10% with a 75% decrease in yield (Farming Ahead, 2003; kondinigroup.com.au). Breeding for DR is considered by Blum (2011a, b) and Araus *et al.* (2008). Evolved mechanisms enable crops to maintain some production even under rather severe water deficits and provide the basis for improvements by selection breeding combined with molecular genetics information (Reynolds *et al.*, 2005). In contrast the implication in part of the GM literature that GM plants are ‘DR’ and thus unaffected by water deficiency is perhaps unintentional, but is certainly uncritical. GM in relation to breeding is briefly considered later.

GM technology has been justified in terms of ability to produce DR crops more rapidly and efficiently than selection breeding and thus to alleviate food shortages (Priyanka *et al.*, 2010b). Particular emphasis has been put on the potential for GM to improve food production in drought-prone areas, for example in developing economies of Africa (Thomson, 2004), although the likelihood of even moderate success is small (Lawlor, 2010). Claims that GM could, and would, achieve DR crops have been substantial. They contrast strongly with the view (Sinclair, 2011; Sinclair *et al.*, 2004) that GM technology is unlikely to achieve advances in enhancing drought resistance. Nonetheless, the potential for rapid achievement of the goals has led to substantial shifts of research funding and teaching towards GM technology. The huge investment, by public organizations and particularly by large companies, in GM has not achieved increased yields under drought (Passioura, 2007). There is still a body of opinion emphasizing the need to understand the effects of water supply and deficits on plant mechanisms—genome to yield—and to find methods, including GM technology, of altering plant and crop responses and thereby improving plant processes, such as photosynthesis (Lawlor and Cornic, 2002, Lawlor

and Tezara, 2009) and crop production (Chaves *et al.*, 2003). The latter is clearly a function not just of the genome (Denby and Gehring, 2005) but of the whole complex plant system (Chaves *et al.*, 2009), as shown by analysis of quantitative trait loci in breeding for DR (Tuberosa *et al.*, 2007). Focus on the minutiae of mechanisms is required for understanding, but consideration of the whole system is also important (Moore *et al.*, 2009). Yet here is a contradiction—very specific interventions in the genome result in DR under laboratory conditions but have not produced, as yet, clear evidence of substantial improvements in crops under drought in the field, and those concerned with crops strongly doubt the ability of GM approaches to give drought resistance.

Scope of the review, aims, and questions

General assessment of the GM literature shows that nearly all studies have achieved ‘DR’ but by a large number of different alterations to the genome. The few exceptions, for example Peng *et al.* (2007), are considered later, and in detail in the [Supplementary data](#) available at *JXB* online. Mutations in genes [e.g. of MAPKKK (Ning *et al.*, 2010)] affect DR but are not considered, as the focus is on GM plants. One view of GM is that it aims for ‘one-size-fits-all’ metabolic solutions to a range of environment–plant interactions, as shown by reviews in Pareek *et al.* (2010): this has been achieved judging from the large number of claims. How is it possible that in such a short time so many GMs have achieved the target of ‘DR’? The apparent success of these predominantly laboratory studies contrasts strongly with the supposedly slow (or no) progress by conventional methods, although DR cultivars are recognized (Degenkolbe *et al.*, 2009). Evidence that DR oilseed rape in the field (Wan *et al.*, 2009; Y. Wang *et al.*, 2009) and, more recently, maize (release in the USA of DR corn; Padgett *et al.*, 2010; see also US Food and Drug Administration, Biotechnology Note (FDA)] give 10–15% more yield under mild drought is significant ‘proof of concept’ now being widely tested in the field in the USA. Yet it is unclear what form of DR has been achieved and how. Claims to have achieved it require evaluation, particularly to understand the mechanisms, as initial impressions from the literature are that physiological mechanisms have not been adequately considered. This review of the ‘GM literature’ is not from a molecular—genomic or metabolic—perspective, which has been done frequently and extensively, but considers application of GM to overcome the deleterious effects of water deficits on physiological processes such as growth, photosynthesis, dry matter production, and water loss, and particularly claims to have produced DR plants. Assessment of the literature suggested that the methods of testing for DR required detailed examination as they have generally been uncritical. Tests are often on small plants (Dalal *et al.*, 2009) under laboratory, controlled-environment, or glass house (e.g. Pellegrineschi *et al.*, 2004) conditions, with few under near-field or field conditions (commercial information is not available) although recognized as essential (Mittler, 2006; Mittler and Blumwald, 2010). A detailed analysis (Salekdeh *et al.*,

2009) considers methods relevant to analysis of responses of GM plants to drought. Only GM with respect to drought is considered, although in the GM literature all ‘stresses’ are regarded as very closely related, sharing common features (Valliyodan and Nguyen, 2008). Against this background, it is appropriate and important, indeed essential, to establish a firm understanding of the achievements of GM in making DR plants, and to evaluate methods and mechanisms to suggest future approaches to improving crop production. More specifically:

- Has drought resistance been achieved and what type?
- From what physiological mechanisms, affecting cell, tissue, organ, or plant, have changes resulted?
- Is it possible to identify the common traits and basic features providing drought resistance?
- How have changes to the genome contributed to drought resistance?
- Under what environmental conditions are the GM plants which have been produced effective?
- What are the limitations, if any, to production under drought and how will future GM approaches improve it?

Analysis of the literature

Reviews and research papers, plus books, concerning GM and drought resistance were selected, using search criteria aiming to be inclusive. Conference proceedings, etc., where information was insufficient to evaluate the methods, were later largely excluded. Analysis and sorting was done electronically and also by extensive non-electronic assessments—reading—focusing not only on the types of GM made, as is the usual feature of the review literature in this subject, but on the methods used for evaluation of DR. This essential information is often minimal and relegated to the ends of papers. Databases used included World of Science, Biosis, and CAB. Titles, key words, and abstracts were examined for terms and synonyms—drought resistant or resistance, drought tolerant or tolerance, and equivalents such as water deficit, water stress, combined with genetic modification, transgenic plants, etc. Checks were made to ensure maximal coverage, and specific searches were made for a wide range of terms, for example proline, heat shock proteins, trehalose, abscisic acid (ABA), etc., related to drought.

Results of searches

Number of publications

There has been an approximately exponential increase in publications on GM for DR since ~1990 to the end of 2011 (Fig. 1), indicating the scientific and applied importance. These are overwhelmingly molecular biological in nature. In addition, as Passioura (2007) pointed out, there are very many patents and applications for DR plants, which he regarded as having limited potential value for crop production in the field under drought, and also as showing the disparity between

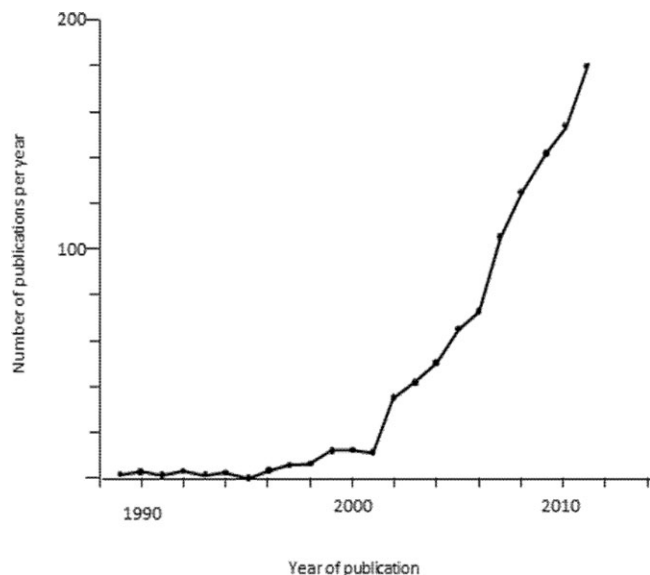


Fig. 1. Number of publications per year related to genetic modification for drought resistance from a comprehensive literature search.

the short-term molecular biological (Pareek *et al.*, 2010) and long-term physiological (Passioura, 2006a) approaches to crop production under drought and the requirements in practice where higher order factors play the major role. The number of studies and years is sufficient, therefore, to draw conclusions and gain an overview of developments, and to assess the relationship between potential and achievements. With so many papers, it is not possible to refer specifically to each, and a selection is presented to illustrate important general points. More detailed analysis is available in the [Supplementary data](#) at *JXB* online.

Types of papers

The papers are separable into reviews (considered briefly) and experimental studies. Both have specific features of importance to understanding development of the literature and how GM has produced DR plants.

Reviews

Reviews provide the reasons for the choice of the gene/metabolic system to be modified: it is generally taken ‘as read’ from earlier literature that understanding of metabolism is sufficiently exact to allow the outcome of GM to be predicted. Great emphasis has been placed on signal transduction to explain DR (Zhang *et al.*, 2002; Manavella *et al.*, 2006; Chae *et al.*, 2010). There is emphasis on the ‘candidate genes’ concept (Toenniessen, 1991; Toenniessen *et al.*, 2003) which provides a starting point for transformation (Pflieger *et al.*, 2001). If expression of a gene is increased or activity enhanced so that the product—enzymatic or other protein or resulting metabolites—increases, then improved performance under drought is expected to result (Bartels *et al.*, 1996; Bohnert and Shen, 1999; Bartels and Hussain, 2008). Discovery is often based on abundance of mRNA (detected by microarrays) or

on changes in metabolites, for example proline, which signify gene expression for enzymes of metabolic pathways. The idea of candidate genes perhaps involved in responses to drought has drifted into considering them ‘drought resistance genes’ which will confer DR if incorporated into a plant, and they become referred to as ‘genes for drought resistance’ (and even ‘drought-resistant genes’), a very misleading terminology. Particularly insidious is the almost universal view that an increased amount or activity of a component (mRNA, protein, or metabolite) as a consequence of water deficit is positive; that is, a direct response to conditions in the organ/cell/tissue and plant (Bartels and Hussain, 2008; X.W. Xiao *et al.*, 2009), and is a pre-programmed response which has the function of protecting the system from drought. Indeed, on occasions, it appears that such changes are ‘designed’ to do so and that increasing or introducing such a gene by GM will achieve this. A decreased amount or activity is ignored, despite the possible effects on metabolism. Specialized ‘model species’, particularly ‘resurrection plants’ with extreme adaptations to environment, have been considered sources of genes conferring DR (Moore *et al.*, 2009) as most of their physiological functions stop rapidly when they are desiccated and rapidly return to normal when rehydrated (Iturriaga *et al.*, 1992; Kranner *et al.*, 2002; Al-Wahaibi, 2004), potentially valuable for crops. Mechanisms for de- and re-activating metabolism involve many processes, such as synthesis of particular sugars and proteins, probably involve complex interactions, and are poorly understood. In no cases are assessments made on the basis of even semi-quantitative models of metabolism, etc., and this topic is greatly neglected in GM analysis as in plant science in general (Assmann, 2010). In microbial systems, such approaches are more advanced (Almaas *et al.*, 2004).

The earlier literature emphasized transformation with one or a few changes to alter key metabolites, for example glycine betaine and proline (Ashraf and Foolad, 2007; Chen and Murata, 2008), or proteins, for example late embryogenesis abundance (LEA) proteins (Cheng *et al.*, 2002), for DR. This did not produce the required DR, leading to emphasis on DR as a complex trait (Valliyodan and Nguyen, 2006; Fleury *et al.*, 2010), although this was not universally accepted (e.g. Blum, 2011a). Direct, specific ‘engineering’ approaches with a gene responsible for cellular component/function have moved to alterations in major regulatory systems (e.g. to signalling components such as transcription factors), or to many genes (with ‘cassettes’ and ‘gene stacking’) to replace metabolic sequences and pathways so that generalized responses are obtained (e.g. De Block *et al.*, 2005; Vinocur and Altman, 2005; Naqvi *et al.*, 2010). The effects may not be clear, with indirect consequences for DR, but expectations that GM plants may be constructed to enhance tolerance to adverse environments are enthusiastically promoted (Zurbriggen *et al.*, 2010) and reviews are very optimistic (Y.-x. Zhang *et al.*, 2007). Physiological consequences have not been considered adequately. Theoretical analyses of the mechanisms proposed would be desirable and have been promoted (Assmann, 2010), as would greater integration of metabolic and physiological measurements and simulation modelling. In this large literature, many metabolic systems and candidate genes have been targeted to achieve DR.

Targeted cell functions Transformations have produced many different types of GM plants (~600 distinct types). They are classifiable into groups targeting different aspects of cell function, although they overlap and their perceived functions change as a consequence of analysis. Only some are considered to illustrate the nature of the physiological changes achieved by specific GM.

- (i) Modifications to decrease cell osmotic potential (π) and thus increase turgor whilst decreasing plant water potential (ψ_p), although later the mechanisms actually affected may change [e.g. detoxification of reactive oxygen species (ROS)]. Mannitol: Karakas *et al.* (1997), Abebe *et al.* (2003), Sickler *et al.* (2007). Proline: Kishor *et al.* (1995), Zhu *et al.* (1998), Vendruscolo *et al.* (2007), Dobra *et al.* (2010), Pospisilova *et al.* (2011), Yue *et al.* (2011). Glycine betaine: effects on π but also protection of proteins and organelles against increasing ionic concentrations: Hanson *et al.* (2000), Hanson and Roje (2001), Zhang *et al.* (2008).
- (ii) Amino acid metabolism affecting protein synthesis: Glutamate: Lightfoot *et al.* (2007).
- (iii) Signalling molecules which alter the balance of cell metabolism: Trehalose: originally considered as an osmolyte, see Paul and Pellny (2003), Paul (2007), Paul *et al.* (2008a, b), Holmstrom *et al.* (1996), Goddijn and van Dun (1999), Goddijn *et al.* (1997), Fernandez *et al.* (2010), Jang *et al.* (2003), Garg *et al.* (2002), Lee *et al.* (2003), Rodriguez-Salazar *et al.* (2009), Karim *et al.* (2007), Iturriaga *et al.* (2009). Phosphatidylinositol: Georges *et al.* (2009), C.R. Wang *et al.* (2008), Perera *et al.* (2008), Khodakovskaya *et al.* (2010), Zhai *et al.* (2012). Ononitol: Sheveleva *et al.* (1997).
- (iv) Protective proteins which accumulate in water-deficient cells and are considered to stabilize protein structure, act as chaperones, etc.: Molecular chaperones: Alvim *et al.* (2001), Reis *et al.* (2011), Valente *et al.* (2009). LEA proteins: Cheng *et al.* (2002), Babu *et al.* (2004), Liu *et al.* (2009b), Bahieldin *et al.* (2005), L.J. Wang *et al.* (2009). RNA chaperone: Castiglioni *et al.* (2008). Cold-shock protein B: (Monsanto, FDA).
- (v) Proteins involved in cell growth and metabolism: Expansins: F. Li *et al.* (2011). Mitochondrial uncoupling protein: Begcy *et al.* (2011).
- (vi) Transport proteins of diverse functions: Aquaporins: Lian *et al.* (2004, 2006), Peng *et al.* (2007), Lin *et al.* (2007), Y. Zhang *et al.* (2007), Hachez *et al.* (2006), Yu *et al.* (2005). Vacuolar H₁ pyrophosphatase: Gaxiola *et al.* (2001), Bao *et al.* (2009). Vacuolar Na⁺/K⁺ antiporter: Asif *et al.* (2011).
- (vii) Regulation of gene expression and protein synthesis: a large group of different GM plants with multiple alterations to metabolism. Transcription factors: Liu *et al.* (1998), Sakuma *et al.* (2006), Belin (2010), Saibo *et al.* (2009), Sakuma *et al.* (2006), Qin *et al.* (2007), Pellegrineschi *et al.* (2004), Lourenco *et al.* (2011), Xiang *et al.* (2008), Hou *et al.* (2009), Cheng *et al.* (2012), J.Y. Zhang *et al.* (2007), Lin *et al.* (2011), Karaba *et al.* (2007),

Abogadallah *et al.* (2011), Oh *et al.* (2005), Chen *et al.* (2008), Hu *et al.* (2006), Nelson *et al.* (2007), Nakashima *et al.* (2007).

- (viii) Phytohormones and related metabolism: Abscisic acid: Schwartz *et al.* (2003), C.F. Zhang *et al.* (2007), Schwartz and Zeevart (2010), Thompson *et al.* (2007). Cytokinins: Werner *et al.* (2010). Farnesyl transferase: Wang *et al.* (2005), Y. Wang *et al.* (2009), Manavalan *et al.* (2012). Isopentenyl transferase: Rivero *et al.* (2007).
- (ix) Energy regulation and signalling: Ascorbate peroxidase: Rossel *et al.* (2006), Badawi *et al.* (2004), Li *et al.* (2009). Retrograde signals: Phosphonucleotide 3'-phosphoadenosine 5'-phosphate (PAP): Estavillo *et al.* (2011). Poly(ADP-ribose) polymerase (PARP): De Block *et al.* (2005), Vanderauwera *et al.* (2007). Poly(ADP-ribose) polymerase glycohydrolase 1: G. Li *et al.* (2011). Dihydroorotate dehydrogenase: Liu *et al.* (2009a).

A more detailed assessment of the literature is provided as **Supplementary data** at *JXB* online to show what was regarded as important in assessing the studies and the reasons for the conclusions, without overburdening the main analysis.

Experimental studies A large proportion of GM studies (~50%) is on *Arabidopsis*, and, more recently and increasingly, on rice (~30%) and oilseed rape and maize (~10% each); some studies consider poplar, soya bean, and cowpea, etc. About 25% are made on callus tissues or very small seedlings under conditions far from 'physiological', with DR tested with osmotica (see later; e.g. Khare *et al.*, 2010). Most studies are on young plants (e.g. Lu *et al.*, 2009): increasingly more are on mature, reproductive plants. Most are grown in controlled environments or glass houses, and in small pots of soil or horticultural compost, with attendant problems (Passioura, 2006b). Recently, a limited number of studies are on mature oilseed rape (Georges *et al.*, 2009), rice (Chen *et al.*, 2009; Gao *et al.*, 2011), and maize (Lightfoot *et al.*, 2007; Padgett *et al.* (2010) in field conditions, with limited physiological information. Analysis of the whole spectrum of GM studies shows that:

- Evidence of DR is based on simple methods, predominantly by comparing GM with wild-type (WT) plants allowed to dry in a small volume of soil, and assessing if the GM plants are stressed later than the WT.
- GM plants develop stress symptoms [wilting, decreased stomatal conductance (g_s), transpiration, and photosynthesis, increased chlorophyll fluorescence, increases and decreases in content of metabolites, etc.] later than the WT, considered to show superior ability of the GM plants to withstand drought (i.e. they are drought resistant).
- GM plants recover from drying faster and better upon re-watering than WT plants, which is claimed to confirm their DR properties.
- Correlations of metabolic and other changes resulting from GM with slower development of stress symptoms and better recovery are regarded as evidence that the metabolic mechanisms targeted are the cause of DR.
- GM plants are, generally, smaller and have impaired functions compared with the WT.

These points require detailed assessment as they are generic, applicable to all the many types of alterations to the genomes and metabolomes of several different species. The types of transgenes employed and the nature of the metabolic systems modified are not detailed above because it is clear from the analysis that a general, physiological, response has resulted in the claimed DR, irrespective of the nature of the transgene. This is an important and surprising conclusion which requires substantiation. Analysis of the literature shows, with considerable certainty (although not unequivocally as crucial evidence is difficult to establish from the literature), that the form of drought resistance achieved is based on plant size and stomatal conductance and their interaction with the environment, largely not as a direct effect of changes in the targeted metabolic processes. To establish and explain this, it is necessary to re-examine what is meant by drought resistance and basic water relations of the plant–soil–atmosphere system, as it is a feature of the literature relating to GM and DR that neither experimental nor review papers (for an exception see [Verslues et al., 2006](#)) explore adequately the concepts relating plants to environment.

Methods of evaluating drought resistance

Methods of applying water deficits and controlling the water status of plants are absolutely central to testing GM plants with the aim of developing ‘DR’ plants. Yet, it is clear that the techniques and criteria for judging the performance of plants are all too often a secondary aspect of GM work; the primary concern is the nature of the transformation. Few GM studies have considered the water status adequately, as [Jones \(2007\)](#) emphasized, although the later GM literature does pay more attention to it. Important comments on the difficulties of interpreting data ([Blum et al., 1996](#)) and requirements for analysis of DR ([Blum, 2000, 2005](#); [Verslues et al., 2006](#)) have not been followed adequately in the GM literature. Therefore, a briefly analysis of methods is given. As the GM literature does not adequately address methods used to assess DR, it is essential to consider, in very simplified form, plant–water relations and soil characteristics. Details are available in the extensive literature ([Kramer and Boyer, 1995](#)). Water moves in the soil–plant–atmosphere system according to physical principles. Total water loss from a plant is determined by the leaf area (LA) and the rate of transpiration per unit LA (T) which depends on atmospheric humidity, air and leaf temperatures, g_s , and the boundary layer conductance. Total available water to the plant depends on the difference in soil water content (Θ_s) between maximum, freely drained and that at which plants cannot extract more water from the volume of soil exploited. Thus, water supply to the plant is determined by Θ_s and soil water potential (ψ_s) and the water transport characteristics (conductivity), and the water potential of the plant (ψ_p) (specifically the roots). To maintain cells and tissues at the water content, ψ_p , and turgor required for growth, water uptake must equal total water loss. If water supply does not match demand, then adjustments in the phenotype (e.g. size, organ characteristics, and metabolism) occur. These include decreased cell water content and turgor, which lowers ψ_p ,

maintaining the gradient of potential from soil to root to leaf and thus water flux. However, smaller turgor also decreases g_s , so slowing T . In addition, it also decreases growth of leaves, and thus total water loss. Changes in metabolism may decrease π of cells, permitting ψ_p to drop but maintain a large turgor. Root systems may grow relatively more than leaves, so enhancing the supply of water. However, changes to leaf area and g_s and to root size and function are only effective at maintaining the water supply as long as there is available water in the soil volume exploited. Interaction between processes is very dynamic, and adjustments to drought proceed at different rates. Regulation of water balance of plants growing in a limited volume of soil (see [Passioura, 2006](#)) which is rapidly depleted of water (as in most GM studies) is generally by decreased g_s and only little by decreased LA. However, mature plants which are stressed may develop smaller LA and also lose functional leaves. Rates of drying and stages of growth are very important and determine responses to drought. When soil water available to mesophytic plants (such as those considered in the GM literature) is depleted, control of cellular water balance is not possible and severe damage may occur. However, in the field with slower water loss and a larger volume of soil to exploit, the LA (or leaf area index, LAI) is often more important than g_s ([Legg et al., 1979](#)), and metabolic adjustment may be significant. Irrespective of the details, an essential feature is that water loss from the plant is determined by LA and leaf surface characteristics (g_s) not by metabolic composition. It is not possible to retain significant water in leaf tissue by increasing content of metabolites, as sometimes suggested ([Bao et al., 2009](#)), although metabolite content may be important in perennating organs, for example meristems. In the GM studies analysed, the LA and g_s are the factors determining water loss, as soil water supply is the same in comparisons of GM and WT plants. Changes to the genome, proteome, and metabolome interact with the environment via effects on plant size and surface characteristics.

Testing plants for drought resistance

In the papers reviewed, transgenic (GM) plants are compared with the WT, parental line. In some studies, transformation controls (i.e. ‘empty vector’ plants), which have been subjected to all transformation and cultural procedures but lack the transgene, are also included. This may allow the effects of the transformation process to be identified. In some cases, comparison is made between a well-watered and droughted transformant ([B.Z. Xiao et al., 2009](#)) not directly with the WT: care is then needed in interpreting what ‘DR’ means and its significance. Often drought treatments are extremely complex, e.g. exposing small plants to polyethylene glycol (PEG) or mannitol for a period, re-watering, then transplanting before photographing ([Priyanka et al., 2010a](#)) and thus difficult to interpret.

Studies based on soil water depletion

This is the predominant method for assessing DR, so brief consideration of the way that water is held in soils and the relationship between water content and matric potential of soils or

horticultural compost is necessary as it is not considered in the GM literature. The topic has been exhaustively described with physical rigour: basic information is given in many text books (White, 1987; Scott, 2000). Water held in spaces (pores or voids) in the matrix is relevant for plants. The proportion of spaces to particles and their geometry (pore diameter, tortuosity, etc.) depend on the type of soil. Coarse sand has relatively large spaces with wide pores, and fine sands have less space, with smaller pores. Clays and silts have very small particles with less space, connected by very small pores. Organic soils and composts—of the type often used in GM studies—generally have many large pores between fibres and few within them. The void volume determines how much water may be held in a given volume of soil. In soil saturated with water, all the space between the particles is occupied, so there is no gas phase. Under gravity, water in saturated soil drains quickly from the largest pores (which fill with air), then more slowly from increasingly smaller pores, because the forces (capillarity and adsorption) holding the water in them is less than the force due to gravity. Eventually the force holding water within the pores equals gravity and no more water drains. The water content of soil (Θ_s) in a pot is then called ‘pot capacity’ and is a useful baseline for starting experiments at known Θ_s and water potential (ψ_s). Soil in the field also drains under gravity: drainage stops at ‘field capacity’. To remove more water from soils at pot or field capacity, ψ_p must be more negative than the ψ_s . Eventually Θ_s cannot be further depleted by the plant, giving the ‘permanent wilting point’. The relationship between Θ_s and ψ_s is extremely non-linear. Figure 2 shows the water content of a typical soil in relation to the potential of water in the matrix, generally called the ‘soil water characteristic curve’. The large decrease in ψ_s occurs over a narrow range of Θ_s . Also, conductance of soil to water movement decreases greatly ($\sim 10^4$ -fold) and non-linearly as Θ_s decreases. The soil water characteristic curve is essential for interpreting plant, specifically GM, responses to drought. A slower rate of total water loss from a GM plant compared with the WT slows the rate at which the transition from wet to dry soil occurs, giving the appearance of a large difference in DR. The characteristic curve also explains why decreasing plant (root) water potential may have limited benefit when roots exploit a small volume of soil. The amount of additional water obtained beyond the transition from wet to dry is generally very small compared with total water loss, so the metabolic cost of increasing the concentration of osmotica in cells to decrease π and thus ψ_p whilst maintaining cellular relative water content (RWC) and turgor is not commensurate with water obtained. However, there may be advantages when roots exploit a large volume (especially greater depth) of soil, as in the field (Kell, 2011). Clearly, this requires analysis under appropriate environmental conditions.

Experiments using soil water depletion

In a large proportion of GM studies, GM and WT plants are grown individually in small pots usually filled with the same volume of potting compost often with large organic matter content or in vermiculite: both have large pot capacity and a very marked transition between wet and dry states (see Fig. 2).

Soil density is not generally controlled so may contribute to variation in plant responses. Pot size is roughly scaled with the plant’s size—*Arabidopsis* is grown in very small pots or with many seedlings together in one pot, and individual or a few rice plants are grown in larger ones. Some studies (e.g. Hou *et al.* (2009); Yue *et al.*, 2011) do not grow GM and WT plants separately but in the same container, assumed to give a more direct comparison, although it is inherently a more complex system than single comparisons and potentially more difficult to evaluate.

Soil is watered and allowed to reach pot capacity (often over several hours in darkness) as a reference point. One group of plants (control or check) is maintained at or near this state. For another group, the droughted plants, watering is stopped: responses of both groups of plants are measured until wilting is observed in the droughted plants. Physiological, metabolic, and other functions are measured [e.g. infrared gas analyser (IRGA) systems are used to determine water vapour loss from which T and g_s are calculated, and CO_2 exchange from which A and respiration/unit LA are obtained]. From A and g_s the substomatal CO_2 concentration (c_i) is calculated. These measurements apply, strictly, only to the conditions within the chamber in which they are measured. Also chlorophyll a fluorescence alone (Hideg *et al.*, 2003), or combined with gas exchange, may be used to assess photosynthetic competence. The advantages of the single-cycle ‘drying-down’ approach to establish drought resistance are ease, apparent simplicity, and clarity of the method. Also soil is the ‘natural’ medium from crop plants, with no problems of root aeration. However, the method gives results which require careful analysis.

Delayed appearance of stress symptoms shown by GM compared with WT plants (Valente *et al.*, 2009) and shown

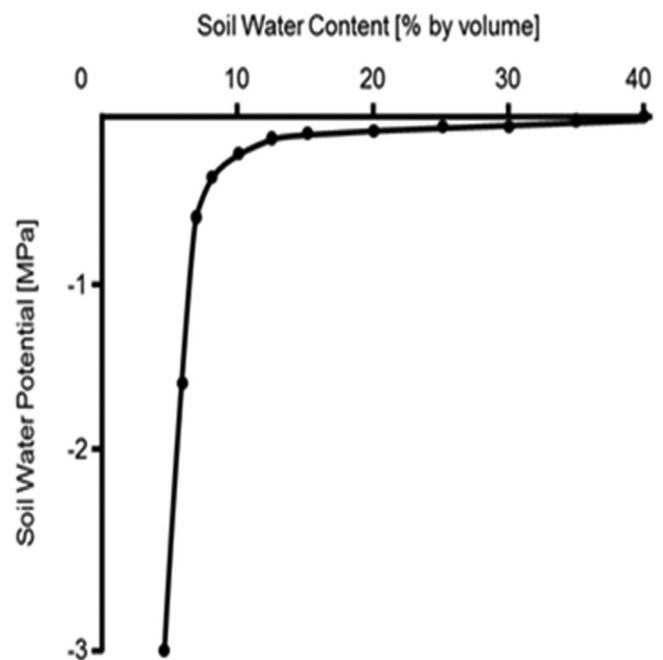


Fig. 2. Soil water (moisture) characteristic curve of a sandy-loam soil, demonstrating the extreme non-linearity which is so important in plant–water relations.

schematically (but based on photographs in the literature) in Fig. 3, is explicable simply as a consequence of maintenance of water uptake by the GM sufficient to balance water loss for a longer period. With cessation of irrigation, water is removed from the soil at a rate depending on the total amount of water lost by the plant to the atmosphere (that from the soil surface is relatively small and is usually ignored). Total water loss from the plant is a function of LA, g_s , and environmental conditions, so is difficult to control, and varies greatly between experiments and during them. In simplified form:

$$\text{Total water loss over period} = T \times \text{total LA} \times \text{period of water loss} = \text{kg m}^{-2} \text{ s}^{-1} \times \text{m}^2 \times \text{s} = \text{kg}.$$

Thus both total LA and T are critical in determining total water loss. IRGA measurements of T are often equated with the rate of water loss from the whole plant, but should not be so used as the conditions and duration are very different and—crucially—do not account for LA. The transpiration rate/unit LA is not only affected by g_s ; cuticular conductance (largely determined by the epidermal surface waxes) may be important when stomata are closed, and trichomes and other structures on the surface may affect the boundary layer conductance and also alter the albedo of the surface (and hence leaf temperature). Differences in plant architecture, for example the compact rosette compared with the erect form in *A. thaliana* or caused by GM-induced alterations to growth, may determine exposure of the leaves to light and alter air movement and thus the boundary layer conductance. Despite the known importance of these factors in water relations and the fact that they may be affected by transformation, the total LA and other characteristics are almost ignored in the GM literature as a potential cause of differences in DR between GM and WT plants.

The rate of soil water supply to the plant (and so of soil water depletion) depends partly on the amount of water available in the soil, on the volume of soil exploited by roots

(ignoring transport of water from outside the root zone as this is not applicable in the small pot studies examined, but important in the field), on ψ_s and ψ_p , and on the resistance to water transport between the bulk soil and the root vasculature.

$$\text{Total water uptake over period} = \text{rate of uptake/unit root surface area} \times \text{total root surface area} \times \text{period of uptake} = \text{kg m}^{-2} \text{ s}^{-1} \times \text{m}^2 \times \text{s} = \text{kg}.$$

Little attention has been paid to these aspects in GM studies, as it is assumed that roots fully exploit the soil and do not limit water supply, but Werner *et al.* (2010) ascribed DR to a larger root system resulting from modification to cytokinin metabolism. However, much attention has been directed towards metabolic changes, for example in metabolites such as proline which might alter plant π and so alter ψ_p and proteins such as aquaporins which may affect the conductance to water flux.

Late wilting and other symptoms of stress in GM compared with WT plants must be caused by a slower rate of total water loss, although it is considered in some studies that the GM plant retains water even when the soil water is depleted (see Gao, 2011). Figure 4A illustrates how Θ_s changes with time for a WT and a GM plant with a smaller rate of total water loss per plant. Small decreases in LA and/or g_s in the GM plant, which are difficult to determine experimentally, may accumulate over several days, resulting in much smaller total water loss. This explains the DR observed in many studies (e.g. Gaxiola *et al.*, 2001; Sakuma *et al.*, 2006; Xiang *et al.*, 2008; Bao *et al.*, 2009). Under 'normal' atmospheric conditions, the soil water characteristic means that ψ_s changes very little initially for GM and WT plants despite decreasing Θ_s , so their RWC and ψ_p remain large until the transition is reached. As the WT dries the soil faster than the GM, the soil very quickly (often in hours) goes from wet to dry, and ψ_p , RWC, and g_s decrease rapidly (Fig. 4B), accompanied by symptoms of stress, for example decreased A and increased non-photochemical chlorophyll a fluorescence (Q. Wang *et al.*, 2008; Woo *et al.*, 2008). Plants

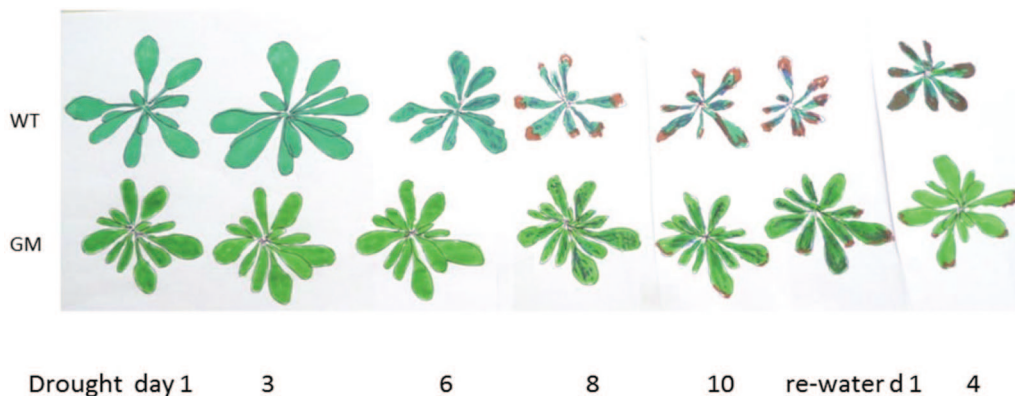


Fig. 3. Development of stress in droughted wild-type (WT) and genetically modified (GM) *Arabidopsis* (schematic, based on photographs in the literature). GM plants often appear similar to the WT, but leaf colour may indicate differences in growth. At day 1 the GM depicted has 20% less leaf area than the WT. However, because the architecture differs, the GM appears larger. The WT may continue to grow faster than the GM during the early period of soil drying. Without water, the WT wilts before day 6 and is severely stressed by day 10. In contrast, the GM loses water more slowly than the WT, so wilts later on day 8 and is not severely stressed even by day 10 when recovery from drought is tested. After 1 day of re-watering, the GM recovers rapidly but the WT is not able to do so even after 4 d.

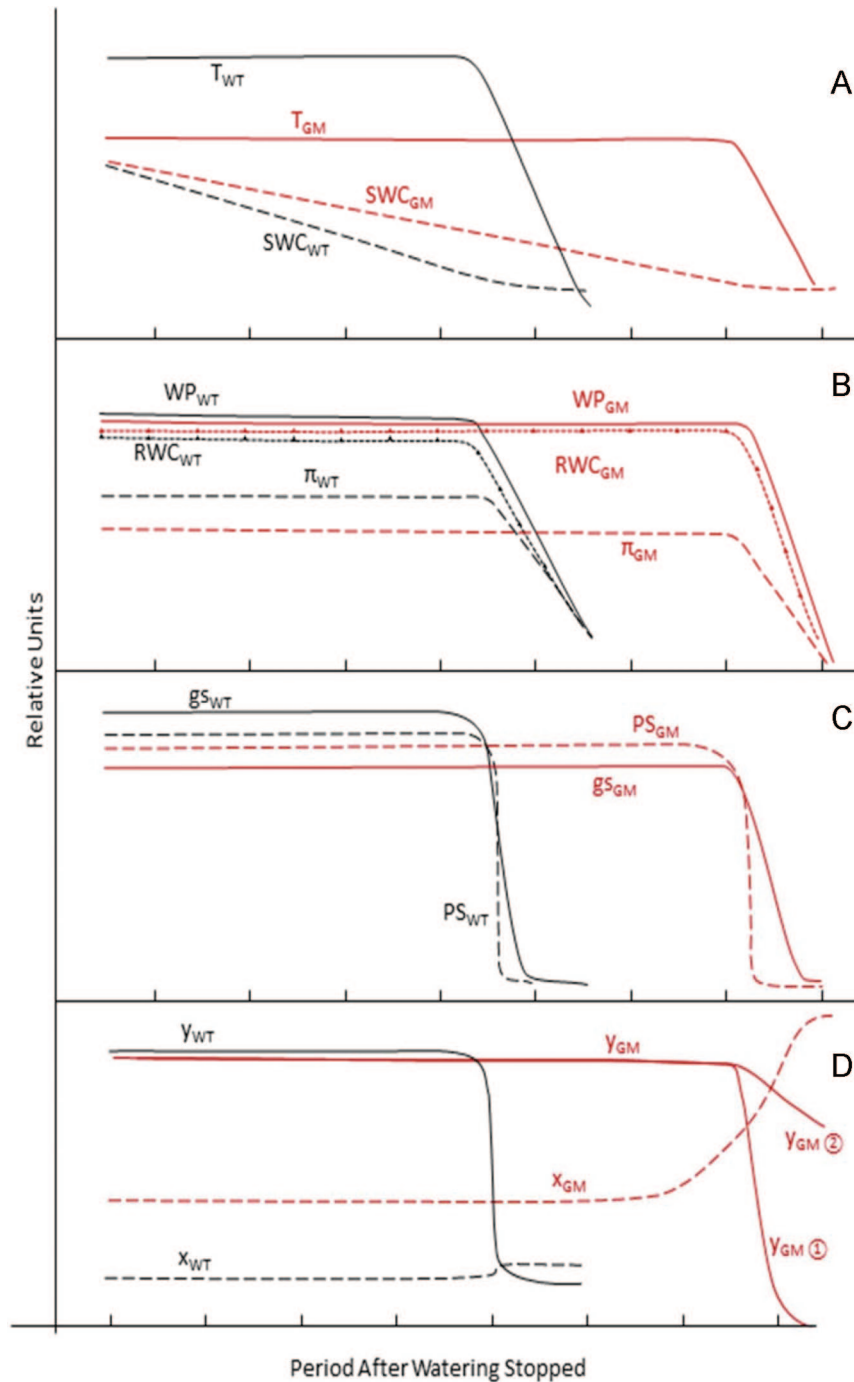


Fig. 4. Schematic of the most frequently used test for drought resistance in the GM literature. Interactions between water loss and soil water for wild-type (WT) and genetically modified (GM) plants are shown. Individual plants are grown in identical volumes of the same soil with full watering, following which, at time 0, watering is stopped and the plants dry the soil. The changes are shown relative to the WT. (A) Water loss (T mass per plant per unit time) and soil water content (shown as SWC , change in volume of water per volume of soil, abbreviated as Θ_s in the text). (B) Water potential (WP , abbreviated as ψ_p in the text), osmotic potential (π), and relative water content (RWC) of leaves. (C) Photosynthesis (PS , abbreviated as A in the text) and stomatal conductance (g_s) of leaves. (D) Metabolites X and Y of leaves. Two different responses of Y in the GM plant are shown (Y_{GM1} and Y_{GM2}).

may be fully watered and turgid one day and very dry and wilted the next, and leaves may die soon after, followed later by meristems. The GM plant undergoes the same process but with a delay, often of several days. As a consequence of the delay, metabolism of the GM plant is not affected when that of the

WT is greatly impaired. Photosynthesis of the GM plant may continue over the period when the WT is stressed (Fig. 4C), resulting in a larger GM than WT plant after a period and an apparent improvement in WUE of GM compared with the WT. Complex conditions and treatments, combined with

limited information about plants, generally make it difficult to evaluate if LA, g_s , or metabolism is responsible for the DR in many studies. As examples, better growth and survival of PARP transgenic *Arabidopsis* (said to grow as well as the WT) after drying, then re-watering, followed by drying (De Block *et al.*, 2005) was attributed to improved pyridine nucleotide metabolism. Later, effects were ascribed to changes in ABA metabolism, suggesting that stomatal behaviour was affected (Vanderauwera *et al.*, 2007). Overproduction of ABA in tomato line sp12 (Thompson *et al.*, 2007) decreased g_s substantially ($300 \text{ mmol m}^{-2} \text{ s}^{-2}$ compared with $550 \text{ mmol m}^{-2} \text{ s}^{-2}$) so improving transpiration efficiency. Also, LA was measured: it was 28% smaller than in the WT at the start of the experiment, so its water loss was less than half, thus conserving soil water. The decrease in ψ_p was delayed by 2 d and remained larger than that in the WT after 5 d, and the LA of the GM plant grew faster than that of the WT (which may have not been optimally watered according to the paper), possibly as a consequence. This work did not claim to have achieved DR plants, in contrast to many such studies. Transformation of soybean (Valliyodan and Nguyen, 2008) may also have resulted in DR based on these methods (Zhou *et al.*, 2008).

Recovery following re-watering is also taken as showing DR. Because all plants are (generally) re-watered once the last plants (the GM) have wilted (see Peleg *et al.*, 2011), the GM plants are only mildly and briefly stressed and so appear to maintain a larger green LA, g_s , A , etc., and recover much better than the WT (Rivero *et al.*, 2007), as illustrated in Fig. 3. Damage due to drought depends on the product of intensity of stress and its duration, so the WT may have been severely stressed for several days compared with the GM plants. Thus, there will be a very strong correlation between delay in wilting and good recovery, and, apparently, with the nature of the GM. Overexpressing a tonoplast membrane aquaporin from *Panax ginseng* (PgTIP1) in *Arabidopsis* (Peng *et al.*, 2007) shows the importance of LA. Under normal conditions, the GM plants developed faster, had larger leaves and roots, and were heavier than the WT. Consequently, in shallow pots, they were less DR than the WT, but in deeper pots they were more DR. This was attributed to deeper rooting and increased water channel activity in the transgenic. The latter overcame the rate limitation in the WT from inadequate aquaporin in both normal conditions and with water deficit (i.e. GM increased the speed of hydration). However, a change in plant size (Lin *et al.*, 2007), combined with the drought treatments, explains the results. Another example is the DR of a GM modified with LEA protein (Cheng *et al.*, 2002; Wang *et al.*, 2006; C.R. Wang *et al.*, 2009). Both the rate of stress onset and recovery serve, wrongly, in GM studies to reinforce the view that a GM has given DR. One of the weakest aspects of GM analyses is using time of appearance of stress symptoms after drought is imposed as a criterion of DR: it is not an adequate criterion to separate ‘drought resistance’ caused by delayed stress due to size from that caused by metabolic adjustments. Strict criteria are required.

Partial and repeated cyclic soil drying In addition to the ‘single cycle soil drying’ method analysed above, other methods have been employed. In partial drying, individual plants are

grown in pots of compost and maintained at a subsaturating Θ_s to give a particular water status, and measurements are made. At the start of an experiment, the pot capacity (mass of soil+plant+water) is determined as the unstressed reference point. This is often taken as the Θ_s of the wettest pots (Giri *et al.*, 2011), which may bias the treatments in favour of plants which have lost least water, as occurs in GM studies (see later discussions). Plants then deplete the soil and, after a period, the mass is again determined. The water content at this state is usually given as a percentage compared with the mass at pot capacity. When a desired percentage water content (e.g. 10, 20, or 30% water per unit mass or volume may be used) is reached, further depletion over a period, usually hours or days, decreases the Θ_s . Then the pot mass is returned to the desired percentage by re-watering back to the required mass. This may be repeated with varying frequency depending on many factors, principally soil volume, plant size, and environment, and, although the deviation from the set point may be large, it is then claimed (X.W. Xiao *et al.*, 2009) that the plant has been maintained at a specific, constant, water status (statistical evaluation is neglected). However, this is not possible. In dry soil, the change in plant water potential (ψ_p), etc. is very large because of the soil water characteristic curve (Fig. 2). Also, to maintain a uniform, subsaturating Θ_s by application of a small volume of water (which does not saturate the whole soil volume) to dry soils is impossible. All that is achieved is a zone of almost saturated soil separated from a zone of dry soil by a narrow intervening transition zone, the wetting front. This is fully established in the soils literature (Scott, 2000), as are implications for plants (Kramer and Boyer, 1995), yet this is too often disregarded in plant studies, including GM. The plant grows in a small volume of soil at large Θ_s (freely available water), and is unstressed for as long as water is sufficient to meet the demand, after which drying occurs and it is stressed, affecting growth, etc. Effects depend on the relative durations of wet and dry periods, and on the severity of the water deficit and stress induced in the plant. It may appear that water applied to the soil surface is distributed uniformly, but it is not: water may run down large voids (e.g. between the pot wall and soil mass which is often large as the soil dries) and give the visual impression of uniform watering. Practical difficulties also arise. Comparison of different plants (e.g. GM compared with the WT) is difficult as watering is often based on the fastest or slowest rates of loss, not on the requirements of each plant (which is laborious and time-consuming) which is essential. If a plant with small LA is compared with one with a large LA, the latter will experience more accumulated stress and so be more badly affected, and the smaller plant will appear more ‘drought resistant’ (e.g. smaller relative decrease in plant growth, and better—more rapid—recovery) than the larger. Interpretation is more difficult than with simple drying, and although there may be similarities to the field—periods of rainfall which wet the soil surface for example—the results may not be directly applicable to it because of large differences in frequency, severity, etc. Partial drying is favoured in automatic systems (Granier *et al.*, 2006; Berger *et al.*, 2010) which weigh pots at set times, calculate water loss,

and apply water to pre-determined mass. Interpretation of the effects on the plants requires considerable caution, but this is ignored.

Osmotic control of plant water status

Comparisons of GM and WT plants and their mechanisms of response to drought are required at the same plant water status (e.g. ψ_p and RWC). Because of the transfer resistances and capacitances in soils, control of plant water to a known, constant, value is extremely difficult and virtually impossible during the transition between well-watered and fully droughted states (see above). By growing GM and WT plants in identical environmental conditions in solutions of solutes such as non-metabolized sugars (e.g. mannitol) or large molecular mass artificial polymers (e.g. PEG) of known osmotic potential, it is possible to alter their water status readily to particular values and maintain them (Lawlor, 1970). Treatment may be applied at specific times, in one step or gradually, and plant water status remains essentially constant (solutions can be renewed easily). Small and large plants may be compared and concentrations may be maintained relatively easily. However, clarity is required over the method: transferring plants from soil to PEG solution, as seems to have been the case in a study of tobacco (Yue *et al.*, 2011), may result in physical damage to roots and uptake of PEG, with deleterious effects, long known (Lawlor, 1970). Osmotica are assumed not to be broken down (but mannitol may be by bacteria) or enter the root, but this may occur. Another disadvantage is immersion of roots in liquid which greatly decreases oxygen supply, so vigorous aeration is essential. Mannitol and PEG are incorporated into agar gel in which callus or very small seedlings (Li *et al.*, 2010) are grown, for example rice in agar containing PEG (Park *et al.*, 2010), or moved (Verslues *et al.*, 2006) with possibility of damage. Mannitol was applied to soil in some studies (Begcy *et al.*, 2011) and appeared to damage WT tobacco more than the GM. GM plants may be favoured if they have smaller LA and g_s than the WT, as the uptake of solute may be smaller. This effect may also be important in the response of plants to salinity. Despite the apparent advantages over drying of soil, limited use of osmotica may be explained by difficulties with experimentation, and it is considered 'unnatural'.

Terminology and definitions of drought, stress, and drought resistance

What is the nature of the drought resistance considered to have been achieved with these many transformations which the above analysis shows to be a consequence of slower water loss? In GM studies 'drought' and 'drought resistance' are approached with their particular, simplified views of how plants interact with their environment despite critical analysis (Verslues *et al.*, 2006). Earlier work in the plant and agronomic sciences (Kramer and Boyer, 1995) paid considerable attention and effort to defining them in theoretical and practical terms, and efforts continue (Salekdeh *et al.*, 2009). Here it is necessary to consider drought, drought tolerance and resistance, and drought stress.

Drought

Drought is defined in many ways, depending on a number of factors, for example country and affected process: see Wilhite (2005) for full discussion of drought, definitions, and consequences. In meteorological terms, drought is the deficiency in water supply (precipitation, i.e. rain, snow) compared with a measure of the supply, such as long-term annual rainfall. In the more agronomic and physiological literature, drought is the water deficit which impairs plant growth and yield compared with the supply required for maximum or optimum growth, etc. The concept is complicated, as a crop may absorb water from the soil or water table, even when rainfall is zero in an area where it is normally good, so there is substantial drought on the first definition but the crop has adequate water. The amount and timing of rainfall relative to evaporative demand are known for most geographical regions, as is soil water and rooting volume for particular crops; thus statistical methods are used to determine probabilities of drought, both timing and severity (Price *et al.*, 2002). Both are very important in relation to developmental stages of plants, are well understood, and must be considered in any meaningful analysis of responses of plants (Witcombe *et al.*, 2008) including GM (Toenniessen *et al.*, 2003). However, in the GM literature, there is little discussion of how drought (timing, duration, and intensity) affects specific processes such as development and growth of vegetative (root and leaf) and reproductive [i.e. flowering, fertilization, seed set, filling, and maturation (Georges *et al.*, 2009)] organs. In terms of crop production, all of these may be crucial. Drought is largely treated as a simple factor—cessation of watering—and focuses on generic changes in metabolic processes, with the implication that they will provide DR under all conditions.

Drought resistance

Drought resistance is used without qualification. From the *Oxford English Dictionary* definition of resistance, DR is the ability (capacity) to oppose drought successfully and to prevent the effects of, and be proof against, water deficit. Thus, a truly DR plant would not be affected by a decrease in water supply, which is unrealistic. DR is a quantitative trait, expressed not as an absolute but relative to a control value, for example response of a GM plant compared with the appropriate controls (WT and empty vector) under a defined drought in comparable conditions. So, a DR plant would produce the same yield as the well-watered WT with optimal water supply (zero water deficit), but with 50% average rainfall it would yield 25, 50, or 75% more than the WT, which itself would produce only 50% of the yield compared with optimal watering. The term 'drought tolerance' is often used: it implies ability to sustain or bear drought without harm or suffering. So a drought-tolerant crop would have the capacity to withstand a water deficit without damage. Again this is quite unrealistic: from crop physiology, it is to be expected that varieties (GM or selection bred) for water-deficient environments will have smaller yield potential than those where water is abundant. Resistance and tolerance have a similar meaning: ability to withstand and be unaffected by drought or water deficit. 'Drought tolerance' also suggests an innate, perhaps metabolic,

ability to overcome drought, and this is discussed later. The GM literature is not characterized by critical quantification of ‘drought’ or ‘drought resistance/tolerance’ which is often treated as a constant, fixed, value inherent in the genome.

Definition of stress

This is the response (usually negative compared with the well-watered control plant) exhibited by the plant’s (cell, organ) functions to decreasing water content and free energy of water in the environment and within the plant (for a discussion of plant–water relations, see [Kramer and Boyer, 1995](#)). Stress is a plant physiological and biochemical phenomenon, involving alterations in structure and function at all levels of organization, from large molecules such as proteins and lipids, and aggregations of them in membranes, to the more complex organelles (chloroplasts, mitochondria) and then to cells, tissues, and organs, through to the whole plant. There are large interactions between these scales and processes.

Types and mechanisms of drought resistance

[Levitt \(1980\)](#) and [Kramer and Boyer \(1995\)](#) clarified and defined many aspects of plant responses to water. Subsequently, terminology has become simplified and less exact. Avoiding unnecessary terminology and definitions for processes which are quantitative and interacting, and considering fundamentally important cellular biology, it may be said that maximum growth and production of plants, and especially of crops with large yield potential, requires that tissue and cellular water contents and ψ_p and π are maintained at or near the maximum (optimum). In all environments, LA and g_s determine water loss, and the root system determines water uptake: these are probably optimized in relation to environmental factors together with the capacity of metabolic processes and their regulation. The GM literature largely focuses on metabolic processes and adopts a simplified terminology. From the above, DR may be attained in several ways which are not distinct, but are quantitative traits ([Blum, 2005, 2011a](#)). [Yue et al. \(2006\)](#) consider three aspects: drought escape, drought avoidance, and drought tolerance. An addition is drought survival. Generally, GM studies do not adequately test or evaluate the type of DR achieved.

(i) Drought escape (DE) is characterized by the timing and duration of growth (phenology) to coincide with water supply which is adequate for optimal production by adapted genotypes. Plants and crops are therefore unaffected by drought which may occur in the area at other times; that is, they ‘escape’. This is extremely important ecologically and in agronomy. In annual crops, such as cereals (but even in perennials), the growth cycle generally coincides with average climate/weather conditions, for example vegetative growth exploits the rainy period in regions with pronounced wet and dry seasons and grain maturation occurs in the dry period. Variations in rainfall patterns may subject crops to drought, so DE is a

quantitative characteristic, subject to statistical variation. It is not considered in the GM literature reviewed.

(ii) Drought avoidance (DA) is shown by plants which grow in periods of drought but maintain water status, generally by the following methods. (a) By restricting water loss (transpiration) and conserving soil water, with a smaller LA and g_s . Here it should be emphasized that in agriculture (as also in natural vegetation) it is the LA per unit area of ground surface, the LAI, and its retention over a period, the LA duration, which are the important features determining water loss over a period. Decreased LA and LAI may arise in the early stages of slowly developing drought by production of fewer, smaller leaves, and later, with more severe drought, by senescence of older ones. In addition to these, a smaller g_s decreases water loss, as do folding, drooping, and rolling of leaves. Generally these changes decrease A and photosynthesis per plant, and there is loss of productivity per unit land area. (b) By increasing water supply, with deeper, denser rooting to exploit water in the soil or from a water table. Again, this is a very important mechanism ecologically. (c) Water storage in organs (stems, trunks) is important ecologically but less so in agriculture. In (a) and (b), it is the size and functions of the plant’s organs which determine the water balance. It must be emphasized that the primary interaction of the plant with the environment is via the size of the organs, as seen in crop responses to water deficits in the field ([Legg et al., 1979](#)). Of course, the surface characteristics (e.g. g_s , cuticular conductance) of the organs are also very important. Metabolic mechanisms, encoded in the genome, determine when organs are made, how many, and their potential size and characteristics, but it is the effect of the environment on the metabolic mechanisms which determines the actual size and characteristics of the plant’s organs. These interact with the water supply. In the GM literature reviewed, it appears that the many metabolic processes modified affect the size and surface characteristics of plant organs, which will be considered later, yet interpretation has focused on metabolic and cellular processes.

The term ‘drought avoidance’ is not particularly appropriate as plants do not ‘avoid’ (= to go out of the way of) drought which may occur, but rather exploit alternative water resources (e.g. stored in the soil) so delaying and minimizing the development of plant/cell water deficits and stress effects. In many GM studies, for example, watering is stopped (i.e. drought is imposed) but plants continue to transpire using water from the soil, so they have not ‘avoided’ drought. ‘Avoidance’ also implies that the mechanism is so effective that plants experience no adverse effects: this is unlikely. ‘Delayed stress onset’ is the term which I suggest should be used to emphasize the condition, as it better encompasses the mechanism. It is a quantitative trait. [Peleg et al. \(2011\)](#) recognized that ‘plants exhibited delayed response to stress’ but ‘delayed stress onset’ is actually a different concept—depletion of water reserve induces water deficit and stress in the plant, rather than ‘stress’ being applied (see [Lawlor, 2009](#) regarding this usage) when it is actually water supply which is stopped leading to a deficit in the soil. Delayed stress onset is a dominant form

of adaptation by current crops, so is its achievement by complex GM technology (apparent in many studies; see Karaba *et al.* 2007) an advantage? It is necessary to emphasize that delayed stress onset should not be identified with a single cellular/metabolic mechanism. Indeed, as the earlier analysis showed, it is a consequence of several gene-encoded mechanisms which, probably in combination, affect the plant's LA, stomata, etc., thus restricting transpiration. Thus adverse water deficits are delayed and may be less severe. Also, roots may exploit a larger volume of soil and so obtain more water but, as mentioned, are hardly considered in the GM studies analysed. In mature plants, adaptation may be rapid, with decreased g_s initially (a characteristic of rapidly developing water deficit which is the predominant form of drought seen in the studies reviewed) followed by senescence of old leaves. In young, growing plants, leaf growth slows and ceases rapidly (as well as or before g_s) followed by senescence of older leaves. So the number and size of organs are affected. These aspects of plant and crop growth are responsible for altering water loss relative to supply over the longer scale (weeks or longer for annual crops). They have evolved within the constraints of water supply and evaporative demand, and it is probably incorrect to view changes in metabolism as a direct route to DR—the route is via LA, g_s , etc. The main feature of GM plants is slower water loss (Peleg *et al.*, 2011) resulting in delayed stress onset.

- (iii) Drought survival (DS) is a form of DR in which cells, tissues, and organs which have ceased growing under drought (quiescent state) are able to maintain key cellular functions and recover rapidly to pre-deficit values with minimal damage, allowing resumption of activity (e.g. photosynthesis). DS of this type may be metabolically very similar to, but should be distinguished from, processes in organs such as meristems and bulbs which have the ability to grow after a quiescent period. Interestingly, the latter indicate that even mesophytic plants have the capacity to develop survival mechanisms, albeit not associated with growth and rapid production. There is considerable emphasis on DS in the GM literature, for example exploiting resurrection plants in selection of candidate genes for DR, but the mechanisms may not be compatible with the large production required of crops. It may be valuable in particular forms of agriculture, allowing recovery after extreme drought.
- (iv) Drought tolerance (DT) is often used to suggest a metabolic mechanism for DR. As DT and DR are often used interchangeably and are poorly defined, the terminology needs to be improved and be linked to processes more closely. The GM literature places great emphasis on metabolic mechanisms to achieve DT, aiming for modifications to the genome which alter the proteome and metabolome in such a way that cellular mechanisms are regulated to maintain the cellular water status or, even more fundamentally, to maintain metabolic activity during water deficits which then do not (or minimally) impair overall functions in cellular or higher organization. Mechanisms considered to confer DT by preserving cellular (tissue) water content and turgor, even when water supply is limiting, include decreasing π with proline or increasing conductance of cells to water with

aquaporins. Particular importance is attached to regulation of metabolism, for example maintenance of A when g_s decreases at small RWC. If potential A is large in bright light but g_s is small, then CO_2 supply may limit A . Energy capture by chlorophyll may exceed energy use, for example in CO_2 assimilation, which increases generation of ROS with consequent adverse effects on photosynthetic and other cellular mechanisms, for example photoinhibition of photosystems (Hideg *et al.*, 2003; Demmig-Adams *et al.*, 2006; Shi *et al.*, 2007) and damage to ATP synthase (see Lawlor and Tezara, 2009). Protective and regulatory mechanisms altered in GM plants include those which increase energy dissipation and regulate the energy balance of cells (e.g. the xanthophyll cycle in photosynthetic tissues) and those preventing production of—or enhancing breakdown of—ROS (Hou *et al.*, 2009; Melchiorre *et al.*, 2009). Increased enzymes such as superoxide dismutase, and metabolic systems such as the ascorbate and glutathione cycles, and those protecting synthesis of cellular proteins (e.g. chaperone proteins) are also targeted. Alterations to energy and reductant metabolism (e.g. De Block *et al.*, 2005; Liu *et al.*, 2009a) fall into this category. These are the types of GM plants outlined earlier.

The GM literature is unclear about what DT does or may do. Is it based on the ability to survive, as in the 'resurrection plants' in which metabolism may adjust very rapidly to cell and tissue drying and remain viable at extremely small water content and very rapidly rehydrate and regain full metabolic function? Or is it the ability of GM plants to remain more (and if so to what degree) or fully productive when tissues/cells become water deficient compared with the WT? What drought and water deficit is DT expected to protect against—moderate or extreme (Padgett *et al.*, 2010)? A truly DT plant, unaffected in growth and production by the water supply, is the form of 'drought resistance' which is the holy grail of current plant science and of GM technology. Plants with such metabolic mechanisms would be able to function without reference to the environment, but this is quite unrealistic. The characteristic could be characterized as constitutive (intrinsic, inherent) metabolic dehydration or stress tolerance. Here 'metabolic' must be considered to include all processes, including those determining growth, size, and function of organs which interact with the environment. The term 'constitutive metabolic dehydration tolerance' will be used, abbreviated to CMDT. CMDT is a quantitative characteristic based on a very complex interaction of many metabolic processes.

Methods of testing for delayed stress onset and constitutive metabolic dehydration tolerance

The GM literature has not unequivocally demonstrated CMDT because the required comparisons of GM plants with the correct controls (WT and empty vector) to distinguish between the differences in size and g_s responsible for delayed stress onset and the metabolic processes leading to CMDT have not been made. An example is given by maize transformed in sense and antisense orientation with a gene coding for phosphatidylinositol-specific

phospholipase C (C.R. Wang *et al.*, 2008). The sense transformant had slightly better performance than the WT and that was better than that of the antisense in pots in the field under drought. Aspects of water relations were modified (decreased π), suggesting that CMDT was enhanced. However, it is not established that delayed stress onset was not responsible as g_s of the antisense was greater than that of the WT and that was greater than that of the sense plants. Delayed stress onset could be responsible as they were not compared at the same water status: critical tests are required. To assess experimentally whether CMDT occurs in GM plants and to analyse the mechanisms, it is essential to remove the effects of delayed stress onset and compare GM and WT plants at the same plant water status over the same duration (time period): this is very difficult to achieve. The GM and associated literature (see Salekdeh *et al.*, 2009) has not identified this crucial point.

Soil water content is not a good basis for comparison as it is not closely coupled with plant water status, due to effects of differences in T , transfer conductances in the soil and plant, and also because roots may have access to wet(ter) soil as the root zone dries unevenly (from the soil surface down), thus greatly affecting interpretation. Also, Θ_s is difficult to measure accurately in drying soil (see soil water characteristic). The study (Merewitz *et al.*, 2011) of metabolite contents of a GM plant with increased endogenous cytokinin illustrates the difficulty of comparison: an RWC of 47% was obtained at 10% Θ_s for WT and 5% for GM plants, suggesting that comparisons at fixed Θ_s did not reflect the tissue water adequately, explaining part of the change in metabolites, such as amino acids. The ψ_s did not reflect a GM plant's greatly improved water status during drought which must have resulted from much decreased g_s (Rivero *et al.*, 2007).

The logical step, made many decades ago in the physiological literature (see Kramer and Boyer, 1995), is to compare metabolic and physiological functions at the same water status of the plant (or organs, tissues, and cells) in terms of the energetics of water (ψ_p and π) and water content (RWC, not water content/unit dry matter). Measurements on plants should be relevant to understanding the effects of GM and their interactions with water deficits, ideally testing clearly defined aspects of potential mechanisms. Examination of the literature indicates that many GM plants show, compared with appropriate controls, a plethora of changes in the genome, proteome, and metabolome, but the relevant measurements of water status are generally limited. Total water loss from plants is not measured during drying under the growing conditions in GM studies, but is essential to determine if delayed stress onset is the cause of the DR of a GM plant or if CMDT has occurred. Because of the complex nature of cellular water relations, there is uncertainty about what is the most useful measure to adopt as a basis for comparison (see Lawlor and Tezara, 2009), so ideally several should be measured and included in analyses of responses. RWC is a relatively easily determined value reflecting internal cellular water: it correlates well with ψ_p and π . The duration of water deficit is important, as effects on metabolism are related to intensity \times duration, so water loss of GM and WT plants should be the same. However, to achieve similar rates

the environment should be manipulated, which is demanding (see above on the use of osmotica). A compromise is to measure frequently, in replicated experiments using 'best practice' experimental methods, physiological, biochemical, and genomic processes across the whole response range. Measurements should aim to establish how transformation has affected a range of cell (proteome, metabolome) and tissue functions, and organ (leaf, stem, and flower) number, size, and structure, and ideally rates of growth and senescence. Unfortunately, even such an important feature as LA is generally neglected. Physiological functions such as A and g_s are important and valuable, especially when combined with chlorophyll fluorescence (Rivero *et al.*, 2007; Woo *et al.*, 2008).

Comparisons of GM and WT plants are generally based on measurements made at fixed times which do not correspond to the same water status in GM and WT plants, so a correlative approach should be adopted. Measurements are made for GM and WT plants frequently over the course of a drying cycle covering the range of plant water status (RWC, ψ_p , turgor, etc.). Data are then plotted as in Fig. 5, and correlated as a function of plant water status by rigorous statistical techniques. Comparison of slopes of regressions, or of fitted curves, provides a true test of difference in response between GM and WT plants and demonstrates specific differences, which may be further analysed. This approach provides the most direct, and informative, link between functions of the genome, proteome, metabolome, and phenotype and water status of the plant independent of the size and rate of water loss. Unfortunately, data sets generally lack detail: studies of metabolites, for example (Li *et al.*, 2009), may not have data on water status. A unique example of proper comparison of experimental data for a WT and a GM plant (in this case a mutant rather than transgenic) from the literature examined is provided by Estavillo *et al.* (2011). Their fig. 3A compares the concentration of PAP in a WT and mutant plant over a range of RWC, an excellent example of data being presented to test directly the effects of water status on metabolism. It shows a constitutive increase in PAP in the GM which did not correlate or interact with water status and suggests that DR was caused by delayed stress onset (see Supplementary data at JXB online).

Effects of GM on plants

Correct phenotyping is essential for comparison of GM and WT plants and the effects of water deficits (Bressan *et al.*, 2009). Established methods of growth analysis are generally destructive and not applied in the GM literature. Current emphasis is on 'high-throughput', automatic, non-destructive methods of phenotyping (Granier *et al.*, 2006; Berger *et al.*, 2010).

Development

Depending on the nature of the GM, development is often modified, for example earlier flowering in *Brassica napus* (Georges *et al.*, 2009), associated with DR and suggesting smaller plants. However, many GM studies are made during vegetative growth and few (Peleg *et al.*, 2011) address developmental and reproductive processes. Timing, duration, and intensity of drought

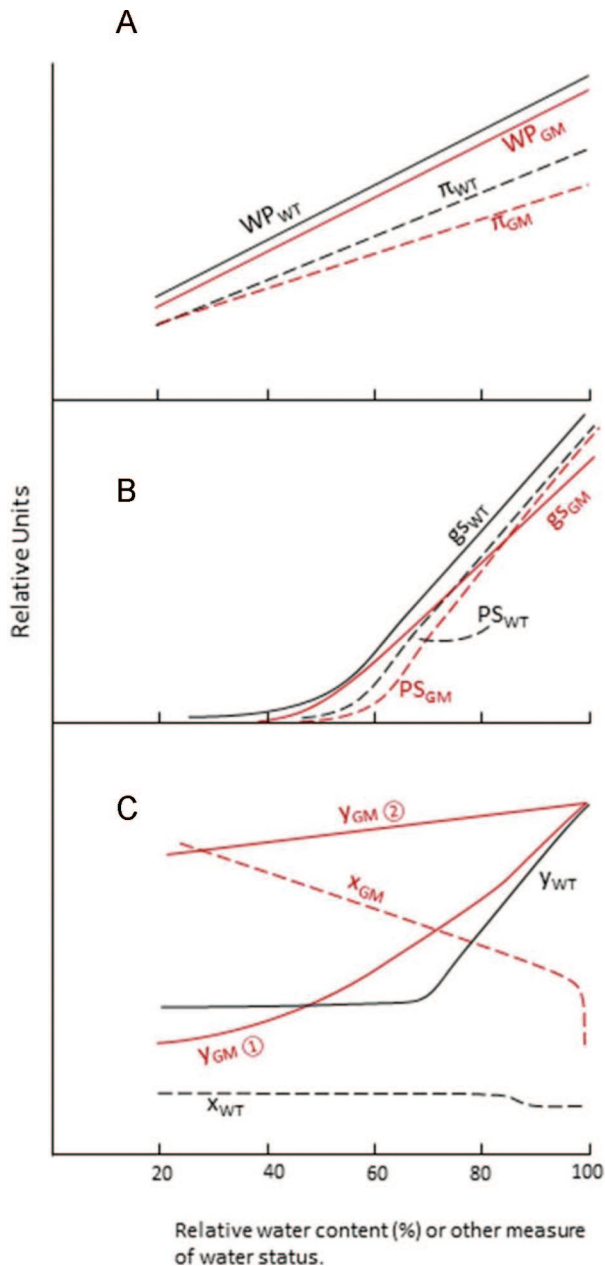


Fig. 5. Comparative plots of the data from Fig. 3. (A) Relative changes in water potential (WP) and osmotic potential (π) of wild-type (WT) and genetically modified (GM) plants as a function of relative water content (RWC) or other potential basis for comparison. (B) Relative changes in stomatal conductance (g_s) and photosynthetic rate (A) as a function of RWC. (C) Relative changes in metabolites X and Y as a function of RWC. The two different responses of Y in the GM plant are shown (Y_{GM1} and Y_{GM2}). From such an analysis, the changes caused by the alterations made to the genome and resulting from the water deficit may be determined. (A) This shows that the GM plant's leaves had a lower π than those of the WT when turgid but not when severely water deficient. (B) This illustrates that g_s and A were little affected by the transformation but greatly so by water deficit: interactions would be difficult to establish. (C) A theoretical situation where compound X is unaffected in the WT but increases greatly before RWC decreases in the GM, suggesting a major alteration in metabolism (with potential regulatory importance?).

relative to those of development are particularly important determinants of yield (e.g. of cereal grain) and will be most important for evaluating GM crops in the field, where applying defined (with respect to timing, duration, and intensity) water deficits to analyse the effects on specific developmental processes of GM plants is complex and difficult. Sampling and measurements are required frequently during a single drying period (erratic watering or rainfall greatly complicates interpretation, necessitating rain-out shelters in many environments) using well-established field methods (e.g. [Legg et al., 1979](#)).

Growth

Decreased growth is apparent in many GM studies ([Kasuga et al., 1999](#); [Karaba et al., 2007](#); [Nakashima et al., 2007](#); [B.Z. Xiao et al., 2009](#); [Lourenco et al., 2011](#)), including vegetative and reproductive organs, so that plants produce less total dry matter and yield. Shoot architecture may be altered: *Arabidopsis* may have more compact rosettes and rice more erect, bunched culms. Multiple modifications in rice ([B.Z. Xiao et al., 2009](#)) decreased yield per plant by 30–50% compared with the WT: the authors commented 'In fact, yield decrease seems to be very frequent in transgenic rice produced by tissue culture'. The relative yield (yield of stressed GM plant/yield of well-watered GM plant) was used as a criterion of DR, and several of the different types of GM plants had relatively larger yield with water deficit than the WT, probably due to the GM plants being smaller, losing less water, and so being less stressed, explicable from the soil water characteristic. However, the GM plants produced less than the WT. The method does not constitute a fair test of DR. In some reviews ([Roy et al., 2011](#)), the nature of DR claimed in studies such as that of [Nelson et al. \(2007](#); see [Supplementary data](#)) and [Rivero et al. \(2007\)](#) is not critically assessed.

Effects on growth, etc. depend on the nature of the GM. An example is that of overexpression of DREB1A with the constitutive 35S *Cauliflower mosaic virus* promoter which severely retarded growth under normal conditions. However, the inducible rd29A promoter had minimal effects on growth (although the evidence is weak) yet the plants had even greater DR ([Ma et al., 2010](#)). From the earlier discussions, the importance of quantification of LA and g_s is apparent and, indeed, decreased A suggests that g_s is smaller than in the WT. More attention in the GM literature to LA and growth would greatly aid interpretation of experiments.

Leaf area and structure

LA must generally be assessed from photographs (which seem obligatory in this literature but are a poor substitute for

Compound Y decreases in GM1 similarly to the WT, but in GM2 behaviour is very different, suggesting major alterations in metabolism with water deficiency (also with potential regulatory importance?). Such information might lead to understanding of the causes of altered whole-plant growth and size and thus of delayed stress onset.

quantitative measurements; Sakuma *et al.*, 2006) and has been quantified in very few studies. Thompson *et al.* (2007) over-expressed 9-*cis*-epoxycarotenoid dioxygenase and increased ABA content in tomato: this slowed early growth so LA was decreased by 28% at the start of a drying-down experiment with plants at the four-leaf stage but not later when LA was increased by 27%. Smaller LA and g_s accounted for a slower decrease in ψ_p in the transgenic. Decreasing cytokinins in tobacco increased root growth and also early shoot growth, and DR was observed (Werner *et al.*, 2010). Testing for DR was based on drying down very young GM and WT seedlings in the same trays for 26 d, observing wilting (which occurred in both types of plants but the frequency in each is unclear and also when it occurred) and then re-watering: recovery after 11 d was very much greater in the GM plants. The roles of the smaller LA and potentially smaller g_s (see below concerning g_s) in the GM in maintaining cellular water status were not established and the increased root growth was considered to explain the DR. In some studies (Jang *et al.*, 2003; Xiang *et al.*, 2008), assertions, without data, that no phenotypic changes resulted from transformation are not acceptable. In many studies, it is difficult to assess the initial size of GM relative to WT plants (Li *et al.*, 2009), particularly when other factors, such as the size of pots, cannot be compared (Begy *et al.*, 2011). Comparisons of growth after a period of drought and recovery are poor. A significant number of GM plants have (or appear to have) less total LA with fewer leaves of smaller area. Laminae are often thicker, with smaller, more closely packed mesophyll cells, and the number of stomata/unit area increases and sometimes their structure is altered (Holmstrom *et al.*, 1996; Goddijn *et al.*, 1997; Fernandez *et al.*, 2010). Transformation with HARDY, a gene with effects on cell growth (Karaba *et al.*, 2007), made cells smaller, giving stronger roots and decreasing LA. This suggests a common mechanism or link, via cell development and expansion, in the disparate types of GM. Few GM plants have increased growth: tomato with decreased inositol (1,4,5)-trisphosphate (InsP3) content is an exception (Khodakovskaya *et al.*, 2010), and this has predictable effects on water loss. Increasing ABA also stimulated growth in tomato (Thompson *et al.*, 2007).

Stomatal conductance

This is an important regulator of water loss, best rapidly measured with IRGA systems under the conditions of growth to minimize effects on stomatal aperture caused by changed conditions. A larger ABA content of tomato leaves decreased g_s more than A , so increasing transpiration efficiency (Thompson *et al.*, 2007). However, in many GM studies, g_s is often not well measured, although it appears to be frequently decreased (Belin, 2010). Water loss of detached leaves by weighing under conditions differing from those of growth is often presented, but is complicated by stomatal closure, changed conditions, and, above all, greatly compromised water status. Active stomata will close more than inactive stomata, so the method tends to underestimate water loss by WT compared with GM plants, obscuring the cause of delayed stress onset. Microscopic measurement of stomatal aperture

(M.R. Li *et al.*, 2011) may indicate responses but cannot substitute for g_s in quantitative evaluations of water relations. Many GM plants have impaired stomatal structure and function (Cui *et al.*, 2008; Gao *et al.*, 2011). Transformation alters stomatal size, density (stomata/unit LA), and index (stomata/epidermal cell), and possibly the mechanism of response to water deficits, etc., so g_s generally decreases. Despite the likely effects on phytohormone metabolism of transformation with a gene coding for isopentenyl transferase, and thus likely to decrease g_s , this was not measured and substantial DR was attributed to altering leaf senescence, the process targeted, although decreased A suggests smaller g_s (Rivero *et al.*, 2007). Decreasing the cytokinin content of tobacco increased root growth and slightly decreased early shoot growth (Werner *et al.*, 2010), but the effects on g_s were not examined, so its role in the DR observed in very young tobacco (with very small GM and WT seedlings in one container) is not established. Smaller g_s may be considered a cause of changes in metabolism (Lourenco *et al.*, 2011), rather than a consequence. Altering basic metabolism may affect much more 'distant' physiology, as shown by changing PARG1 which degrades poly(ADP-ribose) polymers in post-translational modification of some regulatory proteins (G. Li *et al.*, 2011). A mutation in the gene impaired stomatal closure of *A. thaliana* and inhibited growth, but overexpression gave a similar stomatal response and phenotype to the WT. The conclusion that PARG1 has a function in abiotic stress tolerance is only justified in that it affects stomatal function— g_s is actually responsible for regulating water loss. The effects of altering PARP metabolism suggest that changing energy balance (De Block *et al.*, 2005; Vanderauwera *et al.*, 2007; G. Li *et al.*, 2011) affects stomatal development (Bergmann and Sack, 2007), perhaps via ABA synthesis, and so produces DR. It is likely that many GM plants experience delayed stress onset from smaller g_s .

An example of the importance of stomata, if any is needed, and the potential for decreasing g_s is given by a study of the effect of the ERECTA gene in *A. thaliana* (Masle *et al.*, 2005). Mutants selected for large discrimination against ^{13}C , the heavier C isotope ($\delta^{13}\text{C}$), which results from large g_s (i.e. open stomata), have smaller transpiration efficiency (mol CO_2 assimilated/mol H_2O transpired) than the WT. Transforming the mutants with the ERECTA gene restored the WT. With greater abundance of ERECTA, WT performance was improved, attributable to effects on development of the leaves, for example increasing stomatal density, so explaining the increased g_s . This arose because epidermal cells were smaller, but the stomatal index was similar in the WT and mutant. ERECTA probably acts as a master gene which modulates stomatal density through changes in cell expansion, thus affecting mesophyll compactness and cell to cell contact. Also, there were effects on photosynthetic capacity and balancing of the biochemical and stomatal limitations of photosynthesis. Optimization was altered between processes in the leaf under a range of conditions (e.g. vapour pressure), and with drought (however, based on partial re-watering). This indicates that the strategy for improving WUE and DR by genetic modification should be linked to alterations in LA, g_s ,

and photosynthetic capacity. The analysis of ABA transgenics (Thompson *et al.*, 2007) shows the value of isotope (carbon and oxygen) discrimination in analysis of responses to water.

Photosynthesis

Measurements tend to show that A is maintained in GM plants of many types for longer than in the corresponding WT during drying-down experiments. GM plants are then regarded as DR. However, the conclusion that the GM directly improves metabolism and efficiency is questionable unless measurements are made at the same tissue water status (e.g. RWC, see above). This applies also to measurements of fluorescence when the water status of the tissue is not known, and the GM plant may have delayed stress onset (Hideg *et al.*, 2003). Also, in some studies, A may be increased in GM compared with WT plants. If the GM is responsible, directly, for improvements in A , then what is the mechanism? Also, if GM plants grow better under water deficiency than the WT, then their A must be relatively better (or respiration smaller, for which there is no evidence). An explanation, not examined in the literature although evidence is available (Georges *et al.*, 2009), is that the thicker leaves of GM plants have greater amounts of 'photosynthetic machinery' per unit area. This increases the potential and actual rate of A , if CO_2 does not limit, which it probably does not as measurements are generally made in weak light. Even if g_s decreases, small c_i (CO_2 supply) will only limit A in bright light. With light rather than CO_2 limiting growth, this would allow a larger A in a GM plant with small g_s . However, a small g_s would decrease T , giving the greater WUE seen. Comparisons of photosynthetic mechanisms between GM and WT plants over a range of RWC have not been made. However, detailed analysis of effects of increased ABA from GM plants on g_s (Thompson *et al.*, 2007) shows that T is decreased and A maintained, so increasing transpiration efficiency. The value of large A and small T , with increasing biomass and WUE for growth is obvious providing that there are no adverse genotype \times environmental interactions, such as poor control of leaf temperature or inadequate capacity for use of excess energy and prevention of ROS accumulation. These aspects have been considered in general for GM (Mitra, 2001) in relation to water deficits, but not experimentally in the field and certainly not there in relation to GM. Detailed analysis of photosynthesis and related metabolism has not been made in transgenics with putative DR.

The future

To ensure adequate staple food for the increasing human population in the next 20–50 years will require knowledge of GM plants plus assessment of their agronomic needs. Currently, poorly quantified claims to have made 'drought resistant' plants are based on laboratory studies and limited field trials. If GM plants with delayed stress onset prove adequate to increase yields under drought, then current technology and science may be said to have been successful for

practical agriculture. As oilseed rape and maize have recently been released for trials under field conditions, it would seem particularly important to assess their performance critically under a wide range of water availability and specific drought conditions in different environments. The relative efficiency of these crops in carbon assimilation and total biomass and yield production, and water saving and WUE, is of the greatest importance. Clearly, more in-depth analysis of the metabolism and physiology of GM plants than has been done is required to aid GM technology and to improve production of crops in dry environments. Attempting to genetically modify such complex, interacting metabolic systems in plants to achieve greater production under a variable environmental factor such as drought is a formidable challenge, probably requiring a considerable time. It is generally recognized that the genome, proteome, and metabolome interact non-linearly with environment: the consequences in moving from cellular processes to tissues, number, and size of organs and their physiology (physiome), and to a crop, have not been adequately explored but their determination is essential (Fleury *et al.*, 2010).

Common mechanisms, already identified in selection breeding, which might be directed targets (rather than incidental consequences) for GM to maintain or improve productivity (yield) under drought include the following.

- (i) Decreased water loss primarily by smaller LA (including leaf posture, rolling, folding, etc.), then smaller g_s followed by cuticular and boundary layer conductances (affected by leaf rolling, trichomes, etc.). As discussed at length, the GM changes already made have had this effect, resulting in delayed stress onset and also increasing transpiration efficiency. More detailed analysis of already produced plants may clarify the mechanisms operating. However, if LA (i.e. LAI and LAD) is decreased, so, often, is crop production, which depends ultimately on light interception (Monteith, 1965, 1977; Legg *et al.*, 1979). An important point which seems to have been overlooked in assessing the potential of GM plants, as crops, is that crops generally cover the whole land surface (unless sown or planted widely spaced as in drought-prone environments where water loss from the soil surface may be important) and at LAI >3–4, water loss is from the crop. So the characteristics of individual GM plants may not be appropriate for a crop. Well-established crop physiological methods are available to examine crop responses.
- (ii) Increased water uptake, by exploitation of greater soil volume and depth and thus stored water, through larger root systems (Kell, 2011) with greater root area per unit volume of soil (plus improved surface conductance). More extensive rooting has been considered (Song *et al.*, 2009) to improve DR. Werner *et al.* (2010) modified cytokinins and increased root size, which was associated with a greater proportion of seedlings of the GM plant surviving than those of the WT after a period of drying. The possibility that this was due to the slower growth and smaller size of the GM plants was not explored in detail, or under more realistic rooting conditions. The general benefits of increased root

- growth with specific GM will only be apparent if water is available in the soil at an appropriate ψ_s .
- (iii) Regulation of metabolism (mass and energy flows) to allow metabolism, when constrained by water deficits, to continue at, or close to, the well-watered state. Much greater emphasis on understanding CMDT is essential, using GM plants to quantify metabolism under water deficits and to optimize metabolism (especially energy capture versus use). Such optimization of water loss, supply, and metabolism is required to achieve and maintain sustainable productivity under water deficits in target environments. It may also address the problem of genotype \times environmental interactions.
 - (iv) Incorporation of novel genes into crop plants or alteration of gene number and expression may provide starting material for selection breeding using advanced methods combining GM with other techniques, as discussed for carbon isotope discrimination, and with proper physiological evaluation. A greater range of material for selection may be obtained and assessed for field production. In this way, GM technology may converge with 'classical' plant breeding. To quote [Bressan et al. \(2009\)](#) 'For a better understanding and rapid improvement of abiotic stress tolerance, it is important to link physiological and biochemical work to molecular studies in genetically tractable model organisms. With the use of several technologies for the discovery of stress tolerance genes and their appropriate alleles, transgenic approaches to improving stress tolerance in crops remarkably parallels breeding principles with a greatly expanded germplasm base and will succeed eventually'. How yields and CMDT of GM crops such as oilseed rape and maize, which have probably achieved a degree of delayed stress onset DR, respond to a range of drought conditions, from brief and mild to severe and frequent, has yet to be assessed quantitatively.

Application of current technology to integrated programmes of analyses of GM, and also promising non-GM, plants will be required to understand and improve production under drought. Theoretical and experimental studies at all levels of function—gene to organ—using, for example, *in silico* mathematical simulation modelling, will aid understanding ([Semenov and Halford, 2009](#)). Also, use of model species (*Arabidopsis*) plus computer modelling will 'facilitate the development of a virtual plant—a computer model...' and thus lead to engineering of 'the next generation of biotech crops' ([Zhang et al., 2004](#)). Without theoretical advances and improved methods of transformation (to improve insertion of genes into specific sites in the genome), GM may have few advantages over current breeding methods addressing key physiological process ([Reynolds et al., 2005](#)) and using molecular information ([Tuberosa et al., 2007](#); [Thomson, 2008](#)) such as quantitative trait loci and markers ([Kamoshita et al., 2008](#); [Ashraf, 2010](#); [Fleury et al., 2010](#); [Richards et al., 2010](#)). Evidence of successful non-GM routes with improved DR is provided by selection based on carbon isotope discrimination combined with measurements of growth and yield under field conditions ([Richards et al., 2010](#)). Combining GM plants and carbon

isotope analysis, as done very effectively by [Thompson et al. \(2007\)](#), under relevant field conditions with carefully devised and measured water relations studies may provide a rigorous way of detecting CMDT as well as delayed stress onset.

General conclusions from detailed analysis of GM plants

The conclusion of this analysis is that the many alterations to metabolism by GM have modified growth, giving smaller LA and g_s by largely unknown mechanisms, resulting in slower water loss and giving the form of DR to which the term delayed stress onset is applied. The relationships are shown in [Fig. 6](#) in greatly simplified form: the essential point is that the many alterations to the genome have affected metabolism in such a way that growth of organs is, generally, impaired. This may involve known mechanisms, such as ABA synthesis, which decreases g_s and increases transpiration efficiency but may decrease or increase LA depending on the ABA content. Effects of GM on the whole plant may involve less understood mechanisms involving energy and regulatory networks. Also, epigenetic regulation of the genome, discussed by [Madlung and Comai \(2004\)](#), may have consequences for organ size and function. If the 'normal' metabolism is close to optimal, then it is to be expected that altering metabolism by GM, perhaps rather considerably in terms of control and signalling mechanisms, will cause a decrease in growth and g_s . These interact with the environment—which results in the generic mechanism which explains the apparent success of GM to give DR. GM has not unequivocally produced a novel form of DR, despite the claimed potential. The consequences of the many specific changes in gene expression in terms of proteins, metabolites, etc. resulting from GM have not been evaluated sufficiently critically to say that metabolism has been altered to enable functions to continue without interference by cellular water deficits. The putative advantages of GM in terms of metabolic adaptations—called here constitutive metabolic dehydration tolerance—have not been demonstrated, as discussed, despite the apparent potential of GM (and claims made for its achievement) for introducing novel genes and thus metabolic systems for improving metabolic performance with increasing cellular water deficits. Intriguingly, attempts to alter plant size directly have been few, given the importance of the surface area for water loss. Also, there is poor evaluation of the consequence of transformation for development (e.g. earlier flowering) and growth (leaf expansion rates) and duration (e.g. leaf longevity/senescence), which have been neglected, perhaps because they are not important in short duration experiments.

GM is not the specific, targeted, and rapid method for giving DR once envisaged. The early assumptions and claims must be re-evaluated more objectively. Current knowledge is inadequate to enable further targeted refinement of the technology to allow metabolism of cells, tissues, and organs and their physiology under water deficits to be altered to make them less sensitive (or insensitive) to loss of water (i.e. to have CMDT). Therefore, further empirical transformations will be required, but they should be better evaluated physiologically. Given this evaluation of what GM has achieved, what is the novelty of

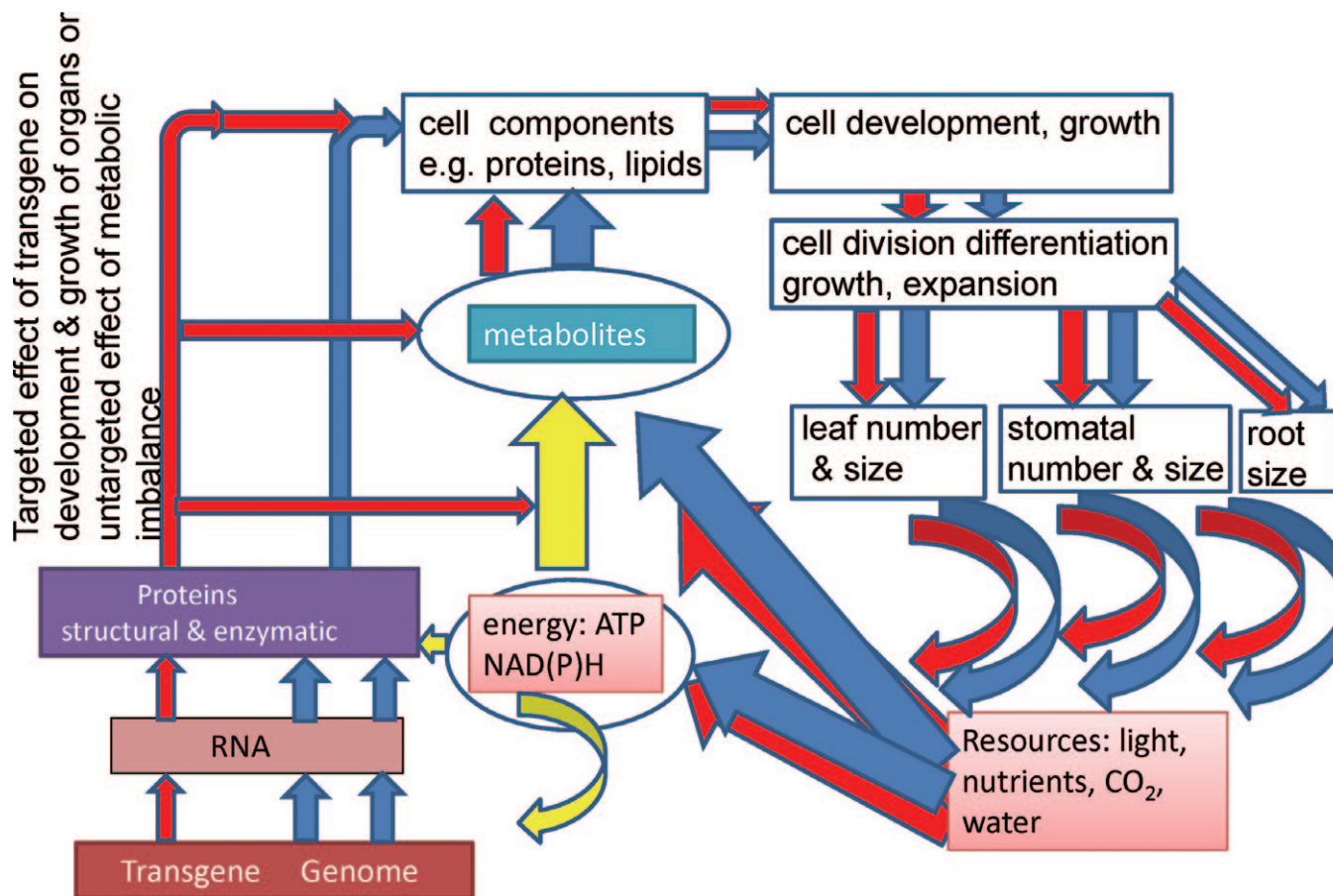


Fig. 6. The form of 'drought resistance' resulting from a considerable number of different genetic modifications is 'delayed stress onset'. This may be explained (and is shown by red arrows in the figure) by effects of transgenes or their interactions with other components of the system, or of the transformation process, on different parts of cellular metabolism. GM may be directed to alter specific processes which directly affect the plant water balance, for example by increasing ABA content to decrease stomatal conductance. However, other transformations directed towards metabolism, for example PARP and energy, may affect ATP and reductant. Such changes might affect growth factors (phytohormones) and the supply of key proteins and metabolites, etc. These changes are postulated to alter the balance between growth processes within the GM plant, development of stomata, etc. Therefore, growth is decreased, resulting in smaller leaf area and decreased stomatal conductance: both slow water loss by the GM plant compared with the WT. Consequently, droughted GM plants experience delayed stress onset compared with the WT.

GM compared with selection breeding? Delayed stress onset via smaller LA and g_s is also a primary mechanism by which species and non-GM crops have adapted, under evolutionary/human selective pressure, to water supply. Endowing current crops with truly CMDT may be very difficult and prolonged. Will GM crops eliminate loss of yield under severe drought? Can GM replace more classical agronomy and selection breeding? Can GM compensate for a long-term decrease in support for classical agronomy and selection breeding? The answer to these three questions is a guarded no.

Supplementary data

Supplementary data are available at *JXB* online.

A more detailed assessment of the literature is provided to show what was regarded as important in assessing the studies and the reasons for the conclusions, without overburdening the main analysis.

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