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Functional reversion to identify controlling genes in multigenic responses: analysis of floral abortion

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Abstract
In many situations, organisms respond to stimuli by altering the activity of large numbers of genes. Among these, certain ones are likely to control the phenotype while others play a secondary role or are passively altered without directly affecting the phenotype. Identifying the controlling genes has proven difficult. However, in a few instances, it has been possible to reverse the phenotype by physiological or biochemical means without altering the genetics of the organism. During this functional reversion, only a few genes may respond, thus identifying those likely to be controlling the phenotype. Floral abortion during a water shortage in maize is an example because the response is inherently multigenic, and the phenotype can be reversed by physiological/biochemical means. A recent analysis used this reversal to reveal that only a few genes are likely to control the abortion phenotype. In maize, these genes coded for a cell wall invertase (Incw2), a soluble invertase (Ivr2), a ribosome-inactivating protein (RIP2), and phospholipase D (PLD1). The invertases appeared to control the normal sugar uptake by the ovaries. Their down-regulation depleted ovary sugar pools and resulted in an up-regulation of the genes for ribosome-inactivating protein and phospholipase. The latter changes appeared to initiate senescence that degraded cell membranes, thus causing irreversible abortion. With these findings, these genes have become targets for preventing abortion. This approach might have value in other contexts with some additional methods.

Key words: Gene expression, invertase, maize (Zea mays L.), ovary, rice (Oryza sativa L.), sucrose, transcription, water deficit, water shortage, wheat (Triticum aestivum L.).

Introduction
One of the largest problems in biology is how to make sense of large numbers of genes whose expression changes when an organism is perturbed. These multigenic responses have become increasingly apparent as genome sequencing and mapping uncover tens if not hundreds of genes affected by simple, frequently occurring stimuli (DeRisi et al., 1997; Zinselmeier et al., 2002; Yu and Setter, 2003; Price et al., 2004). The complexity and sheer numbers of genes lead to long lists of up- or down-regulated genes that indicate the sweep of changes in gene expression. However, identifying specific controlling genes can be elusive.

Plant biology is no exception. Thousands of expressed genes may be operating in a tissue at a particular time, and hundreds of them may change expression when the plant environment changes. Constraints to productivity caused by drought or extreme temperatures, or improvements in yield or product quality have been difficult to pinpoint because suites of genes are typically involved (Calenge et al., 2006). Instead of targeting individual genes, it has been necessary to identify groups of genes usually by mapping regions of the genome active in the response (i.e. quantitative trait loci) or by using methods that do not require detailed knowledge of the controlling genes ( Tanksley, 1993; Koomeeef et al., 2004; Tuberosa and Salvi, 2006).

When detailed knowledge is available and plant responses can be attributed to a few specific genes, advances can be...
rapid. Examples are crops with improved insect or disease resistance that are increasingly popular in the USA and are traceable to one or a few genes (Witham et al., 1996; Hammond et al., 1998; Tang et al., 1999). By incorporating these genes using the tools of molecular biology and plant breeding, marked improvements in crop health have occurred. Another modification involving only a few genes can be seen with weed control. By incorporating a small number of herbicide resistance genes into major crops, the selectivity of herbicides is dramatically improved, typically with less herbicide use. Uniform, relatively weed-free fields are frequently seen and give further evidence of the value of this method.

In contrast, comparable progress with multigenic responses has not been rapid (Tuberosa et al., 2002). An obvious illustration is plant reproduction, which depends on a highly ordered development of male and female florets, release of gametes, and subsequent growth of the embryos. Large numbers of genes are involved, and knocking out individual genes may disrupt the process or be missed due to functional redundancy. Another important issue is the number and magnitude of genotype–environment interactions of major genes that influence reproduction in specific environments. In this situation, more information is needed before reproductive control can be understood.

Recently, a different approach was used to identify controlling genes in a multigenic response. It involves reversing the phenotype using biochemical methods based on knowledge of the function of the organism (Boyle et al., 1991a, b; Zinselmieier et al., 1995a, b, 1999; McLaughlin and Boyer, 2004a, b). By reversing the phenotype while maintaining the other features of the environment and genome constant, specific candidate genes are identified that control the phenotype. This review describes the concepts and some of the findings from this approach.

**Overview of functional reversion**

The approach, which will be termed functional reversion, involves three basic steps. In the first, likely physiological and metabolic functions are identified that are involved in the organism’s response to the perturbation. In the second, changes in gene expression are documented. If the changes are numerous, the third step is initiated and uses the likely physiological and metabolic functions from step one to reverse the phenotype. The reversion occurs without changing the genetic make-up of the organism (e.g. no mutation) or changing the environmental stimuli (i.e. the perturbation remains in place). By using the functional reversion approach, the organism phenotype is returned to the condition before the perturbation occurred, and it is possible to conclude that those genes responding to the reversion must control the phenotype.

In certain respects, functional reversion is analogous to genetic means for identifying controlling genes. For example, if a gene mutates (i.e. the genome is perturbed), the likely gene can be identified either by studies of metabolism or by surveying changes in gene expression. If supplying the missing metabolite or complementing the mutated gene reverses the phenotype, the causative gene is identified. The analysis is classic for identifying the gene that was altered during the mutation. However, if many genes change expression during a perturbation, many metabolites or complementation steps are usually needed before the original phenotype is found. This method becomes laborious or practically impossible. The method is often abandoned without identifying the controlling genes.

A tacit assumption underlying much of biology is that most genes altered in expression during a stimulus are secondary responses that do not control the phenotype or modify it only slightly. In genetic terms, both epistasis and pleiotropy are involved. Single gene mutations can alter the interaction between genes or lead to altered metabolite levels or post-translational events downstream or upstream of the primary mutation, leading to secondary changes in gene expression. With the mutation of a single gene, these multiple changes can modify the phenotype, but complementing the secondary genes does not cause reversion and only the primary complementation is effective. Multigenic events appear to be an extension of this concept and differ only in complexity. Sorting out the controlling genes becomes proportionately more difficult.

**Analysis of floret abortion**

In order to illustrate functional reversion, this review will focus on the failure of reproductive development during a water deficit. There is a large impact on agricultural and ecological production when water is limited because water is required for nearly everything the plant does. This causes the plant response to be inherently multigenic, and partly for that reason it has been difficult to pinpoint the genes controlling the phenotypic response to the shortage. The likelihood of multigenicity first became obvious at the biochemical level when it was possible by the early 1970s to identify large numbers of enzyme reactions that were affected. Todd (1972) reviewed 53 papers assaying 28 different enzymes, all of which showed increased or decreased activity when extracted from plants experiencing water shortages and compared with controls that were supplied with adequate water. More recently, molecular biology methods uncovered hundreds of genes altered by plant dehydration (Bohnert et al., 1995; Bray, 1997; Shinozaki and Yamaguchi-Shinozaki, 1997; Seki et al., 2002).

At about the time of the earlier biochemical work, Salter and Goode (1967) reviewed hundreds of studies documenting the response of crops to irrigation and pointed out that those with valuable reproductive structures are most vulnerable to a water shortage around the time of flowering. The highest return per unit of water can be
obtained by irrigating at this time. However, despite the vulnerability, Salter and Goode (1967) could find few reasons for it. Research on mechanisms of reproductive failure was rare. The review of Salter and Goode (1967) directed attention to this lack of understanding, and a number of studies followed that were focused on the causes of the failure, mostly in grain crops. Recently, this latter work was reviewed by Saini and Westgate (2000) and Boyer and Westgate (2004).

Saini and Westgate (2000) pointed out that early reproduction is highly phasic in grain crops, with each phase showing susceptibility to water deficits. Meiosis, anthesis, pollen fertility, pollination, female fertility, and early zygote development were shown to be susceptible, and their failure diminishes the number of grains the plant produces. Later in development, water deficits tend to reduce grain size rather than number, and size seems to be determined in large part by the available photosynthetic reserves that can be moved to the grain. Large agricultural losses can occur during either phase, but the early ones resulting in fewer grain numbers are especially damaging because they are irreversible and set an upper limit on yield.

Sometimes, the irreversibility can be attributed to a failure of pollination. For example, water deficits during meiosis in wheat and rice prevented pollen from storing starch (Dorian et al., 1996; Sheoran et al., 1996). Without starch to fuel pollen tube growth on the female florets, pollen tubes could not reach the ovule. In maize, water deficits during meiosis in wheat and rice prevented pollen from storing starch (Dorian et al., 1996; Sheoran et al., 1996). Without starch to fuel pollen tube growth on the female florets, pollen tubes could not reach the ovule. In maize, water deficits during meiosis in wheat and rice prevented pollen from storing starch (Dorian et al., 1996; Sheoran et al., 1996). Without starch to fuel pollen tube growth on the female florets, pollen tubes could not reach the ovule. In maize, water deficits during meiosis in wheat and rice prevented pollen from storing starch (Dorian et al., 1996; Sheoran et al., 1996). 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Establishing physiological and metabolic function

Early in the physiological investigation of water deficits, it was found that photosynthesis was often inhibited as water began to be limited (Kramer and Boyer, 1995). Although many other processes are also affected, photosynthesis is one of the most distinguishing features of plants and is critical to their survival. Photosynthesis cannot occur at night, and plants are able to cope with these short interruptions by storing photosynthetic reserves. The reserves are abundant in root and stem apices, floral parts, and other rapidly growing organs, and typically are present as starch or occasionally fructans or sucrose. The reserves gradually break down at night so that the normal activities of the cells can continue. During a water deficit, photosynthesis may be inhibited for days, and the reserves become especially critical because respiration continues to demand substrates. Without a stream of substrate from photosynthesis, many cellular activities cannot continue.

However, in addition, increasing evidence indicates that the reserve status of the cells may serve as a signal that affects gene expression. Koch (1996) and Sheen et al. (1999) review this area and identify several genes that appear to change in expression when the sugar status of the cells changes. This raises the possibility that the sugar status is monitored by the genome (as reflected by transcriptome analysis), and developmental changes could result. The monitoring appears to detect the local sugar status because Schussler and Westgate (1994) found in maize that a larger pool of reserves in the plant as a whole did not help to maintain ovary growth during a water deficit. Kernel number decreased irreversibly, i.e. abortion continued in the female florets. This focused attention on the sugar status of the ovary tissues themselves.

Bearing in mind the dual role as substrate and signal, sucrose fed to stems during a water deficit might cause the stream of substrates to resume but also signal to the developing reproductive structure that metabolism could
proceed. Genes responding to the sugar signal would be the ones controlling the phenotype and preventing abortion. If this scenario is correct, there might be two sugar signals. The first would be a downturn in sucrose content of the ovary cells because sucrose delivery was curtailed by the decreases in photosynthesis during the water deficit. The second would be depletion of glucose as starch was consumed in the ovary tissues. Zinselmeier et al. (1999) found that the ovary wall was loaded with starch, which disappeared during a water deficit around the time of pollination. Starch breakdown would produce glucose, and McLauglin and Boyer (2004a) found a decrease in glucose about the time that starch disappeared during a water deficit. Mäkela et al. (2005) demonstrated that glucose disappeared if maize was shaded or subjected to a water shortage, clearly indicating that the starch was breaking down because of the decreased photosynthesis. However, the starch was a small pool and quickly depleted. Mäkela et al. (2005) calculated that it would be sufficient to supply glucose for about one night but could not make up for days of inhibited photosynthesis during a water shortage.

These possible signalling changes depend on the delivery and location of the sugars. Around the time of pollination, maize ovaries accumulate ~1 mg of dry mass each day, mostly in the form of carbohydrate (Mäkela et al., 2005). The dry mass is delivered by the phloem as far as the pedicel where the phloem terminates (Fig. 1C). At the termination, the bulk flow of sucrose ends except for a strand of phloem that passes around the ovary into the stigma and style (‘silk’) of the floret. In order to enter most of the ovary tissues, sucrose or its breakdown products must find their way without the help of phloem transport. McLauglin and Boyer (2004a) found a large concentration of glucose (Fig. 1B) in the upper pedicel tissues on the day of pollination, and these tissues also possess high activity of acid invertase (Fig. 1A). Invertase hydrolyses sucrose to glucose and fructose, and the presence of glucose unequivocally locates the hydrolytic activity. In view of the location of the glucose in Fig. 1B, sucrose appeared to be hydrolysed by invertase immediately after release from the phloem termini. Because the invertase was detected in fresh sections containing living cells, the assay only detected activity outside of the plasma membrane, indicating that the invertase activity was restricted to the cell walls of the pedicel tissues. No unbound (soluble) invertase was detected in these cells (although it was abundantly present in the nucellus cells, McLauglin and Boyer, 2004a). Therefore, the accumulating glucose appeared to be in the pedicel apoplast to a large extent. The cell wall-bound invertase had activity sufficient to account for nearly all of the hydrolysis of sucrose, and the accumulated glucose represented nearly 40% of the hydrolysis (20% glucose and 20% fructose) while the rest was presumably used by metabolism in the ovaries (McLauglin and Boyer, 2004a; Mäkela et al., 2005).

The concentrated glucose in the pedicel created a steep gradient extending downward into the nucellus, which had a low concentration presumably because metabolism rapidly consumed the glucose there (Fig. 1B). The gradient favoured passive glucose movement toward the nucellus where the embryo sac containing the egg was located. Whether this movement was completely in the symplast or apoplast remains an open question, but the coincidence of the cell wall-bound invertase and glucose in the upper pedicel suggests that the apoplast was involved.

During a water shortage, Mäkela et al. (2005) fed the phloem-mobile dye carboxyfluorescein to the stems of maize and found less movement to the ovary than in controls supplied with water and carrying on rapid photosynthesis. This confirmed that the phloem delivered less sugar to the pedicel during the water shortage. With less sucrose, starch was depleted and the glucose gradient nearly disappeared. With the loss of the gradient, glucose movement to the nucellus probably was curtailed. Schussler and Westgate (1991) reported that ovaries excised from water-deficient maize plants absorbed sucrose less rapidly than controls, confirming that post-phloem transport had been inhibited.

The ovaries also displayed less cell wall-bound invertase activity when the plants were subjected to a water shortage (Zinselmeier et al., 1995b, 1999). All of the downstream metabolites were depleted for starch biosynthesis in the ovaries, which indicated that the loss in invertase formed a metabolic block in addition to the lack of sucrose delivery because of decreased photosynthesis (Zinselmeier et al., 1999).

A similar scenario occurred in wheat when a water shortage occurred around the time of microspore meiosis.
(early boot stage). Fischer (1973) and Saini and Aspinall (1981) found that wheat was particularly susceptible to water shortage for ~2 weeks beginning around meiosis, and sterile pollen was a frequent result. Dorian et al. (1996) found that invertase activity was low in the microspores when water was limiting for the plants. Without invertase activity, sucrose hydrolysis was diminished and starch did not accumulate. Therefore, the failure of reproduction in wheat had a biochemical origin resembling that in maize, except wheat lost pollen viability and maize lost ovary viability.

An intriguing early observation was that pollen sterility could be induced in wheat by exogenous abscisic acid applications (Morgan, 1980; Saini and Aspinall, 1982; Morgan and King, 1984; Waters et al., 1984; Zeng and King, 1986). It might be speculated that the abscisic acid (ABA) caused stomatal closure, inhibited photosynthesis, and decreased the delivery of sucrose to the pollen at a critical time when it was depositing starch. This would suggest that ABA could have a physiological effect similar to that of water shortage.

However, Dembinska et al. (1992) split the roots of wheat so that one half of the system was dehydrated while the other was supplied with water. This enabled the spikelet ABA level to increase via the transport of the hormone produced in the drying roots (Davies and Zhang, 1991) while the wet roots maintained the shoot water status. In the plants with split roots shown in Fig. 2, ABA was elevated to the same degree as when the whole root system was dehydrated. Despite the high ABA in both treatments, grain numbers decreased only if the whole root system was dehydrated. With the whole root system dehydrated, the shoot would have dehydrated and may have inhibited photosynthesis, in contrast to the plants with the split roots where the shoots remained hydrated. It would be useful to know whether photosynthetic activity was affected in these experiments.

Recent reviews propose intriguing interactions between ABA and sugars that might regulate developmental processes in well-watered plants (Finkelstein and Gibson, 2002; León and Sheen, 2003). Setter et al. (2001) reported increasing sugar and ABA concentrations in maize florets during a water deficit. Reports that these compounds increased in concentration in florets of water-deficient maize (Schussler and Westgate, 1995; Zinselmeier et al., 1995b; Setter et al., 2001) raise the possibility that such changes (or their interactions) might signal abortion processes to commence. However, for reasons detailed in the review of Boyer and Westgate (2004), concentrations are difficult to interpret because expression on a dry mass or water content basis can cause concentrations to increase simply because the dry mass or water content decreases. With water shortage, the dry mass (Mäkela et al., 2005) or water content of maize ovaries often decreases.

Other evidence for signalling comes from the response of invertase in the parent plant compared with the ovaries.

In leaves and roots of young maize, Pelleschi et al. (1997), Kim et al. (2000a), and Trouverie et al. (2003) report that invertase activity increased during a water shortage, whereas Zinselmeier et al. (1995b, 1999) found a decreased activity in the ovaries around the time of pollination. This marked contrast implies that the signal to abort development might be lacking (or undetected) in leaves and roots, but present in the ovaries. Under this treatment schedule, the leaves remain viable but the ovaries abort.

**Gene responses**

Given these biochemical and physiological functions, possible changes in gene expression become of interest. Table 1 shows that nearly all the genes known to be involved in sucrose processing in young maize ovaries were down-regulated soon after the water deficits began to affect the plants (McLaughlin and Boyer, 2004b). These were the invertases (Incw1, Incw2, Incw3, Incw4, Ivr1, and Ivr2) and sucrose synthases (SS1 and SS2) in the ovaries, and their down-regulation was measured by comparing mRNA abundance in the ovaries with that before water shortage occurred. Done in this way, down-regulation involved not only less mRNA than in well-watered controls but also an actual decrease in mRNA compared with that before the treatments were imposed. Because the down-regulation commenced (McLaughlin and Boyer, 2004b) before glucose disappeared in the ovaries (McLaughlin and Boyer, 2004a), the signal for the genes may have been low sucrose.

The exceptions to down-regulation were the gene coding for an inhibitor peptide for invertase (Zminh1), which was unaltered in expression, and Ivr1, which was not expressed

**Fig. 2.** Effect of endogenous ABA on grain set in wheat. Plants had split root systems, both sides of which were supplied with water (W/W), one side dehydrated and the other supplied with water (D/W), or both sides dehydrated (D/D). Grain set is shown in the left column, and the ABA concentration in the right column of each treatment. Data are the means ±SE (n=10). Adapted from Dembinska et al. (1992), copyright The American Society of Plant Biologists and reproduced with kind permission.
Gene expression in maize ovaries from water-deficient plants

Change in expression is from a time-course and is shown relative to that before the water deficit was imposed. Sugar-responsive genes are shown in bold type, indicating functional reversion when sugar was supplied by feeding to the stems. These sugar-responsive genes caused phenotype reversion in two-thirds of the ovaries, which developed normally instead of aborting. Adapted from McLaughlin and Boyer (2004b).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
<th>Change in expression</th>
<th>Sugar responsive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell wall invertase 1 (Incw1)</td>
<td>Sucrose processing</td>
<td>Down (early)</td>
<td>No</td>
</tr>
<tr>
<td>Cell wall invertase 2 (Incw2)</td>
<td>Sucrose processing</td>
<td>Down (early)</td>
<td>Yes</td>
</tr>
<tr>
<td>Cell wall invertase 3 (Incw3)</td>
<td>Sucrose processing</td>
<td>No expression</td>
<td>–</td>
</tr>
<tr>
<td>Vacular invertase 1 (Ivr1)</td>
<td>Sucrose processing</td>
<td>No expression</td>
<td>–</td>
</tr>
<tr>
<td>Vacular invertase 2 (Ivr2)</td>
<td>Sucrose processing</td>
<td>Down (early)</td>
<td>Yes</td>
</tr>
<tr>
<td>Sucrose synthase 1 (SS1)</td>
<td>Sucrose processing</td>
<td>Down (early)</td>
<td>No</td>
</tr>
<tr>
<td>Sucrose synthase 2 (SS2)</td>
<td>Sucrose processing</td>
<td>Down (early)</td>
<td>No</td>
</tr>
<tr>
<td>Invertase inhibitor peptide (Zmh1)</td>
<td>Sucrose processing</td>
<td>Constitutive</td>
<td>No</td>
</tr>
<tr>
<td>Bifunctional nuclease 1 (ZmBFN1)</td>
<td>Senescence</td>
<td>Down (early)</td>
<td>No</td>
</tr>
<tr>
<td>Cysteine protease 1 (CCP1)</td>
<td>Senescence</td>
<td>Down (late)</td>
<td>No</td>
</tr>
<tr>
<td>Ribosome-inactivating protein 2 (RIP2)</td>
<td>Senescence</td>
<td>Up (late)</td>
<td>Yes</td>
</tr>
<tr>
<td>Phospholipidase D (PLD1)</td>
<td>Senescence</td>
<td>Up (late)</td>
<td>Yes</td>
</tr>
</tbody>
</table>

(Table 1). Kim et al. (2000b) reported that Incw3 was also not expressed, and Cheng et al. (1996) found that Incw4 had little role to play in maize at this stage of development.

Taken together, the down-regulation of the sucrose-processing genes is consistent with the diminished activity of the cell wall and soluble invertases reported by Zinselmeier et al. (1995b, 1999), Andersen et al. (2002), and McLaughlin and Boyer (2004b). Andersen et al. (2002) report a strong correlation between the decreased activity of these forms of ovary invertase and the expression of their genes. Kim et al. (2000a) and Trouverie et al. (2003) found that increased invertase activity in the leaves was accompanied by increases in mRNA abundance of Ivr2, a gene encoding soluble acid invertase. These results suggest that most of the invertase activity was controlled by gene transcription.

Also shown in Table 1 are four genes involved in breaking down cell components. The gene for ribosome-inactivating protein (RIP2) was strongly up-regulated 2 d after the down-regulation of the sucrose-processing genes. Because this gene codes for an enzyme that breaks down ribosomes, it may have initiated senescence in the ovary tissues. Two days later, phospholipase D (PLD1) was up-regulated, suggesting that membrane breakdown may have been initiated. McLaughlin and Boyer (2004b) found evidence that the plasma membranes were losing integrity at this time. Therefore, the action of these two genes was consistent with an accelerated senescence in the affected ovaries, and McLaughlin and Boyer (2004b) suggest that this action may initiate the irreversible loss in viability found during ovary abortion. Zinselmeier et al. (1999) observed starch losses in ovaries of water-deficient plants and at first thought that the ovaries had simply starved to death. However, the up-regulation of these putative senescence genes argues against a simple starvation hypothesis. The genome actively responded to the lowered availability of sugar.

Genes for two other enzymes, a bifunctional nuclease (Bfn1) and cysteine protease (CCP1), were down-regulated about the time that sucrose-processing genes were down-regulated (Table 1). McLaughlin and Boyer (2004b) suggest that they may function in nucleotide and protein turnover during normal growth, which was not occurring in the aborting ovaries.

These changes in ovary gene expression are summarized in Table 1 which shows that among many expressed genes for sucrose processing (Fig. 3A), there is no way to identify which might be considered candidates for controlling abortion because all were down-regulated during a water shortage (Fig. 3B). Among the genes for breakdown of cellular constituents, two are identified as likely candidates because of their up-regulation (RIP2 and PLD1).

Koonjul et al. (2005) similarly explored gene expression for invertases in wheat microspores when a water shortage occurred during meiosis. There was a down-regulation of a short form of the soluble invertase gene Ivr1 and a later down-regulation of a long form of the gene. Ivr5, another member of the same gene family, was also down-regulated, while Ivr3 was not. Therefore, wheat microspores and maize ovaries experience similar gene actions for invertase during a water deficit, but which ones control microspore development was not determined.

Functional reversion of phenotype

With the above in mind, McLaughlin and Boyer (2004b) fed sucrose to the stems of maize during a water shortage around the time of pollination in order to cause the ovary phenotype to revert (ovary abortion prevented). The sucrose replaced that missing because of inhibited photosynthesis. Large quantities had to be fed (5 g d⁻¹) because photosynthesis is normally rapid in these plants when water is available. By allowing uptake in the transpiration stream, the sucrose was delivered to the
leaves and loaded into the phloem. The phloem delivered the sucrose to the ovaries and other tissues in the parent plant (Zinselmeier et al., 1999). The feeding prevented abortion in about two-thirds of the ovaries, and a large improvement in kernel number was observed at maturity (McLaughlin and Boyer, 2004b).

This functional reversion was specific for ovaries of water-deficient plants. Sucrose fed to adequately watered maize produced no additional kernels (Zinselmeier et al., 1999a). Water in the same amount as fed with sucrose had no effect on the water-deficient plants (the fed amount was too small to alter the plant water status) (Boyle et al., 1991b). Other growth factors, amino acids, salts, auxin, and cytokinin had little activity when tested by Zinselmeier et al. (1995a). Therefore, the ovary response appeared to be specific for sucrose not being delivered to the ovaries by the parent plant or not being processed by the ovaries.

Sucrose feeding maintained sucrose at normal levels in the ovaries and avoided depletion during the water deficit (Zinselmeier et al., 1999). Transport activity to the ovaries appeared to be completely intact and was confirmed by carboxyfluorescein feeding by Mäkelä et al. (2005). As a consequence, ovary fate was determined in the ovaries themselves. The biochemical rationale for feeding the whole plant was based on the concepts of Kacser and co-workers (Kacser and Burns, 1973; Kacser and Porteous, 1987) whose analysis of biochemical control identified features specific to the whole system that were not observable in the individual parts after isolation. The depletion of metabolic pools downstream of ovary invertase was especially significant because it was seen in the whole plant. With this observation, the block in downstream biochemistry could be attributed to invertase with confidence, and the phenotypic reversion could be linked to the invertase.

**Functional reversion of gene expression**

The multigenic nature of the ovary response and inability to identify controlling genes concerned Zinselmeier et al. (2002), who used cDNA microarrays to measure differential gene expression in maize ovaries and pedicel tissues when a water shortage occurred around the time of pollination. More than 1500 genes representing 27 regulatory and metabolic pathways were investigated. Of these, 15–45 showed differential expression 4 d after moderate water shortage was imposed, with some genes down-regulated and others up-regulated. A greater number of genes were differentially expressed as the shortage intensified. Yu and Setter (2003) conducted a similar study but withheld water from plants 5–9 d after pollination. Gene expression profiles from endosperm and pedicel tissues showed that the water deficit affected 79 genes in the pedicel and 56 in the endosperm. Most were up-regulated in the pedicel but down-regulated in the endosperm. Curiously, there were no changes in expression of sucrose-processing genes reported in either study. This may have been because many genes were transiently expressed with a limited lifetime of their mRNA (Seki et al., 2002). The sampling times might have missed critical changes in mRNA abundance that trigger ovary abortion.

With functional reversion of whole plants, however, it is possible to monitor gene expression along an extended time-course and detect transient events. Table 1 summarizes the results of a time-course study from McLaughlin and Boyer (2004b) and shows that, of the nine ovary genes responding to water shortage, only four responded to sucrose feeding during the shortage. These four, identified in Table 1 in bold type, are the candidate genes for controlling phenotype reversion and, by inference, abortion. The other genes were responding to other factors associated with the water shortage. The latter genes are much less likely to be involved in the reversion. Figure 4 summarizes the genes displaying reversion and thus acting as candidates for controlling abortion.

It would be desirable to have a much larger array than the 13 genes in Table 1 to test for the functional reversion of gene expression. Ideally, all of the expressed genes in maize ovaries would be represented in the test and probably would identify more than the four candidates in Table 1. However, the four include two invertase genes, and one (Incw2) encodes an invertase involved in sucrose
biochemistry as shown in Fig. 1. Therefore, a key gene appears to be Incw2. Ivr2 may also be important for encoding the soluble enzyme which was detected only in the nucellus (McLaughlin and Boyer, 2004). Its function may be in processing sucrose escaping hydrolysis in the pedicel. Because the nucellus surrounds the embryo sac containing the egg, changes in Ivr2 expression might initiate or control sugar signals reaching the egg (Andersen et al., 2002).

**Using functional reversion**

While sucrose was diminished in the ovaries during water shortage, it did not disappear (Zinselmeier et al., 1999). The loss of invertase activity prevented the ovaries from scavenging the remaining sucrose, effectively turning off carbon acquisition by the ovaries. When sucrose was fed, cell wall invertase activity increased and carbon acquisition was improved. Functional reversion seemed to indicate that a sugar signal prevented the ovaries from accessing the limited sucrose remaining in the ovaries during the water deficit. Only when sucrose was fed to the parent did some of the genes respond and allow greater access to the sucrose.

At the gene level, this behaviour might be explained from the diversity of members of the gene families. Cell wall-bound acid invertase is coded by a four-gene family, and the two members expressed in the ovaries were both down-regulated during the water shortage (Incwl and Incw2), but only Incw2 underwent functional reversion (Table 1). This may explain why Zinselmeier et al. (1999) and McLaughlin and Boyer (2004) found only a partial reversion in the activity of the enzyme when sucrose was fed to the stems, and a partial reversion of the phenotype (abortion prevented in two-thirds of the ovaries). Whether or not complete phenotype reversion could have been achieved with complete enzyme reversion is an open question.

Nevertheless, the results suggest several strategies for preventing abortion. One might be to enhance sucrose availability to the ovary during the water deficit by improving the photosynthetic activity or sucrose content of the ovaries, which might prevent Incw2 from being down-regulated. A second strategy might be to increase expression by Incw2 or Incwl or both by linking Incwl or Incw2 to the promoter of RIP2, which was massively up-regulated. Cell wall invertase activity might be enhanced during a water deficit where little would otherwise be available.

For plant breeding programmes lacking the resources to create and test transgenic individuals, the strategy might be implemented by selecting for large ears around the time of pollination. This would rely on sufficient variation in invertase activity in existing germplasm but would require only simple measurements of ear length or diameter, or assessments of ear size at pollination by eye in water-limited conditions in the field. For genotypes that display a rapid senescence of leaves, selections might also include evaluations of leaf health. Aparicio-Tejo and Boyer (1983) found that rapid leaf senescence contributed only a small amount to the maintenance of ear growth in maize, and selecting for leaves that stay green would promote photosynthetic activity during and after the water deficit.

An entirely different approach would be to accept diminished sugar processing in the ovaries but prevent accelerated senescence, which may be the irreversible component of abortion. Accelerated senescence was detected as a breakdown of plasma membrane integrity in maize ovaries after the sucrose-processing genes had been down-regulated (McLaughlin and Boyer, 2004). Putative senescence genes RIP2 and PLD1 could have been involved in the losses in membrane integrity, and preventing their up-regulation might avoid the irreversible abortion step. The ovaries would become quiescent but remain viable, and would re-grow when water availability improved. Such a strategy might be implemented by selecting against up-regulated RIP2 or PLD1.

**Other kinds of functional reversion**

The genetic tools of mutation, transformation, and complementation are important for understanding plant function, but functional reversion adds to that capability without altering the genome and thus avoids some side-effects of the genetic methods (epistasis, pleiotropy, gene silencing, and so on). Although functional reversion has been presented with respect to water shortage, it seems likely to be useful in many different contexts when multigenic responses occur. The analysis begins by identifying the physiological attributes of the response, often starting with field observations, followed by identification of biochemical steps likely to be involved in the physiological behaviour. This provides the ‘functional’ knowledge for focusing on genes controlling those steps.

Gene expression is then followed in ways that include those steps as well as many others, ideally including all the expressed genes in the genome. However, even without all of the expressed genes, candidate genes can be identified by focusing on those playing a functional role. From the
biochemical and expression results, phenotype reversion may become possible by supplying a missing metabolite or other factor. For water shortage, for example, other means of creating phenotype reversion might be pressurizing roots to maintain shoot hydration despite dehydrated soil (Passioura, 1988), splitting roots to supply water to the shoot while drying one half of the roots to create a shoot hormone status similar to that when the entire root system is dehydrated (Davies and Zhang, 1991; Dembinska et al., 1992), or grafting to place genetically altered plant parts on unaltered plants, allowing detailed hormone or metabolite regulation to be explored (Holbrook et al., 2002).

In the sucrose feeding detailed here, part of the analysis required an assessment of the reserve status of the floral parts because photosynthesis appeared to be an important physiological component. The reserves can be measured by creating a water shortage sufficient to prevent net photosynthesis. Continued growth then depends on reserves, and fails when reserves are depleted. For example, with a water shortage, the reserve status was measured by allowing the leaf water potential to decrease sufficiently to bring net photosynthesis near zero. Water was then supplied at the beginning of each day in an amount approximating the use on the previous day. By adding the water to the soil in a local region where a few roots could meter it into the plant, the leaf water potential could be kept reasonably uniform and net photosynthesis at zero for weeks. Boyer and McPherson (1975), McPherson and Boyer (1977), Jurgens et al. (1978), Westgate and Boyer (1985b), and Setter et al. (2001) used this technique in the laboratory or field for long-term water shortages in maize.

When reserves are depleted, the plant loses its ability to supply substrate for metabolic processes and must be fed in order to maintain them. While it is a simple matter to provide small amounts of growth regulators, amino acids, and other metabolites to plants, the flux of photosynthetic products is very large and rarely supplied exogenously to whole plants. Because maize deposits ~5 g of dry mass each day around the time of pollination, feeding this amount of substrate was essential to feed all the sinks to ensure that particular sinks such as florets were supplied. It was possible to achieve this uptake by supplying a concentrated sucrose solution to a well cut into the stem and allowing the exposed xylem to absorb the solution (Boyle et al., 1991a).

While the required amount of sucrose can be readily supplied to controls by feeding at sunup, the xylem comes under tension during midday or during a water shortage. Emboli form in the exposed xylem and can block sucrose uptake. It is important to monitor uptake and refresh the solution in the well if this occurs. Air bubbles need to be flushed out of the well or connecting tubing, and a new well may need to be made in order to supply what is needed. In other experiments, it has sometimes been possible to cut leaves under a sucrose solution and achieve sufficient uptake (Spoehr, 1942).

For some types of multigenic responses, the functional reversion may take a different form. Plants small enough to be grown in sterile culture (e.g. Arabidopsis) can sometimes be fed small amounts of metabolites or growth regulators through the roots (bacterial decomposition of the fed materials must be prevented). Gases such as CO2 or O2 might also be supplied to reverse photosynthetic or respiratory losses. Provided that functional reversion occurs, these methods could add to the genetic methods already available for finding the few relevant genes among the many that respond to a stimulus.

References


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