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RESEARCH PERSPECTIVE

Seed genomics: germinating opportunities

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Abstract

With the sequencing of the *Arabidopsis thaliana* genome, the field of plant biology has made a quantum leap. The sequence information available to the community has greatly facilitated the identification of genes responsible for mutant phenotypes and the large-scale analysis of gene expression in *Arabidopsis*. High-throughput laboratory tools for DNA sequencing (genomics), mutant analysis (functional genomics), mRNA quantification (transcriptomics) and protein analysis (proteomics) are being used to scrutinize this model plant. For seed physiologists, the challenge lies in translating this information into physiological processes in seeds from other plant species. Combining more traditional seed biology with the new high-throughput molecular tools should yield breakthroughs in seed science.

Keywords: functional genomics, proteomics, seed biology, transcriptomics

Introduction

With the development of farming by our early ancestors, the value of seeds to start a crop has been an important consideration. Without much knowledge of physiology or genetics, these farmers determined by trial and error which seed characteristics were desirable for optimal performance. In the nineteenth century, Mendel laid the foundation of modern genetics by crossing peas with different seed traits (Mendel, 1866). Characterization and classification of seed proteins based on their physical characteristics, such as solubility in aqueous and non-aqueous solutions, that started over a century ago set the stage for biochemical analyses of seeds (Osborne, 1894).

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Over the past few decades our knowledge of seed physiology has exploded, resulting in the ability to control seed production, storage and treatments in such a manner that is it possible to produce uniform, high-quality seed lots that germinate in a predictable manner under normal growing conditions. Analysing the composition of seeds as they matured and germinated led to many important discoveries, such as the reduction in water content, the rise of abscisic acid (ABA) levels and the appearance of a number of unique proteins, membrane characteristics and sugars during maturation. Correlative studies indicated that these components were required for acquisition of desiccation tolerance, survival during the dry state or subsequent germination.

Due to their high expression levels, sequestration into protein bodies and biochemical properties, the seed storage proteins constitute one of the best characterized groups of seed protein genes to date, as exemplified by the extensive studies performed on the phaseolin gene of French bean (reviewed by Hall *et al*., 1999). Another prominently expressed group of genes during seed maturation encode the late embryogenesis abundant proteins (LEA) (Galau *et al*., 1986; reviewed by Cuming, 1999), which are highly hydrophilic in nature and are thought to alleviate the consequences of water deficit stress within cells by stabilizing cellular components under these conditions.

A number of stress-induced genes that normally play a role in the prevention of protein denaturation during stress are also expressed during seed maturation, such as small heat-shock proteins (Coca *et al*., 1994; Wehmeyer and Vierling, 2000). Levels of specific sugars, such as raffinose, also rise during this phase, and although raffinose levels alone are not correlated to seed storability (Bentsink *et al*., 2000), the cumulative effects of these compounds contribute to desiccation tolerance and the ability to survive long periods in the dry state. The plant hormone ABA plays a central role in the maturation phase. ABA regulates many of the genes that are expressed during

maturation and that are thought to contribute to desiccation tolerance. A subset of these genes, such as the dehydrins, is also expressed in vegetative tissues in response to ABA during drought stress (Cuming, 1999).

Studies of gene expression during germination of tomato have led to the discovery of genes that contribute to cell wall weakening, such as expansin, endo-β-mannanase and polygalacturonase, as well as genes involved in energy metabolism, such as galactinol synthase (Bradford *et al*., 2000), and genes that may play a role in protecting the emerging radicle from microbial pathogens, such as β-1,3-glucanase and chitinase (Wu *et al*., 2001). Since some of these genes were isolated from a differential screen between imbibed wild-type and gibberellic acid (GA)-deficient tomato seeds, many of them are involved in GA-dependent germination processes (Bradford *et al*., 2000). GA is of critical importance in the process of germination (Ritchie and Gilroy, 1998) and can be used to break seed dormancy (Hilhorst, 1995). Although dormant seeds that are imbibed initially share some gene expression characteristics with non-dormant imbibed seeds, a number of genes that seem to be specifically expressed in dormant imbibed seeds have been found. These genes include members of the LEA gene family, a serine–threonine protein kinase, peroxiredoxins and other stress-related ABA-regulated genes (Li and Foley, 1997; Aalen, 1999). This coincides with the role for ABA in controlling seed maturation and maintaining seed dormancy (Hilhorst, 1995). In wheat, pre-harvest sprouting has been correlated with aberrant splicing of the *vp1* transcript. Since *vp1*/*abi3* is an important regulator of seed maturation, mutant proteins produced from these defective transcripts presumably fail to repress precocious germination (Holdsworth *et al*., 2001). GA-dependent expression of α-amylase and proteases in the barley aleurone layer to provide nutrients to the growing embryo has been well documented (reviewed by Ritchie and Gilroy, 1998). Accumulation of β-tubulin and DNA replication are reliable markers for the start of the cell cycle during germination and priming in a number of crops, and can be detected prior to radicle protrusion (de Castro *et al*., 1995; Jing *et al*., 1999).

The advent of genomics techniques now makes it possible to perform gene expression studies during seed maturation, dormancy and germination for large numbers of genes at once, allowing a more global molecular analysis of the processes involved, and increasing the chances of finding additional genes controlling these processes.

Lessons from mutants

The study of *Arabidopsis* mutants that are affected in seed maturation or germination has led to increased insight into the molecular events that govern these processes (recently reviewed by Holdsworth *et al*., 1999; Wobus and Weber, 1999). The balance between maturation and germination is maintained by a number of key regulators, many of which are now known, but the precise molecular control of onset and release of seed dormancy remains an enigma (Koornneef *et al*., 2002). A central role is played by the antagonistic action of ABA and GA, with ABA promoting maturation and dormancy, and GA stimulating germination (Fig. 1). A number of known seed maturation or germination mutants have defects in hormone synthesis and perception of these hormones (Koornneef and Karssen, 1994; McCarty, 1995). Gibberellin-deficient mutants require exogenous gibberellin to germinate, due to a defect in GA biosynthesis (Koornneef and van der Veen, 1980). Recently, a number of mutants have been described that either resemble GA-deficient mutants, with features such as dwarfism and reduced germination due to an insensitivity to GA (i.e. *sly*, and certain alleles of *rga* or *gai*), or that resemble GA-treated plants due to a constitutive GA response even in the absence of GA (such as *spy*). These discoveries have shed light on the complex gibberellin signalling pathways (reviewed by Ritchie and Gilroy, 1998; Sun, 2000; Richards *et al*., 2001).

The role of ABA in promoting maturation and dormancy is illustrated by the *aba1* mutant that germinates precociously due to defects in ABA biosynthesis (Karssen *et al*., 1983), and the behaviour of ABA insensitive (*abi*) mutants that can germinate in the presence of ABA (Koornneef *et al*., 1984; Finkelstein, 1994). The *ABI1* and *ABI2* genes encode highly homologous members of the protein phosphatase 2C family (Leung *et al*., 1994, 1997; Meyer *et al*., 1994), whereas *ABI3*, *ABI4* and *ABI5* encode transcription factors that mediate ABA-induced gene expression in seeds and/or vegetative tissues (Giraudat *et al*., 1992; Finkelstein *et al*., 1998; Finkelstein and Lynch, 2000).

Together with *LEAFY COTYLEDON* (*LEC1*) and *FUSCA* (*FUS3*), the *ABA INSENSITIVE* (*ABI3*) gene controls a number of maturation-related processes in seeds. Mutation of any of these genes results in a developmental shift, suppressing maturation-related events while displaying characteristics that are normally associated with germination. All three mutants exhibit defects in desiccation tolerance, seed storability and have reduced levels of accumulation of maturation-related gene products, such as LEA and storage proteins (Parcy *et al*., 1997). The *ABI3*, *LEC1* and *FUS3* genes encode transcription factors (Giraudat *et al*., 1992; Lotan *et al*., 1998; Luerßen *et al*., 1998) that act in concert to control seed maturation, as illustrated by their ability to act synergistically to induce transient expression of genes that are normally

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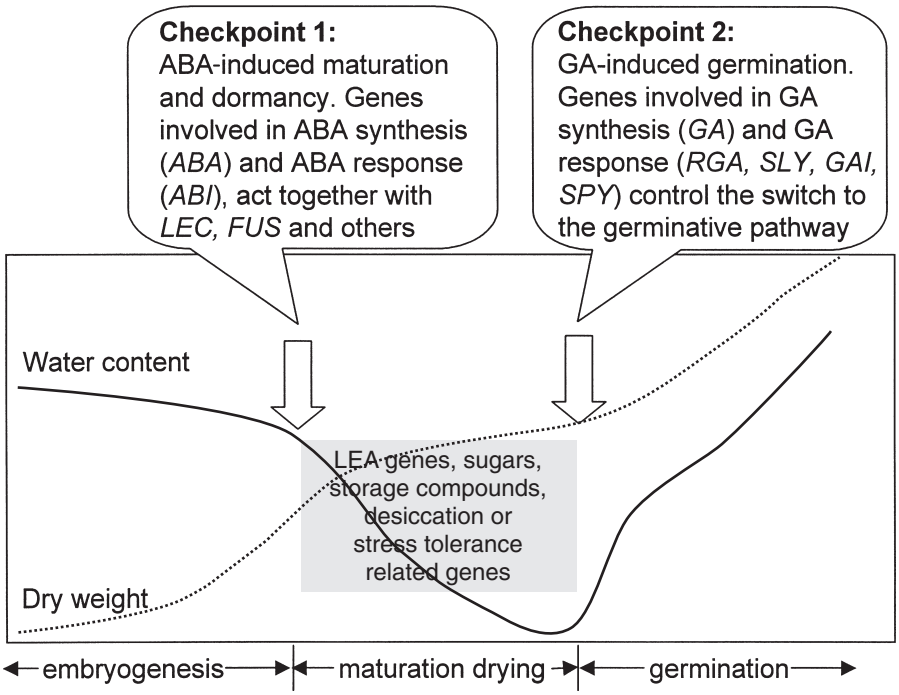

Figure 1. Overview of seed development and germination, and genes known to play a role in these processes. Two main transitions can be distinguished; the first is the switch from embryo development and growth to maturation, and the second from quiescence in the dry state to germination. This overview assumes that seeds are non-dormant and able to germinate when environmental conditions are right. For a recent overview on genes involved in dormancy see Koornneef *et al.* (2002).

expressed during seed maturation (Reidt *et al.*, 2001). Therefore, these genes play a key role in the regulation of the developmental switch between seed maturation and germination. A recently identified mutant at the *COMATOSE* (*CTS*) locus has the opposite phenotype. Mutation of this locus causes a disruption in the transition from maturation to germination, producing seeds that do not after-ripen or respond to GA (Russell *et al.*, 2000).

Using the mutants described above, in combination with high-throughput genomics techniques, should greatly increase our understanding of the complex intersecting pathways regulating seed maturation and germination processes, since the downstream genes that are affected by these mutant loci can be identified using such techniques. Combining phenotypic and gene expression data of double or triple mutants should help elucidate the intricate processes that take place.

Genomics

Now that the *Arabidopsis* genomic sequence is fully available (http://www.arabidopsis.org; Meinke *et al.*, 1998; Arabidopsis Genome Initiative, 2000), focus has shifted from obtaining sequence information to locating genes and understanding their function and regulation. Large-scale sequencing of ESTs (expressed sequence tags, short sequences from randomly selected cDNA clones from various tissues, representing the genes that are expressed in that specific tissue at that specific time) has been performed for a number of species. When enough ESTs are available from different tissues, additional information, such as approximate expression level (indicated by the number of times a given EST is found) and location of expression (indicated by the tissues in which such ESTs were found) from the genes annotated in the genome, can be obtained.

Using bioinformatics programs that predict locations of genes, and matching the predicted genes with EST sequences, the genome of *Arabidopsis* has been predicted to contain approximately 25,000 genes (the Arabidopsis Genome Initiative, 2000). By comparing the sequence of the proteins predicted to be encoded by the *Arabidopsis* genome, based on bioinformatics, with known gene structure and function information from *Caenorhabditis elegans* and human genes, much information about putative functions of *Arabidopsis* genes can be obtained. However, it is clear that many of the known gene categories found in the animal model systems are as yet underrepresented in plants, and that plants

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contain unique classes of genes not found in animals (Assaad, 2001).

In order to understand the functions, behaviour and cross-talk of these genes in regulating such complex physiological processes as seed development and germination, stress responses and hormone signalling, new high-throughput analysis tools are needed. Functional analysis of individual genes can be performed by studying mutants. These can be identified in the large collections of mutants (containing T-DNA inserts, transposons or EMS-induced mutations) that are publicly available for study (in Europe contact http://nasc.nott.ac.uk/). Finding a mutant for a specific gene is relatively easy in mutant collections where a defined DNA insert, such as a T-DNA or transposon, results in the mutation. Using a polymerase chain reaction (PCR) screen, the line with the insert in the specific gene of interest can be identified quickly. In addition to genetic analysis of gene function, the expression levels and patterns of genes can be studied by looking at RNA or protein levels and modifications (Fig. 2). Initiatives to perform high-throughput transcriptional analysis of all predicted genes in the *Arabidopsis* genome using microarrays are under way (Wisman and Ohlrogge, 2000; Zhu and Wang, 2000).

Over 100,000 ESTs from *Arabidopsis* are currently present in the public database (dbEST from the National Center for Biotechnology Information). Since these were randomly obtained from cDNA libraries from various tissues and treatments of *Arabidopsis* plants and cells, many duplications exist in this database. The relative abundance of the different ESTs in cDNA libraries gives an indication of their expression level. Even though a large number of ESTs have been sequenced, there are still many predicted genes in the *Arabidopsis* genome for which no EST has been found yet. The recent sequencing of 10,500 ESTs from developing *Arabidopsis* seeds revealed that about 40% of these were not yet present in the public EST database (White *et al*., 2000). Based on sequence homology of the most abundant gene classes found among these ESTs, genes involved in a number of well-described seed developmental pathways could be identified, such as seed reserve accumulation and acquisition of desiccation tolerance. Among these were storage protein genes, ribosomal and translation factor genes, amino acid, carbohydrate and lipid metabolism genes and desiccation-related genes (White *et al*., 2000). This information is extremely valuable for analysis and engineering of complex pathways such as oil metabolism in plant seeds, where a multitude of genes are involved in the metabolic pathway, and critical genes need to be identified (White and Benning, 2001).

This vast amount of sequence information, soon to be complemented with functional and expression

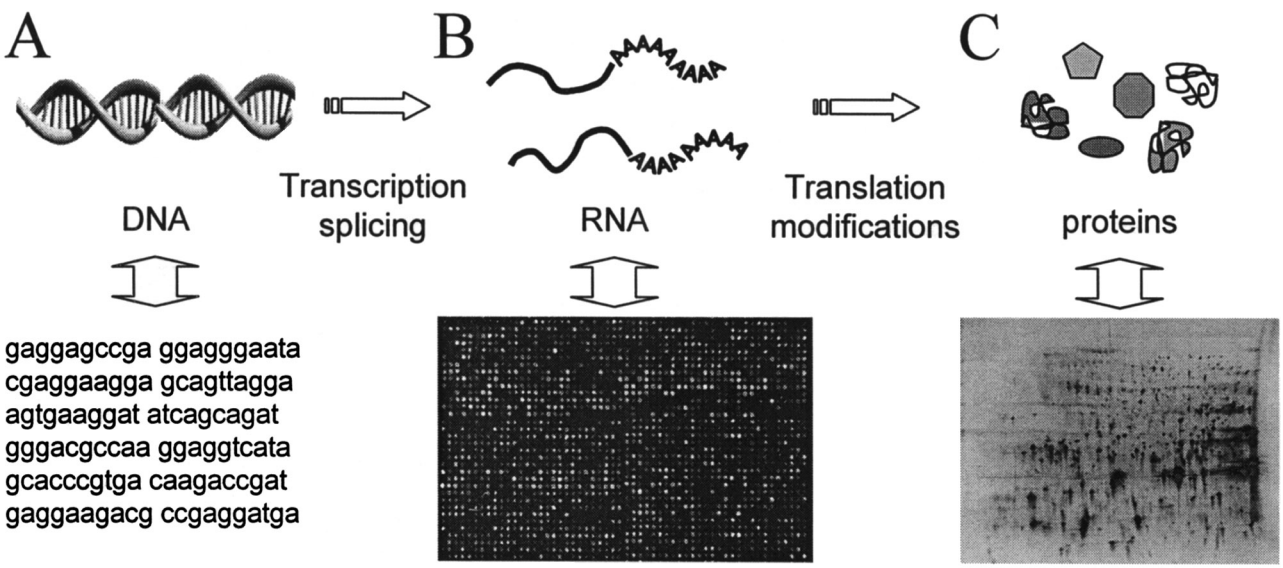

Figure 2. Genetic flow of information from DNA to mRNA to protein, and the tools for their high-throughput analysis. (A) DNA sequencing (ESTs or genomic DNA) reveals gene sequence and structure, genetic variation, and characteristics of regulatory DNA elements. Part of the *Brassica napus* Em6 LEA gene sequence is shown as an example (Vicient *et al*., 1998). (B) cDNA microarrays allow quantitative measurements of RNA levels of hundreds of genes simultaneously. Expression levels of 1500 genes during 15 hours' imbibition in water of *Brassica napus* seeds are shown as an example. (C)The great diversity of proteins and their post-translational modifications can be visualized using two-dimensional gel electrophoresis. The protein pattern of *Brassica napus* seeds after osmopriming and subsequent drying is shown as an example.

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information, will provide detailed molecular insights into most of the basic processes shared by plants, including seed development, dormancy and germination. Large EST sequencing efforts have also been made for crop species. For example, over 200,000 ESTs from soybean, 140,000 from tomato, 108,000 from maize and 92,000 from rice can be found in the public databases (http://www.ncbi.nlm.nih.gov/dbEST). The homology among genes from different plant species allows functional information obtained for *Arabidopsis* genes to be applied to other species. Scientists studying gene expression related to these processes in other species, such as tomato (Bradford *et al*., 2000) and wheat (Holdsworth *et al*., 2001), have already benefited from the advances that have been made with *Arabidopsis*.

Transcriptomics

The invention of DNA microarrays has made it possible to measure the expression levels of thousands of genes simultaneously. The real power of these technologies stems from their ability to measure global changes in gene expression, which can be used to gain a molecular understanding of the underlying physiological processes. Two technologies to accomplish this feat are now commonly available. The high-density oligonucleotide array contains thousands of oligos that are synthesized *in situ* on the surface of a chip. Sets of 10–20 oligos complementary to a single gene can be used to quantify expression of the gene or detect single base-pair mismatches (Lockhart *et al*., 1996). An oligonucleotide array containing oligo probes for more than 8000 *Arabidopsis* genes already exists, and 500 transcription profiles of these genes have been produced and are available to the public (http://www.affymetrix.com/; Zhu and Wang, 2000). A more widely distributed technology for analysing expression of large numbers of genes is the cDNA microarray (Schena *et al*., 1995). In order to prepare a cDNA microarray, cDNA clones representing thousands of genes are robotically spotted on to a 1 cm^2 area on a glass slide. Hybridizing these slides with fluorescently labelled cDNAs derived from mRNAs present in various cell or tissue types allows simultaneous detection of changes in gene expression of all the genes that are spotted on the chip. By hybridizing with a mixture of reference and test samples, each labelled with a differently coloured fluorescent dye, relative changes in gene expression compared to the reference sample can be measured accurately (reviewed by Aharoni and Vorst, 2002). As part of the Arabidopsis Functional Genomics Consortium, microarrays of more than 10,000 *Arabidopsis* ESTs have been produced, and scientists can submit RNA samples to be analysed using this array for a moderate fee. The results of over 200 hybridizations can be found at http://afgc.stanford.edu (Wisman and Ohlrogge, 2000).

The first microarray to be used to study the expression of a large set of plant genes was produced using 1443 random *Arabidopsis* ESTs (Ruan *et al*., 1998). Hybridization of this array with labelled cDNA from four different plant tissues (root, leaf, flower buds and opened flowers) illustrated the large differences in gene expression patterns that could be expected to occur. Tissue localization of a number of genes, known to be specifically expressed in either root, leaf or flower, was confirmed using microarrays, and large differences in relative expression were found for additional genes as well (Ruan *et al*., 1998). Aharoni *et al*. (2000) used a microarray containing cDNAs isolated from strawberry fruit to study gene expression during strawberry ripening, and genes involved in a number of related processes, such as softening, auxin response, colour and flavour formation were analysed. These studies led to the discovery of a novel key gene in strawberry flavour biogenesis during ripening (Aharoni *et al*., 2000)

A cDNA microarray containing 2715 ESTs from developing *Arabidopsis* seeds was used to compare gene expression in seeds, leaves and roots. Initial experiments revealed that 25% of the seed-derived ESTs were preferentially expressed in seeds, as compared to roots and leaves (Girke *et al*., 2000). Many of these ESTs had been found previously to be abundant among the known 10,500 ESTs, confirming that abundance of a given sequence in a large pool of random sequences is an indication of expression level (White *et al*., 2000). Performing hybridization experiments to compare gene expression in seeds and leaves from both *Brassica napus* and *Arabidopsis* gave similar results, indicating sufficient sequence homology to use an *Arabidopsis* microarray for studies of the related species *B. napus* (Girke *et al*., 2000).

In order to study processes that occur during germination prior to radicle protrusion, a cDNA microarray was produced containing 1500 genes from immature (green cotyledon stage), and imbibed (7 and 15 hours after addition of water) *Brassica napus* seeds (van der Geest *et al*., 2001). This array was used to monitor gene expression during maturation, priming or imbibition of *B. napus*, white cabbage and *Arabidopsis* seeds. In the first 15 hours of imbibition of *B. napus* (radicle protrusion occurs on average at 20 hours), about 600 of the genes on the chip dramatically increased their expression, while mRNA levels of about 100 genes on the chip were reduced. Comparisons between germinating seeds and leaves revealed that, although many of the genes on the chip were classified as housekeeping genes, their expression was much higher in germinating seeds than in young developing leaves (van der Geest *et al*., 2001). Such

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results illustrate the usefulness of cDNA microarrays as a tool to study molecular processes in seeds, and their potential for identification of markers for germination, priming or other physiological processes.

In the near future a full-genome *Arabidopsis* microarray will undoubtedly become available, although it may not be complete due to errors in the annotation of genes in the *Arabidopsis* genome. Smaller microarrays will remain useful tools when defined physiological processes need to be studied, since they can contain genes specific to that process and are easier to analyse.

Proteomics

Proteins, their characteristics, abundance and modifications have been the focus of study since the 19th century (Osborne, 1894). Technological advances in the separation, quantification and sequence determination of proteins have facilitated high-throughput, comparative studies of proteins in different plant tissues, treatments and developmental stages. Such studies are important, since the flow of genetic information from gene to protein or metabolite in a plant cell can be modified at many steps. Knowing the gene sequence and the abundance and location of its mRNA still leaves room for post-transcriptional regulation, such as translation efficiency of a given mRNA. Once a protein is produced from the mRNA, it can be processed into a smaller mature protein, targeted to subcellular locations, modified by glycosylation or phosphorylation, physically linked to cellular structures, assembled into a complex structure, or preferentially degraded. It is therefore clear that techniques that allow an inventory of the proteins present in a cell are extremely valuable.

Two-dimensional gel electrophoresis (first separating proteins based on their charge, followed by a separation based on size) allows the resolution of hundreds of proteins in a single gel, and this technique has been perfected over the past 25 years to provide great precision and reproducibility. Comparing protein patterns among different tissues, developmental stages or physiological states can lead to the discovery of proteins that are present, absent or modified in some gels (reviewed by Zivy and de Vienne, 2000). Comparison of protein patterns during different stages of tomato germination revealed that remarkable differences in the protein profiles were observed within 24 hours after water addition (Bergervoet *et al*., 1994). Now that the technology of protein sequencing has progressed to the point where it is possible to micro-sequence picomolar amounts of protein that are extracted from spots on two-dimensional gels, the proteins that differ can also be identified with relative ease.

Using two-dimensional gel analysis of dry, 1-, 2- or 3-day-imbibed, hydroprimed or osmoprimed *Arabidopsis* seed protein samples, it was possible to detect over 1000 individual protein spots in these extracts (Gallardo *et al*., 2001). Proteins were classified according to their occurrence in the different samples, and a large number of proteins observed in different priming treatments or germination stages were sequenced. The large majority of proteins detected in these gels (1251) did not differ between germination stages. These included storage protein subunits, proteases, translation factors and enzymes of metabolic pathways (Gallardo *et al*., 2001). Several proteins that either appeared or disappeared during germination can be used as molecular markers to monitor the progression of germination or priming. These include degradation of biotinylated proteins and cytosolic glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and appearance of myrosinases (Gallardo *et al*., 2001). Since some of the differences found in *Arabidopsis* had previously been found for other species, such as sugarbeet (degradation of biotinylated proteins, and an increase in the solubilization of subunits of the 11S globulin storage proteins; Job *et al*., 2000), cucumber and tomato (tubulin accumulation; de Castro *et al*., 1995; Jing *et al*., 1999), such work on *Arabidopsis* is relevant to the seed community at large.

Future proteomics approaches will be able to build on this protein inventory to study more complex, protein-related questions, such as the role of protein–protein interactions and post-translational modifications in protein activity, stability and regulation, and biochemical characteristics of protein complexes, in order to understand how these contribute to physiological processes of interest (van Wijk, 2001; Kersten *et al*., 2002; Roberts, 2002).

Applications in seed research

Using *Arabidopsis* as a model, genetic mechanisms regulating physiological processes in seeds are being elucidated rapidly. Molecular dissection of the pathways involved in controlling processes such as perception of environmental cues, water availability, hormone levels, growth and development is under way. The tools available for such studies, including a large collection of mutants, microarrays for gene expression studies and other genomics tools, will facilitate applications for seed scientists. At the single-gene level, it is relatively easy to translate the *Arabidopsis* results into meaningful applications for similar genes in crop plants. However, at the whole genome level, these modifications are much more complex (Barnes, 2002; Osterlund and Paterson, 2002). As more plant genomes are sequenced, comparisons among them will make interpretation

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easier. Given their economical importance and the large number of ESTs already available, it can be expected that before long, cDNA microarrays containing most genes will be available for important crops such as corn, rice, soybean and tomato. Ideally, one could envision a microarray that contains oligos that are specific to those regions of genes most highly conserved among different crop species at the DNA level, allowing the use of a single oligo array for multiple crop species. Such new cDNA or oligo arrays for crop species will allow expression analysis of relevant genes during seed development and germination under field conditions.

For the seed community, these tools will make it feasible to study global changes in gene expression instead of focusing on individual genes or seed components. By comparing gene expression profiles, protein patterns and metabolite composition in good- and bad-quality seed lots, aged, primed, dormant or non-dormant seed lots, seeds subjected to various physiological stresses, drying or conditioning treatments, it will be possible to develop molecular markers for these processes that allow quick diagnosis of the physiological condition of a given seed sample. It will then be possible to monitor the progress of the priming or dormancy-breaking process, predict the ability of seeds to respond to stress and have insight into their field performance without complicated lengthy protocols, but rather using a simple biochemical assay. Using genomic tools, discovery of genes that play a critical role in controlling these processes (for example master switch genes that control the transition from the dormant to the non-dormant state or vice versa) will soon follow. Correlations among environmental conditions, agronomic traits, plant performance and gene expression should greatly facilitate selection for desired seed characteristics in breeding programmes, and open possibilities for certification of seed lots based on gene expression. Many of the 'black boxes' that still exist in seed biology await illumination with the new high-throughput technologies of today and tomorrow.

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