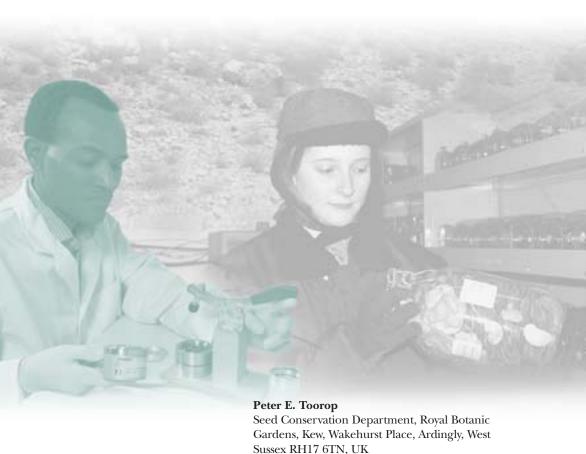


Molecular Biology in Seed Diagnostics



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Introduction

Conventionally, seed quality has been mainly diagnosed through germination tests. One clear disadvantage is that germination is the end point of a large number of processes occurring in the seed prior to emergence of a seedling. Moreover, relatively large numbers of seeds are required for statistical reliability. The development of molecular biology in recent decades has opened doors to using/applying modern tools that may not only require smaller numbers, but also may tell us more about the state the seed is in. For example, if a seed does not complete germination it could be dead, although it could be quiescent or dormant. Testing a range of molecular parameters may exclude one or more of these options. It may also indicate if the problem is in the embryo or in the endosperm, what kind of dormancy is involved, or even how to break it once sufficient data have been gathered.

Polymerase Chain Reaction

The polymerase chain reaction (PCR) has become a routine method in most biological research laboratories. PCR is a strong tool in the detection of DNA sequences, because it detects the presence of target sequences with great sensitivity and specificity. The technique is based on the duplication of nucleotide sequences using oligonucleotides that are designed to bind to a specific region of the sequence of interest. Annealing of these oligonucleotides to single stranded DNA occurs at a temperature that depends on the oligonucleotide sequence. Choosing the annealing temperature is critical for the reaction: too high a temperature will result in poor annealing and low yield, whereas too low a temperature results in non-specific binding. The oligonucleotides act as primers for DNA synthesis, and upon annealing the sequence is duplicated at 72°C which is the optimum temperature for DNA synthesis (Figure 27.1). This reaction is catalysed by a heat-stable enzyme, called Taq DNA polymerase which was originally cloned from Thermus aquaticus, a micro-organism isolated from a hot spring in Yellowstone National Park. Several components are required for this reaction: deoxynucleoside triphosphates (the building stones of the DNA molecule), divalent cations (usually magnesium), monovalent cations (usually potassium), and the appropriate buffer. Upon duplication, the newly formed double stranded DNA sequence is denatured at 94°C, which results in the separation into two single stranded DNA molecules, which form the new templates in the next cycle of DNA polymerisation (Sambrook and Russell, 2001).

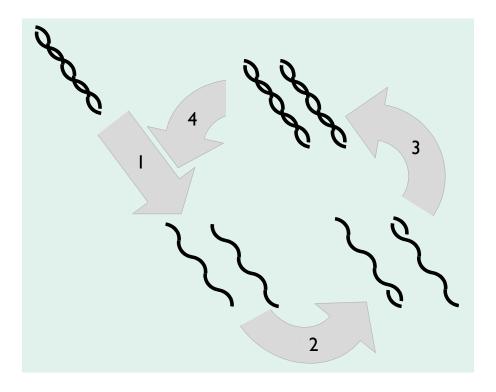


Figure 27.1 In the PCR reaction double-stranded DNA is first melted at 95°, which results in 2 single-stranded DNA molecules (1). Upon lowering the reaction temperature to 55–60°C oligonucleotide primers anneal to the complementary sequence (2). *Taq* DNA polymerase fills in the sequence with nucleotides at 72°C, resulting in double-stranded DNA molecules (3). Upon melting, these function as templates for another cycle of PCR (4). Thus, with every cycle the amount of DNA is duplicated.

Messenger RNA (mRNA) cannot be used directly as a template for a PCR reaction to study expression. Since the *Taq* DNA polymerase can only use DNA as a template, the mRNA first needs to be copied into copy-DNA (cDNA). This reaction requires reverse transcriptase as the catalysing enzyme. The major difference in this reaction is that only one oligonucleotide primer is required for one cycle resulting in one pool of cDNA. Depending on the choice of primer, a specific (set of) cDNAs is produced with a gene-specific primer, or the entire mRNA fraction is copied using an oligo(dT) anchor primer. All plant mRNAs are characterised by a poly(A)⁺ tail at the 3' end of the molecule. Using the oligo(dT) as a universal primer leads to binding to the poly(A)⁺ tail and first-strand cDNA synthesis. This first-strand cDNA can then be used for further PCR-reactions, e.g., to semi-quantitatively determine the relative expression of a gene or, if differences are more extreme, to determine whether the gene of interest is expressed at all.

Molecular tools have been incorporated in the science of medical diagnostics for some time. Techniques like PCR have been widely used to detect infectious diseases, food poisoning, sexually transmitted diseases, viral infections and genetic defects (Lee *et al.*, 1997). For example, upon unravelling the genomic sequence of a gene involved in citrullinemia, an autosomal recessive inherited disease, two novel mutations were found that caused a milder form of argininosuccinate synthetase deficiency. This knowledge was used to design intronic primers for a quick PCR-based diagnosis of the disease that could be applied as prenatal diagnostics (Haberle *et al.*, 2002).

However, in plants application has been limited and is mostly restricted to phytopathology and parasitology to detect the presence of pathogens. Not surprisingly, these studies all use a PCR-based method to diagnose infection, and in two cases the PCR method could differentiate between different pathotypes (Schafer and Wostemeyer, 1992; Freeman and Maymon, 2000; Johnson *et al.*, 2000). In order to detect the presence of viruses or transgenes a quick and simple one step reverse transcriptase PCR (RT-PCR) method has been described (Myslik and Nassuth, 2001). This method takes advantage of two recent developments, the first one being an RNase inhibitor, which can be added to RNA samples to protect the RNA from degradation by RNases. The second development is a one-step RT-PCR method that allows reverse transcriptase and subsequent PCR in one single tube, thus minimising contamination and inaccuracies due to pipetting errors. Of course this method can also be applied to detect expression of any gene of plant origin.

Genes Associated with Germination

The expression of several genes has been associated with germination. One well-studied species, not surprisingly a crop, is tomato, displaying an increase in gene expression and activity of many hydrolytic enzymes during germination that are involved in cell wall hydrolysis (Chen and Bradford, 2000). Most of these enzymes, of which endo-\(\textit{B}\)-mannanase has been best characterised, are expressed in the endosperm during germination and are supposedly involved in weakening and degradation of the endosperm, allowing radicle protrusion. In dormant seeds no active endo-\(\textit{B}\)-mannanase was found, which suggests that the correlation with germination is a causal relationship (Toorop, unpublished results). However, the most obvious attempt to falsify this hypothesis, i.e., application of abscisic acid (ABA) in order to inhibit germination, does not show down-regulation of any of this hydrolytic enzyme (Toorop et al., 1996). ABA has been shown to be synthesised during imbibition of seeds and could, thus, explain delayed

germination or dormancy in some species (Grappin *et al.*, 2000; Schmitz *et al.*, 2002). ABA may decrease the expression of genes that are involved in germination. Using a proteomics approach, α -2,4-tubulin was found to be induced by gibberellins (GAs) and appeared to be strongly associated with the completion of germination (Gallardo *et al.*, 2002; Gallardo *et al.*, 2001). Accumulation of β -tubulin is tightly associated with germination (De Castro *et al.*, 1995). These data strongly imply that cell cycle events are an essential prerequisite for germination. Thus, genes and gene products related to the cell cycle may be potential universal germination markers.

Genes Associated with Dormancy

Not so many genes have yet been associated with dormancy. So far, they have only been shown to correlate with dormancy rather than form a causal relationship, e.g., AtPer1, which codes for a peroxiredoxin (Haslekas et al., 1998) and genes associated with reserve deposition and developmental remnants. AB13, a developmental gene involved in maintaining the seed in a non-germinative state, forms an interesting topic of study, since it is normally transiently expressed during seed-maturation (Parcy et al., 1997). However, in Xanthocyparis nootkatensis its expression has also been found in mature, dormant seeds (Lazarova et al., 2002). In fact, AB13 expression has been found in many other tissues, including apical buds of seedlings, stipules and abscission zones of siliques, and is thought to form part of a molecular network regulating quiescence (Rohde et al., 1999). Not surprisingly, most of this research is carried out with Arabidopsis. Over the last 20 years there has been a near 200-fold increase in publication output, which shows the wide use of this model species. All this accumulated information, not to forget the genome sequencing projects, should allow us to select a number of parameters for further investigation.

Micro-array Technology

A new revolutionary tool that might give more information and perhaps more insight into germination and dormancy is micro-array. This technology uses the same principle for detection of sequences as in dot blotting, a method in which RNA samples are spotted on a carrier membrane and hybridised with a gene of interest. Conventional detection uses a radiolabelled probe for hybridisation, and visualisation through exposure to X-ray. With micro-arrays the situation is inverted (the spots are genes, and the labelled probe is RNA or cDNA from a sample), and the throughput has been increased significantly through miniaturisation. The carrier is now a glass slide and, with a single spot diameter of less then 250 microns, hundreds or even thousands of spots (depending on the spotting technique used) can be placed on a glass slide that has the size of an ordinary microscope slide. Hybridisation is carried out with mRNA or cDNA that is labelled with a fluorescent probe. By using different fluorescent dyes for two independent samples one can image and calculate expression levels in each sample of every individual gene that is spotted. The colour of the dye tells whether the gene is differentially expressed, and the intensity gives information on the level of expression. One can imagine that the amount of information that is generated is vast, and that this technology should unravel genes correlated with dormancy and other seed quality parameters.

Micro-array is not necessarily restricted to research, and could eventually be used as a diagnostics tool to screen seed lots. Once the expression patterns of genes have been assessed, a selection could be spotted on a custom micro-array. Depending on the genes that are spotted, multiple aspects of seed quality can be screened. Since 'the sky is the limit', any physiological parameter could be tested in the same run by spotting marker genes. In the light of *ex-situ* seed conservation, seed quality aspects such as dormancy, longevity, storability, and desiccation tolerance are most appropriate for investigation.

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