Ciências ULisboa | Biologia Vegetal

Fisiologia Molecular do Stress

Laboratory classes
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PCR – Polymerase Chain Reaction

The Polymerase chain reaction (PCR), is a DNA amplification technique which has revolutionized biological research and has become an essential technique in molecular biology and genetic engineering. PCR allows scientists to make unlimited copies of DNA fragments and genes from a single copy of initial DNA.

PCR is based on using the ability of the enzyme DNA polymerase to synthesize a new strand of DNA complementary to the offered template strand. Because DNA polymerase can add a nucleotide only onto a pre-existing 3'-OH group, it needs a primer (a short single stranded oligonucleotide complementary to the DNA fragment that we want to amplify) to which it can add the first nucleotide. This requirement makes it possible to delineate a specific region of template sequence that the researcher wants to amplify. At the end of the PCR reaction, the specific sequence will be accumulated in billions of copies (amplicon or PCR product).

Before performing a PCR, the template DNA is extracted from the biological sample being analysed (e.g. cDNA from grapevine leaves subjected to water stress). In order to amplify the specific DNA or target sequence (e.g. the cDNA encoding the catalase (CAT) gene from grapevine), two primers are design to align to the ends of the target sequence (Figure 1; blue colour). In theory the primers are design to align only to the target gene (ex. CAT gene) in order to have a specific amplification. Primer design is performed by the researcher and is one of the most important factors affecting the quality of the PCR analysis. Unproperly designed primers can align to other DNA sequences in the template DNA generating unspecific amplifications. In this laboratory class we will amplify a sequence of 150 bp of the CAT gene from grapevine. We will use the cDNA synthetized from RNA extracted from Pinot Noir and Touriga Nacional (two grapevine varieties) leaves after dry treatment and control treatment. The CAT gene, which encodes an oxidative stress enzyme, is responsive to drought stress.

Figure 1 represents an overview of a PCR amplification reaction. After denaturation to separate the two DNA strands, the primers hybridize to the DNA template (the CAT cDNA of grapevine) marking that sequence to be copied by the Taq DNA polymerase. Starting from the primer, Taq builds a new strand of DNA in the 5’- 3’ direction, using the DNA template as a guide. Each cycle of the polymerase chain reaction doubles the number of copies of

the gene of interest, so for this experiment, which has 33 cycles, over 17 billion copies of your gene of interest will be made for each starting template (see Figure 1).

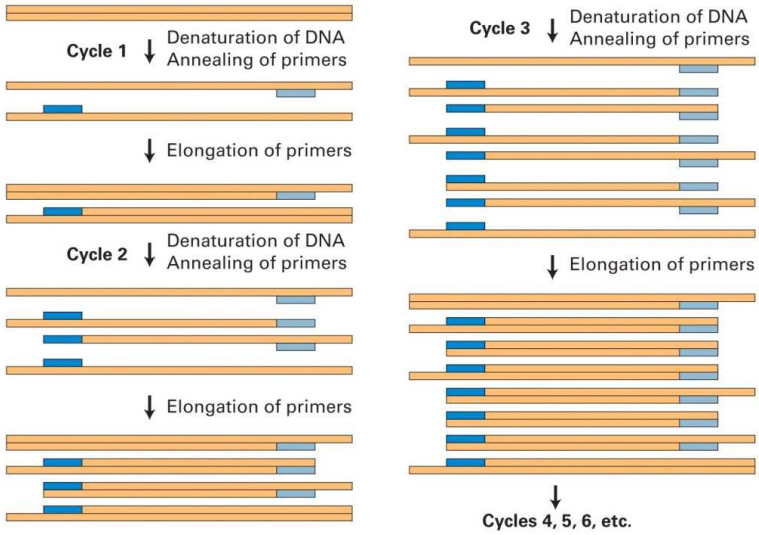

Figure 1: The exponential copying of a gene of interest during the polymerase chain reaction. Primers are in blue.

The key to the polymerase chain reaction is the Taq polymerase that was first purified from the thermophile Thermus aquaticus. A thermophile is an organism that grows at extreme temperature (>100°C). The importance of the Taq polymerase being purified from a thermophile is that the enzyme will not be destroyed at high temperatures required to denature the DNA and allow PCR to begin.

There are three basic steps in PCR (Figure 1 and 2).

Denaturation - First, the template DNA is denatured; the strands of its helix are unwound and separated-by heating to 94-96°C. The high temperature disrupts the hydrogen bonds between the 2 complementary DNA strands, causing their separation.

Annealing - The second step is primer annealing. Short complimentary single stranded oligos are added that bind the denatured DNA and act as origins of replications for later DNA synthesis with Taq. Two primers are used, one for each strand of DNA. The reaction mixture is rapidly cooled to a temperature 45-65º C which allows the primers to bind to their complementary bases on the now single stranded DNAs.

Extension - In the third step, the temperature of the reaction is raised to the optimal temperature for the polymerase (72ºC) and the Taq DNA polymerase adds nucleotides to the hybridized primers, synthesizing new DNA, starting from the primer. The result is two new strands in place of the first, each composed of one of the original strands plus its newly assembled complementary strand.

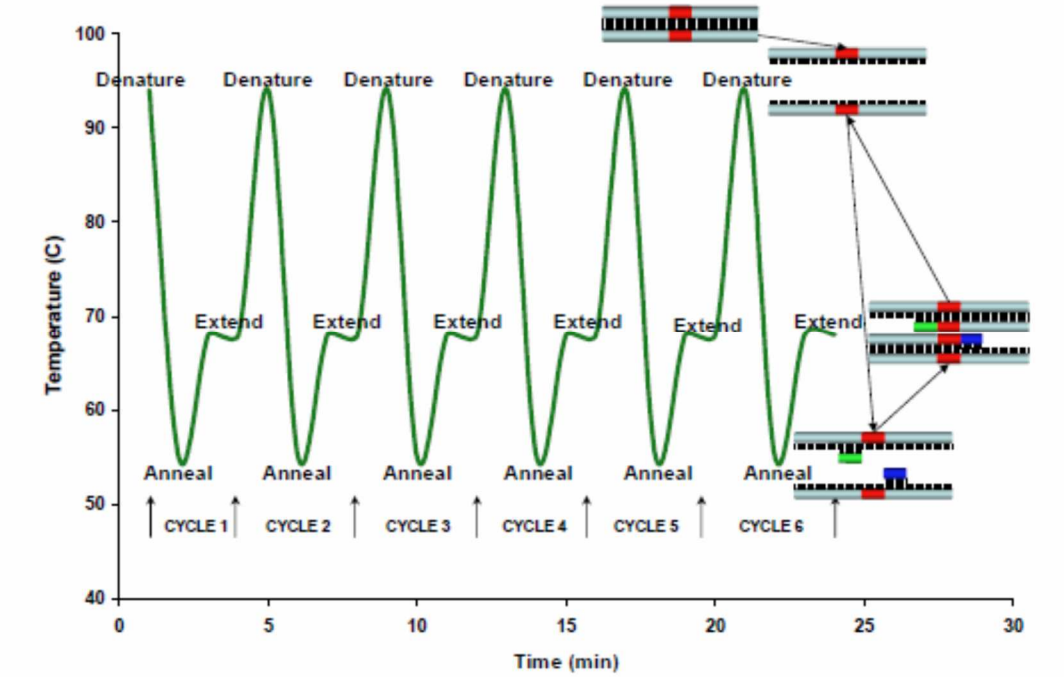

Figure 2: The temperature cycles used during the PCR reaction. The graph depicts the changes in temperature and the resulting effect on the DNA. A schematic of these effects is shown to the right of the graph.

The polymerase chain reaction is able to produce many copies of the genes of interest as the above cycle can be repeated numerous times leading to an exponential increase in the number of new copies.

Each cycle of PCR doubles the amount of DNA, in an exponential relationship (Figure 3). In general, 20-40 cycles produce enough DNA for analysis. After that, the amount of DNA reaches a maximum known as the plateau phase since reaction components begin to be depleted and the activity of Taq starts to decrease.

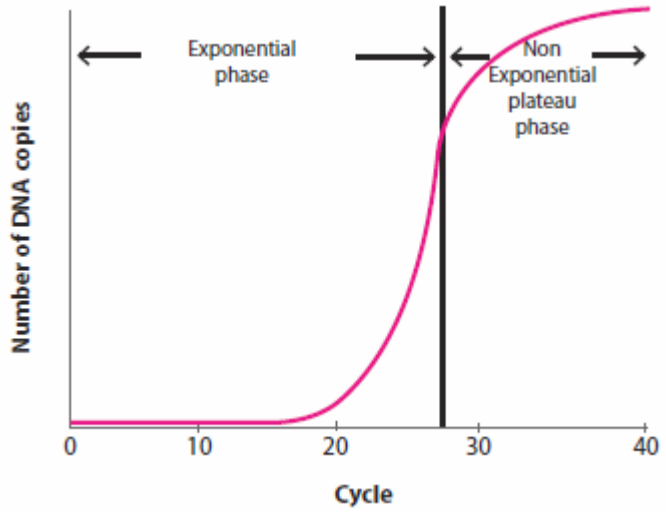

Figure 3 – The exponential phase and the plateau phase of the PCR

To perform a PCR, the template DNA (e.g. cDNA from grapevine) is mixed in a tube with the primers (forward and reverse primers), free deoxynucleotides (dATP, dCTP, dGTP, dTTP; dNTPs), Taq DNA polymerase and a buffer containing Mg^{2+} essential for Taq optimal activity. The tube with the PCR reaction mixture is then introduced in a thermocycler equipment (Figure 4), where the sequential heating/cooling of the denaturation, annealing and extension cycles are performed. The thermocycler tightly regulates the temperature changes required for denaturation, annealing and extension. It also controls the number of cycles. Thermocyclers are fully programmable and allow for rapid heating and cooling and therefore tight control of the PCR.

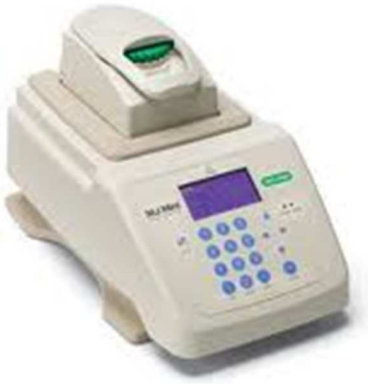

Figure 4 – Thermocycler

Since researchers can specify the primers sequences to target a specific gene of interest (e.g. the CAT gene from grapevine), this method allows the rapid amplification of a selected DNA sequence on the laboratory. The amplified DNA can be used for cloning applications, such as cloning a gene (e.g. CAT from grapevine) in an overexpression vector to be use for plant transformation to investigate the effect of overexpression of that gene on plant resistance to water stress (if the gene is involved in water stress resistance, transformed plants will be more resistant to drought than non-transformed plants). Also, medical diagnostics use PCR for identification of infectious agents, such as covid 19 virus.

Agarose Gel Electrophoresis

After PCR is performed, the result can be analysed by agarose gel electrophoresis. This technique will allow a "visualization "of the PCR amplification reaction regarding the number of amplified regions (one or more PCR products?) and the size of the PCR

products (the size is the expected one based on the predicted amplicon flanked by the primers?).

Agarose gel electrophoresis separates DNA molecules according to size. First, a board of agarose gel is assembled with wells (depressions) on top (Figure 5). The gel is submerged in electrophoresis buffer inside the electrophoresis tank. The PCR products (DNA) are then deposited on the wells and an electrical current is passed through the gel, conducted by the salts in the electrophoresis buffer (e.g. TAE - Tris-acetate-EDTA electrophoresis buffer).

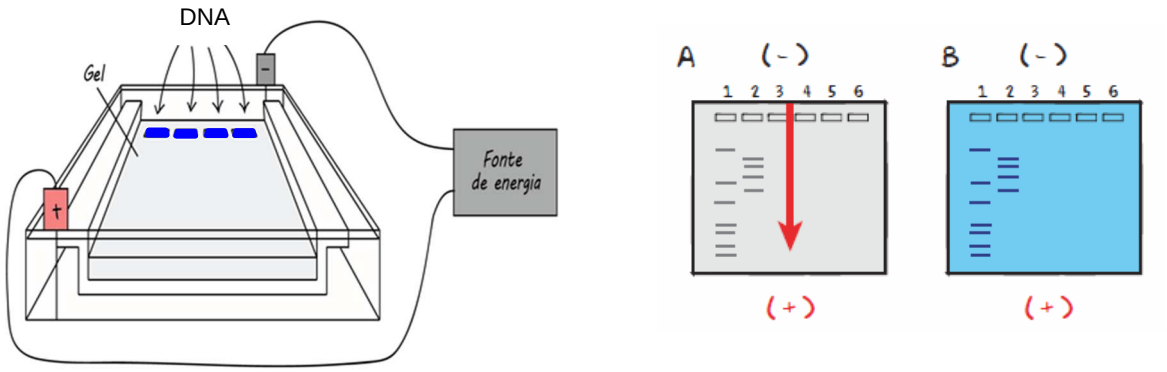

Figure 5 – Overview of agarose gel electrophoresis

Because DNA has a negative charge the current drives the DNA across the gel into the positive electrode (Figure 5A). The agarose gel is a porous matrix where DNA molecules move according to size; smaller molecules take less time to move towards the end of the gel because shorter DNA fragments are not retained by the pores of the gel. Therefore, when we have a mixture of different DNA molecules, shorter molecules will migrate faster and will be deposited as bands at the bottom of the gel. Longer DNA molecules will be deposited as bands at the top of the gel because they have been retained by the pores in the gel matrix.

Before applying the DNA into the wells of the gel, the DNA is mixed with loading buffer (Figure 5; blue colour in the wells). This buffer is composed of glycerol (to enable the deposition of the DNA inside the wells) and bromophenol blue dye (to visualize the DNA solution when loading the samples and monitoring the progression of the electrophoresis run). The loading buffer does not interfere with the electrophoresis.

After the current is stopped, the DNA bands can be visualized using a fluorescent stain that intercalates the DNA molecules and a UV light equipment (Figure 5B). There are several types of stains that intercalate the DNA molecules, emitting fluorescence when the gel is irradiated under UV light (254nm -365 nm) such as ethidium bromide, SybrGreen or GelRed. These agents are added to the agarose when assembling the gel.

If using ethidium bromide which is a suspected carcinogenic, extra safety measures are needed – always wear gloves and lab protective clothes.

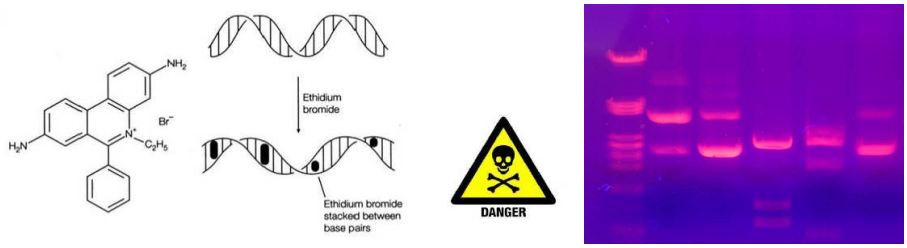

Figure 6 – An overview of an agarose gel visualized with ethidium bromide

To know the approximate size of the PCR product, a DNA molecular weight (MW) marker is added to one of the wells (typically the first well) when the DNA samples are deposited in the wells. This marker is composed of a mixture of DNA fragments with known sizes and can be purchased from biotech companies. By comparing the band from the unknown PCR products with the lanes from the MW marker, we can estimate the size of our PCR product because the MW marker was "run" in the same gel as our samples (Figure 7).

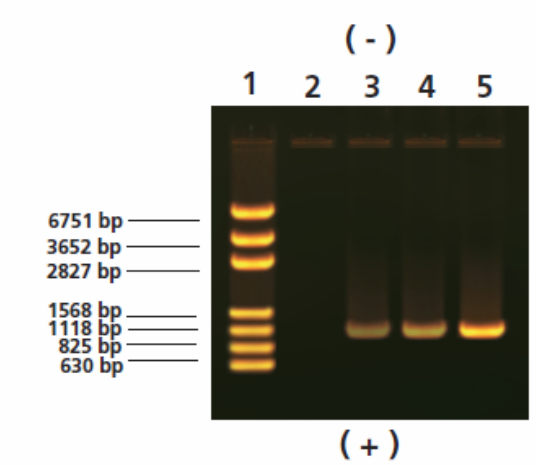

Figure 7 – Overview of an agarose gel with the bands from the MW marker on lane 1. The "unknown" samples are in lanes 3, 4 and 5 (increasing concentrations of the same PCR product). The PCR product analysed has approx. 1118 bp.

Expected results from the amplification of the catalase (CAT) grapevine cDNA:

1 – How many PCR products are expected? Justify your answer.
2 – What is the expected size for the CAT PCR product(s)? Justify your answer.
3 – What are the components of a PCR reaction? How does each component contribute?