Correct version of the article by Bernard Slippers. Apologies for the errors produced in the Yearbook 23 version.

The complexity of flower colour formation: Regulation of anthocyanin production and its relevance to flower colour in *Clivia*

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Background

The colour in *Clivia* flowers is produced by three types of pigments, namely anthocyanins (red), carotenoids (yellow) and chlorophyll (green). The colour in the flowers is formed by the intensity (amount) and pattern of the deposition of these colour-reflecting molecules in a 3D matrix of the cellular layers of the tepal/sepal. The colour we perceive is not formed by the physical mixture of these pigments (like paint), but by the patterns in which the pigments are deposited across the tepal/sepal (and in layers) and the mixture of light reflections from these pigments.

Anthocyanins, carotenoids and chlorophyll are ubiquitous in flowering plants. These pigments are not only involved in reproduction for the formation of colour in flowers, but are produced throughout the tissues and developmental stages, with various critical functions in growth, development and defense. They are pleiotropic, meaning they affect a variety of biological functions in different plant parts. Consequently, the genes and pathways that underlie the production of these pigments are highly conserved amongst plants. While these genes have not been characterized in *Clivia*, they are also expected to be conserved in this genus.

Very few genes are expressed all the time. Virtually all our genes are regulated so that they are only expressed when they are needed. Such regulation happens in various ways, through proteins or molecules binding to the DNA or RNA. This prevents wasteful cellular processes, which would have negative energy consequences for any organism.

The same is true for colour variations in flowers, which in most cases is the consequence of the regulation of expression of genes that underlie the production of colour pigments in certain tissues. The expression of these genes and the processes they control are increased or decreased at certain times and in tissues during development.

In this article, we discuss what is known about the production and regulation of anthocyanins in flowering plants, and what this might mean for colour formation in *Clivia*. For this purpose we specifically refer to a recent international review on the topic by LaFountain and Yuan (2021).

Anthocyanin production

Anthocyanin is part of a group of chemicals or metabolites called flavonoids. These chemical compounds occur widely in plants and are responsible for red, blue and purple colors in flowers, autumn leaves and fruits. They are also present, albeit not always visible, in vegetative tissues, where they have various roles, for example, protecting the photosynthesis machinery from UV radiation, detoxifying harmful oxidants that build up during environmental stresses (such as drought, temperature and more) or to protect plants against pests and pathogens.

Anthocyanins are produced through a highly conserved pathway or process called the anthocyanin biosynthesis pathway (ABP). This pathway includes 10 or more proteins that all play a role, which consecutively converts starting chemicals into the eventual anthocyanin products.

The production of anthocyanins is regulated because the pigments are needed in different tissues of the plant at different times and in different quantities. For this reason, plants have evolved very complex regulatory mechanisms that can respond to a myriad of different internal (eg. development stage) and external (e.g. physical damage, temperature, etc.) signals during the development of the plant.

The regulatory mechanism of anthocyanin production is known as a 'double-negative logic' regulation. This regulatory mechanism is common in plants with molecular processes that require rapid response to environmental or other cues. The double-negative regulation means that there is a two-step process to the regulation of the gene – a primary regulator (often stimulating production continuously) and a secondary regulator (often repressing the primary regular) that responds to various input signals. Signals that suppress the secondary regulator (suppresses the suppressor – thus the 'double negative logic'), indirectly activate the primary regulator/stimulator.

An analogy to understand this is water in our homes (Figure 1). The water comes from a reservoir via a pipeline to our homes. This pipeline is like a primary regulator and is always full of water or 'turned on'. The flow of the water from this pipeline is regulated by a tap, the secondary regulator. The tap, however, needs a hand or a signal to open it– and can be opened fully or partly. The hand that acts on the tap is the 'double negative logic' that allows the water to flow.

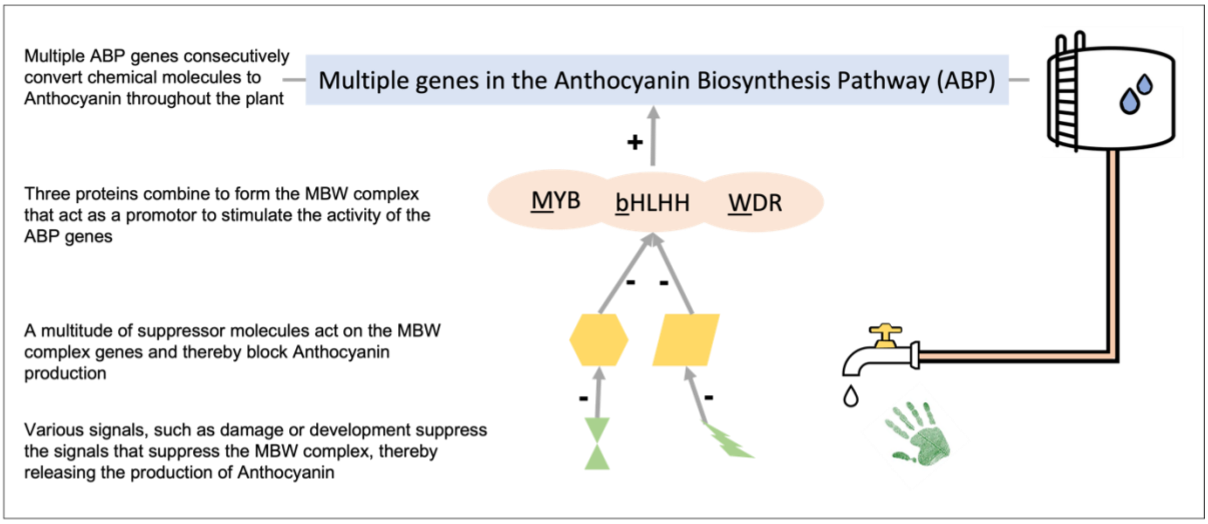

Figure 1. A schematic explanation of elements of the Anthocyanin production and suppression process. An analogy for the process is provided, as water flowing from a reservoir, through a pipe to a tap where its flow can be controlled by an external factors (hand). Colors on the schematic and analogy match parts of the process.

The main or primary regulator of the ABP (Anthocyanin Biosynthesis Pathway) is called the MBW complex (Regulatory complex) and is conserved in all angiosperms studied to date. The complex is made up of different proteins that interact and bind each other. This regulator activates genes in the ABP, and thereby stimulates the production of anthocyanins.

In addition, there is a whole range of secondary regulators, most of which act on the MBW complex, and mostly to suppress its action. By suppressing the MBW complex, these regulators also suppress the production of anthocyanins. Various signals, such as environmental triggers such as temperature, development - chemical processes relating to development (ripening, petal formation, etc), can deactivate the suppressors, and thus release the MBW complex to stimulate anthocyanin production.

In the case of anthocyanin production in flowers, the MBW complex is like a pipeline, while the secondary factors that regulate the MBW complex are like taps. The signals that control these secondary factors are like hands that turn the taps.

While these processes have not been fully studied in *Clivia*, it is expected that *Clivia* would have these same conserved processes as found in all other flowering plants (as explained by LaFountain and Yuan (2021)). Albeit limited, prior work done on *Clivia* and related plants support this hypothesis. This includes work to characterize the pigments, as well as on the genes underlying the production of the pigments in *Clivia* (see for example Snyman, Spies and Viljoen, and Hammett 2006). Quite extensive work has also been done in other Amaryllidaceae, which also support this hypothesis (see for example Wang et al. 2021). The study by Snyman, Spies and Viljoen confirms the presence of key genes in the ABP pathway in *Clivia*, and its close relation to those of other related monocots.

Anthocyanin and colour formation in *Clivia*

It has often been stated that yellow plants have a mutated gene for anthocyanin production in the ABP pathway. Given the general importance of anthocyanin in plants, and its conservation in all plants, it is unlikely that these are mutations in the ABP genes that completely disable its production. No anthocyanin production is likely to lead to developmental issues (weaker plants) and there is no evidence that yellow flowering *Clivia* suffer from any developmental issues. Rather, anthocyanin production is likely only suppressed in some developmental stages in yellow plants, and in these cases clearly in the leaf tissues in early development (green stems) and in the flowers. In the light of the mechanisms described above, the mutations that create this effect are likely in one of the secondary repressors of the MBW complex. This suppressor does not respond in the same way as in orange plants, and thus the MBW complex is not 'released' to stimulate anthocyanin production.

This conclusion is supported by evidence that low levels of anthocyanin production is maintained in at least some yellow *Clivia*. For example, as Snyman, Spies and Viljoen demonstrated the anthocyanin pigments are present in the yellow flowers of Group 2 yellow plants, albeit at least 16 times lower than in a standard orange flowering *Clivia* plant. The analysis by Hammett (2006) did not detect anthocyanin in the flowers, but it is not clear if this is because of less - sensitive methods were used than in the later study by Snyman, Spies and Viljoen, to reveal the very low levels of anthocyanin that they found. Considering all the evidence, it is more likely that anthocyanin production is inhibited, and not altogether disabled by a mutation in a gene in the ABP pathway. This would also explain the red 'bleeding' upon damage to some yellow flowers. In this case the suppressor is inhibited by the damage signal in the flower, thus releasing the MBW complex to stimulate production of anthocyanin around the site of damage. This is a known protective mechanism in other plants as well and demonstrates that anthocyanin production is not completely disabled.

Figure 2. Examples of 'bleeding' in flowers of Group 2 yellow and green plants, which provides an example of a mutation in a regulator influencing the normal production of this chemical in the flower, as opposed to a mutation that made its production impossible under all conditions.
Photos curtesy of Dawie Strydom.

It is common knowledge among *Clivia* breeders that a cross of group 1 and group 2 yellows produce orange plants. In the light of the above discussion, the mutations and complementation of alleles is likely necessary for the regulators and not the ABP genes. A cross between these two lines will then provide a non-mutated regulator of the anthocyanin production from each parent, which then allows for the 'release' of the MBW complex at the right time, allowing anthocyanin to be deposited in the flowers and young stems.

Group 1 Peach could be interpreted as weaker repression of the same gene that is repressed in Group 1 yellows. The repression of the production of anthocyanin in the flower is not released in yellow plants, while it is only activated in a limited manner in peach flowers. The same would be true for Group 2 yellows and peaches. In fact, the shades of color we see from light-peach, to pastel, to orange and to red, can be interpreted at a scale of repression of the MBW complex, which stimulates anthocyanin production. In plants that blush over time, it is possible that there is a time dependent release of the suppression of the MBW complex as the flower matures.

It is, however, also possible that there are changes at cellular, biochemical level that influence our perception of the anthocyanin in the flower. It is known for example that pH and chemical changes or interacting chemicals (such as metal ions) could change the colour variations (Houghton et al. 2021). Such alternatives will require more extensive biochemical analysis of different colour flowers in the future.

We have here focused on the possible influence of the regulation of anthocyanin in *Clivia* flowers. The regulation of carotenoids and chlorophyl production in flowering plants will be explored in a future article, noting that both these types of molecules, like anthocyanin, are critical to the overall development and growth of plants, and are thus likely to be regulated differently, rather than mutated, in different color forms of *Clivia*.

This article aims to simplify some of the complex processes that influence pigment and colour formation in *Clivia* and other plants. Hopefully, this article will make readers aware of the complexity and beauty of this process. The complexity of this process suggests that the diversity of colours we see in *Clivias* at present, is only the tip of the iceberg.

Summary

- Anthocyanins are produced in various tissues and have a variety of roles throughout the life cycle of the plant
- Anthocyanins are produced through a serious of steps involving many genes and regulators (ABP-Anthocyanin Biosynthetic Pathway)
- Yellow, blushing or pastel orange *Clivia* flowers have suppressed anthocyanin production
- Breeding for variation in *Clivia* flower colour does not involve the variation in a single gene, but more likely includes complex variations in ABP genes and their regulators

Some useful terms

DNA – carries the codes for all life in each cell, including structural elements and processes. These codes are long strings of millions of repeats of four basic coding elements (abbreviated ACGT). The code carried in cells in the roots, leaves and flowers are identical, but regulated differently.
Proteins – are both the building blocks and engines of life, some proteins form part of the physical structures of cells, while others perform the processes inside cells.
Genes – are pieces (usually a few 100 to a few 1000 of the building blocks ACGT) of the DNA string in each cell that contain the code for a particular protein.
Allele – Each plant has one copy of each gene from each parent. If these two copies differ from each other, then we called them alleles of the gene.
RNA – The code in a DNA string is transcribed into another molecule called RNA, which in turn contains the code for the order and length of the building blocks of the proteins (called amino acids). Some RNA molecules can also interact with DNA and other RNA molecules.
Gene regulation – How quickly and how often a gene is transcribed to RNA and from there translated to proteins control the extent to which a process is performed in a cell. Various molecules can bind to DNA to speed up or slow down this process, called transcription factors. Various external environmental and internal cellular processes affect whether a gene is 'turned on' or 'turned off'.
MBW complex - Three molecules, called MYB, bHLH, and WDR, that combine to form a larger, complex molecule that inhibit Anthocyanin biosynthesis.
Secondary metabolite pathway – Most products in cells, including the pigments of flowers, are produced through chain of reactions, performed by a set order of enzymes, each of which is produced by a different gene. Each process takes the slightly modified product from the previous process and modifies it further, until the end-product is produced. This is much like a factory process that starts with various ingredients that are mixed and packaged through multiple steps to produce the products we buy off a shelf. An example is the **Anthocyanin Biosynthesis Pathway (ABP)**.

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