Molecular characterisation and physiological adaptations of cowpea to growth temperature

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Abstract

This research focuses on characterising Rubisco activase expression in cowpea, as well as physiological adaptations of cowpea, *Vigna unguiculata,* to different temperatures. Cowpea is a drought-tolerant crop, however, increase in temperature and weather instability due to global warming can produce conditions which would stretch even cowpea tolerance to its limit. Here, physiological adaptations of cowpea to changing temperatures are characterised, and rubisco activase (Rca) expression is determined to aid in understanding temperature effects on cowpea. No previous work has been done to measure Rca expression in cowpea. Whilst some work has been done on characterising cowpea physiological responses to temperature, it has been focusing on long-term effects, and this study aims to quantify physiological adaptations during first four growth stages (until full unfolding of second trifoliate). A process for RNA extraction from cowpea leaves was optimised, a set of primers differentiating between different Rca isoforms was designed and tested, and a set of candidate reference genes was selected and tested for stability. This was used to perform qPCR to determine leaf Rca expression levels. Daily physiological observations were taken to quantify physiological adaptations.

No significant difference in expression between first and second trifoliate leaves was observed for isoforms 1β, 8α, 8ββ, and 10α. There was some difference in expression for isoform 10β, but the scientific significance of this observation is hard to determine. The most expressed isoform was 1β. Isoform 8α was second most expressed followed by 8ββ, 10β and 10α. Physiologically, chlorophyll density tended to be correlated with temperature, whereas leaf size, above ground biomass, and LMA tended to be inversely correlated to growth temperature.

These results could be used for further study into physiological and molecular adaptations of Cowpea to different environmental temperatures.

Literature Review

1.0 Cowpea as an agricultural crop.

Cowpea is an important legume cultivated in the tropical regions across the world. The majority of cowpea is grown in the West African region, with production centred in Nigeria and Niger (figure 1), with the former producing 45% of the total world cowpea. Outside West Africa, large amount of cowpea is grown in Brazil (with 17% of total world production), and, whilst cowpea is grown industrially in USA, India, and South east Asia, their proportion of the total world supply is marginal (Langyintuo et al., 2003).

Natural properties of cowpea make it a prime choice of seed in the region (Callo-Concha et al., 2013; Mishili et al., 2007). Cowpea beans provide a cheap source of protein, and leaves are used as animal fodder. Some varieties are drought tolerant, which is important in the semi-arid savannahs where majority of production takes place. In addition, being a legume cowpea, helps to restore already poor soil in the region. And finally, growing population and urbanisation of west African region, makes it a viable cash crop (Faye et al., 2004).

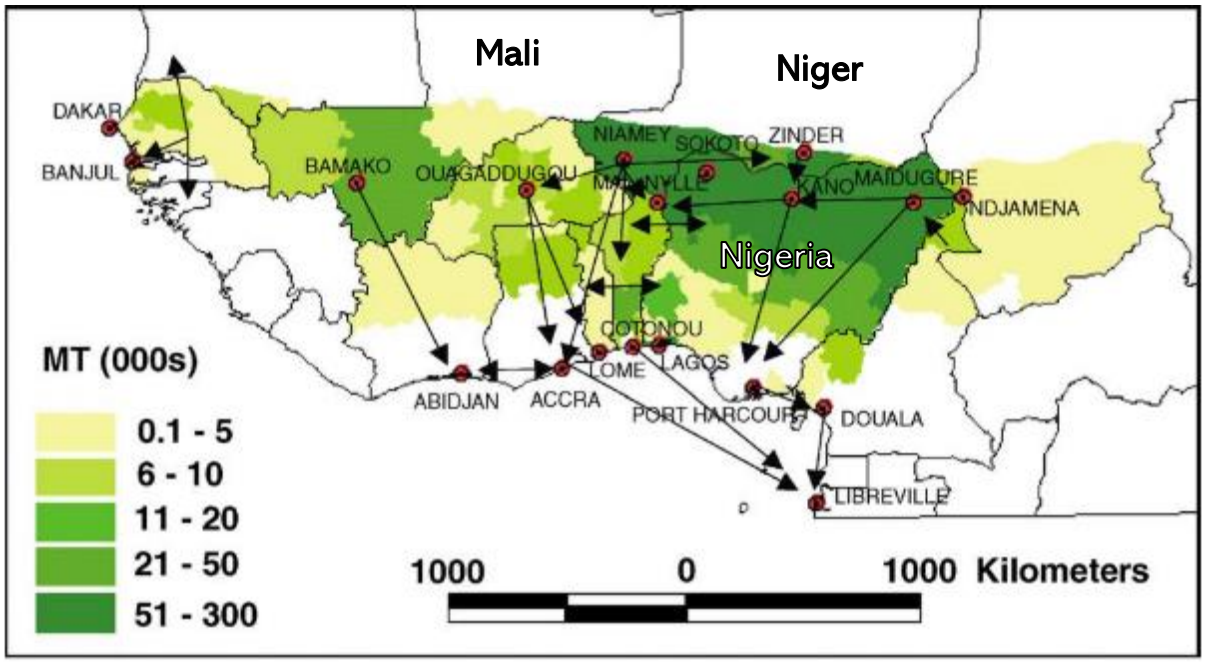


Figure 1. Cowpea production areas (Denoted in green), Main wholesale markets (red dots) and main distribution routes (black arrows) across west Africa. Modified from Langyintuo et al., (2003).

1.1 Challenges of agricultural production

There are several challenges faced by the west African region, which will require improvements to the cowpea. West African region currently experiences a large population growth and growing trend towards urbanisation, with population expected to reach 600 million by 2050(ECOWAS-SWAC/OECD, 2007). Set against a backdrop of poverty, political instability, corruption, sectarian conflicts, institutional weakness, which has now been aggravated by the ecological fragility caused by climate change (Callo-Concha et al., 2013). This provides a unique challenge of increasing cowpea yield and quality in conditions of increasing desertification, and small-scale farming.

In west Africa, Cowpea farming season begins in late July and lasts until October – November (USAID, n.d.). This corresponds with the wet season, which brings water to the arid region, and cools the temperature to 32-28 °C during the day.

1.2 Morphology of Cowpea

Cowpea is a warm-season, annual legume, which exhibits a wide range of growth habits. It has a compound leaf composed of three leaflets. Leaflets exhibit symmetry along a central axis, which leads to a symmetrical terminal leaflet, and asymmetric lateral leaflets which mirror each other. Depending on the species, cowpea may grow short and bushy, or exhibit a tall, vine-like behaviour (Sheahan, 2012). The morphological diversity of cowpea has been previously characterised to aid in the selection efforts (Gerrano et al., 2015; Kouam et al., 2018). Difference in morphology indicates promising lines specialised for grain yield or forage (Gerrano et al., 2015). Though the importance of plant morphology will be discussed in detail in the next section.

Figure 2. Cowpea of IT86D-1010 variety growing at Lancaster university environment centre.

For this experiment, a cowpea variety IT86D-1010 was chosen. IT86D-1010, is a promising high-yield variety which also has demonstrated resistance to biotic stresses (Singh, 2006). The supporting genomic and transcriptomic data for this cultivar was released by Spriggs et al., (2018).

For farmers in west Africa, there is a number of desirable traits a cowpea can possess. Whilst a larger overall bean yield is important, there is (with some exceptions) a premium on varieties that produce larger beans, as consumers are willing to pay more for larger cowpea beans (Mishili et al., 2007). Whilst skin colour, eye colour, as well as skin texture are generally important, there is no general preference for a particular colour or texture across the west African region. Instead, the consumer opinion varies from area to area and market to market (Faye et al., 2004; Mishili et al., 2007). Additionally, as the leaves of cowpea are used as animal fodder, and in many areas around west, south and central Africa for human consumption (Nielsen et al., 1997), there is an advantage to varieties that produce larger canopies.

There is evidence showing that the cowpea growth is significantly affected by the temperature of the surrounding environment, with previous studies showing how temperature affects the flowering time and the time it takes for the plant to ripen (Linnemann, 1994; Craufurd et al., 1997; Hadley et al., 1983; Littleton et al., 1981). Though, Muchow et al.(1993) found that the external air temperature did not affect the radiation use efficiency in the cowpea, soybean and mungbean, as long as the plants remained well-watered. However, this is likely because well-watered plans can use evaporation to cool themselves down, thus maintaining optimal temperature (Burke & Upchurch, 1989).

1.3 Plant physiology

Physiological traits were the first to be observed in plants, and have been recorded as early as the works of Theophrastus (Hort, 1999). With the development of botany as a science, a number of sophisticated systems were developed to quantitatively assess plant traits and accurately compare traits within the same species, and between many different species. (Mokronosov, 1981; Monsi et al., 2005; H. Poorter et al., 2012). Those are commonly referred to as “Soviet”, “Japanese”, and “Western” schools.

Western school measures predominantly whole-organ traits, such as leaf dimensions, leaf mass, as well as stem and root biomass, and a variety of derivative traits, such as specific leaf area, or leaf mass ratio. (Díaz et al., 2016; L. Poorter & Bongers, 2006) Japanese school is overall similar in its approach to the western school, however, it places greater importance on the ratio of photosynthetic/non photosynthetic tissues (Monsi et al., 2005). In contrast, Soviet method, as posited by Mokronosov (1981), places heavy importance on tissue and cell properties, such as relative tissue proportions (similarly to Japanese school), as well as cell size and chlorophyll area per leaf area. They argue that in a number of situations same whole-leaf traits may correspond to different internal structures and functional principles. (Ivanova et al., 2018).

This study works with the methodology described by Poorter et al (2012) to assess the changes in the plant morphology caused by the changes in the external temperature. The overall performance of the plant can be measured by how fast it grows. For this purpose, Relative Growth Rate (RGR) is used. Loveys et al., (2003) showed that plants show a variety of responses in RGR in relation to temperature. It also confirmed findings of Norton et al., (1999) that temperature-induced change in RGR is not correlated with the climate of origin.

Temperature affects not only the overall growth rate of the plant, but also a number of secondary characteristics. One such change is how biomass is allocated between major parts of the plant such as its roots, leaves and stem, which is represented by root mass fraction, leaf mass fraction, and stem mass fraction. Which are measured as dry biomass of the relevant plant part, in relation to the whole plant dry biomass.

Other traits are measured to quantify changes to the individual leaf. Those include: Leaf Area (LA), Leaf Thickness (LT), Leaf Volume Area (LVA), Leaf Mass per Area (LMA), and Leaf Density (LD) (H. Poorter et al., 2012). Those parameters can be used to point to deeper structural changes. For example, changes in LMA may indicate that the internal leaf structure is different (H. Poorter et al., 2009). LMA is affected by temperature, with cold temperatures producing higher LMA (Atkin et al., 2006). As LMA (or its corresponding value SLA) is a component of RGR (Loveys et al., 2003) and is a good predictor for the final RGR (Shipley, 2006), it is a very important trait.

1.4 History of Agriculture with focus on west Africa.

Agriculture first began in the area known as fertile crescent, which encompasses modern day Iraq, Syria, Israel and Jordan. Earliest composite tools for grain harvesting dating back to as early as 23,000 BC were found in the area, showing gradual progression towards sedentary agriculture, which did not happen until 11 000 – 9 000 BC. (Groman-Yaroslavski et al., 2016). Later, agriculture would be independently invented in the Yangtze and yellow river basins, New Guinea highlands, Central Mexico, and Eastern North America. Whilst sub-Saharan Africa is not one of the original agricultural sites, it is the source of many domesticated plants. Development of Agriculture in west Africa followed a different pattern, with many communities preferring pastoralism to settled farming, despite having contacts with people who practiced settled agriculture.(Manning, 2011) With evidence of pastoralism in western Africa dating back to around 6000 BC(Gautier, 1987) However, as the climate begun to change, communities begun to shift towards sedentary agriculture, and by 1650 BC, Kintampo people in Ghana were in possession of domesticated cowpea, domesticated pearl millet, and could exploit oil palms. (D'Andrea et al., 2007)

The second agricultural revolution, sometimes known as British agricultural revolution happened in 17th-19th century. It coincided with, and by and large allowed the industrial revolution. The unprecedented advancements in the productivity came from several key technological improvements:

Crop rotation, Dutch plough, better land management, and improvements in husbandry. Several societal factors such as improvements in trade, farm size, and land enclosure also played an important role(Thompson, 1968)

In the Third agricultural revolution, also known as the Green revolution, advances in yield were made mostly in the selection of semi-dwarfish genes which shortened the growth cycle, often allowing for several harvests where previously this was impossible. Further improvements in technology such as developments of agricultural machinery, wide application of pesticides and synthetic fertilisers, all helped to increase the agricultural yield, and facilitated the explosive population growth seen in the 20th century. (Pingali, 2012)

Due to the socio-economic problems pervasive throughout the African continent, the advances achieved during Second and Green agricultural revolutions, are not fully available, and a sizeable portion of the population is forced to rely on subsistence farming, with relatively primitive techniques. (IFPR, 2013) However, even with complete implementation of those advances, changes in climate and growing population would require further advances in agriculture to ensure food production meet the expected demand. Improving yield efficiency of the cowpea through genetically improving the efficiency of the photosynthesis will help to "futureproof" the crop against climate change and provide for the growing population of the region

2.0 Photosynthesis

Plants obtain the energy required for growth by the process of photosynthesis. A series of computer simulations has shown that increase in the efficiency of leaf photosynthesis can lead to the increase in crop yields. (Ort et al., 2015; Yin & Struik, 2017). Improvement that leads to higher rate of photosynthesis, could range from introducing carbon concentrating mechanisms, increasing Rubisco specificity, or engineering photorespiratory bypass. All of which help to eliminate energy loss originating from the process of photorespiration. Those would be explained in more detail later in the review.

Although usually described as a single process, photosynthesis can be split into two parts, carbon assimilation (Calvin-Benson-Bassham cycle) and light dependent reactions. Those reactions may be localised in the same cellular compartments, as in the case of chloroplasts of C3 plants, where light dependent reactions happen in the thylakoid membrane and carbon fixation happens in adjacent stroma. Alternatively, those reactions can be separated spatially as in the case of C4 metabolism where Light dependent reaction happens in mesophyll cells and carbon fixation happens in bundle sheath cells. or temporally, such as in the case of CAM metabolism, where light dependant reactions occur during the day, and carbon fixation during the night. The reactions are technically independent of each other, but unless there is a biological advantage for them to occur separately, such as in the case of C4 and CAM mentioned above, it is more efficient for them to occur simultaneously.

2.1 Light dependent reactions

Electron transfer reactions, also known as light-dependent reactions occur in the thylakoid membrane in the chloroplasts, and are responsible for capturing energy from sunlight, which is then used to provide the plant with energy source (ATP), and a source of reductive power (NADPH).

Photosystem II captures the energy from the photons, and concentrates it in the reaction centre. The energy from 4 photons is enough to split two molecules of water into one molecule of oxygen, 4 protons and 4 electrons. Electrons, having acquired significant energy from the separation, are then sent through electron transport chain which consists of plastoquinone, cytochrome b6 complex and

plastocyanin. The energy of the electron is used to push protons against the gradient into the thylakoid. Electron is then transferred to Photosystem I, where it receives additional energy from the photons, and is then transferred to ferredoxin and later to ferredoxin – NADP+ reductase, which produces NADPH. The proton gradient produced is used to convert ADP into ATP (Hahn et al., 2018).

2.2 Water splitting & Photosystem II

Photosystem II is an ancient enzyme super complex, which evolved in a single event not long before the great oxygenation 2.45 billion years ago.(Rasmussen et al., 2008). The addition of Photosystem II allowed photosynthesising organisms to utilise water as a source of electrons. Previously, the energy which could be imparted by just one photosystem wasn't enough to cover the difference in redox potential between electrons in the water and NADPH. The ability to utilise widely available water as a source of electrons gave a unique advantage to the cyanobacterial ancestor. This lead to its survival and proliferation both as an individual organism and as an organelle within plants, which paved the way for the oxygenation of the planet. (Cardona et al., 2015)

Photosystem II can be divided into the reaction centre and the outer antenna system. Reaction centre is composed of four membrane intrinsic subunits: PsbA, PsbB, PsbC, PsbD. More specifically PsbA and PsbD form the photochemical reaction centre, whereas PsbB and PsbC for the internal antenna which harvests light and facilitates energy transfer from the outer antennae. The core complex is surrounded by the 12 low molecular mass membrane subunits, which form a belt-like structure. Those subunits play a role in dimerization and stabilisation of the core complex. PsbP, PsbO, and PsbQ, together with reaction centre form the oxygen evolving complex. Outside of those proteins lies the Light Harvesting Complex II. LHCII is a heterotrimer comprised of three primary antenna peptides: Lhcb1, Lhcb2, Lhcb3. A reaction centre might have two to four LHCII heterotrimers bound to it. (Barros & Kühlbrandt, 2009; Gao et al., 2018) The PSII super complex relies on cofactors like Chlorophylls, carotenoids, lipids, plastoquinone, and Mn_4CaO_5 cluster. (Barros & Kühlbrandt, 2009)

2.3 Cytochrome b6 & ATP synthesis.

On the pathway from photosystem II to photosystem I, the electron passes through plastoquinone, cytochrome b6, and plastocyanin. This process does not merely serve to connect two photosystems. Some of the redox potential carried by the electron is used to transport protons across the thylakoid membrane. After this, the electron is transferred to the photosystem I.

The electrochemical gradient in the thylakoid membrane is used by the ATP synthase to restore ATP from ADP and phosphate. ATP synthase is a highly complex protein consisting of two subunits F_0/F_1. Where F_0 generates rotation from the transfer of protons across the thylakoid membrane, and F_1 is responsible for the ATP regeneration. (Shirakihara, 1999)

2.4 Photosystem I & NADPH production

Similar to photosystem II, photosystem I is a large multisubunit membrane complex. Although both photosystems share the same ancestor, they have significant structural differences, particularly in the type of reaction centre they utilise. While photosystem II uses quinone type reaction centre, photosystem I utilises iron-sulphur reaction centre. Although both are required for oxygenic photosynthesis, in the non-oxygenic organisms such photosystems may be present individually, such as type II in purple bacteria or type I in heliobacteria. (Caffarri et al., 2014)

Photosystem I consist of at least 15 core subunits and the light harvesting antenna is made up from 6 different subunits. This level of complexity allows photosystem I to create the highest negative redox

potential found in nature, as well as making complex regulation possible. (Jensen et al., 2007). This high negative redox potential allows for the utilisation of water as the electron source.

2.5 Linear/circular operation mode

At the end of the Photosynthetic electron transfer lies the protein known as Ferredoxin (fd). Fd lies at the intersection of multiple metabolic pathways and can direct electrons it receives from the Photosystem I towards multiple fates. The most well-known pathway is towards the Ferredoxin:NADP(H) oxidoreductase. This process protonates NADP, to form NADPH a reducing agent critical for the carbon fixation, as well as for the number of other biosynthesis reactions. This pathway is widely referred to as the linear pathway.

Alternatively, electrons from the Fd can be returned into the Plastoquinone pool, in the process known as the cyclic electron flow. This prioritises the proton transfer across the thylakoid membrane resulting in the increased ATP synthesis. (Goss & Hanke, 2014)

As a result of the light dependent reactions, a photosynthesising organism obtains conveniently stored ready-to-use energy in the form of ATP and a source of reducing power in the form of NADPH. This is however only a temporary storage of energy, and reducing potential. To produce a long-term molecular store of energy, as well as convenient building blocks for all other molecules, plants utilise CO_2 fixation.

2.6 Carbon fixation

Currently we are aware of 6 autotrophic carbon fixing pathways (Erb, 2011). However, the overwhelming majority of all organic carbon is produced by just one of those 6 pathways – Calvin-Benson-Bassham cycle (CBB), which is utilised by all plants.

Carbon fixation is the first step in the Calvin-Benson-Bassham cycle. The CO_2 molecule is combined with ribulose 1-5 bisphosphate to produce two molecules of 3-phosphoglycerate. 3-Phosphoglycerate is then phosphorylated to produce a molecule of 1-3 Bisphosphoglycerate, which is then further reduced with use of NADPH to produce two molecules of Glyceraldehyde 3-phosphate (G3P). During each 3 cycles CBB produces one extra molecule of G3P than is required to regenerate RuBP required for the cycles to repeat. This extra molecule is directed towards glucose synthesis. This means that for six cycles of CBB one glucose molecule is produced. The remaining five molecules of G3P go through the complicated metabolic pathway to regenerate 3 Ribulose bisphosphate molecules (figure 2). Although, Glucose synthesis is the most commonly cited output of the cycle, other intermediates have the use in metabolism as well. For example, Fructose-6-phosophate is a common intermediate for starch synthesis (Chen et al., 2020; Schreier & Hibberd, 2019), and G3P, among other products of CBB could also be converted to 1-deoxy-D-xylulose 5-phosphate, a precursor to the non-mevalonate pathway, responsible for production of isoprene precursors (Rohmer, 2010). Erythrose-4-phosphate acts as precursor for phenylpropanoid synthesis and, pentose-phosphates act as precursors for nucleotide synthesis (Gontero et al., 2007).

A vital process like CBB cycle calls for a fine regulation (Gontero et al., 2007; Michelet et al., 2013). A major point of control is the carbon-fixation step, and the activity of the Rubisco and its chaperone, Rubisco Activase, which would be described in detail further in the review(Zhang & Portis, 1999a). However, it is not the only enzyme which is regulated in the CBB cycle. Other enzymes are regulated via the help of Glyceraldehyde-3-phosphate dehydrogenase, Phosphoribulokinase, Fructose-1,6-bisphosphatase and Sedoheptulose-1,7-bisphosphatase are all examples of the enzymes which are redox regulated by ferredoxin–thioredoxin system (Michelet et al., 2013). Those are the four of the

main five enzymes (the other being rubisco) which regulation was studied in detail (Gontero et al., 2007), However, newer studies point at other post-translational modifications, such as glutathionylation and nitrosylation, playing a role in the regulation of the cycle.(Michelet et al., 2013)

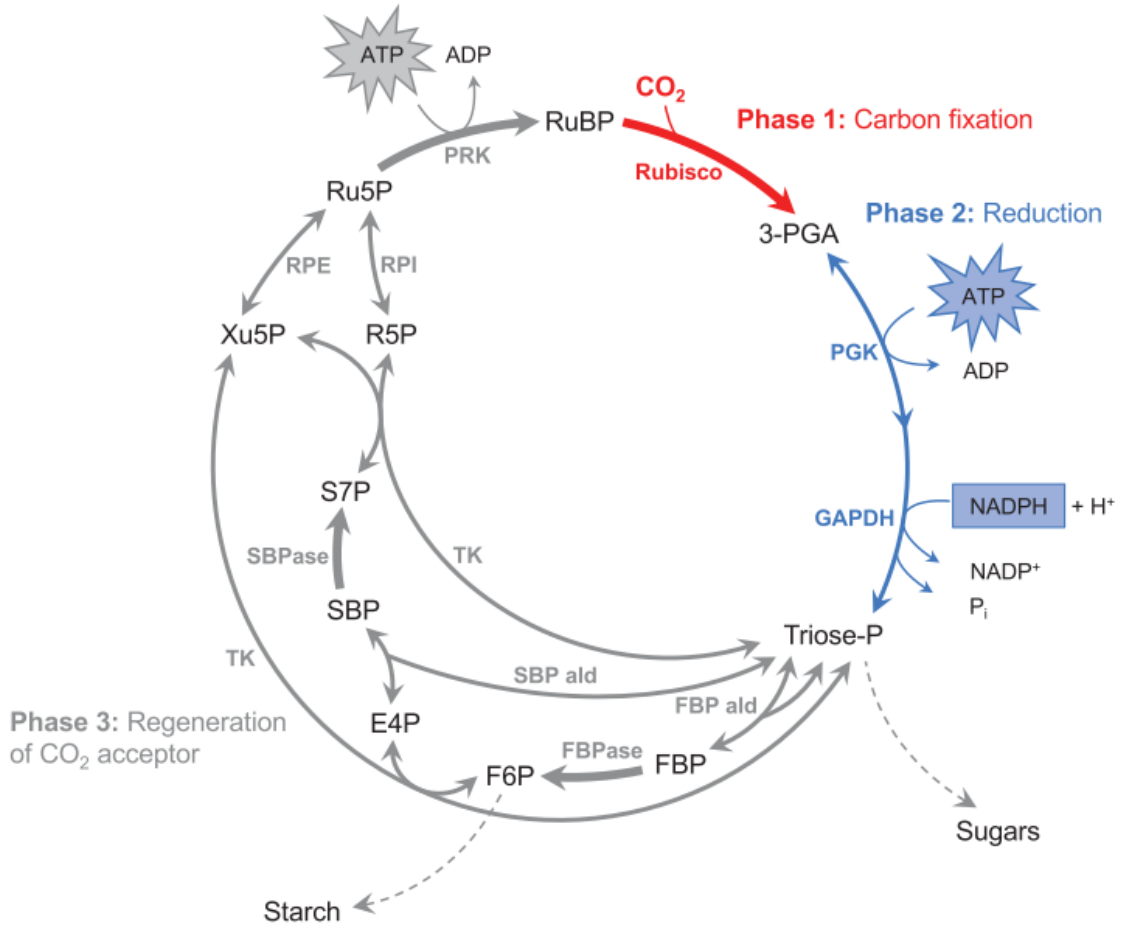


Figure 3. Calvin-Benson-Bassham cycle. Abbreviations: fructose-1,6-bisphosphate (FBP), fructose-1,6-bisphosphatase (FBPase), erythrose-4-phosphate (E4P), sedoheptulose 1,7-bisphosphate aldolase (SBP ald), sedoheptulose-1,7-bisphosphate (SBP), sedoheptulose-1,7- bisphosphatase (SBPase), sedoheptulose-7-phosphate (S7P), transketolase (TK), ribose-5-phosphate (R5P), xylulose-5-phosphate (Xu5P), ribose-5-phosphate isomerase (RPI), ribulose-5-phosphate epimerase (RPE), ribulose-5-phosphate (Ru5P), phosphoribulokinase (PRK). Enzymes which catalyse irreversible reactions are highlighted by a heavy bold arrow (i.e. Rubisco, FBPase, SBPase and PRK). Taken from the paper by Schreier & Hibberd, (2019)

3.0 Rubisco introduction/Evolution.

At the heart of the carbon fixation lies the most abundant enzymes on the planet - Ribulose-1,5-bisphosphate carboxylase/oxygenase, or Rubisco(Bar-On & Milo, 2019)

There are 4 known types of Rubisco, form I, II, III, and IV. Out of them, Type I is most abundant, consisting of 16 subunits, 8 small and 8 large subunits, and is the only type to contain small subunits. Types II and III, consist only of large subunits, with type II forming a dimer, and type III an aggregation of 5 dimers. Type IV rubisco cannot catalyse the CO_2 fixation, and as such are further classified as Rubisco-like-proteins. (Tabita et al., 2008)

3.1 Structure

Cowpea, like all other higher plants, utilises a form I Rubisco, which consists of 8 large (L) and 8 small (S) subunits. The structure is formed by four dimers of the large subunits arranged in the four-fold symmetry to each other. Large subunits are surrounded by 2 small subunit tetramers

In the case of higher plants, L subunits are encoded and translated within the chloroplast, whilst S subunits are encoded in the nucleus and translated in the cytoplasmic ribosomes(Patel & Berry, 2008). While the plant may possess several distinct genes for the S subunit, a non-hybrid plant will possess only a single form of L subunit.

Large rubisco subunits are about 52-55 kDa in size, and contain two distinct domains, a N-terminal domain (residues 1-150), and a C-terminal domain (151-475). To form active sites, two L subunits form a dimer with N terminal of one subunit attaching to C terminal of another. Residues for a single active site are contributed by loops connecting the B-strands to the α-helices of the C-terminal domain of one subunit, and two loop regions in the N-terminal domain of the second L subunit. Consequently each L-subunit dimer possesses two active sites. (Andersson, 1996)

Small subunits are 12-18 kDa in size. They are not strictly required for the reaction, as can be seen in the case of Form II Rubisco which consists exclusively of L subunit dimers. However, in Form I rubisco, S subunits play important role in modifying the reaction, and are arguably necessary as without them, the level of Carboxylation drops to 1% of normal(Andrews, 1988)

3.2 Rubisco Carbamylation

Before carboxylation reaction may take place, Rubisco enzyme needs to be activated: This is achieved by the formation of the carbamate group, via the reaction of CO_2 molecule with the lysine residue (lys210)(Stec, 2012). This CO_2 molecule is distinct from the molecule later incorporated into RuBP. The newly formed carbamate is stabilised by Rubisco forming a complex with the Mg II ion. The resulting complex of Rubisco–CO_2–Mg II is ready to perform carboxylation and oxygenation. It should also be noted that although, Mg II is preferred, activity of the Form I rubisco was observed with other ions, such as Ni II, Co II, Fe II, Mn II, and Cu II. A type of metal ion used, has been observed to influence partitioning between carboxylation and oxygenation.(Schneider et al., 1992).

3.3 Rubisco mechanism

Rubisco, due to the structure of the active site can perform both oxygenation and carboxylation of RuBP. In general, oxygenation is viewed as a wasteful side of the reaction. However, as oxygenation and the subsequent process of photorespiration is deeply interwoven with general energy and nitrogen metabolism, removal of oxygenation reaction may have negative effects. (Busch et al., 2018; Foyer et al., 2009)

Both reactions start with the formation of RuBP–CO_2 complex, which forces RuBP to undergo the enolization reaction. The double bond of the new enediolate is located between the carbon atoms C2 and C3 of RuBP molecule. In the second step, the C2 atom in RuBP is carboxylated to form a six-carbon intermediate. In the third step, the C3 atom of the intermediate is hydrated. The resulting C3-gemdiolate intermediate is split in the next step via scission of the C2-C3 bond and formation of a carbanion intermediate. After protonation of C2 carbon, the reaction ends producing two 3-phosphoglycerate molecules (Cleland et al., 1998; Cummins et al., 2018).

The oxygenation reaction follows an almost identical pathway to the carboxylation reaction with the exception of the second step, where instead of a CO_2 molecule, an O_2 molecule is added. Consequently, once the reaction is complete, the products are one molecule of 3-phosphoglycerate and one molecule of 2-phosphoglycerate. (Cleland et al., 1998; Cummins et al., 2018)

3.4 Inhibition.

Inhibition plays an important role in the short-term regulation of Rubisco. There are two paths for Rubisco inhibition, depending on whether the enzyme is carbamylated or not.

In the first case, non-carbamylated Rubisco active sites bind either RuBP or misfire products (XuBP, D-xylulose-1,5-bisphosphate, KABP, 3-ketoarabinitol-1,5-bisphosphate etc.). After binding the substrate or misfire product, the Rubisco active site adopts a closed conformation. As cleavage of the C2-C3 bond is required to open the active site, Rubisco bound to one of the misfire products will remain inactive until helped by a chaperone (see below). The absence of CO_2 and Mg II when RuBP binds, means that Rubisco is incapable of proceeding with the reaction, thus turning substrate into an inhibitor (Parry et al., 2008).

Carbamylated Rubisco can also be inhibited via the binding of molecules like PDBP, D-glycero-2,3-pentodiulose-1,5-bisphosphate, CABP, 2-carboxy-D-arabinitol 1-phosphate or above mentioned KABP and XuBP, which are produced as a result of catalytic misfire, and the process of *in-vitro* inhibition by them is sometimes called fallover. (Zhu & Jensen, 1991). Another Rubisco Inhibitor is CA1P. It binds exclusively to carbamylated Rubisco, and is used to regulate Rubisco in response to the change in light levels(Berry et al., 1987), though, in some plants amount of CA1P present is too small to effectively regulate Rubisco activity(Moore et al., 1991).

4.0 Rubisco activase.

Rubisco activases (Rca) belong to the super-family of AAA+ chaperones. This is a large family of molecular motors which use energy of ATP binding and hydrolysis to reshape macromolecules (Ammelburg et al., 2006). This makes them perfect candidates for rubisco-reactivation. Although all part of the same superfamily, Rca are split in 3 distinct groups which all arise from different points of evolutionary origin and share little AAA module similarity(Mueller-Cajar, 2017). The three groups of rubisco activases identified to date are Green-type, Red-type, and CbbQO-type. (Mueller-Cajar et al., 2011; Salvucci et al., 1985; Tsai et al., 2015). CbbQO-type Rca is present in proteobacterium, Red-type Rca is present in red algae, proteo-bacteria and phytoplankton, and is also of proteobacterial origin, though distinct from CbbQO-type. The last and of most interest to this research is Green-type Rca. This group of Rubisco activases is present in cyanobacteria, green algae and higher plants, was earliest to be discovered, and most widely studied. Most importantly it is the type of Rca present in Cowpea.

4.1 Structure

The green type Rca's general structure consists of N domain, an AAA+ module and a C-terminal domain. Although overall structure of green type Rubisco activase shows AAA+ fold topology, consisting of nucleotide binding α/β-subdomain, and a-helical subdomain, there are several differences from the classical AAA+ topology. (Stotz et al., 2011) The AAA+ module in the two crystal structures of Rca Molecules from *Nicotiana tabacum* and *Arabidopsis thaliana* has shown structural similarity. (Hasse et al., 2015).

It was shown that Green-type Rca may form a range of oligomers under physiological conditions, ranging from dimers to dodecamers and larger. However, it is common for the AAA+ superfamily to form hexamers (Sysoeva, 2017), and several studies (Shivhare et al., 2019; Stotz et al., 2011) suggest that the oligomeric form required for Rubisco activation is a hexamer. Serban et al. (2018) provided evidence that oligomers larger than hexamers are catalytically inactive, and suggested that they might serve as an energy efficient storage form.

4.2 Rca isoforms.

In higher plants, Rubisco activase is a nuclear encoded enzyme and is often expressed in two isoforms – the longer α form and the shorter β form. The α-form is longer as it possesses an extension at the C-terminus which makes it more sensitive to light changes (Zhang & Portis, 1999a). The different isoforms can arise from alternative splicing of a single gene, or they may be encoded on different genes. Depending on the species, one or both mechanisms are present. Nagarajan & Gill (2018) found that plants can have from 1 to 6 copies of Rca genes. Cowpea has 3 copies of Rca, located on the chromosomes 1, 8 and 10. Rca gene located on chromosome 1 produces a single β isoform of Rca. Rca gene located on chromosome 8 can produce both a longer α isoform and a much shorter ββ – isoform. Rca gene located on chromosome 10 can produce both α and β isoform. In total Cowpea plants possess 5 isoforms of Rca.

4.3 Rca control over photosynthesis.

The level of Rubisco self-inhibition makes Rca a convenient point for the overall regulation of the CBB cycle. One of the important signals to which Rca responds to is the level of light experienced by the plant. This is achieved in two ways: Firstly, the action of Rubisco activase is inhibited by ADP and promoted by ATP. This allows Rubisco activase to sense ADP/ATP ratio, which is increased as ATP production is ramped up in presence of higher light levels (Gardeström & Wigge, 1988).

In addition, the C-terminus extension in longer α-isoforms makes them more sensitive to ADP levels. Reduction of the disulfate bond between Cys-392 and Cys-411 via thioredoxin when light levels enables the α-isoform to be active at physiological levels of ADP (Zhang & Portis, 1999a). This allows Rca to be regulated by the wider redox regulatory pathway in chloroplasts (Buchanan & Balmer, 2005).

Furthermore, presence of free Mg ions in the solution has been shown to increase the rate of ATP hydrolysis by Rubisco activase (Hazra et al., 2015).

4.4. Rca and temperature.

Rubisco activase is highly susceptible to heat stress. Increase in temperature beyond the optimum leads to the enzyme quickly losing catalytic activity (Crafts-Brandner et al., 1997). As it becomes irreversibly denatured, Rca forms high molecular-mass aggregates (Feller et al., 1998). This constrains photosynthesis at elevated temperature, as Rubisco itself is a lot more tolerant to higher temperatures (Crafts-Brandner & Salvucci, 2000).

The level of thermal stability of a particular Rca isoform varies both between species (Henderson et al., 2013), and different isoforms. (Degen, Worrall, et al., 2020; Henderson et al., 2013; Scafaro et al., 2019).

Crafts-Brandner et al., (1997) shows that spinach α isoform is better adapted to higher temperatures. Increased abundance of α isoform in rice is one of the mechanisms utilised to acclimatise to the higher temperatures (Wang et al., 2010a). Contrary, in wheat, both α and β located on the 2nd chromosome have similar thermostability (Scafaro et al., 2019), and the thermotolerant isoform is located on the 1st chromosome is a β (Degen, Worrall, et al., 2020).

The aim of this research project is to characterise how cowpea responds to the changes in the external temperature, with the focus on how it specifically affects the expression levels of Rubisco activase. Phenotypic changes are also characterised to provide context to the changes in Rca expression.

Chapter 1 – Rubisco Activase expression

Abstract

This chapter aims to characterise expression levels of Rubisco activase in the leaves of cowpea, *Vigna unguiculata* L. Cowpea possesses 5 different isoforms of Rubisco activase, however, their relative expression levels are unknown. In this chapter, adequate reference genes were selected, primers capable of distinguishing between different copies of RCA were designed and produced, RNA extraction was optimised, and levels of Rca expression were determined in the first trifoliate and second trifoliate leaf of cowpea grown at optimal conditions. There was no significant difference in expression between first and second trifoliate leaves during the first two vegetative stages of growth, with exception of isoforms 10β. β-isoform of Rubisco activase (Rca) located on chromosome 1 is the most expressed isoform under optimal conditions, followed by 8α, 8ββ, 10β and 10α.

Introduction

The absolute majority of organic carbon compounds on earth are produced as a result of photosynthesis. In virtually all cases, the carbon is fixed via the Calvin-Benson-Bassham pathway, which relies on the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase or rubisco to actually fix inorganic carbon (Andersson & Backlund, 2008). However, Rubisco is prone to self-inhibition, and as such, requires constant presence of the chaperone, Rubisco activase (Rca), to operate at optimum capacity(Mueller-Cajar, 2017; Parry et al., 2008). Plants possess two types of Rca isoforms of differing length with most plants having between 1 and 6 isoforms (Nagarajan & Gill, 2018).

As the regulator of Rubisco activity, Rubisco activase is a natural control hub for the process of carbon fixation as a whole (Hazra et al., 2015). Activity of Rubisco activase can be affected by parameters such as ATP/ADP ratio (Carmo-Silva & Salvucci, 2013), redox status (Zhang & Portis, 1999b), and temperature (Crafts-Brandner et al., 1997). The sensitivity of rubisco activase to each of the parameters is dependent on the isoform type (Zhang & Portis, 1999b), and the specific version of the isoform (Scafaro et al., 2018). This allows plants to adapt to the changing conditions by changing the relative expression levels of Rubisco activase (Law & Crafts-brandner, 2001; Lu et al., 2020).

As first and second trifoliate are sampled at the same stage during their development and both occur at the same stage during plant growth stage, it is reasonable to assume that there would be little difference in Rca expression between them. While it is possible that there is change between fully expanded first trifoliate and fully expanded trifoliate + 1 week, the expectation is that the majority of changes in expression triggered by development would cease once the leaf is fully expanded (Cui et al., 2011; Xie et al., 2012), and so changes associated with maturation are unlikely to be significant.

Normalisation is a prerequisite for the accurate measurement of gene expression. Currently, it is standard to use at least two, but preferably three control genes. The use of control genes allows to control for variation between samples, which could arise either from technical reasons, or due to natural differences in expression between different specimens (Wen et al., 2016). A control gene is required to display little to no variation in expression over the range of the experiment to serve as a reliable reference point. For this reason, housekeeping genes are often selected as candidates for the reference gene. In cases where there is no established set of reference genes, a set of candidates is selected and tested for stability.

Over the years, a number of algorithms have been developed to select most stable genes out of multiple candidates (Andersen et al., 2004; Vandesompele et al., 2002; Pfaffl et al., 2004). Of the three

algorithms examined for this research, Normfinder (Andersen et al., 2004), Genorm (Hellemans & Vandesompele, 2014; Vandesompele et al., 2002) and Bestkeeper (Pfaffl et al., 2004), the latter is the simplest, using pairwise comparison of Standard deviation and Coefficient of variance to select for adequate reference genes (Pfaffl et al., 2004). The other two algorithms, Normfinder and Genorm, illustrate two different approaches towards determining the most stable genes. Normfinder computes intragroup and, where applicable, intergroup variance. The values are then used to produce a single parameter which can be used for selecting appropriate reference genes (Andersen et al., 2004). In contrast, Genorm and the upgraded version GenormM, works by calculating relative gene stability between all the genes present. Each round, a relative comparative stability value M is calculated for all the genes present. The gene which is found to be least stable is eliminated, and the process is repeated once again until only one gene remains. The order of elimination is used to rank genes from most stable to the least (Hellemans & Vandesompele, 2014; Vandesompele et al., 2002). Both methods have their strengths and weaknesses. To ensure best performance, a composite of both rankings was used to determine optimal reference genes.

The aim of this chapter is to measure Rca expression in cowpea grown under optimal temperature conditions, and determine whether there is difference in Rca expression between 1st and 2nd leaf and 1st leaf of two different ages. This required optimising experimental conditions for cowpea growth, sampling, RNA extraction, designing and testing primers differentiating between 5 isoforms of Rca present, selecting reference genes and determining their stability.

Materials and Methods

Plant growth conditions

Forty cowpea (*Vigna unguiculata* L. cultivar IT86D-1010) plants were grown in semi-controlled conditions in a glasshouse in the Lancaster Environment Centre. The temperature was maintained at 30°C during the day and 20°C during the night. The photoperiod was set to 16 hours to expedite plant growth.

Seeds were planted in D40H Deepot cells (produced by Stuewe & Sons, Ontario, USA), lined with verve weed control fabric. The cells were filled with 1:1 mixture of sand and compost (RHS silver sand and Petersfield compost respectively). Seeds were planted directly into watered pots at the depth of 2 cm.

Sample collection

Cowpea plants were distributed in an even pattern and sample groups were selected using Egdar II randomisation algorithm, dividing plants into two sampling groups. Leaf samples (0.5 cm^2) were taken and immediately frozen in liquid nitrogen and stored at -80°C. First group was sampled when plants had just developed one fully-expanded trifoliate leaf, the second group was sampled when two fully-expanded trifoliate leaves had developed, with the second group having both the 1st and 2nd leaves sampled.

RNA extraction.

To extract RNA from the leaf samples, a Macherey-Nagel™ NucleoSpin™ RNA Plant Kit (Macherey-Nagel™ 740949.50) was used, with several modifications made to the standard protocol provided by the manufacturer. Alterations included weight of sample, spin time, and number of treatments, as described below. The details of the original protocol and changes made are illustrated in table 1.

Firstly, optimal amount of frozen leaf powder had to be determined. Manufacturer states that columns can take up to 100 mg of homogenized material, however based on the previous experience

of other colleagues with extracting RNA from wheat leaves, it was known that too much material reduces efficiency. Weights in the range of 10 to 40 mg were tested to determine optimal amounts.

Secondly, spin times in following steps were increased. In step 3 (Filtrate lysate) increased time from 60 seconds to 90 seconds, and in first two washes of step 8 (Wash and dry silica membrane), spin times were increased from 30 to 60 seconds.

Step 7 was repeated twice to decrease DNA contamination. In step 9 (RNA elution), a number of alterations were made. First amount of RNase-free water was reduced to 45 µL from recommended 60. Then, instead of simply eluting RNA once, flow through from the first elution was returned to the column and centrifuged for a second time. In later experiments, the time of second centrifugation was increased to 120 seconds from 60 seconds.

Table 1. Comparison of original protocol proposed by the manufacturer (Macherey-Nagel™ 740949.50) and changes made to optimise RNA extraction.

Steps	**Original Instruction**	**Alterations (initial experiments)**	**Alterations (final experiments)**
1 Homogenize sample	Grind up to 100 mg tissue under liquid N2	Use 15-20 mg of tissue.	Use 25-40 mg of tissue.
2 Lyse cells	Add 350 µL Buffer RA1 and 3.5 µL ß-mercaptoethanol (ß-ME) to 100 mg tissue and vortex vigorously.	----------	----------
3 Filtrate lysate	Place NucleoSpin® Filter in a Collection Tube (2 mL), apply the mixture, and centrifuge for 1 min at 11,000 x g	Increase spin time to 90 seconds	----------
4 Adjust RNA binding conditions	Alternatively, transfer flow-through into a new 1.5 mL microcentrifuge tube, add 350 µL ethanol (70 %), and mix by vortexing (2 x 5 s)	Gently mix by pipetting.	----------
5 Bind RNA	For each preparation take one NucleoSpin® RNA Plant Column (light blue ring) placed in a Collection Tube and load the lysate. Centrifuge for 30 s at 11,000 x g. Place the column in a new Collection Tube (2 mL).	----------	----------
6 Desalt silica membrane	Add 350 µL MDB (Membrane Desalting Buffer) and centrifuge at 11,000 x g for 1 min to dry the membrane.	----------	----------
7 Digest DNA	Apply 95 µL DNase reaction mixture directly onto the center of the silica membrane of the column. Incubate at room temperature for 15 min.	----------	After 15 minutes, spin columns briefly. Add another 95 µL DNase reaction mixture and let incubate for a further 15 minutes.
8 Wash and dry silica membrane	1. Add 200 µL Buffer RAW2 to the NucleoSpin® RNA Plant Column. Centrifuge for 30 s at 11,000 x g. Place the column into a new Collection Tube (2 mL). 2. Add 200 µL Buffer RAW2 to the NucleoSpin® RNA Plant Column. Centrifuge for 30 s at 11,000 x g. Place the column into a new Collection Tube (2 mL). 3. Add 250 µL Buffer RA3 to the NucleoSpin® RNA Plant Column. Centrifuge for 2 min at 11,000 x g to dry the membrane completely. Place the column into a nuclease-free Collection Tube (1.5 mL, supplied).	Increase spin time for sub-steps 1 and 2 to 60 seconds.	

9 Elute RNA	Elute the RNA in 60 µL RNase-free H2O, (supplied) and centrifuge at 11,000 x g for 1 min.	Use 45 µL of RNase-free H2O Put the flow through back in the column, centrifuge for further 60 seconds	Increase time of second spin to 120 seconds.

DNA contamination.

Experiments were performed to determine the best way to reduce DNA contamination from the samples. Two approaches were tested. In the first approach, the RNA extraction protocol was modified by repeating the DNA digestion step. The original manufacturer protocol suggests addition of 95 µL of DNase reaction mixture followed by a 15 minute incubation, then proceeding to wash steps afterwards. In the modified protocol, after the first incubation was over, instead of proceeding to the wash steps, the column is briefly spun, and then an additional 95 µL of DNase reaction mixture is added and allowed to incubate for a further 15 minutes.

The second approach tested, involved separate Invitrogen TURBO DNA-free ™ Kit. In this case, the experiment was performed following the protocol provided by the manufacturer (Invitrogen TURBO DNA-free ™ Kit).

cDNA synthesis.

cDna synthesis was performed using Precision nanoScript™ 2 Reverse Transcription kit, by primer design. The experiment was performed using standard protocol supplied by the producer.

Primers design and reference gene selection.

Geneious 9.0.4 (https://www.geneious.com) software was used for the primer design. Cowpea Genome was accessed from the Phytosome (Goodstein et al., 2012) database. Two sets of primers were designed/selected.

First set was designed to distinguish between different isoforms of VuRca present in the cowpea. Primers were designed with the standard criteria in mind: 19-30 base pairs long, have a melting temperature of 56 °C ±5 °C, an amplicon of about 100-200 base pairs long, have a GC content of about 40-60%, and no complimentary regions. The primers were designed to target regions which would allow differentiation between different isoforms. With the goal to produce primers which could target each isoform, or at the very least have one primer targeting one splicing variant on the chromosome and one primer targeting both variants. Primers were checked against the cowpea genome to ensure they only matched target transcript.

Second set was designed to serve as a reference set for the experiment and used the same design guidelines as the first. Candidate reference genes targeted common housekeeping genes. Those are: VuElf1a, which stands for Elongation factor 1 alpha, and is responsible for delivery of aa-tRNAs to the A site of the ribosome (Sasikumar et al., 2012); VuBAct which stands for β-actin and forms part of Actin cytoskeleton of the cell (Meagher et al., 1999); VuUbi28 a member of the vast plant Ubiqutin family (Meagher et al., 1999); Pp2A is the major phosphatase for microtubule-associated proteins (Janssens & Goris, 2001); PolyP which was identified by Phytozome as putative trehalase (Goodstein et al., 2012); Tua4 codes for the alpha Tubulin (Ludwig et al., 1987).

Primer product verification

Amplified segments were cut out from the gel, cleaned, and sent to the Source Bioscience for sequencing. Results were compared to the sequences initially used for primer design. As some results

proved inconclusive due to damage after extraction from gel, some new amplifications were made, and sent for additional sequencing.

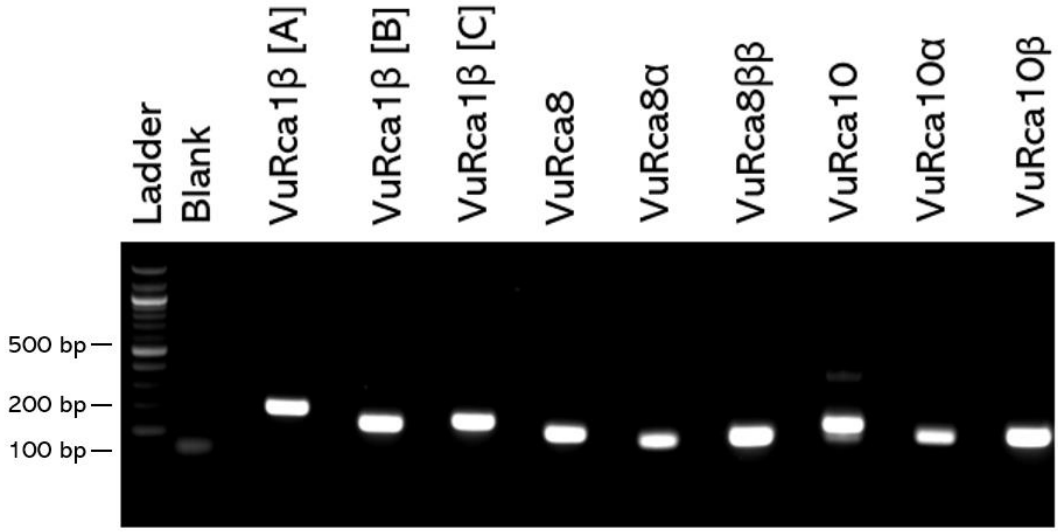


Figure 4. Agarose Gel showing the amplicons produced from the Rca primer pairs tested. All the amplicons were in the expected range. Blank was produced by applying VuRca10β on water instead of cDNA sample.

Gene stability

To identify which of the candidate reference genes were suitable 7 candidate genes and one of the rubisco activase genes were tested. RNA was extracted and then amplified from 10 samples which included samples from fully expanded first trifoliate, a week old fully expanded trifoliate leaf, and second trifoliate. Each primer pair/sample combination was repeated two times. Results were analysed with both Qbase+ software (GenormM algorithm) and Normfinder algorithm. Best keeper was not used due to its inability to account for as much variables as the other two algorithms.

Gene expression between leaves

Three reference genes (PolyP, Pp2A and Elf1a) and cowpea Rca transcripts (Rca1.1B, Rca8, Rca8.2, Rca10.1, Rca10.2) were amplified from 27 samples, 9 for each of three sample types described above. The results were used to calculate the level of gene expression of each Rubisco activase transcripts. The geometric mean of three reference genes was used to normalise for individual expression.

Statistical analysis

Statistical analysis and graph preparation were performed using R (R Core Team & R Foundation for Statistical Computing, 2020) in RStudio (RStudio Team, 2020). Agricolae package (Mendiburu, 2020) was used for analysis and ggplot2 package (Wickham, 2016) was used to prepare figures. Microsoft office excel was used for table preparation. When assessing significance One-way ANOVA was used with the Tukey's post hoc test to compare individual treatments.

Results.

Determining optimal weight of frozen leaf powder.

Increasing the weight of frozen ground leaf powder from Batch 1 to Batch 2 led to nearly three times increase in the RNA concentration and significant increase in the quality of the samples (table 2). Although it is possible that further increase in amount of leaf powder would lead to increase in RNA concentration, no further experimentation was performed for three reasons:

Firstly, from previous experience of other laboratory members with using this kit to extract RNA from wheat, it was known that further increase may lead to diminishing returns or even decrease in RNA concentration due to the clogging of the columns. Secondly, this amount of powder uses about 40% of the ground sample. The remaining sample left after a single extraction could be used to attempt second extraction should the first one fail. And finally, RNA concentration achieved from batch 2 was high enough to perform the experiment, so there was no need for further improvements.

Table 2. Weight of powdered leaves in two batches and resulting RNA concentration and quality. Each batch has 6 samples. Values are the mean of all samples

Table 2 : Weight of powdered leaves and RNA quality				
Batch	Weight	RNA concentration (ng/µl)	Ratio 260/280	Ratio 260/230
1	15.76	138.182	2.01	1.73
2	30.875	342.875	2.015	2.23

Reducing DNA contamination.

From experiments performed on the RNA extracted from batches 1 and 2 it became obvious that there was some DNA contamination, as the amplification in the control without reverse transcriptase was only 3 PCR cycles behind the samples. As each PCR cycle doubles the DNA present, this indicated that total DNA present in the sample made up as much as 1/8 of RNA present. This is enough to interfere with the results of the expression analysis. Experimentation was performed to determine optimal DNA cleaning method.

Table 3. Comparison of the two approaches of cleaning samples from the DNA contamination. Sample quality was assessed using nano-drop via 260/280 and 260/230 ratios, ct values given are means of the two repetitions.

Table 3. Comparison of the two approaches of cleaning samples from the DNA contamination.					
DNA cleaning method	Delay with PP2A primers (qPCR cycles)	Delay with vuRCA1β primers (qPCR cycles)	Sample quality	Difficulty	Time required
Approach 1 (Double Dnase treatment)	8.615	14.705	High	Low	15 minutes
Approach 2 (Turbo DNA-Free treatment)	19.6025	22.635	Medium	High	in excess of 1 hour.

Samples treated by Turbo DNA-free kit (approach 2), were a lot cleaner than samples which were double-treated with DNase during extraction (approach 1) (Table 3). Approach 1 reduced DNA to RNA ratio to 1/388, which pales in comparison to approach 2 which reduced that ratio to 1/600000. However, Approach 1 was chosen, as a method for further RNA extractions. This was due to the fact that Approach 2 was more expensive (requiring separate kit to be purchased), significantly slower, and harder to perform, and most importantly reduced the quality of the RNA produced.

Primer verification and efficiency

After primers were confirmed to be working via PCR, and sequencing proved that the amplified fragments correspond to the target genes, primer efficiency was obtained. Table 4 lists the efficiency of all primers tested. With "pass" column indicating whether the primer is used in later experimentation. All 7 reference genes were used in stability experiments, as it increased chance of finding a suitable set of genes. From the primer pairs targeting Rubisco activase, pairs were selected based on how close their efficiency was to 2.

Table 4: Primer pair efficiency. Primer pair is indicated by the gene they target. The naming system is Vu.GeneName.Chromosome.Size. Reference genes only state gene name. In case where several distinct primer pairs target the same Isoform they are labelled A-Z. ββ at the end of VuRca8ββ indicates an extra-short isoform present in cowpea. Primer pair that ammplify both isoforms produced by gene have no isoform marker at the end.

Table 4: Primer pair efficiency

Primer pair	pass	Primer efficiency
VuPp2A	✓	1.808
VuUbi28	✓	1.845
VuPolyP	✓	1.691
VuBAct	✓	1.979
VuElf1A	✓	1.906
VuTua4	✓	1.755
VuPs2bp	✓	1.718
VuRca1β [A]	×	1.748
VuRca1β [B]	✓	1.845
VuRca1β [C]	×	1.714
VuRca8	✓	2.002
VuRca8α	×	1.908
VuRca8ββ	✓	1.961
VuRca10	×	1.896
VuRca10α	✓	1.938
VuRca10β	✓	1.981

Gene expression stability and selection of reference genes

Results from gene stability experiments were evaluated using three different algorithms for determining gene stability, comparing different rankings (Table 5). PolyP, Elf1A and Pp2a were selected as reference genes as both Genorm and Normfinder ranked them highly in relative stability. Ps2bp was ranked as most stable by Normfinder algorithms, however, due to large disagreement with Genorm algorithm, which ranked Ps2bp as 5, it was decided to not use it in the final experiment. Bestkeeper ranking are provided for comparison but were not used to calculate overall rank, as their methodology only assesses simple standard deviation and variance, whereas other two algorithms use more advanced mathematical methods to determine stability, and as such account for more factors.

Table 5: Reference gene stability analysis

Gene	Bestkeeper Rank	GenormM Rank	Normfinder Rank	Combined Rank
PolyP	1	1	2	1.5
Elf1A	5	2	3	2.5
Pp2A	6	3	4	3.5
Ubi28	7	4	5	4.5
Ps2bp	4	5	1	3
Tua4	2	6	7	6.5
Bact	3	7	6	6.5
Rca8	8	8	8	8

Rubisco activase expression

Rubisco isoform 1β was the most expressed isoform, followed by isoform 8α (Figure 3). The third most expressed isoform was extra-short 8ββ version, followed by two isoforms located on chromosome 10. Isoform 10α was 1000000 less expressed than isoform 1B. There was no significant differences in the expression of isoform Rca 1β, Rca 8α, Rca 8ββ and 10α amongst the different leaves. There was statistical difference in expression of 10β Rca across leaves, with 1st trifoliate just after expansion having significantly larger amount then 1st trifoliate at the time of second trifoliate full expansion, and 2nd trifoliate at full expansion. Tukey's test found no significant difference in expression between 10α in young 1st trifoliate and 10β expression in mature 1st trifoliate and 2nd trifoliate. There was no statistically significant difference in 10α expression between three leaves. However, there was statically significant difference between 10α and 10β expression in mature 1st trifoliate and 2nd trifoliate (Figure 5).

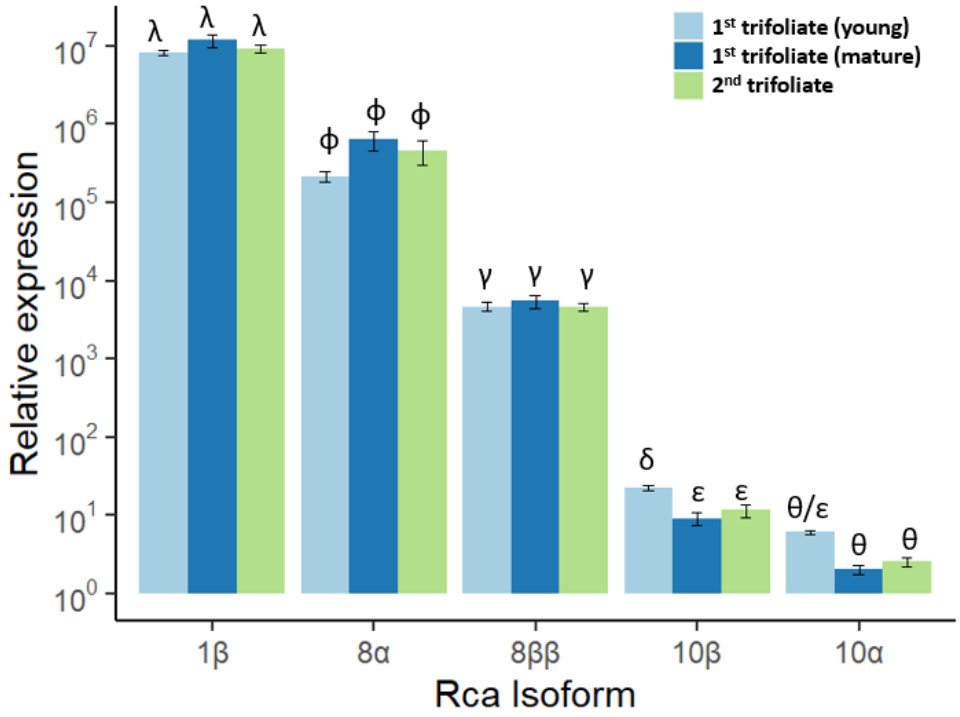


Figure 5. Expression of different RCA isoforms between leaves of cowpea. The y axis is a log scale of difference in expression. Samples were taken from the first trifoliate leaf on the day the leaf reached full expansion are labelled as 1st trifoliate (young), samples taken from the first trifoliate leaf on the day the second trifoliate leaf reached full expansion are labelled as 1st trifoliate (mature), and samples taken from the second trifoliate leaf on the day the leaf reached full expansion are simply labelled as second trifoliate. Error bars were calculated using standard error of the mean. Greek letters (λ, ϕ, γ, δ, ε, θ) denote significant differences between the treatments according to Tukey's test ($P < 0.05$). Samples marked with the same letter are not statistically different from each other.

Discussion

Procedure for extracting RNA from cowpea was optimised, primers and reference genes were chosen and Rca expression levels in different leaves were analysed. With the exception of young 1st trifoliate 10α, 10β, and mature 1st trifoliate 10β and 2nd trifoliate 10β there was significant difference in expression between the rest of Rca isoforms, and no significant difference in isoform expression between different leaves.

Selection of stable reference genes is an important step in accurate qPcr procedure. As algorithms are one-sided, and can be biased, combined ranking system for the reference gene selection allows to offset any potential bias introduced by different methodology. Using combined statistical approaches has become a common practice in the selection of the new reference genes (Monteiro et al., 2013; Tang et al., 2019; Wu et al., 2016; Yang et al., 2014) with combined rankings being used to selectable suitable reference genes from the candidates. Combined statistical approach enabled selection of three reference genes for cowpea gene expression studies. Whilst MIQE guidelines recommend a use minimum two reference genes for any qPCR experiment (Bustin et al., 2009), It is the minimum, and three reference genes are often used for higher accuracy (Tang et al., 2019).

The clear pattern of expression with no statistically significant differences between leaves and statistically significant changes in the expression levels between isoforms was interrupted only for the two isoforms of the Rca gene located on the 10th chromosome: 10α and 10β. Whilst the changes were statistically significant, it should be considered that at optimal growth conditions provided in the glasshouse those genes are 10^6 times less expressed than the dominant Rca isoform, and thus any pattern is unlikely to have consequences on the plant performance or phenotype. Also, due to the ultra-low levels of expression, it is possible that the difference is caused not by genuine expression, but by the inefficient regulation – so called “leaky expression”. Without measuring Rca expression under different conditions the significance or nature of changes is impossible to tell.

For all isoforms except 10β, there was no observable change between the fully expanded first trifoliate, fully expanded second trifoliate, and first trifoliate one week after it is fully expanded. The uniformity of expression between fully expanded first and second trifoliate could be explained by the leaves being at the same stage of development (just reaching full expansion), and thus would be identical in their expression profile.

In this cowpea genotype grown under 30/20 °C conditions, RCA expression was largely unchanged between the first and second trifoliate during the early vegetative stages. This suggests that RCA is not affected by the developmental processes between the fully expanded first trifoliate, and the same trifoliate one week later, in the V2 stage of plant development.

Both soybean and cowpea had β isoform expressed more than an α isoform (Chao et al., 2014), However, in cowpea this difference was a lot more pronounced, with β isoform being expressed 10 times more than the α, whereas in soybean the difference was at most 2-2.5 times more (Chao et al., 2014). This pattern was also observed in wheat, where the most expressed isoform β, with second most expressed being an α followed by another β isoform (Degen, Orr, et al., 2020). Though, unlike wheat where most and second most expressed isoforms are located on the second chromosome, in cowpea the most expressed isoform is located on chromosome 1, whereas second and third most produced isoforms are alternative splicing of gene on chromosome 8.

Whilst it is impossible to predict with any accuracy the exact changes in expression which would happen in response to increased temperature, one thing, which is likely, is that Rca 1β will be downregulated, as the most expressed isoform at optimal temperature was found to be

downregulated at increased temperatures both in wheat and rice (Degen, Orr, et al., 2020; Wang et al., 2010b). It could also be expected that in colder environments some or all rubisco activase will be upregulated together with the other proteins involved in the photosynthesis (Lu et al., 2020).

Chapter 2 –Phenotyping cowpea

Abstract

This chapter aims to characterise the temperature-induced morphological changes to the development of *Vigna unguiculata* L., commonly known as cowpea. Several previous studies have addressed effects of environmental factors, namely temperature, on specific aspects of plant growth. A number of previous studies have characterised phenotypes of different cowpea lines. This chapter aims to combine the two, to characterise temperature induced changes to the phenotype in one cowpea line ITD86-1010. To quantify differences between Cowpea plants growing at different temperatures, leaf traits were measured on a daily basis, from plants in between vegetative stages 1 and 2. Final biomass was measured at the harvest. The observed changes show that cowpea grown under lower temperatures tends to produce larger plants with higher LMA, and lower chlorophyll density.

Introduction

The question of quantifying and comparing plant physiological traits has been a widely discussed topic in botany since its inception. At present, a number of sophisticated systems have been developed to categorise plant physiology and compare plant traits both within and between plant species (Mokronosov, 1981; Monsi et al., 2005; H. Poorter et al., 2012). For this study, methodology described by Poorter et al (2012) was adopted. "Western school" deals primarily with whole-leaf traits, and other whole-organ observations. It is also the method that the majority of previous studies used. Other schools of thought which focus more on tissue composition and internal structure rather than the whole organ can provide insight into how changes in whole-organ traits likely affect internal structure (Monsi et al., 2005). However, any speculations on effects must be undertaken with caution as numerous internal changes can cause similar change in whole-leaf traits (H. Poorter et al., 2009).

As leaf characteristics have been shown to be good predictors for the overall plant performance (L. Poorter & Bongers, 2006), it was hypothesised that plants of cowpea, an important crop for Sub-Saharan African, would show differential allocation of biomass to the leaves when grown at different temperatures. Specifically, it was hypothesised that at warm temperatures plants would have relatively less leaf area, and a decreased leaf to stem ratio.

In response to the changes in the environment plants often change how they allocate biomass (H. Poorter et al., 2012). For this reason, leaf mass fraction, stem mass fraction, and root mass fraction are commonly measured traits to characterise plant growth under differing conditions (Hoang et al., 2020; Ribeiro et al., 2015), frequently by measured by dry biomass.

Other traits to specifically quantify the properties of individual leaves include: Leaf area (LA), leaf thickness (LT), Leaf volume area (LVA), Leaf mass per area (LMA), and leaf density (LD) (H. Poorter et al., 2012). Those parameters can be used to point to deeper structural changes. For example, changes in LMA indicate that the internal leaf structure is different (H. Poorter et al., 2009). In cooler conditions, cell expansion is limited, leading to a large number of cells per leaf volume, increasing density and LMA, and reverse is true in hot conditions LMA is also correlated with relative growth rate, making it a good predictor (Shipley, 2006).

Chemical composition and proportions of the cell constitute another set of important characteristics, affected by temperature. Physiological acclimation to different environmental conditions is accompanied with the metabolic changes in the cell chemistry (Atkin et al., 2006; Usadel et al., 2008).

Chlorophyll density is one such characteristic, which can be easily assessed using non-invasive methods. In general higher chlorophyll correlates with the plant viability, however, some fast growing species prioritise Rubisco production over chlorophyll (L. Poorter & Bongers, 2006).

30 °C is widely regarded as the optimal temperature for the cowpea growth (AFF Republic of South Africa, 2011; USAID, n.d.). However, west African "cowpea belt" is dominated by the savannahs and temperatures in the day may rise to as much as 40 °C. However, planting season is governed by the rain season (USAID, n.d.), which tends to drive temperatures down (Gentilli et al., 2012), but may reach as much as 40 °C (Callo-Concha et al., 2013) For this experiment the range of 22, 30 and 38 °C was chosen as it represents the optimal temperature of growth, the highest temperature setting of the available equipment, and the low temperature equally distanced from the optimal. Although this range was chosen primarily due to hardware limitations, it closely represents the maximum range of temperatures possible in west Africa.

The key objective of this chapter is to quantify and analyse the phenotypic changes in cowpea induced by the changes in temperature.

Materials and Methods.

Plant growth cabinet calibration.

Five Snijder cabinets at the Lancaster Environment Center growth were tested to determine whether they provide similar growth conditions. Light spectrum, intensity and photosynthetic photon flux density were measured using UPRtek PG100N hand held spectrometer. No significant differences were observed between the cabinets in light spectrum. Long term temperature and relative humidity were measured using EasyLog portative Humidity/Temperature meters, set to take readings every 10 minutes. Loggers were placed under white cover to avoid light absorption influencing the results. One cabinet was found to have humidity which heavily deviated from the programmed pattern, and was not used for the experiment.

Plant growth conditions.

Two batches of cowpea (*Vigna unguiculata* L. cultivar IT86D-1010), were grown in fully controlled conditions in the Snijder Plant Growth Cabinets at the Lancaster Environment Centre. Four Snijder cabinets were used to provide 3 different temperature treatments, with cabinets changing treatments between batches. Three temperature regimes selected were 22/18°C, 30/22°C and 38/24°C day/night. The fourth cabinet was set to 38/24°C in the first and 22/18°C in the second batch. Temperatures were chosen to cover the as much of growing range for cowpea as possible while keeping growth at the acceptable rate. The photoperiod was 12 hours, and humidity was set to 60% RH.

Plants were grown in the D40H Deepot cells (produced by Stuewe & Sons), lined with verve weed control fabric. The cells were filled with 1:1 mixture of sand and compost (RHS silver sand and Petersfield compost respectively). Seeds were planted directly into watered pots at the depth of 2 cm.

Each cabinet had 12 plants in the first and 9 in the second batch. Amounting to 48 plants in the first batch and 36 in the second. Plants were grown until the first trifoliate leaf stopped expanding, which was dependent on the temperature and took between 17-25 days on average.

Leaf sampling and in vivo observation

As significant divergence in the phenotype of plants was observed during sampling on the first batch, it was decided to carry out more detailed phenotypic observations on the second batch. Phenotypic observations were taken each day approximately an hour before the end of the photoperiod.

Leaf length was measured by measuring the straight distance between leaf base and apex. Leaf width was measured as the straight line between leaf edges, perpendicular to the midrib and measured at the widest part of the leaf. Chlorophyll Density was measured using an Apogee Instruments handheld MC-100 photometer. Leaf chlorophyll and leaf thickness were measured in the central leaflet.

Leaves were sampled for protein (2 cm^2 of leaf matter) and gene expression analysis (4 cm^2 of leaf matter). Sample tissue was immediately frozen in liquid nitrogen and stored in the -80C freezer awaiting analysis. [Due to the pandemic, these samples were not analysed.] All samples were harvested approximately 4 hours after the beginning of the photoperiod.

Final leaf chlorophyll readings were taken after sample collection, but before leaf area was measured. The process was the same as for the daily measurements described above. Leaf lamina thickness was measured using callipers. Leaf area was measured by passing freshly cut plant leaves through the Li-Cor LI-3100C Area Meter, and final area obtained by adding area removed during sampling. After passing through the area meter, leaves were placed into previously labelled paper bags. Stems, now separate from the leaves, were cut at the ground level, wiped of dirt, and similarly to leaves were placed into pre-labelled paper bags. Bags were then left to dry in the 60 °C oven for more than 72 hours, and were weighed afterwards to determine their weight Approximate development stage was also estimated based on number and size of trifoliate leaves at harvest.

Statistical Analysis.

Phenotypic results were carefully checked and outliers removed. Data for plants grown at 30°C had to be discarded because they were subjected to a heat shock that cooked the leaves after equipment malfunction. Statistical analysis and graph preparation were performed using R (R Core Team & R Foundation for Statistical Computing, 2020), in Rstudio(RStudio Team, 2020). Agricolae package (Mendiburu, 2020) was used for analysis and ggplot2 package (Wickham, 2016) was used to prepare figures. For the majority of the traits, there were a total of 13 samples taken from independent plants grown at 22°C and 9 samples from plants grown at 38°C. The statistical significance of differences between plants grown at different temperatures was investigated using double ended t-test. For some of the measured traits there were only 11 and 4 for 22 °C and 38 °C respectively, Due to measurements being done in leaves of the same developmental stage.

Some phenotypic data analysed was from the first batch of plants grown. As there were no malfunctions that time, those plants included all three treatments. 38°C treatment had 24 plants, and 22°C and 30°C treatments both had 12 plants. When assessing significance One-way ANOVA was used with the Tukey's post hoc test to compare individual treatments.

Results

A significant morphological difference was observed between cowpea grown at different environmental temperatures. When measured at the same stage in development plants grown at 22 °C were visibly larger than their counterparts grown at 38 °C. However, it should be noted that it took plants growing at 22 °C three weeks to reach the same stage cowpea grown at 38 °C reached in two weeks. Those differences were not limited to size alone – a significant variation in morphology was observed. Whole plant changes to biomass allocation between leaves and stems were observed with plants grown at lower temperatures having significant emphasis on leaves (Figure 6C, A2C). This coincides with changes to the relative leaf dimensions, with plants growing at lower temperatures having wider leaves (Figure 8D). Chlorophyll concentration also differed significantly between the plants grown at different temperatures.

Table 6: First trifoliate thickness between cowpea plants grown at 22 °C (n = 18) and 38 °C (n = 9). All measurements are given in mm and correspond to the mean. Significance values were given to show that there is no substantial difference.

Table 6: leaf thickness (mm)		
Growth Temperature	Central leaflet	Side leaflet
38 C	0.21	0.21
22 C	0.18	0.21
Significance	0.112	0.786

One of the characteristics which saw no difference between the different temperature treatments was leaf thickness. Both plants showed 0.21 mm of thickness of the first trifoliate, with t-test showing no difference between temperature treatments (Table 6). This is in line with the previous research by Smith & Nobel (1978), which indicates that the main environmental factor responsible for changes in leaf thickness is light intensity. As cabinets were calibrated to have same levels of light intensity, similar leaf thickness was expected.

Plants grown at the lower and higher temperatures had significantly different rates of daily increase in above ground biomass, measured by dividing final above ground biomass by the number of days it took plants to reach it. Cowpea grown under lower temperature had higher rates of daily biomass increase (Table 7).

Table 7: Daily increase in above ground biomass in plants grown at 38 °C (n = 11) and 22 °C (n = 4). Values presented is the mean final biomass divided by the number of days before maturity.

Table 7: Increase in above grownd biomass		
Temperature	Daily biomass (g/day)	SEM
38	0.029	0.0012
22	0.044	0.0034
	p = 0.005	

Plant morphology.

Overall plant morphology differed significantly between plants grown at 22 °C and 38 °C. Plants grown at 22 °C were larger and had larger leaves in proportion to the stem. In batch 2 total leaf area was a lot larger in plants grown under 22 °C, with average total leaf area being 171 cm^2 ± 11.6 in comparison to average 102 cm^2 ± 4.7 of plants grown at 38 °C (Figure 6A) Similar effect was observed in batch 1 but to a greater extent, with plants grown under 22 °C reaching total leaf area of 300 cm^2 ± 8.6 and plants grown at 38 °C remaining at around 100 cm^2 (88 ± 5.2) . The disparity is likely due to the fact that plants in batch 2 were collected earlier than their counterparts in batch 1. Consequently, plants grown under 22 °C also had greater dry biomass, with an average of 1.1 grams, in comparison, plants grown at 38 °C (Figure 6B), had on average only 0.5 g of dry biomass. The biomass allocation was also more heavily focused on the leaves of plants grown under lower temperature, with average leaf to stem ratio being above 3 (3.1 in first batch, 4.1 in second) for plants grown at 22 °C (Figure 6C, Appendix 2). With plants grown at 38 at having leaf to stem ratios of around 2 (1.9 in first, 2.1 in second batch), and 30 °C plants having leaf to stem biomass ratio of about 2.1 (first batch only).

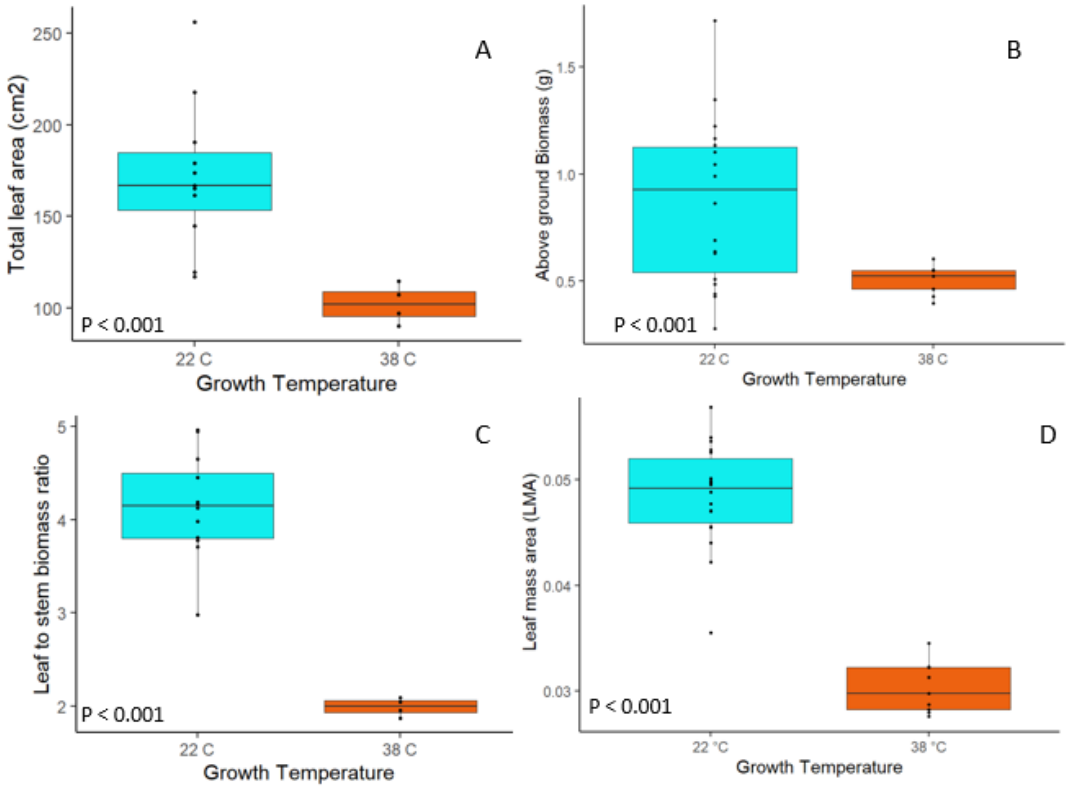


Figure 6. Differences in the morphology between second batch of cowpea grown at 38 °C and 22 °C. All measurements were performed when plants were between stages V1 and V2 of development. (A) Difference in the total leaf area of the plant, measured using LICOR Li 3100c Area Meter immediately after collection. (B) Total above ground dry biomass of the plants. (C) Ratio between leaf and stem biomass. (D) Leaf Mass Area for the plants in the second batch. Significance values were calculated using unpaired T-test. Box plots show upper and lower quartile as well as the median, dots represent individual data points. There were 4 samples at 38 °C and 11 samples at 22 °C.

Leaf Morphology.

There were considerable differences in the morphology of the first trifoliate leaves between plants grown at 22 and 38 °C, with plants grown under lower temperature showing leaves which were much larger. The total leaf area of the 1st trifoliate leaf was considerably larger in plants grown at 22 °C, with the average of 100 cm^2± 8.9 , compared to 40 cm^2 ± 2 of plants grown under 38 °C (Figure 7A). The same pattern continues with both length and width – plants grown at 22 °C were on average 13.8 ± 0.39 cm long and 4.6 ± 0.2 cm wide, whilst plants grown at 38 °C were only 9.8 ± 0.44 cm long and 3.2 ± 0.07 cm wide (Figure 7B, 7C). There was a greater relative increase in leaf width than length, which can be seen from the fact that plants grown at 22 °C had lower leaf length/width ratio (Figure 7D), showing that leaves became wider. Another indicator of the changes in the leaf morphology was the change in the Leaf mass area (LMA) (Figure 6D). Plants grown at 22 °C had higher LMA. Considering that it is known that leaf thickness stayed constant, (Table 6), increase in LMA indicates increase in leaf density, indicating that there is a change in leaf tissue morphology/proportions. However, in absence of leaf dissections, the exact nature of the change is unknown.

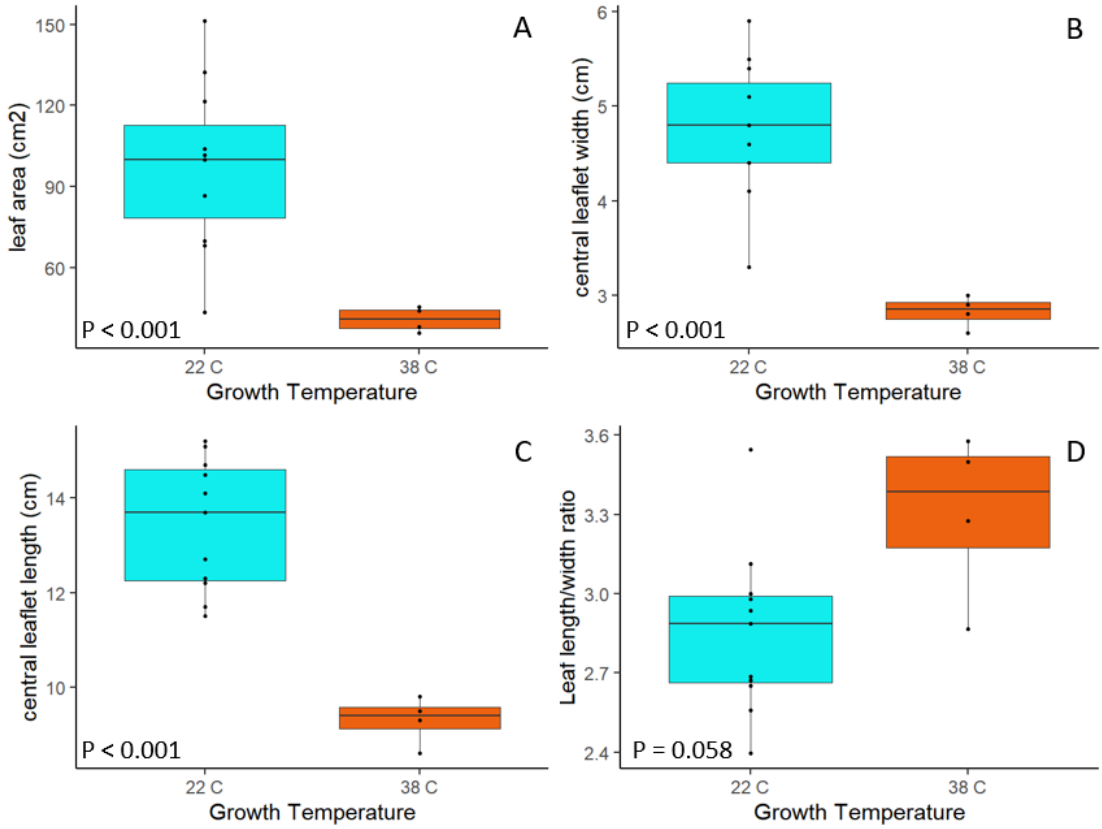


Figure 7. Differences in the 1st trifoliate morphology between Cowpea plants grown at 38 °C and 22 °C. All measurements were performed when plants were between stages V1 and V2 of development. Plants were from the experiment 2 . (A) Surface area of the first trifoliate leaf, measured using LICOR Li 3100c Area Meter immediately after collection. (B) Width of the central leaflet. Measured at the widest part of the leaflet, with ruler perpendicular to midrib. (C) Length of the central leaflet, measured between the apex and the base. (D) Ratio between length and width of the central leaflet. Box plots show upper and lower quartile as well as the median, dots represent individual data points. There were 4 samples at 38 °C and 11 samples at 22 °C. Significance values were calculated using unpaired T-test.

Plant development

By comparing the progress of leaf development before the sampling date, it was possible to compare relative leaf expansion. Although cowpea plants grown under 22 °C took 7 more days to reach full first trifoliate maturity, the rate of first trifoliate elongation was greater in absolute terms in the plants grown under the lower temperature (Figure 8A, 8B). This pattern is a lot more evident in the increase of the central leaflet width (Figure 8C, 8D), which is likely due to the fact that temperature affects leaf width a lot more than it affects leaf length (Figure 8D), and thus contrast is a lot more apparent. This shows that although plants grown at lower temperature take a lot longer to grow, at the end their rate of growth exceeds that of plants grown at higher temperatures.

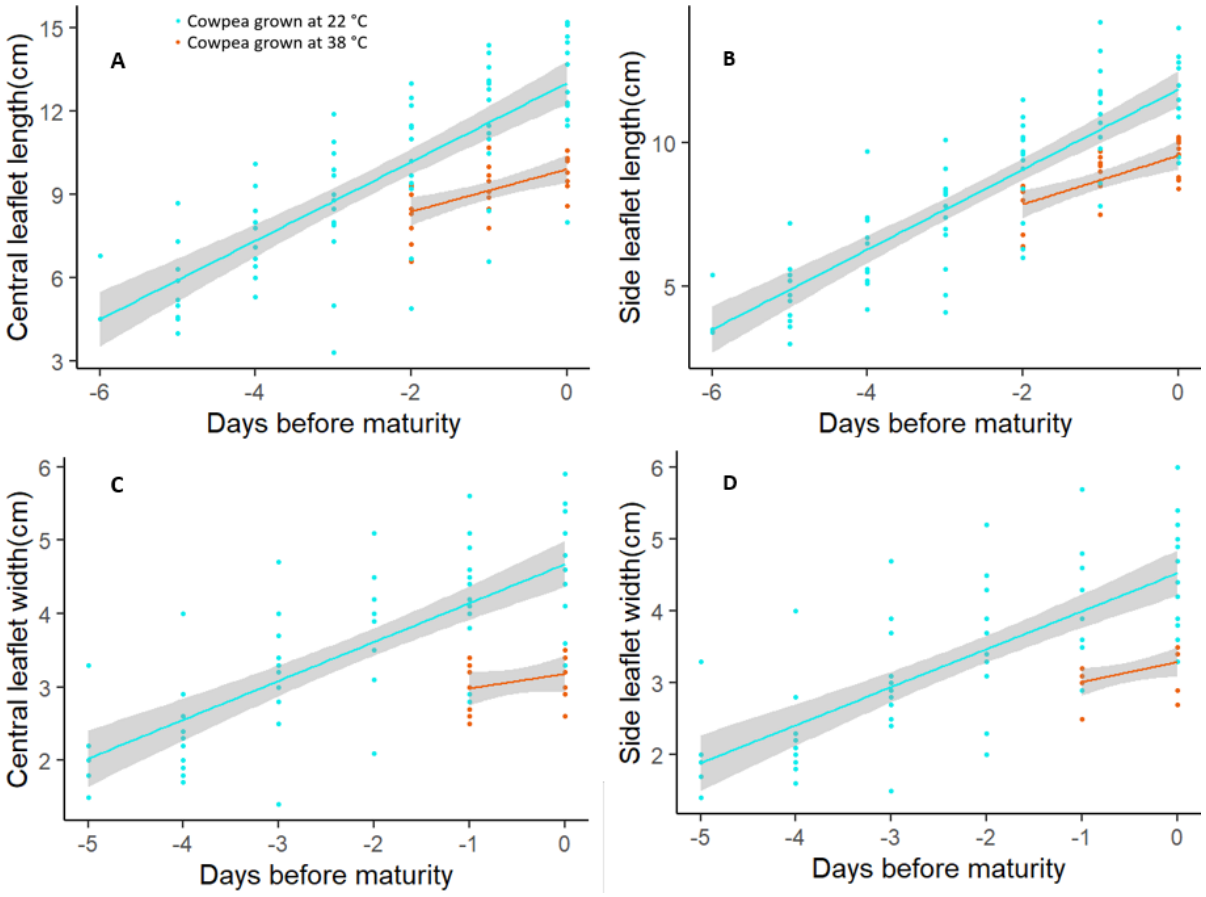


Figure 8. Differences in leaf development between plants grown at 38 °C and 22 °C. All measurements were performed when plants were between stages V1 and V2 of development, with measurements finishing at the point of full expansion of first trifoliate. A) Increase in central leaflet length over time. Measurements taken parallel to the central vein of the leaflet. B) Increase of side leaflet length over time. Measurements taken parallel to the central vein of the leaflet. C) Increase of central leaflet width over time. D) Increase of side leaflet width over time. Width is measured as the widest point parallel to the central vein of the leaflet. Each symbol represents one data point, with 4 samples at 38 °C and 11 samples at 22 °C measured over multiple days. Line represents the smoother line, illustrating the relationship, and the grey area shows the confidence bands.

Chlorophyll Density.

Chlorophyll density was considerably different between the plants grown under 38 °C and 22 °C. Figure 10A shows the increase in chlorophyll density over time, nearly doubling in the space of the week, and appeared to be slowly decreasing as leaves reached maturity, though range of the observations did not allow to assess it properly. The leaves of the plants grown in the 22 °C environment reached a lower average chlorophyll content of 356 ± 35 µmol/m^2 whilst the plants grown at 38 C had 541 ± 3.4 µmol/m^2 (Figure 9A). However, plants growing under 22 °C conditions, had significantly larger first trifoliate leaves (Figure 7A). When absolute amount of chlorophyll per leaf is considered (calculated by multiplying density by area), plants grown under 22 °C had an average of 3.38 ± 0.1 µmol of chlorophyll in their first trifoliate, whereas plants grown under 38 °C had on average 2.63 ± 0.5 µmol of chlorophyll in their first trifoliate, However, this difference was not statistically significant (Figure 9D).

When comparing results from plants grown in batch 1 and batch 2, the same trend for the chlorophyll density to increase with temperature was observed (Figure 9C), however it is also obvious that the plants grown in the first batch had significantly higher chlorophyll density. Only overlap between the two batches at the same temperature happens at 22 °C, However, the statistical value for the comparison is p = 0.051, just missing from the significant difference. The same value corresponds much better with the result from the second batch for 38 °C. It should be noted that the difference between the treatments in the second batch were statistically significant both when analysed using both the T-test and Tukey's test. .

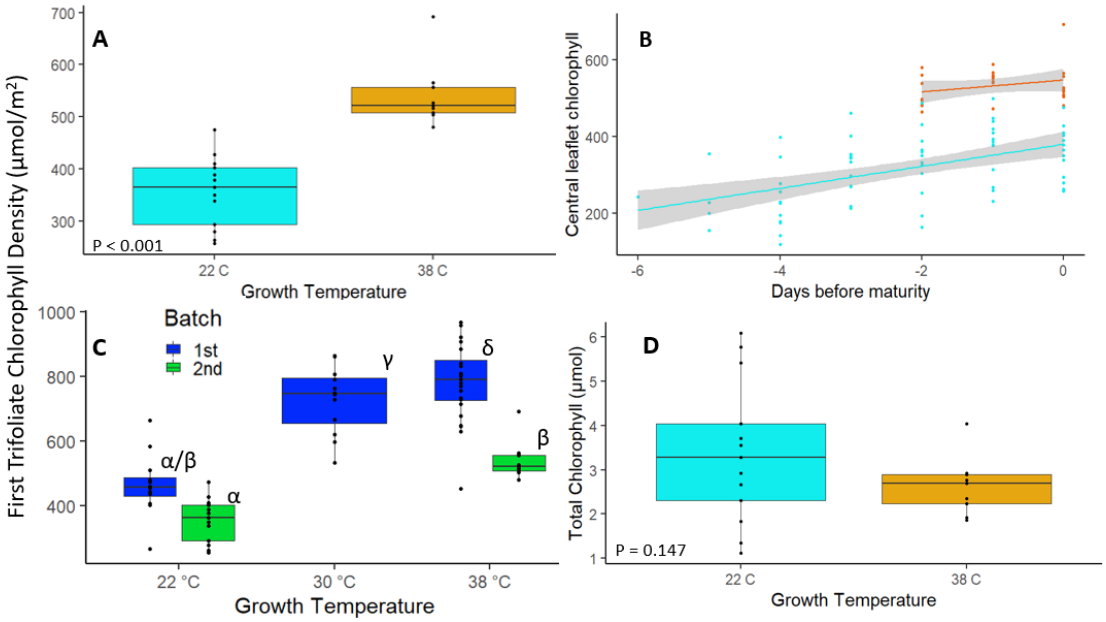


Figure 9. Differences in chlorophyll content between cowpea grown at 38 °C and 22 °C. (A) Final Chlorophyll Density of the first trifoliate leaf, measured in the central leaflet of the first trifoliate leaf, measured using Apogee MC-100 Chlorophyll meter. (B) Increase in the Chlorophyll density over time, using the same method as figure A. The time is given in days before first trifoliate reaches full maturity. (C) Final Chlorophyll Density of the first trifoliate leaf, from plants grown in batch 1 and 2. Measurement methodology is the same as used for graphs A and B. Greek letters (α, β, γ, δ) are used to determine statistically different samples. Samples marked with the same letter are not statistically different from each other. (D) Total chlorophyll contained in the first trifoliate leaf, obtained by combining chlorophyll density and First Trifoliate area. Significance values were calculated using unpaired T-test. Box plots show upper and lower quartile as well as the median, dots represent individual data points. There were 4 samples at 38 °C and 11 samples at 22 °C. For graph (C), significance was determined using ANOVA and later using Tukey's post hock test, groups were determined.

Discussion.

Cowpea plants were grown under different environmental temperatures and phenotypic measurements were performed to observe and quantify the changes induced by the temperature. Significant changes were observed in plant and leaf size, in the leaf proportions as well as chlorophyll concentration.

The first significant observation is the difference in the plant size between the plants grown under 22 and 38 °C conditions, with the former having on average twice the dry biomass and twice the leaf area, when corrected for development stage. Leaves growing larger in response to lower temperature is an effect previously observed by Smith & Nobel (1978), and similarly increase in growth at colder temperatures was observed in *Brassica rapa* by (Si & Thurling, 2001). This could have significant implications on the communities producing cowpea, as the leaves are commonly used as fodder for the animals and in some recipes for human consumption (Cookpad; Sheahan, 2012). This would mean that temperature increase predicted over the next century would negatively affect the yields. This change was observed in the plants that suffered no water shortage. In a real-world scenario, the situation would be compounded by the droughts which are expected to increase in the region with the onset of climate change (Callo-Concha et al., 2013).

In the plants grown under lower temperature conditions, larger proportion of the biomass was allocated towards leaves. This same pattern was observed by Hoang et al. (2020) in Wasabi plants and Ribeiro et al. (2015) in *Ricinus communis* plantlets. Whilst the magnitude and relative levels change from study to study, the overall trend remains the same. The differences could be due to inter-species differences, or dependent on when measurements being taken a lot earlier during life cycle, before plants grown at lower temperatures have time to catch up. This pattern falls into the general change in the plant size and proportions, however, due to the agricultural usefulness of cowpea leaves described above, this effect carries great importance for communities who rely on biomass produced by cowpea for foraging or nutrition.

Although overall shape of the leaves remained the same, leaf proportions changed, with plants grown at lower temperatures having wider leaves. Leaf length and width of cowpea grown under different temperatures fell within the values reported by Animasaun et al., (2015) and (Gerrano et al., 2015) , of 4 to 11 cm for length and 2.6 to 8 cm for width across 34 genotypes. Leaf length to width (L/W) ratio of cowpea grown at 38 °C and 30 °C (2.85) was slightly above range of 1.27 – 2.3 which was calculated from the data of Animasaun et al., (2015) & Gerrano et al., (2015), and significantly different from the L/W ratio of cowpea grown at 22 °C (3.23). The differences between plants grown at 38°C and 30 °C and previously reported parameters could likely be explained by the variation between the species. However, there is a clear change in the leaf proportions induced by cold temperature.

Plants grown at 22 °C had higher LMA than plants grown at 38 °C. This is consistent with previous trends observed by Bjorkman et al. (2018). At the same time, plants grown under different conditions had nearly identical leaf thickness. LMA can be portrayed as a product of Leaf Thickness (LT) and Leaf Density (LD), although some studies prefer more general Leaf Volume Area (LVA) instead of leaf thickness (John et al., 2017; Poorter et al., 2009). As leaf thickness was not affected by growth temperature, leaf density was likely the factor determining LMA in cowpea in the present study. This is in line with previous research finding LD as the main component to LMA response to temperature (Poorter et al., 2009). The difference in LMA is likely caused by the changes in the internal structure of the leaf. In absence of empirical data it is impossible to determine precise nature of such changes, however, previous studies in other species identified temperature induced changes in chemical composition, protein and organelle density, as well as cell size (Atkin et al., 2006; Usadel et al., 2008).

Plants grown in the lower temperature had considerably lower chlorophyll density than their peers grown in the 30 °C and 38 °C cabinet. This is different from soybean, where increase of temperature away from optimum led to gradual decrease in chlorophyll density (Jumrani et al., 2017). The difference in behaviour is likely due to cowpea being a drought-tolerant crop adapted to higher temperatures.(McDonald & Paulsen, 1997)

The trend remained the same between the first and second batch of cowpea. However, it is notable that the first batch had significantly higher chlorophyll content than the second batch. There is no good explanation for this difference, as the change was present in all temperature treatments, across all cabinets and thus could not be accounted for by chamber effects. Temperature/humidity meters also did not detect any differences between the batches.

Despite having lower chlorophyll density, when accounting for the leaf area, plants grown in the 22 °C cabinets had similar amounts of total chlorophyll in their first trifoliate leaf. This would indicate that despite physiological differences, cowpea plants should have approximately equal capacity for light capture. This might explain why cowpea grown under the lower (22 °C) temperatures had lower initial growth rate compared to their counterpart grown under optimal (30 °C), and high (38 °C) temperature conditions. Initially their capacity to capture sunlight would be much lower, however, as plants developed, increase in surface area of the leaf as well as chlorophyll density led to plants grown under lower temperature matching and likely surpassing light capture capacity of plants grown under optimal and high temperatures. This resulted in the fact that plants grown under 22 °C had a higher daily increase in the above ground biomass compared to plants grown at higher temperatures, despite the latter developing at a much quicker rate.

The phylogenetic changes in the cowpea induced by the lowering temperatures include a wide range of yield-increasing effects beneficial to the farming communities. However, this comes at an expense of slower rate of growth, which would likely mean only one harvest during the planting season. In many tropical countries, the number of harvests is determined by the rain, rather than temperature. Cowpea is valued for its ability to thrive in the arid savannahs of the Sub-Saharan West Africa, providing communities with valuable source of protein and fodder, where other plants would struggle. With global climate change leading to the increase in world temperatures, the likely result will be decreased yields for the farmers, which will add to the list of problems in the region.

Conclusion

The effects of temperature on the development of plants are complex and affect plants on all structural levels, from the chemical composition and proportion of amino acids (Usadel et al., 2008) to the organelles and tissues (Atkin et al., 2006; H. Poorter et al., 2009), and to the individual organs and entire organisms (H. Poorter et al., 2012). The cowpea is no exception, and environmental temperature had a profound effect on the development of the plant, significantly affecting its leaves, leading to significant variation in above ground biomass, leaf area and leaf density. This strongly implies changes to the internal structure, but in absence of empirical observations these are merely speculations. Whilst no significant analysis on leaf biochemistry was performed, chlorophyll density and base line Rubisco activase expression were measured which could be used to speculate on the possible internal changes.

As demonstrated in chapter 2, chlorophyll density and leaf size changed significantly with temperature. Plants grown at low temperatures produced large leaves with relatively low chlorophyll density, whilst plants grown at high temperatures yielded small chlorophyll dense leaves. The decrease of leaf size with temperature was already observed in cowpea by Adblusi & Lawanson, (1978). And whilst the increase in chlorophyll content is different from the behaviour observed in closely related soybean(Jumrani et al., 2017), cowpea was previously noted to be the only legume to not decrease in chlorophyll content with increase in temperature (McDonald & Paulsen, 1997).

The result of this inverse relationship is that leaves of plants grown under lower temperature and leaves of plants grown under higher temperature had similar total amount of chlorophyll. The plants grown under lower temperature conditions had slightly higher average total chlorophyll value, but the result was not statistically significant. Accounting for the fact that the plants grown under 22 °C were sampled at slightly lower development stage than their counterparts grown at 38 °C, and that the chlorophyll density tended to increase with the age of the leaf, it is possible that repeating the measurements with a greater and more homogeneous set of plants would show significant difference in the total chlorophyll amounts.

Whilst there is no significant difference between light capturing capacity of plants grown at 22 °C and 38 °C, there is a significant difference in the above ground biomass gain. One possible explanation for this effect is that whilst low temperature decreases the rate of catalysis of Rubisco, it increases its specificity, reducing photorespiration (Brooks & Farquhar, 1985; Usadel et al., 2008), with decreased catalytic rate, offset by the larger quantity of Rubisco present. (Usadel et al., 2008).

In either case, the overall ability of the cowpea to capture light energy remains broadly the same despite the change of temperature. This indicates that cowpea retains the ability to successfully utilise that energy.

If the response to the increased temperature in cowpea is similar to the response in rice and wheat, It is likely that the expression of 1B RCA will decrease. If the behaviour of rubisco activase in soybean is similar to that in spinach and rice, then we would expect α-isoforms (10α and 8α) to be more stable, and be upregulated (Crafts-Brandner et al., 1997; Wang et al., 2010b). However, as shown by wheat (Degen et al., 2020), it is possible that one of the other two present β -isoforms (10 β and 8β) is more thermotolerant and will be upregulated. The fact that isoform 8ββ is an extra-short version compared to other two β isoforms present, introduces further uncertainty.

If cowpea acclimatisation strategy to lower temperatures is similar to that of the watermelon observed by the Lu et al. (2020), then, it is likely that Rubisco activase is upregulated together with

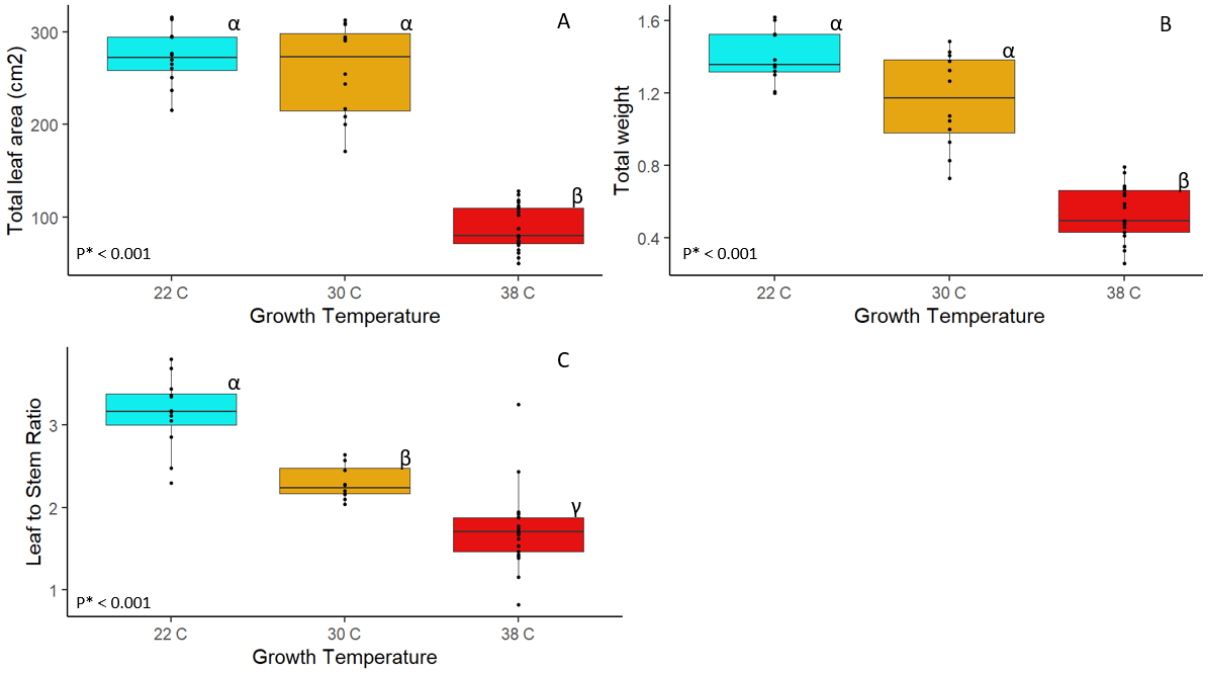
the other proteins involved in the photosynthesis. This is likely one of the causes of increased leaf density, and higher leaf mass to chlorophyll ratio.

In this work, an optimised method for cowpea Rca expression analysis was produced. And the physiological response of cowpea to varying temperatures was quantified and analysed. The methodology developed in chapter 1 can serve as the basis for the future studies into the RCA gene expression in the cowpea at different temperatures, providing a selection of reference genes and the primers for the RCA isoforms present. The physiological analysis suggest that cowpea grown at lower temperatures produces more biomass. Further research into the physiology could focus on investigating the underlying mechanism for the change in LMA, and investigate the effect of growth temperature on grain yield.

Appendix

1: *Vigna unguiculata* Rubisco activase primers.

Gene of interest	primer	Sequence	size(bp)	Tm (Beacon)	Amplicon size
VuRca1β [A]	Forward	CTTGGAAATGCTAACGAAGATGC	23	56.38	210bp
	Reverse	AACTTAGCCTCTTCTCTTTGCAC	23	56.61	
VuRca1β [B]	Forward	CTTGGAAATGCTAACGAAGATGC	23	56.38	156bp
	Reverse	CACAAGTGCCATCACAATTGC	21	56.22	
VuRca1β [C]	Forward	GATGAGTCTGGACAAGCTCTTG	22	56.23	176bp
	Reverse	GCAATCATGTATGCCCTTAAGTC	23	55.59	
VuRca8	Forward	CGATGATGGAACCTGCTTATACA	23	56.35	115bp
	Reverse	GGGATAACAATCCAGTGTGAACT	23	56.35	
VuRca8α	Forward	TGATTTCTTTGGTGCACTCAGG	22	55.83	136bp
	Reverse	CTAGATACATTATTCGATTATCTCATGTCC	30	56.85	
VuRca8ββ	Forward	GCCAATCCATTGGGCTAGAGT	21	57.3	127bp
	Reverse	CTTGGGCTGCTCAAAGGTTG	20	56.67	
VuRca10	Forward	GAGTGGCAGGCTCTGCATTG	20	58.64	177bp
	Reverse	GCTGGTCATCGGAAGTGTCA	20	57.02	
VuRca10α	Forward	GAAACTTCTATGGACAAGCAGCT	23	56.67	134bp
	Reverse	CATCACCTAAAGTGTGTATGTGCA	24	57.19	
VuRca10β	Forward	TGCTTGTCCAAGAGCAAGAGA	21	56.91	144bp
	Reverse	ACAACTTTAGGATCAAATCAAGGGT	25	56.70	



Appendix 2. Differences in the morphology between first batch of cowpea grown at 38 °C, 30 °C and 22 °C in the first batch of plants. All measurements were performed when plants were between stages V1 and V2 of development. (A) Total Surface leaf area measured using LICOR Li 3100c Area Meter immediately after collection. (B) Total dry biomass of the plants, after 2 weeks of drying at 60 °C. (C) Ratio between leaf and stem biomass. P value given at the bottom is the significance value obtained using one-way ANOVA. Small Greek letters (α, β, γ) indicate significant differences between the treatments according to Tukey's test ($P < 0.05$). Samples marked with the same letter are not statistically different from each other.

Bibliography

Adblusi, S. A., & Lawanson, A. O. (1978). Heat-induced changes in dry weight, leaf size and number of chloroplasts per cell in maize and cowpea shoots. *The Journal of Agricultural Science*, *91*(2), 349–357. https://doi.org/10.1017/S002185960004644X

AFF Republic of South Africa. (2011). Production Guidelines for Cowpea. In *~: Vol. 3 MAY*. https://doi.org/10.3389/fphys.2012.00155

Ammelburg, M., Frickey, T., & Lupas, A. N. (2006). Classification of AAA+ proteins. *Journal of Structural Biology*, *156*(1), 2–11. https://doi.org/10.1016/j.jsb.2006.05.002

Andersen, C. L., Jensen, J. L., & Ørntoft, T. F. (2004). Normalization of real-time quantitative reverse transcription-PCR data: A model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer Research*, *64*(15), 5245–5250. https://doi.org/10.1158/0008-5472.CAN-04-0496

Andersson, I. (1996). *Large Structures at High Resolution : The 1 . 6 Å Crystal Structure of Spinach Ribulose-1 , 5- Bisphosphate Carboxylase / Oxygenase Complexed with 2-Carboxyarabinitol Bisphosphate*. 160–174.

Andersson, I., & Backlund, A. (2008). Structure and function of Rubisco. *Plant Physiology and Biochemistry*, *46*(3), 275–291. https://doi.org/10.1016/j.plaphy.2008.01.001

Andrewss, T. J. (1988). *Catalysis by Cyanobacterial Ribulose-bisphosphate Carboxylase Large Subunits in the Complete Absence of Small Subunits *. 2*, 12213–12219.

Animasaun, D. A., Oyedeji, S., Mustapha, O. T., & Azeez, M. A. (2015). Genetic Variability Study Among Ten Cultivars of Cowpea (Vigna unguiculata L. Walp) Using Morpho-agronomic Traits and Nutritional Composition. *Journal of Agricultural Sciences*, *10*(2), 119. https://doi.org/10.4038/jas.v10i2.8057

Anita R. Linnemann, P. Q. C. (1994). *Effects of Temperature and Photoperiod on Phenological Development in Three Genotypes of Bambara Groundnut (Vigna subterranea)*. https://doi.org/https://doi.org/10.1006/anbo.1994.1170

Atkin, O. K., Loveys, B. R., Atkinson, L. J., & Pons, T. L. (2006). Phenotypic plasticity and growth temperature: Understanding interspecific variability. *Journal of Experimental Botany*, *57*(2 SPEC. ISS.), 267–281. https://doi.org/10.1093/jxb/erj029

Bar-On, Y. M., & Milo, R. (2019). The global mass and average rate of rubisco. *Proceedings of the National Academy of Sciences of the United States of America*, *116*(10), 4738–4743. https://doi.org/10.1073/pnas.1816654116

Barros, T., & Kühlbrandt, W. (2009). Crystallisation, structure and function of plant light-harvesting Complex II. *Biochimica et Biophysica Acta - Bioenergetics*, *1787*(6), 753–772. https://doi.org/10.1016/j.bbabio.2009.03.012

Berry, J. A., Lorimer, G. H., Pierce, J., Seemann, J. R., Meek, J., & Freas, S. (1987). Isolation, identification, and synthesis of 2-carboxyarabinitol 1-phosphate, a diurnal regulator of

ribulose-bisphosphate carboxylase activity. *Proceedings of the National Academy of Sciences*, *84*(3), 734–738. https://doi.org/10.1073/pnas.84.3.734

Brooks, A., & Farquhar, G. D. (1985). Effect of temperature on the CO2/O2 specificity of ribulose-1,5-bisphosphate carboxylase/oxygenase and the rate of respiration in the light - Estimates from gas-exchange measurements on spinach. *Planta*, *165*(3), 397–406. https://doi.org/10.1007/BF00392238

Buchanan, B. B., & Balmer, Y. (2005). REDOX REGULATION: A Broadening Horizon. *Annual Review of Plant Biology*, *56*(1), 187–220. https://doi.org/10.1146/annurev.arplant.56.032604.144246

Burke, J. J., & Upchurch, D. R. (1989). Leaf temperature and transpirational control in cotton greenhouse procedures temperature of several points on each test field procedures cotton (Gossypium hirsutum L . Paymaster 404). *Journal of Experimental Botany*, *29*(4), 487–492.

Busch, F. A., Sage, R. F., & Farquhar, G. D. (2018). Plants increase CO2 uptake by assimilating nitrogen via the photorespiratory pathway. *Nature Plants*, *4*(1), 46–54. https://doi.org/10.1038/s41477-017-0065-x

Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M. W., Shipley, G. L., Vandesompele, J., & Wittwer, C. T. (2009). The MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments. *Clinical Chemistry*, *55*(4), 611–622. https://doi.org/10.1373/clinchem.2008.112797

Caffarri, S., Tibiletti, T., Jennings, R., & Santabarbara, S. (2014). A Comparison Between Plant Photosystem I and Photosystem II Architecture and Functioning. *Current Protein & Peptide Science*, *15*(4), 296–331. https://doi.org/10.2174/1389203715666140327102218

Callo-Concha, D., Gaiser, T., Webber, H., Tischbein, B., Müller, M., & Ewert, F. (2013). Farming in the West African Sudan Savanna: Insights in the context of climate change. *African Journal of Agricultural Research*, *8*(38), 4693–4705. https://doi.org/10.5897/ajar2013.7153

Cardona, T., Murray, J. W., & Rutherford, A. W. (2015). Origin and evolution of water oxidation before the last common ancestor of the cyanobacteria. *Molecular Biology and Evolution*, *32*(5), 1310–1328. https://doi.org/10.1093/molbev/msv024

Chao, M., Yin, Z., Hao, D., Zhang, J., Song, H., Ning, A., Xu, X., & Yu, D. (2014). Variation in Rubisco activase (RCAβ) gene promoters and expression in soybean [Glycine max (L.) Merr.]. *Journal of Experimental Botany*, *65*(1), 47–59. https://doi.org/10.1093/jxb/ert346

Chen, C., He, B., Liu, X., Ma, X., Liu, Y., Yao, H. Y., Zhang, P., Yin, J., Wei, X., Koh, H. J., Yang, C., Xue, H. W., Fang, Z., & Qiao, Y. (2020). Pyrophosphate-fructose 6-phosphate 1-phosphotransferase (PFP1) regulates starch biosynthesis and seed development via heterotetramer formation in rice (Oryza sativa L.). *Plant Biotechnology Journal*, *18*(1), 83–95. https://doi.org/10.1111/pbi.13173

Cleland, W. W., Andrews, T. J., Gutteridge, S., Hartman, F. C., & Lorimer, G. H. (1998). Mechanism of rubisco: The carbamate as general base. *Chemical Reviews*, *98*(2), 549–561. https://doi.org/10.1021/cr970010r

Cookpad. (n.d.). *60 Cowpea Recepies*. Retrieved December 22, 2020, from https://cookpad.com/uk/search/cowpeas leaves

Crafts-Brandner, S. J., & Salvucci, M. E. (2000). Rubisco activase constrains the photosynthetic potential of leaves at high temperature and CO2. *Proceedings of the National Academy of Sciences of the United States of America*, *97*(24), 13430–13435. https://doi.org/10.1073/pnas.230451497

Crafts-Brandner, S. J., Van de Loo, F. J., & Salvucci, M. E. (1997). The two forms of ribulose-1,5-bisphosphate carboxylase/oxygenase activase differ in sensitivity to elevated temperature. *Plant Physiology*, *114*(2), 439–444. https://doi.org/10.1104/pp.114.2.439

Craufurd, P. Q., Subedi, M., & Summerfield, R. J. (1997). Leaf appearance in cowpea: Effects of temperature and photoperiod. *Crop Science*, *37*(1), 167–171. https://doi.org/10.2135/cropsci1997.0011183X003700010029x

Cui, H., Zhang, S. T., Yang, H. J., Ji, H., & Wang, X. J. (2011). Gene expression profile analysis of tobacco leaf trichomes. *BMC Plant Biology*, *11*. https://doi.org/10.1186/1471-2229-11-76

Cummins, P. L., Kannappan, B., & Gready, J. E. (2018). *Revised Mechanism of Carboxylation of Ribulose-1 , 5- Biphosphate by Rubisco from Large Scale Quantum Chemical Calculations*. https://doi.org/10.1002/jcc.25343

D'Andrea, A. C., Kahlheber, S., Logan, A. L., & Watson, D. J. (2007). Early domesticated cowpea (Vigna unguiculata) from Central Ghana. *Antiquity*, *81*(313), 686–698. https://doi.org/10.1017/S0003598X00095661

Degen, G. E., Orr, D. J., & Carmo-Silva, E. (2020). Heat-induced changes in the abundance of wheat Rubisco activase isoforms. *New Phytologist*. https://doi.org/10.1111/nph.16937

Degen, G. E., Worrall, D., & Carmo-Silva, E. (2020). An isoleucine residue acts as a thermal and regulatory switch in wheat Rubisco activase. *Plant Journal*, 1–10. https://doi.org/10.1111/tpj.14766

Díaz, S., Garnier, E., Lavorel, S., Poorter, H., Jaureguiberry, P., Cornwell, W. K., Craine, J. M., Gurvich, D. E., Urcelay, C., Veneklaas, E. J., Reich, P. B., Poorter, L., Wright, I. J., Ray, P., Enrico, L., Pausas, J. G., Vos, A. C. De, Buchmann, N., Funes, G., … Cornelissen, J. H. C. (2016). Corrigendum: New handbook for standardised measurement of plant functional traits worldwide (Australian Journal of Botany (2013) 61:3 (167-234) DOI: 10.1071/BT12225). *Australian Journal of Botany*, *64*(8), 715–716. https://doi.org/10.1071/BT12225_CO

ECOWAS-SWAC/OECD. (2007). tlas on Regional Integration R URAL A REAS AND. *Africa*, *May*.

Elizabete Carmo-Silva, A., & Salvucci, M. E. (2013). The regulatory properties of rubisco activase differ among species and affect photosynthetic induction during light transitions. *Plant Physiology*, *161*(4), 1645–1655. https://doi.org/10.1104/pp.112.213348

Erb, T. J. (2011). Carboxylases in natural and synthetic microbial pathways. *Applied and Environmental Microbiology*, *77*(24), 8466–8477. https://doi.org/10.1128/AEM.05702-11

Faye, M., Jooste, A., Lowenberg-DeBoer, J., & Fulton, J. (2004). The influence of cowpea characteristics on cowpea prices in senegal. *Agrekon*, *43*(4), 418–429. https://doi.org/10.1080/03031853.2004.9523658

Feller, U., Crafts-brandner, S. J., & Salvucci, M. E. (1998). *Moderately High Temperatures Inhibit Ribulose-1 , 5-Bisphosphate Carboxylase / Oxygenase (Rubisco) Activase-Mediated Activation of Rubisco 1*. 539–546.

Foyer, C. H., Bloom, A. J., Queval, G., & Noctor, G. (2009). Photorespiratory metabolism: Genes, mutants, energetics, and redox signaling. *Annual Review of Plant Biology*, *60*, 455–484. https://doi.org/10.1146/annurev.arplant.043008.091948

Gao, J., Wang, H., Yuan, Q., & Feng, Y. (2018). Structure and function of the photosystem supercomplexes. *Frontiers in Plant Science*, *9*(March), 1–7. https://doi.org/10.3389/fpls.2018.00357

Gardeström, P., & Wigge, B. (1988). Influence of Photorespiration on ATP/ADP Ratios in the Chloroplasts, Mitochondria, and Cytosol, Studied by Rapid Fractionation of Barley (Hordeum vulgare) Protoplasts . *Plant Physiology*, *88*(1), 69–76. https://doi.org/10.1104/pp.88.1.69

Gautier, A. (1987). Prehistoric men and cattle in North Africa: a dearth of data and a surfeit of models. In A. E. Close & F. Wendorf (Eds.), *Prehistory of arid North Africa: essays in honor of Fred Wendorf* (pp. 163–187). Southern Methodist University Press. http://ls-tlss.ucl.ac.uk/course-materials/ARCLG197_56564.pdf

Gentilli, J., Smith, P. J., Krishnamurti, T. N., Rafferty, J. P., & Young, G. (2012). *West african monsoon*. Encyclopædia Britannica. https://www.britannica.com/science/West-African-monsoon

Gerrano, A. S., Adebola, P. O., Jansen Van Rensburg, W. S., & Laurie, S. M. (2015). Genetic variability in cowpea (Vigna unguiculata (L.) Walp.) genotypes. *South African Journal of Plant and Soil*, *32*(3), 165–174. https://doi.org/10.1080/02571862.2015.1014435

Gontero, B., Avilan, L., & Lebreton, S. (2007). Control of Carbon Fixation in Chloroplasts. *Annual Plant Reviews*, *22*, 187–218. https://doi.org/10.1002/9780470988640.ch7

Goodstein, D. M., Shu, S., Howson, R., Neupane, R., Hayes, R. D., Fazo, J., Mitros, T., Dirks, W., Hellsten, U., Putnam, N., & Rokhsar, D. S. (2012). *Phytozome: a comparative platform for green plant genomics*. https://phytozome.jgi.doe.gov/pz/portal.html

Goss, T., & Hanke, G. (2014). The End of the Line: Can Ferredoxin and Ferredoxin NADP(H) Oxidoreductase Determine the Fate of Photosynthetic Electrons? *Current Protein & Peptide Science*, *15*(4), 385–393. https://doi.org/10.2174/1389203715666140327113733

Groman-Yaroslavski, I., Weiss, E., & Nadel, D. (2016). Composite sickles and cereal harvesting methods at 23,000-years-old Ohalo II, Israel. *PLoS ONE*, *11*(11), 1–21. https://doi.org/10.1371/journal.pone.0167151

HADLEY, P., ROBERTS, E. H., SUMMERFIELD, R. J., & MINCHIN, F. R. (1983). A Quantitative Model of Reproductive Development in Cowpea [Vigna unguiculata (L) Walp.] in relation to Photoperiod and Temperature, and Implications for Screening Germplasm. *Annals of Botany*, *51*(4), 531–543. https://doi.org/10.1093/oxfordjournals.aob.a086497

Hahn, A., Vonck, J., Mills, D. J., Meier, T., & Kühlbrandt, W. (2018). Structure, mechanism, and regulation of the chloroplast ATP synthase. *Science*, *360*(6389), 1–23. https://doi.org/10.1126/science.aat4318

Hasse, D., Larsson, A. M., & Andersson, I. (2015). Structure of Arabidopsis thaliana Rubisco activase. *Acta Crystallographica Section D: Biological Crystallography*, *71*, 800–808. https://doi.org/10.1107/S1399004715001182

Hazra, S., Henderson, J. N., Liles, K., Hilton, M. T., & Wachter, R. M. (2015). Regulation of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) activase: Product inhibition, cooperativity, and magnesium activation. *Journal of Biological Chemistry*, *290*(40), 24222–24236. https://doi.org/10.1074/jbc.M115.651745

Hellemans, J., & Vandesompele, J. (2014). Selection of reliable reference genes for RT-qPCR analysis. *Methods in Molecular Biology*, *1160*, 19–26. https://doi.org/10.1007/978-1-4939-0733-5_3

Henderson, J. N., Hazra, S., Dunkle, A. M., Salvucci, M. E., & Wachter, R. M. (2013). Biochimica et Biophysica Acta Biophysical characterization of higher plant Rubisco activase. *BBA - Proteins and Proteomics*, *1834*(1), 87–97. https://doi.org/10.1016/j.bbapap.2012.09.006

Hoang, N. N., Kitaya, Y., Shibuya, T., & Endo, R. (2020). Growth and physiological characteristics of wasabi plantlets cultured by photoautotrophic micropropagation at different temperatures. *Plant Cell, Tissue and Organ Culture*, *143*(1), 87–96. https://doi.org/10.1007/s11240-020-01898-z

Hort, A. (1999). *Theophrastus: Enquiry Into Plants, Books I-V* (Issue bks. 1-5). Harvard University Press. https://books.google.co.uk/books?id=KHxrtAEACAAJ

IFPR, I. F. P. R. I. (2013). *West African agriculture and Climate change*. https://doi.org/10.1017/CBO9781107415324.004

Ivanova, L. A., Yudina, P. K., Ronzhina, D. A., Ivanov, L. A., & Hölzel, N. (2018). Quantitative mesophyll parameters rather than whole-leaf traits predict response of C3 steppe plants to aridity. *New Phytologist*, *217*(2), 558–570. https://doi.org/10.1111/nph.14840

Janssens, V., & Goris, J. (2001). Protein phosphatase 2A: A highly regulated family of serine/threonine phosphatases implicated in cell growth and signalling. *Biochemical Journal*, *353*(3), 417–439. https://doi.org/10.1042/0264-6021:3530417

Jensen, P. E., Bassi, R., Boekema, E. J., Dekker, J. P., Jansson, S., Leister, D., Robinson, C., & Scheller, H. V. (2007). Structure, function and regulation of plant photosystem I. *Biochimica et Biophysica Acta - Bioenergetics*, *1767*(5), 335–352. https://doi.org/10.1016/j.bbabio.2007.03.004

Jo Vandesompele, Katleen De Preter, Filip Pattyn, Bruce Poppe, Nadine Van Roy, A. D. P. & F. S. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric

averaging of multiple internal control genes. *Genome Biology*. https://doi.org/10.1186/gb-2002-3-7-research0034

Jumrani, K., Bhatia, V. S., & Pandey, G. P. (2017). Impact of elevated temperatures on specific leaf weight, stomatal density, photosynthesis and chlorophyll fluorescence in soybean. *Photosynthesis Research*, *131*(3), 333–350. https://doi.org/10.1007/s11120-016-0326-y

Kouam, E. B., Ngompe-Deffo, T., Anoumaa, M., & Pasquet, R. S. (2018). Preliminary study on character associations, phenotypic and genotypic divergence for yield and related quantitative traits among cowpea landraces (Vigna unguiculata) from the Western Highland Region of Cameroon. *Open Agriculture*, *3*(1), 84–97. https://doi.org/10.1515/opag-2018-0009

Langyintuo, A. S., Lowenberg-DeBoer, J., Faye, M., Lambert, D., Ibro, G., Moussa, B., Kergna, A., Kushwaha, S., Musa, S., & Ntoukam, G. (2003). Cowpea supply and demand in West and Central Africa. *Field Crops Research*, *82*(2–3), 215–231. https://doi.org/10.1016/S0378-4290(03)00039-X

Law, R. D., & Crafts-brandner, S. J. (2001). *High Temperature Stress Increases the Expression of Wheat Leaf Ribulose-1 , 5-Bisphosphate Carboxylase / oxygenase Activase Protein*. *386*(2), 261–267. https://doi.org/10.1006/abbi.2000.2225

Littleton, E. J., Dennett, M. D., Elston, J., & Monteith, J. L. (1981). The Growth and Development of Cowpeas (Vigna Unguiculata) Under Tropical Field Conditions 3. Photosynthesis of Leaves and Pods. *The Journal of Agricultural Science*, *97*(3), 539–550. https://doi.org/10.1017/S0021859600036868

Loveys, B. R., Scheurwater, I., Pons, T. L., Fitter, A. H., & Atkin, O. K. (2003). Growth temperature influences the underlying components of relative growth rate: An investigation using inherently fast- and slow-growing plant species (Plant, Cell and Environment (2002) 25 (975-988)). *Plant, Cell and Environment*, *26*(11), 1927. https://doi.org/10.1046/j.1365-3040.2003.01104.x

Lu, J., Nawaz, M. A., Wei, N., Cheng, F., & Bie, Z. (2020). Suboptimal Temperature Acclimation Enhances Chilling Tolerance by Improving Photosynthetic Adaptability and Osmoregulation Ability in Watermelon. *Horticultural Plant Journal*, *6*(1), 49–60. https://doi.org/10.1016/j.hpj.2020.01.001

Ludwig, S. R., Oppenheimer, D. G., Silflow, C. D., & Snustad, D. P. (1987). Characterization of the alpha-tubulin gene family of Arabidopsis thaliana. *Proceedings of the National Academy of Sciences of the United States of America*, *84*(16), 5833–5837. https://doi.org/10.1073/pnas.84.16.5833

Manning, K. (2011). A developmental history of West African agriculture. *Azania: Archaeological Research in Africa*, *46*(3), 376–380. https://doi.org/10.1080/0067270x.2011.609746

McDonald, G. K., & Paulsen, G. M. (1997). High temperature effects on photosynthesis and water relations of grain legumes. *Plant and Soil*, *196*(1), 47–58. https://doi.org/10.1023/A:1004249200050

Meagher, R. B., McKinney, E. C., & Kandasamy, M. K. (1999). Isovariant dynamics expand and buffer the responses of complex systems: The diverse plant actin gene family. *Plant Cell*, *11*(6), 995–1005. https://doi.org/10.1105/tpc.11.6.995

Mendiburu, F. De. (2020). *' agricolae '* (1.3-3). CRAN. http://tarwi.lamolina.edu.pe/~fmendiburu

Michelet, L., Zaffagnini, M., Morisse, S., Sparla, F., Pérez-Pérez, M. E., Francia, F., Danon, A., Marchand, C. H., Fermani, S., Trost, P., & Lemaire, S. D. (2013). Redox regulation of the Calvin-Benson cycle: Something old, something new. *Frontiers in Plant Science*, *4*(NOV), 1–21. https://doi.org/10.3389/fpls.2013.00470

Mishili, F. J., Fulton, J., Shehu, M., Marfo, K., Kushwaha, S., Jamal, M., Chergna, A., Mali, B., & Lowenberg-DeBoer, J. (2007). *Consumer preferences for quality characteristics along the cowpea value chain in Nigeria, Ghana and Mali*. *February*, 06–17. http://ageconsearch.umn.edu/bitstream/28684/1/wp060017.pdf

Mokronosov, A. T. (1981). *Ontogenetic Aspect of Photosynthesis* (1st ed.). Nauka.

Monsi, M., Saeki, T., & Schortemeyer, M. (2005). On the factor light in plant communities and its importance for matter production. *Annals of Botany*, *95*(3), 549–567. https://doi.org/10.1093/aob/mci052

Monteiro, F., Sebastiana, M., Pais, M. S., & Figueiredo, A. (2013). Reference Gene Selection and Validation for the Early Responses to Downy Mildew Infection in Susceptible and Resistant Vitis vinifera Cultivars. *PLoS ONE*, *8*(9). https://doi.org/10.1371/journal.pone.0072998

Moore, B. D., Kobza, J., & Seemann, J. R. (1991). Measurement of 2-carboxyarabinitol 1-phosphate in plant leaves by isotope dilution. *Plant Physiology*, *96*(1), 208–213. https://doi.org/10.1104/pp.96.1.208

Muchow, R. C., Robertson, M. J., & Pengelly, B. C. (1993). Radiation-use efficiency of soybean, mugbean and cowpea under different environmental conditions. *Field Crops Research*, *32*(1–2), 1–16. https://doi.org/10.1016/0378-4290(93)90017-H

Mueller-Cajar, O. (2017). The diverse AAA+ machines that repair inhibited Rubisco active sites. *Frontiers in Molecular Biosciences*, *4*(MAY), 1–17. https://doi.org/10.3389/fmolb.2017.00031

Mueller-Cajar, O., Stotz, M., Wendler, P., Hartl, F. U., Bracher, A., & Hayer-Hartl, M. (2011). Structure and function of the AAA + protein CbbX, a red-type Rubisco activase. *Nature*, *479*(7372), 194–199. https://doi.org/10.1038/nature10568

Nagarajan, R., & Gill, K. S. (2018). Evolution of Rubisco activase gene in plants. *Plant Molecular Biology*, *96*(1–2), 69–87. https://doi.org/10.1007/s11103-017-0680-y

Nielsen, S. S., Ohler, T. A., & Mitchell, C. A. (1997). Cowpea leaves for human consumption: production, utilization, and nutrient composition. *Advances in Cowpea Research*, 326–332.

Norton, L. R., Firbank, L. G., Gray, A. J., & Watkinson, A. R. (1999). Responses to elevated temperature and CO2 in the perennial grass Agrostis curtisii in relation to population

origin. *Functional Ecology*, *13*(SUPPL. 1), 29–37. https://doi.org/10.1046/j.1365-2435.1999.00005.x

Ort, D. R., Merchant, S. S., Alric, J., Barkan, A., Blankenship, R. E., Bock, R., Croce, R., Hanson, M. R., Hibberd, J. M., Long, S. P., Moore, T. A., Moroney, J., Niyogi, K. K., Parry, M. A. J., Peralta-Yahya, P. P., Prince, R. C., Redding, K. E., Spalding, M. H., Van Wijk, K. J., … Zhu, X. G. (2015). Redesigning photosynthesis to sustainably meet global food and bioenergy demand. *Proceedings of the National Academy of Sciences of the United States of America*, *112*(28), 8529–8536. https://doi.org/10.1073/pnas.1424031112

Parry, M. A. J., Keys, A. J., Madgwick, P. J., Carmo-Silva, A. E., & Andralojc, P. J. (2008). Rubisco regulation: A role for inhibitors. *Journal of Experimental Botany*, *59*(7), 1569–1580. https://doi.org/10.1093/jxb/ern084

Patel, M., & Berry, J. O. (2008). Rubisco gene expression in C4 plants. *Journal of Experimental Botany*, *59*(7), 1625–1634. https://doi.org/10.1093/jxb/erm368

Pfaffl, M. W., Tichopad, A., Prgomet, C., & Neuvians, T. (2004). Determination of most stable housekeeping genes, differentially regulated target genes and sample integrity : BestKeeper. *Biotechnology Letters*, *26*, 509–515. https://doi.org/JCMM395 [pii]\r10.1111/j.1582-4934.2008.00395.x

Pingali, P. L. (2012). Green revolution: Impacts, limits, andthe path ahead. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(31), 12302–12308. https://doi.org/10.1073/pnas.0912953109

Poorter, H., Niinemets, Ü., Poorter, L., Wright, I. J., & Villar, R. (2009). Causes and consequences of variation in leaf mass per area (LMA): A meta-analysis. *New Phytologist*, *182*(3), 565–588. https://doi.org/10.1111/j.1469-8137.2009.02830.x

Poorter, H., Niklas, K. J., Reich, P. B., Oleksyn, J., Poot, P., & Mommer, L. (2012). *Tansley review Biomass allocation to leaves , stems and roots : meta-analyses of interspecific variation and environmental control*. 30–50.

Poorter, L., & Bongers, F. (2006). Leaf traits are good predictors of plant performance across 53 rain forest species. *Ecology*, *87*(7), 1733–1743. https://doi.org/10.1890/0012-9658(2006)87[1733:LTAGPO]2.0.CO;2

R Core Team, & R Foundation for Statistical Computing. (2020). *R: A Language and Environment for Statistical Computingo Title*. https://www.r-project.org/

Rasmussen, B., Fletcher, I. R., Brocks, J. J., & Kilburn, M. R. (2008). Reassessing the first appearance of eukaryotes and cyanobacteria. *Nature*, *455*(7216), 1101–1104. https://doi.org/10.1038/nature07381

Ribeiro, P. R., Zanotti, R. F., Deflers, C., Fernandez, L. G., de Castro, R. D., Ligterink, W., & Hilhorst, H. W. M. (2015). Effect of temperature on biomass allocation in seedlings of two contrasting genotypes of the oilseed crop Ricinus communis. *Journal of Plant Physiology*, *185*, 31–39. https://doi.org/10.1016/j.jplph.2015.07.005

Rohmer, M. (2010). Methylerythritol phosphate pathway. *Comprehensive Natural Products II: Chemistry and Biology*, *1*, 517–555. https://doi.org/10.1016/b978-008045382-8.00702-4

RStudio Team. (2020). *R Studio* (1.3.1056 "water Lily"). RStudio, PBC. http://www.rstudio.com/

Salvucci, M. E., Portis, A. R., & Ogren, W. L. (1985). A soluble chloroplast protein catalyzes ribulosebisphosphate carboxylase/oxygenase activation in vivo. *Photosynthesis Research*, *7*(2), 193–201. https://doi.org/10.1007/BF00037012

Sasikumar, A. N., Perez, W. B., & Kinzy, T. G. (2012). The many roles of the eukaryotic elongation factor 1 complex. *Wiley Interdisciplinary Reviews: RNA*, *3*(4), 543–555. https://doi.org/10.1002/wrna.1118

Scafaro, A. P., Atwell, B. J., Muylaert, S., Van Reusel, B., Ruiz, G. A., Van Rie, J., & Galle, A. (2018). *A Thermotolerant Variant of Rubisco Activase From a Wild Relative Improves Growth and Seed Yield in Rice Under Heat Stress*. *9*(November), 1–11. https://doi.org/10.3389/fpls.2018.01663

Scafaro, A. P., Bautsoens, N., Boer, B. Den, Van Rie, J., & Gallé, A. (2019). A conserved sequence from heat-adapted species improves Rubisco activase thermostability in wheat. *Plant Physiology*, *181*(1), 43–54. https://doi.org/10.1104/pp.19.00425

Schneider, G., Lindqvist, Y., & Branden, C. (1992). *RUBISCO : Structure and Mechanism*.

Schreier, T. B., & Hibberd, J. M. (2019). Variations in the Calvin-Benson cycle: Selection pressures and optimization? *Journal of Experimental Botany*, *70*(6), 1697–1701. https://doi.org/10.1093/jxb/erz078

Serban, A. J., Breen, I. L., Bui, H. Q., Levitus, M., & Wachter, R. M. (2018). Assembly–disassembly is coupled to the ATPase cycle of tobacco Rubisco activase. *Journal of Biological Chemistry*, *293*(50), 19451–19465. https://doi.org/10.1074/jbc.RA118.005047

Sheahan, C. . (2012). Plant Guide for cowpea (Vigna unguiculata). *USDA-Natural Resources Conservation Service*, 5–7.

Shipley, B. (2006). Net assimilation rate, specific leaf area and leaf mass ratio: Which is most closely correlated with relative growth rate? A meta-analysis. *Functional Ecology*, *20*(4), 565–574. https://doi.org/10.1111/j.1365-2435.2006.01135.x

Shirakihara, Y. (1999). ATP synthase. *Tanpakushitsu Kakusan Koso. Protein, Nucleic Acid, Enzyme*, *44*(4 Suppl), 538–545. https://doi.org/10.1146/annurev-biochem-060614-034124

Shivhare, D., Ng, J., Tsai, Y. C. C., & Mueller-Cajar, O. (2019). Probing the rice Rubisco–Rubisco activase interaction via subunit heterooligomerization. *Proceedings of the National Academy of Sciences of the United States of America*, *116*(48), 24041–24048. https://doi.org/10.1073/pnas.1914245116

Si, P., & Thurling, N. (2001). A greater relative growth rate of Brassica rapa L. at low temperatures increases biomass at anthesis. *Australian Journal of Agricultural Research*, *52*(6), 645–652.

Singh, B. (2006). Cowpea breeding at IITA: highlights of advances and impacts. Anais. *Congresso Nacional de Feijão-Caupi. Teresina PI*, *June*.

http://scholar.google.com/scholar?hl=en&btnG=Search&q=intitle:COWPEA+BREEDING+AT+IITA+:+HIGHLIGHTS+OF+ADVANCES+AND+IMPACTS#1%5Cnhttp://scholar.google.com/scholar?hl=en&btnG=Search&q=intitle:Cowpea+breeding+at+IITA:+highlights+of+advances+and+impacts.+Anai

Smith, W. K., & Nobel, P. S. (1978). Influence of Irradiation, Soil Water Potential, and Leaf Temperature on Leaf Morphology of a Desert Broadleaf, Encelia Farinosa Gray (Compositae). *American Journal of Botany*, *65*(4), 429–432. https://doi.org/10.1002/j.1537-2197.1978.tb06089.x

Spriggs, A., Henderson, S. T., Hand, M. L., Johnson, S. D., Taylor, J. M., & Koltunow, A. (2018). Assembled genomic and tissue-specific transcriptomic data resources for two genetically distinct lines of cowpea (Vigna unguiculata (L.) walp). *Gates Open Research*, *2*. https://doi.org/10.12688/gatesopenres.12777.2

Stec, B. (2012). Structural mechanism of RuBisCO activation by carbamylation of the active site lysine. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(46), 18785–18790. https://doi.org/10.1073/pnas.1210754109

Stotz, M., Mueller-Cajar, O., Ciniawsky, S., Wendler, P., Hartl, F. U., Bracher, A., & Hayer-Hartl, M. (2011). Structure of green-type Rubisco activase from tobacco. *Nature Structural and Molecular Biology*, *18*(12), 1366–1370. https://doi.org/10.1038/nsmb.2171

Sysoeva, T. A. (2017). Assessing heterogeneity in oligomeric AAA+ machines. *Cellular and Molecular Life Sciences*, *74*(6), 1001–1018. https://doi.org/10.1007/s00018-016-2374-z

Tabita, F. R., Satagopan, S., Hanson, T. E., Kreel, N. E., & Scott, S. S. (2008). Distinct form I, II, III, and IV Rubisco proteins from the three kingdoms of life provide clues about Rubisco evolution and structure/function relationships. *Journal of Experimental Botany*, *59*(7), 1515–1524. https://doi.org/10.1093/jxb/erm361

Tang, F., Chu, L., Shu, W., He, X., Wang, L., & Lu, M. (2019). Selection and validation of reference genes for quantitative expression analysis of miRNAs and mRNAs in Poplar. *Plant Methods*, *15*(1), 1–15. https://doi.org/10.1186/s13007-019-0420-1

Thompson, F. M. L. (1968). *The Second Agricultural Revolution, 1815-1880*. *21*(1), 62–77.

Tsai, Y. C. C., Lapina, M. C., Bhushan, S., & Mueller-Cajar, O. (2015). Identification and characterization of multiple rubisco activases in chemoautotrophic bacteria. *Nature Communications*, *6*(May), 1–10. https://doi.org/10.1038/ncomms9883

Usadel, B., Bläsing, O. E., Gibon, Y., Poree, F., Höhne, M., Günter, M., Trethewey, R., Kamlage, B., Poorter, H., & Stitt, M. (2008). Multilevel genomic analysis of the response of transcripts, enzyme activities and metabolites in Arabidopsis rosettes to a progressive decrease of temperature in the non-freezing range. *Plant, Cell and Environment*, *31*(4), 518–547. https://doi.org/10.1111/j.1365-3040.2007.01763.x

USAID. (n.d.). *Growing Cowpea in Nigeria*.

Wang, D., Li, X. F., Zhou, Z. J., Feng, X. P., Yang, W. J., & Jiang, D. A. (2010a). Two Rubisco activase isoforms may play different roles in photosynthetic heat acclimation in the rice plant. *Physiologia Plantarum*, *139*(1), 55–67. https://doi.org/10.1111/j.1399-

3054.2009.01344.x

Wang, D., Li, X., Zhou, Z., Feng, X., Yang, W., & Jiang, D. (2010b). *Two Rubisco activase isoforms may play different roles in photosynthetic heat acclimation in the rice plant*. 55–67. https://doi.org/10.1111/j.1399-3054.2009.01344.x

Wen, S., Chen, X., Xu, F., & Sun, H. (2016). Validation of reference genes for real-time quantitative PCR (qPCR) Analysis of avibacterium paragallinarum. *PLoS ONE*, *11*(12), 1–14. https://doi.org/10.1371/journal.pone.0167736

Wickham, H. (2016). *ggplot2: Elegant Graphics for Data Analysis*. Springer-Verlag New York. https://ggplot2.tidyverse.org

Wu, Z. J., Tian, C., Jiang, Q., Li, X. H., & Zhuang, J. (2016). Selection of suitable reference genes for qRT-PCR normalization during leaf development and hormonal stimuli in tea plant (Camellia sinensis). *Scientific Reports*, *6*(December 2015), 1–10. https://doi.org/10.1038/srep19748

Xie, K., Shen, J., Hou, X., Yao, J., Li, X., Xiao, J., & Xiong, L. (2012). Gradual increase of miR156 regulates temporal expression changes of numerous genes during leaf development in rice. *Plant Physiology*, *158*(3), 1382–1394. https://doi.org/10.1104/pp.111.190488

Yang, H., Liu, J., Huang, S., Guo, T., Deng, L., & Hua, W. (2014). Selection and evaluation of novel reference genes for quantitative reverse transcription PCR (qRT-PCR) based on genome and transcriptome data in Brassica napus L. *Gene*, *538*(1), 113–122. https://doi.org/10.1016/j.gene.2013.12.057

Yin, X., & Struik, P. C. (2017). Can increased leaf photosynthesis be converted into higher crop mass production? A simulation study for rice using the crop model GECROS. *Journal of Experimental Botany*, *68*(9), 2345–2360. https://doi.org/10.1093/jxb/erx085

Zhang, N., & Portis, A. R. (1999a). Mechanism of light regulation of Rubisco: A specific role for the larger rubisco activase isoform involving reductive activation by thioredoxin-f. *Proceedings of the National Academy of Sciences of the United States of America*, *96*(16), 9438–9443. https://doi.org/10.1073/pnas.96.16.9438

Zhang, N., & Portis, A. R. (1999b). Mechanism of light regulation of Rubisco: A specific role for the larger rubisco activase isoform involving reductive activation by thioredoxin-f. *Proceedings of the National Academy of Sciences of the United States of America*, *96*(16), 9438–9443. https://doi.org/10.1073/pnas.96.16.9438

Zhu, G., & Jensen, R. G. (1991). Fallover of ribulose 1,5-bisphosphate carboxylase/oxygenase activity decarbamylation of catalytic sites depends on pH. *Plant Physiology*, *97*(4), 1354–1358. https://doi.org/10.1104/pp.97.4.1354