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A molecular approach to understand the transition from embryo development to seed filling in common bean (*Phaseolus vulgaris* L.)

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Dissertation presented to obtain the **Ph.D degree in Plant Sciences**

Plants for Life

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INSTITUTO DE TECNOLOGIA QUÍMICA E BIOLÓGICA ANTÓNIO XAVIER | UNIVERSIDADE NOVA DE LISBOA

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The research described in this thesis was performed under the supervision of:

PhD Supervisor
Professor Doctor Pedro Fevereiro
Head of Plant Cell Biotechnology Laboratory, Instituto de Tecnologia Química e Biológica António Xavier, Universidade Nova de Lisboa, Avenida da República, 2780-157 Oeiras, Portugal

PhD Co-Supervisor
Doctor Susana de Sousa Araújo
MORE CoLAB - Laboratório Colaborativo Montanhas de Investigação, Edifício do Brigantia Ecopark, Avenida Cidade de Leon, 506, 5300-358 Bragança, Portugal
CIMO- Centro de Investigação de Montanha, Campus de Santa Apolónia, 5300-253 Bragança, Portugal

All work was performed at:
Instituto de Tecnologia Química e Biológica António Xavier
Universidade Nova de Lisboa
Av. da República, 2780-157 Oeiras, Portugal

Front Cover: SER 16 *Phaseolus vulgaris* tagged flowers.

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Table of contents

List of most used abbreviations

ABA – Abscisic Acid

ABF – ABA Responsive Element-Binding Factor

ABI3 – Abscisic Acid Insensitive 3

ACO – 1-Aminocyclopropane-1-carboxylic acid oxidase

AGPase – ADP-glucose pyrophosphorylase

AHP – Arabidopsis Histidine-containing Phosphotransfer proteins

AOC – Allene Oxide Cyclase

AOS – Allene Oxide Synthase

ARF – Auxin Response Factor

AUX – Auxin

AUX1 – Auxin Influx Carrier 1

AUX/IAA – Auxin/Indole-3-acetic Acid proteins

BA2H – Benzoic Acid 2-Hydroxylase

BIN2 – Brassinosteroid-Insensitive 2

BR – Brassinosteroid

BSK – Brassinosteroid-Signaling Kinase

Ccx – Carotenes and Xanthophylls

CK – Cytokinin

CM – Chorismate Mutase

COI1 – Coronatine Insensitive 1

CP – Control

CWIN – Cell Wall Invertase

CYP707A – ABA 8'-Hydroxylase

DAA – Days After Anthesis

DEG – Differentially Expressed Gene

DHZ – Dihydrozeatin

e – Embryo

EBF1/2 – EIN3-Binding F-box proteins 1 and 2

EM1 – Early Methionine-Labeled Protein 1

eRF1 – Eukaryotic Release Factor 1

ERF1/2 – Ethylene Response Factor 1 and 2

ET – Ethylene

FAA – Formalin-Acetic acid-Alcohol

FBP1 – Fructose-1,6-bisphosphatase

FC – Fold Change

FGP – Final Germination Percentage

FPKM – Fragments Per Kilobase of transcript per Million mapped reads

FW – Fresh Weight

GA – Gibberellin

GA1 / GA4 – Gibberellic Acid 1 / 4

GA2ox – Gibberellin 2-oxidase

GA20ox2 – Gibberellin 20-oxidase 2

GBSS – Granule-Bound Starch Synthase

GH3 – Gretchen Hagen 3 (Auxin-amido synthetase family)

GI – Germination Index

HS – Heat Stress

IAA – Indole-3-Acetic Acid

IAMT1 – IAA Carboxyl Methyltransferase 1

iP – Isopentenyladenine

IPT – Isopentenyltransferase

ISA – Isoamylase

JA – Jasmonic Acid

JAR1 – Jasmonate Resistant 1

JAZ – Jasmonate ZIM-domain Protein

L1L – LEC1-Like

LEA – Late Embryogenesis Abundant

LEC1 – Leafy Cotyledon 1

LEC2 – Leafy Cotyledon 2

MAPK / MPK6 – Mitogen-Activated Protein Kinase 6

MGT – Mean Germination Time

MYC2 – bHLH-type transcription factor in JA signaling

NCED – 9-cis-epoxycarotenoid dioxygenase

NCED5 – 9-cis-Epoxycarotenoid Dioxygenase 5

PAL – Phenylalanine Ammonia-Lyase

PEL1 – Eukaryotic Release Factor 1 Family Protein

PGM – Phosphoglucomutase

PHB – PHABULOSA

Pho1 – Plastid Starch Phosphorylase

PP2C – Protein Phosphatase 2C

PSYUC10 – YUCCA-like gene

PUL – Pullulanase

RAB18 – Responsive to ABA 18

RING – Really Interesting New Gene (E3 ubiquitin ligase family)

RWC – Relative Water Content

SA – Salicylic Acid

SAM synthetase – S-adenosylmethionine synthetase

SBE – Starch Branching Enzyme

SCP – Seeds from Control Plants

SDW – Seed Dry Weight

SFW – Seed Fresh Weight

SG – Starch Grains

SHSP – Seeds from Heat-Stressed Plants

SL – Seed Length

SnRK1 – SNF1-Related Protein Kinase 1

SnRK2 – SNF1-related protein kinase 2

SOD – Superoxide Dismutase

SPP – Sucrose Phosphate Phosphatase

SPS – Sucrose Phosphate Synthase

SVI – Seedling Vigor Index

T50 – Time to 50% Germination

TAGs – Triacylglycerols

TAR – Tryptophan Aminotransferase

TIR1 – Transport Inhibitor Response 1

TW – Turgid Weight

UDPase – UDP-glucose pyrophosphorylase

UGTs – UDP-glucosyltransferases

ZEP – Zeaxanthin Epoxidase

tZ – Trans-Zeatin

Abstract

Phaseolus vulgaris L. (common bean) is one of the most consumed grain legumes worldwide. It is rich in proteins, carbohydrates, fiber, iron and essential micronutrients. Its agronomic significance extends beyond nutrition, as *P. vulgaris* contributes to sustainable agriculture through its nitrogen-fixing capabilities, improving soil fertility and reducing the need for synthetic fertilizers.

In legumes like *P. vulgaris*, seed development is a finely coordinated process that determines final seed quality and yield. As a non-endospermic species, nutrient storage is confined to cotyledons, making the timing and regulation of seed filling especially critical. The transition from embryogenesis to reserve accumulation is a key developmental checkpoint, yet the precise timing and molecular mechanisms controlling this transition remain not totally understood in *P. vulgaris*, despite the advances made by previous works of our team. This knowledge gap has hindered targeted interventions for crop improvement and stress resilience.

Rising global temperatures pose a serious threat to legume productivity, especially during reproductive and seed developmental stages. Heat stress (HS) can disrupt cellular function, impair seed filling, reduce seed size and number, and ultimately compromise crop yields. Moreover, there is growing evidence that stress effects may be passed on to the next generation through transgenerational memory, yet the mechanisms by which heat stress alters seed development and progeny performance in *P. vulgaris* remain poorly understood.

The work conducted under the scope of this thesis aims to answer still open questions regarding the timing and molecular regulation of seed filling initiation in *P. vulgaris* and how it is affected by heat stress.

In view of our previous findings that suggest that the seed filling program can begin before the 10th days after anthesis (DAA), we investigated whether if the transition to seed fille occurs earlier, particularly if seeds at 10 DAA are still in embryogenesis or have already formed cotyledons. We aimed to determine the timing of this developmental transition and the molecular mechanisms underlying it. We also examined how heat stress affects the timing and duration of seed filling, final seed size and number, storage compound accumulation, and hormone regulation. Finally, we also examined how stress experienced by the parental plants influences germination speed, seedling vigor, seed size, and leaf development in the progeny grown under optimal conditions. These questions are addressed across three integrative chapters.

Chapter II provides a refined developmental timeline for *P. vulgaris* seed filling by analyzing seeds at 6, 10, 14, 18, and 20 DAA using a multidisciplinary approach. Morphological and histological data confirmed that at 10 DAA cotyledons are fully formed, but protein and starch granules have not yet accumulated. Transcriptomic data revealed that key transcription factors such as LEC1 and LEC2 are already highly expressed at 6 DAA, indicating that the molecular program for seed filling is initiated during late embryogenesis. Storage protein genes, such as phaseolin, begin to increase expression at 10 DAA and peak by 18 DAA. Through histology, starch accumulation is only visible after 10 DAA and high amylose content in *P. vulgaris* appears to result from the debranching of amylopectin by ISAIII and PUL at 14 and 18 DAA. Additionally, hormonal analysis of gene expression revealed a phase-specific regulation: auxins, cytokinins, and gibberellins dominate early stages, while abscisic acid, ethylene, and brassinosteroids are upregulated during filling and maturation.

Chapter III investigates how heat stress (32/25°C day/night) disrupts seed development when applied during the same timepoints (6, 10, 14, 18 and 20 DAA). Heat stress shortened the seed filling period, since seed fresh and dry weight peaked at 18 DAA, while under control (25/18°C) seeds continued filling until 30 DAA. HS reduced seed number per plant and triggered premature desiccation, as indicated by the cessation of cotyledon expansion at 18 DAA. Histochemical analyses showed that starch accumulation was impaired under HS, and transcriptomic data confirmed downregulation of key starch biosynthesis genes (e.g., AGPase, GBSS, ISA). Hormonal profiling revealed that ABA and JA levels were significantly reduced at 14, 18, and 20 DAA under HS, while auxin, cytokinin (tZ, DHZ), and salicylic acid concentrations were elevated at 6 and 10 DAA under HS. Ethylene-related genes were upregulated under HS at 14 and 18 DAA. Together, these findings show that HS compresses the developmental timeline, impairs reserve accumulation and reprograms hormonal signalling.

Chapter IV evaluates the transgenerational consequences of parental HS by assessing germination and early seedling traits in progeny seeds grown under optimal conditions. Although the final germination percentage was not affected, seeds produced by HS plants (SHSP) exhibited faster germination, with significantly lower mean germination time (MGT) and time to 50% germination (T50). However, SHSP seedlings displayed reduced seedling vigor index, shorter shoots and roots, lower fresh and dry biomass, and smaller primary leaves compared to seed coming from control plants (SCP). Additionally, SHSP seeds were significantly lighter and shorter, indicating that stress-induced developmental changes persisted into the next generation. These results suggest that parental heat stress during SD alters both germination kinetics and early vegetative development of the progeny which underlying molecular and metabolic mechanisms remain to be understood.

Together, the results from **Chapters II**, **III** and **IV** provide a comprehensive framework for understanding how developmental timing, heat stress and parental environment modulate seed development and germination in *P. vulgaris*. This thesis offers the first high-resolution molecular map of the transition from embryogenesis to seed filling in this species, shows how heat stress compresses the developmental program and has effects on the next generation. The results obtained provide advances in the state of the art of legume seed biology, by providing a deeper understanding of the regulatory networks involved in seed development and how they are modulated by HS, providing new directions for future efforts to optimize storage compound accumulation in legumes. By identifying early-activated transcription factors, hormones and metabolic pathways during SD, we highlight valuable molecular targets for breeding programs aimed at enhancing seed quality traits. In due time, our results could contribute to the development of resilient, nutrient-rich legume cultivars that support sustainable agricultural practices, cope with pressing challenges of Climate Changes, while promoting food security.

Sumário

Phaseolus vulgaris L. (feijão) é uma das leguminosas mais consumidas a nível mundial. Rico em proteínas, hidratos de carbono, fibras, ferro e micronutrientes essenciais. A sua importância agronómica vai além do valor nutricional, sendo que o *P. vulgaris* contribui para uma agricultura sustentável através da fixação de azoto, melhorando a fertilidade do solo e reduzindo a necessidade de fertilizantes sintéticos.

Nas leguminosas como o *P. vulgaris*, o desenvolvimento da semente é um processo altamente coordenado que determina a qualidade final da semente e o rendimento da cultura. Sendo uma espécie com semente não-endospérmica, o armazenamento de nutrientes está confinado aos cotilédones, o que torna o controlo temporal e molecular da fase de enchimento da semente particularmente crítico. A transição da embriogénese para a acumulação de reservas representa um ponto-chave no desenvolvimento, mas o momento preciso e os mecanismos moleculares que a regulam permaneciam ainda pouco compreendidos em *P. vulgaris*, apesar dos avanços alcançados pelos trabalhos anteriores da nossa equipa. Esta lacuna de conhecimento limita a melhoria genética e a resiliência ao stress.

O aumento das temperaturas globais representa uma ameaça significativa à produtividade das leguminosas, sobretudo durante as fases reprodutivas e de desenvolvimento das sementes. O stress por calor (do inglês heat stress, HS) pode perturbar funções celulares, comprometer o enchimento da semente, reduzir o número e o tamanho das sementes e, consequentemente, afetar o rendimento das culturas. Além disso, há evidências crescentes de que os efeitos do stress podem ser transmitidos à geração seguinte através de memória transgeracional, embora os mecanismos através dos quais o HS altera o desenvolvimento das sementes e o desempenho da descendência em *P. vulgaris* permaneçam pouco compreendidos.

O trabalho desenvolvido no âmbito desta tese visa responder a questões ainda em aberto relativamente ao momento e à regulação molecular da iniciação do enchimento da semente *P. vulgaris* e como este processo é afetado pelo HS.

Tendo em conta os nossos resultados anteriores que sugerem que o programa de enchimento da semente pode iniciar-se antes dos 10 dias após a antese (DAA), investigámos se a transição para o enchimento ocorre mais cedo, nomeadamente se as sementes aos 10 DAA ainda se encontram em embriogénese ou se já formaram cotilédones. Pretendemos determinar o momento desta transição e os mecanismos moleculares subjacentes. Analisámos também de que forma o HS afeta o momento e a duração do enchimento da semente, o tamanho e número final das sementes, a acumulação de compostos de reserva e a regulação hormonal. Por fim, avaliámos ainda como o stress experienciado pelas plantas parentais influência a velocidade de germinação, o vigor das plântulas, o tamanho da semente e o desenvolvimento foliar da descendência cultivada em condições ótimas. Estas questões são abordadas em três capítulos integrativos.

O **Capítulo II** estabelece um cronograma detalhado do enchimento da semente em *P. vulgaris*, através da análise de sementes aos 6, 10, 14, 18 e 20 DAA utilizando uma abordagem multidisciplinar. A análise morfológica e histológica confirmou que, aos 10 DAA, os cotilédones estão completamente formados, mas ainda não há acumulação visível de proteínas ou amido. A análise transcriptómica revelou que fatores de transcrição chave como LEC1 e LEC2 já estão fortemente expressos aos 6 DAA, indicando que o programa molecular de enchimento é ativado ainda durante a embriogénese tardia. Genes de proteínas de reserva, como a faseolina, iniciam a sua expressão aos 10 DAA e atingem o pico aos 18 DAA. Através da histologia, a acumulação de amido torna-se visível apenas após os 10 DAA, sendo o seu elevado teor de amilose em *P. vulgaris* aparentemente resultado da desramificação de amilopectina mediada por ISAIII e PUL aos 14 e 18 DAA. Além disso, a análise da expressão de genes relacionados com fitohormonas revelou uma regulação onde: auxinas, citocininas e giberelinas dominam os estágios iniciais, enquanto o ácido abscísico, etileno e brassinosteroides são ativados durante o enchimento e maturação.

O **Capítulo III** investiga como o HS (32/25°C dia/noite) perturba o desenvolvimento da semente quando aplicado nos mesmos estágios (6, 10, 14, 18 e 20 DAA). O HS encurtou o período de enchimento, dado que o peso fresco e seco da semente atingiu o pico aos 18 DAA, enquanto em condições de controlo (25/18°C) o enchimento continuou até aos 30 DAA. O HS reduziu o número de sementes por planta e desencadeou uma desidratação da semente precoce, como evidenciado pela cessação da expansão dos cotilédones aos 18 DAA. As análises histoquímicas mostraram que a acumulação de amido foi prejudicada sob HS, e os dados transcriptómicos confirmaram a diminuição da expressão de genes chave de biossíntese de amido (como AGPase, GBSS, ISA). A análise hormonal revelou uma redução significativa dos níveis de ABA e JA aos 14, 18 e 20 DAA sob HS, enquanto as concentrações de auxinas, citocininas (tZ, DHZ) e ácido salicílico aumentaram aos 6 e 10 DAA. Genes relacionados com o etileno estavam mais expressos aos 14 e 18 DAA sob HS. Estes resultados demonstram que o HS diminui a duração do desenvolvimento, prejudica a acumulação de reservas e reprograma a sinalização hormonal.

O **Capítulo IV** avalia as consequências transgeracionais do HS parental através da análise da germinação e características iniciais da plântula, em sementes da descendência cultivadas em condições ótimas. Embora a percentagem final de germinação não tenha sido afetada, as sementes provenientes de plantas expostas ao HS (SHSP) germinaram mais rapidamente, com tempos médios de germinação (MGT) e tempos para 50% de germinação (T50) significativamente mais baixos. Contudo, as plântulas SHSP apresentaram menor índice de vigor, raízes e caules mais curtos, biomassa fresca e seca reduzida, e folhas primárias mais pequenas em comparação com plântulas de sementes de plantas controlo (SCP). Além disso, as sementes SHSP eram significativamente mais leves e curtas, sugerindo que as alterações no desenvolvimento induzidas pelo stress persistem na geração seguinte. Estes resultados sugerem que o HS parental durante o desenvolvimento da semente altera tanto a cinética de germinação como o desenvolvimento vegetativo inicial da descendência, cujos mecanismos moleculares e metabólicos subjacentes permanecem ainda por esclarecer.

Em conjunto, os resultados dos **Capítulos II**, **III** e **IV** fornecem um enquadramento abrangente para compreender como o tempo de desenvolvimento, o HS e o ambiente parental modulam o desenvolvimento da semente e a germinação em *P. vulgaris*. Esta tese oferece o primeiro mapa molecular de alta resolução da transição da embriogénese para o enchimento da semente nesta espécie, demonstra como o HS comprime o programa de desenvolvimento e que o HS tem efeitos sobre a geração seguinte. Os resultados obtidos representam avanços significativos no estado da arte da biologia da semente em leguminosas, proporcionando uma compreensão mais aprofundada das redes regulatórias envolvidas e da sua modulação pelo HS, abrindo novos caminhos para a otimização da acumulação de compostos de reserva em leguminosas. Ao identificar fatores de transcrição, hormonas e vias metabólicas ativadas precocemente durante o desenvolvimento da semente, destacamos alvos moleculares valiosos para programas de melhoramento visando melhorar características de qualidade da semente. A médio prazo, os nossos resultados poderão contribuir para o desenvolvimento de variedades de leguminosas resilientes, ricas em nutrientes, capazes de suportar práticas agrícolas sustentáveis e enfrentar os desafios prementes das Alterações Climáticas, promovendo a segurança alimentar.

Chapter I

General Introduction

Author’s contributions to the chapter:

Cláudia Lopes wrote this chapter based on the referred bibliography.

Abstract

Common bean (*Phaseolus vulgaris* L.) is one of the most widely consumed legumes worldwide, providing a vital source of protein, carbohydrates, fibers and essential micronutrients. However, rising global temperatures pose a significant threat to its productivity, particularly through heat stress, which affects key developmental processes such as seed germination, seed filling and nutrient remobilization. Heat stress disrupts hormonal regulation, including abscisic acid, gibberellins, auxins and cytokinins, which are essential for maintaining source-sink dynamics, seed viability and stress recovery. Additionally, high temperatures impair photosynthesis, reduce pollen viability and accelerate phenological stages, leading to lower yields and reduced seed quality. Advances in molecular biology have identified heat-responsive genes, epigenetic modifications and antioxidant mechanisms that contribute to thermotolerance in common bean. This chapter synthesizes current knowledge on the physiological, hormonal and molecular responses of *Phaseolus vulgaris* to heat stress. It also explores emerging strategies, including genetic engineering, marker-assisted selection and exogenous applications of phytohormones and biostimulants, to enhance heat resilience. By addressing key research gaps, such as the interplay between hormonal regulation and nutrient remobilization, this chapter aims to provide a roadmap for developing climate-resilient common bean varieties, contributing to global food security in the face of climate change.

Keywords: *Phaseolus vulgaris*; Heat stress; Seed filling; Phytohormonal regulation; Thermotolerance; Source-sink dynamics; Molecular adaptation; Climate resilience.

1. Introduction

Legumes account for 27% of the world's primary crop production, with grain legumes alone contributing 33% of the human dietary (Vance, 2001) with several health benefits (Arnoldi et al., 2014; Bassett et al., 2010; Vaz Patto et al., 2014). Common bean (*Phaseolus vulgaris* L.) is one of the most consumed grain legumes worldwide. Common bean represents an advantage in developing countries for their affordability and long storage life when compared to animal protein (Castro-Guerrero et al., 2016; De La Fuente et al., 2011). In Eastern and Southern Africa common bean consumption per capita is around 40–50 kg per year and it represents 50% of the grain legumes consumed worldwide (Ghanbari et al., 2014; Ia et al., 2012). Common bean seeds are one of the major sources for proteins, carbohydrates, fibers, vitamins, minerals (calcium, potassium, phosphorus, magnesium, iron, zinc and copper) as well as other health-promoting phenolic compounds, crucial for food security and nutrition (McClean et al., 2004; Talukder et al., 2010). Since common bean genome sequence and genetic tools are already available (Collado et al., 2015; Leitão et al., 2017; O'Rourke et al., 2014; Parreira et al., 2016, 2018; Schmutz et al., 2014), this crop represents a suitable model for molecular studies to improve and select the desirable quality traits regarding the common bean seed development.

However, climate change poses a threat to food and feed supplies, increasing the incidence of different abiotic stresses on crops. In particular, it is essential to understand the responses of crops to the heat stress imposed by the increase in the average annual temperature. Global average temperature increased 1.04°C from 2014 to 2018 (IPCC, 2018) and is expected to rise up to 4°C by the end of the current century (Bita & Gerats, 2013). As a result, plant growth and development are being damaged by heat stress (Asseng et al., 2015; Dusenge et al., 2019; P. S. Li et al., 2014; Vadez et al., 2012; Zinn et al., 2010). It was shown that heat stress affects seed germination, growth, development, photosynthesis, flowering and seedling growth (Arshad et al., 2020; Hasanuzzaman et al., 2012; Lal et al., 2021; Teskey et al., 2015; Zhao et al., 2017). Furthermore, the regulation of seed development under heat stress involves complex hormonal interactions, yet the precise roles of abscisic acid (ABA), gibberellins (GA), auxins and cytokinins in these processes are not fully elucidated. Investigating how hormonal pathways influence seed filling, seed viability and stress recovery could provide a deeper understanding of source-sink dynamics under high temperatures. Moreover, there is a growing need for research into the interactions between heat stress and epigenetic modifications, which may have profound implications for plant adaptation across generations. While substantial progress has been made in understanding the direct effects of heat stress on crop physiology and reproduction, many questions remain regarding its long-term impacts on plant fitness, particularly through transgenerational effects. It is critical to evaluate how environmental stresses experienced by parent plants can influence progeny traits, including seed germination, seedling vigor and stress resilience. Understanding these transgenerational responses could reveal novel mechanisms of adaptation and provide valuable insights for breeding climate-resilient crops.

This chapter examines the effect of heat stress on key aspects of plant development, including seed germination, seed filling and hormonal regulation. It also evaluates recent advances in understanding the molecular mechanisms underlying heat tolerance and explores potential strategies to mitigate heat stress in common bean and other legumes. Future research should address how heat stress-induced changes in source-sink relationships, hormonal regulation and epigenetic mechanisms could be leveraged to improve crop resilience. By synthesizing current knowledge, this chapter aims to identify research gaps and highlight opportunities for enhancing crop resilience in the face of climate change.

2. Effect of Heat Stress in Plant Growth

Heat stress is a significant abiotic challenge to plant growth and development, leading to reduced productivity across many crops, including legumes. High temperatures lead to leaf scorching, rolling, senescence and abscission (Rodríguez et al., 2005; Vollenweider & Günthardt-Goerg, 2005). Heat stress can also cause sunburn, discoloration of fruits, flower and fruit abortion (L. W. Young et al., 2004) and retarded growth of shoot and root (Hasanuzzaman et al., 2013; Vollenweider & Günthardt-Goerg, 2005).

During the reproductive and seed grain-filling stages, heat stress significantly impacts seed development and quality in cereal crops, since it reduces photosynthetic capacity, dry matter accumulation and grain yield (Farooq et al., 2011). Studies have shown that elevated temperatures affect phloem loading and sucrose translocation (Soltani et al., 2019), disrupts source-sink activities and induces undesirable biochemical changes in seeds, affecting carbohydrates, proteins, oils and other nutritional components (Kumar et al., 2023).

Legumes are known to be particularly vulnerable to heat stress, especially during their reproductive phase (Saxena et al., 1988; Solh et al., 1994). Species such as chickpea, lentil, pea, and faba bean exhibit sensitivity to elevated temperatures, with chickpea demonstrating comparatively higher critical temperature limits (Saxena et al., 1988). Within the legume family, *P. vulgaris* is notably impacted by heat stress, which disrupts key physiological processes during reproduction and severely limits productivity. High nighttime temperatures are especially harmful, leading to yield reductions of up to 37% (Vargas et al., 2021). In *P. vulgaris*, heat stress has been associated with a decline in pod and seed numbers per plant, along with a decrease in pod length and weight (Konsens et al., 1991). Furthermore, reproductive success is hampered under elevated temperatures, negatively affecting pod and seed set (Gross & Kigel, 1994; Vara Prasad et al., 2002). Heat stress can also induce premature abscission of flowers and pods, exacerbating the negative impact on reproductive output in *P. vulgaris* (Monterroso & Wien, 1990).

These findings emphasize the vulnerability of legumes, particularly during their reproductive stages. Despite these insights, further research is needed to understand the mechanisms underlying the interaction between high temperatures and pollen viability in common bean. Specific attention should be given to how hormonal regulation and epigenetic changes influence reproductive success under heat stress, which could provide pathways for developing more heat-tolerant cultivars. Moreover, in soybean subjected to heat stress, a reduction in seed germination and seedling vigour was observed (Egli et al., 2005). It was observed that the impact of heat stress on photosynthesis in legumes, including common bean, is not only a result of damage to Photosystem II but also due to disrupted source-sink relationships, which compromise nutrient flow to developing seeds (Soltani et al., 2019). This underscores the need for a deeper investigation into how heat-induced photosynthetic changes affect reproductive outcomes and seed quality. Future studies could explore the dynamics of source-sink adjustments under varying heat stress intensities to determine whether altering hormonal pathways or enhancing phloem loading efficiency might mitigate yield losses.

In *Phaseolus vulgaris*, the effects of heat stress were also observed. It was shown that when common bean was subjected to temperatures above 30°C at daytime and temperatures above 22°C during nighttime, the bean yield was reduced (Omae et al., 2012; Rainey & Griffiths, 2005). Vargas et al, (2021) saw a decrease from 90% to 75% of pollen viability under heat stress conditions, in common bean. Moreover, high temperatures reduced the yield by 37% (Vargas et al., 2021). Growth and development were seriously hampered (Hasanuzzaman et al., 2013), pollen tube growth, germination and fertilization were affected by heat stress (Gross & Kigel, 1994; Konsens et al., 1991; Lohani et al., 2022; Omae et al., 2012). Moreover, it was also observed that micro-sporogenisis was obstructed (Porch & Jahn, 2001), leading to yield loss (Rainey & Griffiths, 2005) in common bean. Understanding the link between pollen viability, fertilization success and seed development under high-temperature stress could yield valuable insights for developing improved heat-tolerant cultivars. Additionally, future work could focus on how transgenerational effects of heat stress might influence subsequent generations' reproductive performance, providing a broader perspective on the adaptive potential of common bean under changing climates.

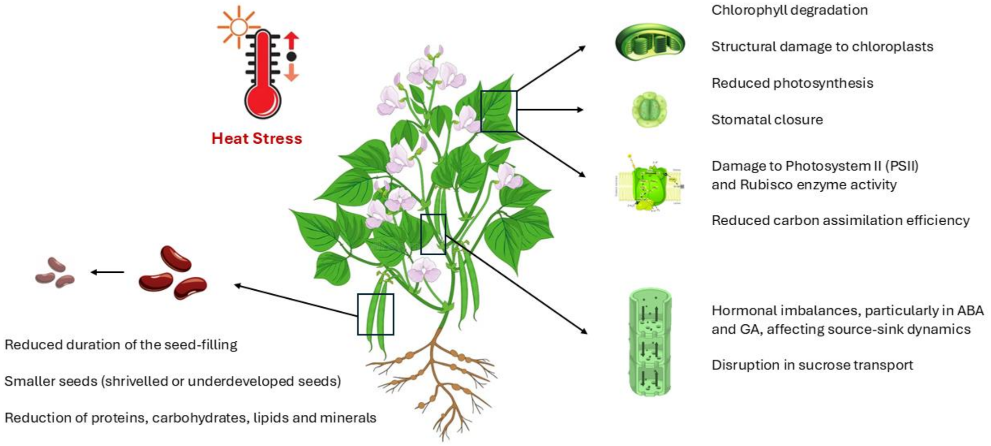

Figure 1- Impacts of heat stress on the physiology and seed quality of common bean (*Phaseolus vulgaris*). Heat stress induces chlorophyll degradation, structural damage to chloroplasts, reduced photosynthesis and stomatal closure.

2.1 Photosynthesis, Leaf senescence and water relations

Heat stress stimulate leaf senescence, which disrupts chloroplasts and damages chlorophyl (M. Farooq et al., 2017; Marchand et al., 2005; Ougham et al., 2008). High temperatures change the structural organization of thylakoids (Bsmrau et al., 1997; Crafts-Brandner & Salvucci, 2002; J. Z. Wang et al., 2009), leading to loss of grana stacking or its sweeling (Rodríguez et al., 2005). High temperatures reduce chlorophyll biosynthesis in wheat and cucumber (Dutta et al., 2009; Mohanty et al., 2006; Tewari & Tripathy, 1998) and can also increase its degradation (Guo et al., 2006; Karim et al., 1999). Recent studies also reveal that heat stress reduces the stability of pigment-protein complexes, contributing to photoinhibition and reduced photosynthetic performance in legumes, including common bean (Jan et al., 2023). Furthermore, a reduction of Rubisco binding protein (RBP) was observed (Sumesh et al., 2008). Recent advancements in transcriptomic studies have revealed that heat stress in legumes, including common bean, induces upregulation of genes encoding heat-shock proteins and antioxidant enzymes, which act to stabilize PSII and mitigate chlorophyll degradation (Soltani et al., 2019). However, additional research is needed to determine how these molecular responses interact with hormonal signaling pathways and their subsequent effects on yield under prolonged heat stress. This gap represents an important opportunity for future studies to link physiological and molecular mechanisms of thermotolerance. Moreover, a reduction in Fv/Fm ratio, stomatal conductance (gs) and net photosynthesis (Pn) was observed in common bean (Traub et al., 2018). These changes may severely impact the photosynthetic activity in plant resulting in a decrease in yield. Future studies could explore whether targeted manipulation of root hydraulic traits or hormonal pathways involved in water transport could improve thermotolerance, particularly in legumes like common bean, where root plasticity may play a pivotal role in maintaining water uptake under stress.

Heat stress decreases leaf water content, conductance of stomata and intercellular CO_2 concentration (Greer & Weedon, 2012) and reduces the water potential (L. W. Young et al., 2004) and enhances evapotranspiration (Gates, 1968). By reducing the number and size of the roots heat stress also affects the supply of water and nutrients (Huang et al., 2012; WAHID et al., 2007). High temperature also reduces the relative water content (RWC) and total water absorption rate (Ashraf & Hafeez, 2004), which results in reduction in final yield. Recent findings in common bean highlight that plants subjected to heat stress exhibited adaptive mechanisms, such as altered root-to-shoot ratios and increased root biomass allocation, which partially compensated for reduced water uptake capacity under high temperatures (Carmenza Muñoz et al., 2021). These insights suggest that a better understanding of root system plasticity could be key to enhancing heat stress resilience. Stefanov et al, 2011 using JIP-test showed that heat stress decreased the total performance index (PItotal), the J, I and P points and that the P maximum (FM) appears earlier in comparison with the control plants, showing that high temperatures influenced the chlorophyll a fluorescence in common bean (Stefanov et al., 2011). Furthermore, severe heat stress caused the decline in photosynthesis associated with cellular lesions, provoked by high temperatures in common bean (Hüve et al., 2011). To address these challenges, future studies could investigate whether the application of protective biostimulants or genetic enhancement of chlorophyll fluorescence traits might mitigate these declines in photosynthetic efficiency.

On the other hand, Soltani et al., (2019) showed that, under heat stress, common bean plants had higher stomatal conductance, which helps decrease leaf temperature and had a decrease in stomata density that were more opened in heat stress condition. Moreover, common bean plants grown at high temperatures showed a reduction in leaf mass and an increase in chlorophyll a, chlorophyll b and carotenoids content. These physiological changes, while potentially beneficial for short-term stress mitigation, highlight a critical gap in understanding the trade-offs between these mechanisms and their impact on seed filling and yield under prolonged heat exposure. Addressing this gap by providing quantitative data on hormonal changes and their interaction with these physiological responses will be extremely important. This increase in chlorophyl content may be due to the overexpression of nitrate reductase (niR and glutamine synthase N-1 (GS)), that are two key enzymes in nitrogen assimilation. Common bean plants grown under elevated temperatures, had a lower ratio of photosynthesis to respiration, that were compensated by a larger leaf area expansion compared with plants grown in control conditions. Also, it was observed an overexpression of heat shock protein 21 (HSP21), abscisic acid-deficient 4 (ABA4) and light harvesting complex photosystem II (LHCB4.3) which confer

PSSII protection under elevated temperatures. Therefore, since in common bean photosynthesis was not severely affected by heat stress, it was suggested that the decrease in seed yield was due to disruption of crucial source-sink relationships (Soltani et al., 2019). However, further research on the link between impact of heat stress in photosynthesis with the damage effects in seed yield is required. This aligns with observations in maize, where grain yield was reduced under heat stress despite unaffected photosynthetic activity due to impaired sink activity (Ordóñez et al., 2018). Future studies that investigate how heat stress impacts the hormonal regulation of seed filling in common bean are needed, being hormones a key determinant of sink strength.

2.2 Seed Germination

Seed germination can be severely affected by heat stress (Mitra & Bhatia, 2008). This may be due to high temperatures ability to induce abscisic acid synthesis and disturb the activity of enzymes that brake starch (Essemine et al., 2010). In chickpea, seeds germinated above 30°C had the seedling growth affected and above 40°C showed a lethal effect in seedlings (Kumari et al., 2018). In soybeans, temperature affects germination rates and seedling vigor, with 25°C promoting better total seedling length compared to 15°C and 35°C (Abd Ghani et al., 2023). Poor seed germination will result in poor seedling vigour, reduced root length, poor stand establishment, lower plant population, leading to reduced final yield (Akman, 2009; Piramila et al., 2012; Toh et al., 2008). Such findings underscore the need to explore the specific molecular pathways by which heat stress disrupts cellular and enzymatic processes during seed germination. Investigating how these mechanisms vary among genotypes could reveal critical insights for enhancing germination resilience. Recent studies confirm that the decline in seed germination under high temperatures is linked to increased reactive oxygen species (ROS) levels, which damage cellular structures and impair embryonic development (M. A. Farooq et al., 2021). This underscores the importance of antioxidant pathways during seed germination under stress, a mechanism further emphasized by recent research focusing on hormonal modulation of these responses. Future research should investigate whether exogenous treatments, such as phytohormones, could enhance seed viability and reduce ROS-induced damage during germination in legumes.

Moreover, studies regarding the impact on germination of seeds produced by plants that faced heat stress are necessary. Emerging evidence in legumes indicates that heat stress also alters hormonal pathways regulating seed dormancy and germination. For example, high temperatures decrease gibberellin biosynthesis while increasing ABA levels in seeds, disrupting the balance necessary for germination inhibition (Toh et al., 2008). Such hormonal imbalances can lead to a decoupling of dormancy release and germination vigor under stress conditions, presenting a critical area for further study. It would be worthwhile to explore how the interplay of GA and ABA influences germination timing and vigor, particularly under fluctuating temperature regimes. Investigating the genetic and epigenetic basis of these hormonal responses could provide new targets for improving seed germination under heat stress. By linking germination metrics, such as mean germination time (MGT) and seedling vigor index (SVI), to specific hormonal imbalances, will fill an important research gap in understanding the legacy effects of heat stress on germination and early seedling development. Further investigation into these transgenerational effects, particularly the role of epigenetic modifications, could reveal adaptive strategies to improve germination success in progeny from stressed parent plants. Research focusing on the role of seed development temperature on future germination performance would provide essential insights for breeding programs. Additionally, it would be important to examine whether pre-sowing treatments, such as seed priming or hormonal applications, could mitigate the adverse effects of parental heat stress on offspring germination. Insights on how seeds from heat-stressed plants exhibit faster germination but reduced seedling vigor suggest a trade-off between germination speed and subsequent growth potential. This highlights the need to optimize seed development conditions to balance these traits, an area that has been underexplored in current research.

3. Effect of Heat Stress in Seed Development

3.1 Seed development in *Phaseolus vulgaris*

Seed development is a key biological process that determines final seed traits such as size, nutrient content, desiccation tolerance, and viability. In crop species like *P. vulgaris*, these attributes are crucial for agronomic performance and human nutrition. Seed development is a complex, tightly regulated process, influenced by both genetic and environmental factors and generally divided into three main stages: embryogenesis, seed filling and maturation/desiccation (Coelho et al., 2008).

Embryogenesis represents the initial stage, where successive cell divisions and morphogenesis events give rise to the embryo, seed coat, and endosperm. In *P. vulgaris*, the embryo passes through the globular, heart, torpedo, and cotyledonary stages (Gallardo et al., 2003). Our previous studies demonstrated that at 10 DAA, seeds are at the late embryogenesis phase (Parreira et al., 2016). At this stage, morphological development is accompanied by intense metabolic activity, characterized by high rates of glycolysis, tricarboxylic acid cycle activity, DNA replication and repair, RNA metabolism and protein synthesis. This is reflected in the high accumulation of proteins associated with these functions, as seen in the proteomic profiles (Figure 2). In parallel, active protein degradation through the ubiquitin/26S proteasome pathway and post-translational modifications occur, suggesting intense protein turnover and regulation (Bostick et al., 2004; Smalle et al., 2004). The embryogenesis stage also involves oxidative stress responses (Kim et al., 2011). Importantly, genome integrity mechanisms, including the accumulation of PROLIFERATING CELL NUCLEAR ANTIGEN (PCNA), histone H2A, and DNA damage-inducible proteins, are activated during this phase to ensure faithful embryo development (Parreira et al., 2018).

Seed filling follows the cessation of intense cell division, marking a shift toward storage reserve accumulation. Between 10 and 20 DAA, *P. vulgaris* seeds undergo rapid biomass accumulation, as shown by the strong increase in proteins involved in major carbon metabolism and storage protein biosynthesis (Parreira et al., 2016). This stage is characterized by the accumulation of storage proteins such as phaseolin, legumin, phytohemagglutinin and alpha-amylase inhibitors, key for seedling nutrition and human dietary protein intake. Concurrently, enzymes related to starch biosynthesis (e.g., sucrose synthase) and regulators of carbohydrate metabolism (e.g., SnRK1 kinases) show high abundance, supporting intense carbon and energy metabolism required for reserve deposition (Braun et al., 2014; Coello et al., 2014). Transcriptomic analyses further revealed that miRNAs accumulate during early seed filling, regulating key transitions by targeting genes involved in development, storage compound allocation, and hormone pathways (Parreira et al., 2021). Together, these mechanisms ensure that cotyledons become the principal storage organs, in the absence of persistent endosperm tissue in *P. vulgaris*.

Seed maturation and desiccation initiate after 30 DAA, leading to seed dehydration and entrance into a quiescent state. This phase is characterized by a decline in cell expansion and biosynthetic activity, accompanied by the activation of protective mechanisms against dehydration stress (Parreira et al., 2016; 2018). Proteomic analyses revealed an increase in proteins related to redox homeostasis (Parreira et al., 2016). Moreover, a notable accumulation of proteasome subunits and proteases suggests that protein degradation processes used to facilitate the mobilization of storage reserves during germination (Tan-Wilson et al., 2012). Simultaneously, chromatin structure and genome stability are safeguarded, with sustained expression of histone variants and DNA damage response proteins (Parreira et al., 2018). Post-translational regulation continues to play a role, with ubiquitination and phosphorylation events modulating proteins crucial for survival during desiccation (Parreira et al., 2016).

In summary, seed development in *P. vulgaris* is a highly dynamic process, orchestrated by the temporal coordination of energy metabolism, biosynthesis, storage accumulation, oxidative stress responses and genome maintenance. The integrated results from Parreira et al. (2016, 2018, 2021) combined with proteomic and transcriptomic analyses, have provided critical insights into each developmental phase. However, despite these advances, important questions remained regarding the precise timing of seed filling initiation. Previous observations suggested that the seed filling program might activate earlier than 10 DAA, but a comprehensive characterization of this transition was still lacking. Furthermore, the impact of heat stress on the transition from embryogenesis to seed filling remains poorly understood, highlighting the need for further investigation.

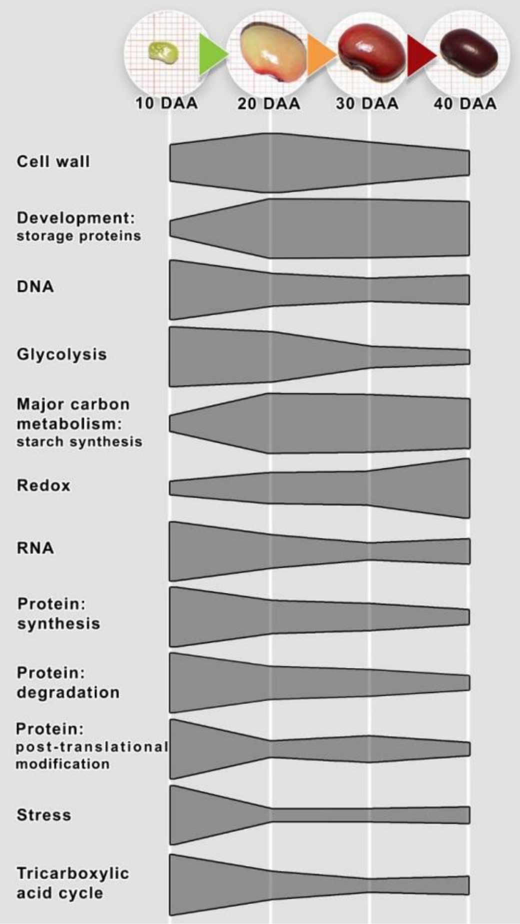

Figure 2- Schematic representation of the main changes in protein abundance profiles during *P. vulgaris* seed development observed by Parreira et al., (2016).

3.2 Heat stress in seed Filling

The seed yield is dependent on the synthesized assimilates from photosynthesis during seed filling and the remobilization of assimilates from vegetative organs to the developing seeds (Plaut et al., 2004; J. Yang & Zhang, 2006). During heat stress, mobilization of stem reserves plays a crucial role to seed (Blum et al., 1994; Rebetzke et al., 2008). Furthermore, starch and glucose synthesis are greatly affected. Under high temperatures, adenosine diphosphateglucose pyrophosphorylase, sucrose phosphate synthase and invertase enzymes activity is reduced (Chaitanya et al., 2001;

Djanaguiraman et al., 2009; Vu et al., 2001). More recently, it has been demonstrated that heat stress disrupts the expression of genes associated with carbohydrate metabolism, including those involved in sucrose cleavage and starch biosynthesis, such as SUSY and AGPase, leading to impaired seed filling in legumes (Ahmed et al., 2023). Understanding how specific enzymes involved in starch and sucrose metabolism respond to heat stress in legumes, particularly in relation to seed filling duration, could reveal key mechanisms to improve resilience. In chickpea (Kaushal et al., 2013) and mung bean (R. Kaur et al., 2015), heat stress inhibited sucrose metabolism in leaves and impaired sucrose supply to developing seeds, causing the seed to shrivel. Heat stress increases seed filling rate by reducing the duration of this stage (M. Farooq et al., 2017; Kaushal et al., 2016; Prasad et al., 2011). Moreover, a reduction of remobilization and translocation of photosynthesis assimilates to developing seed and a reduction in seed filling period in chickpea (Chakrabarti et al., 2013; Kaushal et al., 2013), lentil (Sita et al., 2017), pea, soybean and white lupin (Duthion & Pigeaire, 1991) was observed, resulting in smaller seeds. In cowpea the seed filling period was shortened from 21 days in control conditions to 14 days in heat stress conditions (Nielsen & Hall, 1985). In soybean it was showed that the smaller size during heat stress is also due to the decrease in cotyledon cell number and decrease in cell expansion (Munier-Jolain & Ney, 1998). Expanding such studies to include common bean could provide critical insights into how sucrose transporters and cell division regulators contribute to heat stress-induced reductions in seed size and weight.

In common bean, it was found that sucrose transporter 2 (SUT2) expression was downregulated under heat stress. This gene as a major role in loading sucrose from leaves into phloem and its translocation into sinks (Soltani et al., 2019). Soltani et al, 2019, also suggested that an overexpression of the sucrose 6 (SUS6), β- amylases 3 (BAM3) and β-amylases 5 (BAM5) was possible due to a shift in carbon flow from sucrose export to starch and maltose formation. However, gaps remain regarding how heat stress-mediated hormonal signals interact with carbon partitioning during seed filling, particularly in legumes, which rely on finely tuned source-sink relationships. Further research could elucidate how hormonal signaling pathways are integrated with carbohydrate metabolism during heat stress. It would be beneficial to explore the role of hormonal regulators, such as ABA and GA, in mediating these shifts in carbon allocation. Investigating whether targeted hormonal treatments during seed filling could mitigate these disruptions offers a promising area of future research. In heat stress conditions it was observed a higher number of pods with shriveled seeds and pods with embryos that failed to develop (Vargas et al., 2021). These observations underscore the need for further research into the genetic and physiological mechanisms regulating pod and seed development under heat stress. By combining physiological measurements with transcriptomics, it will be possible to identify candidate genes and pathways that mitigate these developmental defects.

Heat stress during the filling period also deteriorate protein quality. Likewise, heat stress denatured and aggregated globulins, legumin and vicilin (Mession et al., 2013) in pea. Similarly, in soybean, high temperatures adversely impacted globulin, phaseolin (Hernández-Unzón & Ortega-Delgado, 1988) and bconglycinin (Iwabuchi & Yamauchi, 1984). In lentils, albumins and globulins were also affected by heat stress (Sita et al., 2018). In quinoa, the protein and fiber content increased with higher temperatures, however fats and carbohydrates decreased with heat stress (Matías et al., 2021). Further research will address this by profiling heat-induced changes in storage protein composition, identifying potential biomarkers for heat tolerance. To address these challenges, studies could investigate the specific heat-induced changes in protein structure and function in common bean, particularly focusing on phaseolin stability and its role in maintaining seed nutritional quality.

Furthermore, in lentils, during heat stress iron and zink bioavailability was reduced due to increase in phytic acid (Choukri et al., 2022). Despite significant progress in understanding these biochemical impacts, questions remain regarding how heat stress impacts nutrient translocation to seeds in common bean. Further research could provide a novel perspective by integrating these biochemical changes with molecular and physiological traits, offering a comprehensive view of heat stress resilience. Exploring strategies to enhance nutrient partitioning to seeds under stress could mitigate these losses. The reduction of starch synthesis and translocation, the decline of synthesis and the denaturation of seed storage proteins and inhibition of fatty acid synthesis induced by heat stress during seed filling period, reduces the overall seed quality. This underscores the urgency of identifying key molecular targets and pathways that can be manipulated to mitigate these adverse effects. Furthermore, some additional research into the effect of heat stress in the translocation of storage compounds is required. Investigating the epigenetic and molecular mechanisms underlying these processes in common bean could provide a roadmap for breeding thermotolerant varieties with improved seed composition and quality.

4. Hormonal Regulation During Heat Stress

During seed filling period, phytohormone regulation and exchange of signals between the seed and the plant are essential for seed development (I. Ahmad et al., 2021; Locascio et al., 2014; J. Yang & Zhang, 2006). There are several phytohormones, such as Gibberellins, Abscisic acid, Auxin, Cytokinin and Ethylene that regulate seed filling processes (I. Ahmad et al., 2018; Önder et al., 2022). Phytohormones are increasingly recognized as central mediators of plant stress responses, enabling adaptive changes in source-sink relationships and nutrient allocation during heat stress (Fahad et al., 2017).

4.1 Gibberellins

It was already shown that Gibberellins (GA) play a crucial role in early seed development, facilitates growth and enhance seed sink strength in developing seeds (Iqbal et al., 2011; H. Kaur et al., 2021). In developing pea seeds, it was shown that a GA-overexpressor line TG1 that constitutively expresses PsGA3ox1 (converts GA20 to GA1) presented an enhanced parenchymal cell expansion compared to the control and consequently an enhanced starch mobilization and accumulation, occurring earlier in the transgenic line at 16 DAA compared with the control that only started at 20 DAA. It was also observed that a GA deficient mutant (lh2) showed reduced parenchyma cell expansion, seed growth and starch accumulation (Waduthanthri, 2016).

Stress tolerance is promoted by suppressing the growth of vegetative tissues (Abdulaziz S. Alhaithloul et al., 2021). One mechanism to cope with heat stress is to reduce bioactive GA and consequently accumulate DELLA proteins. (H. Kaur et al., 2021) saw that heat stress increased the transcript abundance of GA deactivation gene PsGA2ox1, in pea seeds. Likewise, in rice seeds, heat stress decreased the expression of GA biosynthesis genes (GA2ox2 and GA3ox2) and increased the expression of GA catabolic enzymes genes (GA2OX7 and GA2ox8) (Begcy et al., 2018). Also, in Arabidopsis, GA20ox1, GA20ox2, GA20ox3, GA3ox1 and GA3ox2 genes are suppressed in heat stress (Toh et al., 2008). Nagar et al, 2021, observed a decrease expression in GA biosynthesis genes (CPS, EKS, KO, KAO, GA20ox, GA3ox and GA2ox) under heat stress (Nagar et al., 2021). In the presence of GA during heat stress, the aleurone layer of the endoplasmic reticulum is heat shocked and suppresses secreted proteins. In the absence of GA, the saturation of the aleurone membrane increases and sustain its function during heat stress (Grindstaff et al., 1996). During heat stress, the levels of GA, Auxins and CK were reduced, which led to reduced spikelet fertility, reduced grain size and grain weight in rice (C. Wu et al., 2016). On the other hand, heat-resistant variety had stable levels of this hormones (C.

Wu et al., 2016). A decrease in gibberellins during heat stress, results in decrease of sink strength and affects seed yield. Research is needed to determine whether GA-mediated sink strength is predominantly regulated through biosynthesis, transport, or catabolism during prolonged heat exposure.

4.2 Abscisic acid

Abscisic acid (ABA) promotes abscission/abortion of abnormally developing reproductive structures, stimulates nutrient storage reserves in embryos, induces seed dormancy, desiccation tolerance and prevents precocious germination (Finkelstein et al., 2002; Radchuk et al., 2010). A higher seed filling rate was associated with a high ABA concentration (Qin et al., 2013). ABA accelerates sucrose transport to the cotyledons during seed filling in soybean (B. Liu et al., 2010) and inhibits cell cycle, accelerating seed maturation (Sreenivasulu et al., 2010). Under heat stress, ABA's role in modulating carbon remobilization becomes critical, as evidenced in several legumes. However, the dynamic interplay between ABA and its metabolites, such as phaseic acid, in regulating source-to-sink transitions remains underexplored in common bean. In pea seeds, there are two peaks of ABA accumulation, the first one is when the seed reaches maximum endosperm volume to promote seed storage reserves and inhibit vivipary and the second occurs during seed maturation to promote desiccation tolerance, dormancy and germination inhibition (Finkelstein et al., 2002; T. L. Wang et al., 1987). Under normal conditions, ABA is rapidly catabolized in developing seeds through ABA C-8’ hydroxylation pathway, being crucial its transportation from the maternal tissues during early embryo development to avoid seed abortion and promote growth (Frey et al., 2004). Reduction of ABA levels during seed development in pea led to smaller seeds, with fewer globulin storage proteins and lower dry matter accumulation compared with the wild type (Radchuk et al., 2010).

Under heat stress, seed levels of ABA catabolites phaseic acid (PA) and dihydrophaseic acid (DPA) were reduced, suggesting that under high temperature stress the ABA transport is triggered from the pericarp tissues to the developing seed increasing ABA seed levels (Frey et al., 2004) to promote seed sink strength to facilitate seed growth and development. ABA can induce nitric oxide, an antioxidant that is able to reduce the reactive oxygen species (ROS) generated under heat stress (Song et al., 2008). Exogenous application of ABA induced the activity of antioxidative enzymes (superoxide dismutase, catalase, ascorbate peroxidase and guaiacol peroxidase) and decreased lipid peroxidation induced by heat stress (Dong et al., 2011; Gong et al., 1998). ABA signalling mutants (abi1 and abi2) and ABA biosynthesis mutants (aba1, aba2 and aba3) showed defects in acquired thermotolerance (Larkindale & Huang, 2005). It was further observed that ABA treatment improved tobacco suspension survival and mitigate cell viability reduction induced by heat stress (Z. G. Li & Jin, 2016). Heat stress provokes pollen sterility, which can be improved by ABA treatment, which enhances the content of soluble sugars, starch and nonstructural carbohydrates. Furthermore, ABA treatment increased the expression levels of heat shock proteins (HSP24.1 and HSP71.1), genes related to sugar metabolism and transport, such as sucrose transporters (SUT) genes, sucrose synthase (SUS) genes and invertase (INV) genes. Moreover, increased antioxidant activities and increased content of adenosine triphosphate (Rezaul et al., 2019). Studies in *Phaseolus vulgaris* have highlighted gaps in our understanding of ABA's interaction with stress-induced ROS accumulation. Further exploration of tissue-specific ABA signaling during seed filling in response to temperature gradients could provide insights into its precise contributions to thermotolerance.

4.3 Auxins

Auxins play a key role in regulating seed development, initiating endosperm development, stimulating seed coat development and regulating early embryo patterning (Figueiredo & Köhler, 2018; Robert et al., 2018; Smit & Weijers, 2015). Auxins also increase GA biosynthesis to facilitating seed development and fruit set (Dorcey et al., 2009; Ozga et al., 2009; Tiwari et al., 2012) and increase cytokinin content to regulate endosperm cell division increasing the seed filling rate (S. Ahmad et al., 2020). In common bean, knowledge about how auxin biosynthesis pathways respond to heat stress and whether they sustain early embryogenesis remains limited.

Auxin levels are reduced by heat stress, which can lead to abortion of pollen development (Higashitani, 2013; Ozga et al., 2006). Treatment with auxin reduced lipid peroxidation, damage to membrane integrity and maintained the levels of antioxidant enzymes activity (superoxidase dismutase, catalase and peroxidase) close to the control condition level, in pea (Sergiev et al., 2018). Heat stress decreased the auxin levels by reducing the auxin biosynthesis genes: YUC2, YUC6, SQUAMOSA promo-tor- binding protein- like (SPL), NGATHA (NGA), TERMINAL FLOWER (TFL) (J. Chen et al., 2006; Sakata et al., 2010; L. Sharma et al., 2018). Also, the auxins signalling and transport genes: TRANSPORT INHIBITOR1 (TIR1)/AFB2-AUX/IAA, AUXIN RESPONSE FACTOR (ARF) target gene HALF FILLED (HAF), ARF2, ARF3, ARF11, ARF17, ARF23, PIN-FORMED (PIN1b, PIN5a and PIN10a) were downregulated under heat stress decreasing the auxin levels (L. Sharma et al., 2018). Treatments with auxins during heat stress induced anther cell proliferation and reverse male sterility (Abbas et al., 2018). Further research is required to dissect whether this tissue-specific accumulation could explain reduced embryo abortion in heat-tolerant genotypes. However, in heat stress auxins are accumulated in seed tissues (H. Kaur et al., 2021). In common bean, cultivars less sensitive to heat stress exported more auxins to flowers and young pods (Ofir et al., 1993). H. Kaur et al., (2021) showed that the increase in auxin biosynthesis and transport act to maintain critical processes for maintain seed development and prevent seed abscission.

4.4 Ethylene

An accumulation of ethylene is linked to a decrease of grain yield by accelerating senescence (Hays et al., 2007; Huberman et al., 2013; Savada et al., 2017). Under heat stress, ethylene can promote senescence and resource remobilization while also triggering processes that lead to seed abortion and reduced yield. Despite its negative effects, controlled ethylene signaling in seeds has been proposed as a potential pathway for improving stress resilience.

Ethylene is an important heat stress regulator. Heat stress increased the expression of ethylene biosynthesis gene PsACS2, PsACO3 and the ethylene-signalling gene PsEBF2, in developing pea seeds and vascular tissues of the ovary and an increase in ACC (a mobile precursor to ethylene), facilitating senescence in these tissues (H. Kaur et al., 2021). Conversely, it was observed a reduction in transcript abundance of PsACO2, PsACO3 and PsEBF2 in pericarp tissues with developing seeds during heat stress, suppressing the ethylene evolution, suggesting a mechanism to optimize the resource allocation, during heat stress, to fruits with developing seeds (H. Kaur et al., 2021; Savada et al., 2017). It was shown that ethylene reduces oxidative stress and maintains chlorophyl content (Y. S. Wu & Yang, 2019). The ethylene-insensitive mutant etr-1 showed increased susceptibility to heat stress (Larkindale & Knight, 2002). The ethylene mutant ein2-1 conferred heat stress tolerance, decreasing the electrolyte leakage (Clarke et al., 2009). Furthermore, rice seedlings treated with ACC, an ethylene precursor, improved stress tolerance, with a higher chlorophyll content, increased seedling fresh weight and lower cell damage (Y. S. Wu & Yang, 2019). In pea, Ethylene biosynthesis genes were also upregulated in the ovaries, however in the stigma and petals ethylene was inhibited (Savada et al., 2017), suggesting that during heat stress the ethylene biosynthesis is specific for each tissue. In common bean, heat stress induced a rise in the production of ethylene (Sauter et al., 1990). However, further research should investigate whether modulating ethylene precursors directly impacts seed viability and sink strength in heat-tolerant genotypes.

4.5 Cytokinins

Cytokinins play a key role in seed filling by inducing rapid cell division of endosperm cells (Kong et al., 2015), improving seed filling rate by enhancing sugar import from phloem to endospermic cells (I. Ahmad et al., 2019; Jameson & Song, 2016; Rijavec et al., 2009; J. Yang et al., 2000) and also increases fertilization, seed set and endosperm growth (Morris et al., 1993). Heat stress reduced not only the number of kernels but also the number of endosperm cells (D. Yang et al., 2016). Furthermore, a reduction of cytokinin in heat stress, was due to a reduced transport of cytokinin transport from root to shoot. Besides, an increase activity of cytokinin oxidase enzyme, a degrading cytokinin enzyme, was observed (C. Wu et al., 2017). Moreover, treatment with benzyl adenine (a cytokinin), rescued kernel abortion (Cheikh & Jones, 1994) and cell division was induced leading to high grain filling during heat stress (D. Yang et al., 2016). In legumes, recent advances suggest that external cytokinin applications could be used to mitigate stress-induced seed abortion by enhancing sugar flow to reproductive structures. However, these approaches require optimization to address species-specific differences in hormone transport and response. In *Phaseolus vulgaris*, there is a significant research gap in understanding how endogenous cytokinins mediate stress responses during seed filling. Future research could elucidate cytokinin-regulated pathways that maintain source-sink balance under heat stress, focusing on identifying genes involved in cytokinin biosynthesis, transport and degradation that are crucial for seed development. This could provide new targets for enhancing thermotolerance in common bean.

4.6 Salicylic acid

Salicylic acid (SA) enhances chlorophyll and carotenoids level, improves plant growth, flower induction, ion uptake, thermogenesis (Hayat et al., 2009; Pan et al., 2006; Saleh et al., 2007). SA promotes photosynthesis, proline metabolism and nitrogen assimilation under heat stress (Haydari et al., 2019; Khan et al., 2015; N. Li et al., 2021). Under heat stress, SA is induced and accumulated endogenously in pea (Kaushal et al., 2016; Pan et al., 2006) increasing thermotolerance, maintaining membrane integrity and protecting the Photosystem II complex (Q. Bin Wang et al., 2010; X. Wang et al., 2014; Zandalinas et al., 2018). The dual role of SA, as both a signaling molecule and a direct enhancer of thermotolerance, highlights its potential as a key target in improving crop resilience. It was also showed that SA can reduce ROS accumulation in anthers increasing pollen viability (Feng et al., 2018). A transgenic line that did not permit SA accumulation (NaHG transgenic plants) was sensitive to heat stress. Furthermore, a SA-accumulating mutant (cpr5) showed high thermotolerance (Clarke et al., 2004, 2009). Exogenous application of SA decreased lipid peroxidation induced by heat stress (Larkindale & Knight, 2002), enhancing thermotolerance (Abd-Elkader et al., 2016; Galani et al., 2016; Lin et al., 2019; Senaratna et al., 2000; Shah Jahan et al., 2019; Shi et al., 2006). SA application alleviated the decrease in net photosynthesis rate induced by heat stress (L. J. Wang et al., 2010). Furthermore, common bean seeds treated with SA enhanced heat stress tolerance (Senaratna et al., 2000). This highlights the translational potential of SA applications for improving thermotolerance in legumes like common bean. Further studies focusing on mapping SA biosynthesis and signaling pathways in heat-stressed seeds, with an emphasis on identifying temporal and tissue-specific variations in SA accumulation should be considered. Moreover, further work is required to optimize dosage and timing to achieve maximum benefits without adverse effects.

4.7 Jasmonic acid

Jasmonic acid (JAs) regulates plant growth under both abiotic and biotic stresses (Ding et al., 2022; H. Li et al., 2021; Siddiqi & Husen, 2019), accumulating amino acids and soluble sugars, activating the antioxidant system, regulating stomatal opening and closing, regulating the expression of JA-associated genes (JAZ, AOS1, AOC, LOX2 and COI1) (X. Wang et al., 2020). JA dependent gene expression has a key role in plant acclimation to heat stress (Balfagón et al., 2019). JA was accumulated upon heat stress and JA signalling mutants coi1-1, opr3 and jar1-1cpr5-1 were sensitive to heat stress (Clarke et al., 2009). Thus, establishing that JA provide basal thermotolerance (M. Sharma & Laxmi, 2016). Chen et al, (2006) showed that JA treatment could increase the activity of superoxide dismutase, catalase, peroxidase in grape seedlings under heat stress, while also preserving cell viability under these conditions (Chen., 2006). Additionally, exogenous JA applications have been reported to improve heat stress re-silience in several crops. JA-treated plants exhibit improved photosynthetic rates, enhanced antioxidant enzyme activity and reduced lipid peroxidation (Chen et al. 2006; Clarke et al. 2009). Future studies should focus on determining the optimal timing and concentration of JA treatments to maximize heat stress tolerance, particularly in legumes, where information on JA-mediated regulation of seed filling is limited. This research should also investigate potential crosstalk between JA and other phytohormones, such as abscisic acid and ethylene, during heat stress. The interplay of these hormones may provide insights into complex regulatory networks driving heat resilience in seeds.

4.8 Brassinosteroids

Brassinosteroids (BR) regulate seed filling, stimulating the flow of assimilate from source to sink and increasing assimilation of glucose to starch in the seed (C. Y. Wu et al., 2008). BR stimulates cell elongation, improving growth and biomass of French beans under heat stress (Upreti & Murti, 2004). BR deficient mutants produced smaller seeds in faba bean (Fukuta et al.,

2006) and in pea (Nomura et al., 2007). At the same time, overexpression of BR increased rice seed filling and yield (C. Y. Wu et al., 2008). Treatment with BR rescued seed size in BR deficient mutant det2 (W. B. Jiang et al., 2013). BR regulate seed size by expanding cell cavity and endosperm volume, increasing embryo cell size and number (W. B. Jiang & Lin, 2013). BR confers heat tolerance by inducing the expression of ABA, ET, SA and JA in Arabidopsis (Divi et al., 2010). BR treatment in common bean increased vegetative growth, total yield, total phenolic acids in pods (El-Bassiony et al., 2012). Despite these advances, there is limited research on BR-mediated regulation of seed filling and thermotolerance in legumes. Nevertheless, further investigation on the individual role of each phytohormone on seed filling and thermotolerance in legumes is required to confirm their vital relevance. Moreover, while exogenous BR applications have shown promising results in enhancing heat tolerance, the underlying mechanisms remain unclear. Future studies should aim to elucidate how BR interacts with other phytohormones to coordinate seed filling and stress responses. Additionally, optimizing BR application strategies, including timing and dosage, could provide new tools to mitigate yield losses in legumes under heat stress.

Table 1- Hormonal pathways and their effects under heat stress in common bean (Phaseolus vulgaris L.).

Hormone	Role of hormone on Seed Filling	Effect of Heat Stress	References
Gibberellins	Enhances starch mobilization, parenchymal cell expansion, and seed sink strength.	Reduces bioactive GA levels; suppresses biosynthesis genes (GA20ox, GA3ox); increases deactivation genes (GA2ox); reduces seed size and sink strength.	Kaur et al. (2021); Begcy et al. (2018);

Abscisic Acid	Promotes nutrient remobilization, sucrose transport, and seed maturation.	ABA accumulates under HS, enhancing sink strength and reducing ROS; ABA catabolites (phaseic acid, dihydrophaseic acid) decrease, indicating increased ABA transport to seeds.	Frey et al. (2004); Song et al. (2008); Dong et al. (2011); Rezaul et al. (2019)
Auxins	Regulates endosperm and seed coat development; enhances cytokinin and GA levels to increase seed filling.	HS reduces auxin biosynthesis genes (YUC2, YUC6) and transport genes (PIN1b), leading to pollen sterility and embryo abortion. Auxin accumulation in seeds prevents abscission in heat-tolerant genotypes.	Sergiev et al. (2018); Kaur et al. (2021); Abbas et al. (2018); Ofir et al. (1993)
Ethylene	Facilitates resource remobilization to seeds under controlled levels.	HS increases ethylene synthesis in seeds (PsACS2, PsACO3), but suppresses it in pericarp tissues. Excessive ethylene causes seed abortion; controlled levels reduce oxidative stress and maintain chlorophyll content.	Savada et al. (2017); Hays et al. (2007); Wu & Yang (2019); Clarke et al. (2009)
Cytokinins	Induces cell division in the endosperm and enhances sugar import to seeds.	HS reduces CK levels and transport from roots, increases cytokinin oxidase activity, leading to reduced seed filling, grain weight, and seed abortion.	Cheikh & Jones (1994); Wu et al. (2017); Yang et al. (2016)
Salicylic Acid	Enhances starch synthesis, ROS reduction, and photosynthesis to protect seed viability.	SA accumulates under HS, protecting Photosystem II, increasing antioxidant enzyme activity, and improving seedling survival and yield. Exogenous SA improves heat tolerance in beans and other crops.	Clarke et al. (2004, 2009); Kaushal et al. (2016); Senaratna et al. (2000);
Jasmonic Acid	Activates antioxidant systems, reduces lipid peroxidation, and enhances seed filling.	HS increases JA accumulation; JA treatments improve photosynthesis, antioxidant activity, and cell viability. JA signaling mutants are more sensitive to HS.	Balfagón et al. (2019); Clarke et al. (2009); Chen et al. (2006); Sharma & Laxmi (2016)
Brassinosteroids	Stimulates sugar transport, increases endosperm volume, and regulates seed filling.	BR enhances HSP synthesis, improves antioxidant enzyme activity, and reduces cell damage. BR treatments improve seed size, yield, and thermotolerance.	Wu et al. (2008); Alam et al. (2018);

5. Molecular Regulation of Heat Tolerance

Heat stress induces oxidative stress, causing cellular injury by generating Reactive oxygen species (ROS), like singlet oxygen, superoxide radical, hydrogen peroxide and hydroxyl radical (Asada, 2006; X. Liu & Huang, 2000). Heat stress increases antioxidative activity (APX, CAT, GST, SOD, GR and POX) to reduce oxidative damage (Almeselmani et al., 2009; Balla et al., 2009). The oxidative stress causes peroxidation of membrane lipids and pigments (S. Xu et al., 2006). On the other hand, antioxidants activity also increases under heat stress, promoting plants defence against heat stress damages by scavenge injuring induced by ROS (Nagesh Babu & Devaraj, 2008).

Furthermore, in response to high temperature, plants synthesize Heat shock proteins (HSPs) that prevents denaturation and assist refolding of damaged proteins (Boston et al., 1996), protein transportation, cell cycle control and signalling (F. Wang et al., 2014) and protects the Photosystem II (Neta-Sharir et al., 2005). HSPs along with Heat shock factors (HSFs) are key players in heat stress tolerance in plants (Nadeem et al., 2018). HSPs are mainly localized in chloroplasts, mitochondria, ribosomes, endoplasmic reticulum, cytoplasm and nucleous (Nieto-Sotelo et al., 2002; Ul Haq et al., 2019; K. A. Yang et al., 2006). HSPs are divided in five families, based on their molecular weight: HSP20, HSP60, HSP70, HSP90 and HSP100 (Gupta et al., 2010; Swindell et al., 2007). HSP20 maintains membrane integrity during heat stress (Nakamoto & Vígh, 2007). HSP60 and HSP70 main role is in protein translation, proteolysis, translocation, folding and preventing protein aggregates (DeRocher & Vierling, 1995; D. Mishra et al., 2018; Nagaraju et al., 2021). HSP90 protects plant cells from heat stress effects (W. Li et al., 2013). In maize, HSP101 increased mostly in tassel, embryo, ear, silk and endosperm (T. E. Young et al., 2001). HSFs are divided in HSFA, HSFB and HSFC. HSFAs are essential for transcriptional activation (Scharf et al., 2012). Overexpression of HSFA1 increases heat tolerance (S. K. Mishra et al., 2002). HSFA1 is a transcriptional activator that triggers DREB2A, HSFA2, HSFA7, HSFBs, MBF1C and induced HSP70 and HSP90 increasing thermotolerance (Hahn et al., 2011; X. dong Li et al., 2017). This is a promising area for future research, particularly in characterizing HSPs' role in the reproductive stages. Despite these advances, a significant research gap remains in understanding the role of small HSPs and their specific functions during seed filling in legumes. Future research should explore how HSP dynamics correlate with reproductive resilience in *Phaseolus vulgaris*.

Other stress proteins synthesized in response to heat stress are ubiquitin, as it was observed in soybean (Ortiz & Cardemil, 2001; Sun & Callis, 1997). Also, Cytosolyc Cu/Zn-SOD increases under heat stress, to maintain chloroplast stability (Khanna-Chopra & Sabarinath, 2004). Furthermore, Late embryogenesis abundant (LEA) increased under heat stress in wheat during late grain filling stage (Majoul et al., 2003) and prevent aggregation and protect citrate synthase from desiccation (Goyal et al., 2005). Moreover, dehydrin proteins also increased in heat stress in sugarcane (Wahid & Close, 2007), protecting dehydration and oxidative stress. Also, Mn-POD plays a key role in decreasing oxidative damages (Brown et al., 1993; Iba, 2002). However, more research should be conducted to understand the genetic basis of legumes thermotolerance. Future studies should integrate transcriptomic and proteomic approaches to unravel the specific roles of these proteins in seed filling under heat stress, identifying candidate genes for engineering thermotolerant common bean varieties.

6. Heat Stress Tolerance Strategies

In traditional breeding one of the main challenges to select lines with heat tolerance and greater yield potential, is ensure that they are subjected not only to a high day temperature, but also elevated night temperatures (Mason & Singh, 2014). Another challenge is to make sure that other stresses, like abiotic stresses are not being imposed, making the selection more difficult (Jha et al., 2014). There are several criteria for heat tolerance screening, such as fruit set, pollen germination, pollen tube growth and seed viability (Berry & Uddin, 1988). Additionally, modern breeding programs increasingly integrate high-throughput phenotyping to evaluate heat stress responses under controlled and field conditions. These approaches focus on traits like canopy temperature, staygreen characteristics and photosynthetic efficiency (Bita & Gerats, 2013; Cossani & Reynolds, 2012). Also, assimilate translocation, mesophyll resistance and cellular membrane disorganization are traits to select lines with heat tolerance (H.-H. Chen et al., 1982). Transcriptomic analyses have revealed candidate genes associated with these traits, providing markers for marker-assisted selection (Kudapa et al., 2023). Various genotypes have been develop using breeding techniques in chickpea (Devasirvatham et al., 2013; P. M. Gaur et al., 2013; Krishnamurthy et al., 2011; Yadav et al., 2007), common bean (Porch, 2006), cowpea (Hall, 1990, 2000, 2010; Hall et al., 1993; Lucas et al., 2013; P. N. Patel & Hall, 1988), groundnut (Chauhan & Senboku, 1997), lentil (R. K. Gaur & Sharma, 2014; Kimurto et al., 2014), pea (Srikanthbabu et al., 2002) and faba bean (Siddiqui et al., 2015). However, significant gaps remain in breeding heat-tolerant varieties for legumes, particularly in developing high-yielding genotypes that also retain nutritional quality under heat stress conditions. Selecting lines with heat tolerance, sometimes are associated with undesirable characteristics, for example in tomato heat tolerant genotypes have small fruit and foliar canopy (Scott et al., 1997).

To address these trade-offs, researchers are exploring genome-editing techniques like CRISPR-Cas9 to precisely modify genes involved in heat stress response, allowing for targeted improvement without adverse traits (Zafar et al., 2020). One of the approaches, of some genetics techniques is to enhance the expression of HSPs (A. Singh & Grover, 2008). Other uses of genetic engineering is improving thylakoid membrane stability, chlorophyl content, stomatal conductance, photosynthetic rate, grain filling duration, fruit setting and grain yield (Nagarajan et al., 2010; Reynolds et al., 1994; J. Yang et al., 2002). This can be achieved with the use of molecular markers, such as randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), simple sequence repeats (SSR), restriction fragment length polymorphism (RFLP) and single nucleotide polymorphism (SNP) that can be used to characterize genotypes with heat stress tolerance (Lavania et al., 2015; Pottorff et al., 2014). Also, genome wide association studies (GWAS), microarray and suppression subtractive hybridization (SSH) are important for discovering novel genes related to heat stress (B. Singh et al., 2019).

Another method to achieve thermotolerance, is foliar application or seed treatment with osmoprotectants, inorganic salts and hormones. Also, exogenous application of Ca^{2+} in the CaCl2 stimulated the antioxidant activity inducing heat tolerance (Y. Jiang & Huang, 2001; Kleinhenz & Palta, 2002; Kolupaev et al., 2005). Furthermore, treatments with polyamines, such as spermidine and putrescine, have been shown to mitigate the adverse effects of heat stress by stabilizing membrane integrity and enhancing antioxidant activity (M. A. Farooq et al., 2021). Furthermore, application of Sodium nitroprusside (Suryavanshi et al., 2016) and Potassium nitrate (Chaurasiya et al., 2018) increased grain yield. Moreover, integrating biostimulants with traditional breeding programs may further enhance the physiological resilience of legumes to heat stress (Yamauchi & Yamauchi, 2018). However, further research is needed in legumes regarding seed priming and foliar application on phytohormones, together with genetic engineering approaches, in order to improve seed yield under heat stress conditions. A comprehensive approach combining omics technologies, genome editing and precision agriculture could pave the way for developing climate-resilient common bean varieties.

Table 2- Comprehensive Strategies for Enhancing Heat Stress Tolerance in Common Bean (Phaseolus vulgaris): This table presents an integrative summary of approaches to improve heat tolerance, including traditional breeding, modern genomic techniques, marker-assisted selection, genome editing, and physiological treatments.

Strategy	Description	References
Traditional Breeding	Selection criteria include fruit set, pollen germination, seed viability, and high day and night temperatures. Focuses on traits like canopy temperature, photosynthetic efficiency, and stay-green.	Mason (2014); Jha (2014); Berry & Uddin (1988); Cossani & Reynolds (2012); Bita & Gerats (2013)
Modern Breeding Techniques	Integrates high-throughput phenotyping, transcriptomic analysis, and marker-assisted selection to identify heat-tolerant genotypes in legumes such as chickpea, common bean, and cowpea.	Devasirvatham et al. (2013); Porch (2006); Hall (1990, 1993, 2000, 2010); Kudapa (2023)
Genome Editing	CRISPR-Cas9 used to modify heat-stress response genes, enhance HSP expression, and improve traits like photosynthetic rate, grain filling, and fruit set.	Zafar et al. (2020); Singh & Grover (2008); Nagarajan et al. (2010); Reynolds et al. (1994)
Molecular Markers	Techniques like RAPD, AFLP, SSR, RFLP, and SNP for characterizing heat tolerance; GWAS and microarrays for identifying novel genes.	Lavania et al. (2015); Pottorff et al. (2014); Singh et al. (2019)
Foliar Applications and Priming	Includes treatments with osmoprotectants, Ca^{2+} ($CaCl_2$), melatonin, polyamines (spermidine, putrescine), sodium nitroprusside, and potassium nitrate to enhance heat tolerance.	Jiang & Huang (2001); Farooq et al. (2021); Suryavanshi et al. (2016); Chaurasiya et al. (2018)

This chapter highlights the impact of heat stress in plant development, particularly in seed filling and the role of phytohormones in regulating this process. This role in thermotolerance has been extensively documented, showcasing their importance in enhancing antioxidant activity, maintaining source-sink relationships and protecting vital cellular processes during heat stress. However, further studies are necessary to fully elucidate the complex interactions between phytohormones and environmental stressors to improve common bean seed yield under heat stress conditions. Heat stress poses a significant threat to global food security by reducing pollen viability, seed filling and increasing seed abortion rates, leading to substantial yield losses in legumes. Although advances in molecular biology and genetic engineering have provided valuable insights, challenges remain in translating these findings into practical applications in breeding programs. The urgency for crop improvement, driven by the escalating effects of climate change, requires a multifaceted approach combining traditional breeding, molecular tools and biotechnological innovations. For instance, integrating genome-editing technologies like CRISPR-Cas9 to target key genes associated with thermotolerance and leveraging omics approaches such as transcriptomics, proteomics and metabolomics, can help identify novel targets for improving heat stress resilience. Moreover, addressing research gaps, such as understanding the specific role of phytohormones in regulating nutrient remobilization during heat stress, will be critical. The unique findings from ongoing studies, including those from common bean, highlight the potential of dissecting stress-response mechanisms in legumes to identify genetic markers for heat-tolerant traits. The integration of such findings into breeding programs will enhance the ability to develop high-yielding, climate-resilient legume varieties.

7. Scope of the Thesis

The use of multidisciplinary approaches, combining morphology, histology, phytohormone profiling and transcriptomics, allows researchers to deepen our understanding of key developmental processes in species of agronomic interest. The integration of high-throughput methodologies enables the identification of critical molecular players and regulatory networks with greater specificity and accuracy, providing a deeper overview of the complex biological mechanisms underlying plant development and stress responses. In the context of climate change, advancing knowledge on seed development and stress resilience in crops like *Phaseolus vulgaris* is crucial to ensure future food security.

Limited insights into the detailed timing of the transition from embryogenesis to seed filling, molecular mechanisms and stress responses regulating *P. vulgaris* were available. In particular, there was a lack of comprehensive descriptions regarding the initiation of seed filling, the impact of heat stress on seed development and quality and the potential transgenerational effects of heat stress. As such, several research questions remained unanswered, namely: i) how do measurable morphological and histological characteristics change during the transition from embryogenesis to seed filling occur and what mechanisms regulate it? ii) how does heat stress affect seed developmental transitions, storage compound accumulation and hormonal regulation? and iii) does parental heat stress influence seed and seedling traits in the next generation?

The broad goal of this PhD thesis was to dissect the developmental and molecular mechanisms underlying seed development in *P. vulgaris*, both under optimal and heat stress conditions, and to explore how parental heat stress affects the next generation. To address these gaps in knowledge, several specific objectives were defined:

1. To refine the developmental timeline of seed filling initiation in *P. vulgaris* and characterize the main molecular mechanisms involved. To address this objective, seeds were collected at multiple early stages (6, 10, 14, 18 and 20 DAA) and subjected to morphological, histological and transcriptomic analyses. This was crucial to pinpoint the precise timing of seed filling onset and the associated regulatory pathways.

2. To assess the impact of heat stress on seed developmental transitions, seed size, seed number, storage compound accumulation and hormonal regulation. This objective was addressed by evaluating the seeds of plants exposed to heat stress during reproduction and analyzing their

development using a multidisciplinary approach combining histochemistry, hormone quantification and transcriptomic profiling.

3. To investigate how parental heat stress affected seed germination and seedling vigor. Seeds produced under heat stress were evaluated for germination performance, seedling development and early growth parameters under optimal conditions to determine whether parental stress induces changes across generations.

To address these objectives, **Chapter II** focuses on the developmental timeline of seed filling, expanding the analysis to earlier time points (6, 10, 14, 18 and 20 DAA) using a multidisciplinary approach that includes morphological, histological and transcriptomic methods to further study the transition to seed filling and how it is transcriptionally regulated. **Chapter III** builds upon this developmental framework to assess how heat stress alters seed development at the same stages, combining morphology, histochemistry, hormone profiling and transcriptomic data to uncover the mechanisms by which elevated temperatures disrupt developmental timing, storage compound accumulation and hormonal balance. **Chapter IV** evaluates how heat stress during seed development affects germination speed, seedling vigor, leaf growth and seed size in the next generation, even growing under optimal conditions. Overall, this thesis provides new insights into the molecular and physiological plasticity of seed development in *P. vulgaris* and its sensitivity to heat stress.

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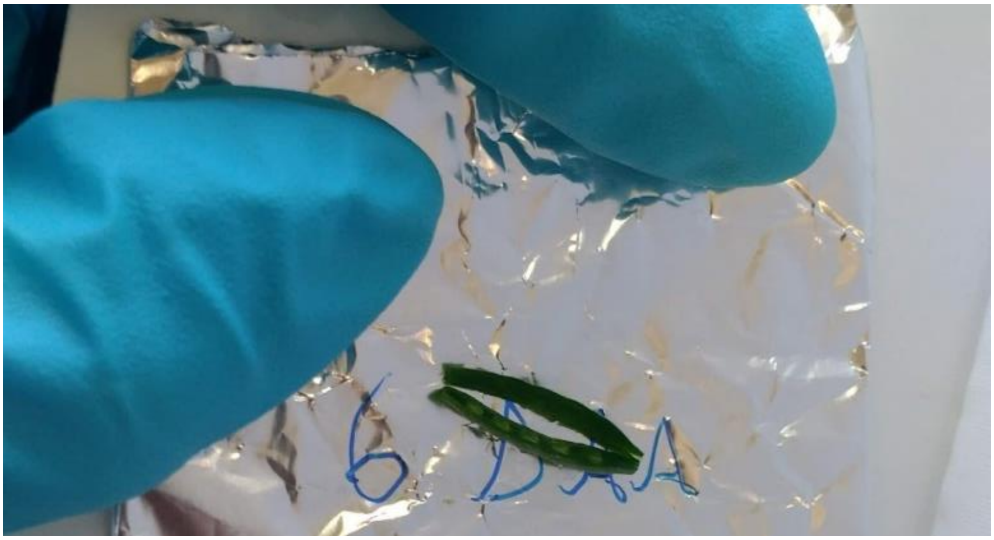
Chapter II

Understanding the transition from embryogenesis to seed filling in *Phaseolus vulgaris* L. non-endospermic seeds



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Cláudia Lopes participated in the experimental setup and design, performed the experiments and corresponding analysis, analysed the data and wrote the chapter.

Abstract

Common bean (*Phaseolus vulgaris* L.) is one of the most consumed grain legumes. These legumes are a major source of proteins and other important nutrients, especially in developing countries. Studying seed development in common bean is crucial for improving yield, nutrition, stress tolerance and disease resistance while promoting sustainable agriculture and food security, with its sequenced genome and available molecular tools making it an excellent research model. Despite advances in studying *P. vulgaris* seed development, the precise timing and molecular regulation of the transition from embryogenesis to seed filling remain poorly understood. Although *P. vulgaris* seeds at 10 days after anthesis (DAA) were previously characterized as being in the late embryogenesis stage, our previous studies suggested that this transition might occur earlier than 10 DAA, prompting us to investigate earlier developmental stages. To accomplish this goal, we conducted a comprehensive analysis at 6, 10, 14, 18 and 20 DAA, integrating morphological, histological, and transcriptomic approaches. Morphological and histochemical data revealed that by 10 DAA, cotyledons are fully formed, but storage compound accumulation is only noticed at 14 DAA, indicating that the transition from embryogenesis to seed filling occurs between 10 and 14 DAA. Transcriptomic analysis further supported this finding, showing upregulation of genes associated with seed storage proteins, starch metabolism, and hormonal regulation at 14 and 18 DAA. This study redefines the developmental timeline of *P. vulgaris* seed filling initiation, bridging a critical knowledge gap in legume seed biology. Given the limited availability of histological studies on early *P. vulgaris* seed development, our findings provide essential insights into the structural and molecular events driving this transition. By refining the timing and regulatory mechanisms of early seed development, this study lays the groundwork for future research aimed at enhancing seed quality and resilience in legumes.

Keywords: Early Seed Filling, *Phaseolus vulgaris* L., Seed histology, Storage compounds, Transcriptome

1. Introduction

The common bean (*Phaseolus vulgaris* L.) is one of the most consumed grain legumes worldwide. *P. vulgaris* represents an advantage in developing countries for their affordability and long storage life when compared to animal protein (De La Fuente et al., 2011; Castro-Guerrero et al., 2016). In Eastern and Southern Africa per capita consumption of common bean is around 40–50 kg per year and represents 50% of the grain legumes consumed worldwide (Ribeiro et al., 2012; Ghanbari et al., 2015). With the massive increase in human population, agriculture faces some challenges and it is estimated that a 30% increase in common bean yield is needed by 2050 (Porch et al., 2013). Seed yield is a multifaceted trait that represents the final outcome of an intricate growth and maturation process that is less known in non-endospermic seeds where endosperm is completely consumed during embryo development. Among the primary contributors to yield, seed dry weight plays a crucial role, thus modulating this trait can significantly influence overall seed yield (Li et al., 2014). *P. vulgaris* develops large and fleshy cotyledons to guarantee seed germination and plantlet development. Indeed, 90% of the total nutritive value of common bean seeds are in the cotyledons (Chávez-Mendoza et al., 2019). With the release of *P. vulgaris* genome sequence and other genetic tools (Collado et al., 2015; Schmutz et al., 2014), this species represents a suitable model for molecular studies aimed at improving and selecting desirable quality traits underlying the development of non-endospermic seed (Leitão et al., 2017; Mendes et al., 2022).

Our team has been devoting major efforts in characterizing the molecular and metabolic processes underlying *P. vulgaris* non-endospermic seed development and maturation. In our previous work, we provide a comprehensive description of proteome dynamics in *P. vulgaris* seed development (Parreira et al., 2016). Through a high-throughput gel-free proteomics approach (LC-MS/MS), we analysed seeds at 10, 20, 30 and 40 days after anthesis (DAA), defining the principal stages of *P. vulgaris* seed development and main metabolic pathways underlying these stages. At 10 DAA, seeds were identified as being at the late embryogenic stage, characterized by a high rate of cell division. By 20 DAA, seeds transitioned into the maturation/filling stage, marked by increased biomass accumulation driven by storage reserve synthesis. At 30 DAA, seed development reached the end of the filling stage, as biomass accumulation reached its peak, marking the onset of seed dehydration. Finally, by 40 DAA, seeds had undergone complete desiccation, finalizing their maturation process.

In a subsequent study, we provided new insights into DNA integrity maintenance during seed development, focusing on DNA damage repair and chromatin remodelling mechanisms that safeguard genome stability (Parreira et al., 2018). Using Massive Analysis of cDNA Ends (MACE) and digital PCR (dPCR), we conducted a qualitative analysis of gene expression dynamics across developmental stages, characterizing the molecular mechanisms governing this process. Our findings shed light on the critical role of DNA integrity maintenance in developing seeds, revealing how DNA repair pathways and chromatin remodelling contribute to genome stability throughout seed development.

In a follow-up study, we addressed a critical knowledge gap by elucidating the role of post-transcriptional regulation mediated by miRNAs in *P. vulgaris* seed development (Parreira et al., 2021). To achieve this, we employed a high-throughput non-coding transcriptomics approach, utilizing small RNA sequencing (sRNA-Seq) in combination with degradome analysis and target prediction algorithms to identify miRNAs and their respective targets. This analysis led to the identification of 72 known miRNAs from 25 miRNA families and 39 novel miRNAs. Our findings revealed that miRNAs highly accumulated during early developmental stages play roles in regulating the end of embryogenesis, delaying the onset of seed maturation, and modulating storage compound synthesis and allocation. Notably, most miRNAs were most abundant at 10 DAA. Other studies have demonstrated that seed filling in Arabidopsis is primarily regulated by the LAFL network, which consists of the key transcription factors LEC1, ABI3, FUS3, and LEC2 (Jo et al., 2019; Lepiniec et al., 2018; Boulard et al., 2017). In our previous work, we observed that at 10 DAA, PHABULOSA (PHB), which promotes the seed maturation program by directly activating LEC2 expression (Tang et al., 2012), was already present at relatively low levels, with only residual LEC2 expression detected (Parreira et al., 2018). This suggests that the seed filling program activation might have occurred earlier than 10 DAA, the earliest time point analysed by our previous LC-MS, MACE and sRNA-Seq.

This study addresses some key questions regarding the timing and molecular regulation of seed filling initiation in *P. vulgaris*. In view of our previous findings suggesting that the seed filling programme can begin before the 10th DAA, the main objective was to test this hypothesis by determining the timing of this developmental transition and the molecular mechanisms underlying it. Specifically, we aimed to answer the following questions:

-Does the transition to seed filling occur before 10 DAA?

-At 10 DAA, is the seed still in the embryogenesis stage, or does it already have well-formed cotyledons?

-What are the main molecular mechanisms and metabolic pathways regulating the transition from embryogenesis to seed filling?

To address these questions, we expanded our analysis to include earlier time points (6, 10, 14, 18 and 20 DAA) and employed a multidisciplinary approach that integrates morphological, histological and transcriptomic analyses. By refining the developmental timeline of seed filling initiation, this study provides a more detailed understanding of *P. vulgaris* non-endospermic seed development, contributing essential knowledge for crop improvement strategies.

2. Material and Methods

2.1. Plant material and growth conditions

In this study, the *Phaseolus vulgaris* genotype SER16, a red-seeded Mesoamerican variety provided by CIAT (International Center for Tropical Agriculture) was used. Recognized for its drought resistance and efficient remobilization of storage compounds to seeds, SER16 serves as an excellent model for research on seed development and stress adaptation (Polania et al., 2016). Additionally, it has been previously utilized in studies conducted by our laboratory (Parreira et al., 2016; 2018; 2021). Seeds were germinated onto water soaked paper in Petri dishes at 27 °C for 2 days, followed by 3 days at 23 °C, always in the dark as described in Parreira et al., (2016). Seedlings were individually transferred to 2.5 l pots containing a (2:1:1) mixture of commercial soil (Compo Sana S.A., Barcelona, Spain), peat and vermiculite, respectively. Seedlings were grown in growth chamber (Fitoclima 5.000 EH, ARALAB, Portugal) with controlled environmental conditions, with 50% humidity, photoperiod of 16/8-h day/night at 25/18 °C, respectively and light intensity of 400 μmol m−2 s−1. Eighty plants were kept in the environmental conditions described above during the full experiment and watered 3 times per week.

P. vulgaris SER16 flowers were tagged and pods/seeds were harvested at 6, 10, 14, 18 and 20 days after anthesis (DAA). Harvested seeds were divided into 3 batches: one immediately frozen in liquid nitrogen and stored at −80 °C for molecular (transcriptomic) analyses; another used to measure seed length, fresh and dry weight to characterize the seed development process, while the remaining batch was fixed for histochemical analyses.

2.2. Morphological characterization of the early steps of seed development

For the morphological analyses, 2 seeds coming from 15 individual plants were used in each of the studied time points (6, 10, 14, 18 and 20 DAA). Immediately after harvesting, seeds were photographed and seed length was measured, using ImageJ (Schneider et al., 2012). Then, those seeds were weighted to quantify the seed fresh weight. Later, after being submitted for 15 days to 70°C the seed dry weight was weighted.

2.3. Histology assays

For the histochemical analysis, 4 biological replicates per time point (6, 10, 14, 18 and 20 DAA) were used. Seeds were submerged in ice-cold FAA fixative solution [FAA: 47.5% ethanol from Carlo Erba (Val de Reuil, France), 3.7% formaldehyde solution from Sigma-Aldrich (St. Louis, MO, USA) and 5% glacial acetic acid from Scharlau (Barcelona, Spain)]. The immersed material was exposed to moderate vacuum for 1 hour to pull the air out of the tissue and force the infiltration of the fixative solution. The material was fixed overnight at 4 °C and then washed with TBS 1X (0.05M Tris-HCl from Carl Roth (Karlsruhe, Germany), 0.15M sodium chloride (NaCl) pH 7.6 from Merck (Darmstadt, Germany). Seeds were sectioned, by first securing the material in the vibratome's block with a drop of Super Glue and set for 15–20 min at room temperature. Transversal sections at the seed hilum level with 25 µm were cut using the vibratome 1000 Plus. Each section was placed in a microscope slide coated with 200µl of poly(lysine) and left to dry. Six sections per fixed seed were stained with Calcofluor White Staining, Coomassie Blue Staining or Periodic acid Schiff to stain cellulose in cell walls, proteins and carbohydrates, respectively (Pellicciari et al., 2017). Images from the sections were captured using a LEICA DM6 B microscope. Calcofluor White Staining images were captured using UV light, while for Coomassie Blue Staining and Periodic acid Schiff a bright-field lighting was used.

For each timepoint studied, Calcofluor slides were used to calculate cotyledon parenchyma cell area and cotyledon section area using ImageJ software (Schneider et al., 2012). To measure the cotyledon parenchyma cell area and cotyledon section area, after the scale is stablished, the cells and the cotyledons, were selected and calculated using ImageJ. Coomassie and Periodic acid Schiff images were converted to a grayscale (16 bit), inverted and the mean grey value for pixel intensity retrieved by the software was used to estimate overall protein and carbohydrate accumulation.

2.4. RNA Extraction, Quantification and Quality Assessment

For total mRNA isolation, frozen seeds were ground to a fine powder in liquid nitrogen using a mortar and pestle. RNA was extracted as described in (Parreira et al., 2018). Traces of DNA contamination were removed with an Ambion® TURBO™ DNase (Life Technologies, Carlsbad, CA, USA) following the manufacturer´s instructions. RNA quantification and purity were assessed using a NanoDrop™ 2000c Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). Moreover, the Qubit® 2.0 Fluorometer (Thermo Fisher Scientific Inc.) with RNA BR Assay Kit was used to quantify the RNA. RNA purity was estimated based on the A260/280 and A260/230 absorbance ratios and was approximately 2 before DNAse treatment. RNA integrity was assessed by electrophoresis in a 2.0% agarose gel, stained with SYBR® Safe (Life Technologies). The absence of DNA contamination was verified by a standard polymerase chain reaction (PCR) using primers for the *P. vulgaris* ACTIN gene, gene ID: Phvul.001G142500 (Supp table 2). RNA samples were stored at -80°C until needed.

2.5. RNA-Sequencing

Twelve RNA-Seq libraries were constructed from three biological replicates from seeds harvested at 6, 10, 14 and 18 DAA. Due to the reduced size of the sampled seeds at 6 DAA, each biological replicate consists of a pool of 200 seeds randomly harvested from 80 plants. For seed samples harvested at 10, 14, 18 and 20 DAA, each biological replicate consists of a pool of 3 to 4 seeds harvested from the same individual. Libraries construction and sequencing was performed by NOVOGENE (Cambridge, UK). Pair-ended cDNA libraries were sequenced on Illumina NovaSeq 6000 platform following manufacturer's recommendations and 150 bp paired-end reads were generated. mRNA was purified from total RNA using poly-T oligo attached magnetic beads and fragmented randomly in fragmentation buffer [NEBNext First Strand Synthesis Reaction Buffer (5X)], followed by cDNA synthesis using random hexamers and reverse transcriptase (RNase H-). Second-strand cDNA synthesis was subsequently performed using buffer (Illumina) with dNTPs, RNase H and *Escherichia coli* polymerase I to generate the second strand by nick-translation. The final cDNA library is ready after a round of purification, adenylation of 3′ ends of DNA fragments, A-tailing, ligation of sequencing adapters, size selection of cDNA fragments of preferentially 150 bp in length with ligated adaptor molecules on both ends were selectively enriched using Illumina PCR Primer Cocktail in a 10 cycle PCR. Library concentration was quantified using a Qubit 2.0 fluorometer (Life Technologies). Insert size was checked on an Agilent 2100 and quantified using qPCR.

2.6. RNA-Seq Bioinformatic Analysis

The original raw data from Illumina are transformed to Sequenced Reads by base calling. Raw data are recorded in a FASTQ file. Raw reads are filtered to remove reads with adapter contamination or reads with low quality. Only clean reads were used in the downstream analyses. The percentage of bases whose correct base recognition rates are greater than 99% and 99.9% (Q20, Q30, respectively), GC content and sequence duplication level of the clean data were calculated. All the downstream analyses were based on the clean data with high quality. Pair-ended clean reads were mapped with HISAT2 (HISAT version 2.1.0; Kim et al 2019) to the reference genome (*P. vulgaris* v2.1, U.S. Department of Energy Joint

Genome Institute, Phytozome v12.0: http://phytozome.jgi.doe.gov/). HISAT2 is a fast and sensitive alignment program for mapping next-generation sequencing reads, enabled effective alignment of RNA-seq reads, particularly, reads spanning multiple exons. Because transcriptome annotation is still incomplete, this RNA-seq study revealed novel genes and transcripts. To do that, Cufflinks Reference Annotation Based Transcript (RABT) assembly method was used to assemble the set of transcript isoforms of each bam file obtained in the mapping step. This was done using 'Cuffcompare' that compares Cufflinks assemblies to reference annotation files and help sort out new genes from known ones.

Genes were considered expressed if they present an average raw number ≥ 100 at least in one of the studied timepoints. The expected number of Fragments Per Kilobase of transcript sequence per Millions base pairs sequenced (FPKM) which takes into account the effects of both sequencing depth and gene length on counting of fragments was also used to establish gene expression abundances and profiles using HTSeq software (Anders et al 2014). Differential expression analysis was performed using the DESeq2 R package (1.12.0; TNLIST, Beijing, China). Pair-wise comparisons between two consecutive timepoints (6 vs 10 DAA, 10 vs 14 DAA and 14 vs 18 DAA) were established. A Benjamini-Hochberg correction, for estimating false discovery rates (FDR) was also applied to the analysis made (Benjamini et al., 1995). Genes which presented a corrected p-value ≤ 0.001 and |Log2(Fold Change - FC) | ≥1 were considered differentially expressed genes (DEGs).

Functional characterization was performed using the MapMan web tools (http://www.plabipd.de/portal/mercator-sequence-annotation). Protein sequences were obtained using BioMart (*Phaseolus vulgaris* genome version 2.1) in Phytozome v.12 (https://phytozome.jgi.doe.gov/) to create a mapping file for the Mercator pipeline. Some genes were also annotated using Phytozome v.12 BLAST.

With the differential expression analysis data, Venn diagrams were generated and obtained from the Venny 2.1 (Oliveros, 2015) and used to infer the overall distribution of differentially expressed genes (DEGs). Heatmaps were constructed using Morpheus online tool, (https://software.broadinstitute.org/morpheus), to show the normalized gene expression among the samples. Clustering was performed based on Euclidean distance and average linkage.

2.7. RT-qPCR validation of RNA-Seq data

Expression of 15 selected genes were analysed by RT-qPCR (Supp table 2) on a Light Cycler® 480 System, using the LightCycler® 480 SYBR Green I Master protocol. These genes were chosen to represent a broad range of differential expression and total transcript counts. 1 µg of total RNA from 3 biological replicates per time point (6, 10, 14 and 18) was reverse transcribed, using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. Primers were designed using the Primer3 software and primer sequences are listed in Supplementary Table 2. PCR amplification efficiencies were tested for all primers for target and reference genes using cDNA two-fold dilution series. Using the geNorm and NormFinder software packages from the GenEx v.5 software (MultiD, Goteborg, Sweden), two reference genes: eukaryotic Release Factor 1 (eRF1) family protein (PEL1; Phvul.010G077790.1) and RING/U-box superfamily protein (XERICO; Phvul.008G190100.1) used by Parreira et al., (2021), were selected for the gene relative expression analysis.

Thermo cycling reactions were carried out following the described conditions: denaturation step at 90°C for 5 min; 45 cycles of amplification at 95°C for 10 s; 10 s at 60°C and 10 s at 72°C. For each reaction, a melting curve (dissociation stage) was performed to detect non-specific PCR products and/or contaminants. A non-template control (NTC), without cDNA, was also included for each primer mix to detect possible contaminations.

2.8. Data availability

RNA-seq data were deposited in the NCBI Sequence Read Archive BioProject under the accession number PRJNA1224322 (SRR32361921, SRR32361920, SRR32361917, SRR32361916, SRR32361915, SRR32361914, SRR32361913, SRR32361912, SRR32361911, SRR32361910, SRR32361919 and SRR32361918).

2.9. Statistical analyses

Data from the morphological analysis (seed length, fresh and dry weight) and from histochemical analysis (mean grey value for pixel intensity in Coomassie and Periodic acid Schiff images) was analysed with IBM SPSS Statistics V25.0 software. Data statistical significance was assessed using one-way analysis of variance (ANOVA) coupled with post-hoc Tukey HSD for mean pairwise comparison. Means were considered significantly different when $P \leq 0.05$.

3. Results

3.1. In *P. vulgaris* SER16 seeds, the accumulation of reserves starts to occur between 10 and 14 DAA

A significant increase in seed length was observed throughout all the time points studied (p-value < 0.05) (Figure 1). No significant differences were observed in the seed fresh weight (SFW) and seed dry weight (SDW) between 6 and 10 DAA samples, although a slight increase in SFW is observed. However, between 10 and 14 DAA a significant increase, with a Fold change (FC) of 8.49 in the SFW and a FC of 7.27 in SDW were observed, suggesting that the accumulation of reserves starts to occur around 10DAA. Between 14 and 18 DAA, there was still a significant increase in SFW (FC=2.41) and SDW (FC= 3.76). Between 18 and 20 DAA, the increase was less prominent with a FC of 1.88 in the SFW and a FC of 1.85 in SDW. Figure

1 illustrates a noticeable increase in seed size over time. Additionally, the same figure highlights a gradual change in seed coat colour transitioning from green to orange until 20 DAA.

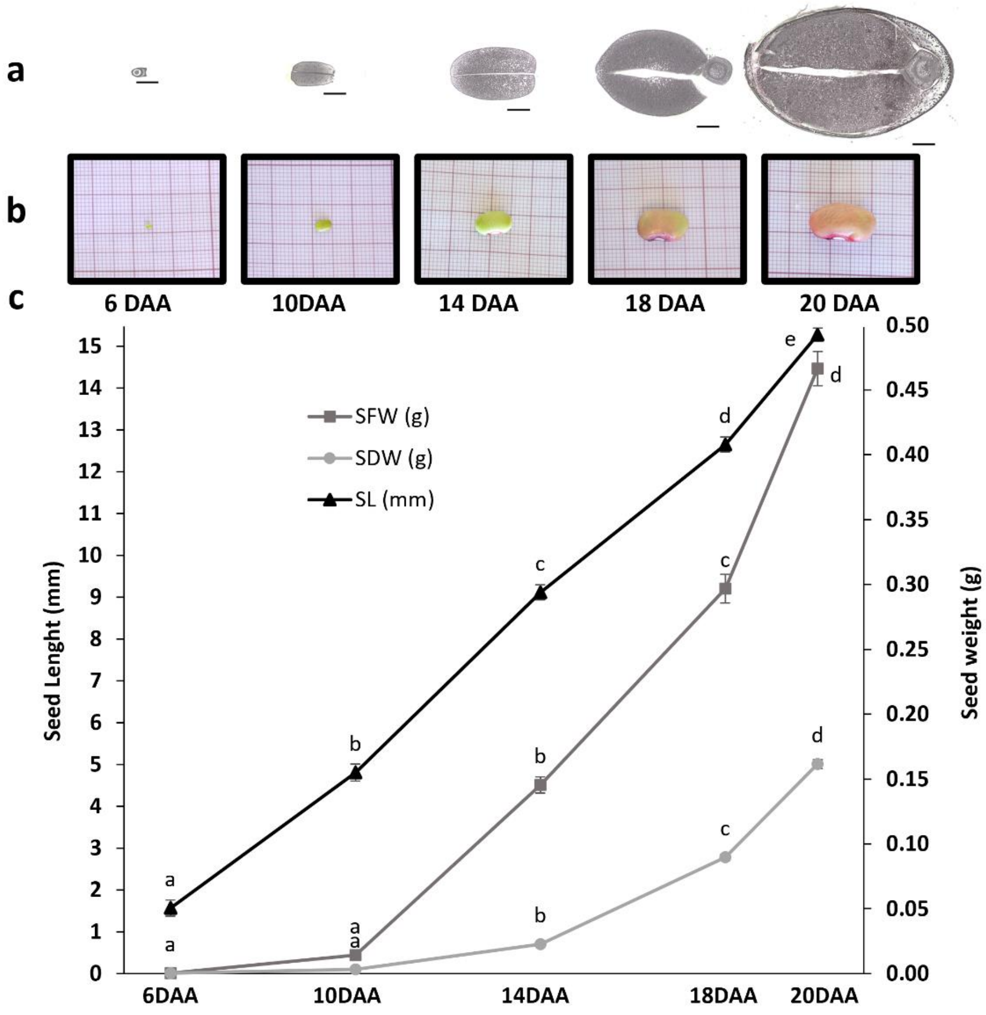

Figure 1- Characterization of seed development in *P. vulgaris* SER16 at 6, 10, 14, 18 and 20 DAA (days after anthesis). The study was focused on the transition from seed embryogenesis to seed filling. a transversal section of seeds at 6, 10, 14, 18 and 20 DAA at the Hilum level, black scale bars indicate 1 mm; b Photographs of seeds at 6, 10, 14, 18 and 20 DAA; c Seed fresh weight (SFW), Seed dry weight (SDW), Seed length (SL). Error bars represent the standard deviation and different letters indicate statistically significant differences between time points ($p < 0.05$).

At 6 DAA the seeds were still under embryogenesis stage since no clear differentiation of cotyledons from the embryo is noticed (Figure 2). At 10 DAA, the cotyledons were clearly developed, in which it was possible to observe cotyledon parenchyma cells, dermal cell complex and vascular bundles (Figure 2). Furthermore, at 10 DAA, the visible stained structures are the cell walls, as detected by Coomassie Blue and Periodic Acid-Schiff staining.

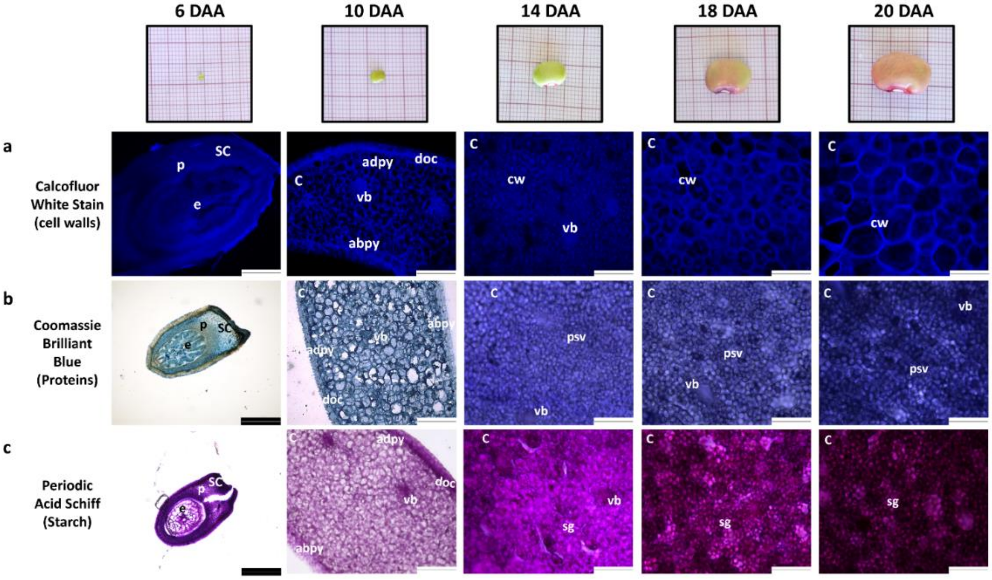

Figure 2- Morphology and histology of developing *P. vulgaris* SER16 seeds harvested at 6, 10, 14, 18 and 20 days after anthesis (DAA). Histological observations of: a cell walls, b protein and c starch accumulation during common bean seed development. The cell walls, proteins and starch were stained with Calcofluor white stain, Coomassie Brilliant Blue and Periodic Acid Schiff, respectively. The white scale bars indicate 124.4 µm and black scale bars indicate 248.9µm; abpy - cotyledon abaxial parenchyma; adpy - cotyledon adaxial parenchyma; C- cotyledon; cw - cell wall; doc - dermal cell complex; e - embryo; p - parenchyma; psv - protein storage vacuoles; SC - seed coat; sg - starch grains; vb - vascular bundle.

By 14 DAA, protein storage vacuoles and starch grains are already visible and are also stained, indicating that starch and storage protein accumulation begins between 10 and 14 DAA (Figure 2b, 2c), suggesting that the transition from embryogenesis to seed filling occurs between 10 and 14 DAA. Due to the staining of protein storage vacuoles and starch grains with Coomassie Blue and Periodic Acid-Schiff, cell walls cannot be easily distinguished in these images at these time points.

Coomassie Blue and Periodic Acid-Schiff images were converted to a grayscale and the mean grey value for pixel intensity was used to estimate overall protein and starch accumulation. Values from 10 DAA correspond to cell walls staining, since no protein storage vacuoles or starch grains were visible (Figure 2b, 2c). Protein and starch accumulation increased significantly from 14 DAA to 18 DAA, followed by a more pronounced rise from 18 DAA to 20 DAA (Figure 3c, 3d). This trend is supported by the mean grey value FC measurements: Coomassie Blue staining shows a FC= 1.08 from 14 to 18 DAA and a FC=1.19 from 18 to 20 DAA. Moreover, Periodic Acid-Schiff staining shows a FC= 1.13 from 14 to 18 DAA and a FC=1.24 from 18 to 20 DAA (Figure 3c, 3d).

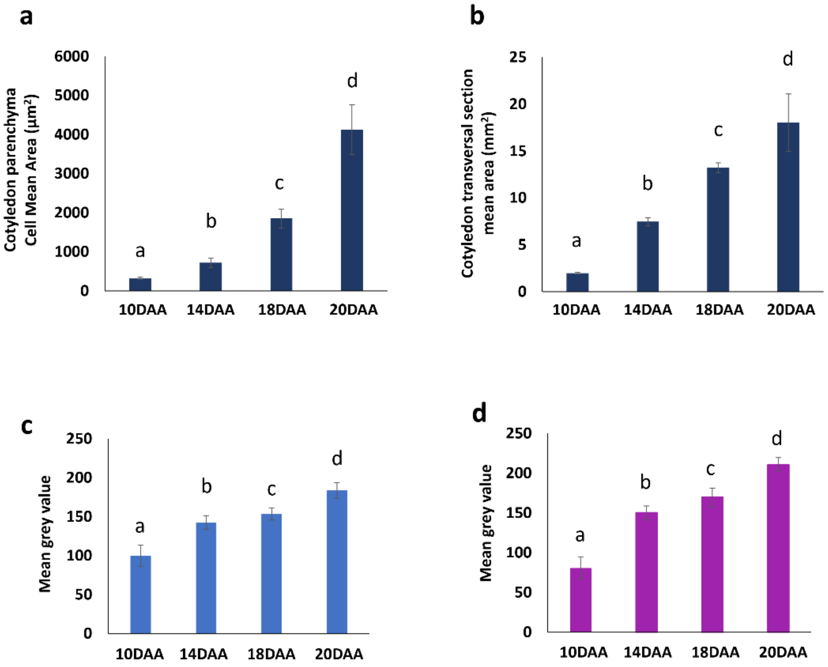

Figure 3- Changes in cotyledon parenchyma cell number and area of *P. vulgaris* SER16 seeds, from 10 to 20 DAA (days after anthesis). a Cotyledon parenchyma cell

mean area. b Cotyledon transversal section mean area. Mean grey value for pixel intensity (obtained using Image J software after converted to a grayscale) for: c Coomassie blue and d Periodic acid Schiff. Different letters above each bar indicates statistically significant differences ($p < 0.05$).

The cotyledon section area, at the hilum level, increased approximately four-fold (FC=3.98) from 10 to 14 DAA and almost doubled (FC=1.70) from 14 to 18 DAA (Figure 1b, 3b). Cotyledon parenchyma cells approximately doubled their area along the time points studied (10 vs 14 FC=2.19; 14 vs 18 FC=2.79; 18 vs 20 FC=2.39) (Figure 3a). These results suggest that from 10 to 14 DAA cells stopped to divide and start to expand to accumulate storage compounds.

3.2. Global Overview of the Gene Expression from late embryogenesis to seed filling

The RNA-Seq of 12 libraries from three biological replicates from seeds harvested at 6, 10, 14 and 18 DAA, produced 884.1 million 150-bp reads, with an average of 68 millions of mapped reads/sample (Table 1). A total of 17191 genes, with ≥ 100 average raw reads at least in one studied timepoint, were considered expressed in this study (Supplementary table 1). Sequences annotated as BOWMAN-BIRK SERINE PROTEASE INHIBITOR FAMILY (Phvul.004G134000; Phvul.004G133900), CONCANAVALIN A-LIKE LECTIN PROTEIN KINASE FAMILY PROTEIN (Phvul.004G158200), LECTIN RECEPTOR KINASE A4.3 (Phvul.004G158100) and RMLC-LIKE CUPINS SUPERFAMILY PROTEIN (Phvul.007G192800) were amongst those with highest total raw read counts, suggesting no ribosomal RNA (rRNA) contamination during library preparation.

Table 2- Characterization of the 12 RNA-Seq libraries generated for developing seeds of *P. vulgaris* SER16.

Sample	*Number of raw reads*	*GC (%)*	*Q20(%)*	*Q30(%)*	*Number of mapped reads*	*Mapped reads (%)*
SD6DAA1	35262765	45.77	97.47	92.73	34733045	98.5
SD6DAA2	31498837	44.67	97.21	92.27	30689952	97.4
SD6DAA3	39111219	44.99	97.50	92.78	38286149	97.9
SD10DAA1	43099217	45.91	97.55	92.88	42453531	98.5
SD10DAA2	39512074	46.76	97.52	92.84	38753821	98.1
SD10DAA3	50089372	46.43	97.58	92.98	49139349	98.1
SD14DAA1	35309229	46.23	97.64	93.08	34644029	98.1
SD14DAA2	37942658	46.13	97.73	93.29	37228676	98.1
SD14DAA3	37260953	46.25	97.72	93.24	36568854	98.1
SD18DAA1	33694888	46.01	97.48	92.77	33030091	98.0
SD18DAA2	34481992	46.51	97.59	93.01	33905182	98.3
SD18DAA3	33312508	45.34	97.52	92.88	32615875	97.9

The most represented functional category was ”Amino acid metabolism” [MapMan BINCODE (BC 13), followed by ”Cell” (BC 31), ”Cell wall” (BC 10), ”DNA” (BC 28), ”Development” (BC 33), ”Lipid metabolism” (BC 11), ”Hormone metabolism” (BC 17) and ”Minor CHO metabolism” (BC 3) (Figure 4).

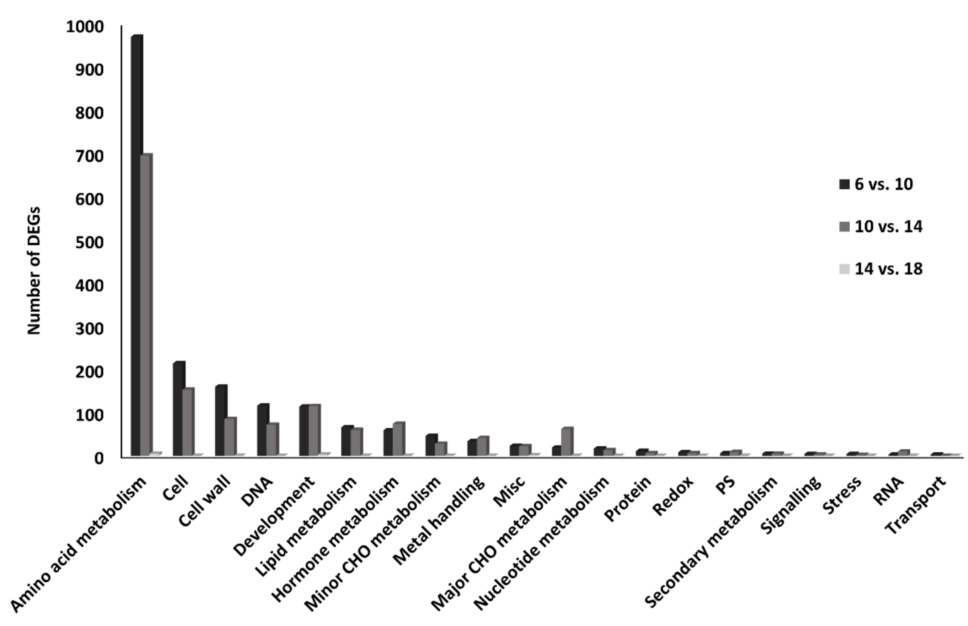

Figure 4- Functional categories of differentially expressed genes (DEGs) in the transition from the embryo development to seed filling in *P. vulgaris* SER16 seeds. The percentage of DEGs in each category were displayed between the main comparison studied (6 DAA vs 10 DAA; 10 DAA vs 14 DAA and 14 DAA vs 18 DAA). The percentage of DEGs changed was calculated by comparison of the number of DEGs in each category in relation to the total of DEGs identified within each comparison.

3.3. Expression of genes involved in seed storage protein accumulation and carbohydrate synthesis

Transcripts annotated as belonging to the Cupin superfamily, some of whose genes encode the phaseolin storage protein, were analysed (Figure 5). A cluster of genes of this family is upregulated at 6 DAA and a second cluster is upregulated at 14 and 18 DAA. Furthermore, most of the amino acid activation, protein synthesis, protein folding and protein post-translational modification genes are upregulated at 6 and 10 DAA, with a smaller cluster upregulated at 14 and 18 DAA (Supplementary Figure 1, 2, 3 and 4).

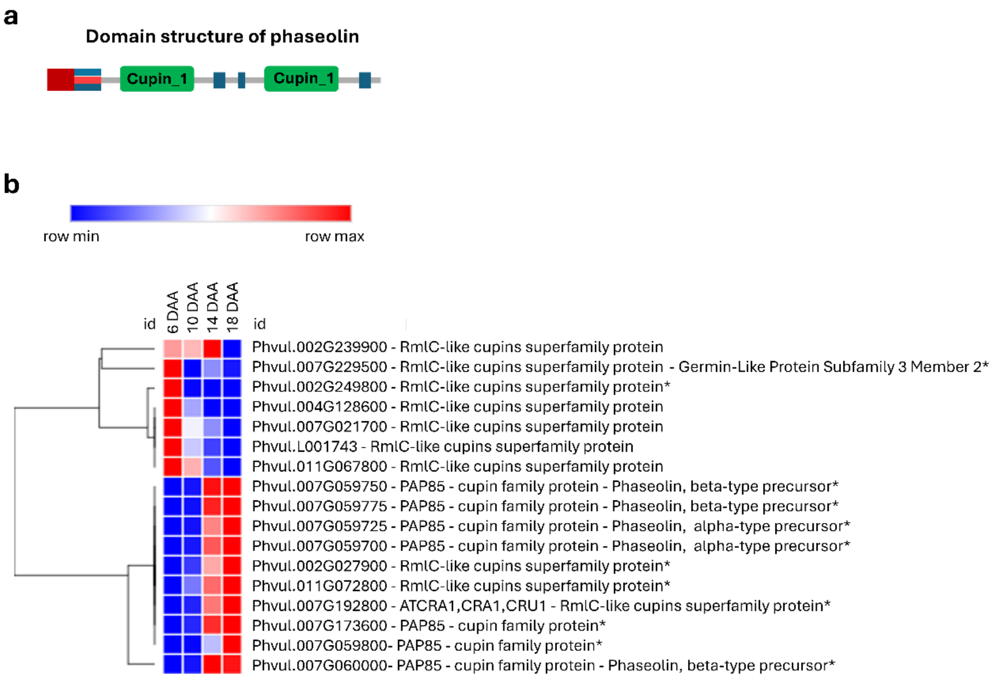

Figure 5- Seed storage protein gene expression. a Domain structure of Phaseolin exhibiting the bi-cupin architecture (Emani et al., 2008); b Heatmap of transcripts related to seed storage proteins at 6, 10, 14 and 18 days after anthesis (DAA). FPKM were clustered using Euclidean distance and an average linkage. Blue colour represents low expression levels (lower FPKM values); White (Middle Colour) represents moderate expression levels and Red represents high expression levels (higher FPKM values). Asterisks indicate differentially expressed genes (DEGs): corrected p-value ≤ 0.001 and |Log2(Fold Change - FC) | ≥1.

The expression of transcripts categorized in MapMan related to genes involved in the sucrose and starch synthesis (Figure 6) was analysed. Among those, Phosphoglumatase (PGM) (EC. 5.4.2.2) was more expressed after 10 and 14 DAA. Additionally, Sucrose phosphate synthase (SPS) (E.C. 2.4.1.14) showed an upregulation at 18 DAA. Starch molecular structure is composed by two polysaccharides: amylose and amylopectin. Granule-bound starch synthase (GBSS) (E.C. 2.4.1.242) is consistently upregulated across all time points. The gene of the 1,4-alpha-glucan branching enzyme (SBE) (E.E. 2.4.1.18) that catalysis the branched structure of the amylopectin, was upregulated after 10 DAA. The gene of Isoamylases (ISO) (E.C. 3.2.1.68) essential for amylopectin crystallization, exhibited increased expression at 14 and 18 DAA. The upregulation of SBE and ISO genes at 14 and 18 DAA, which contribute to amylopectin formation, suggests enhanced carbohydrate accumulation at this stage of seed development.

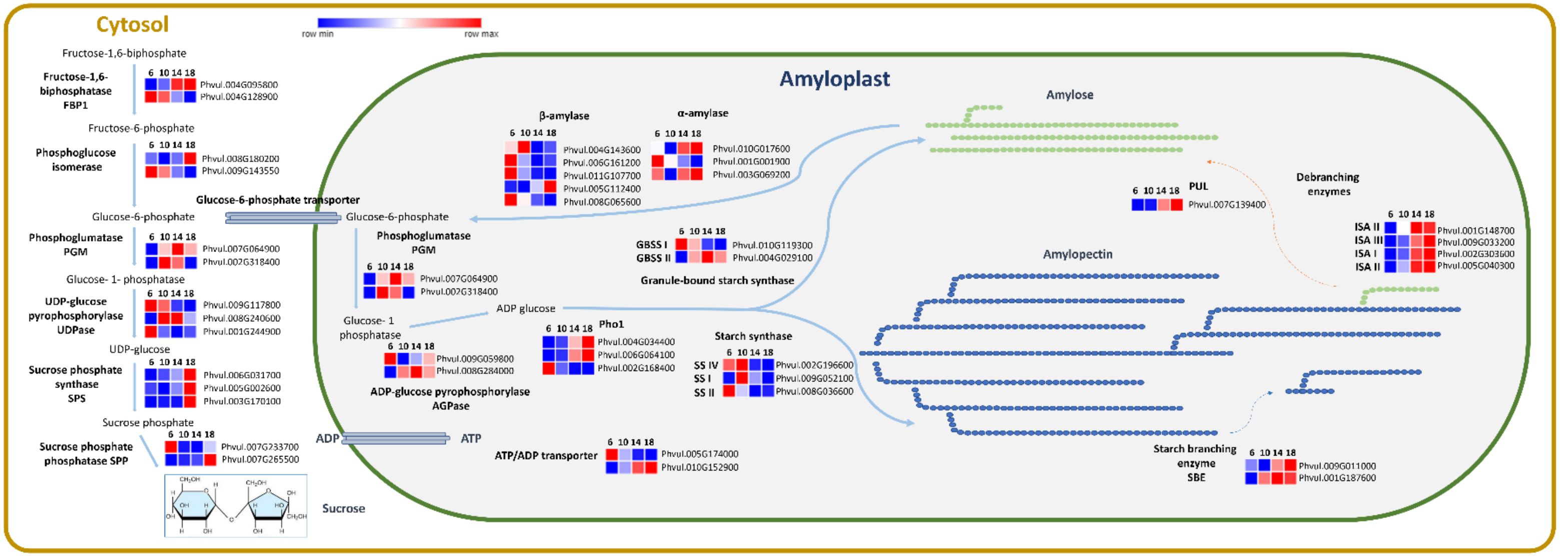
Cytosol
row min
row max
Fructose-1,6-biphosphate
Fructose-1,6-biphosphatase FBP1
6 10 14 18
Phvul.004G095800
Phvul.004G128900
Fructose-6-phosphate
Phosphoglucose isomerase
Phvul.008G180200
Phvul.009G143550
Glucose-6-phosphate transporter
Glucose-6-phosphate
Phosphoglumatase PGM
Phvul.007G064900
Phvul.002G318400
Glucose- 1- phosphatase
UDP-glucose pyrophosphorylase UDPase
Phvul.009G117800
Phvul.008G240600
Phvul.001G244900
UDP-glucose
Sucrose phosphate synthase SPS
Phvul.006G031700
Phvul.005G002600
Phvul.003G170100
Sucrose phosphate
Sucrose phosphate phosphatase SPP
Phvul.007G233700
Phvul.007G265500
Sucrose
ADP
ATP
ATP/ADP transporter
Phvul.005G174000
Phvul.010G152900
Amyloplast
β-amylase
Phvul.004G143600
Phvul.006G161200
Phvul.011G107700
Phvul.005G112400
Phvul.008G065600
α-amylase
Phvul.010G017600
Phvul.001G001900
Phvul.003G069200
Glucose-6-phosphate
Phosphoglumatase PGM
Phvul.007G064900
Phvul.002G318400
Glucose- 1 phosphatase
ADP glucose
ADP-glucose pyrophosphorylase AGPase
Phvul.009G059800
Phvul.008G284000
Pho1
Phvul.004G034400
Phvul.006G064100
Phvul.002G168400
GBSS I
GBSS II
Phvul.010G119300
Phvul.004G029100
Granule-bound starch synthase
Starch synthase
SS IV
SS I
SS II
Phvul.002G196600
Phvul.009G052100
Phvul.008G036600
Amylose
PUL
Phvul.007G139400
Debranching enzymes
ISA II
ISA III
ISA I
ISA II
Phvul.001G148700
Phvul.009G033200
Phvul.002G303600
Phvul.005G040300
Amylopectin
Starch branching enzyme SBE
Phvul.009G011000
Phvul.001G187600

Figure 6- Heatmaps of transcripts of *P. vulgaris* SER16 seeds, categorized in MapMan, related to sucrose and starch synthesis at 6, 10, 14 and 18 days after anthesis (DAA). Fructose 1-6 biphosphatase (FBP1), Phosphoglucose isomerase, Phosphoglumatase (PGM), Sucrose phosphate synthase (SPS), Sucrose phosphate phosphatase (SPP), UDP-glucose pyrophosphorylase (UDP), ADP-glucose pyrophosphorylase (AGPase), Plastid starch phosphorylase (Pho1), Starch synthase (SS), Granule-bound starch synthase (GBSS), Starch branching enzyme (SBE), Isoamylases (ISA), Pullulanase (PUL), α-amylase and β-amylase. Schematic representation of sucrose and starch synthesis adapted from (Qu et al., 2018). FPKM were clustered using Euclidean distance and an average linkage. Blue colour represents low expression levels (lower FPKM values); White (Middle Colour) represents moderate expression levels and Red represents high expression levels (higher FPKM values). Asterisks indicate differentially expressed genes (DEGs): corrected p-value ≤ 0.001 and |Log2(Fold Change - FC) | ≥1.

3.4. Expression of genes involved in the LAFL regulatory Network

Seed development is regulated by the LAFL transcription factors network, which consists of the key genes ABI3 (Abscisic Acid Insensitive 3), FUS3 (FUSCA3), LEC1 (Leafy Cotyledon 1), LEC2 (Leafy Cotyledon 2) and L1L (LEC1-Like) (Figure 7). Among these, LEC1 and LEC2 were upregulated at 6 DAA and FUS3 and ABI3 were upregulated at 10, 14 and 18 DAA. Moreover, ABI5 (ABA Insensitive 5) showed increased expression at 14 and 18 DAA. LEA (Late-Embryogenesis-Abundant) was predominantly upregulated at 18 DAA, with a smaller cluster exhibiting upregulation at 6 and 10 DAA.

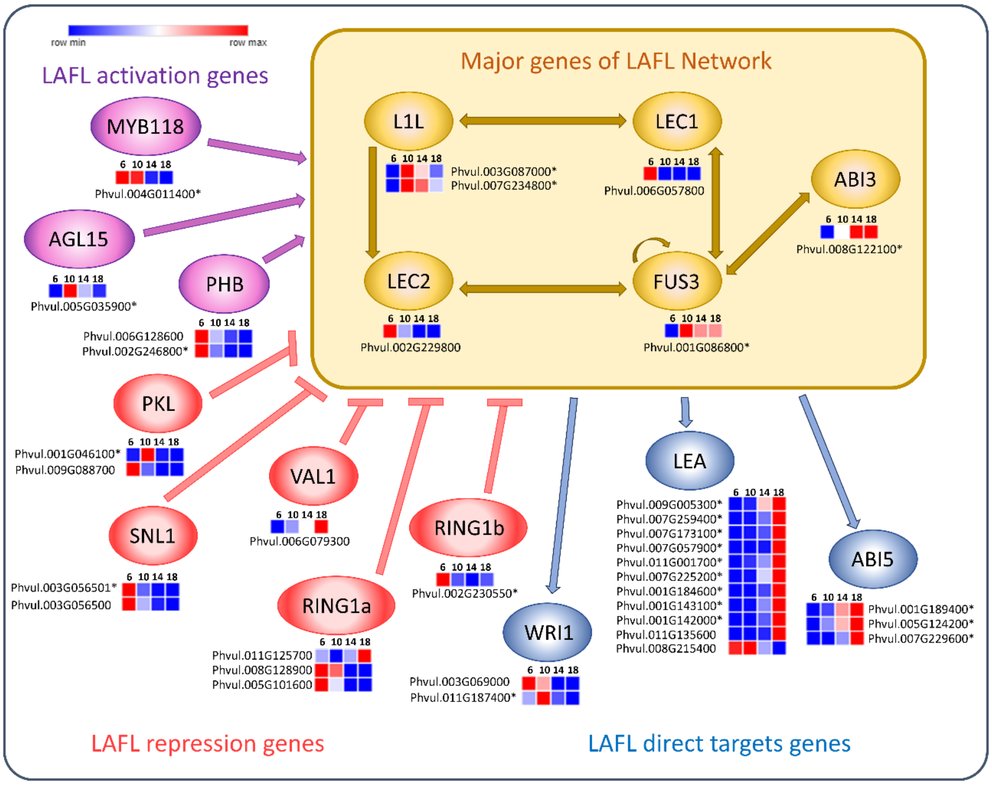

Figure 7- Heatmaps of transcripts of *P. vulgaris* SER16 seeds, categorized in MapMan, related to LAFL network genes found differentially expressed between 6, 10, 14 and 18 days after anthesis (DAA). LAFL activation genes are shown in purple, LAFL repression genes in pink and LAFL direct target genes in blue. The yellow box highlight the major genes of the LAFL network (LEC1, L1L, ABI3, FUS3 and LEC2) and illustrates their regulatory interactions. Schematic representation adapted from (Devic et al., 2016; Jia et al., 2014). FPKM were clustered using Euclidean distance and an average linkage. Blue colour represents low expression levels (lower FPKM values); White (Middle Colour) represents moderate expression levels and Red represents high expression levels (higher FPKM values). Asterisks indicate differentially expressed genes (DEGs): corrected p-value ≤ 0.001 and |Log2(Fold Change - FC) | ≥1.

Genes involved in the metabolism of plant growth regulators, modulated by LAFL Network proteins were also found expressed among the analysed time points (Figure 8). In general, most of these genes are upregulated at 6 DAA. Exceptions are one of the ABA2 genes, involved in ABA synthesis, the IAMT1 gene involved in Auxin catabolism, one of the DWF4 genes, involved in Brassinsteroid (BR) synthesis, two of the BRH1 genes involved in BR signalling and ERF1 a key regulator in Ethylene signalling. These genes were upregulated at 14 or 18 DAA.

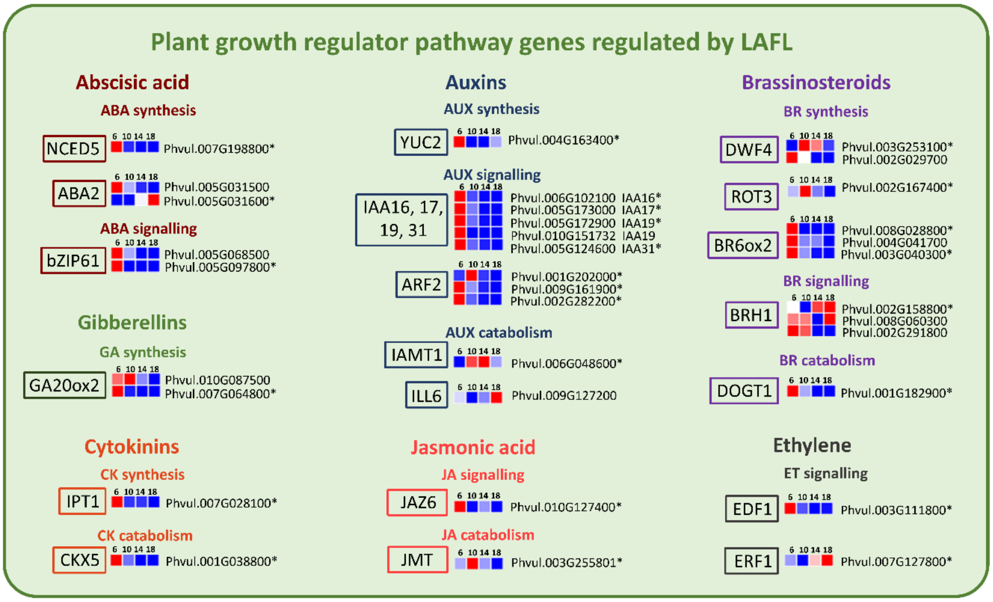

Figure 8- Heatmaps of transcripts of *P. vulgaris* SER16 seeds, categorized in MapMan, related to plant growth regulator pathways, including key components of the abscisic acid (ABA), jasmonic acid (JA), cytokinin (CK), gibberellin (GA), ethylene (ET), auxin (AUX) and brassinosteroid (BR) pathways, which are regulated by the LAFL network, found differentially expressed between 6, 10, 14 and 18 days after anthesis (DAA). FPKM were clustered using Euclidean distance and an average linkage. Blue colour represents low expression levels (lower FPKM values); White (Middle Colour) represents moderate expression levels and Red represents high expression levels (higher FPKM values). Asterisks indicate differentially expressed genes (DEGs): p-value ≤ 0.001 and |Log2(Fold Change - FC) | ≥1.

Genes associated with cell division showed an upregulation at the first time points studied (6 and 10 DAA) of seed development (Supplementary

Figure 5). A smaller subset of these genes exhibited sustained expression at later stages. Furthermore, genes related to cell wall expansion were identified from RNA-Seq data. Those genes showed an upregulation at the first time points studied (6 and 10 DAA) of seed development (Supplementary Figure 6). A smaller group of these genes continued to be expressed during the later stages. Genes related to cell wall synthesis showed an upregulation throughout all the time points studied of seed development (Supplementary Figure 7).

3.5. Validation of RNA Sequencing data by RT-qPCR

A strong positive correlation ($0.79 \leq R^2 \leq 1.00$) was observed between RNA-Seq expression values (FPKM) and the corresponding average RT-qPCR expression levels (relative units) for each of the fifteen selected genes, measured at 6, 10, 14 and 18 DAA (Supplementary Table 3).

4. Discussion

Seed development in *P. vulgaris* SER16 follows a well-orchestrated series of events that include embryogenesis, reserve accumulation and maturation. Our previous studies on seed development from 10 to 40 DAA established a comprehensive molecular and proteomic landscape underlying later stages of development, including the transition from storage compound deposition to desiccation (Parreira et al., 2016; 2018; 2021). However, unresolved questions remained: What are the histological changes underlying the SD stages studied? When the transition from embryogenesis to seed filling does occur? What are the main molecular mechanisms and metabolic pathways regulating the transition from embryogenesis to seed filling? To address this, we expanded our analysis to earlier time points (6, 10, 14, 18 and 20 DAA) using a multidisciplinary approach, integrating morphological, histological and transcriptomic analyses.

4.1 Morphological, histochemical and transcriptomic analysis shows that the transition from late embryogenesis to seed filling begins around 10 DAA

Morphological measures revealed a continuous increase in seed length from 6 to 20 DAA. While seed fresh weight (SFW) and seed dry weight (SDW) exhibited a slight increase from 6 to 10 DAA (Figure 1), a pronounced accumulation was observed from 10 DAA onwards, suggesting a shift towards storage reserve deposition. Histochemical analysis reinforces this developmental progression. As shown in Figure 2, embryogenesis is still in progress at 6 DAA. At 10 DAA, the cotyledons are fully formed, but protein granules are not seen yet, indicating that the transition to seed filling occurs only after 10 DAA.

Moreover, the observed increase in cell area and cotyledon section size between 10 and 14 DAA (Figure 3a, 3b) suggests a transition from active cell division to cell expansion, a hallmark of seed filling where cells allocate space for reserve deposition. The observed upregulation of genes related to cell division and expansion at 6 and 10 DAA (Supplementary Figure 5,6), alongside continuous activation of cell wall synthesis genes (Supplementary Figure 7), aligns with the histological results, reinforcing the hypothesis that the transition from embryogenesis to seed filling is marked by a shift from cell proliferation to expansion.

A similar growth regulation pattern has been described in other studies in chickpea, where early embryogenesis is primarily driven by cell division (Turner et al., 2005; Weber et al., 2005), followed by cotyledon differentiation, which coincides with sucrose uptake, cell expansion, and the accumulation of storage compounds (Dante et al., 2014). According to Coelho et al., (2008), the embryogenesis phase in *P. vulgaris* is characterized by cell division and differentiation, which contribute to the formation of the embryo tissues and the endosperm. The rapid increase in SFW relative to SDW during this phase, observed in our study, further suggests an influx of water that facilitates cell expansion, a process commonly observed during early seed filling stages (Bewley et al., 2013).

4.2 Seed storage reserve accumulation occurs between 10 and 14 DAA

The expression of genes involved in the synthesis and accumulation of seed reserves follows the morphological and histochemical findings. The upregulation of RmlC-like cupins superfamily genes at 6 DAA (Figure 5) may be related to their dual role in embryogenesis and stress response, as some early-expressed storage proteins contribute to protecting the developing embryo (Neutelings et al., 1998; Dunwell et al., 2000). Germin-like proteins were previously identified as a highly homogeneous group of proteins with oxalate oxidase activity and they were found expressed in the embryo axis of *P. vulgaris* during germination (Aubry et al., 2003). Notably, GERMIN-LIKE PROTEIN SUBFAMILY 3 MEMBER 2, expressed at 6 DAA, has been associated with somatic and zygotic embryogenesis and proposed to play a role in desiccation/hydration processes and oxidative stress protection (Neutelings et al., 1998; Khuri et al., 2001). Additionally, Germin-like proteins exhibit superoxide dismutase (SOD) activity, which mitigates oxidative stress during early seed development, ensuring proper cellular function and metabolic stability (Khuri et al., 2001). The early activation of these protective mechanisms suggests that seed development at 6 DAA is already metabolically active, preparing for reserve accumulation and structural modifications essential for seed filling.

However, genes that encode Phaseolin, a major *P. vulgaris* storage protein, start to increase their expression at 10 DAA and are all highly upregulated at 18 DAA, suggesting that the seeds are starting the seed filling stage at 10 DAA, which correlates with the histochemical detection of storage compounds and the significant increase in seed dry weight observed during this period. Such results are also aligned with our previous studies (Parreira et al., 2016), where we found that seed storage proteins as Legumin (UniProKB ID: F8QXP7), Phaseolin (UniProKB ID: Q43632) and Alpha-phaseolin (UniProKB ID: X5CHW7 and X5D3J1) showed a significant accumulation from 10 to 20 DAA.

The upregulation, at 6 DAA of genes involved in protein amino acid activation, synthesis, folding, and post-translational modification (Supplementary Figure 1, 2, 3 and 4) suggests that seeds are already preparing for reserve synthesis and accumulation at that stage. Likewise, Parreira et al., (2016) observed at 10 DAA, a high accumulation of proteins associated with protein synthesis (e.g 40S ribosomal protein S2-4 (UniProKB ID: V7C8K4 (A0A0B2QJI4)) and 60S ribosomal protein L6 (UniProKB ID: V7C7H0 (A0A0B2SQP6)), folding (e.g Chaperone protein ClpC, chloroplastic (UniProKB ID: V7AZ24 (A0A0B2PK21)), targeting and post-translational modification (e.g Ubiquitin carboxyl-terminal hydrolase (UniProKB ID: V7ASH7)), in early seed development. The early activation of these processes is crucial, as seed storage proteins must undergo proper folding and post-translational modifications to ensure functional stability and efficient accumulation during seed filling. This aligns with previous studies showing that post-translational modifications, such as glycosylation and disulfide bond formation, play essential roles in stabilizing storage proteins and optimizing their deposition within protein bodies (Önder et al., 2022). Our findings indicate that even though storage protein accumulation does not begin until 10 DAA, the molecular machinery required for their synthesis is already being established at 6 DAA.

4.3 Starch accumulation starts after 10 DAA and show a complex interplay among different starch biosynthetic enzymes

P. vulgaris seeds have one of the highest percentages of amylose in their starch grains. Amylose contents from 32.40% to 49.73% were reported by Du et al., (2014), while Bajaj et al., (2018) found an amylose content of 49.50% in *P. vulgaris*. The histochemical analysis show that starch starts to accumulate after 10 DAA (Figure 2). The continuous increase in transcription observed for one of the two ATP/ADP transporter genes (Figure 6), which is the more highly expressed isoform, particularly from 10 DAA onward, appears to be consistent with this observation. Previous studies showed that starch accumulation in potato tubers is strongly influenced by alterations in plastidic ATP/ADP transporter activity (Tjaden et al., 1998).

In our study, a higher GBSSI expression at 6 DAA was observed, followed by a decline until 18 DAA. Despite the increase of GBSSII until 14 DAA, it is possible that additional enzymatic or regulatory mechanisms may contribute to amylose biosynthesis. The anticipation of the expression of the GBSSI may be linked to the fact that it may also participate in amylopectin biosynthesis (Pfister et al., 2016). It was shown that GBSS enzymes are involved in amylopectin synthesis by extending its side chains and aiding in the formation of elongated glucan structures (Hanashiro et al., 2008). Similar patterns have been observed in *Brachypodium distachyon*, where GBSSI is highly expressed in immature seeds but decreases in later stages of development (Chen et al., 2014). Moreover, our previous proteome study showed high levels of ISA3 protein (Parreira et al, 2016), along with the increased transcript levels of ISA3 and PUL gene expression between 10 and 20 DAA (Parreira et al, 2018), which further supports the hypothesis that starch debranching enzymes contribute to amylose accumulation. This suggests that GBSS activity alone may not fully account for amylose accumulation, and other enzymatic pathways, to be elucidated, may play a role in the storage of high amylose percentage in *P. vulgaris*.

In *P. vulgaris* seeds, Takashima et al., (2007) showed that PvISAI/II have high activity for amylopectin, whereas PvISAIII shows a preference for dextrin and glycogen over amylopectin and has high specificity for short-chain removal (DP 2 to 4) (Takashima et al., 2007). Although the role of ISAIII in starch biosynthesis remains uncertain, its highest expression at 14 and 18 DAA in our data suggests a potential involvement in amylose synthesis. We propose that ISAIII facilitates this process by modifying the starch granule surface, likely enhancing the availability of elongated glucan chains for GBSS-independent amylose-like molecules formation during these stages.

This is further supported by the increased expression of Pullulanase (PUL) at 14 and 18 DAA, which has been identified as a key enzyme in amylopectin remodelling through debranching, leading to increased amylose content as part of a cold-adaptive response (Thakur et al., 2021).

These findings suggest the interplay between GBSS, SBE, SS, ISA and PUL enzymes may compensate for the decline in GBSS expression from 6 to 18 DAA, ensuring continued amylose accumulation in *P. vulgaris* seeds. It is possible that this corresponds to a different pattern in starch assemblage in relation to other seeds.

4.4 LAFL network regulation in *P. vulgaris* SER16: LEC1/LEC2 initiate embryogenesis, FUS3/ABI3 drive seed filling, and LEA/ABI5 regulate desiccation

The LAFL transcription factors modulate embryo development and maturation (Lepiniec et al., 2018). Our results show an early upregulation of LEC1 and LEC2 at 6 DAA (Figure 7), which is aligned with LEC1 being a central regulator of seed development, controlling embryo morphogenesis, photosynthesis and seed maturation (Jo et al., 2019). In soybean, GmLEC2a expression was found to be highest during the early stages of seed development, followed by a decline as the seeds matured (Manan et al., 2017). Furthermore, Manan et al. (2017) also demonstrated that LEC2 plays a role in regulating carbon partitioning, influencing the synthesis of triacylglycerol, carbohydrates and proteins in soybean. Moreover, in our previous work, we observed that PHABULOSA (PHB) and LEC2 were already present at 10 DAA at relatively low levels (Parreira et al., 2018), suggesting that the peak of LAFL activation occurs before 10 DAA. Indeed, we found MIR166 members could play a role in tuning the levels of several HD-ZIP transcription factors including PHB in *P. vulgaris* SER16 developing seeds (Parreira et al., 2021). The higher expression of LEC1 and LEC2 at 6 DAA supports this finding, indicating that the molecular framework for seed filling is already in place before 10 DAA, even though the histological changes associated with reserve accumulation occur after this date.

Expression of FUS3 and AB13 starts to increase at 10 DAA (Figure 7) correlating with the onset of seed filling, coinciding with the starting of the significant increase in SFW and SDW (Figure 1). This finding aligns with FUS3 playing a crucial role in the acquisition of embryo-dependent seed dormancy, the determination of cotyledonary cell identity, and the regulation of storage compound synthesis and accumulation (Tiedemann et al., 2008). ABI3 loss-of-function in *Medicago* mutants show impaired expression of storage protein genes, LEA genes, secondary metabolism genes, and cell cycle-related genes throughout seed maturation (Lalanne et al., 2021). Additionally, in *Linum usitatissimum*, overexpression of LuABI3-1 or LuABI3-2 significantly increased seed fatty acid and storage proteins (Liu et al., 2023), reinforcing ABI3's role in controlling reserve deposition during seed maturation.

Furthermore, we observed an upregulation of LEA and ABI5 at 14 and 18 DAA (Figure 7), which suggests that desiccation-related mechanisms are progressively activated alongside reserve accumulation, rather than being restricted to the final maturation phase. It was demonstrated in soybean that LEA proteins help protect and stabilize desiccation-sensitive proteins and plasma membranes during periods of dehydration (Shih et al., 2010). Notably, LEA proteins in soybeans were found to be more highly expressed in fully matured dry seeds, reinforcing their potential role in preserving cellular structures during desiccation (Jones et al., 2013). Furthermore, research in *P. vulgaris* has demonstrated that the PvLEA-18 protein responds to dehydration and accumulates in the seed during the final stage of seed development (Colmenero-Flores et al., 1999). The increased expression of ABI5 may be linked to its role as a key regulator of late seed maturation in legumes. This is supported by findings in *Pisum sativum* and *Medicago truncatula,* where abi5 knockout mutants exhibited severe impairments in both seed longevity and dormancy acquisition (Zinsmeister et al., 2016).

Additionally, Zinsmeister et al. (2016) demonstrated that ABI5 regulates LEA protein expression, as abi5 mutants showed a significant reduction in LEA proteins. Two new miRNAs, miR_6 and miR_18 targeting LEAs, EM1 and RAB18 transcripts respectively, were found highly expressed in 5 and 10 DAA, decreasing afterwards in developing *P. vulgaris* SER16 seeds (Parreira et al., 2021). On the same work, opposite profiles were shown for EM1 and RAB18 transcripts expression and protein accumulation. These results reinforce the functional role of LEA proteins in safeguarding cellular integrity during quiescence and acquisition of desiccation tolerance. Additionally, these findings indicate that seed mechanisms related to desiccation are progressively activated in *P. vulgaris* mid-filling, rather than being restricted to the final maturation phase. Additionally, post-transcriptional regulatory mechanisms mediated by miRNAs play role in tuning these responses.

4.5 LAFL-regulated phytohormones orchestrate early seed development and maturation in *P. vulgaris* SER16

A key function of the LAFL network is re-programming of the major plant hormone signaling pathways in the seed (Jia et al., 2014). Our results show that cytokinin, gibberellin, and auxin pathways are actively involved in early seed development, setting the molecular framework necessary for the later transition into seed filling. The upregulation of IPT1 (biosynthesis) and CKX5 (catabolism) at 6 DAA (Figure 8) implies a fine-tuned balance between cytokinin accumulation and degradation, likely contributing to early embryogenesis progression. In another grain legume, pea, an increase in cytokinin levels in the seed coat of transgenic peas was associated with higher expression of cell wall invertase (CWIN) in the seed coat and correlated with an increase in sucrose levels in the cotyledon, influencing nutrient allocation and storage compound accumulation in seeds (Grant et al., 2021). This agrees with our findings in *P. vulgaris* SER16, where the early expression of cytokinin-related genes at 6 DAA likely contributes to the metabolic activity required to establish source-sink relationships before seed filling begins.

The upregulation of GA20ox2 (gibberellin biosynthesis) at 6 and 10 DAA (Figure 8) suggests that gibberellins facilitate early cell expansion and cotyledon differentiation, preparing the seed for subsequent storage compound deposition. In a previous study, GA 20-oxidase genes from French bean were expressed in young seeds, similar to pea, where their highest expression was observed in very young seeds (until 4 DAA) (García-Martínez et al., 1997). Furthermore, in pea seeds, PsGA20ox genes were high in the embryo during early seed development, demonstrating that GA1 plays a key role in stimulating rapid branched parenchyma cell expansion. As the seed starts to mature GA levels start to decrease to limit embryo axis growth and allowing embryo maturation to proceed (Nadeau et al., 2011). The presence of GA20ox2 expression at 10 DAA aligns with histological evidence showing that cotyledons are fully developed at this stage, yet storage compounds have not yet begun to accumulate. This supports the idea that early GA20ox2 upregulation is essential for initial seed expansion and differentiation, reinforcing the developmental shift from active growth to storage phase initiation.

The upregulation of IAA16, IAA17, IAA19, IAA31, and YUC2 (key auxin biosynthesis genes) at 6 DAA (Figure 8) indicates an active auxin signaling network governing early development. Auxins play a crucial role in embryo patterning, determining the apical-basal axis and promoting cell division and elongation (Smit et al., 2015). This aligns with findings in *Phaseolus coccineus*, where the highest concentration of total IAA was detected in early-heart stage embryos, highlighting the importance of auxins in early seed development (Picciarelli et al., 2001). Similarly, in pea, auxins are essential for normal seed size and starch accumulation (McAdam et al., 2017). This is particularly relevant when considering that there were no significant differences observed between 6 and 10 DAA in SFW and SDW, reinforcing the idea that hormonal regulation at this stage is primarily preparing the seed for later expansion and reserve accumulation.

The upregulation of NCED5 (a key enzyme in ABA biosynthesis) and bZIP61 (a regulator of ABA signaling) at 6 DAA (Figure 8) aligns with the early activation of cell division genes and precedes the upregulation of genes associated with storage compound biosynthesis at later stages. In line with statement, we found that in SER16 developing seed, some members of the MIR167 potentially repress NCED1 expression likely contributing to tune ABA levels during *P. vulgaris* seed development (Parreira et al., 2021). These finding support that in *P. vulgaris* SER16, ABA plays an early role in seed maturation initiation and in preparation for seed filling. In soybeans, ABA enhances carbon allocation and partitioning to the seeds (Reinoso et al., 2011). In pea seeds, the SnRK1 kinase was shown to interact with the ABA signaling pathway, serving as a crucial regulator in developmental programming and having a key role in orchestrating the transition from the pre-storage phase to maturation, ensuring proper seed development (Radchuk et al., 2006).

The upregulation of ERF1 (a key regulator in ethylene signaling) and BRH1 (a brassinosteroid-responsive RING-H2 gene) at 14 and 18 DAA (Figure 8) suggests that, in *P. vulgaris* SER16, ethylene and brassinosteroids contribute to the regulation of storage compound accumulation and seed maturation. Brassinosteroids have been shown to enhance grain filling in rice by stimulating the flow of assimilates from source to sink, thereby increasing seed weight and overall yield (Wu et al., 2008). Moreover, in pea seeds, the amount of endogenous active Brassinosteroids increased when seeds were rapidly growing (during seed filling) but dropped significantly once the seeds were fully expanded and green (Nomura et al., 2007). This pattern is consistent with the role of brassinosteroids in promoting growth and development during early seed expansion. Moreover, in support of this, transcriptomic data from our previous study (Parreira et al., 2018) showed that key brassinosteroid-related genes, including ROT3 and BR6ox2, are highly expressed at 10 DAA, with a gradual decline observed at later stages, further suggesting that brassinosteroids play a prominent role during early seed development. These findings support the idea that brassinosteroids regulate seed development through mechanisms that affect both embryo growth and nutrient mobilization, which likely contributes to the sustained increase in seed size and storage compound accumulation observed in *P. vulgaris* SER16.

5. Conclusion

This study provides the first comprehensive transcriptomic dataset, morphological and histolochemical data spanning from 6 to 18 DAA during the early stages of *Phaseolus vulgaris* seed development. These results provide a high-resolution perspective on the intricate and molecular mechanisms governing the transition from late embryogenesis to seed filling. Our findings indicate that while changes related to reserve accumulation occur after 10 DAA, the molecular framework for seed filling is established earlier, starting at the late embryogenesis. Likewise, the preparation for seed desiccation is established during the filling stage. The integration with the results obtained with previous works done in this experimental system (Parreira et al., 2016, 2018, 2021) provided biological evidence that an intricate and complex regulatory network is governing the seed filling mechanisms.

Preparation to seed filling, happening between 6 and 10 DAA, is marked by the early establishment of the molecular framework required for this process. The transcription factors LEC1 and LEC2 were upregulated at 6 DAA, along with cytokinin-related genes, suggesting early regulatory activation. Auxin biosynthesis and ABA-related genes were also more expressed at 6 DAA, likely influencing cell division, elongation before the onset of seed filling. The expression of GA20ox2 at this stage indicates a transition from active growth to storage phase initiation. Additionally, the upregulation of genes involved in protein synthesis, folding, and modification at this stage further supports early molecular preparation for reserve accumulation.

Seed filling stage is characterized by protein and starch accumulation, which begins around 10 DAA. The expression of FUS3 and ABI3 starts to increase at 10 DAA, correlating with the onset of seed filling and coinciding with a significant rise in SFW and SDW. At this stage it is clear the increase of the expression of phaseolin genes, which guarantees the accumulation of this reserve protein. In relation to starch accumulation, high synthesis of amylose is supported by the interplay among GBSS, SBE, SS, ISA and PUL enzymes which compensate for the decline in GBSS expression from 6 to 18 DAA. In particular, there was high expression of ISA III and PUL at 14 and 18 DAA, probably involved in modifying amylopectin and facilitating the formation of GBSS-independent amylose molecules.

During mid-filling, between 14 and 18 DAA, desiccation tolerance genes, including LEA proteins and ABI5, are progressively activated, rather than being restricted to the final maturation phase. At this stage the upregulation of ERF1 and BRH1 at 14 and 18 DAA contributes to the regulation of storage compound accumulation and seed maturation.

These insights significantly advance our understanding of the regulatory networks involved in seed development and lay the groundwork for future efforts to optimize storage compound accumulation in legumes. By identifying early activated transcription factors and metabolic pathways, this study highlights valuable molecular targets for breeding programs aimed at enhancing seed quality traits such as protein content and starch composition. Still functional validation studies are needed to corroborate suggested roles played by selected genes and underlying mechanisms in *P. vulgaris* developing seeds. Furthermore, the molecular framework established here offers a valuable reference for cross-species comparisons in non-endospermic legumes, enabling translational research toward the development of resilient, nutrient-rich legume cultivars that support sustainable agricultural practices.

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Supplementary Material

Supplementary Table 1- RNA-seq data

https://www.dropbox.com/scl/fi/b3wrtw53vaf9pi8cm994x/Chapter-II_Supp1_RNA-Seq-data.xlsx?rlkey=5wdnoputk62tl0eeadaxz2mdg&st=430cfhzt&dl=0

Supplementary Table 2- Primers used for RNA extration and for RT-qPCR

RNA extraction:

Symbol	ID	Annotation	Forward (5'-3')	Tm (ºC)	Reverse (5'-3')	Tm (ºC)
Actin	Phvul.001G142500	Actin 11	TCCTGCTATGTATGTTGCCATC	58.3	GGAACCGTGTGACTGACACCGTCTCCGGAGTC	73.6

RT-qPCR : Genes of interest

Symbol	ID	Annotation	Forward (5'-3')	Tm (ºC)	Reverse (5'-3')	Tm (ºC)	Efficiency
ABI3	Phvul.008G122100	AP2/B3-like transcriptional factor family protein	TTTGCTCGAGAACACTGGTG	58.4	TTCACACCTTGTTGCCTCAC	58.6	0.7771
ABI5	Phvul.001G189400	Basic-leucine zipper (bZIP) transcription factor family protein	CACGGTTGAATTAGAAGCGGAAC	60.4	TCTGAGCGTTGGTTTGAACTC	58.8	0.9387
ADG1	Phvul.008G284000	ADP glucose pyrophosphorylase 1	TATTGGCCGGAACTCTCATGTC	60.2	TCTGTTTCCCTTGCAGCTTC	58.4	0.9704
AGL15	Phvul.005G035900	AGAMOUS-like 15	TACTGGTGCCAGATGTCTCAAC	60.0	CTTCCTCTTGAACACCAGCAG	58.9	0.8432
CRA1	Phvul.007G192800	RmlC-like cupins superfamily protein	TCAGTAACCTCAACAGCCTCAC	60.0	TTGCCCTCGGATCACGTATATC	59.8	0.9530
DGs	Phvul.005G045900	DNA glycosylase superfamily protein	GACCTCAACCTTGAAGAAACGC	60.0	ATCAGTCAAGTTGGCCACAG	58.1	0.8888

EXP15	Phvul.009G186400	expansin A15	TAACACTGCAGGAGGATGGTG	59.7	ATCTGATGCCCCCTCTCTTTTG	60.1	0.8651
EXPB2	Phvul.007G235300	expansin B2	AGGCAATCAGCTACGCAATG	59.0	CCTCGTATTCAACCATCACTGC	59.4	0.7931
ISA3	Phvul.009G033200	isoamylase 3	CATTCACGCTTCATGACAGGAG	59.6	CAAAGTCCTCCGGTGATTTGAG	59.3	0.7905
LEA4-5	Phvul.001G142000	Late Embryogenesis Abundant 4-5	TGGAGAAGACCAAGGCCAATG	60.3	CTCAGCCTGGTTGACCTTTAC	58.6	0.9626
PAP85	Phvul.007G173600	cupin family protein - vicilin-like seed storage protein	GCCAGGAATGCTGTTTGTTG	58.2	TGGAGCTCACAATGTTGTCC	58.1	0.8148
SBE2.2	Phvul.001G187600	starch branching enzyme 2.2	GATGCCTTGTTTGGTGGTTTC	58.2	AAGGGCATAAACCACTGCTG	58.5	0.8502
SUS4	Phvul.003G127500	sucrose synthase 4	AAGTGCAAGGTTGAGCCATC	58.7	AGCCTCTGGGAGTAAATTTGCC	60.6	0.9412
UGP2	Phvul.008G240600	UDP-glucose pyrophosphorylase 2	AAAGTGGCTGGCGATGTTTG	59.7	TTCCTTGTCCGCAATGACAG	58.5	0.8107

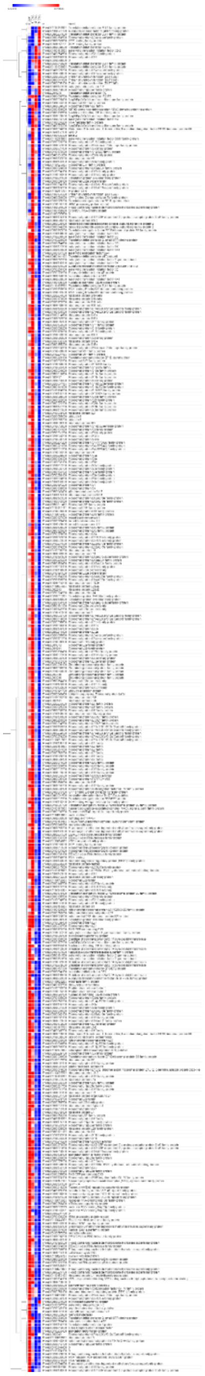
Reference genes

PEL1	Phvul.010G077790.1	Eukaryotic release factor 1 (eRF1) family protein	ACGAAGACAGTTGCGACCTATT	56.35	GGAGCATCCAAAACCTCCCTTA	56.92	0.9480
XERICO	Phvul.008G190100.1	RING/U-box superfamily protein	GATCCCTACCAAACTCCACCTG	57.07	ACACACACTGTCAAACCTGAGT	56.52	0.8898

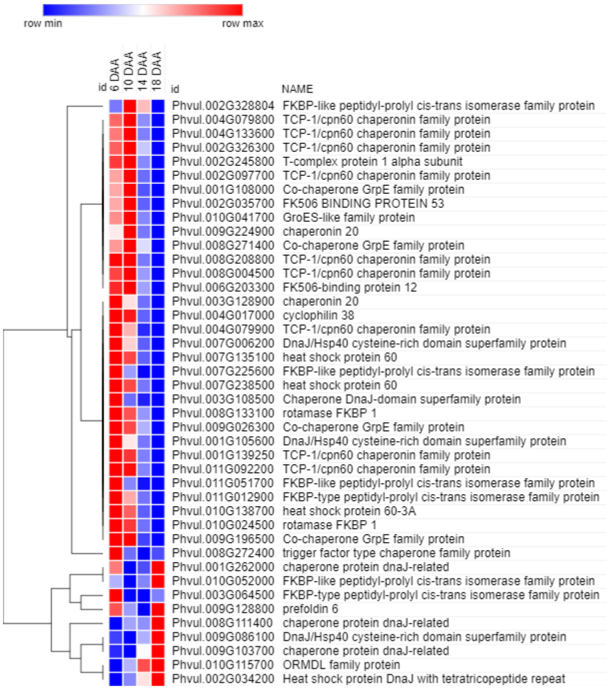
Supplementary Table 3- Correlations between RNA-Seq and RT-qPCR data: correlation values between the FPKM read value for each gene and the average gene expression (RT-qPCR) are depicted for each time point studied. P-value < 0,05

Gene	Dene ID	R^2 (RNA-Seq. vs. RT-qPCR)
ABI3	Phvul.008G122100	93.3%
ABI5	Phvul.001G189400	94.3%
ADG1	Phvul.008G284000	94.5%
AGL15	Phvul.005G035900	99.4%
CRA1	Phvul.007G192800	92.7%
DGs	Phvul.005G045900	79.4%
EXP15	Phvul.009G186400	98.6%
EXPB2	Phvul.007G235300	96.2%
ISA3	Phvul.009G033200	95.8%
LEA	Phvul.001G142000	100.0%
PAP85	Phvul.007G173600	97.8%
PGM	Phvul.002G318400	97.6%
SBE2.2	Phvul.001G187600	97.3%
SUS4	Phvul.003G127500	95.1%
UGP2	Phvul.008G240600	82.1%

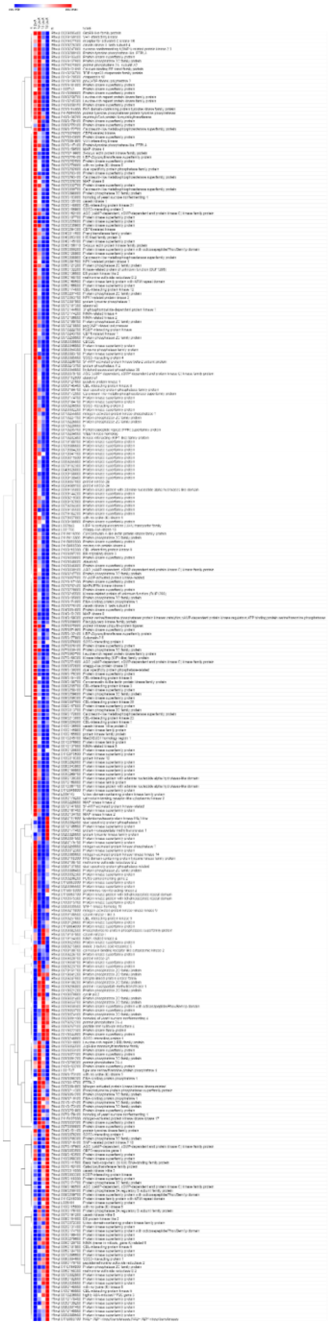
Supplementary Figure 1. Heatmap of transcripts involved in protein amino acid activation between the time points studied - 6, 10, 14 and 18 days after anthesis (DAA). FPKMs were clustered using Euclidean distance and an average linkage.



Supplementary Figure 2. Heatmap of transcripts involved in protein synthesis between the time points studied - 6, 10, 14 and 18 days after anthesis (DAA). FPKMs were clustered using Euclidean distance and an average linkage.



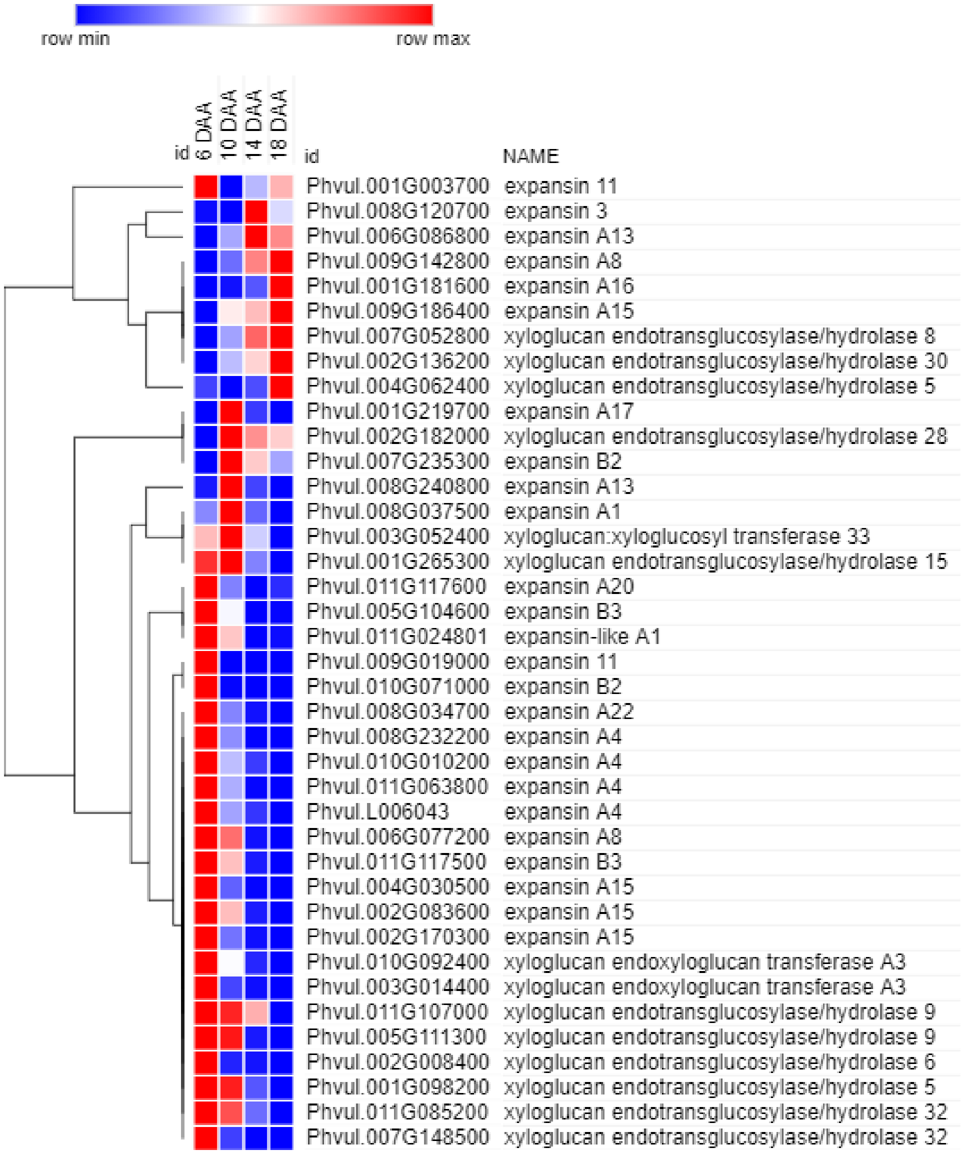
Supplementary Figure 3. Heatmap of transcripts involved in protein folding between the time points studied - 6, 10, 14 and 18 days after anthesis (DAA). FPKMs were clustered using Euclidean distance and an average linkage.



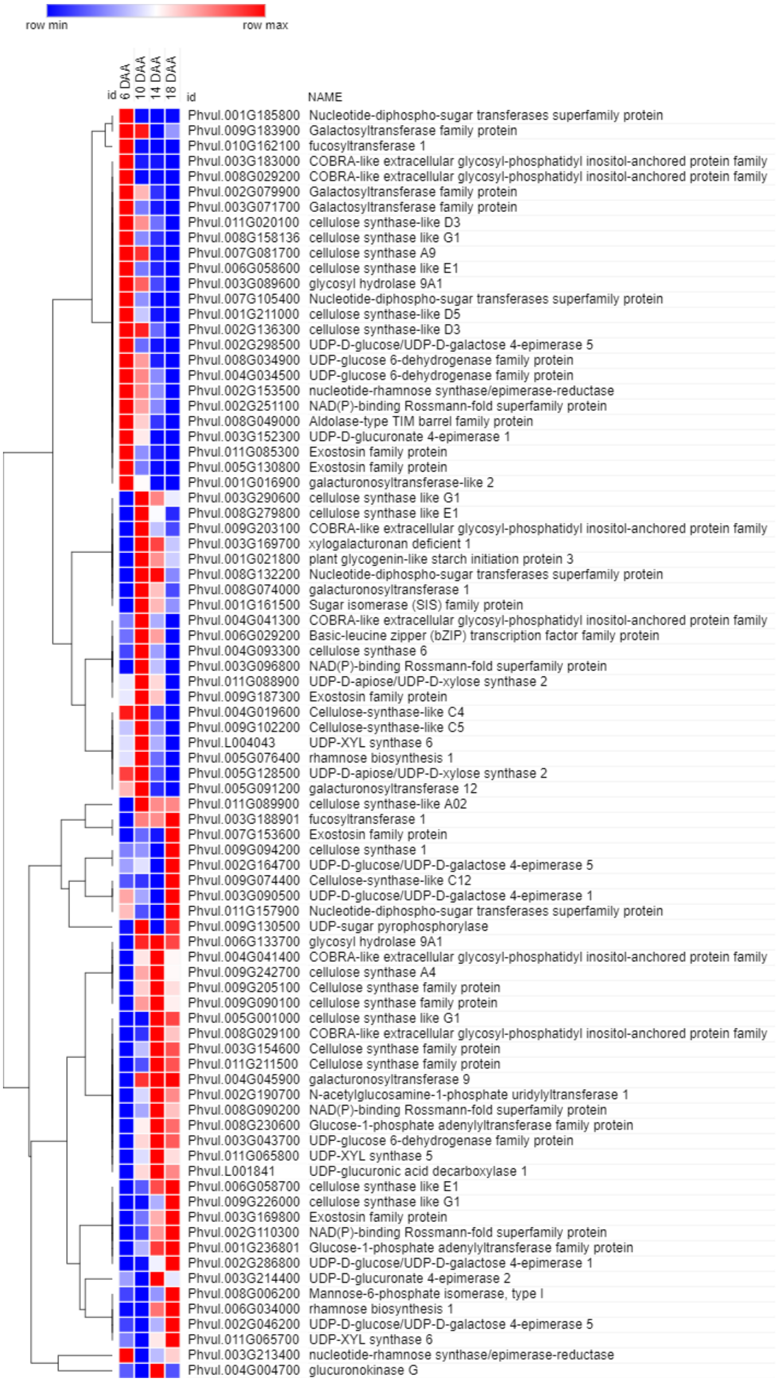
Supplementary Figure 4. Heatmap of transcripts involved in protein post-translational modification between the time points studied - 6, 10, 14 and 18 days after anthesis (DAA). FPKMs were clustered using Euclidean distance and an average linkage.

id	NAME
Phvul.002G031800	Regulator of chromosome condensation (RCC1) family protein
Phvul.002G067400	Regulator of chromosome condensation (RCC1) family protein
Phvul.002G253100	Regulator of chromosome condensation (RCC1) family protein
Phvul.005G083200	Regulator of chromosome condensation (RCC1) family protein
Phvul.001G113200	ankyrin repeat family protein / regulator of chromosome condensation (RCC1) family protein
Phvul.009G175800	ATPase, AAA-type, CDC48 protein
Phvul.001G189700	Tubulin/FtsZ family protein
Phvul.008G230700	Regulator of chromosome condensation (RCC1) family protein
Phvul.002G315300	stomatal cytokinesis defective / SCD1 protein (SCD1)
Phvul.008G115800	Regulator of chromosome condensation (RCC1) family protein
Phvul.002G285500	structural maintenance of chromosomes 5
Phvul.004G113600	Transducin/WD40 repeat-like superfamily protein
Phvul.002G299500	centromere protein C
Phvul.003G076200	mitotic checkpoint family protein
Phvul.004G029000	structural maintenance of chromosome 3
Phvul.004G099000	Regulator of chromosome condensation (RCC1) family with FYVE zinc finger domain
Phvul.004G139200	paralog of ARC6
Phvul.002G037700	Transducin family protein / WD-40 repeat family protein
Phvul.005G172500	centromere protein C
Phvul.006G064300	cell division control 6
Phvul.006G086822	Regulator of chromosome condensation (RCC1) family with FYVE zinc finger domain
Phvul.007G038400	CYCLIN D3;1
Phvul.007G044000	Transducin/WD40 repeat-like superfamily protein
Phvul.007G044700	Transducin/WD40 repeat-like superfamily protein
Phvul.007G149432	ATPase, AAA-type, CDC48 protein
Phvul.001G258800	DNA-binding HORMA family protein
Phvul.001G198300	Transducin family protein / WD-40 repeat family protein
Phvul.001G168400	CLIP-associated protein
Phvul.001G055400	mitotic-like cyclin 3B from Arabidopsis
Phvul.001G000500	cyclin-dependent kinase B2;2
Phvul.001G024900	CYCLIN D3;1
Phvul.011G038600	NAD(P)-binding Rossmann-fold superfamily protein
Phvul.009G037000	MSCS-like 2
Phvul.004G106800	septum site-determining protein (MIND)
Phvul.011G203500	Ribosome recycling factor
Phvul.011G165500	anaphase promoting complex 6
Phvul.011G007300	P-loop containing nucleoside triphosphate hydrolases superfamily protein
Phvul.009G161400	MEI2-like 4
Phvul.009G097500	mitotic-like cyclin 3B from Arabidopsis
Phvul.009G015100	Regulator of chromosome condensation (RCC1) family protein
Phvul.008G253500	cyclin-dependent kinase B1;2
Phvul.008G021500	CDC27 family protein
Phvul.008G019500	MEI2-like 4
Phvul.004G047500	cell division protein ftsH, putative
Phvul.002G059000	alpha/beta-Hydrolases superfamily protein
Phvul.007G192600	Structural maintenance of chromosomes (SMC) family protein
Phvul.008G027700	Regulator of chromosome condensation (RCC1) family protein
Phvul.008G077100	phosphorylethanolamine cytidylyltransferase 1
Phvul.009G067600	CYCLIN D3;2
Phvul.009G196800	Structural maintenance of chromosomes (SMC) family protein
Phvul.007G020500	GTP binding
Phvul.009G036900	MSCS-like 2
Phvul.004G064300	Regulator of chromosome condensation (RCC1) family protein
Phvul.005G009100	prohibitin 3
Phvul.006G049100	homolog of bacterial cytokinesis Z-ring protein FTSZ 1-1
Phvul.007G172400	ribosome recycling factor, chloroplast precursor
Phvul.007G227600	Tubulin/FtsZ family protein
Phvul.001G236500	Regulator of chromosome condensation (RCC1) family protein
Phvul.011G083800	ATPase, AAA-type, CDC48 protein
Phvul.004G154550	MSCS-like 3
Phvul.008G130700	cell division cycle 45
Phvul.008G197100	maternal effect embryo arrest 22

Supplementary Figure 5. Heatmap of transcripts involved in cell division between the time points studied - 6, 10, 14 and 18 days after anthesis (DAA). FPKMs were clustered using Euclidean distance and an average linkage.

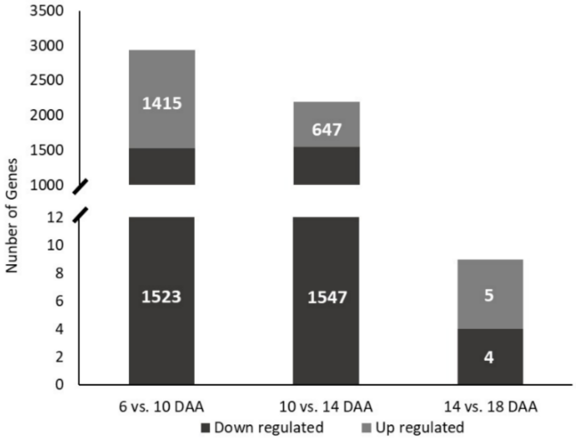


Supplementary Figure 6. Heatmap of transcripts involved in cell wall modification (expansion) between the time points studied - 6, 10, 14 and 18 days after anthesis (DAA). FPKMs were clustered using Euclidean distance and an average linkage.

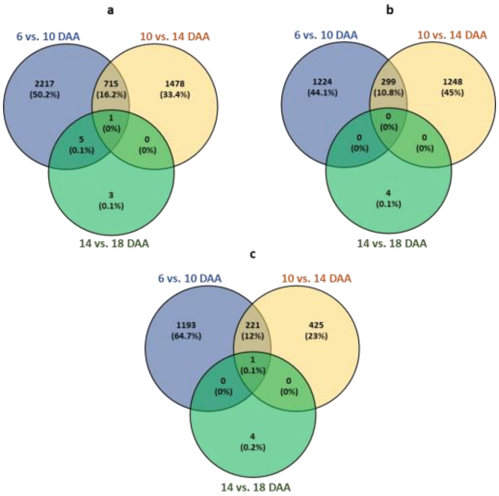


id	NAME
Phvul.001G185800	Nucleotide-diphospho-sugar transferases superfamily protein
Phvul.009G183900	Galactosyltransferase family protein
Phvul.010G162100	fucosyltransferase 1
Phvul.003G183000	COBRA-like extracellular glycosyl-phosphatidyl inositol-anchored protein family
Phvul.008G029200	COBRA-like extracellular glycosyl-phosphatidyl inositol-anchored protein family
Phvul.002G079900	Galactosyltransferase family protein
Phvul.003G071700	Galactosyltransferase family protein
Phvul.011G020100	cellulose synthase-like D3
Phvul.008G158136	cellulose synthase like G1
Phvul.007G081700	cellulose synthase A9
Phvul.006G058600	cellulose synthase like E1
Phvul.003G089600	glycosyl hydrolase 9A1
Phvul.007G105400	Nucleotide-diphospho-sugar transferases superfamily protein
Phvul.001G211000	cellulose synthase-like D5
Phvul.002G136300	cellulose synthase-like D3
Phvul.002G298500	UDP-D-glucose/UDP-D-galactose 4-epimerase 5
Phvul.008G034900	UDP-glucose 6-dehydrogenase family protein
Phvul.004G034500	UDP-glucose 6-dehydrogenase family protein
Phvul.002G153500	nucleotide-rhamnose synthase/epimerase-reductase
Phvul.002G251100	NAD(P)-binding Rossmann-fold superfamily protein
Phvul.008G049000	Aldolase-type TIM barrel family protein
Phvul.003G152300	UDP-D-glucuronate 4-epimerase 1
Phvul.011G085300	Exostosin family protein
Phvul.005G130800	Exostosin family protein
Phvul.001G016900	galacturonosyltransferase-like 2
Phvul.003G290600	cellulose synthase like G1
Phvul.008G279800	cellulose synthase like E1
Phvul.009G203100	COBRA-like extracellular glycosyl-phosphatidyl inositol-anchored protein family
Phvul.003G169700	xylogalacturonan deficient 1
Phvul.001G021800	plant glycogenin-like starch initiation protein 3
Phvul.008G132200	Nucleotide-diphospho-sugar transferases superfamily protein
Phvul.008G074000	galacturonosyltransferase 1
Phvul.001G161500	Sugar isomerase (SIS) family protein
Phvul.004G041300	COBRA-like extracellular glycosyl-phosphatidyl inositol-anchored protein family
Phvul.006G029200	Basic-leucine zipper (bZIP) transcription factor family protein
Phvul.004G093300	cellulose synthase 6
Phvul.003G096800	NAD(P)-binding Rossmann-fold superfamily protein
Phvul.011G088900	UDP-D-apiose/UDP-D-xylose synthase 2
Phvul.009G187300	Exostosin family protein
Phvul.004G019600	Cellulose-synthase-like C4
Phvul.009G102200	Cellulose-synthase-like C5
Phvul.L004043	UDP-XYL synthase 6
Phvul.005G076400	rhamnose biosynthesis 1
Phvul.005G128500	UDP-D-apiose/UDP-D-xylose synthase 2
Phvul.005G091200	galacturonosyltransferase 12
Phvul.011G089900	cellulose synthase-like A02
Phvul.003G188901	fucosyltransferase 1
Phvul.007G153600	Exostosin family protein
Phvul.009G094200	cellulose synthase 1
Phvul.002G164700	UDP-D-glucose/UDP-D-galactose 4-epimerase 5
Phvul.009G074400	Cellulose-synthase-like C12
Phvul.003G090500	UDP-D-glucose/UDP-D-galactose 4-epimerase 1
Phvul.011G157900	Nucleotide-diphospho-sugar transferases superfamily protein
Phvul.009G130500	UDP-sugar pyrophosphorylase
Phvul.006G133700	glycosyl hydrolase 9A1
Phvul.004G041400	COBRA-like extracellular glycosyl-phosphatidyl inositol-anchored protein family
Phvul.009G242700	cellulose synthase A4
Phvul.009G205100	Cellulose synthase family protein
Phvul.009G090100	cellulose synthase family protein
Phvul.005G001000	cellulose synthase like G1
Phvul.008G029100	COBRA-like extracellular glycosyl-phosphatidyl inositol-anchored protein family
Phvul.003G154600	Cellulose synthase family protein
Phvul.011G211500	Cellulose synthase family protein
Phvul.004G045900	galacturonosyltransferase 9
Phvul.002G190700	N-acetylglucosamine-1-phosphate uridylyltransferase 1
Phvul.008G090200	NAD(P)-binding Rossmann-fold superfamily protein
Phvul.008G230600	Glucose-1-phosphate adenylyltransferase family protein
Phvul.003G043700	UDP-glucose 6-dehydrogenase family protein
Phvul.011G065800	UDP-XYL synthase 5
Phvul.L001841	UDP-glucuronic acid decarboxylase 1
Phvul.006G058700	cellulose synthase like E1
Phvul.009G226000	cellulose synthase like G1
Phvul.003G169800	Exostosin family protein
Phvul.002G110300	NAD(P)-binding Rossmann-fold superfamily protein
Phvul.001G236801	Glucose-1-phosphate adenylyltransferase family protein
Phvul.002G286800	UDP-D-glucose/UDP-D-galactose 4-epimerase 1
Phvul.003G214400	UDP-D-glucuronate 4-epimerase 2
Phvul.008G006200	Mannose-6-phosphate isomerase, type I
Phvul.006G034000	rhamnose biosynthesis 1
Phvul.002G046200	UDP-D-glucose/UDP-D-galactose 4-epimerase 5
Phvul.011G065700	UDP-XYL synthase 6
Phvul.003G213400	nucleotide-rhamnose synthase/epimerase-reductase
Phvul.004G004700	glucuronokinase G

Supplementary Figure 7. Heatmap of transcripts involved in cell wall synthesis between the time points studied - 6, 10, 14 and 18 days after anthesis (DAA). FPKMs were clustered using Euclidean distance and an average linkage.

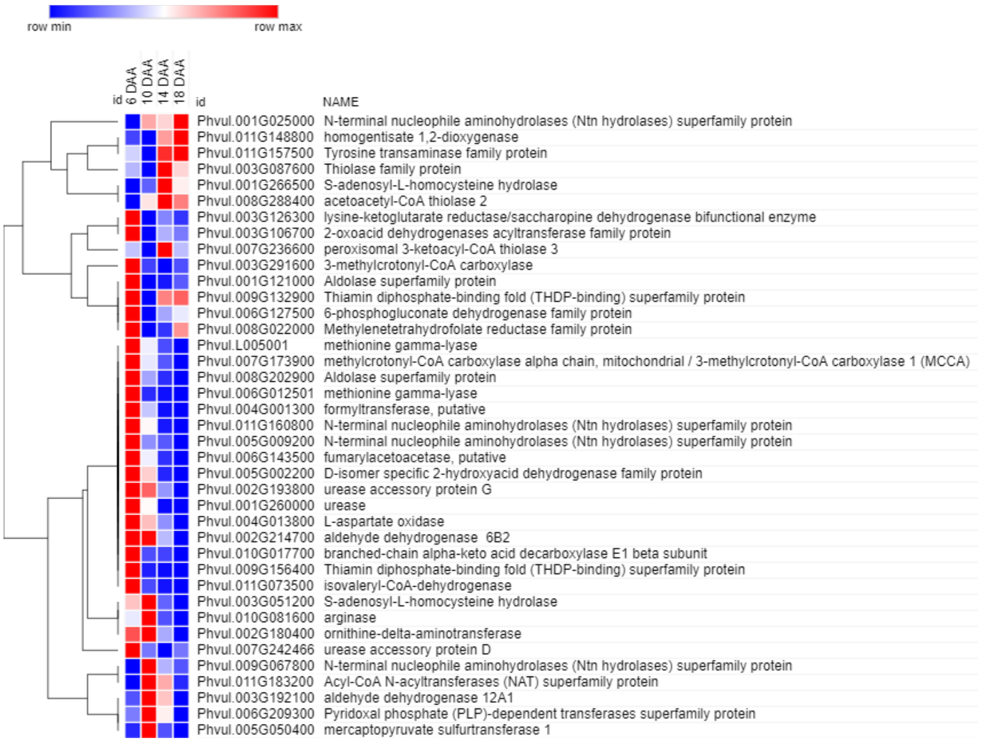


Supplementary Figure 8. Number of up- (grey square) and downregulated (black square) genes in the early stages of developing seeds of P. vulgaris with a minimum of 100 reads and a Log2FC |≥1| and a adj. p value ≤0.001. DAA: days after anthesis.

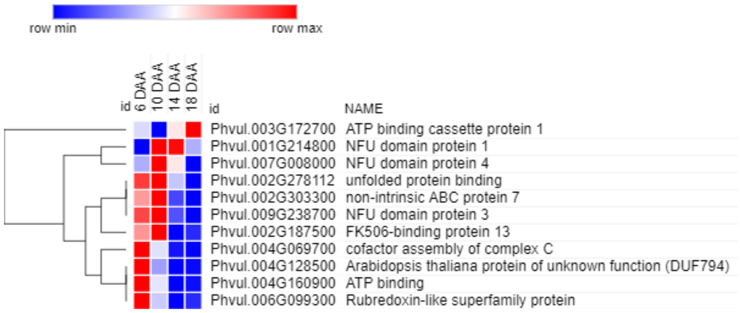


Supplementary Figure 9. Venn diagram analysis of differentially expressed genes identified in the transition from the embryo development to seed filling in common bean seeds. The overlapping regions denote common genes among the comparisons studied – 6 vs. 10, 10 vs. 14, 14 vs 18 days after anthesis (DAA). a Total DEGs; b downregulated genes; c upregulated genes.

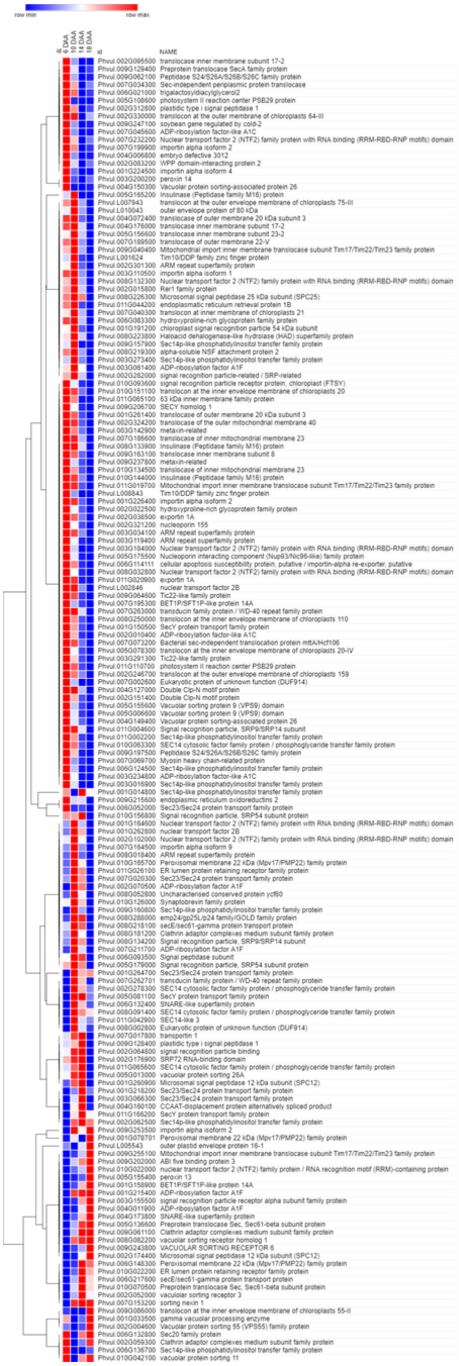
Supplementary Figure 10. Heatmap of transcripts involved in amino acid synthesis between the time points studied - 6, 10, 14 and 18 days after anthesis (DAA). FPKMs were clustered using Euclidean distance and an average linkage.



Supplementary Figure 11. Heatmap of transcripts involved in amino acid degradation between the time points studied - 6, 10, 14 and 18 days after anthesis (DAA). FPKMs were clustered using Euclidean distance and an average linkage.



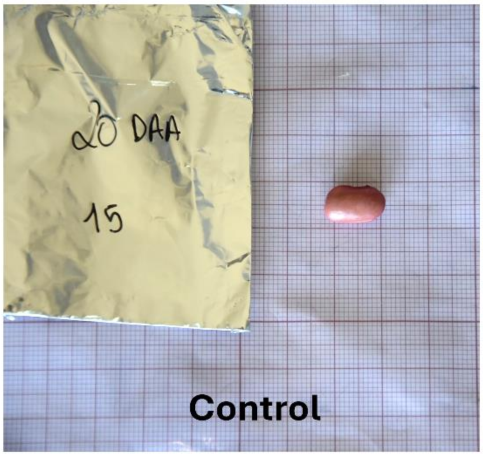
Supplementary Figure 12. Heatmap of transcripts involved in protein assembly between the time points studied - 6, 10, 14 and 18 days after anthesis (DAA). FPKMs were clustered using Euclidean distance and an average linkage.



Supplementary Figure 13. Heatmap of transcripts involved in protein targeting between the time points studied - 6, 10, 14 and 18 days after anthesis (DAA). FPKMs were clustered using Euclidean distance and an average linkage.

Chapter III

Hormonal and transcriptional changes in developing common bean (*Phaseolus vulgaris* L.) seeds under heat stress



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Author contributions to the chapter:

Cláudia Lopes participated in the experimental setup and design, performed the experiments except for UHPLC-MS, analysed the data and wrote the chapter.

Abstract

Common bean (*Phaseolus vulgaris* L.) is a major grain legume worldwide, but rising global temperatures threaten its productivity by increasing the incidence of heat stress (HS). This study investigates the impact of HS on seed filling, focusing on phytohormone-mediated regulation. A combined approach using hormone quantification (UHPLC-MS), transcriptomics (RNA-Seq) and histochemistry was defined. Plants were allowed to set seeds under control (25°C Day/18°C night) and elevated (32°C Day/25°C night) temperature. In seeds developing under HS the filling period was shortened, ending 18 days after anthesis (DAA) and a decrease in seed biomass, cotyledons' area and cotyledons' parenchymal cells area was observed. The number of seeds per plant also decreased and a reduction in the accumulation of storage compounds was observed, with the down regulation of the expression of the main genes of the biosynthesis of starch and storage proteins. HS changed phytohormonal profiles, increasing indole-3-acetic acid (IAA), cytokinin (CK), and salicylic acid (SA) levels at 6 and 10 DAA, potentially driven by the upregulation of auxin transporter genes, cytokinin (IPT), and SA (CM, PAL) biosynthesis genes. Also, at 14, 18 and 20 DAA, seed content of Abscisic acid (ABA) and Jasmonic acid (JA), was significantly lower compared to control, likely due to the upregulation of PP2C, as well as increased expression of JA negative regulator (JAZ). Additionally, HS increased ethylene-related gene expression at 14 and 18 DAA, upregulating biosynthesis and signalling genes. This knowledge establishes a base to guide seed quality improvement in the context of climate change.

Keywords: *Phaseolus vulgaris,* Heat stress, Seed development, Phytohormones, Transcriptomics, Hormonal regulation

1. Introduction

Common bean (*Phaseolus vulgaris* L.) is one of the most important legumes crops worldwide, serving as a primary source of protein, fibber and essential micronutrients for millions of people, especially in Africa and South America southern countries (González-Guerrero et al., 2016; Myers & Kmiecik, 2017). However, common bean is highly susceptible to environmental stresses, with heat stress (HS) posing a significant threat to its productivity (Soltani et al., 2019; Vargas et al., 2021). Heat stress has been shown to reduce pollen viability, leading to lower yields under high-temperature conditions (Kumar et al., 2023; Vargas et al., 2021). It also negatively influences stomatal movement, chlorophyll synthesis and degradation and the functionality of photosynthetic proteins (Khan et al., 2024).

During the reproductive and seed grain-filling stages, heat stress significantly impacts seed development and quality in cereal crops, since it reduces photosynthetic capacity, dry matter accumulation and grain yield (Farooq et al., 2011). Studies have shown that elevated temperatures affect phloem loading and sucrose translocation (Soltani et al., 2019), disrupts source-sink activities and induces undesirable biochemical changes in seeds, affecting carbohydrates, proteins, oils and other nutritional components (Kumar et al., 2023).

Legumes are also highly sensitive to heat stress, particularly during the reproductive phase (Saxena et al., 1988; Solh et al., 1994). Chickpea, lentil, pea and faba bean are susceptible to high temperatures, with chickpea showing higher critical temperature thresholds (Saxena et al., 1988). Among legumes, heat stress significantly impacts *P. vulgaris* productivity, affecting various physiological processes throughout the reproductive phase. High night temperatures appear particularly detrimental, reducing yield by up to 37% (Vargas et al., 2021). In *P. vulgaris*, exposure to heat stress has been shown to reduce the number of pods and seeds per plant, as well as decrease pod length and weight (Konsens et al., 1991). Similarly, reproductive processes such as pod and seed set are negatively affected under high temperature conditions (Gross & Kigel, 1994; Vara Prasad et al., 2002). Additionally, heat stress can trigger premature flower and pod abscission, further compromising reproductive success in *P. vulgaris* (Monterroso & Wien, 1990). Interestingly, tepary bean (*Phaseolus* acutifolius) demonstrates superior heat tolerance compared to common bean, attributed to its ability to regulate stomatal opening and photosynthetic efficiency (Carmenza Muñoz et al., 2021). Additionally, epigenetic mechanisms, such as DNA methylation, histone modification and non-coding RNAs, have been implicated in regulating gene expression under abiotic stress conditions in legumes, including heat stress responses (Yung et al., 2023). Heat stress affects multiple stages of reproductive development, including gametophyte development, fertilization and early seed and fruit development, with male reproductive development being particularly susceptible (Y. Liu et al., 2019). These changes involve complex molecular responses, including the activation of heat shock proteins, antioxidants, metabolites and phytohormones (Y. Liu et al., 2019).

Plant phytohormones play crucial roles in regulating stress responses and seed development. Abscisic acid (ABA) and Gibberellins (GA) are key phytohormones that antagonistically control seed development and germination (reviewed by Kozaki & Aoyanagi, 2022). ABA is essential for normal plant growth and mediates responses to various abiotic stresses. Gibberellins participate in numerous developmental processes, including seed development and germination (reviewed by Vishal & Kumar, 2018). Jasmonic acid (JA), along with other phytohormones like Salicylic acid (SA) and Ethylene (ET), integrates environmental stimuli to regulate plant defence responses against abiotic stresses, including heat stress (reviewed by Li et al., 2021). The intricate crosstalk among these phytohormones and their signalling pathways enables plants to adapt to adverse environmental conditions (reviewed by Verma et al., 2016). Understanding these hormone-mediated regulatory mechanisms is crucial for developing stress-tolerant crops and addressing challenges posed by global climate change (Li et al., 2021; Verma et al., 2016). HS affects reproductive development by disrupting hormone-regulated processes during pollination, early fruit set, and seed formation in legume crops (Ozga et al., 2017). Ofir et al., (1993) reported a strong association between reduced pod and seed numbers under heat stress and a decline in the export of indole-3-acetic acid (IAA) from the flowers. In pea, HS decreases bioactive Gibberellin levels, potentially inhibiting growth-related processes, while increasing Auxin (IAA) and abscisic acid levels, which may enhance seed sink strength (Kaur et al., 2021). Nevertheless, while some knowledge exists about hormone regulation in seed development under heat stress in model plant species, a better understanding of its regulation in non-endospermic seed legumes, particularly in common bean, is crucial to ensure food security under changing climate conditions.

The research conducted herein aimed to address several key questions regarding the effect of heat stress in seed development. Specifically, we aimed to answer the following questions:

-Does HS alter the timing of the transition from embryogenesis to seed filling?

-How does HS affect the duration of seed filling and the final seed size compared to control conditions? Does HS influence the number of seeds per plant?

-Does HS impact the accumulation of storage compounds during seed development?

-How do hormonal profiles change under HS and at which developmental stages do these changes occur? How does HS affect hormonal regulation under HS?

To address these questions, we evaluated the effect of heat stress in *P. vulgaris* SER16 seeds at 6, 10, 14, 18, and 20 DAA using a multidisciplinary approach that integrates morphology, histochemistry, phytohormone quantification and transcriptomic data. By combining these methodologies, we aimed to uncover the mechanisms by which HS disrupts seed formation and storage compounds accumulation. The insights gained from this study will contribute to the broader effort to enhance *P. vulgaris* resilience to climate change and improve strategies for sustaining crop productivity under increasing temperatures.

2. Material and Methods

2.1 Plant material and growth conditions

Seeds of the *P. vulgaris* genotype SER 16 were germinated onto water soaked paper in Petri dishes at 27 °C for 2 days, followed by 3 days at 23 °C, always in the dark as described in Parreira et al., (2016). Seedlings were individually transferred to 2.5 l pots containing a (2:1:1) mixture of commercial soil (Compo Sana S.A., Barcelona, Spain), peat and vermiculite, respectively. Seedlings were grown in growth chamber (Fitoclima 5.000 EH, ARALAB, Portugal) with controlled environmental conditions, with 50% humidity, photoperiod of 16/8-h day/night and light intensity of 400 μmol m−2 s−1, until flower buds appear. Control plants were grown at 25/18°C day/night and heat stress plants grown at 32/25°C day/night. Both conditions were watered 3 times per week.

SER16 flowers were tagged and pods/seeds were harvested at 6, 10, 14, 18 and 20 days after anthesis (DAA). Harvested seeds were divided into 4 batches: two immediately frozen in liquid nitrogen and stored at −80 °C for molecular (transcriptomic) analyses and phytohormone quantification; another used to measure seed length, fresh and dry weight to characterize the seed development process, while the remaining batch was fixed for histochemical analyses.

2.2 Morphological characterization of the early steps of seed development

For the morphological analyses, two seeds from 15 individual plants were used, both from the control (CP) and under heat stress (HS), in each of the time periods studied (6, 10, 14, 18 and 20 DAA). Immediately after harvesting, seeds were photographed and seed length was measured, using ImageJ (Schneider et al., 2012). The seeds were weighted to quantify the seed fresh weight. Later, after being submitted for 15 days to 70°C the seed dry weight was weighted.

2.3 Physiological characterization of plants during seed sampling

To assess the physiological responses of plants under heat stress nine plants grown under control conditions and nine grown under heat stress were measured during sampling. Relative Water Content (RWC) was measured in accordance with (Čatský, 1960), three leaf discs were removed from each plant and their fresh weight (FW) was measured. Leaf discs were placed in water in the dark at room temperature until the next day. The following day, excess water from the discs was removed and the turgid weight (TW) was measured. Finally, the leaf discs were placed for one week in the oven at 70ºC to measure the dry weight (DW). Having the values of FW, TW, and DW, the RWC was calculated through the formula: %RWC=(FW-DW)/(TW-DW) ×100.

Photosynthetic efficiency and performance were measured using a leaf infrared gas-exchange analyzer (LCpro+, (ADC BioScientific, UK)). Stomatal conductance (gs), transpiration (E) and photosynthetic rate (CO2 assimilation rate) (A), Water use efficiency (A/E) parameters were evaluated at 347 PAR of light intensity. Chlorophylls a and b, and carotenes and xanthophylls (Ccx) were quantified according to (Wintermans & De Mots, 1965).

2.4 Histology assays

Seeds from control and heat stress plants were used for histochemical analysis, 4 biological replicates per time point (6, 10, 14, 18 and 20 DAA). Seeds were submerged in ice-cold FAA fixative solution [FAA: 47.5% ethanol from Carlo Erba (Val de Reuil, France), 3.7% formaldehyde solution from Sigma-Aldrich (St. Louis, MO, USA) and 5% glacial acetic acid from Scharlau (Barcelona, Spain)]. The immersed material was exposed to moderate vacuum for 1 hour to pull the air out of the tissue and force the infiltration of the fixative solution. The material was fixed overnight at 4 °C and then washed with TBS 1X (0.05M Tris-HCl from Carl Roth (Karlsruhe, Germany), 0.15M sodium chloride (NaCl) pH 7.6 from Merck (Darmstadt, Germany). Seeds were sectioned, by first securing the material in the vibratome's block with a drop of Super Glue and set for 15–20 min at room temperature. Transversal sections at the seed hilum level with 25 µm were cut using the vibratome 1000 Plus. Each section was placed in a microscope slide coated with 200µl of poly(lysine) and left to dry. Six sections per fixed seed were stained with Calcofluor White Staining, Coomassie Blue Staining or Periodic acid Schiff to stain cellulose in cell walls, proteins and carbohydrates, respectively (Pellicciari & Biggiogera, 2017). Images from the sections were captured using a LEICA DM6 B microscope. Calcofluor White Staining images were captured using UV light, while for Coomassie Blue Staining and Periodic acid Schiff a bright-field lighting was used.

For each timepoint studied, Calcofluor slides were used to calculate cotyledon parenchyma cell area and cotyledon section area using ImageJ software (Schneider et al., 2012). To measure the cotyledon parenchyma cell area and cotyledon section area, after the scale is stablished, the cells and the cotyledons, were selected and calculated using ImageJ. Coomassie and Periodic acid Schiff images were converted to a grayscale (16 bit), inverted and the mean grey value for pixel intensity retrieved by the software was used to estimate overall protein and carbohydrate accumulation.

2.5 Phytohormone quantification

Gibberellins (GA), indolacetic acid (IAA) jasmonic acid (JA) abscisic acid (ABA) salicylic acid (SA), cytokinins: dihydrozeatine (DHZ), isopentyladenine, (iP) and t-zeatine (tZ) were quantified by ultra-high performance liquid chromatography–mass spectrometry (UHPLC-MS) using a Thermo Scientific™ Q Exactive™ Hybrid Quadrupole-Orbitrap mass spectrometer at Institute for Plant Molecular and Cell Biology (IBMCP), Spain. Plant hormone extraction and separation were conducted following the method by (Seo et al., 2011).

2.6 RNA Extraction, Quantification and Quality Assessment

Frozen seeds were ground to a fine powder in liquid nitrogen using a mortar and pestle for total mRNA isolation. RNA was extracted as described in Parreira et al, (2018). Traces of DNA contamination were removed with an Ambion® TURBO™ DNase (Life Technologies, Carlsbad, CA, USA) following the manufacturer´s instructions. RNA quantification and purity were assessed using a NanoDrop™ 2000c Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). Moreover, the Qubit® 2.0 Fluorometer (Thermo Fisher Scientific Inc.) with RNA BR Assay Kit was used to quantify the RNA. RNA purity was estimated based on the A260/280 and A260/230 absorbance ratios and was approximately 2 before DNAse treatment. RNA integrity was assessed by electrophoresis in a 2.0% agarose gel, stained with SYBR® Safe (Life Technologies). The absence of DNA contamination was verified by a standard polymerase chain reaction (PCR) using primers for the *P. vulgaris* ACTIN gene, gene ID: Phvul.001G142500 (Supp table 3). RNA samples were stored at -80°C until needed.

2.7 RNA-Sequencing

Twenty-four RNA-Seq libraries were constructed from three biological replicates from samples harvested at 6, 10, 14 and 18 DAA, twelve RNA-Seq libraries for control conditions and twelve for heat stress conditions. Due to the reduced size of the sampled seeds at 6 DAA, each biological replicate consists of a pool of 200 seeds randomly harvested from 80 plants. For seed samples harvested at 10, 14, 18 and 20 DAA, each biological replicate consists of a pool of 3 to 4 grinded seeds harvested from the same individual. Libraries construction and sequencing was performed by NOVOGENE (Cambridge, UK). Pair-ended cDNA libraries were sequenced on Illumina NovaSeq 6000 platform following manufacturer's recommendations and 150 bp paired-end reads were generated. mRNA was purified from total RNA using poly-T oligo attached magnetic beads and fragmented randomly in fragmentation buffer [NEBNext First Strand Synthesis Reaction Buffer (5X)], followed by cDNA synthesis using random hexamers and reverse transcriptase (RNase H-). Second-strand cDNA synthesis was subsequently performed using buffer (Illumina) with dNTPs, RNase H and *Escherichia coli* polymerase I to generate the second strand by nick-translation. The final cDNA library is ready after a round of purification, adenylation of 3′ ends of DNA fragments, A-tailing, ligation of sequencing adapters, size selection of cDNA fragments of preferentially 150 bp in length with ligated adaptor molecules on both ends were selectively enriched using Illumina PCR Primer Cocktail in a 10 cycle PCR. Library concentration was quantified using a Qubit 2.0 fluorometer (Life Technologies). Insert size was checked on an Agilent 2100 and quantified using qPCR.

2.8 RNA-Seq Bioinformatic Analysis

The original raw data from Illumina was transformed to Sequenced Reads by base calling. Raw data are recorded in a FASTQ file. Raw reads are filtered to remove reads with adapter contamination or reads with low quality. Only clean reads were used in the downstream analyses. The percentage of bases whose correct base recognition rates are greater than 99% and 99.9% (Q20, Q30, respectively), GC content and sequence duplication level of the clean data were calculated. All the downstream analyses were based on the clean data with high quality. Pair-ended clean reads were mapped with HISAT2 (HISAT version 2.1.0; Kim et al 2019) to the reference genome (P.*vulgaris* v2.1, U.S. Department of Energy Joint Genome Institute, Phytozome v12.0: http://phytozome.jgi.doe.gov/). HISAT2 is a fast and sensitive alignment program for mapping next-generation sequencing reads, enabled effective alignment of RNA-seq reads, particularly, reads spanning multiple exons. Because transcriptome annotation is still incomplete, this RNA-seq study revealed novel genes and transcripts. To do that, Cufflinks Reference Annotation Based Transcript (RABT) assembly method was used to assemble the set of transcript isoforms of each bam file obtained in the mapping step. This was done using 'Cuffcompare' that compares Cufflinks assemblies to reference annotation files and help sort out new genes from known ones.

Genes were considered expressed if they present an average raw number ≥ 100 at least in one of the studied timepoints. The expected number of Fragments Per Kilobase of transcript sequence per Millions base pairs sequenced (FPKM) which takes into account the effects of both sequencing depth and gene length on counting of fragments was also used to establish gene expression abundances and profiles using HTSeq software (Anders et al 2014). Differential expression analysis was performed using the DESeq2 R package (1.12.0; TNLIST, Beijing, China). Pair-wise comparisons were done between the same timepoints in control or heat stress conditions (CP6DAA vs HS6DAA, CP10DAA vs HS10DAA, CP14DAA vs HS14DAA, CP18DAA vs HS18DAA). A Benjamini-Hochberg correction, for estimating false discovery rates (FDR) was also applied to the analysis made (Benjamini and Hochberg 1995). Genes which presented a corrected p-value ≤ 0.001 and |Log2(Fold Change - FC) | ≥1 were considered differentially expressed genes (DEGs).

Functional characterization was performed using the MapMan web tools (http://www.plabipd.de/portal/mercator-sequence-annotation). Protein sequences were obtained using BioMart (*Phaseolus vulgaris* genome version

2.1) in Phytozome v.12 (https://phytozome.jgi.doe.gov/) to create a mapping file for the Mercator pipeline. Some genes were also annotated using Phytozome v.12 BLAST.

With the differential expression analysis data, Venn diagrams were generated and obtained from the Venny 2.1 (Oliveros, J.C., 2007-2015) and used to infer the overall distribution of differentially expressed genes (DEG). Heatmaps were constructed using Morpheus online tool, (https://software.broadinstitute.org/morpheus), to show the normalized gene expression among the samples. Clustering was performed based on Euclidean distance and average linkage.

2.9 RT-qPCR validation of RNA-Seq data

Expression of 12 selected genes were analysed by RT-qPCR (Supp table 4) on a Light Cycler® 480 System, using the LightCycler® 480 SYBR Green I Master protocol. These genes were chosen to represent a broad range of differential expression and total transcript counts. 1 µg of total RNA from 3 biological replicates per time point (6, 10, 14 and 18) for control and heat stress conditions, was reverse transcribed, using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) following the manufacturer’s instructions. Primers were designed using the Primer3 software and primer sequences are listed in Supplementary Table 3. PCR amplification efficiencies were tested for all primers for target and reference genes using cDNA two-fold dilution series. Using the geNorm and NormFinder software packages from the GenEx v.5 software (MultiD, Goteborg, Sweden), two reference genes: eukaryotic Release Factor 1 (eRF1) family protein (PEL1; Phvul.010G077790.1) and RING/U-box superfamily protein (XERICO; Phvul.008G190100.1) used by Parreira et al., (2021), were selected for the gene relative expression analysis.

Thermo cycling reactions were carried out following the described conditions: denaturation step at 90°C for 5 min; 45 cycles of amplification at 95°C for 10 s; 10 s at 60°C and 10 s at 72°C. For each reaction, a melting curve (dissociation stage) was performed to detect non-specific PCR products and/or contaminants. A non-template control (NTC), without cDNA, was also included for each primer mix to detect possible contaminations.

2.10 Data availability

RNA-seq data were deposited in the NCBI Sequence Read Archive BioProject under the accession number PRJNA1227159 (SRR32458332, SRR32458331, SRR32458312, SRR32458323, SRR32458322, SRR32458321, SRR32458320, SRR32458319, SRR32458318, SRR32458317, SRR32458330, SRR32458329, SRR32458328, SRR32458327, SRR32458326, SRR32458325, SRR32458316, SRR32458315, SRR32458314, SRR32458313, SRR32458311, SRR32458310, SRR32458309, SRR32458324).

2.11 Statistical analyses

Data from the morphological analysis (seed length, fresh and dry weight) and from histochemical analysis (mean grey value for pixel intensity in Coomassie and Periodic acid Schiff images) was analysed with IBM SPSS Statistics V25.0 software. Data statistical significance was assessed using one-way analysis of variance (ANOVA) coupled with post-hoc Tukey HSD for mean pairwise comparison. Means were considered significantly different when $P \leq 0.05$.

3. Results

3.1 Plant physiological responses: evidence of heat stress without water deficit

Plant physiological parameters measured during seed sampling showed no statistically significant differences between HS and CP conditions across all traits analysed, including relative water content (RWC), photosynthetic rate (A), transpiration rate (E), stomatal conductance (gS), and water use efficiency (Supp. Fig. 1).

Chlorophyll and carotenoid measurements showed slight, non-significant reductions in chlorophyll a and b in plants under HS, with total chlorophyll remaining stable. Significant differences were observed in pigment ratios and carotenoid content, including lower chlorophyll a/b and higher chlorophyll to carotenoid ratios in plants under HS (Supp. Fig. 2).

3.2 Morphological and histochemical changes in seeds: influence of heat stress on development and storage compounds accumulation

In plants under control conditions, seed fresh weight (SFW) increased from 6 DAA, peaking at approximately 30 DAA, followed by a slight decline. In plants under HS, SFW increased until 18 DAA and then began to decline, indicating a shorter period of seed filling. The same pattern was observed for seed dry weight (SDW). This suggests that, in *P. vulgaris* SER16 seeds, HS disrupts the accumulation of storage compounds, leading to a seed reduced final weight compared to seeds in CP. In the CP, seed length (SL) increased rapidly, reaching its maximum around 30 DAA before a slight reduction. In contrast, in plants under HS, SL remained consistently lower than in CP throughout the entire growth period. The difference in SL between the two conditions became more apparent after 18 DAA, with seeds in plants under HS showing significantly shorter lengths during the final stages of development (Fig. 1). Not only HS shortens the seed filling period but also accelerates the onset of the desiccation phase, limiting both biomass accumulation and seed elongation during critical growth stages.

Moreover, HS significantly reduced the number of seeds produced per plant. On average, plants exposed to HS produced 28 seeds per plant, whereas those under CP conditions produced 70 seeds per plant. This substantial reduction in seed production further highlights the detrimental impact of HS on overall plant reproductive success and productivity.

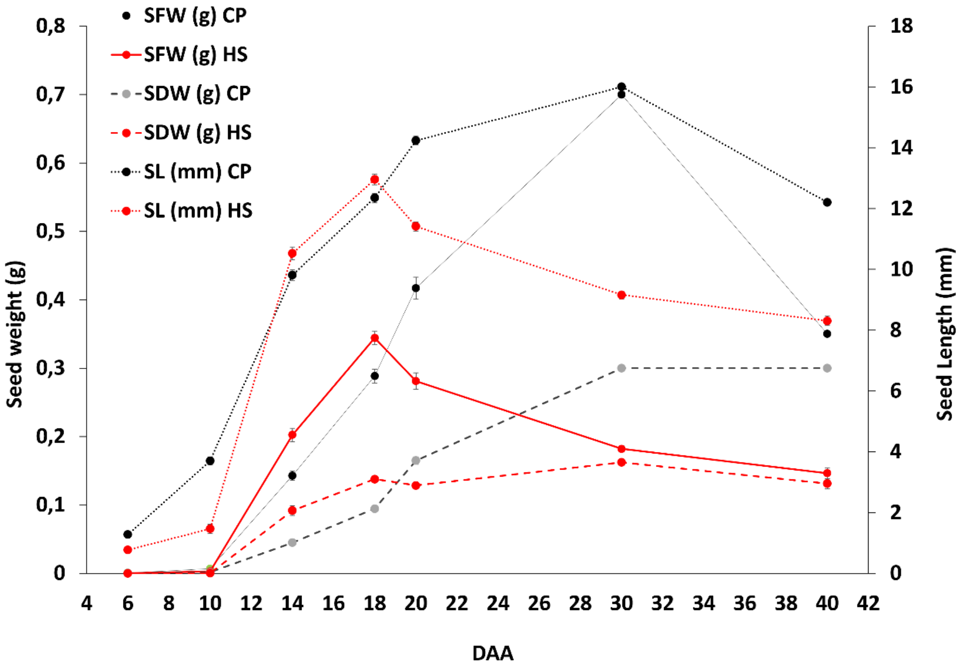

Figure 1- Characterization of seed development in *Phaseolus vulgaris* at 6, 10, 14, 18 and 20 DAA (days after anthesis) during control (CP) (25/18°C day/night) and heat stress (HS) (32/25°C day/night) conditions. Seed fresh weight (SFW), Seed dry weight (SDW), Seed length (SL), Heat Stress (HS), Control (C).

In the seeds of the control plants, the seed coat changes colour from green to orange at 20 DAA. In contrast, in plants under HS, seed coat turns brown at 10 DAA indicating the onset of the desiccation phase, as confirmed by morphological characterization. In addition, in contrast to CP, under HS there is a more rapid increase in seed size and a greater accumulation of storage compounds between 10 and 14 DAA (Fig. 2).

Furthermore, it is possible to observe that from 18 to 20 DAA, under HS, cotyledon cell mean area begins to decrease as the seed is starting to undergo desiccation (Fig. 2D), while under CP cotyledon cell mean area progressively increases until 20 DAA. Despite relevant differences between CP and HS plants, in the cotyledon transversal mean area is only observed at 14 DAA (Fig. 2E).

Heat stress induces a significant ($p<0,05$) higher accumulation of starch and proteins at 14 DAA (Fig. 2F, 2G). However, for starch, this ratio changes at 18 DAA, with a significantly higher accumulation in seeds of CP, that is maintained at 20 DAA (Fig. 2F). As for protein accumulation at 20 DAA, the same amount for both conditions is observed (Fig. 2G).

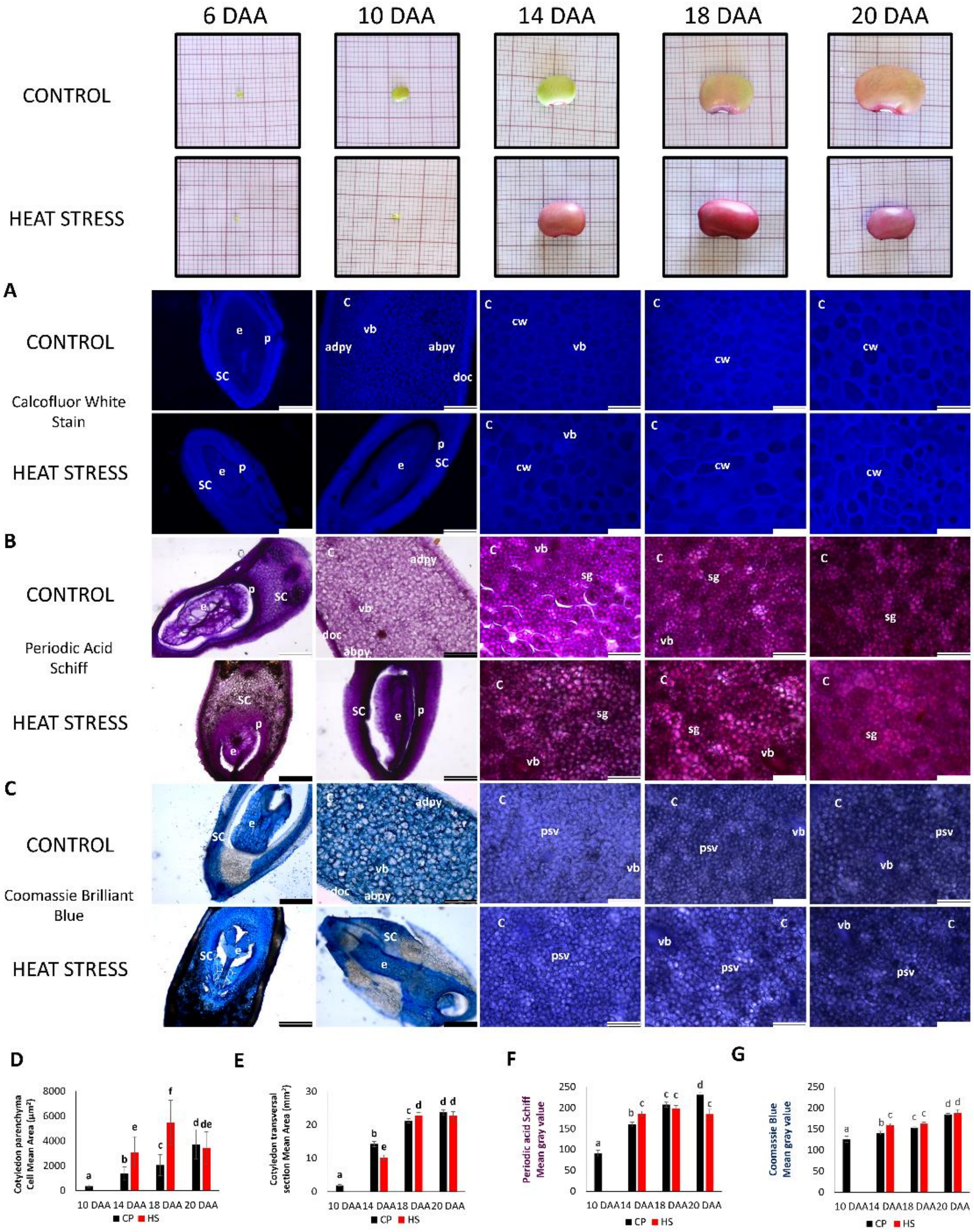

Figure 2- Histochemistry of *Phaseolus vulgaris* seed development under control and heat stress (6, 10, 14, 18 and 20 days after anthesis (DAA)). **A** cell walls, **B** protein

and **C** starch accumulation. The cell walls, proteins and starch were stained with Calcofluor white stain, Coomassie Brilliant Blue and Periodic Acid Schiff, respectively. The scale bar indicate 124,4 µm. abpy - cotyledon abaxial parenchyma; adpy - cotyledon adaxial parenchyma; C- cotyledon; cw - cell wall; doc - dermal cell complex; e - embryo; p - parenchyma; psv - protein storage vacuoles; SC - seed coat; sg - starch grains; vb - vascular bundle. **D** cotyledon parenchyma cell mean area. **E** cotyledon transverse section mean area, **F** mean grey value from Periodic Acid Schiff staining, **G** mean grey value from Coomassie Brilliant Blue staining.

3.3 Heat stress induces a change in the phytohormone's profile

Except for Jasmonic (JA) and Abcisic acid (ABA), the amount of all the other quantified phytohormones decrease to almost 0 ng per g of FW, at 14 DAA, in seeds of plants either under control or HS.

Seeds of CP exhibited a peak of ABA at 14 DAA, followed by a slight decline afterwards, while the concentration of ABA in seeds of plants under HS is significantly lower at 14, 18 and 20 DAA (Fig. 3A). At 6 and 10 DAA, JA levels were low and stable in seeds of CP or plants under HS. However, between 10 and 14 DAA, JA levels in seeds of CP start to increase, continuing to rise until 20 DAA, with significant differences at 18 and 20 DAA compared to seeds of plants under HS. In contrast, JA levels in seeds of plants under HS remained consistently low, showing minimal variation across the different time points (Fig. 3B).

Both GA_4 and GA_1 concentrations are relatively low when compared to other phytohormones, except for iP, and decrease along seed development either on seeds of CP or of plants under HS (Fig. 3C, 3D). Despite, there is a significant difference ($p < 0.05$) at 6 DAA: while in CP, GA_4 seed concentration is significantly higher at this date, the opposite is observed with GA_1, which concentration is significantly higher at 6 DAA.

Indole-3-acetic acid (IAA) levels were higher in seeds of plants under HS than in seeds of CP at both 6 and 10 DAA, with the difference being statistically significant at 10 DAA. After this time point, IAA levels in seeds of

CP or of plants under HS dropped to nearly zero (Fig. 3E). Salicylic acid levels were significantly higher in seeds of plants under HS compared to seeds of CP at both 6 and 10 DAA. After these time points, SA levels in the seeds of plants under HS and in seeds of CP decrease drastically. (Fig. 3F).

Trans-zeatin (tZ) and dihydrozeatin (DHZ) levels followed a similar pattern, having both a spike at 10 DAA in seeds of plants under HS. At that time point those levels are significantly different ($p < 0.05$) from the ones in seeds of CP (Fig. 3G, 3H). Additionally, the level of tZ is also significantly higher at 6 DAA in seeds of plants under HS when compared with seeds of CP.

The levels of Isopentenyladenine (iP) are very low when compared to tZ and DHZ, either in seeds of plants under HS or CP. At 18 DAA there is a significant increase in the concentration of iP in seeds of CP when compared to seeds of plants under HS. This difference is maintained at 20 DAA (Fig. 3I).

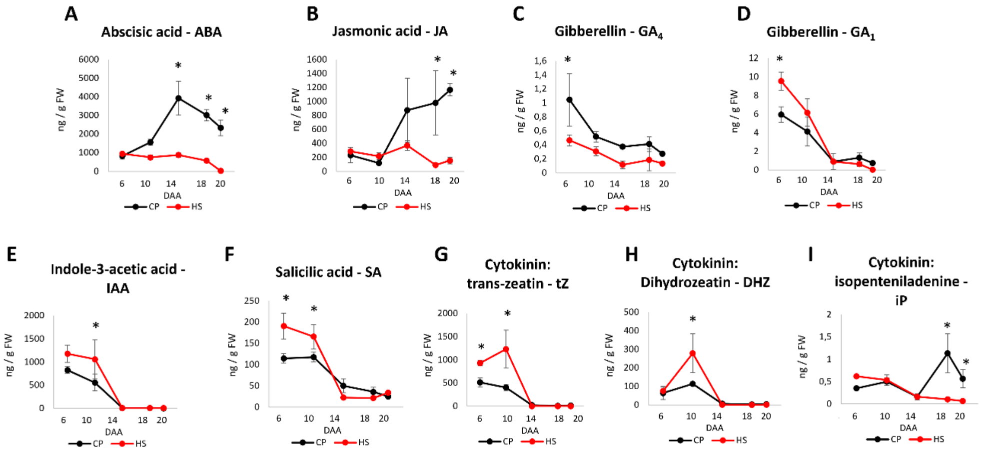

Figure 3- Changes in phytohormone levels in control (CP) and heat-stressed (HS) developing seeds of *Phaseolus vulgaris* at 6, 10, 14, 18 and 20 DAA: **A** abscisic acid (ABA), **B** jasmonic acid (JA), **C** gibberellin GA_4, **D** gibberellin GA_1, **E** indole-3-acetic acid (IAA), **F** salicilic acid (SA), **G** cytokinin trans-zeatin (tZ), **H** cytokinin dihydrozeatin (DHZ) and **I** cytokinin isopentenyladenine (iP). Data points represent the mean concentration (ng/g FW) with standard error bars. Statistically significant differences between CP and HS treatments at the respective time points ($p < 0.05$).

3.4 Transcriptomic analysis of heat stress impact on early seed development

RNA-Seq of 24 cDNA libraries from 6, 10, 14 and 18 DAA seeds of CP and of plants under HS (Supp. Table 1) generated over 1.7 billion 150-bp reads with an average percentage of mapped reads of 94,16% and 93,46%, respectively (Supp. Table 2). Differentially expressed genes were mainly involved in protein, RNA, solute transport, cell wall, lipid metabolism, and phytohormone-related functions (Supp. Fig. 3).

Genes ZEP, NCED and ABA2, that encode enzymes of the ABA biosynthetic pathway, are notably more expressed in seeds of plants under HS at 14 and 18 DAA. Despite, key genes of enzymes of the ABA degradation pathway, such as CYP707A and Abscisate beta-glucosyltransferase, are also more expressed at 14 and 18 DAA in seeds of plants under HS. In addition, within the ABA signalling pathway, PP2C (Protein phosphatase 2C), a negative regulator of ABA signalling, is also upregulated at 14 and 18 DAA under HS (Fig. 4A), further reflecting the decrease in ABA levels in seeds of plants under HS observed in the phytohormone quantification data (Fig. 3A).

Genes PLA, AOC, AOS and OPR3, that encode enzymes of the JA biosynthetic pathway, are more expressed in seeds from CP (Fig. 4B). Furthermore, the gene of the JA transcriptional repressor JAZ is more expressed at 14 and 18 DAA in seeds of plants under HS compared to seeds in CP, which is aligned with the data from phytohormone quantification (Fig. 3B).

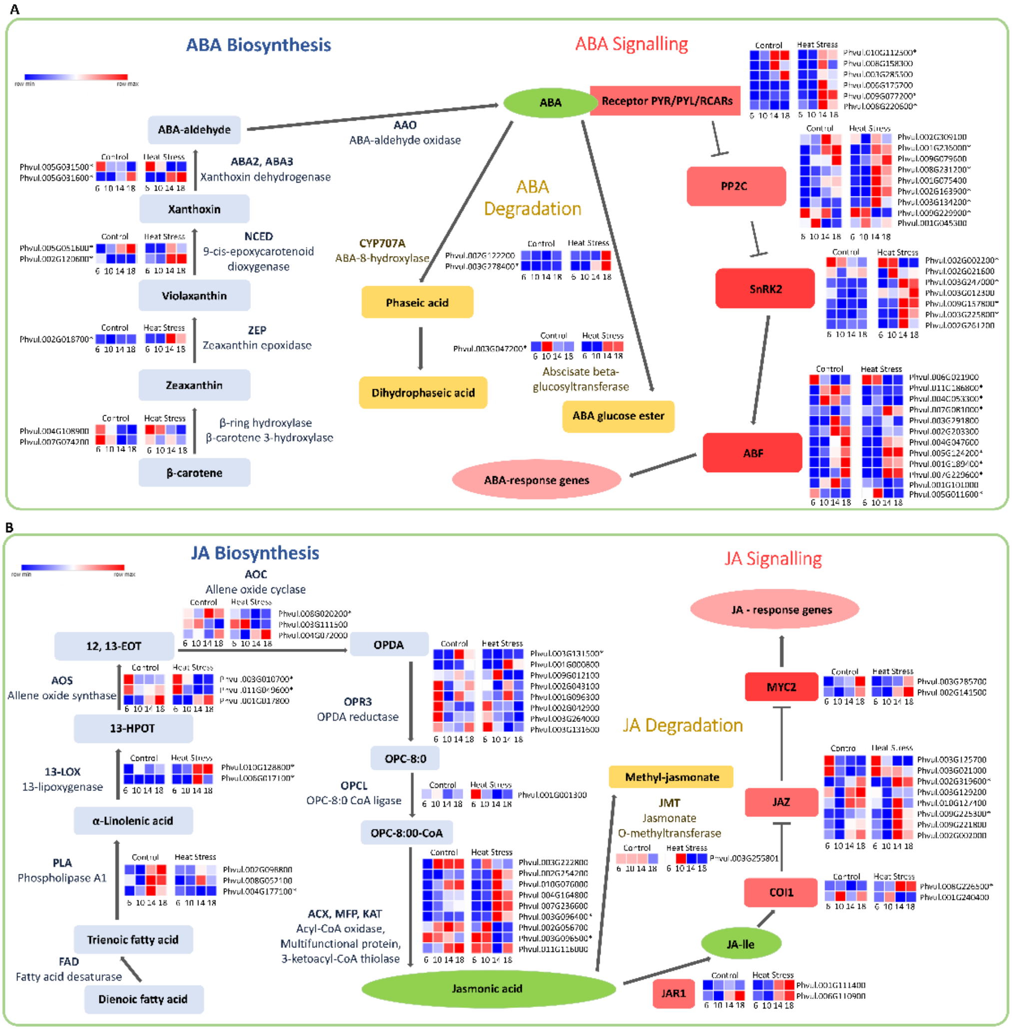

Figure 4- **A** Schematic representation of the ABA biosynthesis, degradation and signalling pathways in seeds at 6, 10, 14 and 18 days after anthesis (DAA) under control and heat stress conditions. The scheme includes key genes involved in ABA biosynthesis, such as β-ring hydroxylase, ZEP, NCED and ABA2. Additionally, key genes involved in ABA degradation, including CYP707A and Abscisate beta-glucosyltransferase, are represented. The signalling pathway is illustrated with the inclusion of the receptor PYR/PYL/RCARs, PP2C, a negative regulator of ABA signalling, SnRK2 and ABF. Adapted from (Sharma & Nayyar, 2016). **B** Schematic representation of the jasmonic acid (JA) biosynthesis, degradation and signalling pathways in seeds at 6, 10, 14 and 18 DAA under control and heat stress conditions. Key genes in the JA biosynthesis pathway include PLA, 13-LOX, AOS, AOC, OPR3,

OPCL and ACX. In the JA signalling pathway, JAR1, COI1, the transcriptional repressor JAZ and the transcription factor MYC2 are represented. Adapted from (Macioszek et al., 2023). FPKM were clustered using Euclidean distance and an average linkage. Blue colour represents low expression levels (lower FPKM values); White (Middle Colour) represents moderate expression levels and Red represents high expression levels (higher FPKM values). Asterisks indicate differentially expressed genes (DEGs): corrected p-value ≤ 0.001 and |Log2(Fold Change - FC) | ≥1.

The genes of the auxin transporter AUX1 are more expressed 6 and 10 DAA in seeds of plants under HS. Moreover, the AUX/IAA genes, which are important regulators of auxin responses, are higly expressed at 6 DAA, particularly in seeds of plants under HS conditions (Fig. 5A), indicating an early increase in auxin response regulation in heat-stressed seeds, as shown by phytohormone quantification (Fig. 3E).

In the cytokinin (CK) biosynthetic pathway, IPT and CYP735A1 exhibit higher expression at 10 DAA under HS conditions, suggesting a more active CK response during the early stages of development in HS-treated plants. This observation aligns with phytohormone quantification data (Fig. 3G, 3H), further supporting the notion of enhanced CK biosynthesis. Additionally, Type-B ARRs, which play a key role in regulating CK signaling, also show increased expression under HS conditions (Fig. 5B).

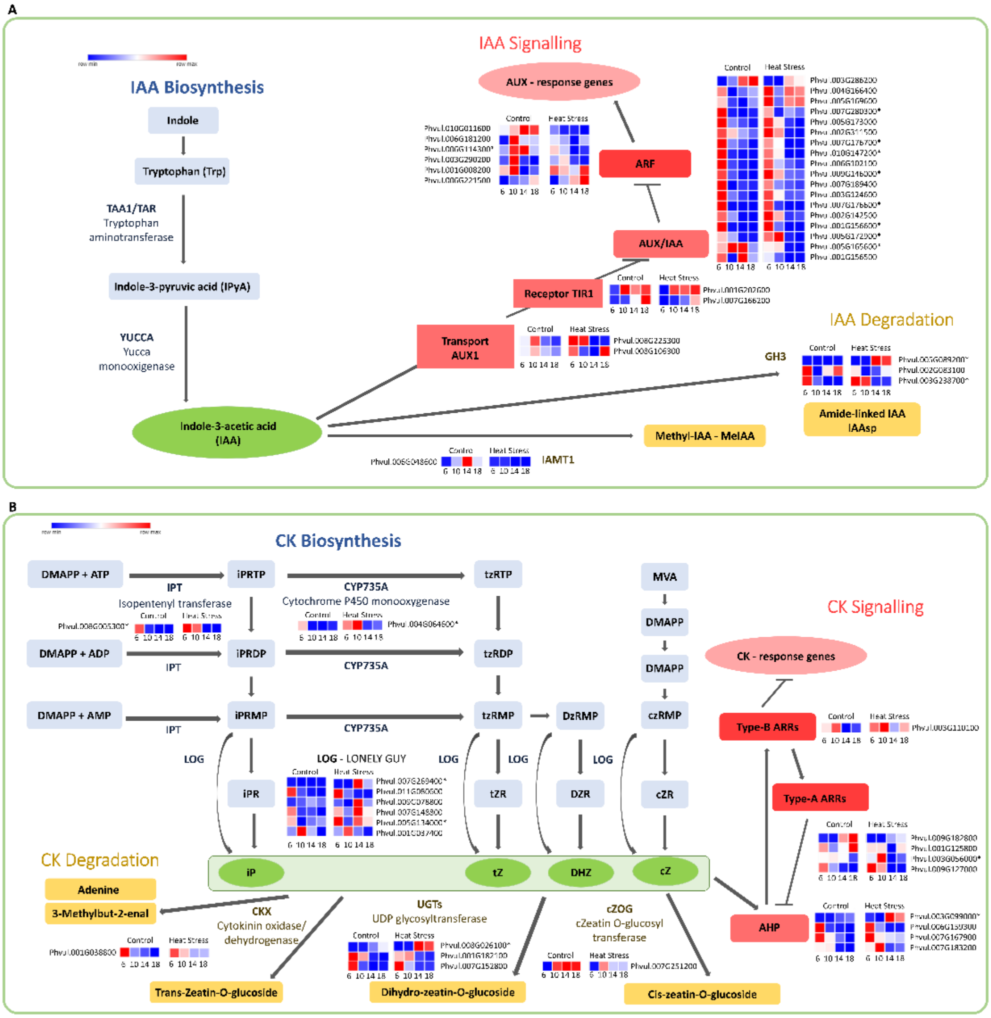

Figure 5- **A** Schematic representation of the auxin (IAA) biosynthesis, transport, signalling and degradation pathways in seeds at 6, 10, 14 and 18 DAA) under control and heat stress conditions. The auxin degradation genes, GH3, are shown. AUX1, involved in auxin transport and the auxin response regulators, receptor TIR1, AUX/IAA and ARF genes are also represented. Adapted from (Olatunji et al., 2017). **B** Schematic representation of the cytokinin (CK) biosynthesis, degradation and signalling pathways in seeds at 6, 10, 14 and 18 DAA under control and heat stress conditions. The cytokinin biosynthesis genes IPT and LOG are shown. Genes involved in cytokinin degradation, like UGTs are also depicted. The cytokinin response AHP is highlighted, along with Type-A ARR and Type-B ARR. Adapted from (Cai et al., 2018). FPKM were clustered using Euclidean distance and an average

linkage. Blue colour represents low expression levels (lower FPKM values); White (Middle Colour) represents moderate expression levels and Red represents high expression levels (higher FPKM values). Asterisks indicate differentially expressed genes (DEGs): corrected p-value ≤ 0.001 and |Log2(Fold Change - FC) | ≥1.

In the GA pathway, biosynthesis genes (GA20ox, GA3ox) are upregulated under HS at 6 and 10 DAA, while degradation (GA2ox) and signaling genes (DELLA) show sustained or increased expression at later stages (Supp. Fig. 4), which reflects the decline observed in phytohormone quantification data (Fig 3C, 3D). Additionally, in the salicylic acid biosynthetic pathway, CM and PAL genes are upregulated at 6 and 10 DAA under HS (Supp. Fig. 5), which is aligned with the data from phytohormone quantification (Fig. 3F).

In the brassinosteroid (BR) signalling pathway, BSK a positive regulator shows higher expression at 14 and 18 DAA under HS. Meanwhile, BIN2, a negative regulator of BR signalling, is more expressed at 14 and 18 DAA under CP conditions (Supp. Fig. 6). Furthermore, in the ethylene pathway, biosynthetic genes (SAM synthetase, ACO) and signalling components (MPK6, EIN2, EIN3, EBF1/2, ERF1/2) exhibit elevated expression at 14 and 18 DAA under HS, suggesting enhanced ethylene response under HS conditions (Supp. Fig. 7).

The storage protein phaseolin genes were more expressed under CP conditions at 18 DAA compared with HS (Fig. 6A). However, at 18 DAA, CP seeds are still undergoing seed filling, whereas HS seeds have already initiated desiccation. Additionally, under CP conditions, genes involved in carbohydrate metabolism are more expressed at 14 and 18 DAA, corresponding to the mid-filling stage of seed development, compared to HS. Examples of these genes include FBP1, Phosphoglucose isomerase, PGM, GBSS, SBE and ISA. However, another set of genes, such as UDPase, SPS and SPP exhibited higher expression in HS compared to CP (Fig. 6B).

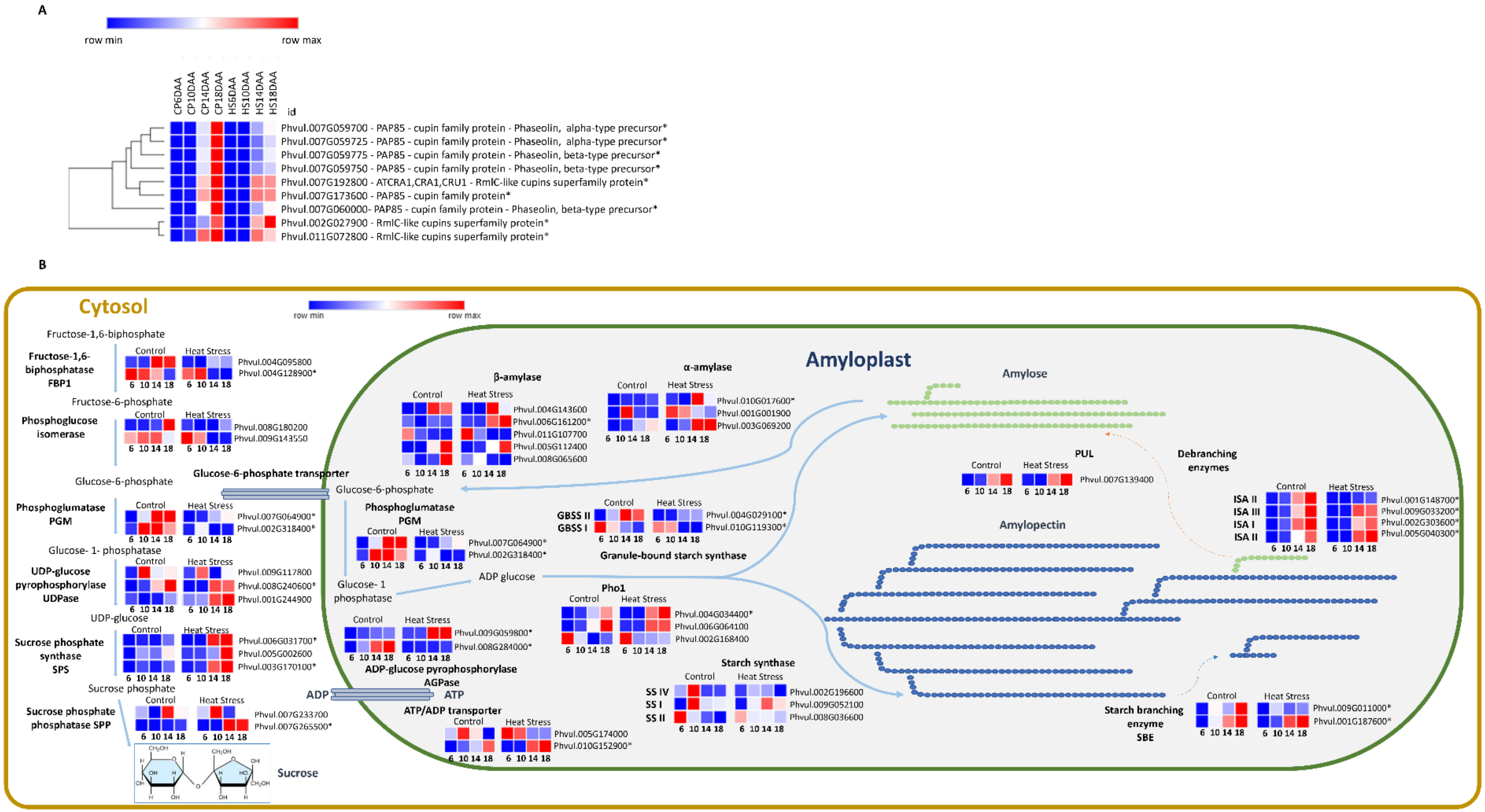
A
row min
row max
CP6DAA
CP10DAA
CP14DAA
CP18DAA
HS6DAA
HS10DAA
HS14DAA
HS18DAA
id
Phvul.007G059700 - PAP85 - cupin family protein - Phaseolin, alpha-type precursor*
Phvul.007G059725 - PAP85 - cupin family protein - Phaseolin, alpha-type precursor*
Phvul.007G059775 - PAP85 - cupin family protein - Phaseolin, beta-type precursor*
Phvul.007G059750 - PAP85 - cupin family protein - Phaseolin, beta-type precursor*
Phvul.007G192800 - ATCRA1,CRA1,CRU1 - RmlC-like cupins superfamily protein*
Phvul.007G173600 - PAP85 - cupin family protein*
Phvul.007G060000- PAP85 - cupin family protein - Phaseolin, beta-type precursor*
Phvul.002G027900 - RmlC-like cupins superfamily protein*
Phvul.011G072800 - RmlC-like cupins superfamily protein*
B
Cytosol
row min
row max
Amyloplast
Fructose-1,6-biphosphate
Fructose-1,6-biphosphatase FBP1
Phvul.004G095800
Phvul.004G128900*
Fructose-6-phosphate
Phosphoglucose isomerase
Phvul.008G180200
Phvul.009G143550
Glucose-6-phosphate
Glucose-6-phosphate transporter
Phosphoglumatase PGM
Phvul.007G064900*
Phvul.002G318400*
Glucose- 1- phosphate
UDP-glucose pyrophosphorylase UDPase
Phvul.009G117800
Phvul.008G240600*
Phvul.001G244900
UDP-glucose
Sucrose phosphate synthase SPS
Phvul.006G031700*
Phvul.005G002600
Phvul.003G170100*
Sucrose phosphate
Sucrose phosphate phosphatase SPP
Phvul.007G233700
Phvul.007G265500*
Sucrose
Control
Heat Stress
6 10 14 18
β-amylase
Phvul.004G143600
Phvul.006G161200*
Phvul.011G107700
Phvul.005G112400
Phvul.008G065600
α-amylase
Phvul.010G017600*
Phvul.001G001900
Phvul.003G069200
Glucose-6-phosphate
Phosphoglumatase PGM
Phvul.007G064900*
Phvul.002G318400*
Glucose- 1 phosphatase
ADP glucose
ADP-glucose pyrophosphorylase AGPase
Phvul.009G059800*
Phvul.008G284000*
ADP
ATP
ATP/ADP transporter
Phvul.005G174000
Phvul.010G152900*
GBSS II
GBSS I
Phvul.004G029100*
Phvul.010G119300*
Granule-bound starch synthase
Pho1
Phvul.004G034400*
Phvul.006G064100
Phvul.002G168400
Starch synthase
SS IV
SS I
SS II
Phvul.002G196600
Phvul.009G052100
Phvul.008G036600
Amylose
PUL
Phvul.007G139400
Debranching enzymes
ISA II
ISA III
ISA I
ISA II
Phvul.001G148700*
Phvul.009G033200*
Phvul.002G303600*
Phvul.005G040300*
Amylopectin
Starch branching enzyme SBE
Phvul.009G011000*
Phvul.001G187600*
CH₂OH
OH
H
HO
O

Figure 6- **A** Heatmap of transcripts related to seed storage proteins at 6, 10, 14 and 18 days after anthesis (DAA) in heat stress (HS) and control conditions (CP). **B** Heatmaps of transcripts categorized in MapMan related to sucrose and starch synthesis at 6, 10, 14 and 18 DAA under CP (25/18°C day/night) and HS (32/25°C day/night) conditions. Fructose 1-6 biphosphatase (FBP1), Phosphoglucose isomerase, Phosphoglumatase (PGM), Sucrose phosphate synthase (SPS), Sucrose phosphate phosphatase (SPP), UDP-glucose pyrophosphorylase (UDP), ADP-glucose pyrophosphorylase (AGPase), Plastid starch phosphorylase (Pho1), Starch synthase (SS), Granule-bound starch synthase (GBSS), Starch branching enzyme (SBE), Isoamylases (ISA), Pullulanase (PUL), α-amylase and β-amylase. Schematic representation of sucrose and starch synthesis adapted from (Qu et al., 2018). FPKM were clustered using Euclidean distance and an average linkage. Blue colour represents low expression levels (lower FPKM values); White (Middle Colour) represents moderate expression levels and Red represents high expression levels (higher FPKM values). Asterisks indicate differentially expressed genes (DEGs): corrected p-value ≤ 0.001 and |Log2(Fold Change - FC) | ≥1.

3.5 Validation of RNA Sequencing data by RT-qPCR

Twelve genes were selected for RT-qPCR validation of sequencing results. A strong positive correlation ($0.76 \leq R2 \leq 0.99$) between RNA-Seq and RT-qPCR data (Supp. Table 4) was evidenced for these genes.

4. Discussion

This study focusses on the response of *P. vulgaris* to HS during the critical transition from embryogenesis to seed filling through an integrative approach combining physiological and histochemical assessments, with phytohormone quantification and gene expression analysis. This multi-faceted methodology allowed us to examine the interaction with complex hormonal signalling and stress-response pathways across developmental stages.

4.1 Inducing heat stress without causing water deficit in *P. vulgaris* SER16

In our study, we selected 25/18°C (day/night) for control plants and 32/25°C (day/night) for heat-stressed plants based on previous research on *P. vulgaris* genotype SER16. The control temperature of 25/18°C aligns with conditions used in earlier studies conducted in our lab, where *P. vulgaris* genotype SER16 was maintained under similar environmental conditions to investigate seed development and physiological responses (Parreira et al., 2016, 2018, 2021). This consistency allows for a direct comparison with previously observed results, ensuring the reliability of our findings. Beyond our lab, several other studies on *P. vulgaris* genotype SER16 have reported similar growing temperature conditions. (Leitão et al., 2021) used 26 ± 2°C (day) and 18 ± 2°C (night), while (Mendes et al., 2022) maintained plants at 25 ± 2°C (day) and 20 ± 2°C (night). Similarly, (Ambachew & Blair, 2021) applied 23 ± 3°C (day) and 20 ± 3°C (night) to grow SER16 genotype. Additionally, (Asfaw et al., 2017), who conducted field-based experiments on SER16, recorded ambient temperature variations ranging from 24–29°C (day) and 9.5–14.1°C (night), further confirming that our control conditions fall within the natural temperature range experienced by this genotype in the field. By ensuring that our control conditions match those used in previous SER16 studies, we maximize the reliability and comparability of our results.

For the HS treatment, we selected 32/25°C (day/night) as an appropriate elevated temperature regime based on previous studies investigating the effects of high temperatures on *P. vulgaris*. (Soltani et al., 2019) found that exposure to 32/25°C disrupted pollen fertility and seed set, leading to reduced productivity. Similarly, (Rainey & Griffiths, 2005) observed increased abscission and reduced pod and seed numbers at 32/28°C. Rose et al., (2023) studied heat-stress breeding lines at 31/24°C, identifying *P. vulgaris* genotypes with higher tolerance under these conditions. Field studies further emphasize the impact of high nighttime temperatures. (Vargas et al., 2021), with field temperatures ranging from 26–38ºC (day) and 16–25ºC (night), reported that night time HS led to a 37% yield reduction in 2016 and 26% in 2017, primarily due to defects in seed formation and grain quality.

Furthermore, our selection of 32/25°C as the HS condition is strongly supported by the Bean Improvement group of CIAT, who also kindly provided the SER16 genotype seeds used in this study and suggested 32ºC as temperature to trigger HS condition in this genotype without inducing flower abortion (Dr. Steve Beebe, personal communication). Their research emphasized that *P. vulgaris* originates from mid- to high-altitude regions and is generally sensitive to high temperatures, particularly night temperatures above 20°C, which significantly reduce pollen fertility and pollination. Their findings indicate that while high night temperatures negatively impact yield, day temperatures exceeding 30°C are also a major limiting factor (Beebe et al., 2012), making 32/25°C a relevant and appropriate level of stress. They further pointed out that high nighttime temperatures at flowering lead to flower and pod abortion, reduced pollen viability, impaired pollen-tube formation, and smaller seed size, all of which are key reproductive traits we aim to investigate under heat stress. By selecting 32/25°C, we ensured that plants experienced significant stress while still maintaining seed production, allowing us to investigate the effects of HS on early seed development, with a particular focus on the transition from embryogenesis to seed filling.

To isolate the effects of HS alone, we ensured that plants were well-watered throughout the study, thereby avoiding water deficit stress. In irrigated soybean, HS alone does not significantly alter photosynthesis or senescence (Ergo et al., 2021). Lack of water is a known factor to significantly lower relative water content (RWC), photosynthetic rate (A), transpiration rate (E) and stomatal conductance (gS) in legumes like mungbean and lentil (Talukdar, 2013). In our study, these parameters remained stable during sampling, confirming that the plants experienced only HS. This allowed us to specifically analyse HS physiological effects.

4.2 HS induce phenotype changes during the development and filling of *P. vulgaris* SER16 seeds

Chlorophyll a and b levels showed no significant change between CP and HS conditions, suggesting that photosynthetic function was largely preserved. In contrast, xanthophylls and carotenes significantly declined under HS, suggesting reduced photoprotection. These findings align with Wentworth et al., (2006), who reported that HS alters pigment composition, leading to chlorophyll degradation and increased xanthophyll accumulation in *P. vulgaris*. Additionally, HS conditions lowered the chlorophyll a/b ratio but increased the total chlorophyll/carotenoid ratio, implying a shift toward protective pigments, a response also noted under stress (Caliandro et al., 2013).

HS alters the timing of the transition from embryogenesis to seed filling. HS also significantly shortened the seed filling period in *P. vulgaris*: under CP, SFW and SDW steadily increased until 30 DAA, whereas under HS, both peaked at 18 DAA before declining, signalling a premature shift to desiccation. Similarly, cotyledon and cell areas expanded until 18 DAA under HS, then shrank, while under CP they continued growing until 20 DAA, reflecting mid-filling progression.

Our results showed a clear reduction in seed number under HS compared to CP conditions. This decline highlights the impact of HS on reproduction, including flower abortion, reduced pollen viability, and impaired fertilization (Lohani et al., 2020). These findings align with previous studies showing male gametophyte development as highly heat-sensitive. In wheat, temperatures above 30°C lead to pollen abortion and reduced fertilization (Ullah et al., 2022). Similarly, high temperatures during seed filling can lower seed numbers by up to 50% (Sita et al., 2018), underscoring the negative effects of HS on reproductive success in *P. vulgaris*.

4.3 *P. vulgaris* SER16 seeds under HS show an impairment in the final content of storage compounds

To assess how HS affects storage compounds accumulation, we analysed histochemical and transcriptomic data. Starch accumulation progressed steadily under CP, whereas HS seeds exhibited significant reduction at 20 DAA. Transcriptomic data further support these findings, as genes like FBP1, Phosphoglucose isomerase, PGM, AGPase, GBSS, SBE, and ISA are more expressed in CP conditions. This disruption in starch accumulation aligns with previous studies in rice, HS significantly reduces the activities of sucrose synthase, starch synthase and granule-bound starch synthase (GBSS) (Ahmed et al., 2015). Similarly, in maize, HS during grain filling leads to premature decline in starch-synthesizing enzyme activity, resulting in reduced kernel size and abbreviated metabolic duration (Singletary et al., 1994).

Histochemical analysis reveals that *P. vulgaris* seeds exposed to HS have comparable levels of seed storage proteins at 20 DAA to those under CP. One possible explanation is that HS reduces seed size primarily by limiting cell proliferation rather than storage compound accumulation. This aligns with observations in soybean, where elevated temperatures decreased the number of cotyledon cells without affecting cell volume, ultimately reducing seed size and growth rate (Tacarindua et al., 2012). However, at 20 DAA HS seeds had reached the desiccation stage and maximum protein accumulation. While CP seeds at 20 DAA were still in the mid-seed-filling phase and will accumulate additional storage compounds until desiccation around 30 DAA. Transcriptomic data showed that genes related to seed storage protein were more expressed under CP conditions at 18 DAA compared with HS. This pattern is consistent with findings in other species. In wheat, high temperatures during grain development shorten the grain-filling period and reduce both starch and protein accumulation (Ullah et al., 2022). Similarly, in lentils, HS inhibits the accumulation of storage proteins and essential minerals in seeds (Sita et al., 2018). In rice, HS decreased grain weight and altered storage protein accumulation (Lin et al., 2010). Furthermore, in maize, high temperatures reduce kernel dry weight and protein content, by repressing synthesis and increasing protein degradation (Monjardino et al., 2005).

4.4 HS changes the phytohormonal concentration profiles along transition from seed embryogenesis to seed filling in *P. vulgaris* SER16

Under HS, GA_1 levels were higher at 6 DAA compared CP. In contrast, GA_4 showed the opposite trend, with higher levels at 6 DAA under CP than HS. However, the highest measured value of GA_1 was around 10 ng/g FW, whereas GA_4 reached only about 1 ng/g FW. Likewise, isopentenyladenine (iP), peaked at around 1.5 ng/g FW. The low levels of isopentenyladenine (iP) observed in our results for *P. vulgaris* seeds may reflect a tissue-specific pattern of cytokinin biosynthesis. Although iP is a bioactive cytokinin, it generally accumulates in shoots, whereas trans-zeatin (tZ)-type cytokinins are more prevalent in roots and developing seeds (Matsumoto-Kitano et al., 2008). This distribution could explain why zeatins, rather than iP, were the predominant cytokinins detected in *P. vulgaris* seeds. A similar trend was reported in chickpea, where iP and its riboside (iPR) were found at low but detectable levels only in older cotyledon extracts, after the cessation of cell division (Jameson, 2023). Furthermore, studies in rice and maize have shown that tZ levels are more closely associated with endosperm cell division, reinforcing the idea that zeatins play a central role in regulating early seed development (Rijavec & Dermastia, 2010). Another possible explanation for the lower levels of iP is that iPRTP, iPRDP, and iPRMP are believed to be transported from the plastids to the endoplasmic reticulum, where they serve as substrates for the synthesis of tZ by CYP735A enzymes (Takei et al., 2004). In contrast, the other measured phytohormones exhibited significantly higher peak values, ranging between 200 and 4000 ng/g FW. Except for Jasmonic and Abcisic acid, the amount of all the other quantified phytohormones decrease to almost 0 ng per g of FW, in seeds of plants either under CP or HS, at 14DAA, showing this time to be a turning point in *P. vulgaris* seed development.

HS reduced ABA and JA levels in seeds at 14, 18, and 20 DAA in *P. vulgaris* seeds. The consistently low ABA levels may result from overexpression of PP2C, a negative ABA signalling regulator, consistent with hormone quantification data. Since ABA promotes storage compound accumulation and desiccation tolerance, its reduction under HS may disrupt these processes, potentially causing premature germination and lower seed viability (Nonogaki, 2019). Similar reductions in ABA under HS have been reported in *Brassica napus*, contributing to embryo defects and reduced dormancy (Mácová et al., 2022). In CP seeds, JA levels remain stable between 6 and 10 DAA, then increase significantly from 14 to 20 DAA, aligning with the upregulation of JA biosynthetic genes observed. Under HS, JA levels remain consistently low, likely due to the upregulation of JA transcriptional repressor JAZ at 14 and 18 DAA under HS. These patterns align with findings in other species, including Arabidopsis, where HS enhances JA catabolism (Zhu et al., 2021) and in rice, where HS suppresses JA biosynthetic genes (Du et al., 2013).

HS increased IAA, CK and SA levels in seeds at 6 and 10 DAA, in *P. vulgaris* seeds. IAA levels peak at 6 DAA under both CP and HS, but was consistently higher under HS, likely due to the overexpression of auxin transporter genes AUX1 at 6 and 10 DAA. Likewise, HS induced the upregulation of auxin biosynthesis genes (TAR, PsYUC10) in pea seeds (Kaur et al., 2021). CKs, tZ and DHZ, also peaked at 10 DAA before declining, with higher levels under HS, possibly driven by early activation of CK biosynthesis genes. This aligns with findings by (Xu et al., 2010), who demonstrated that IPT overexpression enhanced plant thermotolerance. Elevated CK (zeatin) levels under HS may initially enhance cell division during early seed development, potentially contributing to the increased fresh and dry weights observed at 14 and 18 DAA. However, this effect appears to diminish beyond 14 DAA, likely due to the natural decline in CK levels as seed filling progresses. A similar pattern has been observed in white lupine, where CK concentrations peaked early and then dropped sharply as cotyledons expanded (Emery et al., 2000). In lupins, CKs have been shown to stimulate embryo cell division (Staden et al., 1983) and, in fava bean, to help maintain cell division within the developing embryo through a sugar signalling mechanism (Herbers & Sonnewald, 1998). Moreover, Salicylic acid levels are significantly higher under HS at 6 and 10 DAA, likely due to the upregulation of CM and PAL involved in SA biosynthesis. This trend mirrors observations in Arabidopsis, where heat triggers SA biosynthesis as part of stress adaptation (Kusajima et al., 2021) and in pea, where SA accumulation under HS is mediated through the PAL and BA2H pathways (Pan et al., 2006).

HS increased ethylene gene expression at 14 and 18 DAA in *P. vulgaris* seeds, with key ethylene biosynthesis and signalling genes being upregulated under HS. Similar patterns have been observed in wheat, where HS enhanced ethylene production in developing grains (Hays et al., 2007) and in pea, it increased ACC levels and the expression of PsACO2, PsACO3, and the signalling gene PsEBF2, indicating ethylene’s role in promoting senescence under stress (Kaur et al., 2021). Additionally, in Arabidopsis, overexpression of ERF1 has been shown to activate heat tolerance (Cheng et al., 2013), supporting ethylene’s involvement in HS adaptation.

5. Conclusion

This study provides a detailed examination of the physiological, morphological and molecular responses of the non-endospermic seeds of *Phaseolus vulgaris* to HS, particularly during the crucial transition from embryogenesis to seed filling.

HS induces phenotype changes during seed filling by delaying cotyledon formation and shortens the seed-filling period in *P. vulgaris*. SFW and SDW increased until 30 DAA under CP but peaked early at 18 DAA under HS, signalling premature desiccation. Additionally, HS reduces the number of seeds produced per plant. Moreover, HS impaired storage compounds accumulation, significantly decreasing starch content at 20 DAA and downregulating key starch biosynthesis genes (FBP1, phosphoglucose isomerase, PGM, AGPase, GBSS, SBE, and ISA). Moreover, although *P. vulgaris* seeds under HS and CP have similar seed storage protein levels at 20 DAA, HS seeds reach desiccation and peak protein accumulation earlier, while CP seeds continue filling until 30 DAA, with lower storage protein gene expression under HS at 18 DAA compared to CP.

HS changed phytohormonal profiles in *P. vulgaris* seeds. ABA and JA levels were reduced at 14, 18, and 20 DAA under HS, likely due to PP2C and ABA-degrading enzyme upregulation, matching ABA quantification data. In CP seeds, JA levels stay stable from 6 to 10 DAA and rise from 14 to 20 DAA, aligning with increased biosynthetic gene expression (PLA, AOC, AOS). Under HS, JA remains low, likely due to JAZ upregulation at 14 and 18 DAA, suppressing accumulation. HS increased IAA, CK, and SA levels in *P. vulgaris* seeds at 6 and 10 DAA. IAA peaks at 6 DAA under both CP and HS but is higher under HS, likely due to the overexpression of AUX1 transporter. Similarly, CK (tZ and DHZ) peaks at 10 DAA in both conditions but remains higher under HS, possibly due to early IPT upregulation. SA levels are also significantly higher under HS at 6 and 10 DAA, likely driven by biosynthesis genes CM and PAL upregulation. HS increased ethylene gene expression in *P. vulgaris* seeds at 14 and 18 DAA, with key biosynthesis and signalling genes upregulated under HS.

These findings highlight *P. vulgaris* sensitivity to HS during seed development, affecting crop resilience and yield. Identifying the molecular and physiological disruptions caused by HS can guide breeding strategies to improve heat tolerance in legumes, supporting food security in a warming climate.

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Supplementary material

Supplementary Table 1 - RNA-seq data

https://www.dropbox.com/scl/fi/x7gsqoz1onb122bdym875/Chapter-III_Supp1_RNA-Seq-data-Heat-Stress.xlsx?rlkey=kkjgf3d2m5uk4dtacx4itw9m1&st=b1srhs5x&dl=0

Supplementary Table 2 -Characterization of the 24 RNA-Seq libraries generated for comparing control (CP) and heat stress (HS) developing seeds of *Phaseolus vulgaris*. (for additional information)

Sample	*Number of raw reads*	*GC (%)*	*Q20(%)*	*Q30(%)*	*Number of mapped reads*	*Mapped reads (%)*
CP6DAA1	61103584	43.89	97.85	94.11	57555207	94.19
CP6DAA2	58700320	44.40	97.89	93.33	55906460	95.24
CP6DAA3	79473812	44.95	97.31	91.95	75156324	94.57
CP10DAA1	58075586	45.42	98.10	94.40	55738883	95.98
CP10DAA2	64492940	45.28	97.29	91.91	61109919	94.75
CP10DAA3	61992054	44.79	97.88	94.15	58892318	95.00
CP14DAA1	80261174	45.50	97.21	91.77	74617976	92.97
CP14DAA2	65022042	45.42	98.24	94.71	61448821	94.50
CP14DAA3	88739212	45.32	98.22	94.63	83740881	94.37
CP18DAA1	72572610	46.42	98.20	94.62	67409712	92.89
CP18DAA2	72010808	45.81	98.04	94.27	66734647	92.67
CP18DAA3	78180318	45.25	98.11	94.37	72503573	92.74
HS6DAA1	63104774	44.17	97.82	93.77	59791450	94.75
HS6DAA2	83185120	45.48	98.16	94.51	79544884	95.62
HS6DAA3	63131310	44.76	97.42	92.26	59546406	94.32
HS10DAA1	58231314	45.16	97.46	92.21	55444118	95.21
HS10DAA2	60180150	44.77	96.93	91.35	56302063	93.56
HS10DAA3	60619896	44.61	97.62	92.87	57474953	94.81

HS14DAA1	78099364	45.05	97.35	92.01	71279616	91.27
HS14DAA2	81753692	45.73	98.19	94.35	76535204	93.62
HS14DAA3	89320192	45.18	98.12	94.39	83534098	93.52
HS18DAA1	75570408	45.99	97.99	94.03	69452924	91.90
HS18DAA2	83618472	45.73	97.37	92.08	76832035	91.88
HS18DAA3	77227988	45.61	98.00	94.18	70364002	91.11

Supplementary Table 3- Primers used for RNA extration and for RT-qPCR

RNA extraction

	Gene ID	**Annotation**	**Forward (5'-3')**	**Tm (ºC)**	**Reverse (5'-3')**	**Tm (ºC)**
Actin	Phvul.001G142500	Actin 11	TCCTGCTATGTATGTTGCCATC	58.3	GGAACCGTGTGACTGACACCGTCTCCGGAGTC	73.6

RT-qPCR

Genes of interest

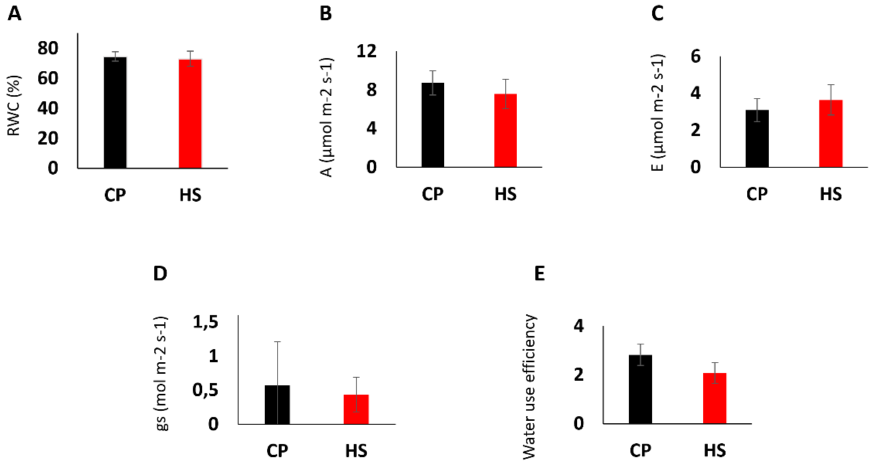
Gene Name	**Gene ID**	**Annotation**	**Forward (5'-3')**	**Tm (ºC)**	**Reverse (5'-3')**	**Tm (ºC)**	**Efficiency**
KAO2	Phvul.006G123500	Ent-kaurenoic acid monooxygenase	TGTGTCCCGGCAATGATCTTG	61.0	TGATCCTTGCCATGCAGTTG	58.8	1.855326494
DELLA	Phvul.008G235500	DELLA	AAGCATGCAGGAAACCTTGC	59.7	AATGTGTGTGCAGCATGTCG	59.8	1.785666589
AUX/IAA	Phvul.005G173000	AUX/IAA	AGATGGTGACTGGATGTTGGTC	60.0	TCTCGGACCTAGAACAATAGCC	59.0	1.722263644
ASB1	Phvul.003G014300	anthranilate synthase component I	CTGATGAACTTGAGGCAACAGC	60.1	TGATGCTCTCCGGATGAAACTG	60.4	1.924245574
AHP	Phvul.006G159300	histidine phosphotransfer	GCAAGAGTACTGCCTTGTGAAG	59.5	CTGAAACTCAGTTCCGTAGGG	58.0	1.797974638
PP2C	Phvul.001G236000	protein phosphatase 2C	TTCTGTTGTTCAGCCTGACC	58.0	CACGAACGGTTTCAGGTAGTAG	58.8	1.744208566

ABA2	Phvul.005G031600	xanthoxin dehydrogenase	CCAGTATTCTTCAGGCTTCCTC	58.2	ACCATGAGTGTGGAAGAGATGC	60.4	1.744208566
PYR/PYL	Phvul.006G175700	PYR/PYL	AAGAAGTGCACCATGCTTGC	59.7	TAACCCGGAAGTGACCATGATC	59.8	1.797974638
TCH4	Phvul.003G147700	Xyloglucan endotransglucosylase/hydrolase	TTTTCAGCCAAGGCAAAGGG	59.2	CTAATAGGAGTGCCATCAACGG	58.6	1.821419095
JAZ1	Phvul.009G225300	Jasmonate Zim-Domain 1	TCCACCGGTTTCTCTTGAAGAG	60.0	ATGTAGCACTCAACCCAAGC	58.2	1.790017543
PR1	Phvul.006G197500	Pathogenesis-related protein 1	ATGCTCAAAGGTACGCGAAC	58.9	AACCCTTCATTTCGCCGTAC	58.6	1.81717454
TGA6	Phvul.001G123300	TGACG motif-binding factor 6	TGGCTATGGGAAAGCTTGGTAC	60.4	GCAGTCGCGAGAAATAGTCG	59.2	1.767475162
Reference genes							
PEL1	Phvul.010G077790.1	Eukaryotic release factor 1 (eRF1) family protein	ACGAAGACAGTTGCGACCTATT	56.35	GGAGCATCCAAAACCTCCCTTA	56.92	1.847214874
XERICO	Phvul.008G190100.1	RING/U-box superfamily protein	GATCCCTACCAAACTCCACCTG	57.07	ACACACACTGTCAAACCTGAGT	56.52	1.89935374

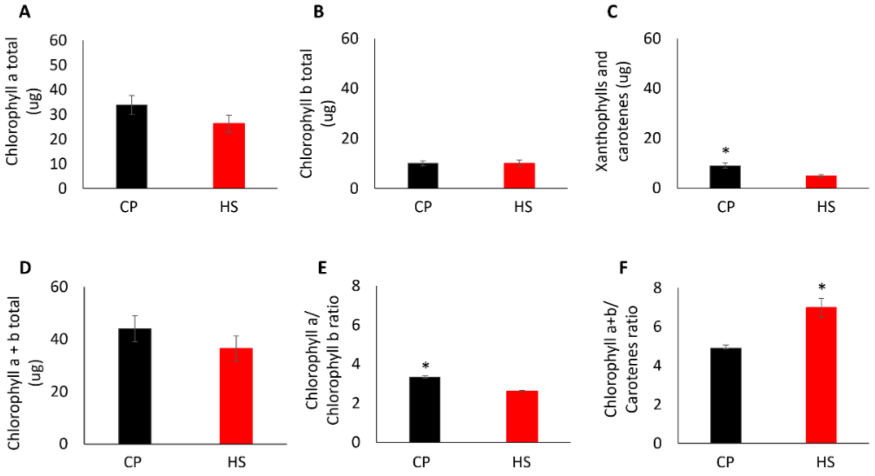
Supplementary Table 4- Correlations between RNA-Seq and RT-qPCR data: correlation values between the FPKM read value for each gene and the average gene expression (RT-qPCR) are depicted for each time point studied. P-value < 0,05

Gene	Pearson	Sig.	R^2 (RNA-Seq. vs. RT-qPCR)	R^2 (RNA-Seq. vs. RT-qPCR)
KAO2	0.988	0.000	0.977	97.7
DELLA	0.869	0.005	0.756	75.6
AUX	0.989	0.000	0.979	97.9
ASB1	0.941	0.000	0.886	88.6
AHP	0.982	0.000	0.965	96.5
TCH4	0.995	0.000	0.990	99.0
PP2C	0.941	0.000	0.886	88.6
ABA2	0.973	0.000	0.947	94.7
PYR	0.995	0.000	0.990	99.0
JAZ1	0.969	0.000	0.938	93.8

PR1	0.989	0.000	0.978	97.8
TGA6	0.981	0.000	0.962	96.2

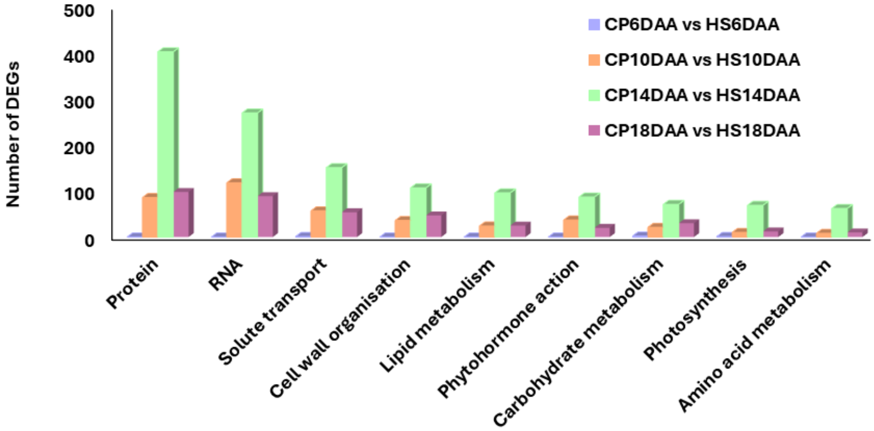


Supplementary Figure 1- Physiological responses of common bean plants under control (C) (25/18°C day/night) and heat stress (HS) (32/25°C day/night) conditions. A Relative water content (RWC), B Photosynthetic rate (A), C Transpiration rate (E), D Stomatal conductance (gS) and E Water use efficiency. Error bars represent standard error of the mean. No statistically significant differences were observed between C and HS conditions across all measured parameters.

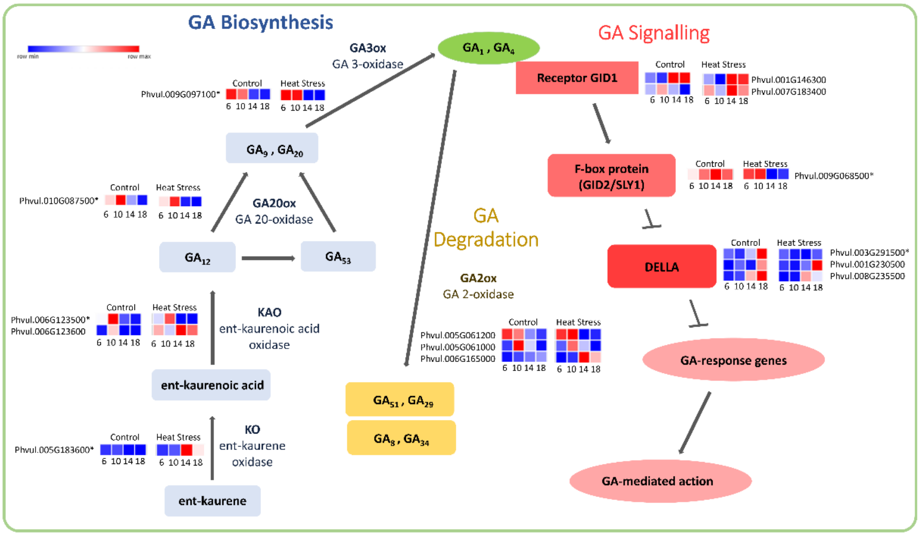


Supplementary Figure 2- Effects of HS on chlorophyll and carotenoid content in plants under control (C) (25/18°C day/night) and HS (HS) (32/25°C day/night) conditions. **A**

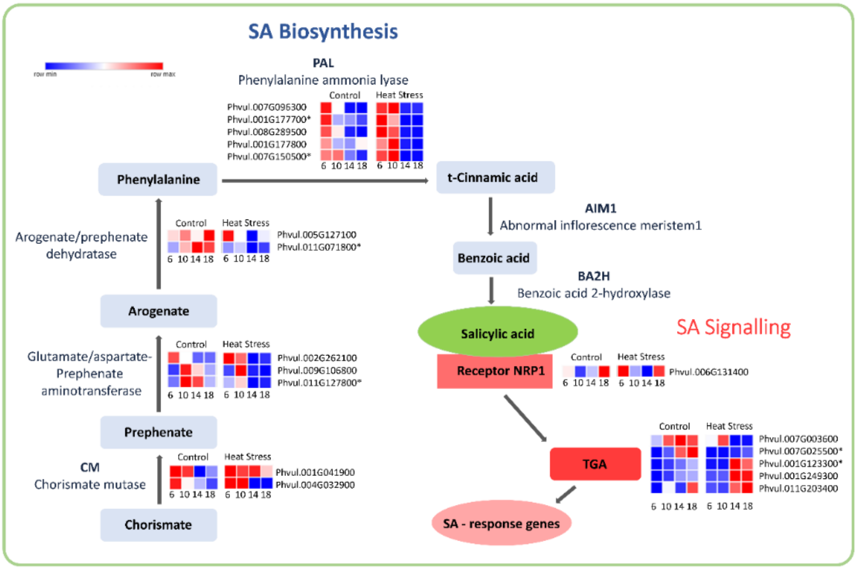
Total chlorophyll a content, **B** Total chlorophyll b content, **C** Xanthophylls and carotenoids content, **D** Total chlorophyll a + b content, **E** Chlorophyll a to chlorophyll b ratio and **F** Chlorophyll a + b to carotenoids ratio. Asterisks indicate statistically significant differences between C and HS conditions ($p < 0.05$). Error bars represent standard error of the mean.



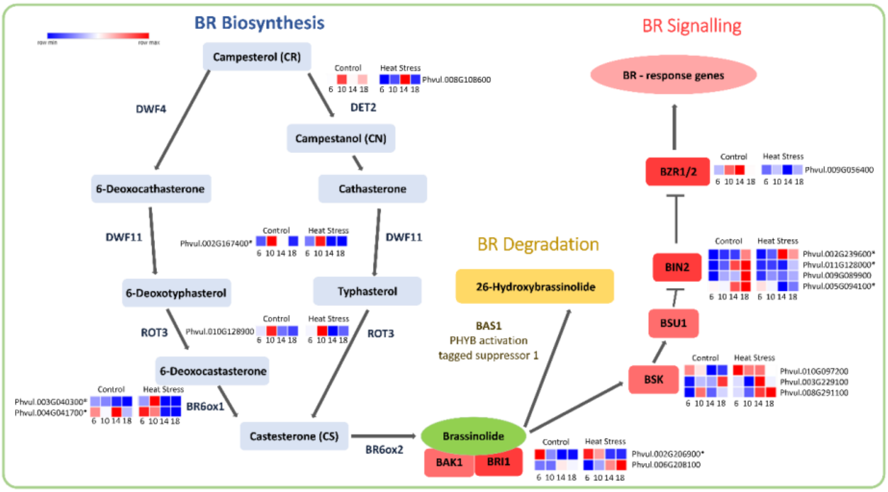
Supplementary Figure 3- Functional categories of differentially expressed genes (DEG) in the transition from the embryo development to seed filling in common bean seeds in heat stress (HS) and control conditions (CP). The percentage of DEG in each category were displayed between the main comparison studied (CP6 vs HS6, CP10 vs HS10, CP14 vs HS14 and CP18 vs HS18 days after anthesis (DAA)). The percentage of DEGs changed was calculated by comparison of the number of DEGs in each category in relation to the total of DEGs identified within each comparison.



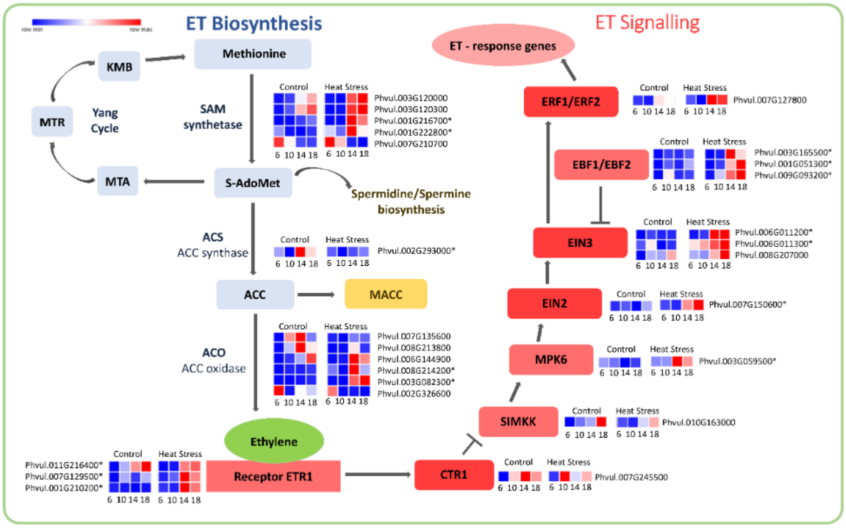
Supplementary Figure 4- Schematic representation of the gibberellin (GA) biosynthesis, degradation and signalling pathways in seeds at 6, 10, 14 and 18 days after anthesis (DAA) under control and heat stress conditions. The GA biosynthesis pathway includes key genes, such as KO, KAO, GA20ox and GA3ox. In the GA degradation pathway, GA2ox genes are represented. The GA signalling pathway features the receptor GID1, GID2/SLY1 and the negative regulators, the DELLA proteins. Adapted from (Chen & Tan, 2015). FPKM were clustered using Euclidean distance and an average linkage. Blue colour represents low expression levels (lower FPKM values); White (Middle Colour) represents moderate expression levels and Red represents high expression levels (higher FPKM values). Asterisks indicate differentially expressed genes (DEGs): corrected p-value ≤ 0.001 and |Log2(Fold Change - FC) | ≥1.



Supplementary Figure 5 - Schematic representation of the salicylic acid (SA) biosynthesis and signalling pathways in seeds at 6, 10, 14 and 18 DAA under control and heat stress conditions. The SA biosynthesis genes, including CM, Glutamate/Aspartate-Prephenate Aminotransferase (G/APa), Arogenate/Prephenate Dehydratase (A/PD) and PAL are depicted. The SA signalling pathway includes the receptor NRP1 and the transcription factor TGA, which plays a crucial role in SA-mediated gene expression. Adapted from (Lefevere et al., 2020). FPKM were clustered using Euclidean distance and an average linkage. Blue colour represents low expression levels (lower FPKM values); White (Middle Colour) represents moderate expression levels and Red represents high expression levels (higher FPKM values). Asterisks indicate differentially expressed genes (DEGs): corrected p-value ≤ 0.001 and |Log2(Fold Change - FC) | ≥1.



Supplementary Figure 6- Schematic representation of the brassinosteroid (BR) biosynthesis, degradation and signalling pathways in seeds at 6, 10, 14 and 18 DAA under control and heat stress conditions. In the BR biosynthesis pathway, DET2, DWF11, ROT3 and BR6ox1 are shown. In the BR signalling pathway, BAK1/BRI1, BSK and BIN2 are represented. Adapted from (Sirhindi, 2013). FPKM were clustered using Euclidean distance and an average linkage. Blue colour represents low expression levels (lower FPKM values); White (Middle Colour) represents moderate expression levels and Red represents high expression levels (higher FPKM values). Asterisks indicate differentially expressed genes (DEGs): corrected p-value ≤ 0.001 and |Log2(Fold Change - FC) | ≥1.



Supplementary Figure 7- Schematic representation of the ethylene (ET) biosynthesis, degradation and signalling pathways in seeds at 6, 10, 14 and 18 DAA under control and heat stress conditions. In the ethylene biosynthesis pathway, key genes such as SAM synthetase and ACO are shown. Simultaneously, in the ethylene signalling pathway, components such as ETR1, CTR1, SIMKK, MPK6, EIN2, EIN3, EBF1/EBF2 and ERF1/ERF2 are presented. Adapted from (Corbineau et al., 2014). FPKM were clustered using Euclidean distance and an average linkage. Blue colour represents low expression levels (lower FPKM values); White (Middle Colour) represents moderate expression levels and Red represents high expression levels (higher FPKM values). Asterisks indicate differentially expressed genes (DEGs): corrected p-value ≤ 0.001 and |Log2(Fold Change - FC) | ≥1.

Chapter IV

Influence of parental heat stress on germination and seedling development in common bean (*Phaseolus vulgaris* L.)

Adapted from:

Cláudia Lopes, Pedro Fevereiro, Susana de Sousa Araújo. Influence of parental heat stress on germination and seedling development in common bean (*Phaseolus vulgaris* L.)

To be submitted shortly.

Author contributions to the chapter:

Cláudia Lopes participated in the experimental setup and design, performed the experiments and corresponding analysis, analysed the data and wrote the chapter.

Abstract

Rising global temperatures pose a major challenge to agriculture, not only by affecting crop performance directly but also by inducing heritable changes that can persist across generations. This study explores the transgenerational effects of parental heat stress in *Phaseolus vulgaris* (common bean), focusing on how it influences germination, seedling development, and reproductive traits in progeny grown under optimal conditions. Seeds derived from heat-stressed parental plants (SHSP) germinated significantly faster than those from control plants (SCP), exhibiting reduced mean germination time (MGT), lower T50, and higher germination index (GI). Despite this accelerated germination, SHSP progeny showed markedly reduced seedling vigor, with shorter roots and shoots, diminished biomass, and smaller leaf area and leaf number. Crucially, these progeny produced seeds that were lighter and shorter than those from SCP, despite being cultivated in non-stressful conditions, indicating a carry-over effect from parental stress. These findings reveal that heat exposure during the parental generation impairs vegetative and reproductive development in the offspring, likely through transgenerational stress memory. The persistence of these effects under optimal conditions suggests an epigenetic basis and highlights the importance of integrating parental environmental history into crop management and breeding strategies under climate change.

Keywords: Heat stress, *Phaseolus vulgaris* L., Transgenerational effects, Germination, Seedling vigor

1. Introduction

Plants, as sessile organisms, have evolved mechanisms to cope with various environmental stressors that can significantly impact their growth, development and reproduction (Kumar et al., 2012; Nawaz et al., 2023).

Understanding how plants sense, respond and adapt to these diverse environmental challenges is crucial for predicting and mitigating the impacts of climate change on ecosystems and ensuring the future survival of plant species (Pagnussat & Gomez-Casati, 2024). Among these, heat stress is particularly problematic due to its capacity to impact crop growth, development and yield by disrupting physiological processes and cellular functions (Prasad et al., 2015). It affects plants at all stages, from germination to harvest, with the reproductive stage being particularly sensitive (Kaushal et al., 2016; Nadeem et al., 2018). Heat stress impairs photosynthesis, respiration, water balance and membrane stability, leading to reduced seed size and yield (Kaushal et al., 2016). The global rise in temperatures due to climate change has exacerbated the occurrence of heat stress, making it a critical concern for agriculture, especially in regions where crops are already near their thermal limits, posing a significant threat to crop production and food security worldwide (Bita & Gerats, 2013; Hassan et al., 2021).

Phaseolus vulgaris L., commonly known as common bean, is one of the most important legumes worldwide (Myers & Kmiecik, 2017). It plays a crucial role in sustainable agriculture, food security and nutrition, particularly in Latin America and Africa (Mukankusi et al., 2019; Perez de la Vega et al., 2017). Additionally, legumes like common bean play a crucial role in sustainable agriculture due to their ability to fix atmospheric nitrogen through symbiotic relationships with rhizobia (Uebersax et al., 2023). This biological nitrogen fixation process reduces the need for nitrogen fertilizers, contributing to environmentally friendly production systems (Uebersax et al., 2023). However, legumes, including common bean, are particularly sensitive to abiotic stresses, especially heat stress. High temperatures, interfere with flower bud formation and pod filling stages (Vargas et al., 2021), causes flower abortion, decreased pod set and smaller seed size in *Phaseolus vulgaris* (D. A. Da Silva et al., 2019). Furthermore, pollen viability is significantly reduced under heat stress, correlating with lower yields (Vargas et al., 2021). In common bean physiological mechanisms affected by heat stress include disrupted source-sink relationships, reduced phloem loading of sucrose and decreased free hexose concentrations (Soltani et al., 2019).

Such effects are not limited to the stressed plants themselves but can also impact the next generation through transgenerational stress responses. Plants can transmit stress responses to their offspring through transgenerational effects, which involve changes in DNA methylation and homologous recombination frequency (Migicovsky & Kovalchuk, 2013). These effects can impact plant phenotypes, with stress timing having a stronger influence than frequency (Deng et al., 2021). While transgenerational responses are not always adaptive, they exhibit genetic variation and correlate with climatic variability, suggesting evolutionary potential (Deng et al., 2021). The mechanisms underlying these effects may involve small interfering RNAs and Dicer-like proteins (Capitão et al., 2011). Heat stress can alter leaf morphology, flowering time and transposon expression in progeny (Migicovsky et al., 2014). Progeny of heat-stressed *Arabidopsis thaliana* exhibit altered leaf morphology, earlier bolting and increased transposon expression (Migicovsky et al., 2014). These changes are associated with modifications in DNA methylation, histone marks and small RNA pathways (Migicovsky & Kovalchuk, 2013). Multigenerational heat stress exposure may lead to increased heat tolerance, higher homologous recombination frequency and more genetic mutations in offspring (Yadav et al., 2022). Transgenerational effects of heat stress can include physiological changes such as altered leaf morphology and flowering time, as well as epigenetic modifications like changes in DNA methylation, histone marks and small RNA expression (Liu et al., 2020; Migicovsky et al., 2014).

Nevertheless, for legumes it remains to be understood how the HS imposed in parental plants during the reproductive phases may impact into the progeny performance, in particular seed germination traits and adaptability of resulting seedlings to harsh conditions. Some studies were conducted to understand the effect of temperature in seed germination and underlying molecular mechanisms. Arabidopsis metabolomics reveal distinct metabolite profiles under moderate (28°C) versus extreme (34-40°C) temperatures during germination, with changes in organic acids, amines and lipids affecting various metabolic pathways (Qian et al., 2023). In soybeans, temperature affects germination rates and seedling vigor, with 25°C promoting better total seedling length compared to 15°C and 35°C (Abd Ghani et al., 2023). It is important to note that these studies analyzed the direct effect of temperature during germination on non-stressed seeds, and not the transgenerational effects arising from parental heat stress. Recent evidence from wheat highlights a clear parental effect: seeds developed under high-temperature stress exhibited enhanced germination capacity at elevated temperatures, mediated by molecular changes such as increased heat shock protein expression and fatty acid remodeling (Toda-Matsunaga et al., 2025). This reinforces the importance of exploring transgenerational responses to environmental stress during seed development, particularly in crops like common bean. Understanding the role of germination temperature in common bean and its impact in moderating these transgenerational effects is crucial for developing effective crop management strategies.

The research conducted herein aimed to address several key questions regarding the parental heat stress effect on germination and seedling development in the progeny (seeds) developed under such conditions. Our hypothesis assumes that HS applied during SD has an effect in the germination performance of resulting seeds. We aimed to answer the following questions, which remain unclear in this important species:

-How does parental heat stress imposed during SD affect seed germination performance?

- Does germination temperature modify the effects of parental heat stress on germination traits?

- Does a HS treatment bring any additional advantage in seedling vigor, early plantlet development and plant morphological traits even if grown under optimal conditions?

- Does parental heat stress experienced during the F1 generation affect the fresh weight and length of F2 seeds developed under optimal conditions?

To accomplish this goal, we characterized the germination process of seeds from both heat-stressed (SHSP) and control plants (SCP) and analysed the main differences in germination traits and related indexes. A morphological characterization of seedling traits originating from these seeds was performed. By understanding these transgenerational effects, this research aims to contribute valuable knowledge towards optimizing common bean production under changing climate conditions.

2. Material and Methods

2.1 Plant material and growth conditions

Seeds of the *P. vulgaris* genotype SER 16 were collected from plants grown at control conditions and from plants grown at heat stress conditions (**Chapter III**). Plants were grown at 2.5 l pots containing a (2:1:1) mixture of commercial soil (Compo Sana S.A., Barcelona, Spain), peat and vermiculite, respectively, in a growth chamber (Fitoclima 5.000 EH, ARALAB, Portugal) with controlled environmental conditions, with 50% humidity, photoperiod of 16/8-h day/night and light intensity of 400 μmol m−2 s−1. Control plants were grown at 25/18°C day/night and heat stress plants grown at 32/25°C day/night. Both conditions were watered 3 times per week. Seeds were collected from plants upon seed desiccation for germination assays.

2.2 Germination assay

Seeds from control plants (SCP) and seeds from heat-stressed (SHSP) of the *P. vulgaris* genotype SER 16 plants were used in this study. SCP and SHSP were germinated onto 9.5 cm Petri dishes covered with a layer of Wathman No.1 paper filter, uniformly moisturized with 2 ml distillated water, found sufficient to conduct germination tests in this species. Five Petri dishes plates, each containing 6 seeds each from both SCP and SHSP were incubated in a controlled growth chamber with different temperatures (25ºC and 32ºC) resulting in four tested experimental conditions. 30 seeds coming from control conditions were germinated at 25ºC and 30 seeds were germinated at 32ºC. 30 seeds coming from SHSP were germinated at 25ºC and 30 seeds were germinated at 32ºC.

Germination was monitored daily over a period of 5 days following the approach described by R. H. Ellis et al., (1985). The time to reach 50% germination (T50) was calculated using the method from Coolbear & McGill, (1990), later adapted by Farooq et al., (2005):

$$T50 = ti + \frac{\left(\frac{N}{2} - ni\right) .\ (ti - tj)}{ni - nj}$$

Where N represents the total number of seeds that germinated and ni and nj denote the cumulative germinated seeds at the consecutive times *ti* and *tj*, when $ni < \frac{N}{2} < nj$.

The final germination percentage (FGP) was determined by:

$$FGP\ (\%) = \frac{n}{N} \times 100$$

Where n is number of germinated seeds and N is number of total seeds sown.

Mean Germination Time (MGT) was calculated using the formula of R.-E. Ellis et al., (1981):

$$MGT = \frac{\Sigma Dn}{\Sigma n}$$

Where n is the number of seeds which were germinated on day D. D is the number of days counted from the beginning of germination.

The germination index (GI) was calculated with the method of the *Association of Official Seed Analysts (AOSA, 1983)*:

$$GI = \frac{G1}{T1} + \frac{G2}{T2} + \ldots + \frac{Gn}{Tn}$$

Where G1, G2, ..., Gn represent the number of seeds germinated on each day and T1, T2, ..., Tn are the corresponding days from sowing to each count.

Germination synchrony was measured using the following expression:

$$Z = \frac{\Sigma \mathrm{Cni,2}}{\mathrm{N}},$$

Where Cni, 2 is calculated as Cni, 2 = $\frac{\mathrm{ni\,(ni-1)}}{2}$ and N = $\frac{\Sigma \mathrm{ni}\ (\Sigma \mathrm{ni}-1)}{2}$, with ni being the count of seeds germinated at each time i. Z = 1 indicates all seeds germinated simultaneously, while Z=0 signifies variability in germination timing according to Ranal & De Santana, (2006).

2.3 Seedling assays

The germinated seedlings were transferred to uncovered trays containing vermiculite five days after germination. Seedlings were grown in a growth chamber (Fitoclima 5.000 EH, ARALAB, Portugal) with controlled environmental conditions, at 25/18 °C day/night with 50% humidity, photoperiod of 16/8-h day/night and light intensity of 400 μmol m^{-2} s^{-1}, the 20 seedlings from each treatment were used to measure root, shoot, primary leaves area seedling length and seedling fresh and dry weight at 9 days after germination.

The seedling vigor index (SVI) was calculated by the following formula (Zhao, 2016):

$$SVI = FGP \times DW$$

Where DW is seedling dry weight.

2.4 Vegetative and Reproductive Traits of F2 Plants

The remaining seedlings were grown at 2.5 l pots containing a (2:1:1) mixture of commercial soil (Compo Sana S.A., Barcelona, Spain), peat and vermiculite, respectively, in a growth chamber (Fitoclima 5.000 EH, ARALAB, Portugal) with controlled environmental conditions, at 25/18 °C day/night with 50% humidity, photoperiod of 16/8-h day/night and light intensity of 400 μmol m^{-2} s^{-1}. After the plants ceased seed production, the total number of leaves was counted, and seed fresh weight and seed length was measured.

2.5 Statistical analysis

Data from the germination parameters and morphological analysis was analysed with IBM SPSS Statistics V25.0 software. Data statistical significance was assessed using one-way analysis of variance (ANOVA) coupled with post-hoc Tukey HSD for mean pairwaise comparison. Means were considered significantly different when $P \le 0.05$.

3. Results

3.1 Seed germination analysis

The germination performance of seeds from both heat-stressed (SHSP) and control plants (SCP) was assessed at two temperatures: 25°C and 32°C (Table 1). The final germination percentage (FGP) was consistently 100% for all treatment combinations, indicating that experimental conditions tested did not affect the final germination rate (Fig. 1).

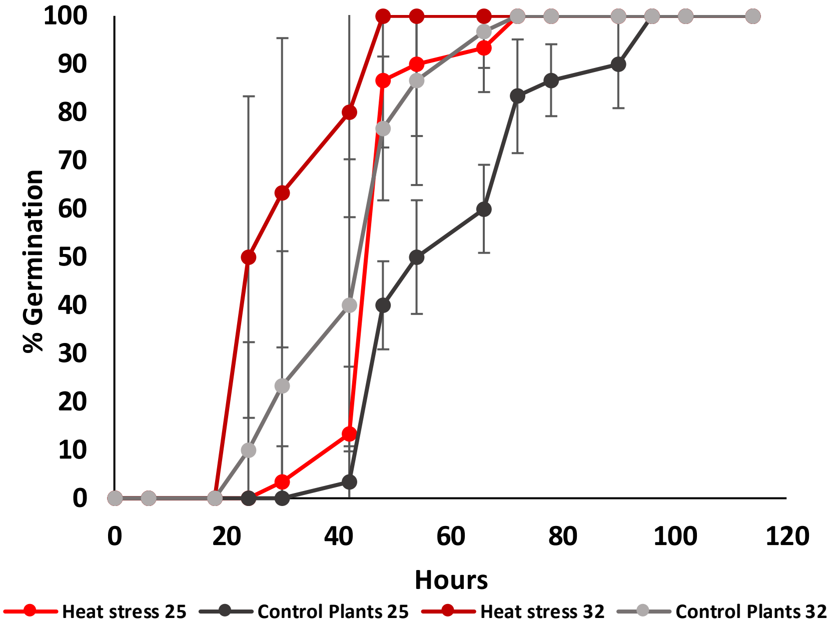


Figure 1- Germination percentage over time of seeds derived from heat-stressed and control plants germinated at 25°C and 32°C.

However, the mean germination time (MGT) showed significant variation across the tested conditions. SHSP germinated faster compared to SCP, especially at 32°C (Table 1). At 25°C, the MGT for SHSP was 49.20 hours, statistically significantly lower than SCP, which exhibited an MGT of 63.00 hours. When germinated at 32°C, SHSP had an MGT of 32.60 hours, much shorter than the 45.40 hours observed for SCP.

The germination index (GI), which reflects the speed of germination, was also higher for SHSP, particularly at 32°C (Table 1). SHSP had a GI of 0.200 at 32°C, compared to 0.144 for SCP. At 25°C, the GI for SHSP was 0.125, while SCP recorded a slightly lower value of 0.101.

The time to 50% germination (T50) followed a similar pattern (Table 1). SHSP reached 50% germination faster than those from SCP, with T50 values of 45.00 and 57.60 hours at 25°C, respectively. At 32°C, SHSP reached 50% germination in 27.70 hours, significantly faster than the 41.12

hours for SCP. Notably, SHSP seeds germinated at 32 °C reached 50% germination almost twice as fast as SCP seeds germinated at 25 °C (Fig. 1).

The Z parameter, which represents the average germination synchrony, scored a high variability among the tested conditions, nevertheless no significant differences were observed (Table 1).

Table 1- Germination traits accessed in seeds from heat-stressed and control plants germinated at 25°C and 32°C. The table shows Final Germination Percentage (FGP), Mean Germination Time (MGT), Germination Index (GI), time to 50% germination (T50) and Z parameter. Values followed by different letters within the same column indicate significant differences between treatments ($p < 0.05$).

Germinated at:	**Seeds from:**	**FGP (%)**	**MGT (hours)**	**GI**	**T50 (hours)**	**Z**
25°C	**Heat stress Plants**	100^{a}	49.20^{b}	0.125^{b}	45.00^{b}	0.520^{a}
25°C	**Control Plants**	100^{a}	63.00^{a}	0.101^{b}	57.60^{a}	0.133^{a}
32°C	**Heat stress Plants**	100^{a}	32.60^{c}	0.200^{a}	27.70^{c}	0.427^{a}
32°C	**Control Plants**	100^{a}	45.40^{b}	0.144^{b}	41.12^{b}	0.280^{a}

3.2 Seedling and plant morphological analysis

The Seedling Vigor Index (SVI) is significantly different between SHSP and SCP (Fig. 2a). At 25°C, SCP exhibited a statistically higher SVI (22.56) compared to SHSP (16.30). A similar trend was observed at 32°C, with SCP showing a significantly higher average SVI (23.23) than SHSP (15.62).

For shoot length, seedlings from SCP had significantly longer shoots compared to seedlings from SHSP at both temperatures (Fig. 2b). At 25°C, seedlings from SCP exhibited an average shoot length of 321.50 mm, compared to 225.86 mm for seedlings from SHSP. A similar pattern was seen at 32°C, where seedlings from SCP produced longer shoots (287.37 mm) compared to seedlings from SHSP (211.86 mm).

The root length followed the same pattern, with seedlings from SCP having significantly longer roots than seedlings from SHSP (Fig. 2c). At 25°C, seedlings from SCP had a root length of 160.84 mm, whereas seedlings from SHSP had a root length of 113.28 mm. At 32°C, seedlings from SCP had a root length of 154.31 mm, while seedlings from SHSP had a significantly shorter root length of 108.13 mm.

The root/shoot ratio did not show significant differences between treatments at either temperature, indicating that heat stress did not alter the relative biomass allocation between roots and shoots (Fig. 2d).

Total seedling length was significantly greater in seedlings from SCP compared to seedlings from SHSP at both temperatures (Fig. 2e). At 25°C, the total seedling length for seedlings from SCP was 482.34 mm, while seedlings from SHSP had a significantly shorter seedling length of 339.15 mm. Similarly, at 32°C, seedlings from SCP produced longer seedlings (441.68 mm) compared to seedlings from SHSP (319.99 mm).

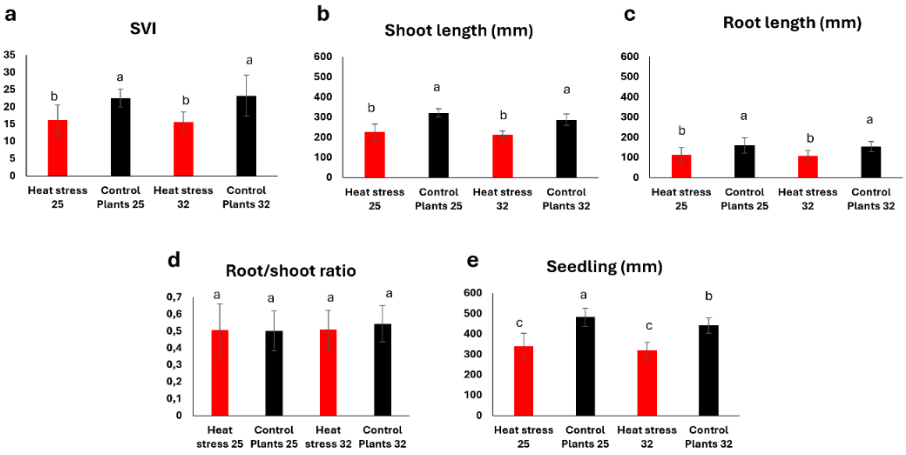


Figure 2- Seed vigour index (SVI) and seedling growth parameters of seeds from heat-stressed and control plants germinated at 25°C and 32°C. **a** Seedling Vigor

Index (SVI), **b** Shoot length, **c** Root length, **d** Root/shoot ratio and **e** Total seedling length. Different letters above the bars indicate statistically significant differences between treatments ($p < 0.05$).

For seedling shoot fresh weight, seedlings from SCP produced significantly heavier shoots than seedlings from SHSP at both temperatures (Fig. 3a). At 25°C, seedlings from SCP had an average shoot fresh weight of 1.63 g, which was almost double that of seedlings from SHSP (0.90 g). At 32°C, the shoot fresh weight of seedlings from SCP was also significantly higher (1.39 g) compared to seedlings from SHSP (0.85 g).

The root fresh weight followed a similar trend (Fig. 3b). At 25°C, seedlings from SCP had significantly heavier roots (0.32 g) than seedlings from SHSP (0.09 g). This difference persisted at 32°C, where seedlings from SCP had a root fresh weight of 0.20 g, significantly greater than the 0.07 g observed in seedlings from SHSP.

The total seedling fresh weight showed clear differences between the groups (Fig. 3c). At 25°C, seedlings from SCP had the heaviest seedlings with a total fresh weight of 1.95 g, compared to 0.99 g for seedlings from SHSP. At 32°C, seedlings from SCP were still significantly heavier (1.59 g) than those from seedlings from SHSP (0.92 g).

Regarding shoot dry weight, seedlings from SCP also exhibited higher values than seedlings from SHSP across both temperatures (Fig. 3d). At 25°C, seedlings from SCP had a shoot dry weight of 0.18 g, while seedlings from SHSP had a significantly lower value of 0.13 g. This trend was also observed at 32°C, where seedlings from SCP had a shoot dry weight of 0.19 g, compared to 0.13 g in seedlings from SHSP.

The root dry weight also showed significant differences between treatments (Fig. 3e). At 25°C, seedlings from SCP had a root dry weight of 0.05 g, which was notably higher than the 0.03 g of seedlings from SHSP. Similarly, at 32°C, seedlings from SCP had significantly higher root dry weight (0.05 g) compared to seedlings from SHSP (0.03 g).

Total seedling dry weight was significantly higher in seedlings from SCP compared to seedlings from SHSP at both temperatures (Fig. 3f). At 25°C, the dry weight of seedlings from SCP was 0.23 g, compared to 0.16 g in seedlings from SHSP. At 32°C, seedlings from SCP again exhibited higher seedling dry weights (0.23 g) than seedlings from SHSP (0.16 g).

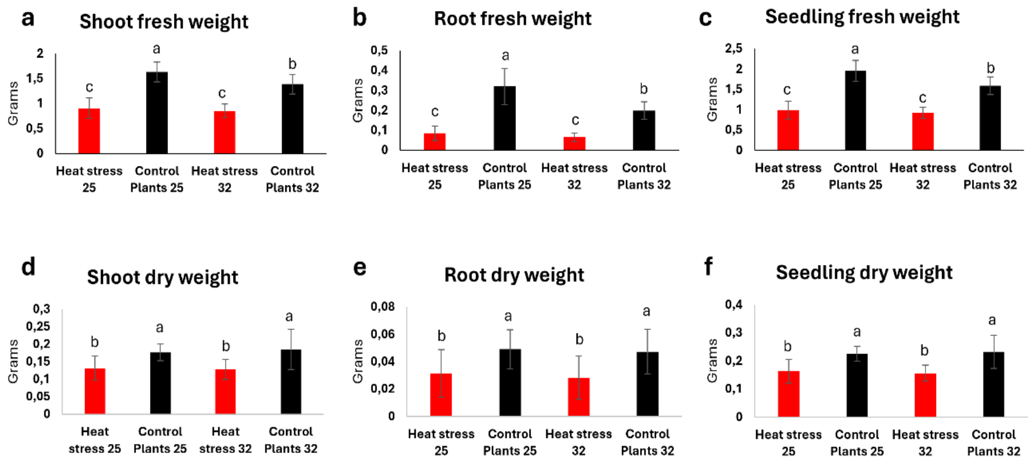


Figure 3- Shoots, roots and seedlings fresh and dry weight measurements from seeds of heat-stressed and control plants germinated at 25°C and 32°C. **a** Shoot fresh weight, **b** Root fresh weight, **c** Seedling fresh weight, **d** Shoot dry weight, **e** Root dry weight and **f** Seedling dry weight. Different letters above the bars indicate statistically significant differences between treatments ($p < 0.05$).

For primary leaf area, seedlings from SCP consistently showed significantly larger leaves compared to SHSP at both temperatures (Fig. 4a). At 25°C, seedlings from SCP had a mean leaf area of 2119.67 mm², significantly larger than the 1136.88 mm² observed in SHSP. A similar trend was seen at 32°C, where the leaf area of seedlings from SCP (1998.78 mm²) was also significantly greater than that of SHSP (953.45 mm²).

The leaf number followed the same pattern as leaf area, with plants from SCP producing significantly more leaves than SHSP (Fig. 4b). At 25°C, plants from SCP produced an average of 20 leaves, whereas SHSP produced only 13 leaves. At 32°C, plants from SCP had 19 leaves on average, compared to 12 leaves for SHSP.

Seed fresh weight showed significant differences between the treatments (Fig. 4c). At both temperatures, seeds from plants from SCP were significantly heavier than those from SHSP. At 25°C, seeds from plants from SCP had an average seed fresh weight of 0,289 g, compared to 0.240 g in SHSP. This difference persisted at 32°C, where seeds from plants from SCP plants had a seed fresh weight of 0.309 g, significantly higher than the 0.229 g observed in SHSP.

The seed length also varied significantly between the treatments (Fig. 4d). At 25°C, seeds from plants from SCP had a significantly longer average length (11.38 mm) compared to SHSP (9.31 mm). This trend was even more pronounced at 32°C, where plants from SCP produced seeds with an average length of 11.85 mm, compared to 8.99 mm in SHSP.

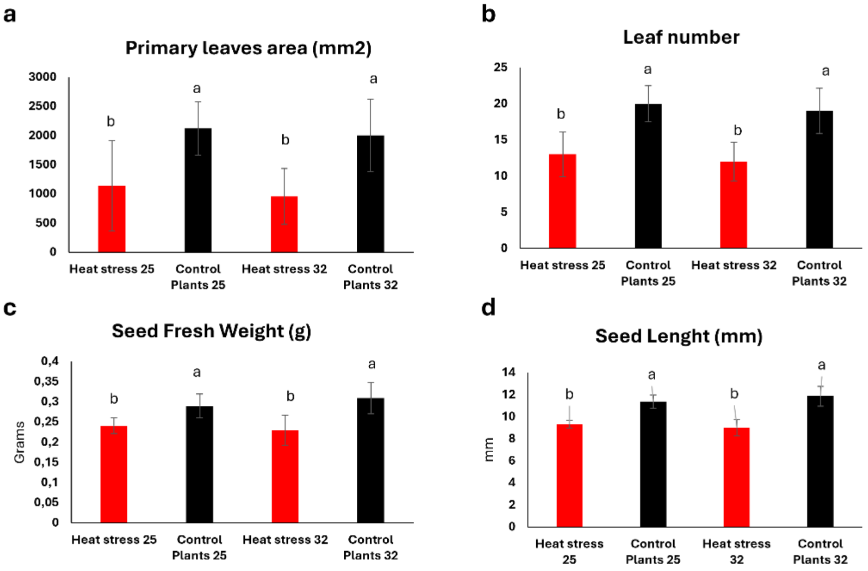


Figure 4- **a** Primary leaf area, **b** leaf number, **c** seed fresh weight and **d** seed length of plants from heat-stressed and control plants germinated at 25°C and 32°C. Different letters above the bars indicate statistically significant differences between treatments ($p < 0.05$).

4. Discussion

This study aimed to assess how parental HS imposed during seed development affects the germination behavior and early plant development of the resulting progeny. We hypothesized that HS during parental reproductive phases could alter seed germination performance, potentially modulated by the temperature during germination, and might impact seedling vigor and morphological traits. Our results show that parental HS indeed favored faster germination in the progeny, particularly when germination occurred at 32 °C. However, this advantage in germination speed was accompanied by a marked reduction in seedling vigor and plant morphological development, with progeny from HS plants exhibiting significantly smaller shoots, roots and leaf area compared to those derived from control plants grown at 25 °C.

4.1 *Phaseolus vulgaris* parental heat stress leads to progeny faster seed germination

Although SCP and SHSP did not show differences in the final germination percentage (FGP) of the seeds, they significantly influenced other parameters. Notably, SHSP consistently showed faster germination, as reflected by lower mean germination time (MGT) and faster time to reach 50% germination (T50) compared to SCP. These findings are aligned with previous research, which showed that higher temperatures generally accelerate germination in species such as citrus and tomato (Mobayen, 1980). This trend is further supported by observations in *Lathyrus sativus* (Sofades accession), where seeds exposed to 35 °C demonstrated a reduced MGT compared to the control, suggesting that moderate heat stress can enhance germination speed in heat-resilient varieties (Pagano et al., 2025).

In *Genista tinctoria* and *Calluna vulgaris*, earlier germination in offspring has been attributed to a transgenerational response aimed at avoiding unfavorable environmental conditions previously experienced by the parent generation—particularly during vulnerable early developmental stages (Walter et al., 2016). Additionally, earlier germination may confer a competitive advantage ecological over later-germinating individuals particularly during field establishment where resources can be limited (Walter et al., 2016). Rising temperatures will result in faster germination in tomato landraces (Sousaraei et al., 2022), supporting our observations in SHSP.

Parental heat stress appears to induce developmental acceleration in progeny of many species. For instance, in Arabidopsis, offspring of heat-stressed parents exhibited accelerated flowering (Deng et al., 2021; Groot et al., 2017) and bolted earlier than progeny from non-stressed parents (Migicovsky et al., 2014). In our results we have observed that seeds resulting from plants grown under heat stress germinated under stress twice faster that the ones resulting from their counterparts grown under “optimal” conditions. Indeed, the SHSP germinated even faster at 32°C, exhibiting lower MGT and T50, further reinforcing the hypothesis that heat stress during seed development influences germination speed. Previous studies have shown that moderate heat stress (35°C for 24–48 hours) during early seed development in rice (*Oryza sativa*) resulted in higher germination rates, likely due to altered abscisic acid (ABA) sensitivity and increased starch accumulation (Begcy et al., 2018). Similarly, Pagano et al. (2025) reported that *Lathyrus sativus* seeds subjected to hydropriming and then challenged with 40 °C heat waves exhibited improved germination speed compared to unprimed seeds, highlighting how pre-sowing thermal conditions can modulate germination dynamics in stress-resilient legumes.

One possible explanation for the accelerated germination observed in SHSP is transgenerational stress memory, a phenomenon where environmental stress experienced by parent plants influences the phenotype and performance of their progeny (Lämke & Bäurle, 2017). This memory is often mediated by epigenetic mechanisms, including small RNAs, histone modifications, and DNA methylation. One of the limitations of our presented study is that we did not explored the molecular mechanisms behind the observed phenotypes. Many molecular mechanisms can be proposed to have particular action in the responses observed and might be explored in future studies. In particular, it would be interesting to examine how parental heat stress might influence the expression profiles of key genes regulating seed development, including those involved in the transition from embryogenesis to seed filling, reserve compound accumulation and hormonal signaling pathways. Building on our previous work, which focused on the LAFL (LEC1, ABI3, FUS3, LEC2) regulatory network and hormone-mediated control of SD, future research could explore whether the expression of these candidate genes is maintained, altered, or reprogrammed in seeds derived from SHSP. Investigating the epigenetic regulation and transcriptional memory of these SD-related genes could provide crucial insights into how environmental stress during the parental generation impacts seed physiology and vigor in subsequent generations.

In *Brassica rapa*, heat stress induces heritable changes in small RNA profiles, with the endosperm and pollen showing the most significant alterations (Bilichak et al., 2015). Similarly, in Arabidopsis, stress memory is transmitted primarily through female gametes and involves DNA methylation and small RNAs (Boyko & Kovalchuk, 2010). Furthermore, progeny of heat-stressed *Brassica rapa* plants exhibit changes in the expression of noncoding tRNA fragments and small nucleolar RNA fragment (Byeon et al., 2019). Liu et al. (2021) identified miRNA-mRNA networks that confer enhanced heat tolerance through regulation of ROS detoxification and growth-related pathways.

Another likely factor contributing to the enhanced germination of SHSP is hormonal regulation, particularly the interaction between ABA and gibberellins (GA). These hormones play opposing roles in seed dormancy and germination, with ABA promoting dormancy and GA stimulating germination (Bicalho et al., 2015; Vishal & Kumar, 2018). Heat stress might decrease ABA levels (**Chapter III**), leading to a more rapid breakdown of dormancy and faster germination, particularly at higher temperatures like

32°C. Our data support this hypothesis, since phytohormone quantification revealed that seeds from control plants exhibited a peak in ABA concentration at 14 DAA (days after anthesis), followed by a gradual decline. In contrast, seeds from heat-stressed plants displayed significantly lower ABA concentrations at 14, 18, and 20 DAA, suggesting that heat stress disrupts the normal accumulation pattern of ABA during seed development. Moreover, PP2C (Protein Phosphatase 2C), a negative regulator of ABA signalling, is also upregulated at 14 and 18 DAA under HS, further reflecting the decrease in ABA levels in seeds of plants under HS (**Chapter III**). This molecular signature, increased ABA degradation and repression of ABA signaling, supports the observed lower ABA levels in HS seeds and offers a mechanistic explanation for the faster germination behavior seen under higher temperature conditions.

Importantly, the physiological, biochemical and molecular disturbances observed during seed development under heat stress (**Chapter III**) likely preconditioned the seeds for the germination behaviors recorded here. Our previous results showed that heat stress during seed development reduced the seed filling period, impaired the accumulation of storage compounds, and altered hormonal profiles, notably lowering ABA and JA levels while increasing IAA, CK, and SA concentrations early in development. These developmental alterations meant that seeds produced under heat stress were already intrinsically different from those produced under control conditions. As a consequence, the accelerated germination (lower MGT and T50) observed in SHSP was not solely a direct effect of germination temperature but largely reflected the physiological state imposed during seed formation. Germination temperatures acted primarily to modulate these intrinsic differences, rather than being their primary cause. This connection highlights that the disruptions identified during seed development have a lasting impact on seed performance and stress resilience in the progeny.

Together, these data suggest that parental heat stress alters ABA homeostasis and signaling during seed development, promoting earlier dormancy release and accelerated germination in the progeny. This mechanism is supported by studies in tomato, where seeds with higher GA levels and lower ABA content exhibited faster germination rates (Yang et al., 2015). Furthermore, heat stress can impact seed germination through changes in enzyme activity, respiration, and antioxidant responses (G. P. Silva et al., 2020). Moreover, DNA methylation has been identified as a key regulator of germination-related genes under heat stress conditions (Malabarba et al., 2021). These findings suggest that SHSP's faster germination may result from molecular changes induced by heat stress, particularly those affecting seed metabolism and dormancy-breaking mechanisms. Additionally, reactive oxygen species (ROS) have emerged as key regulators in the control of seed dormancy and germination. Controlled ROS production during imbibition participates in the perception of environmental signals and triggers cellular events required for germination, such as hormonal regulation and cell expansion. Specifically, ROS can directly oxidize negative regulators of germination, promote GA biosynthesis, and stimulate cell wall loosening at the radicle tip, facilitating radicle protrusion (Bailly, 2019). Another interesting mechanism to be explored in this experimental system would be the characterization of the molecular profiles of the genes involved in DNA damage detection and repair. In the model legume *Lathyrus sativus*, DNA repair and antioxidant genes such as LsSOG1, LsOGG1, LsFPG, and LsAPX were upregulated in response to hydropriming and heat stress, indicating that seeds activate protective mechanisms that facilitate genome integrity and oxidative stress mitigation during germination (Pagano et al., 2025).

4.2 *Phaseolus vulgaris* seedlings grown at optimal temperature condition are affected by parental heat stress

Despite the faster germination, seedlings resulting from the offspring of HS plants showed generally reduced size and biomass parameters, when compared to control plants, irrespective to the temperature studied. The SCP consistently produced seedlings with higher seedling vigor index (SVI), longer shoots, longer roots, and greater overall seedling length compared to SHSP, at both 25°C and 32°C. While the root-to-shoot ratio remained stable, the absolute values for root and shoot length were significantly reduced in SHSP seedlings, indicating that parental heat stress negatively affected overall seedling growth potential. Additionally, the lower fresh and dry biomass observed in SHSP seedlings further illustrates the detrimental impact of heat stress on seedling vigor and development, even when grown under optimal conditions. These findings support the idea that the effects of heat stress on parental plants persist into the next generation, impairing early seedling establishment. Moreover, seedling primary leaf area was significantly reduced in seedlings from SHSP, indicating that heat stress experienced by the parent plants led to impaired leaf development in the next generation.

The transgenerational impact of heat stress on root and shoot growth has been observed in various plant species. In wheat, parental heat stress led to reduced shoot and root growth, as well as a decline in root numbers in the offspring (Grass & Burris, 2011). Similar transgenerational effects have been reported in durum wheat (Liu et al., 2020), *Polygonum persicaria* (Herman et al., 2012), maize (Chukwudi et al., 2021) and soybean (Wijewardana et al., 2019), where stress experienced by parent plants resulted in growth reductions in progeny. Notably, it was observed in *Polygonum persicaria* that the effects of environmental stress can be cumulative, with offspring of both stressed grandparents and parents showing the most pronounced developmental constraints (Herman et al., 2012).

Our findings lead us to postulate that smaller seedlings with reduced shoot and root systems are less capable of resource acquisition, thereby decreasing their productivity (Souza et al, 2014). For example, in tomato, heat stress disproportionately affects root systems, impairing nutrient uptake and leading to yield penalties and reduced fruit quality (Giri et al., 2017). Likewise, in Arabidopsis, progeny of heat-stressed plants showed a marked reduction in rosette diameter, reinforcing the idea that early vegetative growth is sensitive to parental stress history (Groot et al., 2017). While the effects of HS in vegetative and seed progeny are well marked in common bean under controlled conditions studies, more studies with a higher number of seeds and under field conditions are needed to better understand if these phenotypes are maintained and bring any advantage when considering plant performance, namely productivity.

4.3 Seed production and plant development in *Phaseolus vulgaris* are affected by parental heat stress, even when the offspring are grown under optimal temperature conditions

Leaf number was significantly reduced in plants from SHSP, indicating that heat stress experienced by the parent plants led to impaired leaf development in the next generation. In Arabidopsis, progeny of heat-stressed plants showed changes in leaf size and bolting as compared with the progeny of control plants. progeny of heat-stressed plants exhibits fewer but larger leaves and earlier bolting (Migicovsky et al., 2014).

A possible mechanism underlying reduced leaf development in SHSP is hormonal imbalance, particularly involving cytokinins, which play a key role in leaf formation and expansion. In maize, heat stress during kernel development reduced cytokinin levels and increased kernel abortion, while exogenous cytokinin application improved thermotolerance (Cheikh & Jones, 1994). Similarly, in rice, high temperatures during the early reproductive stage decreased panicle cytokinin abundance and spikelet number in heat-susceptible varieties (Wu et al., 2017).

In addition to leaf development, our results also show that seeds from SHSP had lower fresh weight and reduced seed length compared to SCP.

This suggests that parental heat stress may have altered seed development processes, leading to smaller seeds with potentially lower energy reserves for germination and early seedling growth. The reduction in seed size in SHSP progeny is consistent with findings in rice, who reported that heat stress during early seed development resulted in moderately smaller seeds and led to malformed or non-viable seeds (Begcy et al., 2018). In *Brassica napus*, high temperature stress during flowering decreased seed production and weight, with both male and female reproductive organs affected (Young et al., 2004). Elevated temperatures during seed development can decrease seed size, number and fertility in cereals, legumes and vegetable crops (Reed et al., 2022). The mechanisms behind these transgenerational effects may involve changes in seed storage metabolites, epigenetic modifications and alterations in microRNA expression (Hatzig et al., 2018; Liu et al., 2020). Understanding these effects is crucial for developing climate-resilient crop varieties and maintaining food security.

5. Conclusion

In conclusion, this study pinpoints new avenues for better studying the transgenerational effects of heat stress on seed germination, seedling vigor and overall plant development in *Phaseolus vulgaris*. Although heat stress did not impair the final germination percentage, it significantly accelerated germination rates, particularly at higher temperatures, indicating a potential developmental acceleration mechanism. However, this faster germination did not translate into improved performance in post-germination traits.

Across all measured parameters, progeny from SHSP showed reduced growth potential: lower seedling vigor index, shorter roots and shoots, reduced biomass, and smaller primary leaves. Such reductions in seedling biomass and leaf area are likely to impact future plant competitiveness, nutrient uptake and yield potential, with long-term implications for crop fitness and productivity. Still more studies are needed to better elucidate these aspects.

Seeds produced by SHSP plants were consistently lighter and shorter compared to SCP seeds, even though the F2 seedlings were grown under optimal conditions. This suggests that developmental constraints were already established during seed development in the parental generation under heat stress and that these constraints were carried over to the progeny. Specifically, parental plants were exposed to heat stress during seed development, leading to modifications in seed traits. Although the resulting seeds (F2 generation) were cultivated under favorable, non-stressful conditions, they did not fully recover the typical phenotypic traits observed in seeds from control plants. This persistent difference indicates that heat stress during the parental seed development phase can induce lasting alterations, likely at the physiological, metabolic, or epigenetic level, that influence progeny performance independently of the environment in which they are grown.

These findings confirm that parental heat stress can induce a heritable physiological state that affects seed and seedling traits beyond the stressed generation. The expression of these effects in stress-free conditions suggests a form of stress memory, likely mediated by epigenetic mechanisms or altered seed provisioning. This highlights the importance of considering parental environmental history when assessing crop performance and resilience, especially under climate change scenarios where heat stress is increasingly common.

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Chapter V

General Discussion, Conclusions and Future Perspectives

Author’s contributions to the chapter:

Cláudia Lopes wrote this chapter based on the previous sections and the referred bibliography.

1. An overview

Phaseolus vulgaris is one of the most important grain legumes cultivated globally and serves as a staple food for over 500 million people. It is a good source of proteins, carbohydrates, dietary fibres, iron, zinc and essential amino acids, making it a vital source of high-quality nutrition, particularly in regions with limited access to animal protein (**Chapter I, section 1**). Beyond its dietary significance, *P. vulgaris* plays a crucial role in sustainable agriculture, as a nitrogen-fixing legume, it enhances soil fertility, reduces reliance on synthetic fertilizers, and supports agroecological balance (**Chapter I, section 1**).

As result of the contributions made by the previous research work undertaken by our team (Parreira et al 2016, 2018, 2021), as well as the research works herein enclosed (Lopes et al., 2025), *P. vulgaris* is now a suitable model to study seed development (SD) in a non-endospermic legumes. We evidenced that seed development in *P. vulgaris* is a complex and tightly regulated process with profound agronomic, nutritional, and developmental importance. Unlike endospermic seeds such as cereals, non-endospermic seeds, including those of soybean, pea and lentil, store nutrients primarily in the cotyledons. This places greater importance on the timing and coordination of developmental events such as cell expansion, reserve accumulation and desiccation. In *P. vulgaris*, seed development follows three overlapping stages: embryogenesis, seed filling (reserve accumulation) and maturation/desiccation. Embryogenesis begins shortly after fertilization, marked by rapid cell division and tissue patterning, along with the activation of genes responsible for genome stability and DNA repair. This is followed by the filling stage, where the cotyledons serve as the main site of metabolic activity, accumulating key storage reserves like proteins, starch and lipids. The final phase involves seed desiccation, metabolic quiescence and the buildup of protective molecules such as LEA proteins and dehydrins.

The transition from embryogenesis to reserve accumulation represents a key developmental stage in seed formation, marked by extensive transcriptional reprogramming, hormonal signalling changes and shifts in nutrient allocation to support rapid biomass accumulation. However, the precise timing and regulation of this transition remained unclear in *P. vulgaris*. To address this, **Chapter II** investigates whether seed filling begins before 10 DAA and identifies the molecular mechanisms underlying this transition. In the next section the main achievements and advances on the current state-of-the art will be summarized.

Environmental stresses, particularly heat stress (HS), can severely disrupt this transition, leading to impaired embryo development, altered storage reserve synthesis, and reduced seed viability. As global temperatures continue to rise, episodes of HS during this vulnerable phase are becoming more frequent, threatening both seed quality and yield stability. Little is known about how HS affects the double fertilization process and subsequent embryogenesis and seed setting in legumes. Indeed, most of the studies have mainly addressed the effects of HS on pollen germination and elongation (Liu et al., 2019). Non-endospermic seeds may be especially sensitive due to their reliance on cotyledon-driven nutrient storage and tightly constrained developmental timeline. Understanding how HS interferes with molecular and physiological processes during this transition is therefore essential. To address this gap of knowledge **Chapter III** builds on the developmental insights from **Chapter II** by examining how HS disrupts this process. **Chapter IV** investigates whether these disruptions carry over to the next generation, offering a complete picture of both immediate and inherited effects.

Insights gained from studying *P. vulgaris* seed development under HS conditions can inform targeted breeding strategies aimed at improving stress resilience, maintaining seed nutritional value and ensuring stable yields in a changing climate, particularly in regions where legumes serve as primary sources of protein and micronutrients.

This thesis provides a comprehensive exploration of seed development in *P. vulgaris*, with a focus on the molecular mechanisms governing the transition from embryogenesis to seed filling, the effects of HS on this transition and the transgenerational consequences of parental heat exposure. In **Chapter II**, we established a developmental and transcriptomic baseline by generating the first high-resolution dataset spanning 6 to 18 DAA in *P. vulgaris*. Building on this foundation, **Chapter III** demonstrated that HS disrupts the developmental trajectory by shortening the filling period and impairing the accumulation of storage compounds, particularly starch. Hormonal profiling and transcriptomic data revealed impaired key pathways, including reduced abscisic and jasmonic acid levels and early increases in auxin, cytokinin and salicylic acid. Finally, **Chapter IV** extended this work by showing that seeds produced under HS carry transgenerational effects into the next generation: although germination was accelerated, progeny seedlings displayed reduced vigor, biomass and leaf development under optimal conditions. These findings collectively demonstrate that *P. vulgaris* seed development is finely tuned, highly sensitive to HS and capable of transmitting environmental cues across generations, insights that are crucial for breeding climate-resilient legume crops.

2. Understanding the transition from embryogenesis to seed filling in *Phaseolus vulgaris* L. non-endospermic seeds

Our previous studies described the *P. vulgaris* SER16 molecular and proteomic regulation from 10 to 40 DAA (Parreira et al., 2016, 2018, 2021). However, fundamental questions remained to be answered regarding the molecular mechanism governing the earliest phases of seed development, particularly when the transition from embryogenesis to seed filling begins and what molecular programs regulate this shift. In **Chapter II**, we focused on the earlier stages of development, from 6 to 20 DAA, applying morphological, histochemical and transcriptomic tools to better understand the processes underlying the shift from embryogenesis to seed filling.

Our data demonstrate that seed development at 6 DAA is still marked by embryogenesis, with active cell division. At 10 DAA, cotyledons are fully formed, yet storage protein granules are not yet visible, indicating that seed filling initiates only after this point. Morphologically, seed fresh weight and dry weight begin to increase substantially after 10 DAA, corresponding to the onset of reserve deposition. This shift is accompanied by a transition from cell division to cell expansion, supported by transcriptomic upregulation of cell expansion and wall modification genes between 10 and 14 DAA, consistent with observations in chickpea and *P. vulgaris* (Turner et al., 2005; Weber et al., 2005).

Although storage compound accumulation begins visibly after 10 DAA, the molecular machinery is already established by 6 DAA. Notably, RmlC-like cupin superfamily and germin-like protein genes, known to play dual roles in embryogenesis and oxidative stress protection (Khuri et al., 2001; Neutelings et al., 1998), are already expressed at 6 DAA. Likewise, genes related to protein activation, synthesis, folding, and post-translational modifications, critical steps in ensuring storage protein stability and accumulation (Önder et al., 2022), are activated early, suggesting that molecular programming for seed filling is initiated well before phenotypic markers emerge.

The accumulation of starch follows a similar pattern. While histochemical staining confirms that starch begins accumulating after 10 DAA, early upregulation of GBSSI was observed, suggesting that transcriptional control precedes actual deposition. Expression of debranching enzymes such as ISA3 and pullulanase at 14 and 18 DAA supports the notion that *P. vulgaris* employs a complex enzymatic network for amylose and amylopectin biosynthesis, potentially compensating for declining GBSS levels during this period (Hanashiro et al., 2008; Takashima et al., 2007; Thakur et al., 2021).

LEC1 and LEC2, master regulators of embryogenesis and early seed maturation, are already upregulated at 6 DAA, aligning with their known roles in promoting cotyledon differentiation and establishing the molecular basis for reserve accumulation (Jo et al., 2019; Manan et al., 2017). FUS3 and ABI3 expression begins to rise at 10 DAA, coinciding with the onset of visible seed filling and the rapid increase in SFW and SDW. LEA proteins and ABI5, involved in desiccation tolerance, are expressed progressively from 14 DAA onward, indicating that seed maturation programs are engaged well before the final desiccation phase.

Our data also reveal the expression of phytohormone genes regulated by the LAFL network. At 6 DAA, genes involved in cytokinin, auxin, ABA, and GA biosynthesis are upregulated, suggesting an active hormonal network preparing the seed for reserve accumulation. This aligns with findings in legumes, where auxin and cytokinin modulate early embryonic patterning and source-sink establishment (McAdam et al., 2017; Picciarelli et al., 2001). From 14 DAA onward, genes involved in ethylene (ERF1) and brassinosteroid (BRH1) signalling are upregulated, reflecting their known functions in coordinating storage compound accumulation and late maturation (Nomura et al., 2007; Wu et al., 2008).

In summary, **Chapter II** redefines the developmental timeline of seed filling in *P. vulgaris* by demonstrating that molecular preparations for reserve accumulation begin as early as 6 DAA. This developmental baseline sets the stage for understanding how environmental stresses, such as heat, disrupt these finely tuned processes, as explored in **Chapters III** and **IV**.

3. Hormonal and transcriptional changes in developing common bean (*Phaseolus vulgaris* L.) seeds under heat stress

Building on the developmental framework established in **Chapter II**, **Chapter III** investigated how HS affects seed development in *P. vulgaris* during the sensitive transition from embryogenesis to seed filling. Using the same time points, from 6 to 20 DAA, we adopted a comprehensive strategy combining morphological, histochemical, hormone quantification and transcriptomic profiling to dissect the effects of elevated temperatures.

A critical point of comparison lies in the control (CP) datasets used in **Chapters II** and **III**, which were both conducted under identical temperature conditions (25/18°C day/night). This consistency not only reinforces the methodological rigor of the study but also allows for a direct comparison of developmental benchmarks between stressed and non-stressed seeds. Indeed, control plants in **Chapter III** displayed phenotypic, transcriptomic and hormonal profiles remarkably similar to those described in **Chapter II**. Seed fresh and dry weight accumulation continued steadily until 30 DAA, and histological observations confirmed sustained cotyledon growth and storage compound accumulation, consistent with the timeline established in **Chapter II**. Furthermore, hormonal trends in CP seeds were also comparable across chapters, for instance, the early expression of auxin and cytokinin biosynthesis genes between 6 and 10 DAA were observed in both datasets.

Seeds under HS reached maximum fresh and dry weight by 18 DAA, compared to 30 DAA in CP, indicating an early onset of maturation. Cotyledon and cell areas followed a similar pattern, expanding until 18 DAA under HS before declining, while growth continued under CP until at least 20 DAA. Moreover, HS significantly reduced seed number, consistent with known impacts of elevated temperature on male fertility and reproductive success (Lohani et al., 2020; Sita et al., 2018).

Storage compound accumulation was also affected. Histochemical analyses and transcriptomic data indicated impaired starch accumulation in HS seeds by 20 DAA, with lower expression of key starch biosynthetic genes such as AGPase, GBSS, and ISA compared to controls. This mirrors findings in cereals like rice and maize, where HS disrupts carbohydrate metabolism during grain filling (Ahmed et al., 2015). In contrast, seed storage protein levels at 20 DAA were similar between treatments, suggesting that HS shortened cell division rather than reducing protein accumulation. However, transcriptional data revealed that storage protein genes were more highly expressed in control conditions at 18 DAA, supporting the notion that HS leads to early but impaired filling, an outcome also reported in soybean, lentil, rice and maize (Lin et al., 2010; Monjardino et al., 2005; Tacarindua et al., 2012).

Under HS, ABA and JA concentrations were markedly reduced at 14, 18 and 20 DAA, a trend supported by the upregulation of negative regulators such as PP2C and JAZ. These changes likely compromise maturation-related processes in HS, as both ABA and JA are essential for storage reserve accumulation and desiccation tolerance (Nonogaki, 2019). Conversely, early developmental stages (6 and 10 DAA) under HS exhibited elevated levels of auxin (IAA), cytokinins (tZ, DHZ), and salicylic acid (SA), along with the upregulation of their biosynthetic and transporter genes, suggesting enhanced cell division and stress signalling during early seed formation (Jameson, 2023; Miyawaki et al., 2004), aligned with the shortening of seed filling period and the acceleration of the onset of the desiccation phase in HS. Ethylene-related genes were also more expressed at later stages under HS, consistent with ethylene’s role in stress-induced senescence and adaptation (Hays et al., 2007).

Together, these findings illustrate that HS significantly disrupts the transcriptional and hormonal coordination required for normal seed development in *P. vulgaris*. While the early molecular framework for filling is present by 6 DAA, elevated temperatures compress the developmental timeline, trigger premature desiccation, and alter reserve deposition patterns. Hormonal crosstalk is reprogrammed under HS, with early peaks in growth-promoting signals followed by reduced maturation-associated hormones at later stages. These effects collectively point to a loss of temporal regulation and highlight the vulnerability of early seed development to heat stress.

Chapter III not only confirms the sensitivity of *P. vulgaris* to HS but also identifies key hormonal and transcriptional markers that may be targeted for breeding thermotolerant cultivars.

4. Influence of parental heat stress on germination and seedling development in common bean (*Phaseolus vulgaris* L.)

The findings of **Chapter IV** build directly on the developmental timeline established in **Chapter II** and the HS effects described in **Chapter III**, offering a comprehensive view of how HS experienced during seed development can have consequences on the next generation. While **Chapters II** and **III** focused on the transition from embryogenesis to seed filling and its sensitivity to elevated temperatures, **Chapter IV** extends this analysis to a transgenerational scale by examining the germination under supra-optimal and optimal stress conditions and seedling performance of seeds developed under heat stress (SHSP) when transferred and allowed to grown under optimal conditions.

Despite no difference in final germination percentage (FGP), SHSP consistently germinated faster than SCP (seeds from control plants), showing significantly lower mean germination time (MGT) and time to 50% germination (T50). One of the most interesting finding was that we observed that seeds developed under HS were capable of germinating twice faster than the one developed under defined optimal temperature conditions. This shift toward earlier germination mirrors previous observations in citrus, tomato and rice, where either higher environmental temperatures or developmental HS led to accelerated germination dynamics (Begcy et al., 2018; Mobayen, 1980). Such changes are thought to reflect an adaptive transgenerational response, where progeny attempt to escape environmental cues similar to those experienced by their parents, such as HS during early development (Sousaraei et al., 2022). In our study, this acceleration was even more pronounced at 32°C, suggesting that SHSP have altered thermal sensitivity. Again, it remains to be understood, if this pronounced acceleration in HS generated seeds relates to a decreased dormancy or if HS is triggering any “priming-like” mechanism responsible for the faster germination observed.

This faster germination may be driven by changes in ABA-GA hormonal balance, where reduced ABA levels or enhanced GA biosynthesis lower dormancy thresholds (Bicalho et al., 2015; Vishal & Kumar, 2018). Such hormonal shifts were documented under HS by us in **Chapter III** and are further supported by earlier studies linking heat exposure during seed development with hormonal reprogramming and increased germination rates (Yang et al., 2015). In addition, molecular mechanisms such as epigenetic memory, including altered DNA methylation, histone modification and small RNA expression, have been implicated in regulating seed dormancy and germination following parental stress (Bilichak et al., 2015; Boyko & Kovalchuk, 2010; Byeon et al., 2019; Malabarba et al., 2021).

However, this developmental acceleration comes at a cost, well observed in morphological traits observed in seedlings. Despite faster germination, SHSP seedlings exhibited significantly reduced vigor: shorter shoots and roots, smaller leaf area and lower fresh and dry biomass compared to SCP, across both optimal (25°C) and elevated (32°C) germination temperatures. **Chapter IV** builds upon and reinforces the findings of **Chapter III** by demonstrating that the disruptions caused by heat stress during seed development — specifically the shortened seed filling period, the reduced accumulation of storage reserves, and the altered hormonal balance (notably lower ABA and JA levels) — have functional consequences for the next generation. In particular, reduced ABA and JA during seed development are likely to have impaired desiccation tolerance and reserve mobilization capacity in the progeny, explaining the observed reductions in seedling vigor. Thus, the physiological deficits accumulated during seed maturation under heat stress directly translate into weaker early growth performance in SHSP seedlings.

These results are consistent with reports in wheat, maize and soybean, where parental heat or drought stress negatively impacted progeny growth and architecture (Chukwudi et al., 2021; Grass & Burris, 2011; Wijewardana et al., 2019). In our case, SHSP not only had lower biomass and root/shoot length but also fewer primary leaves, further reducing their growth potential and resource acquisition capacity. This is particularly critical in *P. vulgaris*, where cotyledon reserves serve as the primary nutrient source during early seedling establishment due to the absence of an endosperm. As demonstrated in **Chapter III**, seeds produced under heat stress conditions were significantly smaller and accumulated lower levels of storage compounds compared to control seeds. This reduction in cotyledon biomass and reserves likely impaired the capacity of SHSP seedlings to sustain vigorous early growth, thereby contributing to the diminished seedling vigor observed.

Chapter IV also demonstrated that the F2 seeds produced by plants derived from SHSP were consistently smaller and lighter than those produced by SCP plants. This observation aligns with our findings in **Chapter III**, where parental heat stress during seed development disrupted starch accumulation and limited seed filling, resulting in smaller and lighter F1 seeds. Thus, both the F1 seeds (parental generation) and the F2 seeds (progeny generation) exhibited reduced size and weight, highlighting the lasting and transgenerational impact of heat stress on seed development.

These results suggest that the physiological consequences of HS during seed development are not confined to the developmental window itself but persist into the next generation as altered seed resource allocation. Similar reductions in seed size and quality have been documented in rice, *Brassica napus* and other crops when parental plants experienced elevated temperatures during reproduction or early seed development (Reed et al., 2022; Young et al., 2004). The mechanisms likely include both hormonal imbalances and epigenetic changes that influence seed composition and developmental potential (Hatzig et al., 2018). Due to time and budget constraints concomitants with the last months of my PhD fellowship, we were not able to pursue molecular studies to uncover possible mechanisms which can governing the observed phenotypes. Still, based on our results and comprehensive bibliographic research we speculate about possible mechanisms acting on this. In the future, it would be very interesting to explore this aspect, particularly how HS modulated the expression of genes involved in DNA sensing and repair and antioxidant responses, hallmarks of germination and seed vigour (Pagano et al., 2025).

Chapter IV extends and bring another additional layer of information about the about the effects of HS in SD, which were conducted in earlier **Chapters II** and **III**. **Chapter IV** demonstrated that the effects of such disruptions extend into the next generation through changes in germination, seedling morphology and vegetative growth. It also underscores the importance of integrating transgenerational effects into breeding and agronomic strategies to enhance crop resilience in a warming world.

5. Concluding remarks

This thesis offers a comprehensive and novel contribution to the field of legume seed biology by unravelling the tightly regulated and environmentally sensitive process of seed development in *P. vulgaris*. It delivers the first high-resolution developmental timeline of the transition from embryogenesis to seed filling in this species, using an integrative approach that combines morphology, histology, transcriptomics, hormone profiling and physiological measurements. These findings provide fundamental insights into how molecular, hormonal and cellular events unfold in a non-endospermic seed system - knowledge that was previously lacking. By coupling this baseline with HS experiments and transgenerational assessments, this work advances our understanding of how early developmental events are affected by elevated temperatures and how those effects are transmitted to the next generation, which will be relevant for future molecular breeding approaches to enhance legume´s resilience towards Climate Change while securing food security.

Crucially, this thesis reveals that the molecular program for seed filling is already underway during late embryogenesis, redefines the timing of reserve accumulation and identifies key hormonal pathways that orchestrate developmental progression. It also demonstrates that HS leads to premature desiccation, impairs storage compound accumulation and alters hormone profiles in a stage-specific manner. In a broader context, it highlights that these stress-induced changes persist into the next generation, leading to reduced seedling vigor, altered morphology and evidence of stress memory. Together, these discoveries provide a new developmental and molecular framework for future studies in seed biology and open up concrete avenues for improving heat resilience in legumes. One of the main limitations of this work is the lack of functional validation for “candidate genes” identified in our studies, which would corroborate key roles suggested by us. Despite the availability of genomic and genetic resources, research in *P. vulgaris* still faces a critical drawback: a reduced number of tools for functional gene validation. Despite the efforts made, an efficient transformation system to obtain stable transgenic common bean plants is still lacking, likely due to the *P. vulgaris* recalcitrance towards in vitro regeneration (Moura et al., 2024).

Nevertheless, several promising future directions emerge. One major opportunity lies in applying CRISPR/Cas9 gene editing to dissect the roles of key genes identified in this work, such as LEC1, LEC2, ABI5, PP2C, GH3, AUX1, IPT, JAZ and COI1, to determine their specific contributions to HS responses during seed development. Targeted deletion of these genes in mutant lines could reveal whether manipulating auxin, cytokinin, ABA or JA signalling pathways can enhance seed resilience under elevated temperatures. Recent work has confirmed the feasibility of CRISPR/Cas9-based genome editing in *P. vulgaris*. For example, de Koning et al., (2023) developed a hairy root transformation system that achieved up to 70% editing efficiency using sgRNAs targeting raffinose family oligosaccharide biosynthesis genes. This platform enabled rapid evaluation of guide RNAs and promoters and provides a practical entry point for functional genomics in *P. vulgaris*.

Another critical step is to extend transcriptomic and hormonal profiling to the entire seed development, from early embryogenesis through to full maturation and desiccation. While this thesis focused on the early to mid-developmental from 6 to 20 DAA, later stages remain underexplored. Characterizing gene expression and hormone dynamics during late filling and desiccation will clarify how these final phases are regulated and how they interact with early developmental cues. Quantifying phytohormones throughout all stages, especially ABA, ethylene, and GA, which are central to seed dormancy and desiccation, can reveal how hormonal shifts prepare the seed for maturation and storage and how they may be affected by HS.

Future studies should also include spatial resolution of molecular processes within seeds. Performing tissue-specific RNA-seq in distinct seed compartments, such as cotyledons, seed coat and pod tissues, could help uncover the maternal contributions to seed development, especially under HS. This approach would clarify the transport dynamics and regulatory signals that originate from maternal tissues and modulate seed filling and stress responses.

Complementing this, immunolocalization of phytohormones within the seed tissues would reveal the cellular and subcellular localization of key hormones such as ABA, auxin and cytokinin during development and stress conditions, helping to identify hormone signalling hotspots and their roles in coordinating development.

To understand how transcriptional changes translate into metabolic and functional responses, proteomic analyses should be incorporated to quantify actual protein abundance and post-translational modifications (PTMs) under control and HS conditions. Given the importance of protein folding and stabilization during seed filling, detecting PTMs such as phosphorylation or glycosylation could provide mechanistic insights into heat tolerance or susceptibility.

In addition, stable isotope tracing using ^{13}C and ^{15}N-labelled compounds would allow tracking of maternal nutrient (carbon and nitrogen) flow into developing seeds under HS, shedding light on source-sink regulation and the impact of temperature on nutrient partitioning.

Expanding the genetic scope of this work is also essential. Including additional *P. vulgaris* genotypes with known differences in heat sensitivity would allow comparative studies to identify genotype-specific molecular and physiological traits. This would help determine what changes at the molecular level in tolerant *versus* susceptible varieties, providing concrete targets for marker-assisted selection and introgression into breeding programs.

To further investigate transgenerational effects, performing transcriptomic analysis on the progeny seeds (seeds studied in **Chapter IV**) would offer deeper insights into how heat stress-induced changes are inherited. Identifying the specific genes that are differentially expressed in the offspring of heat-stressed plants would enable breeders to select for epigenetic or transcriptional markers associated with resilience. This could also help uncover which metabolic or regulatory pathways remain perturbed in the next generation, even under non-stress conditions.

This thesis provides a robust conceptual and experimental framework for studying HS and seed development in legumes. It not only generates new knowledge and tools for functional genomics but also establishes a framework for breeding strategies that account for both direct and transgenerational effects of stress, an essential step toward securing food systems in a warming world.

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ITQB NOVA, Avenida da República, 2780-157 Oeiras, Portugal | Tel +351 214 469 100 | itqb.unl.pt