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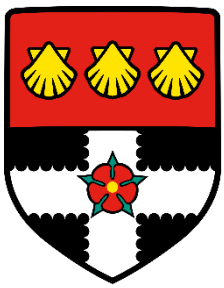
School of Agriculture, Policy and Development

Post-anthesis heat stress in wheat: Is the reduction in grain size a consequence of premature maturation of the outer layers of the grain?

By Richard Ian Kino

Submitted in accordance with the requirements for the degree of Doctor of Philosophy



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"Let me tell you something you already know. The world ain't all sunshine and rainbows. It's a very mean and nasty place and I don't care how tough you are, it will beat you to your knees and keep you there permanently if you let it. You, me or nobody is gonna hit as hard as life. But it ain't about how hard you hit, it's about how hard you can get hit and keep moving forward, how much you can take and keep moving forward… that's how winning is done! Now if you know what you're worth, now go out and get what you're worth but you've got to be willing to take the hits and not pointing fingers saying you ain't where you wanna be because of him or her or anybody. Cowards do that and that ain't you! You're better than that!"

Rocky Balboa

Abstract

High post-anthesis (p.a.) temperatures reduce mature grain weight and consequently yield in wheat. However, the mechanisms behind this reduction are still not entirely known. It has recently been suggested that a premature maturation of the pericarp under high p.a. temperatures could result in a loss of cell wall plasticity in the layer which may restrict endosperm expansion and reduce grain weight.

In order to test this hypothesis, 4 controlled environment experiments were performed using the wheat cultivar *Cadenza.* The effect of high p.a. temperatures on various aspects of wheat grain development were investigated using methods including grain dimension and weight analysis, immunofluorescence microscopy of endosperm cells in addition to the distribution and form of cell wall homogalacturonan (HG) and RNA-Seq analysis of the transcriptome of high p.a. temperature treated grain.

Mature grain weight, length and width were reduced by a high p.a. temperature treatment (35/15°C) applied for 4-days or more from 6-days after anthesis (daa). In addition, endosperm cell number and size were significantly reduced by a similar high p.a. temperature treatment duration applied from 6daa. Normal cell wall HG methyl-esterification status and distribution in the grain during development appeared to be accelerated by high p.a. temperatures suggesting a potential loss of cell wall plasticity in the pericarp and endosperm. RNA-Seq of high p.a. temperature treated grains revealed down-regulation of genes involved in cell wall expansion, including endoglucanase and β-expansin genes in the outer-pericarp at 10 and 14daa, coinciding with the early stabilisation of maximum grain moisture content, height and length in high p.a. temperature treated samples; traits closely associated with the pericarp during development. These results lend support to the overall hypothesis and help elucidate the interaction between grain layers under high p.a. temperatures, which may help molecular biologists and plant breeders develop more climate resilient cultivars.

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This work is dedicated to Lucy; I couldn't have done it without you....

Declaration of original authorship

Declaration: I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.

Richard Ian Kino

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Abbreviations

ABA- Abscisic acid

ARA - Arabinose

AX – Arabinoxylan

cDNA – Complimentary DNA

CesAs – Cellulose synthases

CPM – Counts per million

Daa – Days after anthesis

DEGs – Differentially expressed genes

DGE – Differential gene expression

EST – Expressed sequence tag

EXPs – Expansins

FDR – False discovery rate

FTIR – Fourier-transform infrared spectroscopy

GA – Glucuronic acid

GLA = galacturonic acid

GLU – Glucuronoxylan

GMM – Glucomannan

GO – Gene ontology

GT - Glycosyltransferase

HG - Homogalacturonan

HXL – Heteroxylan

LACs – Long-chain acyl-COA synthetase II enzyme

LSDs – Least significant differences

Log CPM – Log counts per million

Mab – Monoclonal antibody

MHa - Million hectares

MLG – Mixed linkage-(1-3),(1-4)-β-glucan

Mt - Million Tonnes

NSLTPs – Non-specific lipid transfer proteins

NSPs - Non-starch polysaccharides

OGAs - Oligogalacturonides

P.a – Post-anthesis

PAR – Photosynthetically active radiation

PCA – Principal component analysis

PCR – Polymerase chain reaction

PMEs – Pectin methyl-esterases

PMEIs – Pectin methyl-esterase inhibitors

PhA – Phenolic acid

qPCR – Quantitative polymerase chain reaction

qRT-PCR – Quantitative reverse transcription polymerase chain reaction

QTL – Quantitative trait loci

RG – Rhamnogalacturonan

RH – Relative Humidity

RNA-Seq – RNA sequencing

ROS – Radical oxygen species

SnRK1 – SNF1-related protein kinase1

T6P – Trehalose-6-phosphate

TGW – Thousand grain weight

USD – United States Dollar

Vpd – Vapour pressure defecit

WE-AX – Water extractable arabinoxylan

WU-AX - Water un-extractable arabinoxylan

XGA – Xylogalacturonan

XTHs – Xyloglucan endotransglucosylase/hydrolases

XYG – Xyloglucan

XYL – Xylose

Chapter 1 : Introduction

1.1 An introduction to wheat

Common wheat (*Triticum aestivum*) is one of the most important global crops in terms of human consumption and economic value. Global production is second only to maize with approximately 760.1 million tonnes (mt) produced in 2016 compared to 1026 mt of maize (FAO, 2017b). However, wheat is grown on a larger area of land globally than any other commercial food crop with 220.1 million hectares (mha) harvested in 2016 compared to 159.8 and 187.9 mha for rice and maize respectively (FAOSTAT 2017) (Fig.1.1).

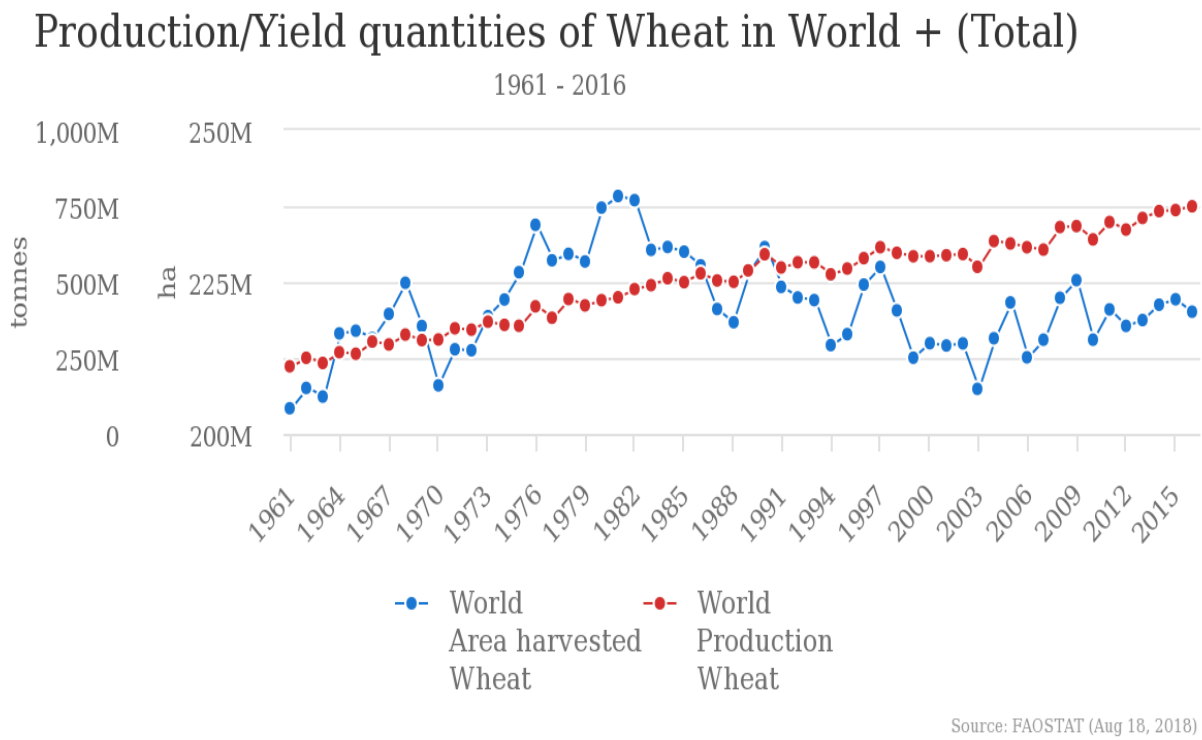


Figure 1.1 Global wheat production (mt) and area harvested (mha) 1961-2016 (FAOSTAT 2017)

Wheat production occupies 22% of cultivated land globally with the greatest levels of production occurring in the temperate regions of both the northern and southern hemispheres (Leff *et al.*, 2004). The climatic adaptability of the crop has led to wheat being grown in a variety of latitudes ranging from 40°S in Argentina to 65°N in Finland (Fig. 1.2).

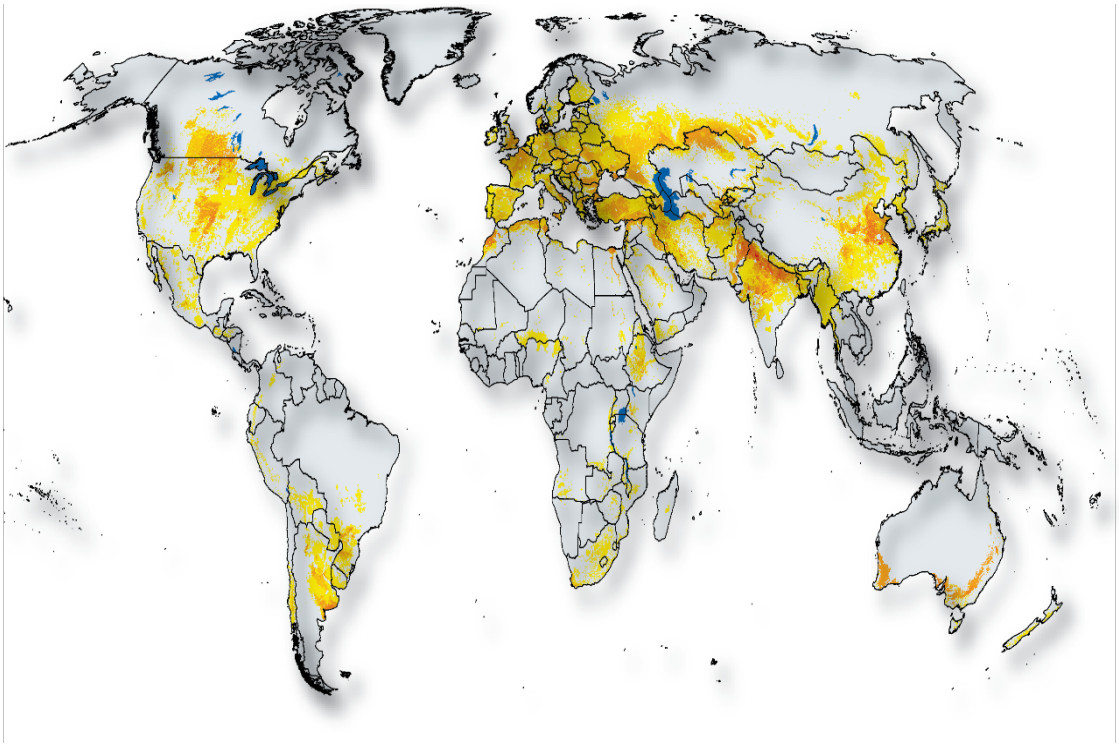


Figure 1.2 Map of wheat growing areas around the world. Darker areas indicate areas of higher production (CGIAR 2017)

Of all the major cereals since the 1960s, wheat has had the biggest increase in demand as a food source (FAO, 2003). This has been partly attributed to an increased desire for a 'western' lifestyle and its associated dietary habits in developing countries (Shewry and Hey 2015). Globally, 171 mt of wheat is forecast to be traded in 2017/2018 (FAO, 2017a) and the market value of annual wheat trading is approximately 50 billion USD (CGIAR, 2017). With 66.7 kg/year of wheat being consumed globally per capita (FAO, 2017a), it provides around 20% of the required protein and calorific intake for the global population (Shiferaw *et al.*,

2013). Therefore, wheat is critically important in supporting and feeding the global population.

1.1.1 Global population increases

The global population is predicted to increase to approximately 9.8 billion by 2050, a 0.4 billion increase on previous estimates (UN, 2017). In order to support this potential population, it is predicted that global food production will have to increase by 50 – 100% by 2050 (Southgate 2009; Royal Society 2009; Parry and Hawkesford 2010). Current global wheat yields are increasing at an annual rate (0.9%) below that needed (~2.4%) to meet the required increase in global food production (Ray *et al.*, 2013). Therefore, wheat yields will have to be improved either through increased land allocation for growth or through increased productivity *via* improved agronomics, and plant breeding techniques. However, any yield increases will have to be achieved whilst overcoming the challenge of increased risk of abiotic stresses, including extreme temperatures that can have a severe detrimental effect on wheat yields.

1.1.2 Global temperatures and wheat yields

Global land and surface temperatures have increased on average by 0.85°C between 1880 and 2012 at a rate of approximately 0.065°C per decade. This has in part been due to increased anthropogenic CO^2 emissions (Canadell *et al.*, 2007). The rate of climate change is increasing with a further rise in temperature of 0.3 - 0.7°C predicted by the year 2035 (IPCC, 2014). It has been suggested that an increase in global average temperatures may improve wheat yields at certain mid-latitude locations by improving growing season conditions and inducing earlier flowering (Gornall *et al.*, 2010). However, an increase in average global temperatures is likely to result in more severe and frequent extreme temperature events such as heatwaves and exceptionally hot days, which can have

dramatic negative effects on wheat yields (IPCC, 2014) negating any potential improvement. A climate change modelling study by Asseng *et al.* (2015) predicted that every additional 1°C rise in global mean temperature will result in a 6% reduction in global wheat yields. In addition, a study by Gourdji *et al.* (2013) predicted that by 2030, 11% of global wheat growing areas will experience extremely high temperatures on more than 5 days during the crop's critical reproductive phase resulting in heat stress. Heat stress in a plant can be considered as irreversible damage to its growth and development, caused by exposure to temperatures that are above the optimum homeostatic level for a sufficient period of time (Wahid *et al.*, 2007). For wheat, optimum growth temperatures vary depending on the variety, genotype and growth stage. However, temperatures between 15°C and 23°C are generally optimum for most stages of the plant's development (Vignjevic *et al.*, 2015) whilst temperatures above 47.5°C and below -17°C are typically lethal (Porter and Gawith 1999).

1.2 Post-anthesis heat stress and wheat yields

1.2.1 Effect of high post anthesis temperatures on grain filling

One stage of wheat development that has been shown to be significantly negatively affected by extreme high temperatures is post-anthesis (p.a.), particularly during the early stages of grain filling (Gooding *et al.*, 2003). During this period, which is critical in determining the final yield of the crop, photoassimilates, nutrients and other compounds are relocated from the leaves, stem and roots of the plant to the rapidly expanding grain post-fertilisation. The optimum temperature for wheat growth during the grain filling period ranges from 12°C to 21°C depending on the variety and genotype (Porter and Gawith 1999; Farooq *et al.*, 2011) whilst the minimum and maximum temperatures at which grain filling can still occur at are around 9.2°C and 35.4°C respectively (Porter and Gawith 1999).

Temperatures above this range during grain filling can result in a number of detrimental effects on wheat yield caused by a variety of physiological responses. Exposure to high p.a. temperatures have been shown to significantly decrease the duration of grain filling, (Hunt *et al.*, 1991; Calderini *et al.*, 1999b; Guedira *et al.*, 2002; Zahedi and Jenner 2003; Hurkman and Wood 2011; Altenbach 2012; Rakszegi *et al.*, 2014) resulting in smaller final grain weights which, along with grain number, is the main factor determining yield obtained from the wheat crop.

In many studies, it has been shown that the rate of grain filling and grain development increases as a result of exposure to high p.a. temperatures (Gooding *et al.,* 2003; Wan *et al.*, 2008; Dias and Lidon 2009; Talukder *et al.*, 2014; Song *et al.*, 2015). Hurkman and Wood (2011) found that a high temperature applied at anthesis in a hard red spring wheat *(cv. Butte 86)*, increased the speed of maturation in wheat with grains reaching physiological maturity by 21 days after anthesis (daa) under a 37°C (day)/28°C(night) temperature regime compared to 42daa under 24°C/17°C conditions. However, the increase in the rate of grain filling under high p.a. temperatures is not sufficient to compensate for the reduced duration of the grain filling period brought about by earlier maturation of the grain, and therefore final grain weight and yield are still reduced (Zahedi and Jenner 2003).

1.2.2 Effect of high p.a. temperatures on grain number

Grain number in wheat is determined during fertilisation and anthesis, however, kernel abortion can occur as a result of high p.a. temperature during early grain development (Plaut *et al.*, 2004; Hays *et al.*, 2007). High p.a. temperatures have been shown to reduce grain number even with a single day's exposure in four Australian bread wheat cultivars with varying levels of thermotolerance (Talukder *et al.* 2014). However, other studies have observed that high p.a. temperature has no effect on mature grain number (Stone and

Nicolas 1994; Wardlaw 1994). This variation has been attributed to genotypic differences between cultivars in their tolerance to high p.a. temperatures (Porter and Gawith 1999; Spiertz *et al.*, 2006; Farooq *et al.* 2011) and these differences have been shown to persist even at extremely high temperatures (40°C) (Stone and Nicolas 1998). Therefore, being able to determine the influence of genotypic differences on any observed response is important when trying to establish the effect of high p.a. temperatures on yields from different varieties. In order to further understand the physiological mechanisms through which high p.a. temperatures reduce wheat yield it is important to understand the biology of the wheat plant, and the structure of the grain.

1.3 The wheat plant

1.3.1 Origin

Wheat, as well as all other cereal crops, belongs to the grass family *(Poaceae)* which contains around 10,000 species and is one of the largest families of flowering plants. Wheat was first cultivated approximately 10,000 years ago in a region of central Asia known historically as the 'fertile crescent'. Modern bread wheat *(T.aestivum)* is an allohexaploid variety (genome: AABBDD, 21 pairs of chromosomes) that developed from multiple historical hybridization events with the most recent event occurring between the cultivated 'emmer' wheat (genome: AABB) and a wild diploid variety *(Aegilops tauschii)* (genome: DD) approximately 8,000 years ago (Marcussen *et al.*, 2014). *T.aestivum* cultivation spread to temperate regions across the globe and its genomic complexity has enabled the development of approximately 25,000 different varieties that are adapted to various climates (Ewert 2005).

1.3.2 Biology and development

T.aestivum is a predominantly self-pollinating variety that utilises the C3 photosynthetic pathway and takes approximately 4-8 months to grow from seed to maturity depending on the variety and the time of planting. The species can be divided into both winter and spring varieties with the name referring to the season when the seeds are sown. Spring varieties flower as soon as physiologically possible resulting in a much shorter time to maturity (~4 months) whilst winter varieties require a period of cold vernalisation before they flower giving an overall growth period of around 8 months. The wheat plant grows from a single seeded caryopsis (grain) that requires imbibition in order to germinate. Imbibition triggers a hormonal response in the embryo of the grain that induces the synthesis of hydrolytic enzymes that break down the proteins, starch and cell wall polysaccharides stored in the endosperm to provide energy for the new seedling. Immediately after imbibition, roots grow before the coleoptile emerges, reaches the surface and establishes a seedling (AHDB 2018; cerealsdb.uk.net). Following seedling establishment, a number of leaf primordia are initiated, the number depending on the variety and environmental conditions. During the subsequent growing period (5-7 weeks) a number of side shoots, known as tillers also develop; these are able to grow to similar heights as the main tiller and produce fertile ears. Stem elongation in the main shoot of the plant occurs following the production of the terminal spikelet within the stem apex at Zadok stage 30 (Zadoks *et al.*, 1974) and coincides with growth of leaves, tillers and roots (Patrick 1972). The stem extends via expansion of the stem internodes and during this process the developing ear is pushed up through the growing stem eventually emerging through the last formed leaf (flag).

At the point of emergence, the wheat ear is fully developed with male anthers and female stigmas. Flowering occurs a few days after the ear emerges from the flag leaf. During this time pollen is shed via pores in the anthers and collected by the feathery stigmas of the unfolded carpels within fertile florets. Inside the carpels and within the embryo sac, a double fertilisation event takes place whereby one pollen nucleus fuses with the egg cell which divides to give the diploid embryo. Following this initial fertilisation, the outer maternal layers (carpels) begin to swell and surround the newly formed zygote. At the same time a second pollen nucleus fuses with two female polar nuclei within the embryo sac to produce a triploid endosperm nucleus. The wheat grain then begins to develop in stages that have been categorised as, the cellularisation stage (0-6daa), the expansion stage (6-14daa), the grain filling stage (14-28daa) followed by the maturation and desiccation stage (28-40daa) after which the grains are ready for harvest (Shewry *et al.* 2012) (Fig. 1.3).

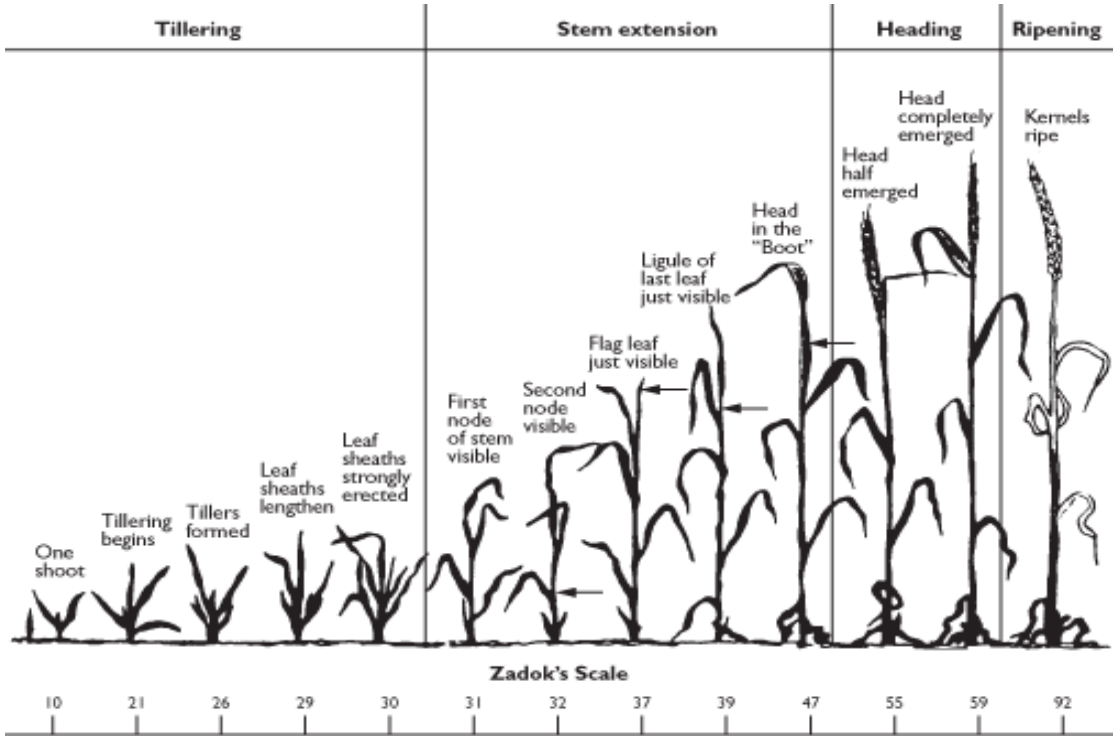


Figure 1.3. Developmental stages of *T.aestivum* with Zadok's Scale reference (Zadoks *et al.*, 1974). Image created by Mike Cowbrough (2016)

1.4 The wheat grain

1.4.1 Developmental stages

1.4.1.1 Cellularisation

Following the double fertilisation event, the triploid endosperm nucleus remains in a state of free nuclear division for 3-4 days producing a layer of coenocytic endosperm. Cellularisation is the formation of distinct, individual cells via separation from a multinucleate cell and this process proceeds in the early wheat grain from around 3-6daa until the central cell cavity is filled with cells (Sabelli and Larkins 2009). This process is initiated in the coenocytic endosperm by the development of anticlinal cell walls and an open-ended alveolation process that separates the nuclei of the coenocytic endosperm and proceeds from the outer periphery of the coenocytic endosperm towards the central vacuole (Sabelli and Larkins 2009; Shewry *et al.*, 2012). Following this process, from 5-10 daa, the internal layers of the endosperm are determined with the transition, in terms of gene expression, from cell proliferation to differentiation beginning in the crease region of the grain before spreading outward (Drea *et al.*, 2005). After the cellularisation phase and the partitioning of the coenocytic endosperm into individual cells, cell division then continues inwards within the endosperm via rapid periclinal mitotic divisions until around 14daa (Bechtel *et al.*, 2009) which overlaps with the next stage of grain development, grain filling.

1.4.1.2 Grain filling

During the grain filling period, the dry weight of the grain increases in a nearly linear manner as photosynthates and other assimilates are formed, translocated, portioned and accumulated in the endosperm cells with the largest rate of filling occurring between 14-28

daa (Wang *et al.*, 1997; Shewry *et al.*, 2012). In addition, the early stages of the grain filling phase are accompanied by a period of rapid net water deposition within the grain (Schnyder and Baum 1992). Water acts as an important medium for transporting photoassimilates and other nutrients to the developing grain as well as providing a suitable environment for metabolic processes (Xie *et al.*, 2015). Photosynthates and assimilates are transported to the developing grain from three primary sources: photosynthesis in the flag-leaf (Evans 1983) pre-anthesis reserves in tissues such as the stem (Gebbing and Schnyder 1999) and ear photosynthesis (Tambussi *et al.*, 2007; Sanchez-Bragado *et al.*, 2016).

The amount of grain filling that takes place is in part determined by a combination of 'sink' and 'source' capacity. The term 'sink' refers to the final location of transported photosynthates and other assimilates; in the case of wheat, the storage tissue of the wheat grain endosperm. 'Source' capacity refers to the ability of tissues to fixate carbon dioxide and photosynthesise, typically in the vegetative parts of the plant (Fig. 1.4). The relative contribution of the different source tissues in providing photosynthates to the developing grain has been studied in some detail, albeit with little consensus due to differences in methodology. A recent study in 6 advanced bread wheat lines, used different methodologies to determine the relative contribution of the vegetative tissues to grain filling and suggested that ear photosynthesis may be a larger contributor than the flag leaf (Sanchez-Bragado *et al.*, 2016) which had previously been identified as contributing the largest proportion of photosynthates to the developing grain (Stoy 1963).

Despite the various contributions of assimilates and photosynthates from other parts of the wheat plant, a number of studies have suggested that the potential size of the developing grain is sink limited and determined by the amount of starch synthase activity that takes place within the wheat grain rather than the amount of photosynthesis within the plant (Labuschagne *et al.*, 2009; Borrill *et al.*, 2015). The sink capacity of the wheat grain is also in-

part determined by the number of cells within the endosperm (Schnyder and Weiß 1993). High p.a. temperature during the rapid cell division phase can significantly reduce the number of endosperm cells, therefore, reducing sink capacity (Yang *et al.*, 2016), however the cause of this reduction in endosperm cell number is still to be determined.

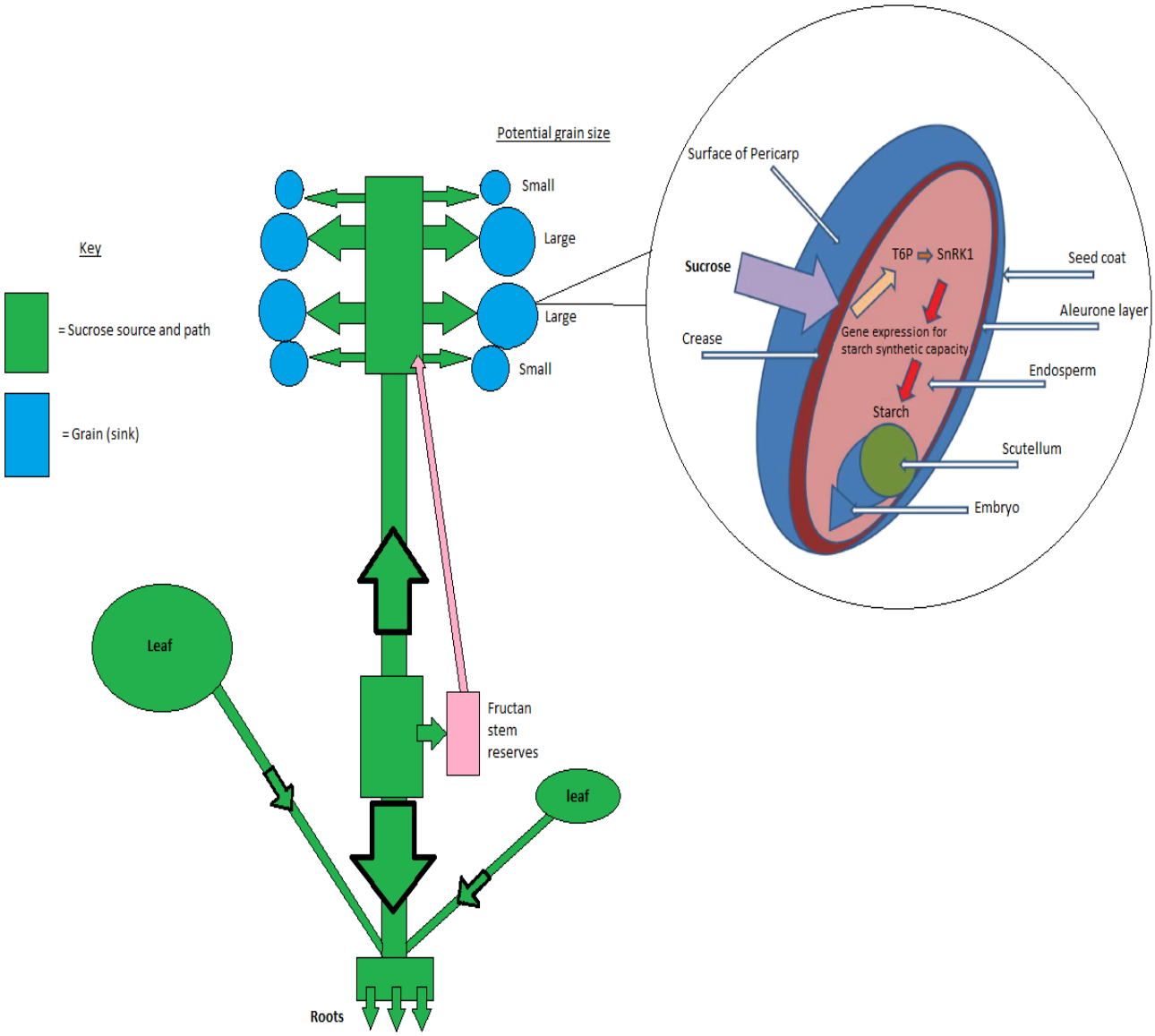


Figure 1.4 System diagram of sink/source relationship in wheat plants (Lawlor and Paul 2014). High p.a. temperature during the grain filling period 11-14daa results in increased contribution of fructan stem reserves to the developing grain (Housley 2000) as the photosynthetic capacity of source tissues is decreased through structural damage to chloroplasts. Fructan stem reserves act as a measure of the supply and demand for assimilates between sink and source tissues i.e. excess assimilate is stored in stem reserves. Trehalose-6-phosphate (T6P) is expressed in wheat grains and regulates the activity of SNF1-related protein kinase1 (SnRK1), which activates or represses gene expression for proteins of basic metabolism and acts as a signal of sucrose availability to the grain (Lawlor and Paul 2014). Image reproduced with permission of the original author.

1.4.1.3 Maturation

Beyond 28daa, the rate of protein and starch deposition in the endosperm is greatly reduced with grain filling ending completely at around 35daa (Shewry *et al.*, 2012). From 26-28daa onward, the endosperm cells begin to rapidly desiccate and mature, coinciding with the attainment of maximum dry weight of the grain, and a grain moisture content of between 37-45% (Schnyder and Baum 1992; Calderini *et al.*, 2000; Pepler *et al.*, 2006; AHDB 2018). Following this desiccation period, the grain is considered ripe and ready for harvest when below 20% moisture content (AHDB 2018) although post-harvest drying may take place in order to standardise grain water content across a harvest and optimise post-harvest grain quality.

1.4.2 Wheat grain structure

The wheat grain is a complex single seeded fruit structure that contains a number of different layers with unique structural and chemical compositions which underpin specific functions. The main tissues include the starchy endosperm which makes up the majority of the mature dry weight of the grain (~82-83%), the germ (embryo) (~ 3% of mature grain dry weight) and the outer grain layers also known as the bran which include the pericarp, seed coat, nucellar layers and aleurone (~ 13-14% of mature grain dry weight) (Barron *et al.*, 2007) (Fig.1.5).

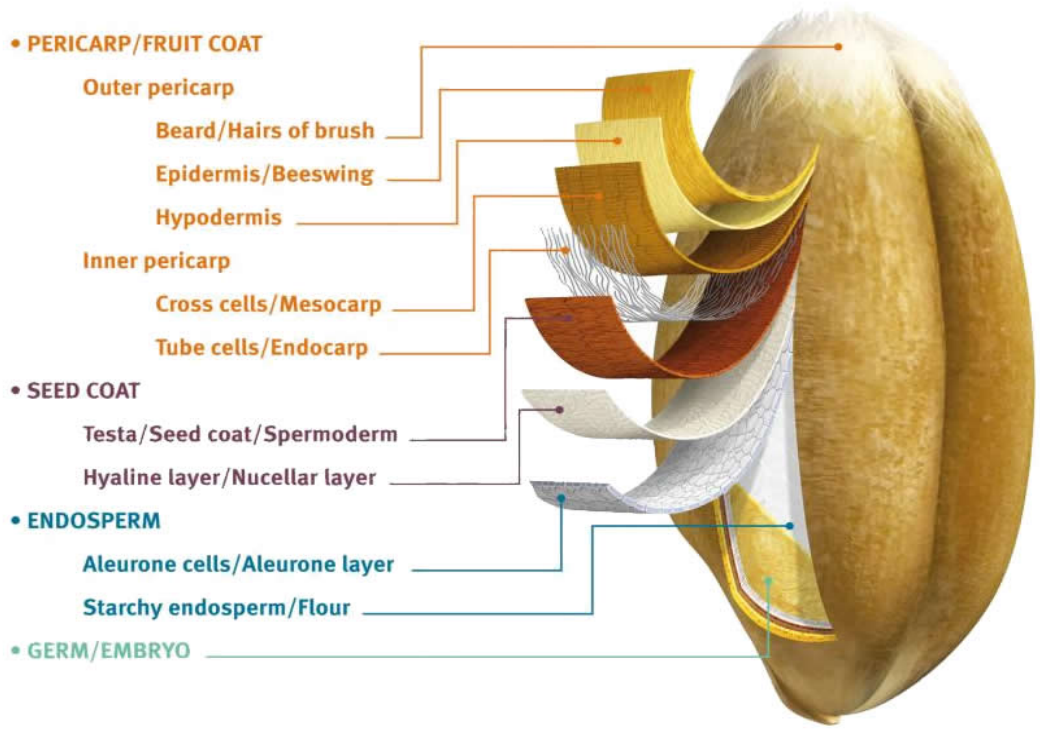


Figure 1.5. Diagram of a fully developed wheat grain with separate layers identified (nabim.co.uk)

1.4.2.1 Pericarp

The pericarp is the ripened ovary wall and consists of a number of individual layers and cell types: the outer epidermis, the hypodermis, the parenchyma, intermediate cells, cross cells and tube cells. These different layers undergo a variety of changes such as degeneration and compression over the course of grain development from fertilization until maturity (www.cerealsdb.uk.net). The pericarp performs a number of functions in the wheat grain's development including regulation of water intake, physical protection of the grain, starch storage and photosynthetic contribution from chloroplasts in the pericarp itself (Bechtel *et al.*, 2009; Xiong *et al.*, 2013b).

The cell walls of the pericarp layers primarily consist of approximately 30% cellulose, 12% lignin and 40% arabinoxylan (AX) (Stone and Morell 2009) with a high content of phenolic acids, mainly in the form of ferulic acid bound to cell wall polymers (Barron *et al.* 2007).

1.4.2.1.1 Outer Epidermis

The outer epidermis is the outermost layer of the wheat grain covering the whole grain. This cuticularised layer acts as a protective barrier to the wheat grain (Ring and Selvendran 1980; Selvendran *et al.*, 1980,) and has been shown to contain proteins that provide resistance to bacterial and fungal colonisation (Jerkovic *et al.*, 2010). The cell walls of the outer epidermis contain approximately 25-30% cellulose, 60% heteroxylan (HXL) (a form of xylan where some of the xylose residues are replaced by glucuronic acid), 0.5% phenolic acid (PhA), 12% lignin and 6% protein.

1.4.2.1.2 Hypodermis

The mature hypodermis lies just beneath the outer epidermis and forms the outer pericarp alongside the outer epidermis and parenchyma cells. Both the hypodermis and the outer epidermis have no intercellular spaces and are composed of closely adhering thick walled cells (Pomeranz 1982). The cell walls of the hypodermis contain 29% cellulose, 6% mixed linkage –(1-3),(1-4)-β-glucan (MLG) , 64% HXL, 0.45% PhA, 8.3% lignin and 9.2% protein (Selvendran *et al.*, 1980). The hypodermis, along with the outer epidermis, provides a protective layer to the endosperm and germ of the wheat grain (Gwirtz 2006).

1.4.2.1.3 Parenchyma

The thin-walled parenchyma are situated beneath the hypodermis and are important for providing structural support and protection to the developing endosperm and embryo (Bechtel *et al.*, 2009). The parenchyma also contains abundant plastids and starch granules

highlighting the role of this tissue as a storage depot for starch which provides energy for pericarp survival and provides additional nutrients to the developing endosperm (Yu *et al.*, 2015). The parenchyma degenerates during the latter stages of development and the starch granules contained within are then disintegrated into nutrients that are absorbed by the endosperm (Zheng *et al.*, 2017). The cell walls of the parenchyma have the same constituents, and in similar proportions to the cell walls of the hypodermis.

1.4.2.1.4 Intermediate cells

Intermediate cells lie below the thin walled parenchyma at the brush and embryo ends of the wheat grain in a thin discontinuous layer. Their function and origin are still under debate, however, since they display numerous projections on their surface, it has been suggested they may aid in connecting cross cells to intermediate cells (Bradbury *et al.* 1956).

1.4.2.1.5 Cross cells

Cross cells are located beneath the intermediate cell layer. The cells are orientated at 90° to the long axis of the wheat grain and are closely packed together with varying levels of thickness across the wheat grain. Cross cells have been shown to contain large amounts of starch reserves and chloroplasts up until around the 20daa stage, giving the grain its green appearance up until this point (Morrison 1976). The presence of these chloroplasts in cross cells suggests that the tissue functions in the fixation of carbon dioxide respired from the developing endosperm (Morrison 1976). Cross cells make up around 6.2% of the total bran dry weight and their cell walls contain 3% Rhamnose, 32% AX, 30% xyloglucan (XYG), 2% galactose, 20% glucose and 12% uric acid (Parker *et al.*, 2005).

1.4.2.1.6 Tube Cells

Tube cells make up the inner epidermis of the pericarp and form an incomplete layer beneath the cross cells in a narrow band on the dorsal side of the grain (Bechtel *et al.*, 2009). The cell walls of tube cells have been shown to become lignified towards maturity of the grain whilst the tube cells themselves were also shown to contain abundant starch reserves although with fewer chloroplasts than in cross cells (Morrison 1976). Tube cells have a similar composition and ratio of polysaccharides to cross cells and in addition, both cell types have proportionately less arabinose (ARA) and xylose (XYL) than the outer bran layers (outer epidermis, hypodermis and parenchyma).

1.4.2.2 Seed Coat

The seed coat (testa) and nucellar layer form a complete covering around the seed and operate cooperatively via the micropyle in the latter stages of wheat grain development to regulate water relations between the pericarp and the endosperm (Bechtel *et al.*, 2009; Rathjen *et al.*, 2009).

1.4.2.2.1 Testa

The testa is the outermost layer of the true seed derived from the inner integument of the embryo sac (Morrison 1975). The testa has a cuticle which adheres to cross cells through gaps in the tube cells of the pericarp. Beneath this cuticle the testa has two extremely thin layers that are so compressed they are referred to as one tissue known as the 'colour layer' (Bechtel *et al.*, 2009). The wheat grain testa has also been shown to secrete the cell wall pectic domain homogalacturonan (HG) in both methyl-esterified and de-methyl-esterified forms and store this polymer within vesicles beneath the cuticle of the testa (Chateigner-Boutin *et al.*, 2014). The cell walls of the testa are very similar in composition to the cell walls

of the hypodermis, parenchyma, cross cells and tube cells although with some variation in the polysaccharides embedded in the cell wall. At 11daa the testa has been shown to contain HG, MLG, cellulose, AX, glucuronoxylan (GLU), p-coumaric acid, XYG, mannan and callose (Chateigner-Boutin *et al.*, 2015).

1.4.2.2.2 Nucellar layer

The nucellar layer (also known as the hyaline layer) is a crushed layer of empty epidermal cells at grain maturity located between the testa and the endosperm that completely surrounds the endosperm and the embryo. This layer is derived from the degeneration of the nucellus, the central portion of the ovule, following the cellularisation phase as the endosperm and embryo expand, crushing the nucellus (Bechtel *et al.*, 2009). The nucellar layer also connects with the nucellar projection which aids in the transport of nutrient solution from the vascular strand to the developing endosperm. The cell walls of the nucellar layer are very similar to the testa, however, at 11daa the nucellar layer contains rhamnogalacturonan (RG) and not GLU or XYG (Chateigner-Boutin *et al.*, 2015)

1.4.2.3 The endosperm

The wheat grain endosperm is the most economically and nutritionally important part of the wheat grain (Jerkovic *et al.*, 2010). Originating from the fusion of a pollen reproductive nucleus with two polar nuclei of the central cell in the embryo sac, the triploid endosperm is the main storage tissue of the wheat grain holding protein and starch reserves (Lopes and Larkins 1993). The three main storage proteins in the wheat grain are albumins, globulins and prolamins (with approximate percentages equalling 9, 5 and 85% respectively of total grain storage protein content (Bewley *et al.*, 2013). These storage proteins provide carbon, nitrogen and sulphur to the developing wheat seedling upon germination. The

wheat grain endosperm also contains non-starch polysaccharides (NSPs), vitamins, minerals and lipids (Keshun and Rosentrater 2011).

1.4.2.3.1 Aleurone

The aleurone is the outermost layer of the endosperm and in wheat is only one cell thick. The tissue derives from differentiation of the peripheral layer of cells produced during cellularisation early in endosperm development (Becraft 2007). Aleurone cells are normally fully differentiated at around 12daa, although endosperm cells destined to become aleurone cells can be identified at around 6-8daa by the presence of a large number of small vacuoles (Bechtel *et al.*, 2009). Aleurone cells have thick cell walls that consist of approximately 65% AX, 29% MLG, 2% glucomannan (GMM) and 2% cellulose (Bacic and Stone 1981). The AX of the aleurone cell wall in wheat is highly esterified and cross-linked with ferulic and di-ferulic acid with additional esterification by p-coumaric acid and acetyl groups (Shewry *et al.*, 2012). In comparison to the cell walls of the starchy endosperm, the ferulic acid of the aleurone cell walls is significantly less dimerized, with around 12-16% dimerization compared to 50-60% in the starchy endosperm (Antoine *et al.*, 2003), although the overall amount of ferulic acid is much higher in the aleurone than in the starchy endosperm (Philippe *et al.*, 2007).

The cells of the aleurone layer are still alive in the mature grain unlike the endosperm cells (Bewley *et al.* 2013) following the reduction in grain moisture content. The ability of the aleurone to desiccate during seed drying while remaining functional has been linked to the expression of abscisic acid (ABA) during maturation which induces aleurone cells to acquire desiccation tolerance (Becraft and Yi 2011). During germination the embryo produces gibberellin which induces the secretion of amylases and proteases by the aleurone. These enzymes break down starch and proteins in the endosperm releasing amino acids and free

sugars to the growing seedling (Becraft and Yi 2011). In addition, minerals, fatty acids and amino acids also accumulate in aleurone cells making it an important layer in terms of human nutrition (Brouns *et al.*, 2012; Xiong *et al.*, 2013a).

1.4.2.3.2 Starchy Endosperm

Starch is deposited in granules embedded in a protein matrix within the endosperm and accounts for 65-75% of the total weight of the wheat grain (Laudencia-Chingcuanco *et al.*, 2006). Starch granules in the endosperm store carbohydrates produced by photosynthesis and transported to the grain. During germination, these starch granules are broken down by amylases releasing glucose molecules that supply the developing wheat grain with energy. The cell walls of the starchy endosperm contribute only around 2-3% of white flour dry weight, yet have important effects on the structural properties of flour and represent an important source of dietary fibre (Topping 2007). Mature starchy endosperm cell walls consist of approximately 70% AX, 20% MLG, 7% GMM and 4% cellulose (Mares and Stone 1973; Bacic and Stone 1980). The cellulose content in the endosperm cell wall is much lower than in the bran layers which are made up of ~30% cellulose (Stone 2012). AX in the starchy endosperm is only composed of ARA and XYL, lacks glucuronic acid (GA) sidechains, acetylation and presents much less feruloylation than the bran layers of the grain. In addition, AX of the starchy endosperm cell wall is mostly water un-extractable AX (WU-AX) (~75% of total endosperm AX) (Saulnier *et al.*, 2007b) whilst in wheat bran, cell walls contain around 90% WU-AX (Maes and Delcour 2002).

Pellny *et al.* (2012) used RNA-sequencing (RNA-Seq) in order to establish the transcriptome of isolated endosperm tissue from 5 stages during grain filling in the spring bread wheat *cv. Cadenza,* and identify some of the candidate genes responsible for the synthesis of cell walls of starchy endosperm cells. One of the most highly expressed gene transcripts they

identified between 10-28daa was a glycosyltransferase (GT) transcript belonging to the GT47 family that they suggested as being implicated in xylan extension. They also found low abundances of transcripts from genes in the acyl-CoA transferase BAHD family, a group postulated to control feruloylation of AX, which supports the observation of low amounts of feruloylation in the cell wall of the starchy endosperm. In addition, they identified transcripts involved in the synthesis of pectin within the starchy endosperm, a polysaccharide that had not previously been reported as present within the starchy endosperm cell wall. Following this study Chateigner-Boutin *et al.* (2014) confirmed the presence of pectin within the starchy endosperm cell wall of grains from the winter wheat *cv. Recital*, through a combination of enzymatic unmasking of the cell wall and subsequent immuno-labelling of HG.

1.4.3 Expansion of the grain layers

Coordinated grain growth and development is controlled and regulated by a number of gene expression pathways and molecular signalling. Martínez-Barajas *et al.* (2011) revealed in *cv. Cadenza*, that throughout development, the wheat grain exhibits dramatic changes in the expression of T6P which is a sugar signal that regulates growth, metabolism and development. Expression of T6P varied dramatically with a 178-fold decrease in expression from 1 to 45daa in the whole grain, with tissue-specific variation also occurring during the course of development. T6P inhibits the central SnRK1; a signalling protein that functions to conserve energy and carbon supplies by switching off biosynthetic processes in response to carbon and energy limitation. Therefore, these two signalling molecules work in tandem throughout wheat grain development to regulate the growth mechanisms and development patterns of the wheat grain. Expansion of wheat grain layers is facilitated by structural modifications to the cell walls of cells within these layers. Cell wall components

are generally conserved among the different layers within the wheat grain although the ratios of their different components can vary.

1.4.4 Wheat grain cell wall components

Plant cell walls typically consist of a cellulose microfibril matrix that is embedded in a matrix of complex polysaccharides that are classified as being either pectins or hemicelluloses (Fig. 1.6). Pectins are cell wall polysaccharides that are soluble in aqueous buffers and help regulate cell wall porosity whilst also creating hydrated gels that push cellulose microfibrils apart during expansion and hold these microfibrils in place during a cessation in cell wall expansion (Cosgrove 2005). Hemicelluloses are cellulose binding polysaccharides that interact with cellulose microfibrils to form a strong layer. Cellulose microfibrils consist of bundles of MLG chains that are synthesised by large complexes of cellulose synthases (CesAs) in the plasma membrane (Cho *et al.*, 2017). Pectins and hemicelluloses are synthesised in the golgi apparatus and then delivered to the cell wall via secretory vessels (Cosgrove 2000). Cell walls in the various layers of the grains of wheat and other cereals have unique compositions that differentiate them from cell walls of other plant seeds but also from the vegetative parts of the same cereal plant. Cell wall polysaccharides make up around 12% of the total mature wheat grain and provide an important source of dietary fibre in addition to having important impacts on the end-use properties of wheat (Toole *et al.*, 2010). The main cell wall polysaccharide in wheat grain is AX, a hemicellulose which constitutes around 65-70% of total cell wall material in the wheat grain (Toole *et al.*, 2010).

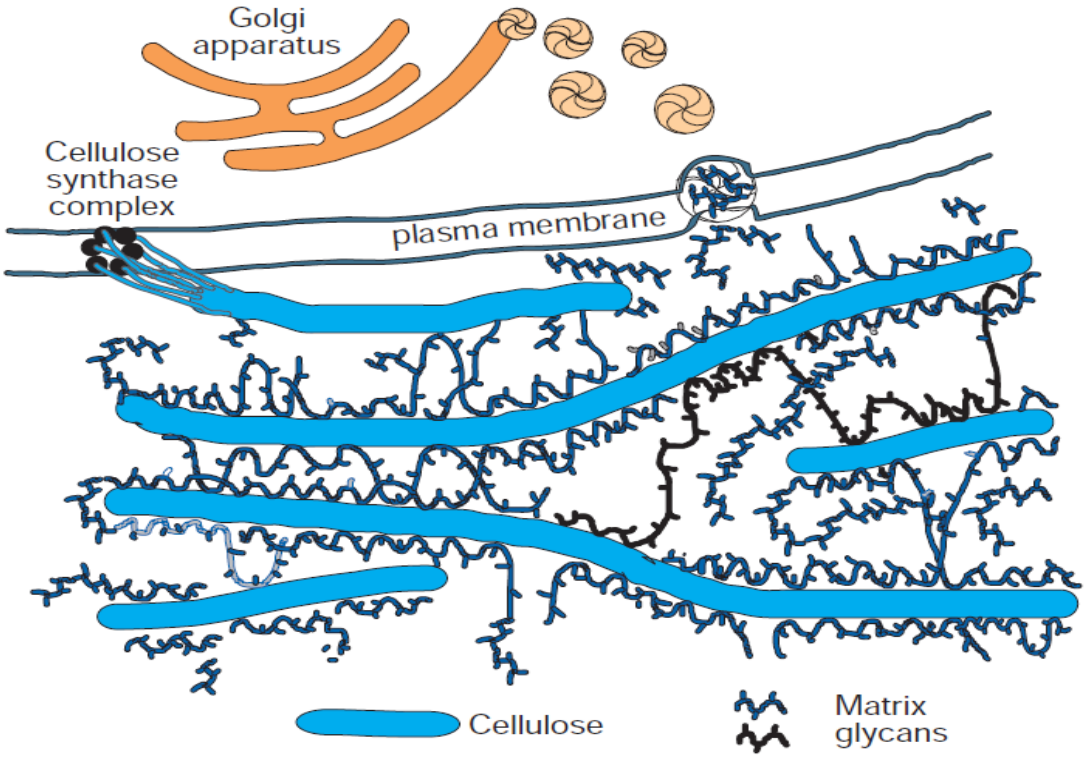


Figure 1.6 Diagram of the assembly of plant cell walls (Cosgrove 2000)

Until recent studies by Pellny *et al.* (2012) and Chateigner-Boutin *et al.* (2014), pectins were considered minor components of wheat grain cell walls, if present at all. However, their presence may indicate important roles in determining cell wall properties and signal sensing within the wheat grain.

1.4.4.1 Arabinoxylan (AX)

AXs are formed of a linear backbone of (1→4)-linked β-D-xylopyranosyl units (XYL). XYL represents approximately 50% of the constitutive sugars and there is a wide variety of side-chains present on the main chain on either of the 02, 03 positions or both. In addition, acetic, hydroxycinnamic, ferulic and *p*-coumaric acids can form esters with the AX molecule (Saulnier *et al.*, 2007a) (Fig.1.7). Depending on the tissue, its location within the grain and stage of development, cell wall AX can present different characteristics. This is partially

dependent on the degree of arabinolysation with removal of arabinose residues decreasing the solubility of the AX polymer (Toole *et al.* 2007). The structure and solubility of AX from tissues and cell layers within the wheat grain expresses both temporal and genotypic variation in both spring and winter wheat varieties. This suggests that AX content and structure could be a suitable target for selective breeding due to the influence of AX structure on dough mixing, baking as well as wheat and flour end-use quality (Li *et al.*, 2009).

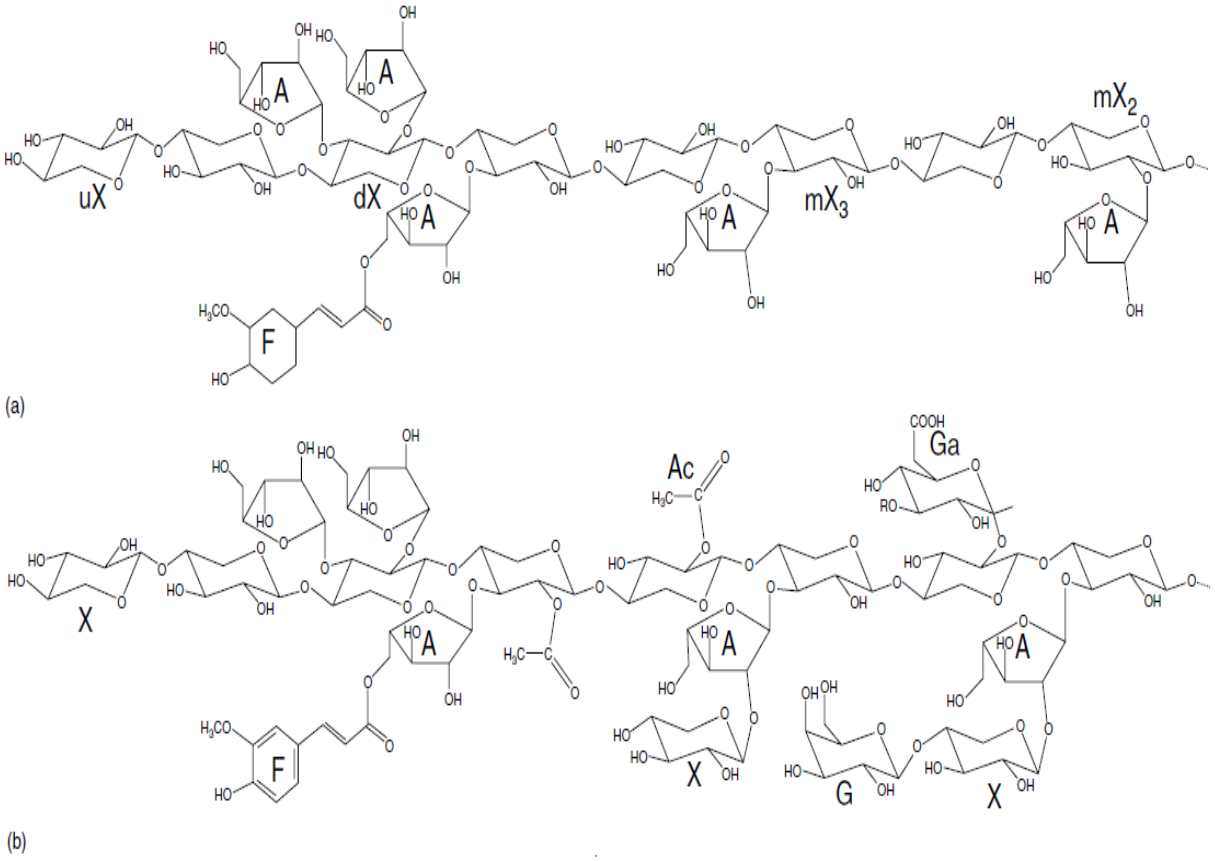


Figure 1.7 Main structural features of AX from endosperm (a) and outer tissues (b) of cereal grains. A: arabinose; X: xylose; G: galactose; GA: glucuronic acid; F: ferulic acid; uX: unsubstituted xylose; dX: disubstituted xylose; mX_3: O3 monosubstituted xylose; mX_2 O2 monosubstituted xylose. (Taken from Saulnier *et al.* (2007a))

1.4.4.1.1 Cross-linking of AX

Cross-linking of AX refers to oxidative coupling of ester groups of adjacent AX chains during cell-wall development *in vivo*. This cross-linking creates polymeric covalent bonds that bind these chains and tighten cell walls (Burr and Fry 2009). PhA residues, principally ferulic acid ether, link to the *O-5* position of some *O-3* mono-substituted ARA residues of the xylan backbone at the C5 position and are implicated in cell wall strengthening. These PhAs form dimers through oxidative reactions, enabling covalent inter-molecular cross-linking between AXs and between AX and lignin (Obel *et al.*, 2003). Feruloylation increases in the cell walls of aleurone cells wall during the grain filling period which coincides with a structural change in the aleurone cell morphology into large isodiametric, thick-walled cells. Therefore cross-linking of AX, particularly with ferulic acid, has been suggested as a major developmental process in establishing cell wall structure (Philippe *et al.*, 2006a).

1.4.4.1.2 AX and high temperatures

AX concentration and structure in wheat grain has also been shown to be modified by the environment and in particular, abiotic stresses on wheat. Rakszegi *et al.* (2014) grew three different varieties of winter wheat *(cvs. Plainsman V, MV Magma* and *Fatima 2)* in controlled experiments under a combination of heat, drought and heat and drought, applied to the plant from 9daa up to 24daa in order to further explore the relationship between growing conditions and the structure of dietary fibre in wheat whole grain. They found that combined heat and drought stress increased the total amount of AX whilst a heat stress alone (8 hours at 35°C) resulted in the highest proportion of WE-AX out of the treatments. In addition, both heat and drought stress treatments resulted in high proportions of unsubstituted AX oligosaccharides such as XYL and xylobiose. Therefore, it is clear that environmental stresses such as high temperature can affect the structure and

distribution of important cell wall polysaccharides such as AX which may have implications for the development and growth of wheat grain.

1.4.4.2 (1-3) (1-4)-β-d-glucan (MLG)

MLGs are the second most prominent NSPs within the cell walls of the wheat grain accounting for around 20% of the starchy endosperm cell wall and around 29% of the aleurone cell wall (Bacic and Stone 1980). They consist of linear, unbranched polymers in which β-*d*-glucopyranosyl residues are joined by both (1-3) and (1-4) glucosidic linkages. Single (1-3) linkages are separated by two or more (1-4) linkages and regions of these (1-4) linkages in groups of 2-3 predominate the structure. They are generally soluble in water due to their extended chain formation and form elastic gel networks that exhibit broad melting transitions (Stone and Morell 2009). MLGs are deposited in all thin cell walls of the endosperm following the cellularisation phase and are then extensively deposited in the cell walls of the aleurone at the beginning of the cell differentiation phase (Philippe *et al.*, 2006b). Rakszegi *et al.* (2014) showed that, in contrast to total AX, heat and drought stress reduced total grain MLG content compared to the control treatment with a combination of heat and drought stress causing the biggest reduction (24.5-47.9% reduction across cultivars).

1.4.4.3 Cellulose

Cellulose is a homopolymer comprised of (1-4) linked β-*d*-glucopyranose units found in the cell walls of all grain layers making up around 2% of the total dry weight of the mature grain. Within the starchy endosperm, cellulose makes up around 2% of the NSPs, however, the proportion greatly increases in the cell walls of the outer pericarp where it makes up approximately 30% of NSPs in the cell wall. The long, ribbon-like formation of cellulose enables the polymers to align parallel to one another and form stable microfibrillar

aggregates. AX and MLG are thought to interact non-covalently with these aggregates via the cellulose-like regions of their molecules, contributing to the control and cohesiveness of the cell walls within the wheat grain (Stone and Morell 2009).

1.4.4.4 Pectins

Pectin is a complex polysaccharide composed of different, covalently linked domains that include HG, xylogalacturonan (XGA), and RG-I and RG-II (Mohnen, 2008). The different pectic domains have distinct structures that impact on their function within cell walls. HG is a polymer of α-d(1,4)-linked-galactopyranosyluronic acid (GalpA) residues whilst XGA consist of a galacturonic acid (GLA) backbone substituted with XYL. RG-1 is formed of a backbone of repeating disaccharide units of rhamnose and GLA with arabinans and galactans acting as side chains whilst RG-2 has a GLA backbone with complex side chains (Chateigner-Boutin *et al.*, 2014). Of all the pectic domains, HG is the most abundant, constituting approximately 65% of total pectin found in plant cell walls (Wolf *et al.*, 2009). The GalpA residues that make up the backbone of the HG domain are often methyl-esterified at the C6 position and in some species of plant can be acetyl-esterified at the 0-2 or 0-3 position (Fig. 1.8). In addition, HG can be de-methyl-esterified by the action of pectin methylesterases (PMEs) which remove these methyl-esters and impact the biological function and structural properties of the pectin molecule by affecting the capacity of pectic chains to bind to one another within cell walls (Caffall and Mohnen 2009). Therefore, the methyl-esterification status of HG has been suggested to affect the structure of cell walls during development (Wolf *et al.*, 2009) and has been implicated in regulating the development of cell walls within the wheat grain (Chateigner-Boutin *et al.*, 2014).

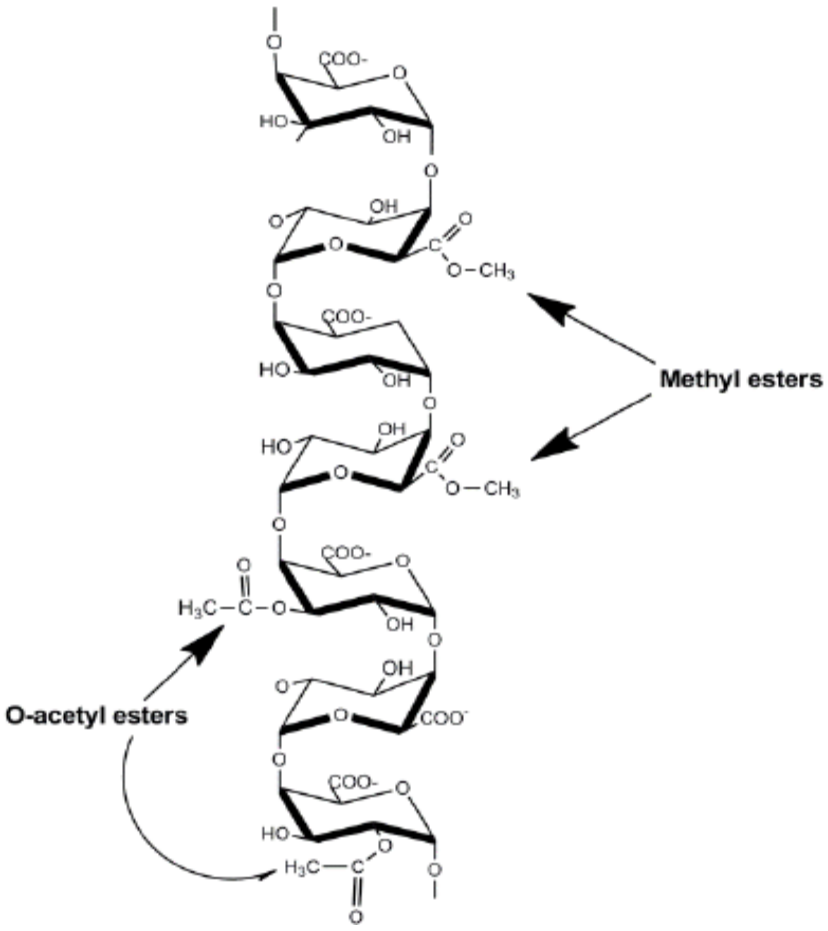


Figure 1.8 Structural diagram of Homogalacturonan (Ochoa-Villareal *et al.* 2012)

1.4.5 Grain growth

Wheat grain size is determined by a number of factors including genotype, environmental conditions and both biotic and abiotic stresses during development. The growth and increase in wheat grain size during development is facilitated by modification of cell walls of the different grain layers to accommodate the growth of the endosperm tissue within the grain. Identifying the processes that affect and regulate these changes will provide a better understanding of size determinants in the wheat grain and potentially provide the understanding to increase wheat yields through selective breeding.

1.4.5.1 Methods of grain expansion during development

During the cellularisation period, initial grain growth occurs through rapid mitotic divisions of the periclinal cells of the coenocytic endosperm. The rapid increase in the number of cells and the subsequent crowding that takes place within the syncytium results in initial expansion of the early grain (Bechtel *et al.*, 2009). Following the completion of cellularisation and the cell division that takes place within the endosperm, subsequent grain expansion is driven by the rapid uptake of water into the grain that accompanies the deposition of starch and protein into the endosperm. A net increase in the amount of water within endosperm cells during grain-filling results in turgor driven pressure being exerted on the endosperm cell wall resulting in expansion and an increase in the cell size. The expansion of the endosperm then exerts pressure on the surrounding seed coat and bran layers leading to their compression (Kutschera and Niklas 2007; Bechtel *et al.*, 2009). Once grain filling has ended and the maturation and desiccation phase begins, the wheat grain reduces in moisture content and consequently the size of the grain is slightly reduced due to a reduction in the amount of turgor pressure.

1.5 Wheat grain and water content dynamics

Water plays a vital role in the development of the wheat grain, such as driving cell expansion through turgor pressure, acting as a transport medium through which solutes are delivered to the developing grain, and providing a suitable environment for metabolic processes to occur. The water content of the wheat grain has been shown to be closely associated with the developmental stage of the grain, exhibiting distinct patterns that coincide with developmental transcriptomic shifts in the grain e.g. cellularisation to grain-filling. This observed relationship has led to suggestions that grain water content acts as an important developmental signal within the grain (McIntosh *et al.*, 2007; Wan *et al.*, 2008).

1.5.1 Grain water content patterns during development

As previously mentioned, the initial growth of wheat grain is driven by rapid net deposition of water that results in turgor driven expansion of the maternal pericarp layers and drives endosperm cell expansion and differentiation until around 10-12daa. Following this period, the grain moisture content increases at a slower rate before reaching a maximum level at around 15daa (Lizana *et al.*, 2010). After this point, grain water content stabilises for 2-3 weeks (Schnyder and Baum, 1992) before dropping as the grain begins the maturation and desiccation phase. Prior to this dehydration the grain 'prepares' for this event through the synthesis of an array of molecules that enable the preservation of the structural integrity of the various organelles, membranes and proteins so that they can persist through this drying event (Bewley *et al.*, 2013). Wan *et al.* (2008) confirmed this in their transcriptomic analysis of the developing wheat grain from the bread-making winter wheat variety *cv. Hereward*, where they clearly saw a shift in transcript abundance and expression around the point of maturity including increased expression of two transcription factors *TaEmBP* and *TaVP1.* Known targets of both transcription factors include genes encoding the early methionine proteins that are involved in protecting cells against tissue damage during seed desiccation by retaining a high degree of hydration (Morris *et al.*, 1990).

The maximum water content of a wheat grain and the point at which this trait is reached is believed to be determined by the volumetric restrictions imposed through cell layer development and endosperm cell division (Schnyder and Baum 1992). Schnyder and Baum (1992) investigated the relationship between developmental changes in grain water content and dry matter accumulation in nine spring wheat cultivars and found that there was a close relationship between the two. In addition, they observed that maximum water content of grains was attained relatively early in grain development at the stage when approximately one third of maximum final dry weight had been obtained and that it was

possible to accurately predict final grain weight based on the maximum water content of a grain. This observation led to the postulation that volumetric restrictions on potential endosperm expansion, reflected by the maximum water content of grains could be causally involved in the determination of the sink capacity of wheat grains. Therefore, exploring whether high p.a. temperature affects endosperm cell number and size could elucidate whether cell wall modifications during development result in a reduction of the final sink capacity of wheat grains through this mechanism.

1.5.2 Grain dimensions and water content

The principle grain dimensions (height, width, length) attain their maximum values at different stages during the grain's development. The dimensions of a wheat grain determine the size potential of the individual grain and therefore are an important yield determinant that could be a targeted trait for improvement by breeders. Understanding how grain dimension and water content dynamics interact will provide further insight into the normal development of the wheat grain and allow for the effect of high p.a. temperatures on these aspects to be determined. Hasan *et al.* (2011) conducted a field experiment in two high yielding spring wheat cultivars, *(cvs.Bacanora T88* and *Kambara),* to study the physiological determinants of grain weight by measuring various traits, both at pre-anthesis (carpel weight) and p.a. (grain dimensions, volume and dry matter). They observed that grain length was the first trait to reach its maximum and stabilise compared to width and height. Their observation that grain length stabilised before maximum water content was reached and the close relationship between grain length and final grain weight led them to postulate that grain length is critical for the determination of grain moisture content and therefore grain weight determination. However, it is unclear through which cellular processes grain length potential is controlled.

The dynamics of grain dimensions and the stages at which they reach their maximum values may differ between genotypes. Gegas *et al.* (2010) studied the genetic basis of phenotypic diversity in wheat grain morphology in several different recombinant populations of doubled haploid winter wheat varieties and a modern and primitive bread wheat. They found that grain size is largely independent of grain shape with length/weight ratios showing little correlation with thousand grain weight (TGW) or grain area. In another study investigating grain weight determination, Xie *et al.* (2015) examined a large number of genotypes from a bread wheat x spelt mapping population and analysed a number of key traits (e.g. carpel size at anthesis, grain dimensions and dry matter accumulation). They also observed that grain length was the first trait to stabilise in the development of the wheat grain. However, they observed that maximum and final grain dimensions were most positively associated with grain height and volume. In addition, they observed that grain water accumulation strongly contributed to maximum and final grain dimensions, in particular height and volume. Regarding the genetic basis of these traits they found 249 quantitative trait loci (QTLs) coinciding among final grain weight, carpel size and grain dry matter accumulation on 16 chromosomes, indicating tight linkages of functionally related genes. Their observation that grain width, height and volume all stop expanding almost simultaneously while grains start to lose water suggests that grain water may function as an incentive for grain dimension establishment. With the important role of grain water content in the expansion of the wheat grain layers and the grain filling period, understanding how grain water content is affected at different stages of development in the wheat plant will allow for internal changes in the wheat grain structure to be deduced and the effect of high p.a. temperature on grain development to be better understood.

1.5.3 Cell wall modification and facilitating proteins

The cell walls within different grain layers are therefore vital in controlling and regulating the expansion and growth of the wheat grain at different stages of development. Not only must cell walls facilitate cellular expansion but they must also provide rigidity and strength once expansion has taken place in order to prevent further growth and maintain the cellular structure. Cell walls within the wheat grain that experience high amounts of tensile stress during grain development expand *via* a mechanism of carefully regulated polymer 'creep'. This is where the matrix of the cell wall yields, allowing the cellulose microfibrils to move apart. This 'loosening' of the cell walls is initiated via the synthesis and subsequent function of particular cell wall proteins, including EXPs and xyloglucan endotransglucosylase/hydrolase (XTHs) (Cosgrove 2005). Therefore, the cell walls within the wheat grain require modification at various stages of development in order to execute the required function at the time. These modifications occur during development but can be triggered by environmental cues.

1.5.3.1 Expansins (EXPs)

EXPs are a class of pH-dependant proteins found within the cell walls of various flowering plants that facilitate the loosening of cell walls during plant cell expansion (Cosgrove 1997; Cosgrove 2000). EXPs are hypothesised to disrupt the hydrogen bonding of glycans to the surface of cellulose microfibrils or to other glycans. Following this disruption, turgor pressure results in slippage in the points of polymer adhesion and leads to progressive and controlled expansion of the cell wall (Cosgrove 2000). The pH level required for EXP activity, and consequently cell wall expansion in plant cells, is between 4.5 and 6 (Cosgrove 2005). A plant hormone, auxin, is believed to lower plant cell pH and activate EXPs through the activation of a proton pump bound to the plasma membrane of cells causing the pH of

nearby cell walls to fall (Lodish *et al.* 2000). Yang *et al.* (2016) observed in two winter wheat varieties, *(cvs.Wennong 6* and *Jimai 20)* that elevated temperatures (35-36°C) applied from 1-5daa resulted in increased levels of auxin within grains from 3-15daa implying an increase in cell wall acidity and therefore EXP activity during this period. However, they also observed variation in grain auxin levels and yield responses to elevated temperatures between the cultivars used in the study, therefore implicating endogenous hormone levels and the consequent activation of EXPs as a determinant in the ability of different wheat cultivars to withstand high p.a. temperatures.

EXPs have a molecular mass of around 26,000 and consist of two domains: an N-terminal domain (~15kDA) with distant sequence similarity to the catalytic domain of the family-45 endoglucanases; and a C-terminal domain (~10kDA) that is related to a family of grass pollen allergens of unknown function (Cosgrove 1997). In addition, two general classes of EXPs can be defined: α-EXPs and β-EXPs. α -EXPs can induce cell wall extension in several plant tissues, while the role and function of β-EXPs are less well understood. However, at least one β-EXP has been shown to induce the extension of cell walls in maize coleoptiles and silks (Li *et al.*, 2002). The distinction between α and β-EXPs was determined following the initial sequencing of EXP complementary DNAs. This revealed a sequence similarity to a group of grass pollen allergens called group-1 allergens which came to be referred to as β-EXPs (Cosgrove 2000). These β-EXPs are found predominantly in the cell walls of grasses. It is suggested that due to the unique structure and composition of cell walls of the *Poacae* family that the α-EXPs and β-EXPs perform a similar function in facilitating cell wall expansion although by acting through different mechanisms.

In wheat grain, EXPs have been shown to be involved in the coordinated growth and development of the different wheat grain layers post-anthesis. Lizana *et al.* (2011) studied the dynamics of grain dry-matter accumulation, water uptake and grain size alongside the

expression of EXPs during grain development in the spring wheat cultivar *cv. Bacanora*, characterising their spatial expression pattern in the pericarp of the developing grain at 5 and 10daa. They found that, within the pericarp, expression of 5 unique EXP sequences and three consensus sequences were highest between the 6-11daa stage. In addition, they observed that expression of these EXPs sequences dramatically reduced once maximum grain length had been attained, suggesting a strong interaction between these two factors. Using *in-situ* hybridisation, they observed that at 5daa EXPs were preferentially located within the pericarp whereas at a subsequent stage of 10daa EXP expression was observed in both the endosperm and pericarp. This temporal variation in EXP expression between the two tissues suggested that their growth is highly coordinated and that EXP expression is a requisite to grain layer expansion in the early stages of wheat grain development.

EXPs have also been shown to exhibit variable expression in response to environmental conditions. Wan *et al.* (2008) conducted a study of the wheat grain transcriptome during development using microarray analysis. They found that at 21daa, a probe-set for a similar β-EXP 1a precursor was upregulated 6.6x in response to drought conditions in controlled environment experiments. However further study is needed to elucidate the role played by EXPs in wheat grain development at high p.a. temperatures.

1.5.3.2 Xyloglucan endotransglucosylase/hydrolases (XTHs)

XTHs are a class of cell wall enzyme that cleave xyloglucan chains endolytically before joining the revealed ends to either H_20 or other XYG free ends and are believed to be involved in cell wall extension (Rose *et al.*, 2002). Liu *et al.* (2007) isolated 3, 6 and 5 genes from the α-EXPs, β-EXPs and XTH families, respectively, from hexaploid drawf wheat seedlings *(cv. Lin 982015)* and studied mRNA expression using real-time qRT-PCR and expressed sequence tag (EST) data mining. They found that hexaploid wheat has the

largest gene family of XTHs when compared against Arabidopsis, rice and maize, and that within wheat, XTH expression was higher than both α and, β-EXPs in the cell elongation stage of the wheat plant's growth. Muñoz and Calderini (2015) confirmed the expression of XTH5 in the epidermal layer of the wheat grain using qPCR from isolated tissue from two tetraploid *(cvs. Corcolen, Quc 3587-2007)* and hexaploid *(cvs. Fritz-Baer* and *Impulso-Baer)* varieties and observed that peaks in expression level coincided with the early phase of grain enlargement when the water content rapidly increases. These observed peaks in expression level also coincided with the lengthening of the pericarp epidermal cells, therefore indicating the importance of XTHs in the expansion and development of the wheat grain.

1.5.4 *Wheat grain cell wall modification under heat stress*

Wheat plants subjected to high p.a. temperatures have been shown to exhibit differences in the contents of their cell walls and their structure. Whilst much focus has been placed on the effect of high temperatures in reducing wheat yields through reduced mobilization of photosynthates during the grain filling period, the relationship between high temperature and cell wall changes within the wheat grain, and whether this may physically limit potential grain size, has not been fully explored.

Toole *et al.* (2007) used FTIR spectroscopy to study environmental effects on endosperm cell wall composition and development in two hard-milling bread wheat varieties, *(cvs. Spark* and *Rialto)*. They grew the varieties in polyethylene covered tunnels that provided different growing temperatures, one giving cool wet conditions (~18°C and full irrigation) until the end of seed maturity, and a hot/dry regime (~30°C with a restricted water supply) with both growth regimes implemented from 14daa. Grains were collected at 14, 22, 26 and 30daa. They found that AX structure within the endosperm cell walls shifted from being

highly-branched with arabinose residues to being lowly-branched and that the switch occurred earlier in wheat plants that were grown at elevated temperatures. Highly-branched AX is more water-soluble than lowly-branched and Toole *et al.* (2007) postulated that an endosperm cell wall containing highly-branched AX may be able to retain water and stretch during the grain filling period whereas a shift to lowly-branched AX may result in bound water being released and endosperm cell walls becoming more rigid. Therefore, this study demonstrates the modifying effect of high p.a. temperature on cell wall components within the grain and suggests modification of AX as instrumental to changes in the structure and function of cell walls during wheat grain development.

1.6 Pericarp restriction of grain expansion under high temperature

Several physiological responses to p.a. heat stress have been identified as causal to the observed reduction in yield and grain quality, including reduced chlorophyll content (Wang *et al.*, 2015), reduced root biomass (Ferris *et al.*, 1998) and reduced mobilization of photoassimilates (Dias and Lidon, 2009). However, it has recently been suggested that morphological changes in the outer, maternal layers of the grain (pericarp and testa) in response to high p.a. temperatures, may represent an additional contributing factor to reduced wheat yield (Lizana *et al.* 2010). More specifically, that high p.a. temperatures could result in a premature decrease in plasticity of the pericarp which in turn would restrict endosperm expansion and therefore reduce average grain weight and yield. The influence of maternal grain layers in determining the final grain size has been well studied, with coordination between the endosperm pericarp and embryo required during normal grain development (Li and Li 2015). In *A.thaliana,* a cytochrome (KLUH) expressed in the inner integument of the developing ovule was shown to control the extent of cell proliferation in

the tissue which determined the size potential of the seed coat (Adamski *et al.*, 2009). The size of the seed coat was then highly correlated with final mature seed size suggesting a strong degree of maternal control on final seed size. In wheat, Brinton *et al.* (2017) identified a stable QTL in a double haploid mapping population (resulting from a winter wheat cross: *cvs. Charger x Badger*) that was associated with a 6.9% increase in grain weight with longer grains whose morphology was associated with increased pericarp cell length. Their results suggested that the gene underlying the locus regulated cell expansion in the pericarp, providing genetic evidence that pericarp cell expansion affects final grain size and weight.

The theory that structural responses in the outer maternal layers to environmental stresses could result in an impact on final grain weight has been suggested in the past with a significant relationship being found between pre-anthesis carpel weight and final grain weight with lower carpel weights pre-anthesis and final grain weight both being observed in wheat subjected to high temperatures (Calderini, 1999b; Hasan *et al.*, 2011; Xie *et al.*, 2015). In addition, high p.a. temperatures have been shown to reduce wheat grain sink capacity through a reduction in the number and size of endosperm cells and starch synthesis sites (Kaur *et al.*, 2011). However, it is not clear whether this reduction in sink capacity in the storage tissue is the result of physical imposition by the pericarp on the endosperm's expansion under high p.a. temperatures or through differential gene expression (DGE) affecting cellular division and intracellular signalling.

Most of the research concerning the influence of the maternal tissue on final grain weight under high temperature has studied this relationship at either pre-anthesis (Calderini 1999a) or at anthesis (Calderini 1999b) and to our knowledge there is no study that has explored the effect of high temperature on the structural relationship between the endosperm and the pericarp p.a.. However, it stands to reason that the endosperm and the pericarp must

undergo synchronised expansion in normal grain development. Whether a premature maturation of the pericarp occurs under high p.a. temperature conditions and imposes mechanical restrictions on the ability of the endosperm to expand provides the basis for this study.

In addition, one cell wall component within the wheat grain that has not been studied under high p.a. temperature conditions is the pectic domain HG. Wheat endosperm cell walls have only recently been shown to contain pectic domains including HG (Chateigner-Boutin *et al.*, 2014) and it is believed that the methyl-esterification status of HG within the cell walls of the wheat grain could affect the ability of cell walls to expand. Therefore, any changes in the distribution and form of HG within the outer layers of the wheat grain under high p.a. temperature conditions may affect the normal development and growth of the wheat grain.

1.7 Transcriptomic analyses

In order to determine the effect of an environmental treatment on gene expression within an organism, investigation of the transcriptome *via.* high-throughput methods such as microarray analysis or RNA-Seq can provide insight into the effect of abiotic stresses on an organism's biology and development. Both of these methods have been used previously in investigations into the development of the wheat grain during grain filling (Wan *et al.* 2008, Pellny *et al.* 2012). Whilst these methods could provide a valuable resource to investigate the effect of high p.a. temperatures on the development of the wheat grain and the interaction between grain layers, there are significant differences between microarray analysis and RNA-Seq in their application and the information they provide.

1.7.1 Microarray vs RNA-SEQ analysis

Microarray analysis is used to detect the expression of thousands of genes at the same time and utilises slides printed with thousands of spots that contain DNA sequences or genes at known locations on the slide. These sections of DNA act as probes to detect gene expression. mRNA is typically collected from both an experimental and reference sample. The mRNA from each sample is then converted to cDNA and labelled with a different colour probe for each sample. The two samples are then mixed together and allowed to bind to the microarray slide and the DNA probes on the slide in a process called hybridization. The analysis then involves scanning the microarray slide to measure the expression for each gene between the two samples based on the predominance of the colour probe attached to each sample. This allows changes in the simultaneous expression of many genes to be visualised between RNA samples in response to a treatment or condition (Sealfon and Chu, 2011).

RNA-Seq also involves the targeting and isolation of mRNA from samples and the production of a cDNA library from fragmented RNA through the addition of sequencing adaptors and amplification of cDNA via PCR. However following the production of this cDNA library, these fragments are attached vertically to a flow cell which is then analysed and sequenced using nucleotide specific fluorescent probes which determines each sequence of nucleotides for the cDNA fragments attached to the cell.

RNA-Seq arguably holds a number of advantages over microarray analysis; microarray analysis is dependent on detecting transcripts that correspond to existing genomic sequencing information in the form of known sequences and genes used as probes on the slides during analysis. However, RNA-Seq is not dependent on existing sequencing information and therefore is able to be used to investigate the expression of known

transcripts but also aid in the identification of novel transcripts with unknown functions. RNA-Seq also avoids issues such as cross-hybridisation that can affect the accuracy of microarray analysis, whilst RNA-Seq has also been shown to be superior to microarray in the detection of low abundance transcripts, identifying critical isoforms in genes and allowing the detection of genetic variants (Zhao *et al*., 2014). RNA-Seq also can quantify a broader dynamic range of expression levels than microarray analysis which allows for a larger resolution of the degree of DGE between samples and the detection of more differentially expressed genes with higher fold-change.

Despite these technical advantages, microarray analysis is still widely used due to the established data analysis protocols of its output and the lower operational costs of the analysis both in its completion and the storage of the output data. The choice of method is largely governed by the design of the experiment, the nature of the investigation performed and the financial resources available to the investigator.

1.7.2 RNA quality

One important aspect of both microarray analysis and RNA-Seq is the quality of the RNA sample used to produce the cDNA library for both analyses. The quality of an RNA sample is determined by both its integrity and purity. These two facets are important in an RNA sample to ensure the accuracy, reproducibility and relevance of downstream analyses performed such as microarray and RNA-Seq. The purity of an RNA sample typical refers to amount of undesirable biomolecules in the RNA sample such as genomic DNA, DNAses RNAses and proteases that can have negative effects on the transcriptomic analyses performed. These biomolecules can persist in RNA samples where usual removal and purification techniques have failed during an RNA extraction protocol. DNAses for example

can degrade the cDNA library created whilst RNAses can degrade the RNA before the analysis has taken place.

RNA integrity refers to the degradation of the RNA itself. Unlike DNA which has a double helix structure, RNA is single stranded and is therefore more unstable and susceptible to degradation. Degraded RNA can significantly affect sequencing and microarray results via uneven gene coverage and 3'-5' transcript bias in favour of the 3' end of transcripts which significantly affects the results of the performed sequencing and can lead to misinterpretation of RNA-Seq data particularly with regards to DGE (Kukurba and Montgomery, 2015). Shorter reads resulting from RNA degradation can be lost in favour of longer reads during RNA-Seq resulting in partial coverage of the transcriptome. RNA integrity can be assessed visually via gel electrophoresis or *via* a bioanalyser to asses for the ratio between 28s and 18s ribosomal sub-units in RNA, an indicator of RNA integrity. Whilst high RNA integrity is important to achieve the greatest amount of information from RNA-Seq and microarray analysis, a study by Gallego Romero *et al.* (2014) suggested that valuable information can still be obtained from samples with similar levels of degradation via transcript normalisation techniques.

1.8 Aims of project

The effect of high p.a. temperature on pericarp and endosperm interaction during development and whether structural modifications occur that place mechanical restrictions on grain size and weight potential has not been investigated. Therefore, the aim of this PhD project was to test the overall hypothesis that high p.a. temperatures result in a premature maturation of the pericarp of the grain in respect to the endosperm resulting in a loss of plasticity in these outer layers that consequently reduces grain size and weight.

1.8.1 Project design summary

Four controlled environment experiments using high p.a. temperatures were conducted over the course of this PhD project, each with individual hypotheses and experimental aims. These experiments are presented in three separate experimental chapters (Chapters 3, 4, 5). When combined, these experiments sought to provide evidence to either prove or disprove the hypothesis that high p.a. temperatures result in a premature maturation of the pericarp of the grain with a subsequent loss of plasticity that may restrict endosperm expansion. Here, these experimental chapters are briefly described with their intended learning outcomes in overview in order aid in the comprehension of this thesis:

Chapter 3: *The effect of high post-anthesis temperature on grain dimensions and yield.*

This chapter details the attempts made to determine the severity of a high p.a. temperature treatment required to elicit a p.a. heat stress response in *cv. Cadenza* through either a reduction in grain weight and/or grain number over two separate experiments (Experiments 1 and 2). Once a treatment had been determined, the structural response of the cell walls of the different grain layers under high p.a. temperatures could be investigated. In addition, this chapter contains the outcomes of experiments investigating the effect of high p.a. temperatures on grain dimensions at maturity (Experiment 2) and during development (Experiment 4), as well as an experiment investigating whether high p.a. temperatures result in a negative effect on grain size when experienced throughout the grain filling period (Experiment 4). These experiments would help reveal more about the potential interaction of the different grain layers in determining grain size under high p.a. temperatures and what other developmental processes of grain development may be impacted.

Chapter 4: *Effect of high p.a. temperature on wheat grain endosperm cells and homogalacturonan distribution and form*

This chapter details an experiment (Experiment 3) on the effect of high p.a. temperature on endosperm cell number and size in *cv. Cadenza* wheat grain. In order to test the overall hypothesis that a premature maturation of the pericarp, resulting from high p.a. temperature, may restrict endosperm expansion, it was necessary to test the effect of high p.a. temperature on these two aspects determining the size of the endosperm. Exploring where in the endosperm and when during development any negative effect may have occurred from high p.a. temperatures would aid in determining whether a premature maturation of the pericarp impacted on endosperm size dynamics when considered with the results of other chapters. In addition the effect of high p.a. temperatures on the form and distribution of cell wall HG within *cv. Cadenza* grain was also investigated in an experiment (Experiment 2). This experiment presents the first investigation, to our knowledge, of the effect of high p.a. temperature on this cell wall constituent only recently discovered in wheat grain (Pellny *et al.* 2012; Chateigner-Boutin *et al.* 2014). As the methyl-esterification status of HG has implications for cell wall expansion, if this was found to be altered in the pericarp or endosperm of the grain under high p.a. temperature this would provide evidence towards the overall hypothesis of project.

Chapter 5: *Effect of high p.a. temperature on the transcriptome of the wheat grain*

In this chapter and experiment investigating the effect of high p.a. temperature on grain moisture content in *cv. Cadenza* during development is detailed (Experiment 2). The aim of this experiment was to determine a suitable p.a. stage following high p.a. temperature for subsequent RNA-Seq analysis by identifying when p.a. high temperatures resulted in a significant difference in moisture content between treatments. These results would then

help determine a suitable sampling stage for RNA-Seq analysis in a subsequent experiment which is also discussed in this chapter (Experiment 3). In addition the effect of high p.a. temperature on grain filling rate was also assessed. RNA-Seq analysis on high p.a. temperature treated and control grains was conducted in Experiment 3 in order to determine whether high p.a. temperatures resulted in the differential expression of genes between treatments and whether genes involved in cell wall synthesis or modification were differentially expressed. Comparison of data with existing RNA-Seq data sets from wheat endosperm and pericarp allowed for tissue specific determination of gene expression patterns. The results of this analysis would help support or contradict the overall hypothesis of the project that high p.a. temperature results in a premature maturation and a loss of plasticity in the pericarp potentially restricting endosperm expansion.

Therefore, in summary, the following major objectives were targeted during this study:

- To determine the minimum duration and severity of high p.a. temperature treatment that significantly affects mature grain weight and size in *cv. Cadenza* and determine the treatment's effect on grain size dimensions at maturity and during development
- To determine the effect of high p.a. temperature on the cellular structure of the endosperm in addition to HG form and distribution within the different grain layers during development and whether they affect grain size and weight determination
- To investigate whether high p.a. temperatures result in earlier maturation and loss of plasticity in the pericarp in respect to the endosperm using information from the transcriptomic profile of the tissues.

Chapter 2: Materials and methods

2.1 Introduction

In total, four controlled environment experiments were performed during this PhD project all using the British spring bread wheat cultivar *cv. Cadenza* (bred by Cambridge Plant Breeders Ltd). The use of this spring wheat cultivar allowed for the relatively quick production of experimental material due to the shorter developmental time from germination to maturity. Whilst no longer widely grown commercially in the UK, the variety has been used in a number of breeding and mapping programs and was crossed with the variety *cv. Avalon* to produce a genetic reference population as a tool for the genetic improvement of wheat, as part of the Wheat Genetic Improvement Network (WGIN) (http://www.wgin.org.uk/). In addition, the utilisation of 1200 mutant varieties of *cv. Cadenza* and the resequencing of their exomes *via.* next generation sequencing as part of the wheat_tilling project (http://www.wheat-tilling.com/) provides a valuable database of information with which any genetic results from this study could be compared against.

All experiments in this project involved the application of a high p.a. temperature and the sampling of grain from a range of p.a. stages, although the treatments and design of each experiment varied. Some experiments had multiple aims, with the same experimental material being used in some cases for multiple investigations (Table 2.1) (Fig 2.1).

Table 2.1 Summary of experiments performed

Experiment	High p.a. temperature treatment used	Purpose	Analysis performed
Experiment 1	32°C (4 hours/day-for 2 days). Applied at different p.a. stages	1. Induce a high p.a. temperature stress response measurable as a decrease in yield.	1. Mature grain weight and dimensions. 2. Immuno-fluorescence microscopy on grain sections at different p.a. stages
Experiment 2	35°C (10 hours/day-different durations). From 6daa.	1. Induce a high p.a. temperature stress response measurable as a decrease in yield. 2. Determine the point in grain development where significant differences in grain moisture content occur between treatments. 3. Determine the effect of high p.a. temperature on the form and distribution of HG within cell walls across the grain	1. Mature grain weight and dimensions. 2. Grain moisture content, fresh weight and dry weight. 3. Immuno-fluorescence microscopy.
Experiment 3	35°C (10 hours/day-different durations). From 6daa.	1. Investigate the effect of high p.a. temperature on the wheat grain transcriptome 2. Investigate the effect of high p.a. temperature treatment on endosperm cell number and size.	1. RNA-Seq analysis 2. Immuno-fluorescence microscopy and image analysis
Experiment 4	1. 35°C (10 hours/day-4days). Applied at different stages p.a. 2. 35°C (10 hours/day-4days. Applied at 6daa.	1. Investigate the effect of an established high p.a. temperature stress inducing treatment applied at different stages p.a. 2. Investigate the effect of an established high p.a. temperature treatment on grain dimensions during development.	1. Mature grain weight 2. Grain dimensions during development

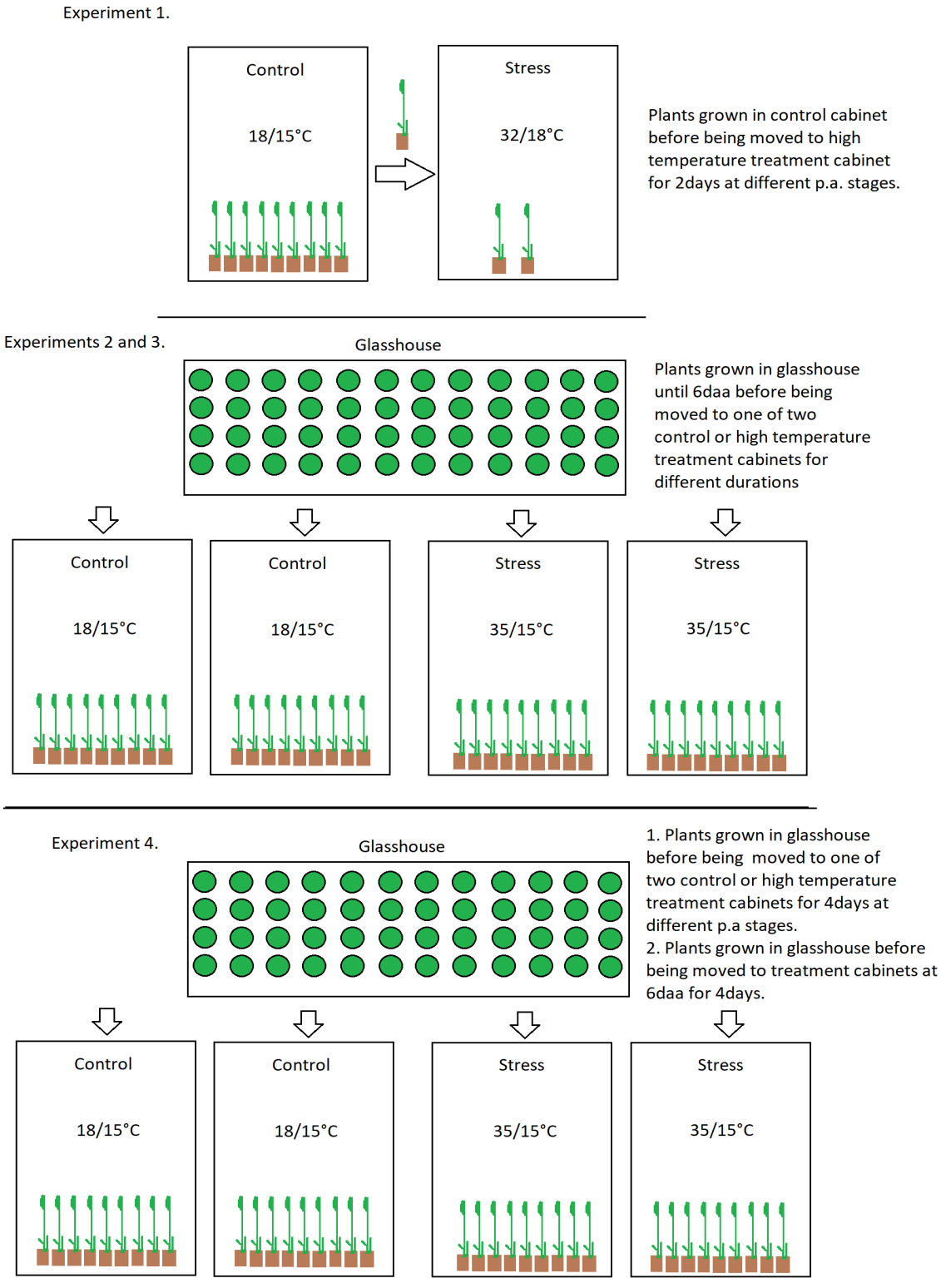


Figure 2.1 Diagram of experiments performed and treatments used in the project

2.1.1 Location of research

Growth of experimental material took place at the University of Reading's 'Plant Environment Laboratory' in Shinfield, Reading, UK for experiment 1 conducted in 2014 while all subsequent experimental material was grown at the University of Reading's Harbourne site.

2.2 Experiment 1: Plant material growing conditions (October 2014)

91 seeds were sown into a germination tray containing a standard soil mix with a 4:2:4:0.02 composition (gravel, sharp sand, vermiculite (Sinclair, Medium 2.0-5.0mm pH (6.0-7.0), Nitrogen fertiliser (Osmocote Pro: 17-11-10+2 MgO+TE (17-2.8-8.3+1.2 Mg+TE)). This seed tray was then left indoors for a 10-day period and watered twice daily by hand. After this period, 80 seedlings at around Zadok's stage 13, (Zadoks, 1974) were transplanted to disinfected 1L pots, (130mm, 5" Bullwell, Richard Sankey and Sons), one plant per pot, filled with the standard mix and placed within a 4'-6" x 4'-6" (1.37m x 1.37m) Saxcil™ controlled environment growth cabinet. The growth cabinet was set to conditions of 18°C (day)/15°C (night) and a photoperiod of 16-hours (5.00-21.00) with overhead lighting providing ~700µmol/m^2/s photosynthetically active radiation (PAR). The cabinet was programmed to provide a relative day humidity of 50% and a night humidity of 70%. A high temperature treatment cabinet was also prepared that had been programmed with the same 16-hour photoperiod but rose from a resting night time temperature of 18°C to 32°C between 5.00-11.00 before returning to 18°C between 15.00-21.00, therefore providing 4-hours of high p.a. temperature treatment between 11.00 to 15.00. The high temperature treatment cabinet also had the same day/night relative humidity (RH). Plants within both cabinets were watered at 9.00 and 17.00 via a soil irrigation system for 60 seconds.

During the early stem extension phase of development, the wheat plants received a 4.5L application of nitrogen fertilizer (Miracle-Gro™ 'All purpose' (6:3:6(UK version) + micronutrients)) at their roots using a watering can with a fine rose head. Each wheat plant was trimmed down to three tillers (primary, secondary and tertiary) and had dead leaves removed. The plants were individually monitored around the heading growth phase in order to record and tag the location and date of first anthesis on the head on each tiller. Anthesis was defined as the moment when the middle third of the ear had anthers protruding.

2.2.1 Experiment 1: Experiment design (October 2014)

Plants were separated into 5 different treatment groups (Control, 4, 10, 16, 22 daa). These groups referred to the daa that a high temperature treatment was applied with the control treatment group receiving no high p.a. temperature exposure. These treatment groups were based on the date of anthesis on the primary tiller of each plant. These treatment stages were chosen in order to subject the wheat to a high temperature treatment of 32°C at separate critical stages in its development (e.g. cellularisation (4daa), early and middle grain filling (10-16daa), onset of desiccation (22daa)). A 32°C temperature treatment was selected as this temperature has previously been shown to reduce grain weight through reducing the duration of the grain filling period (Hunt *et al.*, 1991). Individual plants were allocated a treatment using a random number generator (www.random.org). The plants within each treatment group were then also randomly assigned a collection time (7, 13, 19, 25, 31daa and maturity) using the same random number method. Plants were determined as having reached physiological maturity as the point when the peduncle on the main tiller turned yellow (Hanft and Wych 1982; Isidro *et al.*, 2011). Each plant was used for only one treatment and one collection in order to prevent any negative response to cumulative collections. Three individual plant replicates were used for each treatment and collection

time, resulting in 72 plants being used in the experiment in total (Table 2.2). For the high p.a. temperature treatment, each plant was transferred to the high temperature cabinet and left for a 48-hour period before being returned to the original growth cabinet. Therefore, each plant in the high temperature cabinet received two 4-hour applications of 32°C in the 48-hour period.

Table 2.2 The treatment groupings of Experiment 1.

Treatment initiation stage	Collection points	Number of plants
Control (No treatment)	7,13,19,25,31daa + Maturity	n=18
4daa	7,13,19,25,31daa + Maturity	n=18
10daa	13,19,25,31daa + Maturity	n=15
16daa	19,25,31daa + Maturity	n=12
22daa	25,31daa + Maturity	n=9

2.3 Experiment 2: Plant material growing conditions (September 2015)

Another experiment aimed at inducing a high p.a. temperature stress response in wheat was performed using an increased temperature and range of durations of exposure (number of days spent within the treatment cabinet). Another aim of this experiment was to determine a suitable period during grain development for transcriptomic profiling. This period would be determined by observing when significant differences in moisture content, fresh and dry grain weight occur between control and high p.a. temperature treated plants.

In addition, grains were collected to determine the effect of high p.a. temperature on the form and distribution of cell wall HG across the wheat grain under high p.a. temperatures.

504 seeds were sown into 75well (5ml) germination trays containing a standard soil mix (75% medium grain peat, 12% sterilised loam, 3% medium grade vermiculite 10% sand and 3.5kg Osmocote plus 3-4 month per m^3) and left in a glasshouse for approximately 2 weeks, being watered by hand twice daily. Following this period, 350 seedlings were transplanted from these germination trays into 1.5L B.E.F size 6 pots (15cm diameter) containing ~950g of the standard soil mix with one seedling per pot. Pots were then placed on benches in a glasshouse with additional overhead lighting set to a 16-hour photoperiod (5.00-21.00). Light received at the canopy averaged ~450 /m^2/s PAR. Temperature and (RH) in the glasshouse was recorded by two Gemini™ tinytags (TGU4500) (+/-0.5°C). The average day time temperature of the glasshouse was 19°C with the average night time temperature being 17°C over the growing period (September-December 2015). During the same growing period the highest temperature recorded was 31°C and the lowest recorded temperature was 8°C. The average RH over the growing period in the glass house was 73%. Four controlled environment growth cabinets (Weiss Technik™ HGC 1514) were prepared with two being used for the high p.a. temperature treatment and two for the control treatment. The high p.a. temperature treatment cabinets were programmed to day conditions of 50% humidity, 35°C temperature and a 16-hour photoperiod (5.00-21.00). The night conditions were: 70% humidity and 15°C. There was also a ramping effect within the high temperature treatment cabinets with the temperature increasing from the night time temperature of 15°C to the treatment temperature of 35°C in 4 hours from the start of the light period at 5 am, equating to an increase in temperature of 0.083°C per minute. There was also a ramping of the temperature at the end of each light period with the temperature then decreasing from 35°C to 15°C between 19.00-21.00 (a temperature

decrease of 0.166°C per minute). Therefore, the high p.a. temperature treatment in each of the two cabinets was imposed for 10 hours per day between 9.00am and 19.00. The two control cabinets were set to the same photoperiod and RH as the high p.a. temperature treatment cabinets for both night and day, although the day/night temperature used in the control cabinets was 18/15°C. There was no ramping programmed into the control cabinets due to the small difference in temperatures between day and night. Average amount of light provided was ~550 /m^2/s PAR at the canopy level.

During their growth in the glasshouse the plants were sprayed periodically with Alto Elite (Chloroathanol + cyprocanole) at a rate of 5ml per litre of water in order to treat and control powdery mildew infection. Plants were also sprayed with Aphox™ (pirimicarb) at a rate of 0.5g per litre in order to control for aphid infestation. Plants were watered daily by the glasshouse technical staff between 9.00-11.00. Once the plants had reached approximately Zadok stage 25 (Zadoks *et al.*, 1974) plastic collars were inserted into every pot in order to help support upward growth and reduce entanglement that may have occurred due to close proximity of the plants. Plants were left with their tillers so as not to induce a physical stress response through removing them. Plants were monitored daily from the heading stage onwards so that the date of anthesis of main tillers could be recorded.

2.3.1 Experiment 2: Experiment design (September 2015)

In the pilot transcriptomic analysis experiment, plants were grouped into 4 cohorts, with each cohort consisting of 16 plants that had all reached anthesis on the same day. Each cohort was randomly split in half with 8 plants being used for the high p.a. temperature treatment and 8 being used as the control. All plants within the cohorts were grown in the

glasshouse until 6daa and were then transferred to either the high temperature treatment cabinets or the control treatment cabinets (Fig.2.2)

A randomised block design was used with plants allocated to one of 8 collection times p.a. (4, 6, 8, 10, 12, 14, 16, 18daa). Plants allocated to collections at 4 and 6daa were sampled whilst in the glasshouse before the high temperature was applied in order to obtain an early development measurement of grain moisture content. Each cabinet was split into 6 separate regions to avoid location bias and each set of 8 plants were randomly allocated a region within the cabinets and a position of the pot within that region. The region of the cabinet and the position of the pot within that region acted as blocking levels for statistical analysis of the data. Each individual plant was subjected to one treatment and one collection in order to remove any potential effects of cumulative collection. Once a plant had been sampled from, it was left in its respective control or treatment cabinet until all plants from within the same cohort had been sampled. This was to try and reduce any changes to the surrounding growing environment for the remaining plants that may have occurred from removing plants from the cabinet. Following the final collection at 18daa the entire cohort was returned to the glasshouse.

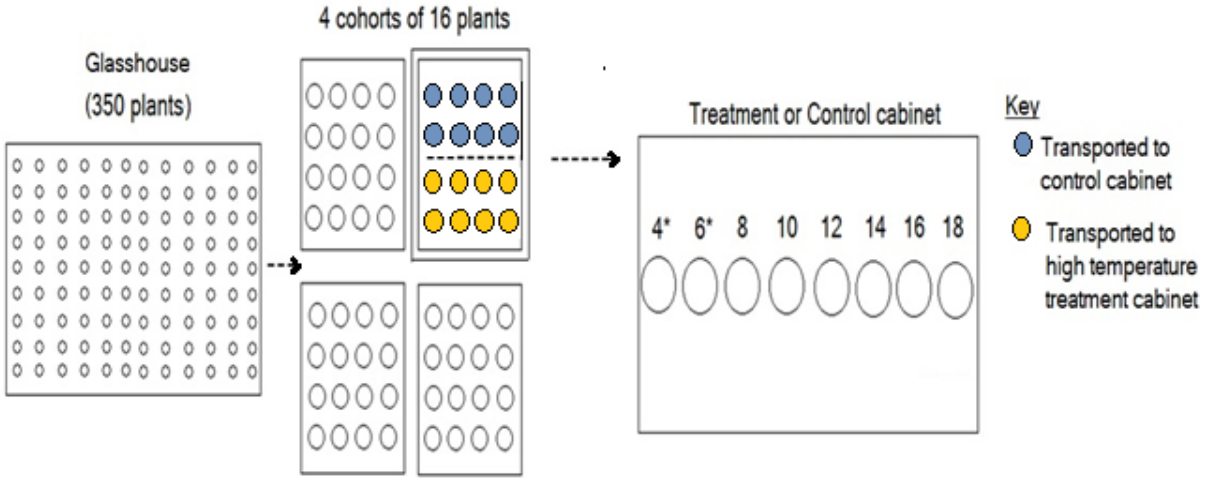


Figure 2.2 Diagram of experimental design for the transcriptomic pilot experiment. * Collections at 4 and 6daa took place in the glasshouse outside of the treatment.

The use of four cohorts provided 4 individual plant replicates for each treatment and collection time resulting in 64 plants being used in the experiment in total ((temperature treatments = 2) x (collection points = 8) x (cohorts = 4) =64). Plants were watered in cabinets twice a day between 9.00-10.00 and between 17.00-18.00, with the aim of keeping the soil moist for both control and heat-treated plants.

For the repeat of the high p.a. temperature stress experiment, 8 cohorts of 7 plants that flowered on the same day were used. The 7 plants of each cohort were grown in the glasshouse until 6daa before they were transferred into the controlled environment growth cabinets. One plant from each of the cohorts was transferred to one of the two control treatment cabinets with the remaining 6 plants being transferred to one of the two high p.a. temperature treatment cabinets as a group. These 6 plants in the high temperature treatment cabinets were randomly assigned a treatment duration of either 2, 4, 6, 8, 10, 12 days. Following a treatment, plants were transferred to the control treatment cabinet until the maximum 12-days of high temperature treatment within the cohort had been

completed. Then the whole cohort was returned to the glasshouse and grown until maturity. This was to ensure that no plants from the cohort received exposure to a high temperature that may have been present in the glasshouse whilst the remaining plants in the cohort were still receiving their treatment. Once they had reached maturity, heads from the primary, secondary and tertiary tillers were collected.

2.4 Experiment 3 -Plant material growing conditions (July 2016)

The aim of this experiment was to produce grain material for RNA-Seq and endosperm cell analysis. The design and parameters of this experiment were based on the results of the second experiment where differences in moisture content and fresh weight were determined between high temperature treated and control plants at different developmental stages.

Plant material for the transcriptomic and endosperm cell analysis experiment was prepared and grown in the same manner as experiment 2. Light received at the canopy averaged ~450 /m^2/s PAR. The average daily temperature during the growing period (July-September) was 24.5°C, although there were two weeks, one during the booting stage (12/08/2016-19/08/2016) and one during the booting-anthesis stage (23/08/2016 – 30/08/2016) where the temperatures in the glasshouse exceeded the high p.a. treatment temperature of 35°C for around 3-4 hours on 5 days out of each week. However, all cohorts experienced this temperature, meaning there was no variation between cohorts in their exposure to high temperatures before the p.a. treatment. The highest temperature recorded in the glasshouse was 46°C, which was reached for a half hour period. The lowest temperature recorded was 17.1°C. The average RH of the growing period was 67%.

2.4.1 Experiment 3 – Experiment design (August 2016)

The same number of cohorts and blocking structure employed in the second experiment was used in this third experiment to produce the grain material for the transcriptomic analyses. However, each cohort in this experiment consisted of 14 plants that had flowered on the same day. Plants again were grown in the glasshouse until 6daa when half of each cohort (7 plants) were transferred to one of two control cabinets, and half to one of the two high temperature treatment cabinets. The 4 controlled environment cabinets used had exactly the same conditions as in the second experiment. The collection points in this experiment were 4, 6, 10, 14, 18, 22, 26daa and therefore the collections at 4daa and 6daa were performed in the glasshouse before the cohort were moved to the treatment cabinets. After the 18daa collection all the plants within a cohort were transferred from their respective controlled environment cabinet to the glass house meaning that collections at 22 and 26daa were performed in the glasshouse.

For the endosperm cell analysis experiment conducted alongside the collection of grain material for transcriptomic analyses, a cohort structure was also used. Eight cohorts of plants that flowered on the same day were used, with each cohort consisting of 8 plants. All plants within a cohort were transferred to either a high temperature treatment cabinet or control treatment cabinet at 6daa. The maximum high p.a. temperature treatment imposed on plants in these cohorts was 12-days. Grain collections took place at 12, 16, 25, 31daa. Therefore, collection of grains at 12 and 16daa was performed within the controlled environment cabinets whereas the collections at 25 and 31daa took place in the glasshouse.

2.5 Experiment 4 – Plant material growing conditions (May 2017)

The aim of this experiment was to measure the dimensions of wheat grain at different developmental stages and compare them in control plants and plants that had undergone a 4-day high p.a. temperature initiated at 6daa. In addition, plants were grown with the aim of investigating the effect of a 4-day high p.a. temperature exposure applied at different p.a. stages on mature grain weight in order to determine whether the effect of a high p.a. temperature is only apparent at certain stages.

Plant material for experiment 4 was produced in the same manner as in experiments 2 and 3. Light received at the canopy averaged ~450 /m^2/s PAR. The average temperature during the growing period (June-August) was 24.4°C, although there were 2 weeks where the temperature in the glasshouse exceeded 35°C for approximately 3 hours per day, one during the stem elongation period (13/06/2017-20/06/2017) and the second during the booting stage (1/07/2017-8/07/2017). However, similarly to the third experiment, all plants within the cohorts experienced these high temperature exposures. The highest temperature reached was 51°C on the 18/06/2017 for a half hour period. The lowest temperature recorded was 14.8°C and the average RH over the growing period was 61.4%.

2.5.1 Experiment 4 – Experiment design (May 2017)

The plants were split into treatments based on the cohort structure previously used: 4 cohorts of 14 plants were used for grain dimensions measurement, 7 cohorts of 8 plants were used to determine the effect of a 4-day high p.a. temperature treatment at different stages. Four controlled environment cabinets were configured exactly as in experiment 2 and 3. For the grain dimension experiment, cohorts were transferred from the glasshouse

to cabinets at 6daa, with half of the cohort being used in the control treatment cabinets and half in the high temperature treatment cabinets. Cohorts were left in their respective treatment cabinet for 4 days before being transferred back to the glasshouse at 10daa. Plants within each cohort and treatment were collected at 6, 8, 10, 14, 18, 22, 26daa either from within the cabinet or glasshouse depending on their allocated collection point. Therefore, collections at 6daa in a cohort were performed in the glasshouse before the rest of the cohort were moved to the treatment cabinets in order to obtain early grain dimension measurements.

For the experiment looking at the effect of high p.a. temperature treatment at different stages p.a. on mature grains, cohorts of 8 plants that had flowered on the same day were grown in the glasshouse until different stages p.a. where they were transferred to the either a control or high temperature treatment-controlled environment cabinet. Each cohort was subjected to a 4-day treatment before being returned to the glasshouse until harvest maturity. The different p.a. stages that treatment was applied from were 4, 6, 8, 11, 15, 18, 22daa, with one cohort being allocated to each treatment.

2.6 Vapour pressure deficit

In experiments 2,3 and 4, 4 Weiss Technik™ HGC 1514 growth cabinets were used with 2 being programmed to provide a control environment (18/15°C and 50/70% RH) and 2 to provide a high temperature treatment (35/15°C and 50/70% RH). The difference between temperatures in control and high p.a. temperature treatment cabinets programmed to the same RH meant that plants were subjected to different vapour pressure deficits (vpd). Vpd is the deficit between the air moisture content and how much moisture the air can hold when saturated. When temperatures increase, the amount of moisture the air can hold increases. As vpd increases plants need to transpire more and therefore draw up more

water via their roots. This increased pressure can lead to nutrient toxicity or desiccation and death. In the control cabinets, the day time vpd = 1.032 kPa whilst the night time vpd = 0.511kPa. In the high temperature treatment cabinets, the day time vpd =2.970kPA, whereas the night time vpd was the same as in the control cabinets. Due to the technical limitations of the cabinets and the environments they can accurately produce it was not possible to match vpds. Therefore, watering was conducted twice a day during the treatment and completed on a visual basis with the aim of keeping the soil adequately moist in both treatments. This was performed in order to try and remove the effect of the difference in vpd and to try and isolate temperature, not water availability, as the only variable.

2.7 Grain collection

In each experiment where grains were collected during development (experiments 2,3,4), the collection was performed in the same manner both in the controlled environment cabinets or glasshouse depending on the design of the experiment. Collections were also performed at approximately the same time between ~10.00am – 12.00pm. Using nitrile gloves, grains were collected from the outside 2 florets (2nd and 4th floret) on the central four spikelets of each side of the ear resulting in 16 grains being collected in total from each plant.

For collection of grains at harvest maturity from all experiments where that was required, entire heads were collected from main tillers when the peduncle had turned yellow which has been used as a visual measure of physiological maturity (Hanft and Wych 1982; Isidro *et al.*, 2011). In experiments 2 and 4, heads were also collected from the 2nd and 3rd tillers in the same manner. Mature heads were then threshed by hand and individual grains counted. Grains were then dried for 48-hour period in grain ovens at 70°C.

2.7.1 Determination of fresh weight, dry weight and moisture content.

In experiment 2 and 3, where grains were collected for fresh weight, moisture content and dry weight analysis in addition to transcriptomic analysis, the 16 grains collected from an ear were weighed collectively in a perforated, pre-weight 5ml Eppendorf ™ tube using a Kern PCB250-3 weighing scale. The perforated Eppendorf ™ tube was then placed in a decanter containing LN_2 before being transferred to an Eppendorf ™ Cryotube F570h -80°C freezer. Frozen grains were then freeze dried for a 4-day period using an Edwards™ Modulyo PB1/201 freeze drier before being weighed again in order to determine the total combined grain dry weight. The combined dry weight could then be subtracted from the combined fresh weight in order to determine the total combined moisture content (ml) of the grains. The total amounts were then divided by the number of collected grains (16) in order to determine an average grain amount for each measure. In addition, the average percentage moisture content of grains was calculated using the following equation:

$$mc_{\%} = \frac{sample_{fwt} - sample_{dwt}}{sample_{fwt}} \times 100$$

where $sample_{fwt}$ is the average fresh weight of a grain and $sample_{dwt}$ is the average grain weight of the same sample after drying.

2.7.2 Determination of mature grain weight

In order to determine the average mature grain weights collected in experiments 1,2 and 4, grains collected from whole heads were counted and then weighed collectively using a Denver Instrument™ SI-234 weighing scale before being divided by the total number of grains per head in order to give an average individual grain weight.

2.7.3 Grain dimension measurements

In experiments 1 and 2, grains collected at maturity were analysed using a Marvin seed analyser ™ (GTA Sensorik GmbH, Germany) which counted the number of grains per head and measured the length, width and area per grain. Once individual grain dimensions had been calculated for each grain in a head it was possible to calculate an average grain dimension per head. Mature grains collected from experiment 2 were also measured for their average volume via ethanol displacement. In this protocol a pre-weighed density bottle was filled with 100% ETOH and weighed. The ETOH was then removed before 10 seeds from a sample were added to the bottle which was then filled with ETOH and re-weighed. From these measurements it was possible to calculate the mass and volume of ETOH in the bottle with and without seeds and therefore the combined volume of the seeds. This combined value was then divided by 10 in order to give the average volume per seed in each sample. In experiment 4, grain dimensions (length, width, height) were calculated at 6, 8, 10, 14, 18, 22, 26daa using a set of Halfords's ™ Electron digital callipers (0.02mm±) before being frozen in LN_2.

2.8 Grain collection for microscopy

In experiments 1, 2 and 3, grains were collected for microscopy. In experiment 1 approximately 10 grains were collected from the central third of the ear from the outer 2

florets within spikelets of each plant from each collection point (7,13,19,25, 31daa and maturity) within each treatment allocation. In experiment 2, approximately 10 grains were collected from either the 1st or 3rd floret within spikelets from the central third of the ear. Grains were collected at 8,14 and 18daa after grains had been collected from the 2nd and 4th florets for the transcriptomic pilot experiment. In experiment 3, approximately 10 grains were also collected from either the 1st or 3rd florets within spikelets from the central third of each ear. The grains collected for microscopy from the 3rd experiment were sampled at 12,16,25 and 31daa to be used in the analysis of endosperm cell number and area.

2.8.1 Grain sample preparation for fixation and microscopy

Grains taken from each plant were removed from their surrounding glumes. For each plant, 5 grains were sectioned using a double-edged razor blade (TAAB B253/1). Transverse medial sections of wheat grain (approximately 1mm thick) were cut from each of the 5 grains and placed in fixative solution (4% paraformaldehyde (TAAB P001/1) + 2.5% glutaraldehyde (TAAB G011/3) (in 0.1M Sodium phosphate buffer, pH 7.4) for 4 hours before being washed in 0.1M sodium phosphate buffer solution. The grains were then dehydrated through a graded ethanol series before being gradually infiltrated with LR White resin medium grade (TAAB L012) (25, 50, 75, and 100%) for a period of 2-hours at each step. Polyethylene capsules (TAAB C094) containing one section in un-polymerised resin per capsule were then placed in a 60°C oven and left for a period of 24 hours in order to fix the sample in the resin ready to be cut with a Reichert-Jung™ Ultracut microtome.

2.8.2 Immunofluorescence microscopy

In order to view grain sections via immunofluorescence microscopy, 1µm sections were cut via the microtome using glass knives prepared on site and collected in drops of distilled

water on 8-well (6mm diameter per well) diagnostic slides (Gerhard Menzel Diagnostic Slides, 25 x 75 x 1, Teflon X2XER201B#) coated with 1% v/v poly-L-lysine hydrobromide (Sigma-Aldrich, P1399) and dried on a hot plate at 40°C. The poly-L-lysine treatment of the slides aided in section cohesion to the slide. Each slide had wells (1, 2, 5, 6) containing repeat sections from a high temperature treated sample with wells 3, 4, 7, 8 containing sections from a control treatment sample (Fig 2.3). Sections on the slides were incubated in 3% BSA in PBS-T (pH 7.0, 40µl per well, 0.05% Tween 20) for 30 minutes in a wet box before being incubated with a primary monoclonal antibody diluted (1:5) in PBS containing 1% BSA 0.05% Tween for 2 hours (Table 2.3).

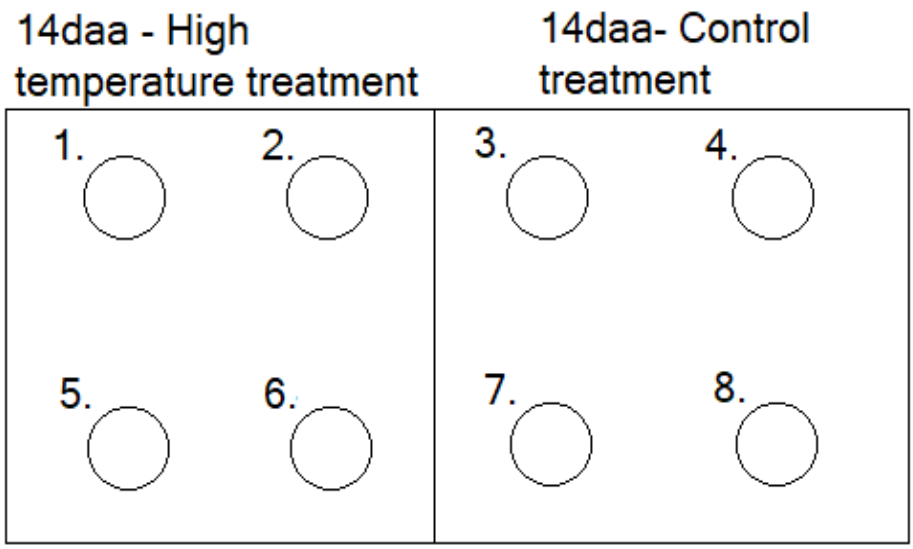


Figure 2.3 Example slide design for immunofluorescence microscopy.

Table 2.3 Monoclonal antibodies (MAbs) used for immunofluorescence microscopy

Antibody	Antigen	Reference	Supplier
JIM7	Partially methyl-esterified homogalacturonan	(Clausen *et al.*, 2003)	Plantprobes.net
LM19	Un-esterified homogalacturonan	(Verhertbruggen *et al.*, 2009)	Plantprobes.net
LM20	Methyl-esterified homogalacturonan	(Verhertbruggen *et al.*, 2009)	Plantprobes.net
LM21	Heteromannan	(Marcus *et al.*, 2010)	Plantprobes.net

Slides were then rinsed 3 times for 5 mins with PBS-T before being incubated in the dark with the secondary antibody diluted 1:40 in 1% BSA in PBS-T (Thermofisher, AlexaFluor 633® - Anti-rat IG, A21094) for 2 hours. Slides were then rinsed twice with PBS-Tween, 2 times with PBS and 3 times with water. Slides were then viewed under a Zeiss™ Axioimager-A2 microscope and photographed with an AxioCam MRm camera at 1500ms exposure. The immunofluorescence analysis slides were replicated 3 times with different grain sections from separate high p.a. temperature and control treated plants.

2.8.2.1 Enzymatic unmasking

A dual enzymatic unmasking pre-treatment was applied to sections used in the investigation into the effect of high p.a. temperatures on HG form and distribution. This pre-treatment was applied prior to the preparation for immunofluorescence microscopy in order to remove AX and MLG that dominate the cell walls of the wheat grain and can mask other cell wall polysaccharides (Chateigner-Boutin *et al.*, 2014). 50µl of β-Xylanase

(Prozomix, PRO-E0062) and Lichenase (Lichenase 26A, NZYtech, CZ00121) solution (20U of β-Xylanase and 40U of Lichenase in 50µl of 50mM PBS buffer pH7.0) was applied to each section. Slides were then incubated overnight in a wetbox at 37°C.

2.8.2.2 Immunofluorescence microscopy for endosperm cell number and area

Grains from experiment 3 were processed in the same manner as other samples used for immunofluorescence microscopy. Four 1µm sections were cut from an individual embedded grain at 12,16 and 25daa from treated and untreated samples and placed on a slide with 50µm spacing between cut sections (Fig 2.4). This process was repeated so that in total 6 individual grains from 6 individual plants were compared against one another from treated and untreated samples at 12,16 and 25daa. 50µm gaps were left between cut sections in order to obtain a representative cross-section.

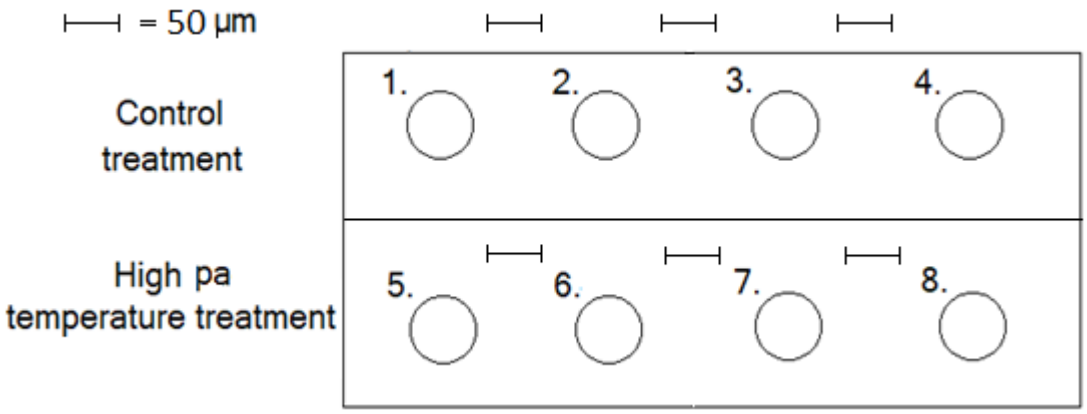


Figure 2.4 Example slide design for analysis of endosperm cell number and area using immunofluorescence microscopy.

Sections were then treated with the primary antibody LM21 which binds to heteromannan in the cell walls of the endosperm accurately revealing their presence under immunofluorescence microscopy.

2.9 Analysis of endosperm cell number and area.

Images of wheat grain sections collected from experiment 3 and incubated with LM21 were collected in sequence at a 5x magnification from making sure to capture the whole cross section. Images were then analysed using the ImageJ™ software (Schneider *et al.*, 2012). The analysis input protocol performed on ImageJ™ for each image is included within the appendix (Appendix 1.) Before the analysis input was performed, the images in sequence were trimmed based on the previous image so that the same cells were not analysed or measured twice. From each slide the average number and area (μm^2) of endosperm cells were calculated from grain sections in wells 1-4 that experienced the control treatment and sections from wells 4-8 from a grain subjected to high p.a. temperature treatment. The average cell number and size from treated and untreated samples from each of the 6 slides were then compared against one another with lobe vs lobe comparisons and 'dorsal' region vs 'dorsal' region comparisons *via* general ANOVA.

2.10 RNA extraction

Total RNA was extracted from freeze-dried material collected from experiment 3 at 6,10 and 14daa with three extractions being performed on grain samples taken from 6daa prior to the initiation of the treatment and 3 extractions being performed per treatment (high temperature/control) for grains at 10 and 14daa. Therefore, 15 RNA extractions were performed in total. Approximately 100mg of frozen, freeze-dried grain material was weighed out and ground into a fine powder in LN_2 using a sterile pestle and mortar. Extractions were then performed via phenol/chloroform extraction. 700µl of RNA extraction buffer + 2% β-mercapto-ethanol heated to 65°C was added to the finely ground material. The extraction buffer consisted of 2% CTAB, 2% PVP, 100mM Tris HCl, 25mM EDTA, 2.0M NaCl and 0.5g/l spermidine and made up using RNA-free water before being autoclaved.

Once the buffer had been added the Eppendorf ™ tubes containing the samples were then inverted and vortexed for 5 seconds to properly mix before being added to a pre-warmed heating block (50°C) for 3 minutes at 400 rpm. Samples were then vortexed thoroughly again before being returned to the heat block and then incubated for 25 minutes with mixing by inversion every 5 minutes. Following incubation an equal volume of Chloroform: Isoamyl alcohol (24:1) was added before being mixed manually and then centrifuged at 14600 rpm for 10 minutes. Following centrifugation approximately 500µl of supernatant was removed and transferred to a new Eppendorf tube from each sample without disturbing the interphase layer. Following this step an equal volume of Chloroform:Isoamyl alcohol was added before being mixed thoroughly by shaking by hand before being centrifuged at 14,600 rpm for 10 minutes. Then approximately 300µl of supernatant was removed and transferred to a new 1.5ml Eppendorf tube before 1/3 LiCl (8M) was added. Samples were then put in a cool room (8°C) and left overnight. The next day a cooled centrifuge was prepared (4°C) and samples spun at 14,000 rpm for 60 minutes in order to pellet the RNA. The supernatant was then removed via pouring and 450µl of RLT buffer from a Qiagen™ RNeasy Mini Kit (74104)) + 10µl β-mercapto-ethanol was added before being vortexed vigorously to dissolve the pellet. The 460µl was then added to an RNA shredder column and taken through the RNA clean-up steps as described in the Qiagen™ protocol. A DNA removal step was also performed using a Qiagen™ RNase free DNase kit (79254). Following the RNA clean up steps and DNA removal, RNA pellets were re-suspended in 80µl of RNA-free water before 2µl was taken for measurement on a nanodrop in order to assess purity and concentration. Based on this reading 1µg of RNA was run from each sample on a 2% ETBR TAE agarose gel at 80v for 40 minutes in order to assess RNA integrity. Samples were then stored at -80°C until then were transferred to a plate to be sent to the Earlham Institute, Norwich, UK frozen on dry ice for analysis, quality control and

RNA-Sequencing. Prior to sending, samples were also assed via an Invitrogen™ Qubit 4 Fluorometer in order to double-check the RNA quantities in the samples.

2.10.1 cDNA library preparation

Additional quality control was performed on the 15 RNA samples by the Earlham Institute according to their quality control pipeline. The integrity of samples was analysed using an Agilent Bioanalyzer 2100 ™ and 14 of the samples gave a RNA integrity number (RIN) score of between 4.2-5.4 and 1 sample scoring 7.0. Those samples that scored below 7 were deemed unsuitable for the high throughput pipelines of the Earlham Institute. The quality check report is attached in supplementary information (Appendix 2). Following discussion, the samples were deemed suitable for attempted sequencing via another platform largely due to there being abundant RNA present in the samples. cDNA library preparation proceeded and all libraries apart from the sample that had a high RIN score produced libraries that were of low concentration with evidence of adapter dimer. Following further discussion, the samples were deemed as potentially suitable to be run on an Illumina HiSeq 2500™ sequencer at a read metric of 2x125bp (paired-end reads) with an expected output of 13-16 million reads per sample. Adapter dimer was removed from the most affected libraries before libraries were pooled together. The concentration of the pooled libraries was then validated via q-PCR which matched closely to concentrations obtained via Qubit. Of the 15 samples, only 1 sample extracted from the material at 6daa produced a library that was of insufficient quality to proceed to sequencing. Therefore, in total, 14 samples proceeded to sequencing (2x6daa, 3x10daa (control), 3x10daa (treated), 3x14daa(control), 3x 14daa(treated).

2.10.2 RNA-Seq data analysis

Sample read files were downloaded via the Aspera Faspex ™ file transfer system in the form of fastq files. These files were then downloaded and processed along with data from other experiments via the 'Galaxy' online software interface (www.usegalaxy.org). Reads from the samples were mapped against the *T.aestivum* genome using the TGACv1 gene assembly produced by the Earlham Institute (Clavijo *et al.*, 2017). Via the galaxy software, samples' read data underwent fastqc and trimmomatic processing in order to remove adapters before undergoing gene alignment using HISAT2, an alignment program for mapping. RNA-Seq data from previous studies (Pearce *et al.*, 2015; Pellny personal communication *unpublished*), was also uploaded to the galaxy platform and processed in the same manner as the collected samples. These data were used as reference to which the sampled data from this PhD project was compared against. Data from Pearce *et al.* (2015) allowed for pericarp and endosperm-specific genes to be determined within the samples as in their study they had dissected grains at 12daa into endosperm, inner and outer pericarp tissues and performed RNA extraction on the individual layers. In addition, Pellny's data (personal communication,*unpublished*) extracted from pure starchy endosperm enabled further specificity of tissue specific genes to be identified from within the samples.

2.11 Statistical analyses

Grain data collected from experiments were analysed using Genstat™ 17th edition (VSN International (2011) (GenStat for Windows 17th Edition. VSN International, Hemel Hempstead, UK. www.genstat.co.uk). In all cases data was checked for normality assumptions and was shown in all cases to demonstrate a pattern of normal distribution. In the majority of the experiments, data were analysed via general ANOVA with a blocking structure of cohort/cabinet/position of the cohort within the cabinet/ position of the pot

within the cabinet position/ pot number. Cabinets were not included within the blocking structure of experiment 2 where the effect of high p.a. temperature on mature grain weight was investigated as this unbalanced the analyses. The analysis of mature grain dimensions and their relationship to mature grain weight were analysed via linear regression analysis with the same blocking structure as the general ANOVAs. In experiments 3 and 4 the number of tillers when treatment begun was included as a co-variate in the analyses. Significant differences between results were determined by obtaining the least significant differences (LSDs) of the means between data and comparing this against the sample means. Where the difference between compared sample means was greater than the LSD value of the means, this was taken to indicate a significant difference ($p=<0.05$) and *vice versa* with differences between samples means lower than the LSD value of the means.

For the RNA-Seq data, comparative analysis was performed using the Rstudio statistical software package (R Core Team, 2013) using the advanced linear model function to identify genes that were differentially expressed between treated and control samples at the different developmental stages. All differential gene expression analysis was performed using a false discovery rate (FDR) of 0.05. The code used in the analysis for this project is included in the supplementary data (Appendix 3).

Chapter 3: The effect of high post-anthesis temperature on grain dimensions and yield

3.1 Introduction

High p.a. temperatures have been widely shown to reduce wheat yields by shortening the duration of the grain filling period, resulting in the production of smaller grains (Hunt *et al.*, 1991; Calderini *et al.*, 1999b; Hurkman and Wood 2011; Talukder *et al.*, 2014). This effect has been attributed to a number of impaired physiological processes including reduced photosynthetic activity in the plant as a result of loss of green area (Vignjevic *et al.*, 2015) and reduced starch deposition in the grain due to reduced activity of starch synthases (Jenner, 1994).

However, the susceptibility of wheat to high p.a. temperatures varies between cultivar and genotype with a range of temperatures having been shown to result in a reduction in grain size and grain number (Porter and Gawith 1999; Spiertz *et al.*, 2006; Farooq *et al.*, 2011). In addition, the period of susceptibility of wheat grain development to high p.a. temperatures has been demonstrated to exhibit varietal differences (Tashiro and Wardlaw, Barnabas *et al.* 2008). Therefore, this genotypic variability means that it is important to establish the p.a. temperature required to induce a heat-stress response in yield in the cultivar used in this project, *cv. Cadenza* and at what stage p.a. the cultivar may be most susceptible to such an effect. To our knowledge there have been no studies that have used *cv. Cadenza* when

investigating the effects of high p.a. temperatures on wheat grain and yield. Once a high p.a. temperature treatment that induces a reduction in grain weight has been determined, then the effect of high p.a. temperatures on the pericarp of the developing wheat grain can be investigated. In addition, determining at what stage during p.a. development grain weight may be most susceptible to high p.a. temperatures in *cv. Cadenza* will help determine what grain development processes may be affected by high p.a. temperatures e.g. cell division, starch synthesis in the later stages of grain filling, and whether the development of the pericarp may be negatively affected.

As previously discussed, a close relationship between the size of the maternal carpels and final grain weight has been observed (Calderini, 1999; Calderini and Reynolds 2000) leading to suggestions that the maternal layers of the wheat grain may affect the determination of grain weight, possibly by constraining grain expansion (Lizana *et al.*, 2010). The wheat grain endosperm develops in intimate contact with the pericarp tissues, meaning that a high degree of coordination between these genetically distinct tissues is essential for grain development (Becraft and Gutierrez-Marcos, 2012). Therefore, any potential structural changes induced by high p.a. temperatures in either tissue is likely to impact on the other.

The maximum length, width, and height of wheat grain attained during development are highly correlated with final grain weight (Gegas *et al.*, 2010; Hasan *et al.*, 2011; Xie *et al.*, 2015). Grain length has previously been shown to be the first dimension to attain its maximum during development at around 15daa (Lizana *et al.*, 2010; Hasan *et al.*, 2011) and has a strong correlation with final grain weight indicating that grain length may be important in determining final grain weight. In support of this suggestion, a study by Brinton *et al.* (2017) identified a stable QTL on chromosome 5A in a double haploid winter wheat cross that increased grain length by 4.04% on average and was closely associated with TGW in addition to pericarp cell length. The latter association lends support to the

hypothesis that the development of the pericarp is crucial to determining final grain size. However, a study by Gegas *et al.* (2010) in multiple mapping double haploid populations and a number of ancestral species, showed that maximum grain width was the grain dimension that had the closest association with final grain weight. Therefore, it appears that there are genotypic differences in the establishment of the wheat grain dimensions and their association with final grain weight.

The establishment of grain dimensions under high p.a. temperatures have previously been investigated and show similar variation between varieties and genotypes. Sun *et al.* (2017) observed in 11 spring wheat genotypes, that wheat grain length was the most negatively affected grain dimension at maturity following exposure to a 10-day high p.a. temperature treatment (34/16°C) applied from anthesis whilst Grass and Burris (1995) found, in two Moroccan spring wheat varieties, *(cvs. Marzak* and *Oum-rabia),* that under high p.a. temperatures (36/29°C) applied from 2-10daa until maturity, grain width was reduced by 33% whilst grain length was not affected. Whilst these studies demonstrate variation in the response of grain dimensions to high p.a. temperatures, they also highlight the issue of variation between experiments in when high p.a. temperatures are applied and the response of the wheat grain. In addition, to our knowledge there have been no studies that have measured grain dimensions subjected to high p.a. temperatures during the course of grain development. Based on the knowledge that high p.a. temperatures accelerate grain development (Nicolas *et al.* 1984; Wan *et al.* 2008) one may expect exposure to high p.a. temperatures will result in grain dimensions reaching their maximum value and stabilising earlier than in control grains. Therefore, the effect of high p.a. temperatures on the stabilisation and maximum values of grain dimensions during development will be investigated.

Further investigation into the effect of high p.a. temperatures on the morphological development of the wheat grain will provide greater insight into the relationship between grain size and dimensions. Whilst potential constraint of wheat grain expansion by the maternal grain layers has been suggested, no studies have investigated the effect of high p.a. temperature on this potential interaction and whether high p.a. temperatures result in a premature maturation of the pericarp that constrains endosperm expansion and reduces yield.

The series of experiments contained within this chapter therefore aimed to test the hypotheses: that exposure to high p.a. temperature reduces mature grain weight, dimensions and/or number resulting in decreased yield in *cv. Cadenza,* that the cultivar is susceptible to the negative effects of high p.a. temperature on grain size throughout the grain filling period and that grain dimensions reach their maximum value and stabilise earlier under high p.a. temperatures. From testing these hypothesis it is hoped that the following experimental aims will be reached:

- determining the minimum length of and severity of a high p.a. temperature treatment required to induce a heat stress response in the form of reduced grain size or number
- determining when during p.a. development, high temperatures have the most negative impact on final wheat grain yields
- analysing how high p.a. temperatures affect grain growth dynamics during development and maturity.

3.2 Results

3.2.1 Minimum high p.a. temperature treatment required to induce a reduction in grain weight and number

3.2.1.1 Initial attempt at inducing a heat-stress response

In experiment 1 aimed at testing the hypothesis that high p.a. temperature reduces mature grain weight number, dimensions and/or grain size, a high p.a. temperature treatment of 32°C was applied for 4 hours per day over a 2-day period at different developmental stages (4,10,16 and 22daa) before grains were collected at maturity. No significant difference between average mature grain weights from the primary tillers of high p.a. temperature treated and control plants was observed (ANOVA, df =4, f=0.07, p=>0.05) Average mature grain weights collected from the secondary and tertiary tillers of high p.a. temperature treated and control plants were also measured and showed no significant difference between treatments (ANOVA, df =4, f=0.92, p=>0.05). Average grain numbers of any of the three tillers when taken individually and compared against one another, e.g. 2nd tiller vs 3rd tiller, were not significantly affected by treatment (ANOVA, df =4, f=3.37, p=>0.05) and when combined the average grain weights of the whole plant and grain number (all three tillers combined) were also not affected (ANOVA, df =4, f=1.04, p=>0.05). Therefore, this result indicates that the high p.a. temperature treatment applied during this initial experiment was insufficient to cause a reduction in mature grain weight or number and did not support the hypothesis of the experiment.

3.2.1.2 Second attempt at inducing a heat stress response

In experiment 2, which reattempted to induce a heat stress response in yield and re-test the initial hypothesis, a higher temperature (35°C) was used for an increased number of hours per day (10hrs). In addition, different durations of treatment were applied ((control (no treatment), 2, 4, 6, 8, 10, 12 days of treatment) from 6daa. The duration of the high p.a. temperature treatment had a significant effect on mature average grain weight in the primary tillers (ANOVA, df =6, f=3.54, p=<0.05 (Fig.3.1). Average mature grain weight in the primary tillers was reduced in those plants that were subjected to 4, 6, 8, 10 and 12 days of treatment based on comparison of the least significant differences (LSDs) of the means against the overall mean for each treatment. However, there was no significant difference between the control treatment group and those subjected to 2 days of high p.a. temperature treatment according to the LSDs of the means. Average grain number from the primary tillers was unaffected by high p.a. temperature (ANOVA, df =6, f=0.53, p=>0.05) (Table 3.1) (Fig.3.1). Average mature grain weights from the secondary[1.] and

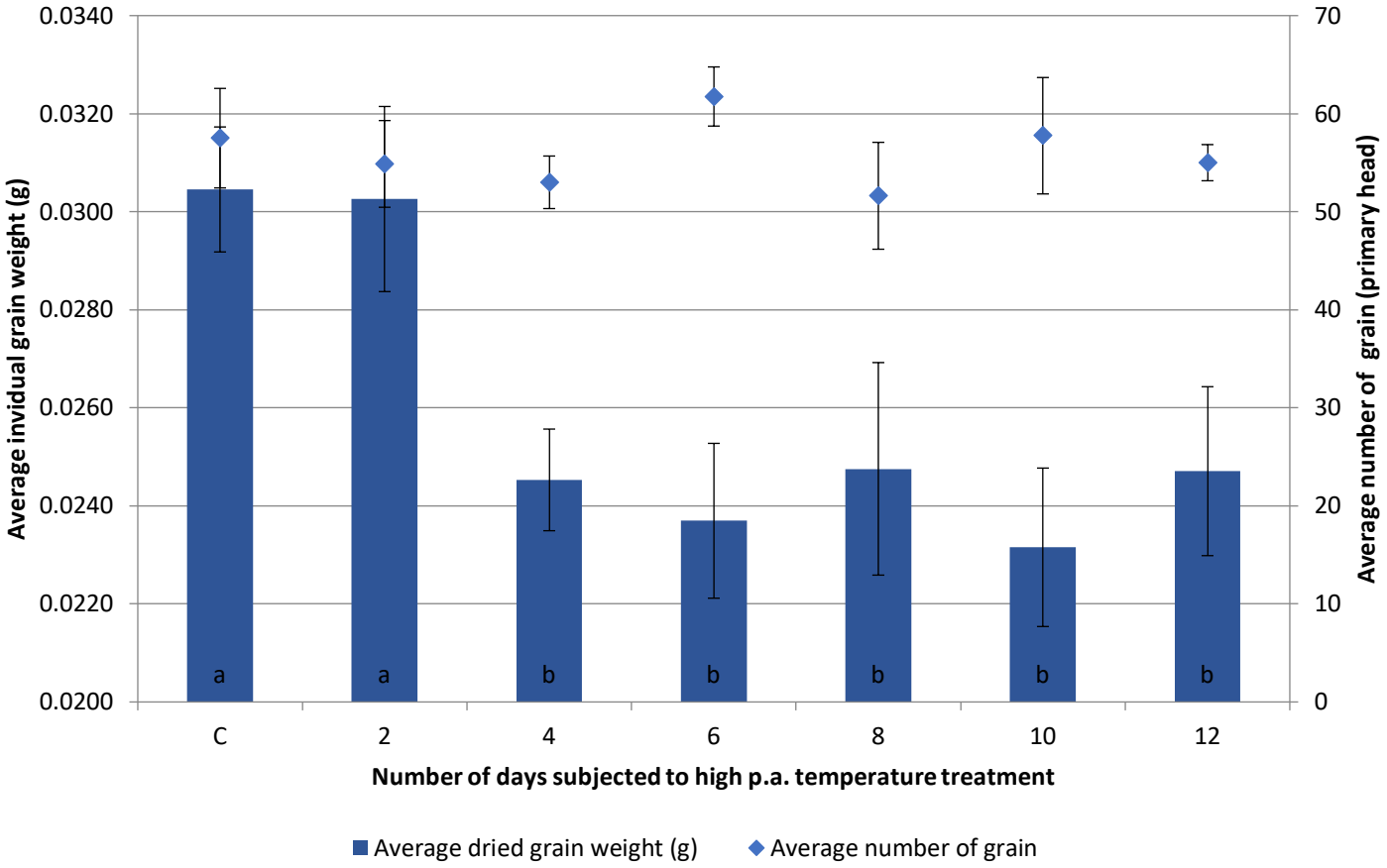


Figure 3.1. Average mature dried grain weights and grain numbers from primary tillers exposed to different high p.a. temperature treatment (35°C) durations from 6daa. Error bars represent the standard error of the means of 8 biological replicates. The 'C' label on the x-axis represents the control/no-treatment group. Labelling with different letters indicates significant differences between groups according to the LSDs between the means at $p<0.05$. Treatments with the same letter had no significant difference between them.

tertiary[2.] tillers were also significantly affected by treatment duration (ANOVA, df=6, f=3.57[1.],3.03[2.] p=<0.05). However, in secondary and tertiary tillers there was no significant difference in average mature grain weight between the control treatment and those plants subjected to a 2, 6 or 12day treatment duration, based on comparisons of the LSDs of the means against the control mean. However, there was a significant difference between the control treatment and those plants subjected to 4, 8 and 10 days of treatment (Table 3.1).

In the secondary [1.] and tertiary [2.] tillers average grain number did not significantly differ between treatments (ANOVA, df =6, f=0.84[1.],1.97[2.], p=>0.05) (Table 3.1). When the average mature grain weight and grain number from the primary, secondary and tertiary tillers were combined for each plant and compared, there was no significant difference in average grain number between treatments (ANOVA, df =6, f=1.14, p=>0.05). However, there was a significant effect of treatment duration on the average mature grain weight (ANOVA, df=6, f=6.89, p=<0.001) with treatments of 4, 6, 8, and 10 days all producing lower average mature grain weights across the three tillers. There was no significant difference between the control treatment and those plants treated for 12days according to the LSDs of the means. In all comparisons the covariate effect of the number of tillers that each plant had when the high p.a. temperature was applied did not significantly affect grain weight response (p=<0.05). Therefore the results of this experiment support the hypothesis that mature grain weight is reduced in *cv. Cadenza* under high p.a. temperatures although grain number is unaffected.

Table 3.1 Summary of average mature grain weights and numbers collected from primary, secondary and tertiary tillers of plants subjected to different durations of a high p.a. temperature (35°C) from 6daa in experiment 2. Averages of 8 biological replicates.

Average mature grain weights (g) and average grain numbers per tiller (mean ± s.e.m)								
Treatment (days of treatment)	Average mature grain weight – primary tiller*	Average grain number - primary tiller	Average mature grain weight – secondary tiller *	Average grain number – secondary tiller	Average mature grain weight – tertiary tiller *	Average grain number – tertiary tiller	Average mature grain weight – 1,2,3 tiller *	Average grain number – 1,2,3 tiller
Control– 0	0.0304±(0.0012)	58±(5)	0.0239±(0.0009)	62±(3)	0.0254±(0.0017)	61±(4)	0.0196±(0.0007)	59±(3)
2-days	0.0302±(0.0018)	55±(4)	0.0227±(0.0009)	58±(6)	0.0220±(0.0015)	55±(3)	0.0185±(0.0006)	56±(4)
4-days	**0.0245* ᵛ±(0.001)**	53±(3)	**0.0185* ᵛ±(0.0012)**	51±(3)	**0.0176* ᵛ±(0.0024)**	46±(6)	**0.0154* ᵛ± (0.0007)**	51±(3)
6-days	**0.0236* ᵛ±(0.0015)**	62±(3)	0.0209±(0.0024)	51±(3)	0.0243±(0.0019)	48±(3)	**0.0170* ᵛ±(0.0010)**	53±(2)
8-days	**0.0247* ᵛ±(0.0021)**	52±(5)	**0.0198* ᵛ±(0.0009)**	53±(5)	**0.0205* ᵛ±(0.0013)**	50±(2)	**0.0158* ᵛ±(0.0006)**	50±(4)
10-days	**0.0231* ᵛ±(0.0016)**	58±(6)	**0.0189* ᵛ±(0.0014)**	44±(10)	**0.0200* ᵛ±(0.0027)**	52±(6)	**0.0138* ᵛ±(0.0009)**	51±(4)
12-days	**0.0247* ᵛ±(0.0016)**	55±(2)	0.0245±(0.0009)	54±(4)	0.0252±(0.0012)	48±(3)	0.0183±(0.0011)	53±(2)

***** = effect of treatment duration p value<0.05,

***ᵛ** =significantly lower than the control based on comparisons of LSDs of means

3.2.2 Effect of timing of the high p.a. temperature treatment application

Following the observation in experiment 2 that 4 days of high p.a. temperature was sufficient to reduce average mature grain weight when applied at 6daa, experiment 4 sought to test the hypothesis that grain weight in *cv. Cadenza* was susceptible to the negative effects of high p.a. temperature throughout the grain filling period. Therefore a 4-day treatment of high p.a. temperature was applied at either 4, 6, 8, 11, 15, 18 and 22daa and the average mature grain weights of the main tillers measured. Overall, high p.a. temperature treatment significantly reduced average mature grain weight (ANOVA, df =6, f=9.41, p=<0.05).

However, there was no significant interaction between daa and high p.a. temperature treatment (ANOVA, df =6, f=0.58, p=>0.05) indicating that there was no difference in the negative effect of the high p.a. temperature on grain weight based on when it was applied. When comparing the LSDs of the means, there were significant differences between control and high p.a. temperature treated grains subjected to a 4-day high p.a. temperature treatment at 6, 8, 11, 18 and 22daa with mature grain weight being significantly lower in heat treated plants (Fig.3.2) therefore supporting the hypothesis of the experiment. However, there was no significant reduction in grain weight between treatments applied at 4daa according to comparisons of the LSDs of the means.

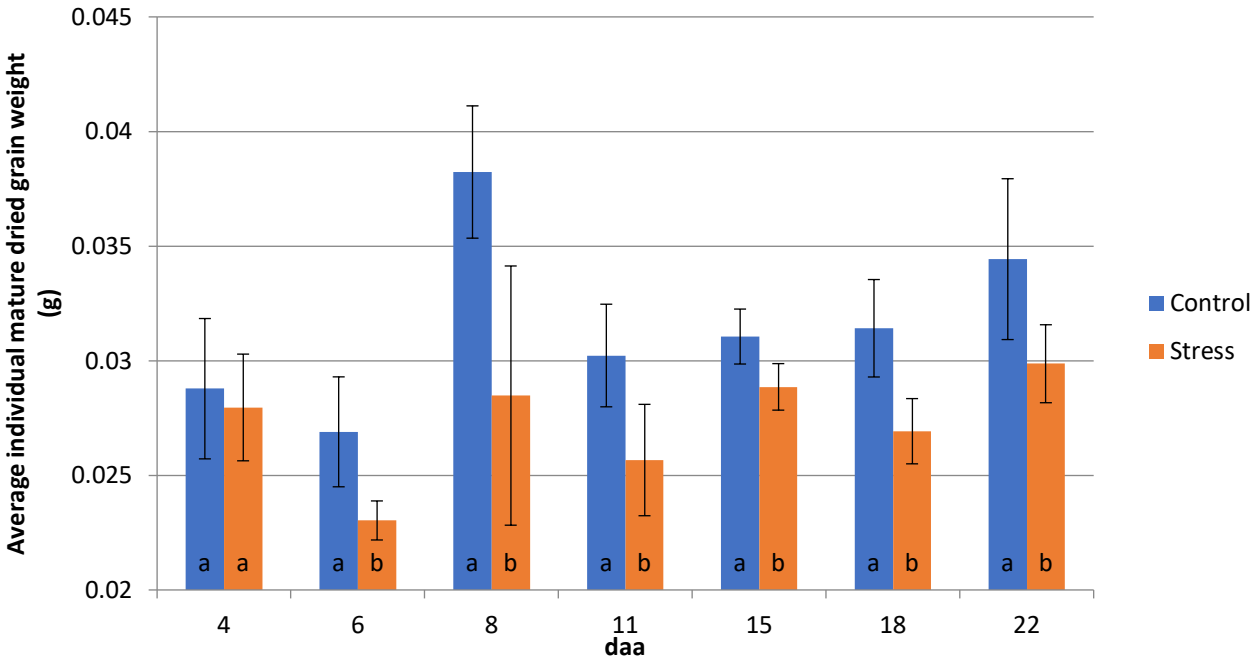
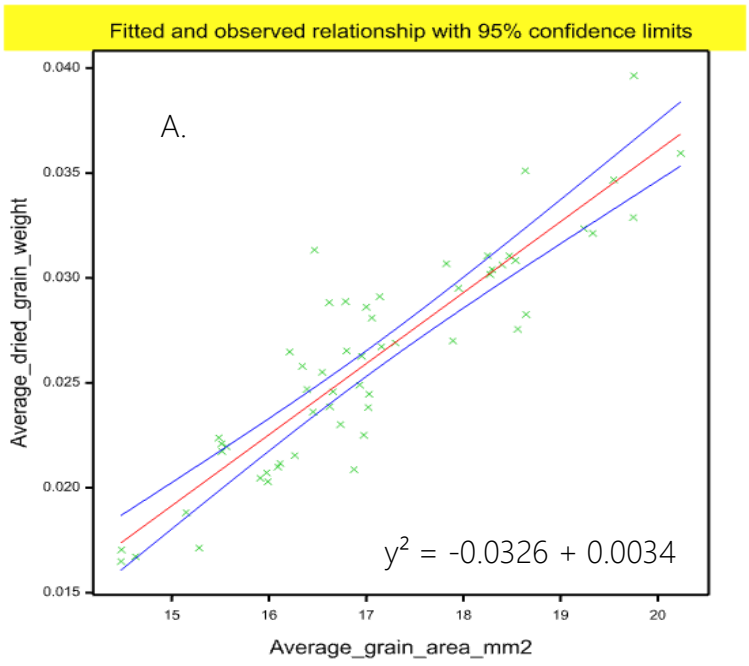


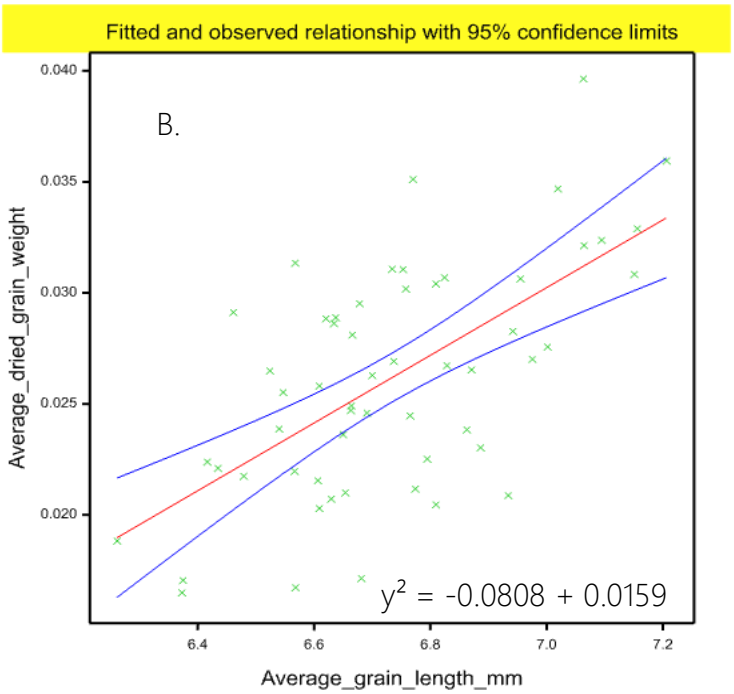
Figure 3.2. Average mature dried grain weights from primary tillers exposed to a 4-day high p.a. temperature treatment (35°C) at different developmental stages p.a. in experiment 4. Error bars denote the standard error of the mean of 7 biological replicates. Labelling with different letters indicates significant differences between control and high p.a. temperature treatments applied at the same daa according to the LSDs between the means at $p<0.05$. Treatments with the same letter had no significant difference between them.

3.2.3 Effect of high p.a. temperature on mature grain dimensions

In experiment 1, mature grain dimensions (length[1], width[2], area[3]) from the primary, secondary and tertiary tillers were measured. There was no significant effect of treatment on any of the measured dimensions in the mature grain from any of the tillers (ANOVA, df =4, f=0.04[1],1.09[2], 0.52[3] p=>0.05). In experiment 2, the same dimensions of mature grain from the primary tillers were measured. Treatment duration had a significant effect on average grain area (mm^2) (ANOVA: df= 6, F=6.57, p=<0.001) with all average grain areas

from high p.a. temperature treated plants being significantly lower than the control treatment, therefore supporting the hypothesis that exposure to high p.a. temperature reduces mature grain dimensions in *cv. Cadenza*. There was no significant difference between the control and the 2-day high p.a. temperature treatment (Table 3.2) according to the LSDs of the means. A linear regression analysis showed that final mature average grain weight and grain area were highly correlated (linear regression analysis: df= 1, 54, F=233.67, p=<0.001) (Fig.3.3A). Average mature grain length (mm) was also significantly affected by treatment duration (ANOVA: df= 6, 36 F=5.25, p=<0.001) with all treatment durations of 4-days or longer producing reduced average mature grain lengths. Mature grain length and grain weight were highly correlated (linear regression analysis: df= 1, 54 F=34.58, p=<0.001) (Fig.3.3B). Average mature grain width (mm) followed the same trend and was significantly affected only by treatment duration of 4-days or longer (ANOVA: df= 6, 36 F=3.12, p=<0.001). In addition, mature grain weight and grain width were highly correlated (linear regression analysis: df= 1, 54 F=355.68, p=<0.001) (Fig.3.3C).





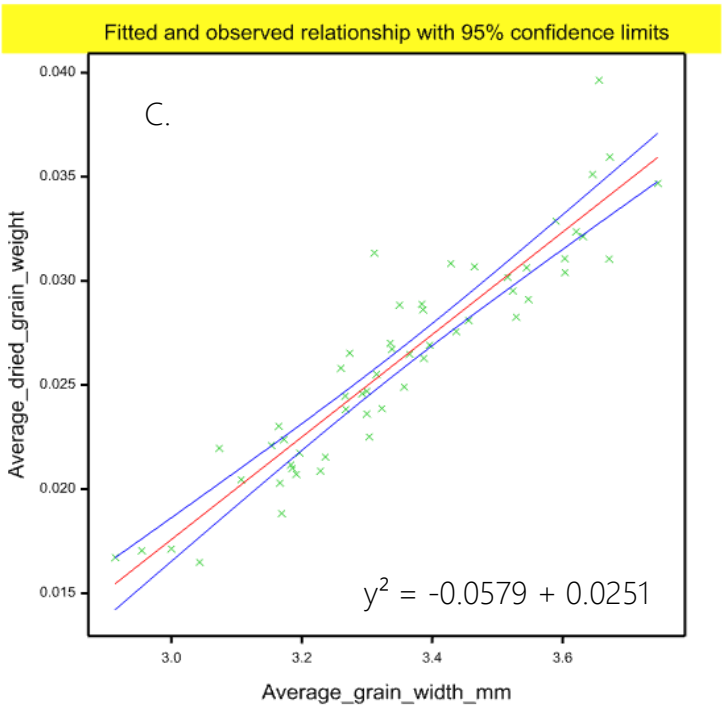


Figure 3.3 Regression analysis with lines of best fit of average mature grain area (mm^2) (A.), average mature grain length (mm) (B.) and average mature grain width (mm) (C.) against average mature grain weight from the primary tiller in experiment 2 (n = 57)

Average mature grain volume was also compared between treatments. However, there was no significant effect of treatment duration (p=>0.05) on average mature grain volume (ANOVA, df =4, f=1..52' p=>0.05. In all comparisons the covariate effect of the number of tillers each plant had when the high p.a. temperature was applied had no effect (p=<0.05).

Table 3.2 Summary of average mature grain area, length and width collected from primary tillers of plants subjected to different durations of a high p.a. temperature (35°C) from 6daa in experiment 2. Averages of 8 biological replicates.

Average mature grain dimensions and grain numbers from the primary tiller (mm) (mean ± s.e.m)				
Treatment (days of stress)	Average number of grain	Average mature grain area (mm^2)*	Average mature grain length (mm)*	Average mature grain width (mm)*
Control – 0	58±(5)	18.78±(0.33)	6.984±(0.05)	3.548±(0.05)
2-days	55±(4)	18.19±(0.51)	6.867±(0.08)	3.479±(0.06)
4-days	53±(3)	**16.95* ^{V}±(0.27)**	**6.698* ^{V}±(0.05)**	**3.328* ^{V}±(0.03)**
6-days	62±(3)	**16.20* ^{V}±(0.41)**	**6.602* ^{V}±(0.07)**	**3.250* ^{V}±(0.06)**
8-days	52±(5)	**16.34* ^{V}±(0.40)**	**6.620* ^{V}±(0.04)**	**3.281* ^{V}±(0.08)**
10-days	58±(6)	**16.15* ^{V}±(0.34)**	**6.698* ^{V}±(0.05)**	**3.223* ^{V}±(0.06)**
12-days	55±(2)	**16.91* ^{V}±(0.34)**	**6.719* ^{V}±(0.07)**	**3.334* ^{V}±(0.05)**

***** = effect of treatment duration p value>0.05,

*** ^{V}** =significantly lower than the control based on comparisons of LSDs of means.

3.2.4 Grain dimensions during development under high p.a. temperatures.

Grain dimensions (length, width, height) (Fig.3.4) during development under high p.a. temperatures were also measured in experiment 4 alongside the experiment that aimed to

test the effect of applying a 4-day high p.a. temperature treatment at different stages p.a. on mature grain weight. This experiment sought to test the hypothesis that exposure to high p.a. temperatures would result in the grain dimensions reaching their maximum value and stabilisation earlier than in control grains. Grain dimensions were measured at 6, 8, 10, 14, 18, 22, 26 daa following a 4-day high p.a. temperature treatment applied at 6daa to determine which dimension attained its maximum value first and how the development of these dimensions compared between control and high p.a. temperature treated samples.

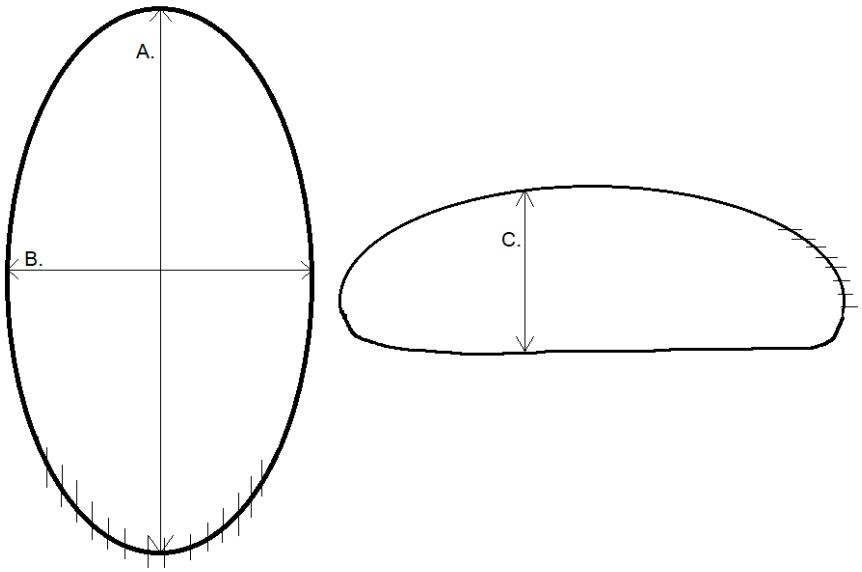


Figure 3.4. Diagram indicating where dimension measurements (A. Length, B. Width, C. Height) were measured on wheat grain using electronic callipers in Experiment 4.

Overall, there was a significant reductive effect of treatment on average grain length (ANOVA, df =1, f=97.02, p=<0.002) (Tab.3.3). However, there was no significant interaction between treatment and daa (ANOVA, df =6, f=0.81, p=>0.05) and the only significant differences in grain length occurred between treatments at 18 and 26daa according to the LSDs of the means (Table 3.3) (Fig. 3.5). Maximum grain length was measured at 22daa and at 18daa in heat treated and control grains respectively. However, according to

comparisons between the LSDs of the means, grain lengths recorded at 10 and 14daa in high p.a. temperature and control treated samples respectively were not significantly

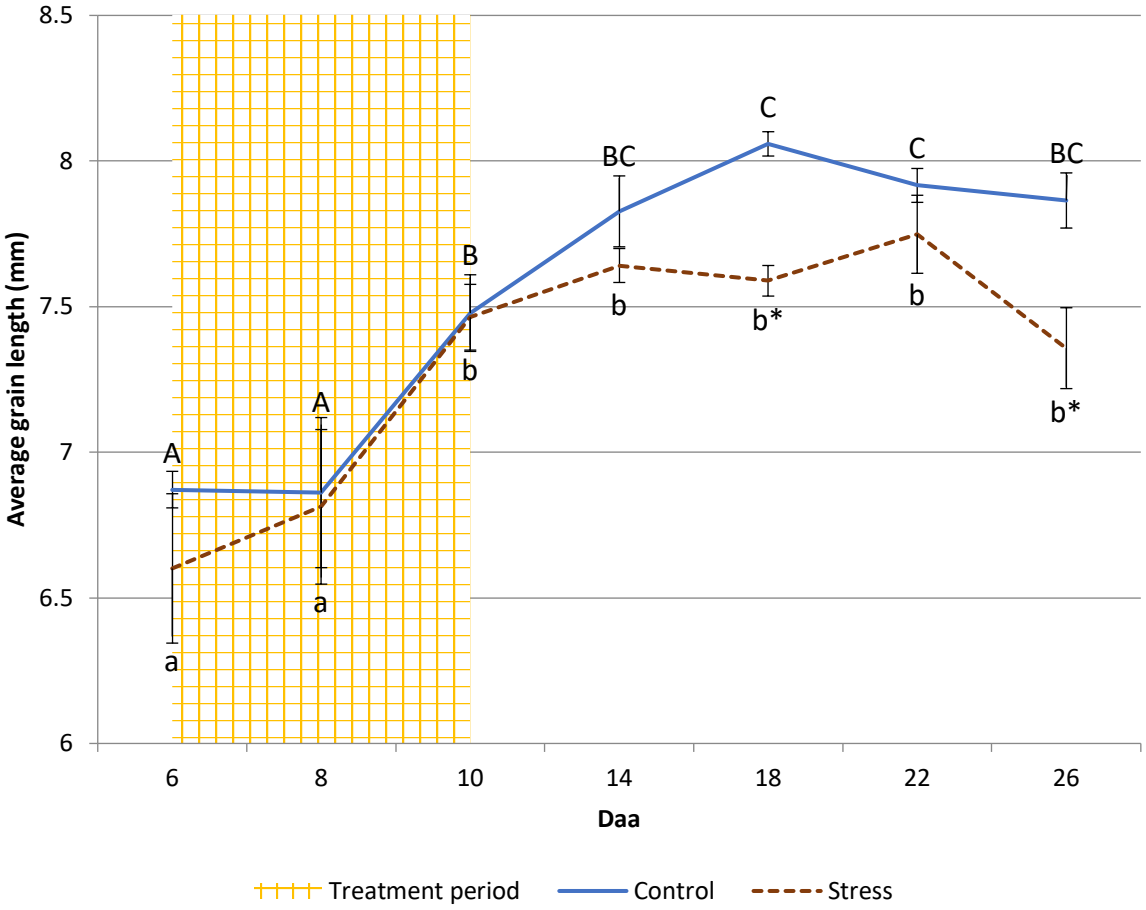


Figure 3.5 Average length of grain (n=16) from primary tillers exposed to a 4-day treatment (35°C) at 6daa and measured at different stages p.a. Error bars denote the standard error of the mean of 4 biological replicates. Labelling with different letters (uppercase=control, lowercase=high p.a. temperature treatment) indicates significant differences in grain length between daa stages according to the LSDs between the means at $p<0.05$. Daa stages with the same letter had no significant difference between them. Letters with an * indicate significant differences between the high p.a temperature treatment and control at the same daa stage according to the LSDs between the means at $p<0.05$.

different to the grain lengths measured at 18daa and 22daa. Therefore this suggests that the maximum grain length is attained at around 10daa in high p.a. temperature treated grains and at 14daa in control grains.

Average grain width was significantly affected by treatment overall (ANOVA, df =1, f=433.01, p=<0.001) and there was a significant interaction between treatment and developmental stage (ANOVA: df= 6 F=4.28, p=<0.001) showing that the differences between grain sizes at different stages p.a. could be attributed to the different treatments (Fig.3.6) (Table 3.3). Average grain widths at 14, 22, 26 daa were significantly lower in the

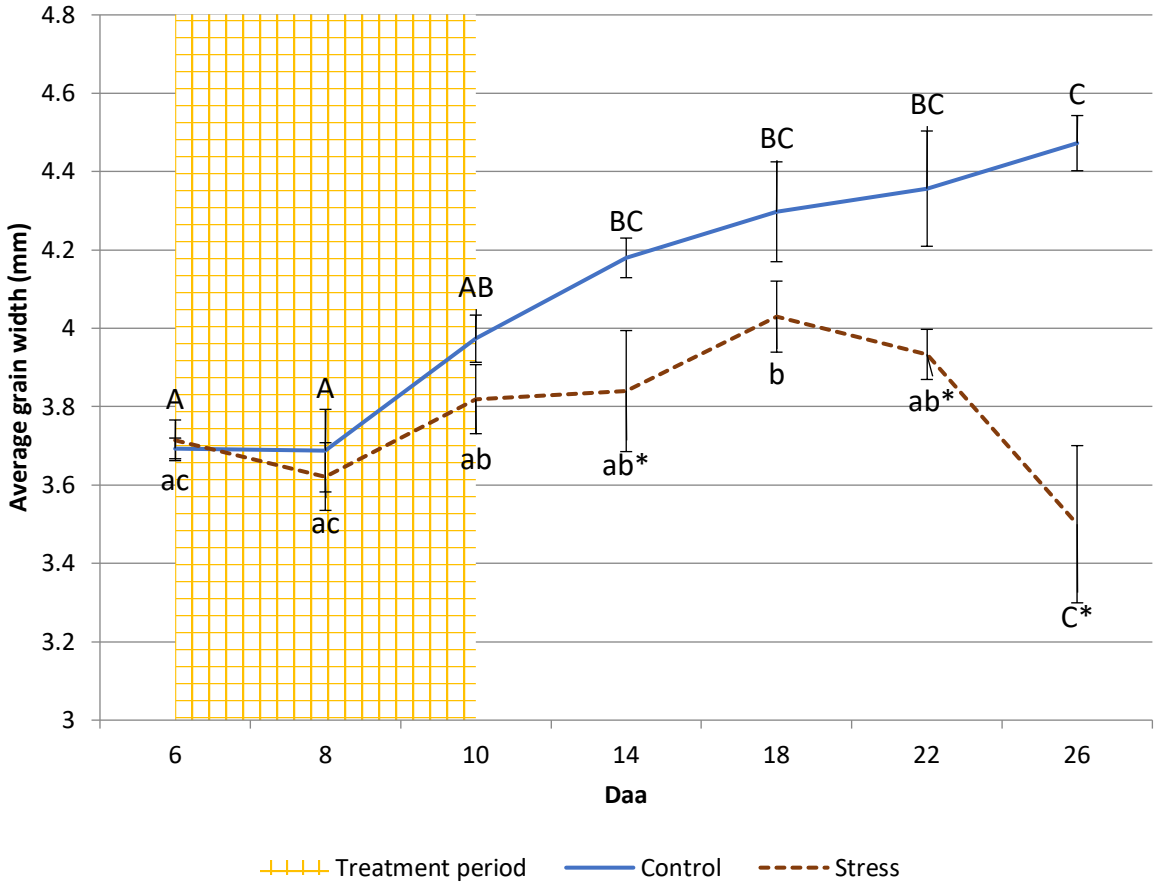


Figure 3.6 Average width of grain (n=16) from primary tillers exposed to a 4-day treatment (35°C) at 6daa and measured at different stages p.a. Error bars denote the standard error of the mean of 4 biological replicates. Labelling with different letters (uppercase=control, lowercase=high p.a. temperature treatment) indicates significant differences in grain width between daa stages according to the LSDs between the means at p<0.05. Daa stages with the same letter had no significant difference between them. Letters with an * indicate significant differences between the high p.a temperature treatment and control at the same daa stage according to the LSDs between the means at p<0.05.

high p.a. temperature treated samples according to the LSDs of the means. In the high p.a. temperature treated samples grain width attained its maximum value at 18daa whereas in the control samples grain width appeared to be increasing up to and potentially beyond the last developmental stage measured (26daa).

Average grain height was also significantly reduced by treatment (ANOVA, df =1, f=46.46, p=<0.001) p=<0.05), however, in the same manner as length, there was no significant interaction between treatment and daa stage, despite grain heights being significantly lower at 14, 1822 and 26daa when the LSDs of the means were compared (ANOVA, df =6, f=1.71, p=>0.05). In high p.a. temperature treated plants, the maximum average grain height was attained at 18daa. However, there was not a significant difference between the average grain heights in high p.a. temperature treated grains sampled at 10,14,18 or 22daa according the LSDs of the means, indicating that the maximum grain height in heat treated samples stabilised at around 10daa. In the control samples maximum average gran height was attained at 26daa. However, similarly to the high p.a. temperature treated samples, there was not a significant difference between the average grain heights of control grains sampled at 18, 22 and 26daa based on the LSDs of the means. Therefore, it appears that grain height reaches it maximum in control samples around 18daa and stabilises although sampling at a further stage would have confirmed whether average grain height begins to decline beyond 26daa in control samples (Fig. 3.7) (Table 3.3). Therefore these results support the experimental hypothesis that high p.a. temperatures result in the maximum values of *cv. Cadenza* grain dimensions being attained and stabilising earlier than in control grains.

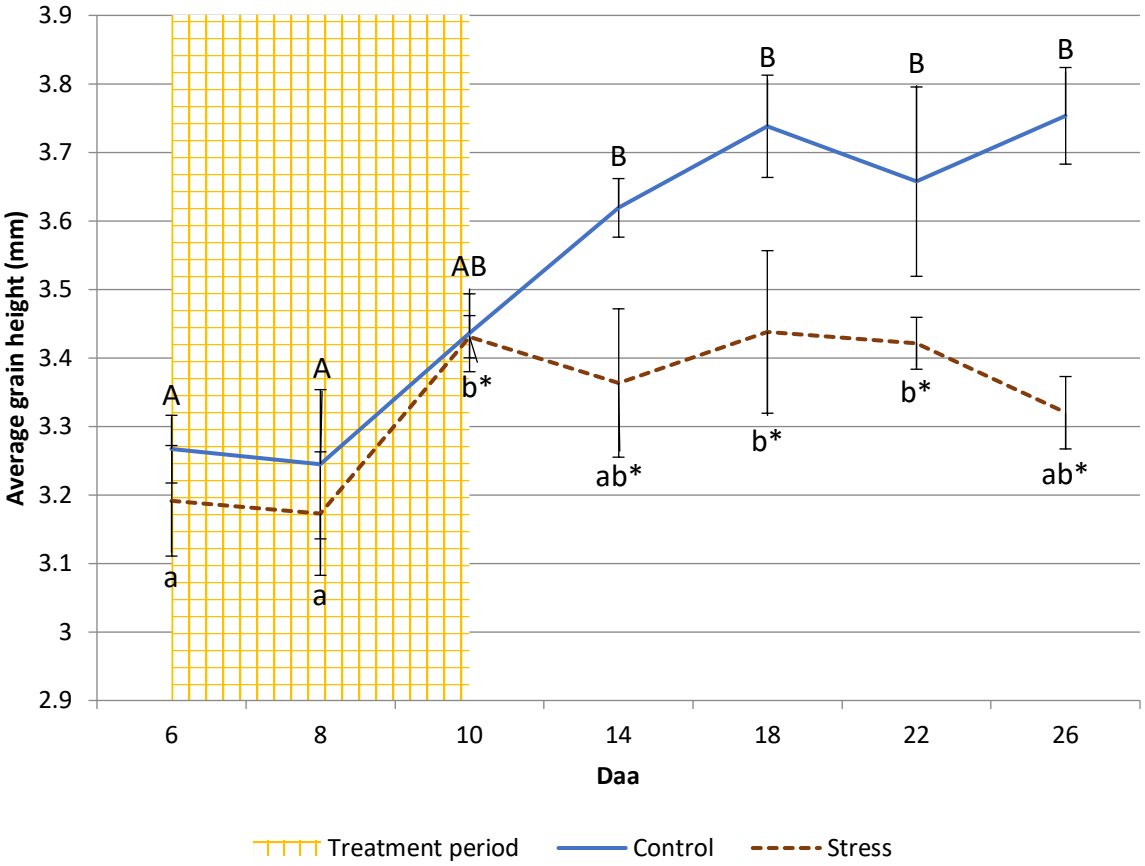


Figure 3.7 Average height of grain (n=16) from primary tillers exposed to a 4-day treatment (35°C) at 6daa and measured at different stages p.a. Error bars denote the standard error of the mean of 4 biological replicates. Labelling with different letters (uppercase=control, lowercase=high p.a. temperature treatment) indicates significant differences in grain height between daa stages according to the LSDs between the means at $p<0.05$. Daa stages with the same letter had no significant difference between them. Letters with an * indicate significant differences between the high p.a temperature treatment and control at the same daa stage according to the LSDs between the means at $p<0.05$.

Table 3.3 Summary of average grain dimensions (length, width, height) (mm) from primary tillers of plants subjected to 4-day durations of a high p.a. temperature (35°C) from 6daa and measured at different stages p.a. in experiment 4. Averages of 4 biological replicates. C = Control treated samples, T= High p.a. temperature treated samples

Average dimensions of grain from primary tillers (mm) (mean ± s.e.m)						
Developmental stage (daa)	Average grain length (mm)		Average grain width (mm) *		Average grain height (mm)	
Treatment	C	T	C	T	C	T
6daa	6.879±(0.062)	6.624±(0.256)	3.688±(0.026)	3.694±(0.051)	3.267±(0.049)	3.211±(0.080)
8daa	6.861±(0.257)	6.813±(0.265)	3.688±(0.105)	3.622±(0.086)	3.245±(0.108)	3.173±(0.090)
10daa	7.478±(0.131)	7.464±(0.112)	3.974±(0.060)	3.819±(0.088)	3.437±(0.056)	3.431±(0.030)
14daa	7.827±(0.121)	7.641±(0.058)	4.180±(0.050)	**3.840* ^{v}±(0.154)**	3.619±(0.042)	**3.363* ^{v}±(0.108)**
18daa	8.059±(0.041)	**7.589* ^{v}±(0.052)**	4.298±(0.127)	4.030±(0.090)	3.738±(0.074)	**3.438* ^{v}±(0.118)**
22daa	7.916±(0.058)	7.748±(0.133)	4.357±(0.147)	**3.933* ^{v}±(0.063)**	3.657±(0.138)	**3.421* ^{v}±(0.037)**
26daa	7.865±(0.094)	**7.357* ^{v}±(0.133)**	4.473±(0.070)	**3.500* ^{v}±(0.200)**	3.753±(0.070)	**3.320* ^{v}±(0.052)**
P.a. stage dimension measurements stabilised	14daa	10daa	26daa	18daa	18daa	10daa

***** = effect of treatment at different developmental stage (interaction) p value<0.05,

*** ^{v}** =significantly lower than the control based on comparisons of LSDs of means.

3.3 Discussion

3.3.1 High p.a. temperature stress response in Cadenza

Previous studies that have examined the effect of high p.a. temperatures on wheat yield, either in the field or in controlled environments have used a variety of high p.a. temperature treatment durations ranging from a single day (Talukder *et al.*, 2014) to a treatment lasting from anthesis until maturity (Altenbach *et al.*, 2003). In addition, treatments have been applied at different developmental stages p.a., ranging from ~5daa (Al-Khatib and Paulsen 1984) to 15daa (Stone *et al.*, 1995). Therefore, when conducting high p.a. temperature treatment experiments there are three main experimental factors that can be modified; the duration of the treatment, the temperature used and the developmental stage at which it is applied. Experiment 1 sought to determine the high p.a. temperature required to induce a reduction in the average mature grain weight in addition to establishing whether there was a difference in effect depending on when the treatment was applied.

A high p.a. temperature of 32°C applied for 4-hours per day over a 2-day period at different stages p.a. was not sufficient to induce a reduction in mature grain weight. This result contrasts similar studies where mature grain weight has been shown to be negatively affected by the application of a 30-32°C p.a. temperature treatment in a range of cultivars (Sofield *et al.*, 1977), although the treatment in that study was applied from anthesis until maturity. In addition, the high p.a. temperature treatment applied in the initial experiment did not significantly reduce final grain number in the primary, secondary or tertiary tillers supporting a previous study by Dias and Lidon (2009) who had previously shown grain number to be unaffected by high p.a.

temperatures in two bread *(cvs. Sever* and *Golia)* and durum *(cvs. TE 9306* and *Acalou)* varieties.

The lack of any significant effect implies that the treatment used in experiment 1 was not severe enough to negatively affect the mature grain weight or grain number. A single day treatment of high p.a. temperature (35°C) similar in duration to the treatment used in experiment 1 (10h) has been shown to be sufficient to reduce final grain weight and number when applied between 7-10daa for 3 hours in four Australian commercial bread wheat genotypes (Talukder *et al.*, 2014). However, there is a high amount of genotypic variation in the ability of *T.aestivum* varieties to withstand high p.a. temperatures, with some temperatures proving detrimental to yields of some varieties but not others (Dias and Lidon, 2009; Farooq *et al.*, 2011).

Therefore, a second attempt to induce a p.a. heat stress response was made in experiment 2 using a higher temperature (35°C) and a longer treatment exposure of 10-hours per day. In addition, the treatment was applied for different durations (2, 4, 6, 10, 12 days) from 6daa. 6daa was used as the start point for treatments in this second attempt as logistical and experimental constraints meant that it was only feasible to vary one treatment aspect. Therefore, the duration of the treatment rather than the stage at which it was applied was changed. The early grain filling phase from around 6daa-10daa has been used as a starting period for treatments in previous studies investigating the effect of high p.a. temperature and has been shown to be a particularly susceptible period to high p.a. temperature resulting in reductions in mature grain weight (Tashiro and Wardlaw; 1990 Tashiro and Wardlaw; 1990b; Plaut *et al.*, 2004; Spiertz *et al.*, 2006; Talukder *et al.*, 2014).

In experiment 2, a high p.a. temperature treatment (35°C/ 10-hours per day) of 4-days or greater applied from 6daa reduced average mature grain weight across primary, secondary and tertiary tillers, although there was no reduction in average grain number per tiller. Therefore, overall yield per plant was reduced in those plants that had experienced a treatment duration of 4-days or longer. In a similar manner to experiment 1, it was found that a 2-day treatment, albeit with a longer treatment duration per day, was insufficient to reduce mature grain weight, contrasting the results of Talukder *et al.* (2014). Spiertz *et al.* (2006) had also observed that in wheat *(cv. Lavett)*, a 3-day heat treatment of 38°C reduced grain number when applied at 10 and 14daa. The results of Talukder *et al.* (2014) and Spiertz *et al.* (2006) in combination with the observation from this study demonstrate the genotypic variation present in wheat's susceptibility to high p.a. temperatures with regards to the number of grains produced per head and grain weight.

From the second attempt to induce a high p.a. temperature heat stress response in yield, it is apparent that there is not a proportional effect between the duration of a high p.a. temperature treatment and the reduction in mature grain weight. Observed reductions in mature grain weight following 4-days of treatment were very similar to those observed in plants that experienced 6, 8, 10 and 12 days of high p.a. temperature treatment suggesting that there is a limit in the reduction of grain weight under high p.a. temperature. This result partially supports findings by Stone *et al.* (1995) who found in the bread wheat cultivar, *cv. Oxley*, that, following a 5-day heat shock event (40/16°C) applied from 15daa, there was no progressive decline in mature individual kernel mass when plants were removed from the heat shock and placed in an environment with one of three moderately high temperatures (21/16°C, 27/22°C, 30/25°C). Stone *et al.* (1995) therefore suggested that when brief periods of

high p.a. temperature occur, yield potential is decreased by a more or less a fixed extent. The results from experiment 2 lend support to their observation although the methodologies between the two studies differ slightly in that in the experiment in this investigation, the treatment temperature remained the same and just duration of the treatment changed. The results of our experiment also support the findings of Nuttall *et al.* (2018) who when investigating the cumulative effects of multiple high p.a. temperature treatments on grain weight during the grain filling phase, found after 4-days of cumulative exposure to a high p.a. temperature treatment of 35/15°C for 6 hours per day that individual grain weight in *cv. Yipti* was reduced by 15% and that this reduction plateaued from this point onward with further durations of treatment. However, the high p.a. temperature treatment applied by Nuttall *et al.* (2018) differs slightly to that used in experiment 2 as it was applied from anthesis and following each day's exposure to a high p.a. temperature treatment, they returned their plants to the glasshouse to rest for a day and be watered before being returned to the high p.a. temperature treatment cabinet for subsequent high p.a. temperature treatments. Despite this variation in treatments, the similar reductions in individual grain weight observed between both our study and the study of Nuttall *et al.* (2018) following 4-days of high p.a. temperature treatment, either applied consecutively or days apart suggest that this duration of exposure may be a threshold beyond which sensitivity to high p.a. temperature declines.

In the context of global wheat production, temperatures such as these described, i.e. 4 consecutive days or more of approximately 35°C around the grain-filling period, are likely to be more frequently experienced in continental areas with temperate conditions e.g. India, Australia, Russia, China (Gourdji *et al.* 2013). A crop modelling study by Asseng *et al.* (2011) attributed a predicted 30% reduction in wheat yield

between growing seasons in 1971 and 2002 in Cunderdin, WA, Australia, to the occurrence of 8 days of maximum temperatures >34°C in the period of flowering to maturity. The results of their modelling indicated that this scenario was likely the result of increases in average global temperatures, also predicted by the IPCC (2014). They also predicted that the average frequency of days with temperatures >34°C during grain filling is expected to increase by 1 day for each 1.6°C increase in average maximum temperature and that this scenario is applicable in a number of large wheat producing areas around the world including India, China and Russia. Within India and other areas of South Asia, significant yield losses are already suffered each year as a result of temperatures exceeding 35°C for extended periods during grain filling stages in mega environments such as the North western and eastern gangetic plain ,peninsular India and the plains of Bangladesh and Nepal (Mondal *et al.,* 2015). The occurrence of these high p.a. temperature events that exceed 35°C is predicted to increase (IPCC 2014) and therefore understanding the high p.a. temperature stress response of wheat and the mechanisms behind it are important to develop new heat tolerant wheat cultivars.

3.3.2 Effect of high p.a. temperature exposure at different stages of grain development

Experiment 4 aimed to test the effect on mature grain weight of the established 4-day high (35°C) temperature treatment found to induce a heat stress response in the form of reduced mature grain weight, when applied at different stages p.a. during grain filing. There were significant differences between average mature grain weights in control and treated plants when the high p.a. temperature treatment was applied for 4days at 6, 8, 11, 18 and 22daa with the greatest difference occurring between

treatments initiated at 8daa. However, there was not a significant difference in average mature grain weights between treatments initiated at 4daa or 15daa according to the LSDs of the means. Whilst there was not a significant difference in mature grain weight between treatments applied from 15daa the value of the difference between the means was only marginally smaller than the LSD (0.00239 vs. 0.00286), suggesting that this difference was very nearly significant. Therefore, it is unlikely to indicate an exception to the trend considering that treatment initiation stages either side of 15daa (11 and 18daa) exhibited a significant difference between treatments. The lack of a significant difference between treatments applied at 4daa suggests that there is a distinct period of susceptibility of *cv. Cadenza* to high p.a. temperatures from 6daa onwards and that exposure during the main period of cellularisation in the grain (4-8daa), prior to the beginning of rapid endosperm cell division does not result in a reduction in grain weight. Tashiro and Wardlaw (1990) observed that a 2-day high p.a. temperature treatment of 36/31°C applied at 3daa to *cv. Banks* resulted in shrunken grain which appears to contrast the results obtained here in *cv. Cadenza,* although the night time temperature used in their study was considerably higher than that used in this experiment. Tashiro and Wardlaw (1990) suggested that their observation could be the result of abnormal nuclear cell division in the developing endosperm and therefore potentially indicates variability between cultivars in the period of susceptibility to high p.a. temperatures in addition to the temperature used. Large changes in gene transcript abundance within the wheat grain occur between distinct phases of development with some of the mostly highly expressed transcripts at 6, 8 and 10daa being associated with cell division, endosperm and pericarp development and storage protein transcripts from around 12daa onwards (Wan *et al.*, 2008). Therefore, the observation that exposure to high p.a.

temperatures applied from 6daa onwards reduces mature grain weight suggests that high p.a. temperature impacts on these processes.

The observation that a 4-day high p.a. temperature treatment significantly reduced mature grain weight when applied at different stages from 6daa onwards supports results observed in a winter bread wheat cultivar, *(cv. Hereward)* that has a similar endosperm composition as *cv. Cadenza* (hard), by Gooding *et al.* (2003), particularly with regard to the latter treatment stages. Gooding *et al.* (2003) observed that an elevated temperature treatment (28 /20°C) applied before the end of the linear phase of grain growth (15-28daa) reduced final mean grain weight. They attributed this reduction to an early ending of the grain filling period that can continue until around 28daa (Shewry *et al.*, 2012). Barnabas *et al.* (2008), citing Nicolas *et al.*, (1984) and Blum (1998) also suggested that reductions in grain weight in response to high temperature stress during the early grain filling period can mainly be attributed to a reduced number of endosperm cells whilst, high p.a. temperatures later on in the grain filling period can reduce grain weight through the impairment of starch synthesis. Whilst the impact of high p.a. temperature on starch synthesis was not investigated as part of this PhD project, the effect of high p.a. temperatures on wheat endosperm cell number was investigated and is reported in a subsequent chapter (Chapter 4.). A future experiment that investigated the effect of the determined 4-day high p.a. temperature treatment on the activity of enzymes involved in starch synthesis later on in the grain-filling process, e.g. 12-15 days onwards, in *cv. Cadenza* may reveal whether this process is behind the reduction observed in this study of grain weight in the later stages of grain filling.

These results therefore provide, to our knowledge, the first indication of the response of *cv. Cadenza* grain yield to high p.a. temperatures both in terms of the susceptibility

of different developmental p.a. stages of the cultivar to high p.a. temperatures and the severity of the high p.a. temperature treatment required to induce a reduction in grain weight. This information, coupled with the fact that grain number in *cv. Cadenza* was unaffected by high p.a. temperatures adds to the knowledge base of the cultivar and may aid crop modellers in creating more accurate yield predictions for the variety with increasing temperatures. However, the use of only controlled environment experiments using this cultivar in this project do limit the application of these results to field. A repeat experiment using *cv. Cadenza* in the field with high p.a. temperatures would be useful to confirm the response of the cultivar observed in this study. This potential limitation is discussed further in subsequent chapters.

When analysing the results from the experiment that sought to determine the susceptibility of *cv. Cadenza* to a 4-day high p.a. temperature treatment at different stages p.a., the pre-anthesis environmental conditions must be considered and included as another potential limitation to the conclusions drawn. Temperatures in the glasshouse over the growing season varied largely with some temperatures greater than 40°C being experienced. Exposure to high temperatures pre-anthesis have been shown to alleviate the effects of p.a. high temperatures on grain reduction through reducing damage to the photosynthetic capacity of the flag leaf through inducing the up-regulation of antioxidant enzyme related genes in plants that received exposure to high temperature pre-anthesis (Wang *et al.*, 2011). In addition, stem reserve mobilisation aiding grain starch accumulation, has been shown to increase in wheat plants exposed to high temperature at both pre- and p.a. rather than in plants subjected to high p.a. temperatures alone. This was attributed to increased activity of fructan catalysing enzyme activities (Wang *et al.*, 2011; Wang *et al.*, 2012).

Therefore, the differences in grain weight between control and treated plants subjected to treatments at the same stage when studied in isolation provide the most insight as both groups experienced the same conditions in the glass house both before and after their exposure to the treatment. However, there was no significant difference between the mature grain weight of the control grains from experiment 2 where grains did not experience high pre-anthesis temperatures and experiment 4 where plants did. In addition, the mature grain weights were reduced to a similar level in both experiments under high p.a. temperatures. Therefore, the lack of a significant difference in mature grain weights between experiments partially supports findings by Wollenweber *et al.* (2003) who observed a lack of an additive interaction between a high temperature event (25°C for four days) applied at the double ridge stage and a subsequent high temperature event in the spring wheat cultivar, *cv. Chablis* (35°C for four days applied at anthesis). However, the conditions of their experiment differ from ours in the severity of the temperatures experienced and the stage when they were experienced by the wheat plant. A repeat of the experiment with no pre-exposure to high temperatures could provide a clearer indication of the susceptibility of the cultivar to high p.a. temperature treatments at different developmental stages. In addition, the pre-exposure to high p.a. temperatures will be taken in to consideration when discussing the results of subsequent experiments in this project.

3.3.3 Effect of high p.a. temperature on mature grain dimensions

In experiment 1, mature grain dimensions (length, width and area) were not significantly affected by high p.a. temperature treatment. In experiment 2, the average length, width and area of mature grain from primary tillers were significantly

reduced by a high p.a. temperature treatment greater than 4-days. These results followed closely the pattern of average mature grain reduction from the same experiment in that there was only a significant difference in treatments greater than 4-days. Average mature grain width, area and length were all highly positively correlated with mature grain weight although grain width was the most highly correlated dimension. This observation matches the pattern observed by Gegas *et al.* (2010), who investigated the genetic basis of grain size and shape variation in a winter wheat germplasm pool and reported that grain width and area were more closely associated with mature grain weight than length. However, they also observed that length/width ratios showed no significant interaction or correlation with mature grain weight which led them to postulate that grain shape is independent of grain size (average grain weight and area). More recently, the close association between mature grain weight and mature grain dimensions have been demonstrated (Xie *et al.*, 2015) which appear in contrast to the observations of Gegas *et al.* (2010). In addition, the results of this investigation, that mature grain weight was significantly correlated with grain width, area, and height also support the findings of Xie *et al.* (2015) and suggest that grain shape is not independent of grain size in *cv. Cadenza*. However, in contrast to the study by Gegas *et al.* (2010), only the single cultivar was used in this experiment, therefore limiting the scope of the conclusions to the *cv. Cadenza* variety alone in terms of the response of mature grain dimensions under high p.a. temperatures.

Despite grain width, length and area all being significantly affected by treatment duration, average mature grain volume was not significantly affected by the high p.a. temperature treatment. Mature grain volume has previously been shown to be closely associated with final grain weight (Millet and Pinthus 1984) as well as both

length and width (Lizana *et al.* 2010; Hasan *et al.*, 2011). Therefore, the lack of a significant interaction between treatment duration and average mature grain volume is notable but may be attributable to sampling variation. When assessing the average mature grain length, width and area, all grains from a head were electronically scanned using a Marvin Seed analyser, whereas in order to calculate average mature grain volume, 10 grains per head were randomly selected from mature heads and measured using an ethanol volume displacement method. As grain sizes have been shown to vary significantly in size within heads (Stoddard 1999), this may account for the lack of significant interaction between average mature grain volume and a high p.a. temperature treatment.

3.3.4 Grain dimensions during development under high p.a. temperatures.

Following the establishment of a 4-day high p.a. temperature treatment as being sufficient to reduce mature grain weight in experiment 2, this treatment was then used to determine the effect of high p.a. temperature on grain dimensions during development in experiment 4. Grain dimensions (length, width and height) were measured at (6, 8, 10, 14, 18, 22 26) after being subjected to the high p.a. temperature from 6daa (grains collected at 6daa and 8daa had therefore received 0 and 2-days of high p.a. temperature treatment respectively).

The results of this experiment support previous observations that grain length is the first dimension to attain its maximum value during development (Lizana *et al.*, 2010; Hasan *et al.*, 2011; Xie *et al.*, 2015) with maximum grain lengths stabilising at 10 and 14daa in high p.a. temperature treated and control grains respectively. In high p.a. temperature treated grains both length and height stabilised at 10daa followed by

grain width at 18daa. The stabilisation of grain height at the same time as length in high p.a. temperature treated grains contrasts the establishment of wheat dimensions under normal growing conditions where grain length has been observed to stabilise well in advance of height (Lizana *et al.*, 2010). In control grains, length was the first dimension to stabilise followed by height at 18daa and width at 26daa. In addition, the observation that all measured grain dimensions stabilised earlier in high p.a. temperature treated grain may support the earlier findings of Nicolas *et al.* (1984) and Wan *et al.* (2008) who observed that high p.a. temperatures accelerate grain development.

The observation that grain length and height begin to stabilise under high p.a. temperatures at 10daa, following the same minimum duration of high p.a. temperature treatment exposure required to reduce mature grain weight, as observed in experiment 2 (i.e. 4-days) potentially indicates the role of these two dimensions in determining grain weight potential under high p.a. temperature conditions. Grain length has been suggested to be a key determinant of final grain weight due to the close association between the two traits and the stabilisation of grain length before other grain dimensions (Hasan *et al.* 2011). In addition, a close association between the expansion of the pericarp and grain length has been previously determined. Lizana *et al.* (2010) observed peaks in expression of 5 putative EXPs isolated from pericarp tissue just prior to the stabilisation of grain length, whilst Brinton *et al.* (2017) identified a robust QTL associated with a 6.9% increase in grain weight that was driven by an increase in grain length, associated with increased pericarp cell length. Therefore, the stabilisation of grain length following the same duration of high p.a. temperature treatment exposure shown to reduce mature grain weight potentially supports the overall hypothesis of the project that high p.a.

temperature results in a premature maturation of the pericarp, reducing grain weight. In addition, these results potentially indicate an interaction between grain length and height in regulating grain dimensions under high p.a. temperatures. Therefore, these results contribute to our understanding of the establishment of wheat grain dimensions during development and how they respond to high p.a. temperatures. The observation that grain height stabilised at the same time as grain length under high p.a. temperatures could provide a future avenue of investigation as to the cause of this premature stabilisation. Through the utilisation of QTL analysis, similar to that employed by Brinton *et al.* (2017) it may be possible to establish a causal relationship between this grain dimension and grain weight under high p.a. temperatures and investigate the genetic basis behind it. Improved understanding of the factors influencing grain size and consequently grain weight could potentially lead to the development of new varieties with bigger grain and subsequently higher yield.

As with the experiment that aimed to determine the susceptibility of mature grain weights to different p.a. stages to a high temperature treatment of 4-days, wheat plants in this experiment experienced high temperatures (>40°C) in the booting-anthesis stage. However, both control and treated plants from each cohort were exposed to the same high temperatures prior to the beginning of the experiment. Therefore, any differences between control and treated plants at a developmental stage can logically be assumed to result from the high p.a. temperature treatment applied.

3.4 Conclusion

These experiments have determined the high p.a. temperature required in order to induce a reduction in mature grain weight in *cv. Cadenza* as well as the response of

the dimensions of the grain during both development and at maturity to high p.a temperatures, therefore contributing to our understanding of the grain's development under stressful abiotic conditions. Due to the high amounts of genotypic and varietal variation in tolerance to high p.a. temperatures, determining a treatment that induces a heat stress response was important in order to investigate the structure of grain layer cell walls under high p.a. temperature. The results obtained have shown that high p.a. temperature reduces grain weight when applied at nearly any point during the linear phase of grain filling (6-22daa), suggesting that multiple processes that determine grain weight are impacted upon under high p.a. temperature. In addition, it is apparent that there is not at additive effect of high p.a. temperature on mature grain weight and that the negative impact of high p.a. temperatures is limited to a point, suggesting potential structural changes in the grain's sink capacity. The stabilisation of grain length and height following a high p.a. temperature treatment period sufficient to reduce grain weight at maturity supports the view that these traits may be involved in determining the final grain size. Due to the close association between pericarp development and grain length, it is possible that cellular modification of the pericarp under high p.a. temperature accounts for the early stabilisation of grain length. In order to further investigate the cause of the reduction in grain weight following high p.a. temperature, the cellular components of the endosperm and pericarp will be investigated via immuno-fluorescence microscopy. In addition, comparative transcriptomic analyses of developing grain following a high p.a. temperature treatment may reveal tissue specific gene expression pathways that potentially regulate grain size development during the early stages of grain filling.

Chapter 4: Effect of high post-anthesis temperature on wheat grain endosperm cells and homogalacturonan distribution and form.

4.1 Introduction

The exact mechanisms through which high p.a. temperatures reduce grain weight during development are still unclear. Many studies have focused on the effect of high p.a. temperatures on 'sink-source' relationships within the wheat plant with high p.a. temperatures having been shown to reduce leaf area index and photo-assimilate supply (Kobata *et al.*, 2012) as well as reducing the activity of starch synthase within the wheat grain and subsequent starch deposition in the latter stages of grain development (Keeling *et al.*, 1993). It is therefore apparent that the establishment of final grain weight in wheat is a dynamic process that can be affected by high p.a. temperature induced modifications to a number of physiological processes. How these processes impact on one another during development of the wheat grain under high p.a. temperature is still an area of investigation.

One main determinant of the final weight and size of the wheat grain is the size of the endosperm. The endosperm, where most of the grain protein and starch are deposited, acts as the main storage tissue of the wheat grain and is often referred to as a photo-

assimilate 'sink'. The sink capacity of a cereal grain is determined by the number of endosperm cells (Gleadow *et al.*, 1982), their size and the number of starch granules initiated per endosperm cell which defines the potential number of sites for starch deposition (Jones *et al.*, 1985; Nicolas *et al.*, 1985). Endosperm cell numbers in both heat tolerant and susceptible wheat cultivars, *cvs. WH730* and *UP2565* have been shown to be significantly reduced under high p.a. temperatures (Kaur *et al.*, 2011). However, the mechanisms through which high p.a. temperatures affect cell division and size in the endosperm are not entirely known.

Endosperm cell division under normal growing conditions is regulated by a number of hormones including cytokinins, auxins, gibberellins and abscisic acids (Hansen and Grossmann 2000; Yang *et al.*, 2003). Cytokinin levels are closely associated with endosperm cell division and number (Morris *et al.*, 1993; Cheikh and Jones 1994). Banowetz *et al.* (1999) investigated the effect of high p.a. temperature on cytokinin accumulation in developing winter wheat grain *(cv. Stephens)* and observed that a 7-day high temperature treatment (35/25°C) applied from anthesis resulted in a reduction in grain cytokinin content and growth rate. However, the reduction in grain growth rate was only observed after 5daa whereas the reduction in cytokinin content was observed after 1daa. This led Banowetz *et al.* (1999) to suggest that either cytokinin accumulation greatly exceeds that needed for endosperm cell division, or that reduced accumulation has no effect on endosperm cell numbers. They concluded that cytokinin content is not solely responsible for high p.a. temperature related reductions in grain size but that it may become limiting under those conditions.

Yang *et al.* (2016) also investigated the role of cytokinins and other endogenous plant hormones such as zeatin riboside, giberrellin, indole-3-acetic acid and ABA in wheat grain growth dynamics under high p.a. temperatures. They observed that an exogenous, daily

application of cytokinin from 1-3daa on two winter wheat varieties *(cvs. Wennong-6* and *Jimai-20)* undergoing a high p.a. temperature treatment (>34°C) applied from 1-5daa, resulted in an increase in endosperm cell division and grain filling rate compared to those plants that just received the high p.a. temperature treatment, where endosperm cell number was reduced. Cytokinin application also increased endosperm cell division and cell number in control plants grown at ambient temperatures (~32°C). In addition, they observed that zeatin riboside content was significantly decreased by high p.a. temperature during the early stages of grain development whilst gibberellin content increased. They concluded that this reduction in zeatin riboside content reduced grain yield during the early stages of development by reducing the rate of endosperm cell division and total endosperm cell number (i.e. reducing grain size).

This project previously observed in experiment 2 (Chapter 3-3.2.1.2) that a 4-day high p.a. temperature treatment applied from 6daa was the minimum treatment duration that resulted in a significantly reduced mature grain weight. Longer durations of treatment resulted in significant reductions in mature grain weight, however, these reductions were not significantly different to the reduction observed following the 4-day treatment. This result suggests that the maximum negative effect of high p.a. temperature on final grain size is limited, potentially by a defined effect on the endosperm in terms of cell number and/or size. Therefore, investigating the effect of a similar high p.a. temperature treatment on these components that make up the size of the endosperm in *cv. Cadenza* would help elucidate whether this is the case.

How endosperm cell division and pericarp development are coordinated under high p.a. temperatures is still not fully understood. Whilst the role of endogenous hormones in regulating endosperm cell division has been demonstrated, the interaction between the pericarp and endosperm cell division and size has not been investigated. Signalling

between the endosperm and pericarp tissues of *A.thaliana* have previously been observed during development (Weijers *et al.*, 2003) whilst the thickness of the pericarp in wheat grain at 28 and 35daa has been shown to decrease under high p.a. temperature and drought conditions in wheat alongside a reduction in endosperm cell size (Kaur *et al.* 2011). The size of a seed is determined by the co-ordinated growth of the embryo, endosperm and maternal tissues (Adamski *et al.*, 2009). Therefore, it is likely that there is a significant degree of interaction between the endosperm and pericarp in their response to high p.a. temperatures due to the close coordination in expansion between the two tissues (Becraft and Gutierrez-Marcos, 2012).

Endosperm cell size has previously been shown to be reduced in the bread wheat variety *cv. Warrigal,* under combined high p.a. temperature (28/20°C) and drought conditions applied from 10-20daa, which correspond to late cell division stage in wheat (Nicolas *et al.*, 1984) but the cause of the reduction is relatively unknown. The imposition of a physical restriction on endosperm cell expansion through changes to the cell wall composition of both the endosperm and pericarp grain layers has been postulated as a potential control of endosperm size in wheat grain during development (Chateigner-Boutin *et al.*, 2014). Changes in pericarp and endosperm cell wall composition may be induced by exposure to high p.a. temperatures. Toole *et al.* (2007) showed in two hard milling bread wheat cultivars that the progression of AX structural modification in endosperm cells from highly branched polymers with arabinose sub-units (WE-AX) to lowly branched (WU-AX), was accelerated under high p.a. temperature conditions (20-30**℃**) and restricted water availability. They postulated that this accelerated restructuring under high p.a. temperature may result in a premature increase in rigidity in the endosperm cell wall resulting in a shortened grain filling period. However, Rakszegi *et al.* (2014) found that high p.a. temperatures resulted in an increase in the amount of WE-AX within the endosperm cell wall, with the difference in

observations between the studies being attributed to potential genotypic differences and variation in environmental conditions between growing sites. Therefore, the effect of high p.a. temperatures on the cell wall composition of different wheat grain layers still remains to be determined.

One cell wall polymer that has been proposed to control wheat grain layer expansion dynamics, is the pectic domain homogalacturonan (HG) (Chateigner-Boutin *et al.*, 2014). Distribution of pectic domains within the wheat grain and particularly within the endosperm were not discovered until an initial study by Pellny *et al.* (2012) in which RNA-Seq conducted on pure starchy endosperm from *cv. Cadenza* wheat grain, revealed the expression of GT gene families (GT47) involved with the synthesis of pectin. The presence of pectin was then confirmed by Chateigner-Boutin *et al.* (2014) who through using an enzymatic treatment of lichenase and xylanase removed the AX and MLG that dominate the cell walls of the starchy endosperm masking the presence of other cell wall polysaccharides. The removal of these cell wall polymers revealed the presence of both RG and HG and other pectic domains. From subsequent monosaccharide analysis, pectin is now thought to comprise approximately 5-6% of the total cell wall content of the wheat grain (Palmer 2014). HG is the primary pectic domain found within plant cell walls constituting approximately 60-65% of total cell wall pectin and the biosynthesis and modification of this polysaccharide within plant cells walls has been shown to play a vital role in regulating plant development, cell adhesion and organ development (Wolf *et al.*, 2009). Structural modification of HG involves the removal of methyl-ester groups from the C-6 region of the polymer which is catalysed by pectin-methylesterases (PMEs). HG is synthesised in the subcellular golgi apparatus before being exported in a methyl-esterified form to the outer layers of the grain in the early stages of development (Northcote and Pickett-Heaps 1966; Chateigner-Boutin *et al.*, 2014). The methyl-esterification status of HG is

believed to play a major role in cell wall growth in plants and tissue development. General consensus is that methyl-esterified HG in cell walls facilitates the expansion of the cell wall through disruption of covalently linked Ca^{2+} ion bridges that connect HG molecules and promote the formation of the 'egg-box' model structure, named so due to the zig zag shaped linkages between HG chains (Grant *et al.*, 1973). The presence of these 'egg-box' structures are believed to induce pectin gel formation and thus strengthen and stiffen cell walls (Wolf *et al.*, 2009). Disruption of these Ca^{2+} ion bridges would allow for turgor driven expansion of the cell walls to occur. Since the discovery of pectin within the wheat grain, there have been no studies, to our knowledge, that have examined the pattern of HG synthesis and modification in the cell walls of the different wheat grain layers under high p.a. temperature treatment and how this may affect the normal progression of wheat grain development. Any changes in HG form and distribution under high p.a. temperatures in the outer layers of the grain may indicate maternal based control of final grain size in wheat, either by acting as an intracellular signal affecting grain development or through the imposition of a physical restriction on endosperm expansion via a premature increase in cell wall rigidity.

Therefore, the aim of this study was to test the experimental hypotheses that: high p.a. temperatures result in a reduction in endosperm cell size and number in *cv. Cadenza* during development and that the methyl-esterification profile and distribution of cell wall HG in the wheat grain is also significantly affected by high p.a. temperatures during the course of grain development. By testing these hypotheses, the experiments contained within this chapter also sought to:

- investigate whether the previously observed fixed reduction in mature grain weight imposed by exposure to a high p.a. temperature treatment of 4-days or longer was due to a reduction in endosperm cell size and number.

- explore whether a high p.a. temperature treatment effects the HG methyl-esterification form and distribution within the wheat grain during development and if so is the normal pattern of HG distribution and methyl-esterification status accelerated under high p.a. temperature.
- analyse in which area of the wheat grain cross-section, endosperm cell size is most affected by high p.a. temperatures.

4.2 Results

4.2.1 Endosperm cell number and size (area µm^2)

The effect of high p.a. temperature on endosperm cell number and size during development of the wheat grain was examined as part of this project in order to test the hypothesis that high p.a. temperatures result in a reduction in endosperm cell size and number during development in *cv. Cadenza*. Previous studies have analysed the number of endosperm cells through the counting of separated endosperm nuclei *via* the use of a haemocytometer (Nicolas *et al.*, 1985; Commuri and Jones 2001). In this study the monoclonal antibody, LM21 that labels heteromannan found in the cell walls of the endosperm, was used in conjunction with imaging analysis software ImageJ™. This allowed for the number of endosperm cells to be counted accurately whilst also providing measurements of the size of the cells and their exact location within the endosperm. Cross sectional average endosperm cell number and area (µm^2) was measured in experiment 3 (Chapter 2 - 2.9) from grains collected at 12, 16 and 25daa and that had been subjected to 6, 10 and 12 days of high p.a. temperature or control temperature (Fig.4.1).

4.2.1.1 Endosperm cell number

The number of endosperm cells in grains sampled at 16daa was significantly lower in grains exposed to high p.a. temperatures than in control grains (ANOVA, df=1, f=8.53, p=<0.05), whilst there was no significant difference in the number of endosperm cells between treatments at 12daa (ANOVA, df=1, f=6.57, p=>0.05) (Fig.4.2). The number of endosperm cells significantly increased between 12 and 16daa in control grains based on the LSDs of the means whereas the number of cells between high p.a. temperature treated grains at 12 and 16daa was not significantly different. LM21 labelling in the endosperm was partially lost in high p.a. temperature treated grains sampled at 25daa (Fig.4.5) making endosperm cell number analysis challenging using the same method. Therefore, endosperm cell numbers were compared between grains subjected to control temperatures sampled at 16 and 25daa in order to determine whether endosperm cell division had ended prior to 25daa. There was no significant difference in the number of endosperm cells between grains collected at 16 and 25daa grown under control conditions according to the LSDs of the means. Therefore the results of this experiment support the experimental hypothesis that high p.a. temperatures result in a reduction in endosperm cell number in *cv. Cadenza* during development, although only at 16daa in this study.

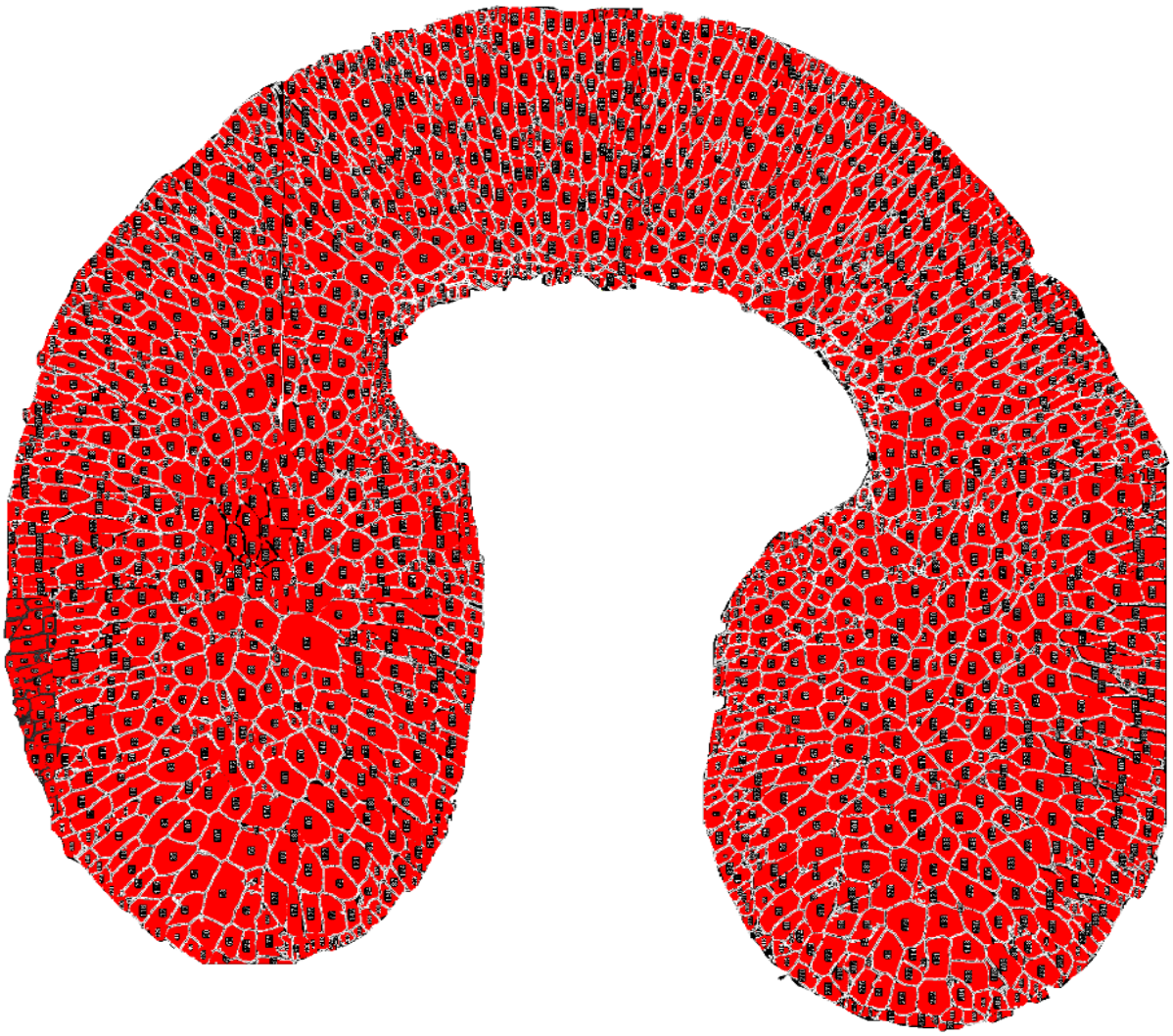


Figure 4.1. Example of cross-section endosperm cell analysis. Individual regions were photographed following immunofluorescence labelling with primary antibody LM21 and secondary antibody Alexa Fluor 633 at 5x magnification. Photographs were then digitally analysed using ImageJ software for cell number and size. Analysed images were then stitched together to create a compound image. Above image is of a grain sampled at 16daa subjected to high p.a. temperature (35/15°C) for 10hours per day from 6daa (10days of treatment).

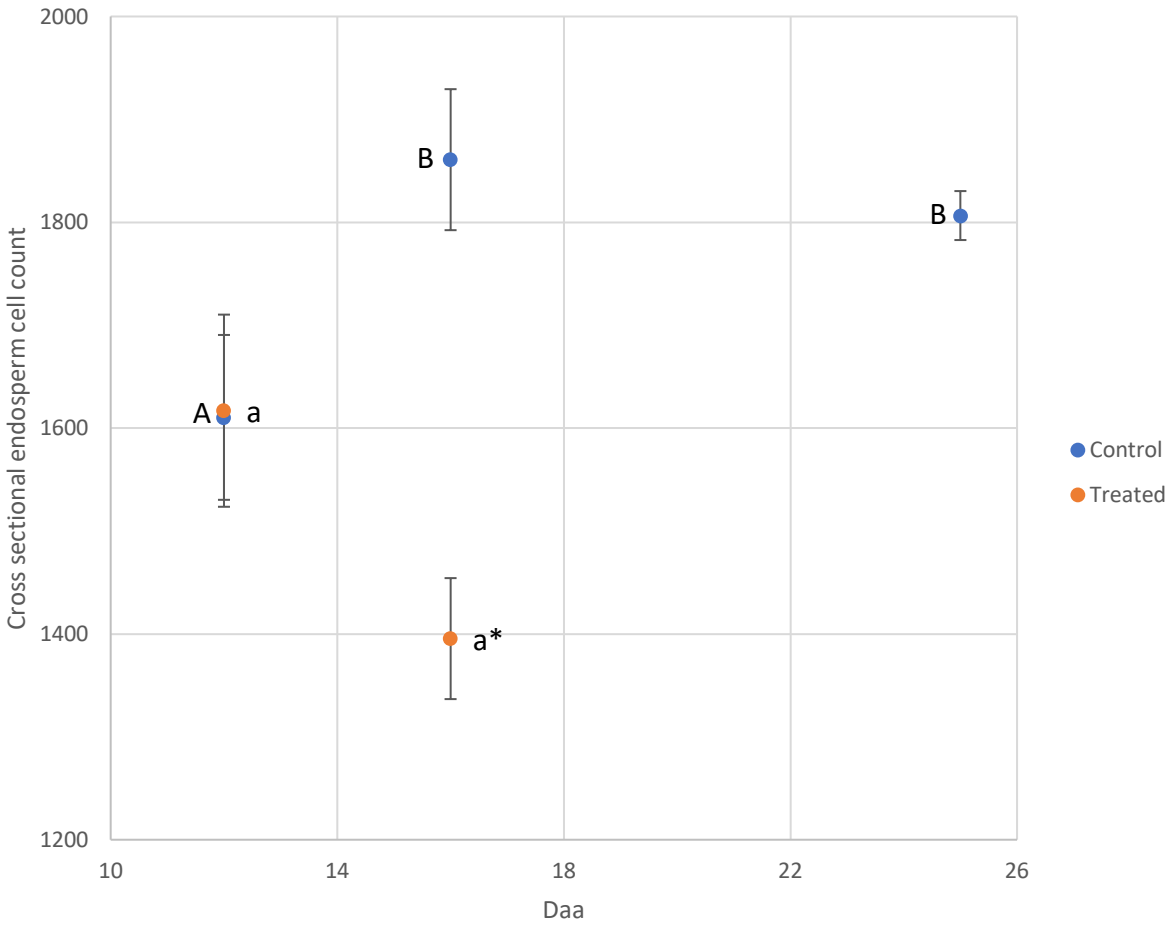


Figure 4.2. Average cross-section endosperm cell count from grains collected at 12, 16 and 25daa from the primary tillers of plants exposed to different high p.a. temperature treatment (35°C) durations from 6daa in experiment 3. The x axis labelling refers to the daa when the grains were sampled. Error bars represent the standard error of the mean of 6 biological replicates. Labelling with different letters (uppercase=control, lowercase=high p.a. temperature treatment) indicates significant differences in endosperm cell numbers in cross sections between daa stages according to the LSDs between the means at $p<0.05$. Daa stages with the same letter had no significant difference between them. Letters with an * indicate significant differences between the high p.a temperature treatment and control at the same daa stage according to the LSDs between the means at $p<0.05$.

4.2.1.2 Endosperm cell size (area µm^2)

Average endosperm cell size was compared and calculated from two separate regions in each endosperm cross-section. These regions were termed the 'dorsal' region which consisted of the endosperm cells above the crease and pigment strand that were between the two 'lobes' of the wheat grain cross section (Fig. 4.3).

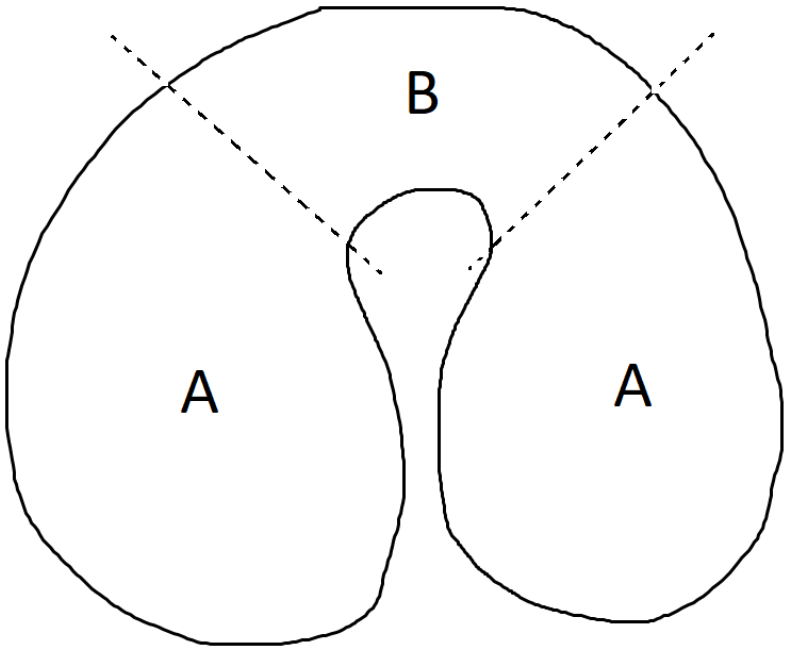


Figure 4.3. Diagram of regional cross-section endosperm cell area analysis. Regions labelled (A) were termed the 'lobe' regions whilst the region labelled (B) was termed as the 'dorsal' region.

High p.a. temperature treatment had no significant effect on average endosperm cell area in the lobe regions of grains sampled at 12[1.] and 16daa[2.] (ANOVA, df=1, f=8.67[1.],8.54[2.] p=>0.05) or in the dorsal region in grain sampled at 12daa. High p.a. temperature significantly reduced endosperm cell area in the dorsal region of grains sampled at 16daa (ANOVA, df=1, f=40.67, p=<0.05) (Fig. 4.4). There was no significant difference in average endosperm cell area between control grains sampled at 16 and 25daa in either the dorsal or lobe region. Therefore these results were in support of the hypothesis that high p.a.

temperatures reduce endosperm cell size in *cv. Cadenza,* although it is apparent that this effect only occurs in the dorsal region at 16daa.

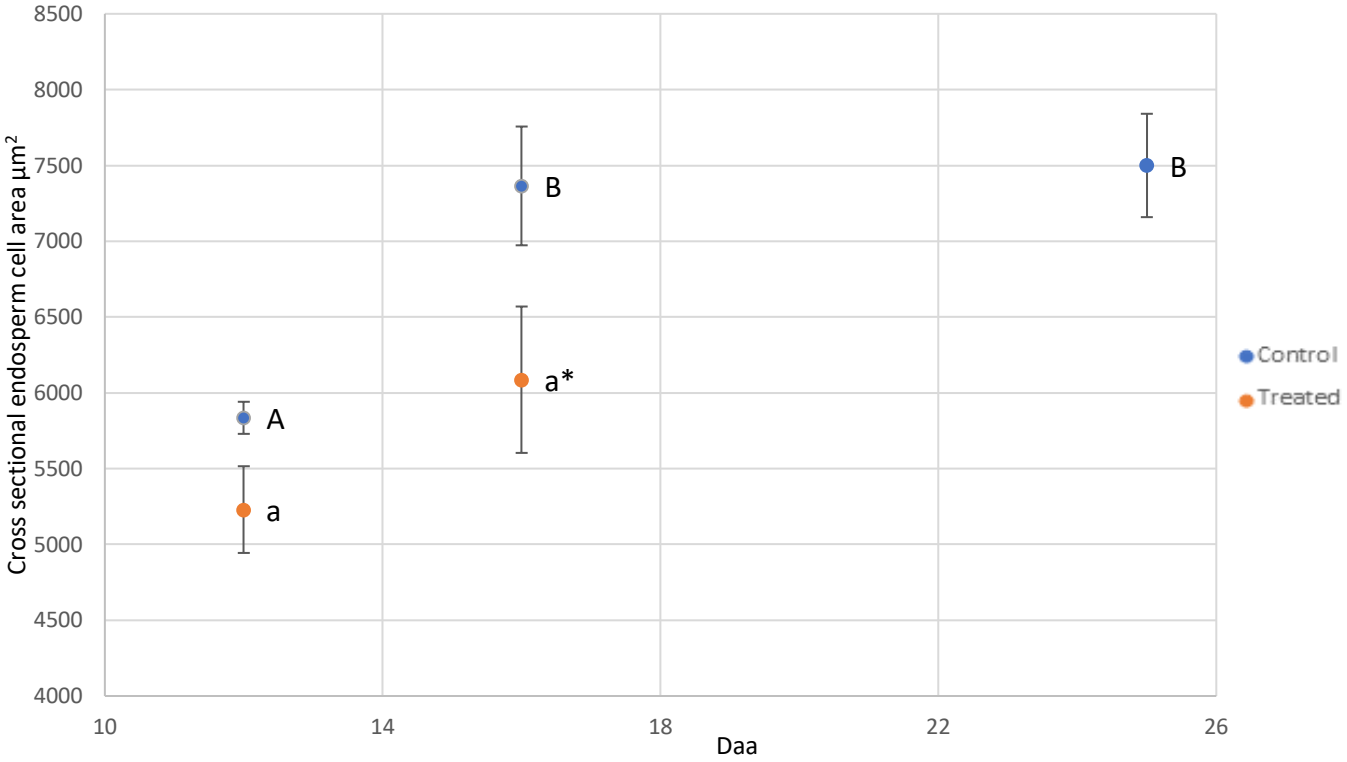


Figure 4.4. Average cross-section endosperm cell area from the dorsal region of grains collected at 12, 16 and 25daa from the primary tillers of plants exposed to different high p.a. temperature treatment (35°C) durations from 6daa. The x axis labelling refers to the daa when the grains were sampled. Error bars represent the standard error of the mean of 6 biological replicates. Labelling with different letters (uppercase=control, lowercase=high p.a. temperature treatment) indicates significant differences in dorsal area endosperm cell size in cross sections between daa stages according to the LSDs between the means at $p<0.05$. Daa stages with the same letter had no significant difference between them. Letters with an * indicate significant differences between the high p.a temperature treatment and control at the same daa stage according to the LSDs between the means at $p<0.05$.

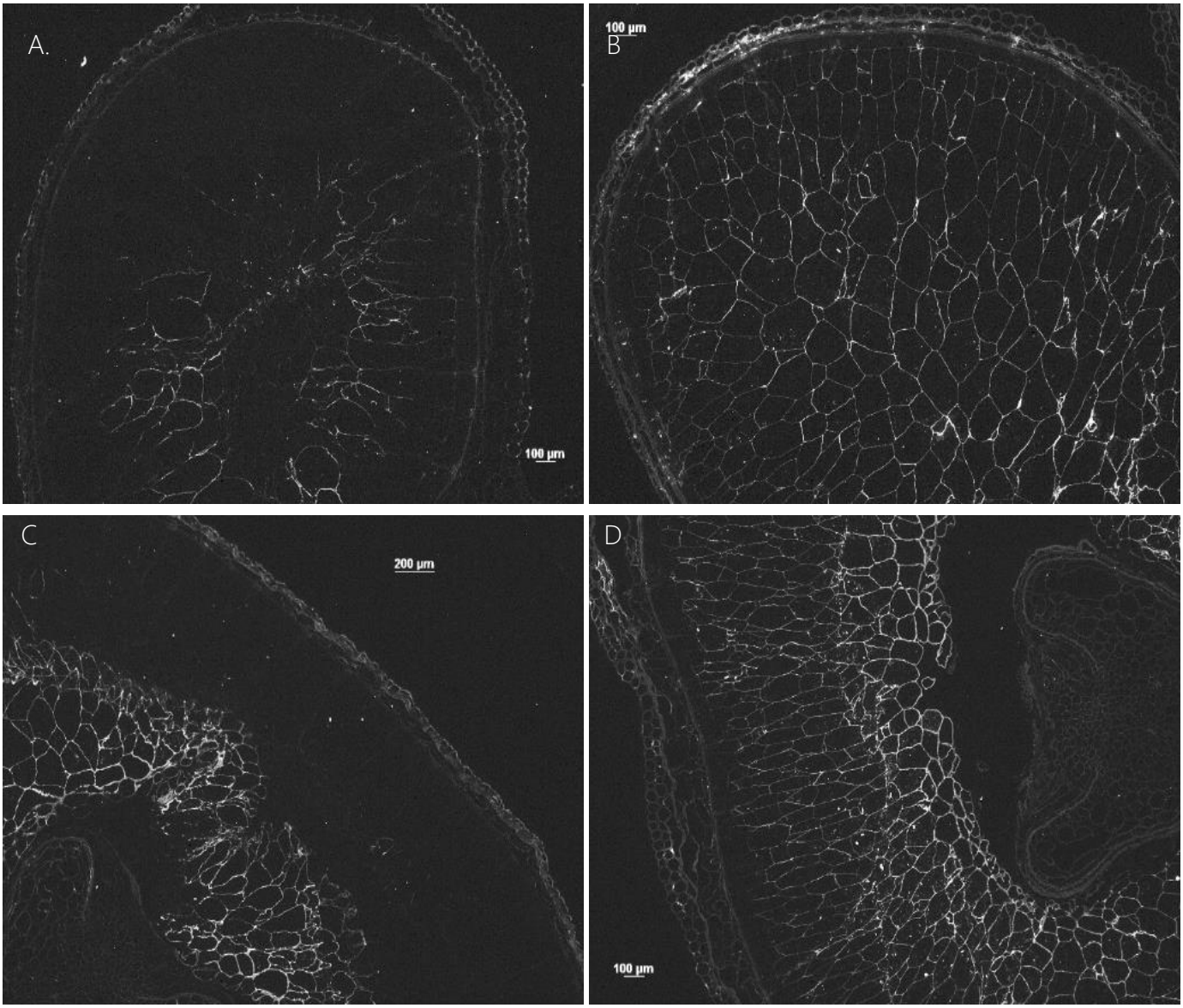


Figure 4.5 Images of grain sections sampled at 25daa subjected to high p.a. temperature (A and C) and control temperature treatments (B and D) from 6daa until 18daa. Labelling with LM21 was reduced in both the 'lobe' (A.) and 'dorsal' (C.) regions of grains subjected to high p.a. temperatures compared to grains subjected to the control treatment.

4.2.2 Effect of high p.a. temperature on homogalacturonan methyl-esterification and distribution

The effect of high p.a. temperature on HG methyl-esterification status and distribution was investigated in grains collected at 8, 14 and 18daa from experiment 2, which had been subjected to control and high p.a. temperature treatments from 6daa (2, 8 and 12days of treatment exposure respectively). This experiment sought to test the hypothesis that the methyl-esterification profile and distribution of cell wall HG in the wheat grain is significantly affected by high p.a. temperatures during the course of grain development. Pectic epitopes, including HG, in the wheat grain have been previously shown to be partially masked in some tissues by the presence of more abundant cell wall polysaccharides such as MLG and AX (Chateigner-Boutin *et al.* 2014). These polysaccharides can prevent labelling of a desired epitope with a monoclonal antibody through steric interference, which reduces the probability of an antibody coming into contact with an epitope. Chateigner Boutin *et al.* (2014) and Palmer (2015) demonstrated the successful removal of AX and MLG from wheat grain sections using a combined enzymatic treatment of lichenase and xylanase (referred to as LX from here on in), with the former degrading MLG and the latter degrading AX. In this study immuno-labelling of sections with HG monoclonal antibodies JIM7, LM19 and LM20, that label partially methyl-esterified, un-methyl-esterified and methyl-esterified HG respectively, was performed on both enzyme-treated and non-enzyme-treated samples in order to confirm that some of the epitopes are masked i.e. the antibody signal appears/increases after treatment with enzymes. HG is exported to the cell wall in a fully methyl-esterified form. Subsequently the methyl-esterification status of HG can be changed through the action of PMEs that catalyses the removal of methyl-esters from the C6

position of the galacturonic acid residue. Therefore the monoclonal antibodies bind to the different forms of HG undergoing this process.

4.2.2.1 Unmasking of homogalacturonan epitopes following an enzymatic dual treatment

Comparisons between grain sections labelled with JIM7 that had and had not received the dual enzymatic pre-treatment revealed differences in labelling when sampled at 8, 14, and 18daa in both high p.a. and control temperature treated grains (Table 4.1). At 8daa, in grains that had received the LX treatment, labelling of the endosperm was observed in control samples whilst only weak labelling of the endosperm cell walls was observed in high p.a. temperature treated samples (Fig.4.6). In both high p.a. and control samples, the labelling of the pericarp, nucellus epidermis, cross cells, early aleurone cells and nucellar projection were all improved by the LX treatment. At 14daa the LX treatment revealed significant JIM7 labelling of the starchy endosperm cells in both high p.a. temperature treated samples and control samples. In high p.a. temperature treated samples labelling of the aleurone and nucellar projection were also revealed following the LX treatment (Fig.4.6). At 18daa similar results were observed between sections that had and had not received the LX treatment in both control and high p.a. temperature treated sections. In both treatments, labelling of the aleurone and starchy endosperm was revealed following the LX treatment although only partially in the endosperm of high p.a. temperature treated samples (Fig.4.6)

Labelling with LM19 was observed in the nucellar projection of high p.a. temperature treated grain at 8daa following the LX treatment, whilst in control grains labelling was revealed in the cross cells following the enzyme treatment (Fig. 4.7). Labelling of the pericarp was improved in both high p.a. temperature and control grains following the LX

treatment. At 14daa, labelling with LM19 increased in the outer most periclinal cell walls of the aleurone and the nucellar projection of high p.a. temperature treated grain following LX treatment (Fig.4.7), whilst labelling of the nucellus epidermis and nucellar projection were improved following the LX treatment in control grain sections (Table 4.1). At 18daa, in control samples, labelling of the nucellus epidermis, starchy endosperm and nucellar projection were revealed following the LX treatment. The only difference between high p.a. temperature treated grains that did and did not receive the LX treatment at 18daa was that labelling of the nucellar projection was revealed following the enzyme treatment. There was no labelling of any grain sections with LM20 at 8daa, 14daa or 18daa suggesting that either the monoclonal antibody failed to bind successfully to the epitope or that fully methyl-esterified HG was not present in the grain sections sampled.

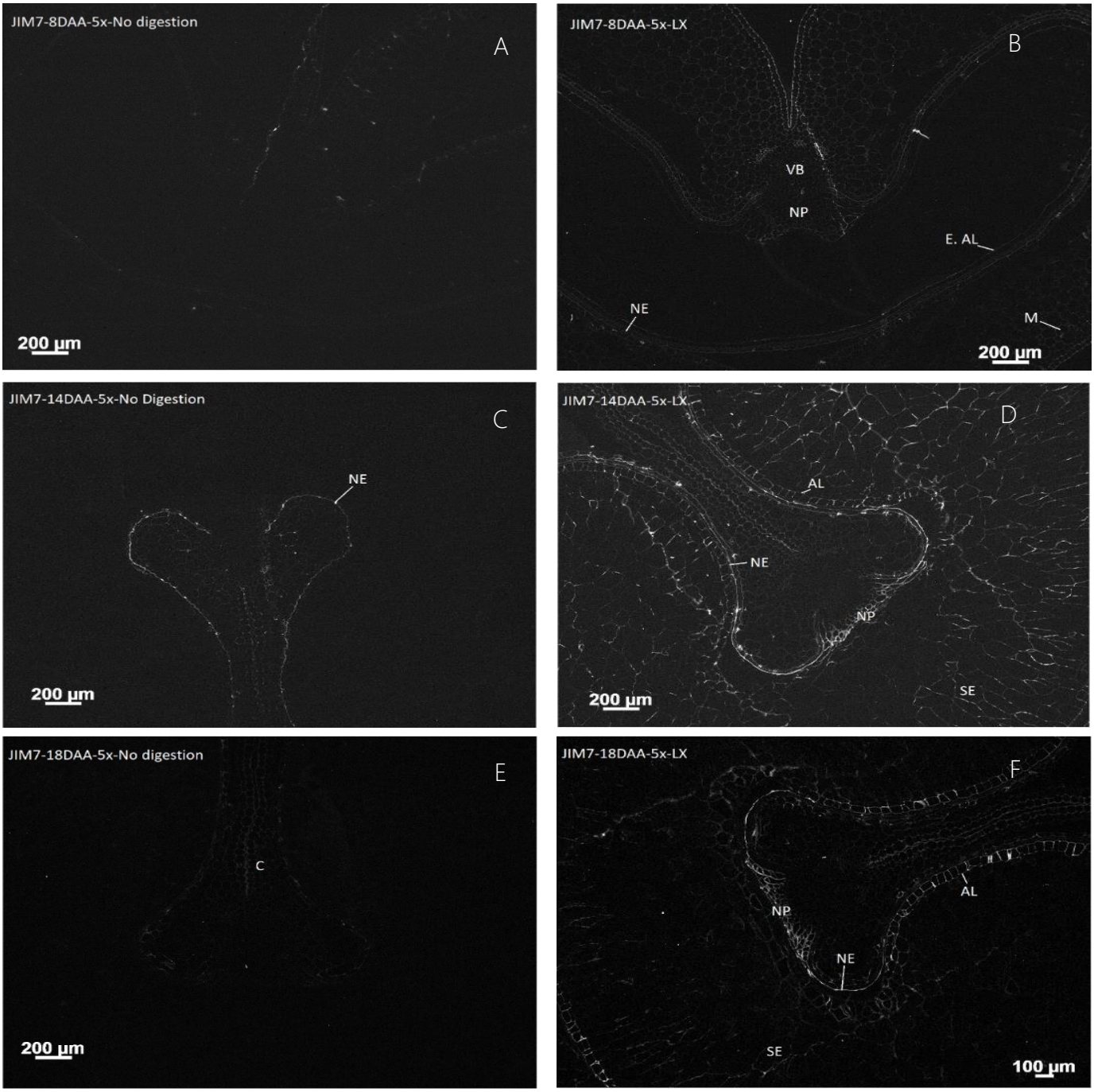


Figure 4.6 High p.a. temperature treated (35°C) sections from 8 (A, B), 14 (C, D) and 18daa (E, F) labelled with JIM7 at 5x magnification. Sections in images A, C, E did not receive the dual enzymatic treatment whilst images B, D, F did receive the LX dual enzymatic treatment. The LX treatment revealed greater labelling that in the non-enzyme treated images. (NE=nucellar epidermis, NP=nucellar projection, SE=starchy endosperm, AL=aleurone, M=maternal pericarp, VB= Vascular bundle)

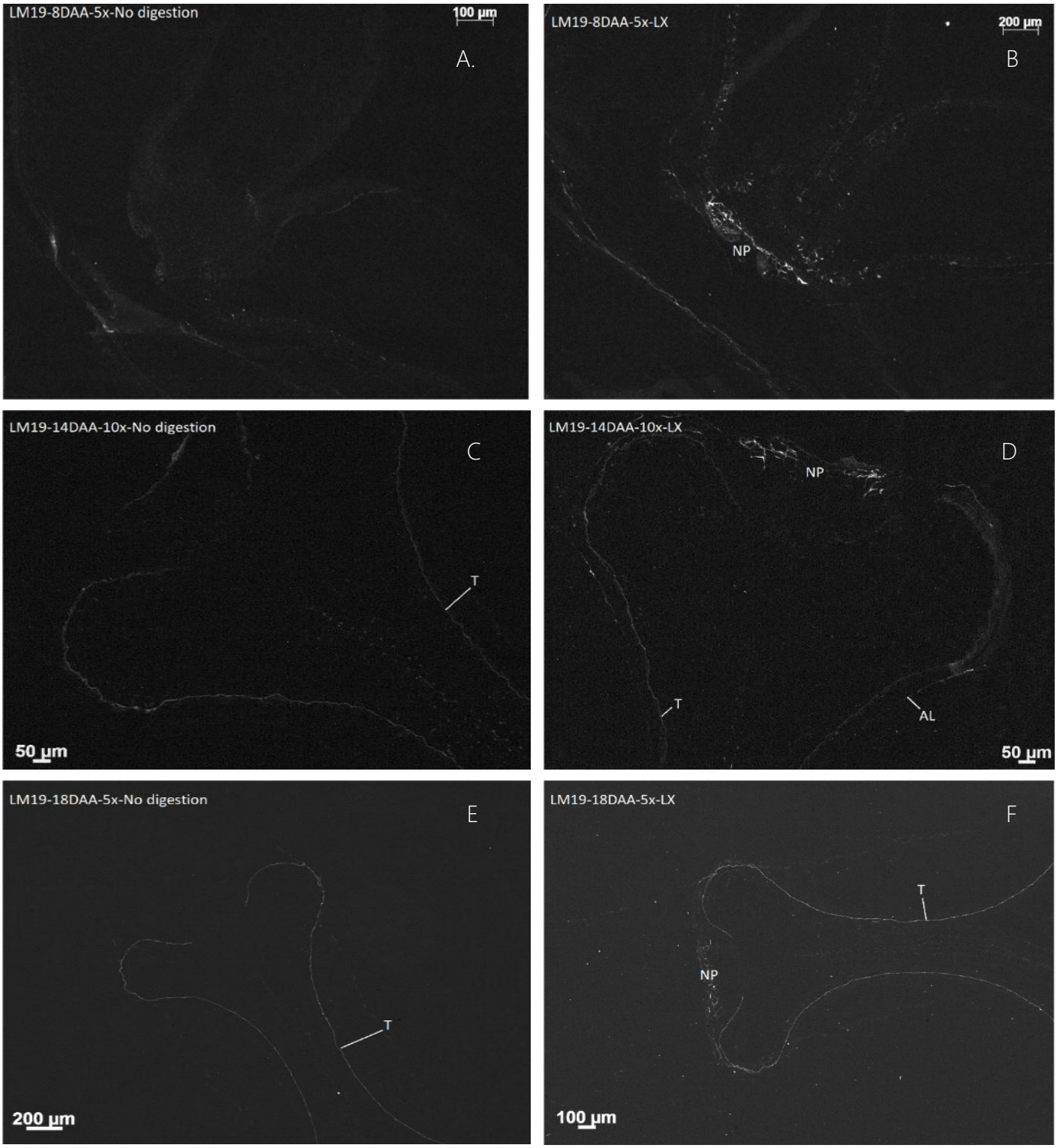


Figure 4.7 High p.a. temperature treated (35°C) sections from 8 (A,B), 14 (C,D) and 18daa(E,F) labelled with LM19. Sections in images A,C,E did not receive the dual enzymatic treatment whilst images B,D,F did receive the LX dual enzymatic treatment. The LX treatment revealed greater labelling than in the non-enzyme treated images. (NP=nucellar projection, T=testa, AL=aleurone)

4.2.2.2 Distribution and methyl-esterification of homogalacturonan under high p.a. temperature.

Exposure to high p.a. temperatures resulted in differences in the distribution and methyl-esterification status of HG within the wheat grain at 8, 14 and 18daa in LX treated samples (Table. 4.1) therefore supporting the experimental hypothesis. JIM7 binds to partially methyl-esterified HG and at 8daa was observed in the endosperm cell walls towards the lobe regions in both high p.a. temperature treated and control grain with stronger labelling in control sections. Labelling of the pericarp, nucellar epidermis, testa and cross cells was observed in both high p.a. temperature treated and control sections. In addition, labelling was observed in both treatments in the early aleurone cells with slightly weaker labelling of the anticlinal cell walls compared to the periclinal cell walls (Fig. 4.8).

At 14daa JIM7 labelling was clearly observed in the endosperm cell walls in both high p.a. temperature treated and control sections albeit with a slightly stronger signal in high p.a. temperature treated sections (Fig. 4.9). Labelling was observed in both high p.a. temperature treated and control sections in the outer pericarp, testa, cross cells and nucellar epidermis. Aleurone cells were labelled in high p.a. temperature treated samples but not in control grain sections.

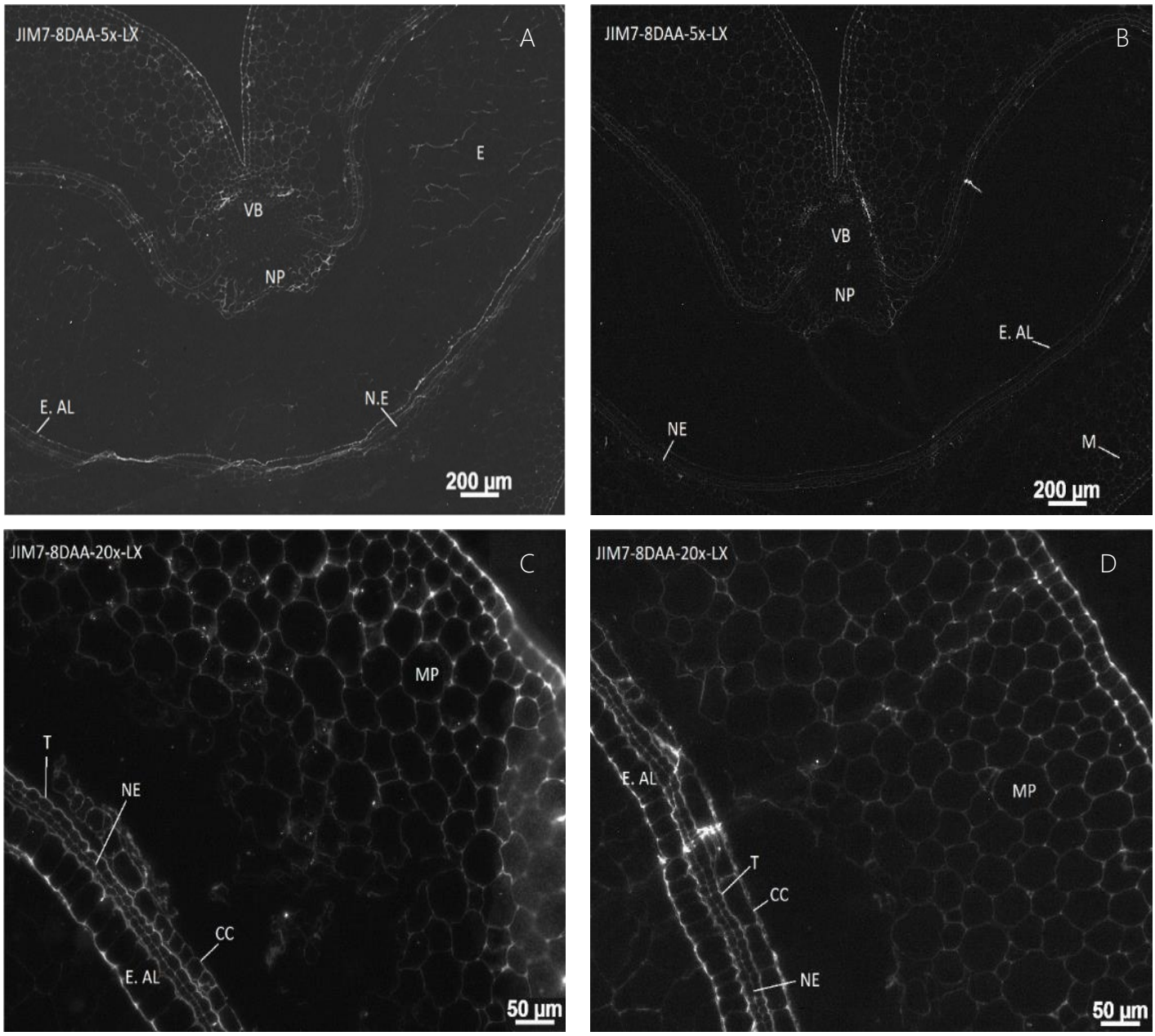


Figure 4.8 Images of control (A, C) and high p.a. temperature (35°C) (B, D) treated sections at 8daa labelled with JIM7 following a dual enzymatic treatment observed at 5x (A, B) and 20x (C, D) magnification and 1500ms exposure. (VB= vascular bundle, NP= nucellar projection, E= endosperm, E. AL= early aleurone, NE= nucellar epidermis, CC= cross cells, T= testa, MP/M= maternal pericarp).

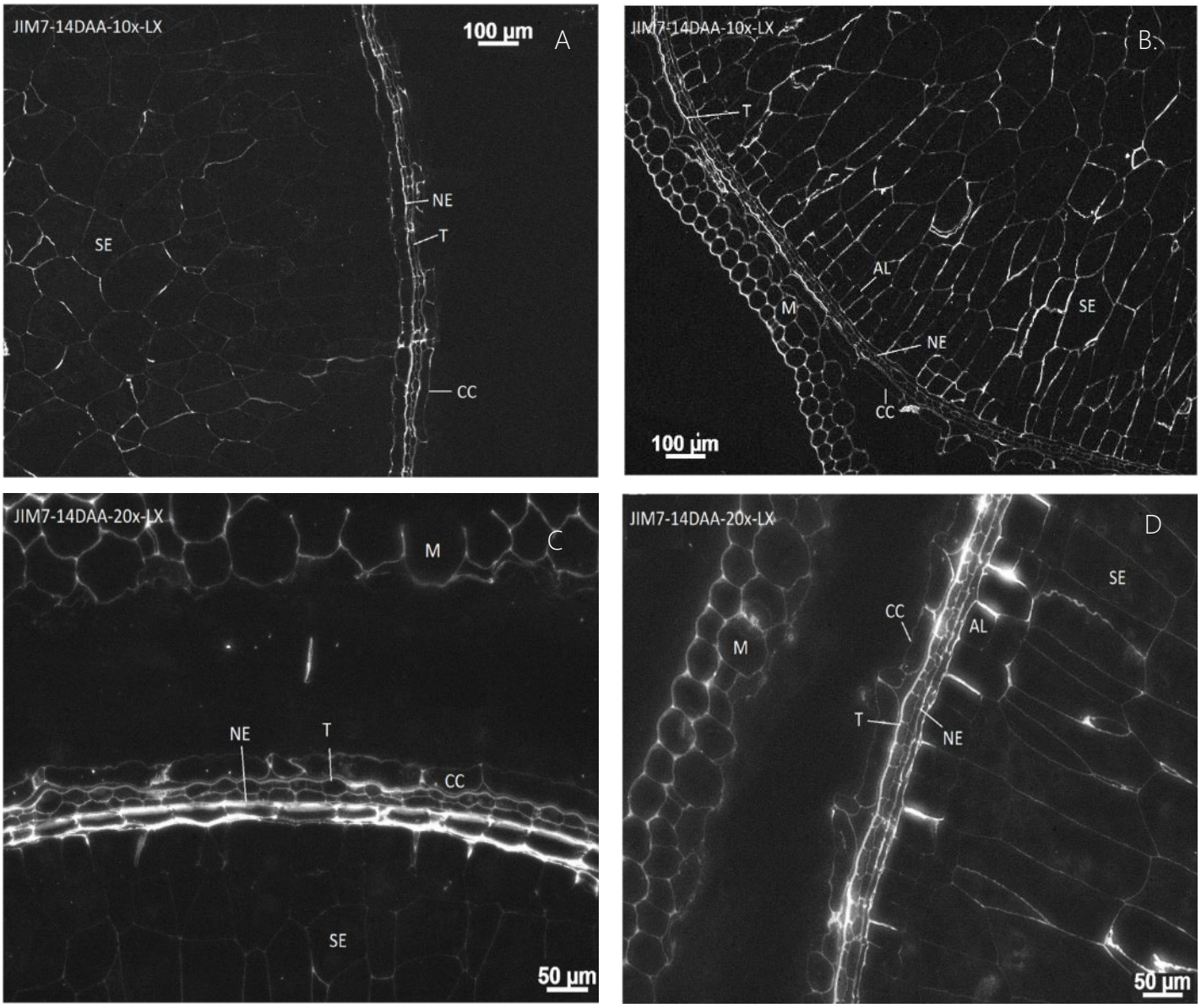


Figure 4.9 Images of control (A, C) and high p.a. temperature (35°C) (B, D) treated sections labelled at 14daa with JIM7 following a dual enzymatic treatment observed at 10x (A, B) and 20x (C, D) magnification and 1500ms exposure. (SE= starchy endosperm, AL= aleurone, NE= nucellar epidermis, CC= cross cells, T= testa, M= maternal pericarp).

At 18daa labelling with JIM7 was significantly reduced in the cell walls of starchy endosperm cells of high p.a. temperature treated sections compared to control sections (Fig. 4.10). In addition, signal in the nucellar epidermis and testa was lost in high p.a. temperature treated samples but retained in samples grown under control temperatures.

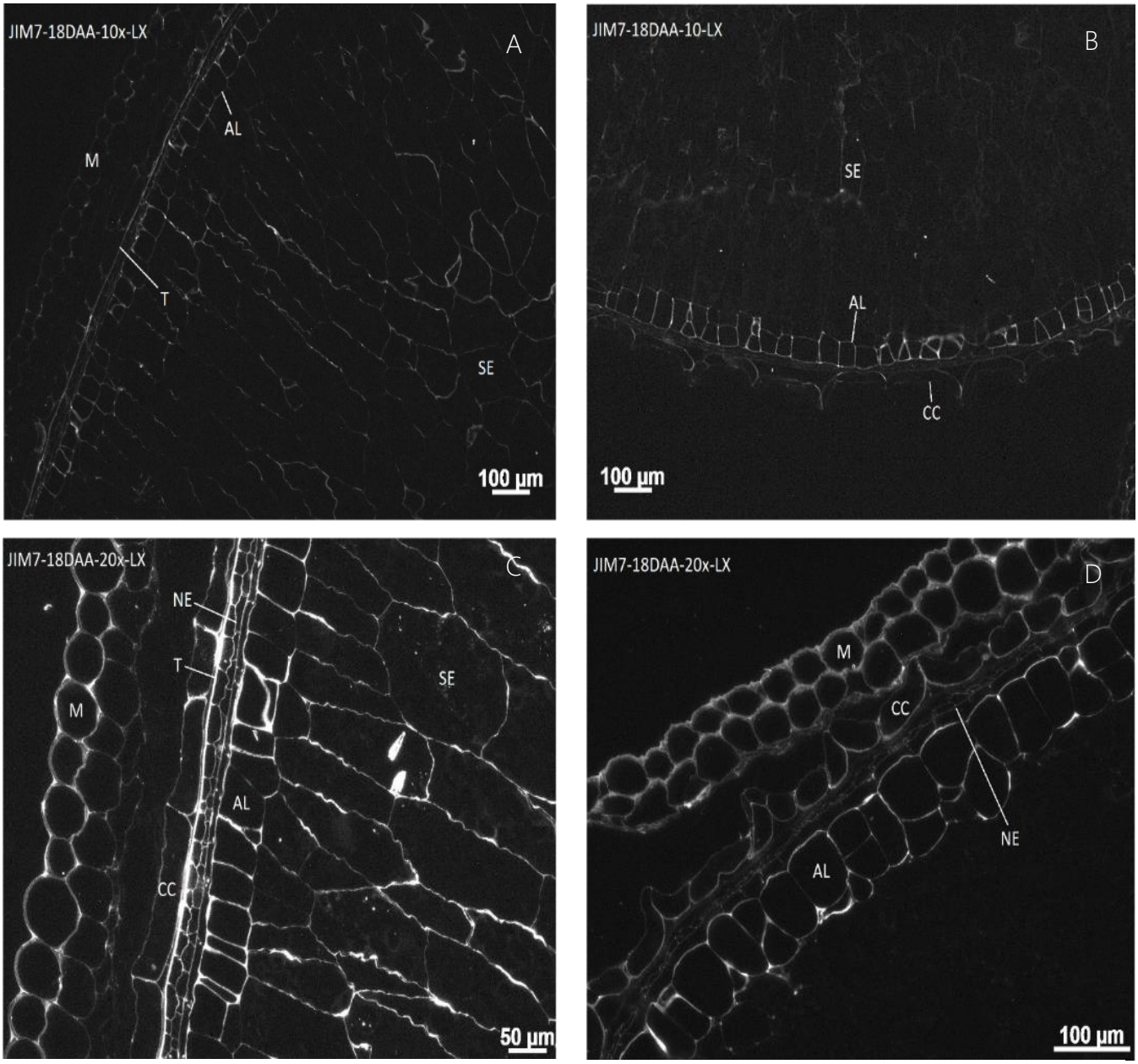


Figure 4.10 Images of control (A, C) and high p.a. temperature (35°C) (B, D) treated sections labelled at 18daa with JIM7 following a dual enzymatic treatment observed at 10x (A, B) and 20x (C, D) magnification and 1500ms exposure. (SE= starchy endosperm, AL= aleurone, NE= nucellar epidermis, CC= cross cells, T= testa, M= maternal pericarp).

LM19 binds to de-methyl-esterified HG and at 8daa labelling of the cross cells, nucellar epidermis, the innermost periclinal cell walls of the early aleurone and nucellar projection was observed in both high p.a. temperature treated and control grains (Fig.4.11).

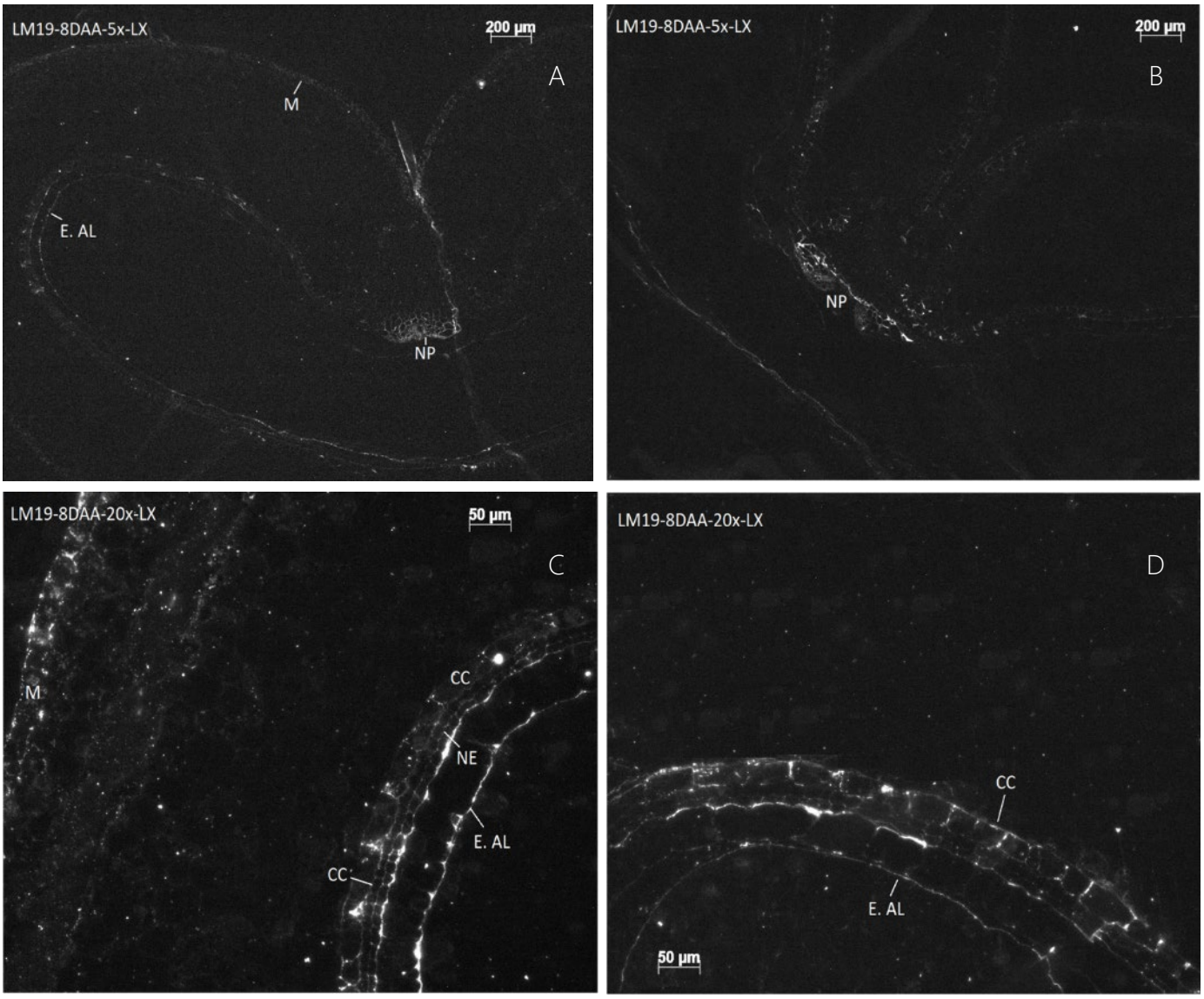


Figure 4.11 Images of control (A, C) and high p.a. temperature (35°C) (B, D) treated sections at 8daa labelled with LM19 following a dual enzymatic treatment observed at 5x (A, B) and 20x (C, D) magnification and 1500ms exposure. (VB= vascular bundle, NP= nucellar projection, E. AL= early aleurone, NE= nucellar epidermis, CC= cross cells, T= testa, MP= maternal pericarp).

At 14daa labelling with LM19 was observed in the pericarp and nucellar projection of sections subjected to high p.a. temperature treatment with weaker labelling of the same tissue observed in control sections. In both high p.a. temperature treated and control grain sections significant labelling was observed in the testa and small vesicles within the testa.

In addition, within control sections there was labelling with LM19 of the nucellar epidermis that wasn't observed in grain sections subjected to high p.a. temperatures (Fig.4.12).

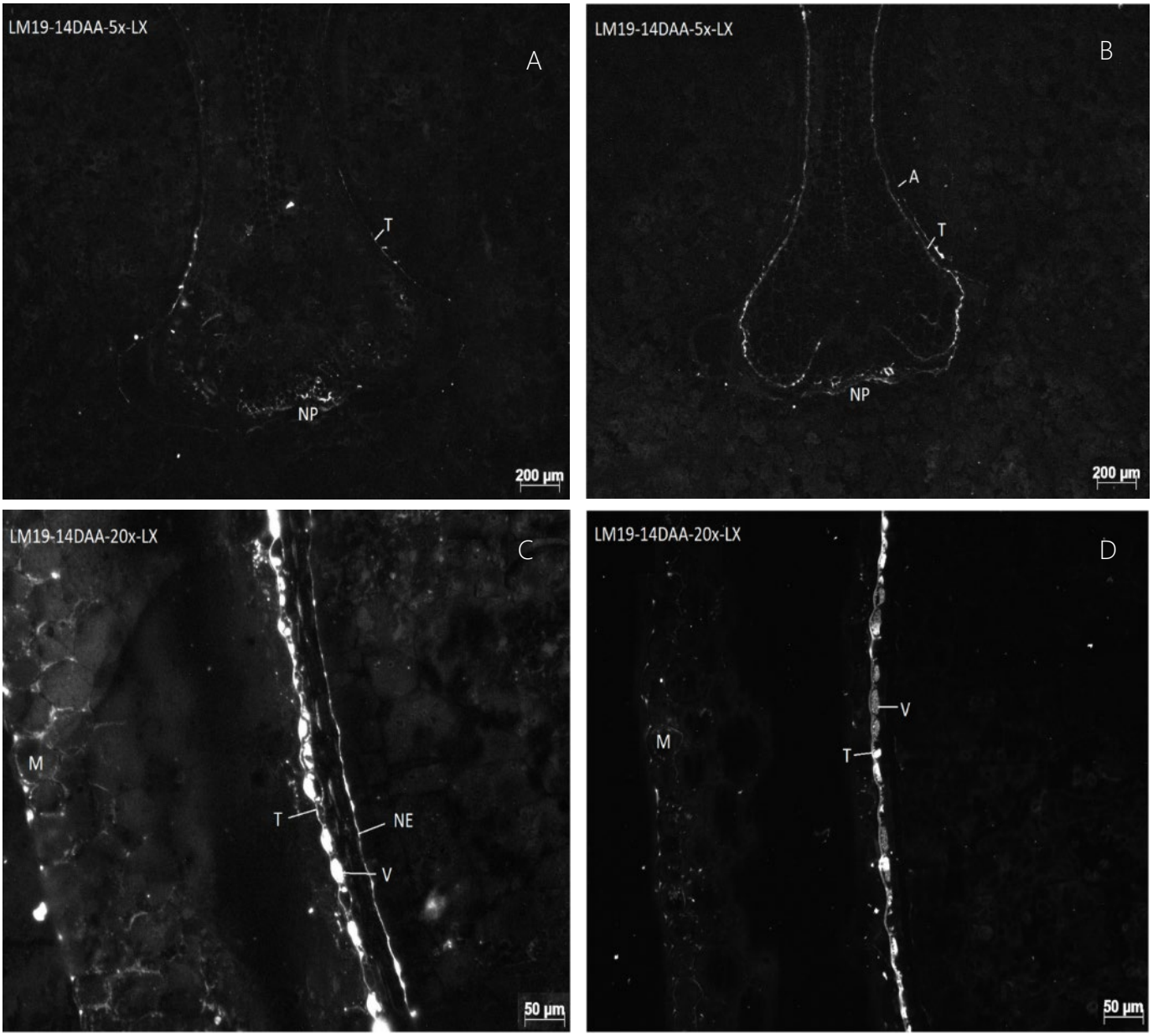


Figure 4.12 Images of control (A, C) and high p.a. temperature (35°C) (B, D) treated sections labelled at 14daa with LM19 following a dual enzymatic treatment observed at 5x (A, B) and 20x (C, D) magnification and 1500ms exposure. (NP= nucellar projection, T= testa, M= maternal pericarp, V= vesicle).

At 18daa, LM19 labelling was observed in both high p.a. temperature and control sections in the nucellar projection and the testa. In addition, there was sporadic labelling of the cell walls of the starchy endosperm cells in control grains (Fig.4.13).

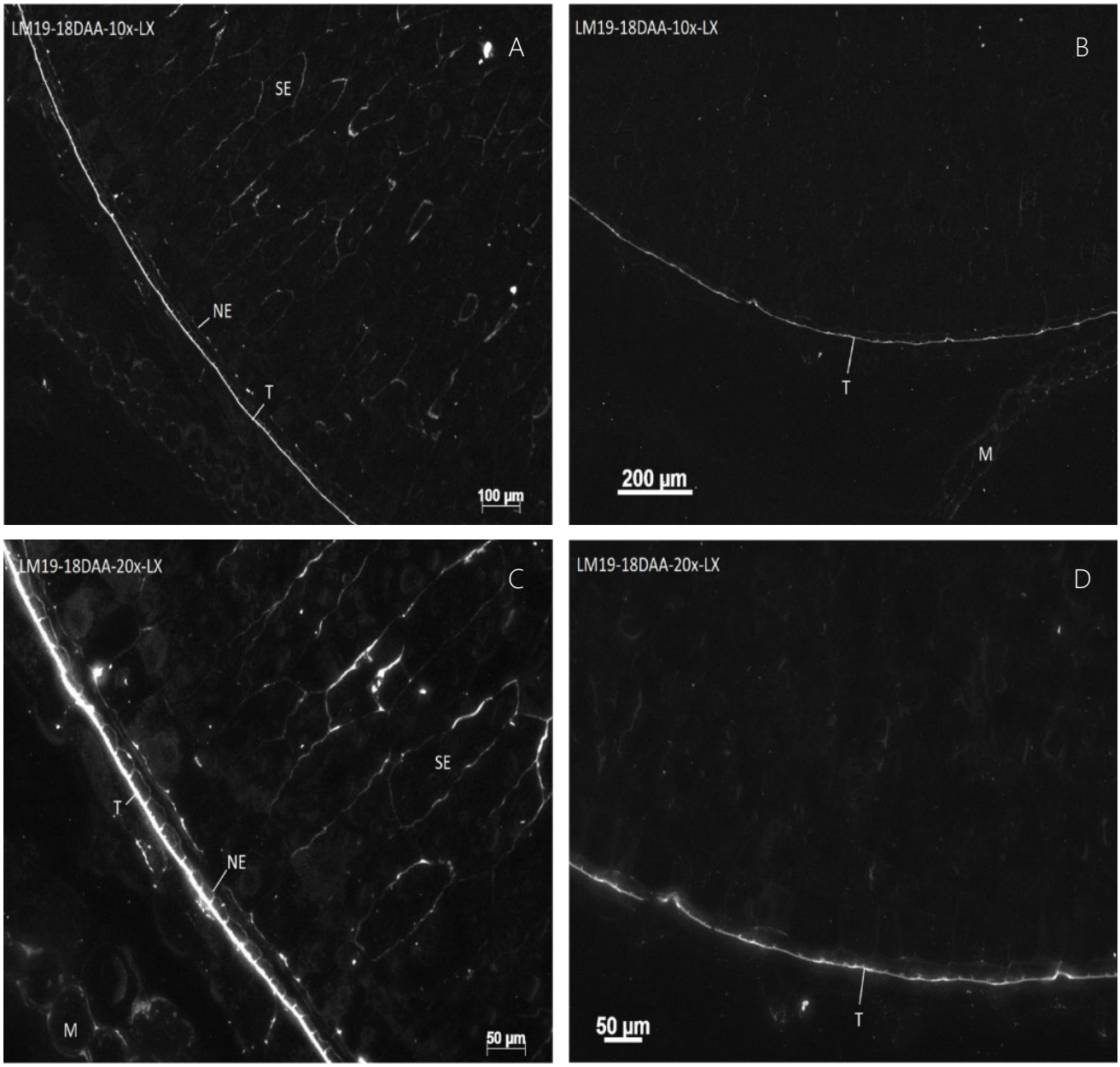


Figure 4.13 Images of control (A, C) and high p.a. temperature (35°C) (B, D) treated sections labelled at 18daa with LM19 following a dual enzymatic treatment observed at 10x (A, B) and 20x (C, D) magnification and 1500ms exposure. (AL= aleurone, T= testa, M= maternal pericarp, NE = Nucellar Epidermis).

These results compliment the findings of Palmer (2015) who observed strong labelling of the nucellar projection at 20daa in addition to the endosperm transfer cells and those cells nearest the crease cavity and Chateigner-Boutin *et al.* (2014) who observed strong labelling of the testa and sporadic labelling of the endosperm cells at 17daa.

Table 4.1 Antibodies used and immunofluorescence labelling results obtained in wheat grain tissues (C = Control temperature treated, S= High p.a. temperature treated, n.d= no digestion) *Although LM20 was used there was no labelling of sections

Antibody	JIM 7				LM19			
Epitope	Partially methyl-esterified HG				De-methyl-esterified HG			
Treatment	C (n.d)	C (LX)	S (n.d)	S (LX)	C (n.d)	C (LX)	S (n.d)	S (LX)
8daa (2-days of treatment)								
Pericarp	-/+	+	-/+	+	-	-/+	-	-/+
Nucellus epidermis	-/+	+	-/+	+	+	+	+	+
Testa	-/+	+	-/+	+	-/+	-/+	-/+	-/+
Endosperm	-	+	-	-/+	-	-	-	-
Early Aleurone	-/+	+	-	+	-/+	+	+	+
Cross Cells	-/+	+	-/+	+	-	+	+	+
Nucellar projection	-	+	-	+	+	+	-	+
14daa (8-days of treatment)								
Outer pericarp	+	+	+	+	-	-/+	-/+	-/+
Cross cells	-/+	+	-/+	+	-	-	-	-
Testa	+	+	-/+	+	-/+	+	+	+
Nucellus Epidermis	+	+	+	+	-	+	-	-
Aleurone	-	-	-	+	-	-	-	-/+
Starchy endosperm	-	+	-	+	-	-	-	-
Nucellar projection	-/+	+	-	+	-	-/+	-	+
18daa (12-days of treatment)								
Outer pericarp	-	+	-/+	+	-/+	-/+	-/+	-/+
Cross cells	-	+	-	+	-	-	-	-
Testa	+	+	-	-	+	+	+	+
Nucellus Epidermis	-/+	+	-	-/+	-	-/+	-	-
Aleurone	-	+	-	+	-	-	-	-
Starchy endosperm	-	+	-	-/+	-	+	-	-
Nucellar projection	-/+	+	-/+	+	-	+	-	+
(+) epitope detected (-) epitope not detected (-/+) weak epitope detection								

4.3 Discussion

This study sought to investigate the effect of high p.a. temperature on the sink capacity of the wheat grain and explore a possible link between sink capacity and the form and distribution of cell wall HG during grain development. The aim was to investigate whether the previously observed reduction in grain weight under high p.a. temperature (Chapter 3-3.2.1.2) was the result of reduced endosperm cell division and size and whether these may be affected by high p.a. temperature induced modification of cell wall HG. This study revealed that high p.a. temperature resulted in reduced endosperm cell numbers at 16daa and affected the distribution and methyl-esterification status of cell wall HG in different grain layers with implications for cell wall expansion that may affect final endosperm cell size.

4.3.1 Effect of high p.a. temperature on wheat grain sink capacity.

4.3.1.1 Endosperm cell number

In this study, a high p.a. temperature treatment of 10-days resulted in a reduced number of endosperm cells in grains sampled at 16daa supporting previous studies that also observed a reduction in endosperm cell number under high p.a. temperatures (Kaur *et al.* 2011; Yang *et al.* 2016). However, there was no significant difference in the number of endosperm cells between treatments at 12daa, and a loss of LM21 signalling, which binds to heteromannan, in the endosperm cell walls of high p.a. temperature treated grain at 25daa prevented comparisons between treatments being made at that stage. Therefore, our understanding into the effect of high p.a. temperature on the sink capacity of the wheat grain during development and how this relates to the observed reductions in mature grain weight in

experiment 2 (Chapter 3- 3.2.1.2) remain somewhat limited. However, the observed results still provide insight into the effect of high p.a. temperature on the sink capacity of the wheat grain and provide useful information to be taken into consideration for any repeat experiment using a similar method of analysis.

The lack of an effect of high p.a. temperature on endosperm cell numbers at 12daa has a number of possible explanations. Endosperm cell division in wheat has been shown to continue until around 16daa under normal growing conditions (Wardlaw 1970; Briarty *et al.*, 1979) and this was supported by the observation in this experiment that endosperm cell number was not significantly different between 16 and 25daa in control grains. It is possible that any negative effect of high p.a. temperature on endosperm cell number is not distinguishable at 12daa due to fact that endosperm cell division is still occurring in both control and high p.a. temperature treated grain. Endosperm cell division may end prior to or soon after 12daa in high p.a. temperature treated grain whilst continuing up until 16daa in control grain and additional sampling stages may have confirmed this. However, this observation could also be attributable to an increased duration of the high p.a. temperature treatment in grains collected at 16daa compared to 12daa (4-days longer). The observations of this experiment support that of Yang *et al.* (2016) in that they observed that endosperm cell numbers measured in two bread wheat cultivars *(cvs. Wennong 6 and Jimai 20)* subjected to high p.a. temperatures (~36°C) from 1-5daa did not begin to significantly differ between treatments until around 15daa in both cultivars. Therefore these results would seem to suggest that even with a range of high p.a. temperature treatment periods, e.g. 1-5daa in Yang *et al.* (2016) and 6-12daa for grains sampled at 12daa in this study, endosperm cell number may only begin to show differences around the 15-16daa stage.

Nicolas *et al.* (1984) observed in bread wheat *cv. Warrigal,* that the duration of endosperm cell division was shortened in wheat grains that had been subjected to an elevated high

p.a. temperature treatment (28℃/20℃) for 10days from either 3-13daa or 10-20daa. However, they also found that this shortened duration of cell division was compensated for by a higher rate of cell division, resulting in the final number of endosperm cells being unaffected by high p.a. temperature. Due to a lack of sampling stages prior to 12daa we are unable to say wherther the rate of endosperm cell division has been affected by high p.a. temperature treatment. Yang *et al.* (2016) used a higher temperature (>34℃) than Nicolas *et al.* (1984), applied from 1-5daa, and in contrast they observed a reduction in endosperm cell number and cell division rate. However, both Nicolas *et al's.* (1984) and Yang *et al's.* (2016) studies vary from ours in that their high p.a. temperature treatment was applied at an earlier stage. Whilst the observation that endosperm cell numbers at 12daa show no significant difference may suggest that the rate of endosperm cell division up to that point is the same in both treatments, it is possible that the rate of endosperm cell division has increased under high p.a. temperatures prior to this stage before potentially plateauing.

When this experiments results are compared against the observed reductions in mature grain weight in experiment 2 (Chapter 3 – 3.2.1.2), the durations of high p.a. temperature treatment experienced at 12 and 16daa here in experiment 3, are the same as the 6 and 10-day treatment duration in experiment 2, as both were applied at 6daa. The similar level of mature grain weight reduction between a 6 and 10-day high p.a. temperature treatment (Chapter 3 -Fig.3.1) and the non-significant difference between endosperm cell numbers at 12 and 16daa in high p.a. temperature treated plants according to comparisons of the LSDs of the means potentially indicate a causal interaction between the results observed in both experiments. However a repeat experiment that incorporated both the analysis of mature grain weight and endosperm cell number over an increased number of sampling stages,

following one high p.a. temperature treatment application would enable this to be investigated.

The loss of LM21 signalling from some endosperm regions in grains subjected to high p.a. temperature treatment at 25daa potentially indicates the accelerating effects of high p.a. temperature on grain development and the composition of the endosperm cell wall. Pellny *et al.* (2012) observed LM21 labelling of endosperm cell walls at 28daa in the same cultivar, *cv. Cadenza*, grown under similar temperature conditions to the control treatment in this experiment (18/15℃). Therefore, due to the observation that LM21 labelling was widespread in the endosperm cell walls at 25daa in control treated grain and the observations of Pellny *et al.* (2012), it appears that high p.a. temperatures potentially result in a reduction in the amount of heteromannan contained in endosperm cell walls and possibly the earlier maturation of endosperm cell development. Mannans have structural diversity based on their backbone structure and presence of galactose side chains. In addition, plant cell wall mannans have dual roles functioning as storage and structural polysaccharides. In a structural polysaccharide capacity, mannans perform a similar role to XYGs, crosslinking cellulose microfibrils in primary plant cell walls, and are structurally important in thickened walls, potentially determining the firmness and flexibility of the wall (Schröder *et al.*, 2009). Immunofluorescence labelling of wheat grains with LM21 beyond the p.a. stages sampled in this experiment and Pellny *et al.* 2012 (25 and 28daa respectively) would confirm the developmental profile of mannan expression in endosperm cell walls throughout the latter stages of wheat grain development.

As with experiment 4, wheat plants from experiment 3 experienced periods of high temperature exposure pre-anthesis (>40℃) that were not experienced by plants in experiment 2. Therefore, this potentially creates another limitation to the conclusions drawn. A potential mitigating effect of pre-anthesis high temperature exposure on

endosperm cell division has not been previously reported in the literature. In addition, in this experiment significant differences were observed between treatments applied p.a. on plant material that had experienced the same high temperatures pre-anthesis. Therefore, exposure of plants to high pre-anthesis temperatures may have had little effect on the results obtained in this experiment. This potential limitation also applies to the analysis of endosperm cell size and will be discussed further in subsequent chapters.

Whilst the relationship between endosperm cell number and mature grain weight under high p.a. temperature was not investigated in this study, previous studies have demonstrated that endosperm cell number is significantly correlated with final grain weight in both wheat (*cv.* Warrigal) (Nicolas *et al.* 1984) and barley (Cochrane and Duffus, 1983). It is therefore somewhat surprising that more focus has not been placed on increasing endosperm cell number in newly bred wheat varieties in order to increase yield. Most of the recent genetic gains in wheat yields, particularly in UK winter wheat varieties have arisen through enhanced grain numbers per spikelet and grains per spike (Shearman *et al.* 2005; Phillip *et al.* 2018). An incomplete understanding of the genetic mechanisms underlying endosperm cell number in wheat has contributed to a lack of selective breeding for this specific trait. However the previously shown close association between endosperm cell number and mature grain weight (Nicolas *et al.* 1984, Cochrane and Duffus 1983) in addition to the reduction in both traits observed in this project (experiments 2 and 3) suggest that this could be a potential mechanism through which to improve heat tolerance and yields in heat susceptible cultivars. The apparent lack of an effect of high p.a. temperatures on endosperm cell numbers until 16daa observed in this study therefore helps provide more information as to the response of wheat grain to high p.a. temperatures and provides a more specific window of p.a. development (e.g. 12-16daa) where further genetic studies can take place in order to investigate potential gene targets

that regulate endosperm cell number. This could ultimately lead to the development of wheat varieties that produce larger grains under high p.a. temperatures.

4.3.1.2 Endosperm cell size

There is little research concerning spatial and regional variation in cell size within the endosperm and how this may be affected by high p.a. temperature. A reduction in the average size of endosperm cells in the dorsal region of grains subjected to high p.a. temperature at 16daa may explain the earlier observation that wheat grain width was significantly reduced by high p.a. temperature at maturity (Chapter 3 – 3.2.3.). A reduction in endosperm cell size in the dorsal region of the wheat grain is likely to result in reduced expansion and growth in the width of the grain whereas fluctuations in the size of endosperm cells in the lobe regions of the grain may contribute to changes in both height and volume due to their spatial arrangement (i.e. contribute less than dorsal region endosperm cells across the width growth axis of the wheat grain). The observation that high p.a. temperatures affected endosperm cell size in the dorsal region of the grain is similar to findings by Morita *et al.* (2005) who observed that high p.a. night time temperatures resulted in reductions in the size of central endosperm cells in rice grains in the same region observed in this experiment. It was previously reported in this project (Chapter 3 – 3.2.4) that grain width was significantly reduced in grains subjected to a 4 day high p.a. temperature treatment from 6daa, when sampled at 14daa with a significant interaction between temperature treatment and daa. (Chapter 3 – 3.2.3). In contrast, grain length and grain height had no significant interaction between temperature treatment and daa, suggesting that width is the dimension most significantly affected by high p.a. temperature. An increased number of sampling stages for endosperm cell size may reveal a correlation between the two traits.

The cause of the reduction of dorsal region endosperm cell sizes at 16daa may be attributed to increased nutritional needs of the developing embryo within the grain under high p.a. temperatures. (Wallwork *et al.*, 1998) observed in malting barley *(Hordeum vulgare)* that high p.a. temperatures (35° C) applied for 5-days during grain filling resulted in an increase in embryo growth rate and size which they suggested may have been associated with an increase in nutrient availability from hydrolysis of endosperm cell contents below the dorsal aleurone. However, no such interaction has been reported to occur in wheat.

Another possible explanation regarding the observed differences in endosperm cell size at 16daa in the dorsal region of the grain relates to the process of endoreduplication. Endoreduplication is the process by which cells increase their ploidy and is initiated by the transition from the mitotic cell cycle to the endocycle where DNA replication occurs without subsequent chromosome separation and cytokinesis (Kobayashi, 2019). The process has been shown to be significantly correlated with endosperm cell size in rice (*Oryza Sativa L.*), with endosperm cells with higher ploidy levels being significantly larger than those with smaller ploidy levels (Kobayashi, 2019). High temperatures have been shown to negatively impact the process of endoreduplication in maize *(Zea mays L.*) (Engelen-Eigles, 2001). Engelen-Eigles *et al.* (2001) subjected maize to a high temperature treatment (35°C) of either 4 or 6 days duration at different developmental stages post pollination and observed that the most deleterious effect of the treatment on endoreduplication occurred during the mitotic phase of the endosperm cell cycle, restricting entry of the mitotic cells into the endoreduplication phase. It is possible that the observed reduction in endosperm cell size in the dorsal region of wheat grain at 16daa in this experiment may result from reduced endoreduplication rates in these cells under high p.a. temperatures. A potential explanation as to why this may occur in the dorsal region of the grain and not the lobe regions at this

stage, could be that gene controlled modifications and cessation of endosperm cell cycle activity has been shown to occur in the crease and central regions of the endosperm tissue prior to the peripheral areas of the endosperm (Drea *et al.* 2005). A study that investigates the effect of high p.a. temperature on endoreduplication in the endosperm of wheat grain and the relationship between this process and endosperm cell size may reveal more information as to the cause of the spatial variation of endosperm cell size under high p.a temperatures observed in this study.

4.3.2 Effect of high p.a. temperature on HG form and distribution

In this study the effect of high p.a. temperature on the distribution and structure of cell wall HG within the wheat grain was examined in grains obtained in experiment 2 in order to investigate whether any induced changes may affect endosperm cell size and potentially grain size and weight. Methyl-esters attached to the galacturonic acid (Gal-A) backbone of HG are believed to result in the loosening of plant cell walls through disrupting the formation of calcium bridges between HG molecules. In contrast, reduced methyl-esterification of HG is believed to result in cell wall stiffening (Wolf *et al.*, 2009; Chateigner-Boutin *et al.*, 2014).

However, recent reviews by Levesque-Tremblay *et al.* (2015) and Hocq *et al.* (2017) have suggested that the nature of HG methyl-esterification and the effect on cell wall dynamics are more complex. Levesque-Tremblay *et al.* (2015) cited papers by Markovič and Kohn (1984) and Moustacas *et al.* (1991) when suggesting that the reduced methyl-esterification of HG in cell walls can increase cell wall elasticity rather than decrease it and that the resulting effect is dependent on the mode of de-methyl-esterification (removal of methyl-esters) performed by pectin methyl-esterases (PMEs). One mode is the random de-methyl-

esterification of Gal-A residues by PMEs under low pH conditions (Hocq *et al.*, 2017). Random de-methyl-esterification by PMEs can free protons promoting the activity of polygalacturonases (Moustacas *et al.*, 1991) that in-turn break down pectin and contribute to cell wall loosening. A second observed mode of PME action is a linear 'blockwise' action of de-methyl-esterification of large stretches of Gal-A residues that allows for the formation of calcium bridges, facilitating the development of a strong gel-like structure. Hocq *et al.* (2017) in a similar manner to Levesque-Tremblay *et al.* (2015) cited a study by Peaucelle *et al.* (2015) who used atomic force microscopy (AFM) on *arabidopsis* hypocotyl and observed that a decrease in stiffness of the walls was associated with increased de-methyl-esterification of pectin. Hocq *et al.* (2017) suggested that a feedback mechanism exists in the action of PMEs whereby PME activity on HG results in an increase in cell apoplast acidity. Increased acidity in the apoplast can then decrease the activity of free PMEs and promote the random de-methyl-esterification of HG by PMEs leading to increases in cell wall elasticity. In addition, decreased pH results in increased activity of polygalacturonases that can contribute to decreased cell wall plasticity.

Therefore, it is apparent that the relationship between the degree of methyl-esterification of HG and cell wall plasticity is more complex than previously thought, with a high degree of control and interaction between HG and cell wall modifying enzymes such as PME and polygalacturonases. Further investigation into the effect of high p.a. temperature conditions on HG methyl-esterification in the cell walls of the wheat grain using AFM techniques and measuring intracellular pH levels would reveal whether the changes in HG methyl-esterification observed in this study under high p.a. temperatures are likely to result in increased or decreased cell wall elasticity.

In this experiment JIM7, a primary antibody that binds to partially methyl-esterified HG was revealed to be widely present in the cell walls of the endosperm of wheat grain sections at

14 and 18daa following enzymatic unmasking with lichenase and xylanase. The lower amounts of labelling of partially methyl-esterified HG in the endosperm of high p.a. temperature treated sections compared to control sections sampled at 18daa may indicate increased stiffening of the cell walls of endosperm. Palmer (2015) observed that endosperm cell wall labelling with JIM7 in *cv. Cadenza* grains grown in normal temperature conditions was widespread at 20daa. Therefore, a reduction in labelling with JIM7 in grains subjected to high p.a. temperatures suggests a premature reduction in the amount of partially-methyl-esterified HG within the cell walls of the endosperm that may potentially limit endosperm cell size in the later stages of grain development.

JIM7 labelling of high p.a. temperature treated grain sections at 14daa revealed different labelling patterns of the aleurone cell walls with no labelling observed in control grain sections. However, labelling of the aleurone cells in both high p.a. temperature treated sections and control sections were observed at 18daa. In addition, Palmer *et al.* (2015) observed labelling with JIM7 of the aleurone cells in *cv. Cadenza* at 20daa. Therefore, the presence of JIM7 labelling in aleurone cell walls of high p.a. temperature treated grains at 14daa appear to support an acceleration of the normal HG profile of the developing grain, although observations of further developmental stages would confirm this effect.

Despite the reduction of labelling with JIM7 in the cell walls of the endosperm under high p.a. temperature conditions at 18daa, there was no corresponding increased labelling of endosperm cell walls in high temperature treated grain sections treated with LM19 that one might expect to coincide with a decrease in the amount of partially-methyl-esterified HG. Therefore, this observation suggests that HG within the cell walls of the endosperm undergoes clearly defined stages of methyl-esterification and that a change in the amount of methyl-esterification of HG may not automatically ensure a corresponding change in the amount of labelling with a monoclonal antibody that signals the opposite methyl-

esterification status. Another possibility is that the total HG content of cell walls within the wheat grain is reduced under high p.a. temperatures. In a study analysing the total pectin content of a variety of food waste streams, Müller-Maatsch *et al.* (2016) also showed that through their analysis they were able to accurately determine the proportion of cell wall pectin that is HG as well as the predominant methyl-esterification status of this HG content. Using a combination colorimetric assays and NMR spectroscopy on extracted cell wall pectin they were able to determine the uronic acid content of the pectin from which they were able to deduce the proportion that is HG, and the acetyl and methyl-esterification status of the HG. By following a similar protocol to Müller-Maatsch *et al.* (2016), the total HG content of cell walls within wheat grain layers could be determined. This information would allow for us to confirm whether the observed changes in the HG profile of the wheat grain under high p.a. temperatures found in this study are the result of increased acceleration of the HG profile or a reduction in total HG content within the grain. In addition, such analysis could be used to assess the HG content between heat tolerant and susceptible wheat cultivars to determine whether the HG and methyl-esterification status differs significantly between the two and whether the make-up and content of this pectic domain is important in regulating the response of wheat yields to high p.a. temperature.

Reduction in the level of HG methyl-esterification in plant cell walls under several abiotic stresses has been suggested as a tolerance mechanism in order to prevent cell wall degradation under sub-optimal environmental conditions (Gall *et al.*, 2015). However, in the maintenance of cell wall integrity this modification is likely to result in decreased expansion capability within the wheat grain. Chateigner-Boutin *et al.* 2014 observed heterogenous labelling of *cv. Recital*, wheat grain tissue cell walls with LM20 and observed widespread labelling in the endosperm from 17daa until well into maturity. However, Palmer (2015) observed contrasting results from a similar developmental stage in *cv. Cadenza* where

labelling with LM20 labelling was observed only in the pigment strand and vascular bundle. The absence of any signalling with LM20 in this study that also uses *cv. Cadenza* from similar p.a. developmental stages is therefore unexpected. One possible explanation may be that a later stage of 20daa was sampled in Palmer (2015) compared to the latest stage sampled in this study i.e. 18daa. However, based on previous observations made in this study that high p.a. temperature appears to accelerate the cell wall composition profile, one may expect to see the same labelling in this study in high p.a. temperature treated grains at 18daa as observed in Palmer (2015) at 20daa. The lack of signal may be due to the effect of high p.a. temperature on HG within the cell walls of the grain in that any entirely methyl-esterified HG is removed. As previously suggested, analysis of total HG content from different grain layers and their methyl-esterification status may provide further support towards this hypothesis.

It was also observed that in high p.a. temperature treated samples at 18daa, JIM7 labelling was lost from the testa and nucellar epidermis whilst being present in the same tissues in control grain sections. The reduction in the amount of partially methyl-esterified HG in these two maternal seed layers under high p.a. temperature suggest a reduction in the plasticity of the cell walls of these two layers at 18daa that is not observed at 14daa. It is possible that this structural change induced by high p.a. temperature in the cell wall of a maternal grain layer at 18daa results in a reduction in the potential of endosperm cell growth as a result of increased resistance to turgor driven cell wall expansion due to the increased rigidity a loss of partially methyl-esterified HG would bring.

LM19 labelling at 14 and 18daa was mainly confined to the outer pericarp, nucellar projection and testa. At 14daa there was strong labelling of the testa and vesicles within the testa in both high p.a. temperature and control treated sections. This supports observations by Chateigner-Boutin *et al.* (2014) who observed large deposition of de-methyl-esterified

HG within vesicles below the testa at a similar stage p.a. (11daa). In addition, there was also stronger labelling of the nucellar projection in heat treated sections than in control sections at 14daa. At 18daa strong labelling of the testa in both high p.a. temperature treated and control sections was observed whilst labelling of the endosperm was observed in just control grains. The lack of de-methyl-esterified HG labelling in the endosperm cells walls of heat treated grain samples at 18daa compared to control sections does not fit with the trend of accelerated development under high p.a. temperature as endosperm cell wall labelling with LM19 was observed at later p.a. stages (23 and 40daa) in Chateigner-Boutin *et al.* and Palmer (20daa) (2015). Either labelling of endosperm cell walls with LM19 in grain subjected to high p.a. temperature occurs between 14 or 18daa in a relatively small developmental window, or the lack of labelling may indicate a cessation of cell wall restructuring in the endosperm cells of high p.a. temperature treated samples with de-methyl-esterified HG. Studies into the distribution of de-methyl-esterified HG across a wider range of p.a. stages would provide further information as to the effect of high p.a. temperature on HG methyl-esterification status within the endosperm cell wall.

The causes of the changes observed in this study, where high p.a. temperatures appear to result in a lack of signalling of LM19 in the endosperm and premature removal of partially methyl-esterified HG from the endosperm, testa and nucellar epidermis, need to be investigated. Based on knowledge of the role of PMEs and pectin methyl-esterase inhibitors (PMEIs) in modifying cell wall HG (Wolf *et al.*, 2009) it is most likely high p.a. temperature results in varying levels of expression of these two enzymes. However, the interaction between modified cell walls and sink storage capacity, in addition to the coordination and signalling mechanisms within the wheat grain's response to high p.a. temperature conditions is unknown. The breakdown of HG by polygalacturonases and pectate lyases are believed to result in the production of pectic oligogalacturonides (OGAs) (Cote *et al.*, 1998)

and this breakdown of HG is controlled by distinct patterns of de-methyl-esterification (Wakabayashi *et al.*, 2003). OGAs have been shown to act as signalling molecules counteracting the effects of auxin during plant development (Ridley *et al.*, 2001). Auxin is an important multi-functional hormone that has been shown to function in the regulation of the early endosperm development of maize and Arabidopsis, where plants with reduced auxin production showed impaired endosperm nuclear mitosis and migration (Forestan *et al.*, 2010; Bernardi *et al.*, 2012). Therefore, there may be a potential link between the observed reduction in endosperm cell number in this investigation and the changes in the labelling of cell wall HG. A future experiment that investigated the effect of high p.a. temperature on OGA production and cell wall HG methyl-esterification status from an increased range of earlier p.a. stages in *cv. Cadenza* would help determine whether such a relationship exists.

Grains sampled at 8daa following 2 days of high p.a. temperature treatment, displayed negligible change between treatments in LM19 or JIM7 labelling. This result may suggest that the HG observed in cell walls at 8daa is newly synthesised and therefore may not exhibit any differences in methyl-esterification status under high p.a. temperatures at this stage, as partially supported by Chateigner-Boutin *et al.* (2014) who observed that LM19 labelling wasn't apparent until around 11daa in *cv. Recital*. In addition, this observation may reflect the previous finding that 2-days of high p.a. temperature treatment was insufficient to negatively impact mature grain weight (Chapter 3 – 3.2.1.2). Further investigation into the effect of high p.a. temperature on cell wall HG in the early p.a. stages would help clarify the HG profile in early wheat grain development.

Despite the results of this investigation regarding the effect of high p.a. temperature on cell wall HG in the wheat grain, it is important to reiterate that pectin is still a minor component of the wheat grain cell wall (~5-6%) (Palmer 2014) and that HG comprises approximately

65% of this pectin content (Wolf *et al.* 2009). However, the presence of pectin in the cell walls of both rice and wheat endosperm suggest that it may be an important component of the cell walls of monocot endosperms. As suggested by Palmer (2014), the detection of pectin in cell walls of other cereal species endosperm would confirm the importance of pectin in the establishment and formation of endosperm cell walls in cereals. However, this investigation provides the first insight into the response of HG methyl-esterification status and distribution under high p.a. temperatures. Based on our knowledge of the function of HG in other species, i.e. facilitation of cell wall expansion and cell wall binding, modification of HG under high p.a. temperatures may have implications on grain development and final grain size. Therefore further investigation in to the function of HG in the cell walls of the wheat grain and the response of other pectic domains such as RG and XGA to high p.a. temperatures will reveal more about the function of pectin in the cell walls of wheat grains. Following on from the results of this study, the use of a variety of wheat cultivars with differing levels of thermotolerance in future experiments investigating the effect of high p.a. temperatures on HG, will help elucidate whether HG plays a role in determining grain size in wheat.

Finally, one structural observation was noted when comparing microscopy images between treatments at 14 and 18daa. There appeared to be morphological changes in the cross cells under high p.a. temperatures that have been associated with the beginning of the ripening phase in some of the earliest observations made on the tissue. Percival (1922), in a rare commentary on the morphology of cross cells, suggested that when adapting themselves to the shrinking grain that cross cells in the pericarp become 'crumpled' or bent leaving intracellular spaces below them. Crumpling of the cross cells was observed at 14daa and 18daa (Fig.4.9, Fig.4.10) in sections subjected to high p.a. temperatures but not in control treated sections. There are to our knowledge no other studies that have investigated the

morphology of cross cells under high p.a. temperatures in wheat grain. The physical change in the cross cells structure in the pericarp layer therefore may provide a structural indication of advanced grain development induced by high p.a. temperatures. Further investigation into the morphology of cross cells under high p.a. temperatures will provide more information regarding their significance in signalling altered grain development.

4.4 Conclusion

This study has shown using a novel method of endosperm cell counting that high p.a. temperatures imposed from 6daa for a period of 10-days result in a significant reduction in the number of endosperm cells within the wheat grain, limiting its sink capacity. In addition, endosperm cell size was shown to be significantly reduced within the dorsal region of the grain, potentially causing the previously observed reduction in grain width. A reduction in endosperm cell number and cell size under high p.a. temperatures are likely to contribute to the observed reduction in grain weight in wheat by shrinking the most economically significant component of the grain; the endosperm. When experienced, these responses in the endosperm to high p.a. temperatures may require changes to the end-use of bread wheats such as *Cadenza*, from milling to industrial and livestock uses in order to maximise economic return for producers. It was also observed that high p.a. temperatures resulted in differences in HG distribution and its methyl-esterification status within the different wheat grain layers with an overall trend of accelerating the HG profile of cell walls. The loss of partially methyl-esterified HG from the cell walls of the nucellar epidermis and testa under high p.a. temperatures may result in reduced expansion capacity of the cell walls of the pericarp which may in turn reduce endosperm cell expansion in the later stages of grain development. However, in order to determine whether changes in the methyl-esterification status of HG impacts endosperm cell division, further investigation into earlier development stages and the levels of hormones involved in regulating cell division is needed. This would

provide more information as to the interaction between high p.a. temperatures, cell wall modifications and grain size.

Chapter 5: Effect of high post-anthesis temperature on the transcriptome of the wheat grain

5.1 Introduction

Advances in transcriptomic analyses and technology have made high-throughput techniques such as RNA-sequencing (RNA-Seq) more accessible and affordable. RNA-Seq allows for a 'snap-shot' to be obtained of the gene expression in a sampled tissue at a given point in time. By comparing RNA-Seq data sampled from tissues under different treatments or developmental timepoints, it is possible to determine the effect of a treatment on the pattern of gene expression and compare gene expression between developmental stages. RNA-Seq and microarray profiling have previously been completed on wheat grain, both at the whole grain (Wan *et al.* 2008) and individual tissue scale (Pellny *et al.* 2012; Pfeifer *et al.*, 2014; Pearce *et al.* 2015) with exposure to high temperature being shown to accelerate the normal trends in transcriptomic expression which is consistent with the acceleration of overall grain development under high temperature (Altenbach and Kothari 2004; Wan *et al.* 2008).

It was previously observed in this project that a 4-day high temperature treatment of 35°C applied from 6daa for 10 hours per day, was sufficient to significantly reduce mature grain weight with no further reductions in grain weight observed in those plants subjected to longer durations of high temperature treatment (Chapter 3 - 3.2.1.2). Therefore, this

observation suggested that at around 10daa, following 4-days of high temperature treatment, that a physiological constraint on grain weight had been imposed by exposure to high p.a. temperature. In addition, It was observed in experiment 3 that a similar high p.a. temperature treatment resulted in a significant reduction in endosperm cell number that became apparent at 16daa (Chapter 4 - 4.2.1.1). However, the mechanisms behind this reduction in endosperm cell number were unclear with endogenous hormones such as cytokinin being a suggested as a possible control in addition to changing methyl-esterification levels of HG in the cell walls of the maternal layers. Therefore, in order to further investigate the cause of the observed reduction in mature grain weight following 4-days of high p.a. temperature treatment applied from 6daa, RNA-Seq analysis of grains subjected to high p.a. temperatures around this stage will provide more information about the genetic mechanisms underlying this effect.

The underlying hypothesis tested in this project is that high p.a. temperatures result in a premature maturation and loss of plasticity in the pericarp that may lead to physical constraint of the expansion of the endosperm. Such an effect may occur following the differential expression of genes that encode proteins involved in cell wall synthesis and expansion under high p.a. temperatures. Therefore, the aim of this experiment was to conduct RNA-Seq on grains subjected to either a high p.a. temperature or control treatment in order to obtain evidence that may support or contradict this hypothesis. The moisture content of a wheat grain has been previously shown to relate closely to its developmental stage, leading to suggestions that the water status of the grain acts as a major signal for control of grain development (Wan *et al.*, 2008). In addition, maximum grain moisture content has also been suggested as a reliable indicator of maximum grain volume (Egli 1998) which may in turn determine final grain dry weight (Schnyder and Baum 1992). In experiment 2, investigating the effect of high p.a. temperature on average mature

grain weight, grains were also collected from separate plants in another experiment and analysed for their fresh weight, dry weight and moisture content and different stages p.a. in order to determine a potential developmental window of interest where high p.a. temperatures resulted in significant differences in the moisture content of treated and un-treated plants. Such a window may indicate a shift in gene expression between the two treatments and an acceleration of development. These results will be discussed in this chapter alongside the reduction in mature grain dry weight in order to justify the p.a. time points chosen for transcriptomic analyses.

The availability of pre-existing transcriptomic profiles of individual wheat grain layers from both the pericarp (Pearce *et al.* 2015) and endosperm (Pellny, personal communication unpublished) enable interpretations to be made regarding RNA-Seq analysis performed on RNA extracted from whole wheat grains. Through identifying those transcripts that are likely to be predominantly expressed in one grain layer or another, and comparing whole grain data against these data sets, it is possible to filter RNA-Seq data from whole grain RNA and estimate the effect of a high p.a. temperature treatment on specific layers within the wheat grain with a greater degree of confidence. The differential expression of any genes involved in cell wall synthesis or modification in either the pericarp or endosperm and the stage at which any such differences may occur will reveal more about the transcriptomic response of wheat grain to high p.a. temperatures and whether high p.a. temperate does result in a loss of plasticity in the outer layers of the grain in *cv Cadenza.*

The experiments in this chapter therefore aimed to the hypotheses that: high p.a. temperatures result in a significant reduction of grain moisture content during development in *cv. Cadenza* and that significant differences in gene expression occur

between high p.a. temperature treated and control grain samples. Through testing these hypothesis the following experimental aims were targeted:

- compare RNA-Seq data collected from whole grains subjected to high p.a. temperature and control conditions against existing tissue specific data.
- determine the effect of high p.a. temperature on the developmental patterns of genes likely to be predominantly expressed in the outer pericarp or endosperm.
- identify genes with a potential role in cell wall modification that are differentially expressed under high p.a. temperatures in both the endosperm and outer pericarp.
- determine the effect of high p.a. temperatures on grain moisture content and dry weight accumulation

5.2 Results

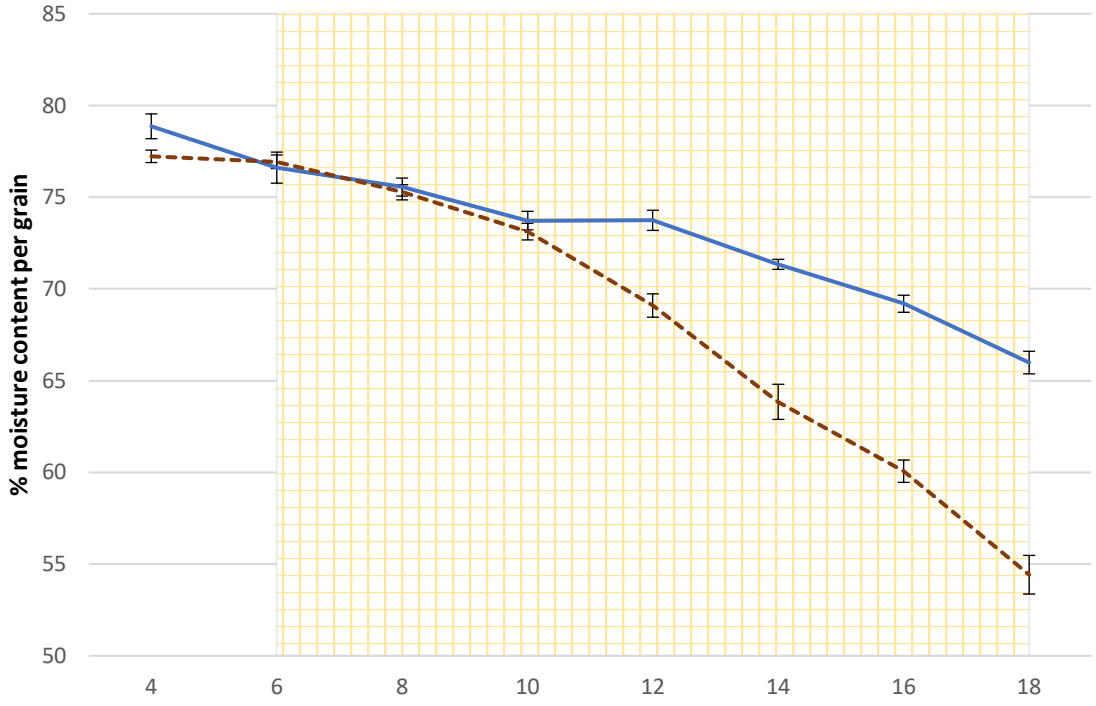
5.2.1 Effect of high p.a. temperatures on grain moisture and dry weight

In experiment 2, a pilot experiment was performed for a subsequent transcriptomic experiment in experiment 3. The aim of that pilot experiment was to determine a period during the development of the wheat grain where high p.a. temperature resulted in a significant difference in average grain moisture content between treated and untreated plants. Significant differences in grain moisture content would indicate a change in developmental progress of the wheat grain and help identify a candidate sampling period for transcriptomic analysis using RNA-seq. In addition, due to the close association of grain moisture content with grain volume (Egli, 1998), the point at which any differences occur could potentially indicate a restriction in the volume of the grain and the endosperm by the maternal layers. Percentage moisture content was also calculated in order to show the

relationship between dry matter accumulation and moisture content between treatments. Percentage grain moisture content was significantly affected by high p.a. temperature (ANOVA, df=1, f=309.30, p=<0.001)[1.] in addition to total grain moisture content (ANOVA, df=1, f=65.50, p=0.004)[2.] with a significant interaction effect of daa in both (ANOVA, df=7, f=28.03 [1.] 14.86 [2.], p=<0.001). Percentage grain moisture content was significantly reduced in high p.a. temperature treated grains from 12daa onwards (Fig. 5.1.A) whilst total moisture content was significantly reduced from 14daa onwards (Fig 5.1.B) according to the LSDs of the means. However, there was no significant difference between the dry weight of grains between treatments during the sampling period in this experiment (4-18daa) (ANOVA, df=1, f=1.12, p=>0.05).

Based on the results of this transcriptomic pilot experiment and the results of the other investigation in experiment 2 that demonstrated that exposure to 4-days of high p.a. temperature was sufficient to reduce mature grain weight when applied from 6daa (Chapter 3 – 3.2.1.2), the developmental time-points of 6, 10 and 14days were chosen for sampling in experiment 3 where grains were collected and whole grain RNA sampled. The observation that 4-days of high p.a. temperature exposure applied from 6daa was sufficient to reduce mature grain weight but that total grain moisture content did not begin to significantly differ until 14daa makes the developmental stages of 10 and 14daa good candidates for transcriptomic analysis. The delay in the effect of high p.a. temperature treatment on grain moisture content from the beginning of the high p.a. temperature application may indicate that the sensitivity of cell wall modifications to heat precedes the point at which they have an effect on endosperm expansion.

A.



B.

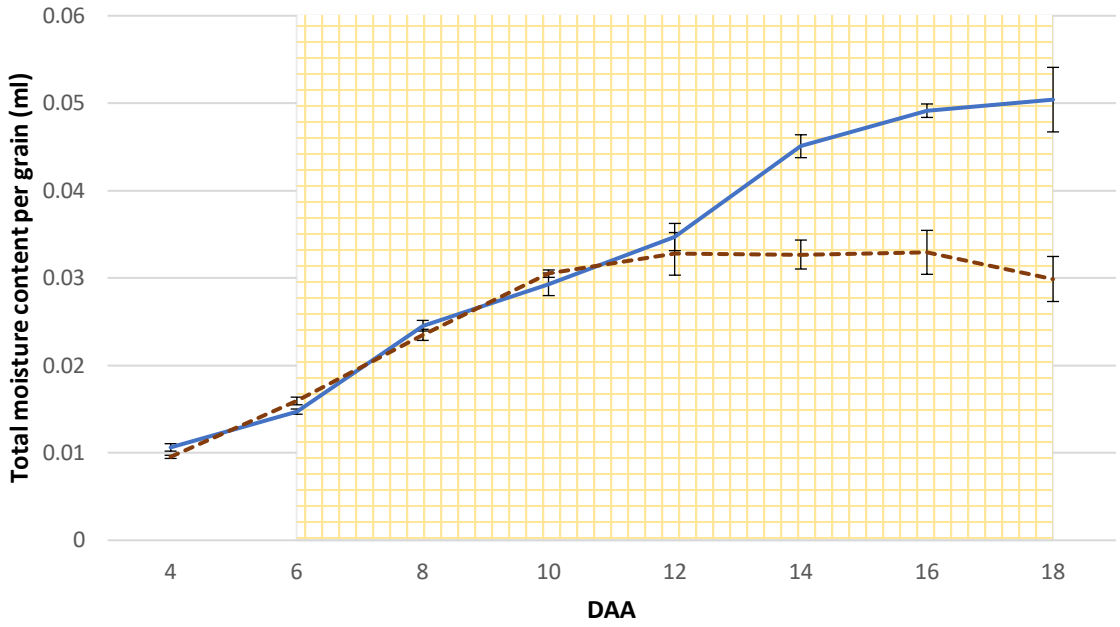


Figure 5.1 Average percentage moisture content (A) and total moisture content (B) of grains (n=16) collected from primary tillers exposed to high p.a. temperature treatment (35°C) from 6daa until 18daa in a pilot transcriptomic experiment. Error bars represent the standard error of the mean of 4 biological replicates.

A similar pattern for moisture content was observed, in experiment 3, where wheat grains were collected for total RNA extraction. Percentage grain moisture content was significantly affected by high p.a. temperature treatment (ANOVA, df=1, f=1048.70, p=<0.001) [1.] in addition to total grain moisture content (ANOVA, df=1, f=224.80, p=<0.001) [2.] with a significant interaction effect of daa in both (ANOVA, df=6, f=65.53 [1.] 34.82 [2.], p=<0.001). Percentage grain moisture content and total moisture content was significantly reduced in high p.a. temperature treated grains from 14daa onwards (Fig. 5.2.) according to the LSDs of the means.

In addition, there was no significant difference between high p.a. temperature treated and control grains in their dry weight (ANOVA, df=1, f=4.12, p=>0.05), however there was a significant interaction of daa between treatments (ANOVA, df=6, f=121.9, p=<0.001) with a significant difference occurring between treatments at the 26daa stage (Fig. 5.3). Therefore, grains were collected from plants subjected to 4 and 8daa of p.a. high temperature exposure (at 10daa and 14daa) respectively in addition to grains collected at 6daa that were not subjected to any treatment. The grains collected at 6daa were used as measures of early grain development whose transcriptional profiles could be compared against the grains collected at 10daa and 14daa. The results of these experiments into the effect of high p.a. temperature on grain moisture content in *cv. Cadenza*, therefore not only help determine a sampling window of interest for RNA-Seq analysis but also are in support of the experimental hypothesis that high p.a. temperatures result in a significant reduction in grain moisture content during development.

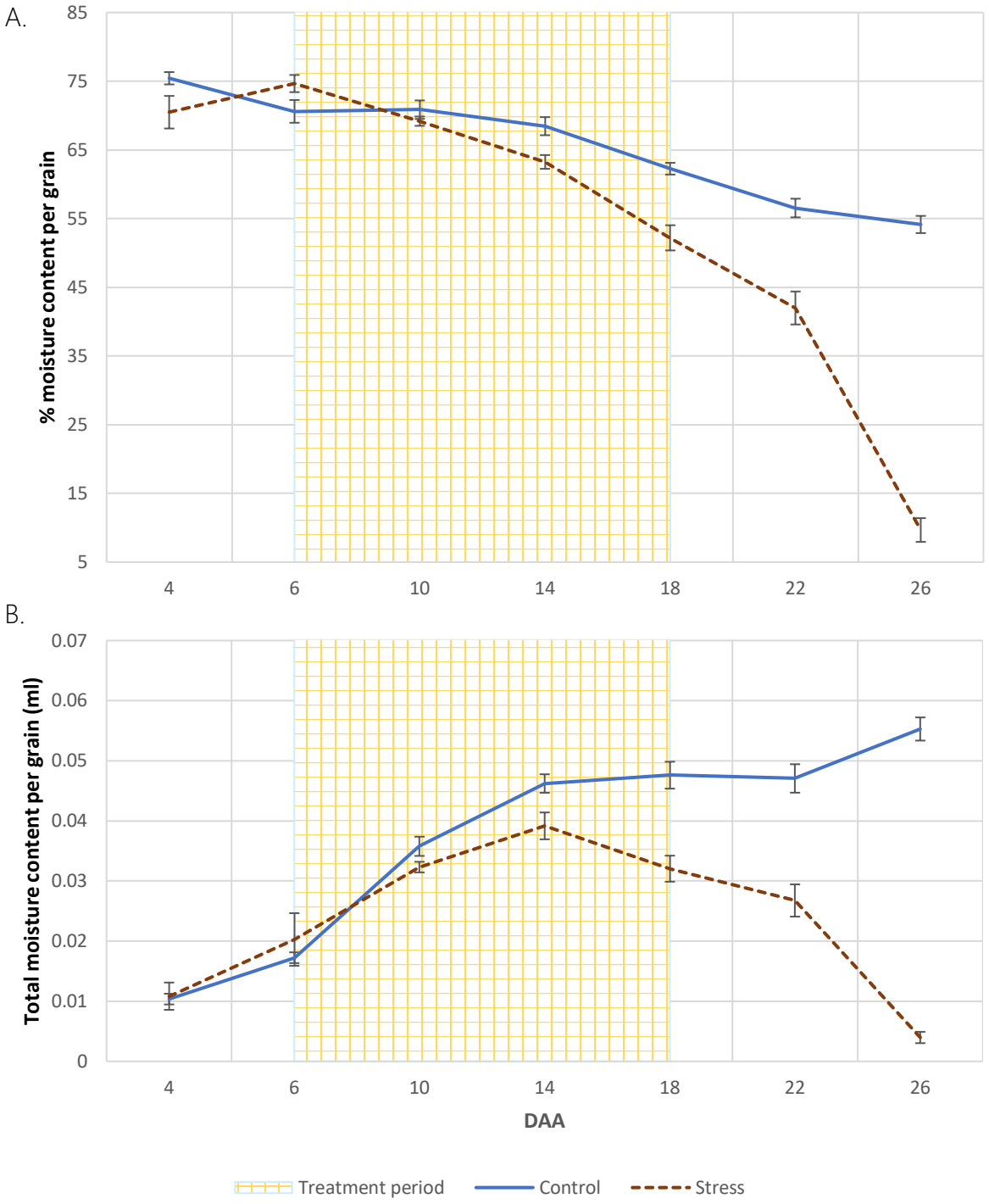


Figure 5.2 Average percentage moisture content (A) and total moisture content (B) of grains (n=16) collected from primary tillers exposed to high p.a. temperature treatment (35°C) from 6daa until 18daa in an experiment where grains were taken for transcriptomic analysis at 6,10 and 14daa. Error bars represent the standard error of the mean of 4 biological replicates.

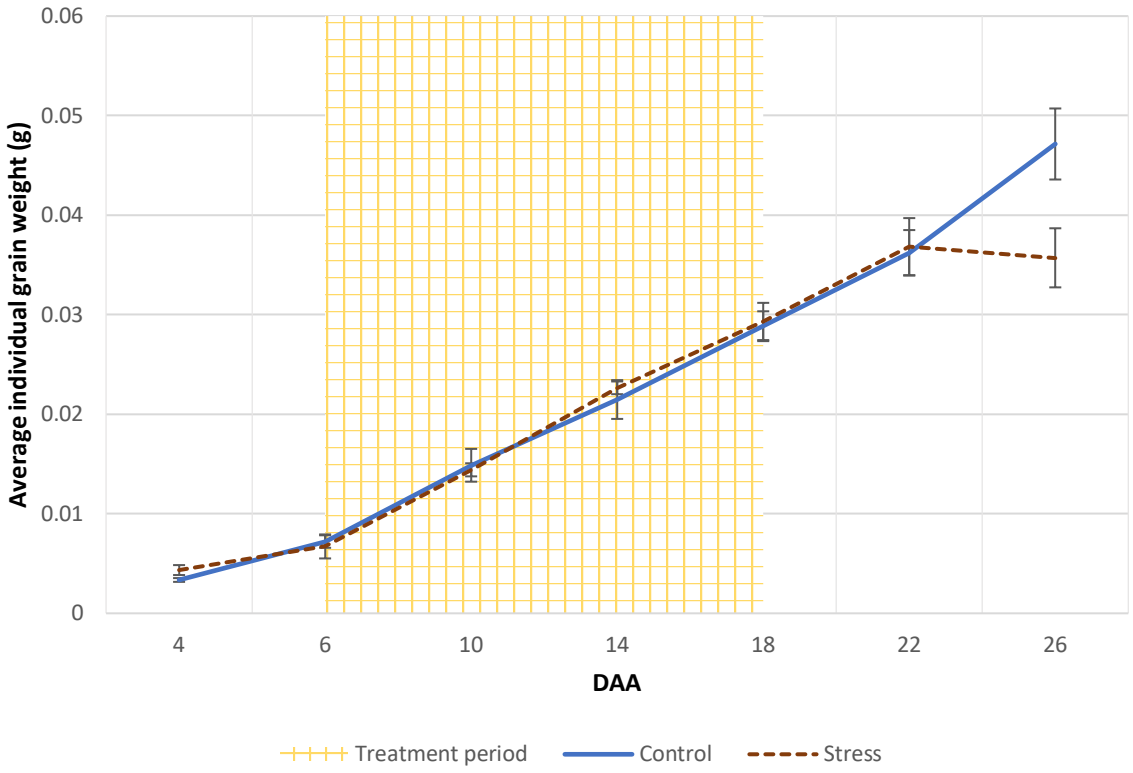


Figure 5.3. Average individual grain dry weight (n=16) collected from primary tillers exposed to high p.a. temperature treatment (35°C) from 6daa to 18daa in experiment 3. Error bars represent the standard error of the mean of 4 biological

5.2.2 Wheat grain transcriptomics: data quality and overall statistics

RNA-Seq reads from grain samples were mapped against the *T.aestivum* genome using the TGACv1 gene assembly produced by the Earlham Institute (Clavijo *et al.*, 2017). Read data underwent fastqc (Andrews 2010), and trimmomatic (Bolger *et al.* 2014), in order to remove adapters before undergoing gene alignment using HISAT2, an alignment program for mapping (Kim *et al.*, 2015) (Table 5.1).

Following mapping of the reads obtained from the RNA-Seq analysis, the number of reads aligned uniquely to each gene within the genome were counted using the featurecounts

programme (Liao *et al.*, 2014) in the R data analysis package (R, 2008). The data were then normalised to account for variation in sequencing depth and log counts per million (Log-CPM) was used as the measure of gene expression (Law *et al.*, 2014) as it is a widely used measure in transcriptomic experiments for absolute gene expression levels and can be compared across experiments.

Table 5.1 RNA quality scores and RNA-Seq statistics for 14 sequenced samples. RIN=RNA integrity number, S1, S2…=Stress sample, C1, C2…=Control sample. Raw reads = Number of 125BP paired end reads, Filtered reads = Number of pure reads filtered for adapters and bad reads, Overall alignment rate = Percentage of filtered reads that were successfully aligned to the reference genome.

Sample id	Sample id in Fig 5.4	RIN score	Raw reads	Filtered Reads	Overall alignment rate (%)
6daa_1	13.	4.5	6,654,796	6,611,383	90.76
6daa_2	14.	4.4	16,212,825	16,019,024	86.18
10daa_C1	10.	4.4	6,698,890	6,660,264	92.35
10daa_C2	12.	5.2	5,976,952	5,946,620	90.31
10daa_C3	11.	5.0	6,878,379	6,842,927	89.44
10daa_S1	7.	7.0	69,824,687	69,091,046	92.77
10daa_S2	9.	5.4	11,867,987	11,751,171	89.66
10daa_S3	8.	4.7	11,560,437	11,388,192	86.49
14daa_C1	4.	4.3	4,650,052	4,544,391	90.71
14daa_C2	5.	4.7	24,286,842	23,926,252	93.81
14daa_C3	6.	4.4	5,175,351	5,085,325	79.10
14daa_S1	1.	4.2	8,064,011	7,897,691	90.60
14daa_S2	3.	5.2	12,008,425	11,882,894	92.69
14daa_S3	2.	4.3	6,223,486	6,195,569	90.65

The data from the 14 analysed samples were then plotted using principal component analysis (PCA). The data showed clear grouping of samples in treatment groups, indicating similar levels of gene transcription between samples within the same treatment (Fig. 5.4) with PC1 (p.a. developmental stage) accounting for 78% of the total variance around the PCs and PC2 (effect of high p.a. temperature treatment) accounting for 9% of the total

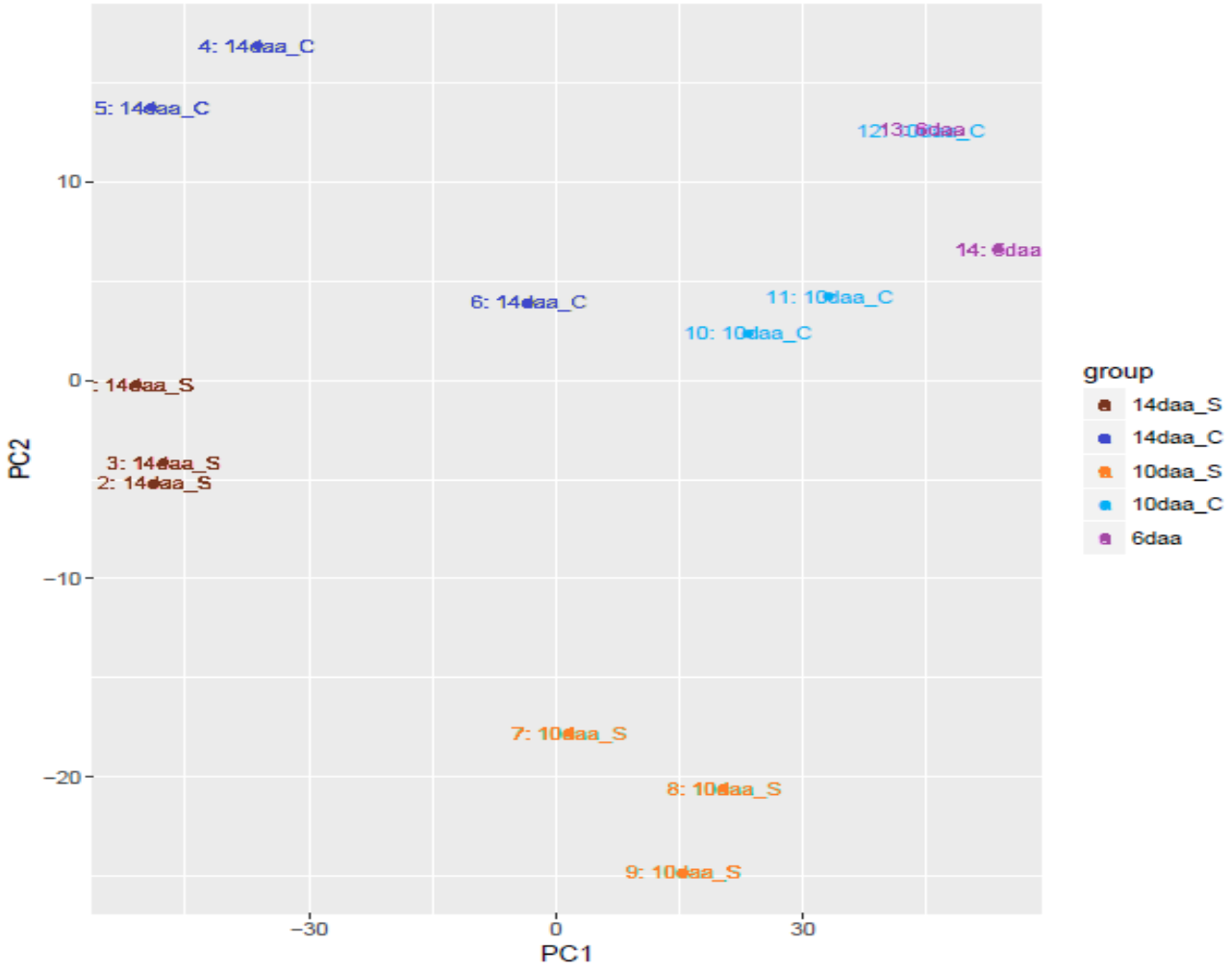


Figure 5.4 Principal component analysis of the transcript counts for the 14 samples analysed by RNA-SEQ. PC1 on the X axis captures the greatest variation (p.a. developmental stage) and accounts for 78% of the total variance around the PCs whilst PC2 on the Y axis captures the second greatest variation (effect of high p.a. temperature treatment) and accounts for 9% of the total variance around the PCs. Samples with similar transcription patterns group together.

variance around the PCs. Sample 7 (10daa_S1), which produced the library with the most reads and was the only sample to produce a RIN score of 7 prior to sequencing, grouped closely with the other two samples from the same treatment level. This indicated that despite the larger library, the transcription profile of those reads was similar to the other samples with smaller libraries Indicating that sequencing depth was not a major factor influencing expression patterns found. The significance of the variation in RIN scores between samples will be addressed in the discussion.

Points for control samples had lower PC1 values the later their daa stage, and treated samples had lower PC1 values than controls; indicating that PC1 is primarily reflecting grain development and that temperature treatment is accelerating development. This means expression of the set of genes used in PC1 respond mostly to temperature by accelerating development. The results of this PCA analysis therefore support earlier studies (Altenbach and Kothari 2004; Wan *et al.* 2008) that observed that high temperature accelerates development. PC2 separated control and high p.a. temperature treated samples completely suggesting that the expression of the genes in PC2 are affected by temperature in a different way than just accelerated development e.g. heat stress responses.

The number of differentially expressed genes (DEGs) was identified between the three sampled developmental stages independent of treatment using pairwise comparisons via the DESEQ2 statistical programme (Love *et al.*, 2014) and a false discovery rate (FDR) of 0.05. This analysis revealed that the largest number of DEGs (21476) occurred between samples at 10 and 14daa with 11288 genes significantly up-regulated and 10188 genes significantly down-regulated. Between 6 and 14daa there were 14845 DEGs with 7703 genes being significantly up-regulated and 7142 significantly down-regulated. Between 6 and 10daa, there were 2053 DEGs with 1060 genes significantly up-regulated and 993 significantly down-regulated (Table 5.2).

5.2.3 Effect of high p.a. temperature treatment on whole grain gene expression

The effect of high p.a. temperature treatment on DGE between the sampled developmental stages was analysed in order to test the hypothesis that high p.a. temperatures result in significant differences in gene expression between high p.a. temperature treated grain and control grain. At 10daa, 4981 DEGs were found between control and high p.a. temperature treated samples with 2349 genes being significantly up-regulated and 2632 genes being significantly down-regulated. At 14daa, there were 3652 DEGs between control and high p.a. temperature treated samples with 1996 genes significantly up-regulated and 1657 genes significantly down-regulated. Between high p.a. temperature treated and control sample averages across all developmental stages 12,857 DEGs were found (Table 5.2). Therefore these results supported the experimental hypothesis.

Table 5.2 Results of DEG analysis between contrasts using DESEQ2 and pairwise comparisons with an FDR of 0.05

Contrasts	Total number of DEGs	Number of significantly up-regulated genes	Number of significantly down regulated genes	Total DEG number as a % of known wheat genes, (107,891) (IWGSC 2018)
6 vs 10daa	2053	1060	993	1.90
10 vs 14daa	21476	11288	10188	19.90
6 vs 14daa	14845	7703	7142	13.75
10daa-C vs 10daa-S	4981	2349	2632	4.61
14daa-C vs 14daa-S	3652	1996	1657	3.38
C vs S (across all timepoints)	12857	*	*	11.91

There were a number of shared DEG between the three comparisons (Heat vs Control at 10 and 14daa and between heat vs control averages across all timepoints (6,10,14)) (Fig.5.5). The expression pattern of the most significant gene for each contrast was measured (Table 5.3) in order to determine the gene most affected by high p.a. temperature at each stage. Between high p.a. temperature treated and control samples at 10daa the most significant DEG encoded an ATPase whose expression was significantly increased under high p.a. temperatures (Fig.5.6). This gene was also the most significant DEG between the average expression values of high p.a. temperature treated samples versus control samples. At 14daa the most significant DEG transcribed an undefined, functional non-coding RNA whose transcription was significantly increased under high p.a. temperature treatment. (Fig.5.6)

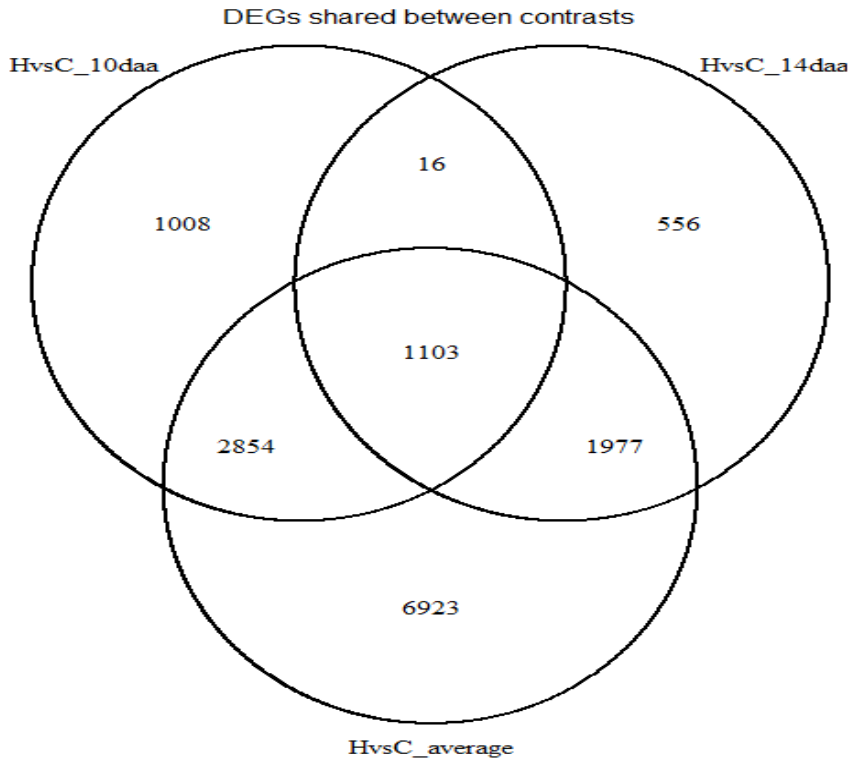
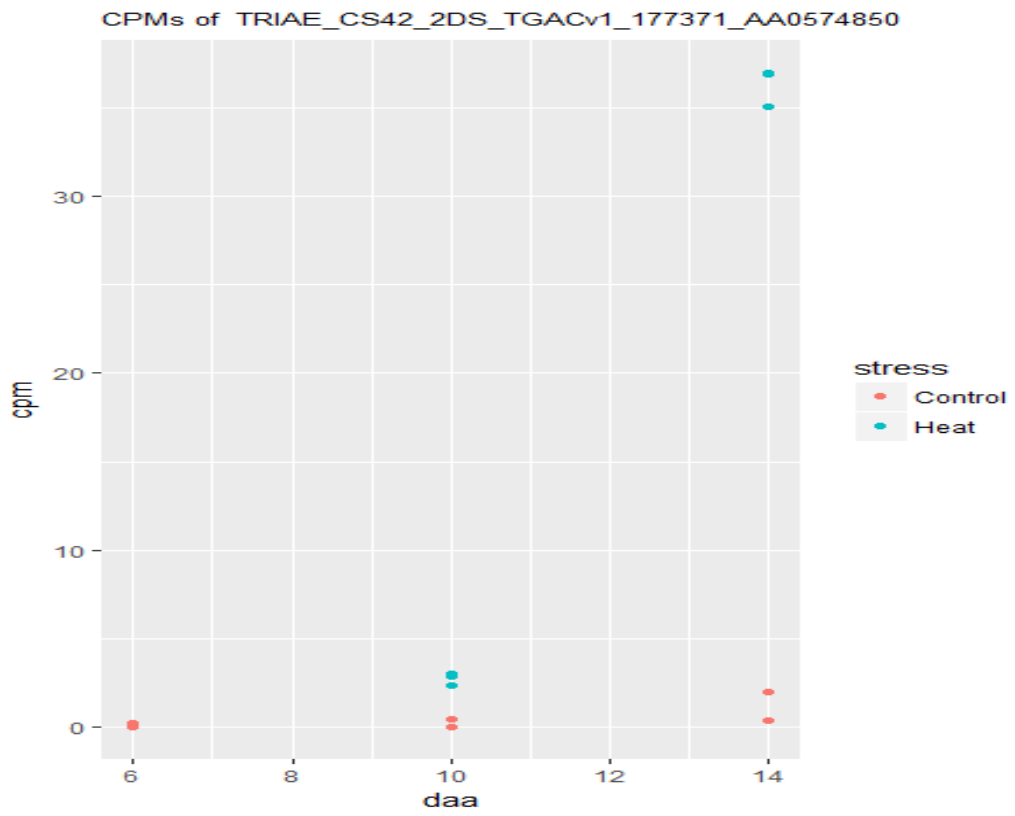


Figure 5.5 A Venn diagram showing the number of DEGs between treated (H) and control samples (C) unique to and shared between developmental stages 10 and 14daa and the average gene expression of samples across all developmental stages from treated and control

Table 5.3 Most significant DEGs between heat and control treated samples at different developmental stages. LogFC = Log fold change, LogCPM = Log counts per million, LR= Likelihood ratio, -Log10(p) = Log10 adjusted to the p value (0.05) FDR= False discovery rate

Contrast	Gene id	Protein encoded	LogFC	LogCPM	LR	-Log10(p)	FDR
Heat vs Control (10daa)	TRIAE_CS42_4DS_TGACv1_361664_AA1171230	ATPase	9.42309	5.91100	112.1152	3.37125	2.12479
Heat vs Control (14daa)	TRIAE_CS42_2DS_TGACv1_177371_AA0574850	Non-coding RNA (unknown function)	4.76441	3.15380	129.6499	4.88803	3.08078
Heat vs Control (Average)	TRIAE_CS42_4DS_TGACv1_361664_AA1171230	ATPase	8.16660	5.91100	238.4372	8.61921	5.43243

A



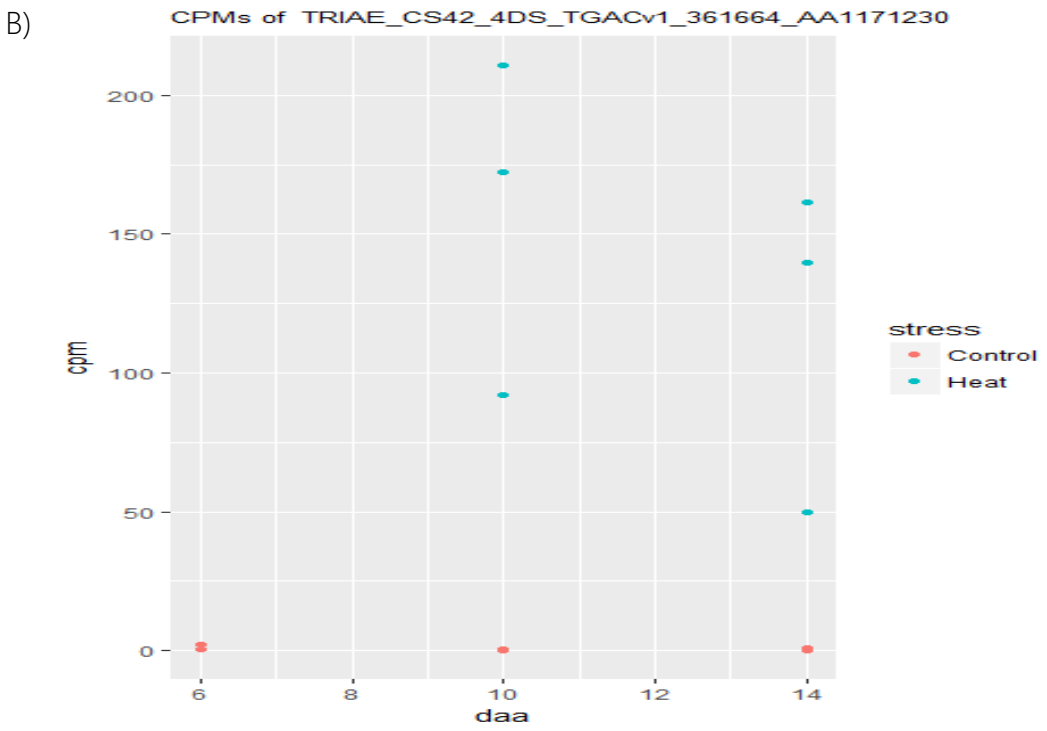


Figure 5.6 Expression values (counts/reads per million transcripts (CPM)) for the two most significant DEG at A) 10daa and B) 14daa. 3 replicates per developmental stage per treatment apart from 6daa (2 replicates).

5.2.4 Differential gene expression of genes associated with individual grain layers under high p.a. temperature treatment

In order to determine gene expression particular to an individual grain tissue, the RNA-Seq data collected from RNA extracted from whole grains were analysed for genes whose expression had previously been shown to be predominantly expressed in either the outer grain layers or endosperm. This specificity of expression information was derived from two experiments; (endosperm and outer seed/coat pericarp tissues collected at 12daa from bread wheat, *(cv. Holdfast),* grain grown under similar conditions (18/15°C day/night) (Pearce *et al.* 2015) (Fig.5.7) and pure starchy endosperm data from *cv. Cadenza* wheat grains (Pellny, personal communication *unpublished.)* also grown under similar conditions (18-20/15°C day/night).

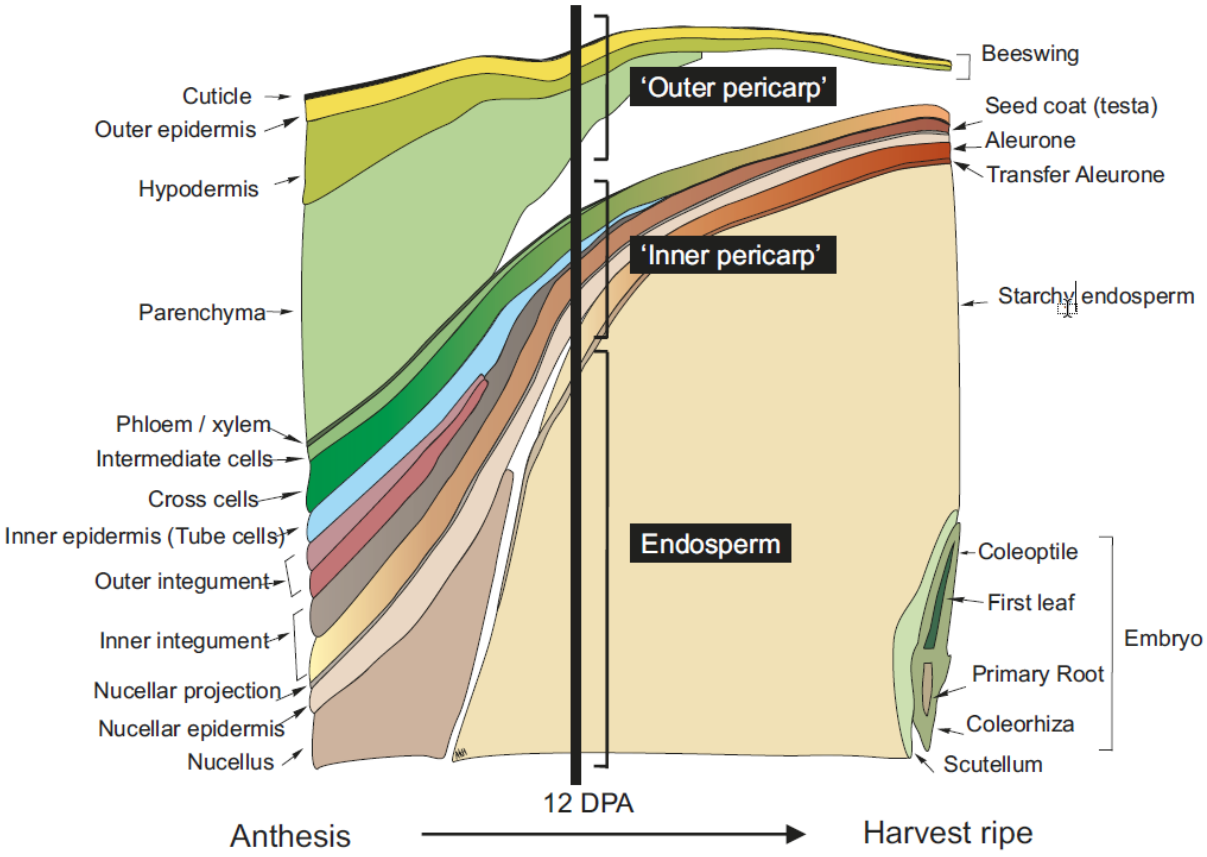


Figure 5.7 Diagram showing the three dissected groups of tissues (endosperm, inner pericarp and outer) and the wheat grain layers included within those groups that form the reference RNA-SEQ data sets ((Pearce *et al.*, 2015) the whole grain RNA-Seq data was compared against. Image reproduced with permission of the original owner, Dr. Allison Huttly, Rothamsted Research Institute.

Transcriptome data from the inner pericarp were not used due to the close-adherence of the aleurone layer to the inner pericarp at 12daa. This is likely to cause cross-contamination between the endosperm and inner pericarp tissues making identification of tissue specific gene expression difficult. Initial analysis of differential gene expression between the outer pericarp and endosperm RNA-Seq reference data sets (Pearce *et al.*, 2015) revealed 39823 DEGs between the two tissues. This list of genes was then filtered to identify DEGs that were

tissue specific, in that they were only expressed in either the outer pericarp or the endosperm. This filtering was performed by selecting DEGs with an expression value higher than 5 counts per million (CPM) in the tissue with the highest expression and a 8 log-fold change between the two tissues This filtering was performed in order to remove very lowly expressed transcripts which may have been affected by cross contamination between the two tissue samples. 1183 DEGs passed this filtering criteria to give two gene lists: an outer pericarp specific list of 638 genes and endosperm-specific list of 545 genes.

An additional filtering step was taken in order to confirm that none of the genes determined to be predominantly expressed in the outer pericarp were also expressed in endosperm. The list of outer pericarp predominant DEGs were contrasted with RNA-Seq data obtained from dissected pure starchy endosperm collected at 17daa (Pellny, personal communication *unpublished*). DEGs determined to be from the outer pericarp that had a measure of expression of more than 40 reads in 80 million in the starchy endosperm data list were assumed to be non-specific to the outer pericarp and were therefore discarded. 175 DEGs from the list of outer pericarp-predominant DEGs met this threshold therefore reducing the number of pericarp specific predominant DEGs from 638 to 463. Following these filtering steps, the list of DEGs for the outer pericarp did not contain any transcripts for glutenin or gliadin proteins, which are known to be unique to the endosperm.

The overlap of these final two tissue-specific lists (endosperm: 545 genes; outer pericarp: 463 genes) with the list of DEGs determined from between high p.a. temperature treated and control samples and developmental stages was then determined. This enabled the identification of genes likely to be predominantly expressed in either the outer pericarp or endosperm whose expression was significantly affected by high p.a. temperature and between developmental stages. Of the 463 DEGs determined to be predominantly expressed in the outer pericarp, 166, were differentially expressed at 10daa and 92

differentially expressed at 14daa of which 48 genes were differentially expressed at both 10daa and 14daa. Only 7 genes likely to be predominantly expressed in the endosperm (out of 545 DEGs) were differentially expressed at 10daa while 39 were differentially expressed at 14daa with 2 genes being differentially expressed at both 10 and 14daa. This shows that gene expression in the outer pericarp is more affected by temperature than gene expression in endosperm during this period of grain development, in line with previous findings (Wan *et al.*, 2008).

The number of DEGs between the two tissues that were significantly differentially expressed between 10 and 14daa with a significant interaction of heat treatment was then determined. DEG analysis performed on the RNA-Seq data that had not undergone any tissue-specific filtering revealed that there were 115 DEGs between samples at 10 and 14daa that also experienced significant differential expression between high p.a. temperature treated and control samples. When compared against the tissue specific outer pericarp and endosperm gene lists, of these 115 DEGs, 12 genes predominantly expressed in the outer pericarp and had a significant interaction with both developmental stage and heat treatment (Fig.5.8). In comparison, only 1 DEG was found in the endosperm predominant list, thought to encode a seed-type vacuolar processing enzyme, differentially expressed at the two developmental stages but whose expression was also significantly affected by treatment.

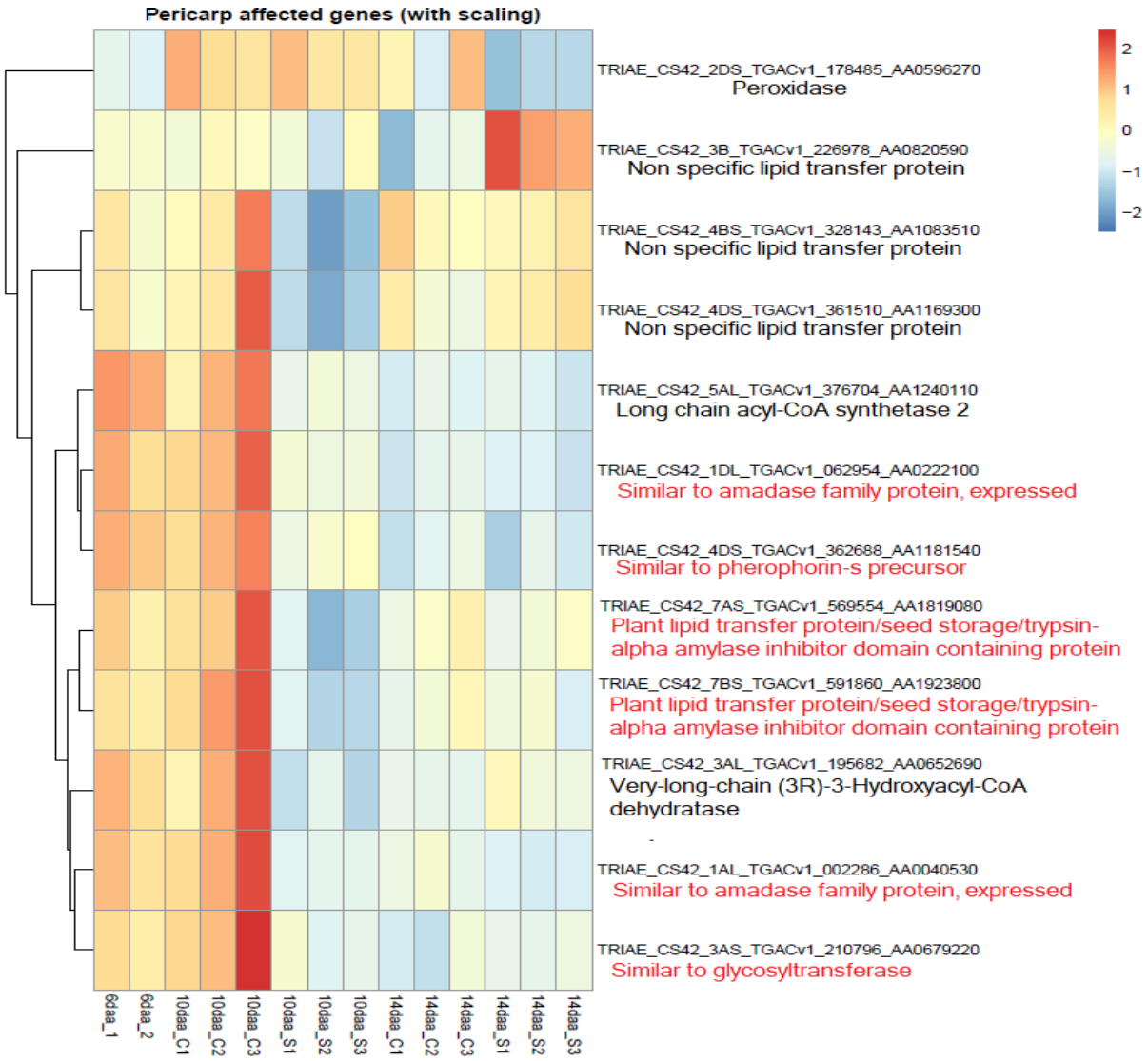


Figure 5.8 Heat map of significant pericarp specific DEGs between 10 and 14daa with a significant interaction of high p.a. temperature treatment. Rows are DEGs whilst columns are samples (C=control, S= stressed). Relative abundances have been scaled per row using z-score scaling and clustered globally in order to group genes with similar expression profiles. In order to scale via 'z-scores', read counts for a gene from the samples had the mean calculated and then subtracted from each sample score for that gene. The standard deviation is then calculated and then each data point divided by this standard deviation. This ensures that the expression data for a gene between samples are tightly grouped regardless of the variation resulting in fewer shades of colour being used. The scale bar represents fold-change in expression based on CPM values. Gene products in red represent available rice (*Sativa japonica, Oryza sativa)* orthologues where gene annotations were not available from the Wheat genome.

The above approach identifies the handful of genes that are significantly affected using stringent criteria, but to identify effects on expression profiles within the tissue-specific sets, it is more useful to look at gene clusters. A k-means clustering algorithm was performed in R studio (R studio team 2015) on the 463 outer pericarp predominant DEGs and 545 endosperm predominant DEGs in order to group these genes in terms of similarity of expression patterns across developmental time-points and between control and high p.a. temperature treatments. K-means clustering arranges a group of RNA-SEQ expression data based on each gene's expression mean across treatments which is then calculated and added to a group/cluster with a gene with the nearest mean. Following the formation of this cluster the new mean of that cluster is then recalculated. This process continues through all data points/genes until there is no more movement in the cluster means. This form of clustering allows for large groups of genes to be visualised in their expression patterns and for patterns to be discerned. The endosperm and pericarp predominant DEGS were then divided into 5 clusters each according to the k-means algorithm (Fig. 5.9).

A)

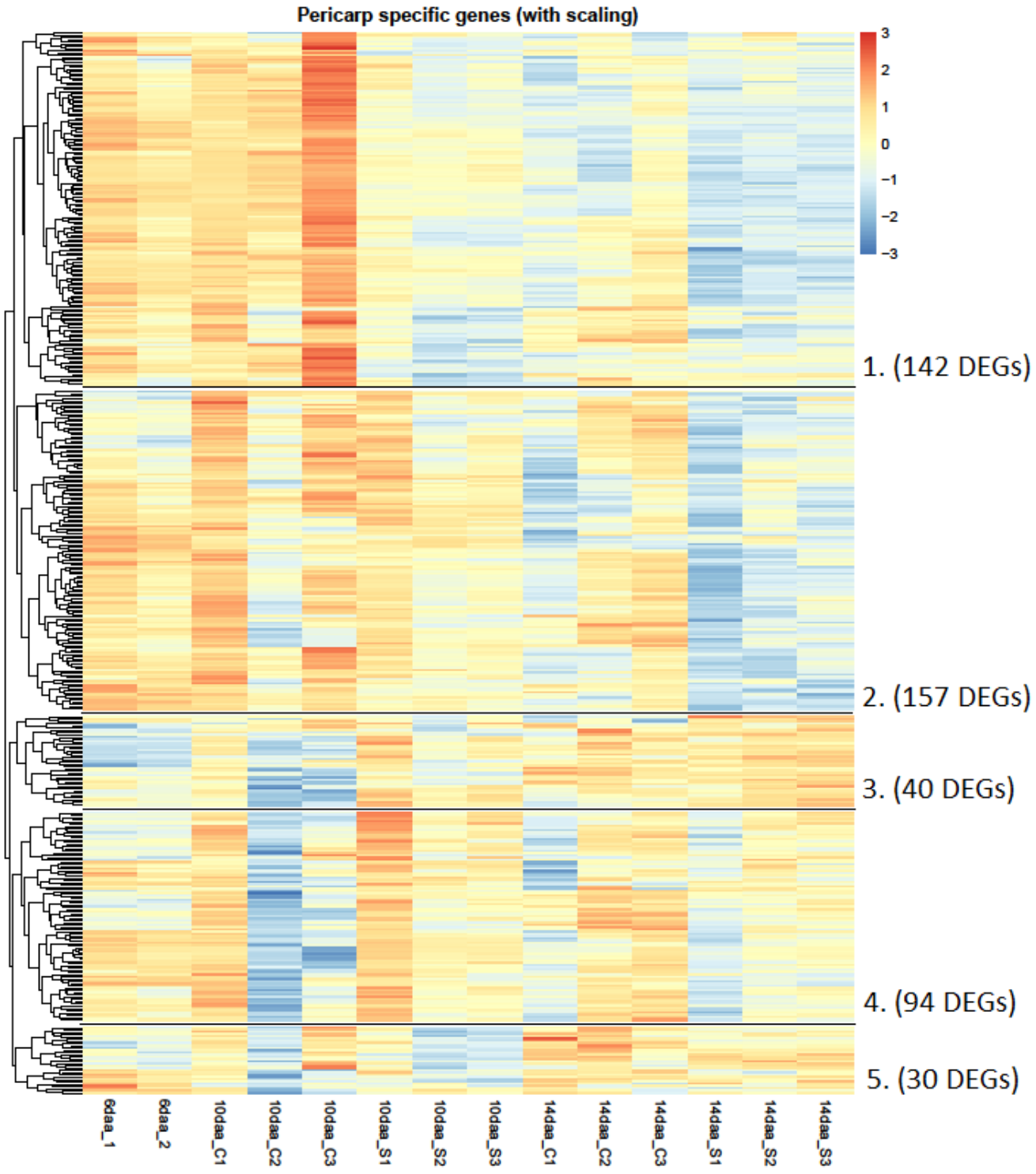


Figure 5.9. A) K-means clustering (n=5) of pericarp predominant DEGs (n=463). Relative expression per gene representative of Log CPM. Numbered sections of the heat-map represent the 5 gene clusters.

B)

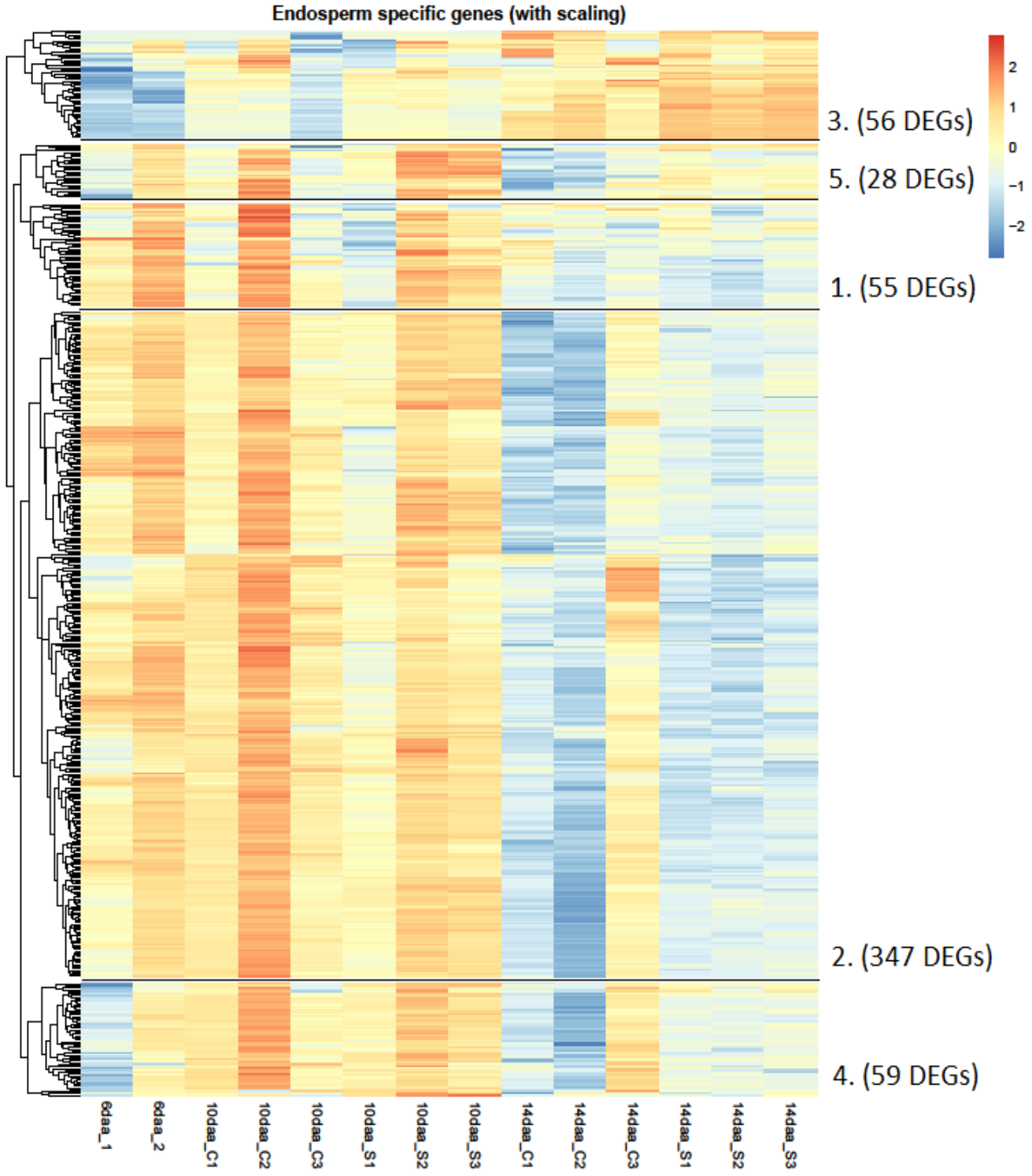


Figure 5.9 B) K-means clustering (n=5) of endosperm specific DEGs (545). Relative expression per gene representative of Log CPM. Numbered sections of the heat-map represent the 5 gene clusters.

CPM values were then collected for each gene from the pericarp and endosperm predominant list of DEGs from the RNA-Seq analysis performed in this experiment. CPM values per gene were calculated for each sample from the different developmental stages and treatments before being log transformed. The median of the log CPM values for each of the five clusters from both the pericarp and endosperm were calculated in each sample. The median value was chosen rather than the mean as this is often the preferred measure for gene clusters to avoid a large influence of extreme expression values.

5.2.5 Expression clusters from Pericarp and Endosperm specific gene lists.

To test the hypothesis that development is differently affected in pericarp and endosperm by high p.a. temperature, only gene expression clusters whose average expression changes between stages are useful. The median log CPM values for each cluster were compared between high p.a. temperature treated and control samples at 10 and 14daa using a 2-way ANOVA. There was a significant difference in expression values between those genes in outer pericarp cluster 2 between control and high p.a. temperature treated samples (2-way ANOVA, f=26.159, p=<0.001) and between 10 and 14daa (2-way ANOVA, df=1, f=24.20 p=<0.001,) with gene expression within this cluster down-regulated under high p.a. temperature at both 10 and 14daa. However, there was no significant interaction between the treatment and developmental stage on gene expression within the cluster (p=>0.05) (Fig. 5.10).

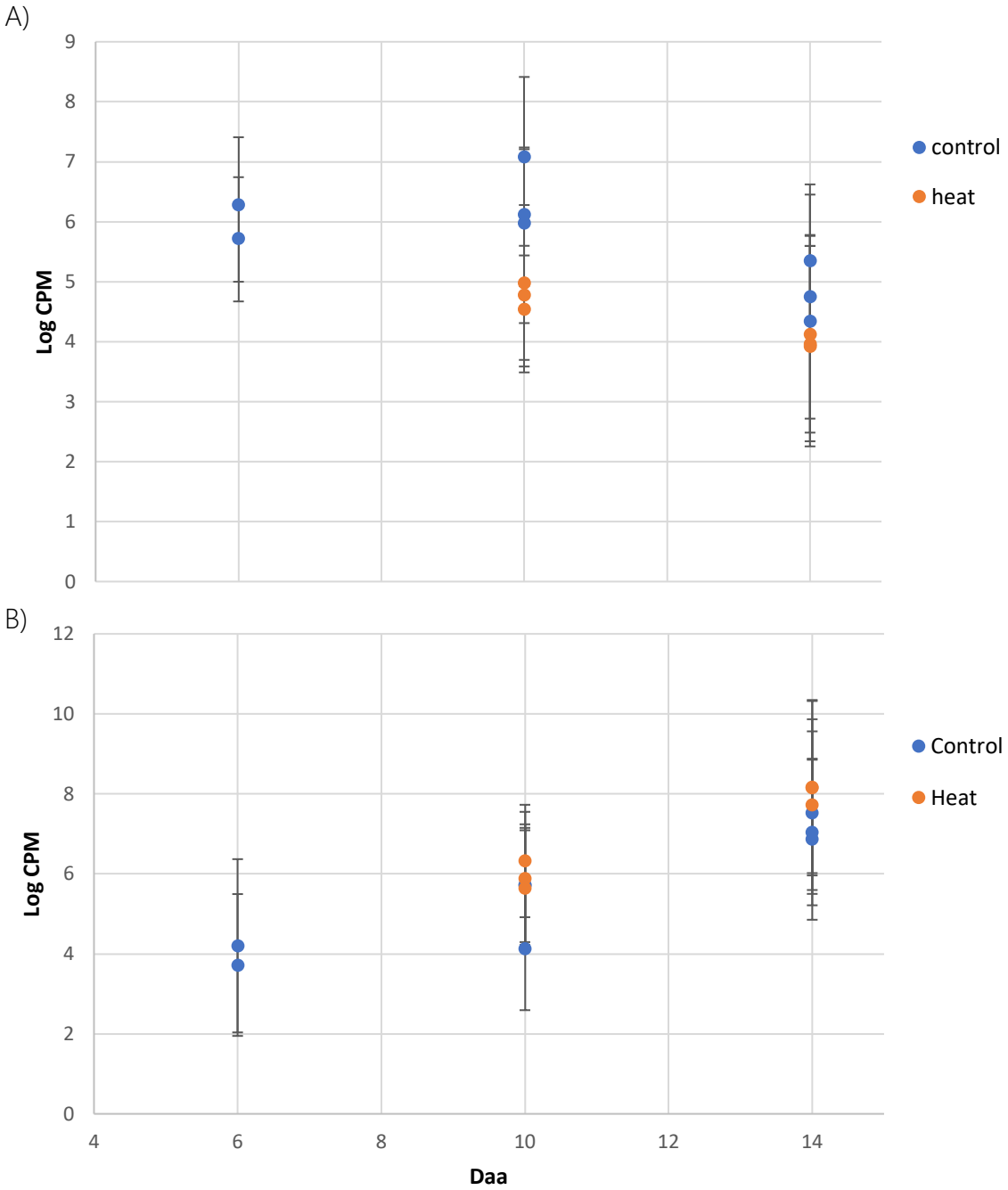


Figure 5.10 Median log CPM values from A) outer pericarp cluster 2 and B) endosperm cluster 3, between control and high p.a. temperature (35°C) treated RNA-SEQ samples at 10 and 14daa and in control treated samples at 6daa. The 6daa after anthesis stage was included as an early developmental point of reference. (Outer pericarp cluster 2 (n=157), endosperm cluster 3 (n=56). Error bars represent 1 standard error.

Expression of genes within endosperm cluster 3 was also significantly up-regulated under high p.a. temperature treatment (2-way ANOVA, df=1, f=6.98, p=>0.001) and between 10 and 14daa (2-way ANOVA, df=1, f = 42.35, p=>0.001) although there was no significant interaction between the developmental stage and the effect of treatment on the gene expression within the cluster (p=>0.05) (Fig 5.10).

It can be seen from the profiles that high p.a. temperature treatment at 10daa tends to resemble the 14daa control implying that expression declines earlier in outer pericarp cluster 2 and rises earlier in endosperm cluster 3 under high p.a. temperature. However, resolution of the data does not allow to say whether or not this acceleration is greater in the outer pericarp.

5.2.6 Gene ontology of significant pericarp and endosperm clusters

Through investigating the annotation of genes within clusters specifically affected by development and high p.a. temperature treatment, evidence can be gained on which processes, e.g. cell wall changes in pericarp, are accelerated by high p.a. temperatures. K-means clustering analysis allows for genes with similar expression patterns genes to be grouped. From these groups it is possible to perform Gene ontology analysis (GO) that allows for common functional pathways or biological processes that a group of genes may be involved in to be determined. Functional analysis of the genes within each cluster were performed using the Knetminer™ web tool developed by the Bioinformatics department of Rothamsted Research Institute (Hassani-Pak *et al.*, 2016). Knetminer is a suite of open-source software tools allowing for the integration and visualisation of large biological datasets. The tool also allows for the visualisation of functional pathways between specific

genes from a dataset in addition to identifying key genes from large datasets that are involved in a user specified function, e.g. cell wall modification.

The outer pericarp genes of cluster 2 were cross-referenced in the Knetminer software with the search term 'cell wall'. Of the 157 genes within the cluster, 120 of the genes registered a relevance score on the Knetminer knowledge database indicating an association of those genes with the term 'cell wall'. The relevance scores given to a gene by the Knetminer software indicate the strength of the association between a gene and the user specified search term with high scores representing a high association and low scores representing a low association. The relevance scores of the 120 outer pericarp genes with the search term 'cell wall' ranged from 0.05-548.00. From the 120 genes, those with the 10 highest relevance scores were investigated at greater detail (Table 5.4). Due to the fact that the relevance scores beyond these top 10 were significantly lower, these were excluded from further investigation for the purposes of this experiment. These genes have been previously shown to be directly involved in cell wall modification, assembly and functioning. In comparison, when the 56 endosperm predominant genes in endosperm cluster 3 were cross referenced with the term 'cell wall' 43 of the genes registered a relevance score, however the strength of the association between endosperm cluster 3 with the search term was lower with relevance scores ranging from 0.12-39.22.

Within the outer pericarp cluster 2 genes that registered the highest relevance scores with the search term 'cell wall', three of the genes contained encoded for endoglucanases, particularly within the GLU2 family. Endoglucanases break down cell wall cellulose and are involved in the remodelling of primary plant cell walls through the cleaving of β-glycosidic linkages of cellulosic chains resulting in polymer creep. In addition, other genes that scored the highest relevance scores with the search term 'cell wall' are also involved in the remodelling of the primary cell wall. β-EXPs, for example act in a similar fashion to

endoglucanases in that they disrupt linkages between cellulose microfibrils thereby facilitating expansion of cell walls.

GO term enrichment using Blast2GO™ (Conesa *et al.*, 2005) was also performed on the outer pericarp cluster 2 and endosperm cluster 3. In the analysis, the reference genome used in the RNA-Seq analysis of this experiment (TGAC_v1.) was compared against the genes within outer pericarp cluster 2 using Fischer's exact test. This analysis allowed for the statistical determination of overrepresentation of genes within the sample set to be deduced through comparison against all the available potential genes contained within the available reference genome. The analysis then references genes from the sample list against a database of GO-terms that represent a number of biological processes within plant development. In outer pericarp cluster 2, the biological process with the highest degree of gene overrepresentation was 'cuticle development' (GO id: 0042335). Table (5.5), (Fig.5.11). In endosperm cluster 3, genes involved in 'the killing of cells of other organisms' (GO id: 0031640) were the most significantly overrepresented genes (Table 5.6) (Fig. 5.12).

In order to compare the expression patterns of the genes attributed to the most significant GO terms from outer pericarp cluster 2 and endosperm cluster 3, between treatments, the expression pattern of these genes was compared against publicly available data from wheat-expression.com (Borrill *et al.*, 2016). Within outer pericarp cluster 2, six genes registered as being attributable to the GO term 'cuticle development' whilst within endosperm cluster 3, five genes were attributable to the GO term 'killing of cells of other organisms'. Each of these genes attributable to each GO term was analysed in the wheat-expression web tool and their expression values compared from available RNA-Seq data from analysis performed on whole grain from 15, 25 and 35daa (Barrero *et al.*, 2015).

The expression pattern of each gene was compared between the RNA-Seq data collected in this experiment against the available data of (Barrero *et al.*, 2015) who had performed RNA extraction on three seeds collected from five sets of near-isogenic lines (NILs) at 15,25,35daa (Fig 5.13-5.14). The RNA-Seq data at 15daa from Barrero *et al.,* (2015) was compared against the 14daa data collected in this project as these were considered comparable due to the similarity in developmental time. In addition, the RNA-Seq expression data from 25, and 35daa was analysed in order to have a developmental time frame of gene expression from 15daa-35daa to observe the normal pattern of expression for each gene during development.

In the endosperm cluster 3 subset, the five genes that were attributed to the GO term 'killing of cells of other organisms' were gradually up-regulated in the reference wheatexpression.com data set. High p.a. temperature treatment resulted in increased up-regulation of those genes compared to in control treated grains (Fig. 5.13). In the pericarp cluster 2 subset, the six genes attributed to the GO term 'cuticle development' had different expression profiles through grain development based on the wheat-expression data.,TRIAE_CS42_7DS_TGACv1_621594_AA2020330, TRIAE_CS42_7AS_TGACv1_569554_AA1819080 and TRIAE_CS42_7BS_TGACv1_591860_ AA1923800 appear to plateau between the 15-25daa stage before being upregulated at around 35daa, whilst the other 3 genes appear to be progressively down-regulated through grain development (Fig.5.14). Increased down-regulation following high p.a. temperature treatment results in lower expression of the 6 genes compared to control treated grains.

Table 5.4 Knetminer output of the ten highest relevance scores of genes from pericarp specific cluster 2 cross-referenced with the search term 'cell wall'. *Unknown protein encoded.

Accession number	Gene name	Protein encoded	Protein Function	Relevance score
TRIAE_CS42_4AL_TGACv1_289281_AA0968300	GLU2/KOR3	Endoglucanase	cell wall assembly	544.88
TRIAE_CS42_4DS_TGACv1_363068_AA1183220	GLU2/KOR3	Endoglucanase	cell wall assembly	544.88
TRIAE_CS42_4BS_TGACV1_330027_AA1105540	GLU2/KOR3	Endoglucanase	cell wall assembly	537.96
TRIAE_CS42_7DL_TGACv1_603404_AA1983010	XTH17	Xyloglucan endotransglucosylase	cell wall reconstruction and expansion	248.43
TRIAE_CS42_7AL_TGACv1_556014_AA1752680	XTH	Xyloglucan endotransglucosylase	cell wall reconstruction and expansion	234.03
TRIAE_CS42_1DL_TGACv1_062395_AA0213780	NIP1-2	Nod26-like-intrinsic-protein/Aquaporin	facilitate water transport in and out of cells	122.99
TRIAE_CS42_6AS_TGACV1_485265_AA1542080	EXPB5	Beta-expansin	facilitate cell wall expansion	82.19
TRIAE_CS42_3AL_TGACV1_195682_AA0652690	PRP2	proline rich protein	specify cell-type specific wall structures	47.57
TRIAE_CS42_U_TGACv1_641891_AA2106790	PAS2	n/a*	Involved in cell division and differentiation	47.37

Table 5.5. Top 20 Ranked GO term enrichment results from Fischer exact test of comparisons between the genes contained within pericarp specific cluster 2 and the reference wheat genome (TGACv1). Results were reduced to provide the most specific GO terms. Genes involved in cuticle development were the most significant overrepresented genes within pericarp specific cluster 2. FDR= Corrected p-value by False Discovery Rate control according to (Benjamini and Hochberg, 1995). P-value= p-value without multiple testing corrections., NR Test = Number of genes within test set (pericarp specific cluster 2) attributed to a GO term. NR Test = Number of genes within the reference set (TGACv1) genome attributed to the GO term.

Nr	Tags	GO ID	GO Name	GO Category	FDR	P.Value	Nr Test	Nr Reference	Non Annot Test	Non Annot Reference
1	OVER	GO:0042335	Cuticle development	BIOLOGICAL_PROCESS	4.288039E-10	5.355362E-14	6	9	151	103715
2	OVER	GO:0006869	Lipid transport	BIOLOGICAL_PROCESS	4.460791E-9	1.114223E-12	11	278	146	103446
3	OVER	GO:0008289	Lipid binding	MOLECULAR_FUNCTION	1.180415E-5	5.896914E-9	11	640	146	103084
4	OVER	GO:0010143	Cutin biosynthetic process	BIOLOGICAL_PROCESS	8.402359E-5	5.246883E-8	5	59	152	103665
5	OVER	GO:0031957	Very long-chain fatty acid-CoA ligase activity	MOLECULAR_FUNCTION	9.008642E-5	6.750575E-8	3	3	154	103721
6	OVER	GO:0008810	Cellulase activity	MOLECULAR_FUNCTION	1.633677E-4	1.428218E-7	5	73	152	103651
7	OVER	GO:0030245	Cellulose catabolic process	BIOLOGICAL_PROCESS	1.728555E-4	1.727044E-7	5	76	152	103648
8	OVER	GO:0004467	Long-chain fatty acid-CoA ligase activity	MOLECULAR_FUNCTION	8.695962E-4	1.520463E-6	3	12	154	103712
9	OVER	GO:0008171	O-methyltransferase activity	MOLECULAR_FUNCTION	8.695962E-4	1.49887E-6	6	222	151	103502
10	OVER	GO:0046658	Anchored component of plasma membrane	CELLULAR_COMPONENT	3.699999E-3	8.31772E-6	7	462	150	103262
11	OVER	GO:0050832	Defense response to fungus	BIOLOGICAL_PROCESS	3.969823E-3	1.239485E-5	6	324	151	103400
12	OVER	GO:0015689	Molybdate ion transport	BIOLOGICAL_PROCESS	3.969823E-3	1.202201E-5	3	26	154	103698
13	OVER	GO:0010311	Lateral root formation	BIOLOGICAL_PROCESS	3.969823E-3	1.202201E-5	3	26	154	103698
14	OVER	GO:0015098	Molybdate ion transmembrane transporter activity	MOLECULAR_FUNCTION	3.969823E-3	1.202201E-5	3	26	154	103698
15	OVER	GO:0001676	Long-chain fatty acid metabolic process	BIOLOGICAL_PROCESS	4.61596E-3	1.787121E-5	3	30	154	103694
16	OVER	GO:0009505	Plant type cell wall	CELLULAR_COMPONENT	1.142783E-2	5.28075E-5	6	422	151	103302
17	OVER	GO:0071555	Cell wall organisation	BIOLOGICAL_PROCESS	1.993692E-2	1.095662E-4	9	1209	148	102515
18	OVER	GO:0009809	Lignin biosynthetic process	BIOLOGICAL_PROCESS	2.694615E-2	1.649009E-4	3	66	154	103658
19	OVER	GO:0006633	Fatty acid biosynthetic process	BIOLOGICAL_PROCESS	3.237343E-2	2.142865E-4	6	548	151	103176
20	OVER	GO:0016746	Transferase activity, tnrasferring acyl groups	MOLECULAR_FUNCTION	3.841486E-2	2.638712E-4	10	1672	147	102052

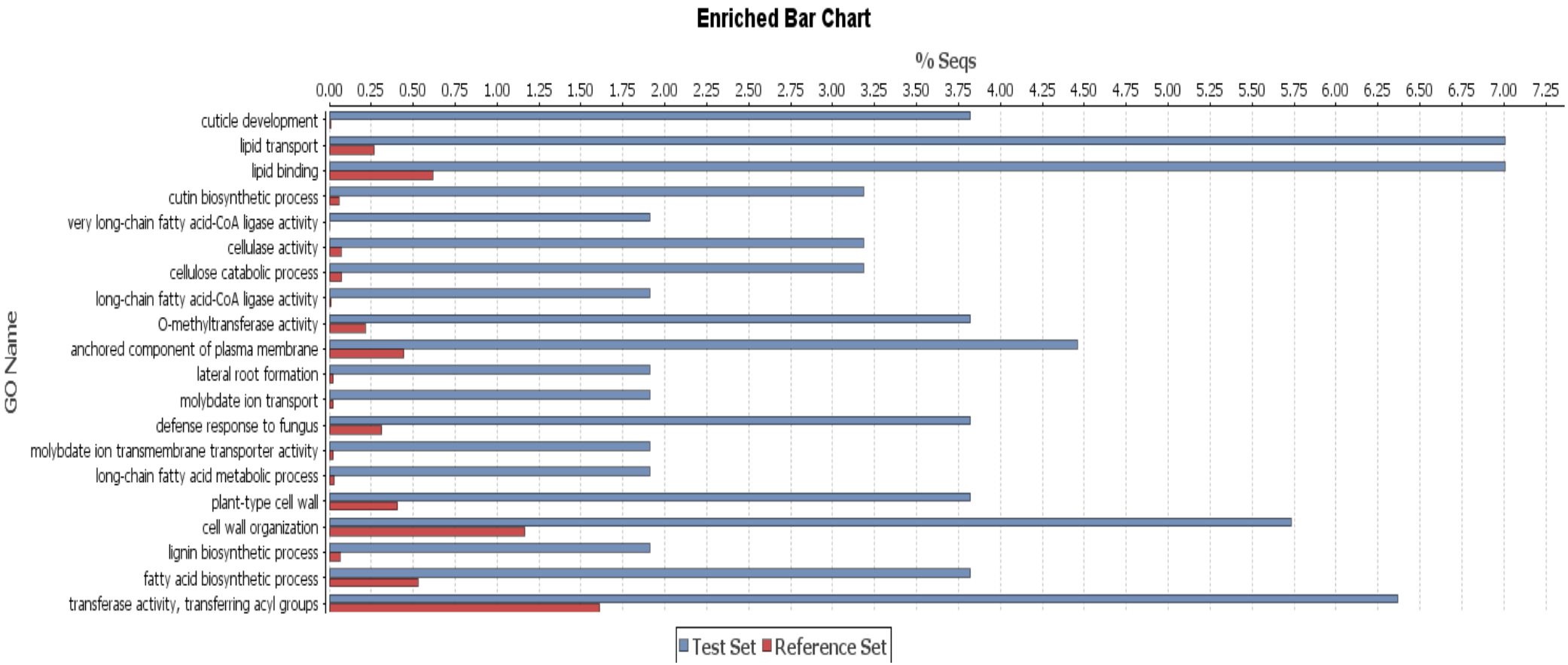


Figure 5.11 Enrichment bar chart of significant GO terms attributed to genes within outer pericarp cluster 2 and the percentage of sequences annotated with these terms. Y-axis shows significantly enriched GO terms and the X-axis gives the relative frequency of each term.

Table 5.6. Top 14 Ranked GO term enrichment results from Fischer exact test of comparisons between the genes contained within endosperm specific cluster 3 and the reference wheat genome (TGACv1). Results were reduced to provide the most specific GO terms. Genes involved in killing of cells of other organisms were the most significantly overrepresented genes within pericarp specific cluster 2. FDR= Corrected p-value by False Discovery Rate control according to (Benjamini and Hochberg, 1995). P-value= p-value without multiple testing corrections.NR Test = Number of genes within test set (pericarp specific cluster 2) attributed to a GO term. NR Test = Number of genes within the reference set (TGACv1) genome attributed to the GO term.

Nr	Tags	GO ID	GO Name	GO Category	FDR	P.Value	Nr Test	Nr Reference	Non Annot Test	Non Annot Reference
1	OVER	GO:0031640	Killing of cells of other organism	BIOLOGICAL_PROCESS	3.023153E-6	1.132691E-9	5	79	51	103746
2	OVER	GO:0016998	Cell wall macromolecule catabolic process	BIOLOGICAL_PROCESS	3.234567E-4	2.423804E-7	4	92	52	103733
3	OVER	GO:0006032	Chitin catabolic process	BIOLOGICAL_PROCESS	4.259435E-4	7.447495E-7	4	123	52	103702
4	OVER	GO:0008051	Chitin binding	MOLECULAR_FUNCTION	4.259435E-4	6.989537E-7	4	121	52	103704
5	OVER	GO:0045300	Acyl-[acyl-carrier-protein] desaturase activity	MOLECULAR_FUNCTION	4.259435E-4	7.277985E-7	3	29	53	103796
6	OVER	GO:0009082	Branched-chain amino acid biosynthetic process	BIOLOGICAL_PROCESS	4.267152E-4	9.592698E-7	3	32	53	103793
7	OVER	GO:0004568	Chitinase activity	MOLECULAR_FUNCTION	4.267152E-4	8.689879E-7	4	128	52	103697
8	OVER	GO:0012511	Monolayer-surrounded lipid storage body	CELLULAR_COMPONENT	4.267152E-4	9.592698E-7	3	32	53	103793
9	OVER	GO:0050832	Defense response to fungus	BIOLOGICAL_PROCESS	4.423256E-4	1.049605E-6	5	325	51	103500
10	OVER	GO:0003984	Acetolactate synthase activity	MOLECULAR_FUNCTION	3.729792E-3	1.280829E-5	2	8	54	103817
11	OVER	GO:0045735	Nutrient reservoir activity	MOLECULAR_FUNCTION	5.16659E-3	1.871251E-5	4	283	52	103542
12	OVER	GO:0000272	Polysaccharide catabolic process	BIOLOGICAL_PROCESS	2.862141E-2	1.251092E-4	4	465	52	103360
13	OVER	GO:0004034	Aldose 1-epimerase activity	MOLECULAR_FUNCTION	3.73424E-2	1.678939E-4	2	33	54	103792
14	OVER	GO:0010155	Regulation of proton transport	BIOLOGICAL_PROCESS	3.744504E-2	1.777085E-4	2	34	54	103791

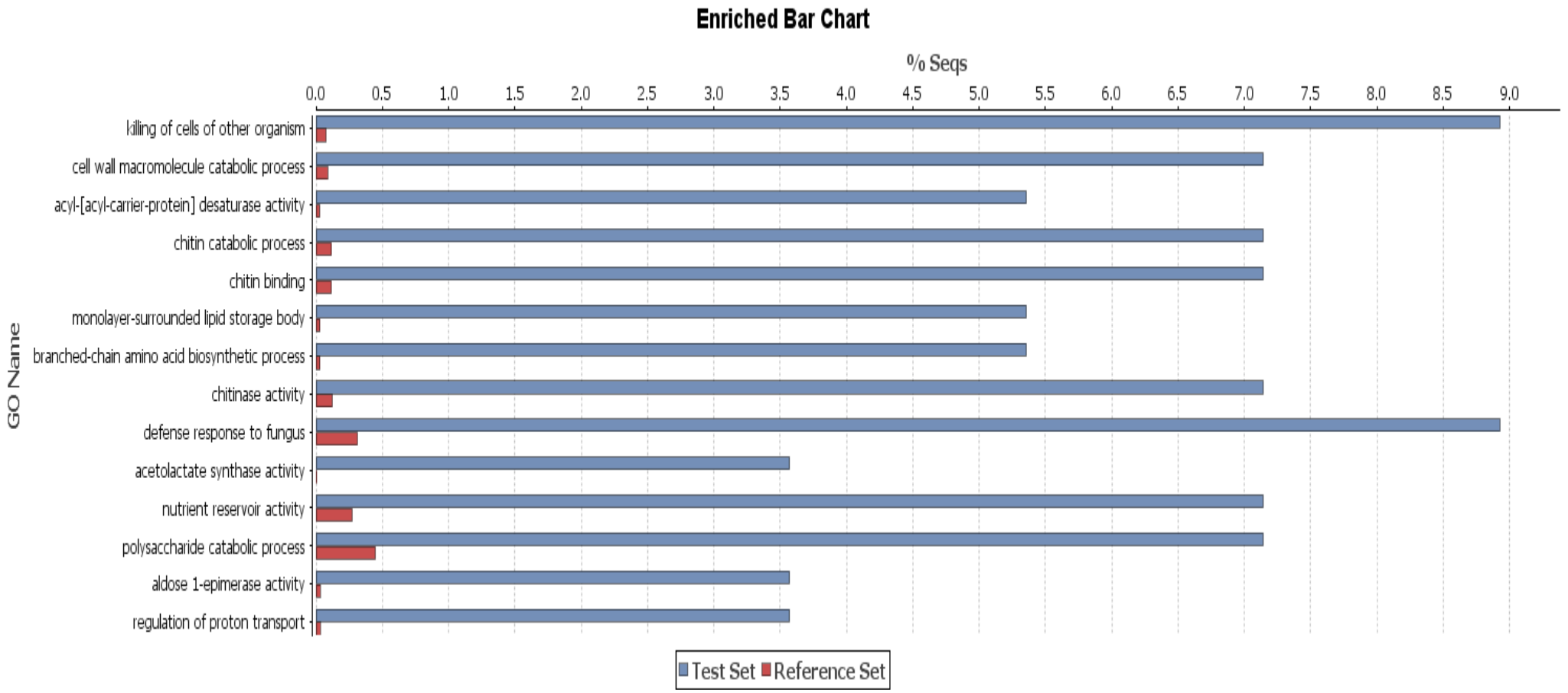


Figure 5.12 Enrichment bar chart of significant GO terms attributed to genes from endosperm specific cluster 3 and the percentage of sequences annotated with these terms. Y-axis shows significantly enriched GO terms and the X-axis gives the relative frequency of each term.

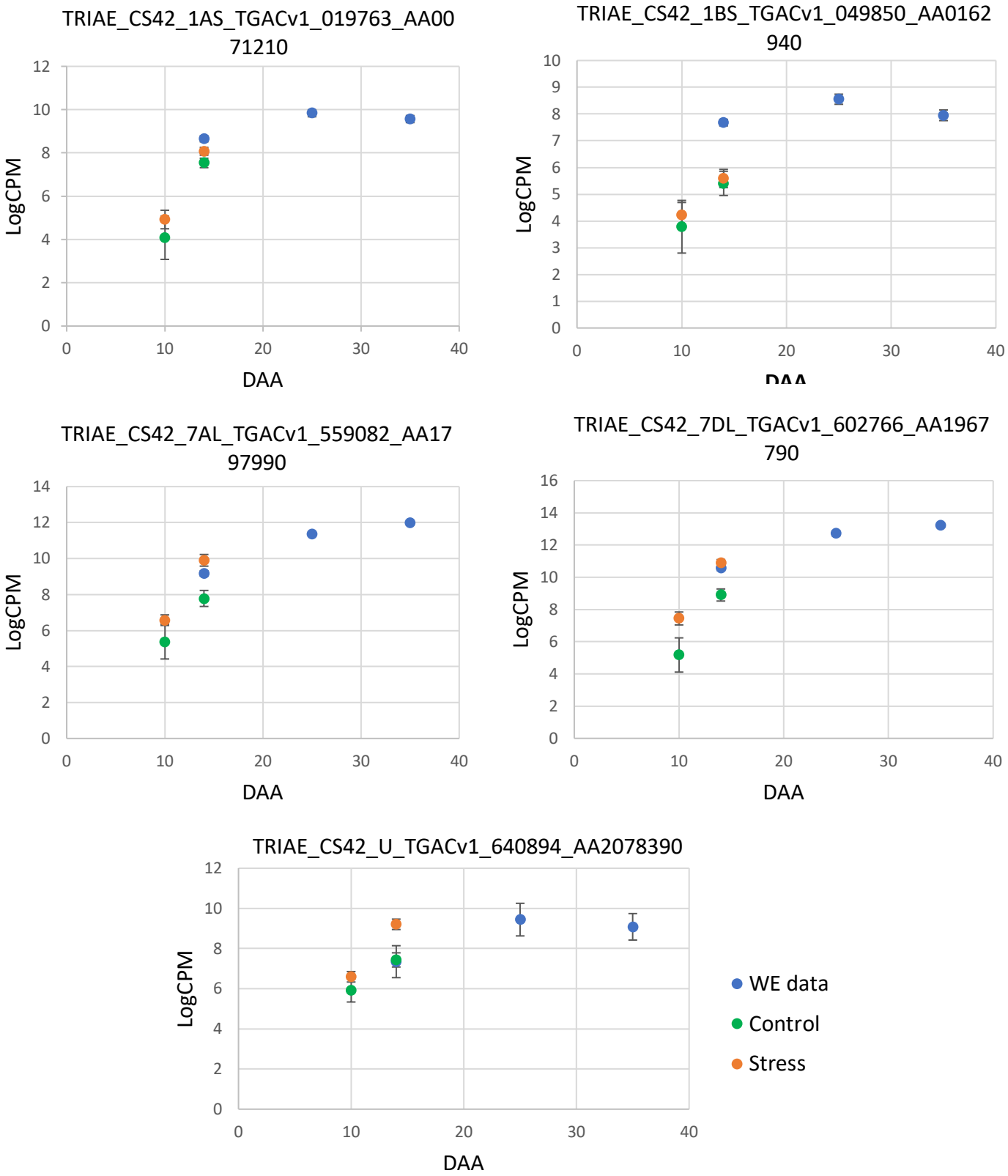


Figure 5.13 Relative expression amounts of genes attributed to the GO term 'killing of cells of other organisms' from the subset of endosperm predominant genes (cluster 3) from publicly available RNA-Seq data from wheat-expression.com (WE data) and data collected from samples exposed to high p.a. temperature treatment and control conditions at 14/15,25 and 35daa. Error bars represent 1 standard error.

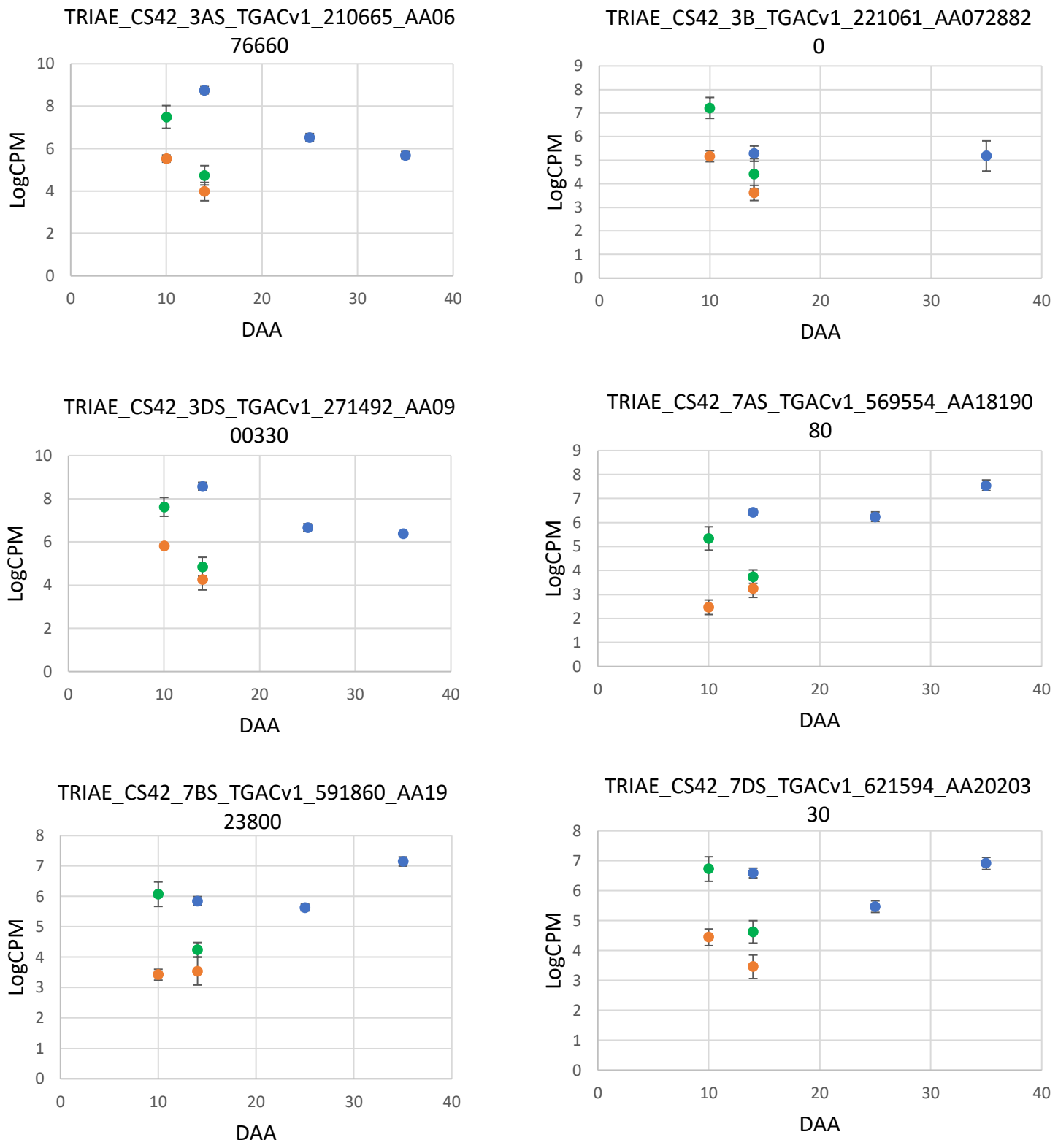


Figure 5.14 Relative expression amounts of genes attributed to the GO term 'cuticle development' from the subset of outer-pericarp predominant genes (cluster 2) from publicly available RNA-Seq data from wheat-expression.com (WE data) and data collected from samples exposed to high p.a. temperature treatment and control conditions at 14/15,25 and 35daa. Error bars represent 1 standard error.

5.3 Discussion

The aim of this study was to investigate whether high p.a. temperatures resulted in the differential expression of genes involved in cell wall modification that may result in the restriction of potential endosperm growth by the pericarp. We examined the effect of high p.a. temperature treatment on the expression of genes found primarily in the outer pericarp and endosperm tissues of the wheat grain by carrying out RNA-Seq analysis from whole grains and by comparing and filtering RNA-Seq data obtained against previous tissue specific data sets. The results from this experiment revealed that high p.a. temperatures resulted in the decreased expression of genes involved in the development of the outer cuticle in the wheat grain potentially indicating the premature maturation of this outer layer. In addition, genes involved in cell wall expansion were down-regulated in the outer-pericarp in high p.a. temperature treated samples consistent with the hypothesis that high p.a. temperature does result in reduced expansion in the cell walls of the outer pericarp.

One factor that must be considered in the discussion of the results of the RNA-Seq analysis is the initial quality of the RNA supplied for analysis. RNA quality is largely dependent on the degree of degradation the molecule has undergone with this process forming an intrinsic part of cellular metabolism and gene expression regulation. Whilst electrophoresis gels can be used to provide a visual assessment of the integrity of sample RNA, a more reproducible and accurate method is to assess the integrity of extracted RNA using a bioanalyzer. Most bioanalyzers use an algorithm to produce a RIN score that is inferred on the intensity ratio of two main ribosomal RNA subunits, 18S and 28S, in addition to factoring in the presence of degradation products. This score provides a metric to assess the extent of degradation in RNA samples with scores ranging from 0 (completely degraded) – 10 (completely intact) (Schroeder *et al.*, 2006). The RNA samples used in this analysis produced RIN scores ranging from 4.2-7.0 (Table 5.1). The consequences of

sequencing RNA samples with relatively low RIN scores (e.g. 3-6) depends on the method used to isolate mRNA in the total RNA sample e.g Poly-A tail selection, rRNA depletion, prior to the construction of the cDNA library. In this study poly-a tail selection was performed and one of the potential effects of performing this procedure on RNA samples with lower RIN scores is the creation of 3' bias in the results of any RNA-Seq analysis. During the priming of cDNA synthesis from the poly-A tails found at the end of the 3' end of the RNA strand degradation of RNA can result in the 5' end of the RNA strand becoming detached from the poly-A tail, meaning it is no longer accessible, and therefore not converted into cDNA for subsequent sequencing. This 3' bias can result in overrepresentation of 3' untranslated regions which may harbour repetitive or low complexity sequences which may result in a lower number of uniquely mapped reads and therefore a reduced library complexity. In addition (Sigurgeirsson *et al.*, 2014) demonstrated that a reduced cDNA library complexity, resulting from sequencing RNA samples with a low RIN score, can lead to increased rates of read duplication. In addition they also demonstrated the increased 3' bias and the subsequent loss of full transcript length can lead to the occurrence false positives in DEG analysis. Similarly, (Gallego Romero *et al.*, 2014)) observed a significant effect of RNA sample degradation on the estimated relative gene expression in samples in addition to a significant loss of library complexity and variation in gene expression between samples with varying RIN scores. However, both (Sigurgeirsson *et al.*, 2014) and (Gallego Romero *et al.*, 2014) suggested that meaningful results can still be obtained from sequencing RNA samples with relatively low RIN scores so long as these are taken into consideration in the analysis and that the RIN score variation between sequenced samples is not too great. In this experiment, the majority of samples had a RIN score ranging from 4.2-5.4, meaning that the inter sample variation was similar between the majority of sequenced samples, therefore potentially minimising the distortion that may occur in the DEG analysis. In addition, the high starting quantities of RNA

provided for sequencing meant that it was possible to proceed to library construction and still obtain meaningful results albeit with reduced sequencing depth. Therefore due to the close similarity between samples in RIN score, the DEG analysis performed in this experiment was unlikely to be significantly affected, although the probable loss of sequencing depth must be considered as a potential limitation to the analysis.

5.3.1 Grain moisture content and grain filling rate during development under high p.a. temperature

Grain moisture content has been shown to be closely associated with the developmental stage of the wheat grain (McIntosh *et al.*, 2007; Wan *et al.*, 2008,). Therefore, a candidate sampling period for RNA-Seq analysis was determined using changes in moisture content under high p.a. temperatures as a measure to determine when high p.a. temperature resulted in a change in transcriptomic development. Total grain moisture content in both the pilot transcriptomic experiment and the experiment from which the RNA samples were collected was significantly lower at 14daa under high p.a. temperatures. Maximum moisture content of the wheat grain has been previously reported as a reliable indicator of final grain weight (Pepler *et al.*, 2006) and maximum grain volume (Egli 1998). Therefore, based on the earlier observation that a 4-day high p.a. temperature treatment reduced mature grain weight when applied from 6daa (Chapter 3 - Fig.3.1) and the results observed in the pilot transcriptomic experiment that total grain moisture content did not significantly differ between treatments until 14daa, the developmental stages of 6, 10 and 14daa were used in experiment 3 where grains were sampled for RNA-Seq analysis. The rate of dry matter accumulation between high p.a. temperature treated and control grains did not significantly differ in either experiment 2 or 3, supporting a study by (Gooding *et al.* 2003) who observed that elevated day/night temperatures (20/28°C) had no effect on grain filling rate in winter wheat *(cv. Hereward)*, although the temperature used in their study was lower

than that used in this project. This lack of an effect of high p.a. temperature on grain filling rate in experiment 2 or 3 was in contrast to Dias and Lidon (2009) who observed increases in grain filling rate in a variety of *T.aestivum* genotypes when subjected to a high p.a. temperature treatment from anthesis until maturity (31/20°C). In addition, our results were also in contrast to Tashiro and Wardlaw (1989) who observed that grain filling rate in wheat (*cv. Banks)* decreased when exposed to day time temperatures greater than 30°C when applied from 7daa until maturity. However, in accordance with Sofield *et al.* (1977) and Tashiro and Wardlaw, (1989) there was an earlier cessation of grain filling in experiment 3, with individual grain dry weight peaking in high p.a. temperature treated grain at 22daa whilst it continued to increase in control grain (Fig. 5.3). This observation indicates a premature termination to the grain filling process under high p.a. temperature as previously observed (Hunt *et al.* 1991; Hurkman and Wood, 2011) as the wheat plant's development accelerates to complete its reproductive cycle under adverse environmental conditions. However, the variation in the observed effect of high p.a. temperatures on grain filling rates between studies supports the previously observed genotypic variation between cultivars in their response to high p.a. temperatures (Hunt et al., 1991).

As discussed previously, the wheat plant material from which the RNA samples were taken experienced high pre-anthesis temperatures during their growing period compared to the pilot transcriptomic experiment, a fact which must be taken into consideration. However, there was no significant difference between the total grain moisture contents or dry weights from grains from both experiments at the same developmental stage. In addition, high p.a. temperatures reduced grain moisture content at the same developmental stage both in the pilot experiment that didn't experience high temperatures prior to anthesis and experiment 3 from which the RNA samples were collected. These results suggest that prior exposure to high temperatures during the pre-anthesis growing period does not lessen the impact of subsequent high p.a. temperature treatment and therefore, it is likely that the

environmental conditions experienced by the wheat prior to anthesis do not impact significantly on the results of this RNA-Seq analysis.

5.3.2 Differential gene expression between developmental stages

Within the developing wheat grain, clear shifts in gene expression have been shown which mark the change to a new developmental phase of the grain (Wan *et al.*, 2008). The period between 10-14daa marks the end of the rapid cell division phase of grain development and the beginning of the grain-filling period. This developmental shift is apparent in the DEG analysis data of this experiment where the largest number of DEGs (21476) occurred between 10daa and 14daa when developmental stage was compared independently of temperature treatment. Interestingly, the number of DEGs was higher between 10daa and 14daa than between 14daa and 6daaa, potentially indicating the notable shift in development between the end of rapid cell division and the beginning of the grain filling period as observed by Wan *et al.*, (2008) in *cv. Hereward,* who also observed large changes in gene expression between 10-12daa. However, this may also be partially explained by the fact that at 6daa, there were only two RNA-Seq samples from plants that had not been subjected to a high p.a. temperature treatment, whereas at both 10 and 14daa there were 3 RNA-Seq samples for both control and high p.a. temperature treatments at each stage. Therefore, in the 10 vs 14daa comparison, 12 RNA-Seq samples were compared for DGE (6 vs 6) whereas in both 6vs10daa and 6vs14daa comparison, 8 RNA-Seq samples were compared (2 vs 6). The relatively low number of DEGs between 6 and 10daa (2053) compared to between 6 and 14daa (14845) again appears to indicate the scale of developmental shift that occurs in transcriptomic expression between the end of the cellularisation period and grain filling.

5.3.3 Outer pericarp gene expression under high p.a. temperature

High p.a. temperature has been shown to accelerate the normal pattern of gene expression within the wheat grain (Altenbach and Kothari 2004; Wan *et al.*, 2008).The largest number of DEGs between high p.a. temperature treated and control samples occurred at 10daa (4981) compared to 14daa (3652) indicating that exposure to high p.a. temperatures from 6daa results in the biggest change in gene expression at 10daa. When filtering of the DEGs between high p.a. temperature and control samples was performed to identify those that were tissue specific using the two existing studies, 12 outer pericarp predominant genes showed significant differences in expression between 10 and 14daa with a significant interaction of heat treatment. A number of these genes have been shown to perform roles in maintaining membrane functionality and cuticle development under high p.a. temperature:

5.3.3.1 Lipid transfer proteins

Of the 12 outer pericarp genes that showed a significant difference in expression between 10 and 14daa with a significant interaction of heat treatment, 5 encoded lipid transfer proteins (LTPs) and were significantly down-regulated at 10daa in samples exposed to high p.a. temperature treatment. 3 of these 5 LTPS were then up-regulated at 14daa with 2 remaining at similar expression levels between 10 and 14daa. LTPs are a group of highly divergent, small basic proteins with eight Cys residues at conserved positions and aid in the solubilizing of lipids facilitating their movement between membranes. In addition, LTPs are believed to have roles in cell defence, cutin development, water permeability of cell membranes and cell wall extension (Kader 1996; Nieuwland *et al.*, 2005; Salminen *et al.*, 2016). Nieuwland *et al.* (2005) isolated LTPs from tobacco leaves and wheat grains and observed that these proteins were able to enhance the extension of cucumber hypocotyl

cell walls by maintaining cell expansion in a logarithmic manner. The authors proposed a similar mode of action between LTPs and EXPs in facilitating cell wall expansion with the difference being that LTPs do not require low pH to become active unlike EXPs.

Of the 5 genes encoding LTPs in our data, 3 of those genes encoded non-specific LTPs (NSLTPs). NSLTPs play an important role in maintaining cell function and mediating responses to stress during plant development and aid in the formation of the surface cuticle layer protecting plants from desiccation under heat stress (Liu *et al.*, 2015). Transcript levels for NSLTPs in wheat have been previously shown to increase from around 12daa until the late stages of grain development in the US spring wheat *cv. Butte-86* (Altenbach *et al.*, 2008) under high p.a. temperatures (37/28°C). The results of Altenbach *et al.* (2008) lend support to the results of this study in that NSLTPs encoding genes that were significantly differentially expressed between developmental stages with a significant interaction of temperature are up-regulated at 14daa. The down-regulation of NSLTPs at 10daa under high p.a. temperatures followed by up-regulation at 14daa could be indicative of the multiple functions of NSLTPs: membrane permeability may be decreased in the grain under high p.a. temperature before NSLTPs are increasingly expressed to aid in cuticle development. The genetic pathway and feedback mechanisms of NSPTLs and the role they play in cell wall extension under abiotic stress conditions is still not entirely known. Therefore, further examination of the role of NSPTLs in grain development under high p.a. temperatures is required.

5.3.3.2 Peroxidase

Another gene that was differentially expressed between 10 and 14daa with a significant interaction of temperature treatment was a gene encoding an un-specified, haem-b class III Peroxidase. Peroxidases are a family of enzymes that detoxify radical oxygen species (ROS) that can generate and accumulate in plants under heat stress conditions. An imbalance in

the amount of ROS can cause oxidative damage to cellular components, impairing metabolic activities and damaging cellular membranes (Suzuki *et al.*, 2011). The class III peroxidase gene was down-regulated in high p.a. temperature treated samples at 14daa. This initially seems counter-intuitive due to likelihood of increased ROS in the high p.a. temperature treated samples compared to the control samples. However, peroxidases consist of 3 major families and play a role in a number of biological processes. Class III peroxidases are secreted glycoproteins known to be involved in a broad range of physiological processes including regulation of cell elongation and cell wall modification (Passardi *et al.*, 2004; Passardi *et al.*, 2005). Class III peroxidases can perform dual functions within plant cell walls, regulating either cell wall stiffening or relaxation. They are able to form rigid walls by either forming strong intracellular bonds by oxidising aromatic cell wall compounds (e.g. lignin), or generate ROS that can break covalent bonds in cell wall polymers such as hydroxyl radical (OH°) (Schopfer *et al.*, 2001; Francoz *et al.*, 2015). Attempts have been made using reverse genetics to determine the function of specific class III peroxidases (Shigeto and Tsutsumi 2015), however the exact function of the peroxidase identified in this analysis (PER2/PER42) has not been determined. Cai and Lashbrook (2008) observed up-regulation of PER2/42 transcripts at abscission zones when studying organ detachment in *A.thaliana.* Whilst they were not able to determine the role of PER2/42, they hypothesised that the peroxidase, in conjunction with other cell wall modifying proteins facilitated the entry of pectin modifying proteins during the period where they observed the greatest pectin modification. This proposed function may link the observed changes in the HG methyl-esterification status of pericarp layers in this project with the down-regulation of this peroxidase at 14daa. In addition, PER2/PER42 has been determined as the closest relative to a peroxidase identified in Tobacco plants implicated in lignin biosynthesis (Blee *et al.*, 2003; Yokoyama and Nishitani, 2006) where down regulation of the peroxidase resulted in a reduction of cell wall lignin content. Lignin is a component of the inner and

outer pericarp cell wall comprising around 8.3 and 12% of the total cell wall components (Ring and Selvendran 1980; Selvendran *et al.*, 1980). A corresponding reduction of cell wall lignin content in the pericarp layers of the wheat grain may be expected to result in a decrease in cell wall rigidity, however the lignin biosynthesis pathway is complex with multiple gene interactions that can be affected by high temperatures (Gall *et al.*, 2015). Investigation into the lignin content of the outer layers of the wheat grain with differential expression of PER2/PER42 would provide further information as to the effect of this peroxidase on the lignin biosynthesis pathway in wheat grain.

Heat stress inducing temperatures throughout grain development can damage cellular membranes through denaturation of the cellular components. Membrane lipid composition is a crucial factor in membrane temperature tolerance. Lipid peroxidation under high temperatures can result in damage to cellular membranes in Chinese spring wheat leaves during the booting stage (Djanaguiraman *et al.*, 2018) and it has been reported during the grain-filling stage where high temperatures have been shown to increase membrane damage and decrease antioxidant levels (Balla *et al.*, 2013). Djanaguiraman *et al.* (2018) suggested that the activity of antioxidant enzymes like peroxidases may be down-regulated under high temperatures exacerbating the accumulation of ROS typical under heat stress conditions. The down-regulation of antioxidant enzymes under heat stress conditions has been previously observed in model species such as *Arabidopsis thaliana* (Mittler *et al.*, 2004). Whilst the result observed by Djanaguiraman *et al.* (2018) was in wheat leaves during booting, it partially supports the observed result from this study that peroxidase expression is decreased under high p.a. temperature conditions in wheat grain at 14daa. The down-regulation of a class III peroxidase could potentially lead to the build-up of damaging ROS that would require increased expression of genes that aid in the maintenance of cell membrane functioning which is observed in this study with the up-regulation of NSLTPs at 14daa in treated samples.

The significant differential expression of PER2/PER42 between treatments and daa in the outer pericarp indicates an effect of high p.a. temperatures on this gene implicated in regulating cell wall plasticity. However, the effect of the down-regulation of this gene at 14daa on the cell walls of the pericarp is not clear. In order to induce stiffening of cell walls, peroxidases require excess amounts of ROS that act as a co-substrate in the same tissue enabling cross-linking of cell wall phenolic compounds and glycoproteins (Tenhaken 2015). Therefore, determining whether the down-regulation of PER2/PER42 results in increased stiffening or loosening of the cell walls in the outer pericarp is difficult. Further study investigating the level of ROS production in the outer pericarp under high p.a. temperature treatment would provide further insight into the role and function of PER2/PER42 in the tissue.

5.3.3.3 Long-chain-acyl-CoA synthetase II enzyme (LACs)

Another gene also significantly differentially expressed between developmental stages and between heat treatments in the outer pericarp encoded a long-chain acyl-CoA synthetase II enzyme (LACs) (Fig 5.8). LACs are a group of enzymes involved in the plant lipid biosynthesis pathway and are utilised in the transport of fatty acids from the endoplasmic reticulum to the plastid (Jessen *et al.*, 2015). Within this RNA-SEQ experiment LACs expression was significantly down-regulated in high p.a. temperature treated samples compared to control samples at 10daa. The down-regulation of the LACs enzyme under high p.a. temperature compared to the up-regulation of NSLTPs suggests a complex relationship between molecular components in the plant lipid pathway and cuticle development under high p.a. temperature treatment. Therefore the down-regulation of LACs under high p.a. temperature stress may indicate a reduction in intracellular lipid trafficking as more resources are diverted towards the formation of the cuticle and the required transport of lipids across cellular membranes.

5.3.4 Gene ontology and functional analysis

5.3.4.1 Knetminer analysis

Following the K-means clustering analysis, outer pericarp cluster 2 and endosperm specific cluster 3 were found to be significantly affected by temperature treatment (Fig 5.10). In addition, 9 of the 12 outer pericarp predominant genes shown to be differentially expressed between treatments and with a significant interaction of daa were contained within the outer pericarp cluster 2. Only the gene encoding peroxidase (TRIAE_CS42_2DS_TGACv1_178485_AA0596270) and an NSLTP (TRIAE_CS42_3B_TGACv1_226978_AA0820590) were contained within different clusters (1 and 3 respectively). In addition, following the K-means clustering of both the outer pericarp and endosperm specific DEGs, the heat maps produced revealed that the expression of DEGs appeared to vary significantly between some samples at the same daa stage and treatment (e.g. 10daa_C3 (Fig 5.9.A) and 10daa_C2 and 14daa_C3 (Fig.5.9.B). Due to the complex nature of RNA-Seq analysis, variation in DEG expression between similar samples can be introduced at numerous stages during the process due to the number of procedural steps that take place, e.g. RNA extraction and handling, cDNA library construction etc. (Kukurba and Montgomery, 2015). Whilst the normalisation procedures employed during this study sought to minimise the amount of variation between samples, some variation is inevitable. Therefore, the inclusion of biological replicates (3-per stage and treatment) in this experiment helps add confidence to the results of the DEG analysis and subsequent k-means clustering. Therefore whilst the cause of variation in expression observed in some samples is unknown, the impact of this variation is reduced due to the replication utilised within the experiment.

The analysis performed within the Knetminer web tool on the genes contained within outer pericarp cluster 2 revealed key genes associated with the search term “cell wall” including

endoglucansases, xyloglucanase, an aquaporin and β-EXP. Endoglucanases are a group of membrane-bound enzymes that hydrolyse cell wall polysaccharides with contiguous (1,4)-β -glycosyl residues in their chain such as XYG and 1,3:1,4 -β-glucanases. They catalyse glycosidic chains and are implicated in the breakdown of plant cell walls during development (Buchanan *et al.*, 2012). In addition, endoglucanases are important for normal cellulose synthesis which is an essential component of cell walls in wheat grain. Over expression of endoglucanases have been shown to result in increased internode extension in rice plants (Zhou *et al.*, 2006) and have been demonstrated to be part of the cellulose synthase complexes in *A.thaliana* (Vain *et al.*, 2014) . The KORRIGAN gene, a specialised member of the endoglucanases, have been shown to be expressed in the expanding cell walls of *A.thaliana* (Nicol *et al.*, 1998). In particular the KOR2 and KOR3 genes, have been shown to exhibit distinct differences in expression patterns in *A.thaliana* compared to the more widely expressed KOR gene with KOR3 postulated to act in cell wall biosynthesis due to high expression in the bundle sheath cells of leaf tissue (Molhoj *et al.*, 2001). Therefore, the down regulation of three KOR3 genes in the outer pericarp cluster 2 suggests a reduction in the amount of cell wall assembly and potential expansion in the outer layers of the pericarp under high p.a. temperature.

Of the highest scoring pericarp cluster 2 genes from the Knetminer analysis webtool, two genes encoded members of the xyloglucan endotransglucosylase (XTH) family. These enzymes cleave XYGs which are the major hemicellulosic polymers of dicot plants that bind to cellulose microfibrils in plant cell walls and are therefore heavily implicated in cell wall remodelling and expansion through polymer creep. Many XTH's experience increased expression under abiotic stresses such as cold, salt and drought stress (Tenhaken 2015) as plants try to maintain necessary cell wall growth under adverse abiotic conditions. A review of studies looking at the effect of heat stress on plant cell formation conducted by Gall *et al.* (2015) largely studied transcriptomic and proteomic data and found that XTH expression

tended to increase under heat stress conditions. Some XTH's have been shown to be down-regulated under drought conditions in the awns of Barley spikes (Abebe *et al.*, 2010), however, there is little information regarding the effect of high p.a. temperature on XTH expression in wheat grain under high p.a. temperature. The increased reduction in expression of XTH genes observed in pericarp cluster 2 under high p.a. temperatures also suggest a reduction in the expansion of the cell walls within the outer pericarp. However, as XTH expression in primary plant cell walls is thought to increase in response to high p.a. temperatures, the down regulation of XTHs in both control and treated samples between 10 and 14daa suggest that within the outer pericarp, cell wall expansion is reduced between 10 and 14daa but to a greater extent under high p.a. temperature.

Finally, EXPB5 was also contained within outer pericarp cluster 2 and was one of the highest scoring genes attributed to the search term "cell wall" in the Knetminer web tool. EXPB5 encodes a β-EXP which have previously been shown to be crucial cell wall proteins that facilitate cell wall expansion via disruption of hydrogen bonds between cellulose microfibrils and cross-linking glycans allowing cellular expansion under turgor driven pressure (McQueen-Mason *et al.*, 1992; Cosgrove 2000). Lizana *et al.*, (2010) found in the spring wheat cultivar *(cv. Bacanora),* that expression of 5 unique α-EXP sequences derived from wheat grain pericarp RNA, decreased rapidly in the developing grain from 25daa. In addition α-EXP expression was previously studied by Lin *et al.*, (2005) in wheat cultivars *cvs. Bau-3338* and *Bau-6554,* who observed that expression of two of the EXPs studied by Lizana *et al.* (2010) (*TaExpA2, TaExpA8)* peaked between 8-12daa before rapidly declining. Whilst these studies show the importance of α-EXP genes in the early stages of wheat grain development, there is less information regarding the function and presence of β-EXPs in wheat grains.

α and β EXPs make up the two largest families of EXP genes and share only around 20% amino-acid identity (Cosgrove, 2000). However, both groups appear to destabilise non-

covalent load bearing contacts between cellulose microfibrils in plant cell walls (Park and Cosgrove 2012). EXPB5, the β-EXP gene within outer pericarp cluster 2 that scored highly within the Knetminer tool, has been shown to be specifically expressed in the tapetum of *A.thaliana* in addition to mature pollen grains, where they are likely to aid in pollen penetration and expansion (Lou *et al.*, 2017). In terms of the action of EXPB5 in the cell wall expansion of other grass species, an orthologue of EXPB5, OsEXPB5 has been shown in rice to be involved in root hair growth (Won *et al.*, 2010). However there is little information pertaining to the specific action of EXPB5 in wheat grain cell walls.

Overall, the presence of these genes in outer pericarp cluster 2 suggest that the expression pattern of this cluster may be representative of changes in the cell wall of the outer pericarp under high p.a. temperatures. The down regulation of the genes contained within outer pericarp cluster 2 between 10 and 14daa with a greater degree of down-regulation in high p.a. temperature treated samples may indicate an increasing rigidity of the cell walls of outer pericarp in high p.a. temperature treated samples.

One limitation that must be considered with regards to the use of the Knetminer webtool to identify particular genes and processes from clusters is potential research and user bias. Whilst the relevance scores provided by the Knetminer webtool provide a basis for the identification of genes involved in particular biological processes and the ability to quickly sift through large gene clusters for genes of interest, the Knetminer database contains only existing genetic knowledge for a number of organisms, including *T.aestivum*. Therefore, un-annotated genes that may play a significant role in the development and modification of cell walls are unlikely to appear in any Knetminer search results. In addition, if only the genes with the highest relevance scores are investigated further as to their role in biological systems (e.g. Top 10) then this may create a cycle of select knowledge, whereby the genes with the most information available (and therefore highest relevance scores) are investigated further, leading to the creation of further knowledge which then feeds into the

search tool. This could lead to important and relevant genes being excluded due to a lack of investigation. The Knetminer search tool and it's efficacy is also dependent on the user defined search term and as a result is susceptible to user bias, in attempts to find a connection between genes and an objective of an investigation (Hassani-Pak 2017). Therefore, whilst the Knetminer tool provides a useful resource, these limitations must be taken into consideration.

5.3.4.2 GO term analysis

GO term enrichment for outer pericarp cluster 2 revealed that the most significant GO term was 'cuticle development' (GO id:0042335) (Table 5.5). The cuticle is a complex extracellular material containing, cutin, waxes and polyesters of hydroxyl and fatty acids (Chateigner-Boutin *et al.*, 2014) that acts primarily as a permeability barrier involved in regulating water loss and protecting plant organs from dirt, bacteria and other microorganisms (Muller and Riederer, 2005). During the early stages of anthesis the developing wheat grain contains a number of osmophilic, cuticles or cuticular membranes, some of which are gradually removed p.a. by enzymatic degradation (Morrison, 1975). Two cuticles persist in the developing grain up to maturity, one being found on the outermost layer of the grain, the outer epidermis and the other being positioned on the surface of the nucellar epidermis. As only RNA-Seq data from the outer pericarp layers and endosperm layers were contrasted and used in the filtering RNA-Seq data from whole grains collected in this experiment, gene expression relating to the development of the inner cuticle is likely to be excluded. The down-regulation of genes contained within outer pericarp cluster 2 at 10daa in high p.a. temperature treated samples compared to control samples indicates a clear effect of high p.a. temperature treatment on the expression of genes involved in cuticle development.

Previous studies have shown that the cuticle of wheat leaves and the specific wax composition is important in regulating drought tolerance in different cultivars (Bi *et al.*, 2017). In terms of the cuticle found on the outer epidermis of the wheat grain, the cuticle forms from around 7daa and reaches is maximum thickness by around 17daa (Morrison, 1975), where it remains structurally unchanged until maturation apart from becoming appressed to the inner cuticle. Therefore, one would expect genes involved in the formation of the cuticle to be down-regulated in samples from around 14-17daa as the cuticle finishes its development, prior to a cessation of growth as the tissue matures. The down-regulation of the genes contained within outer pericarp cluster 2 that registered as being attributed to 'cuticle development' at 10daa in high p.a. temperature treated samples supports the hypothesis of an increased acceleration of development under high p.a. temperature within the outer pericarp. With the cuticle's important role in regulating moisture loss in plant tissues, one may have expected a potential up-regulation of genes involved in cuticle development as the wheat grain attempts to combat water loss under high temperature conditions. However, the results suggest that the expression of genes involved in cuticle development do not respond to the onset of high p.a. temperatures as a responsive defence mechanism against potential heat stress but rather their expression profile is accelerated under high p.a. temperature conditions as shown in the general gene expression patterns of whole grain under high p.a. temperatures.

The results of the GO term analysis complement the set of genes identified as being significantly differentially expressed between developmental stages with a significant interaction of high p.a. temperature. 5 genes encoding lipid transfer proteins were shown to be differentially expressed between 10 and 14daa and these proteins are important in the formation and synthesis of the cuticle. 'Lipid transport' and 'Lipid binding' were the next two most significant GO terms associated with the genes contained within pericarp specific

cluster 2 suggesting the close association between the development of the cuticle and lipid transport and binding.

Outer pericarp degeneration, determined by a loss of greenness has been previously been shown to occur prior to the termination of rapid net water accumulation in spring wheat grain *(cv. Sappo)* (Schnyder and Weiß, 1993). Therefore, the degeneration of the outer pericarp was postulated to be causally related to the determination of the wheat grain sink capacity due to previous findings that the end of endosperm cell division and the cessation of net water accumulation closely coincide (Nicolas *et al.*, 1985). The termination of rapid net water accumulation in sampled grains from both experiment 2 and 3 occurred earlier in high p.a. temperature treated grains compared to control grains at around 10daa in high p.a. temperature treated grains (Fig.5.1-5.2). Therefore, the down-regulation of genes heavily involved in cuticle development under p.a. high temperature at 10daa which continues at 14daa may indicate a premature maturation of the outer pericarp and in particular the cuticle as it nears the end of its development in the outer-pericarp in heat treated grains. However, in the expression data from Barrero *et al.*, (2015) at 15,25 and 35 daa, the expression levels for the genes involved in cuticle development remain considerably higher than in the control treated grains from this experiment. This variation may be the result of varietal differences or due to the fact genes involved in cuticularisation of the inner pericarp layers such as the testa and inner epidermis (Bechtel *et al.* 2009) were likely to have been excluded due to the tissue specific filtering method used in our experiment. RNA-Seq data from untreated de-embryonated whole grains at later stages of development was not available on the wheat-expression.com website but the availability of such data would allow for further investigation into the effect of high p.a. temperature on cuticle development.

The most significant GO term derived from the genes contained within endosperm predominant cluster 3 was 'The killing of cells of other organisms' (GO id: 0031640). The

genes contained within endosperm predominant cluster 3 experienced increased up-regulation under high p.a. temperatures compared to the control samples. Previous studies have shown a large degree of functional overlap in defence associated genes that respond to both biotic and abiotic defence mechanisms (Narsai *et al.*, 2013). In addition, another transcriptomic study based on transcriptome microarray analysis of grains from a Chinese bread wheat cultivar *(cv. Jimai 20)* found that genes associated with defence against biotic and abiotic stresses were all up-regulated during the grain filling period, particularly between 11-15daa (Yu *et al.*, 2016). The findings of this previous study support the results obtained from the RNA-Seq data from this experiment, whereby the defence associated genes contained within endosperm predominant cluster 3 are up-regulated between 10 and 14daa in both treatments although to a higher degree in those samples subjected to high p.a. temperatures. In addition, the authors also saw in their GO term analysis the significant overrepresentation of genes associated with cell wall macromolecule catabolic process that were also up-regulated in their data in groups subjected to drought stress. Yu *et al.* (2016) suggested that the close association and frequent annotation of genes associated with stress response and cell wall metabolism indicates the importance of these functional groups during grain development.

5.3.5 Future experiments

Therefore, it appears that high p.a. temperature accelerates the expression profile of select gene clusters predominantly expressed in the outer pericarp and endosperm that show a significant overrepresentation of genes involved in cuticle development and defence genes. In addition, high p.a. temperatures result in the down-regulation of a number of genes in the outer pericarp involved in the regulation of cell wall growth. The transcriptomic microarray study conducted by Wan *et al.* (2008) on the bread wheat cultivar *cv. Hereward,* observed that under elevated temperatures the expression profile of probesets likely to be predominantly expressed in the endosperm and pericarp was accelerated with endosperm

probeset expression increasing up until around 14daa before decreasing steadily towards maturity whilst pericarp probesets were down-regulated steadily from around 6daa. The experimental conditions used in Wan *et al.* (2008) differ from the conditions used in this experiment in that the elevated temperature conditions in their experiment were imposed from 14daa. In addition, transcripts were allocated a tissue specific categorisation in Wan *et al.* (2008) by comparison of their affymetrix target sequences against known sequences determined from a Barley transcriptomic experiment that analysed separate pericarp endosperm and embryo tissues, (Sreenivasulu *et al.*, 2006) and the in-situ hybridisation database constructed in wheat grain by Drea *et al.* (2005). In contrast, genes in this study were determined as being predominantly expressed in either the endosperm or outer-pericarp through filtering the results of the RNA-Seq conducted in this study against the wheat grain tissue specific data of Pearce *et al.* (2015) which arguably provides a more relevant means to determine tissue specificity of genes expressed in the grain. Only 1 of 5 gene clusters from both the endosperm specific genes and the pericarp specific genes were found to be significantly affected by high temperature treatment whilst the expression of other gene clusters were unaffected by high p.a. temperature in this study. The results observed by Wan *et al.* (2008) of accelerated down-regulation of pericarp specific transcripts under high p.a. temperature conditions somewhat complements the findings of this study where genes within outer pericarp cluster 2 are steadily down-regulated between 10 and 14daa. In terms of similarity between the function of gene clusters identified as predominantly expressed in the pericarp by Wan *et al.* (2008) and those contained within outer-pericarp cluster 2 in this study direct comparisons are difficult to make. Differences in methodologies in determining tissue specificity of transcripts between the studies, the challenge of combining microarray and RNA-Seq data due to biases arising from signal differences, in addition to the labelling of the transcripts, all contribute to this difficulty. However, one cluster of genes (2_3(760)) observed by Wan *et al.* (2008) that showed similar

expression patterns and that was comprised of genes predominantly expressed in the pericarp and that was down-regulated between 10 and 14 days had roles largely based in regulating stress, and defence, protein synthesis, senescence and catabolism. The down-regulation of genes involved in protein synthesis in the results of Wan *et al.* (2008) could be indicative of the down-regulation of cell wall modifying proteins observed in this study albeit under the encompassing term of protein synthesis. Further in depth re-analysis of the Wan *et al.* (2008) data set and particularly their cluster (2_3(760)) may lead to identification of common genes affected by high p.a. temperatures in both studies.

In addition, one area of investigative transcriptomic analysis that has become increasingly popular and accessible via RNA-Seq analysis is the occurrence of alternative splicing events. Alternative splicing is an RNA editing mechanism that provides a valuable source of variety to the proteome of an organism by enabling different varieties of protein to be produced from a single gene. This occurs in the cell during translation through the action of the pre-mRNA, splicosome protein complex which removes introns and splices available exons on an RNA strand. This complex can achieve alternative splicing through a number of methods including exon skipping, mutually exclusive exons, and alternative 5' and 3' donor sites. The high resolution and sensitivity of RNA-Seq allows for the identification of isoforms formed by alternative splicing events by calculating abundances through the utilisation of expectation maximisation algorithms or statistical software that can calculate the occurrence of alternative splicing events through analysis of exons and splice sites on transcripts (Park *et al.*, 2018). Due to time constraints, investigation into alternative splicing, particularly in the identified candidate genes for cell wall plasticity, was not performed. However, this would be useful future investigation to perform with the RNA-Seq results obtained in this experiment. Alternative splicing has been previously observed in wheat seedlings undergoing heat stress (40°C) at 1 and 6 days after germination, with 3576 genes shown to exhibit alternative splicing under these conditions (Liu *et al.*, 2018). The authors

suggested that this could provide a coping mechanism to the plant to produce different and more resilient proteins under stressful abiotic conditions in order to maintain vital processes. Investigation of alternative splicing into the identified candidate genes for reduced cell wall plasticity would allow us to determine whether the DGE between treatments is occurring in the same or different isoforms and reveal more about the function of these genes in the regulating the development of the grain under high p.a. temperatures.

One further avenue of investigation that would be useful to perform is validation of the some of the DEGs identified in this RNA-Seq analysis via methods such as qRT-PCR or high-throughput RT-PCR. Whilst RNA-Seq is useful for identifying patterns in gene expression for a large number of genes transcribed in an organism, in order to confirm the observed expression patterns of genes identified as potentially having a key role in a particular gene pathway or biological process further validation of their expression patterns is desirable. In the case of this experiment, confirming the expression patterns of the proposed candidate genes postulated to reduce cell wall plasticity in the outer-pericarp would add strength to this hypothesis. Successful qRT-PCR analysis of the expression of select genes is dependent on the careful design of primers for the action of the reverse-transcriptase, and the establishment of reference genes to compare select gene expression against. In addition it is preferable to analyse the expression patterns of a mixture of both up-regulated and down-regulated genes from samples. Similarly as with the lack of investigation into alternative splicing, due to time constraints, further validation of the identified potential candidate genes was not performed in this project. A future experiment that utilised q-RT-PCR to assess the expression patterns of these genes in a range of cultivars with varying levels of thermotolerance could provide more insight into whether these genes are implicated in the varying responses to high p.a. temperatures between cultivars in terms of grain size and development.

5.4 Conclusion

From this RNA-Seq analysis it is apparent that high p.a. temperature treatment results in acceleration of the expression profiles of select groups of genes likely to be predominantly expressed in either the endosperm or outer pericarp. In the outer pericarp, the expression of genes involved in the formation of the cuticle and associated lipid-binding and transport genes are significantly affected by exposure of grain to high p.a. temperatures with accelerated down-regulation in high p.a. temperature treated samples suggesting the earlier formation of the cuticle in the outer layers of the wheat grain. The increased up-regulation of genes involved in defence in the endosperm suggest there may be functional overlap between genes that respond to abiotic and biotic stress during wheat grain development that coincide with structural changes to the cell wall. The down-regulation of genes in the outer pericarp including endoglucanases, xyloglucanases an EXP and peroxidase under high p.a. temperature suggests a premature loss of plasticity in the outer pericarp cell walls that would coincide with a reduction in grain moisture content and endosperm cell division. Therefore these genes are viable candidates for further investigation and validation in order to determine their role in regulating the response of the pericarp cell wall under high p.a. temperatures. Further transcriptomic analyses from individual grain layers under high p.a. temperature treatment and investigation into the levels of important cellular substrates such as ROS will shed further light on the effect of high p.a. temperature on the regulation of grain expansion.

Chapter 6: Discussion

6.1 General discussion

The predicted increase in global population during the 21st Century (UN, 2017) is likely to be accompanied by increased frequency and severity of extreme temperature events such as heat waves and acute high temperature events (IPCC, 2014) that can have a significant negative effect on cereal crops' yield (Gourdji *et al.,* 2013; Vignjevic *et al.*, 2015; Asseng *et al.*, 2015). Therefore, in order to overcome these considerable challenges facing adequate global food production, a better understanding of the effect of high temperatures on important cereal crops such as wheat is required. Understanding the mechanisms through which high temperatures affect wheat yield may improve current crop modelling analysis and enable the breeding of new varieties that are more resilient to high temperatures.

This project has utilised a number of methods to investigate whether the widely observed reduction in grain weight following exposure to high p.a. temperatures (Hunt *et al.*, 1991, Calderini *et al.*, 1999b; Gooding *et al.*, 2003, Talukder *et al.*, 2014) is caused by the premature maturation of the pericarp that may in turn constrain endosperm expansion. Maternal tissue control of final grain size and weight in wheat has previously been suggested (Calderini *et al.*, 1999, Calderini *et al.*, 1999b; Lizana *et al.* 2010; Hasan *et al.,* 2011), but this potential interaction under high p.a. temperature has not been investigated in depth. This PhD project provides further insight into the relationship between the maternal pericarp and grain weight determination under high p.a. temperatures.

6.2 Key findings

The results contained within this PhD project lend support to the original hypothesis that high p.a. temperatures result in a premature maturation of the wheat grain pericarp with a consequent loss of plasticity and expansion capability in the cell walls of the tissue. Experimental results from chapters 3 and 5, which when analysed in combination, appear to support existing studies on the interaction between the wheat grain pericarp, grain dimensions and mature weight determination (Lizana *et al.*, 2010; Brinton *et al.*, 2017). We observed in experiment 4 that maximum average grain length and height were significantly smaller in high p.a. temperature treated samples and were the first dimensions to stabilise at around 10daa, earlier than in control grains (Chapter 3 -3.24 – Figures 3.5 and 3.7). Following the stabilisation of maximum grain length and height, both dimensions began to decrease slightly from 22daa towards maturity as also observed by Lizana *et al.* (2010) in *cv. Bacanora*.

The stabilisation of the maximum value of these dimensions under high p.a. temperatures at around 10daa, appears to coincide with the point at which maximum average grain moisture content stabilises in experiment 2 (~10daa) and slightly precede the same point in experiment 3 (~12-14daa) (Chapter 5 – 5.2.1 – Figures 5.1 and 5.2). Lizana *et al.* (2010) and Hasan *et al.* (2011) in cultivars *Bacanora-T88* and *Kambara,* also observed that maximum grain length stabilised just prior to maximum grain moisture content, although in contrast they observed that maximum grain length stabilised before maximum grain height. Therefore, the results of our study potentially indicate an interaction between grain length and height under high p.a. temperatures that had not yet been established. Stabilised maximum grain length and moisture content have previously been shown to be closely associated with final grain weight (Schnyder and Baum 1992; Lizana *et al.* 2010; Hasan *et al.* 2011; Xie *et al.* 2015). In addition, the close association between peak expression of 5 α-

EXPs isolated from pericarp tissue and grain length stabilisation (Lizana *et al.* 2010), as well as the significant interaction observed between pericarp cell length, grain length and thousand grain weight (TGW) (Brinton *et al.* 2017), suggests that grain length is an important determinant of final grain weight and is determined by the cellular expansion of the pericarp.

As for the potential cause of this earlier stabilisation of grain length and height in plants subjected to high p.a. temperatures at 10daa, in our RNA-Seq analysis we observed that a number of genes involved in regulating cell wall expansion, predominantly expressed in the outer pericarp, were contained within a gene cluster (outer pericarp cluster 2) significantly down-regulated in high p.a. temperature treated grain at 10daa (Chapter 5 -5.25,5.26, Table 5.4). These genes included 3 that encoded an endoglucanase, KOR3, 2 that encoded a xyloglucan endotransglucosylase and 1 that encoded a β-EXP. In addition, we observed significant down-regulation of 5 LTPs at 10daa under high p.a. temperature, that have previously been demonstrated to enhance cell wall expansion in cucumber hypocotyls, with a similar mode of action to that postulated for EXPs (Nieuwland *et al.*, 2005). The down regulation of these genes predominantly expressed in the outer pericarp at 10daa under high p.a. temperatures, rather than at 14daa, as observed in control samples, is consistent with the hypothesis that high p.a. temperatures result in a premature loss of plasticity in the outer layers of the grain. Whilst the down-regulation of these genes involved in cell wall expansion coincides with the stabilisation of maximum grain moisture content, length and height, a direct link between the down-regulation of these genes and reduced mature grain weight has not been established in this project.

However, the fact that these genes are down-regulated following the same duration of high p.a. temperature treatment (4-days) required to result in a reduction in mature grain weight (Chapter 3 – 3.2.1.2 – Fig.3.1), in addition to coinciding with the stabilisation of

maximum grain moisture content, length and height, suggests a strong degree of interaction between these genes and traits in the determination of final grain weight under high p.a. temperature. Therefore, we suggest that these genes should be considered as strong candidates for future studies further investigating the role of the pericarp in grain development under high p.a. temperatures, which addresses an outstanding research aim in the field identified by both Hasan *et al.* (2011) and Brinton *et al.* (2017). Further confirmation of the expression patterns of these candidate genes via methods such qRT-PCR and western blotting between high p.a temperature treated samples and control grains would help strengthen the proposed relationship between these genes and the regulation of grain development under high p.a temperatures.

The significant reduction in mature grain weight observed in experiment 2 (Chapter 3 – Fig.3.1) between plants that had either received 0 or 2-days of high p.a. temperature treatment and those that had received a treatment of 4-days or longer, (with no significant further reductions with increased duration of exposure), suggested a potential structural change in the sink capacity of the grain induced by the treatment, either through a reduction in endosperm cell number or their size. Maternal tissue control of seed size and endosperm sink capacity has been well studied (Radchuk *et al.*, 2011; Li and Li, 2015), however, a mechanistic restriction of endosperm cell division or physical constraint of endosperm cell size by maternal grain tissue has not yet been established. We observed that endosperm cell number was reduced by high p.a. temperatures at 16daa, following 10-days of treatment (Chapter 4 – Fig 4.2), but we were unable to determine at what stage endosperm cell division ceased in high p.a. temperature treated samples and whether this coincided with the observed stabilisations of maximum grain length, height and moisture content under high p.a. temperature conditions. Endosperm cell division has previously been shown to end around the same time as rapid net water accumulation in the grain

(Nicolas *et al.*, 1985). Therefore, the stabilisation of maximum grain water content at around 10-14daa in high p.a. temperature treated samples potentially indicates the point at which endosperm cell division ends in grains exposed to high p.a. temperatures. This is partially supported by the fact that there was no significant difference between endosperm cell numbers in high p.a. temperature treated grains at 12daa and 16daa according to the LSDs of the means (Chapter 4 – 4.2.1.1). A repeat experiment with an increased number of sampling stages analysing endosperm cell number and grain moisture content could confirm whether the cessation of net water deposition in high p.a. temperature treated grains precedes the termination of endosperm cell division.

Our investigation into the effect of high p.a. temperature on the form and distribution of cell wall HG in wheat grain sections treated with a dual enzyme treatment to unmask partially methyl-esterified HG and de-methyl-esterified HG, provided evidence in support of the hypothesis that high p.a. temperature results in a premature loss of plasticity in the cell walls of the pericarp. However, it was observed that the HG profile of both the endosperm and pericarp cell walls were significantly affected by high p.a. temperature (i.e. not the pericarp alone). Labelling for partially methyl-esterified HG was lost from the testa and nucellar epidermis under high p.a. temperatures at 18daa (Chapter 4 – Fig 4.10) whereas in a previous study using the same cultivar (Palmer, 2015), the signal persisted until 20daa. In the endosperm cell walls, partially methyl-esterified HG labelling was weaker at 18daa under high p.a. temperatures (Chapter 4 – Fig. 4.10) indicating a potential reduction in plasticity of the endosperm cell wall as well.

De-methyl-esterified HG distribution and labelling was also significantly affected by high p.a. temperature although a potential pattern of the effect was harder to discern. LM19 labelling was absent in the cell walls of the nucellar epidermis at 14daa and 18daa in high p.a. temperature treated samples. In addition, LM19 labelling was absent in the endosperm

cell walls of high p.a. temperature treated samples at 18daa but present in control samples. As previously suggested (Chapter 4 – 4.3.2. – Paragraph 9) it may be that labelling with LM19 occurs in high p.a. temperature treated grain in a relatively small developmental window or that the amount de-methyl-esterified HG in the cell walls of these tissues is reduced to a negligible amount under high p.a. temperatures. This would potentially imply the increased activity of PMEs on the cell walls of these tissues under high p.a. temperatures and could present a further avenue of investigation into the potential role of cell wall HG in grain development under high p.a. temperatures.

There was little evidence to suggest that the changes in methyl-esterification status of cell wall HG observed in this investigation may have affected endosperm cell division, potentially through the production pectic oligogalacturonides (OGA) production that inhibit the exogenous plant hormone auxin (Wakabayashi *et al.*, 2003). This was evidenced by the fact that there was negligible change in the distribution and methyl-esterification status of cell wall HG at the earliest sampling stage of 8daa following 2-days of high p.a. temperature exposure. However, Chateigner-Boutin *et al.* (2014) observed labelling of methyl-esterified HG with LM20 in the pericarp as early as 2daa in *cv. Recital*. Therefore, further investigation into the effect of high p.a. temperatures on HG status and its involvement in the production of OGAs, particularly in the initial p.a. stages of grain development, are needed to better determine how cell wall HG may impact on wheat grain sink capacity.

The results of experiment 4 (Chapter 3 – 3.2.2) showed that the established 4-day high p.a. temperature resulted in reductions in mature grain weight when applied from 6daa at various p.a. stages. The potential of the pericarp to interact with and determine the size and weight of the wheat grain is minimal towards the latter stages of grain development (e.g. 18 and 22daa onwards) when the layers of the pericarp begin to degenerate and

become compressed (Chateigner-Boutin *et al.,* 2018). This result therefore supports suggestions by Blum (1998) and Yang *et al.* (2004) that the observed reductions in grain weight under high p.a. temperatures applied in late grain filling are the result of impaired starch synthesis in the grain, either through the limited supply of assimilates for the grain or direct effects on photosynthetic processes. Both of these mechanisms could be investigated in future experimentation into the relationship of the pericarp and grain weight under high p.a. temperatures.

A 4-day high p.a. temperature treatment applied at 4daa did not reduce mature grain weight, suggesting that in *cv. Cadenza,* high p.a. temperatures only reduce mature grain weight if applied for 4-days or longer once the internal structure of the endosperm is established (i.e. from 6daa). This result potentially strengthens the hypothesis of maternal interaction with endosperm size determination and cell division as from 6daa onwards both the pericarp and endosperm expand in a coordinated fashion (Adamski *et al.* 2009), whereas prior to this stage initial endosperm cellularisation is still occurring. In addition, this result would contradict the previously mentioned hypothesis that high p.a. temperature induced modifications to cell HG may affect early nuclear divisions in the endosperm via the production of OGAs and inhibition of auxin in early grain development. This observed result that high p.a. temperatures only reduced mature grain weight when applied for a duration greater than 4-days from 6daa onwards therefore helps to define the most susceptible period of *cv. Cadenza* to high p.a. temperatures and could be incorporated into new or existing crop models to improve predictions into the effect of high p.a. temperatures on wheat yields.

6.3 Limitations of the study

Whilst this project has presented novel findings regarding the effect of high p.a. temperature on wheat grain development and specifically the endosperm and pericarp tissue, a number of limitations present in both the analysis and implementation of experiments must be acknowledged when discussing the results. One limitation that has been discussed previously was the exposure of experimental material from experiments 3 and 4 to temperatures greater than the p.a. treatment temperature (35°C) during the booting and booting-anthesis stages when grown in the glasshouses. Reductions in mature grain weight were similar in comparable treatments, i.e. 4-days of high p.a. temperature treatment applied from 6daa, between experiments 4 and 2 that had and had not received a pre-anthesis exposure to high temperature (Chapter 3 – Fig 3.1 and Fig. 3.2). In addition, reductions in grain moisture content were also similar in experiment 2 and experiment 3 (Chapter 5 – Fig.5.1 and Fig 5.2). These results suggest that any effect of pre-anthesis exposure may have been negligible on the results observed, therefore suggesting a lack of priming or acquired thermotolerance to high p.a. temperatures in wheat that has previously been observed (Wang *et al.* 2012; Fan *et al.*, 2018). A repeat of experiments 3 and 4 that avoided any pre-anthesis exposure may confirm whether this is the case if similar results were observed.

Another limitation that must be considered in this project is the comparison and combining of results from separate experiments that are then discussed in context with the overall hypothesis of the project. An ideal experiment that measured mature grain weight, grain dimensions and moisture content during development, in addition to providing grain for transcriptomic and immuno-fluorescence microscopy analysis following a 4-day high p.a. temperature treatment applied from 6daa would help better determine the effect of high p.a. temperature on any potential interaction between the endosperm and pericarp in

determining final grain weight. However, in terms of logistics this is not feasible due to the time required to collect samples and perform analysis in addition to the number of plants and cabinets required for such an experiment.

The sub-optimal quality of the majority of RNA extracts, which is a fairly normal occurrence in the case of wheat endosperm tissue ((Wang *et al.*, 2012), meant that samples were not suitable for RNA-Seq analysis with an Illumina HISEQ 4000 ™sequencer, which is able to sequence read lengths of 2 x 150bp, but were instead sequenced using the Illumina HISEQ 2000 sequencer using a read metric 2 x 125bp (paired end reads). Whilst there are a number of protocols available for the extraction of RNA from wheat whole grain, time and logistical constraints meant that it was impossible to try a variety of methods to extract high quality RNA samples. The addition of an α-amylase treatment step prior to the removal of RNA from barley endosperm has been shown to be effective in removing starch in Barley grains, which can negatively affect the quality and integrity of extracted RNA (Betts *et al.*, 2017). The addition of such a step to the RNA extraction protocol utilised in this study may have improved RNA quality and could be trialled with future extractions using the same method. Whilst the sequencer used did not significantly affect the data obtained, the number of reads produced was significantly lower in the samples of lower RNA quality (Chapter 5 – 1.2.2). Whilst normalisation methods used in the analysis corrected for this variation between the number of reads between samples, had all samples produced a high number of reads this may have identified differential expression of more genes with shallow coverage. In addition, larger read counts may have led to the identification of more DEGs between treatments and potentially have allowed for the identification of rare transcripts of genes potentially affected by the high p.a. temperature treatment that may have been relevant to the experimental aims. For example, no PMEs or PMEIs were identified as being significantly up or down regulated under high p.a. temperature in the RNA-Seq analysis,

despite the observations made in chapter 4 that high p.a. temperature treatment resulted in variations in the methyl-esterification status of cell HG in the wheat grain. It is possible that increased sequencing depth may have identified differential expression of PMEs or PMEIs that impact on HG form and function. The development of more effective methods for the isolation of high purity RNA from wheat endosperm and pericarp should improve in the future to improve upon the results reported in this project.

As previously mentioned, the filtering method used in the analysis of the RNA-Seq data only used existing transcriptomic data obtained from isolated outer pericarp and endosperm tissue (Fig. 5.7) (Pearce *et al.*, 2015) in addition to pure starchy endosperm tissue (Pellny, personal communication *unpublished*). These data sets were then cross filtered with the RNA-Seq data obtained in this project obtained from whole grain. Whilst transcriptomic data was available for the inner pericarp, this was not used due to the likelihood of cross contamination between tissues due to the close adherence of the inner pericarp to the endosperm and the difficulty in separating the two layers. Therefore, it is likely that genes involved in regulating cell wall plasticity expressed in the inner pericarp are excluded from this analysis. Were the transcriptomic profile of the inner pericarp analysed as well, it may have revealed the differential expression of an increased number of cell wall proteins involved in regulating cell wall expansion. Isolation of pure tissue sections and subsequent transcriptomic analysis of these tissues in the inner pericarp would improve the filtering method used and allow for further investigation into the effect of high p.a. temperature on the transcriptomic profile of the inner pericarp.

Another limitation of the study is that all four experiments were conducted within controlled environments. Experimentation in controlled environments is a valuable method of investigating the effect of different abiotic treatments on plant species due to the fact that aspects such as temperature and water supply can be closely controlled and the

variability in the growing environment is reduced e.g. light and moisture availability, soil nutrient content and structural heterogeneity. However, the results obtained from controlled environment investigations are as a result often limited in their application and relevance to growing and harvesting crops in the field, even in the same cultivar. The negative effects of high p.a. temperature on grain size has been well established both in the field (Ferris *et al.* 1998, Calderini *et al.* 1999, Hasan *et al.* 2011) and in controlled environment experiments (Gooding *et al.* 2003, Plaut *et al.* 2004) in a number of wheat cultivars including in this project. Hasan *et al.* (2011) conducted their investigation into wheat carpel size and length in the two high yielding spring wheat varieties *cvs. Bacanora T88, and Kambara* in a field based experiment in Chile and observed that grain length stabilised prior to grain moisture content. In a similar manner it was also observed in this controlled environment experiment focused project that grain length stabilised at around 10daa, just prior to total grain moisture content in high p.a. temperature treated grains. Therefore this somewhat suggests that the results observed in the controlled environment experiments of this project may be replicated in field based experiments. Further field based experimentation on the effect of high p.a. temperatures on grain size and development in *cv. Cadenza,* including transcriptomic experimentation, if logistically possible, will help improve the relevance and application of these results to end-users e.g. crop-breeders and farmers.

One final limitation of this project is that the entirety of the experiments were conducted using *cv. Cadenza*, a bread-wheat developed in the UK. Whilst the results of this project provide insight to the response of this cultivar to high p.a. temperatures, whether the identified candidate genes and accelerated expression profile of HG in the grain would be replicated in other cultivars with varying degrees of thermotolerance is unknown. A recent study by (Begcy *et al.*, 2018) examined the impact of a 48h period of high temperature

treatment (35°C) imposed during heading, on different physiological aspects of two sets of summer wheat germplasms from Australia and Europe. They found that the Australian varieties, particularly *cv. RAC875*, were more tolerant of high temperatures and that photosynthetic and transpiration rates were reduced to a much greater extent in the European varieties in addition to pollen viability. Were a heat tolerant cultivar such as *cv. RAC875* to be grown alongside *cv. Cadenza* in a repeat of the experiments conducted in this project, this could potentially indicate whether the identified potential candidate genes do underlie grain response to high p.a. temperatures, if significantly different levels of gene expression were observed in the heat resistant cultivar.

6.4 Future Work

The results of this project provide the basis for both future experimental and theoretical work into the effect of high p.a. temperature on the pericarp of the wheat grain and its interaction with both the endosperm and grain weight. Whilst the reduction in endosperm cell number under high p.a. temperature observed in experiment 3 indicates that the sink capacity of the grain is reduced in *cv. Cadenza,* endosperm sink potential is also dependent on the activity of enzymes involved in the starch synthesis pathway and the initiation of starch granules in endosperm cells (Jones *et al.* 1985). A future experiment that analysed the effect of the treatment used in experiment 3 on the activity of enzymes such as starch synthases and the number of starch deposition sites would help confirm whether the reduction in endosperm cell number and size in the dorsal region of the grain at 16daa also impacts on these determinants of endosperm sink potential.

The utilisation of a novel endosperm cell analysis method that incorporated the use of immuno-fluorescence microscopy and image analysis software in experiment 3 provides scope for future work using this method. Whilst this method allowed for the accurate

counting and size analysis of endosperm cells from wheat grain sections, in addition to being able to identify the location of particular endosperm cells within the cross section, the analysis was a time-consuming process and not yet fully optimised or automated via the software. Differences in image quality meant that it was sometimes difficult for the software to automatically analyse all endosperm cell present in an image, meaning that the remainder had to be selected and measured manually. The development of a high throughput image analysis software method for the quantification of endosperm protein gradients in wheat (Savill *et al.*, 2018) may have application potential for analysis of endosperm cell number and size in future experiments. Furthermore, modification of the analysis input used in this experiment (Appendix 1.) could potentially increase the throughput of this analysis allowing for a larger number of sampling stages to be analysed quickly. In addition, the loss of LM21 labelling in some regions of the endosperm in high p.a. temperature treated sections at 25daa meant that we were unable to identify effects on the size at number of endosperm cells between treatments at this stage. The identification of a specific stain for an endosperm cell wall constituent throughout grain development would allow for a more detailed study about the effects of high p.a. temperature on endosperm cell division throughout development.

The genes involved in cell wall modification that were down-regulated under high p.a. temperature at 10 and 14daa in the RNA-Seq analysis, including a peroxidase, LTPs, endoglucanases and a β-expansin are all good candidates for future investigations into the genetic underpinnings of wheat grain size and weight determination under high p.a. temperature. However, in order to confirm the role of these candidate genes in determining wheat grain size response to high p.a. temperatures, functional validation is required, to demonstrate that differences in the expression of these genes result in different phenotypic effects. One method would be to conduct a molecular assay such as in-situ

hybridization using the candidate transcripts identified in this study to locate the expression of these genes in the wheat grain at a more tissue specific level and reveal more about their potential role under high p.a. temperatures. In situ-hybridization has previously been successfully used in wheat to identify the role of the crease region in the grain in establishing the developmental patterning of gene expression throughout grain development in *cv. Savannah* (Drea *et al.* 2005). In addition, the use of biological assays that use techniques such as RNAi experimentation, tnt1 retrospansposon mutagenesis, or genome editing methods such as CRISPR/Cas9 can be used to functionally validate candidate genes. For example, RNAi experimentation that targeted down-regulation or silencing of these genes in future experiments analysing the response of grain weight to high p.a. temperatures may aid in the establishment of phenotypic data that help us better understand whether these genes are directly involved in regulating the determination of grain weight and development under high p.a. temperatures. In addition, often one of the cheapest and easiest methods to validate proposed candidate genes is to attempt to replicate observed results in another dataset of similar ancestry. The resequencing of the exome of 1200 *cv. Cadenza* mutants as part of the Wheat_Tilling project (www.wheat-tilling.com/) and the subsequent availability of mutant seeds for purchase provides a valuable resource in which to potentially validate the candidate genes identified in this study in closely related mutants. For example a hypothetical mutant lines identified from the database that expressed one of the candidate genes in high amounts could be back-crossed multiple times to the wildtype (e.g. *cv. Cadenza)* in order to obtain a line that is homozygous for the mutation of interest with a reduced load of background mutations. Through this method it would be possible to effectively produce NILs with and without the mutation of interest and then compare their phenotype under high p.a. temperatures. This would enable functional validation of the candidate genes identified in this study by

assessing their specific role in potentially establishing heat resistant or heat tolerant phenotypes in *cv. Cadenza* and would provide an interesting avenue of future investigation.

Further investigation into the effect of high p.a. temperature conditions on an increased range of sampling stages and the distribution of other cell wall pectic domains such as RG, XGA would provide more information regarding the role of HG and pectin within the wheat grain under high p.a. temperature. The exclusion of the inner pericarp tissues from the RNA-Seq analysis may account in fact for the lack of any differential expression of PMEs and PMEIs that antagonistically regulate the removal of methyl-esters from HG within the wheat grain and which may have been expected based on the observed changes in HG methyl-esterification status in experiment 2. Therefore, as previously mentioned, the isolation of pure inner pericarp tissue and the extraction of RNA in a future experiment using similar conditions may reveal an effect of high p.a. temperature on these two enzymes. In addition, despite being the largest cell wall constituent in the pericarp (around 25-30%), the effect of high p.a. temperature on arabinoxylan (AX) and ferulic cross-linking of AX was not investigated in this study. Toole *et al.* (2007) demonstrated in cultivars *Spark* and *Rialto* that elevated temperatures (>30°C) applied from 14daa resulted in endosperm cell wall AX shifting from being highly-branched with arabinose residues to being lowly-branched earlier that in wheat plants that were grown at elevated temperatures. Toole *et al.* (2007) postulated that this change may have resulted in the endosperm cell walls becoming more rigid earlier under high p.a. temperatures and may have contributed to the early cessation of grain filling under high temperatures. Investigation into the structure of pericarp cell wall AX under high p.a. temperatures, both in terms of branching with arabinose residues and ferulic cross linking of AX chains using FT-IR or immunofluorescence microscopy, may confirm the potential effect of a premature loss of

plasticity in the pericarp under high p.a. temperature and its possible effect on grain weight determination.

6.5 Conclusion

The results of this project add further information to a growing understanding of the complex response of wheat grain to high p.a. temperatures during the grain filling period. High p.a. temperatures were shown to reduce mature grain weight, accelerate the HG profile of the developing grain and result in the down-regulation of genes directly involved in cell wall modification in the outer pericarp with the latter 2 results being consistent with the hypothesis of a loss of plasticity in the outer layers. Whilst these results do lend support to the original hypothesis, the reduction of grain size following the application of high p.a. temperatures applied at different stages in grain development demonstrate the complex interaction between high p.a. temperatures and normal grain development as well showing that the observed reductions in mature grain weight under high p.a. temperatures are not the result of perturbations to the normal development of the pericarp alone. Further study into the role of cell wall pectin and the differentially expressed genes affecting pericarp cell wall plasticity identified in this project will reveal more about the interaction between high p.a. temperatures and the pericarp in grain weight determination, and ultimately may assist breeders in developing more climate resilient wheat cultivars in the future.

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Zhou, H. L., He, S. J., Cao, Y.-R., Chen, T., Du, B. X., Chu, C. C., Zhang, J. S. and Chen, S. Y (2006). OsGLU1, A Putative Membrane-bound Endo-1,4-ß-D-glucanase from Rice, Affects Plant Internode Elongation. *Plant Molecular Biology*, **60**: 137-151.

Appendix

1.1 Appendix 1. – ImageJ analysis input

```
run("Auto Threshold...");
//run("Brightness/Contrast...");
run("Apply LUT");
run("Close");
//setTool("freehand");
setBackgroundColor(0, 0, 0);
run("Clear", "slice");
run("Auto Threshold...", "method=Otsu white");
run("Analyze Particles...", "size=0-50000 show=Overlay display add in_situ");
run("Analyze Particles...", "size=0-50000 show=[Overlay Masks] display add in_situ");
roiManager("Measure");
```

Earlham Institute

Decoding Living Systems

QC report for ENQ-2268 | PRO1625

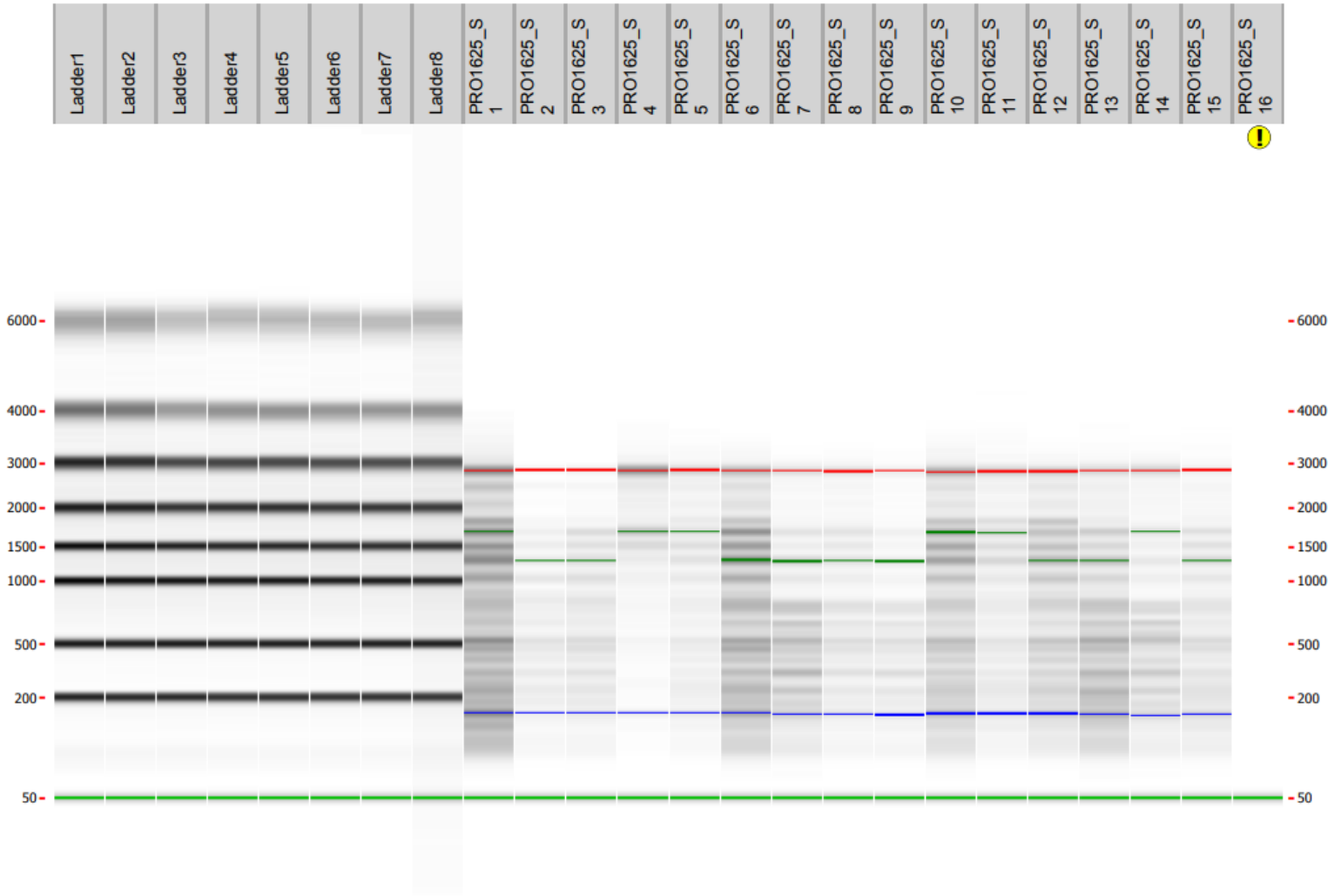
QC threshold criteria: For a sample to pass QC we ask for >2µg material. Samples can not be any less concentrated than 20ng/µl. The RIN/RQS value must be greater than 7 to denote high quality RNA.

Sample ID					RNA Platereader		DNA Platereader		RNA GX		Vol. of sample (µl)	Total Amount (µg)	Overall Pass/Fail
Sample ID	Sample Name	Sample Alias	Customer Ref	Well ID	ng/µl	Pass/Fail	ng/µl	Percentage DNA	RQS	Pass/Fail			
1	SAM35037	PRO1625_S1	22-282-6daa	A1	562.5	Pass	49.2	9	4.5	Fail	55	30.9	Fail
2	SAM35038	PRO1625_S2	1-276-6daa	B1	130.1	Pass	0.7	1	4.2	Fail	55	7.2	Fail
3	SAM35039	PRO1625_S3	2-67-6daa	C1	192.0	Pass	2.3	1	4.4	Fail	55	10.6	Fail
4	SAM35040	PRO1625_S4	7-81-10daa-S	D1	171.6	Pass	1.7	1	7.0	Pass	55	9.4	Pass
5	SAM35041	PRO1625_S5	8-46-10daa-S	E1	177.0	Pass	2.1	1	5.4	Fail	55	9.7	Fail
6	SAM35042	PRO1625_S6	11-187-10daa-S	F1	464.3	Pass	22.1	5	4.7	Fail	55	25.5	Fail
7	SAM35043	PRO1625_S7	5-280-14daa-S	G1	275.0	Pass	5.3	2	4.2	Fail	55	15.1	Fail
8	SAM35044	PRO1625_S8	6-324-14daa-S	H1	169.5	Pass	2.1	1	5.2	Fail	55	9.3	Fail
9	SAM35045	PRO1625_S9	18-296-14daa-S	A2	136.6	Pass	1.3	1	4.3	Fail	55	7.5	Fail
10	SAM35046	PRO1625_S10	4-219-10daa-C	B2	387.2	Pass	15.2	4	4.4	Fail	55	21.3	Fail
11	SAM35047	PRO1625_S11	17-65-10daa-C	C2	183.8	Pass	2.7	1	5.2	Fail	55	10.1	Fail
12	SAM35048	PRO1625_S12	16-177-10daa-C	D2	315.4	Pass	8.2	3	5.0	Fail	55	17.3	Fail
13	SAM35049	PRO1625_S13	23-297-14daa-C	E2	340.3	Pass	10.9	3	4.3	Fail	55	18.7	Fail
14	SAM35050	PRO1625_S14	13-221-14daa-C	F2	213.9	Pass	3.4	2	4.7	Fail	55	11.8	Fail
15	SAM35051	PRO1625_S15	20-234-14daa-C	G2	177.9	Pass	3.0	2	4.4	Fail	55	9.8	Fail
16	SAM35052	PRO1625_S16	RNAse free water	H2	<Min	N/A	<Min	N/A	N/A	N/A	55	N/A	Fail

These samples are concentrated and have plenty of material to proceed into library construction.

Unfortunately, the material itself is not high quality enough to pass our QC. We look for an RQS value >7 to denote that the RNA is high quality and free from degradation. This is typified by distinct ribosomal peaks with little background degraded RNA on the electropherogram. Your traces are attached.

This RNA is unsuitable for our high throughput RNA pipelines. Our low throughput pipelines may be more accommodating though as the samples have failed our QC, we cannot guarantee their downstream success or guarantee that they will yield good sequencing data.
If you are interested in pursuing our low throughput pipelines, please let us know. This will incur an increase in cost and turnaround time, though will give your samples a greater chance at success. A member of the team can explain in greater detail should you wish to pursue this avenue.



LabChip GX Software Version 4.1.1613.0

Datafile Created Version: 4.1.1613.0
Analysis Version: 0
2017-03-23_04-42-31.gxd

Modified:
Printed: 2017-03-24 09:09:46 AM
Firmware Version: 1.04.0.28266L
Instrument Name: N78396

1.3 Appendix 3. – R statistical analysis input for RNA-Seq data

```
rm(list=ls())

library("edgeR")

setwd(file.path("//salt", "wheat_rnaseq", "RichardKino"))

##############################

#Analysis of  samples

##############################

#19/12/2017 This is where we started off to get all my samples listed nicely

counts <- read.table("all_samples_counts.tab", row.names = 1, comment="", as.is=T)

colnames(counts) <- counts[1,]

row_names <- rownames(counts)[-1]

counts <- counts[-1,]

counts <- sapply(counts, as.numeric)

rownames(counts) <- row_names

sampleInfo <- data.frame(row.names = colnames(counts),

                         time = factor(c(rep("six_daa",2),rep("ten_daa",6), rep("fourteen_daa",6)), levels=c("six_daa",
"ten_daa", "fourteen_daa")),

                         stress = factor(c(rep("Control", 5), rep("Heat", 3), rep("Control", 3), rep("Heat", 3)), levels =
c("Control", "Heat")))

sampleInfo$group <- factor(paste(sampleInfo$time, substr(sampleInfo$stress,1,1), sep = "_"), levels =
c("six_daa_C", "ten_daa_C", "ten_daa_H", "fourteen_daa_C", "fourteen_daa_H"))

#Create design matrix using the previous information

design <- model.matrix(~0+sampleInfo$group)

colnames(design) <- levels(sampleInfo$group)

rownames(design) <- row.names(sampleInfo)


#Create DGEList object of the experiment - 19/12/2017 -As R is a statistical package it was able to discern this.
Threw up nearly 40k tho

y <- DGEList(counts = counts, group = sampleInfo$group)

#Filter low expressed genes according to the manual (Those that do not have 3 or more samples with cpm>=1)

keep <- rowSums(cpm(counts)>1) >= 3
```

```
y <- y[keep, , keep.lib.sizes=FALSE]

################################################################################
###

#Normalization - 19/12/2017 -This step normalises for bias in the samples allowing you to identify DEG from all

y <- calcNormFactors(y)

plotMDS(y, main="MDS Plot", col=as.numeric(y$sample$group)+1, xlim=c(-4.5, 4.5))

#Dispersion estimation

y <- estimateDisp(y,design)

fit <- glmFit(y, design)

#19/12/2017 These contrasts were comparing Genes on the basis of the different treatments. The final aim was to find DEG's that have a significant interaction with stage but are also affected by treatment as these are the most interesting genes

contrasts_stress <- makeContrasts(HvsC10=ten_daa_H-ten_daa_C, HvsC14=fourteen_daa_H-fourteen_daa_C, levels = design)

contrasts_time <- makeContrasts(time_10vs6=(ten_daa_H+ten_daa_C)/2-six_daa_C, time_10vs6=(fourteen_daa_H+fourteen_daa_C)/2-six_daa_C, time_14vs10=(fourteen_daa_H+fourteen_daa_C)/2-(ten_daa_H+ten_daa_C)/2, levels = design)

contrasts_interaction <- makeContrasts(interaction_14vs10=(fourteen_daa_H-fourteen_daa_C)-(ten_daa_H-ten_daa_C), levels = design)

contrasts_additive <- makeContrasts(additive_14vs10=(fourteen_daa_H+fourteen_daa_C)-(ten_daa_H+ten_daa_C), levels = design)


lrt_HvsC10daa <- glmLRT(fit, contrast = contrasts_stress[,1])

lrt_HvsC14daa <- glmLRT(fit, contrast = contrasts_stress[,2])

lrt_time_10vs6 <- glmLRT(fit, contrast=contrasts_time[,1])

lrt_time_14vs6 <- glmLRT(fit, contrast=contrasts_time[,2])

lrt_time_14vs10 <- glmLRT(fit, contrast=contrasts_time[,3])

lrt_interaction_14vs10 <- glmLRT(fit, contrast=contrasts_interaction[,1])

lrt_additive_14vs10 <- glmLRT(fit, contrast=contrasts_additive[,1])

deg_HvsC10daa <- topTags(lrt_HvsC10daa, n=Inf, p.value=0.05)

deg_HvsC14daa <- topTags(lrt_HvsC14daa, n=Inf, p.value=0.05)

deg_time_10vs6 <- topTags(lrt_time_10vs6, n=Inf, p.value=0.05)

deg_time_14vs6 <- topTags(lrt_time_14vs6, n=Inf, p.value=0.05)
```

```
deg_time_14vs10 <- topTags(lrt_time_14vs10, n=Inf, p.value=0.05)

deg_interaction_14vs10 <- topTags(lrt_interaction_14vs10, n=Inf, p.value=0.05)

deg_additive_14vs10 <- topTags(lrt_additive_14vs10, n=Inf, p.value=0.05)

#Write significant genes to a file

write.table(deg_HvsC10daa, "deg_HvsC_10daa.tab", quote = F, sep = "\t")

write.table(deg_HvsC14daa, "deg_HvsC_14daa.tab", quote = F, sep = "\t")

write.table(deg_time_10vs6, "deg_time_10vs6.tab", quote = F, sep = "\t")

write.table(deg_time_14vs6, "deg_time_14vs6.tab", quote = F, sep = "\t")

write.table(deg_time_14vs10, "deg_time_14vs10.tab", quote = F, sep = "\t")

write.table(deg_interaction_14vs10, "deg_interaction_14vs10.tab", quote = F, sep = "\t")

write.table(deg_additive_14vs10, "deg_additive_14vs10.tab", quote = F, sep = "\t")

#Clustering of the pericarp and endosperm specific genes

library("pheatmap")

#13.02.2018 DEg interaction read table to file

deg_interaction_14vs10 <- read.table("deg_interaction_14vs10.tab", row.names = 1, comment="", as.is=T)

#Use Till Pelny's pure endosperm data to filter out non-pericarp specific genes - 19/12/2017 - The average for tills reads was 80 million.

endosperm_counts <- read.table("pericarp_specific_genes_in_pellny_data_set_endo.txt", row.names = 1, comment="", as.is=T)

pericarp_specific_strict_filter <- rownames(endosperm_counts[rowMeans(endosperm_counts/80)<0.5,])
#Removed genes from the pericarp list with more than 40 reads on average in till's endosperm data (80mill avg)

#code to find strictly filtered pericarp specific genes within the list

intersect(pericarp_specific_strict_filter, rownames(deg_interaction_14vs10))

intersect(endosperm_specific_genes,rownames(deg_interaction_14vs10))

#19.12.2017 -This is when we looked at the heat maps of the newly filtered pericarp and endosperm groups and split them into clusters. These were then written to tables which I can then put through biomart and find the gene annotation. I also have cpm values in excell so I can plot them

pericarp_specific_genes <- scan(file.path("Pericarp_specific_genes","pericarp_specific_genes.txt"), what="character", skip=1)

endosperm_specific_genes <- scan(file.path("Endosperm_specific_genes","endosperm_specific_genes.txt"), what="character")

#13.02.2018 - This is the version that works for my computer directory (below)
```

```
endosperm_specific_genes <- scan("endosperm_specific_genes.txt", what="character")
interaction_genes <-intersect(pericarp_specific_strict_filter, rownames(deg_interaction_14vs10))
logcmp <- cpm(y, prior.count = 2, log = T)
logcpm_pericarp_specific_genes <- logcmp[rownames(logcmp) %in% pericarp_specific_strict_filter,]
logcpm_endosperm_specific_genes <- logcmp[rownames(logcmp) %in% endosperm_specific_genes,]
logcpm_interaction_genes <- logcmp[rownames(logcmp) %in% interaction_genes,]
pericarp_cluster <- pheatmap(logcpm_pericarp_specific_genes, scale = "row",
                    main="Pericarp specific genes (with scaling)", show_rownames=F,
                    cluster_cols = F, cutree_rows = 5)
pericarp_cluster_genes <- cutree(pericarp_cluster$tree_row, 5)
pericarp_specific_cluster1 <- names(pericarp_cluster_genes)[pericarp_cluster_genes == 1]
write(pericarp_specific_cluster1, "pericarp_specific_cluster1.txt")
pericarp_specific_cluster2 <- names(pericarp_cluster_genes)[pericarp_cluster_genes == 2]
write(pericarp_specific_cluster2, "pericarp_specific_cluster2.txt")
pericarp_specific_cluster3 <- names(pericarp_cluster_genes)[pericarp_cluster_genes == 3]
write(pericarp_specific_cluster3, "pericarp_specific_cluster3.txt")
pericarp_specific_cluster4 <- names(pericarp_cluster_genes)[pericarp_cluster_genes == 4]
write(pericarp_specific_cluster4, "pericarp_specific_cluster4.txt")
pericarp_specific_cluster5 <- names(pericarp_cluster_genes)[pericarp_cluster_genes == 5]
write(pericarp_specific_cluster5, "pericarp_specific_cluster5.txt")
endosperm_cluster <- pheatmap(logcpm_endosperm_specific_genes, scale = "row",
                    main="Endosperm specific genes (with scaling)", show_rownames=F,
                    cluster_cols = F, cutree_rows = 5)
endosperm_cluster_genes <- cutree(endosperm_cluster$tree_row, 5)
endosperm_specific_cluster1 <- names(endosperm_cluster_genes)[endosperm_cluster_genes == 1]
write(endosperm_specific_cluster1, "endosperm_specific_cluster1.txt")
endosperm_specific_cluster2 <- names(endosperm_cluster_genes)[endosperm_cluster_genes == 2]
write(endosperm_specific_cluster2, "endosperm_specific_cluster2.txt")
endosperm_specific_cluster3 <- names(endosperm_cluster_genes)[endosperm_cluster_genes == 3]
write(endosperm_specific_cluster3, "endosperm_specific_cluster3.txt")
```

```
endosperm_specific_cluster4 <- names(endosperm_cluster_genes)[endosperm_cluster_genes == 4]
write(endosperm_specific_cluster4, "endosperm_specific_cluster4.txt")
endosperm_specific_cluster5 <- names(endosperm_cluster_genes)[endosperm_cluster_genes == 5]
write(endosperm_specific_cluster5, "endosperm_specific_cluster5.txt")
pheatmap(logcpm_interaction_genes, scale="row",
        main="Genes with interaction effect between 14 and 10 daa (with scaling)",
        show_rownames=F, cutree_rows = 5)
#13.02.2018 creating the updated heat map
pheatmap(logcpm_interaction_genes, scale = "row",
main="Pericarp affected genes (with scaling)",cluster_cols = F)
#Venn diagram
deg_10daa_ids <- rownames(deg_time_10vs6)
deg_14daa_ids <- rownames(deg_time_14vs6)
deg_average_ids <- rownames(deg_time_14vs10)
area1 <- length(deg_10daa_ids)
area2 <- length(deg_14daa_ids)
area3 <- length(deg_average_ids)
nab <- intersect(deg_10daa_ids, deg_14daa_ids) #A & B (MAYBE IN C TOO)
n12 <- length(nab)
nbc <- intersect(deg_14daa_ids, deg_average_ids) #B & C (MAYBE IN A TOO)
n23 <- length(nbc)
nac <- intersect(deg_10daa_ids, deg_average_ids) #A & C (MAYBE IN B TOO)
n13 <- length(nac)
nabc <- intersect(nab, deg_average_ids) #A & B & c
n123 <- length(nabc)
library("VennDiagram")
g <- draw.triple.venn(area1, area2, area3, n12, n23, n13, n123,
                      category = c("time_10vs6", "time_14vs6", "time_14vs10"), ind = F)
library("gridExtra")
#x11()
```

```
grid.arrange(gTree(children = g), top="DEGs shared between contrasts")
#dev.off()
##############################################
#Analysis of Simon Vaughan's grain tissue data
##############################################
counts <- read.table("SV_tissues_counts.tabular", row.names = 1, comment="", as.is=T)
row_names <- rownames(counts)
counts <- sapply(counts, as.numeric)
rownames(counts) <- row_names
colnames(counts) <- c("Red_Endo_1", "Red_Endo_2", "Red_Endo_3",
                      "White_Endo_1", "White_Endo_2", "White_Endo_3",
                      "Red_InnerPericarp_1", "Red_InnerPericarp_2", "Red_InnerPericarp_3",
                      "White_InnerPericarp_1", "White_InnerPericarp_2", "White_InnerPericarp_3",
                      "Red_OuterPericarp_1", "Red_OuterPericarp_2", "Red_OuterPericarp_3",
                      "White_OuterPericarp_1", "White_OuterPericarp_2", "White_OuterPericarp_3")
sampleInfo <- data.frame(row.names = colnames(counts),
                         colour = factor(rep(c(c(rep("red",3)), c(rep("white",3))), 3), levels=c("red", "white")),
                         tissue = factor(c(rep("endosperm", 6), rep("inner_pericarp", 6), rep("outer_pericarp", 6)), levels =
c("endosperm", "inner_pericarp", "outer_pericarp")))
design <- model.matrix(~sampleInfo$colour+sampleInfo$tissue)
rownames(design) <- row.names(sampleInfo)
#Create DGEList object of the experiment
y <- DGEList(counts = counts, samples=sampleInfo)
#Filter low expressed genes according to the manual (Those that do not have 3 or more samples with cpm>=1)
#### ONLY SELECT THIS IF FILTERING ACCORDING TO COUNTS IN ROW 16 IS DISABLED ####
#keep <- rowSums(cpm(counts)>1) >= 3
#y <- y[keep, , keep.lib.sizes=FALSE]
##############################################################################
####
#Normalization
y <- calcNormFactors(y)
```

```
plotMDS(y, main="SV grain colour and tissue experiment", col=as.numeric(y$sample$tissue)+1, xlim=c(-5, 5))

#Dispersion estimation

y <- estimateDisp(y,design)

fit <- glmFit(y, design)

#contrasts <- makeContrasts(whitevsRed=, HvsC14=fourteen_daa_H-fourteen_daa_C,
average_HvsC=(ten_daa_H+fourteen_daa_H)/2-(six_daa_C+ten_daa_C+fourteen_daa_C)/3, levels = design)

#lrt_red_vs_white <- glmLRT(fit, contrast = c(0,1,0,0))

#lrt_inner_vs_endosperm <- glmLRT(fit, contrast = c(0,0,1,0))

lrt_outer_vs_endosperm <- glmLRT(fit, contrast = c(0,0,0,1))

#lrt_pericarp_vs_endosperm <- glmLRT(fit, contrast = c(0,0,1,1))


#deg_red_vs_white <- topTags(lrt_red_vs_white, n=Inf, p.value=0.05)

#deg_inner_vs_endosperm <- topTags(lrt_inner_vs_endosperm, n=Inf, p.value=0.05)

deg_outer_vs_endosperm <- topTags(lrt_outer_vs_endosperm, n=Inf, p.value=0.05)

#deg_pericarp_vs_endosperm <- topTags(lrt_pericarp_vs_endosperm, n=Inf, p.value=0.05)

#Write significant genes to a file

write.table(deg_outer_vs_endosperm, "deg_outer_vs_endosperm_SV.tab", quote = F, sep = "\t")
```