THE IMPACT OF COLD AND TRICHOSTATIN A TREATMENT ON THE TRANSCRIPTOME OF WHEAT MICROSPORES

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THE IMPACT OF COLD STRESS AND TRICHOSTATIN A ON THE TRANSCRIPTOME OF WHEAT MICROSPORES

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DEDICATION

To my husband, Bradie,

thank you for loving and supporting me through this whole process and believing in me.

ABSTRACT

Obtaining doubled haploids plants from isolated microspores is valuable for plant breeders. Microspores generally require stress treatment to enter androgenesis and produce doubled haploids. RNA-seq analysis was performed on cultured microspores that have been subjected to a three-week cold treatment and exposure to the histone deacetylase inhibitor Trichostatin A. Results point to dramatic transcriptional reprogramming after cold pre-treatment; upregulation in transcription, cytoplasmic turn over, signal transduction through kinase cascades, and chromatin remodelling supporting a change in cell fate. Cold-stressed microspores are then able to respond to TSA treatment with transient genome-wide upregulation of several thousand genes, not seen in unstressed microspores. Pathways upregulated include mitotic cell cycle phase transition genes, transcription, and DNA recombination and repair. These pathways may explain the increase in androgenesis seen after application of TSA. Results shed light on the behavior of wheat microspores in culture and pave the way for improved androgenesis and DH production.

PREFACE

Within Chapter 1 (1.2), Table 1.1. was taken and altered from a review article I co-authored with Kyle Bodell, Fengying Jiang, and John D. Laurie titled “Doubled haploid production through microspore culture.” Data was collected and compiled by me and KB in the review article. I have altered the table included for clarity and to add additional data.

Kyle Bodell also assisted with RNA-Seq data analysis in R – specifically with ChromoMap and writing scripts for Venn diagram creation and troubleshooting R coding.

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LIST OF ABBREVIATIONS

AC	Agriculture Canada
BARD1	BRCA-1 Associated Ring Domain protein
BBM	BABY-BOOM
BER	Base excision repair
BOI	Botrytis Susceptible1 Interactor
BP	Biological Process
BRG2	BOI – related gene 2
CDPK	Calcium-dependent protein kinases
CIMC	Wheat microspore culture media
CPP	Cell penetrating peptide
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CWSWS	Canada western soft white spring wheat
DE	Differentially expressed transcript
DH	Doubled haploid
DMSO	Dimethyl sulfoxide
DSB	Double strand break
F1	First filial generation
F2	Second filial generation
GBSSI	Granule-bound starch synthase I
GO	Gene Ontology
HDAC	Histone deacetylase
HR	Homologous recombination
HSP	Heat shock protein(s)
IMC	Isolated microspore culture
IRAK	Interleukin-1 receptor-associated kinase

KEGG	Kyoto Encyclopedia of Genes and Genomes
LEC1	Leafy cotyledon 1
MAPK	Mitogen-activated protein kinase
MCS	Multi-cellular structure(s)
MDE	Microspore derived embryo(s)
MRE	Meiotic recombination protein 11
PARP	Poly(ADP-ribose) polymerases
PCD	Programmed cell death
PSK-α	Phytosulfokine alpha
RB	Retinoblastoma protein
RNA	Ribonucleic Acid
RNAi	RNA interference
RNA-Seq	RNA Sequencing
ROS	Reactive oxygen species
SE	Somatic embryogenesis
SEA	Singular enrichment analysis
TF	Transcription factor
TSA	Trichostatin A
QTL	Quantitative Trait Loci
VCP	Valosin containing protein
WUS	WUSCHEL
YDA	Mitogen-activated protein kinase kinase kinase YODA

CHAPTER 1 – GENERAL INTRODUCTION

1.1. Background

Wheat, (*Triticum aestivum* L.) is an economically important cereal crop, one of the "Big 5" of monocots and a staple of human diet (Haberer, Mayer, & Spannagl, 2016). Indicating its importance, wheat was grown on 44% of the available agricultural land in 2018 (FAOSTAT). In 2009 wheat provided 20% of the protein in the human diet (Braun, Atlin, & Payne, 2010). Wheat is grown on the majority of agriculturally available land in Canada with 23,260,000 acres of land seeded with wheat in 2021, this includes winter wheat planted in fall of 2020 (Statistics Canada, 2021). Canada is among the top 10 producers of wheat; in 2019 Canada produced 4.22% of the world's wheat with a yield of 3.35 t/ha (http://www.fao.org/faostat/en/#data/QC/visualize).

With the increase in global population and increased challenges of climate change it is important to study staple crops in the hopes of improvements to yield, stress tolerance, and disease resistance. Hawkesford et al. (2013) suggests a multidisciplinary approach to crop improvement focusing on yield improvement, protecting yield potential, and increasing land use efficiency and sustainability. Improving yield requires more than simply increasing biomass produced per area but instead focusing on trait stability over varying environmental conditions and years, increasing carbon fixation, manipulating photosynthesis to increase biomass, increasing hardiness to elongate growth season which increases the number of grains produced, selecting for grain number instead of grain weight in production of new cultivars, improving root systems without excess increase of root biomass, improving efficiency of fertilizer uptake and use by plants, and maintaining

the protein quality of grain for down-stream uses (Hawkesford et al., 2013). Protecting yield potential is mainly focused on the hardiness of the wheat plant towards both abiotic and biotic stresses (Hawkesford et al., 2013) and has been a large focus in wheat improvement for breeders and breeding programs.

In conventional breeding programs 6-7 cycles of selection are usually required to achieve acceptable homozygous levels which can take over 10 years (Hussain, Khan, Ali, & Shaukat, 2012; Seguí-Simarro, 2010). Homozygosity in breeding programs permits precise phenotyping, gene functional studies and mapping (Yan et al., 2017) and maintenance of the line and its traits over generations. Phenotyping – analyzing, quantifying, or predicting the phenotype of an organism – is combined with genomic technology for marker-assisted selection of plants for breeding programs. Precise phenotyping allows breeders to select combinations of ideal traits in parental lines required for crossing in hybrid cultivar creation. Homozygosity also makes precise phenotyping easier as it eliminates dominance masking of traits.

Doubled haploid (DH) production is an important tool in plant breeding programs. DH plants create homozygous lines in a single generation, accelerating breeding programs by facilitating earlier release of new cultivars with desirable traits (Hussain et al., 2012; Seguí-Simarro, 2010). The use of DHs in breeding programs also eliminates the need for and problems associated with back crossing and inbred lines (Hussain et al., 2012). DH plants result from chromosome doubling within haploid cells, resulting in plants that are homozygous at both alleles within the entire genome. DH plants can be used for parental lines in hybrid crosses or used to created the homozygous cultivars that are required to maintain the line in general cultivation (Hussain et al., 2012). Gametes are

used as starting material to create new plants from a single, haploid parental genome. In wheat-maize hybridization, wheat ovaries are pollinated with maize pollen – known as a "wide cross" (Laurie & Bennett, 1986) – which leads to a incompatibility response and elimination of the maize genome, resulting in a haploid plant after embryo rescue and culture (Laurie & Bennett, 1988; Niu et al., 2014). In anther or isolated microspore culture (IMC) the immature pollen cells are cultured to create new haploid plants (explored in more detail in 1.2.). Haploid plants can be induced to double chromosomes spontaneously or through chemical treatment with colchicine, an antimitotic agent that interferes with microtubules, preventing segregation of chromosomes in mitotic cells (Eng & Ho, 2019).

1.2. Microspores and Androgenesis

Normal pollen development is split into two steps; microsporogenesis and microgametogenesis (Figure 1.1.). Although microsporogenesis and microgametogenesis are continuous there are seven developmental stages associated with it in cereal model species *Brachypodium distachyon* (Sharma, Singh, & Bhalla, 2015). During microsporogenesis the microsporocyte (microspore mother cell) undergoes meiosis and generates a tetrad consisting of four haploid microspores that are held together by a callose envelope. The callose envelope degrades, releasing the haploid microspores that continue to develop their exine wall that was started at the tetrad stage. Vacuolated microspores (Figure 1.1.) undergo volume expansion, intine formation, and cytoplasmic changes prior to an asymmetric division giving rise to a generative cell and a vegetative cell (Pollen Mitosis I). The cells differ in size and the generative cell is enclosed by the vegetative cell. The generative cell will go on to divide again (Pollen Mitosis II) to

produce sperm cells for double fertilization of the polar nuclei and egg cell forming the endosperm and embryo, respectively. The vegetative cell goes on to form the pollen tube that delivers the sperm cells.

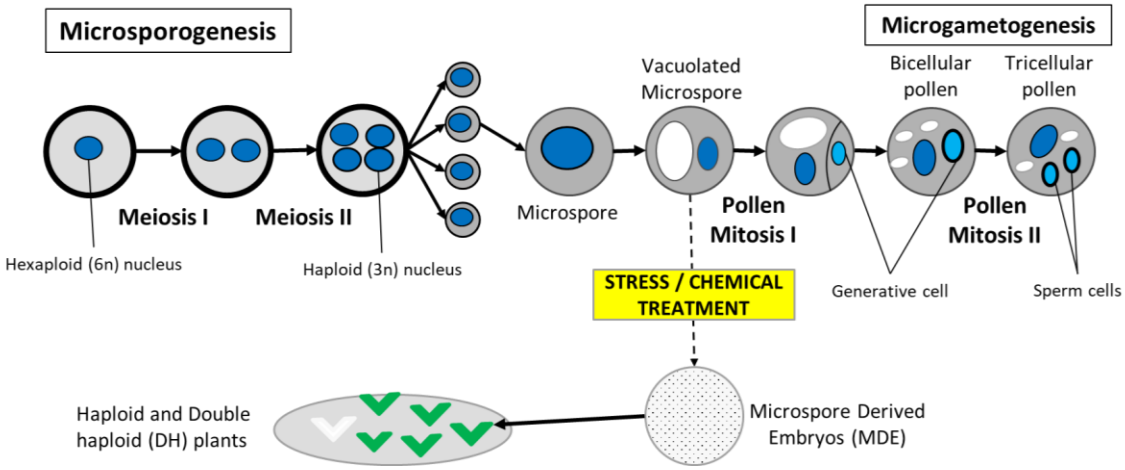

Figure 1.1. Overview of normal pollen development showing microsporogenesis and microgametogenesis modified from University of Leicster and (Maraschin et al., 2006; Seguí-Simarro, 2010). Vacuolated microspores at the mid- to late-uninucleate or early binucleate stage prior to the completion of pollen mitosis I can be diverted from pollen development through stress and/or chemical treatment to form microspore derived embryos (MDE) which develop into haploid and doubled haploid (DH) plants, some of which may be albino (white), lacking functional chloroplasts.

Androgenesis or microspore embryogenesis refers to the process of regenerating a plant from a haploid microspore cell (Figure 1.1). Microspores are isolated at the mid to late uni-nucleate stage or early binucleate stage; prior to the end of first gametophyte mitosis (Pollen Mitosis I, Figure 1.1.) and the commitment to pollen development (Bhowmik, Dirpaul, Polowick, & Ferrie, 2011; Segui-Simarro & Nuez, 2008; Soriano, Li, & Boutilier, 2013). When microspores at the correct stage are exposed to stress and/or chemical treatment, they can be altered from their normal cell fate of pollen development towards an androgenic pathway, creating a microspore derived embryo (MDE) within culture that can develop into a haploid or DH plant through spontaneous or chemically

induced chromosome doubling. The first stage in androgenesis is generally believed to be a symmetric division as opposed to the normal asymmetric division associated with Pollen Mitosis I. Androgenesis through IMC is the fastest way to produce DHs (Seguí-Simarro, 2010). Although somatic embryogenesis (SE) has been studied for decades, androgenesis is not well understood and many cultivars are not easily changed towards an embryonic cell fate (Seifert, Bössow, Kumlehn, Gnad, & Scholten, 2016). Many reviews are dedicated to the induction and improvement of androgenesis (Hosp, de Faria Maraschin, Touraev, & Boutilier, 2007; Ikeuchi et al., 2019; Seguí-Simarro, 2010; Segui-Simarro & Nuez, 2008; Soriano et al., 2013; Testillano, 2019; Zur, Dubas, Krzewska, & Janowiak, 2015).

IMC first requires the isolation of microspores at the correct stage from the anthers of the plant. The isolation process varies by species and is usually some combination of blending, straining, and in some cases density gradient separation through centrifugation. Once microspores are isolated, they are plated in media and incubated to produce MDE in the dark at temperatures that also vary by species and genotype. MDE are transferred to new media and placed under light for germination. In both isolation and germination stages media composition varies by species and cultivar and many studies have focused on these optimizations (Chu, Hill, & Brule-Babel, 1990; Cistué, Romagosa, Batlle, & Echávarri, 2009; Patel, Darvey, Marshall, & Berry, 2004; Wang et al., 2019; Zhao, Gervais, Simmonds, Newcom, & Simmonds, 2005). The specific protocol followed in this study is described in Chapter 2 (2.2.).

When first published in wheat, regeneration of plants from IMC could only be accomplished with temperature stress (heat) treatment prior to isolation and after isolation

co-culture with barley ovaries (Mejza, Morgant, DiBona, & Wong, 1993). Since then, co-culture with wheat ovaries continues to be an important factor in culture for the regeneration of MDE and green plants within wheat IMC (Broughton, 2008; Lu et al., 2008; Patel et al., 2004; Zheng, Weng, Liu, & Konzak, 2002). Ovaries are thought to support androgenesis in cultured microspores however regeneration in closely related cereal crop barley *(Hordeum valgare)* is possible without ovary co-culture (Kasha, Simion, Oro, & Shim, 2003). In some wheat cultivars ovary co-culture was able to be bypassed with media additives, although regeneration was not as efficient as in co-cultured microspores (Letarte, Simion, Miner, & Kasha, 2006). Currently stress treatment is still required to induce androgenesis using a variety of stresses including starvation, heat, cold, or a combination of stresses.

Currently the resulting number of MDE and regenerated plants from IMC varies between species and cultivars. In wheat cultivars Chris, Pavon 76, and WED202-16-2, Zheng et al. (2015) discovered 44%-62% of microspores failed to reach a multicellular structure with some never dividing. Of the microspores that reached a multicellular stage 54%-58% developed into MDEs depending on the cultivar (Zheng, Bieren, & Griggs, 2015). A study on enlarged microspores in barley IMC in cultivar Igri found that 11% developed into “embryo-like structures” (MDEs) following an embryogenic pathway, and 36% followed sporophytic divisions developing into multicellular structures and dying in culture (mostly between day 7 and 10), while 53% failed to be reprogrammed and died within the first few days after isolation (Maraschin, van Bergen, Vennik, & Wang, 2008; Maraschin, Vennik, Lamers, Spaink, & Wang, 2005). These studies highlight that reprogramming is the largest barrier to MDE production, both in terms of microspores

dying early in culture and microspores continue gametophytic divisions towards the microgametophyte instead of initiating embryogenic division patterns.

Along with the variation in regeneration of MDEs the percentage that germinate into green plants also varies by species and cultivar. In contrast to MDE number the number of green plants that result from IMC is more heavily studied (Gajecka et al., 2020; Guo & Pulli, 2000; Jacquard et al., 2009; Larsen, Tuvesson, & Andersen, 1991; Li & Devaux, 2003; Liu, Zheng, & Konzak, 2002; Liu, Zheng, Polle, & Konzak, 2002; Oleszczuk, Sowa, & Zimny, 2004; Patel et al., 2004; Ritala, Mannonen, & Oksman-Caldentey, 2001; Salmenkallio-Marttila, Kurten, & Kauppinen, 1995). High percentage and number of green plants regenerated from IMC is the goal of IMC and therefore the focus of improvements. There are two problems associated with green plant production from MDEs, no germination and germination of albino plants. Albino plants lack functional chloroplasts (leading to their white colour) and therefore will not photosynthesize or survive once removed from the media and transferred into soil. Albinos represent a large obstacle in many cultivars and has proven to be a complex issue involving many factors (Ankele, Heberle-Bors, Pfosser, & Hofinger, 2005). Low levels of DNA within plastids early in culture along with plastid differentiation and development into amyloplasts and has been linked to albino regeneration levels (Caredda, Doncoeur, Devaux, Sangwan, & Clément, 2000). However the phenotypes linked to albino regeneration in the previous study are likely the result of some other cause early on, even before microspore isolation and do not apply to all cultivars with issues of albinism (Caredda, Devaux, Sangwan, Proult, & Clément, 2004). Studies have attempted to understand the complex problem of albinism by working to identify some of the genes

that may be involved in plastid differentiation to chloroplasts, and Gajecka et al. (2020) identified GBSSI gene (Granule-bound starch synthase I) expression level in early uni-nucleate microspores as a good marker of green plant regeneration potential from IMC in barley. Table 1.1. outlines the resulting percentage of green plants resulting from several studies on wheat, barley and triticale cultivars under different treatments.

Table 1.1. Percentage of green plants resulting in a range of species, cultivars and treatments in anther culture and isolated microspore culture.

Species, Cultivar	Treatment	Green Plant (%)	Reference
Wheat, Chris	No Ovaries (Control)	86.7	(Hu, 1997)
Wheat, Chris	Co-culture with ovaries	86.2	(Hu, 1997)
Wheat, WED 202-16-2	No media added to Pre-treatment (Control)	70	(Liu, Zheng, & Konzak, 2002)
Wheat, WED 202-16-3	NPB98 medium added to Pre-treatment	89	(Liu, Zheng, & Konzak, 2002)
Barley, Igri	Normal Anther Culture	88	(Caredda et al., 2004)
Barley, Cork	Normal Anther Culture	3, 2	(Caredda et al., 2004), (Jacquard et al., 2009)
Barley, Cork	Copper sulphate pre-treatment (Anther Culture)	20	(Jacquard et al., 2009)
Barley, Tamparkorn	Normal IMC	97.5	(Gajecka et al., 2020)
Barley, Mercada	Normal IMC	11	(Gajecka et al., 2020)
Triticale, Wanad	190-2M medium (Control)	25	(Żur et al., 2009)
Triticale, Wanad	Starvation inducing medium	50	(Żur et al., 2009)
Triticale, AC Alta	CHB3 medium	59	(Eudes & Amundsen, 2005)
Triticale, AC Alta	NBP99 medium	40	(Eudes & Amundsen, 2005)
Triticale, AC Alta	NBP99 + Ficoll	45	(Eudes & Amundsen, 2005)
Triticale, AC Certa	CHB3 medium	83	(Eudes & Amundsen, 2005)
Triticale, AC Certa	NBP99 medium	89	(Eudes & Amundsen, 2005)
Triticale, AC Certa	NBP99 + Ficoll	92	(Eudes & Amundsen, 2005)

As products of meiosis, microspores and IMC represent vast potential in DH plant production; through homologous recombination (HR) they have the potential to provide a population of diverse combinations of traits within genotypes of interest to breeders and researchers within a single generation. For instance, F1 plants that are genetically diverse

can produce an F2 that is a vastly diverse population of haploid and DH plants through IMC. This is especially helpful in producing parental lines for breeding programs, through androgenesis generating a population of DH plants with differing combinations of traits from a single paternal genome only takes a single generation. IMC can also be used to preserve the homozygosity in genomes of cultivars in breeding programs.

As single cell culture IMC also represents an ideal platform for innovation in breeding programs and genetic studies as the application of transgenic protocols such as bombardment, electroporation, cell penetrating peptides (CPPs), and *Agrobacterium*-mediated transformation (Shariatpanahi & Ahmadi, 2016) and avoid issues such as chimeras in transgenic plants.

Although there has been much published work to improve androgenesis, cultivar differences prevent application of most published methods for improvement. Each new cultivar must be optimized individually in terms of morphological staging for microspore stage, stress treatment, culture media composition, and chemical treatments. The low and variable yield of green plants in combination with the large amount of effort needed for cultivar specific optimization and tissue culture practices currently prevents a more widespread use of microspore technology in wheat. An improvement of the understanding of androgenesis and work towards elimination of optimization for each new cultivar would lead to more wide-spread adoption of this technology and aid research and development in cereal crops.

1.3. Review of Previous Studies on Gene Expression in Androgenesis

Previous studies have been done to measure gene expression of microspores in culture in an effort to learn about androgenesis induction along with the genes and

pathway involved. Differentially expressed transcripts (DEs) involved in androgenesis induction and progression may help us to not only understand what is occurring in cultured microspores but identify targets that can possibly be used to improve androgenesis.

In the study of SE transcripts such as BBM1 and WUSCHEL2 (WUS2) (Lowe et al., 2016), LEAFY COTYLEDON1 (LEC1) (Lotan et al., 1998), and LEC2 (Stone et al., 2001) have been specifically implicated in the induction of embryogenesis. Within rapeseed BBM1, LEC1, and LEC2 were identified at 2-3 days in IMC (Malik et al., 2007). Interestingly a study on rapeseed MDEs showed that their transcriptome differed from zygotic embryos (Joosen et al., 2007).

Barley, a cereal crop closely related to wheat has been a well studied cereal for androgenesis induction. Bélanger, Marchand, Jacques, Meyers, and Belzile (2018) identified pathways such as stress response, embryo development, epigenetic reprogramming, and proliferation in barley microspores over time in culture. Glutathione S-transferase and heat shock protein (HSP) genes were found to be upregulated while ribosomal subunit protein genes were downregulated between day 0 and 2 (Bélanger et al., 2018). BBM1 was also found newly and significantly expressed at day 5 (Bélanger et al., 2018). KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways downregulated in salt-stress barley microspores were translation and ‘folding, sorting, and degradation’ (Liu et al., 2016). Stress treatment to induce androgenesis in barley microspores saw upregulation of sugar and starch hydrolysis, proteolysis, stress response, inhibition of programmed cell death (PCD), and signaling and downregulation of starch biosynthesis

and energy production (Maraschin et al., 2006). Autophagy has also been found to induce cell death in stressed barley microspores (Bárány et al., 2018).

Wheat microspores have not been the focus of many transcriptomic studies thus far. In the study of wheat microspores histone methylation and acetylation, as well as many other transcripts previously implicated in embryogenesis and a large number of novel transcripts were enriched in upregulated DEs in androgenesis while biosynthetic processes were downregulated (Seifert et al., 2016). A study on Quantitative trail loci (QTL) analysis showed two major QTLs, one on chromosomes 1B and another on 7B that explained 53% of the green plantlet variation (Nielsen et al., 2015).

Triticale is a wheat-rye hybrid crop. Transcriptional studies in triticale have been lacking, possibly due to the complex nature of its genome. However, analysis of the expression of a specific subset of 13 genes in several different triticale cultivars of varying responsiveness to androgenesis showed altered expression correlating to responsiveness (Zur et al., 2014).

A recent study in soybean compared cold-shocked to fresh microspores and found HSPs, cytochrome P450s, cell wall modifications, and cell proliferation were upregulated and saccharide metabolism, vacuolar transport, and other pollen-related developmental processes were downregulated immediately after isolation (Hale, Phipps, Rao, Wijeratne, & Phillips, 2020).

1.4. Chemical Treatment for Androgenesis Induction and Improvement

Considering the obstacles associated with androgenesis, it is no surprise that chemical treatments have been applied to microspore culture in efforts to improve the

resulting number of green plants. Many chemical treatments and culture additives tried thus far have been shown to induce or improve androgenesis in microspores.

Addition of organelle antioxidants, proline or glutathione were able to improve green plant regeneration in both wheat and triticale androgenesis, even in recalcitrant genotypes (Asif et al., 2013). Treatment with caspase 3 inhibitor, DEVD-CHO showed a decrease in PCD, improving androgenesis (Rodríguez-Serrano et al., 2011; Sinha & Eudes, 2015). Sinha and Eudes (2015) also reported the positive impact of antioxidant peptide SS-31 treatment on androgenesis in triticale.

Many chemical treatments induce epigenetic changes, unsurprisingly as chromatin organization and gene expression are considered important for androgenesis and recovery of DH plants (Berenguer et al., 2017; Li et al., 2014; Solís, El-Tantawy, Cano, Risueño, & Testillano, 2015; Yang et al., 2010; Zhang et al., 2016). For example, M 2-hydroxynicotinic acid, benzotriazole-5-carboxylic acid and violuric acid monohydrate were able to induce MDE or calli production in fresh wheat microspores in the absence of cold stress (Zheng, Liu, Weng, Polle, & Konzak, 2001). BIX-01294, which inhibits methylation of histone H3K9, was able to stimulate cell reprogramming, totipotency and embryogenesis induction when applied early on in culture (Berenguer et al., 2017; Wang et al., 2019). 5-azacytidine, which leads to reduction in DNA methylation levels in the genome, showed an improvement in androgenesis and SE (Fraga et al., 2012; Nowicka et al., 2019; Solís et al., 2015). 5-azacytidine has also been combined with Trichostatin A (TSA) to improve mitosis in maize (Yang et al., 2010). Scriptaid, a histone deacetylase (HDAC) inhibitor also showed improved regeneration of green plants from androgenesis

in wheat (Wang et al., 2019). TSA and Sodium butyrate were also reported to improve regeneration of plants from immature embryo culture in wheat (Bie et al., 2020).

TSA is a HDAC inhibitor that was purified from mycelia of *Streptomyces hygroscopicus* originally used as an anti-fungal agent (Tsuji, Kobayashi, Nagashima, Wakisaka, & Koizumi, 1976). Histone acetylation is associated with less compact (open) chromatin and promotion of gene expression through increased availability of DNA for transcription factors (TFs) and transcription proteins; reviewed by Salvador and Luesch (2012). The removal of acetyl groups from the histone tails by HDACs generally causes closing of the chromatin structure and a decrease in transcript abundance from that region (Salvador & Luesch, 2012).

Treatment of TSA was shown to induce totipotency in Arabidopsis anther culture (Li et al., 2014) and in wheat IMC (Wang et al., 2019) and specifically in wheat cultivar AC Andrew, showing an increase in the yield of both embryo-like structures and green plants in cold-stressed microspores treated with TSA (Jiang et al., 2017). Another recent study in wheat found TSA improve regeneration when combined with several different stresses in studied cultivars and induce androgenesis in the absence of stress in highly responsive cultivars studied (Castillo et al., 2020). Preliminary data from Jiang and colleges also showed the application of TSA to fresh, non-stressed AC Andrew microspores resulted in a small number of MDE while fresh microspores with TSA application did not result in any MDEs (unpublished data). TSA has also been shown to improve survival of barley microspores in culture as well as resulted in earlier exine rupture and more synchronized formation of multi-cellular structures (MCS) (Pandey et al., 2017).

Recently TSA was also shown to aid in SE in Arabidopsis explants, with upregulation seen in YUCCA genes (YUC1 and YUC10), LEC1, LEC2, BBM, and stress response (Wójcikowska et al., 2018). In the study from Castillo et al. (2020) Quantitative RT-PCR on wheat microspores treated with TSA showed higher expression of two markers for early MDE development at 3 days in culture.

1.5. The Transcriptomic Study of Wheat Microspores in the Presence and Absence of Cold-stress and TSA Treatment

In wheat microspores cold stress treatment is required for the regeneration of plants through IMC. Cold-stressed microspores treated with TSA showed improved regeneration of plants compared to cold-stress alone (Jiang et al., 2017) and application of TSA to fresh microspores showed a small number of resulting MDEs and green plants in preliminary data (unpublished).

After these results we had several questions to explore. What is the switch that induces androgenesis when microspores are cold stressed before isolation? What is the mechanism by which TSA acts to improve androgenesis in cold stressed microspores? And is the same mechanism involved when TSA treatment induces androgenesis and regeneration of green plants in fresh microspores?

In the present study wheat microspores were isolated and RNA-Seq analysis was completed with the intention of learning about androgenesis induction and answering the questions regarding the impact of cold-stress and TSA treatment on the transcriptome. As well as to identify potential targets for improvement of androgenesis in IMC with CRISPR/dCas9 gene expression manipulation in the future.

CHAPTER 2 – METHODS AND MATERIALS

2.1. Donor Plant Growth Conditions

Wheat variety 'AC Andrew', a Canadian soft white spring wheat (CSWSW) (Reg. no. CV-936, PI 632907) was developed by the Lethbridge Research Centre, Agriculture and Agri-Food Canada (AAFC) and was bred to be adapted to the irrigated regions of southern Alberta and Saskatchewan (Sadasivaiah, Perkovic, Pearson, Postman, & Beres, 2004). AC Andrew was chosen for this study as previous paper from our lab showed it to be responsive to TSA treatment (Jiang et al., 2017). Seeds were planted two seeds per pot in Nursery pots (LISTO Nursery #1, 2.8 L) with Cornell mix and grown in growth cabinets with 18 hours of full light (intensity 300 uE $m^{-2}s^{-1}$) at a temperature of 15ºC in the day and 12ºC at night and watered daily. Plants were sprayed with Intercept and NOVA for mildew and aphids at the three-leaf stage, three weeks after planting.

2.2. Cold Treatment, Microspore Isolation and Culture

There were four treatments in this study (Figure 2.1.), the presence or absence of cold-stress and the presence or absence of TSA treatment (described in 2.3.). For treatments with cold stress (Stress & Stress+TSA) the tillers had extra leaves removed and were covered in aluminum foil and stored with their bases in water at 4°C for 21±2 days before microspore isolation. For treatments without stress (Fresh & Fresh+TSA) the spikes were used the same day as collection from growth cabinets.

Tillers were collected from plants when microspores are at the mid- to late-uninucleate stage. For IMC, the spikes were first removed from the boot, their awns trimmed and then sterilized for 5 minutes in 10% commercial bleach and rinsed four

times with autoclaved distilled water. Florets were transferred in a laminar flow hood to a Waring blender with Extraction Buffer (Jiang et al., 2017) and blended twice for seven seconds on low speed. The slurry was filtered through 1 mm pore size strainer then filtered again through a 100 µm sterile mesh and centrifuged at 100 g at 4ºC for 5 minutes. Cells were then washed twice with 10 µM of SS-31 peptide in CIMC Wash (Jiang et al., 2017), centrifuging each time at the same conditions. Cells were suspended in 6 mL of 20% Maltose solution with 1 mL of CIMC Wash layered on top and centrifuged at 4ºC at 100 g for 13 minutes. The resulting band of cells at the interface was collected and cells were counted using a hemocytometer to estimate the cell concentration.

AC Andrew microspores were plated in 4 mL of CIMC Wash in Petri dishes (60x15 mm) with 100,000 cells per dish, 25 µL of Phytosulfokine alpha (PSK-α) and 4-6 ovaries. Ovaries were collected from tillers when the ovary is swollen and hairy and the anthers are light green. It is important that ovaries were collected at the correct stage as ovary co-culture is necessary for MDE production in culture. Cultures with microspores and ovaries were stored at 28ºC in the dark.

2.3. Trichostatin A Application

TSA application followed protocol described by Jiang et al. (2017). Briefly, TSA dissolved in DMSO was added to microspores at the final washing step of isolation in CIMC Wash for a final concentration of 0.5 µM and incubated at room temperature for 10 minutes. Tubes were spun down at 100 g at 4ºC for 5 minutes, CIMC Wash was replaced, and cells are plated as previously described (2.2.).

2.4. Cell Sample Collection

AC Andrew microspores were collected for RNA at time points of interest for the four treatment groups (Stress, Fresh, Stress + TSA, Fresh + TSA); 0-, 3-, and 48-hours following isolation; see Figure 3.1 for illustration of the samples collected.

200,000 cells were collected in 2 mL tough grinding tubes with as much liquid as possible removed and 6-7 metal beads, immediately frozen in liquid nitrogen and stored at -80ºC for subsequent RNA extraction. 200,000 cells was chosen as it showed the optimal amount of cell crushing and resulted in acceptable RNA quality and quantity in trials (results not shown).

For 48-hour time points samples were enriched for viable cells using a standard lab protocol. Briefly, all cells and media was removed from dishes, combined into a single 50 mL Falcon tube and spun at 200 g for 10 minutes to pellet cells. The pellet was resuspended, and suspension was poured into a 15 mL tube and spun down at the same conditions as before. The cell pellet was then re-suspended in 7 mL of 30% Maltose solution to isolate the larger, more swollen live cells at 48 h. As before 1mL of CIMC wash was layered on top to separate via centrifugation for 13 minutes at 200 g. The resulting layer of cells at the interface between the wash and maltose solutions was collected, counted and frozen in aliquots of 200,000 cells per tube as previously described.

2.5. Cell Shearing and RNA Extraction

Cells were physically sheared using the Precellys 24 homogenizer (Bertin Instruments) cooled by the Cryolys (Bertin Instruments). Tubes were spun at 6500g for

10 seconds four times at a temperature between -5ºC and -10ºC then tubes were immediately returned to liquid nitrogen and stored at -80ºC for RNA isolation and RNA quality was determined with the Agilent Bioanalyzer 2000.

RNA was isolated using NucleoSpin RNA Plant (Machery-Nagel, cat. no. 740949) with modifications, 2 samples were combined after addition of Lysis Buffer resulting in 700 µL total, centrifugation times were increased by 30 seconds, and RNA was eluted in 50 µL of RNase-free water. DNA degradation was performed on column following kit protocols and then repeated on eluted RNA using TURBO DNase (Invitrogen, ThermoFisher Scientific, cat. no. AM2238). RNA was cleaned up using NucleoSpin RNA Cleanup XS (Macherey-Nagel, cat. no. 740903) with modifications. Briefly, centrifugation times were increased by 30 seconds, incubation times on column were doubled, and RNA was eluted twice in the same 30 µL. RNA quality and concentration was checked using Biolanalyzer2000, following kit protocols.

RNA samples were sent to BGI Americas for sequencing. RNA with RINs 6.1 and higher were sent in 15 µL of RNase-free water in concentrations of at least 44ng/µL. RNA Sequencing was completed via platform DNBSeq with 150bp paired end reads and 20 million clean reads per sample.

2.6. Data Analysis

Preliminary data analysis was performed by BGI. Low quality reads and reads with adaptors were removed using internal SOAPnuke v1.5.2. Reads were mapped to the *Triticum aestivum* genome (Chinese Spring Wheat IWGSC RefSeq v1.1, Ensembl Plant) (Appels et al., 2018; Howe et al., 2019) using HISAT2 v2.0.4. StringTie v1.0.4,

Cuffcompare from Cufflinks tools v2.2.1, and CPC v0.9-r2 were used for novel transcript prediction. FPKM stands for Fragments Per Kilobase of transcript per Million mapped reads and was calculated with the following formula.

$$\mathrm{FPKM} = \frac{(1000000 * \mathrm{C})}{\mathrm{N} * \mathrm{L}/1000}$$

C represents the amount of fragment which mapped to the specific transcripts,

N represents the amount of fragment which mapped to any transcripts.

L represents the base amount of the specific transcripts.

Gene expression analysis was done through Bowtie2 v2.2.5 and RSEM v1.2.12. Differential expression analysis was completed with DESeq2, with a cut off for fold change of >/= 2.00 and adjusted p-value of </= 0.05. The schematic of comparisons for differential expression analysis is shown in Figure 2.1

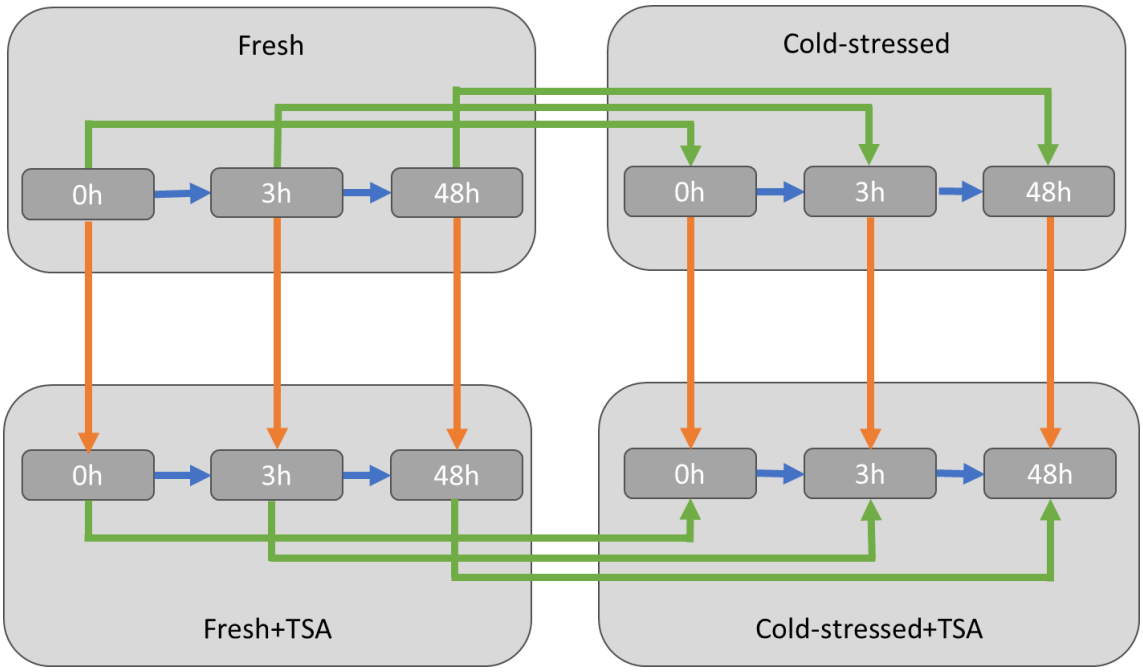

Figure 2.1. Schematic of the comparisons between treatment groups and time points for the RNA-Seq analysis.

Using R version 4.0.3 (R Core Team, 2020) and packages dplyr (Wickham, François, Henry, & Müller, 2021) and chromoMap (Anand, 2019) in Rstudio version 1.4.1106 (RStudio Team, 2021) images of the DEs mapped to the wheat genome were created.

DE lists were compared using Venn Diagrams created with Gene Venn (Pirooznia, Nagarajan, & Deng, 2007) and in RStudio using VennDiagram package (Chen & Boutros, 2011). R package UpSetR (Conway, Lex, & Gehlenborg, 2017) was used for similar comparisons between a larger number DE lists, allowing to visualize the number of intersecting and unique DEs in all comparisons between treatments or within the same treatment over timepoints studied.

DE lists were input into AgriGO v2 (Tian et al., 2017) for singular enrichment analysis (SEA) using the full gene list from our study as the reference. PANTHER classification system (Mi, Muruganujan, Casagrande, & Thomas, 2013; Mi et al., 2019) was also used for GO analysis. To visualize GO terms and remove redundant terms REVIGO (Supek, Bošnjak, Škunca, & Šmuc, 2011) was used with SEA p-values and set to small (0.5 cut off) with all other parameters as default. KEGG pathways were analyzed using KEGG Mapper v4.3 (Kanehisa & Sato, 2020).

CHAPTER 3 – THE IMPACT OF COLD STRESS ON THE TRANSCRIPTOME OF AC ANDREW MICROSPORES IN CULTURE

3.1. Introduction

The requirement for cold-stress treatment prior to microspore isolation indicates that the treatment is altering microspores to allow for androgenesis induction in culture. The cold-stress treatment involves more stress than simply cold-stress; there is also nutrient starvation, absence of light, and osmotic stress. All of these are combined into the "Stress" or "cold-stress" treatment names for simplicity and ease of reading. The purpose of this comparison was to explore how cold stress treatment impacts the transcriptome of wheat microspores prior to and early on in culture. Through this study and in the comparison to previous transcriptomic studies, genes that are differentially expressed and possibly important early on in culture can be identified for future study.

We hypothesized that the transcriptome would be altered by cold, anticipating genes and pathways involved in preventing cell death, responding to the stress and culture conditions, and changing cell fate towards embryogenesis would be present early on in culture.

Gene expression levels were compared by treatment and time point, freshly isolated microspores were compared to microspores that had been treated with cold stress for 21± 2 days in 4°C in the dark immediately following isolation, as well as 3 hours, and 48 hours after isolation. A schematic outlining the comparisons (Figure 2.1.) and methods for microspore isolation, sample collection, and RNA-Seq analysis can be found in Chapter 2.

3.2 Results

Before focusing on the comparison between fresh and stressed microspores a general overview of the results for all treatments will be provided. The other comparisons will be explored in more detail in Chapter 4.

3.2.1. General RNA-Seq Results

107,891 high-confidence (HC) protein-coding loci were identified in the RefSeq 1.1 annotation by BGI. A total of 94,181 genes were identified through RNA-Seq within the four treatments. Of that 87,149 were known genes and 7,034 were novel genes identified by BGI and not matching to any existing known genes. 170,176 novel transcripts were identified in which 122,143 were previously unknown splicing event for known genes, and 7,096 of them were novel coding transcripts. Of the 7096 novel coding transcripts, 471 related to known proteins or unnamed protein products in *Triticum aestivum*.

The number of reads in each sample (Table 3.1.) ranged drastically from 6,120,316 in Stress+TSA 48 h (1) to 27,149,918 in Stress 3 h (3). However, the number of expressed genes in each sample did not have as wide of a range with 57,306 (Fresh+TSA 48 h) being the lowest, 73,601 (Stress+TSA, 3 h) being the highest and an average of 63,487 (Figure 3.2.).

In addition to similar number of genes between samples, the transcriptomes were remarkably similar with 12,262 of the 94,181 genes expressed at 5 or more FPKM in all samples. The average read mapping rate to genes was 68.07% (Table 3.1.) and the average read mapping rate to the genome was 82.33%.

Table 3.1. Statistics of the reads for all samples.
F = Fresh, FT = Fresh+TSA, S = Stress, S = Stress+TSA

Sample	Total Clean Reads	Mapping Ratio	Known Genes	Novel Genes	Known Transcripts	Novel Transcripts
F0 h_1	22113066	70.16	54395	4918	55938	39993
F0 h_2	22917212	71.65	55304	4950	57136	40897
F0 h_3	23556508	71.34	55376	5030	57197	41525
F3 h_1	24479806	71.05	56456	5244	58690	43569
F3 h_2	24874084	69.5	55961	5263	57646	43350
F3 h_3	21389488	72.03	54290	4947	55811	40199
F48 h_1	21459590	68.52	59809	5271	62660	44736
F48 h_2	24612374	65.55	61152	5368	63991	46023
F48 h_3	24741382	65.95	60638	5395	63539	46043
F+TSA0 h_1	24385928	68.88	56189	5210	57993	43261
F+TSA0 h_3	24946348	69.6	57772	5281	59929	43886
F+TSA0 h_4	26574974	68.25	55569	5337	57244	43812
F+TSA3 h_1	24218962	68.86	58162	5221	60043	43674
F+TSA3 h_2	22867516	69.08	56240	5086	57591	41674
F+TSA3 h_3	22938138	69.31	55905	5052	57361	41503
F+TSA48 h_1	7500558	69.13	53020	4286	52905	33788
F+TSA48 h_2	24868440	66.37	61672	5511	64964	47412
F+TSA48 h_3	24406344	64.17	61762	5495	64622	46987
S0 h_1	25692252	67.56	59697	5496	62537	46615
S0 h_2	25081162	67.01	59219	5446	61720	45849
S0 h_3	25578646	69.91	59346	5451	62211	46031
S3 h_1	25796458	66.87	60069	5604	62832	47819
S3 h_2	14985024	67.09	56560	5127	58008	42919
S3 h_3	27149918	67.98	59168	5611	61930	47673
S48 h_1	7316058	66.31	53218	4327	53145	34977
S48 h_2	20146728	64	60819	5374	63142	44734
S48 h_3	22299042	63.97	61203	5380	63993	45641
S+TSA0 h_2	23703588	68.58	58475	5358	61034	45720
S+TSA0 h_3	25217568	67.79	58727	5455	61723	46730
S+TSA0 h_5	25435982	72.77	58118	5315	61182	46177
S+TSA3 h_1	23675250	67.52	61450	5436	63404	45422
S+TSA3 h_2	23553550	64.15	68013	5588	71387	49017
S+TSA3 h_3	23257930	64.46	67598	5619	70839	48952
S+TSA48 h_1	6120316	67.89	53207	4216	52654	33043
S+TSA48 h_2	6743122	68.43	53983	4293	53712	33867
S+TSA48 h_3	21026148	68.99	59739	5291	62441	44272

Within the known and novel genes discovered (Table 3.1.) the range in gene expression level distribution (Figure 3.2.) had similar proportions between the different

samples. The largest number of genes were seen between 1 and 10 FPKM (not inclusive) in each sample. This indicates that the majority of genes in microspores were expressed at a moderate level.

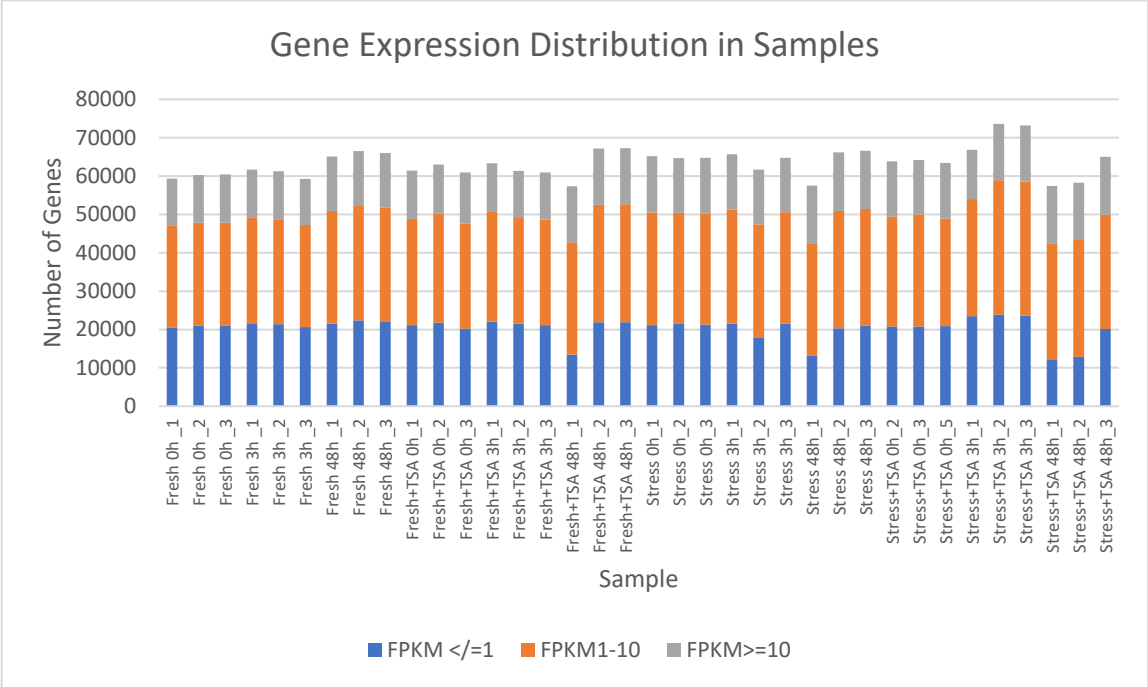

Figure 3.1. The distribution of expression of genes over each sample, separated by less </= 1 FPKM (blue), 1 - 10 FPKM (orange), and =/> 10 FPKM (grey).

3.2.2. General Differential Expression Analysis

In differential expression analysis 25,748 genes were considered differentially expressed between at least one of the two treatments in at least one time point. Within the same treatment only 27,910 genes were considered differentially expressed in at least one comparison between time points within at least one treatment. In comparing those lists of DEs unsurprisingly, there is a large overlap of 18,217 genes differentially expressed both between and within treatment (Figure 3.2.). Illustrating how similar the transcriptomes are both between treatments and within the same treatments over time in culture 58,740

of the 94,181 genes were not considered to be differentially expressed in any comparison. Of those 58,740 not DEs, 3,356 were novel and 55,384 were known genes.

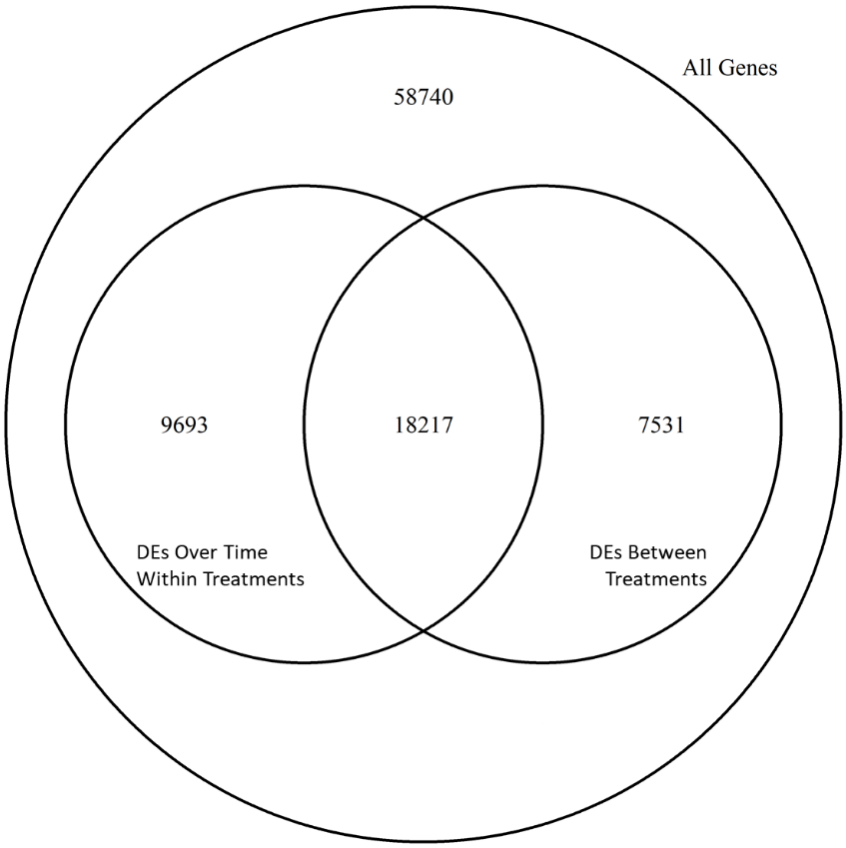

Figure 3.2. Venn diagram showing the number of genes that were considered to be differentially expressed in the different comparisons (either between treatments or within the same treatments over different time points or in both) and the number of genes that were not differentially expressed in any comparisons (only seen in the list of all genes, not in any DE list).

The number of DEs ranged from 74 (Stress vs Stress+TSA, 0 h) to 13,430 (Fresh vs Stress, 0 h) with an average of 5,475 (Table 3.2.). Fresh+TSA vs Stress+TSA showed a comparable number of DEs at 0 h to Fresh vs Stress 0 h (Table 3.2.) indicating the impact of cold-stress on the transcriptome. The percentage of expressed transcripts that were considered DEs was relatively low within each time point and treatment (Table

3.2.), averaging 5.81% with as low as 0.08% (Stress vs Stress+TSA, 0 h) and as high as 14.26% (Fresh vs Stress, 0 h). The transcriptomes are overall remarkably similar both between treatments and between time points within the same treatment.

Table 3.2. The percentage of genes considered to be differentially expressed compared to the total number of genes

Comparison Between		Total DEs	% Considered DEs
Treatment 1	Treatment 2		
Fresh 0 h	Fresh 3 h	650	0.69%
Fresh 0 h	Fresh 48 h	10009	10.63%
Fresh 0 h	Fresh+TSA 0 h	1279	1.36%
Fresh 0 h	Stress 0 h	13430	14.26%
Fresh 3 h	Fresh 48 h	7012	7.45%
Fresh 3 h	Fresh+TSA 3 h	182	0.19%
Fresh 3 h	Stress 3 h	8751	9.29%
Fresh 48 h	Fresh+TSA 48 h	106	0.11%
Fresh 48 h	Stress 48 h	9120	9.68%
Fresh+TSA 0 h	Fresh+TSA 3 h	493	0.52%
Fresh+TSA 0 h	Fresh+TSA 48 h	9404	9.99%
Fresh+TSA 0 h	Stress+TSA 0 h	13352	14.18%
Fresh+TSA 3 h	Fresh+TSA 48 h	10206	10.84%
Fresh+TSA 3 h	Stress+TSA 3 h	5030	5.34%
Fresh+TSA 48 h	Stress+TSA 48	2248	2.39%
Stress 0 h	Stress 3 h	266	0.28%
Stress 0 h	Stress 48 h	7558	8.02%
Stress 0 h	Stress+TSA 0 h	74	0.08%
Stress 3 h	Stress 48 h	8113	8.61%
Stress 3 h	Stress+TSA 3 h	2715	2.88%
Stress 48 h	Stress+TSA 48	930	0.99%
Stress+TSA 0 h	Stress+TSA 3 h	6570	6.98%
Stress+TSA 0 h	Stress+TSA 48	6967	7.40%
Stress+TSA 3 h	Stress+TSA 48	6945	7.37%

Between the three time points the number of DEs was largest between 0 h versus 48 h, and 3 h versus 48 h. As seen in Figure 3.3. A, the number of DEs between 0 h and 3 h is very low in both fresh and stressed, up and down, ranging from 105 (Stress 0 h vs 3 h Down) to 537 (Stress 0 h vs 3 h Up). The largest number of DEs is seen in upregulated

genes between 0 h and 48 h in fresh microspores with 6932. Stressed material surprisingly has less DEs with its largest number seen in 3 h versus 48 h with 4585 upregulated and 3528 downregulated. Interestingly the numbers in cold-stressed microspores between 0 h and 48 h are similar to 3 h versus 48 h with 4036 upregulated and 3522 downregulated. The two treatments show the same pattern of DEs over time (Figure 3.3. B) with an increased number of both up- and down- regulated genes over time post isolation.

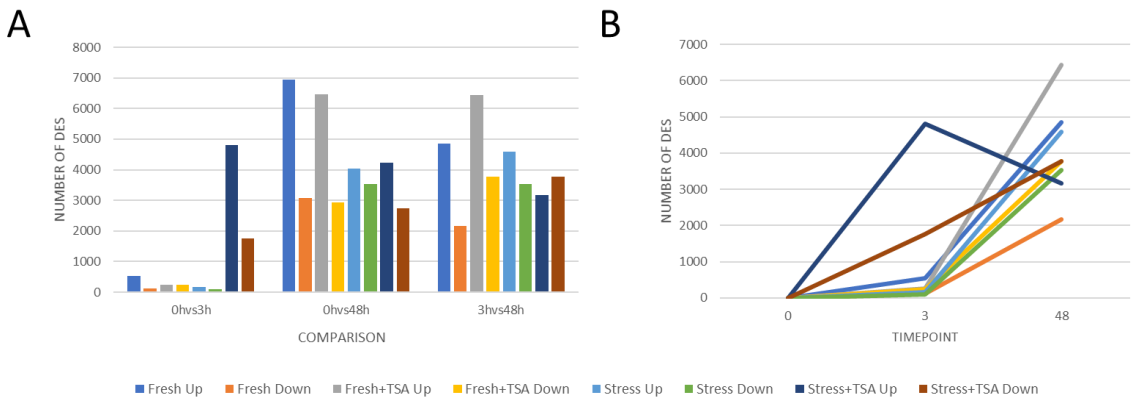

Figure 3.3. A) The number of DEs up or down regulated between time points within each treatment. B) The DEs graphed over time in culture for each treatment, time points are 0 h, 3 h, and 48 h post isolation.

UpSetR is a tool in R that allows for Venn diagram-like analysis of DEs between more than 3 comparisons; comparing what treatment(s) DEs are differentially expressed in. Within Figure 3.4., the vertical bar graphs along the top represent the number of genes that are differentially expressed within the comparisons marked by dots on the bottom of the image, while the horizontal bars on the left show the number of DEs that were present in total within that single comparison. In Figure 3.4., the categories were labelled as follows: AA = Fresh, AB = Fresh+TSA, AC = Cold-Stress, AD = Stress+TSA, 1 = 0 h, 2 = 3 h, 3 = 48 h.

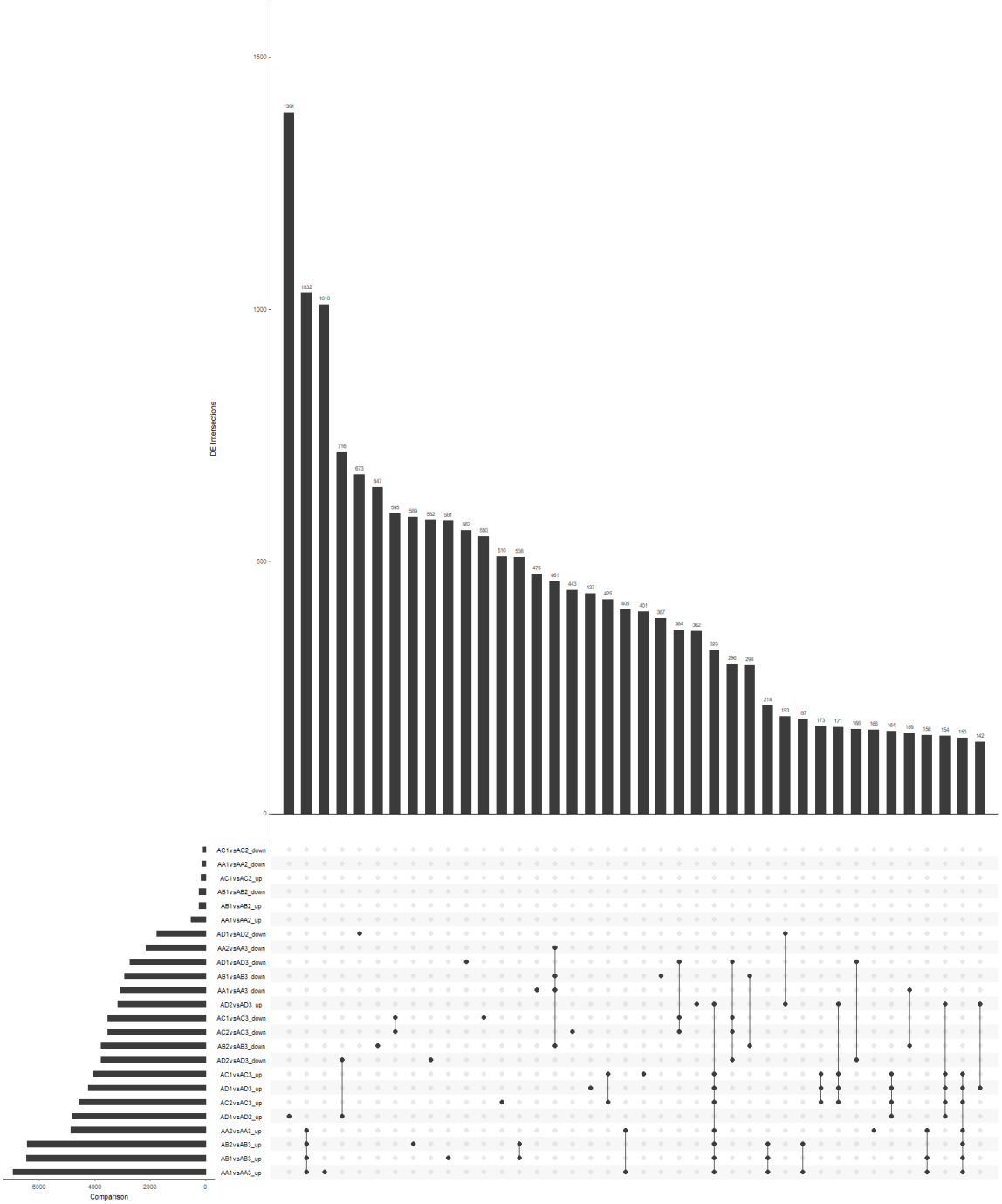

Figure 3.4. UpSetR image of comparisons within the same treatment over time in culture. Lists of DEs were separated by Up- or Down-regulation and plotted to determine DE overlap between treatments (Vertical bar graph) and which treatments that number of DEs are seen in (bottom dots). The horizontal bar graph illustrated the number of shared DEs in that treatment comparison.

DEs that were changing over time within treatments and intersected in treatments related to MDE production were identified. For example, DEs that overlapped in “up” (increasing over time in culture) in Stress, and Stress+TSA and possibly Fresh+TSA were of interest. Of these genes, the ones with interesting biological functions were identified and represent possible targets for improving androgenesis (discussed in more detail in Chapter 5). Only a portion of these will be explored in this thesis.

TraesCS4D02G018100, a BOI (Botrytis Susceptible1 Interactor) and BOI-related Gene 2 (BRG2) ortholog, decreased in expression over time between 0 h and 48 h in all treatments: 1.6-fold in Fresh, 1.4-fold in Fresh+TSA, 3.2-fold in Stress, and 2.56-fold in Stress+TSA. BOI genes act in the regulation of PCD, attenuating the process. BRG2 in particular is a E3 ubiquitin-protein involved in protein degradation in *Arabidopsis thaliana* (The UniProt Consortium, 2020).

TraesCS7B02G259500, an MRE11 ortholog was increased between 0 h and 48 h as well as between 3 h and 48 h in all treatments. Between 0 h and 48 h it decreased in expression 2.8-fold in Fresh, 4.7-fold in Fresh+TSA, and 2.7-fold in both Stress and Stress+TSA. MRE11 is involved in double strand break (DSB) repair and processing (The UniProt Consortium, 2020). Meanwhile, TraesCS7A02G369000, another MRE11 ortholog, had only increased within cold-stress between 3 h and 48 h.

Mitogen-activated protein kinase kinase kinase YODA (YDA) is involved in the mitogen-activated protein kinase (MAPK) signalling cascade that regulates first cell fate decisions in early embryo (Garcia et al., 2019; Lukowitz, Roeder, Parmenter, & Somerville, 2004; Samakovli et al., 2021; Samakovli et al., 2020; The UniProt

Consortium, 2020). YDA orthologs TraesCS6B02G279300 increased 1.6-fold over time in Fresh, Fresh+TSA, and Stress treatments between 3 h and 48 h. TraesCS6A02G245000 increased 1.3-fold between 3 h and 48 h in Fresh and Fresh+TSA. TraesCS6D02G217100 increased 1.5-fold in Fresh+TSA and 1.0-fold in Stress between 3 h and 48 h. TraesCS6D02G227300 only increased over time in Fresh+TSA but increased by 1.5-fold between 0h and 48 h. And TraesCS2D02G404700 only increased over time in Stress but increased 1.36-fold between 0 h and 48 h. Interestingly YDA related terms were not differentially expressed in Stress+TSA comparisons.

GBSSI, identified as relating to green plant regeneration potential by Gajecka et al. (2020) was differentially expressed within cold-stress treatment in our study. TraesCS4A02G418200 and TraesCS7A02G070100 both decreased only within Stress between 0 h and 48 h, 1.9-fold and 1.7-fold respectively. GBSSI is involved in starch and glycan biosynthesis (The UniProt Consortium, 2020).

3.2.3. Differential Expression Between Fresh and Cold-Stressed

Comparing fresh to stressed microspores the number of DEs is highest at 0 h, immediately after isolation, with 13,430 DEs and 7930 of those being upregulated in stress (Figure 3.5. A). The numbers are lower at 3 hours and 48 hours post isolation with 8751 and 9120 total DEs respectively. At each time point the majority of DEs are upregulated in cold stress, suggested relaxed chromatin in cold stress. Those DE's mapped to the chromosomes at each time point show a random distribution between the two treatments (Figure 3.5. B). When the list of DEs were compared using a Venn diagram shows some overlap in DEs between time points with the highest similarity

being between 0 h and 3 h unsurprisingly with 4141 DEs in common (Figure 3.5. C) and 2928 DEs that were at all three time points.

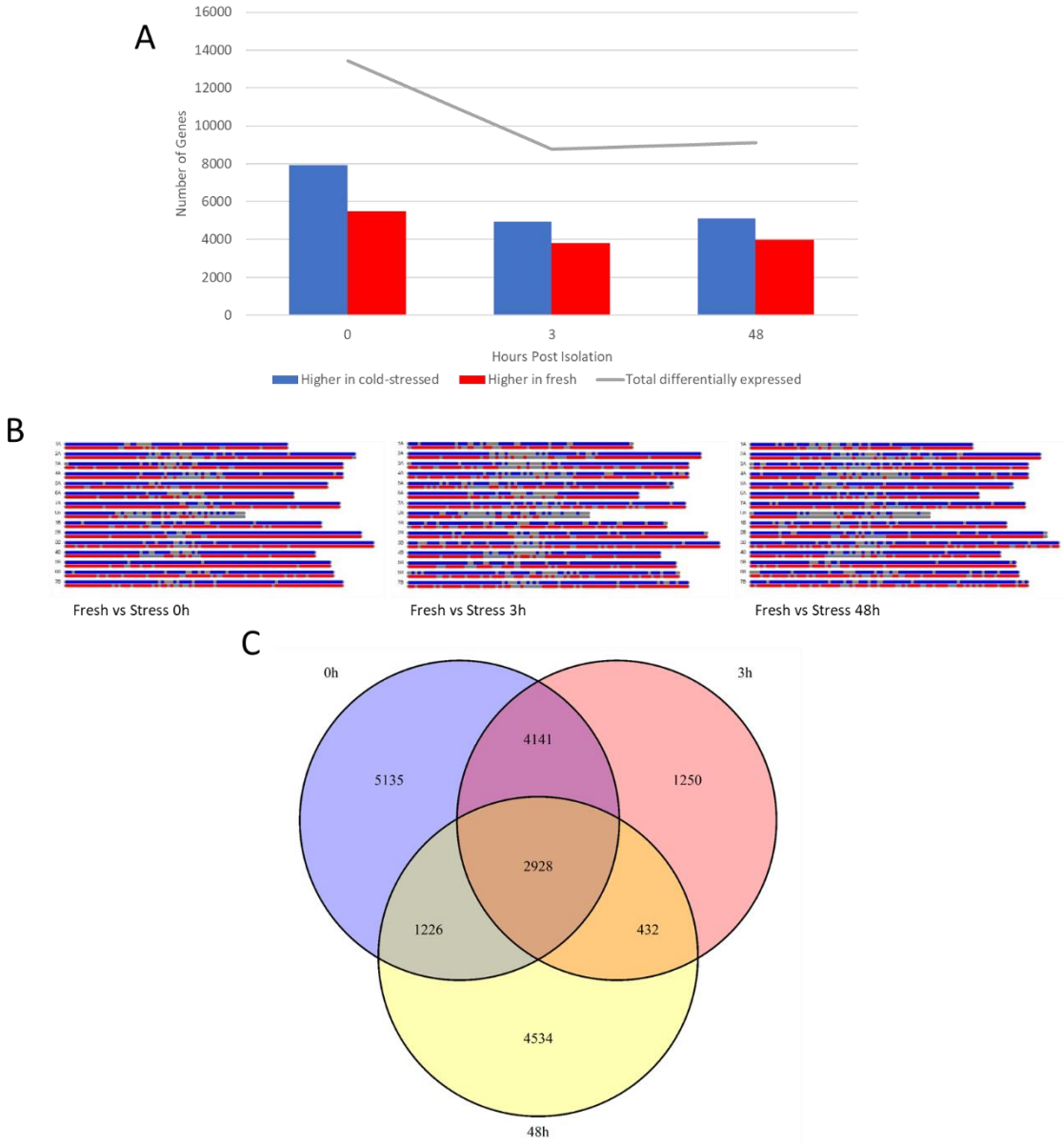

Figure 3.5. The impact of cold stress on AC Andrew microspore transcriptome. **A)** The number of DEs either higher in cold-stressed (blue) or higher in fresh (red) in comparisons of fresh and stressed microspores. **B)** ChromoMap showing the mapping location of DEs higher in stress (blue) and higher in fresh (red) on the *Triticum aestivum* genome. **C)** Venn diagram showing the number of DEs at each time point of comparison between fresh and cold-stressed microspores and the number of overlapping DEs when each time point is compared.

Just as in the comparison within treatments that comparison between fresh and cold-stressed microspores at 0 h, 3 h, and 48 h resulted in identification of genes of interest for androgenesis.

BRCA-1 Associated Ring Domain (BARD1) orthologs TraesCS2A02G384900 and TraesCS2B02G401900 both showed 2-fold increased expression at 48 hours in cold stressed microspores compared to fresh. BARD1 is involved in DNA repair as well as WUSHEL (WUS) regulation and expression within the organizing center (Han, Li, & Zhu, 2008).

Double strand break (DSB) repair protein MRE11 ortholog TraesCS7B02G259500 (also mentioned in DEs within treatments, Chapter 3, 3.2.2.) was increased 1.96-fold at 48 h between cold-stressed and fresh microspores. While TraesCS7A02G369000 was 2.4-fold higher in cold stressed microspores compared to fresh at 48 hours.

Hypoxia up-regulated 1 (HYOU1) ortholog TraesCS6D02G257000 was found to be expressed 2.2-fold lower in cold-stressed microspores when compared to fresh at 48 h. HYOU1 is involved in cryoprotection likely through protein folding as a chaperone and is triggered by hypoxia (The UniProt Consortium, 2020).

TraesCS4A02G418200 (GBSSI ortholog, also mentioned in DEs within treatments, Chapter 3, 3.2.2.) was also 1.35-fold lower in cold stress treatment compared to fresh at 3 h but not differentially expressed at 48 h. Although Gajecka et al. (2020) does not study the change in culture conditions, and focused on the levels before IMC these are still possibly interesting findings.

3.2.4. GO Terms Enriched in Genes not Differentially Expressed in any Comparison

Gene ontology (GO) singular enrichment analysis (SEA) was completed on the 55,384 known genes that were not differentially expressed in any comparison completed in this study. These genes were enriched in 411 biological process GO Terms. After removal of redundant terms, they were plotted in semantic space to aid in visualization of enrichment using REVIGO (Figure 3.6.).

GO terms enriched in the list of genes not considered to be DEs include immune system process (GO:0002376), induced systemic resistance (GO:0009682), phenylpropanoid metabolic process (GO:0009698), polysaccharide localization (GO:0033037), organ growth (GO:0035265), regulation of organ growth (GO:0046620), (obsolete) oxidation-reduction process (GO:0055114), cell killing (GO:0001906), cell recognition (GO:0008037), extracellular structure organization (GO:0043062), apoptotic process (GO:0006915), secondary metabolic process (GO:0019748), flavonoid biosynthetic process (GO:0009813), flavonoid metabolic process (GO:0009812), and pigment biosynthetic process (GO:0046148). Chloroplast RNA processing (GO:0031425) was also enriched in the gene list not differentially expressed, which was interesting.

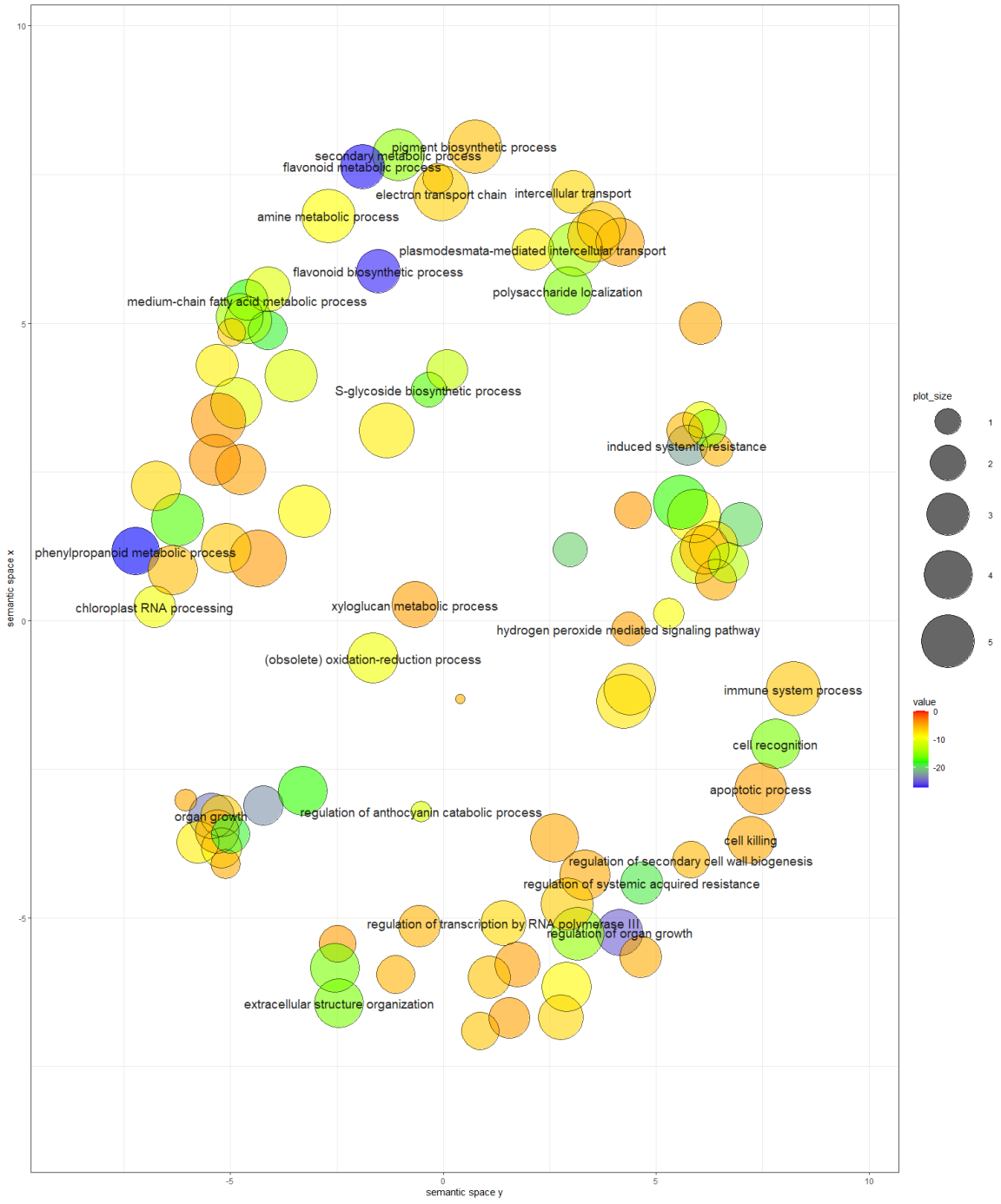

Figure 3.6. Semantic plot of biological processes enriched GO terms in the 55,384 genes that were not considered differentially expressed in any comparison.

3.2.5. Enriched GO Terms for Genes Higher in Cold-Stressed Microspores Compared to Fresh

As seen previously many DEs were higher in cold-stressed microspores at each comparison between fresh and stressed microspores. GO Terms that were enriched in those DEs were ranked by p value (-log[p-value]). Immediately following isolation GO terms that were enriched in DEs upregulated cold-stressed microspores were mostly pollen related with cell tip growth (GO:0009932), pollen tube growth and development (GO:0009860 & GO:0048868), developmental cell growth (GO:0048588), cell development (GO:0048468), morphogenesis involved in differentiation (GO: GO:0000904), plant-type cell wall modification (GO:0009827), response to stress in ER (GO:0034976) and from high light intensity (GO:0009644) and reactive oxygen species (ROS) (GO:0000302 & GO:0006979), and intracellular signal transduction (GO:0035556) (Figure 3.8.). Amylopectin biosynthesis and metabolism was higher in cold-stressed microspores at this time point. REVIGO was used to remove redundant terms and plot them according to similarity (Figure 3.7.)

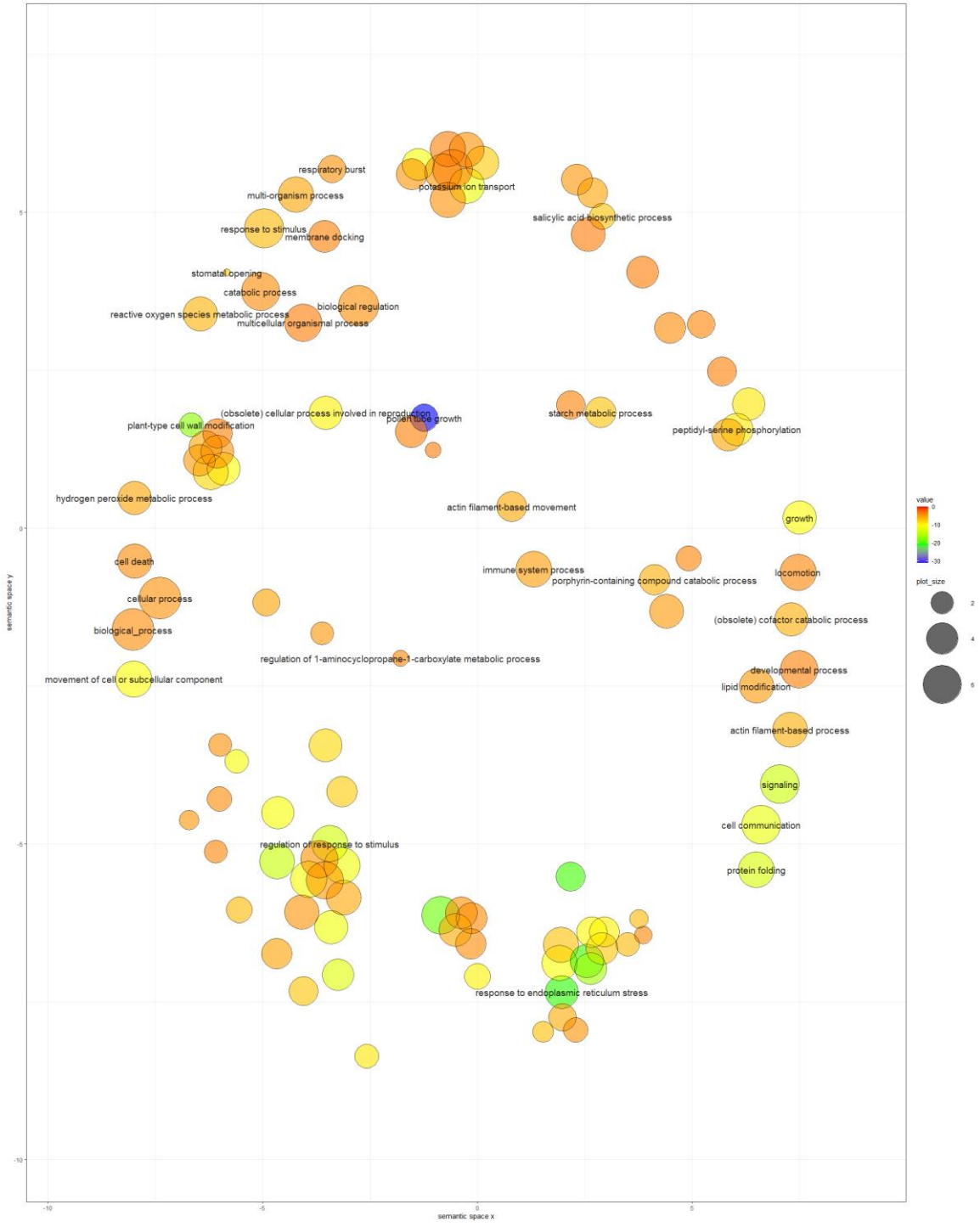

Figure 3.7. Semantic space plot for enriched GO Terms using REVIGO at 0 h in the comparison between Fresh and Cold-Stressed microspores.

At 3 hours post isolation the microspores have both experienced a short time in culture conditions, however the GO terms upregulated in stressed microspores are, as expected, still quite similar to those of 0 h. Pollen tube related (GO:0009860, GO:0009932, GO:0048868), developmental related (GO:0048588, GO:0048468), differentiation (GO: GO:0000904), plant-type cell wall modifications (GO:0009827), and intracellular signal transduction (GO:0035556) are still prevalent GO Terms. Interestingly, response to light and stress are no longer present in the top GO Terms and have been replaced by regulation of receptor activity (GO:0010469), regulation of cation channel activity (GO:2001257), and regulation of potassium ion transmembrane transport (GO:1901379) and inward rectifier potassium channel activity (GO:1901979) (Figure 3.8.) Amylopectin biosynthesis and metabolism continued to be increased in cold-stressed microspores.

By 48 hours after isolation the GO Terms upregulated in stressed microspores is vastly different than 0 h and 3 h. The top terms are nucleosome assembly and organization (GO:0006334 & GO:0034728), DNA packaging and chromatin related terms (GO:0006323, GO:0031497, GO:0065004, GO:0071824, GO:0006333, GO:0051276, GO:0070828), DNA conformational change (GO:0071103) (Figure 3.8.). DNA replication, negative regulation of transcription, and cell proliferation are also now present in the GO terms although in the top 30, not top 15. This is vastly different than the top GO terms upregulated for 0 h and 3 h which were pollen related, signalling and response.

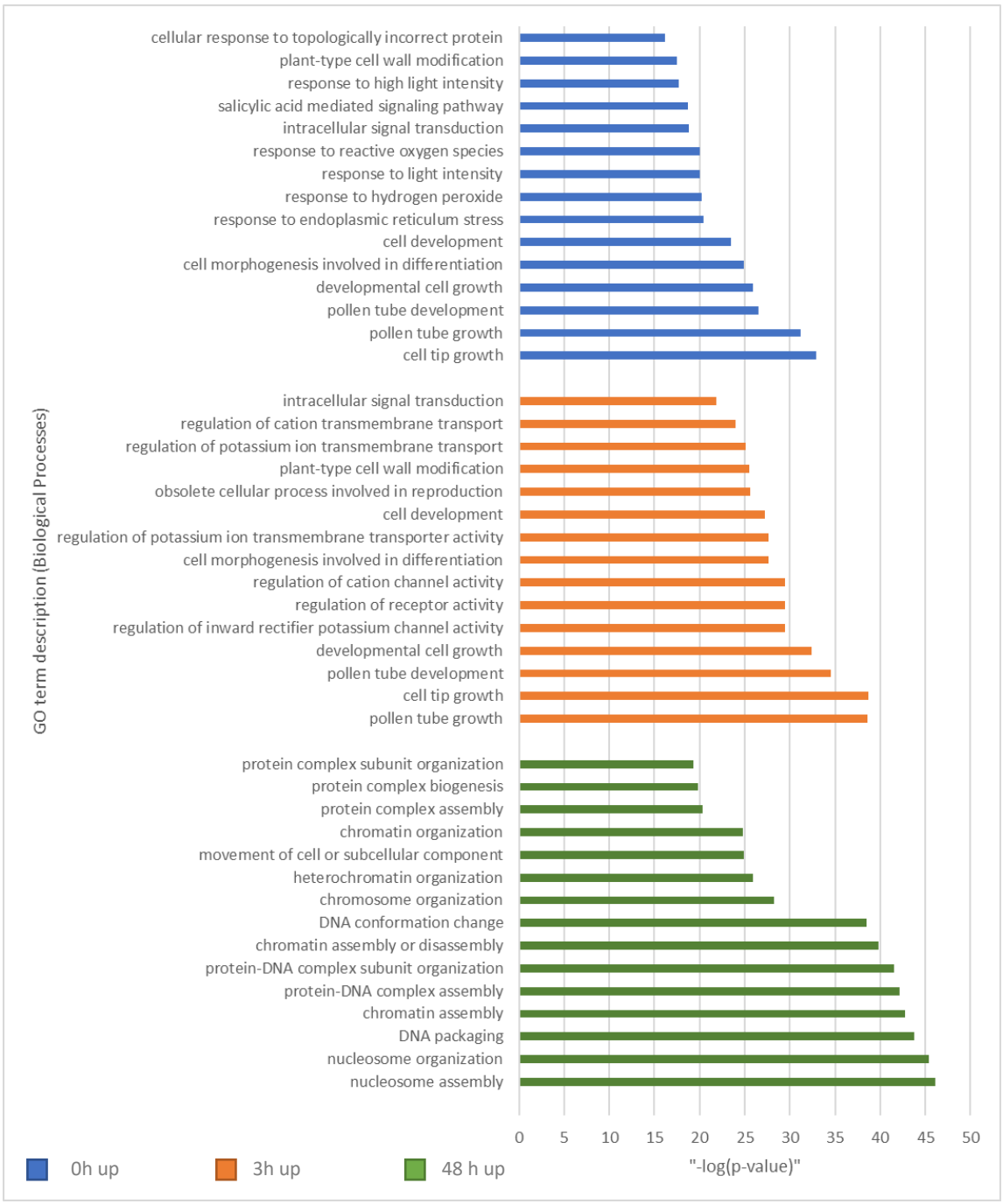

Figure 3.8. Biological Process GO Terms enriched in DEs higher in cold-stressed microspores at 0 (blue), 3 (orange), and 48 (green) hours post isolation, ranked by p-value (-log[p-value]).

3.2.6. GO Terms Enriched in Genes Expressed Lower in Cold-Stressed Compared to Fresh

GO terms enriched in genes expressed at lower levels in stressed microspores immediately following microspore isolation include chromosome and chromatin organization (GO:0051276 & GO:0006325), gene and chromatin silencing (GO:0016458 & GO:0006342), mitotic cell cycle process (GO:1903047 & GO:0000278), cytokinesis related terms (GO:0032506 & GO:1902410), methylation (GO:0032259 & GO:0043414), epigenetic negative regulation of gene expression (GO:0045814), and histone modification (GO:0016570) (Figure 3.9.). Pectin metabolism and biosynthesis were lower in cold-stressed compared to fresh microspores.

As with enriched terms higher in cold-stress at 3 hours post isolation GO terms enriched from genes expressed higher in fresh were similar to 0 h terms. Many of the terms were related to chromatin and chromosome organization (GO:0006325 & GO:0051276), negative regulation of gene expression (GO:0016458, GO:0006342 & GO:0045814), histone modification (GO:0016570), and mitotic cell cycle (GO:1903047) (Figure 3.8.). Some of the additional GO terms included histone phosphorylation (GO:0016572), regulation of DNA replication (GO:0006275), monosaccharide metabolic process (GO:0005996) cell proliferation (GO:0008283), and ribonucleoside monophosphate metabolic process (GO:0009161). Pectin biosynthesis also continued to be decreased in cold-stressed at this time point.

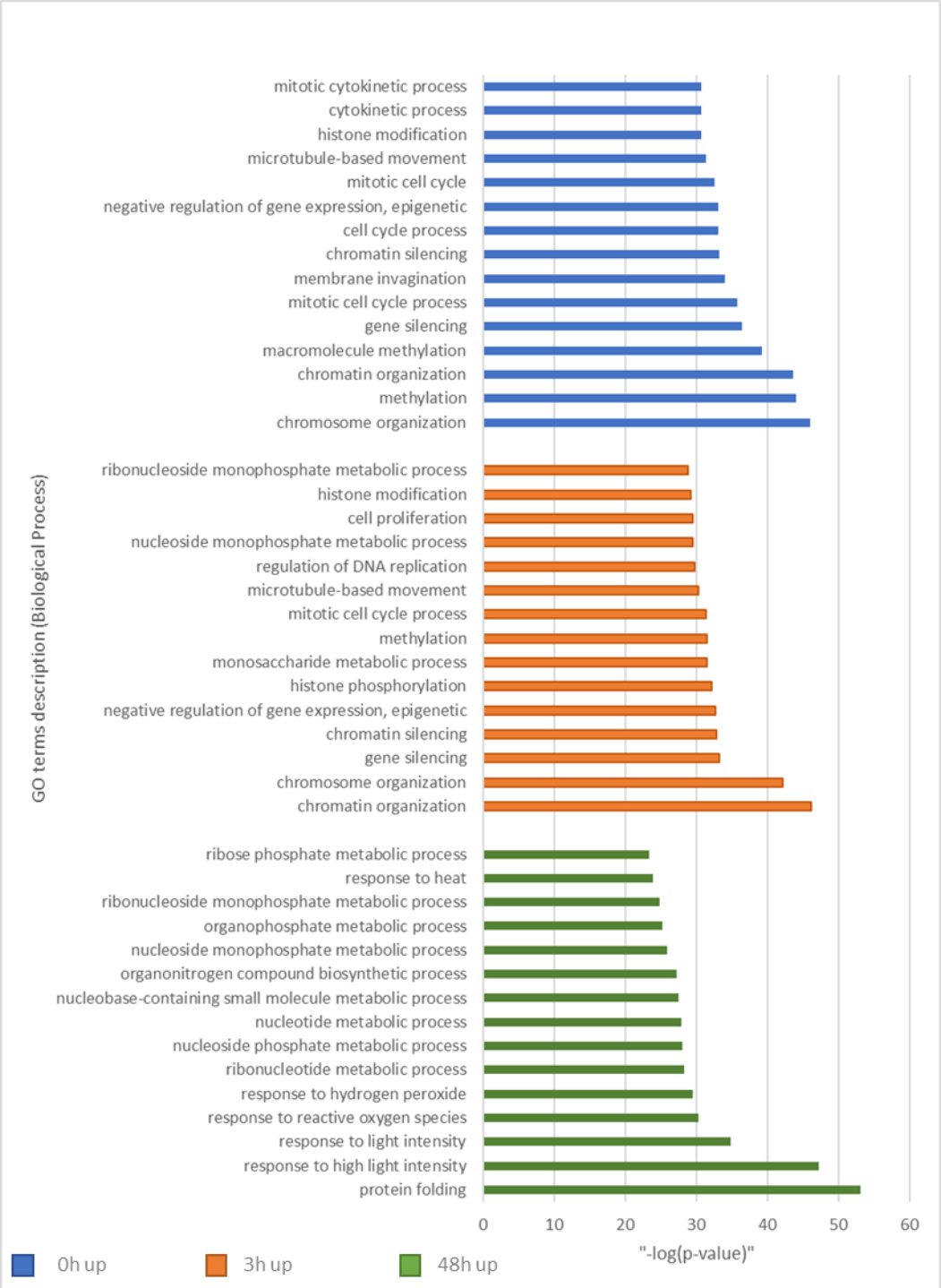

Figure 3.9. Biological Process GO Terms enriched in DEs lower in cold-stressed microspores when compared to fresh microspores at 0 (blue), 3(orange), and 48 (green) hours. Biological process GO terms were sorted for the top 15 for each time point, ranked by p-value (-log[p-value]).

At 48 hours post isolation, most of the GO terms downregulated are mostly metabolic processes related to nucleotides and ribonucleotides (Figure 3.9.). Response to high light intensity (GO:0009644 & GO:0009642) and ROS (GO:0000302) that were upregulated in stress at the earlier time points are now downregulated in cold stress treatment. Response to heat (GO:0009408) and hydrogen peroxide (GO:0042542) as well as protein folding (GO:0006457) are also downregulated at 48 h.

3.2.7. GO Terms Enriched in all Time Points Between Fresh and Cold-Stressed Microspores

Of the 2928 DEs at all three time points there were four patterns of expression in cold-stressed microspores; lower at 0 and 3 hours then higher at 48 hours, higher at 0 and 3 hours then lower at 48 hours, higher at all time points, and lower at all time points.

For DEs higher in fresh at 0- and 3 h then higher in cold-stressed at 48 h the biological process GO terms associated were chromatin (GO:0006325) and chromosome organization (GO:0051276), gene silencing (GO:0016458), cell proliferation (GO:0008283) and related terms. Molecular functions included DNA binding (GO:0003677), protein heterodimerization activity (GO:0046982), and hydrolase activity acting on anhydrides, in phosphorus-containing anhydrides (GO:0016818).

DEs higher in cold early in culture and later higher in fresh microspores were associated with amino acid activation (GO:0043038), tRNA aminoacylation for protein translation (GO:0006418), amide and peptide biosynthetic process (GO:0043604 & GO:0043043), and translation (GO:0006412). Molecular functions included ligase activity, forming carbon-oxygen bonds (GO:0016875), aminoacyl-tRNA ligase activity

(GO:0004812), ligase activity, and forming aminoacyl-tRNA and related compounds (GO:0016876).

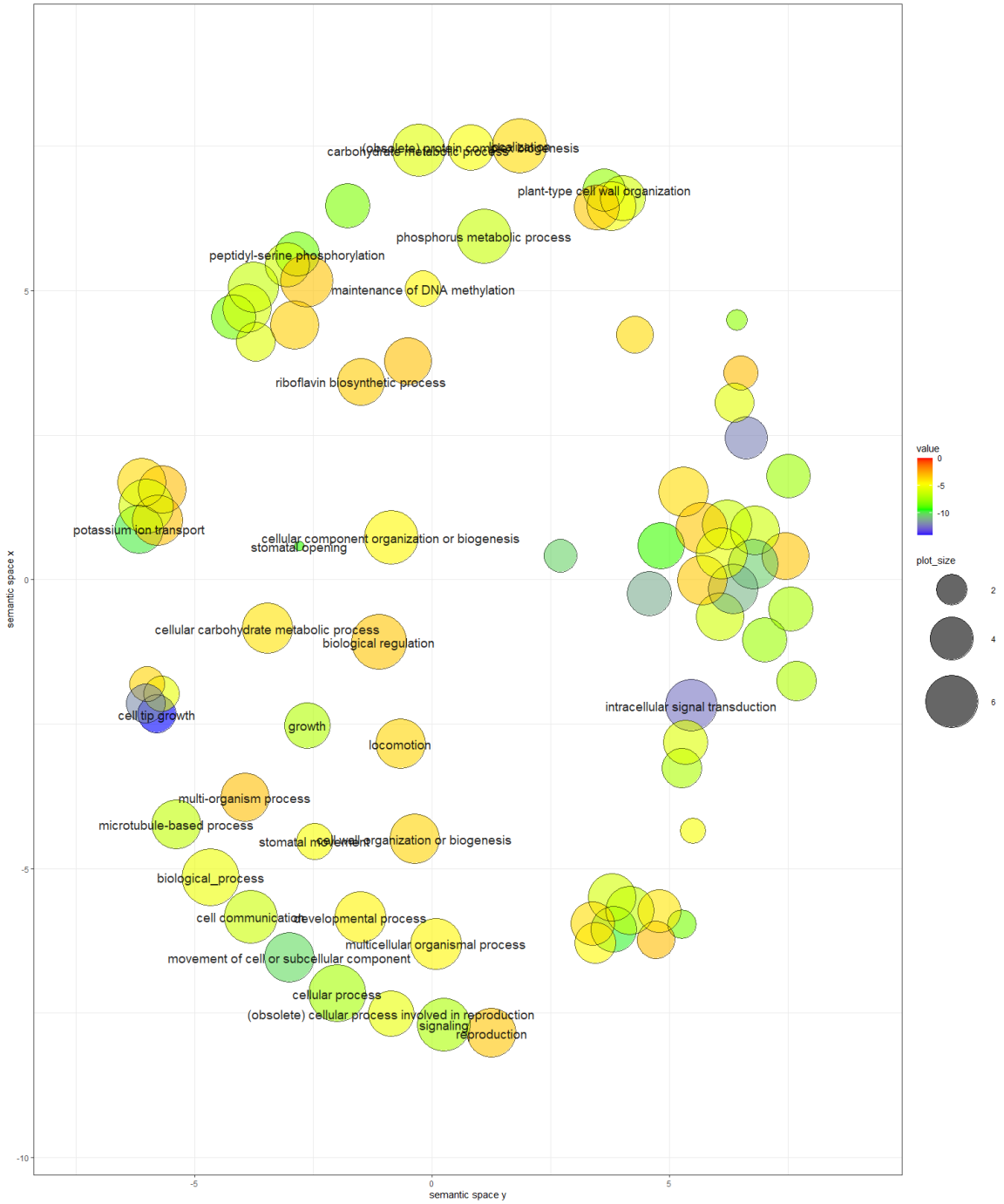

Figure 3.10. Semantic plot of GO terms higher in cold-stress at all three time points.

DEs higher in cold-stressed microspores at all time points studied were associated with cell tip growth (GO:0009932), intracellular signal transduction (GO:0035556), regulation of inward rectifier potassium channel activity (GO:1901979), regulation of receptor activity (GO:0010469), pollen tube growth and development (GO:0009860 & GO:0048868), cell differentiation (GO:0030154), negative regulation of cell death (GO:0060548), and cell development (GO:0048468). Molecular functions include calcium-dependent protein serine/threonine kinase activity (GO:0009931), calmodulin binding (GO:0005516) calmodulin-dependent protein kinase activity (GO:0004683), and ATPase activity, coupled to transmembrane movement of ions, phosphorylative mechanism (GO:0015662). (Figure 3.10.)

DEs lower in cold-stressed microspores at all time points in the study were associated mostly with metabolic processes such as nucleoside monophosphate (GO:0009123), ribonucleotide (GO:0009259), ribonucleoside monophosphate (GO:0009161). Molecular functions of DEs downregulated at all times include copper ion binding (GO:0005507), isomerase activity (GO:0016853), transmembrane transporter activity (GO:0022857). Within cell part chloroplast (GO:0009507) and mitochondrion (GO:0005739) were downregulated in stress at all time points and decreased over time in culture for stressed microspores between 3 h and 48 h. (Figure 3.11.)

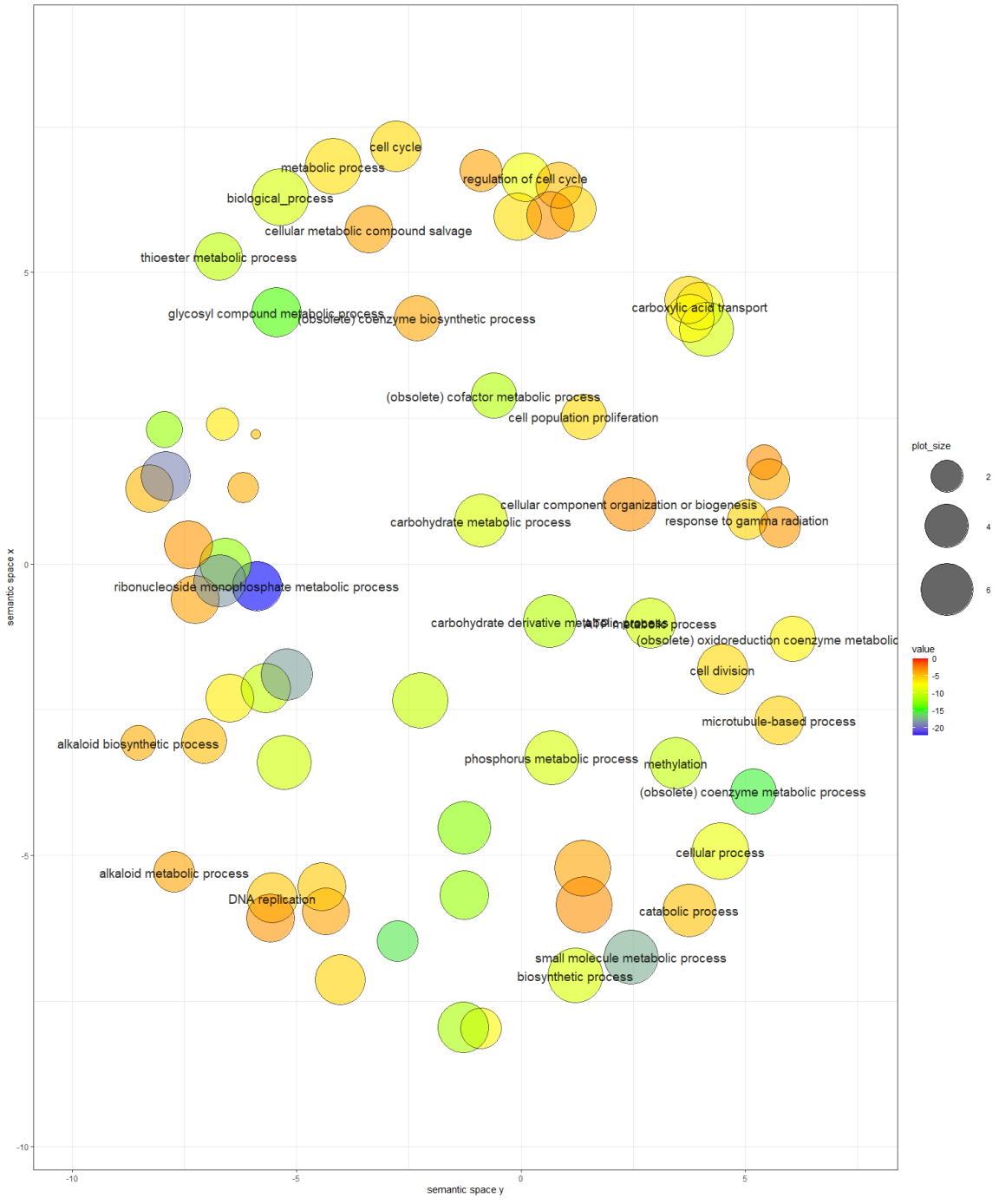

Figure 3.11. Semantic plot of GO terms lower in cold-stress at all three time points.

3.2.8. GO Terms Enriched Over Time in Cold-Stressed Microspores

Within cold-stressed microspores over time in culture downregulated DEs between 3 h and 48 h include protein folding (GO:0006457), ribosome biogenesis (GO:0042254), ribonucleoprotein complex biogenesis (GO:0022613), organophosphate metabolic process (GO:0019637), mitochondrial transport (GO:0006839), ncRNA metabolic process (GO:0034660), protein targeting to mitochondrion (GO:0006626), mitochondrion organization (GO:0007005), rRNA metabolic process and processing (GO:0016072 & GO:0006364), and response to high light intensity (GO:0009644). Upregulated DEs between 3 h and 48 h include nucleosome assembly (GO:0006334), DNA packaging (GO:0006323) and many other chromatin related terms, chromatin and gene silencing (GO:0006342 & GO:0016043), organelle organization (GO:0006996), negative regulation of RNA biosynthetic process and of nucleic acid-templated transcription (GO:1902679 & GO:1903507), and cell proliferation (GO:0008283). 0 h and 3 h were too similar for GO analysis to be completed however 32 of the 161 upregulated DEs were HSP20 family proteins.

3.2.9. KEGG Pathway Analysis

In KEGG pathway analysis several pathways were altered between fresh and cold stressed microspores. MAPK signalling and plant specific MAPK signalling were impacted. Several WRKY, interleukin-1 receptor-associated kinase (IRAK), and MAPK proteins were altered in expression pattern. At 0 h it appears that stress has more expression in pathways leading to pathogenic responses, cell death, and ROS (Figure

3.12.). Cold stress also shows higher expression of genes related to cold and salt stress tolerance.

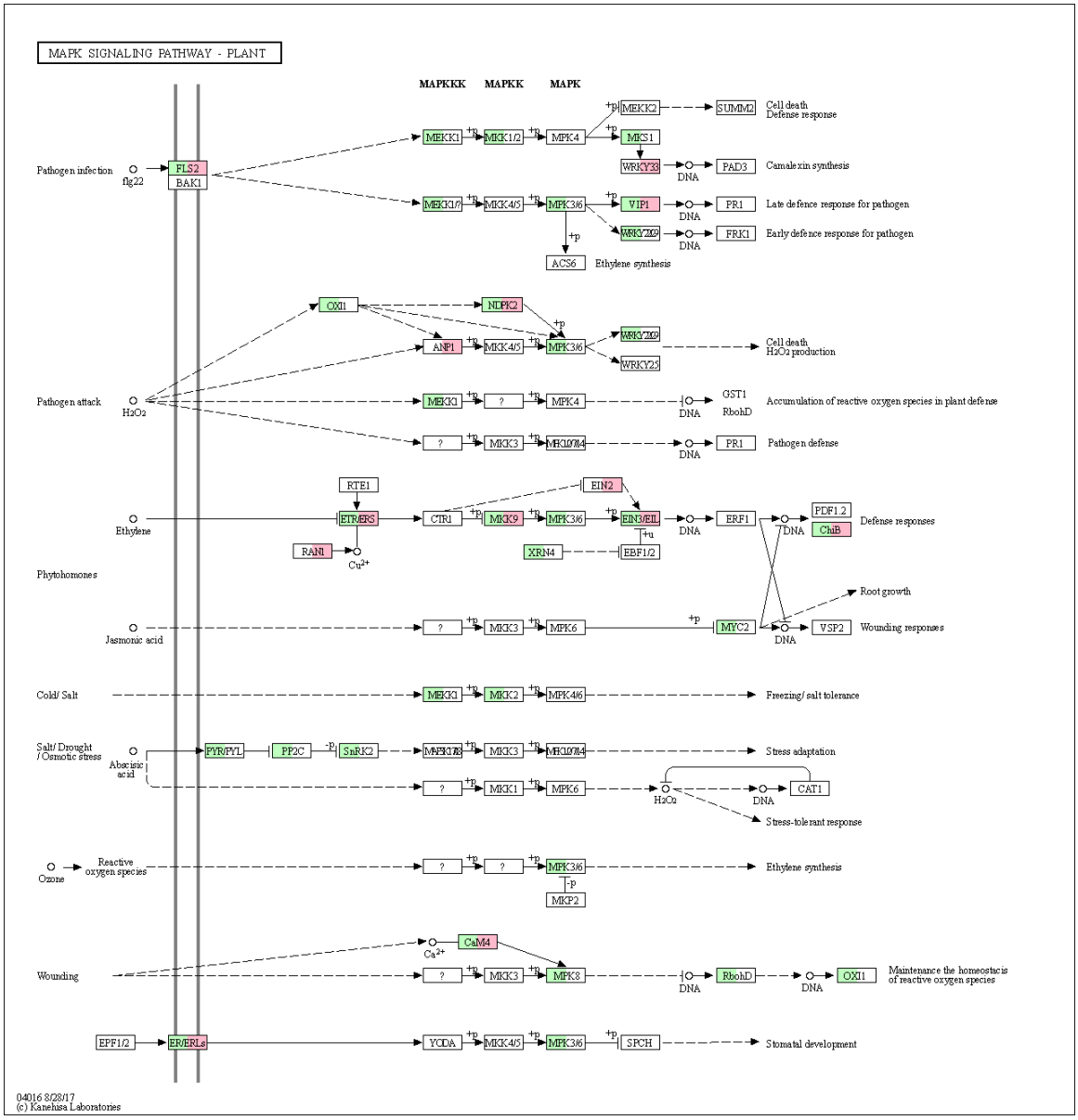

Figure 3.12. Plant Specific MAPK signalling pathway with difference in fresh and cold stressed highlighted. Green is higher in cold stress while red is higher in fresh.

In the apoptotic pathway at 0 h, fresh microspores have higher levels of cathepsin while cold-stressed microspores had higher levels of Poly(ADP-ribose) polymerases PARP, indicating low synthesis of poly(ADP-ribose).

Hormone pathways showed AUX1 was higher in cold stressed microspores are 0 h as well as genes involved in the Jasmonic and Salicylic pathways (Figure 3.13.). Most other paths within hormone signalling were mixed, showing both treatments having higher expression within those part, such as ARF or AUX/IAA.

The cell cycle (Figure 3.14) also showed a mix of genes that were differentially expressed between the two treatments with no real discernible outcome of a difference between the two treatments cell cycle. However, the senescence pathway (Figure 3.15.) illustrates higher expression of retinoblastoma protein (RB) and E2F leading to cell cycle arrest in fresh microspores. As well as higher expression of RAD50 and RAD9 indicating DNA damage in fresh microspores. Fresh microspores also show increased expression of genes related to long-patch base excision repair (BER) such as PCNA, DpoI, Lig and Lig1, and Polε. Increased expression of PARP in cold stressed microspores could indicate short-patch BER.

Interestingly p97, valosin containing protein (VCP) a translational endoplasmic reticulum ATPase was higher in fresh microspores compared to cold stressed. VCP is related to protein ubiquitination, export of misfolded proteins, and Golgi stack fragmentation in mitosis, and seems to play important roles within stress (The UniProt Consortium, 2020).

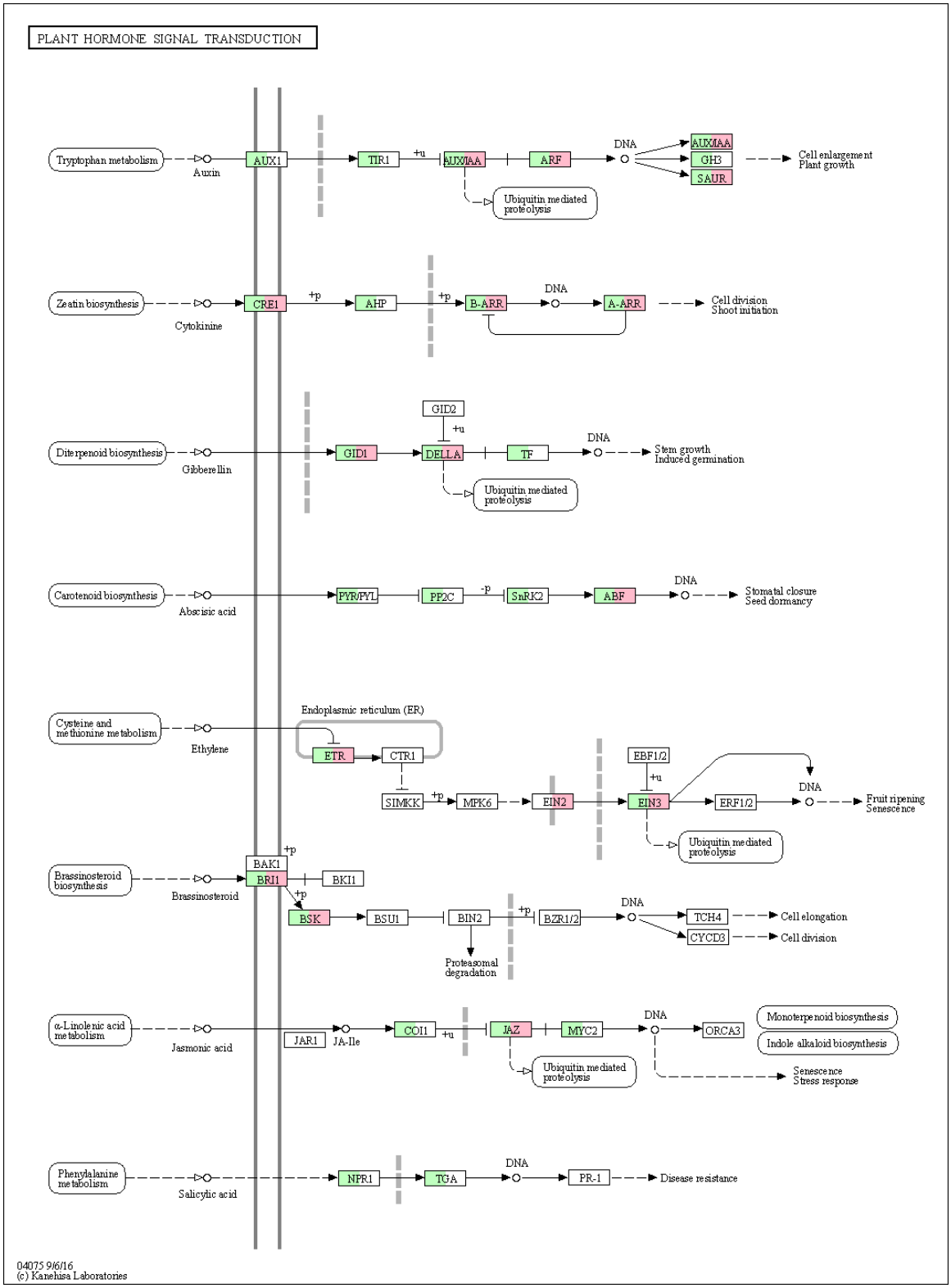

Figure 3.13. Plant Hormone pathways from KEGG mapper for fresh comparison with cold stress at 0 h. Green boxes are higher in cold stress while red boxes are higher in fresh microspores.

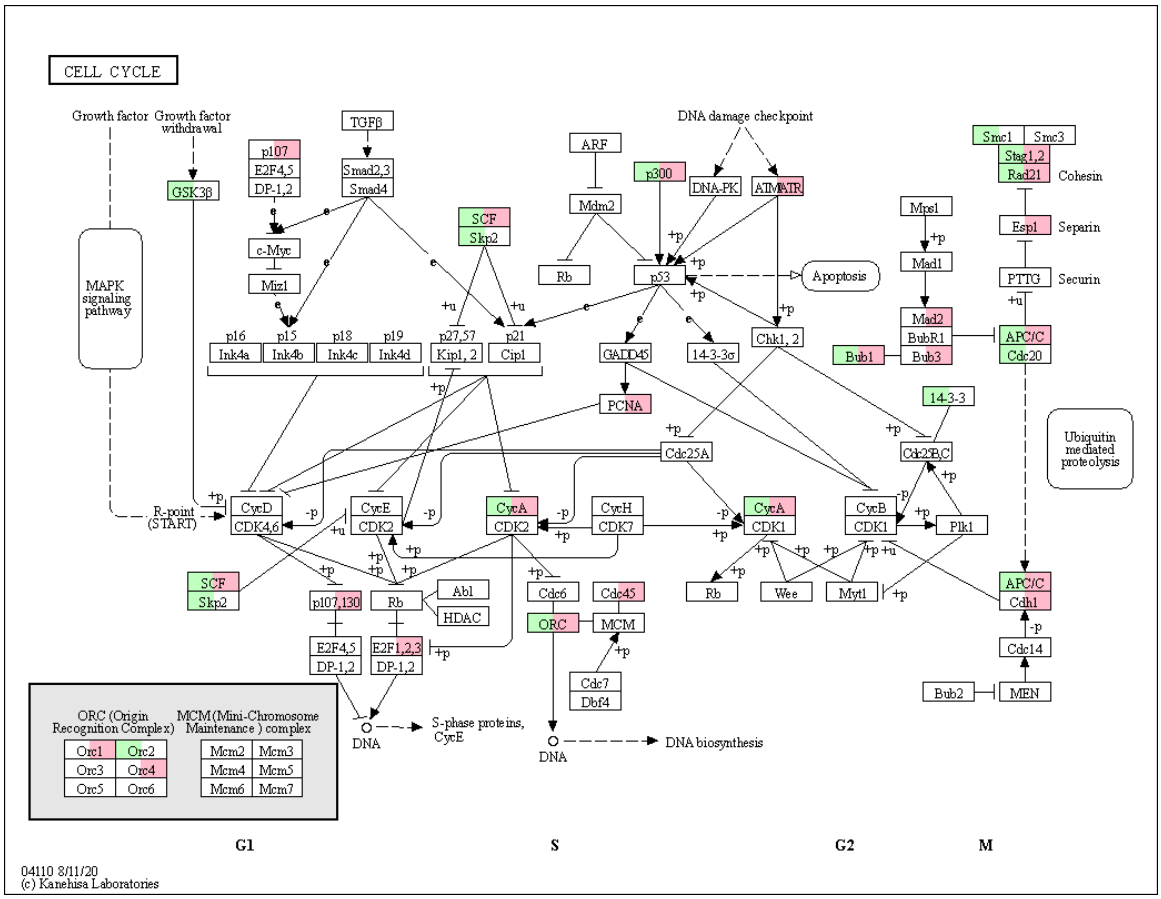

Figure 3.14. KEGG pathway for cell cycle, in the comparison between fresh and cold-stressed microspores at 0 h, green cells are higher in stressed while red are higher in fresh.

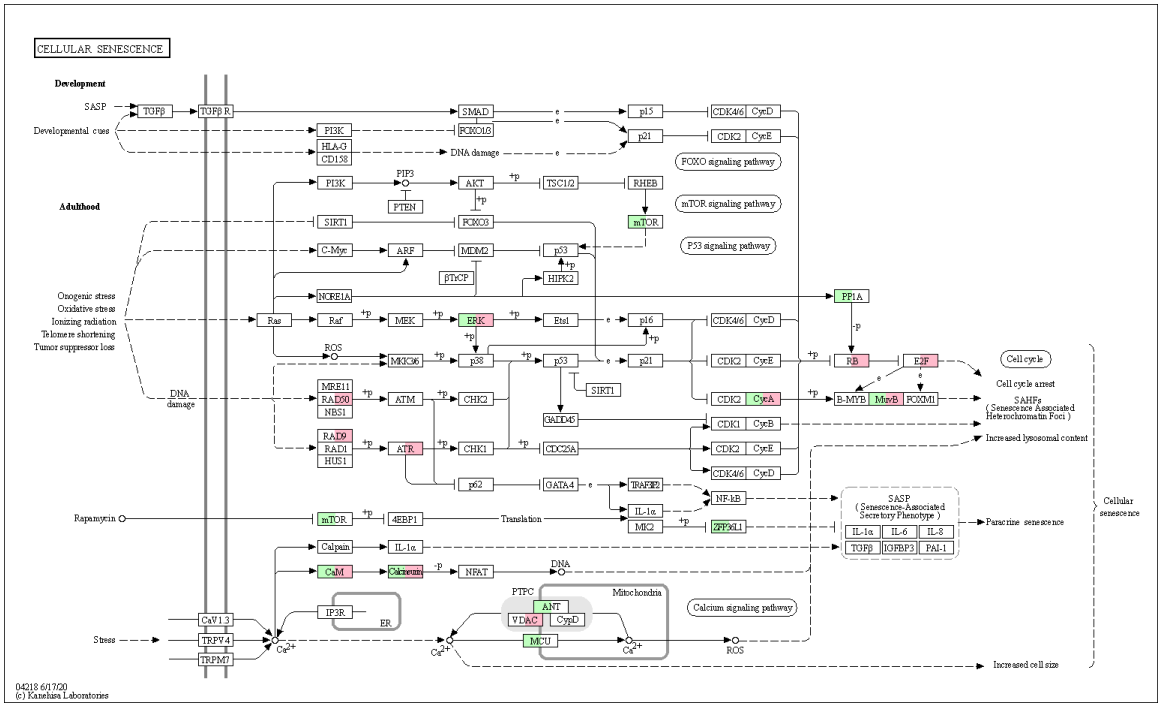

Figure 3.15. KEGG pathway of senescence highlighted for the differences between fresh and cold stressed microspores at 0 h, green in higher in cold stress while red is higher in fresh.

By 48 hours changes started to appear to the MAPK signally pathways, WRKY33 which leads to Camalexin biosynthesis was higher in cold stress (Figure 3.16.). Ethylene synthesis which is involved in both growth and senescence depending on timing and concentration (Iqbal et al., 2017) was higher in fresh. Stress adaptation and cold tolerance were higher in cold stress. CAT1 which inhibits hydrogen peroxide formation was higher in cold as well as the pathway regulating ROS.

Within the plant hormone pathway zeatin formation leading to cell division was higher in cold stress (Figure 3.17.) at 48 hours. However, AUX1 was higher in fresh. EBF1/2 was higher in cold stressed, leading to a decrease in fruit ripening and senescence.

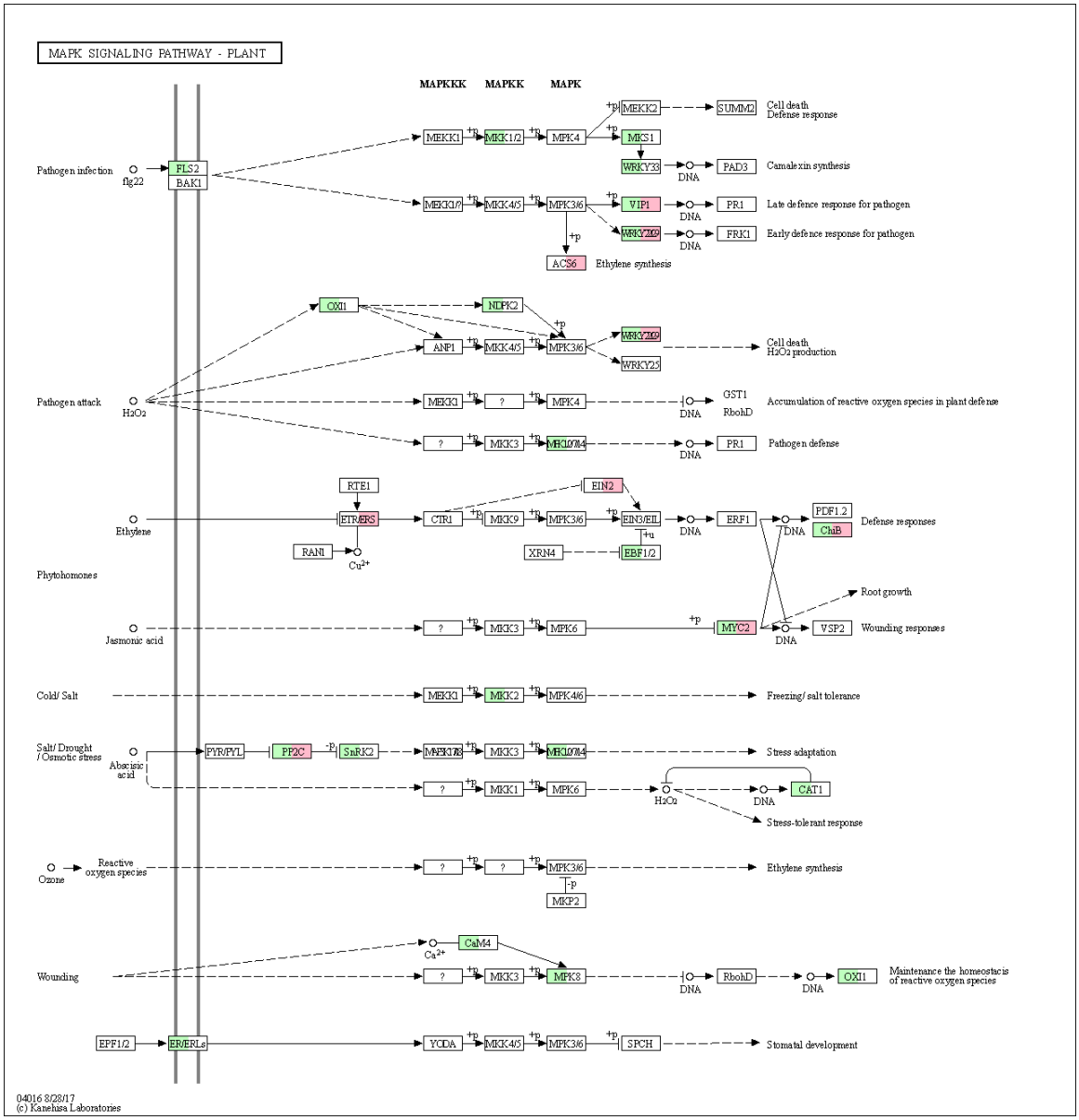

Figure 3.16. KEGG pathway for plant specific MAPK signalling comparing cold stress and fresh at 48 h. Green are higher in cold stress and red are higher in fresh.

Interestingly, by 48 h cathepsin was now higher in cold stressed microspores compared to fresh microspores while PARP continued to be higher in stress. The autophagy pathway also showed higher expression in cold stress at 48 h related to vesicle induction as well as elongation and closure of vesicle.

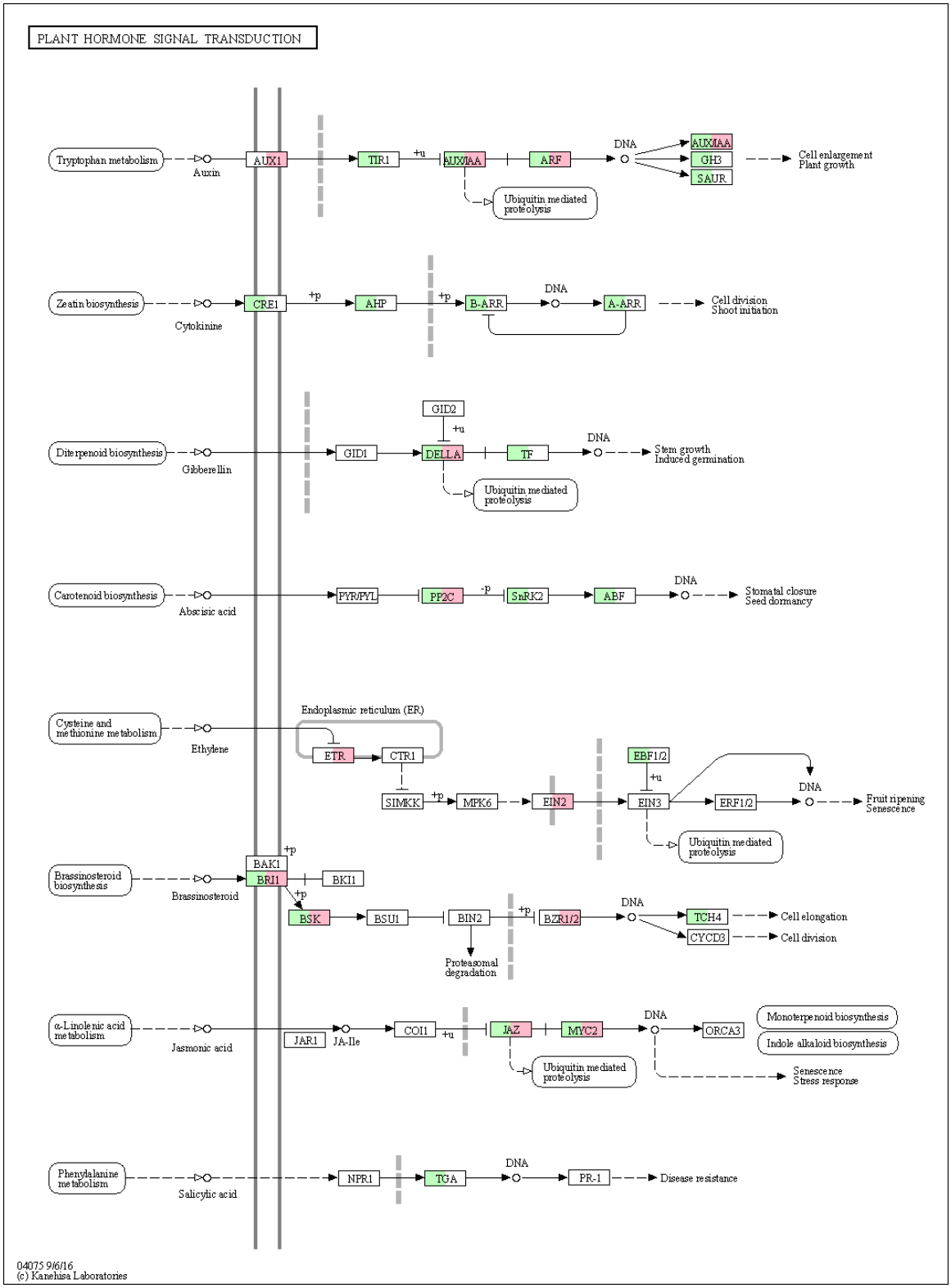

Figure 3.17. Plant Hormone signalling at 48 h in the comparison between fresh and cold stressed microspores. Green are higher in cold stress while red are higher in fresh.

Cell cycle (Figure 3.18.) showed an increase in expression related to mini chromosome maintenance (MCM), which are involved in progression through the cell cycle (Forsburg, 2004; Lei, 2005; Tuteja, Tran, Dang, & Tuteja, 2011). Origin recognition complexes (ORC) are also upregulated in stress at 48 hours as well as Cyclin A which is generally shown to increase within cells in S and G2 phase and plays a role in cell cycle progression during those phases (Oakes et al., 2014).

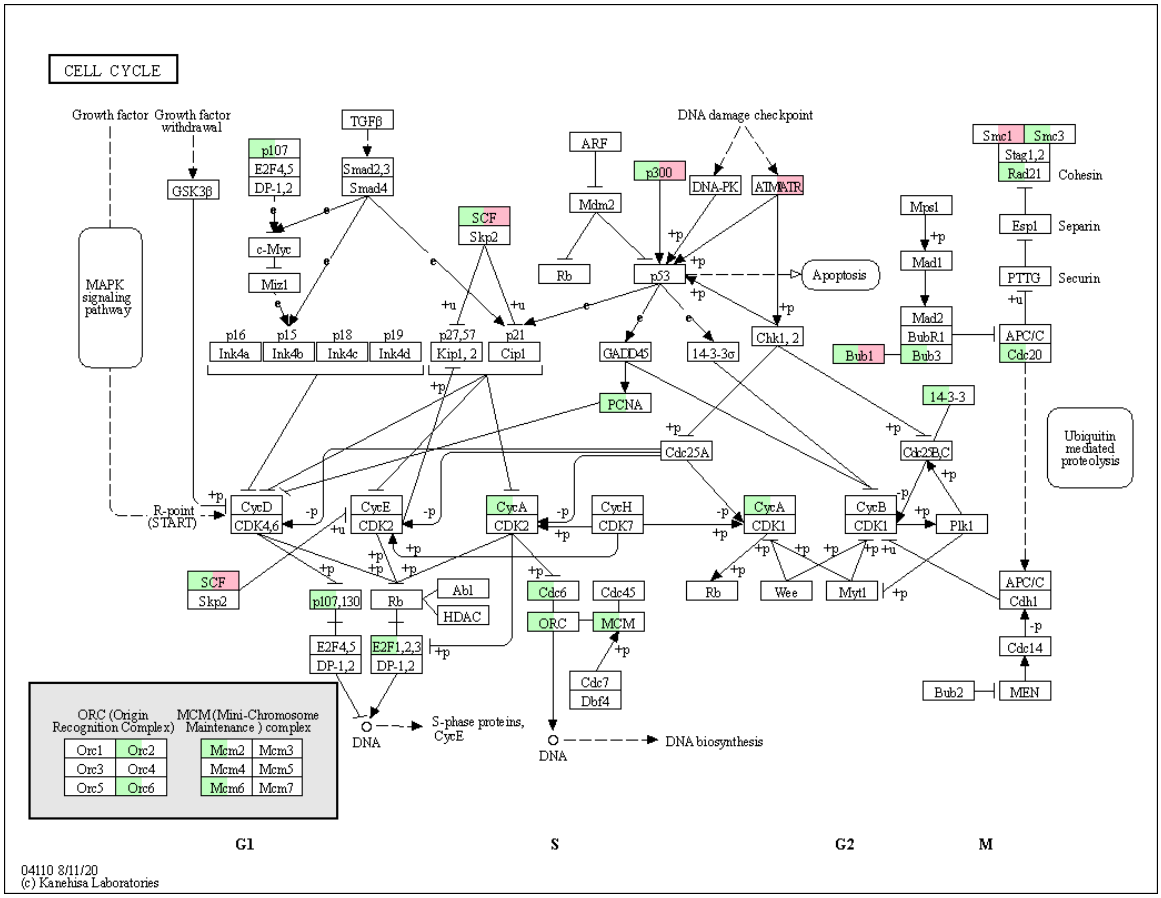

Figure 3.18. KEGG pathway for cell cycle at 48 hours post isolation comparison between fresh and cold-stressed microspores, green cells are higher in stressed while red are higher in fresh.

Interestingly, cold stress also shows an increase in cell cycle arrest at 48 h (Figure 3.19.) with RB representing an S phase entry block (Giacinti & Giordano, 2006). E2F is a

TF that activates may genes involved in cell cycle progression and DNA replication and repair (Ahlander & Bosco, 2009; Ren, 2002; Shen, 2002). The increase in RB is likely due to the higher expression of PP1A seen in stress at 0 h (Figure 3.15.) which is now lower in cold stressed microspores at 48 h.

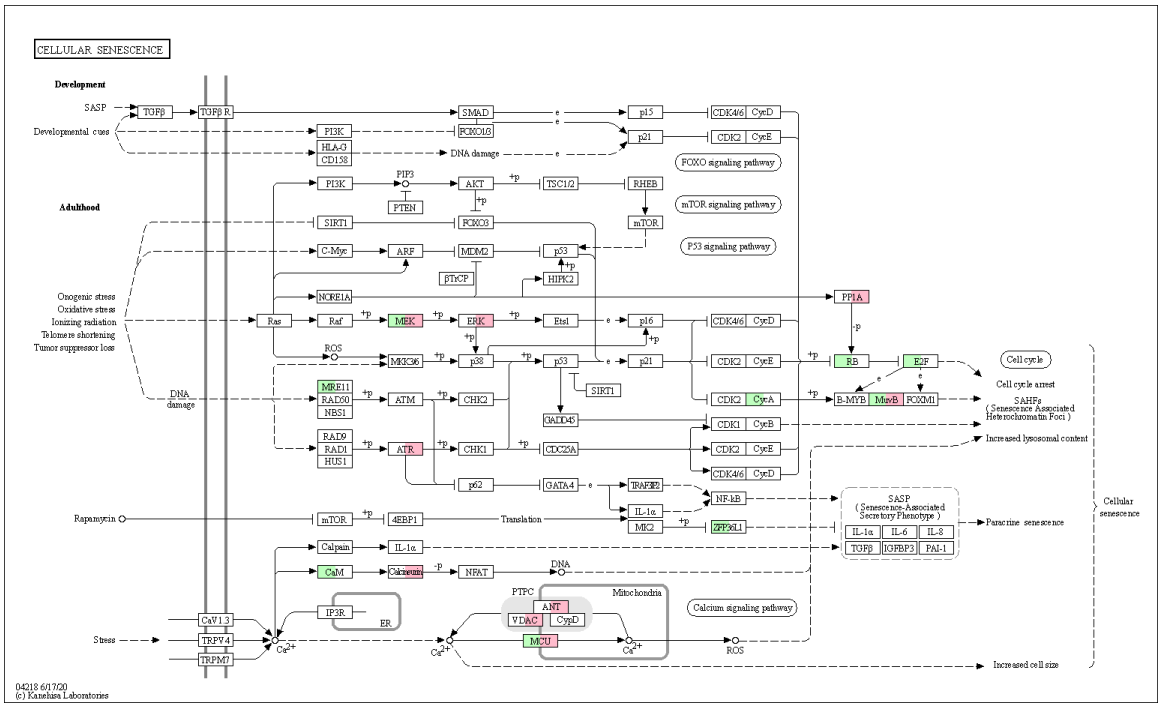

Figure 3.19. KEGG pathway for senescence in cold stress compared to fresh microspores at 48 h. Green is higher in cold stress and red is higher in fresh.

3.3. Discussion

3.3.1. High Number of Novel Transcripts Related to Alternative Splicing

The high number of novel transcripts seen was interesting, a high number of novel genes was also found in another study on androgenesis in wheat microspores, indicating the possibility of androgenesis induction specific transcripts (Seifert et al., 2016).

A partial explanation for the high number of novel transcripts is the early assembly of the wheat genome. Many of the 7096 novel transcripts we found were similar to known, uncharacterized, predicted or hypothetical proteins in closely related species or model organisms such as *Aegilops tauschii, Triticum urartu, Hordeum vulgare, Oryza brachyantha, Brachypodium distachyon, Secale cereale, Medicago truncatula, Arabidopsis thaliana, and Zea may.* These proteins have not been incorporated into the *Triticum aestivum* genomic assembly version that was used for our study but may be added in the future. Also of note, 471 of the 7096 novel transcripts related to known proteins or unnamed protein products in *Triticum aestivum*. As more transcriptomic studies, de novo assemblies, and comparisons are completed in wheat the genomic assembly will change and improve and the number of novel transcripts found when mapping will decrease. In the time after these samples were sequenced in 2020, an updated version of the genome has been released in spring of 2021 (Zhu et al., 2021), and mapping to this new release would likely result in a lower number of both novel genes and novel transcripts.

Alternative splicing of known transcript isoforms was found in 122,143 of the 170,176 novel transcripts in our study. Alternative splicing has been well documented as being involved in environmental response, cell development, and evolution (Shang, Cao, & Ma, 2017; Staiger & Brown, 2013; Syed, Kalyna, Marquez, Barta, & Brown, 2012; Szakonyi & Duque, 2018). The high number of alternatively spliced transcripts in our study is in line with the knowledge base that up to 70% of transcripts are alternatively spliced in higher plants (reviewed by Szakonyi and Duque (2018)) and the aforementioned newness of the wheat genomic assembly. Alternative splicing can alter

protein sequence and function or mRNA stability and are cell type specific (reviewed by Szakonyi and Duque (2018)).

With all of this, it is indeed plausible that alternative splicing is also involved in androgenesis induction. At this time though, we have not fully explored the potential causes and implications of the large number of novel transcripts this study has found nor the role and extent of alternative splicing that could be specifically related to androgenesis induction. Functional analysis of novel genes were not performed specifically, however since they were mapped to KEGG terms by BGI they are included in all KEGG pathway analysis.

3.3.2. GO Terms Enriched in Genes Not Differentially Expressed

Isolated microspores experience several stresses throughout the isolation process and within culture conditions. Maltose, used in density-gradient centrifugation likely results in osmotic stress in microspores. Cell culture conditions can also be osmotically stressful as well as the temperature shock all isolated microspores experience when isolated on ice then cultured at 28ºC. With these stresses it is not a surprise to see apoptosis, extracellular structure organization, and secondary metabolites being enriched in all treatments.

It is interesting however that apoptosis was seen in all treatments when we would have expected to see more differences in apoptosis between the treatments in this study as fresh microspores will all die in culture while cold stressed microspores will go on to produce MDEs. This may potentially be due to our enrichment for live microspores at 48 h.

Flavonoid metabolic process was interesting to see within microspores in culture. These secondary metabolites have antioxidant activity within cells (Wen et al., 2017) and may be enriched to improve cell survival.

Chloroplast RNA processing being among the top enriched GO terms in these genes that were not differentially expressed is also of interest, indicating that even after prolonged cold stress and other treatments the maturation of chloroplast RNA is not impacted, and chloroplast specific RNA is still being expressed although it does not unfortunately aid in the understanding of albinism development.

3.3.3. Transcriptomic Changes Were Seen Over Time in Culture in All Treatments

In the comparisons over time within each treatment the highest number of DEs were seen between 0 h and 48 h as well as 3 h and 48 h, this suggests that after 3 hours in culture the transctipomes of microspores largely haven't changed from when first isolated. After 2 days in culture though, there is a large change in the transcriptomes when compared to earlier time points with many genes increasing or decreasing in expression level.

The downregulation of BRG2 at 48 hours may indicate that protection from PCD within the live-enriched cells is no longer required at this point in culture. Since the majority of cells die within culture in the first 2 days, BOI and BOI-related genes such as BRG2 may play an important role in cell survival early on. BOI in *Arabidopsis thanliana* was shown to interact with a TF that in implicated in stress and pathogen response (Luo et al., 2010).

YDA and the associated MAPK signalling cascade involved in cell fate and early embryogenic divisions (Garcia et al., 2019; Lukowitz et al., 2004; Samakovli et al., 2021; Samakovli et al., 2020; The UniProt Consortium, 2020) showing increase over time in multiple homeologs within the wheat genome over all comparisons is worth further investigation. MAPK signalling is very complex and is involved in the polarity in the early embryo, with YDA initiation (Garcia et al., 2019). YDA controls interleukin-1 receptor-associated kinase (IRAK) (Garcia et al., 2019) which are generally involved in the initiation of kinase signalling cascades (Dardick & Ronald, 2006). Many IRAKs are seen in cellular defense and stress responses as well (Dardick & Ronald, 2006) and there were many IRAKS seen in our study as well. What was especially interesting was the lack of change seen in YDA genes in Stress+TSA comparisons. This makes YDA an interesting target for further research.

The repair of DSBs in cells is vital to their survival and proliferation. The increase seen in MRE11 orthologs by 48 h in all treatments could indicate that culture conditions are inducing DSBs however, MRE11 is involved in more than DSB repair. MRE11 has been implicated in plants genomic stability, telomere extension, and repairing collapsed replication forks (Puizina, Siroky, Mokros, Schweizer, & Riha, 2004). Its involvement in genomic stability may improve the survival of cells within culture conditions.

GBSSI, was identified as changing in expression over time in cold stressed microspores in our study. Gajecka et al. (2020) linked initial expression levels of GBSSI in barley to green plant production potential, however they did not explore changes in expression over time in culture. GBSSI is involved in starch and glycan biosynthesis (The UniProt Consortium, 2020). The decrease in starch biosynthesis has been linked to

androgenesis in other studies (Hale et al., 2020; Maraschin et al., 2006; Seifert et al., 2016). It is interesting that the decrease was not seen in Stress+TSA, possibly indicating that the maintenance of the level of expression and preventing the decrease in expression may improve resulting number of green plants in IMC.

3.3.4. Cold-Stress Results in Transcriptomic Changes in Microspores Seen at the Start of Culture with High Numbers of Differentially Expressed Genes

Prolonged stress treatment with cold leads to transcriptomic changes to adapt to the low temperature environment. Cells need to adjust their physiology, membrane rigidity, protein folding, as well as deal with ROS and damage, and slow down metabolism and growth (Chinnusamy, Zhu, & Sunkar, 2010; Chinnusamy, Zhu, & Zhu, 2007). Cold leads to accumulation of hydrogene peroxide (Chinnusamy et al., 2010) which is likely the cause of enrichment of GO terms for ROS and specifically hydrogene peroxide at 0 h and 3 h in cold-stressed. Cytoskeleton reorganization and cytosolic Ca2+ influx takes place and are sensed by (calcium-dependent protein kinases) CDPKs, phoshatase and MAPKs, which signal to turn on transcriptional cascades (Solanke & Sharma, 2008). Calcium signalling and MAPK signalling are involved in embrogenic pathway (Ahmadi, Ahmadi, & Teixeira da Silva, 2018). Intracellular calcium levels increase in microspores committed to androgenesis, leading to deposition of callose related to membrane permeability (Ahmadi et al., 2018). Kinases related to disease resistance are upregulated in cold environments (Walbot, 2011). The high number of genes that were seen expressed at higher levels in cold-stress could be due to a specific response to Calcium signalling and kinase cascades related to cold, suggesting that more genes are needed for survival in the cold. The increase in calcium associated with

microspores androgenesis could be resulting from poly(ADP)ribose. ADP-ribose stimulates calcium channels and the increase in intracellular calcium leveles and increased levels of intracellular calcium could promote cell death (Pascal & Ellenberger, 2015). Cold-stressed microspores at 0 h showed enrichment of PARP in the apoptotic pathway which is related to low levels of poly(ADP)ribose (Pascal & Ellenberger, 2015) and likely a stop in the accumulation of excess calcium intracellularily. Although PARP is implicated in PCD, excess calcium is as well (Pascal & Ellenberger, 2015) so this may be preventing or inducing cell death depending on the calcium levels within individual cells.

Interestingly expression of most pollen-specific genes is not changed in cold stress and the COR genes implicated in cold tolerance of leaf tissues are not induced significantly in the pollen (Chinnusamy et al., 2007). Also ROS from the cold stress may play an important role in regulation of polarized growth of root hairs and pollen tubes, through controlling cell wall rigidity and cell signalling involving calcium and MAPK cascades (Rivas-San Vicente & Plasencia, 2011). This may explain the enrichment of pollen related terms early on in cold stressed microspores. Under normal development the microspores would reorganize their cytoplasm, increase in size, migrate their nucleus and prepare for division (Chaturvedi et al., 2021; Sharma et al., 2015). With ROS the polarized growth, change in cell wall rigidity in combination with MAPK signalling may play an important role in allowing for a change in cell fate. These changes are cytoskeletal, membrane related, cytoplasmic, and involve the cell wall. In addition to these changes is the cellular response to cold described previously.

Immediately after isolation enriched GO terms for DEs that were higher in cold-stressed microspores are supporting the hypothesis that microspores are changing during the cold treatment. There are signs of cell growth, development and morphogenesis, as well as signs of adapting to the stressful environment (ER and ROS). The adaptation to stress in cold-stressed microspores is supported by the decrease in erichment of stress response at 3 h. This suggests that microspores were stressed during the 3 weeks cold treatment and are more easily adapted to the cell culuture conditions and ambient temperature. Intracellular signal transduction was also prevalent at 3 hours, potentially signalling towards the change in cell fate or towards the adaptation seen to new environmental conditions.

Autophagy was higher in cold-stressed microspores at 0 h. Autophagy has been implicated as involved in reprogramming of microspores, through recycling of cell parts (Ahmadi et al., 2018; Bárány et al., 2018; Berenguer et al., 2021; Corral-Martínez, Parra-Vega, & Seguí-Simarro, 2013; Perez-Perez et al., 2019). Although some level of autophagy seems to be necessary for the change in cell fate associated with androgenesis induction, suppression of autophagy improves androgenesis results (Bárány et al., 2018; Berenguer et al., 2021; Perez-Perez et al., 2019). This is because autophagy can be helpful or harmful to cells mainly dependant upon the level of autophagy. Autophagy of chloroplasts for example, can be used for cell survival and reduction of oxidative damage in starvation conditions (Kikuchi et al., 2020). Autophagy can also induce programmed cell death (Bárány et al., 2018; Liu et al., 2005; Minina, Bozhkov, & Hofius, 2014; Patel, Caplan, & Dinesh-Kumar, 2006; Perez-Perez et al., 2019; van Doorn et al., 2011).

Enriched terms of genes expressed higher in fresh microspores immediately following microspore isolation include chromosome and chromatin organization, gene and chromatin silencing, mitotic cell cycle process, epigenetic negative regulation of gene expression and histone modification supports the changes seen in microspores after prolonged cold-stress. At the start of culture fresh microspores are still on the microgametogenesis pathway towards Pollen Mitosis I, showing cell cycle progress and chromatin organization while cold-stressed microspores are not. Cold stressed microspores are responding to the cold stress they were exposed to as well as the media and microspore isolation protocol. Microspores from cold stress are not enriched for any noticeable pattern within the cell cycle at the start of culture, indicating that they are no longer focused on and committed to pollen development, but instead to growth and cell wall modification and intracellular signalling. Higher cathepsin levels were also seen in fresh microspores at the start of culture. Cathepsin has been linked to apoptosis (Bárány et al., 2018; Chwieralski, Welte, & Bühling, 2006). This could support preliminary findings of more rapid cell death in fresh microspores (not shown).

With the changes to environment seen in cold stressed microspores, at 3 h histone phosphorylation was lower in cold-stress indicating closing of chromosome confirmation and a decrease in expression levels (Rossetto, Avvakumov, & Côté, 2012). Histone phosphorylation can also be a signal for DNA breakage or in the regulation of growth factors (Rossetto et al., 2012). Histone phosphorylation has also been implicated in mitosis (Ito, 2007). This enrichment in histone modification supports the DNA conformational change and packaging seen higher in cold-stressed at 48 hours and may be the start of a change in cell fate.

3.3.5. By Two Days in Culture Cold-Stressed Microspores Show Signs of Change of Cell Fate Towards Androgenesis

At 48 h nucleosome assembly and organization, DNA packaging and chromatin related terms, and DNA conformational change being highly enriched in cold-stressed microspores points towards a switch in cell fate towards androgenesis. These major genomic changes are not seen in fresh microspores and likely indicate that the switch is occurring this early in culture. Cell proliferation and DNA replication were also highly enriched, likely towards a symmetric division related to androgenesis.

The enrichment of protein folding showed a pattern of high expression in cold-stress, later decreasing and expressed lower in cold-stress compared to fresh. This pattern supports again that microspores exposed to the prolonged cold-stress prior to isolation were more adapted to the culture conditions at 48 h. This is likely due to expression of genes that chaperone protein folding in cold-stress, that would be unnecessary once cold is removed. Translation showed the same pattern as protein folding, likely due to issues with both processes in cold-stress and early in culture, decreasing by 2 days.

Metabolic processes and response to stress were lower in cold stressed by 2 days which is supported by previous transcriptomic studies in microspores (Hale et al., 2020; Seifert et al., 2016). Mitochondrial and mitochondrion terms also decreased over time in culture in cold stress which is understandable with the decrease in metabolic processes. Unlike previous studies (Hale et al., 2020; Seifert et al., 2016) ribosomal terms such as ribosome biogenesis were not downregulated in cold stress compared to fresh, however they did decrease over time in culture between 3 h and 48 h in cold stress microspores.

Chloroplast and mitochondrion were downregulated at all times and decreasing in stress over time as well (3 h vs 48 h down) possibly due to enrichment during the 3 weeks of cold stress. Chloroplast and mitochondrion were found to be swollen in cold (Stefanowska, Kuraś, & Kacperska, 2002), which may be due to hyperactivity in response to cold stress. With the removal of that cold stress the cells may be adjusting and decreasing the transcripts relating to those organelles.

The upregulation of WRKY transcription factors through the MAPK signalling pathway is involved with stress response (Bakshi & Oelmüller, 2014; Zheng et al., 2020). WRKY33 induces camalexin biosynthesis (Mao et al., 2011) which involves cytochrome P450 (Lemarié et al., 2015). Hale et al. (2020) found cytochrome P450s to be upregulated in stress treated microspores. Several cytochromes were also found in this study as well as the enrichment in cold stress for camalexin biosynthesis.

As discussed earlier, RB expression leads to cell cycle arrest but also inhibits gene expression and apoptosis in humans (Ahlander & Bosco, 2009). RB has also been implicated to be involved in heterochromatin organization and regulation of E2F (Ahlander & Bosco, 2009; Giacinti & Giordano, 2006; Ren, 2002; Shen, 2002). E2F is a TF that regulated many cell cycle related genes and progresses the cell cycle (Ahlander & Bosco, 2009; Giacinti & Giordano, 2006; Ren, 2002; Seguí-Simarro, 2010; Shen, 2002). The expression of both RB and E2F could indicate upcoming cell cycle arrest for G1/S checkpoint (Giacinti & Giordano, 2006) and that cold stressed cells are preparing for mitosis. The increased expression of MCM related genes could also indicate preparation for mitosis as they are essential for DNA replication (Forsburg, 2004; Lei, 2005; Tuteja et al., 2011).

As discussed earlier, the involvement of BARD1 in WUS expression localization (Han et al., 2008) represents an interesting target for improvement within IMC. BARD1 is also involved in DNA repair (Reidt, Wurz, Wanieck, Chu, & Puchta, 2006; Tarsounas & Sung, 2020). So BARD1 may be increased over time to prepare for WUS localization or for DNA replication and repair, or for both functions. Of note, WUS ortholog TraesCS2A02G491900 was present in genes identified in this study but was part of the genes not differentially expressed in any comparison. BARD1 could be involved in the change of cell fate towards androgenesis or be a good target for improvement.

HYOU1 is a part of the HSP70 family (Junprung et al., 2019) and there is some controversy in the involvement of HSP70 proteins in androgenesis. Many studies have found upregulation of HSPs and specifically HSP70s correlated with androgenesis (Su et al., 2020; Testillano, 2019). However several papers have found HSP70 not required for androgenesis induction (Seguí-Simarro, Testillano, & Risueno, 2003; Zhao, Newcomb, & Simmonds, 2003), and likely plays an indirect role, possibly in inhibition of apoptosis (Segui-Simarro & Nuez, 2008). HYOU1 was lower in cold stressed microspores at 48 h and is likely mostly involved in cell survival in culture however with this function it could still be useful in improving androgenesis through preventing cell death. It is worth further exploration.

3.3.6. Cold-Stressed Microspores are Different than Fresh Microspores, Those Differences Account for Response to Androgenesis Induction

There were many differences seen between fresh and cold stressed microspores at all time points compared. Cell wall and cytoskeleton are the earliest observed differences

in androgenesis induction and represent differences induced by cold stress on microspores.

Cell wall formation was among the highest enriched categories of GO terms early on in culture. Pectin content within the cell wall composition changes in response to exogenous stimulus (Houston, Tucker, Chowdhury, Shirley, & Little, 2016). More fragile and plastic cell wall was associated with microspore commitment to androgenesis, allowing for cell growth (Corral-Martínez, Driouich, & Seguí-Simarro, 2019). Transcription of pectin was higher in fresh and amylopectin was higher in cold stressed microspores in our study. This could indicate that cold stressed microspores already have altered cell walls from cold stress treatment or are in the process of altering it early on in culture.

Cytoskeletal related terms were higher in cold stressed microspores in comparison to fresh microspores. Cellular reorganization has been implicated in androgenesis induction in several studies (Dubas, Wędzony, Petrovska, & Salaj, 2010; Hale et al., 2020; Maraschin, Vennik, et al., 2005; Méndez-Hernández et al., 2019; Parra-Vega, Corral-Martínez, Rivas-Sendra, & Seguí-Simarro, 2015; Sanchez-Diaz, Castillo, & Valles, 2013; Seifert et al., 2016; Sharma et al., 2015; Straatman, Nijsse, Kieft, van Aelst, & Schel, 2000). Dubas et al. (2010) specifically mentioned reorganization of mitochondria and at all times in our study mitochondrion localization was high is cold stressed microspores.

There were also terms that seemed unrelated to androgenesis induction but have been found in other studies. Response to chitin was higher in cold stressed microspores at

all 3 times in our study. Chitinase has been demonstrated to influence cell fate determination during somatic embryogenesis and has been found enriched in androgenesis (Bandupriya & Dunwell, 2015; Hale et al., 2020). Embryogenic meristem was also higher in cold stressed microspores at all time points which is similar to previous findings (Hale et al., 2020).

Overall, prolonged cold treatment on microspores results in differences that influence their response to culture conditions for induction of androgenesis. Changes are seen transcriptionally, metabolically, and physically in cold stressed microspores. The change in cell growth, cell wall rigidity, ROS signalling, calcium signalling, and MAPK signalling present in cold stressed microspores at the start of culture likely play an important role in microspores reprogramming.

CHAPTER 4 – THE IMPACT OF TRICHOSTATIN A TREATMENT ON THE TRANSCRIPTOME OF AC ANDREW MICROSPORES IN CULTURE

4.1 Introduction

As previously reviewed in Chapter 1 (1.4.), TSA an HDAC inhibitor, leads to a generalized increase of histone acetylation across the genome which is associated with open chromosome configuration and general upregulation of genes. Previous work has shown that TSA application increases the number of MDE in microspore cultures and resulting green plant numbers of AC Andrew when applied to cold-stressed microspores (Jiang et al., 2017). The Enabling Technologies laboratory at AAFC Lethbridge took this further, by performing the same experiment on fresh microspores, with the hopes of TSA being able to circumvent the prolonged cold treatment. Application of TSA to fresh microspores isolated without stress treatment showed a small number of resulting MDEs that were able to germinate and go on to produce green plants (unpublished data). The possibility of inducing androgenesis in fresh microspores and eliminating the need for stress treatment would decrease the time to regenerate haploid and DH plants through IMC. We wanted to explore the impact TSA had on the transcriptome of fresh microspores in hopes of identifying pathways and genes that are important for androgenesis induction and the changing of cell fate.

Based on the response of fresh microspores to TSA, we hypothesized that TSA might have the general effect of upregulating genes and that this ability would be similarly exerted on stressed microspores. In stressed microspores, the response to TSA

through general upregulation of genes could be exaggerated by the stress preconditioning; some of which would be helpful towards increased androgenesis.

4.2. Results

4.2.1. General RNA-Seq Results

For an overall summary of the RNA-Seq results and number of transcripts and mapping rate for Fresh and Stress samples see Chapter 3 (3.3.2.) (Table 3.1., Figure 3.2., & Table 3.2.).

4.2.2. Differential Expression Between Treatments with TSA Application

Within cold-stressed microspores treated with TSA an increase of DEs was seen, mostly upregulated between 0- and 3 h samples (Figure 3.4.A). In comparison, all other treatments had much lower numbers of DEs between 0 and 3 hours. Stressed microspores treated with TSA resulted in 6570 DEs total, 4807 of which were upregulated DEs while the other treatments all had less than 700 total DE's between 0 and 3 hours. This is also illustrated in Figure 4.1.B where DEs are shown over time, there is a spike seen in upregulated DEs at 3 hours in Stressed+TSA while the other treatments show very few DEs. Interestingly the number of upregulated DEs decreases between 3- and 48-hours in Stress+TSA while it increases in all other DE category.

When treatments were compared the largest number of DEs was seen in Fresh+TSA versus Stress+TSA at 0 hours with 13,352 DEs (Figure 4.1.A and B), indicating again that stress has the largest impact on the transcriptome. Over time in culture Fresh+TSA and Stress+TSA become more similar, with lessening DEs as time

goes on. Interestingly, Fresh and Fresh+TSA have low numbers of DEs from 1279 DEs at 0 h and declining to 182 at 3 h and 106 at 48 h, indicating not much of a difference seen between fresh microspores and fresh treated with TSA. TSA application to stressed material did however show an impact to the transcriptome, at 0 h Stress and Stress+TSA had only 74 DEs total while at 3 hours there were 2715 DEs with 2486 upregulated. Also of note, with averages of 71,234 transcripts expressed in Stress+TSA at 3 h and 64,046 in Stress at 3 h and 61,888 in Fresh+TSA at 3 h there is a sizeable number of genes only expressed in Stress+TSA at 3 h, although most of those transcripts were less than 10 FPKM (Figure 3.2. & Table 3.2.). At 48 hours the number of DEs between Stress and Stress+TSA had decreased down to 930 in total, indicating a transient response to TSA.

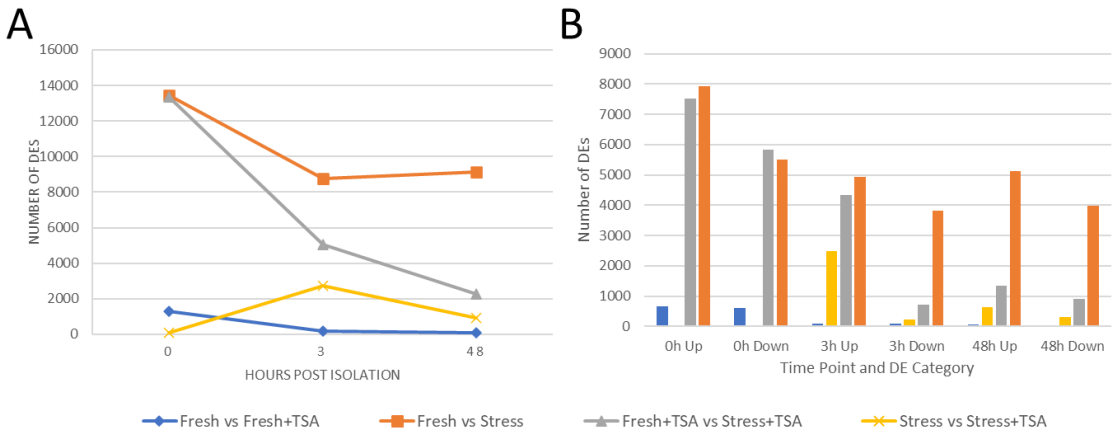

Figure 4.1. The impact of TSA treatment on the transcriptome. **A)** The total number of DEs between treatments over time. **B)** The number of DEs between treatments separated by up or down regulation.

When the DEs were mapped to the genome (Figure 4.2. A-C) the small difference between Fresh and Fresh+TSA at 0 h can be seen with it decreasing over time with 3 h and 48 h showing only a small number of spots (A, 0-48 h L-R). In Stress versus

Stress+TSA (B) there is very little difference at 0 h (leftmost), and the increase in upregulated DEs (blue) is striking at 3 h (middle) and a lessened but still present difference at 48 h (rightmost). The majority of the large number of DEs at 3 hours is mostly genes higher is Stress+TSA. The differences seen at all 3 time points in Fresh+TSA versus Stress+TSA (C) are similar to the patterns seen in Fresh versus Stress seen in Chapter 3 (Figure 3.5.B).

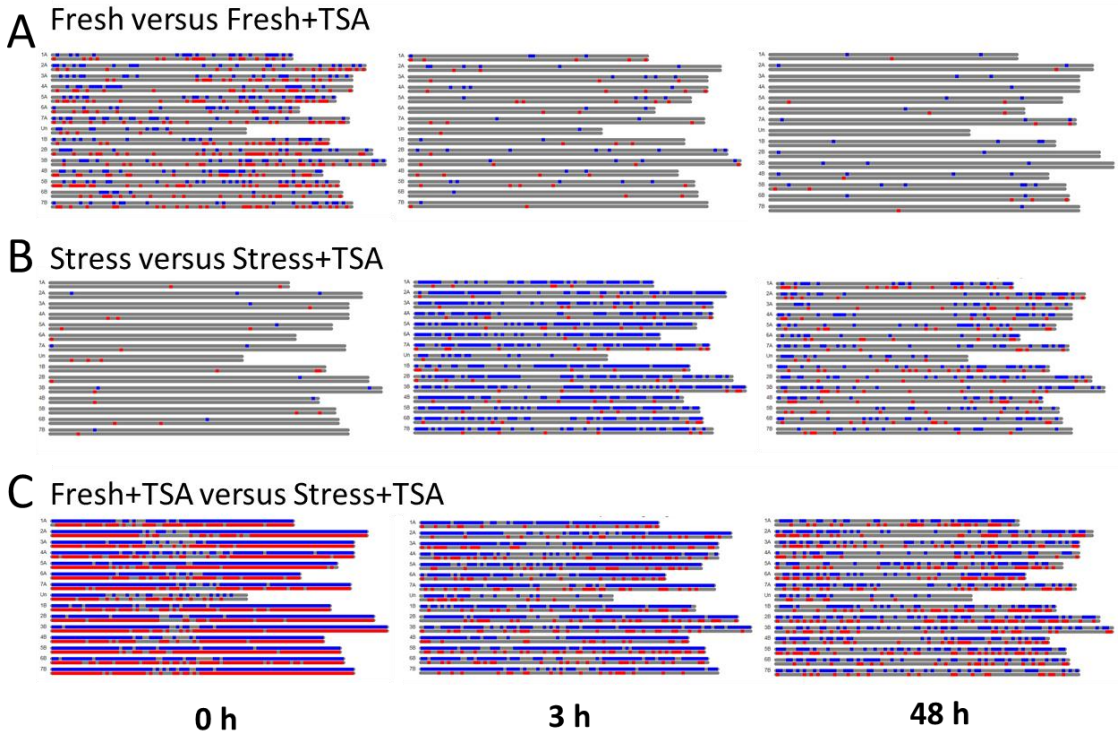

Figure 4.2. ChromoMaps showing where DE's map to in the genome in comparisons blue represents DEs higher in the second treatment and red were higher in the first treatment **A)** Fresh versus Fresh+TSA, **B)** Stress versus Stress+TSA, and **C)** Fresh+TSA versus Stress+TSA). (L-R 0 h, 3 h, 48 h).

The comparisons between treatments were also input into UpSetR and is seen in Figure 4.3. Again, the vertical bar graphs along the top is the number of DEs present

within the comparisons marked by dots on the bottom of the image, while the horizontal bars on the left show the total number of DEs within that single comparison. The categories were labelled as follows: AA = Fresh, AB = Fresh+TSA, AC = Cold-Stress, AD = Stress+TSA, 1 = 0 h, 2 = 3 h, 3 = 48 h, “up” indicates the DE was higher in the second treatment listed while “down” indicates lower expression of the DEs in the second treatment listed (Figure 4.3.).

Cold-stress treatment seems to have the largest influence on the microspore transcriptome, the highest categories of unique DEs were seen in Fresh vs Stress and Fresh+TSA vs Stress+TSA (Figure 4.3.) In Fresh vs Stress the number of DEs that were only seen in the individual comparisons were quite high with 1500 at 0 h up, 1437 at 48 h up, 1367 at 48 h down, 333 at 3 h up, and 284 at 3 h down (figure 4.3.). The same pattern was seen in Fresh+TSA vs Stress+TSA with 1402 unique DEs at 0 h up, 1367 at 0 h down, 595 up at 3 h, and 205 down at 3 h (Figure 4.3.). 697 DEs were downregulated in both Fresh vs Stress and Fresh+TSA vs Stress+TSA 0 h (Figure 4.3. “AA1vsAC1_down” and “AB1vsAD1_down”). 1057 DEs were downregulated in Fresh vs Stress and Fresh+TSA vs Stress+TSA 0 h as well as Fresh vs Stress at 3 h (Figure 4.3. “AA1vsAC1_down”, “AB1vsAD1_down”, and “AA2vsAC2_down”). This is interesting and supports the increased genome-wide expression in Stress+TSA at 3 hours.

TSA application in cold-stressed material also seems to make a novel impact on the transcriptome with 807 DEs only seen in the comparison between Stress vs Stress+TSA at 3 h (“AC2vsAD2_up” in Figure 4.3.).

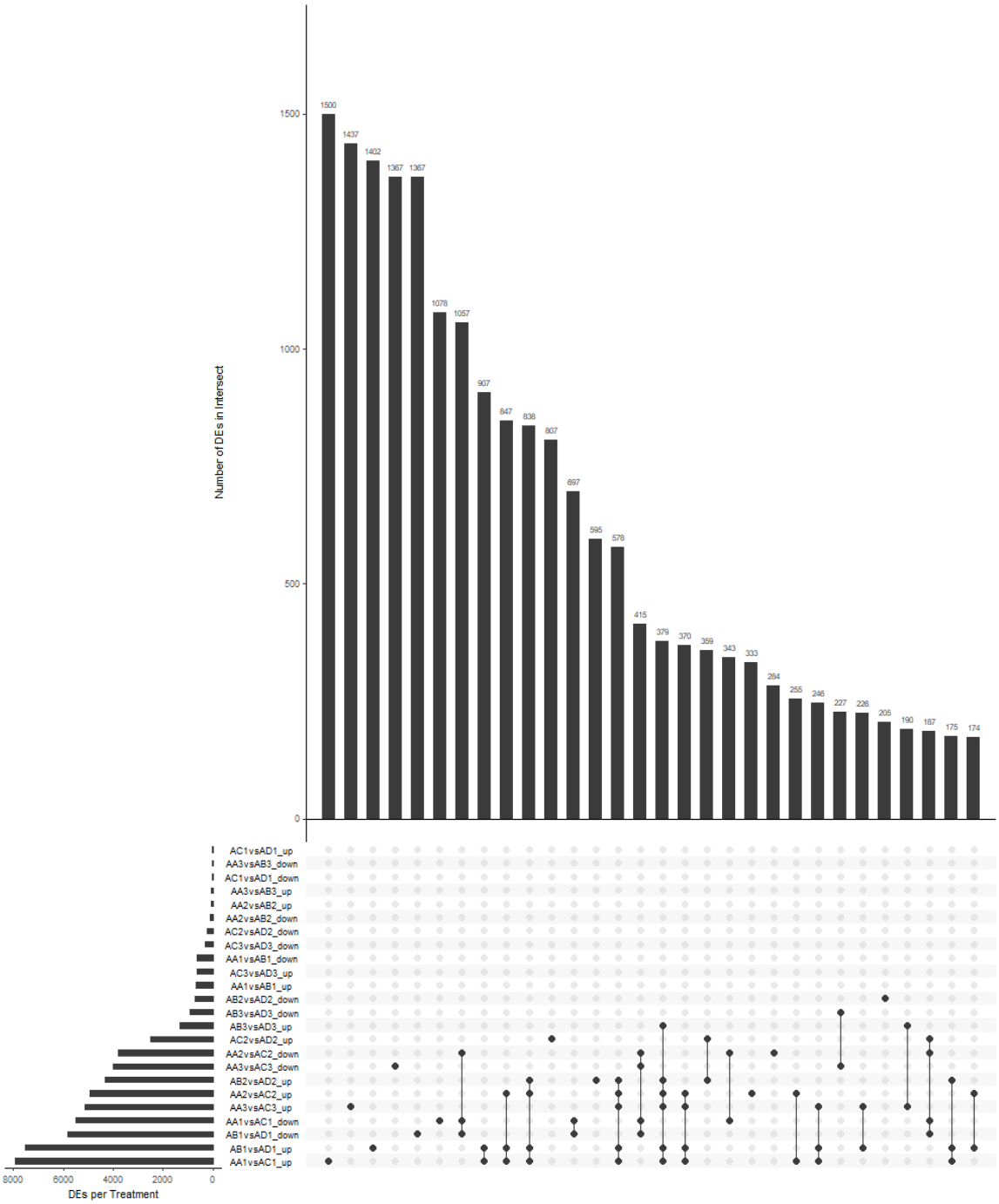

Figure 4.3. Image created using UpSetR in RStudio. Lists of DEs were separated by Up- or Down-regulation and plotted to determine DE overlap between treatments (Vertical bar graph) and which treatments that number of DEs are seen in (bottom dots). The horizontal bar graph illustrated the number of shared DEs in that treatment comparison.

Several genes that were seen altered in cold stress compared to fresh in the absence of TSA treatment (discussed in Chapter 3) were also seen in the presence of TSA. BARD1 orthologs were shown to be impacted by both cold-stress and TSA. TraesCS2A02G384900 showed 2-fold increased expression at 48 hours in Stress+TSA compared to Fresh+TSA. TraesCS2B02G401900 was expressed 1.3-fold higher in Stress+TSA compared to Fresh+TSA at 0 h and 48 h as well as at 3h in Stress+TSA compared to Stress. TraesCS6D02G257000 (HYOU1 ortholog) was found to be expressed 1.6-fold lower in Stress+TSA compared to Fresh+TSA. GBSSI ortholog, TraesCS4A02G418200 was interestingly 1.4-fold higher in Fresh compared to Fresh+TSA at 0 h and 1.8-fold higher in Stress+TSA compared to Stress at 48 h.

4.2.3. GO Terms Enriched in Cold-stressed Microspores Treated with TSA Compared to Cold-stressed

Of the 74 DEs at 0 h between Stress and Stress+TSA did not have statistically significant enrichment of any GO terms. This shows how similar the two treatments are at this time point, before TSA impacts the transcriptome.

The 2486 DEs higher in Stress+TSA at 3 h between Stress and Stress+TSA were enriched in 466 biological processes mostly related to DNA (Figure 4.4.). The GO terms were ranked by p-value and the top 15 terms included mostly DNA reorganization terms; nucleosome assembly (GO:0006334), nucleosome organization (GO:0034728), chromatin organization and assembly (GO:0006325 & GO:0031497), DNA packaging (GO:000632), chromosome organization (GO:0051276), heterochromatin organization (GO:0070828), and DNA conformation change (GO:0071103).

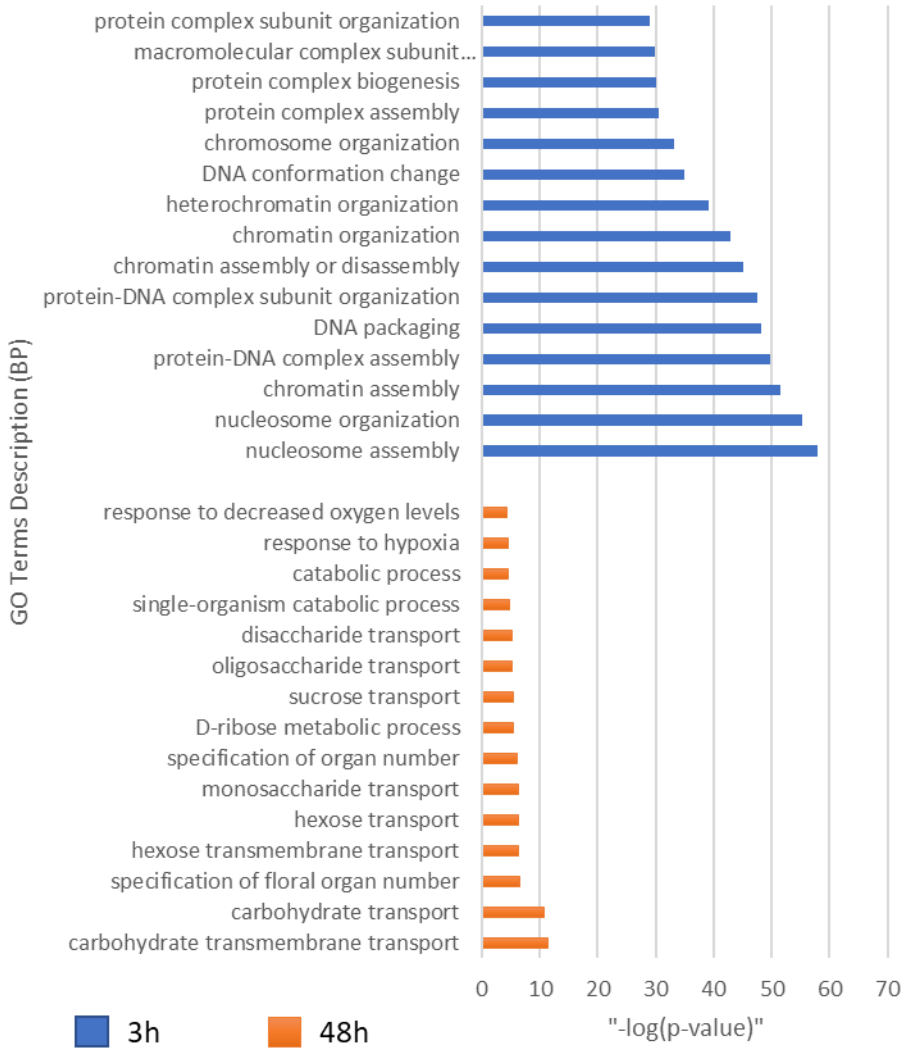

Figure 4.4. GO term descriptions for upregulated DEs at 3 hours and 48 hours in Stress and Stress+TSA.

At 48 h the 18 Biological Processes GO terms enriched for DEs higher in Stress+TSA were more related to transport (Figure 4.4.); carbohydrate transmembrane transport (GO:0034219), carbohydrate transport (GO:0008643), hexose transmembrane transport (GO:0035428), hexose transport (GO:0008645), sucrose transport (GO:0015770), monosaccharide transport (GO:0015749), and disaccharide transport (GO:0015766). Metabolic and catabolic process were also prevalent in the top 15 GO

terms by p-value (GO:0006014, GO:0009056, GO:1901575, GO:0044712). Interestingly specification of floral organ number (GO:0048833) was top 3 GO term and specification of organ number (GO:0048832) was 7th.

The 807 DEs that were only found in Stress and Stress+TSA 3 hours (Figure 4.3.) relate to 394 biological process GO terms which is similar in size to what is created from DE lists much larger in number, such as Fresh and Stress at the same time point with 346 Biological Process GO terms from 4943 DEs upregulated. The GO terms from those 807 DEs include protein autophosphorylation (GO:0046777), hormone-mediated signaling pathway (GO:0009755), microtubule cytoskeleton organization (GO:0000226), xylem and phloem pattern formation (GO:0010051), cell division (GO:0051301), transmembrane receptor protein serine/threonine kinase signaling pathway (GO:0007178), regulation of ion transport (GO:0043269), regionalization (GO:0003002), response to lipid (GO:0033993), and histone modification (GO:0016570). These and the rest of the other top 25 GO terms ranked by p-value can be see in Figure 4.5.

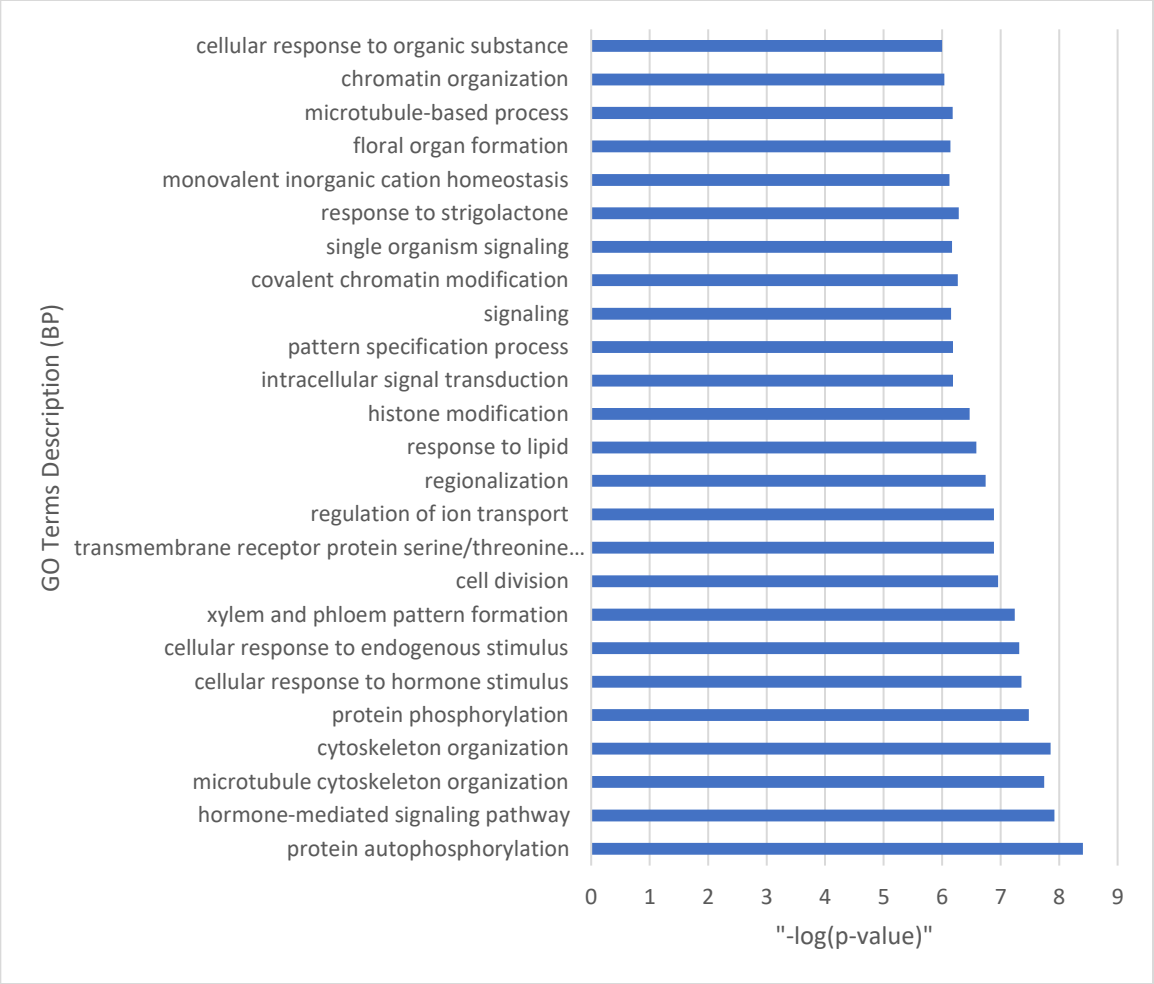

Figure 4.5. Top 25 GO Terms from 807 DEs that were only found in Stress vs Stress+TSA 3 h upregulated.

The GO terms with p-values from SEA were plotted using REVIGO (Supek et al., 2011) to show level of enrichment and to remove redundant terms (Figure 4.6.). The plot in semantic space mitotic cell cycle, protein autophosphorylation, plant-type cell wall modifications, cytoskeletal organization, cell cycle, cell division, signalling, carbohydrate biosynthesis, regulation of ion transport, and response to endogenous stimulus were enriched in cold-stressed microspores treated with TSA at 3 hours.

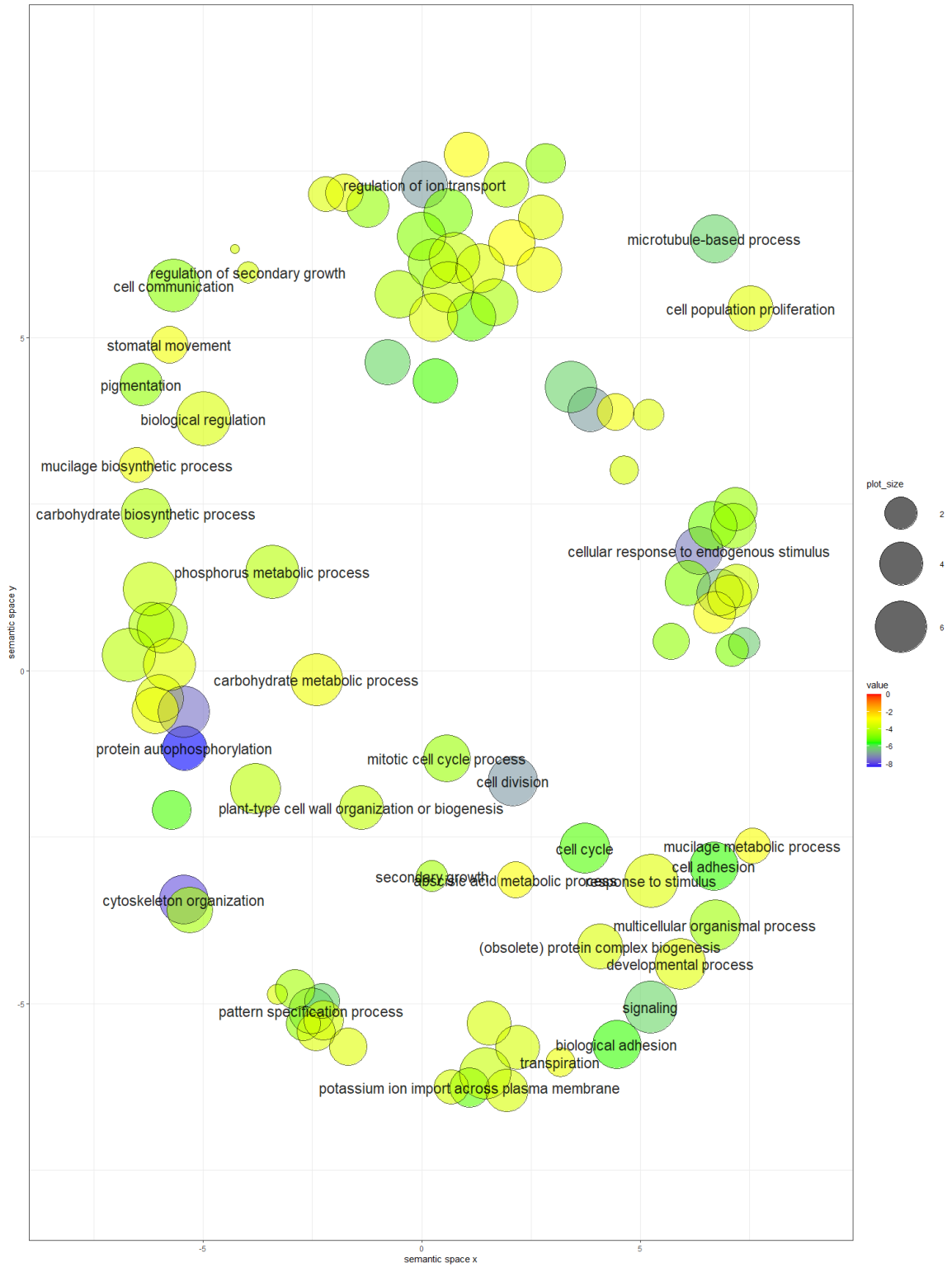

Figure 4.6. Scatterplot of GO terms associated with 807 DEs only found in Stress vs Stress+TSA at 3 h upregulated.

4.2.4. GO Terms Enriched in Genes Higher in Fresh Microspores Treated with TSA Compared to Fresh Microspores

At 0 h the GO terms for DEs list that were higher in Fresh+TSA when compared to Fresh microspores were enriched in 46 biological process categories. The top 15 ranked by p-value are seen in Figure 4.7. The GO terms enriched include: response to high light intensity (GO:0009644), protein folding (GO:0006457), response to light intensity (GO:0009642), response to hydrogen peroxide (GO:0042542), response to heat (GO:0009408), response to reactive oxygen species (GO:0000302), response to oxidative stress (GO:0006979), heat acclimation (GO:0010286), response to temperature stimulus (GO:0009266), response to endoplasmic reticulum stress (GO:0034976), response to light stimulus (GO:0009416), response to radiation (GO:0009314), response to inorganic substance (GO:0010035), response to abiotic stimulus (GO:0009628), and response to oxygen-containing compound (GO:1901700).

The list of enriched GO terms for DEs that were higher in Fresh+TSA was considerably smaller, with only 12 terms at 3 hours (Figure 4.7.). These terms were cellular response to starvation (GO:0009267), cellular response to phosphate starvation (GO:0016036), cellular response to nutrient levels (GO:0031669), response to starvation (GO:0042594), response to nutrient levels (GO:0031667), cellular response to extracellular stimulus (GO:0031668), cellular response to external stimulus (GO:0071496), response to extracellular stimulus (GO:0009991), protein oligomerization (GO:0051259), cell communication (GO:0007154), dephosphorylation (GO:0016311), and response to external stimulus (GO:0009605).

By 48 hours the DE list of genes that were higher in Fresh+TSA was much smaller and was not enriched in any biological processes GO terms. However, some molecular functions were enriched: glucan endo-1,3-beta-D-glucosidase activity (GO:0042973) beta-glucosidase activity (GO:0008422), glucosidase activity (GO:0015926), hydrolase activity, hydrolyzing O-glycosyl compounds (GO:0004553), and hydrolase activity, acting on glycosyl bonds (GO:0016798).

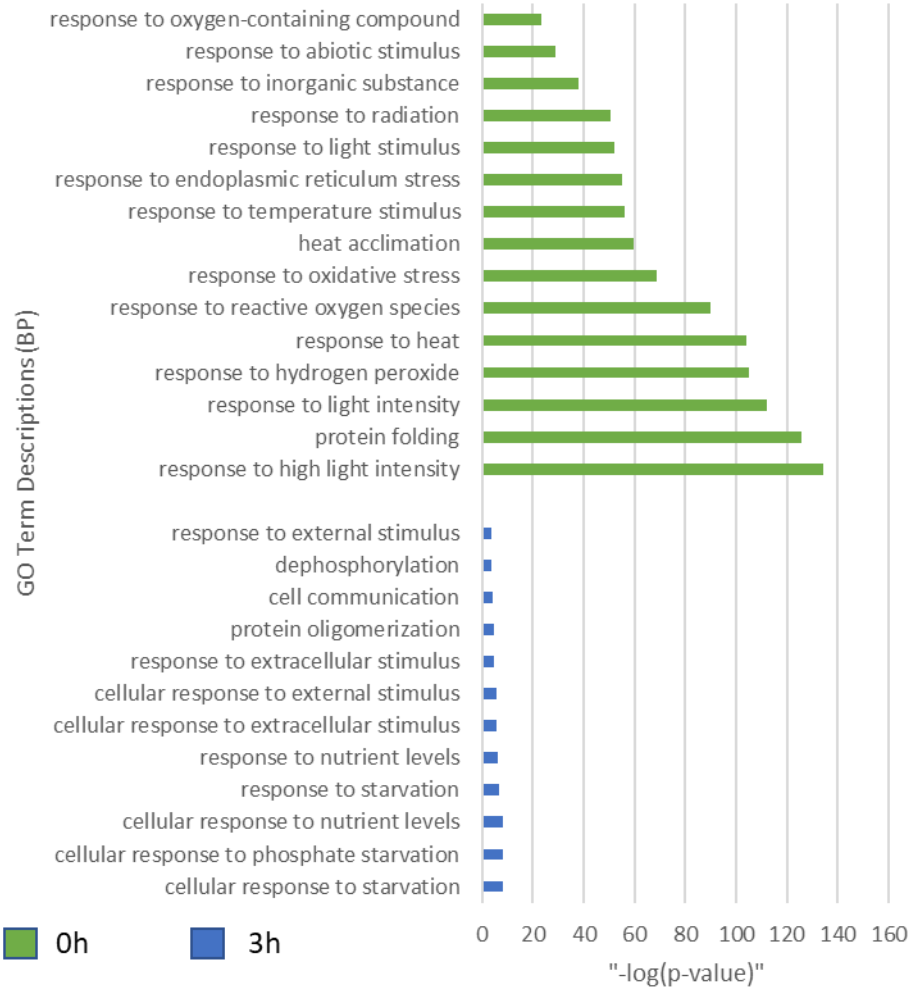

Figure 4.7 GO Terms Descriptions upregulated in fresh microspores treated with TSA compared to fresh microspores. Terms are ranked by p-value (-log[p-value]).

4.2.5. GO Terms Enriched in Genes Higher in Stress+TSA Compared to Fresh+TSA

In the comparison between Stress+TSA and Fresh+TSA at 0 h DEs that were higher in Stress+TSA were enriched in 507 Biological Process GO Terms (Figure 4.8.). The GO terms were similar to Fresh versus Stress without TSA treatment at 0 h (Chapter 3, 3.2.4.1.) and included cell tip growth (GO:0009932), pollen tube growth (GO:0009860), developmental cell growth (GO:0048588), cell development (GO:0048468), cell morphogenesis involved in differentiation (GO:0000904), pollen tube development (GO:0048868), intracellular signal transduction (GO:0035556), developmental growth involved in morphogenesis (GO:0060560), cell morphogenesis (GO:0000902), cell growth (GO:0016049), unidimensional cell growth (GO:0009826), plant-type cell wall modification (GO:0009827), multi-multicellular organism process (GO:0044706), pollination (GO:0009856), cell differentiation (GO:0030154).

At 3 h 400 Biological Process GO terms were enriched in the DEs higher in Stress+TSA when compared to Fresh+TSA (Figure 4.8.). Terms were again similar to Stress versus Fresh without TSA treatment at 3 h however not as similar as 0 h. Terms included, cell tip growth (GO:0009932), developmental cell growth (GO:0048588), response to oxygen-containing compound (GO:1901700), pollen tube growth (GO:0009860), cell development (GO:0048468), intracellular signal transduction (GO:0035556), response to osmotic stress (GO:0006970), cell growth (GO:0016049), developmental growth involved in morphogenesis (GO:0060560), cell morphogenesis involved in differentiation (GO:0000904), regulation of potassium ion transport (GO:0043266), regulation of metal ion transport (GO:0010959), response to alcohol (GO:0097305), and response to endogenous stimulus (GO:0009719).

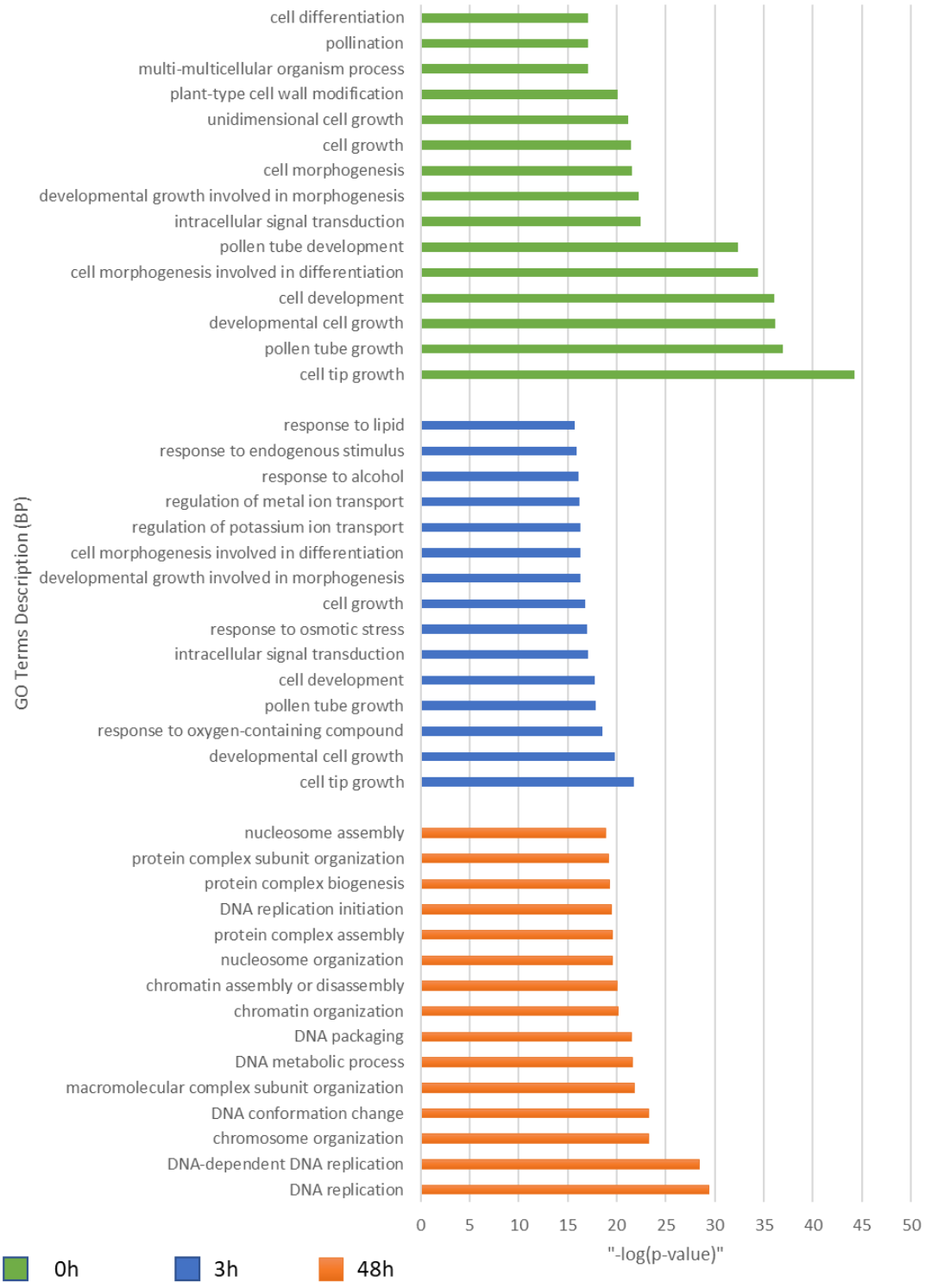

Figure 4.8. Enriched biological process GO Terms from genes that are expressed higher in Stress+TSA compared to Fresh+TSA, ranked by p-value (-log[p-value]).

At 48 h 395 Biological Process GO Terms were enriched in the genes higher in Stress+TSA when compared to Fresh+TSA (Figure 4.8.). Similar GO Terms were seen in Stress versus Fresh (Chapter 3, 3.2.4.1.). Many of the most substantial enrichment was seen in DNA related terms including DNA replication (GO:0006260), DNA-dependent DNA replication (GO:0006261), chromosome organization (GO:0051276), DNA conformation change (GO:0071103), macromolecular complex subunit organization (GO:0043933), DNA metabolic process (GO:0006259), DNA packaging (GO:0006323), chromatin organization (GO:0006325), chromatin assembly or disassembly (GO:0006333), nucleosome organization (GO:0034728), protein complex assembly (GO:0006461), and DNA replication initiation (GO:0006270).

4.2.6. GO Terms Enriched in Genes Higher in Stress Compared to Stress+TSA

Genes higher in Stress at 0 h were only enriched in 7 Biological Process GO terms (Figure 4.9.), which is unsurprising given how few genes were differentially expressed in that comparison. The GO Terms enriched in Stress were response to decreased oxygen levels (GO:0036293), response to hypoxia (GO:0001666), response to oxygen levels (GO:0070482), regulation of hydrogen peroxide metabolic process (GO:0010310), regulation of reactive oxygen species metabolic process (GO:2000377), systemic acquired resistance, salicylic acid mediated signaling pathway (GO:0009862), and systemic acquired resistance (GO:0009627).

At 3 h the genes that were expressed higher in Stress compared to Stress+TSA were interestingly enriched in 2 Biological Process GO terms, both related to RNA

(Figure 4.9.), mRNA processing (GO:0006397) and mRNA metabolic process (GO:0016071).

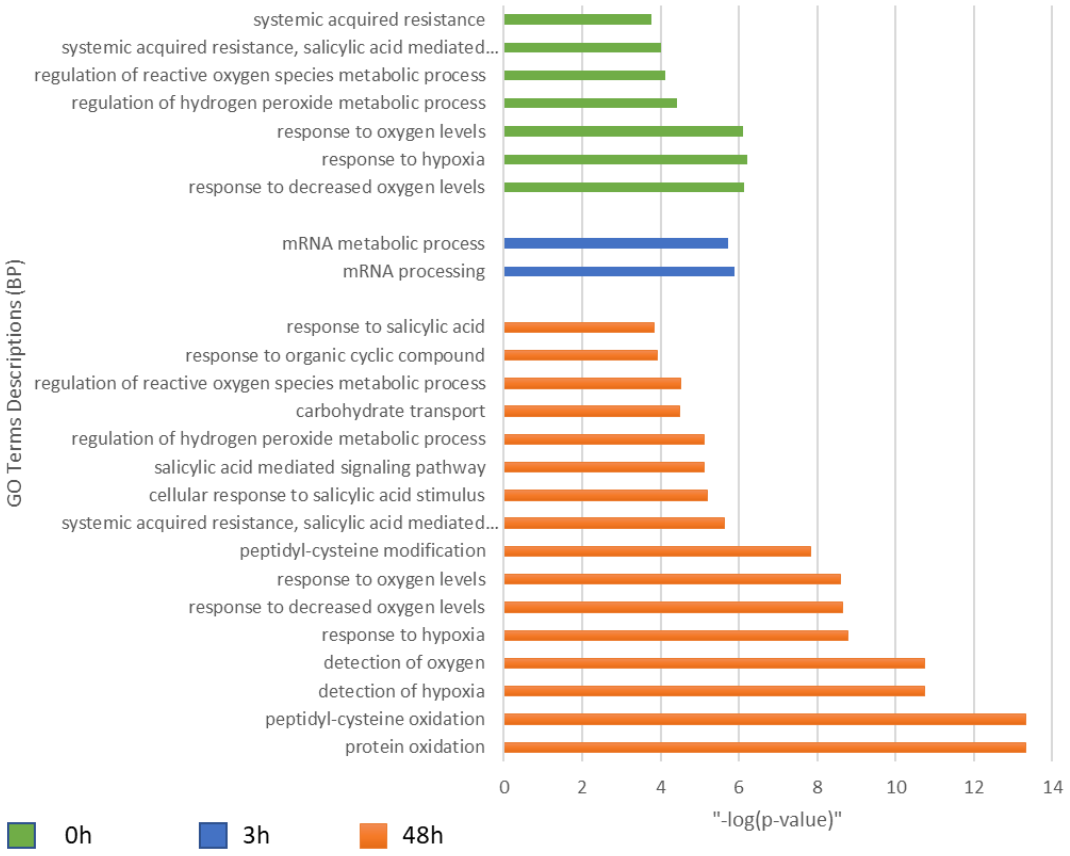

Figure 4.9. Enriched biological process GO Terms from genes that are expressed higher in Stress compared to Stress+TSA, ranked by p-value (-log[p-value]).

At 48 h the genes higher in Stress when compared to Stress+TSA were enriched in only 16 categories of Biological Process GO Terms (Figure 4.9.). These GO terms were: protein oxidation (GO:0018158), peptidyl-cysteine oxidation (GO:0018171), detection of hypoxia (GO:0070483), detection of oxygen (GO:0003032), response to hypoxia (GO:0001666), response to decreased oxygen levels (GO:0036293), response to oxygen levels (GO:0070482), peptidyl-cysteine modification (GO:0018198), systemic

acquired resistance, salicylic acid mediated signaling pathway (GO:0009862), cellular response to salicylic acid stimulus (GO:0071446), salicylic acid mediated signaling pathway (GO:0009863), regulation of hydrogen peroxide metabolic process (GO:0010310), carbohydrate transport (GO:0008643), regulation of reactive oxygen species metabolic process (GO:2000377), response to organic cyclic compound (GO:0014070), and response to salicylic acid (GO:0009751).

4.2.7. GO Terms Enriched in Genes Higher in Fresh Compared to Fresh+TSA

In the comparison between Fresh and Fresh+TSA, the DEs that were higher in Fresh at 0 h were enriched in 163 biological process GO terms. Some of the most enriched by p-value include: carbohydrate metabolic process (GO:0005975), starch metabolic process (GO:0005982), cation transmembrane transport (GO:0098655), inorganic cation transmembrane transport (GO:0098662), inorganic ion transmembrane transport (GO:0098660), hydrogen ion transmembrane transport (GO:1902600), microtubule-based movement (GO:0007018), monovalent inorganic cation transport (GO:0015672), starch biosynthetic process (GO:0019252), carbohydrate transport (GO:0008643), transmembrane transport (GO:0055085), proton transport (GO:0015992), and hydrogen transport (GO:0006818).

At the 3 h time point genes higher in Fresh were enriched in 45 biological process GO terms. Those 45 GO terms include many RNA-related terms: mRNA metabolic process (GO:0016071), cellular macromolecule metabolic process (GO:0044260), macromolecule metabolic process (GO:0043170), mRNA processing (GO:0006397), RNA metabolic process (GO:0016070), nucleic acid metabolic process (GO:0090304),

nucleobase-containing compound metabolic process (GO:0006139), cellular aromatic compound metabolic process (GO:0006725), gene expression (GO:0010467), primary metabolic process (GO:0044238), cellular metabolic process (GO:0044237), RNA processing (GO:0006396), RNA splicing (GO:0008380).

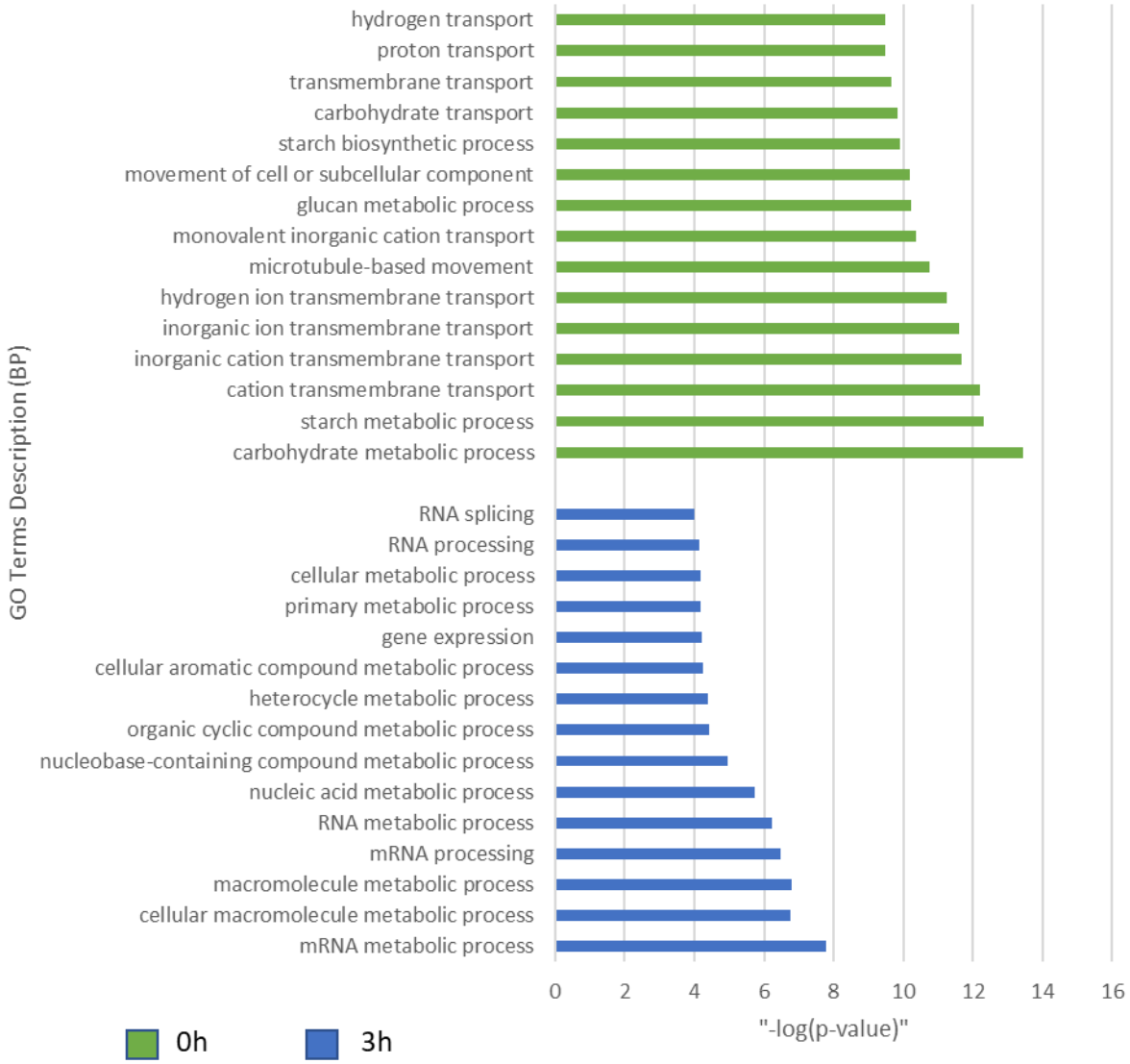

Figure 4.10. Enriched biological process GO Terms from genes that are expressed higher in Fresh versus Fresh+TSA, ranked by p-value (-log[p-value]).

At 48 hours, similar to higher in Fresh+TSA, DEs that were higher in Fresh were not enriched in any biological process GO terms. There were also no other categories of GO terms enriched in that comparison. It is interesting that at this point the transcriptomes are so similar.

4.2.8. GO Terms Enriched in Genes Higher in Fresh+TSA Compared to Stress+TSA

275 Biological Process GO Terms were enriched in the DEs that were expressed at higher levels in Fresh+TSA when compared to Stress+TSA (Figure 4.11.). Many of those terms were similar to those seen higher in Fresh compared to Stress (Chapter 3, 3.2.6.). Terms enriched include nucleosome assembly (GO:0006334), nucleosome organization (GO:0034728),chromatin assembly or disassembly (GO:0006333), chromatin assembly (GO:0031497), protein-DNA complex assembly (GO:0065004), DNA packaging (GO:0006323), DNA conformation change (GO:0071103), protein-DNA complex subunit organization (GO:0071824), chromatin organization (GO:0006325), protein folding (GO:0006457), chromosome organization (GO:0051276), heterochromatin organization (GO:0070828), RNA methylation (GO:0001510),IMP metabolic process (GO:0046040), and methylation (GO:0032259).

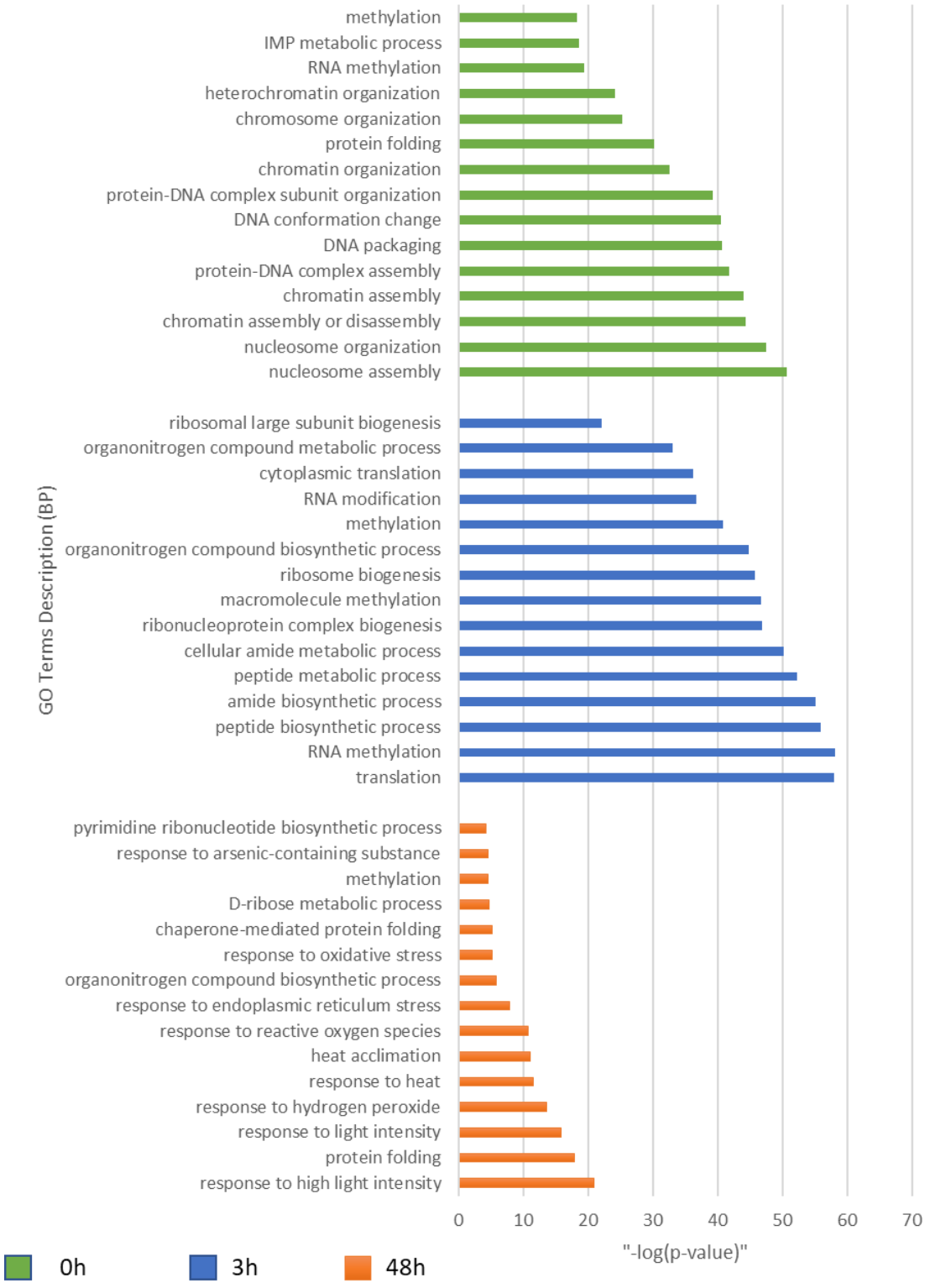

Figure 4.11. Enriched biological process GO Terms from genes that are expressed higher in Fresh+TSA compared to Stress+TSA, ranked by p-value (-log[p-value]).

At 3 h 221 Biological Process GO terms were enriched in DEs that were higher in Fresh+TSA in comparison to Stress+TSA (Figure 4.11.) Those GO terms included translation (GO:0006412), RNA methylation (GO:0001510), peptide biosynthetic process (GO:0043043), amide biosynthetic process (GO:0043604), peptide metabolic process (GO:0006518), cellular amide metabolic process (GO:0043603), ribonucleoprotein complex biogenesis (GO:0022613), macromolecule methylation (GO:0043414), ribosome biogenesis (GO:0042254), organonitrogen compound biosynthetic process (GO:1901566), methylation (GO:0032259), RNA modification (GO:0009451), cytoplasmic translation (GO:0002181), organonitrogen compound metabolic process (GO:1901564), and ribosomal large subunit biogenesis (GO:0042273).

At 48 h genes higher in Fresh+TSA were enriched in 31 Biological Process GO terms. Some of the GO terms similar to Fresh vs Stress (Chapter 3, 3.2.6.) include response to high light intensity (GO:0009644 & GO:0009642), response to hydrogen peroxide (GO:0042542), response to heat (GO:0009408), heat acclimation (GO:0010286), response to reactive oxygen species (GO:0000302) (Figure 4.11.). Other GO terms enriched were protein folding (GO:0006457), response to endoplasmic reticulum stress (GO:0034976), organonitrogen compound biosynthetic process (GO:1901566), and response to oxidative stress (GO:0006979).

4.2.9. KEGG Pathways

The comparisons between Fresh and Fresh+TSA showed very little impact on KEGG pathways. The only things of note were Cathepsin being higher in fresh compared

to Fresh TSA at 0 h and AUX1 being higher in fresh while other growth factors were higher in Fresh+TSA. At 3 h and 48 h there was no difference in KEGG pathways.

Fresh+TSA compared to Stress+TSA showed the same findings as Fresh versus Stress at 0 h (Chapter 3). However, at 48 h Stress+TSA had higher expression in many categories of the cell cycle (Figure 4.12.) in addition to higher expression of E2F. These together strongly indicate that Stress+TSA is progressing through the cell cycle at 48 h.

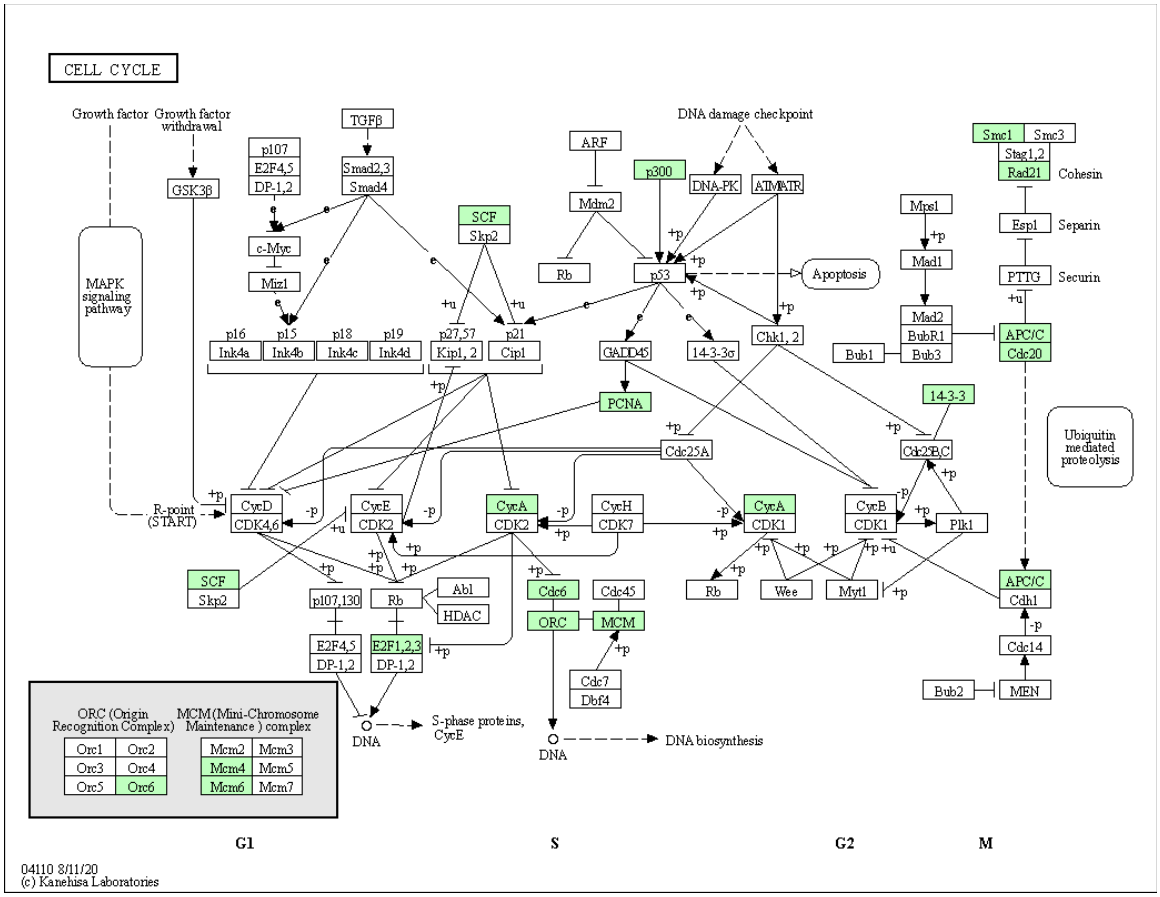

Figure 4.12. KEGG cell cycle pathway compared between Fresh+TSA and Stress+TSA at 48 h. Green is higher in Stress+TSA.

In the general upregulation at 3 h in Stress+TSA cell cycle genes were impacted and expressed at a higher level when compared to Stress (Figure 4.13.). This may indicate

progression or preparation for progression through the cell cycle occurring as early as 3 hours in Stress+TSA.

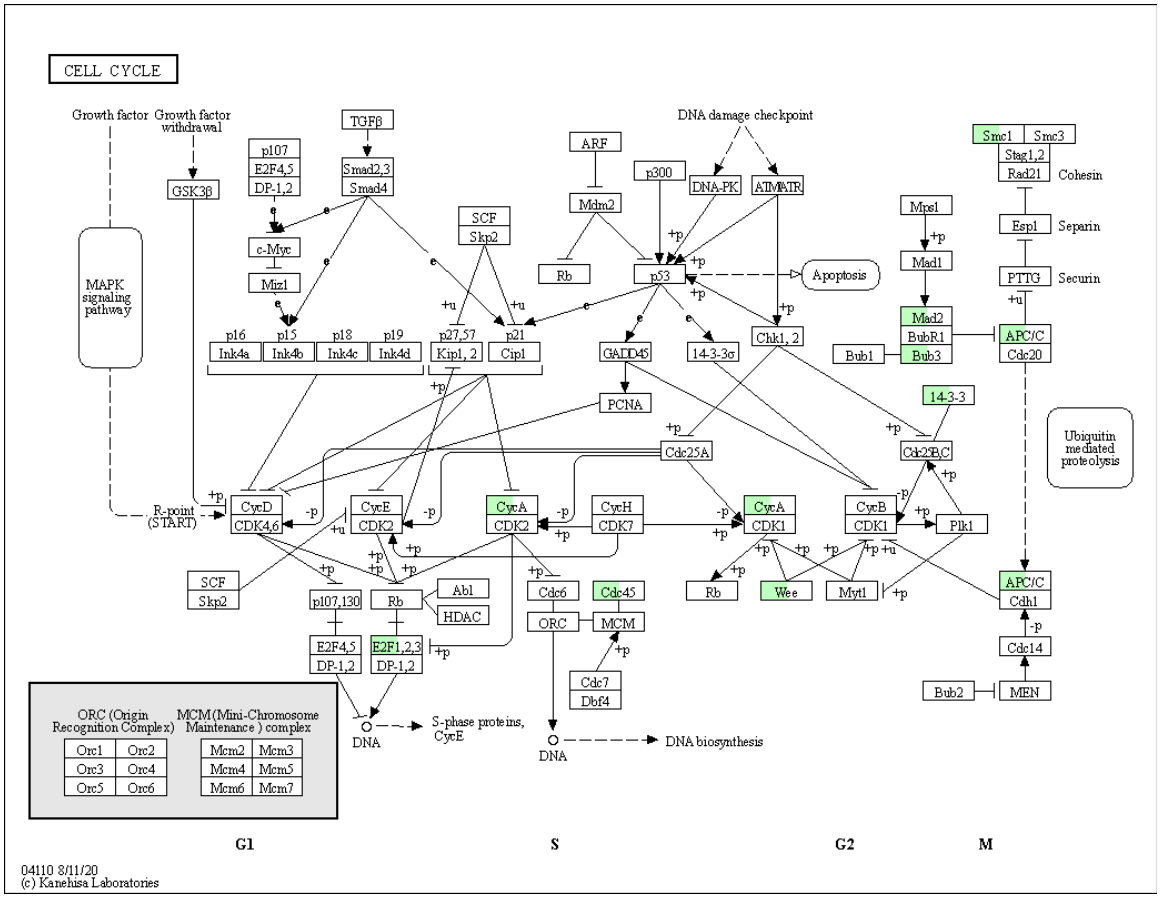

Figure 4.13. Cell Cycle KEGG Pathway genes at 3 hours, green boxes are expressed higher in Stress+TSA in comparison to Stress.

TSA application in cold-stressed microspores showed altered expression patterns within genes involved in the cell cycle (Figure 4.14 & 4.15.). In Cold-stressed microspores treated with TSA cyclin A was decreased over time in culture, which suggests a shift from S/G2 towards M phase in the cell cycle (Oakes et al., 2014). In comparison to the increase in cyclin A, this suggests that Stress+TSA is progressing

along the cell cycle towards cell division through mitosis more rapidly than cold-stressed without TSA treatment.

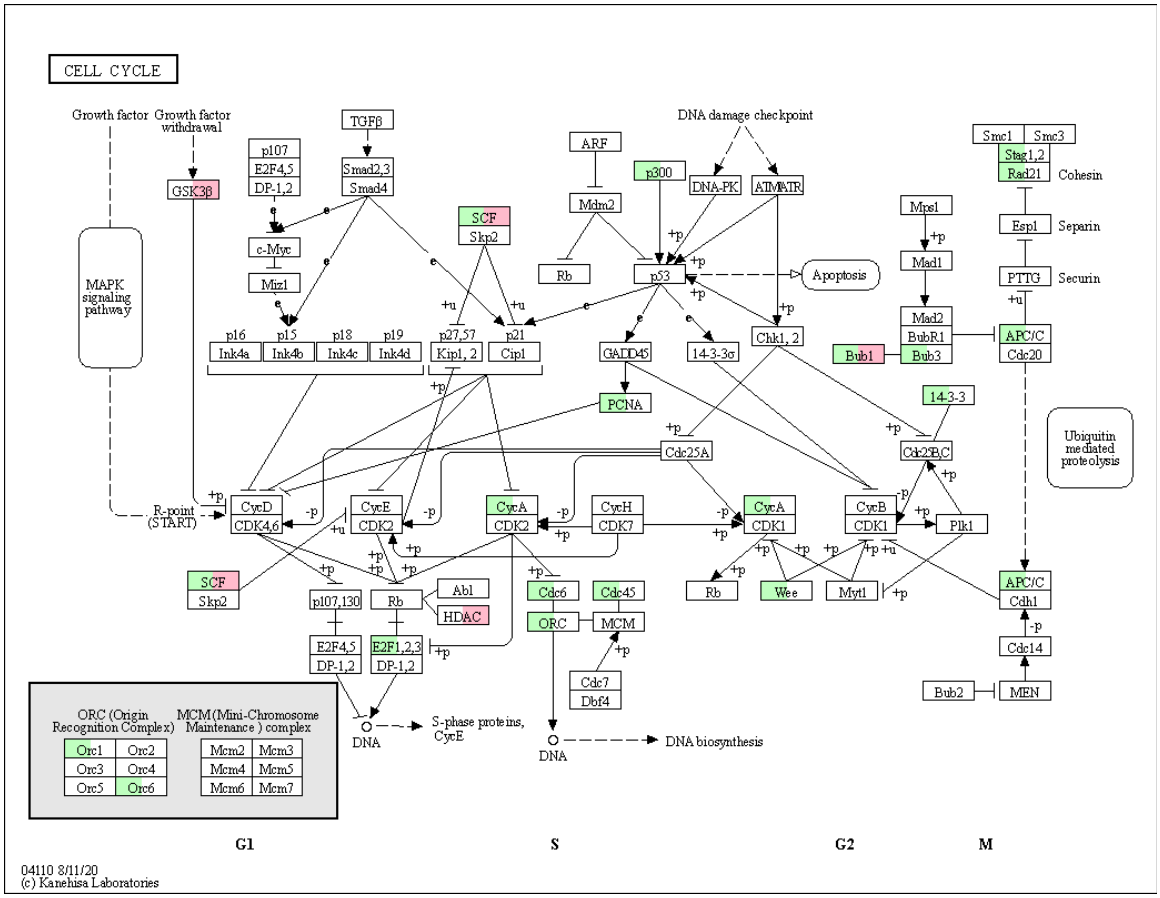

Figure 4.14. Cell Cycle KEGG pathway change in expression over time in comparison between 3 hours and 48 hours in Stress. Green cells increased in expression between 3 h and 48 h while red cells decreased in expression between 3 h and 48 h.

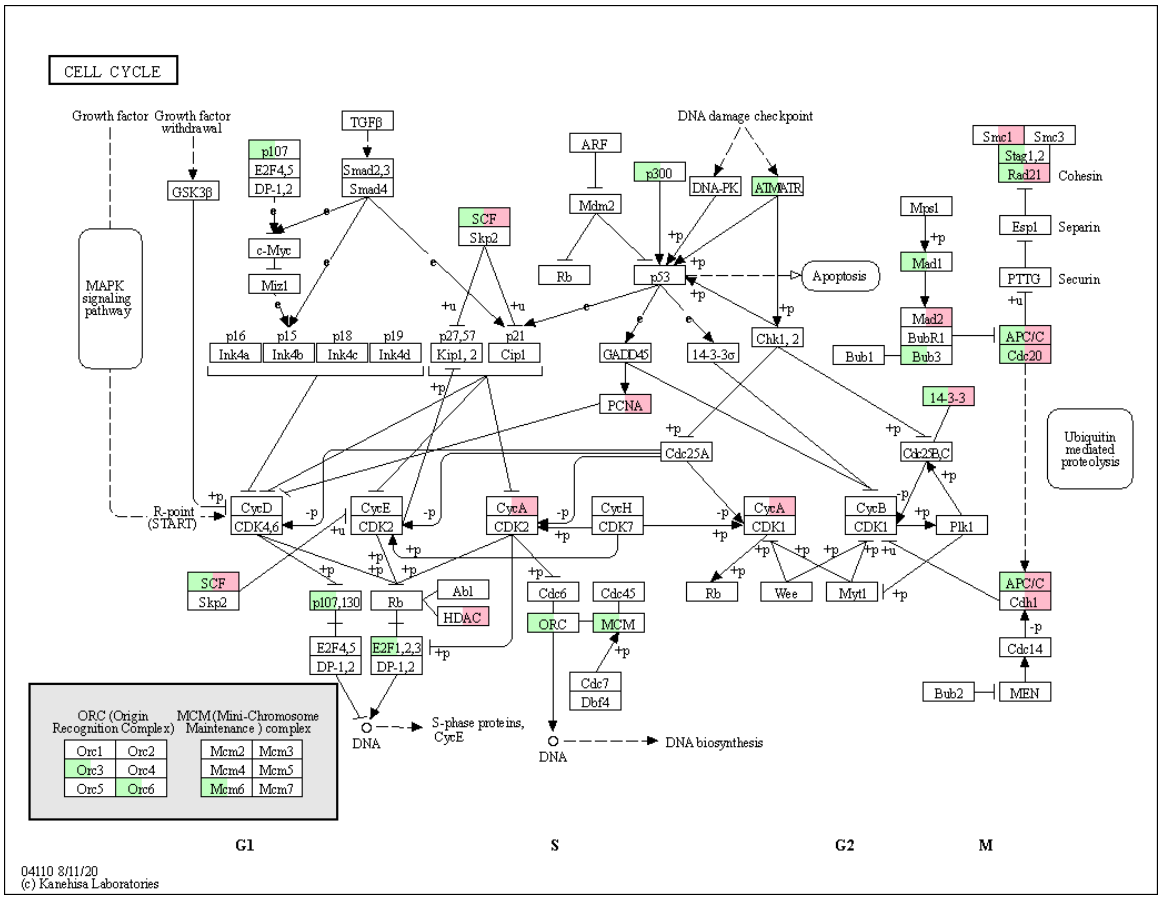

Figure 4.15. Cell Cycle KEGG pathway change in expression over time in comparison between 3 hours and 48 hours in Stress+TSA. Green cells increased in expression between 3 h and 48 h while red cells decreased in expression between 3 h and 48 h.

4.3. Discussion

4.3.1. TSA Application to Cold-Stressed Microspores Shows a Transient and Sizable Increase in Expression Compared to Cold-Stress

The application of TSA did not show an impact on stressed microspores at 0 h. Interestingly at 0 h Stress without TSA was enriched for hypoxia and ROS response. At 3 h though, the majority of DEs were higher in Stress+TSA and related to chromosome organization and DNA conformational change. These support a change in cell fate and were also seen in another study on wheat microspores (Seifert et al., 2016). mRNA was

enriched in stress compared to Stress+TSA which supports a continuing of the same cell fate in stressed microspores. By 48 h carbohydrate transport, metabolic and catabolic processes and floral organ number and specific organ number were higher in Stress+TSA. Cold stress on the other hand had enrichment in terms for responses to stresses.

Several genes relating to cell cycle were expressed at higher rates in Stress+TSA at 3 h when compared to Stress. Over time in culture – between 3 hours and 48 hours – Stress+TSA shows altered expression related to cell cycle compared to cold-stressed. The changes seen in Stress+TSA supported cell cycle progression. This supports findings that stressed microspores treated with TSA resulted in earlier exine rupture compared to stressed microspores in the absence of TSA treatment (Pandey et al., 2017).

The enrichment for floral organ and specific organ number at 3 h and 48 h higher in Stress+TSA was interesting. The same factors that influence floral organ identity also control the expression of many genes involved in the response to different phytohormones, such as auxin, cytokinin, gibberellins, and Jasmonic acid (Thomson, Zheng, & Wellmer, 2017). These hormones have also been shown to mediate control of meristematic activity and pattern formation (Thomson et al., 2017). This may mean that instead of floral development specifically it could more broadly be related to response to phytohormones, or meristem related. Terms related to meristems were enriched in cold stress treated with TSA. And specifically embryogenic meristem at 3 hours in Stress versus Stress+TSA.

Increased histone acetylation has also been implicated in reprogramming required for androgenesis induction (Ahmadi et al., 2018). The application of TSA leads to increased histone acetylation and appears to have sped up the induction of androgenesis.

4.3.2. In the Absence of TSA in Cold-Stressed Microspores Shows Enrichment for Responses to Stress and Salicylic Acid

Cold-stressed microspores treated with TSA showed signs in their enriched GO terms that they were able to quickly overcome oxidative stress and hypoxia. Stress+TSA microspores showed a more rapid reduction of stress response enrichment over time and a lower expression level of the genes associated with those terms when compared to cold-stressed microspores without TSA treatment. One such example is enrichment of salicylic acid responses and signalling pathways in Stress when compared to Stress+TSA; salicylic acid is involved in pathogen and stress response but also negatively regulates plant growth and development when expressed at high levels (Koo, Heo, & Choi, 2020; Wang et al., 2018). Salicylic acid led to increased levels of Abscisic acid and hydrogen peroxide (Wang et al., 2018). Salicylic acid also decreases mitochondrial function and respiration, and is implicated in interference with AUX related pathways (Rivas-San Vicente & Plasencia, 2011).

There is a tight and complex relationship between Salicylic acid, ROS, and MAPK cascades (Rivas-San Vicente & Plasencia, 2011). As discussed earlier (Chapter 3 3.3.3.), MAPK signalling is important for stress response. MAPK cascades are also important mediators of the interplay between Salicylic acid, other phytohormones, and ROS signalling in cell growth regulation. (Rivas-San Vicente & Plasencia, 2011). At 3

hours cold stressed microspores treated with TSA showed increased expression of several factors in the MAPK signalling pathway in comparison to cold stress alone. This may mean that there is indeed improved response to stress in cold stress microspores treated with TSA compared to cold stress.

This salicylic pathway along with MAPK signalling are interesting potential targets for improvement of androgenesis.

4.3.3. Cold-Stress versus Cold-Stress+TSA Showed Differential Expression of Genes Not in Other Comparisons

The 807 genes only seen differentially expressed in Stress vs Stress+TSA 3 h were related to many biological processes such as cytoskeletal organization, cell division, histone modification, and plant-type cell wall modification, hormone mediated signalling. Protein autophosphorylation, signalling cascades and endogenous stimulus response were also seen enriched. These are pathways that are important to androgenesis induction. The increased expression of these pathways due to TSA treatment would make sense to not only improve androgenesis induction but also speeding it up.

Cytoskeletal organization changes, cell wall alterations, and modification to histones are important steps in commitment to androgenesis or somatic embryogenesis (Borg & Berger, 2015; Corral-Martínez et al., 2019; De-la-Pena, Nic-Can, Galaz-Avalos, Avilez-Montalvo, & Loyola-Vargas, 2015; Maraschin, de Priester, Spaink, & Wang, 2005; Pandey et al., 2017; Parra-Vega et al., 2015; Rodriguez-Sanz et al., 2014). Hormones and hormone signalling is also important for androgenesis and somatic

embryogenesis (Iqbal et al., 2017; Pérez-Pérez, El-Tantawy, Solís, Risueño, & Testillano, 2019; Zur et al., 2015).

The increased expression of cell division specifically may lead to earlier mitosis and MCS production in culture. This supports findings from Pandey et al. (2017) that microspores treated with TSA ruptured the exine sooner and were more synchronous. MCM were also higher at Stress+TSA 3 h, supporting the thought that cell cycle progression was faster in cold-stress with TSA.

4.3.4. Fresh Microspores Respond to TSA with Less Intensity Than Cold-Stressed Microspores

The response to TSA at 0 h in fresh microspores was mostly enriched for stress responses in comparison to fresh. Interestingly, starch biosynthesis and transmembrane transport, carbohydrate metabolism and energy transport was down in Fresh+TSA compared to Fresh which was similar to what was found in soybean stressed microspores compared to fresh (Hale et al., 2020). At 3 h the DE list was smaller and mostly response to nutrient level and starvation and cell communication in Fresh+TSA while mostly RNA related but not really associated with any recognizable process in the absence of TSA. By 48 h the number of DEs was low, comparable to how similar Stress and Stress+TSA were at 0 h. The only enrichment was seen in glucosidase and hydrolase activity. The low number of DEs between Fresh and Fresh+TSA at 3 h and 48 h may suggest that the TSA affect in fresh microspores is very brief/transient. Since very few embryos develop from this Fresh+TSA, the cells that respond are rare and pooled cells would have a higher number of cells not responding to TSA.

The enrichment for β-Glucosidases which function in hydrolysis of glycosidic bonds and are linked to defense, cell wall lignification, cell wall b-glucan turnover, and phytohormone activation (Ketudat Cairns & Esen, 2010). Glucan endo-1,3-beta-glucosidase specifically has been linked to plant fungal defense and carbohydrate metabolism in barley (The UniProt Consortium, 2020).

4.3.5. Fresh+TSA versus Stress+TSA is Similar to Fresh versus Stress

The same pattern of enrichment in pollen related terms at 0 h and switch to enrichment in DNA organization and cell cycle seen in Stress+TSA compared to Fresh+TSA was the same pattern that was observed in Stress compared to Stress explored in Chapter 3. The same differences were also seen with increased response to chitin and higher amylopectin stressed microspores at 0 h. Cytoskeletal terms and cell wall modifications were also enriched and higher in stress at all time points.

By 48 h stress response was higher in Fresh+TSA while MCM, DNA replication and helicase activity were higher in Stress+TSA. This was similar to fresh versus stress (Chapter 3) however, Stress+TSA showed enrichment of terms involved in starting S phase DNA replication while stress without TSA was in DNA confirmational changes and packaging. This supports findings of TSA improving the time taken for exine rupture (Pandey et al., 2017) and suggest that increased histone acetylation is indeed improving reprogramming (Ahmadi et al., 2018).

CHAPTER 5 – GENERAL DISCUSSION

5.1. Introduction

In the present study the impact of prolonged cold stress and TSA application were explored for transcriptomic changes that can be linked to androgenesis early in wheat microspores culture. As reviewed earlier in Chapter 1 and Chapter 3, stress pre-treatment is necessary to induce androgenesis in wheat cultivar AC Andrew. Fresh microspores without any treatment were unable to produce MDEs (reviewed in Chapter 1, 1.4 – unpublished data). The application of TSA to AC Andrew microspores improved the androgenic response and increased the number of MDEs and green plants in cold-stressed microspores (Jiang et al., 2017)(reviewed in Chapter 1 and 3). TSA application also resulted in a small number of MDEs and green plants in fresh microspores (Chapter 1, 1.4 – unpublished data). Transcriptomic comparisons between these 4 treatments over the 3 times points early on in culture were used to explore: the induction of androgenesis, the impact of TSA on cold-pretreated microspores that improves their response to androgenesis, and how MDEs result from fresh microspores treated with TSA.

Overall our transcriptomic results are consistent with other studies on the induction of androgenesis showing a general pattern of decreasing expression in carbohydrate biosynthesis, metabolic processes and protein folding along with increasing expression of chitinase, embryogenic meristem, HSPs, cell wall modification, signalling, proteolysis, epigenetic changes, histone modification, and proliferation (Bélanger et al., 2018; Hale et al., 2020; Maraschin et al., 2006; Seifert et al., 2016).

Our findings were also consistent with the findings of transcriptomic studies on somatic embryogenesis. In a study comparing four developmental stages of somatic embryogenesis, Chen et al. (2020) found increasing expression of hormone signalling, a large number of genes related to alternative splicing, plant-pathogen interaction, signal transduction, and chitinases. These findings are consistent with our overall findings of induction of androgenesis in cold-stressed microspores. In the same study they found an increase in expression for metabolic processes (Chen et al., 2020), which was opposite to the findings of other androgenesis induction studies, including our findings. Higher expression of genes associated with hormone signalling such as AUX/IAA and ARF were again similar to our study but more pronounced in another study on direct somatic embryogenesis comparing zygotic embryos to early and developed somatic embryos (Kang et al., 2021). Kang et al. (2021) also found higher expression of many known TFs that have previously been associated with somatic embryogenesis; these were not found differentially expressed in our study. Overall, there is some overlap between somatic embryogenesis and androgenesis however their similarities and differences are still being explored.

Through our study and the comparison to other studies we have learned things in addition to the questions we set out to answer. Firstly, prolonged treatment with cold changes the microspores to better respond towards an androgenic change in cell fate, as well as allowing the microspores to respond more dramatically to TSA treatment (explored in 5.2.). And secondly, the difference in response to TSA is likely due to the initial state of the chromatin at the time of TSA application (explored in 5.3.). With these in mind there are limitations to this study and possible methods for improving androgenic

response based on what we currently know including possible genes of interest for improvement (explored in 5.4.).

5.2. Cold-Stress Preconditions Microspores to Respond to TSA

The impact of cold-stress led to similar large numbers of DEs at 0 h in both Fresh vs Stress and Fresh+TSA vs Stress+TSA. These changes were to microspores composition and occurred in response to conditions prior to the isolation of microspores. High ROS during cold stress treatment likely leads to cell wall modifications, cell growth, cell wall rigidity, ROS signalling, calcium signalling, and MAPK signalling (Rivas-Sendra, Calabuig-Serna, & Seguí-Simarro, 2017). The combination of these changes preconditioned microspores to respond to TSA treatment with the transient increase in genome expression at points from all over the genome. Overall cold stress results in changes in microspore cell morphology, chemistry and epigenetic states (as discussed in Chapter 3) that allow them to respond differently to TSA, compared to fresh microspores. It is because of this large change in microspores during cold stress treatment that the Fresh to Stress comparisons are so similar in DE number at time 0 h. That is, the cold stress cause large changes, but the TSA is only minor. Therefore, the changes to the transcriptome that result from cold exposure as well as TSA application must be important for androgenesis induction.

TSA treatment to cold stressed microspores showed improved androgenesis, likely due to increased expression of gene involved in cell division, DNA packaging changes, DNA replication initiation and MAPK signalling. The improved response to

stress and decrease in salicylic acid in cold-stressed microspores treated with TSA at 2 days was also likely important for improvement of androgenesis response.

In the absence of cold-stress pre-treatment, TSA application led to a less drastic response with fewer DEs was seen in comparison to the number of DEs resulting from cold-stressed microspores. However, some of the changes must be enough for the small numbers of MDEs and green plants that we observed in our preliminary data. The response to stresses seen at 0 h and 3 h in Fresh+TSA along with cell communication at 3 h, and glucosidase activity at 48 h may explain the change in cell fate seen in fresh microspores treated with TSA and not in fresh microspores alone (as discussed in Chapter 4). The low response of fresh microspores also suggests that the shock from anthers grown at normal growth conditions to CIMC wash is much smaller than the shock from cold treated tillers to CIMC wash media. The low response of fresh microspores to TSA indicates that cold stress treatment prior to TSA application was important for a larger, more dramatic response.

5.3. Chromatin Organization is Important for Cellular Response to TSA

Based on our data we concluded that stress makes microspores more receptive to TSA's impact. TSA inhibits HDACs and its use has been implicated in somatic embryogenesis and androgenesis induction (Castillo et al., 2020; Jiang et al., 2017; Wang et al., 2019; Wójcikowska et al., 2018). Regions of the genome that are expressed during the prolonged period of cold, dark, and nutrient starvation may have acetylated histones, which are associated with open chromosome structure (reviewed in Chapter 1 and 4) or open chromatin capable of being acetylated and these regions are impacted by TSA at the

start of culture. As previously discussed (Chapter 1 and 4), HDACs removal of acetyl groups from histones results in repressive chromosome structure (Verdin & Ott, 2015). Many HDACs act in response to specific environmental and cellular factors, HDA9 for example responds to stress conditions, negatively regulating the cellular response through repressive chromosome structure in targeted regions (Zheng et al., 2020). Inhibition of HDAC activity leads to an accumulation of acetylated histones in the genome and results in increased expression in those regions (Marks, Richon, & Rifkind, 2000).

The mode of action for TSA is preventing the de-acetylation of histones, meaning that the existing chromosome structure provides the initial canvas for alteration. The chromosomal regions with open structure remain open – allowing transcription – when they would otherwise be closed – preventing transcription. Therefore, chromatin organization/state is important for a response to TSA as TSA does not switch on new genes; it simply interferes with the silencing of them.

The initial chromosomal state also explains the difference in response seen in fresh microspores treated with TSA. The initial chromosomal organization in fresh microspores would reflect the pollen development of the microspores as they are progressing through microgametogenesis (Process is outlined in Chapter 1, 1.2, & Figure 1.1.). While the prolonged cold treatment would result in changes to chromatin structure to facilitate a response to the stresses and change in environment (as discussed in 5.2.). Based on this knowledge the resulting small number of MDEs in fresh microspores treated with TSA is both surprising and unsurprising. It is unsurprising that the majority of cells in culture would not experience a change in cell fate as a result of TSA application, being that the transcripts that would likely be associated with open

chromosomal structure would be related to pollen development. What was surprising was the lack of drastic and general upregulation seen; TSA should have resulted in a number of DEs in the comparison between Fresh and Fresh+TSA even if related to pollen development. However as outlined in Chapter 4 (4.2.7.), there was only a small number of DEs seen in the comparison and a pattern of increased upregulation was not seen in the time points sampled. With this data the resulting MDEs in Fresh+TSA are surprising however, as discussed in Chapter 4 and 5.2. the small number of responding cells may mean that transcriptomic changes are hidden by the vast majority of cells that are not responding to TSA.

5.4. Future Improvements to Microspores

One of the shortcomings in our approach lies in the fact that the transcriptome is not necessarily an accurate representation of what is occurring within the proteome of the cell. A study on the transcriptome and proteome in heat stressed tomato plants found very little correlation between them (Ding et al., 2020). For example, heat stress within tomato pollen showed a more drastic response within the proteome when compared to the transcriptome (Keller, Consortium, & Simm, 2018). The differences between the transcriptome and proteome is due to the level of translation of the mRNA transcripts, representing another layer of control within the cell in addition to transcription (Merchante, Stepanova, & Alonso, 2017). The level of translation can be influenced by many pathways influencing mRNA transcript structure and stability, ribosomal binding and re-initiation or shunting, small-RNA and associated silencing complexes, RNA binding proteins, or other currently unknown mechanisms (reviewed by Merchante et al. (2017)). Combined transcriptomic and proteomic analysis on microspores would be

beneficial to aid in the understanding of androgenesis induction in terms of proteins and functional mRNA active within the cell and potentially contributing to the switch in cell fate.

In addition to the messenger RNA transcripts studied here there are non-coding RNAs within cells that carry out specific functions in addition to the silencing functions listed in the previous paragraph. Small non-coding RNA such as small interfering RNAs (siRNAs) and microRNAs (miRNAs) may play an important role in the reprogramming of microspores (Bélanger et al., 2020; Seifert et al., 2016). The siRNAs have suggested involvement in silencing of stress response, chromatin modification, and DNA methylation (Seifert et al., 2016), while miRNAs were associated with regulation of transcription factors important to embryogenesis induction (Bélanger et al., 2020) and showed expression patterns suggesting involvement in androgenesis induction (Seifert et al., 2016). The addition of small and non-coding RNA analysis would improve our understanding of androgenesis as well as provide valuable information on the impact TSA has on small and non-coding RNA levels within cells.

Through our transcriptomic analysis we have identified large numbers of genes in several different pathways, however more work is needed to determine which of these are important for inducing androgenesis. This could be accomplished through functional studies altering expression of transcripts and genes of interest within pathways of interest for androgenic induction and green plant regeneration. With this in mind, the alteration of gene expression leaves a transgenic free path for improvement to androgenesis. Targeted editing to alter and improve the androgenic response of cultivars is possible with Clustered Regularly Interspersed Palindromic Repeats (CRISPR) combined with

nucleases to induce cuts within the DNA and provide a template for repair with an altered sequence (Bhowmik et al., 2018; Ferrie, Bhowmik, Rajagopalan, & Kagale, 2020; Nishitani et al., 2016; Svitashev, Schwartz, Lenderts, Young, & Mark Cigan, 2016; Tak et al., 2017; Wang et al., 2015; Yang, Wu, Tang, Liu, & Dai, 2017). If CRISPR is combined with a de-activated nuclease and a strong promoter protein, it can be used to increase expression of targeted genes instead of editing their sequence (Gao et al., 2016; Lowder et al., 2018). Using CRISPR up-regulation provides a useful means to study the impact of gene expression on androgenesis and possibly improve the resulting number of MDEs and green plants, especially in recalcitrant lines. Additionally, the repression of some genes may improve androgenesis or aid in our understanding of its induction. For altering expression in a repressive manner RNA interference (RNAi) is an excellent tool to silence and often degrade transcripts to prevent translation into proteins (Fang & Qi, 2016; Joga, Zotti, Smagghe, & Christiaens, 2016; Setten, Rossi, & Han, 2019; You et al., 2020).

It is our hope that a few key players can be identified, but it could also turn out to be that many hundreds or thousands might be necessary for a proper developmental shift to embryogenesis. A list of potential genes identified in this study as potential targets for gene expression alteration experiments is seen in Table 5.1. BARD1 in particular shows an interesting pattern of expression in the comparisons from this study and given that and its function within cells for DNA repair and WUS expression localization it makes a compelling target to explore how its altered expression might impact androgenesis. Using CRISPR in conjugation with a highly potent TF to increase expression within IMC would hopefully lead to improved androgenesis response and possibly improved response time

in microspores. In addition to the potential targets this study identified, there is also the possibility of using CRISPR and RNAi technologies in combination with IMC when future experiments identify novel and/or key regulators of androgenesis. Corral-Martínez et al. (2020) for example identified basal and apical embryo cell fate transcripts that are associated with embryogenic callus development into suspensor-bearing embryos in culture. These genes might also benefit culture through promoting early exine rupture when upregulated.

Table 5.1. Possible targets for gene expression alteration for improvement of androgenesis

Gene	Wheat ortholog(s)
BRG2	TraesCS4D02G018100
YDA	TraesCS6B02G279300 TraesCS6A02G245000 TraesCS6D02G217100 TraesCS6D02G227300 TraesCS2D02G404700
MRE11	TraesCS7B02G259500 TraesCS7A02G369000
GBSSI	TraesCS4A02G418200 TraesCS7A02G070100
BARD1	TraesCS2A02G384900 TraesCS2B02G401900
HYOU1	TraesCS6D02G257000

In this study we had hoped to find genes and pathways that may impact albino regeneration as many believe the issues with albinism occur early on (Ankele et al., 2005;

Caredda et al., 2004; Caredda et al., 2000; Gajecka et al., 2020; Kumari, Clarke, Small, & Siddique, 2009; Makowska & Oleszczuk, 2014; Ritala et al., 2001). Although we identified several GO terms related to organelles, we did not find any genes or pathways definitively linked to albinism; however, they might have been present and not differentially expressed or they are impacted at time points outside of our sampling, prior to culture or in response to regeneration conditions at the MDE germination stage.

5.5. Conclusion

We were able to address several questions with this study. One of the goals of this study was to improve our understanding of the initial switch towards androgenesis within wheat microspores. We were able to support our idea that the switch occurs early on in IMC, within the first 2 days. This switch was marked by a decrease in stress response and an increase in signalling, DNA packaging and organization, and cell cycle progression. We were also able to detect TSA's impact on the transcriptome of cold-stressed microspores and identify possible improvement to androgenic response with increased gene expression in areas of cell division, DNA organization, signalling, hormones, and cell wall modifications. We were unable to find evidence of the same pathways increasing in expression after TSA treatment in fresh microspores, however we found gene expression of stress response and β-Glucosidases may explain the small number of MDEs seen in this treatment.

Our results add to the knowledge base towards understanding androgenesis induction in the hopes of improvement in the future for faster, more efficient creation of DH plants through IMC for downstream applications.

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