Characterizing gene expression responses to colchicine in *Dactylis smithii*

by

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Abstract

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Tetraploid plant species are preferred in certain agricultural cases over their diploid counterparts for larger biomass and increased resistance to abiotic stresses. Naturally occurring tetraploids are thought to result through a breakdown in chromosome segregation during mitosis. In the lab, they can be produced synthetically using colchicine, a microtubule inhibitor. To better understand the mechanism of action of colchicine in chromosome doubling, Zhou et al. (in preparation) treated the roots of *Dactylis smithii* seedlings with colchicine, sampled the roots and leaves, and characterized changes in gene expression by RNA sequencing. The objectives of this study were to use quantitative PCR to verify changes in gene expression of 11 target genes that were identified in this RNA sequencing experiment, and to characterize the target gene expression responses to 24 hour colchicine treatment in two accessions of *D. smithii*. The expression of 5 genes in the UK accession was verified with 5 hour transcriptome data from Zhou et al. (in preparation). Three genes were expressed consistently between two accessions (distinct populations) of the *D. smithii* species. These genes, which function in mRNA splicing, membrane function, and as aquaporins, are candidates for additional research regarding their potential role in the process of chromosome doubling via colchicine. Additional research could use qPCR to assess gene expression in the *D. smithii* leaf samples to compare the effect of cell type and location. Future directions could also include assessing the changes in gene expression of *D. mariana*, a natural tetraploid subspecies of orchardgrass, compared to the synthetic tetraploid specimen produced by Zhou et al. (in preparation).

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Table of Contents

List of Tables

List of Figures

1. Introduction

1.1 Polyploidy in Plants

The ploidy of an organism describes its total number of complete unique chromosome sets. For example, the cells of animals and most plants are diploid, containing two copies of each chromosome. At fertilization, one complete set of chromosomes was inherited from the egg, and the second set from the sperm. Polyploidization, in contrast, describes the occurrence of more than two complete sets of chromosomes. Tetraploids, organisms with four chromosome sets, are the most common form of polyploidy (Comai, 2005). Polyploidization plays a significant role in the evolutionary history of angiosperms (Glover et al., 2016; Borrill et al., 2015), where an estimated 30 to 70 percent of angiosperms are polyploid (del Pozo & Ramirez-Parra, 2015; Sattler et al., 2016).

Polyploid plants can arise in one of two ways: via allopolyploidy, or more commonly, autopolyploidy (del Pozo & Ramirez-Parra, 2015). Allopolyploids are created through the hybridization of genomes from two different species, while autopolyploids result from the duplication of a single plant's genome (Tamayo-Ordóñez et al., 2016; Comai, 2005). Genome doubling in autopolypoloids can form due to somatic doubling (somatic cells replicate their genome in preparation for cell division, but fail to divide the replicated chromosomes into two cells during mitosis) or by the fusion of unreduced gametes (reproductive cells fail to reduce their chromosome number by half during meiosis; Sattler et al., 2016; Otto, 2007). These unreduced gametes can then fuse together or fertilize a reduced gamete to form a cell with more than the two sets of chromosomes that is typical of diploid cells (Sattler, 2016). The production of unreduced gametes is the

most common cause of polyploidy and its high frequency is a heritable genetic characteristic of many plant species (Sattler et al., 2016). The incidence of polyploidy is also affected by a range of environmental factors like temperature, herbivory, wounding, water deficiency, and nutrient shortage (Sattler et al., 2016).

Many polyploid plant species are important crop sources, such as peanuts (*Arachis hypogaea*), oats (*Avena sativa*), coffee (*Coffea arabica*), cotton (*Gossypium hirsutum*), tobacco (*Nicotiana tabacum*), and bread wheat (*Triticum aestivum*; Glover et al., 2016). Polyploidy provides advantages to some food crops over their diploid counterparts. For example, some varieties of polyploid plants may have seedless fruit, greater resistance to pests and pathogens, improved adaptation capacity in terms of biotic and abiotic stresses, as well as larger fruit, flowers or leaves (Tamayo-Ordóñez et al., 2016; del Pozo & Ramirez-Parra, 2015). These traits can be advantageous for the volume and quality of food and livestock crops, as well as the range of environments in which they can be grown (Wang et al., 2015).

Polyploidization has been artificially induced in many crop plants to yield desirable physiological and morphological characteristics (Tamayo-Ordóñez et al., 2016). Aside from morphological advantages noted above, polyploidy can be used by plant breeders to restore fertility to otherwise sterile hybrids that are then able to properly pair their chromosomes during meiosis with an additional copy of their genome (Sattler et al., 2016). Breeders can also use polyploidy to transfer desirable traits between two species that would not normally be able to crossbreed because of differing ploidy levels. In this case, a polyploid hybrid is used as an intermediate in crosses with both species to transfer the desired traits from one to the other (Sattler et al., 2016). Improved tolerance to abiotic

stresses related to water, temperature, and salinity conditions is also an asset to tetraploid crop species (del Pozo & Ramirez-Parra, 2015). In contrast to these benefits, artificially inducing polyploidy for these purposes may result in infertility, erratic fruit bearing, brittle wood, watery fruit, smaller plant height, or fewer fruits per plant (Tamayo-Ordóñez et al., 2016). Thus, there is a tradeoff between the beneficial and disadvantageous traits associated with polyploidy, and the outcome of polyploidization for breeding purposes can vary.

Some of the consequences of polyploidization that could be responsible for these changes in physiological and morphological characteristics are: larger cell sizes (termed the 'gigas' effect); increased heterozygosity; greater opportunity for mutations; compensation for deleterious mutations; ability to acquire genes from other plants through hybridization; greater plasticity for adaptations; and higher metabolite diversity (Sattler et al., 2016; Bretagnolle & Thomas, 2001; Borrill et al., 2015; Otto, 2007). Polyploidy has also been shown to alter gene expression by silencing, up-, or down-regulating the expression of duplicated genes; a phenomenon that appears to be organ specific in some cases, and may be a reliable or more random occurrence depending on the gene (Adams & Wendel, 2005; Otto, 2007). Studying the outcomes of polyploidization is important for maximizing the beneficial traits in polyploid crops while decreasing their negative effects (Tamayo-Ordóñez et al., 2016).

1.2 Artificial Polyploidy via Colchicine

To synthetically produce tetraploid crop species, as well as facilitate the study of polyploid gene expression in a lab setting, colchicine has been used to artificially produce tetraploids from diploid plants (Tamayo-Ordóñez et al., 2016; Dhooghe et al., 2011).

Colchicine is an alkaloid compound that is isolated from the seeds and bulbs of the autumn crocus (*Colchicum autumnale*; Dhooghe et al., 2011). It is one of the most commonly used antimitotic agents, which prevents microtubule polymerization during cell division such that chromosomes are not able to separate and cell division is prevented (Leung et al., 2015; Cahill, 2016). This doubles the genetic contents of the cell, which can then divide normally after the colchicine treatment has ceased. All of the daughter cells of the original polyploid cell will contain double the normal number of chromosomes (Cahill, 2016).

Colchicine has also had a history of therapeutic uses. It was first used in to relieve joint and arthritis pain, and then more popularly as a treatment for acute gout (Malkinson, 1982). It has since been used to treat a number of other health conditions, such as familial Mediterranean fever, Behcet's disease, pericarditis, coronary artery disease, and inflammatory conditions (Leung et al., 2015). However, colchicine is extremely toxic at high doses, which limits its use in therapeutic treatments of humans and animals (Leung et al., 2015).

Colchicine solution can be applied to the roots or an entire plant either by growing small seedlings in a greenhouse medium that contains colchicine, by exposing microspores to colchicine in their isolation media, by directly injecting buds with colchicine, or by wiping a cotton swap soaked in colchicine along apex or axillary buds of the plant (Mohammadi et al., 2011). Successful induction of tetraploidy depends upon the concentration and duration of exposure to colchicine. As one would expect from a toxic substance, high doses (>1000 mg/L) and longer treatment times (36-48 hours) correspond with reduced plant survival and regeneration (Mohammadi et al., 2011). A

study testing the optimal colchicine parameters with wheat (*Triticum aestivum L.*) found the best results using a concentration of 1 mM colchicine for 48 hours (Hansen & Anderson, 1998). Temperature is also a factor, as one study using oilseed rape (*Brassica napus L.*) observed better results at a temperature of 8°C than 24°C (Mohammadi et al., 2011). It is hypothesized that an even longer treatment time may yield greater chromosome doubling, but at the cost of negatively affecting embryo formation and regeneration. Therefore, it is suggested to increase the reaction temperature for quicker cell division during longer treatment times (Hansen & Anderson, 1998).

To date, the majority of information regarding colchicine's mechanism of action has focused on its inhibition of the mitotic spindle. It does so through binding tubulin molecules into a complex that halts the elongation of microtubule polymers (Leung et al., 2015). At particularly high concentrations, colchicine can even degrade the microtubule structure in addition to preventing its growth (Leung et al., 2015). Furthermore, through its strong affinity for tubulin molecules, its actions also inhibit cytokinesis and chromosome distribution (Malkinson, 1982).

Aside from disruption of the mitotic spindle during cell division, colchicine has also been noted to inhibit adenosine uptake, affect fluid transport in the cell membrane, and bind to cell membranes in a way that prevents cell secretion (Malkinson, 1982). In an attempt to learn more about colchicine's mechanism of action, outside of its effect on the mitotic spindle, Zhou et al. (in preparation) used RNA sequencing transcriptome data to identify a number of genes and gene families that appeared to alter their expression in response to colchicine treatment in the *Dactylis smithii* grass species.

1.3 Studying Gene Expression

Examining gene expression can identify the genes that are actively being used by cells in a particular situation. The total of all mRNA that is being expressed from those active genes is referred to as the cell's 'transcriptome' (Lockhart & Winzeler, 2000). Changes in normal cell functioning as well as influences from the external environment evoke physiological changes that affect gene expression, therefore changing the nature of the transcriptome (Lockhart & Winzeler, 2000). The dynamic nature of transcriptomes allows us to better understand the role that gene expression plays in the regulatory mechanisms, cellular functions, and biochemical pathways that help create morphological and phenotypic variation within organisms (Lockhart & Winzeler, 2000).

Studying transcriptomes for changes in the expression levels of various genes can also make use of quantification analysis. One of the most accurate methods to measure changes in mRNA content is with real-time polymerase chain reaction (RT-PCR), also known as quantitative PCR (qPCR). This technique is used to quantify the amount of template DNA (and by extension, the amount of RNA isolated from the cells) present at the beginning of the PCR reaction, by measuring the rate of DNA production across repeated amplification cycles of PCR. Through the use of fluorescent dyes that bind to nucleic acids, the level of fluorescence detected is directly proportional to the total amount of DNA present (Mirmajlessi et al., 2015). In this way, fluorescence is measured after each PCR cycle to provide the exact concentration of DNA produced at each stage of the amplification process (Mirmajlessi et al., 2015). This information is recorded using a data software program with built-in analysis options, which facilitates comparisons of the expression of particular genes between samples. Samples are compared using

threshold cycle (Ct) values, which is the cycle number at which there is enough DNA to detect fluorescence past a set threshold value. Higher Ct values indicate a smaller amount of template DNA, and therefore initial RNA from the source tissues, because more PCR cycles were required to elevate fluorescence past the threshold. Lower Ct values imply the opposite, meaning the gene was more highly expressed by cells of the initial sample.

Compared to traditional PCR, qPCR does not require any post-PCR processing (such as gel electrophoresis) and it can amplify relatively short DNA fragments in the range of 70-100 bp in length, which provides greater reaction efficiency and sensitivity (Mirmajlessi et al., 2015). Disadvantages consist of a high equipment cost, greater sensitivity to contamination due to errors during RNA/DNA preparation, and a more complicated data analysis process (Biosistemika, 2016). Pros and cons aside, qPCR has quickly become a standard lab protocol that provides a relatively simple and more precise method of measuring the RNA content of cells and tissues.

1.4 Research Goals and Test Species

Dactylis smithii was used in this study to further investigate additional genes affected by the process of colchicine chromosome doubling. *Dactylis smithii* is a subspecies of the *Dactylis glomerata* grass species, which is more commonly known as Orchardgrass (Cole, 2015). It is part of the Poaceae family of grasses, and is widely distributed in temperate regions (Hamstra, 2016; Tuna et al., 2004). One of its primary uses is for hay production in forage crops to feed livestock, predominantly in North America, Europe, and eastern and southeastern Asia (Huang et al., 2015). It is also a good plant for ground cover in nesting sites, stabilizing the ground against erosion, and providing a source of nitrogen to the soil (Grant, 2016).

Dactylis is a useful test species because polyploidy is very common within this genus. The *Dactylis glomerata* species alone is divided into several subspecies, including a hexaploid, three tetraploid, and 15 diploid taxa (Bretagnolle & Thomas, 1996). *Dactylis smithii* is a diploid subspecies but has a natural tetraploid counterpart. This tetraploid form occurs through the process of autopolyploidy via the fusion of unreduced diploid gametes (Bretagnolle & Thomas, 1996).

The first objective of this study is to characterize the overall response of target genes to colchicine over the course of 24 hours in two accessions of *D. smithii*. The target genes being investigated were chosen based on the previous research of Zhou et al. (in preparation) that identified changes in gene expression in response to colchicine treatment of *D. smithii*. Based on comparing transcriptome data with curated databases, these target genes have been associated with alpha-tubulin, oxidation-reduction, kinetochore structure and attachment, transcription regulation, mRNA slicing, membrane formation, cell motility, water transport via aquaporins, and photosystem II assembly. The second objective is to use quantitative PCR procedures to verify the relative expression of the target genes previously detected by RNA sequencing and transcriptome data analysis of *D. smithii* treated with colchicine (Zhou et al., in preparation).

More information about the genes that are differentially expressed in synthetic tetraploids could provide additional insight into the mechanism of colchicine chromosome doubling. Research regarding the genetic characteristics of this mechanism could also be applied to the field of agronomics by using colchicine to more effectively induce tetraploid plants capable of enhancing the biomass and range of environments in which certain crop species are available to livestock.

2. Materials and Methods

2.1 Plant Materials & Experimental Treatments

To investigate the effects of chromosome doubling via colchicine treatment on gene expression in *Dactylis smithii,* a grass species, we investigated the change in gene expression of the root samples from colchicine-treated and untreated (control) plants. The same plant materials from two randomly chosen accessions (PI 237607 from Spain and PI 441032 from the United Kingdom) of the grass species, *Dactylis glomerata* subsp. *smithii* Link, used by Zhou et al. (in preparation) were also used for this research project. The *D. smithii* seeds were germinated and grown in the greenhouse at Saint Mary's University, Halifax, NS, Canada. For complete rearing details, see Zhou et al. (in preparation). After one month, the seedlings were separated into treatment and control groups; the entire root system of the control group was put into water while that of the treatment group was placed in a solution of 2.5 mM colchicine (0.1%, w/v) mixed with 2% dimethyl sulfoxide. Root tissues from subsets of roughly a dozen plants each were sampled from the control and treatment groups at 0, 5, and 24 hours, respectively, and then snap frozen and stored in liquid nitrogen prior to subsequent experiments.

RNA was extracted from the root tissues sampled by Zhou et al. (in preparation). After RNA extraction, the quality of the RNA samples from each tissue was assessed on agarose gels, and by measuring its concentration and purity using NanoDrop spectrophotometry. Following this, quantitative PCR (qPCR; also called real-time PCR or RT-PCR) was used to measure changes in gene expression levels of the RNA samples.

In order to perform qPCR, reverse transcription cDNA synthesis was first used to build DNA strands complementary to the extracted RNA. From these, the exact sequence

of each particular gene was selected by primers during DNA amplification. A total of 25 primer sets were designed by Kai Zhou as part of his research on the plant response to colchicine treatment for chromosome doubling using the *D. glomerata* subs. *smithii* grass species (Zhou et al., in preparation), and were used to select the relevant housekeeping and target genes pertinent to this study. Regular polymerase chain reaction (PCR) and gel electrophoresis procedures were used to assess and predict the ability of the primers to adequately amplify their gene product from the cDNA template during qPCR. Based on their performance, the starting selection of 25 primers sets was reduced to 2 housekeeping and 11 target genes with the most consistent performance. Details of all primer sets and their corresponding genes are listed in Table 2. After cDNA synthesis, qPCR was then performed on all root samples to measure gene expression and primer efficiency.

2.2 RNA Extraction

First, RNA extraction was performed at room temperature on all root samples of the Spain and UK *Dactylis smithii* accessions in accordance with Zhou et al. (in preparation) using the TRI Reagent (Sigma-Aldrich, USA) protocol, with some modifications (Chomczynski and Sacchi, 1987). RNase enzymes, capable of degrading RNA sequences, are ubiquitous; therefore, all tools, pipette tips, and reaction tubes were RNase-free, gloves were worn at all times, and the workspace was cleaned prior to extraction using 0.1% DEPC water or 10% ethanol solution.

Homogenization

Liquid nitrogen and an autoclaved metal stir rod were used to powder 0.1 g of each sample of root tissue within a 15 mL centrifuge tube. The stir rod was sterilized

between samples to prevent contamination. 1 mL of Trizol Reagent was added to each sample and the sample was vortexed until well mixed. Samples were then left to sit at room temperature for 5 min.

Phase Separation

0.2 mL of chloroform (per 1 mL Trizol Reagent) was added to each centrifuge tube, which was then shaken vigorously for 15 seconds and left to sit at room temperature for 2-3 min. The samples were then centrifuged at 12 000 x g and 4°C for 15 min. 400 µL of the resulting clear layer of supernatant, containing the RNA, was pipetted at a 45-degree angle into new 1.5 mL Eppendorf reaction tubes. While the volume of supernatant varied depending on the RNA content of the source tissue, all samples had the same amount of supernatant removed.

Precipitation

An equal volume of 100% isopropyl alcohol was combined with the supernatant in the new centrifuge tubes. These tubes were then placed into the freezer at 4°C for at least 30 min. Following this, the tubes were centrifuged at 12 000 x g and 4°C for 10 min.

Wash

After centrifugation, the supernatant was discarded and 1 mL of 75% ethanol (per 1 mL of Trizol Reagent) was added to the tubes. The tubes were shaken or vortexed to thoroughly wash the RNA pellet before being placed in the centrifuge at 4°C and 7500 x g for 5 min.

The above steps were repeated to wash the pellet a second time. Upon completing the second wash, the supernatant was removed using a pipette instead of pouring it out so

as to remove as much of the liquid as possible. The tubes were left to air dry at room temperature until the pellets were opaque and no obvious liquid remained (~10-15 min).

Resuspension

The RNA pellets in each tube were resuspended in 50 µL of RNase-free water by passing it up and down through the pipette tip multiple times. The tubes were placed in a heat block set to approximately 55-60°C for 10 min, and then were stored at -70°C.

2.3 RNA Qualitative Assessment

Gel electrophoresis was used to confirm that no DNA or protein contamination remained. The RNA products were loaded into a 1% agarose gel using 5.0 µL of loading dye alongside a DNA ladder. The gel was run in 1x TBE buffer at 180 volts for 20 min using a Fisher Biotech electrophoresis system. To view the bands of RNA, the gel was stained in a solution of 0.1% ethidium bromide (EtBr) for 15 min and photographed under the UV light of a SynGene bioimaging system using GeneSnap software.

2.4 RNA Quantitative Assessment

A NanoDrop 2000 Spectrophotometer (Thermo Scientific, USA) was used to quantify the RNA concentration to determine the volume required for cDNA synthesis. These measurements were taken using RNase-free water as a blank and 2 µL of each RNA sample.

2.5 Reverse Transcription cDNA Synthesis

DNase I Treatment

To eradicate any traces of contamination due to genomic DNA or residual proteins, the RNA samples were first treated using an RNase-free DNase kit by Quiagen (USA). The amount of RNA needed for 20 µL of a 0.1 µg/µL solution was determined

from NanoDrop quantifications. The appropriate volume of each RNA sample was pipetted into 0.2 mL Eppendorf tubes, along with 0.2 µL DNase I and 2 µL DNase Buffer and water to 20 µL. The tubes were briefly centrifuged to mix the liquids and then incubated in a Bio-Rad T100 Thermal Cycler for 10 min at 37°C followed by a standing temperature of 4°C. Lastly, 0.2 µL of EDTA was added to all tubes (to protect the RNA at high temperatures) before vortexing and returning them to the thermal cycler for 10 min at 75°C to inactivate the DNase I enzyme.

To provide a more robust purification of the RNA samples, the standard DNA elimination reaction that comes with the QuantiTect RT kit was performed in addition to the separate DNase I treatment already performed.

New 0.2 mL Eppendorf reaction tubes were prepared with 10 µL of RNA (0.1 µg/µL), 2 µL of gDNA Wipeout Buffer, and 2 µL of RNase-free water for a total volume of 14 µL. The tubes were briefly centrifuged to mix the reagents and incubated in the same thermal cycler for 8 min at 42°C. Samples were then placed on ice before performing reverse transcription.

cDNA Synthesis

Following the DNase I treatments, the purified RNA samples were used to create complementary cDNA strands using a QuantiTect Reverse Transcription (RT) Kit (Qiagen, USA). To tubes used in the above reaction, 1 µL of RT Primer Mix, 4 µL of Quantiscript RT Buffer, and 1 µL of Quanitscript RT enzyme were added for a total reaction volume of 20 µL. The tubes were then incubated in the thermal cycler for 30 min at 42°C and another 3 min at 95°C. The completed cDNA products were stored in a freezer at -20°C until use in later PCR reactions.

2.6 Primer Optimization

Regular PCR was employed prior to qPCR procedures to optimize primers to amplify a single DNA gene product. Gel electrophoresis was also used, as previously described, to view PCR products and ascertain which primers were successfully amplifying a single gene.

A 96-well PCR plate was set up so that each well contained 10 μL of Taq (2x) Mastermix (including Taq enzyme, dNTPs, and Buffer solution), 4 μL of cDNA template, 4 μL of primers, and 2 μL of ddH_2O. Primers were diluted to a 5 μM concentration, and template cDNA was diluted by 10% with nuclease-free water.

The PCR reaction, involving the denaturation, annealing, and extension phases of DNA replication, took place in a Bio-Rad T100 Thermal Cycler over the course of the following cycles: 3 min at 95°C; 35 cycles of 30 s at 95°C, 30 s at 60°C, and 30 s at 72°C; followed by a final 5 min at 72°C extension; and an infinite standing temperature of 12°C to end the run.

2.7 qPCR Reaction

qPCR was performed for all root cDNA samples in triplicates (both control and treated samples at 0 h, 5 h, and 24 h) using a total of 13 primers sets that showed good PCR results.

The qPCR procedures to measure gene expression levels were carried out in 96-well PCR plates in accordance with Zhou et al. (in preparation). Each reaction-well contained 7.5 μL of 2x SYBR Green ROX qPCR Mastermix (QIAGEN, USA), 1 μL of cDNA template (10% dilution), 1.5 μL of each primer (forward and reverse), and 3.5 μL of nuclease-free water for a final mixture volume of 15 μL. An ABI PRISM 7000

Sequence Detection System (Applied Biosystems) was used to detect the results after 10 min at 95°C; 40 cycles of 15 s at 95°C and 1 min at 60°C; 15 s at 95°C; 20 s at 60°C; and 15 s at 95°C.

The efficiency of all 25 primer sets was also measured using five dilutions of the UK 0h template cDNA (0.1, 0.01, 0.001, 0.0001, and 0.00001) as well as a no-template-control using ddH2O in place of cDNA. qPCR was performed as described above.

3. Results

3.1 RNA Extraction and Primer Verification

The quantitative and qualitative assessment of the isolated RNA samples is summarized in Table 1. NanoDrop technology measured absorbance for the samples at 260 and 280 nm. The 260/280 absorbance ratio is an indicator of the purity of RNA, where values below 1.8 are likely due to sample contamination with DNA, proteins, salts, ethanol, or phenol. Concentration of the RNA samples was also measured for use in the cDNA synthesis protocol.

From the results of regular PCR and gel electrophoresis used to test the selectivity of 25 primer sets on both the 0 h UK and Spain cDNA samples, 13 primer sets were chosen based on good results in both gels for use in qPCR trials. The other primers were excluded due to the presence of multiple DNA bands, in which case it was unclear which DNA product was the intended gene to be selected, or lack of a clear PCR product. The 13 primers chosen were D1, D8, D9, D10, D11, D15, D16, D17, D18, D19, D22, D24, and D25; they correspond respectively to genes that code for actin, positive regulation of cell proliferation, alpha-tubulin, kinetochore attachment, oxidation-reduction, transcription regulation, photosystem II assembly, mRNA splicing, membrane regulation,

cell motility, unknown function, kinetochore protein, and aquaporin NIP. Table 2 shows a complete list of the primer sets and their associated products/functions.

Table 1. NanoDrop concentrations and absorbance ratios for UK and Spain *D. smithii* root samples.

Root Samples	Concentration (ng/μL)	260/280 (nm)	260/230 (nm)
UK - 0 h	158.8	1.92	1.69
UK - WT5h	132.6	1.82	2.17
UK - CH 5h	118.4	1.83	2.13
UK - WT 24h	120.2	1.80	1.95
UK - CH 24 h	108.2	1.86	1.98
Spain - 0h	120.8	1.85	1.99
Spain - WT 5h	73.3	1.74	1.80
Spain - CH 5h	119.0	1.81	1.73
Spain - WT 24h	163.4	1.80	1.94
Spain - CH 24h	190.1	1.87	1.85

Note: WT = Water, control treatment. CH = Colchicine, experimental treatment.

Table 2. Primer sets used to isolate housekeeping and target genes of interest.

Gene	Oligo Name	Sequence (5' to 3')	Abbreviation
Actin	C83951F	CAAGGTCCAAACGAAGGATAG	*D1
	C83951R	CGGTCGAACAACTGGTATTG	
Actin	C63128F	GGATCAGCAAGCAGGAGTATG	D2
	C63128R	AGAAAGGGTGTAACGCAACTAA	
CYP2	C69121F	GTCGTAGAGGACGAAGTTGATG	D3
	C69121R	CATCCGCAAACATGGCAATC	
ABC	C167974F	AGAGGCTAGAGAAGGTGTATGA	D4
	C167974R	TTCTGTGTGGGTCGATTCTG	
EF2	C156461F	GCTTTCCCACAATGTGTGTTT	D5
	C156461R	CTCCTTCAATCCCTTCCTCTTAC	
EF1	C157432F	TCATGCCCTACGTTGGTATAATC	D6
	C157432R	CTTCCTGTAGTGTCTTCCACATC	
GAPDH	C26026F	GGTGTGAACCATGAGAAGTATGA	D7
	C26026R	GAGTCCTTCCACGATACCAAAG	
Positive regulation of cell proliferation	C100325F	CAGGAACAGGAAGGGATGATG	*D8
	C100325R	CAGATCTTGGCGTGAGGATAAA	
Alpha-tubulin	C101064F	CAGAGTCAAACCAGGAGTCAG	*D9
	C101064R	CCAAGCAGAGGTAGCGTAAA	
Kinetochore attachment to spindle microtubule	C102023F	CCGCAATGACGAGAGTTACA	*D10
	C102023R	CACTGTCACACCACCTTCTATC	
Oxidation-reduction	C96405F	TCTCCTTCCCGCTGTATT	*D11
	C96405R	ACTGAGCCTCTCTTTCAGTAG	
Unknown function	C74797F	CATGAACGACGCTGGTATGA	D12
	C74797R	CTGCGTTGCCCATAACATAAC	
Unknown function	C76031F	TTATCAGGGACCGATGTGTATTT	D13
	C76031R	CGAAGGACGAGGAAACATCA	
Unknown function	C76599F	AGGATATGTGACTGGGTATCAG	D14
	C76599R	GGTCGTCGTCATATTTGGATTAC	
Transcription regulation	C101317F	TGCAGTTGGAACAATGATGTATG	*D15
	C101317R	CCGCAGGAGAGGAAGAAATAG	
Photosystem II assembly	C93529F	TCTGGATGGACGGAGAGAAA	*D16
	C93529R	GATTCATCATCACCGGCTACA	
mRNA splicing	C49608F	CGTACAAGCAACACACTGGATA	*D17
	C49608R	TTGTTCTGTGCCTGCCTATG	
Membrane	C71496F	CTCCAGTGCTCCTTGTTCTT	*D18
	C71496R	GCCTCCTGTAACGGGTATTC	
Cell motility	C89024F	GTTACTGATGCCACCCTTATCT	*D19
	C89024R	CAGCGATGCCGTCCTATAAA	
Unknown function	C166741F	GTCCTGCTATGAGGTTCCATAAA	D20
	C166741R	GCACCAGACGGTAAAGTGTAT	
Unknown function	C86996F	GGGATCCATGGAGGAATTCTG	D21

	C86996R	TTCTACCCTCTTCCGTTCCT	
Unknown function	C27495F	CAGTACCTGACGTTGGCTATAC	*D22
	C27495R	GTGCTTGCGATTGCTTCTAAC	
Unknown function	C76820F	CTTGTCTCCTCCAACTTCTAACC	D23
	C76820R	TTACAGGCAACGCACACA	
Kinetochore protein	C101667F	ACTCCACTACAAAGCAGGTAAA	*D24
	C101667R	CACGAAGTCCCATCCAAGAATA	
Aquaporin NIP	C88478F	GCGATGTGAATGTGCGTAATAA	*D25
	C88478R	GATACATCCAGCTCCAGCATAG	

*Indicates primer sets that performed most consistently and were therefore used for data analysis.

3.2 qPCR Results

Primer efficiency was calculated for 11 target genes and 2 housekeeping genes according to Pfaffl (2001) using the following equation: $E = 10^{(-1/slope)}$, where E is the primer efficiency, and slope is calculated from standard curves plotting 5 sample concentrations against their corresponding Ct values. The results of the primer efficiency calculations are shown in Table 3. Of the two housekeeping genes used in data collection (D1 and D8), D1 was selected for analysis of the qPCR expression data because its efficiency fell within the preferred range of 1.8-2.2.

Table 3. Primer efficiencies and standard curve slope values for 11 primer sets.

Primer	Slope	Efficiency (E)*
D1	-3.5203	1.9234
D8	-2.3904	2.6202
D9	-2.9344	2.1917
D10	-2.7642	2.3002
D11	-3.2557	2.0284
D15	-3.0265	2.1400
D16	-3.681	1.8692
D17	-3.5135	1.9258
D18	-3.4528	1.9481
D19	-2.7482	2.3114
D22	-3.339	1.9929
D24	-2.0394	3.0927
D25	-7.274	1.3724

*$E = 10^{(-1/slope)}$

For each of the UK and Spain DNA root samples tested using the 13 primer sets, consisting of 3 replicates for each, qPCR trials resulted in the detection of cycle threshold (Ct) values. These Ct values, representing the cycle number at which DNA amplification produces a fluorescence signal sufficient to pass an arbitrary threshold concentration, were averaged with standard deviations (see Table 4). Certain replicates were removed before calculating averages due to irregular dissociation curves that did not display clear peaks that would indicate a single DNA product had been amplified.

Table 4. Average Ct values and standard deviations for the UK and Spain *D. smithii* samples at 0, 5, and 24 h after colchicine treatment. (WT = Water, control treatment. CH = Colchicine, experimental treatment.)

UK Samples										
Ct values	Avg	SD	Avg	SD	Avg	SD	Avg	SD	Avg	SD
Primer	0h	0h	WT 5h	WT 5h	CH 5h	CH 5h	WT 24h	WT 24h	CH 24h	CH 24h
D1	23.58	0.27	21.00	7.97	18.23	4.24	32.33	0.31	27.14	0.09
D8	27.87	0.15	13.87	1.94	20.85	6.03	33.74	0.00	32.60	0.16
D9	25.84	0.22	14.38	0.83	17.51	4.36	30.95	0.00	31.07	0.32
D10	26.57	0.11	24.56	8.49	21.06	4.21	37.39	0.67	29.85	0.32
D11	23.85	0.21	17.39	0.41	17.42	0.52	38.19	0.71	29.93	0.10
D15	24.65	5.06	16.84	0.30	18.74	2.93	29.34	0.21	26.74	0.15
D16	30.16	0.12	16.68	0.48	17.67	1.48	33.62	0.01	32.49	0.23
D17	25.64	0.08	23.30	5.12	18.91	4.68	24.03	0.14	26.42	0.12
D18	23.24	0.15	18.84	5.80	17.07	0.46	32.20	0.00	29.05	0.22
D19	24.23	0.09	16.06	0.48	24.18	3.44	30.79	0.00	26.90	0.18
D22	21.88	0.16	17.74	5.00	26.72	4.63	36.49	0.38	36.09	1.59
D24	29.76	0.15	17.61	6.44	20.38	0.00	33.92	0.00	33.97	1.44
D25	31.00	0.84	26.66	9.64	19.42	8.28	30.57	0.54	33.14	0.15
Spain Samples										
Ct values	Avg	SD	Avg	SD	Avg	SD	Avg	SD	Avg	SD
Primer	0h	0h	WT 5h	WT 5h	CH 5h	CH 5h	WT 24h	WT 24h	CH 24h	CH 24h
D1	32.36	0.75	25.83	0.19	25.29	0.11	26.51	0.04	25.24	0.13
D8	32.49	0.70	30.85	0.26	30.28	0.26	31.99	0.33	33.06	0.30
D9	32.23	0.35	30.09	0.27	27.78	0.33	30.84	0.32	31.00	0.12
D10	32.89	0.80	31.38	0.05	29.77	0.46	31.73	1.26	30.28	1.16
D11	36.67	0.00	31.78	0.20	28.02	0.15	33.41	0.52	34.16	0.00
D15	30.96	0.45	25.82	0.17	25.24	0.06	28.07	0.05	26.40	0.10
D16	33.27	0.48	31.68	0.16	29.59	0.33	31.87	0.66	29.22	0.35
D17	28.35	0.21	26.35	0.09	25.65	0.22	25.43	0.17	24.88	0.00
D18	30.71	0.66	27.15	0.31	26.69	0.26	27.84	0.30	28.07	0.13
D19	30.19	0.22	29.00	0.28	27.87	0.12	26.65	0.13	31.24	0.12
D22	33.58	0.35	25.50	0.07	25.57	0.17	33.66	0.56	26.94	0.11
D24	32.82	0.43	29.57	0.32	29.58	0.27	33.34	0.50	31.76	0.44
D25	31.25	0.42	30.78	0.29	29.34	0.21	30.79	0.56	32.01	0.44

Relative expression of each target gene (9 primer sets) at 0, 5, and 24 h was then calculated according to Pfaffl (2001) using the following equation:

$$ratio = \frac{(E_{target})^{\Delta CP_{target}(control-sample)}}{(E_{ref})^{\Delta CP_{ref}(control-sample)}}$$

E_{target} is the efficiency of the target gene, and ΔCP_{target} is the difference between the Ct values of the control (WT) and colchicine (CH) treated samples amplified with the target gene. E_{ref} and ΔCP_{ref} were calculated in the same way using the efficiency and Ct values from the D1 reference gene. Ratios of expression for the 9 target genes relative to the expression of the reference gene were calculated and expressed as fold change (summarized in Table 5 and plotted in Figures 1 & 2).

Table 5. Relative expression ratios and fold change of target genes at 0, 5, and 24 h after colchicine treatment.

UK	**0h**		**5h**		**24h**	
Primer	Expr. Ratio	Fold change	Expr. Ratio	Fold change	Expr. Ratio	Fold change
D9	1	1	0.0139	0.0853	0.0304	0.9060
D10	1	1	2.9944	18.3305	17.8647	532.3924
D11	1	1	0.1595	0.9767	11.5769	345.0055
D15	1	1	0.0386	0.2362	0.2431	7.2437
D16	1	1	0.0879	0.5383	0.0680	2.0260
D17	1	1	2.9014	17.7610	0.0070	0.2089
D18	1	1	0.5318	3.2556	0.2738	8.1597
D19	1	1	0.0002	0.0011	0.8704	25.9399
D22	1	1	0.0003	0.0020	0.0442	1.3159
D24	1	1	0.0072	0.0440	0.0317	0.9460
D25	1	1	1.6178	9.9034	0.0149	0.4435
Spain	**0h**		**5h**		**24h**	
Primer	Expr. Ratio	Fold change	Expr. Ratio	Fold change	Expr. Ratio	Fold change
D9	1	1	4.3052	6.1381	0.3867	0.8858
D10	1	1	2.6636	3.7977	1.4578	3.3394
D11	1	1	10.0505	14.3294	0.2567	0.5881
D15	1	1	1.0977	1.5650	1.5508	3.5526
D16	1	1	2.5947	3.6994	2.2982	5.2647
D17	1	1	1.1078	1.5794	0.6271	1.4365
D18	1	1	0.9510	1.3558	0.3745	0.8579
D19	1	1	1.8038	2.5718	0.0093	0.0213
D22	1	1	0.6657	0.9491	45.0441	103.1851
D24	1	1	0.7009	0.9993	2.6024	5.9615
D25	1	1	1.1058	1.5765	0.2964	0.6790

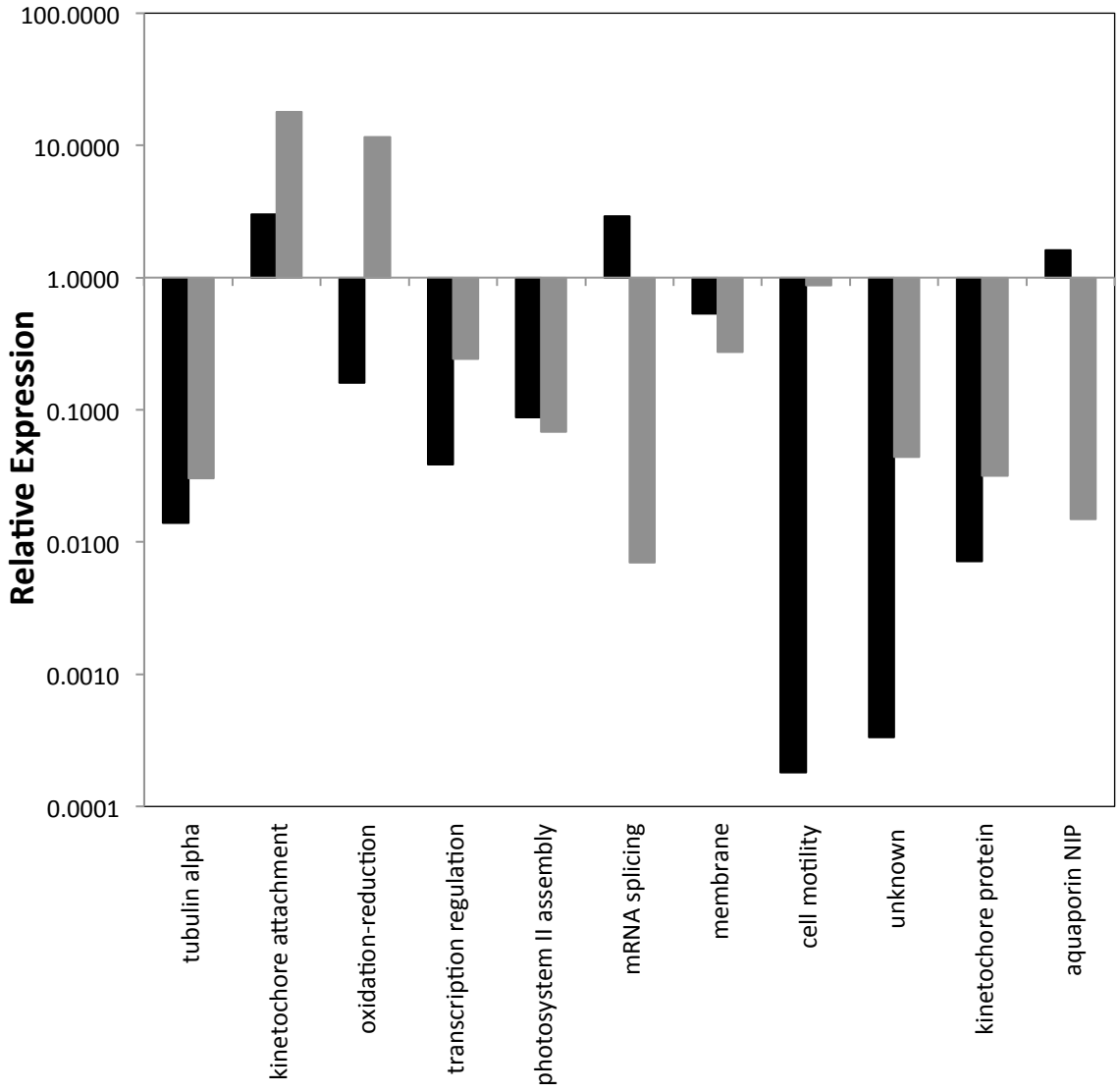

Figure 1. Expression values of 11 target genes after colchicine treatment in *D. smithii* samples (UK accession) relative to control group samples and actin housekeeping gene expression after 5 h (black) and 24 h (grey).

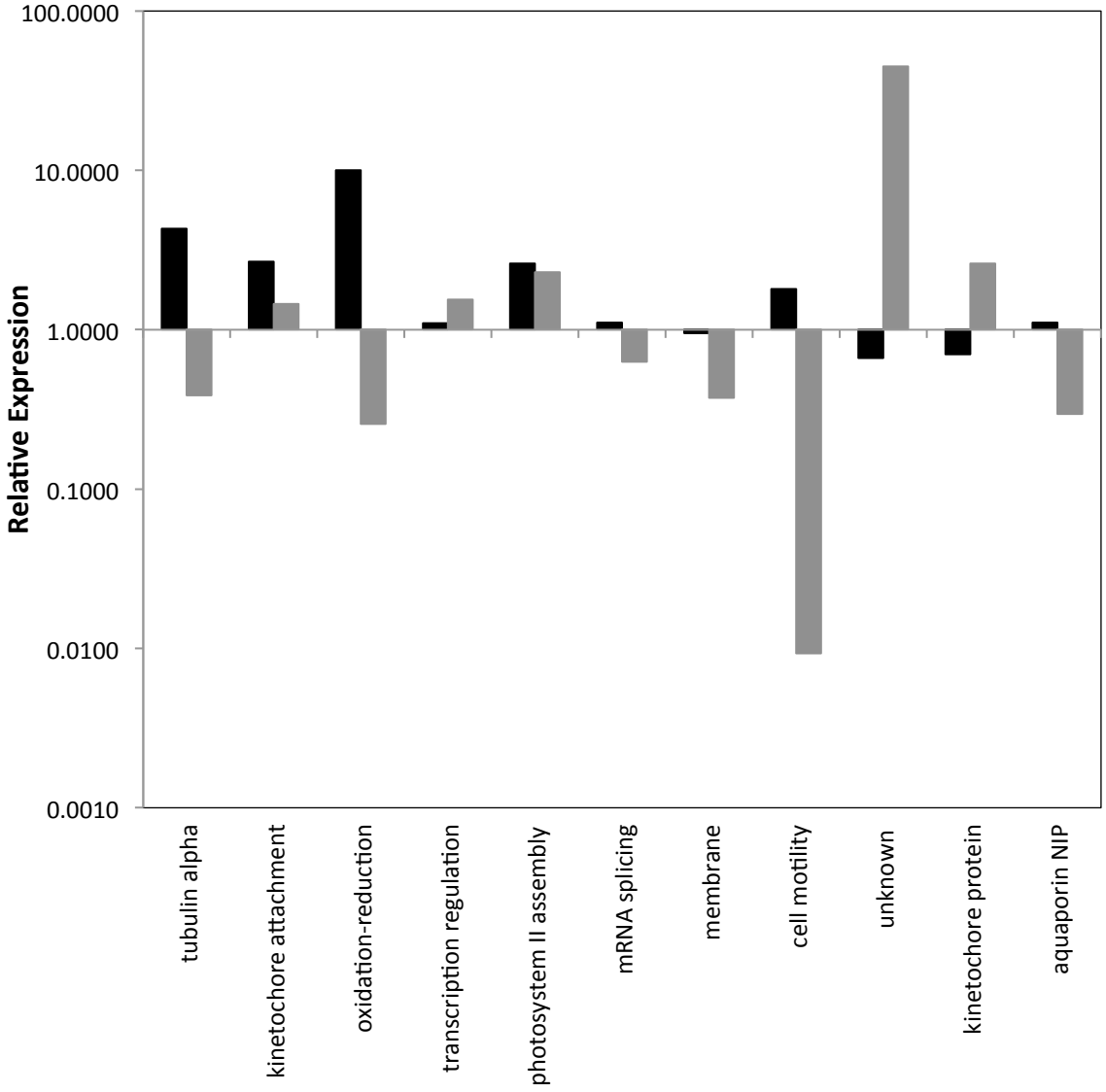

Figure 2. Expression values of 11 target genes after colchicine treatment in *D. smithii* samples (Spain accession) relative to control group samples and actin housekeeping gene expression after 5 h (black) and 24 h (grey).

To ensure accurate qPCR results, the expression of each gene was recorded using 3 technical replicates of each sample. However, plant sample replicates were not sufficient to perform formal statistics. Nevertheless, general trends in the expression data can still be observed, and are summarized in Table 6.

The counts of how many genes were either up- or down-regulated differed between the 5 h and 24 h time points (Table 6). Looking at figures 1 and 2, it is also apparent that some genes that started out up-regulated after 5 hours could end up down-regulated after 24 hours, and vice versa. This switching of gene expression occurred more often in the Spain accession than the UK accession, with 7 and 3 genes respectively that behaved this way (Table 6). The magnitude of up- and down-regulation that occurred also varied between the accessions. Most notably, down-regulation at 5 hours was roughly 7 times larger in the UK accession than in Spain (Table 6). Overall, there were only 3 genes that had similar patterns of relative expression at both 5 h and 24 h in both the UK and Spain accessions. The predicted functions of these 3 proteins are mRNA splicing, membrane function, and aquaporins (Figures 1 & 2).

Table 6. General trends in gene expression after 5 and 24 hours of colchicine treatment. Described first is the number of genes that were either up- or down-regulated after 5 h and 24 h of colchicine treatment. $\bar{x}$ indicates the mean relative expression of the group of genes that were up- or down-regulated at the 5 h and 24 h time periods, and is presented with standard error. "# switched" refers to the number of genes in the UK and Spain accessions that switched their expression from up- to down-regulated (or vice versa) between the 5 h and 24 h sampling points.

	UK					Spain				
Time	# up reg.	$\bar{x}$ up reg.	# down reg.	$\bar{x}$ down reg.	# switched	# up reg.	$\bar{x}$ up reg.	# down reg.	$\bar{x}$ down reg.	# switched
5h	3	2.50 ± 0.63	8	0.10 ± 0.17	3 out of 11 genes	8	3.09 ± 2.82	3	0.77 ± 0.13	7 out of 11 genes
24h	2	14.72 ± 3.14	9	0.18 ± 0.26		5	10.59 ± 17.2	6	0.33 ± 0.18	

In comparison to the RNA sequencing transcriptome data from Zhou et al. (in preparation), the expression of five genes at the 5 h time point was verified in the UK accession using qPCR (Table 7). These genes correspond to kinetochore attachment, mRNA splicing, membrane function, cell motility, and kinetochore proteins. The relative expression of eight genes in the Spain accession also happens to be consistent with the UK transcriptome data from Zhou et al. (in preparation) at the 5 h time point (Table 7). The function of these eight genes are predicted to be associated with alpha-tubulin, kinetochore attachment, oxidation-reduction processes, transcription regulation, photosystem II assembly, mRNA splicing, membrane function, and kinetochore proteins.

Table 7. Relative gene expression measured by qPCR of 10 target genes in comparison to RNA sequencing transcriptome data 5 h after colchicine treatment. Transcriptome data is from the *D. smithii* UK accession, while qPCR data is for the UK and Spain accessions of *D. smithii*. Up-regulation is represented with a "+" and down-regulation is represented with a "–".

	Relative Expression		
Gene function	UK (transcriptome) 5hCH vs. 5hWT	UK (qPCR) 5hCH vs. 5hWT	Spain (qPCR) 5hCH vs. 5hWT
alpha-tubulin	+	−	+
kinetochore attachment	+	+	+
oxidation-reduction	+	−	+
transcription regulation	+	−	+
photosystem II assembly	+	−	+
mRNA splicing	+	+	+
membrane	−	−	−
cell motility	−	−	+
kinetochore protein	−	−	−
aquaporin NIP	−	+	+

4. Discussion

In contrast to animals, many plant species are polyploid with more than two copies of their genome. Typically occurring through autopolyploidy or allopolyploidy, polyploidy is thought to be associated with increased vigour, a wider phenotypic range, functional redundancy, mutational robustness, and increased rates of evolution and adaptation (Van de Peer et al., 2009). These characteristics could all contribute to a reduced risk of extinction for polyploid species compared to their diploid relatives. In the case of the *Dactylis* genus, the tetraploid form (*Dactylis glomerata* subsp. *glomerata*) is commonly favoured as a forage grass over its diploid relatives due to a selective advantage, less restricted range, resistance to lower temperatures, and larger plant size with earlier flowering times (Stewart & Ellison, 2011; Lindner & Garcia, 1997a). Therefore, mitotic inhibitors such as colchicine have been used to synthetically produce this tetraploid species for its use as a crop species. Regarding colchicine's mechanism of action, much is already known about its role in the failure of the mitotic spindle (Leung et al., 2015; Dumontet & Sikic, 1999). There is a need for continued research on this topic to identify what other genes and cellular functions are involved in the process of colchicine chromosome doubling.

The first objective of this study was to characterize the long-term response of genes to colchicine over the course of 24 hours in both accessions of *D. smithii*. The second objective was to verify the results of RNA sequencing and transcriptome data analysis performed by Zhou et al. (in preparation). Based on those results, it was predicted that genes involved in the processes of oxidation-reduction, kinetochore structure and attachment, transcription regulation, mRNA slicing, membrane formation, cell motility,

water transport, and photosystem II assembly would show changes in expression in response to the application of colchicine in *D. smithii*. The exact responses of up and down regulation demonstrated by Zhou et al. are listed in Table 7.

Some samples had increased expression after 5h and then decreased expression after 24h, or vice versa (Table 6). For instance, this was especially prominent in the relative expression of genes associated with oxidation-reduction and aquaporins (Figures 1 & 2). This change in gene expression across the 24 hour time period could be due to how long it takes for the expression of each gene to be regulated in response to colchicine. It is difficult to know at exactly what point each gene changes its expression in relation to its normal levels. Furthermore, the implications of expression patterns of the genes with unknown functions cannot be determined. Regardless, colchicine presents a limitation to the length of time that a plant can be treated before the toxic nature of the chemical interferes with successful propagation of the plant's offspring. (Mohammadi et al., 2011).

Additional variation in relative gene expression was also observed between genes of both accessions (Table 6). For example, the genes involved in photosystem II assembly and transcription regulation were consistently down-regulated in the UK accession but up-regulated in the Spain accession (Figures 1 & 2). The variation in gene expression between these two populations of *D. smithii* could be due to the differing conditions of their microenvironments that cause the same genes to be differentially expressed. Wide variation in the genetic diversity of plant species has been seen even within small geographical ranges, as in the case of Mount Carmel in Israel (Nevo, 1995). One side of this mountain is a drier, more tropical and heterogeneous environment that receives

ample sunlight, while the opposing side is a more temperate and moist environment. Thus, biodiversity and genetic variation of organisms is seen between these regions as well as at their interface (Nevo, 1995). In the context of the two *Dactylis* accessions used in this project, UK is a mild and temperate environment compared to the hotter, more arid climate of Spain to the south. Within Spain alone there are Mediterranean, coastal, and semiarid regions, and subspecies of *Dactylis* throughout Spain have been known to hybridize and exhibit morphological and cellular variation (Lindner & Garcia, 1997b). Therefore, this environmental variation presents a need to test the expression of these genes within more accessions of this species to get a better consensus of the general expression patterns that result from colchicine treatment.

The first objective, to characterize the overall response of target genes to 24 hour colchicine treatment in two accessions of *D. smithii*, was successfully met through the results of this research project. The general trends in relative expression for each of the 11 target genes can be seen in Figures 1 and 2. Genes associated with mRNA splicing, membrane function, and aquaporins showed the same changes in gene expression within both accessions. In contrast, the genes involved in alpha-tubulin formation, kinetochore proteins and attachment, oxidation-reduction, transcription regulation, photosystem II assembly, and cell motility showed differences in up-regulation and down-regulation between the two accessions. The three genes that responded the same across both accessions are suggested as candidate genes for additional research regarding their involvement in the process of colchicine chromosome doubling. Aquaporins related to water exchange between the cell and exterior environment are likely involved in the cell's response to stress induced by colchicine (Afzal et al., 2016). Regulation of the cell

membrane is involved in the formation of the actin plate that forms during cytokinesis (Ebine & Ueda, 2015; Higaki et al., 2008). Genes responsible for mRNA splicing could also be expected to alter their expression due to changes in mRNA stability related to microtubule depolymerization that results from colchicine treatment (Wilson & Hunt, 2015).

The qPCR results also fulfilled the second research objective; to verify the 5 hour change in gene expression previously detected by Zhou et al. (in preparation) using RNA sequencing and transcriptome data analysis. Shown in Table 7, the qPCR relative expression of five genes in the UK accession was confirmed with the transcriptome expression data presented by Zhou et al. (in preparation). Although not from the same accession, the expression of eight genes from the Spain accession also matched the gene expression of the transcriptome data for *D. smithii* UK samples at 5h (Table 7). qPCR is regarded as a more precise way to assess the gene expression levels that were detected from the transcriptome data, as performing RNA sequencing is a single analysis, whereas qPCR can incorporate technical replicates.

Overall, gene expression varied between the 5h and 24h time periods, as well as between the two accessions for the same gene. Only the genes responsible for membrane function, mRNA splicing, and aquaporins showed similar expression patterns over 24 hours in both accessions. Additionally, the expression of five genes was confirmed in the UK accession compared to transcriptome data, while consistent expression of eight genes in the Spain accession was also identified (Table 7).

In light of colchicine's history as an antimitotic drug for therapeutic treatments and genome duplication, its role in compromising the function of the mitotic spindle is fairly

well established (Leung et al., 2015; Dumontet & Sikic, 1999; Cook & Louden, 1952). The research described in this thesis has provided a preliminary study of additional genes that could be targeted in future research to add to the knowledge of what genes are involved in the process of colchicine chromosome doubling. The trends of up- and down-regulation identified in the 11 genes investigated in this study provide insight into additional *Dactylis* genes affected by colchicine, which can be studied further through the use of additional accessions and biological replicates to clarify the aforementioned trends in gene expression. Colchicine is also one type of abiotic stress; therefore, the results of this study are also informative regarding how this plant species responds to this form of stress at the cellular level.

Future research on this topic could explore the gene expression of leaf samples that were also taken from the control and colchicine treated *D. smithii* plants used for this study. These results would allow for a comparison of the root and leaf cells to indicate how expression differs between these two cell types and locations. For instance, are the same genes affected by colchicine, and to the same degree? Is there a delayed or diluted response due to the time it takes colchicine to travel from the roots to the leaves? Another avenue of research could also assess the changes in gene expression of *D. mariana*, which is a natural tetraploid subspecies of orchardgrass. This could provide comparative insight regarding the genetic characteristics of the wild (*D. mariana*) and synthetic tetraploids that were derived from colchicine treated *D. smithii* specimen cultivated by Zhou et al. (in preparation).

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