Evaluation of gene expression responses to colchicine in *Dactylis smithii* at different tissues

by

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Abstract

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Environmental stresses have had a major impact on gene expression and evolutionary adaptation as a whole. What has been discovered is that polyploids have a genetic advantage compared to their diploid relatives in allele selectivity. Naturally occurring polyploids can occur through allopolyploids where two species hybridize their genomes, but more commonly autopolyploidy occurs when either chromosomes within an organism fail to separate during cell division, or when fertilization occurs, gametes do not separate correctly. What has been recently discovered is that diploids can be artificially doubled into polyploids using colchicine. Colchicine is an antimitotic agent that stops microtubules from separating chromosome sets during cell division in an organism. In this study, colchicine was used to treat diploid *Dactylis smithii* roots from two different accessions (UK and Spain). qPCR was then used to differentiate gene activity between the root and leaf samples and then compared with the RNA sequence transcriptome provided by Zhou et al. (2017). The comparison showed some similarities in up- and down- regulation for all areas, but also provided insight on trends where the expression of a gene changed between 5 and 24 hours after treatment. Future research could look at a comparison between another known autopolyploid and compare the gene activity between the two.

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I. Introduction

I.1 *Environmental Stress and Plants*

Environmental stress also known as abiotic stress, including drought, high salt, and extreme temperatures cause significant crop losses, while also causing detrimental damage to a variety of plant species globally. Of the major environmental stressors, water stress is considered to be the most extensive cause of plant deterioration. However, multiple stressors including salinity, and high temperature also cause the same physiological damage to the plants as water stress. (Smirnoff, 1998). Abiotic stress as well as normal natural mutations can cause physiological changes that in turn can influence gene expression. Overall, the genes expressed might noticeably change, but what is not necessarily visible are the changes in transcriptome (Lockhart & Winzeler, 2000). The interesting thing about this is that these changes can be both beneficial and detrimental to a plants survival in response to external stressors.

I.2 *Polyploidy versus Diploid in Plants*

A plant's ability to adapt to abiotic stresses is highly dependent on its activation of molecular networks, including those that regulate gene expression (Vinocur & Altman, 2005). The presence of these molecular networks and their changes have been well documented in polyploidy plant species, and can be analysed when comparing diploid and polyploidy transcriptome; which is the total of all mRNA that is being expressed by active genes (Lockhart & Winzeler, 2000). The ploidy of a plant or organism can be described as the number of chromosome sets in their cells. Specifically, this study is interested in diploid and the differentially expressed genes when treated with an antimitotic agent often used for chromosome doubling. For diploid, most animals and the majority of plants have two sets of chromosomes, where they received one set of chromosomes from the maternal side and the other from the

paternal. For example, when animals procreate and the offspring receive a set of chromosomes from the egg of the mother, and sperm from the father. The option of polyploidy that will be considered in this study is tetraploid as colchicine can be used to double chromosomes. Tetraploid's are the most common form of polyploidy overall, when there are four sets of chromosomes in an organism's cells (Comai, 2005). Whole genome duplication (WGD) in polyploid plants has demonstrated to be significant in a plant's ability to adapt to stress compared to diploid plants who hybridize (Jackson & Chen, 2010).

It has been noted that diploid species may develop to be polyploidy via allopolyploidy, or autopolyploidy (Del Pozo & Ramirez-Parra, 2015). Autopolyploids are much more common than allopolyploids where they arise through the duplication of one plant's genome (Tamayo-Ordóñez et al., 2016) through somatic doubling. Somatic doubling is when one plant enters cell division but fails to separate the replicated chromosomes during mitosis, or fusion and unreduced gametes, which occurs when gametes do not properly detach during meiosis. Unreduced gametes is the most common to naturally occur in the wild as it becomes a genetic characteristic of many species (Otto, 2007; Sattler et al., 2016). Allopolyploids on the other hand occur when two different species hybridize their genomes (Tamayo- Ordóñez et al., 2016). A key characteristic of polyploids is their allele selectivity ability. There have been multiple studies completed supporting the significant divergence genetically between diploid and polyploidy plants. Saminathan et al. (2015) explores the difference between diploid and tetraploid sweet watermelon where tissues from the leaves, stem, and fruit were compared. The leaves and the stem both showed increased levels of alternative splicing for the tetraploid plant. Alternative splicing is important in regulating plant processes such as development, flowering time, and how the plant deals with abiotic and biotic environmental stresses. Liu and Sun (2017) also support

this statement in their study on barley that is focused on the differences between diploid and tetraploid, and concluded tetraploids can retain more water under salt stress than their diploid ancestor.

Polyploid plant species have proven to both benefit and be at a disadvantage when it comes to environmental stress response. In the circumstance of food crops such as the peanut (*Arachis hypogaea*), barley (*Hordeum bulbosum*), coffee (*Coffea Arabica*) and bread wheat (*Triticum aestivum*) (Glover et al., 2016), being polyploidy adds particular advantages that diploids do not have. For example, polyploid plants could have greater resistance to pests and pathogens, and improved adaptation in regards to abiotic stresses, and increased or decreased flower and leaf size depending on their needs (del Pozo & Ramirez-Parra, 2015;Tamayo-Ordóñez et al., 2016). Having the allele selectivity to meet their needs under stress allows polyploidy crop species to increase their quality and quantity of product while also increasing their persistence even when under undesirable conditions (Wang et al., 2015). However, some disadvantages of polyploidy species are that they can become infertile, be shorter in height, and even develop less fruit (Tamayo-Ordóñez et al., 2016).

Although polyploids generally have a greater ability to tolerate stress compared to diploids, it has been noted that the molecular basis of these mechanisms has been understudied (Liu & Sun, 2017), due to previously limited research models that did not analyze at the molecular level (Zhu, 2001). Zhou et al. (2017), provides RNA sequence transcriptome data with samples treated with colchicine that allows for further molecular insight into the difference of gene expression, whether a gene is up- or down-regulated between diploid and polyploidy samples.

I.3 *Creation of Polyploidy with Colchocine*

To further the knowledge of molecular mechanisms and a plant's response to stress, biotic or abiotic, colchicine has been used to artificially alter diploid's into polyploid plants (Tamayo-Ordóñez et al., 2016). Colchicine is a compound that originates from the seeds and bulbs of the autumn crocus (*Calchicum autumnale*) and has been used for both medicinal and research purposes for many years (Dhooghe et al., 2011). Colchicine's history in the medical field has been for therapeutic practice in joint pain relief for arthritis, treatment for gout (Malkinson, 1982), pericarditis, coronary artery disease and even inflammatory. However, due to its extreme toxicity at high doses, its use has been decreased for medicinal use and in recent years has been more commonly used for research (Leung et al., 2015). Colchicine has the ability to change diploid's into polyploid's by preventing microtubule polymerization during cell division, therefore colchicine is termed as an "antimitotic agent". This means that during mitosis, the spindles will not complete their job of pulling the chromosomes apart, and might even degrade the spindles entirely (Cahill, 2016). Colchicine does this by binding the tubulin molecules in a complex, and this complex acts as an inhibitor to the elongation of spindles (Leung et al., 2015).

To apply colchicine as a treatment in a manageable amount (to avoid toxicity) to a diploid plant, a cotton swab can be used to rub a small amount on the apex or axillar buds, or by applying a very small amount to a greenhouse medium that the seedling is being grown in (Mohammadi et al., 2011). It is important to use small amounts to avoid negative impacts due to toxicity cause by high doses (>1000mg/L) and long treatment times (36-48 hours). These negative impacts include degradation of the spindles altogether, prevent growth, inhibit endosine

uptake, affect the polarity of the cell membrane, and even prevent cell secretions (Malkinson, 1982; Mohammadi et al., 2011).

As an antimitotic agent, colchicine binds to the tubulin heterodimer creating a complex that causes the breakdown of microtubules (Garl, 1978). Microtubules are the largest components of the cytoskeleton, centrosomes and mitotic spindles and are critical in the success of proper cell division. Changing the function of microtubules can lead to molecular and morphological changes within an organism (Janke & Bulinski, 2011) which can cause both advantageous and disadvantageous results.

Optimizing the dose and affectivity of colchicine can induce overall physiological changes to diploid plants and allows for further research and understanding of both colchicine activity mechanisms, and overall gene regulation mechanism of diploids when subjected to an antimitotic agent in an attempt to double chromosomes. Using both colchicine and the RNA sequence transcriptome in this study may improve the overall molecular understanding of how plants have adapted throughout history to the ever-changing abiotic stresses.

I.4 *Gene Expression*

Using the RNA sequence transcriptome (total mRNA expressed) data provided by Zhou et al. (2017) to identify the active gene response to colchicine treatment of *Dactylis smithii* plants, gene expression can be examined at different levels and time periods after colchicine treatment. Using colchicine as a treatment in this study will enhance our knowledge of how gene expression mechanisms, cellular functions, pathways, and physiological changes adapt in the environment through lab manipulation (Lockhart & Winzeler, 2000). Being able to reference the already prepared transcriptome for variations in regulations allows for the use of quantification analysis with real-time polymerase chain reaction (RT-PCR), also known as quantitative PCR

(qPCR). Not only can the amount of template DNA be determined with this method, but also the amount of RNA isolated from the diploid. The qPCR concludes these results by repeating cycles of amplification and seeing the amount of DNA produced through fluorescent dyes. There is a designated threshold, and when the fluorescent dye has reached the desired level, the threshold cycle (Ct) value is recorded. High Ct values imply a low amount of template where low Ct values indicate high amounts of template DNA (Mirmajlessi et al., 2016).

For the objectives and goals of this study, qPCR is more appropriate for analysis compared to regular PCR as it can amplify very small fragments of DNA and in turn allows for greater sensitivity and more efficient results without having to do further post-PCR action such as gel electrophoresis (Mirmajlessi et al., 2016). However, on top of these positives, there are also disadvantages found in the high cost of equipment, greater sensitivity to contamination, and an increase in the time allocated to analysis. Using the qPCR requires the use of NanoDrop technology in order to measure the level of contaminants (ideally NanoDrop concentrations would be >1.8) before proceeding with the amplification (Biosistemika, 2016).

I.5 *Goals and Importance of Research*

The subspecies that will be used for the analysis of diploid gene expression following the treatment of colchicine is *Dactylis smithii* of the *Dactylis glomerata* grass species, more commonly known as Orchardgrass (Cole, 2015). In the wild, the *D. glomerata* species undergoes autoploidization actively and many sibspecies are polyploids ranging from diploid to hexaploid. This makes *D. glomerata* and *D. smithii* the ideal candidate for lab manipulation because these phenotypes and physiological changes can also be seen naturally (Bretagnolle & Thompson, 1996), which provides a point of reference for the comparison.

The objective of this study is to apply colchicine to diploid *D.* smithii leaves and roots

from the United Kingdom (UK) and Spain, in order to analyze differential gene expression (up- and down-regulation) following treatment using qPCR. The second objective is to compare Zhou et al. (2017)'s already prepared RNA sequence transcriptome data with our qPCR gene expression results over the course of 24 hours at varying time intervals. A third objective is to identify trends between time periods comparing the control and treatment samples and possible trends independent of time periods following treatment. The primers chosen and the gene functions were also pre-determined by Zhou et al. (2017). The gene functions under observation are cell proliferation, alpha-tubulin, kinetochore attachment, oxidation-reduction, transcription regulation, photosystem II assembly, mRNA splicing, membrane, cell motility, a protein with unknown function, kinetochore protein, and aquaporin NIP. Of the gene functions being analyzed, it is noted that mRNA splicing, membrane, cell motility, kinetochore protein and aquaporin NIP are all essential in the cytoskeleton and cell division within an organism. These results will provide further insight on colchicine's effects on plants and implications if colchicine were to be used to artificially create polyploids as it is already used in plant breeding programs in an attempt to double chromosomes (Zhou et al., 2017). Understanding trends within time periods and types of samples as well as those outlying such criteria will provide further information on the mechanisms and possibly the nature of naturally occurring evolutionary adaptation in *D. smithii*, *D. glomerata*, and possibly polyploids in general.

II. Methods

II.1 *Dactylis smithii Samples & Treatments*

Two accessions were randomly chosen for the analysis of gene expression for the *Dactylis smithii* subspecies, (PI 441032) from the United Kingdom, and (PI 237607) from Spain. Each accession was subjected to control (water) and colchicine (2.5mM mixed with 2% dimethyl

sulfoxide) treatments for both leaf and root samples. The UK leaf samples were tested at 1, 5, 10, and 24 hours, and Spain leaf samples were tested at 0.5, 1, 2, 10, and 24 hours. The UK root samples were tested at only 5 hours, and the roots from Spain were tested at 5 and 24 hours after colchicine treatment. At each time period, samples were taken and immediately frozen in 15mL centrifuge tubes with liquid nitrogen and either used right away for RNA isolation or stored in -80°C for later use. The 11 chosen primer sets designated by both Zhou (2017) and Fleet (2017) can be viewed in table 1, where of the 11 target genes, D1 was chosen as the housekeeping gene. All primers and corresponding genes were tested after cDNA synthesis with qPCR to determine efficiency, refer to table 2 for more details.

II.2 *RNA Isolation*

After being flash-frozen, all leaf and roof samples from the UK and Spain accessions were prepared for RNA isolation at room temperature. A previously autoclaved metal rod was used to crush samples into a fine powder while being sterilized between each sample. When all large sections of leaves and roots were grinded down, the TRIzol Reagent protocol (Sigma-Aldrich, USA) was completed.

Homogenization

Roughly 2mL (or enough to cover your grinded sample) of TRIzol Reagent was added to each tube and was then centrifuged for 10 minutes at 12,000rpm before sitting at room temperature for 5 minutes. The clear supernatant was then transferred to a new 1.5mL Eppendorf reaction tube (ERT) with a sterile pipette.

Phase Separation

Following the transfer of the supernatant, 0.4mL of chloroform was added (roughly 0.2mL per 1mL of TRIzol Reagent) to the tube and shaken for 15 seconds. The tubes were then

centrifuged for 15 minutes at 4°C, 12,000rpm. 400uL of clear layer following centrifugation was then pipetted into a new 1.5mL ERT. This clear layer contained the desired RNA.

Precipitation

An equal ratio of isopropyl alcohol (1mL per 1mL) was added to the ERT with the new clear layer containing RNA. The tubes were then left at room temperature for 10 minutes before being centrifuged for another 10 minutes at 4°C, 12,000rpm. At this point, the RNA was visible as a white streak/pellet at the bottom of the tube.

Pellet Wash

To wash the pellet remaining after precipitation, the clear liquid was removed using a pipette to ensure the maximum amount of liquid was removed without disturbing the pellet. 2mL of 75% ethanol was then added to each tube and vortexed. Following the vortex, the tubes were then centrifuged for 5 minutes at room temperature, 7,500rpm and then left to air-dry for 5-10 minutes or until there was no visible liquid on or surrounding the pellet.

Resuspension

To resuspend the pellet, 50uL of RNase-free water was added to the tube and moved back and forth like the motion of a teeter-totter. Samples were then placed in the water bath at 55-60°C for 15 minutes. Following incubation all samples were placed in the -80°C freezer until further use.

II.3 *Testing RNA Sterility*

To ensure the RNA was not contaminated during the isolation procedure, a gel electrophoresis test was conducted. 5uL of RNA products for each sample were loaded into the well of a 1% agarose gel mixed with a drop of dye. The gel was placed in a Fisher Biotech electrophoresis system with 1x TBE buffer at 140 volts for 30 minutes. The gel was then placed

in a 0.1% ethidium bromide (EtBr) solution for another 30 minutes to ensure the bands would be visible. Following the time in the EtBr solution, the gel was placed in the SynGene bioimaging system where UV light and the GeneSnap software was used to take a photo of the bands.

II.4 *Reverse Transcription*

To further the purification of RNA for all samples, an RNase-free DNase kit (Quiagen, USA) was used. Following NanoDrop concentrations determined after gel electrophoresis, the amount of RNA needed was concluded at a ratio of 20uL per 0.1ug/uL solution. The designated volumes of RNA were then pipetted into a 0.2mL ERT, with 0.2 uL DNase I, 2uL DNase Buffer and DEPC water creating a total volume of 20uL. Tubes were centrifuged for 2 minutes at 10,000rpm and then incubated in the water bath for 10 minutes at 37°C before being placed in the 4°C freezer. After 5 minutes at 4°C, 0.2uL of EDTA was added to each sample. EDTA is a solution that protects the RNA from being denatured at high temperatures. Following the addition of EDTA, to inactivate the DNase I enzyme, the samples were incubated at 75°C for 10 minutes and then placed on ice before completing reverse transcription. The RNA samples were used to create complementary cDNA strands using the QuantiTect Reverse Transcription (RT) Kit (Qiagen, USA). 1uL of RT Primer Mix, 4uL of Quantiscript RT Buffer, and 1 µL of Quanitscript RT enzyme were all added to each of the sample tubes used above to create a total volume of 20uL. Samples were then placed in the water bath at 42°C for 30 minutes, and another 3 minutes in the Thermal Cycler at 95°C. The products of this procedure were then stored in the -20°C freezer until further use.

II. 5 *qPCR Reaction*

Every leaf and root sample for both UK and Spain accessions as controls and treatments went through a qPCR analysis, UK leaf samples were tested at 1, 5, 10, and 24 hours, and Spain

leaf samples were tested at 0.5, 1, 2, 10, and 24 hours. The UK root samples were tested at only 5 hours, and the roots from Spain were tested at 5 and 24 hours after colchicine treatment. Each sample was tested with a total of 11 primer sets.

A 96-well PCR plate was used where each reaction had 7.5uL of 2x SYBR Green ROX qPCR Mastermix (Qiagen, USA), 1uL of template cDNA at a 10% dilution, 1.5uL of both the forward and reverse primer (see table 1 for sequences), and 3.5uL of nuclease-free water; creating a final volume of 15uL. Following the combination of this mixture, the ABI PRISM 7000 Sequence Detection System (Applied Biosystems) was used record results following the programmed cycles of: 10 min at 95°C; 40 cycles of 15s at 95°C and 1 min at 60°C; 15s at 95°C; 20s at 60°C; and 15 s at 95°C.

All work was completed under the fume hood and was cleaned with 70% ethanol prior to, and after work was completed. Appropriate aseptic lab wear including glasses, a lab coat and gloves were worn at all times.

III. Results

III.1 *RNA Extraction*

RNA extraction using TRI reagent resulted in isolated RNA that underwent quantitative and quality assessment using NanoDrop technology. NanoDrop measured the absorbance for each sample at 260/280nm. The purity of the RNA was determined by the ratio given where everything at 1.8 and lower were likely contaminated by DNA, protein, or other elements during isolation. Fortunately, this did not occur and no further purification was needed.

III.2 *Primer verification*

Primer verification PCR was used to determine which primers were efficient in binding a single gene. The 12 primers chosen were D1, D2, D3, D4, D5, D6, D7, D8, D9, D10, D11, and

D12. Respectively, the genes each code for are cell proliferation, alpha-tubulin, kinetochore attachment, oxidation-reduction, transcription regulation, photosystem II assembly, mRNA splicing, membrane, cell motility, unknown function, kinetochore protein, and aquaporin NIP. Table 1 provides a full list of the primers, their sequences, oligo name, and gene function.

Table 1. Forward (F) and reverse (R) primers used to isolate housekeeping and target genes.

Gene	Oligo Name	Sequence (5' to 3')	Abbreviation
Positive regulation of cell proliferation	C100325 (F)	CAGGAACAGGAAGGGATGATG	D1
	C100325 (R)	CAGATCTTGGCGTGAGGATAAA	
Alpha-tubulin	C101064 (F)	CAGAGTCAAACCAGGAGTCAG	D2
	C101064(R)	CCAAGCAGAGGTAGCGTAAA	
Kinetochore attachment to spindle microtubule	C102023 (F)	CCGCAATGACGAGAGTTACA	D3
	C102023 (R)	CACTGTCACACCAACCTTCTATC	
Oxidation-reduction	C96405 (F)	TCTCCTTCCCGCTGTATT	D4
	C96405 (R)	ACTGAGCCTCTCTTTCAGTAG	
Transcription regulation	C101317 (F)	TGCAGTTGGAACAATGATGTATG	D5
	C101317 (R)	CCGCAGGAGAGGAAGAAATAG	
Photosystem II assembly	C93529 (F)	TCTGGATGGACGGAGAGAAA	D6
	C93529 (R)	GATTCATCATCACCGGCTACA	
mRNA splicing	C49608 (F)	CGTACAAGCAACACACTGGATA	D7
	C49608 (R)	TTGTTCTGTGCCTGCCTATG	
Membrane	C71496 (F)	CTCCAGTGCTCCTTGTTCTT	D8
	C71496 (R)	GCCTCCTGTAACGGGTATTC	
Cell motility	C89024 (F)	GTTACTGATGCCACCCTTATCT	

	C89024 (R)	CAGCGATGCCGTCCTATAAA	D9
Unknown function	C27495 (F)	CAGTACCTGACGTTGGCTATAC	D10
	C27495 (R)	GTGCTTGCGATTGCTTCTAAC	
Kinetochore protein	C101667 (F)	ACTCCACTACAAAGCAGGTAAA	D11
	C101667 (R)	CACGAAGTCCCATCCAAGAATA	
Aquaporin NIP	C88478 (F)	GCGATGTGAATGTGCGTAATAA	D12
	C88478 (R)	GATACATCCAGCTCCAGCATAG	

III.3 *Primer Efficiency*

Using the equation given by Pfaffl (2001)'s: $E = 10^{(-1/slope)}$, primer efficiency (E) was calculated following qPCR results for 12 primers. Table 2 provides a more detailed account of these results where the slope value was calculated from standard curves plotting 5 sample concentrations against their Ct values.

Table 2. The standard curve slope and efficiency values for 12 chosen primers.

Primer	Slope	Efficiency ($E=10^{(-1/slope)}$)
D1	-2.3904	2.6202
D2	-2.9344	2.1917
D3	-2.7642	2.3002
D4	-3.2557	2.0284
D5	-3.0265	2.1400
D6	-3.6810	1.8692
D7	-3.5135	1.9258
D8	-3.4528	1.9481
D9	-2.7482	2.3114
D10	-3.3390	1.9929
D11	-2.0394	3.0927
D12	-7.2740	1.3724

III.4 *qPCR Results*

For every root, and leaf sample at both UK and Spain accessions using the designated primers, cycle thresholds were determined through qPCR results. The resulting Ct values consist of the cycle number where DNA was amplified enough that a fluorescent signal, over the threshold, was detected. This value was then averaged with the standard deviation. Table 3 gives a complete list of average Ct values and standard deviations for UK leaf samples for 0, 1, 5, 10, and 24 hours. Table 4 provides a list of Spain leaf samples at 0.5, 1, 2, 10, and 24 hours. Where table 5 provides a full list of UK root samples at 0 and 5 hours and table 6 a list of Spain root samples at 0, 5, and 24 hours.

Table 3. Average Ct values and standard deviations for the UK *D. smithii* leaf samples at 0, 1, 5, 10, and 24 hours after colchicine treatment

UK Leaf Samples

Ct values	**Avg**	**SD**	**Avg**	**SD**	**Avg**	**SD**	**Avg**	**SD**	**Avg**	**SD**
Primer	**0h**	**0h**	**WT 1h**	**WT 1h**	**CH 1h**	**CH 1h**	**WT 5h**	**WT 5h**	**CH 5h**	**CH 5h**
D1	33.17	0.41	36.62	0.66	37.08	1.20	34.28	0.58	30.19	1.56
D2	32.79	0.94	29.80	0.30	34.32	0.69	31.88	0.44	30.92	0.39
D3	32.82	0.71					32.70	0.52	29.22	0.74
D4	30.78	2.55					31.39	3.70	28.45	0.51
D5	30.78	0.15	28.47	0.49	31.58	0.83	31.39	0.25	28.45	0.34
D6	37.88	2.12					36.79	0.65	35.28	0.76
D7	32.26	0.86	28.93	0.23	35.82	0.22	33.52	0.50	29.70	0.31
D8	32.69	0.78	28.66	2.17	30.42	1.28	31.46	0.36	27.51	0.31
D9	29.95	0.49	28.77	0.17	32.49	0.57	31.46	0.36	27.51	0.31
D10	32.24	1.96								
D11	32.96	0.36					32.48	0.54	28.29	0.06
D12	31.10	0.65					30.71	0.56	27.37	0.26

UK Leaf Samples								
Ct values	**Avg**	**SD**	**Avg**	**SD**	**Avg**	**SD**	**Avg**	**SD**
Primer	**WT 10h**	**WT 10h**	**CH 10h**	**CH 10h**	**WT 24h**	**WT 24h**	**CH 24h**	**CH 24h**
D7	37.02	0.02	35.27	1.00	32.07	0.47	37.10	0.93
D8	29.48	0.20	33.36	1.02	31.66	0.57	35.78	0.49
D9	34.15	4.88	36.71	2.15	33.25	0.91	36.87	1.97
D10	34.90	1.83	36.64	1.24				
D11	34.93	0.47	34.82	0.32				

Table 4. Average Ct values and standard deviations for the Spain *D. smithii* leaf samples at 0.5, 1, 2, 10, and 24 hours after colchicine treatment.

Spain Leaf Samples								
Ct values	**Avg**	**SD**	**Avg**	**SD**	**Avg**	**SD**	**Avg**	**SD**
Primer	**WT 0.5h**	**WT 0.5h**	**CH 0.5h**	**CH 0.5h**	**WT 1h**	**WT 1h**	**CH 1h**	**CH 1h**
D1	35.11	1.10	34.78	0.66	36.50	2.28	33.33	0.59
D2	37.94	2.43	35.03	0.86				
D3	33.52	1.01	33.15	1.37	34.03	0.55	32.00	0.51
D5	29.88	0.33	29.84	0.32	30.81	0.98	28.80	0.88
D7	33.91	0.66	32.57	0.39	34.16	0.51	31.76	0.33
D8	29.48	0.39	28.64	0.30	29.49	0.16	29.06	0.39
D9	31.89	0.70	30.37	0.15	29.56	0.38	28.67	1.06
D10					37.56	1.33	36.06	2.63
D11	34.13	1.15	32.75	1.03	34.56	0.90	33.25	0.19

Spain Leaf Samples												
Ct values	**Avg**	**SD**	**Avg**	**SD**	**Avg**	**SD**	**Avg**	**SD**	**Avg**	**SD**	**Avg**	**SD**
Primer	**WT 2h**	**WT 2h**	**CH 2h**	**CH 2h**	**WT 10h**	**WT 10h**	**CH 10h**	**CH 10h**	**WT 24h**	**WT 24h**	**CH 24h**	**CH 24h**
D1					34.79	0.23	35.82	0.87				
D3	31.86	0.67	31.78	0.33	30.29	0.08	36.63	2.24				

D5									33.46	0.78	30.34	0.28
D7	35.18	3.87	34.57	0.96	35.47	0.44	36.24	0.49				
D8	32.04	0.47	30.05	0.09	30.57	0.40	34.43	1.48				
D9	34.13	3.17	31.13	0.39	33.06	4.29	36.69	2.63				
D11	35.41	3.78	32.78	0.03	34.85	0.11	37.24	0.09				
D12	33.61	0.84	33.87	0.21								

Table 5. Average Ct values and standard deviations for the UK *D. smithii* root samples at 0, and 5 hours after colchicine treatment.

UK Root Samples

Ct values	Avg	SD	Avg	SD	Avg	SD
Primer	**0h**	**0h**	**WT 5h**	**WT 5h**	**CH 5h**	**CH 5h**
D1	26.15	0.09	33.98	0.36	33.00	0.27
D2	24.57	0.20	32.54	0.43	31.46	1.69
D3	26.36	0.72	33.59	1.28	32.70	0.66
D5	24.35	0.12	28.98	0.16	27.17	0.05
D6	30.79	1.76	34.60	1.82	31.47	1.75
D7	26.29	0.14	25.97	0.19	25.73	0.26
D8	24.18	0.27	29.69	0.54	31.38	0.32
D9	25.43	0.34	28.91	0.25	33.04	1.02
D10	25.28	0.23	24.78	0.13	34.73	0.42
D11	26.90	0.43	33.94	0.29	35.01	0.78
D12	26.54	0.41	32.06	0.67	33.68	0.80

Table 6. Average Ct valyes and standard deviations for Spain *D. smithii* root samples at 0, 5, and 24 hours after colchicine treatment.

Spain Root 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	32.44	0.20	35.82	1.17	32.31	0.45	32.09	0.86	31.83	0.72
D2	30.98	0.19	34.27	1.92	31.09	0.21	30.34	0.28	33.69	2.94
D3	30.11	0.92	33.92	0.70	32.82	0.47	31.90	0.48	33.33	0.94
D5	25.88	0.29	27.77	0.29	28.22	0.35	27.34	0.24	28.92	0.28
D6	33.02	0.83	34.67	1.65	34.02	1.22	32.67	0.79	32.90	0.58
D7	33.18	1.99	32.05	1.85	30.34	2.17	30.63	1.93	29.70	1.66
D8	29.65	0.53	34.73	2.42	30.54	0.54	30.64	0.56	31.83	0.56
D9	29.46	1.53	34.02	1.28	30.95	0.20	29.52	0.13	33.39	0.55
D10	32.95	1.74	34.01	0.40	31.69	0.68	37.87	1.30	33.21	1.36
D11	33.06	1.00					33.96	1.01	33.63	1.33
D12	32.93	0.09	33.00	0.14	32.96	1.47				

11 primers were used for the calculation of relative expression, D1, D2, D3, D4, D5, D6, D7, D8, D9, D11 and D12 at 0, 1, 5, 10, and 24 hours for UK leaf samples (table 7), and 0.5, 1, 2, 10 and 24 hours for Spain leaf samples (table 8). For root samples, UK was tested at 0 and 5

hours (table 9) and Spain root samples were tested at 0, 5, and 24 hours (table 10). Relative expression values were calculated using Pfaffl (2001)'s equation:

$$\text{Ratio} = \frac{(E_{target})^{\Delta CPtarget(control^{-}sample)}}{(E_{ref})^{\Delta CPref(control^{-}sample)}}$$

The equation consists of efficiency of the target gene (E_{target}), and the difference between Ct values of the control and colchicine treated samples ($\Delta CPtarget$). ΔCP_{ref} and E_{ref} were calculated the same as above except compared with the housekeeping gene D1 instead of the control. Plotted values for UK leaf samples can be seen in Figure 1, Spain leaves in Figure 2, UK root samples plotted in figure 3, and Spain root samples in figure 4.

Table 7. Relative expression ratios for UK leaf samples at 0, 1, 5, 10, and 24 hours after colchicine treatment.

Expression Ratio	**UK Leaf Samples**				
Primers	**0h**	**1h**	**5h**	**10h**	**24h**
D1	1	1.5564	1.3089		
D2	1	0.3759	0.2314		
D3	1		0.4524		
D4	1	2.0094	1.1243		
D5	1	0.1710	0.2809		
D6	1		0.6269		
D7	1	0.0190	1.4162	2.8491	1.2886
D8	1	0.4173	1.2452	2.8803	1.1236
D9	1	0.7893	0.8102	9.5175	4.2119
D11	1		0.8950	31.5420	
D12	1		1.0432	20.9526	

Table 8. Relative expression ratios for Spain leaf samples at 0.5, 1, 2, 10, and 24 hours after colchicine treatment.

Expression Ratio	**Spain Leaf Samples**				
Primer	**0.5h**	**1h**	**2h**	**10h**	**24h**
D1	0.4594	2.2433		9.4245	
D2	1.5986				
D3	0.4767	0.6707	0.0172	0.5621	
D5	0.7047	0.7449			1.1299
D6			0.0812		
D7	1.4651	1.3243	0.0216	43.7768	
D8	0.9619	0.4047	0.0696	1.0969	
D9	1.0055	0.6282	0.0412	2.4106	
D10		0.8089			
D11	1.0813	0.6912	0.0509	3.2807	
D12			0.0034		

Table 9. Relative expression ratios for UK root samples at 0, and 5 hours after colchicine treatment.

Expression Ratio	**UK Root Samples**	
Primers	**0h**	**5h**
D1	1	2.0685
D2	1	2.0871
D3	1	1.8008
D5	1	1.8551
D6	1	10.3726
D7	1	2.0132
D8	1	0.6467
D9	1	0.1876
D10	1	0.0098
D11	1	0.5036
D12	1	0.3163

Table 10. Relative expression ratios for Spain root samples at 0, 5, and 24 hours after colchicine treatment.

Expression Ratio	**Spain Root Samples**		
Primer	**0h**	**5h**	**24h**
D1	1	9.0084	1.7881
D2	1	6.5211	0.3016
D3	1	2.1975	0.8421
D5	1	0.7750	0.7461
D6	1	1.6311	1.2851
D7	1	0.9382	2.6205
D8	1	3.9715	0.7448
D9	1	1.5611	0.1988
D10	1	0.9240	19.8624
D11	1		1.8394
D12	1	1.2116	

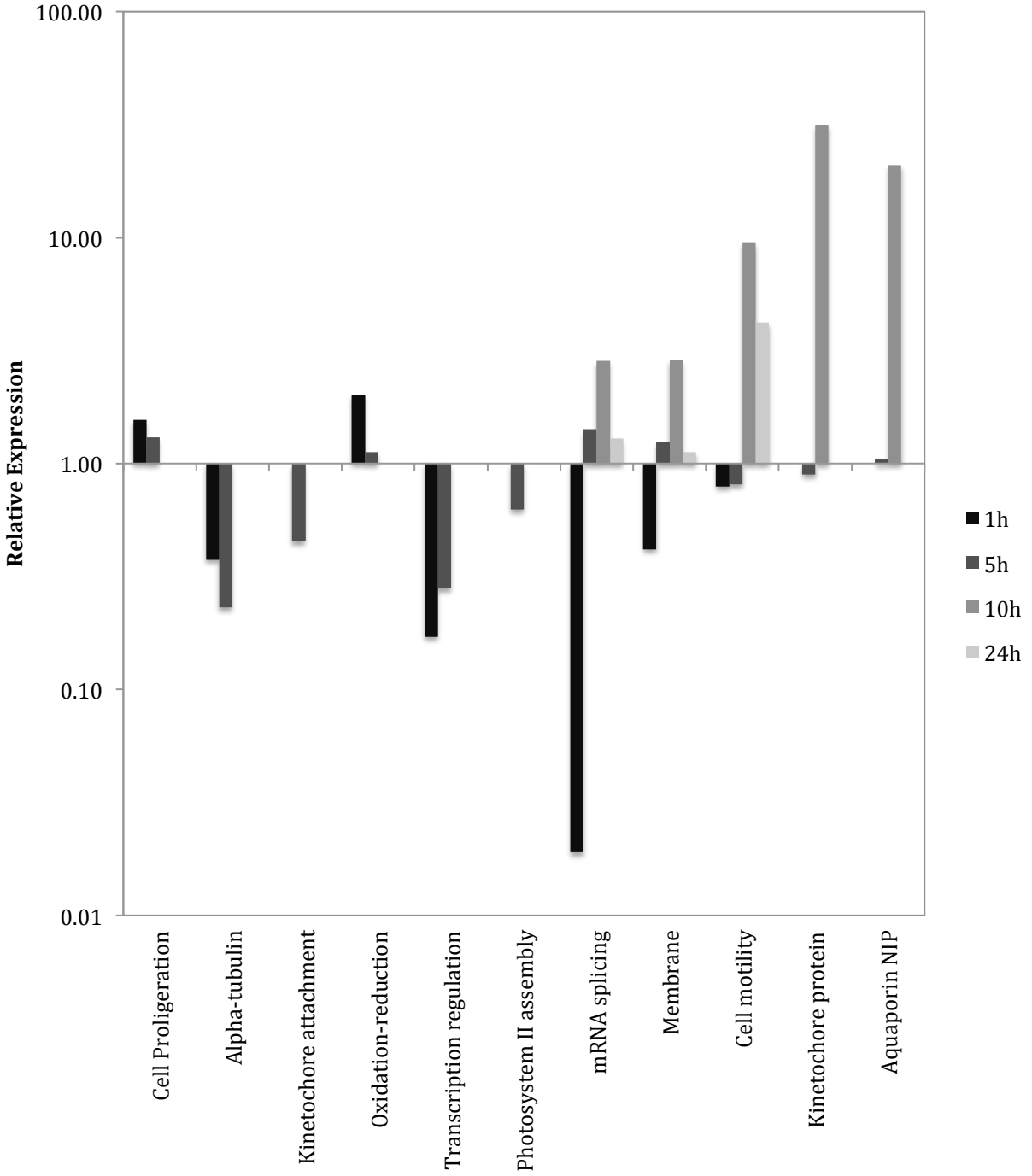

Figure 1. Expression values of 12 target genes in *D. smithii* leaf samples of the UK accession following treatment with colchicine relative to control group (y=1) and actin housekeeping gene after 1, 5, 10 and 24 hrs.

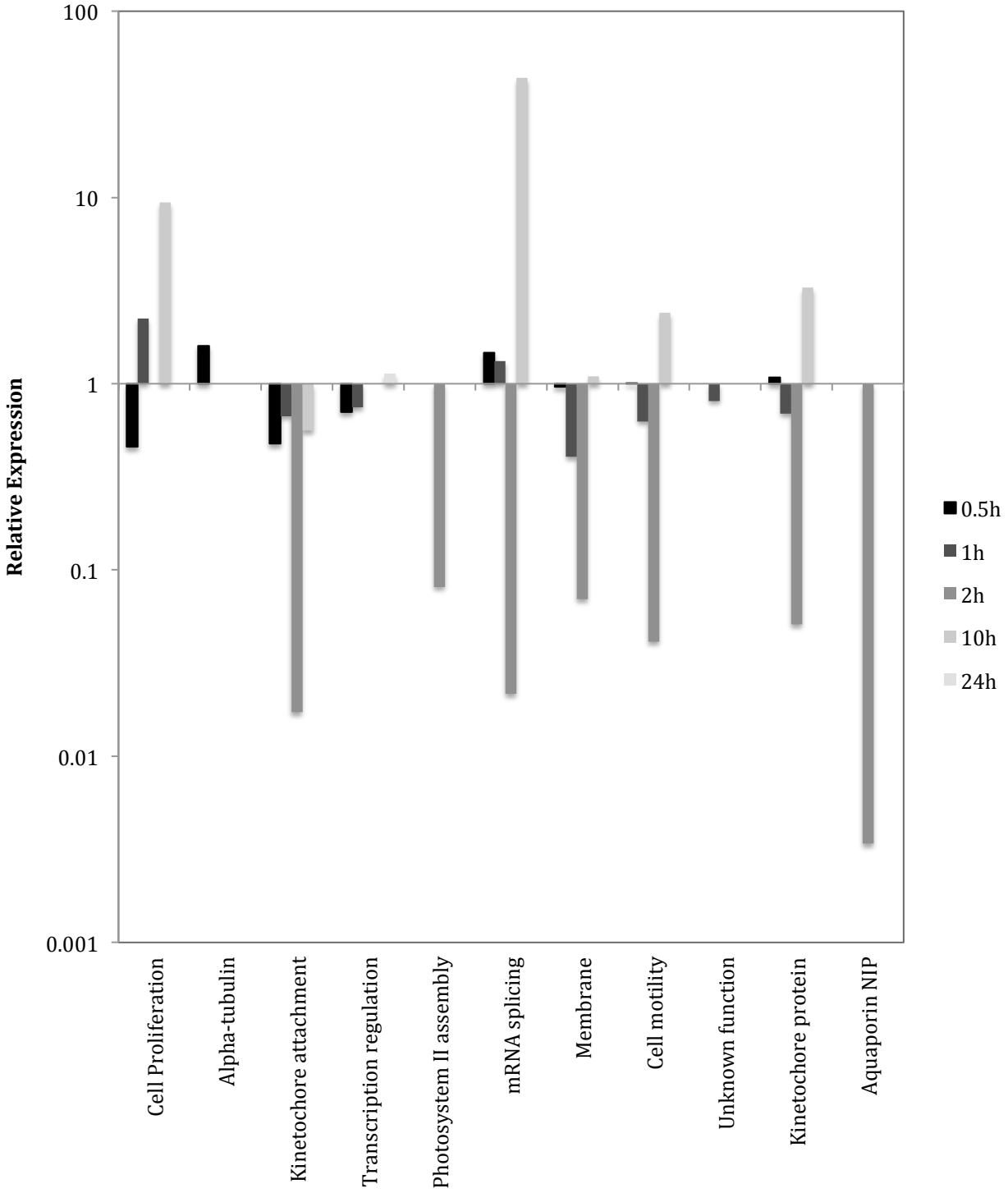

Figure 2. Expression values of 11 target genes in *D. smithii* leaf samples of the Spain accession following treatment with colchicine relative to control group (y=1) and actin housekeeping gene after 0.5, 1, 2, 10 and 24 hrs.

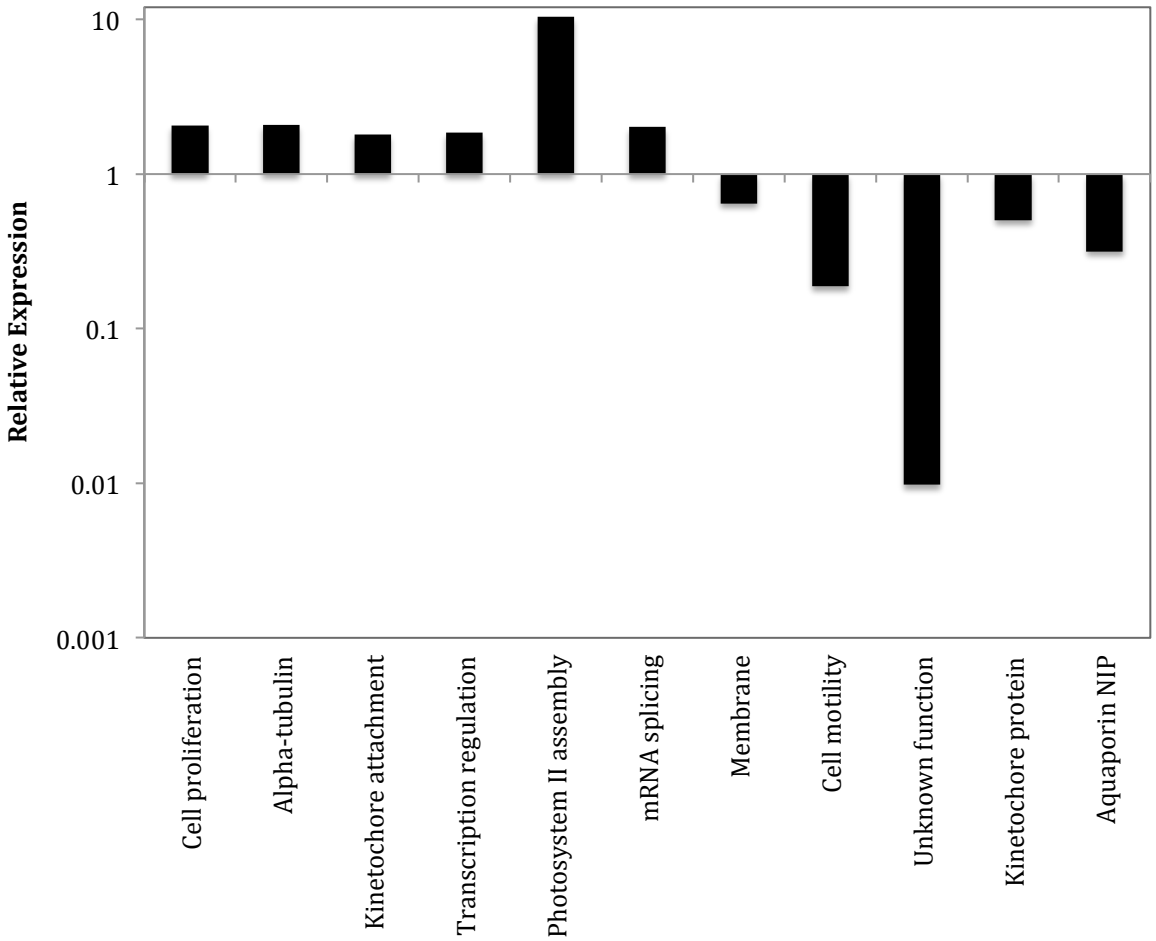

Figure 3. Expression values of 11 target genes in *D. smithii* root samples of the UK accession following treatment with colchicine relative to control group (y=1) and actin housekeeping gene after 5hrs.

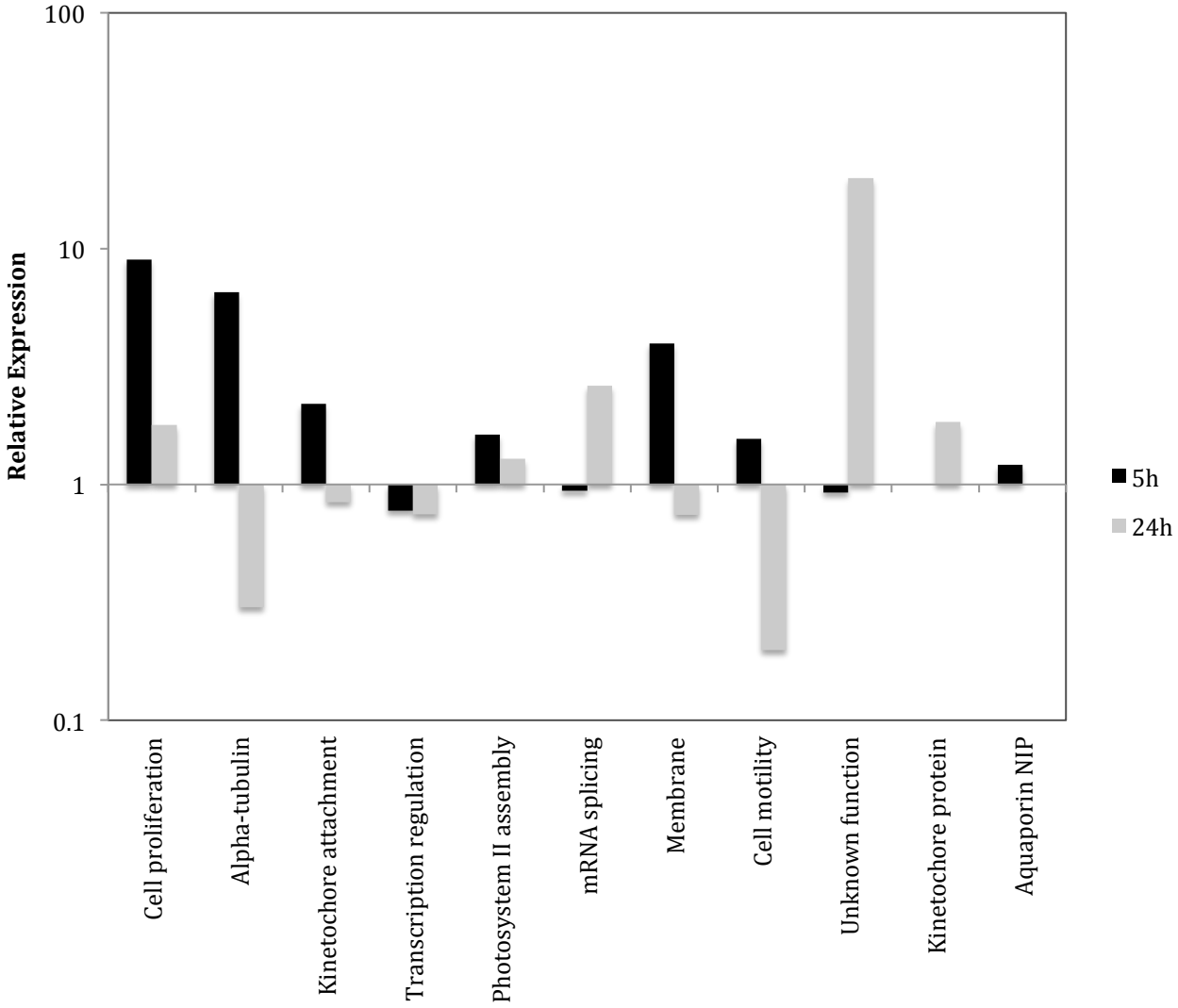

Figure 4. Expression values of 11 target genes in *D. smithii* root samples of the Spain accession following treatment with colchicine relative to control group (y=1) and actin housekeeping gene after 5 and 24hrs.

Data was analyzed to determine what genes were up- or down-regulated in comparison to Zhou et al.'s RNA sequencing transcriptome data, as seen in table 11. Compared to the transcriptome data, 5 of the UK leaf samples matches the regulation of our qPCR data, 10 matched for the UK root samples, and 6 matched for the Spain root samples. Overall, the qPCR yielded 5 up-regulated genes and 6 down-regulated genes for the UK leaves, 6 up-regulated and

5 down-regulated for the UK root samples, and 7 up-regulated and 3 down-regulated for Spain root samples; all measured 5 hours after the colchicine treatment.

Table 11. Relative gene expression as shown by qPCR results of 11 genes in comparison to RNA sequencing transcriptome. "+" is used to represent up-regulation where "-" is representative of down-regulation. Each sample and its regulation are in relation to its expression 5 hours after colchicine treatment compared to the control (water).

	Relative Expression			
Gene Function	UK Transcriptome (5h)	UK qPCR Leaves (5h)	UK qPCR Root (5h)	Spain qPCR Root (5h)
cell proliferation	+	+	+	+
alpha-tubulin	+	-	+	+
kinetochore attachment	+	-	+	+
oxidation-reduction	+	+	+	-
transcription regulation	+	-	+	+
photosystem II assembly	+	-	+	-
mRNA splicing	+	+	-	+
membrane	-	+	-	+
cell motility	-	-	-	-
kinetochore protein	-	-	-	N/A
aquaporin NIP	-	+	-	+

Analyzing the different time periods together (referencing figures 1-4), it is possible to see different trends. Within the figures, there are genes that show up-regulation at 5 hours post-treatment, then at 24 hours they are down-regulated, and vice versa. An overall trend of more up-regulation in UK leaf samples and more down-regulation in Spain leaf samples is apparent. A more detailed list of trends not necessarily categorized by hours can be seen in table 12.

Table 12. Trends seen in Figures 1, 2, and 4 (not categorized by hour because hourly data is not consistent in all)

	UK Leaf (figure 1)	Spain Leaf (figure 2)	Spain Root (figure 4)
Only one sample per primer set:	D3, D6	D2, D6, D10, D12	D11, D12
No samples per primer set:	D10	D4	D4
Most primers have a net:	Increase (6/9)	Increase (7/7)	Decrease (7/9)
Switch from down to	D7, D8, D9, D11	D1, D5, D7, D8, D9,	D6, D9

up:		D11	
Switch from up to down:	None	D7, D11	D2, D3, D7, D8
Net increase in expression:	D5, D7, D8, D9, D11, D12	All	D7, D10
Net decrease in expression:	D1, D2, D4	None	D1, D2, D3, D5, D6, D8, D9
Final up regulated	D1, D4, D7, D8, D9, D11, D12 = 7	D1, D2, D5, D7, D8, D9, D11 = 7	D1, D6, D7, D19, D22, D24, D25 =7
Final down regulated	D1, D2, D3, D5, D6 = 5	D3, D5, D6, D10, D12 = 5	D2, D3, D5, D8, D9 =5

IV. Discussion

Exploring the molecular networks and gene expression in plants opens up a window to both the studies of evolutionary adaptation, conservation, and gene regulation mechanisms. Plants are ideal to study in comparison to animals because adaptations can be seen much faster, can be manipulated more easily in lab environments and share some key functions with animals themselves, while having complex mechanisms that have helped unravel the history of evolutionary adaptation through diploid's and polyploid's of the same species. Generally the diploid to polyploidy transition is seen through the more common occurrence of autopolyploidy compared to allopolyploidy (Van De Peer et al., 2009). Animals are only diploid, where they have two chromosome sets, while plants have some species that are diploid where the subspecies is polyploidy, like the chosen species for this study, *Dactylis smithii*. *Dactylis glomerata* is the tetraploid form within the *Dactylis* genus and is favoured over its diploid relatives because of the allele selectivity which allows it to have less restricted range, greater ability to withstand abiotic stressors and thus an overall increase in survival ((Lindner & Garcia, 1997; Stewart & Ellison, 2011). Due to the ample amount of opportunities to study this species as a diploid, tetraploid or even hexaploid, and having the transcriptome data available, using colchicine to cause molecular changes similar to that of artificially producing a polyploidy response from a diploid subspecies

is a great way to study gene expression and possibly adaptation regulation.

The objective of this study is to apply colchicine to diploid *D.* smithii leaves and roots from the United Kingdom (UK) and Spain, in order to analyze differential gene expression (up- and down-regulation) following treatment using qPCR. The second objective is to compare Zhou et al. (2017)'s already prepared RNA sequence transcriptome data with our qPCR gene expression results over the course of 24 hours at varying time intervals. A third objective is to identify trends between time periods comparing the control and treatment samples and possible trends independent of time periods following treatment. For the first objective, colchicine showed up- or down-regulation with all gene functions studied, none showed to be the same as the control.

The second objective, to compare Zhou et al. (2017)'s RNA sequence transcriptome data to our qPCR results showed that 5/11 for UK leaves, 10/11 for UK roots, and 6/10 for Spain roots. The assumption as to why all did not match are that the species accessions greatly vary, which means some genes may be more beneficial in UK compared to Spain and vice versa. The same is assumed for roots and leaves in general, and some genes may not be as important for leaves as they are for roots and vice versa.

The third objective, to note other visible trends yielded multiple results. It is also important to note that a trend seen throughout the results is an up-regulation following treatment in mRNA splicing, membrane, cell motility, kinetochore protein, and aquaporin NIP. As mentioned earlier, these gene functions are directly related to a cell's cytoskeleton that has already been manipulated by the colchicine. This trend was noted after the 10 hour mark and continued into the 24hr period for the samples that were collected. The likely cause of this is that a cell's life cycle is up to 20 hours. Changes may not have been in effect until cell division had

occurred and this could have been a limitation for our results as when samples were analyzed, not all may have passed through a full life cycle. Other trends independent of the time periods were noted in table 12. The most notable, and interesting trend as mentioned above is that some gene regulation under colchicine treatment began with up- regulation and by the 24 hour mark was down-regulated and vice versa (refer to figures 1-4). It is thought that the first few hours following the treatment shows the organism adjusting to the molecular changes, and by the 24 hour mark we are able to see the final change in genetic activity. It is also important to note that in the UK accession for leaf samples, the mRNA splicing was extremely down-regulated at 1 hour post-treatment. mRNA splicing is important in gene expression during development and environmental response. Being down-regulated may be a result of *D.smithii* stopping the production of development in order to respond to the colchicine. It also could be explained as a shock response as the down-regulation only occurred after one hour and was up-regulated in every other sample period.

Colchicine presents the issue of toxicity, and although we can manipulate the plant into becoming polyploidy for a short period of time with low doses, prolonged exposure to the compound will actually degrade the plant and kill it. It would be interesting if it were possible to find a dose, or a dosage rate that could create a more permanent reaction to the treatment and be measured over days or weeks compared to a max of 24 hours.

The results of the qPCR in this study along with the RNA sequence transcriptome have provided further information on colchicine's effect on diploid plants in relation to gene activity. Colchicine's role as an antimitotic agent while compromising the function of the microtubule spindles and their ability to separate the chromosome sets was further exemplified and increases the knowledge of its ability to possibly act as a way to artificially create poluploid's from diploid

species. This may also provide an understanding into the naturally occurring evolutionary adaptation of polyploid's at the gene activity level of different tissues.

Future research could build on this study and explore the gene expression of multiple autopolyploidy species in more detail, and possibly aim to find a way to test colchicine's abilities over a longer period of time to see if there are more permanent results and to ensure most cells have gone through a life cycle. The comparison between two entirely different species like *D. smithii* and *Hordeum bulbosum* (barley) could also shed light on the truth behind evolutionary adaptation, and how we could possibly manipulate plant species, specifically those involved with agricultural crops to decrease the global crop yield loss (Smirnoff, 1998).

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