The University of South Bohemia in České Budějovice
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Conservation and diversification of PRC2 subunits in moss

Bachelor thesis

Sonja Hanl

Supervisor: Ahamed Khan, Ph.D.

Co-supervisor: Iva Mozgová, Ph.D.

Institute of Plant Molecular Biology, Biology Centre CAS

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Annotation

This bachelor thesis delves into the conservation and diversification of Polycomb Repressive Complex (PRC2) subunits in the model moss *Physcomitrium patens*. Through *in-silico* studies, gene expression analysis, and yeast two-hybrid experiments, it highlights the evolutionary relationships, gene expression patterns in distinct developmental phases, and interactions of PRC2 subunits. The findings extend our comprehension of the role that PRC2 plays in moss development and establish a platform for further exploration, extending our understanding of PRC2 engagement in plant development.

Declaration

I declare that I am the author of this qualification thesis and that in writing it I have used the sources and literature displayed in the list of used sources only.

Date 14. 12. 23 Signature Sonja Hanl

České Budějovice

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Abstract

This bachelor thesis delves into the conservation and diversification of PRC2 subunits, examining the moss species *Physcomitrium patens* (Pp), which is a well-established bryophyte model species providing insights into early land plant evolution. The study involved *in-silico* identification and classification of PRC2 subunits in plants, as well as an expression analysis of *P. patens* PRC2 subunit genes at different developmental stages (protonema and leafy gametophyte) and time points using quantitative reverse transcription PCR (qRT-PCR) and semi-qRT-PCR. Additionally, interactions between PRC2 subunits of moss and *Arabidopsis thaliana* were investigated using the yeast two-hybrid technique.

The phylogenetic analysis confirmed that PRC2 subunits are clustered into four distinct clades {E(z), Su(z), Esc, and P55}, and revealed that moss PRC2 subunits share a close relationship with counterparts from other plant species, suggesting their conservation across different lineages. Gene expression analysis showed noticeable differences in the gene expression of *PpCLF* and *PpEMF2* between protonema and leafy gametophyte, indicating their potential roles in maintaining specific developmental states. Yeast two-hybrid experiments confirmed the physical interaction of moss PpCLF with *Arabidopsis* (*At*) CLF and AtEMF2, supporting functional conservation of interaction surfaces throughout land plant evolution.

Overall, the study highlights the evolutionary conservation of the PRC2 core subunit proteins in land plant evolution. This research advances our understanding of moss PRC2 subunit expression patterns in two distinct developmental stages and aids in analysing the conservation of the PpCLF interaction surface with the AtPRC2 subunits, providing valuable knowledge that paves the way for future research.

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1. Introduction

1.1. PcG Proteins and Epigenetic Regulation in Cellular Development

Eukaryotes, like other organisms, have genomes made of deoxyribonucleic acid (DNA), which forms a double helix. The expression of traits is influenced by both the sequence and three-dimensional structure of the DNA helix. It is believed that while the DNA sequence contributes to this process, the regulated gene expression is the key mechanism facilitating the generation of traits. Eukaryotic DNA, which is wrapped around an octamer of histone proteins, forms nucleosomes, the fundamental structural components of chromatin. Due to the DNA being tightly packed in nucleosomes and the possibility of higher order chromatin structure, complex transcriptional control machinery is needed for transcriptional regulation (Paro et al., 2021).

A single cell, called zygote, forms after fertilization in eukaryotic organisms and, via division and differentiation of the cell, multicellular tissues as well as organs with varying functions and morphology are formed (Khanday & Sundaresan, 2021). During the complex process of development, cells must maintain distinct transcriptional networks which determine their identities, even after each division. Their cellular memory ensures the preservation of developmental decisions and specific functions (Paro et al., 2021; Wolpert et al., 2015). The determining factor for differentiation into specialized cells with different characteristics has been studied in *Drosophila melanogaster*, where homeotic genes specify the development of different body parts. The group of genes influencing cellular identities is categorized into two groups: the Polycomb group (PcG) and the Trithorax group (TrxG). The first descriptions of two PcG genes, Extra sex combs (Esc) and Polycomb (Pc), were provided by Lewis and colleagues in *Drosophila melanogaster* (Lewis, 1947; Lewis, 1978). PcG mutant cells reactivate specific Hox genes inappropriately, leading to the transformation of one body segment into another (Struhl, 1983; Jurgens, 1985). This implies that PcG functions to maintain the repressed state of Hox genes in *D. melanogaster* (Pirrotta, 1997).

One of the functions of TrxG proteins is to counteract Polycomb group (PcG)-mediated gene repression through TrxG-mediated H3K4me3 and other post-translational modifications associated with TrxG complexes. PcG proteins are responsible for maintaining stable gene expression patterns across cell divisions and play a pivotal role in controlling cell proliferation (Paro et al., 2021). In *Drosophila*, PcG mutations, resulting in the ectopic formation of

developmental structures, have been observed. These mutations are referred to as homeosis or homeotic transformations (Deutsch, 2010). In plants, homologous PcG proteins have been discovered, where genetic mutations frequently lead to those homeotic transformations (Chanvivattana et al., 2004; Goodrich et al., 1997). Thus, PcG proteins that function as transcriptional regulators seem to be well conserved throughout evolution and they are crucial for cellular development (Whitcomb et al., 2007).

The concept of epigenetics was first mentioned by Conrad Waddington (1942), who described how genes and their products interact, thereby influencing the expression of phenotypic traits. Over time, the term epigenetics has been redefined and is nowadays described as the collective heritable phenotypic changes resulting from processes occurring independent of the DNA sequence (Tollefsbol, 2017). Epigenetics involves changes to chromosomal regions that can mark, signal, or sustain altered activity states without changing the DNA sequence. These alterations are essential for regulating gene expression and ensure consistency throughout multiple cell generations (Bird, 2007). The principal methods of epigenetic regulation involve RNA-based mechanisms (small and long non-coding RNA processes), DNA methylation, and histone modifications. Histone methylation, including the trimethylation of histone H3 on lysine 27 (H3K27me3), is a crucial post-transitional modification, which involves the addition of three methyl groups on the amino group of lysine residues. These modifications are substantial for various cellular processes such as gene silencing (Zhang et al., 2021).

1.2. Comparison of PRC2 Composition in *Drosophila* and *Arabidopsis*

The proteins of the PcG constitutes the Polycomb repressive complex 1 (PRC1) and the Polycomb repressive complex 2 (PRC2). These protein complexes act as silencers of genes due to histone modifications (Kim & Sung, 2014). By studying *Drosophila* embryos, scientists succeeded in identifying the four core subunits of PRC2: Enhancer of Zeste (E(z)), Extra Sex Combs (Esc), NURF-55 (P55) and Suppressor of Zeste 12 (Su(z)12) (Müller et al., 2002). These PRC2 subunits exhibit different functions. E(z), and its homologs, are histone methyltransferases (HMTases) and play an important role in development and regulation of genes (Holoch & Margueron, 2017). E(z) contains a SET (Su(var)3-9, Enhancer-of-zeste and Trithorax) domain acting as an enzyme for histone methylation whose activity is boosted by Esc (Cao et al., 2002). Esc generally functions as a stabilizer and enhancer of catalytic activity of PRC2. The two subunits Su(z)12 and Nurf55 are required for nucleosome binding

(Margueron et al., 2009) and play crucial roles in mediating interactions with accessory subunits within PRC2. In mammals, Su(z)12, for instance, contributes to chromatin binding and regulation. It exhibits two unique structural platforms defining distinct classes of PRC2 complexes for chromatin binding. Notably, *Drosophila* and mammals have a conserved PRC2 core. Additionally, Su(z)12 it is involved in interactions with accessory subunits like Aebp2 and Phf19, as well as nucleosome binding facilitated by Jarid2 and Aebp2 (Chen et al., 2018). Altogether, the PRC2 subunits play an essential role in maintaining H3K27me3 marks, ensuring the stability of chromatin structure during cell division. (Margueron et al., 2009; Steffen and Ringrose, 2014).

As a plant model, *Arabidopsis* has been extensively used in plant biology research to understand the basic mechanisms of several important molecular and physiological processes (Meinke et al., 1998). *Arabidopsis* belongs to the mustard family (Brassicaceae) as a representative of this simple angiosperm, or flowering plant. Its genome, consisting of approximately 120 megabases in haploid state, comprises of five chromosomes with an estimated number of 20,000 genes. In plants, the corresponding orthologs subunits of PRC2 have been identified in the model crucifer *Arabidopsis* (Figure 1). The three orthologs of E(z) are *CURLY LEAF* (CLF*), MEDEA* (MEA) and *SWINGER* (SWN) (Chanvivattana et al., 2004; Goodrich et al., 1997; Grossniklaus et al., 1998; Luo et al., 1999). *Arabidopsis* also has three Su(z)12 orthologs, namely Fertilisation independent seed (FIS2), *EMBRYONIC FLOWER 2* (EMF2) and *VERNALISATION 2* (Gendall et al., 2001; Luo et al., 1999; Yoshida et al., 2001), and one single homolog for Esc which is *FERTILIZATION INDEPENDENT ENDOSPERM* (FIE) (Ohad et al., 1999). As for P55, *MULTICOPY SUPPRESSOR OF IRA 1-5* (MSI1-5) are homologous and MSI1 was found to be in association with the PRC2 complex of *Arabidopsis* (Ach et al., 1997; Pazhouhandeh et al., 2011). *Arabidopsis* PRC2 can be categorized into the three distinctive Su(z)12 homologous complexes FIS2, EMF2 and VRN2. MSI1 and FIE constitute common subunits being constitutively expressed in all PRC2 subunits, whereas MEA and FIS2 have restricted expression in seed tissues, the female gametophyte (Kim & Sung, 2014) and early embryo (Simonini et al., 2021).

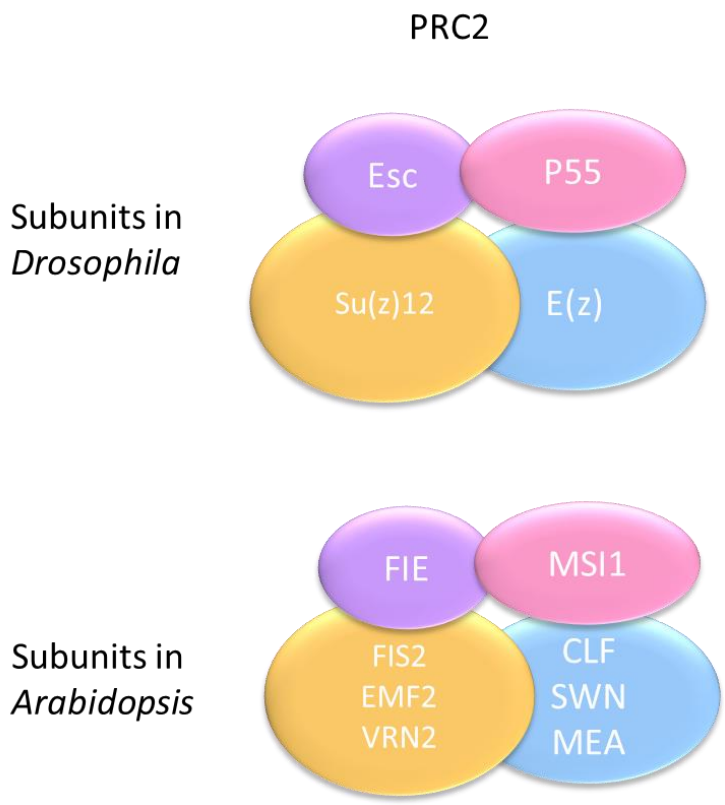

Figure 1: Conserved components of PRC2 between Drosophila and Arabidopsis. Core components of Drosophila PRC2 and the homologous subunits in Arabidopsis are depicted in corresponding colours.

1.3. Functions of PRC2 Subunits in Plants

PRC2 is responsible for the trimethylation of histone H3 on lysine 27 (K27) (Figure 2), (H3K27me3) (Simon & Kingston, 2013). The hallmark histone modification H3K27me3 is crucial for PcG silencing. (Grossniklaus & Paro, 2014; Nekrasov et al., 2007). The activity of methyltransferase (MTase) is contained in the PcG complex, more precisely, the Esc/E(z) complex (Czermin et al., 2002). The product H3K27me3 is a functional chromatin mark that is associated with silencing by PcG proteins (PcG silencing), which means the repression of gene expression (Simon & Kingston, 2013).

The PcG complex involves multiple proteins and their interactions with nucleosomes to establish and sustain the silenced state (Pirrotta, 1997). In addition, PRC2 in plants responds quickly to developmental or environmental stimuli. Its role in regulating developmental transitions in plants is crucial (Grossniklaus & Paro, 2014). While the function of H3K27me3

has been extensively studied, its precise roles in various biological processes continue to be an active area of research.

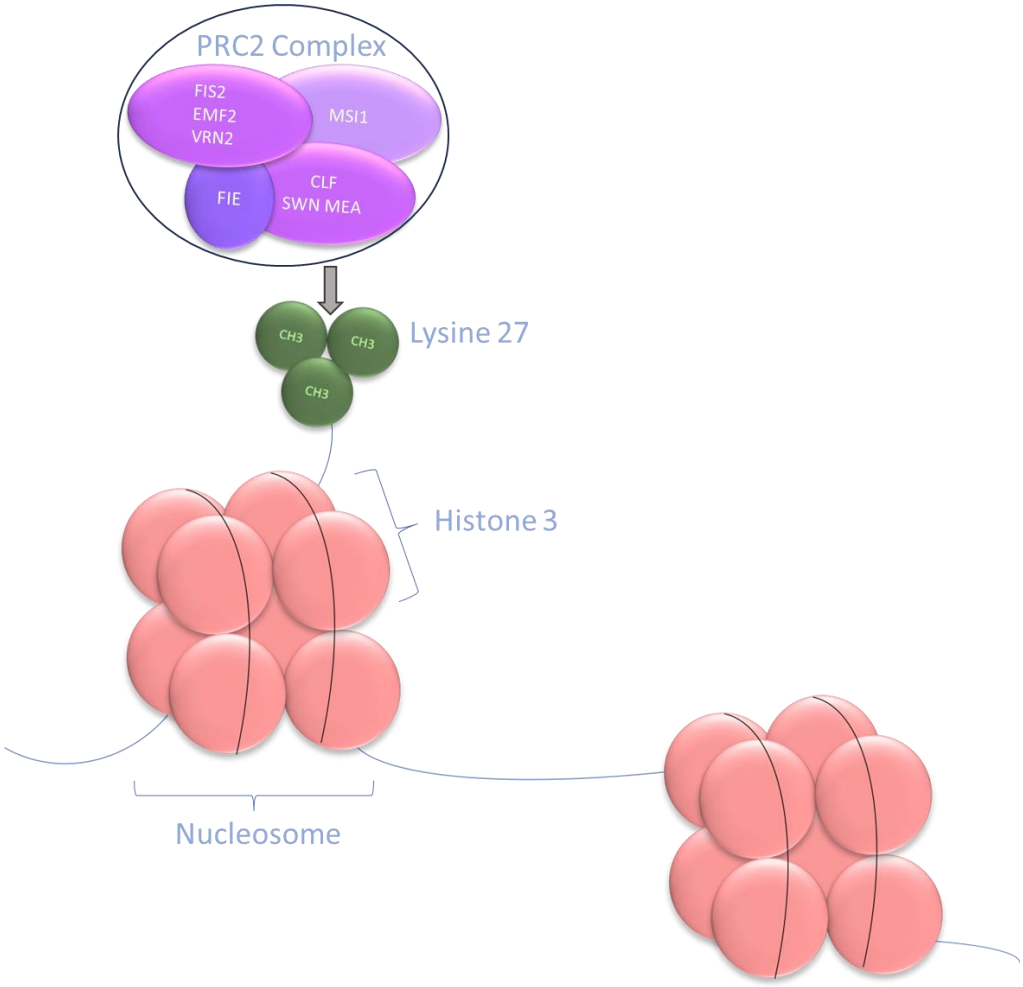

Figure 2: Methylation of histone H3 at lysine 27 (H3K27me3) by PRC2. This complex maintains gene transcriptional repression and plays an essential role in the maintenance of cellular identity as well as normal organismal development.

To date, numerous investigations have affirmed the role and functionality of PcG proteins in flowering plants. Evidence suggests that PRC2 is conserved throughout the green lineage. Mutant lines of PRC2, particularly those impacting catalytic subunits like CLF and SWN in *Arabidopsis*, display inherent defects in vegetative development, characterized by inadequately differentiated shoot organs. Additionally, these mutants form ectopic callus-like structures, within which the presence of cotyledon-like organs and somatic embryos has been observed (Mozgova et al., 2017). Both, CLF and SWN, play an important part in regulating plant development and might play a significant role in suppressing seed maturation in the seedling stage (Shu et al., 2019) Expression or repression of homeotic genes is regulated by CLF in

Arabidopsis. CLF represses the AGAMOUS (AG) and PISTILLA (PI) genes, two floral homoeotic genes found in vegetative tissue. The AG gene specifies the identity of stamen and carpel in *Arabidopsis* (Goodrich et al., 1997; Yanofsky et al., 1990; Yoshida et al., 2001). It is suggested that EMF2 and CLF operate within the same pathway for phase transitions by regulating the appropriate expression of floral homeotic genes (Yoshida et al., 2001). PRC2 proteins not only control phase transitions in seed plants. In mosses, similar results have been documented, where the removal of *P. patens CLF* (*PpCLF*) led to apogamy with the deletion lines showing the development of a sporophyte-like body originating from gametophytic cells (Okano et al., 2009). *PpFIE* similarly demonstrates exclusive expression in the apical gametophytic cell, and the absence of PpFIE hinders gametophyte development in the absence of fertilization. This suggests a conserved function between PpFIE and AtFIE (Mosquna et al., 2009).

In *Arabidopsis*, seed development is, to a large extent, regulated by MEA, FIE, and FIS2. These PcG proteins control the proliferation of embryo and endosperm (Köhler et al., 2003a; Simonini et al., 2021). For instance, the formation of an endosperm is prevented by FIS2 when no fertilization occurs. Furthermore, FIS2 inhibits proliferation of the endosperm after fertilization. EMF2 and FIS2 seem to have homologous structure and functions. EMF2 plays a significant role in the development of the sporophyte. In *Arabidopsis*, EMF genes were found to have an influence on phase transitions and shoot development. It is presumed that EMF genes are also involved in repressing the reproductive development in *Arabidopsis*. Mutations in EMF genes – EMF1 (connected to PRC1) and EMF2 – leading to loss of their function induce *Arabidopsis* to omit its vegetative shoot growth, causing the plant to flower directly. A range of early-flowering characteristics were observed when EMF2 was mutated, providing evidence for the effect of EMF2 on phase transitions (Grossniklaus et al., 1998; Luo et al., 1999; Yoshida et al., 2001).

In plant reproduction, the FIE gene plays an important role because it suppresses genes involved in the development of endosperm in the female gametophyte before fertilization. Studies show that the FIE protein often behaves analogously to WD Polycomb proteins in insects or other animals. Similar to other WD Polycomb proteins, for example in *Drosophila*, complexes between FIE and other PcG genes are formed in *Arabidopsis*, which causes repression of gene transcription (Ohad et al., 1999). Embryogenesis is maternally regulated by the PcG gene MEA, expressed in the endosperm of *Arabidopsis*. It affects the growth during embryonic development, causing excessive growth and thus death during seed desiccation. (Grossniklaus

et al., 1998). In addition, expression of MEA during early embryo formation impacts on developmental patterning and cell identity specification of the embryo (Simonini et al., 2021). MSI1 plays an important role in initiating seed development and also in the further progression (Köhler et al., 2003b). It shows a high expression in flowers and floral buds and is also assumed to be expressed in fruits (Hennig et al., 2003; Köhler et al., 2003b). MSI1 interacts with both, MEA and FIE, and is a crucial member of the MEA-FIE-MSI1 complex, which also plays an essential role in seed development. (Köhler et al., 2003b). The physical interaction of the RPD3-like histone deacetylase HDA6 with MSI1 was shown trough yeast-two hybrid experiments. Both partake in controlling the flowering time through histone modifications including H3K27me3 (Xu et al., 2022).

RETINOBLASTOMA RELATED 1 (RBR1), a plant counterpart to the human tumor suppressor Retinoblastoma protein (pRb) that is vital for seedling development via PRC2-mediated H3K27me3, interacts with PRC2, including FIS2, as part of a regulatory circuit involved in reproductive development and cellular differentiation. This interaction contributes to the repression of specific genes during seed development. Furthermore, RBR1 collaborates with MSI1 to down-regulate DNA METHYLTRANSFERASE 1 (MET1), resulting in the activation of MET1 targets, including FIS2, during female gametogenesis emphasizing the importance of FIS2 in the context of gene regulation and developmental processes, particularly in reproductive development (Godwin & Farrona, 2022).

PcG plays a significant role in processes affecting cellular memory, for instance vernalization in various plant species (Whitcomb et al., 2007). The term vernalization refers to promoting flowering of a plant on the basis of prior exposure to cold temperatures for a longer period of time. The PcG protein VRN2 is, among other functions, essential for the gene silencing of a flowering repressor gene (Sung & Amasino, 2004). It responds to vernalization with stimulation of floral transitions by repressing FLOWERING LOCUS C (FLC), a powerful repressor of flowering, in *Arabidopsis* (De Lucia et al., 2008; Kim et al., 2009). Amongst the plant kingdom, multi-protein complexes of PcG proteins, which are evolutionally well conserved, are crucial for regulating plant developmental processes (Kim & Sung, 2014; Molitor & Shen, 2013).

1.4. *Physcomitrium patens* as a Model Organism

As non-seed plants, mosses hold significant importance as model organisms in the fields of plant development, genetics, and evolution. Their value in research was notably heightened

through the previous acknowledgment as the most primitive land plants (Cove et al., 1997). However, recent research proposes a reduction in complexity in the evolutionary path of bryophytes (Harris et al., 2022). The life cycle of moss involves two stages: the haploid (one set of chromosomes) gametophyte stage and the diploid (two sets of chromosomes) sporophyte stage. The gametophyte produces gametes via mitosis, which subsequently combine to form zygotes that grow into the sporophytes (Figure 3). The sporophyte depends on the gametophyte for maintenance and nutrient supply. It produces spores via meiosis which germinate to form new gametophytes. In the majority of moss species, spores germinate into a filamentous structure, referred to as protonema, which comprises chloronema and caulonema. This development then leads to the production of leafy gametophytes, within which spores are formed (Figure 3) (Cove et al., 1997; Wettstein, 1924). Often, scientists focus on the gametophytic stage of moss as the haploid gametophyte stage allows for direct observations of recessive mutations (Cove et al., 1997).

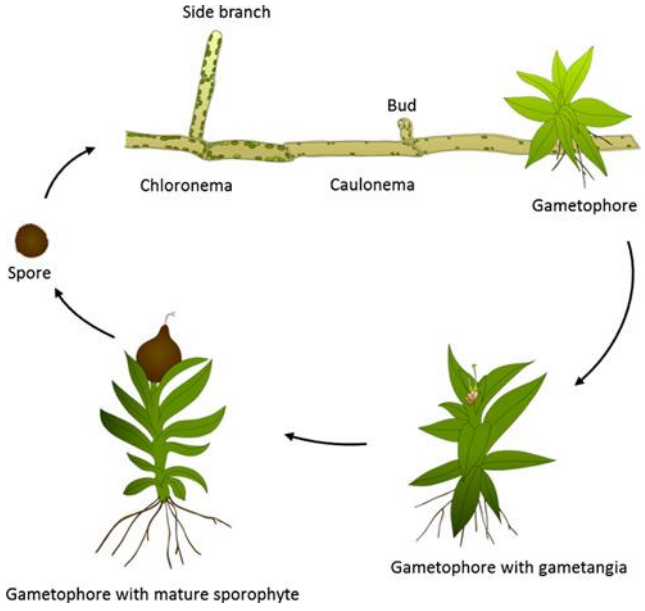

Figure 3: The P. patens life cycle (Image acquired from Lang et al., 2018).

Within the class of bryophytes, several species, including *P. patens*, have been selected for genome sequencing due to their placement in the sister lineage of vascular plants, making them valuable candidates compared to other well-known model systems. Along with other nonvascular plants, such as *Marchantia polymorpha* (Berger et al., 2016; Bowman et al., 2016), *P. patens* has, ever since its discovery, been a valuable model species for research regarding nonvascular plant biology, evolutionary development inquiries and reprogramming of stem cells (Rensing et al., 2020). In addition, due to the presence of apical stem cells, *P. patens* can

help investigate conserved characteristics and evolutionary processes regulating stem cell maintenance in plants (Hata & Kyozuka, 2021). Since *P. patens* is phylogenetically situated between seed plants and algae, this moss species can be valuable for both, developmental and evolutionary studies (Reski, 1998; Xiao et al., 2011). In research, various ecotypes of *P. patens* are utilized, with the most frequently used type being Reute (Hiss et al., 2017).

Similar to *Arabidopsis*, the protein complex PRC2 is conserved in *P. patens*, playing an essential role in gene regulation by chromatin modifications. Genes, such as *PpFIE* and *PpCLF* are important factors in stem cell regulation. Mutants lacking these genes showed defects in gametophore bud development and occasionally produced sporophyte-like structures (Mosquna et al, 2009; Okano et al, 2009). While PRC2 genes appear to suppress stem cell activity in mosses, their impact on apical cells can differ depending on the specific type of apical cell (Hata & Kyozuka, 2021).

Recognizing the vital role of PRC2 proteins, their importance extends across plant evolution, demonstrating functional conservation from ancestral to modern plants in crucial epigenetic regulatory processes. Investigating the function of PcG in early land plants can provide valuable knowledge to help understand land plant evolution as well as the evolution of distinct cell types and multicellularity. Additionally, knowledge about the PcG function contributes to the comprehension of phase transitions and gene regulation. Furthermore, examining the expression patterns of PRC2 subunits in different developmental stages provides crucial information on their dynamic roles throughout the plant life cycle and their involvement in various developmental processes. In addition, gaining insight into interactions among PRC2 subunits can elucidate shared mechanisms of PRC2 composition and, ultimately, mechanisms of repression. By addressing these questions, this thesis aims to contribute to the broader knowledge of the complex roles of PRC2 proteins in plant evolution, delving into their expression patterns, subunit interactions, and contributions to epigenetic regulation.

2. Aims

The aim of this thesis is to perform *in-silico* identification and classification of PRC2 subunits in plants with focus on bryophytes, specifically the moss '*Physcomitrium patens'*. Furthermore, this thesis aims to determine the expression of *P. patens* PRC2 subunit-transcripts in two distinct developmental stages and tissues – the filamentous protonema and leafy gametophyte – at various time points. Finally, this thesis aims to analyse mutual interactions of moss PRC2 subunits as well as interactions with *Arabidopsis* PRC2 subunits.

3. Materials and Methods

3.1. Phylogeny Reconstruction of PRC2 Subunits

A phylogenetic tree provides insight into the evolutionary relationship between proteins within the plant lineage (Morrison, 1996). By creating a phylogenetic tree, the relationship of PRC2 subunits from *Drosophila* and different species of Viridiplantae, including *Physcomitrium patens*, was studied. Viridiplantae are comprised of green algae and land plants (Becker, 2007). First, the *Arabidopsis* PRC2 sequence was used as a template in NCBI Protein BLAST[1] to obtain different PRC2 sequences for selected species based on sequence homology. Subsequently, protein sequences of the different PRC2 subunits from various plant species, as well as sequences from *Drosophila melanogaster* were gathered. For this, the websites NCBI (National Center for Biotechnology Information) (Sayers et al., 2022), NCBI BLAST (Basic Local Alignment Search Tool) (Altschul et al., 1990) and Phytozome v13 (Goodstein et al., 2012) were utilised. In the course of this project, the following protein sequences were examined:

Table 1: Selected species for phylogeny reconstruction.

1	*Arabidopsis thaliana*
2	*Capsella grandiflora*
3	*Brassica rapa FPsc*
4	*Gossypium raimondii*
5	*Zea mays*
6	*Oryza sativa*
7	*Brachypodium distachyon*
8	*Selaginella moellendorffii*
9	*Physcomitrella patens*
10	*Chalamydomonas reinhardtii*
11	*Drosophila melanogaster*

[1] https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins

The BLAST search was executed by first searching for the desired subunit in the intended species using the NCBI database based on blastp (Protein BLAST). Protein sequences were selected and subsequently inserted into the BLAST search and, to ensure the collected sequences were suitable for the alignment, the query coverage in percent and the percent identity of each sequence was then examined with NCBI BLAST (Protein BLAST). The threshold for query coverage and percentage identity was set to be greater than 95% with a BLAST E-value of 0.0.

Aligning sequences is essential for building phylogenetic trees and helps in detecting similarities and differences of the aligned sequences to allow construction of a phylogenetic tree. Utilising the software MEGA11 (Molecular Evolutionary Genetics Analysis version 11) (Tamura et al., 2021), the alignment was done using "ClustalW" (Thompson et al., 1994). In the last step, a phylogenetic tree was constructed. For the construction of the phylogenetic tree, the "neighbour joining method" was applied. This method is based on identifying pairs of operational taxonomic units (OTUs), the so-called neighbours that minimize branch length during the stepwise clustering of OTUs (Saitou & Nei, 1987).

3.2. Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) of *P. patens* cDNA

3.2.1. Moss Cultivation and Collection

For the purpose of analysing the expression of transcribed PRC2 genes of *P. patens* (ecotype Reute), moss was cultivated on MM-plates (BCDAT plates). The preparation of these agar plates was done according to Table 2. Solution B, C and D, as well as ammonium tartrate, calcium chloride and alternative TES, had previously been prepared according to Tables 3-8. The mixture for the MM-plates was prepared by adding all ingredients to a glass bottle and sterilized by autoclaving for 15 minutes at approximately 120 °C. Subsequently, the mixture was cooled down to approximately 40-50 °C, before pouring the solution into the petri dishes. The plates were cooled down to solidify and stored upside down at room temperature.

To avoid contamination, the whole procedure was performed under a fume hood. Sterilized tweezers were used to transfer small amounts of protonema from previously grown *P. patens* onto fresh agar plates. These plates were stored in a cultivation room allowing *P. patens* to grow under bright fluorescent bulbs (16 hours light, 8 hours dark) and constant temperature (25 °C).

Table 2: Preparation of MM-plates for P. patens cultivation.

Reagents	Amount / mL or g
Distilled water	800 mL
Solution B	10 mL
Solution C	10 mL
Solution D	10 mL
Ammonium tartrate (500 mM)	10 mL
Calcium chloride (100 mM)	10 mL
Alternative TES (Tris(hydroxymethyl)-aminomethane-EDTA-salt solution)	1 mL
Distilled water	Filled up to 1000 mL
Agar	8 g

Table 3: Preparation of Solution B for MM-plates.

Reagents	Amount / mL or g
$MgSO_4 \cdot 7H_2O$	25 g
dH_2O	Filled up to 1000 mL

Table 4: Preparation of Solution C for MM-plates.

Reagents	Amount / mL or g
KH_2PO_4	25 g
dH_2O	700 mL
4M KOH	pH adjusted to 6.5 (approximately 15 mL)
dH_2O	Filled up to 1000 mL

Table 5: Preparation of Solution D for MM-plates.

Reagents	Amount / mL or g
KNO_3	101 g
$FeSO_4 \cdot 7H_2O$	1.25 g
dH_2O	Filled up to 1000 mL

Table 6: Preparation of 500mM Ammonium Tartrate (100x) for MM-plates.

Reagents	Amount / mL or g
Ammonium Tartrate	92.05 g
dH_2O	Filled up to 1000 mL

Table 7: Preparation of 100mM Calcium Chloride (100x) for MM-plates.

Reagents	Amount / mL or g
$CaCl_2$	14.7 g
dH_2O	Filled up to 1000 mL

Table 8: Preparation of Alternative TES (1000x) for MM-plates.

Reagents	Amount / mL or mg
$CuSO_4 \cdot 5H_2O$	55 mg
H_3BO_3	61 mg
$CoCl_2 \cdot 6H_2O$	55 mg
$Na_2MoO_4 \cdot 2H_2O$	25 mg
$ZnSO_4 \cdot 7H_2O$	55 mg
$MnCl_2 \cdot 4H_2O$	389 mg
KI	28 mg
dH_2O	Filled up to 1000 mL

After the desired period of growth (7 days for protonema and 21 days for leafy gametophyte), *P. patens* was collected from the petri dishes. Protonema and leafy gametophyte were collected separately. At 7 days of growth, protonema was scraped off from the surface of MM-medium in the petri plate, the leafy gametophyte was cut off using scissors and collected separately at 21 days. Both *P. patens* samples were gathered in separate Eppendorf tubes. Care was taken to prevent mixing of both tissues. The collected samples were flash-frozen in liquid nitrogen and stored at -80 °C.

To replicate and achieve higher confidence in the results, the whole procedure of cultivation and collection of *P. patens* samples was done twice, one time with the conditions given above, and the second time collecting protonema after 7 days, and both tissue types after 14, 21 and 28 days of growth.

3.2.2. RNA Isolation and Total RNA Extraction

For the isolation of RNA, working under semi-sterile to sterile conditions is essential to avoid contamination and RNA degradation. The aim was to isolate RNA of *P. patens* using the Thermo Scientific MagMAX ™ Plant RNA Isolation Kit (Thermo Scientific, cat no. A33784). Total RNA isolation was performed according to the manufacturer´s instructions. Briefly, the collected *P. patens* samples (after 7, 14, 21, and 28 days) were transferred to sterile Eppendorf tubes and approximately 10 glass beads were added to break the samples in a bead beater homogenizer (Silamat S6, Ivoclar Vivadent AG, cat no. 0091011) by shaking the tube for 15 seconds. Lysis buffer solution was prepared (20 µL 2M DTT in 1 mL lysis buffer) and 300 µL of this buffer were added to each sample. The samples were then vortexed briefly, incubated for 5 minutes at 56 °C and centrifuged for 10 minutes. After centrifugation, the supernatant was carefully transferred to a sterile 1.5 mL tube. For the binding of RNA to magnetic beads, 12.5 µL of resuspended MagJET beads, followed by 200 µL of ethanol (96 %), were added to each sample. In order to uniformly distribute the beads, the samples were vortexed and briefly spun for 1-2 seconds. They were then placed on a magnetic rack to separate the beads from the liquid. After waiting 2 minutes, the supernatant was discarded, and the beads were washed with 350 µL of wash buffer 1. The samples, containing the beads and the wash buffer, were vortexed, briefly spun and placed on the magnetic rack, where they were left for another 2 minutes. Afterwards, the supernatant was removed and the samples were dried at room temperature for 5 minutes. DNase I solution was prepared (Table 9) and 100 µL were added to each tube.

Table 9: Preparation of DNase I solution.

Reagents	Volume / µL
2X DNase I Buffer	50
Reconstituted DNase I	1
Manganese Chloride Solution	10
Nuclease free water	39

Subsequently, the samples were vortexed briefly and incubated in the thermoshaker at 37°C for 25 minutes. 75 µL of rebinding buffer and 200 µL of ethanol (96 %) were added to the samples to cause rebinding of RNA to the beads. The samples were then vortexed and centrifuged as before and placed on the magnetic rack for 2 minutes. Thereupon, the supernatant was discarded, and the samples were washed two times with 350 µL of wash buffer 1. As before,

the sample was vortexed, spun and put on the magnetic rack. After 2 minutes, the supernatant was removed and the same procedure was repeated two more times, using wash buffer 2. After carefully removing the supernatant, the tubes were dried on the rack for 5 minutes and then removed from the rack. For elution, 25 µL of nuclease-free water were added, the samples were vortexed so that the beads were uniformly spread, centrifuged for 1-2 seconds, slowly and carefully placed on the magnetic rack, and left there for 2 minutes. The supernatant containing purified total RNA was collected. The concentration of each eluate was measured using the NanoDrop Spectrophotometer (product no. ND-1000) – by selecting the RNA concentration analysis with the 260/280 ratio 2. The isolated RNA was stored at -80 °C.

The lower quantity of the sample and the use of glass beads to break down the sample in the homogenizer possibly do not lead to sufficient quality of RNA for constructing the cDNA library. Therefore, an additional RNA isolation was performed with the samples collected after 7 (protonema) and 21 days (leafy gametophyte) in order to obtain a higher RNA concentration. This time, mortar and pestle were used for crushing the *P. patens* tissues. For this, liquid nitrogen was added repeatedly to both, the protonema and leafy gametophyte sample, and the mixture was ground thoroughly until a powder-like texture was obtained. Similar to the total RNA isolation, a lysis buffer was prepared and, subsequently, blended with the powdered tissue samples. The mixture was then transferred to sterile Eppendorf tubes. RNA was isolated as described above and was stored at -80 °C. Later, this RNA was used for gel electrophoresis to analyse the RNA products and cDNA synthesis. 1% agarose gel was prepared for gel electrophoresis by heating a mixture of 1 g of agarose and 100 ml TAE (Tris-acetate-EDTA) buffer. The TAE buffer was prepared by diluting 50x TAE buffer with distilled water to 1X TAE. After cooling down, one drop of ethidium bromide was added, resulting in a concentration of 0.5 µg mL^{-1}, and the mixture was cast on the gel electrophoresis tray with a well-forming comb. The gel was loaded with the samples (GeneRuler 1kb DNA Ladder (Thermo Scientific); 3 µL TriTrack DNA Loading Dye (Thermo Fisher Scientific)) and a voltage of 95 V was applied for 30 minutes.

3.2.3. First Strand cDNA Synthesis

Isolated RNA, prepared as described in 3.2.2., was used to perform cDNA synthesis. Due to RNA instability, complementary DNA (cDNA) is useful for further studying gene transcription using quantitative reverse transcription PCR (qRT-PCR). The cDNA synthesis was performed in a sterile Eppendorf tube, on ice.

First strand cDNA synthesis was carried out using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, cat no. K1621) following the manufacturer´s protocol. For both samples (7 days protonema and 21 days leafy gametophyte), a reaction was prepared as specified in Table 10. The samples were mixed, incubated at 65 °C for 5 minutes to allow for more precise annealing of hexamer primers to templates, put on ice, spun down briefly, and put back on ice again. Afterwards, the reaction mixture (Table 11) was added to each tube giving a total volume of 20 µL. Then, the samples were incubated at 25 °C for 5 minutes followed by incubation at 42 °C for 60 minutes. The reaction was finally heated to 70 °C for 5 minutes to terminate the reaction. The obtained cDNA was stored at -80 °C.

Table 10: Reaction mixture for cDNA synthesis containing RNA.

Substance	Volume / µL
RNA sample	10
Random Hexamer primer	1
nuclease-free water	1

Table 11: Reaction mixture added to RNA sample.

Substance	Volume / µL
5x Reaction buffer	4
RiboLock RNase Inhibitor (20 U μL^{-1})	1
10mM dNTP Mix	2
RevertAid M-MuL V RT (200 U μL^{-1})	1

3.2.4. Semi-qRT-PCR and Real-Time PCR

The goal of the polymerase chain reaction (PCR) was the amplification of the transcript of specific genes followed by analysing the products by agarose electrophoresis. For this purpose, a total of 12 mixtures were prepared, 6 with protonema cDNA as a template, and 6 with leafy gametophyte cDNA. The cDNA samples were diluted in a ratio of 1:2 with Milli Q H_2O (10 µL cDNA stock, 20 µL MilliQ H_2O). The reactions for the PCR were prepared according to Table 12, using the primers specified in Table 13. The PCR was then carried out applying the cycling parameters specified in Table 14. The gel electrophoresis was performed according to

Section 3.2.2., with the difference that this time, 1% agarose gel was prepared, and the separation was carried out for 25 minutes at 80 mA.

qRT-PCR was performed with the CFX Connect Real-Time PCR Detection System (1855201) using the primers listed in Table 13, except for *Su(z)_EMF2-LIKE isoform X3*, since it showed no amplification in the semi-qRT-PCR. The *ACTIN 5* gene, as well as the *E3 UBQ-LIGASE* gene (Table 17) were used as reference genes for the qRT-PCR. For each of the primer pairs of the selected subunits and the two reference genes, three PCR technical replicates for each growth stage, and three technical replicates for negative control were carried out, resulting in a total of 54 individual reactions. The cycling conditions are given in Table 16 with a total number of 40 cycles. From the obtained results, the mean normalized values of Relative Normalized Expression (MNE-values) of all tested genes were calculated for both reference genes by applying the $2^{-\Delta\Delta C_T}$ method (Livak & Schmittgen, 2001). The determined values were used to assess the difference in expression of PRC2 subunits in the two different developmental stages. The quality of the amplified products from the qRT-PCR was assessed by the melting analyses as part of the qPCR protocol and was further tested by performing 1% agarose gel electrophoresis. The gel was prepared according to Section 3.2.2., and 15 µL of sample were loaded per well.

Further, the gene expression of the studied genes *Esc_ FIE2-LIKE, MSI1-LIKE, MSI4-LIKE, E(Z) CLF-LIKE* and *EMF2-LIKE, isoform X1* in different *P. patens* tissues was investigated using the *P. patens* Gene Model lookup Database on the website *Physcomitrella* Expression Atlas Tool[2] (PEATmoss). PEATmoss is a database search of existing gene expression experiments unifying the available data on *P. patens* expression (Fernandez-Pozo et al., 2020).

[2] https://peatmoss.plantcode.cup.uni-freiburg.de

Table 12: PCR reactions comprising a total volume of 25 µL each.

Substance	Volume / µL
Water (Milli-Q)	19.8
10x Buffer (DreamTaq)	2.5
200 mM Forward Primer	0.5
200 mM Reverse Primer	0.5
2.5 mM dNTP	0.5
Template (cDNA)	1
Enzyme (Taq DNA Polymerase, DreamTaq, Thermo Scientific, cat no. EP0703)	0.2

Table 13: Primers used for PCR.

Gene	Primer	Accession number	Primer sequence 5´ - 3´
Su(z)_EMF2-LIKE isoform X3	Pp_219	XM_024522973.1 _F	GGGAGCTTTCGGTGAGAACT
	Pp_220	XM_024522973.1 _R	TGGTACGTCTGCATTTGGCT
Esc_ FIE2-LIKE	Pp_221	XM_024507506.1 _F	AGATTGTGCTATGGGAGCCG
	Pp_222	XM_024507506.1 _R	GACTTGCACTGGGGATGTGA
MSI1-LIKE	Pp_223	XM_024504334.1_F	CAGATCTTCCAGGGGCATGT
	Pp_224	XM_024504334.1_R	TGTTGCTAGCACCCATTCGT
MSI4-LIKE	Pp_225	XM_024540013.1_F	GTCCAGTGGTGCCCAGATAG
	Pp_226	XM_024540013.1_R	CCAAATCTGCAAAGTGCCCC
E(Z) CLF-LIKE	Pp_227	XM_024504837.1_F	AGTGCAATGTGAACGGCAAG
	Pp_228	XM_024504837.1_R	CTTTCCGCCCGTTTGACATC
EMF2-LIKE, isoform X1	Pp_229	XM_024509601.1_F	CGGCAGAGGCGGTTGG
	Pp_230	XM_024509601.1_R	TGGAGCACTTGAAGCACCAT

Table 14: Semi-qRT-PCR parameters (28 cycles).

PCR-step	Temperature / °C	Time / min or s
Initialization	95	1 min
Denaturation	95	30 s
Annealing	60	30 s
Extension	72	30 s
Final elongation	72	5 min
Final hold	4	∞

Table 15: qRT-PCR reactions comprising a total volume of 25 µL each.

Substance	Volume / µL
Water (Milli-Q)	10.4
Forward Primer (200 mM)	0.3
Reverse Primer (200 mM)	0.3
qPCR Master Mix (containing Cheetah™ Taq hotstart DNA polymerase, Evagreen®, cat no. 31003)	3
Template (cDNA)	1

Table 16: qRT-PCR parameters with a total of 40 cycles.

PCR-step	Temperature / °C	Time / min or s
Initialization	95	12 min
Denaturation	95	15 s
Annealing	60	20 s
Extension	72	20 s
Melt curve	65–95°C in 0.5 °C increments	each 0.05 s
Final hold	4	∞

Table 17: Primer details of reference genes ACTIN 5 (ACT5) and E3 UBQ-LIGASE (E3).

Reference gene	Primer	Primer sequence 5´ - 3´
E3	Pp_91	TGAACTGATGGGACTAGAGG
	Pp_92	TCTTTGCTTACTCACGATGAC
ACT5	Pp_33	ACCGAGTCCAACATTCTACC
	Pp_34	GTCCACATTAGATTCTCGCA

3.3. Yeast Two-Hybrid Analysis of Interactions Between PRC2 Subunits

The Yeast Two-Hybrid (Y2H) assay is a well-established technique for detecting protein-protein interactions in vivo. In the Y2H assay, a bait protein is expressed by fusing it with the Gal4 DNA-binding domain (DNA-BD). Simultaneously, other proteins of interest, designated as prey proteins, are expressed by fusing them with the Gal4 activation domain (AD) (Fields & Song, 1989; Chien et al., 1991). When the bait and prey fusion proteins interact, they bring the DNA-BD and AD into proximity, activating the transcription of three independent reporter genes responsible for encoding ADE2 (adenine), HIS3 (histidine), and MEL1 (α-galactosidase) (Figure 4).

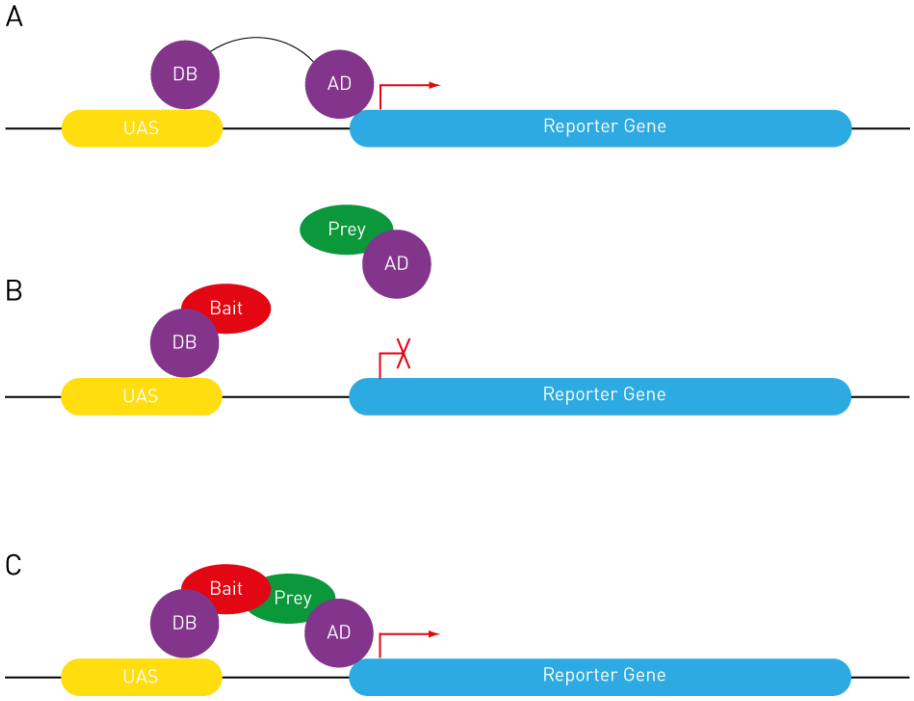

Figure 4: Principle of Y2H. The bait and prey proteins are fused separately to the binding domain (BD) and activation domain (AD) (Figure 4A). If bait-prey interactions occur (Figure 4C), RNA polymerase is activated by the complex and the reporter gene is expressed (Image acquired from Yang, 2023).

In our Y2H analysis we investigated the interactions of *P. patens* PRC2 subunits with the PRC2 orthologs from *A. thaliana*. Initially, yeast growth media (YPDA broth: yeast peptone dextrose adenine) were prepared and solidified in plates for further experimentation. Culture media were made by mixing 2 g agar, 5 g YPDA broth (Yeast Peptone Dextrose, Millipore Sigma, cat no. Y1375) and 0.4 mg adenine with 100 mL distilled water. The Erlenmeyer flask containing the prepared medium was sealed with a cotton plug, covered by aluminium foil, and subsequently sterilized in an autoclave. Under the fume hood, the cooled YPDA medium was carefully poured onto plates and left to solidify. Yeast cells (AH109 strain) were streaked on these plates to create colonies. The spreading over the medium was done using sterile loops carrying the yeast cells.

Gateway cloning provides an alternative to restriction cloning without the need for restriction enzymes. In a recombination process, consisting of two steps, an insert is transferred into a vector utilizing integration and excision processes with the attachment sites *attL* and *attB*. During the BP reaction, the first step of the recombination process, an entry clone with the DNA inserts, bordered by *attL* sites, is created. Subsequently, the LR reaction generates an expression clone with the DNA insert bordered by *attB* sites. This process is also shown in the scheme in Figure 5 (SnapGene, n.d.).

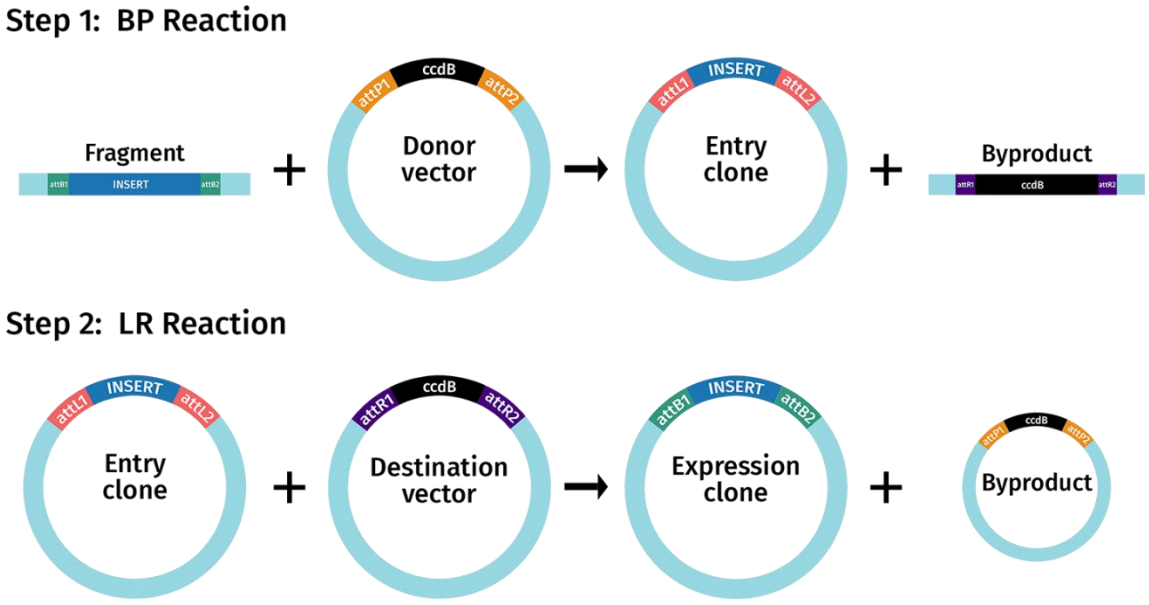

Figure 5: Scheme of Gateway cloning demonstrating the two-step recombination process (SnapGene, n.d.).

Gateway® technology (Invitrogen) was used to generate constructs to be used in Y2H. The *P. patens* PRC2 catalytic subunit (*PpCLF*) coding sequence (CDS), including the stop codon, was cloned into the Gateway pDONR221 ™ vector to create the entry clones. In particular, the

CDSs were amplified using primers containing *att*B site. The amplified products were then incubated with the pDONR221™ vector and BP Clonase ™ enzyme for 3-4 hours at 25 °C. The mixture was prepared according to Table 18. The primer details for the cloning of PpCLF are given in Table 19. The *Arabidopsis* subunits were used from the laboratory database.

Table 18: BP reaction.

Substance	Amount / µL or ng
attB PCR product (gene specific amplicons)	300-500 ng (6-8 µL)
pDONR221	200-400 ng (2-4 µL)
Enzyme (BP Clonase ™)	2 µL
Water (Milli-Q)	filled up to 10-12 µL

Table 19: PpCLF primer details.

Gene	Primer	Primer sequence 5´ - 3´
PpCLF	P.pCLF_F_attB5 (P.p170)	AAAAAGCAGGCTTCATGGCGTCCTCCAGCT
	P.pCLF_R_attB6 (P.p171)	AGAAAGCTGGGTTTTTAAGCAACTTTCTGTGCTCGTCC

Subsequently, proteinase K was added to the reactions, which were then incubated at 37 °C for 10 minutes. The resulting reactions were transformed into transformation-competent modified *E. coli* strain (TOP10) using the Heat Shock Method. The competent cells were thawed on ice for approximately 20-30 minutes, then plated on agar plates containing kanamycin (50 µg/mL) and incubated at 37°C. In a Falcon tube, 5 µL of DNA were combined with 100 µL of competent cells and mixed gently. The mixture was incubated for 30 minutes and subsequently heat-shocked by placing the tubes in a water bath (42°C) for 45 seconds. Subsequently, the tubes were placed on ice for 3 minutes. 1 mL of LB medium was added to the bacteria and the mixture was incubated and shaken for 45 minutes at 37°C. 50 µL of the mixture were plated onto Luria Broth (LB composition: 10 g peptone, 5 g yeast extract, 0.5 g NaCl per litre) plates containing kanamycin with the final concentration of 50 µg mL^{-1}. The cells were incubated overnight at 37 °C to allow for the colonies with the construct recombined into the pDONR221 vector to form.

Negative selection of non-transformed colonies was done by the presence of the *ccdB* killer gene present in the Gateway® cassette that was recombined out of the pDONR221 vector. A few individual colonies were screened using gene-specific primers (Table 20) through colony PCR to identify positive clones. Colony PCR is performed similarly to normal PCR with the

difference that a colony is added as a template instead of a plasmid. The positive clones were then incubated in 10 mL of LB medium overnight, and the plasmids were extracted using the GeneJet Plasmid Miniprep Kit (Thermo Scientific, cat no. K0503) following the manufacturer´s protocol.

Briefly, the samples were subjected to centrifugation, and the resulting pellets were reconstituted in 250 µL of resuspension buffer. Following this, 250 µL of lysis buffer were added, and the mixtures were incubated at room temperature for 5 minutes. After adding 300 µL of neutralization solution, the samples were vigorously mixed and subsequently centrifuged at 13,000 rpm for 10 minutes. The supernatant obtained was then transferred to filter tubes and centrifuged for 45 seconds at 13,000 rpm. Following this, wash buffer was added, followed by another centrifugation using the same settings. The remaining wash buffer was removed by spinning the filter tubes for two additional minutes. Finally, 30 µL of elution buffer were pipetted into the tubes, which were subsequently centrifuged for 2 minutes, collecting the eluate in clean Eppendorf tubes. The concentrations of the isolated plasmids were measured using the NanoDrop Spectrophotometer. This plasmid isolation step was essential to obtain purified plasmid DNA constructs before their transformation into yeast cells.

For generation of Y2H expression constructs the CDSs of the genes encoding the proteins of interest are fused with the DNA-binding domain (BD) or the activation domain (AD) of a transcription factor. To do this, the acquired pDONR221-PpCLF clones were used as entry clones for subcloning into Y2H-specific destination vectors, namely pGADT7-activation domain, using the LR Gateway® cloning technique with the LR reaction given in Table 20.

Table 20: LR reaction.

Substance	Amount / µL or ng
pDONR221	200 ng (1-2 µL)
Destination vector (pGBKT7)	200-400 ng (2-4 µL)
Enzyme (LR Clonase ™)	2 µL
Water (Milli-Q)	filled up to 10-12 µL

The entry vector (pDONR221-PpCLF) and the destination vector (pGBKT7) were kept with the LR recombination enzyme to perform the LR reaction discussed above by being incubated at 25 °C for 2-3 hours, after which they were transferred into transformation-competent TOP10 cells, similarly to the BP reaction described earlier. Constructs containing the CDSs of *A. thaliana* PRC2 subunits, including pGBKT7-*AtCLFΔSET*, pGBKT7-*AtEMF2*, pGBKT7-

AtMSI1, were obtained from the lab plasmid database. The resulting clones, integrated into yeast-specific vectors, were subsequently utilized for interaction screening through the Y2H system.

Before every transformation of yeast cells, it is necessary to freshly prepare the yeast transformation-competent cells. To do this, 6 mL YPAD medium were inoculated with yeast strains (AH109 strain) and incubated overnight by shaking at 30 °C. The preculture was then sub-cultured in 1:10 into 100 mL YPAD and incubated for 3-4 hours until an OD_{600} of 0.6 was reached. The cells were transferred to a 50 mL Falcon tube and centrifuged for 5 minutes at 1500 rpm. The cells were washed once with 30 ml of TE buffer (Table 21) and spun at 1500 rpm for 5 minutes. Subsequently, the supernatant was discarded, and the cells were resuspended in approximately 2 mL of TE/LiAc buffer (Table 22) to make the competent cells ready for transformation. The plasmids, in combination with the interaction setups, were mixed in an Eppendorf tube at a ratio of 1:1, with a final concentration of 200-300 ng/µl. Following that, 10 µl of carrier DNA (Herring Testes carrier DNA, Deoxyribonucleic acid sodium salt from salmon testes, Sigma-Aldrich Merck, cat no. D1626) was added from the stock concentration of 10 mg mL^{-1} and mixed with the plasmids. Subsequently, the plasmid, along with carrier DNA, was boiled at 100 °C for 6 minutes and cooled down on ice. Next, 100 µl of yeast competent cells were added to the same Eppendorf tube containing plasmid and carrier DNA, followed by adding 600 µL of PEG/LiAc buffer (Table 23). The mixture was vortexed and then incubated for 30 minutes at 30 °C. The tubes were inverted every 10 minutes for mixing the sample. Subsequently, 35.5 µL dimethyl sulfoxide (DMSO) were added and tubes were mixed by inverting. After 20 minutes of heat shock at 42 °C in a water bath, the cells were centrifuged for 5 seconds and resuspended in 100 µL TE buffer. 100 µL of the mixture were plated on minimal medium +HA (SD/+Ade/+His/-Leu/-Trp) and incubated for 2-3 days at 30 °C. The yeast transformation and screening plates were prepared by dissolving one package of SD broth (SD/-Ade/-His/-Leu/-Trp Broth, TAKARA, cat no. 630322) with or without adenine and histidine (3 mL each), along with 10 g of agar (Sigma Aldrich, cat no. L-2897), in 500 mL of distilled water, using a sterile magnetic stirrer.

To set up the final interaction on interaction screening plates with low stringency, +A-H (SD/ -Leu/-Trp/+Ade/-His), three colonies were cultured from each transformed plate in 1 ml of liquid broth (+HA in liquid) and incubated overnight on a 30°C shaker with a speed of 200 rpm. The next day, the samples were centrifuged at 1500 rpm for 5 minutes, and the OD values were measured. For all samples, the OD was maintained at 0.2 by diluting with sterilized TE buffer.

Then, a series of 1:10 serial dilutions were performed by adding 20 µL of stock cells to 180 µL of TE buffer each time, ranging from 10-fold to 1000-fold dilution. Subsequently, 5 µL of each dilution was spotted onto the screening plate +HA and +A-H plates using a multichannel pipette. Once the spots dried, the plates were stored at 30°C and incubated for 4-6 days to allow the cell to grow depending on the strength of the protein-protein interaction.

On the +AH media, growth is expected, independent of an occurring interaction between the two selected subunits as it only allows the selection for two plasmid transformations in a single yeast cell, whereas the +A-H media should only show growth in case of the two subunits interacting (weak interaction). Meanwhile, no growth should be observed on +A-H media, when empty vectors are used as a negative control.

Table 21: Preparation of TE by combining all components and autoclaving.

Reagents	Volume / mL
0.5 M EDTA	1
1 M Tris pH 8	5
Water	Filled up to 500 mL

Table 22: Preparation of TE/LiAc by combining all components and autoclaving.

Reagents	Amount / mL or g
0.5 M EDTA	0.2 mL
1 M Tris pH 8	1 mL
0.1 M LiAc	1.02 g
Water	Filled up to 100 mL

Table 23: Preparation of PEG/LiAc by combining all components and autoclaving.

Reagents	Amount / mL or g
0.5 M EDTA	0.2 mL
1 M Tris pH 8	1 mL
0.1 M LiAc	1.02 g
40 % PEG 4000	40 g
Water	Filled up to 100 mL

4. Results

4.1. Phylogeny reconstruction of the PRC2 subunits in selected plant species

A phylogenetic tree was constructed to investigate the evolution and separation of PRC2 subunits in multiple representative plant species and identify points of divergence into distinct clades. The phylogenetic tree revealed four distinct clades, Enhancer of Zeste (E(z)), Extra Sex Combs (Esc), NURF-55 (P55) and Suppressor of Zeste 12 (Su(z)12), representing the PRC2 subunits, highlighted in different colours (Figure 6).

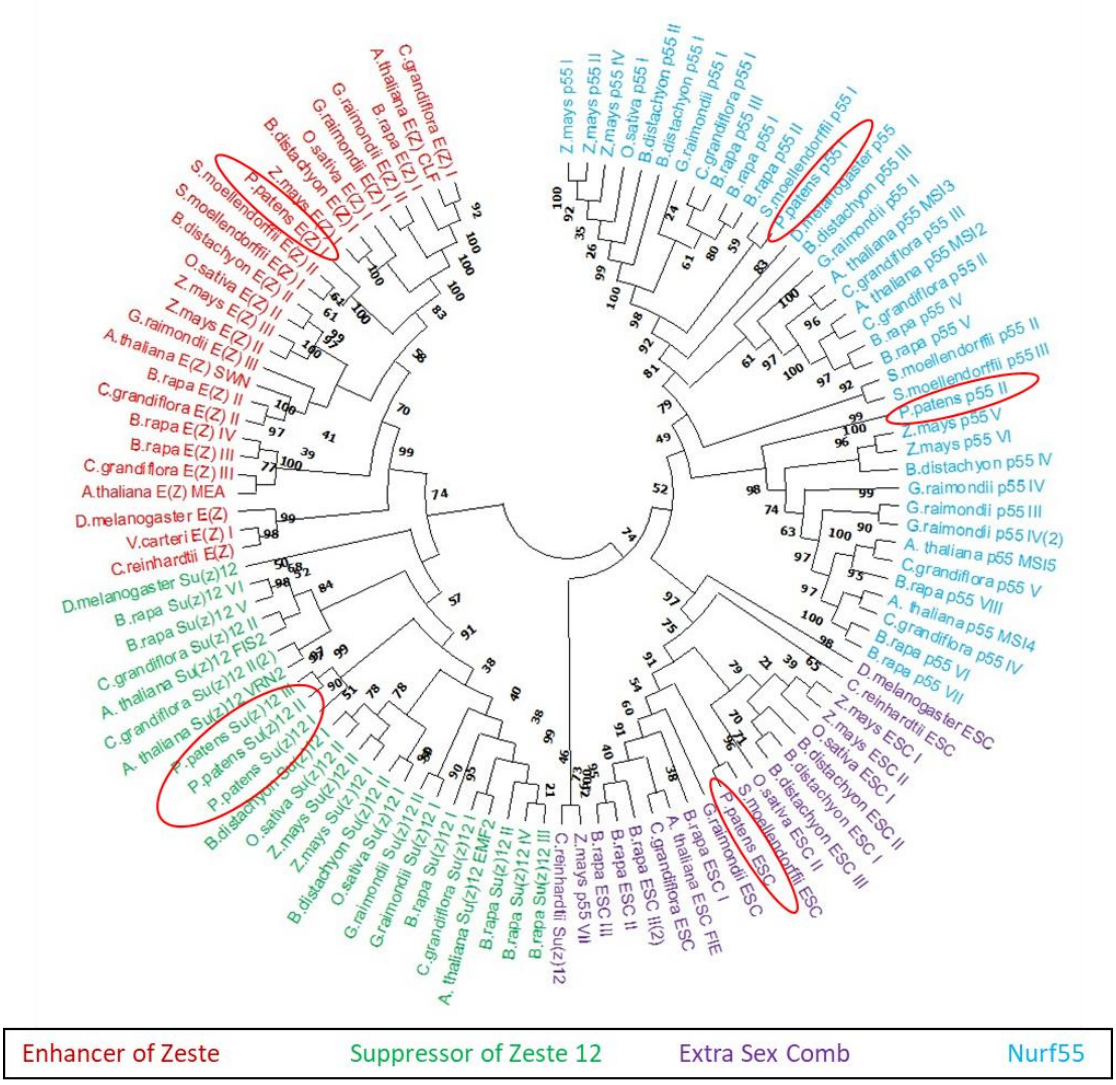

Figure 6: Phylogenetic tree of PRC2 proteins in representative plant species. The different protein sequences were aligned using ClustalW, and the phylogenetic tree was constructed using MEGA11 software with the neighbour joining method. The PRC2 subunits are clustered into four different clades, namely Enhancer of zeste (E(z)), Suppressor of zeste (Su(z)), Extra sex comb (Esc) and Nurf55/P55. Similarly, the moss PRC2 subunits are also found in the same cluster with the four different clades. Su(z) has three and P55 two homologues of PRC2 subunits of Physcomitrium patens, whereas the other subunits each have one.

Following the phylogenetic tree, the copy numbers of PRC2 subunits in each selected plant species were investigated. The variations in and distribution of PRC2´s core components across this evolutionary spectrum is displayed in Figure 7. The number of selected species, along with the accession numbers of each PRC2 subunit and their corresponding subunit are shown in Table 24.

In contrast to *Drosophila*, *P. patens* displayed a distinctive pattern with 3 copies of Su(z)12, 2 copies of P55, and 1 copy each of Esc and EZH suggesting a unique configuration in *P. patens* compared to *Drosophila*. *Arabidopsis*, a higher plant species, demonstrated a more complex distribution, featuring 5 copies of P55, 1 copy of Esc, and 3 copies each of EZH and Su(z)12. This combination points to a potential functional diversification of PRC2 subunits in the evolution of *Arabidopsis*.

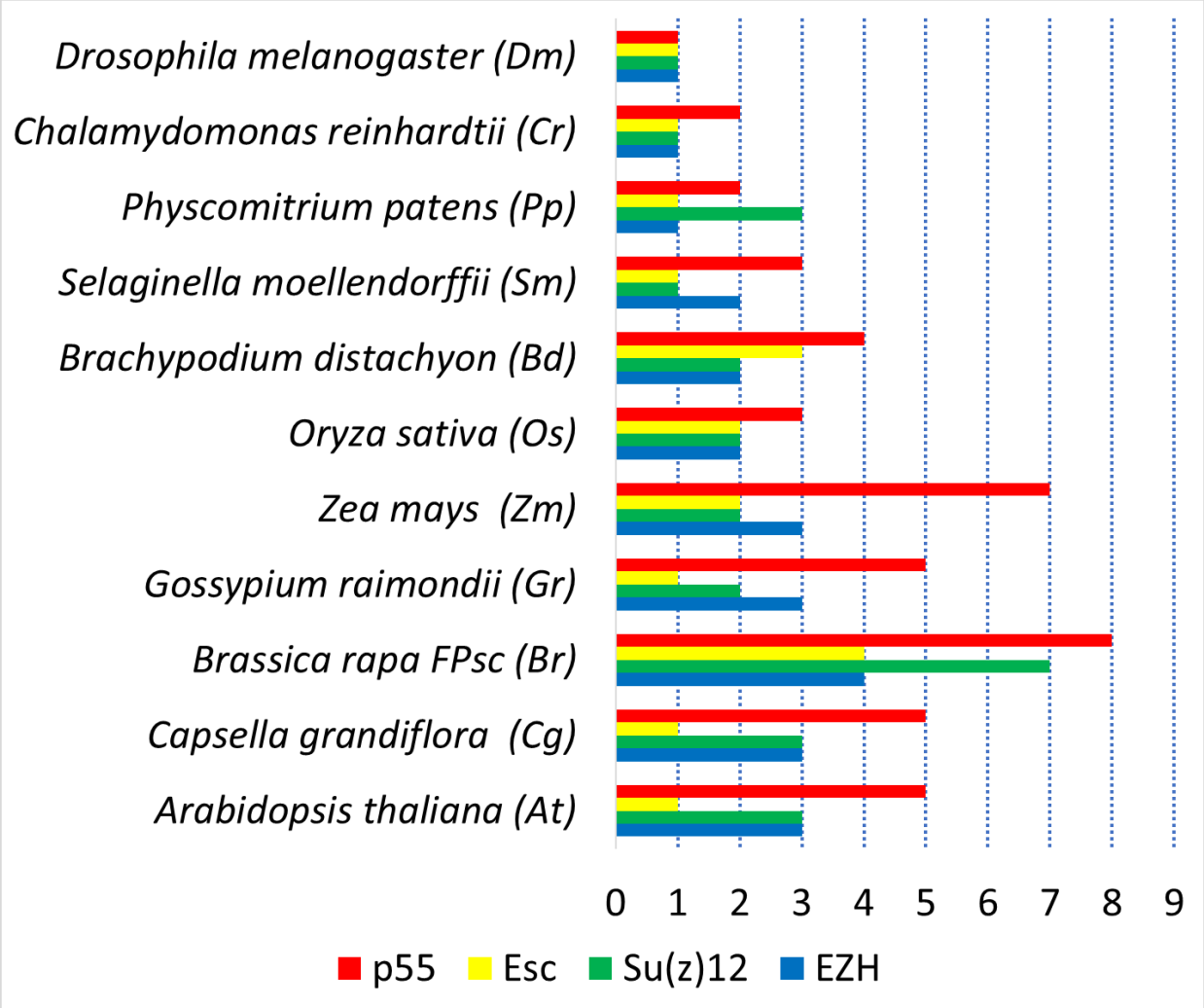

Figure 7: Number of PRC2 subunits in representative plants. Comparative visualisation showing the copy numbers of PRC2 subunit-encoding genes in the selected plant species across the green linage.

Table 24: Corresponding protein IDs of the sequences used in the phylogenetic tree.

Species	**EZH**	**PRC2-Su(z)12(EMF2)**	**PRC2-FIE**	**PRC2-MSI**
Arabidopsis thaliana (At)	NP_179919.1	NP_851168.1	NP_188710.1	NP_200631.1
	NP_567221.1	NP_565815.2		NP_179269.1
	NP_563658.1	NP_567517.1		NP_195231.1
				NP_565456.2
				NP_194702.2
Capsella grandiflora (Cg)	XP_006295423.1	XP_023639926.1	XP_006297945.1	XP_006282322.1
	XP_023636245.1	XP_023634371.1		XP_006297756.1
	EOA39212.1	XP_006295553.1		EOA16600.1
				XP_006300000.1
				EOA16474.1
Brassica rapa FPsc (Br)	XP_048633615.1	RID40579.1	XP_009110247.1	KAH0888789.1
	RID43252.1	CAG7892785.1	XP_009145589.1	RID41235.1
	RID40047.1	RID69385.1	RID63128.1	RID74040.1
	RID49101.1	RID69386.1	RID63131.1	RID44192.1
		KAG5389375.1		RID52879.1
		RID79083.1		RID44322.1
				RID48201.1
				RID77895.1
Gossypium raimondii (Gr)	KJB43777.1	XP_012466954.1	KJB81803.1	XP_012464258.1
	XP_012456470.1	XP_012472074.1		KJB12145.1
	Gorai.008G139100			KJB54377.1
				XP_012450880.1
				XP_012452224.1
Zea mays (Zm)	AQK80560.1	ONM09369.1	NP_001105182.1	XP_008665098.1
	AQL07170.1	ONM54143.1	NP_001105181.1	NP_001105556.1
	ONL97252.1			XP_020393498.1
				ONM38798.1
				AQK97793.1
Oryza sativa (Os)	XP_015644234.1	XP_015636445.1	XP_015649120.1	NP_001405311.1
	NP_001404538.1	BAF24739.2	XP_015649883.1	XP_015612432.1
				XP_015621921.2
Brachypodium distachyon (Bd)	XP_014752952.1	XP_010233774.1	KQJ94987.1	PNT66915.1
	XP_010228709.1	XP_014758742.1	XP_010234289.1	XP_003562012.1
			XP_003571503.1	XP_003576779.1
				XP_003569664.1
Selaginella moellendorffii (Sm)	EFJ18941.1	XP_002971532.2	XP_002966477.1	EFJ17852.1
	XP_002987466.1			EFJ07085.1
				EFJ05929.1
Physcomitrium patens (Pp)	XP_024360605.1	XP_024365369.1	XP_024363274.1	XP_024360102.1
		XP_024378746.1		XP_024395780.1
		XP_024376716.1		
Chlamydomonas reinhardtii (Cr)	XP_042915213.1	XP_042920472.1	XP_042915313.1	XP_001696907.1
				XP_042914781.1
Drosophila melanogaster (Dm)	NP_001137932.1	NP_652059.1	NP_524354	XP_017064818.1

4.2. Expression of *Physcomitrium patens* PRC2 Genes

To analyse the expression PRC2-subunit-encoding genes in the different developmental stages of *P. patens*, total RNA was isolated from the protonema and leafy gametophyte samples collected after 7, 14, 21, and 28 days of growing. The RNA concentrations were measured (Table 25), and a gel electrophoresis was performed (Figure 8).

Table 25: Measured concentrations of isolated total RNA from P. patens in ng μL^{-1}. P. patens samples of protonema and leafy gametophyte were collected after 7, 14, 21 and 28 days.

Tissue type	Growth period (days)	Concentration (ng μL^{-1})
Protonema	7	47.7
Protonema	14	38.3
Leafy gametophyte	14	33.4
Protonema	21	24.7
Leafy gametophyte	21	35.7
Protonema	28	53.9
Leafy gametophyte	28	27.7

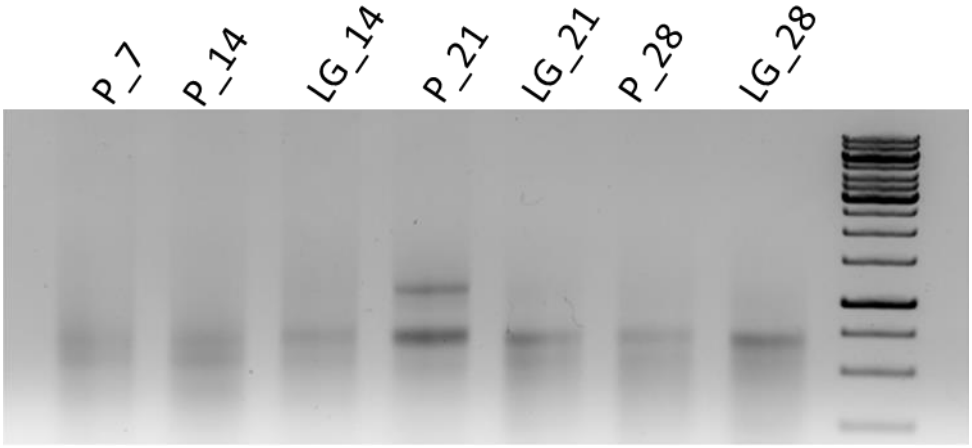

Figure 8: Gel electrophoresis for total RNA extraction from two different developmental conditions (P_Protonema, LG_leafy gametophytes) of P. patens after different growing periods (7, 14, 21, and 28 days) (200 ng loaded).

Due to the low concentrations, or, possibly, low quality of total isolated RNA obtained at various time points, the cDNA synthesis did not proceed as intended and the experiment needed to be repeated. A subsequent RNA isolation was conducted using a larger quantity of *P. patens* collected at days 7 (protonema) and 21 (leafy gametophyte). The resulting concentrations were measured and are presented in Table 26, while gel electrophoresis was performed as illustrated in Figure 9.

Table 26: Measured concentrations of isolated RNA from P. patens in ng μL^{-1}. The protonema sample of P. patens was collected after 7 days of growing and the leafy gametophyte sample after 21 days.

Sample	Growth period / days	Concentration / ng μL^{-1}
Protonema	7	168.4
Leafy gametophyte	21	582.5

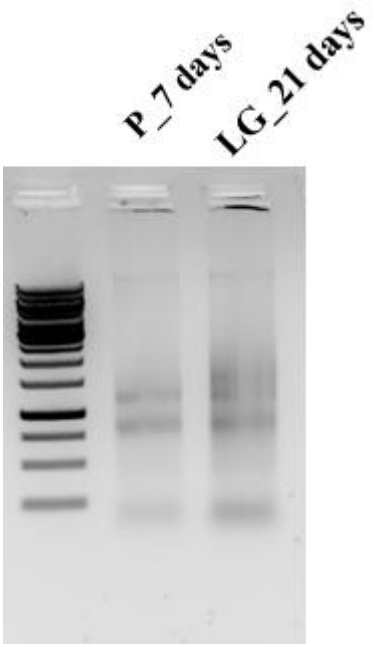

Figure 9: Isolated RNA from P. patens in two different tissue types (growth stages) after 7 days (P_Protonema) and 21 days (LG_Leafy gametophyte) (500 ng loaded).

The isolated RNA of days 7 (protonema) and 21 (leafy gametophyte) was further used for the cDNA synthesis. Thereupon, semi-qRT-PCR was performed using the synthesized random-primed cDNA and gene-specific primers. The amplicon was analysed by gel electrophoresis (Figure 10). Since *Su(z)_EMF2-LIKE isoform X3* showed no expression in either of the two developmental stages, it was not considered for further experiments within the scope of this thesis. Additionally, the expression of *Esc_FIE2-LIKE* seems very low at both developmental stages. Meanwhile, the protonema expression of *E(Z) CLF-LIKE* and *EMF2-LIKE, isoform X1*, seems to be higher in comparison to the leafy gametophyte stage and the other genes in general.

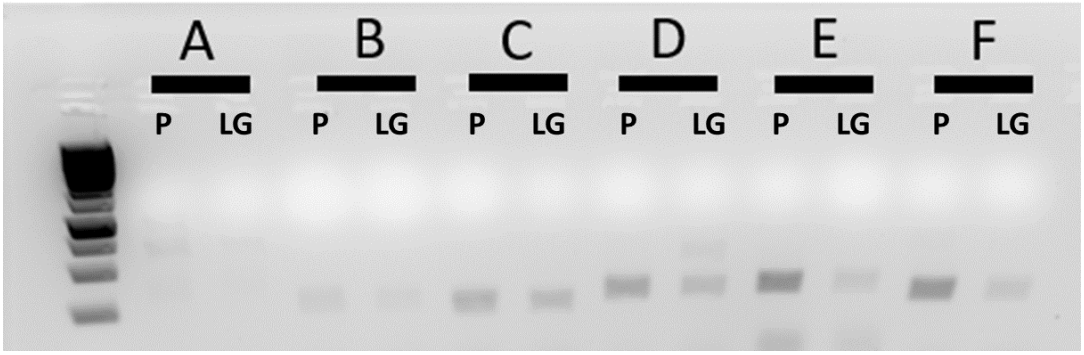

Figure 10: Gel electrophoresis for semi-qRT-PCR (28 cycles) (15 µL loaded). This figure displays the transcription level of different subunits of P. patens in protonema (P, 7 days) and leafy gametophyte (LG, 21 days) stages. In the given order, primers for the genes Su(z)_EMF2-LIKE isoform X3 (A), Esc_ FIE2-LIKE (B), MSI1-LIKE (C), MSI4-LIKE (D), E(Z) CLF-LIKE (E) and EMF2-LIKE, isoform X1 (F) were used..

qRT-PCR was performed using the same primers as for the semi-qRT-PCR, except for *Su(z)_EMF2-LIKE isoform X3*, which was omitted because it seemed not to be transcribed in the semi-qRT-PCR (Figure 10). *ACTIN 5* (Figure 11a) and *E3 UBQ_LIGASE* (Figure 11b) were used as reference genes. The MNE-values, displayed in Figure 11, were calculated from the obtained data from the qRT-PCR. *E(Z) CLF-LIKE* and *EMF2-LIKE, isoform X1* both show a higher expression in protonema compared to leafy gametophyte (Figure 11). Therefore, the genes *ESC_ FIE2-LIKE* (B), *MSI1-LIKE* (C), *MSI4-LIKE* (D), *E(Z) CLF-LIKE* (E) and *EMF2-LIKE, isoform X1* (F), as well as both reference genes, *ACT5* and *E3 UBQ-LIGASE*, were inspected (Figure 12).

No notable difference in the expression of *Esc_ FIE2-LIKE, MSI1-LIKE,* and *MSI4-LIKE, E(Z)* in the two different developmental stages at different time points is displayed in the graphs. However, the transcript expression of *CLF-LIKE* and *EMF2-LIKE, isoform X1* differs in the two developmental conditions (protonema and leafy gametophyte).

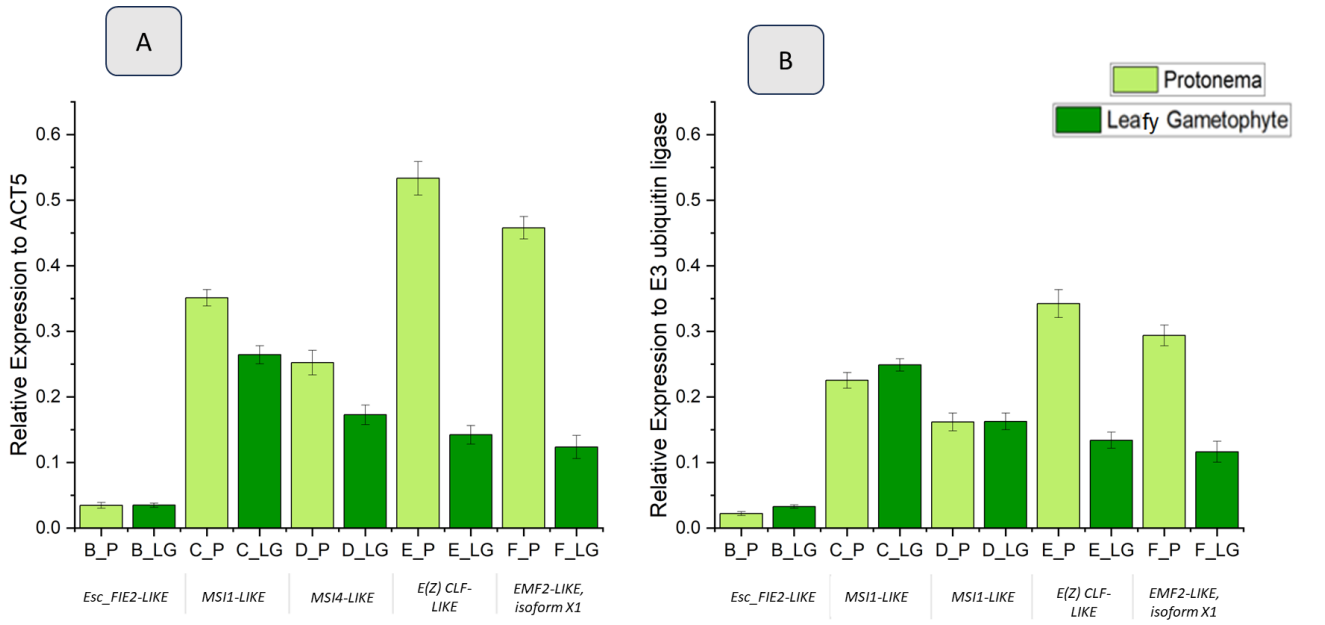

Figure 11: MNE-values of Relative Normalized Expression with ACT5 (11A) or E3 ubiquitin ligase (11B) as a reference gene. The MNE-values of Relative Normalized Expression were calculated using the values obtained from qPCR.

After the qPCR was done, the protonema and leafy gametophyte samples were further examined in a gel electrophoresis analysing *Esc_ FIE2-LIKE, MSI1-LIKE, MSI4-LIKE, E(Z) CLF-LIKE and EMF2-LIKE, isoform X1*. As expected, the negative control shows no expression. Only for E(Z) CLF LIKE (E), a lower expression in the negative control can be observed.

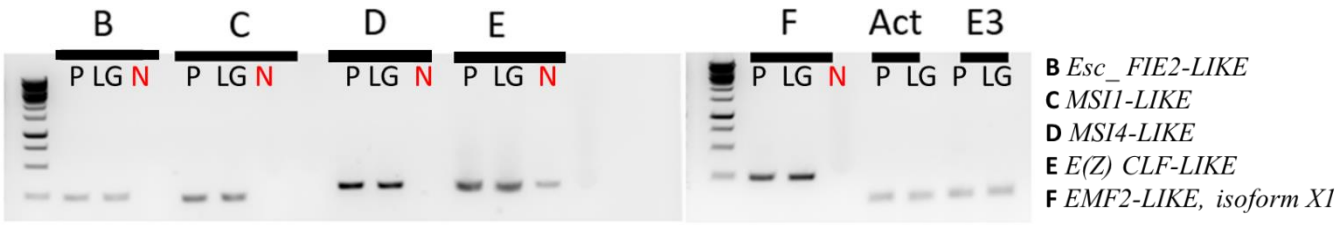

Figure 12: Gel electrophoresis for qRT-PCR (15 µL loaded). For the genes Esc_ FIE2-LIKE (B), MSI1-LIKE (C), MSI4-LIKE (D), E(Z) CLF-LIKE (E) and EMF2-LIKE, isoform X1 (F), the protonema (P) and leafy gametophyte (LG) samples, as well as the negative control (N, in red), were analysed. In the case of Actin and E3, only protonema and leafy gametophyte samples were analysed.

Utilizing PEATmoss, we conducted an analysis of gene transcription levels for the moss PRC2 subunit across various developmental and growth conditions of *P. patens*. PEATmoss automatically provides results for two ecotypes (Reute and Gransden) across different tissue

types such as protonema, gametophores, leaflets, spores, and sporophytes. Additionally, it includes different growth conditions on various growing media, including BCD liquid medium (Bsl), BCD solid medium (Bsq), Knop liquid medium, and Knop solid medium (Ksq). The results were visually represented through a heatmap, generated using the available transcriptome RPKM values for each gene. This heatmap illustrates the normalized gene-level RNA sequencing expression data in RPKM (reads per kilobase million).

In Reute (wild type), the protonema stage of *EMF2-LIKE isoform X1* seems to be slightly higher expressed compared to the gametophores and adult gametophores. For *MSI1-LIKE*, the protonema stage of Reute shows a considerably stronger gene transcription expression compared to the gametophore. *MSI1-LIKE* exhibits an overall comparatively higher expression in various tissue types. Moreover, the transcript expression of *CLF-LIKE* does not seem to vary much between the protonema and gametophore.

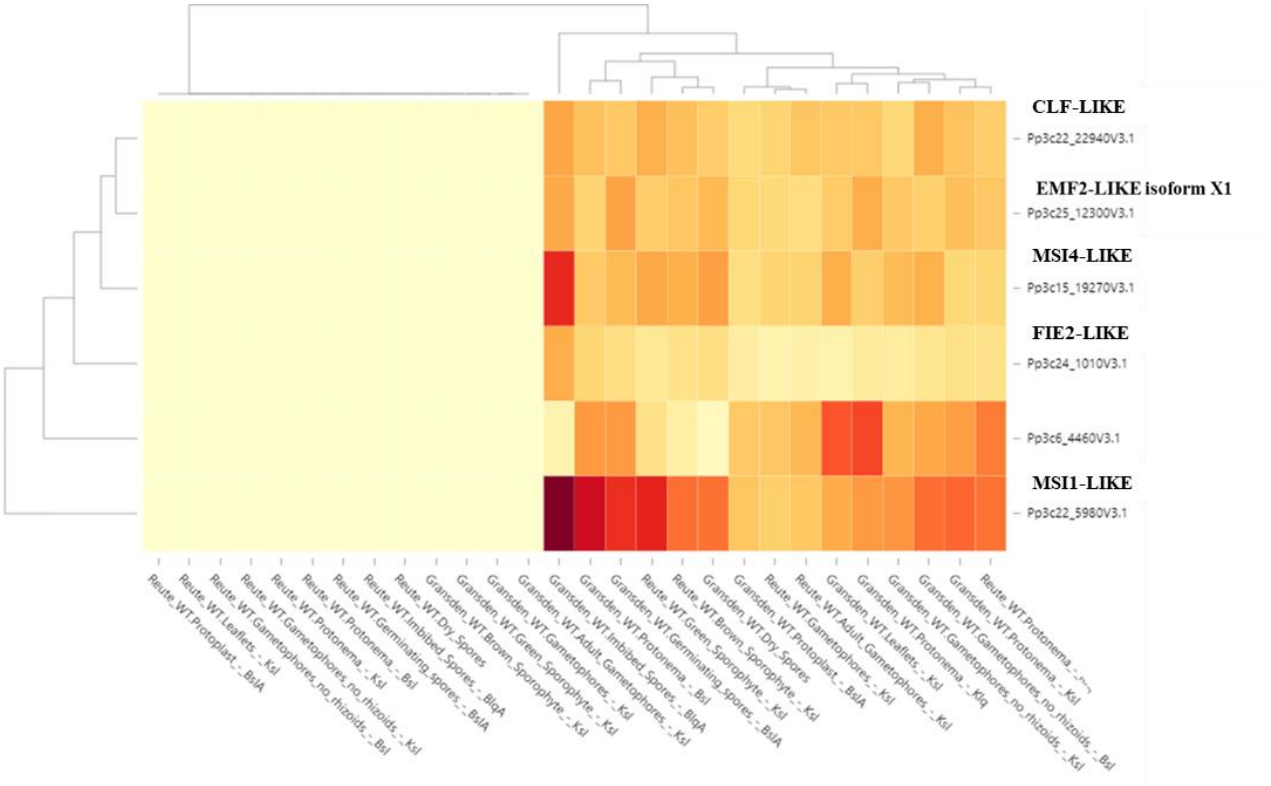

Figure 13: Expression-based heat map of the moss PRC2 genes (Esc_ FIE2, MSI1-LIKE, MSI4-LIKE, E(Z) CLF-LIKE and EMF2-LIKE, isoform X1) various developmental and growth condition. The colours represent gene expression as follows: Dark red for high expression, light orange for low expression, and yellow for no data available.

4.3. Interactions between *P. patens* PRC2 and *Arabidopsis* PRC2 Subunits

The Y2H assay was performed to detect possible interactions between selected PRC2 subunits from *P. patens* and *A. thaliana*. Due to limited time, we focused on testing whether PpCLF has retained the ability to interact with *A. thaliana* PRC2 subunits (*AtCLFΔSET, AtEMF2* and *AtMSI1*) in the course of evolution. Due to lack of time and to learn the technique, only a few interactions – interactions of the subunits *AtCLFΔSET, AtEMF2* and *AtMSI1* of *A. thaliana* with the *P. patens* subunit PpCLF – were tested (Figure 14). For these interactions, the vectors *pGADT7_PpCLF* and *pGBKT7_AtCLFΔSET*, *pGBKT7_AtEMF2*, *pGBKT7_AtMSI1* were used. Yeast growth was monitored on two different types of media {+AH and +A-H}, all of them without Leucine (Leu) and Tryptophan (Trp). Yeast cells transformed with empty vectors were used as a negative control to observe no growth on the selection plate.

All combinations, including the empty vectors, show growth on the +AH medium. As for the +A-H plate, growth can be observed for all non-empty vectors as well as the empty vector pGADT7 combined with pGADT7_MSI1 indicating autoactivation of *AtMSI1*.

The obtained results demonstrate interactions between PpCLF and AtCLFΔSET, as well as PpCLF and AtEMF2. However, no conclusions could be drawn regarding the interaction of PpCLF with AtMSI1 due to significant autoactivation of *AtMSI1* in the negative control. These findings suggest that PpCLF may retain a conserved PRC2 interaction surface throughout plant evolution, underscoring the significance of complex PRC2 interactions in regulating cellular and molecular functions in plants.

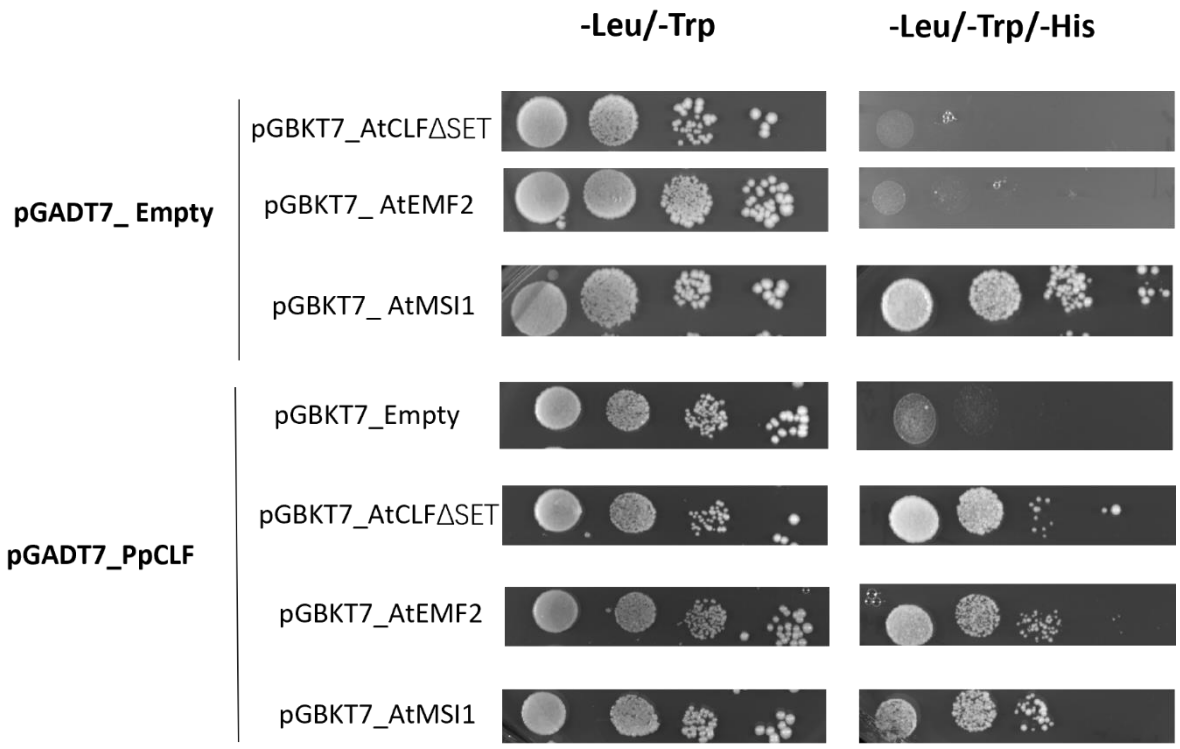

Figure 14: Yeast colony growth on selection media after Y2H assay. The Y2H shows growth on the plates containing adenine and histidine for all tested interactions. For the empty pGADT7 vector, Arabidopsis MSI1 (pGBKT7_AtMSI1) shows growth on the plate containing only adenine, whereas CLF (pGBKT7_AtCLFΔSET) and EMF2 (pGBKT7_AtEMF2) show no growth.

5. Discussion

5.1. Objective Evaluation and Research Methods

In this study, we aimed to investigate the dynamics of the PRC2 subunits within *P. patens* through examining evolutionary patterns, gene transcription and protein interactions. A phylogenetic analysis of PRC2 subunits across various plant species, including *P. patens*, was implemented *in-silico* to observe evolutionary patterns of PRC2 across diverse plant species. Additionally, we analysed the levels of several *P. patens* PRC2 subunit-transcripts in the filamentous protonema and leafy gametophyte by performing qRT-PCR and calculating the MNE-values of Relative Normalized Expression using the $2^{-\Delta\Delta C_T}$ method (Livak & Schmittgen, 2001). Finally, we aimed to study physical interactions of PpCLF with AtCLFΔSET, AtEMF2 and AtMSI1 respectively, as well as mutual interactions of *P. patens* PRC2 subunits by performing Y2H assays. As we delve into the results, this discussion aims to unravel the significance of our findings, connecting observed patterns and their potential implications for the broader field of plant evolution.

5.2. Conclusions from Phylogenetic Analysis of PRC2 Subunits

The phylogenetic analysis of PRC2 subunits across various plant species, including *P. patens*, revealed that the subunits can be grouped into four distinct clades {E(z), Su(z), Esc, and P55}. Notably, the *P. patens* PRC2 subunits were found to cluster together with their counterparts from various plant species within these clades. This finding demonstrates a high degree of conservation in the PRC2 subunits among different plant lineages, indicating their fundamental roles in various biological processes.

CLF orthologs, which are also the most ancient, are the only representative E(z) homolog in *P. patens* (Pereman et al., 2016). This matches our results with only one homolog of E(z) in *P. patens* (Figure 6). Our observations in *P. patens* highlight the presence of one subunit in each, E(z) and Esc, as well as the existence of three Su(z) homologs and two P55 homologs (Figure 7). These findings resonate with Vijayanathan et al. (2022), displaying the distribution of *P. patens* PRC2 subunits with experimental evidence of one E(z) and one Esc subunit and the prediction of three Su(z) homologs and two P55 homologs. In alignment with the findings presented by Huang et al. (2016), demonstrating the copy numbers of homologous proteins in *P. patens*, amongst other plant species, our study contributes further evidence about the distribution of PRC2 core components in *P. patens*, which solidifies our findings and

contributes to a more comprehensive understanding of the structure of *P. patens* PRC2. The distinctive pattern of *P. patens*, with 3 copies of Su(z)12, 2 copies of P55, and 1 copy each of Esc and EZH, suggests a unique configuration in *P. patens* in comparison to *Drosophila*. The more complex distribution of 5 copies of P55, 1 copy of Esc, and 3 copies each of EZH and Su(z)12 in *Arabidopsis* suggests a potential functional diversification of PRC2 subunits in the evolution of *Arabidopsis.*

5.3. Reviewing Gene Expression Patterns in *P. patens* Development

In the experiments analysing the transcript expression of various genes {*Su(z)_EMF2-LIKE isoform X3, Esc_FIE2-LIKE, MSI1-LIKE, MSI4-LIKE, E(Z) CLF-LIKE* and *EMF2-LIKE, isoform X1}*, intriguing trends regarding the expression of various PRC2 subunits in *P. patens* were observed.

In *A. thaliana*, *CLF* and *SWN* are assumed to play an important part in repressing seed maturation during the seedling stage by directly targeting essential maturation genes (Shu et al., 2019) indicating the importance of these genes in plant development. Also, qRT-PCR experiments in various organs of the plant species *Ginko biloba* (*G. biloba*) have shown that the *EMF* gene contributes to the maintenance of the vegetative development and the repression of flower development (Zhou et al., 2021). The impact of CLF and EMF2 on plant development in diverse plant species possibly indicates similar behaviour in the *P. patens* genes *E(Z) CLF-LIKE* and *EMF2-LIKE, isoform X1*.

Since the gel electrophoresis for semi-qRT-PCR showed no expression in either of the two developmental stages of *Su(z)_EMF2-LIKE isoform X3* (Figure 10), the gene was excluded from the subsequent experiments. Due to time limitation, the semi-qRT-PCR was not repeated in order to test for a false negative result. Future research could further investigate this to rule out the possibility of a false negative result for *Su(z)_EMF2-LIKE isoform X3.*

As shown by qRT-PCR of the representative genes, followed by application of the $2^{-\Delta\Delta C_T}$ method (Livak & Schmittgen, 2001), the transcription level of *Esc_FIE2-LIKE, MSI1-LIKE*, and *MSI4-LIKE* did not differ between the two different developmental stages (Figure 11). This suggests that these particular subunits may not play a critical role in the transition regulation between the protonema and leafy gametophyte stages. Conversely, notable differences in the transcript amount of *E(Z) CLF-LIKE* and *EMF2-LIKE, isoform X1* between the two different tissue types can be observed with a higher expression in the protonema stage. The higher expression of these genes in protonema indicates their potential involvement in maintaining the

developmental state of the protonema and their significance during the early stages of moss development. The difference in expression in the two different developmental stages could be explained by the developmentally simpler tissue of protonema in comparison to the more complex leafy gametophyte. In protonema tissue, fewer cell types would be present, so if the gene is expressed, it would be expressed in all cells. In contrast, the leafy gametophyte stage is more complex and has more diverse cell types. Here, the expression of the gene may be diluted in the bulk sample due to the presence of non-expressing cells, contributing to a seemingly lower average transcript level. This difference highlights the possible importance of these genes in shaping the early stages of *P. patens* development. Evidently, the genes *E(Z) CLF-LIKE* and *EMF2-LIKE, isoform X1*, showing considerable change in expression throughout *P. patens* development, from the early protonema stage to the more complex leafy gametophyte tissue, might present promising candidates for further functional characterization and studies in the field of evolutionary developmental biology.

While working with PEATmoss, it becomes evident that a direct comparison of gene expression with our qRT-PCR results is not feasible. The conditions for transcriptome data and growth medium did not precisely match our experimental setup. Nevertheless, our observations with PEATmoss revealed variations in the expression of certain PRC2 subunits under different developmental conditions. This insight underscores the dynamic nature of moss PRC2 gene expression across various developmental stages. It is important to note that drawing conclusive inferences directly from PEATmoss can be challenging due to the complexity of the data, which includes different cell types and growth mediums, and even lacks available data for certain conditions.

5.4. Conclusions from the Y2H assay

The molecular interaction of AtCLFΔSET and AtEMF2 has been observed through both, Y2H assays and in-vitro binding assays, demonstrating the binding of the CLF C5 domain to the EMF2 VEFS domain (Chanvivattana et al., 2004). Our Y2H results indicate that PpCLF can physically interact with AtCLFΔSET and AtEMF2. Similar to the *CLF-EMF2* interaction in Arabidopsis (Chanvivattana et al., 2004), the interaction of PpCLF with AtEMF2 meets our expectations. The Y2H assay also demonstrates the physical interaction of PpCLF and AtCLFΔSET. These interactions suggest the functional relevance of these subunits and possibly supports the concept of conservation of their roles across different plant species as shown by

Okano et al. (2009). Overall, the Y2H experiments provided valuable insights into the conservation of interaction potential between PRC2 subunits of *P. patens* and *A. thaliana*.

While the interaction of PpCLF and AtEMF2 was anticipated, the observed interaction of PpCLF and AtCLFΔSET is rather unexpected. So far, there is no indication that E(z) is involved in homodimerization, which would theoretically support our findings. Therefore, we suggest further research in order to provide evidence for this CLF-CLF interaction.

The PpCLF-AtEMF2 and PpCLF-AtCLFΔSET interactions were indicated by the observed growth on media used as positive control (+AH), growth on +A-H, and the lack of growth for the negative control using empty vectors pGADT7 and pGBKT7 (pGADT7_empty; pGBKT7_empty) on +A-H media. The interaction between PpCLF and AtMSI1, however, also showed growth for negative control due to the autoactivation of the MSI1-binding construct. So-called auto-activators (AAs) cause this false-positive by activating the reporter genes, even in the absence of an interacting protein partner (Shivhare et al., 2021). Consequently, a PpCLF-AtMSI1 interaction could neither be confirmed nor denied. Therefore, further research is needed to resolve this issue and to gain a comprehensive understanding of the interactions between these subunits. The ability of CLF to interact with itself and other PRC2 components might be a crucial aspect of its regulatory function.

Overall, the findings of this study provide valuable insights into the expression patterns and interactions of PRC2 subunits in *P. patens*. These results contribute to understanding the functions of PRC2 subunits and pave the way for further investigations into the regulatory mechanisms controlling moss development and the conservation of PRC2 subunits in plant evolution.

5.5. Limitations and Future Research

Future research is necessary to evaluate the transcript expression of *Su(z)_EMF2-LIKE isoform X3* in the protonema and leafy gametophyte stage. The possibility of a false negative cannot be excluded, and this should be investigated, possibly by repetition of the experiment. Alternatively, the possibility of *Su(z)_EMF2-LIKE isoform X3* being a pseudogene could be explored. Moreover, with the gained experience, I suggest to also re-analyse the transcription of *E(Z) CLF-LIKE* due to the false positive in gel electrophoresis for qRT-PCR (Figure 12) in order to explain this issue, potentially caused by contamination. In general, I would recommend

repeating semi-qRT-PCR with varying numbers of cycles, which might also give better results for *Su(z)_EMF2-LIKE, isoform X1* and *Esc_FIE2-LIKE* which shows rather low expression (Figure 10). Additionally, the focus on a specific moss species may limit the generalizability of our findings. Therefore, including other moss species or ecotypes in further research could allow for the formulation of more general conclusions.

In order to make a final conclusion about the observed interactions of PpCLF with AtCLFΔSET and AtEMF2 (Figure 14), conducting similar interaction analyses using alternative techniques such as co-immunoprecipitation (co-IP) and Bimolecular Fluorescence Complementation (BiFC) to validate the interaction between these subunits is needed. Regarding the testing of the interaction between PpCLF and AtMSI1, employing different interaction approaches is highly advisable. Due to the tendency of Y2H to generate false positives, opting for a distinct approach is preferable. To address auto-activators in Y2H assays, we highly recommend the use of 3-aminotriazole (3AT), a competitive inhibitor of the HIS3 gene product, to inhibit yeast growth for negative control setups (Shivhare et al., 2021).

To better understand the assumed interaction of PpCLF and AtCLFΔSET, we suggest exploring the possibility that CLF proteins have the ability to form homodimers. We could further investigate, which region of CLF serves as a domain or region for the homodimerization by experimental approaches such as structural analysis or domain mapping. Furthermore, we recommend utilizing Y2H assays to test specific regions of CLF for interactions. Moreover, mutual interactions between *P. patens* subunits could be investigated in future research. This part of the objective was not covered within the scope of this thesis due to the limited time frame. Gaining knowledge about mutual *P. patens* interactions could provide essential information about the functional relevance of the tested subunits.

6. Conclusion and Future Perspectives

In conclusion, this study successfully explored the conservation and diversification of PRC2 subunits in the model moss *Physcomitrium patens*. PRC2 subunits in moss were identified, including putatively novel genes encoding some of the subunits. Phylogenetic analysis revealed that PRC2 subunits are highly conserved across various plant species including *P. patens*. The gene expression analysis indicated that *E(Z) CLF-LIKE* and *EMF2-LIKE, isoform X1* exhibit differences in expression at two developmental stages of *P. patens*, suggesting their importance in maintaining specific developmental states. Additionally, the yeast two-hybrid experiments demonstrated physical interactions of PpCLF with AtCLFΔSET and AtEMF2, suggesting a high level of conservation of the E(z) and Su(z)12 subunit interaction surfaces in evolution of the green lineage.

The findings of this study contribute to our understanding of PRC2 subunit conservation and diversification in *P. patens* and highlight potential roles of specific subunits in early developmental stages. However, further research is needed to fully understand the functional significance of these subunits in *P. patens* and other plant species. Future studies could investigate the epigenetic modifications associated with the differential expression of PRC2 subunits in different developmental stages, which may provide valuable insights into the regulatory mechanisms governing *P. patens* development. Within the scope of this work, no conclusions about mutual interactions of PRC2 subunits in *P. patens* were made due to time constraints, suggesting the need for future studies to explore these interactions thoroughly. Moreover, since the interaction between *P. patens* and *A. thaliana* PRC2 subunits was only partially explored in this study, further investigations should be conducted to elucidate the functional implications of these interactions.

Overall, this study advances our knowledge of moss developmental biology and PRC2 subunit function, presenting new opportunities for future research in plant growth and development. The conservation and diversification of PRC2 subunits in *P. patens* and other plant species continue to be intriguing areas of study, which might help in understanding fundamental processes of plant growth and development.

7. References

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