Identification of plant-specific components of the Polycomb Group pathway and their roles in the regulation of *Arabidopsis thaliana* development

Volume 1 of 1

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Declaration

I certify that this thesis is my own work, and that I have not used this work in the course of another degree, either at National University of Ireland Galway, or elsewhere.

Part of this research was published in the following publications:

March, E., & Farrona, S. (2018). **Plant Deubiquitinases and Their Role in the Control of Gene Expression Through Modification of Histones**. Frontiers in Plant Science, 8(January), 1–14. https://doi.org/10.3389/fpls.2017.02274

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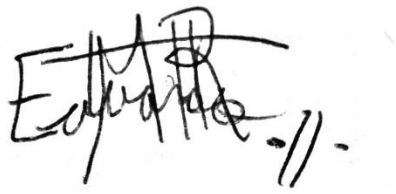

Eduardo March

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Communications, publications and awards

Congresses attended as speaker:

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Summary of contents

The knowledge about *Arabidopsis thaliana* developmental life cycle is deep in broad strokes. Nevertheless, the mechanisms that fine tune these phase transitions are still poorly described. One of these mechanisms is the epigenetics, that consist in the precise temporal and -spacial control of genes to respond to specific endogenous or exogenous cues. This control is mediated by different players of diverse nature that together or antagonistically produce a transcriptional change in one or more genes.

In this research project I partially characterized FORGETTER 1 (FGT1) and UBIQUITIN PROTEASE 5 (UBP5), both partners of PWWP-DOMAIN INTERACTOR OF POLYCOMBS 1 (PWO1) a Polycomb Group (PcG) pathway member involved in the maintenance of nuclear morphology and necessary for the repression of some PcG target genes.

FGT1 and UBP5 physically interact with subunits of PRC2 and a subunit of the Histone Deacetylase Complex 6. In addition, I demonstrated how FGT1 and UBP5 are necessary for *Arabidopsis thaliana* development. Specifically, FGT1 is involved in the repression of *FLOWERING LOCUS C (FLC*), the main floral repressor, suggesting a novel role of FGT1 in inducing flowering, one of the main developmental traits.

On the other hand, in the absence of a functional UBP5 the plant displays a pleotropic phenotype, such as loss of shoot apical dominance, delay in germination and delay in flowering. These results suggest that both proteins play an important role in Arabidopsis development.

Taking together, this research will contribute to depict the developmental epigenetic regulation of *Arabidopsis thaliana* and increase our knowledge in the complex network of proteins that operates within the context of the chromatin regulation.

Abbreviations

β-ME - β- mercaptoethanol

°C - degree Celsius

3AT - 3-amino-1,2,4-triazole

aa- amino acids

AD - activation domain

BD- binding domain

bp - base pair

BSA - bovine serum albumin

Cas - CRISPR associated

cDNA - complementary DNA

cm - centimeters

Col-0 - ecotype Columbia (Accession of *Arabidopsis thaliana*)

CRISPR - clustered regularly interspaced short palindromic repeats

DAG - days after germination

DAI - days after infiltration

DNA - deoxyribonucleic acid

DTT - ditiotreitol

EDTA - ethylenediaminetetraacetic acid

g- G-force

g -gram

GA - gibberellic acid

GFP - green fluorescent protein

h - hour

HRP - horseradish Peroxidase

IP - immuno precipitation

IR - intron retention

Kb - kilobase

L - liter

LB - lysogeny broth

LD - long day

M - molar

mg - milligram

min - minute

mL - milliliter

mm - millimeter

mM - minimolar

PAGE - Polyacrylamid gelectrophoreses

PBS - phosphate-buffered saline

PCR - polymerase chain reaction

PMSF - phenylmethylsulfonyl fluoride

PTM - post-translational modifications

PVDF - Polyvinylidenfluoride

q-PCR - quantitative real time - PCR

RNA- ribonucleic acid

S. cerevisiae - *Sacharomices cerevisiae*

SD - short day

SD (Y2H) - synthetic defined medium

SDS - sodiumdodecylsulfate

sgRNA - single guide RNA

siRNA - small interference RNA

T-DNA - transfer DNA

Tris - Tris(hydroxymethyl)aminomethane

TSS - transcriptional start site

V - volt

WB - western blotting

WT - Wild type

Y2H - Yeast two-hybrid

Zt - Zeitgeber time

$\mu E\ m^{-2}\ s^{-1}$ - μEinsteins $m^{-2}\ s^{-1}$

µL - microliter

µM - micromolar

Introduction

1. Introduction

1.1 Epigenetics

Almost 80 years have passed since Conrad Waddington proposed the use of the term epigenetics to refer to the developmental mechanisms in which changes in the genotype relate with changes in the phenotype constituting what Waddington called "the kernel of the whole problem of development" (Waddington, 1942). Years later, Waddington showed how an environmental stimulus can produce phenotypic changes and can be assimilated into a population in *Drosophila melanogaster* (Drosophila) (Waddington, 1956; Waddington, 1953). Since then, the definition of epigenetics has undergone changes, but, nevertheless, continues to maintain that central core between (epi)genotype and phenotype relation. We can currently define epigenetics as the study of the mechanisms that produce stable and heritable changes in gene expression patterns without changes in the DNA sequence (Wolffe & Matzke, 1999). These specific epigenetic modifications may act at several levels, such as modifications related to chromatin remodeling, modifications in noncoding RNAs, histones variants, DNA methylation and post-translational histone tail modifications (Allis & Jenuwein, 2016). In *Arabidopsis thaliana* (Arabidopsis), as in other organisms, epigenetics regulation is essential for plant development and for the response of the plant to the environment (Pikaard & Mittelsten Scheid, 2014).

1.2 Chromatin structure and the histone code

Chromatin is an evolutionary-conserved structure necessary to compact and protect the genetic information within the nucleus and also play a key role in controlling gene expression. Chromatin is basically a DNA-protein complex formed by double-stranded DNA and histones. The simplest structural organization unit of the chromatin is the nucleosome. The nucleosome is formed by 146 base pairs (bp) of DNA enclosing an octamer of histones (two H2A, two H2B, two H3 and two H4). Nucleosomes are connected between them every 200 bp by a linker DNA that interacts with the H1, forming the classic "pearl necklace" structure (Kornberg, 1974). The next packaging level is known as the solenoid, a spiral-like structure composed by the wrapping of six nucleosomes that form a 30 nm fibre which, if it continues compacting in higher-order structural levels, form a chromosome (Finch & Klug, 1976).

Considering chromatin condensation and organization within the nuclear space, we can describe two chromatin conditions: (i) euchromatin and (ii) heterochromatin. Euchromatin is less compacted and enriched in genes. In addition, the loci in euchromatin tend to group in defined foci. On the other hand, the heterochromatin is a high compacted chromatin with low genes-content and tend to sub localize in the nuclear periphery (Fransz et al., 2002; Gordon et al., 2015; Trojer & Reinberg, 2007).

More than 200 protein post-translational modifications (PTMs) have been discovered (Minguez et al., 2012). The N-terminal region of histones, known as histone tails, can suffer some of these PTMs, generally called epigenetic marks, that will impact on gene expression (Allfrey et al., 1964). These modifications are covalent, and mostly reversible, chemical bonds that are going to promote the recruitment of other proteins, "readers". In addition, they affect the condensation of the chromatin fibers hindering or facilitating the transcriptional machinery access by steric hindrance, acting together or sequentially and generating what has been called the "histone code". However, on the contrary to the genetic code, the histone code is not universal and the meaning of specific histone marks and its impact on gene expression may vary between species. In addition, the presence or absence of specific of these marks can promote the transition between euchromatin to heterochromatin and *vice versa* (Strahl & Allis, 2000).

There are more than 30 different epigenetic marks (**Figure 1**) (Berger, 2007; Pfluger & Wagner, 2007). The main modifications are acetylation, phosphorylation, methylation, β-N-acetylglucosamination and ubiquitination (Bannister & Kouzarides, 2011); however, other many exist and new ones are still being discovered, such as direct histone tail clipping (Azad et al., 2018) . The deposition of these marks is mediated by enzymes that are normally part of protein complexes (Rando & Ahmad, 2007).

Histone acetyltransferases (HATs) mediate the deposition of acetyl residue on the lysines of the histones 3 and 4 tails, a mark related with chromatin unfolding and gene expression (Wang et al., 2014). Whereas histone deacetylases (HDACs) remove these residues (Probst, 2004).

Phosphorylation, mark linked with gene activation, is carried out by kinases and removed by phosphatase enzymes. The kinases are over-represented in Arabidopsis, with 1,019 putative-coding genes in the Arabidopsis genome against the 119 of the *S. cerevisiae* genome (Wang et al., 2003). They phosphorylate mostly serines and threonines on the H3 (Houben et al., 2007) and also on the H1 (Bigeard et al., 2014).

The β-N-acetylglucosamination (O-GlcNac) is the deposition of sugar residue in serines and threonines amino acids of proteins in general. As histone PTMs, these modifications have a relatively high turnover and were found affecting H2A, H2B and H4 of metazoan *in vivo* (Sakabe et al., 2010). In Arabidopsis, these marks seem to be necessary for the proper repression of specific genes (Xing et al., 2018).

Ubiquitination is a post-translational modification that consists of the covalent binding of the small polypeptide ubiquitin to a target protein, either singly or sequentially (polyubiquitination; (Akutsu et al., 2016)). The process starts with the activation of inactive ubiquitin carried out by the E1 (ubiquitin-activating) enzyme in an ATP-dependent manner. The active ubiquitin is transferred from the E1 to the E2 (ubiquitin-conjugating) enzyme that acts as an intermediate and, finally, the E3 (ubiquitin ligase) enzyme mediates the deposition of the active ubiquitin to the target protein, mainly on a lysine residue. Protein ubiquitination can be direct or indirect depending on the E3 that mediates this process (Haas et al., 1982; Ishikura et al., 2010; Zheng & Shabek, 2017). At histone level, histones H2A and H2B can be monoubiquitynated. The enzymes that mediate the removal of ubiquitin are the deubiquitinases, which I will further discuss in chapter 1.5 together with the role of this histone mark in transcriptional regulation.

Methylation of histone tails occurs on lysines and arginines. Unlike acetylation and phosphorylation, methylation does not affect the histone charge. Methylation can occur as mono-, di- or trimethylation depending on the number of methyl groups added to the histone tail. The deposition of these marks is mediated by the histone methyltransferases (HMTs) and the removal by histone demethylases (HDMs). The result of histone methylation on gene expression depends on the number of methyl groups and the histone residue which is modified (Liu et al., 2010).

1.2.1 Histone Post-Translational Modifications cross-talk

All the organisms are subjected to a chain of constant stimuli. To react against a particular stimulus animal cells have evolved well-defined molecular pathways to tolerate or adapt to that specific stimulus and, eventually, animals can simply move to escape from the negative stress. On the other hand, plants are sessile organism and hence need a higher developmental plasticity compare with other non-sessile organisms. To fulfil this quick adaptability requirement (development, external stresses, etc), live forms need to change very quickly their proteome and, therefore, their transcriptome (Strahl & Allis, 2000). For instance, this quick response is essential in the activation of immediate-early genes after the stimulus. Although PTMs have been described individually, indeed deposition or removal of one of these marks can have an antagonistic or synergistic effect on other marks and, subsequently, on transcription (Minguez et al., 2012).

Figure 1. **Summary of the most common histone PTMs in Arabidopsis and their predominant effect on gene transcription.** Orange for Histone 2A modifications; purple for Histone 2B modifications, blue for Histone 3 modifications and green for histone 4 modifications. (Berger, 2007; Su et al., 2017; Xu et al., 2008; Zhang et al., 2007).

We can talk about different scenarios (Bannister & Kouzarides, 2011) : (i) competitive antagonism, as it happens with H3K9 that can be acetylated or methylated; (ii) dependency, when a PTM depends on a previous one (Xing et al., 2018); (iii) binding disruption, the binding ability of an effector is disrupted by a PTM due to steric hindrance; (iv) binding impairment, an effector cannot interact with its substrate due to another previous modification (Lindroth et al. , 2004); (v) synergy, when two or more PTMs coordinate for the same aim, as it happens between Histone 2A monoubiquitination (H2Aub) and the trimethylation of the lysine 27 of the histone 3 (H3K27me3) in order to maintain gene repression.

1.3 Polycomb Repressive Complexes (PRCs)

Polycomb group (PcG) proteins, identified as repressors of the homeotic *HOX* genes in Drosophila, are a highly evolutionary conserved group of proteins that can form four main kinds of complexes: Polycomb Repressive Complex 1 (PRC1), PRC2, Pho Repressive complex (PhoRC) and Polycomb Repressive DeUBiquitinase (PR-DUB) complex (Grimaud et al., 2006; Jamieson et al., 2013; Li et al., 2007; Müller et al., 2002). Roughly, the main role of these complexes is the precise repression of a specific target loci for a certain time.

In metazoans, PRC1, PRC2 and PR-DUB are directly linked with histone PTMs (Scheuermann et al., 2012). While PhoRC is necessary, with its subunits Pleiohomeotic (Pho) or Pleiohomeotic like (Phol) and Scm-like with four MBT domain-containing protein 1 (Sfmbt1) for recognition and DNA-binding to Polycomb response elements (PREs) (Alfieri et al., 2013; Klymenko et al., 2006). This PRE binding is necessary for direct PRC1 and indirect PRC2 recruitment to specific target loci guided by the Sex comb on midleg protein (Scm) (Frey et al., 2016; Kim et al., 2005; Schuettengruber et al., 2009; Wang et al., 2004). In vertebrates, PRC1, PRC2 and PR-DUB are conserved with some variations that I will discuss later. In addition, the mammalian Pho ortholog is the PcG Ying yang1 (YY1) transcriptional factor that interacts with all the subunits of the inositol auxotroph 80 (INO80) chromatin remodeling complex and mediates PCR2 subunit recruitment, although is not contemplated as a member of a putative mammalian PhoRC complex, being the only PcG complex not conserved in mammals (Brown, 2003; Hauri et al., 2016; Lu et al., 2018; Wilkinson et al., 2006).

In plants only two of these complexes are conserved and have so far been described (**Figure 2**). PRC1, which mediates gene repression *via* deposition of H2Aub (Merini & Calonje, 2015), and PRC2, which mediates the deposition of H3K27me3, a conserved histone PTM for gene repression in eukaryotes (Mozgova et al., 2015; Schatlowski et al., 2008). These two molecular activities are also shared with the respective animal PRC1 and 2.

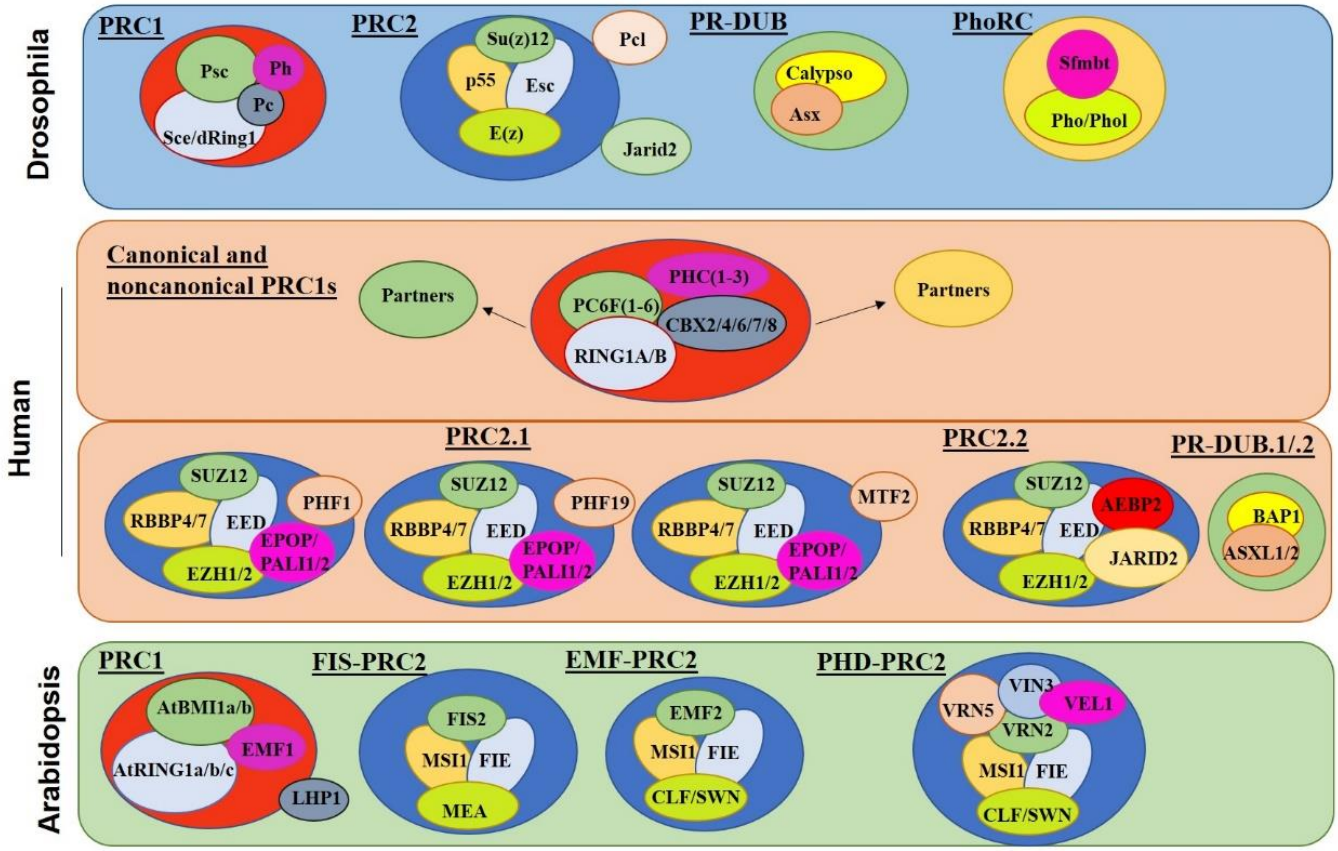

Figure 2. Polycomb complexes in Drosophila, Human and Arabidopsis. Drosophila PRC1: Posterior sex comb (Psc), Polyhomeotic (Ph), Polycomb (Pc) and Sex combs extra (Sce), also called dRing1. **Drosophila PRC2**: Suppresor of zeste 12 (Su(z)12, protein p55 (p55), Extra sex combs (Esc), Enhancer of zeste (E(z), Polycomb-like (Pcl) and jumonji/ARID domain-containing protein 2 (Jarid2). **Drosophila PR-DUB**: CALYPSO and Additional sex combs-like protein (Asx). **Drosophila PhoRC**: Pleiohomeotic (Pho) or Pleiohomeotic like (Phol) and Scm-like with four MBT domain-containing protein 1 (Sfmbt1). **Human PRC1**: RING1A, RING1B, Polycomb group RING fingers 1-6 (PCGF1-6), Chromobox protein homologs (CBX2/4/6/7/8), **Human PRC2.1 and PRC2.2**: Orthologs of Drosophila PRC2 plus Enhancer of Zeste 1 (EZH1), Embryonic ectoderm development (EED), Retinoblastoma-binding protein 4 (RBBP4) or RBBP7, PHD finger protein 1 (PHF1), PHF19, or Metal-response element-binding transcription factor 2 (MTF2), Elongin BC and Polycomb repressive complex 2-associated protein (EPOP) or Polycomb associated ligand-dependent nuclear receptor corepressor isoform 1 (PALI1) and Adipocyte Enhancer-binding protein (AEBP2). **Human PR-DUB1 and PR-DUB2**: tumor suppressor BRCA-1-associated protein 1 (BAP1) and Additional sex combs-like protein (ASXL) 1 and ASXL2. **Arabidopsis PRC1**: AtBMI1a/b/c, AtRING1a/b, LIKE HETEROCHROMATIN 1 (LHP1) and EMBRYONIC FLOWER 1 (EMF1). **Arabidopsis PRC2**: CURLY LEAF (CLF) or SWINGER (SWN) or MEDEA (MEA), MULTICOPY SUPRESSOR OF IRA 1 (MSI1), EMBRYONIC FLOWER 2 (EMF2), VERNALISATION 2 (VRN2) and VRN5, FERTILISATION INDEPENDENT SEED (FIS2), FERTILIZATION INDEPENDENT ENDOSPERM (FIE) and VERNALISATION INSENSITIVE 3 (VIN3), and VERNALIZATION5/VIN3-LIKE 1 (VEL1).

1.3.1 PRC2

PRC2 was first discovered in flies (Lewis, 1978) and includes four main subunits encoded by single copy genes: *Enhancer of zeste (E(z)), Suppresor of zeste 12 (Su(z)12), Extra sex combs (Esc)* and *protein p55 (p55)* (Cao et al., 2002; Czermin et al., 2002; Müller et al., 2002). Despite being evolutionary conserved, we can observe appreciable differences in terms of complex composition, recruitment, specificity and activity between Drosophila, humans and Arabidopsis PRC2s.

In Drosophila and humans, the PRC2 catalyze the deposition of mono-, di- and trimethylation on the lysine 27 of the histone 3 tail (Ebert et al., 2004; Montgomery et al., 2005). However, in Arabidopsis the mono- and dimethylation deposition are not PRC2 conserved and are in charge of other methyltransferases (Lindroth et al., 2004).

In humans, two PRC2 subcomplexes had been described, the PRC2.1 and the PRC2.2 (**Figure 2**) (Hauri et al., 2016).

Both PRC2 subcomplexes share the core PRC2, formed by one of the orthologues of Drosophila E(z), Enhancer of Zeste 1 (EZH1) or EZH2, the ortholog of Su(z)12, SUZ12, the ortholog of Esc, Embryonic ectoderm development (EED), and a histone-binding protein, Retinoblastoma-binding protein 4 (RBBP4) or RBBP7 as stabilizing factors that also are part of several protein complexes (Hauri et al., 2016).

Human PRC2.1 is formed by the core PRC2 and one of the Polycomb-like (PCL) homologs, PHD finger protein 1 (PHF1), PHF19, or Metal-response element-binding transcription factor 2 (MTF2), in combination with Elongin BC and Polycomb repressive complex 2-associated protein (EPOP**)** or Polycomb associated ligand-dependent nuclear receptor corepressor isoform 1 (PALI1) (Hauri et al., 2016). MTF2 recruits PRC2 to an unmethylated CpG island in a PRE-like DNA manner (Li et al., 2017); whereas PHF1 and PHF19 recruit PRC2 by reading H3K36me3 and stimulating the catalytic activity of PRC2 (Hunkapiller et al., 2012; Sarma et al., 2008). On the other hand, PRC2.2 is formed by the core PRC2 as well as Adipocyte Enhancer-binding protein (AEBP2) and jumonji/ARID domain-containing protein 2 (JARID2) that stimulates PRC2 activity, PRC2 stability *via* RBBP4/7 interaction and PRC2 nucleosome binding capacity (Kasinath et al., 2018; Son et al., 2013).

In Arabidopsis, PRC2 deposition of H3K27me3 is conserved (Lafos et al., 2011). PRC2-mediated repression is dynamic, changing throughout time depending on environmental and endogenous factors, such as age. H3K27me3, the PRC2 epigenetic hallmark, directly affects approximately 13.5%-31% of protein coding genes depending on published studies carried out with different plant material and it has been related with the regulation of developmental phase transitions (Lafos et al., 2011; Turck et al., 2007; Vergara & Gutierrez, 2017; Zhang et al., 2007). The main developmental phase transitions that PRC2 affects are seed maturation (Köhler et al., 2003; Roszak & Köhler, 2011), germination (Müller et al., 2012), seedling to vegetative transition (Bouyer et al., 2011), flowering (Bastow et al., 2004; Jiang et al., 2008) and gametophytic development (Chaudhury et al., 1997).

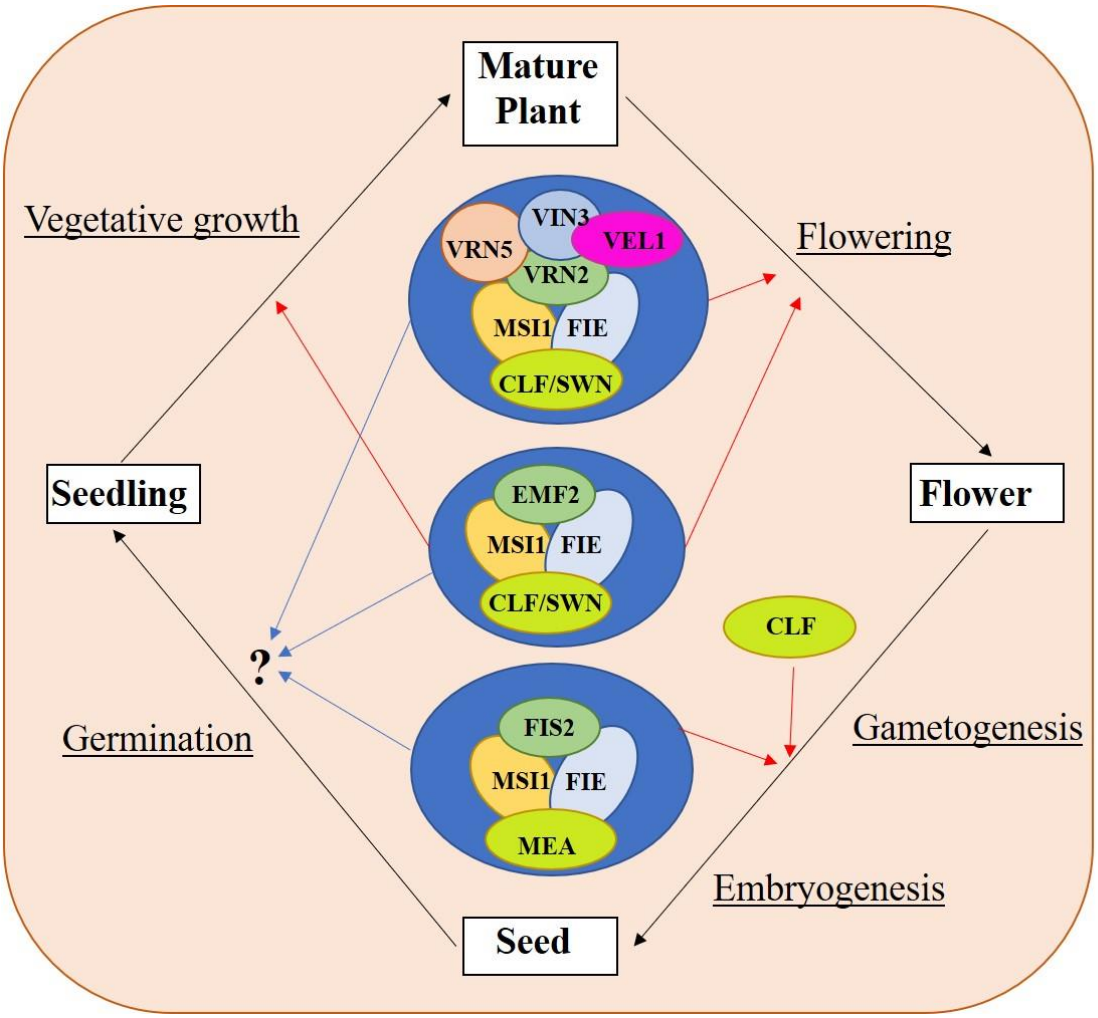

Figure 3. PRC2 subcomplexes regulate Arabidopsis developmental phase transitions. Different PRC2 participate in several developmental stages sharing some core subunits, but with other specific subunits depending on where and when the complex is repressing gene expression through the deposition of H3K27me3. EMF2-subcomplex mediates both, vegetative shoot growth and flowering (Schönrock et al., 2006; Yoshida et al., 2001). FIS-subcomplex is involved in gametogenesis, embryogenesis and endosperm development (Roszak & Köhler, 2011; Spillane et al., 2000). PHD-subcomplex affects flowering *via* vernalization, repressing *FLC* (De Lucia et al., 2008). The PRC2 subcomplex or subcomplexes involved in germination are not clear since defects in this developmental phase transition were found in the mutants of the two conserved subunits among the three subcomplexes, *fie* and *msi1* (Bouyer et al., 2011; Köhler et al., 2003; Hennig et al., 2003). Finally, CLF participates in the embryogenesis (Liu et al., 2016). Red arrows represent described PRC2-subcomplexes actions, blue arrows represent unknown players (Adapted from Henning and Derkacheva, 2009). **Arabidopsis PRC2**: CURLY LEAF (CLF) or SWINGER (SWN) or MEDEA (MEA), MULTICOPY SUPRESSOR OF IRA 1 (MSI1), EMBRYONIC FLOWER 2 (EMF2), VERNALISATION 2 (VRN2) and VRN5, FERTILISATION INDEPENDENT SEED (FIS2), FERTILIZATION INDEPENDENT ENDOSPERM (FIE) and VERNALISATION INSENSITIVE 3 (VIN3), and VERNALIZATION5/VIN3-LIKE 1 (VEL1).

Drosophila PRC2 histone methyltransferase catalytic subunit E(z) has three orthologs in Arabidopsis PRC2: CURLY LEAF (CLF), SWINGER (SWN) and MEDEA (MEA), all of which contain the characteristic SET domain that has lysine methyltransferase activity (Chanvivattana, 2004; Goodrich et al., 1997; Grossniklaus et al., 1998). Nevertheless, these catalytic subunits may play different and more specific roles within the complex. For instance, at the *FLC* locus after vernalization SWN is involved in nucleation while CLF is involved in spreading of H3K27me3 over the locus (Yang et al., 2017). In addition, PRC2 is composed by one of the five orthologs of p55, MULTICOPY SUPRESSOR OF IRA 1-5 (MSI1-5), specifically MSI1, and associates with another, MSI4, also call FVE (Köhler et al., 2003; Hennig et al., 2003; Pazhouhandeh et al., 2011).

Three Su(z)12 orthologs are present in Arabidopsis, EMBRYONIC FLOWER 2 (EMF2) (Yoshida et al., 2001), VERNALISATION 2 (VRN2) (Gendall et al., 2001) and FERTILISATION INDEPENDENT SEED (FIS2) (Luo et al., 1999). The final component is an Esc ortholog called FERTILIZATION INDEPENDENT ENDOSPERM (FIE) (Spillane et al., 2000).

The catalytic subunits of PRC2 seem critical since *clf,swn* double mutant plants present dramatic pleiotropic phenotype and develop callus-like structures due to mis-regulation of several genes produced by general H3K27me3 deposition and chromosomal structure changes (Chanvivattana et al., 2004; Feng et al., 2014; He et al., 2012). Thus, PRC2 epigenetic regulation is essential to mediate plant development and cell lineage. In Arabidopsis, PRC2 subcomplexes can be distinguished, depending on the composition, with specific functions (**Figure 3**). These subcomplexes can only partially regulate same target genes (Makarevich et al., 2006). The three PRC2 subcomplexes in Arabidopsis can be mainly divided by the orthologs of Su(z)12 and E(z), as the other two core subunits are shared by all of them: the FIS complex (FIS and MEA); EMF complex (EMF2 and CLF/SWN) and the VRN complex (VRN2 and CLF/SWN). This last complex is also known as PHD-PRC2 because it also contains a Plant Homeodomain (PHD)-containing orthologs of the human PHF1 (PRC2.1): VERNALISATION 5 (VRN5), VERNALISATION INSENSITIVE 3 (VIN3), and VERNALIZATION5/VIN3-LIKE 1 (VEL1) (Chanvivattana, 2004; De Lucia et al., 2008; Luo et al., 2009).

1.3.2 PRC1

In Drosophila, the PRC1 is composed by Posterior sex comb (Psc), Polyhomeotic (Ph), Polycomb (Pc) and Sex combs extra (Sce), also called dRing1 (Peterson et al., 2004).

Whereas in humans, Ring1A and Ring1B PRC1 subunits, have E3 ubiquitin ligase activity, regulating H2AubK119 (H2AubK118 in Drosophila) deposition after recruitment through a H3K27me3-dependent manner (Buchwald et al., 2006; Cao et al., 2005).

This canonical PRC1 has been related with chromatin compaction (Levine et al., 2002). In addition to the canonical PRC1, we can distinguish four non-canonical PRC1 complexes in humans (**Figure 2**) based on the presence of different orthologs of the Drosophila Psc, the Polycomb group RING fingers (PCGF) family that are recruited dependently on or independently of H3K27me3 and show specific interaction to different partners (Chittock et al., 2017; Gao et al., 2012; Trojer et al., 2011).

In addition to the aforementioned Sce orthologs, RING1A/1B and PCGF1-6 proteins, PRC1 complexes also are formed by orthologs of Ph, called Polyhomeotic-like proteins (PHC1-3). Five different Pc orthologs and Chromobox protein homologs (CBX2/4/6/7/8) that display differential affinities for H3K27 also formed part of non-canonical PRC1s (Bernstein et al., 2006; Ma et al., 2014). The different noncanonical PRC1s are also distinguished by the specific different interacting-partners for each of them, similarly as it happens for PRC2.1 (Chittock et al., 2017; Hauri et al., 2016).

In Arabidopsis, there are three orthologs of Bmi1 (AtBMI1a, AtBMI1b, AtBMI1c) and two Ring1A/1B (AtRING1a and AtRING1b) (Sanchez-Pulido et al., 2008). The five PRC1 RING-fingers orthologs of Arabidopsis can monoubiquitinate H2A.1K121 isoform *in vitro* and AtBMI1a/1b are necessary for H2Au *in vivo*. Whether AtRING1a/1b directly catalyze H2Aub *in vivo* has not been unveiled yet (Bratzel et al., 2010). In addition, there is a protein that share one of the binding domains with the Drosophila PRC1 subunit Pc, LIKE HETEROCHROMATIN 1 (LHP1). EMBRYONIC FLOWER 1 (EMF1) is a functional analogue of the Drosophila PRC1 subunit Psc (Beh et al., 2012; Calonje et al., 2008; Gaudin et al., 2001).

In Arabidopsis, PRC1 subcomplexes have not been characterized so far. However a putative EMF1 complex (EMF1c) composed by EMF1, LHP1 and a demethylase involved in the regulation of the flowering time trough *FLOWERING LOCUS T* (*FT*) repression has been proposed as a putative PRC1 (Wang et al., 2014) . Nevertheless, some PRC1 subunits seems to have more than one role and display multi-interaction patterns, such as LHP1 and EMF1 (Jullien et al., 2008; Hennig et al., 2003). In addition, there are some PRC1 target genes that require H2Aub deposition to be repressed and other that do not depend on this mark, suggesting the existence of different combination of subunits. The identification of new putative-complexes in Arabidopsis may be just a matter of time (Yang et al., 2013a).

1.3.3 Hierarchical model, PcG interactors and recruitment

PRC1 and PRC2 can interact in the repression of a specific locus consecutively. PRC2 adds the H3K27me3 mark and then LHP1 reads it and stabilizes the repression *via* PRC1-mediated H2Aub deposition. This sequential process is known as the hierarchical model (Simon & Kingston, 2013). However, the hierarchical model is one possibility since the evidences indicate that these two complexes can act independently (Zhou et al., 2017) as well as in reverse hierarchy (Comet & Helin, 2014; Merini et al., 2017; Tavares et al., 2012; Yang et al., 2013a).

A positive feedback-loop between PRC2 and PRC1 is also possible. In Drosophila, PRC2 recruits PRC1 through deposition of H3K27me3 that stimulates deposition of H3Kub1 and this in turn stimulates PRC2 activity (Chittock et al., 2017). In humans this loop is conserved between PRC2.2 and some PRC1 subcomplexes (Kalb et al., 2014). So far, only two of the PRC1 subcomplexes share the requirement for H3K27me3 loci occupancy, suggesting that in humans the rest of them may repress independently of PRC2 activity (Hauri et al., 2016).

Regarding Polycomb interactors, PcG proteins require other partners that are going to support the PcG complexes in one way or another, such as mediating in gene repression or recruiting the complexes to the specific loci and/or at the specific time. For example, the human PRC2 have 14 described associated-proteins (Bowers et al., 2010; Dietrich et al., 2012; Hauri et al., 2016).

In Arabidopsis the number of discovered and characterized partners is smaller compared to humans or Drosophila. However, in recent years novel interactors of PcG complexes have been discovered. These interactors are mainly proteins, but we can find interactors of different nature as COLD ASSISTED INTRONIC NONCODING RNA (COLDAIR), interactor and recruiter of CLF to *FLC* locus after vernalization; the component of the replication machinery DNA polymerase ε, which interacts with CLF, MSI1 and EMF2, maintains the PRC2-mediated repression during replication by direct interaction to CLF, SWN and LHP1 (Del Olmo et al., 2016; Heo & Sung, 2011; Yang et al., 2017).

Regarding LHP1, it has been proposed to be both part of the PcG pathway and a connector of PcG components. This protein physically interacts with the RINGs-like and EMF1 subunits of PRC1; however, H2Aub deposition is mostly independent of LHP1. It also interacts with EMF-PRC2 trough MSI1 and CLF, possibly directly interacting to H3K27me3 through its chromodomain and promoting H3K27me3 spreading at several specific loci (Derkacheva et al., 2013; Turck et al., 2007; Wang et al., 2016; Zhou et al., 2017). In addition, *lhp1* defective mutant plants display changes in the chromosomal architecture related with loss of H3K27me3, similar effect compared to the one observed in the *clf; swn* double mutant, suggesting that it could be more connected to PRC2 rather than PRC1 (Feng et al., 2014; Veluchamy et al., 2016). Finally, LHP1 seems to play a dual transcriptomic role, also working as a gene activator, affecting, among others processes, auxin biosynthesis (Rizzardi et al., 2011; Veluchamy et al., 2016).

Another few examples of interactors of PcG members in Arabidopsis are, the complex formed by ASYMMETRIC LEAVES 1 (AS1) and AS2, both proteins interact *in vivo* with CLF, recruiting EMF-PRC2 subcomplex in the leaves to repress KNOTTED-LIKE FROM ARABIDOPSIS THALIANA 1 (KNAT1/BP) and KNAT2, members of the class I KNOTTED1-like homeobox (KNOX) gene family involved in the shoot apical meristem stem cells balance among others (Lodha et al., 2013).

BLISTER (BLI), is a CLF direct interactor required for the proper repression of specific PRC2 target genes, although it can also affect plant development independently of PRC2 repression (Schatlowski et al., 2010). UPWARD LEAF 1 (UCL1), which ubiquitinates CLF for degradation via ubiquitin-26S proteasome pathway. A similar control system has been described in human cells in which UBIQUITIN SPECIFIC PROTEASE 7 (USP7) and USP11 control the degradation of the catalytic subunits of PRC1 through the proteasome pathway (Jeong et al., 2011; Maertens et al., 2010).

Different mechanisms for recruitment of PcG complexes have been described and its conservation and impact on PcG recruitment seem to vary among organisms. In Drosophila, where PcG PREs were firstly described (Simon et al., 1993; Zink et al., 1991), a large number of PREs cis-elements, compared to other organisms, has been characterized as well as DNA binding factors acting in trans able to recruit PcG proteins that must follow three requirements: 1) recruit PRC2 complex, 2) disseminate H3K27me3 in the surrounding genetic areas and 3) repress PRC2-linked markers (March & Farrona, 2017; Simon & Kingston, 2009). On the other hand, our knowledge of PRCs recruitment mechanisms in humans is less clear as PREs are not conserved between Drosophila and humans, even in common and well described PcG targets such as the *HOX* genes (Ringrose, 2007). The D11.12 element is consider the first human PRE-like described (Woo et al., 2010). In addition, some epigenetic marks are subordinated to the presence of another mark related to a specific chromatin state. This PTM crosstalk seem to be also relevant for regulation of PRC2 activity, as PRC2 show preference to be recruited and repress dense chromatin (Grau et al., 2011; Yuan et al., 2012).

In Arabidopsis, recruitment of PRCs has been a focus on recent years. The first described PRE-like is present in the *LEAFY COTYLEDON2* (*LEC2*) locus, a gene involved in seed development (Berger et al., 2011). However, this putative PRE does not conserve the characteristic structure of Drosophila PREs. The Plant homo domain (PHD)-containing ALFIN1-like (ALs) proteins were also described has a PcG complex recruiter, interacting *in vivo* with PCR1 complex subunits AtBMI1b and AtRING1a. AL6 is necessary in the deposition of H3K27me3 through its interaction to LHP1 on specific loci (Molitor et al., 2014). In 2017, 170 putative-PREs identified at PRC2-target genes were proposed (Xiao et al., 2017).

Focusing in the putative PREs from three PRC2 targets, *AGAMOUS (AG), SEPALLATA 3 (SEP3)* and an uncharacterized gene *At5G61120,* Xiao et al described DNA motives present at them. Considering these motives, the putative PRE-motif-binding transcriptional factors were more than 200, of which two of them were confirmed as a PRC2 recruiters, APETALA 2 (AP2)-like and BASIC PENTACYSTEINE (BPC).

Another publication recently identified TELOMERE-REPEAT-BINDING FACTORS (TRBs) 1-3 as a CLF/SWN-mediated PRC2 recruiters through direct binding to telobox DNA motif (Zhou et al., 2018). EARLY BOLTING IN SHORT DAYS (EBS) and its homolog SHORT LIFE (SHL) have been the recent proteins involved in PcG repression so far. EBS is able to read H3K4me2/3 whit its bromo-adjacent homology (BAH) domain and H3K27me3 with its PHD domain. It was hypothesized that this dual reading ability could play a role in switching gene expression (Yang et al., 2018). This hypothesis was confirmed by Li et al, who showed that EBS and SHL, interactors of EMF1 and AtBMI1a, in association with LHP1 play a critical role in whole-genome expression patterns (Li et al., 2018).

1.4 PWWP family

Looking for new partners of PRC2, PWWP-DOMAIN INTERACTOR OF POLYCOMBS1 (PWO1) protein was identified (Hohenstatt et al., 2018). The Proline and Tryptophan (PWWP) motif/domain is present in several eukaryotic proteins and has been proposed to be a protein-protein and DNA-protein interacting domain (Qiu et al., 2002; Stec et al., 2000).

PWO1 interacts *in vivo* with the three methyltransferases of PRC2, CLF, MEA and SWN, and mobilizes CLF to specific foci in the nucleus. In addition, PWO1 is necessary for repression of some PcG target genes such as *FUSCA3* (*FUS3*), *SEP3*, *AG* and *FLC*, all of them MAD-box transcriptional factors. It interacts with histone 3 through its PWWP domain and is involved in the maintenance of the nuclear morphology, controlling nuclear size, suggesting that PWO1 is a novel histone reader involved in Arabidopsis development and nuclear structure (Hohenstatt et al., 2018; Mikulski et al., 2019).

PWO1 is a plant specific member of the PWWP family, included in the Royal Superfamily, which in Arabidopsis is composed by 16 PWWP-containing proteins (Alvarez-Venegas & Avramova, 2012; Maurer-stroh et al., 2003). Recently, PWO1 and its closest homologs PWO2 and 3, have been proposed to be part of a novel multifunctional complex, the PWWPs- EPCRs-ARIDs-TRBs (PEAT) complex (Tan et al., 2018). This complex is formed by PWWP proteins, AT-rich interaction domain-containing proteins (ARIDs) and enhancer of Polycomb-related (EPCRs).

The current model proposes that the PEAT complex is involved in histone deacetylation and heterochromatin condensation and facilitates heterochromatin silencing interacting with both, histone acetyltransferases and deacetylases *in vivo*.

PWWP domain protein 1 (PDP1), member of the PWWO family, interacts with FVE, meantime PDP2/3 interact with FVE and MSI5 in a PcG complex-like form of LHP1 and regulate flowering (Kenzior & Folk, 2015; Zhou et al., 2018). Indeed, Y2H data showed an interaction between FVE and PWO1 (Hohenstatt's thesis, unpublished).

A novel interactor of PWO1 is FORGETTER 1 (FGT1) (Mikulski et al, 2019). FGT1 is a plant homeodomain (PHD) protein involved in heat-shock memory. In addition to the PHD domain, FGT1 has other domains involved in chromatin remodeling or translocation of macromolecules called Helicase C-like domain and ATPases Associated with diverse cellular Activities (AAA domain) (Brzezinka, et al., 2016). FGT1 homologs are found in other plant species and orthologues in metazoans (Gazave et al., 2009). In animals, the orthologue of FGT1 is STRAWBERRY NOTCH (SNO). *SNO* genes encode conserved nuclear proteins that are involved in the regulation of cell-cell interactions, cellular identity, apoptosis, embryogenesis, cell proliferation and differentiation (Coyle-Thompson & Banerjee, 1993; Majumdar et al., 1997; Takano et al., 2010). Many proteins with PHD zinc fingers have been reported in the last years in several organisms (Li & Li, 2012). These proteins act as epigenomic H3 tail readers in eukaryotes (Bienz, 2006; Sanchez & Zhou, 2011). In addition, they can also bind to non-histones proteins (Musselman & Kutateladze, 2011). Thus, PHD proteins play a critical role in several developmental stages in Arabidopsis due to the recognition of H3 tail post-translational modifications and have been involved in regulation of the meiosis process, embryonic meristems initiation, seed development, flowering and vernalization (Mouriz et al., 2015).

1.5 DUB superfamily in Arabidopsis

The importance of the deposition of some epigenetic marks has already been discusses in this introductory chapter, specifically H3K27me3 and H2Aub, marks deposited respectively by PRC2 and PRC1. Considering the last one, ubiquitination of histones, can yield different results: increasing or reducing the gene expression.

Protein ubiquitination can be direct or indirect depending on the E3 (ubiquitin ligase) enzyme that mediates this process, mainly on a lysine residue (Haas et al., 1982; Ishikura et al., 2010; Zheng & Shabek, 2017). On the other hand, deubiquitination is carried out by an evolutionary conserved group of proteins known as ubiquitin deconjugating enzymes. Comprising one of the biggest super families, the deubiquitinase superfamily (DUB) counter the action of E3 ligases. Ubiquitination/deubiquitination is a highly dynamic process that is ultimately essential for many processes including cell homeostasis, signal transduction, transcriptional gene regulation, protein degradation and endocytosis among others (Frappier & Verrijzer, 2011; Hershko & Ciechanover, 1998; Yan et al., 2000).

DUBs have three molecular roles: i) generation of ubiquitin monomers (Chung & Baek, 1999); ii) regeneration of ubiquitin during the decomposition of ubiquitin-protein conjugates in the 26S proteasome (Amerik & Hochstrasser, 2004); and iii) deubiquitination of conjugates by releasing intact both the ubiquitin and the target to prevent the degradation of the pre-targeted protein (Taya et al., 1999).

This superfamily has five families: ubiquitin-specific proteases (USPs), also called ubiquitin-specific-processing proteases (UBPs) in Arabidopsis, ubiquitin carboxy-terminal (UCH) proteases, the ovarian tumor proteases (OTUs), the Machado-Joseph disease protein domain proteases or Josephine (MJD) family and the JAB1/MPN^{+}/MOV34 (JAMMs) proteases.

The first four families are cysteine proteases while the JAMM family are zinc metalloisopeptidases. The DUB family in Arabidopsis contains an estimated 64 members (Yan et al., 2000). However, many of the putative members are still uncharacterized and their molecular activities are still poorly understood.

Special attention is required for the UBP family, as in the last years several members of this family have been linked with epigenetic regulation through histone modifications. Arabidopsis UBP members have redundant functions, but also specific roles in plant development (Liu et al., 2008). The family of UBPs, which possess a highly similar sequence to human USPs proteins, has 27 members in Arabidopsis divided in 14 subfamilies based on specific protein domains (Yan et al., 2000; Zhou et al., 2017). All UBPs in Arabidopsis contain a UBP domain (although these vary in length depending on the protein) and one or more domains that are speculated to be involved in protein-protein interactions (Komander et al., 2009).

1.5.1 DUBs in Arabidopsis and their role in epigenetics

As was mentioned before, PRC1 mediates the deposition of the transcriptional repressive mark H2Aub on lysine 121 in Arabidopsis, 119 in humans and 118 in Drosophila through its catalytic subunits RINGs and BMI in Arabidopsis (**Figure 4 A**) (Sanchez-Pulido et al., 2008). On the other hand, H2A deubiquitination is usually associated with transcriptional activation (Nakagawa et al., 2008).

In humans, MYSM1, a member of the JAMM DUB subfamily was the first enzyme reported to affect H2Aub levels (**Figure 4 B**). *mysm1* mutant in human embryonic kidney cell lines (HEK293T) displays an accumulation of H2Aub. Changes in the levels of H2Aub also alter the enrichment of other epigenetic marks. For instance, H2A deubiquitination relates with an increase of H1 phosphorylation, which is related with gene activation (Zhu et al., 2007). Phylogenetic analyses demonstrate that in plants there is not a clear candidate with similar sequence to MYSM1 (March & Farrona, 2018).

In HeLa cells, USP16 deubiquitinates H2A *in vitro* and *in vivo* and *USP16* knock-down RNA line shows accumulation of H2Aub, which affects the cell growth ratio and regulates the expression of a HOX gene, *HOXD10* (Joo et al., 2007). The closer proteins in Arabidopsis showing high sequence similarity are UBP1 and UBP2 (March & Farrona, 2018). Nevertheless, the role of these two proteins in H2A deubiquitination have not been characterized.

Human USP3 affects cell cycle progression and at a molecular level mediates the deubiquitination of H2Aub and γ-H2AX under DNA damage response (Nicassio et al., 2007; Sharma et al., 2014). However, a clear USP3-like candidate in Arabidopsis UBPs is not detected (March & Farrona, 2018).

The UCH family member CALYPSO, a Drosophila PcG member and catalytic subunit of PR-DUB, is involved in H2Aub deubiquitination. CALYPSO binds to Additional sex combs (ASX) to constitute the PR-DUB complex. PR-DUB mutants display an accumulation of H2Aub and mis-regulation of homeotic genes, indicating the essential role of this complex in the dynamics of H2Aub (Alonso et al., 2007; Scheuermann et al., 2010). The PR-DUB complex counteracts PRC1-mediated H2Aub deposition, even though it mediates PcG repression by un resolved mechanism (Chittock et al., 2017; Scheuermann et al., 2012). In Drosophila, PR-DUB and PRC1 share the same target genes (Schuettengruber et al., 2009), something that does not follow the same rule in humans (Hauri et al., 2016).

The CALYPSO orthologue in humans is the tumor suppressor BRCA-1-associated protein 1 (BAP1) that along with transcription-related proteins mediates H2Aub deubiquitination (Sahtoe et al., 2016). Recently in humans, two PR-DUB subcomplexes have been described, PR-DUB.1 and PR-DUB.2 (**Figure 2**) (Hauri et al., 2016). This differentiation comes from the ability of BAP1 to interact with Additional sex combs-like protein (ASXL) 1 and ASXL2, but not with both of them at the same time, as well as with several partners including transcriptional factors, chromatin associate proteins and transcriptional co-regulators that implicate BAP1 in other non-PcG mediated processes (Dey et al., 2012; Hauri et al., 2016; Lee et al., 2014). Thus, ubiquitination and deubiquitination of H2A is a highly dynamic process in crosstalk with PRC-related activities as well as with other epigenetic marks in order to fine-tune gene expression patterns. In contrast to PRC1 and PRC2, a functional PR-DUB complex and CALYPSO ortholog has not been described in Arabidopsis or any other plant species.

In Arabidopsis, UBP12 has been characterized as a new PcG member since it displays an *in vivo* H2Aub deubiquitinase activity, regulating also H3K27me3 enrichment levels at some loci in interaction with LHP1 and UBP13 (Derkacheva et al., 2016). In addition, UBP12 and UBP13 can deubiquitinate polyubiquitinated ORESARA 1 (ORE1), a transcriptional factor involved in leaf senescence in a direct way, mode of action as its human ortholog USP7 (Maertens et al., 2010; Park et al., 2019). These results suggest that UBP12 may be part of a novel-unknown-complex with, at least, partial PR-DUB complex activity (**Figure 4 B**). Finally, UBP12 and UBP13 also contribute to gene silencing in heterochromatin, participating in the PcG repression, sharing this function with the Drosophila USP7, member of several complexes with multiple functions, suggesting that UBP12/13 may play an undescribed role (Derkacheva et al., 2016; Kim & Sixma, 2017).

While H2Aub is a repressive transcriptional mark, H2B monoubiquitination (H2Bub) generally plays a role in transcriptional activation. In Arabidopsis this mark is deposited on the K143 of the H2B tail, but in other organisms H2Bub occurs on different lysine residues of the same histone. Similar to the situation in humans and yeasts, genome-wide distribution of H2Bub in Arabidopsis relates with other active epigenetic marks, such as H3K4me3 and H3K36me3 (Roudier et al., 2011).

Radiation sensitivity protein 6 (Rad6) was the first yeast protein reported to have E2 activity in the deposition of the H2Bub *in vitro* and *in vivo* (Robzyk et al., 2000) (**Figure 4 C**). Rad6 co-operates with the E3 enzyme Bre1, which is essential for H2Bub *in vivo*. In the monoubiquitination of H2B several support complexes are needed. The Paf1 complex is necessary for proper H2Bub since defective single mutants of components of this complex showed a loss of H2Bub (Wood et al., 2003) (**Figure 4 C**). The activity of the Bur1/Bur2 (BUR) cyclin-dependent protein kinase complex is also required since the defective mutant of Bur2, which encodes one of the two complex components, shows a decrease in H2Bub (Wood et al., 2005) (**Figure 4 C**). In humans these factors and their functions are conserved (Kim et al., 2005; Zhu et al., 2005). In Arabidopsis, both, Rad6 (UBC1, UBC2 and UBC3) and Bre1 (HUB1 and HUB2) play a repressive role in the control of flowering time through the activation of *FLC,* by the deposition of ubiquitin from UBC1/2 to H2B guided by the E3 HUB1/2 (Xu et al., 2009). Subsequently, H2Bub enrichment on *FLC* promotes the deposition of H3K4me3 and H3K36me3 (Cao et al., 2008).

Considering the role of H2Bub, it is obvious that removal of this mark entails a reduction of transcription. In yeast, Ubp8 and Ubp10 deubiquitinate H2Bub. Ubp10 acts independently, while Ubp8 is a subunit of the Spt-Ada-Gcn5-acetyltransferase (SAGA) complex (Gardner et al., 2005) (**Figure 4 D**). Orthologues of the yeast Ubp8 exist in Drosophila (Nonstop) and humans (USP22), as well as other SAGA complex subunits (Zhang et al., 2008; Weake et al., 2008).

The Arabidopsis SAGA complex shares some functions with the SAGA complex of other organisms, specifically the control of gene expression through histone acetylation (Moraga & Aquea, 2015; Kim et al., 2015). On the other hand, the relation between the SAGA complex and H2B deubiquitination differs compared to the situation in other organisms. In Arabidopsis, the H2Bub deubiquitination activity involves UBP22, which in association with SAGA-associated factor 11 (SGF11) and ENCHANCER OF YELLOW (ENY) 2, both orthologs of the SAGA complex in other organisms, compose the deubiquitination module (DUBm) of the SAGA complex **(Figure 4 D)** (Pfab et al., 2018). This DUBm has H2B deubiquitination activity, being able to deubiquitinate in a SAGA complex-independent way, an undescribed activity in other organisms (Nassrallah et al., 2018; Pfab et al., 2018). Antagonistically to DUBm function, the C3D complex component DE-ETIOLATED 1 (DET1)- DDB1-Associated-1 (DDA1) protein interacts with SGF11 *in vivo* to mediate DUBm degradation in a ubiquitin-mediated process affecting, indirectly, H2Bub levels (Nassrallah et al., 2018).

Drosophila USP7, in a complex with the guanosine 5-monophosphate synthetase (GMPS), also mediates H2Bub deubiquitination and contributes to homeotic gene silencing guided by Pc in Drosophila (Van Der Knaap et al., 2005) (**Figure 4 D**). In humans, USP7 and USP11 physically interact with members of PRC1 *in vivo*, such as Mel18, Bmi1 and Ring1. USP7 deubiquitinates H2A and H2B *in vitro* and changes in Bmi1 and Ring1 ubiquitin levels were reported in *USP7* and *USP11* overexpression lines. *usp7* and *usp11* mutants in human fibroblast result in de-repression and loss of PRC1 binding to the tumor suppressor *INK4α* locus (Maertens et al., 2010). These results suggest that USP7 and USP11 have a double role in PRC1 functions, as direct partners of PRC1 as well as regulating the levels of ubiquitin in PcG members *per se*. The closer proteins in Arabidopsis showing high sequence similarity are UBP12 and UBP13, nevertheless, this activity has not been described in them.

UBP26 and OTUBAIN-LIKE DEUBIQUITINASE 1 (OTLD1) also have H2Bub deubiquitination activity in Arabidopsis (**Figure 4 D**). *UBP26* was identified as a suppressor of mutations affecting *REPRESSOR OF SILENCING1 (ROS1)*, which encode a DNA demethylase involved in suppressing gene silencing (Sridhar et al., 2007). *ubp26* shows higher levels of H2B monoubiquitination (H2Bub) as well as decreased non-CpG DNA methylation. These results indicate that UBP26 may deubiquitinate H2B (**Figure 4 D**) and furthermore that this post-translational modification is required for the deposition of the repressive mark H3K9me2, which in turn is needed for gene silencing through DNA methylation in heterochromatin (Sridhar et al., 2007). Mutations in *UBP26* arrest embryo development, similar to some PcG members mutants, upregulating the expression of PcG target gene *PHERES1 (PHE1)* due to low enrichment of H3K27me3 at the *PHE1* locus (Luo et al., 2008).

The role of UBP26 in controlling flowering time has also been shown through its activity in the regulation of *FLC*. In particular, UBP26 affects *FLC* expression due to H2Bub deubiquitination of *FLC* chromatin and *ubp26* mutant displays an early flowering phenotype as well as higher global level of H2Bub (Schmitz et al., 2009). Methylation levels of H3K36 at *FLC* also decreases in *ubp26*; whereas, H3K27me3 levels increase. Thus, these results suggest that UBP26 might regulate *FLC* expression by decreasing the repressive mark H3K27me3 and increasing H3K36me3 through H2B deubiquitination (Derkacheva et al., 2016). Finally, it was shown that the PcG target gene AT1G80160 is also upregulated in the *ubp26* mutant (Derkacheva et al., 2016). Taken together, these data show that UBP26 plays an important role in the regulation of the expression of loci located in both heterochromatin and euchromatin, being necessary for H3K27me3 and H3K9me3 at specific loci.

Finally, OTLD1 was found to interact with the histone lysine demethylase KDM1C *in planta*. Indeed, OTLD1 has H2B deubiquitination activity *in vitro* and the KDM1C-OTLD1 complex represses gene expression by H2Bub deubiquitination (Krichevsky et al., 2011).

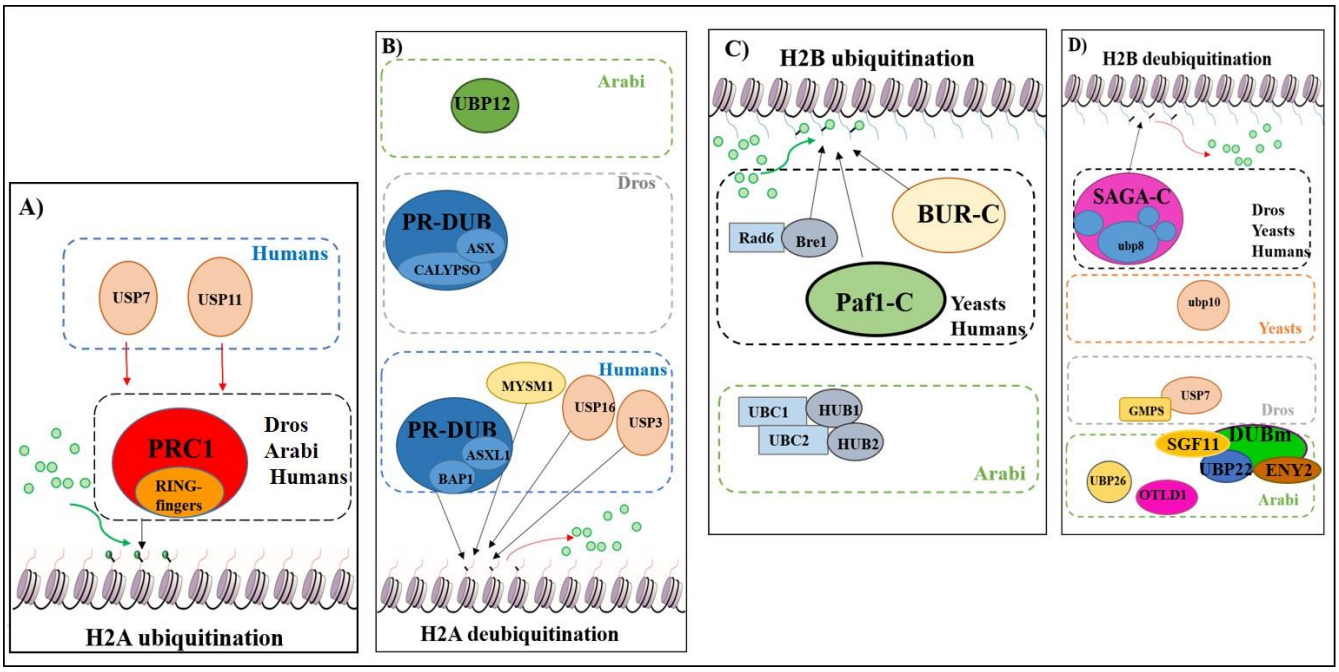

Figure 4. Schematic model showing the regulation of DUBs and support complexes involved in H2A and H2B monoubiquitination/deubiquitination. The figure represents four processes, H2A monoubiquitination, H2A deubiquitination, H2B monoubiquitination and H2B deubiquitination. Ubiquitin is represented by small green circles. **(A)** H2A monoubiquitination. UBIQUITIN-SPECIFIC PROTEASE 7 (USP7) and USP11. PRC1 function is conserved in eukaryotes; in humans two additional USPs have been described in this process. **(B)** H2A deubiquitination. UBIQUIRIN PROTEASE 12 (UBP12), CALYPSO and Additional sex combs-like protein (Asx); tumor suppressor BRCA-1-associated protein 1 (BAP1) and Additional sex combs-like protein (ASXL) 1, UBIQUITIN-SPECIFIC PROTEASE 3 (USP3), USP16 and Histone H2A deubiquitinase MYSM1 (MYSM1). The role of PR-DUB has been described in humans and flies, as well as three additional DUBs. The protein involved in this process in Arabidopsis is UBP12. **(C)** H2B monoubiquitination. Radiation sensitivity protein 6 (Rad6), E3 ubiquitin-protein ligase BRE1 (Bre1); UBIQUITIN CARRIER PROTEIN 1(UBC1), UBC2, HISTONE MONO-UBIQUITINATION 1(HUB1) and HUB2. Bre1 and two complexes (BUR-C and Paf1-C) are involved in the deposition of ubiquitin on the H2B. In Arabidopsis only the orthologues of Bre1 and its partner Rad6, have been described in relation to H2Bub. **(D)** H2B deubiquitination. Ubiquitin protease 8 (ubp8), ubp10, UBP22, UBP26, Ubiquitin-specific protease 7 (USP7), SAGA-associated factor 11 (SGF11), ENCHANCER OF YELLOW 2 (ENY2), guanosine 5-monophosphate synthetase (GMPS) and OTUBAIN-LIKE DEUBIQUITINASE 1 (OTLD1). Several DUBs are involved in this process in different organisms. Arabidopsis has a module of a SAGA-like complex (DUBm) involved in this process that has H2B deubiquitination activity in a SAGA-independent way. Adapted from (March & Farrona, 2018).

Objectives

2. Objectives

The aim of this project is the molecular characterization of UBP5 and FGT1 as putative novel PcG members in the regulation of Arabidopsis development and the study of how UBP5 and FGT1 genetically and physically interacts with others PcG members to regulate gene expression.

The results of this project will contribute to a better understanding the pathways and mechanisms that control plant development, as well as reveal novel characteristics and roles of these two proteins.

Materials and Methods

3. Materials and methods

3.1 Plant material

3.1.1 Accessions

For the elaboration of this research all the *Arabidopsis thaliana* (Arabidopsis) plants were Col-0 genetic background (**Table 1**). Seeds used in this research were obtained from the Nottingham Arabidopsis Stock Centre (NASC) or others research laboratories and some have been generated during this particular research.

Table 1. Genotypes used in this research project.

Name	Locus	Description
fgt1-4	At1g79350	T-DNA insertional mutant (Meinke et al., 2008)
EMB1144	At1g48850	T-DNA insertional mutant (Meinke et al., 2008)
fgt1-1	At1g79350	Point mutation (Brzezinka et al., 2016)
fgt1-5	At1g79350	Deletion mutant developed in this study
flc-3	At5g10140	Deletion; fast neutron (Michaels & Amasino, 1999)
Col FRI SF2	At4g00650	Natural functional allele (Lee and Amasino, 1995)
fve-3	At2g19520	Deletion, fast neutron (Ausín et al., 2004)
ubp5-1	At2g40930	T-DNA insertional mutant (Meinke et al., 2008)
pwo1-1	At3g03140	Sail_342_C09
ubp5-2	At2g40930	Deletion mutant developed in this study

3.1.2 Conditions

The growth conditions for Arabidopsis were similar in walk-in chambers and in chambers for *in vitro* culture. Day length conditions used were Long Day (LD) conditions, 16 hours (h) under cold white fluorescent lamps (120 μE m^{-2} s^{-1}) and 8h of dark and Short Days (SD) conditions, 8 h of light (120 μE m^{-2} s^{-1}) and 16 h of dark. In both cases with temperatures between 19ºC for dark and 21ºC for light periods. For the vernalization requirement the plants were growth during 8 weeks at 8h of light (120 μE m^{-2} s^{-1}) and 16 h of dark with a constant temperature of 4ºC. In all the growth conditions the humidity was between 65% and 70%. For stratification the seeds were immerse in water or sowed on Murashige and Skoog (MS) plates and kept in dark at 4ºC during 3 days.

The seeds of Arabidopsis were sowed on soil pots containing 5:1:1 proportion of compost, vermiculite and perlite respectively. For *in vitro* culture a MS medium with 7g/L of plant agar was used. In both cases, the seeds were disinfected in columns with two washes of 2 minutes (min) of ethanol, first 70% ethanol and second 96%, followed by a centrifugation at 9391 g for 2 min.

Nicotiana benthamiana seeds were sowed on soil, the same 5:1:1 compost and grown in LD conditions.

3.1.3 Phenotypic analysis

To measure the flowering time in LD and SD conditions I quantified the total leaf number (rosette and cauline) at the time in which the first flower opens due to the relation with the flowering time (Pouteau, 2004). Number of plants was at least 10 plants/genotype.

3.1.4 Plant transformation

The method used to generate transgenic Arabidopsis plants was the floral dip (Clough & Bent, 1998). 4 mL of saturated *Agrobacterium tumefaciens* (Agrobacterium) culture with our plasmid of interest grown during 36-48 h was used to inoculated 400 mL of liquid lysogeny broth (LB) media plus specific antibiotics during 24 h (DO_{600} = 3-4). After the pellet collection by centrifugation, the bacterial culture was resuspended in a media of 5% sucrose and 0.01% silwet till DO_{600} = 1. Once the plants flowered, they were immersed in the *Agrobacterium* solution twice during 2 min.

For the transient transformation of *Nicotiana benthamiana* an over-night co-culture of Agrobacterium with our plasmid of interest (**Table 2**) and with an Agrobacterium clone carrying the pCB301-P19 plasmid to inhibit the RNA silencing guided by siRNA *in planta* was prepared (Win & Kamoun, 2004). *Agrobacterium* cells were harvested by centrifugation and resuspended in induction media composed of 10 mM MgCl2, 10 mM MES and 150 µM acetosyringone. The cultures were inoculated after 3 h in induction media in a final density of DO_{600} = 0.3 by injection in the abaxial part of the leaf with a syringe. One and two days after infiltration (DAI) the plants were sprayed with 20 µM β-estradiol, 0.1% Tween®-20 to induce the production of our chimeric proteins, due to the plasmids that I used (**Table 2**) have a β-estradiol inducible promoter.

Table 2. Expression vectors with the cassettes of interest fused to a heterologous epitope.

Vector	Experiment	Description
pMDC7-FGT1-mCherry	CoIP	From D.Schubert
pMDC7-UBP5-mCherry	CoIP	From D.Schubert
pMDC7-GFP-SWNΔSET	CoIP	GFP-SWN without SET domain

3.2 Microorganisms

3.2.1 Strains and preparation

For this research I used the following microorganisms and strains: *E. coli* DH5α and DB3.1 heat-shock competent cells, Agrobacterium GV3101 electro-competent cells and AGL0 strains and *Saccharomyces cerevisiae* AH109.

For *E. coli*, 0.5 -2 µL of the construction was added to a 50 µL aliquot of competent cells. After 20 min on ice the aliquot was immerged in a water bath at 42ºC during 45 sec. After 2 min at 4ºC 950 µL of liquid LB was added and incubate at 37ºC during 1 h. After a centrifugation to collect the bacteria, the pellet was resuspended in 200 µL of liquid LB and plated with the proper antibiotic/s.

For the *Agrobacterium* transformations an 1800 V electroporation were carried out, followed by a recovery stage in liquid LB at 28ºC during 2 h.

3.3 Nucleic acids analysis

3.3.1 Plant genomic DNA extraction

To extract genomic plant DNA a young leaf of a seedling was enough. The samples were frozen in liquid N_2 with glass bead and crushed in a tissuelyser. 400 µL of DNA extraction buffer was added (200 mM TRIS-HCl pH 8; 250 mM NaCl; 25 mM EDTA; 0.5% SDS). Centrifugated 5 min 15871 g, transferred the supernatant to another 1.5 mL tube and mixed with 320 µL of 2-propanol. After a 15 min 15871 g centrifugation, the samples were washed with 70% ethanol and the final pellets were resuspended in water. This protocol is based on (Edwards et al., 1991).

3.3.2 Plant RNA extraction and relative expression analysis

Total 0.1 g of 9 days after germination (DAG)-old seedlings grown *in vitro* under LD condition plus 2 days of stratification were collected at Zeitgeber time (Zt) 16 and ground in liquid N_2. The RNA extraction was performed with E.Z.N.A Plant RNA Kit (OMEGA) following the manufacturer's instruction. A DNAse treatment of 2 µg of RNA was performed before cDNA synthesis with RevertAid Reverse Transcriptase (Thermo Fisher). Real time quantitative PCR (q-PCR) was performed in Bio Rad CFX96 device using Takyon for SYBR assay (Eurogentec). All the qPCRs of this research were done with cDNA from seedlings.

The housekeeping gene used as control was *UBIQUITIN-CONJUGATING ENZYME* (*UBC)21* (*At5G25760*) (Czechowski et al., 2005). Relative enrichment was calculated using the comparative Ct method (Livak & Schmittgen, 2001).

3.3.3 Thermal Asymmetric interlaced PCR (TAIL-PCR)

The TAIL-PCR consist in up to three specific conditions-nested-PCRs using specific primers for the T-DNA borders, right (RB) and left (LB), and Arbitrary Degenerate (AD) primers that are going to bind interspecifically to the DNA. Using as a template the PCR product of the previous PRC the final sample is enriched in random fragments from one of the borders to an unknow T-DNA flanking sequence. The conditions for the nested-PCRs were previously described in (Liu & Whittier, 1995).

To carry out the TAIL-PCR 3 left border (LB) primers and other 3 right border (RB) primers for the vector pCSA104 were used together with combinations of other 3 arbitrary degenerate (AD) primers (**Table 3**) (McElver et al., 2001).

3.3.4 Oligonucleotides

The following **tables 3-6** summarize the oligonucleotides used to carry out this research, clustered by experiment.

Table 3. Primers used to verify, by TAIL-PCR, the T-DNA flanking regions, FAIRE and introns retention.

Gene	Sequence (5'->3')	Experiment	Information
TAIL_LB1	GCCTTTTCAGAAATGGATAAATAGCCTTGCTTCC	TAIL-PCR	
TAIL_LB2	GCTTCCTATTATATCTTCCCAAATTACCAATACA	TAIL-PCR	
TAIL_LB3	TAGCATCTGAATTTCATAACCAATCTCGATACAC	TAIL-PCR	
TAIL_RB1	ATTAGGCACCCCAGGCTTTACACTTTATG	TAIL-PCR	
TAIL_RB2	GTATGTTGTGTGGAATTGTGAGCGGATAAC	TAIL-PCR	
TAIL_RB3	TAACAATTTCACACAGGAAACAGCTATGAC	TAIL-PCR	
TAIL_AD1	CTCGAGTATCGAGTT	TAIL-PCR	
TAIL_AD2	GGTCGACAGACATGAA	TAIL-PCR	
TAIL_AD3	TGTGCAGAATCATAGA	TAIL-PCR	
KNAT1.Fw2.FAIRE	TCATGGCTTCAACATCGCTT	FAIRE	
KNAT1.Rv2.FAIRE	AACAACCGAGAATTGCTTCCG	FAIRE	
KNAT1.Fw1.FAIRE	GCAGAGACAGACGGTGTTGA	FAIRE	
KNAT1.Rv1.FAIRE	GAGCTCCACCTGATGTGGTT	FAIRE	
HSP22_TSS-393_F	GACACAAGCATGGCAAGCCAA	FAIRE	(Brzezinka et al., 2016)
HSP22_TSS-393_R	TGACCTCTATTGCCCTATG	FAIRE	(Brzezinka et al., 2016)
HSP22_TSS-8_F	GCTAGAACAATCTCAATATC	FAIRE	(Brzezinka et al., 2016)
HSP22_TSS-8_R	GATGGTTAGTCTCAATTCTC	FAIRE	(Brzezinka et al., 2016)
fgt1-1_Fw_amplicon (A)	ATGTCCGCTTTGGGGGTATT	Intron retention	Genomic PCR *fgt1-1* A+B
fgt1-1_Rv_amplicon (B)	AAAGGGACTTTCCTGCGGTC	Intron retention	IR *fgt1-1* spliced C+D
fgt1-1_IR_Fw_ExonJunct19_20 (C)	TAGACTACTCTTCACTAATCTCGGT	Intron retention	
fgt1-1_IR_Rv_exon20 (D)	AACAATAGAGGCAAAGCGGC	Intron retention	
fgt1-1_IR_Fw_Intron19 (E)	AGTTCTTTTGTTTTGGTCCTGTCA	Intron retention	IR *fgt1-1* unspliced E+D
fgt1-1_IR_Fw_Intron19 (F)	AGTTCTTTTGTTTTGGTCCTGTCA	Intron retention	
fgt1-4_ExonJunc20_21_Fw (G)	ACCGCAGGGCTGGGC	Intron retention	IR *fgt1-4* spliced G+H
fgt1-4_Exon21_Rv (H)	TGCCTCGATACATCACCATCAA	Intron retention	
fgt1-4_Intron20_Fw_IR (I)	AAACCTTGCCATGTGTTTTGTC	Intron retention	IR *fgt1-4* unspliced I+H
fgt1_exon19_Fw_semi (J)	AGGGTTCACTTGACTTTAGAGCTT	Semi qPCR	J+D

Table 4. Primers used to measure the relative expression of the tested genes. Most of them were designed with QuantPrime (Arvidsson et al., 2008).

Gene	Sequence (5'->3')	Experiment
UBC FW	CTGCGACTCAGGGAATCTTCTAA	RT-qPCR
UBC RV	TTGTGCCATTGAATTGAACCC	RT-qPCR
KNAT1-FW	CACATCCTCAACAATCCTGATGGG	RT-qPCR
KNAT1-RV	TGGTTCTTGAGTTCCCGATCTTCG	RT-qPCR
KNAT2-FW	CGTTCGACGAGGCTACAACTTTC	RT-qPCR
KNAT2-RV	ACCGCACCATCATCTGAAAGAG	RT-qPCR
KNAT6-FW	TCATCTGACGAGGAACTGAGTGG	RT-qPCR
KNAT6-RV	TTGAGGTCCCGGTCTTCACATC	RT-qPCR
STM-fw	ACCTTCCTCTTTCTCCGGTTATGG	RT-qPCR
STM-RV	GCGCAAGAGCTGTCCTTTAAGC	RT-qPCR
FLC_Fw	AGCCAAGAAGACCGAACTCA	RT-qPCR
FLC_Rv	TTTGTCCAGCAGGTGACATC	RT-qPCR
EMB1144_Fw	TCCTTGTGTTGTTCCACGAGCTG	RT-qPCR
EMB1144_Rv	ACAAATGGCATTGTGCGTATTGCG	RT-qPCR
FT_Fw	CTGGAACAACCTTTGGCAAT	RT-qPCR
FT_Rv	AGCCACTCTCCCTCTGACAA	RT-qPCR
COR15a_Fw	ACCTCAACGAGGCCACAAAGAAAG	RT-qPCR
COR15a_Rv	CGCTTTCTCACCATCTGCTAATGC	RT-qPCR
SOC1_FW	TTCGCCAGCTCCAATATGCAAG	RT-qPCR
SOC1_RV	TGCTGACTCGATCCTTAGTATGCC	RT-qPCR
FVE_fw	GGCCTTCACTCTCTTGCAGATG	RT-qPCR
*FVE_rv_*	AGACGCTGGCGATTCTTGTAGG	RT-qPCR
AGL24_fw	TGGATCCACCTTCTACTCATCTCC	RT-qPCR
AGL24_rv	AGATCCTCTCCTCTCAGTTTCCG	RT-qPCR
AGAMUS_Fw	TCACCAGCACAACCTTACCTTCC	RT-qPCR
AGAMUS_Rv	TGGTACGCCGTGATTGCTGTTG	RT-qPCR
WUS_Fw	TCATCACGGTGTTCCCATGCAG	RT-qPCR
WUS_Rv	CCCGTTATTGAAGCTGGGATATGG	RT-qPCR
CLV3_Fw	TAAGGACTGTTCCTTCGGGACCTG	RT-qPCR
CLV3_Rv	TCTTGGCTGTCTTGGTGGGTTC	RT-qPCR

Table 5. Primers used to genotype.

Gene	Sequence (5'->3')	Experiment
Frigida_Fw	AGATTTGCTGGATTTGATAAGG	Genotyping
Frigida_Rv	CTTGATGTTGGTCGATGATG	Genotyping
fve-3_Fw	TCGGATTCAGGTATTATGTCCAA	Genotyping
fve-3_Rv	TCACTTAAACCCAGAAATCGAGA	Genotyping

Table 6. Primers designed to develop the targeted mutagenesis specific vectors as well as the primers to genotype the deletion *in* Arabidopsis.

Gene	Sequence (5'->3')	Experiment
FGT1_guide3-BsF	ATATATGGTCTCGATTGGACTTTAGCTGACATACACGTT	FGT1double-guide vector construction
FGT1_guide3-F0	TGGACTTTAGCTGACATACACGTTTTAGAGCTAGAAATAGC	
FGT1_guide1-R0	AACGCCCTGCAGTACACCACACCAATCTCTTAGTCGACTCTAC	
FGT1_guide1-BsR	ATTATTGGTCTCGAAACGCCCTGCAGTACACCACACCAA	
UBP5_guide6-BsF	ATATATGGTCTCGATTGTACGGGGGTGGTCCAACTCGTT	UBP5 double-guide vector construction
UBP5_guide6-F0	TGTACGGGGGTGGTCCAACTCGTTTTAGAGCTAGAAATAGC	
UBP5_guide20-R0	ACCAGTCGGAGGAGTTGCTTTTCAATCTCTTAGTCGACTCTAC	
UBP5_guide20-BsR	ATTATTGGTCTCGAAACAGTCGGAGGAGTTGCTTTTCAA	
P3_CAS9_FGT1_F	GCATTGCATAACTTGGAATGGA	Genotyping
P3_CAS9_FGT1_R	AGCATAGTTGAGGCACCAGAC	Genotyping
P3_CAS9_FGT1_F	AGTCAGATGTGTAGCTCTACCAAG	Genotyping
P3_CAS9_FGT1_Rv	ACTTCAGGCAAACTCTCCAGG	Genotyping

3.3.5 CRISPR/CAS9 double guide targeted mutagenesis

The construction of the double RNA guides (gRNAs) vector for CRISPR/Cas9 directed deletion was developed as explained in (Xing et al., 2014) supplementary information; the two gRNA-expressing modules, AtU6-26p and AtU6-U26t, were cloned from the pCBC-DT1T2 (**Table 7**). The gRNAs were designed in CRISPR-P (Lei et al., 2014).
The final vector used was named P3-Cas9-mCherry (**Appendix) (Table 7)** (Mc Hale et al., unpublished). This vector is composed by the *Cas9* sequence under an egg cell-specific promoter cloned from the pHE401 vector (Wang et al., 20015) followed by a mCherry under a seed maturation-specific promoter, the At2S3 promoter cloned from the pHDE-35SCas9-mCherry vector (Gao et al., 2016). The P3-Cas9-mCherry allows to activate the Cas9 exclusively at egg-cell stage and then follow the putative mutation at seed stage.

Table 7. CRISPR/CAS9 Intermediary and final vector backbones used in this research.

Vector	Experiment	Description
pCBC-DT1T2	Targeted mutagenesis	gRNAs module vector ((Xing et al., 2014)
P3-Cas9-mCherry	Targeted mutagenesis	Final vector (McHale et al., unpublished)

3.3.6 Formaldehyde Isolation of Regulatory Elements (FAIRE)

FAIRE is a technique that allow us to isolate nucleosome-depleted regions (NDRs) of the chromatin; these regions are important specific regions where proteins, involved in the control of gene regulation, may bind.

The samples cultivation, collection and fixation were done by Sara Farrona. The samples used were Col-0 and *fgt1-4* seedlings collected after 17 days on MS plates at 20ºC in LD conditions.

The FAIRE was carried out as previously described by (Omidbakhshfard et al., 2014) after fixation step. For the isolation and sonication of the chromatin the samples were grinded in a pre-cooled mortar with liquid N_2.

The powder was resuspended in crosslinking buffer 15 mins on ice (400 mM sucrose (20 mL of 2M stock), 10mM Tris-HCl, pH 8.0 (1 mL of 1M stock), 5mM β-ME (35 µL of 14.3 M stock), 0.1 mM PMSF (50 µL of 0.2 M stock). Add 1 tablet of Complete® Protease Inhibitor Cocktail to 50 mL crosslinking buffer immediately before use. The samples were filtered through 2 layers of Miracloth two times followed by a 20 min of centrifugation at 2880 g 4ºC. The pellets were resuspended in 1 mL resuspension buffer (250 mM sucrose (1.25 mL of 2 M stock), 10mM Tris-HCl, pH 8.0 (100 µL of 1M stock), 10mM MgCl2 (100 µL of 1 M stock), 1% Triton X-100 (0.5 mL of 20% stock), 5 mM β - ME (3.5 µL of 14.3M stock), 0.1mM PMSF (5 µL of 0.2M stock). Immediately before use dissolve half a Complete® Protease Inhibitor Tablet in resuspension buffer. The samples were centrifuged for 20 mins at 2880 g 4ºC. After 2 more centrifugations removing the supernatant and adding 1 mL resuspension buffer each time the pellets were resuspended in 300 µL of pre-cold buffer 3 (1.7 M Sucrose (8.2 mL of 2 M stock), 10 mM Tris-HCl, pH 8.0 (100 µL of 1 M stock), 0.15% Triton X-100 (75 µL of 20% stock), 2 mM MgCl2 (20 µL of 1M stock), 5 mM b- ME (3.5 uL of 14.3 M stock), 0.1mM PMSF (5 µL of 0.2M stock). Immediately before use dissolve half a Complete® Protease Inhibitor Tablet in Buffer 3. The samples were centrifuged 70 mins 4ºC at 16000 g.

After centrifugation the samples were sonicated in the Bioruptor® (Diagenode), medium power 15 sec ON/OFF 20 min. After sonication, the samples were centrifuged 10 min 4ºC at 16000 g.

For the isolation of the NDRs, a triple phenolization in a final volume of 600 µL was carried out. After the isolation, 0.1 volume of 3M sodium acetate was added, then 2.5 volumes of absolute ethanol and 1µL of glycogen. The samples were kept at -80ºC 1h for precipitation followed by a centrifugation at 16000 g 4ºC for 45 mins. The pellets were washed in 70% ethanol, then the pellets were dried for 10 mins at room temperature. The pellets were resuspended in TE buffer (10 mM Tris-HCl, pH 8.0 (100 µL of 1 M stock), 1 mM EDTA (10 µL of 1 M stock). These samples can be used now in qPCR. Primer used as a positive control come from (Brzezinka et al., 2016) (**Table 3)**.

3.3.7 Intron retention

The experiments carried out to detect an intron retention were done following the procedure and schemes of (Marquardt et al., 2014). The cDNAs were obtained as explain in 3.3.2 from Col-0, *fgt1-1* and *fgt1-4;* 9 days-old seedlings grown on MS on LD conditions at 20ºC. Primers were designed to confirm an intron 19 retention in *fgt1-1* allele and an intron 20 retention in *fgt1-4* allele. The primer and the combination of them can be found in (**Table 3**).

3.4 Immunoassays

3.4.1 Antibodies

Table 8 shows the antibodies that were used in this study.

Table 8. Antibodies used in the CoIP experiment.

Name	Company	Host species	purpose	remarks
anti-mCherry	Clontech	Rabbit	WB/IP	1:1K
anti-GFP	Roche	Mouse	WB/IP	1:5K
anti-rabbit HRP	Sigma	Goat	WB	1:20K
anti-mouse HRP	Millipore	Goat	WB	1:80K

3.4.2 Protein extraction and Co-immunoprecipitation (CoIP)

As mentioned before, the plasmid used for this experiment have a β-estradiol inducible promoter. The leaves co-infiltrated with the proper plasmid combinations have to be sprayed 1 and 2 DAI to produce our chimeric proteins. After 6 h from the second induction with 20 µM β-estradiol, 0.1% Tween®-20 the samples were frozen in liquid N_2. The samples were ground in a liquid N_2 pre-cold mortar followed by 20 min at 4ºC in a shaker in 10 mL of protein extraction buffer (10% glycerol, 150 mM NaCl, 2.5 mM EDTA, 20 mM TRIS-HCl pH 8, 1% Triton and Complete® EDTA-free protease inhibitor cocktail (1 tablet/50 mL; Roche). After resuspension, samples were filtered by a two Miracloth (Calbiochem®) layers and centrifuge at 4ºC 15 min 3220 g.

After the centrifugation the supernatants were transfer to a new 15 mL tube, and the extracts were taken, mixed with SDS buffer (0.3 M Tris-HCl (pH 6.8); 10 % (w/v) SDS; 30 % (v/v) glycerol; 0.6 M DTT; 0.01 % (w/v) bromophenol blue) and heated at 95ºC 5 min. Co-IPs were carried out incubating the samples with 30 µL of slurry of agarose beads protein-A during 4h at 4ºC in a rotating wheel and the specific antibodies (see **table 7**). After 4 h incubation, a centrifugation at 4ºC, 2 mins 500 g was carried out to precipitate the beads. The beads were washed 3 times with protein extraction buffer. The washed beads were resuspended in 20 µL of protein extraction buffer and 5 µL of SDS buffer before to be heated at 95ºC 5 min. The samples were chilled on ice 2 mins and centrifuge at 500 g, 2 mins before be loaded in the SDS-PAGE gel.

3.4.3 SDS-PAGE

10% SDS-PAGE gels were used and prepared as described in (Sambrook et al., 1989). 10% - 12% separating gels were used depending on the molecular weight of the proteins of interest in each experiment and run in running buffer 5X used (37.75g TRIS ultrapure, 235g Glycine, 125 mL SDS 10% and molecular H2O water till 5L). The gels were run at 120V 5 mins followed by 1h at 180V.

3.4.4 Western blot

Wet transfers were carried out as described in (Sambrook et al., 1989) with a 0.45 µM PVDF membrane (10 x 6.7 cm)(Immobilon®, Millipore). The PVDF membranes were activated in absolute methanol 30 s, then washed in transfer buffer 1X (100 mL; buffer A 10X 100 mL, 200 mL absolute methanol, 700 mL molecular H2O); buffer A 10X (30g TRIS ultrapure, 144g Glycine, molecular water till 1 L). The transfer to the membrane were carried out at 4ºC, overnight at 25V in a shaker. After the transfer, the membranes were blocked 1h at room temperature in blocking solution (PBS at 3% of BSA). After this first incubation, the membranes were rinsed 5 mins in a PBS 1X Tween®-20 0.1% solution followed by a wash in PBS 1X 1 min. The secondary antibody was incubated 1 time in PBS 1X and 3% BSA solution for 30 mins. After the incubation, the membrane was washed 4 times, 5 mins each wash, in PBS 1X Tween®-20 0.1% solution followed by a wash in PBS 1X 1 min. Finally, 1 mL of Super Signal™ West Pico PLUS Chemiluminescent Substrate was incubated with the membranes for 5 mins on dark. The signal was detected in a Syngene G:Box iChemi XR UV/White Light Gel Documentation System.

3.5 Yeast two hybrid assay

The Y2H system detects protein-protein interactions based on the yeast growth in the absence of a specific amino acid. The medias used lack one or more of these amino acids and the yeast is only able to grow if the Gal4-BD and the Gal4-AD domains interacts due to a protein-protein interaction. The system also allows to distinguish between weak or strong protein-protein interaction based on the yeast growth level and the media composition.

The *S. cerevisiae* AH109 competent cells were obtained as previously described (Gietz & Schiestl, 2007). For Yeast two hybrid (Y2H) experiments yeast were co-transformed with the plasmids listed in **table 9**. The negative controls as well as PWO1-BD + SWNΔSET-AD positive control (Hohenstatt et al., 2018). 3 µL of culture were plated at the same concentration on drop-out media (minimal medium) in absence of leucine and tryptophan (SD-L-W) or more restrictive media without histidine (SD-L-W-H) in serial dilutions. Growth was analyzed after 3 to 4 days growing at 28ºC.**Table 9**. Final vectors used in the Y2H assay.

Table 9. Expression vectors with the cassette of interest fused to the Gal4 activation domain and/or binding domain.

Vector	Experiment	Description
pGADT7-FGT1	Y2H	GAL4-Activation domain
pGADT7-UBP5	Y2H	GAL4-Activation domain
pGADT7-PWO1	Y2H	GAL4-Activation domain
pGADT7-SWNΔSET	Y2H	GAL4-Activation domain; SWN without the SET domain
pGADT7-FVE	Y2H	GAL4-Activation domain
pGBKT7-FGT1	Y2H	GAL4-Binding domain
pGBKT7-UBP5	Y2H	GAL4-Binding domain
pGBKT7-PWO1	Y2H	GAL4-Binding domain
pGBKT7-EMF2	Y2H	GAL4-Binding domain
pGBKT7-FVE	Y2H	GAL4-Binding domain
pGBKT7-PWO1_F1	Y2H	GAL4-Binding domain; PWO1 (a.a 1 to 290)
pGBKT7-PWO1_F2	Y2H	GAL4-Binding domain; PWO1 (a.a 353 to 541)
pGBKT7-PWO1_F3	Y2H	GAL4-Binding domain; PWO1 (a.a 633 to 769)

3.6 Bio-Software and Statistics

In order to carry out this research the following software and data bases were used:

SnapGene Viewer® software (from GSL Biotech, available at snapgene.com), SerialCloner (http://serialbasics.free.fr/Serial_Cloner.html), MEGA (Kumar et al., 2016), Primer-BLAST (Jian et al., 2012), TAIR (https://www.arabidopsis.org/), PLAZA (Van Bel et al., 2018), UniProt (https://www.uniprot.org/), TAIR GO terms (Berardini et al., 2004), R (https://www.r-project.org/) and BAR e-plant; attribution 4.0 International (CC BY 4.0) (Waese et al., 2017) mainly.

The RNA Seq was carried out by Illumina sequencing technology (Beijing Genome Institute, Shenzhen, China) from samples taken by Sara Farrona. The samples used were Col-0 and *fgt1-4* seedlings collected after 17 days on MS plates at 20ºC in LD conditions. For the analysis of RNA Seq, the raw data was trimmed 5' and 3'ends in Trim galore and FastQC (Andrews S, 2010). The clean reads were aligned to the reference genome (TAIR10) with Tophat2 tool (Kim et al., 2013) in Galaxy open source (Blankenberg et al., 2010). Tophat2 uses the short read aligner Bowtie2 (Langmead & Salzberg, 2012). Data post-processing, sort and index, was carried out with Samtool (Li et al., 2009). The assembling and transcript analysis were carried out with Rsubread – featureCounts (Liao et al., 2014) based on read counts from sequencing experiments from RNA-seqs.

The Differential Gene Expression (DGE) was done with edgeR (Robinson et al., 2010) a package of Bioconductor based on Poisson model. The data were selected by the False Discovery Rate (FDR) ≤ 0.05 and then by log Fold Change to filter the candidates. The Fisher's exact test to compare the DGE list versus the H3K27me3 target genes (Lafos et al., 2011) was carried out in Virtual Plant1.3 (Katari et al., 2010). The Gene Ontology terms were obtained through the online tools from TAIR (https://www.arabidopsis.org/tools/bulk/index.jsp) The data obtained by q-PCR and the flowering time were analyzed with Student's t-test for comparing two means, p-value < 0.05, <0.01 and <0.001.

Results

4. Results

Once PWO1 was characterized as a new PcG member interacting with CLF and SWN *in planta* among others PcG members, a proteomic study screening looking for *in vivo* PWO1 interactors was carried out in the laboratory of Prof. Schubert (Mikulski et al., 2019). Based on the peptide output, the second most abundant protein found interacting with PWO1 was EMBRYO-DEFECTIVE 1135 (EMB1135), also described as FGT1. EMB1135/FGT1 protein has three domains; an ATPases Associated with diverse cellular Activities (AAA domain) and a Helicase C4, domains involved in chromatin remodeling or translocation of macromolecules and a PHD domain (**Figure 5**) (Brzezinka et al., 2016).

4.1 Characterization of the putative embryo defective T-DNA line *emb1135*

Four T-DNA lines were investigated in *EMB1135/FGT1*; however, three of them presented germination, segregation and loss of resistance problems and, therefore, were not further analyzed:

SalkSeq_17372 (intron 4) – germination problems

Gabi_811B05 (intron 4) – germination problem, wild type phenotype

Salk_041012 (intron 7) – low germination, the few that germinated died

Another T-DNA insertion line was obtained from the SeedGenes database, an Oklahoma State University – Syngenta collaboration project to discover essential genes involved in Arabidopsis development (http://seedgenes.org/) (McElver et al., 2001). In this collection our gene of study was catalogued as *EMB1135*, a gene required for embryo development. The mutant line in *EMB1135* from this collection presents a T-DNA insertion in the intron 20. Although this mutant allele was named as *emb1135* by the SeedGenes database, from now on it will be mentioned as *fgt1-4* following the nomenclature by Brzezinka et al. 2016) (**Figure 5**).

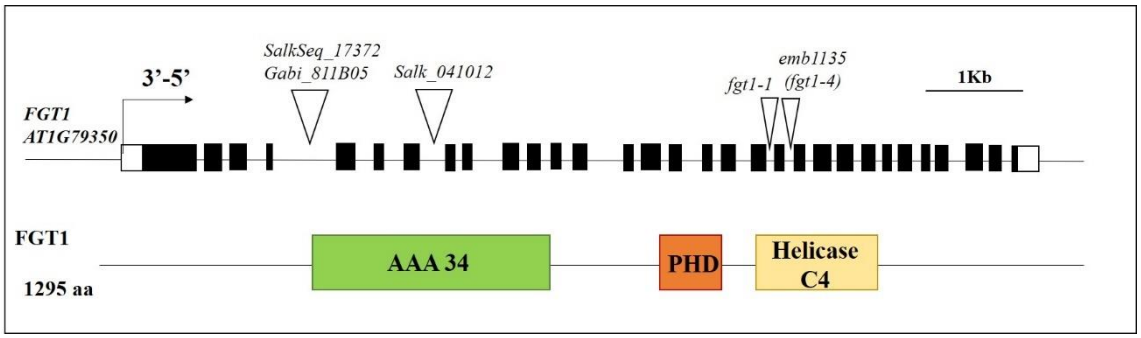

Figure 5. Schematic representation of *FGT1* locus and FGT1 protein. Top) *FGT1* locus with the T-DNA insertions used in this research. Bottom) FGT1 protein scheme and its domains.

4.1.1 *fgt1-4* T-DNA line presents a pleiotropic phenotype

Even though the majority of the *fgt1-4* allele seeds do not germinate, the few that do display multiple and severe phenotypical changes including low grow ratio, loss of apical dominance and 1% of them develop callus-like structures that remind to the PCR2 *clf, swn* double mutant (**Figure 6**) (Chanvivattana et al., 2004). This severe developmental phenotype fits with our hypothesis that FGT1 could be involved in PcG-related epigenetic mechanisms based on the interaction with PWO1.

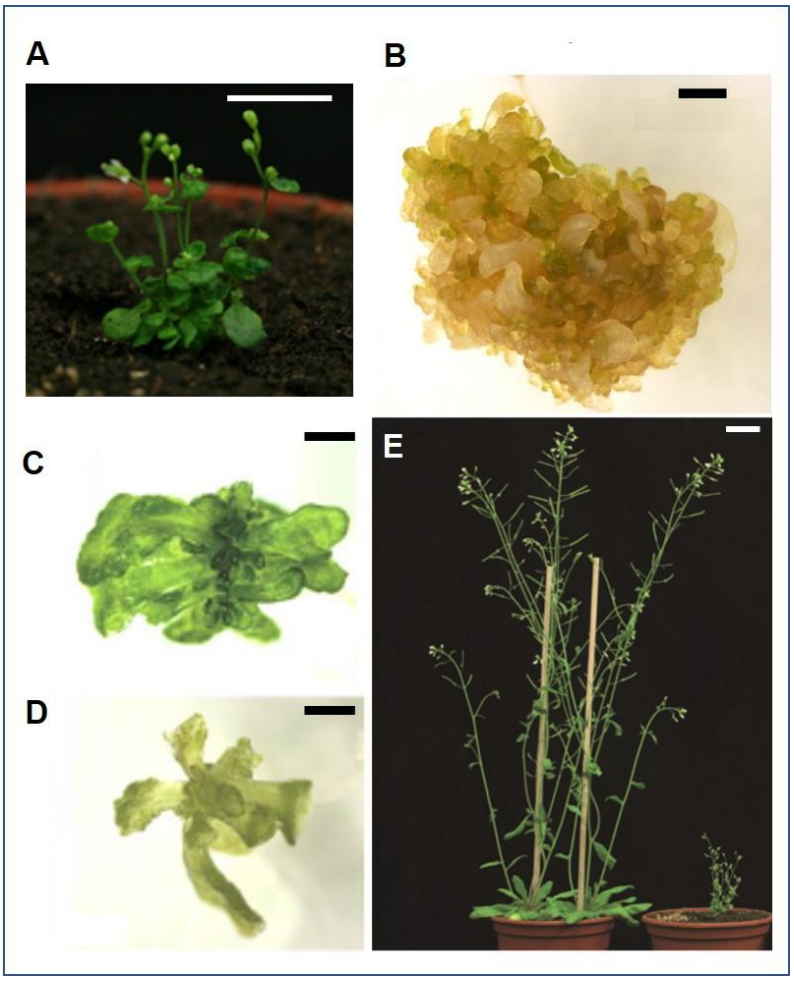

Figure 6. Pleiotropic phenotypes of *fgt1-4*. A) Loss of apical dominance. **B)** Callus-like structure. **C-D)** Developmental abnormalities at seedling stage. **E)** Overall plant growth reduction (left, WT plant; right, *fgt1-4*). Black bar = 1mm; white bar = 1cm.

4.1.2 FGT1 regulates H3K27me3 target genes

In order to unveil the genes affected in the absence of a functional FGT1 RNA-seq experiments were carried out. 3182 genes (FDR ≤ 0.05),11% of the Arabidopsis genome, genes were found mis-regulated in *fgt1-4* mutant plants compared to Col-0 (**Figure 7**). RNA Seq was validated by q-PCR of top 10 genes down- and up-regulated (data not shown). Filtering by 2-Fold Change (2FC) in order to finely analyze the differential gene expression (DGE), the list is reduced to 157 mis-regulated genes.

Specifically, upregulation of master genes of the shoot apical meristem (SAM) development were detected, supporting *fgt1-4* mutant phenotypes. Using the Gene Ontology (GO) terms, which allow to cluster genes by three different categories, in this case only two categorizations could give us useful information about what kind of processes and which cellular structures are affected in *fgt1-4* (Berardini et al., 2004). We found that impairment of *FGT1* is affecting genes that are found involved in membranes and the nucleus functions and 33% are related to stress (**Figure 7**).

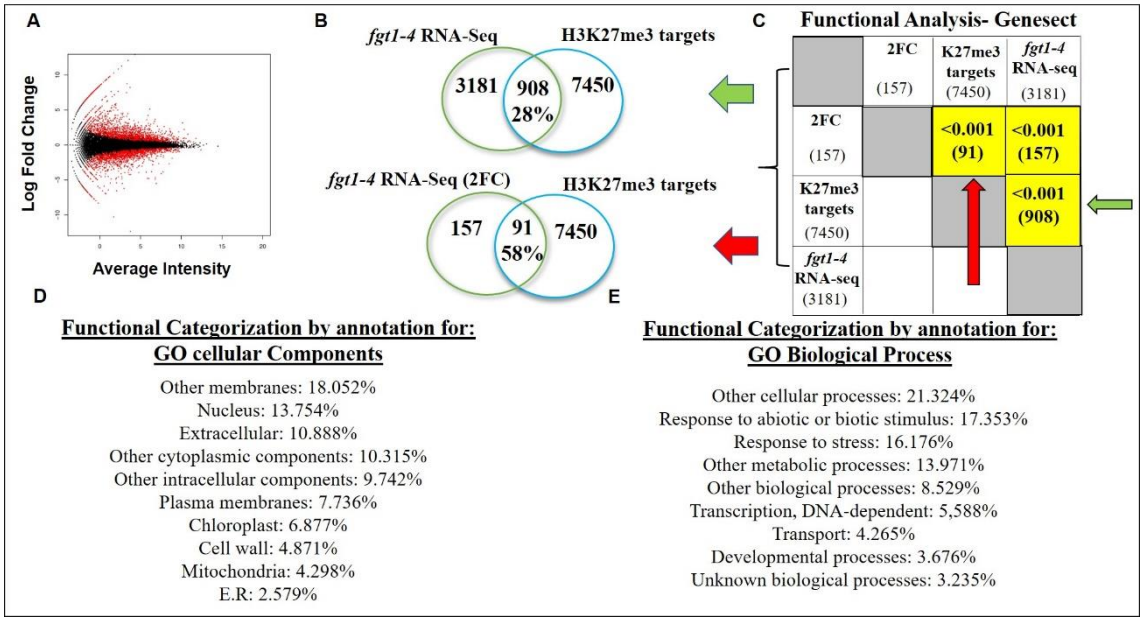

Figure 7. FGT1 significantly shares target genes with the PcG pathway. **A)** Intensity ratio (M) / average intensity (A) plot of all read peaks from the comparison between *fgt1-4* and Col-0 wild type after normalization by MA norm. X-axis is the A value, which represents the average intensity. Y-axis is the log 2FC value, which represents the difference of the intensity. **B)** Venn diagrams representing the overlap between *fgt1-4* mis-regulated genes and H3K27me3 target genes. Upper panel, all mis-regulated genes; bottom panel, 2FC mis-regulated genes in the RNA-Seq. **C)** Statistical significance of the overlap between two groups of genes based on Fisher's exact test. **D-E)** Top 10 GO terms classifying the genes mis-regulated in *fgt1-4* by cellular component and biological function of the 2FC genes. H3K27me3 target genes list from (Lafos et al., 2011).

Considering the putative relationship between FGT1 and the PcG pathway, a meta-analysis to compare *fgt1-4* mis-regulated genes to H3K27me3 target genes (Lafos et al., 2011) was carried out. This meta-analysis indicated that 28% of *fgt1-4* mis-regulated genes are indeed H3K27me3 target genes. Strikingly, when only the 2-Fold Change is considered the number of H3K27me3 enriched genes mis-regulated in *fgt1-4* significantly increases up to 58% (**Figure 7**). This data indicated a possible role of FGT1 in the repression mediated by the PcG pathway.

4.1.3 SAM stem cell identity genes are mis-regulated in the *fgt1-4* T-DNA line

As mentioned before, one of the phenotypes was the development of multiple ectopic SAMs (**Figure 8).** The Arabidopsis SAM development is a well characterized process and the repression of key genes involved in SAM regulation is guided by PRC2 (Xu & Shen, 2008). To confirm the role of FGT1 in the regulation of SAM development the relative expression of specific SAM genes was measured. Master genes involved in SAM initiation and maintenance were mis-regulated in the *fgt1-4* mutant plants compare to Col-0 (**Figure 8**).

Among these mis-regulated genes, we found *Class I KNOTTED-like homeobox (KNOX)* transcriptional factors family members *KNAT1, KNAT2* and *SHOOTMERISTEMLESS* (*STM*). These three *KNOX* genes are expressed in the SAM, participating in the stem cells pool establishment (Lincoln et al., 1994; Long et al., 1996; Scofield et al., 2008). These three genes are essential for the SAM maintenance and, as mentioned before, are PcG target genes (Lafos et al., 2011; Ori et al., 2000).

As *KNOX* genes have very specific and limited spatial expression patterns, different constructs using the promoters of *KNAT2* and *KNAT6* fused to the reporter *GUS* gene were used. In addition, the promoter of *CLAVATA 3 (CLV3)*, which is involved in SAM maintenance, was also fused to GUS and used in these analyses.

With these lines, a different expression patterns as well as localization patterns were found. In the *fgt1-4* mutant, ectopic SAMs at 20 days compared with Col-0 background can be detected marked by mis-regulation of *CLV3* (**Figure 8 A-B, E-F**). In addition, while at 10 days no differences can be observed comparing *KNAT2::GUS* construction in Col-0 and *fgt1-4* background, at 20 days *KNAT2* shows ectopic expression in others tissues such as the roots (**Figure 8 C, G**). On the other hand, 10 days is sufficient to detect differences in *KNAT6* expression pattern in *fgt1-4* backgrounds, showing ectopic expression in the cotyledons (**Figure 8 D, H**).

In summary, the presence of several SAMs in *fgt1-4* mutant plants can be explained by the up-regulation and ectopic expression of these genes as it is observed in the RNA Seq, qPCRs and GUS stain assays results.

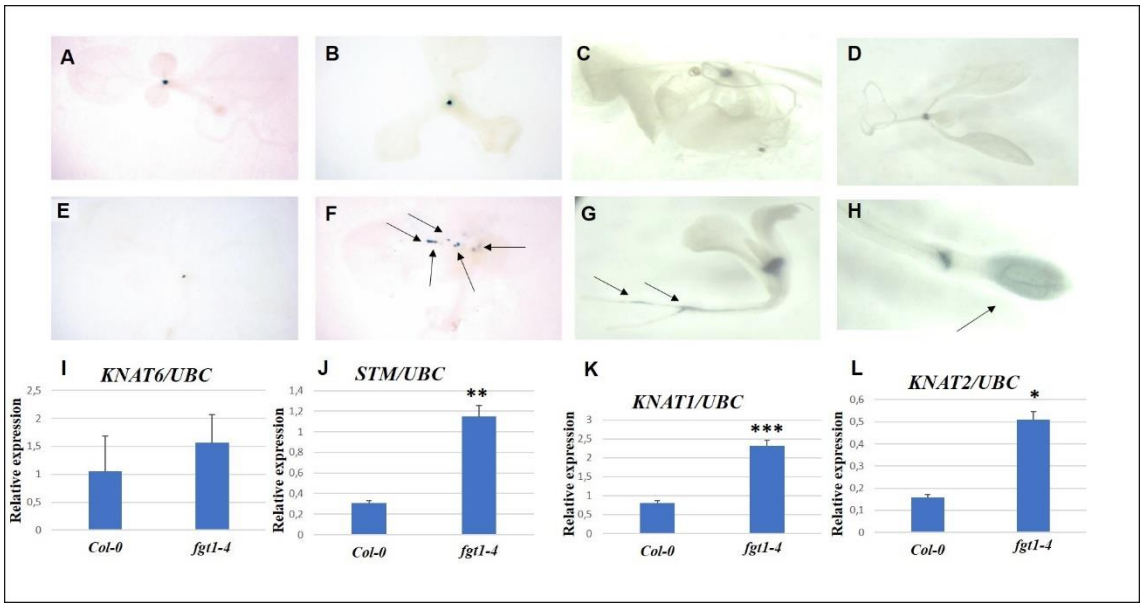

Figure 8. Effects in the SAM regulation in *fgt1-4*. A) 10 day-old p*CLV3::GUS* seedling in Col-0 background. **B)** 20 day-old p*CLV3::GUS* seedling in Col-0. **C)** 20 day-old p*KNAT2::GUS* seedling in Col-0. **D)** 10 day-old p*KNAT6::GUS* seedling in Col-0. **E)** 10 day-old p*CLV3::GUS,fgt1-4* seedling. **F)** 20 days-old p*CLV3::GUS, fgt1-4* seedling; arrows indicate putative-ectopic SAMs. **G)** 20 days-old p*KNAT2::GUS, fgt1-4* seedling. **H)** 10 days-old p*KNAT6::GUS, fgt1-4* seedling. **I** *STM* relative expression in Col-0 and *fgt1-4* normalized against the constitutively-expressed *UBC21* gene. **J)** *STM* relative expression in Col-0 and *fgt1-4* normalized against the constitutively-expressed *UBC21* gene. **K)** *KNAT1* relative expression in Col-0 and *fgt1-4* normalized against the constitutively-expressed *UBC21* gene. **L)** *KNAT2* relative expression in Col-0 and *fgt1-4* normalized against the constitutively-expressed *UBC21* gene. Error bars represent standard error. Asterisk indicates significance level P-value ≤ 0.05; two asterisks indicate P-value ≤ 0.01; three asterisks indicate P-value ≤ 0.001.

4.1.4 Two *fgt1* mutants, two phenotypes

During the development of this research a publication characterizing the role of FGT1 was published (Brzezinka et al., 2016). FGT1 is required for heat-shock (HS) memory regulation at *HEAT-STRESS-ASSOCIATED (HAS) 32*, *HEAT SHOCK PROTEIN (HSP) 18.2* and *HSP22.0 loci*. FGT1 associates with chromatin remodeling complexes of the imitation SWI (ISWI) and SWItch/Sucrose Non-Fermentable (SWI/SNF) classes to mediate the nucleosome occupancy and promoting gene expression. Nevertheless, despite the loss of memory to heat stress, the *fgt1-1* allele, a chemical mutagenesis induced mutant, did not show any further developmental phenotype as observed for the *fgt1-4* T-DNA mutant.

4.1.4.1 Splicing variants detected in both mutant lines

In order to validate the *fgt1-4* and the *fgt1-1* mutant lines, intron retention experiments were carried out. *fgt1-1* has a C to T mutation in the intro 19 splicing acceptor site that ultimately produce a premature stop codon (Brzezinka, et al., 2016). Nevertheless, a final confirmation of this statement was not found in the publication. On the other hand, the T-DNA on *fgt1-4* localizes at the end of exon 20 just before the splice donor site. Hence, we hypothesized that this mutant allele may also produce a putative splicing variant as it has been proposed for the *fgt1-1* mutant allele. Therefore, intron retention experiments were carried out in both lines to check this hypothesis (Marquardt et al., 2014). In the *fgt1-1* mutant two different transcript populations were identified in semiquantitative RT-PCR to detect *fgt1-1* cDNA, one corresponding to unspliced and the other one to spliced *FGT1* transcript versions (**Figure 9 A**). A q-PCR were carried out in order to confirm this result. In the q-PCR data it is possible to detect an increase of both, the spliced and unspliced transcript, suggesting that the *fgt1-1* mutant is not a null mutant allele, even showing *FGT1* over-expression (**Figure 9 B**).

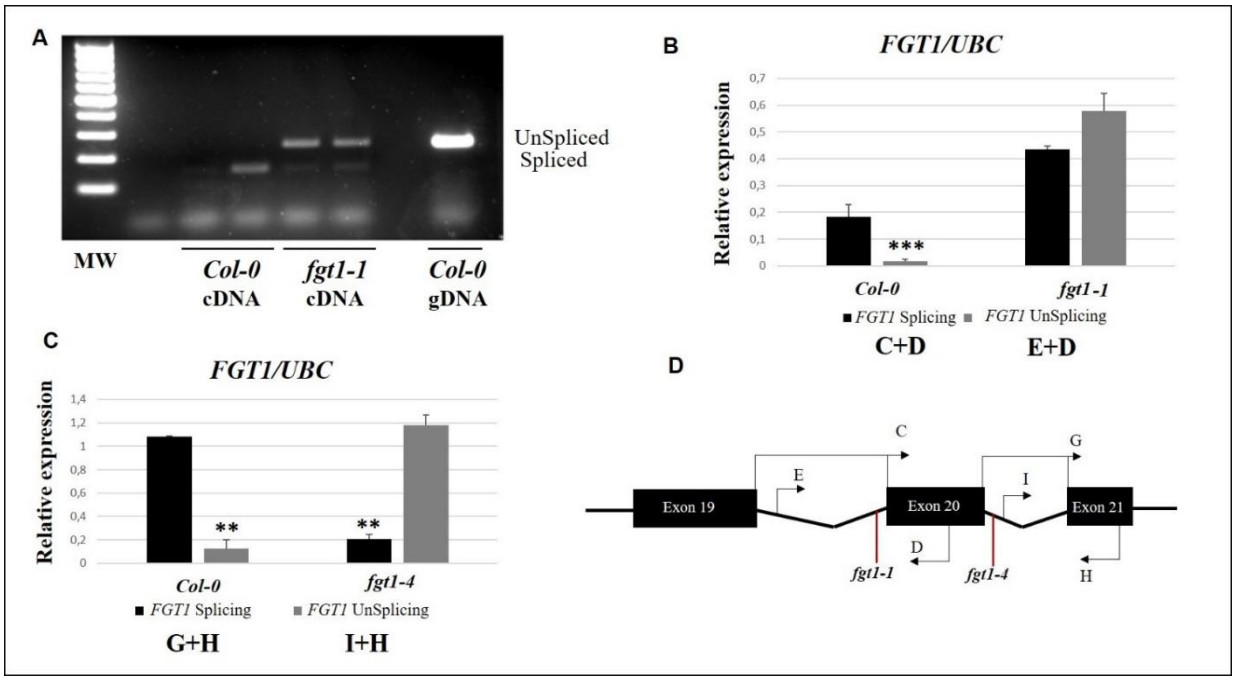

Figure 9. *fgt1-1* displays two mRNA populations of *FGT1* and *fgt1-4* an intron 20 retention. A) Semiquantitative RT-PCR confirming the intron retention (312pb) in *fgt1-1* cDNA as well as spliced transcript in *fgt1-1* mutant (160pb). cDNA samples were prepared by duplicates. **B)** Quantitative RT-PCR showing *FGT1* splicing alterations between Col-0 and *fgt1-1*. **C)** Quantitative RT-PCR showing retention of intron 20 in *fgt1-4* background compared to Col-0. Error bars represent standard error. Two asterisks indicate P-value ≤ 0.01; three asterisks indicate P-value ≤ 0.001. **D)** Scheme showing the primers position used in IR experiments in B) and C). Primers C and G are expanding an exon-junction. The letters in the primers correspond to the same letters used in Table 3.

Regarding *emb1135/fgt1-4* mutant, an intron retention event affecting intron 20 was detected relating with a very low abundance of spliced transcript, what in principle could explain the dramatic phenotype found in this line (**Figure 9 C**).

4.1.5 *fgt1-4* T-DNA line affects nucleosome position in *KNAT1* locus

One of the effects of *fgt1-1* mutant is the repositioning of nucleosomes in, at least, some HS loci, as *HSA32, HSP22.0* and *HSP18.2*, before and during a heat shock (Brzezinka et al., 2016). Other type of experiments to compare the mutant lines was to measure if the *fgt1-4* mutant line affects the nucleosome position as it has been proposed for the *fgt1-1* mutant. To check this possibility, a FAIRE-qPCR was done in *fgt1-4* and Col-0. The FAIRE technique allows us to detect the specific regions in the genome, nucleosome-depleted regions, where proteins involved in the control of gene expression bind to the DNA sequence.

Using the same primers used by Brzezinka and colleagues on *HSP22* locus, 393 and 8 base pairs from the TSS, as a control, an alteration of nucleosome position was also detected in *fgt1-4* mutant compared to Col-0 before HS as published before (**Figure 10;** Brzezinka et al, 2016). After this confirmation, despite the absence of FGT1 direct chromatin binding data to the *KNOX* loci but considering the mis-regulation of *KNOX* genes, a FAIRE qPCR was carried out on the mis-regulated SAM master genes described in *fgt1-4*. To localize a putative-nucleosome in these loci an indirect relation was used based on H3 distribution data bases (http://epigenomics.mcdb.ucla.edu/H3K27m3/, https://www.arabidopsis.org/). Theoretically, where a histone 3 is located, we expect to find a nucleosome. Hence, overlapping the H3 map to the selected loci we should be able to stablish in which regions of the genes it is possible to find a nucleosome and check if these nucleosomes have undergone a change in their position by qPCR. Several mis-regulated loci were tested (*KNAT1, KNAT2, KNAT4, STM*) but only significant nucleosome position changes were detected at the *KNAT1* locus (**Figure 10**).

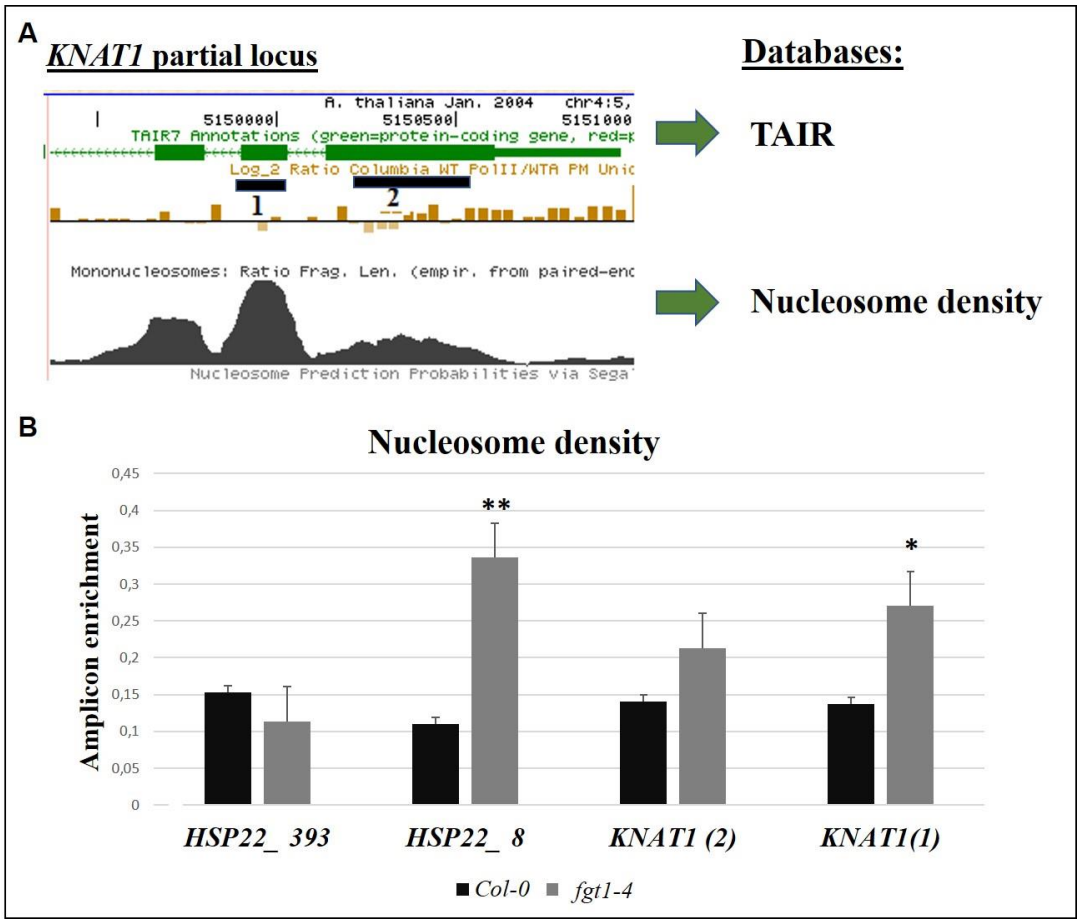

Figure 10. Nucleosome position changes detected in *HSP22* and *KNAT1*. A) Overlap between the data bases used in order to localize a putative nucleosome position. **B)** Amplicon enrichment detected in *fgt1-4* mutant background against *Col-0* accession using the FAIRE-qPCR assay. Error bars represent standard error. Asterisk indicates a significance level P-value ≤ 0.05; two asterisks indicate P-value ≤ 0.01. HSP22 primers used as control come from (Brzezinka et al., 2016).

4.1.6 *fgt1-4* T-DNA line presents a second T-DNA insertion

Considering the previous data two hypotheses were still plausible to explain the phenotypic differences between the *fgt1-1* and the *fgt1-4* alleles : (i) *fgt1-1* is a weak mutant allele due to a partial intron retention and *fgt1-4* is a strong mutant allele due to a major intron retention; (ii) *fgt1-1* is a single mutant exclusively affected in the *FGT1* gene, whereas in the *fgt1-4* mutant line other loci are affected. To check this second hypothesis the *fgt1-4* mutant line was tested by a Thermal Asymmetric Interlaced (TAIL) PCR. This method had previously been used to validate the T-DNA insertion in this mutant line (McElver et al., 2001). Nevertheless, the confirmation had exclusively been done using primers for the Left Border (LB) of the T-DNA. Therefore, the experiment was repeated by including primers for both borders. Four PCR bands obtained by the LB primer were sequenced and confirmed a flanking region of *FGT1* exon 20 (**Figure 11**).

However, when Right Border (RB) primers were used for amplification, a second insertion was discovered in the *EMB1144* (At1G48850) gene. This gene was also described in the same SeedGenes project as the *EMB1135/FGT1* gene (Bryant et al., 2011). *EMB1144* encodes a chorismate synthase involved in the synthesis of aromatic amino acids and, hence, may have a strong impact on the Arabidopsis proteome.

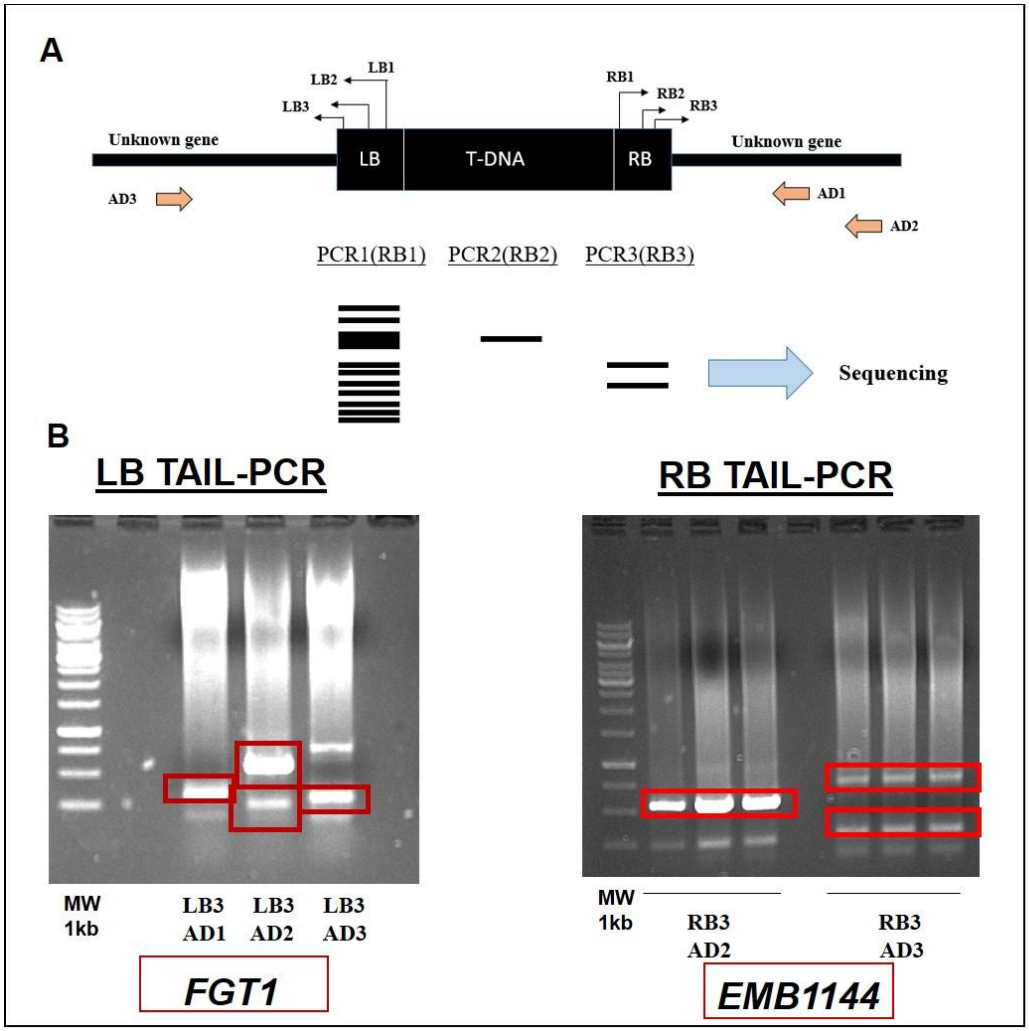

Figure 11. *fgt1-4* line presents a second T-DNA insertion. A) Schematic representation of the TAIL-PCR experiment. **B)** Left; sequenced PCR products of LB TAIL-PCR marked by red boxes. Right; sequenced PCR products of RB TAIL-PCR marked by red boxes. The RB TAIL-PCR was repeated twice with different biological replicates.

4.1.7 *EMB1144* is knocked-down in *fgt1-4*

In order to confirm the T-DNA insertion in the *EMB1144* locus a series of PCR were carried out using gDNA from the *fgt1-4* line as template. The T-DNA insertion was located by sequencing in the putative-promoter region of the *EMB1144* gene (**Figure 12**). In addition, to detect a putative mis-regulation of *EMB1144* expression a qPCR was done. The results show how even if the T-DNA insertion is located before the coding region, it has a strong impact on *EMB1144* expression, approximately 80% reduction. Similar results were also observed in the RNA-seq data, suggesting that the *fgt1-4* line is a null allele for *FGT1* and a knock-down for *EMB1144* (**Figure 12**).

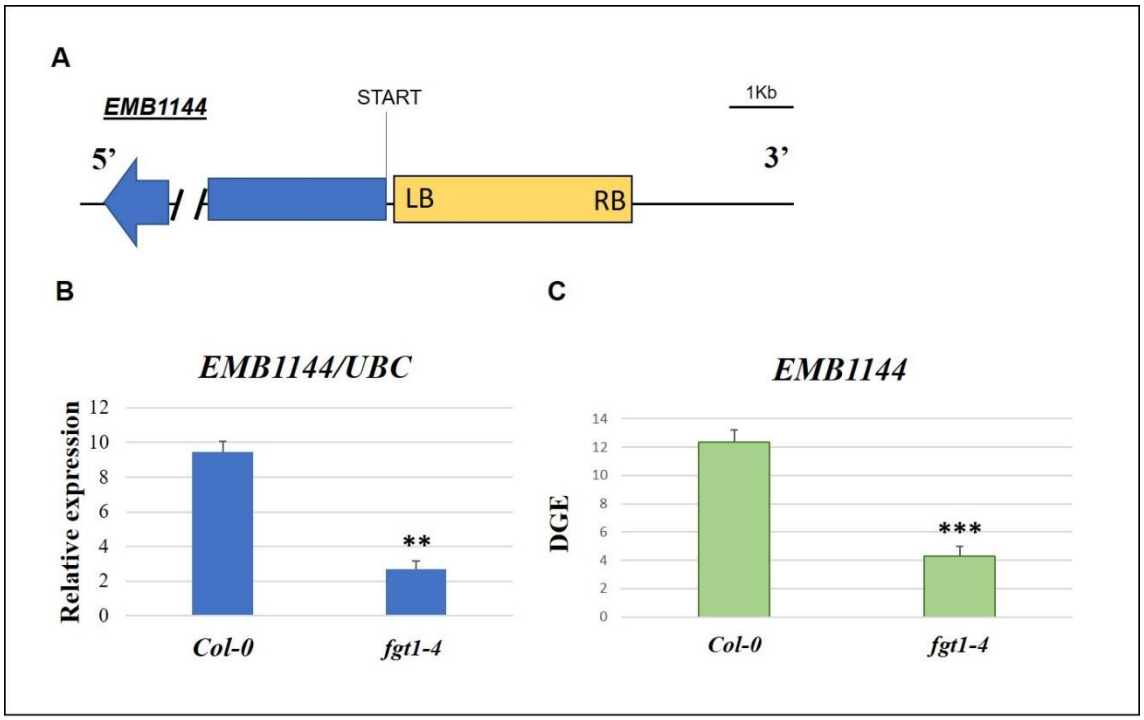

Figure 12. *fgt1-4* line displays a knock-down on *EMB1144*. A) Schematic drawing of *EMB1144* locus indicating the position of insertion for the T-DNA in *fgt1-4*. **B)** qPCR measuring the relative expression of *EMB1144*, normalized against the constitutively-expressed *UBC21* gene in *Col-0* and *fgt1-4* background. **C)** DGE of *EMB1144* in *fgt1-4* line compared to Col-0 between the four biological replicates used in the RNA-seq, with a 1.5-fold change difference. Error bars represent standard error. Two asterisks indicate a significance level P-value ≤ 0.01; three asterisks indicate P-value ≤ 0.001.

In addition, attempts to complement the *fgt1-4* line with a p*FGT1::FGT1-GFP* transgene were unsuccessful despite the characteristic FGT1-GFP nuclear signal was observed in these lines (data not shown; Brzezinka et al., 2016).

Taken together, these data were definitive to confirm our second hypothesis: the severe developmental phenotypes observed in the *fgt1-4* T-DNA insertion line are indeed due to a second insertion in *EMB1144*.

4.2 FGT1 protein interactions and novel role in flowering

A CRISPR/Cas9 deletion mutant for *FGT1* was generated. The *fgt1* CRISPR/Cas9 line (*fgt1-5*) was developed as described in (Wang et al., 2015) using as final vector a modified version of the double guide RNA (gRNA) with a Cas9 driven by an egg-cell specific promoter and with a mCherry reporter cassette driven by a seed maturation-specific At2S3 promoter (Gao et al., 2016; McHale et al., unpublished). This vector allows to produce a double deletion that ultimately will produce the deletion of a considerable fragment of the locus. The *fgt1-5* mutant line has a final 2.6Kb deletion, truncating the three conserved domains described in FGT1 (**Figure 13**). The *fgt1-5* mutant plants do not show any developmental phenotype.

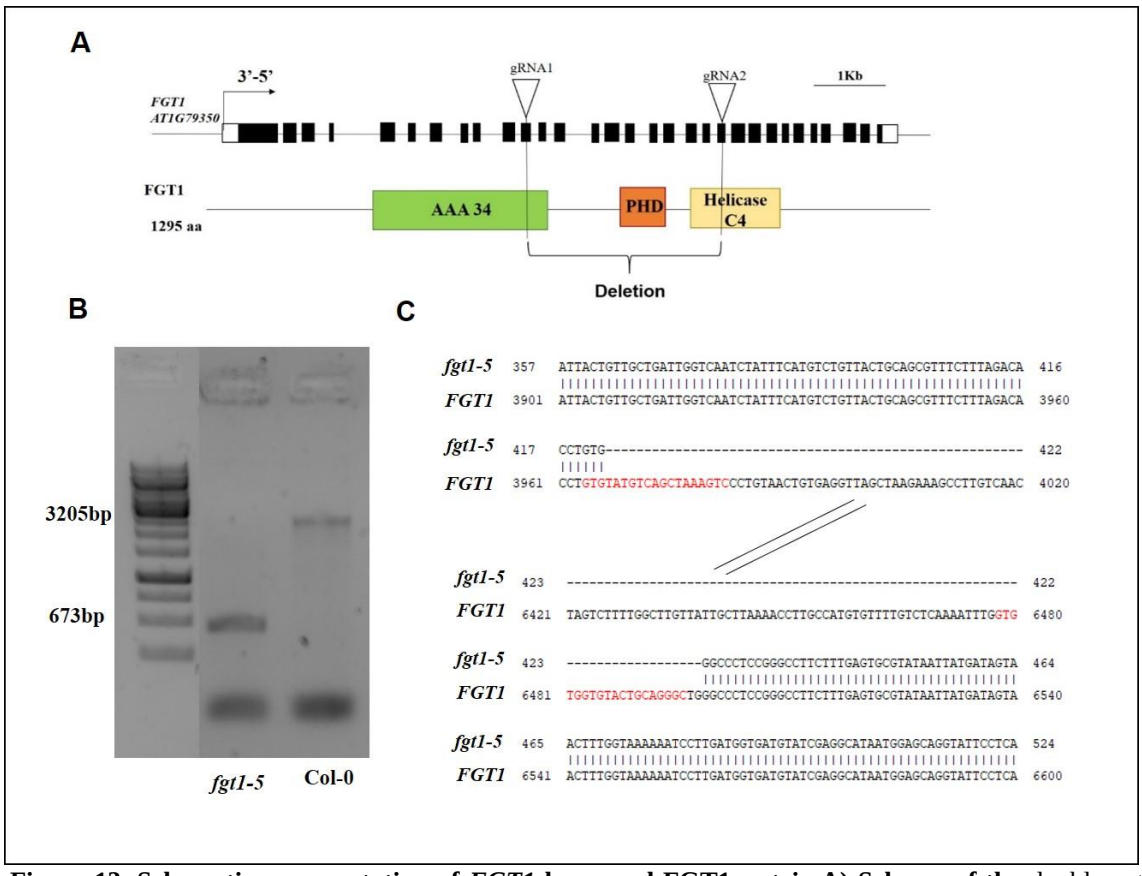

Figure 13. Schematic representation of *FGT1* locus and FGT1protein.A) Scheme of the double cut that generates a final 2.6Kb deletion on *FGT1* locus. **B)** Agarose gel showing a shorter PCR band due to the deletion. **C)** Sequence confirming the cut around the sgRNAs sites (red). The two lines represent the deletion.

4.2.1 FGT1 interacts with PWO1 and PcG members in yeast and *in planta*

In order to unveil new protein-protein interactions, an *in vivo* assay was carried out through yeast two hybrid (Y2H) experiments. For these experiments, the cDNA of FGT1 was cloned to the Gal4-DNA-binding domain (Gal4-BD) and used as bait against PcG members fused to the Gal4-activation domain (Gal4-AD). The catalytic subunits of PRC2 SWN and CLF were truncated at the C-terminal SET domains (CLF/SWNΔSET) as this domain reduces the sensitivity of the interaction (Chanvivattana et al., 2004).

The *in planta* interaction previously detected by LC-MS/MS between PWO1 and FGT1 (Mikulski et al., 2019) was confirmed by Y2H. In addition, a novel *in vivo* interaction between FGT1 and SWN was detected as well (**Figure 14 A**). Nevertheless, no interactions were detected between FGT1 and others PcG members as CLF, EMF2, VRN2, MSI1, and LHP1 (data not shown).

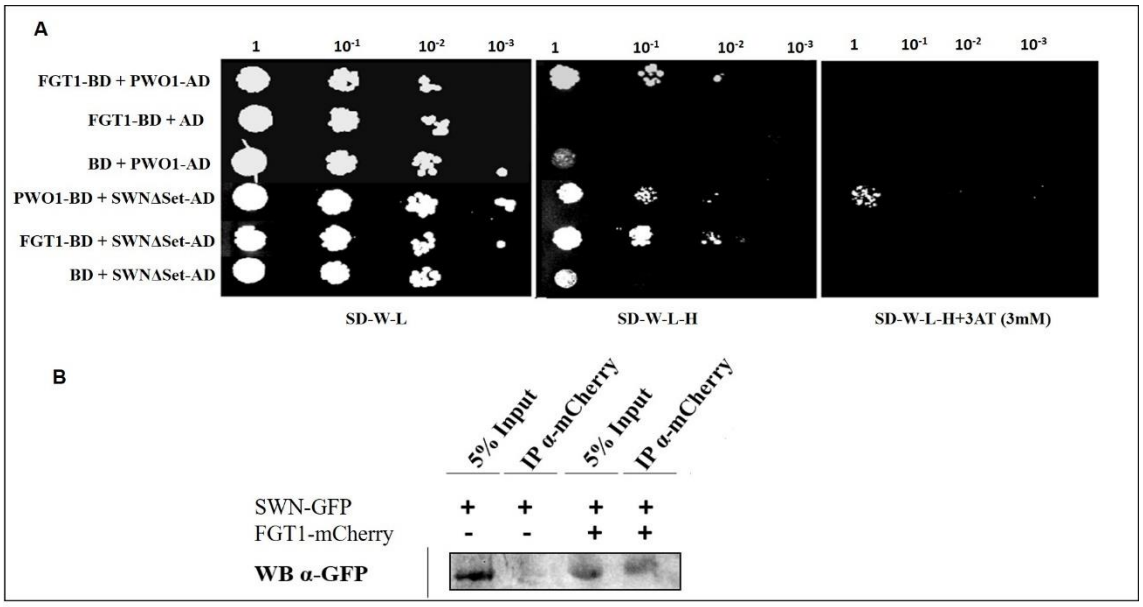

Figure 14. Y2H experiments showing interactions between FGT1 and PcG members. A) Interaction between FGT1-PWO1 and FGT1-SWNΔSET. The proteins were cloned in the two vectors of the Y2H system and co-transformed in different combinations growing onto –LW and –LWH selective media supplemented with 3mM of 3AT. Interaction between PWO1-BD and SWNΔSET-AD was used as positive control of the Y2H (Hohenstatt et al., 2018). **B)** *N. benthamiana* plants were co-infiltrated with *pMDC7-FGT1-mCherry* and *pMDC7-GFP-SWNΔSET*, or *pMDC7-FGT1-mCherry* only. Immunoprecipitation was performed with anti-mCherry antibody, and proteins were detected by western blot using anti-GFP. Input = immunoprecipitated samples.

In order to confirm the interaction between FGT1 and SWN a *in planta* co-immunoprecipitation (Co-IP) was carried out in transient expression experiments using *N. benthamiana* plants co-expressing FGT1-mCherry and GFP-SWNΔSET fusion proteins. This interaction was indeed confirmed **(Figure 14 B)**.

4.2.2 FGT1 cannot bind PWO1 fragments and do not form homodimers

Once FGT1-PWO1 interactions was confirmed by Y2H, we investigated if FGT1 has a binding preference to a specific region/domain of PWO1. Y2H assays using FGT1-AD/-BD against different PWO1 fragments-AD/BD mapping the PWO1 cDNA were carried out. PWO1 fragments correspond to the N-terminal PWWP domain-containing region, a central region with an NLS and a third fragment with the C-terminal region of PWO1. The Y2H results suggest that FGT1 cannot bind to any of these fragments (**Figure 15**).

In addition, PWO1 forms *in vivo* homodimers (Hohenstatt et al., 2018). To check if FGT1 forms homodimers too, a Y2H protein-protein interaction experiment was done with FGT1 fused to the Gal4-BD against FGT1 Gal4-AD. No interaction was detected suggesting that FGT1 do not form a dimer with itself (data not show).

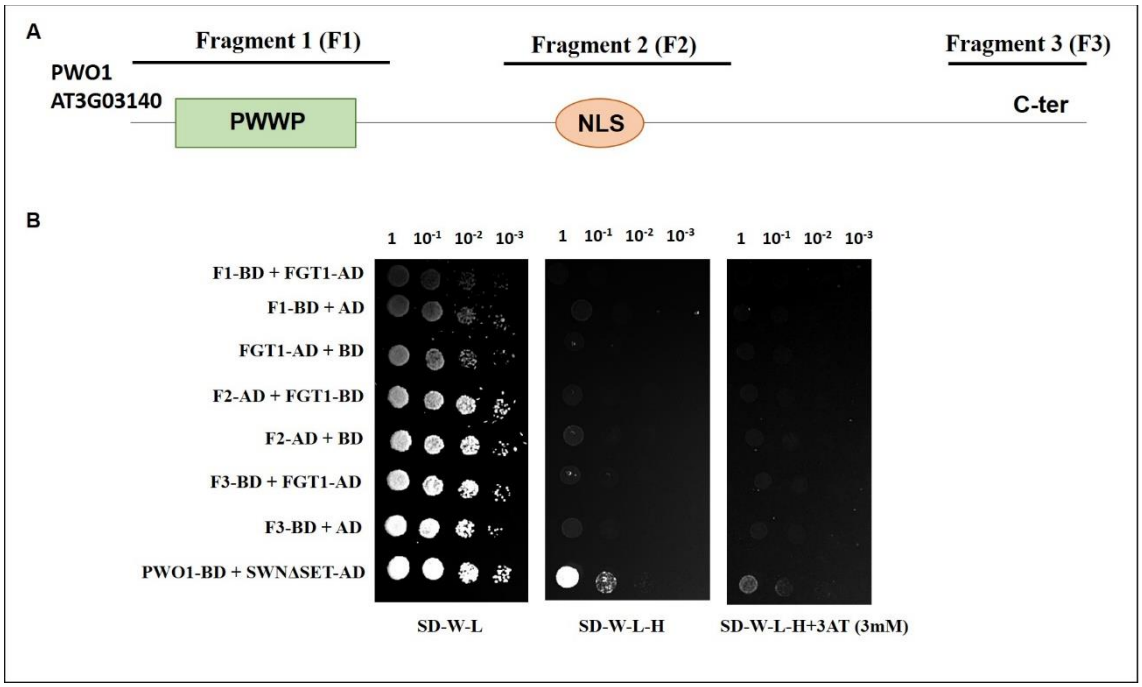

Figure 15. FGT1 does not interact with PWO1 fragments. A) Schematic representation of PWO1 protein and the fragments used to this protein-protein interaction experiment. **B)** The protein fragments were cloned in the two vectors of the Y2H system and co-transformed in different combinations growing onto –LW and –LWH selective media supplemented with 3mM of 3AT. Interaction between PWO1-BD and SWNΔSET-AD was used as positive control of the Y2H (Hohenstatt et al., 2018).

4.2.3 FGT1 interacts with FVE *in vivo*

Interaction between FGT1 and FVE was also detected by Y2H (**Figure 16**). As mentioned before, FVE is part of the autonomous pathway that regulates flowering time (Ausín et al., 2004), interacting with PRC2 to promote flowering due to repression of *FLC* (Pazhouhandeh et al., 2011). This is relevant because we know that FVE interacts with PWO1 as well as other PWO1 family members (Hohenstatt's thesis 2012; Zhou et al., 2018). In addition, the *pwo1* single mutant develops an early flowering phenotype due to the mis-regulation of *FLC* (Hohenstatt et al., 2018).

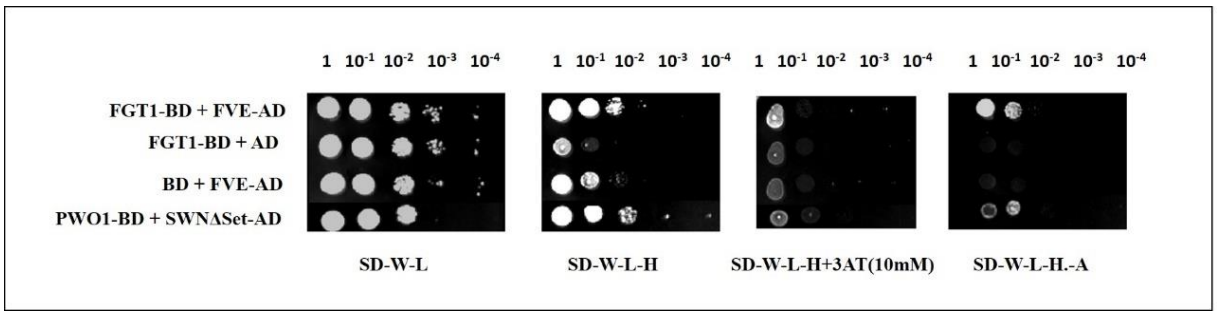

Figure 16. FGT1 interacts *in vivo* with FVE. The proteins were cloned in the two vectors of the Y2H system and co-transformed in different combinations growing onto –LW and –LWH selective media and –LWHA.

4.2.4 FGT1 may be involved in the flowering-freeze tolerance crosstalk mediated by FVE

fve mutant alleles were also identified in a screening for freeze tolerance mutants because in absence of a functional FVE several coding genes for the late embryogenesis abundant (LEA) proteins are overexpressed (Kim et al., 2004). In Arabidopsis, LEA proteins are involved in freezing response, being one of the most studied the *COLD-REGULATED (COR)15a*, that is overexpressed in *fve* plants (Hincha & Thalhammer, 2012; Sowemimo et al., 2019). To determine if FGT1 may be related to FVE functions, *COR15a* relative expression was measured without freeze or cold exposure.

As it is shown in **figure 17**, the relative expression level of *COR15a* is up-regulated compared to Col-0 in the absence of a functional FGT1 protein in the *fgt1-5* mutant line, similarly as in the *fve* mutant, suggesting that FGT1 may be involved in regulation of FVE target genes. On the other hand, *fgt1-1* mutant line does not show a significant mis-regulation, suggesting that in this mutant line, *COR15a* expression is not affected.

To exclude a role of FGT1 at *FVE* genetic level, the *FVE* relative expression was measured as well (**Figure 17**). No alteration was detected compared with Col-0 suggesting that the FGT1-FVE crosstalk is at protein level.

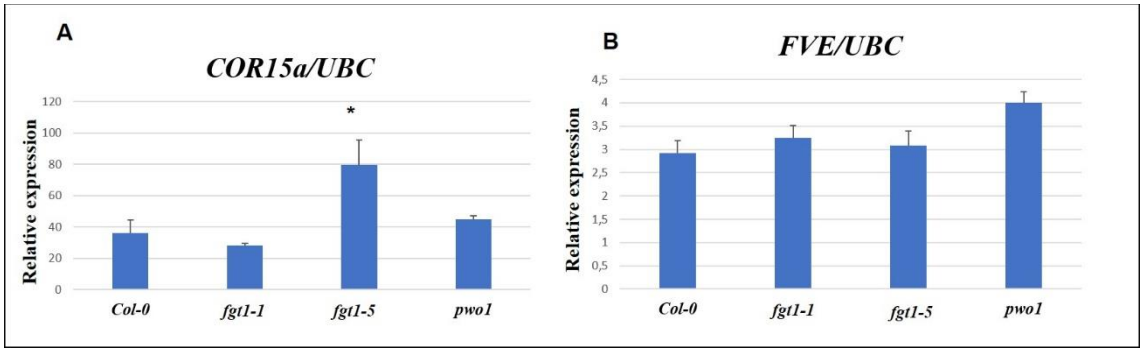

Figure 17. The *COR15a* cold stress gene is up-regulated in *fgt1-5* plants. Relative expression of *COR15a* and *FVE* at Zt 8, normalized against the constitutively expressed *UBC21* gene. Error bars represent standard error. Significance level is P-value ≤ 0.05.

4.2.5 FGT1 repress *FLC* expression but do not presents flowering time alterations

FGT1 interacts with FVE as well as with PWO1. In addition, FGT1 affects the relative expression of the FVE target gene *COR15a*. The second most studied Arabidopsis gene after *PHYTOCHROME B*, is *FLC*, a gene that is regulated by PWO1, PRC2 and FVE among others (Crevillén & Dean, 2011; Hohenstatt et al., 2018). Although, a flowering time alteration was not previously detected or described for *fgt1* mutant allele, a *FLC* relative expression level was carried out. In *fgt1-5* mutant line an increase of *FLC* expression compare to Col-0 accession was detected (**Figure 18 A**). Upregulation of *FLC* usually leads to a delay in flowering time because the plant has an overexpression of the main flowering repressor *FLC*. However, in long day conditions (LD, 16 hours of light and 8 hours of darkness) although no alteration was detected in flowering time compared to Col-0 for *fgt1-5* but *fgt1-1* mutant lines flowered significantly earlier (**Figure 18 B**). Regarding short day conditions (SD, 8 hours of light and 16 hours of darkness), no alteration was detected (**Figure 18 C**). Therefore, the flowering phenotype of *fgt1-5* mutant plants is indistinguishable from Col-0 accession plants in both analyzed conditions. This result suggests that FGT1 may be involved in the regulation of other(s) player(s) of the flowering time pathway downstream of FLC.

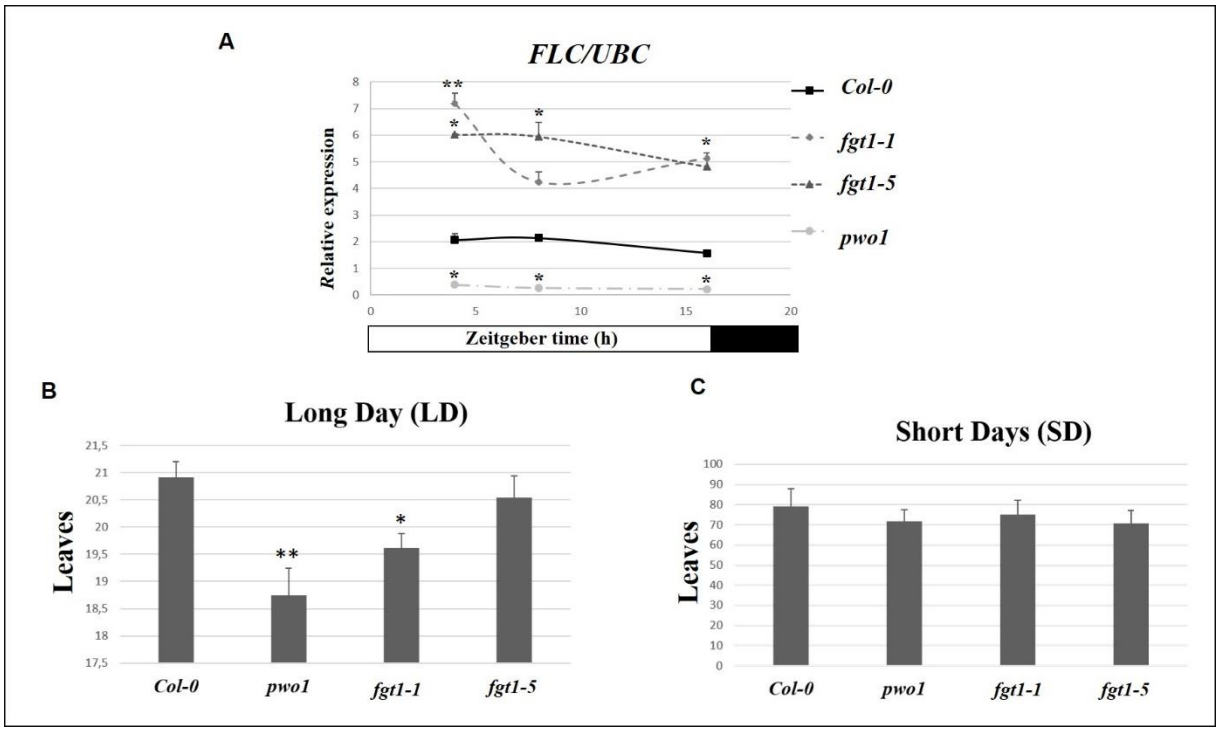

Figure 18. Flowering time analysis on *fgt1* mutant lines. A) Relative expression of *FLC* at different Zeitgeber times (4, 8 and 16 hours) in long day conditions**.** *FLC* expression normalized against the constitutively expressed *UBC21* gene. Error bars represent standard error. **B)** Flowering time measurement on LD conditions; n= 30/genotype. **C)** Flowering time measurement on SD conditions; n= 30/genotype. Col-0 (wild type control), *pwo1*, *fgt1-1* and *fgt1-5*. Asterisk indicates significance level P-value ≤ 0.05; two asterisks indicate P-value ≤ 0.01.

4.2.6 *SOC1* expression is not altered in the *fgt1-5* mutant

In order to unveil why flowering time is not altered in *fgt1-4* mutants despite *FLC* overexpression, measurement of the relative expression of FLC downstream targets was done (**Figure 19 A- D**). The two main FLC targets are *FLOWERING LOCUS T* (*FT)* and *SUPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1)*, known as floral integrators (Bloomer & Dean, 2017). Ultimately, these two proteins activate flower development. In addition, *SHORT VEGETATIVE PHASE* (*SVP)* relative expression was analyzed. SVP is also a direct repressor of *SOC1* and *FT* acting as partner of FLC (Mateos et al., 2015), receiving the exogeneous cue that comes from the ambient temperature (Blümel et al; 2015).

In **Figure 19 A**, *FT* relative expression is down-regulated in *fgt1-5* mutant plants as expected in plants with a high relative level of *FLC*. On the other hand, *SOC1* relative expression levels does not shows a significant decrease in *fgt1-5* seedlings. In addition, *SVP* relative expression levels are not altered in the *fgt1-5* mutant, suggesting that FGT1 is not involved in *SVP* regulation.

Finally, *AGAMOUS-LIKE 24 (AGL24)* a flowering activator downstream of FLC that form a positive feedback loop with SOC1 displays an expected downregulation in a *FLC* overexpression scenario (Torti & Fornara, 2012).

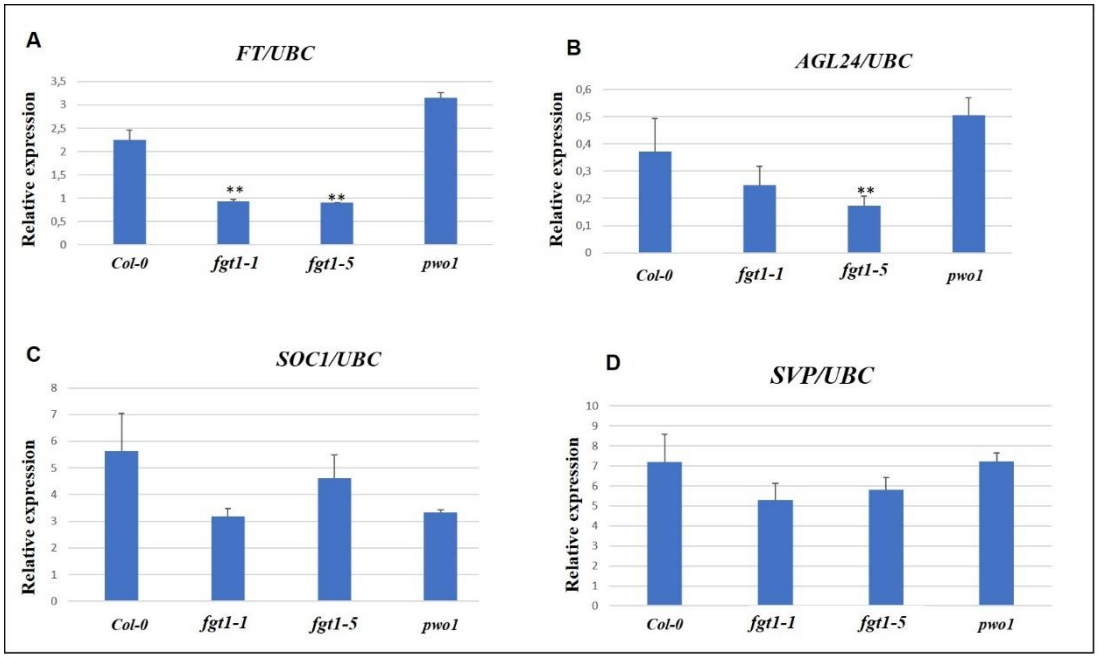

Figure 19. FGT1 affects expression patterns of other flowering genes. Relative expression of the floral integrators normalized against the constitutively expressed *UBC21* gene at Zt 8. Error bars represent standard error, Asterisk indicates significance level P-value ≤ 0.05; two asterisks indicate P-value ≤ 0.01. **A)** *FT*. **B)** *AGL24*. **C)** *SOC1*. **D)** *SVP*.

4.3 UBP5 regulates Arabidopsis development

As mentioned before, based on proteomic analyses using PWO1-GFP as bait, FGT1 was the second most abundant interactor of PWO1 (Mikulski et al, 2019). The most abundant interactor of PWO1 was UBP5 (At2G40930) (Mikulski et al, 2019). The analysis of three *ubp5* T-DNA mutant lines (*Salk_152779, GABI_957C09* and *Salk_044292*) indicated different abnormalities and segregation problems. Another available *ubp5* mutant line was obtained from the same collection as *emb1135* (Syngenta), identified as *pigment defective embryo 323* (*pdp323/ubp5-1*).

Following the same approach as for the *fgt1-4* line, a TAIL-PCR was carried out in order to confirm a single insertion in this line. However, only one border of the T-DNA was detected in the *UBP5* locus through this method (data not shown), whereas the presence of the other border could not be confirmed despite more than 6 different randomly degenerate primers were used. Hence, we decided to apply the CRISPR/Cas9 double guide system to develop a novel *ubp5* deletion (from the 250 to the 804 aa) mutant allele (*ubp5-2*) (**Figure 20**).

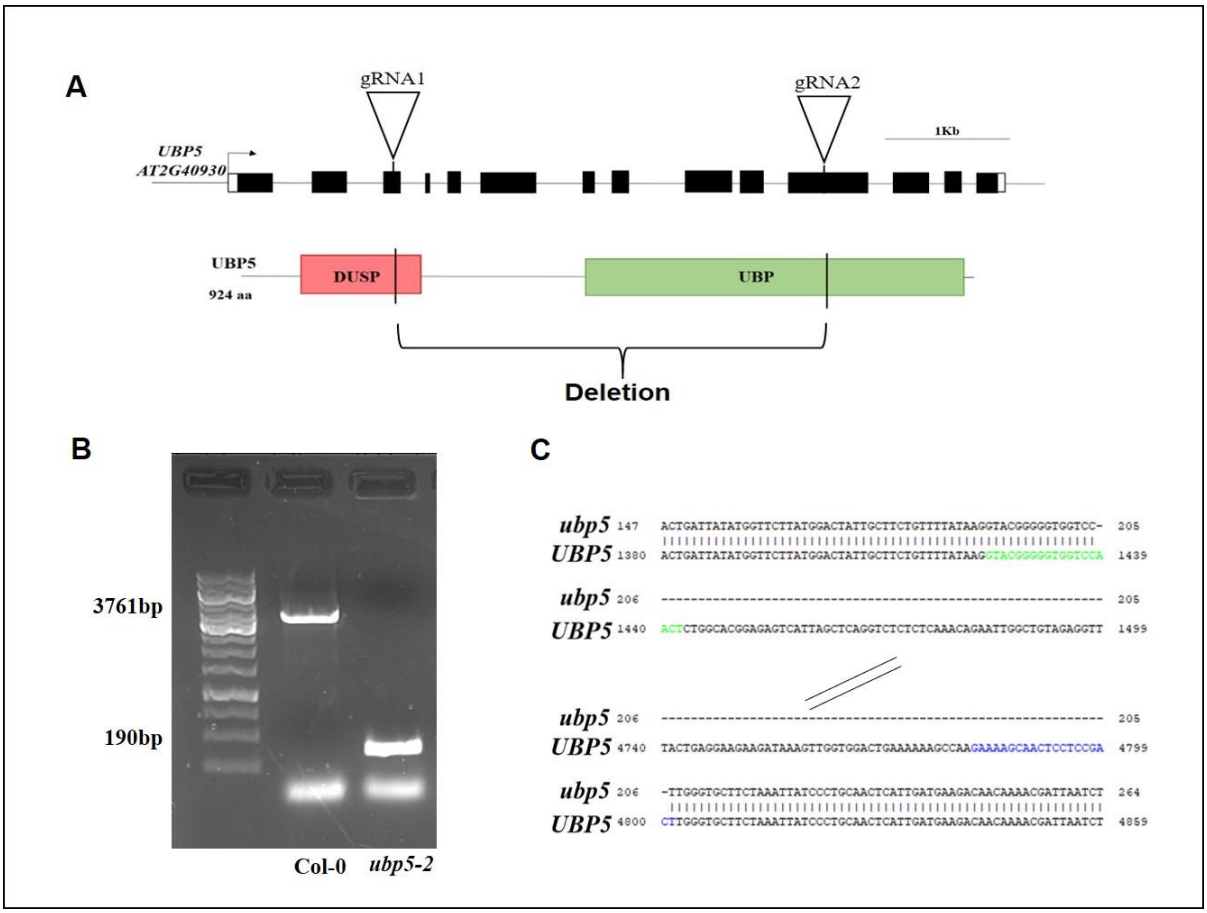

Figure 20. Generation of *ubp5-2* deletion mutant allele. A) Representation of the *ubp5-2* single mutant; the expected deletion generated in the UBP5 protein and the affected domains (marked with black line). Red box, domain present in ubiquitin-specific proteases (DUSP) domain; green box, ubiquitin protease (UBP) domain**. B)** Gel demonstrating the deletion in the *ubp5-2* allele. **C)** Sequencing confirming the Cas9 cut around the sgRNAs regions (represented as green and blue colors). The two lines represent the deletion.

4.3.1 *ubp5-2* presents pleiotropic phenotypes

ubp5-2 plants display a pleiotropic phenotype including: delay in germination and growth, shorter roots, loss of phyllotaxis, leaves without trichomes or abnormal trichome patterning, alteration of leaf shape, dwarfism and loss of apical dominance (**Figure 21**).

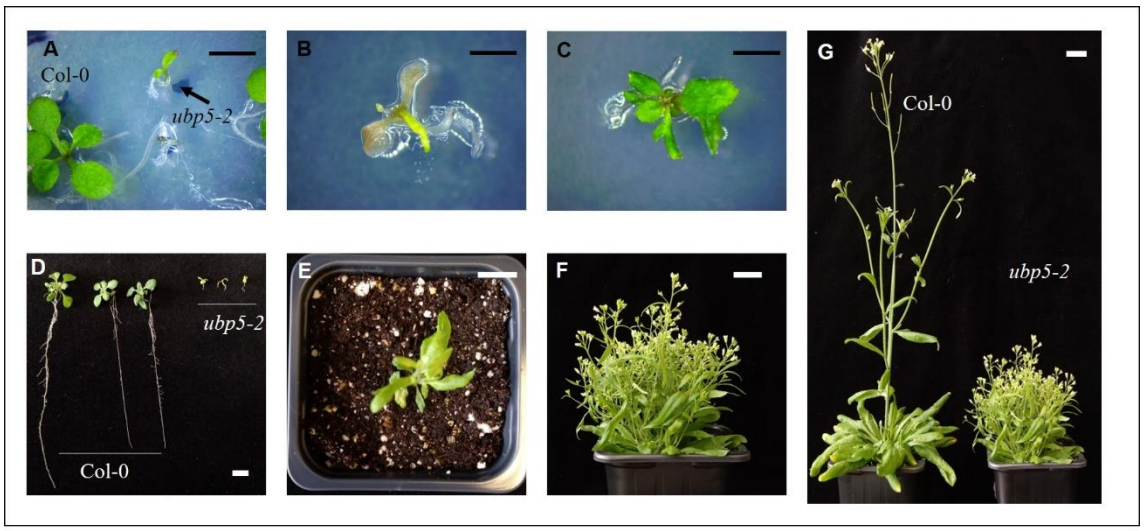

Figure 21. Phenotypic characterization of *ubp5-2* mutant. A) Delay in germination. **B-C)** Leaf malformation. **D)** Root shortening. **E)** Loss of phyllotaxis. **F)** Loss of apical dominance. **G)** Left WT plant, right *ubp5-2* plant showing decrease in overall size. Note: plants were of different age as *ubp5-2* shows a pronounce delay in growth (21-day-old WT plant, 33-day-old *ubp5-2* plant)**.** Black bars = 1mm; white bars = 1cm.

This phenotype suggests that UBP5 is involved in several developmental processes, with an emphasis in germination and postembryonic development. Once the plants reach adult stage, a delay in flowering time was also observed. To confirm this result, flowering time was quantified by the total leaf number (rosette) produced by the plants prior to bolting. (**Figure 22**) showing a significant difference. These results suggest that UBP5 is essential for proper plant development.

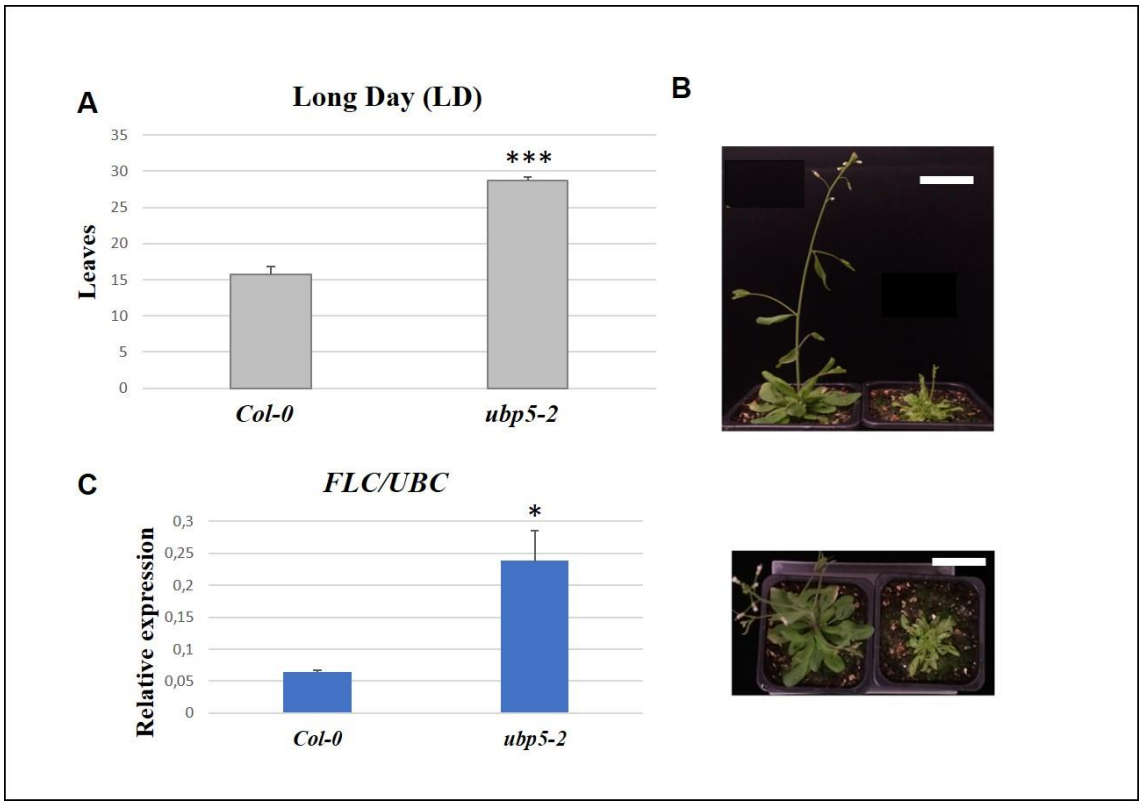

Figure 22. *ubp5-2* shows late flowering phenotype. A) Flowering time in *ubp5-2* compared with Col-0 as number of rosette leaves prior to bolting. **B)** Developmental comparation between Col-0 and *ubp5-2* after flowering. **C)** Relative expression of *FLC* in Col-0 and *ubp5-2* normalized against the constitutively expressed *UBC21* gene at Zt 8, 17 DAG. Error bars represent standard error, Significance level is P-value ≤ 0.05; three asterisks indicate P-value ≤ 0.001 Note: for the q-PCR, samples were collected at same developmental stage; Col-0 9 days-old, *ubp5-2* 16 days-old.

4.3.2 UBP5 is necessary for SAM dominance, SAM normal initiation and SAM maintenance.

One of the phenotypes is the loss of apical dominance, since we cannot appreciate a clear unique SAM; by contrast we observe several small shoots. As mentioned before, the SAM initiation is partially controlled by *WUS* and *CLV3* that form a feedback loop of regulation to maintain a pool of stem cells and differentiating the external layers (Schoof et al., 2000). These two master genes were up-regulated in *ubp5-2* plants (**Figure 23**). In addition, based on transcriptomic analysis, *UBP5* is highly expressed in the WUS and CLV3 tissue specific region in the SAM (Yadav et al., 2009), suggesting a key regulatory role of UBP5 in this process of SAM initiation (**Figure 23**).

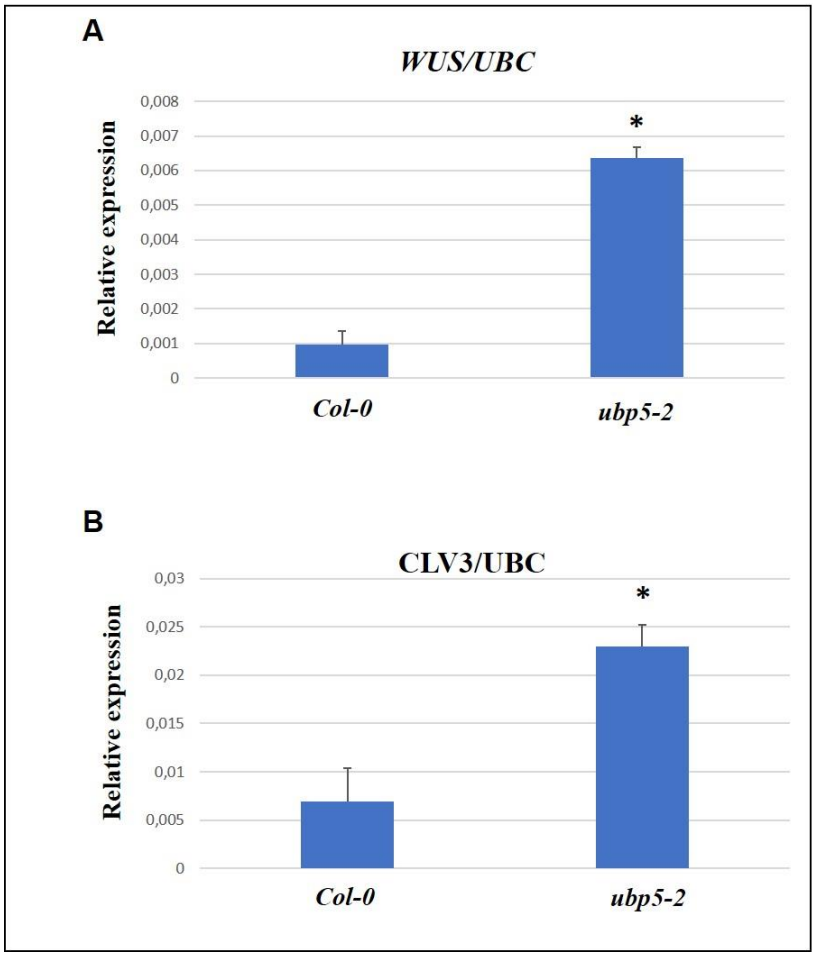

Figure 23. *WUS* and *CLV3* are mis-regulated in *ubp5-2* plants. A-B) Relative expression measurement of *CLV3* and *WUS* in Col-0 and *ubp5-2 plants* normalized against the constitutively expressed *UBC21* gene at Zt 8. Error bars represent standard error. Significance level is P-value ≤ 0.05.

KNAT1/2 and *STM* were also measured. These three genes are required for the establishment of the stem cell pool and are critical for SAM maintenance. The three of them were up regulated in *ubp5-2* plants (**Figure 24**). This up regulation plus the one of *WUS* and *CLV3* explains may probably be the reason for the development of several SAMs in *ubp5-2* mutants **(Figure 24**).

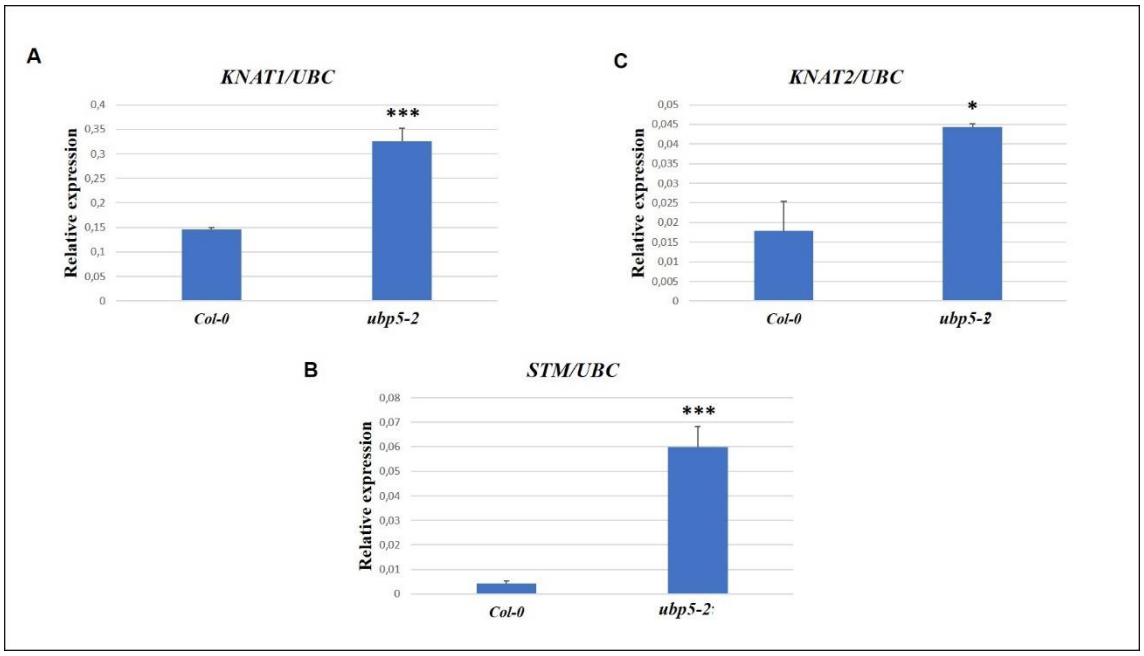

Figure 24. UBP5 is necessary for the proper expression of SAM master genes. The values indicate the relative expression of *STM, KNAT1* and *KNAT2* in these mutants normalized against the constitutively expressed *UBC21* gene at Zt 8. Error bars represent standard error, asterisk indicates significance level P-value ≤ 0.05; two asterisks indicate P-value ≤ 0.01; three asterisks indicate P-value ≤ 0.001.

On the other hand, *KNAT6*, another gene encoding a KNOX class I transcriptional factor that works redundantly with STM in the maintenance of stem cells, was not mis-regulated (**Figure 25**) (Belles-Boix et al., 2006). Other genes related with the observed phenotypes were not mis-regulated, including the *Cyclin-dependent protein kinase 1;1* (*CYCB1*), which encodes a protein involved in the cell cycle control that works as growth effector **(Figure 25**).

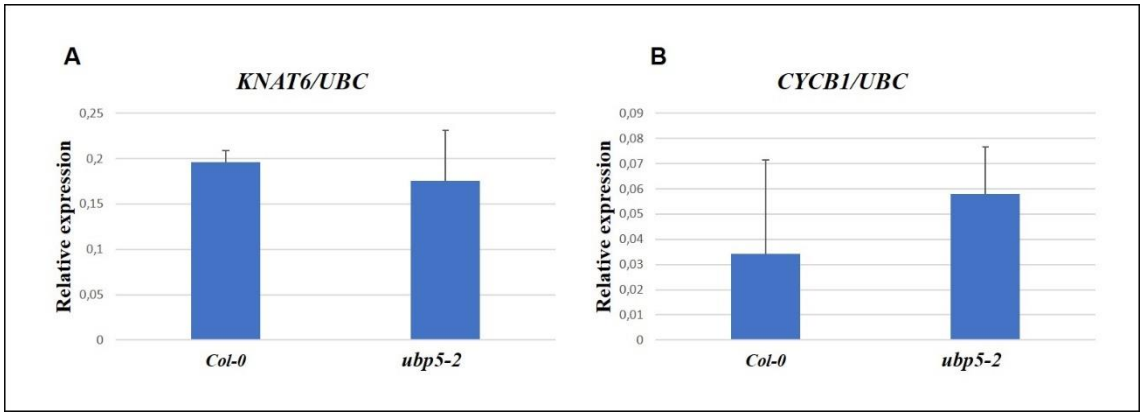

Figure 25. UBP5 do not affects *KNAT6* and *CYCB1;1* expression. The values indicate the relative expression of *KNAT6* and *CYCB1;1* in this mutant normalized against the constitutively expressed *UBC21* gene at Zt 8. Error bars represent standard error.

4.3.3 UBP5 interacts with PWO1 and PcG members *in vivo* and *in planta*

In order to confirm the previous *in planta* UBP5-PWO1 interaction observed through a proteomic study, Y2H assays were carried out. We confirmed the interaction between UBP5 and PWO1 as well as a novel interaction to FGT1 (**Figure 26 A, B**). UBP5 was checked against several PcG members (i.e. VRN2, EMF2, TLF2/LHP1, MSI1, SWN and CLF). These experiments reported positive interactions between UBP5 and EMF2 and SWN (see **Figure 26 B, C**).

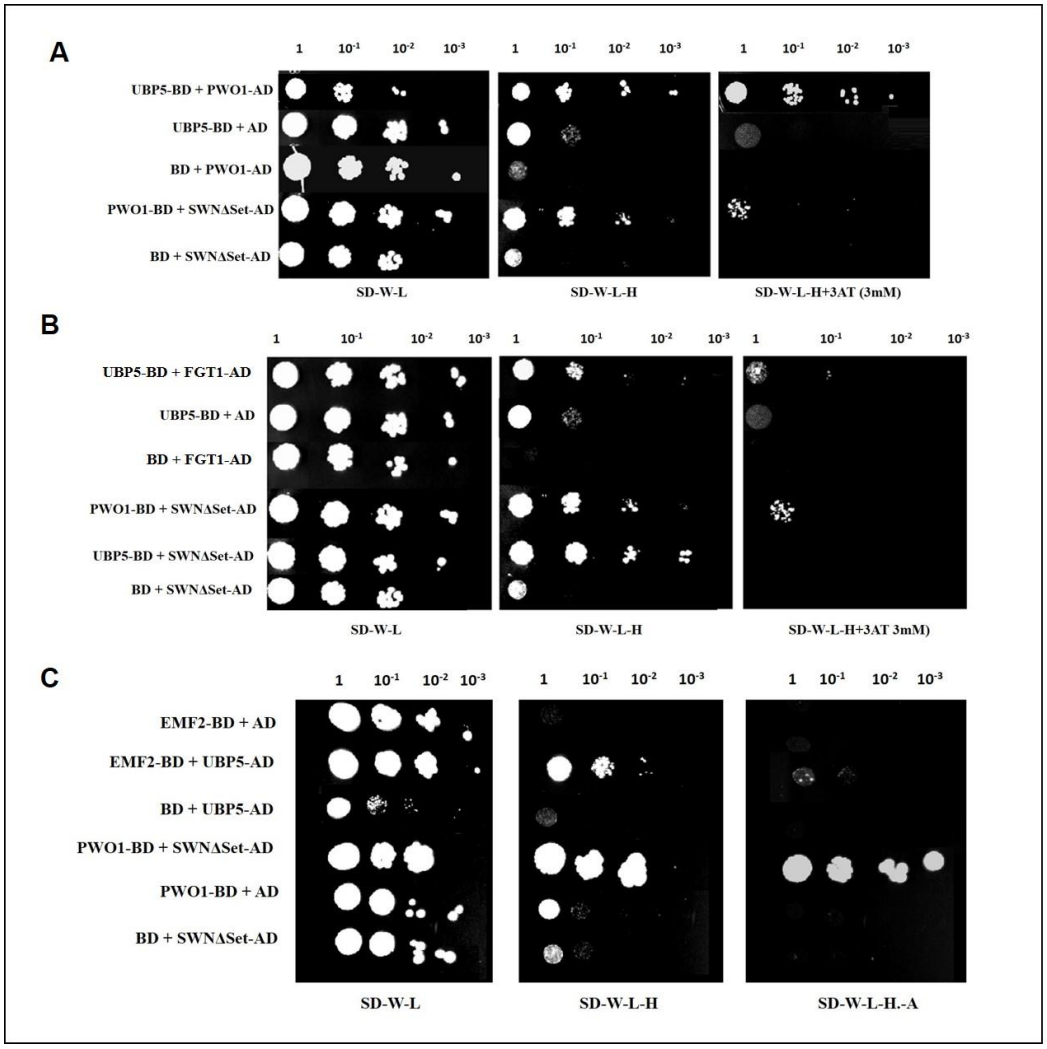

Figure 26. Y2H experiments showing interactions between UBP5, FGT1 and PcG members. **A)** Interaction between UBP5 and PWO1. The proteins were cloned in the two vectors of the Y2H system and co-transformed in different combinations growing onto –LW and –LWH selective media supplemented with 3mM of 3AT. **B)** Interaction between UBP5-FGT1 and UBP5-SWN. The proteins were cloned in the two vectors of the Y2H system and co-transformed in different combinations growing onto –LW and –LWH selective media supplemented with 3 mM of 3AT. **C)** Analysis of EMF2-UBP5. The proteins were cloned in the two vectors of the Y2H system and co-transformed in different combinations growing onto –LW and –LWH selective media supplemented with 3 mM of 3AT. **D)** Interaction between FGT1 and UBP5 with SWN. The interaction between PWO1-BD and SWNΔSet-AD was used as positive control of the Y2H (Hohenstatt et al., 2018).

The interaction between UBP5 and SWN was also confirmed by co-IP experiments in *N. benthamiana* plants co-expressing UBP5-mCherry and GFP-SWNΔSET fusion proteins (**Figure 27**). SWN co-immunoprecipitated with UBP5, revealing a physical connection between these two proteins.

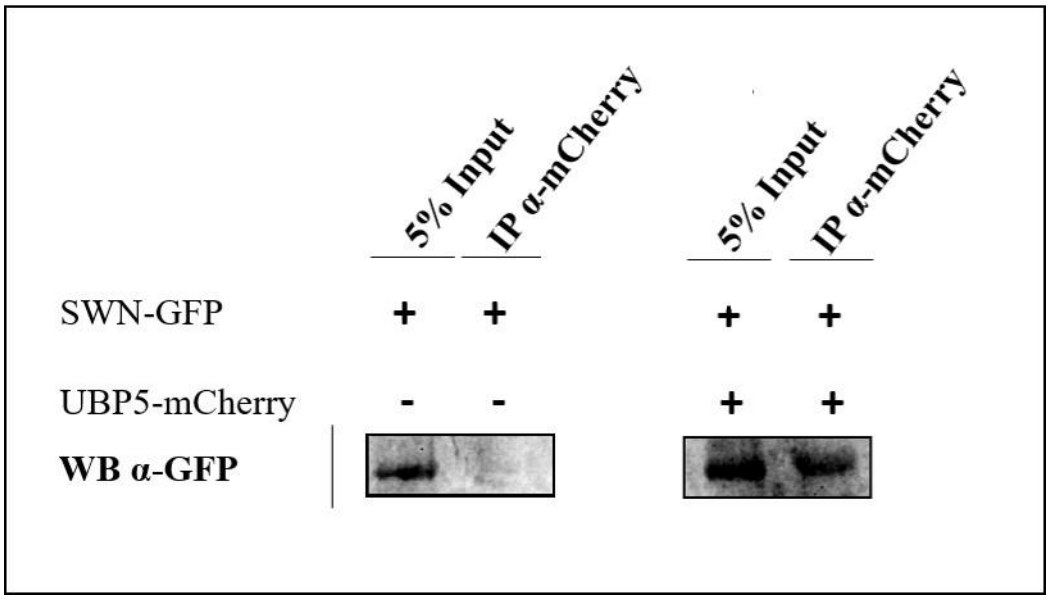

Figure 27. SWN interacts and co-immuno-precipitates UBP5. *N. benthamiana* plants were co-infiltrated with pMDC7- SWNΔSET-GFP and pMDC7-UBP5-mCherry. Immunoprecipitation was performed with anti-mCherry antibody, and proteins were detected by western blot using anti-GFP. IP = immunoprecipitated samples.

4.3.3.1 UBP5 interacts with PWO1 N- and C- terminal regions and do not form homodimers

In order to discover whether the interaction between UBP5 and PWO1 depends on a specific region of PWO1, Y2H experiments between UBP5 and the three fragments spanning the *PWO1* cDNA were carried out (**Figure 28**). UBP5 interacts with the full-PWO1 but is also able to interact with the N-terminal PWWP fragment as well as with the C-terminal. In addition, UBP5 does not form homodimers (data not shown).

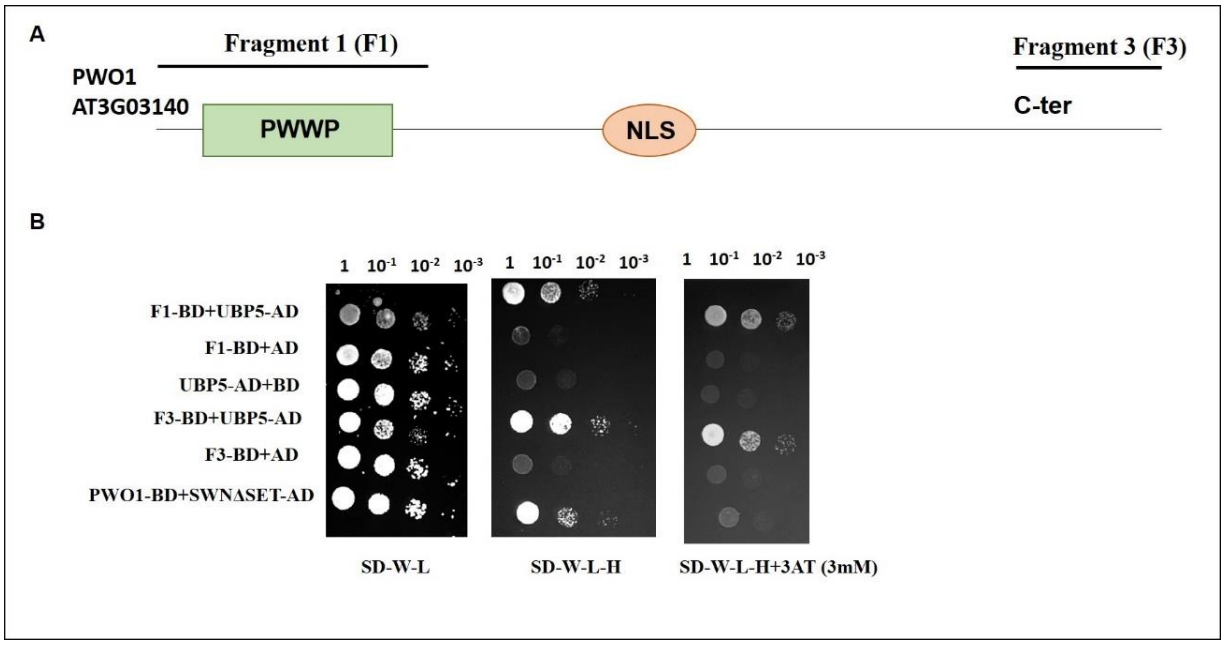

Figure 28. UBP5 interacts with PWO1 N-terminal and C-terminal protein regions. A) Schematic representation of PWO1 protein and the fragments used to this protein-protein interaction experiment. **B)** The protein fragments were cloned in the two vectors of the Y2H system and co-transformed in different combinations growing onto –LW and –LWH selective media supplemented with 3mM of 3AT. Interaction between PWO1-BD and SWNΔSET-AD was used as positive control of the Y2H (Hohenstatt et al., 2018).

4.3.4 UBP5 interacts with FVE *in vivo*

Considering that UBP5 interactors PWO1 and FGT1 interacts with FVE, FVE-UBP5 interaction was also cheeked by Y2H. In this assay, an interaction between UBP5 and FVE was detected (**Figure 29**).

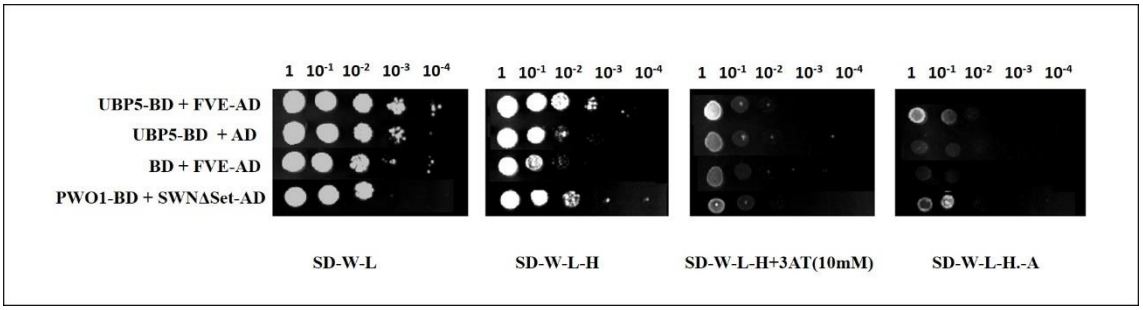

Figure 29. UBP5 interacts with FVE. UBP5 interacts with FVE. The proteins were cloned in the two vectors of the Y2H system and co-transformed in different combinations growing onto –LW and –LWH selective media and –LWHA.

Discussion

5. Discussion

The PcG pathway contributes to Arabidopsis developmental phase transitions, repressing a large number of genes when and where they are needed. On the other hand, little is known about how exactly PcG members can carry out this tremendous task, suggesting the involvement of other players in this process of gene repression (Mozgova et al., 2015; Wassef & Margueron, 2016). The discovery of new PcG proteins interactors is currently under intensive investigation. For instance, PWO1, a new PcG interactor, has been involved in the regulation of several PcG target genes (Hohenstatt et al., 2018), BLISTER (BLI) is involved in PcG gene repression and cellular differentiation (Schatlowski et al., 2010) or SCARECROW (SCR) recruits LHP1 to *MAGPIE* (Cui & Benfey, 2009). In this search of PcG proteins interactors, FGT1 and UBP5 were found in a proteomic study revealing a novel *in vivo* interaction with PWO1.

The aim of this doctoral thesis is the molecular characterization of these two novel interactors of PWO1 and its putative relationship to the PcG pathway.

5.1 *fgt1-4* phenotype cannot be attributed to *FGT1* mutation

FGT1 was firstly described as an essential protein for embryo development (EMB1135) (McElver et al., 2001). The mutant allele from this collection was initially used in this research (e*mb1135*); from now on called *fgt1-4*. Not all the *fgt1-4* mutants show embryo-defective phenotype and a few seeds germinated, producing seedlings that display strong phenotypes, which remind of the strong *clf,swn* double mutant plants (**Figure 6**). In order to get a general view of the kind of genes mis-regulated in this mutant line a transcriptomic experiment was carried out. Based on RNA-seq assays, between these 3182 genes, which represent 11% of Arabidopsis genes, genes related to stress were significantly enriched in the list of *fgt1-4* mis-regulated genes (**Figure 7**). Among the mis-regulated genes, some of the key transcription factors involved in SAM development were also found, including *CLV3, WUS* and the *KNOX* genes among others.

This mis-regulation may explain certain characteristics of the *fgt1-4* phenotypes, such as loss of apical dominance. In the SAM, the KNOX transcriptional factors participate in the establishment of the stem cells pool (Scofield et al., 2008). CLV3 promotes the cell lineage to organ initiation and is the repressor of *WUS* homeobox gene (Kayes & Clark, 1998; Laux et al., 1996; Schoof et al., 2000). The WUS/CLV3 negative feedback loop controls the stem cell pool in the SAM (Schoof et al., 2000). In addition, PRC1 and PRC2 have already been shown to be involved in the regulation of the *KNOX* genes (Xu & Shen, 2008).

CLV3 and the KNOX genes *KNAT1, KNAT2, KNAT6* and *STM* are up-regulated in *fgt1-4* based on relative expression experiments and marker lines, confirming the RNA-seq analysis (**Figure 8**). All these results in principle suggested that FGT1 could control the SAM development, participating in the initiation process and in the SAM maintenance, processes also regulated by PRC2.

FGT1 is a single copy gene in Arabidopsis that has a homologs in other species, animals and plants (Gazave et al., 2009). However, apart from the conserved AAA and Helicase C-like domains, *FGT1* encoded one more domain compared to animals, a PHD domain (Brzezinka et al., 2016). The PHD domain acts as a epigenomic H3 tail reader, conferring to the PHD-containing proteins an important role in development (Mouriz et al., 2015).

In flies, PRC2 works with the PHD-containing protein Pcl, which is required for generate high levels of H3K27me3, supporting PRC2 in the repression of PcG target genes (Nekrasov et al., 2007). In Arabidopsis the PHD-containing proteins VEL1-3 have the same role increasing H3K27me3 levels in vernalization as well as in the photoperiodic pathway, promoting flowering (De Lucia et al., 2008; Sung et al., 2006). On the other hand, the contribution of other PHD-containing proteins to PRC2 activities in other phase transitions have not been reported.

As mentioned before, in *fgt1-4* RNA-seq analysis, 28% of the mis-regulated genes are H3K27me3 target genes, including the *KNOX* and *CLV3* genes. These results in principle suggested a putative novel link between FGT1 and PRC2 in the control of Arabidopsis development, particularly the SAM development.

Nevertheless, during the molecular characterization of *fgt1-4* a publication describing the putative molecular role of FGT1 was published (Brzezinka et al., 2016). In this publication they used a different mutant allele (*fgt1-1*) that does not present the same phenotype as did our mutant allele of study. Indeed, the results provided by PCRs, TAIL-PCRs and splicing variants (intron retention) experiments concluded that *fgt1-1* is not a null allele and that *fgt1-4* has a second insertion in the putative promoter region, -108bp, of *EMB1144*, a gene that encodes a key enzyme involved in synthesis of aromatic amino acids (**Figures 9, 11 and 12**). Thus, these results mean that the transcriptomic data as well as the phenotypes observed in *fgt1-4* cannot be exclusively attributed to the mutation on *FGT1* locus. This was further confirmed by the unsuccessful complementation of the *fgt1-4* mutant with a *FGT1::GFT1-GFP* transgene. Therefore, these results and the *fgt1-4* allele must be handled with extreme care when drawing conclusions about the role of FGT1 in Arabidopsis development.

5.2 FGT1 as a new Polycomb interactor

FGT1 modifies the nucleosome position at the start site, promoting gene expression of its target genes under a Heat Stress (HS) in a mediated process by the SWI/SNF and ISWI families members (Brzezinka et al., 2016). On the other hand, FGT1 physically interacts *in vivo* with PcG members such as SWN and PWO1 (**Figures 14 and 15**) suggesting that despite the role of FGT1 promoting gene expression, a novel role of FGT1 in gene repression may arise in the future. In addition, the protein-protein interaction experiments were mostly performed with PRC2 subunits, except FVE and LHP1, so whether FGT1 further interacts with PRC1 components is still an unanswered question. Further research in this direction combined with chromatin immunoprecipitation will shed some light into the dark. In addition, these results suggest that FGT1 may have a function related with gene repression through its relationship to the PcG pathway and due to *FLC* overexpression in the *fgt1-5* mutant that I will discuss later on. Whether this interaction is as a mediator, supporter, effector or as a repression-mediator still requires to be investigated.

5.3 FGT1 and its role in other abiotic stresses

Regarding non-PcG member interactors of FGT1, we detected an interaction between FGT1 and FVE (**Figure 16**), bringing a new research perspective to my project. On the other hand, in *fgt1-5* mutants, *FVE* expression level is not altered (**Figure 17**), suggesting that *FGT1* is not epistatic over *FVE* and they may cooperate at post-translational level. In addition to FGT1, FVE interacts *in vivo* with the proteins encoded by At3G54760, At1G48560, At3G48190, *PDP1* (At5G27650), *PDP2* (AT3G09670) and *PDP3* (AT5G40340), PWWP protein family members, specifically through the PWWP domain (Kenzior & Folk, 2015; Zhou et al., 2018b). Strikingly PWO1, also interacts with FVE (Hohenstatt's thesis 2012), suggesting that PWWP-containing proteins can be part of a putative complex that mediate a crosstalk between PRC2 and HDAC in regulation of developmental processes.

FVE regulates the cold response through the C-repeat dehydration-responsive element-binding factor/dehydration-responsive element-binding protein (CBF/DERB) pathway (Cheng et al., 2017; Kim et al., 2004). In this context, in *fve* mutants, several coding genes for the LEA proteins, a cluster of proteins conserved in prokaryotes and eukaryotes, are overexpressed (Kim et al., 2004). In Arabidopsis they are necessary for the final stage of embryogenesis during seed development and are also found in vegetative tissues involved in response to freezing temperatures (Hincha & Thalhammer, 2012). One of the most studied LEA proteins in Arabidopsis is COR15a that accumulates in the chloroplast stroma under freezing temperatures to increase the freezing tolerance of the leaves. *COR15a* is overexpressed in *fve* mutants without cold or freezing stress due to an increase in H3 acetylation (Sowemimo et al., 2019). Together these data suggest that FVE may be involved in the recruitment of several complexes or playing an essential role as a scaffold subunit. Similar mis-regulation of *COR15a* in the *fgt1-5* mutant compared to Col-0 was observed (**Figure 17**), suggesting that FGT1 may be involved in FVE functions. In order to corroborate this hypothesis, further research is needed and a *fgt1-5;fve-3* double mutant line is presently under study for this purpose.

A main topic of research in plant science is the interactions between different stresses, as plants are rarely affected by single stresses. The genetic pathways to respond to specific abiotic stresses, such as temperature changes, osmotic stress or light, usually share some of their intermediaries (Ishitani et al., 1997; Kim et al. 2002; Xiong et al., 1999). Downstream of these stresses, different epigenetic factors will produce changes in gene expression, that ultimately will induce the response to the specific stress (Mozgova et al., 2019).

As mentioned before, FGT1 is involved in HSM control, but there is no information about FGT1's role in the response to other stresses (Brzezinka et al., 2016). The interaction with PWO1 and FVE suggests that a further research should be carried out to unveil if FGT1 plays a role in others abiotic stresses response mediated by PcG pathway and /or HDAC.

5.4 FGT1 participates in flowering time regulation

As mentioned before, the PcG pathway and FVE regulate flowering time in Arabidopsis in an independent and dependent way (Ausín et al., 2004; Chanvivattana et al., 2004; Pazhouhandeh et al., 2011). CLF regulates *FT*, *FLC* and *FLC* relatives genes expression, mediating the deposition of H3K27me3 (Jiang et al., 2008). On the other hand, *fve* mutant plants display an increase of H3 and H4 acetylation and decrease of H3K27me3 and H3K4me3 on *FLC* locus, changes explained by its interaction with histone deacetylase 6 (HDA6) and FLOWERING LOCUS D (FLD), a lysine specific demethylase 1 (LSD1) type (Yu e al., 2016; Yu et al., 2011). The decrease of H3K27me3 may be due to the association of PRC2 with the Cullin4 (CUL4) and Damage Binding protein 1A (DDB1A) and DDB1B ubiquitin E3 ligases, members of the DDB1 and CUL4-associated factors (DCAFs) family, in an FVE-dependent manner. In addition, PDP1, PDP2, PDP3 and PWO1, interactors of FVE, have been involved in the regulation of flowering time thought H3K27me3 changes on *FLC* locus. Although with opposite effect between PWO1 and PDP1, PDP2 and PDP3 (Hohenstatt et al., 2018; Zhou et al., 2018b). These results suggest that FVE may be a member of putative PcG-like complexes (Lee & Zhou, 2007; Pazhouhandeh et al., 2011).

fgt1-5 mutants show an increase of *FLC* relative expression compared to Col-0 that does not relate with the phenotypic analysis of flowering time (**Figure 18**). This result generates specific questions such as; (i) why does the relative high expression of *FLC* not promote changes in the flowering phenotype of *fgt1-5* plants? (ii) why is *FLC* upregulated in the *fgt1-5* mutant plants? and (iii) why *fgt1-1* allele displays an early flowering phenotype but not *fgt1-5*?

Regarding the first question, this scenario suggests that in *fgt1-5* other gene(s) downstream of *FLC* might be mis-regulated as well, covering the up-regulation of *FLC*, as happened with CLF, that is necessary for *FLC* and *FT* direct repression (Chanvivattana et al., 2004). To check this hypothesis several down-stream genes were measured (**Figure 19** and data not shown). Whereas *FT* and *AGL24* showed the expected expression levels under a *FLC* overexpression situation, *SOC1* seem to escape this regulation, suggesting that in *fgt1-5* mutants, despite the increase in *FLC* expression, *SOC1* cannot be properly repressed. Another possibility could be that FGT1 may be directly necessary for *SOC1* repression independently of FLC. Therefore, in order to elucidate if *FGT1* is epistatic over *FLC* in the *SOC1* regulation, a *fgt1-5,flc-3* double mutant plant is under construction.

Another possibility to explain how, despite an increase in *FLC* expression, a late flowering phenotype was not observed, could be the gibberellic acid (GA) dependent flowering pathway. GAs promote flowering acting in parallel to the photoperiod pathway. Mutations of the GA pathway are only perceptible in short day conditions and almost imperceptible in LD conditions, suggesting that in short days conditions, the GA pathway is the most important one (Galvão et al., 2015). This hypothesis has been discarded because as shown in **Figure 18** *fgt1-5* mutant plants do not show an alteration of flowering time compared to Col-0 accession plants in SD conditions, hence FGT1 may not be involved in GA-mediated control of flowering time.

Regarding the second question, previous results showed that FGT1 is an interactor of PcG proteins (i.e. PWO1, SWN) (**Figure 14**). PRC2 acts both, dependently and independently of the vernalization pathway in *FLC* repression (Chanvivattana et al., 2004; Pazhouhandeh et al., 2011). Thus, fgt*1-5;swn-7*, *fgt1-5;clf-28* and *fgt1-5;pwo1* double mutant plants are presently segregating to check a putative mis-regulation (epistatic, additive) of the flowering time mediated by PRC2. In addition, to check a putative role of FGT1 in the vernalization pathway, a *fgt1-5;FRI+* double mutant line is as well currently under study. *FRIGIDA (FRI)* is the active allele of the main *FLC* activator

through the vernalization pathway (Blümel et al., 2015). *FRI* locus present a natural inactive allele in Col-0, due to this reason Col-0 plants do not have a vernalization requirement, hence an active allele needs to be introduced in order to study the activation of *FLC* mediated by FRI. In addition, FRI has been proposed to be part of an uncharacterized complex, FRI-complex (FRI-C), as a scaffold subunit (Choi et al., 2011).

Regarding the third question, *fgt1-1* mutants show higher *FLC* and lower *FT* expression compared to Col-0 and display early flowering phenotype (**Figures 18 and 19**). Since two different transcripts can be found in *fgt1-1* mutants (**Figure 9**) this line could be considered as an overexpression allele and the different processes regulated by FGT1 will suffer alterations compared to wild type and to a null allele. Those scenarios are relatively common, such as it happened with SWR1-complex protein (SWC) 4 knock-down line (*swc4*i). SWC4 is a subunit of the SWR1-complex (SWR1-C), one of the complexes involved in the H2A.Z turnover, histone variant related, among other processes, with gene activation and repression (Jarillo & Piñeiro, 2015). SWR1-C acts as a *FLC* activator, nevertheless, the *swc4*i mutants shown an overexpression of *FLC*, similar expression of *FT* and a decrease of *SOC1* expression compared to Col-0, all these added to an early flowering phenotype (Gómez-Zambrano et al., 2018). In conclusion, for genes that are involved in multiple processes the utilization of knock-down lines can produce unexpected phenotypes. Another possibility is that these genes are regulated independently, as it occurs with the FGT1 interactor BRAHMA (BRM). BRM regulates *FT, SOC1*; independently of *FLC, CO* and *FLC* (Brzezinka et al., 2016; Farrona et al., 2011).

5.5 UBP5 is essential for proper development

Until recent years, the characterization of the DUB family had mainly been carried out from a biochemistry point of view and analyzing the activity of its members as ubiquitin proteases. In this context, UBP5 shows specificity for ub-ub linkages, suggesting that UBP5 may work in a post-translational process, showing a deubiquitinase *in vivo* activity (Rao-Naik et al., 2000). Nevertheless, the molecular role of UBP5 has never been described.

The *ubp5-2* line has demonstrated the importance of UBP5 in the regulation of plant development since the *ubp5-2* mutant plants show a pleiotropic phenotype, affecting several developmental processes (e.g., altered phyllotaxy, lack of apical dominance, overall reduced plant size) (**Figure 21**).

Among these phenotypes, a late-flowering phenotype and high *FLC* relative expression are observed in *ubp5-2* mutants (**Figure 22**), as well as, an impaired activity of the SAM. In fact, UBP5 is involved in the control of the SAM development, as *CLV3, WUS, KNAT1* and *KNAT2* are up-regulated in *ubp5-2* plants (**Figure 23 and 24**). In addition, *UBP5* has its highest expression levels in the SAM, during the embryogenesis, the seed maturation and flower development considering the Eplant viewer based on DNA microarray and RNA-Seq experiments (**Figure 23**) (Klepikova et al., 2016; Nakabayashi et al., 2005; Schmid et al., 2005).

ubp5-2 plants also showed a delay in germination (**Figure 21).** One key transcriptional factor involved in seed dormancy is the PcG target gene *DELAY OF GERMINATION1 (DOG1)* (Alonso-Blanco et al., 2003; Footitt et al., 2015). PRC1 and PRC2 mutants show delayed germination due to the mis-regulation of *DOG1* (Bouyer et al., 2011; Müller et al., 2012). *DOG1* expression peaks during seed maturation, relating this level to seed dormancy; in addition, *DOG1* expression level is stimulated by low temperatures (Bentsink et al., 2006; Chiang et al., 2011). HDACs can also affect *DOG1* expression since *hda19* mutant seeds have a reduced dormancy but HISTONE DEACETYLASE 2B (HD2B), that is also stimulated by low temperatures, promote seed dormancy (Wang et al., 2013; Yano et al., 2013). During the writing of this doctoral thesis, the relative expression of *DOG1* in *ubp5-2* was compared to Col-0 by a member of the Farrona lab. *DOG1* is down-regulated in *ubp5-2*, suggesting that the increase of dormancy in *ubp5-2* seeds is not mediated by *DOG1*. On the other hand, *ABSCISIC ACID INSENSITIVE (ABI) 3*, *ABI4* and *ABI5* are up-regulated in *ubp5-2* seeds compared to Col-0. ABI3 is a B3 domain transcriptional factor that is involved in the transition from embryogenesis (seed maturation) to the seedling stage through ABA-arrested seed germination (Nambara et al., 2000; Parcy et al., 1997). ABI4 and ABI5, downstream of ABI3, participate in seed germination (Lopez-Molina et al., 2002; Yan et al., 2019).

Nevertheless, these three transcriptional factors are not only involved in seed germination; ABI4 and ABI5 promote *FLC* expression in an independent way; ABI3 also is a negative regulator of flowering time (Hong et al., 2019; Shu et al., 2018, 2016; Wang et al., 2013). In addition, ABI4 is also involved in lateral root development (Mu et al., 2017).

These results suggest that these factors are involved in some of the most important Arabidopsis developmental phase transitions, hence understanding how UBP5 regulate the expression of these genes may provide crucial information to understand its role in plant development.

It has been previously reported that HDA6, HDA19 and PRC2 directly repress *ABI3* and *ABI4* (Lafos et al., 2011; Mu et al., 2017; Ryu et al., 2014; Tanaka et al., 2008). Therefore, two plausible hypotheses are that UBP5 participates in the control of these developmental processes through repression of the *ABI* genes through a HDA6 and/or PRC2-mediated pathway. The direct interaction of UBP5 with components of PRC2 (SWN, EMF2) (**Figures 26 and 27**) and FGT1 supports the second hypothesis. On the other hand, recent genome-wide transcriptomic analysis of *hda19* and *fve* single mutants do not report a mis-regulation of *ABI* genes, suggesting that it will be very important to confirm these results in future experiments (Yu et al., 2016).

5.6 Understanding UBP5 functions

During last years, new promising studies are discovering novel molecular functions of DUB members and their relationship with epigenetics (review in March & Farrona, 2018). In this research, novel protein-protein interactions between UBP5 and members of PcG pathway (i.e. SWN, EMF2, PWO1), FGT1 and FVE were detected (**Figures 28 and 29**). UBP5 interactions to other chromatin-related proteins suggest that these proteins may co-participate in a specific unknown-process. We hypothesize that UBP5 would support PRC2 in other developmental processes based on the pleiotropic phenotype found in *ubp5-2* mutants and because SWN, redundantly with CLF, co-regulates different processes, such as seed maturation, juvenile to adult leaf transition or hormonal signaling pathways; in addition, SWN have its own specific target genes involved in lipid storage, cell wall modification and post-embryonic development (Shu et al., 2019).

Regarding the putative role of UBP5, three main hypotheses are currently taken into account:

(i) UBP5 may directly be necessary for proper PRC2-mediated repression as it was reported for UBP12 and UBP13, other members of the family (Derkacheva et al., 2016).

(ii) The human protein with highest sequence similarity to UBP5 is the UBIQUITIN-SPECIFIC PROTEASE (USP) 4. USP4 is involved in the indirect epigenetic regulation at histone level through direct deubiquitination of HDAC2 (Li et al., 2016). Basically, USP4 stabilizes HDAC2 at post-translational level, removing the ubiquitin to avoid the degradation of the complex. Therefore, a similar activity for UBP5 in plants may occur in which UBP5 would stabilize other chromatin related proteins such as HDAC6 and PRC2. Recently, UBP5 has been found co-immunoprecipitating with members of the PEAT complex, specifically with EPCR1, ARID2, TRB1 (Tan et al., 2018). This complex is involved in heterochromatin silencing through histone deacetylation and heterochromatin condensation; in addition, the PEAT complex negatively regulates the production of small interfering RNAs and DNA methylation. These results suggest that UBP5 could directly participate in the PEAT complex activities.

(iii) UBP5, which has deubiquitinase *in vivo* activity (Rao-Naik et al; 2000), may mediate the H2Aub and/or H2Bub deubiquitination, epigenetic marks that are catalyzed in metazoans by PR-DUB and PRC1 (Merini et al., 2017; Nassrallah et al., 2018). In addition, in mammals, others USPs like USP3 and USP16 can mediated the H2Aub independently of PR-DUB (March & Farrona, 2018). Even if this hypothesis is the less probable because the deubiquitination of -H2Aub is linked with gene activation, it must be checked as previous results connect UBP5 with PRC2.

All these hypotheses will be considered for future work of the Farrona research group and are currently being tested.

5.7 Perspectives

The use of omics techniques will help to discover direct interactors and direct target genes of FGT1 and UBP5, necessary for their full characterization. In addition, the protein-protein interactions discovered in this research between FGT1 and UBP5 with chromatin-related proteins will contribute to some of the current open question in epigenetics, such as the crosstalk between epigenetic marks and the pathways for recruitment of epigenetic complexes.

An open question is whether FGT1, UBP5 and/or PWO1 are necessary for the proper FVE activities (including recruitment, chromatin binding ability or repression trough deacetylation) that could add new knowledge to the role of FVE in flowering regulation and to the cold tolerance, in which FVE mediates a still non fully understood activity in the regulation of the cold response pathway. Preliminary results from our colleagues in the Schubert's lab (University Freie Berlin, Germany) showed that PWO1 and FGT1 may be involved in cold stress response. These preliminary results suggest that a putative PWO1-FGT1 complex may be involved in, at least, more than one abiotic stress response mediated by epigenetic regulation.

A final question remains unanswered, which is the connection between FGT1 and UBP5 to the regulation of genetic expression?

FGT1 was found interacting with chromatin remodelling complexes and was proposed to play a role as activator of gene expression (Brzezinka et al., 2016). On the other hand, in this research I demonstrated that FGT1 also binds to subunits of the repressive complexes (PRC2, HDAC6), suggesting that FGT1 may be also involved in gene repression or, at least, in regulating the repressive activity of these complexes. In addition, a novel complex was recently described, the PEAT complex (Tan et al., 2018). The PEAT complex is former by enhancer of polycomb-related proteins (EPCR1-2), PWWP domain-containing proteins (PWO1-3), AT-rich interaction domain-containing proteins (ARID2-4), and telomere repeat binding proteins (TRB1-2). It has been hypothesized that this complex is necessary for heterochromatin formation, DNA methylation and PRC2 recruitment due to its subunits TRB1-2, which are a direct PRC2 recruiters (Zhou et al., 2018).

Among the proteins identified interacting with the PEAT complex UBP5 was also present. In addition, FGT1 interacts with UBP5 and PWO1, interactor and subunit of the PEAT complex respectively. Therefore, these results suggest that FGT1 and UBP5 may also be required for the proper repression of specific genes.

Finally, considering results from our phenotypic analyses of the single *fgt1-5* and *ubp5-2* mutants, it is probable that both UBP5 and FGT1may also regulate partially different gene sets as part of alternative complexes. Nevertheless, further research is needed to address this question.

Conclusions

6. Conclusions

1- *fgt1-1* is not a null-allele since the results from IR experiments shown how *fgt1-1* produce two different population of *FGT1* transcripts, spliced and unspliced, suggesting that this allele is not a null allele.*fgt1-4* is a double T-DNA mutant allele since the results from TAIL-PRCs shown how this mutant allele carry at least an extra T-DNA insertion in the putative promoter region of *EMB1144* locus.

2- FGT1 interacts with PcG members *in vivo* as were shown by Y2H and Co-IP, interacting with SWN and PWO1.

3- FGT1 interacts with FVE and UBP5 *in vivo* in Y2H, suggesting that FGT1 and UBP5 may be part of a novel putative complexes as well as with a member of the HDAC6, highlighting this relation between FGT1, UBP5 and gene repression.

4- FGT1 participates in the regulation of flowering pathway since relative expression levels of FLC were altered in *fgt1-5*.

5- UBP5 is essential for Arabidopsis development since in *ubp5-2* mutant allele a pleotropic phenotype was reported, suggesting that UBP5 is important for the proper Arabidopsis development affecting several processes and developmental phases transition.

6- UBP5 participates in the regulation of flowering pathway, affecting the relative expression patterns of *FLC* and *SOC1*. *FLC* is up-regulated meantime *SOC1* do not show alterations, suggesting that UBP5 may participates in the repression of these genes.

7- UBP5 regulates the SAM initiation and development. Several genes involved in this process are up-regulated; including CLV3, WUS, KNAT1, KNAT2 and STM in *ubp5-2* mutant allele, suggesting that UBP5 participates in the control of these gene expression patterns.

8- UBP5 interacts with PcG members in vivo in Y2H and Co-IP, specifically UBP5 physically interacts with SWN, EMF2, and PWO1, suggesting that UBP5 may co-participates with PRC2.

9- UBP5 interacts with FVE has happened with FGT1, interacting in vivo by Y2H, creating a new link between UBP5 and gene repression through this interaction with a member of the HDAC6.

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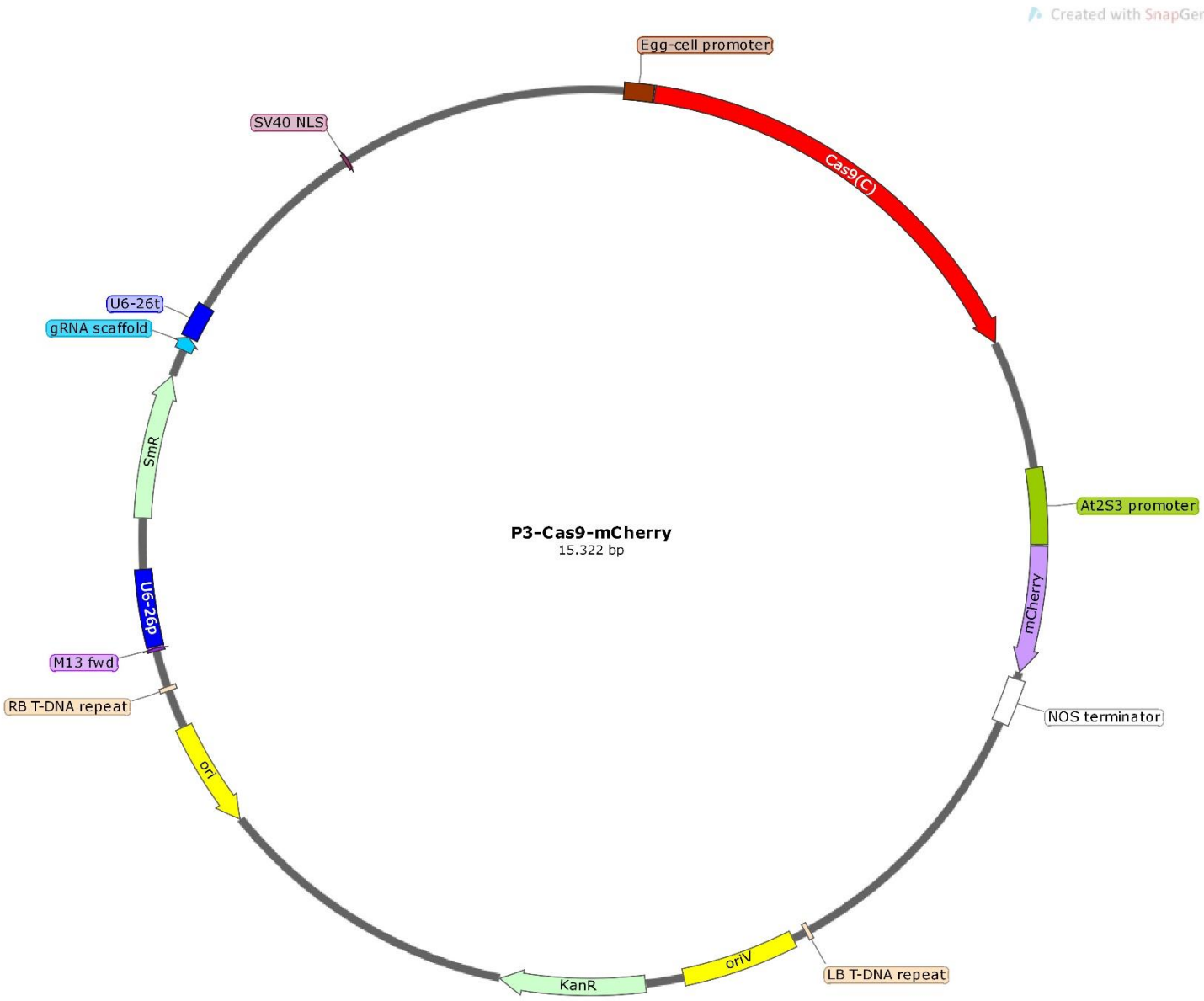
Appendix

P3-Cas9-mCherry vector map and sequence.



Appendix 1. Map generated with SnapGene viewer, available at snapgene.com

>P3Cas9mCherry.

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