**Palacký University Olomouc**

**Faculty of Science**

**Department of Cell Biology and Genetics**

# Analysis of ploidy and reproductive development in the mutant lines of SMC5/6 complex in Arabidopsis

**Master thesis**

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**Supervisor: Assoc. prof. Aleš Pečinka**

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# ZADÁNÍ DIPLOMOVÉ PRÁCE

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**Diaz M., Pecinka A. (2018). Scaffolding for Repair: Understanding Molecular Functions of the SMC5/6 Complex. Genes 9: 36.**
**Ishida T., Fujiwara S., Miura K., Stacey N., Yoshimura M., Schneider K., Adachi S., Minamisawa K., Umeda M., Sugimoto K. (2009). SUMO E3 ligase HIGH PLOIDY2 regulates endocycle onset and meristem maintenance in Arabidopsis. Plant Cell 21: 2284-2297.**
**Ishida T., Yoshimura M., Miura K., Sugimoto K. (2012). MMS21/HPY2 and SIZ1, two Arabidopsis SUMO E3 ligases, have distinct functions in development. PloS One 7: e46897.**
**Liu M., Shi S., Zhang S., Xu P., Lai J., Liu Y., Yuan D., Wang Y., Du J., Yang C. (2014). SUMO E3 ligase AtMMS21 is required for normal meiosis and gametophyte development in Arabidopsis. BMC Plant Biology 14: 153.**

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# SUMMARY

The key regulators of chromatin structure, dynamics and function are the Structural maintenance of chromosomes (SMC) complexes. In eukaryotes there are at least three such complexes: cohesin, condensin and SMC5/6 complex. The SMC5/6 complex is involved in diverse mechanisms responsible for the maintenance of post-replicative chromosome structure. However, its functions still remain very poorly understood. The aim of this thesis was to analyze defects in the reproductive development of SMC5/6 complex mutants in Arabidopsis. The analysis included evaluation of seed development, analysis of pollen viability using FDA staining assay, measurements of pollen area in ImageJ software, reciprocal crossing and ploidy measurements using flow cytometry. It was shown that *NSE2/HPY2/MMS21* mutants caused paternally inherited abnormal seed phenotype and the pollen viability was reduced. Measurements of the pollen area revealed that *HPY2* mutants also produced two cohorts of differently sized pollen, which indicated that the ploidy level of these mutants could be affected. Following ploidy measurements proved that *HPY2* mutants lead to triploid offspring by producing diploid male gametes. Obtained results suggested that the SMC5/6 complex is involved in basic control of the ploidy level. This novel function of the SMC5/6 complex has not yet been proposed even in the animal kingdom.

# Bibliografické údaje

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# SOUHRN

Klíčovými regulátory struktury, dynamiky a funkce chromatinu jsou Structural maintenance of chromosomes (SMC) komplexy. U eukaryot existují alespoň tři takové komplexy: kohezin, kondenzin a SMC5/6 komplex. SMC5/6 komplex je zapojen v různých mechanizmech zodpovědných za udržení post-replikativní struktury chromozomu. Jeho funkce však nadále zůstávají jen velmi málo pochopeny. Cílem této práce bylo analyzovat defekty v reprodukčním vývoji mutantů SMC5/6 komplexu u huseníčku. Analýza zahrnovala hodnocení vývoje semen, analýzu životnosti pylových zrn pomocí FDA barvení, měření velikosti pylových zrn s využitím programu ImageJ, reciproká křížení a měření ploidie metodou průtokové cytometrie. Bylo prokázáno, že *NSE2/HPY2/MMS21* mutanti způsobili paternálně děděný abnormální fenotyp semen a sníženou životnost pylových zrn. Měření velikosti pylových zrn odhalilo, že *HPY2* mutanti rovněž produkovali dvě skupiny různě velkých pylových zrn, což naznačovalo, že by u těchto mutantů mohla být ovlivněna úroveň ploidie. Následná měření ploidie prokázala, že mutace *HPY2* vede ke vzniku triploidního potomstva v důsledku produkce diploidních samčích gamet. Získané výsledky naznačují, že SMC5/6 komplex je zapojen v základní kontrole úrovně ploidie. Tato nová funkce SMC5/6 komplexu nebyla dosud navržena ani u zvířat.

## Declaration:

I hereby declare that I elaborated this master thesis independently under the supervision of Assoc. prof. Aleš Pečinka, using only information sources listed in the References chapter.

Date: ……

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I would like to thank my supervisor, Assoc. prof. Aleš Pečinka, for the patient guidance and advice he has provided throughout my studies. I am very grateful to M.Sc. FenYang for generosity with her time and sharing her expertise at the laboratory.

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| ASAP1 | Arabidopsis SNI associated protein 1 |
| CAP | Condensin associated protein |
| CDCA5 | Sororin/cell-division-cycle-associated 5 |
| DIC | Differential interference contrast |
| DIF1 | Damage-regulated import factor 1 |
| DSB | DNA double-strand break |
| EMS | Ethyl methanesulfonate |
| FCM | Flow cytometry |
| FDA | Fluorescein diacetate |
| HPY2 | High ploidy 2 |
| KITE | Kleisin interacting tandem element |
| MAGE | Melanoma antigen gene |
| MCD1 | Mitotic chromosome determinant 1 |
| MIM | Hypersensitive to MMS, irradiation and MMC |
| MMS21 | Methyl methanesulfonate-sensitive protein 21 |
| MRE11 | Meiotic recombination 11 |
| NASC | Nottingham Arabidopsis Stock Centre |
| NSE | Non-SMC element |
| PTM | Post-translational modification |
| RAD | Radiation-sensitive |
| REC8 | Recombination 8 |
| RING | Really interesting new gene |
| SA | Stromalin |
| SCC | Sister chromatid cohesion |
| SGOL1 | Shugoshin-like 1 |
| Siz/PIAS | SAP and mIZ-finger/Protein inhibitor of activated STAT |
| SMC | Structural maintenance of chromosomes |
| SNI1 | Suppressor of NPR1, inducible 1 |

| | |
|---|---|
| STAG | Stromal antigen |
| SUMO | Small ubiquitin-like modifier |
| SYN1 | Synapsin 1 |
| TRAX | Translin-associated factor-X |
| *ttn* | *TITAN* mutant |
| WAPL | Wings apart-like protein homolog |
| WH | Winged-helix |

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# 1 INTRODUCTION

Maintenance of genome integrity is essential for a proper course of the reproductive development, during which substantial changes in chromatin structure and dynamics occur. The key regulators of chromatin structure, dynamics and function are the Structural maintenance of chromosomes (SMC) complexes. One of the three SMC complexes in eukaryotes, the SMC5/6 complex, is involved in various mechanisms responsible for the maintenance of post-replicative chromosome structure. Nevertheless, very little is still known about its functions and involvement in reproductive development, especially in plants.

For the other two SMC complexes, cohesin and condensin, it has already been shown in Arabidopsis several years ago that mutants exhibit an abnormal seed phenotype also known as the *titan* seed phenotype. However, the results of a recent study on the SMC5/6 complex suggest that all three SMC complexes could be involved in seed development (Díaz *et al*., 2019). Therefore, as part of my thesis I focused on further analysis of defects in the reproductive development of SMC5/6 complex mutants in Arabidopsis, specifically of two different mutants of NSE2/HPY2/MMS21 subunit. HPY2 is a SUMO E3 ligase that has already been suggested to play an important role in the development of both the male and the female gametophyte in Arabidopsis (Liu *et al*., 2014).

# 2 AIMS OF THE THESIS

The aim of the thesis was to analyze defects in the reproductive development of SMC5/6 complex mutants. The theoretical introduction will describe the state-of-the-art of this complex and its functions. The experimental part will include:

- Evaluation of seed development
- Analysis of pollen viability using FDA staining assay
- Measurements of pollen area in ImageJ software
- Reciprocal crossing
- Plant genotyping
- Plant ploidy measurements using flow cytometry

# 3 THE CURRENT STATE OF KNOWLEDGE

## 3.1 Organization and stability of the eukaryotic genome

The eukaryotic nuclear genome is organized into linear DNA molecules known as chromosomes. For chromosomal DNA to fit in the cell nucleus and to ensure its different accessibility, DNA is compacted at several levels. Chromatin is a structure consisting of DNA interacting with histone and non-histone proteins. Chromatin folding is a very dynamic process enabling chromatin structural changes during the cell cycle and responses to developmental and environmental stimuli (Li *et al*., 2002).

The primary chromatin unit is called nucleosome which is a cylindric structure of 5.5 nm in height and 11 nm in diameter that is composed of 145–147 bp of DNA wrapped in 1.75 turns of left-handed supercoil around a histone core (Richmond *et al*., 1984). Histones are small basic proteins and one nucleosome is formed by an octamer of two copies of each of the four histone proteins: H2A, H2B, H3 and H4. The unstructured histone N-terminal parts (tails) are protruding out of the nucleosome core and are targets of the post-translational modifications (PTMs) that modulate nucleosome stability and accessibility of the associated DNA. The presence of different histone variants (histones differing somewhat from the canonical variants in their amino acid composition and function) also contributes to nucleosome diversity and dynamic regulation of chromatin functions. Histone PTMs include for example acetylation, methylation, sumoylation, ubiquitylation, phosphorylation, ADP-ribosylation, deimination and proline isomerization. Some of these modifications remain currently unknown in plants. PTMs influence cellular processes such as DNA repair and replication, transcription and chromosome condensation (reviewed in Cutter and Hayes, 2015; Kouzarides, 2007). Another important component of the nucleosome is a linker histone H1 that, compared with the core histones, is less conserved across eukaryotic species.

Specific interactions between nucleosomes lead to the formation of higher-order chromatin structure referred to as 30-nm fiber. For this helical structure, two alternate models are proposed. Whereas the solenoid model predicts a one-start helix in which interactions occur between consecutive nucleosomes (Robinson *et al*., 2006), the zigzag model predicts two-start helix and interactions between alternate nucleosomes (Schalch *et al*., 2005). Chromatin fibers are then folded into distinct functional domains, such as the topologically associated domains, that regulate expression of comprised genes,

the timing of DNA replication and propagation of chromatin state along with the DNA. Finally, the entire chromosomes form distinct chromosome territories that may occupy specific nuclear positions based on their gene and repetitive DNA content and distribution (reviewed in Dixon *et al*., 2016; Sexton and Cavalli, 2015).

## 3.2 The importance of DNA damage repair

All living organisms are constantly exposed to various endogenous and exogenous DNA-damaging factors that challenge genome stability. DNA damages, if not repaired, affect DNA stability, replication, and transcription and thus growth, development, and reproduction. Over time, eukaryotes have developed several mechanisms to detect DNA damage, arrest the cell cycle, repair DNA lesions and/or activate programmed cell death (reviewed in Yoshiyama *et al*., 2013). Specific DNA repair pathways are chosen based on the cell type, its proliferation state, cell cycle stage and the type of lesion (reviewed in Britt, 1999). Although most of the repair pathways are to some extent conserved between the major eukaryotic groups, each of them may have different hierarchy and preference of their use.

## 3.3 From DNA to chromosomes with SMC complexes

The key regulators of chromosome organization, stability, and function are the ring-shaped Structural maintenance of chromosomes (SMC) complexes. The core of the SMC complexes is formed of two SMC proteins and a kleisin protein (Figure 1). SMC proteins comprise intramolecular coiled-coils whose stable dimerization is secured by the hinge at one end. The dimerization of the heads at the other end is ATP-dependent, with the heads responsible for DNA binding activity. The kleisin bridges the heads of the two SMC subunits and closes the ring. Besides that, each complex contains several regulatory subunits. Whereas in most bacteria, only one type of SMC complex is known that is involved in chromosome condensation and segregation (reviewed in Graumann and Knust, 2009; Strunnikov, 2006), in eukaryotes there are at least three such complexes: cohesin, condensin and SMC5/6 complex (reviewed in Uhlmann, 2016).

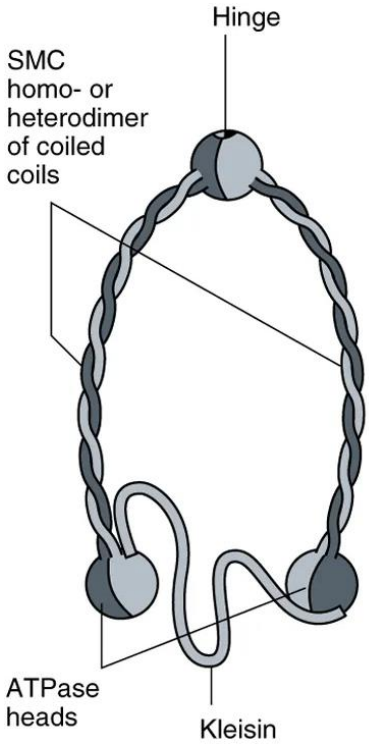


**Figure 1:** General architecture of SMC complexes. SMC complexes contain two SMC proteins, the SMC complex in prokaryotes being a homodimer and in eukaryotes a heterodimer. SMC subunits are connected at the hinge at one end and they carry ATPase heads at the other. Kleisin bridges the heads and completes the ring shape (modified from Eeftens and Dekker, 2017).

The best characterized of the three complexes is cohesin. Cohesin complex includes two SMC proteins (SMC1 and SMC3) and function-dependent alternative kleisin proteins RAD21/SCC1/MCD1 or STAG/SA/SCC3 (Figure 2). Cohesin mediates sister chromatid cohesion and therefore has an impact on meiotic recombination and chromosome segregation during anaphase. During these processes, cohesin associates with several proteins such as Sororin/cell-division-cycle-associated 5 (CDCA5), PDS5A and PDS5B, Wings apart-like protein homolog (WAPL), Shugoshin-like 1 (SGOL1) and Separase (reviewed in Onn *et al*., 2008; Peters *et al*., 2008). It was first shown in yeast that cohesin participates in DNA-damage response (Watrin and Peters, 2006) and DNA repair (Sherwood *et al*., 2010). Cohesin also directly regulates gene expression and development (Dorsett, 2010).

Condensin plays an important role in chromosome organization and compaction. There are two types of condensin: condensin I and condensin II. Both condensin complexes share common heterodimeric backbone, made of SMC2 and SMC4, but differ in the subunits called Condensin associated proteins (CAPs) (Figure 2). It was revealed that condensin II establishes the chromosome axis around which condensin I can arrange

chromatin loops (Green *et al*., 2012). Another study shows that, unlike condensin I, condensin II is required in early prophase. In contrast to condensin II, condensin I is required for complete cohesin dissociation, chromosome shortening and timing of progression through prometaphase and metaphase (Hirota *et al*., 2004). Nevertheless, the different roles of the two condensin complexes in chromosome organization and compaction are still poorly understood.

The third complex is SMC5/6, which is the main object of my thesis. Therefore, its architecture and functions are described in more detail in the following chapter.

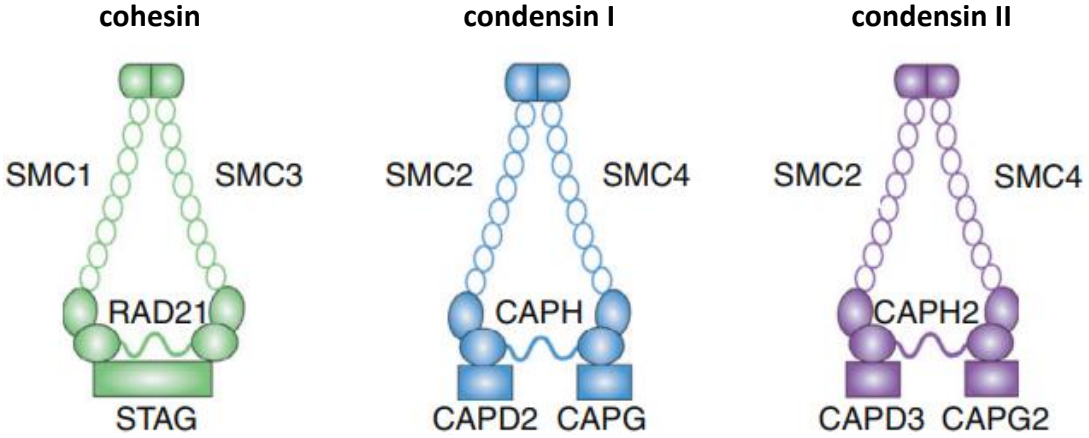


**Figure 2:** Architecture of SMC complexes: cohesin, condensin I and II (modified from Dowen and Young, 2014). **CAP** – Condensin associated protein; **RAD21** – Radiation-sensitive 21; **STAG** – Stromal antigen

## 3.4 The structure and function of SMC5/6 complex

SMC5/6 complex heterodimer is formed by SMC5 and SMC6 proteins. Besides that, this complex contains at least six additional subunits named Non-SMC elements (NSEs) (Figure 3). NSE1 is associated with the SMC heads and contains a "really interesting new gene" (RING)-like domain typical for ubiquitin E3 ligases (Pebernard *et al*., 2008) However, the potential E3 ligase function of NSE1 is still debated. NSE2 is a small ubiquitin-like modifier (SUMO) E3 ligase that mediates sumoylation of several substrates of SMC5/6 complex, including itself (Andrews *et al*., 2005). NSE1 and NSE3 are kleisin interacting tandem winged-helix (WH) elements (KITE) proteins (Palecek and Gruber, 2015). NSE4 is a member of a kleisin family that also contains a WH motif in its structure (Palecek *et al*., 2006). Whereas the aforementioned subunits are evolutionarily conserved in eukaryotes, several subunits (sometimes popularly named as the NSE5 and NSE6) are unique in the major evolutionary branches. Yeast NSE5 and NSE6 comprise helical HEAT repeats that allow dynamic protein-protein interactions (Yoshimura and Hirano,

2016). They were proposed to be involved in loading SMC5/6 complex to the sites of DNA damage. Three subcomplexes are sometimes distinguished within the SMC5/6 complex: the SMC5-SMC6-NSE2 subcomplex, the NSE1-NSE3-NSE4 subcomplex and the NSE5-NSE6 subcomplex.

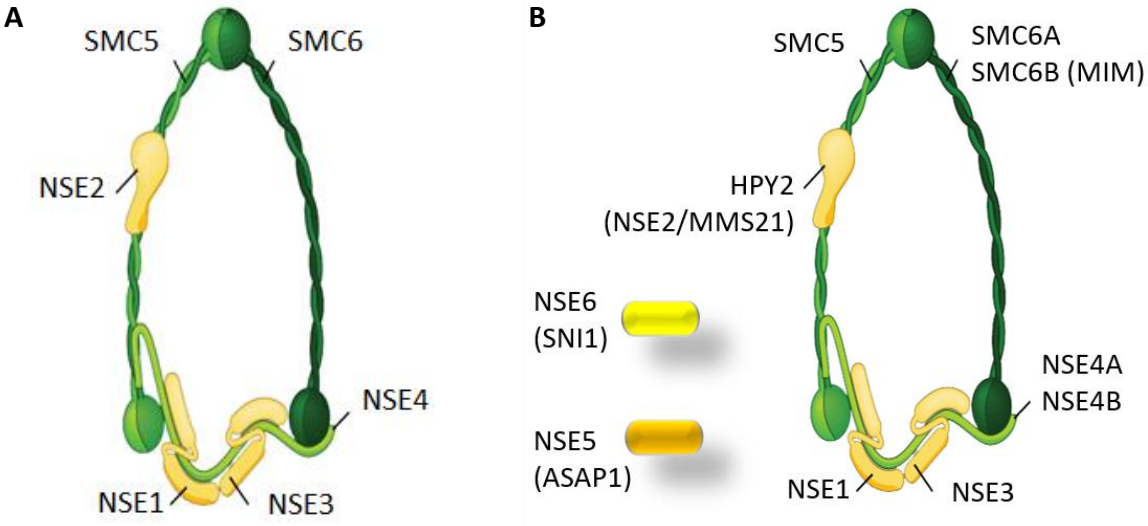


**Figure 3:** Architecture of SMC5/6 complex **A)** without species-specific positions of NSE5 and NSE6 **B)** in *Arabidopsis thaliana*, where the locations of ASAP1 and SNI1 (putative functional orthologs of yeast NSE5 and NSE6) remain unknown (modified from Haering and Gruber, 2016). Arabidopsis specific names: **ASAP1** – ARABIDOPSIS SNI ASSOCIATED PROTEIN 1; **HPY2** – HIGH PLOIDY 2; **MMS21** – METHYL METHANESULFONATE-SENSITIVE PROTEIN 21, **MIM** – HYPERSENSITIVE TO MMS, IRRADIATION AND MMC, **SNI1** – SUPPRESSOR OF NPR1, INDUCIBLE 1

### 3.4.1 The SMC5-SMC6-NSE2 subcomplex

The SMC5-SMC6-NSE2 trimer represents the core of the SMC5/6 complex. NSE2 is stably bound to the coiled-coil segment of SMC5 subunit and, as previously mentioned, it is a SUMO E3 ligase. SUMO is a small ubiquitin-like protein that is attached to target proteins during the process of sumoylation. Sumoylation is a reversible post-translational modification in eukaryotes that affects a protein's structure and subcellular localization and is involved in a variety of different cellular processes. What cellular processes NSE2 participates in is still largely unknown. Already identified targets of NSE2 include SMC6, NSE3 and NSE4 in fission yeast (Andrews *et al.*, 2005); SMC5 and KU70 in budding yeast (Zhao and Blobel, 2005); cohesin subunits SA2 and SCC1 (McAleenan *et al.*, 2012); Translin-associated factor-X (TRAX) (Potts and Yu, 2005) and some components of the telosome/shelterin complex in humans (Potts and Yu, 2007). NSE2 is also capable of auto-sumoylation, which means it probably regulates its own activity. It was shown in humans that NSE2 is required for DNA repair (Potts and Yu, 2005) and the timing of the Topo2a-dependent arrest in G2 (Deiss *et al.*, 2019). Moreover, it was recently

discovered that the sumoylation of SMC5 by NSE2 was upregulated by replication fork damage and involved in a bypass of DNA lesions (Zapatka *et al*., 2019). In Arabidopsis, NSE4 was the only identified target of NSE2, although this could be caused largely by the DNA damage-free experimental conditions (Rytz *et al*., 2018).

NON-SMC ELEMENT 2/HIGH PLOIDY 2/METHYL METHANESULFONATE-SENSITIVE PROTEIN 21 (NSE2/HPY2/MMS21) in Arabidopsis contains Protein inhibitor of activated STAT (Siz/PIAS) RING domain that has proved to be crucial for its SUMO E3 ligase activity (Huang *et al*., 2009; Ishida *et al*., 2009; Zhao and Blobel, 2005). Although NSE2/HPY2/MMS21 is not essential for Arabidopsis, the mutant has a strong developmental phenotype including small growth, stem fasciations, leaf deformations, partial sterility and reduced number of seeds that may not develop properly (Liu *et al*., 2014). Arabidopsis genome also contains two SMC6 homologs: SMC6A and SMC6B (Watanabe *et al*., 2009).

### 3.4.2 The NSE1-NSE3-NSE4 subcomplex

This highly conserved subcomplex is located at the head region of the SMC5/6 complex and it is essential for the regulation of DNA binding activity and bridging the SMC heads. The RING-like motif of NSE1 enables the formation of NSE1-NSE3-NSE4 trimer, ensures the integrity of the whole complex and its DNA repair activity (Pebernard *et al*., 2008). NSE3 is homologous to the Melanoma antigen gene (MAGE) family, which is divided into two groups. Whereas type I MAGE genes are usually overexpressed in different types of human cancers, type II MAGEs are expressed in normal tissues. Several members of the MAGE family can associate with NSE1, but only the human MAGE-G1 (type II MAGE and NSE3 homolog) was confirmed to interact with the SMC5/6 holocomplex and to stimulate NSE1 ubiquitin ligase activity (Feng *et al*., 2011; Doyle *et al*., 2010;). In Arabidopsis, only a single NSE3 homolog is found. NSE4 was found to be responsible for bridging the SMC heads, the interaction of subunits within the subcomplex and interaction with SMC5. Arabidopsis genome contains two NSE4 paralogs, NSE4A and NSE4B, that vary in function (Díaz *et al*., 2019; Duan *et al*., 2009; Palecek *et al*., 2006).

### 3.4.3 The NSE5-NSE6 subcomplex

The NSE5-NSE6 subcomplex represents the non-conserved part of the SMC5/6 complex. Its position within the complex seems to be species-specific. Whereas in budding yeast this subcomplex binds to the hinge (Duan *et al*., 2009), in fission yeast it binds to the heads at the base of the SMC5/6 complex (Palecek *et al*., 2006). The ARABIDOPSIS SNI ASSOCIATED PROTEIN 1 (ASAP1) and SUPPRESSOR OF NPR1, INDUCIBLE 1 (SNI1) were identified as the missing parts of the SMC5/6 complex and putative functional homologs of NSE5 and NSE6 in Arabidopsis. However, their exact location within the SMC5/6 complex is still unknown. Interestingly, it was reported that both NSE5 and NSE6 were not essential in fission yeast (Sergeant *et al*., 2005) but in budding yeast and Arabidopsis are required for their viability (Yan *et al*., 2013; Zhao and Blobel, 2005). Whereas the homozygous *ASAP1* mutant dies in an early stage of development (Yan *et al*., 2013), the *SNI1* mutant is characterized by a small growth and reduced fertility in Arabidopsis. Despite its unresolved functional role, the NSE5-NSE6 subcomplex is most likely involved in a process of SMC5/6 complex localization and/or multimerization.

## 3.5 Plant sexual reproduction

Sexual reproduction, compared with asexual, represents an evolutionarily advanced way that ensures diversity within the species, allows the organisms to evolve and thus increases their chance of survival in the changing environment. Flowering plants (angiosperms) make up about 90 % of all living plant species and can reproduce in both ways, sexually and asexually (Paton *et al*., 2008). Sexual reproduction in angiosperms begins with the production of male and female gametes by meiosis.

### 3.5.1 Gametogenesis in flowering plants

Meiosis is a conserved type of cell division that reduces ploidy through the segregation of homologous chromosomes and creates new genetic combinations of parental genomes. For the proper course of meiosis, the cohesion of sister chromatids is crucial. The sister chromatids are associated from DNA replication in S phase until anaphase II, with the association mediated by the cohesin complex. A mutation of SYN1/DIF1/AtREC8, the Arabidopsis RAD21 homolog, affects both male and female fertility (Bai *et al*., 1999; Bhatt *et al*., 1999). It gives rise to chromosome alterations

that lead to the formation of the meiotic products of different size and shape, and of variable ploidy.

Chromosome pairing and synapsis is essential for proper separation of homologous chromosomes at anaphase I. The synaptonemal complex, that is evolutionarily conserved in plants and animals, promotes chromosome synapsis and homologous recombination in prophase I. Homologous recombination is the exchange of genetic material between paired homologs in which double-strand breaks (DSBs) are deliberately produced and subsequently repaired. The resulting crossovers are necessary for maintaining homolog interactions until anaphase I (reviewed in Gray and Cohen, 2016). For example, deficiency of AtRAD50 and AtMRE11 resulted in chromosome fragmentation, increased sensitivity to DNA damage and reduced fertility (Gallego *et al*., 2001; Puizina *et al*., 2004).

Unlike in animals, in plants, the meiotic products undergo several rounds of mitosis to form multi-cellular haploid gametophyte. The correct progression and regulation of meiotic and mitotic cycles is required for proper gametophyte development. Mutants involved in meiotic cycle progression and regulation are sporophytic mutants that, only if they are homozygous recessive, exhibit reduced male and/or female fertility. Mutants involved in mitotic cycle regulation affect male and/or female gametophyte development (reviewed in Liu and Qu, 2008).

The male gamete is called a sperm cell and it is produced in the stamen (Figure 4). The part of the stamen known as the anther contains diploid microsporocyte that undergoes meiosis to form four haploid microspores. Each of the microspores then undergoes asymmetric mitotic cell division to form bi-cellular pollen composed of a larger vegetative cell and a smaller generative cell. The generative cell divides one more time to form two sperm cells. The vegetative cell plays a key role in pollen tube formation and sperm cell delivery. The tri-cellular pollen grain is also termed the male gametophyte (reviewed in McCormick, 1993).

The female gamete is called an egg cell. The egg cell is located in an ovule, the ovule being contained in the ovary at the base of the pistil (Figure 4). First, the diploid megasporocyte produced inside the nucleus of the ovule undergoes meiosis to create four haploid megaspores of which three usually degenerate, then the nucleus of the remaining megaspore divides three times by mitosis to form an eight-nucleate embryo sac,

also called the female gametophyte, with four nuclei at the micropylar pole and four nuclei at the chalazal pole. In the next step, the nuclei are cellularized, forming three antipodals in the chalazal region and two synergids and the egg cell in the micropylar region. The two remaining polar nuclei fuse in the central part of the embryo sac into the diploid nucleus that will, upon fertilization, give rise to the triploid endosperm (reviewed in Yang and Sundaresan, 2000).

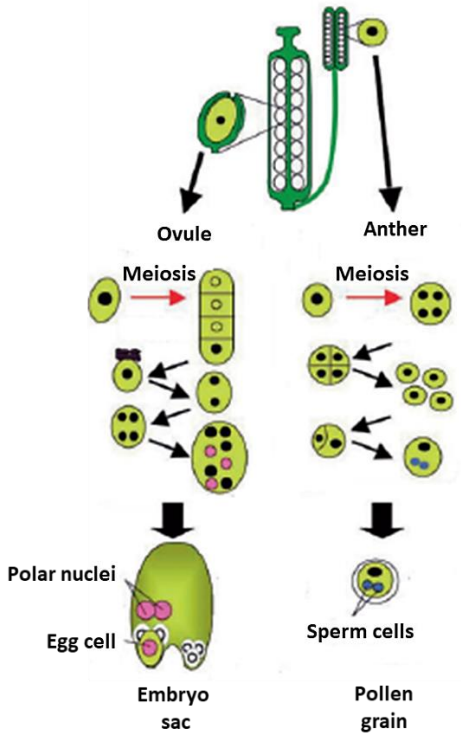


**Figure 4:** Gametogenesis in flowering plants. The female gamete is called an egg cell which is contained in the embryo sac. The embryo sac is also referred to as the female gametophyte. The male gamete is called a sperm cell which is contained in the pollen grain, also known as the male gametophyte (modified from Scott and Spielman, 2006).

### 3.5.2 Double fertilization in flowering plants

Fertilization is preceded by a pollination process in which mature pollen grain is transferred to the stigma of a flower. Compatible pollen grain then hydrates and germinates the pollen tube that grows through the pistil until it reaches the ovule. Double fertilization, characteristic of flowering plants, is ensured by two sperm cells contained in the pollen tube (Figure 5). One of the sperm cells fuses with the haploid egg cell to form a zygote which further develops into an embryo. The other sperm cell fuses with the diploid nucleus of the embryo sac to form a triploid nutritive tissue endosperm. After fertilization, the ovule develops into a seed and the ovary surrounding the ovules develops into a fruit (reviewed in Pereira and Coimbra, 2019).

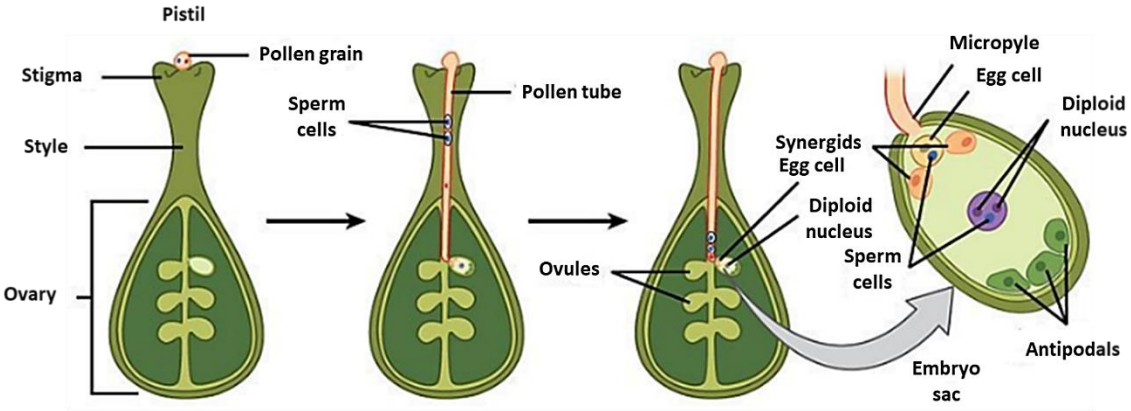


**Figure 5:** Double fertilization in flowering plants. Once a pollen grain lands on the stigma of a flower, it germinates the pollen tube that grows through the pistil until it reaches an ovule. The pollen tube penetrates an opening in the ovule called a micropyle. One of the released sperm cells fertilizes an egg cell to form a diploid zygote which further develops into an embryo. The second sperm cell fertilizes the diploid nucleus of an embryo sac to form a triploid endosperm that later becomes a nutrition source for the growing embryo (modified from Panawala, 2017).

### 3.5.3 The role of SMC complexes in plant reproduction

Maintaining genome integrity is essential for the proper course of reproductive development, during which dramatic changes in chromatin structure and dynamics occur. These changes influence replication, transcription and progression through meiotic and mitotic cell cycles. Therefore, germ cell development is strictly regulated. Defects resulting from misregulation can lead to infertility or incorrect gametophyte development. The key regulators of chromatin structure, stability and function are the SMC complexes.

Meiosis and mitosis are essential processes of gametophyte development. Cohesin maintains the association of sister chromatids established during replication until anaphase. Premature disruption of the association can cause aneuploidy, chromosomal translocations, and defects in DNA repair. Condensin is required for proper chromosome condensation and separation throughout meiosis, mitosis and embryo development (Schubert, 2009; Siddiqui *et al*., 2003).

SMC5/6 complex is involved in various mechanisms responsible for the maintenance of post-replicative chromosome structure, partially through PTMs of target proteins and also through establishing DNA-damage dependent cohesion. Together with cohesin, the SMC5/6 complex participates in DNA replication and homologous recombination. During DNA replication, the SMC5/6 complex is involved in stabilization and restart of replication fork in case of topological stress occurrence. Also, it helps to reduce

superhelical tension generated in the course of replication (reviewed in Palecek, 2019). SMC5/6 complex also participates in DSB repair by promoting sister chromatid alignment and homologous recombination (Watanabe *et al*., 2009). Not only that SMC5/6 complex maintains genome stability by promoting homologous recombination, it was also found to prevent aberrant recombination between repetitive DNA sequences (reviewed in Potts, 2009).

My host laboratory recently found that the *NSE4A* subunit, one of the two *NSE4* paralogs in Arabidopsis, is essential for plant fertility (Díaz *et al*., 2019). Partial loss of NSE4A function leads to abnormal seed phenotype including noncellularized endosperm and embryos that do not develop beyond the torpedo stage. For NSE2/HPY2/MMS21 in Arabidopsis it was already suggested that it has an important role during both male and female gametophyte development (Liu *et al*., 2014). Therefore, I followed up on these studies and focused on further analysis of the defects in the reproductive development of *hpy2* mutant plants.

# 4 MATERIAL AND METHODS

## 4.1 Biological material

### Plants

Two different mutants of *HPY2* (Figure 6) were used to analyze ploidy and reproductive development in Arabidopsis. *hpy2-1* is ethyl methanesulfonate (EMS) mutant allele that was isolated in the laboratory of Prof. Keiko Sugimoto, RIKEN Center for Sustainable Resource Science, Japan. It is a single nucleotide substitution from cytosine to thymine in the fourth exon, resulting in premature translational stop codon. *hpy2-2* is a T-DNA insertion mutant (SAIL 77 G06) ordered from the Nottingham Arabidopsis Stock Centre (NASC). Arabidopsis (Columbia accession) wild-type was also ordered from NASC.

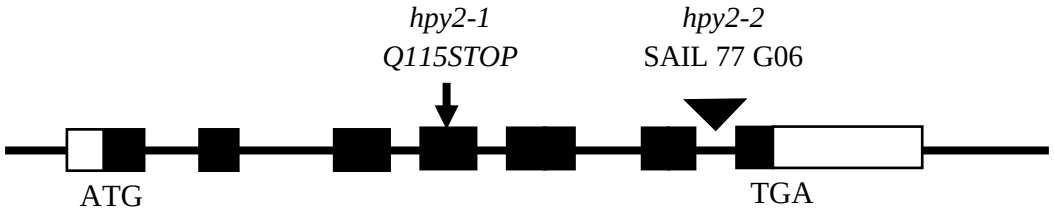


**Figure 6:** The gene model of Arabidopsis *HPY2*. White boxes represent untranslated regions and the black ones represent exons. Introns are shown as lines. The arrow points to the site of single nucleotide substitution of the *hpy2-1* mutant allele. The arrowhead points to the site of T-DNA insertion of the *hpy2-2* mutant allele.

### Growth conditions

For cultivation in soil, seeds were put on the surface of moist soil, stratified for two days at 4 °C in dark, then the pots were moved to the air-conditioned chamber with controlled long-day conditions (16 h light / 8 h dark cycle, 21 °C day and 19 °C night temperature). For *in vitro* growth, Arabidopsis seeds were surface sterilized (70% ethanol with 0.5% TritonX-100 v/v) for 10 min and washed three times with sterile water. Dried seeds were sown on ½MS agar medium (Murashige and Skoog), stratified in dark for two days at 4 °C and then cultivated in the climatic chamber (Percival) under 16 h light / 8 h dark cycle, 21 °C day and 19 °C night temperature.

## 4.2 List of used chemicals and solutions

### Chemicals

- Acetic acid 99% p.a. (Lach-ner, cat. no. 10047-A99)
- Acetone (VWR, cat. no 67-64-1)
- Agar (HiMedia, cat. no. RM201)
- $Ca(NO_3)_2 \cdot 4H_2O$ (Lachema)
- Citric acid (Lachema)
- Colchicine (Sigma-Aldrich, cat. no. C3915)
- DAPI stock solution (Sigma-Aldrich, cat. no. D9542)
- DNeasy Plant Mini Kit (Qiagen, cat. no. 69104)
- Dream*Taq* Hot Start DNA Polymerase (cat. no. EP1703)
- EDTA (ethylenediaminetetraacetic acid) (Sigma-Aldrich, cat. no. E5134)
- Ethylalcohol 96% (Lach-ner, cat. no. 20025-A96)
- FDA (fluorescein diacetate) (Sigma-Aldrich, cat. no. F7378)
- GeneRuler 1 kb Plus DNA Ladder, ready-to-use (Thermo Fisher Scientific, cat. no. SM1333)
- Isopropanol (Lach-ner, cat. no. 67-63-0)
- $KNO_3$ (Lachema)
- KOH p.a. (Lach-ner, cat. no. 10003-AP3)
- MES monohydrate (Duchefa Biochemie, cat. no. M1503)
- $MgSO_4 \cdot 7H_2O$ p.a. (Lach-ner, cat. no. 30807)
- MOPS (3-(N-morpholino)propanesulfonic acid) (Sigma-Aldrich, cat. no. M1254)
- MS mixture (Duchefa Biochemie, cat. no. M0231)
- $Na_2HPO_4 \cdot 12H_2O$ (Lach-ner, cat. no. 30061-AP0)
- NaCl p.a. (Lach-ner, cat. no. 30423)
- NaOH p.a. (Lach-ner, cat. no. 10006-AP2)
- PCR agarose (Top-Bio, cat. no. P045)
- PCR ethidium bromide (Top-Bio, cat. no. P047)
- Q5 High-Fidelity DNA Polymerase (BioLabs, cat. no. M0491)
- SDS (sodium dodecyl sulfate) (Duchefa, cat. no. 151-21-3)
- Sucrose p.a. (Lach-ner, cat. no. 40135-AP0)
- Tris (Sigma-Aldrich, cat. no. T1503)

- Triton X-100 (Sigma-Aldrich, cat. no. X100)
- Tween 20 (Sigma-Aldrich, cat. no. P2287)

**Solutions and their preparation**

**½MS medium** (pH 5.8): 2.2 g MS mixture, 10 g sucrose, 1 g MES monohydrate. Dissolve in 800 ml of distilled water and adjust pH with KOH. Make up to 1000 ml by adding distilled water and sterilize by autoclaving. To prepare a solid medium with 0.6% agar (w/v), add 6 g of agar per 1000 ml of medium.

**1x TAE buffer**: Mix 20 ml of 50x TAE buffer and make up to 1000 ml by adding distilled water.

**50x TAE buffer**: Dissolve 242 g of Tris in 800 ml of distilled water, add 57.1 ml of 99% acetic acid and 100 ml of 0.5 $mol \cdot l^{-1}$ EDTA (pH 8,5). Make up to 1000 ml by adding distilled water.

**BK buffer S15 MOPS** (pH 7.5): 0.127 $mmol \cdot l^{-1}$ $Ca(NO_3)_2 \cdot 4H_2O$, 0.081 $mmol \cdot l^{-1}$ $MgSO_4 \cdot 7H_2O$, 0.1 $mmol \cdot l^{-1}$ $KNO_3$, 15% sucrose (w/v), 10 $mmol \cdot l^{-1}$ MOPS (pH 7.5). Store at -20 °C.

**DAPI stock solution**: 0.1 $mg \cdot ml^{-1}$ DAPI. Filter through a 0.22-µm filter and store at - 20 °C in 1 ml aliquots.

**Edwards buffer**: 200 $mmol \cdot l^{-1}$ Tris (pH 7.5), 250 $mmol \cdot l^{-1}$ NaCl, 25 $mmol \cdot l^{-1}$ EDTA, 0.5% SDS (w/v).

**FDA stock solution**: 2 $mg \cdot ml^{-1}$ in acetone.

**FDA-buffer mixture**: 1 µl FDA stock solution, 1 ml BK buffer S15 MOPS.

**Otto I solution**: 0.1 $mol \cdot l^{-1}$ citric acid, 0.5% Tween 20 (v/v). Filter through a 0.22-µm filter and store at 4 °C.

**Otto II solution**: 0.4 $mol \cdot l^{-1}$ $Na_2HPO_4 \cdot 12H_2O$. Filter through a 0.22-µm filter and store at room temperature (18–25 °C). Dissolving can be speeded up by heating. Fluorochrome can be added before final volume adjustment. In that case, the solution should be stored in darkness.

## 4.3 List of equipment

- Analytical scale A 200S (Sartorius)
- Centrifuge 5415 D (Eppendorf)
- Centrifuge Jouan BR4i, fixed angle rotor AM 50C.13 (Thermo Fisher Scientific)
- Centrifuge MiniStar Silverline (VWR)
- Centrifuge myFuge Mini (Benchmark Scientific)
- Flow cytometer CyFlow Space (Sysmex), FloMax software (Partec)
- Horizontal gel electrophoresis system (Bio-Rad)
- Image processing and analysis – Adobe Photoshop CS5 (Adobe) and ImageJ
- Laboratory fume hood M 1800 (Merci)
- Laminar box MSC 1.2 Advantage (Thermo Fisher Scientific)
- Magnetic stirrer VS-C7 (VWR)
- Microscope Olympus, type IX81 inverted, equipped with a DSU (Disc Scanning Unit) and digital monochromatic CCD camera (CCD-ORCA⁄ ER), CellR software (Olympus)
- NanoDrop ND-1000 Spectrophotometer, ND-1000 V3.8.1 software (Thermo Fisher Scientific)
- pH meter InoLab pH 7110 (WTW)
- Scale Scout SC4010 (Ohaus)
- Sequence data analysis – Geneious software
- Shaker VV3 (VWR)
- Thermocycler C1000 Touch (Bio-Rad)
- Transilluminator InGenius LHR, GeneSnap software (Syngene)

## 4.4 Methods

### 4.4.1 Staining with fluorescein diacetate

One way to distinguish living cells from dead ones is by detecting the functional metabolism of living cells. For this purpose, fluorescein diacetate (FDA) was chosen, whose metabolic product is used as an indicator of metabolic activity and indirectly as a marker of cell viability. FDA is a non-fluorescent and non-polar molecule that readily crosses the cell membrane. In living cells, FDA is hydrolyzed to fluorescein, which is highly polar and therefore accumulates in the cell. It is also a fluorescent molecule that emits green fluorescence when excited by light at 488 nm wavelength.

Analysis of pollen viability using FDA staining was performed according to the protocol published by Xiyan Li in 2011.

First, about 1 ml of opened flowers was collected into a 15 ml falcon tube containing 3 ml BK buffer S15 MOPS, and the sample was 5 min vortexed to release pollen. Then, the tube was centrifuged (2600 g, 5 min) and the supernatant was removed. In the end, the pollen pellet was re-suspended in 20 µl FDA-buffer mixture and the suspension was transferred to a microscopic slide and covered with a coverslip. The fluorescence was observed after 10 min of staining using an inverted microscope IX81 (Olympus) at 488 nm excitation and 510 nm emission wavelengths.

### 4.4.2 Polyploidization of Arabidopsis seedlings

To prepare tetraploid wild-type plants for pollen area measurement, the polyploidization of Arabidopsis seedlings was performed.

The seedlings were grown *in vitro* for two weeks. Then, the seedlings were sunk in the solution of 0.1% Colchicine in tap water and were kept in dark for 2 h. Colchicine solution was poured away and seedlings were washed with copious amounts of tap water. Subsequently, the seedlings were transferred to soil. To select polyploids, the larger seeds were selected and grown in soil. Ploidy was analyzed using flow cytometry (FCM).

### 4.4.3 Ploidy measurements using flow cytometry

FCM is a method used to analyze optical properties such as fluorescence intensity of microscopic particles in a suspension. These particles are individually measured at high speed. FCM is used in a wide range of applications, with ploidy analysis being one of the most common in plants.

Whether the ploidy level was affected in mutants was examined using FCM. Sample preparation was performed according to the protocol published by Doležel *et al*., 2007.

A small amount of plant tissue (1–2 young leaves) was placed in the center of a plastic Petri dish. Then 0,5 ml of ice-cold Otto I solution was added and the tissue was immediately chopped in the buffer with a sharp razor blade into very fine slices. The homogenate was mixed by pipetting up and down several times. In the next step, the homogenate was filtered through a 42-µm nylon mesh into a labeled sample tube and left approximately 1 min at room temperature. Then 1 ml of Otto II solution, which contained DAPI fluorochrome (4 $\mu g \cdot ml^{-1}$), was added to the nuclear suspension. The sample was gently shaken and nuclear DNA content was subsequently analyzed using flow cytometer CyFlow Space (Sysmex). First, the relative fluorescence of G0/G1 nuclei from a reference plant with known ploidy was set on channel 100. Then, samples of unknown ploidy were analyzed. The fluorescence of 3–5000 nuclei was analyzed during each measurement.

### 4.4.4 Genotyping

After the reciprocal crossing of wild-type with mutant, all offspring had to be genotyped to confirm that there was no self-pollination and they were all heterozygotes. For detection of the *hpy2-2* mutant allele, PCR-based genotyping using Dream*Taq* Hot Start DNA Polymerase was performed. Template DNA for PCR was isolated according to the protocol published by Edwards *et al*., 1991.

A small piece of leaf was collected into a 1.5 ml tube. Then, 100 µl of Edwards buffer were added and the leaf was homogenized using a pestle. The sample was centrifuged (2250 g, 20 min) and 80 µl of supernatant were transferred into the new tube. Subsequently, 80 µl of isopropanol was added and tube content was mixed by inverting 3–4 times. The sample was centrifuged (2250 g, 10 min), the supernatant was removed and 80 µl of 70% ethanol were added. After that, the sample was centrifuged one more

time (2250g, 10 min), the supernatant was removed and the excess of ethanol was drained by keeping the tube upside down on a paper towel for approximately 5 min. In the next step, the tube was turned upright and dried at room temperature (incubation at 55 °C speeds up drying). Finally, 50 µl of nuclease-free water were added and the sample was gently vortexed.

Components of the PCR (final concentration): 1x Dream*Taq* Green Buffer, 200 $\mu mol \cdot l^{-1}$ dNTP mix, 0.5 $\mu mol \cdot l^{-1}$ forward primer, 0.5 $\mu mol \cdot l^{-1}$ reverse primer, template DNA (<1 µg), 0.025 $U \cdot \mu l^{-1}$ Dream*Taq* Hot Start DNA Polymerase, nuclease-free water. PCR conditions are given in Table 1.

**Table 1:** PCR conditions for genotyping (Dream*Taq* Hot Start DNA Polymerase).

| Process | Temperature [°C] | Time | Number of cycles |
|---|---|---|---|
| Initial denaturation | 95 | 60 s | 1 |
| PCR amplification | 95 | 30 s | 33 |
| | 58 | 30 s | |
| | 72 | 60 s | |
| Final elongation | 72 | 5 min | 1 |

The PCR products were then separated by horizontal electrophoresis in a gel prepared from 1% (w/v) agarose, 1x TAE buffer and 0.5 $\mu g \cdot ml^{-1}$ ethidium bromide. Electrophoresis was performed at 80 V at room temperature. For approximate DNA fragment size determination, GeneRuler 1kb Plus DNA Ladder was used. The obtained PCR products were visualized under UV light with a wavelength of 360 nm.

Primers for detection of the wild-type allele:

forward 5'-TTAGCAGATCCAGTTCGCAGG-3'

reverse 5'-TTGTTCAACGAGCGCATCTC-3'.

Primers for detection of the *hpy2-2* mutant allele:

forward 5'-TTAGCAGATCCAGTTCGCAGG-3'

reverse 5'-TCGAGTACAGGGACTGGGAA-3'

For detection of the *hpy2-1* mutant allele, Sanger sequencing was performed. First, template DNA was isolated using DNeasy Plant Mini Kit according to the instructions of the manufacturer. Then, PCR using Q5 High-Fidelity DNA Polymerase was performed. DNA fragments amplified by PCR were subsequently sent to SEQme (https://www.seqme.eu/en/) for sequencing. Obtained sequences were compared to wild-type sequence using Geneious software.

Components of the PCR (final concentration): 1x Q5 reaction buffer, 200 $\mu mol \cdot l^{-1}$ dNTP mix, 0.5 $\mu mol \cdot l^{-1}$ forward primer, 0.5 $\mu mol \cdot l^{-1}$ reverse primer, template DNA (<1 µg), 0.02 $U \cdot \mu l^{-1}$ Q5 High-Fidelity DNA Polymerase, nuclease-free water. PCR conditions are given in Table 2.

**Table 2:** PCR conditions for amplification of the *hpy2* allele (Q5 High-Fidelity DNA Polymerase).

| Process | Temperature [°C] | Time | Number of cycles |
|---|---|---|---|
| Initial denaturation | 98 | 30 s | 1 |
| PCR amplification | 98 | 10 s | 36 |
| | 58 | 30 s | |
| | 72 | 30 s | |
| Final elongation | 72 | 2 min | 1 |

Primers for detection of the *hpy2-1* mutant allele:

forward 5'-CAAAGCTCAAGGCTACACCTTCCTCAG-3'

reverse 5'-AACTGGCGCATCAAATGATC-3'

# 5 RESULTS

## 5.1 *hpy2* causes paternally inherited abnormal seed phenotype

The *hpy2* mutant plants have a strong developmental phenotype in Arabidopsis. Both *hpy2-1* and *hpy2-2* are characterized by small growth, shorter root and shorter siliques (Figure 7).

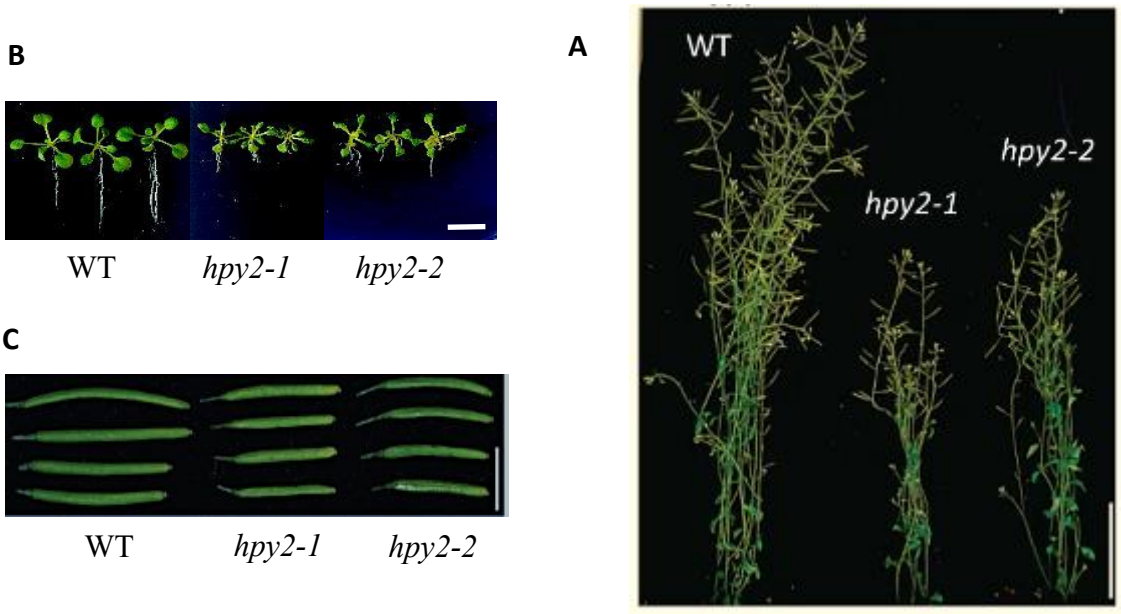


**Figure 7:** Developmental phenotypes of *hpy2* plants. **A)** 4-week-old plants, Bar = 1 cm **B)** seedlings, Bar = 0.5 cm **C)** siliques, Bar = 7 cm.

The *hpy2* plants also produce aborted ovules and abnormal seeds (Figure 8, Table 3). The abnormal seeds are either white or brown and at later stages also shrunken. Furthermore, the phenotype of the seeds from the reciprocal crossing was analyzed (Figure 9, Table 3). The results showed that aborted ovules were produced no matter whether *hpy2* mutation was inherited from the maternal or the paternal plant. Interestingly, the abnormal seed phenotype was paternally inherited. It indicated that *hpy2* produces pollen which subsequently affect seed development.

**Table 3:** The proportion of aborted ovules and abnormal seeds produced by *hpy2* plants after self-pollination and after reciprocal crossing with wild-type.

| ♀ | ♂ | **Number of analyzed seeds** | **Normal seeds [%]** | **Aborted ovules [%]** | **Abnormal seeds [%]** |
|---|---|---|---|---|---|
| WT | WT | 1424 | 94.4 | 2.8 | 2.8 |
| *hpy2-1* | *hpy2-1* | 1343 | 34.7** | 49.2** | 16.1** |
| *hpy2-2* | *hpy2-2* | 1253 | 30.8** | 45.8** | 23.4** |
| *hpy2-1* | WT | 919 | 37.3** | 60.7** | 2.0 |
| *hpy2-2* | WT | 629 | 38.1** | 58.2** | 3.7 |
| WT | *hpy2-1* | 1369 | 56.5** | 29.1* | 14.4** |
| WT | *hpy2-2* | 783 | 36.9** | 41.6** | 21.5** |

**Significantly different from wild-type (* P<0.05, ** P<0.01).

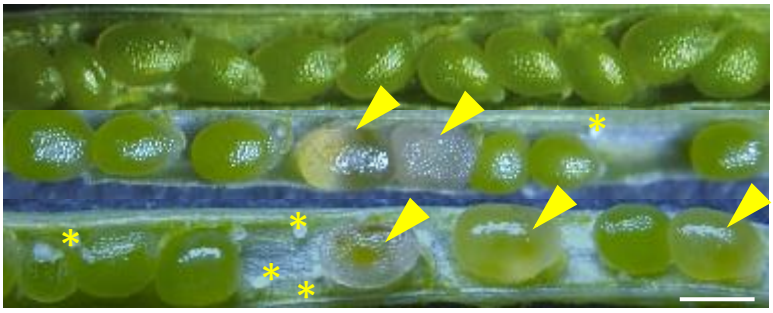


**Figure 8:** Seed development of *hpy2* plants. Asterisks point to aborted ovules and arrowheads point to abnormal seeds. Bar = 0.5 mm.

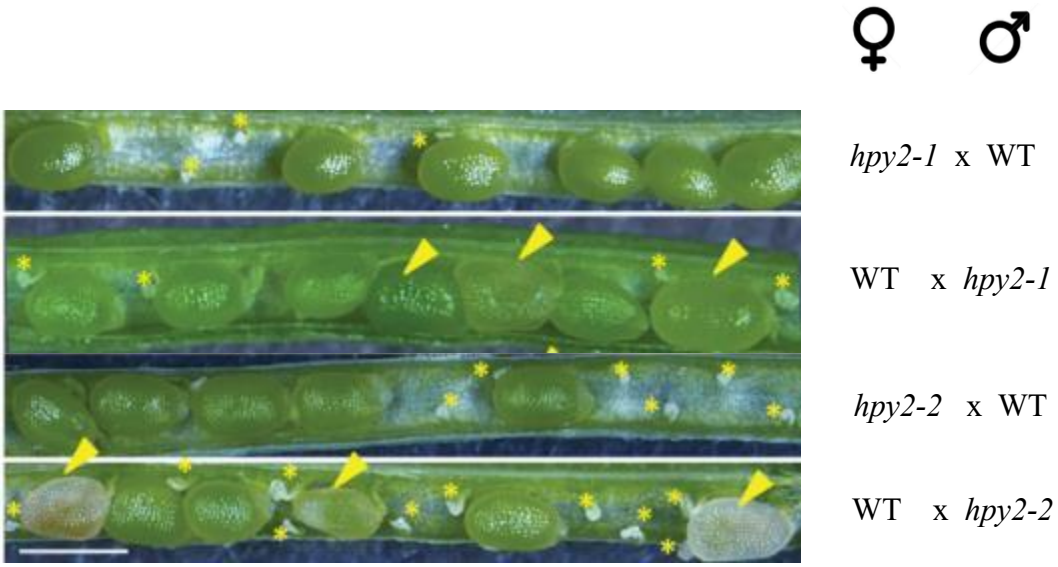


**Figure 9:** Seed development after the reciprocal crossing of *hpy2* plants with wild-type. Asterisks point to aborted ovules and arrowheads point to abnormal seeds. Bar = 0.4 mm.

## 5.2 Pollen viability of *hpy2* is reduced

To check whether the pollen development is affected in *hpy2*, the viability of pollen was tested first. Here, FDA staining was used to distinguish viable pollen grains from the dead ones. Compared to wild-type, the *hpy2* plants showed reduced pollen viability (Figure 10, Table 4). Besides, we noticed that pollen grains of *hpy2* vary considerably in size and therefore pollen area was subsequently measured.

**Table 4:** Viability of *hpy2* pollen determined with the use of FDA.

| | **Number of analyzed pollen** | **Viability [%]** |
|---|---|---|
| **WT** | 1504 | 83.4 |
| ***hpy2-1*** | 503 | 63.6* |
| ***hpy2-2*** | 609 | 52.4** |

**Significantly different from wild-type (* P<0.05, ** P<0.01).

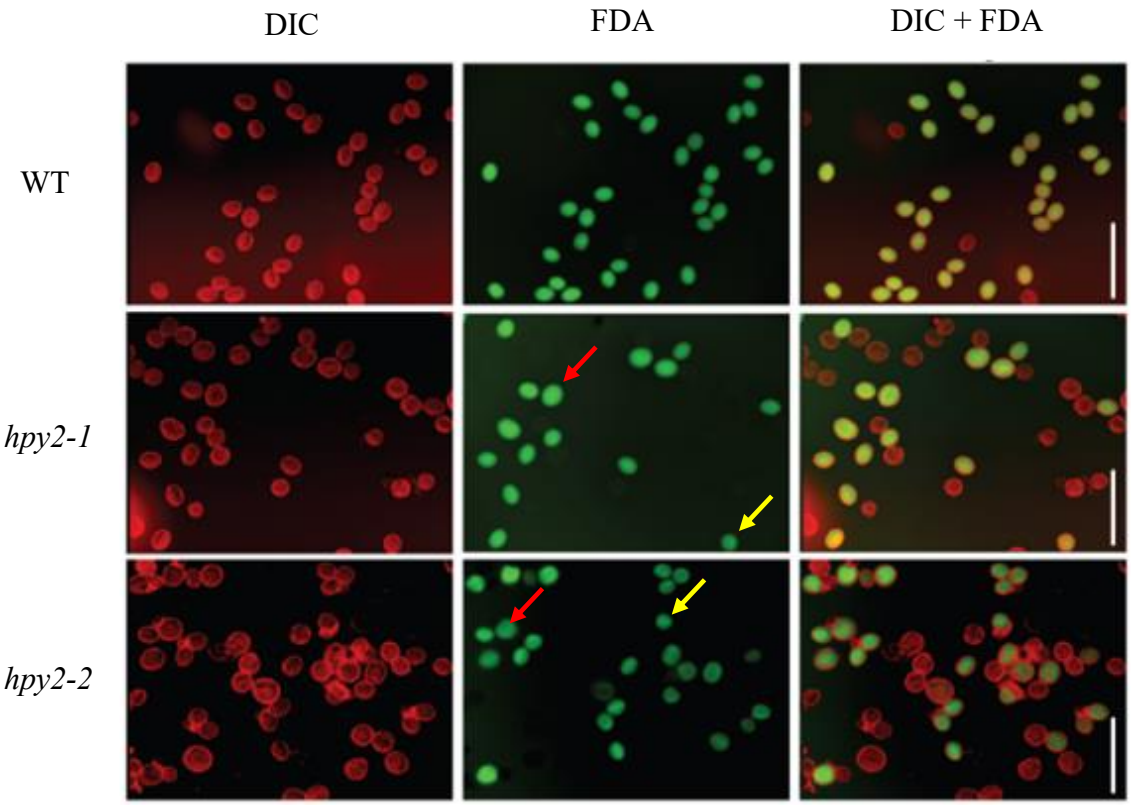


**Figure 10:** Analysis of pollen viability of *hpy2* using FDA staining. Yellow arrows point to normally sized pollen. Red arrows point to larger pollen whose ploidy level could be affected. Originally black/white DIC images were pseudocolored as red. Pseudocolored DIC images and merged DIC + FDA images were created in Adobe Photoshop CS5 (Adobe). Bar = 100 µm

## 5.3 HPY2 mutants produce two cohorts of differently sized pollen

DIC images from the viability analysis were used for pollen area measurements in ImageJ software. The obtained data were processed into the violin plots that are graphic representations of the distribution of pollen area in wild-type and *hpy2* mutant plants.

Whereas diploid plants produce haploid gametes containing only one set of chromosomes, tetraploid plants produce haploid gametes that contain two sets of chromosomes. Since cell size correlates with genome size, the pollen size of diploid and tetraploid plants is different. There is only one peak in diploid and tetraploid wild-type, respectively. In diploid wild-type, the pollen area maximum is around 440 $\mu m^2$ and in tetraploid wild-type around 660 $\mu m^2$. However, both *hpy2* mutants produced bimodal pollen area distributions (Figure 11). Whilst maximum of the first peak corresponds approximately to the maximum of diploid wild-type, maximum of the second peak corresponds approximately to the maximum of tetraploid wild-type. These results thus suggested differences in the ploidy level of *hpy2* male gametes.

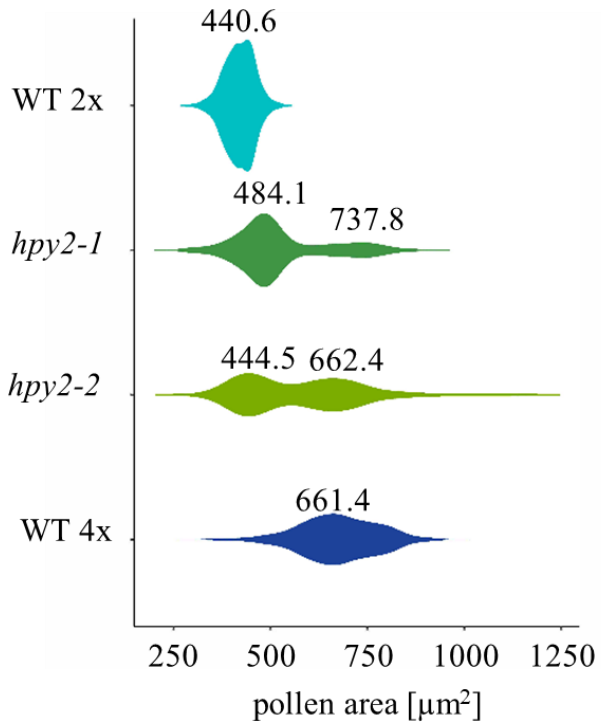


**Figure 11:** Violin plots showing the distribution of pollen area of wild-type and *hpy2*. The number above each violin indicates the density maxima.

## 5.4 HPY2 mutants lead to triploid offspring by producing diploid male gametes

To verify the hypothesis that *hpy2* plants produce male gametes of different ploidy, the ploidy measurement of the mutant plants was performed using FCM. First, the ploidy level of the offspring generated by diploid *hpy2* plants was measured after self-pollination. If both the male and the female gamete were haploid, the offspring would be diploid. However, the obtained results showed that while diploid wild-type plants produced only diploid offspring, diploid *hpy2* plants also produced triploid offspring that accounted for approximately 10 % (Figure 12, Table 5).

Then, the ploidy measurement was performed in the F1 generation after the reciprocal crossing of diploid *hpy2* plants with diploid wild-type. Triploid offspring were detected only when the mutant offered pollen grains (Table 6). This strongly indicated that triploid offspring were a result of the fusion of haploid female gamete and diploid male gamete.

**Table 5:** The proportion of diploid and triploid offspring produced after the self-pollination of diploid *hpy2* plants.

| **Diploid ♀** | **Diploid ♂** | **Number of offspring** | **Diploid offspring [%]** | **Triploid offspring [%]** |
|---|---|---|---|---|
| WT | WT | 110 | 100 | 0 |
| *hpy2-1* | *hpy2-1* | 380 | 92.4 | 7.6 |
| *hpy2-2* | *hpy2-2* | 253 | 85 | 15 |

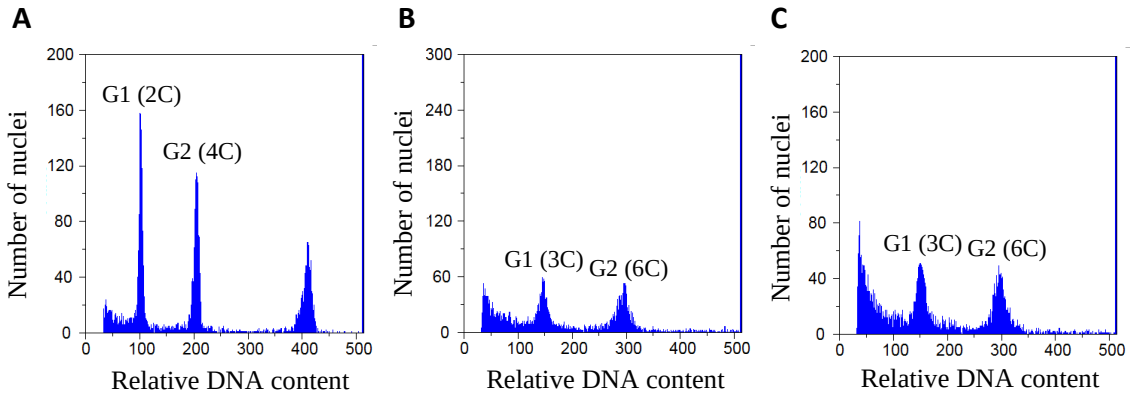


**Figure 12:** Representative histograms of relative DNA contents obtained after flow cytometry analysis of DAPI-stained suspension of nuclei of **A)** diploid wild-type **B)** triploid *hpy2-1* **C)** triploid *hpy2-2*.

**Table 6:** The proportion of diploid and triploid offspring produced after the reciprocal crossing of diploid *hpy2* plants with diploid wild-type.

| **Diploid** ♀ | **Diploid** ♂ | **Number of offspring** | **Diploid offspring [%]** | **Triploid offspring [%]** |
|---|---|---|---|---|
| WT | *hpy2-1* | 270 | 93.3 | 6.7 |
| WT | *hpy2-2* | 78 | 94.9 | 5.1 |
| *hpy2-1* | WT | 43 | 100 | 0 |
| *hpy2-2* | WT | 93 | 100 | 0 |

# 6 DISCUSSION

It was already described in several studies that mutations in Arabidopsis cohesin and condensin genes lead to a *titan* seed phenotype caused by alterations in chromosome dynamics and cell division (Liu *et al*., 2002; Liu and Meinke, 1998). Embryos of the *titan* (*ttn*) mutants exhibit differences in cell size, morphology and viability. The *ttn* mutants also give rise to large polyploid nuclei in endosperm, which is a defining feature common to all of these mutants. However, the results of a recent study (Díaz *et al*., 2019) on the SMC5/6 complex suggest that all three SMC complexes could be involved in seed development. That study showed that partial loss of function of the *NSE4A* subunit leads to defects similar to the *titan* seed phenotype. Our study regarding *HPY2* showed that non-functional subunit also caused abnormal seed phenotype and confirms the importance of the SMC5/6 complex during seed development. However, specific mechanisms by which the SMC5/6 complex affects seed development are still unknown.

As already mentioned, the loss of function from HPY2 and partial loss of function from NSE4A lead to disruption of proper seed development. Homozygous *nse4a* null mutant plants die. Partial loss of function *nse4a-2* plants are initially smaller but recover, show reduced seed production and are hypersensitive to specific types of DNA damage. In contrast, loss of function from NSE4B is not lethal and mutant plants do not have any obvious abnormal phenotype (Díaz *et al*., 2019). Non-functional NSE1 and NSE3 are both lethal in Arabidopsis. These two subunits are required for early embryo and seedling development (Li *et al*., 2017). The phenotype of SNI1 mutant plants resembles that of *hpy2* mutants. Homozygous *asap1* plants die in an early stage of development (Yan *et al*., 2013).

What we further discovered during our research was that the abnormal seed phenotype of *hpy2* mutant plants is paternally inherited. Therefore following experiments were focused on the analysis of the male gametophyte. Measurement of the pollen area of *hpy2* revealed striking differences in pollen grain size, indicating a possible effect on the ploidy level. In one study, a pollen size-based screen was used to identify Arabidopsis EMS mutants producing diploid pollen (De Storme and Geelen, 2011). Therefore, the question arises as to why *hpy2* wasn't also identified? The possible reason for that could be a significantly reduced ability of *hpy2* plants to grow or the lower number of diploid

pollen grains produced by *hpy2* plants, which could be below the detection limit in a high throughput screen.

Nevertheless, we proved that *hpy2* plants produce triploid offspring due to the fusion of haploid female and diploid male gamete. This result suggested that the SMC5/6 complex is involved in basic control of the ploidy level, although specific mechanisms remain unclear. This new function of the SMC5/6 complex has not yet been suggested even in the animal kingdom.

Diploid male gametes are probably produced as a result of defective meiosis. Meiosis reduces the number of chromosomes by half to give rise to the haploid gametes. It seems likely that the problem lies in disrupted chromosome segregation that leads to unreduced (diploid) gametes. The reason for that might be that the SMC5/6 complex interferes with the cohesin pathway. It is known that cohesin is sumoylated by NSE2 in the presence of DNA damage in yeast and humans (McAleenan *et al*., 2012; Potts *et al*., 2006). Could NSE2 mediated sumoylation also play a role in a proper cleavage of cohesin during anaphase? It remains unknown whether the SMC5/6 complex has such function in plants.

The fusion of a diploid male gamete with a haploid female gamete leads to the triploid offspring. The process of polyploidization usually provides both advantages and disadvantages for the plant. However, triploid plants are generally unstable and have lower fitness. Triploids are often sterile or their fertility is significantly reduced. Their seeds also have lower germination rates that often depend on the source of diploid gamete (Ramsey and Schemske, 1998). Interestingly, it was shown that the germination rate is lower when the male gamete is diploid, which could be related to an imbalance of maternally and paternally imprinted genomes in the endosperm (Haig and Westoby, 1991).

The question still remains, why only male gametes were produced as diploid? One possible explanation is that the number of ovules produced by the plant is significantly lower than the number of produced pollen and the mechanisms that regulate female gametogenesis are therefore more strict. Hence, the defects created during female gametogenesis often lead to the abortion of the ovule. In contrast, male gametes are produced in excess and a relatively low percentage of the healthy ones can still lead to almost normal fertility in plants.

Do other viable mutants of the SMC5/6 complex also produce diploid male gametes? This and the other questions mentioned above are important for the future. Answers to these questions could help us to better understand the functions of SMC5/6 complex in plants.

In summary, our work demonstrates that the SMC5/6 complex is important during reproductive development in Arabidopsis and also suggests a brand new function of this complex in basic control of the ploidy level. Above all, working on this project provided me the opportunity to gain valuable experience in the field of plant genetics from colleagues who are knowledgeable and willing to share their expertise. I learned new methods, approaches and also improved in both data presenting and scientific writing in English, which I think will be very useful for my future work.

# 7 CONCLUSION

The aim of the thesis was to analyze defects in the reproductive development of Arabidopsis SMC5/6 complex mutants, specifically of two mutant lines of the gene encoding the NSE2/HPY2/MMS21 subunit. It was shown that *HPY2* mutants cause paternally inherited abnormal seed phenotype and decreased number of viable pollen. *HPY2* also leads to the production of triploid offspring as a result of the fusion of haploid female gamete and diploid male gamete. This is the first study providing evidence of the SMC5/6 complex playing a role in basic control of the ploidy level. In order to better understand the function of the SMC5/6 complex, studies focused on specific mechanisms standing behind this newly discovered fact will be necessary.

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