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Physical mapping of *Ph2* region in hexaploid wheat

Ph.D. Thesis

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Olomouc 2020

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Acknowledgements

I would like to express my gratitude to my supervisor Mgr. Jan Bartoš, Ph.D., for his professional leadership, advice and patience. My thanks also belong to the head of the laboratory prof. Ing. Jaroslav Doležel, Dr.Sc., for giving me the opportunity to work in the Centre of Plant Structural and Functional Genomics. I would like to thank the rest of the employees of the laboratory for creating a friendly atmosphere in the workplace.

Declaration

I hereby declare that I have written the Ph.D. thesis independently under the supervision of Mgr. Jan Bartoš, Ph.D., using the sources listed in references with no conflict of interest.

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This work was supported by the Czech Science Foundation (grant award 17-05341S) and Ministry of Education, Youth and Sports of the Czech Republic (award LO1204 from the National Program of Sustainability I).

Bibliographical identification

Author's name:	Mgr. Radim Svačina
Title:	Physical mapping of *Ph2* region in hexaploid wheat
Type of Thesis:	Ph.D. thesis
Department:	Department of Cell Biology and Genetics
Supervisor:	Mgr. Jan Bartoš, Ph.D.
The year of presentation:	2020

Abstract:

Bread wheat (*Triticum aestivum* L.) is one of the most essential cultivated crops around the globe. It is a staple food for about 40 % of population and together with corn and rice constitutes the bedrock of plant agriculture. Hexaploid wheat emerged from two distinct hybridization events among three diploid species, thus giving rise to its three subgenomes A, B and D. Consequently, its genome has a huge size (~ 16 Gb) with a high content of repetitive sequences (85 %), making the identification of genes with agronomical value a challenging task. However, such complex nature of hexaploid wheat allows a creation of various aneuploid stocks, missing fragments or even entire chromosomes, creating an opportunity to facilitate gene cloning.

The existence of three homoeologous sets of chromosome in bread wheat genome generate a vulnerability towards incorrect chromosome pairing during meiosis, endangering the creation of healthy gametes and thus its fertility. Consequently, as many other polyploid species, wheat developed a genetic control of correct chromosome pairing which is being regulated by *Pairing homoeologues* (*Ph*) genes. The most significant gene of this group is located on the chromosome 5B and is called *Ph1* which was recently indetified as *TaZIP4-B2*. A different gene with a lower effect on chromosome pairing was mapped through use of radiation mutant *ph2a* to a distal 121 Mb of the short arm of chromosome 3D and is called

Ph2. Together with *Ph3* and some other minor genes, they contribute to correct chromosome pairing and recombination during meiosis.

Usually, genes are kept in a population by being beneficial to its host or through high linkage to this gene, however there are known exceptions, such as gametocidal genes. The gametocidal genes ensure inheritance through induction of genomic aberrations to gametes lacking them, causing total or partial sterility. In hexaploid wheat, chromosomes containing gametocidal genes are being used for creation of mostly terminal chromosome deletion lines that can be utilized as material for various purposes.

The main goal of this thesis was the use of terminal deletion lines of chromosome 3D to delimit the *Ph2* gene location with subsequent selection of candidate gene(s) and their functional validation using TILLING population. Novel 113 deletion lines for chromosome 3D were developed and subsequently screened by 84 markers alongside the entire chromosome length to determine the deletion size. The deletion lines in the area of interest were crossed with rye and phenotyped for *Ph2* gene presence. Through this approach, we delimited the *Ph2* gene locus from original 121 Mb to 12.3 Mb region of the short arm of chromosome 3D, reducing the number of potential candidate genes from 1577 to 88. Out of these 88 genes, only a single one was mutated in EMS-induced *ph2b* mutant. This gene encodes a DNA mismatch repair protein TaMSH7-3D. TILLING population of bread wheat 'Cadenza' cultivar carrying EMS-induced point mutations was exploited for selection of seven mutants of *TaMSH7-3D* gene. These mutants were crossed with *Aegilops variabilis*, showing a similar *Ph2* deleterious phenotype. Through this study, we show that *Pairing homoeologous 2* encodes a mismatch repair protein TaMSH7-3D, thus solving a half-century-old question.

Keywords: Bread wheat, *Triticum aestivum*, *Pairing homoeologues*, *Ph2*, deletion line, TILLING.

Number of Pages/Appendices: 77/X

Language: English

Bibliografická identifikace

Jméno autora:	Mgr. Radim Svačina
Název práce:	Fyzické mapování *Ph2* regionu u pšenice seté
Typ práce:	Disertační práce
Katedra:	Katedra buněčné biologie a genetiky
Školitel:	Mgr. Jan Bartoš, Ph.D.
Rok obhajoby:	2020

Abstrakt:

Pšenice setá (*Triticum aestivum* L.) je jednou z nejdůležitějších kultivovaných plodin na světě. Jedná se o základní potravinu pro přibližně 40 % lidské populace a spolu s kukuřicí a rýží tvoří základ rostlinné výroby. Hexaploidní pšenice vznikla na základě dvou různých hybridizací mezi třemi diploidními druhy, jež daly původ jejím třem subgenomům A, B a D. Následkem toho má její genom značnou velikost (~ 16 Gb) s vysokým obsahem repetitivních sekvencí (85 %), což činí identifikaci agronomicky významných genů složitým úkolem. Takto složitá struktura pšeničného genomu však umožňuje tvorbu různých aneuploidních linií, usnadňujících klonování genů.

Existence tří homoeologních sad chromozomů pšenice seté s sebou nese náchylnost k nesprávnému chromozomálnímu párování během meiozy, což potenciálně ohrožuje tvorbu zdravých gamet, a tudíž i fertilitu. Proto si pšenice, jako mnoho jiných polyploidních druhů, vyvinula genetickou kontrolu správného chromozomálního párování, která je regulována „*Pairing homoeologous*“ (*Ph*) geny. Nejdůležitější gen této skupiny, který se nazývá *Ph1*, se nachází na chromozomu 5B, a byl nedávno identifikován jako *TaZIP4-B2*. Další gen, s nižším vlivem na chromozomální párování, byl mapován pomocí radiačního mutanta *ph2a* na distálních 121 Mb krátkého ramene chromozomu 3D a je nazývám *Ph2*. Společně s *Ph3* a dalšími minoritními geny přispívají ke správnému chromozomálnímu párování a rekombinaci během meiozy.

Geny jsou obvykle udržovány v populaci v důsledku jejich prospěšnosti hostiteli nebo vysokou vazbou na takový gen, nicméně jsou známy výjimky jako například gametocidní geny. Gametocidní geny si zajišťují svůj přenos do potomstva díky tvorbě genomických aberací v gametách, do kterých nebyly přeneseny, což způsobuje jejich úplnou nebo částečnou sterilitu. U hexaploidní pšenice se chromozomy obsahující gametocidní geny využívají při tvorbě delečních linií, které mohou být použity jako materiál pro různé aplikace.

Hlavní cíl této práce bylo využití delečních linií chromozomu 3D pro mapování genu *Ph2* s následnou selekcí kandidátních genů a jejich funkční validací pomocí TILLING populace. Bylo vytvořeno 113 nových delečních linií, které byly následně testovány 84 markery po celé délce chromozomu, pro zjištění velikosti delece. Přítomnost genu *Ph2* byla analyzována křížením delečních linií s žitem a jejich fenotypováním. Tímto přístupem byl *Ph2* gen zamapován do regionu o velikosti 12,3 Mb, oproti původním 121 Mb krátkého ramene chromozomu 3D. Množství potenciálních kandidátních genů bylo sníženo z 1577 na 88. Jen jediný z identifikovaných 88 genů nese deleci v EMS mutantovi *ph2b*. Tento gen kóduje ‘DNA mismatch repair’ protein TaMSH7-3D. Sedm mutantů tohoto genu bylo vybráno z TILLING populace pšeničného kultivaru ‘Cadenza’ nesoucí bodové mutace. Tito mutanti byli kříženi s *Aegilops variabilis*, kdy analýza fenotypu *Ph2* odpovídala jeho delečnímu projevu. Touto studií bylo prokázáno, že *Pairing homoeologous 2* kóduje ‘DNA mismatch repair’ protein TaMSH7-3D, čímž byla zodpovězena půl století nevyřešená otázka.

Klíčová slova: Pšenice setá, *Triticum aestivum*, *Pairing homoeologues*, *Ph2*, deleční linie, gametocidní.

Počet stran/příloh: 77/X

Jazyk: Anglický

CONTENTS

1 **LITERATURE OVERVIEW** 11
1.1 Polyploidy 11
1.2 Bread wheat (*Triticum aestivum* L.) 13
1.2.1 Formation of bread wheat genome 15
1.2.2 Contents of bread wheat genome 16
1.3 Chromosome pairing in bread wheat 18
1.3.1 *Pairing homoeologous 1 (Ph1)* 19
1.3.2 *Pairing homoeologous 2 (Ph2)* 21
1.3.3 *TaMSH7-3D* 23
1.3.4 Chromosome pairing in hybrids of wheat and its *ph* mutants 23
1.3.5 Utilization of *Ph* genes in wheat breeding 24
1.4 Mapping through deletion lines 25
1.5 Gametocidal genes 26
1.6 EMS-induced mutagenesis and TILLING 29
2 **AIMS OF THE THESIS** 31
3 **RESULTS** 32
3.1 Original publications 37
3.1.1 Development of deletion lines for chromosome 3D of bread wheat 38
3.1.2 *Pairing homoeologous 2* (*Ph2*) encodes the mismatch repair protein MSH7-3D that inhibits homoeologous recombination in wheat 39
3.1.3 Chromosome pairing in polyploid grasses 40
3.2 Published abstracts – poster presentations 41
3.2.1 Development of deletion lines for physical mapping of *Ph2* gene in bread wheat 42
3.2.2 Development of chromosome deletion lines for *Ph2* gene mapping in bread wheat 44
3.2.3 *Ph2* gene mapping through development and phenotyping of deletion lines in bread wheat 46
3.2.4 Towards identification of *Ph2*, a gene controllong homoeologous chromosome pairing in bread wheat 48
3.2.5 *Ph2* gene phenotype scoring in wheat-rye hybrids with terminal deletions of 3D chromosome 49

3.2.6 Wheat-rye hybrids with chromosome deletions analysed for *Ph2* gene phenotype 51
3.2.7 *Ph2* gene mapping through phenotyping of wheat-rye hybrid deletion lines 53
3.3 Published abstracts – oral presentations 55
3.3.1 Development of chromosome deletion lines for *Ph2* gene mapping in bread wheat 56
4 **CONCLUSION** 58
5 **REFERENCES** 60
6 **LIST OF ABBREVIATIONS** 74
7 **LIST OF APPENDICES** 77

1 LITERATURE OVERVIEW

1.1 Polyploidy

Polyploidy plays a major role in evolution, especially in plants (Lewis *et al.*, 1980). It is a state of an organism having one or more extra sets of chromosomes coexisting in its cell nuclei, relative to its ancestral state. In plants, polyploidy represents one of the main adaptation mechanisms, as all angiosperms underwent at least one round of whole-genome duplication (WGD) (Jiao *et al.*, 2011). Based on the origin of individual subgenomes, polyploidy can be classified into two main groups, namely autopolyploidy and allopolyploidy. Autopolyploids contain more than two copies of the identical chromosome sets, called homologous, while allopolyploids emerge through gaining one or more extra sets of more or less related chromosomes, called homoeologous. The latter group is in some literature divided into two classes based on the level of homology between individual sets, namely true allopolyploids and segmental allopolyploids, the latter having higher homology (Winterfeld *et al.*, 2012). The existence of more than two sets of chromosomes generate a risk of reduced fertility, since it hampers a chromosome recognition and formation of bivalents during meiosis, making an adaptation to this new state a necessary step (Jauhar, 2003).

The polyploid formation can be accomplished through several hypothesised ways. The most obvious one presumes that the chromosome doubling can be executed through nondisjunction in mitosis, however it is usually achievable only through use of chemicals and is not frequently observed in natural populations (Ramsey and Schemske, 1998; Tamayo-Ordóñez *et al.*, 2016; Pelé *et al.*, 2018). On the other hand, a creation of unreduced gametes is rather common process with a frequency of 0.1 to 2 % (Kreiner *et al.*, 2017; Pelé *et al.*, 2018), raising with different stress conditions. With regard of unreduced gametes, the polyploid formation can be achieved through a single-step, involving two unreduced gamates or in a two-step, creating a triploid bridge after combination of a reduced and unreduced gamete

(Figure 1; Husband, 2004). Another two-step process takes into account a combination of two different reduced gametes into a homoploid individual which is usually sterile. The fertility could be restored through chromosome doubling, combination of its two unreduced gametes or a triploid bridge (Mason and Pires, 2015).

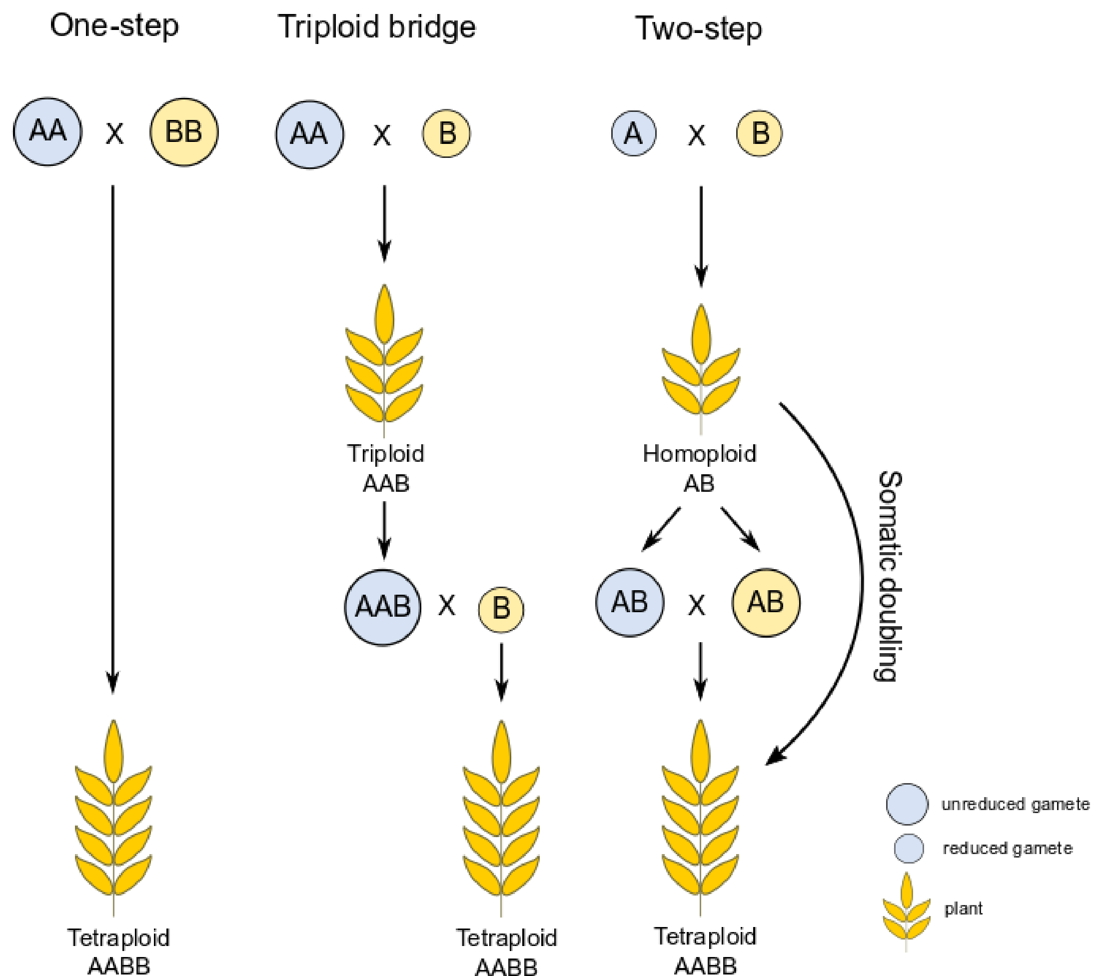

Figure 1 I Hypothesised pathways leading to polyploidy. Polyploidy can be established via several ways, most often through unreduced gamete formation and subsequent fertilization. In case of the one-step pathway, two unreduced gametes merge, resulting directly in a polyploid species. More steps are usually needed, where the reduced gamete merges with an unreduced gamete, forming a triploid bridge that needs an additional reduced gamete in subsequent generations to form a polyploid. The final depicted option is the two-step pathway, through a homoploid hybrid, which needs a somatic doubling event or unreduced gamete formation to establish a polyploid state (Svačina *et al.*, 2020a).

Polyploid species are evolutionary remarkably successful and can often colonize areas with extreme conditions, in opposition to its diploid progenitors (Ehrendorfer, 1980). One of the main advantages of polyploid state is a gene redundancy, which offers an opportunity to diversify the extra gene copies to gain new traits with no negative effect of losing its original function (Ha *et al*., 2009). In case of allopolyploid species, a fixed heterosis plays a major role in evolutionary success, providing a lasting combination of parental traits (Comai, 2005; Osborn *et al*., 2003). However, the polyploid state comes with many obstacles, regularly connected to a progression of meiosis. One of the substantial issues is the chromosome pairing and recombination in meiosis. The polyploid species contain three or more chromosome sets, ranging in homology, creating a danger of multivalent formation, hampering the chromosome segregation, often leading to aneuploidy and reduced fertility (Ramsey and Schemske, 2002). Even though some species maintain fertility while forming multivalents, other adaptation mechanisms exist. In autopolyploids, a reduction of number of crossing-overs to a single one per chromosome pair enforces a creation of rod bivalents between random homologues (Lloyd and Bomblies, 2016). On the other hand, the known adaptive mechanisms in allopolyploids usually implement more stringent recognition of homoeologues, as the chromosome sets are not entirely identical; these mechanisms are mostly controlled genetically (Jenczewski and Alix, 2004).

1.2 Bread wheat (*Triticum aestivum* L.)

Bread wheat (*Triticum aestivum* L.) is among the most important crop plants, as it represents a main source of food intake for a large portion of population. Taxonomically, it is a monocotyledonous species, which belongs to a family Poaceae, subfamily Pooideae and the tribe Triticeae. It emerged from two distinct hybridization events between three progenitors, resulting in its allohexaploid nature, consisting out of three closely-related subgenomes A, B and D ($2n = 6x = 42$; AABBDD). Its genome has therefore considerable size ~16 Gb (Doležel *et al*., 2018), containing a high amount of repetitive DNA sequences, which is about 85 % (IWGSC, 2018).

Many of cereal crops as well as wheat domestication and foundation of modern agriculture come from the Fertile Crescent, emerging about 10 000 years ago (Heun *et al*., 1997; Lev-Yadun *et al*., 2000; Riehl *et al*., 2013; Salamini *et al*., 2002). Early farming efforts were founded on utilization of diploid wild wheat species, mainly from genus *Triticum* and *Aegilops*, however as agriculture developed, these crops were being substituted by domesticated diploid and polyploid varieties (Riehl *et al*., 2013; Salamini *et al*., 2002). One of these crops is the hexaploid bread wheat, which plays a crucial role in development of our civilization, as it represents a main source of food for about 40 % of population and provides 20 % of total intake of proteins and calories globally. It started to spread out of the Fertile Crescent, as the polyploid nature enables wheat to adapt to many different climate conditions. Nowadays it is being cultivated almost world-wide, ranging from as north as Norway and Russia to as south as Argentina, however in tropical countries, its cultivation is restricted to higher altitutes (Dubcovsky and Dvorak, 2007). As a result, in the year of 2019, wheat was being cultivated on an area larger than 220 million ha, with a global production of almost 767 million tonnes (Figure 2; OECD, 2020).

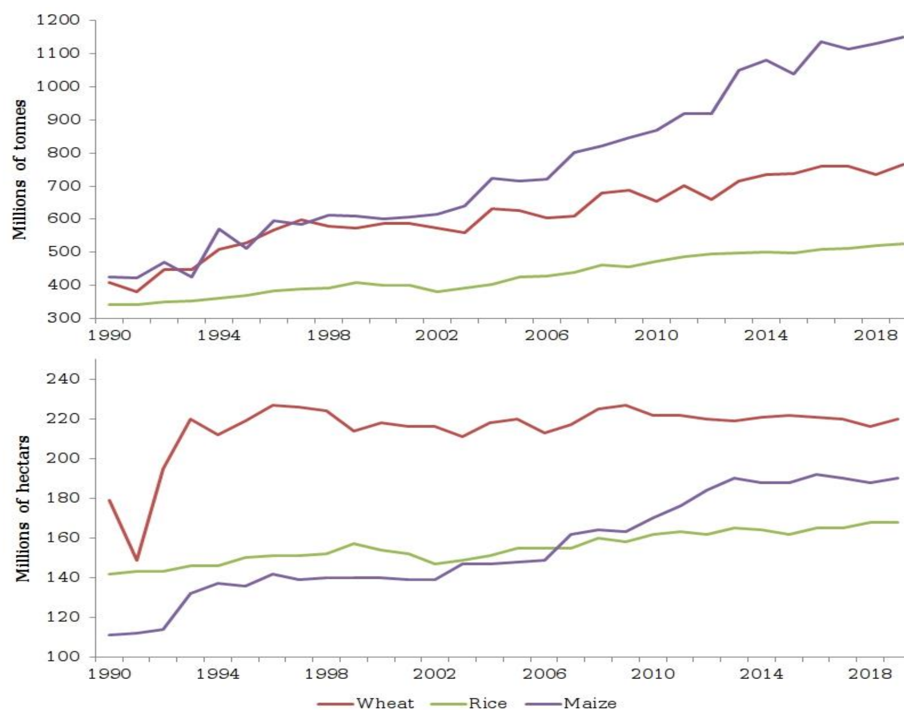

Figure 2 | Comparison of wheat, rice and maize production and cultivation area between years 1990 and 2019 (OECD, 2020).

1.2.1 Formation of bread wheat genome

The bread wheat subgenomes originated from three diploid progenitors from the tribe Triticeae, all with the same chromosome base number of seven (IWGSC, 2014). The two hybridization events combined *T. urartu*, a donor of genome AA, an unknown closely-related species to *Ae. speltoides*, progenitor of genome BB, and *Ae. tauschii*, goatgrass carrying genome DD (Salamini *et al.*, 2002; Petersen *et al.*, 2006). The first hybridization event emerged between *T. urartu* and a close relative of *Ae. speltoides*, resulting in tetraploid emmer wheat *T. turgidum* (2n = 4x = 28; AABB) (Figure 3; Marcussen *et al.*, 2014), which through continuous cultivation and breeding gave rise to *T. turgidum* spp. *durum*, wheat used for pasta production (IWGSC, 2014). The subsequent hybridization arose between allotetraploid *T. turgidum* and diploid goatgrass *Ae. tauschii*, resulting in allohexaploid bread wheat *T. aestivum* (2n = 6x = 42; AABBDD). Various studies deduced different approximate dates of these two events, however Marcussen *et al.* (2014) suggested the first to be <0.82 MYA and the second <0.43 MYA (Figure 3).

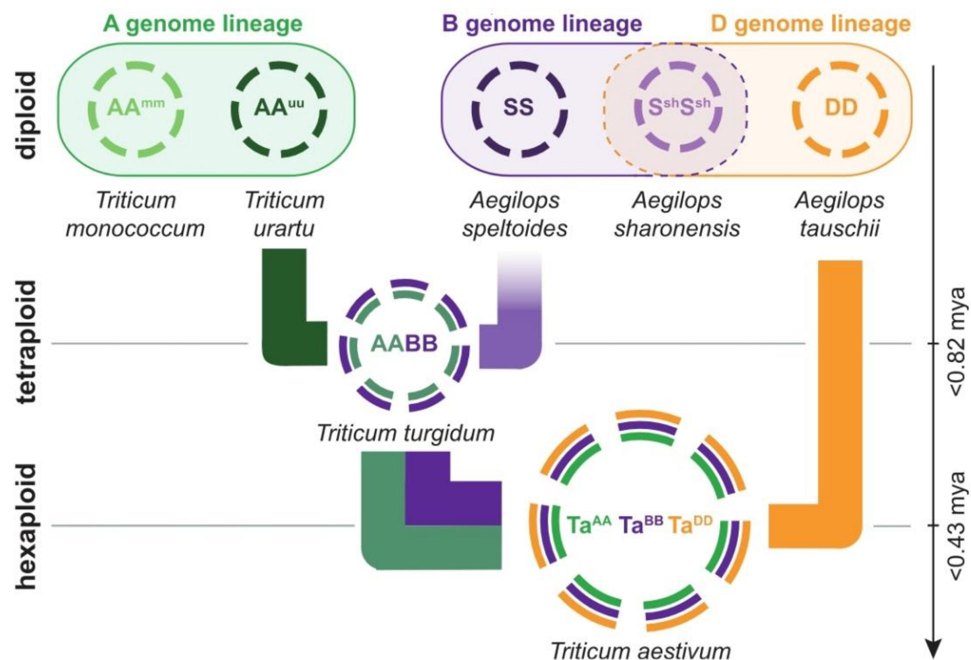

Figure 3 I Bread wheat evolution scheme through two distinct hybridization events. The first hybridization event happening between *T. urartu* and a close relative of *Ae. speltoides*, resulting in tetraploid *T. turgidum*. The second hybridization took place between *T. turgidum* and diploid goatgrass *Ae. tauschii*, resulting in allohexaploid bread wheat *T. aestivum* (2n = 6x = 42; AABBDD) (IWGSC, 2014).

1.2.2 Contents of bread wheat genome

The analysis of bread wheat genome was until lately hampered by non-existence of annotated high-quality reference sequence, due to its high genome size and repetitive sequence content. However, IWGSC (2018) succeeded by providing both reference sequence and annotation representing all 21 chromosomes of hexaploid bread wheat variety ‘Chinese Spring‘, presenting distribution of coding and non-coding elements across all three subgenomes. This accomplisment thus allowed more precise analyses of key elements of individual A, B and D subgenome and subsequent detailed comparison.

The bread wheat genome contains 85 % of repetitive sequences, more or less equally distributed along all three subgenomes. More precisely, 3 968 974 copies of transposable elements, belonging to 505 families were found alongside individual subgenomes (IWGSC, 2018). The amount of repetitive sequences plays a major role in size of wheat subgenomes. About 64 % of the size difference between A and D genome is caused by a lower copy number of gypsy retrotransposon in the latter. On the other hand, in case of difference of genetic material amount between A and B subgenomes, about 40 % is caused by a low-copy DNA segments (Figure 4; IWGSC, 2018).

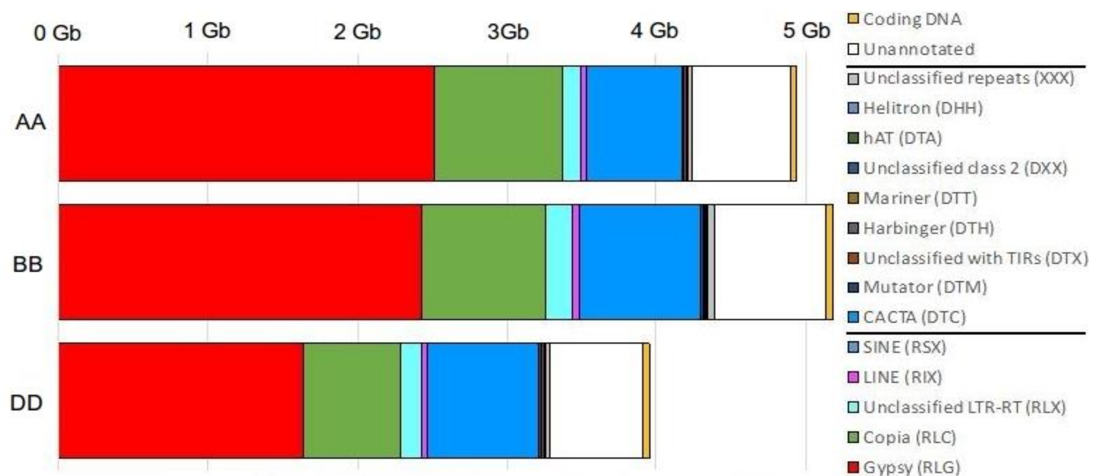

Figure 4 I A composition of genome content in bread wheat. Various repetitive sequences and coding DNA content in different subgenomes (edited from IWGSC, 2018).

The number of genes in wheat was deduced using two different annotation pipelines, resulting in 107 891 high-confidence genes, relatively equaly distributed across the A, B and D subgenomes, namely 35 345, 35 643 and 34 212 respectively (Figure 5). However, the annotation resulted in additional 161 537 low-confidence genes, partially exhibiting gene models, their fragments or orphans. Moreover, 2 691 of high-confidence and 675 of low-confidence genes were found in unassembled sequences (IWGSC, 2018). The function was predicted in 90 919 (82.1 %) and transcriptional activity was found in 94 114 (85 %) of high-confidence genes, while the latter was found only in 49 % of low-confidence genes (Ramírez-Gonzales *et al*., 2018). The further analysis of 181 036 of both high-confidence and low-confidence genes showed that 113 653 (about 63 %) are present in all three subgenomes in a form of homoeologues, together called „triads“. All three subgenomes show similar number of loss of homoeologous genes, namely 10.7 %, 10.3 % and 9.5 % in A, B and D subgenomes respectively (IWGSC, 2018).

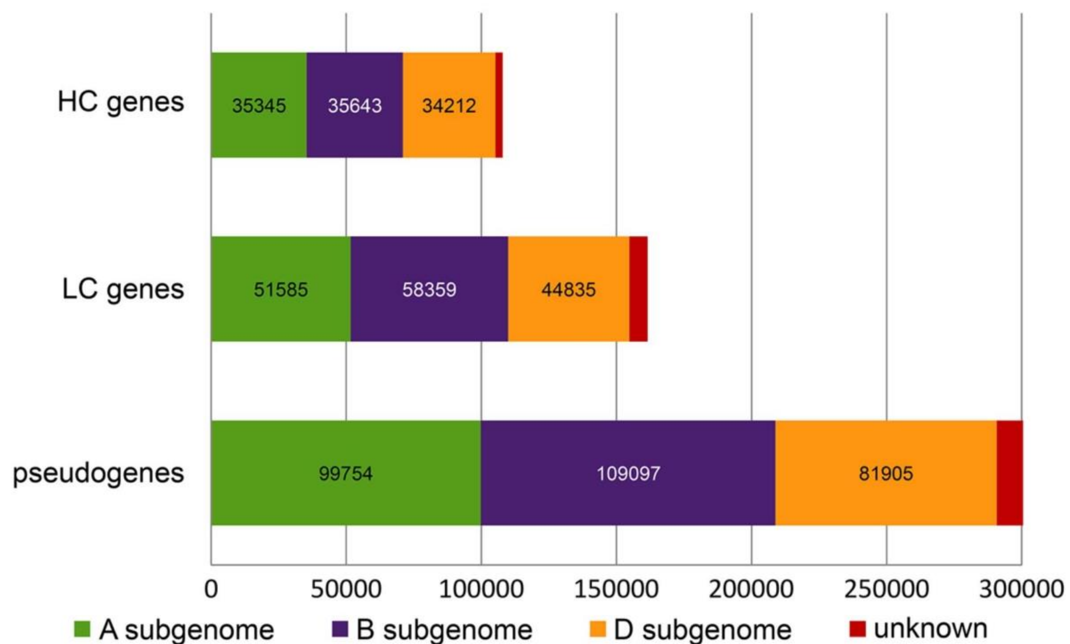

Figure 5 I The predicted number of high-confidence and low-confidence genes, together with pseudogenes across bread wheat subgenomes and unassembled sequences (IWGSC, 2018).

1.3 Chromosome pairing in bread wheat

As it was said before, the bread wheat genome consists of three highly similar subgenomes originating from three closely-related species. High homology between the homoeologous chromosomes hampers the correct chromosome pairing through risk of creation of multivalents during the first meiotic division. In general however, the meiosis of wheat is fully diploid-like, with formation of 21 bivalents in methaphase I (Martínez *et al.*, 2001a; Martínez *et al.*, 2001b). To ensure the correct chromosome recombination and consistency of meiotic division, wheat had to develop a genetic control mechanism ensuring a higher stringency of chromosome recognition to allow a proper development of gametes (Sears and Okamoto, 1958; Riley and Chapman, 1958).

Bread wheat is considered a model for analyses concerning chromosome pairing in meiosis because of its trait to tolerate a creation of various aneuploid stocks, allowing scientists of the last century to map regulation genes controlling meiosis to entire chromosomes or even chromosome arms (Naranjo and Benavente, 2015). Several genes control chromosome pairing and recombination in wheat. However, the most well-studied control mechanism is *Pairing homoeologous* (*Ph*), nonetheless, up to this day, the exact way of its function is still unknown. The first gene of this control mechanism is called *Ph1*, which was localized to a long arm of chromosome 5B by Sears and Okamoto (1958) and Riley and Chapman (1958). The study of its deleterious phenotype in wheat and its hybrids concluded that this gene has the highest effect on chromosome pairing (Martínez *et al*., 2001a; Martínez *et al*., 2001b; Naranjo *et al*., 1987; 1988; Naranjo and Maestra, 1995; Maestra and Naranjo, 1997; Maestra and Naranjo, 1998). Another gene of this control mechanism is located on a short arm of chromosome 3D, called *Ph2*, having a less distinctive mutant phenotype than *Ph1* (Mello-Sampayo, 1971). The last and least effective gene out of this control mechanism is called *Ph3* and is located on a short arm of chromosome 3A (Driscoll, 1972; Mello-Sampayo and Canas, 1973). However, the location of *Ph2* and *Ph3* on the same arm of homoeologous chromosomes and their similar function leads to conclusion that these two genes might be orthologues. In

general, the mutant phenotype of *Ph* genes in metaphase I in meiosis show a lower number of ring bivalents and increased number of univalents, rod bivalents and multivalents, resulting in lower number of chiasmata, compared to wild-type (Table 1; Martínez *et al*., 2001a; Martínez *et al*., 2001b). However in hybrids, where there are no pairs of homologous chromosomes, the *ph* mutants display higher number of chiasmata, resulting from higher number of chromosome associations compared to wild-type (Table 2; Naranjo *et al*., 1987; 1988; Naranjo and Maestra, 1995; Maestra and Naranjo, 1997; Maestra and Naranjo, 1998).

Table 1 I Chromosome pairing in *ph1* and *ph2* mutants in hexaploid and tetraploid wheat; 6x WT = hexaploid wheat, 4x WT = tetraploid wheat (Martínez *et al*., 2001a; Martínez *et al*., 2001b).

Genotype	Chromosome number	Univalents	Rod bivalents	Ring bivalents	Multivalents	Chiasmata/cell
6x WT	42	0.02	1.48	19.50	0	40.49
ph1b	42	2.76	4.76	14.5	0.77	34.22
ph2b	42	0.48	2.95	17.78	0	38.57
4x WT	28	0.04	0.34	13.64	0	27.62
ph1c	28	0.94	3.69	9.46	0.19	23.16

1.3.1 *Pairing homoeologous 1 (Ph1)*

Ph1 is the most significat gene affecting diploid-like chromosome pairing in wheat. The existence of this gene was first proposed over 60 years ago by Sears and Okamoto (1958) and Riley and Chapman (1958), while studying meiotic behaviour of bread wheat haploids lacking chromosome 5B. In these lines, they observed formation of both bivalents and trivalents, despite the non-existence of any chromosome homologue pair. Even though the existence of *Ph1* has been known over a half of a century, the molecular mechanism has been partially uncovered only recently (Rey *et al*., 2017). Subsequent efforts to map *Ph1* were performed using *ph1b* mutant (Sears, 1977), specifying further the location of this gene on chromosome 5B. Another round of delimiting the location of *Ph1* was performed by Gill *et al*. (1993) through exploitation of deletion lines, narrowing down the region to about 70 Mb. However, the size of the region was only indicative, since it was estimated through cytogenetic approach, that's why it was later re-estimated by molecular techniques to be only 54.6 Mb (Gyawali *et al*., 2019). Since the

proposition of *Ph1*, there were countless studies analysing its mutant phenotype in both euploid wheat and its hybrids, all showing disturbed diploid-like behaviour, with creation of multivalents in pollen mother cells (Riley and Chapman, 1958; Riley, 1960; …), often leading to creation of aneuploidy or other genomic aberrations in its progeny (Sanchez-Morán *et al*., 2001).

The function of *Ph1* gene is not reserved to hexaploid wheat only. Its phenotype was observed also in tetraploid *Triticum* species, such as in *T. timopheevi* subsp. *timopheevi* (Feldman, 1966) or *T. turgidum* subsp. *durum* (Dvorak *et al.*, 1984), while for the former, a mutant *ph1c* was developed with similar phenotype as *ph1b* in hexaploid wheat (Jauhar *et al*., 1999). The study of effectivity of both *Ph1* orthologs in hybrid with *Ae. peregrina* was performed by Ozkan and Feldman (2001), who exchanged the 5B chromosome of hexaploid wheat by tetraploid wheat chromosome 5B. The replacement resulted in a higher level of homoeologous associations in meiosis, which led to conclusion that the tetraploid *Ph1* gene functions with a lower strength.

The way of function of the *Ph1* gene was further deduced by Martín *et al.* (2014), who stated that it operates in two phases of first meiotic division – promoting homologous synapsis in early prophase I and subsequently influencing a crossing-over formation. Martín *et al.* (2017) proposed that *Ph1* works as a homologous pairing promoter, rather than suppressor of homoeologous pairing as it was originally thought (Holm and Wang, 1988). This statement is supported by the occurence of univalents in *ph1b* mutant, as well as incorrect chromosome pairing occurring in only about half of studied mutant meiocytes (Martín *et al*., 2017).

The attempts to find a single gene responsible for *Ph1* phenotype in ethylmethanesulphonate (EMS) mutant population failed, because Griffiths *et al.* (2006) was unsuccessful to identify any line showing sufficient *ph1b*-like phenotype. The region of its presence was narrowed down to a 2.5 Mb area of the long arm of 5B chromosome, carrying a set of *CDK2*-like and methyl-transferase genes duplicated from a chromosome 3B (Griffiths *et al*., 2006; Al-Kaff *et al*., 2007; Martín *et al*., 2017). In the region, there were identified two candidate genes

responsible for *Ph1* phenotype proposed by different research groups, namely *C-Ph1* (Bhullar *et al*., 2014) and *TaZIP4-B2* (Chelysheva *et al*., 2007; Shen *et al*., 2012; Rey *et al*., 2017). However, deletion lines lacking *C-Ph1* did not show the *ph1b*-like phenotype and furthermore, this gene is specific to tapetal cells (Al-Kaff *et al*., 2007; Wang *et al.,* 2003). *TaZIP4-B2*, a paralog of *ZIP4* from *Arabidopsis* and rice is handling homologous crossing-overs (Chelysheva *et al*., 2007; Shen *et al*., 2012; Rey *et al*., 2017). The utilization of EMS and CRISPR mutants of *TaZIP4-B2* in a hybrid of wheat and *Ae. variabilis* resulted in a higher number of homoeologous chromosome pairing, however the level of multivalent and univalent formation was not the same as in *ph1b* variant (Rey *et al*., 2017; 2018), nonetheless this gene remains the strongest candidate. Further analyses are necessary to uncover the molecular mechanism of *Ph1*.

1.3.2 *Pairing homoeologous 2 (Ph2)*

Another gene ensuring a homologous chromosome pairing in wheat is located on chromosome 3D (Mello-Sampayo, 1968; 1971). It was discovered through observation of multivalent formation in pentaploid hybrids between hexaploid wheat lacking chromosome 3D and *T. durum* or *Aegilops* spp. (Mello-Sampayo, 1968; 1971). This gene is called *Ph2* and has a weaker effect than *Ph1*. The further mapping was performed through utilization of two derived mutants, an X-ray mutant carrying a large deletion on chromosome 3D *ph2a* (Sears, 1982) and a chemically induced mutant *ph2b* carrying point mutations caused by ethyl methanesulfonate (EMS) (Wall *et al*., 1971). The *Ph2* gene was mapped to a distal 80 Mb of a short arm of chromosome 3D through studies exploiting *ph2a* mutant and synteny with rice (Sutton *et al*., 2003). However, using molecular markers, Svačina *et al*. 2020b demonstrated that the deletion size is larger than previously believed, encompassing about 125 Mb of the terminal part of short arm of chromosome 3D (Chapter 3 Results). Even more precise analysis using a high-density SNP genotyping array (35K SNP Affymetrix Axiom®) of *ph2a* mutant showed that the deletion breakpoint is at 121 Mb, containing about 1577 annotated genes (Chapter 3 Results; IWGSC, 2018; Serra *et al*., 2020).

The course of action seems to differ between *Ph1* and *Ph2* genes, as each of these genes takes effect in different stages of meiosis (Benavente *et al*., 1998; Martínez *et al*., 2001a). The result of mutation of *Ph2* gene is different in hexaploid wheat, as it only shows a slight raise of univalent formation in comparison with *Ph1* mutation, which also increases a creation of multivalents (Table 1; Martinez *et al*., 2001a; Sanchez-Moran *et al*., 2001). Its mutant phenotype is clearer in hybrids with closely-related species, where it causes homoeologous chromosome recombinations (Sears 1977; 1982), which is why it was studied mainly in this configuration. Hybrids of *ph2* mutant and rye are frequently used in scientific studies, as only negligible level of background homoeologous chromosome recombinations are observed in its non-mutant variants that could influence the consistency of results. Prieto *et al*. (2005) observed the expected behaviour of *Ph2* mutation in wheat x rye hybrid, a higher level of homoeologous chromosome recombination. However, using GISH, his team proved that almost only wheat-wheat associations form, while wheat-rye and rye-rye are rare. In case of *Ph1* mutation, the hybrids show higher numbers in all types of associations compared to *Ph2* mutation (Table 2). According to these studies, the effect of *Ph2* gene seems to have importance in both euploid wheat, where it prevents creation of univalents and in case of missing homologues, it suppresses a formation of chromosome associations between homoeologues (Table 1; Table 2; Martinez *et al*., 2001a; Sanchez-Moran *et al*., 2001; Prieto *et al*., 2005).

Martinez *et al*. (2001a) and Prieto *et al*. (2005) suggest that *Ph2* gene works in different stages of meiosis than *Ph1*, as it affects a progression of synapsis, however it is possible that both genes cooperate in their ways of function (Boden *et al*., 2009). The attempts to identify the gene responsible for the *Ph2* phenotype, a number of candidates was selected, a *TaMSH7-3D*, a homologue of DNA mismatch repair gene in yeast (Dong *et al*., 2002), *WM5* (Thomas, 1997) and *WM1* genes (Ji and Langridge, 1994; Whitford, 2002), however *Ph2* gene was not identified up to this day.

Table 2 I Number of chromosome-arm associations in hybrids of euploid wheat 'Chinese Spring' and *ph1b* and *ph2b* mutans with rye (Prieto *et al.*, 2005).

Genotype	CS x rye	*ph2b* x rye	*ph1b* x rye
Chromosome number	28	28	28
Wheat-wheat	0.48	1.68	7.14
Wheat-rye	0.08	0.08	0.59
Rye-rye	0.02	0.04	0.05
Total	0.58	1.8	7.78

1.3.3 *TaMSH7-3D*

TaMSH7-3D is one of the candidate genes potentially responsible for *Ph2* phenotype (Dong *et al*., 2002). The *MSH7* gene is probably derived through replication and divergence of *MSH6*-like gene in early plant evolution (Culligan, 2000; Culligan and Hays, 2000). It is a plant specific homologue of *MutS 7* gene in yeast and belongs to the DNA mismatch repair family (MMR) (Dong *et al*., 2002). The MSH proteins are highly conserved and play a crucial role in initial steps of MMR pathway, by recognizing the base-base mismatches and insertion/deletion mutations that were created during DNA replication (Reyes *et al.*, 2016). MSH7 acts in a heterocomplex with MSH2, while in *Arabidopsis*, studies showed that it recognizes base mismatches A/A, C/A, G/A, G/G and partially G/T (Culligan and Hays, 2000; Wu *et al.*, 2003; Gómez and Spampinato, 2013).

1.3.4 Chromosome pairing in hybrids of wheat and its *ph* mutants

The effect of mutations of *Ph* genes on phenotype is mostly being studied either in haploids or in interspecific hybrids between closely-related species, since its impact is much more easily scored in the absence of homologous chromosomes. The level of homoeologous chromosome associations is usually scored cytogenetically and in both *ph1* and *ph2* mutants varies based on the level of homology between the sets of chromosomes, the higher the homology, the greater number of associations (Naranjo *et al*., 1987; 1988; Naranjo and Maestra, 1995; Maestra and Naranjo, 1997; 1998).

In some cases, the homology of subgenomes of hybrids between wheat and phylogenetically close species is too high, consequently the *Ph* system of wheat

starts to fail the recognition of homologues even in non-mutant plants. An example of this phenomenon is a hybrid of wheat and *Ae. speltoides*, which is related to a donor of B genome (Table 3; Dvorak and Zhang, 1990). On the opposite end, the combination of wheat and rye show the smallest level of chromosome pairing among the studied cases, this can be explained by the fact, that genus *Aegilops* diverged from wheat only 2.5 – 5 million years ago, while rye about 7 million years ago, making rye more distant relative (Table 3; Huang *et al.*, 2002). The higher level of chromosome pairing is however not always caused by the homology of involved subgenomes, as there have been shown number of examples of suppressors of *Ph* gene activity, as in study of Riley (1960), Dover and Riley (1972), Dvorak *et al*. (2006), Koo *et al*. (2017) and Liu *et al*. (2011).

Table 3 I Chromosome pairing numbers of associations in hybrids of wheat and *Ph* mutants with closely-related species, all having 28 chromosomes (Naranjo *et al*., 1987; 1988; Naranjo and Maestra, 1995; Maestra and Naranjo, 1997; 1998).

Hybrid	Univalents	Rod bivalents	Ring bivalents	Multivalents	Chiasmata/ cell
WT x *S. cereale*	26.31	0.80	0.03	0.01	0.88
ph2b x *S. cereale*	19.23	3.4	0.57	0.51	5.26
ph1b x *S. cereale*	11.76	2.33	2.36	2.16	12.35
WT x *Ae. longissima*	24.55	1.59	0.06	0.05	1.81
ph2b x *Ae. longissima*	14.93	5.8	0.58	0.55	7.44
ph1b x *Ae. longissima*	3.48	4.4	2.99	2.86	18.28
WT x *Ae. sharonensis*	25.21	1.18	0.03	0.03	1.29
ph2b x *Ae. sharonensis*	10.16	5.58	1.42	1.13	11.17
ph1b x *Ae. sharonensis*	4.37	3.74	3.79	2.39	17.93
WT x *Ae. speltoides*	3.97	4.9	3.11	2.61	17.79
ph2b x *Ae. speltoides*	3.25	3.41	3.28	3.2	19.41
ph1b x *Ae. speltoides*	2.53	3.36	4.29	2.68	20.08

1.3.5 Utilization of *Ph* genes in wheat breeding

In the past, the crossing of wheat was solely based on selection for a higher grain yield, domestication traits and resistance to biotic and abiotic stress. This process resulted in a number of landraces that are locally adapted to the place of its origin (Kiszonas and Morris, 2018). Later, the wheat breeders started to combine beneficial traits in existing cultivars and through phenotyping selected individuals

with higher quality for bread and pasta production. With the uprise of 'DNA era', the crossing started to rely on identification of genes responsible for agronomically important phenotypes, with use of marker assisted selection (Sorrells, 2007). In any way, crossing of cereals partially relies on utilization of diversity of related domesticated and non-domesticated species. In case the related species has high enough chromosomal homology, the introgression to wheat can be performed solely by hybridization of such species and subsequential backcrossing to wheat and selection of introgressed individuals that underwent homoeologous recombination (Friebe *et al.*, 1996). However, in case of more distant relatives, the recombination does not take place, as the homology is too low for the meiotic apparatus of wheat. To overcome this obstacle, the mutation of *Ph1* gene is being utilized for decreasing the stringency of homologue recognition, allowing the recombination process to pass even between homoeologous chromosomes (Friebe *et al.*, 2012). The utilization of *ph1* mutant to introgress a beneficial trait from related species to wheat was performed in countless studies (Niu *et al.*, 2011; Han *et al.*, 2016; Able and Atienza, 2014; Ayala-Navarrete *et al.*, 2013; Marais *et al.*, 2010). This method of breeding is however very laborious and demanding, as the recombinations are partially random and to select beneficial and precise combinations, large populations need to be screened and analysed to isolate new cultivar.

1.4 Mapping through deletion lines

Mapping through deletion lines is a type of physical mapping, where a position of a mapped genetic element is characterized by so-called "bins". Deletion bin is a unit representing an interval between two closest deletions in distinct lines. This kind of approach is dependent on a mapping population, while the acquired resolution of mapped genetic element is directly proportional to the number of lines with different deletion sizes.

This kind of approach is mainly used for qualitative gene/marker mapping and integration of physical maps in sequencing projects. Deletion mapping is best suited for traits with distinctive phenotype, where mapping population provides information of location of responsible gene, such as in cloning efforts of *Ph2* gene in this thesis.

There have been numerous studies utilizing deletion mapping for various purposes. As an example, the speltoid suppression gene (Q) and β-amylase (β-Amy-A2) in common wheat were identified to an approximate location on a long arm of chromosome 5A (Tsujimoto and Noda, 1990). Another example of deletion line use is delimiting the *Ph1* gene location on chromosome 5B (Gill *et al*., 1993).

1.5 Gametocidal genes

The ability of wheat to tolerate aneuploidy enables a creation of various stocks, such as deletion, substitution and addition lines. In the past century, a lot of research was performed to study the effect of alien chromosomes (often from genus *Aegilops*) introduced to wheat. Endo and Tsunewaki (1975) and Maan (1975) were experimenting with hybrids between wheat and *Aegilops* and noticed that back-crossing to wheat did not remove certain chromosomes, later called gametocidal (Gc). Moreover, Finch *et al.* (1984) observed chromosomal aberrations in the gametes of these lines. These genetic units were also observed earlier and were at first called as "pollen killer" (Cameron and Moav, 1957; Loegering and Sears, 1963) and "gamete eliminator" (Rick, 1966; Sano, 1990), however nowadays they are most frequently called gametocidal genes/chromosomes (Endo, 1990; 2007). After introgression to wheat, these genetic units are being inherited in a selfish manner through induction of genomic aberrations in gametes where it is absent and are otherwise dispensable to the host (Endo, 1990; 2007); this effect is inherently only visible when dealing with gametes of monosomic addition line of gametocidal chromosome.

Additional gametocidal chromosomes were discovered in various species from the genus *Aegilops*. These chromosomes belong to genomes C, S and M and homoeologous groups 2, 3, 4, 6 and have different effects when introgressed into wheat genome (Tsujimoto, 2005). The severity of gametocidal action is both dependent on the involved Gc chromosome and the variety of host wheat plant. The chromosome 2C from *Ae. cylindrica* introduced to wheat can be used as an example. Once introduced to the cultivar 'Jones Fife', it causes a complete sterility of gametes lacking the 2C gametocidal chromosome. However in case of a host cultivar

‘Chinese Spring’, the gametocidal action is only partial, and the gametes lacking the 2C chromosome survive, however often carrying terminal chromosomal deletions, as shown in Figure 6 (Endo, 1988). The gametocidal genes seem to have a double function to operate in its absence. The first function is to induce genomic damage in gametes that lack this gene, however in the same time preventing it in gametes that contain it (Friebe *et al.*, 2003), not much is known about gametocidal chromosomes otherwise.

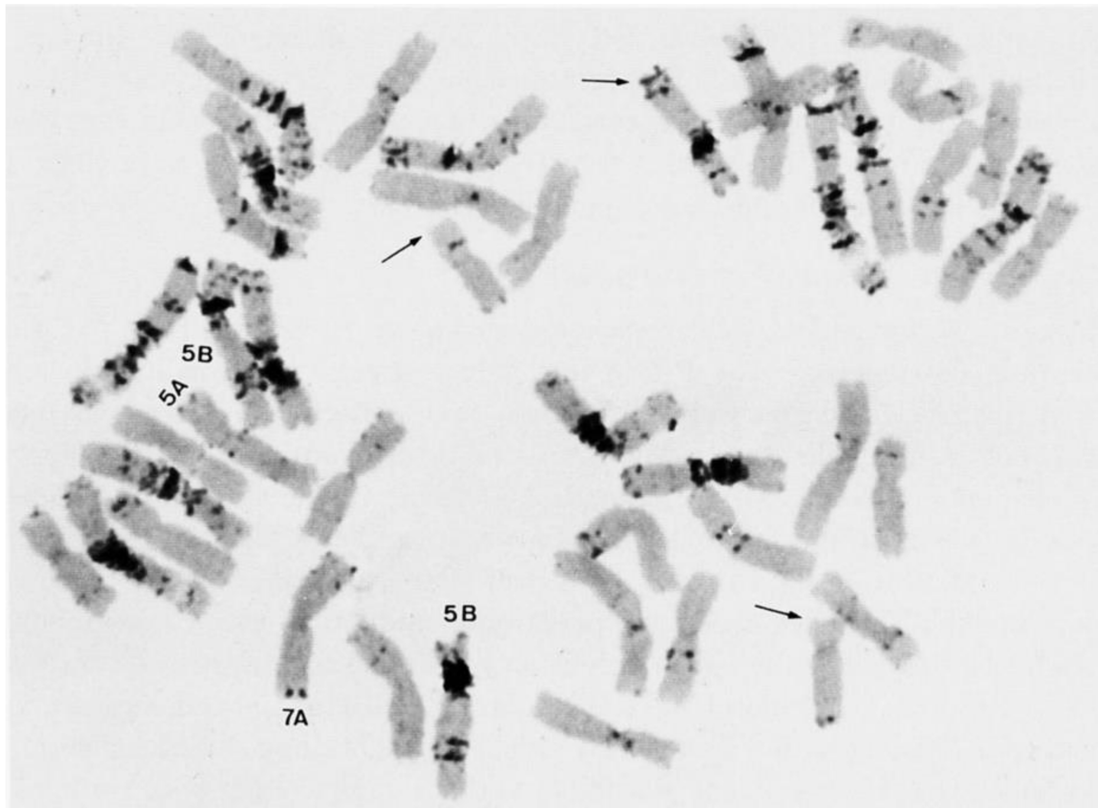

Figure 6 | C-banding of a wheat variety ‘Chinese Spring’ with terminal deletions. The deletions are on chromosome arms 5AL, 7AL and 5BL (shown by arrows) induced by 2C gametocidal chromosome. The non-aberrant chromosomes are shown by in picture text of 5A, 5B (trisomic) and 7A (Endo, 1990).

The wheat deletion lines have been utilised as a genomic tool for decades, because of its easy and straightforward use. Such deletion lines can be produced by various ways, irradiation—such as *ph2a* mutant in wheat (Sears, 1982)—and usage of gametocidal chromosomes (Endo, 1988). Endo and Gill (1996) used 2C gametocidal chromosome monosomic addition line in bread wheat (6x = 2n = 43; AABBDD + 2C′) to create 436 deletion lines. These lines carry random terminal

deletions across all chromosomes that were subsequently fixed in homozygous/hemizygous configuration. As an example, all 12 lines from this study carrying a deletion on chromosome 3D are shown in Figue 7. These deletion lines were utilised as a powerful tool for mapping numerous genes and markers (Sourdille *et al*., 2004).

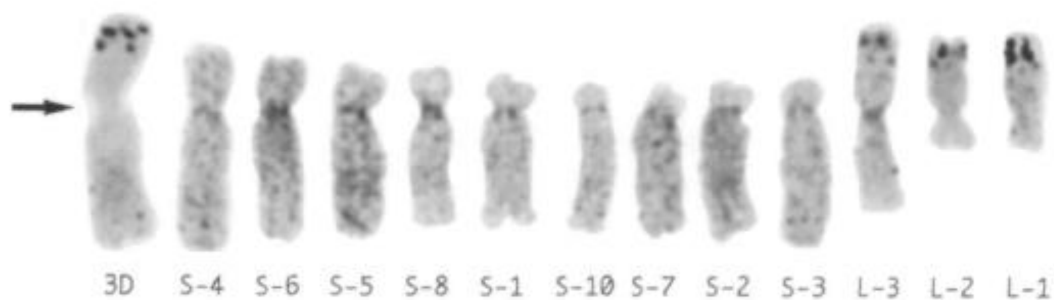

Figure 7 I Deletion lines of chromosome 3D of wheat variety 'Chinese Spring'. The chromosomes were stained by C-banding, the centromere is shown by arrow (Endo and Gill, 1996).

However, for mapping a single gene with sufficient resolution, much higher number of deletion lines varying in aberration sizes with a special focus on a single chromosome is necessary. As an example, the *Ph2* gene is known to be located in the terminal 121 Mb region of short arm of chromosome 3D (Chapter 3 Results; IWGSC, 2018; Serra *et al*., 2020). However, there was never created any material with sufficient resolution to map this gene further. For this purpose, a crossing schedule developed by Endo and Gill (1996) can be modified for creation of single-chromosome deletion lines (Figure 8; Svačina *et al*., 2020b). The first crossing takes place between 2C gametocidal chromosome monosomic addition line in bread wheat (male; 6x = 2n = 43; AABBDD + 2C′) and wheat nulli-tetrasomic lines lacking chromosome 3D with tetrasomic constitution either for chromosome 3A or 3B (female; 6x = 2n = 42; AABBDD − 3D″ + 3A″/3B″). The gametes lacking 2C gametocidal chromosome from addition line carry terminal deletions on various number of chromosomes. However, after fertilization of gamete from nulli-tetrasomic lines, all aberration apart from those on chromosome 3D will be masked by a healthy chromosome. The resulting plant will therefore carry aberrant chromosome 3D in a monosomic constitution. Subsequent self-fertilization is thus

necessary to ensure a stable inheritance. After characterizing this material using molecular markers, this tool can be used for many purposes, such as gene and marker mapping, or as a mutant for absent gene(s).

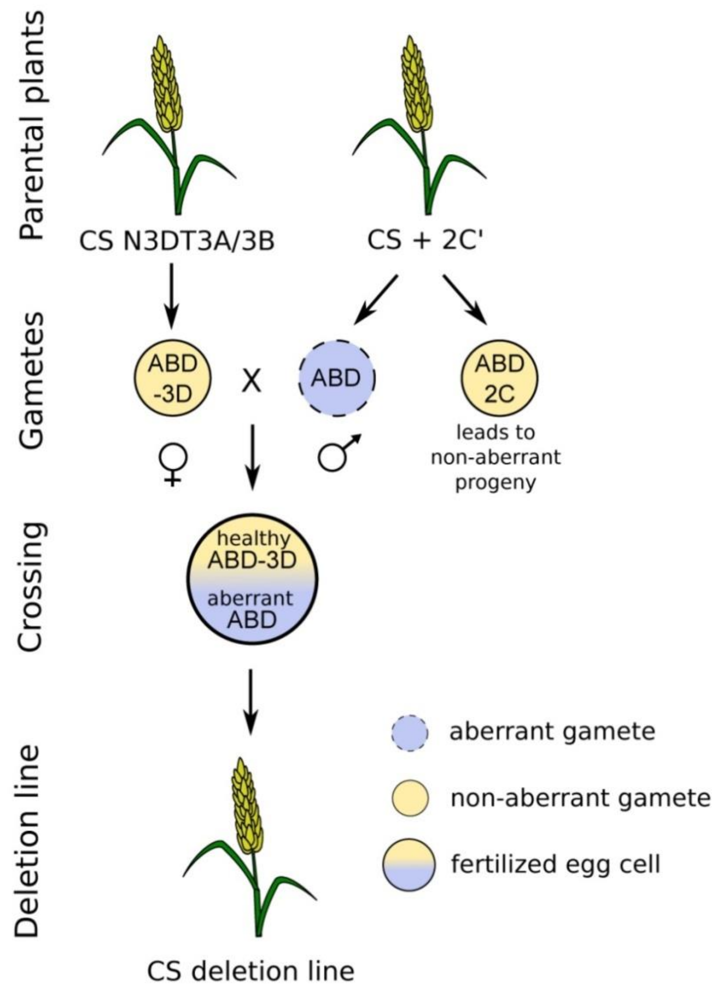

Figure 8 I Crossing scheme for creation of chromosomal deletion lines in 'Chinese Spring' variety of wheat. The creation of deletion lines is performed by crossing 2C monosomic addition lines of 'Chinese Spring' with nulli-tetrasomic lines lacking 3D chromosome. The progeny lacking the 2C gametocidal chromosome will potentially carry a deletion on chromosome 3D (Svačina *et al.*, 2020b).

1.6 EMS-induced mutagenesis and TILLING

Ethyl methanesulfonate (EMS) is a mutagenic compound widely used in induction of point mutations in genetic studies. Its genotoxicity is mediated through creation of O^6-methylguanine adducts mispairing with thymine during replication, mainly leading to transitions from GC to AT (Figure 9; Kim *et al.*, 2006). Other

mutations can be observed, such as transversions, frameshifts and deletions, however these are relatively rare (Bökel, 2008).

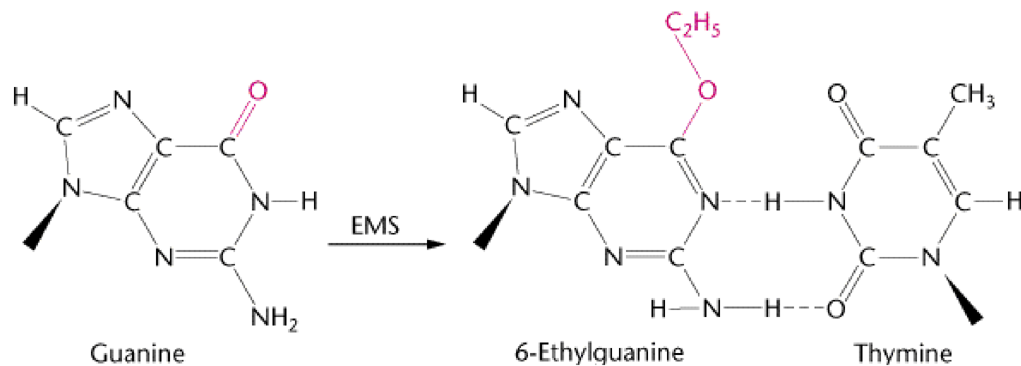

Figure 9 I EMS mutagenesis mechanism. Random mutations are created through guanine alkylation, resulting in nucleotide substitutions (Klug and Cummings, 1997).

EMS is mainly used for creation of mutants used for TILLING (Targeting Induced Local Lesions IN Genomes), which is a method in reverse genetics that combines chemical mutagenesis with high-througoutput genome-wide screening for point mutation in candidate genes (Serrat *et al.*, 2014). One of applications of this technique is the identification of loss-of-function phenotype with subsequent analysis of candidate gene(s) on a sequence level. The main disadvantage of this method is creation of random point mutations throughout the whole genome, thus analysis of several different individuals with same or similar loss-of-function phenotype is needed for conclusive gene identification. Nevertheless, many genes were identified through this approach, such as s leaf rust resistence gene *Rph1* in barley (Dracatos *et al.*, 2019), genes *Pinb*, *Waxy*, *Agp2* and *SSIIa-A*, playing a key role in kernel hardness and starch biosynthesis in wheat (Li *et al.*, 2017) and a mutated gene *FAD2B* responsible for higher oleate content in peanut (Fang *et al.*, 2012).

2 AIMS OF THE THESIS

I Development and characterization of deletion lines for chromosome 3D of bread wheat

The first aim of the thesis was the development of terminal deletion line stock for the chromosome 3D of bread wheat using system involving gametocidal chromosome 2C of *Ae. cylindrica*. This procedure constisted of crosses between wheat and its addition lines carrying 2C chromosome of *Ae. cylindrica* in monosomic constitution. The created deletion lines were scored with molecular markers to determine deletion sizes with subsequent FISH analysis as a control.

II Deletion mapping of *Ph2* gene located on the short arm of chromosome 3D

The second aim of this work was delimitation of *Ph2* locus area. The phenotype of this gene is more visible in haploid hybrids between wheat and closely-related species, such as rye. The deletion lines were crossed with rye, with subsequent scoring of number of homoeologous chromosome associations in flowering stage of this hybrid, more precisely in metaphase I. The locus of *Ph2* gene was mapped to an area between closest deletions with contrasting phenotypes.

III Candidate gene(s) selection and validation using TILLING population of wheat

The candidate gene(s) were derived by combining the positional information from deletion mapping and exome sequencing of *ph2b* EMS mutant. The available TILLING population of wheat was exploited for validation of gene(s) in the deletion area that carry a mutation in *ph2b* mutant. The TILLING lines with point mutations in candidate gene(s) were crossed with *Ae. variabilis* to score number of homoeologous chromosome associations.

3 RESULTS

The first goal was to develop a set of deletion lines to delimit the area of *Ph2* gene locus. The deletion lines were produced by gametocidal system (Endo and Gill, 1996). In this procedure, monosomic addition line of chromosome 2C from *Ae. cylindrica* on wheat 'Chinese Spring' background (6x = 2n = 43; AABBDD + 2C′) and the nulli-tetrasomic lines (6x = 2n = 42; AABBDD − 3D″ + 3A″/3B″) crosses were performed (see Figure 8 in section 1.5). These crosses yielded 6169 seeds in F1 generation potentially carrying a terminal deletion on 3D chromosome in monosomic constitution. These plants were germinated and screened with STS markers positioned in terminal ends on chromosome 3D using PCR to identify lines carrying a deletion. In total, 113 lines were developed, 43 (39.13 %) of the lines had a deletion on the short arm and 68 (60.87 %) carried a deletion on the long arm of chromosome 3D, while two lines had deletion on both arms. These numbers correspond with arm-length ratio of 0.393 (240 Mb) for 3DS and 0.607 (370 Mb) for 3DL, suggesting that deletion sites occur randomly (IWGSC, 2018; Svačina *et al.*, 2020b).

To make this material usable for physical mapping, the whole set of deletion lines was characterized using STS molecular markers designed from reference sequence developed by IWGSC (2018). In total, 84 markers distributed along the entire schromosome 3D were designed and used for characterization of whole set of deletion lines. The size of terminal deletions ranged from 6.5 to 357 Mb, while the size of deletion bins (the region between two adjacent deletion breakpoints) ranged from 0.15 to 50 Mb (Svačina *et al.*, 2020b). The deletion size was verified on the number of lines using FISH (Figure 10).

The *Ph2* gene was originally mapped to a distal 80 Mb of a short arm of chromosome 3D (Sutton, *et al.*, 2003). STS markers however showed that the deletion size is larger than previously believed, encompassing about 125 Mb of the terminal part of short arm of chromosome 3D (Svačina *et al.*, 2020b). Subsequent analysis using a high-density SNP genotyping array (35K SNP Affymetrix Axiom®)

of *ph2a* mutant showed that the deletion breakpoint is at 121 Mb, containing about 1577 annotated genes (IWGSC, 2018; Serra *et al*., 2020).

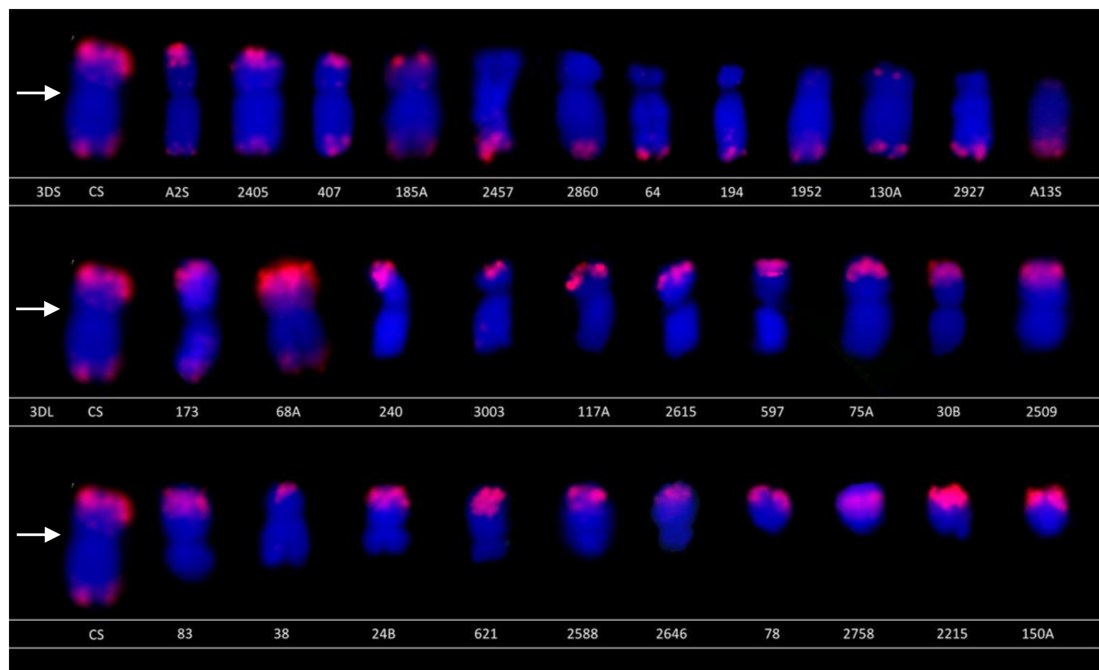

Figure 10 I Selected lines characterized by FISH to confirm the deletion size. The chromosomes were labeled using $(GAA)_n$ microsatellite (green) and Afa repeat (red) to distinguish chromosome 3D from other chromosomes. Only Afa repeat is present on chromosome 3D. Arrow shows the location of centromere (Svačina *et al*., 2020b).

All the deletion lines were self-pollinated to fix chromosome 3D carrying deletion to disomic constitution, to increase stability of this material. The number of chromosome 3D was detected using ddPCR technique, where primer set and probe on chromosome 4A acted as a reference (disomic in all lines). The disomic constitution of chromosome 3D was established in 102 lines out of 113 (Svačina *et al*., 2020b).

A subset of 32 deletion lines was selected that carried a deletion in area of interest of 121 Mb of terminal part of short arm of chromosome 3D (size of deletion in *ph2a* mutant) (Serra *et al*., 2020). These deletion lines were crossed with rye, since the *ph2* mutant phenotype is easily distinguishable in wheat-rye haploid (ABDR) hybrids (Mello-Sampayo and Canas, 1973). The progeny of these crosses were checked for 3D deleted chromosome presence. Cytogenetic analyses were performed

in 21 haploid hybrids to score a number of chiasmata in metaphase I in anthers of each line, calculated from the number of univalents, bivalents (rod and ring) and multivalents (Serra *et al.*, 2020). The average number of chiasmata in wheat-rye hybrids indicate that in non-mutant lines, chromosomes rarely associate (0.38 ± 0.10 chiasma/meiocyte), however the number of chiasmata was increased in *ph2a* hybrid (3.10 ± 0.13 chiasmata/meiocyte) (Figure 11; Serra *et al.*, 2020), which is in agreement with previous studies (Sears, 1982). The analyses of homoeologous chromosomal associations revealed that in terminal deletion line hybrids with deletion sizes higher than 79.2 Mb, the chiasmata frequency is increased (ranging from 2.6 to 3.92 chiasmata/meiocyte). Conversely, in individuals with deletion shorter than 64.9 Mb, frequency is lower than 2 chiasmata per meiocyte (Figure 11; Serra *et al.*, 2020). These differences in chiasmata frequency indicate that *Ph2* gene is located in 14.3 Mb area ranging between deletion lines contrasting in phenotype with deletion sizes 64.9 and 79.2 Mb on the short arm of chromosome 3D.

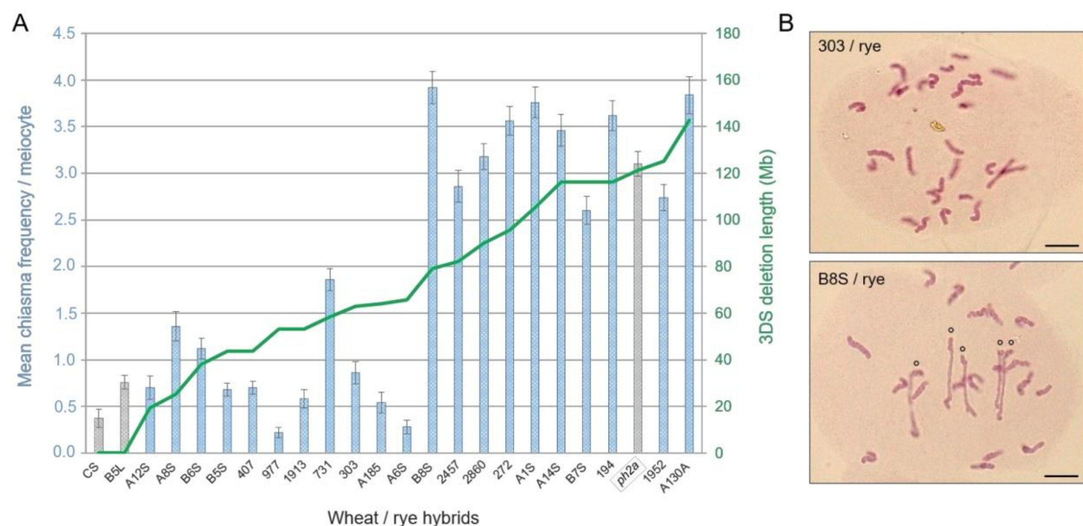

Figure 11 I Deletion mapping of Ph2 gene using wheat-rye hybrids carrying various 3DS deletions. (A) Frequency of chiasmata per meiocyte in WT, *ph2a* control and various mutant hybrids of wheat chromosome 3D mutants and rye. Histogram bars show the mean ± standard error (B) Meiocytes with contrasting *Ph2* phenotypes, 303/rye having a *Ph2* locus and B8S/rye lacking *Ph2* locus. Dots are showing rod bivalents, scale bars represent 10 µm (Serra *et al*., 2020).

To identify the potential candidate genes for *Ph2*, we executed exome capture of EMS-induced mutant *ph2b*, performed at INRA GDEC (Clermont-Ferrand,

France) (Serra *et al.*, 2020). The mutant *ph2b* carries point mutations alongside its whole genome, while one of those mutations disrupted the effect of *Ph2* gene (Wall *et al.*, 1971). The comparison of genic areas between *ph2b* and WT 'Chinese Spring' wheat showed 59 SNPs within the 121 Mb deletion of *ph2a* mutant. Using this approach, number of candidates was reduced to 24 (Serra *et al.*, 2020). However, only one candidate (*TraesCS3D02G119400*) was present in 14.3 Mb area deduced from deletion mapping. This gene encodes a DNA mismatch repair protein TaMSH7-3D. In *ph2b* mutant, RNA seq was used to verify its function being disrupted by a SNP compromising correct splicing, leading to a premature STOP codon (Figure 12; Serra *et al.*, 2020).

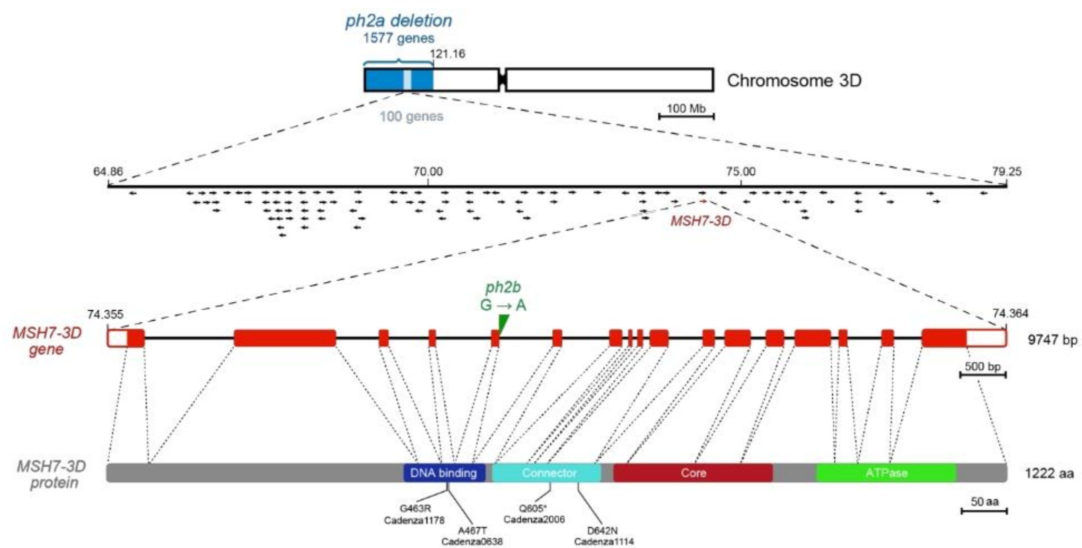

Figure 12 I Schematic representation of *TaMSH7-3D* gene, a candidate for *Ph2* phenotype. The first two lines of schematic overview shows a *ph2a* mutation on chromosome 3D and a location of *TaMSH7-3D* location in this area. The second two lines depict exons of *TaMSH7-3D*, with a location of mutation in *ph2b* mutant and its functional domains and the point mutations in used 'Cadenza' TILLING lines (Serra *et al.*, 2020).

To validate the *TaMSH7-3D* gene to be responsible for *Ph2* phenotype, TILLING population of wheat EMS mutants of 'Cadenza' cultivar was exploited (www.wheat-tilling.com) (King *et al.*, 2015; Krasileva *et al.*, 2017). In cooperation with INRA, GDEC (Clermont-Ferrand, France), 7 possible mutants for *TaMSH7-3D* were selected to analyse whether the phenotype will correspond to *ph2a/b* mutants. These EMS mutants and WT were crossed with *Ae. variabilis*, since 'Cadenza'

cultivar is not cross-compatible with rye. In the haploid hybrid progeny, the frequency of chiasmata was scored, based on numbers of univalents, ring and rod bivalents and multivalents in metaphase I of anthers. The hybrid of WT 'Cadenza' and *Ae. variabilis* showed on average 1.10 (± 0.09) per meiocyte at metaphase I (Serra *et al*., 2020). Out the seven mutant hybrids, four showed increased frequency of chiasmata in metaphase I, Cadenza2006 x *Ae. variabilis* had the strongest mutant phenotype of 6.07 ± 0.17 chiasmata on average, which is a 5.52-fold increase (Serra *et al*., 2020). The hybrids of Cadenza0638, Cadenza1178 and Cadenza1114 and *Ae. variabilis* showed 2.21, 1.90 and 2.37-fold increase in chiasmata frequency respectively. This difference can be attributed to the type of mutation in these hybrids, Cadenza2006 having a premature STOP codon, while others having amino acid substitution (Figure 13; Serra *et al*., 2020). This analysis functionally validates the *TaMSH7-3D* as a gene responsible for *Ph2* phenotype.

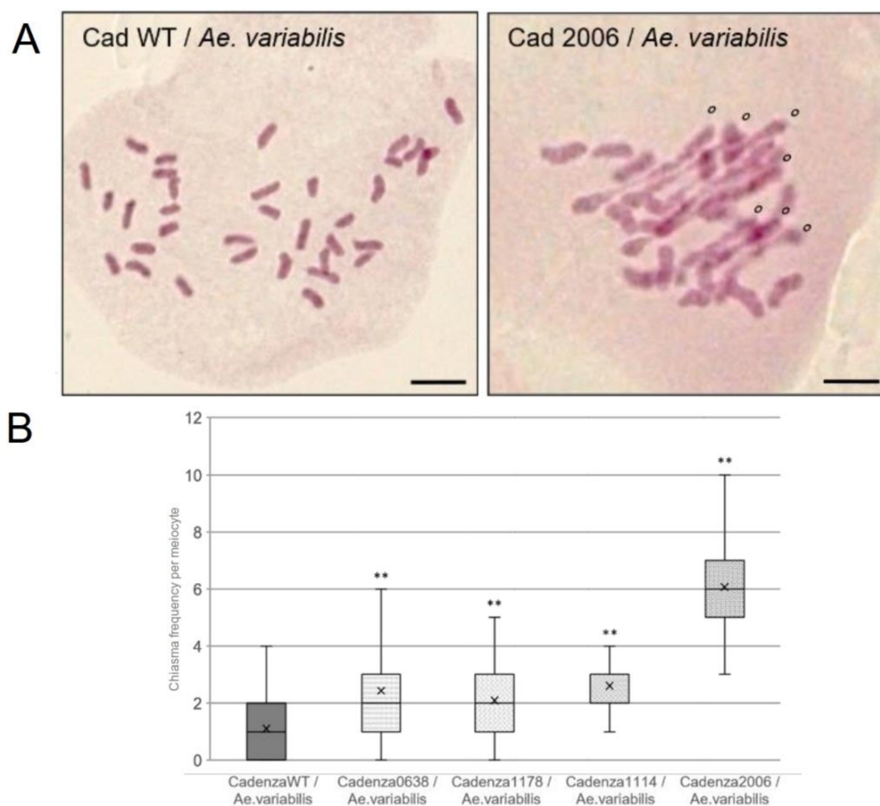

Figure 13 I Chiasmata frequency in different *TaMSH7-3D* mutant hybrids of 'Cadenza' wheat cultivar and *Ae. variabilis*. (A) An effect of *TaMSH7-3D* mutation on homoeologous chromosome chiasmata frequency in metaphase I. (B) Average number of chiasmata in WT and different Ta*MSH7-3D* mutants The significance indicator ** report a p value p < 0.001 (Serra *et al*., 2020).

3.1 Original publications

3.1.1 Development of deletion lines for chromosome 3D of bread wheat

(Appendix I)

3.1.2 *Pairing homoeologous 2* (*Ph2*) encodes the mismatch repair protein MSH7-3D that inhibits homoeologous recombination in wheat

(Appendix II)

3.1.3 Chromosome pairing in polyploid grasses

(Appendix III)

3.1.1 Development of deletion lines for chromosome 3D of bread wheat

Svačina, R., Karafiátová, M., Malurová, M., Serra, H., Vítek, D., Endo, T.R., Sourdille, P., Bartoš, J.

Frontiers in Plant Science **10**: 1756, 2020

doi: 10.3389/fpls.2019.01756

IF: 4.298

Abstract:

The identification of genes of agronomic interest in bread wheat (*Triticum aestivum* L.) is hampered by its allopolyploid nature (2n = 6x = 42; AABBDD) and its very large genome, which is largely covered by transposable elements. However, owing to this complex structure, aneuploid stocks can be developed in which fragments or entire chromosomes are missing, sometimes resulting in visible phenotypes that help in the cloning of affected genes. In this study, the 2C gametocidal chromosome from Aegilops cylindrica was used to develop a set of 113 deletion lines for chromosome 3D in the reference cultivar Chinese Spring. Eighty-four markers were used to show that the deletions evenly covered chromosome 3D and ranged from 6.5 to 357 Mb. Cytogenetic analyses confirmed that the physical size of the deletions correlated well with the known molecular size deduced from the reference sequence. This new genetic stock will be useful for positional cloning of genes on chromosome 3D, especially for *Ph2* affecting homoeologous pairing in bread wheat.

3.1.2 *Pairing homoeologous 2* (*Ph2*) encodes the mismatch repair protein MSH7-3D that inhibits homoeologous recombination in wheat

Serra, H., Svačina, R., Baumann, U., Whitford, R., Sutton, T., Bartoš, J., Sourdille, P.

Nature Communications, 2020

Manuscript submitted for publication

IF: 12.121

Abstract:

Meiotic recombination is a critical process for plant breeding, as it is the basis for creating novel allele combinations that can be exploited for crop improvement. In wheat, a complex allohexaploid that has a diploid behaviour, meiotic recombination between homoeologous or alien chromosomes is suppressed through the action of several loci. Here we report positional cloning of *Pairing homoeologous 2* (*Ph2*), first demonstrating that it encodes a DNA mismatch repair protein MSH7-3D, thus solving a half-century-old question. Similar to *ph2*, we show that by mutating *MSH7-3D*, it induces a substantial homoeologous recombination increase (up to 5.5 fold) in wheat-wild relative hybrids, which is also associated with a slight reduction in homologous recombination. This data reveals a role for *MSH7-3D* in meiotic stabilisation of allopolyploidy and opens an opportunity to improve wheat's genetic diversity through alien gene introgression, a major bottleneck facing crop improvement.

3.1.3 Chromosome pairing in polyploid grasses

Svačina, R., Sourdille, P., Kopecký, D., Bartoš, J.

Frontiers in Plant Science **11**: 1056, 2020

doi: 10.3389/fpls.2020.01056

IF: 4.298

Abstract:

Polyploids are species in which three or more sets of chromosomes coexist. Polyploidy frequently occurs in plants and plays a major role in their evolution. Based on their origin, polyploid species can be divided into two groups: autopolyploids and allopolyploids. The autopolyploids arise by multiplication of the chromosome sets from a single species, whereas allopolyploids emerge from the hybridization between distinct species followed or preceded by whole genome duplication, leading to the combination of divergent genomes. Having a polyploid constitution offers some fitness advantages, which could become evolutionarily successful. Nevertheless, polyploid species must develop mechanism(s) that control proper segregation of genetic material during meiosis, and hence, genome stability. Otherwise, the coexistence of more than two copies of the same or similar chromosome sets may lead to multivalent formation during the first meiotic division and subsequent production of aneuploid gametes. In this review, we aim to discuss the pathways leading to the formation of polyploids, the occurrence of polyploidy in the grass family (Poaceae), and mechanisms controlling chromosome associations during meiosis, with special emphasis on wheat.

3.2 Published abstracts – poster presentations

3.2.1 Development of deletion lines for physical mapping of *Ph2* gene in bread wheat
(Appendix IV)

3.2.2 Development of chromosome deletion lines for *Ph2* gene mapping in bread wheat
(Appendix V)

3.2.3 *Ph2* gene mapping through development and phenotyping of deletion lines in bread wheat
(Appendix VI)

3.2.4 Towards identification of *Ph2*, a gene controllong homoeologous chromosome pairing in bread wheat
(Appendix VII)

3.2.5 *Ph2* gene phenotype scoring in wheat-rye hybrids with terminal deletions of 3D chromosome
(Appendix VIII)

3.2.6 Wheat-rye hybrids with chromosome deletions analysed for *Ph2* gene phenotype
(Appendix IX)

3.2.7 *Ph2* gene mapping through phenotyping of wheat-rye hybrid deletion lines
(Appendix X)

3.2.1 Development of deletion lines for physical mapping of *Ph2* gene in bread wheat

Svačina, R., Bartoš, J., Karafiátová, M., Sourdille, P., Endo, T.R., Doležel, J.

In: Proceedings of the "13th International Wheat Genetics Symposium". Tulln, Austria, 2017

Abstract:

Bread wheat (*Triticum aestivum* L.) is an allohexaploid species. Its genetic information consists of 3 subgenomes (A, B and D), formed by hybridisation of three progenitors. Hybridisation between 3 close-related species caused a coexistence of highly similar homoelogous chromosomes. Mechanisms of precise chromosome pairing had to be developed, so diploid-like behavior is secured. Homologous pairing of chromosomes in wheat is primarily controlled genetically by *Ph* genes. *Ph2* gene was located on a short arm of chromosome 3D. Mutant plants of this gene *ph2a* and *ph2b* were observed and only small effect on homoelogous pairing suppression was witnessed. On the other hand, removal of this gene caused pairing of wheat and alien chromosomes in hybrids with close-related species. These findings suggest much potential of *Ph2* gene for introgression of alien genes into wheat genome, which could be used as a new breeding tool.

Certain alien chromosomes introduced into wheat are inherited preferably by causing sterility in gametes, in which it were absent, therefore chromosomes like these have been named "gametocidal". The mechanism of causing sterility is by inducing genomic rearrangements. Gametes carrying only semi-lethal genomic rearrangements can be used to transfer aberrations into progeny. By using monosomic addition of 2C gametocidal chromosome derived from *Aegilops*

cylindrica into 'Chinese Spring' cultivar, it is possible to create deletion lines of wheat.

We have been continuously extending a set of deletion lines for a short arm of chromosome 3D. The obtained deletion lines are being characterised by a set of molecular markers up to average resolution of 5 Mbp, focusing on distal 80 Mbp of short arm of 3D chromosome, which is the identified area of *Ph2* gene presence. The aim of this project is to narrow down region of *Ph2* gene through deletion mapping. Eighteen novel lines with terminal deletion of short arm of chromosome 3D have been developed so far. In the frame of the project we would like to map the *Ph2* gene physically to a region smaller than 5 Mb, followed by more precise mapping using a set of radiation deletion lines.

3.2.2 Development of chromosome deletion lines for *Ph2* gene mapping in bread wheat

Svačina, R., Karafiátová, M., Sourdille, P., Endo, T.R., Doležel, J., Bartoš, J.

In: Abstracts of the "Olomouc Biotech 2017. Plant Biotechnology: Green for Good IV". Olomouc, Czech Republic, 2017

Abstract:

Bread wheat (*Triticum aestivum* L.) emerged by hybridization of three closely related species. Its genome thus consists of three subgenomes, and the coexistence of similar homoelogous chromosomes led to the evolution of diploid-like system of chromosome pairing. Homologous pairing in wheat is controlled mainly genetically by *Ph* genes. One of the genes, *Ph2*, was mapped to distal 80 Mb of the short arm of chromosome 3D. Mutants for the gene were developed and only a small effect on homoelogous chromosome pairing suppression was observed. On the other hand, pairing between wheat and alien chromosomes was observed after the removal of *Ph2* gene in hybrids with closely related species. This phenomenon suggests a possibility of using *Ph2* gene as a new breeding tool to facilitate introgression of alien genes into wheat gene pool.

Some gametocidal chromosomes introduced into wheat are inherited preferably by causing sterility of gametes in which they are absent. The sterility is caused by the ability of the chromosomes to induce genomic rearrangements. In some cases, the changes are not lethal, providing an opportunity to transfer aberrant chromosomes into progeny. Gametocidal chromosome 2C from *Aegilops cylindrica* can be used to develop deletion lines after monosomic introduction into wheat cultivar 'Chinese Spring'.

We have established a set of chromosome deletion lines for the short arm of chromosome 3D. The set is being continuously expanded and the newly obtained deletion lines are characterized by molecular markers. We focus preferentially on the distal 80 Mb region of the arm, where *Ph2* gene is believed to be located. The goal of the project is to narrow down the *Ph2* gene region to 5 Mb, so that more precise mapping using radiation deletion lines can be initiated.

3.2.3 *Ph2* gene mapping through development and phenotyping of deletion lines in bread wheat

Svačina, R., Malurová, M., Karafiátová, M., Sourdille, P., Endo, T.R., Doležel, J., Bartoš, J.

In: Abstracts of the "EUCARPIA Breeding cereals for sustainable agriculture". Clermont-Ferrand, France, 2018

Abstract:

Wheat (*Triticum aestivum* L.) emerged by hybridization of three closely-related species. Thus its genome consists of three highly-similar sub-genomes (A, B and D) called homoeologues. The coexistence of similar homoeologous chromosomes led to establishment of a diploid-like system of homologous chromosome pairing mainly genetically controlled by two *Ph* (for *Pairing homoeologous*) genes: *Ph1* and *Ph2*. *Ph2* was mapped to a distal region of 80 Mbp on the short arm of chromosome 3D. Mutants for this gene were developed and it was observed that *Ph2* has only a small effect on homoeologous wheat-chromosome pairing suppression. On the other hand, pairing of wheat and alien chromosomes was witnessed after removal of *Ph2* gene in hybrids derived from crosses between wheat and closely related species such as rye (*Secale cereale*). This discovery suggests a capability of *Ph2* gene to be used as a new breeding tool by introgression of alien genes into wheat gene pool. Positional cloning of *Ph2* would thus be of interest. However, the size of the actual deletion (80 Mb) hampers the identification of any candidate and it would be useful to reduce the deletion to a maximum of a few Mb.

The goal of the project is to scale down the *Ph2* gene region by deletion mapping up to 5 Mbp radius, so more precise mapping by radiation deletion lines can be performed. We have established a new set of deletion lines for a short arm of chromosome 3D, which is being currently extended. We focused on a distal 80 Mb

part of a short arm of chromosome 3D, which is the pinpointed area of *Ph2* gene presence. We used the 2C gametocidal chromosome from *Aegilops cylindrica* as a tool for the development of the deletion lines after monosomic introduction into 'Chinese Spring' cultivar of wheat. Some gametocidal chromosomes introduced into wheat are inherited preferably by causing sterility of gametes in which they are absent. The sterility is caused by the ability of these chromosomes to induce genomic rearrangements. In some cases, these changes are not lethal, thus giving the opportunity to transfer aberration into progeny.

The novel deletion lines were characterized by molecular markers. These lines will now be crossed with rye to see if we observe the *ph2* mutant corresponding phenotype.

3.2.4 Towards identification of *Ph2*, a gene controllong homoeologous chromosome pairing in bread wheat

Serra, H., Svačina, R., Bartoš, J., Sourdille, P.

In: Abstracts of the "EMBO Workshop on Meiosis". La Rochelle, France, 2019

Abstract:

Improvement of bread wheat varieties through introgression of original alleles derived from related species relies on meiotic recombination between homoeologous chromosomes. One of the two main genes controlling homoeologous recombination in this species is *Ph2* (*Pairing homoeologous 2*). Inactivation of this gene results in increase frequency of chromosome pairing during meiosis of hybrids between wheat and close-related species. Although this locus has been described decades ago, the Ph 2 gene is still unidentified and only two mutants are available (*ph2a*, distal deletion of the short arm of the chromosome 3D (3DS) and *ph2b*, EMS mutant).

Characterizing *Ph2* is of main interest to contribute to the improvement of introgression efficiency of new alleles at loci bearing genes of agronomical interest.

3.2.5 *Ph2* gene phenotype scoring in wheat-rye hybrids with terminal deletions of 3D chromosome

Svačina, R., Bartoš, J., Sourdille, P., Serra, H., Malurová, M., Karafiátová, M., Endo, T.R., Doležel, J.

In: Abstracts of the "International Conference on Polyploidy". Ghent, Belgium, 2019

Abstract:

Wheat (*Triticum aestivum* L.) developed through hybridization of three related species, resulting in coexistence of three highly similar sub-genomes in its nuclei. The development of diploid-like chromosome pairing during meiosis is necessary to allow formation of viable gametes. In wheat, this system is being enforced genetically by means of Ph genes, mainly *Ph1* and *Ph2*. In absence, both *Ph1* and *Ph2* lead to increased level of homoeologous chromosome associations in metaphase I in hybrids, while the former has higher effect over the latter. Analysis of a mutant *ph2a* has narrowed down the position of *Ph2* gene to a distal 80 Mb of a short arm of chromosome 3D. However, the size of such deletion hampers the identification of candidates and therefore it would be useful to reduce the deletion size.

The goal of our project is to reduce the *Ph2* gene region through deletion mapping, so that analysis of candidate genes can be performed. We have established a new set of deletion lines for a short arm of chromosome 3D. We utilized the 2C gametocidal chromosome from A. cylindrica to develop the deletion lines after monosomic introduction into wheat cv. 'Chinese Spring'. Through this tool, we are able to induce non-lethal terminal deletions to chromosomes which can be transferred into progeny.

The novel deletion lines were characterized using molecular markers and crossed with rye for subsequent observation of *ph2* mutant phenotype on a haploid background in meiocytes isolated from young anthers. Recently, 27 various deletion lines in the *ph2a* deletion area were crossed with rye and analyzed. Through this study, we managed to narrow down the region of *Ph2* gene to an area varying from 63 – 67 Mb to 77 – 79 Mb, containing 86 – 133 genes.

3.2.6 Wheat-rye hybrids with chromosome deletions analysed for *Ph2* gene phenotype

Svačina, R., Bartoš, J., Sourdille, P., Serra, H., Malurová, M., Karafiátová, M., Endo, T.R., Doležel, J.

In: Abstracts of the "Olomouc Biotech 2019. Plant Biotechnology: Green for Good V". Olomouc, Czech Republic, 2019

Abstract:

Bread wheat (*Triticum aestivum* L.) developed through hybridization of three related species, resulting in coexistence of three highly similar subgenomes in its nuclei. The diploid-like system of chromosome pairing during meiosis had to be developed to allow formation of viable gametes. In wheat, it is being controlled genetically through *Ph* genes, mainly *Ph1* and *Ph2*. In absence, both *Ph1* and *Ph2* cause higher level of homoeologous chromosome associations in metaphase I in haploid cells, while the former has higher effect over the latter. Phenotyping of an X-ray mutant *ph2a* have narrowed down the position of *Ph2* gene to a distal 80 Mb of a short arm of chromosome 3D. However, the size of such deletion hampers the identification of candidates and therefore it would be useful to reduce the deletion size as much as possible.

The goal of the project is to scale down the *Ph2* gene region through deletion mapping up to 5 Mb area, so that analysis of candidate genes can be performed. We have established a new set of deletion lines for a short arm of chromosome 3D. We utilized the 2C gametocidal chromosome from Aegilops cylindrica to develop the deletion lines after monosomic introduction into 'Chinese Spring' cultivar of wheat. Through this material, we are able to create non-lethal terminal deletions on chromosomes which can be transferred into progeny.

The novel deletion lines were characterized using molecular markers, crossed with rye for easier observation of *ph2* mutant phenotype on a haploid background. Up to this day, 34 various deletion lines in the *ph2a* deletion area were crossed with rye and analysed.

3.2.7 *Ph2* gene mapping through phenotyping of wheat-rye hybrid deletion lines

Svačina, R., Bartoš, J., Sourdille, P., Serra, H., Malurová, M., Karafiátová, M., Endo, T.R., Doležel, J.

In: Abstracts of the "22nd International Chromosome Conference". Prague, Czech Republic, 2019

Abstract:

Wheat (*Triticum aestivum* L.) emerged through hybridization of three related species. As a result its genome consists of three highly-similar sub-genomes (A, B and D). The coexistence of similar chromosomes led to establishment of a diploid-like system of chromosome pairing during meiosis which is controlled genetically by Ph (for *Pairing homoeologous*) genes, mainly *Ph1* and *Ph2*. In absence, both *Ph1* and *Ph2* cause higher level of homeologous chromosome associations in metaphase I, while the former has higher effect over the latter. Phenotyping of an X-ray mutant *ph2a* have narrowed down the position of *Ph2* gene to a distal 80 Mb of a short arm of chromosome 3D. However, the size of such deletion hampers the identification of any candidate and therefore it would be useful to reduce the deletion to a maximum of a few Mb.

The goal of the project is to scale down the *Ph2* gene region through deletion mapping up to 5 Mb area, so that analysis of candidate genes can be performed. We have established a new set of deletion lines for a short arm of chromosome 3D. We focused on a distal 80 Mb part of a short arm of chromosome 3D, which is the pinpointed area of *Ph2* gene presence. We used the 2C gametocidal chromosome from Aegilops cylindrica as a tool for development of deletion lines after monosomic introduction into 'Chinese Spring' cultivar of wheat. In this case, these deletions are

mostly terminal and are not lethal, thus giving the opportunity to transfer aberration to progeny.

The novel deletion lines were characterized using molecular markers. These lines were crossed with rye for easier observation of *ph2* mutant corresponding phenotype on a haploid background. Up to this day, 34 various deletion lines in the *ph2a* deletion area were crossed with rye and will be sown shortly for meiotic behavior analysis.

3.3 Published abstracts – oral presentations

3.3.1 Development of chromosome deletion lines for *Ph2* gene mapping in bread wheat

3.3.1 Development of chromosome deletion lines for *Ph2* gene mapping in bread wheat

Svačina, R., Bartoš, J., Sourdille, P., Karafiátová, M., Endo, T.R., Doležel, J.

In: Abstracts of the "14th Student Conference of Experimental Plant Biology". Bratislava, Slovakia, 2017

Abstract:

Wheat (*Triticum aestivum* L.) emerged by hybridization of three close-related species. Its genome thus consists of three subgenomes, therefore the coexistence of similar homoelogous chromosomes led to the evolution of diploid-like system of chromosome pairing. Homologous pairing in wheat is ensured genetically by Ph genes. One of the genes, *Ph2*, was mapped to distal 80 Mb of the short arm of chromosome 3D. Mutants for the gene were developed and only a small effect on homoelogous chromosome pairing suppression was observed. On the other hand, pairing between wheat and alien chromosomes was witnessed in hybrids with closely related species lacking the *Ph2* gene. This phenomenon suggests a possibility of using *Ph2* gene as a new breeding tool to facilitate introgression of alien genes into wheat gene pool.

Some gametocidal chromosomes introduced into wheat are inherited preferably by causing sterility of gametes in which they are absent. The sterility is caused by the ability of the chromosomes to induce genomic rearrangements, such as terminal chromosomal deletions. In some cases, the changes are not lethal, providing an opportunity to transfer aberrant chromosomes into progeny. Gametocidal chromosome 2C from *Aegilops cylindrica* can be used to develop deletion lines after monosomic introduction into wheat cultivar 'Chinese Spring'.

We have established a set of chromosome deletion lines for the short arm of chromosome 3D. The set is being continuously expanded and the newly obtained deletion lines are characterized using STS molecular markers. We preferentially focus on the distal 80 Mb region of a short arm of chromosome 3D, where *Ph2* gene is located. The goal of the project is to narrow down the *Ph2* gene region to 5 Mb, so that more precise mapping using radiation deletion lines can be initiated.

4 CONCLUSION

The *Ph2* gene in bread wheat affects homoeologous chromosome associations in meiosis (Mello-Sampayo, 1968). This gene is located on chromosome 3D (Mello-Sampayo, 1968) and its position was delimited through *ph2a* X-ray deletion mutant (Sears, 1982) to terminal 80 Mb of the short arm of the chromosome based on syntney with rice (Sutton *et al*., 2003). Throughout this study however, it was found that the size of *ph2a* deletion is 121 Mb (Serra *et al*., 2020).

The physical mapping of *Ph2* gene was performed through deletion lines. We developed 113 deletion lines for chromosome 3D of bread wheat (Svačina *et al.*, 2020b). Out of all developed deletion lines, a subset of 32 carried a deletion in the area of interest, 21 lines were succesfully crossed with rye. In the haploid wheat-rye hybrid progeny, a number of chiasmata in metaphase I was scored in anthers. Through this aproach, we delimited the area of *Ph2* locus to 14.3 Mb region on the short arm of chromosome 3D (Serra *et al*., 2020).

EMS-induced mutant *ph2b* carries point mutations throughout its whole genome, one or more of these mutations responsible for *Ph2* gene malfunction (Wall *et al*., 1971). The exome capture of the mutant *ph2b* provided information of candidate genes for *Ph2* phenotype. The comparison between genic areas of *ph2b* and WT 'Chinese Spring' wheat revealed only one candidate (*TraesCS3D02G119400*) that carried a point mutation and was present in 14.3 Mb area delimited by deletion mapping. This gene encodes a DNA mismatch repair protein TaMSH7-3D.

TILLING population of 'Cadenza' cultivar carrying EMS-induced point mutations (King *et al*., 2015; Krasileva *et al*., 2017) was exploited for selection of seven mutants of *TaMSH7-3D* gene. These mutants were crossed with *Ae. variabilis* for number of chiasmata scoring in metaphase I in anthers of the progeny. The hybrid of WT 'Cadenza' and *Ae. variabilis* showed on average 1.10 (± 0.09) chiasmata per meiocyte (Serra *et al*., 2020). Out the seven mutant hybrids, four showed increased frequency of chiasmata in metaphase I, Cadenza2006 x *Ae. variabilis* having the

strongest mutant phenotype of 6.07 ± 0.17 chiasmata per meiocyte on average, which is a 5.52-fold increase (Serra *et al*., 2020). Through this analysis, we functionally validated the *TaMSH7-3D* as a gene responsible for *Ph2* phenotype.

Cloned *Ph2* gene could provide a valuable tool to increase wheat genetic pool, opening new possibilities for enrichment of wheat diversity through alien introgression in breeding programmes. Up to this day, mainly mutants of 'Chinese Spring' with either large background mutation (*ph2a*) or with high number of point mutations across whole genome (*ph2b*) are available for this gene. With knowledge of *TaMSH7-3D* being responsible for *Ph2* phenotype, a precise mutant can be created to be exploited without any background genomic damage in various elite cultivars used for breeding. As the *Ph2* gene has a smaller effect than *Ph1*, its mutant can be used to induce introgressions in a smaller scale in more related hybrids, where *Ph1* gene mutant would cause a lot of background recombinations. On the other hand, in distant relatives, where *Ph1* mutant only is not capable of inducing recombinations between homoeologous chromosome sets, a combination of both *Ph1* and *Ph2* mutations can enlarge the variety of relatives possible to gain genetic diversity from.

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6 LIST OF ABBREVIATIONS

3DL	long arm of wheat chromosome 3D
3DS	short arm of wheat chromosome 3D
5AL	long arm of wheat chromosome 5A
5BL	long arm of wheat chromosome 5B
7AL	long arm of wheat chromosome 7A
Agp2	ADPglucose pyrophosphorylase
C-banding	centromere banding
CDK2	cyclin-dependent kinase 2
CENH3	centromeric histone 3
ChIP-seq	chromatin immunoprecipitation sequencing
C-Ph1	candidate *Pairing homoeologous 1*
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CS	wheat cultivar ‘Chinese Spring’
CSS	chromosome survey sequence
ddPCR	digital droplet polymerase chain reaction
DNA	deoxyribonucleic acid
EMS	ethyl methanesulfonate
FAD2B	delta-12 fatty acid desaturase
FISH	fluorescence *in situ* hybridization
Gb	gigabase pairs
Gc	gametocidal
GDEC	Génétique Diversité Ecophysiologie des Céréales

GISH	genomic in situ hybridization
Hi-C	chromosome-conformation-base mapping
INRA	Institut national de la recherche agronomique
IWGSC	International wheat genome sequencing consortium
kb	kilobase pairs
Mb	megabase pairs
MMR	mismatch repair
MSH	DNA mismatch repair protein
MSH2	DNA mismatch repair protein 2
MSH6	DNA mismatch repair protein 6
MSH7	DNA mismatch repair protein 7
MutS 7	DNA mismatch repair protein MutS 7
MYA	million years ago
OECD	Organisation for Economic Co-operation and Development
PCR	polymerase chain reaction
Ph	*Pairing homoeologous*
Ph1	*Pairing homoelogous 1*
Ph2	*Pairing homoelogous 2*
ph1a	mutant a of *Pairing homoeologous 1*
ph1b	mutant b of *Pairing homoeologous 1*
ph1c	mutant c of *Pairing homoeologous 1*
ph2a	mutant a of *Pairing homoeologous 2*
ph2b	mutant b of *Pairing homoeologous 2*
Ph3	*Pairing homoeologous 3*
Pinb	Puroindoline-B

Rph1	Resistance to Phytophthora 1 protein
SNP	single-nucleotide polymorphism
SSIIa-A	starch synthase
STS	sequence-tagged site
TaMSH7	bread wheat version of DNA mismatch repair protein 7
TaZIP4-B2	bread wheat version of major meiotic crossover gene
TILLING	Targeting Induced Local Lesions IN Genomes
Waxy	granule-bound starch synthase 1
WGD	whole-genome duplication
WM1	leucine-rich repeats protein 1
WM5	leucine-rich repeats protein 5
WT	wild-type
ZIP4	major meiotic crossover gene
β-Amy	β-Amylase

7 LIST OF APPENDICES

Original publications

Appendix I	Development of deletion lines for chromosome 3D of bread wheat
Appendix II	*Pairing homoeologous 2* (*Ph2*) encodes the mismatch repair protein MSH7-3D that inhibits homoeologous recombination in wheat
Appendix III	Chromosome pairing in polyploid grasses

Published abstracts – poster presentation

Appendix IV	Development of deletion lines for physical mapping of *Ph2* gene in bread wheat
Appendix V	Development of chromosome deletion lines for *Ph2* gene mapping in bread wheat
Appendix VI	*Ph2* gene mapping through development and phenotyping of deletion lines in bread wheat
Appendix VII	Towards identification of *Ph2*, a gene controllong homoeologous chromosome pairing in bread wheat
Appendix VIII	*Ph2* gene phenotype scoring in wheat-rye hybrids with terminal deletions of 3D chromosome
Appendix IX	Wheat-rye hybrids with chromosome deletions analysed for *Ph2* gene phenotype
Appendix X	*Ph2* gene mapping through phenotyping of wheat-rye hybrid deletion line

APPENDIX I

Development of deletion lines for chromosome 3D of bread wheat

Svačina, R., Karafiátová, M., Malurová, M., Serra, H., Vítek, D., Endo, T.R., Sourdille, P., Bartoš, J.

Frontiers in Plant Science **10**: 1756, 2020

doi: 10.3389/fpls.2019.01756

IF: 4.298

frontiers in Plant Science

ORIGINAL RESEARCH
published: 28 January 2020
doi: 10.3389/fpls.2019.01756

Development of Deletion Lines for Chromosome 3D of Bread Wheat

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The identification of genes of agronomic interest in bread wheat (*Triticum aestivum* L.) is hampered by its allopolyploid nature (2n = 6x = 42; AABBDD) and its very large genome, which is largely covered by transposable elements. However, owing to this complex structure, aneuploid stocks can be developed in which fragments or entire chromosomes are missing, sometimes resulting in visible phenotypes that help in the cloning of affected genes. In this study, the 2C gametocidal chromosome from *Aegilops cylindrica* was used to develop a set of 113 deletion lines for chromosome 3D in the reference cultivar Chinese Spring. Eighty-four markers were used to show that the deletions evenly covered chromosome 3D and ranged from 6.5 to 357 Mb. Cytogenetic analyses confirmed that the physical size of the deletions correlated well with the known molecular size deduced from the reference sequence. This new genetic stock will be useful for positional cloning of genes on chromosome 3D, especially for *Ph2* affecting homoeologous pairing in bread wheat.

Keywords: wheat, deletion line, homoeologous pairing, *Ph2*, gametocidal

OPEN ACCESS

Edited by:
*Luigi Cattivelli,
Council for Agricultural and Economics Research, Italy*

Reviewed by:
*Andrea Brandolini,
Council for Agricultural and Economics Research, Italy
Adam Lukaszewski,
University of California, Riverside, United States*

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Specialty section:
This article was submitted to Plant Breeding, a section of the journal Frontiers in Plant Science

Received: *25 October 2019*
Accepted: *16 December 2019*
Published: *28 January 2020*

Citation:
Svačina R, Karafiátová M, Malurová M, Serra H, Vítek D, Endo TR, Sourdille P and Bartoš J (2020) Development of Deletion Lines for Chromosome 3D of Bread Wheat. Front. Plant Sci. 10:1756. doi: 10.3389/fpls.2019.01756

INTRODUCTION

Bread wheat (*Triticum aestivum* L.) is one of the most important cultivated crops. It emerged through two distinct hybridization events between three diploid species, resulting in its allohexaploid nature. The genetic material consists of three closely related subgenomes, namely A, B, and D (Huang et al., 2002), which generate the genomic plasticity necessary for bread wheat to grow under a wide range of climatic conditions. Moreover, bread wheat tolerates the creation of aneuploid lines, such as nullisomic, substitution, deletion, and many other types. However, the three sets of homoeologous chromosomes create a vulnerability to incorrect chromosome pairing during meiosis, possibly resulting in aberrant gametes. Therefore, to maintain the pairing behavior during meiosis, a system developed in wheat that is enforced genetically by *pairing homoeologues* (*Ph*) genes. In this control, the most effective genes are *Ph1* and *Ph2. Ph1* is on the 5B chromosome and has a major influence on homoeologous chromosome pairing (Riley and Chapman, 1958; Sears and Okamoto, 1958). *Ph2* is on the short arm of chromosome 3D (Mello-Sampayo, 1971) and has less of an effect compared with *Ph1*. Despite some attempts at positional cloning (Sutton et al., 2003), *Ph2* has not been formally identified to date. Other genes contribute to the control of homoeologous pairing but have only minor influence, such as *Ph3*, which is on the short arm of chromosome 3A and is possibly a homoeologous variant of *Ph2* (Driscoll, 1972; Mello-Sampayo and Canas, 1973).

Genes are usually maintained in a population by benefiting their hosts or alternatively, by high linkage to such a gene (a phenomenon called linkage-drag). However, there are exceptions to this rule, such as transposable elements, B chromosomes, and gametocidal genes/chromosomes. These genetic units use various "selfish" behaviors to either preserve their existence in the population or to increase their number. The gametocidal genes or chromosomes secure their inheritance to progeny through induction of genomic aberrations and consequent total or partial sterility in gametes lacking them. In wheat, this phenomenon is observed in substitution and addition lines with alien chromosomes from the genus *Aegilops*. The backcrossing of hybrids to wheat between the two species does not remove certain chromosomes of *Aegilops* from the genome of progeny (Endo and Tsunewaki, 1975; Maan, 1975), and chromosomal aberrations are observed in some gametes of such hybrids (Finch et al., 1984). Gametocidal chromosomes originate from the *Aegilops* genomes C, S, and M, and the magnitude of their effect in wheat varies with the type of gametocidal chromosome and the genotype of the wheat background. Whereas some chromosomes cause complete sterility of gametes that lack them (e.g., $2S^{lo}$, $2S^{sh}$, T2B-$2S^{sp.au}$, $4S^{lo}$, $4S^{sh}$, and $4S^{sh}$#2); others generate only semi-lethal changes and make it possible to transfer the aberrations to progeny (Endo, 1990; Endo, 2007).

The 2C gametocidal chromosome from *Aegilops cylindrica* has been introduced to the *T. aestivum* 'Chinese Spring' background and is being exploited to create mostly terminal deletions of wheat chromosomes. Hereafter, this procedure will be called the "2C gametocidal system" (Endo and Gill, 1996). Tsujimoto (1993) showed that telomeric regions are quickly rebuilt after chromosome breakage and that chromosome stability allows this system to be used as a genetic tool. Endo and Gill (1996) produced 436 deletion lines across all chromosomes using this approach, with subsequent establishment of deletion chromosomes in homozygous/hemizygous constitutions. This resource has been a powerful tool in mapping the position of various genes and markers (Sourdille et al., 2004).

The 2C gametocidal system can be used to create a series of aberrations in any chromosome of wheat. However, the judicious use of existing aneuploid stocks can increase the efficiency and ease in selecting aberrations targeting specific chromosomes. If the monosomic addition line 2C is crossed as male to a nulli-tetrasomic line lacking the targeted chromosome, the recovered aberrations will be monosomic and hence easily detectable by PCR-based techniques. The selection of disomic/homozygous aberrations is performed following self-pollination of the plants carrying the aberrations, but the presence of the additional copy of a homoeologue inherited from the nulli-tetrasomic may complicate the transmission patterns of the targeted chromosome.

In this study, the 2C gametocidal system was used to develop a set of deletion lines for chromosome 3D in wheat to map the position of the *Ph2* gene. This gene was previously mapped using a *ph2a* mutant carrying a terminal deletion on chromosome 3D that was estimated to be approximately 80 Mb (Sutton et al., 2003).

MATERIALS AND METHODS

Plant Material and Crosses

The deletion lines were derived from crosses between the monosomic addition line of chromosome 2C from *A. cylindrica* in the hexaploid wheat cultivar Chinese Spring (CS) background (6x = 2n = 43; AABBDD + 2C′) used as male and the hexaploid CS wheat nulli-tetrasomic lines lacking chromosome 3D with tetrasomic constitution either for chromosome 3A or 3B (6x = 2n = 42; AABBDD − 3D″ + 3A″/3B″) used as female (**Figure 1**). The 2C gametocidal chromosome induces chromosomal breakages in gametes where it is not transferred, resulting

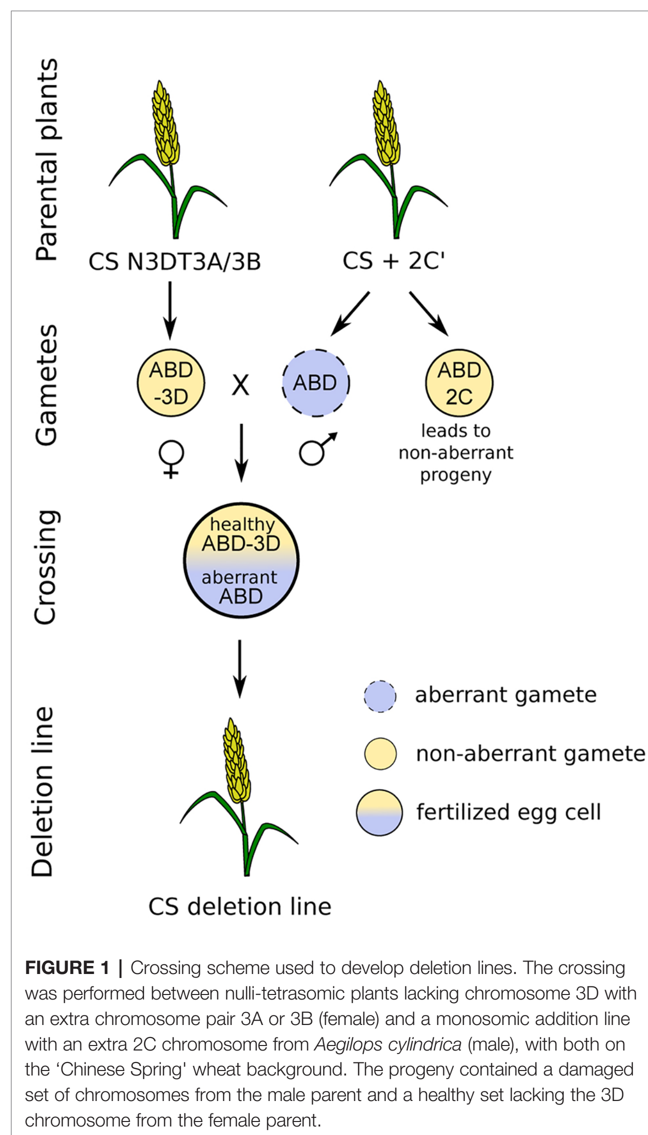

FIGURE 1 | Crossing scheme used to develop deletion lines. The crossing was performed between nulli-tetrasomic plants lacking chromosome 3D with an extra chromosome pair 3A or 3B (female) and a monosomic addition line with an extra 2C chromosome from *Aegilops cylindrica* (male), with both on the 'Chinese Spring' wheat background. The progeny contained a damaged set of chromosomes from the male parent and a healthy set lacking the 3D chromosome from the female parent.

mostly in terminal deletions. The crosses with nulli-tetrasomic lines lacking a pair of 3D chromosomes ensure that a potentially aberrant 3D chromosome from the 2C addition-line parent will be in the progeny in a monosomic state and that a deletion will not be masked by an entire 3D chromosome from the female parent. The plants were cultivated in growth chambers under the following conditions: a 16/8 h light/dark photoperiod, temperatures of 20 °C during the day and 16 °C at night, and 60% humidity.

Identification of Plants With Deletion on the 3D Chromosome

The seeds acquired from the crosses were germinated in pots and cultivated for 2 weeks. Thereafter, DNA was isolated from a part of a young leaf by using a magnetic beads protocol (Sbeadx mini plant kit, LGC, Teddington, United Kingdom). The DNA was used to identify the deletion lines in the F1 generation. Molecular markers were designed for the distal ends of both arms of chromosome 3D, with a marker located in the centromeric area as a control for chromosome presence; primer details are shown in **Table 1**. The PCR was performed in 20 µl (1× PCR buffer, 1.5 mM MgCl, 200 µM dNTPs, 1 µM primers, 20 ng of DNA, 0.4 U/20 µl Taq DNA polymerase) under the following conditions: initial denaturation at 95 °C for 10 min; 35 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and elongation at 72 °C for 50 s; followed by a final extension at 72 °C for 10 min. The PCR was scored for the presence/absence of a specific product on 1.2% agarose gel. The plants carrying a deletion on chromosome 3D (lacking either or both 3DS and 3DL-specific PCR products) were replanted into larger pots and cultivated under the following conditions: a 16/8 h light/dark photoperiod, temperatures of 20 °C during the day and 16 °C at night, and 60% humidity. Plants were grown until seed harvest.

Characterization of Sizes of Deletions

The deletion lines of chromosome 3D were characterized using a set of STS molecular markers covering the entire chromosome. In addition to the deletion lines, the X-ray-induced deletion mutant *ph2a* (Sears, 1950) was also characterized. Eighty-four was the final number of markers (**Supplementary Table 1**), of which 58 were on the short arm and 26 were on the long arm of the chromosome. The characterization was performed using presence/absence scoring and agarose gel electrophoresis separation as described above.

The primers for analysis were designed using the reference sequence of the wheat genome (IWGSC, 2018). The sequence was masked for annotated repetitive sequences. The loci for primer design were selected to cover the chromosome as evenly as possible with the priority in the distal 125 Mb of the short arm of chromosome 3D. The regions not masked with repeats (10–30 kb) were aligned using BLASTn against reference sequences of chromosomes 3A and 3B, and the corresponding regions were compared to depict the 3D-specific polymorphisms. Those polymorphisms were used to design 3D-specific primers (**Supplementary Table 1**). The primers were tested on *T. aestivum* 'Chinese Spring' as the positive control, a nulli-tetrasomic line lacking chromosome 3D as the negative control, and water as the blank.

TABLE 1 | Sequences and localization of primers used for identification of lines carrying a deletion on the short arm, long arm, or both arms of chromosome 3D.

Oligo ID	Sequence 5′–3′	Localization
3D_0.3Mb_F	TTAGTGGATCGAGGATTGTG	distal 3DS
3D_0.3Mb_R	TCGGTGACTAGTGTGTTTCTG	
3D_610.2Mb_F	GCAACAGAAGAAGAAAATACTGCT	distal 3DL
3D_610.2Mb_R	GTGCATCATATCTATGGTCTATC	
3D_253.4Mb_F	TATGCGTTTGGAGTAGTTCTTGT	3D centromere
3D_253.4Mb_R	CTCATCTCAGGCTGTCTAATTAA	

Identification of Deletion Lines With the 3D Chromosome in a Disomic State

Each deletion line was self-pollinated to increase seed stocks and to induce a disomic constitution of the 3D chromosome carrying a deletion. The upcoming generation comprised nullisomics, monosomics, and disomics for the analyzed chromosome. Therefore, screening with molecular markers was necessary to select the stable lines carrying the 3D chromosome with deletion in disomic constitution. First, the entire population was screened using the PCR marker on the centromere of the 3D chromosome (see above) to eliminate all nullisomics. The plants carrying the 3D chromosome were selected for droplet digital PCR (ddPCR) analysis. The ddPCR analysis was performed using ddPCR™ Supermix for Probes (no dUTP) (Bio-Rad, Hercules, USA) according to manufacturer's instructions with a 60 °C annealing/extension phase. The reference and target primers and TaqMan® probes (Thermo Fisher Scientific, Waltham, USA) used for chromosomes 4A (disomic in all lines) and 3D are listed in the **Table 2**.

Fluorescent *in Situ* Hybridization of Selected Lines

Selected lines carrying a deletion on the 3D chromosome were characterized cytogenetically using fluorescent *in situ* hybridization (FISH). Mitotic metaphase chromosomes were obtained from synchronized root tip meristems (Vrána et al., 2012). Synchronized roots were fixed in 90% ice-cold acetic acid for 10 min and then washed three times with 70% ethanol and stored at −20 °C in 70% ethanol. Chromosome preparations using the drop technique were performed according to Danilova et al. (2012). The individual chromosomes in the wheat karyotype were identified using the combination of two FISH probes: $(GAA)_n$ microsatellite (FITC) and Afa repeat (Cy3) (Pedersen and Langridge, 1997; see **Supplementary Figure 1**). The probes were labeled *via* PCR, and FISH was performed under the conditions described in Kubaláková et al. (2003). The signals were observed using a Zeiss Axio Imager Z2 fluorescent microscope (Carl Zeiss, Jena, Germany) equipped with a CCD camera. At least five copies of the 3D chromosome per line were characterized by measurement of the deleted arm and whole chromosome length by using MicroImage software version 4.0 (Olympus, Shinjuku, Japan). The deletion size on chromosome 3D was estimated on the basis of the fragment length value (Endo and Gill, 1996).

RESULTS

From the F1 generation, 6169 seeds formed by crosses between the monosomic addition line of chromosome 2C from *A. cylindrica* and the nulli-tetrasomic lines (6x = 2n = 42; AABBDD − 3D″ + 3A″/3B″) were analyzed. The plants carrying a deletion on chromosome 3D were detected using STS markers designed for the terminal ends of 3D chromosomal arms. In total, 113 deletion lines were developed (**Supplementary Table 2**). All identified plants in the F1 generation carried a 3D chromosome with a deletion in monosomic constitution. More precisely, 43 (39.13%) of the lines carried a deletion on 3DS, 68 (60.87%) carried a deletion on 3DL, and two lines carried a deletion on both arms (for the schematic layout, see **Supplementary Figures 2**, **3**, and **4**). These numbers corresponded to the length–arm ratio of 0.393 (240 Mb) for the short arm and 0.607 (370 Mb) for the long arm (IWGSC, 2018).

A set of self-pollinations established a disomic constitution of deleted chromosomes in individual lines. The number of 3D copies was analyzed using a ddPCR protocol with specific primers and the TaqMan probe system comparing the number of events on the analyzed chromosome (3D) with that on the reference chromosome (4A). The disomic constitution of deletion chromosomes was successfully established in 102 of the 113 lines.

The whole set of deletion lines was characterized using 84 STS molecular markers evenly distributed along the entire 3D chromosome (**Supplementary Table 1**). The size of the deletions ranged from 6.5 to 357 Mb, and the size of the deletion bins (the region between two adjacent deletion breakpoints) ranged from 0.15 to 50 Mb. Some deletions seemed to have the same breakpoint; however, this was most likely caused by insufficient resolution of molecular markers in that particular region. The length of chromosome arm deletions and the number of missing genes in individual lines, as well as the differences in missing genes among the lines, are summarized in **Supplementary Table 2**.

The deletion lines of chromosome 3D were produced to map the position of the *Ph2* gene that was localized on this chromosome by Mello-Sampayo (1971). The position of this gene was further delimited using an X-ray-induced deletion mutant *ph2a* (Sears, 1982), and therefore, this mutant was included in the analysis as a control. The size of the deletion in the *ph2a* mutant was previously estimated to affect approximately 80 Mb in the terminal part of the 3DS using synteny with the rice chromosome (Sutton et al., 2003). However, the screening by molecular markers showed this deletion to be larger by approximately 40 Mb, because the breakage point was between 120 and 125 Mb.

FISH analysis of selected deletion lines representing various lengths of deletions was performed to cytogenetically characterize the material (**Figure 2**). The 3D chromosome was identified using the Afa repeat family (Pedersen and Langridge, 1997). Among the 32 selected deletion lines, 12 had the breakage on the 3DS and 20 had the breakage on the 3DL. In all cases, the size of deletion determined by molecular markers was confirmed by cytogenetic observation.

TABLE 2 | Sequences of primers and probes used for determination of 3D chromosome number in the ddPCR assay. The TaqMan (taq) probes were either labelled by FAM (4A chromosome; used as a reference) or VIC (3D chromosome; target).

Oligo ID	Sequence and modifications 5′–3′	Amplicon length [bp]
Ta-4A_F	ATTTTGGGTCCTTGTTGTTATC	181
Ta-4A_R	ACACGCATGAAGTGTATAATGC	
Ta-4A_taq	FAM-AAGAACTTCACACACGAACTCGGA-QSY	
Ta-3D_F	CTCATCTCAGGCTGTCTAATTAA	167
Ta-3D_R	CATAGATCCCTCCTTGAAGGA	
Ta-3D_taq	VIC-CCTCACTCAAGCACCACATCG-QSY	

DISCUSSION

The deletion lines were produced using the gametocidal system described by Endo and Gill (1996). The 2C gametocidal chromosome causes terminal chromosomal deletions in the gametes that lack it. However, these aberrations are usually not lethal because of the compensation by the other two homoeologous chromosomes. Thus, deletions can be transferred into progeny (Endo, 1988). Endo and Gill (1996) derived 436 plants *via* this system and characterized the deletions cytogenetically using a C-banding protocol. Of the 436 plants, 12 of them carried a deletion on chromosome 3D. In this study, 113 novel deletion lines for chromosome 3D were generated, increasing substantially the number of chromosome 3D deletion lines that are available for use in various applications.

Because the deletion lines were primarily produced to map the *Ph2* gene, the marker resolution was highest on the short arm of chromosome 3D. The 58 markers divided the short arm into segments ranging from 100 kb to a maximum of 29 Mb in the centromeric area. Owing to the high marker resolution, only a single or a few deletion breakpoints were assigned in each segment (**Supplementray Table 2**). The short arm of chromosome 3D comprises 1,949 annotated genes (IWGSC, 2018), and in this study, the estimated number of genes deleted in individual lines was 194–1,927, with the number of genes in each deletion bin ranging from 7 to 276. By contrast, the resolution achieved with 26 markers on the long arm of the chromosome was lower than that on the short arm, with segments ranging from 3 to 50 Mb. The long arm of chromosome 3D carries 3,369 genes (IWGSC, 2018), and the number of genes deleted in individual lines ranged from 306 to 3,351, with each deletion bin comprising between 76 and 468 genes (see **Supplementray Table 2**). The resolution of deletion bins in the area of the *Ph2* gene (distal 125 Mb of the short arm) ranged between 1.5 and 12 Mb, with an average of 6 Mb, limiting the number of potential candidate genes to between 7 and 276.

To produce single chromosome deletion lines *via* the 2C gametocidal system, a cross is performed with one parent a nulli-

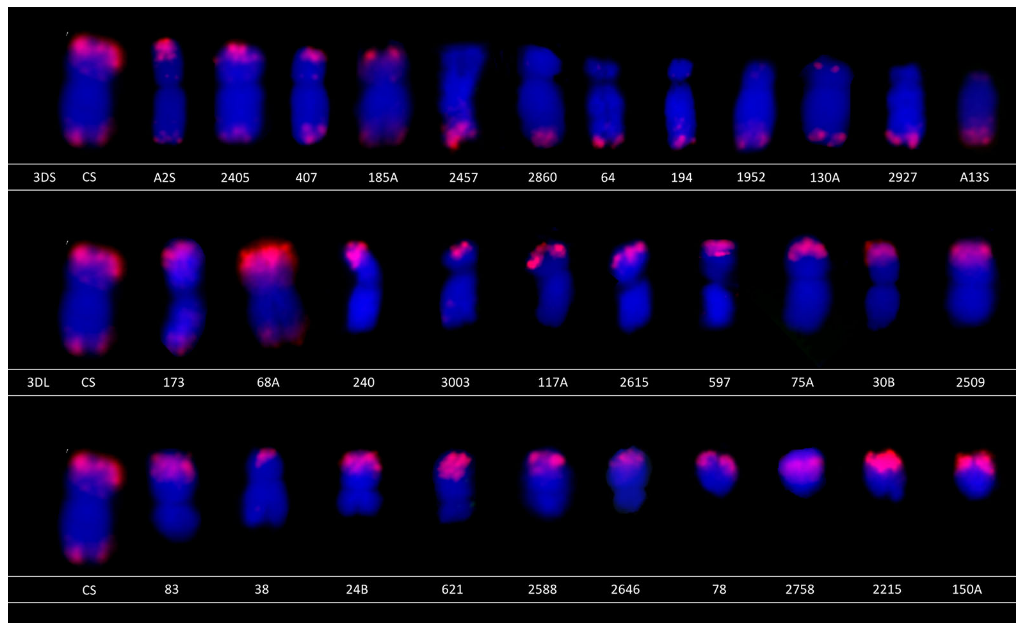

FIGURE 2 | Characterization of selected lines using fluorescence *in situ* hybridization (FISH). The selected lines were characterized using FISH to confirm the deletion size. The chromosomes were labeled using $(GAA)_n$ microsatellite (green) and Afa repeat (red) to distinguish chromosome 3D from other chromosomes. Note that only Afa repeat is present on chromosome 3D.

tetrasomic line lacking a chromosome of interest. The resulting progeny carry the deleted chromosome of interest in a monosomic constitution. Because the gametes produced by the progeny may or may not contain the deleted chromosome, the lines are unstable for direct use, making it unreliable material for seed stock enlargement, crossing, or physical gene mapping. Therefore, self-pollination of this material is recommended to accumulate the deleted chromosome in a disomic constitution. The self-pollination of a plant carrying a chromosome in the monosomic state can produce nullisomic, monosomic, or disomic progeny for the respective chromosome. However, the proportion of transmission to progeny of such a chromosome is shifted by various irregularities in univalent behavior in meiosis. In *Nicotiana tabacum*, the univalent elimination of different monosomic chromosomes occurs at the same frequency, fluctuating around 75% (Olmo, 1935). In wheat, however, the univalent elimination seems to have greater variability, depending on which chromosome is in a monosomic state (Morrison and Unrau, 1952; Tsunewaki and Heyne, 1960). In the material in this study, nullisomics occurred more frequently than expected in progeny of monosomic deletion lines. Univalent behavior during meiosis can explain the unexpected proportions of nullisomics, monosomics, and disomics in progeny. Because univalents lag behind the bivalents while being pulled to the poles at anaphase I, they are therefore excluded from newly formed nuclei and are preserved in the cytoplasm as micronuclei (Sears, 1950; Tsunewaki and Heyne, 1960).

In this study, the 2C gametocidal system was used to develop novel deletion lines for chromosome 3D in common wheat (Endo and Gill, 1996). The deleted chromosome was successfully fixed in disomic constitution in most of the material to ensure the stable inheritance of the chromosome of interest, which greatly improves further use of the deletion lines. The new material will be useful to clone genes of agronomic interest, such as *Ph2*, a gene involved in homoeologous pairing in bread wheat (Mello-Sampayo, 1971).

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

TE, PS, and JB designed the study. RS and TE crossed plants. RS, HS, and DV performed PCRs and ddPCR screening. MK and MM characterized the deletion lines using FISH. RS, PS, and JB wrote the manuscript. All authors approved the manuscript.

FUNDING

This work was supported by the Czech Science Foundation (grant award 17-05341S) and the ERDF project "Plants as a

tool for sustainable global development" (CZ.02.1.01/0.0/0.0/16_019/0000827). We acknowledge the Investment for the Future programme BREEDWHEAT (project ANR-10-BTBR-03) funded by the French Government and managed by the Research National Agency (ANR) for providing markers.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.01756/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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APPENDIX II

Pairing homoeologous 2 (_Ph2_) encodes the mismatch repair protein MSH7-3D that inhibits homoeologous recombination in wheat

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Nature Communications, 2020

Manuscript submitted for publication

IF: 12.121

Pairing homoeologous 2 (Ph2) **encodes the mismatch repair protein MSH7-3D that inhibits homoeologous recombination in wheat**

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Key words: Meiosis, Chromosome pairing, Homoeologous Recombination, Crossover, *Ph2*, Mismatch repair, *MSH7*, *Triticum aestivum*

Abstract

Meiotic recombination is a critical process for plant breeding, as it creates novel allele combinations that can be exploited for crop improvement. In wheat, a complex allohexaploid that has a diploid-like behaviour, meiotic recombination between homoeologous or alien chromosomes is suppressed through the action of several loci. Here we report positional cloning of *Pairing homoeologous* 2 *(Ph2)* and functional validation of the wheat DNA mismatch repair protein MSH7-3D as a key inhibitor of homoeologous recombination, thus solving a half-century-old question. Similar to *ph2* mutant phenotype, we show that mutating MSH7-3D induces a substantial increase in homoeologous recombination (up to 5.5 fold) in wheat-wild relative hybrids, which is also associated with a reduction in homologous recombination. This data reveals a role for MSH7-3D in meiotic stabilisation of allopolyploidy and provides an opportunity to improve wheat's genetic diversity through alien gene introgression, a major bottleneck facing crop improvement.

Introduction

Crop wild relatives provide a valuable source of novel genes and allelic variants for abiotic stress tolerance, disease resistance and quality traits that are important for breeding, particularly in the context of human population growth and a changing climate. Remarkable progress has been made over the last 80 years, with notable boosts in the 1970's and 1980's [1], in knowledge and resulting methodology to allow utilisation of wild relatives in wheat breeding. However, an important challenge still facing breeders now is the ability to routinely perform DNA-introgression, a process by which distantly related chromosomes exchange genetic information that is passed onto progeny. The transfer of chromatin between pairing maternal and paternal chromosomes relies on recombination, a process which occurs in all sexually reproducing species during meiosis [2].

The genetics of chromosome pairing and meiotic recombination is complicated by the allopolyploid nature of many crops, a widespread feature in the plant kingdom [3]. For example, hexaploid bread wheat (*Triticum aestivum* L., AABBDD 2n = 6x = 42), which derives from two successive interspecific crosses involving three diploids [4,5], has three sets of related homoeologous chromosomes. Genetic and cytogenetic studies have revealed the presence of several pairing homoeologous (*Ph*) loci that ensure wheat behaves as a diploid during meiosis, with only homologous chromosomes of the same sub-genome (AA, BB or DD) pairing and recombining. The two main loci controlling homoeologous recombination are located on chromosome-arms 5BL and 3DS, named *Ph1* and *Ph2* respectively [6–9].

Ph1 mutant analysis (*ph1b*) has identified a 2.5 Mbp region deleted on chromosome 5BL [10]. This region contains a duplicated 3B-chromosome segment carrying an additional copy of the meiotic gene *ZIP4* and a heterochromatin tandem repeat block, inserted within a cluster of *CDK2-like* genes [10–13]. Recent evidence now points to *TaZIP4-B2* as being responsible for the effect of this locus on homoeologous recombination [14,15]. Although the exact mode of action is unknown, *TaZIP4-B2* seems to act as a focal point, facilitating physical interactions between components of the chromosome axis and crossover machinery [12].

In comparison to *Ph1*, the causative gene sequence for *Ph2* is yet to be determined. Analysis of the irradiation-mutant *ph2a* in comparison to the syntenic region on rice chromosome 1 estimated the deletion to be at least 80 Mb in size [16], but more likely to span a 120 to 125 Mb region [17] on the terminal portion of 3DS. Research aiming to identify *Ph2* has resulted in the isolation of a number of candidate

59 meiotic genes from this region on 3DS. These include the genes *WM1* [18,19], *WM3* [20], *WM5* [21] and
60 *TaMSH7* [22,23]. Despite these attempts, the region and its candidate meiotic gene content was deemed
61 too large and complex to confidently identify the *Ph2* causative sequence using the *ph2a* deletion mutant
62 alone. The chemically induced *ph2b* mutant [24], thought to contain either a point mutation or small lesion
63 at *Ph2*, offered the prospect of identifying the causative gene sequence.

64 Identifying the genetic control and underlying mechanism of action of *Ph2* would provide valuable
65 knowledge, and enable novel resources to be developed for introgressing alien sequences from related
66 species into bread wheat. The use of *ph2* mutation could be of particular interest to breeders and
67 geneticists as it induces only a minimal disruption to endogenous homologous chromosome pairing [25–
68 27] but reinforces *ph1b*'s effect of promoting homoeologous recombination in some crosses [28].

69 Here we report the positional cloning of *Ph2* from a 121.16 Mb candidate region on 3DS. Based on the
70 analysis of a set of specifically created new 3DS deletion mutants [17] combined with exome sequencing
71 and transcriptome analysis of *ph2a* and *ph2b* mutants versus wild-type, we identified *TaMSH7-3D*, a
72 gene encoding a plant specific DNA mismatch repair protein. Using four independent Ethyl
73 methanesulfonate (EMS) generated *msh7-3D* mutants crossed with wheat wild relative *Aegilops*
74 *variabilis*, we demonstrate that *msh7-3D* mutants recapitulate the *ph2* phenotype with a highly significant
75 (up to 5.5-fold) increase in homoeologous recombination and a reduction in homologous recombination.
76 This data suggests that, in addition to *Ph1*, *TaMSH7-3D* is an attractive target for facilitating alien gene
77 introgression in pre-breeding and breeding programs.

78 ## Results

79 ### Molecular characterisation of *ph2a* and *ph2b* mutations

80 The *Ph2* pairing homoeologous locus is located on chromosome-arm 3DS within the terminally deleted
81 region of irradiation-mutant *ph2a* [9,26]. Marker-based analysis of *ph2a* recently revealed that minimum
82 deletion size is 120 Mb with a maximum of 125 Mb [17]. To precisely delineate the deletion breakpoint,
83 *ph2a* was genotyped using a high-density SNP genotyping array (35K SNP Affymetrix Axiom®).
84 Genotyping data showed the deletion breakpoint on chromosome 3D located between markers AX-
85 178057815 and AX-178057206 at the coordinates 120.722.379 and 121.539.725, respectively
86 (Supplementary Figure S1 A). This was further refined using exome capture of *ph2a*: the deletion
87 breakpoint is around the coordinate 121.163.000, upstream of the gene *Traes3D01G153800*
88 (Supplementary Figure S1 B). Using the newly available Chinese Spring Reference Genome v1.0 [29],
89 we identified 1577 genes within the deleted *ph2a* region.

90 To identify possible candidate genes for *Ph2*, we performed an exome capture of the EMS induced *ph2b*
91 mutant, in which a point mutation was proposed as being responsible for the observed phenotype [24].
92 Comparison between *ph2b* exome sequence and the Chinese Spring reference genome highlighted 165
93 single nucleotide differences within the 121.16 Mb deleted *ph2a* region. These consisted mainly of G to
94 A and C to T transitions, as would be expected from alkylation by EMS treatment. We detected 59 SNPs
95 within genic regions (including 5' and 3' UTR and potential promoter regions), among these 36 were
96 exonic mutations (13 synonymous, 21 non-synonymous and 2 non-sense mutations) and one likely to
97 affect transcript splicing (Supplementary Table S1). Considering only those genes that contain exonic
98 SNPs predicted to result in either non-synonymous amino acid changes, protein truncations (premature
99 STOP codons) or alternate splicing, the total number of *Ph2* candidate genes was reduced to 24.

100

101 ### *Ph2* locates within a 14.3-Mb region on 3DS

102 Since these 24 candidate genes were dispersed over the entire length of the chromosomal region
103 deleted in *ph2a*, we then sought to delineate *Ph2* spatially. With this purpose, we developed a series of
104 113 wheat deletion lines carrying terminal deletions of chromosome 3D [17]. Among these lines, a subset
105 of 32 that possessed 3DS deletions ranging in size from 6.5 to 142.6 Mb were selected. The region

106 between each adjacent deletion breakpoint of the tiled series did not exceed 14.3 Mb. Since the mutant
107 phenotype for *Ph2* is easily discernible in ABDR haploid hybrids [30], each selected deletion line (in 3D
108 monosomic constitution) was crossed with rye (RR, 2n = 14) and hybrids carrying a mutant 3D
109 chromosome (terminal deletion) were selected in the progeny using 3D specific markers. We screened
110 for the presence of *Ph2* by characterizing meiotic behavior at metaphase I of 22 ABDR hybrids (each
111 carrying a 3D chromosome with a terminal deletion). While haploid sets of wheat and rye homoeologous
112 chromosomes rarely associated in wild-type hybrids (0.38 ± 0.10 chiasma / meiocyte), mean chiasma
113 frequency was significantly increased in the *ph2a* mutant context (3.10 ± 0.13 chiasmata / meiocyte),
114 indicating that formation of chiasmata between non homologous chromosomes occurs more frequently
115 in the absence of *Ph2* (Figure 1; Supplementary Table S2) [26]. Cytogenetic analyses of the generated
116 wheat 3D-deletion line / rye hybrids revealed that individuals carrying a 3DS terminal deletion of 79.2
117 Mb or more exhibit a high chiasma frequency (ranging from 2.60 to 3.92 chiasmata / meiocyte), similar
118 to that observed for *ph2a* (Figure 1; Supplementary Table S2). However, hybrids carrying a 3DS terminal
119 deletion shorter than 64.9 Mb (minimal breakage position of the A6S line) showed less than 2 chiasmata
120 / meiocyte indicating the presence of *Ph2* and its ability to inhibit homoeologous recombination within
121 these lines. Taken together, these data clearly demonstrate that *Ph2* is located within a 14.3 Mb genetic
122 interval ranging from 64.9 to 79.2 Mb on chromosome-arm 3DS.

123

124 ***TaMSH7-3D* is a unique candidate for *Ph2***

125 Among the 24 candidate genes identified by *ph2b* exome sequencing, only one located between
126 positions 64.9 and 79.2 Mb on 3DS. This gene, *TraesCS3D02G119400*, contains 17 exons and 16
127 introns with a total length of 9747 bp and encodes the DNA mismatch repair protein TaMSH7-3D (Figure
128 2). In *ph2b*, a G to A transition was detected at position 74.359.312 and confirmed by Sanger
129 sequencing. It affects the first nucleotide of the splicing pattern GTAAGT at the junction between exon
130 5 and intron 5 and is predicted to compromise correct splicing of the transcript. No other unique mutation
131 from *ph2b*-derived sequences was detected for this gene's A or B homoeologues
132 (*TraesCS3A02G117500* and *TraesCS3B02G136600*, respectively) nor for previously identified potential
133 candidates for *Ph2* : the *WM1* gene family (*TraesCS3D02G034300, TraesCS3D02G034500,*
134 *TraesCS3D02G034700, TraesCS3D02G034900, TraesCS3D02G035200, TraesCS3D02G035100*)

135 [18,19], *WM3* (*TraesCS3D02G152900*) [20] or *WM5* (*TraesCS3D02G140300*) [21]. We were unable to confirm
136 the presence of three previously identified SNPs in the *TaMSH7-3D* coding sequence of *ph2b*, none of
137 which were deemed to result in a non-functional or malfunctioning protein [23]. To determine whether the
138 G to A SNP we identified here affects intron-5 splicing (leading to a predicted loss of protein function
139 and therefore the *ph2* phenotype), we performed a high-depth RNAseq from wild-type and *ph2b* mutant
140 anthers staged from pre-meiotic interphase to metaphase I. RNAseq data from *ph2b* confirmed the
141 presence of the splice junction mutation and showed that this mutation leads to the use of a downstream
142 splice site. This results in a frame-shift and thereby creates a premature in-frame STOP codon
143 (Supplementary Figure S2). This STOP codon is predicted to result in a truncated, non-functional
144 TaMSH7-3D protein missing major functional domains, specifically the core domain and the C-terminal
145 ATPase domain (Figure 2). Taken together, deletion-line mapping combined with exome and
146 transcriptome sequencing of the *ph2b* mutant identifies *TaMSH7-3D* as a unique candidate for *Ph2*.

147

148 **Validation of *TaMSH7-3D* as the causative gene for *Ph2***

149 To functionally validate that *TaMSH7-3D* affects homoeologous recombination, we took advantage of
150 the Targeting Induced Local Lesions In Genome (TILLING) population of 1200 wheat mutant lines of
151 the variety Cadenza and the corresponding databases cataloguing mutations identified through exome
152 sequencing (www.wheat-tilling.com) [31,32]. Screening by BLAST search identified 127 possible mutants
153 for *TaMSH7-3D* (*Traes_3DS_72259A292.1*) within the population. We selected seven mutant lines with
154 either a high probability of being knocked-out (Cadenza2006; stop codon gained) or carrying missense
155 mutations likely to affect different regions of protein coding (Supplementary Table S3). Considering the
156 wheat variety Cadenza does not produce viable F1's when crossed with rye, the selected *TaMSH7-3D*
157 mutants, as well as a wild-type Cadenza (Cad wt), were crossed with the wheat wild relative *Aegilops*
158 *variabilis* (UUSS, 2n = 4x = 28). The frequencies of univalents, rod and ring bivalents as well as
159 multivalents were scored at meiotic metaphase I in the resulting F1 wheat / *Ae. variabilis* hybrids and
160 were used to calculate total chiasma frequency per cell. Cad wt / *Ae. variabilis* hybrids exhibited on
161 average 32.79 (± 0.18) univalents and 1.10 (± 0.09) rod bivalents corresponding to a mean chiasma
162 frequency of 1.10 (± 0.09) per meiocyte at metaphase I (Supplementary Table S4). Among the seven
163 generated *Tamsh7-3D* Cadenza / *Ae. variabilis* hybrids, four showed a clear increase in homoeologous
164 recombination at meiotic metaphase I (Figure 3A, 3B, 3C, Supplementary Table S4). Cadenza2006 /

165 *Ae. variabilis* hybrids exhibited the strongest phenotype with a 5-fold increase in bivalent number (5.29
166 ± 0.15 rod and 0.26 ± 0.04 ring bivalents per meiocyte on average) and the presence of multivalents
167 (0.12 ± 0.03). This is associated with a highly significant 5.52-fold increase in mean chiasma frequency
168 (6.07 ± 0.17; F-test, $p = 3.81 \times 10^{-11}$) (Figure 3C; Supplementary Table S4). Cadenza0638, Cadenza1178
169 and Cadenza1114 / *Ae. variabilis* hybrids displayed an intermediate phenotype with a 2.21, 1.90 and
170 2.37-fold statistically-significant increase in mean chiasma frequency, respectively (Figure 3C;
171 Supplementary Table S4). The absence of a full-length TaMSH7-3D protein (and to a lesser extent,
172 amino acid substitution within TaMSH7-3D protein) thus induces a substantial increase in genome-wide
173 homoeologous recombination within the hybrid context. This data clearly demonstrates that TaMSH7-
174 3D inhibits recombination between homoeologous chromosomes.

175 Moreover, a previous study reported a slight reduction in homologous recombination efficiency in wheat
176 in the absence of *Ph2* [27]. We first confirmed that chiasma frequency is significantly reduced in *ph2b*
177 relative to Chinese Spring wild-type (37.48 ± 0.26 versus 40.98 ± 0.14, respectively; F-test, $p = 3.77 \times$
178 10^{-7}) due to an observed increase in univalent and rod bivalent frequencies for *ph2b* (Figure 3E, 3F;
179 Supplementary Table S5). As expected, *Tamsh7-3D* TILLING mutant Cadenza2006 exhibited a similar
180 phenotype (mean chiasma frequency of 37.58 ± 0.19) (Figure 3D, 3E, 3F; Supplementary Table S5).
181 Interestingly, rare trivalents and quadrivalents were also observed in *ph2b* and Cadenza2006 meiocytes
182 (but not in those of a wild-type background), revealing that homoeologous recombination occurs in
183 wheat in the absence of *Ph2 / TaMSH7-3D*, *albeit* in the presence of *Ph1*. Among the three lines carrying
184 amino acid changes within TaMSH7-3D and likely affecting protein function, Cadenza1114 exhibited a
185 more significant reduction in homologous recombination efficiency relative to Cadenza0638 and
186 Cadenza1178 (Figure 3E, 3F; Supplementary Table S5). The corresponding mutations are predicted to
187 affect different regions of the protein as shown by TaMSH7-3D protein modelling: *Tamsh7-3D*-G463R
188 (Cadenza1178) and *Tamsh7-3D*-A467T (Cadenza0638) are located within the mismatch-binding
189 domain, whilst *Tamsh7-3D*-D642N (Cadenza1114) is located within the connector domain
190 (Supplementary Figures 3 and 4) and could thus differentially impact phenotype.

191 We then crossed together the two EMS mutants exhibiting the strongest phenotypes (Cadenza2006 and
192 Cadenza1114) and confirmed that homologous recombination efficiency is significantly reduced in the
193 generated F1 compared to wild-type (mean chiasma frequencies: 39.20 ± 0.17 and 40.94 ± 0.09,
194 respectively; F-test, $p = 1.85 \times 10^{-9}$) (Supplementary Table S5). This result eliminates the possibility of

195 background recessive mutation elsewhere in the genome being a contributor to the phenotypes
196 observed.

197 Taken together, these data demonstrate that *TaMSH7-3D* loss-of-function mutants have the capacity to
198 recapitulate the *ph2* phenotype with a substantial increase in homoeologous recombination in wheat /
199 *Ae. variabilis* hybrids and a slight reduction of homologous recombination in wheat. These findings
200 reveal a key role for *TaMSH7-3D* in inhibiting recombination between homoeologous chromosomes and
201 consequently, in assuring accurate chromosome segregation during meiosis.

202

203 ***Tamsh7-3D* reduces pollen viability but does not affect plant fertility**

204 To assess whether meiotic behaviour disorders caused by *Tamsh7-3D* were associated with changes
205 in fertility, we performed Alexander staining of pollen and scored the proportion of viable versus non-
206 viable grains. Compared to wild-type, Cadenza2006 showed a slightly higher proportion of non-viable
207 pollen (p = 6 x 10^{-6}, pairwise t-test with correction for multiple testing) (Supplementary Figure S5,
208 Supplementary Table S6). We also measured seed-set and observed that seed number per spike in
209 Cadenza2006 was not significantly reduced compared to wild-type (p = 0.43, pairwise t-test with
210 correction for multiple testing) (Supplementary Figure S5, Supplementary Table S7). These data
211 demonstrate that *TaMSH7-3D* loss-of-function does not significantly affect wheat fertility (as comparable
212 seed sets are observed in the mutants) although this mutation does disturb proper homologous
213 recombination (and induces homoeologous recombination events in some meiocytes). These results
214 are in agreement with studies of tomato and Arabidopsis, in which reduced *MSH7* expression or MSH7
215 loss-of-function (respectively) do not affect seed number [33,34].

216

217 ***TaMSH7-3D* is expressed in anthers during meiotic prophase I**

218 To precisely determine *TaMSH7-3D* expression over the course of early meiosis, transcript-profiling
219 using a sub-staged meiotic time series was performed on whole-wheat anthers. Four meiotic stages
220 were analysed: late leptotene, zygotene / pachytene, diplotene / diakinesis and metaphase I. RNA-seq
221 data revealed that *TaMSH7-3D* (as well as *TaMSH7-3A* and *3B* homoeologues) is expressed for the
222 entirety of prophase, which is in agreement with a role of *TaMSH7-3D* in control of homoeologous

223 recombination at meiotic prophase I (Figure 4). *TaMSH7-3D* expression however, is not restricted to
224 meiosis considering similar transcript abundance was detected for each of the 3A, 3B and 3D
225 homoeologous copies in leaf, root and stem before flowering (Figure 4). To investigate whether an
226 absence of the pairing homoeologous gene *Ph1* could be compensated by an overexpression of *Ph2 /*
227 *TaMSH7-3D*, we compared *TaMSH7-3D* expression in wild-type versus the *ph1b* mutant background
228 using RNA-seq data previously generated [35]. No significant change in *TaMSH7-3D* transcript abundance
229 (or *TaMSH7-3A* and *3B)* was observed between wild-type and *ph1b* anthers at prophase I
230 (Supplementary Figure S6). This data indicates that the absence of *Ph1* does not feedback to cause
231 significant changes in *TaMSH7* expression during meiosis in wheat.

232

233 ***TaMSH7-3D* is more highly conserved than 3A and 3B homoeologues, both among wild and**
234 **domesticated wheats**

235 Comparison of *TaMSH7-3D* with its homoeologous copies revealed they share more than 97%
236 sequence identity at the nucleotide sequence level, with *TaMSH7-3A* being equidistant to *TaMSH7-3B*
237 and *TaMSH7-3D*. This is reflected in their deduced protein sequences, TaMSH7-3B and 3D are 96.3 %
238 identical to TaMSH7-3A and 97.2 % identical to each other (Supplementary Table S8). ~25 % of amino
239 acid differences between the homoeologues are concentrated within the region from amino acid 760 to
240 880, thus in the MutS domain III corresponding to the core domain of the proteins (Supplementary Figure
241 S7).

242 Exome sequencing data revealed a high level of conservation of *TaMSH7-3D* among 436 bread wheat
243 accessions studied. Only two haplotypes exist with the three identified SNPs exclusively localised within
244 introns (Supplementary Table S9). In contrast, *TaMSH7-3A* and *3B* genes (inclusive of promoter
245 regions) are more diverse with 12 and 30 polymorphisms identified within this population, respectively
246 (Supplementary Table S9). By data mining NCBI's small read archive, querying the 10+Genome Project
247 data and DAWN [36], we identified 14 accessions that contain a 28 bp deletion in *TaMSH7-3A*, likely
248 leading to a non-functional truncated protein (Supplementary Figure S8, Supplementary Table 10).
249 Pedigree analysis allowed us to deduce the most likely ancestor as Red-Fife, from which the deletion
250 was transmitted (Supplementary Figure S9). Considering only polymorphisms located within exons, we
251 calculated the number of variants found in the 436 wheat lines within each gene and reported it relative
252 to exon length. 13.69, 6.74 and 0 variants per 10 kb of exons were found in *TaMSH7-3A, TaMSH7-3B*

253 and *TaMSH7-3D*, respectively. Consistent with the low genetic diversity of the D-genome, *TaMSH7-3D*
254 is very well conserved within this collection. The *TaMSH7-3A* and *TaMSH7-3B* copies are however
255 highly polymorphic: *TaMSH7-3A* sits within the top 30% of most diverse A genome derived genes and
256 *TaMSH7-3B* within the top 20% of most diverse B-genome derived genes. Taken together, these data
257 demonstrate that *Ph2* / *TaMSH7-3D* is more highly conserved than its homoeologues among wild and
258 domesticated wheats, consistent with a major role for this gene in homoeologous recombination
259 inhibition.

260 MSH7 is also highly conserved more broadly amongst the grasses. Bread wheat MSH7 proteins indeed
261 show more than 70% amino acid identity with MSH7 homologues of all studied *Poaceae* species
262 (Supplementary Figure S10, Supplementary Table S11). Protein sequence alignment of MSH7
263 homologues revealed that the main functional domains of the protein (MutS domain I, II, III and V) are
264 particularly well conserved although the remaining regions of the protein display lower level of amino
265 acid identity across species (Supplementary Figure S11). This observation is in agreement with MSH7
266 function also being required for genome stability in more distantly related species.

Discussion

By 1952, it had become clear that corresponding bread wheat chromosomes derived from each subgenome were genetically very closely related, as observed through tetrasomy and nullisomy [37]. However the inability of these chromosomes to recombine during meiosis remained a paradox until a role for genetic suppressors was highlighted [38]. In this study, we report on the identification and functional validation of the key homoeologous chromosome pairing suppressor *Ph2*, through a combination of high-throughput exome and transcriptome sequencing of known mutants (*ph2a* and *ph2b*), cytogenetic analyses of both a 3DS deletion line series and independent EMS-induced mutants. We demonstrate that: (1) *Ph2* locates within a 14.3-Mb region ranging from 64.9 to 79.2 Mb on 3DS; (2) *TaMSH7-3D* is the only gene localized within this region that contains an EMS-derived SNP susceptible to affect protein sequence in *ph2b*; (3) additional mutants of *TaMSH7-3D* recapitulate the *ph2* phenotype in regards to homologous and homoeologous recombination; and (4) we were able to exclude all previously proposed candidates for *Ph2* (not localized within the 14.3-Mb newly refined *Ph2* locus and not mutated in *ph2b*) except for *TaMSH7-3D* which had been fortuitously identified. Taken together, these data point to *TaMSH7-3D* being the causative gene for *Ph2*, thus solving a half-century-old question.

TaMSH7 (*MutS homolog 7*) is a plant specific member of the DNA mismatch repair (MMR) family. These highly conserved proteins play an essential role in maintaining genome stability by assuring the initial step of the MMR pathway, *i.e.* recognition of base-base mismatches and insertion/deletion mispairs generated during DNA replication and recombination [39]. MSH7 forms a heterodimer with MSH2 and the protein complex allows specific recognition of single-base mismatches including G/G, G/A, A/A and C/A mispairs and to a lesser extent G/T, as shown by biochemical studies of Arabidopsis MSH2-MSH7 (MutSγ) complex [40–42]. The two heterodimeric complexes MSH2-MSH3 (MutSβ) and MSH2-MSH6 (MutSα), present in yeast, animals and plants, have different mismatch recognition properties and abilities to support MMR. MSH2-MSH3 senses large (2-16 nucleotides) insertion/deletion loops and interstrand crosslinks, whereas MSH2-MSH6 recognizes single-base mismatches, including oxidative mispairs (dihydro-8-oxoguanine), methylated mispairs (O^6meG:T and O^6meG:C) and small (1-2 nucleotides) insertion/deletion loops [43,44]. MSH7 appears to have arisen early in plant evolution, most likely *via* duplication and divergence from a MSH6-like gene present in a primitive plant [40,45], with this

296 extra DNA lesion recognition protein likely contributing to efficient repair of various DNA damage caused
297 by constant environmental exposure, for which plants are naturally subjected [42].

298 We show that the absence of a functional TaMSH7-3D induces a 5.5-fold genome-wide increase in
299 chiasma frequency in a bread wheat / *Ae. variabilis* hybrid context (Figure 3A, 3C), providing evidence
300 that this protein acts as a key inhibitor of homoeologous recombination. This finding is in line with a
301 previous study assessing how frequently alien chromatin of wild tomato (*Solanum lycopersicoides*) is
302 introgressed into cultivated forms (*Solanum lycopersicum*) following *MSH7* silencing [33]. This study
303 demonstrated a modest yet significant increase of 16.1% in recombination rate between these divergent
304 chromosomes. In Arabidopsis, loss of AtMSH7 (*msh7* T-DNA insertion line) was observed to increase
305 meiotic homologous recombination rate by 97% relative to wild-type at the subtelomeric 420 genetic
306 interval as assessed using a fluorescent seed reporter line [46]. This data contrasts with a slight but
307 significant reduction in genome-wide homologous recombination frequency observed for *Tamsh7-3D* in
308 wheat (Figure 3D, E, F; Martinez *et al.*, 2001). In some *Tamsh7-3D* / *ph2* wheat meiocytes, such a
309 reduction in homologous recombination is associated with the presence of multivalents resulting from
310 homoeologous recombination (Figure 3E; Supplementary Table S5; [26]). This observation suggests
311 that in a wild-type context, TaMSH7-3D plays a role in recombination partner choice (homologous *vs*
312 homoeologous) likely through promoting destabilization of recombination intermediates established
313 between homoeologous chromosomes. These intermediates could be less stable than those
314 established between homologous sequences because of the presence of mismatches. A role for MMR
315 proteins in recognizing mismatches created in heteroduplex DNA, following DNA-strand exchange and
316 promoting dissociation of strand invasion events - a process known as heteroduplex rejection - has
317 indeed been reported [47]. In rice, MSH7 interacts with MEICA, an orthologue of FLIP known to be a
318 partner of FIGL1 [48]. FIGL1/FLIP is a conserved complex that regulates the strand invasion step of
319 meiotic recombination [49]. Direct interaction between these two proteins is thus consistent with a role for
320 TaMSH7-3D during this critical step. By preventing divergent DNA sequences from recombining,
321 TaMSH7-3D would play a crucial role in assuring the diploid-like meiotic behavior of polyploid bread
322 wheat required for accurate chromosome segregation during meiosis. In diploid species, MSH7 may
323 also be involved in limiting ectopic (non-allelic) recombination, a driver of highly deleterious
324 chromosomal rearrangements, and could potentially provide an immediate advantage to newly formed
325 allopolyploids by assuring meiotic stability and consequently, fertility.

326 Identification of the two main genes controlling homoeologous recombination in bread wheat, *TaZIP4-*
327 *B2* [14] and *TaMSH7-3D* (this study), now offers a possibility of deciphering their direct mode of actions
328 and interactions. Recent data from G. Moore laboratory revealed that TaZIP4-B2 promotes homologous
329 bivalent formation by preventing recombination intermediates established between homoeologous
330 chromosomes from becoming crossovers [12,50]. In contrast to MMR proteins, there is no indication of
331 ZIP4 involvement in the inhibition of pairing between homoeologous (divergent) DNA sequences. This
332 thus suggests that TaMSH7-3D and TaZIP4-B2 could act sequentially with different modes of action
333 and consequently that homoeologous recombination is controlled by a multilayered mechanism in
334 polyploid bread wheat. *ph1* was found to be twice as strong as *ph2* [38] and an additive effect in promoting
335 homoeologous recombination has been reported, for example in wheat / *Aegilops* hybrids [28]. Combining
336 *Tazip4-B2* and *Tamsh7-3D* mutations may therefore offer an opportunity to further improve the efficiency
337 and ease of introgression of wild relative chromosomal segments into wheat, providing new
338 opportunities for the development of genetically unique and desirable wheat varieties. Exploitation of
339 *Tazip4-B2* and *Tamsh7-3D* EMS-derived double mutants that are in the elite background Cadenza, are
340 likely to be of particular interest to pre-breeders compared to previously available Chinese Spring
341 mutants (*ph1b*, *ph2a*), as time required to move an introgression into a breeding relevant genotype is
342 reduced. Additionally, the utilisation of point mutations is likely to avoid possible meiotic instability that
343 can be induced by large chromosomal deletions.

344 *TaMSH7-3D* has two highly similar homoeologous copies on chromosomes 3A and 3B, *TaMSH7-3A*
345 and *TaMSH7-3B*, with which it shares 97.77 % and 97.96 % identity, respectively (Supplementary Table
346 S8). Because of possible functional redundancy and their genomic locality, it is reasonable to assume
347 that *TaMSH7-3A* and *TaMSH7-3B* could correspond to the homoeologous pairing suppressors
348 previously identified on 3AS [30,51] and 3BS [52]. This also takes into consideration that the loss of both 3AS
349 and 3DS (*Ph2*) was observed to result in a level of homoeologous pairing similar to that caused by the
350 deficiency of 5B (*Ph1*) [30]. An interesting question is what could be the cause for differences in phenotypic
351 severity observed between homoeologues (3DS > 3AS > 3BS) [52]? As *TaMSH7-3A*, *TaMSH7-3B* and
352 *TaMSH7-3D* show comparable RNA abundance in wheat anthers during early meiosis (Figure 4), a
353 difference derived from transcriptional level is unlikely. Although TaMSH7-3A, TaMSH7-3B and
354 TaMSH7-3D proteins are very similar (> 96.3 % of sequence identity), mutation prediction algorithms
355 have hinted to potentially deleterious amino acid substitutions between homoeologues (*e.g.* L877S in

356 TaMSH7-3B and R855H in TaMSH7-3A). However, these predictions are indicative and require
357 experimental validation. Additionally, a shared 28 bp deletion predicted to lead to a non-functional
358 TaMSH7-3A protein in 14 wheat related accessions (Supplementary Figures S8 and S9, Supplementary
359 Table S10) indicates that *TaMSH7-3A* has degenerated into a pseudogene. This could potentially reflect
360 progressive duplicated gene loss – which is particularly rapid for meiotic genes – following
361 polyploidization events described in Angiosperms [53]. Generation of CRISPR/Cas9 mutants for one or
362 more *TaMSH7* copies will allow confirmation of their relative impact on homoeologous recombination as
363 well as determine their combinatorial effects, opening an opportunity to further refine exotic chromatin
364 introgression into elite wheats.

365 Taking advantage of newly available genetic and bioinformatic resources, this research has answered
366 a 50-year-old question on the causative agent for *Ph2* by identifying TaMSH7-3D. This work provides
367 new and fundamental insights into the molecular control of meiotic recombination in allopolyploids and
368 opens a path towards more efficient and flexible access to genetic diversity, a major bottleneck currently
369 facing crop improvement.

370 ## Material and methods

371

372 ### Plant material and growth conditions

373 Plant material used in this study included the following: wild-type hexaploid wheat (*Triticum aestivum*
374 cv. Chinese Spring and cv. Cadenza); Chinese Spring *ph2a* and *ph2b* mutants; 32 Chinese Spring 3D-
375 deletion lines (from Svačina *et al.*, 2020) and 7 Cadenza *Tamsh7-3D* mutant lines (Cadenza0638,
376 Cadenza0998, Cadenza1035, Cadenza1114, Cadenza1138, Cadenza1178, Cadenza2006
377 (Supplementary Table S3; www.wheat-tilling.com)). Wild-type cv. Chinese Spring, *ph2a*, *ph2b* and the
378 3D-deletion lines were crossed with rye (*Secale cereale*; RR, 2n = 14; var. Dankowski nove) to produce
379 wheat / rye haploid hybrids (ABDR, n = 28). Wild-type cv. Cadenza and the *Tamsh7-3D* mutant lines
380 were crossed with *Aegilops variabilis* (UUSS, 2n = 4x = 28) to produce wheat / *Ae. variabilis* haploid
381 hybrids (ABDUS, n = 35). Plants were grown in a controlled-environment room with the following
382 conditions: 16h light / 8h night photoperiod at 20°C day and 15°C night, with 70% humidity.

383

384 ### Cytological analysis

385 Young spikes were collected from 7 to 10-week-old plants and carefully dissected to isolate anthers. For
386 each dissected floret, one of the three developmentally equivalent anthers was squashed in aceto-
387 carmine staining solution and meiocytes visualised using a ZEISS Optima microscope. When meiocytes
388 at metaphase I were identified (for pairing analysis) or other defined stages (RNA analysis), the two
389 remaining anthers were either fixed in 100% ethanol/acetic acid 3:1 (v/v) for 48h and then subsequently
390 transferred to 70% ethanol or snap frozen in liquid N_2 for later RNA-based analyses. Fixed anthers can
391 eventually be stored at 4°C for a few months. For cytological analysis of meiocytes at metaphase I,
392 pollen mother cells (PMC) were released from the anther by crushing it on a slide in a drop of aceto-
393 carmine staining solution. Anther debris was carefully removed, and a coverslip placed on the slide. The
394 slides were then heated until separation of the chromosomes and aceto-carmine solution replaced by
395 acetic acid 45%. Coverslips were then vertically pressed to spread out the chromosomes. Chromosome
396 configurations of ~50 PMC per anther were analysed under a ZEISS Axio Observer Z1 inverted
397 microscope. For each cell, the number of univalents, rod bivalents (pair of chromosomes linked by a
398 unique chiasma), ring bivalents (pair of chromosomes linked by two chiasmata), trivalents (three

399 chromosomes linked by two chiasmata) and quadrivalents (four chromosomes linked by three or four
400 chiasmata) were counted. Frequency of chiasmata (the cytological manifestation of meiotic crossovers)
401 was then calculated. Significant differences between mutant and corresponding wild-type control
402 chiasma frequencies were assessed using unilateral F-test of equality of variances.

403

404 **Exome capture and bioinformatic analysis**

405 *DNA extraction and sequencing*

406 DNA was extracted from young leaves of wild-type bread wheat cv. Chinese Spring, *ph2a* and *ph2b* as
407 previously described [54]. Purified DNA samples were send to Arbor Biosciences (USA) for whole exome
408 capture and sequencing. Genomic DNA was sonicated using a Q800R sonicator (Qsonica, CT, USA)
409 and size-selected using SPRI beads to modal insert lengths of roughly 400 bp. Then 200 ng of the
410 resulting processed DNA was converted to Illumina Truseq-style libraries using in-house chemistry and
411 six cycles of dual-8bp-barcode indexing amplification. Target enrichment reactions were performed in
412 singleplex using 750 ng of each library and the Arbor Wheat Exome Beta probe set. The enrichment
413 procedure followed the standard myBaits version 4.0 manual (https://arborbiosci.com/wp-
414 content/uploads/2018/04/myBaits-Manual-v4.pdf), but with 0.75 µL IDT xGen Universal Blocking Oligos
415 (Integrated DNA Technologies, USA) *in lieu* of 0.5 µL Block A. Following capture clean-up as described
416 in the myBaits user manual, libraries were submitted for 150 bp paired-end sequencing on a partial
417 NovaSeq S4 lane (Illumina). Following sample de-multiplexing using both barcodes per library, read
418 pairs were taken to bioinformatic analysis.

419 *Preprocessing of raw reads*

420 Read qualities were inspected with FASTQC version 0.11.4 [55] before and after quality and adapter
421 trimming. For the exome capture data, trimming was achieved with Trimmomatic version 0.36 [56], using
422 the following parameters: -phred33 LEADING:5 TRAILING:5 SLIDINGWINDOW:4:20 MINLEN:50.

423 *Sequence alignment to CS Ref1.0*

424 Cleaned exome capture reads were aligned to the CS Ref v1.0 by Bowtie2 version 2.3.0 [57] allowing a
425 2% mismatch rate with the following parameters: --end-to-end --very-sensitive --n-ceil L,0,0.1 --rdg 3,3
426 --rfg 3,3 --no-unal --mp 6,6 --np 4 --no-mixed --score-min L,0,-0.12. After alignment PCR duplicates

427 were detected and removed from bam files using an in-house Java application Wheatbio.jar
428 (https://github.com/CroBiAd/TILLinG-mutant).

429 *SNP and Indel calling*

430 After read alignment, a pileup file was generated from the bam files of the exome capture data using the
431 mpileup command (--min-MQ 20 -B -f) in samtools version 1.4.1 [58]. The pileup file was subsequently
432 used to identify SNPs and indels located in the *ph2a* demarcated deletion region. To determine the
433 effect of the detected polymorphisms the CS Ref v1.0 annotation, specifically the high-confidence gene
434 models, was relied upon. To avoid calling false positive polymorphisms, we demanded that each SNP
435 or indel position was supported by a read depth of ≥ 4. To predict the consequences of mutations on
436 protein sequences, we used SNPeff [59] and the annotation from CS Ref v1.0 choosing the longest
437 predicted splice-form.

438

439 **RNA sequencing and processing**

440 Anthers cytologically determined to be at metaphase I or earlier were pooled for RNA extraction
441 according to [60]. These purified early-meiotic anther RNA samples derived from *ph2b* and Chinese
442 Spring were submitted to the Australian Genome Research Facility (AGRF, Australia) for library
443 preparation and sequencing on the Illumina NovaSeq 6000 instrument. Stranded cDNA was generated
444 from poly-adenylated RNA by TruSeq stranded mRNA library kits (Illumina). Samples were sequenced
445 to give 150 bp paired-end reads aiming at around 130 Mill reads/sample.

446 RNASeq raw data was processed with fastp version 0.19.7 [60] within minimum length requirement of 60,
447 trimming of poly G and removal of the first 10 bases. After trimming we had 144,818,345 clean paired-
448 end reads for *ph2b* replicate 1 and 141,336,841 reads for *ph2b* replicate 2. For the wild-type Chinese
449 Spring we obtained 130,912,185 and 135,248,954 reads for the two replicates, respectively. Trimmed
450 RNASeq reads were aligned using STAR version 2.5.3 [61] to CS Ref v1.0 with the following parameters:
451 --outFilterMismatchNoverLmax 0.02 --outFilterMatchNminOverLread 0.98 --outFilterMultimapNmax 5
452 --outFilterMultimapScoreRange 0 --outFilterScoreMinOverLread 0 --alignEndsType Local --
453 alignIntronMax 10000 --alignMatesGapMax 10500 --alignSoftClipAtReferenceEnds No --

454 outSJfilterOverhangMin 35 20 20 20 --outSJfilterCountTotalMin 10 3 3 3 --outSJfilterCountUniqueMin
455 5 1 1 1.
456

457 **Genetic diversity and phylogenetic analysis**

458 The MSH7-3A and 3B sequences were used as queries in BLASTN searches against available genomic
459 sequence data generated by the Wheat Initiative's 10+ Genomes project (https://webblast.ipk-
460 gatersleben.de/wheat_ten_genomes/) and for the UK varieties
461 https://wheatis.earlham.ac.uk//grassroots-portal/blast. Australian varieties were inspected for variation
462 in MSH7-3A and 3B in DAWN (http://crobiad.agwine.adelaide.edu.au/dawn/jbrowse/). Sequences for
463 Cadenza and Paragon were retrieved and trimmed to exon2 for CLUSTALW alignment. Public database
464 were searched for the presence of the 28 bp deletion by either using the full MSH7-3A sequence or for
465 NCBI-SRA data with a 300 bp subsequence covering the deleted region. In addition we queried DAWN
466 [36] at position chr3A:87373944..87374105 for the presence of the deletion in Bioplatforms Australia
467 sequenced varieties (https://data.bioplatforms.com/organization/bpa-wheat-cultivars). Pedigree
468 information was retrieved from GRIS (wheatpedigree.net) when available or the literature otherwise.

469 Grass sequences homologous to MSH7 were identified by BLAST searches against a range of
470 databases (Supplementary Table S10). Whenever gene models were non-existent or incomplete,
471 putative homologs were manually derived from genomic DNA, and their protein sequences deduced.

472 DNA and protein sequences were aligned by MUSCLE in Geneious and percentage identities
473 calculated. The unrooted phylogenetic tree was inferred using PhyML v20160115 ran with model JTT
474 and parameters: --nclasses 4 –f m –alpha e –pinv e –bootstrap 100 –o tlr [62]. Branch supports are
475 computed out of 100 bootstrapped trees.

476 **Acknowledgments**

477 We warmly acknowledge Isabelle Lhommet and Amelie Jeanneau for technical support and Hélène
478 Rimbert, Wandrille Duchemin and Jonathan Kitt for bioinformatics support. We thank Peter Langridge
479 (University of Adelaide) for providing the *ph2a* and *ph2b* mutants, and for the support provided to the
480 Australian authors over many years of meiosis research in his laboratory. We also thank Eric Jenczewski
481 (INRAE, IJPB, Versailles) for critical reading of the manuscript and helpful discussion. This work was
482 supported by EU-funded AgreenSkills+ fellowship, ANR CROC (CE19-2014), the Bettencourt-Schueller
483 Foundation, the Czech Science Foundation (grant award 17-05341S) and the ERDF project "Plants as
484 a tool for sustainable global development" (CZ.02.1.01/0.0/0.0/16_019/0000827).

485

486 **Author contributions**

487 HS, RS, UB, RW, TS, JB and PS conceived and designed the experiments. HS, RS and UB performed
488 the experiments. HS, RS, UB, RW and PS analysed the data. HS and RW wrote the manuscript with
489 inputs from UB and PS. All authors approved the manuscript.

490

491 **Competing interests**

492 The authors declare no competing interests.

493

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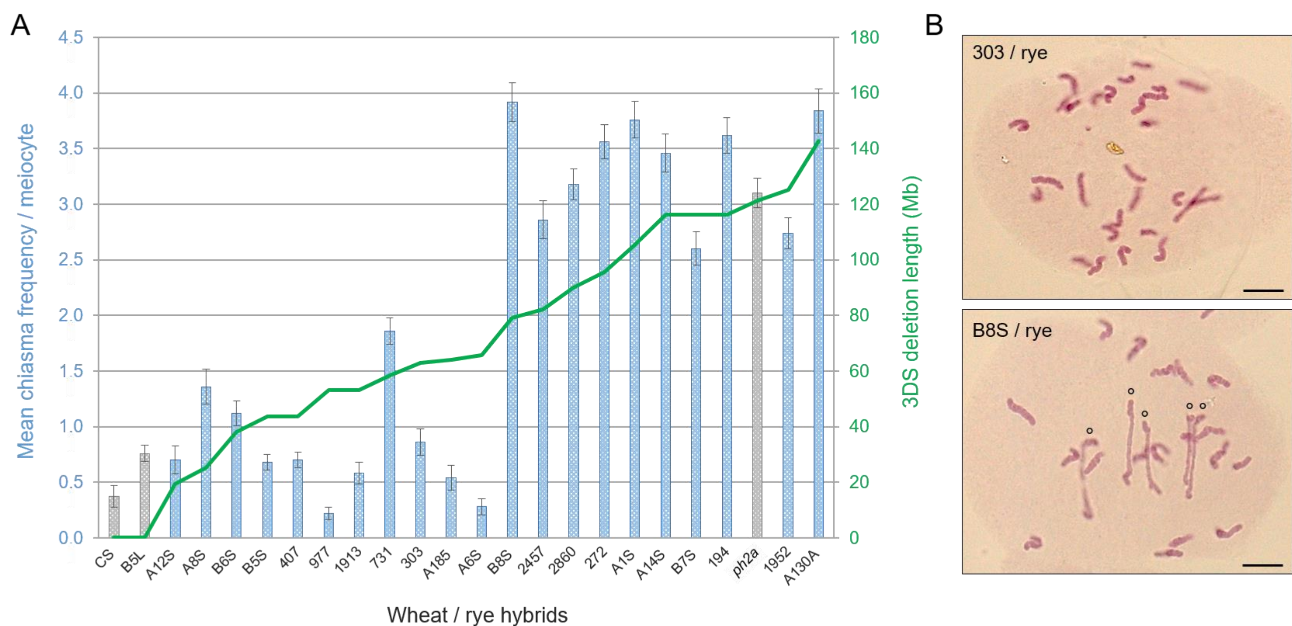
636

637

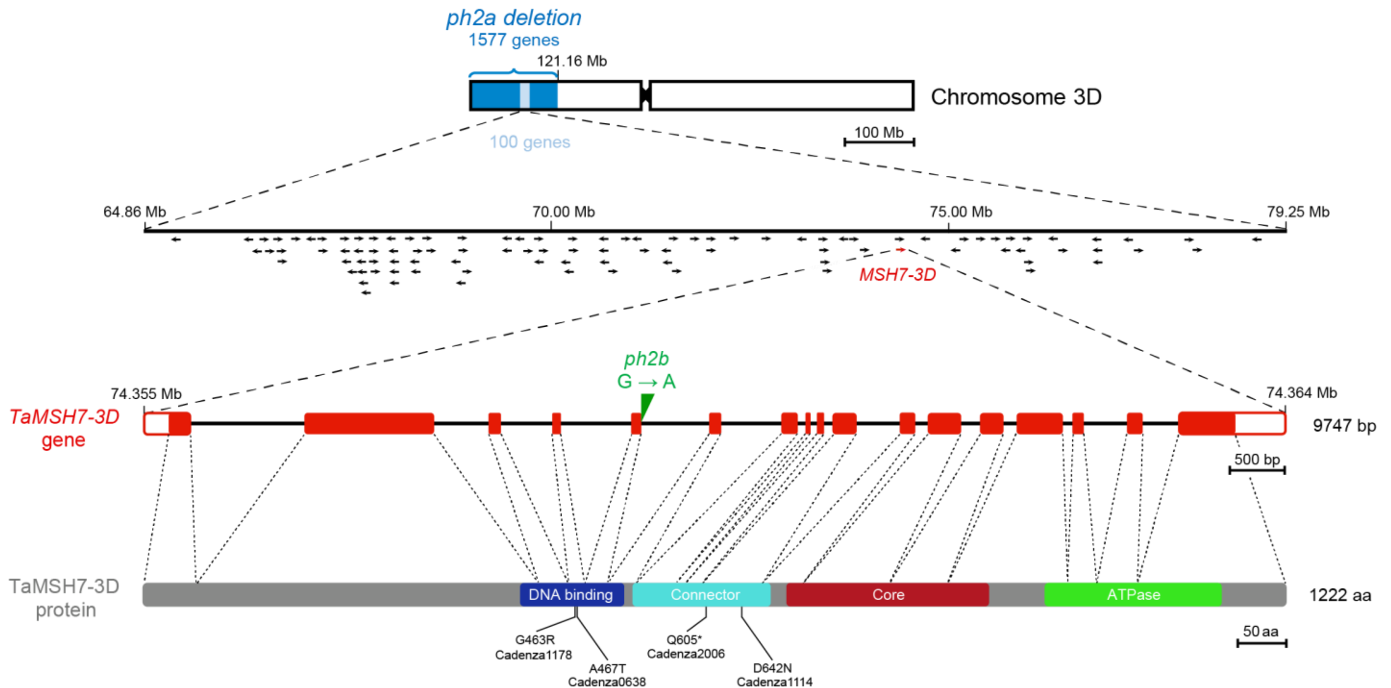
638 **Figures**

639

640 **Figure 1. Physical mapping of *Ph2* using wheat / rye hybrids carrying 3DS terminal deletions.**
641 Meiotic phenotypes at metaphase I of 24 wheat / rye hybrids were analysed cytogenetically. This
642 analysis includes three wheat / rye hybrid controls (in grey) derived from: wild-type wheat cv. Chinese
643 Spring (CS), CS carrying a 357 Mb deletion of 3DL but no 3DS deletion (B5L) and CS *ph2a* mutant
644 (*ph2a*). (A) Quantification of mean chiasma frequency per meiocyte for each hybrid. Histogram bars
645 represent the mean ± standard error of mean. The green line indicates length (in Mb) of the 3DS terminal
646 deletion carried by each hybrid. (B) Representative meiocytes at metaphase I of "303 / rye" and "B8S /
647 rye" haploid hybrids showing chromosome configurations. "303 / rye" meiocyte exhibits 28 univalents
648 while "B8S / rye" shows 18 univalents and 5 rod bivalents (o). Scale bars represent 10 μm for both
649 panels.



650 **Figure 2. Positional cloning of *Ph2* identified *TaMSH7-3D* as the causative agent.** Schematic
651 representation of chromosome 3D showing *ph2a* deletion (dark blue). Further deletion-line mapping
652 localised *Ph2* to a 14.3 Mb genetic interval (light blue) containing 100 genes (represented by arrows).
653 Among them, the only gene identified to contain an exonic SNP predicted to either result in a non-
654 synonymous amino acid (aa) change, protein truncation or alternate splicing in *ph2b* is *TaMSH7-3D*
655 (TraesCS3D02G119400) at the coordinates 74.355.077 – 74.364.823 (highlighted in red). The G to A
656 transition at position 74.359.312 in *ph2b* sequence is shown (in green) in the gene structural schematic
657 for *TaMSH7-3D*. *TaMSH7-3D* contains 17 exons (red rectangles) and 16 introns (black lines) with a total
658 length of 9747 bp. 5' and 3' UTR's are represented by white rectangles. The schematic representation
659 of the TaMSH7-3D protein is shown below. Regions encoding predicted protein domains are highlighted
660 by coloured rectangles: N-terminal mismatch-recognition domain (aa 405-515), connector domain (aa
661 525-672), core domain (aa 689-905) and C-terminal ATPase domain (aa 967-1154) containing a Walker
662 A motif. Thin vertical lines below indicate positions of the aa changes in the four TILLING *Tamsh7-3D*
663 mutants used in this study and * represents stop mutation.



664 **Figure 3. Independent mutations in *MSH7-3D* promote homoeologous recombination and reduce**
665 **homologous recombination, similar to *ph2*.** (A, B, C) Meiotic chromosome phenotype at metaphase
666 I of wheat cv. Cadenza / *Aegilops variabilis* haploid hybrids. Hybrids have 35 homoeologous
667 chromosomes and the presence of bivalents and/or multivalents at metaphase I are therefore markers
668 of homoeologous recombination (*i.e.* crossovers established between homoeologous chromosomes).
669 (D, E, F) Meiotic chromosome phenotype at metaphase I of wheat cv. Chinese Spring (CS) or Cadenza.
670 In a wild-type (WT) context, most homologous chromosome pairs are connected by two distal
671 crossovers that form ring bivalents at metaphase I. The presence of rod bivalents and/or univalents
672 reveals a reduction in homologous recombination efficiency. (A, D) Chromosome configurations of
673 representative meiocytes at metaphase I. Open circles and asterisks indicate rod bivalents and
674 univalents, respectively. Scale bars represent 10 μm for all panels. (B, E) Stacked bar graphs showing
675 mean proportions of each metaphase I chromosome configuration (univalent pairs, rod and ring
676 bivalents, multivalents). (C, F) Box plots showing minimum, first quantile, median (horizontal middle
677 line), third quantile and maximum count of chiasma frequency per meiocyte. Mean values are
678 represented by a cross. To test for differences between each mutant and corresponding wild-type
679 control, unilateral F-tests of equality of variances were performed. The significance indicators * and **
680 report a p value of $0.01 < p < 0.001$ and $p < 0.001$, respectively.

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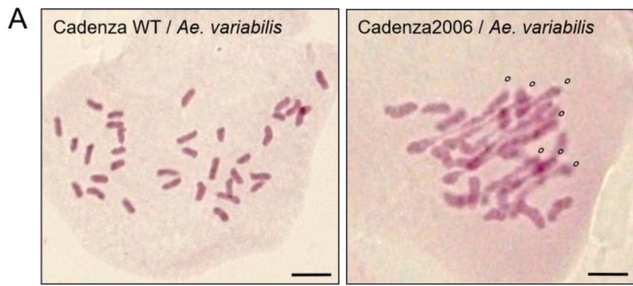
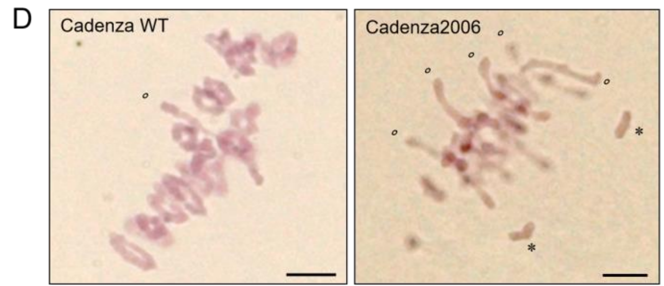
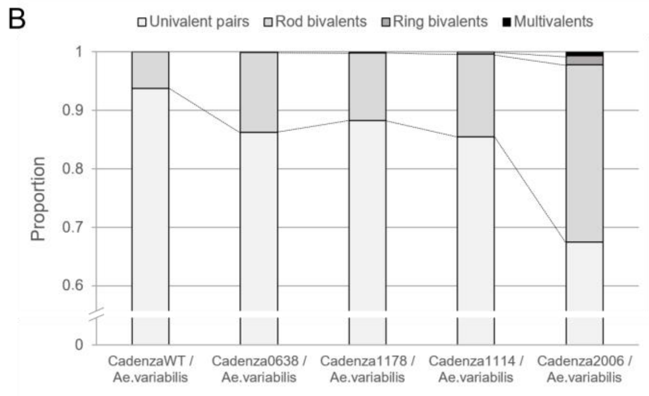
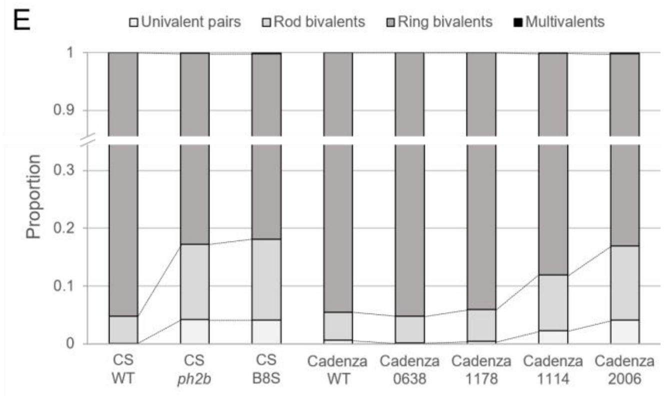
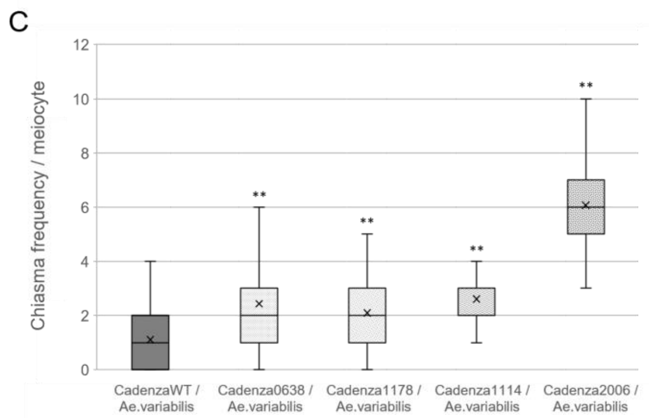
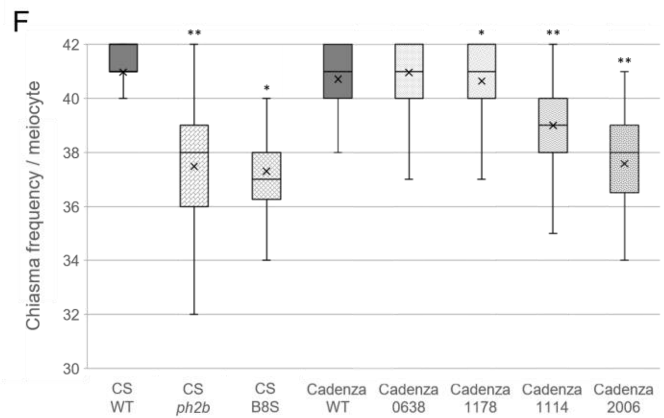




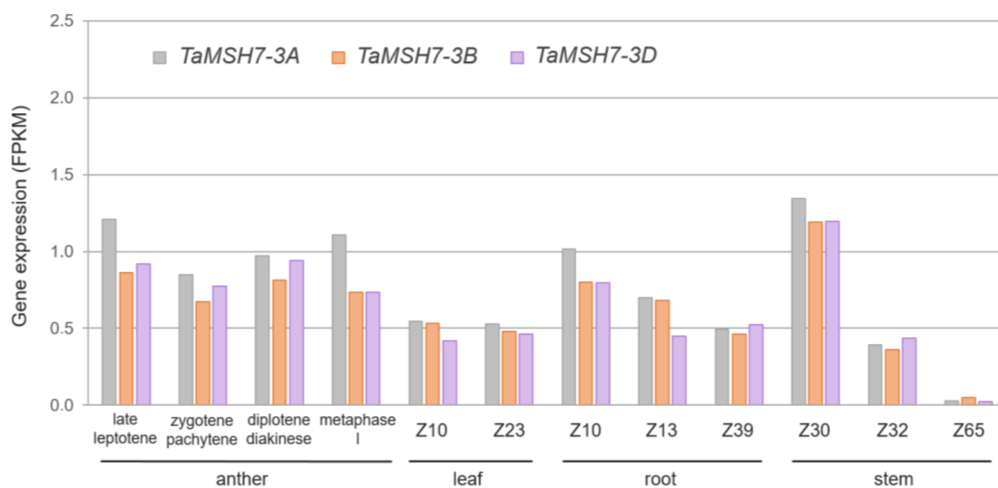








682 **Figure 4. *TaMSH7-3A*, *TaMSH7-3B* and *TaMSH7-3D* genes are expressed in anthers at early
683 meiosis and in somatic tissues.** Relative expression (in FPKM: Fragments per kilo base of transcript
684 per million mapped reads) of the three *TaMSH7* homoeologues in wild-type wheat anthers, leaves, roots
685 and stems at various developmental stages. Stages according to Zadoks scale [63]: Z10, seedling; Z13:
686 three leaves unfolded; Z23, main shoot and three tillers; Z30, pseudostem erection; Z32, two nodes;
687 Z39, flag leaf ligule and collar visible, Z65, half of flowering complete. Gene expression in anthers was
688 obtained following the method described by Lloyd *et al.*, 2014 [53] and data is available at http://wheat-
689 urgi.versailles.inra.fr/Seq-Repository/Expression. Gene expression in leaves, roots and stems is from
690 Pingault *et al.*, 2015 [64].



APPENDIX III

Chromosome pairing in polyploid grasses

Svačina, R., Sourdille, P., Kopecký, D., Bartoš, J.

Frontiers in Plant Science **11**: 1056, 2020

doi: 10.3389/fpls.2020.01056

IF: 4.298

frontiers in Plant Science

REVIEW
published: 09 July 2020
doi: 10.3389/fpls.2020.01056

Chromosome Pairing in Polyploid Grasses

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Polyploids are species in which three or more sets of chromosomes coexist. Polyploidy frequently occurs in plants and plays a major role in their evolution. Based on their origin, polyploid species can be divided into two groups: autopolyploids and allopolyploids. The autopolyploids arise by multiplication of the chromosome sets from a single species, whereas allopolyploids emerge from the hybridization between distinct species followed or preceded by whole genome duplication, leading to the combination of divergent genomes. Having a polyploid constitution offers some fitness advantages, which could become evolutionarily successful. Nevertheless, polyploid species must develop mechanism(s) that control proper segregation of genetic material during meiosis, and hence, genome stability. Otherwise, the coexistence of more than two copies of the same or similar chromosome sets may lead to multivalent formation during the first meiotic division and subsequent production of aneuploid gametes. In this review, we aim to discuss the pathways leading to the formation of polyploids, the occurrence of polyploidy in the grass family (Poaceae), and mechanisms controlling chromosome associations during meiosis, with special emphasis on wheat.

Keywords: chromosome pairing, homoeologous pairing, meiosis, Poaceae, polyploidy

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Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Plant Cell Biology, a section of the journal Frontiers in Plant Science

Received: *27 April 2020*
Accepted: *26 June 2020*
Published: *09 July 2020*

Citation:
Svačina R, Sourdille P, Kopecký D and Bartoš J (2020) Chromosome Pairing in Polyploid Grasses. Front. Plant Sci. 11:1056. doi: 10.3389/fpls.2020.01056

INTRODUCTION

Poaceae (grasses) is a large family of monocotyledonous flowering plants that includes ~10,000 diverse species divided into 12 subfamilies, 51 tribes, and 80 subtribes (Soreng et al., 2015). This family includes the cereals, bamboos, as well as natural and cultivated grasses, and its members are found worldwide except in ice-covered areas. Their economic importance derives mainly from their utilization for food and feed production, but they also have ecological and aesthetic roles in ecosystems and for humanity. For example, maize (*Zea mays*), rice (*Oryza sativa*), and wheat (*Triticum aestivum*) together provide >50% of the calories consumed by all humans. Sugarcane (*Saccharum officinarum*) remains the major source of human-consumed sugar and is increasingly used for biofuel production. Ryegrasses (*Lolium* spp.), fescues (*Festuca* spp.), and bluegrasses (*Poa* spp.) are cultivated as fodder crops and for amenity purposes (i.e. sports, private and industrial lawns). Bamboos (Bambuseae) are used to construct elaborate scaffolds and the straws of cereals can serve as insulation in buildings or as raw material for paper production. All these uses make the Poaceae species a priority choice for enhancing both their quality (i.e., protein, lipid or sugar

contents; cooking-quality, and digestibility, among others) and quantity (yield of grain and straw, biomass production).

Besides their great economic importance, species of the Poaceae family also serve as excellent model organisms for evolutionary studies (Kellogg, 2001). According to the pollen fossil record, grasses arose 55–70 million years ago (MYA; Jacobs et al., 1999). With ever more sequenced genomes (for details see https://bioinformatics.psb.ugent.be/plaza/), a detailed investigation of the evolutionary fate of duplicated chromosomal blocks led to the proposition of an ancestral karyotype for grasses, one structured in seven protochromosomes that contained 16,464 protogenes (Murat et al., 2014). This ancestral genome then further evolved, through the fusion and fission of chromosomes, gene duplication events as well as deletions, and chromosomal inversions and translocations. Moreover, interspecific hybridization and polyploidization (whole genome duplication; WGD) are two other key mechanisms of speciation in the Poaceae. All these phenomena have contributed to the extensive genome diversity extant within the family, including its variability in basic chromosome numbers and a wide range of polyploidy levels (Keeler, 1998). In this review, we highlight the nature of polyploidy in grasses, using wheat as a model, with a special focus on chromosome pairing during meiosis.

POLYPLOIDY

Polyploidy plays a significant role in the evolution of higher plants, in that all angiosperms apparently underwent at least one round of WGD in their evolutionary history (Jiao et al., 2011). Polyploids can be categorized based on their origin. *Autopolyploids* possess three or more copies of the same chromosome set; by contrast, the multiple chromosome sets in *allopolyploids* are of different origin, due to the involvement of interspecific hybridization. Yet a strict boundary between these two categories is not always evident, such that a third (intermediate) group called segmental allopolyploidy is sometimes recognized in plants (Winterfeld et al., 2012). In general, autopolyploids often exhibit the formation of multivalents during meiosis and polysomic inheritance in their progeny. By contrast, allopolyploids with distant parental genomes usually exhibit formations of bivalents from homologous chromosomes (i.e., diploid-like pairing behavior), leading to disomic inheritance (Ramsey and Schemske, 1998). Nevertheless, allopolyploids sometimes carry chromosome sets that are not identical, but divergence of their sequence is insufficient to avoid the pairing of homoeologs (i.e., chromosomes originating from two related parental genomes with substantial homology); hence, they must employ an additional mechanism to ensure diploid-like behavior. Jauhar (2003) suggested that stable meiotic behavior and genome stability in allopolyploid species is achievable only after establishing a mechanism to ensure homologous chromosome recombination and segregation.

Autopolyploids

For a long time, autopolyploids were believed to suffer from various evolutionary disadvantages, leading to the conviction that autopolyploidy is rare in nature and often represents an evolutionary dead end (Clausen et al., 1945; Stebbins, 1971). This view, however, contrasts with their widespread utilization in crop production, for which many autopolyploids including potato, banana, watermelon, and sugarcane are of high economic importance. The proportion of autopolyploidy among plant species can only be debated so far, given that many autopolyploids have escaped recognition, being morphologically similar to their progenitors and concealed among common diploid taxa (Soltis et al., 2007). Recently, Barker et al. (2016) inferred that autopolyploids might be as frequent as allopolypoids among vascular plants. The Poaceae family contains many known autopolyploid species, such as *Andropogon gerardii*, a dominant grass of the tallgrass prairie (Keeler and Davis, 1999), several *Brachiaria* species (Gallo et al., 2007), the forage crop *Hordeum bulbosum* (Eilam et al., 2009), the sugarcane plant *S. spontaneum* (Wang et al., 2010), in addition to several *Avena* species (Ladizinsky, 1973).

Allopolyploids

Allopolyploids result from the hybridization of two more or less related species, such as *Psidium guineense* (Marques et al., 2016), wheat (*T. aestivum*) or the common oat (*Avena sativa*). Genomes inherited by allopolyploids vary in chromosomal homology, based on congeniality of parental species. In the case of hybridization between distantly related species, chromosomal homology can be low enough to not pair up during meiosis, frequently having different basic number of chromosomes. Conversely, allopolyploids that originated from the cross between closely related species carry chromosomes with much higher degree of homology. Accordingly, their homoeologous chromosomes have the potential to pair and recombine during meiosis (Ramsey and Schemske, 1998; Sun et al., 2017). Bread wheat is a typical example of an allopolyploid; it originated from two distinct interspecific hybridizations among three related diploid species that diverged 5–7 MYA (Marcussen et al., 2014). The first hybridization event occurred <0.82 MYA, between *T. urartu* and an as of yet unknown species from the *Sitopsis* section, closely related to *Aegilops speltoides*, which resulted in the development of a tetraploid species that further evolved into cultivated tetraploid wheat (*T. turgidum* ssp. *durum*; BBAA; Marcussen et al., 2014). The second hybridization took place more recently, between this newly developed tetraploid and *Ae. tauschii* (DD), resulting in hexaploid *T. aestivum* ($2n = 6x = 42$; BBAADD; Huang et al., 2002; Petersen et al., 2006; Marcussen et al., 2014). Similarly, oats (*Avena* spp.) also comprise diploid, tetraploid, and hexaploid species, either as auto- or allopolyploids. The allopolyploid oats behave diploid-like during meiosis despite having partial homology between their parental genomes (Thomas, 1992). Besides evolutionarily old allopolyploids, relativey recent allopolyploidazion events are evident in nature. For example, about 150 years ago, the two natural hybrids *Spartina* × *neyrautii* and *S.* × *townsendii* emerged through crosses between European *S. maritima* and *S. alternifolia*, the latter introduced from America. While the homoploid hybrid *S.* × *townsendii* is mostly sterile, chromosome doubling gave rise to the fertile allotetraploid

species *S. anglica* (Hubbard, 1968) which spread rapidly throughout salt marshes in Western Europe (Gray et al., 1990; Thompson et al., 1991; Baumel et al., 2001; Salmon et al., 2005). As such, the polyploidization found in *S. anglica* may represent a way by which interspecific hybridization can foster evolutionary success.

Pathways Leading to Polyploidy

There are several routes leading to the formation of a polyploid individual. The first way is *via* chromosome doubling because of non-disjunction during mitosis. However, this way is rarely observed under natural conditions and is usually achieved only by exposure to chemical agents (Ramsey and Schemske, 1998; Tamayo-Ordóñez et al., 2016; Pelé et al., 2018). The more likely mechanism operating is that through the generation of unreduced gametes. The frequency of their production usually varies from 0.1% to 2% (Kreiner et al., 2017; Pelé et al., 2018) but this increases in response to stress, such as drought, low or high temperatures, and physical damage (Mason et al., 2011; Pécrix et al., 2011; De Storme et al., 2012; Vanneste et al., 2014; Kreiner et al., 2017; Van de Peer et al., 2017). This fact indicates polyploid formation could accelerate in periods of intensive environmental disturbances and rapid changes (Soltis et al., 2007). Polyploidy can be achieved in a single step process by fusing two unreduced gametes, through a so-called triploid bridge, or *via* a pathway involving two steps (**Figure 1**). The triploid bridge is expected to more commonly occur than the one-step pathway, due to the low probability of fusion of two unreduced gametes in natural populations (Husband, 2004). The two-step pathway of allopolyploid formation first involves generation of a homoploid hybrid. Such an individual would either require a somatic doubling event, fusion of its two unreduced gametes, or involvement of the triploid bridge to restore its fertility (Mason and Pires, 2015). Alternatively, when the progenitors are autopolyploids, an allopolyploid can emerge immediately through the fusion of their standard (i.e., reduced) gametes (Pelé et al., 2018).

Polyploid species usually revert to a diploid state during evolution. The first part of this process, called *cytogenetic diploidization*, results in the formation of species, whose polyploid origin might be hidden by disomic inheritance and diploid-like meiosis. This step occurs rather rapidly after polyploid formation either by establishment of genetic control mechanism similar to Ph system in wheat (see below) or extensive chromosomal rearrangements. Over millions of years *genomic diploidization* continues. The content of the genes, which has doubled by polyloidization, is gradually returned towards one copy for each gene. For example, maize underwent an ancient WGD ~10 MYA. Since then, it has not only become cytogenetically diploid but also undergone extensive gene loss causing many genes to revert to a single-copy status in the genome (Renny-Byfield et al., 2017).

Advantages and Risks of Polyploidization

The question still stands: what is the main evolutionary advantage of polyploid formation in plants? While it may appear to have little impact on particular species (Meyers and Levin, 2006), it can also represent a significant evolutionary tool for improving possibilities of adaptation (Otto and Whitton, 2000). For example, gene redundancy offers an opportunity to better resist deleterious mutations and to diversify the extra copies of genes in subsequent evolution; in this way, new traits may be acquired without the adverse effects of losing the original genes' function (Ha et al., 2009). From comparative analysis of collinear genes in syntenic regions of wheat and its diploid relatives Akhunov et al. (2013) confirmed the increased gene diversification conferred by polyploidy. Besides gene redundancy, allopolyploids can also benefit from the advantages of heterosis immediately upon their formation (Osborn et al., 2003; Comai, 2005), which can foster a greater biomass and accelerated development. Similarly, autopolyploidy might result in higher biomass of plants (Stebbins, 1971) and seed size, the latter enabling a more rapid rate of early development, such as in *Triticum* and *Aegilops* species (Villar et al., 1998; von Well and Fossey, 1998). All these effects of polyploidization could contribute to faster colonization of new niches, including extreme habitats (Ehrendorfer, 1980). At the chromosomal level, the existence of extra chromosomal set(s) represents a significant fitness advantage for tolerating large rearrangements in the genome that would normally lead to fatal consequences in diploid progenitors.

Clearly then, polyploid species are evolutionarily successful. In many cases (e.g., *T. aestivum*) they can grow in broad geographical areas and occupy a range of habitats (Feldman and Levy, 2005; Dubcovsky and Dvorak, 2007) as well as colonize extreme environments, like *S. anglica* has done (Hubbard, 1968; Gray et al., 1990; Thompson et al., 1991; Baumel et al., 2001; Salmon et al., 2005). Van de Peer et al. (2009) argued the higher competitiveness of polyploids could be explained by an ability to produce more diverse phenotypes than diploid species. Finally, it is worth noting that many staple crops are in fact polyploid species, and humankind has been using artificial polyploidization techniques and wide hybridization as a tool for their breeding and crop improvement. The use of wild relatives to enhance crops dates back to the early 1940s but gained prominence during the 1970s and 1980s (Hajjar and Hodgkin, 2007). Specifically, allopolyploidization is implemented to widen the target species' genetic diversity or to introgress beneficial alleles from relatives into cultivated crops. For example, while the natural genetic diversity of elite sown material is significantly lower than that observed in its landraces, breeding programs have introduced new sources of diversity into wheat's cultivars. To date, novel alleles have been introgressed from more than 50 related species representing 13 genera, highlighting the importance of these alien introgressions for improved wheat breeding (Wulff and Moscou, 2014). Perhaps the most well-known case is the rye (*Secale cereale*) 1RS translocation that harbors genes involved in a plant's resistance to multiple diseases (*Pm8*/*Sr31*/*Lr26*/*Yr9*) and its yield enhancement. Other examples of introgressions include that of *Sr36*/*Pm6* from *T. timopheevii*, *Lr28* from *Ae. speltoides*, and *Pch1* and *Sr38*/*Lr37*/*Yr17* from *Ae. ventricosa*, which provided resistance to severe diseases such as

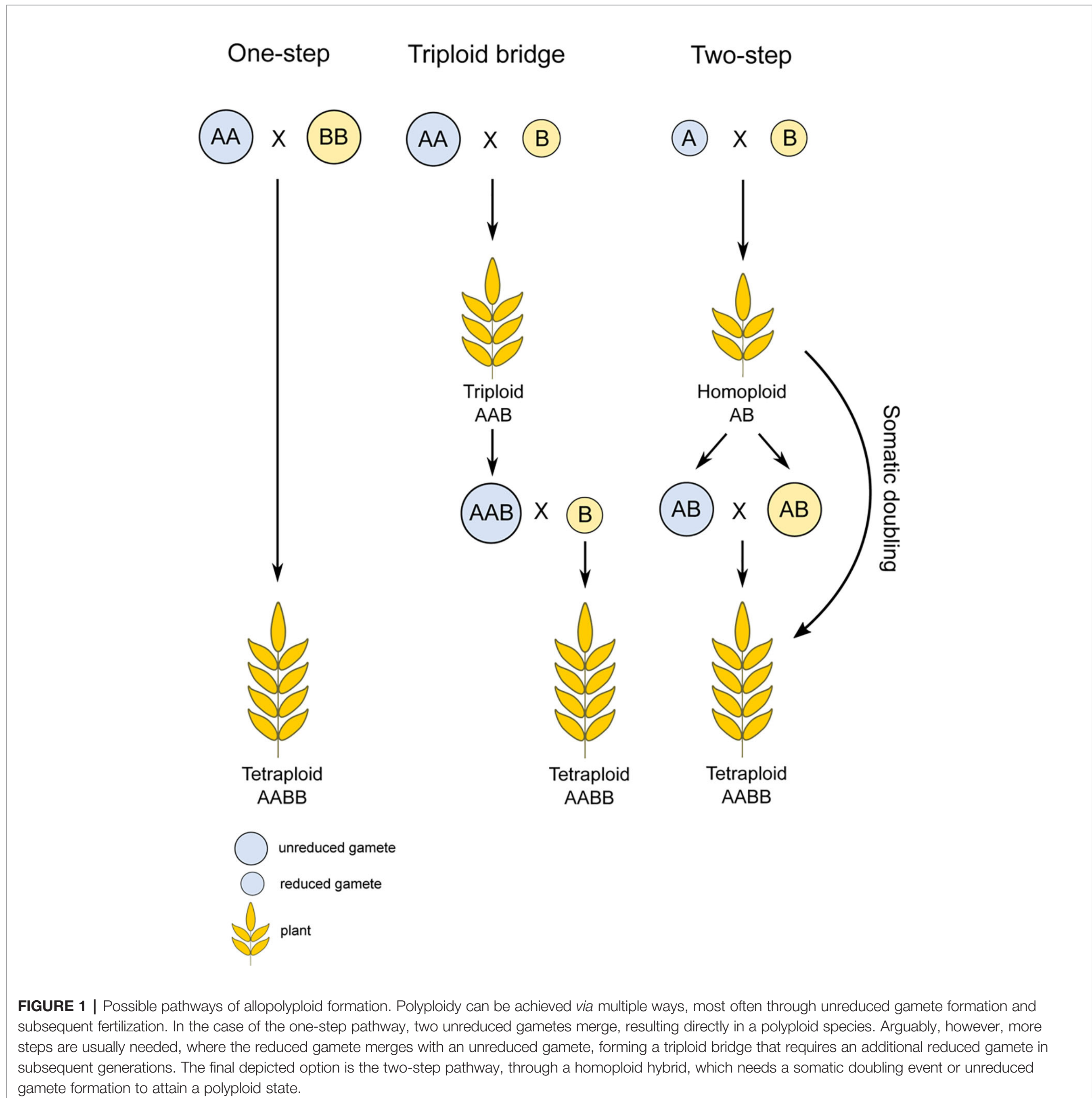

FIGURE 1 | Possible pathways of allopolyploid formation. Polyploidy can be achieved *via* multiple ways, most often through unreduced gamete formation and subsequent fertilization. In the case of the one-step pathway, two unreduced gametes merge, resulting directly in a polyploid species. Arguably, however, more steps are usually needed, where the reduced gamete merges with an unreduced gamete, forming a triploid bridge that requires an additional reduced gamete in subsequent generations. The final depicted option is the two-step pathway, through a homoploid hybrid, which needs a somatic doubling event or unreduced gamete formation to attain a polyploid state.

stem and leaf rust and powdery mildew. Some of these introgressions were implemented gobally in commercial lines; for example, the 1RS.1BL translocation now found in 10% of the world's genetic wheat diversity (Balfourier et al., 2019).

Nontheless, in addition to its positive impacts, polyploidy may have negative aspects. Perhaps the most obvious issue is the presence of more than one pairing partner in meiosis. Unless it is properly processed, it could result in multivalent formation and the production of aneuploid gametes, and thus, lower fertility or complete sterility (Ramsey and Schemske, 2002). Among the adaptive mechanisms described for autopolyploids, there is one based on a reduction in the number of cross-overs to one per chromosome pair, thereby ensuring only bivalents form from any two random homologs (Lloyd and Bomblies, 2016). This mechanism was observed in natural accessions of autotetraploid *Arabidopsis arenosa* (Carvalho et al., 2010; Pecinka et al., 2011; Yant et al., 2013; Pelé et al., 2018). By contrast, recognition of homologous chromosomes is critical for diploid-like pairing in allopolyploids. In allopolyploids containing distinct genomes, it is usually maintained by sequence variation between

homoeologous chromosomes. In allopolyploids containing closely-related genomes, homolog recognition seems to be genetically controlled (Jenczewski and Alix, 2004). However, some allopolyploid and homoploid hybrids do not necessarily display significantly reduced fecundity, despite the pairing of homoeologous chromosomes. In such case, aneuploidy, chromosome rearrangements, and the predominance of one of the parental genomes could be observed, as described for ×Festulolium hybrids (Kopecký et al., 2006). Hereon, we focus on mechanisms controlling chromosome pairing in some crops belonging to the grass family (Poaceae).

CONTROL OF CHROMOSOME PAIRING IN POLYPLOID GRASSES

Meiosis is a crucial process for sexual reproduction and gamete formation. It ensures reduction of genetic material to half resulting in restoration of normal chromosomal constitution in progeny. As noted above, some allopolyploids have evolved molecular mechanisms that govern homologous chromosome pairing. Such regulators were observed and identified in several species, including those of *Triticum*, *Avena*, and *Festuca*. The origin of the genes responsible for regulating chromosome pairing is not known yet, however. Nonetheles, several hypotheses explaining the possible emergence of such mechanisms have been proposed.

The first hypothesis works by presuming the presence of these pairing regulators in diploid progenitors (Waines, 1976; Jenczewski and Alix, 2004). In this model, a stable allopolyploid would emerge after a rare event, in which the appropriate combination of such genes is achieved (Waines, 1976). Indeed, several regulators acting as suppressors of homoeologous chromosome pairing were believed to exist in diploid relatives of allopolyploids, such as *Lolium* spp., *Hordeum vulgare* (Gupta and Fedak, 1985), *Hirschfeldia incana* (Eber et al., 1994), *Secale cereale* (Riley and Law, 1965), *Elytrigia elongata* (Dvorak, 1987), *Triticum monococcum* (Shang et al., 1989), and *Ae. tauschii* (Attia et al., 1979). In *Lolium*, the pairing suppressors were found present in some accessions of *L. multiflorum* and *L. perenne*, where they influenced the number of chiasmata during the first meiotic division of their homoploid hybrid. This chiasma reduction was accounted for exclusively by homoeologous pairing, as revealed by artificially tetraploidized hybrids (Evans and Aung, 1985; Jenczewski and Alix, 2004). Another example of how chromosome-pairing control is induced through a combination of genotypes or genes was found in rice. Generally, rice intersubspecific autotetraploid hybrids display meiotic instability such as chromosome lagging and the formation of univalents and trivalents (Cai et al., 2007). Yet two lines PMeS-1 and PMeS-2 were distinguished as being stable, presumably due to the presence of one or more active meiotic regulator *PMeS* (polyploid meiosis stability) genes (Cai et al., 2007). These two lines display regular meiotic behavior, with bivalents and quadrivalents. The existence of genetic chromosome pairing *PMeS* control was confirmed by the persistent meiotic stability of the two lines even after several generations (Xiong et al., 2019).

The second hypothesis posits that the regulators of chromosome pairing emerge during or immediately after the formation of polyploids, by a mutation or multiple, successive mutations (Riley and Law, 1965; McGuire and Dvořák, 1982). This can happen *via* conversion of a gene that promotes chromosome pairing in the diploid progenitor into a repressor in the polyploidy individual (Riley and Kempanna, 1963; Feldman, 1966b). This phenomenon was described in hexaploid wheat, where a mutation in a pairing promoter gene on the long arm of its chromosome 5D caused a reduction of homoeologous chromosome paring in several interspecific hybrids. Such mutations provide a more pronounced effect than does being 5D nullisomic, which suggests the mutation is antimorphic, changing the gene's function from pairing-promotion to suppression (Viegas et al., 1980). Those authors argued that this allele more likely arose from spontaneous mutation of a pairing-promoter known to be located on 5DL than from the transfer of *Ph1* from chromosome 5B.

The third hypothesis proposes that such regulators of chromosome pairing could be transferred *via* accessory B chromosomes (Riley et al., 1973; Sears, 1976). Early allopolyploid species would have depended on the presence of a B chromosome(s), until the gene was transferred to an A chromosome by translocation, with the subsequent loss of the B chromosome from the karyotype (Jenczewski and Alix, 2004). Many studies have investigated the role of B chromosomes in the repression of homoeologous pairing (Evans and Macefield, 1972; Evans and Macefield, 1973; Aung and Evans, 1985). It seems that one or more B chromosomes from a specific source could complement one copy of the aforementioned homoeologous-pairing suppressor into a functional complex. Evans and Aung (1986) found homoeologous pairing dramatically reduced in the hybrids of *F. arundinacea* × *L. perenne* carrying B chromosomes. Also, the average number of chromosome arms joined by chiasmata is reduced in the presence of B chromosomes in a diploid meadow fescue when compared to the control plants lacking B chromosomes (Kopecký et al., 2009). In the hybrids of *Ae. mutica* and *Ae. speltoides*, the B chromosomes can also complement a missing *Ph1* locus (Dover and Riley, 1972). Mechanisms controling chromosome pairing in allopolyploids seems to be specific among individual taxa, with very little known of the molecular pathways contributing to this phenomenon. In this respect, the best-elucidated molecular mechanism concerning the *Ph* genes is that of hexaploidy wheat (*T. aestivum*), which we describe in greater detail later on.

Apart from specific genetic systems to ensure proper chromosome pairing in particular species, various other (more general) genes are involved during process of meiosis that could increase the frequency of cross-overs between homologous chromosomes while suppressing them between homoeologs. Recently, Gonzalo et al. (2019) studied the effect of *MSH4* upon homo- and homoeologous cross-overs, by using the EMS (ethylmethanesulphonate) mutant population in *Brassica napus*. They discovered that, when the *MSH4* gene returns to a single

copy status, the frequency of homologous cross-overs remained at the same frequency, whereas that of homoeologous cross-overs decreased drastically compared with the presence of two functional copies of the gene. Gonzalo et al. (2019) also studied the copy numbers of other genes of the synapsis-initiation complex (SIC, or alternatively ZMM-pathway) vis-à-vis diploid relatives, deducing that the acquisition of additional copies of such genes through small-scale duplications is a rare event; an example its occurrence is *ZIP4* in wheat (Rey et al., 2017). Furthermore, the rapid reduction in the number of copies for ZMM genes in many species after whole genome duplication—namely for *MSH4*, *MSH5*, *MER3*, and *ZIP4*—supports the hypothesis that ensuring fewer copies of such genes could be a general process of meiotic stabilization (Lloyd et al., 2014; Gonzalo et al., 2019). Another study found no evidence for an increased loss of those genes after polyploidization in hexaploid wheat (including *MSH4*), in that most meiotic genes were retained in three homoeologous variants at similar expression levels (Lloyd et al., 2014). However, because wheat underwent its two hybridization events rather recently (Marcussen et al., 2014), the potential ZMM pathway gene reduction cannot be ruled out. Alternatively, the machinery established *via Ph* genes might have weakened the selective pressure for fewer copies of these genes.

Chromosome Pairing in Wheat

Allohexaploid bread wheat (*T. aestivum* L.; 2n = 6*x* = 42; BBAADD) can serve as a model plant for meiotic behavior analyses of allopolyploids. Despite the coexistence of three highly similar genomes, its meiotic behavior is strictly diploid-like, with 21 bivalents between homologous chromosomes forming in metaphase I of meiotic division. It has been known for more than 60 years that bread wheat developed genetic control of precise formation of homologous chiasmata, which is enforced by *Ph* (pairing homoeologous) genes (Sears and Okamoto, 1958; Riley and Chapman, 1958). The hexaploid nature of wheat allowed for the development of various aneuploid stocks, permitting the identification of several key genes involved in the regulation of meiosis (Sears and Okamoto, 1958; Sears, 1976; Sears, 1977; Sears, 1982; Sears, 1984).

It was proposed that premeiotic chromosome associations in interphase nucleus also play role in homolog recognition (Brown and Stack, 1968; Comings, 1968; Loidl, 1990; Aragón-Alcaide et al., 1997; Schwarzacher, 1997; Mikhailova et al., 1998; Martínez-Pérez et al., 1999). Nevertheless, different studies disagree in the extent and role of premeiotic chromosome associations, where they start and how long they last (Schwarzacher, 1997; Mikhailova et al., 1998; Martínez-Pérez et al., 1999). However, all these studies partially agree with Feldman (1966a), who suggested that *Ph1* controls spatial organization of chromosomes in premeiotic interphase nuclei. In wheat, the arrangement of chromosomes in interphase nuclei is done through distribution of centromeres and telomeres in opposite sides of nuclei into Rabl configuration (Fussell, 1987), whereas this configuration is being maintained in premeiotic cells (Naranjo, 2015). This organization plays a role in the recognition of homologs, as it reduces the homolog search and simplifies the subsequent alignment (Pernickova et al., 2019). The telomeres are then recruited to the nuclear envelope and form a telomere bouquet (Dawe, 1998; Harper et al., 2004), which is believed to be essential for homolog identification and initiation of synapsis (Bass et al., 2000; Scherthan, 2001; Bass, 2003; Harper et al., 2004; Scherthan, 2007). The molecular mechanisms driving these changes are, however, mostly unknown.

Formation of chiasmata in wheat is driven by both suppressors and promoters, of which several have already been identified. The most important gene regulating homologous chiasmata is *Ph1* (*Pairing homoeologous 1*), located on the long arm of chromosome 5B (Sears and Okamoto, 1958; Riley and Chapman, 1958). Another gene affecting chromosome behavior during meiosis, called *Ph2*, is located on the short arm of chromosome 3D but it exerts a weaker effect than does *Ph1* (Mello-Sampayo, 1971). The least effective regulator, *Ph3*, is located on the short arm of chromosome 3A (Driscoll, 1972; Mello-Sampayo and Canas, 1973). Similar effects of *Ph2* and *Ph3* genes and their location on the same chromosomes of different parental genomes suggest these two genes are probably paralogs. During metaphase I of meiosis, *ph* mutants typically display fewer ring bivalents (with two or more chiasmata) and more univalents, rod bivalents and multivalents when compared to the wild type (**Table 1**).

Pairing Homoeologous 1 (Ph1)

Among those genes controlling chiasmata formation during meiosis in wheat, *Ph1* has the strongest effect on ensuring the correct recognition of homologous chromosomes. Although the presence of this control element was discovered over 60 years ago, its molecular effect was uncovered in part only recently. Its existence was first proposed by Sears and Okamoto (1958) and Riley and Chapman (1958) in haploid lines of wheat lacking chromosome 5B, in which the formation of both bivalents and trivalents had been observed. This contrasted with the meiotic behavior of lines carrying a copy of 5B. Subsequent gene mapping was carried out using the *Ph1* mutant called *ph1b* (Sears, 1977), which helped to delimit the gene's location. Later

TABLE 1 | Comparison of chromosome associations in hexaploid and tetraploid wheat plants and particular *ph* mutants during metaphase I (Martínez et al., 2001a; Martínez et al., 2001b).

Genotype	Chromosome number	Univalents	Rod bivalents	Ring bivalents	Multivalents	Chiasmata per cell
Hexaploid WT	42	0.02	1.48	19.50	0.00	40.49
ph1b	42	2.76	4.76	14.5	0.77	38.57
ph2b	42	0.48	2.95	17.78	0.00	34.22
Tetraploid WT	28	0.04	0.34	13.64	0.00	27.62
ph1c	28	0.94	3.69	9.46	0.19	23.16

mapping, by Gill et al. (1993), used deletion lines to narrow down the genome region harboring the gene, which was cytogenetically estimated to be ~70 Mb. A more recent estimate of this deletion's length put its at 54.6 Mb (Gyawali et al., 2019). Countless studies have shown that when *Ph1* is missing, the chiasmata formation is no longer strictly diploid-like and chromosomes will form multivalents in more than 50% of pollen mother cells (Riley and Chapman, 1958; Riley, 1960). Work by Sánchez-Morán et al. (2001) confirmed that stark irregularities, such as aneuploidy and genomic rearrangements, are observable in lines lacking *Ph1*.

The *Ph1* locus is present in tetraploid wheat plants as well, such as *T. turgidum* subsp. *durum* (Dvorak et al., 1984) and *T. timopheevi* subsp. *timopheevi* (Feldman, 1966b). In the latter, a mutant for this particular gene was developed, called *ph1c*, having a similar phenotype as the hexaploid mutant *ph1b*, i.e., increased homoeologous chromosome chiasmata in metaphase I (Jauhar et al., 1999). In a comparative study assessing the effectiveness of *Ph1* gene in tetraploid and hexaploid wheat, Ozkan and Feldman (2001) crossed *Ae. peregrina* with hexaploid wheat and derivative lines, wherein chromosome 5B was replaced by its variant from tetraploid wheat (either from *T. turgidum* subsp. *dicoccoides* or *T. timopheevi* subsp. *Timopheevi*). With 5B from tetraploid wheat present, a higher frequency of homoeologous chromosome associations was observed in hybrids relative to the presence of endogenous 5B, indicating the tetraploid variant of *Ph1* gene might operate with lower effectiveness. Interestingly, once *Ph1* is introgressed from wheat into related species, its ability to modify chromosome bahavior is also preserved in the host genome (**Figures 2A, B**; Lukaszewski and Kopecký, 2010).

The *Ph1* regulator probably acts in multiple ways during meiosis. In early prophase I, it promotes the formation and subsequent correction of synapses (Holm, 1986; Martínez et al., 2001a), but later on, it affects the frequency of cross-over formation (Martín et al., 2014). Originally, the *Ph1* gene was thought to function as a suppressor of homoeologous synapses (Holm and Wang, 1988), but the current view is that it works primarily by promoting and stabilizing homologous synapses (Martín et al., 2017). During metaphase I in hexaploid wheat, ring bivalents are predominantly formed between homologous chromosomes, with some rod bivalents occurring in all meiocytes (Martín et al., 2014). In the *ph1b* mutant, only ~50% of meiocytes wil display similar meiotic behavior with increased frequency of rod bivalents; in the other half, variable numbers of multivalents and univalents were instead detected. This means that roughly half of the meiocytes display chiasmata only between homologous chromosomes (Martín et al., 2014). Similarly, other studies could not find homoeologous chiasmata in significant fractions of meiocytes in other *Ph1* mutants (Roberts et al., 1999; Al-Kaff et al., 2008; King et al., 2016). This suggests the promotion of homologous synapses is the main function of the *Ph1* gene, rather than suppression of homoeologous ones (Martín et al., 2017). This hypothesis is further supported by the higher occurrence of univalents in *ph1b* mutants than in the wild type or *ph2b* mutant (**Table 1**).

Griffiths et al. (2006) performed a screen for a *ph1*-like phenotype in the population of EMS mutants. Yet they failed to find an individual showing the full *ph1b*-like phenotype. This indicates the *Ph1* phenotype might not be under the control of a single gene. The *Ph1* locus was further narrowed down to a 2.5-Mb region on the long arm of the 5B chromosome (Griffiths et al., 2006), which contains a duplicated segment from chromosome 3B composed of a cluster of *Cdk2*-like kinases and methyl-transferase genes (Griffiths et al., 2006; Al-Kaff et al., 2008; Martín et al., 2017). The Cdk-like kinases in the locus show close homology to the mammalian Cdk2, which is essential for homologous chromosome recognition and recombination (Ortega et al., 2003; Viera et al., 2009). Two groups of researchers disagree on which of the genes located in this particular region is the one responsible for promotion of homologous chiasmata. Bhullar et al. (2014) proposed *C-Ph1* (RAFTIN1-like protein containing BURP domain) to be a putative *Ph1* gene, but deletion lines for *C-Ph1* locus failed to produce the same phenotype as the *ph1b* mutant (Al-Kaff et al., 2008). Moreover, the rice homolog and wheat paralog of this gene were already shown to be specific to tapetal cells (Jeon et al., 1999; Wang et al., 2003). The other group proposed a different candidate, a paralog of *ZIP4*. The encoded protein affects the homologous cross-overs in *Arabidopsis* and rice, supporting the assumption that this gene could be responsible for the *Ph1* phenotype (Chelysheva et al., 2007; Shen et al., 2012; Rey et al., 2017). Both EMS and CRISPR mutations for this gene (named *TaZIP4-B2)* promoted homoeologous cross-overs in hybrids between wheat and *Ae. variabilis* (Rey et al., 2017; Rey et al., 2018). But these hybrids did not show the same extent of multivalent formation or an increase in univalents as typically observed in hybrids between the *ph1b* mutant and *Ae. variabilis*. Nevertheless, these results do suggest the *TaZIP4-B2* plays an important role in the control of homoeologous pairing in wheat (Rey et al., 2017; Rey et al., 2018; Naranjo, 2019). The putative additional effector in this region has yet to be identified.

Pairing Homoeologous 2 (Ph2)

Another gene, called *Ph2*, has a weaker effect (than *Ph1*) on homologous chromosome pairing in wheat. That gene was assigned to chromosome 3D by Mello-Sampayo (1968; 1971) who observed multivalent formation in metaphase I in the absence of chromosome 3D in pentaploid hybrids between *T. aestivum* and *T. durum*, as well as in hybrids between *T. aestivum* and *Aegilops*. Two *Ph2* mutants were since developed; the X-ray-induced mutant *ph2a* carrying a large deletion (Sears, 1982), and the chemically-induced (EMS) mutant *ph2b* (Wall et al., 1971). Using both mutants, the *Ph2* phenotype was studied and the locus narrowed down, using synteny with rice, to a terminal 80 Mb of the short arm of chromosome 3D (Sutton et al., 2003). More recently, however, Svačina et al. (2020) showed that this deletion in the *ph2a* mutant is actually larger than expected, comprising about 125 Mb terminal part of the short arm of chromosome 3D.

The *Ph2* gene operates in a different way than does *Ph1* (Benavente et al., 1998; Martinez et al., 2001a). Both Martinez et al., (2001a) and Sánchez-Morán et al. (2001) evaluated the effect of its mutations in hexaploid wheat, finding no visible

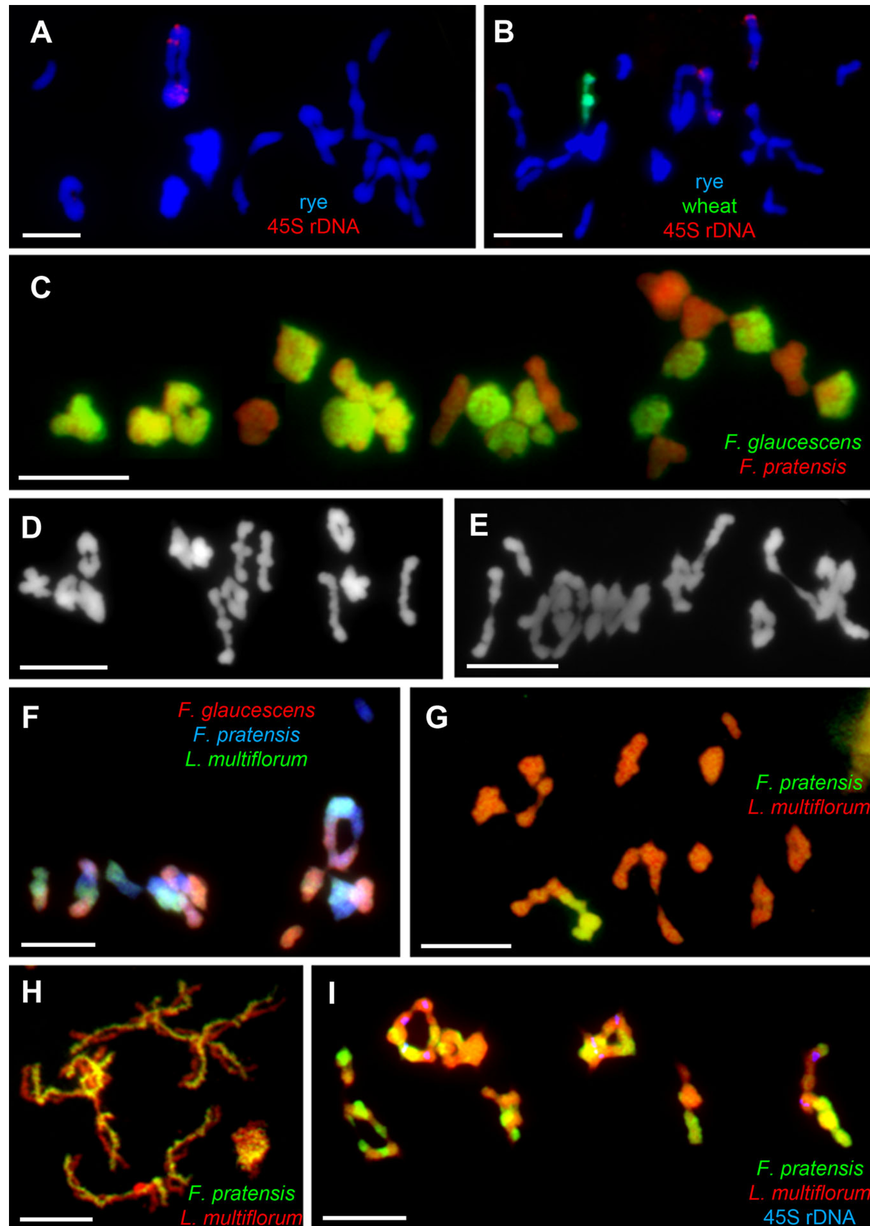

FIGURE 2 | Chromosome assocaitions in allo- and autopolyploids from the Poaceae family. Chromosome pairing in autotetraploid rye (2n = 4*x* = 28, RRRR) differs depending on the presence or absence of *Ph1* located on the introgressed 5BL chromosome arm of wheat. In **(A)**, trivalents and quadrivalents are commonly observed in the control line (2I+4II+2III+3IV), in **(B)**, multivalent chromosome formation is reduced in the line (6I+7II+2IV), where 5B and 5BL are introgressed. In both **(A, B)**, genomic DNA of *Triticum aestivum* was labeled with digoxigenin (green coloring), 45S rDNA was labeled with biotin (red), and genomic DNA of *Secale cereale* served as blocking DNA; all chromosomes counterstained with DAPI (blue). In **(C)**, the chromosome-pairing control system similar to that of *Ph1* found in allohexaploid *Festuca arundinacea* (2n = 6*x* = 42) hampers the associations of homeologous chromosomes and multivalent formation (21II). Genomic DNA of *F. glaucescens* was labeled with digoxigenin (green), while genomic DNA of *F. pratensis* was used as blocking DNA; all chromosomes were counterstained with DAPI (red pseudocolor). In **(D)**, the homoeolog suppressor was probably inherited from one of the progenitors, *F. glaucescens*, as this species also forms only bivalents during meiosis (14II). Conversely, in **(E)**, multivalent formation was detected in the autotetraploid form of the other progenitor, *F. pratensis* (2I+7II+3IV). The system is hemizygous-ineffective, thus allowing for promiscuous homeologous chromosome associations in tetraploid hybrids of *F. arundinacea* × *Lolium multiflorum*, where only one copy of the gene(s) is present **(F)**. Here, genomic DNA of *F. glaucescens* was labeled with biotin (red coloring) and that of *L. multiflorum* labeled with digoxigenin (green), while that of *F. pratensis* was used as blocking DNA; all chromosomes were counterstained with DAPI (blue). In **(G)**, homeologous chromosomes of *F. pratensis* and *L. multiflorum* pair freely in the substitution lines (1I+8II+1III+2IV) as well as in diploid *Festuca* × *Lolium* hybrids (7II), as seen in diplotene shown in **(H)**, due to the absence of any chromosome pairing system and the phylogenetic relationship of both genomes. Note many chiasmata between homeologous chromosomes. This results in frequent homeologous recombinations and massive chromosome rearrangements in successive generations **(I)**, as can be seen in the tetraploid *L. multiflorum* × *F. pratensis* cv. 'Sulino' (7IV). In panels **(G–I)**, genomic DNA of *F. pratensis* was labeled with digoxigenin (green coloring), while genomic DNA of *L. multiflorum* served as blocking DNA and all chromosomes were counterstained with DAPI (red pseudocolor).

influence upon homoeologous chiasmata when *Ph1* is present and *Ph2* absent, apart from a slight increase in univalent formations. Earlier, Sears (1977; 1982) had shown that in hybrids of wheat and closely related species, moderate frequency of homoeologous chiasmata happened in the absence of *Ph2* but in the presence of *Ph1*. In the case of wheat-rye hybrids lacking the *Ph2* locus, Prieto et al. (2005) also observed an intermediate number of homoeologous chiasmata; however, according to their GISH analysis, the chromosome associations only occur between wheat chromosomes, whereas wheat–rye associations were rare similarly to the wild-type hybrid. This contrasts with the *ph1b* mutant, for which some frequency of wheat–rye associations was detectable (refer to **Table 2**; Prieto et al., 2005). These findings suggest to us that *Ph2* plays a diminished functional role when homologous chromosomes are present (**Table 1**). Yet, in the absence of homologs, it may well suppress associations among homoeologous chromosomes. Furthermore, researchers discovered that *Ph2* has a different function to that of *Ph1* as it is not involved in recognition of homologous chromosomes but instead affects the progression of synapsis (Martinez et al., 2001a; Prieto et al., 2005). We should also not overlook possible cooperation between *Ph1* and *Ph2* in their modes of action, as suggested by the work of Boden et al. (2009).

The *ph2a* mutant has been exploited in trying to identify candidate genes underlying its phenotype. Many have been proposed, such as *TaMSH7*, the homolog of the *MSH6* DNA mismatch repair gene in yeast (Dong et al., 2002), in addition to the *WM5* (Thomas, 1997) and *WM1* gene family members (Ji and Langridge, 1994; Whitford, 2002). Sutton et al. (2003) used comparative genetics to further identify the putative genes involved in the *Ph2* phenotype; however, no clear candidate producing a mutant phenotype similar to the *ph2a* has been identified.

TABLE 2 | Number of chromosome-arm associations in metaphase I in haploid hybrids derived from the crossing of rye with euploid wheat (CS, 'Chinese Spring') and *ph1b* and *ph2b* mutants (Prieto et al., 2005).

Genotype Chromosome number	CS × rye 28	*ph2b* × rye 28	*ph1b* × rye 28
Wheat–wheat	0.48	1.68	7.14
Wheat–rye	0.08	0.08	0.59
Rye–rye	0.02	0.04	0.05
Total	0.58	1.80	7.78

Meiotic Behavior in Hybrids of *ph* Mutants and Wild-Type Wheat With Closely Related Species

The pairing of homoeologous chromosomes is mostly studied in haploids or interspecific hybrids, that is, in the absence of homologous chromosomes, the natural partners for pairing. The extent of chromosome associations during metaphase I of meiosis, in hybrids of wild-type hexaploid wheat or *ph2b* and *ph1b* mutants with various relatives, will differ based on the degree of homology between the genomes involved. The frequency of homoeologous chromosome chiasmata increases when there is a closer phylogenetic relationship of the parents. The fewest homoeologous associations were observed in the hybrids between hexaploid wheat and rye (**Table 3**; Naranjo et al., 1987; Naranjo et al., 1988). This can be explained by the fact that lineages towards wheat and rye split about 7 MYA while *Aegilops* diverged from wheat 2.5–5.0 MYA (Huang et al., 2002). Accordingly, the *Aegilops* chromosomes are more closely related to wheat chromosomes than those of rye. The highest frequency of homoeologous chromosome associations was observed in the hybrid of hexaploid wheat and *Ae. speltoides* (Maestra and Naranjo, 1998; **Table 3**); the latter is a species closely related to the donor of the B genome in wheat, and thus highly similar to one of the wheat genomes (Huang et al., 2002; Petersen et al., 2006). These observations suggest the *Ph* system's recognition of homologous chromosomes begins to fail with increasing homology between genomes in the hybrid, resulting in homoeologous chromosome chiasmata. Alternatively, there may exist genes that suppress or interfere with the *Ph* system in certain species used for hybridization with wheat (see below).

Homoeologous Chromosome Associations in the Presence of *Ph* Genes

Ph genes ensure that only homologous chromosome chiasmata occur in polyploid wheat during meiosis. However, the functioning of these genes can be suppressed in some hybrids, resulting in increased homoeologous chromosome associations; e.g., in hybrids of *T. aestivum* with *Ae. speltoides* or *Ae. mutica*

TABLE 3 | Associations of homoeologous chromosomes in metaphase I in various hybrids of wild-type wheat (WT) and *ph1b* and *ph2b* mutants with closely related plant species (Naranjo et al., 1987; Naranjo et al., 1988; Naranjo and Maestra, 1995; Maestra and Naranjo, 1997; Maestra and Naranjo, 1998).

Hybrid	Chromosome number	Univalents	Rod bivalents	Ring bivalents	Multivalents	Chiasmata percell
WT × rye	28	26.31	0.80	0.03	0.01	0.88
ph2b × rye	28	19.23	3.4	0.57	0.51	5.26
ph1b × rye	28	11.76	2.33	2.36	2.16	12.35
WT × *Ae. longissima*	28	24.55	1.59	0.06	0.05	1.81
ph2b × *Ae. longissima*	28	14.93	5.8	0.58	0.55	7.44
ph1b × *Ae. longissima*	28	3.48	4.4	2.99	2.86	18.28
WT × *Ae. sharonensis*	28	25.21	1.18	0.03	0.03	1.29
ph2b × *Ae. sharonensis*	28	10.16	5.58	1.42	1.13	11.17
ph1b × *Ae. sharonensis*	28	4.37	3.74	3.79	2.39	17.93
WT × *Ae. speltoides*	28	3.97	4.9	3.11	2.61	17.79
ph2b × *Ae. speltoides*	28	3.25	3.41	3.28	3.2	19.41
ph1b × *Ae. speltoides*	28	2.53	3.36	4.29	2.68	20.08

(Riley, 1960; Dover and Riley, 1972; Dvorak et al., 2006a). For the wheat × *Ae. speltoides* hybrid, Dvorak et al. (2006b) identified two suppressors on chromosomes 3S (*Su1-Ph1*) and 7S (*Su2-Ph1*) that affected homoeologous chromosome associations, varying from 7.0 to 16.4 chiasmata per cell. The *Su1-Ph1* was introgressed into both hexaploid and tetraploid wheat, opening new possibilities in inducing homoeologous chromosome recombinations for introgression into wheat (Li et al., 2017). This phenomenon can also be observed in lines where only a single chromosome was introgressed into the wheat background. In particular, the presence in wheat of chromosome 5U from *Ae. umbellulata* (Riley et al., 1973), or that of chromosome 5E from *Elytrigia elongata* (Dvorak, 1987), promotes homoeologous chromosome chiasmata with the formation of trivalents and bivalents in the haploids (ABD + 5U; ABD + 5E). This outcome suggests that introducing some alien chromosomes can suppress the functioning of *Ph* genes (Koo et al., 2017). Another case of homoeologous chromosome associations in the presence of *Ph* genes was reported on by Liu et al. (2011), who observed frequent recombination between $5M^g$ and 5D chromosomes in substitution lines containing $5M^g$ from *Ae. geniculata*. Later, Koo et al. (2017) used two different $5M^g$ chromosomes from different accessions in the wheat background and observed differential associations between $5M^g$ and 5D in both lines, for which chiasmata between $5M^g$ and 5D were detected in 6.7% and 21.7% of ensuing meiocytes. This might have been caused by the presence of genes located on the particular alien chromosome either actively promoting homoeologous chromosome chiasmata or repressing *Ph1*. Additionally, homoeologous associations probably occurred only between the $5M^g$ and 5D chromosome, as no multivalent was detected (Koo et al., 2017). In another example, homoeologous barley chromosomes fully associated in pairs in the presence of *Ph1* (Martín et al., 2017; Calderón et al., 2018). However, these homoeologous chromosomes did not cross-over, suggesting that *Ph1* does not prevent chromosome pairing between homoeologs, but supresses its recombination (Calderón et al., 2018).

In a natural population of the Chinese landrace of hexaploid wheat 'Kaixianluohanmai' (KL), another gene promoting homoeologous chiasmata in wheat–alien hybrids (presumably in presence of *Ph*) was posited (Luo et al., 1992). Meiosis is regular and normal in KL wheat by itself, as in other wheat landraces (Fan et al., 2019), but a moderate frequency of homoeologous chromosome associations occurs in hybrids of KL wheat with rye and *Aegilops variabilis* (similar as that between *ph1b* × rye and *ph2b* × rye hybrids) (**Table 4**; Luo et al., 1992; Liu et al., 1998; Liu et al., 2003; Xiang et al., 2005). In hybrids arising between KL wheat and *Psathyrostachys huashanica*, the frequency of homoeologous chromosome chiasmata even exceeded that of the *ph1b* × *P. huashanica* hybrid (Kang et al., 2008). This locus, named *phKL*, is most probably not allelic to either *Ph1* or *Ph2* (Liu et al., 2003; Hao et al., 2011). The analysis of monosomics did show that a locus on chromosome 6A in KL might be responsible for the *phKL* phenotype (Liu et al., 1997). However, using two mapping populations, Fan et al. (2019) recently identified a QTL locus possibly responsible for homoeologous associations on chromosome arm 3AL.

TABLE 4 | Chromosome associations in metaphase I in hybrids derived from crossings of rye with the wheat KL landrace, "Chinese Spring" (CS), and the Chinese Spring *ph1* (*CSph1b*) and *ph2* (*CSph2a*) mutants (Hao et al., 2011).

Genotype	Number of associations per cell			
	Rod	Ring	Multivalent	Chiasmata
KL × rye	4.73	0.20	0.11	5.40
CS*ph1b* × rye	4.85	1.87	0.47	9.53
CS*ph2a* × rye	1.74	0.00	0.02	1.78
CS × rye	0.54	0.00	0.00	0.54

Chromosome-Pairing Regulators in Other Poaceae Taxa

Bread wheat is undoubtedly the most studied and well-understood species concerning the mechanism of homologous chromosome recognition in the Poaceae family. Nonetheless, clues to the presence of similar machinery has been observed in other grass species, namely in *Avena* spp. (Ladizinsky, 1973), *Oryza* spp. (Cai et al., 2004), *Festuca* spp. (Jauhar, 1993), polyploid *Hordeum* spp. (Gupta and Fedak, 1985), or *Alopecurus* spp. (Murray et al., 1984). Several examples of chromosome associations in allo- and autopolyploids from the Poaceae family are shown in **Figure 2**.

The genus *Festuca* comprises over 500 species having a wide range of ploidy levels, from diploids to dodecaploids (Loureiro et al., 2007). Agriculturally most important are those species from the subgenus *Schedonorus* comprising broad-leaved fescues, the majority of which are polyploids, from tetraploids to decaploids (Kopecký et al., 2008b). Molecular and cytogenetic analyses have revealed that all these studied polyploid species arose from interspecific hybridization (Humphreys et al., 1995; Catalán and Olmstead, 2000; Hand et al., 2010; Ezquerro-López et al., 2017); hence, they are of allopolyploid origin. All these allopolyploid species—including the tetraploids *F. mairei*, *F. apennina*, and *F. glaucescens*, hexaploid *F. arundinacea*, and octoploids *F. arundinacea* subsp. *atlantigena* and decaploid *F. arundinacea* var. *letourneuxiana*—possess diploid-like pairing behavior during meiosis, with bivalent formation (reviewed in Jauhar, 1993). Jauhar (1975) had proposed the existence of a homoeologous-pairing suppressor in tall fescue (*F. arundinacea*, 2n = 6*x* = 42; FpFpFgFgFg'Fg') (**Figure 2C**). He found frequent multivalent formations in haploid plants of tall fescue (2n = 3*x* = 21) and speculated on the haplo-insufficiency or hemizygous-ineffectivity of the system: meaning that two copies of such gene(s) must be present for the induction of strict homologous pairing. This differentiates the fescues' system from *Ph1* of wheat and the regulator found in oats (Jauhar, 1993). Another difference is that *Ph1* can supress homeologous recombination and/or promote homologous ones, while the control system in tall fescue seems to be responsible for the formation of homologous bivalents. Colchicine-induced dodecaploid wheat was able to form quadrivalents composed of four homologous chromosomes, whereas only homologous

bivalents formed in the synthetically derived dodecaploid tall fescue plant (Jauhar, 1975).

Where the gene(s) underpinning diploid-like pairing system is located on one or more particular chromosomes or even subgenomes of tall fescue plants remains unknown. In tetraploid tall fescue (FpFpFgFg'), homoeologous chromosomes form chiasmata frequently; moreover, the frequent formation of quadrivalents was recorded in colchicine-induced autotetraploids of *F. pratensis* (**Figure 2E**; Kopecký et al., 2009). Thus, one of the subgenomes originating from *F. glaucescens* must harbor the responsible gene(s) (**Figure 2D**). In early work, Jauhar (1975) analyzed a set of monosomic lines of tall fescue and found one line with disrupted diploid-like behavior, probably due to an absence of the chromosome carrying the gene(s) for diploid-like pairing behavior. Unfortunately, this line was lost over time and so it cannot be further investigated. Later, Kleijer and Morel (1984) speculated that disruption of strictly homologous associations in a single plant is more likely to be only a consequence of normal variation among plants. The system may also interfere with other systems present in the genus, or in closely related genera. A high frequency of quadrivalents was observed in the tetraploid *Lolium multiflorum* × *F. arundinacea* hybrid (LmFpFgFg') (**Figure 2F**), which exceeded that of quadrivalents in tetraploid *F. arundinacea* (FpFpFgFg') (Kopecký et al., 2009).

The origin of the system in polyploid fescues is not known, but several scenarios are plausible. It could have developed in a currently unknown diploid species, which served as a progenitor of all recent polyploid species. Alternatively, such a system arose in an early-day polyploid (presumably an allotetraploid), since involved in the evolution of other allopolyploids. Support for both scenarios lies in the fact that the system in all species has the same (rare) attribute: haplo-insufficiency. The third possible scenario involves multiple origins of the system in different species during their evolutionary history. Or, the system is the outcome of two scenarios combined. It does seem that the systems found in various species are compatible in some hybrid combinations yet dysfunctional in others. Eizenga et al. (1990) found that multivalents were rare in the hybrids of tall fescue and giant fescue (*F. gigantea*). Similarly, hybrids of *F. mairei* × *F. glaucescens* show preferential formation of bivalents with a very low frequency of multivalents (nine quadrivalents and one trivalent among 200 PMCs [pollen mother cells]) (Malik and Thomas, 1967). By contrast, the hybrids of Continental and Mediterranean morphotypes of tall fescue all display high levels of multivalent formation (Kopecký et al., 2019), suggesting incompatibility of the two regulatory systems, or some epistatic effects. Therefore, we cannot unambiguously clarify if the system evolved once or twice (or even more times). However, if it did develop just once, the system diverged in different species during evolution to reach a level of incompatibility, as evinced from the analyses of interspecific hybrids.

The genus oat (*Avena* spp.) consists of diploid, tetraploid, and hexaploid species, including the important crop *A. sativa*. Polyploid oats include both auto- and allopolyploid forms, whose diploid-like behavior in meiosis is preserved despite partial homology between their genomes, suggesting the existence of a *Ph*-like system (Thomas, 1992). Oats comprise four cytologically distinct genomes (A, B, C, and D), however the genomes B and D occur only in polyploid taxa (Leggett and Thomas, 1995). Similar to wheat, the system found in tetraploid and hexaploid oats is hemizygous effective and haplo-sufficient, and susceptible to dosage effects and genetic repressibility. The locus that contains the gene(s) for meiotic regulation is likely localized to the A genome (Jauhar, 1977). Unfortunately, surpisingly little is known about the genes whose activity maintains homologous chromosome pairing in oats, apart from their existence being proven by increased associations among homoeologous chromosomes in some nulli-haploid *A. sativa* lines (Gauthier and McGuinnis, 1968).

POLYPLOIDY AND HOMOEOLOGOUS CHROMOSOME PAIRING IN PLANT BREEDING

Besides its key role in plant speciation, polyploidization and hybridization are popular tools in plant breeding. The most straightforward agronomical effect of polyploidy is an increased cell size, potentially resulting in larger organs, including fruits, roots, flowers, leaves, and seeds (Stebbins, 1950). Another frequent consequence of polyploidy is sterility, which generally has an agronomically negative effect; however, for seedless fruit production it can be a desirable trait, as in triploid seedless watermelon (Crow, 1994). The fixation of heterozygosity in allopolyploid species often leads to heterosis, resulting in higher vigor of the hybrids compared with their diploid progenitors, such as in hexaploid wheat *T. aestivum* (Sattler et al., 2016). Wide hybridization coupled to whole genome duplication is commonly used to merge beneficial inheritable traits from both parents, namely in the introgression of a chromosome segment carrying genes for a desirable trait from the wild relative to elite crop cultivars, or for simply widening the gene pool. One of the most promising artificially developed hybrids is Triticale, which originated from the crossing of wheat and rye with a subsequent chromosome doubling (Meister and Tjumjakoff, 1928).

One of the key components for the successful utilization of wide hybridization in plant breeding is the control of homoeologous chromosome associations. In countless studies, the *ph1b* mutant of wheat has been used to induce homoeologous chromosome recombinations between chromosomes of wheat and related species, for transferring desirable traits into the wheat genome (Marais et al., 2010; Niu et al., 2011; Ayala-Navarrete et al., 2013; Rey et al., 2015a; Rey et al., 2015b; Han et al., 2016; King et al., 2019). After the introgression of the chromosomal segment from a related species, it is necessary to immediately re-activate the *Ph1* gene to avoid risking the rapid elimination of the

segment. Nevertheless, some hybrids without meiotic regulation but with homoeologous chromosome pairing can be valuable also and remain relatively stable. Complementary attributes of ryegrasses (i.e., high yield and nutrition) and fescues (i.e., abiotic stress tolerance) can be combined in their hybrids called Festulolium. In last 50 years, many agriculturally successful cultivars have been released *via* several breeding programs (Ghesquière et al., 2010). To do this, the breeders often used tetraploid parents for the initial mating. Such F1 Festulolium hybrids are all allotetraploids and possess two sets of chromosomes from both parental species. One would presume that homologous chromosome associations would be the predominant mode of action due to variation in the DNA sequence. The repetitive elements from these two genera diverged sufficiently that it is now possible to distinguish chromosomes of *Festuca* from those of *Lolium* by genomic *in situ* hybridization (GISH) (Thomas et al., 1994). Yet, frequent formation of homoeologous chromosome chiasmata has been detected in F1 hybrids, as well as in monosomic and disomic substitution lines of *L. multiflorum* × *F. pratensis* (**Figures 2G, H**; Kopecký et al., 2008a). Such massive homoeologous associations and recombination leads to highly variable karyotypes differing from plant to plant (**Figure 2I**). An outcrossing mode of reproduction augments this variability within each population of hybrids over subsequent generations. Consequently, both high variability and heterosis ensue within the bred plant material. It is nevertheless possible to uniform the breeding material at a phenotypic level to the extent that it passes DUS tests for registration as a commercial cultivar. While the proportion of parental genomes was relatively stable in subsequent generations of three commercial hybrids (Kopecký et al., 2008a), substantial variability was found within populations of each generation of those cultivars.

Besides those amphiploid (or allotetraploid) cultivars, introgression breeding may also be used to develop Festulolium cultivars. Doing this involves at least one round of backcrossing of F1 hybrids with one of the parental species (usually *Lolium*), giving rise to plants similar to the parental species but with improved characteristics, such as frost tolerance or higher survivorship (reviewed in Kopecký et al., 2008b). Karyologically, these plants usually carry only one or few chromosome segments of *Festuca*. Such introgression lines are usually highly unstable and the introgressed segment(s) is/are often lost in subsequent generations (Kopecký et al., 2019). Accordingly, implementing any system capable of preventing associations of homoeologous chromosomes is arguably desirable to stabilize the genomic composition of hybrids. In amphiploids, immediate introgression of the system would be required to keep both parental subgenomes intact. To date, most cultivars have originated from the cross of *L. multiflorum* × *F. pratensis*, though none of the parents carry a homoeologous suppressor. Instead, tetraploid wild relatives, such as *F. glaucescens*, *F. apennina* and *F. mairei*, which possess a meiotic regulator hampering homoeologous pairing, should be considered for future crosses as they are known for their tolerance to biotic and abiotic stress, which might complement the high yield and nutrition traits of ryegrasses. In this respects, first attempts have been made and the cultivar of *L. multiflorum* × *F. glaucescens 'Lueur'* was registered in France (Ghesquière et al., 2010) and other similar cross combinations are used in breeding programs in both the UK and Czech Republic. Considering the haplo-insufficiency of the system found in polyploid fescues, evidently the F1 hybrids will possess some level of homoeologous associations. Still, it should be possible to select F2 plants that have two copies of the gene(s) of the system and then intercross them. Doing this should facilitate the stabilization of the hybrid genome in successive generations. For the corresponding introgression lines, the segment carrying the gene(s) of the system must be present among the introgressions. Thereafter, haploidization, followed by either spontaneous or induced chromosome doubling, should result in the establishment of plants having two copies of such gene(s) required for its/their functionality as the homoeologous pairing suppressor(s). Clearly, though, further investigation of chromosome behavior in fescues is necessary if we hope to foster genetically stable grass hybrids.

We envisage that with more knowledge of the mechanisms responsible for correct chromosome associations, the efficient employment of targeted interspecific hybridization techniques will become available in the near future. Perhaps the most challenging task is the developing and operating of an "OFF" and "ON" switch to control recombination of homoeologous chromosomes. It would be immensely helpful for breeders to switch "OFF" the system in wheat and other allopolyploids with an established and functional regulatory system for introgressing the specific segment from a wild relative. Once the segment is transferred, the switch to "ON" would then stabilize the segment and permit its proper transmission into successive generations. Similarly, introgression of the system into a hybrid (originally lacking the regulator) with desirable combinations of parental chromatin would assist in further stabilizing the hybrid genome composition. To conclude, additional research broadening our knowledge of the mechanisms governing meiotic chromosome behavior in allopolyploids is necessary to ensure further success in future breeding of grass plants.

AUTHOR CONTRIBUTIONS

RS, PS, DK, and JB wrote the manuscript.

FUNDING

This work was supported by the Czech Science Foundation (grant award 17-05341S) and the ERDF project "Plants as a tool for sustainable global development" (CZ.02.1.01/0.0/0.0/16_019/0000827).

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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APPENDIX IV

Development of deletion lines for physical mapping of *Ph2* gene in bread wheat

Svačina, R., Bartoš, J., Karafiátová, M., Sourdille, P., Endo, T.R., Doležel, J.

In: Proceedings of the "13th International Wheat Genetics Symposium". Tulln, Austria, 2017

Development of deletion lines for physical mapping of *Ph2* gene in bread wheat

Radim Svačina[1], Miroslava Karafiátová[1], Pierre Sourdille[2], Takashi R. Endo[3], Jaroslav Doležel[1], Jan Bartoš[1]

[1] Institute of Experimental Botany AS CR, Centre of Plant Structural and Functional Genomics – Šlechtitelů 31, Olomouc – Holice, 783 71, Czech Republic
[2] INRA UMR 1095 – Génétique, Diversité, Ecophysiologie des Céréales – 5 chemin de Beaulieu 63039, Clermont-Ferrand Cedex 2, France
[3] Faculty of Agriculture, Ryukoku University, 1-5 Yokotani, Seta Oe-cho, Otsu, Shiga 520-2194, Japan

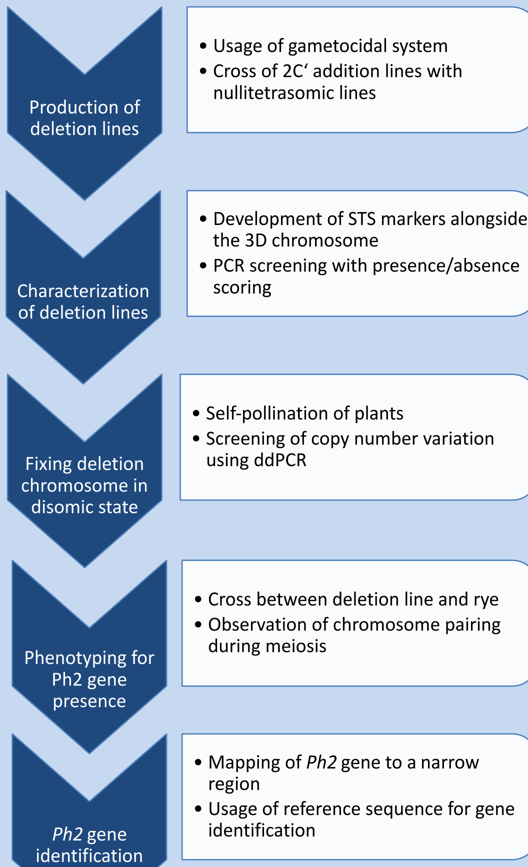
Introduction

Bread wheat (*Triticum aestivum* L.) is an allohexaploid species. Its genetic information consists of 3 subgenomes (A, B and D), formed by hybridization of three progenitors, therefore mechanisms of precise chromosome pairing had to be developed. Homologous pairing of chromosomes in wheat is primarily controlled genetically by *Ph* genes. *Ph2* is one of these genes and was located on a short arm of chromosome 3D. Removal of this gene caused pairing of wheat and alien chromosomes in hybrids with close-related species, while pairing between wheat chromosomes remained untouched. These findings suggest much potential of *Ph2* gene for introgression of alien genes into wheat genome, which could be used as a new breeding tool.

Some gametocidal chromosomes introduced into wheat are inherited preferably by causing sterility of gametes in which they are absent. The sterility is caused by the ability of the chromosomes to induce genomic rearrangements. In some cases, the changes are not lethal, providing an opportunity to transfer aberrant chromosomes into progeny. Gametocidal chromosome 2C from *Aegilops cylindrica* can be used to develop deletion lines after monosomic introduction into wheat cultivar 'Chinese Spring'.

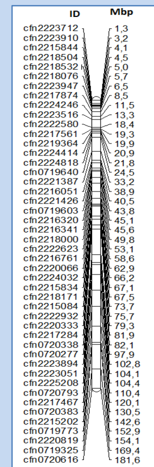
We have established a set of chromosome deletion lines for the short arm of chromosome 3D. The set is being continuously expanded and the newly obtained deletion lines are characterized by molecular markers. We focus preferentially on the distal 80 Mb region of the arm, where *Ph2* gene is believed to be located. The goal of the project is to narrow down the *Ph2* gene region to 5 Mb, so that more precise mapping using radiation deletion lines can be initiated.

Methods and material

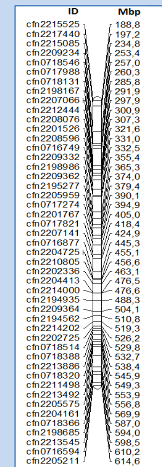


Results

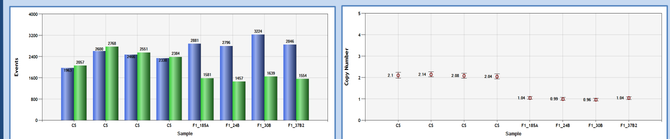
- Set of markers for a short arm of chromosome 3D



- Set of markers for a long arm of chromosome 3D



- Raw data output of ddPCR screening
- Calculated copy number variation after ddPCR screening



- Size of deletions on chromosome 3D in developed lines

Conclusion

- Up to date, we have developed 51 deletion lines, out of which 28 for a long arm and 23 for a short arm of chromosome 3D.
- These lines were characterized using 96 markers developed alongside the whole chromosome with special focus on distal 80 Mb region of a short arm.
- The deletion lines in the area of interest are being crossed with rye, so phenotyping of *Ph2* gene can be performed.
- After the area of Ph2 gene presence is narrowed down, radiation deletion lines will be used to get even more precise gene location.
- The reference sequence of chromosome 3D will be used to identify the candidate gene(s).

Acknowledgements

This work has been supported by the Czech Science Foundation (grant award 17-05341S) and the Ministry of Education, Youth and Sports of the Czech Republic (award LO1204 from the National Program of Sustainability I).

APPENDIX V

Development of chromosome deletion lines for *Ph2* gene mapping in bread wheat

Svačina, R., Karafiátová, M., Sourdille, P., Endo, T.R., Doležel, J., Bartoš, J.

In: Abstracts of the "Olomouc Biotech 2017. Plant Biotechnology: Green for Good IV". Olomouc, Czech Republic, 2017

Development of chromosome deletion lines for *Ph2* gene mapping in bread wheat

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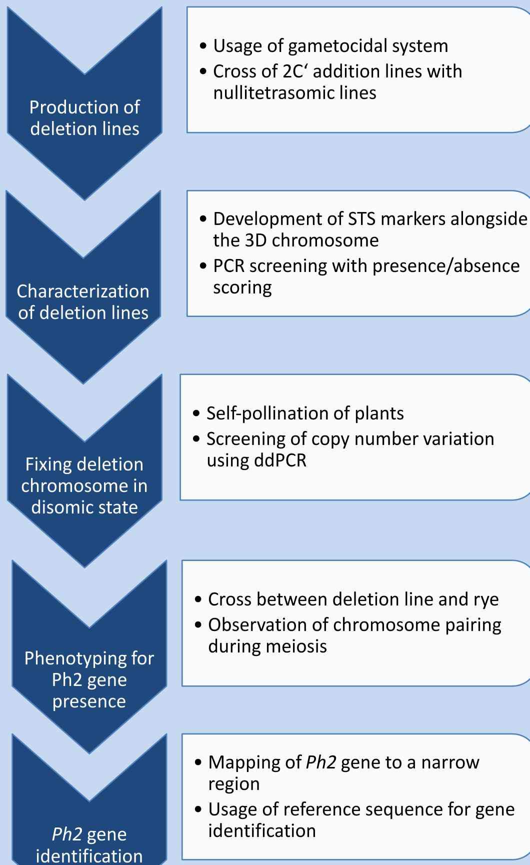
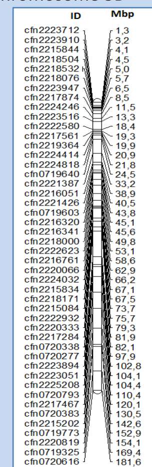
Methods and material

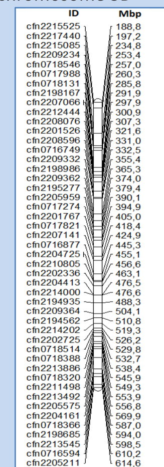


Results

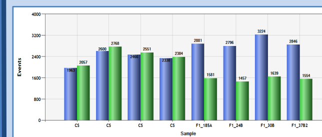
- Set of markers for a short arm of chromosome 3D



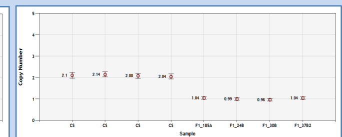
- Set of markers for a long arm of chromosome 3D



- Raw data output of ddPCR screening



- Calculated copy number variation after ddPCR screening



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Acknowledgements

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APPENDIX VI

Ph2 gene mapping through development and phenotyping of deletion lines in bread wheat

Svačina, R., Malurová, M., Karafiátová, M., Sourdille, P., Endo, T.R., Doležel, J., Bartoš, J.

In: Abstracts of the "EUCARPIA Breeding cereals for sustainable agriculture". Clermont-Ferrand, France, 2018

Ph2 gene mapping through development and phenotyping of deletion lines in bread wheat

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Methods and material

Production of deletion lines
- Usage of gametocidal system
- Cross of 2C' addition lines with nullitetrasomic lines

Characterization of deletion lines
- Development of STS markers alongside the 3D chromosome
- PCR screening with presence/absence scoring

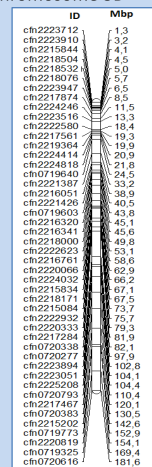
Fixing deletion chromosome in disomic state
- Self-pollination of plants
- Screening of copy number variation using ddPCR

Phenotyping for Ph2 gene presence
- Cross between deletion line and rye
- Observation of chromosome pairing during meiosis

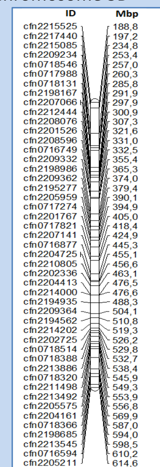
***Ph2* gene identification**
- Mapping of *Ph2* gene to a narrow region
- Usage of reference sequence for gene identification

Results

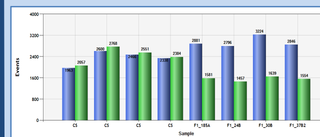
- Set of markers for a short arm of chromosome 3D



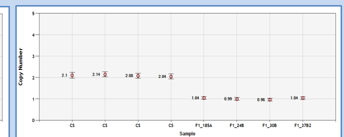
- Set of markers for a long arm of chromosome 3D



- Raw data output of ddPCR screening



- Calculated copy number variation after ddPCR screening



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APPENDIX VII

Towards identification of *Ph2*, a gene controllong homoeologous chromosome pairing in bread wheat

Serra, H., Svačina, R., Bartoš, J., Sourdille, P.

In: Abstracts of the "EMBO Workshop on Meiosis". La Rochelle, France, 2019

Towards identification of ***Ph2***, a gene controlling homoeologous pairing in bread wheat

Heïdi Serra[1], Radim Svačina[2], Jan Bartoš[2] & Pierre Sourdille[1]

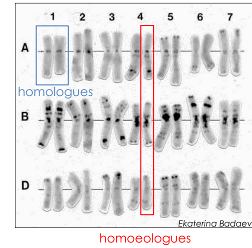
[1] Genetics, Diversity et Ecophysiology of Cereals, UMR 1095 INRA/UCA, 5 Chemin de Beaulieu, 63000 Clermont-Ferrand, France
[2] Institute of Experimental Botany, Centre of Plant Structural and Functional Genomics, Slechtitelu 31, Olomouc, Holice 78371, Czech Republic

heidi.serra@inra.fr

Introduction

Improvement of bread wheat varieties through **introgression** of original alleles derived from related species relies on meiotic recombination between homoeologous chromosomes. One of the two main genes controlling **homoeologous recombination** in this species is ***Ph2*** *(Pairing homoeologous 2)*. Inactivation of this gene results in increase frequency of chromosome pairing during meiosis of hybrids between wheat and close-related species. Although this locus has been described decades ago, the *Ph2* gene is still unidentified and only two mutants are available (*ph2a*, distal deletion of the short arm of the chromosome 3D (3DS) and *ph2b*, EMS mutant).

Characterizing *Ph2* is of main interest to contribute to the improvement of introgression efficiency of new alleles at loci bearing genes of agronomical interest.



Triticum aestivum L.
2n = 6x = 42

Results

❶ *Ph2* gene location on chromosome arm 3DS

✓ Characterization of the region deleted in the *ph2a* mutant using a set of 3D specific SNP markers:

121 Mb ⇔ 1577 genes

✓ Screening and characterization of wheat 3D deletion lines produced through gametocidal system and crossing with rye. Cytogenetic analysis of homoeologous pairing at metaphase I of wheat / rye haploid hybrids:

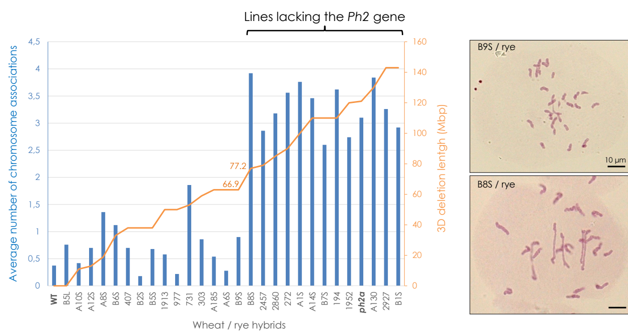


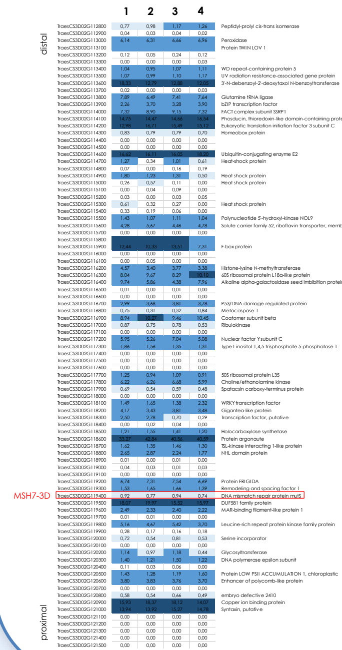
Figure 1. Homoeologous crossover frequency is increased in wheat / rye lines carrying a 3DS terminal deletion with a length > 77.2 Mb compared to wild-type (WT) hybrid control (> 50 meiocytes at metaphase I analysed per genotype).

✓ The *Ph2* gene is located within the 12,3 Mb genetic interval ranging from 66.9 to 77.2 Mb on chromosome arm 3DS. Identification of the genes present in this interval using the new anchored and annotated sequence of wheat genome:

12,3 Mb ⇔ 88 genes

❷ Meiotic expression and putative functions of the candidate genes

✓ Determination of candidate gene expression at early meiosis using our mRNA-Seq data for a sub-staged meiotic time series of whole-wheat anthers:



52 genes are expressed during meiotic prophase

MSH7-3D is the best candidate for *Ph2*

- Expressed during meiotic prophase in anthers

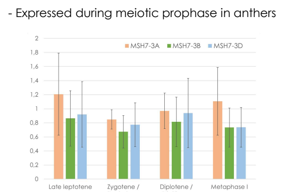


Figure 3. Meiotic expression of MSH7-3A, 3B & 3D in anthers (FPKM; error bar: 95% confidence interval)

- Forms heterodimers with MSH2, an anti-rec. protein in Arabidopsis (Culligan & Hays, 2002)

- Has a minor suppressor effect on homoeologous recombination in tomato (Tam et al, 2011)

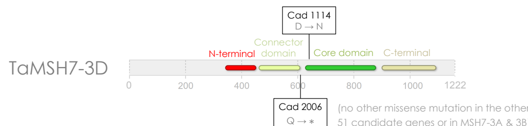
Repress recombination between divergent sequences?

Figure 2. Gene expression in wheat anthers at 4 meiotic stages
1. Late leptotene
2. Zygotene / Pachytene
3. Diplotene / Diakinese
4. Metaphase I

FPKM < 0,5
0,5 < FPKM < 1
1 < FPKM < 10
FPKM > 10

❸ Impact of *Tamsh7-3D* mutations on chromosome pairing

✓ Among the 7 *msh7-3D* mutants selected from the Cadenza TILLING population of the John Innes Centre (UK), 2 exhibit reduced homologous recombination at metaphase I (figure 4).



✓ Resequencing of the *ph2b* EMS mutant revealed an undescribed mutation at the splice junction exon5/intron likely leading to splice-site disruption and expression of a truncated MSH7-3D protein.

⇒ Three independent EMS mutants carrying missense or nonsense mutations within *MSH7-3D* exhibit similar phenotypes indicating that **MSH7-3D** is very likely responsible of the phenotype.

➢ Effect on homologous recombination

Line	n	Univalents	Rod bivalents	Ring bivalents	Chiasma frequency
Cad WT	50	0,28 ± 0,10 (0-2)	1 ± 0,14 (0-4)	19,86 ± 0,13 (17-21)	**40,72** ± 0,15 (38-42)
Cad 1114-3	55	1,38 ± 0,19 (0-8)	2,07 ± 0,17 (0-4)	18,24 ± 0,18 (15-21)	**38,55** ± 0,24 * (33-42)
Cad 2006-1	42	2,24 ± 0,29 (0-8)	3 ± 0,29 (0-9)	16,88 ± 0,30 (11-20)	**36,76** ± 0,38 * (31-41)
Cad 2006-2	26	1,38 ± 0,33 (0-6)	3,15 ± 0,24 (1-6)	17,15 ± 0,26 (15-20)	**37,46** ± 0,28 * (34-41)
CS WT	50	0,04 ± 0,04 (0-2)	0,98 ± 0,13 (0-4)	20,00 ± 0,13 (17-21)	**40,98** ± 0,14 (38-42)
CS B8S	52	1,73 ± 0,14 (0-4)	2,94 ± 0,15 (0-6)	17,19 ± 0,17 (14-21)	**37,33** ± 0,21 * (34-40)

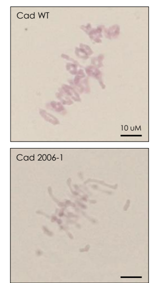


Figure 4. Homologous CO frequency at metaphase I is reduced in *Tamsh7-3D* mutant lines compared to wild-type (WT) control. Mean ± standard error of the mean. F-test, * p < 0,05

➢ Effect on homoeologous recombination (experiment ongoing)

Conclusion

Although the meiotic phenotype of the *msh7-3D* wheat / rye hybrids need to be confirmed, all these data strongly suggest that TaMSH7-3D is the *Ph2* gene and together with *Ph1*, play a major role in limiting pairing and recombination to homologous chromosomes in polyploid bread wheat. Many questions remain unanswered and especially, what is the precise mechanism by which MSH7-3D controls homologous and homoeologous recombination in polyploid bread wheat?

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APPENDIX VIII

***Ph2* gene phenotype scoring in wheat-rye hybrids with terminal deletions of 3D chromosome**

Svačina, R., Bartoš, J., Sourdille, P., Serra, H., Malurová, M., Karafiátová, M., Endo, T.R., Doležel, J.

In: Abstracts of the "International Conference on Polyploidy". Ghent, Belgium, 2019

Ph2 gene phenotype scoring in wheat-rye hybrids with terminal deletions of 3D chromosome

Radim Svačina[1], Jan Bartoš[1], Heïdi Serra[2], Pierre Sourdille[2], Miroslava Karafiátová[1], Magdaléna Malurová[1], Takashi R. Endo[3], Jaroslav Doležel[1]

[1] Institute of Experimental Botany AS CR, Centre of Plant Structural and Functional Genomics – Šlechtitelů 31, Olomouc – Holice, 783 71, Czech Republic
[2] INRA UMR 1095 – Génétique, Diversité, Ecophysiologie des Céréales – 5 chemin de Beaulieu 63039, Clermont-Ferrand Cedex 2, France
[3] Faculty of Agriculture, Ryukoku University, 1-5 Yokotani, Seta Oe-cho, Otsu, Shiga 520-2194, Japan

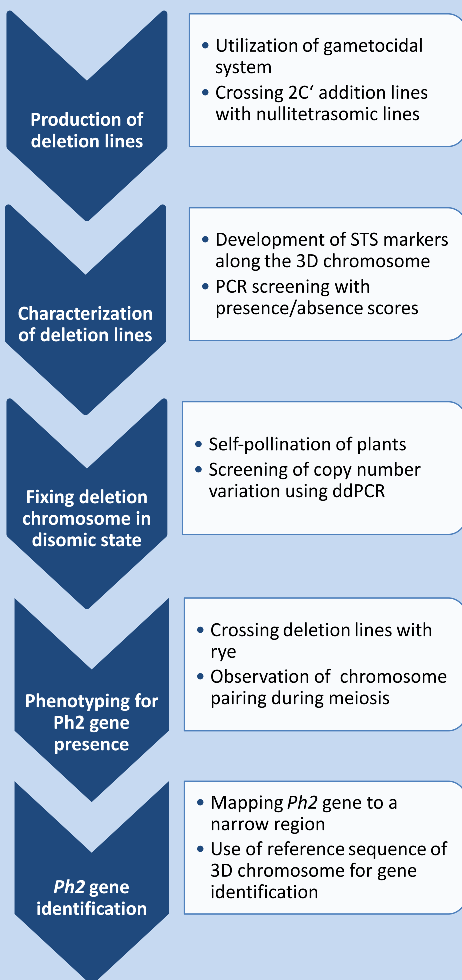
Introduction

Bread wheat (*Triticum aestivum* L.) is an allohexaploid species. Its genetic information consists of 3 closely-related subgenomes (A, B and D), formed by hybridization of three progenitors, therefore mechanisms of precise chromosome pairing had to be developed. Homologous pairing of chromosomes in wheat is primarily controlled genetically by *Ph* (pairing homologue) genes. *Ph2* is one of these genes and is located on a short arm of chromosome 3D. Removal of this gene results in intermediate pairing of wheat chromosomes in hybrids with closely-related species.

Some gametocidal chromosomes introduced into wheat are inherited preferably by promotion of genomic rearrangements in gametes in which they are absent, usually causing their sterility. In some cases, the changes are not lethal, providing an opportunity to transfer aberrant chromosomes into progeny. Gametocidal chromosome 2C from *Aegilops cylindrica* can be used to develop deletion lines after monosomic introduction into wheat cultivar 'Chinese Spring'.

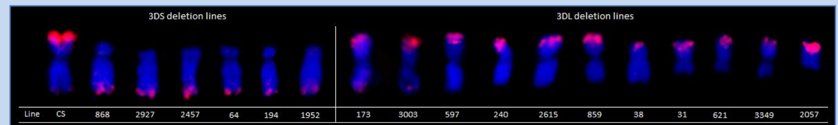
We have established a set of chromosome deletion lines for the chromosome 3D. The obtained deletion lines were characterized by molecular markers and crossed with rye to score the *Ph2* mutant phenotype. We focus preferentially on the distal 121 Mb region of the short arm, where *Ph2* gene is believed to be located. The goal of the project is to narrow down the *Ph2* gene region to a few megabases, so that candidate genes can be selected from reference genome sequence annotation and mutants can be utilised for their verification.

Material and methods

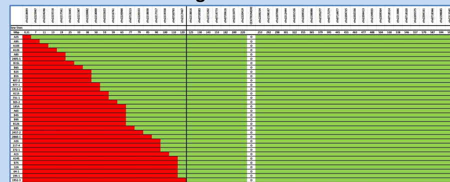


Results

FISH of selected 3D deletion lines



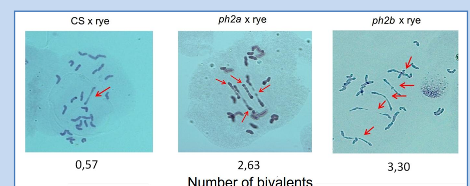
Deletion line sizes in region of interest



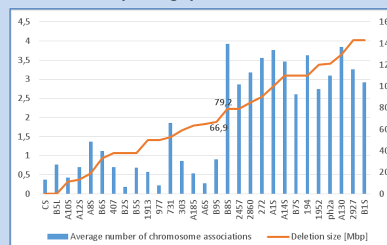
- 35 of lines carry deletion in region of interest (distal 121 Mb).
- These lines were crossed with rye for chromosome pairing behaviour observation.

Ph2 mutant phenotype

- Mutants of *Ph2* gene show different number of chromosome associations in meiosis.
- The mutant phenotype is being observed on hybrid haploid background.

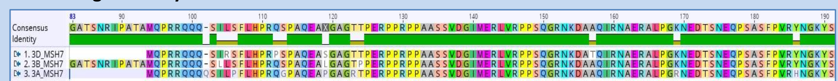


Chromosome pairing quantification



- Chromosome pairing in wheat-rye hybrids with various deletion lines was observed and quantified.
- The Ph2 gene presence was narrowed down to a region from 66,9 to 79,2 Mb of chromosome 3D.
- Selection of candidates from annotated genes and usage of mutants to observe the corresponding phenotype.
- MSH7 gene is a strongest candidate, EMS mutant analysis is in progress.

Candidate gene analysis



Conclusion

- To date, we have developed 122 deletion lines, out of which 73 affect the long arm, 47 the short arm and 2 both arms of chromosome 3D; 35 of these deletions are in the region of interest.
- The lines were characterized using 96 markers developed along the whole chromosome with a special focus on the distal 121 Mb region of the short arm.
- The deletion lines in the area of interest were crossed with rye and the phenotyping of *Ph2* gene has been performed.
- The *Ph2* gene presence was narrowed down to a region from 66,9 to 79,2 Mb of chromosome 3D.
- The annotated genes on chromosome 3D were used to verify the strongest candidate gene *MSH7*, EMS mutant analysis is in progress.

Acknowledgements
This work has been supported by the Czech Science Foundation (grant award 17-05341S) and the Ministry of Education, Youth and Sports of the Czech Republic (award LO1204 from the National Program of Sustainability I).

APPENDIX IX

Wheat-rye hybrids with chromosome deletions analysed for *Ph2* gene phenotype

Svačina, R., Bartoš, J., Sourdille, P., Serra, H., Malurová, M., Karafiátová, M.,
Endo, T.R., Doležel, J.

In: Abstracts of the "Olomouc Biotech 2019. Plant Biotechnology: Green for Good V". Olomouc, Czech Republic, 2019

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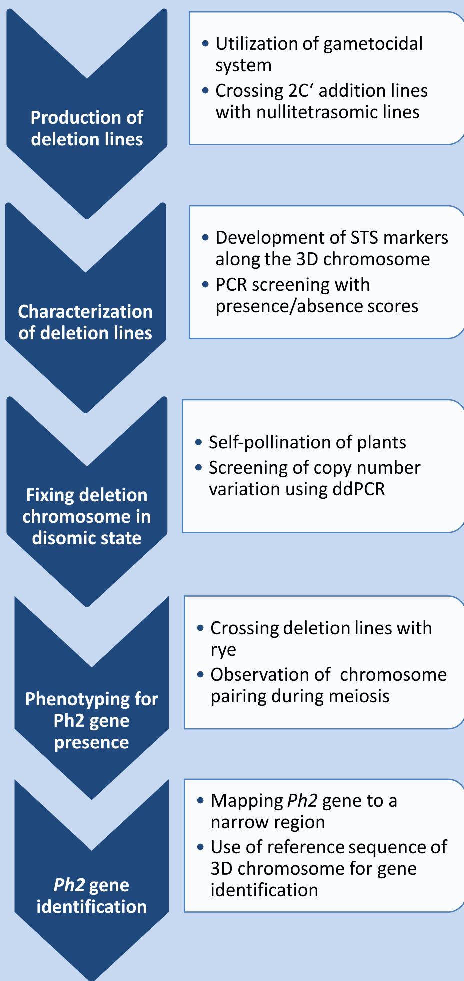
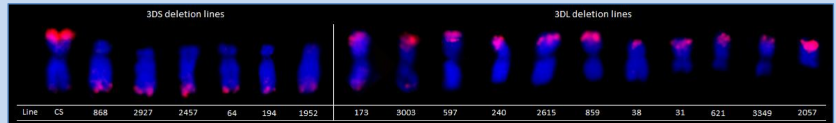
We have established a set of chromosome deletion lines for the chromosome 3D. The obtained deletion lines were characterized by molecular markers and crossed with rye to score the *Ph2* mutant phenotype. We focus preferentially on the distal 121 Mb region of the short arm, where *Ph2* gene is believed to be located. The goal of the project is to narrow down the *Ph2* gene region to a few megabases, so that candidate genes can be selected from reference genome sequence annotation and mutants can be utilised for their verification.

Material and methods



Results

FISH of selected 3D deletion lines



Deletion line sizes in region of interest

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- These lines were crossed with rye for chromosome pairing behaviour observation.

Ph2 mutant phenotype

- Mutants of *Ph2* gene show different number of chromosome associations in meiosis.
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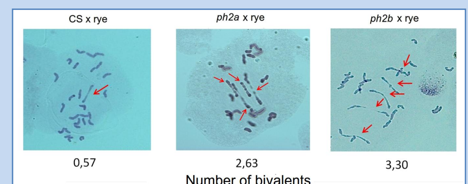
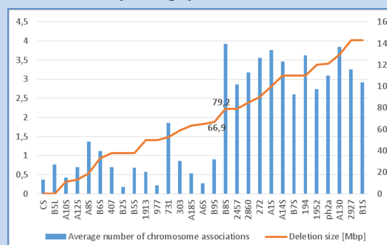


Chromosome pairing quantification



- Chromosome pairing in wheat-rye hybrids with various deletion lines was observed and quantified.
- The Ph2 gene presence was narrowed down to a region from 66,9 to 79,2 Mb of chromosome 3D.
- Selection of candidates from annotated genes and usage of mutants to observe the corresponding phenotype.
- MSH7 gene is a strongest candidate, EMS mutant analysis is in progress.

Candidate gene analysis

Conclusion

- To date, we have developed 122 deletion lines, out of which 73 affect the long arm, 47 the short arm and 2 both arms of chromosome 3D; 35 of these deletions are in the region of interest.
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APPENDIX X

_Ph2_ gene mapping through phenotyping of wheat-rye hybrid deletion lines

Svačina, R., Bartoš, J., Sourdille, P., Serra, H., Malurová, M., Karafiátová, M., Endo, T.R., Doležel, J.

In: Abstracts of the "22nd International Chromosome Conference". Prague, Czech Republic, 2019

Ph2 gene mapping by phenotyping wheat-rye hybrid chromosome deletion lines

Radim Svačina[1], Heïdi Serra[2], Pierre Sourdille[2], Miroslava Karafiátová[1], Magdaléna Malurová[1], Takashi R. Endo[3], Jaroslav Doležel[1], Jan Bartoš[1]

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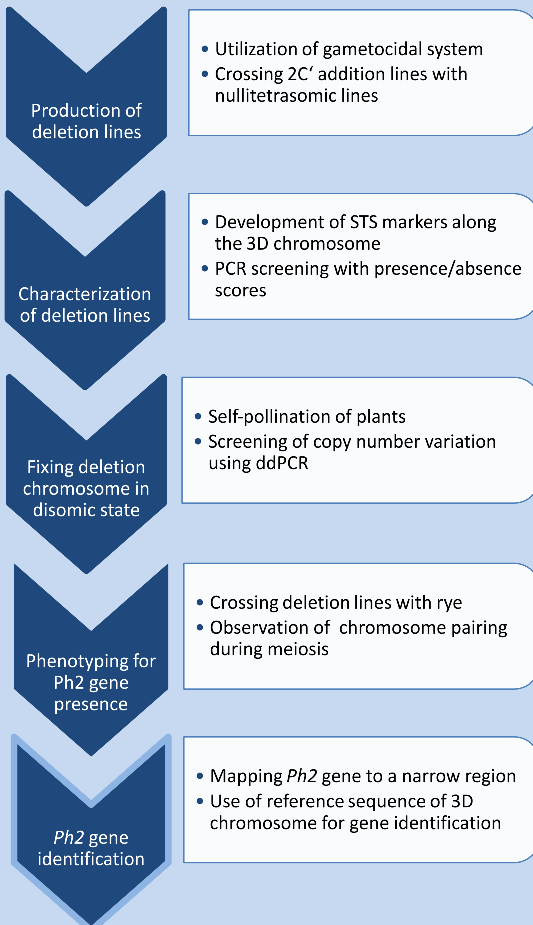
Some gametocidal chromosomes introduced into wheat are inherited preferably by promotion of genomic rearrangements in gametes in which they are absent, usually causing their sterility. In some cases, the changes are not lethal, providing an opportunity to transfer aberrant chromosomes into progeny. Gametocidal chromosome 2C from *Aegilops cylindrica* can be used to develop deletion lines after monosomic introduction into wheat cultivar 'Chinese Spring'.

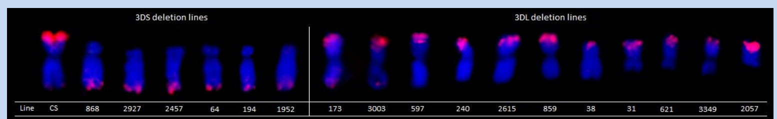
We have established a set of chromosome deletion lines for the chromosome 3D that is being used for phenotyping of the *Ph2* gene. The obtained deletion lines were characterized by molecular markers and crossed with rye to score the *Ph2* mutant phenotype. We focus preferentially on the distal 121 Mb region of the short arm, where *Ph2* gene is believed to be located. The goal of the project is to narrow down the *Ph2* gene region to a few megabases, so that candidate genes can be selected from reference genome sequence annotation and mutants can be utilised for their verification.

Methods and material



Results

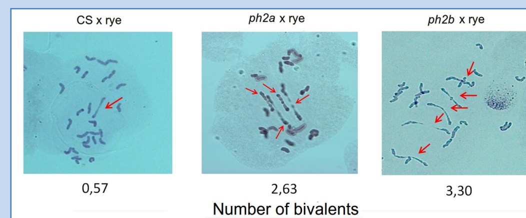
Size of deletions on chromosome 3D in selected lines



Deletion lines in region of interest

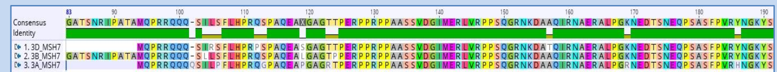
- 35 of lines carry deletion in region of interest (distal 121 Mb).
- These lines were crossed with rye.
- The chromosome pairing behaviour will be observed shortly.

Ph2 mutant phenotype



- Mutants of *Ph2* gene show different number of chromosome associations in meiosis.
- The mutant phenotype is being observed on hybrid haploid background.

Candidate gene analysis



- Phenotyping of wheat-rye hybrid chromosome deletion lines will narrow down *Ph2* gene position to a few megabases.
- Selection of candidates from annotated genes and use of mutants to observe the corresponding phenotype.

Conclusion

- To date, we have developed 122 deletion lines, out of which 73 affect the long arm, 47 the short arm and 2 both arms of chromosome 3D; 35 of these deletions are in the region of interest.
- The lines were characterized using 96 markers developed along the whole chromosome with a special focus on the distal 121 Mb region of the short arm.
- The deletion lines in the area of interest were crossed with rye, so that phenotyping of the *Ph2* gene can be performed.
- The annotated reference sequence of chromosome 3D will be used to identify the candidate gene(s).
- Mutants for the candidates will be used to check the phenotype and validate the gene.

Acknowledgements

This work has been supported by the Czech Science Foundation (grant award 17-05341S) and the Ministry of Education, Youth and Sports of the Czech Republic (award LO1204 from the National Program of Sustainability I).

Palacký University Olomouc

Faculty of Science

Department of Cell Biology and Genetics

and

Institute of Experimental Botany of the Czech Academy of Sciences

Centre of Plant Structural and Functional Genomics

Centre of Region Haná for Biotechnological and Agricultural Research

Radim Svačina

Physical mapping of *Ph2* region in hexaploid wheat

P1527 – Molecular and Cellular Biology

Summary of Ph.D. Thesis

Olomouc 2020

Ph.D. thesis was carried out at the Department of Cell Biology and Genetics, Faculty of Science, Palacký University Olomouc, in years 2016–2020.

Candidate: **Mgr. Radim Svačina**

Supervisor: **Mgr. Jan Bartoš, Ph.D.**

Reviewers: ....

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The evaluation of the Ph.D. thesis was written by ....
....

The summary of the Ph.D. thesis was sent for distribution on ....

The oral defence wil take place on .... in front of the commission for the Ph.D. study of the program Molecular and Cell Biology in ....

The Ph.D. thesis is available in the Library of the biological departments of the Faculty of Science of Palacký University Olomouc, Šlechtitelů 11, Olomouc-Holice.

Prof. RNDr. Zdeněk Dvořák, DrDC. *et* Ph.D.
Chairman of the Commission for the Ph.D.
thesis of the study program Molecular and Cell Biology
Department of Cell Biology and Genetics, Faculty of Science
Palacký University Olomouc

CONTENTS

1 Introduction 4
2 Aims of the thesis 6
3 Material and methods 7
4 Summary of results 9
5 Summary 11
6 References 12
7 List of author's publications 16
7.1 Original publications 16
7.2 Published abstracts of posters 17
8 Souhrn 18

1 Introduction

Bread wheat (*Triticum aestivum* L.) is among the most important crop plants, as it represents a main source of food intake for a large portion of population. Taxonomically, it is a monocotyledonous species, which belongs to a family *Poaceae*, subfamily *Pooideae* and the tribe Triticeae. It emerged from two distinct hybridization events between three progenitors, resulting in its allohexaploid nature, consisting out of three closely-related subgenomes A, B and D (2n = 6x = 42; AABBDD). Its genome has therefore considerable size ~16 Gb (Doležel *et al.*, 2018), containing high amount of repetitive DNA sequences, which is about 85 % (IWGSC, 2018).

High homology between the homoeologous chromosomes hampers the correct chromosome pairing through risk of creation of multivalents during the first meiotic division. In general however, the meiosis of wheat is fully diploid-like, with formation of 21 bivalents in methaphase I (Martínez *et al.*, 2001a; Martínez *et al.*, 2001b). To ensure the correct chromosome recombination and consistency of meiotic division, wheat developed a genetic control mechanism called *Pairing homoeologues (Ph)* ensuring a higher stringency of chromosome recognition to allow a proper development of gametes (Sears and Okamoto, 1958; Riley and Chapman, 1958). The first gene of this control mechanism is called *Ph1*, which was localized to a long arm of chromosome 5B by Sears and Okamoto (1958) and Riley and Chapman (1958). The study of its deleterious phenotype in wheat and its hybrids concluded that this gene has the highest effect on chromosome pairing (Martínez *et al.*, 2001a; Martínez *et al.*, 2001b; Naranjo *et al.*, 1987, 1988; Naranjo and Maestra, 1995; Maestra and Naranjo, 1997; Maestra and Naranjo, 1998). Another gene of this control mechanism is located on a short arm of chromosome 3D, called *Ph2*, having a less distinctive mutant phenotype than *Ph1* (Mello-Sampayo, 1971). The attempts to identify the gene responsible for *Ph2* phenotype resulted in number of candidates a *TaMSH7-3D*, a homologue of DNA mismatch repair gene in yeast (Dong *et al.*, 2002), *WM5* (Thomas, 1997) and *WM1* genes (Ji and Langridge, 1994; Whitford, 2002), however *Ph2* gene was not identified up to this day.

TaMSH7-3D is one of the candidate genes potentially responsible for *Ph2* phenotype (Dong *et al.*, 2002). The *MSH7* gene is probably derived through replication and

divergence of *MSH6*-like gene in early plant evolution (Culligan, 2000; Culligan and Hays, 2000). It is a plant specific homologue of *MutS 7* gene in yeast and belongs to the DNA mismatch repair family (MMR) (Dong *et al*., 2002). The MSH proteins are highly conserved and play a crucial role in initial steps of MMR pathway, by recognizing the base-base mismatches and insertion/deletion mutations that were created during DNA replication (Reyes *et al.*, 2016). MSH7 acts in a heterocomplex with MSH2, while in *Arabidopsis*, studies showed that it recognizes base mismatches A/A, C/A, G/A, G/G and partially G/T (Culligan and Hays, 2000; Wu *et al.*, 2003; Gómez and Spampinato, 2013).

2 Aims of the thesis

I Development and characterization of deletion lines for chromosome 3D of bread wheat

The first aim of the thesis was the development of terminal deletion line stock for the chromosome 3D of bread wheat using system involving gametocidal chromosome 2C of *Ae. cylindrica*. This procedure constisted of crosses between wheat and its addition lines carrying 2C chromosome of *Ae. cylindrica* in monosomic constitution. The created deletion lines were scored with molecular markers to determine deletion sizes with subsequent FISH analysis as a control.

II Deletion mapping of *Ph2* gene located on the short arm of chromosome 3D

The second aim of this work was delimitation of *Ph2* locus area. The phenotype of this gene is more visible in haploid hybrids between wheat and closely-related species, such as rye. The deletion lines were crossed with rye, with subsequent scoring of number of homoeologous chromosome associations in flowering stage of this hybrid, more precisely in metaphase I. The locus of *Ph2* gene was mapped to an area between closest deletions with contrasting phenotypes.

III Candidate gene(s) selection and validation using TILLING population of wheat

The candidate gene(s) were derived by combining the positional information from deletion mapping and exome sequencing of *ph2b* EMS mutant. The available TILLING population of wheat was exploited for validation of gene(s) in the deletion area that carry a mutation in *ph2b* mutant. The TILLING lines with point mutations in candidate gene(s) were crossed with *Ae. variabilis* to score number of homoeologous chromosome associations.

3 Material and methods

The presented work is focused on physical mapping and identification of gene responsible for *Ph2* phenotype in wheat. This gene was previously mapped using *ph2a* mutant (Sutton *et al.*, 2003). However, the mapped area is too large to effectively identify a gene responsible for *Ph2* phenotype. To delimit the location of this gene, development of novel deletion lines was necessary. Deletion lines were produced using gametocidal system developed by Endo and Gill (1996), modified for creation of single-chromosome deletion lines (Svačina *et al*., 2020). The modified gametocidal system consists of crossing between 2C chromosome monosomic addition line in bread wheat (male; 6x = 2n = 43; AABBDD + 2C′) and wheat nulli-tetrasomic lines lacking chromosome 3D with tetrasomic constitution either for chromosome 3A or 3B (female; 6x = 2n = 42; AABBDD − 3D″ + 3A″/3B″). The resulting plants lacking 2C gametocidal chromosome from addition line carry terminal deletions on various number of chromosomes, while lacking healthy chromosome 3D. The lines carrying deletion on chromosome 3D were identified and characterised using PCR with set of markers alongside the entire chromosome.

Lines carrying a deletion in region containing *Ph2* gene were selected and crossed with rye, since the *ph2* mutant phenotype is easily distinguishable in wheat-rye haploid (ABDR) hybrids (Mello-Sampayo and Canas, 1973). Cytogenetic analyses were performed in haploid hybrids to score a number of chiasmata in metaphase I in anthers of each line, calculated from the number of univalents, bivalents (rod and ring) and multivalents (Serra *et al*., 2020). Using this approach, the location of *Ph2* gene was further delimited.

The mutant *ph2b* carries point mutations alongside its whole genome, while one or more of those mutations disrupted the effect of *Ph2* gene (Wall *et al*., 1971). Hence exome capture of this mutant was performed and combined with data from deletion mapping (Serra *et al*., 2020) identifying the candidate gene.

The validation of candidate gene was performed through TILLING population of wheat EMS mutants of 'Cadenza' cultivar (King *et al*., 2015; Krasileva *et al*., 2017). Several possible mutants for *TaMSH7-3D* were selected to analyse whether the phenotype will correspond to *ph2a/b* mutants. These EMS mutants and WT were crossed with *Ae.*

variabilis, since ‘Cadenza’ cultivar is not cross-compatible with rye. In the haploid hybrid progeny, the frequency of chiasmata was cytogenetically scored, based on numbers of univalents, ring and rod bivalents and multivalents in metaphase I of anthers.

4 Summary of results

The *Ph2* gene was originally mapped to a distal 80 Mb of a short arm of chromosome 3D (Sutton *et al*., 2003). STS markers however showed that the deletion size is larger than previously believed, encompassing about 125 Mb of the terminal part of short arm of chromosome 3D (Svačina *et al*., 2020). Subsequent analysis using a high-density SNP genotyping array (35K SNP Affymetrix Axiom®) of *ph2a* mutant showed that the deletion breakpoint is at 121 Mb, containing about 1577 annotated genes (IWGSC, 2018; Serra *et al*., 2020).

To delimit the area of *Ph2* gene locus, 113 novel deletion lines for chromosome 3D of wheat were produced by gametocidal system (Svačina *et al*., 2020). The whole set of deletion lines was characterized using 84 STS molecular distributed alongside the entire chromosome. The size of terminal deletions ranged from 6.5 to 357 Mb, while the size of deletion bins varied between 0.15 and 50 Mb (Svačina *et al*., 2020).

A subset of 32 deletion lines that carried a deletion in area of 121 Mb of terminal part of short arm of chromosome 3D was selected and crossed with rye (Serra *et al*., 2020). Cytogenetic analyses were performed in 21 haploid hybrids to score a number of chiasmata in metaphase I in anthers of each line, calculated from the number of univalents, bivalents (rod and ring) and multivalents. The average number of chiasmata in wheat-rye hybrids indicate that in non-mutant lines, chromosomes rarely associate (0.38 ± 0.10 chiasma/meiocyte), however the number of chiasmata was increased in *ph2a* hybrid (3.10 ± 0.13 chiasmata/meiocyte) (Serra *et al*., 2020). The analyses of homoeologous chromosomal associations revealed that in terminal deletion line hybrids with deletion sizes higher than 79.2 Mb, the chiasmata frequency is increased (ranging from 2.6 to 3.92 chiasmata/meiocyte). Conversely, in individuals with deletion shorter than 64.9 Mb, frequency is lower than 2 chiasmata per meiocyte. These differences in chiasmata frequency indicate that *Ph2* gene is located in 14.3 Mb area ranging between deletion lines contrasting in phenotype with deletion sizes 64.9 and 79.2 Mb on the short arm of chromosome 3D (Serra *et al*., 2020).

The mutant *ph2b* carries point mutations alongside its whole genome, while one of those mutations disrupted the effect of *Ph2* gene (Wall *et al*., 1971), hence exome capture of this mutant was performed. The comparison of genic areas between *ph2b* and WT 'Chinese Spring' wheat showed 59 SNPs within the 121 Mb deletion of *ph2a* mutant. However, only one candidate (*TraesCS3D02G119400*) was present in 14.3 Mb area deduced from deletion mapping. This gene encodes a DNA mismatch repair protein TaMSH7-3D. In *ph2b* mutant, RNA seq was used to verify its function being disrupted by a SNP compromising correct splicing, leading to a premature STOP codon (Serra *et al*., 2020).

To validate the *TaMSH7-3D* gene to be responsible for *Ph2* phenotype, TILING population of wheat EMS mutants of 'Cadenza' cultivar was exploited (www.wheat-tilling.com) (King *et al*., 2015; Krasileva *et al*., 2017). Seven possible mutants for *TaMSH7-3D* were selected to analyse whether the phenotype will correspond to *ph2a/b* mutants. These EMS mutants and WT were crossed with *Ae. variabilis*, and their progeny the frequency of chiasmata was scored, based on numbers of univalents, ring and rod bivalents and multivalents in metaphase I of anthers. The hybrid of WT 'Cadenza' and *Ae. variabilis* showed on average 1.10 (± 0.09) per meiocyte at metaphase I (Serra *et al*., 2020). Out the seven mutant hybrids, four showed increased frequency of chiasmata in metaphase I, Cadenza2006 x *Ae. variabilis* had the strongest mutant phenotype of 6.07 ± 0.17 chiasmata on average, which is a 5.52-fold increase (Serra *et al*., 2020). The hybrids of Cadenza0638, Cadenza1178 and Cadenza1114 and *Ae. variabilis* showed 2.21, 1.90 and 2.37-fold increase in chiasmata frequency respectively. This difference can be attributed to the type of mutation in these hybrids, Cadenza2006 having a premature STOP codon, while others having amino acid substitution (Serra *et al*., 2020). This analysis functionally validates the *TaMSH7-3D* as a gene responsible for *Ph2* phenotype.

5 Summary

The goal of this work was the physical mapping and identification of the *Ph2* gene in bread wheat. It affects homoeologous chromosome associations in meiosis and is located on the short arm of chromosome 3D. The physical mapping of *Ph2* gene was performed through deletion lines. We developed 113 deletion lines for chromosome 3D of bread wheat. Lines carrying a deletion in terminal 121 Mb of short arm of chromosome 3D were crossed with rye. Through scoring chiasmata in metaphase I in its progeny, we delimited the area of *Ph2* locus to 14.3 Mb region on the short arm of chromosome 3D.

EMS-induced mutant *ph2b* carries point mutations throughout its whole genome, one or more of these mutations responsible for *Ph2* gene malfunction. The information provided by exome capture of this mutant and deletion mapping revealed only one candidate (*TraesCS3D02G119400*) a DNA mismatch repair protein TaMSH7-3D.

TILLING population of 'Cadenza' cultivar carrying EMS-induced point mutations was exploited for functional validation of *TaMSH7-3D* gene. Hybrids of its mutants were utilised for scoring of number of chiasmata in metaphase I in anthers. Cadenza2006 x *Ae. variabilis* had the strongest mutant phenotype of 6.07 ± 0.17 chiasmata per meiocyte on average, which is a 5.52-fold increase compared to WT. Through this analysis, we functionally validated the *TaMSH7-3D* as a gene responsible for *Ph2* phenotype.

Cloned *Ph2* gene could provide a valuable tool to increase wheat genetic pool, opening new possibilities for enrichment of wheat diversity through alien introgression in breeding programmes. With knowledge of *TaMSH7-3D* being responsible for *Ph2* phenotype, a precise mutant can be created to be exploited without any background genomic damage in various elite cultivars used for breeding.

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7.2 Published abstracts of posters

Svačina, R., Bartoš, J., Karafiátová, M., Sourdille, P., Endo, T.R., Doležel, J.: Development of deletion lines for physical mapping of *Ph2* gene in bread wheat. In: Proceedings of the "13th International Wheat Genetics Symposium". Tulln, Austria, 2017.

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8 Souhrn

Název práce: Fyzické mapování *Ph2* regionu u pšenice seté

Cílem této práce bylo fyzické mapování a identifikace *Ph2* genu pšenice seté. Tento gen ovlivňuje asociaci chromozomů v meioze a nachází se na krátkém rameni chromozomu 3D. Fyzické mapování *Ph2* genu bylo provedeno využitím delečních linií. Celkově bylo vyvinuto 113 delečních linií pšenice seté. Linie nesoucí deleci v distálních 121 Mb krátkého ramene chromozomu 3D byly kříženy s žitem. V jejich potomstvu byl analyzován počet chiasmat v metafázi I, čímž jsme upřesnili region lokusu *Ph2* genu na 14,3 Mb krátkého ramene chromozomu 3D.

Mutant *ph2b*, vytvořený pomocí EMS, nese bodové mutace v jeho celém genomu, přičemž jedna nebo více těchto mutací jsou zodpovědné za selhání funkce genu *Ph2*. Informace získané sekvenováním exonů tohoto mutanta a delečním mapováním odhalilo pouze jediného kandidáta (*TraesCS3D02G119400*), "DNA mismatch repair" protein TaMSH7-3D.

TILLING populace 'Cadenza' kultivaru, nesoucí bodové mutace způsobené EMS, byla využita pro funkční validaci *TaMSH7-3D* genu. Hybridi nesoucí mutaci v tomto genu byli využiti pro analýzu počtu chiasmat v metafázi I prašníků. Hybrid Cadenza2006 x *Ae. variabilis* měl nejsilnější mutantní fenotyp (průměrně 6,07 ± 0,17 chiasmat na meiocyt), což je 5,52 násobný počet v porovnání s nemutantní formou. Touto analýzou jsme funkčně potvrdili *TaMSH7-3D* jako gen zodpovědný za *Ph2* fenotyp.

Klonovaný gen *Ph2* může poskytnout cenný nástroj k rozšíření genetických zdrojů pšenice, čímž může dojít k obohacení její variability pomocí introgrese z příbuzných druhů ve šlechtitelských programech. Poznatek, že *TaMSH7-3D* je zodpovědný za *Ph2* fenotyp, může být použit k vytvoření precizního mutanta bez dalších genomických aberací. Takový mutant může být využíván při šlechtění různých elitních kultivarů.