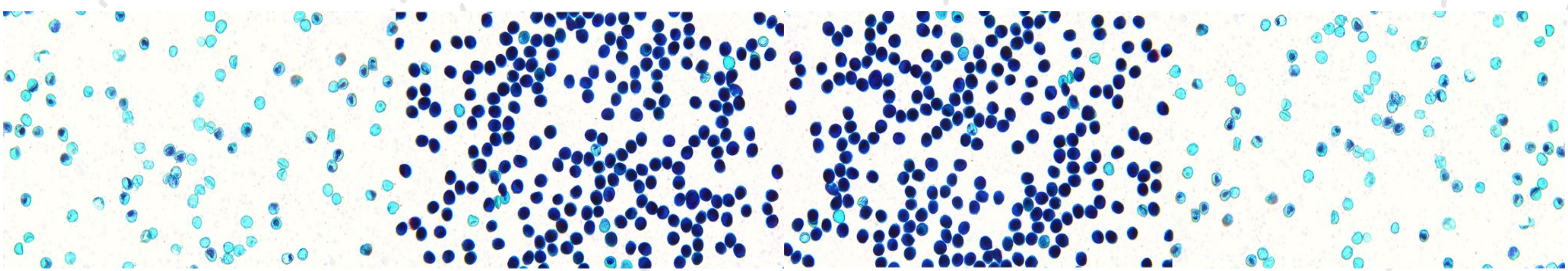
Deciphering the genetic basis of CMS and fertility restoration in wheat

Dr Joanna Melonek

IWGSC Webinar 25th March 2021



Shortly about us and the project…

THE UNIVERSITY OF WESTERN AUSTRALIA

SEEK WISDOM

The Australian Research Council Centre of Excellence in Plant Energy Biology (PEB) is focused on better understanding the way in which plants capture, convert and use energy in response to environmental change

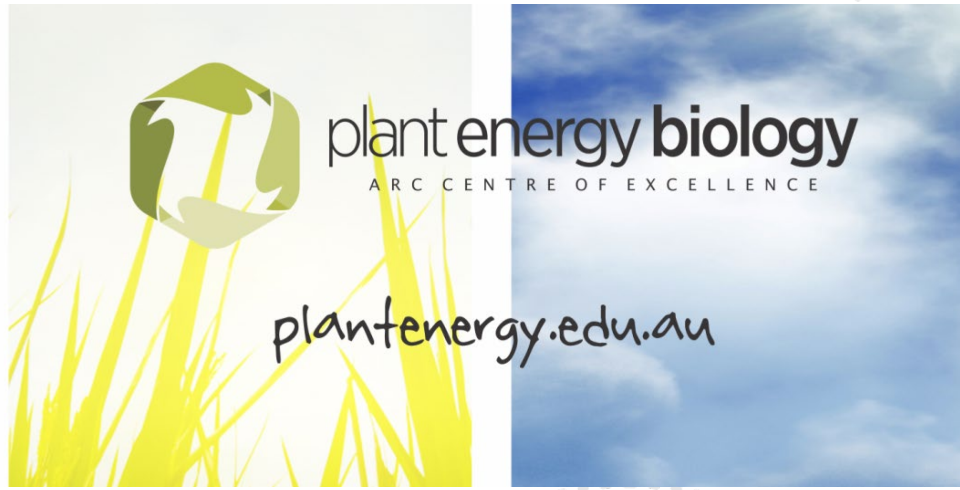


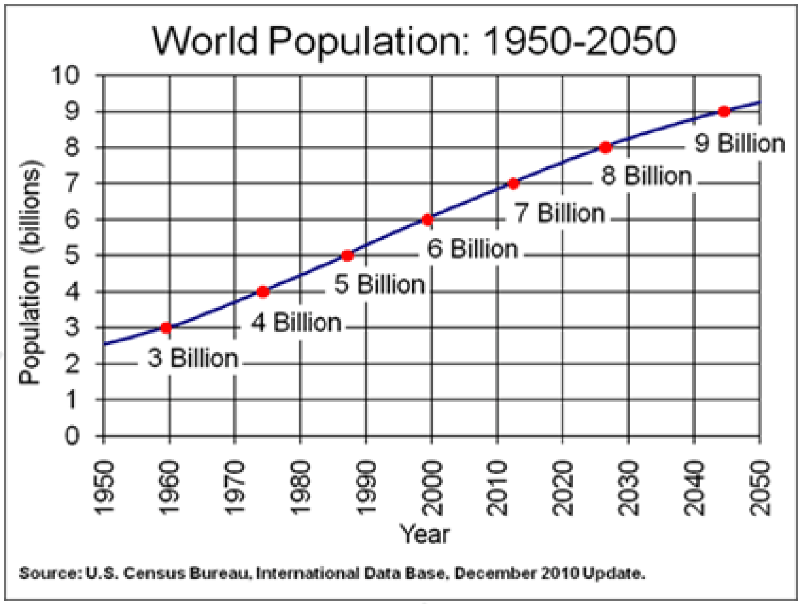
Photo: Ian Small and myself

Successful collaboration with LG since 2015

Limagrain

Pascual Perez

By 2050 crop production needs to more than double to feed the growing human population



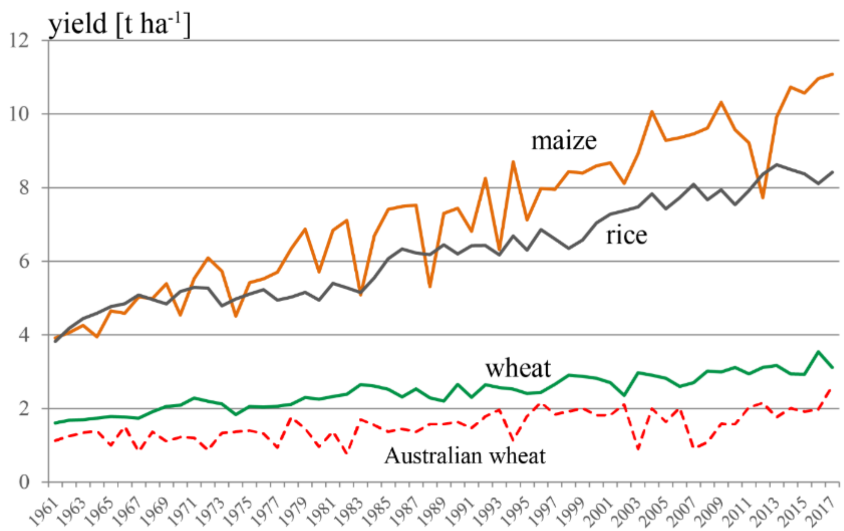


Figure 1. Wheat yield gains are much slower compared to maize and rice.
Data: faostat.fao.org

Hybrids as a way to increase yield in wheat

Why should we grow hybrids?

Agriculture:
Better plant varieties

Food security:
- Higher and more consistent yield
- More safety to secure global food supply

Excellence of hybrids

Plant breeding:
Higher flexibility in stacking multiple traits

Environment:
- Higher energy efficiency
- Better use of available resources
- Better adaption to climate change

plant energy biology

The potential of hybrid breeding in wheat has been recognised since the 19th century!

limits. It would carry on in the Far East the work already performed in British India and Burmah.

A SPANISH Expedition under Capt. Cervera has been exploring Adrar in the Western Sahara. Capt. Cervera describes Port Rio de Oro, where he landed from the Spanish cruiser *Ligera*, as rather difficult of entry, but, once entered, as secure from all winds, with good anchoring ground, and from 10 to 30 metres' depth of water. "Rio de Oro" is a misnomer, as there is only one well of fresh water, and that very dirty. There are, however, good wells in the interior, and at four days' journey there is a running spring. The Expedition proceeded, between latitude 22° and 23°, south-eastwards 425 kilometres through an arid country of gneiss and granite, and struck the boundary of Adrar. The population is composed of four tribes—the Uled Delim, speaking and nearly all capable of writing pure Arabic, mixed with a few words of Berber origin. These tribes are nomadic, moving their tents from well to well for the pasture of their dromedaries, goats, and sheep. The capital of Adrar is Aatar, not Wadan, as hitherto believed. Wadan lies more to the south.

"*HYBRID*" *WHEAT*

IT is probably not generally known that the cereal from which we obtain our bread corn is almost invariably self-fertilised in nature, and that only a skilful expert can perform the delicate operation involved in the cross-breeding of wheat. The anthers, when near maturity, must be removed from a number of wheat-flowers, and on the following day the pollen of the male parent must be placed on the stigma. The opening of the glumes, however, is dependent on the swelling of the "lodicules," which only occurs when the temperature of the atmosphere is not less than about 75°. Below that minimum the florets will not open so as to expose the reproductive parts to the operator. The angle of opening of the glumes corresponds to this swelling, and when fertilisation has been performed the lodicules shrivel up and the glumes again close over the pistil. It had long been obvious that half a dozen different varieties of wheat, blossoming at the same time, may be grown in adjacent fields or in contiguous rows without the occurrence of interbreeding, in spite of the clouds of pollen which sunshine and warmth develop at the time of blossoming; and considering the remarkable results from the cross-fertilisation of numerous plants in gardens, it seems surprising that the same process should not have been applied to wheat. Many years ago a well-known selector and "improver" of cereals, the late Mr. Patrick Sheriff, tried some experiments in this direction. His usual method of improvement consisted in the selection and careful cultivation of "sports," and he was approaching the end of his career when his earliest attempts at cross-breeding were made. The increased vigour of wheat, the moulding of the ear, the production of a larger and fuller ear, with superior grain, earlier maturity, and the modification of the straw so as to render it stronger, or shorter, and less liable to become laid as in the present season, are all improvements which may certainly be accomplished in regard to this cereal, just as analogous modifications have beeen effected in animals and some other plants by the recognised methods of breeders.

The wheat-crop of the United States reaches at present 50,000,000 quarters, or four times that of England, and this may in some measure account for the numerous experiments in cross-breeding by scientific American farmers, and especially by some of the professors of agriculture in the colleges of that country. The same remark applies to France, where the cultivation of wheat is relatively far more important than in England, and where the noted seed-firm of Vilmorin are now in the midst of the work of cross-breeding. But even in England, disheartened as farmers may be as regards wheat-culture, their prospects might certainly be improved if the average production of this cereal could be increased, its quality improved, and its liability to disease and injury from indifferent weather diminished. Both growers and consumers, therefore, have an interest in the undertaking of Messrs. Carter and Co., the seedsmen, who for several years past have been engaged in the cross-breeding of wheat at their trial-grounds and gardens at Forest Hill. The collection of different sorts of wheat at this establishment includes varieties from every country which exports this grain to England. Some of them are not hardy, and the wretched appearance of the growing specimens of Persian and Indian varieties was probably due to their depreciation in our climate. Some of the colonial and other sorts were excellent, but none could compare to the so-called hybrids.

The operations commenced in 1882 by the sowing of a number of the best English and American varieties, and in the following summer twenty crosses were effected by experts who are usually employed by the firm in delicate manipulations of a similar kind in connection with garden vegetables and flowers. In the following autumn the hybrids, as they are usually called for convenience, were sown between the rows of the male and female parents for the sake of comparison, and in the succeeding year the mixture of the breeds became apparent. In one plot, for example, the female parent was a short-strawed velvet-chaffed variety, and the male a very large, bearded, and tall American wheat, and the offspring attained a stature exceeding that of the former by a foot, with smooth chaff, and stout thick-set ears bearing minute awns at the apex of the chaff of each grain. This last-named peculiarity, the occurrence of defensive points in serrated order from top to bottom of the ear, may be referred to as one of the many advantageous peculiarities which have been developed in the course of the experiments, and it has gained for the new variety the appropriate name of "Bird-proof."

Another of the cross-breeds, having the earliest of English varieties, Talavera, for one of its parents, was almost ready for cutting this year on July 21, when we inspected the new sorts, a very early date in the case of a late backward harvest. Another has the grains very firmly set, and therefore not liable to shell out even when the crop is dead ripe, as it usually is before the time of cutting in New Zealand, where this wheat will probably prove popular.

Another of the crosses proved to be a wheat with shorter straw than any other variety in cultivation, and this too will prove a valuable modification, since neither soil nor season, however productive of straw they might be in certain years, could throw the crop down. Now does it surprise the experts that the offspring of two parents which are both of average height, should prove to be a dwarf in regard to the length of its straw, since they have had occasion to observe the same thing in the breeding of peas—two sorts of peas, each 4 feet high, and requiring the support of sticks, having produced a very useful seedling of 2½ feet, which requires no such artificial assistance.

We cannot attempt a detailed description of the numerous other peculiarities—some of them promising to be highly advantageous—which have been developed in the course of these wholesale experiments. But we may here observe that the most tiresome part of the business has proved to be the fixing of the types after the crossing had been accomplished. The work, however, has proved sufficiently successful to encourage the experimenters to undertake the cross-breeding of barley as well as wheat, and to lead them to anticipate a large demand for their new varieties, not only in this country, but in the colonies.

H. E.

DR. AUGUST WEISMANN ON THE IMPORTANCE OF SEXUAL REPRODUCTION FOR THE THEORY OF SELECTION [1]

IN NATURE, vol. xxxiii. p. 154, was given an article on Prof. Weismann's most interesting and important memoir on "The Continuity of the Germ-Plasma considered as the Basis of a Theory of Heredity." The present memoir also abounds with interest, and may be regarded as following naturally from the former one as a continuation and further elaboration of some of the questions raised in it. The main aim of the memoir is to establish the position that the process of sexual reproduction is the prime agent by which all the varied differentiations of the complicated phyla of the Metazoa has been brought into existence. A strong part of the argument is devoted to the establishment of the position that peculiarities acquired by the parent are not transmitted to the offspring, and to showing that the hypothesis that such acquired peculiarities are transmitted is not necessary for the explanation of the known phenomena of heredity and the mode of origin of the series of organic forms. It will be remembered that the assumption of this position forms an important and necessary factor in the theory of the

[1] "Die Bedeutung der sexuellen Fortpflanzung für die Selektions Theorie." (Jena: G. Fischer, 1886.)

E., H. *"Hybrid" Wheat* . *Nature* **34,** 629 (1886).

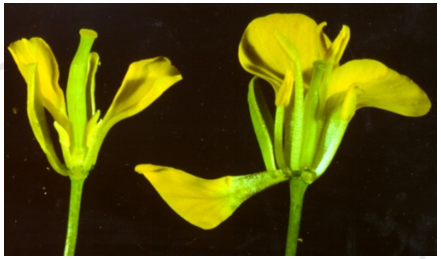
"Hybrid" Wheat drawing by Vilmorin the funder of Vilmorin & Cie, A seed producing company with a long history in France that now belongs to Groupe Limagrain.

Hybrid production requires a way to block self-pollination to exploit heterosis

Mechanical emasculation of plants

Chemical treatment of flowers

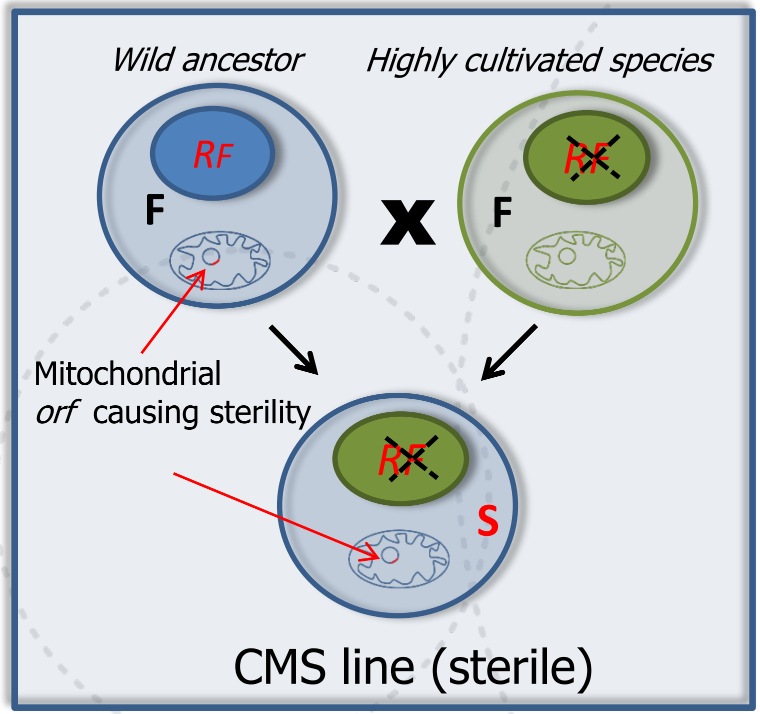
Cytoplasmic male sterility (CMS)



CMS in rapeseed Greg Brown, Mc Gill University

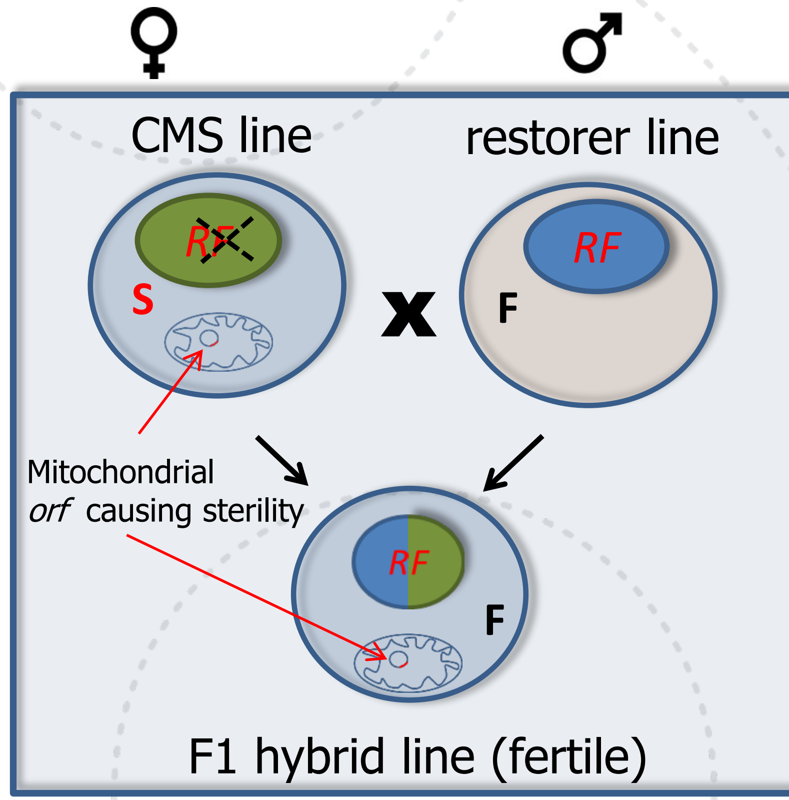
Cytoplasmic male sterility (CMS)

CMS – is a natural phenomenon that widely occurs in plants and can be used in hybrid breeding



no pollen
no self-pollination

F-fertile
S-sterile



lots of pollen
self-pollination

CMS-based hybrid seed production systems are used in rice, maize, sorghum and canola

Hybrid canola using the OGU-INRA CMS system

♀ CMS line

♂ restorer line

x

F1 hybrid line fully fertile

https://www.graincentral.com/cropping/oilseeds/dont-think-twice-its-all-right-australias-canola-exports/

Flowering canola on Ivan Lee's farm at Bulyee, near Corrigin, WA.

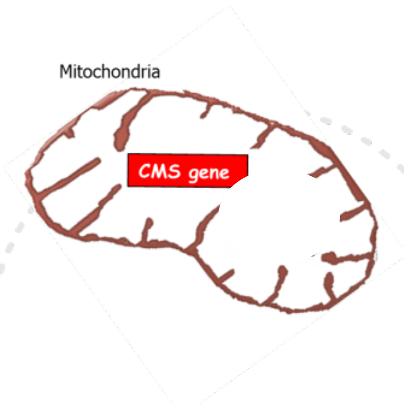
45% yield increase in hybrids compared to conventional lines!

Hybrid varieties are missing in wheat

T-CMS in bread wheat

Hybrid system based on *T. timopheevii* cytoplasm (T-CMS)

T. timopheevii



Full sterility

CMS line

Restorer gene

Partial fertility

restored line

Incomplete fertility restoration due to lack of a strong restorer gene

Hybrid system based on *T. timopheevii* CMS (T-CMS):

- So far, 9 restorer genes have been described to be involved in fertility restoration in wheat:

Locus	Chromosome	Source	Reference
→ *Rf1*	1A	*T. timopheevii*	Bahl and Maan (1973); Maan (1985)
Rf2	7D	*T. timopheevii*	Livers (1964); Bahl and Maan (1973)
→ *Rf3*	1B	*T. spelta var. duhamelianum*	Tahir & Tsunewaki (1969)
Rf4	6B	*T. aestivum*	Maan (1985)
Rf5	6D	[(*T. timopheevii x Ae. tauschii*) x 'Canthatch'3]F2	Yen et al. (1969)
Rf6	6U	Translocation line '2114' (T6AL.6AS-6U)	Ma et al. (1995)
Rf7	7B	*T. timopheevii*	Sinha (2013): Bahl & Maan (1973)
Rf8	2DS	*T. aestivum 'PWR4099'*	Sinha et al. (2013)
Rf9	6A		Shahinnia et al. 2020

- The restoration of fertility by single *Rf* gene is not complete - hope in stacking!
- Majority of cloned *Rf* genes belong to the family of the pentatricopeptide repeat (PPR) proteins.

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Family of the pentatricopeptide repeat proteins

PPR proteins are specific to Eukaryotes and the family is highly expanded in plants

Organism	# of genes	# of PPRs
Homo sapiens	37,490	6
Drosophila melanogaster	17,087	2
Caenorhabditis elegans	20,673	2
Schizosaccharomyces pombe	5,010	2
Saccharomyces cerevisiae	6,304	5
Trypanosoma brucei	16,757	19
Cyanidioschyzon merolae	4,772	10
Arabidopsis thaliana	32,641	496
Oryza sativa	91,992	475
Vitis vinifera	28,352	534
Ralstonia solanacearum	5,118	1
Synechocystis sp	3,169	0
Rickettsia prowazekii	834	0

REVIEW

RNA Biology 10:9, 1433–1438; September 2013; © 2013 Landes Bioscience

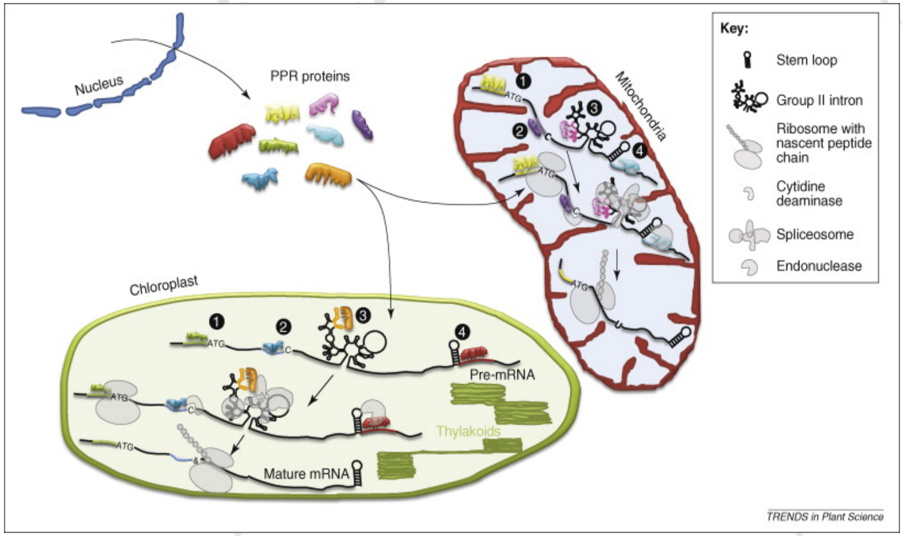
Human pentatricopeptide proteins

Only a few and what do they do?

Robert N Lightowlers[1] and Zofia MA Chrzanowska-Lightowlers[2,*]

[1]The Wellcome Trust Centre for Mitochondrial Research; Institute for Cell and Molecular Biosciences; Newcastle University; The Medical School; Framlington Place; Newcastle upon Tyne, UK; [2]The Wellcome Trust Centre for Mitochondrial Research; Institute for Ageing and Health; Newcastle University; The Medical School; Framlington Place; Newcastle upon Tyne, UK

PPRs are RNA binding proteins located in chloroplasts and mitochondria …



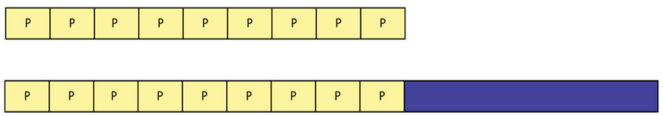
Modified from: Schmitz-Linneweber and Small. 2008. Trends in Plant Science. 13:P663-670

… where they regulate gene expression by splicing, editing, stabilizing or directly cleaving RNA.

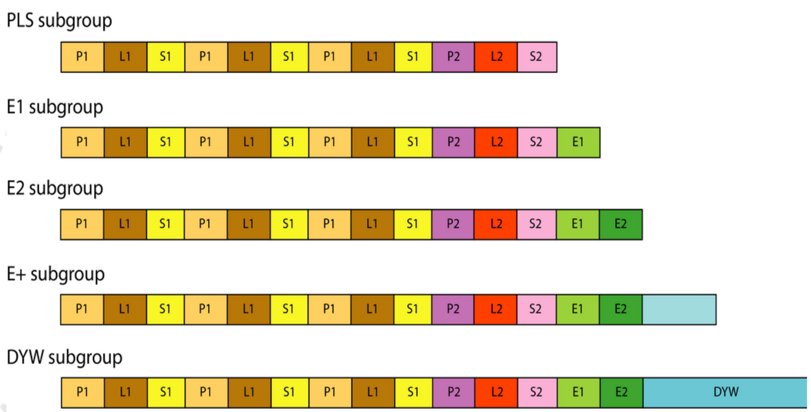
Family of the pentatricopeptide repeat proteins

PPR proteins are made of tandem repeats of 31-36 amino acids

P subfamily

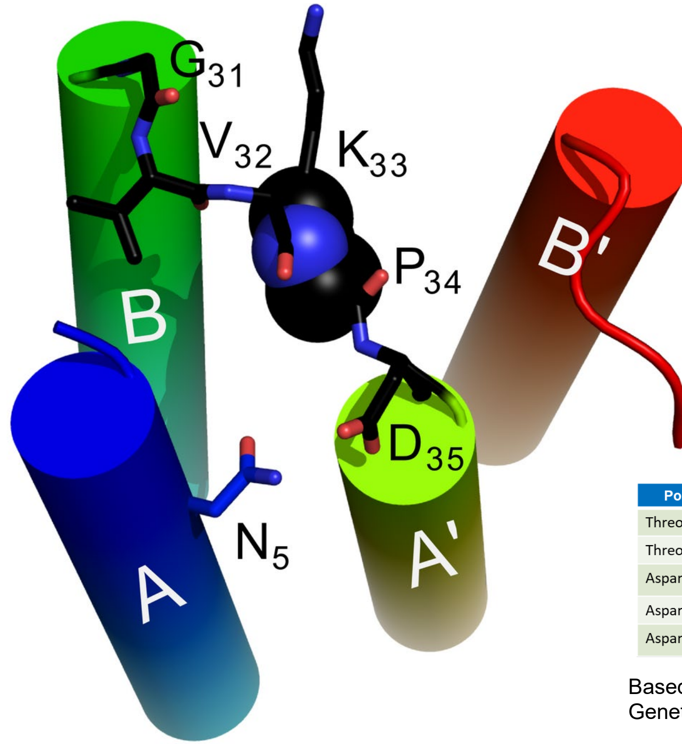


PLS subfamily



Modified from: Cheng et al 2016. Plant J 85 (4): 532-547

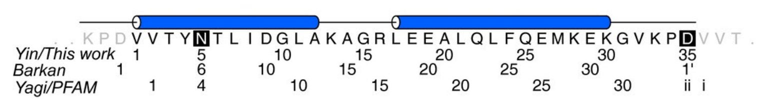
PPR tracts bind RNA via modular recognition mechanism



PPR code

Position 5	Position 35	Binds to
Threonine (T)	Aspartate (D)	G
Threonine (T)	Asparagine (N)	A
Asparagine (N)	Aspartate (D)	U > C
Asparagine (N)	Asparagine (N)	U = C
Asparagine (N)	Serine (S)	U < C

Based on: Barkan et al. PLoS Genet. 2012. 8(8):e1002910



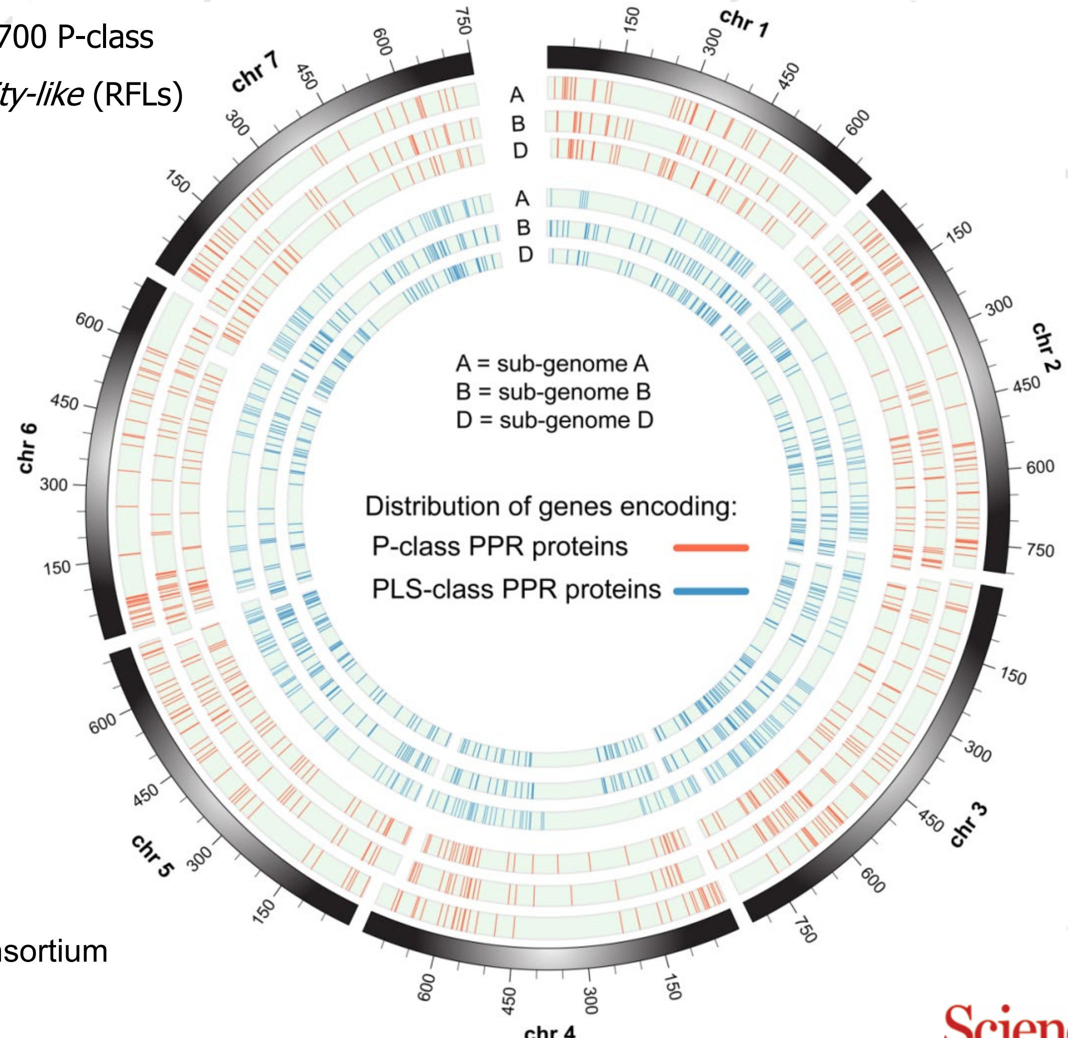
https://ppr.plantenergy.uwa.edu.au/ppr/

Based on: Gutmann et al. Mol Plant 2020.13(2):215-230

Identification of PPRs in the IWGSC RefSeq v1.0 reference genome

Genome-wide distribution of genes encoding PPR proteins in wheat

- 1686 pentatricopeptide repeat (PPR) genes/~700 P-class
- 206 genes were identified as *restorer-of-fertility-like* (RFLs)

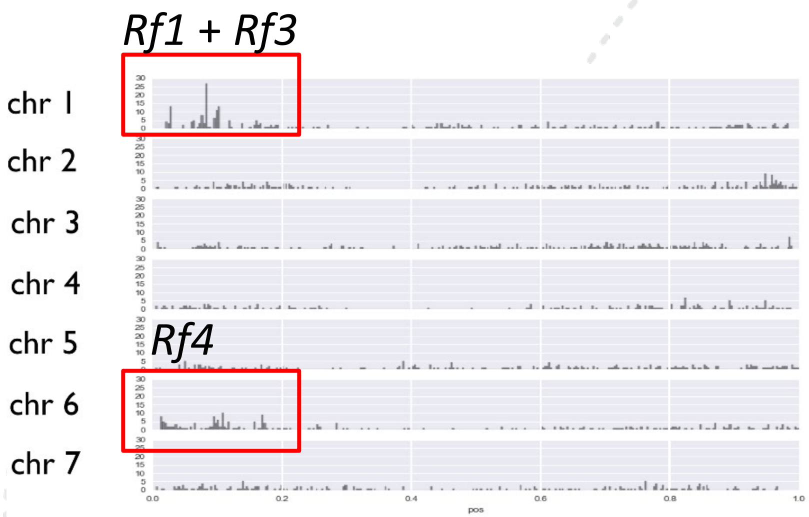


The International Wheat Genome Sequencing Consortium (IWGSC) et al. Science 2018;361:eaar7191

Science AAAS

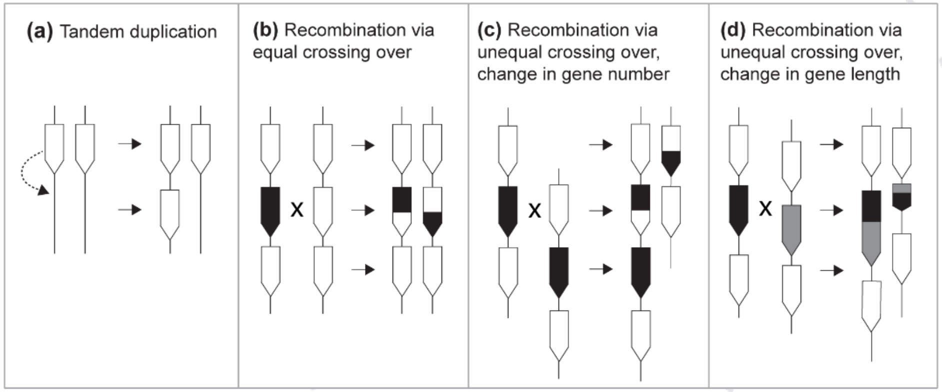
Expansion of RFL genes in wheat

RFL genes are located in high-density clusters



Modified from: IWGSC. Science 2018. 361:eaar7191

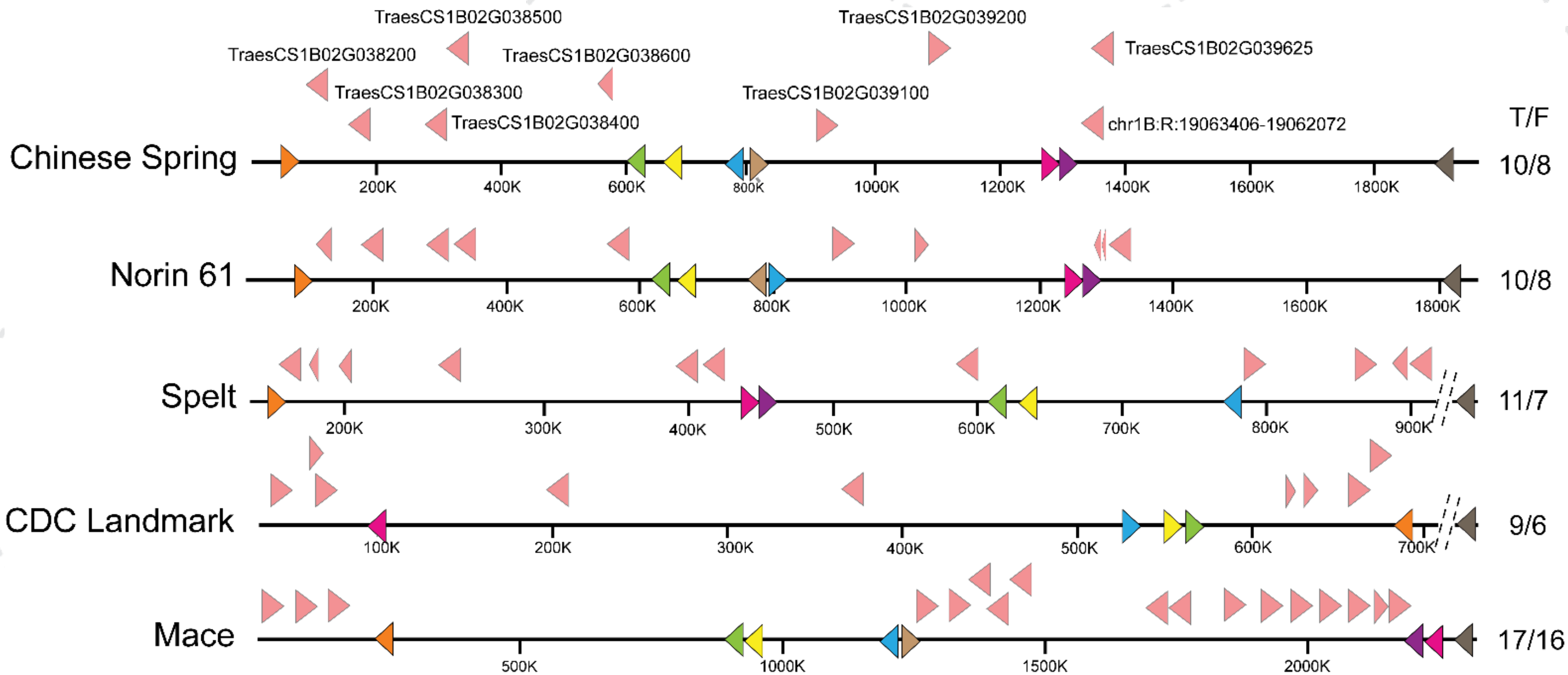
Mechanisms contributing to the plasticity of RFL genes



Modified from: Melonek et al 2016 Sci Reports 2016. 6:35152.

- RFL genes are organised in clusters on chromosomes 1, 2 and 6
- RFL clusters show much higher gene density compared with other PPR genes
- *Rf1* and *Rf3* map to the cluster on chr 1, *Rf4* maps to the cluster on chr 6

Structural diversity within RFL cluster in wheat pan-genome



Modified from: Walkowiak et al. Nature. 2020. 588(7837):277-283.

High RFL sequence diversity in wheat varieties seen in wheat pangenome

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Deciphering the molecular basis of CMS and fertility restoration in wheat

Part I. Cloning the sequences of Rf1 and Rf3 restorer genes

Mapping genomic locations of the restorer genes

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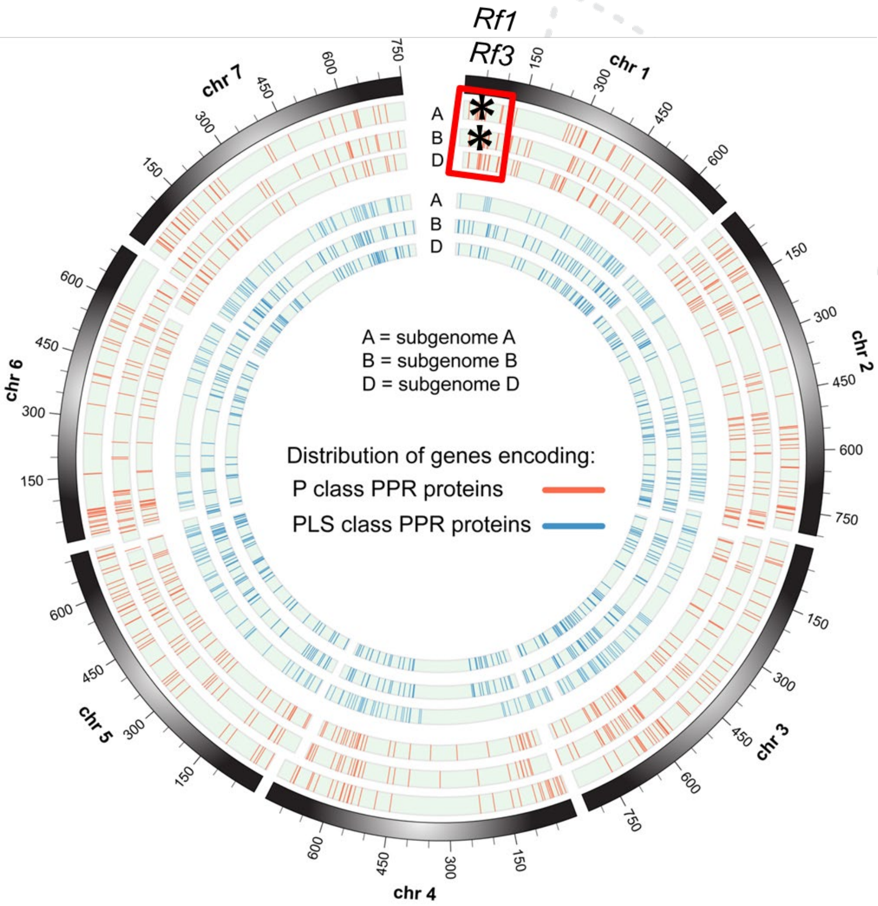
Pierrick Varenne

Limagrain



Rf1 and *Rf3* map to a cluster on chr 1A and 1B

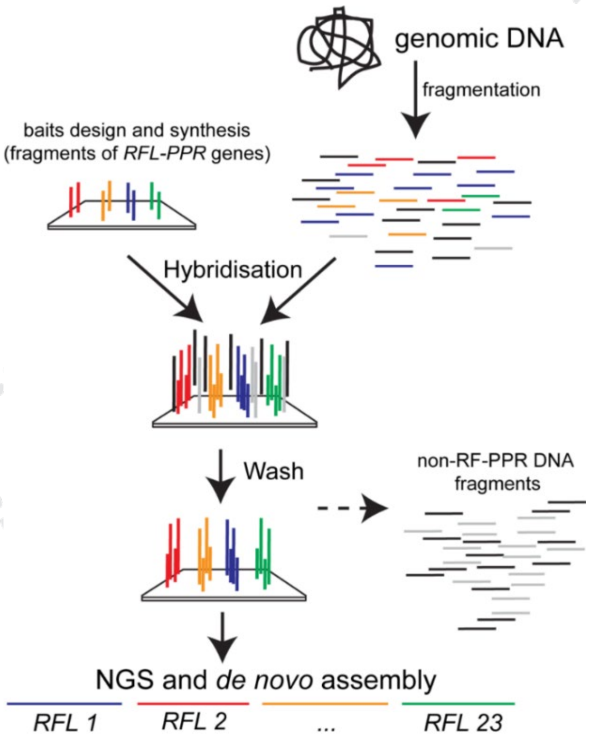
PPR capture approach to identify RFL genes in commercial wheat genotypes

Jean-Philippe Pichon

Jorge Duarte

Limagrain

RFL capture approach:



Summary of RFL capture experiment

#	restoration status	Accession name	Number of orthologous groups with at least one RFL from the accession	Number of RFL ORFs > 350 aa assigned to orthologous groups
1	weak *Rf3* restorer	Chinese Spring	205	161
2	maintainer	Anapurna	202	156
3	maintainer	Fielder	212	138
4	*Rf1*	R197	219	174
5	*Rf1*	R0932E	221	183
6	*Rf3*	R0946E	237	171
7	*Rf3+Rf1*	R0934F	215	174
8	*Rf3*	Primepi	215	162
9	*Rf1*	*Triticum timopheevii*	129	114
	TOTAL:		397 (non-redundant)	1433

Modified from: Melonek et al. Nature Communications. 2021.12(1):1036

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Mapping and anchoring the genomic regions carrying *Rf1* and *Rf3* in the IWGSC Refseqv1.0 genome

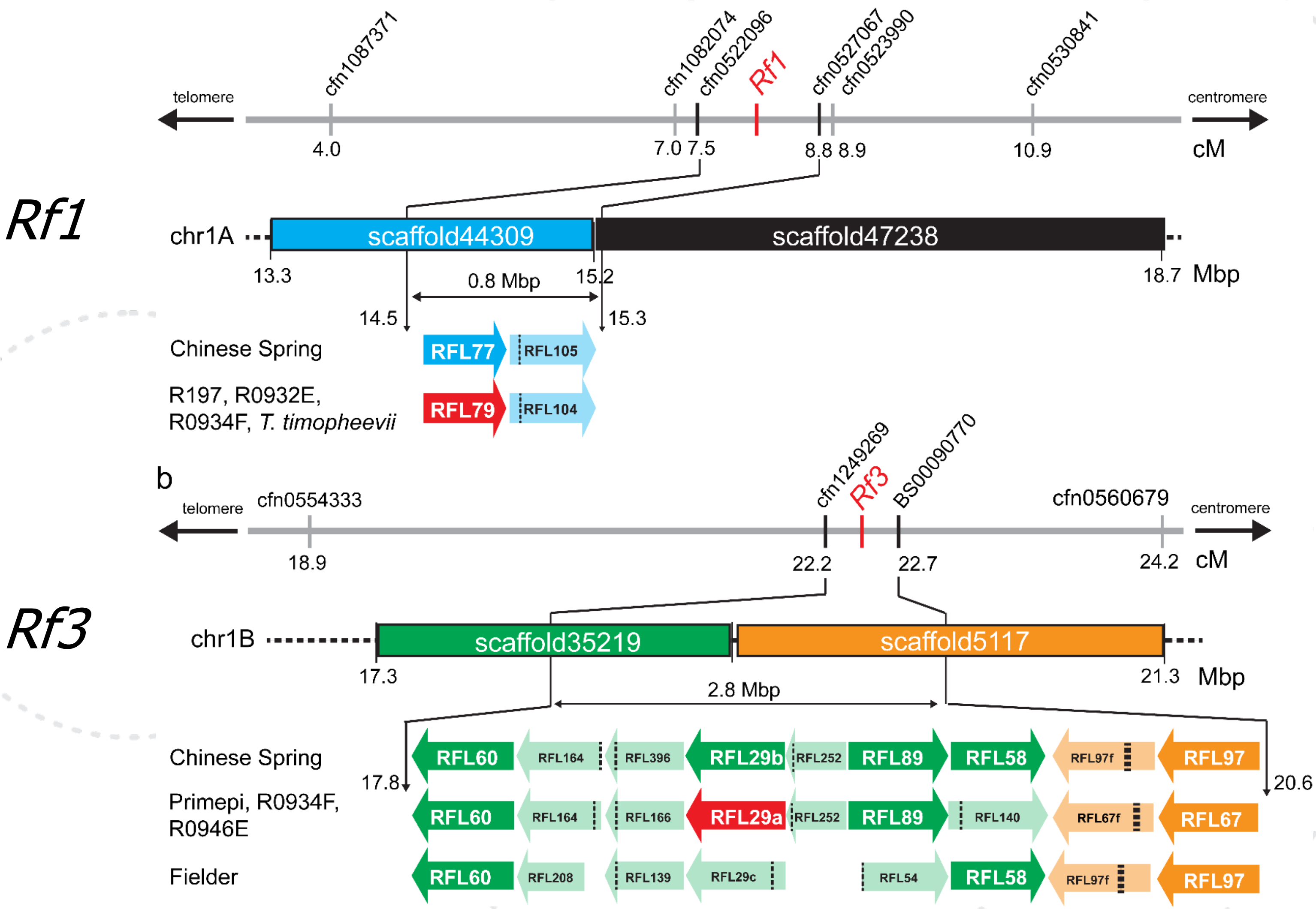
Selection of candidate RFL groups based on restoring status of analysed wheat accessions and location within the *Rf1* interval

RFL gene	Protein size (aa)	Gene located within *Rf1* interval (*in silico* mapping with	Gene located within *Rf1* mapping interval (genetic mapping (nb of markers))	Restoring genotype							
				Maintainer		*Rf1* restorer		*Rf3* restorer		*Rf1* + *Rf3*	*Rf1* restorer
				Chinese Spring	Anapurna	R197	R0932E	R0946E	Primepi	R0934F	*T. timopheevii*
RFL1	988	no	n.a.	0	0	1*	1	0	0	0	0
RFL56	804	no	n.a.	0	0	1	1*	0	0	0	1
RFL59	813	no	n.a.	0	0	1	2	0	0	0	1
RFL73	813	no	n.a.	0	0	1	1	0	0	0	1
RFL74	813	no	n.a.	0	0	1	1	0	0	1	0
RFL79	808	no	yes (4)	0	0	1	1	0	0	1	1
RFL93	775	no	no	0	0	1	1	0	0	0	0
RFL104	757	yes	yes (4)	0	0	1	1	0	0	1	1
RFL129*	693	no	no	0	0	1	1	0	0	1	0
RFL185*	524	yes	yes (4)	0	0	1	1	0	0	1	1
RFL268*	382	yes	yes (4)	0	0	1	1	0	0	1	1

Modified from: Melonek et al. Nature Communications. 2021.12(1):1036

Mapping and anchoring the genomic regions carrying *Rf1* and *Rf3* in the IWGSC RefSeq v1.0 genome

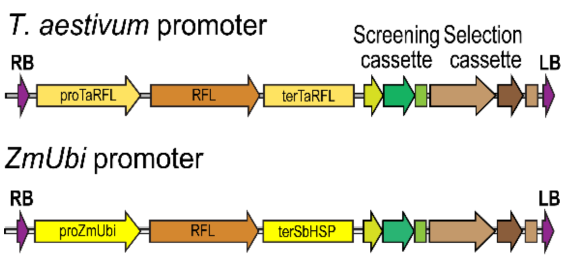
Genetic map of the *Rf1* and *Rf3* loci in wheat



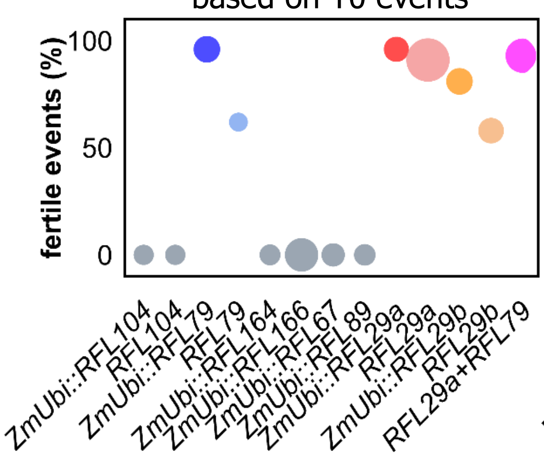
Modified from: Melonek et al. Nature Communications. 2021.12(1):1036

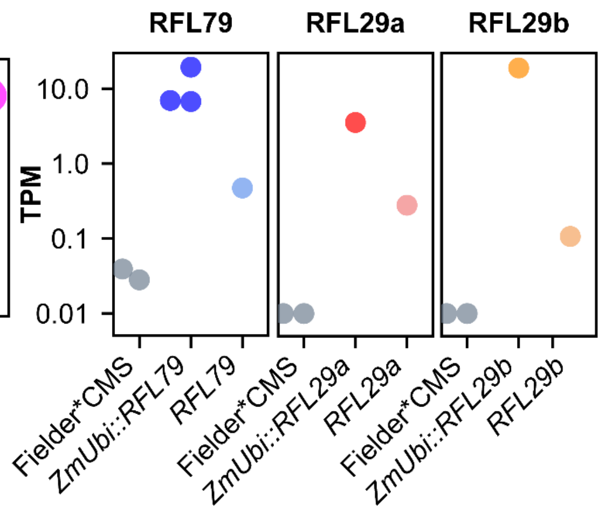
Testing candidate *Rf* genes by stable transformation of wheat plants

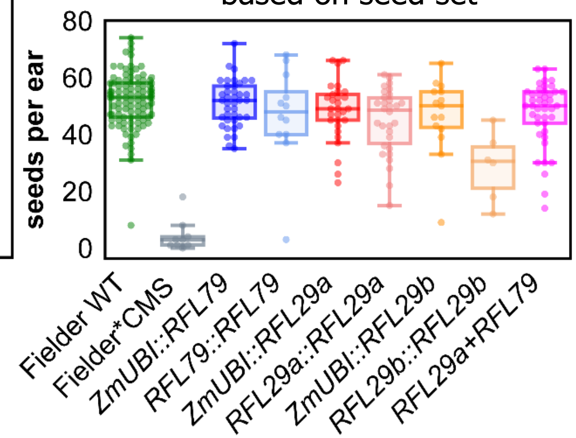
Laurent Beuf   Jerome Martin



Fielder   Fielder*CMS   *ZmUbi::RFL79*   *RFL79*   *ZmUbi::RFL29a*   *RFL29a*   *ZmUbi::RFL29b*   *RFL29b*



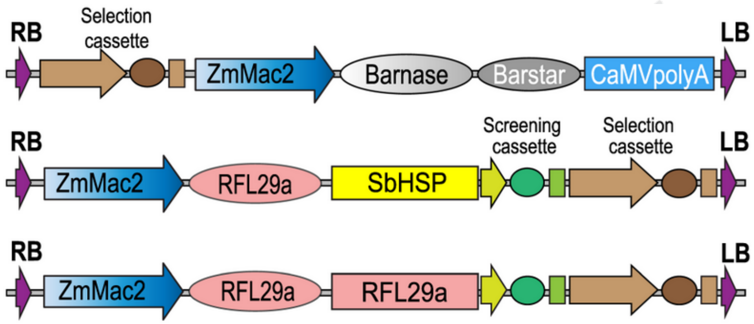




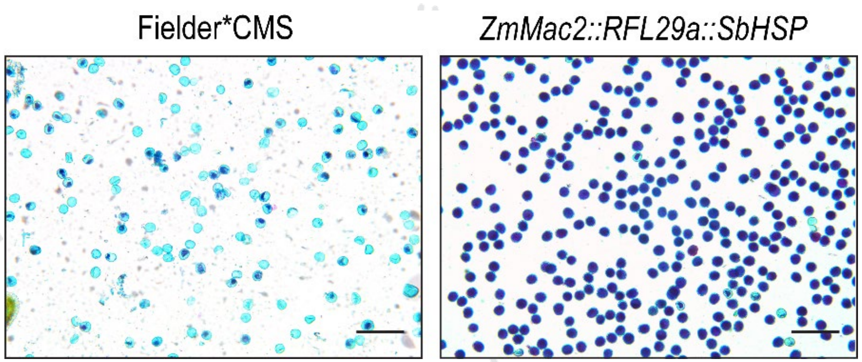
RFL29 => *Rf3*   RFL79 => *Rf1*   Rf3 > Rf1

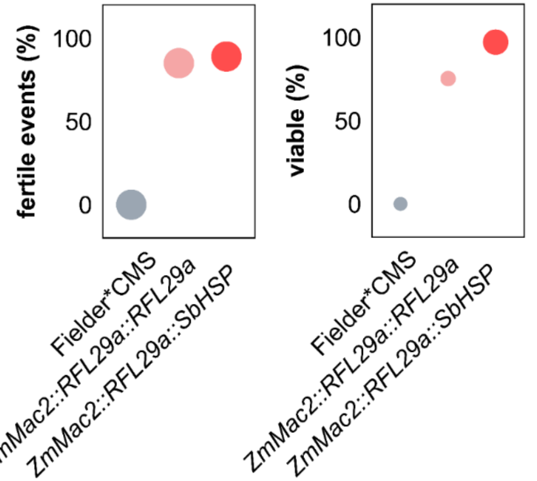
Rf gene expression in tapetum cells is sufficient to restore fertility of T-CMS plants

Testing expression driven by tapetum specific *ZmMAC2* promoter



NB1 *ZmMac2::Barnase*





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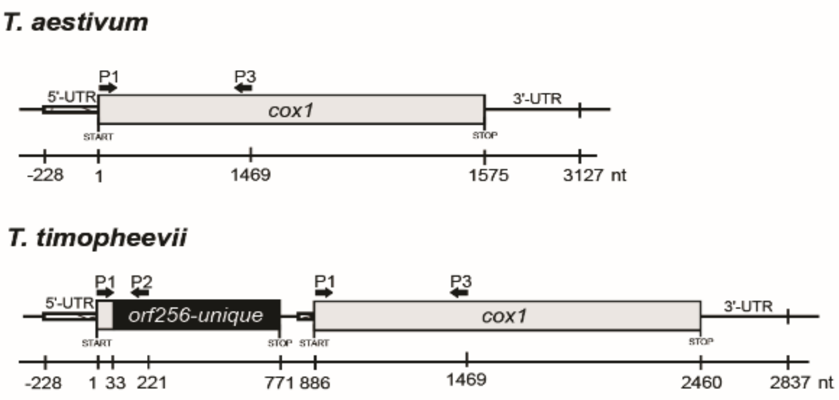
Deciphering the molecular basis of CMS and fertility restoration in wheat

Part II. Identification of CMS-inducing gene

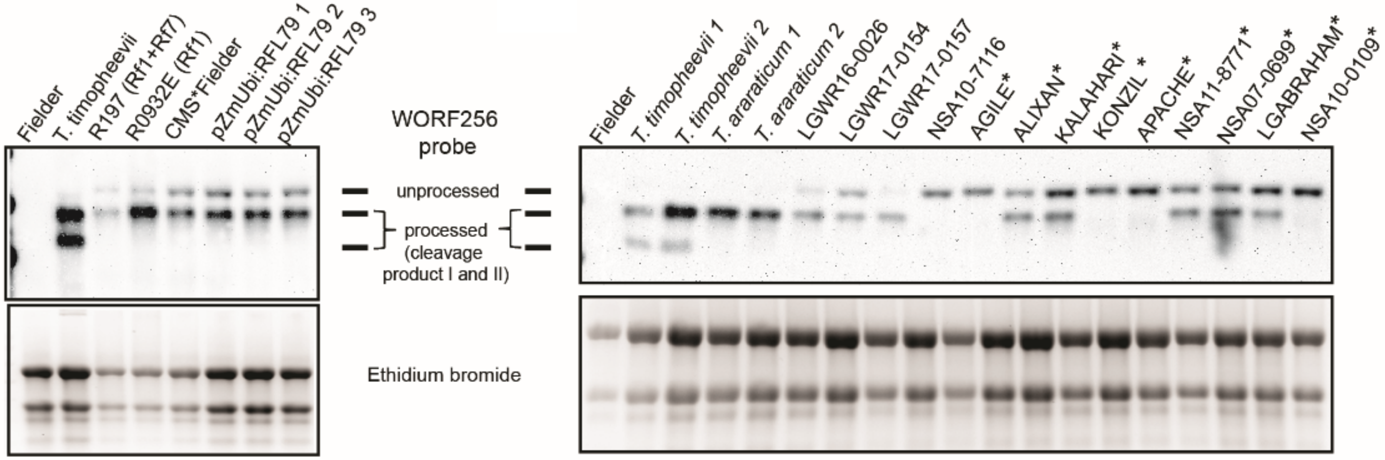
Cleavage of *orf256* does not correlate with fertility restoration phenotype



Based on: Song and Hedgcoth. 1993. 37(2):203-9

Photo credit: Limagrain

Northern blot

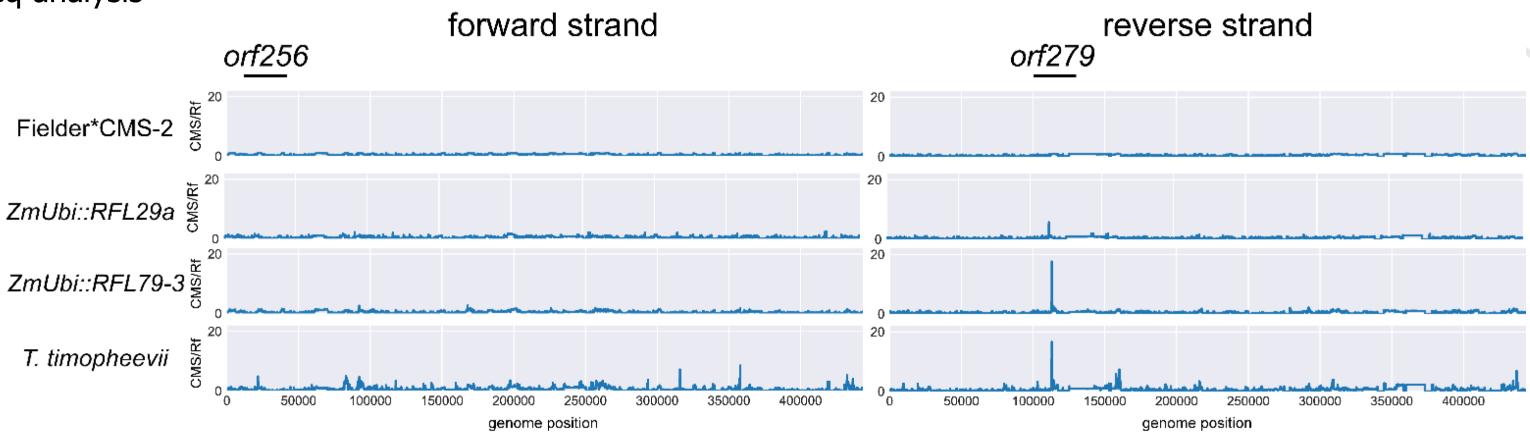


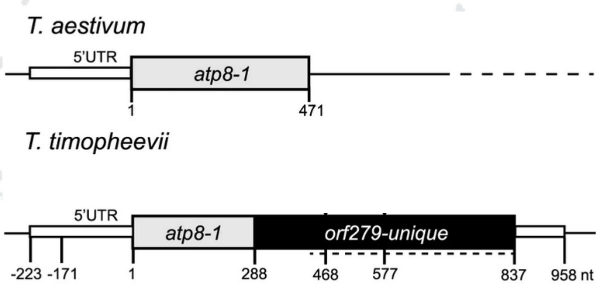
orf256 is not the cause of sterility in T-CMS wheat

Orf279 as basis for sterility caused by T-CMS

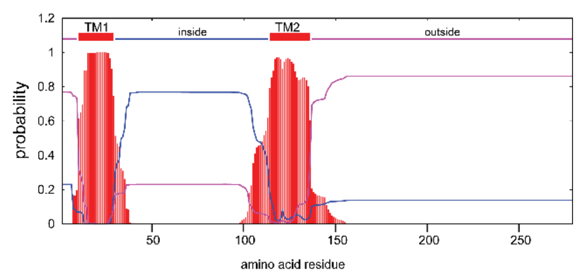
RNAseq analysis

read-depth ratio CMS vs restored line

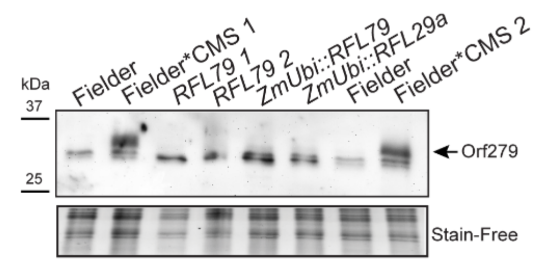




Prediction of transmembrane domains



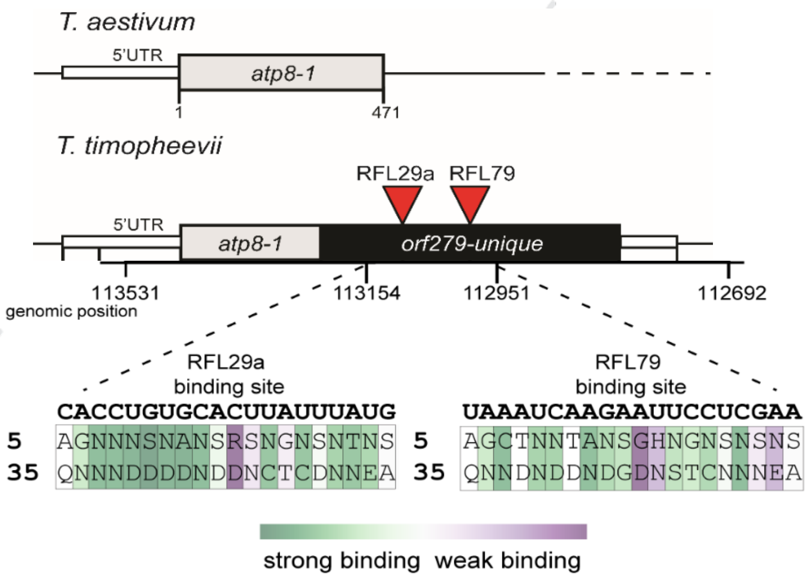
Immunological detection of Orf279



Modified from: Melonek et al. Nature Communications. 2021.12(1):1036

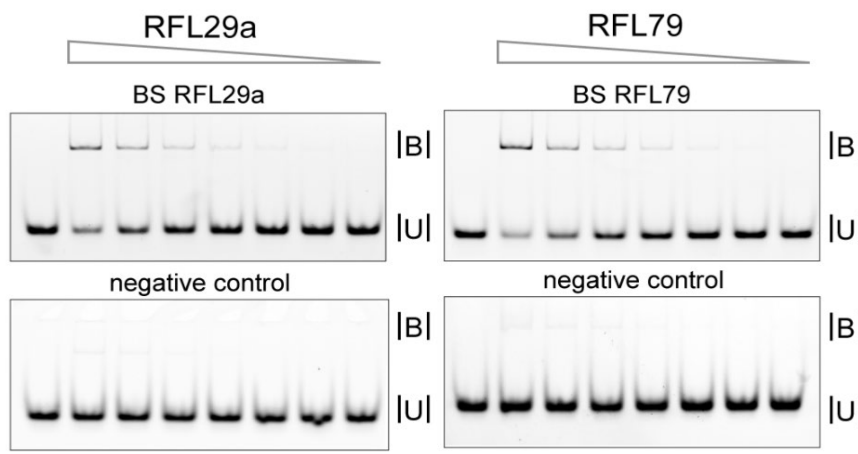
Rf1 and Rf3 bind to *orf279* RNA at two different positions

Prediction of Rf1 and Rf3 binding sites within *orf279* RNA



RNA-binding assay REMSA

Kalia Bernath-Levin

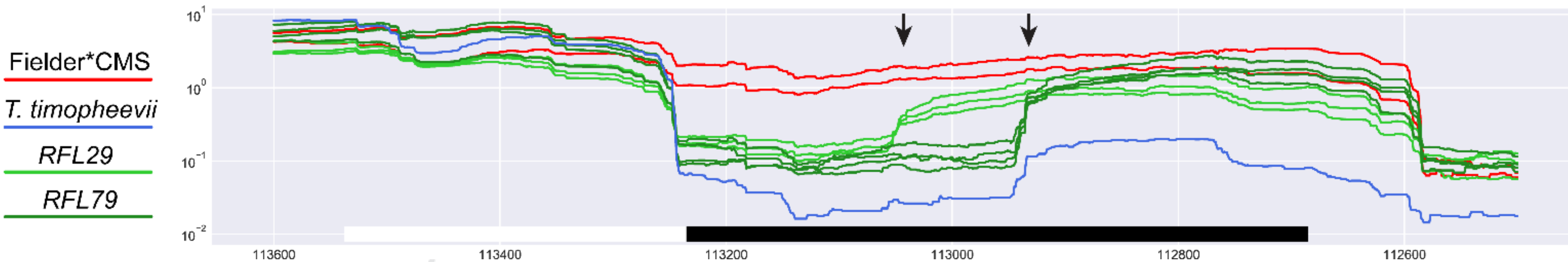


Modified from: Melonek et al. Nature Communications. 2021.12(1):1036

Rf1 and Rf3 induce cleavage of *orf279* at two different positions

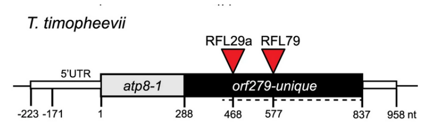
RNAseq analysis

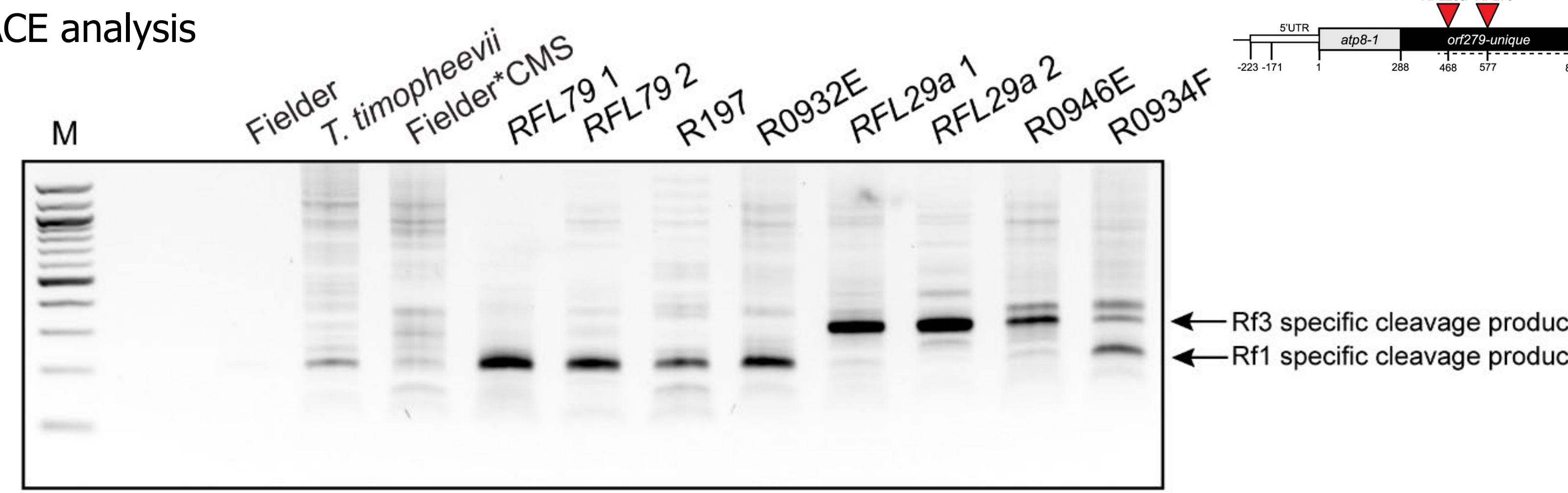
read-depth coverage across *orf279* transcript



atp8 region *orf279*-unique region

5′- RACE analysis





Modified from: Melonek et al. Nature Communications. 2021.12(1):1036

Summary and conclusions:

- We have cloned the *Rf1* and *Rf3* restorer genes in wheat.
- We have discovered that previously described *orf256* is not the cause of T-CMS.
- Instead we have identified *orf279* as the mitochondrial gene causing T-CMS in wheat.
- Both *Rf1* and *Rf3* bind to *orf279* and induce its cleavage.
- Neither *Rf1* nor *Rf3* alone provide complete fertility restoration.
- We will investigate why *T. timophevii* is fully fertile.
- We will clone further restorer genes.

PhD students

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Gilang Bintang Fajar Suhono

Thank you to our collaborators!

Hybrid wheat project

Pascual Perez
Laurent Beuf
Jean-Philippe Pichon
Jorge Duarte
Jacques Rouster
and the team

Tristan Coram

Annotation of the PPR family in the wheat reference genome

Kellye Eversole
Rudi Appels

Nils Stein
Martin Mascher

Curtis Pozniak
Sean Walkowiak

PPR and mTERF families in rye

International Rye Genome Sequencing Consortium

Tim Rabanus-Wallace
Nils Stein

Viktor Korzun

Bernd Hackauf

Hybrid sorghum project

Emma Mace
David Jordan

Robert R Klein

Photo credit: Limagrain

We are looking for collaborations!

Our genomics approach will work in all crops for which enough sequence data can be obtained!

For more information please contact:

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Professor Ian Small ian.small@uwa.edu.au

Or visit our website:

https://plantenergy.edu.au/opportunity/collaborate

We will advertise a PhD position later in the year!

Contact us if interested!

Thank you for your attention!