UNIVERSITÀ DEGLI STUDI DELLA TUSCIA

DIPARTIMENTO DI SCIENZE AGRARIE E FORESTALI

D.I.Ver.So. Digitali, Innovativi, Verdi, Sostenibili

Nutritional improvement of wheat using TILLING, transgenesis and genome editing

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Presentation outline:

1. Realization of wheat lines with lower amounts of α–amylase/trypsin inhibitors (ATI)
2. Production of high amylose (high resistant starch) wheats
3. Development of lines potentially forming low-acrylamide in final products

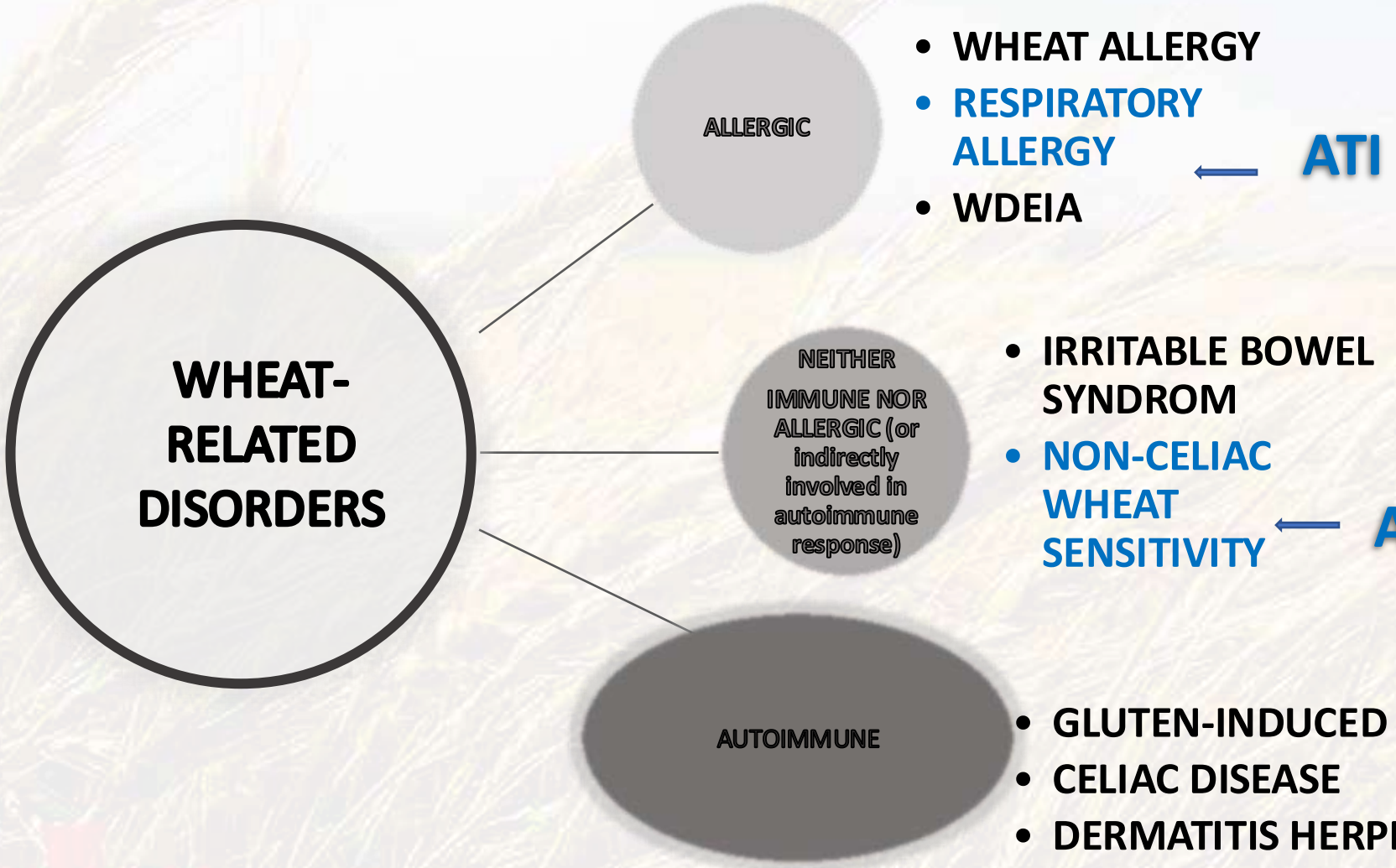
Presentation outline:

1-Realization of wheat lines with lower amounts of α–amylase/trypsin inhibitors (ATI)

Wheat Amylase Trypsin Inhibitors (ATI)

- A complex family of non-gluten wheat proteins involved in pest resistance
- All with a conserved secondary/tertiary structure and a divergent primary structure
- Tetramers (CM proteins), dimers (0.19, 0.28) and monomers (0.19, 0.53) are present
- Nutritional activators of innate immunity
- Activate toll-like receptor 4 (TLR4)
- Promote intestinal and extra-intestinal inflammation
- Resistant to intestinal proteases
- Strong immunogenic potential even after baking

WHEAT RELATED DISORDERS



- WHEAT ALLERGY
- RESPIRATORY ALLERGY ← ATI
- WDEIA

- IRRITABLE BOWEL SYNDROM
- NON-CELIAC WHEAT SENSITIVITY ← ATI

- GLUTEN-INDUCED ATAXIA
- CELIAC DISEASE
- DERMATITIS HERPETIFORMIS

GLIADINS AND GLUTENINS

We need to know WHO makes WHAT

- Is there an influence of all types of food processing?
- Are the enzyme inhibiting properties related to this pathology?
- Why ATI present in non gluten-containing cereals are different?
- Are all ATI responsible of this pathology?

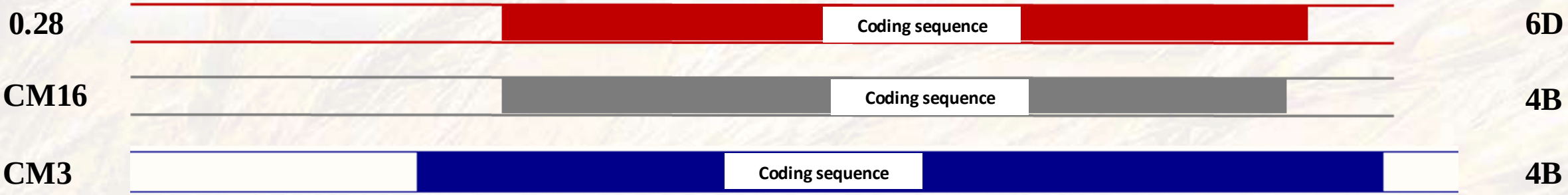
Plant breeding procedures, either classical or advanced, may allow to answer the some of these questions

- Using silent versions of specific *ATI* genes it is possible to understand WHO (*specific gene/s or all?*) makes WHAT (*is there a different degree in the inflamatory response caused by different ATI genes? Is there a relationship with enzyme inhibiting properties?)*

Target ATI genes for silencing

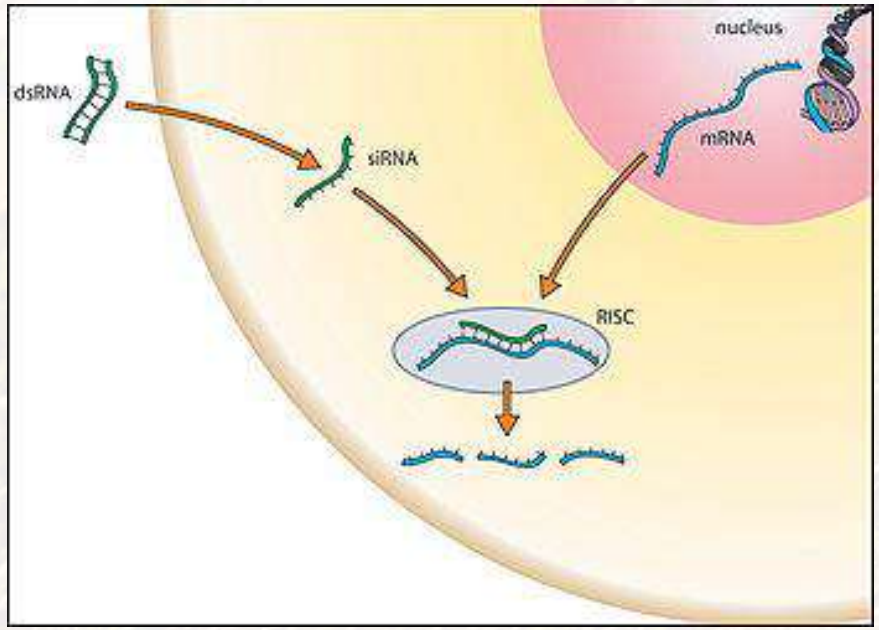
Durum wheat
cv *Svevo (AABB)*

Bread wheat
cv *Bobwhite (AABBDD)*

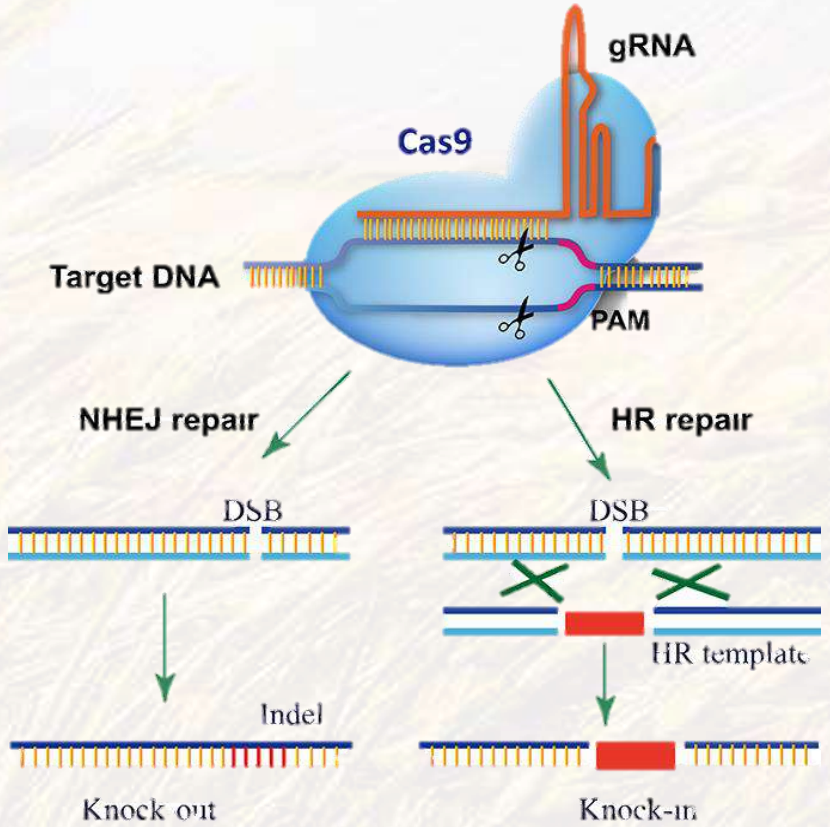


We have used two different silencing procedures

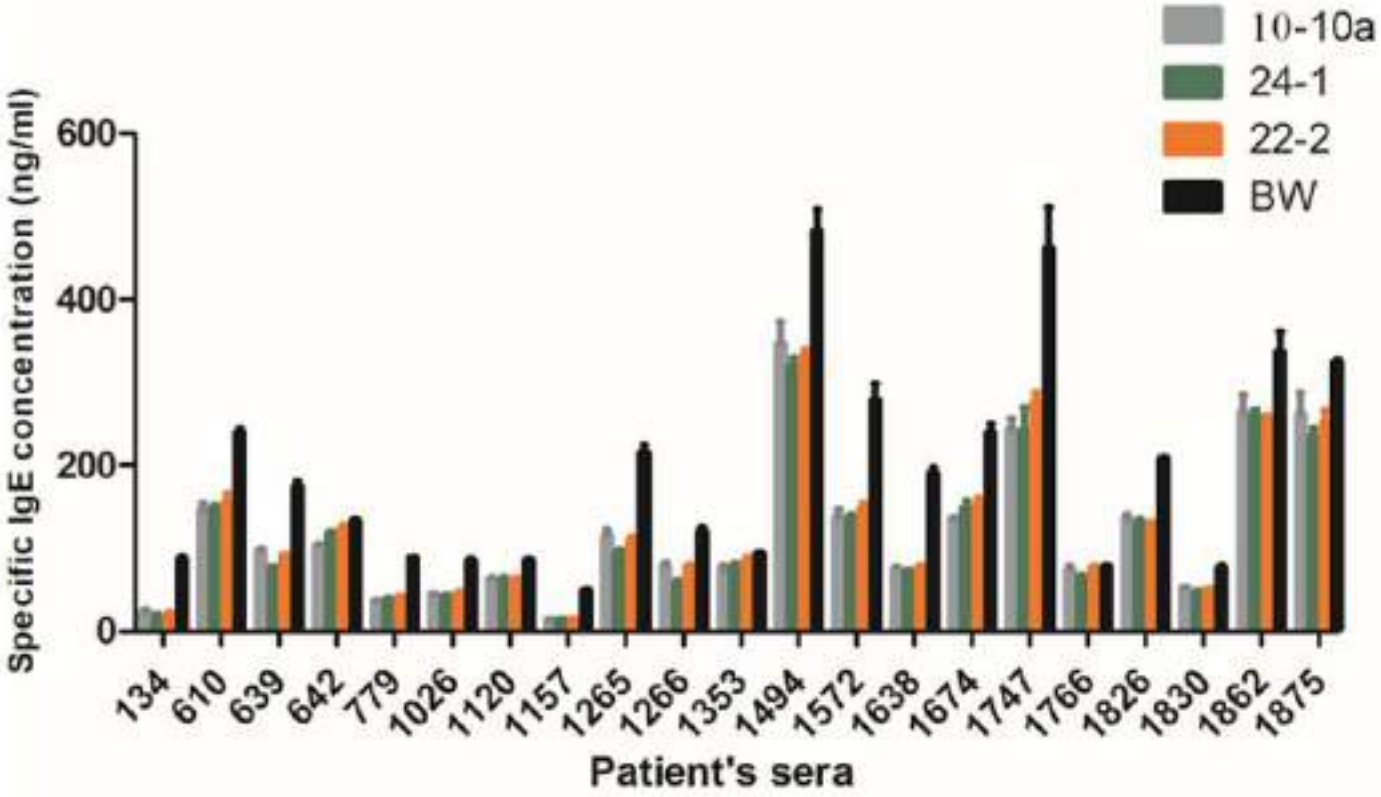
RNA interference



Genome editing by means of CRISPR-Cas9

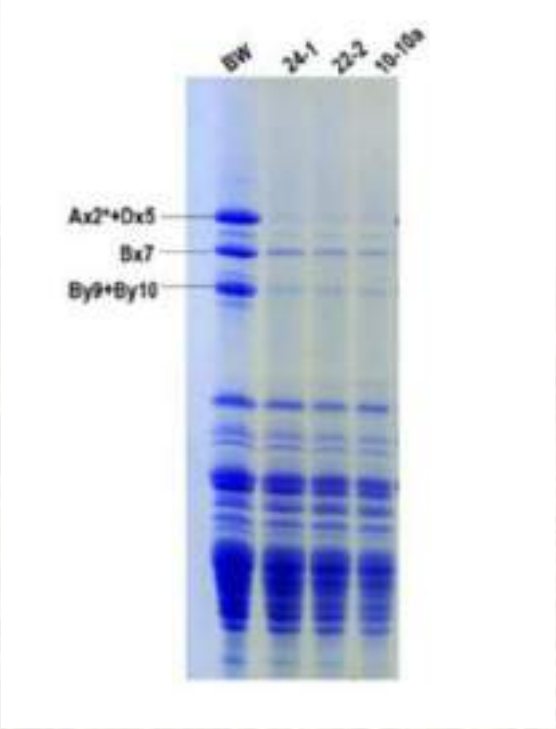


ELISA TESTS BY USING SERA OF WHEAT ALLERGIC PATIENTS



Almost all patients sera show a lower amount of IgE against the soluble proteins extracted from the RNAi silenced lines with respect to those extracted from the reference untransformed genotype

Kernel protein expression is highly affected in the three RNAi silenced lines show with respect to the reference untransformed genotype



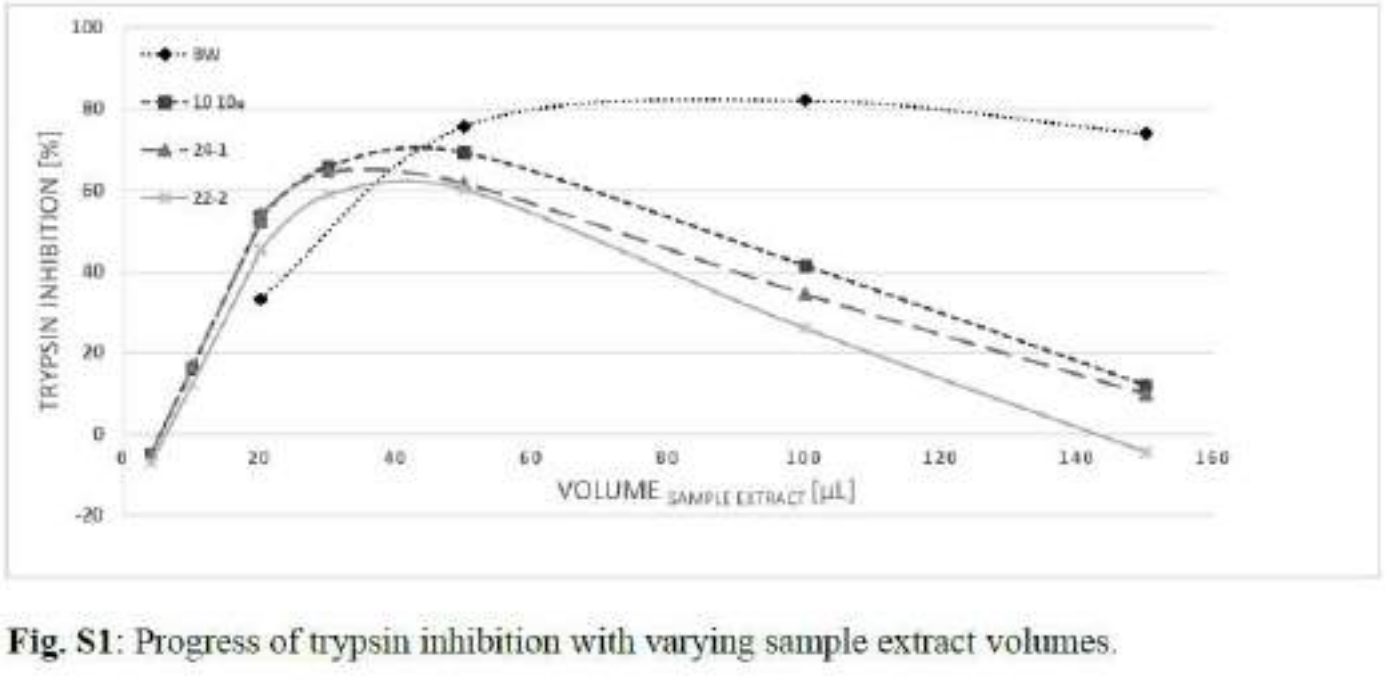
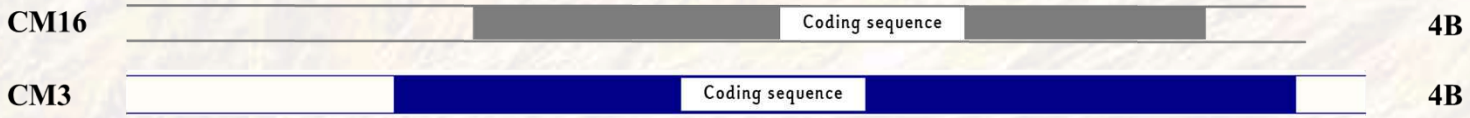
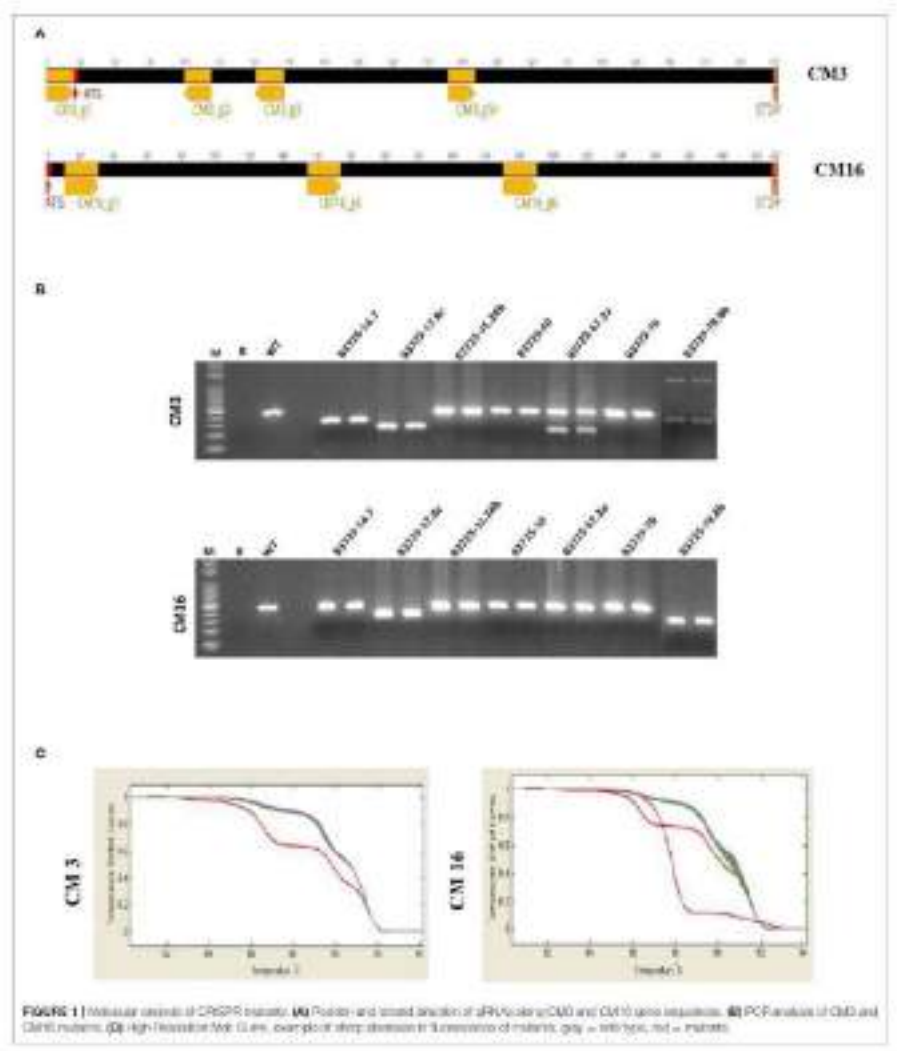


Fig. S1: Progress of trypsin inhibition with varying sample extract volumes.

Silencing of *CM3* and *CM16 α-amylase/trypsin inhibitors (ATI)* genes in durum wheat cv Svevo by using simultaneous multiplex CRISPR-Cas9 editing

Durum wheat
cv *Svevo (AABB)*





Camerlengo F, Frittelli A, Sparks C, Doherty A, Martignago D, Larré C, Lupi R, Sestili F and Masci S (2020) CRISPR-Cas9 Multiplex Editing of the α-Amylase/Trypsin Inhibitor Genes to Reduce Allergen Proteins in Durum Wheat. *Front. Sustain. Food Syst.* 4:104. doi: 10.3389/fsufs.2020.00104

Testing TLR4-stimulating bioactivity in vitro of wheat soluble proteins extracts using a HeLa TLR4 dual luciferase reporter cell line

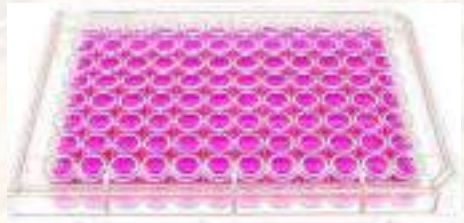
ATI can trigger TLR4 by direct interaction, and provoke innate immunity, related to NCWS

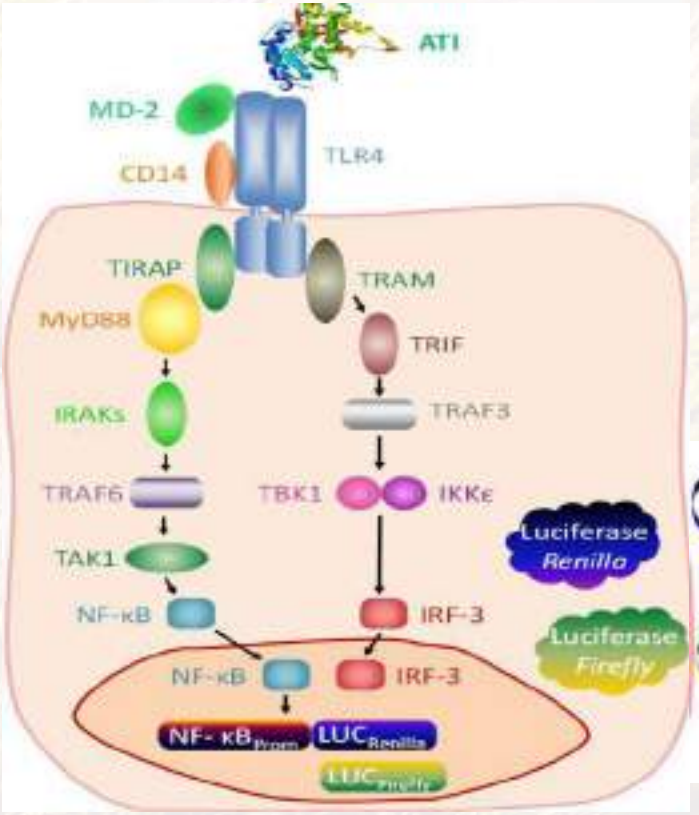
Methodology

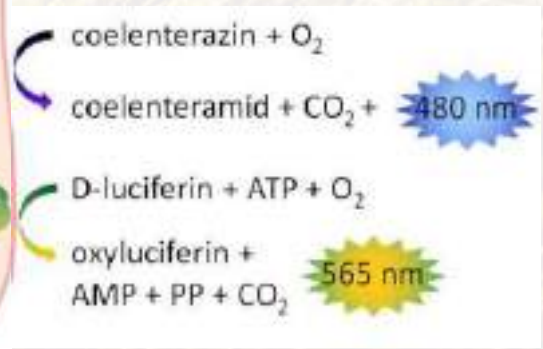
ATIs extracted from flour

7h incubation of ATIs with cells

Bioactivity test using dual reporter TLR4 transfected Hela cells



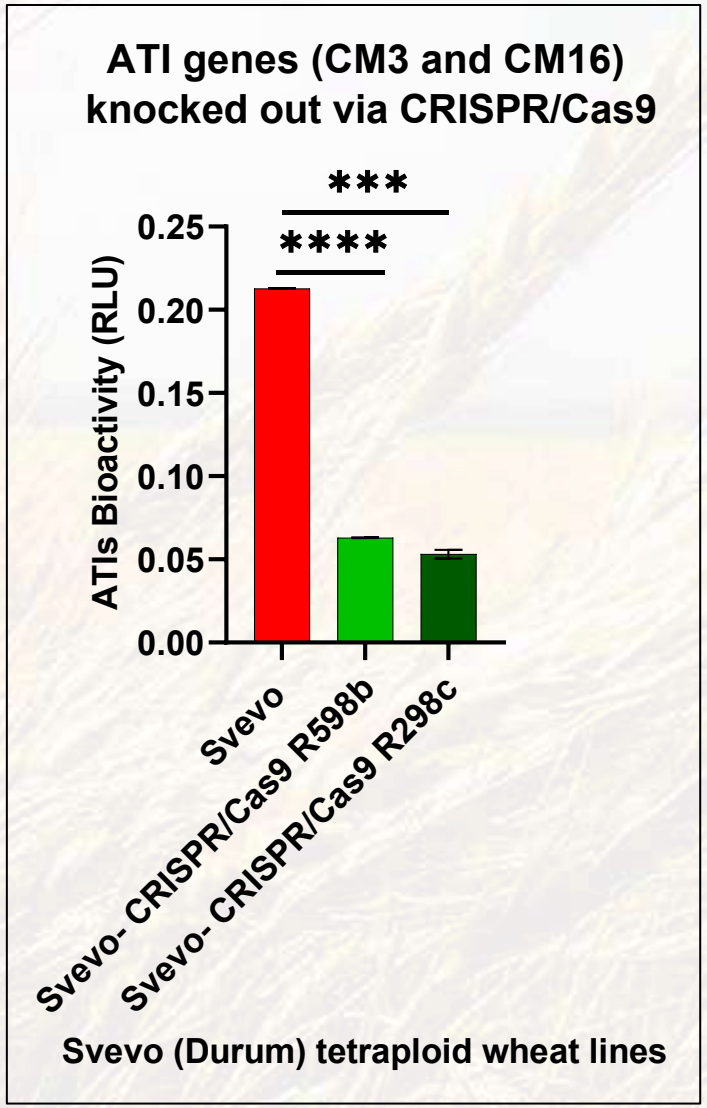


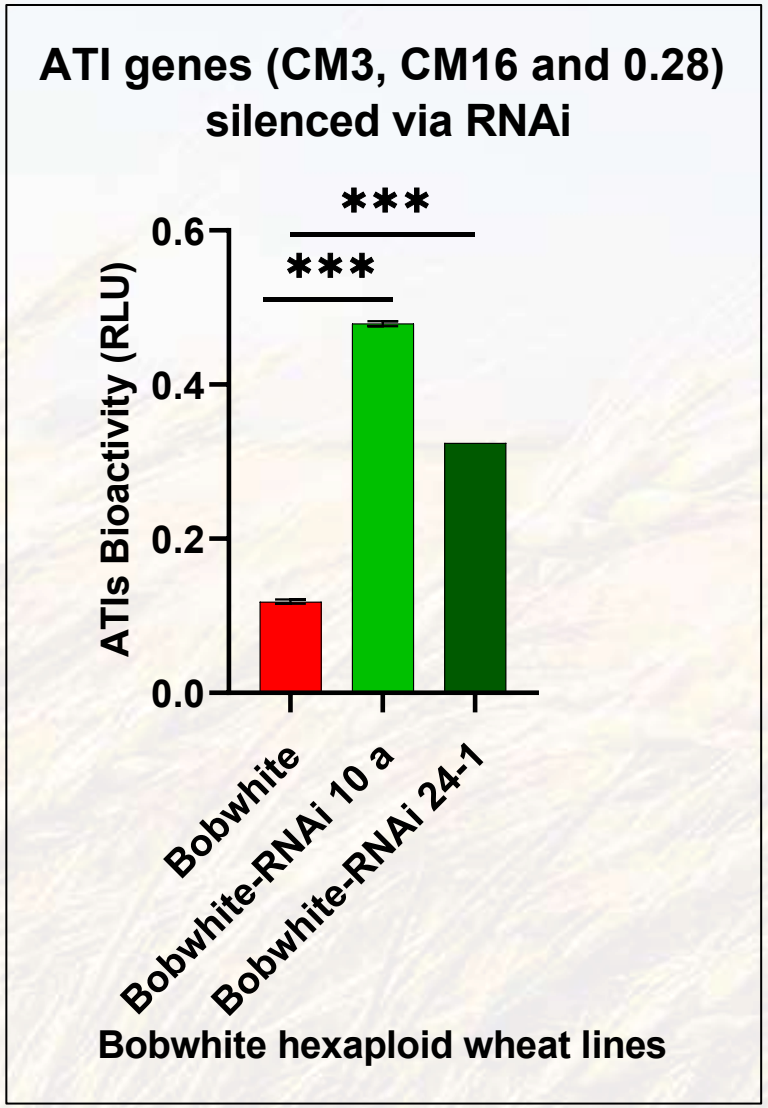


Cell lysis, **measurement**

Luciferase activities
Renilla/Firefly
(Bioactivity)

TLR4- stimulating bioactivity testing of RNAi edited and CRISPR/Cas9 knock- out wheat kernels





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Absolute and relative quantitation of amylase/trypsin-inhibitors by LC-MS/MS from wheat lines obtained by CRISPR-Cas9 and RNAi

Sabrina Geisslitz[1,2], Shahidul Islam[3], Lukas Buck[1], Clemens Grunwald-Gruber[4], Francesco Sestili[5], Francesco Camerlengo[5], Stefania Masci[5] and Stefano D'Amico[6]*

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Quantitation of wheat proteins is still a challenge, especially regarding amylase/trypsin-inhibitors (ATIs). A selection of ATIs was silenced in the common wheat cultivar Bobwhite and durum wheat cultivar Svevo by RNAi and gene editing, respectively, in order to reduce the amounts of ATIs. The controls and silenced lines were analyzed after digestion to peptides by LC-MS/MS with different approaches to evaluate changes in composition of ATIs. First, a targeted method with stable isotope dilution assay (SIDA) using labeled peptides as internal standards was applied. Additionally, four different approaches for relative quantitation were conducted, in detail, iTRAQ labeled and label free quantitation (LFQ) combined with data dependent acquisition (DDA) and data independent acquisition (DIA). Quantitation was performed manually (Skyline and MASCOT) and with different proteomics software tools (PLGS, MaxQuant, and PEAKS X Pro). To characterize the wheat proteins on protein level, complementary techniques as high-performance liquid chromatography (HPLC) and gel electrophoresis were performed. The targeted approach with SIDA was able to quantitate all ATIs, even at low levels, but an optimized extraction is necessary. The labeled iTRAQ approach

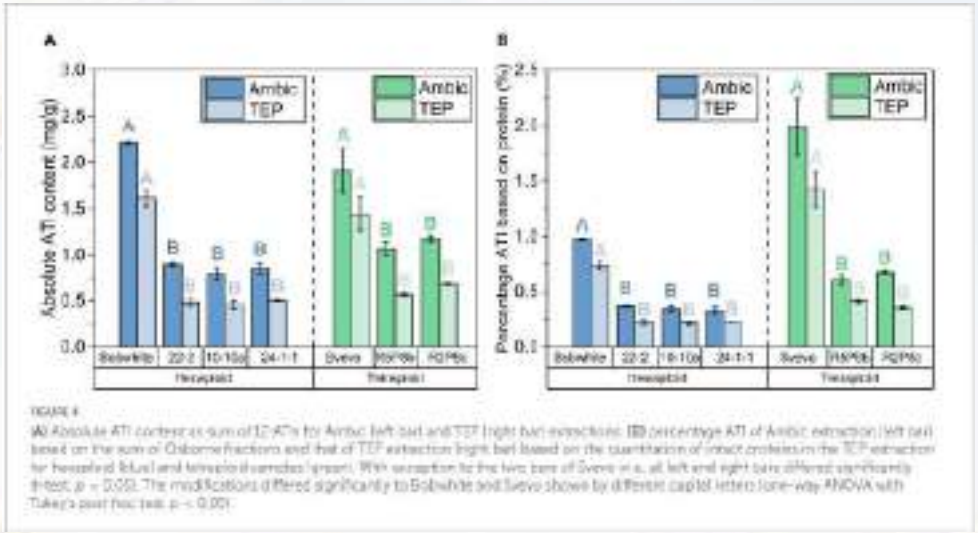
Bread wheat RNAi silenced plants: Silencing of CM3, CM16 and 0.28

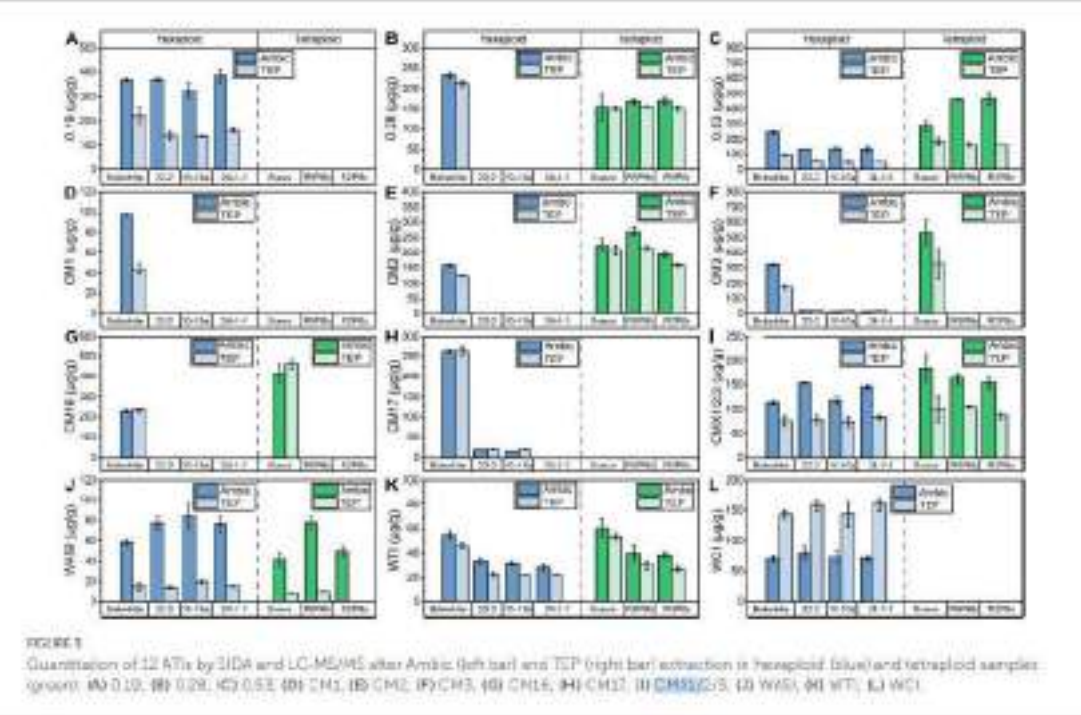
- Lead to undetectable amounts of 0.53, WTAI, CM2, CM1 and CM17
- Higher amounts of CMX1/2/3

Durum genome edited lines: Knock-out of CM3 and CM16

- The amounts of CM2 did not vary significantly, whereas that of CMX1/2/3 slightly decreased
- High decrease of bioactivity

Thus, besides targeted ATI, the observed changes in bioactivities (especially in the bread wheat RNAi silenced materials) are due to CMX1/2/3? Or have other trypsin inhibiting proteins an important role?





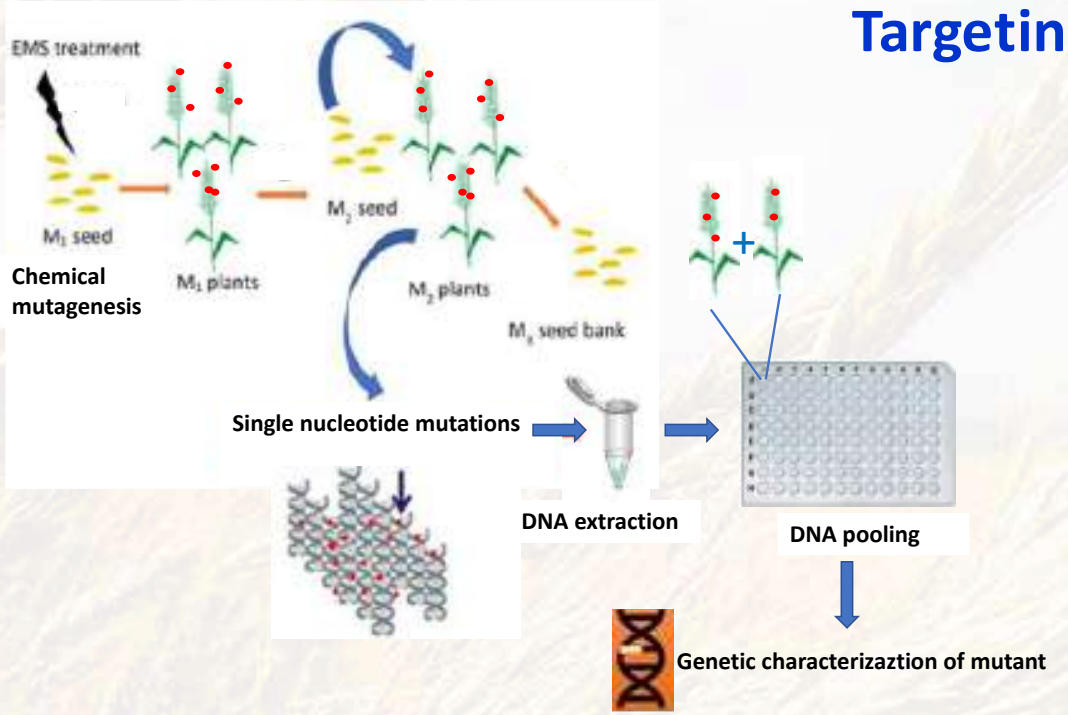
- Genome editing is the most promising tool for evaluating the role of ATI in triggering NCWS, because no major pleiotropic effects have been observed

- New lines silenced in different members of the *ATI* gene family are needed in order to understand the role of each polypeptide in triggering this pathology

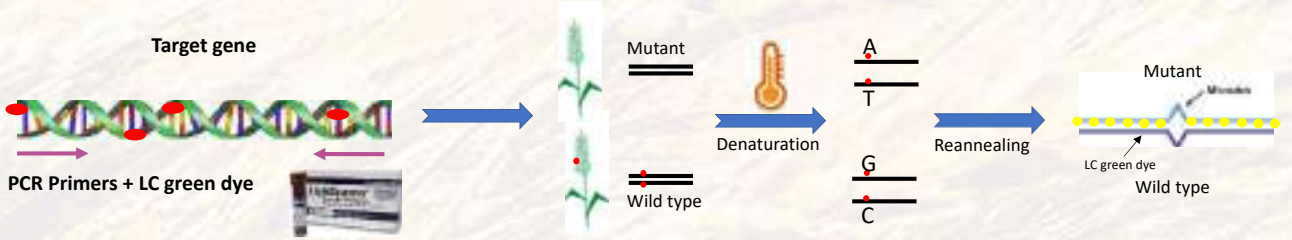
- If genome editing will be (hopefully) considered a non-GM procedure, silenced lines, produced in wheat varieties of common use, may be directed addressed to the production of foods suitable for NCWS patients

TILLING

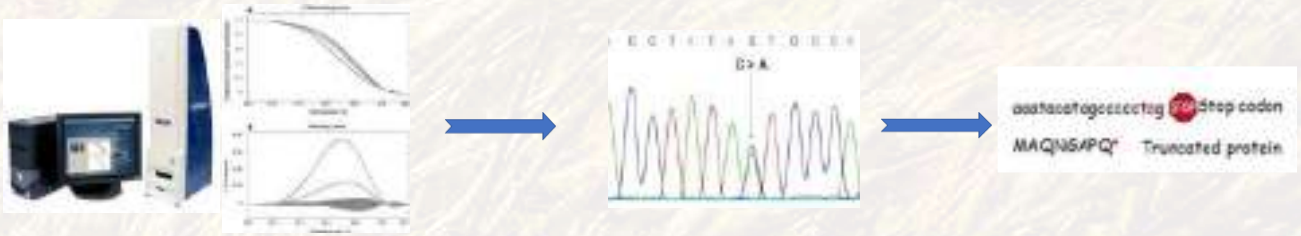
Targeting Inducing Local Lesion IN Genomes



- Reverse genetic approach to identifying chemically induced mutations in known genes
- Highly successful in isolating mutations in both common and durum wheat for several genes



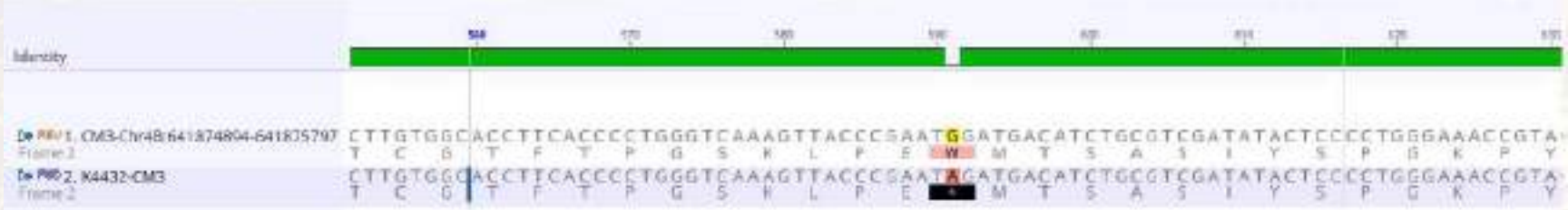
- Suitable method for inducing mutations in desired genes to target useful traits for breeding programs



K0265

TILLING → Kronos TILLING population → Kronos 0265 0.19 / Kronos 4432 CM3 → K0265 x K4432 F_1 → K0265 x K4432 F_2

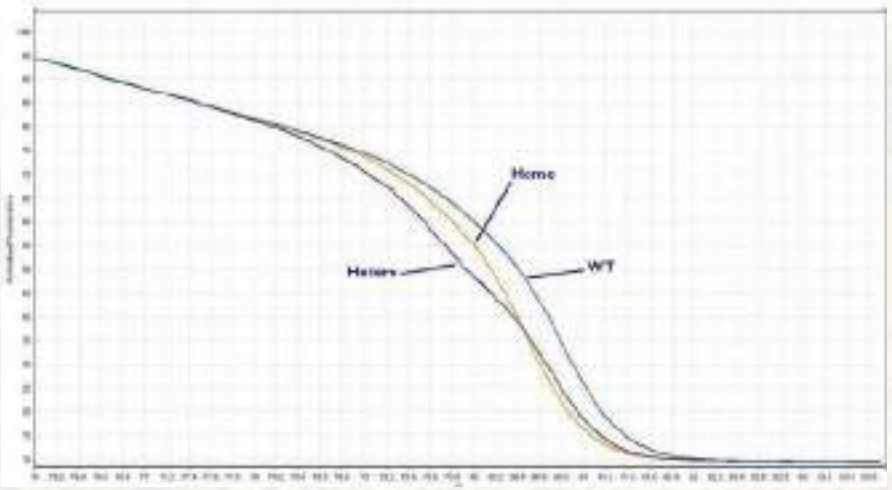
K4432



K0265 x K4432 F_1

K0265 x K4432 F_2

- 21 F1 plants verified with HRM



Dihybrid cross (gene linkage)

A and *a* represent one trait, and *B* and *b* represent a different trait that is linked to inheritance of *A* or *a*.

	AB	Ab	aB	ab
AB	AABB	AABb	AaBB	AaBb
Ab	AABb	AAbb	AaBb	Aabb
aB	AaBB	AaBb	aaBB	aaBb
ab	AaBb	Aabb	aaBb	aabb

Dominant for *A* and *B* = 9/16

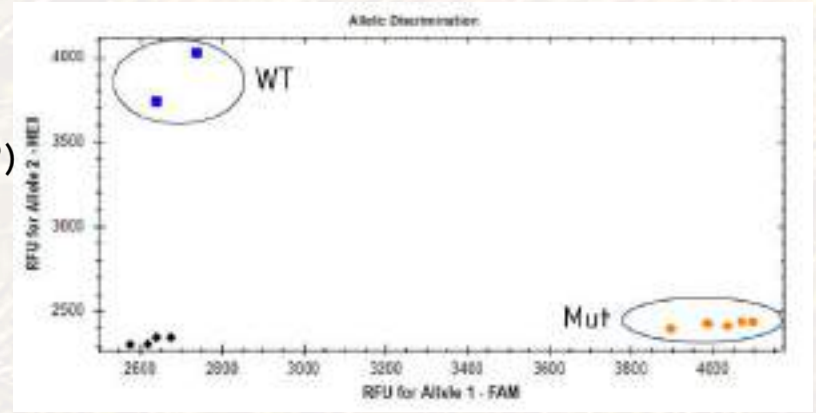
Recessive for *a*, dominant for *B* = 3/16

Dominant for *A*, recessive for *b* = 3/16

Recessive for *a*, recessive for *b* = 1/16

- ~100 seeds to obtain ~5 double mutants to use per phenotypization

Kompetitive Allele Specific PCR (KASP)



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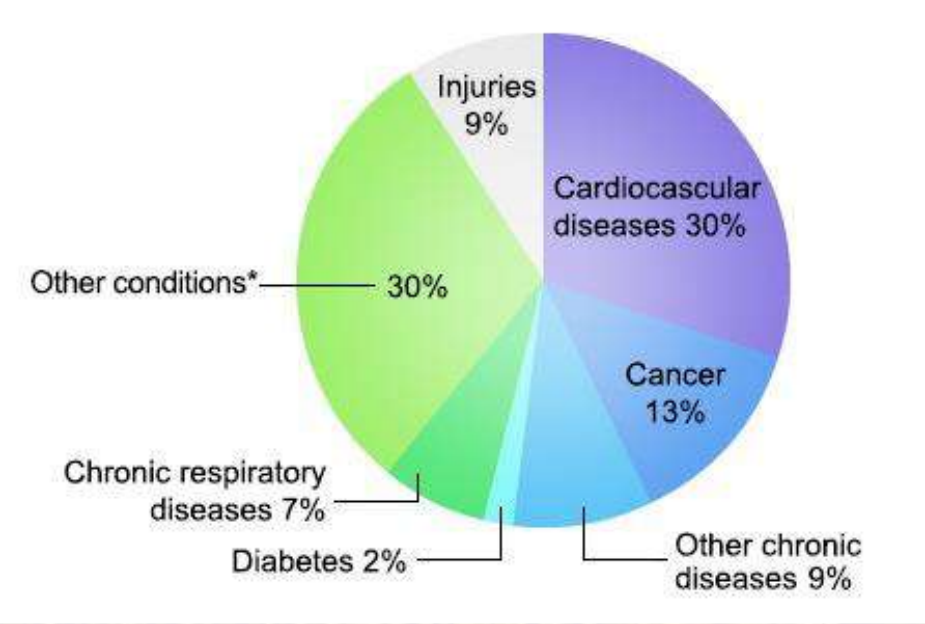
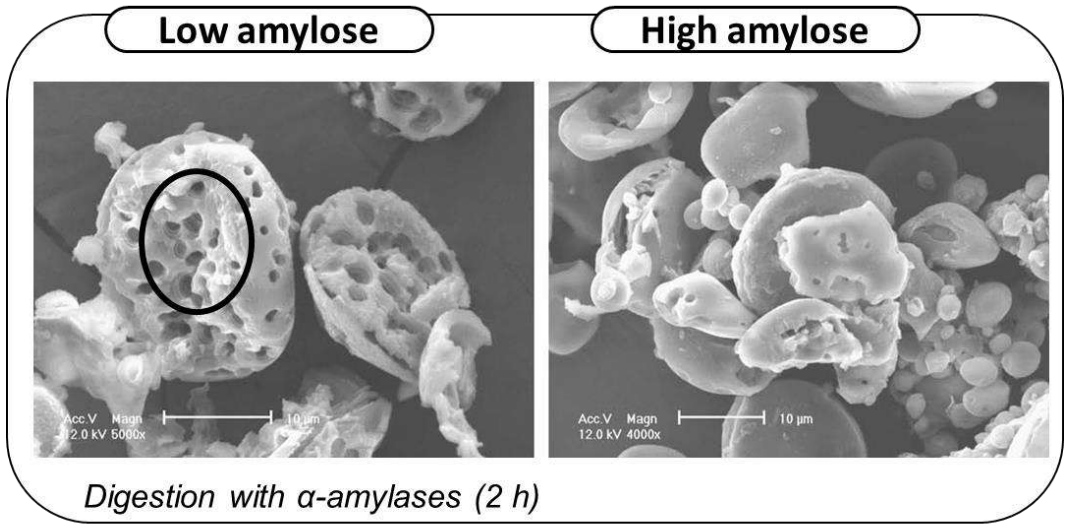
Presentation outline:

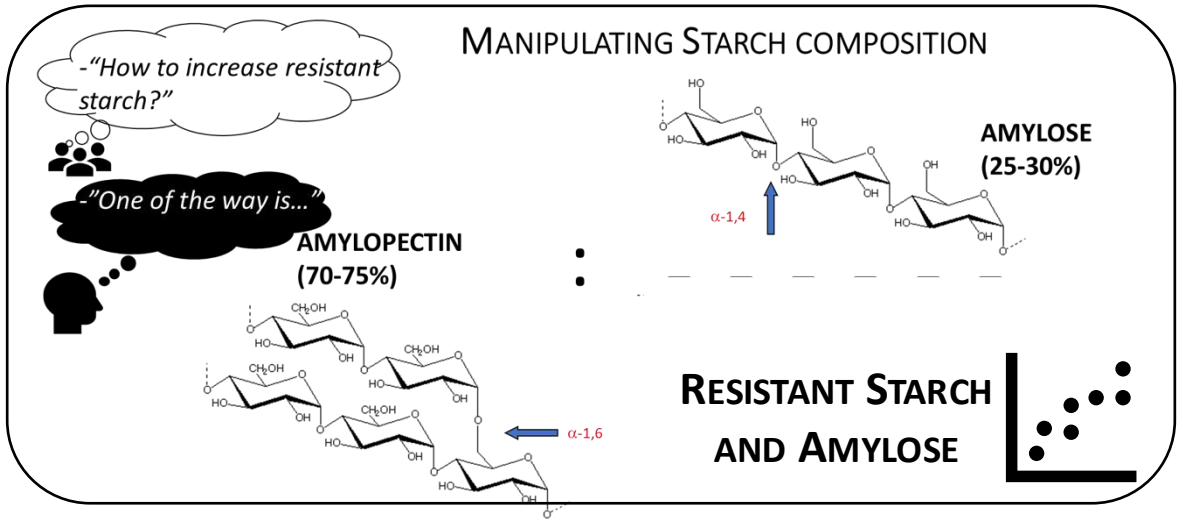
2-Production of high amylose (high resistant starch) wheats

NONCOMMUNICABLE DISEASES

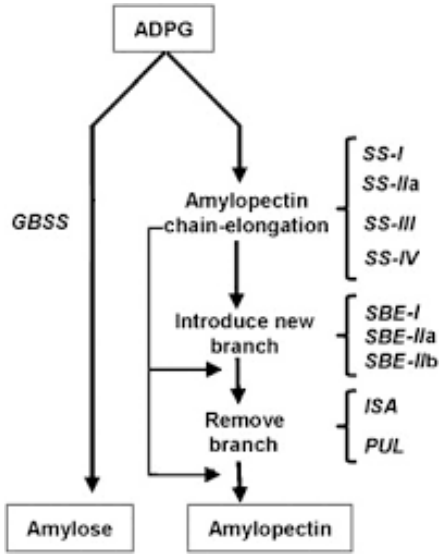


- Noncommunicable diseases (NCDs) kill 41 million people each year, equivalent to 74% of all deaths globally
- Diabetes and cardiovascular diseases are mostly affected by food



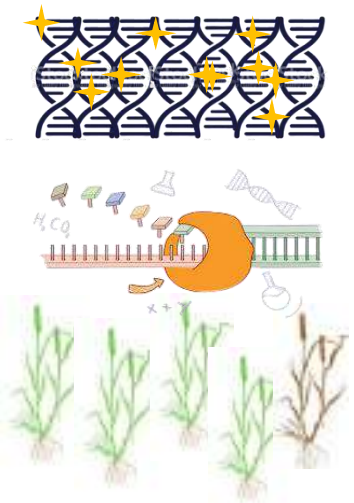


Starch Biosynthesis



SBEIIa

TILLING In Wheat



High Amylose Wheat

Svevo	≈ 27%	↑ **Amylose**
*SBEIIa**	≈ 52%	
Svevo	≈ 0.8%	↑ **RS**
*SBEIIa**	≈ 6.5%	

Svevo pasta

Svevo HA Pasta

Wholemeal pasta

foods

MDPI

Article

Can Manipulation of Durum Wheat Amylose Content Reduce the Glycaemic Index of Spaghetti?

Mike Sissons [1,*], Francesco Sestili [2], Ermelinda Botticella [2], Stefania Masci [2] and Domenico Lafiandra [2,*]

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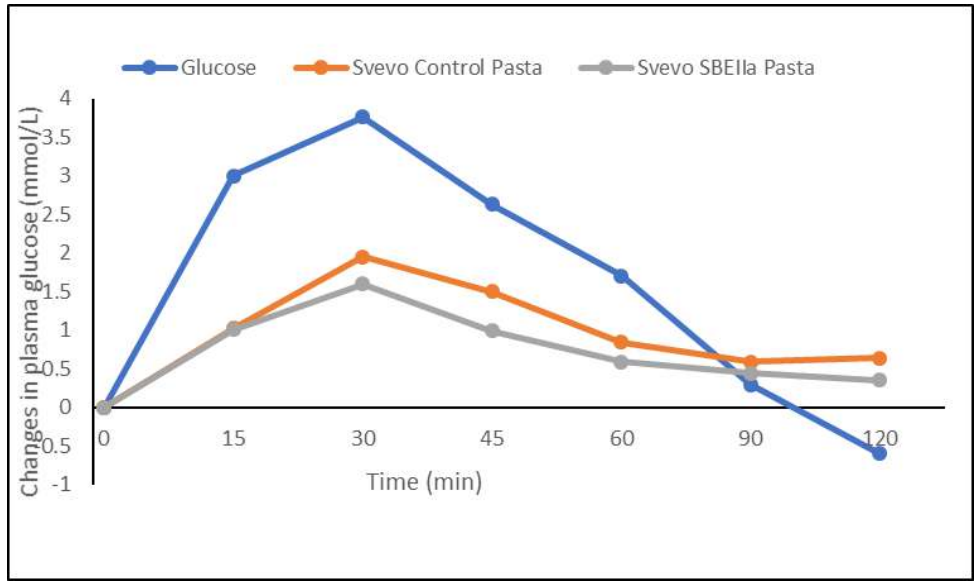

Test food	Portion size (g)	Available carbohydrate in test portion (g)
Glucose (ref)	51.4	50.0
Svevo Pasta	67.5	50.0
Svevo HA Pasta	76.6	50.0

50 g available carbohydrate (n=10 subjects)
Conducted by SUGiRS, Univ. SYD

Glycemic Index of pasta

Test food (amylose %)	GI	Glycemic load
Svevo (25%)	48±4a	18
Svevo HA (55%)	38±3b	12
Glucose	100±0c	

Reaching the same target with a biotech (RNAi) approach: silencing of *SbeIIa* gene

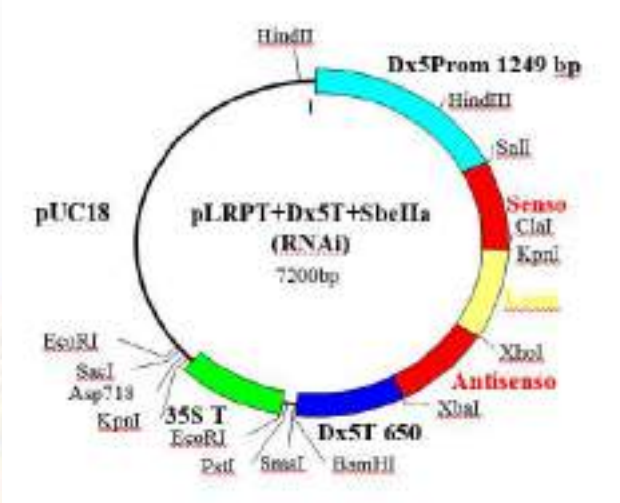

Table 2: Seed weight, total starch, amylose and protein content of RNAi transgenic lines and control plants.

Lines	100 grain weight	Starch content %	Amylose content %	Protein content%
Svevo	4.14 ± 0.14	59.80 ± 0.10	24.50 ± 0.70	18.36 ± 0.13
MJ16-112	3.90 ± 0.12	50.98 ± 0.68	75.05 ± 0.36	18.95 ± 0.36
MJ16-114	3.74 ± 0.10	44.91 ± 2.36	43.50 ± 2.52	16.16 ± 0.30
MJ16-119	4.06 ± 0.22	45.17 ± 1.47	32.40 ± 0.39	17.38 ± 0.16

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Francesco SESTILI

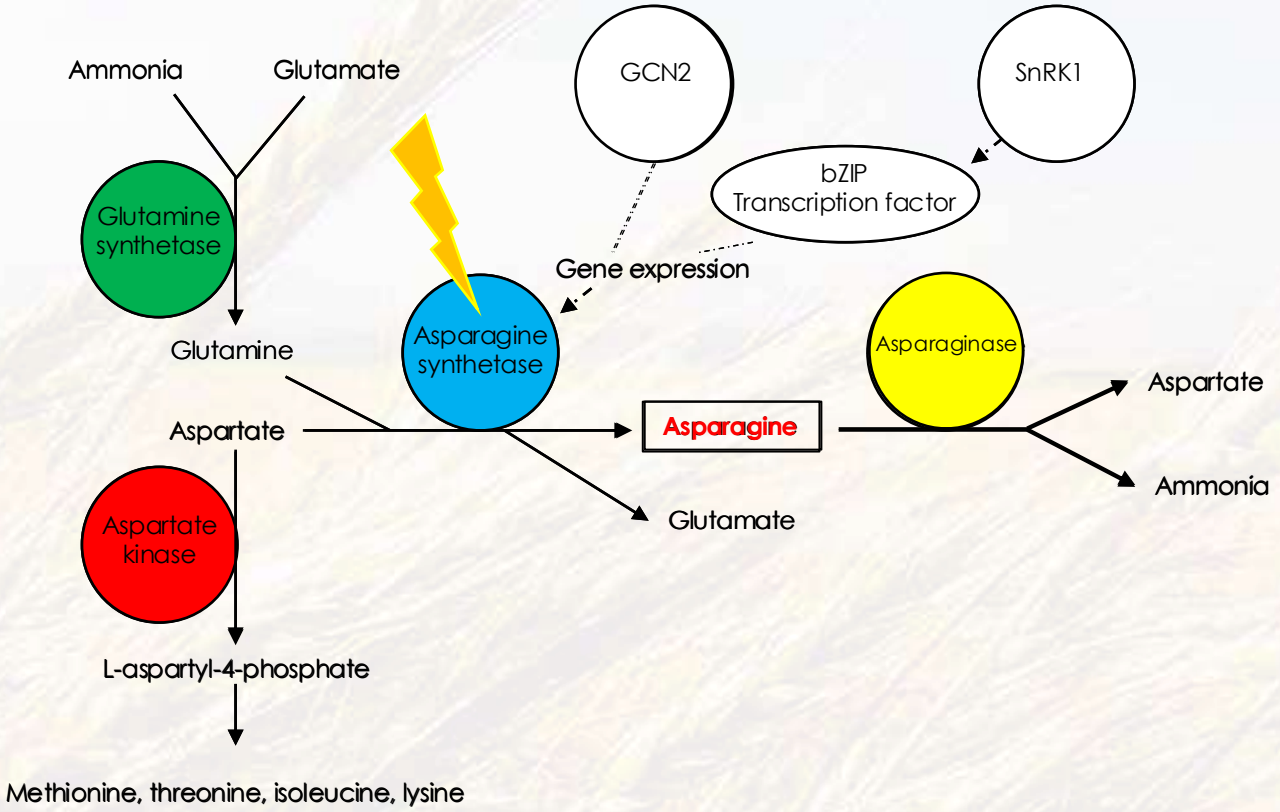
NSW Department of Primary Industries (Australia)
Mike SISSONS

Grandi Molini Italiani (Italy)
Mirko VOLPATO

Presentation outline:

3-Development of lines potentially forming low-acrylamide in final products

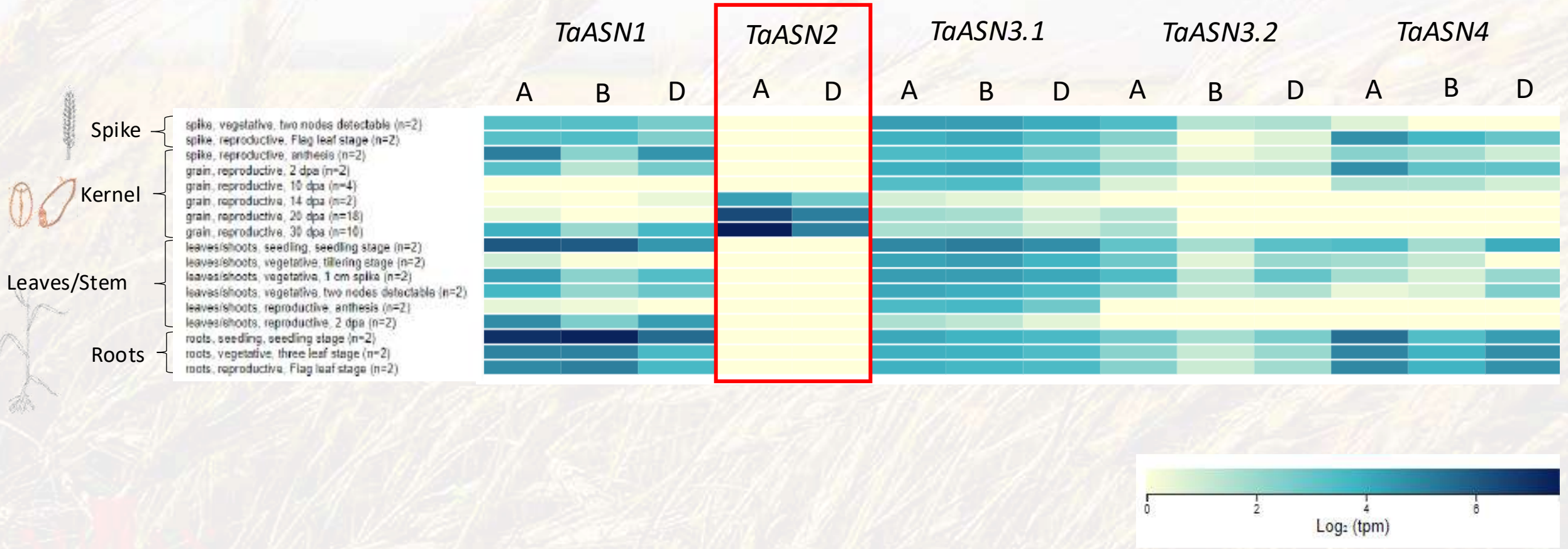
Reduction of fAsn by silencing *TaASN2* genes



- Development of a low-fAsn bread wheat by TILLING coupled with KASP analysis.

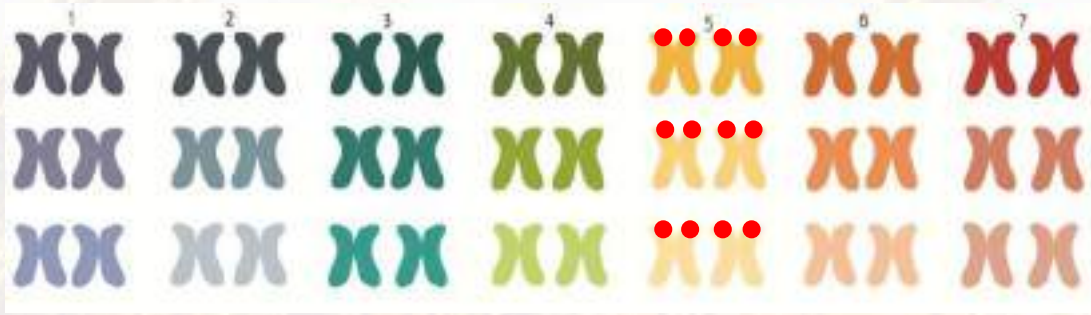
- Five genes encoding asparagine synthetase have been identified in bread wheat

- *TaASN2* gene is expressed specifically in the kernel, starting from embryo development (> 10 days post anthesis). Its expression levels exceed those of other *TaASN* genes in any other tissue from the intermediate stage of kernel development (14-20 dpa).



Bread Wheat Genome (AABBDD)

3 subgenomes and 3 homeoalleles of each gene:
TaASN-A2, TaASN-B2 and *TaASN-D2*

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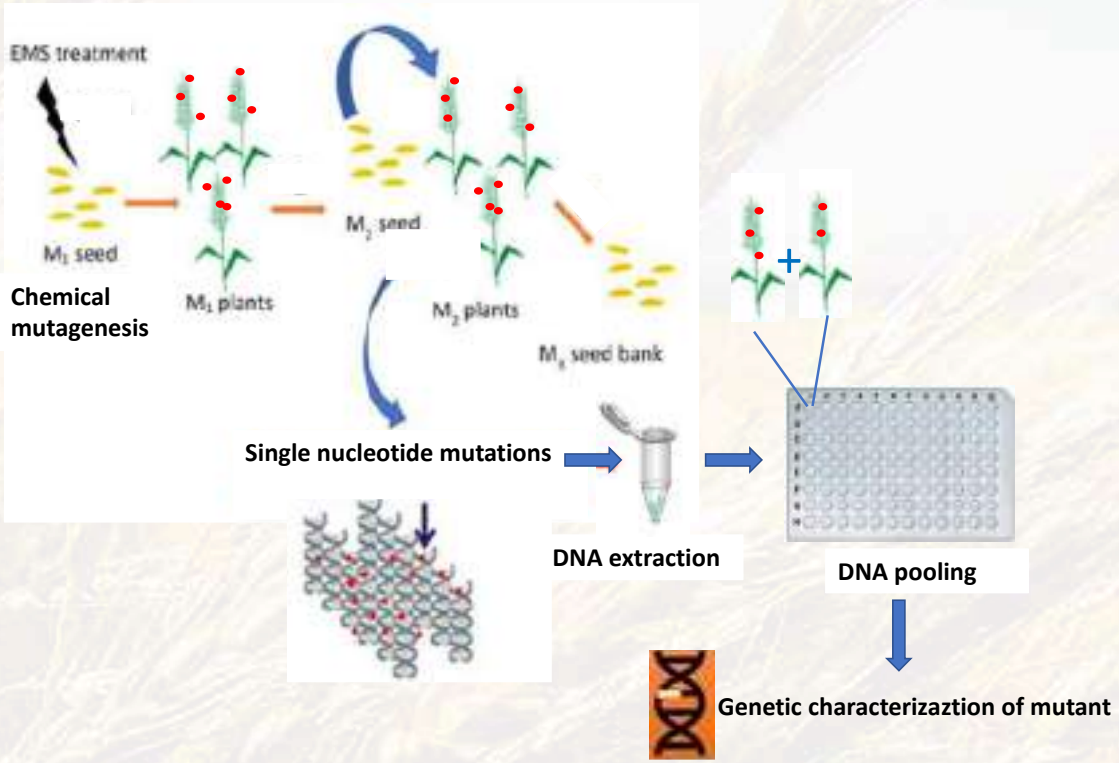
Reduced free asparagine in wheat grain resulting from a natural deletion of *TaASN-B2*: investigating and exploiting diversity in the asparagine synthetase gene family to improve wheat quality

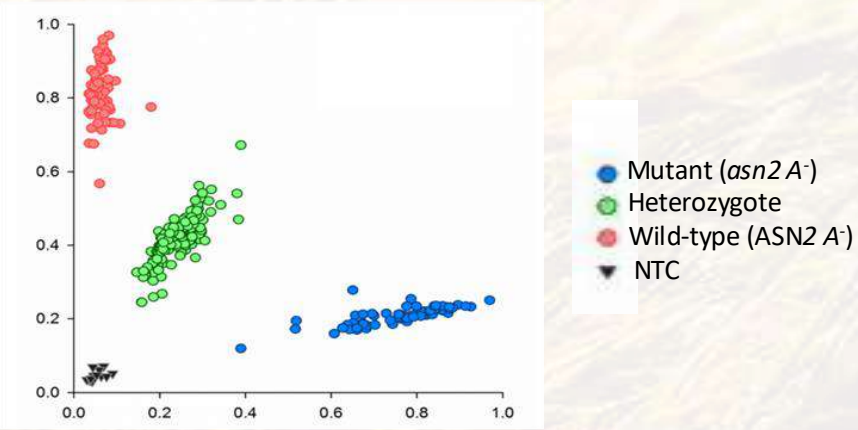
Protein	IWGSC RefSeq v1.1 ID
TaASN-A1	*TraesCS5A02G153900*
TaASN-B1	*TraesCS5B02G152600*
TaASN-D1	*TraesCS5D02G159100*
TaASN-A2	*TraesCS3A02G077100*
TaASN-B2	Not present
TaASN-D2	*TraesCS3D02G077300*
TaASN-A3.1	*TraesCS1A02G382800*
TaASN-B3.1	*TraesCS1B02G408200*
TaASN-D3.1	*TraesCS1D02G390500*
TaASN-A3.2	*TraesCS1A02G422100*
TaASN-B3.2	*TraesCS1B02G453600*
TaASN-D3.2	*TraesCS1D02G430300*
TaASN-A4	*TraesCS4A02G109900*
TaASN-B4	*TraesCS4B02G194400*
TaASN-D4	*TraesCS4D02G195100*

- Development of a low-fAsn bread wheat variety by TILLING approach coupled with KASP (Kompetitive Allele Specific PCR) analysis.

TILLING



KASP



1

TaASN-A2^{-}

X

TaASN-D2^{-}

Screening KASP genotyping

TaASN-A2^{-}D2^{-}

14 Double Mutant Lines

2

TaASN-A2^{-}D2^{-} x *TaASN*-B2^{-}

Strength Wheat

1) Rebelde (3 lines)

Bread-making

2) Sy Cicerone (4 lines)

Biscuit Maker

3) Canaletto (15 lines)

Participants to this research

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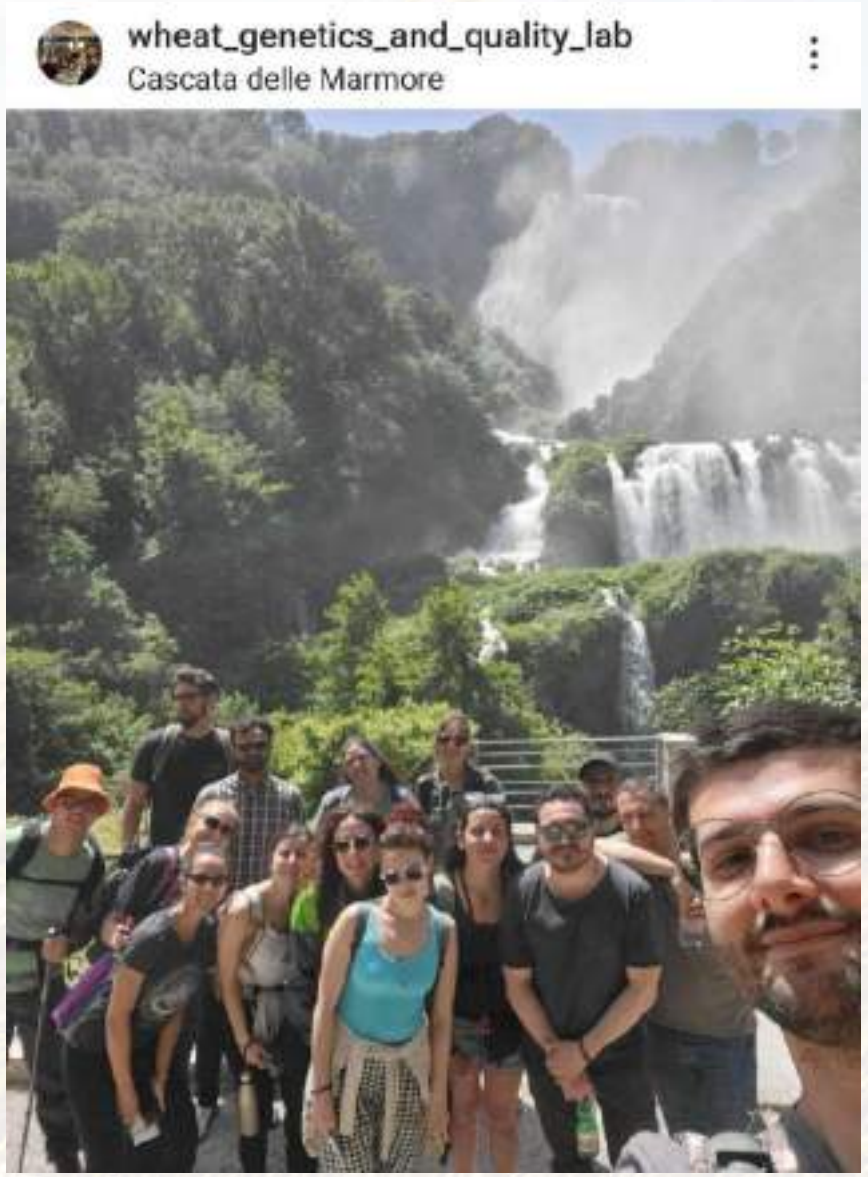
Francesco SESTILI


wheat_genetics_and_quality_lab
Cascata delle Marmore