CRISPR/Cas9 editing to produce low asparagine, low acrylamide wheat: out of the lab and into the field

Nigel G. Halford
Rothamsted Research

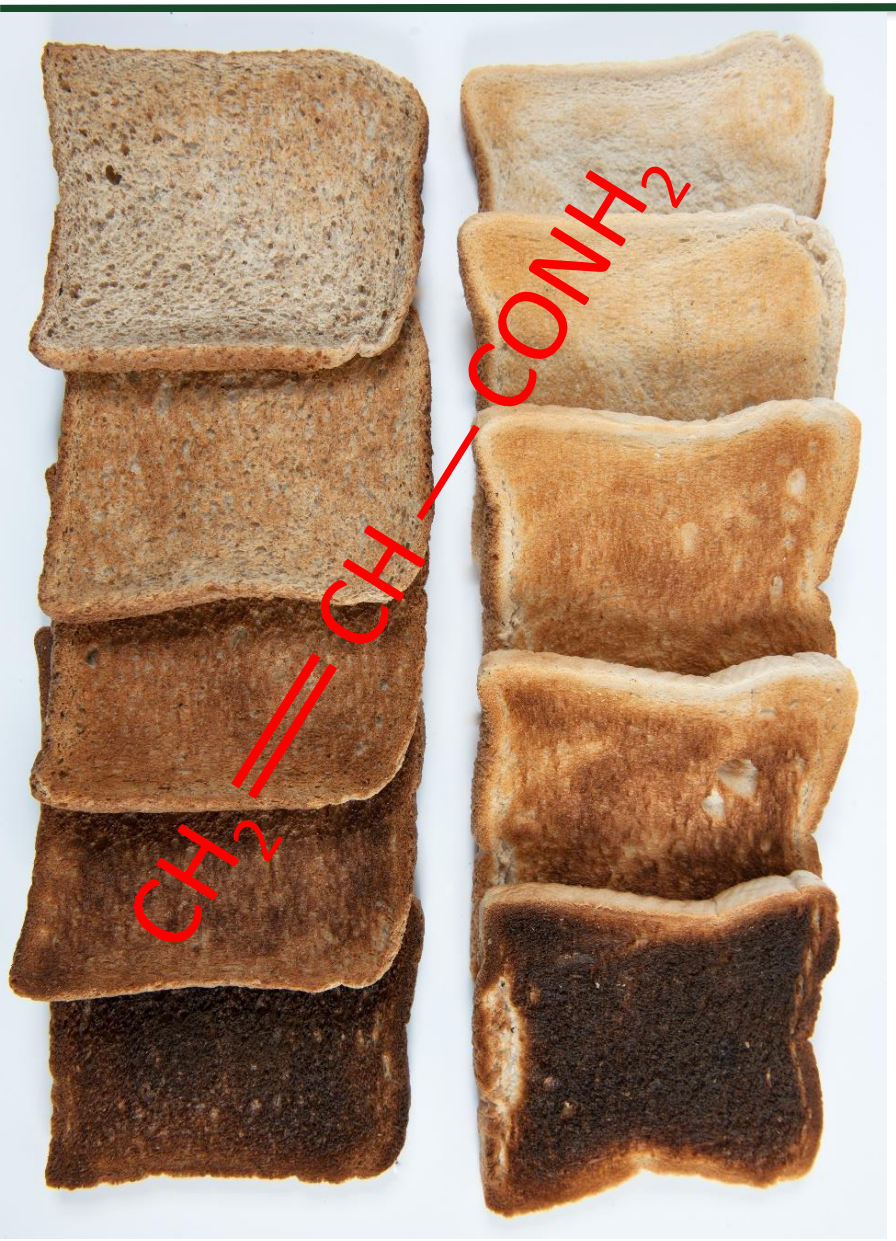
9th Plant Genomics & Gene Editing Congress Europe
April 11th – 12th, 2022

ROTHAMSTED RESEARCH

UKRI Biotechnology and Biological Sciences Research Council

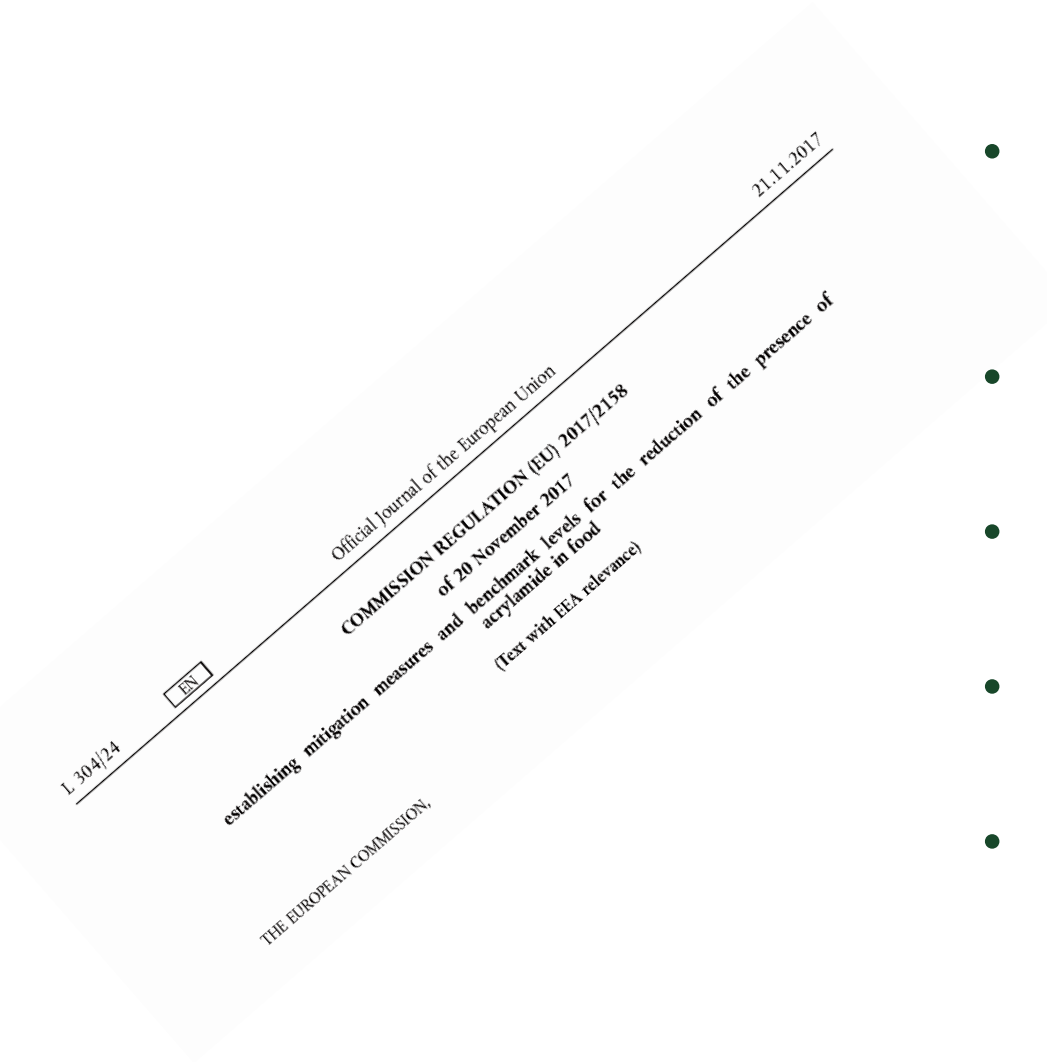
Lawes Agricultural Trust

Acrylamide



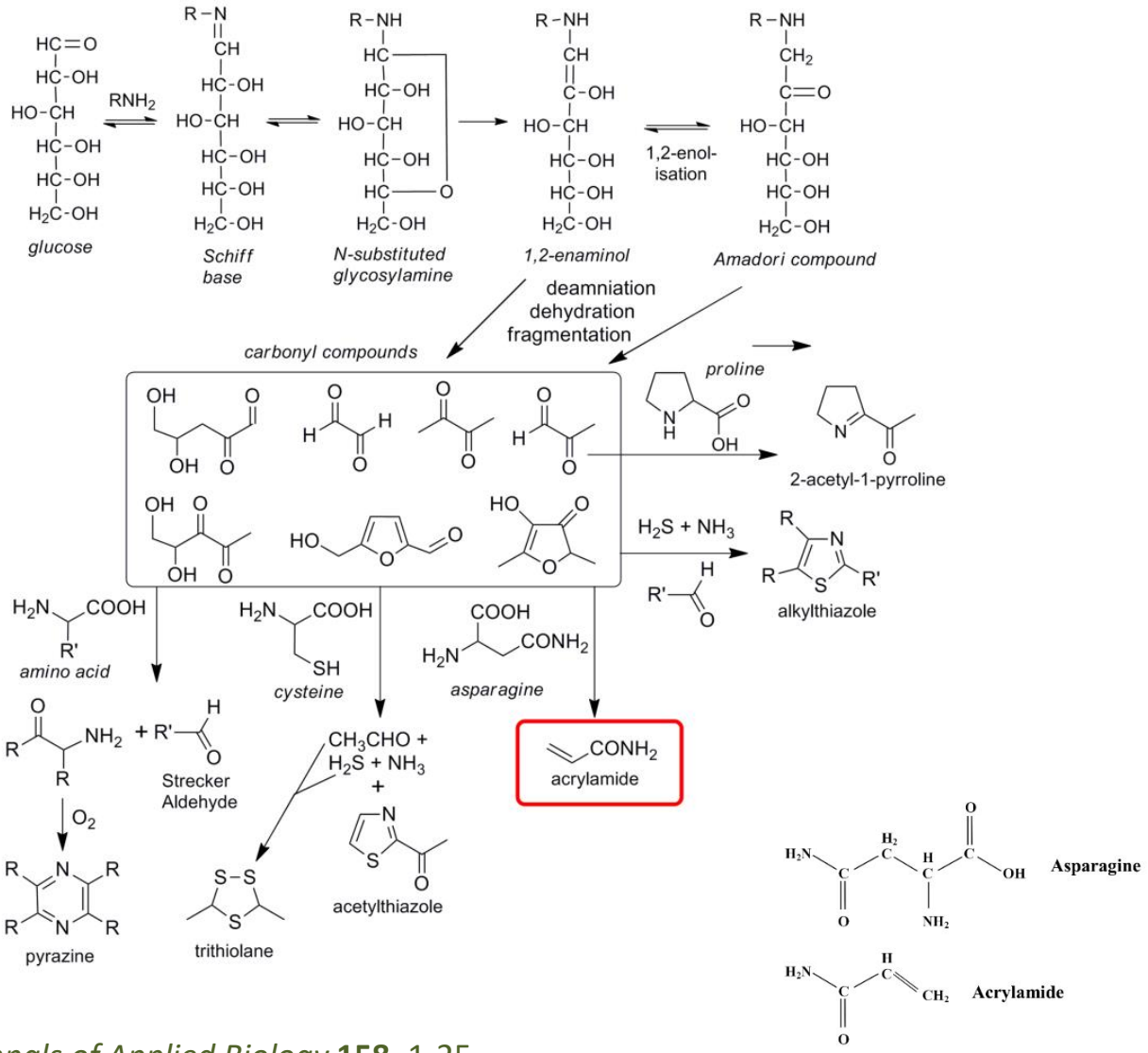
- The processing contaminant, acrylamide, was unexpectedly found in cooked foods, mainly those derived from plants, in 2002.
- Acrylamide causes cancer in rodents and is regarded as a probable (Group 2a) carcinogen in humans. It also affects development, and at high doses the nervous system and fertility.
- Fried, baked and roasted potato and cereal products and coffee are the major contributors to dietary intake in Europe.
- Bread (especially toasted), breakfast cereals, biscuits, cereal snack products, cakes, pies, batter, as well as chips (French fries), crisps, roast potatoes, and all types of coffee are all affected.

Commission Regulation (EU) 2017/2158: New risk management measures for acrylamide in Europe



- All food businesses to monitor the levels of acrylamide in their products and to keep a record of the mitigation measures they apply
- Compulsory mitigation measures (Codes of Practice), many of which reflect the FoodDrinkEurope Acrylamide Toolbox
- Benchmark Levels set for different food products, replacing Indicative Values.
- Mitigation measures to be reviewed if the levels of acrylamide in a product are not below the Benchmark Level.
- Threat of Maximum Levels with regulatory enforcement for sectors not showing sufficient progress: the European Commission has already started this process.

Acrylamide forms in the Maillard reaction



Free amino acids react with reducing sugars (glucose, fructose and maltose) at high temperature in the Maillard reaction to produce a plethora of flavour, aroma and colour compounds.

Acrylamide is formed when free asparagine participates in the final stages of the reaction.

This is the predominant route for the generation of acrylamide during cooking.

Free (soluble, non-protein) asparagine and reducing sugars can therefore be regarded as precursors of acrylamide, but other free amino acids can play a part, positively and negatively.

$$CH_2 = CH - CONH_2$$

Annals of Applied Biology **158,** 1-25
Journal of Experimental Botany **63**, 2841–2851

Free (non-protein) asparagine concentration determines acrylamide formation in heated flour produced from wheat and rye grain

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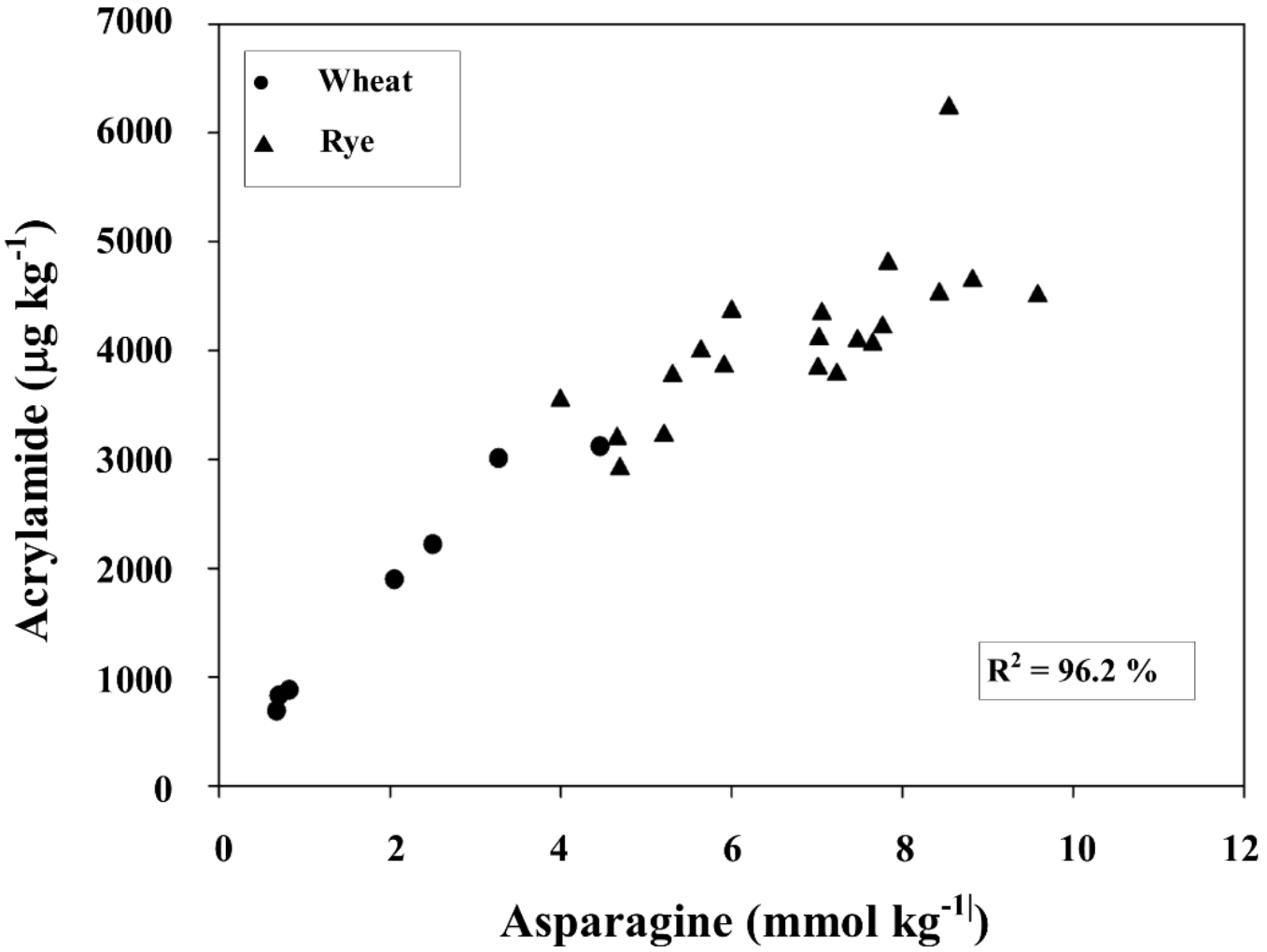

Acrylamide formation (µg/kg) plotted against free asparagine concentration (mmol/kg) in wheat and rye flour heated at 180 ˚C.

A consideration of free asparagine concentration in the raw material forms part of the cereal products Code of Practice.

Journal of Cereal Science **59**, 382-392.

Varietal differences in wheat

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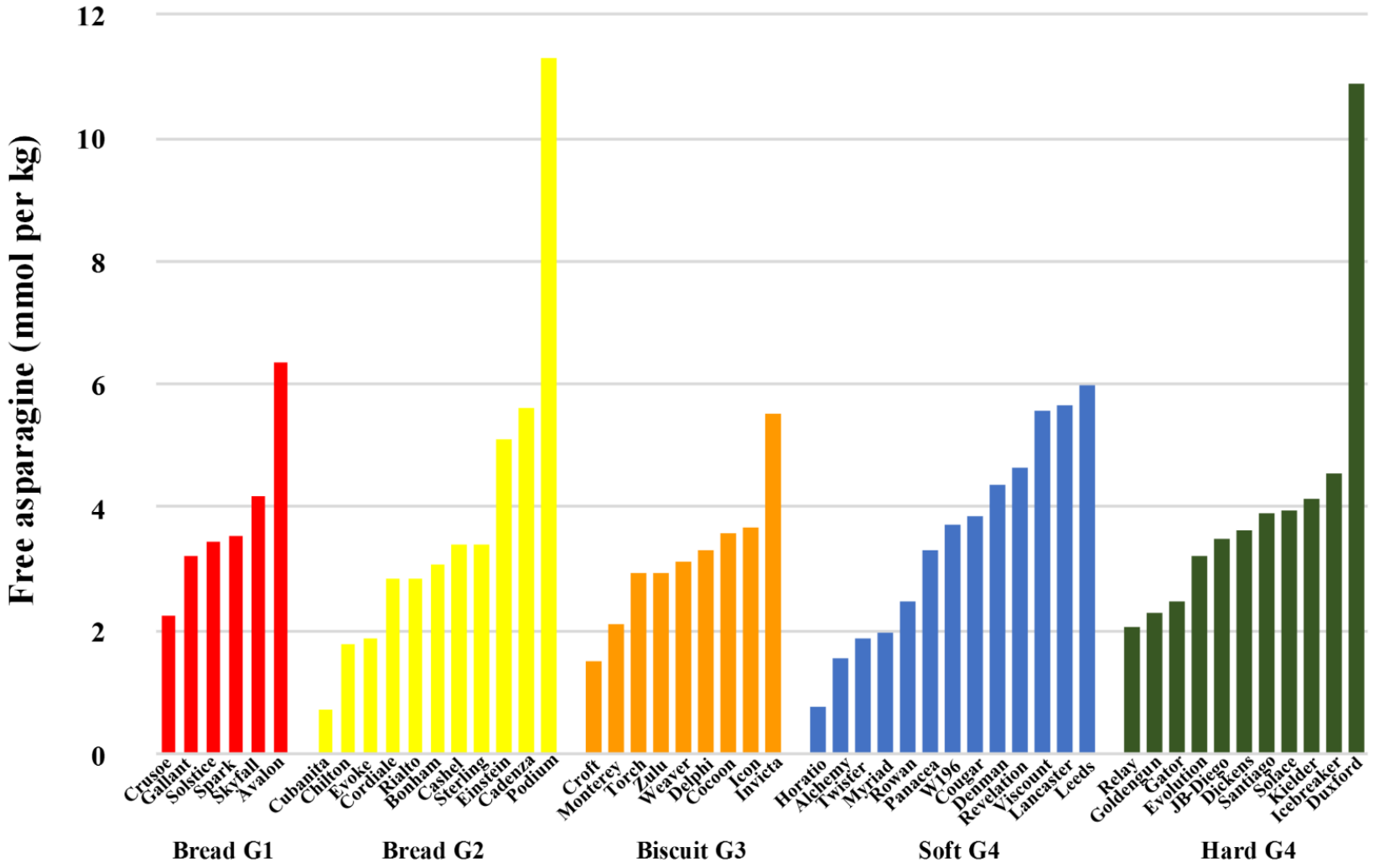
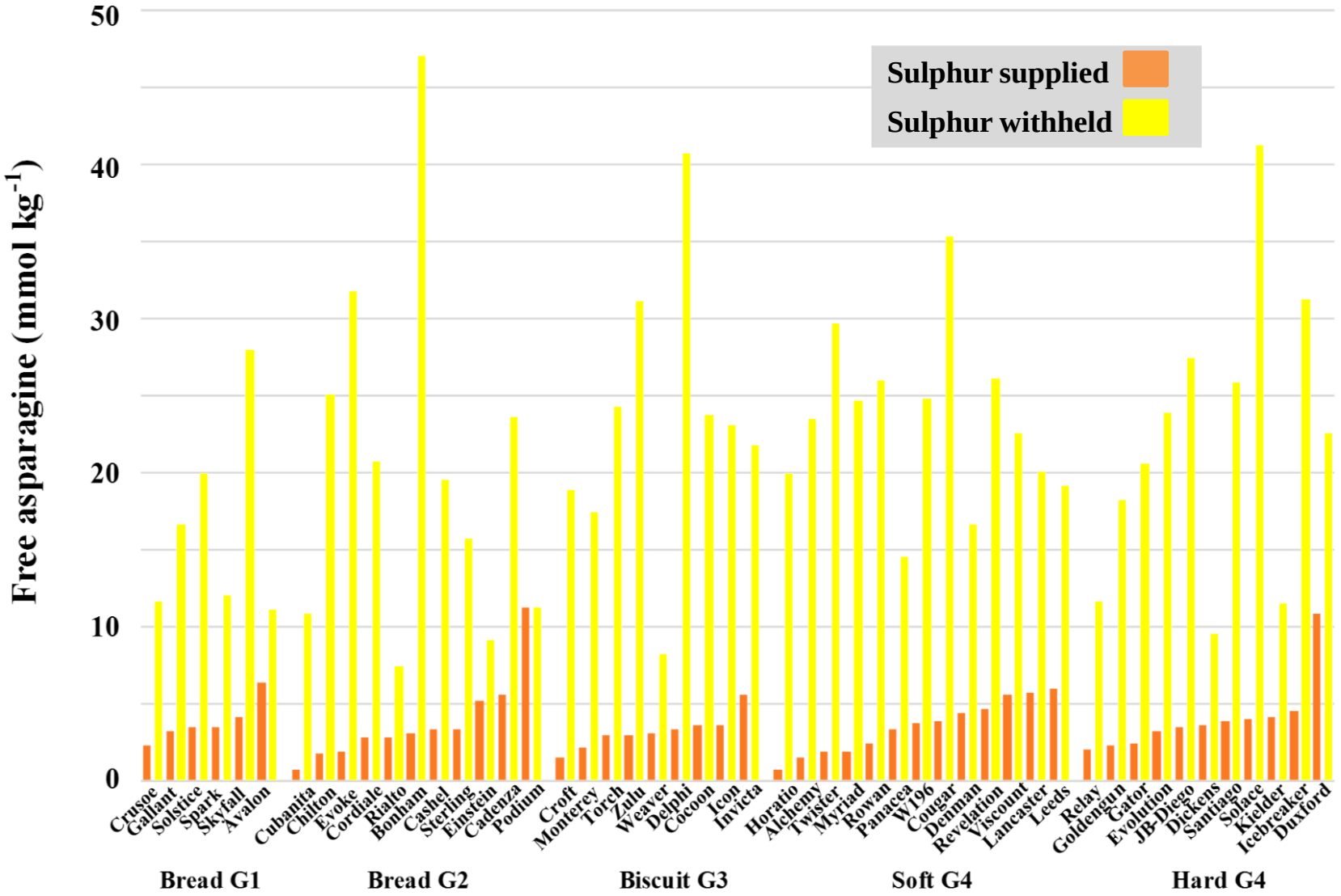


Free asparagine concentration in grain of winter wheat varieties from the UK's AHDB Recommended List grown in a field trial in the UK

Food Chemistry **239**, 304-313

Crop management: the importance of supplying wheat with sufficient sulphur



Mean free asparagine concentration in the grain of 50 varieties of winter wheat grown in a field trial in 2012-2013. Data are shown from split-plots in which wheat in half the plot was supplied with nitrogen and sulphur (brown bars), while the wheat in the other half was supplied with nitrogen but not sulphur (yellow bars).

Ensuring sulphur sufficiency is also included in the European Commission's compulsory Code of Practice for cereals.

Food Chemistry **239**, 304-313

Field trials aimed at refining our advice on N and S application and other minerals

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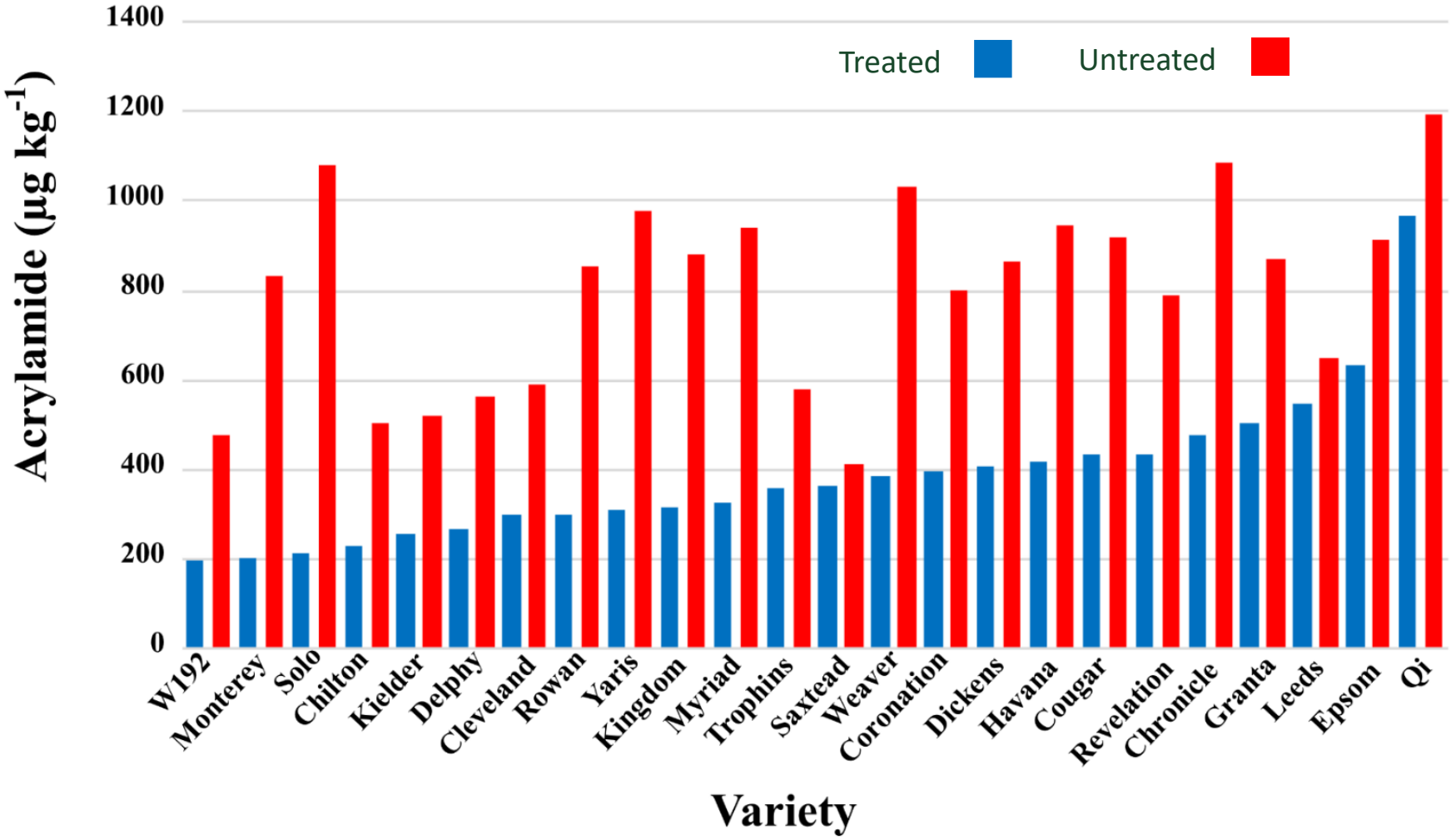
- Woburn UK, 2019-2022
- Different ratios of N and S
- Also looking at phosphate and potassium

See Joe Oddy's poster.

Crop management: Acrylamide formation in flour from wheat grown with or without fungicide treatment

ROTHAMSTED RESEARCH



Untreated plots showed visible infection by *Septoria tritici*, Yellow rust and Brown rust.

To follow 'good phytosanitary practices to prevent fungal infection' has been included in the European Commission's compulsory Code of Practice for wheat products

J. Agric. Food Chem. **64**, 9689-9696

Aims

ROTHAMSTED RESEARCH

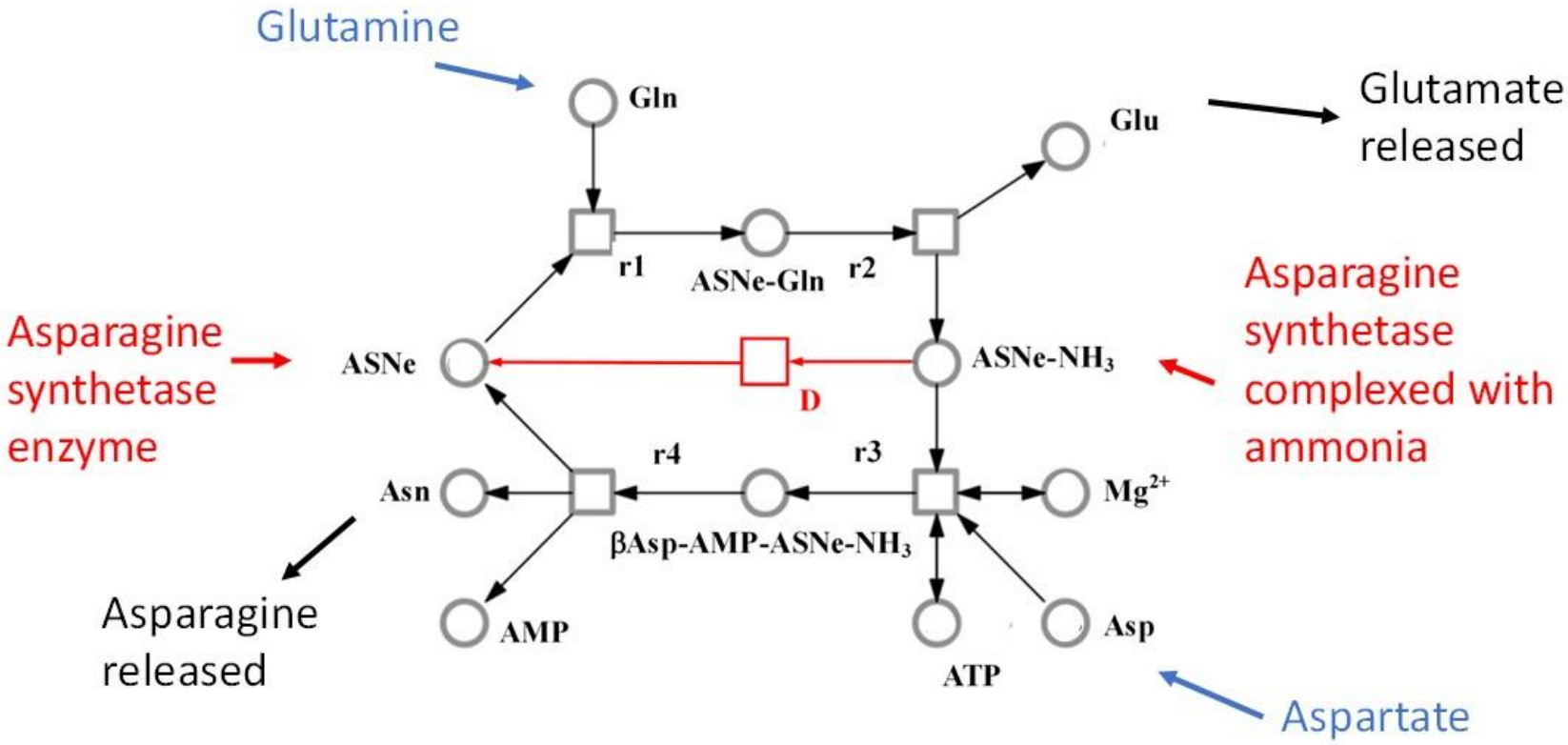
- To achieve a step reduction in free asparagine concentration in wheat grain without affecting other quality traits, yield, disease resistance etc.

- Longer term:

To uncouple asparagine concentration from the effects of nutrition (N and S) and pathogens.

To re-engineer other aspects of amino acid metabolism in wheat grain for biofortification, using low asparagine wheat as a starting point.

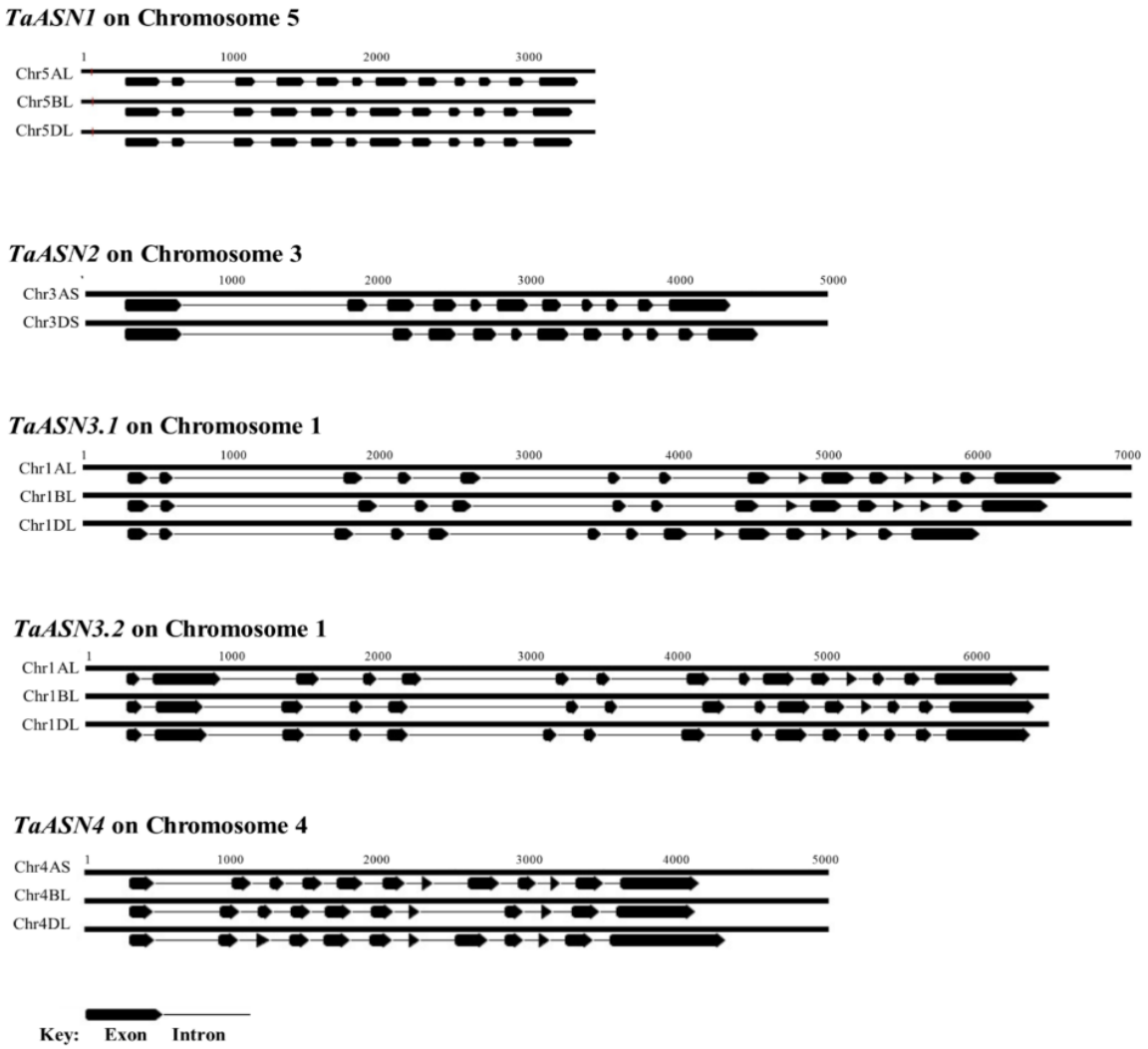
Genetic targets: asparagine synthetase



Asparagine synthetase catalyses the ATP-dependent transfer of an amino group from glutamine to aspartate to produce glutamate and asparagine.

Frontiers in Plant Science **8**, 2237

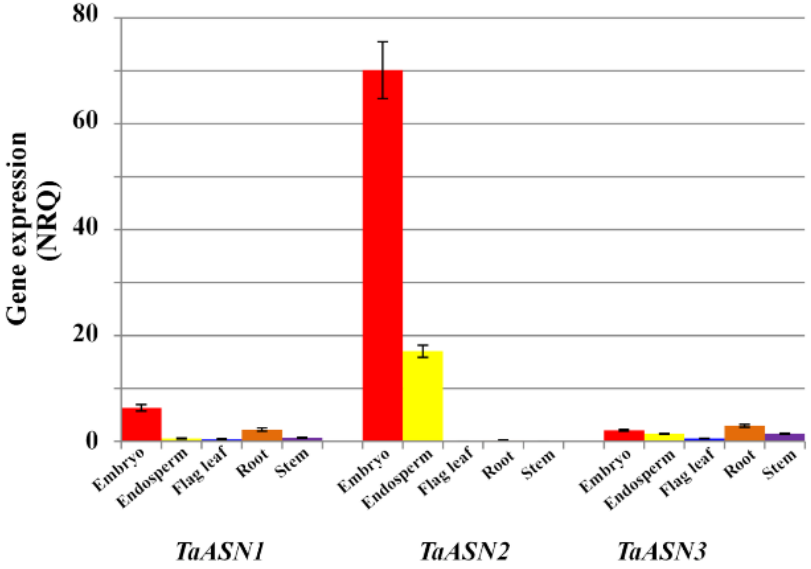
Genetic targets: Wheat asparagine synthetase genes



- Five genes per genome: *TaASN1*, *TaASN2*, *TaASN3.1*, *TaASN3.2* and *TaASN4.*
- Each gene is present as a single copy.
- Some varieties lack a *TaASN2* gene on Chromosome 3B.
- This gene family structure is unique to the Triticeae.

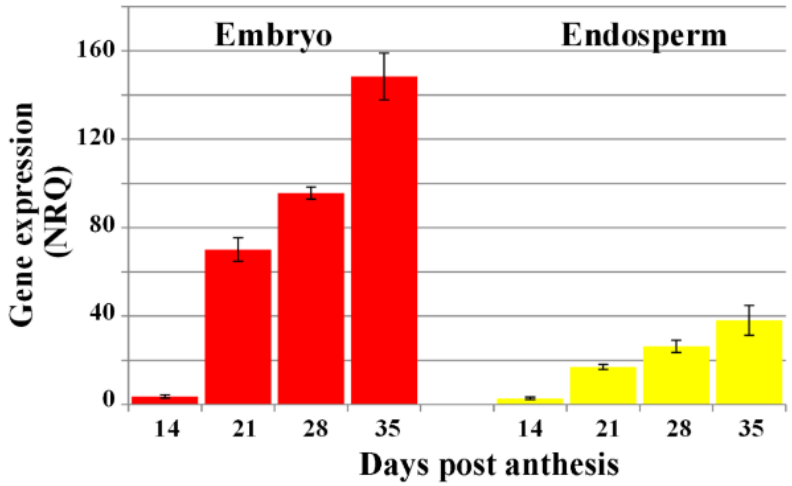
Frontiers in Plant Science **8**, 2237

Differential expression of asparagine synthetase genes in different tissues of wheat



Expression (NRQ means and standard errors) of *TaASN1*, *TaASN2* and TaASN3 in different tissues of wheat.

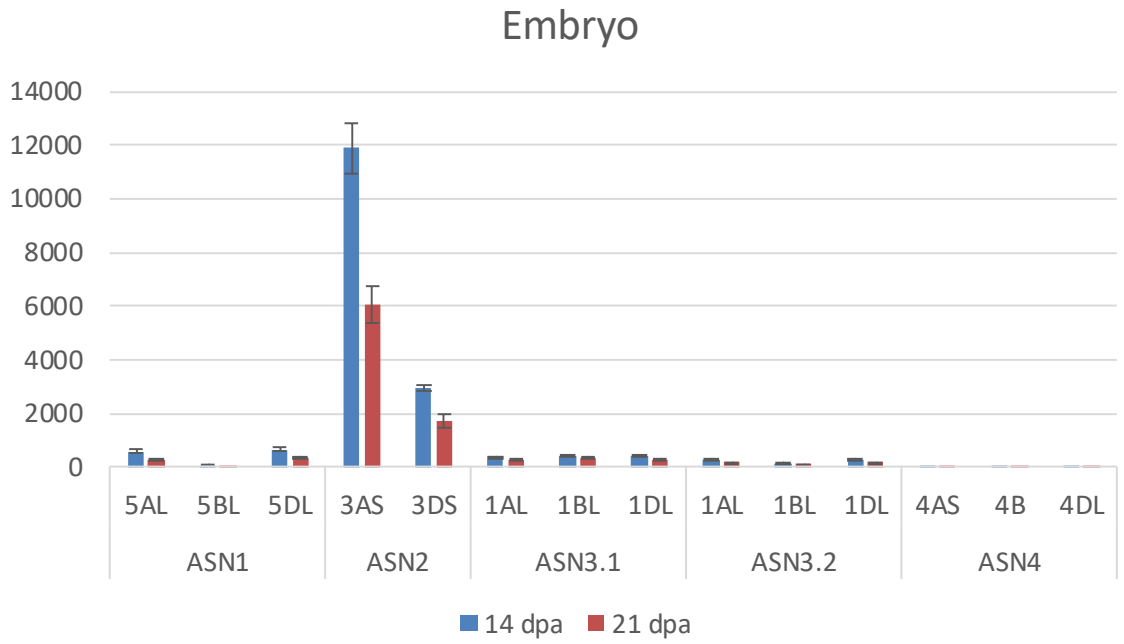
TaASN2 is an obvious target for genetic intervention

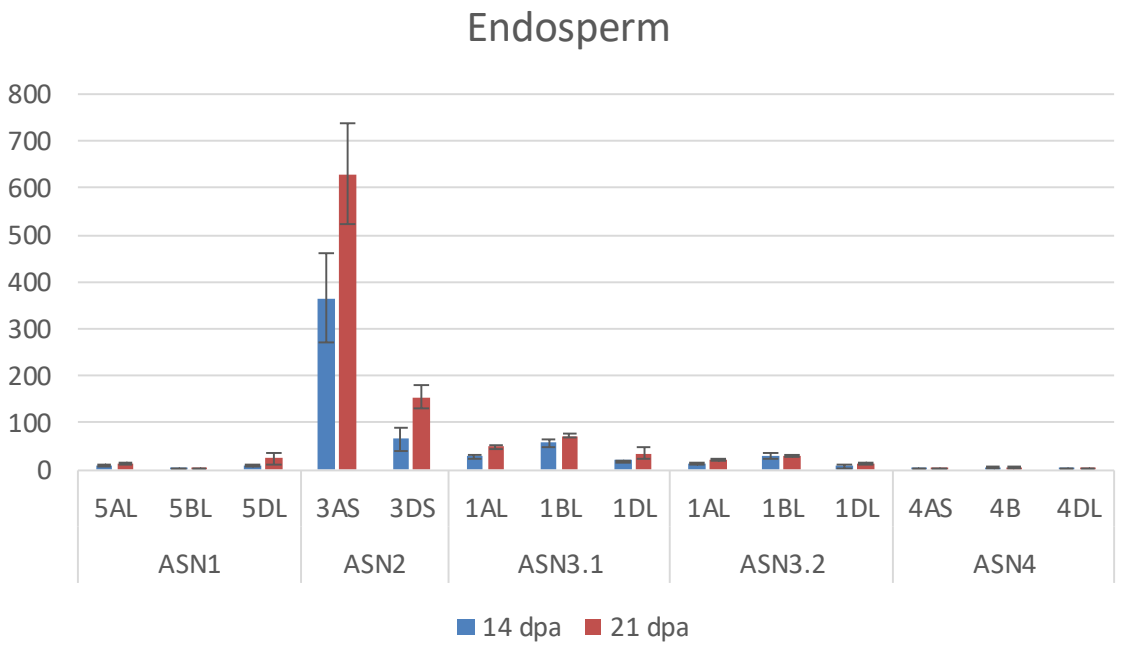


Expression of *TaASN2* in the embryo (bran fraction) and endosperm (white flour fraction) through grain development.

Journal of Cereal Science **68**, 122-131

Asparagine synthetase gene expression: RNA-seq data





- Higher levels of expression (> 10-fold difference) in total asparagine synthetase gene expression in the embryo than in the endosperm.
- *TaASN2* was the most highly expressed in both tissues.
- The A genome *TaASN2* gene was much more highly expressed (> 3-fold difference) than the D genome copy. The *TaASN-B2* gene is absent in these genotypes.
- Reaffirmed *TaASN2* as the best target! Knocking out the *TaASN-A2* gene on its own may be effective.

BMC Genomics **20**, 628.

A natural deletion in Chromosome 3B of variety Chinese Spring includes the *TaASN-B2* gene

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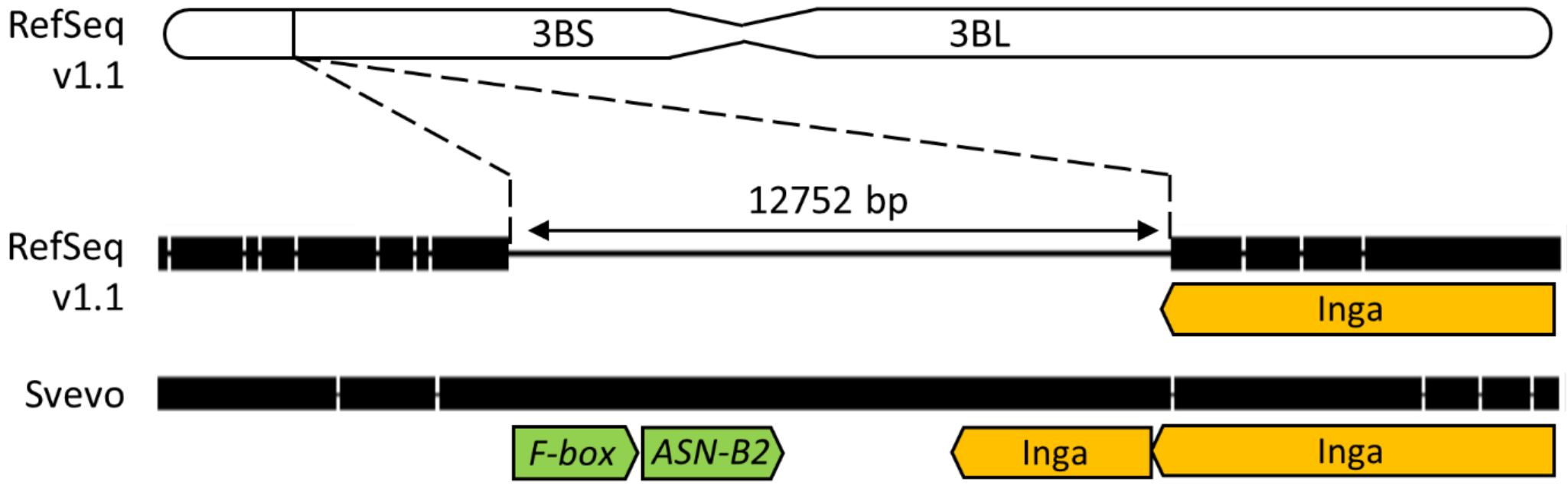
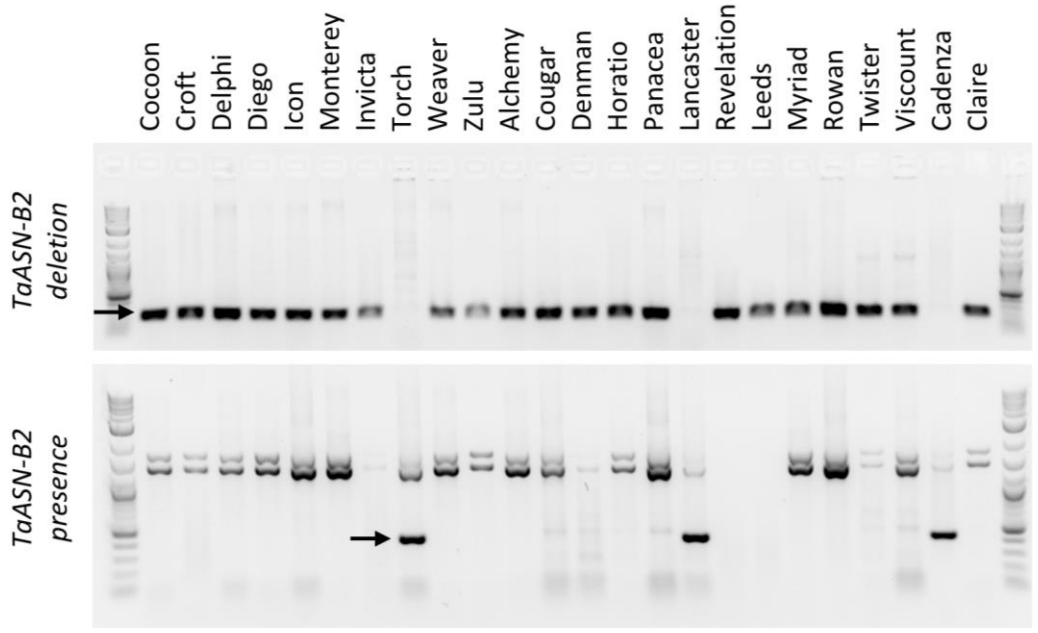


Alignment of the region of Chromosome 3B of variety Chinese Spring where the *TaASN-B2* gene has been deleted, and alignment with the corresponding region in variety Svevo, in which the *TaASN-B2* gene is present.

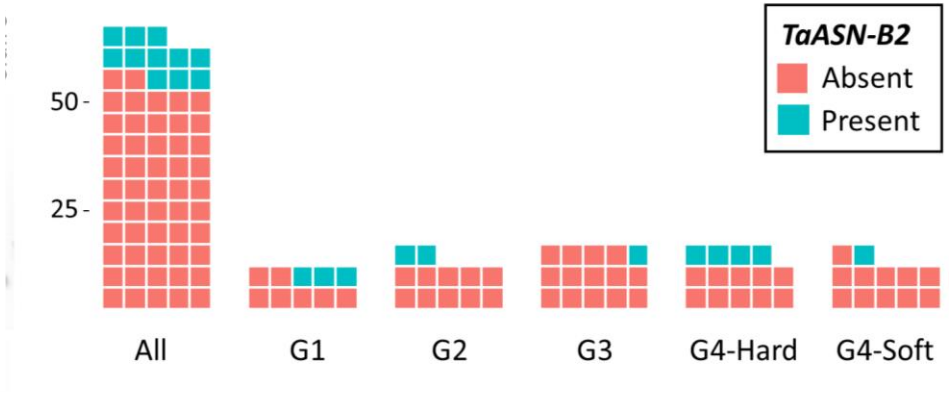
Joe Oddy

BMC Plant Biology **21**, 302.

Screening for the presence/absence of the *TaASN-B2* gene in UK wheat varieties



Top. Example of an electrophoresis gel of PCR products from assays to distinguish the presence and absence of *TaASN-B2* in a collection of UK wheat varieties. Varieties Cadenza and Claire were used as controls for TaASN-B2 presence and absence, respectively.

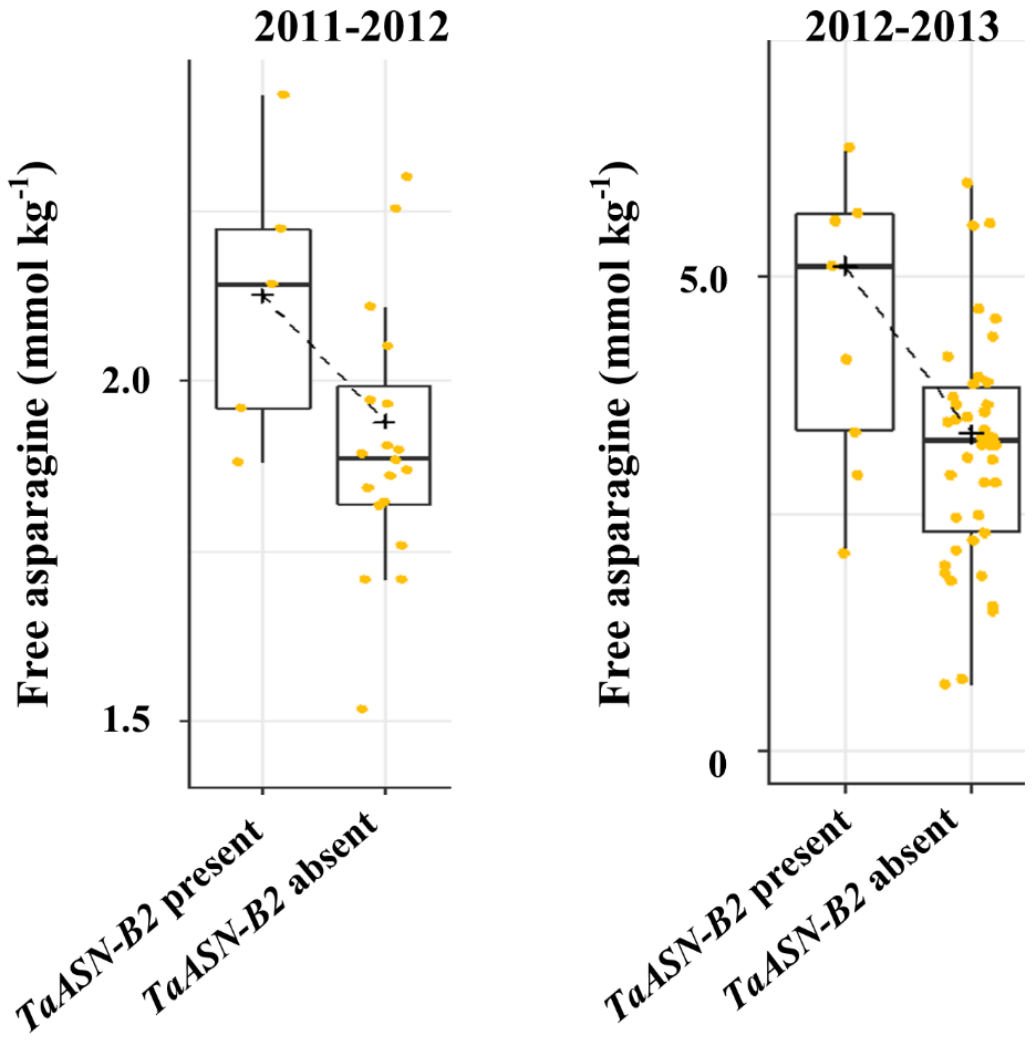


Bottom. Diagram showing the frequency of the *TaASN-B2* deletion in 63 UK wheat varieties, separated into UK Flour milling groups: G1 (breadmaking), G2 (breadmaking potential), G3 (soft/biscuit), G4 (feed/other).

- Most but not all UK wheat varieties lack a *TaASN-B2* gene.
- The deletion is present in some but not all wild wheat relatives.

BMC Plant Biology **21**, 302.

Effect of the *TaASN-B2* deletion on grain free asparagine levels in two year's of field trials



- The presence of the *TaASN-B2* gene was associated with higher free asparagine concentrations.
- This breaks down under sulphur deficiency (not shown).
- See Joe Oddy poster for more on this and work to identify QTL for asparagine concentration

BMC Plant Biology **21**, 302.

Knocking out *TaASN2* to produce low asparagine wheat: Genome editing with CRISPR-Cas9

ROTHAMSTED RESEARCH

Four gRNAs were designed to target the first exon of *TaASN2*

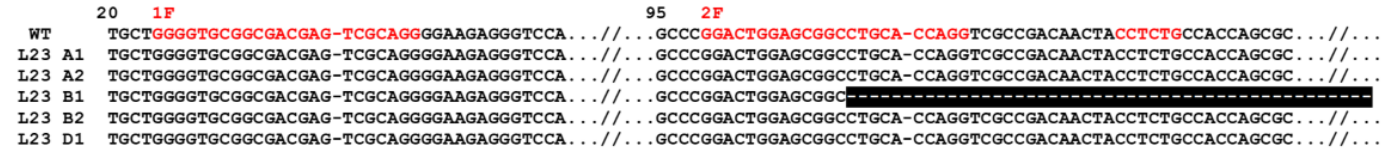
```
                    1F -> GGGGTGCGGCGACGAGTCGCAGG
  1 ATGTGCGGCATACTAGCGGTGCTGGGGTGCGGCGACGAGTCGCAGGGGAAGAGGGTCCACGTGCTAGAGCTCTCGCGCAG
  1 M  C  G  I  L  A  V  L  G  C  G  D  E  S  Q  G  K  R  V  H  V  L  E  L  S  R  R

                  2F -> GGACTGGAGCGGCCTGCACCAGG
 81 GCTCAAGCACCGGGGCCCGGACTGGAGCGGCCTGCACCAGGTCGCCGACAACTACCTCTGCCACCAGCGCCTCGCCATCA
 28  L  K  H  R  G  P  D  W  S  G  L  H  Q  V  A  D  N  Y  L  C  H  Q  R  L  A  I  I

            GGAGGCCGCTGGTCGGCGAGATG <- 3R
161 TCGACCCGGCCTCCGGCGACCAGCCGCTCTACAACGAGGACAAGTCCATCGCCGTTGCCGTCAACGGGGAGGTCTACAAC
 55   D  P  A  S  G  D  Q  P  L  Y  N  E  D  K  S  I  A  V  A  V  N  G  E  V  Y  N

                                          GGCCTGGCCGTCACTGACGCTCC <- 4R
241 CATGAGGAGCTTCGGGCACGGCTCTCCGGACACAGGTTCCGGACCGGCAGTGACTGCGAGGTCATCGCCCATCTG
 81 H  E  E  L  R  A  R  L  S  G  H  R  F  R  T  G  S  D  C  E  V  I  A  H  L
```

Sarah Raffan

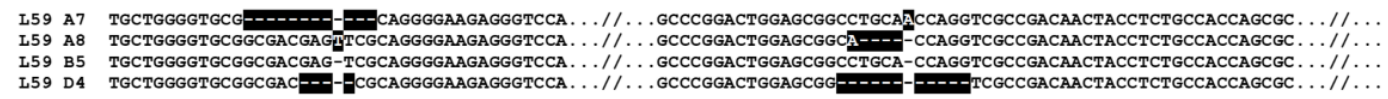
A gene encoding the 4 gRNAs was introduced into wheat by particle bombardment, along with a gene encoding the Cas9 nuclease and a selective marker gene (*Bar*).

Plant Biotechnology Journal **19**, 1602–1613.

Types of editing induced in our wheat plants by CRISPR



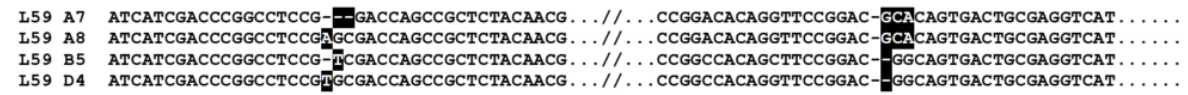
Line 23: Total null



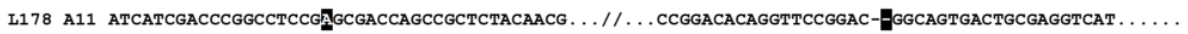
Line 59: Total null

Line 178: A genome null

Line 23: Total null



Line 59: Total null



Line 178: A genome null

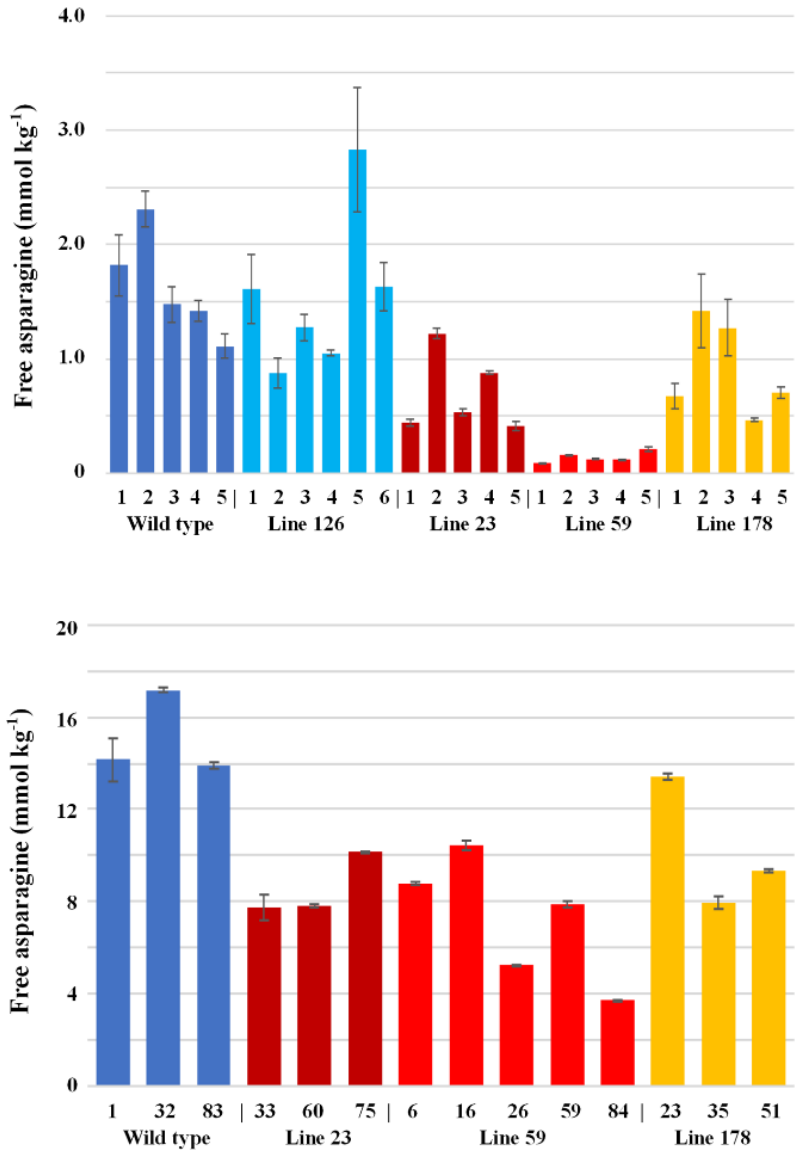
Most of the edits are deletions, but there are substitutions and single base insertions as well.

Most of these edited genes encode highly truncated, dysfunctional proteins.

Line 59 also has an edit in the *TaASN-B1* gene; i.e. it is a partial knockout for *TaASN1*: see Sarah Raffan's poster

Plant Biotechnology Journal **19**, 1602–1613.

Free asparagine levels in grain of control and edited *TaASN2* knockout lines



T2 top and T3 (bottom) seed. Free asparagine and total free amino acid levels were higher in the T3 generation, possibly due to stress.

Lines 23 and 59 are total knockouts

Line 178 is a partial knockout

Line 126 is not edited.

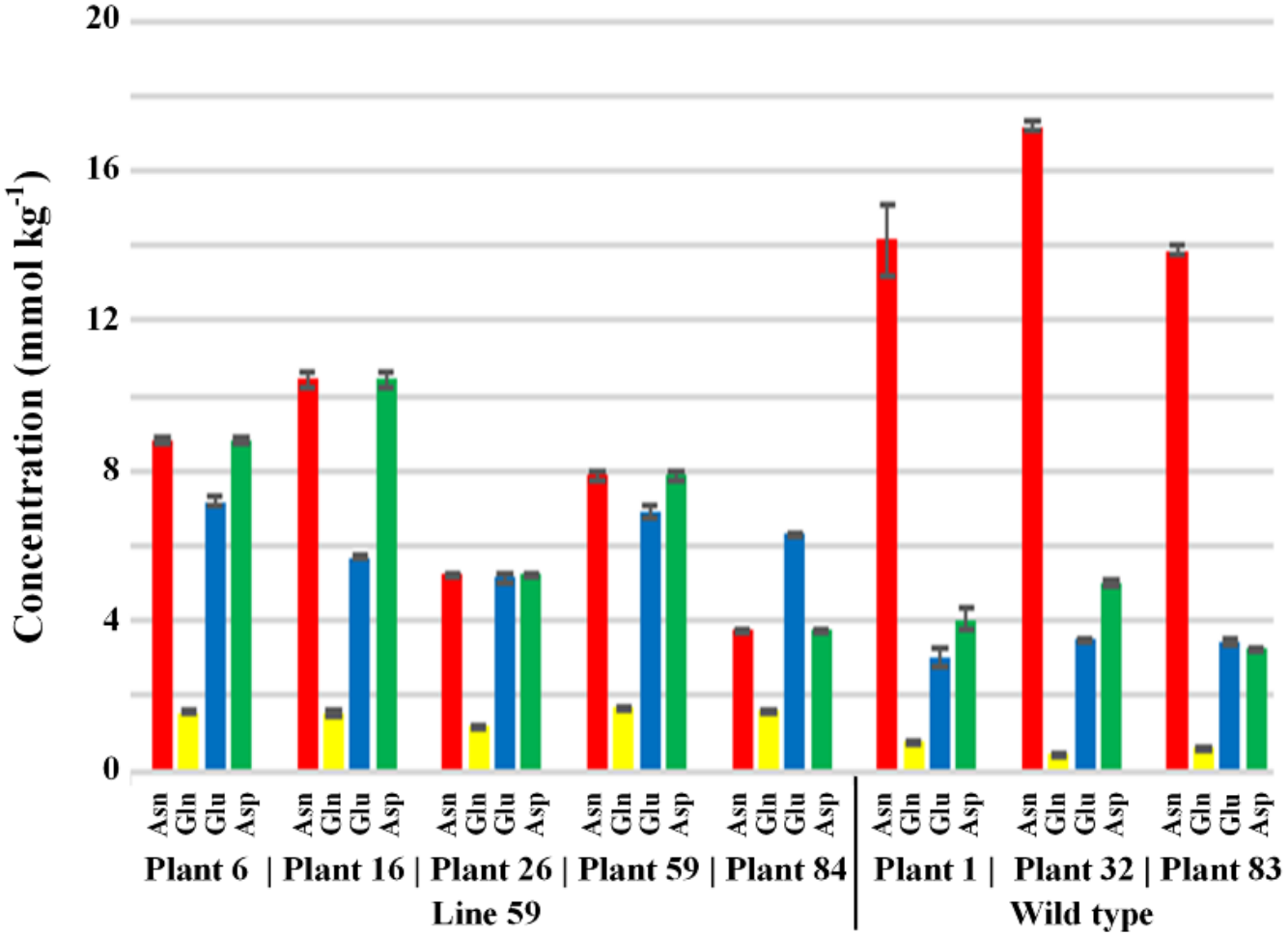
Line 59 is also a partial *TaASN1* knockout.

Currently they still contain some transgenes but these are being removed by selfing and crossing and we now have some plants from line 59 that are transgene-free.

Plant Biotechnology Journal **19**, 1602–1613.

The ratios of free asparagine to free glutamine, glutamate and aspartate are altered in the CRISPR lines

ROTHAMSTED RESEARCH



The graph shows the means and standard errors of free asparagine, glutamine, glutamate and aspartate concentrations in wholemeal flour prepared from the grain of edited wheat plants of line 59 and wildtype controls.

Plant Biotechnology Journal **19**, 1602–1613.

CRISPR editing of *TaASN2*

ROTHAMSTED RESEARCH

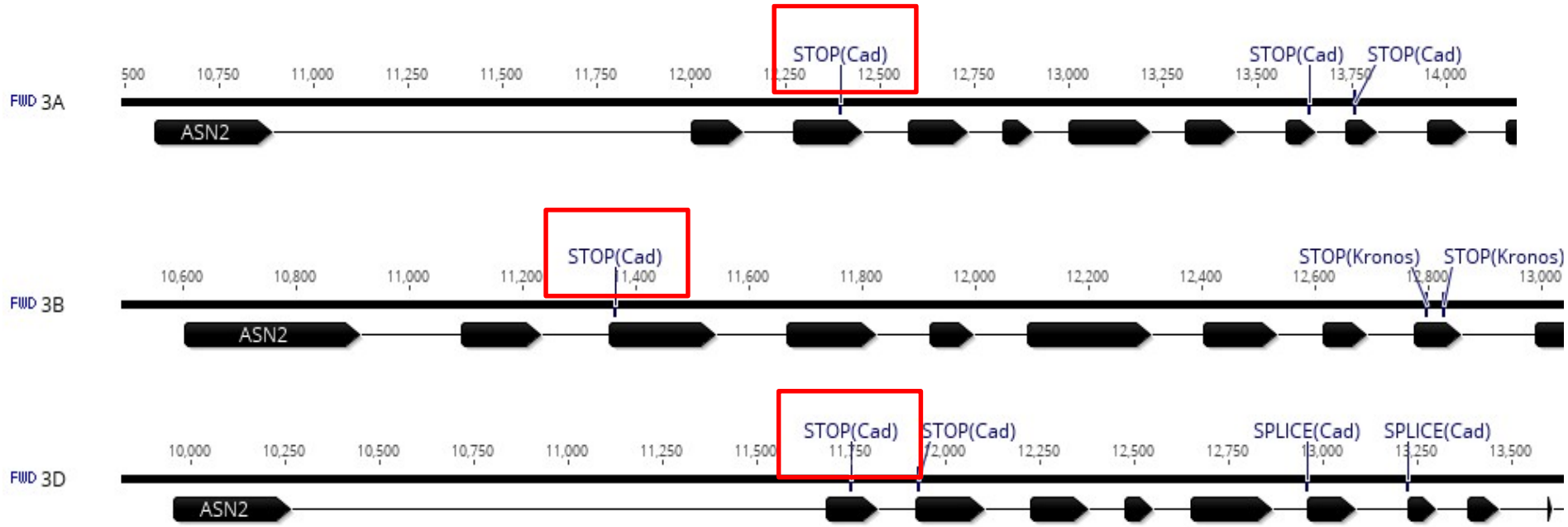
- Our study achieved a high mutation rate, with 11 lines derived from 14 selected T0 plants showing editing. We put this down to the simultaneous introduction of four gRNAs along with a wheat-optimised *Cas9* gene.
- It required NGS analysis to characterise the edits in the plants.
- Editing continued beyond the T0 generation, but only in some of the lines, and we also saw evidence of somatic editing. We conclude that several generations and segregating away of the *Cas9* gene may be required to achieve stability.
- Most of the edits were deletions, the longest of which was of 173 base pairs. However, there were some insertions as well, all of a single base pair, and some substitutions.
- The wheat asparagine synthetase gene family lent itself to the aims of the study. Non-Triticeae cereals, such as maize and rice, do not have an equivalent of the *TaASN2* gene.
- Poor germination was seen in some of the low asparagine lines but this could be overcome by application of asparagine and was not seen in every generation. This requires further investigation.

Plant Biotechnology Journal **19**, 1602–1613.

Knocking out *TaASN2*: stacking mutations from TILLING lines

ROTHAMSTED RESEARCH

- Target Induced Local Lesions In Genome (TILLING)
- Two EMS-mutagenised populations, in tetraploid durum wheat cv 'Kronos' and hexaploid bread wheat cv 'Cadenza', which have been exome-sequenced and characterised
- Developed as part of a joint project between the University of California Davis, Rothamsted Research, The Earlham Institute, and John Innes Centre
- Lines have been identified carrying mutations in the *TaASN2* genes in each of the A, B and D genomes
- The mutations are being stacked in variety Claire by our partners at RAGT to generate a null *TaASN2* line (Claire already lacks *TaASN-B2*).



Thanks to Andy Phillips, Rothamsted

Europe's first CRISPR wheat field trial

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Britain to grow cancer-cutting wheat for making healthier bread

Rhys Blakely Science Correspondent

British scientists will grow a new strain of wheat using a revolutionary form of genetic editing as part of an effort to make healthier toast.

The trials will be the first in Europe for a wheat that has been genetically edited using a tool known as Crispr. It comes as the government consults on whether to use post-Brexit freedoms to break with European Union rules that have limited the use of the technology in agriculture.

The trial, which had to be approved by the government, will be run by Rothamsted Research in Hertfordshire, which will grow the crop outside. The grain has been gene-edited to reduce levels of asparagine, a naturally occurring amino acid. When wheat is used to make bread and toast, asparagine is converted into acrylamide, which is thought to be carcinogenic.

Professor Nigel Halford, leading the project, said the aim was to produce healthier wheat without changing the taste, that would not be considered to be genetically modified (GM).

GM was originally used as a label for crops where genes had been transferred from one organism into another, sometimes across non-related species, in a way that does not happen naturally. The Crispr tool enables the genetic material of a plant to be precisely edited without new material being added.

Scientists have argued that Crispr is fundamentally different from conventional GM, in part because the changes that result could occur through natural mutation.

In 2018 the EU Court of Justice ruled that Crispr-edited crops should be subject to the same stringent regulations as conventional GM organisms, which some scientists said would slow research.

For the wheat in Hertfordshire,

Continued on page 2, col 3

Department for Environment Food & Rural Affairs

Victoria Prentis MP
Parliamentary Under Secretary of State

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Our ref: 21/R08/01

3rd August 2021

ENVIRONMENTAL PROTECTION ACT 1990, SECTIONS 111 AND 112:

CONSENT TO RELEASE GENETICALLY MODIFIED ORGANISMS REFERENCE 21/R08/01

1. In accordance with section 111 of the Environmental Protection Act 1990, the Secretary of State for Environment, Food and Rural Affairs hereby grants consent to Rothamsted Research to perform the release of the genetically modified organisms described in **paragraph** 2, in accordance with the particulars set out in **paragraph 3**, and subject to the limitations and conditions set out in the **Schedule** attached.

2. Genetically Modified Organism to be released:

The genetically modified organisms (GMOs) are wheat *Triticum aestivum* plants that have been gene edited using CRISPR-Cas9 to contain point mutations in the *TaASN2* asparagine synthetase gene.

3. Particulars of the consent to release:

Maximum size of the release: The area sown with the GM wheat must not exceed 1500 square metres and may take the form of one or more plots within the trial site.

(a) Purpose of the release:

To investigate the effect of knocking out the *TaASN2* gene on free asparagine accumulation in wheat grain in the field.

(b) Location of the release ("trial site"):

Regulation of genome edited crops

ROTHAMSTED RESEARCH

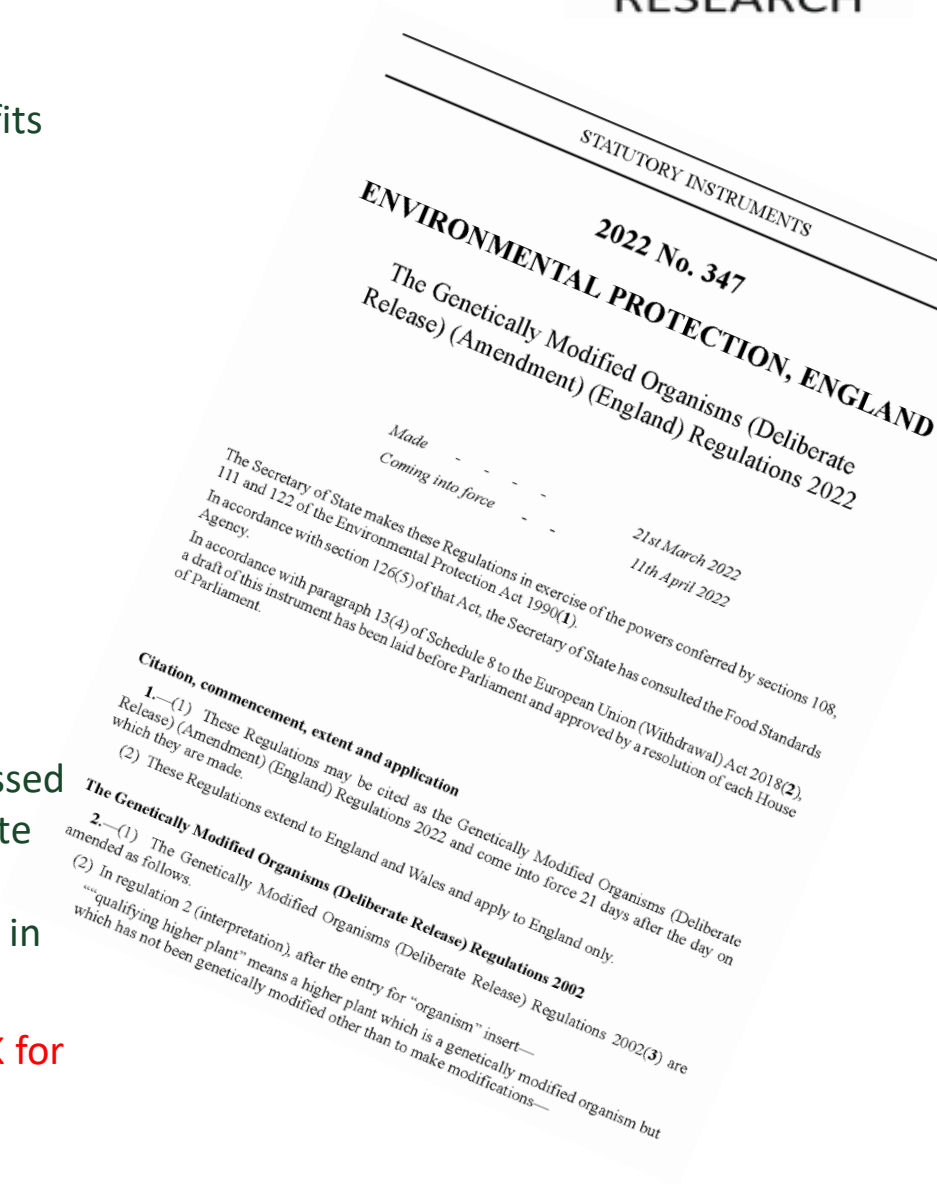
- The EU currently has no formal regulations on the genome editing of crops, but as a result of European Court of Justice ruling on case C-528/16 (25th July 2018), gene edited plants have to be treated like transgenic plants, and edited genes as if they were transgenes.

- Many other regulatory authorities around the world have taken a very different view (USA, Brazil, Argentina, Japan, Canada, Chile, Colombia, Israel, China, India for example).

UK: Changes to regulations on genome editing

ROTHAMSTED RESEARCH

- The UK currently operates under legacy regulations from the EU.
- UK Government announcement, 29th September 2021: Plans to unlock potential benefits of gene editing set out.
- As a first step the government will change the rules relating to gene editing to cut red tape and make research and development easier.
- Research scientists will still need to notify Defra of any trials involving GE plants.
- This first step applies to research and development only; for any products that are authorised for market, existing GMO rules would apply.
- The government will also consider the appropriate measures needed to enable gene edited products to be brought to market safely and responsibly.
- In the longer term, this will be followed by a review of its approach to GMO regulation more broadly.
- The Statutory Instrument (SI) on removing GE field trials from GMO regulation was passed last month and comes into effect today: The Genetically Modified Organisms (Deliberate Release) (Amendment) (England) Regulations 2022 (legislation.gov.uk/uksi/2022/347/regulation/2/made), so things are already in motion in England.
- Probably the first positive step in the regulation of agricultural biotechnology in the UK for 25 years.



STATUTORY INSTRUMENTS

2022 No. 347

ENVIRONMENTAL PROTECTION, ENGLAND

The Genetically Modified Organisms (Deliberate Release) (Amendment) (England) Regulations 2022

Made - - - - 21st March 2022

Coming into force - - 11th April 2022

The Secretary of State makes these Regulations in exercise of the powers conferred by sections 108, 111 and 122 of the Environmental Protection Act 1990(1).

In accordance with section 126(5) of that Act, the Secretary of State has consulted the Food Standards Agency.

In accordance with paragraph 13(4) of Schedule 8 to the European Union (Withdrawal) Act 2018(2), a draft of this instrument has been laid before Parliament and approved by a resolution of each House of Parliament.

Citation, commencement, extent and application

1.—(1) These Regulations may be cited as the Genetically Modified Organisms (Deliberate Release) (Amendment) (England) Regulations 2022 and come into force 21 days after the day on which they are made.

(2) These Regulations extend to England and Wales and apply to England only.

The Genetically Modified Organisms (Deliberate Release) Regulations 2002

2.—(1) The Genetically Modified Organisms (Deliberate Release) Regulations 2002(3) are amended as follows.

(2) In regulation 2 (interpretation), after the entry for "organism" insert—

""qualifying higher plant" means a higher plant which is a genetically modified organism but which has not been genetically modified other than to make modifications—

UK: Changes to regulations on genome editing

ROTHAMSTED RESEARCH

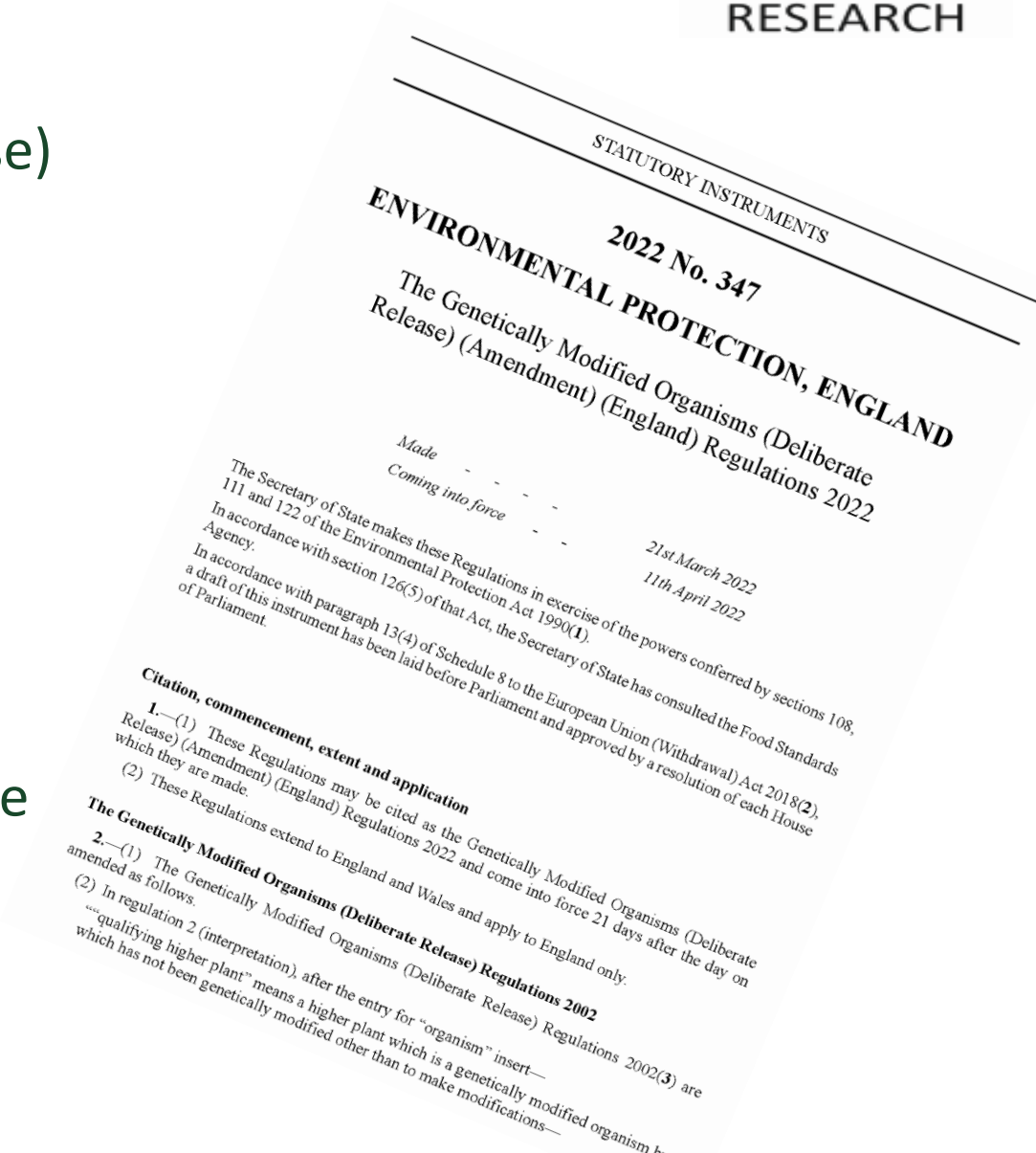
The Genetically Modified Organisms (Deliberate Release) Regulations 2002(3) are amended as follows.

(2) In regulation 2 (interpretation), after the entry for "organism" insert—

qualifying higher plant means a higher plant which is a genetically modified organism but which has not been genetically modified other than to make modifications:

(a) that could have occurred naturally, or

(b) that could have been made using one or more of the techniques set out in regulation 5(2);" [these include polyploidy induction, mutagenesis and cell/protoplast fusion].



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Concluding remarks

- Crop management best practice and the use of low asparagine wheat varieties are important aspects of regulatory compliance with respect to the acrylamide content of wheat products.
- Further step reductions in the free asparagine concentration of wheat grain are possible using genome editing and presumably also chemical mutagenesis.
- Effects on yield, N content, grain weight, grain protein etc. have still to be determined.
- This is important because genetic and agronomic approaches to solving the acrylamide problem could eventually lead to massive savings for food businesses, enabling them to comply with evolving regulations on acrylamide without costly changes to production lines or reduction in product quality.

Acknowledgements: Current low acrylamide wheat team

ROTHAMSTED RESEARCH

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