March 2018

Project Report No. PR591

Genetic improvement of wheat to reduce the potential for acrylamide formation during processing

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This is the final report of a 48 month project (20103748) which started in October 2011 and was extended for one year to October 2016. The work was funded by BBSRC Stand-alone LINK grant BB/I020918/1.

CONTENTS

1. Abstract

Free asparagine concentration, which is the determining factor for acrylamide-forming potential in cereals, was measured in grain from wheat grown in field trials in 2011-2012 and 2012-2013. There were 25 varieties in 2012 and 59 in 2013, with eleven present in both trials. Eight varieties were identified as having consistently low free asparagine concentration. There was a differential response of varieties to sulphur, and much higher levels of free asparagine in 2012-2013 versus 2011-2012. A key conclusion of this study was that, given the short commercial lifespan of some wheat varieties, information on free asparagine concentration should be made available when a variety is launched. The effect of fungicide application on free asparagine accumulation in wheat grain was also investigated. Flour was analysed from 24 varieties grown in adjacent plots that were treated in identical fashion except that fungicide was applied to one and not the other. Lack of fungicide resulted in visible infection by *Septoria tritici*, yellow rust and brown rust. Free asparagine concentration was much lower in the fungicide-treated wheat than the untreated wheat, resulting in less acrylamide formation in flour when it was heated. The study showed disease control by fungicide application to be an important crop management measure for mitigating the problem of acrylamide formation in wheat products. Asparagine synthesis occurs by the amidation of aspartate, catalysed by asparagine synthetase, and the expression of asparagine synthetase (*TaASN*) genes in wheat was therefore studied. The expression of three genes, *TaASN1-3*, was measured in different tissues and in response to nitrogen and sulphur supply. The expression of *TaASN2* in the grain during mid to late development was the highest of any of the genes in any tissue. Both *TaASN1* and *TaASN2* increased in expression through grain development, and in the grain of field-grown plants during mid-development in response to sulphur deprivation. However, only *TaASN1* was affected by nitrogen or sulphur supply in pot-based experiments, showing complex responses. A possible regulatory motif was found in the promoter of *TaASN1* genes from several cereal species. As the study was completed, a fourth gene, *TaASN4*, was identified from recently available genome data. Phylogenetic analysis showed that other cereal species have similar asparagine synthetase gene families to wheat. *TaASN1* and *TaASN2* were used to produce their encoded proteins in *Escherichia coli*. The proteins were shown to react with two monoclonal antibodies raised to distinct epitopes. The reaction catalysed by asparagine synthetase was modelled using publicly-available data from various species to generate a series of differential equations to describe the reaction stages. The TaASN1 and TaASN2 proteins were purified and found to be active, synthesising asparagine and glutamate from glutamine and aspartate. Data from the reactions was entered into the model, enabling values to be determined for kinetic parameters within the differential equations. A network describing asparagine metabolism was developed and used to identify networks of genes responding to stress in wheat. The network is also being used to filter RNAseq datasets to enable the comparison of high and low asparagine genotypes.

2. Introduction

Acrylamide (C_3H_5NO) is a processing contaminant produced predominantly in the Maillard reaction at the high temperatures generated by frying, baking, roasting or high-temperature processing (Halford *et al*. 2011). It is classified as a Group 2A carcinogen and the latest report from EFSA's Expert Panel on Contaminants in the Food Chain (CONTAM) stated that the margins of exposure for dietary acrylamide indicated a concern for neoplastic effects (CONTAM, 2015). Cereal products are major contributors to dietary acrylamide intake (CONTAM, 2015) and all wheat products are affected, including bread, flatbreads, biscuits, snacks, breakfast cereals, pies, pastries, pancakes, cakes and batter.

The European Commission issued 'Indicative Values' for the presence of acrylamide in food in 2011, the year in which this project started, and revised them downwards for many product types in 2013 (European Commission 2013). Indicative Values are not regulatory limits or safety thresholds, but if a product is found to exceed the Indicative Value the relevant food safety authority should take action to ensure that the manufacturer addresses the problem. However, the European Commission has just approved strengthened risk management measures including compulsory Codes of Practice and the renaming of Indicative Values as Benchmark Levels, with reduced Benchmark Levels for many products (European Commission 2017: https://ec.europa.eu/info/law/better-regulation/initiatives/ares-2017-2895100_en). The new regulations also include a specific reference to the setting of Maximum Levels for acrylamide in certain foods, stating that this should be considered following the entry into force of the new regulations, which is expected in April 2018.

Acrylamide forms in the Maillard reaction, a complex series of non-enzymic reactions between free (non-protein) amino acids and reducing sugars. It forms principally via the deamination and decarboxylation of free asparagine (Mottram *et al*. 2002): free asparagine and reducing sugars can, therefore, be regarded as its precursors (in fact the carbon skeleton is derived entirely from asparagine). In the case of wheat, fructose, glucose and maltose account for almost all the reducing sugar content. Free asparagine concentration is the major determinant of acrylamide formation in cereal products and was, therefore, the focus for this study.

The project was a BBSRC-funded stand-alone LINK, involving Rothamsted Research (PI Nigel Halford and Researcher Tanya Curtis), and the John Innes Centre (PI Simon Griffiths), with support from a consortium of companies and organisations: AHDB, PepsiCo, Nestle, Weetabix, Kelloggs, United Biscuits, CEEREAL, Con-Agra, Cereal Partners Worldwide, Saaten Union, Lantmännen, the Snack, Nut and Crisp Manufacturers Association and the Association of Cereal Food Manufacturers. The original objectives were:

1. Identify genotypes/varieties of wheat with 'high' and 'low' acrylamide-producing potential.
2. Identify the site for synthesis of the free asparagine that accumulates in wheat grain under normal conditions and under stress conditions such as sulphur deprivation.

3. Obtain metabolite and gene expression profiles for cultivar Spark and doubled haploid (DH) line SR3, which differ significantly in free asparagine concentration, in order to identify the key enzymes and genes that determine free asparagine concentration in wheat grain.
4. Add to and characterise Quantitative Trait Loci (QTL) for free asparagine concentration, and identify genetic markers for low free asparagine concentration in wheat grain.
5. Use targeted mutagenesis (TILLING) and genetic modification to change the activity of enzymes identified in 3.
6. Assess the impact of reductions in acrylamide-forming potential of grain on performance in industrial processes.

3. Results

3.1. Effects of fungicide treatment on free amino acid concentration and acrylamide-forming potential in wheat

This study has been published and the paper will accompany this report.

3.1.1. Abstract

Acrylamide forms from free asparagine and reducing sugars during frying, baking, roasting, or high-temperature processing, and cereal products are major contributors to dietary acrylamide intake. Free asparagine concentration is the determining factor for acrylamide-forming potential in cereals, and this study investigated the effect of fungicide application on free asparagine accumulation in wheat grain. Free amino acid concentrations were measured in flour from 47 varieties of wheat grown in a field trial in 2011−2012. The wheat had been supplied with nitrogen and sulphur and treated with growth regulators and fungicides. Acrylamide formation was measured after the flour had been heated at 180 °C for 20 min. Flour was also analysed from 24 (of the 47) varieties grown in adjacent plots that were treated in identical fashion except that no fungicide was applied, resulting in visible infection by *Septoria tritici*, yellow rust, and brown rust. Free asparagine concentration in the fungicide-treated wheat ranged from 1.596 to 3.987 mmol kg^{-1}, with a significant ($p < 0.001$ to $p = 0.006$, F test) effect of variety for not only free asparagine but all of the free amino acids apart from cysteine and ornithine. There was also a significant ($p < 0.001$, F test) effect of variety on acrylamide formation, which ranged from 134 to 992 μg kg^{-1}. There was a significant ($p < 0.001$, F test) correlation between free asparagine concentration and acrylamide formation. Both free asparagine concentration and acrylamide formation increased in response to a lack of fungicide treatment, the increases in acrylamide ranging from 2.7 to 370 %. Free aspartic acid concentration also increased, whereas free glutamic acid concentration increased in some varieties but decreased in others, and free proline concentration decreased. The study showed disease control by fungicide application to be an important crop management measure for mitigating the problem of acrylamide formation in wheat products.

3.1.2. Key findings

The study showed fungicide application and, by inference, crop disease to have a profound effect on the free asparagine concentration and acrylamide-forming potential of grain from a range of commercial wheat varieties (Figure 1).

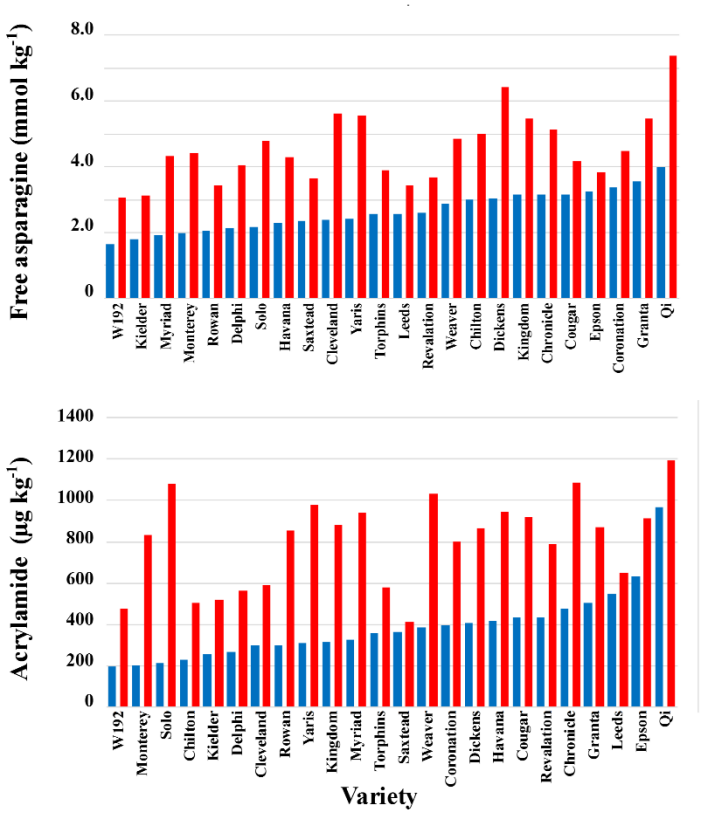

Figure 1. Graphs showing the mean free asparagine concentration (top) and acrylamide formation in heated flour (180 °C for 20 min) (bottom) in 24 varieties of wheat grown in a field trial in 2011−2012. The plants were all supplied with nitrogen (200 kg ha^{-1}) and sulphur (30 kg ha^{-1}), but plots were either treated with fungicides (blue, n = 3) or left untreated (red, n = 1). Reproduced from: Journal of Agricultural and Food Chemistry 64, 9689-9696. Refer to accompanying manuscript for statistical analysis.

As a result of the study, we proposed that effective disease control through fungicide application be adopted as a second crop management measure, alongside ensuring sulphur sufficiency, to mitigate the problem of acrylamide formation in wheat products. This has been incorporated into the European Commission's Code of Practice for cereal products. We also recommended that regulatory authorities take the consequences for acrylamide-forming potential into account when considering the risks and benefits of fungicide usage.

3.2. Effects of variety, year of cultivation and sulphur supply on the accumulation of free asparagine in the grain of commercial wheat varieties

This study has been published and the paper will accompany this report.

3.2.1. Abstract

Free asparagine concentration, which is the determining factor for acrylamide-forming potential in cereals, was measured in grain from wheat grown in field trials in the United Kingdom in 2011–2012 and 2012–2013. There were 25 varieties in 2012 and 59 in 2013, with eleven present in both trials. The trials were split-plot, with half of each plot supplied with sulphur and the other half not. The varietal means (mmol per kg) for free asparagine in the sulphur-fed wheat ranged from 1.521 to 2.687 in 2011–2012 and 0.708 to 11.29 in 2012–2013. Eight varieties were identified as having consistently low free asparagine concentration. There was a differential response of varieties to sulphur, and much higher levels of free asparagine in 2012–2013 versus 2011–2012. Given the short commercial lifespan of some wheat varieties, it is concluded that information on free asparagine concentration should be made available when a variety is launched.

3.2.2. Key findings

The study showed large differences in free asparagine concentration between varieties grown in the trials, for example ranging from under 1 mmol kg^{-1} to almost 12 mmol kg^{-1} in the 2012-2013 trial (Figure 2). It also identified eight varieties from the 73 that were included in the study that have shown consistently low free asparagine concentration in the grain in field trials conducted for this study and in trials at different locations in different years in the UK. These were Claire, Cocoon, Cordiale, Croft, Delphi, Horatio, Monterey and Myriad. Cordiale is a nabim-defined Group 2 wheat (breadmaking potential), while Croft, Claire, Cocoon, and Monterey are Group 3 (soft, biscuit type), and Horatio and Myriad are soft Group 4 types. Clearly there is a predominance of soft wheats on this list, but all four groups contained varieties with a wide range of free asparagine concentration and our analysis did not reveal significant differences in free asparagine between the groups overall ($p = 0.133$, F-test). Choosing varieties for low acrylamide-forming potential simply on the basis of their classification as soft is therefore simplistic and probably ineffective, although there is anecdotal evidence from food industry sources that this is common practice.

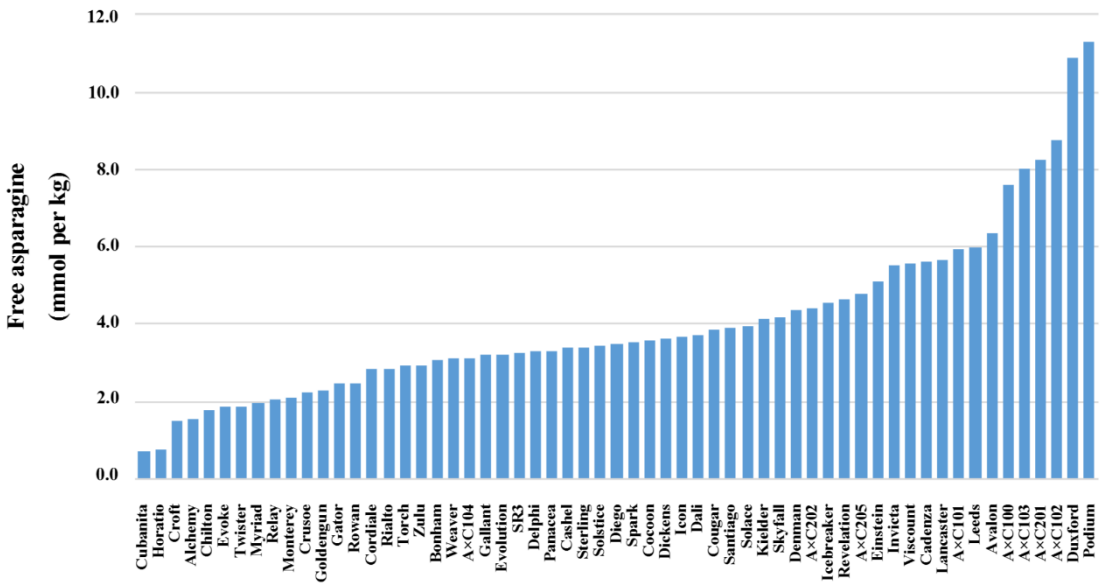

Figure 2. Graph showing the mean free asparagine concentration in the grain of 59 varieties/genotypes of wheat grown in a field trial in 2012–2013, supplied with nitrogen at a rate of 140 kg per hectare (in two applications) and sulphur at 40 kg per hectare. Reproduced from: Food Chemistry 239, 304-313. Refer to accompanying manuscript for statistical analysis.

3.2.3. Additional field trials and recommendations on varietal differences

Field trials were conducted in the two subsequent seasons, and included sulphur as well as varietal trials. Data was also compiled for the Watkins and Gediflux collections of diverse wheat genotypes. These data are being prepared for publication. Our conclusions with respect to varietal differences are as follows:

- Some wheat varieties have been in the bottom half of the rankings on asparagine concentration over several field trials, and some are consistently in the top half.
- Most of the ‘low’ varieties are soft wheats, but selecting varieties for low asparagine simply on the basis that they are soft would be simplistic and potentially counter-productive (some soft varieties are high in asparagine).
- Other varieties may join the ‘low’ list but we do not have enough information on them.
- We would support the inclusion of information on grain asparagine concentration in the UK’s Recommended List variety descriptions, but there are still some problems to resolve.
- Free asparagine concentration is very sensitive to environmental factors, and the ranking breaks down if sulphur supply is insufficient or as a result of disease. As a result, there is still too much uncertainty for end-users to trust variety rankings.
- The turnover in varieties in the UK is so rapid that, by the time enough is known about asparagine concentrations, the variety may no longer be available. The only solution to this

is for testing to be carried out by breeders during variety development. This is currently not done.

- Biscuit (soft) wheat cultivation in the UK has declined dramatically in recent years, partly because Claire, a stalwart of that market, is susceptible to new strains of rust. So some varieties may not be available in sufficient quantities anyway.
- Excluding varieties in the 'high' group from processes in which there is a high risk of acrylamide formation could be a sensible course of action in the short term.

3.3. Structure and expression of the asparagine synthetase gene family of wheat

This study has been published and the paper will accompany this report.

3.3.1. Abstract

Asparagine is an important nitrogen storage and transport molecule, but its accumulation as a free amino acid in crops has implications for food safety because free asparagine is a precursor for acrylamide formation during cooking and processing. Asparagine synthesis occurs by the amidation of aspartate, catalysed by asparagine synthetase, and this study concerned the expression of asparagine synthetase (*TaASN*) genes in wheat. The expression of three genes, *TaASN1-3*, was studied in different tissues and in response to nitrogen and sulphur supply. The expression of *TaASN2* in the embryo and endosperm during mid to late grain development was the highest of any of the genes in any tissue. Both *TaASN1* and *TaASN2* increased in expression through grain development, and in the grain of field-grown plants during mid-development in response to sulphur deprivation. However, only *TaASN1* was affected by nitrogen or sulphur supply in pot-based experiments, showing complex tissue-specific and developmentally changing responses. A putative N-motif or GCN4-like regulatory motif was found in the promoter of *TaASN1* genes from several cereal species. As the study was completed, a fourth gene, *TaASN4*, was identified from recently available genome data. Phylogenetic analysis showed that other cereal species have similar asparagine synthetase gene families to wheat.

3.3.2. Key findings

Initially, three wheat asparagine synthetase genes were identified, but a fourth, hitherto uncharacterised gene, was identified from genome data at the end of the study. Phylogenetic analysis showed that all of the cereal asparagine synthetases identified to date fall into three clusters. The proteins that were annotated as TaASN1 and TaASN2 clustered together in the phylogenetic analysis, but because the expression analyses had shown the genes encoding these two proteins to be differentially regulated this cluster was annotated as Group 1/2, the others being Groups 3 and 4. This is consistent with the pattern reported for maize (Todd *et al*., 2008) and barley (Avila-Ospina *et al*., 2015).

The expression of *TaASN2* in the embryo and to a lesser extent the endosperm during mid to late grain development was by far the highest of any of the genes in any tissue (Figure 3). This suggests that most grain asparagine is likely to have been synthesised *in situ*, and that genetic interventions should be targeted at *TaASN2*. It also encourages optimism that such interventions could be successful.

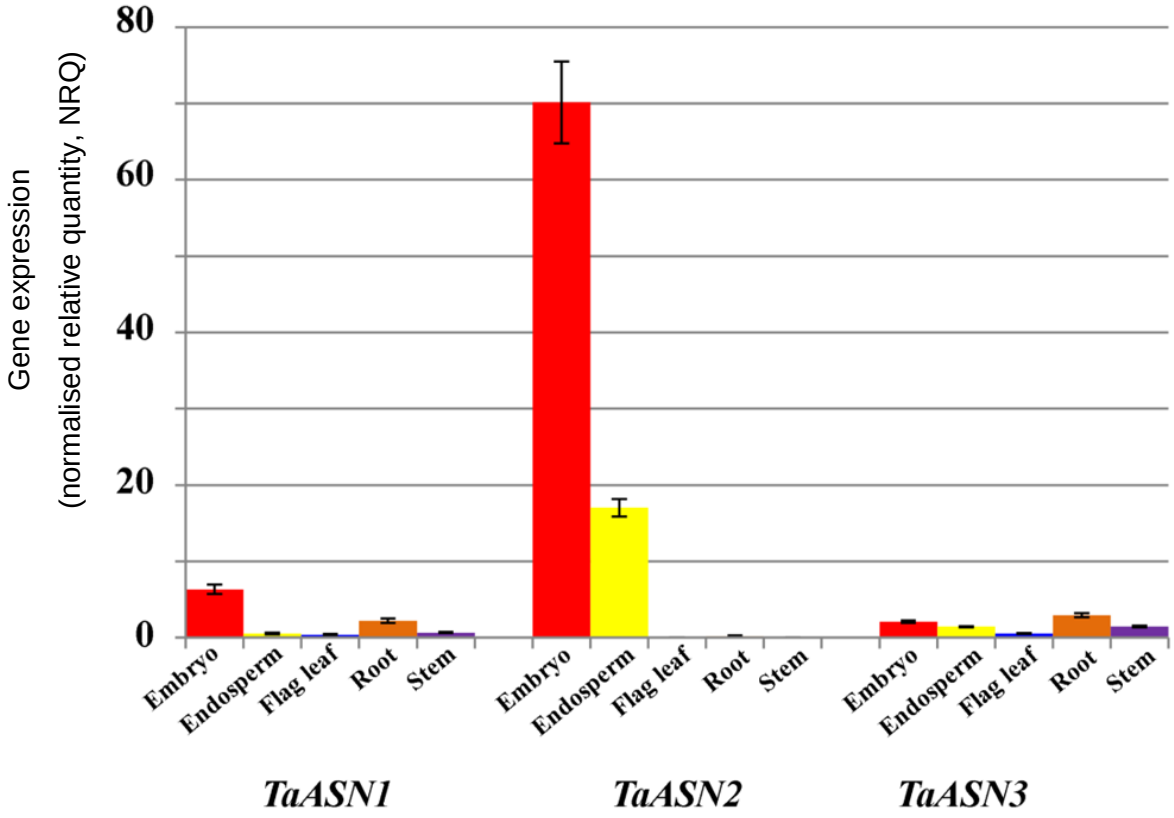

Figure 3. Differential expression of asparagine synthetase genes (*TaASN1*, *TaASN2* and *TaASN3*) in different tissues of wheat (*Triticum aestivum*) cv. Cadenza plants grown with nitrogen and sulphur supplied. Expression was measured at 21 days post-anthesis by real-time PCR and the graphs show the NRQ means and standard errors. Reproduced from: Journal of Cereal Science 68, 122-131. Refer to accompanying manuscript for statistical analysis.

The problem of acrylamide formation in baked wheat products is exacerbated by the sensitivity of asparagine metabolism to nutrient supply (Curtis *et al*., 2009, 2014b; Granvogl *et al*., 2007; Muttucumaru *et al*., 2006). We have suggested previously that wheat uses free asparagine as a grain nitrogen store when deficiencies in other minerals, particularly sulphur, inhibit protein synthesis (Halford *et al*., 2012) and this is one example of how nutritional stress can affect crop composition (Halford *et al*., 2015). We have previously shown *TaASN1* to be up-regulated in the leaves of wheat seedlings in response to sulphur deprivation (Byrne *et al*., 2012), and in the

present study both *TaASN1* and *TaASN2* expression increased in the grain during mid-development in field-grown plants in response to sulphur deprivation. However, only *TaASN1* responded to nitrogen or sulphur in the pot-based experiments, yet it did not show the clear response that had been reported for seedlings. Indeed, it showed a complex series of tissue-specific and developmentally-changing responses. The response of *TaASN1* gene expression to nutrient supply may involve a promoter element that is a perfect match for the so-called N-motif or GCN4-like motif (Shewry *et al*., 2003). This element was found in *TaASN1* genes from bread wheat, *Ae. tauschii*, barley and *B. distachyon*.

3.4. Construction of a network describing asparagine metabolism in plants and its application to the identification of genes affecting asparagine metabolism in wheat under drought and nutritional stress

This study has been published and the paper will accompany this report.

3.4.1. Abstract

A detailed network describing asparagine metabolism in plants was constructed using published data from Arabidopsis (*Arabidopsis thaliana*) maize (*Zea mays*), wheat (*Triticum aestivum*), pea (*Pisum sativum*), soybean (*Glycine max*), lupin (*Lupus albus*) and other species, including animals. Asparagine synthesis and degradation is a major part of amino acid and nitrogen metabolism in plants. The complexity of its metabolism, including limiting and regulatory factors, was represented in a logical sequence in a pathway diagram built using yED graph editor software. The network was used with the Unique Network Identification Pipeline in the analysis of data from 18 publicly-available transcriptomic data studies. This identified links between genes involved in asparagine metabolism in wheat roots under drought stress, wheat leaves under drought stress, and wheat leaves under conditions of sulphur and nitrogen deficiency. The network represents a powerful aid for interpreting the interactions not only between the genes in the pathway but also between enzymes, metabolites and smaller molecules. It provides a concise, clear understanding of the complexity of asparagine metabolism that could aid the interpretation of data relating to wider amino acid metabolism and other metabolic processes.

3.4.2. Key findings

The study represented the construction of a detailed and comprehensive asparagine metabolism network (Figure 4). The network comprised stimuli, enzymes, genes and small molecules, including asparagine itself, glutamine, aspartate and glutamate, and energy molecules, including ATP, ADP and NADH. It was hand-curated using data from existing databases and literature from a range of species, but was applied to the analysis of transcriptomic data from wheat to identify genes involved in asparagine metabolism under conditions of drought stress and nutrient

deficiency. This also served as a validation exercise because it identified interactions between genes in the network.

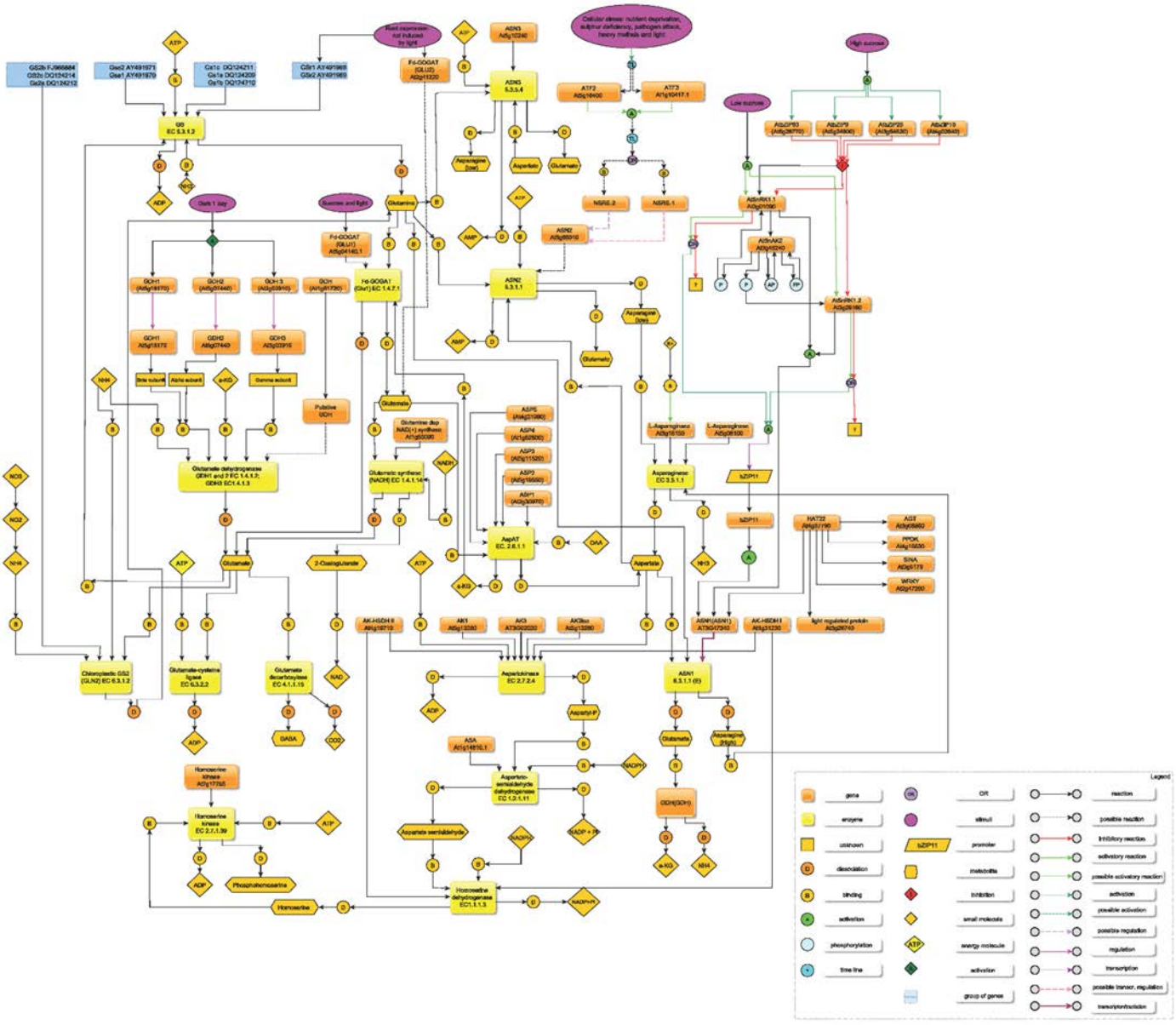

Figure 4. Network describing asparagine metabolism, created with yED Graph Editor Version 3.2.0.1 (yWorks, Tübingen Germany). Reproduced from: Food and Energy Security, e00126, Supplementary File S1: https://onlinelibrary.wiley.com/action/downloadSupplement?doi=10.1002%2Ffes3.126&attachmentId=2190837826

3.5. Genomic, biochemical and modelling analyses of asparagine synthetases from wheat

This study has been published and the paper will accompany this report.

3.5.1. Abstract

Asparagine synthetase activity in cereals has become an important issue with the discovery that free asparagine concentration determines the potential for formation of acrylamide, a probably carcinogenic processing contaminant, in baked cereal products. Asparagine synthetase catalyses the ATP-dependent transfer of the amino group of glutamine to a molecule of aspartate to generate glutamate and asparagine. Here, asparagine synthetase-encoding polymerase chain reaction (PCR) products were amplified from wheat (*Triticum aestivum*) cv. Spark cDNA. The encoded proteins were assigned the names TaASN1, TaASN2, and TaASN3 on the basis of comparisons with other wheat and cereal asparagine synthetases. Although very similar to each other, they differed slightly in size, with molecular masses of 65.49, 65.06, and 66.24 kDa, respectively. Chromosomal positions and scaffold references were established for *TaASN1*, *TaASN2*, and *TaASN3*, and a fourth, more recently identified gene, *TaASN4*. *TaASN1*, *TaASN2*, and *TaASN4* were all found to be single copy genes, located on chromosomes 5, 3, and 4, respectively, of each genome (A, B, and D), although variety Chinese Spring lacked a *TaASN2* gene in the B genome. Two copies of *TaASN3* were found on chromosome 1 of each genome, and these were given the names *TaASN3.1* and *TaASN3.2*. The TaASN1, TaASN2, and TaASN3 PCR products were heterologously expressed in *Escherichia coli* (*TaASN4* was not investigated in this part of the study). Western blot analysis identified two monoclonal antibodies that recognized the three proteins, but did not distinguish between them, despite being raised to epitopes SKKPRMIEVAAP and GGSNKPGVMNTV in the variable C-terminal regions of the proteins. The heterologously expressed TaASN1 and TaASN2 proteins were found to be active asparagine synthetases, producing asparagine and glutamate from glutamine and aspartate. The asparagine synthetase reaction was modelled using SNOOPY® software and information from the BRENDA database to generate differential equations to describe the reaction stages, based on mass action kinetics. Experimental data from the reactions catalysed by TaASN1 and TaASN2 were entered into the model using Copasi, enabling values to be determined for kinetic parameters. Both the reaction data and the modelling showed that the enzymes continued to produce glutamate even when the synthesis of asparagine had ceased due to a lack of aspartate.

3.5.2. Key findings

The study established that there are single copies of *TaASN1*, *TaASN2*, and *TaASN4*, and two of *TaASN3*, and identified their chromosomal locations. The relatively simple structure of the gene family means that genetic interventions to reduce free asparagine accumulation and thereby

acrylamide-forming potential in wheat grain are more likely to be successful. It also showed that wheat asparagine synthetase enzymes, TaASN1 and TaASN2, can be expressed in *E. coli* and analysed biochemically.

Modelling of the reactions catalysed by TaASN1 and TaASN2 showed the two enzymes to be biochemically very similar (Figure 5). This, together with the expression data showing *TaASN2* gene expression rising to 10 times that of *TaASN1* by mid-development, reaffirms the conclusion that TaASN2 is the major enzyme synthesizing asparagine in wheat grain, and therefore an appropriate target for genetic interventions to reduce free asparagine accumulation.

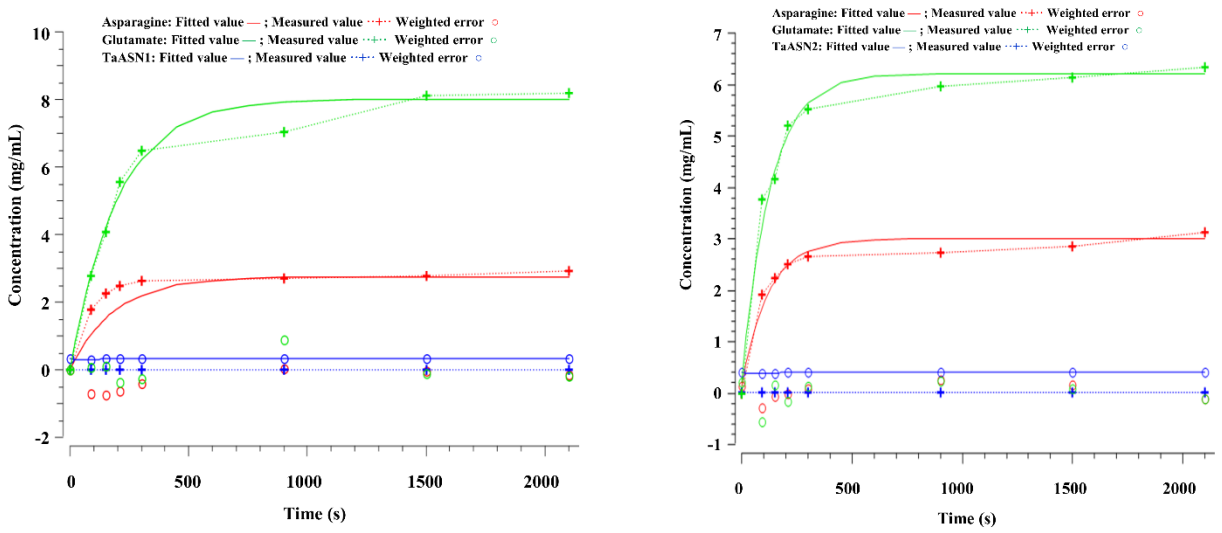

Figure 5. Time-series plots for parameter estimation against experimental data for TaASN1 (left) and TaASN2 (right), illustrating the similarity in activity of the two enzymes. Reproduced from: Frontiers in Plant Science 8, 2237.

3.6. Gene expression profiling (RNAseq) of cultivar Spark and doubled haploid (DH) line SR3,

Gene expression profiling (RNAseq) was carried out for cultivar Spark and doubled haploid line SR3 from the Spark × Rialto mapping population generated by the John Innes Centre Wheat Genetics Group. SR3 has a lower concentration of free asparagine in the grain than either of its parents (Figure 6). RNA was prepared from embryo and endosperm separately, at 14 and 21 days post-anthesis, in plants grown under glass and either supplied with or deprived of sulphur. Four reps were prepared for each genotype × treatment × tissue × timepoint, making 64 samples in all. The RNA was sent to GATC for RNAseq analysis, with 15M 125bp PE reads/sample and a strand specific cDNA library. The huge RNAseq datasets are currently being analysed in collaboration with the Rothamsted bioinformatics team and a paper prepared for publication. The data will be

used to refine the model of asparagine metabolism, identify the key enzymes and genes that determine free asparagine concentration in wheat grain, locate the predominant site of synthesis of free asparagine in the grain under different sulphur feeding regimes, and identify additional targets for genetic intervention.

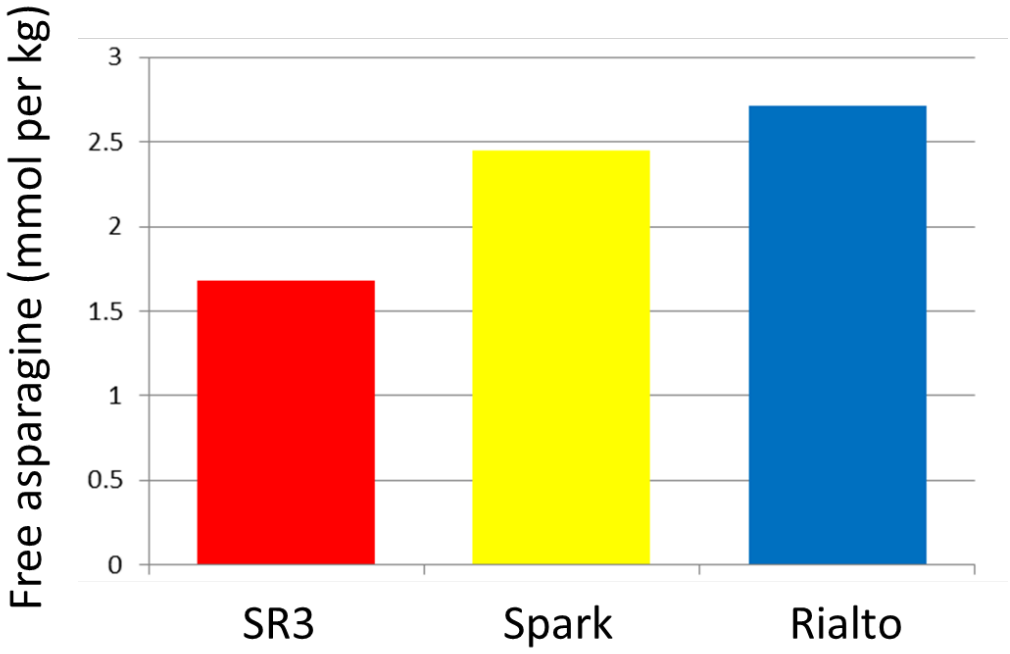

Figure 6. Free asparagine concentration in flour from wheat cv Spark and Rialto, and doubled haploid line SR3. Plotted from data produced by Curtis *et al*. (2009).

3.7. QTL/genetic markers

Data on free asparagine and other amino acid concentrations were obtained from three years of field trials at Rothamsted of the Spark × Rialto mapping population supplied by John Innes Centre, from 2007-8 (predating the present project) to 2009-10. The grain from the different genotypes showed a good spread of asparagine concentration, from 1.6 to 25 mmol per kg. Grain from the Avalon × Cadenza population was also analysed, but this showed a narrower range of concentrations. In 2013, it became clear from analysis of QTL controlling thousand grain weight that a genotype mix-up in the Spark × Rialto population must have occurred. DNA was prepared from seedlings from the three trials where seeds still germinated. Genotyping with 8 discriminatory SNP markers revealed that a larger number (about 32 %) of the Spark × Rialto genotypes were either contaminated or not correct due to a mix-up in JIC multiplication plots. It was unfortunately impossible to resolve the problem, so the data from 2008-9 and 2009-10 could not be used.

The QTL that were identified from the 2007-8 trial were quite weak and without further data to narrow and confirm them are unlikely to be of high interest for breeders. Mainly, a QTL on

chromosome 5A was detected, which influences the concentration of several free amino acids, including asparagine, explaining between 7 and 11 % of the variation observed, with the decreasing allele on Spark. No asparagine-specific QTL were detected.

Analysis of free amino acid concentrations in the Avalon × Cadenza population detected a minor QTL for asparagine concentration on chromosome 1A, with a decreasing effect from Avalon, and a QTL for glutamine concentration was mapped to a similar position. However, no major QTL (that is explaining more than 5-6 % of the variation observed) was detected.

The John Innes Centre team developed a mapping population from a cross between two soft wheat varieties, Claire (low asparagine) × Robigus (high asparagine). This population was the first of its kind. Two methods were employed to ensure success of the population development, the Doubled Haploid (DH) method and the Single Seed Descent (SSD) method. The DH population contains 176 lines, while the SSD population consists of 160 lines.

3.8. TILLING and GM

TILLING

Mutant wheat populations have been produced by colleague Dr Andy Phillips at Rothamsted and are held by the JIC Wheat genetics group. Complete genomic data for these populations became available recently, enabling the identification of mutant lines carrying mutations in each of the *TaASN2* genes. These are being stacked within a follow-up SW-DTP CASE PhD studentship, also involving AHDB.

GM

Virus-induced gene silencing (VIGS) was used to manipulate asparagine synthetase gene expression in wheat, but this was not successful. However, CRISPR-Cas9 genome editing has been used within the context of the SW-DTP CASE PhD studentship to create ‘knockouts’ for *TaASN2*, using the data obtained within the present project. Plants have been regenerated and are being analysed.

The TILLING and CRISPR-Cas mutagenesis approaches have the potential to effectively solve the acrylamide problem, but this depends on how the plants respond to the intervention, and, in the latter case, how genome edited plants are regulated in Europe.

3.9. Low free asparagine wheat in industrial processes

Low free asparagine genotype SR3 was bulked up at Saaten Union to enable its performance to be assessed in real food systems by food industry members of the consortium. Seed was provided to Saaten Union by the John Innes Centre in 2012 and grain was produced for these analyses in

2013. However, the genotype that was supplied turned out to be SR2, not SR3, and SR2 is not a low asparagine genotype. This part of the project was therefore severely delayed. Confirmed SR3 seed was bulked up and supplied to Saaten Union for planting in autumn 2014, with grain available for analysis from August 2015. A no-cost extension to the project was granted so that the food industry partners could complete their analyses.

Product trials were conducted by Pladis (UB) and Weetabix. Pladis used flour produced at Lantmaanen and there were problems with the milling, so the results should be considered in that light. Although the free asparagine concentration in SR3 was lower than in the Pladis standard flour, the acrylamide content of the biscuit product was similar. However, the acrylamide content was about half that typically associated with that product. It was noted that there were additional sources of asparagine (egg and malt) in the recipe, as well as ammonium bicarbonate (required for the finished product to be of acceptable quality).

Weetabix made a product from whole, un-milled grains. Free asparagine concentration was confirmed to be lower in SR3 than in their standard grain samples that were used for comparison. Acrylamide levels after cooking correlated with free asparagine concentration but this relationship broke down in the finished product.

The overall conclusions from these studies were that the relationship between free asparagine formation and acrylamide formation was more complex in commercial products than in standard heated flour tests, and that the addition of additional sources of asparagine or ammonium bicarbonate could nullify any advantage of using a low asparagine genotype. Note that the use of ammonium bicarbonate is not allowed in the compulsory Code of Practice for bakery products drawn up by the European Commission; this will be in place by April 2018.

3.10. Output

3.10.1. Refereed papers

Curtis, T.Y and Halford, N.G. (2014) Food security: The challenge of increasing wheat yield and the importance of not compromising food safety. *Annals of Applied Biology* **164**, 354-372. DOI:10.1111/aab.12108

Curtis, T.Y., Postles, J. and Halford, N.G. (2014) Reducing the potential for processing contaminant formation in cereal products. *Journal of Cereal Science* **59**, 382-392. DOI: 10.1016/j.jcs.2013.11.002.

Halford, N.G., Curtis, T.Y., Chen, Z. and Huang, J. (2015) DARWIN REVIEW: Effects of abiotic stress and crop management on cereal grain composition: implications for food quality and safety. *Journal of Experimental Botany* **66**, 1145-1156, doi: 10.1093/jxb/eru473

Curtis, T.Y. and Halford, N.G. (2016) Reducing the acrylamide-forming potential of wheat. *Food and Energy Security* **5**, 153-164, doi: 10.1002/fes3.85.

Curtis, T.Y., Powers, S.J. and Halford, N.G. (2016) Effects of fungicide treatment on free amino acid concentration and acrylamide-forming potential in wheat. *Journal of Agricultural and Food Chemistry* **64**, 9689-9696, doi: 10.1021/acs.jafc.6b04520.

Gao, R., Curtis, T.Y., Powers, S.J., Xu, H., Huang, J. and Halford, N.G. (2016) Food safety: Structure and expression of the asparagine synthetase gene family of wheat. *Journal of Cereal Science* **68**, 122-131, doi: 10.1016/j.jcs.2016.01.010.

Curtis, T.Y., Powers, S.J., Wang, R. and Halford, N.G. (2018) Effects of variety, year of cultivation and sulphur supply on the accumulation of free asparagine in the grain of commercial wheat varieties. Food Chemistry **239**, 304-313, doi: 10.1016/j.foodchem.2017.06.113.

Curtis, T.Y., Bo, V., Tucker, A. and Halford, N.G. (2018) Construction of a network describing asparagine metabolism in plants and its application to the identification of genes affecting asparagine metabolism in wheat under drought and nutritional stress. *Food and Energy Security* **7**, e00126. doi:10.1002/fes3.126.

Xu, H., Curtis, T.Y., Powers, S.J., Raffan, S., Gao, R., Huang, J., Heiner, M., Gilbert, D. and Halford, N.G. (2018) Genomic, biochemical and modelling analyses of asparagine synthetases from wheat. *Frontiers in Plant Science* **8**, 2237, doi: 10.3389/fpls.2017.02237.

3.10.2. Popular articles

Frood, Arran. Under the bar: Acrylamide and Food Safety. BBSRC News: http://www.bbsrc.ac.uk/news/food-security/2013/130206-f-acrylamide-and-food-safety.aspx

Global Food Security: http://www.foodsecurity.ac.uk/research/current/acrylamide-and-food-safety.html

A shortened version of this, edited by Tracey Duncombe, appeared in BBSRC Business Magazine, Spring 2013

3.10.3. Book chapters

Halford, N.G. and Curtis, T.Y. (2016) Acrylamide in Cereals: The Problem and Potential Genetic and Agronomic Solutions. In: Biotechnology of Major Cereals, ed. Jones, H. CAB International (CABI), Wallingford, Chapter 13, pp. 165-178.

Halford, N.G. and Curtis, T.Y. (2016). Reducing the Acrylamide-Forming Potential of Wheat, Rye and Potato: A Review. In: ACS Symposium Series, Vol. 1237, Browned Flavors: Analysis, Formation, & Physiology, ed. Granvogl, M., American Chemical Society, Washington DC, pp. 35-53. Chapter 4, DOI: 10.1021/bk-2016-1237.ch004

3.10.4. Conference papers

Curtis, T and Halford, N.G. (2013) Advances in reduction of acrylamide-forming potential in wheat. Aspects of Applied Biology 116, Acrylamide, furans and other food-borne contaminants, from plant science to food chemistry, 131-136.

3.10.5. Conference abstracts

Curtis, T. and Halford, N. (2012) Genetic and environmental factors controlling acrylamide formation in wheat products. UK PlantSci 2012, Norwich.

Curtis, T., Halford, N.G., Gao, Q. and Gilbert, D. (2012) Modelling asparagine synthetase function in wheat using Petri nets. Computational Methods in Systems Biology, 3rd - 5th October 2012, Brunel University.

3.10.6. Sequence database entries

KY937995
Triticum aestivum asparagine synthetase-1 (TaASN1), cds.
KY937996
Triticum aestivum asparagine synthetase-2 (TaASN2), cds.
KY937997
Triticum aestivum asparagine synthetase-2 (TaASN2), cds.

3.10.7. Conference presentations by the PI

The 21st Conference of the International Plant Growth Substances Association (IPGSA), Shanghai, China, June18th to 22nd, 2013. Interactions between sugar, amino acid and stress signalling pathways in plants, and the implications for food quality and safety.

Acrylamide workshop, European Commission, Directorate General for Health and Consumers, Unit E3: Chemicals, Contaminants and Pesticides. Brussels, 13th-14th January 2014. Genetic and agronomic approaches to acrylamide reduction: progress and prospects for potato, wheat and rye.

Monogram Annual Meeting, Reading, March 2014. Food safety: Reducing the acrylamide-forming potential of wheat and rye.

SIK meeting, Gothenburg, March 2014. Genetic and agronomic approaches to acrylamide reduction: progress and prospects for potato, wheat and rye.

Agricultural Genomics, London, April 2014. Genomics Approaches to Improving the Food Safety of Wheat, Rye and Potato.

9th International Workshop on Sulfur Metabolism in Plants: Molecular Physiology and Ecophysiology of Sulfur, Freiburg, Germany, April 2014. Effect of sulphur availability on the acrylamide-forming potential of wheat, rye and potato.

Cereal Partners Worldwide, Grain Summit, Orbe, Switzerland, June 2014. Genetic and agronomic approaches to acrylamide reduction in wheat and rye.

SELECTBIO on-line, October 2014: Advances in Plant Genomics. Processing contaminants: a problem that will not go away for the food industry and on which plant scientists and breeders must engage.

NABIM Research and Development Workshop, December 2nd 2014.

Food, Nutrition and Agricultural Genomics Congress, London, April 20th – 21st 2015.

Campden-BRI Cereals, Milling and Baking Members Interest Group Meeting, May 6th 2015.

Federation of Bakers Workshop on Acrylamide, May 11th 2015.

SELECTBIO on-line, October 2015: Advances in Plant Genomics (APG 2015).

Wheat – genetic improvement of end use quality. 9-10 February 2016. Association of Applied Biologists, Rothamsted Research, Harpenden, Herts.

Food Security Annual Conference 2016 – Lancaster University, 19th – 20th April 2016. Acrylamide and food safety.

Food Safety 2016: 2nd International Conference on Food safety and Regulatory Measures. London, June 6th-8th 2016. Session on 'Formation and analysis of food-borne toxicants'. Reducing the acrylamide-forming potential of wheat, rye and potato.

2nd Global Summit on Plant Science, London, October 6th – 8th 2016. Genetic and agronomic approaches to reducing the acrylamide-forming potential of wheat.

Speaker and session chair, BIT's 7th World Gene Convention-2016 (WGC-2016), November 3rd – 5th, 2016, Shanghai, China. Crop improvement for food safety: reducing the acrylamide-forming potential of wheat, rye and potato.

BBSRC showcase, Campden BRI, 6th July 2016. Genetic improvement of wheat to reduce the potential for acrylamide formation during processing.

Healthgrain Forum 2017 Spring Workshop, Leuven, Belgium, 2nd – 3rd May 2017

254th American Chemical Society National Meeting & Exposition, Washington DC, USA, August 2017. Session on Food-Borne Toxicants.

Royal Society of Chemistry, Acrylamide Update, 16th November 2017, Reading.

3.10.8. Knowledge exchange

The PI presented a webinar to CEEREAL Technology Group on the acrylamide issue: 12th June 2017.

The PI was an 'Expert speaker' (at the invitation of CEEREAL/FoodDrinkEurope) at a meeting of the European Commission's Civil Dialogue Group: CDG ARABLE CROP, 4th July 2017, DG-Agri, Rue de la Loi, Brussels.

The PI held meetings with CEEREAL, Brussels, to discuss the acrylamide issue and possible grant applications, 29th November 2017.

3.10.9. Public engagement

The PI was interviewed on the acrylamide issue by Meera Senthilingam for CNN, 23rd January 2017. On-line news and broadcast in the US. Quotes from this interview were picked up by >60 other media outlets.

The PI participated in BBC Radio 4 You and Yours programme on Thursday 13th June 2013 on the acrylamide issue.

The PI was interviewed by Volker Mrasek for Deutschlandfunk, WDR (West German Radio) and NDR (North German Radio) on the acrylamide issue, 22nd August 2013.

The PI was interviewed for Newstalk ZB (New Zealand) 23rd January 2017.

3.10.10. Follow-up funding

BBSRC Pathfinder, BB/P017541/1: Automated analysis of free amino acids for acrylamide reduction in wheat-based food matrixes: applications in food production and commercial testing" (IP Pragmatics). 17/11/16 to 16/02/17. £16728.

South West Biosciences Doctoral Training Partnership CASE studentship: Genome editing for low acrylamide wheat. Partners: Agriculture and Horticulture Development Board, KWS UK Ltd., Saaten Union UK Ltd, RAGT Seeds Ltd; Syngenta UK Ltd, and Limagrain UK Ltd.

AHDB. Effect of sulphur fertilisation on the acrylamide-forming potential of wheat. Home Grown Cereals Authority Project Report No. 2170001. July to October 2013. £7978.

4. Discussion

The study achieved the following:

- Identification of low and high asparagine wheat varieties. The use of low asparagine varieties for cereal products is included in the European Commission's compulsory Code of Practice, where wheat is grown to contract.

- Assessment of variation in asparagine concentration in a wide range of commercial and non-commercial genotypes.
- New information on the asparagine synthetase gene family in wheat and identification of a target for genetic intervention.
- Valuable tools for the study of asparagine synthetase in wheat, including cloned genes, expressed proteins and monoclonal antibodies.
- Huge datasets on gene activity that will inform the modelling of free asparagine metabolism in wheat and the genetic control of asparagine accumulation.
- First soft wheat mapping population produced.
- A model for asparagine metabolism that is far more detailed than anything else in the scientific literature.
- Identification of wheat plants carrying mutations in TaASN2, the most highly expressed asparagine synthetase gene in the grain.
- Data on the effect of reduced concentration of free asparagine and other amino acids on food processing and product quality.
- Assessment of effect of fungicide treatment on asparagine accumulation. To follow 'good phytosanitary practices to prevent fungal infection' has been included in the European Commission's compulsory Code of Practice for wheat products.
- As a result of a short, associated project funded by AHDB, advice on sulphur supply to wheat has been updated, with a recommendation of 20 kg S per hectare for all wheat destined for human consumption (Project Report No. 525). Ensuring sulphur sufficiency is also included in the European Commission's compulsory Code of Practice for cereals.

5. References

Other than those given in section 3.10.1

Avila-Ospina, L., Marmagne, A., Talbotec, J. and Krupinska, K. (2015) The identification of new cytosolic glutamine synthetase and asparagine synthetase genes in barley (*Hordeum vulgare* L.), and their expression during leaf senescence. *Journal of Experimental Botany* **66**, 2013-2026.

Byrne, E.H., Prosser, I., Muttucumaru, N., Curtis, T.Y., Wingler, A., Powers, S. and Halford, N.G. (2012) Overexpression of GCN2-type protein kinase in wheat has profound effects on free amino acid concentration and gene expression. *Plant Biotechnology Journal* **10**, 328-340.

Curtis T.Y., Muttucumaru, N., Shewry, P.R., Parry, M.A., Powers, S.J., Elmore, J.S., Mottram, D.S., Hook, S. and Halford, N.G. (2009) Effects of genotype and environment on free amino acid levels in wheat grain: implications for acrylamide formation during processing. *Journal of Agricultural and Food Chemistry* **57**, 1013-1021.

Curtis, T., Halford, N. G., Powers, S. J., McGrath, S. P. and Zazzeroni, R. (2014) Home Grown Cereals Authority Project Report No. 525. Agriculture and Horticulture Development Board, Stoneleigh Park, Kenilworth, UK, 2014.

European Commission (2013) Commission recommendation of 8 November 2013 on investigations into the levels of acrylamide in food. European Commission, Brussels.

European Commission (2017) Establishing mitigation measures and Benchmark Levels for the reduction of the presence of acrylamide in food. European Commission, Brussels.

European Food Safety Authority Panel on Contaminants in the Food Chain (CONTAM) (2015) Scientific opinion on acrylamide in food. *EFSA Journal* **13**, 4104.

Granvogl, M., Wieser, H., Koehler, P., Von Tucher, S. and Schieberle, P. (2007) Influence of sulfur fertilization on the amounts of free amino acids in wheat. Correlation with baking properties as well as with 3-aminopropionamide and acrylamide generation during baking. *Journal of Agricultural and Food Chemistry* **55**, 4271–4277.

Halford, N.G., Curtis, T.Y., Muttucumaru, N., Postles, J., Elmore, J.S. and Mottram, D.S. (2012) The acrylamide problem: a plant and agronomic science issue. *Journal of Experimental Botany* **63**, 2841-2851.

Halford, N.G., Curtis, T.Y., Muttucumaru, N., Postles, J. and Mottram, D.S. (2011) Sugars in crop plants. *Annals of Applied Biology* **158**, 1-25.

Mottram, D.S., Wedzicha, B.L. and Dodson, A.T. (2002) Acrylamide is formed in the Maillard reaction. *Nature* **419**, 448-449.

Muttucumaru, N., Halford, N.G., Elmore, J.S., Dodson, A.T., Parry, M., Shewry, P.R. and Mottram, D.S. (2006) The formation of high levels of acrylamide during the processing of flour derived from sulfate-deprived wheat. *Journal of Agricultural and Food Chemistry* **54**, 8951-8955.

Shewry, P.R., Halford, N.G. and Lafiandra, D. (2003) The Genetics of Wheat Gluten Proteins. *Advances in Genetics* **49**, 111-184.

Todd, J., Screen, S., Crowley, J., Penga, J., Andersen, A., Brown, T., Qi, Q., Fabri, B. and Duff, S.M.G. (2008) Identification and characterization of four distinct asparagine synthetase (*AsnS*) genes in maize (*Zea mays* L.). *Plant Science* **75**, 799-808.