



Office Use Only

Project Code	
Project Type	

FINAL REPORT 2024

Final reports must be submitted using the online application form at www.sagit.com.au with this Word document attached **within two months** after the completion of the Project Term.

PROJECT CODE	UA420
PROJECT TITLE	Enhancing grain production and quality traits for bread wheat

PROJECT DURATION <i>These dates must be the same as those stated in the Funding Agreement.</i>					
Project start date	1/07/2020				
Project end date	31/05/2024				
SAGIT Funding	2020/21		2021/22		2022/23

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PROJECT REPORT: Please provide a clear description for each of the following:

Executive Summary (200 words maximum)

The project built on our discovery that microRNA resistant alleles of *HOMEODOMAIN2* on the A and D subgenomes (*HB-A2* and *HB-D2*) increase spikelet number and grain protein content (GPC) in wheat. The project also included a missense allele of *RAMOSA-D2* (*RA-D2*), which was tested because it increases spikelet number and yield under certain field conditions. We identified these alleles in the Cadenza mutant population; the project aim was to introduce the identified alleles into elite Australian cultivars and test if they increase GPC and yield-related traits in these backgrounds.

MicroRNA resistant alleles of *HB-A2* and *HB-D2*, and the missense *RA-D2* allele, were introduced into Mace (AGT), Rockstar and Sheriff (Intergrain). BC₂F₄ germplasm was grown in two field trials based in Roseworthy; one field trial was performed by the University of Adelaide and the second by Australian Grain Technologies (AGT).

The field trials supported our previous work:

- Lines with the mutant alleles of *HB-A2* and *HB-D2* produced grain with more protein than wild-type siblings.
- In some cases, these mutant lines produced more grain.
- Regarding *RA-D2*, some lines expressing the mutant allele produced more grain per spike (weight) than the wild-type siblings, but there was no effect on GPC.

Project objectives

The project aim was to introduce genetic diversity that will help breeders improve grain quality and yield-related traits of wheat. The work is following three specific objectives:

- 1) Introduce alleles that increase grain protein content (GPC) and floret fertility into elite bread wheat varieties.
- 2) To measure the effect of these alleles on yield-related traits and grain protein content.
- 3) Generate advanced germplasm using the alleles from Objective 1 for use as pre-breeding lines by commercial breeding programs for delivery of the improved traits to market.

Overall Performance

We achieved all the major project objectives, including 1) the introduction of GPC alleles into elite varieties, 2) analysis of the effect of these alleles on GPC and floret fertility, and 3) generation of advanced germplasm for use as pre-breeding lines by commercial breeding companies. The only component that is still ongoing is analysis of GPC from plants grown under controlled environment conditions; this work has not been completed yet because of staff leaving the position and because of equipment limitations.

The personnel who have worked on this project include: Scott Boden, Kara Levin, Julian Qu, Hui Zhou, Suong Cu, and Apriadi Situmorang (University of Adelaide), and James Walter, Cathrine Ingvordsen and Takara Fort (AGT). The main difficulties encountered have related to staff turnover due to the relatively low job security of this position, and some delays in access to growth facilities during the Covid-19 pandemic.

KEY PERFORMANCE INDICATORS (KPI)

KPI	Achieved	If not achieved, please state reason.
Generate NILs for three genes in elite backgrounds (Obj. 1)	Yes <input checked="" type="checkbox"/> No <input type="checkbox"/>	
Test GPC and nutrient levels in grain of NILs	Yes <input checked="" type="checkbox"/> No <input type="checkbox"/>	This KPI has been partially achieved, as we have not yet measure macro-nutrient levels. This was not completed due to staffing issues and

Examine inflorescence development and floret fertility of NILs	Yes <input checked="" type="checkbox"/> No <input type="checkbox"/>	
Test for NUE using glasshouse trials	Yes <input checked="" type="checkbox"/> No <input type="checkbox"/>	We have performed these experiments but are still waiting to complete the analysis of grain quality. We determined based on the phenotypes of yield-related traits that the plants did not display improved NUE.
Analyse NUE, GPC and yield related traits in NILs under field-based trials.	Yes <input checked="" type="checkbox"/> No <input type="checkbox"/>	We could not perform NUE analysis under field conditions due to limitations in seed quantities needed for field trials. The GPC and yield related traits were evaluated.
Generate advanced RILs in elite backgrounds	Yes <input checked="" type="checkbox"/> No <input type="checkbox"/>	
Annual progress reports submitted to SAGIT	Yes <input checked="" type="checkbox"/> No <input type="checkbox"/>	
Final report submitted to SAGIT	Yes <input checked="" type="checkbox"/> No <input type="checkbox"/>	

TECHNICAL INFORMATION (Not to exceed **three** pages)

Allele information: The variant alleles used in this project included microRNA resistant alleles of *HB-A2* and *HB-D2*, and a missense allele of *RA-D2* (Table 1). The *HB-A2* and *HB-D2* alleles are semi-dominant because they cause *HB-A2* and *HB-D2* to be expressed more highly and the missense allele of *RA-D2* changes alanine to threonine at position 91 (A91T). The alleles were identified in mutant lines of the Cadenza TILLING population from the John Innes Centre.

Gene	Line of origin	Mutation	KASP marker sequences (SNP, common)
<i>HB-A2</i>	WCAD1761	G>A, 580 bp	GATTGGGTCCCCGATGCCT[G/A], ACAAGAACCCTCCAGAATTGGT
<i>HB-D2</i>	WCAD1290	G>A, 575 bp	TGAATTGGCAACTGACCTTCATC[C/T], TGTAAGAAGCTTGTACATGTCTC
<i>RA-D2</i>	WCAD1591	G>A, 271 bp	GCAACAATTTACTCAAACGGG[C/T], TGGATCACAAGGAAGGAGGA

Germplasm development: The *HB-A2*, *HB-D2* and *RA-D2* alleles were introduced into the elite Australian cultivars of Mace (AGT), Rockstar and Sheriff (Intergrain) via marker assisted selection using KASP PCR (Table 1). The process involved crossing each Cadenza TILLING line to Mace, Rockstar and Sheriff, and heterozygous F₁ plants were backcrossed twice to the respective elite parent line to generate BC₂F₁ families; at least 3-4 independent families were carried through for each cross. Segregating BC₂F₂ families were genotyped to select plants that contained either the mutant or wild-type reference allele for the respective genes; these plants were grown through two more generations to produce BC₂F₄ seed, which included a round of bulking in either the glasshouse or a summer nursery (performed by AGT). This work completed the work for **Objective 1**. We used the BC₂F₃ plants to cross to the elite parent line to generate advanced BC₃F₂ lines, which have been genotyped by KASP PCR. These seed are available to pre-breeding programs of commercial companies, and this works completes **Objective 3**.

Field trials and trait analysis: Two replicated field trials were performed in Roseworthy/Reeves Plains, with one trial conducted by the University of Adelaide and the other by AGT. The University trial was conducted as a double row trial in a randomized block design prepared by Dr Julian Taylor (University of Adelaide) and the AGT trial was conducted in plots in a randomized block design prepared by Dr James Walter and Dr Cathrine Ingvordsen (AGT). For each trial, we measured the **following traits**: flowering time, height, spikelet number per spike, floret fertility, grain number per spike, grain weight per spike, thousand grain weight, total paired spikelets, and grain protein content.

WCAD1290 (HB-D2) allele

Analysis of the *HB-D2* allele was performed to address the following questions:

- Do both the homozygous and heterozygous mutant lines form paired spikelets in the elite Australian backgrounds? If they do form paired spikelets, is the phenotype too strong that it would limit the usage of these lines by commercial breeders?
- Do the homozygous mutant plants display the leaf curling phenotype observed in the Cadenza background when grown under field conditions?
- What are the phenotypes of plants that are heterozygous for the microRNA resistant *HB-D2* allele, relative to those that homozygous for the mutant allele?
- Does the microRNA resistant allele improve yield-related traits and grain protein content in the elite Australian backgrounds?

Regarding the paired spikelet phenotype, the heterozygous and homozygous mutants formed secondary spikelets in each of the three elite backgrounds (Figure 1). As observed with the Cadenza TILLING lines, the phenotype was semi-dominant, with homozygous mutants displaying a stronger phenotype than heterozygous genotypes (e.g. cv. Mace and Rockstar). The phenotype appeared stronger in the cv. Rockstar background. While all genotypes formed secondary spikelets, the AGT breeders were not too concerned about presence of the trait, and would consider these lines in their breeding program if the grain protein content was higher than wild-type siblings.

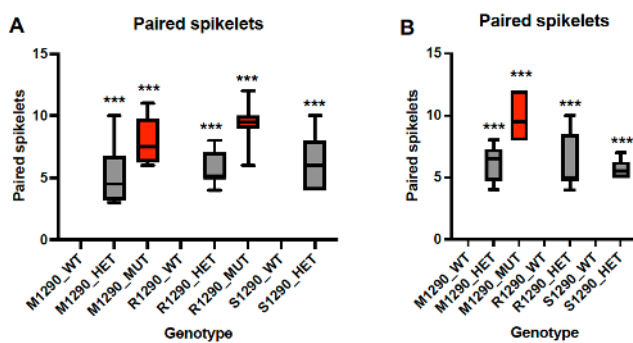


Figure 1: MicroRNA resistant alleles promote paired spikelet development. Heterozygous (grey) and homozygous (red) mutants formed paired spikelets in the (A) University and (B) AGT trial. *M1290*, *R1290* and *S1290* are Mace, Rockstar and Sheriff genotypes, respectively. In the boxplots, each box is bound by the lower and upper quartiles, the central bar is the median, whiskers show minimum and maximum values of 8-10 biological replicates. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Results: Homozygous mutant lines formed curled leaves in the Mace and Rockstar backgrounds; however, this was less noticeable in the AGT trial because the plants had a longer vegetative growth phase as it was sown approximately 3 weeks earlier than the University trial. In both trials, the curled leaf phenotype did not prevent head emergence and leaves of heterozygous mutants did not curl. The yield-component traits that we measured for these genotypes included rachis nodes per spike (spikelet number), floret fertility, grain number per spike, grain weight per spike, and thousand grain weight. These measurements were complemented by analysis of grain protein content.

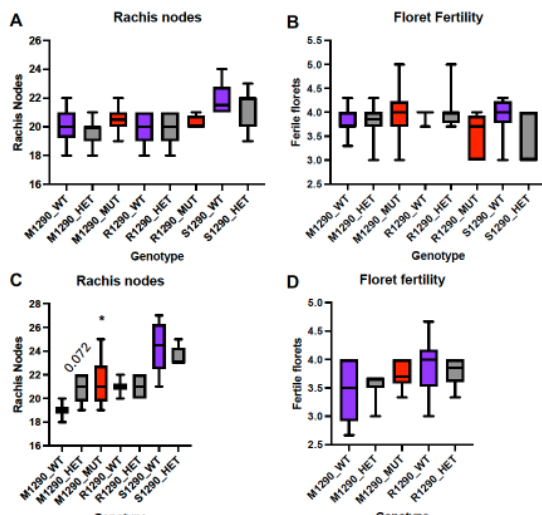


Figure 2: Analysis of rachis node and floret fertility in *HB-D2* lines. Summary of rachis nodes (A, C) and floret fertility (B, D) in the heterozygous (grey) and homozygous (red) mutants, relative to wild-type siblings (purple), in the (A-B) University and (C-D) AGT trials. *M1290*, *R1290* and *S1290* are Mace, Rockstar and Sheriff genotypes, respectively. In the boxplots, each box is bound by the lower and upper quartiles, the central bar is the median, whiskers show minimum and maximum values of 8-10 biological replicates. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Results: For rachis nodes and floret fertility, the only significant difference that we detected was for the heterozygous and homozygous genotypes in the Mace

background, which produced more nodes/spikelets that the wild-type sibling.

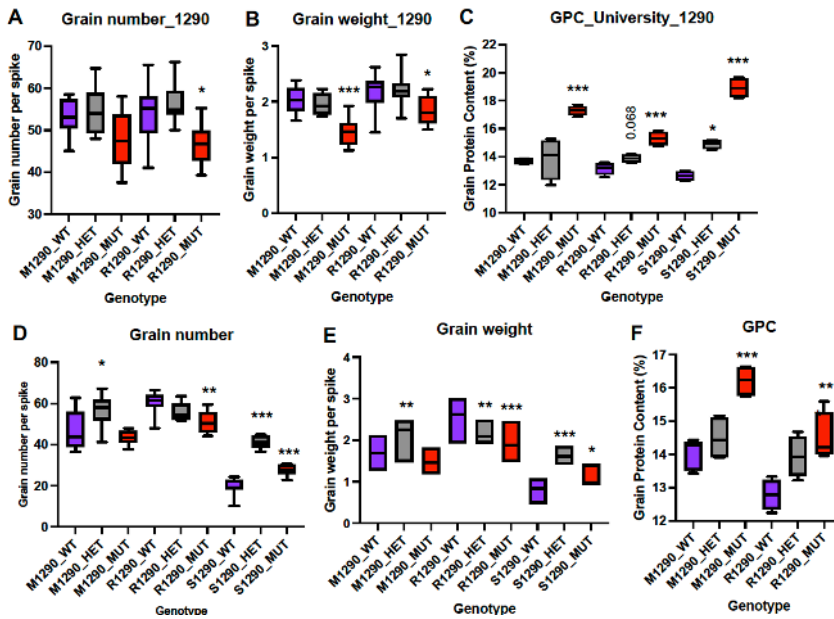


Figure 3: Analysis of yield-related traits and GPC in *HB-D2* lines. Summary of grain number (A, D) grain weight (B, E) and GPC (C, F) in the heterozygous (grey) and homozygous (red) mutants, relative to wild-type siblings (purple), in the (A-C) University and (D-F) AGT trials. *M1290*, *R1290* and *S1290* are Mace, Rockstar and Sheriff genotypes, respectively. In the boxplots, each box is bound by the lower and upper quartiles, the central bar is the median, whiskers show minimum and maximum values of 8-10 biological replicates for grain number and weight, or 4 replicates for GPC. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

the homozygous mutant lines produced grain with more protein than the wild-type sibling lines. Similarly, the heterozygous mutants produced grain with more protein, although the difference was not always significant. The homozygous mutant did tend to produce fewer grains than wild-type siblings; however, this was not always the case, especially for the Mace and Sheriff genotypes. We did not detect any substantial differences in grain number or weight for the heterozygous genotypes, which is consistent with our previous results in the Cadenza genotype. In some cases, the Sheriff lines flowered and matured late, which prevented trait analysis for these genotypes.

WCAD1761 (*HB-A2*) allele

Our analysis of the *HB-A2* involved similar traits as those for *HB-D2*, except we only phenotyped homozygous mutant and wild-type lines, without any analysis of heterozygous genotypes.

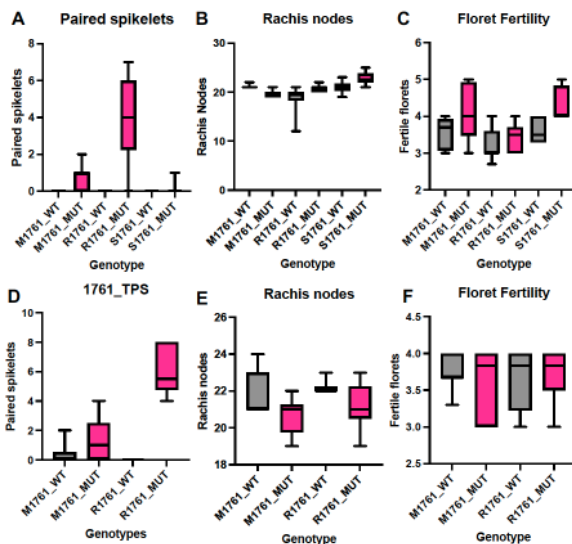


Figure 4: Analysis of spike-related traits in *HB-A2* lines. Summary of paired spikelet (A, D), rachis nodes (B, E) and floret fertility (C, F) in homozygous (pink) mutants, relative to wild-type siblings (grey), in the (A-C) University and (D-F) AGT trials. *M1761*, *R1761* and *S1761* are Mace, Rockstar and Sheriff genotypes, respectively. In the boxplots, each box is bound by the lower and upper quartiles, the central bar is the median, whiskers show minimum and maximum values of 8-10 biological replicates. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Results: The microRNA resistant allele of *HB-A2* promoted paired spikelet development in Mace, Rockstar and Sheriff, but most secondary spikelets were infertile and no concern to breeders. There was no significant effect on rachis node number or floret fertility.

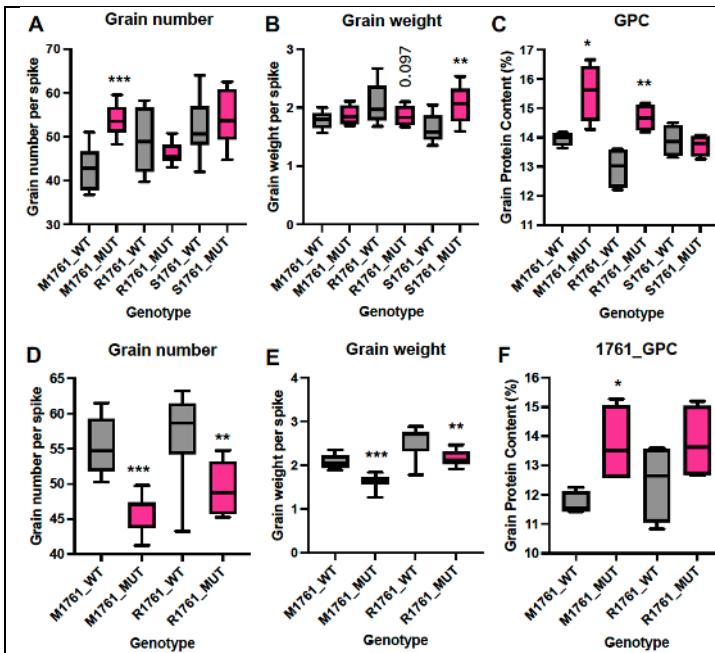


Figure 5: Analysis of yield-related traits and GPC in *HB-A2* lines. Summary of grain number (A, D) grain weight (B, E) and GPC (C, F) in the homozygous (pink) mutants, relative to wild-type siblings (grey), in the (A-C) University and (D-F) AGT trials. *M1761*, *R1761* and *S1761* are Mace, Rockstar and Sheriff genotypes, respectively. In the boxplots, each box is bound by the lower and upper quartiles, the central bar is the median, whiskers show minimum and maximum values of 8-10 biological replicates for grain number and weight, or 4 replicates for GPC. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Results: In both trials, the mutant genotypes in the Mace and Rockstar backgrounds produced grain with more protein than wild-type siblings. In the University trial, the extra protein was produced without a compensatory reduction in grain number or

weight, with mutant lines producing more grain in the Mace background. In the AGT trial, however, the mutant lines did produce less grain in the Mace and Rockstar backgrounds. Therefore, while this result is encouraging, we will need to investigate these traits further in this year's field trials.

WCAD1591 (RA-D2) allele

Our analysis of the *RA-D2* allele focused on the grain yield-related traits, including grain number and weight per spike, rachis nodes and paired spikelets. We anticipated that the *RA-D2* allele would more likely affect yield-related traits rather than GPC. The data is not shown due to space limitations.

In summary, our analysis showed that:

- Grain number and weight tended to increase in the University trial, but this was not replicated in the AGT trial.
- The mutant allele did promote paired spikelet development in the Mace and Rockstar backgrounds, and there was no significant effect on rachis node numbers.
- Due to technical limitations, we only measured GPC in the AGT trial – we did not detect any differences in GPC.

CONCLUSIONS REACHED &/OR DISCOVERIES MADE (Not to exceed one page)

Our research has provided preliminary results that microRNA resistant alleles of *HB-A2* and *HB-D2* increase grain protein content in wheat without compromising yield potential. These outcomes indicate that the beneficial effects provided by the mutant alleles identified in the Cadenza TILLING background are maintained in the backgrounds of elite Australian cultivars (Mace, Rockstar and Sheriff). Our analysis of the *RA-D2* allele indicates that it may increase the number of grain produced by a wheat plant, thereby raising yield potential. These preliminary discoveries are being investigated through a second round of field trials in 2024.

INTELLECTUAL PROPERTY

The project has generated advanced backcrossed germplasm that contains the microRNA resistant alleles of *HB-A2* and *HB-D2*, as well as a mutant allele of *RAMOSA-D2*. The original germplasm that contained these alleles were from the Cadenza TILLING population, namely *WCAD1761*, *WCAD1290* and *WCAD1591*, respectively. The background information about these alleles was reported in our article published in *Science Advances*, and this knowledge is therefore in the public domain and free of any intellectual property.

We obtained a material transfer agreement to use these alleles for research purposes. The University of Adelaide legal team advised me that the non-commercial clause of the MTA should not apply to lines derived from a cross using the original Cadenza TILLING lines, and that the progeny derived from crosses to the Australian cultivars should be excluded. Nonetheless, given that our data indicates the alleles improve GPC and yield-related traits in the Australian cultivars, it would be prudent to obtain the commercial MTA to cover the commercial use of these alleles in Australian

breeding programs. We will therefore apply for this commercial-based MTA from the John Innes Centre and Rothamsted Research.

The germplasm generated in this project includes the following: Mace, Rockstar and Sheriff backgrounds that contain the microRNA resistant alleles of *HB-A2* and *HB-D2* and a missense allele of *RA-D2* at the BC₂F₄₋₅ and BC₃F₃ generations.

APPLICATION / COMMUNICATION OF RESULTS

Main findings suitable for communication to farmers

We have increased protein content in wheat grains from elite Australian varieties by 11-26% using variant alleles of genes that control vascular development in the stem and spike.

A statement of potential industry impact

We have demonstrated the potential for microRNA resistant alleles of *HB-A2* and *HB-D2* to increase grain protein content in wheat without compensatory losses in grain yield. This outcome provides potential benefit to industry given that protein content is a major determinant of wheat grain sale price that will ultimately benefit consumers due to the higher nutritional content.

Publications and extension articles delivered as part of the project

Work of the SAGIT-funded project contributed to our research article published in the high impact journal, *Science Advances*. The citation for this article is “Dixon, L.E., et al... Boden, S.A. (2022). MicroRNA-resistant alleles of *HOMEBOX DOMAIN-2* modify inflorescence branching and increase grain protein content of wheat. *Science Advances* 8, eabn5907.”

Regarding extension work, the SAGIT-funded project has contributed to the research project of a joint PhD student between the University of Adelaide and Nottingham University, which has contributed to two articles published in *Plant Communications* (<https://doi.org/10.1016/j.xplc.2023.100716>) and *Food and Energy Security* (<https://doi.org/10.1002/fes3.498>).

We also intend to write a research article about our results from this project once we have collected the data from field trials in 2024 – we will notify SAGIT of this outcome when the article is published.

Suggested path to market

We are currently working with the pre-breeding team at AGT to further evaluate the ability of these alleles to increase grain protein content and yield related traits. If data from the 2024 field trials supports the outcomes of the 2023 trials, then we will offer this information and germplasm to commercial wheat breeders to test under their own conditions and in their own germplasm. We also intend to communicate these results at field days or annual meetings and grains updates.

POSSIBLE FUTURE WORK

We will complete the analysis of GPC in grain of plants grown under glasshouse conditions.

We have sown a second round of field trials in partnership with AGT and the University’s field trial team, with the objective of obtaining a second year of field trial data to confirm the alleles increase grain protein content using larger yield plots in multiple environments. These field trials are being funded *in-kind* by AGT and from my ARC Future Fellowship funds – we will communicate outcomes of the field trials to SAGIT at completion.

Other future work may include testing other alleles for these genes that are available in the Cadenza TILLING population.