Engineering high α-amylase levels in wheat grain lowers Falling Number but improves baking properties

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Summary
Late maturity α-amylase (LMA) and preharvest sprouting (PHS) are genetic defects in wheat. They are both characterized by the expression of specific isoforms of α-amylase in particular genotypes in the grain prior to harvest. The enhanced expression of α-amylase in both LMA and PHS results in a reduction in Falling Number (FN), a test of gel viscosity, and subsequent downgrading of the grain, along with a reduced price for growers. The FN test is unable to distinguish between LMA and PHS; thus, both defects are treated similarly when grain is traded. However, in PHS-affected grains, proteases and other degradative processes are activated, and this has been shown to have a negative impact on end product quality. No studies have been conducted to determine whether LMA is detrimental to end product quality. This work demonstrated that wheat in which an isoform α-amylase (TaAmy3) was overexpressed in the endosperm of developing grain to levels of up to 100-fold higher than the wild-type resulted in low FN similar to those seen in LMA- or PHS-affected grains. This increase had no detrimental effect on starch structure, flour composition and enhanced baking quality, in small-scale 10-g baking tests. In these small-scale tests, overexpression of TaAmy3 led to increased loaf volume and Maillard-related browning to levels higher than those in control flour when baking improver was added. These findings raise questions as to the validity of the assumption that (i) LMA is detrimental to end product quality and (ii) a low FN is always indicative of a reduction in quality. This work suggests the need for a better understanding of the impact of elevated expression of specific α-amylase on end product quality.

Introduction
Wheat is a major source of carbohydrate and protein in human diets and with approximately 240 million ha harvested worldwide; in both 2011 and 2012 (FAO Stat), wheat is the largest crop, by area, ahead of both maize and rice with 220 and 200 million ha, respectively.

In wheat, starch makes up over 70% of total grain dry weight and is made of glucose residues linked by α-1,4 glucosidic bonds and branched via α-1,6 glucosidic linkages (Ball and Morell, 2003). Reserve starch is accumulated during seed development and rapidly metabolized during germination to fuel the first stages of the seedling growth. Starch degradation requires the synergetic action of different amylolytic enzymes with the arsenal of enzymes mobilized to digest starch varying between species and starch type (Blennow and Engelsen, 2010; Lloyd et al., 2005; Ritte et al., 2002, 2006; Streb et al., 2012).

α-Amylase (EC 3.2.1.1) is an endo-hydrolase belonging to the glycoside hydrolase 13 family and is considered to be one of the primary enzymes responsible for starch degradation (Majzlova et al., 2013). The roles and number of these α-amylase isoforms vary across the plant kingdom.

In cereals, barley has at least four classes of α-amylase, HvAMY1 to HvAMY4 (Radchuk et al., 2009), while rice contains three classes OsAMY1, OsAMY2 and OsAMY3, with multiple isoforms of each present (Huang et al., 1992). In both barley and rice, all the classes have been shown to be expressed at different grain developmental stages and in various tissues (Hwang et al., 1999).

In wheat, three isoforms of α-amylase have been identified to date (Barrero et al., 2013). The two major isoforms, TaAMY1 and TaAMY2, have been extensively characterized and isolated based on their isoelectric point (pI) (Ainsworth et al., 1985). The TaAmy1 locus is a multigene family present on the long arm of Chromosome 6 and encodes high PI α-amylases in the aleurone layer of the mature grain during germination. The TaAmy2 locus encodes a single gene located on the long arm of the Chromosome 7 and produced a low PI α-amylase in the pericarp of the developing grain. Until very recently, very little was known about the third isoform TaAmy3, beyond that it is encoded on Chromosome 5 and the presence of mRNA throughout grain development implied a role in grain maturation (Baulcombe et al., 1987).

Preharvest sprouting (PHS) is a genetic defect in which the grain germinates prior to harvest. This defect is usually triggered by rainy weather when grains reach maturity and results in severe losses for the farmers (Gubler et al., 2005). During PHS, the suite of degradative enzymes involved in germination, including TaAMY1 and TaAMY2 and several proteases, are expressed. This results in loss of functionality for end uses such as baked products and noodles as the proteases and amylases digest the seed storage proteins and the starch.

Late maturity α-amylase (LMA) is a condition seen in some wheat varieties in which TaAmy1 is expressed in a small
percentage of the cells in the aleurone layer in response to environmental triggers (Mares and Mrva, 2008). The TaAMY1 protein remains present and active in the aleurone during grain development, but does not have an impact on grain morphology and size. Interestingly, a similar phenomenon has been described in rice where a temperature stress during grain maturation leads to increase in α-amylase activity causing grain chalkiness (Hwang et al., 1999).

In the case of both LMA and PHS, the expression of α-amylases results in low starch gel viscosity which can be measured as a low Falling Number (FN), a decrease in starch viscosity using Rapid Visco Analyser (RVA) or Stirring Number test (SN) (Chang et al., 2002; Codina et al., 2012; Mares and Mrva, 2008). Because of the link between degradative enzymes and reduced flour quality, it has been assumed that high levels of α-amylase in the grain, regardless of the form or source, lower baking quality. As the FN test is a simple surrogate for total α-amylase measurement, samples with a low score (below 250 s) are downgraded.

Despite grain with low FN being downgraded and excluded from milling grits, bakers have long made use of addition of α-amylases either in the form of malt flour or modern commercial enzyme preparations. Grains with insufficient α-amylase, typically indicated by FNs above 400 s, will yield bread with poor loaf volume unless α-amylase is added to the formulation to compensate (Posner and Hibbs, 2005). Enzymatic transformation of flour dough is a regular occurrence in modern bakeries (Bae et al., 2014; Sanz-Penella et al., 2014). In fact, addition of exogenous α-amylases helps generation of fermentable sugars, stimulates yeast fermentation and therefore increases bread loaf volume and shelf life (Eugenia Steffolani et al., 2012). Common best baking practices highlight the necessity of adding baking improver containing amylases and xylanases into the flour and water mixture to help improve baking quality (Blaszczak et al., 2004).

Therefore, investigations are required to determine the impact of specific α-amylases expressed during grain development on wheat bread quality. Previously, we developed lines engineered to overexpress TaAMY3 (A3OE) targeted to the amyloplasts in the endosperm of the grain and demonstrated an increase of total α-amylase activity in harvested grains from 2 to 2000-fold compared to negative segregant controls. Unexpectedly, increased activity did not have a significant impact on starch content but led to an increase of soluble carbohydrate (mainly sucrose and glucose) and triacylglycerol (TAG) in the endosperm of dry grain (Whan et al., 2014a). While the overexpressed α-amylase in our lines is a different isoform to that involved in LMA, we used these lines to study the impact of elevated levels of α-amylase in the flour on baking quality. The objectives of this study were to: (i) characterize the composition of the flour from A3OE lines, (ii) study the impact of a high level of endogenous α-amylase on flour viscosity and FN test and (iii) understand the impact of overexpression of an α-amylase on small-scale baking quality to provide preliminary evidence as to whether elevated levels of endogenous α-amylase in the mature grain impact baking quality or not.

Results

Flour composition

Grains from three A3OE lines (A4, A10 and A17) that showed between 40 and 100-fold higher levels of α-amylase than their negative segregants (A4N, A10N and A17N) (Whan et al., 2014a) were selected and milled for further study along with a tissue culture control (TCC) line and a control flour (CF). Compositional analysis of the flour was performed to confirm whether the phenotype seen in whole grains, and wholemeal flour in the previous study (Whan et al., 2014a) was present in the refined white flour.

Analysis of the carbohydrate composition and content of white flours revealed that the A3OE flours contained between two and fivefold higher levels of soluble carbohydrates compared to their respective negative segregants. Both glucose and sucrose levels increased between 1.5 and 3.5 fold (Figure 1a,b).

Triacylglycerol composition was also altered in the A3OE transgenic lines with the positive lines exhibiting a decrease in levels of linoleic acid (C18:2) and an increase in monounsaturated oleic acid (C18:1) (Figure 1c,d). A3OE lines A4, A17 and A10 showed approximately 2%, 2.8% and 6.3% conversion, respectively, compared to the controls. No other significant changes were detected in the TAG profile of these lines (Table S1).

Total flour protein contents were comprised between 14.27% (A10N) and 16.68% (A4N) and did not show any significant difference that could be linked to the presence of the transgene (Table S2). For two of the events (A4 and A17), the negative segregants had larger unextractable polymeric proteins (%UPP) than their positive counterparts, indicating larger polymer size and therefore stronger flours. In the case of A10, no significant differences were found (Figure 1e,f). A slight decrease in the proportion of the Bx high molecular weight glutenin subunit (HMW-GS) was observed in the positive lines A4 and A17 when compared with their negative counterparts. In the case of A10, the opposite occurred. Notably, this line (A10) showed an increase in the amount of the Dx HMW-GS when compared with A10N (Table S2).

Analysis of mineral ion content of the flours did not reveal any significant or consistent changes between A3OE lines and negative controls (Table S3).

Starch characterization

The presence of high levels of glucose and sucrose within the white flour A3OE lines is consistent with our previous studies on whole grains, which demonstrated no reduction in starch content, despite partial digestion of the starch granules in mature grains (Whan et al., 2014a) was present in the refined white flour A3OE lines is consistent with our previous studies on whole grains, which demonstrated no reduction in starch content, despite partial digestion of the starch granules in mature grains (Whan et al., 2014a). To investigate whether structural modifications occurred in starch structure, amylase content and chain length distribution analysis with or without preliminary β-amylolysis treatment revealing internal chains were performed. No significant differences were observed in starch amylase content or chain length distribution (Figure S1).

Differential scanning calorimetry analysis of the flours revealed that all of the lines displayed the same endothermic profile with onset (T0), peak (Tc) and conclusion (Tf) on average of 61, 70.6 and 80.7 °C, respectively (Table 1). However, the A3OE lines demonstrated a low gelatinization enthalpy (ΔH) (1.6 J/g on average) compared to the controls (2.14 J/g on average) (p = 0.04).

The RVA was also used to investigate the flour pasting properties of these lines. Figure 2 and Table 2 illustrate the complete RVA characteristics including peak viscosity, breakdown, final viscosity, trough and setback from the eight lines. The negative segregants and TCC had very similar profiles with peak and final viscosity at 114 and 200 Rapid Visco Unit (RVU) on average, respectively (Figure 2, Table 2). The profiles of the A3OE were selected and milled for further study along with a tissue culture control (TCC) line and a control flour (CF). Compositional analysis of the flour was performed to confirm whether the phenotype seen in whole grains, and wholemeal flour in the previous study (Whan et al., 2014a) was present in the refined white flour.

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Figure 1 Compositional changes in A3OE flour. Positive lines (blue) compared to negative and tissue culture controls (red). Major differences in soluble carbohydrate including glucose (A) and sucrose (B) are expressed in mg of sugar per g dry. Differences in Lipid content including Oleic (C) and linoleic (D) are expressed in percentage dry weight. Differences in unextractable polymeric proteins (UPP) (e) and (f) are expressed in percentage of total protein.
lines were very different compared to the controls, with peak viscosity and final viscosity near the detection limit (14.5 and 7.25 RVU on average). When silver nitrate an inhibitor of α-amylase was added to the A10 line, the RVA pasting profile (Table S5) was similar to the controls, demonstrating that the altered profile of the A3OE lines in the absence of silver nitrate is due to α-amylase activity.

The absence of a clear peak viscosity and the exceptionally low final viscosity of the A3OE lines are similar to the pasting profile of severely sprouted grain. Sprouting of grain is normally determined using the FN test during grain collection to prevent sprouted grain from contaminating sound, nonsprouted grain in the grain distribution and trading chain. The FN value itself is the time taken to agitate and to allow the stirrer to fall a measured distance (Table 1).

Table 1 Gelatinization properties of A3OE and control flours

| Sample             | Gelatinization endotherm |
|--------------------|--------------------------|
|                    | Onset temp. | Peak temp. | Final temp. |
|                    | Average SE | Average SE | Average SE |
| A4                 | 61.80      | 71.80      | 81.10      |
| A4.N               | 55.10      | 69.30      | 82.95      |
| A10                | 63.85      | 71.80      | 79.75      |
| A10N               | 61.65      | 70.10      | 79.80      |
| A17                | 61.55      | 70.40      | 78.90      |
| A17N               | 59.85      | 69.60      | 80.35      |
| Tissue culture control | 59.90  | 69.40      | 80.40      |
| A3OE pos average   | 60.79      | 70.68      | 80.50      |
| A3OE neg average   | 58.87      | 69.67      | 81.03      |

Temperatures (are expressed in °C). The enthalpy of gelatinization (delta H) is expressed in Joules per g (J/g). Results displayed are the mean of three independent assays.

P-values (P < 0.05) were determined with Student’s t-test (two-tailed).

Figure 2 Rapid Visco Analyser comparison between A3OE and controls flours. Positive lines (blue) compared to negative and tissue culture controls (red). Baking control CF is shown in green. Viscosity is expressed in Rapid Visco Unit (RVU). Peak viscosity, breakdown (peak–trough), setback (final–trough) and final viscosity are indicated in grey.
distance through a hot aqueous flour or meal gel undergoing liquefaction due to the action of endogenous α-amylase (AACCI Method 56-81.03). Consequently, low FNs are indicative of significant α-amylase activity and therefore assumed to identify the presence of sprouted grain.

The RVA SN test provides similar information to the FN test, and so SN evaluations were undertaken of the A3OE lines to determine how these lines would be classified by grain handlers. The α-amylase activity of these flours was also determined directly. Similarly to the results seen in the A3OE whole grain (Whan et al., 2014a), flours of our positive lines exhibited between 40 and 90-fold increases in total α-amylase activity (Figure 3a).

The SN values were converted into eFN using the calculation method developed by Ross et al. (1987). All the negative segregants and TCC were considered as sound grain with FN between 358 and 376 s. All the A3OE lines had eFN <120, with the lowest being 58 s (Figure 3b). This would result in the A3OE lines being classified as severely sprouted, segregated as unfit for milling or baking and only fit for feed wheat.

Baking studies

To investigate the impact of the elevated α-amylase levels in the flour on end product quality, small-scale rheological and baking trials were conducted. Surprisingly, the A3OE lines displayed similar profiles to their negative segregants in both Z-arm and mixograph studies (Table S4). The A3OE lines had lower water absorption levels and shorter dough development times (DDT), reduced by almost 50%, and decreased stability compared to the negative segregants. There was insufficient flour to undertake replicate Z-arm water absorption measurements to allow an assessment of the significance of these differences in DDT. Whereas the mixograph mixing times of the A3OE lines were longer than the negative segregants, however, these differences were not statistically significant. There were also no significant differences in the breakdown in dough structure with overmixing 3 and 8 min beyond optimal mixing time between the A3OE lines and their negative segregants.

In small scale, 10-g loaf baking tests, the A3OE lines had significantly greater loaf volume than the control lines without baking improver (Figure 4). When baking improver was added, the loaf volume of A3OE lines was not significantly altered; however, the specific loaf volume of control lines was maintained in the absence of baking improver addition, while in the controls, there was a reduction in loaf volume from 6 to 4.6 mL/g (Figure 4a,b,e) and colour (Figure 5a,c,e) in the absence of baking improver.

To understand the mechanism by which the altered grain composition of the A3OE lines enhanced baking properties, experiments were conducted in which equivalent amounts of each component equivalent to the A3OE levels were added to the baking mix of a selected negative segregant (A17N) individually or in combination (e.g. 3% sucrose, 0.5% glucose and commercial α-amylase to increase activity 40-fold). We selected commercial

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Table 2 Viscosity properties of A3OE and control flours

|       | Peak viscosity | Trough | Peak time | Pasting temp. | Breakdown | Setback | Final viscosity |
|-------|----------------|--------|-----------|---------------|-----------|---------|----------------|
| A4    | 12.67          | 4.25   | 5.85      | 50.05         | 8.42      | 1.58    | 5.83           |
| A4N   | 108.75         | 83.92  | 8.52      | 50.05         | 24.83     | 109.50  | 193.42         |
| A10   | 13.83          | 3.58   | 4.98      | 50.00         | 10.25     | 2.00    | 5.58           |
| A10N  | 121.33         | 92.00  | 8.72      | 49.90         | 29.33     | 119.42  | 211.42         |
| A17   | 17.75          | 6.50   | 7.85      | 50.00         | 11.25     | 3.83    | 10.33          |
| A17N  | 101.58         | 73.67  | 8.52      | 49.95         | 27.92     | 94.75   | 168.42         |
| Tissue culture control | 128.00  | 97.67  | 8.85      | 50.00         | 30.33     | 123.58  | 221.25         |
| CF    | 209.50         | 136.42 | 8.58      | 50.00         | 73.08     | 149.50  | 285.92         |

Viscosity is expressed in Rapid Visco Unit (RVU). Peak time is expressed in minutes and pasting temperature in °C. Results displayed are the mean of two independent assays.

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Figure 3 α-Amylase activity and Falling Number of A3OE and control flours. Total α-amylase activity measured against extractable protein (Unit/g flour) in flour. Equivalent Falling Number (FN) based on RVA Stirring test. FN is expressed in second. Positive lines (blue) compared to negative and tissue culture controls (red). RVA, Rapid Visco Analysers.

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α-amylase from *Aspergillus oryzae* as this α-amylase is used extensively in bakeries. Loaf volume and loaf colour were compared to the same flour negative segregant with or without addition of baking improver (Figure 6a,b). Overall, the results confirmed that addition of α-amylase improved loaf volume by 22%. However, sucrose and glucose had no effect on loaf volume as their respective volumes remained identical to their controls either with or without presence of additional α-amylase. While α-amylase was the only component affecting loaf volume, the increase in volume upon addition of exogenous α-amylase was not as marked as that seen in the A3OE lines, 22% compared to 56% on average.

Colour analysis revealed that added α-amylase or glucose alone had no effect on crust colour (a*, b* nor L*). Sucrose addition resulted in a higher a* and b* and a decrease in L*. However, the combination of the three components lead to a more pronounced increase of a* and b* associated with a strong decrease in L*. It was interesting to note that despite being in accordance with our previous baking test, the values were of a lesser magnitude than when the A3OE lines were used probably highlighting the importance of the nature of the α-amylase used.

**Discussion**

In this study, we characterized the flour and starch properties and initiated a preliminary baking quality study of wheat lines in which an α-amylase (TaAMY3) was overexpressed in the amyloplast endosperm during grain development and maturation.

**Overexpressing TaAMY3 in flour enhanced bread loaf volume**

The enhancement of loaf volume and crust colour, in the absence of baking improver in the A3OE lines, is the most striking result of this work. In effect, the body of research has generated a ‘self-improved’ flour that is produced *in planta*.

Interestingly, no major changes in starch structure and flour composition were detected with two exceptions: a slight alteration in the TAG profile and a small change in the amount of % UPP.

Triacylglycerol composition was altered with a shift of the oleic/linoleic acid ratio. A similar shift in this ratio has been described in response to cold stress and involves the enzyme FAD2-1 (Zhang et al., 2012). Interestingly, high levels of sucrose and soluble sugar have also been also implicated as a key component of the cold stress response mechanism in plants (Lunn et al., 2014). This alteration might be an illustration of the increase of sucrose and/or soluble sugar in our engineered TaAMY3 lines.

The relationship between flour protein content and loaf volume has been shown to be linear in some studies (Finney and Barmore, 1948; Wrigley and Bekes, 2002) and is used in industry as a parameter to help predict the suitability of a flour for different end products. No significant difference in protein content could be established between the A3OE lines and their respective negative controls (Table S2). Thus, changes in total protein content between the lines cannot be considered a factor in the enhancement of loaf volume and crumb colour in the absence of baking improver in the A3OE lines.

In two of the A3OE lines, the %UPP was reduced by just over 10%, while in the third line, a decrease in %UPP was observed but was not significant (Table S2). Dough strength has been shown to be positively correlated with %UPP (Gupta et al., 1993), and thus, a reduction in %UPP in the A3OE lines would be expected to reduce dough strength and negatively impact baking properties. Indeed, Zhang et al. (2008) have demonstrated strong positive correlations between %UPP and specific volume of Chinese steamed breads, and the volume and oven spring of pan breads. The decrease in %UPP along with the low FN, a universally accepted industry measure of low quality, for the A3OE lines would be expected to result in a reduction in baking quality. However, it is known that addition of α-amylase can improve baking quality (Steffolani et al., 2012). The beneficial effects of α-amylase in addition to bread quality has been postulated to result from the amylase decreasing the viscosity of the dough, via starch hydrolysis, facilitating expansion of gas
bubbles in the dough during fermentation and baking and so contributing to a greater baked loaf volume (Selman and Sumner, 1947). Further analysis needs to be performed in order to understand how proteins and starch interact in a high α-amylase flour context.

Despite having no effect on starch endothermic properties, dramatic increases in total α-amylase activities lead to dramatic effects on the flour paste viscosity. Thus, A3OE flours exhibited extremely low viscosities and final viscosity probably due to the digestion of the starch matrix preventing granule swelling and inhibiting gel formation. Interestingly, the viscosity profiles were largely restored when the α-amylase inhibitor silver nitrate was added to the A3OE lines paralleling similar findings by Bhattacharya and Corke (1996) on the affect of silver nitrate on pasting properties. This strongly suggests that the starch degradation and loss of pasting viscosity of the A3OE lines occurs only under the hydrated conditions of processing. This hypothesis is supported by the observations of no fundamental alteration in the polysaccharide structure of the A3OE lines.

Overexpressing TaAMY3 in the endosperm during grain development unexpectedly generated ‘self-improved’ flour. We hypothesized that the increased α-amylase activity was responsible for the increase in loaf volume. Increased hydrolase activity would increase the rate of starch degradation during dough development and the early stages of the baking process and provide an additional source of fermentable sugars for the yeast.

Figure 5  Baking performance of A3OE and control lines: Loaf colour (a–d) Representative set of bread loaves produce from negative segregant segregants without baking improver (a), with baking improver (b) compared to A3OE lines without baking improver (c) and with baking improver (d). (e) CIELAB colour channel values of bread loaf produced using A3OE flour (blue) compared to negative and tissue culture controls (red). CIELAB colour space parameters are a* (red), b* (yellow) and L* (dark/light).
Figure 6  Effect of additive on bread performance. (a) Volume of bread loaf produced using A3OE-negative segregant flour with or with addition of glucose, sucrose, α-amylase individually or in combination and baking improver. Specific Volume is expressed in mL per g. (b) CIELAB colour channel values of bread loaf produced using A3OE-negative segregant flour with or with addition of glucose, α-amylase, sucrose, baking improver or glucose, sucrose and α-amylase combined. Specific colour channels are a* (magenta), b* (yellow) and L* (dark/light).

to metabolize. This rapid increase in fermentation would accelerate CO₂ production and therefore increase loaf volume (Rosell et al., 2001). This is supported by the results from the addition of commercial α-amylase to flour from a negative control, which increased loaf volume (Figure 6).

Engineering TaAMY3 in flour enhanced the Maillard reaction

The Maillard reaction is a biochemical reaction in which bread crusts or baking products get their golden brown surfaces (Noguchi et al., 1982). At oven temperatures and low moisture, sugar reacts with proteins in the baking product causing the change of colour. These reactions also generate the characteristic aroma associated with the ‘baked fresh’ sensation. This caramelization process also adds flavours, improves moisture retention and prolongs shelf life (Deblander et al., 2015). The effect of sucrose addition on fermentative activity of yeast and consequently on bread quality including colour and flavour has been previously described (Voica and Codina, 2009). The A3OE flour analysis confirmed the increase of glucose and sucrose in the flour as described in A3OE grain (Whan et al., 2014a). In the flour, sucrose and glucose reached levels of 3% and 0.5% of the total flour dry weight, respectively. In addition, A3OE loaves showed significant modification of their CIELAB colour values towards the Maillard characteristic browning. It is logical that an increase of at least 3.5% in soluble carbohydrate content would enhance crust caramelization enhancing loaf crust browning. The addition of equivalent amount of sucrose to 3% (w/w) detected in the A3OE led to a significant browning of the loaf surface (Figure 6), confirming the role of additional sucrose in enhancing loaf crust colour. Interestingly, the addition of glucose alone to 0.5% (w/w) had little impact on crust colour, and it remains to be determined whether it is the type or amount of added carbohydrate that is important for loaf crumb colour. Interestingly, the browning effect was most pronounced when α-amylase, sucrose and glucose were added suggesting that simple sugars present in the flour are supplemented by the products of starch breakdown as processing continues.

TaAMY3, 3% sucrose, 0.5% glucose: the perfect recipe?

While addition of exogenous sugars or α-amylase from Aspergillus oryzae identified the key components responsible for the increases in loaf volume and Maillard browning, two key points needed to be raised.

1. The addition of both soluble carbohydrates combined with commercial α-amylase had a more pronounced effect on loaf crust colour. These observations suggested a synergetic effect of the three components on loaf properties. Previous studies have already highlighted the importance of sucrose in baking properties. Sucrose addition from 1% to 3% was shown to increase gelatinization temperature of starch (Kim and Walker, 1992). While sucrose levels of 3% (w/w) were found in the A3OE flours, no clear effect on starch DSC properties were observed. Voica and Codina (2009) determined the optimal addition of sucrose for the best baking practice to be comprised between 2% and 3% of the flour dry weight. They described sucrose as an enhancer of yeast fermentation. In fact, glucose and sucrose have been described as primary target for yeast during fermentation process [for review Verstrepen et al. (2004)]. Briefly, exposure of high level of sucrose and glucose triggered a cascade of reaction including active fermentation and repressed alternative pathways such as neoglucogenesis, respiration, stress response mechanism and cell division in yeast cell named the Crabtree effect (Meneses et al., 2002). When prolonged, this Crabtree effect condemns the yeast to a short-life-spanned burnout if high sugar concentrations are maintained. However, industrial processes allow initial high concentration of sucrose and glucose for inducing sugar signalling cascade and stimulate fermentation (Calado et al., 2003). It would be of interest to measure the fermentation kinetics of the yeast in the presence of A3OE flour to assess the potential stimulation of high sucrose level on yeast performance.
However, high sucrose content in grain is thought to have a role in reduction of starch degradation and specifically to α-amylase activity via the trehalose-6-phosphate pathway (Lunn et al., 2014b; Martins et al., 2013; Whan et al., 2014a,b). In addition, studies from Rosell et al. (2001) described a clear reduction of α-amylase activity in vitro in presence of 3 mM of sucrose. This inhibition suggested a direct interaction between the protein and the disaccharide. Interestingly, they noted that α-amylases from wheat or barley were less affected by the sucrose inhibition than fungal or bacterial α-amylase suggesting different specificity between amyloglucosidases from different origins.

2. Despite a clear increase in loaf volume and improved crust colour development when commercial fungal α-amylase was added, the scale of the modification was inferior to the result observed when the TaAMY3 was involved. This suggests a better specificity of the wheat α-amylase for baking purposes. α-amylase has been described as ‘best known amylolytic enzyme’ (Janecek et al., 2014) and the endo-hydrolase family includes over 133 groups types of hydrolyases (Majzlova et al., 2013). Many types of α-amylases have been used to improve bread making. Rosell et al. (2001) demonstrated that enzyme activities and origins strongly affected the bread making process conditions. In our attempt to identify the key factor responsible for the changes in baking properties, we chose a commercial α-amylase from Aspergillus oryzae. The rationale was to select an α-amylase used in bakeries to ensure wheat starch digestion. However, it is possible that the commercial amylase did have a higher degradation rate than the TaAMY3 in our experimental conditions and therefore completely hydrolysed more of the starch matrix before the loaf could reach its full expansion. TaAMY3 may have a slower degradation rate than could liberate soluble sugars without being detrimental to the starch/protein matrix. These results demonstrate the large variation in α-amylase activity with the process conditions and the importance of its knowledge in the selection of the appropriate α-amylase for specific product end uses.

Due to their involvement in two of the main genetic quality defects causing substantial economic loss to growers, wheat α-amylases are more often studied with a view to minimizing or mitigating their impact, rather than understanding the impact on end product quality. Therefore, the biochemical specificity of individual isoforms of wheat α-amylase in cereal starch degradation still remains unclear.

What is the real impact of grain α-amylase increase on baking quality?

Preharvest sprouting is genetic defect that causes the grain to germinate in the head before ripening, resulting in the induction of amylolytic and proteolytic enzymes (for review, see Mares and Mrva, 2014). LMA is a genetic that has been primarily detected in United Kingdom and particularly in Australia where it has been identified in a number of countries (Barnard and Smith, 1987; Mares and Mrva, 2008). However, LMA has now been identified in a number of countries (Barnard and Smith, 2012; EnNian et al., 2011; Mohler et al., 2014; Ross et al., 1987). LMA occurs in certain wheat genotypes and is characterized by abnormally high levels of the high PI α-amylase 1 (TaAMY1) in the aleurone layer of the grain during wheat grain development. Induction of the high level of TaAMY1 is in response to complex environmental effects (in particular temperature shocks between 26 and 30 days after anthesis), and it remains present through to grain maturity. As discussed previously, during grain receival testing and quality assessment for grain trading, flour viscometric assays (FN or RVA SN tests) (Hagberg, 1961) are used. As both PHS and LMA result in elevated α-amylase activity in the grain, both conditions result in a reduced FN and thus a reduction in perceived grain quality and thus value.

When grain is impacted by PHS not only is the starch matrix disrupted due to the activity of amylases, but the gluten matrix is also impaired by the activity of proteases (Ichinose, 2008). The combination of these two leads to sticky or gummy bread crumb baking the bread unpalatable and therefore results in flours that are unacceptable for the desired end product quality (Ariyama and Khan, 1990; Buchanan and Nicholas, 1980; Greenaway, 1969). Interestingly, despite previous studies demonstrating a negative effect of sprouted grains on the quality of many end products (Edwards et al., 1989; Orth and Moss, 1987), very recent work suggests a beneficial effect of using sprouted grains as additive on loaf volume and baking quality contradicting historical studies (Richter et al., 2014; Sanz-Penella et al., 2014) which parallels earlier usage of malt flour in baking formulations. According to Mares and Mrva (2008), samples affected by LMA displayed a FN somewhere between 150 s and the acceptable threshold (220–300 s depending on the wheat grade and growing region (Posner and Hibbs, 2005), therefore do not meet end-user standards as it is assumed that they will also be unsuitable for baking, despite the fact that LMA does not result in induction of proteases in the grain.

FN estimation of our TaAMY3 lines, in which AMY3 was overexpressed in the endosperm, classified the samples as heavily sprouted and therefore would be most certainly be discarded as the general opinion would have classified them with high LMA lines or as being severely sprouted with poor baking quality. These findings present a challenge for the wheat industry, as FN and RVA SN tests are simple and cheap, but nonspecific, and are possibly misclassifying valuable wheat lines as having poor baking quality. Although the beneficial baking effects of A3OE have only been assayed in small-scale bread, the results warrant further investigation in large-scale bread making systems. While small-scale baking process used in study allows for the evaluation of baking performance when only small quantities of flour are available, it does not allow the appraisal of information such as crumb texture. Large-scale baking trials will be required to assess the effect of A3OE lines on bread crumb texture.

Nevertheless, the results provided in this manuscript demonstrate that an increase in endogenous α-amylase, in unsprouted grain, has a positive impact on baking properties, and new methods to discriminate between LMA and PHS are required.

We acknowledge that the α-amylase involved in our study (TaAMY3) differs from the LMA involved α-amylase (TaAMY1) by its nature and its location (amylolytic for TaAmy3 and aleuronic for TaAMY1). However, there are numerous similarities between our TaAMY3-engineered lines and the LMA-affected lines. Following the definition of LMA by Mares and Mrva (2014), and unlike PHS, LMA and A3OE lines are defined by increased activity of a specific α-amylase isoform during the final stage of the grain development. In both cases, the increase in activity does not have any significant detrimental effect on grain morphology, development or germination. The flour pasting viscosity is restored with the addition of α-amylase inhibitors such as silver nitrate highlighting the fact that degradations only occurs during processing. Therefore, it is reasonable to question...
the assumption that detrimental effects due to stochastic expression of a particular α-amylase alone on baking quality and end products.

If agricultural production is to increase in an environment of growing pressures on land use, reduced availability of inputs and increasing climate uncertainty, the results present in this report should reinforce the need for a thorough investigation into the effect of LMA on wheat end product quality. At present, large numbers of otherwise excellent advanced wheat lines are being discarded as LMA susceptible because current industry testing methodology cannot discriminate between the two distinctly different conditions LMA and PHS.

**Experimental procedures**

**Plant growth**

The A3OE lines and tissue culture control (TCC) were developed in the Bobwhite 26 (BW26) background as described in Whan et al. (2014a,b). An additional generation of selected stable events were grown in glasshouses at CSIRO Agriculture Flagship, Canberra, Australia, under natural light on a diurnal temperature cycle of 14/25 °C. A3OE lines and their negative controls were glasshouse grown in 1-cubic-metre simulated field plot trials as described in Ral et al. (2012). The simulated plot trials were watered automatically at a rate equivalent of 10 mm of water every 3 days.

**Milling**

Wheat grain from the A3OE lines and their negative segregants, together with a tissue culture control (TCC) and a baking control flour (CF) of Australian wheat variety Sunco, were milled on a Metefém QC-109 laboratory roller mill (Metefém Co. Ltd., Hungary) to produce white flour. Grain was conditioned to 15% moisture content overnight prior to milling.

**Viscosity of starch and flour**

Differential scanning calorimetry (DSC) was performed using a DSC 8000 (Perkin Elmer is an international distributor). following the protocol described in Ral et al. (2008). Results displayed are the mean of three independent assays.

Starch pasting properties were analysed using RVA type 4 (Perten Instruments Australia Pty Ltd, Sydney, NSW, Australia). A 9% (w/v) starch sample suspension was equilibrated at 50 °C for 2 min, heated to 95 °C over 6 min, maintained at temperature for 4 min, cooled to 50 °C over 4 min and finally maintained at 50 °C for 5 min. A constant rotating speed of the paddle (160 rpm) was used throughout the analysis.

**Stirring test**

Equivalent Falling Numbers (eFN) of the A3OE lines flour were estimated using the RVA SN test (AACC 28-08-01). The FN was estimated from the measured SN using the formula of Ross et al. (1987). Results displayed are the mean of three for the three independent transgenic events and their negative segregants.

**α-Amylase assay**

α-Amylase activity was determined in 10-mg wholemeal samples and 2–6 ground developing grain samples. The CERALPHA kit (Megazyme International Ireland, Bray Business Park, Bray, Co. Wicklow, Ireland.) was used, with the manufacturer’s protocol adapted for 96-well format and with appropriate dilutions.

Results displayed are the mean of three independent assays of four biological replicates for the three independent transgenic events and their negative segregants.

**Carbohydrate measurement**

Fifty milligram of flour aliquots were extracted three times in 400 μL boiling 80% ethanol each. Total soluble sugars, total sucrose, free fructose and free glucose were measured as described by Campbell et al. (1999). All spectrophotometric measurements were performed using a Thermo Multiscan Spectrum plate reader (Thermo Electric Corporation, Finland) after appropriate dilutions. Results displayed are the mean of three technical replicates for the three independent events and their negative segregants.

**Amylose content**

Amylose content was measured using a small-scale (1 mg starch) iodine adsorption method on flour as described in Mohammdkhanii et al. (1998). A microtitre plate reader was used to compare the samples to standards based on a calibration curve; the percentage amylose content was estimated. The absorbance was read at 620 nm on three samples per replicate analysis and averaged. Results displayed are the mean of three independent assays of four biological replicates.

**Starch chain length distribution**

Chain length distribution of wheat endosperm starch was analysed by fluorophore-assisted carbohydrate electrophoresis as described by Morell et al. (1998). Chain length distribution studies were performed in duplicate for the three independent events and their negative segregants.

**Triacylglycerol measurement**

Triacylglycerol from embryos and endosperms of dry grains from T3 plants were extracted and analysed following the method described in Vanhercke et al. (2013). Each biological replicate contained tissues from five grains. Three technical replicates of three biological replicates were performed for each independent events and their negative segregants.

**Size-exclusion HPLC: %unpolymerized protein determination**

Unextractable polymeric proteins is obtained following the method described in Cavanagh et al. (2010). Protein extracts were subjected to HPLC using a Dionex Ultimate 3000 system. Twenty microlitres of each sample was injected into the system that was fitted with a BioBasic SEC-300 column (Thermo Scientific). Flow rate was 1.0 mL/min. Detection was performed at 214 nm.

Two different %UPP measurements were performed (‘s2’ and ‘s4’) that varied in the width (as indicated by time in the x axis of the chromatogram) of the glutenin soluble (no sonication) peaks (pk1 + pk2 in s2 or pk1 + pk2 + pk3 + pk4 in s4).

**Reversed-phase HPLC: proportion of glutenin subunits**

The method of Naem and Sapirstein (2007) was used for the qualitative/quantitative analysis of individual subunits of glutenins. Protein extracts (gliadins and glutenins) were subjected to HPLC using a Dionex Ultimate 3000 HPLC system that was fitted with a Zorbax 300SB-C8 column. The column was maintained in an oven at 60 °C throughout the run. Flow rate was 0.2 mL/min. A gradient of water/acetonitrile was used to selectively release the proteins adsorbed to the solid phase of the column. Detection was
performed at 214 nm. Analysis of each sample was performed in triplicate for each independent event and negative segregant.

Baking

Water absorption

The optimal water absorption of the A3OE flour was determined using a 4-g 2-arm micro-doughLAB mixer (Perten Instruments Australia PTY Ltd).

Mixograph

The A3OE flours were mixed on a 10-g mixograph (National Manufacturing Co., Lincoln, NE) to determine the optimal mixing conditions for each blend. Doughs were prepared using the optimal water absorption, and 2% salt was added. The formulation was adjusted to maintain a constant dough mass of 17.5 g. MixSmart version 1.0.438 (AEW Consulting, Lincoln, NE) was used to collect and analyse the mixing curves.

10-g Rapid dough test baking method

The A3OE lines were subjected to small-scale 10-g rapid dough bread making (Mann et al., 2005; Shogren and Finney, 1984). A 35-g mixograph was used to mix the flour and ingredients to peak dough development time as determined previously using the 10-g mixograph. Flour and ingredients were added to attain a constant dough mass of 22 g according to the formulation of 100% flour, 2% salt, 2% vegetable oil, 1.5% dry yeast and 1.5% improver. The dough development time as determined previously using the 10-g mixograph was used to collect and analyse the mixing curves. Following mixing, the dough was proofed at 40 °C and 85% RH, firstly, for an intermediate proof of 15 min and, then following scaling and moulding, a final proof of 1 h. Between the intermediate and final proofs, the dough was scaled into two 10-g dough pieces which were then separately moulded and placed into loaf pans, and so proofs, the dough was scaled into two 10-g dough pieces which were then separately moulded and placed into loaf pans, and so two loaves were baked from each mixed dough. The proofed loaves were baked in a Rotel oven (Moffat, Mulgare, Vic., Australia) at 190 °C for 15 min. Following depanning and cooling on a wire rack, loaves were weighed and the volumes determined by displacement by rapeseed displacement. In the first baking experiment, the A3OE lines were baked over 5 days, with eight bakes per rack, loaves were weighed and the volumes determined by displacement by rapeseed displacement. In the first baking experiment, the A3OE lines were baked over 5 days, with eight bakes per day, according to a partially replicated (p-rep) experimental spatially randomized design using the DiGGer design generation software (Coombes et al., 2002). Each line was either baking in duplicate or triplicate, depending upon flour availability within the p-rep baking design. The second baking experiment was also conducted under a p-rep design, over 2 days, under the sample baking conditions and replication levels as the first baking experiment.

Bread crust colour

Bread crust colour was assessed using an adapted version of GrainScan software and colour reported based on the CIELAB colour space parameters as described in Whan et al. (2014a,b).

Statistical analysis

All statistical analyses, except for mixograph tests, were performed with ASReml (Butler et al., 2007), in the R statistical language environment (R Core Team, 2013). For carbohydrate measurements on flours (total starch, total sucrose and free glucose), a model was fitted treating presence of the construct and the genetic background as fixed effects. Where observations were made on flours, and assays were extended over multiple plates, (α-amylase activity and sucrose concentration), nongenetic variation due to experimental processes, such as day of extraction and plate location, were fitted as random effects. Predicted means and standard errors were derived from the ASReml-R predict method.

Mixograph analyses were compared using ANOVA and Tukey’s honest significant differences test for multiple comparisons.

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Supporting information
Additional Supporting information may be found in the online version of this article:

Figure S1 Starch content and chain length distribution comparisons between A3OE positive and isogenic controls.
Table S1 Tri Acyl Glycerol (TAG) analysis and comparison of A3OE (A4, A10 and A17) to their isogenic negative segregants (A4N, A10N and A17N) and Tissue culture control (TCC).
Table S2 Protein profiling analysis and comparison of A3OE flours (A4, A10 and A17) to their isogenic negative segregants (A4N, A10N and A17N) and Tissue culture control (TCC).
Table S3 Ionomic analysis and comparison of A3OE flour (A4, A10 and A17) to their isogenic negative segregants (A4N, A10N and A17N) and Tissue culture control (TCC).
Table S4 Mixograph and flour parameters for A3OE flour (A4, A10 and A17), their isogenic negative segregants (A4N, A10N and A17N) and Tissue culture control (TCC).
Table S5 Rapid Visco Analyser profiles of A3OE positive (A10), negative (A4N) and Tissue culture control (TCC) flours in presence or absence of the Silver Nitrate (α-amylase inhibitor).