Near-isogenic lines of desi chickpea (*Cicer arietinum* L.) that differ in milling ease: differences in chemical composition

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Abstract Milling performance is an important attribute for desi chickpea and other pulses, as varieties that are more difficult-to-mill lead to processing yield loss and damage to the resulting split cotyledons (dhal) such as chipping and abrasion which are unattractive to the consumer. Poor milling performance leads to poor dhal quality and therefore lower prices and profitability along the pulse value chain. The Pulse Breeding Australia Chickpea Program identified near-isogenic desi lines that differed in seed shape and milling yields, however it was unknown whether this was due simply to a difference in physical forces on the seed during milling, mediated by seed shape, or whether there were underlying differences in chemical composition that could explain these differences. The two isolines differed in the composition of their seed coat, cotyledons and adjoining surfaces. Some of these differences were in agreement with previous research on composition of easy- and difficult-to-mill samples. These differences suggest that biochemical adhesive or cohesive mechanisms at the interface of seed tissues involve pectic polysaccharides and lignin-mediated binding.

Keywords Pulse (grain legume) · Chemical composition · Milling performance · Chickpea (*Cicer arietinum*) · Non starch polysaccharides · Minerals

Introduction

Chickpea is an important food crop which is consumed in many countries around the world. Desi chickpea consumption is predominantly in the Indian sub-continent, and commonly in the form of dhal. The production of dhal from desi chickpea seed involves milling; a process involving seed coat removal and cotyledon splitting. Dhal yield is an important quality trait that processors aim to optimise but research has shown that a significant amount of variation in dhal yield exists between genotypes in chickpea (Agrawal and Singh 2003; Wood et al. 2008) and other pulses (Black et al. 1998; Singh et al. 1992; Wang 2008). This reduction in dhal yield is due to seeds that are not successfully dehulled or split, plus losses due to chipping and abrasion and, additionally, results in a lower quality dhal that is visually unacceptable to the consumer.

Differences in composition between easy- and difficult-to-mill chickpeas that may influence milling difficulty have been identified previously (Wood et al. 2014a, b, c). However, those contrasting genotypes were identified from breeding lines developed by the Australian National Breeding Program and, being essentially a random set, could not be used for genetic analysis. The more recent availability of near-isogenic desi lines differing for a major gene influencing seed shape (Knights et al. 2011) enabled examination of the effects of seed shape on milling quality. Wood et al. (2012) demonstrated differences in milling yield, however it is unknown whether this was due simply to a difference in physical forces on the seed during

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milling, mediated by seed shape, or whether there were differing adhesive mechanisms at the interface of seed tissues.

In this study the chemical composition of various seed fractions from isogenic lines contrasting in seed shape were examined to determine if compositional differences supported any of the previously published explanations and to understand compositional difference influenced by this single gene of difference.

Materials and methods

Samples

Two isogenic lines ('Rounded' and 'Angular') were selected from 9113-13N-2, an F5 breeding line derived from the single cross between breeding lines 8507-28H and 946-31. They differ by a single gene (Knights et al. 2011) and were previously observed to differ significantly for milling performance; the Angular isolate being more difficult-to-mill; Fig. 1 (Wood et al. 2012). Seed was harvested from a genotype evaluation trial grown at Tamworth, Australia, allowed to equilibrate to moisture contents of 10–11% (at 21 °C) and then stored in sealed containers at 4 °C.

Sample preparation

Seed tissue fractions were prepared as previously described by (Wood et al. 2014a). Briefly, seed was passed repeatedly through the "pitter" component of an SK Engineering mill (SK Engineering and Allied Works, India) to remove the seed coat fraction. The remaining fraction consists of whole dehulled seeds and split cotyledons. Both remaining fractions were abraded separately in a tangential abrasive dehulling device (TADD; Venables Machine Works Ltd,

Canada) for 5 min to obtain the intermediate fractions IF(whole) and IF(splits), respectively. The remaining central parts of the cotyledons after TADD abrasion constitute the Cotyledon Core fraction. All fractions were ground to a flour in a Newport Scientific 5100 Mill (Newport Scientific Pty Ltd, Australia) fitted with a 0.5 mm screen. The entire fractionation process was repeated in triplicate.

In summary, the tissue fractions examined in this study are:

- Seed coat—ground to a flour;
- IF(whole)—cotyledon flour abraded from dehulled, whole seeds (the abaxial surface adjacent to the seed coat);
- IF(split)—cotyledon flour abraded from dehulled, split seeds (containing flour from the adaxial surface at the inter-cotyledon junction and some flour from the abaxial surface)
- Core cotyledon—inner cotyledon remaining after abrasion to obtain the intermediate fractions, ground to a flour.

Compositional Analysis

The methodologies used to analyse composition have been previously described in detail (Wood et al. 2014a, b, c). Briefly, moisture content was determined gravimetrically after drying (105 °C, 24 h); protein content was determined by Dumas combustion method CCD Official Method 02-03 (2003); total starch, enzyme-susceptible and resistant starch were determined using the Megazyme total starch kit (Megazyme International, Ireland); total free sugars, non-starch polysaccharides and lignin were determined by the Uppsala method as modified by (Choct et al. 1999); lignin was measured gravimetrically according to (van Soest 1963); uronic acid was determined using the method of (Scott 1979); ash was determined using the

Fig. 1 Photograph of seeds viewed from the base and their respective sphericity (Φ) determinations: **a** isogenic line 'rounded', **b** isogenic line 'angular' (image reproduced with permission from Wood et al. 2012.)

Ash—Basic method (AACC Method 08-01); mineral composition was determined according to the standard digestion method outlined in the Milestone Application Note 032 (Milestone Inc. 2000) and analysed by ICP-OES; fatty acid composition was determined using the direct methylation method of (Outen et al. 1976) with modifications (Wood et al. 2014a).

Statistical analysis

The data were analysed with Genstat (VSN International Ltd, UK). Predicted means for each genotype across fraction classifications are reported with differences determined using least significant difference (LSD) with a significance level of 0.05; except where particular fractions were one or more order of magnitudes different and in those cases individual standard deviations are reported. Principal component analysis (PCA) was used as an additional tool to examine relationships between the isoline fractions.

Results and discussion

Sample fractionation

The pitting process was used to remove the seed coat from the desi seeds and resulted in some seed splitting into their cotyledons. Ease of seed coat removal and splitting of the seeds occurred more readily for the Rounded isoline compared to the Angular isoline, consistent with the milling differences reported in Wood et al. (2012). Table 1 shows the percentage of each seed fraction (by weight) for the two chickpea genotypes. The Angular isoline had significantly ($P < 0.01$) higher percentage seed coat than the Rounded isoline. Although this difference may contribute to the lower dehulling efficiency (DE) of the Angular isoline, it would not cause the larger differences observed for splitting yield (SY) which is influenced mostly by inter-cotyledon adhesion.

Major components of isogenic line fractions

Table 1 shows the major components of each fraction for the isogenic lines. The composition of whole seeds was similar to the composition of genotypes previously reported (Wood et al. 2014a, d); 52% carbohydrate (35% starch, 11% NSP, 4% free sugars and 2% uronic acid), 24% protein, 10% moisture, 4% lipid, 3% ash, and 2% lignin. The isogenic lines had higher protein and lignin contents and lower ash and lipid contents than Australian chickpea genotypes previously (Wood et al. 2014a, d). This could be due to a genetic difference or an effect of environment

Table 1 Composition of whole seed and fractions from desi isolines with contrasting seed shape

Isoline	Fraction	Fraction as % of initial seed weight	Moisture content (%)	Protein (%)	Ash (%)	Lipid (%)	Total starch (%)	Total NSP (%)	Free sugars (%)	Lignin (%)	Uronic acid (%)
Rounded	Whole	100.00 ± 0.00 ^a	10.03 ^e	22.88 ^h	2.67 ^f	3.84 ^e	36.39 ^d	10.89 ± 0.16 ^d	4.32 ^d	2.26 ± 1.55 ^b	2.02 ± 0.05 ^d
Angular	Whole	100.00 ± 0.00 ^a	9.80 ^f	24.69 ^f	2.75 ^e	3.40 ^g	34.40 ^e	11.68 ± 0.14 ^c	3.92 ^e	2.10 ± 1.55 ^b	2.30 ± 0.07 ^c
Rounded	Seed coat	13.63 ± 0.23 ^f	11.19 ^a	2.24 ± 0.04 ^a	3.99 ^b	0.05 ± 0.01 ^h	0.35 ± 0.03 ^f	46.72 ± 0.48 ^b	0.24 ± 0.02 ^g	12.17 ± 4.86 ^a	7.00 ± 0.19 ^a
Angular	Seed coat	16.33 ± 0.44 ^e	10.64 ^b	2.25 ± 0.03 ^a	4.16 ^a	0.05 ± 0.02 ^h	0.34 ± 0.05 ^f	50.38 ± 0.43 ^a	0.28 ± 0.02 ^f	11.47 ± 4.86 ^a	5.36 ± 0.16 ^b
Rounded	Core cotyledon	61.29 ± 0.89 ^b	10.36 ^d	23.88 ^g	2.22 ⁱ	4.44 ^a	45.39 ^a	4.99 ^g	3.93 ^e	0.25 ^c	0.64 ^h
Angular	Core cotyledon	58.64 ± 0.78 ^c	10.47 ^c	26.82 ^e	2.42 ^h	3.94 ^d	43.77 ^b	4.80 ^h	4.49 ^d	0.11 ^f	0.69 ^g
Rounded	IF(whole)	21.47 ± 0.87 ^d	9.48 ^h	28.81 ^c	3.28 ^c	3.94 ^d	36.95 ^d	5.58 ^f	5.86 ^a	0.44 ^c	0.92 ^f
Angular	IF(whole)	21.08 ± 0.45 ^d	10.01 ^e	31.44 ^a	2.90 ^d	3.73 ^f	34.89 ^e	5.87 ^e	5.27 ^b	0.46 ^c	0.98 ^e
Rounded	IF(split)	23.82 ± 1.28 ^d	9.74 ^g	27.81 ^d	2.56 ^g	4.23 ^b	38.74 ^c	5.65 ^f	5.00 ^c	0.38 ^d	1.00 ^e
Angular	IF(split)	23.39 ± 1.10 ^d	9.43 ⁱ	30.32 ^b	2.74 ^e	3.99 ^c	36.70 ^d	5.82 ^e	5.20 ^{bc}	0.47 ^c	0.99 ^e
	LSD ^a	NA	0.03	0.10	0.02	0.04	0.62	0.13	0.24	0.08	0.03

Different letters within a column are significantly different ($P < 0.05$)

^a Least significant difference (LSD) applies to all values in the column, except for where the values are much larger or smaller for which a Standard Deviations (SD) are shown after the values

since they were harvested from a different trial. The more difficult-to-mill Angular line contained higher amounts of protein, ash, total NSP and uronic acid than the rounded line, and lower amounts of lipid, starch, free sugars and lignin.

The composition of seed coats was 55% carbohydrate (48% NSP, 6% uronic acid, <1% starch and <1% free sugars), 12% lignin, 11% moisture, 4% ash, 2% protein and <1% lipid. This amounts to 9% less carbohydrate than the chickpeas analysed in (Wood et al. 2014a, d) and 9% higher lignin, so that the sum of components was equal for both groups (approximately 85%). This supports the assumption that the unaccounted 15% of seed coat weight is due to highly bound components that were not extracted by the methods used, most likely lingo-cellulosic and tannin compounds. Compared to the previous Australian samples (Wood et al. 2014a), the seed coats of the two isogenic lines had much higher lignin contents and much lower uronic acid contents, and lower total NSP, ash and free sugar contents. Protein contents were similar. Small amounts of starch, lipid and free sugars were recorded in the seed coat fraction, similar to results reported by Wood et al. (2014a). The protein and lipid contents of the seed coat fractions were consistent for the isogenic lines. However, the Angular isoline contained higher ash, total NSP and free sugar contents than the Rounded isoline, and lower total starch, lignin and uronic acid contents. Lignin content in the seed coat fraction decreased as the milling difficulty increased (Wood et al. 2014a, d) and this trend was mirrored in the isogenic lines: the more difficult-to-mill Angular isoline contained less lignin in the seed coat than the Rounded isoline.

The major components of the core cotyledon fraction of the isogenic chickpea lines was 55% carbohydrate (45% starch, 5% NSP, 4% free sugars and 1% uronic acid), 24–27% protein, 10% moisture, 4% lipid, 2% ash and <1% lignin. These components accounted for 96–98% of the cotyledon weight. The core cotyledon fractions of the two isogenic lines were higher in protein and slightly lower in ash, lipid, lignin and uronic acid contents than genotypes previously (Wood et al. 2014a, d). The Angular isoline cotyledons contained higher amounts of protein, ash, and free sugars than the Rounded isoline, and lower amounts of starch and lipid. The cotyledons of both isolines contained only small amounts of lignin and uronic acid.

Major components of the intermediate fractions

The major components of the intermediate fractions derived from dehulled whole seeds and derived from dhal are also shown in Table 1. The composition of these intermediate fractions was approximately 48–51% carbohydrate (35–39% starch, 6% NSP, 5–6% free sugars and

1% uronic acid), 28–31% protein, 10% moisture, 4% lipid, 3% ash and <1% lignin, which together accounted for 95% of the fraction weight, similar to other Australian genotypes (Wood et al. 2014a, d). The starch and lipid are more concentrated in the cotyledon core, whereas the protein, ash, total NSP, free sugars, lignin and uronic acid were more highly concentrated in the peripheral layers of the cotyledons, in agreement with the previous study (Wood et al. 2014a, d). The intermediate fractions of the isogenic lines had higher protein content and lower ash, lipid, lignin and uronic acid content compared to those of the other three desi genotypes (Wood et al. 2014a, d). The Angular isoline contained higher amounts of protein, total NSP and lignin than the Round line for both intermediate fractions, and lower amounts of lipid and starch. The Angular isoline contained more uronic acid and less ash and free sugars than the Rounded isoline in the IF(whole) fraction, but not in the IF(split) fraction. These results agree with the previous finding that the difficult-to-mill genotypes contained higher protein and lower starch contents in the intermediate fractions (Wood et al. 2014a, d).

Detailed composition whole seeds and seed fractions of isogenic lines

Lignin composition

The lignin contents for the whole seeds were similar for the isogenic lines (Table 1). Most lignin is located in the seed coat. The difficult-to-mill Angular isoline contained lower lignin contents in the seed coat and core cotyledon fractions, and higher content in the IF(split) fraction. This supports the results from Wood et al. (2014a): the difficult-to-mill genotypes contained less lignin in the seed coat. Lignin was assumed to improve milling efficiency by increasing the rigidity and brittleness of the seed coat, thereby facilitating cracking and detachment by physical forces (Wood et al. 2014a).

Amino acid composition

The individual amino acid fractions for the two isolines are detailed in Supplementary Table A. The sum of individual amino acids generally mirrored the total protein content for each sample. The most abundant amino acids in whole seeds were glutamic acid > aspartic acid > - arginine > leucine and lysine, a trend that generally applied for all the cotyledonary fractions of both isolines. The Angular isoline contained higher arginine in the whole seed and core cotyledon fraction than for the Rounded isoline, but all other amino acids were similar. The seed coat fraction contained only small amounts of protein, comprised mainly of glutamic acid (48 and 39% for the

Angular and Rounded isolines respectively), and contents of the other amino acids differed markedly between the isolines. The amino acid concentrations were adjusted to a percentage of the total amino acid content to allow direct comparisons between the isolines for each fraction (data not shown). The IF(whole) fraction of the more difficult-to-mill Angular isolate had higher arginine and proline contents and lower glutamic acid and tyrosine contents than the Rounded isolate. The IF(split) fraction of the Angular line had higher arginine and glutamic acid contents and lower valine and aspartamine contents. The only similarity with results from Wood et al. (2014b) were that the more difficult-to-mill genotypes contained higher arginine contents in their IF(split) fractions. The two isolines had similar cysteine contents in their intermediate fractions which does not support the involvement of lectins in adhesion proposed in Wood et al. (2014c). The possible role of lectins in adhesion cannot be dismissed, however, since the location of the cysteine residues is also important and would require further investigation to determine.

Mineral composition

Table 2 shows the individual minerals that comprise the ash component of the isogenic lines. Similar to results in (Wood et al. 2014b), potassium was the most abundant mineral in whole seeds, followed by phosphorus. All fractions of the seed contained high levels of potassium. The seed coat comprised calcium > potassium > magnesium, whereas the cotyledonary fractions comprised potassium > phosphorus > sulphur. There were large differences between the isogenic lines for certain minerals in particular fractions. For example, the sodium content for the Rounded isolate was double that of the Angular isolate in the seed coat fraction and 1.3 times higher in the core cotyledon fraction; for the IF(whole) fraction was half as much and for the IF(split) fraction it was about one-third. Despite these large differences, the whole seeds of both isolines contained similar amounts of sodium. Similarly, the zinc content in the core cotyledon of the Angular line was 1.6 times that of the Rounded line, and the IF(split) 1.2 times higher, yet the Angular line contained less zinc in the IF(whole) fraction and nil in the seed coat.

It is unclear why these differing storage patterns for sodium and zinc exist. Plants have adapted to limit the transfer of sodium from the soil to the plant, hence the content in seeds of most plants is relatively low (Subba Rao et al. 2003). Genotypic differences in sodium uptake have been identified in other plant species, such as tomato (Subba Rao et al. 2003). Sodium ions can be toxic to cells, so are mainly located within vacuoles of maturing seeds. As seeds mature and dry, water moves out of seeds through the funiculus, via xylem transport, back into the plant

Table 2 Mineral composition of whole seed and fractions of desi isolines with contrasting seed shape

Genotype	Fraction	Al (μg/g)	B (μg/g)	Ca (%)	Cu (μg/g)	Fe (μg/g)	K (%)	Mg (%)	Mn (μg/g)	Mo (μg/g)	Na (μg/g)	P (%)	S (%)	Zn (μg/g)
Rounded	Whole seed	3.55 ^b	8.46 ^b	0.19 ^b	6.58 ^g	27.71 ^g	0.88 ^f	0.12 ^b	38.97 ^c	0.00 ^a	187.07 ^c	0.24 ^e	0.18 ^e	14.86 ^g
Angular	Whole seed	0.00 ^c	5.37 ^f	0.16 ^b	6.59 ^g	30.36 ^f	0.89 ^{ef}	0.11 ^{bc}	35.99 ^d	0.00 ^a	164.67 ^e	0.25 ^{de}	0.19 ^{de}	20.68 ^f
Rounded	Seed coat	0.00 ^c	7.92 ^c	0.93 ^a	1.69 ^h	8.64 ^h	0.46 ^h	0.31 ^a	131.21 ± 3.74 ^a	0.00 ^a	220.19 ^b	0.02 ^f	0.02 ^f	0.14 ⁱ
Angular	Seed coat	0.00 ^c	7.89 ^c	0.88 ^a	1.08 ⁱ	8.40 ^h	0.61 ^g	0.30 ^a	126.80 ± 3.12 ^b	0.00 ^a	109.19 ^g	0.02 ^f	0.02 ^f	0.00 ^j
Rounded	Core cotyledon	0.00 ^c	9.89 ^a	0.05 ^c	6.91 ^f	27.98 ^g	0.95 ^d	0.08 ^d	20.40 ⁱ	0.00 ^a	227.61 ^a	0.27 ^{ce}	0.21 ^d	14.06 ^h
Angular	Core cotyledon	0.00 ^c	5.71 ^e	0.05 ^c	7.23 ^e	31.04 ^e	0.93 ^{de}	0.08 ^d	21.15 ^h	0.00 ^a	176.28 ^d	0.29 ^c	0.21 ^d	22.98 ^e
Rounded	IF(whole)	33.66 ^a	4.57 ^g	0.05 ^c	12.56 ^a	51.92 ^a	1.17 ^a	0.10 ^{bcd}	26.81 ^e	0.00 ^a	68.02 ⁱ	0.36 ^a	0.28 ^{ab}	35.55 ^a
Angular	IF(whole)	0.00 ^c	2.71 ⁱ	0.06 ^c	9.20 ^b	47.45 ^b	1.09 ^b	0.09 ^{cd}	25.88 ^e	0.00 ^a	134.94 ^f	0.34 ^{ab}	0.28 ^{ab}	30.13 ^b
Rounded	IF(split)	0.00 ^c	7.56 ^d	0.07 ^c	7.96 ^d	37.76 ^d	1.03 ^c	0.10 ^{bcd}	23.59 ^g	0.00 ^a	89.58 ^h	0.29 ^{cd}	0.24 ^c	24.35 ^d
Angular	IF(split)	0.00 ^c	4.24 ^h	0.07 ^c	8.58 ^e	42.80 ^c	1.04 ^c	0.10 ^{bcd}	24.93 ^f	0.00 ^a	228.89 ^a	0.31 ^{bc}	0.26 ^{bc}	28.24 ^c
	LSD ^a	0.12	0.08	0.05	0.09	0.39	0.05	0.03	1.29	NS	3.23	0.05	0.03	0.27

Different letters within a column are significantly different ($P < 0.05$)

NS not significant at $P < 0.05$

^a Least significant difference (LSD) applies to all values in the column, except for where the values are much larger or smaller for which a standard deviations (SD) are shown after the values

(Hamilton and Davies 1988). It is possible that mobile ions, like sodium, may be transported with water during seed drying. Differences in the periods of seed filling (inflow) or seed drying (outflow) between these isolines may explain these unusual mineral distributions. While no differences in seed filling/drying have been noticed for these lines, they have not been specifically examined either. Increased inflow and outflow of water in pea causes a wrinkled seed type (Bhattacharyya et al. 1993) and a similar mechanism may confer the angular seed shape in these isolines (Knights et al. 2011) and possibly these unusual mineral distributions. However, amylose contents of the isolines were roughly similar (Wood et al. 2012), suggesting this analogy does not fit. An alternative explanation is that sodium can replace potassium in cell vacuoles (Subba Rao et al. 2003), although no relationship between sodium and potassium was evident. In this way, sodium acts as an osmoticum to increase cell turgor, possibly resulting in larger cotyledon cells in the Rounder line which may explain the plumper seed shape.

The Rounded isoline was the only one to contain aluminium, which was only found in the IF(whole) fraction and whole seed. The Rounded line also contained 1.8 times the amount of boron in the IF(split) fraction and in the IF(whole) fraction, 1.7 times the amount of boron and 1.4 times as much copper. These results contrast with the trend reported in Wood et al. (2014b).

Table 3 summarises the major differences and similarities in mineral composition of the various fractions for the difficult-to-mill isogenic line and genotypes from the previous study of Wood et al. (2014b). The difficult-to-mill genotypes from the previous study were higher in boron, aluminium and magnesium in their seed coat fractions. In contrast, the difficult-to-mill Angular isoline contained more potassium in the seed coat. The difficult-to-mill genotypes of the previous study were higher in boron, calcium, copper, iron, potassium, magnesium, manganese, molybdenum, sodium and zinc in their IF(whole) fraction (Wood et al. 2014b), however in this study the Angular isoline only contained more sodium in this fraction. The difficult-to-mill genotypes were higher in aluminium, iron and manganese in the IF(split) fraction, whereas the

Angular isoline contained more sodium, zinc, manganese, sulphur, iron and copper in this fraction than the Rounded isoline.

The elements in abundance for all of the difficult-to-mill genotypes were sodium and calcium in the IF(whole) fraction and iron and magnesium in the IF(split) fraction. Of these, sodium and iron have no obvious roles in adhesion (Wood et al. 2014b). Sodium has been discussed above. Iron is known to be important for redox reactions and as a bridging agent between some enzymes and their substrate in plant metabolism (Marschner 2002). Within legume seeds, iron is stored in the plastids as ferritin (Lobreaux and Briat 1991) and/or in vacuole globoids in association with phytate (Lanquar et al. 2005). The iron within pea cotyledons is greatly reduced within the first 7 days of germination, presumably for use by the developing seedling (Lobreaux and Briat 1991). Since both iron and sodium appear to be fully enclosed within plastids and/or vacuoles, they are unlikely to play a direct role in adhesion. Hence, these results support the theory that manganese is the element most likely contributing to intercotyledon adhesion. As detailed in Wood et al. (2014b), manganese can bind strongly with phenolic and carboxylic groups to strengthen cell walls, particularly in the seed coat and at the adaxial cotyledon regions.

Fatty acid composition

The fatty acid composition of the different fractions of the two isolines can be found in Supplementary Table B. The lipid of both isogenic lines was mainly composed of unsaturated long chain fatty acids: C18:2 (linoleic; ω 6) and C18:1 (oleic; ω 9), in agreement with results of other Australian chickpea genotypes (Wood et al. 2014b). The majority of the lipid was located in the core cotyledon. There were no large differences in the content of fatty acids between the isolines for any fraction. There was very little lipid content in the seed coat of both isolines, and this was mainly (60%) saturated fatty acids C16:0 (palmitic). This differs from the C18:2 > C16:0 > C18:1 > C18:0 prevalence of fatty acids in the seed coats of the genotypes in (Wood et al. 2014b). No large differences in fatty acid

Table 3 Major mineral differences of the difficult-to-mill angular isogenic line and difficult-to-mill genotypes from Wood et al. (2014b)

Difficult-to-mill genotypes	Seed coat	Core cotyledon	IF(whole)	IF(split)
Angular isoline	Higher K Lower Na, Mn and no Zn	Higher Fe, Zn Lower Na, B	Higher Na Lower B, Cu, Fe, K, Zn and no Al	Higher Na, Zn, Mn, Fe, Cu Lower B and no Al
From Wood et al. (2014b)	Higher B, Al, Mg	No trends	Higher B, Ca, Cu, Fe, K, Mg, Mn, Mo, Na, Zn	Higher Al, Fe, Mn
Similarities	Nil	Nil	Higher Na	Higher Fe & Mn

contents were evident between the cotyledonary fractions of the isolines. However, examination of the PCA biplot for lipids showed that the Rounded isoline contained more Total Fat than the Angular isoline for every seed fraction except the seed coat and that Total Fat contributed almost 46% of the variance, with the combination of C18:0, C18:1 and C18:3 accounting for around 38% of the variance.

Starch composition

The starch composition for the whole seeds and their fractions of the isogenic lines can be found in Supplementary Table B. The total starch in whole seeds comprised 96% susceptible starch for the Rounded isoline and 99% for the Angular isoline. The similar, but slightly higher amylose content of the Angular isoline (Wood et al. 2012) may be attributable for this difference, since amylose is less susceptible to amolytic degradation than amylopectin (Thorne et al. 1983). The starch derived from all cotyledonary fractions had similar ratios of susceptible to resistant starch which was similar to the ratio for whole seed. The seed coat contained very little starch, with a higher portion of resistant starch. The Rounded isoline had slightly higher starch content in all fractions than the Angular line.

Free sugar residues and NSP free sugar composition

Table 4 shows the residues of the free sugar as well as the free sugar residues of Total NSP, soluble NSP and Insoluble NSP fractions. The galactose and glucose residues accounted for more than 90% of the total free sugars in the whole seeds and all cotyledonary fractions. This finding is in agreement with results of other Australian chickpeas genotypes (Wood et al. 2014c). The ratio of galactose to glucose was also similar, approximately 2:1.

The most abundant sugar residues from total NSP in whole seeds were mainly glucose followed by arabinose, in similar proportion to other Australian chickpea genotypes (Wood et al. 2014c). Similarly, the seed coat NSP comprised mainly glucose, whereas the cotyledonary fractions comprised mostly arabinose.

The NSP glucose contents for the Rounded and Angular isolines were similar. Glucose and fructose condense (1:1) to create sucrose (a disaccharide). In peas, wrinkled pea was found to have almost twice the sucrose content of the round pea, causing higher water inflow/outflow in the seed and subsequent wrinkling on drying (Bhattacharyya et al. 1993). The similarity of glucose contents for Rounded and Angular desi seeds does not support a similar mechanism operating in angular-seeded chickpeas, as suggested by Knights et al. (2011).

Proportion of soluble and insoluble NSP

Table 5 shows the solubility of total NSP. Similar to results in the previous study, 94–97% of the total NSP in the whole seeds and all fractions of both isogenic lines was insoluble (Wood et al. 2014c). The difficult-to-mill Angular isoline contained more soluble and insoluble NSP in all fractions except the core cotyledon fraction where it contained less insoluble NSP.

Sugar residues of soluble and insoluble NSP

Table 4 also shows the composition of sugar residues from soluble NSP and insoluble NSP. The seed coat had the highest content of total soluble NSP. The soluble NSP was composed of distinctly different sugar residues in the seed coat compared to the cotyledonary fractions. The seed coat composition also differed between the isogenic lines. The difficult-to-mill Angular isoline contained more total soluble NSP, with the order of abundance galactose > xylose and arabinose > rhamnose > glucose (in the approximate ratio of 6.5:5.3:1 respectively), similar to genotypes in the previous study (Wood et al. 2014c). In contrast, the Rounded isoline contained less galactose and more mannose than the Angular isoline, resulting in xylose, arabinose and galactose being most abundant, followed by rhamnose and mannose (approximate ratio of these groups were 4:1 respectively). PCA biplots revealed that Component 2 (accounting for 24% variance) separated all the cotyledonary fractions of the two isolines (Fig. 2). As Component 2 is mainly controlled by mannose, ribose and arabinose contents, this means that the difficult-to mill Angular isoline cotyledonary fractions contained more of these soluble NSP residues than the Rounded isoline.

The cotyledonary fractions of soluble NSP comprised mainly galactose and arabinose, similar to other Australian chickpeas (Wood et al. 2014c), however, the minor sugar residues differed: mannose > ribose > glucose for the isogenic lines, whereas for the genotypes in Wood et al. (2014c) it was glucose > mannose. The difficult-to-mill Angular isoline contained higher residues of galactose, arabinose and ribose in the IF(whole) fraction than the Rounded line, similar to the difficult-to-mill genotypes in the previous study (Wood et al. 2014c).

The seed coat had the highest concentrations of insoluble NSP, which was dominated by glucose residues derived from cellulose. The whole seeds of the isolines contained more mannose than the genotypes in the previous study (Wood et al. 2014c), and this was derived mainly from the seed coat. The cotyledonary fractions produced insoluble NSP residues in the order arabinose > glucose > galactose > xylose. Arabinose abundance in the

Table 4 Free sugar composition (g/kg) and free sugar composition of NSP fractions (g/kg) of whole seed and fractions of desi isolines with contrasting seed shape

Genotype	Fraction	Rhamnose	Fucose	Ribose	Arabinose	Xylose	Mannose	Galactose	Glucose	Total free sugars
<i>Free sugar composition (g/kg)</i>										
Rounded	Whole seed	0.00	0.00	0.00	0.49	0.00	2.11	27.24	13.31	43.29
Angular	Whole seed	0.26	0.00	0.00	0.40	0.00	1.86	24.44	12.25	39.22
Rounded	Seed coat	0.32	0.00	0.00	0.41	0.00	0.22	0.75 ± 0.02	0.79 ± 0.01	2.38 ± 0.16
Angular	Seed coat	0.39	0.00	0.00	0.39	0.00	0.15	0.97 ± 0.03	0.99 ± 0.02	2.82 ± 0.18
Rounded	Core cotyledon	0.00	0.00	0.00	0.29	0.00	1.68	24.87	12.60	39.29
Angular	Core cotyledon	0.16	0.00	0.07	0.69	0.00	1.97	25.27	16.82	44.94
Rounded	IF(whole)	0.40	0.00	0.09	0.62	0.00	3.06	34.31	20.16	58.60
Angular	IF(whole)	0.38	0.00	0.08	0.57	0.00	2.36	32.16	17.17	52.67
Rounded	IF(split)	0.37	0.00	0.00	0.48	0.00	2.09	31.64	15.81	49.96
Angular	IF(split)	0.38	0.00	0.00	0.49	0.00	2.54	31.66	17.13	52.01
	LSD ^a	0.04	NS	0.03	0.05	NS	0.08	0.14	0.11	2.37
Genotype	Fraction	Rhamnose	Fucose	Ribose	Arabinose	Xylose	Mannose	Galactose	Glucose	Total NSP
<i>Total NSP sugar composition (g/kg)</i>										
Rounded	Whole seed	1.02	0.36	0.31	34.53	9.55	5.21	7.36	63.89 ± 0.82	108.94 ± 1.62
Angular	Whole seed	1.42	0.35	0.51	35.40	10.03	6.00	8.01	69.32 ± 0.89	116.76 ± 1.44
Rounded	Seed coat	3.25 ± 0.16	0.00	0.10	20.06	45.02 ± 0.61	27.27 ± 0.34	19.86 ± 0.21	404.97 ± 1.46	467.15 ± 4.83
Angular	Seed coat	3.88 ± 0.14	0.58	0.00	20.78	43.75 ± 0.72	31.01 ± 0.38	24.96 ± 0.24	436.33 ± 1.49	503.83 ± 4.31
Rounded	Core cotyledon	0.02	0.00	0.35	37.72	3.11	0.51	5.16	9.45	49.86
Angular	Core cotyledon	0.49	0.25	0.38	36.82	2.93	1.04	5.07	9.23	47.97
Rounded	IF(whole)	0.65	0.32	0.45	38.56	3.98	1.22	6.15	12.07	55.77
Angular	IF(whole)	0.71	0.37	0.59	41.04	3.88	1.25	6.47	11.90	58.67
Rounded	IF(split)	0.00	0.00	0.40	42.13	3.69	1.20	5.95	10.70	56.45
Angular	IF(split)	0.69	0.36	0.48	41.51	3.92	1.22	6.35	11.35	58.21
	LSD ^a	0.05	0.04	0.06	1.05	0.09	0.12	0.07	0.16	1.34
Genotype	Fraction	Rhamnose	Fucose	Ribose	Arabinose	Xylose	Mannose	Galactose	Glucose	Total soluble NSP
<i>Soluble NSP sugar composition (g/kg)</i>										
Rounded	Whole seed	0.22	0.00	0.31	1.36	1.17	0.51	1.54	0.41	4.90
Angular	Whole seed	0.45	0.00	0.30	1.65	1.25	0.51	1.86	0.44	5.75
Rounded	Seed coat	1.52 ± 0.34	0.00	0.10	4.48 ± 0.35	4.64 ± 0.48	1.07	4.37 ± 0.42	0.51	14.83 ± 0.81
Angular	Seed coat	3.44 ± 0.46	0.00	0.00	5.77 ± 0.40	5.85 ± 0.51	0.33	6.72 ± 0.49	1.22	20.73 ± 0.83
Rounded	Core cotyledon	0.02	0.00	0.35	0.79	0.07	0.51	0.98	0.36	2.74
Angular	Core cotyledon	0.03	0.00	0.38	0.85	0.08	0.52	0.99	0.33	2.83
Rounded	IF(whole)	0.04	0.00	0.45	1.08	0.11	0.53	1.17	0.42	3.38
Angular	IF(whole)	0.00	0.00	0.59	1.54	0.00	0.54	1.23	0.34	3.78
Rounded	IF(split)	0.00	0.00	0.40	1.16	0.00	0.52	1.17	0.40	3.26
Angular	IF(split)	0.00	0.00	0.48	1.29	0.11	0.52	1.21	0.37	3.55
	LSD ^a	0.03	NS	0.04	0.07	0.08	0.05	0.07	0.06	0.16
Genotype	Fraction	Rhamnose	Fucose	Ribose	Arabinose	Xylose	Mannose	Galactose	Glucose	Total insoluble NSP
<i>Insoluble NSP sugar composition (g/kg)</i>										
Rounded	Whole seed	0.80	0.36	0.00	33.17	8.38 ± 0.07	4.71 ± 0.05	5.82	63.48 ± 0.85	104.04 ± 0.94
Angular	Whole seed	0.97	0.35	0.21	33.75	8.78 ± 0.07	5.49 ± 0.05	6.15	68.88 ± 0.89	111.01 ± 0.97
Rounded	Seed coat	1.73	0.00	0.00	15.58	40.38 ± 0.42	26.20 ± 0.23	15.49 ± 0.07	404.47 ± 1.42	452.32 ± 2.42
Angular	Seed coat	0.44	0.58	0.00	15.01	37.90 ± 0.46	30.68 ± 0.25	18.24 ± 0.09	435.11 ± 1.56	483.10 ± 2.67
Rounded	Core cotyledon	0.00	0.00	0.00	36.93	3.04	0.00	4.18	9.09	47.12
Angular	Core cotyledon	0.46	0.25	0.00	35.97	2.85	0.52	4.08	8.90	45.14
Rounded	IF(whole)	0.60	0.32	0.00	37.48	3.86	0.69	4.98	11.65	52.38
Angular	IF(whole)	0.71	0.37	0.00	39.50	3.88	0.71	5.25	11.56	54.90
Rounded	IF(split)	0.00	0.00	0.00	40.97	3.69	0.68	4.78	10.30	53.19

Table 4 continued

Genotype	Fraction	Rhamnose	Fucose	Ribose	Arabinose	Xylose	Mannose	Galactose	Glucose	Total insoluble NSP
Angular	IF(split)	0.69	0.36	0.00	40.22	3.81	0.70	5.13	10.98	54.65
	LSD ^a	0.06	0.04	0.02	0.22	0.05	0.03	0.13	0.17	0.19

NS not significant at $P < 0.05$

^a Least significant difference (LSD; $P < 0.05$) applies to all values in the column, except for where the values are much larger or smaller for which a Standard Deviations (SD) are shown after the values

Table 5 Soluble, insoluble and total NSP content (g/kg) and uronic acid composition (g/kg) of whole seed and fractions of desi isolines with contrasting seed shape

Genotype	Fraction	Total soluble NSP	Total insoluble NSP	Total NSP	Soluble uronic acid	Insoluble uronic acid	Total uronic acid
Rounded	Whole seed	4.90 ^d	104.04 ± 0.94 ^d	108.94 ± 0.90 ^d	3.97 ± 0.19 ^c	16.26 ± 0.41 ^d	20.22 ± 0.46 ^d
Angular	Whole seed	5.75 ^c	111.01 ± 0.97 ^c	116.76 ± 0.94 ^c	3.82 ± 0.16 ^c	19.18 ± 0.45 ^c	22.99 ± 0.65 ^c
Rounded	Seed coat	14.83 ± 0.81 ^b	452.32 ± 1.42 ^b	467.15 ± 1.57 ^b	17.31 ± 0.67 ^b	50.65 ± 1.54 ^a	67.96 ± 1.88 ^a
Angular	Seed coat	20.73 ± 0.83 ^a	483.10 ± 1.67 ^a	503.83 ± 1.68 ^a	21.70 ± 0.64 ^a	31.88 ± 1.38 ^b	53.59 ± 1.64 ^b
Rounded	Core cotyledon	2.74 ^h	47.12 ⁱ	49.86 ^h	0.24 ^h	6.11 ⁱ	6.35 ^h
Angular	Core cotyledon	2.83 ^h	45.14 ^j	47.97 ⁱ	0.27 ^h	6.63 ^h	6.90 ^g
Rounded	IF(whole)	3.38 ^g	52.38 ^h	55.77 ^g	0.56 ^e	8.61 ^g	9.17 ^f
Angular	IF(whole)	3.78 ^e	54.90 ^e	58.67 ^e	0.61 ^d	9.23 ^f	9.84 ^e
Rounded	IF(split)	3.26 ^g	53.19 ^g	56.45 ^g	0.38 ^g	9.64 ^e	10.02 ^e
Angular	IF(split)	3.55 ^f	54.65 ^f	58.21 ^f	0.43 ^f	9.43 ^{ef}	9.89 ^e
	LSD	0.16	0.19	1.34	0.04	0.29	0.33

Different letters within a column are significantly different ($P < 0.05$)

^a Least significant difference (LSD) applies to all values in the column, except for where the values are much larger or smaller for which a standard deviations (SD) are shown after the values

fractions followed the order of IF(split) > IF(whole) > core cotyledon. Glucose was highest in the IF(whole) > IF(split) > core cotyledon. Insoluble xylose and mannose residues were equally high in both intermediate fractions. The Rounded isoline contained no rhamnose or fucose in the cotyledon core or IF(split) and no mannose in the core cotyledon. The IF(whole) fraction of the difficult-to-mill Angular isoline contained more insoluble NSP sugar residues than the Rounded isoline, particularly galactose, arabinose, rhamnose and fucose. The only residue where it contained less was glucose. Similarly, the difficult-to-mill genotypes in the previous study were higher in all the insoluble NSP sugar residues to some extent (Wood et al. 2014c). Rhamnose and galactose were the main insoluble residues that were at substantially higher concentrations for all the difficult-to-mill genotypes in the previous study (Wood et al. 2014c) and for the Angular isoline in this study.

Uronic acid composition

Table 5 shows that the total uronic acid contents of the seeds of the isogenic lines were much lower than the contents reported previously (Wood et al. 2014a). The seed coat fraction accounted for most of the uronic acid in seeds, and the intermediate fractions contained more total uronic acid than the cotyledon core. The Rounded isoline contained 14% more total uronic acid in the seed coat than the Angular isoline, while the content in the cotyledonary fractions was similar. No definitive links between uronic acid content and milling difficulty were established previously, although the IF(whole) fraction from the kabuli genotype had a uronic acid content 4 times higher than that of the desi genotypes (Wood et al. 2014a). The Angular isoline had a slightly higher total uronic acid content in this fraction, indicating a possible association between pectins and seed coat adherence.

Fig. 2 PCA biplot showing the soluble NSP sugar residues of the cotyledonary fractions of the desi isolines with contrasting seed shape (Comp. 1 = 67.9% variance, Comp. 2 = 23.7% variance). Key: odd numbers = rounded isoline; even numbers = angular isoline; 1 and 2 = whole seed; 3 and 4 = cotyledon core; 5 and 6 = IFwhole; 7 and 8 = IFsplit

For both isolines the insoluble uronic acid comprised 81–84% of the total uronic acid in whole seed and >95% of total uronic acid in the cotyledonary fractions. The seed coat of the Angular isoline contained only 60% insoluble uronic acid (compared to 75% insoluble in the Rounded isoline), and contained more soluble uronic acid. This suggests that the seed coat of the more difficult-to-mill Angular isoline may be less rigid (due to much lower insoluble uronic acid content) and more sticky (due to more soluble uronic acid). The IF(split) fraction of the Angular isoline contained a much higher proportion of soluble uronic acid than the Rounded isoline, suggesting soluble pectins may be involved in the adhesion between cotyledons. The solubility of the total uronic acids was not measured for the genotypes referred to in the previous study by Wood et al. (2014a).

Overall

It is unclear whether differences observed between the isogenic lines in this study and the chickpea genotypes in the previous study (Wood et al. 2014a, b, c) were due to genetic or environmental influence. There were, however,

some common relationships between their chemical composition and the degree of milling difficulty, despite genotypic and environmental effects. The overall similarities between the difficult-to-mill isoline in this study and the difficult-to-mill genotypes examined previously are summarised in Table 6.

These common attributes are characteristic of the difficult-to-mill genotypes examined in this study and suggest the following processes/relationships/causalities:

- Pectic polysaccharides play a role in seed coat adhesion, evinced by higher soluble NSP and uronic acid contents in the IF(whole) fraction of the difficult-to-mill genotypes;
- Higher lignin and insoluble uronic acid contents (indicating higher insoluble pectic polymer contents) in the seed coats of easy-to-mill genotypes increase the rigidity/brittleness of the seed coat, leading to improved ease of seed coat cracking and removal.
- Strengthening of cell walls on the abaxial surface of the cotyledons, adjacent to the seed coat, increases milling difficulty. This is indicated by higher insoluble NSP contents, especially the sugar residues arabinose,

Table 6 Compositional attributes common to all the difficult-to-mill angular isoline and the difficult-to-mill genotypes from Wood et al. (2014a, b, c)

	Seed coat	Abaxial cotyledon surface	Adaxial cotyledon surface
Compositional similarities of difficult-to-mill genotypes	Lower lignin Lower insoluble uronic acid	Higher protein Higher Na Higher soluble NSP (esp. galactose, arabinose and ribose) Higher insoluble NSP (esp. rhamnose and galactose) Lower starch Higher total and insoluble uronic acid	Higher arginine Higher Fe, Mn Higher lignin Higher soluble uronic acid

galactose, rhamnose and fucose in the IF(whole) fractions of the more difficult-to-mill genotypes,

- Strengthening of cell walls on the adaxial surfaces of the cotyledons and possible inter-cotyledon binding, evinced by higher manganese and lignin contents in the IF(split) fraction of the more difficult-to-mill genotypes;
- A proteinaceous material functions in adhesion at the abaxial and adaxial junctions, evinced by a higher content of total protein in the IF(whole) fractions of the more difficult-to-mill genotypes, particularly arginine in the IF(split) fractions. This protein could be derived from lectins, known to contribute to cell to cell adhesion in animal tissues. Higher concentrations of protein and manganese were higher in the difficult-to-mill isoline and genotypes from (Wood et al. ref) which suggest the presence of lignin-mediated binding between the seed coat and cotyledons.
- Higher sodium and iron contents in the IF(whole) and IF(split) fractions (respectively) were associated with increased milling difficulty, although no likely mechanism is known.

Conclusion

The isogenic lines were remarkably different in their chemical composition despite only a single gene difference. This suggests that this gene, whilst controlling seed shape, may also have other influences in the seed, including possible involvement in mineral absorption, transport or sink into the seed and the deposition of other components in seed tissues. Examination of the different fractions showed clear differentiation in the distribution of some chemical components. There was no evidence to support a role for sucrose accumulation in conferring different seed shapes in desi type chickpea, as is postulated to occur in pea. Rather, sodium is proposed as an osmoticum, inducing

larger cotyledon cells within the Rounded isoline and conferring the characteristic plumper shape.

It was significant that many of the associations of chemistry and milling quality identified in one set of genotypes have now been confirmed in a second, unrelated set of test genotypes (isogenic lines), especially as there were likely to have been major environmental effects (different years and trials). Attributes common to the difficult-to-mill genotypes suggest that pectic polysaccharides are involved in seed coat adhesion, physical plasticity of the seedcoat against a more rigid cotyledon periphery and potentially lignin-mediated binding. These relationships are worthy of further investigation to confirm or further explain any direct or indirect modes of action underpinning the differences in milling performance.

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