Chapter 6
Epigenetic Variation Amongst Polyploidy Crop Species

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Abstract Many agronomically important crop species such as wheat are (or were once) polyploid, with at least one round of whole genome duplication occurring before domestication. This genetic buffering or redundancy allows for sequence divergence, and in turn the development of functional variations between duplicated genes (homoeologues). Homoeologues may encode proteins with different properties and plant breeders have successfully used this genetic resource to introduce new genetic diversity into breeding populations. However duplicated genes are also subject to extensive epigenetic control and are therefore not always equally expressed. The preferential bias in the expression or the silencing of a specific homoeologue may be heritable and can be stable across many generations. There is also mounting evidence to suggest that selective homoeologue expression occurs in response to stresses such as salinity and may be specific to individual pathways or processes. Importantly, this type of epigenetic variation may segregate within a breeding population and is readily observed in newly synthesised polyploid hybrids.

It is now known that heritable phenotypic characteristics are determined by a combination of both genotype and epigenotype. Therefore the epigenome of polyploid crop species such as wheat and cotton represents a potent new source of diversity for agronomically important traits such as those linked to abiotic stress, secondary metabolite synthesis and fibre development. This text describes the characterisation of epigenetic variation in polyploidy crop species and its potential for exploitation by breeders for crop improvement.

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6.1 Background and Context

With an ever increasing global population, the need to provide a secure food supply has never been greater. It is therefore a grand challenge to crop breeders and agronomic scientists to maximise yields and make best use of agricultural resources available. Although substantial gains in productivity have been achieved in the years since the beginning of the last century, yields of a number of important crop species have plateaued in recent decades (see Grassini et al. 2013). During the 1800s, average UK wheat yields were in the order of approximately 1 t/ha, this figure now stands at 9 t/ha today (source: Rothemstead Research). Improvements in agronomic technologies such as mechanised cultivation and the development of new and better fertilisers all contributed to a year-on-year rise in yields; however, advances in the science of crop genetics and marker assisted breeding have contributed to the dramatic increase in the quantity and quality of wheat.

It has been suggested that a regional increase of just by 2 t/ha for African farmland would tangibly impact on global food security (Professor Martin Parry, Rothamsted Research) and although the UK production levels remain significantly higher than the global average, it is an aspiration to double output within the next 20 years (source: Biotechnology and Biological Sciences Research Council). To achieve these ambitious aims a number of issues will need to be resolved; the need to identify and capture new sources of diversity within wheat breeding populations is one such challenge. Although thus far a successful strategy, the breeding and interbreeding of a narrow panel of elite wheat's has resulted in a 'genetic bottleneck', resulting in a breeding population with limited potential for new desirable traits. This chapter discusses a potentially valuable new source of tractable diversity; a facet of biology that underpins developmental growth and abiotic stress responses. Although epigenetics is more widely studied in model organisms or human disease biology, this area of research may be productive for the improvement of polyploidy crop species.

6.2 Wheat as a Crop and Evolutionary History

The evolution of hexaploid wheat *Triticum aestivum* (genome formula AABBDD) can be traced to three diploid species: *T. urartu* (A genome), a species closely related to *Aegilops speltoides* (B genome) and *Ae. tauschii* (D genome) (Kihara 1944; McFadden and Sears 1946; Sarker and Stebbins 1956; Dvorak et al. 1993). Molecular clock-based studies have indicated that T. urartu and *Ae. speltoides* hybridised to form alloploid T. turgidum (AB) approximately 0.5 million years ago, while the integration of the *Ae. tauschii* to form *T. aestivum* occurred approximately 8000 years ago (Huang et al. 2002). Archaeological evidence suggests that tetraploid (emmer) was the predominant dietary grain in pre-9500BC in this region, while the consumption of hexaploid grains began approximately 9500–7500 years ago (Harris 1998; Kislev 1984). As no wild forms of hexaploid wheat have yet been

identified, it is likely that hexaploid hybrids naturally occurred at the margins of cultivated emmer and were then selected by early agriculturalists; presumably as this hybrid possessed superior traits compared to tetraploid emmer.

6.3 Wheat Polyploidy

Commercially cultivated wheat is predominantly either tetraploid or hexaploid, although the diploid *T. monococcum* is still sporadically cultivated in some parts of the Middle East (Salimi et al. 2005; Vallega 1995). Tetraploid durum wheat has two complete groups of seven chromosomes and its grain is typically suited to the manufacture of pasta. Hexaploid wheat has three groups of seven chromosomes, and it is commonly used for bread making. Hence it is often referred to as bread wheat.

Allopolyploidy is genetically unstable and over evolutionary time, most polyploidy species eventually revert to diploidy through various processes of genomic re-arrangements or deletions. Wheat is able to maintain three intact diploid genomes largely due to the action of genes such as Ph1, a gene which maintains diploid-like chromosome pairing (Riley and Chapman 1958). *T. aestivum* is just one of the many species to undergo speciation through polyploidy and as many as 80% of all known angiosperms are thought to have experienced a ploidy event(s) at some stage of their evolutionary history (Masterson 1994). Although it is difficult to precisely determine when and how many rounds of duplication and reorganisation may have occurred within the evolutionary history of a species, through the use of comparative mapping, etc., it is well established that polyploidy is a common and ancient phenomenon in plants (Brubaker et al. 1999; Gaut and Doebley 1997).

As the different parent genome donor species of hexaploid wheat probably descend from a common progenitor (Zohary and Feldman 1962), their constituent genomes although differing in size and structure are highly homologous in content. Therefore a functional consequence of an increase in ploidy is multiple copies of genes with near identical sequence. Over time, the accumulation of random mutations led to a divergence in sequence between duplicates derived from a single 'ancestor' gene (Feldman et al. 1997); in turn this allows for a functional divergence of the gene product (see Blanc and Wolfe 2004).

6.4 Gene Duplication and Fate of Duplicated Genes

The homology between the three genomes (A, B and D) has been subjected to sequence analysis using a range of techniques. These approaches include in silico sequence alignment, EST mapping and most recently whole genome sequence alignment (Gill et al. 1991, 2004; Somers et al. 2003; Qi et al. 2004; Brenchley et al. 2012). Historical approaches used to comparatively asses the structural relationship between each homoloeogous chromosome included meiotic chromosome pairing (Chapman

and Riley 1970), mapping (Erayman et al. 2004) and aneuploid analysis (Sears 1954), and fluorescent in situ hybridisation. The level of single nucleotide polymorphisms between homoeologous coding regions is estimated to occur at 1 in every 24 bases (Somers et al. 2003); however, the consequence to the transcriptome or ultimately the proteome of this sequence variation remains essentially unexplored.

In addition to mutation, sequence deletion has also shaped the diversity that exists between homoeologous gene sequences. Cryptic polyploids, such as maize, are thought to have evolved from ancient polyploids by a process of pseudogene formation followed by sequence loss. In a study investigating the fate of duplicated maize genes, Lai et al. (2004) suggested that within as little as 5 million years, approximately 50% of duplicated genes were lost through deletion. Deletions are also a common occurrence in established polyploids and may impact on important agronomic traits, e.g. a polymorphism for a puroindoline A deletion (or for a point mutation in puroindoline B) in the hexaploid wheat D genome dramatically affects grain hardness (Giroux and Morris 1998). Research investigating gene deletions in the D genome of *T. aestivum* suggests that as little as 0.17% of the D genome has been deleted during the past 8500 years and that deletions in established wheats occur at low frequencies (Dvorak et al. 2004). Surprisingly some loci were deleted from all three genomes, indicating a predisposition for the deletion of specific sequences (Dvorak et al. 2004). This research suggests that deletions occur gradually in established polyploids rather than as a rapid loss of sequence following hybridisation (Dvorak et al. 2004). Homoeologue deletion may negatively impact on the potential for each remaining homoeologues to become co-opted for a specific function or recruited into a specific pathway.

Homoeologous genes are by nature near identical in sequence and it is therefore logical to assume that homoeologues should be expressed at relatively similar levels (Gottlieb 2003). Early techniques such as enzymatic staining suggested however this assumption may not be correct for all genes. Using this technique to profile protein levels for a group of wheat isoenzymes, researchers unexpectedly found that of 54 sets of genes for which a genetic profile had been elucidated, 42 showed co-expression of all three homoeoalleles, but for 12 sets the product of only one homoeoallele could be identified (data extracted from Mcintosh et al. 1998). Similar variation in expression has also been reported amongst the Glu-1 homoeologues, a set of genes encoding an important class of seed storage protein (Flavell and O'Dell 1990). This work suggests that although homoeologues may possess near identical sequence homology, they are not always equally expressed (see review by Doyle et al. 2008).

6.5 Silencing in Crop Polyploidy Species

Early studies investigating epigenetic regulation or gene silencing in hexaploid wheat suggested that a bias in the expression or the silencing of individual homoeologues was a fairly rare occurrence. With little evidence to suggest that silencing was widespread, it was not considered an important factor in the organisation and

regulation of genes within the genome of polyploidy species (Hart 1996). However, as gene expression in wheat and other polyploids have been more extensively researched, estimates of the levels of silencing have been revised upwards. Kashkush et al. (2002) estimated that between 1 and 5% of genes in newly synthesised wheat hexaploids are silenced. This is comparable with the work by He et al. (2003), which estimated by cDNA-AFLP analysis that about 7–8% of genes are silenced in established wheats. He et al. (2003) suggested that genes located on the D genome may be silenced at a higher frequency than equivalents located on either the B or the A genomes. This may be due to the evolutionary history of wheat in which the D genome progenitor species hybridised with an established AB polyploidy species. The hypothesis would therefore be that silencing is directed at the 'invading' sequence. An alternative hypothesis suggests that any bias in the frequency of silencing may be due to an as yet unknown structural characteristic of the D genome itself (He et al. 2003).

Exploiting large collections of EST data, Mochida et al. (2003) concluded that silencing affected 11 of 90 sets of homoeoalleles tested (12%). Using an SSCP platform, Adams et al. (2003) suggested that about 25% of genes may be silenced in established tetraploid cotton. The authors (2004) also identified a similar difference between *de novo* and established cotton hybrids; using cDNA-AFLP they were able to demonstrate that about 5% of all genes are silenced in a newly synthesised cotton allotetraploid. In our study using SSCP and seedling leaf tissue of 'CS', at least one homoeolocus was silenced for 27% of the genes expressed (Bottley et al. 2006). This represents 9% of the total number of homoeologues (52 homoeologues of a total of 582) present. The frequency of silencing was numerically greatest in the D genome, although this was not statistically significantly as assessed by a chi-squared test in our experiments. Collectively, this work suggests that not all silencing is imposed immediately after hybridisation but that some silencing may gradually accumulate over evolutionary time.

In addition to the discovery that at least some homoeologues may be silenced after polyploidisation, Kashkush et al. (2002) amongst others also described a phenomenon whereby homoeologue activation occurred in newly synthesised polyploids. Transcriptionally silent sequences in diploid/tetraploid parent lines can become active in the polyploid progeny, occurring at a frequency of ~0.2% of all genes (Kashkush et al. 2002). It should be noted that two thirds of activated transcripts showed a high degree of sequence homology to transposable elements (Kashkush et al. 2003).

Genes identified as possessing silent homoeologues in hexaploid wheat have a diverse range of functions, e.g. ABC transporter genes to Rubisco subunits (He et al. 2003; Kashkush et al. 2002). The absence of a link between function and silencing particularly in newly synthesised polyploids is consistent with the theory of 'genomic shock' as opposed to a functionally controlled regulatory process. This model however contradicts data which suggests that silencing accumulates gradually. The most likely hypothesis is that some silencing or a bias in the expression occurs immediately after hybridisation and then new layers of regulation and complexity accumulate over many generations.

6.6 Frequency of Polyploidy Associated Silencing in Model Species

Silencing associated with polyploidy is widespread and not limited to cereal and fibre crops. Experiments using polyploids lines derived from model species, such as hybrids synthesised from *Arabidopsis thaliana* and *Cardaminopsis arenosa*, demonstrate that this phenomenon is a common feature associated with a change in ploidy. However although silencing occurs in *Arabidopsis* polyploids, the patterns and frequencies of silencing are markedly different to those identified for hexaploid wheats or tetraploid cotton. Comai et al. (2000) showed that contrary to the preferential silencing of the wheat D genome (He et al. 2003), silenced transcripts in the *Arabidopsis thaliana* × *Cardaminopsis arenosa* hybrid map at an equal frequency to both the *Arabidopsis* and *Cardaminopsis* genomes. Also the frequency of silencing is estimated to be in the region of 0.4%, differing from hexaploid wheat by ~10-fold (Comai et al. 2000). Differences in frequencies of silencing identified between polyploids generated artificially in the lab using Arabidopsis spp. and those hybrids originating from the hybridisation of diverse progenitor wheat *spp* may relate to the level of homology present in the sequences of merging genomes. *Arabidopsis* and *Cardaminopsis* are highly similar, only divergent in sequence for 5% of coding regions (Comai et al. 2000). Both size and genome homology are therefore likely to be important factors governing the overall frequency of silencing and will likely impact on the ability to derive new sources of epigenetic variation through the formation of synthetic hybrids.

6.7 Patterns of Silencing

Where tested, a significant proportion of cotton homoeologues appear to be differentially transcribed/silenced, importantly however this bias in expression may be linked to discrete organs or tissues (Adams et al. 2004). Further that in some instances, silencing may be associated with a specific process such as the preferential expression of the A genome in cotton fibre filament production (Yang et al. 2006). In silico analysis of pistil wheat in EST libraries identified that of 54 genes tested, over half showed a bias or silencing of expression; however, this figure was substantially lower in equivalent data sets obtained from emerging spike tissue (Mochida et al. 2003). Using an SSCP approach we were able to demonstrate that tissue specific silencing is widespread in hexaploid wheat (Bottley et al. 2006). In some instances silencing could be detected in only one tissue, conversely in other examples homoeologues were silenced in both root and leaf tissue. More unusually, in the instance of the gene FtsZ which encodes a plastid division protein, the A genome homoeologue was silenced in the leaf and the D homoeologue was silenced in the root. This may represent the subfunctionalisation of these homoeologues, i.e. the A genome homoeologue is in the process of being recruited as a root specific gene.

Differences in the expression of homoeologues amongst different tissues are informative. If the A genome homoeologue is silenced in leaf tissue but expressed in the root tissue of the same plant, this absence of expression cannot be explained by homoeologue deletion or inactivation by transposition or mutation. In most instances where a homoeolocus is silenced in leaf tissue but expressed in root tissue, this is likely due to tissue specific regulation. Research by authors such as Yang et al. (2006) also further suggests that this process is not merely a random consequence of gene duplication, rather an evolutionary process which serves to recruit duplicates into different functions or pathways as described above.

6.8 Consequences to Pathways and Enzymes

The consequence of bias or the selective expression of only one homoeologue is not necessarily trivial. Nomura et al. (2005) showed that the enzymatic properties of the homoeologous biosynthetic TaBx isozymes were specific to each homoeologue. To summarise, the enzymatic activity of each homoeologue protein differs by two fold between the A and B genome copies and a difference of up to 13 fold between the A and D genome copies. Thus the properties of TaBx enzymes which populate the proteome can be significantly affected by the identity or relative levels of the homoeologous transcripts that are transcribed; it is unlikely therefore that each homoeologue contributes equally to a pathway or process.

6.9 Silencing as a Stress Response

The experiments described above established the prevalence of silencing in a number of different agronomically important crop species. These data are also suggestive that homoeologue specific regulation plays a substantive role in specific pathways and processes (e.g. Yang et al. 2006). In Lui and Adams (2007) demonstrated that a bias or silencing of different homoeologues formed part of an abiotic stress response for one gene. It had already been well established that diploid species initiate stress responses which result in rapid and genome wide changes in gene expression (e.g. Ouyang et al. 2007), and polyploidy species respond in a similar manner (Kawaura et al. 2008). It had also been established that genes may be differentially regulated between sensitive and tolerant varieties in response to different stresses (Gulick et al. 2005), although a genetic explanation seemed the most likely cause. The data was first published in 2007, then subsequent works were published by Dong and Adams (2011), Chaudhary et al. (2009), etc., all suggest that a bias or the silencing of individual homoeologs in tetraploid cotton is a common feature of the polyploid cotton stress response, e.g. the relative levels of up to 70% of all homoeologue transcripts may be altered by some stresses.

A similar pattern of selective expression has been observed in polyploidy wheat. Where tested, the expression of the individual RAD50 DNA damage repair homoeologues is not equal; the B genome copy accounts for ~70% of the transcript pool in tetraploid wheat and ~60% in hexaploid wheat (Pérez et al. 2011). Stresses such as drought can elicit variation in the relative transcription of homoeologues of the cell wall invertase gene family (Webster et al. 2012), while we observed stress specific silencing for a broad range of different genes (8.9% of 112 genes tested) could be induced by salt stress (Bottley 2013). In our study an identical silencing response was observed in more than one cultivar tested and in some instances the same silencing profile could be obtained through the exposure of seedlings to a second distinct stress, e.g. cold. Cumulatively this data suggests that this bias in the expression of these homoeologues represents a generic stress response across a range of polyploidy species. Work by researchers such as Shoeva et al. (2014) is beginning to characterise these types of stress responses through the dissection of the relative expression of homoeologues encoding stress-linked proteins or metabolites, e.g. the expression of different homoeologue transcripts linked to the Chalcone pathway.

It is possible that the selective expression of homoeologues located to one genome as opposed to another is reflective of the relative stress tolerant properties of the progenitor species. In a simple model this may fit with the proposed mechanism of homoeologue specific regulation proposed by Udall and Wendel (2006), e.g. in a simplified model, a stress specific transcription factor has a greater affinity for the promoter of homoeologue A compared to homoeologue B. This promoter sequence of homoeologue A may have evolved under a greater selection pressure of stress exposure due to the environment experienced by the plant A. It is possible that this type of epigenetic response differs amongst varieties of wheat; however, further research is required to establish how variations in the epigenome can be exploited to develop polyploidy crop species with greater stress resistance properties.

6.10 Segregation and Differences Between Varieties and Transgenerational Stability

Patterns of gene expression amongst different wheat varieties are not uniform. Using a microarray platform, Gulick et al. (2005) demonstrated that for two wheat varieties 65 of 947 genes tested are differentially regulated. Although this study was unable to differentiate between the relative levels of each homoeologue transcript, it demonstrates that variations in the expression amongst varieties of the same species are not uncommon. Intriguingly research investigating the distribution of methylation using methylation sensitive enzyme experiments suggests that methylation is more frequently polymorphic amongst 20 accessions of the cotton polyploidy *Gossypium hirsutum* than equivalent genetic diversity (Keyte et al. 2006). This suggests a candidate mechanism which underpins differences in expression between varieties and it is worth mentioning in this section that methylation can be both stable and heritable.

Where tested, profiles of silencing differed amongst a panel of 16 different wheat varieties, and cultivars commonly used to generate most commercially grown crop lines (see Bottley and Koebner 2008). Plants were profiled to identify silencing in both leaf and root tissue and no variety showed the same homologous expression profile when each were tested for the expression of 15 genes. Although overall frequencies of silencing were similar in each cultivar, each line possessed a unique pattern of silencing. Some homoeologues were silenced rarely, whereas other homoeologues were silenced frequently and silenced in more than one variety.

In order to understand the heritability of this silencing, the expression of a homoeolog identified as silenced in only one parent line was profiled in the progeny of a cross between the varieties Avalon and Cadenza. The same homoeologue was identified as silent in a number of offspring, although the trend favours a ratio where expression was more common than silencing. Interestingly a small but significant variation in the percentage of silenced homoeologues has been identified between two replicates of the same variety of tetraploid cotton (Adams et al. 2003). Although initially attributed by the authors to be an artefact of the cDNA-AFLP technique employed, it is possible that this represents a layer of intra-species variation not yet fully appreciated.

Although in some instances silencing is stochastic, research investigating hexaploid wheat, tetraploid cotton and artificially generated Arabidopsis hybrids has proven that silencing may be stable and heritable across many generations (Bottley et al. 2006; Adams et al. 2003; Wang et al. 2004). It should be noted that where silencing has previously been documented to be unstable or random, this may reflect unrecorded changes in abiotic stress or subtle variations in growth conditions which are then reflected in profiles of transcription (discussed above). Conversely it may be suggested that a heritable pattern of expression merely reflects the same response by the same genotype to the same conditions, rather than heritable transgenerational silencing.

To summarise, patterns of silencing are not always identical amongst cultivars or varieties of the same species, may be heritable and can segregate within breeding populations (Bottley and Koebner 2008). With this in mind, it is likely that within the panel of elite wheat's there exists a substantial amount of 'untapped' epigenetic variability. This is also likely to be true for other polyploidy species such as cotton. As described above the consequence of this type of epigenetic control is not without consequence and it is likely that silencing or a bias in the expression of different homoeologues forms an intrinsic part of a polyploidy specific stress response. Therefore it is not unreasonable to suggest that each variety possesses a unique epigenetic-type in addition to genotype, and that this layer of epigenetics may segregate differently within breading populations.

6.11 Newly Synthesised Polyploids

The rates of silencing identified in newly synthesised polyploidy plants differ markedly from the frequencies observed for established polyploid equivalents. 'Genomic shock' has been proposed as a possible driver for polyploidy decay (McClintock

1984) and may in-part explain the phenomenon of homoeologue specific silencing; in this model, genomic instability occurs immediately upon hybridisation, and is followed by a period of stabilisation (reviewed by Chen and Ni 2006). Intriguingly polyploidy may also lead to the re-activation of previously silenced genes; this phenomenon, although not as frequent as silencing, has been documented in wheat, cotton and Arabidopsis polyploids (Kashkush et al. 2002; Adams et al. 2003; Wang et al. 2004).

Using a cDNA-AFLP platform to assay the frequency of silencing in newly synthesised cotton polyploids, approximately 5% of 2000 transcripts were identified as silent (Adams et al. 2004). A similar figure was observed for newly synthesised wheat hexaploids polyploids using the same technique—an estimate of between 1 and 5% of genes were silenced in these lines (Kashkush et al. 2002). The frequency of silencing for tetraploid *Arabidopsis* hybrids was substantially lower (0.4%) than tetraploid cotton equivalents, which likely reflects the importance of the composition of the relative genomes rather than a consequence of mere duplication (Comai et al. 2000).

Using an SSCP platform, we profiled the expression of 36 genes amongst a panel of number of newly synthesised polyploidy wheats (data unpublished). Genes were tested for expression in hybrid root and leaf tissue and equivalent material obtained from six parental lines each with differing backgrounds (diploid, e.g. *Aegilops tauschii* spp. *strangulata* and *tetraploid T. turgidum* spp. durum cv. *carthlicum*). We identified rates of silencing in these newly synthesised wheat hexaploid lines which ranged from ~5 to 10%. Interestingly, in some instances silencing was maintained, i.e. present in both the parent and the hybrid; however, in other examples silencing was only observed in the newly synthesised line. One possible explanation is that this variation in the rate of silencing which is observed amongst newly synthesised plants is reflective of the degree of homology which exists between the different parental lines. This data together with the data recorded for other polyploidy crop species suggests that the process of forming new hybrids may introduce epigenetic variation, a new diversity within the epigenome distinct from the originating progenitor plants.

6.12 Exploiting Epigenetics as an Agronomic Tool

Epigenetic variation may shape phenotype. A few important examples of this have been described in the literature for diploid species, e.g. the colourless non-ripening phenotype tomato epimutant described by Manning et al. (2006); a dramatic example where an epi-polymorphism alone determines an alternate ripening process. It is therefore not controversial to suggest that selecting for epigenetic variation or the incorporation of techniques such as epimarkers may have a role in exploiting the epigenetic diversity which already exists within breeding populations of polyploid crop species. It is likely that epigenetic variation may determine agronomically important traits such as fibre production in cotton or drought stress in wheat. It is

possible that some epigenetic modifiers are stochastic and therefore not amenable for use as a breeding resource; however, it is equally likely that patterns of silencing represent a valuable resource if they can be exploited. Although further research is required to fully understand the mechanisms which determine and regulate homoeologue specific silencing, it is becoming clear that in polyploidy species the blend in the expression of different genomes may represent an important resource for crop breeders.

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