

Developing genetic resources for pre-breeding in *Brassica oleracea* L.: an overview of the UK perspective

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Abstract The vegetable brassicas are an important crop worldwide and are of significant commercial value. In order to ensure our targets for food security are met it is important that these crops are continually improved to increase sustainability of production, increase nutritional quality and reduce waste. Development of resistances against both biotic and abiotic stress are recognised as being key. Plant breeding plays a vital role in addressing these issues through the development of new and improved varieties. This continued improvement is becoming evermore dependent on our ability to identify and introgress beneficial alleles from ‘exotic’ germplasm into elite breeding material. Increasingly, more diverse germplasm such as those found in genebanks is being screened for beneficial allelic variation, however, plant breeders often find it difficult to make use of such material due to the time required to remove undesirable characteristics from progeny due to linkage drag. This article describes how we have attempted to overcome this and develop resources that make the diversity available within the *Brassica oleracea* gene pool more accessible.

Introduction

Brassica vegetables within the U.K. are a significant crop and although the land area occupied by them is relatively small compared to the broad acre crops such as wheat and oilseed, their relative value is high. In terms of quantity-

produced world wide, brassicas exceed 76.1 million tonnes, with the UK producing over 479 thousand tonnes with a farm gate value of £60 million (FAOSTAT 2010). Vegetable brassicas have received a great deal of attention in the past five to ten years as scientific studies have demonstrated that there is a real basis to the claims that they are a “super food”. Governments worldwide are now viewing both food security and health as areas of prime importance, which is helping to raise consumer awareness. Both the medical profession and consumers are as a result paying more attention to the nutritional content of their diet; added to this the UK government has promoted the consumption of at least five fruit or vegetable portions per day as part of a healthy diet. There has been some debate as to the efficacy of this campaign and it is seen that further evidence of the benefits of a diet rich in vegetables and fruits is required. The horticultural brassicas are beginning to provide the evidence for such a policy. This is due to their ability to deliver vitamins, minerals and antioxidants, while research is ongoing to demonstrate how they might act to reduce the risk of cancer and other diseases (Walley and Buchanan – Wollaston 2011; Traka and Mithen 2009).

Because of their commercial value, breeding within the horticultural brassicas has been seen as important to the UK industry and development of genetic resources has been recognized as playing a vital role in this programme. Within the UK this has been carried out at Wellesbourne since the formation of the National Vegetable Research Station in 1949. Since then research has focused on agronomy, nutrition, crop improvement, pests and disease, and weed control. This has resulted in the Wellesbourne site becoming a centre of excellence for *Brassica* genetics. More recently, this first class facility was incorporated into the newly formed School of Life Sciences at the University of Warwick; this amalgamation fosters the continuum between academia and translational crop genetics. This

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article describes the resources being developed in the Warwick Crop Centre, which is based at the Wellesbourne site, and is maintaining the resources developed over the past 60 years.

Capturing genetic variation

The Warwick Genetic Resources Unit (WGRU) is part of the Crop Centre and is a genebank that holds the UK vegetable seed collections. Its remit is the collection, conservation, characterization and documentation of a range of crops and their wild relatives. It holds the UK collections of vegetable germplasm including brassicas, alliums, lettuce, carrot, radish and celery (<http://www.warwick.ac.uk/go/gru>). The genebank holds over 6,000 accessions of brassica crops and represents an invaluable resource of alleles that could offer solutions to many of the problems of concern to plant breeders. Crop domestication and subsequent breeding efforts have resulted in modern crops with great variation in morphology but with a restricted genetic base sometimes referred to as ‘allelic canalization’ (Fig. 1).

The reduced range of allelic diversity captured in the current domesticated gene pool may not be sufficient to respond to the challenges of local and, to a greater extent, global food security where phenotypes that are capable of resisting biotic and abiotic stress may be required (Reeves et al. 2012). One of the best routes to introduce vigour into breeding programs is through the production of F1 hybrids, which allows the increased mixing and interaction

between alleles causing hybrid vigour. Often breeders find genebank collections hard to deal with because of the abundance of material that makes selection difficult. To address this problem, diversity core collections have been produced where the aim is to represent the majority of the genetic variation in Genebank collections in a smaller, more manageable number of lines. Brassica diversity core collections are now available in France, the Netherlands, Korea and the UK. However, the difficulty with such collections is that most of the accessions are effectively variable populations. This means that using them for trait genetic analysis and genotyping is confounded by plant-to-plant variation and heterozygosity of individual plants. As such it can be difficult to correlate results and to identify the specific allele responsible for a trait difference.

Recently, the concept of a core collection has been developed further by the generation of homozygous lines from the starting, or founder lines of the core collections. These new collections are termed Diversity Fixed Foundation Sets (DFFS) (Pink et al. 2008). Lines within a DFFS are thus much more suited to trait genetics research, as experimental replication using genetically identical plants will greatly facilitate the dissection of the heritable component of a trait from the environmental effects. In addition, the polyploid ancestry of the *Brassica* genome, which is manifested as a high percentage of gene replication, makes it difficult to differentiate allelic variation at one locus from sequence differences between homologues in heterozygous germplasm. However, in the homozygous DFFS lines, apparent gene polymorphisms can now be attributed to differences between replicated loci. The selection of a reference sample from the available diversity is illustrated in Figure 2.

The *B.oleracea* DFFS (BoDFFS) collection was developed with 376 founder accessions based on the EU GENRES core collection (Leckie et al., 1996). These were mostly sourced from genebanks, but also included 25 fixed founder lines to encompass the parents of a number of mapping populations and other available DH lines. The collection was subdivided into 4 subsets of 94 to represent the diversity within the species sub-taxa and crop type, as

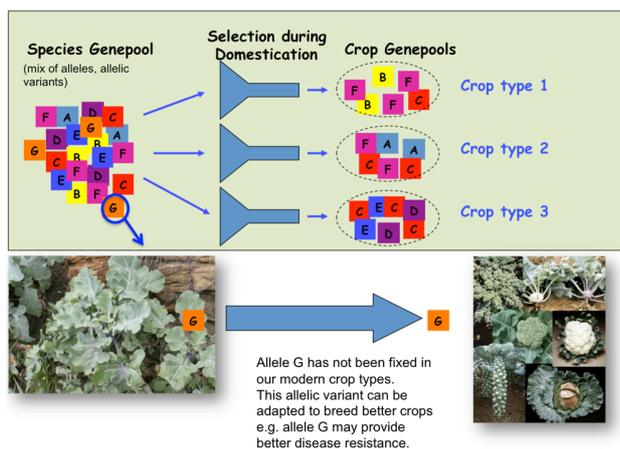


Fig. 1 Crop domestication may have only captured a subset of the alleles present in a species gene pool and left ‘useful’ alleles behind. This is referred to as the founder effect. In the example, alleles have been captured during domestication to form crop types (broccoli, cauliflower, kale), but allele G was not incorporated into the domesticated genetic base, therefore allele G may be a variant that can enhance a desired phenotype

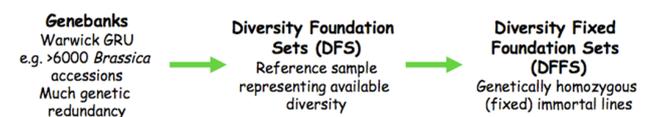


Fig. 2 The schematic illustrates the principle behind capturing the genetic diversity in the form of Diversity Foundation Sets, then fixing this diversity in homozygous Diversity Fixed Foundation Sets

well as variation in eco-geographic origin. In order to generate homozygous lines from the genebank accessions, plants were grown to flowering and subjected to microspore culture. DH lines have been produced both at Wellesbourne and through collaboration with the breeding industry. It has not proven feasible to produce DH lines for all the founders due to variation in their responsiveness to microspore culture. The desired outcome is the largest subset of fixed lines for which sufficient seed can be generated.

To extend the genetic base of our diversity sets we have also created the *Brassica* C genome Diversity Fixed Foundation Set (BCgDFFS). The BCgDFFS was established from 89 founder accessions, representing 14 species closely related to *B. oleracea*. With the prior knowledge that many of the founder accessions have long generation times and possessed strong self-incompatibility, an alternative strategy for generating fixed lines was taken with the aim of creating a more user-friendly resource. When the founder lines eventually flowered they were crossed with a microspore responsive, self-compatible rapid cycling *B. oleracea* line, this strategy helps overcome the problems of self-incompatibility and long generation times that can be experienced with wild *Brassica* accessions (Pink et al. 2008). This helps overcome the problems of self-incompatibility and long generation time that is often experienced within wild *Brassica* accessions. The F1s from the majority of these crosses flowered without vernalisation and have been subjected to microspore culture. To date, DH lines have been produced, representing 12 wild species. Another set of DH lines is also currently being generated. These DH lines will have undergone one round of recombination between the wild species genome and the rapid cycling *B. oleracea* parent. As a result their genomes will represent a mixed patchwork of the two parental genomes.

The establishment of genetic resources such as the diversity collection and the DFFS collections provide a reservoir of valuable alleles; however, there is still a long way to go if we are to make these resources fully accessible for use in crop improvement. In humans we are beginning to gain a picture of human diversity through programmes such as the human HapMap project. In this project thousands of individuals are being screened with a large number of markers. However to reduce cost these markers have been directly selected to represent specific haplotype blocks rather than using all possible SNPs. This may miss some of the variation that is present but does reduce the cost to an acceptable level (Altshuler et al. 2010). The understanding of recombination and where the breakpoints or recombination hotspots are within the

genome, are increasing our ability to identify causative agents of disease such as copy number variants (Altshuler et al. 2010).

Within plant breeding the focus has until recently been on the assessment of phenotypes associated with a trait of commercial interest. This was partly due to the cost of molecular techniques, associated marker development and the requirement of highly skilled individuals. The marker of choice has with time moved from AFLPs to SSRs and, with the advent of high throughput genome sequencers, single nucleotide polymorphisms (SNPs) have become the latest marker of choice (Baird et al. 2008). This is because large numbers of SNPs can now be identified relatively quickly at a reasonable cost. In the past, identification of sufficient suitable markers for use within a given population has proved difficult since often markers that were polymorphic in one cross were monomorphic, and thus not informative in another. The recent development in sequencing technologies has helped resolved the difficulties in generating suitable markers. This has been followed by the development of high throughput SNP genotyping platforms such as the Goldengate and Infinium assays (Illumina), Kaspar (KBioscience) and Sequenom (Perkel 2008). The cost of SNP genotyping has now come down to a point where breeders consider it cost effective. This has meant that the use of marker-assisted breeding is becoming more prevalent within breeding programs.

The screening of diversity collections including the DFFS collections with a large panel of SNP markers can help us to understand the degree of duplication that is present within gene bank collections, and has also helped in our understanding of how the available genetic diversity has arisen. It also means that breeders can focus on regions of the genome that they are most interested in when selecting material from genetic collections (Paterson 2006). An example of the value of genetic markers to understanding genetic diversity is presented in Figure 3.

The diverse collections provide a valuable source for genetic material for use in breeding programs but they are only one tool that is available to plant breeders who are interested in identifying alleles underlying a trait of interest. A specific application of molecular markers has been to examine both the chloroplast and nuclear genome of *B. oleracea* and its wild relatives, along with *B. rapa* and the amphidiploid *B. napus* (Allender and King 2010). The nuclear genome of the latter species is composed of the C genome of *B. oleracea* and the A genome of *B. rapa*, but the ancestral genotypes involved in the creation of *B. napus* as a species had not been identified. Chloroplast

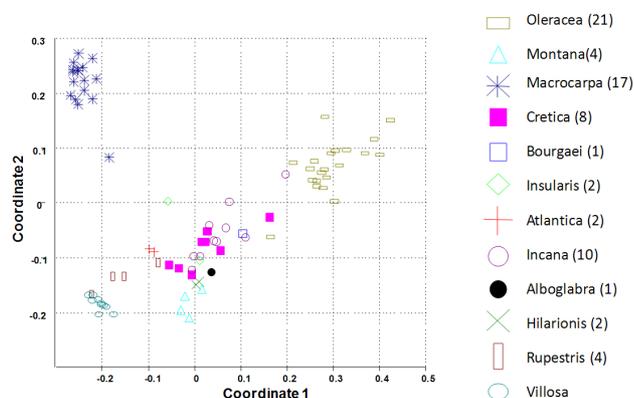


Fig. 3 Principal co-ordinates analysis of genetic diversity within the BCgDFFS founder lines generated using 536 SNP markers (GoldenGate assay) showing species-specific clustering of lines. Brassica species are on the left side: *B. oleracea*, *B. montana*, *B. macrocarpa*, *B. cretica*, *B. bourgaei*, *B. insularis*, *B. atlantica*, *B. incana*, *B. alboglabra*, *B. hilarionis*, *B. rupestris*, *B. villosa*

SSR markers showed that the majority of *B. napus* possessed a chloroplast haplotype only found in certain crop types of *B. rapa*. This suggested that *B. oleracea* or a related species was the original pollen donor, since the chloroplast genome is transmitted through the maternal line.

Mapping populations

Applied trait based research in vegetable brassicas relies on the presence of genetic variation that segregates for the phenotype of interest. This variation is usually captured in a mapping population that has been genetically characterised in the form of genotype data for genetic markers for the individuals within the population. In most cases the marker data will have been used to construct a linkage map, and the genotype data aligned with the map data to provide information about the relative recombination within the population. Combining these data with the phenotype data allows quantitative trait loci (QTL) to be mapped relative to the linkage map. This forms the starting point for line selection and subsequent backcross programmes to further define and resolve the QTL.

For *B. oleracea*, the creation of mapping populations is hampered by the presence of a self-incompatibility system that makes the fixation of progeny by single seed descent (SSD) difficult in the presence of strong S-alleles. When lines can be taken through SSD the recovery of useable numbers of viable seed is often reduced.

Doubled haploid populations

The vegetable brassicas (*B. oleracea*) generally respond well to both anther culture and microspore culture. Microspore culture is the preferred route in many cases due to the ease of use and the generally greater number of androgenic lines recovered (Lichter 1989), however the embryo yield is genotype dependent (Takahata and Keller 1991; Duijs et al. 1992). When doubled haploid lines are generated for use in hybrid production, the time savings can be substantial, two years compared to eight or more (Dias 2001; Pink et al. 2008). Doubled haploid lines have the advantage of eliminating residual heterozygosity and heterogeneity and as a result the assessment of quantitative traits is simplified. As already stated above, DH lines can be replicated for phenotype assessment, minimising the environmental variation associated with trait analyses. Several *B. oleracea* mapping populations and associated linkage maps are available for which we curate the seed collections at Warwick: BolTBDH (Iniguez-Luy et al. 2009), BolNGDH (Sebastian et al. 2000), BolAGDH (Bohuon et al. 1996; Sebastian et al. 2000), BolMGDH (Walley et al. 2012), Table 1.

The BolTBDH population is the reference mapping population for *B. oleracea* genomics. The population was derived from the cross between the Chinese Kale ‘TO1000’ and the broccoli line ‘Early Big’. TO1000 is a rapid cycling line and like Early Big does not require vernalisation. In addition, virtually all the DH lines in the population set seed well making the population an attractive resource to use. TO1000 is also the reference *B. oleracea* line used for the International sequencing project (*B. oleracea* sequencing consortium 2011). The BolNGDH population is a unique population derived from a cross between a DH cauliflower (CA25; ex cv Nedcha) and a DH Brussels sprout line (AC498; ex cv Gower) (Sebastian et al. 2000). The BolAGDH population was created using microspore culture on the F1 of a cross between ‘A12Dhd’, a Chinese kale DH line and the broccoli DH line ‘GDDH33’ (derived from the cultivar Green Duke). This population has been well characterised (Bohuon et al. 1996; Sebastian et al. 2000) and the AGSL, a population of substitution lines (backcross inbred lines) were created from it (Ramsey et al. 1996; Rae et al. 1999) as an additional resource for trait fine-mapping.

These populations have been used to map QTL for a range of economically important traits, including: flowering time (Bohuon et al. 1998; Rae et al. 1999), and the mineral content in leaves (Broadley et al. 2008), calcium and magnesium variation, the link between shoot yield and phosphorus use efficiency (Hammond et al. 2009), shoot

Table 1 Summary of the *B. oleracea* fixed mapping populations that are curated by Warwick Crop Centre

Population name	Cross	Crop types	Type	No. lines	Seed available	Lines curated	Reference
BolTBDH*	TO1000DH3 × Early Big	Chinese kale × Calabrese	DH	150	Yes	Yes	Iniguez-Luy et al. (2008)
BolAGDH	A12DHd × GDDH33	Chinese kale × Calabrese	DH	100	Yes [^]	Yes	Bohuon et al. (1996); Sebastian et al. (2000)
BolAGSL	A12DHd × GDDH33 substitution lines	Chinese kale × Calabrese	BILs	63	Yes	Yes	Ramsay et al. (1996); Rae et al. (1999)
BolMGDH	GDDH33 × MarDH34	Calabrese × Calabrese	DH	-	**	Yes	Walley et al. (2011)

*The BolTBDH population is the reference mapping population for *B. oleracea*. The parental line TO1000DH3 is the reference genome for the *B. oleracea* sequencing project.

[^]Seed for some accessions in this population are being bulked, therefore these accessions will not be available.

**Seed for this population is currently not available; seed are being bulked up.

DH = Doubled Haploid; BILs = Backcross Inbred Lines

zinc concentration (Broadley et al. 2010), potassium use efficiency (White et al. 2009), water use efficiency (Hall et al. 2005) and circadian rhythm variation (Salathia et al. 2007). Bettey et al. (2000) carried out a quantitative genetic analysis of seed vigour and seedling pre-emergence, this led to the mapping of QTL for germination efficiency and the development of a hypothesis for seedling establishment in both the NGDH and AGDH populations (Finch-Savage et al. 2010). The NGDH population has been used to characterize a number of morphological traits (Sebastian et al. 2002).

Broccoli breeding programmes continue to improve a wide range of traits including aspects of head morphology and appearance, quality attributes such as nutritional content (Moreno et al. 2006; Jeffery and Araya 2009), pest and disease resistance (Darling et al. 2000; Farinhó et al. 2004) and post harvest shelf life (Wurr et al. 2002; Jeffery et al. 2003). These traits are all influenced by environmental interactions (Wurr et al. 2002), making phenotype selection difficult and expensive. The BolMGDH population was developed as a tool for broccoli research. It is an intra-crop population from two broccoli ‘Calabrese’ lines, MarDH34 derived from the cultivar Marathon and GDDH33 (Walley et al. 2012). Intra-crop crosses reduce the number of polymorphic markers that can be used for genetic map production, compared to intraspecific crosses (e.g. BolAGDH and to a lesser extent BolTBDH). However, the available inter- insert “Broccoli” and intraspecific mapping populations do not segregate for head morphological traits. The

BolMGDH population has the advantage over other *B. oleracea* mapping populations because it does segregate for head morphologies, which makes it attractive for broccoli research. This population has a genetic map and has been used to identify QTL for yield related traits (head size, weight loss through storage) and variation in leaf morphology (Walley et al. 2012). The population is currently being used to quantify broccoli head shelf life traits. The QTL identified for these traits can be integrated directly into broccoli breeding programmes, reducing linkage drag that would be associated with non-target crop type populations.

The future

The increasing awareness of the beneficial phytochemicals present within crop types of the vegetable brassicas has advanced many of these to ‘super-food’ status. Modern breeding techniques within the Brassicaceae are pushing forward at an increasing pace, as new molecular tools based on next generation sequencing become more accessible. To make best use of these technological advances, we still need to capture and understand the allelic variation that is contained within exotic sources of germplasm. The creation of genetic resources, such as the *B. oleracea* diversity fixed foundation sets, opens the door to the flow of allelic variation required for directed breeding necessary to further improve public health, and in the medium to long term, addresses issues relating to food security.

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