

# Chapter 15

## Bioenergy Trees: Genetic and Genomic Strategies to Improve Yield

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### Global Drivers for Increasing Bioenergy from Trees

The recently concluded COP21 climate negotiations committed UN signatories to restrict global temperature increases to ‘well below’ 2 °C above pre-industrial levels and to aspire towards 1.5 °C (United Nations 2015). If such ambitious climate and GHG emissions targets are to be met and future energy security assured, it is essential that renewable and sustainable alternative energy sources are developed and utilised on a global scale. Bioenergy and bioenergy with CCS will be a central part of that commitment, since these technologies feature strongly in many forward scenarios for reduced and negative carbon emissions, as described in the work leading

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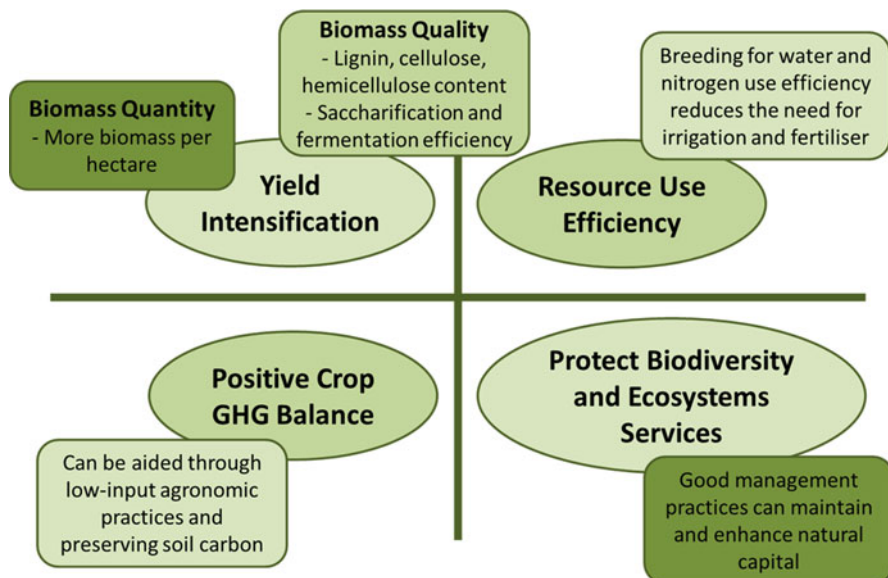
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to COP21 (Fuss et al. 2014). Many outstanding issues remain on delivery however, the concept of ‘land sparing’ (increasing agricultural yields and reducing farmland area to allow the spare land to be used for climate change mitigation and biodiversity conservation) could enable high-yield feedstocks to produce significant, low-impact lignocellulosic resources (Lamb et al. 2016). Sources of lignocellulosic biomass under active consideration over the past decade include energy grasses (David and Ragauskas 2010; Jørgensen 2011), crop residues (Gomez et al. 2008) and fast growing trees cultivated under short rotation coppice or short rotation forestry (Hinchee et al. 2009; Tullus et al. 2012).

## **Prioritisation of the Sustainable Intensification of Biomass-Tree Cultivation**

Bioenergy trees are prime examples of second-generation (2G) bioenergy feedstocks (as defined by Manning et al. 2015) in which lignocellulosic biomass is harvested from dedicated perennial species (Somerville et al. 2010). Such crops must be appropriate to the climate and region in which they are grown and able to grow on marginal lands thus minimising competition with food crops or the destruction of high-nature-value ecosystems. They should require few inputs; both to minimise the economic and energy costs of their cultivation and management and to reduce the environmental impacts associated with the fertilisers and pesticides necessary for intensive farming. A review by Manning et al. (2015) suggested that farmland biodiversity and the provision of regulating and cultural ecosystem services could even be improved by perennial biomass crop cultivation if appropriately managed across the landscape. Benefits (in addition to provisioning services such as low carbon energy) include the provision of habitat corridors between and within intensively farmed land areas; harbouring biodiversity including pollinators and other insect populations; preventing soil erosion and buffering water sources against nutrient run-off and sedimentation (Manning et al. 2015). Land use change modelling reported by Milner et al. (2015) supports this idea and proposes that planting perennial lignocellulosics, in temperate landscapes at least, can enhance the provision of a basket of ecosystem services. A review by Don et al. (2012) reported that soils under dedicated perennial crops emit significantly less  $N_2O$  than soils under conventional arable cultivation. They also have the potential to sequester more carbon, though the effect of transition from grassland to perennial biomass cropping can be neutral or even slightly negative (Don et al. 2012; Harris et al. 2015). The net greenhouse gas balance of bioenergy crops depends strongly upon good land management practices (Davis et al. 2013) and maintaining soil-based ecosystem services is an essential aspect of sustainable agricultural intensification (Schulte et al. 2014). Figure 15.1 provides an overview of the ‘more-from-less’, paradigm for such sustainable intensification; increasing yield in a given area without degrading the land or resorting to energy intensive cultivation practices (Allwright and Taylor



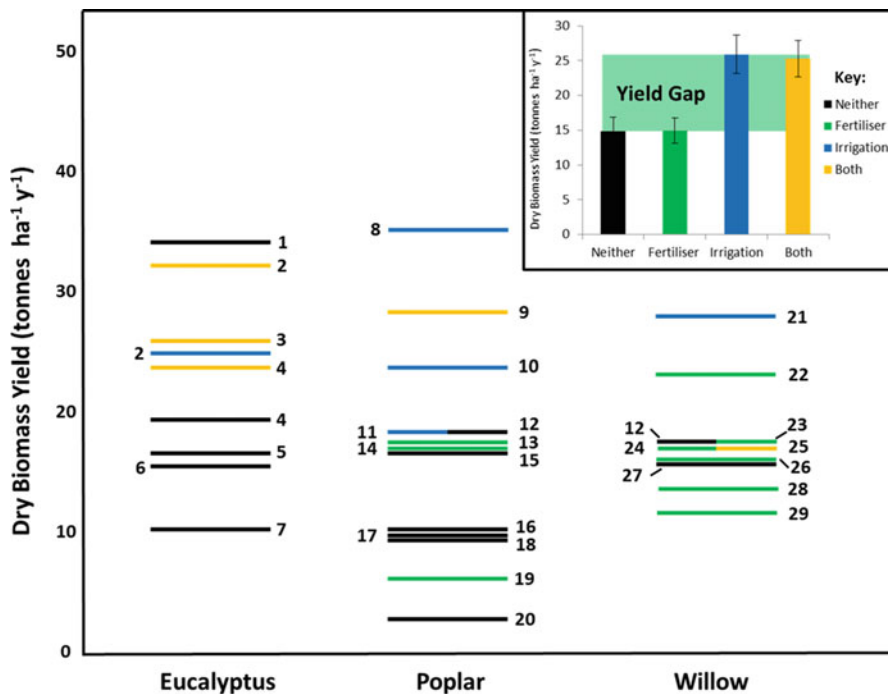
**Fig. 15.1** More from less—sustainable intensification. The figure illustrates how breeding targets to fill the yield gap through yield intensification should be a high priority. However, this yield intensification must make full consideration of the wider inputs required to achieve high yield and that more efficient plants with respect to resources such as water and nitrogen are required. In addition, yield intensification must occur alongside assessment of the GHG balance of the crop system such that lower GHG emissions can be targeted; perhaps through management practices related to the preservation of soil carbon. Finally, a basket of ecosystem services that includes biodiversity protection must be delivered from future multi-functional landscapes

2016). This essential principle drives bioenergy tree breeding and development and thus the remainder of this chapter is focussed on research in three important species: poplar (Verlinden et al. 2015), willow (Stolarski et al. 2013) and eucalyptus (Freeman et al. 2013).

The primary goal of bioenergy tree breeding and development is the sustainable intensification of biomass production. In addition to breeding for yield traits, this also means targeting feedstock quality to increase the efficiency of conversion to liquid fuels and decrease emissions across the whole life cycle of the system. Resource use efficiency with respect to water and nutrients is also a priority to ensure a low-input crop without high irrigation or fertigation needs. Good agronomic practices are also an essential aspect of making high yielding bioenergy trees sustainable. The crop GHG balance can be improved through the preservation of soil carbon stocks and biodiversity and ecosystems services may be preserved and enhanced by good land management.

## Unravelling the Yield Gap is Central to the Sustainable Intensification of Bioenergy

Bioenergy trees have the potential to achieve high yields in sustainable, low input agronomic systems and could allow significant reductions in GHG emissions compared to conventional fossil fuels (Zanchi et al. 2012). However, these species still require investment and research effort to overcome the yield gap between typical biomass harvests and their true genetic potential (Allwright and Taylor 2016). The yield gap is widely acknowledged in the breeding and development of food crops (Affholder et al. 2013; Kassie et al. 2014). It is the difference between the potential crop yield under optimal, non-limiting conditions (water, nutrients, pest control) and the average yield under typical field conditions (Mueller et al. 2012; Van Ittersum et al. 2013). A yield gap may result from one, or a combination, of genetic (G), environmental (E) or management (M) factors and failure to address these factors may result in yield stagnation (Licker et al. 2010; Ray et al. 2012). We have already outlined that a central aim for the development of bioenergy trees is sustainable intensification (Fig. 15.1) and understanding the nature of  $G \times E \times M$  interactions underpinning the yield gap can help drive this. For example, through enhanced water and nutrient resource management (Mueller et al. 2012; Bredemeier et al. 2015); the optimisation of soil pH (Tilman et al. 2011); protecting and increasing soil organic carbon stocks (Powlson et al. 2011) or improving soil aeration which is important to fine root development and growth (Weltecke and Gaertig 2012). Figure 15.2 (modified from Allwright and Taylor 2016) demonstrates significant yield gaps for all three tree species; although this figure generally draws on data from small research-scale yield plots since those are the only data widely available at present for these crops for such an analysis. With this caveat, poplar and eucalyptus show a greater range of values than willow with larger maximum biomass yields reported. Poplar trials range from 3 to 35  $t^{-1}ha^{-1}y^{-1}$  (mean 16.1), eucalyptus trials from 10.5 to 34  $t^{-1}ha^{-1}y^{-1}$  (mean 22.4) and willow trials from 11.6 to 27.5  $t^{-1}ha^{-1}y^{-1}$  (mean 17.3). It can be seen that the highest yields are generally achieved in trials in which irrigation or fertigation are supplied, yet potential yields are rarely reached. There is however, an exception in the case of the highest yielding eucalyptus trial whose small experimental plot size means, in the words of the authors, ‘commercial yields are likely to be considerably lower’ (Sims et al. 1999). In practice therefore, the yield gap may be greater than indicated by the shaded region of the chart as commercial yields fall short of those reported in experimental plots and trials (Nonhebel 2002) where only climatic conditions may be limiting. In the remainder of this chapter, we explore the potential for high-throughput phenotyping and transcriptomics, forward genetics (association studies and genomic selection), reverse genetics and genetic modification/genome editing as tools to help close the yield gap and drive the sustainable intensification of the cultivation of biomass trees as part of the molecular breeding pipeline.



**Fig. 15.2** Reported biomass yields reveal a yield gap for biomass trees. Poplar, willow and eucalyptus show wide variation in their biomass yields. Where more than one value is reported in a publication those given here are the maximum reported oven-dry biomass yields ( $t\ ha^{-1}\ y^{-1}$ ) for the best performing sites, genotypes and years or coppice cycles within each study. The inset bar chart displays the mean yield and standard error for all the trials shown, pooled across feedstocks for each management practice. Numerical citations adjacent to each bar correspond to a single published field trial: **1.** (Sims et al. 1999) **2.** (Shankwar and Srivastava 2015) **3.** (Minhas et al. 2015) **4.** (Guo et al. 2006) **5.** (de Andrade et al. 2013) **6.** (Herrero et al. 2014) **7.** (Müller et al. 2005) **8.** (Scaracia-Mugnozza et al. 1997) **9.** (Pontailleur et al. 1999) **10.** (Carmona et al. 2015) **11.** (Rae et al. 2007) **12.** (Labrecque and Teodorescu 2005) **13.** (Fortier et al. 2010) **14.** (Nassi O Di Nasso N et al. 2010) **15.** (Verlinden et al. 2015) **16.** (Dillen et al. 2013) **17.** (Truax et al. 2012) **18.** (Nielsen et al. 2014) **19.** (Bungart and Hüttl 2004) **20.** (Bungart 1999) **21.** (Adegbidi et al. 2001) **22.** (Labrecque and Teodorescu 2003) **23.** (Volk et al. 2011) **24.** (Stolarski et al. 2013) **25.** (Kopp et al. 2001) **26.** (Stolarski et al. 2011) **27.** (McElroy and Dawson 1986) **28.** (Serapiglia et al. 2013) **29.** (Adegbidi et al. 2003). (Modified from Allwright and Taylor 2016)

### Three Tree Species for Biomass Production

There is now an extensive knowledge and technology foundation for the improvement of poplar, willow and eucalyptus. These include phenotyping facilities, genetic mapping, genetic modification and advanced molecular breeding. The publication of the poplar genome in 2006 (Tuskan et al. 2006) was followed by that of eucalyptus in 2011 (Myburg et al. 2011) while the willow genome is still in progress.

All three species are of commercial significance and have been subject to extensive QTL mapping over more than two decades for traits of interest including biomass yield (Rae et al. 2009), wood quality (Brereton et al. 2010) and pest resistance (Alves et al. 2012). This depended upon the development and curation of mapping populations in all three species. More recently, genotyping-by-sequencing (GBS) and association mapping for higher resolution identification of candidate genes for bioenergy traits have been conducted in poplar and eucalyptus (Porth et al. 2013a, b; Silva-Junior et al. 2015). Genetic transformation protocols are established for all three species; there have been extensive field trials of transgenic poplar (Van Acker et al. 2014) and commercial transgenic eucalyptus is now a reality in Brazil (Ledford 2014). Table 15.1 provides an overview of the state of progress in these species while a more detailed discussion of how these resources fit together in a systems biology approach to molecular tree breeding is provided below.

## A Systems Biology Approach to Molecular Tree Breeding

Systems biology may be broadly defined as the use of computational approaches to understand complex biological systems, using functional data from the cellular to organism perspective. As such it has much to offer tree breeding and is generally considered as the integration of ‘omics’ data, such as data from genomics, proteomics and metabolomics with data from the phenotyping. Figure 15.3 is an illustrated overview of how a systems biology approach might aid the discovery of links between genes and traits. Conducting the quantity of phenotyping required now represents a significant challenge and a bottleneck relative to the ability to obtain molecular data for genotyping. An exception to this is the ability to procure high-throughput RNA-Seq data which is now revolutionising eQTL approaches. Both reverse and forward genetics can be of value to the acceleration of the breeding pipeline. Reverse genetics seeks to elucidate a specific gene’s function through mutagenising its DNA sequence and observing the phenotypic outcome. By contrast, forward genetics seeks to map the genetic basis of a specific trait of interest by seeking a statistical relationship between genetic markers and that phenotype. In general, reverse genetics approaches are valuable for understanding the basis of traits controlled by a small number of genetic loci of large effect while forward genetics approaches are better suited to understanding polygenetic traits with multiple small effect loci. One powerful forward genetic technique is genomic selection. Here the complex nature of a polygenetic trait such as yield is explicitly recognised, with 100s or 1000s of SNP molecular markers used together to establish breeding values and obviate significance testing in association and linkage studies (Beaulieu et al. 2014). In contrast, there is also now powerful evidence that CRISPR/Cas genome editing (a cutting edge reverse genetics approach) may be deployed in tree crops such as poplar (Zhou et al. 2015) where mutation breeding could be precisely deployed, again accelerating the breeding pipeline. The following sections give more detail of progress made to date for our target bioenergy trees.

**Table 15.1** Biological and technical resources for the development of poplar, willow and eucalyptus

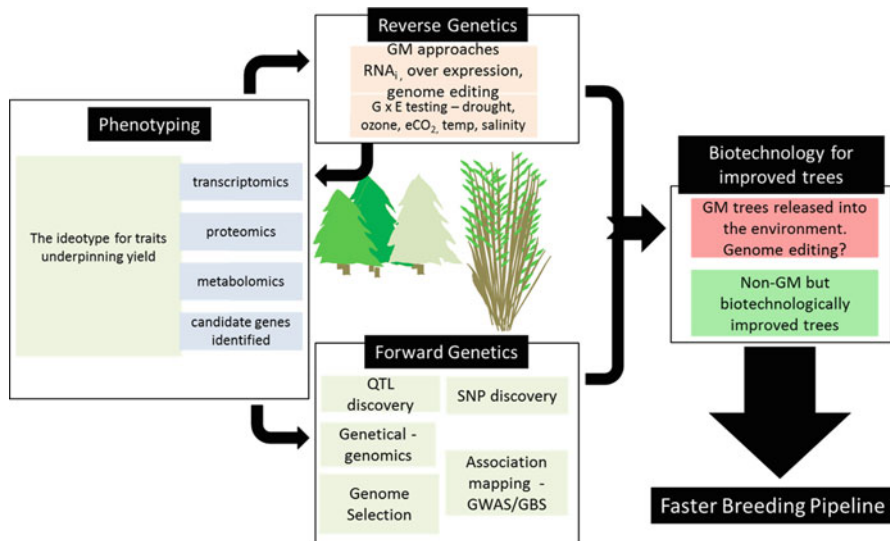
Resource	Poplar	Willow	Eucalyptus
Genome sequence	Fully sequenced genome (Tuskan et al. 2006) and some next generation sequencing (NGS).	In progress.	Fully sequenced genome (Myburg et al. 2011).
Genomic mapping	Genetic linkage maps for several <i>Populus</i> species (Cervera et al. 2001; Yin et al. 2002; Wang et al. 2011).	Extensive linkage mapping (Berlin et al. 2010; Hanley and Karp 2014).	Extensive linkage mapping (Grattapaglia and Sederoff 1994; Brondani et al. 2006; Hudson et al. 2012).
QTL mapping	Extensive QTL mapping including for biomass distribution (Wullschlegel et al. 2005), biomass yield (Rae et al. 2007, 2009) and wood quality (Novaes et al. 2009).	QTL mapping for rust resistance (Hanley et al. 2011; Samils et al. 2011); enzymatic saccharification (Brereton et al. 2010) and coppicing response (Salmon et al. 2014).	QTL mapped for growth (Freeman et al. 2009, 2013); flowering time (Missaggia et al. 2005); wood quality (Rocha et al. 2007) and rust resistance (Alves et al. 2012).
Advanced genotyping	Multiple reports of GBS in poplar utilising restriction enzyme (Schilling et al. 2014), random sheering (Slavov et al. 2012) and transcriptome resequencing (Geraldes et al. 2011) based methods. In the latter instance more than 500,000 single nucleotide polymorphisms (SNPs) were called and assisted in developing a 34 K genotyping array for <i>P. trichocarpa</i> (Geraldes et al. 2013).	Candidate gene cloning (Serapiglia et al. 2011) and transcriptome resequencing (Liu et al. 2013) but no GBS reported at this time.	Population genetic analysis and phylogeny reconstruction with 8000 markers across several taxa (Steane et al. 2011). A 60 K SNP chip was recently constructed from whole genome resequencing of 240 genotypes from 12 species (Silva-Junior et al. 2015) and employed for analysis of linkage disequilibrium, recombination and nucleotide diversity (Silva-Junior and Grattapaglia 2015).

(continued)

Table 15.1 (continued)

Resource	Poplar	Willow	Eucalyptus
Genome wide association studies (GWAS)	Candidate gene approaches have been employed in poplar (Wegrzyn et al. 2010). A 34 K genotyping array for <i>P. trichocarpa</i> has permitted a number of GWAS to be conducted in a widely utilised association population for this species. Large numbers of associations have been identified for wood quality (Porth et al. 2013a, b); rust resistance (La Mantia et al. 2013) and phenology, ecophysiology and biomass traits (McKown et al. 2014a, b).	Association populations have been established (Karp et al. 2011). In one diverse population, SSR markers were employed to assess genetic diversity and population structure to investigate the potential for future GWAS (Berlin et al. 2014).	Candidate gene approaches for growth and wood quality traits (Thavamanikumar et al. 2011, 2014). 60 K SNP chip available as of this year (Silva-Junior et al. 2015).
Genetic modification (GM)	Extensive glasshouse (Stewart et al. 2009; Wilkerson et al. 2014) and some field trials (Van Acker et al. 2014), predominantly addressing lignin content/structure or other aspects of wood chemistry.	Genetic transformation reported (Xing and Maynard 1995) but no trials of transgenic material published.	Extensive research with transgenic lines (Navarro et al. 2011; Yu et al. 2013) and the world's first commercial GM tree deployment in Brazil (Ledford 2014; FuturaGene 2015).
Deployment	Widely cultivated in temperate regions.	Limited deployment primarily in Canada and northern Europe.	Widely cultivated in southern hemisphere.





**Fig. 15.3** Systems biology for optimised biomass tree breeding. Phenotyping and ‘omics’ technologies, linked to the development of both forward and reverse genetic approaches, are proposed as a mechanism to deliver the yield improvement required for sustainable intensification. (Modified from Sims et al. 2006)

## Phenotyping: The Bottle Neck for Molecular Breeding

In order to make genetic gains to increase the productivity of biomass trees, it is necessary to thoroughly assess the phenotypes of large numbers of existing and emerging genotypes. Given the latest approaches use association rather than mapping populations, the number of individual genotypes and replicates can soon lead to very large and unwieldy experiments with several thousand plants (Porth et al. 2013a, b; McKown et al. 2014a, b). Phenotyping throughput is still limited and now stands as the major bottleneck for breeding programs. To this end, there is increased interest in developing high-throughput phenotyping platforms such as those which make up the International and European Plant Phenotyping Networks (<http://www.plant-phenotyping.org/> and <http://www.plant-phenotyping-network.eu/>). These facilities include both controlled environment and field set-ups and generally increased throughput is reliant on the utilisation of imaging and remote sensing technologies (Table 15.2).

The facility at IPK Gatersleben (Leibniz-Institut für Pflanzengenetik und Kulturpflanzenforschung, Germany) combines a high-throughput controlled environment phenotyping platform (IPK LemnaTec Scanlayzer) with GC-MS for metabolite profiling. This accommodates plants of small–large size with a capacity for up to 4608 plants to be grown in parallel. The system has enabled the detailed evaluation of stress-related metabolic and phenotypic traits in crops such as lentil. In this case, drought and salinity stress were the focus. Four accessions were int-

**Table 15.2** Phenotyping and environmental monitoring capabilities of major global phenotyping platforms including downstream proteomics and transcriptomics

Environmental monitoring	Air and soil temperature
	Humidity
	Light quality and intensity
	Quantification of CO <sub>2</sub> , O <sub>3</sub> , NO <sub>x</sub> and other trace gases
	Micro-meteorological variables
Biomass traits	Total biomass and growth dynamics, stem height and diameter
	Leaf area, Leaf Area Index, leaf growth rate
	Root structure and growth (number, length, density and architecture)
Water relations	Pot water balance
	Soil water potential
	Root–soil interactions
	Photosynthesis and transpiration
Remote sensing/imaging	Visible for green biomass estimates and plant architecture (2D and 3D)
	Hyperspectral and multi-spectral
	Near infra-red (NIR) for moisture content
	Far infra-red (FIR) for transpiration rates and apex temperature
	Fluorescence—bulk and chlorophyll
	Raman spectrometry
Biochemistry and ‘omics’	Cell/tissue processes
	Nitrogen content
	Metabolite profiling
	Thermogravimetric profiling
	GC/MS analysis of pyrolytic gas composition
	Proteomics profiling
	Transcriptomics

ensively phenotyped in order to link drought and salinity tolerance to observed metabolic differences (Muscolo et al. 2015). Phenotyping platforms which utilise controlled environments allow high-precision plant phenotyping and support the study of the genetic basis of these traits. However, for traits that are subject to high Gx $\mathcal{E}$  interaction, field phenotyping to measure plants in conditions more similar to the target commercial environment is required. For example, DIAPHEN is a field-based platform, comprising imaging tools carried by drones and phenomobiles, managed by the Institut National de la Recherche Agronomique (INRA, France). These systems are GPS-equipped and have the capacity to frequently crop physiological parameters, for example green area cover and canopy activity, in field plots of medium–large plants with a throughput of up to 100 plots h<sup>-1</sup>. The platform has been successfully used to identify genetic determinants of the drought response in an apple tree hybrid population consisting of 122 genotypes (Virlet et al. 2015).

Although substantial advances have been made in plant phenotyping in recent years (Grobkinsky et al. 2015), the gap between the relatively low-throughput of high accuracy, controlled environment platforms and the higher throughput, lower precision phenotyping which can be achieved in the field remains a challenge and several ongoing, large-scale projects are currently working on the problem of up-scaling phenotyping approaches to crop scale. High-precision, high-throughput phenotyping is necessary to support crop breeding and management.

## Metabolomics, Proteomics and Transcriptomics

In addition to traditional morpho-physiological phenotyping, individuals and populations can be characterised based on their metabolome, proteome and transcriptome. Using these ‘omics’ approaches allows physiological phenotypes to be linked to the underlying metabolome, proteome and transcriptome. This is a valuable tool to elucidate the molecular and genetic basis of yield and underpin the breeding effort.

In eucalyptus, the drought stress response of two contrasting genotypes was examined at a metabolomic and physiological level (Shvaleva et al. 2006). These *Eucalyptus globulus* genotypes were found to differ in physiological drought avoidance mechanisms. The metabolomic analyses indicating that glutathione reductase plays a central role in response to drought. Similarly, proteomics can be applied alongside traditional phenotyping to unravel the molecular basis of traits of interest. For example, xylem development is important as it impacts downstream bioethanol production. In *Populus* a proteomic method was used to identify co-expressed proteins in the secondary xylem and generate transgenic trees based on this analysis for field evaluation (Jia et al. 2011). In this way, proteomic and genomic-informed breeding strategies can be developed which utilise these rapidly advancing technologies to support breeding.

It is now possible to carry out whole-transcriptome sequencing in which expressed mRNA sequences are reverse transcribed and the cDNA (complementary DNA) sequenced to provide the entire coding region of the genome. This is a powerful and increasingly achievable tool to characterise the genetic control of traits of interest; such as yield under stress conditions. For example, a comparative transcriptomic approach has been used to identify genes with conserved expression patterns in the woody tissues of *Populus trichocarpa* and *Eucalyptus grandis* (Hefer et al. 2015). This identified conserved multi-gene orthologous gene clusters involved in secondary cell wall biosynthesis as well as species-specific gene regulation which allows xylem specialisation. Similar transcriptomic approaches have been employed in a number of plant species and many of these have been able to construct transcript correlation networks which can be linked to phenotypic traits (Porth et al. 2013a, b; Gehan et al. 2015; Vining et al. 2015). In *P. balsamifera* a high level of network module preservation was again present however organisation within modules and the central hub genes (highly interconnected genes at the centre of a network res-

possible for modulating a trait of interest) was found to vary between genotypes (Hamanishi et al. 2015). Through this transcriptome analysis, one of the six genotypes was found to have a large and distinct transcriptomic drought response while also exhibiting the smallest metabolomic response. The transcripts in this hub are likely to play a central role in regulating the drought response. This shows the power of these transcriptome-based strategies to determine critical gene hubs and gene connectivity at the genotype, organ and tissue levels. Furthermore, modelling approaches can be taken based on these identified gene hubs whereby phenotypic predictions can be made based on the alteration of genes in the hub network. The combination of transcriptomic network analysis and predictive modelling has further scope to be extended to other bioenergy tree species and to inform breeding programs for industrially-important bioenergy traits.

In the past, ‘omics’ approaches have been costly and constrained by both technologies, for example for protein identification or high-throughput sequencing, and the bioinformatics pipelines that must deal efficiently with large amounts of data. However, these are now rapidly decreasing in cost and accessibility. While it is not always straightforward; it is now possible to use these approaches as part of a powerful multi-omics strategy which can be linked with traditional phenotypic data to underpin breeding efforts.

## **Reverse Genetics: Proof of Concept or Direct Release of Biotechnologically Enhanced Trees?**

Reverse genetic approaches such as gene knockouts or overexpression are required for proof of concept studies to confirm the function of putative candidate genes from QTL or GWAS analyses (Prado et al. 2014) but may themselves also produce new trees of valuable and distinctive phenotypes for direct commercialisation (Fig. 15.3). An example of this is seen in the successful launch of the world’s first commercial, transgenic forest tree in Brazil this year (FuturaGene 2015). FuturaGene’s GM eucalyptus has shown 20% increases in biomass yield in field trials over a 10-year period and is ready for harvest after 5½ years instead of the usual 7 (Ledford 2014). The overexpressed protein (derived from a gene sequence identified from the model plant, *Arabidopsis*) accelerates growth by enhancing cell wall expansion; however, its identity remains a commercial secret. Overexpression of stress-responsive genes in eucalyptus can improve salt and cold tolerance (Navarro et al. 2011; Yu et al. 2013). The overexpression of several stress-related genes by gene stacking resulted in poplar with increased salt and pest tolerance (Polle and Chen 2015). Cold tolerance is a key breeding priority for eucalyptus to extend its growth range, sustain consistent yields and be commercially competitive in emerging bioenergy markets (Yu and Gallagher 2015). Reverse genetics can also assist in systems biology approaches to understand more complex pathways. For example, Vanholme et al. (2012) used *Arabidopsis* loss-of-function mutants for each of the ten genes in the lignin biosynthesis pathway to understand the responses to perturbations in this

pathway. Low-lignin transgenic trees (generally knockouts or RNAi knockdowns) are of great research interest because they have the potential to yield a feedstock that is less recalcitrant to enzymatic saccharification (Studer et al. 2011). Van Acker et al. (2014) demonstrated improved saccharification and ethanol yield from field-grown GM poplar deficient in the lignin biosynthetic enzyme cinnamoyl-CoA reductase. Unfortunately, blunt reductions in lignin content can have negative consequences for yield (Van Acker et al. 2014) and pest resistance (Polle et al. 2013) and other, more novel, transgenic approaches are being investigated. One route is the heterologous expression (i.e. stimulating gene expression in cells that do not normally express the gene) of thermophilic, cell wall degrading enzymes *in planta* (Jung et al. 2012). These enzymes can be activated by mild temperature increases post-harvest and can decrease the energy and financial costs of the conversion of wood to ethanol. Poplar especially is known as an efficient bioreactor for the expression of foreign enzymes (Kim et al. 2012). Another exciting GM approach for reduced recalcitrance without impacting fitness has been reported by Wilkerson et al. (2014). They successfully incorporated a transferase gene into poplar which introduced ester linkages into the lignin backbone. These ester bonds can be readily hydrolysed by a mild, alkaline pre-treatment, aiding processing. In recent years, highly targeted, sequence-specific genome editing has become more feasible in eukaryotes through the development of CRISPR/Cas technology (Gaj et al. 2013). In a key development for bioenergy trees this technology has now been successfully used in poplar to target a lignin biosynthetic enzyme (4-coumarate:CoA ligase) and further innovations using this method are likely to follow (Zhou et al. 2015). Finally, tilling is a powerful and high-throughput reverse genetic approach to elucidate gene function in a mutagenised population using a mismatch endonuclease to detect the induced mutations (SNPs or indels). Ecotilling is closely related but seeks to identify polymorphisms in natural populations and evaluate their effects on genes of interest and phenotypic significance (methodology and development reviewed by Barkley and Wang 2008). Ecotilling has been successfully employed in food crops (Yu et al. 2012) and has potential for accelerating the domestication of forest trees (Harfouche et al. 2012). Marroni et al. (2011) reported the detection of rare alleles in poplar using NGS and believe that this methodology could drive next generation ecotilling in this species; allowing function to be ascribed to these low frequency variants.

## From Trees to Genes: Forward Genetics

Forward genetic techniques seek to understand the genetic basis of a phenotype and identify genomic regions, markers and/or candidate genes linked with the trait of interest (Fig. 15.3). Forward genetic approaches are of particular value for elucidating quantitative, polygenic traits. Before the revolution in cost-efficient, high-throughput, next-generation sequencing (Mardis 2011), genetic marker density was generally limited and research was focussed on broad QTL mapping which can be

achieved with only a few hundred SSLR or microsatellite markers. QTL have been mapped for yield in both eucalyptus (Freeman et al. 2013) and poplar (Wullschleger et al. 2005; Rae et al. 2007). Rae et al. (2009) identified five robust QTL hotspots for yield in short rotation coppice (SRC) poplar explaining 20% of final biomass yield in the mapping population. In willow, QTL have been mapped for rust resistance; a major willow pathogen and responsible for commercial losses (Hanley et al. 2011; Samils et al. 2011). Expression QTL (eQTL) mapping is a more recent development, also known as 'genetical genomics' (Joosen et al. 2009). This approach considers gene expression (quantified levels of given mRNA transcripts) as a quantitative trait and maps this expression data as QTL (Ingvarsson and Street 2010). This can permit the identification of causal genes underpinning the phenotype of interest. Genetical genomics has been widely employed for several years in a number of plant and animal species including model organisms such as *Drosophila*, yeast and mice (Joosen et al. 2009). In the model plant *Arabidopsis* the technique has been useful for understanding the genetic basis of complex responses such as genotype-by-environment interactions (Joosen et al. 2013) and genetic regulatory networks (Terpstra et al. 2010). In bioenergy research genetical genomics has been applied in the biodiesel crop jatropha for oil production traits (Liu et al. 2011) and in poplar for leaf shape variation (Drost et al. 2015). Another genetical genomics approach that has been employed in poplar is a form of bulk segregant analysis with microarray expression data. Street et al. (2006) identified extreme genotypes for drought tolerance traits in response to soil drying and used microarrays to identify differentially expressed genes between these groups. They were able to identify promising candidate genes whose differential expression co-located with traditionally mapped QTL for these drought-specific traits. More recently, with the availability of NGS approaches to provide high-throughput DNA marker data, eQTL mapping has become relatively cheap and much more tractable to elucidate the link between phenotypes and their underlying resolution at the genomic level (Majewski and Pastinen 2011). These technologies are now being applied to plant improvement and combined with QTL-Seq approaches (Takagi et al. 2013). Using NGS for RNA-Seq can offer significant new potential to resolve traits in trees in future in a more time and cost-effective manner.

Association mapping is a more powerful forward genetic approach for elucidating the genetic basis of qualitative and quantitative traits in species of interest; seeking statistical associations between SNPs and phenotypes of interest within a population (Ingvarsson and Street 2010). The finesse with which a trait can be mapped is dependent on the rate of decay of linkage disequilibrium (LD), i.e. the non-random association of alleles at different loci. Since linkage is a major contributor to LD, LD declines with physical distance (Flint-Garcia et al. 2003). Outbreeding species (including poplar, eucalyptus and willow), which have a higher effective degree of recombination than inbreeders (Gaut and Long 2003), can achieve higher resolution association mapping but concomitantly require a higher marker frequency (Neale and Kremer 2011). The need for high marker density meant that initial association studies in bioenergy trees tended to take a candidate

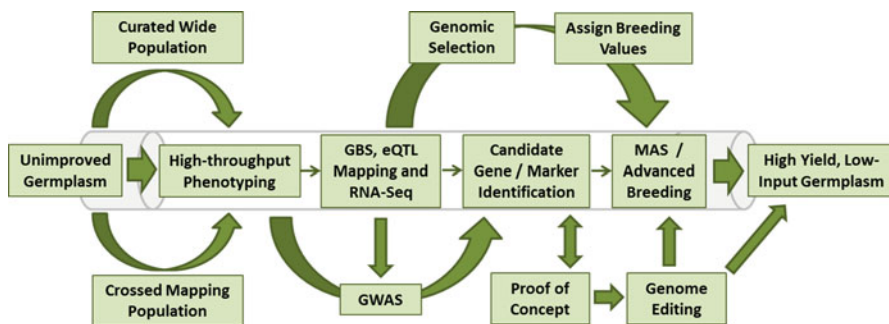


Fig. 15.4 The advanced molecular breeding pipeline

gene approach which are not genome-wide but useful for narrowing down genes of interest within a broader QTL region or identifying candidates within a group of genes of putatively similar function (Teare 2011). In both poplar (Wegrzyn et al. 2010; Guerra et al. 2013) and eucalyptus (Thavamanikumar et al. 2011, 2014) candidate gene approaches have been employed for wood quality traits with robust trait-marker associations identified. In poplar these have been superseded by the development of a 34,000 SNP array for *P. trichocarpa* with SNPs drawn from 3543 candidate genes for a variety of valuable bioenergy traits (Geraldès et al. 2013). This ‘chip’ has been employed in a number of GWAS in poplar in the past 2 years identifying hundreds of trait-marker associations for key traits including biomass yield (McKown et al. 2014a, b), wood quality (Porth et al. 2013a, b) and rust tolerance (La Mantia et al. 2013). Eucalyptus researchers are now pursuing a similar path with the recent publication of a 60,000 SNP chip that will permit GWAS in this species (Silva-Junior et al. 2015). Associations can then feed into the molecular breeding pipeline (Fig. 15.4) and marker-assisted selection (MAS), as seen in many crop plants (Miedaner and Korzun 2012), for the advanced breeding of superior bioenergy trees.

Beginning with unimproved germplasm curated in a natural, wide or mapping population, advanced molecular breeding may proceed through high-throughput phenotyping for traits of interest. In parallel; GBS, GWAS, genomic selection, transcriptome sequencing and/or eQTL mapping can allow the identification of candidate genes or markers for the phenotyped traits which may also serve as high value targets for GM proof of concept studies and genome editing. Collectively these techniques feed directly into advanced, marker-assisted selection and breeding programmes for novel, high yield, low-input feedstocks.

As NGS reduces genotyping costs and marker numbers and density increase there is the potential to move towards GS in bioenergy trees. GS assigns breeding values to individuals based on genome-wide markers of sufficient density to permit the assumption or knowledge that all relevant genomic regions are in LD with some of the genotyped SNPs (single nucleotide polymorphisms or single base changes in the DNA sequence) (Grattapaglia and Resende 2010). A modelling study from

Resende et al. (2012) suggests that GS could accelerate the domestication of forest trees by increasing selection efficiency resulting in a faster breeding cycle. This has huge potential for biomass poplar, willow and eucalyptus where trees take several years to reach reproductive maturity and traditional breeding can take decades. GS has recently been shown to be effective in interior spruce (Gamal El-Dien et al. 2015) using markers obtained through GBS; with ongoing research to identify the best breeding groups to deploy this technology in white spruce (Beaulieu et al. 2014).

## The Breeding Pipeline

Trees are long-lived and largely out-breeding species and it is therefore difficult to make rapid improvements through breeding and selection (Harfouche et al. 2011; Allwright and Taylor 2016). In addition many tree species are dioecious (single sexed), making the selection of specific crosses difficult and genetic research complex. These lifecycle limitations have major impacts on the breeding cycles for woody plants and have been partially overcome in the past by the extensive use of vegetatively propagated or clonal material, as in the three species considered here (Liesebach and Naujoks 2004; Meilan et al. 2002; Stape et al. 2008). Recently, protoplast fusion has been introduced as a novel technique for the production of enhanced poplar germplasm (Hennig et al. 2015); however, the technique is still in its infancy.

This short review has highlighted several approaches that are combining next-generation DNA sequencing technologies with high-throughput phenotyping approaches to overcome this bottleneck in the next decade with accelerated breeding cycles possible. All pipelines begin with the collection and curation of novel germplasm material (Fig. 15.4) and future efforts to fulfil the necessity for sustainable intensification (Fig. 15.1) are likely to involve collection from extreme climate sites. The value of wild germplasm cannot be overestimated and has proved to be of central importance in recent breeding efforts in both rice (Arbelaez et al. 2015) and tomato (Blanca et al. 2015). Recent advances now mean that this material is tractable with large GWAS studies enabling the rapid development of links between traits and genes and the development of molecular markers with which to pursue MAS. The difficulty with this approach for trees is their outbreeding nature, although rare variants have been identified using a modified pooled multiplexing (the simultaneous sequencing of many DNA samples tagged for their identification, thus speeding DNA sequencing whilst reducing cost) approach that identifies rare variants of functional genes underpinning lignin production in poplar (Marroni et al. 2011). More promising are the genomic selection tools where training and validation populations are used to calculate genotypes' breeding values from mul-



multiple markers in relation to traits of interest. Such techniques offer significant potential to reduce breeding time since selections can be made in a fraction of the time required to follow the growth and performance of a breeding population using routine harvest and assessment methods. Alongside genomic selection, genome editing has also been shown as a proven technology for poplar (Fan et al. 2015; Zhou et al. 2015) and offers a route for the rapid assessment of individual genes that might emerge from the breeding pipeline and high-throughput phenotyping. In many respects, genomic selection and genome editing offer two contrasting routes to the production of improved, high yielding biomass material for the future bioenergy landscape and both should be considered over the coming decades.

## Conclusions

Tree breeding for bioenergy is important as woody lignocellulosic crops can contribute to efforts to fulfil global commitments to reduced emissions and the move towards a low carbon economy. Most future energy scenarios highlight a significant role for energy from biomass, including through co-firing; biomass burning with CCS and biomass for liquid biofuels. However, the supply of high yielding, sustainable feedstock cultivars of biomass tree species is hampered by the biology of trees. This review has highlighted the importance of high-throughput phenotyping and new molecular technologies that can be deployed to significantly accelerate the breeding pipeline, without the necessity to produce a ‘GM’ tree; helping to address the current yield gap and increase potential yields in these important lignocellulosic crops.

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